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Alba Martínez Albiñana

Thesis advisors: María Luisa Gil and Alberto Yáñez

Role of PRRs (TLR2 and Dectin-1) in hematopoietic stem and progenitor cell differentiation: implications in protection against *Candida albicans* infection

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Doctoral thesis presented by:

Alba Martínez Albiñana

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La Dra. **Mª Luisa Gil Herrero**, catedrática de la *Universitat de València*, y el Dr. **Alberto Yáñez Boyer**, investigador Ramón y Cajal de la *Universitat de València*,

CERTIFICAN:

Que el trabajo presentado por Alba Martínez Albiñana, titulado: "**Role of PRRs (TLR2 and Dectin-1) in hematopoietic stem and progenitor cell differentiation: implications in protection against *Candida albicans* 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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Fdo. Dra. Mª Luisa Gil Herrero

Fdo. Dr. Alberto Yáñez Boyer

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Pompeu Fabra

ABBREVIATIONS

APC	Antigen Presenting Cell
ATTC	American Type Culture Collection
BCG	Bacille Calmette-Guérin
BM	Bone Marrow
CARD9	Caspase Recruitment Domain-containing protein 9
CCL	CC Chemokine Ligand
CCR	CC Chemokine Receptor
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CFU	Colony Forming Unit
CMP	Common Myeloid Progenitor
CSF	Colony-Stimulating Factor
CTL	Cytotoxic T Lymphocyte
CXCL	CXC Chemokine Ligand
CXCR	CXC Chemokine Receptor
DAMP	Damage-Assotiated Molecular Pattern
DC	Dendritic Cell
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
FBS	Fetal Bovine Serum
FcR	Fragment crystallizable region Receptor
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Granulocyte-Monocyte Progenitor

G-CSF	Granulocyte Colony-Stimulating Factor
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem and Progenitor Cell
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
IL	Interleukin
IRF	Interferon-Regulatory Factor
KO	Knockout
Lin	Lineage
LKS	Lin^- c-Kit^+ $Sca\text{-}1^+$
LPS	Lipopolysaccharide
LT-HSC	Long-Term repopulating HSC
MSC	Mesenquimal Stem Cell
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
moDC	Monocyte-derived Dendritic Cell
MPP	Multi-Potent Progenitor
MyD88	Myeloid Differentiation Factor 88
NF-κB	Nuclear Factor kappa B
NK	Natural killer
NLR	NOD-like Receptor
NOD	Nucleotide-binding Oligomerization Domain
OD	Optical Density
OVA	Ovalbumin
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline

PGE_2	Prostaglandin E_2
PLM	Phospolipomannan
PMA	Phorbol 12-Myristate 13-Acetate
PRR	Pattern Recognition Receptor
RIG-I	Retinoic acid Inducible gene I
RLR	RIG-I-like Receptor
ROS	Reactive Oxygen Species
RT	Room Temperature
SCF	Stem Cell Factor
ST-HSC	Short-Term repopulating HSC
Syk	Spleen Tyrosine Kinase
TC	Tissue Culture-treated
TCR	T Cell Receptor
Th	T helper
TLR	Toll-Like Receptor
TNF-α	Tumor necrosis Factor α
Treg	T regulatory
TRIF	TIR-domain-containing adapter-inducing Interferon-β
WT	Wild Type

CONTENTS

ABSTRACT

Detection of infection by hematopoietic stem and progenitor cells (HSPCs) is essential to replace myeloid cells consumed during the immune response. HSPCs express some functional pattern recognition receptors involved in the recognition of *Candida albicans*. In this context, our group has previously demonstrated that *C. albicans* yeasts induce proliferation and differentiation of HSPCs via TLR2 and Dectin-1. In the present PhD thesis, we used *in vitro* and *ex vivo* models of HSPC differentiation to investigate the functional consequences for mature myeloid cells of exposure of HSPCs to PAMPs or *C. albicans* yeasts.

In vitro experiments show that murine HSPCs continuously exposed to TLR2 or TLR4 ligands, in homeostatic conditions, generate macrophages with a diminished ability to secrete TNF-α and IL-6 (tolerized phenotype), whereas Dectin-1 or *C. albicans* yeasts stimulation leads to the generation of macrophages that secrete higher amounts of these pro-inflammatory cytokines (trained phenotype). In these conditions, transient exposure of HSPCs to TLR agonists is sufficient to generate macrophages with a tolerized phenotype, which is partially reversed by subsequent exposure to *C. albicans* yeasts. However, only TLR2 signaling in HSPCs impacts the inflammatory function of macrophages they produce in inflammatory conditions, and subsequently exposure to *C. albicans* yeasts do not reverse but reinforce this altered phenotype. TLR2 and Dectin-1 signaling in HSPCs also have functional consequences for the antigen presenting cells (APCs) derived from them, which exhibit an altered expression of histocompatibility complex class II molecules (signal one) and costimulatory molecules (CD40, CD80 and CD86) (signal two), as well as an altered cytokine production (TNF-α, IL-6, IL-12 p40 and IL-2) (signal three). These changes in the three key signals that APCs deliver to naïve T cells provoke that APCs from TLR2/Dectin-1-programed HSPCs prime enhanced Th1 and Th17 responses in $CD4^+$ T cell cocultures.

C. albicans systemic infection and *in vivo* TLR2 agonist treatment also alter cytokine production and fungicidal activity of the *ex vivo* macrophages derived from murine HSPCs in homeostatic conditions. Unexpectedly, an extended TLR2 agonist treatment generates macrophages with a trained phenotype. This prolonged treatment also causes an expansion of HSPCs and myeloid cells in the spleen, and drastically reduces the fungal burden in the kidney and the spleen during systemic *C. albicans* infection. This protection is abrogated by immunodepletion of HSPCs. In addition, HSPCs produce *in vitro* cytokines and chemokines in response to a TLR2 ligand or *C. albicans* yeasts, and these secretomes can induce differentiation of HSPCs towards the myeloid lineage.

Taken together, these data assign an active role to HSPCs in sensing pathogens during infection and in contributing to host protection by diverse mechanisms.

INTRODUCTION

1 Immunity to *Candida albicans* invasive infections

The genus *Candida* is a polyphyletic group that includes more than 300 disparately related species, which are found in a diverse range of environmental niches. Despite their distant evolutionary origin, various members of the genus have evolved mechanisms to successfully colonize the human body, effectively promoting their tolerance by the host immune system to avoid clearance; therefore, these species constitute part of the normal microbiota that resides throughout the mucosal surfaces of the gut and oral or vaginal cavities (Hube, 2009). As bacteria associated with our bodies, it has been also described that commensal fungal communities (the mycobiome) play a key role in maintaining immune homeostasis in healthy individuals (Underhill and Iliev, 2014). However, when normal host defenses are impaired, this delicate balance may turn into a fungal transition from commensalism to opportunism and *Candida* species can become a serious agent of infection in these conditions (Calderone, 2012; Wheeler *et al.*, 2017).

Manifestation and severity of candidiasis, a broad term that refers to infections caused by fungi of the *Candida* genus, range from superficial mucocutaneous infections to severe disseminated infections depending on the nature and extent of the impairment of host immune responses (Calderone, 2012; Perlroth *et al.*, 2007). Superficial infections are common and tend to resolve naturally or with minimal intervention. Nevertheless, invasive diseases, which refer to bloodstream infections (candidaemia) or deep-seated infections, are hard to treat and mortality rates can be up to 40%. The lack of quick and accurate diagnostic procedures, together with the emergence of resistances to the limited antifungal agents available contribute to this high number of deaths (Pappas *et al.*, 2018; Pfaller and Diekema, 2007; Yapar, 2014). There are well-described risk factors associated with invasive candidiasis, some of them intrinsic to the host or the disease state, whereas others are the result of medical treatment (Pappas *et al.*, 2018). Thus, in adult patients, systemic candidiasis frequently arises from either colonization of foreign material, such as intravenous catheters, or from fungi translocation from the gut into the blood. The three major predisposing factors for *Candida* sepsis due to dissemination from the gut are: (i) previous intestinal colonization with *Candida* spp., (ii) host defects in phagocytic cell function or numbers, and (iii) disruption of intestinal barrier. This dissemination of *Candida* commonly occurs in cancer patients who receive chemotherapy, as the combination of chemotherapy-induced neutropenia, mucositis and treatment with antibiotic cocktails

results in a highly permissible environment through which *Candida* can disseminate to the bloodstream (Wheeler *et al*., 2017).

From all the species included in the genus *Candida*, only about twenty can cause human disease. Of these, more than 90% of invasive infections are caused by the five most common pathogens, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei*. Based on the frequency of isolation and virulence, the most prominent species among these five is *C. albicans*, due to its morphological flexibility and ability to adhere to biological and inert surfaces. In the United States and northwestern Europe, the second most common species is *C. glabrata,* whereas in Latin America, Southern Europe, India and Pakistan, *C. parapsilosis* and/or *C. tropicalis* are much more frequently encountered. *C. krusei* is the least common of the five major *Candida* spp., and it is most often found among patients with underlying hematological malignancies. *C. glabrata* is also generally more common among individuals older than 60 and among recipients of solid organ transplant. In the last decades, the diagnosis of non-albicans candidaemia has increased and other *Candida* species have been described as emerging pathogens able to cause disease in humans (i.e. *C. dubliniensis*, *C. guilliermondii*, *C. kefyr, C. famata*, *C. lusitaniae*, *C. auris,* among others) (Guinea, 2014; Pappas *et al.*, 2018; Perlroth *et al.*, 2007).

Host resistance to candidiasis involves the coordinated action of both innate and adaptive host immune responses, which are triggered following fungal recognition by immune and non-immune cells. Defense mechanisms are initially triggered by an inflammatory response mediated by the innate immune system. This response also induces and modulates the adaptive immune responses, which in turn regulates signals from the innate system. As the most common *Candida* spp. causing candidiasis, the major factors that characterize *C. albicans* as an opportunistic pathogen, as well the most relevant aspects of these defense responses to *C. albicans* are described next.

1.1 *C. albicans*, an opportunistic pathogen

1.1.1 Fungal cell wall

The fungal cell wall is a complex dynamic structure that protects cells from osmotic pressure in hypotonic media and is responsible for its morphology. Moreover, as the outermost cellular structure, the *C. albicans* cell wall plays an essential role in the host-microorganism interactions and therefore in the pathogenicity of the fungus (Arana *et al*., 2009; Gozalbo *et al*., 2004; Poulain and Jouault, 2004; Ruiz-Herrera *et al*., 2006). This cell

wall is a multilayered structure mainly composed by polysaccharides, while proteins represent only 20 % of the cell wall dry weight and lipids 1-7 %. The inner layer is based on a core structure of β-D-glucose polysaccharide with 1-3 β-glycosidic bonds (β-(1,3)-glucan) covalently linked to a β-(1,4)-linked polymer of N-acetylglucosamine (chitin) towards the inside of the cell wall, and itself attached towards the outside to branched β-1,6-glucan (**Figure 1**). These polymers, by forming hydrogen bonds between adjacent polysaccharide chains, constitute a three-dimensional network of microfibrils that accounts for the cell wall rigidity. Besides being close to the cell membrane in an inner layer, those skeletal components can become exposed to the surface in budding yeast cells, as a scar is left on the mother cell after separation. Furthermore, differential surface exposure and structural differences of β-glucans have also been described between the two main growth forms of *C. albicans,* yeasts and hyphae (see 1.1.2), thus modulating their immunological properties (Gantner *et al.*, 2005; Lowman *et al.*, 2014).

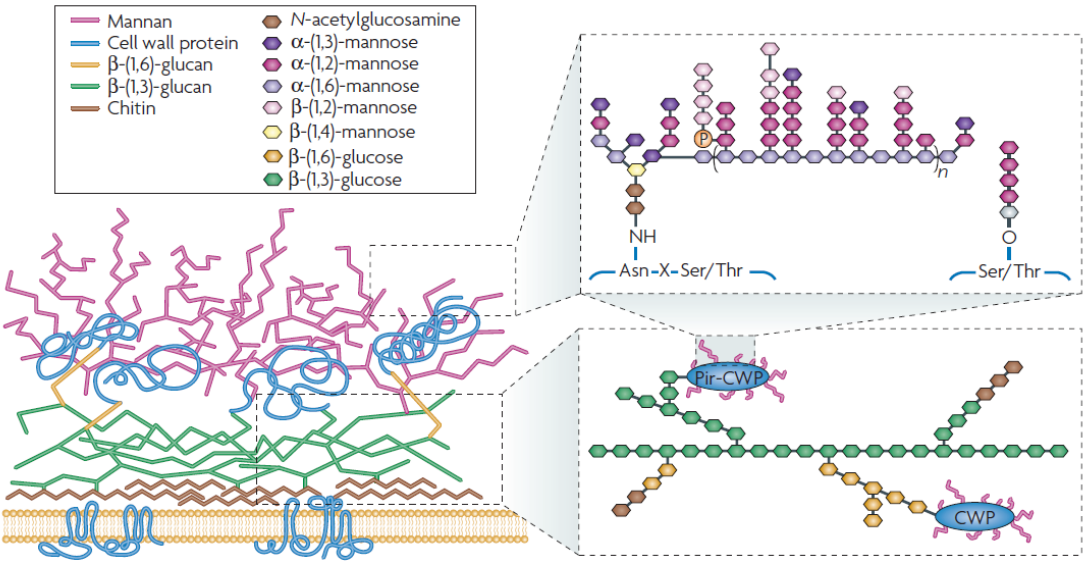


FIGURE 1 | The structure of the *Candida albicans* cell wall. The schematic diagram shows the major components of the cell wall and their distributions. Glucans and chitin (the microfibrillary polysaccharides) constitute the main structural components and they are distributed in internal layers, with chitin located near the plasma membrane. Proteins and mannoproteins (cell wall proteins, CWP) are predominant in the outer layer. Different covalent bonds, formed between some mannoproteins and polysaccharide chains and between different mannoproteins, contribute to the organization of the cell wall. The insets show the structure of the glucan and mannan components (Netea *et al.*, 2008).

In addition to the glucan and chitin skeleton, the *C. albicans* cell wall contains an outer surface that mainly comprises glycosylated proteins. These cell wall proteins are covalently associated with mannan through *N*-glycosidic or *O*-glycosidic linkages (mannoproteins). Linked *O*-mannans are linear polysaccharides linked to serine/threonine residues that, in *C. albicans*, consist of one to five mannopyranose units almost exclusively α-(1,2)-linked.

Otherwise, *N*-mannan is a complex structure, linked to asparagine residues, comprising up to 150 mannose sugars arranged as an α-(1,6)-linked backbone with side chains of α-(1,2)-, α-(1,3)-mannose and phosphomannan. Mannoproteins could also be classified, depending on how they are attached to the glucan network, in two types: protein with internal repeats (Pir) proteins and glycosylphosphatidylinositol (GPI) proteins (**Figure 1**). Pir proteins are directly associated with β-1,3-glucan, while GPI proteins used a GPI anchor remnant to interact with β-1,6-glucan. GPI proteins in eukaryotes are usually anchored into the plasma membrane, although in *C. albicans* it has been shown that some of them are redirected to the cell wall network. In this case, the GPI anchor is cleaved and the protein with the remnant part of the anchor is then transported to the outer layer of the fungal cell wall (Richard and Plaine, 2007). Mannoproteins are dominant in the external surface, but they expand the entire cell wall structure, and some are secreted to the extracellular medium (Arana *et al.*, 2009; Gozalbo *et al.*, 2004; Ruiz-Herrera *et al.*, 2006).

Other *C. albicans* cell wall component that contribute actively to fungal-induced pathogenesis is phospolipomannan (PLM), a mannosylated sphingolipid with a glycan fraction composed of a long chain of β-1,2-linked mannoses. This hyper-mannosylation seems crucial to relocate PLM from the plasma membrane to the cell wall, and for its secretion to the environment (Fradin *et al.*, 2015).

1.1.2 Virulence factors

C. albicans does not act as a passive element during infectious process but actively participates in the establishment and progress of the infection by expressing a set of putative virulence factors (Calderone, 2012). These virulence traits include morphological transition between yeast and hyphal forms, hydrolytic enzyme production, cell surface expression of invasins or adhesins, ability to develop structured microbial communities or biofilms, phenotypic switching, antigenic variability, and immunomodulation of host responses. Additionally, a rapid adaptation to fluctuations in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and robust stress response machineries are fitness attributes that also support the ability of *C. albicans* to infect host niches. Some of them are essential to *C. albicans* pathogenicity and represent promising targets for vaccine and antifungal drug development (Höfs *et al.*, 2016; Mayer *et al.*, 2013; Poulain, 2015).

One of the most studied *C. albicans* virulence factors is polymorphism. *C. albicans* is a polymorphic fungus that usually growths as ovoid-shaped budding yeast, but under specific

environmental conditions, it can form germ tubes to develop hyphae. Further detected morphologies include elongated ellipsoid yeast cells with constrictions at the septa (pseudohypae), spore-like structures generated from hyphae under stress conditions (chlamydospores) or white and opaque cells formed during switching (see below) (Calderone, 2012; Sudbery *et al*., 2004). The transition between yeast and hyphal forms is termed dimorphism and it was traditionally considered as a virulence factor because: (i) commensal *C. albicans* only growths as yeast whereas both yeast and hyphal forms are detected in infected tissues (Calderone, 2012), and (ii) expression of fungal virulence factors (e.g. adherence or antigen expression) is related with morphology, as they are coregulated with the yeast-to-hypha transition, enabling the hyphae to be better equipped to develop the infectious process and to overcome the host immune responses (Lo *et al*., 1997). Moreover, it has been shown that there are differences in cell wall organization and composition between budding yeasts and hyphae (such as chitin and β-glucan content, structure and exposure to cell surface, or mannan structure and mannoprotein expression) that may contribute to the increased ability of hyphae to infect host cells (Gozalbo *et al.*, 2004; Höfs *et al.*, 2016; Ruiz-Herrera *et al.*, 2006). In fact, monomorphic mutants unable to form hyphae exhibit impaired virulence in mice (Lo *et al.*, 1997). However, it has been proposed that both growth forms are important for pathogenicity: the hyphal form would be more adapted to adhesion and invasion while the smallest yeast form could represent the form primarily involved in dissemination (Noble *et al.*, 2010).

Furthermore, *C. albicans* may perform phenotypic switching, a strain-dependent process which consist in the reversible generation of genetic variants easily distinguishable by the morphology of the colonies they form; switching is associated to genome rearrangements that result in the generation of genetic variants with an altered expression of numerous virulence and immunomodulatory factors, affecting both cell and colony morphologies. The best studied switching is the White/Opaque transition. Strains homozygous at the mating type locus (*MTL* a/a or *MTL* α/α), following mitotic recombination or duplication of the homologous chromosome, can switch from the normal yeast form (white cells/colonies) to an elongated cell form termed "opaque"; this opaque cells are less resistant than the white cells, but are the mating-competent form of *C. albicans*: they can mate with other opaque cells to produce recombinant progeny. White/Opaque switching may occur spontaneously at a relatively low frequency and may be involved in fungal survival during infection by generation of fungal cells better adapted for invasion and/or immune evasion (Soll, 2009).

C. albicans secrete a variety of enzymes, such as proteases, phospholipases and others, that can damage host cell structures and contribute to infection, reason why these enzymes are considered virulence factors. The best characterized are the secreted aspartyl proteinases (SAPs), proteolytic enzymes that can degrade proteins with immunological functions (such as complement or immunoglobulins) therefore favoring fungal evasion of host defenses, or mucin, whose degradation promotes an active fungal-driven penetration into gastrointestinal mucosa (Naglik *et al*., 2004). In addition, it has been also described that *C. albicans* hyphal form can also produce a cytolytic peptide toxin named candidalysin. When accumulated at sufficient concentrations, candidalysin interacts with the cell membrane to form pore-like structures that results in membrane damage (Moyes *et al.*, 2016; Naglik *et al.*, 2019). However, candidalysin seems to play a dual role in *C. albicans* pathogenesis: on one hand it directly damages host cells, but on the other hand it is an immunomodulatory molecule that is sensed by the host to initiate a protective response (see section 1.2). Thus, the outcome of infection is dictated by this virulence immunomodularory balance, namely damage induction versus immune protection (Naglik *et al.*, 2019).

Effective *C. albicans* yeasts adherence and invasion of endothelial and epithelial cells enable their dissemination into the bloodstream (Sheppard and Filler, 2014). Host-pathogen interactions are mainly mediated by a set of specialized cell wall proteins and mannoproteins, termed adhesins, which also participate in adherence to other microorganisms, to *C. albicans* cells, or to abiotic surfaces (de Groot *et al*., 2013; Gozalbo *et al*., 2004). Once adhered, *C. albicans* can utilize two different mechanisms in order to invade host cells: induced endocytosis and active penetration (Naglik *et al*., 2014; Sheppard and Filler, 2014; Zakikhany *et al.*, 2007). For inducing endocytosis, the fungus expresses specific proteins termed invasins that mediate binding to host ligands, thereby triggering the engulfment of the fungal cell (yeast or hyphae) into the host cell. This is a passive process, killed cells are even taken up, whereas active penetration is a fungal-driven process that requires viable *C. albicans* hyphae (Dalle *et al*., 2010). The capacity of *C. albicans* for effective adherence to inert materials also facilitates biofilm formation on implanted medical devices such as central venous catheters or prostheses, which represents a major source of long-term candidaemia (Desai *et al.*, 2014).

1.2 Initial recognition of *C. albicans*: innate immune responses

When systemic candidiasis arises from dissemination of fungal cells from the gut to the bloodstream, the first line of defense against fungal infections is the epithelium. It provides a physical barrier between the internal environment and the external environment that contains pathogens. Epithelial cells are able to restrict fungal invasion by a variety of defense mechanisms that appear to be activated depending on the degree of tissue damage and invasion by *C. albicans*. In the presence of yeast cells, epithelial cells produce antimicrobial peptides with potent antifungal activity, such as β-defensins, cathelicidin or alarmins. This response suggests that the uppermost epithelial layers are able to maintain the commensal status of *C. albicans* without further activation of immune responses (Lilly *et al.*, 2010; Naglik *et al.*, 2014; Yano *et al.*, 2012).

A mechanism to discriminate between the yeast (commensal) and hyphal (invasive) forms of *C. albicans* has been described in epithelial cells from mucosal surfaces (Moyes *et al.*, 2010; Moyes *et al.*, 2015; Naglik *et al.*, 2014). These epithelial cells orchestrate an innate response to *C. albicans*, inducing a biphasic production of pro-inflammatory cytokines and chemokines (Moyes *et al*., 2010). The first phase is independent of hyphal formation and involves a low pro-inflammatory response that, added to the antifungal effect of resident macrophages, may be sufficient to stop tissue invasion and damage, thus maintaining commensalism and low fungal burdens. If this early and transient response fails to avoid fungal proliferation, a second phase is triggered. This second phase, which is dependent on fungal burden and hyphae formation, involves a higher production of pro-inflammatory cytokines and chemokines by epithelial cells, inducing neutrophil and monocyte recruitment, as well as the secretion of antimicrobial peptides. The production of candidalysin by the hyphal form also induces this second phase that favors fungal clearance or reduction of fungal burden below the threshold level of activation, and thus a return to the commensal state. Such biphasic response will allow epithelial tissues to remain quiescent under low fungal burdens while responding strongly to damage-inducing hyphae when burden increases (Moyes *et al.*, 2015; Naglik *et al.*, 2014).

Finally, it is worth to note that normal microbiota of mucosal surfaces is also part of the first line of antimicrobial defense, as its members compete with pathogens for nutrients and space (Muraille, 2015). There is increasing evidence indicating that host microbiota also influences fungal colonization and antifungal immune responses (Oever and Netea., 2014; Romani *et al.*, 2015). As an example, some bacterial species (*Pseudomonas aeruginosa*, *Enterococcus faecalis*) are able to inhibit hypha development of *C. albicans*, and others

(lactobacilli) inhibit fungal adhesion and growth, thus protecting gut mucosa from *C. albicans* proliferation. This complex relationship between microbiota and *C. albicans* is clearly deduced by the fact that treatment with antibacterial antibiotics with wide spectrum is a risk factor contributing to *C. albicans* colonization, and disturbances in normal microbiota can lead to mucosal infections in otherwise healthy hosts (Calderone, 2012; Kennedy and Volz, 1985; Oever and Netea, 2014).

When *C. albicans* is able to deal with mucosal immunity and reach the bloodstream, the first line of defense against the pathogen is the innate immune system. Pathogen recognition by phagocytes is the initial step in activating a rapid immunological response to fight against the pathogen. Phagocytes can kill the pathogen and induce an inflammatory response that modulates the adaptive immune responses, thus coordinating both arms of the immune system. The cellular and molecular bases of protective innate responses against *C. albicans,* which are described below, have been elucidated by the discovery of human genetic disorders in immune factors associated with susceptibility to fungal infections and studies using mouse models of systemic candidiasis (Duggan *et al.*, 2015; Lionakis, 2014).

1.2.1 Innate immune responses

1.2.1.1 Serum factors

Although humans with genetic deficiencies in components of the complement system do not show increased risk for systemic *Candida* infections, considerable evidence from both murine studies and *in vitro* assays with human cells indicate an important role of complement in antifungal immunity (Kozel, 1996; Mullick *et al*., 2004; Speth *et al.*, 2008; Tsoni *et al.,* 2009). *C. albicans* cell wall is a strong inducer of the three pathways of complement activation: (i) mannan on the cell surface is recognized by a mannan-binding protein which activates the lectin pathway, (ii) mannan on the cell surface is recognized by anti-mannan antibodies that activate the classical pathway, and (iii) the cell wall is targeted by C3b to initiate the alternative pathway. All three pathways converge on the rapid formation of C3 convertase, giving rise to the C3b cleavage product that opsonizes and promotes *C. albicans* phagocytosis by neutrophils and macrophages via complement receptor 3 (CR3) (Tsoni *et al.*, 2009). Anti-mannan antibodies also act as opsonins promoting fungal cell detection by phagocytes through immunoglobulin-γ fragment crystallizable region receptors (FcγRs) (Kozel, 1996; Speth *et al*., 2008). Furthermore, the activation of complement system also generates anaphylatoxins (cleavage products C3a and C5a) that enhance pro-inflammatory cytokine production, inflammatory cell recruitment

and antimicrobial activity of phagocytes (Cheng *et al.*, 2012). In fact, mice lacking the C5a precursor molecule C5 or the C3a/b precursor C3 are highly susceptible to invasive *C. albicans* infection (Mullick *et al.*, 2004; Tsoni *et al.*, 2009). However, activated complement is unable to kill *C. albicans* hyphae during infection, probably due to the thick fungal cell wall that may block the formation of the membrane attack complex (C5-C9) and the direct lysis of *C. albicans* (Kozel, 1996; Speth *et al.*, 2008).

Mouse models of infection have shown that fungal cells in the bloodstream bind and activate platelets, which in turn produce immune mediators, such as the CXC chemokine ligand 4 (CXCL4 or PF4) and the CC chemokine ligand 5 (CCL5 or RANTES), which have antifungal activity; also platelet-enriched plasma causes inhibition of *C. albicans* growth. However, the possible role of platelets in antifungal host defense is far from being elucidated (Drago *et al.*, 2013; Robert *et al.*, 2000).

1.2.1.2 Myeloid phagocytes: neutrophils and monocytes/macrophages

Host defense against systemic candidiasis depends mainly on the ingestion and killing of opsonized and non-opsonized fungal cells by myeloid phagocytes (neutrophils, monocytes and macrophages). In addition to the effector responses, secretory responses of phagocytes to *C. albicans* are also critical for the development of a protective host response. Phagocytes use a variety of surface receptors for recognizing opsonins or pathogen-associated molecular patterns (PAMPs) on the fungal surface (see section 1.2.2).

Neutrophils

During candidaemia, neutrophils have a crucial role in protection against the pathogen, as they constitute the most potent immune cell population in killing *C. albicans* and the only immune cells able to inhibit hyphae development from yeast cells. In fact, neutropenia is a major risk factor for disseminated candidiasis in humans, and mouse models of neutropenia have shown an increased susceptibility to disseminated candidiasis (Brown, 2011; Lionakis, 2014; Miramón *et al.*, 2013). In mice, these phagocytes are phenotypically identified by the presence of two surface markers: CD11b (a general myeloid lineage marker) and Ly6G (a specific neutrophil marker).

C. albicans killing by phagocytes involves intra- and extracellular, as well as oxidative and non-oxidative mechanisms. Intracellular mechanisms require the internalization of fungal cells into the phagosome. In neutrophils, this nascent phagosome fuse with cytoplasmic granules that contain antimicrobial proteins (defensins, lactoferrin, elastase,

gelatinase, lysozyme or myeloperoxidase, amongst others) in order to kill and remove the engulfed pathogen (Brown, 2011; Miramón *et al*., 2013). Moreover, upon phagocytosis, a characteristic production of copious amounts of oxidants does occur. This oxidative burst involves formation, via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and myeloperoxidase, of reactive oxygen species (ROS: superoxide radical, hydrogen peroxide, hypochlorous acid) with a strong oxidative and damaging properties (Fang, 2004; Segal *et al*., 2012). These oxidative mechanisms are critical for killing fungal cells after phagocytosis (Aratani *et al.*, 2002). Besides, following engulfment of the pathogen, phagocytes also express nitric oxide-generating enzymes, as inducible nitric oxide synthase (iNOS) involved in production of reactive nitrogen species (RNS: nitric oxide an peroxynitrite, which is decomposed in nitrogen dioxide and hydroxyl radical), which also contribute to the killing of *C. albicans* phagocytized cells. Although NO^- production has been studied most extensively in macrophages, iNOS is expressed in both polymorphonuclear and mononuclear phagocytes. However, the amount of ROS produced is greater in neutrophils than in macrophages, and macrophages generally produce considerably more RNS levels than neutrophils (Fang, 2004).

Based on studies with neutrophils from patients with defined genetic defects, it has been shown that human neutrophils have two independent pathways for *C. albicans* killing: (i) a ROS-dependent mechanism, required for clearance of opsonized *C. albicans* cells, that depends on the FcγR pathway, and (ii) a ROS-independent pathway involved in the killing of non-opsonized fungal cells, that depends on CR3 and the signaling proteins phosphoinositide 3-kinase and caspase recruitment domain-containing protein 9 (CARD9) (Gazendam *et al*., 2014) (**Figure 2, p. 21**).

For extracellular elimination of pathogens, neutrophils can release the content of cytoplasmic granules to the extracellular environment in a process called degranulation (Murphy and Weaver, 2017). Besides, the neutrophilic NADPH oxidase complex is also assembled on the cytoplasmic membrane to secret superoxide radical into the extracellular space (Segal *et al.*, 2012). In addition to those mechanisms, neutrophils use a phagocytosis-independent mechanism to deal with the pathogen: the release of chromatin containing antimicrobial proteins, known as neutrophil extracellular traps (NETs) (Papayannopoulos and Zychlinsky, 2009). This DNA-containing fibril structures bind to and neutralize extracellular *C. albicans* hyphae, providing a mechanism to deal with this fungal morphotype that is too big to be efficiently phagocytosed (Urban *et al.*, 2006).

In mouse models of systemic candidiasis, a rapid clearance of the fungus from the bloodstream within the first hours of injection does occur, but fungus also quickly

disseminate to peripheral organs, such as spleen, liver, or kidney. In the early stage of infection, the timing of neutrophil recruitment to the site of infection results crucial for an optimal host defense. Thus, the liver and spleen are able to recruit significant numbers of neutrophils within the first critical 24 h, period post-infection to successfully control fungal proliferation and prevent *Candida* filamentation. Instead, the lack of efficient signals for rapid neutrophil recruitment in the kidney is associated with the inability of the organ to control fungal overgrowth and pseudohyphal formation (Lionakis *et al.*, 2011). Neutrophils migrate toward the site of infection by detecting gradients of chemotactic factors that guide direct cell movement toward infected tissues where those factors are present in higher concentrations. There are a multitude of chemokines that can direct neutrophil recruitment, including ligands of the two main chemokine receptors expressed on mice neutrophils, CC chemokine receptor type 1 (CCR1) and CXC chemokine receptor type 2 (CXCR2) (Murphy and Weaver, 2017). It has been shown that early during fungal infection, neutrophils are recruited via CXCR2 detecting chemokines such as CXC chemokine ligand type 1 (CXCL1) and CXC chemokine ligand type 2 (CXCL2) (Kanayama *et al*., 2015). However, CCR1 is necessary for neutrophil trafficking to the kidney at later times after infection, but this late neutrophil recruitment was shown to correlate with pathological consequences and contribute to mortality. Thus, it should be noted that despite their essential antifungal properties, excessive neutrophil recruitment and/or activation can also exert adverse effects linked to potent pro-inflammatory activity of neutrophils (Lionakis *et al.*, 2012) (**Figure 2, p. 21**).

Monocytes and macrophages

Macrophages are sentinels found in virtually all tissues of adult mammals that maintain tissue homeostasis by eliminating/repairing damaged cells and tissues. Furthermore, they orchestrate innate immunity by phagocytosing microorganisms and coordinating inflammatory responses (Murphy and Weaver, 2017). Mouse macrophages are phenotypically identified by the presence of the myeloid surface marker CD11b and the specific macrophage marker F4/80. Classically, it was thought that macrophages were entirely generated by bone marrow (BM) hematopoiesis, giving rise to blood monocytes that become macrophages in tissues. However, new evidence showing that some macrophages have self-renewal capacity and can be derived from early hematopoiesis in the yolk sac points out some questions about definitions, functions and relationship between macrophages and monocytes. Monocyte contribution to resident macrophages is highly tissue-dependent and varies from no contribution for brain microglia to complete

monocyte origin for intestinal macrophages. Other locations as peritoneum, kidney or spleen contain macrophages derived from both sources (Italiani and Boraschi, 2014).

Resident macrophages are able to internalize and kill *C. albicans* cells, as well as produce inflammatory mediators that recruit and activate other immune cells [monocytes, neutrophils or Natural killer (NK) cells] at the site of infection (Brown, 2011; Lionakis, 2014; Miramón *et al.*, 2013). These observations, together with early *in vivo* studies in mice showing that clodronate-induced depletion of mononuclear phagocytes results in accelerated tissue fungal proliferation and increased mortality, remark the important role of these cells in antifungal host defense (Bistoni *et al.*, 1986; Bistoni *et al.*, 1988). Monocytes and macrophages appear to be relevant for anti-*Candida* defense in dissemination sites. It has been shown that early during infection, inflammatory Ly6C^{high} monocytes are rapidly recruited to peripheral organs (Lionakis *et al.*, 2011). This migration is dependent on CC chemokine receptor type 2 (CCR2) and results crucial for fungal clearance in the kidney and subsequent mice survival (Ngo *et al.*, 2014). Interestingly, Domínguez-Andrés *et al*. (2017) proposed an NK-mediated mechanism based on cytokine production through which spleen inflammatory monocytes will boost the candidacidal potential of kidney neutrophils (see next section, 1.2.1.3). In addition, Lionakis *et al*. (2013) demonstrated that resident kidney monocytes and macrophages, characterized as CX_3C chemokine receptor 1 (CX_3CR1) positive cells, promote *Candida* control and host survival due in part to their location at the site of infection that facilitates a very early contact with fungal cells (**Figure 2, p. 21**).

The killing mechanisms that macrophages use to eliminate fungal cells are very similar to those described for neutrophils. However, as macrophage do not present cytoplasmic granulocytes, phagosomes formed after microbial engulfment follow the endocytic maturation pathway to acquire lytic activity by phagolysosome formation. Moreover, they possess a characteristic acidic pH that promotes activity of hydrolytic enzymes such as cathepsin D (Vieira *et al.*, 2002). Similarly to neutrophils, it has been shown that murine macrophages are also able to form extracellular traps in response to *C. albicans*, although their function could be retaining the invading microbes at the site of infection rather than significantly killing them (Liu *et al.*, 2014).

Besides the candidacidal activity, mononuclear phagocytes release several key mediators which orchestrate the inflammatory response to infection. Recognition of fungal cells by a variety of surface receptors, mainly Dectin-1 and TLR2, triggers a signaling cascade that induces the expression of inflammatory genes mediated by transcription factors such as nuclear factor kappa B (NF-κB) or AP-1 (extended explanation on section 1.2.2). The most relevant cytokines produced by macrophages in response to *C. albicans* are

TNF-α, IL-6, IL-1β and IL-18 (Brown, 2011; Lionakis, 2014; Miramón *et al.*, 2013). TNF-α is essential for controlling systemic *Candida* infections, as TNF-α knockout (KO) mice or wild type (WT) mice treated with specific neutralizing antibodies against TNF-α become more susceptible to infection (Marino *et al.*, 1997; Steinshamn and Waage, 1992). This cytokine regulates phagocyte recruitment by inducing the expression of adhesion molecules in endothelial cells and activates oxidative burst to promote microbial killing (Murphy and Weaver, 2017). Furthermore, it has been shown that the use of TNF-α antagonists for the treatment of rheumatoid arthritis and other autoimmune diseases is associated with an increased incidence of candidiasis among patients (Filler *et al.*, 2005). Similarly, it has been described that IL-6 and IL-1β KO mice are more susceptible to disseminated candidiasis, due to an impaired recruitment of neutrophils (van Enckevort *et al.*, 1999; Vonk *et al.*, 2006). On the other hand, prophylactic treatment of *C. albicans*-infected mice with recombinant murine IL-18 decreased mortality and outgrowth of yeasts in the kidneys by increasing the levels of type II interferon (IFN), (known as IFN-γ), therefore promoting an adaptive response against the pathogen (see section 1.3) (Stuyt *et al.*, 2004; Stuyt *et al.*, 2002). Early during infection, macrophages are also able to produce chemokines, such as CXCL1 and CXCL2, which induce neutrophil recruitment to the site of infection (Kanayama *et al.*, 2015) (**Figure 2, p. 21**). The signaling pathways leading to production of cytokines and chemokines will be deeply explained in section 1.2.2.

1.2.1.3 NK cells

Innate lymphoid cells are emerging as important effectors of innate immunity. As neutrophils or inflammatory monocytes, NK cells (considered a prototypical innate lymphoid cell population) are rapidly recruited at the site of infection. There, pro-inflammatory cytokines and chemokines generated in response to the pathogen bind to their receptors on NK cells, leading to their activation and to a rapid production of IFN-γ. NK cells can also secrete IFN-γ after directly recognizing pathogen components through TLRs, although whether NK cells are stimulated directly by *C. albicans* cells remains unclear. In this context, it has been described that inactivated *C. albicans* cells inhibit activation of murine purified NK cells *in vitro*, and therefore, this may be considered as a mechanism of fungal immune evasion or to avoid hyperinflammation in immunocompetent host (Murciano *et al.*, 2006). In accordance with these results, depletion of NK cells in immunocompetent mice was found to be protective against *C. albicans* infection due to the attenuation of systemic inflammation. In contrast, the absence of NK cells in T/B cell-deficient mice led to an increased susceptibility to systemic candidiasis demonstrating

that NK cells are an essential and non-redundant component of anti-*C. albicans* host defense in immunosuppressed hosts (Quintin *et al.*, 2014).

In contrast to murine NK cells, human NK cells are activated following detection of yeasts, leading to degranulation, release of GM-CSF, TNF-α and IFN-γ, and fungal damage. This direct antifungal activity of NK cells has been mainly attributed to secreted perforin (Voigt *et al.*, 2014). Moreover, using a mouse model of systemic candidiasis, it has been shown that GM-CSF production by activated NK cells in the spleen is also required to boost the *C. albicans* killing capacity of neutrophils and therefore to control the infection. Mechanistically, Dectin-1-mediated recognition of *C. albicans* by recruited inflammatory monocytes to the spleen induces the type I IFN-dependent production of IL-15, which plays a pivotal role in the activation and GM-CSF release by splenic NK cells (Domínguez-Andrés *et al.*, 2017). IL-23 secreted by dendritic cells (DCs) is also essential to induce GM-CSF release by NK cells. In response to *C. albicans*, a signaling pathway mediated by the spleen tyrosine kinase (Syk) is activated in DCs to produce IL-23, which in turn acts on NK cells (Whitney *et al.*, 2014).

The scheme shown in **Figure 2** summarizes the effector mechanisms of myeloid phagocytes neutrophils and macrophages) to control *C. albicans* invasion in infected tissue, which have been described in this section; the role of DCs and NK cells in promoting neutrophil activation is also depicted in Figure 2

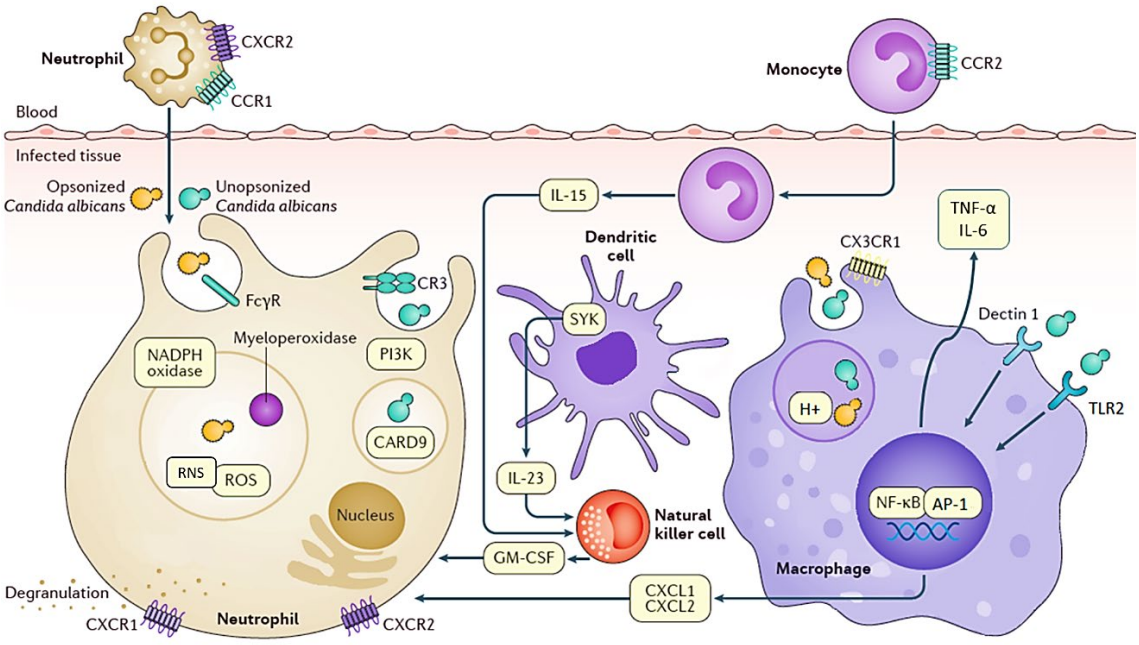


FIGURE 2 | Effector mechanisms of myeloid phagocytes to control *C. albicans* invasion in infected tissues. Adapted from Pappas *et al.*, (2018).

1.2.2 PRRs involved in *C. albicans* detection

Candida sensing is based on a complex set of interactions involving a variety of host receptors, mainly pattern recognition receptors (PRRs), which recognize fungal ligands considered microbe-specific molecular signatures (pathogen associated molecular patterns or PAMPs) (Miramón *et al.*, 2013; Netea *et al.*, 2008; Poulain and Jouault, 2004). Main *C. albicans* PAMPs are cell wall components. Due to the localization of mannoproteins in the outermost part of this cell structure, mannan detection would be expected to be one of the first steps in the recognition of fungal cells by the host. Nevertheless, the presence of β-glucans and chitin, in particular at the level of the bud scar, is also likely to influence *C. albicans* sensing (Gantner *et al.*, 2005). β-glucans can directly stimulate leukocytes *in vitro*, a similar situation that may occur *in vivo*, as it has been described that β-glucans are released into the circulation during systemic fungal infections (Obayashi *et al.*, 1995). Intracellular fungal components, such as nucleic acids (DNA, RNA) that are secreted following phagocytosis and killing of fungal cells, are also ligands for some intracellular PRRs. It should be noted that expression and cell surface exposition of some fungal PAMPs may differ between the yeast and hyphal forms of *C. albicans*, a phenomenon that determines significant differences in the immune responses triggered by both fungal morphotypes (Arana *et al.*, 2009; Poulain, 2015; Poulain and Jouault, 2004).

The most relevant families of PRRs involved in *C. albicans* sensing are 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) (Netea *et al.*, 2008). Following recognition of their respective ligands, these receptors activate different signal pathways leading to initiate the innate immune responses against the pathogen. These signaling pathways are well known for TLRs and CLRs, whereas are still poorly understood for NLRs and RLRs (Hardison and Brown, 2012; Kawasaki and Kawai, 2014; Kumar *et al.*, 2011; Osorio and Reis e Sousa, 2011).

1.2.2.1 Toll-like receptors (TLRs)

TLRs are type I membrane proteins characterized by an ectodomain containing leucine-rich repeats that is responsible for recognition of PAMPs, and a cytoplasmic domain, homologous to the cytoplasmic region of the IL-1 receptor (TIR-domain), which is required for downstream signaling. These receptors constitute a family of PRRs expressed by most immune cell types (innate cells such as neutrophils, monocytes, macrophages, DCs, as well as B-/T-lymphocytes and NK cells), and non-immune cells, such as epithelial and endothelial

cells. Based on their subcellular localization, TLRs can be classified into two groups: those located at the plasma membrane (TLR1, TLR2, TLR4, TLR5 and TLR6) or those located in endocytic compartments inside the cells (TLR3, TLR7, TLR8 and TLR9) (Kawasaki and Kawai, 2014; Kumar *et al*., 2011) (**Figure 3, p.27**).

Among TLRs, the plasma membrane-bound TLR2 (which forms homodimers and heterodimers with TLR1 and TLR6) and TLR4 recognize mannan moieties associated to the fungal cell wall; β-1,2 mannosides present in PLM and mannoproteins constitute the major ligand for TLR2, whereas *O*-linked mannosyl residues, which are accessible on the yeast surface, are the ligand for TLR4. On the other hand, endosomal TLR9, TLR7 and TLR3 sense microbial nucleic acids (DNA and RNA) released following endocytosis and pathogen degradation. TLR9 recognizes DNA containing unmethylated CpG motifs (the high rate of methylation and low frequency of CpG motifs in mammalian DNA avoids its recognition by TLR9), whereas TLR7 and probably TLR3 recognize fungal RNA. Restriction of some TLRs to endosomal membrane is also critical for discriminating between self and non-self nucleic acids (Fradin *et al.*, 2015; Kawasaki and Kawai, 2014; Yáñez *et al.*, 2019).

Upon ligand recognition, TLRs activate intracellular signaling pathways leading to the induction of inflammatory cytokine genes, as *TNF-α*, *IL-1β*, *IL-6* and *IL-12.* Signal transduction starts with the recruitment of a set of intracellular TIR-domain-containing protein adaptors that interact with the cytoplasmic TIR domain of the TLRs. Myeloid differentiation factor 88 (MyD88) is the universal adaptor molecule, shared by all TLRs, except TLR3, that triggers inflammatory pathways through activation of the transcription factors NF-κB and AP-1, which in turn induce the expression of inflammatory cytokines. TRIF (TIR-domain-containing adapter-inducing IFN-β) is crucial for the induction of type I IFN genes and type I IFN-inducible genes by TLR3 and TLR4, through the activation of the transcription factor IFN-regulatory factor 3 (IRF3), whereas the transcription factor IRF7 induces type I IFN genes and type I IFN-inducible genes through TLR7 and TLR9 signaling (Kawasaki and Kawai, 2014). TLR-mediated signaling is essential for host protection against candidiasis: MyD88 $^{-/-}$ mice are extremely susceptible to *C. albicans* infections; deficiencies in TLRs, mainly TLR2, and others in a minor extent (TLR4, TLR2 coreceptors, TLR3, TLR7 and TLR9) also cause impaired immune responses to *C. albicans* infection, both in mouse and/or human, although the role of some receptors (as TLR9) may be redundant (Netea *et al.*, 2008; Villamón *et al*., 2004a; Villamón, *et al.*, 2004b; Yáñez *et al.*, 2019). Recognition of fungal RNA by TLR7 has a non-redundant role in host defense against candidiasis, as it is partially required for IL-12 production by an IRF1-dependent pathway (Biondo *et al.*, 2012).

1.2.2.2 C-type lectin receptors (CLRs)

CLRs are part of a heterogeneous superfamily of soluble and transmembrane proteins defined by a characteristic protein region with carbohydrate binding properties (C-type lectin domain). Thus, CLRs play critical roles in *C. albicans* sensing by innate immune cells, as they recognize specific carbohydrate domains of the fungal cell surface (Hardison and Brown, 2012; Netea, *et al.*, 2015a; Poulain and Jouault, 2004) (**Figure 3, p. 27**). These receptors belong to the family of non-catalytic tyrosine-phosphorylated receptors that share a similar signaling pathway involving phosphorylation of tyrosine residues. CLRs have an activating motif known as immunoreceptor tyrosine-based activating motif (ITAM) in their intracellular tail, consisting of YXXL tandem repeats, or otherwise they can interact with ITAM-containing adaptor proteins. When phosphate groups are added to the tyrosine(Y) residue of the ITAM by membrane-anchored tyrosin kinases, mainly Syk, a signaling cascade is generated within the cell that leads to the production of pro-inflammatory cytokines and chemokines or mediates phagocytosis (Hardison and Brown, 2012; Kumar *et al.*, 2011; Osorio and Reis e Sousa, 2011).

Major ligands for CLRs are β-glucans and mannans. The receptors that participate in the recognition of β-glucans are mainly Dectin-1 and CR3. Both are also the major CLRs involved in phagocytosis of non-opsonized fungal cells. Dectin-1 is a transmembrane receptor expressed by myeloid phagocytes (macrophages, monocytes, DCs and neutrophils), whose extracellular portion specifically recognizes β-(1,3)-glucan (Brown and Gordon, 2001; Brown *et al.*, 2002). The intracellular portion contains a hemi-ITAM involved in signaling that, in contrast to the ITAM motif, consists of a single tyrosine within an YXXL motif. On the other hand, CR3 belongs to the family of β_2 (CD18) integrins and forms a heterodimeric complex containing one β_2 chain (CD18) and one αM chain (CD11b). This receptor is unique among integrins as, in addition to the conventional binding domain for C3b, it also contains a polysaccharide binding lectin-like domain to which β-(1,3)-glucan can bind (Thornton *et al*., 1996). Their role in *C. albicans* detection has been mainly associated to human neutrophils (van Bruggen *et al.*, 2009).

Several membrane receptors have been described to directly recognize mannans (Hardison and Brown, 2012; Netea, *et al*., 2015a; Poulain and Jouault, 2004; Osorio and Reis e Sousa, 2011). Dectin-2, mainly expressed by macrophages, neutrophils and DCs, is the functional receptor for *N*-linked α-mannan residues on the yeast and hyphal cell wall, and forms heterodimers with Dectin-3, a CLR that recognizes α–mannans on the surface of *C. albicans* hyphae. Consequently, the simultaneous and differential recognition of mannans

from yeasts and hyphae by Dectin-2 and Dectin-3, also with the different accessibility of β-glucans to Dectin-1 (previously explained in section 1.1), may account for differences in the immune responses triggered by yeast and hyphal forms of *C. albicans*. Highly branched *N*-linked mannosyl chains are also recognized by the mannose receptor (MR) expressed on macrophages; MR also participates in recognition of fungal chitin. DC-SIGN (dendritic cell-specific-ICAM-grabbing non-integrin) is a CLR present on myeloid cells, including DCs, which also recognizes *N*-linked mannan. Although in a minor extent than β-glucan receptors, DC-SIGN, MR and Dectin-2 are also involved in the phagocytosis of non-opsonized fungal cells. Mincle (macrophage inducible Ca^{2+}-dependent lectin) is another member of the CLR family, expressed on monocytes/macrophages and neutrophils, which is involved in *C. albicans* recognition, although its ligand has been not well characterized yet. Galectin-3 is an S-type lectin receptor that recognizes β-mannan domains present in both PLM and mannoproteins. Mannan-binding lectin (MBL) is a soluble serum CLR that binds mannan moieties on fungal surfaces. MBL binding on fungal surface initiates the lectin pathway of complement activation, promoting phagocytosis of *C. albicans* by neutrophils. Furthermore, MBL may act as an opsonin directly recognized by complement receptor 1 (CR1) on the surface of neutrophils (Brouwer *et al.*, 2008; Li *et al.*, 2012).

Dectin-1 induces intracellular signals leading to (i) secretion of inflammatory cytokines through NF-κB activation (through Syk1/CARD9- or Raf-1-mediated signaling pathways), (ii) Syk1-mediated activation of the NLRP3 inflammasome that generates bioactive IL-1β and IL-18 following caspase activation, and (iii) phagocytosis and production of ROS (Hardison and Brown, 2012). It has been shown that Dectin-1 signaling is only activated by particulate β-glucans, clustering the receptor in a synapse-like structure (phagocytic synapse) which represents a mechanism to distinguish direct microbial contact from detection of soluble ligands (Goodridge *et al.*, 2011). In order to avoid the strong immune response induced by Dectin-1 signaling, *C. albicans* hyphae mask glucan exposure by mannan/mannoproteins (Gantner *et al.*, 2005; Lowman *et al.*, 2014). However, it has been demonstrated that NETs cause unmasking of the *C. albicans* hyphal β-glucans and trigger changes in the fungal cell wall architecture that promote immune recognition by Dectin–1 and probably by other host receptors. This remodeling of the cell wall architecture enhances host responses and points out the concept that pattern recognition during infection is a dynamic process that depends on the host-pathogen cross-talk (Hopke *et al.*, 2016). *In vivo* studies with Dectin-1 $^{-/-}$ mice showed contradictory results about their susceptibility to systemic candidiasis (probably due to strain-specific differences in glucan exposure), whereas human Dectin-1 deficient patients showed clinically mucocutaneous infections but not invasive fungal infections (Drummond and Brown, 2011; Ferwerda *et al.*,

2009). In fact, human neutrophils are capable of phagocytizing and killing *C. albicans* through a Dectin-1-independent pathway (Gazendam *et al*., 2014). Interestingly, CARD9 deficiency is associated with susceptibility to invasive candidiasis, both in mouse and humans, probably because CARD9 mediates signal transduction pathways downstream of CLRs other than Dectin-1 (Drewniak *et al.*, 2013; Gross *et al.*, 2006).

Dectin-2 $^{-/-}$ mice were more susceptible to systemic candidiasis, and phagocytosis of *Candida* cells by macrophages lacking Dectin-2 was moderately decreased (Ifrim *et al.*, 2016). Mincle, which associates with FcγR and signals through Syk/CARD9, appears to have a protective role during candidiasis due to cytokine production, but is not involved in phagocytosis (Wells *et al.*, 2008). MR mediates several antifungal activities such as phagocytosis of yeast by DCs, although its role in protection against candidiasis appears to be redundant (Hardison and Brown, 2012). Interestingly, chitin sensing by MR dampens inflammatory responses through the induction of anti-inflammatory IL-10 production, thus indicating that chitin recognition plays a critical role for immune homeostasis (Wagener *et al*., 2014). DC-SIGN recognition of *C. albicans* leads to *in vitro* release of cytokines and activation of the respiratory burst, probably in collaboration with Dectin-1, although its role *in vivo* during infection has not been reported (Takahara *et al.*, 2011).

1.2.2.3 Cytosolic PRRs: NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs)

In addition to detect PAMPs on the cell surface or in the lumen of endosomes or lysosomes, there is a cytosolic detection system to sense infection, which includes NLRs and RLRs. NLRs detect fungal ligands released from phagolysosomes and/or damage associated molecular patterns (DAMPs), such as heat-shock proteins (Hsps), generated by host cellular damage during infection (Kumar *et al.*, 2011). In particular, it has been described that NOD2 is involved in the immune responses to chitin-derived components (Wagener *et al*., 2014). Besides, certain NLRs function as components of the inflammasome and some of them are known to be involved in the response to *C. albicans* (NLRP3, NLRC4, NLRP10) (Hise *et al.*, 2009; Joly and Sutterwala, 2010; Tomalka *et al*., 2011) (**Figure 3, p.27**).

Inflammasomes are protein complexes composed of NLRs and non-NLR proteins. These protein complexes associate with an inactive form of caspase-1 (procaspase-1) and promote its proteolytic activation to yield caspase-1, which, in turn, promotes proteolysis of the zymogen form of the IL-1 family cytokines, as IL-18 and IL-1β. This production of mature IL-18 and IL-1β can be also mediated by a non-canonical activation process that is dependent on caspase 8 and caspase 11. Thus, the production of pro-inflammatory IL-1β differs from that of other pro-inflammatory cytokines as it involves two steps: (i)

transcriptional induction of pro-IL-1β, as an inactive precursor, downstream PRRs, and (ii) proteolytic cleavage by caspase-1 to release bioactive IL-1β (Kumar et al., 2011; Murphy and Weaver, 2017). Activation of NLRP3 by *Candida* hyphae or SAPs triggers the assembly of the inflammasome, with the subsequent caspase activation and pro-IL-1β cleavage. Thus, *C. albicans* transition from yeast to hyphae has been shown to be necessary for NLRP3 inflammasome activation (Joly *et al.*, 2009). When pro-IL-1β is released in the inflammatory environment where neutrophils are present, it can be also cleaved by neutrophil-derived serine proteases, such as proteinase 3. *C. albicans* itself can also contribute to produce bioactive IL-1β as fungal-derived proteases can generate host-derived active IL-1β *in vitro*, and probably during infection, leading to activation of the immune system. Therefore, at the site of infection, mature IL-1β can be also produced in an inflammasome-independent manner (Netea *et al*., 2015b).

MDA5, a member of the RLRs important for viral recognition, has been also described to play a role in immune responses to *C. albicans*, although the fungal ligand responsible for MDA5 activation has been not yet characterized (Jaeger *et al.*, 2015) (**Figure 3, p.27**).

1.2.2.4 Colaboration between different PRRs in *C. albicans* recognition

C. albicans cells express various PAMPs and their expression may change among strains and morphotypes. Therefore, recognition of *C. albicans* by immune cells is a complex process that may involve the simultaneous or sequential activation of different PRRs. Consequently, collaboration among receptors in fungal recognition and crosstalk between intracellular signaling pathways may lead to the final tailored immune responses generated (Lionakis, 2014; Netea *et al.*, 2006; Yáñez *et al.*, 2019) (**Figure 3**).

As an example, some lectin receptors such as Dectin-1, Galectin-3 and SIGNR1 (a murine C-type lectin homologue of the human DC-SIGN) have been identified as TLR2 coreceptors to design a collaborative recognition or to modulate ligand specificity (Ferwerda *et al.*, 2008; Gantner *et al.*, 2003). Similarly, Dectin-1 and SIGNR1 may also collaborate with TLR4 in fungal recognition, and Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human macrophages (Ferwerda *et al*., 2008). In some cases, a 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 (Esteban *et al.*, 2011; Jouault *et al*., 2006). In addition, this complex network of *C. albicans* sensing receptors allows immune cells to respond to (i) whole fungal cells through interaction between surface PAMPs and PRRs, (ii) fungal ligands generated following phagocytosis and fungal destruction (such as 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 NLRs.

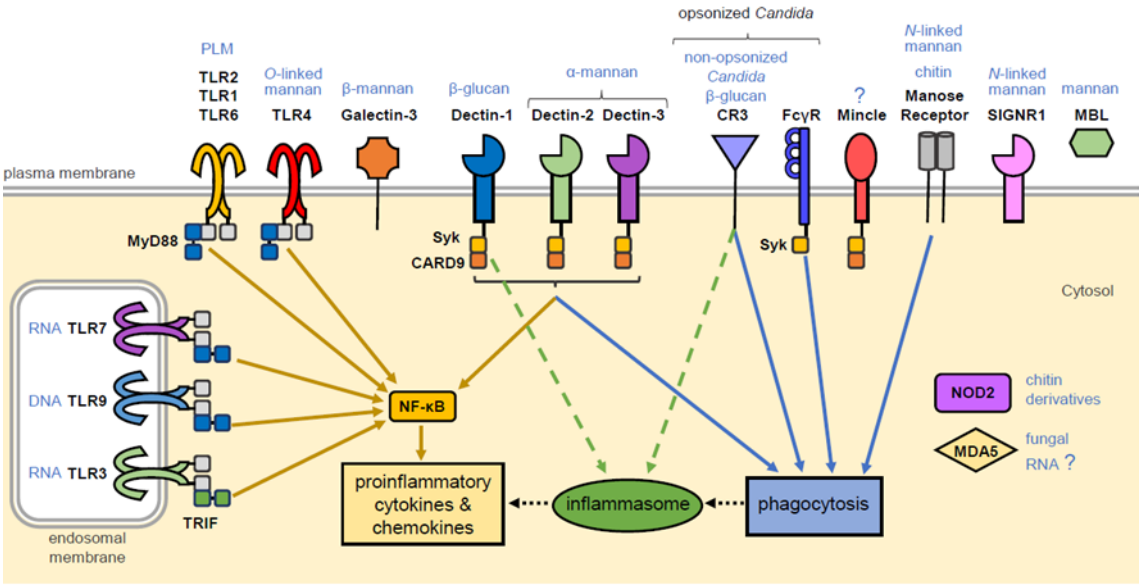


FIGURE 3 | *C. albicans* sensing by innate immune cells. *C. albicans* yeasts and/or hyphae are sensed by a variety of pattern recognition receptors (PRRs) that recognize fungal ligands considered microbe-specific molecular signatures (pathogen associated molecular patterns or PAMPs). Extracellular fungal ligands (basically cell wall components) are recognized by a diversity of plasma membrane receptors: Toll-like receptors (TLRs) (mainly TLR2 and TLR4); C-type lectin receptors [Dectin-1, Dectin-2 and Dectin-3, Mannose receptor and others: Mincle (macrophage inducible Ca^{2+}-dependent lectin) and the murine C-type lectin homologue of the human SIGNR1 (dendritic cell-specific-ICAM-grabbing non-integrin)], Galectin-3 and complement receptor 3 (CR3). Intracellular fungal ligands (DNA, RNA), released upon phagocytosis and killing, involves recognition by TLRs located at the endosomal membrane. Other cytosolic receptors (NOD2, MDA5) also contribute to detection of fungal-derived ligands and serum Mannan-binding lectin (MBL) contributes to *C. albicans* detection by binding mannan moieties and promoting opsonization. Receptors for complement fragments and particularly FcγR are also involved in the recognition of *C. albicans* opsonized cells. The simultaneous recognition of various PAMPs triggers complex signal transduction pathways leading to activation of immune responses. Signaling though TLR/MyD88 and Dectin-1/Syk/CARD9 converge in the transcription factor NF-κB that activates expression of pro-inflammatory cytokines, whereas signaling through Dectins, CR3, Mannose receptor and FcγR induces phagocytosis of fungal cells. The inflammatory response is also generated by inflammasomes that induce caspase-1-mediated activation of pro-IL-1β, a key cytokine in immunity against *C. albicans*. CARD9, caspase recruitment domain-containing protein 9; IL-1β, interleukin-1β, MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa B; Syk, spleen tyrosine kinase; TRIF, TIR-domain-containing adapter-inducing IFN-β (Yáñez *et al.*, 2019).

1.3 Adaptive immune responses to *C. albicans*

In most cases, activation of innate responses by epithelial cells, phagocytes (macrophages and neutrophils) and NK cells is sufficient to restrict fungal tissue invasion from the colonized surface, therefore preventing disseminated infection. In other cases, innate immune mechanisms fail to control fungal infection and activation of adaptive immune responses are required to deal with the pathogen. The adaptive immune system is

composed of B and T lymphocytes expressing highly specific antigenic receptors. Each lymphocyte carries cell-surface receptors of a single specificity, generated by the random somatic recombination of VDJ elements and the expression of *RAG1* and *RAG2* genes, a feature unique to these cells. This recombination process produces a vast diversity of lymphocytes, each bearing a distinct receptor, so that the total repertoire of receptors can recognize virtually any antigen. When a recirculating naïve lymphocyte encounters its specific foreign antigen in peripheral lymphoid tissues, it is induced to proliferate, and its progeny then differentiate into effector cells that can eliminate the infectious agent. A subset of these proliferating lymphocytes differentiates into memory cells, ready to respond rapidly to the same pathogen if it is encountered again (see section 3) (Murphy and Weaver, 2017).

T lymphocytes (T cells) constitute an integral component of the host adaptive immunity in response to *C. albicans* infections that provide both direct and indirect mechanisms to control fungal proliferation. Activation of both $CD8^+$ (cytotoxic T lymphocytes, CTL) and $CD4^+$ (T helper cells, Th) T cells is controlled by DC populations. Tissue resident DCs can detect and phagocytose *C. albicans*. Following exposure to pathogens and/or inflammatory mediators, DCs are transformed into mature DCs that migrate efficiently from peripheral tissues into draining lymph nodes. At this location, DCs activate antigen-specific naïve T cells, ultimately leading to both T cell expansion and differentiation of effector cells. Despite CTLs have a role in protection against candidiasis, the major mechanism of adaptive immunity to *C. albicans* is the development of Th cell responses (Lee and Iwasaki, 2007; Richardson and Moyes, 2015). The elevated prevalence of oropharyngeal candidiasis in AIDS/HIV^+ patients where $CD4^+$ T cells are depleted clearly shows the paramount importance of Th cell responses (Fidel, 2011).

1.3.1 Dendritic cells

Although other immune cells are capable of antigen presentation (including macrophages and B cells), DCs are the main antigen presenting cells that have the ability to translate the early danger signals received by the innate immune system after microbial invasion to the adaptive arm of the immune system (Lee and Iwasaki, 2007; Murphy and Weaver, 2017). In general terms, DCs can be identified by their expression of CD11c and histocompatibility complex (MHC) class II molecules (MHCII). DC activation is a complex process that can be even more intricate considering the various DC populations, which differ in localization, function and phenotype. The three main populations of DCs that have been described are: classical DCs (cDCs), plasmocytoid DCs (pDCs) and monocyte-derived DCs

(moDCs) (Hochrein and O'Keeffe, 2008). Morphologically, cDCs exhibit typical plasmatic extensions called dendrites and their main function is to present antigens to T cells, whereas pDCs do not exhibit dendrites and their main purpose is to produce high levels of IFN-α in response to viral infections, before becoming mature DCs that activate specific T cells for viral antigens. Moreover, pDCs are constantly located in lymphoid organs while cDCs reside in peripheral tissues and transport antigens to lymphoid organs (Hochrein and O'Keeffe., 2008; Lande and Gilliet., 2010). On the other hand, moDCs are produced from blood monocytes during inflammatory responses against pathogens to replace tissue resident DCs after the inflammation (Domínguez and Ardavín, 2010).

DCs are able to phagocytose and kill fungal cells (although less efficiently than macrophages), but their major role is the activation of Th cell responses through the processing and presentation of fungal antigens to naive $CD4^+$ T cells, which can develop to four different subsets [Th1, Th2, Th17 and T regulatory (Treg) cells] (Richardson and Moyes, 2015). In general terms, in order to effectively initiate an adaptive immune response, DCs must deliver three key signals to naïve T cells: (i) presentation of antigen via MHC molecules to the TCR, (ii) upregulation of costimulatory signals such as CD40, CD80 and CD86, and (iii) production of cytokines that regulate T cell polarization, such as IL-6 or IL-12. Development of each specific Th cell subset depends on the cytokines and the microenvironment present during $CD4^+$ T cell priming by DCs at lymph nodes (Lee and Iwasaki, 2007). Cytokine milieu drives differentiation to one specific Th subset while inhibits development of the others, polarization that is critical for the outcome of the infection (Claudia *et al.*, 2002).

TLR/MyD88 mediated signaling (through TLR2, TLR4, and endosomal TLR9) is involved in mounting a Th1 response. Despite DCs from MyD88-deficient mice are able to phagocytose fungal cells similarly to WT DCs, MyD88 is essential for IL-12 production and the subsequent antifungal Th1 differentiation. However, individual TLRs may contribute differentially to these responses (Bellocchio *et al.*, 2004; Yáñez *et al*., 2019). Furthermore, recognition of *C. albicans* by CLRs, such as Dectin-1, induces production of pro-inflammatory cytokines through Syk/CARD9-dependent and Syk-independent Raf-1 pathway that converge on NF-κB to drive Th1 and, particularly, Th17 polarization (Gringhuis *et al.*, 2009; Richardson and Moyes, 2015; Robinson *et al*., 2009). Initial differentiation toward Th17 phenotype is driven by IL-1β, whereas IL-23 signaling is involved in maturation and terminal differentiation of Th17 cells (Ferwerda *et al.*, 2009). IL-23 and IL-6 are released by DCs following recognition of *C. albicans* mannan, and favor Th17 differentiation (Richardson and Moyes, 2015; Smeekens *et al.*, 2010). IL-1β production involves the canonical NLRP3

inflammasome/caspase-1 and caspase 8, which are activated upon recognition of fungal β-glucan by Dectin-1 and CR3 on DCs, and drive protective Th1 and Th17 cellular responses to disseminated candidiasis (Ganesan *et al.*, 2014; van de Veerdonk *et al.*, 2011). Despite NLRP10 is not involved in innate pro-inflammatory cytokine production, NLRP10-deficient mice showed a profound defect in *Candida*-specific adaptive Th1 and Th17 responses, indicating a role for this inflammasome in the generation of adaptive immune responses to fungal infections (Eisenbarth *et al.*, 2012; Joly *et al*., 2012).

Overall, signals triggered upon recognition of different PAMPs are integrated to define particular Th responses (**Figure 4**). The engagement of distinct receptors leads to disparate downstream signaling events that ultimately determines cytokine production, costimulation and Th responses. Consequently, selective challenge of receptors can be exploited for driving DCs toward a biased protective Th differentiation priming, with important implications in the design of DC-based strategies for developing vaccines (Iannitti *et al.*, 2012, Wang et al, 2015)

Type I IFNs (IFN-α and IFN-β), which are known to inhibit viral replication and mediate protection against viral infection, also play a role in anti-*Candida* host defense. However, their impact on immune responses is controversial as some studies show that type I IFNs are detrimental for fungal clearance, while others reveal that IFN-β favors immune responses to eliminate fungal cells.

Using mouse models, it has been shown that DCs are able to mount a type I IFN response against several *Candida* spp. This response requires phagosomal TLR7-mediated IFN-β signaling, but in *C. glabrata* infections promotes persistence of fungal cells in the host. Moreover, IFN-β production inhibits fungal clearance in mice infected with *C. parapsilosis* (Bourgeois *et al.*, 2011; Patin *et al.*, 2016). Similarly, mice lacking a functional type I IFN receptor showed a remarkable protection against invasive *C. albicans* infections and this detrimental role for type I IFNs is associated with a reduced recruitment and activation of inflammatory monocytes and neutrophils (Mayer *et al.*, 2013; Stifter and Feng, 2015). On the contrary, type I IFNs have been also described to be beneficial for the immune responses to *C. albicans*. Production of IFN-β by DCs, which is largely dependent on Dectin-1 and Dectin-2 signaling (via Syk and IRF5), is crucial for immunity to *C. albicans* by promoting the mobilization of neutrophils to the kidney (Biondo *et al.*, 2011; del Fresno *et al.*, 2013). More recently, 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. The type I IFNs act in an autocrine manner via their receptor (IFNAR) to promote the presentation of exogenous antigen on MHCI molecules, surface expression of the costimulatory molecules CD40 and

CD86, and the release of other cytokines, including IL-12 p70, IL-2, IL-6, and TNF-α (Hassanzadeh-Kiabi *et al*., 2017). Consequently, these results support a protective role of type I IFNs during candidiasis (**Figure 4**).

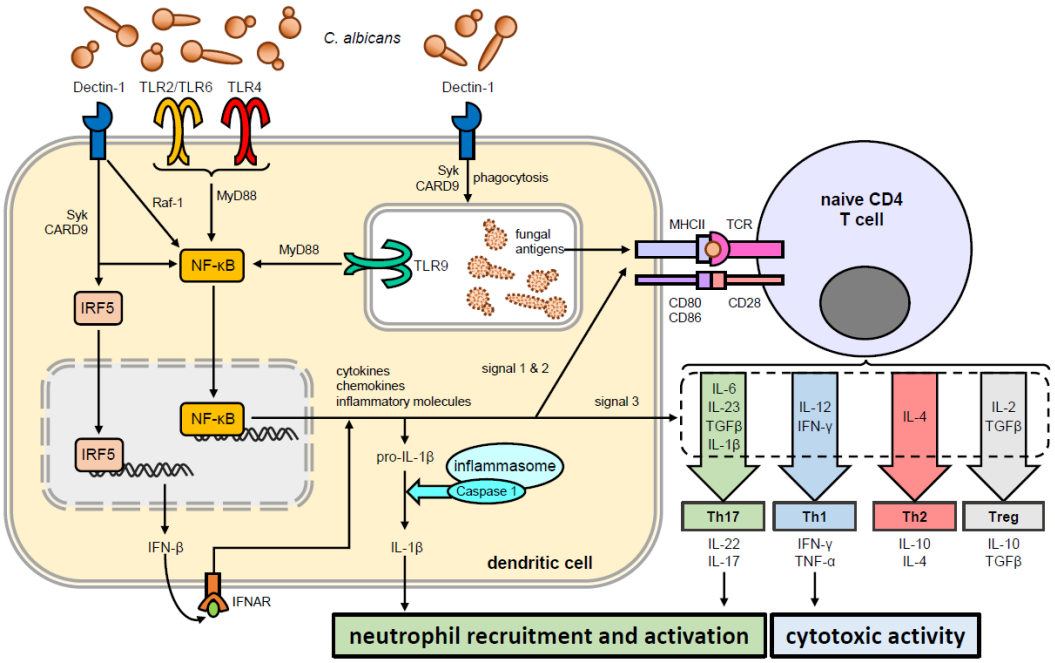


FIGURE 4 | Dendritic cell activation of T helper responses. This figure summarizes the process by which dendritic cells induce T helper (Th) responses through the processing and presentation of fungal antigens to naïve $CD4^+$ T cells, which can develop to four different subsets [Th1, Th2, Th17 or T regulatory (T reg) cells)] depending on the cytokines and the microenvironment. Th1 and Th17 responses are associated to immune protection, promoting cytotoxic activity of $CD8^+$ T cells and neutrophil recruitment and activation, respectively. IFN, interferon; IFNAR, type I IFNs receptor; IRF5, interferon-regulatory factor 5; MHCII, histocompatibility complex class II; TCR, t cell receptor; TNF-α, tumour necrosis factor α. (Yáñez *et al*., 2019)

Overall, the role of type I IFNs in response to *Candida* infections might differ among fungal species, and the specific roles of type I IFNs in *C. albicans* infections requires further research. During bacterial infections, low level of type I IFNs may be required at an early stage to initiate cell-mediated immune responses, whereas high concentrations have immunosuppressive effects, such as the reduction of responsiveness of macrophages to activation by IFN-γ (Stifter and Feng, 2015). It should be noted that type I IFN genes are also induced through signal transduction pathways initiated by some TLRs (TLR4 and TLR2 via IRF3, and TLR7 and TLR8 via IRF5), although their involvement in the development of adaptive responses to *C. albicans* infection remains to be determined. Furthermore, findings obtained by integrating transcriptional analysis and functional genomics indicate that type I IFNs pathway is a main signature of *C. albicans*-induced inflammation and plays a crucial

role in anti-*Candida* host defense in humans, probably by eliciting antifungal responses of macrophages and NK cells (Smeekens *et al*., 2013).

Production of IFN-γ is crucial in determining the effectiveness of the immune responses against pathogens. Its importance is highlighted by the fact that mice and humans with defects in IFN-γ or IFN-γ receptor show profound deficiencies in their responses to certain pathogens, including bacteria, viruses and fungi, such as *C. albicans* (Gozalbo *et al.*, 2014). Although research had been mainly focused on the production of IFN-γ by T cells and NK cells, an early study reported the production of IFN-γ by BM-derived macrophages (Munder *et al.*, 1998). Later studies described the molecular mechanism of IFN-γ production by myeloid cells, providing evidence of the role of myeloid-derived IFN-γ in host defense against intracellular pathogens (Frucht *et al.*, 2001).

1.3.2 $CD4^+$ T cells: Th and T reg responses

Th1 responses have been considered protective against both mucosal and disseminated infection. Th1 cells produce several cytokines, but the most representative is IFN-γ. This cytokine has stimulatory effects on the phagocytosis and killing of *C. albicans* by neutrophils and macrophages. It also causes autocrine upregulation of IL-12 receptor, which in turn renders the Th cells more sensitive to IL-12, thus maintaining differentiation to the Th1 phenotype (Gozalbo *et al*., 2014; Richardson and Moyes, 2015).

Th17 lymphocytes secrete numerous cytokines, including IL-17 and IL-22. IL-17 induces neutrophil recruitment and activation, and IL-22 enhances epithelial barrier function by promoting the production of β-defensins by epithelial cells (De Luca *et al*., 2010; Huang *et al*., 2004). *C. albicans*-specific Th17 cells also produce IFN-γ, a cytokine that activates effector antifungal activities of phagocytes, as above mentioned (Zielinski *et al.*, 2012). Th17 response is critical for protection against *C. albicans* infection at most mucosal surfaces, and chronic mucocutaneous candidiasis often develops in patients with disorders in Th17-mediated antifungal responses, whereas these patients do not show increased susceptibility to invasive candidiasis (Ferwerda *et al.*, 2009; Hernández-Santos and Gaffen, 2012; Smeekens *et al.*, 2013). Overall, it is accepted that while mucosal infections predominantly induce polarization of adaptive immunity to protective Th17 responses, systemic candidiasis are still considered to induce predominantly Th1 responses (Richardson and Moyes, 2015).

Th2 responses are generated in an anti-inflammatory environment (e.g. production of IL-4 by DCs in response to *C. albicans* hyphae). Anti-inflammatory cytokines produced by

Th2 cells (IL-10, IL-4) inhibit Th1/Th17 development and deactivate phagocytic effector cells. Consequently, Th2 response has been considered as non-protective against infections. However, some Th2 cells are required for the maintenance of a balanced non-deleterious pro-inflammatory Th1/Th17 response, and to restore the non-inflammatory status following fungal clearance (Mencacci *et al.*, 2001; Netea *et al.*, 2015b).

The observation that Th effectors can produce other cytokine patterns not fitting the pre-conceived definitions of Th1/Th2 or Th17 subsets has led to the description of additional Th cell lineages, such as Th9 and Th22. It is known that Th9 cell subset, which specifically produced IL-9, can mediate tumor immunity and participates in autoimmune and allergic inflammation, but the knowledge about its function in fungal infections is still emerging (Borghi *et al*, 2014). During a respiratory fungal infection in mice, Th9 cells have been associated with failure to clear fungal pathogens while promoting asthma. Moreover, in experimental leaky-gut mouse models, *Candida*-driven IL-9 production in the gut was reported to contribute to the loss of barrier integrity, fungal dissemination, and inflammation. In these mouse models, IL-9 deficiency also promoted gut dysbiosis, suggesting that the functions of IL-9 might also involve the regulation of the microbiota (Speakman *et al.*, 2020). Th22 cells producing only IL-22 but neither IFN-γ nor IL-17 have been identified in humans (Borghi *et al.*,2014).

Treg cells maintain peripheral tolerance and limit the effector responses to control excessive pro-inflammatory responses leading to immune-mediated tissue damage. However, the role of Treg cells during candidiasis has been not unequivocally established. The immunosuppressive effects of Treg cells can be blocked by TLR-activated DCs leading to a Th1 response (Lee and Iwasaki, 2007). Besides, Treg cells also express TLRs, and the presence of TLR ligands (e.g. during a *C. albicans* infection) cause temporarily expansion and abrogation of the suppressive phenotype of Treg cells, enabling the enhancement of immune responses (pro-inflammatory Th1 response). Later during infection, upon pathogen clearance, the expanded Treg cells regain their immunosuppressive activity to restore the immune balance (Sutmuller *et al*., 2006a; Sutmuller *et al.*, 2006b). Th17 and Treg cells are reciprocally regulated during T cell differentiation and can act cooperatively against *C. albicans*, although the final response appears to be dependent on the infection site: Treg cells enhances Th17 protective responses to oropharyngeal candidiasis, while reduces resistance in systemic infections, supporting that Th responses to invasive and mucosal infections are different (Whibley and Gaffen, 2014; Whibley *et al.*, 2014).

1.3.3 $CD8^+$ T cells

Murine models of infection indicate that $CD8^+$ cells also have a role in protection against candidiasis, both mucosal and disseminated infections. The main antimicrobial effector mechanisms of $CD8^+$ T cells are cytotoxicity (by production and release of cytotoxic granules containing perforins and granzymes) and cytokine production (TNF-α and IFN-γ). The role of cytotoxicity in host defense against fungal infections is not well delineated, whereas the activity of cytokines is better understood. The protective effect is most probably due to IFN-γ, whose production is induced by IL-12 (Gozalbo *et al.*, 2014). As previously cited, activation of CTLs can be promoted by DCs through autocrine type I IFNs signaling upon recognition of fungal β-glucan by Dectin-1 (Hassanzadeh-Kiabi *et al.*, 2017).

1.3.4 Humoral responses: antibodies

Soluble (humoral) proteins, mainly complement and antibodies, also contribute to defense against candidiasis as components of the innate and adaptive immune responses, respectively. While cellular adaptive responses play a major role in host defense against *C. albicans* infection, the contribution of adaptive humoral immune mechanisms, mediated by antibodies secreted by B lymphocytes, play a relatively minor role in immune protection against the fungus (Netea *et al.*, 2015a; Richardson and Moyes, 2015).

Although binding of antibodies to fungal surface antigens triggers activation of the classical complement pathway (see section 1.2.1.1), the role of anti-*Candida* antibodies in host defense against candidiasis is paradoxical. Cell wall associated components (glucans, mannans, and mannoproteins) are major *Candida* antigens, as well as secreted enzymes (such as SAPs) or cytosolic fungal proteins (such as Hsps and glycolytic enzymes). Anti-*Candida* specific antibodies against each antigen interfere with its function in fungal biology, and therefore affect the host-pathogen interactions by inhibiting/neutralizing a specific fungal virulence factor (Gozalbo *et al.*, 2004; Martínez *et al.*, 1998). In fact, it has been shown that passive immunization using different anti-*Candida* antibodies are able to confer protection in animal models of mucosal and/or disseminated infection and in patients with invasive candidiasis (Wang *et al.*, 2015, Xin 2016). Thus, Mycograb®, a monoclonal antibody that binds to the immunodominant epitope of *C. albicans* Hsp90, enhances the percentage of patients with invasive candidiasis treated with amphotericin B that achieved complete mycological resolution (Wang *et al.*, 2015).

Despite these observations, B cell deficiency in mice does not confer increased susceptibility to *C. albicans* infection, and patients with agammaglobulinemia or

hypogammaglobulinemia do not show increased susceptibility to fungal infection, indicating that humoral response during infection has a very modest role in host protection. However, the use of some purified antigens [e.g. glucan or agglutinin-like sequence protein 3 (Als3)] in vaccination strategies, in conjunction with suitable carrier proteins and adjuvants capable of eliciting the production of antigen-specific antibodies, confer limited protection against candidiasis. Therefore, and due to the prevalence of fungal infections and their increased resistance to antifungal therapies, eliciting protective antibodies through vaccination remains as a viable strategy for improving resistance to *C. albicans* infections (Iannitti *et al.*, 2012, Moragues *et al.*, 2014; Richardson and Moyes, 2015; Wang *et al.*, 2015).

2 Demand-adapted hematopoiesis during infection

Hematopoietic stem and progenitor cells (HSPCs) in the BM are responsible for the maintenance of both steady state and stress-adapted hematopoiesis (Chavakis *et al.*, 2019; King and Goodell, 2011; Rieger and Schroeder, 2012; Zhao and Baltimore, 2015). Physiopathologic conditions that disturb the hematopoietic equilibrium, such as bleeding or severe systemic infection, induce demand-adapted hematopoietic responses that offset cell losses and increase cellular output to meet the specific needs during emergency situations (Boettcher and Manz, 2017; Kobayashi *et al.*, 2016). Acute infection usually triggers the mobilization of myeloid cells, in particular neutrophils and monocytes, from the BM to infected tissues, and this is accompanied by the proliferation and differentiation of HSPCs to maintain the supply of myeloid cells killed by the invading microbes or consumed during the immune response. Emergency myelopoiesis may consist of granulopoiesis (particularly neutrophil production), monopoiesis (generation of monocytes and macrophages) or both, depending on the specific microbe as well as the route and the severity of infection (Boettcher and Manz, 2016; Boettcher and Manz, 2017; Kobayashi *et al.*, 2016). By contrast, the adaptive immune system meets this demand by clonal expansion of mature T and B cells in secondary lymphoid organs. Therefore, in order to favor myeloid cell production, inflammatory cytokines secreted during infection-induced myelopoiesis reduce the expression of growth factors for lymphopoiesis. Besides, these cytokines also reduce the expression of retention factors for BM lymphocytes, inducing their mobilization to secondary lymphoid organs (King and Goodell, 2011; Zhao and Baltimore, 2015).

The essential first step in the initiation of demand-adapted hematopoiesis is the detection of the pathogenic organism (e.g, bacteria, virus, parasite, or fungi). Consequently, the cell type that triggers the process needs to: (i) have a high probability of encountering the pathogen, (ii) be equipped with the molecular machinery for pathogen sensing, and (iii) be able to translate pathogen detection into emergency myelopoiesis (Boettcher and Manz., 2016, Boettcher and Manz, 2017). Host mature cells in infected tissues, including both non-hematopoietic and hematopoietic cells, initially recognize pathogens by detection of PAMPs through PRRs, an event that elicits several molecular cascades for proper immune responses. Thus, these cells release pro-inflammatory cytokines, such as TNF-α, IL-1β, or IL-6, which reach the BM niche through peripheral circulation and then act stimulating myelopoiesis (Chiba *et al.*, 2018; Kumar *et al.*, 2011). However, new perspectives on demand-adapted hematopoiesis came when reports began to emerge demonstrating that

murine and human HSPCs express functional PRRs, including TLRs, and that TLR/PRR-mediated signals provoke cell cycle entry and myeloid differentiation (Nagai *et al.* 2006, Cannova *et al.*, 2015; Yáñez *et al.*, 2013a). Thus, depending on the developmental stage of the pathogen-sensing cell type, two major mechanisms for initiating demand-adapted myelopoiesis can be considered: indirect and direct mechanisms. According to a model of indirect initiation, mature cells present in the BM or the peripheral tissues would act as pathogen sensors stimulating myelopoiesis via secretion of soluble factors. By contrast, in a model of direct initiation, pathogen recognition by HSPCs would directly induce enhanced proliferation and myeloid-biased differentiation (Boettcher and Manz, 2016, Boettcher and Manz, 2017).

In contrast to the clear role of TLR signaling in demand-adapted hematopoiesis (Yáñez *et al.*, 2013a) (see section 2.2), the function of TLRs under normal homeostatic conditions is not yet entirely clear. It has been shown that commensal microbiota promotes steady-state myelopoiesis by specifically maintaining granulocyte-monocyte progenitor's proportions and enhancing their differentiation into mature myeloid cells in the BM (Khosravi *et al.*, 2014). Other studies have demonstrated that TLR-mediated innate pro-inflammatory signaling plays important functions in generating embryonic hematopoietic stem cells (HSCs), but whether and how TLR signaling initiates these inflammatory environments remains to be determined (Cannova *et al.*, 2015; Luis *et al.*, 2016; Zhao and Baltimore, 2015).

Collectively, all these studies highlight the fundamental role that inflammatory signals play in the ontogeny and maintenance of HSPCs, at steady state or stress-adapted hematopoiesis. However, if this inflammation is chronically sustained may lead to HSPC loss and eventually BM failure, or even it may increase propensity to acquire clonal genetic lesions which may result in leukemia. Therefore, it is critical to understand how HSCs balance their response to inflammatory signals, harmonizing the need to effectively fight immune stresses and regenerate the hematopoietic system with regulating proliferative cell divisions that may ultimately select for transformative genetic mutations (Luis *et al.*, 2016).

2.1 Steady state hematopoiesis

The mammalian blood system contains more than ten different mature cell types (lineages) with various functions. Aside from a few exceptions (e.g., memory T cells, some tissue macrophages), most of the mature cell types have a finite life span, and so they must be constantly replenished to ensure homeostatic peripheral blood cell counts. Blood is one

of the most fast-regenerative tissues, as millions of blood cells are replaced with new ones each second during life. Despite their functional differences, all blood cell types are generated from HSCs that reside mainly in the BM, the major site of adult hematopoiesis. HSCs, therefore, are defined by its capacity to continuingly giving rise to all the blood cell lineages due to their differentiation potential (Rieger and Schroeder, 2012; Seita and Weissman, 2010).

In adult mammals, immune cell production is hierarchically organized with HSCs at the apex of numerous progenitor cell stages with increasingly restricted lineage potential (Cheng *et al*., 2020; Rieger and Schroeder, 2012; Seita and Weissman, 2010). The first *in vivo* evidence for the existence of HSCs was based on the rescue of lethally irradiated recipient mice by BM transplantation, followed by detection of hematopoietic colonies in the spleens of recipient mice (Till and McCulloch, 1961). Thus, the gold standard for identification of HSCs has traditionally been to conduct BM transplantation and demonstrate generation of all blood lineages in mice. Besides, these functional assays revealed that HSCs are heterogeneous concerning their repopulating capacity as they are composed by different subpopulations. As transplantation is impractical for experimental manipulation, surrogate methods have been developed to better study the relationship between HSCs and their progenies, as well as the stepwise differentiation process (Challen *et al*., 2009; Seita and Weissman, 2010). Weissman and colleagues first described HSC-enriched cells by using the combination of several surface markers (Spangrude *et al.*, 1988). Since then, the identification of multiple surface markers has enabled the isolation of defined HSCs, as well as multi-/oligo-/unipotent progenitor populations, establishing the immunophenotype-based tree-like hierarchy model of developing blood cells (Cheng *et al.*, 2020; Rieger and Schroeder, 2012).

Mouse HSPCs from the BM are defined by their lack of expression of markers of differentiated cells. A cocktail of antibodies specific for antigens termed “lineage markers” (Lin; typically CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119) can be used to selectively eliminate mature hematopoietic cells from complex (BM, spleen) samples. The remaining Lin^- cells can then be enriched for specific stem or progenitor cell populations (**Figure 5**). Sorting Lin^- c-Kit^+ Sca-1^+ (LKS^+) cells enriches for cells with hematopoietic-reconstituting activity. In the classical model, HSCs can be divided into two subpopulations according to their self-renewal capacity: long-term (LT)-HSCs and short-term (ST)-HSCs. LT-HSCs are a minor (about 10 % of LKS^+ cells), quiescent population in BM and have full long-term reconstitution capacity following serial transplantation in mice (Morrison and Weissman, 1994). On the other hand, ST-HSCs only have a short-term

reconstitution ability. LT-HSCs differentiate into ST-HSCs, and subsequently, ST-HSCs differentiate into multipotent progenitors (MPPs), which have no detectable self-renewal ability, although they still retain the LKS$^+$ phenotype. The LKS$^-$ fraction contains oligopotent lineage committed progenitors. By immunophenotyping it was shown that MPPs give rise to progenitors committed either to the lymphoid or to the myeloid lineages, (CLPs and CMPs, respectively). A second split appears as CMPs can give rise to megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-monocyte progenitors (GMPs). DCs in this model can be derived either from CLPs or CMPs (Rieger and Schroeder, 2012; Seita and Weissman, 2010, Yáñez et al. 2017).

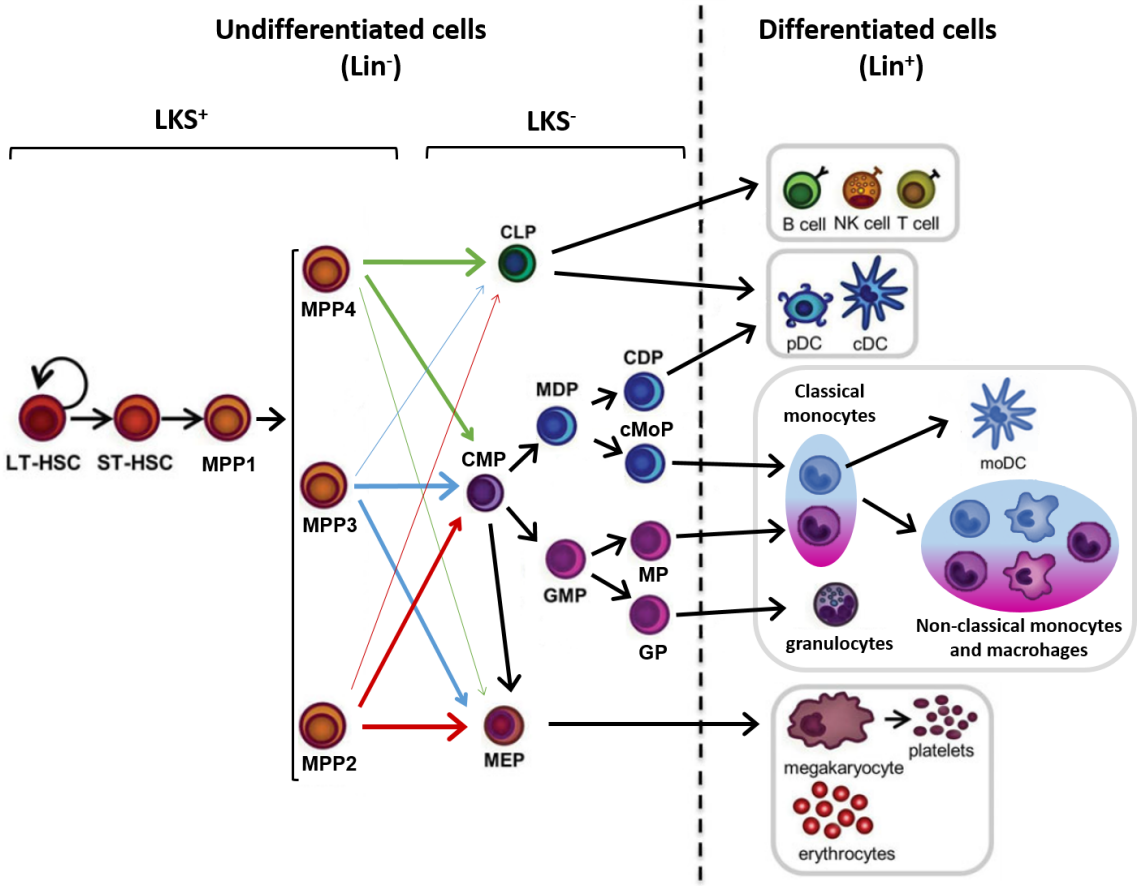


FIGURE 5 | The mouse hematopoietic tree. Hematopoiesis is initiated by long-term hematopoietic stem cells (LT-HSCs), which have the capacity for self-renewal and give rise to proliferating short-term HSCs (ST-HSCs). These cells produce multipotent progenitors, which have been classified in four subsets depending on their lineage-biased potential: MPP1 (multiple lineages), 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. CMP are also able to produce MEPs, as well as granulocyte-monocyte progenitors (GMPs) and monocyte-dendritic cell progenitors (MDPs). GMPs give rise to monocyte committed progenitors (MPs) and granulocytes committed progenitors (GPs), whereas MDPs give rise to common dendritic cell progenitors (CDP) and monocyte committed progenitors (cMoPs). Functionally distinct subsets of classical monocytes are produced by both MPs and cMoPs, and consequently, derived non-classical monocytes and macrophages may also exhibit functional differences; monocyte-derived DCs (moDCs) also arise exclusively from cMoPs. Mouse hematopoietic stem and progenitor cells are defined by their lack of expression of markers of differentiated cells (Lin$^-$ cells). From Lin$^-$ cells, c-Kit$^+$ Sca-1$^+$ (LKS$^+$) fraction contains HSCs and MPPs, whereas c-Kit$^+$ Sca-1$^-$ fraction (LKS$^-$) includes oligopotent lineage-committed progenitors. Adapted from Yánez *et al.*, (2013).

Although the classical model has been very useful for understanding how the multi-potent HSCs differentiate into these diverse functional types, it oversimplifies the complexity of HSPCs as the model is only based on the expression of a few surface markers and transplantation assays using bulk cells. With advances in single cell technology and genetic mouse models, this classical model has been challenged and new types of HSPCs have been identified according to their lineage biases (Giladi *et al*., 2018). These new strategies have uncovered the presence of more heterogeneity than formerly thought in the most primitive HSC population. It was observed that MPPs are a heterogeneous population that could be divided into four subpopulations with different lineage-biased potential: MPP1 have multiple lineage reconstitution ability, while MPP2 is a megakaryocyte/erytrocyte-biased MPP subset, MPP3 is a myeloid-biased subset, and MPP4 is a lymphoid-biased subset (Cheng *et al*., 2020) (**Figure 5**).

Heterogeneity and hierarchy within myeloid progenitors have also been checked to better understand the role of hematopoiesis in producing the huge variety of myeloid functional phenotypes described in the last decades. Myeloid cell differentiation is probably the most recently characterized process of blood cell lineage production, although some aspects concerning mature myeloid subsets heterogeneity are still unknown. Regarding the hierarchically organized production of myeloid cells from HSPCs, it has been observed that CMPs can give rise to GMPs and to monocyte and dendritic cell progenitors (MDPs) (Zhu *et al.*, 2016). Thus, both GMPs and MDPs can produce monocytes, while GMPs are committed to produce neutrophils via granulocytes progenitors (GPs), wheres MDPs yield cDCs and pDCs via common DC progenitors (CDPs) (Yáñez *et al*., 2015). A hierarchical relationship (CMP–GMP–MDP) was presumed to underlie monocyte differentiation, although Yáñez et *al*. (2017) clearly demonstrate that mouse MDPs arise from CMPs independently of GMPs and that both produce monocytes via not identical monocyte–committed progenitors (cMoP and MP, derived from MDP and GMP, respectively) (**Figure 5**). Monocytes can be generally classified into two subsets: classical monocytes, also termed "inflammatory" monocytes ($Ly6C^+$ in mice), and non-classical monocytes or "patrolling" monocytes ($Ly6C^-$). It has been shown that, in the steady state, $Ly6C^+$ monocytes are precursors of tissue-macrophages and $Ly6C^-$ monocytes, a functional end stage considered as blood-resident macrophages. Moreover, inflammatory monocytes express high levels of CCR2 and low levels of CX_3CR1, whereas patrolling monocytes show the reversed pattern (Italiani and Boraschi, 2014). Distinct subsets of classical monocytes are produced by both GMPs and MDPs, so non-classical monocytes and macrophages derived from them may also exhibit functional differences. One of these subsets of classical monocytes (neutrophil-like subset) is only

produced by GMPs, whereas moDCs arise exclusively from cMoP-derived monocytes (Yáñez *et al.*, 2017) (**Figure 5**).

The balance between HSCs self-renewal and differentiation must be tightly regulated to enable both the generation of differentiated cells and the accurate maintenance of the right HSC pool size. The homeostasis of hematopoietic system is a highly dynamic and tightly regulated orchestration of intrinsic programs and extrinsic signals from the microenvironment, often referred to as "niche". The niche promotes a variety of juxtacrine (cell-cell or cell-matrix) and paracrine (via cytokines, chemokines, and growth factors) interactions involving HSCs that are required for their appropriate behavior. BM provides the environment for sustained HSC function and HSCs rapidly lose their self-renewal capacity once isolated from their *in vivo* niche in the trabecular bone area of BM, near to sinusoids and blood vessels (Rieger and Schroeder, 2012; Wei and Frenette, 2018). Interestingly, it has been proposed that various progenitors could have specific niches in the BM (Wei and Frenette, 2018). Many different cell types within the BM are now known to form part of the niche, including non-hematopoietic/stromal cells and hematopoietic cells. Mesenquimal stem cells (MSCs) and endothelial cells are in close proximity and release soluble factors, such as CXCL12 or Stem cell factor (SCF, c-Kit ligand), that act directly on HSCs and promote their survival. Besides, other cell types, such as cells of the neural system, including sympathetic nerves and Schwann cells, osteolineage cells (e.g. osteoblasts and osteocytes) and adipocytes may act indirectly through actions on proximal stroma (Wei and Frenette, 2018). In addition to the stromal niche components, HSCs' own progeny has been shown to regulate HSC behavior. A network of innate immune cells (neutrophils and resident macrophages), BM-resident memory T cells and most recently, megakaryocytes, have been shown to have important regulatory functions on the hematopoietic niche (Casanova-Acebes *et al*., 2014; Wei and Frenette, 2018).

HSC populations are heterogeneous in their reconstitution efficacy and their lineage patterns. Importantly, these patterns are conserved through serial transplantations, indicating the existence of stable inheritable stem cell intrinsic programs. Accumulated knowledge has demonstrated the importance of numerous intrinsic factors in regulating HSC self-renewal, although their exact interplay remains to be unrevealed (Li *et al.*, 2019; Rieger and Schroeder, 2012). However, the molecular mechanisms that explain how HSPCs manage to establish lineage-committed stage are better defined. The differentiation from a multipotent progenitor towards a specific lineage involves a global change of gene expression. This lineage choice and commitment include both the expression of lineage-specific genes and the repression of those genes specific for other lineages. One

excellent example is the switch from high levels of the transcription factor GATA2, mainly expressed in early progenitors, to high levels of GATA1, which precedes erythropoiesis from HSCs. GATA2 induces GATA1 expression, which in turn activates its own expression and represses GATA2 (Rieger and Schroeder, 2012). However, gene regulation depends on the interplay of a variety of elements, including transcription factors, epigenetic modifiers, and post-transcriptional control mechanisms. In light to recent data obtained by high-throughput techniques at the single-cell level, detailed information has been achieved concerning intrinsic programs for HSPCs differentiation suggesting that these processes are more complex and less sequential than previously appreciated (Cheng *et al*., 2020; Giladi *et al.*, 2018).

2.1.1 Myeloid cell differentiation

Similarly to the tightly regulated self-renewal and differentiation of HSCs, the differentiation of a multipotent progenitors to a specific lineage is also controlled by intrinsic and extrinsic mechanisms (Rieger and Schroeder, 2012). As previously mentioned, the intrinsic mechanisms lead to an induction and maintenance of lineage-affiliated genetic programs. The lineage-specific transcription factors that regulate the broad gene expression switch to myeloid lineages are mainly PU.1, IRF8 and C/EBP family of transcription factors (Giladi *et al*., 2018; Yáñez *et al*., 2015; Zhu *et al*., 2016). According to consensus myeloid differentiation models, PU.1 is the master regulator of all myeloid lineages. *PU.1* is expressed at low levels in CMPs, and its expression increases in their subsequent more committed progenitors to give rise to monocytes or neutrophils. C/EBPα is also an important regulatory transcription factor that cooperates with PU.1 to promote myeloid identity. On the other hand, *Irf8* is expressed in the GPs and MPs lineage-committed progenitors, but not in GMPs, and regulates their survival and differentiation to promote monocyte and suppress neutrophil production (Yáñez *et al*., 2015). Previously, it was demonstrated that IRF8 binds to and inhibits the transcriptional activity of C/EBPα, thereby preventing neutrophil differentiation (Kurotaki *et al.* 2014). More recently, using a novel approach based on high-throughput techniques at the single-cell level, Giladi *et al.* (2018) have revealed that C/EBPα regulates the entry into all myeloid fates, while IRF8 and PU.1 participate in later differentiation towards monocyte or granulocyte fates, respectively. The study shows that IRF8 is essential for monocyte development, in accordance with previous studies, whereas PU.1 is not necessary for granulocyte differentiation but for further neutrophil maturation (Giladi *et al*., 2018).

As previously mentioned, the differentiation from progenitors to mature cells also depends on post-transcriptional control mechanisms and epigenetic regulation. The study of epigenetic events during hematopoiesis has revealed that both DNA and histone modifications are important in this process of cell production. Histone acetylation, one of the best-studied histone modifications, generally correlates with the level of gene expression, and is determined by the relative activity of histone acetyltransferases and histone deacetylases. Concerning myeloid differentiation, downstream of CMPs, histone deacetylase 1 (*HDAC1*) expression is downregulated by C/EBP transcription factors to induce the production of myeloid cells, in particular granulocytes. Distinct DNA methylation patterns have been also related to myeloid cell differentiation (Álvarez-Errico *et al*., 2015). On the other hand, some studies have reported a role for small non-coding RNAs or microRNAs (miRNAs), such as the miR-106a or miR-223, in regulating monocyte development and monocyte differentiation to macrophages (Zhu *et al*., 2016). Moreover, using a model of transdifferentiation from pre-B cells to macrophages, it has been shown that C/EBPα induce the expression of miR-34a and miR-223, which target and downregulate *Lef1*, a gene that encodes for a lymphoid transcription factor, whose ectopic expression delays transdifferentiation (Rodríguez-Ubreva *et al*., 2014).

A vast body of literature demonstrates that myelopoiesis is also regulated by extrinsic signalling from members of the colony-stimulating factor (CSF) superfamily, which has essentially three canonical members namely macrophage (M)-CSF, granulocyte (G)-CSF, and GM-CSF (Hamilton and Achuthan, 2013). These myelopoietic growth factors provide proliferation and survival signals to HSPCs, as well as they are critically involved in the process of lineage specification as demonstrated by the ability of G-CSF and M-CSF to instruct bipotent GMPs to differentiate either into granulocytes or macrophages, respectively (Rieger *et al*., 2009).

The importance of the CSF system for steady state myelopoiesis is most clearly revealed using mice deficient for the respective *CSF* genes. Among these, M-CSF receptor (M-CSF-R) and M-CSF KO mice have the most dramatic phenotype as they exhibit skeletal, sensory, and reproductive abnormalities caused by severe deficiencies in tissue macrophages and osteoclasts (Chiba *et al*., 2018). M-CSF is broadly expressed and regulates the generation of monocytes and macrophages from lineage-committed progenitors and even acts on HSCs through activation of PU.1 (Mossadegh-Keller *et al*., 2013). On the other hand, studies in G-CSF $^{-/-}$ mice showed a 70-90 % reduction in circulating neutrophils, indicating an essential role of G-CSF in homeostatic granulopoiesis. Unlike M-CSF, several publications suggest that G-CSF may be mainly acting on myeloid-restricted progenitors but

not on HSCs (Boettcher and Manz, 2017; Mossadegh-Keller *et al*., 2013). GM-CSF was originally discovered as a protein capable of generating both granulocyte and macrophage colonies from myeloid precursor cells *in vitro*. Nevertheless, analyses of GM-CSF $^{-/-}$ mice have failed to detect a major defective hematopoietic phenotype (except for the absence of alveolar macrophages and a reduction in a subset of DCs in non-lymphoid tissues), indicating that GM-CSF is largely redundant for steady-state myelopoiesis (Becher *et al*., 2016). As well as G-CSF, GM-CSF do not induce increased *PU.1* expression in HSCs (Mossadegh-Keller *et al*., 2013).

2.2 Infection-induced hematopoiesis

2.2.1 Indirect pathogen sensing and consequences in hematopoiesis

According to a model of indirect initiation of demand-adapted hematopoiesis during infection, mature cells would act as pathogen sensors stimulating myelopoiesis via secretion of soluble factors such as CSFs or cytokines. Mononuclear phagocytes (monocytes and macrophages) act as the primary pathogen-sensing cell type during systemic infections and they can release a great variety of inflammatory cytokines. Therefore, these cells may be the cell type that indirectly stimulates HSPCs, although this notion has never been conclusively proven by *in vivo* experiments. However, Kwak *et al.* (2015) have revealed that BM mature myeloid Gr1^{+} cells (including granulocytes and monocytes as well as their late-stage precursor cells) play a critical role in mediating emergency granulopoiesis following heat-inactivated *Escherichia coli* injection *in vivo*. This mechanism implies the production of ROS that externally regulate the proliferation and differentiation of adjacent GMPs (**Figure 6A**). By contrast, BM chimeric mice with TLR4 $^{-/-}$ hematopoietic cells on a WT non-hematopoietic background were able to mount an emergency myelopoiesis (production of Gr1^{+} cells) in response to the TLR4 ligand Lipopolysaccharide (LPS) (Boettcher *et al.*, 2012). In fact, the same study showed that TLR4-expressing non-hematopoietic cells are indispensable for the myelopoietic responses induced by LPS. From the various non-hematopoietic cells that have been shown to express TLRs, which made them also candidate as pathogen-sensing cell types during emergency myelopoiesis, Boettcher *et al.* (2014) demonstrated that emergency myelopoiesis in response to LPS is mediated by TLR4-MyD88 signaling in endothelial cells and subsequent G-CSF release (**Figure 6B**).

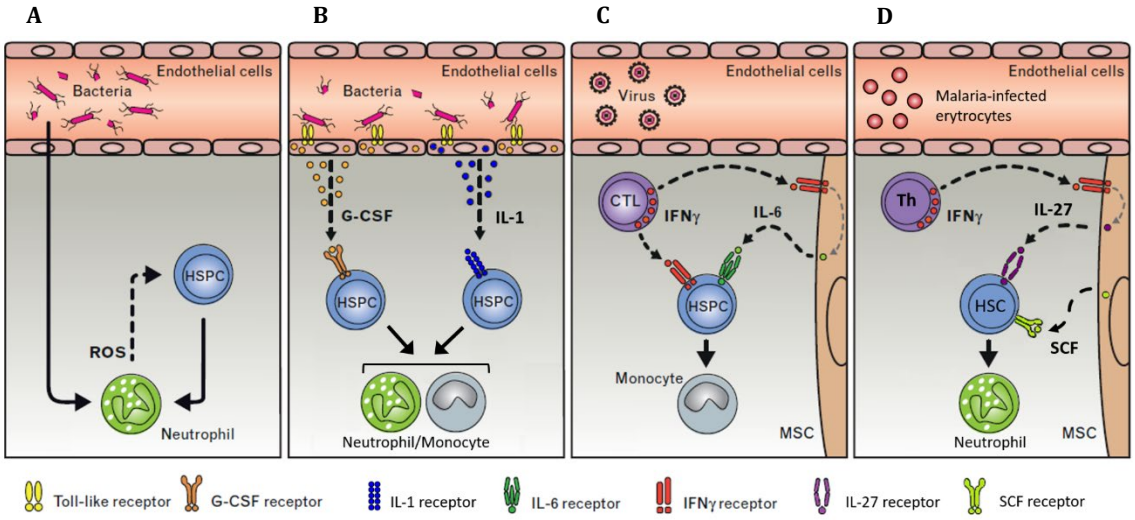


FIGURE 6 | Pathways for indirect sensing of pathogen signals and translation into emergency myelopoiesis. Adapted from Boettcher *et al.*, 2015.

Plasma levels of the myelopoietic growth factors G-CSF and GM-CSF rise rapidly during infection, to replace myeloid cells consumed fighting against the pathogen. As in steady-state, CSFs role in emergency myelopoiesis was studied using respective KO mice. Concerning G-CSF $^{-/-}$ mice, there are conflicting results possibly due to the different experimental models used. In response to *C. albicans*, G-CSF $^{-/-}$ mice, as similarly infected WT mice, developed a profound and sustained neutrophilia, suggesting that G-CSF is dispensable for mounting an emergency granulopoietic response in this model (Basu *et al.*, 2000). By contrast, as mentioned above, emergency myelopoiesis induced by LPS was fully dependent on G-CSF, although in response to *E. coli* infection it was not totally dependent on G-CSF, since a granulopoietic response was still detectable (Boettcher *et al.*, 2014). Interestingly, ROS-induced emergency granulopoiesis in response to *E. coli* infection was independent of G-CSF (Kwak *et al.*, 2015), thus showing a cooperation between both G-CSF-dependent and -independent mechanisms following infection with *E. coli*. On the other hand, different infection models using GM-CSF $^{-/-}$ mice showed that GM-CSF seems to be involved in maintaining an appropriate emergency hematopoietic response once earlier response mechanisms are being overcome, such as during later stages of acute infection or during chronic infection (Becher *et al.*, 2016).

As CSFs, other cytokines released by pathogen-sensing cells can also induce emergency hematopoiesis (Chiba *et al.*, 2018; King and Goodell, 2011). IL-1, a key pro-inflammatory signal that rapidly augments host innate immunity, has also a role in regulating emergency hematopoiesis, although its effect depends on the time of exposure. Upon infection or injury, IL-1 is produced at elevated levels in the BM microenvironment, in particular by endothelial

cells that form an essential component of the HSC niche. There, IL-1 accelerates HSC proliferation and instructs HSCs towards myeloid differentiation by inducing PU.1 via NF-κB signaling downstream of the IL-1 receptor. While this response is advantageous in acute inflammation, it is ultimately detrimental in situations of chronic exposure because IL-1 promotes uncontrolled HSC division, loss of self-renewal capacity and eventual exhaustion of the HSC pool. However, these damaging consequences are fully reversible upon IL-1 withdrawal, indicating that IL-1 effects are essentially transient and require continuous exposure to negatively impact HSC function (Pietras *et al.*, 2016) (**Figura 6B**).

MSCs from BM niche are also able to support HSPC expansion through active production of cytokines. Schurch *et al.* (2014) found that CTLs induced myelopoiesis during viral infection by migrating to the BM niche and producing IFN-γ, which was then detected by MSCs. Consequently, MSCs secreted IL-6 that, in turn, increased MPP proliferation and myeloid differentiation predominantly along the monocytic lineage (**Figure 6C**). Following a similar mechanism, blood-stage malaria infection induces IFN-γ production by $CD4^+$ T cells and NK cells, which may stimulate MSCs to produce the cytokine IL-27. It has been demonstrated that IL-27, which belongs to the IL-16/IL-12 family of cytokines, promotes the expansion of HSCs and their differentiation into myeloid progenitors in synergy with SCF. Thus, IL-27 acts on HSCs to promote emergency myelopoiesis resulting in enhanced production of neutrophils to remove malaria infected red blood cells (Furusawa *et al*., 2016) (**Figure 6D**). In both described mechanisms (during viral or malaria infection), IFN-γ has an indirect effect mediating secretion of other cytokines such as IL-27 or IL-6 by MSCs. In addition, several studies have demonstrated that IFN-γ is also able to directly act on HSPCs (de Bruin *et al*., 2014). However, its impact on HSC stemness is controversial as IFN-γ can have both stimulating or suppressing effects on HSC proliferation and reconstitution, and the outcome of this balance may be context-dependent (*in vitro* or *in vivo* stimulation, the use of different mouse infection models, etc.). Nevertheless, it is firmly accepted that IFN-γ production during infection is very important for tightly orchestrating myelopoiesis, promoting monopoiesis while inhibiting neutrophil development. It has been proposed that IFN-γ acts through upregulation of the Suppressor of cytokine signaling (SOCs) proteins, which impair the signaling of several cytokines and growth factors. IFN-γ induces expression of SOCS3 in GMPs and thereby inhibits G-CSF-induced activation of STAT3, an essential transcription factor for emergency granulopoiesis. On the other hand, IFN-γ also causes increased expression of the master regulator of all myeloid lineages, PU.1, and the monocyte-promoting transcription factor IRF8 in the same myeloid progenitors (de Bruin *et al.*, 2012).

HSPCs also express both type I and type II IFN receptors, and therefore, in addition to IFN-γ, IFN-β and particularly IFN-α can influence the response of HSPCs to inflammation (Chavakis *et al.*, 2019). It has been demonstrated that murine HSCs efficiently proliferate in response to *in vivo* treatment with the TLR3 ligand and type I IFN inducer polyinosinic:polycytidylic acid (polyI:C) or with IFN-α itself (Essers *et al.*, 2009). However, as described for chronic exposure to IL-1, chronic administration of IFN-α also results in impaired HSC repopulation capacity (Essers *et al.*, 2009). In addition, type I IFNs are able to induce apoptosis in HSCs, intensifying the suppressive effects of type I INFs on HSC function (Pietras *et al.*, 2014). On the other hand, it has been shown that type I IFNs drives emergency myelopoiesis in mice with transgenic overexpression of TLR7 (Buechler *et al.*, 2013).

The scheme shown in **Figure 6** summarizes the indirect mechanisms for translating signals from pathogen sensing into in emergency myelopoiesis (granulopoiesis, monopoiesis or both), described in this section.

2.2.2 Direct pathogen sensing by HSPCs

PRR expression by HSPCs and their role in emergency myelopoiesis were first reported in 2006. A pioneering work from Nagai *et al*. (2006) demonstrated that murine HSCs, as well as lineage-restricted progenitors, expressed TLR4 and/or TLR2 and that upon *in vitro* exposure to their respective ligands, WT but not MyD88 KO HSCs entered cell cycle and acquired myeloid lineage markers. Since then, a solid body of evidence has demonstrated that mice and human HSPCs express most TLRs and their stimulation induce proliferation and myeloid differentiation *in vitro* (Yáñez, *et al.*, 2013a). Moreover, mouse HSPCs *ex vivo* pre-stimulated with LPS, and then transplantated under the renal capsule and subjected to *in vivo* LPS stimulation were able to locally generate clusters of myeloid cells in the kidney (Massberg *et al.*, 2007). The expression of other PRRs by HSPCs has also been described. For example, NOD2 is expressed by human $CD34^+$ cells (HSPCs) and stimulation of NOD2 with muramyl dipeptide is sufficient to trigger differentiation to myeloid cells (Sioud and Floisand, 2009). More recently, the endoplasmic adaptor protein STING, which recognizes cyclic bacteria– or virus–derived DNA, was added to the collection of PRRs expressed in HSPCs (Kobayashi *et al*., 2015; Kobayashi *et al*., 2016).

Subsequent studies focused on determining whether HSPCs may be stimulated directly by pathogenic microbes. In this context, our group has studied the interaction between *C. albicans* and murine HSPCs, describing that inactivated yeasts or hyphae induce LKS^+ cells to proliferate and differentiate toward the myeloid lineage in a TLR2/MyD88-dependent

manner (Yáñez *et al*., 2010; Yáñez *et al*., 2009). Challenge of LT-HSCs with *C. albicans* yeasts also induces their proliferation, as well as the upregulation of myeloid progenitor markers through a TLR2/MyD88-dependent signaling pathway. TLR2/MyD88 signaling also promotes, upon challenge with yeasts, the differentiation of CMPs and GMPs into cells with a morphology of mature myeloid cells expressing CD11b, F4/80, and Gr-1. These myeloid-like cells display functional properties, as they are able to (i) phagocytose *C. albicans* yeasts and (ii) release pro-inflammatory cytokines upon *in vitro* stimulation (Yáñez *et al.*, 2010).

It has been shown that *in vitro* stimulation of human and mouse HSPCs with TLR agonists induce the production of specific myeloid subsets depending on the agonist used (Bieber and Autenrieth, 2020; Yáñez, *et al.*, 2013a). For example, human $CD34^+$ cells develop into $CD11c^+$ cells upon NOD2 stimulation (Sioud and Floisand, 2009), whereas stimulation of mouse CMPs with a TLR7/8 ligand leads to the generation of macrophages. The TLR7/8 ligand promote the repression of genes that encode for transcription factors associated with granulocyte, erythroid and megakaryocyte differentiation, and upregulate the expression of the myeloid-specifying transcription factor PU.1 (Buechler *et al*., 2016). Our group has also demonstrated that TLR2, TLR4 and TLR9 stimulation of mouse Lin^- and LKS^+ cells induce their differentiation towards macrophages (Megías *et al*., 2012). Indeed, Nagai *et al.* (2006) showed that LT-HSC and myeloid progenitors (GMP and CMP) stimulated with Pam_3CSK_4 (synthetic version of the bacterial lipopeptide, detected by TLR1/TLR2 heterodimers) or LPS produced monocytes or macrophages, while lymphoid progenitors stimulated with the same TLR agonists produced DCs.

The specific myeloid subsets that are produced following *in vitro* exposure of mouse HSPCs (Lin^- cells) to *C. albicans* have been also determined. Inactivated *C. albicans* yeasts induced their differentiation into moDCs, via TLR2/MyD88- and Dectin-1-dependent pathways **(Figure 7A)**. Interestingly, the response to *C. albicans* yeasts was more similar to the response to curdlan (a pure Dectin-1 ligand) than to Pam_2CSK_4 (a pure TLR2/TLR6 ligand), as Pam_2CSK_4 promoted differentiation to macrophages rather than moDCs (Yáñez *et al*., 2011), indicating that Dectin-1 plays a key role in the response of HSPCs to *C. albicans*. Dectin-1 is not expressed on HSCs, but Lin^- cells express detectable levels of Dectin-1 (Yáñez *et al.*, 2011), indicating that it is turned on in differentiating progenitors prior to the acquisition of lineage markers. The moDCs generated *in vitro*, in response to inactivated yeasts, are functional as they have acquired the capability to secrete TNF-α and have fungicidal activity, and therefore could participate in innate immunity against *C. albicans*.

All these data strongly support the notion that TLR signaling programs early progenitors to generate functional mature cells to deal with the fungal pathogen.

In vivo myeloid differentiation from HSPCs has been demonstrated using a wide variety of infection models or by stimulating with purified TLR ligands (Bieber and Autenrieth, 2020; Buechler *et al*., 2016; Nagai *et al*., 2006; Yáñez, *et al.*, 2013a; Yáñez *et al*., 2011). However, direct *in vivo* interaction of pathogens and/or their components with TLRs on HSPCs during infection is more difficult to demonstrate, as HSPCs can also respond to other stimuli, including inflammatory cytokines generated by mature cells following direct pathogen recognition. To solve this issue, our group used the experimental approach next explained to address whether TLR agonists can directly stimulate HSPCs *in vivo*. Purified Lin^- or LKS^+ cells from the BM of C57BL/6-Ly5.1 mice ($CD45.1^+$) were transplanted into TLR2 $^{-/-}$, TLR4 $^{-/-}$, or MyD88 $^{-/-}$ mice ($CD45.2^+$), which were then injected with pure ligands for TLR2, TLR4, or TLR9 (Pam_3CSK_4, LPS, and CpG ODN), respectively. In this model, KO mouse cells are not capable of recognizing or responding to the injected TLR pure ligands; therefore, any responses observed in the transplanted cells must be due to direct recognition the TLR agonists by the donor HSPCs. Transplanted HSPCs were detected in the BM and the spleen of recipient mice and, in response to TLR ligand injection, these cells differentiated preferentially into macrophages, demonstrating unequivocally that HSPCs can recognize directly the TLR agonists *in vivo*, and that the engagement of these receptors induces macrophage differentiation (Megías *et al.*, 2012). In this context, it has been also described that HSPCs injected directly into *Staphylococcus aureus* infected wounds of TLR2 KO or WT mice were able to produce granulocytes equivalently, whereas TLR2 KO HSPCs differentiate less efficiently than WT HSPCs to towards neutrophils in a WT *S. aureus* infected wound environment (Granick *et al*., 2013) **(Figure 7B)**.

A similar *in vivo* transplantation approach with CD45.1/CD45.2 mice was used to study the effect of *C. albicans* infection on HSPCs. Transplanted Lin^- cells were detected in the spleen and BM of recipient mice, and they differentiated preferentially to macrophages in response to both viable and inactivated yeasts in a TLR2-dependent but TLR4-independent manner (Megías *et al*., 2013) **(Figure 7A)**. These results indicate that TLR2-mediated recognition of *C. albicans* by HSPCs helps to replace and/or to increase cells that constitute the first line of defense against the fungus, and suggest that TLR2-mediated signaling leads to programming of early progenitors to rapidly replenish the innate immune system and generate the mature cells most urgently needed to deal with the pathogen.

It is widely accepted that cytokines released by mature immune cells in infected tissues reach BM niche and induce myelopoiesis. However, new studies have suggested that HSPCs

could also alter their neighbors' fate by secreting soluble factors in response to infectious stimuli. Zhao *et al*. (2014) performed a single-cell cytokine analysis to illustrate that ST-HSCs and MPPs, but not LT-HSCs, secrete a variety of cytokines after stimulation with LPS and Pam_3CSK_4 in a NF-κB-dependent manner. Surprisingly, HSPCs produced huge amounts of cytokines, greater than those produced by mature myeloid cells, and IL-6 was the most prominently detected. Moreover, they were able to demonstrate that effects on myelopoiesis in response to LPS were partially dependent on IL-6 production. Interestingly, based on their response to LPS, HSPCs could be divided in largely two distinct populations: (i) one subset of HSPCs responding to TLR stimulation by rapidly turning on NF-κB and producing copious amounts of cytokines and (ii) a neighboring HSPC subset with cytokine receptors that can undergo rapid proliferation and differentiation in response to cytokine stimulation (Zhao *et al.*, 2014) **(Figure 7C)**.

Similarly, Buechler *et al.* (2016) demonstrated that CMPs directly sense a TLR7/8 ligand and then activate intracellular signaling pathways leading to the production of IFN-β, which could act in an autocrine and/or paracrine manner to promote macrophage differentiation (**Figure 7D**). This TLR-induced differentiation depended on and was amplified by type I IFNs, but IFN–β itself did not drive mature cell development (Buechler *et al*., 2016). Therefore, these results are in concordance with the observation that type I IFNs modulate HSCs behavior *in vivo* (Essers *et al*., 2009), where DAMPs could be present and may also be recognized by TLRs, but not *in vitro* when used alone (Pietras *et al.,* 2014). Moreover, Granick *et al.* (2013) reported that HSPCs produce prostaglandin E_2 (PGE_2) in *S. aureus*-infected wounds, leading to local granulocyte accumulation (**Figure 7B**). In this case, PGE_2 itself did induce proliferation and granulocyte differentiation of HSPCs (Granick *et al*., 2013).

Demand-adapted hematopoiesis in response to acute infection is beneficial as it meets the increased demand for innate immune cells consumed fighting against the pathogen. However, this adaptation of HSPCs towards myelopoiesis may also cause impairment of their function and exhaustion if HSPC activation is chronically sustained (Luis *et al*., 2016). Using similar approaches to those used by our group to address whether TLR agonists can induce HSPC differentiation *in vivo*, several studies have demonstrated that systemic LPS exposure enhances HSC proliferation in a direct TLR4-dependent manner (Herman *et al.*, 2016; Liu *et al.*, 2015; Takizawa *et al.*, 2017). Similar to chronic exposure to IL-1 or type I IFNs, chronic low-dose LPS treatment also results in a reduced HSC repopulation capacity (Esplin *et al*., 2011; Liu *et al.*, 2015). Takizawa *et al.* (2017) demonstrated that this impaired repopulating capacity of HSCs is a direct effect mediated via TLR4-TRIF, but not MyD88

signaling. Thus, while direct TLR4 activation in HSCs might be beneficial for controlling systemic infection inducing emergency myelopoiesis, prolonged TLR4 signaling has detrimental effects and may contribute to inflammation-associated HSPC dysfunction (Takizawa *et al.*, 2017).

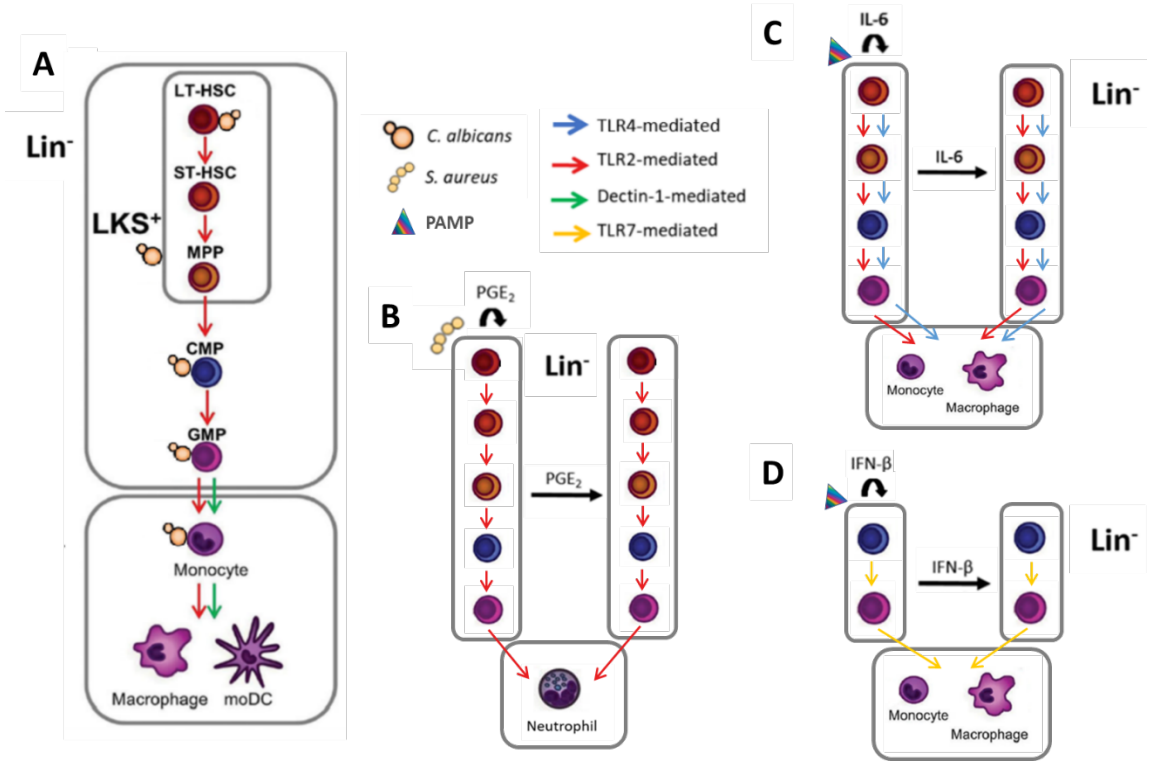


FIGURE 7 | Direct PAMP or pathogen sensing by HSPCs and translation into emergency myelopoiesis. **(A)** *C. albicans* interacts *in vitro* with different subsets of mouse hematopoietic stem and progenitor cells (HSPCs), inducing the differentiation of these cells towards the myeloid lineage in a TLR2-dependent manner. Fungal cells also induce TLR2 and Dectin-1 dependent production of moDCs by Lin⁻ cells *in vitro* and TLR2-dependent macrophage production by transplanted HSPCs upon *C. albicans* infection *in vivo*. **(B)** *Staphylococcus aureus* interacts with HSPCs and induces TLR2-dependent granulopoiesis. In response to bacteria, HSPCs also produce prostaglandin E_2 (PGE_2) that mediates myeloid differentiation in an autocrine or paracrine manner. **(C)** TLR2 and TLR4 ligands (depicted as PAMP) activate HSPCs and promote their myeloid differentiation, a process partially mediated by IL-6 secreted by different subsets of HSPCs (except LT-HSCs). **(D)** A TLR7/TLR8 ligand (depicted as PAMP) activates CMPs and promote their myeloid differentiation in part by inducing them to secrete IFN-β. Adapted from Yáñez *et al.*, 2013.

In the steady state, a small number of HSPCs is continuously mobilized to the periphery, enabling them to detect local infections and rapidly differentiate into immune cells at specific sites by extramedullary hematopoiesis (Massberg *et al.*, 2007). Thus, the migratory pool of HSPCs might act as a source of highly versatile HSPCs that can respond to infection signals locally within tissues before these signals reach the BM. In fact, acute infections induce a dramatic increase in the number of HSPCs that egress from BM to peripheral organs, particularly to spleen. Thus, although the BM is the site for most hematopoietic activities in adult mice and humans, extramedullary hematopoiesis can occur

mostly in the spleen, when the individual is under severe stress, as during infection. Therefore, it is suggested that some common features shared between the BM and the spleen might constitute a critical HSC niche component, although the cellular and molecular nature of spleen HSC niche is elusive (Wei and Frenette, 2018)

HSPCs are held within the BM by adhesive interactions and chemoattraction provided mainly by CXCL12/CXCR4 signaling, although SCF also contribute with its chemotactic activity. Molecules that modulate the extracellular matrix in the BM niche, such as metalloproteases, induce the release of HSPCs into circulation. Then, HSPCs migrate by detecting the CXCL12 gradient that guide directional cell movement toward BM, where this factor is present in higher concentrations (Ciriza *et al*., 2013). Early studies revealed that G-CSF, whose plasma levels rise rapidly during infection, has an ability to mobilize HSPCs from the BM into the blood (Hamilton and Achuthan, 2013). In fact, nowadays, in order to obtain hematopoietic progenitors for allogenic transplantation, healthy donors are treated with G-CSF and progenitors are purified from peripheral blood samples. Furthermore, it has been shown that this effect is mimicked and enhanced by yeast-derived β-glucan. Both G-CSF and/or yeast-derived β-glucan induce a proteolytic BM microenvironment that leads to the degradation of adhesive interactions between chemokines and their receptors, thus promoting HSPC mobilization (Cramer *et al.*, 2008).

3 Innate immune memory

The vertebrate immune system has conventionally been divided into innate and adaptive arms: the first one reacts rapidly and non-specifically to pathogens, whereas the latter one responds in a slower but specific manner. Other property that has been classically used to discriminate between innate and adaptive host defense mechanisms is the capacity to induce immunological memory, as for a long time it was assumed to be an exclusive hallmark of the adaptive immune response. Immunological adaptive memory has been defined as long-term acquired memory against specific antigens, leading to persistent antibody production and/or more efficient (that is, quicker and/or enhanced) activation of T cells and B cells upon a second encounter with the pathogen (Murphy and Weaver, 2017). Nevertheless, a growing body of evidence has challenged this dogma, indicating that innate immune cells can also exhibit adaptive characteristics.

In plants and invertebrates, which do not have adaptive immunity, resistance to infection following previous exposure to the same or unrelated infectious stimulus has been reported (Netea *et al.,* 2020; Netea *et al.*, 2016). In higher vertebrates, early experimental studies using certain models of vaccination described that mice showed homologous or heterologous (non-specific) protection against pathogens due to an enhanced macrophage activation (Bistoni *et al*., 1986; van 't Wout *et al*., 1992). Also, at that time, the role of macrophages in induction of endotoxin tolerance was demonstrated. In this context, it was observed that a first exposure to LPS (also referred as endotoxin) protects mice against LPS-induced lethality by generating, conversely to the previous described effect, a lower inflammatory response that prevents tissue damage (Freudenberg and Galanos, 1988). Although these effects were not initially considered attributable to innate memory, this idea has become increasingly evident with time (Cassone, 2018; Seeley and Ghosh, 2017). From these observations until now, lots of studies have reinforced the initial notion that innate immunity can be modulated by former encounters with microbes or PAMPs in mice and humans, a property that has been generally termed innate immune memory (**Figure 8**). Innate immune memory becomes relevant particularly in the context of vaccination, as it mediates in part the heterologous beneficial effects that have been reported for certain live attenuated vaccines (Goodridge *et al*., 2016). Despite the differences between innate and adaptive immune memory, both fulfil the same major function that is the induction of a quicker and stronger response against pathogens that improves host survival upon a second infection. Recently, it has been proposed a unifying model of immune memory that reflects

an evolutionary continuum link between innate and adaptive immune memory (Netea *et al.*, 2019).

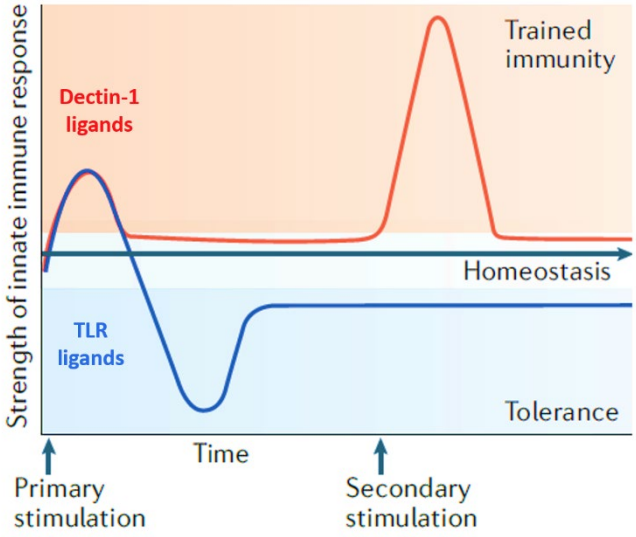


FIGURE 8 | Trained immunity and tolerance: two opposite functional programmes of innate immune memory. Infections induce inflammation and the activation of innate immune effector mechanisms. Concomitant to a pro-inflammatory response, anti-inflammatory mechanisms are provoked to limit the inflammatory response and return to homeostasis. However, this encounter with microbes or PAMPs induces changes in innate immune cells that are maintained in time and modulate secondary responses to homologous or heterologous stimulation. Dectin-1 ligands generate trained immunity, a response that involves an enhance macrophage activation to subsequent stimuli, whereas TLR ligands generate tolerance, which is characterized by a lower inflammatory response that prevents tissue damage upon secondary stimulation (Adapted from Pappas *et al.*, 2018).

The evident renewed interest in the field of innate immune memory in the last decade has prompted the scientific community to rethink the terminology used for describing the various innate memory phenomena (Boraschi and Italiani, 2018). Regarding the induced responses, in the case of decreased responses to a second challenge upon a first exposure to a TLR stimuli as LPS, the term "endotoxin tolerance" has been widely used (Biswas and López-Collazo, 2009; Cavaillon and Adib-Conquy, 2006; Medvedev *et al.*, 2006). On the other hand, the term "trained immunity" has been initially proposed for the enhanced responses to a second challenge following exposure to certain microorganisms or PAMPs (Netea *et al.*, 2011). However, the term "training" is under debate as it could be expected to result in a different response from the initial one, either higher or lower (Boraschi and Italiani, 2018, Netea *et al.*, 2020). Furthermore, it should be noted that the term "endotoxin tolerance" only refers to the final outcome of the response because this phenomenon is the result of a general reprogramming, with some effector mechanisms being decreased, while others increased or not changed (Foster *et al.*, 2007). From this point of view, the concept of trained immunity may be used as a synonymous of innate immune memory and consequently, the

last reviews in the field considered trained immunity as the general adaptation of innate immune cells rather than a particular functional programme acquisition (Netea *et al*., 2020). Despite this new definition of trained immunity, we herein will use the term innate immune memory to describe the functional reprogramming of innate immune cells that include both: an enhanced response (trained immunity) or a diminished response (tolerance) to subsequent stimulus in comparison to the primary cell response.

3.1 Endotoxin tolerance

While detection of pathogens and/or endotoxins by innate immune cells triggers a robust and essential inflammatory reaction, this process needs to be tightly regulated to protect the host from uncontrolled inflammation that leads to the manifestation of pathological states such as sepsis. Injection of high doses of LPS into animals induced the systemic production of inflammatory cytokines that led to tissue damage, dysregulation of body temperature and lethality, reason why injection of high doses of LPS has been used as an experimental model of septic shock. It was demonstrated that a pretreatment with low doses of LPS prevented animals from LPS-induced lethality, and that monocytes/macrophages were the principal cells responsible for the induction of endotoxin tolerance *in vivo*. Thus, LPS tolerance can be modeled *in vitro* using cultured monocytes or macrophages to study the molecular mechanisms underlying this decrease in the production of pro-inflammatory cytokines. LPS is recognized by TLR4 and after prolonged LPS stimulation, defects in TLR4 signaling have been observed at the level of the receptor, signaling molecules, and transcription factors, due to attenuated expression levels or protein function (Biswas and López-Collazo, 2009; Cavaillon and Adib-Conquy, 2006; Seeley and Ghosh, 2017).

This unresponsiveness against subsequent challenges with the same stimulus can be also induced by other TLR agonists, such as TLR2 ligands, whose tolerance effect has been proved *in vitro* and *in vivo* (Medvedev *et al*., 2006). Moreover, early studies of tolerance noted that treatment with one form of bacterial pyrogen would often confer tolerance to other bacterial pyrogens, phenomenon that it was referred as heterotolerance or crosstolerance. For example, early *in vitro* or *in vivo* exposure to TLR2 ligands attenuated the production of inflammatory cytokines, such as TNF-α or IL-6, following reexposure to LPS (Sato *et al.*, 2000; Wang *et al.*, 2002). Ifrim *et al.* (2014) also showed that polyI:C (a TLR3 ligand) and flagellin (a TLR5 ligand) induced crosstolerance to LPS in human peripheral blood mononuclear cells.

However, as mentioned before, endotoxin tolerance is far from being an overall unresponsiveness effect. Transcriptional characterization of tolerized macrophages upon LPS restimulation showed a profound altered response compared to unexposed cells, characterized not only by downregulation but also by upregulation of targeted genes. Most of the downregulated genes ("tolerizable" genes) encoded pro-inflammatory mediators, such as IL-6 or IL-1β, whereas upregulated genes ("non–tolerizable" genes) included genes involved in pathogen recognition and antimicrobial response such as lipocalin 2, a protein involved in innate immunity by binding to bacterial siderophores (Foster *et al.*, 2007). The existence of "non-tolerizable" genes has also been related to cross-tolerance, as prior stimulation with TLR2 ligands can selectively potentiate subsequent IFN-β production in response to TLR4 ligands. This effect is mediated by the ubiquitin ligase TRAF3, which is uniquely positioned at a common node in the IFN-inducing pathways downstream of both TLRs (Perkins *et al.*, 2013). This general reprogramming resulting in homotolerance or crosstolerance between stimuli suggests that reduced activity of signal transduction mediators is unlikely to be responsible for the core tolerance phenotype. In addition, as it will be better explained in section 3.3, several epigenetic mechanisms have been described to induce this functional reprogramming of myeloid cells.

The ability of LPS tolerance to reduce inflammatory damage in *in vivo* models of septic shock is fairly well established, although the relevance of the "non-tolerizable" genes remains somewhat unclear. However, data from several *in vivo* tolerization assays show that tolerance induced by TLR4 or TLR2 agonists is also a form of innate immune memory that may benefit the host in resisting subsequent infections. Thus, mice pretreated with LPS are less susceptible to infection by *Cryptococcus neoformans* (Rayhane *et al*., 2000), as well as mice pretreated with TLR2 ligands are less susceptible to acute polymicrobial peritonitis, polymicrobial sepsis (by cecal ligation and puncture) or coinfection by *S. aureus* and *Salmonella typhimurium* (Feterowski *et al*., 2005; Wang *et al.*, 2002). *In vitro* models have also demonstrated that endotoxin-tolerized monocytes exhibit an increased phagocytic ability coupled with a conserved capacity to kill internalized pathogens, albeit with an impaired antigen presentation capacity (del Fresno *et al*., 2009).

Tolerance induced by exposure to TLR4 and TLR2 ligands also affects DCs, which show diminished expression of the pro-inflammatory cytokines IL-12, TNF-α and IL-6, but 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 mediator of DC anti-inflammatory activity (Albrecht *et al.*, 2008; Fallarino *et al.*, 2015).

3.2 Trained immunity

Many studies in mice have documented that immunization with certain live microorganisms or microbial components that activate innate immune cells confers protection against subsequent lethal infection in a non-specific manner. This protective effect has been described for various PAMPs: (i) resistance to infection by *S. aureus* is induced by fungal β-glucans treatment, (ii) the peptidoglycan component muramyl dipeptide (NOD2 ligand) protects against *Streptococcus pneumonia* and *Toxoplasma gondii*, (iii) CpG treatment protects against *E. coli* meningitis, and (iv) flagellin protects against *Streptococcus pneumoniae* and rotavirus (Goodridge *et al.*, 2016). Bacille Calmette-Guérin (BCG) vaccine (that is a *Mycobacterium bovis* live strain) was reported to protect mice against secondary infections with *C. albicans* or *Schistosoma mansoni* through T-cell independent mechanisms (Tribouley *et al.*, 1978), involving activated tissue macrophages (van't Wout *et al*., 1992). Similarly, it has been shown that mice vaccinated with an attenuated strain of *C. albicans* (PCA2) were protected from systemic candidiasis caused by a virulent *C. albicans* strain, as well as from a lethal challenge with other *Candida* species, other fungal genera (*Aspergillus* and *Cryptococcus*), and even bacteria (*S. aureus*) (Bistoni *et al.*, 1986). This protection, which could also be induced in athymic mice, was dependent on macrophages and pro-inflammatory cytokine production, both being prototypical innate immune components (Bistoni *et al.*, 1986; Bistoni *et al*., 1988; Vecchiarelli *et al*., 1989).

It is worth to note that PCA2-immunized mice remained chronically infected by the immunizing agent, as BCG causes a mild chronic infection for up to one month after vaccination. Research has long neglected the study of chronic infection by silent pathogens, such as *herpesviruses* or *cytomegalovirus*, but it has now become clear that persistence of a pathogen can affect the ability of the immune system to react to a new unrelated infection. This phenomenon has been termed the "Mackaness effect" in reference to the seminal work of Mackaness in 1964 demonstrating cross-protection among three intracellular bacteria: *Listeria monocytogenes*, *Brucella abortus* and *Mycobacterium tuberculosis* in mice (Muraille, 2015). A later study showed that herpesvirus latent infection can confer beneficial T cell-independent protection against *L. monocytogenes* and *Yersinia pestis*, owing to sustained systemic macrophage activation (Barton *et al.*, 2007). Indeed, these observations seem to be examples of trained immunity, although it has been recently highlighted the need to distinguish such examples of protection from immune memory (Netea *et al.*, 2019). Importantly, during induction of immune memory, between the first infection and the reinfection, the immune status functionally returns to a low basal state. In contrast, the functional immune status in chronic infections does not return to the low basal state

existing before the insult. Therefore, this process has been called "immune differentiation" and it is defined as a form of adaptation through long-term changes in immune responses determined by a constant change in the environmental conditions or due to a chronic infection, leading to a new functional state (Netea *et al*., 2019).

Quitin *et al.* (2012) got to reproduce the results from Bistoni *et al.* (1986) using other protective model of immunization based on preinjection of a low/non-lethal dose of the same virulent *C. albicans* strain used for the second lethal challenge, instead of using the non-virulent PCA2 strain. Also, by vaccinating T/B cell-defective Rag1-deficient and CCR2-deficient mice, they showed that vaccine-induced protection was T cell-independent but monocyte-dependent, as Rag1 KO mice but not CCR2 KO mice were as resistant as WT mice to a lethal systemic candidiasis (Quintin *et al.*, 2012). As LPS tolerance, trained immunity can be modeled *in vitro* using cultured monocytes or macrophages to study the molecular mechanisms underlying this myeloid-induced protection. It has been shown that *C. albicans* and β-glucans primed *in vitro* the production of pro-inflammatory cytokines, such as TNF-α and IL-6, in response to several stimuli after a resting period of up to two weeks (Quintin *et al.*, 2012). Moreover, in a human trial, vaccination with BCG resulted in enhanced IL-6, TNF-α and IL-1β production by monocytes for up to three months after vaccination when cells were stimulated *in vitro* with *M. tuberculosis*, *S. aureus*, or *C. albicans* (Kleinnijenhuis *et al.*, 2012). This long-lasting effect has opened a debate about the possible difference between priming and innate immune memory. Netea *et al.* (2019) define priming as a term to describe increased responses to a secondary stimulus, although it is often an acute process that does not involve long-term memory effects (that would persist for months or years) (Netea *et al.*, 2019). Nevertheless, the immunological phenotype of trained immunity in humans after BCG vaccination has been proven after three months and even one year (Kleinnijenhuis *et al.*, 2014; Kleinnijenhuis *et al.*, 2012).

Similar to LPS tolerance and in contrast to adaptive immune responses, epigenetic reprogramming of transcriptional pathways, rather than gene recombination, mediates trained immunity (see section 3.3). Therefore, by exploring the epigenetic profiling and validating the most relevant information with transcriptional analysis, it has been proved that trained monocytes reinforce some innate immune signaling pathways. As expected, among them are those leading to pro-inflammatory cytokine production, by upregulating the expression of CLRs and TLRs, such as Dectin-1, TLR2 and TLR4, as well as downstream adaptors as MyD88 (Quintin *et al.*, 2012; Saeed *et al.*, 2014). In addition to immune signaling pathways, several studies have revealed extensive rewiring of metabolic pathways in myeloid cells upon activation (O'Neill and Pearce, 2016). The importance of cellular

metabolism for proper innate immune responses suggests that similar mechanisms may play a role in the long-term functional changes in monocytes and macrophages during innate immune memory. Epigenetic profiling of β-glucan-trained monocytes identified an increase of activation marks in the promoters of genes encoding enzymes involved in glycolysis (such as hexokinase or pyruvate kinase) and its master regulator mTOR (mammalian target of rapamycin), as well as glycolytic genes that are targets of the transcription factor HIF-1α (hypoxia inducible-1α). These observations were validated by biochemical studies in trained monocytes that revealed an elevated rate of aerobic glycolysis with a reduced basal respiration rate. The Dectin-1/AKT/mTOR/HIF-1α pathway was responsible for this metabolic shift, known as "Warburg effect" (Cheng *et al.*, 2014). Regarding lipid metabolism, enhanced cholesterol biosynthesis is also an important hallmark of β-glucan-trained monocytes (Netea *et al.*, 2020).

The cellular bases for the protection induced by trained immunity during bacterial and fungal infections reside in the functional reprogramming of myeloid cells, mainly macrophages and monocytes. However, it has been shown that NK cells can also respond more vigorously after previous encounters with pathogens. Indeed, studies of Cytomegalovirus infection have reported that NK cell activation induces a protective response against reinfection by rapidly degranulating and releasing cytokines (Sun and Lanier, 2009). The heterologous protective effects of BCG vaccination have also been linked with activation of NK cells. In this context, BCG-vaccinated individuals showed an enhanced pro-inflammatory cytokine production by NK cells in response to mycobacteria or other unrelated pathogens. Moreover, experimental studies in mice have shown that NK cells participate in the non-specific protection conferred by BCG vaccination against *C. albicans* infection (Kleinnijenhuis *et al.*, 2014). However, the specificity of the NK memory immune responses is a complex issue. It has been demonstrated in mice that NK cell memory induced by Cytomegalovirus or hapten sensitization was specific to the priming agent. This specific NK memory has also been evidenced in primates, which once vaccinated, exhibit NK cells able to efficiently lyse antigen-specific but not antigen-unspecific targets five years after vaccination (Goodridge *et al.*, 2016; Netea *et al*., 2020; Netea *et al*., 2016). As these adaptive NK cell responses more closely resemble T cell than trained immune macrophage responses, they may represent an evolutionary bridge between T cell memory and trained myeloid cell responses (Netea *et al*., 2019).

Recent work has shown that DCs can also exhibit immune memory responses. DCs isolated from mice exposed to *C. neoformans* displayed strong IFN-γ production and enhanced pro-inflammatory cytokine responses on subsequent challenge, which is

indicative of memory response. These effects were dependent on epigenetic changes and were impaired by the treatment of mice with histone methyltransferase inhibitors (Hole *et al*., 2019). On the other hand, the trained immunity concept has been recently proposed for non-immune cell types, such as stromal and epithelial cells. In tissues particularly exposed to the external environment containing pathogens, such as skin, lung and gut, epithelial and stromal cells can possess the capacity to modulate their responses to successive encounters with the pathogen (Cassone, 2018).

A relevant issue to be considered regarding innate immune memory is the lifespan of innate immune cells, particularly monocytes and macrophages derived from them. As mentioned before, human trained monocytes can be observed in the circulation for at least three months after BCG vaccination (Kleinnijenhuis *et al*., 2014; Kleinnijenhuis *et al.*, 2012). This observation suggests that reprogramming also takes place at the level of progenitor cells to account for the persistence of modified populations of the relatively short-lived monocytes/macrophages. Indeed, the effects of microbial exposure on innate immune memory can be transferred from HSPCs to their progeny. For instance, HSPCs that are exposed to TLR2 agonists generate macrophages that produce lower amounts of inflammatory cytokines and ROS (Yáñez, *et al*., 2013b).

3.3 The molecular bases of innate immune memory: epigenetic reprogramming

The central feature of innate immune memory is the ability of PAMP-exposed cells to mount a qualitatively and quantitatively different transcriptional response compared to unexposed cells when challenge with subsequent stimuli. The molecular bases of this altered responsiveness is only partially understood, but evidence supports the convergence of multiple regulatory mechanisms that lead to an epigenetic reprogramming of myeloid cells after being initially exposed to PAMPs, which may explain the long-lasting effects of pretreatments on gene expression. These regulatory mechanisms include changes in chromatin organization, DNA methylation and transcription of non-coding RNAs, such as miRNAs and/or long non-coding RNAs (lncRNAs) (Domínguez-Andrés *et al.*, 2020; Netea *et al.*, 2020; Netea *et al*., 2016; Seeley and Ghosh, 2017).

Modulation of pro-inflammatory cytokine production is one of the most characteristic traits of innate immune memory. In myeloid cells, inflammatory gene transcription is strictly regulated to be highly induced, but only under certain specific conditions. Thus, upon a primary stimulation with PAMPs, many loci containing inflammatory genes in a

repressed configuration gain in accessibility by chromatin changes, resulting in the activation of gene expression (that is hundreds of times higher than baseline levels) in a short window of time. This activation of gene expression, driven by the recruitment of stimulation-responsive transcription factors, such as NF-κB or AP-1, is accompanied by chromatin organization changes that include covalent histone modifications and nucleosome remodeling. It has been demonstrated that, in trained monocytes, all of these changes are only partially removed after cessation of the stimulus, allowing the maintenance of an activated gene expression after subsequent challenges (Domínguez-Andrés *et al*., 2020; Netea *et al*., 2020; Netea *et al*., 2016; Seeley and Ghosh, 2017)(**Figure 9**).

Histone acetylation is one of the best-studied histone modifications and it generally correlates with the levels of gene expression, whereas the effect of histone methylation depends on the exact residue targeted and the degree of methylations. Regarding the role of these mechanisms in mediating trained immunity, some studies have described a new class of enhancers that undergo H3K4 methylation and H3K27 acetylation during the first encounter with the stimulus. However, when the stimulus has ceased, only H3K4me1 persists at these latent enhancers and constitutes the basis for a faster and enhanced response after second stimulation (Álvarez-Errico *et al*., 2015; Netea *et al*., 2016). In this context of reestimulation, stable enrichment in H3K4me3 at the promoters of pro-inflammatory genes has been also associated with monocyte training in different experimental models, such as by β-glucan stimulation or BCG vaccination (Kleinnijenhuis *et al.*, 2012; Quintin *et al.*, 2012). Moreover, the acquisition of H3K27ac is another key epigenetic mark that accompanies trained immunity, mainly observed in distal enhancers (Saeed *et al.*, 2014) (**Figure 9**).

The contribution of DNA methylation changes to trained immunity has been less explored, given the presumed stability of this type of modification. However, new studies have suggested that individuals able to undergo monocyte reprogramming after BCG vaccination displayed a wide loss of DNA methylation among promoters of genes belonging to immune pathways compared to individuals characterized as non-responders (Álvarez-Errico *et al*., 2015; Netea *et al*., 2020) (**Figure 9**).

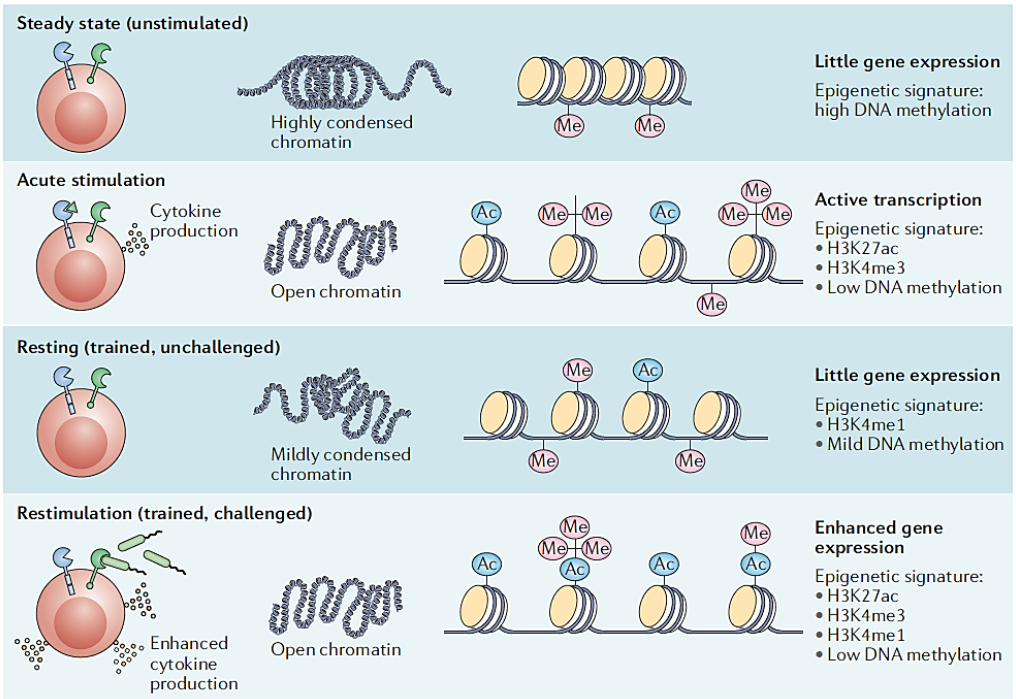


FIGURE 9 | Epigenetic reprogramming underlying the induction of trained immunity. Highly condensed chromatin blocks expression of pro-inflammatory genes in steady state (unstimulated cells); upon first stimulation with PAMPs, epigenetic changes (acetylation, methylation) causes opening of chromatin structure allowing upregulation of gene expression. Following this former stimulation, epigenetic signatures/marks are in part maintained in resting (trained, unchallenged) cells, which allow a stronger upregulation upon subsequent restimulation (trained challenged cells). See text for further details. (Netea *et al*., 2020).

On the other hand, endotoxin tolerance has been associated with the lack of the transcription-activating H3K4me3 mark. However, as already mentioned in section 3.1, the restimulation of tolerant macrophages with LPS produces two main types of gene-expression programmes: one set of "tolerizable genes" show diminished or abolished expression, whereas a second group on "non-tolerizable genes" increase their expression or remain unchanged. The transcription-activating H3K4me3 mark is imprinted on the promoters of both group of genes but is only maintained on the promoters of "non-tolerizable" genes. Therefore, the failure to maintain active histone marks may be behind the lack of responsiveness in this innate immune memory phenomenon (Foster *et al*., 2007; Seeley and Ghosh, 2017).

Various epigenetic modifications are closely associated with metabolic processes, since histone-modifying enzymes require metabolites as substrates or cofactors. Thus, metabolic rewiring that accompanies trained immunity may influence the epigenomic reprogramming of myeloid cells after being initially exposed to PAMPs. Trained monocytes accumulate the Krebs-cycle metabolite fumarate, which influences epigenetic reprogramming by downregulating the activity of histone KDM5 demethylases (Netea *et al.*, 2020). Links

between metabolic and epigenetic changes have also been demonstrated in LPS-induced tolerance, in which histone deacetylases coordinates a switch from glucose to fatty acid oxidation (Netea *et al*., 2016).

Furthermore, the non-coding portion of the genome has been shown to play an integral role by regulating the transcription of inflammatory genes. This significant portion of the genome is transcribed into a highly diverse family of RNAs, including miRNAs and lncRNAs. miRNAs bind to the complementary sequences of target mRNAs and promote their degradation or more directly arrest translation, whereas lncRNAs act at various stages of the transcriptional program to either amplify or repress gene activity (Domínguez-Andrés *et al.*, 2020; Seeley *et al.*, 2017). Regarding the role of miRNA in innate immune memory, miR-146a was the first miRNA described to be upregulated in tolerized monocytes and able to partially induce LPS desensitization, effect that has been related to its ability to downregulate the NF-κB pathway (Cavaillon and Adib-Conquy, 2006). By contrast, miRNAs known as activators of the inflammatory response, such as miR-155, may also contribute to trained immunity, possibly due to the repression of phosphatases that negatively regulate transducers of several signaling pathways (Netea *et al*., 2020; Netea *et al.*, 2016).

The contribution of lncRNAs in the regulation of trained immunity has been explored using novel bioinformatic tools. These studies have revealed that chromatin is folded into DNA loops, which are spatially segregated into topologically associated domains, probably involved in gene regulation (Domínguez-Andrés *et al.*, 2020). It was previously shown that based on their secondary structure, lncRNAs can interact with other proteins, allowing chromatin machinery to be delivered (or sequestered from) appropriate sites (Seeley and Ghosh, 2017). Chromosomal looping within topologically associating domains has been shown to bring distally located genes that encoded for lncRNAs adjacent to target genes, to regulate their transcriptional activation. For example, chromosomal looping is used by a specific lncRNA to direct an epigenetic remodeling complex across the CXCL chemokine promoters, that enables the H3K4me3 epigenetic priming of these promoters to upregulate their expression. Importantly, this mechanism also modulates other key genes of the trained innate immune response such as IL-6 and IL-1β (Domínguez-Andrés *et al.*, 2020). Furthermore, differential recruitment of nucleosome remodeling components to gene promoters in naïve and tolerized cells has also been described to contribute to changes in LPS-induced gene expression (Seeley and Ghosh, 2017).

OBJECTIVES

In accordance with the background information provided in the Introduction, the general objectives of this PhD thesis have been the following:

1. **Studying the effects of HSPC exposure to PAMPs on the antifungal phenotype of the macrophages they generate.**

   1.1. Analyzing the consequences of PRR signaling in HSPCs for the macrophages they produce *in vitro* in M-CSF or GM-CSF cultures.

   1.2. Analyzing the consequences of systemic candidiasis and *in vivo* TLR2 agonist exposure for the macrophages they produce *ex vivo* in M-CSF cultures.

2. **Studying the effects of HSPC exposure to PAMPs on their antifungal response.**

   2.1 Analyzing the *in vivo* role of HSPCs against *C. albicans* infection in a model of extended TLR2 agonist treatment.

   2.2 Analyzing the effector mechanisms of HSPCs in response to PAMPs.

3. **Studying the effects of PRR signaling in HSPCs on the maturation of the APCs they generate, as well as on their ability to activate and polarize $CD4^+$ T cell responses.**

MATERIALS AND METHODS

1 Mice

C57BL/6 mice were purchased from Envigo or The Jackson Laboratory, whereas OVA-specific-TCR-transgenic (OT-II) mice were purchased only from The Jackson Laboratory. TLR2 $^{-/-}$ mice (C57BL/6 background) were provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) and bred and maintained at the animal production service facilities (SCSIE, University of Valencia).

Animals of both sexes between 8 and 24 weeks' old were used. The studies were carried out in strict accordance with the "Real Decreto 1201/2005, BOE 252" for the Care and Use of Laboratory Animals of the "Ministerio de la Presidencia," Spain. Moreover, the protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Valencia, Generalitat Valenciana (Permit Numbers: 2014/072 type 2, 2017/VSC/PEA/00004; 00024; 00084 and 2019/VSC/PEA/0126). The studies with OT-II mice were carried out at Cedars-Sinai Medical Center and protocols were approved by its Animal Care and Use Committee.

2 Culture media

2.1 Culture media for fungi

YPD media	
Yeast extract	10 g
Peptone	20 g
Glucose	20 g
Distilled water pH 6-7	1 liter

Sabouraud Dextrose Agar media	
Pancreatic digest of casein	5 g
Peptid digest of animal tissue	5 g
Dextrose	40 g
Agar	15 g
Distilled water pH 5.6 ± 0.2	1 liter

Simplified synthetic Lee media	
$(NH_4)_2SO_4$	5 g
$MgSO_4$. $7H_2O$	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 pH 6,8-7	1 liter

2.2 Culture media for murine cells

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

RPMI complete media for macrophage differentiation	
RPMI 1640 with GlutaMAX™ I (Gibco)	
Heat-inactivated fetal bovine serum (FBS) (Gibco)	5 %
Penicillin-streptomycin stock solution (Gibco)	1 %
SCF (Preprotech)	20 ng/ml
Or M-CSF (Miltenyi Biotec)	50 ng/ml
Or GM-CSF (Preprotech)	50 ng/ml

RPMI complete media for APC differentiation and culture	
RPMI 1640 with GlutaMAX™ I (Gibco)	
Heat-inactivated fetal bovine serum (FBS) (Gibco)	10 %
Penicillin-streptomycin-glutamine stock solution (Gibco)	1 %
GM-CSF (Preprotech)	20 ng/ml

RPMI complete media for T cell culture	
RPMI 1640 with GlutaMAX™ I (Gibco)	
Heat-inactivated fetal bovine serum (FBS) (Gibco)	10 %
Penicillin-streptomycin-glutamine stock solution (Gibco)	1 %
Sodium Pyruvate (Gibco)	1mM
2-Mercaptoethanol (Sigma-Aldrich)	50 µM

Serum-free media for HSPC culture	
StemPro™-34 SFM (Gibco)	
L-glutamine (Gibco)	2 mM
Penicillin-streptomycin stock solution (Gibco)	1 %
SCF (Preprotech)	20 ng/ml
Fms-like tyrosine kinase 3 (Flt3) ligand (Preprotech)	100 ng/ml

3 Microbial stimuli

Fungal strains used in this thesis were: *C. albicans* ATCC 26555, a virulent strain commonly used in studies with this fungus; *C. albicans* PCA2, a low-virulence non-germinative strain, provided by Dr. Cassone (Istituto Superiore di Sanità, Roma, Italia); and *Candida glabrata* CECT1448, one of the most prominent non-albicans *Candida* specie.

As *in vitro* stimuli, we used pure TLRs and Dectin-1 ligands, as well as inactivated yeasts prepared as described below. In particular, the PAMPs used were: the TLR2 ligand Pam_3CSK_4, (1 μg/ml or 100 ng/ml), the TLR4 ligand Ultrapure *Escherichia coli* LPS (100 ng/ml) and the Dectin-1 agonist depleted zymosan (1-100 μg/ml), inactivated *C. albicans* ATCC 26555 yeasts (1:7.5 or 1:12, murine cell: yeast ratio) and inactivated *C. albicans* PCA2 yeasts (1:12 murine cell: yeast ratio). Pure PRR agonists were purchased from Invivogen. The concentrations used on each assay will be indicated in their respective sections. The TLR2 ligand Pam_3CSK_4 was also used for *in vivo* stimulation of HSPCs (see section 6).

Inactivated *C. albicans* PCA2 and *C. albicans* ATCC 26555 used as *in vitro* stimuli were prepared as follows. Firstly, cells were cultured in endotoxin-free YPD media, at 28 °C with shaking, up to the late exponential growth phase (OD $_{\lambda 600}$ 0.6-1). Cellular growth was determined by measuring the optical density (OD) at a wavelength of 600 nm in a Heλios spectrophotometer (Thermo Fisher Scientific). Then, fungal cells were resuspended in endotoxin-free water, and maintained for 3 h at 28°C with shaking, and afterwards at 4 °C for 24 h (starved yeast cells). Those yeast cells were inoculated in symplified synthetic Lee media (in 5 times more volume than the one used in water incubation) and incubated for 3 h at 28 °C. For inactivation, yeast cells were resuspended (20×10^6 cells/ml) in BD Cytofix™ Fixation Buffer (BD Bioscience) containing 4 % paraformaldehyde and incubated for 30 min at room temperature (RT). After treatment, fungal cells were extensively washed in phosphate-buffered saline (PBS) (Gibco) to eliminate the inactivating agent, cell concentration was determined by microscopically counting and cells were maintained at -80 °C as dry pellet.

Viable fungal cells used for *in vitro* killing assays (*C. albicans* PCA2 and *C. glabrata* CECT1448) were obtained culturing cells in endotoxin-free YPD media, at 28 °C with shaking, up to the late exponential growth phase. Fungal cells were collected, washed with endotoxin-free water twice and brought to the desire cell density in RPMI complete media. For *in vivo* assays (*C. albicans* ATCC 26555), cells were cultured in endotoxin-free YPD media, at 28 °C with shaking, up to the late exponential growth phase. Then, fungal cells

were resuspended in endotoxin-free water, and maintained for 3 h at 28 °C with shaking, and afterwards at 4 °C for 48 h (starved yeast cells). Finally, those cells were resuspended in PBS to the desired concentration for their injection to mice.

4 Isolation of HSPCs

HSPCs were purified from mouse BM as Lin^- cells by immunomagnetic cell sorting with MicroBeads. Firstly, to obtain murine BM, C57BL/6 mice were euthanized by cervical dislocation and animal surface was sprayed with 70 % ethanol. Next, mice were placed on its back, an incision of the skin was made in the ankle of both lower extremities and the skin covering them was removed (**Figure 1A**). The muscles were cut off using scissors and the acetabulum was dislocated form the hip joint, while avoiding breaking the femur head (**Figure 1B**). The femur was separated from the tibia at the knee joint and the remaining muscles from the bones were removed using tissue paper (**Figure 1C**). Each bone was rinsed with 70 % ethanol and then placed in a clean 5 cm plate. The following steps were performed under sterile conditions, so bones were introduced in a tissue culture hood. After waiting a few minutes to ensure ethanol evaporation from the surface of the bones, inside a clean sterile 5 cm plate, the epiphyses of each bone were cut off. Using a 25-gauge needle and a 10 ml syringe filled with pre-cooled PBS, femurs and tibias were flushed onto a 50 ml tube (**Figure 1D** and **1E**). Cell suspension was filtered using a 70 μm filter and then centrifuged at 450 g for 5 min.

HSPCs were purified from BM cell suspension by magnetic cell separation using the "Lineage Cell Depletion Kit, mouse" and the QuadroMACS™ Separator (both from Miltenyi Biotec). Cells were magnetically labeled with an antibody cocktail against a panel of mature immune lineage antigens [CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119] following a two-step procedure. BM cell suspension was first labeled with the cocktail of biotinylated antibodies followed by labeling with anti-biotin MicroBeads. Cell sorting was performed in a MACS column placed in a powerful permanent magnet that induces a high-gradient magnetic field; cells being labeled with MACS MicroBeads are retained in the column while unlabeled cells flow through it. Following this principle, effluent that contains the enriched Lin^- cell fraction was collected. Cell suspension was centrifuged at 450 g for 5 min, cell pellet was resuspended in complete cell culture media (different depending on each assay) and cells were counted using the Countess ™ II FL Automated Cell Counter

(Thermo Fisher Scientific). The yield of Lin^- using this method was approximately 0.7-1.5 million per 8-24 week-old C57BL/6 mouse.

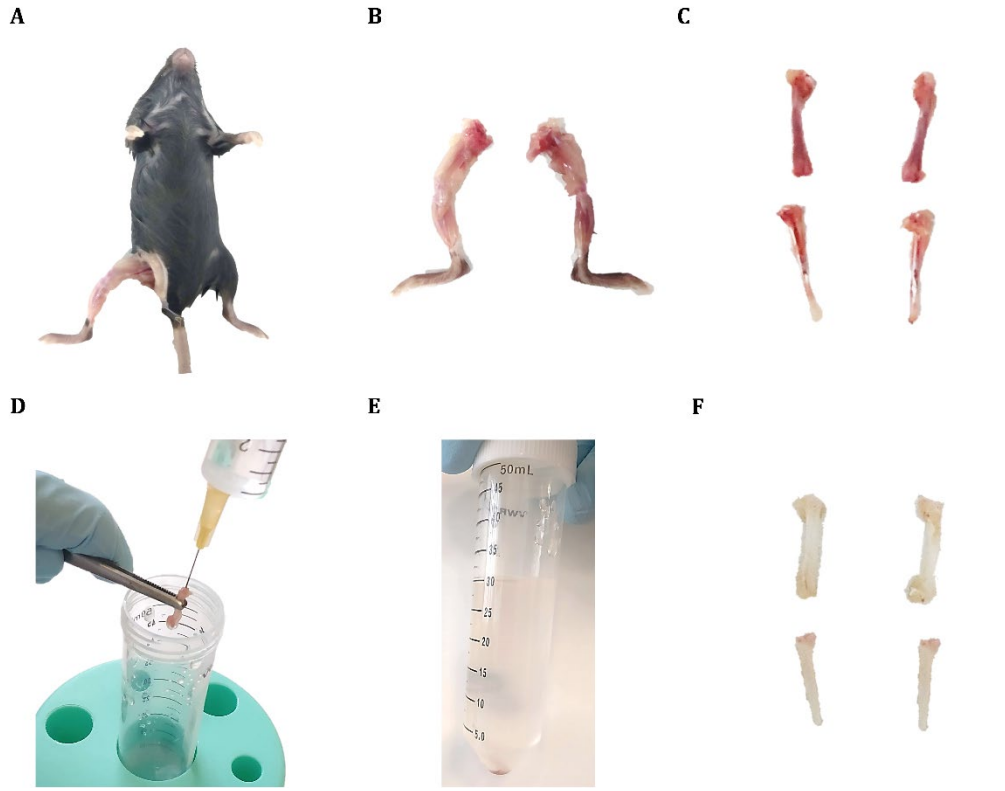


FIGURE 1 | Mouse bone marrow (BM) isolation. Set of images showing the different stages of mouse BM isolation process. Blanching of bones, showed on image F, indicated that cells have been sufficiently scraped.

5 *In vitro* differentiation of HSPCs to macrophages or APCs

Macrophages were obtained from HSPCs culturing them in a RPMI complete media containing the myelopoietic growth factors M-CSF or GM-CSF. Moreover, SCF was added to support the survival of HSPCs (cytokine concentrations detailed on section 2.2). 2×10^5 Lin^- cells (M-CSF cultures) or 1×10^5 (GM-CSF cultures) were plated in 4 ml of RPMI complete medium, in a 55 mm non-tissue culture-treated (non-TC) plate, and cultured at 37°C in a 5 % CO_2 atmosphere for seven days. At day seven, adherent cells were harvested discarding cell media and adding precooled MACS buffer [PBS + 5 % FBS + 2mM EDTA (Gibco)] for gently scraping them. Collected macrophages were counted and replated in 96-well plates with RPMI complete medium at different cell densities for cytokine, phagocytosis or killing

assays (detailed in each section). Cells were also labeled with antibodies to analyze the expression of some surface molecules (see flow cytometry section).

For APC differentiation, 5×10^5 Lin^- cells were plated in 20 ml of RPMI complete media in the presence of GM-CSF (media composition detailed in section 2.2), in a 15 cm non-TC plate, and cultured at 37 °C in a 5 % CO_2 atmosphere. The media was changed on day three as follows: the existing media containing non-adherent cells was reduced to 5 ml and 15 ml fresh RPMI complete media was added. APCs were collected on day six and for collection, media containing non-adherent cells were aspirated and discarded. Then PBS + 2 mM EDTA was added to the plate and cells were incubated for 20 min at 37 °C in a 5 % CO_2 atmosphere. Adherent cells were then harvested washing the plate several times with the PBS + 2 mM EDTA added, promoting cells to detach from the surface. Collected APCs were counted and replated with RPMI complete media at different concentrations for cytokine production, APC and T cell coculture or flow cytometry assays (detailed in each section). Cells were rested for 4 h prior to stimulation.

6 *In vitro* and *in vivo* HSPC stimulation protocol

Stimulation of HSPCs during the *in vitro* macrophage differentiation process was performed following two strategies: continuous exposure or transient exposure to stimuli (**Figure 2A**). Continuous exposure was based on the presence of PRR agonists or inactivated *C. albicans* cells throughout the whole culture period necessary for myeloid differentiation. Lin^- cells differentiated in the presence of M-CSF or GM-CSF were continuously exposed to 1 μg/ml Pam_3CSK_4, 100 ng/ml LPS, 1 μg/ml depleted zymosan or inactivated *C. albicans* ATCC 26555 yeasts (1:7.5 murine cell: yeast ratio). Otherwise, HSPCs were transiently exposed to 1 μg/ml Pam_3CSK_4 or 100 ng/ml LPS, both soluble ligands, by adding these agonists for the first 24 h of culture and then removing them by thoroughly washing the cells. Myeloid cells differentiated from HSPCs in the absence of microbial components were used as control cells.

In APC differentiation from HSPCs, the effect of *in vitro* PRR exposure was evaluated following the transient strategy (**Figure 2B**). Lin^- cells were plate at 5×10^5 cells /1 ml in 24-well non-TC plates in the presence or absence (control) of TLR2 or Dectin-1 agonists for 24 h. Then, the PRR agonists were eliminated by washing twice the cells and “clean” cells

were plate in a 15 cm non-TC plate. The TLR2 ligand, as a soluble ligand, will be entirely removed after washing the cells, as mentioned before. Moreover, we checked that depleted zymosan particles (the Dectin-1 ligand used) were fifty percent eliminated in the first wash and completely eliminated at differentiation day three, after changing the cell culture media (**Figure 2C**).

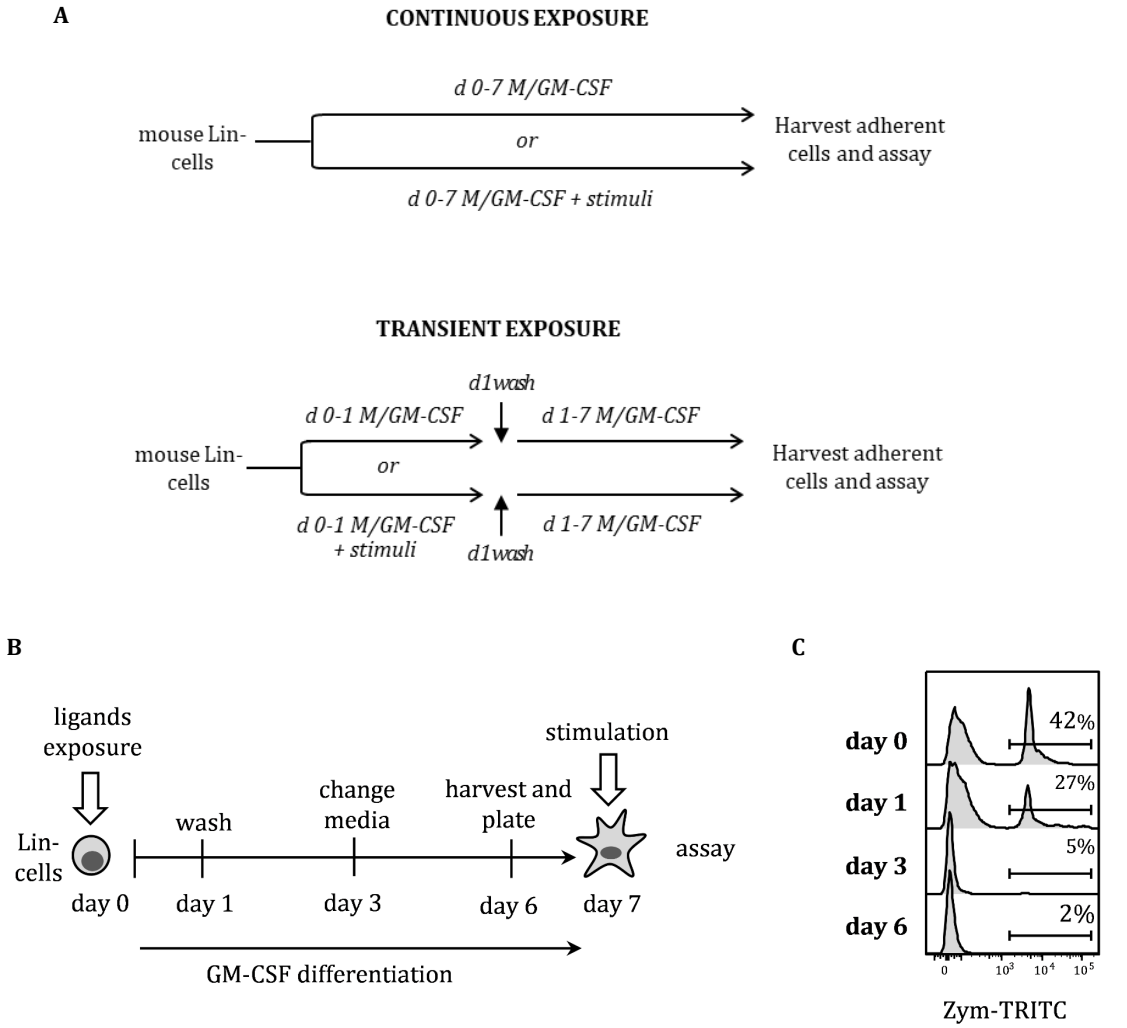


FIGURE 2 | Continuous or transient exposure of HSPCs to PAMPs during *in vitro* differentiation towards macrophages or Antigen Presenting cells (APCs). (**A**) Purified Lin⁻ cells (HSPCs) from BM of C57BL/6 mice were cultured with Macrophage-Colony Stimulating Factor (M-CSF) or Granulocyte and Macrophage-Colony Stimulating Factor (GM-CSF) or for seven days to induce macrophage production, in the presence or absence of different microbial stimuli for the entire seven days (continuous exposure) or the first 24 h only (transient exposure). The microbial stimuli used were 1 μg/ml Pam_3CSK_4, 100 ng/ml Lipopolysaccharide (LPS), 1 μg/ml depleted zymosan or inactivated *C. albicans* yeasts (1:7.5 murine cell:yeast ratio). At day seven, adherent cells were harvested and analyzed. Non-exposed cells were used as control cells. (**B**) Lin⁻ cells were exposed to 100 ng/ml Pam_3CSK_4 or 10 μg/ml depleted zymosan for 24 h. Then, cells were washed twice to eliminate the stimuli and replated. At day three, media was changed, and at day six, adherent cells were harvested and replated. Mature APCs cells were stimulated for 24 h and then, they were analyzed. (**C**) Fluorescent zymosan particles were used for HSPC stimulation following the schematic protocol exposed in Figure 2B. At day zero, those particles represent 42 % of the total events detected using flow cytometry. At day 1, after washing cells twice, this percentage diminished to 27 % and at day three, after changing cell culture media, only 5 % of detected events were fluorescent zymosan particles.

We also evaluated the effect of *in vivo* HSPC stimulation on the phenotype of the macrophages derived from them. For that purpose, mice were treated with Pam_3CSK_4 or challenged with *C. albicans* ATCC 26555. Mice exposure to Pam_3CSK_4 was performed following two strategies: short treatment or extended treatment with the TLR2 ligand. For the short treatment model, C57BL/6 mice were injected intravenously (i.v.) with one dose of Pam_3CSK_4 (100 µg) (**Figure 3A**). For the extended treatment, we used a model previously described by Hernan *et al*. (2016); C57BL/6 mice were given 100 µg of Pam_3CSK_4 by intraperitoneal injection (i.p.) at days zero, three and five (**Figure 3B**). In both cases, C57BL/6 mice injected with one or three doses of PBS were used as control mice. 24 h after the last dose of Pam_3CSK_4, mice were euthanized and HSPCs were purified to differentiate them into macrophages in the presence of M-CSF, as it has been explained in section 5. Then, the functional phenotype of mature macrophages was analyzed performing cytokine and killing assays (detailed in each section).

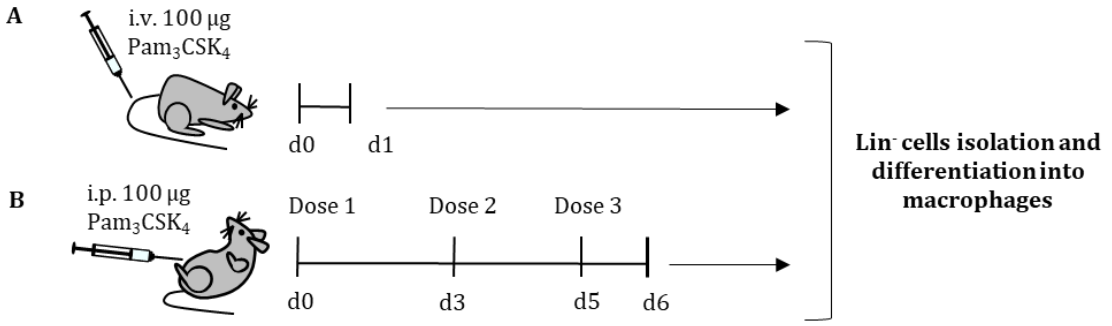


FIGURE 3| *In vivo* Pam_3CSK_4 stimulation protocol. C57BL/6 mice were treated with Pam_3CKS_4 following the indicated schedule. 24 h after the last dose of Pam_3CKS_4, mice were euthanized to isolate HSPCs and differentiate them into macrophages in M-CSF cultures. Then, functional phenotype of mature myeloid cells was evaluated.

7 *C. albicans* infection model

We used an experimental infection model of systemic candidiasis based on i.p. injection of *C. albicans* ATCC 26555, as previously described by our group (Villamón *et al.*, 2004b). To *in vivo* expose HSPCs to *C. albicans*, C57BL/6 mice were challenged with 45×10^6 starved yeasts in 200 µl of PBS (prepared as described in section 3). Moreover, susceptibility to infection was also analyzed in non-exposed and Pam_3CSK_4-exposed mice following an extended treatment. For these experiments, mice were injected with 30×10^6 starved yeasts. To assess the tissue outgrowth of the microorganism, the fungal burden in the kidney and the spleen at 24 h or 72 h post-infection was determined. The organs 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 °C and expressed as CFUs per gram of tissue.

Moreover, HSPCs were purified from BM of infected and non-infected mice to differentiate them into macrophages in the presence of M-CSF, as it has been explained in section 5. In order to prevent potential fungal growth in cultures, differentiation was performed in the presence of 2.5 μg/ml amphotericin B. Then, the functional phenotype of mature macrophages was analyzed performing cytokine and killing assays (detailed in each section) (**Figure 4**).

In order to deplete c-Kit progenitors in Pam_3CSK_4-exposed mice and evaluate the effect of this depletion on the susceptibility to *C. albicans* infection, 500 μg of the anti-c-Kit antibody ACK2 (eBioscence) or isotype control (rat IgG2b, clone eB149/10H5, from eBioscence) were given to mice i.p. two days before infection.

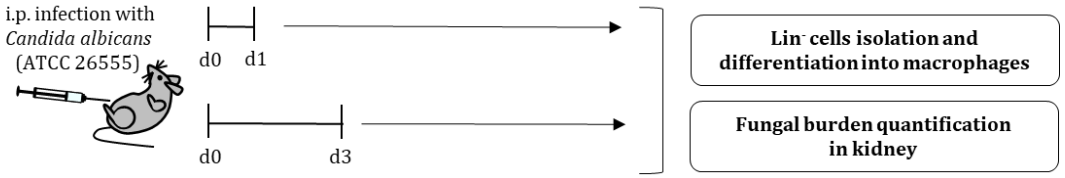


FIGURE 4 | *C. albicans* infection model. C57BL/6 mice were challenged with 45 × 10^6 *C. albicans* ATCC 26555 i.p. Day one or day three post-infection, mice were euthanized in order to (i) isolate HSPCs and differentiate them into macrophages in M-CSF cultures and (ii) quantify fungal burden in kidney. To prevent potential fungal growth factor in M-CSF cultures, differentiation was performed in the presence of 2.5 μg/ml of amphotericin B. Then, the functional phenotype of mature macrophages was analyzed. Fungal burden quantification was performed by homogenizing the organs in 1 ml of PBS and plating dilutions of the homogenates on Sabouraud dextrose agar. The colony forming units (CFUs) were counted after 24 h of incubation at 37 °C.

8 Isolation of splenocytes and peritoneal macrophages

Resident peritoneal macrophages were harvested from C57BL/6 mice under sterile conditions from mice euthanized by cervical dislocation. Once sprayed with 70 % ethanol, animals were placed in a dissection table on their backs and 10 ml RPMI complete media were injected into the peritoneal cavity. Next, the abdomen was massaged to distribute cell media equally around the cavity. Mice were placed on their backs and a longitudinal cut was made in the outer ventral skin of the mice. To obtain a sterile pocket, the skin was separated from the peritoneum to expose the inner skin lining the peritoneal cavity. Then, a small cut

was made in the peritoneum to recover the cell media injected with a plastic sterile Pasteur pipette. Cell suspension recovered was spun down at 450 g at 4 °C for 10 min, and obtained cells were counted and plated at a density of 1.5×10^5 cells in 200 µl of RPMI complete media per well, in 96-well flat-bottom TC plates. To avoid adhesion of peritoneal macrophages to plastic, the whole process was performed using cell media at 4 °C and maintaining cells on ice. Peritoneal macrophages were allowed to adhere for 5 h at 37 °C in a 5 % CO_2 atmosphere. Then, the non-adherent cells were removed by changing the media, and the adherent macrophages were cultured for 72 h prior to be challenged with the indicated stimuli for cytokine production measurement (see section 12).

Splenic leukocytes (splenocytes) were obtained from control or Pam_3CSK_4-treated mice. Spleens were taken under sterile conditions from mice euthanized by cervical dislocation and placed in a 5 cm plate. 10 ml of MACS buffer were injected into the organs using a 25-gauge needle and cell suspension obtained was filtered using a 70 µm filter. Then, each spleen was cut in small pieces and that pieces were placed in the filter. Using the piston of a sterile syringe, pieces were mashed, and the filter was washed with MACS buffer. Cell suspension was centrifuged at 450 g for 5 min, and erythrocytes were lysed with BD FACS™ lysing solution (BD Bioscience). Splenocytes were washed, counted, and plated in 24-well plates at a density of 2.5×10^5 cells in 0.5 ml RPMI complete media for cytokine production and killing assays (detailed in each section).

9 Obtainment of secretomes produces by HSPCs

Lin^- cells were cultured at a density of 5×10^4 cells in 250 µl of a serum-free medium (media composition detailed in section 2.2) in 96-well TC plates, and challenged for 72 h with 1 µg/ml Pam_3CSK_4 or 2.5×10^5 inactivated *C. albicans* yeasts. Then, culture supernatants (secretomes) were collected and used for (i) measuring cytokine production and (ii) evaluating their effect on HSPC differentiation or cytokine production by peritoneal macrophages. As control secretomes, culture supernatants produced by HSPCs without stimuli were used. The effect of secretomes from HSPCs stimulated with Pam_3CSK_4 was determined on HSPCs or peritoneal macrophages from TLR2 $^{-/-}$ mice to avoid direct activation by Pam_3CSK_4.

10 $CD4^+$ T cells purification and coculture with APCs

8×10^4 DCs were plated in 48-well TC plates with RPMI complete medium. When cocultured with OT-II T cells, 2 to 4 h after plating, DCs were given OVA peptide 323-339 (7 µg/ml). After 20 to 40 min OVA peptide was added to cultures, cells were stimulated with 100 ng/ml Pam_3CSK_4 or 100 µg/ml depleted zymosan. On the other hand, when cocultured with WT T cells, 2 to 4 h after plating, DCs were stimulated with 9.6×10^5 inactivated *C. albicans* PCA2 yeasts or 9.6×10^5 inactivated *C. albicans* ATCC 25666 yeasts. After 18 to 24 h of stimulation with PAMPs or fungal cells, supernatants were aspirated off and wells were washed twice with PBS.

For sorting $CD4^+$ T cells, spleens were taken from OT-II or WT mice and splenocytes were obtained as described in section 8. Negative selection of $CD4^+$ naïve T cells was performed using the "Negative selection CD4 kit" from Stem Cell Technologies (Seattle, WA, USA). Unwanted cells were targeted for removal with biotinylated antibodies directed against non-$CD4^+$ T cells and streptavidin-coated magnetic particles (RapidSpheres™). Labeled cells were separated using an EasySep™ magnet without the use of columns and desired cells were poured off into a new tube.

T cell proliferation was measured by assessing dilution of CFSE (Carboxyfluorescein succinimidyl ester), a fluorescent dye that is cell permeable and covalently couples to intracellular molecules, being retained within cells for extremely long periods. CFSE labeling is progressively halved with each cell division and can be used to measure up to 8 cell divisions. CFSE (Invitrogen Molecular Probes) (10 nM) diluted in PBS was added to cells for 9 min at RT in the dark. Then, 5 ml pre-cooled T cell media (media composition detailed in section 2.2) were added in order to stop staining reaction, and cells were maintained on ice for 5 min. T cell media contained sodium pyruvate, added as a carbon source in addition to glucose, and 2-mercaptoethanol, a reducing agent used to stabilize culture media by avoiding the oxidation of certain unstable compounds. T cells were washed twice with T cell media and counted.

CFSE-labeled $CD4^+$ T cells were then added to washed APC cultures (see above) at a concentration of 4×10^5 per well in 400 µl T cell media. Thus, DCs and $CD4^+$ T cells were cocultured at a 1:5 ratio. After 3 (OT-II T cells) or 4 (WT T cells) days of coculture with APCs, T cells were lifted by pipetting and resuspended in T cell media. Cells were then replated for cytokine production measurement and stained for surface molecule expression

analysis (see detailed protocol in sections 12 and 11, respectively). T cells replated for cytokine production measuring were restimulated with 50 ng/ml PMA (Phorbol 12-Myristate 13-Acetate) (Sigma-Aldrich) and 500 ng/ml Ionomycin (Sigma-Aldrich) for 24 h. PMA is a small organic compound which diffuses through the cell membrane into the cytoplasm, omitting the "need" of surface receptor stimulation for activating T cells. Ionomycin, a calcium ionophore that synergizes with PMA, is used to trigger calcium release, which is needed for nuclear factor of activated T cells (NFAT) signaling that induces the synthesis of IL-2 by T cells. The cytokine IL-2 provides proliferation and survival signaling to T cells (Chatila *et al.*, 1989). Cell-free supernatants were collected and production of IL-17A and IFN-γ was analyzed by enzyme-linked immunosorbent assay (ELISA), with ELISA kits purchased from Biolegend and eBioscience, respectively.

11 Immunophenotyping analysis by flow cytometry

For APC surface molecule expression analysis, APCs were plated at 2.5×10^5 cells /500 µl RPMI complete media in 24-well non-TC plates. Two to 4 h after plating, cells were stimulated with 100 ng/ml Pam_3CSK_4, 100 µg/ml depleted zymosan, 3×10^6 inactivated *C. albicans* PCA2 yeasts or 3×10^6 inactivated *C. albicans* ATCC 26555 yeasts for 18 to 24 h. Then, supernatants were aspirated off and PBS + 2mM EDTA was added to the wells, left on cells at RT for 10 min. Adherent cells were then harvested washing each well several times with the PBS + 2 mM EDTA added, promoting cells to detach from the plastic surface. Adherent cells were centrifuged 450 g for 5 min, cell pellets were resuspended in 90 µl of MACS buffer and 10 µl of FcR blocking reagent (Miltenyi Biotec) was added to each sample for 10 min at 4 °C. This reagent is used to block receptors that recognize the fragment crystallizable region of antibodies (FcRs), thereby avoiding unspecific unions. Antibodies (**Table 1**) were added to cells at 1/100 dilution for 15 min at 4 °C in the dark. Cells were then washed with MACS buffer with 10 % FBS to eliminate extra antibody and spun down at 450 g for 3 min. Cell pellets were resuspended in PBS + 25 % BD Cytofix™ Fixation Buffer (BD Bioscience) and analyzed by flow cytometry.

For surface molecule expression analysis of total BM cells, splenocytes, HSPCs or macrophages, ≤ 10^6 cells were labeled following the same steps described above (from incubation with FcR blocking reagent to sample fixation). To analyze total BM cells and

splenocytes, erythrocytes were previously lysed using BD FACS™ lysing solution (BD Bioscience). Antibodies used are indicated below (**Table 1**).

For T cell surface molecule expression and proliferation assay, the staining buffer used was composed by PBS + 0.09 % Sodium Azide + 5 % FBS + FcR blocking reagent. T cells were centrifuged at 450 g for 3 min, cell pellets were resuspended in 50 µl of staining buffer and cells were incubate at 4 °C for 10 min. Then, antibodies (**Table 1**) were added to cells at a concentration of 1/200, preparing the antibody staining cocktail in 50 µl final volume of staining buffer, for 20 min at 4 °C in the dark. Cells were then washed with MACS buffer and spun down at 450 g for 3 min. Cell pellets were resuspended in PBS + 25% BD Cytofix™ Fixation Buffer (BD Bioscience) and counting beads (Thermo Fisher) were added to quantify T cell number.

Flow cytometry analyses were performed on a LSR Fortessa analyzer (BD Biosciences) and the data were analyzed with FlowJo 10 software.

TABLE 1| List of murine antibodies used for flow cytometry experiments

APC surface molecules				
Surface molecule	**Fluorochrome**	**Clone**	**Company**	**Isotype control**
CD11b	BV510	M1/70	BD Bioscience	Rat IgG2b, κ
CD11c	BUV395	N418	BD Bioscience	Armenian Hamster (AH) IgG2
CD40	PE	$3^{-/-}23$	Biolegend	Rat IgG2a, κ
CD80	PerCP-Cy5.5	16-10A1	Biolegend	AH IgG
CD86	FITC	GL1	Biolegend	Rat IgG2a, κ
MHCII	Pacific Blue	M5/114.15.2	Biolegend	Rat IgG2b, κ

HSPC surface molecules				
Surface molecule	**Fluorochrome**	**Clone**	**Company**	**Isotype control**
CD117 (c-Kit)	APC	2B8	BD Pharmingen	Rat IgG2b
CD11b	FITC	M1/70	BD Pharmingen	Rat IgG2b

Macrophage surface molecules				
Surface molecule	**Fluorochrome**	**Clone**	**Company**	**Isotype control**
CD11b	PE-Cy7	M1/70	BD Pharmingen	Rat IgG2b
F4/80	PerCP-Cy5.5	BM8	eBioscience	Rat IgG2a, κ
Ly6C	PE	AL-21	BD Pharmingen	Rat IgM, κ
MHCII	FITC	M5/114.15.2	eBioscience	Rat IgG2b
TLR2	PE	6C2	eBioscience	Rat IgG2b, κ
TLR4/MD2	FITC	MTS510	ENZO	Rat IgG2a, κ

Splenocytes and bone marrow surface molecules				
Surface molecule	**Fluorochrome**	**Clone**	**Company**	**Isotype control**
Biotin	PE	REA746	Miltenyi Biotec	REA Control (S) antibodies
CD117 (c-Kit)	PE-Vio770	3C11	Miltenyi Biotec	Rat IgG2bκ
CD11b	PE	M1/70	eBioscience	Rat IgG2b
CD11c	APC	N418	Miltenyi Biotec	Hamster IgG
Lineage antigens (Cocktail)	Biotin		Miltenyi Biotec	
Ly6C	APC-Cy7	AL-21	BD Pharmingen	Rat IgM, κ
Ly6G	BUV395	1A8	BD Bioscience	Rat (LEW) IgG2a, κ
MHCII	PerCP-Cy5.5	M5/114.15.2	BD Pharmingen	Rat IgG2b
T cell surface molecules				
Surface molecule	**Fluorochrome**	**Clone**	**Company**	**Isotype control**
CD4	BV510	RM4-5	BD Bioscience	Rat IgG2a, κ
CD44	APC	IM7	Biolegend	Rat IgG2b, κ
CD69	PE	H1.2F3	BD Bioscience	AH IgG1, λ3

12 Cytokine measurements

In vitro differentiated macrophages were plated at a density of 5 × 10^4 cells in 200 µl RPMI complete media, while APCs were plated at 5 × 10^4 cells in 100 µl, both in 96-well TC plates. Both cell types, as well as peritoneal macrophages, splenocytes and HSPCs, were challenged with the indicated stimuli for 18 to 24 h and cell-free supernatants were then collected. Levels of the pro-inflammatory cytokine TNF-α were determined in culture supernatants from splenocytes and peritoneal macrophages, while levels of the pro-inflammatory cytokines IL-6 and/or TNF-α were measured in culture supernatants from *in vitro* differentiated macrophages and HSPCs. ELISA kits used for these assays were purchased from eBioscience. Culture supernatants from APCs were tested for TNF-α, IL-6, IL-12 p40 and IL-2 release using commercial ELISA kits purchased from Biolegend. At least triplicate samples were analyzed in each assay.

In the secretomes produced by HSPCs, 40 cytokines were determined using a mouse cytokine array (RayBio Mouse inflammation antibody array C1) according to the manufacturer's instructions (RayBiotech).

13 *C. albicans* phagocytosis assay

Cells of *C. albicans* PCA2 were obtained as previously described (see section 3) and they were made fluorescent by FITC labeling. Briefly, yeasts were resuspended in PBS (Gibco) containing 0.1 mg FITC per ml, at a concentration of 5 × 10^6 yeasts/ml, and incubated at RT for 2 h. FITC was eliminated by extensive washing with PBS (Gibco) and homogeneous distribution of FITC labeling was confirmed by flow cytometry. Macrophages were plated at a density of 1 × 10^5 cells in 200 µl RPMI complete medium per well in a 96-well TC plate and challenged with FITC-labeled yeasts at a 1:5 ratio (murine cell: yeast). Yeasts were settled onto macrophages by centrifugation and incubated for 30 min. Cells were then labeled with PerCP-Cy5.5-anti-F4/80 antibody and analyzed by flow cytometry. To differentiate cell surface-adherent yeasts from the internalized ones, 0.2% trypan blue was added to quench extracellular fluorescence. Macrophages were gated based on their F4/80 expression and the extent of phagocytosis was assessed as the percentage of green (FITC) cells, as well as by the mean intensity of green fluorescence.

14 *C. albicans* killing assay

The assay was performed with M-CSF-derived macrophages, GM-CSF-derived macrophages or with splenocytes. Macrophages were plated in 96-well TC plates at a density of 2 × 10^5 cells in 150 µl of RPMI complete media. They were challenged with viable *C. albicans* PCA2 yeasts or *C. glabrata* yeasts (prepared as indicated in section 3) at a 1:3 ratio (murine cell: yeast), settled onto macrophages by centrifugation, and incubated for 1 h. Splenocytes were plated in 24-well TC plates at a density of 2.5 × 10^6 cells in 0.5 ml of RPMI complete media. They were challenged with 1 × 10^5 viable *C. albicans* PCA2 yeasts, settled onto the cells by centrifugation and incubated for 4 h. As a control, yeast cells were inoculated in culture medium without murine cells. At least triplicate samples were analyzed in each assay. After co-incubation, samples were diluted in water, plated on Sabouraud dextrose agar, and incubated overnight at 37 °C to determine CFUs. Colonies were counted, and killing percentages were determined as follows:

$$\%\ killing = \left(1 - \frac{CFUs\ sample\ at\ t1\ or\ 4h}{CFUs\ control\ at\ t1\ or\ 4h}\right) \times 100$$

C. albicans PCA2 and *C. glabrata*, both non-germinative *Candida* species, were chosen for killing assays to facilitate determination of CFUs after the incubation period, as no germ tube (hyphae) aggregates are formed. At least triplicate samples were analyzed in each assay.

15 Statistical analysis

Statistical differences were determined using one-way ANOVA followed by Dunnett's test for multiple comparisons and two tailed Student's *t-test* for dual comparisons. Data are expressed as mean ± SD. Significance was accepted at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ levels

RESULTS AND DISCUSSION

Innate immune responses result crutial in the early defense against pathogens, but phagocytes are rapidly consumed dealing with the invading microorganisms. Thus, in order to offset this cell loss and ensure an effective immune response, hematopoiesis switches from balance production of all blood cell lineages to promote myelopoiesis. Moreover, HSPCs also respond to infection egressing to peripheral tissues, thus generating myeloid cells directly in the site of infection. PRRs are not only expressed in terminally differentiated innate immune cells but also in early hematopoietic progenitors, indicating that direct sensing of microbial components by HSPCs may play a role in this demand-adapted hematopoiesis during infection (Boettcher and Manz, 2016; Cannova *et al.*, 2015; Yáñez *et al.*, 2013a).

In this context, our group has previously shown: (i) that inactivated *C. albicans* yeast cells induce *in vitro* proliferation and differentiation of mice HSPCs towards the myeloid lineage in a TLR2- and Dectin-1-dependent manner (Yáñez *et al.*, 2010; Yáñez *et al.*, 2011; Yáñez *et al.*, 2009) and (ii) that signaling through TLR2 on HSPCs does occur *in vivo* and induces their differentiation towards macrophages in response to both pure ligands and *C. albicans* infection (Megías *et al.*, 2013; Megías *et al.*, 2012). Other authors have shown that production of both PGE_2 and IL-6 by HSPCs is mediated by TLR2-signaling and that these molecules promote HSPCs proliferation and differentiation, in an autocrine/paracrine manner (Granick *et al.*, 2013; Zhao *et al.*, 2014).

However, although "PRR-derived" cells exhibit myeloid cell characteristics, it is not clear whether they are functionally equivalent to myeloid cells derived from HSPCs in the absence of PRR stimulation. Our previous studies indicate that transient exposure of Lin^- cells to the TLR2 ligand Pam_3CSK_4 results in the generation of macrophages with a reduced ability to produce inflammatory cytokines (Yáñez *et al.*, 2013b).

Considering the previous exposed facts, the main aim of the present PhD project was to characterize the phenotype of mature myeloid cells generated by HSPCs in the presence or absence of several PAMPs (pure ligands or *C. albicans* yeasts) by using *in vitro* and *ex vivo* assays. We studied the possible changes in their antimicrobial activities or their ability to activate and polarize $CD4^+$ T cell responses. The antimicrobial response of HSPCs after PAMPs exposure was also analyzed.

CHAPTER 1

Functional phenotype of macrophages derived from *in vitro* or *in vivo* exposed HSPCs to PAMPs

1 Phenotype of macrophages derived from HSPCs exposed to TLR2, TLR4 and Dectin-1 ligands in M-CSF cultures

1.1 Macrophage yield and surface molecules expression

We investigated the consequences of *in vitro* exposure of HSPCs to PAMPs during differentiation by comparing the phenotype of the macrophages they produce in homeostatic conditions. To study this, Lin^- cells were cultured with M-CSF in the presence or absence (control) of different PRR agonists: Pam_3CSK_4 (which only activates TLR2), LPS (which only activates TLR4), depleted zymosan (a Dectin-1-activating *Saccharomyces cerevisiae* cell wall preparation that has been treated with hot alkali to remove its TLR-stimulating properties), or *C. albicans* ATCC 26555 yeasts (which activate several PRRs, but principally TLR2 and Dectin-1). In these conditions, PRR agonists are present throughout differentiation (**Figure 1A**, continuous exposure) most closely reproducing the *in vivo* situation during an ongoing infection, when HSPCs in the BM or infected tissues may interact with the microorganisms or their products. We also investigated the consequences of exposure of HSPCs to soluble TLR2 or TLR4 agonists prior to differentiation. This transient exposure model (**Figure 1A**, transient exposure) enables us to define the phenotype of macrophages generated by previously exposed HSPCs, and thus determine whether TLR signaling in HSPCs influences the phenotype of macrophages produced after clearance of an infection.

After HSPC differentiation, adherent cells were harvested, and macrophage yields were assessed by cell counting (**Figure 1B**). Pam_3CSK_4 treatment boosted the yield of M-CSF-derived adherent cells, following both continuous and transient exposure, whereas LPS only enhanced the yield following continuous exposure. The increased yield of M-CSF derived cells induced by Pam_3CSK_4 transient exposure is consistent with our previous report (Yáñez *et al.*, 2013b). However, neither depleted zymosan nor *C. albicans* yeast treatment changed the number of adherent cells generated in M-CSF cultures (**Figure 1B**). These results indicate that soluble TLR2 and TLR4 agonists but not particulate Dectin-1 agonists or inactived *C. albicans* yeasts induce the proliferation and/or improve the survival of HSPCs.

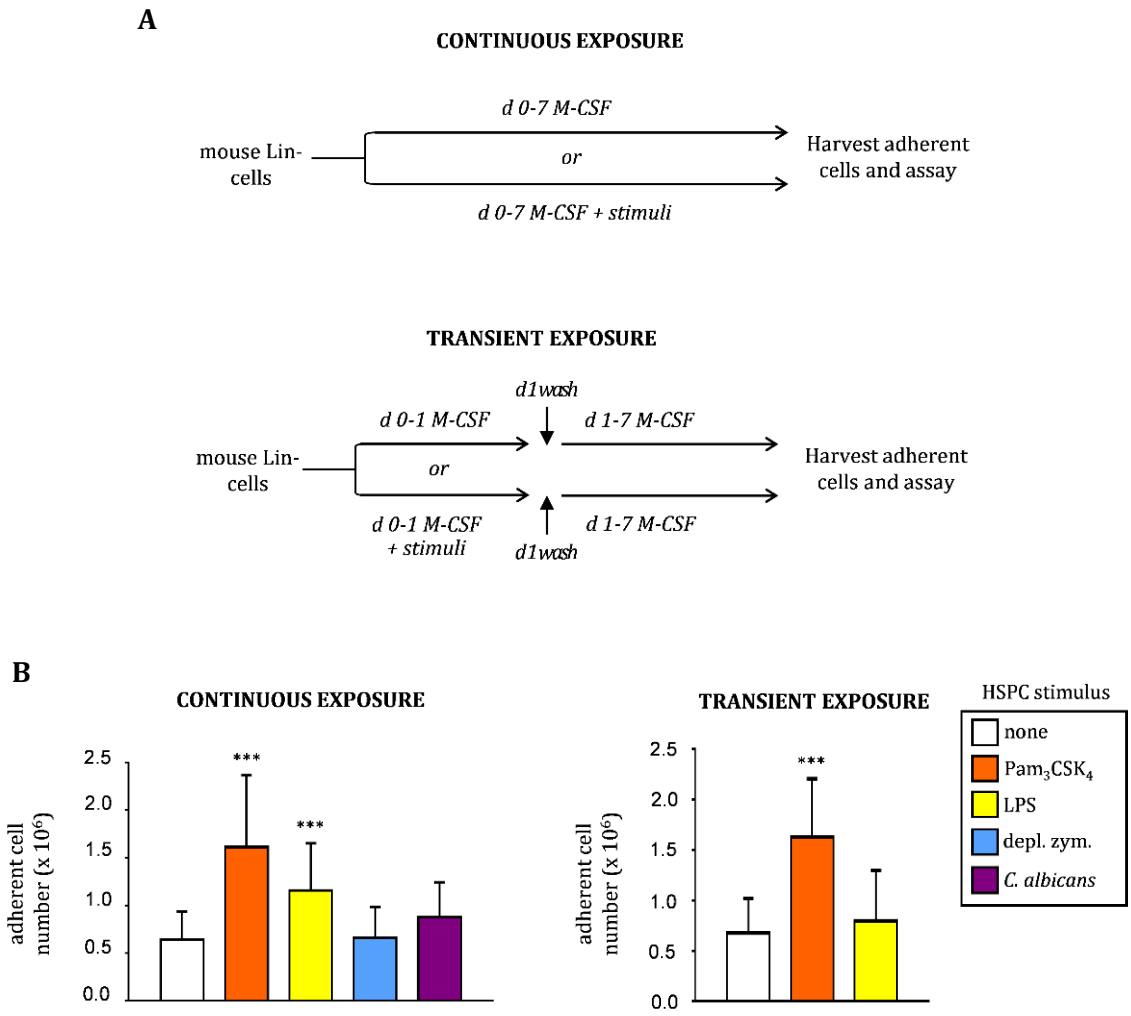


FIGURE 1 | Continuous or transient exposure to different stimuli (PAMPs) during *in vitro* differentiation of HSPCs into macrophages. (A) Schematic protocol. Purified Lin$^-$ cells from BM of C57BL/6 mice were cultured with 20 ng/ml Stem cell factor (SCF) and 50 ng/ml M-CSF for seven days to induce macrophage production, in the presence or absence of different microbial stimuli for the entire seven days (continuous exposure) or the first 24 h only (transient exposure). The microbial stimuli used were 1 µg/ml Pam_3CSK_4, 100 ng/ml LPS, 1 µg/ml depleted zymosan or inactivated *C. albicans* yeasts (1:7.5 murine cell:yeast ratio). **(B)** At day seven, adherent cells were harvested and counted. The figure shows mean values of total cells with standard deviation (SD) from at least ten independent experiments. *** $P < 0.001$ with respect to cells derived from unstimulated HSPCs (M-CSF only) for each condition (continuous or transient).

Next, we analyzed the phenotypic surface molecules of the HSPC-derived macrophages in M-CSF cultures by multicolor flow cytometry (**Figure 2**). Analysis of CD11b and F4/80 expression allowed for the identification of mature macrophages (CD11b$^+$ F4/80$^+$), which, as expected, represented the majority of obtained cells with more than 75%. Among these cells, only 4.9 % expressed the inflammatory monocyte marker Ly6C, 10.8 % expressed MHCII and 4.7 % were double positive for both markers, results consistent with those observed in resting macrophages. However, when Lin$^-$ progenitor cells were cultured with M-CSF in the presence of TLR ligands, these percentages changed. The CD11b$^+$ F4/80$^+$ macrophages generated in the presence of LPS and Pam_3CSK_4 exhibited a higher percentage

of Ly6C$^+$ MHCII$^-$ cells (from 4.9% to 77% and 30%, respectively) and a lower percentage of MHCII$^+$ cells (from 15.5 to 3.5%). Furthermore, LPS induced an increase of Ly6C expression in Ly6C$^+$ MHCII$^-$ cells, as the fluorescence mean intensity of the indicated surface marker was increased comparing to M-CSF-derived cells (4429 versus 1151). Neither depleted zymosan nor *C. albicans* yeast treatment altered the percentages of CD11b$^+$ F4/80$^+$ macrophages that express Ly6C or MHCII (**Figure 2,** continuous exposure). Interestingly, although LPS induced a greater phenotypic change than Pam_3CSK_4 upon continuous exposure, transient exposure to LPS did not change the percentage of cells that express Ly6C or MHCII, whereas transient exposure to Pam_3CSK_4 induced a similar increase in Ly6C$^+$ MHCII$^-$ macrophages than continuous exposure to this TLR2 ligand (**Figure 2,** transient exposure).

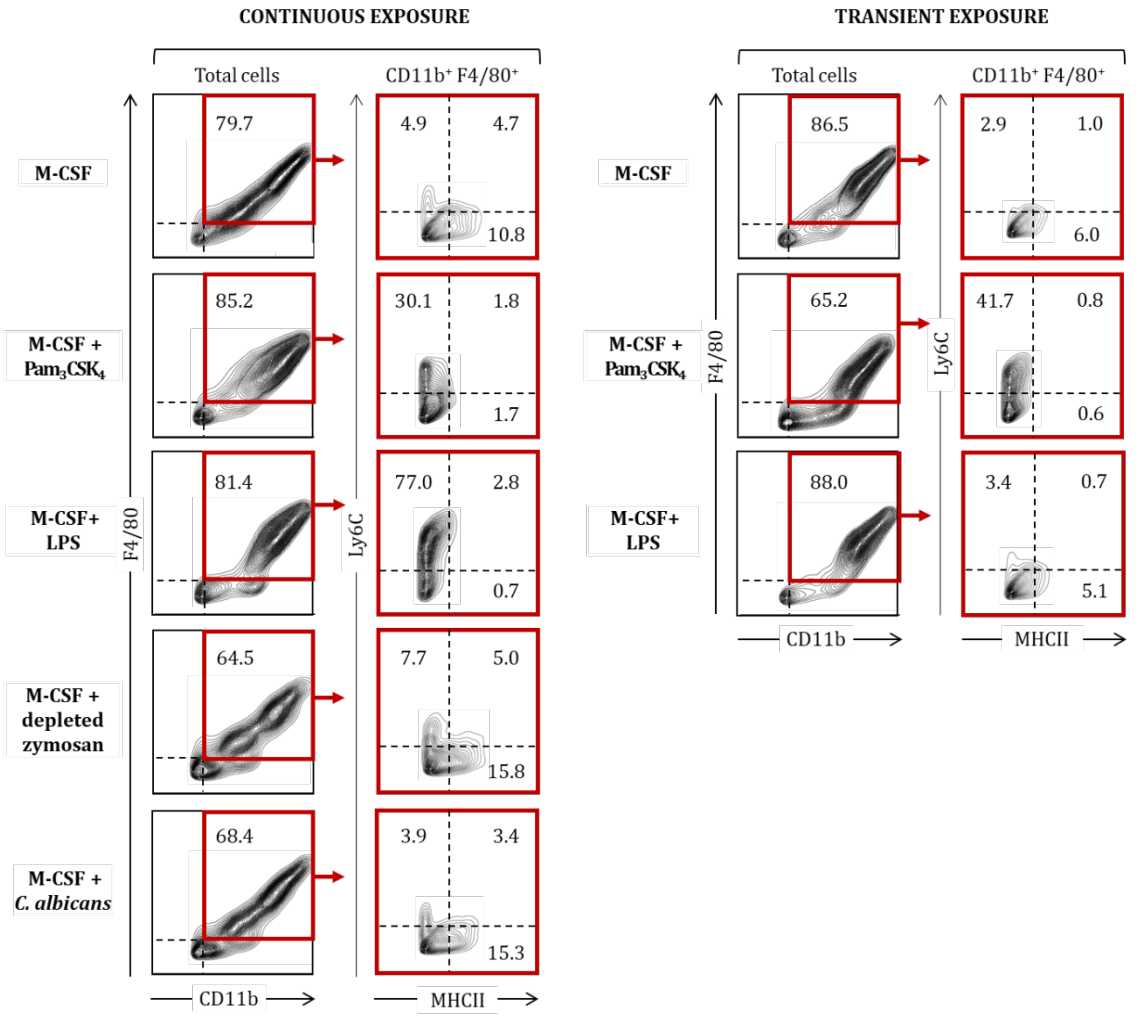


FIGURE 2 | Flow cytometry analysis of adherent cells produced by HSPCs under the same conditions as in Figure 1A. Myeloid cells obtained from HSPCs following continuous or transient exposure to the different microbial stimuli were labeled with antibodies and analyzed by flow cytometry. Macrophages were gated as CD11b$^+$ F4/80$^+$ cells and were subsequently analyzed in a MHCII versus Ly6C plot. The indicated numbers refer to the percentages of cells analyzed in each contour-plot. Results shown are representative of three independent experiments.

Overall, these results demonstrate that transient exposure of HSPCs to a TLR2 agonist is sufficient to increase the yield of M-CSF-derived cells and also to increase the generation of Ly6C^{+} MHCII^{-} macrophages. A TLR4 agonist induced the same changes but only following continuous exposure, whereas neither the yield nor the surface molecules of M-CSF derived macrophages were changed by a Dectin-1 agonist or *C. albicans* yeasts.

1.2 Cytokine production: TNF-α and IL-6

To investigate whether the exposure of HSPCs to PAMPs influences the antifungal functions of HSPC-derived macrophages, we firstly tested their ability to secrete pro-inflammatory cytokines in response to TLR agonists. For this, adherent cells obtained from HSPCs in M-CSF cultures were counted, and equal numbers of macrophages were stimulated with TLR agonists for 24 h; then, supernatants were harvested and tested for TNF-α and IL-6 release. Results reveal that pro-inflammatory cytokine production in response to Pam_3CSK_4 or LPS was significantly diminished in macrophages generated from HSPCs exposed (transiently or continuously) to Pam_3CSK_4, compared to control macrophages (derived from unexposed HSPCs) (**Figure 3**). Similarly, macrophages generated from LPS-exposed HSPCs also produced lower pro-inflammatory cytokines than control macrophages. However, it should be noted that exposure to Pam_3CSK_4 had a greater impact on cytokine production than exposure to LPS, particularly following transient exposure. On the other hand, interestingly, TNF-α and IL-6 secretion in response to TLR agonists was increased in macrophages generated from HSPCs in the presence of *C. albicans* yeasts or depleted zymosan, compared to control macrophages.

Taken together, these results indicate that exposure of HSPCs to PRR agonists may profoundly alter the ability of the macrophages derived from them to produce pro-inflammatory cytokines. Soluble TLR2 and TLR4 ligands cause a reduction in cytokine production while particulate Dectin-1 agonists and yeasts provoke an increased response.

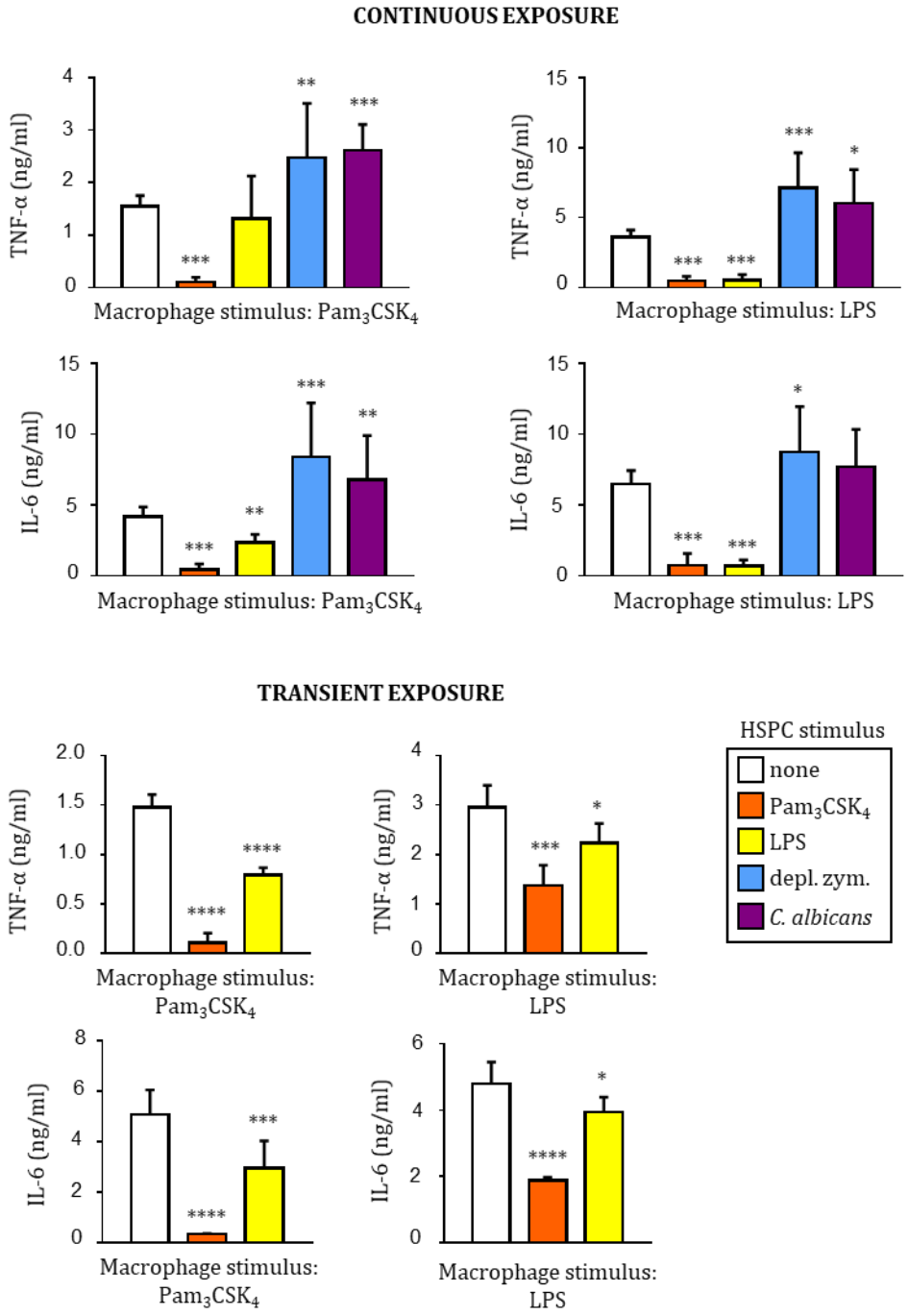


FIGURE 3 | Cytokine production by adherent cells generated by HSPCs in the same conditions as in Figure 1A. Macrophages obtained from HSPCs following continuous or transient exposure to different microbial stimuli were challenged with 100 ng/ml Pam_3CSK_4 or 100 ng/ml LPS for 24 h. TNF-α and IL-6 levels in cell-free culture supernatants were measured by ELISA. Results are expressed as means ± SD of pooled data from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ with respect to cytokine production by cells derived from unstimulated HSPCs (M-CSF only) for each condition (continuous or transient).

Our group previously demonstrated that HSPCs transiently exposed to Pam_3CSK_4 produce soluble factors that act in a paracrine manner to influence the function of macrophages produced by unexposed HSPCs in M-CSF cultures (Yáñez *et al.,* 2013b). The identity of these factors is not known, but candidates include IL-6 and PGE_2 that are both induced by TLRs in HSPCs and are able to induce myeloid differentiation in a paracrine manner (Granick *et al.*, 2013; Zhao *et al.*, 2014). These findings prompted us to study whether the reduced inflammatory responsiveness of macrophages produced by HSPCs exposed to Pam_3CSK_4 or LPS (transient exposure) may be at least in part due to produced IL-6 or PGE_2. We therefore measured TNF-α production in response to Pam_3CSK_4 stimulation by macrophages generated from HSPCs transiently exposed to Pam_3CSK_4, in the continuous presence or absence of indomethacin (to block PGE_2 secretion) or an IL-6 neutralizing antibody. The blockade of neither PGE_2 nor IL-6 reversed the reduced inflammatory responsiveness of macrophages produced by Pam_3CSK_4-exposed HSPCs (**Figure 4**).

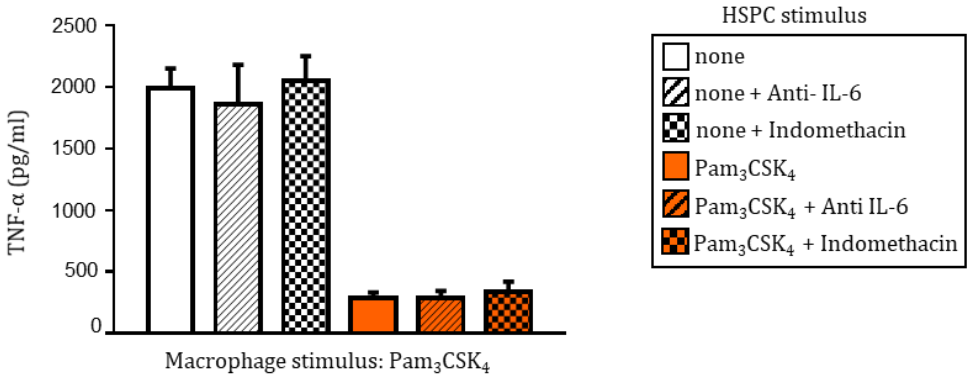


FIGURE 4 | Cytokine production by adherent cells obtained from HSPCs transiently exposed to Pam_3CSK_4, in the presence or absence of an IL-6 neutralizing antibody or indomethacin, in M-CSF cultures. Purified Lin^- cells from BM of C57BL/6 mice were cultured with 20 ng/ml SCF and 50 ng/ml M-CSF for seven days to induce macrophage production, in the presence or absence of 1 μg/ml Pam_3CSK_4 for the first 24 h (transient exposure) and an IL-6 neutralizing antibody (1 μg/ml) or indomethacin (10 μM, added at day zero and at days one, three and five). At day seven, adherent cells were harvested, counted, replated and challenged with 100 ng/ml Pam_3CSK_4 for 24 h. TNF-α concentration, in cell-free culture supernatants, was measured by ELISA. Results are expressed as means ± SD of pooled data from two experiments.

In vitro models of LPS-tolerance have demonstrated that after prolonged LPS stimulation, macrophages show defects in TLR4 signaling at the level of the receptor, signaling molecules, and transcription factors, due to attenuated expression levels or protein activity (Biswas and López-Collazo, 2009; Cavaillon and Adib-Conquy, 2006; Seeley and Ghosh, 2017). Regarding these observations, we next determine whether the altered production of cytokines in response to Pam_3CSK_4 or LPS may be due to modulation of the

expression of the receptors themselves (TLR2 and TLR4, respectively). HSPC-derived macrophages, gated as CD11b$^+$ F4/80$^+$ cells, were analysed for TLR2 and TLR4 expression by flow cytometry (**Figure 5**).

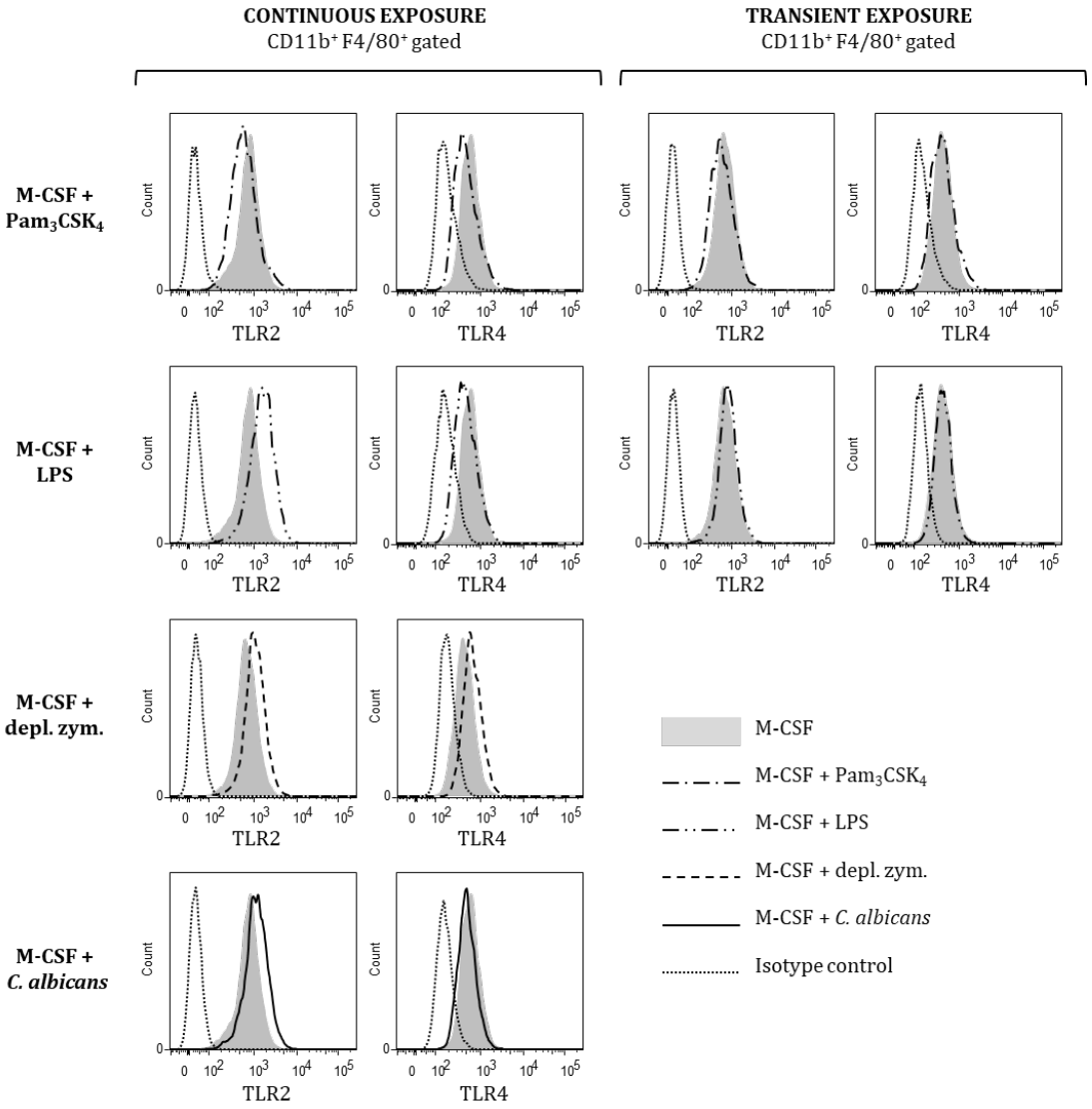


FIGURE 5 | TLR2 and TLR4 expression on adherent cells produced by HSPCs under the same conditions as in Figure 1A. Myeloid cells produced by HSPCs following continuous or transient exposure to different microbial stimuli were labeled with antibodies and analyzed by flow cytometry. Macrophages were gated as CD11b$^+$ F4/80$^+$ cells and were analyzed for the Mean Fluorescent Intensity (MFI) of TLR2 and TLR4. Results shown are representative of two independent experiments.

As expected, M-CSF derived macrophages expressed both TLR2 and TLR4. Macrophages generated in the continuous presence of Pam_3CSK_4 exhibited lower TLR2 and TLR4 expression, whereas transient exposure to Pam_3CSK_4 induced a slight decrease in TLR2 and no changes in TLR4 expression. Interestingly, although LPS increased TLR2 and decreased TLR4 levels upon continuous exposure, transient exposure to LPS did not change TLR2 or TLR4 expression. On the other hand, continuous exposure to depleted zymosan

induced an increase in both TLR2 and TLR4 expression, whereas the continuous presence of *C. albicans* yeasts only provoked increased TLR2 expression. These results demonstrate that exposure of progenitors to TLR and Dectin-1 agonists during differentiation modulates TLR2 and TLR4 expression, and therefore this may contribute to the altered cytokine production in response to Pam_3CSK_4 or LPS. However, the observed changes in TLR expression cannot fully explain the profoundly altered cytokine responses, particularly in transiently-exposed macrophages. For example, transient exposure to Pam_3CSK_4 did not change TLR4 expression, and yet these macrophages produced significantly lower amounts of cytokines in response to LPS.

1.3 Fungicidal activity

To further characterize the antifungal function of macrophages generated from PAMP-stimulated HSPCs in M-CSF cultures, we measured the ability of differentiated cells to internalize and kill yeast cells. Firstly, macrophages were challenged with inactivated FITC-labeled yeasts at a 1:5 ratio (murine cell: yeast) for 30 min. Phagocytosis of the yeast cells by macrophages (gated as F4/80 positive cells) was analysed by flow cytometry and expressed as the percentage of cells that contain at least one internalized yeast, as well as the mean channel fluorescence intensity that indicates the extent of phagocytosis per cell (**Figure 6**).

C. albicans uptake by macrophages generated from HSPCs transiently exposed to TLR agonists was not altered compared to uptake by control macrophages (derived from unexposed HSPCs). However, the macrophages generated from HSPCs in the continuous presence of Pam_3CSK_4 showed a slight but significant decrease in the percentage of phagocytosis, while the macrophages generated from HSPCs in the continuous presence of LPS exhibited an increased mean fluorescence intensity. The presence of *C. albicans* yeasts or depleted zymosan during differentiation in M-CSF cultures changed neither the percentage nor the extent of phagocytosis of HSPC-derived macrophages. These results indicate that exposure of progenitors to TLR agonists during differentiation modestly modulates macrophage phagocytic capacity.

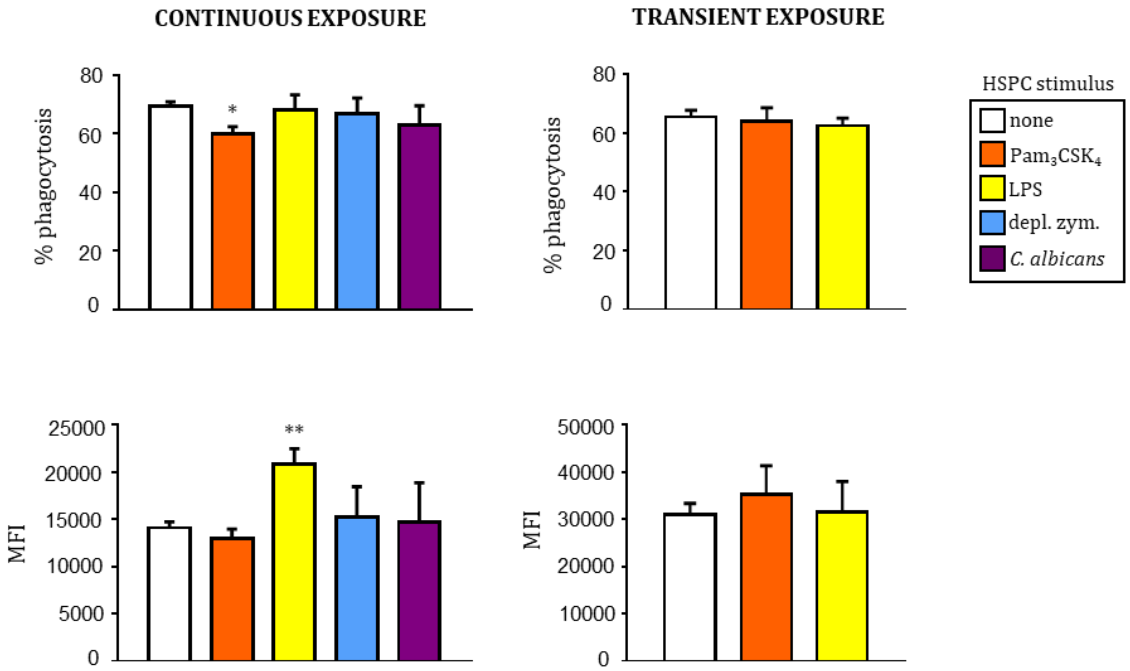


FIGURE 6| Phagocytosis of *C. albicans* yeasts by adherent cells produced by HSPCs in the same conditions as in Figure 1A. Macrophages obtained from HSPCs following continuous or transient exposure to different stimuli were challenged with FITC-labeled yeast at a 1:5 ratio (murine cell:yeast) for 30 min. Afterward, cells were labeled with anti-F4/80 antibody and analyzed by flow cytometry. Macrophages were gated based on their F4/80 expression, and the extent of phagocytosis was assessed as means ± SD of pooled data from three experiments. * $P < 0.05$ and ** $P < 0.01$ with respect to the phagocytic capacity of cells produced by unstimulated HSPCs (M-CSF only) for each condition (continuous or transient).

Finally, HSPC-derived macrophages were challenged with viable *C. albicans* PCA2 yeasts, at a 1:3 ratio (murine cell: yeast) for 1 h, to determine their fungicidal activity (**Figure 7**). Control macrophages (derived from unexposed HSPCs) were able to kill a significant percentage of *C. albicans* yeasts: CFUs after coculture were reduced to 70% (a roughly 30% decrease). Continuous exposure of progenitors to Pam_3CSK_4 or *C. albicans* yeasts generated macrophages with higher fungicidal activity: CFUs were reduced to 41% and 48%, respectively. This represents a relative increase in the fungicidal activity of HSPC-derived macrophages induced by HSPC exposure to Pam_3CSK_4 or *C. albicans* cells of roughly 36% and 60%, respectively, as compared to control macrophages. Therefore, macrophages derived in the presence of a TLR2 agonist or *C. albicans* yeasts possess an increased fungicidal activity, which is not due to an increased capacity to internalize yeasts (**Figure 6**). In contrast, neither continuous exposure of HSPCs to LPS or depleted zymosan, nor transient exposure to Pam_3CSK_4 or LPS changed the fungicidal activity of HSPC-derived macrophages.

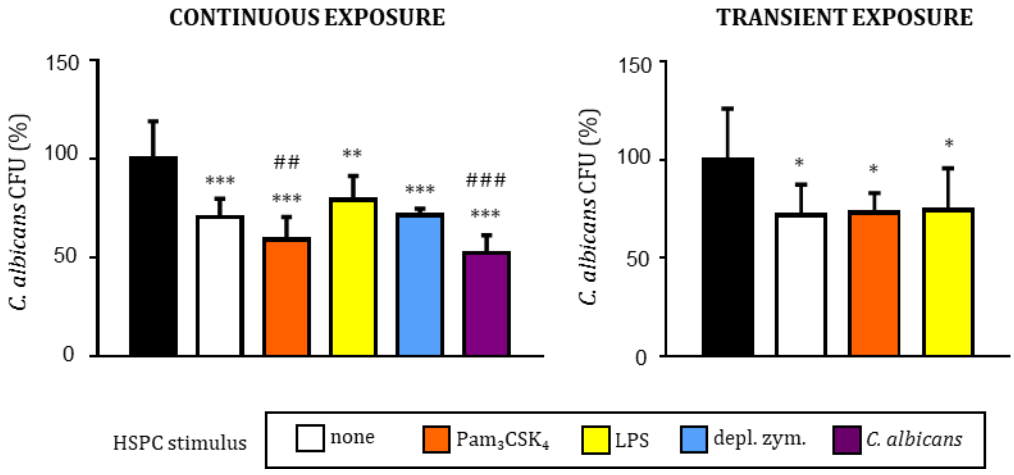


FIGURE 7 | Fungicidal activity of adherent cells produced by HSPCs under the same conditions as in Figure 1A. Macrophages obtained from HSPCs following continuous or transient exposure to different stimuli were challenged with viable PCA2 yeasts at a 1:3 ratio (murine cell:yeast) for 1 h. *C. albicans* cells were also inoculated in culture medium without murine cells (control, black bar). After incubation, samples were diluted, plated on Sabouraud dextrose agar and incubated overnight at 37 °C; the colonies were counted and expressed as % CFUs as compared to CFUs in the control sample at time 1 h. Triplicate samples were analyzed in each assay. Data represents means ± SD of pooled data from three experiments. * $P < 0.05$ and ** $P < 0.01$ and *** $P < 0.001$ with respect to control, ## $P < 0.01$ and ### $P < 0.001$ with respect to fungicidal capacity of cells produced by unstimulated HSPCs (M-CSF only) for each condition (continuous or transient).

To confirm the increased fungicidal activity of macrophages generated from HSPCs in the presence of *C. albicans* cells, we also tested their ability to kill yeasts of *Candida glabrata* CECT1448 strain (**Figure 8**). While *C. albicans* is the most frequently detected species in fungal invasive infection, non-albicans *Candida* have an increasing role particularly in high-risk populations, with *C. glabrata* being the most prominent of these species in United States and north western Europe (Pappas *et al.*, 2018).

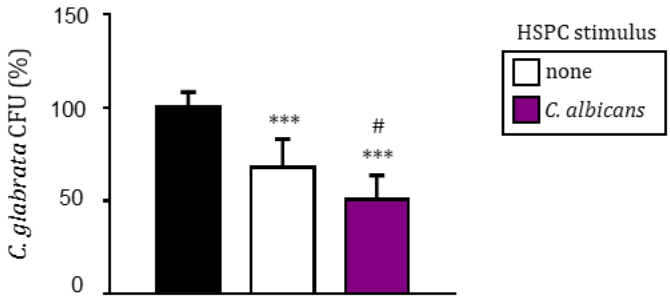


FIGURE 8 | Fungicidal activity against *Candida glabrata* of macrophages produced by HSPCs exposed to *C. albicans* yeasts in M-CSF cultures. Purified Lin^- cells from BM of C57BL/6 mice were cultured with 20 ng/ml SCF and 50 ng/ml M-CSF for seven days to induce macrophage production, in the presence or absence of inactivated *C. albicans* yeasts. At day seven, adherent cells were harvested, plated at equal numbers and challenged with viable *C. glabrata* yeasts at a 1:3 ratio (murine cell:yeast) for 1 h. *C. glabrata* cells were also inoculated in culture medium without murine cells (control, black bar). After incubation, samples were diluted, plated on Sabouraud dextrose agar and incubated overnight at 37 °C; the colonies were counted and expressed as % CFUs as compared to CFUs in the control sample at time 1 h. Triplicate samples were analyzed in each assay. Data represents means ± SD of pooled data from three experiments. *** $P < 0.001$ with respect to control, # $P < 0.05$ with respect to fungicidal capacity of cells produced by unstimulated HSPCs (M-CSF only).

Control macrophages (derived from unexposed HSPCs) were able to kill a significant percentage of *C. glabrata* yeasts: CFUs after coculture were reduced by 32%. Continuous exposure of the progenitors to *C. albicans* yeasts generated macrophages with a greater ability to kill *C. glabrata* yeasts: CFUs were reduced by 49% (i.e. 53% better at killing than control macrophages). Therefore, the fungicidal activity against *C. glabrata* was similar to that against *C. albicans*, confirming the higher fungicidal activity of M-CSF-derived macrophages generated from HSPCs in the presence of *C. albicans* cells.

2 Phenotype of macrophages derived from HSPCs exposed to TLR2, TLR4 and Dectin-1 ligands in GM-CSF cultures

2.1 Surface molecules expression

Levels of the myelopoietic growth factors G-CSF and GM-CSF rise rapidly during infection to replace myeloid cells consumed fighting against the pathogen (Boettcher and Manz, 2016). Therefore, we decided to use GM-CSF to induce *in vitro* macrophage differentiation from HSPCs and studied the functional consequences for generated macrophages of the presence of PRR agonists during differentiation. Lin^- cells were cultured with GM-CSF in the presence or absence (control) of different PRR agonists: Pam_3CSK_4, LPS, depleted zymosan or *C. albicans* ATCC 26555 yeasts. Stimulation of HSPCs was performed following both strategies used in the M-CSF differentiation model: continuous exposure, stimulating HSPCs with all mentioned PRR agonists for the seven days of differentiation, or transient exposure, stimulating cells with the soluble agonists only for the first 24 h (**Figure 9A**).

Adherent cells were harvested after seven days of culture and the phenotypic surface molecules of the GM-CSF-derived adherent cells were analyzed by multicolor flow cytometry (**Figure 9**). It has been described that the output from culturing mouse BM cells with GM-CSF is heterogeneous and comprises different $CD11b^+$ mature myeloid cells including granulocytes, macrophages and dendritic cells (Helft *et al*., 2015). In our culture conditions, the mature $CD11b^+$ adherent population obtained (GM-CSF-derived macrophages) was heterogeneous in terms of expression of MHCII and Ly6C, as roughly 30% cells were $Ly6C^+$ $MHCII^-$, 22% cells were $Ly6C^-$ $MHCII^+$ and 45% cells were $Ly6C^+$ $MHCII^+$.

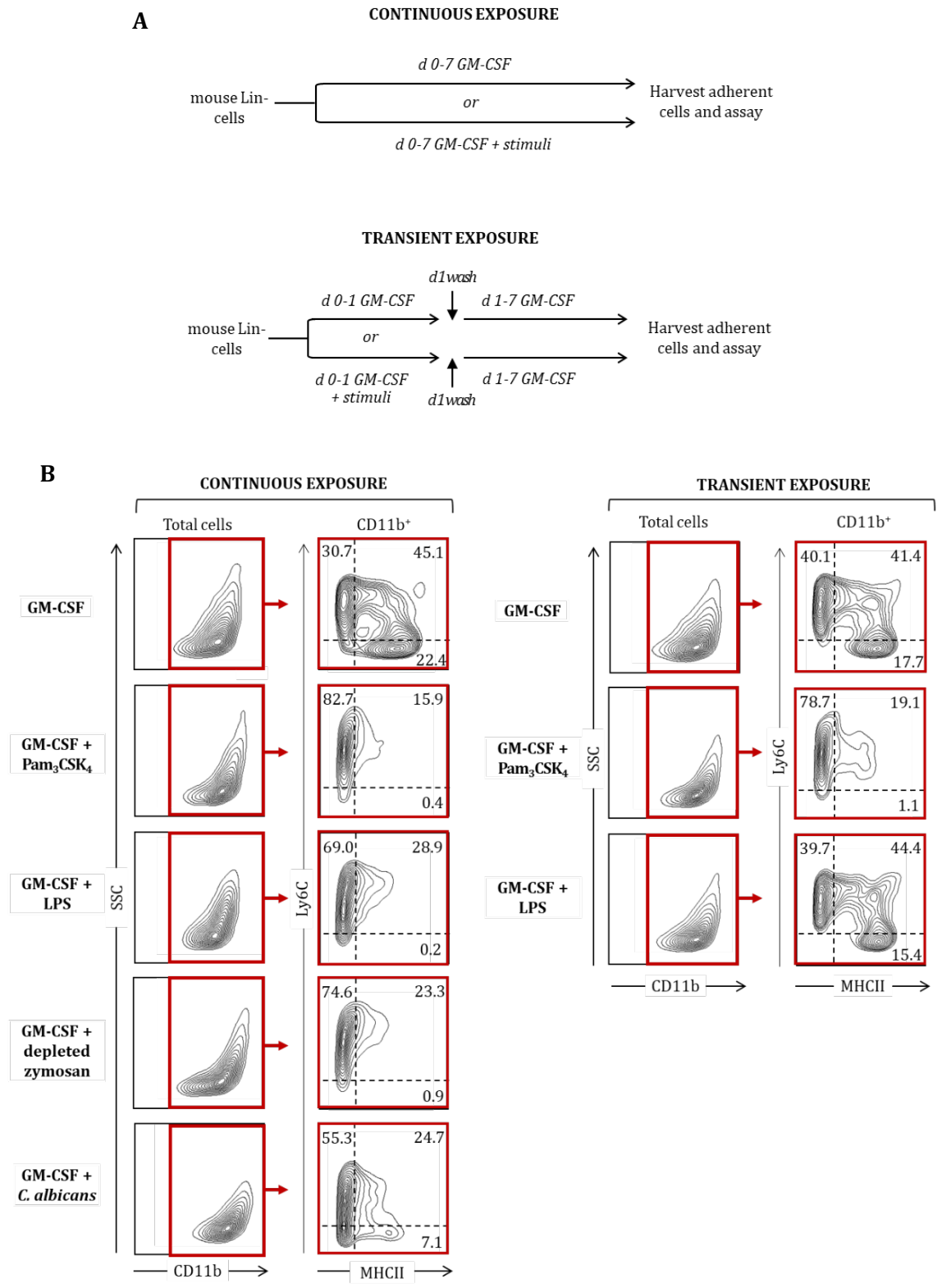


FIGURE 9 | Flow cytometry analysis of adherent cells produced by HSPCs continuously or transiently exposed to PAMPs in GM-CSF cultures. (A) Schematic protocol. Purified Lin⁻ cells from BM of C57BL/6 mice were cultured with 20 ng/ml SCF and 50 ng/ml GM-CSF for seven days to induce macrophage production, in the presence or absence of different microbial stimuli for the entire seven days (continuous exposure) or the first 24 h (transient exposure). The microbial stimuli used were 1 μg/ml Pam_3CSK_4, 100 ng/ml LPS, 1 μg/ml depleted zymosan or inactivated *C. albicans* yeasts (1:7.5 murine cell:yeast ratio). **(B)** At day seven, adherent cells were harvested, labeled with antibodies, and analyzed by flow cytometry. Macrophages were gated as $CD11b^+$ cells and were subsequently analyzed in a MHCII versus Ly6C plot. The indicated numbers refer to the percentages of cells analyzed in each contour-plot. Results shown are representative of three independent experiments.

However, the presence of TLR ligands during myeloid differentiation from Lin^- progenitors with GM-CSF changed the expression of Ly6C and MHCII in the macrophages they produced. The $CD11b^+$ macrophages generated in the presence of LPS and Pam_3CSK_4 exhibited a higher percentage of $Ly6C^+$ cells (from 30% to 83% and 69%, respectively) and a lower percentage of $MHCII^+$ cells (from 22% to 0.4% and 0.2%, respectively). The percentage of $Ly6C^+$ $MHCII^+$ cells also decreased (from 45% to 16% and 29%, respectively). Depleted zymosan and *C. albicans* yeast treatment changed the percentages of GM-CSF-derived macrophages that express Ly6C and/or MHCII in a similar way (**Figure 9**, continuous exposure), although in a lower extent than Pam_3CSK_4 and LPS. It was particularly noted when HSPCs were exposed to yeasts, as the percentage of $MHCII^+$ cells remained at 7%. Interestingly, although LPS and Pam_3CSK_4 induced similar phenotypic changes upon continuous exposure, transient exposure to LPS did not change the percentage of single or double positive cells for Ly6C and/or MHCII, whereas transient exposure to Pam_3CSK_4 induced similar changes than continuous exposure (**Figure 9**, transient exposure).

In summary, transient exposure of HSPCs to the TLR2 agonist Pam_3CSK_4 in GM-CSF cultures is sufficient to promote the generation of macrophages with an increased Ly6C expression and a decreased MHCII expression. The TLR4 agonist LPS also induce this phenotypic change but only following continuous exposure, similarly to both the Dectin-1 agonist depleted zymosan and *C. albicans* yeasts.

2.2 Cytokine production: TNF-α and IL-6

Next, we wondered whether the exposure of HSPCs to PAMPs in GM-CSF cultures influences the ability of generated macrophages to secrete pro-inflammatory cytokines in response to TLR agonists (**Figure 10**). Results show that TNF-α and IL-6 production in response to Pam_3CSK_4 or LPS was significantly diminished in macrophages generated from HSPCs exposed (transiently or continuously) to Pam_3CSK_4 compared to control macrophages (derived from unexposed HSPCs). Interestingly, macrophages generated from LPS-exposed HSPCs, either following a transient or continuous exposure, produced similar pro-inflammatory cytokine levels to control macrophages. On the other hand, TNF-α and IL-6 secretion in response to Pam_3CSK_4 was slightly increased in macrophages generated from HSPCs in the presence of depleted zymosan, whereas *C. albicans* yeasts did not change the production of cytokines by macrophages in response to neither Pam_3CSK_4 nor LPS. These results demonstrate that exposure of HSPCs to a TLR2 agonist (continuous or

transient) during myeloid differentiation in the presence of GM-CSF causes a reduction in the ability of the macrophages derived from them to produce pro-inflammatory cytokines. However, neither differentiation in the presence of a soluble TLR4 ligand nor a particulate Dectin-1 agonist or inactivated yeasts provokes a consistent altered cytokine production by GM-CSF-derived macrophages.

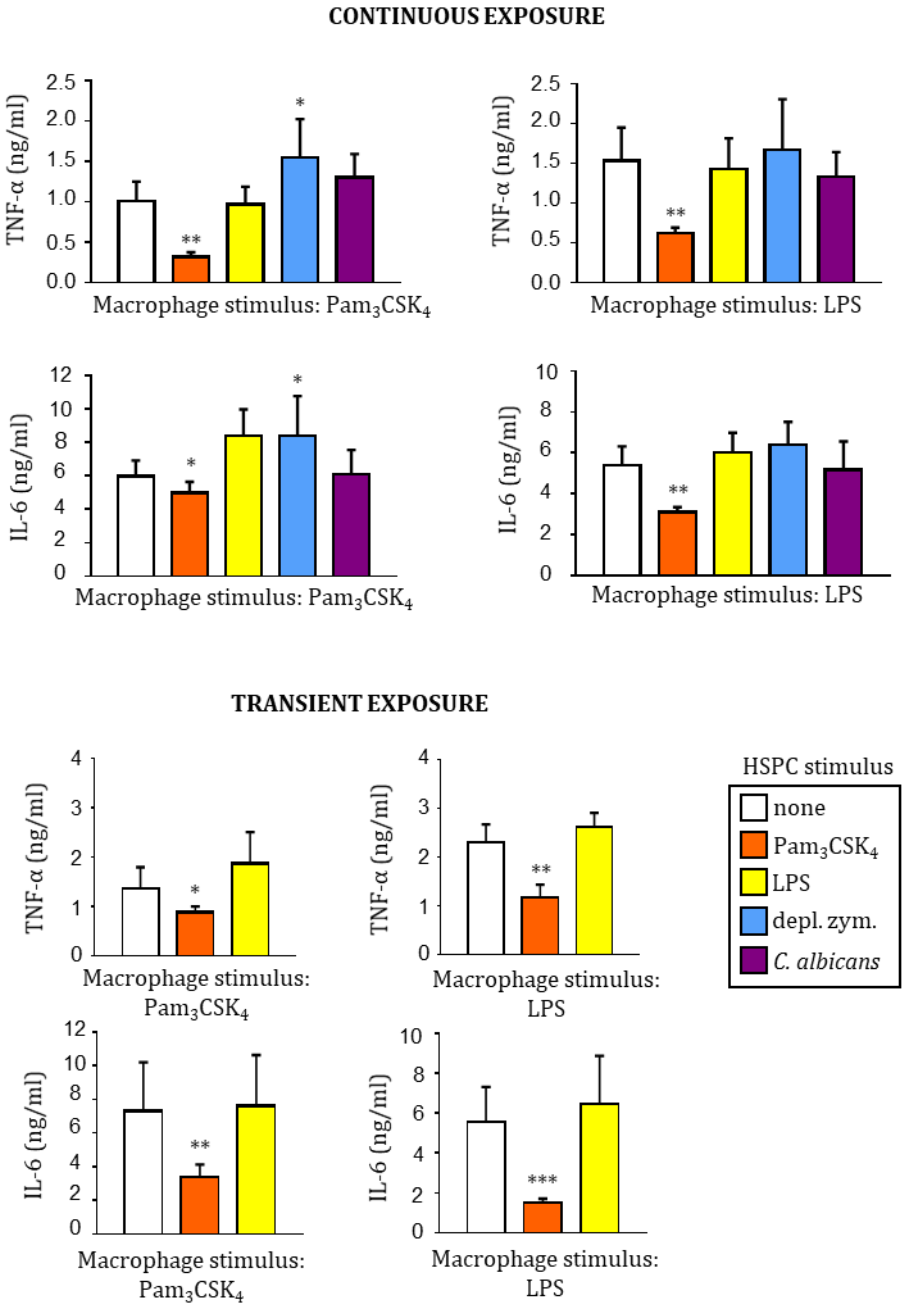


FIGURE 10 | Cytokine production by macrophages generated from HSPCs under the same conditions as in Figure 9A. Macrophages obtained as in Figure 9A were plated at equal numbers and challenged with 100 ng/ml Pam_3CSK_4 or 100 ng/ml LPS for 24 h. TNF-α and IL-6 levels in cell-free culture supernatants were measured by ELISA. Results are expressed as means ± SD of pooled data from three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ with respect to cytokine production by cells derived from unstimulated HSPCs (GM-CSF only) for each condition (continuous or transient).

2.3 Fungicidal activity

To further characterize the antifungal properties of GM-CSF-differentiated macrophages, we measured their ability to internalize and kill *C. albicans* yeasts using the same experimental assay performed with M-CSF-differentiated macrophages. Macrophages differentiated from HSPCs with GM-CSF were able to reduce *C. albicans* CFUs to roughly 30% after coculture (**Figure 11**), much more than M-CSF-differentiated macrophages (**Figure 7**). As the presence of inactivated *C. albicans* yeasts during macrophage differentiation with M-CSF generate macrophages with an increase fungicidal activity (**Figure 7**), we tested whether this functional phenotypic changes also occured when macrophages were obtained in GM-CSF cultures. However, the presence of *C. albicans* yeasts during differentiation of macrophages from HSPCs in GM-CSF cultures did not modify the ability of generated macrophages to kill yeasts (**Figure 11**).

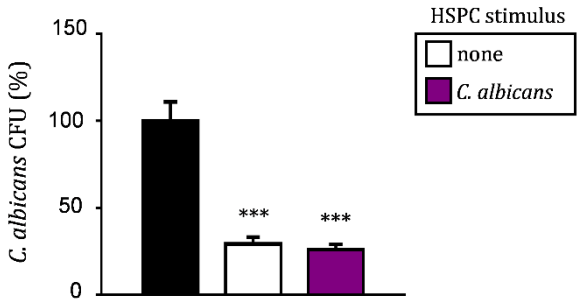


FIGURE 11 | Fungicidal activity of adherent cells produced by HSPCs exposed to *C. albicans* yeasts in GM-CSF cultures. Macrophages obtained as in Figure 9A were plated at equal numbers and challenged with viable *C. albicans* yeasts at a 1:3 ratio (murine cell:yeast) for 1 h. *C. albicans* cells were also inoculated in culture medium without murine cells (control, black bar). After incubation, samples were diluted, plated on Sabouraud dextrose agar and incubated overnight at 37 °C; the colonies were counted and expressed as % CFUs as compared to CFUs in the control sample at time 1 h. Triplicate samples were analyzed in each assay. Data represents means ± SD of pooled data from three experiments. *** $P < 0.001$ with respect to control.

3 Macrophages phenotype after sequential signaling of different PAMPs during *in vitro* differentiation from HSPCs

3.1 Macrophage functional phenotype in M-CSF cultures

It has been shown that Dectin-1 ligand β-glucan reverses LPS tolerance in human macrophages, restoring their ability to produce pro-inflammatory cytokines (Novakovic *et al.*, 2016). These results prompted us to investigate whether specific fungal ligands or *C. albicans* yeasts can reverse the functional consequences of TLR2 activation in HSPCs on the macrophages produced from them. For these experiments, Lin^- cells were cultured with M-CSF in the presence or absence of Pam_3CSK_4 on day 0, washed thoroughly to remove the Pam_3CSK_4 on day 1, and then cultured with M-CSF to induce macrophage differentiation for further six days in the presence or absence of depleted zymosan or *C. albicans yeasts* (**Figure 12A**). Adherent harvested cells were then counted, equal numbers of macrophages were plated, and cells were challenged with Pam_3CSK_4 or LPS to assess their ability to produce pro-inflammatory cytokines.

In accordance with our previous data, TNF-α and IL-6 levels in response to both TLR agonists decreased in M-CSF-derived macrophages generated from HSPCs transiently exposed to Pam_3CSK_4 in comparison to control macrophages (derived from unexposed HSPCs) (**Figure 12B**). Here, we showed that differentiation of Lin^- cells with M-CSF in the presence of fungal stimuli following a transient Pam_3CSK_4 challenge partially reversed the Pam_3CSK_4-induced macrophage phenotype; generated macrophages produced higher levels of pro-inflammatory cytokines than macrophages derived from Pam_3CSK_4-exposed HSPCs, but still significantly minor amounts than control macrophages (derived from unexposed HSPCs). Moreover, this partial reversion of the Pam_3CSK_4 effect was stronger in response to *C. albicans* than to depleted zymosan. In fact, the reduced production of IL-6 by macrophages derived from Pam_3CSK_4-exposed HSPCs was not affected by subsequent exposure to depleted zymosan during differentiation.

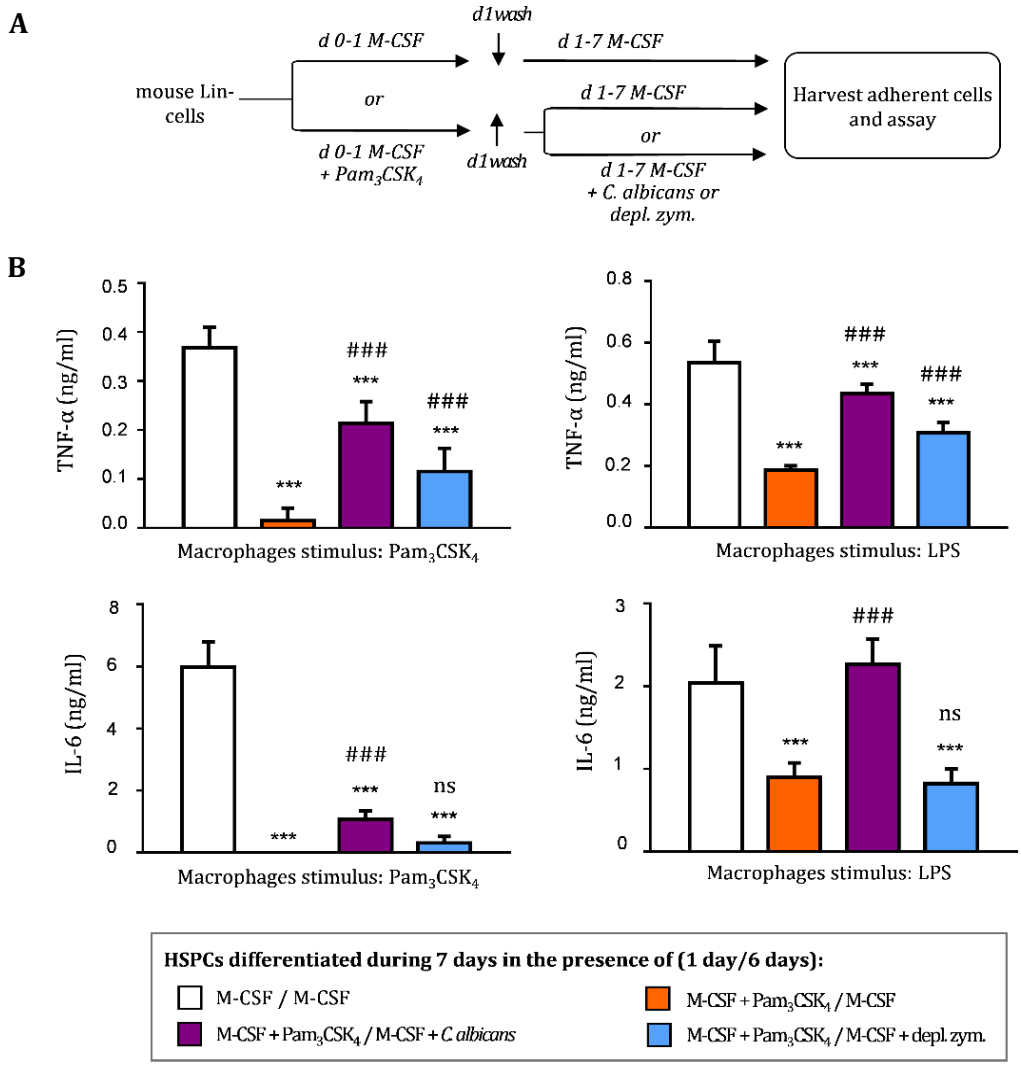


FIGURE 12| Cytokine production by M-CSF-derived macrophages obtained from HSPCs under the conditions in the schematic protocol. (A) Purified Lin⁻ cells from BM of C57BL/6 mice were cultured on day 0 with 20 ng/ml SCF and 50 ng/ml M-CSF in the presence or absence of 1 µg/ml Pam_3CSK_4. Cells were washed thoroughly on day 1 and then cultured with M-CSF for further 6 days in the presence or absence of 10 µg/ml depleted zymosan or inactivated *C. albicans* yeasts (1:7.5 murine cell: yeast ratio). **(B)** Adherent cells were harvested and challenged with 100 ng/ml Pam_3CSK_4 or 100 ng/ml LPS for 24 h. TNF-α and IL-6 levels in cell-free supernatants were measured by ELISA. Results are expressed as means ± SD of pooled data from two experiments. ns: non-significant, *** $P < 0.001$ with respect to cytokine production by control cells (macrophages derived from HSPCs differentiated with M-CSF only) and ### $P < 0.001$ with respect to cytokine production by cells derived from HSPCs transiently exposed to Pam_3CSK_4.

3.2 Macrophage functional phenotype in GM-CSF cultures

As the presence of *C. albicans* yeasts during differentiation from HSPCs partially reverse the effect induced by the TLR2 ligand Pam_3CSK_4 in M-CSF-derived macrophages, we next investigate whether this enhanced inflammatory responsiveness may also occur in GM-CSF-derived macrophages. Lin⁻ cells were cultured with GM-CSF in the presence or

absence of Pam_3CSK_4 for 24 h (transient exposure). Then, after removing Pam_3CSK_4 by washing, HSPCs were cultured with GM-CSF for further 6 days in the presence or absence of depleted zymosan or *C. albicans* yeasts. As expected, the production of TNF-α and IL-6 in response to both Pam_3CSK_4 and LPS was significantly diminished in macrophages generated from HSPCs transiently exposed to Pam_3CSK_4, compared to control macrophages (derived from unexposed HSPCs) (**Figure 10**). However, neither depleted zymosan nor *C. albicans* yeasts reversed the lower production of cytokines induced by Pam_3CSK_4 exposure of HSPCs. Rather, they even further decreased TNF-α and IL-6 production in response to Pam_3CSK_4, while they did not modify cytokine production in response to LPS. Therefore, *C. albicans* yeasts reinforce the phenotype induced by Pam_3CSK_4 exposure of HSPCs in GM-CSF-derived macrophages, while partially reversing it in M-CSF-derived macrophages.

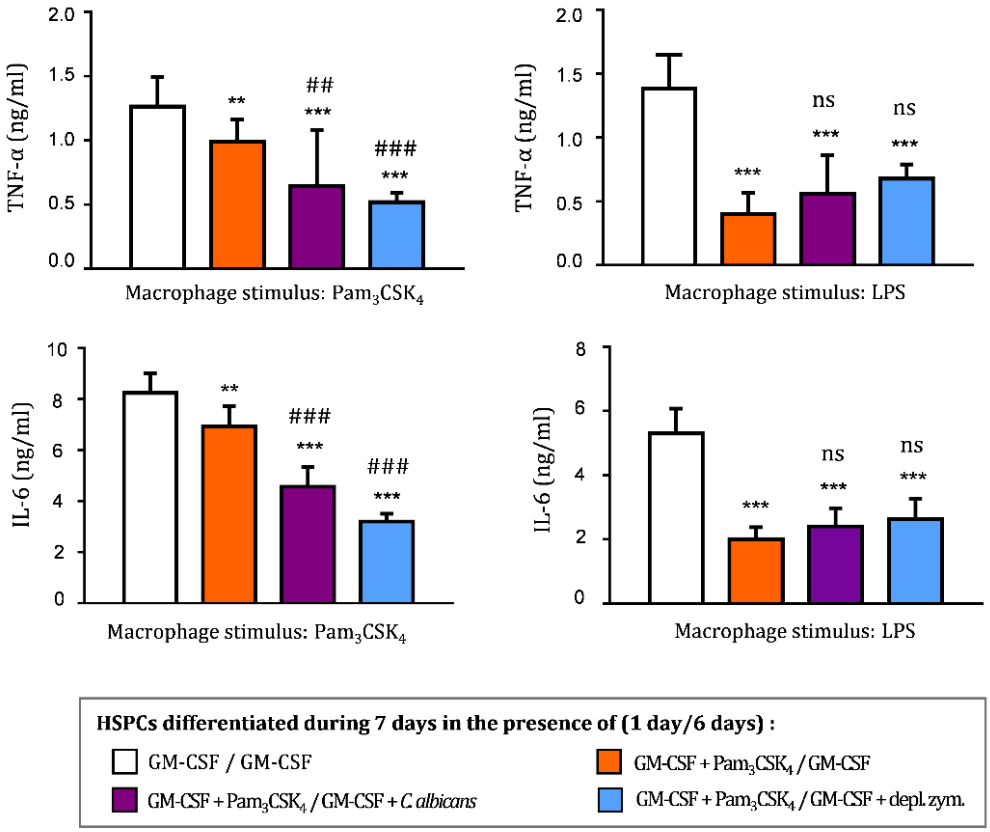


FIGURE 13 | Cytokine production by GM-CSF-derived macrophages obtained from HSPCs following the same PRR stimulating protocol as in Figure 12A. Purified Lin^- cells from BM of C57BL/6 mice were cultured on day 0 with 20 ng/ml SCF and 50 ng/ml GM-CSF for 24 h in the presence or absence of 1 µg/ml Pam_3CSK_4. Cells were washed thoroughly on day 1 and then cultured with GM-CSF for further 6 days in the presence or absence of 10 µg/ml depleted zymosan or inactivated *C. albicans* yeasts (1:7.5 murine cell: yeast ratio). Adherent cells were harvested and challenged with 100 ng/ml Pam_3CSK_4 or 100 ng/ml LPS for 24 h. TNF-α and IL-6 levels in cell-free supernatants were measured by ELISA. Results are expressed as means ± SD of pooled data from two experiments. ns: non-significant, ** $P < 0.01$ and *** $P < 0.001$ with respect to cytokine production by control cells (macrophages derived from HSPCs differentiated with GM-CSF only) and ## $P < 0.01$ and ### $P < 0.001$ with respect to cytokine production by cells derived from HSPCs transiently exposed to Pam_3CSK_4.

4 Phenotype of macrophages *ex vivo* derived from HSPCs *in vivo* exposed to systemic candidiasis or to a TLR2 ligand

4.1 Systemic candidiasis

As it has been reported in the previous sections of this thesis project, *in vitro* detection of TLR2 or Dectin-1 ligands (including inactivated yeasts of *C. albicans*) by HSPCs impacts the antimicrobial function of the macrophages they produce. The observed *in vitro* effect of *C. albicans* on HSPCs may be of biological relevance *in vivo* during infection. Therefore, we now sought to determine whether HSPCs may sense microorganisms *in vivo* using a mouse model of systemic candidiasis, and whether this may alter the function of the macrophages they produce *ex vivo*. C57BL/6 mice were infected via intraperitoneal injection of a virulent strain of *C. albicans* (45×10^6 yeasts per mouse). At days one or three post-infection, Lin^- cells were purified from mice BM and differentiated into macrophages in M-CSF cultures in the presence of 2.5 μg/ml amphotericin B to prevent potential fungal growth. To assess the tissue outgrowth of the microorganism in infected mice, the fungal burden was determined in the kidney, the target organ in this invasive model of candidiasis. The dose of yeasts injected resulted in a low and high number of CFUs at days one and three, respectively. The *ex vivo* differentiated macrophages were counted and plated at equal cell numbers for stimulation with TLR agonists to assess their ability to produce TNF-α (**Figure 14**). Cytokine production in response to Pam_3CSK_4 was significantly increased in macrophages generated from low-infected mice (day one post-infection) compared to macrophages generated from control uninfected mice (control macrophages), whereas macrophages generated from high infected animals (day three post-infection) produced lower cytokine levels than control macrophages. TNF-α production in response to LPS was significantly diminished in macrophages generated from both low- and high-infected mice compared to control macrophages.

To further characterize the antifungal function of HSPC-derived macrophages generated from infected animals, we measured the macrophages ability to kill yeast cells *in vitro* using the same experimental assay performed with *in vitro* differentiated macrophages. In these conditions, control macrophages were able to kill a significant percentage of yeasts, similar to the percentage of yeasts killed by macrophages obtained from high-infected mice. However, macrophages differentiated from HSPCs purified from

low-infected animals possessed an increased fungicidal activity (roughly 80% as compared to 65% of killing by control macrophages, i.e., 23% greater than control macrophages (**Figure 14**).

Therefore, early during the infection, with low fungal burden levels, HSPCs give rise to macrophages trained in response to Pam_3CSK_4 and with higher fungicidal activity. Interestingly, when the infection reaches high fungal burden levels, HSPC-derived macrophages become tolerized, as they have a diminished ability to produce TNF-α, whereas they keep up their fungicidal capacity. These data collectively indicates that HSPCs sense the infection *in vivo* and this profoundly alters the functional phenotype of the macrophages *ex vivo* derived from them.

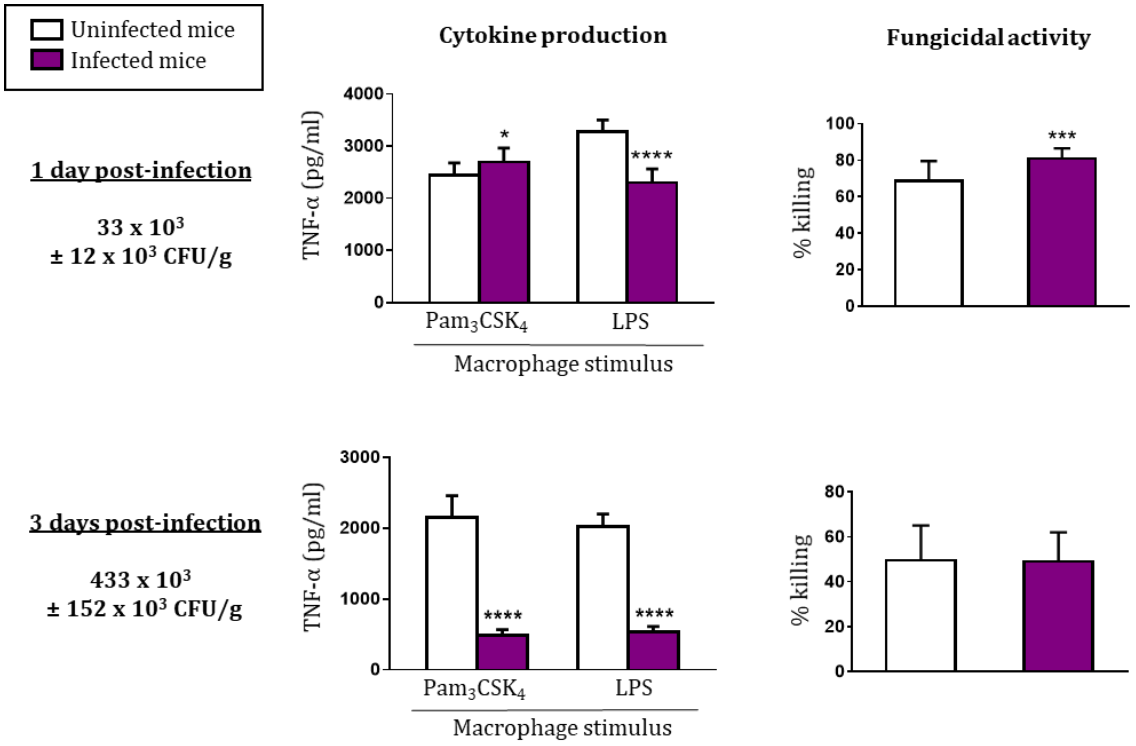


FIGURE 14 | Cytokine production and fungicidal activity of *ex vivo* HSPC-derived macrophages from *C. albicans* infected mice. C57BL/6 mice were injected intraperitoneally with 45 × 10^6 yeasts of *C. albicans* ATCC 26555. At day 1 or 3 post-infection, mice were sacrificed to assess the outgrowth of the yeasts in the kidney, and to isolate the BM. The fungal burden in the kidneys is expressed as CFUs per gram of tissue. Lin^- cells were purified from BM and cultured for seven days with medium containing 20 ng/ml SCF, 50 ng/ml M-CSF and 2.5 μg/ml amphotericin B. At day seven, adherent cells were harvested and for cytokine assays, they were challenged with Pam_3CSK_4 (100 ng/ml) or LPS (100 ng/ml) for 24 h. TNF-α levels in cell-free culture supernatants were measured by ELISA. For fungicidal activity determination, macrophages were challenged with viable PCA2 yeasts at a 1:3 ratio (murine cell:yeast) for 1 h. *C. albicans* cells were also inoculated in culture medium without murine cells (control). After incubation, samples were diluted, plated on Sabouraud dextrose agar and incubated overnight art 37 °C; CFUs were counted and killing percentages were determined as follows: % killing = [1 – (CFUs sample at t = 1 h)/(CFUs control at t = 1 h)] × 100. Triplicate samples were analyzed in each assay. Results are expressed as means ± SD of pooled data from two experiments. * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$ with respect to macrophages derived from control uninfected mice.

4.2 TLR2 agonist exposure: short treatment and extended treatment

Next, we wondered whether *in vivo* exposure of HSPCs to Pam_3CSK_4 might alter the function of the macrophages they produce *ex vivo*. Firstly, we used a short treatment model by intravenously injecting mice with one dose of the ligand. One day later (24 h), Lin^- cells were purified from BM and cultured with M-CSF for 7 days, for macrophage differentiation, in the presence or absence of inactivated *C. albicans* yeasts. HSPC-derived macrophages from untreated mice were used as control macrophages. The harvested adherent cells were then plated for stimulation with TLR agonists to assess their ability to produce TNF-α. The production of TNF-α in response to Pam_3CSK_4 or LPS was significantly diminished in macrophages generated from HSPCs exposed to the TLR2 ligand *in vivo*, compared to control macrophages (**Figure 15**, plain white and colored bars).

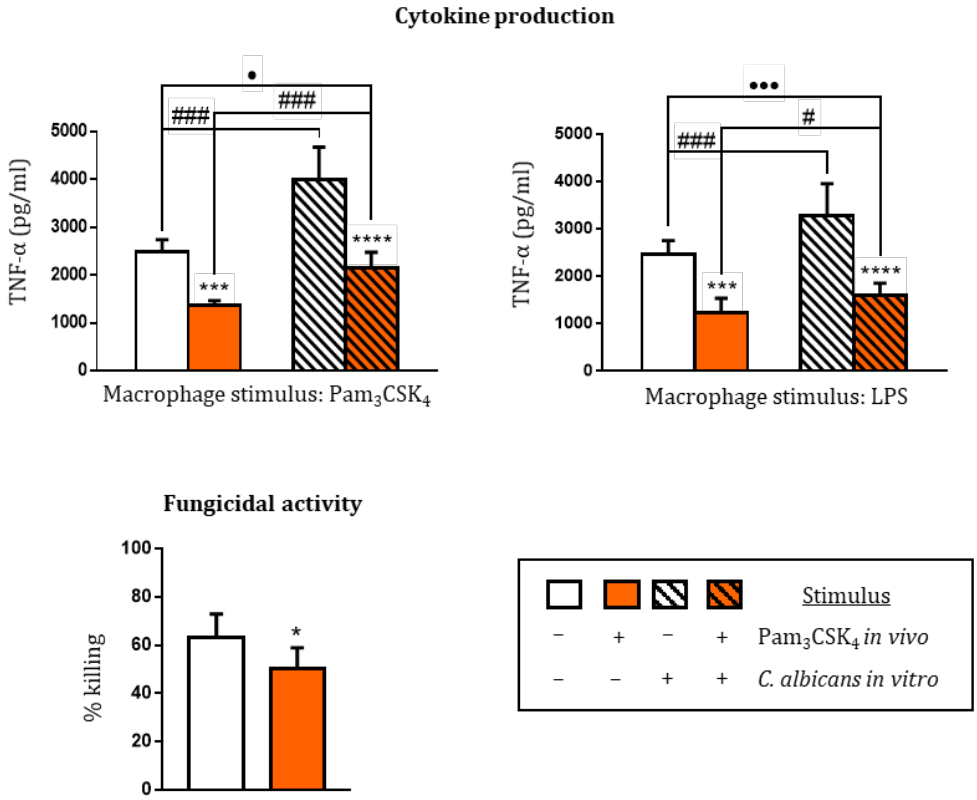


FIGURE 15 | Cytokine production and fungicidal activity of *ex vivo* HSPC-derived macrophages from mice treated with one dose of Pam_3CSK_4. C57BL/6 mice were injected intravenously with 100 µg of Pam_3CSK_4 and Lin^- cells were recovered from BM on day 1 for *ex vivo* differentiation to macrophages. Lin^- cells were cultured during seven days with media containing 20 ng/ml SCF and 50 ng/ml M-CSF, in the presence or absence of inactivated yeasts of *C. albicans* (1:7.5 murine cell:yeast ratio). At day seven, adherent cells were harvested and for cytokine assays and fungicidal activity determination, they were challenged as indicated in Figure 14. Triplicate samples were analyzed in each assay. Results are expressed as means ± SD of pooled data from two experiments. * $P<0.05$, *** $P < 0.001$ and **** $P <0.0001$ with respect to macrophages derived from control untreated mice, # $P < 0.05$ and ### $P < 0.001$ with respect to cytokine production by macrophages derived from HSPCs differentiated with M-CSF only, in the absence of inactivated yeasts, and · $P < 0.05$ and ··· $P < 0.001$ with respect to macrophages derived from control untreated mice.

Consistent with our previous data reported in this thesis project, *in vitro* HSPC differentiation in the presence of *C. albicans* leads to the generation of macrophages that produce higher levels of TNF-α (**Figure 15**, grated white bars). Interestingly, differentiation of Lin^- cells in the presence of fungal stimuli partially reversed the effect of *in vivo* Pam_3CSK_4 signaling in HSPCs (**Figure 15**, grated colored bars), as those macrophages produced higher cytokine levels than HSPC-derived macrophages from Pam_3CSK_4-treated mice (**Figure 15**, plain colored bars) but still significantly minor amounts than control macrophages (**Figure 15**, plain white bars). In addition, macrophages were challenged with viable yeasts in order to determine their fungicidal activity. Results show that the tolerized macrophages generated from HSPCs exposed to the TLR2 ligand *in vivo* have a diminished ability to kill yeast cells *in vitro*, as compared to control macrophages (**Figure 15**). Therefore, a short *in vivo* exposure to a TLR2 agonist results in M-CSF-derived macrophages with a less ability to produce pro-inflammatory cytokines, which can still respond to *in vitro* training by *C. albicans* yeasts but have decreased their fungicidal activity.

These findings prompted us to investigate whether this macrophage phenotype, induced by a short systemic Pam_3CSK_4 exposure, might also occur after an extended Pam_3CSK_4 treatment. For this purpose, we used a model previously described by Herman *et al.* (2016), for our *ex vivo* assays. C57BL/6 mice were given 100 μg of Pam_3CSK_4 by intraperitoneal injection at days 0, 3 and 5. One day (24 h) after the final dose (day 6), Lin^- cells were purified from the BM and differentiated into macrophages with M-CSF, in the presence or absence of inactivated *C. albicans* yeasts. In this scenario of extended treatment with the TLR2 ligand, TNF-α production was significantly higher in response to Pam_3CSK_4 or LPS, compared to control macrophages (generated from HSPCs from control animals) (**Figure 16**, plain white and colored bars). These macrophages, generated from HSPCs exposed to the TLR2 ligand *in vivo*, showed no significant differences in their ability to kill yeast cells *in vitro*, as compared to control macrophages (**Figure 16**, plain white and colored bars). The presence of fungal cells during differentiation further increased the increased TNF-α response to Pam_3CSK_4, but not to LPS stimulation (**Figure 16**, grated colored bars). Therefore, after an extended *in vivo* TLR2 agonist exposure, HSPCs give rise to M-CSF-derived macrophages with an increased ability to produce pro-inflammatory cytokines, but a similar fungicidal activity than control macrophages.

Our data indicate that HSPCs sense the TLR2 agonist *in vivo*, and that this profoundly alters the functional phenotype of the macrophages *ex vivo* derived from them. The ability of HSPC-derived macrophages to produce inflammatory cytokines is dependent on the extent of HSPC exposure to Pam_3CSK_4 challenge, as short exposure (one dose) generates

macrophages that produce lower amounts of cytokines, while an extended treatment (three doses) generates macrophages that produce higher amounts of cytokines than macrophages derived from unexposed HSPCs. Furthermore, the fungicidal activity is lower for short exposure or similar for extended treatment, compared to that of control macrophages.

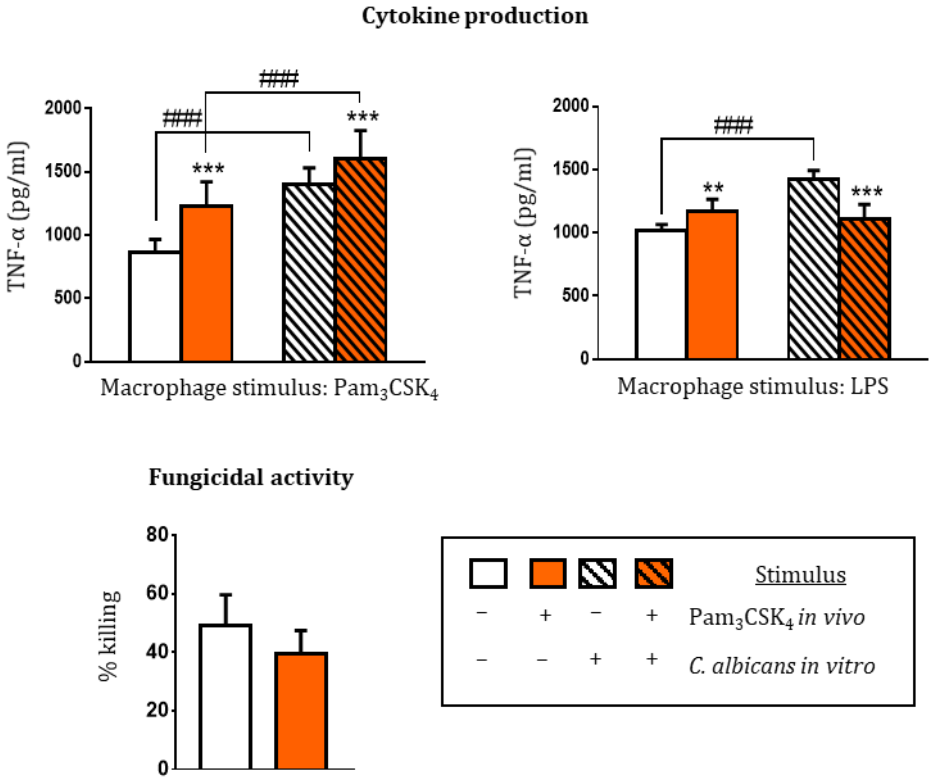


FIGURE 16 | Cytokine production and fungicidal activity of *ex vivo* HSPC-derived macrophages from mice treated with several doses of Pam_3CSK_4. C57BL/6 mice were given 100 µg of Pam_3CSK_4 by intraperitoneal injection at days zero, three, and five (three doses), and 24 h after the final dose, mice were sacrificed to isolate the BM. Lin^- cells purified from BM were cultured during seven days in medium containing 20 ng/ml SCF and 50 ng/ml M-CSF in the presence or absence of inactivated yeasts of *C. albicans* (1:7.5 murine cell:yeast ratio). At day seven, adherent cells were harvested and for cytokine assays and fungicidal activity determination, they were challenged as indicated in figure 14. Triplicate samples were analyzed in each assay. Results are expressed as means ± SD of pooled data from two experiments. ** $P < 0.01$ and *** $P < 0.001$ with respect to macrophages derived from control untreated mice, ### $P < 0.001$ with respect to cytokine production by macrophages derived from HSPCs differentiated with M-CSF only, in the absence of inactivated yeasts.

5 Discussion

The supply of myeloid phagocytes, both in the steady state as well as during a demand-adapted response to infection, is primarily obtained by myelopoiesis from HSPCs. Various mechanisms govern HSPC responses to infection, including cytokine signaling, niche function and direct sensing of PAMPs by HSPCs themselves (Boettcher and Manz, 2016; Chavakis *et al.*, 2019; Kobayashi *et al.*, 2016; Zhao and Baltimore, 2015). Thus, additional perspectives on hematopoiesis during infection have come from the discovery that HSPCs express functional TLRs and that TLR signals provoke cell cycle entry and myeloid differentiation both *in vitro* and *in vivo* (Cannova *et al.*, 2015; Yáñez *et al.*, 2013a). Our previous studies show that PRR-mediated recognition of *C. albicans* by HSPCs may help replace and/or increase cells that constitute the first line of defense against the fungus (Megías *et al.*, 2013; Yáñez *et al.*, 2010; Yáñez *et al.*, 2011; Yáñez *et al.*, 2009). The fact that HSPCs sense pathogens indicates a new role for TLRs because they may be involved in instructing immune cell development following direct detection of microbes by HSPCs, leading to the generation of innate immune cells that fight against the pathogen. However, a study of the functional properties of the generated myeloid cells is essential to discern whether pathogens may modulate HSPC responses to evade the immune system or, alternatively, this mechanism may be protective, allowing a rapid generation of cells in a pathogen-specific manner, which are better prepared to deal with the infection.

In this study, we first performed an *in vitro* model of HSPC differentiation by using M-CSF, a factor produced by the host in homeostatic conditions, to investigate the functional consequences for macrophages generated in the presence of different PRR agonists or *C. albicans* yeasts. The exposure of HSPCs to Pam_3CSK_4 (continuous or transient) or LPS (continuous) increased the yield of M-CSF-derived macrophages, while the exposure to depleted zymosan or *C. albicans* cells did not, indicating that TLR agonists, but not Dectin-1 agonist or yeasts, induce proliferation and/or survival of the progenitors during differentiation. These results are in agreement with our previous report in which we show that Pam_2CSK_4 (a synthetic version of bacterial lipopepetide that is detected by TLR6/TLR2 heterodimers, instead of detection by TLR1/TLR2 heterodimers as Pam_3CSK_4) induces the proliferation of LT-HSCs *in vitro* under defined conditions of culture (serum-free, stromal cell-free cultures) (Yáñez *et al.*, 2010). In those conditions (specifically designed to prevent differentiation), *C. albicans* yeasts are also able to induce HSC proliferation in a TLR2/MyD88-dependent manner, although under our current experimental conditions

(M-CSF and serum), differentiation seems to be favored over proliferation of progenitors in response to *C. albicans.*

The same stimuli that increased the yield of M-CSF-derived macrophages (continuous/transient Pam_3CSK_4 or continuous LPS) also modified the expression of the surface molecules Ly6C and MHCII. TLR signaling increased the percentage of cells that expressed the inflammatory monocyte marker Ly6C and are negative for MHCII, a phenotype corresponding to inflammatory macrophages that play a clear role in defense against pathogens. To investigate this further, we assessed whether PRR signaling modulates anti-fungal functions such as cytokine production, phagocytosis and killing of yeasts. In this context, our group have already shown that macrophages derived from HSPCs exposed to a TLR2 agonist produced lower levels of inflammatory cytokines (Yáñez *et al.*, 2013b). Here we found the same phenotype after LPS treatment (continuous or transient) whereas the opposite phenotype (increased TNF-α and IL-6 production) was observed following depleted zymosan or *C. albicans* yeast treatment. It should be noted that depleted zymosan and *C. albicans* yeasts are particulate ligands while Pam_3CSK_4 and LPS are soluble molecules, and these different physical properties may be sensed by the progenitors. Although the profoundly altered ability to produce cytokines may be partially due to the observed changes in TLR expression, other mechanisms regulating signal transduction from TLRs must also contribute to this phenotype.

Endotoxin tolerance is a well-known process whereby innate immune cells (particularly monocytes and macrophages) that previously responded to LPS display a reduced ability to produce inflammatory cytokines upon subsequent stimulation (Biswas and López-Collazo, 2009; Cavaillon and Adib-Conquy, 2006). Our data indicate that this tolerance could be applied not only to mature myeloid cells but to HSPCs too, as transient exposure of Lin^- cells to LPS results in the generation of mature macrophages with a reduced ability to produce inflammatory cytokines. Therefore, detection of soluble LPS by HSPCs during systemic infections may lead to the generation of tolerized macrophages. Heterotolerance also occurs in HSPCs, as HSPCs exposed (transiently or continuously) to Pam_3CSK_4 or LPS generate macrophages with a diminished ability to produced pro-inflammatory cytokine in response to both TLR agonists.

Regarding to the ability of killing yeasts, we found that macrophages derived in the presence of the TLR2 agonist or *C. albicans* cells possessed higher fungicidal activity than control M-CSF-derived macrophages. In fact, macrophages derived in the presence of Pam_3CSK_4 killed yeasts better, even though they showed a reduction in phagocytosis. In contrast, these macrophages exhibited a reduced ability to produce inflammatory cytokines

(tolerized phenotype). In this context, Pam_3CSK_4 tolerance in macrophages has also been associated with an enhanced antimicrobial activity against gram-positive and gram-negative bacteria, due to an accelerate phagosome maturation with upregulation of membrane-trafficking regulators and lysosomal enzymes (Liu *et al.*, 2017). In this study, an enhanced bacteria phagocytosis by Pam_3CSK_4-tolerized macrophages was detected, unlike our results showed a reduction of *C. albicans* phagocytosis by HSPC-derived macrophages. These differences might be explained by the use of different microorganisms to study the antimicrobial ability of tolerized-macrophages, added to the fact that exposure to the TLR2 agonist prior to or during myelopoiesis did not have precisely the same effects on macrophage function as exposure after differentiation (Yáñez *et al.*, 2013b). It is interesting to note that TLR2 signaling reduced cytokine production and increased fungicidal activity, while the Dectin-1 agonist increased cytokine production but did not affect the fungicidal ability. However, *C. albicans* yeasts that signal via both receptors induced higher cytokine and higher fungicidal activity. These results indicate that combinatorial signaling by multiple receptors on HSPCs shapes their responses. Therefore, functional properties of macrophages produced during infection may depend on the specific molecular composition of the pathogen (the combination of PRRs triggered), added to other signals the HSPCs receive.

Our results are in line with a report showing that candidiasis affords protection against reinfection by inducing a functional reprogramming of monocytes, leading to an increased cytokine production in response to the secondary challenge (Quintin *et al.*, 2012). This phenomenon termed trained immunity, as well as endotoxin tolerance, proves that innate immune cells can display some memory characteristics and respond in a different way upon reexposure to the same or heterologous stimuli. The central feature of this innate immune memory is the ability of PAMP-exposed cells to mount a different transcriptional response compared to unexposed cells. Moreover, it has been demonstrated that epigenetic and transcriptional programs of monocyte differentiation to macrophages distinguish tolerant and trained macrophage phenotypes (Saeed *et al.*, 2014). Although in our continuous exposure experiments it is possible that both progenitor cells and differentiated monocyte/macrophages respond to PRR ligands, results obtained upon transient exposure indicate that this concept of "trained immunity" may apply not only to differentiated cells but also to HSPCs. Therefore, PRRs may instruct immune cell development and function following direct detection of PAMPs by HSPCs. Furthermore, training at the level of HSPCs would provide a mechanism for sustained production of trained monocytes after clearance of the pathogen.

HSPCs have been reported to produce copious amounts of diverse cytokines through NF-κB signaling in response to TLR stimulation. Among cytokines produced by HSPCs, IL-6 is a particularly important paracrine regulator of myeloid differentiation and HSPC proliferation, which induces rapid myeloid cell recovery during neutropenia (Zhao *et al.*, 2014). Other authors have shown that HSPCs secrete PGE_2 upon TLR2-mediated recognition of *S. aureus*, molecule that promotes HSPC proliferation and differentiation to the myeloid lineage in an autocrine/paracrine manner (Granick *et al.*, 2013). Consequently, we tested the possible role of IL-6 and PGE_2 in conferring phenotypic properties during differentiation, but blockade of these mediators did not reverse the diminished cytokine production of the macrophages produced by TLR-stimulated HSPCs. Future studies are required to define the mechanisms underlying the effects of HSPC exposure to PAMPs on macrophage function.

Thus far, our data show that detection of PAMPs by HSPCs impacts the antimicrobial function of the macrophages they produce in homeostatic conditions (M-CSF-derived macrophages). This is the first experimental evidence showing that HSPC activation in response to *C. albicans* leads to the generation of macrophages better prepared to deal with the infection: they produce higher levels of inflammatory cytokines (trained phenotype) and are better at killing *C. albicans* and *C. glabrata* yeasts than macrophages derived from unexposed HSPCs.

Next, we decided to study the effect of PRR stimulation in HSPCs on the functional phenotype of myeloid cells *in vitro* derived from them by using factors produced by the host during infection/inflammatory conditions (GM-CSF-derived macrophages). The exposure of HSPCs to TLR2, TLR4, or Dectin-1 agonists, as well as *C. albicans* inactivated yeasts, during the whole differentiation process, increased the percentage of cells that exhibit a "more pro-inflammatory" phenotype ($Ly6C^+$ $MHCII^-$). Interestingly, transient exposure (before differentiation) to Pam_3CSK_4 induced the same phenotype clearly indicating that TLR2 signaling on HSPCs impacts the phenotype of the macrophages they produce during infection/inflammation.

To study the impact of PRR signaling in HSPCs on the functional phenotype of macrophages obtained in GM-CSF cultures, we focused on their ability to produce inflammatory cytokines. Here, we found that GM-CSF-derived macrophages generated from HSPCs exposed to a TLR2 agonist (transiently or continuously, during differentiation) produced lower levels of inflammatory cytokines upon being challenged with TLR ligands. However, cytokine production by GM-CSF-derived macrophages was not modified by challenging HSPCs with LPS, depleted zymosan, or *C. albicans* yeasts. These results contrast

with the previous results obtained with M-CSF cultures, as LPS exposure during HSPC differentiation generates M-CSF-derived macrophages with a tolerized phenotype and *C. albicans* activation leads to the generation of trained macrophages. Furthermore, the fungicidal ability of GM-CSF-derived macrophages was not affected by the presence of inactivated *C. albicans* yeasts during macrophage differentiation. These results suggest that in the presence of M-CSF (in homeostatic conditions or very early during infection), different PRR activation of HSPCs may differentially influence the function of the macrophages they produce, while in the presence of GM-CSF (inflammatory conditions during the infection), only TLR2 activation of HSPCs determines the tolerized phenotype.

Endotoxin tolerance in macrophages is a mechanism that avoids sustained activation, which can lead to extensive tissue damage and manifestation of pathological states such as sepsis (Biswas and López-Collazo, 2009; Cavaillon and Adib-Conquy, 2006). In clinical studies, the magnitude and the persistent nature of this tolerized state is associated with increased mortality and nosocomial infections. In this context, it has been reported that Dectin-1 activation can reverse macrophage tolerance *ex vivo*, providing a potential therapeutic approach to reverse the tolerized phenotype in patients with sepsis (Novakovic *et al.*, 2016). Therefore, we hypothesized that fungal ligands may be capable of reversing the tolerized phenotype of macrophages obtained from HSPCs transiently exposed to Pam_3CSK_4. However, our results show that fungal ligands reinforced the tolerized phenotype of GM-CSF-derived macrophages but partially reversed the tolerized phenotype of M-CSF-derived macrophages. Overall, these results indicate that activation of HSPCs with *C. albicans* cells may generate macrophages with a different phenotype, depending on the growth factor (M-CSF or GM-CSF) that induces their differentiation as well as on the previous stimuli that progenitors received. Although further studies will be required to define the mechanisms underlying the effects that exposing HSPCs to PAMPs has on macrophage function, epigenetic reprogramming probably underlies these effects as it has been described in several models for innate immunity memory (Netea *et al.*, 2020; Saeed *et al.*, 2014).

Therefore, our results show that *in vitro* detection of PAMPs by HSPCs impacts the antimicrobial function of macrophages they produce either in homeostatic conditions (M-CSF-derived macrophages) or inflammatory conditions (GM-CSF-derived macrophages). However, tolerized or trained phenotype of HSPC-derived macrophages, regarding pro-inflammatory cytokine production, depends on the combinatorial signals that HSPCs receive (PRRs and CSFs), as well as on the timing of HSPC activation by the different stimuli.

Finally, we used an *ex vivo* model to further investigate whether HSPCs may sense *C. albicans* or their PAMPs during infection or *in vivo* exposure, respectively, and whether this signaling impacts the antimicrobial function of the macrophages they produce *ex vivo*. The direct interaction of *C. albicans* with HSPCs may involve yeast cells and/or fungal derived PAMPs in BM. In a previous report, we demonstrated the presence of viable yeasts in the BM of mice infected with the low virulent strain PCA2 (Yáñez *et al.*, 2011), and it is well known that some fungal PAMPs, such as mannan and glucan, are present in the blood of patients with systemic candidiasis (López-Ribot *et al.*, 2004; Obayashi *et al.*, 1995).

In these experiments, we have shown that early during candidiasis, *ex vivo* cultured HSPCs gave rise to macrophages with a trained phenotype in their cytokine response to a TLR2 ligand and with a higher fungicidal activity. However, when the infection progressed to high fungal burden levels, *ex vivo* HSPC-derived macrophages became tolerized in their cytokine response, while they kept up their fungicidal capacity. We can deduce from these observations that differences in the phenotype of generated macrophage depending on the stage of infection could be a beneficial effect for the host during the infection; in the first stages of the infection, inflammatory cytokines and phagocytes with high fungicidal capacity are needed, whereas when the fungal invasion progresses, a "cytokine storm" could be harmful, therefore the generated macrophages are tolerized then. The reasons why the generated macrophages exhibit these opposite responses (training or tolerance) during the infection are not obvious. However, they may be related to the increasing pathogen loads during the infection, to the progressive tissue damage and/or to the impairment of the immune cell function. Thus, immune cells shift from fighting against the-pathogen (training phenotype) toward maintenance and repair activities, which probably induces a phenotype of immune tolerance to the pathogen.

The fact that the pathogen dose plays a key role in determining hormetic responses has been recently proposed for mature innate immune cells (Bauer *et al.*, 2018). Our results suggest that this hormetic response also occurs in HSPCs during infection. A tolerized response would only develop when the early trained response is not able to deal with infection and, therefore, pathogen burden increases. Although the *in vitro* generated phenotype in our *ex vivo* model may not exactly reproduce the *in vivo* situation, our results clearly indicate that HSPCs sense the infection *in vivo*, which profoundly alters the functional phenotype of macrophages derived from them *ex vivo*. Supporting this idea, it has been shown that intravenous vaccination with BCG educates HSCs to generate trained monocytes/macrophages that protect mice against tuberculosis (Kaufmann *et al.*, 2018).

In the opposite direction to trained immunity, TLR2 agonist tolerance is a phenomenon that avoids sustained production of inflammatory mediators by innate immune cells (mainly monocytes and macrophages), which can be harmful to the host (Medvedev *et al.*, 2006). We have shown that a short systemic Pam_3CSK_4 exposure *in vivo* resulted in tolerized HSPC-derived macrophages, being this tolerized phenotype partially reversed by *C. albicans* yeasts presence during the *in vitro* HSPC differentiation. These results are consistent with the previous ones obtained using an *in vitro* HSPC stimulating protocol, in which macrophages produced by HSPCs *in vitro* exposed to Pam_3CSK_4 (prior to or during macrophage differentiation) manifested a tolerized phenotype that could be partially reverse by fungal ligands. However, an extended systemic Pam_3CSK_4 exposure *in vivo* generated HSPC-derived macrophages that produce higher amounts of cytokines (trained macrophages). Therefore, the *ex vivo* tolerized or trained phenotype depends both on the dose and the timing of the different signals (direct TLR2-mediated signaling and cytokines released by different cell types in response to Pam_3CSK_4) that HSPCs receive *in vivo*. It should be noted that a TLR2 agonist exposure and a systemic *C. albicans* infection are not comparable models. Therefore, the observed opposite responses of HSPCs to Pam_3CSK_4, switching from tolerance (one dose) to training (extended exposure), cannot be related to pathogen load, as the response to Pam_3CSK_4 involves only one PRR (TLR2) and occurs in the absence of deleterious effects associated to infection. In addition, the fungicidal activity of *ex vivo* generated macrophages was dependent on the dose and the timing of the signals that HSPCs receive *in vivo*. Even though, there is no clear relationship between tolerized or trained macrophages and their killing activity.

In conclusion, our data demonstrate that the concept of innate immune memory, already described for monocytes and macrophages, also occurs in HSPCs, and suggest that the tailored manipulation of these responses may serve as an immunotherapeutic approach to boost innate immune responses to infection. Moreover, these memory-like innate immune responses probably occur during infections other than candidiasis, and development of training or tolerance may depend on the severity of the infection and its time-course (microbial burden and associated deleterious effects), as well as on the specific pathogens.

CHAPTER 2

Antifungal responses of HSPCs exposed to PAMPs

1 *In vivo* role of HSPCs against *C. albicans* infection in a model of extended TLR2 agonist treatment

1.1 HSPCs and myeloid cells in the spleen of TLR2 agonist treated mice

Despite the fact that hematopoiesis in adult mice takes place mainly in the BM, it can also occur in the spleen. This extramedullary hematopoiesis happened in the steady state and it is enhanced under stress conditions such as inflammation or infection. As previously described by Herman *et al*. (2016), we observed that the spleens of mice treated with several doses of the TLR2 agonist Pam_3CSK_4 were enlarged and possessed higher numbers of leukocytes in comparison to the spleens of control mice (untreated with Pam_3CSK_4). The percentage and the total number of Lin^- c-Kit^+ cells had also significantly increased in treated mice (**Figure 17**).

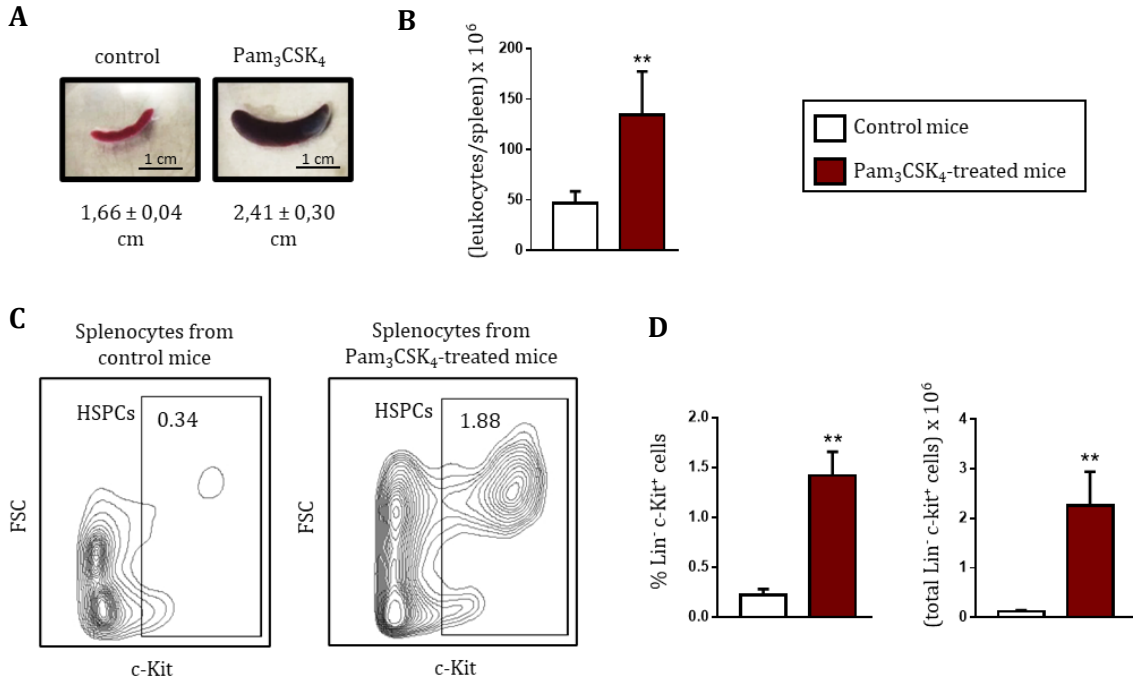


FIGURE 17 | Effect of extended *in vivo* Pam_3CSK_4 treatment on the number of splenocytes and splenic HSPCs. C57BL/6 mice were given 100 μg of Pam_3CSK_4 by intraperitoneal injection at days 0, 3, and 5 (three doses), and 24 h after the final dose, mice were sacrificed to obtain the spleen. **(A)** Appearance and size of spleens from control and treated mice are shown in the image. **(B)** After isolation of splenocytes and red blood cell lysis, cell number was obtained. **(C)** Cells were labeled with an anti-c-Kit antibody and a cocktail of antibodies against "lineage markers" and analyzed by flow cytometry. The lineage negative population was gated, shown in forward scatter (FSC) versus c-Kit^+ contour plots, and subgated as c-Kit^+ cells. The indicated numbers refer to the percentages of total splenocytes. **(D)** Frequency and absolute numbers of Lin^- c-Kit^+ cells in the spleens are indicated. Representative plots and bar graphs of data expressed as means ± SDs from two experiments (2 mice per condition and experiment). ** $P < 0.01$ with respect to cells from untreated mice.

We have previously shown that this Pam_3CSK_4 treatment altered the functional phenotype of BM HSPC-derived macrophages, results that prompted us to evaluate the functional phenotype of spleen HSPC-derived macrophages. Lin^- cells from spleens of Pam_3CSK_4-treated mice were cultured with M-CSF to induce differentiation to macrophages; once differentiated, macrophage cytokine production or fungicidal activity were assessed following the same experimental procedure performed with BM HSPC-derived macrophages (**Figure 18**). BM HSPC-derived macrophages from untreated mice were used as controls, since very few c-Kit^+ cells were found in spleens from control mice. TNF-α production in response to Pam_3CSK_4 was significantly higher in macrophages generated from spleen HSPCs exposed to the TLR2 ligand *in vivo*, compared to control macrophages. However, these trained macrophages were almost unable to kill yeasts *in vitro*.

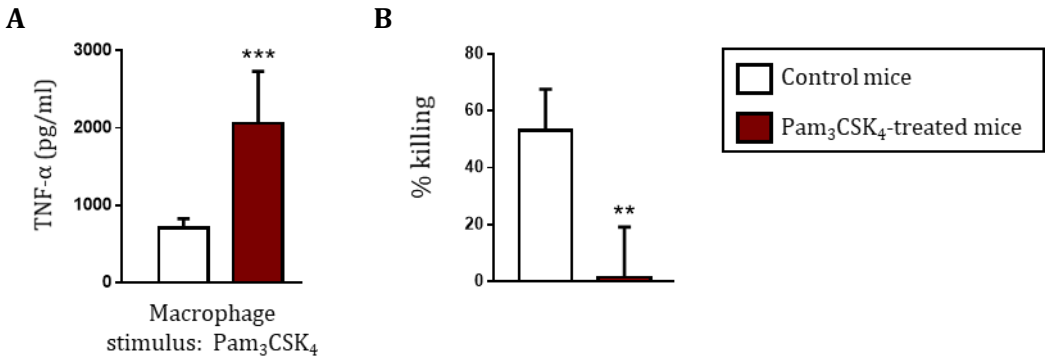


FIGURE 18 | Cytokine production and fungicidal activity of macrophages *ex vivo* derived from spleen HSPCs purified from mice treated with several doses Pam_3CSK_4. C57BL/6 mice were given 100 μg of Pam_3CSK_4 by intraperitoneal injection at days zero, three, and five (three doses), and 24 h after the final dose, mice were sacrificed to obtain the spleen. Lin^- cells were recovered for *ex vivo* differentiation culturing them with 20 ng/ml SCF and 50 ng/ml M-CSF. At day seven, adherent cells were harvested. **(A)** Macrophages were challenged with 100 ng/ml Pam_3CSK_4 for 24 h and TNF-α levels in cell-free culture supernatants were measured by ELISA. **(B)** For fungicidal activity determination, macrophages were challenged with viable PCA2 yeasts at a 1:3 ratio (murine cell:yeast) for 1 h. *C. albicans* cells were also inoculated in culture medium without murine cells (control). After incubation, samples were diluted, plated on Sabouraud dextrose agar and incubated overnight at 37 °C; CFUs were counted and killing percentages were determined as follows: % killing = [1 – (CFUs sample at t = 1 h) / (CFUs control at t = 1 h)] × 100. Triplicate samples were analyzed in each assay. Results are expressed as means ± SD of pooled data from two experiments. ** $P < 0.01$ and *** $P < 0.001$ with respect to macrophages derived from untreated mice.

Direct sensing of TLR2 ligands induce proliferation and myeloid differentiation of HSPCs *in vivo* (Megías *et al*., 2012). Therefore, due to the increase of HSPCs numbers in the spleen of Pam_3CSK_4-treated mice, we examined the number of mature myeloid cells in this organ after treatment. Both the percentage and the total number of $CD11b^+$ cells were significantly increased in Pam_3CSK_4-treated mice compared to control mice, showing an expansion of mature myeloid cells in the spleen. This increase in $CD11b^+$ cells stems from an increase in the absolute number of neutrophils, monocytes, cDCs and macrophages. Among all these cell types, the population that stood out the most was a population of

macrophages ($CD11b^+$, $MHCII^+$, $Ly6C^-$ and $CD11c^-$) that raised from 1.5% in control mice to 13.6% in Pam_3CSK_4-treated mice (**Figure 19**).

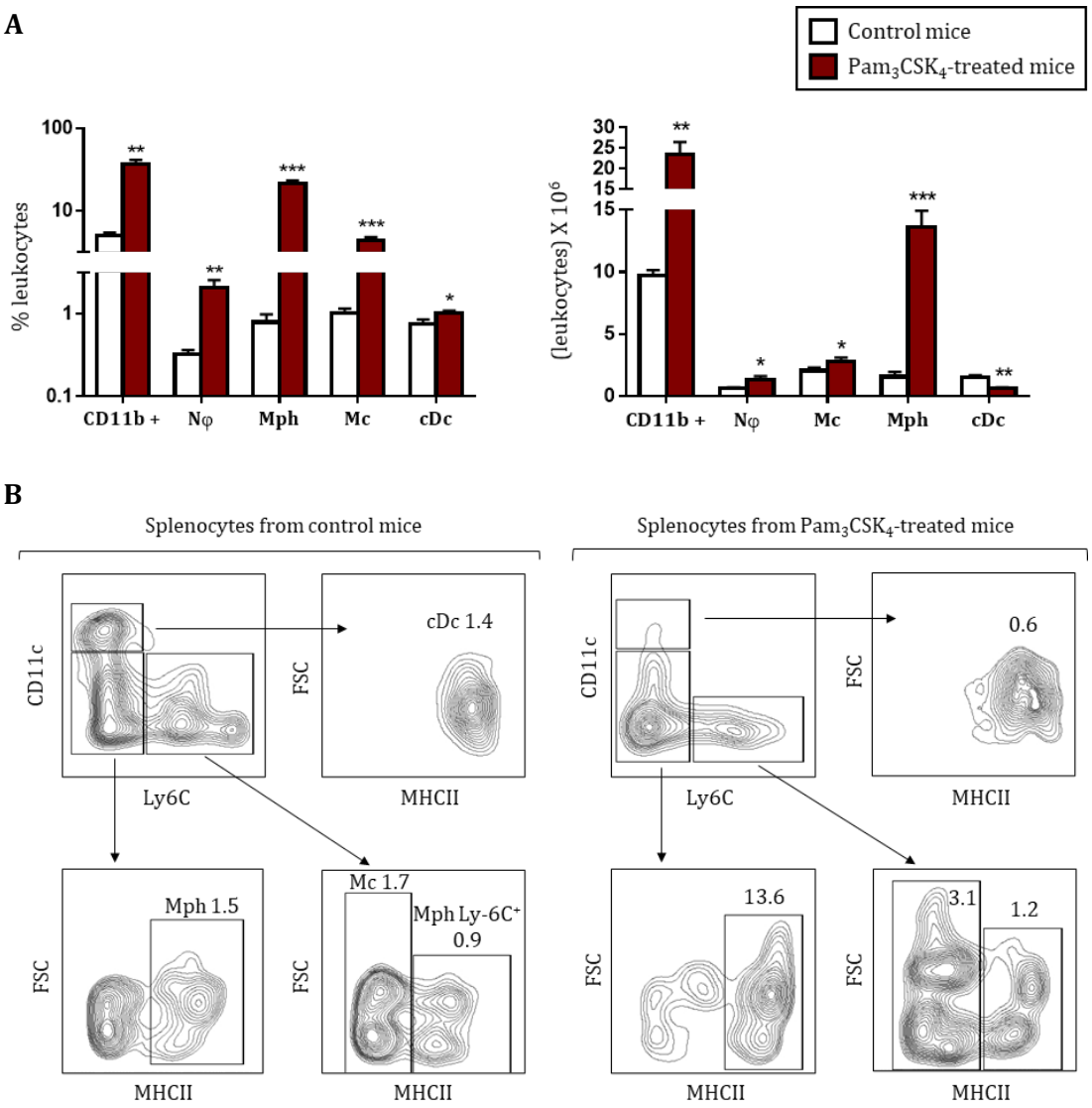


FIGURE 19 | Flow cytometry analysis of myeloid splenic cells from mice following an extended Pam_3CSK_4 treatment. C57BL/6 mice were given 100 µg of Pam_3CSK_4 by intraperitoneal injection at days 0, 3, and 5 (three doses), and 24 h after the final dose, mice were sacrificed to obtain the spleen. After isolation of splenocytes and red blood cell lysis, cell number was obtained. Cells were labeled with anti-CD11b, anti-CD11c, anti-Ly-6G, anti-Ly-6C and anti-MHCII antibodies and analyzed by flow cytometry. **(A)** Frequency and absolute numbers of myeloid cells per spleen were determined by flow cytometry. Results are expressed as means ± SD of pooled data from two experiments (two mice per condition and experiment). **(B)** The gating strategy for mature myeloid cells analysis from $CD11b^+$ $Ly6G^-$ splenic cells is shown using representative contour plots and the indicated numbers refer to the percentages of total splenocytes. Nφ (neutrophils), cDc (classical dendritic cells), Mph (macrophages), Mc (monocytes). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ with respect to control cells (leukocytes from untreated mice).

Next, we analyzed cytokine production and fungicidal activity of total splenocytes from control and Pam_3CSK_4-treated mice (**Figure 20**). *In vitro* production of TNF-α in response to Pam_3CSK_4 or LPS was significantly diminished in total splenocytes from

Pam_3CSK_4-treated mice compared to control mice splenocytes, indicating that a TLR2 agonist treatment induce tolerance in spleen mature myeloid cells. However, splenocytes from Pam_3CSK_4-treated mice showed higher fungicidal activity than splenocytes from control mice. This result correlates with the higher number of mature myeloid cells (mostly phagocytic cells) in the spleen of treated mice but may also indicate a higher fungicidal activity of these cells due to the TLR2 ligand treatment. Therefore, after an extended *in vivo* TLR2 agonist exposure, myeloid cells in the spleen expand, and they are tolerized to pro-inflammatory cytokine production but competent to kill *C. albicans* cells *in vitro*.

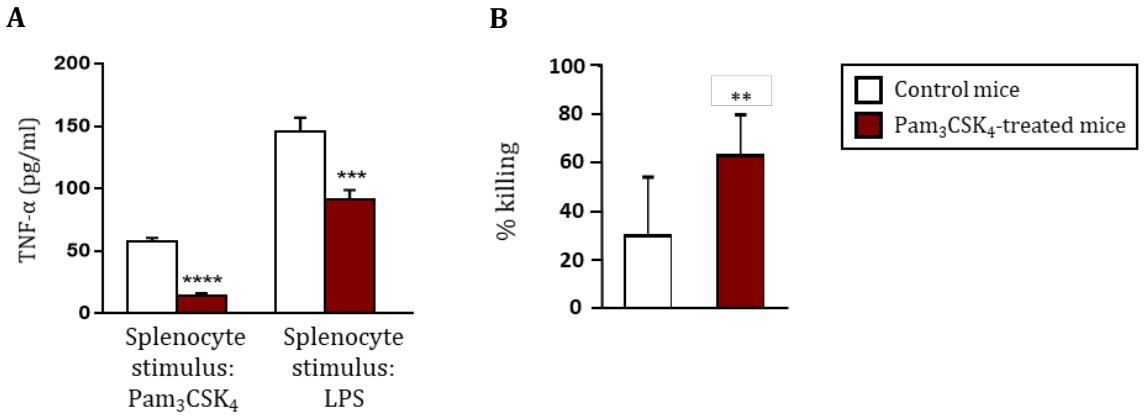


FIGURE 20 | Cytokine production and fungicidal activity of splenocytes from mice treated with several doses of Pam_3CSK_4. C57BL/6 mice were given 100 µg of Pam_3CSK_4 by intraperitoneal injection at days 0, 3, and 5 (three doses), and 24 h after the final dose, mice were sacrificed to obtain the spleen. **(A)** For cytokine assay, splenocytes were challenged with 100 ng/ml Pam_3CSK_4 or 100 ng/ml LPS for 24 h. TNF-α levels in cell-free culture supernatants were measured by ELISA. **(B)** For fungicidal activity determination, splenocytes were challenged with $1 \cdot \times 10^5$ viable PCA2 yeasts for 4 h. *C. albicans* cells were also inoculated in culture medium without murine cells (control). After incubation, samples were diluted, plated on Sabouraud dextrose agar and incubated overnight at 37 °C; CFUs were counted and killing percentages were determined as follows: % killing = [1 – (CFUs sample at t = 4 h) / (CFUs control at t = 4 h)] × 100. Triplicate samples were analyzed in each assay. Results are expressed as means ± SD of pooled data from two experiments. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ with respect to control cells (splenocytes from control mice).

1.2 Influence of the TLR2 agonist treatment on *in vivo* susceptibility of mice to invasive candidiasis

Due to the remarkable effect of an extended TLR2 agonist treatment on the phenotype of macrophages produced *ex vivo*, we next addressed the possibility that this treatment could influence the *in vivo* susceptibility to invasive candidiasis. C57BL/6 mice were given 100 µg of Pam_3CSK_4 by intraperitoneal injection at days 0, 3 and 5, and 24 h after the final dose, mice were infected via intraperitoneal injection (30×10^6 yeasts per mouse) of *C. albicans* yeasts. Four days post-infection, kidney or spleen fungal burden were determined (**Figure 21**). CFUs in both organs significantly decrease in Pam_3CSK_4-treated

mice in comparison to those in control mice. This result clearly indicates that an extended TLR2 agonist treatment protects mice from tissue invasion during systemic *C. albicans* infection.

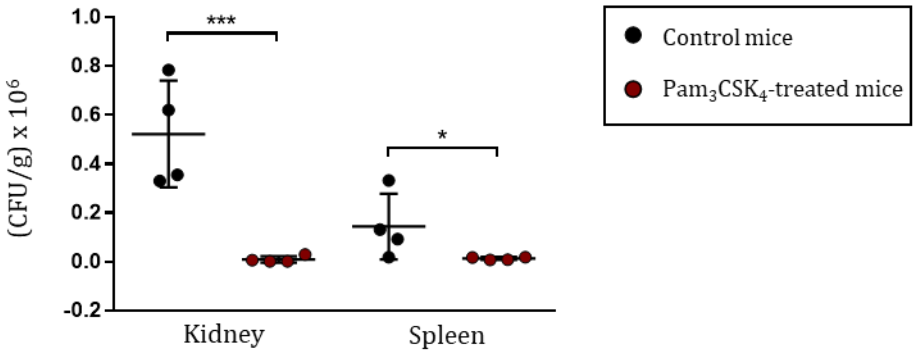


FIGURE 21 | Susceptibility to systemic *C. albicans* infection of mice following an extended Pam_3CSK_4 treatment. C57BL/6 mice were given 100 µg of Pam_3CSK_4 by intraperitoneal injection at days 0, 3, and 5 (three doses), and 24 h after the final dose, mice were injected intraperitoneally with 30 × 10^6 yeasts of *C. albicans* ATCC 26555. Four days post-infection, mice were sacrificed to assess the outgrowth of the yeasts in the kidney and the spleen. The fungal burden is expressed as CFUs per gram of tissue. Results are expressed as means ± SD of pooled data from two experiments (n=2 mice each group per experiment). * $P < 0.05$ and *** $P < 0.001$ with respect to control mice (infected and not treated with Pam_3CSK_4).

1.3 Effect of HSPC immunodepletion on *in vivo* susceptibility of TLR2 agonist treated mice to invasive candidiasis

To determine whether HSPCs are required for TLR2 agonist mediated protection against tissue invasion, we immunodepleted c-Kit^+ progenitors in Pam_3CSK_4-treated mice before *C. albicans* infection. For this purpose, we used the monoclonal antibody ACK2, as it has been previously reported that the complete inhibition of c-Kit signaling by *in vivo* ACK2 administration leads to a rapid but transient depletion of BM HSCs (95% depletion at 48 h post-injection, with a maximum of depletion at day 9, and a recovery to normal levels at day 23) (Czechowicz *et al.*, 2007). Considering these data, in our model of Pam_3CSK_4 extended treatment, mice were given 500 µg of ACK2 or its isotype control, by intraperitoneal injection, at day 4 (48 h before infection). As shown in **Figure 22A**, the injection of ACK2 at day 4 resulted in a significant reduction of Lin^- c-Kit^+ progenitors at day 6, both in BM (roughly 56% reduction) and in spleen (roughly 75% reduction), as compared to isotype control injected animals. Despite of HSPC depletion, similar number of mature myeloid cells were found in the spleen of immunodepleted mice compared to isotype control injected mice.

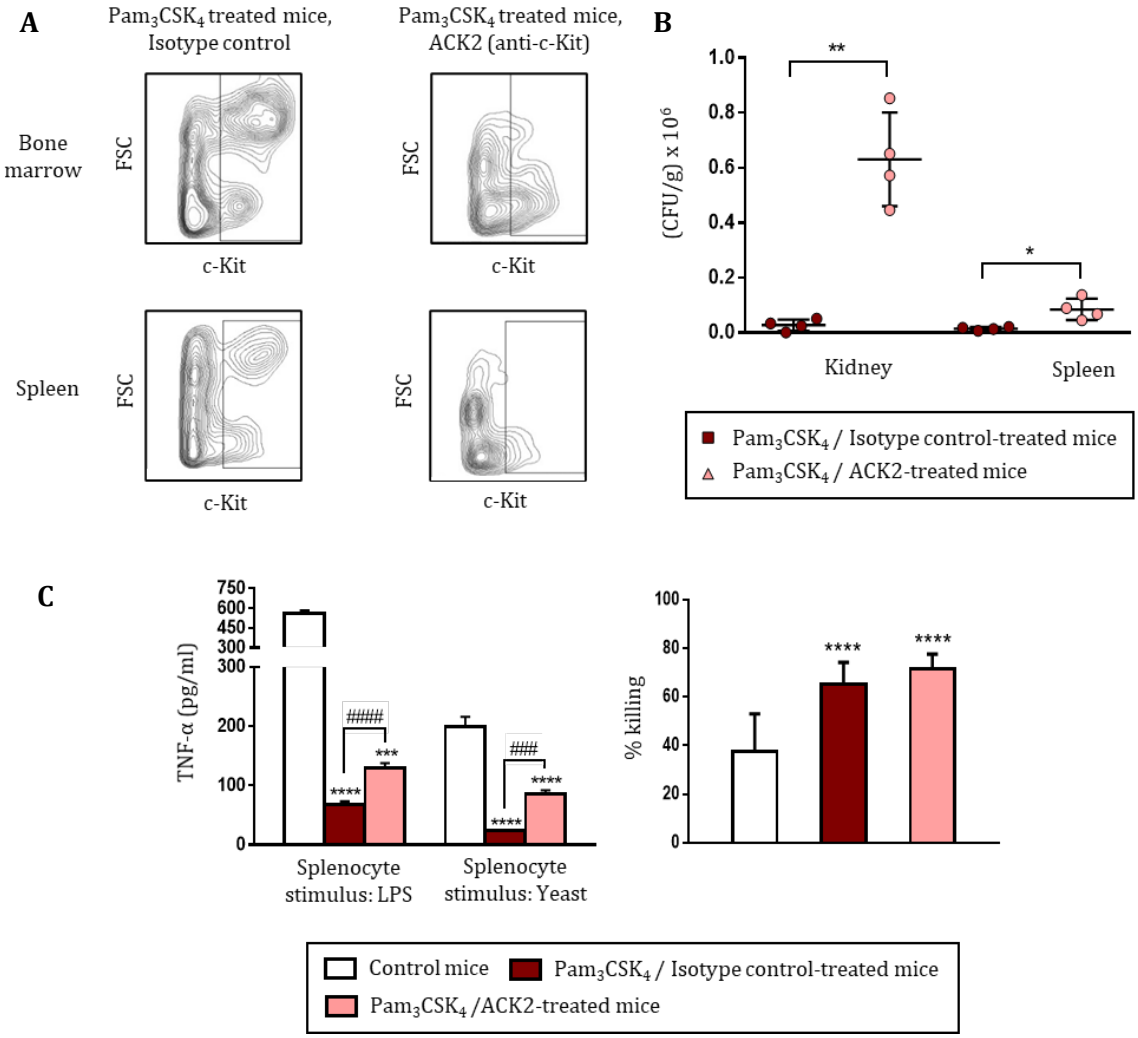


FIGURE 22 | Immunodepletion of c-Kit⁺ progenitors in Pam3CSK4-treated mice: susceptibility to candidiasis and effect on cytokine production by splenocytes. C57BL/6 mice were given 100 μg of Pam3CSK4 by intraperitoneal injection at days 0, 3, and 5 (three doses), and 500 μg of the anti-c-Kit antibody ACK2 or isotype control at day 4. One day (24 h) after the final dose of Pam3CSK4, mice were injected intraperitoneally with 30 × 10^6 yeasts of *C. albicans* ATCC 26555 or were sacrificed to BM or spleen isolation. Four days post-infection, mice were sacrificed to assess the outgrowth of the yeasts in kidney and spleen. **(A)** Flow cytometry analysis of Lin^- c-Kit^+ cells in BM and spleen. **(B)** Fungal burden in kidneys and spleens are expressed as CFUs per gram of tissue. Results are expressed as means ± SD of pooled data from two experiments (n=2 mice each group per experiment). * P < 0.05 and ** P < 0.01 with respect to control mice (treated with Pam3CSK4 and isotype control). **(C)** TNF-α production and fungicidal activity of splenocytes. For cytokine assays, splenocytes were challenged with 100 ng/ml LPS or inactivated yeasts and cell-free culture supernatants were measured by ELISA. For fungicidal activity determination, splenocytes were challenged with 100,000 viable PCA2 yeasts and incubated for 4 h. After incubation, samples were diluted, plated on Sabouraud dextrose agar and incubated overnight at 37 °C; CFUs were counted and killing percentages were determined as follows: % killing = [1 – (CFUs sample at t = 4 h) / (CFUs control at t = 4 h)] × 100. Triplicate samples were analyzed in each assay. Results are expressed as means ± SD of pooled data from two experiments. *** *P* < 0.001 and **** *P* < 0.0001 with respect to control cells (splenocytes from control mice), and ### *P*< 0.001 and with #### *P*< 0.0001 respect to cytokine production by splenocytes from mice treated with Pam3CSK4 and isotype control.

We then assessed kidney or spleen fungal burden in Pam_3CSK_4/ACK2 or Pam_3CSK_4/isotype control-treated mice at day four post *C. albicans* infection (**Figure 22B**).

ACK2-treated mice showed a marked increase in CFUs in both organs, as compared to isotype control injected mice, clearly demonstrating that depletion of c-Kit$^+$ progenitors in Pam_3CSK_4-treated mice abrogates protection against tissue invasion during invasive candidiasis. Therefore, we conclude that Pam_3CSK_4-induced protection against candidiasis is, at least, partially mediated by the expansion of hematopoietic progenitors. *In vitro* cytokine production and fungicidal activity of total splenocytes from control, Pam_3CSK_4/ACK2 or Pam_3CSK_4/isotype control-treated mice were also analyzed (**Figure 22C**). As expected, mature myeloid cells in the spleen of Pam_3CSK_4/isotype control-treated mice were tolerized, as TNF-α production in response to LPS or inactivated yeasts was significantly diminished in total splenocytes in comparison to splenocytes from untreated mice. Interestingly, splenocytes from ACK2-treated animals produced significantly higher amounts of TNF-α than splenocytes from isotype control-treated mice, although still lower levels than splenocytes from untreated mice. The increased in fungicidal activity induced by the Pam_3CSK_4 treatment was similar in ACK2-treated mice and isotype control treated mice. In conclusion, depletion of c-Kit$^+$ progenitors in Pam_3CSK_4-treated mice increases cytokine production by splenocytes without modifying their potentiated fungicidal activity.

2 Effector mechanisms of HSPCs in response to PAMPs

2.1 *In vitro* soluble factors production: HSPC secretomes

Our findings indicate that HSPCs sense Pam_3CSK_4 and *C. albicans* yeasts *in vivo*, and subsequently may contribute to protect the host against candidiasis. These results prompted us to investigate potential mechanisms through which HSPCs could protect mice against infection. Since it has been described that HSPCs produce cytokines in response to LPS and Pam_3CSK_4 (Zhao *et al.*, 2014), we profiled the pro-inflammatory molecules secreted by HSPCs in response to Pam_3CSK_4 and *C. albicans* yeasts. Lin$^-$ cells were cultured in a serum-free medium, in the absence or presence of Pam_3CSK_4 or inactivated yeasts, and 3 days later culture supernatants (secretomes) were collected and analyzed (**Figure 23A**). TNF-α and IL-6 levels were measured in secretomes using quantitative ELISAs (**Figure 23B**). Cytokine levels in control secretomes, that is secretomes produced by HSPCs in the absence of PAMPs, were undetectable. Consistent with the previous report by Zhao *et al.* (2014), HSPCs produced IL-6 in response to Pam_3CSK_4. On the other hand, in response to

inactivated *C. albicans* yeasts, HSPCs produced high amounts of TNF-α. Next, 40 cytokines were determined in secretomes using a mouse cytokine detection array. Relative intensity values from stimulated HSPC secretomes in comparison to non-stimulated HSPC secretomes were analyzed and only those cytokines whose values were significantly different among secretomes are depicted in **Figure 23C**. We found that HSPCs produced higher levels of CCL2, CCL3, and CCL9 in response to Pam_3CSK_4 than unstimulated HSPCs, while only CCL3 and CCL9 were higher in the yeast secretome. Taken together, these data demonstrate that HSPCs are capable of producing pro-inflammatory cytokines and chemokines in response to different PRR ligands.

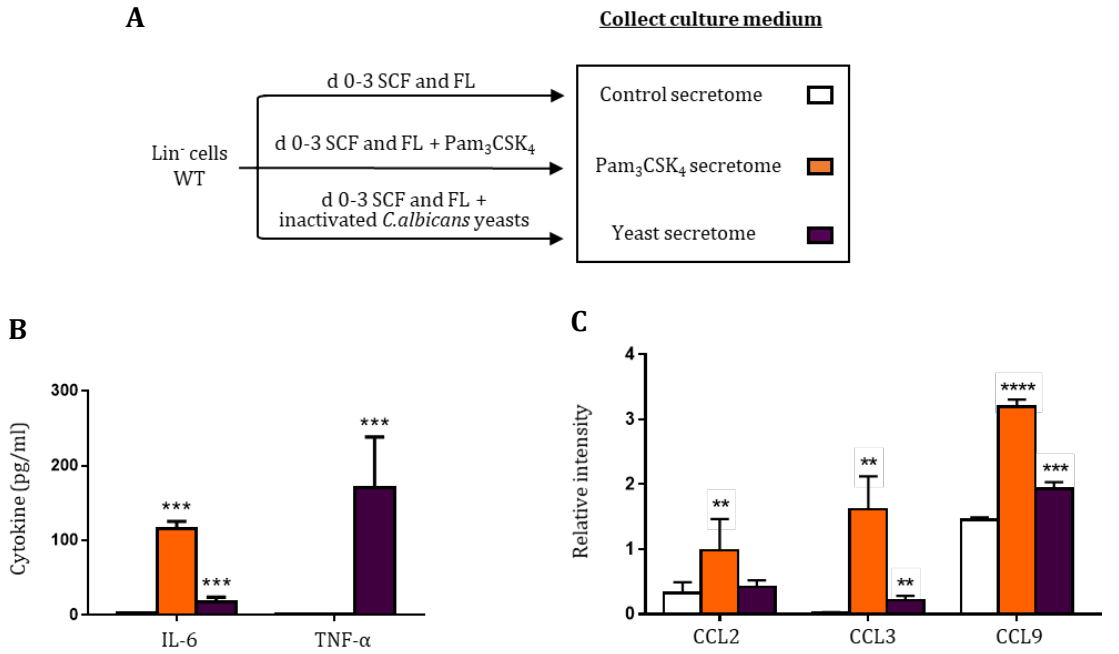


FIGURE 23 | Cytokine production by HSPCs in response to Pam_3CSK_4 and *C. albicans* yeasts. (A) Schematic protocol to obtain secretomes. Purified Lin^- cells from BM of C57BL/6 mice were cultured in a serum-free medium containing 100 ng/ml Fms-like tyrosine kinase 3 ligand (FL) and 20 ng/ml SCF, in the presence or absence of 1 μg/ml Pam_3CSK_4 or inactivated *C. albicans* yeasts for 3 days. Then, secretomes (conditioned media) were collected. **(B)** TNF-α and IL-6 levels in secretomes were measured by ELISA. **(C)** CCL2, CCL3, and CCL9 levels were determined using a mouse cytokine array. Data are expressed as means ± SD of pooled data from two experiments. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$with respect to control secretomes.

2.2 Influence of HSPC secretome on *in vitro* differentiation of untreated HSPCs and cytokine production by resident macrophages

The above data demonstrate that HSPCs produce several pro-inflammatory cytokines and chemokines in response to a TLR2 agonist and *C. albicans* yeasts. Hence, we wondered whether these secretomes may play some role in regulating the function of surrounding cells, such as HSPCs or resident macrophages. To study the effects of secretomes from Pam_3CSK_4-stimulated HSPCs, we used cells from TLR2 $^{-/-}$ mice to avoid stimulation by soluble Pam_3CSK_4 present in secretomes We examined the effect of secretomes on HSPC differentiation (**Figure 24A**).

The effect of secretomes on HSPC differentiation was initially examined. Lin^- cells were cultured in this "conditioned media" for 3 days, and expression of CD11b (as a marker of myeloid differentiation) and c-Kit (as a marker of progenitor cell) was analyzed by flow cytometry. Lin^- cells were also cultured with LPS or inactivated *C. albicans* yeasts, as positive controls of differentiation (**Figure 24B**). Results showed that Lin^- cell differentiation pattern in the presence of control secretomes was similar to that in Lin^- cells cultured in fresh medium. However, the presence of Pam_3CSK_4 secretomes or yeast secretomes increased the percentage of $CD11b^+$ cells and accordingly, decreased the percentage of c-Kit^+ cells (roughly 70 % higher and 30 % less in both conditions than in control secretomes, respectively). Therefore, HSPC secretomes in response to *C. albicans* and Pam_3CSK_4 induce myeloid differentiation of HSPCs.

Finally, we studied whether secretomes had an impact on cytokine production by tissue resident macrophages (**Figures 24C**). Resident peritoneal macrophages from TLR2$^{-/-}$ mice or C57BL/6 mice were used to test Pam_3CSK_4 secretomes or yeast secretomes, respectively. TNF-α production in response to LPS was significantly decreased in the presence of Pam_3CSK_4 secretomes in comparison to control secretomes, whereas yeast secretome did not alter cytokine levels produced by macrophages in response to Pam_3CSK_4 or LPS.

Taken together, these results demonstrate that detection of PAMPs by HSPCs defines the secretome they produce, and therefore its impact on HSPC differentiation and macrophage function. Thus, these observations support the idea that direct pathogen sensing by HSPCs plays an active role in regulating the immune responses against infection.

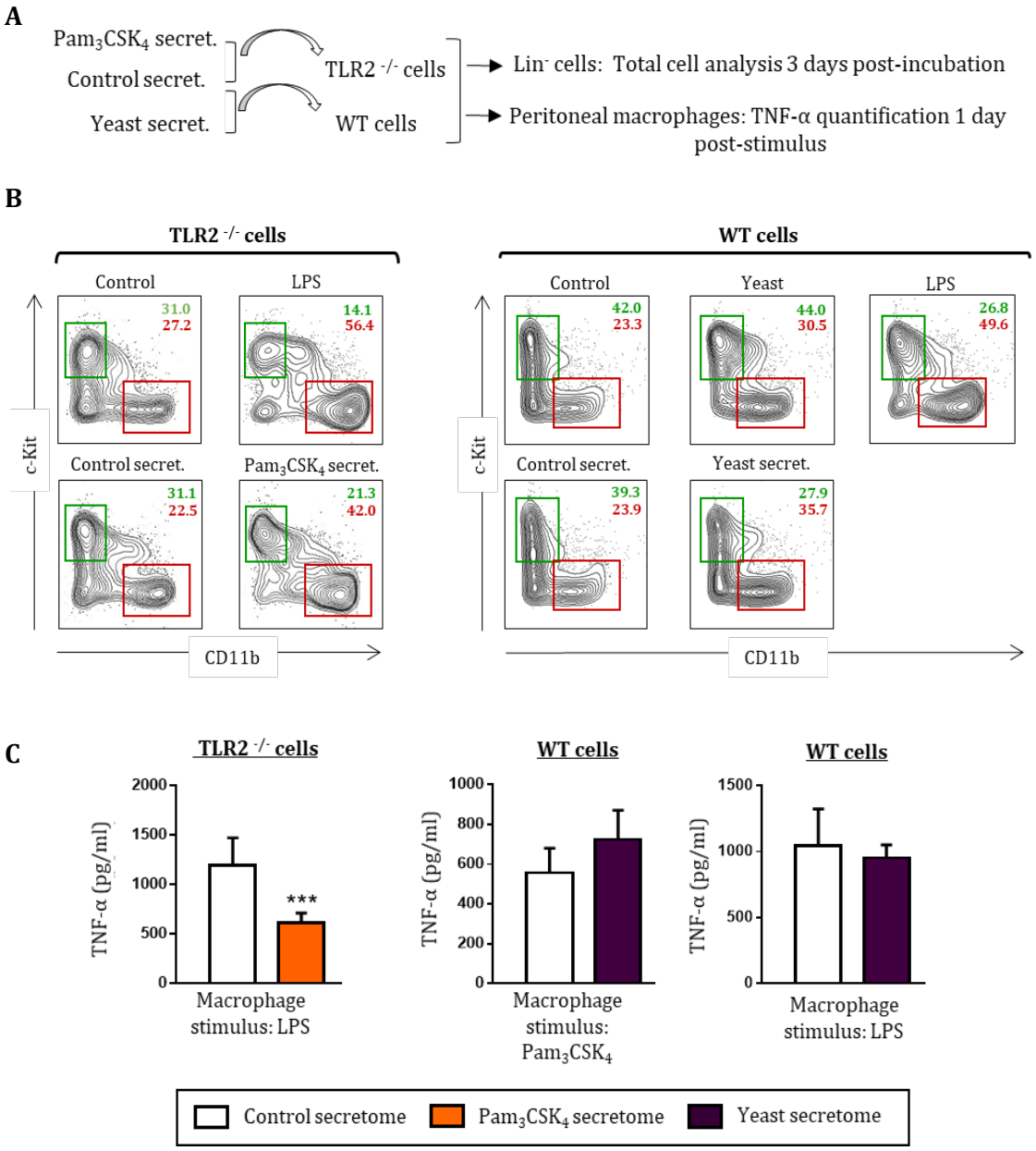


FIGURE 24 | HSPC secretomes in response to Pam_3CSK_4 or *C. albicans* induce myeloid differentiation and modulate cytokine production by peritoneal macrophages. (A) Schematic protocol to study the effect of secretomes (obtained as indicated in Figure 23A) on HSPC differentiation and TNF-α production by peritoneal macrophages. HSPCs or peritoneal macrophages from $TLR2^{-/-}$ mice were used to study the effect of the secretomes from HSPCs stimulated with Pam_3CSK_4. **(B)** Purified Lin^- cells from BM of C57BL/6 mice were cultured for 3 days, labeled with antibodies, and analyzed by flow cytometry. Cells were gated as c-Kit or CD11b positive cells. The indicated percentages refer to total analyzed cells. Results shown are from one representative of two independent experiments. **(C)** Resident peritoneal macrophages were challenged with 100 ng/ml Pam_3CSK_4 or 100 ng/ml LPS for 24 h. TNF-α levels in cell-free culture supernatants were measured by ELISA. Triplicate samples were analyzed in each assay. Results are expressed as means ± SD of pooled data from two experiments. ***$P < 0.001$ with respect to cytokine production by peritoneal macrophages in the presence of control secretome. Secret.: secretome.

3 Discussion

Accumulated evidence has emerged supporting a role for HSPCs in fighting against infection, although mechanisms governing the response of HSPCs to infection are poorly understood (Boettcher and Manz, 2017; Chavakis *et al*., 2019; Yáñez *et al.*, 2013a). Various mechanisms had been involved, including cytokine signaling, egressing to peripheral tissues, and PAMP's sensing directly by HSPCs (Kobayashi *et al.*, 2016). As Hernan *et al*. (2016) described, an extended exposure to the TLR2 agonist Pam_3CSK_4 leads to an expansion of spleen HSPCs, a cell-non-autonomous effect that is in part mediated by hematopoietic and non-hematopoietic cell production of G-CSF and TNF-α. However, even when Pam_3CSK_4-treated mice were injected with the specific neutralizing antibodies against G-CSF and TNF-α, they still exhibited higher amounts of HSPCs than non-treated mice. This residual HSPCs expansion may be due to cell-autonomous TLR2 signaling in HSCs and GMPs. In addition, HSCs in the spleen were significantly less quiescent upon Pam_3CSK_4 treatment, suggesting that both local proliferation and migration may underlie the increase in splenic HSPCs after an *in vivo* exposure to a TLR2 agonist (Hernan *et al.*, 2016).

Using the extended TLR2 agonist treatment model described by Hernan *et al.* (2016), we show that splenic HSPCs, similarly to BM HSPCs, generate *in vitro* macrophages with a higher ability to produce pro-inflammatory cytokines. Even though, it is difficult to predict the influence of extended Pam_3CSK_4 treatment on susceptibility to infection. We hypothesized that the substantial accumulation of HSPCs and mature myeloid cells in spleen could protect mice against candidiasis, and that HSPCs may contribute to this protection, probably by providing an additional site for the generation of effector cells during infection. Results clearly indicate that an extended TLR2 agonist treatment strongly protects mice from tissue invasion during systemic *C. albicans* infection, as the fungal burden in the kidney and in the spleen of treated mice was drastically reduced. This *in vivo* TLR2 agonist exposure induces an expansion of myeloid cells in the spleen, being macrophages the most amplified population; thus, it would be possible to suggest that myeloid cells could be responsible for protection. However, immunodepletion of c-Kit^+ progenitors in Pam_3CSK_4-treated mice abrogates protection against tissue invasion during candidiasis, despite the similar amount of spleen myeloid cells than that in isotype control injected mice, at the moment of infection. In conclusion, the protective effect against candidiasis in TLR2 agonist-treated mice is at least partially dependent on HSPCs. In accordance with these observations, recently *in vivo* studies have demonstrated that BGC vaccination or pretreatment with LPS reprograms

HSPCs to have protective capacities against secondary infections, effect in part mediated by an enhanced myeloid cell production (Kaufmann *et al.*, 2018; de Laval *et al.*, 2020).

These results are in line with Granick *et al.* (2013), which reported that proliferation of HSPCs in skin wounds in response to *S. aureus* is TLR2-mediated and contributes significantly to the production of neutrophils and resolution of local infection, supporting a role for TLR2 signaling in the regulation of extramedullary hematopoiesis. Other authors have also described that systemic infection of mice with *E. coli* induces a dramatic mobilization of functional HSPCs to spleen, and that mobilized HSPCs give rise to neutrophils and monocytes (Burberry *et al.*, 2014). Interestingly, mobilized HSPCs contribute to limiting secondary infection, proposing that HSPC mobilization itself constitute a host mechanism against infection. These experiments were performed transplanting total splenocytes but not purified HSPCs from infected mice to recipients that were then challenged with *E. coli*. As they demonstrated that G-CSF was essential for HSPC accumulation in spleen, mice injected with a specific neutralizing antibody against G-CSF were used to obtain "control" splenocytes. Similarly, mice treated with a cyclic bacteria-derived DNA that is recognized by the receptor STING also exhibit a HSPC expansion in the spleen (Kobayashi *et al.*, 2015).

However, the possibility that protection against candidiasis in our model of Pam_3CSK_4-treated mice may be also mediated by mature myeloid cells cannot be completely excluded. Supporting this possibility, it has been shown that tolerance induced by one dose of a TLR2 ligand protects mice against acute polymicrobial peritonitis or coinfection by *S. aureus* and *S. typhimurium*, mainly by promoting an increase in circulating granulocytes and a recruitment of those cells into the peritoneal cavity (Feterowski *et al*., 2005; Wang *et al.*, 2002). Moreover, either circulating neutrophils or peritoneal macrophages exhibited and enhanced recognition, ingestion, and intracellular killing of *S. aureus* and *S. typhimurium* in TLR2 agonist-tolerized mice (Wang *et al.*, 2002).

TLR2 and Dectin-1 are two of the most relevant PRRs involved in *C. albicans* detection (Miramón *et al.*, 2013; Netea *et al*., 2008; Poulain and Jouault, 2004). As we have seen, an extended treatment with the TLR2 agonist Pam_3CSK_4 leads to an expansion of spleen HSPCs and protects mice against candidiasis, effect that is abrogated by immunodepletion of HSPCs, suggesting their protective role against infection in this model. Early studies also demonstrated that mice are less susceptible to systemic *C. albicans* infection following exposure to the Dectin-1 ligand β-glucan, process that it is mainly mediated by myeloid cells (Bistoni *et al.*, 1986). This innate memory phenomena, referred as trained immunity, has been described to involve long-term effects that would persists for months or even one year

(Kleinnijenhuis *et al*., 2014; Kleinnijenhuis et *al.*, 2012). Regarding the long-lasting effects of trained immunity, Mitroulis *et al*. (2018) have recently described a modulation of myeloid progenitors in the BM of β-glucan treated mice (Mitroulis *et al.*, 2018). However, early studies showed that β-glucans exhibit potent stimulating effects on hematopoiesis by enhancing the production of hematopoietic factors and inducing a mobilization of HSPCs from the BM into the blood (Hofer and Pospisili, 2011). As it has been described that during systemic fungal infections β-glucans are released into peripheral blood (Obayashi *et al.*, 1995), HSPC mobilization induced by β-glucans may act also in the context of infection. It will be interesting to see whether extramedullary hematopoiesis, as in extended TLR2 treatment, has a role in *C. albicans*- or β-glucan-induced trained immunity.

Even though the HSPC effector mechanisms to protect against candidiasis in Pam_3CSK_4-treated mice are not well defined yet, our data point toward the involvement of diverse mechanisms. In agreement with Zhao *et al.* (2014), we confirm here that HSPCs can respond directly to TLR ligands by producing cytokines to coordinate immune responses. Moreover, we show for the first time that HSPCs also produce the CCL2, CCL3 and CCL9 chemokines in response to different PRRs ligands. Chemokines involved in the early recruitment of innate immune cells to the sites of infection are crucial for local control of fungal infections (Pappas *et al.*, 2018). Consequently, CCL2, CCL3, and CCL9 produced by HSPCs may activate and attract neutrophils, monocytes and DCs, therefore contributing to protection against infection. It has been described that HSPCs, mainly the myeloid committed progenitors CMPs and GMPs, express CCR2 that can guide these progenitors to sites of inflammation (Si *et al.*, 2010); therefore, it could be speculated that HSPCs may induce their own recruitment. *In vitro* TLR ligation on common dendritic progenitors has also been reported to modulate their chemokine receptor expression, and consequently favors their migration from bloodstream to inflammatory/infection sites (Schmid *et al.*, 2011). In line with these results, Massberg *et al*. (2007) demonstrated that TLR stimulation blocks HSPC egress from inflamed tissues, which may favor extramedullary hematopoiesis (Massberg *et al.*, 2007).

Besides chemokine production, we show that secretomes produced by HSPCs in response to the TLR2 ligand Pam_3CSK_4 and *C. albicans* yeasts induce HSPCs myeloid differentiation. Zhao *et al.* (2014) described that among the cytokines produced by HSPCs in response to LPS and Pam_3CSK_4, IL-6 is a particularly important regulator of myeloid differentiation. Our results suggest that other molecules produced by HSPCs in response to *C. albicans* may induce myeloid differentiation, as IL-6 was barely found in the yeast secretome. Secretomes acting in a paracrine or autocrine manner on HSPCs may mediate a

rapid myeloid cell recovery during candidiasis. Finally, in addition to acting on HSPCs, secretomes may also modulate the function of other surrounding cells, such as macrophages. In this context, we found that the Pam_3CSK_4 secretome reduces TNF-α production by peritoneal macrophages. In agreement with this *in vitro* effect, the production of cytokines by splenocytes from Pam_3CSK_4/ACK2-treated mice (c-Kit^+ depleted mice) is increased as compared to Pam_3CSK_4/isotype control-treated mice. Therefore, the Pam_3CSK_4-mobilized HSPCs may contribute to protection against infection, not only by myeloid cell replenishment but also by secreting molecules that recruit and activate leukocytes, and modulate the phenotype of mature myeloid cells. Future studies will be required to identify the molecules and the mechanisms responsible for the different secretome functions.

In conclusion, our data show that extended systemic exposure to a TLR2 agonist leads to the expansion of splenic HSPCs, effect that is at least partially responsible for protection against tissue invasion during systemic *C. albicans* infection. HSPCs produce cytokines and chemokines in response to a TLR2 agonist or *C. albicans* yeasts, and these secreted molecules induce myeloid differentiation of HSPCs and modulate cytokine production by peritoneal macrophages. These results support the hypothesis that HSPCs can sense pathogens during infection and contribute to protect the host by several mechanisms. A better understanding of the signals that influence HSPCs during infection may lead to new therapeutic strategies for anti-infection intervention.

CHAPTER 3

Phenotype of APCs derived from HSPCs exposed to TLR2 or Dectin-1 agonists and its impact on their modulation of $CD4^+$ T cell responses

1 Surface molecule expression and cytokine production by APCs derived from HSPCs exposed to TLR2 or Dectin-1 agonists

1.1 Surface molecule expression: MHCII, CD40, CD80, CD86

To investigate the functional consequences of HSPC exposure to a TLR2 or Dectin-1 agonist on the APCs they produce, we designed the following experimental approach. Briefly, Lin^- cells were purified from mouse BM and cultured with GM-CSF to induce differentiation to APCs. Lin^- cells were treated with Pam_3CSK_4 or depleted zymosan or untreated (none) for the first 24 h of culture (day 0), and then washed thoroughly to remove any remaining microbial components prior to continued culture with GM-CSF to derive APCs (**Figure 25A**). We verified that β-glucan particles were mostly absent in the cultures at day 3 by using fluorescently labeled zymosan (see Materials and Methods, section 6). On day 6, adherent cells were recovered from the cultures, counted, plated overnight and stimulated (day 7) with Pam_3CSK_4, depleted zymosan, or unstimulated. We then assessed their expression of surface molecules involved in antigen presentation and T cell activation.

Expression of MHCII (required for signal 1) and costimulatory molecules (CD40, CD80 and CD86; signal 2) on $CD11b^+$ $CD11c^+$ cells was assessed by flow cytometry. In all conditions, $CD11b^+$ $CD11c^+$ cells represented about 80 % of total adherent cells analyzed (**Figure 25B**). As expected, day 7 stimulation of APCs with Pam_3CSK_4 or depleted zymosan upregulated the expression of most of these surface molecules in comparison to unstimulated APCs (**Figure 25C**). Therefore, $CD11b^+$ $CD11c^+$ cells highly upregulated MHCII and CD80 following day 7 stimulation with both PRRs, whereas Pam_3CSK_4 stimulation slightly induced CD86 and CD40 expression and depleted zymosan stimulation highly induced CD86 but not CD40 expression (**Figure 25D**). Interestingly, day 0 treatment of HSPCs with Pam_3CSK_4 increased the expression of MHCII, CD80 and CD86 on day 7-stimulated APCs, while depleted zymosan treatment of HSPCs on day 0 induced more subtle changes in day 7-stimulated APCs (**Figure 25C** and **25D**).

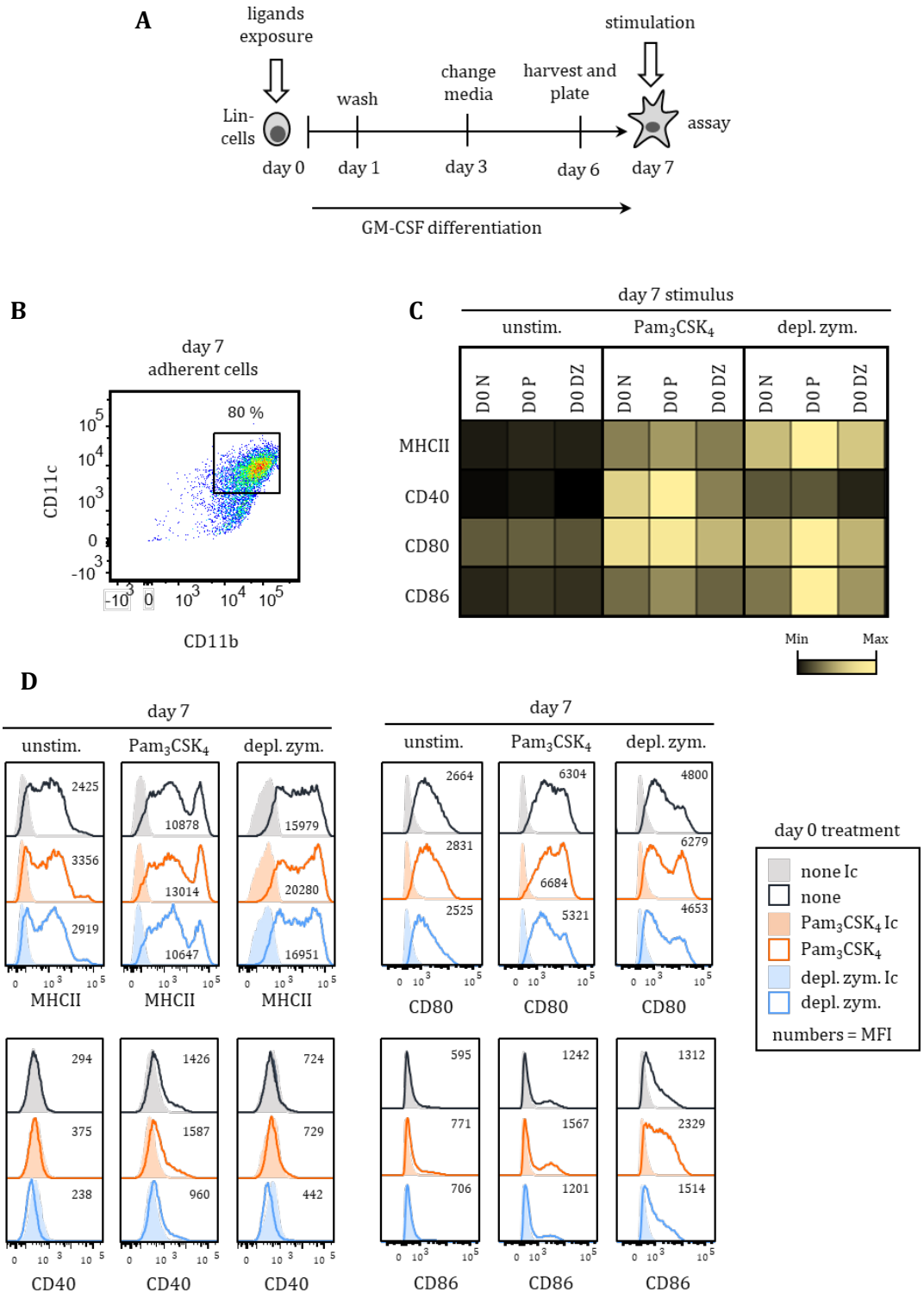


FIGURE 25 | Flow cytometry analysis of APCs generated from HSPCs transiently stimulated with Pam_3CSK_4 or depleted zymosan. (A) Schematic protocol. Purified Lin^- cells from BM of C57BL/6 mice were treated with 100 ng/ml Pam_3CSK_4, 10 µg/ml depleted zymosan, or nothing (none) during the first 24 h of culture, washed thoroughly to remove any remaining stimuli and then continued in culture with GM-CSF for a further six days to derive APCs. At day six, adherent cells were recovered from the cultures, counted and plated at equal numbers for 24 h, and then stimulated with Pam_3CSK_4, depleted zymosan or nothing (unstimulated) for 24 h to assess the expression of MHCII and costimulatory molecules (CD40, CD80 and CD86) on $CD11b^+$ $CD11c^+$ APCs by flow cytometry. **(B)** CD11b and CD11c expression by the adherent cells recovered from the cultures at day seven. **(C)** Colormap based on min-max values per row using the MFI values obtained with the specific antibodies and the isotype controls. (DO, day 0; N, none; P, Pam_3CSK_4; DZ, depleted zymosan). **(D)** Histograms and MFIs corresponded to data shown in colormap. Results shown are from one experiment that is representative of three-five independent experiments.

1.2 Cytokine production: TNF-α, IL-6, IL-12 p40, IL-2

Next, production of pro-inflammatory cytokines (signal 3) was assessed in the culture supernatants of day 7 APCs (**Figure 26**). Day 0 treatment of HSPCs with Pam_3CSK_4 or depleted zymosan increased the production of TNF-α, IL-6 and IL-12 p40 by day 7-stimulated APCs, with the exception of Pam_3CSK_4-stimulated APCs derived from day 0 Pam_3CSK_4-exposed HSPCs, which produced significantly less IL-6. As previously described, IL-2 was only secreted in response to depleted zymosan (Hassanzadeh-Kiabi *et al.*, 2017), and surprisingly only the treatment of HSPCs with Pam_3CSK_4 boosted its production. Overall, these data illustrate that HSPC treatment with Pam_3CSK_4 or depleted zymosan modifies the T cell-activating signals one, two and three of the APCs derived from them.

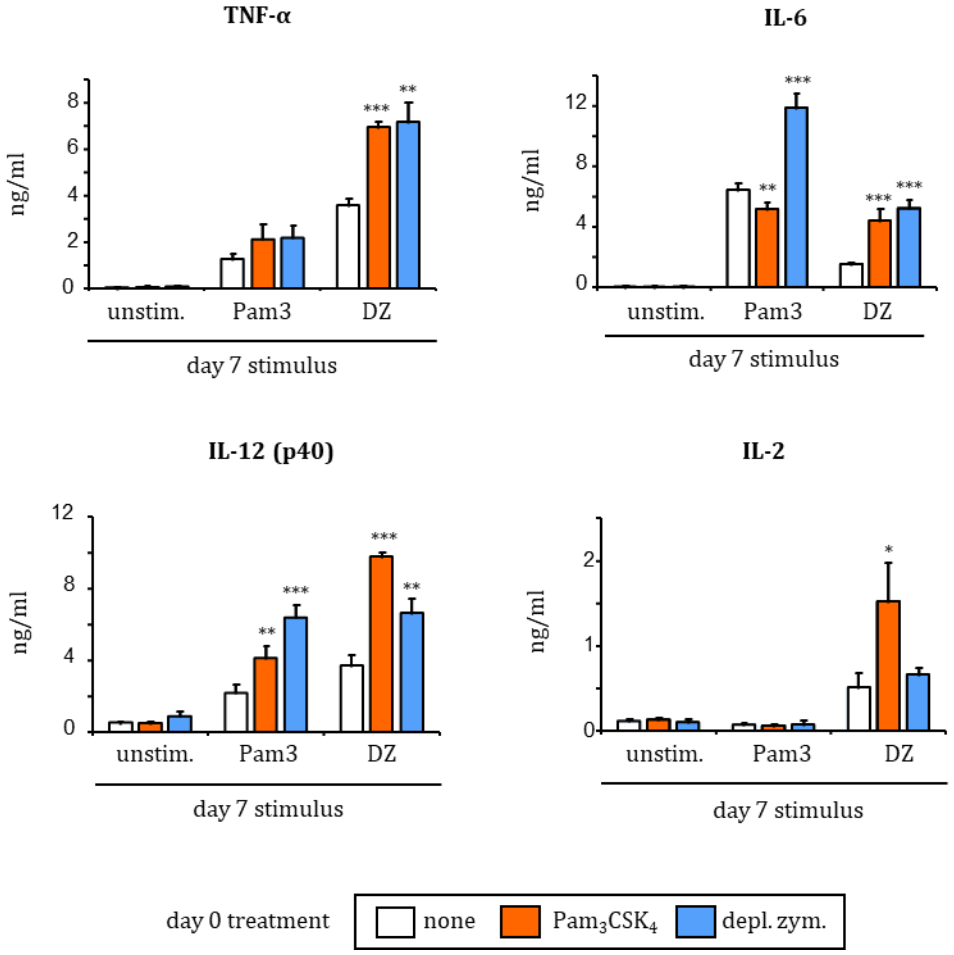


FIGURE 26 | Cytokine production of APCs generated from HSPCs transiently stimulated with Pam_3CSK_4 or depleted zymosan. APCs derived from HSPCs under the same conditions as in Figure 25A were plated at equal numbers for 24 h, and then stimulated with Pam_3CSK_4, depleted zymosan or nothing (unstimulated) for 24 h to assess cytokine production (TNF-α, IL-6, IL-12 p40 and IL-2) in the supernatants by ELISA. Data represent means ± SD of triplicate cultures, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with respect to day 0 none. Results shown are from one experiment that is representative of three independent experiments.

2 OVA-specific CD4⁺ T cell responses induced by APCs derived from HSPCs exposed to PAMPs

2.1 CD4⁺ T cell proliferation

We next examined whether the changes observed in the APC phenotype could impact the proliferation and activation of CD4⁺ T cells. To address this, day 7 APCs were loaded with an OVA peptide and cocultured with CFSE-labeled OVA-specific CD4⁺ T cells isolated from OT-II transgenic mice (**Figure 27**).

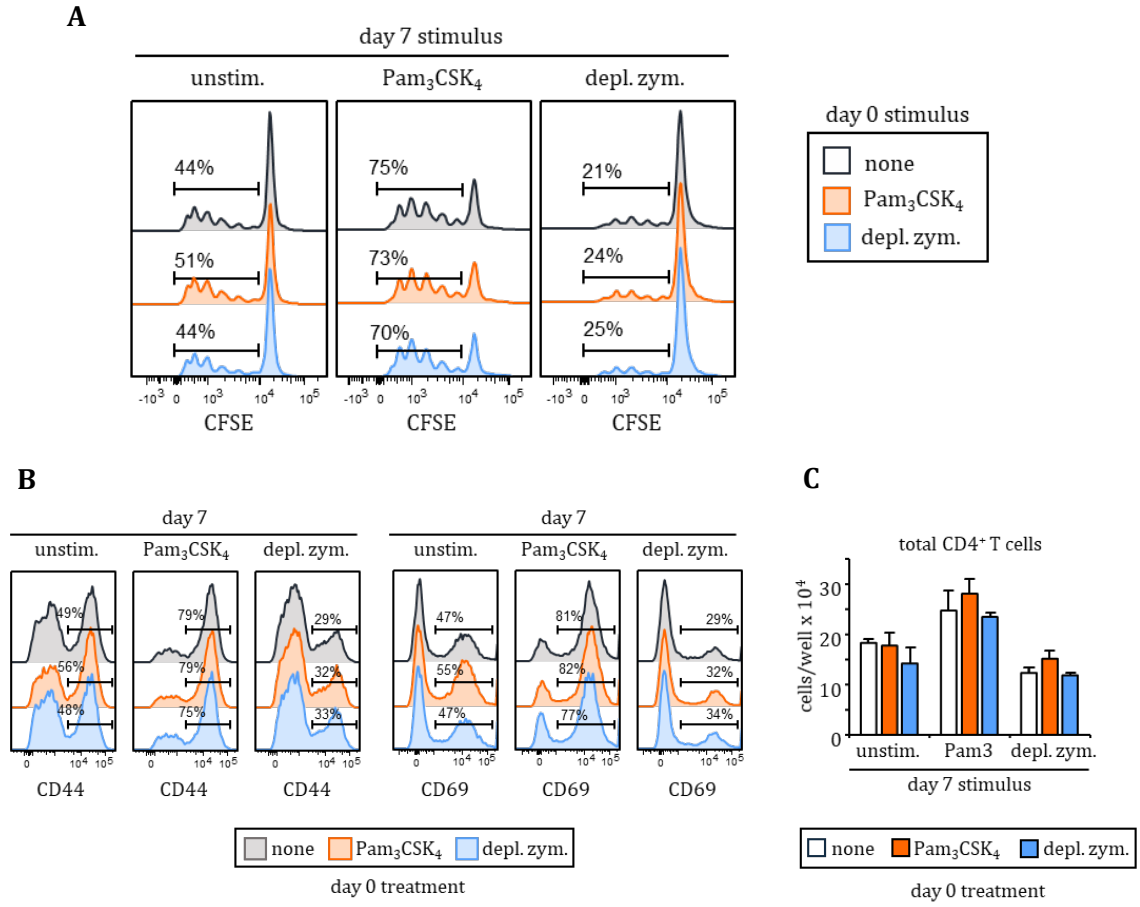


FIGURE 27 | Ability of APCs derived from HSPCs transiently stimulated with Pam3CSK4 or depleted zymosan to induce CD4⁺ T cell proliferation in OVA-specific CD4⁺ T cell cocultures. APCs derived from HSPCs under the same conditions as in Figure 25A were plated at equal numbers for 4 h, and then stimulated with Pam3CSK4, depleted zymosan or nothing (unstimulated) for 24 h. APCs were also loaded with the OVA323-339 peptide for 24 h and cocultured with CFSE-labeled CD4⁺ T cells isolated from OT-II mice at 1:5 ratio (APC:T cell). Following three days of coculture, T cells were harvested for flow cytometry analysis. **(A)** Proliferating CD4⁺ T cells. **(B)** Histograms and % of CD4⁺ T cells expressing CD44 or CD69 from cocultures. **(C)** Fold increase of total CD4⁺ T cell numbers, data represent means ± SD of three independent experiments, * $P < 0.05$, ** $P < 0.01$ with respect to day 0 none. Results shown are from one experiment that is representative of three independent experiments.

T cells primed by day 0 control APCs stimulated with Pam_3CSK_4 (day 7) were more proliferative and expressed higher levels of the activation markers CD44 and CD69 than T cells primed by unstimulated APCs (**Figure 27A** and **27B**). Conversely, day 7 stimulation of APCs with depleted zymosan decreased T cell proliferation and downregulated CD44 and CD69. Day 0 Pam_3CSK_4 or depleted zymosan treatment of HSPCs did not cause significant changes in T cell proliferation or activation marker expression (**Figure 27A** and **27B**), although T cell numbers increased modestly in the cocultures with stimulated APCs derived from day 0 Pam_3CSK_4-treated HSPCs (**Figure 27C**).

2.2 Cytokine production: IFN-γ and IL-17A

To evaluate Th1 and Th17 responses, we measured IFN-γ and IL-17A production by the $CD4^+$ T cells (**Figure 28**). Consistent with previous reports (LeibundGut-Landmann *et al.*, 2007; Sieling *et al.*, 2003), Pam_3CSK_4-stimulated APCs induced IFN-γ production by $CD4^+$ T cells, whereas depleted zymosan-stimulated APCs induced IL-17A production by $CD4^+$ T cells. APCs derived from HSPCs treated with both agonists more potently stimulated IFN-γ production by $CD4^+$ T cells in all the conditions studied, whereas only APCs derived from depleted zymosan-treated HSPCs significantly increased IL-17A production by $CD4^+$ T cells. These results indicate that HSPCs programed by microbial ligands can produce trained APCs capable of priming more strongly Th1 and Th17 responses in $CD4^+$ T cell cocultures.

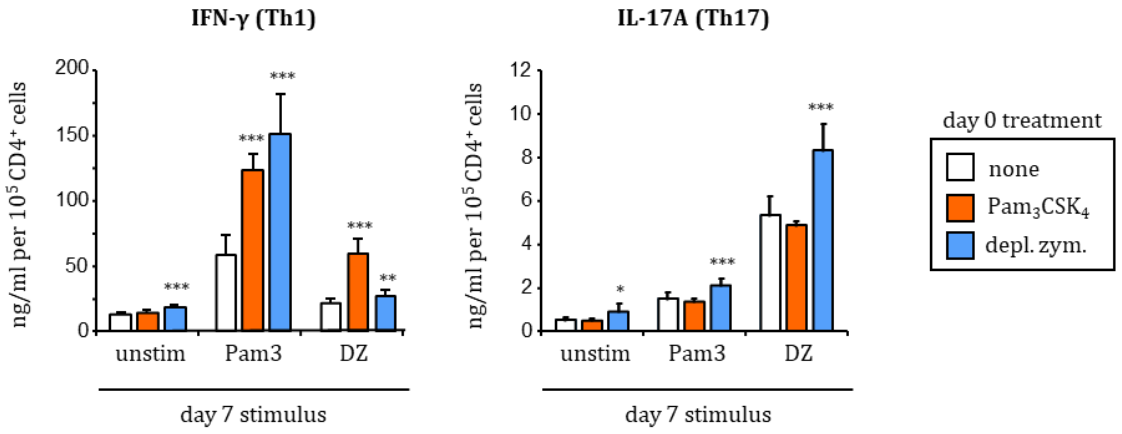


FIGURE 28 | Ability of APCs derived from HSPCs transiently stimulated with Pam_3CSK_4 or depleted zymosan to enhanced Th1 and Th17 responses in OVA-specific $CD4^+$ T cell cocultures. APCs derived from HSPCs under the same conditions as in Figure 25A were plated at equal numbers for 4 h and then stimulated with Pam_3CSK_4, depleted zymosan or nothing (unstimulated) for 24 h. APCs were also loaded with the $OVA_{323-339}$ peptide for 24 h and cocultured with CFSE-labeled $CD4^+$ T cells isolated from OT-II mice at 1:5 ratio (APC:T cell). Following three days of coculture, T cells were harvested and cytokine production (IL-17A and IFN-γ) was assessed after 24 h of restimulation with PMA (Phorbol 12 -Myristate 13-Acetate) and ionomycin. Cytokine data was normalized by $CD4^+$ T cell numbers (shown in Figure 27) and represent means ± SD of triplicate cultures, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with respect to day 0 none. Results shown are from one experiment that is representative of three independent experiments.

3 Surface molecule expression and cytokine production by *C. albicans* stimulated APCs derived from PAMP-exposed HSPCs

3.1 Surface molecule expression: MHCII, CD40, CD80, CD86

In order to investigate the functional responses of APCs derived from Pam_3CSK_4/depleted zymosan-programed HSPCs to intact microorganisms (which signal through several PRRs simultaneously), we used inactivated yeasts from a non-virulent (PCA2) and a virulent (ATCC 26555) strain of *C. albicans* as day 7 APC stimuli (**Figure 29**). APC expression of MHCII (signal one), CD80 and CD86 (signal two) on $CD11b^+$ $CD11c^+$ cells increased upon stimulation with both strains of *C. albicans*, and interestingly, unlike stimulation with pure PRR ligands, CD40 was highly upregulated. Consistent with the previous results, day 0 treatment of HSPCs with Pam_3CSK_4 further increased the expression of these proteins. In contrast, depleted zymosan treatment of HSPCs at day 0 did not change the induction of these APC surface molecules by the non-virulent strain but decreased their induction by the virulent strain.

A

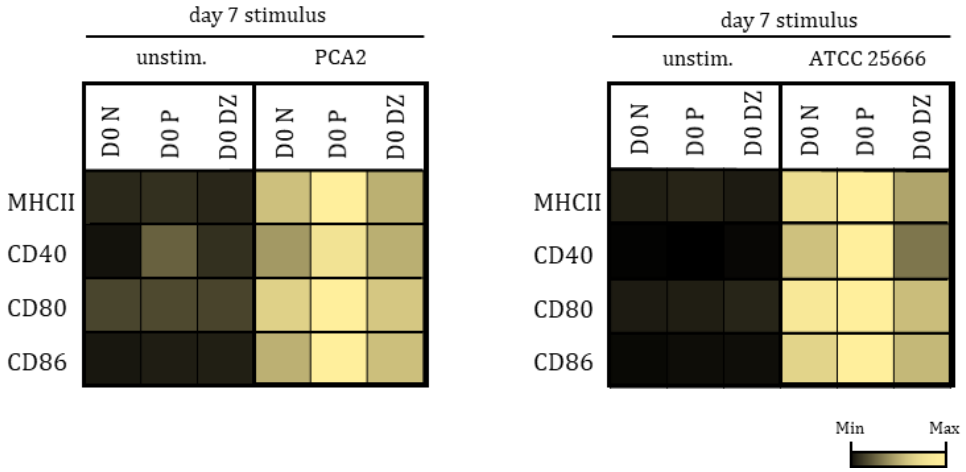


FIGURE 29 | Flow cytometry analysis of APCs generated from HSPCs transiently stimulated with Pam_3CSK_4 or depleted zymosan, following stimulation with *C. albicans*. APCs derived from HSPCs under the same conditions as in Figure 25A were plated at equal numbers for 24 h, and then stimulated with inactivated yeasts of *C. albicans* from the non-virulent strain PCA2 or the virulent strain ATCC 26555, or nothing (unstimulated) for 24 h to assess the expression of MHCII and costimulatory molecules on $CD11b^+$ $CD11c^+$ APCs by flow cytometry. **(A)** Colormap is based on min-max values per row using the MFI values obtained with the specific antibodies and the isotype controls. (DO, day 0; N, none; P, Pam_3CSK_4; DZ, depleted zymosan). **(B)** Histograms and MFIs (numbers) corresponding to data shown in colormaps. Results shown are from one experiment that is representative of three independent experiments.

B

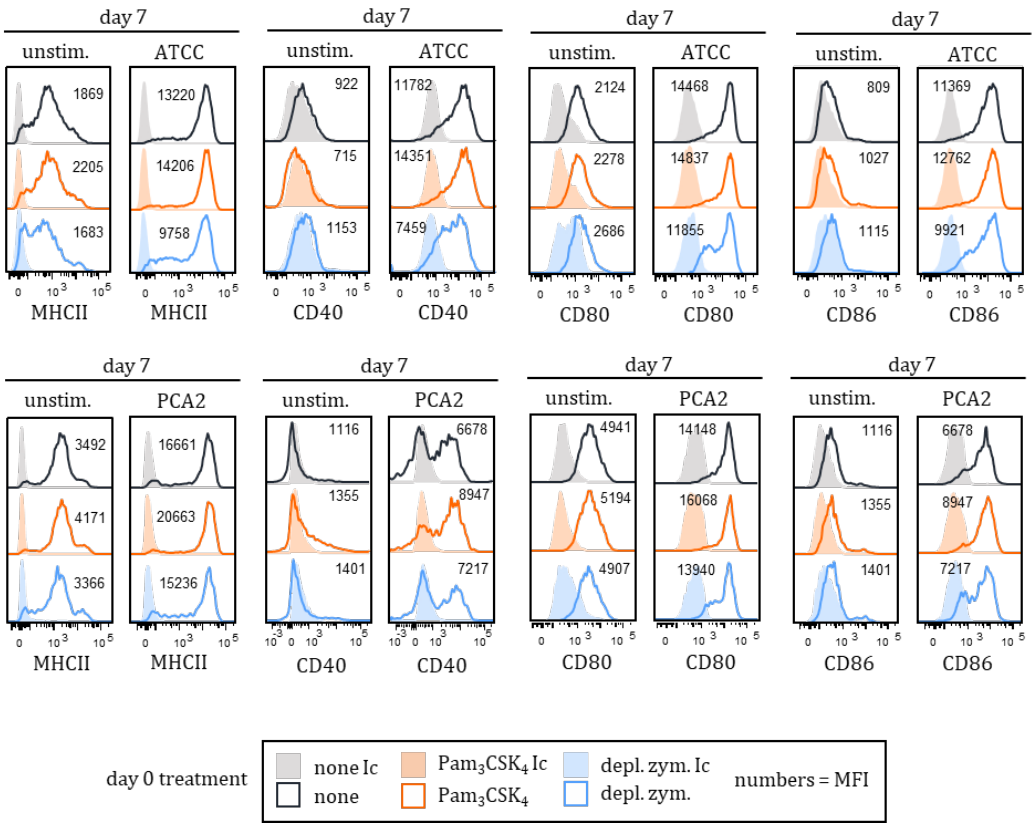


FIGURE 29 | Flow cytometry analysis of APCs generated from HSPCs transiently stimulated with Pam_3CSK_4 or depleted zymosan, following stimulation with *C. albicans*. Continuation.

3.2 Cytokine production: TNF-α, IL-6, IL-12 p40, IL-2

Next, production of the pro-inflammatory cytokines (signal three) was assessed in the culture supernatants of day 7 APCs (**Figure 30**). Day 0 treatment of HSPCs with Pam_3CSK_4 increased the production of IL-6 and IL-2 by APCs stimulated with both the non-virulent and virulent strains, and increased IL-12 p40 production by APCs stimulated with the virulent strain, but did not have any effect on TNF-α production by either strain. On the other hand, day 0 treatment of HSPCs with depleted zymosan potently augmented the production of IL-6 by APCs stimulated with both the non-virulent and virulent strains, and TNF-α and IL-12 p40 in response to the virulent strain, but did not impact IL-2 production.

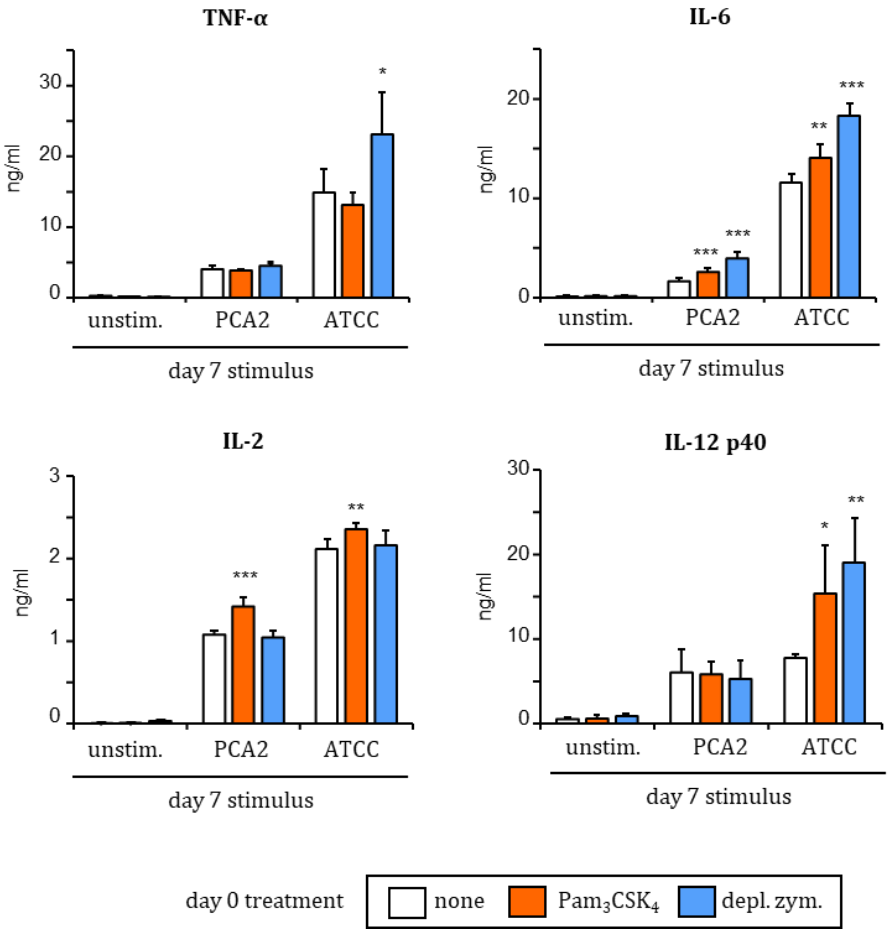


FIGURE 30 | Cytokine production of APCs generated from HSPCs transiently stimulated with Pam_3CSK_4 or depleted zymosan, following stimulation with *C. albicans*. APCs derived from HSPCs under the same conditions as in Figure 25A were plated at equal numbers for 24 h, and then stimulated with inactivated yeasts of *C. albicans* from the non-virulent strain PCA2 or the virulent strain ATCC 26555, or nothing (unstimulated) for 24 h to assess cytokine production (TNF-α, IL-6, IL-12 p40 and IL-2) in the supernatants by ELISA. Data represent means ± SD of triplicate cultures, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with respect to day 0 none. Results shown are from one experiment that is representative of three to independent experiments.

4 *C. albicans*-specific $CD4^+$ T cell responses induced by APCs derived from HSPCs exposed to PAMPs

4.1 $CD4^+$ T cell proliferation

We next evaluated how *C. albicans* stimulation of APCs derived from programed HSPCs modifies the proliferation of $CD4^+$ T cells isolated from naïve C57BL/6 mice. Under these conditions, *C. albicans* cells act as multi-antigen sources, as well as activators of APC PRRs (**Figure 31**).

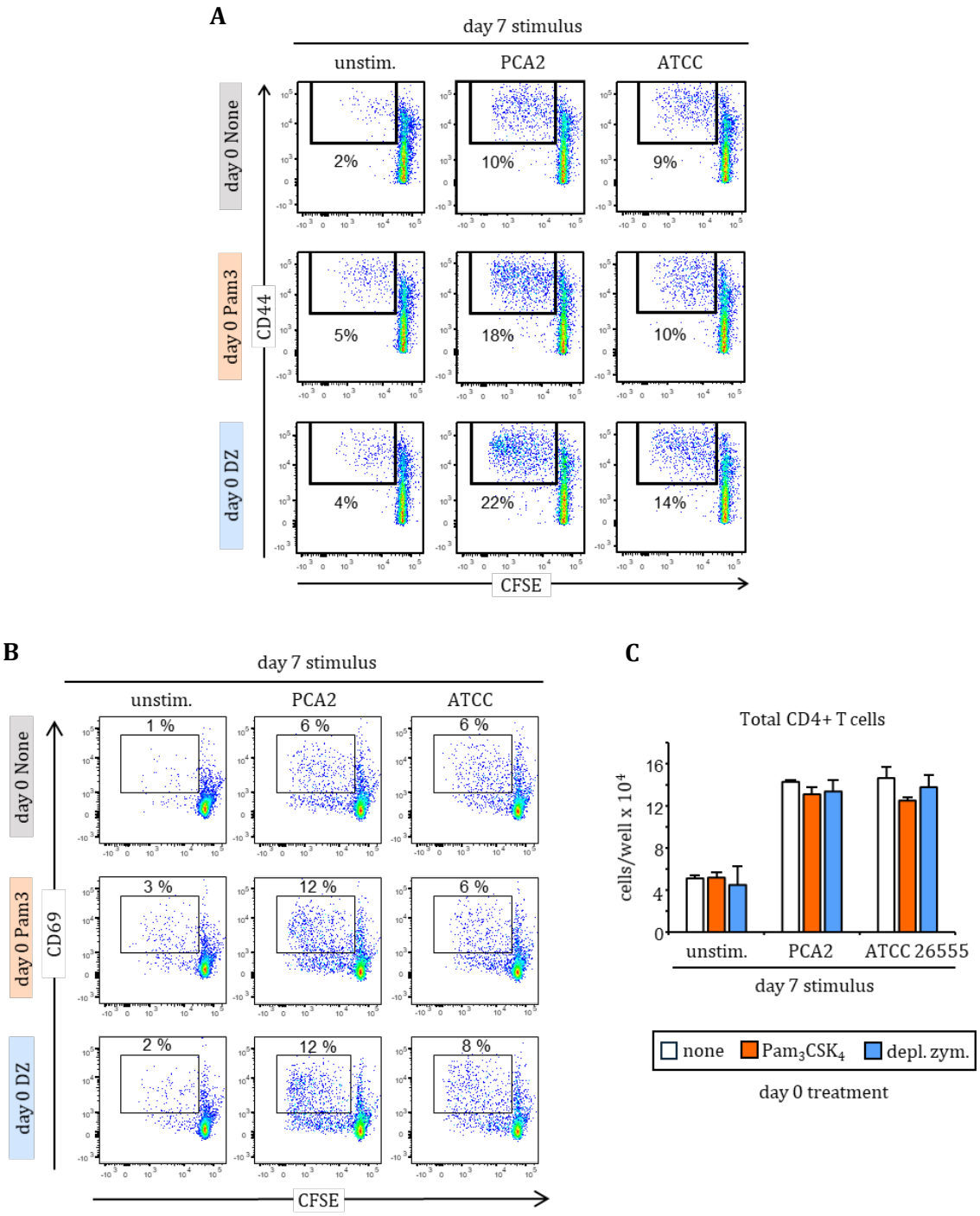


FIGURE 31 | Ability of APCs derived from HSPCs transiently stimulated with Pam_3CSK_4 or depleted zymosan to induce $CD4^+$ T cell proliferation after *C. albicans* stimulation. APCs derived from HSPCs under the same conditions as in Figure 25A were plated at equal numbers for 4 h, and then stimulated with inactivated yeasts of *C. albicans* from the non-virulent strain PCA2 or the virulent strain ATCC 26555, or nothing (unstimulated) for 24 h. Then, APCs were cocultured with CFSE-labeled $CD4^+$ T cells isolated from naïve C57BL/6 mice at 1:5 ratio (APC:T cell). Following four days of coculture, T cells were harvested for flow cytometry analysis. **(A)** Proliferating $CD4^+$ T cells expressing CD44. **(B)** Proliferating $CD4^+$ T cells expressing CD69. Results shown are from one experiment that is representative of three independent experiments. **(C)** Fold increase of total $CD4^+$ T cell numbers. Data represent means ± SD of three independent experiments.

Day 7 stimulation of APCs with both strains of *C. albicans* increased total T cell numbers and the percentage of proliferating $CD44^+$ and $CD69^+$ $CD4^+$ T cells in comparison to unstimulated APCs (**Figure 31A** and **31B**). Day 0 treatment of HSPCs with Pam_3CSK_4 and depleted zymosan modestly enhanced T cell proliferation when APCs were stimulated with the non-virulent strain, although T cell numbers in the cultures did not change, and there was little or no effect on T cell proliferation when APCs were stimulated with the virulent strain (**Figure 31C**).

4.2 Cytokine production: IFN-γ and IL-17A

To evaluate Th1 and Th17 responses, we measured IFN-γ and IL-17A production by the $CD4^+$ T cells after coculturing them with *C. albicans*-stimulated APCs (**Figure 32**). Interestingly, IFN-γ production by $CD4^+$ T cells was induced by stimulating APCs with both strains of *C. albicans*, and treatment of HSPCs with Pam_3CSK_4 and depleted zymosan enhanced IFN-γ production when APCs were stimulated with the non-virulent strain, but not the virulent strain. Moreover, IL-17A production by $CD4^+$ T cells, which was greater upon APC stimulation with the virulent strain than the non-virulent strain, was clearly enhanced by treating HSPCs with both Pam_3CSK_4 and depleted zymosan at day 0 (**Figure 32**). Taken together, these data show that APCs derived from HSPCs programed by microbial ligands have an improved ability to induce Th1, Th17 or both responses, depending on the pathogen strain the APCs are responding to.

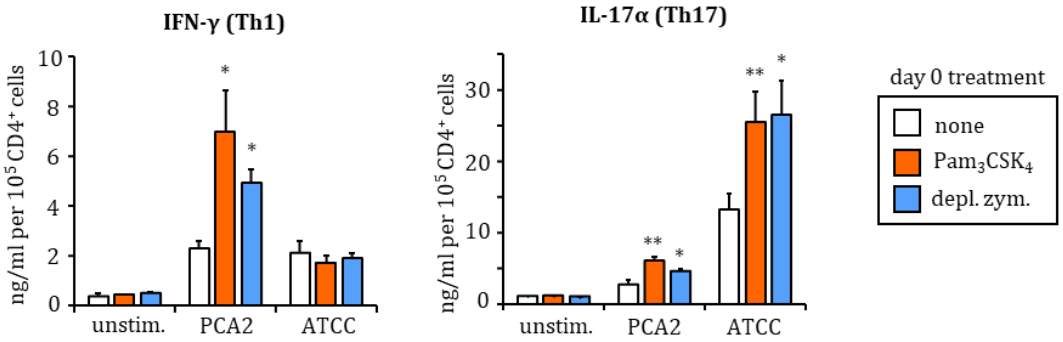


FIGURE 32 | Ability of APCs derived from HSPCs transiently stimulated with Pam_3CSK_4 or depleted zymosan to enhanced Th1 and Th17 responses in $CD4^+$ T cell cocultures after *C. albicans* stimulation. APCs derived from HSPCs under the same conditions as in Figure 25A were plated at equal numbers for 4 h, and then stimulated with inactivated yeasts of *C. albicans* from the non-virulent strain PCA2 or the virulent strain ATCC 26555, or nothing (unstimulated) for 24 h. Then, APCs were cocultured with CFSE-labeled $CD4^+$ T cells isolated from naïve C57BL/6 mice at 1:5 ratio (APC:T cell). Following four days of coculture, T cells were harvested and cytokine production (IL-17A and IFN-γ) was assessed after 24 h of restimulation with PMA and ionomycin. Cytokine data was normalized by $CD4^+$ T cell numbers (shown in Figure 31) and represent means ± SD of triplicate cultures, * $P < 0.05$, ** $P < 0.01$ with respect to day 0 none. Results shown are from one experiment that is representative of three independent experiments.

5 Discussion

Innate immune memory has raised lots of interest in the scientific community since it was discovered, using certain models of vaccination, that mice showed protection against the same or unrelated pathogens due to an enhanced activation of innate immune cells (mainly monocytes and macrophages) (Bistoni *et al*., 1986; van 't Wout *et al*., 1992). Mechanistically, the protection conferred by myeloid training may be due to: (i) enhanced non-specific innate immune responses of monocytes or macrophages derived from them, e.g. increased cytokine production, which could boost the recruitment and activation of other immune cells, contributing to pathogen clearance (Netea *et al*., 2020); (ii) HSPC programing, which increases myelopoiesis and improves the functional phenotype of the macrophages produced (Kaufmann *et al.*, 2018; Mitroulis et al., 2018), explaining the long-lasting effects of trained immunity; and (iii) modification of the T cell priming potential of APCs, which could enhance adaptive T cell responses, thus bridging innate training with an improved adaptive response. The latter idea has not been formally demonstrated, although some previous have supported it (Sánchez-Ramón *et al.*, 2018). It has been described that BM-derived APCs from mice exposed to the filarial nematode glycoprotein ES-62 prime anti-inflammatory responses (Goodridge *et al.*, 2004). Moreover, BM progenitors from mice exposed to UV radiation or the inflammatory mediator PGE_2 produce DCs that are defective in their ability to prime T cells responses (Goodridge 2014).

We therefore decided to study whether stimulation of HSPCs with Dectin-1 or TLR2 ligands could alter the functional phenotype of the APCs derived from them and subsequently have an impact in T cell activation. We used GM-CSF to derive APCs from HSPCs as it is the most widely used growth factor to study the biology of these cells (Lutz *et al.*, 2017). While M-CSF drives macrophage differentiation, GM-CSF induces the development of monocyte-derived cells with a much higher antigen presentation capacity. More importantly, GM-CSF has been used for the development of dendritic cell vaccines by culturing peripheral blood cells from patients *in vitro* for autologous transplantation, although there is still some debate about naming these APCs “dendritic cells” (Ginhoux *et al.*, 2016; Lutz *et al.,* 2017). GM-CSF cultures have been considered to be heterogeneous (Helft *et al*., 2015, Lutz *et al.,* 2017; Na *et al.*, 2016). However, by starting from HSPCs, instead of total BM, and in our culture conditions, about 80% of the cells are $CD11b^+$ $CD11c^+$, which depending of their activation state can show differences in the expression of some functional molecules.

Here we show that induction of MHCII (required for antigen presentation; signal one) and costimulatory molecules (CD40, CD80 and CD86; signal two) on APCs is mostly enhanced by stimulation of HSPCs with a TLR2 agonist, while stimulation with a Dectin-1 ligand decreases or does not change them. We also observed changes in the levels and types of cytokines produced by APCs (signal 3), which are dependent on the combination of the stimuli used for HSPC programing and for APC stimulation. Interestingly, while HSPCs stimulated with a TLR2 agonist give rise to tolerized macrophages in M-CSF cultures (Yáñez *et al.*, 2013b; Megías *et al.*, 2016), here we show that GM-CSF-induced differentiation of HSPCs stimulated with a TLR2 agonist gives rise to trained APCs (enhanced response). Antigen uptake and presentation could also be influenced by HSPC programing. Although, previous studies showed minor changes in the phagocytic ability of macrophages derived from programed HSPCs (Yáñez, *et al.*, 2013b; Megías *et al.*, 2016), we cannot discard differences in *C. albicans* cells uptake by APCs. However, here we also employ OVA peptide as an antigen, which can be directly loaded into the MHCII molecules. In this case, T cell responses would not be affected by differences in antigen uptake and presentation by APCs.

In order to prevent hyper-activation and regulate the duration of the immune response, mechanisms of feedback control are required. Suppressor of cytokine signaling (SOCS) proteins and the mammalian sterile 20-like kinase (MST1) have been identified as a negative feedback loop to cytokine signaling, thus regulating $CD4^+$ T cell differentiation. In the context of candidiasis, it has been demonstrated that SOCS1 silencing can promote *in vitro* maturation of DCs after exposure to *C. albicans*, which produce higher levels of IL-12 resulting in an enhanced $CD4^+$ Th1 differentiation (Shi *et al.*, 2015). This effect also occurs *in vivo* and increase mouse survival in a systemic candidiasis mouse model (Shi *et al.*, 2018). On the other hand, SOCS3 and MST1 negatively regulates Th17 differentiation, as DCs deficient in these proteins promote IL-6 secretion (Li *et al.*, 2017; Shi *et al.*, 2019). Therefore, it will be interesting to see whether activation of PRRs in HSPCs lead to modulation of these negative regulators in APCs.

Our results show that different Th1 and Th17 outcomes may be achieved by varying the set of PRR ligands used for HSPC programing and for APC stimulation, although future experiments will be needed to better define the factors contributing to Th1 and Th17 polarization. As we have seen, PRR ligands induce the activation and maturation of APCs, which upregulate MHCII and costimulatory molecules, as well as produce cytokines that modulate Th polarization. Interestingly, depending on the strain of *C. albicans* we used to stimulate APCs, a different Th response was observed. Regardless of the stimulus used to treat HSPCs, IFN-γ production was only enhanced when APCs derived from programed

HSPCs were stimulated with a nonvirulent strain, while more potent IL-17 production was achieved after stimulating APCs derived from programed HSPCs with a virulent strain of *C. albicans.* In addition to studying the molecular mechanisms that alter the functional phenotype of APCs derived from PAMP-stimulated HSPCs, further studies will be necessary to know whether these APCs are also able to induce better T cell responses *in vivo*. It will also be interesting to evaluate whether HSPC programing could influence $CD8^+$ T cell responses and whether innate immune memory impacts the generation of long-lasting memory T cells.

CONCLUSIONS

1. Continuous *in vitro* exposure of HSPCs to PAMPs determines the antifungal phenotype of macrophages differentiated with M-CSF: TLR2 or TLR4 agonist exposure generates macrophages with a diminished ability to secrete TNF-α and IL-6 (tolerized), whereas Dectin-1 or *C. albicans* yeasts stimulation leads to the generation of macrophages that produce higher amounts of these pro-inflammatory cytokines (trained). Moreover, TLR2 agonist exposure and *C. albicans* stimulation give rise to macrophages with an enhanced fungicidal activity.

2. Transient *in vitro* exposure of HSPCs to a TLR2 or TLR4 agonist in M-CSF cultures is sufficient to generate tolerized macrophages. The tolerized phenotype induced by a TLR2 agonist exposure can be partially reversed by subsequent stimulation to a Dectin-1 agonist or inactivated *C. albicans* yeasts.

3. Transient and continuous *in vitro* exposure of HSPCs to a TLR2 agonist in GM-CSF cultures generate tolerized macrophages, a phenotype reinforced by subsequently exposure to a Dectin-1 agonist or *C. albicans* yeasts.

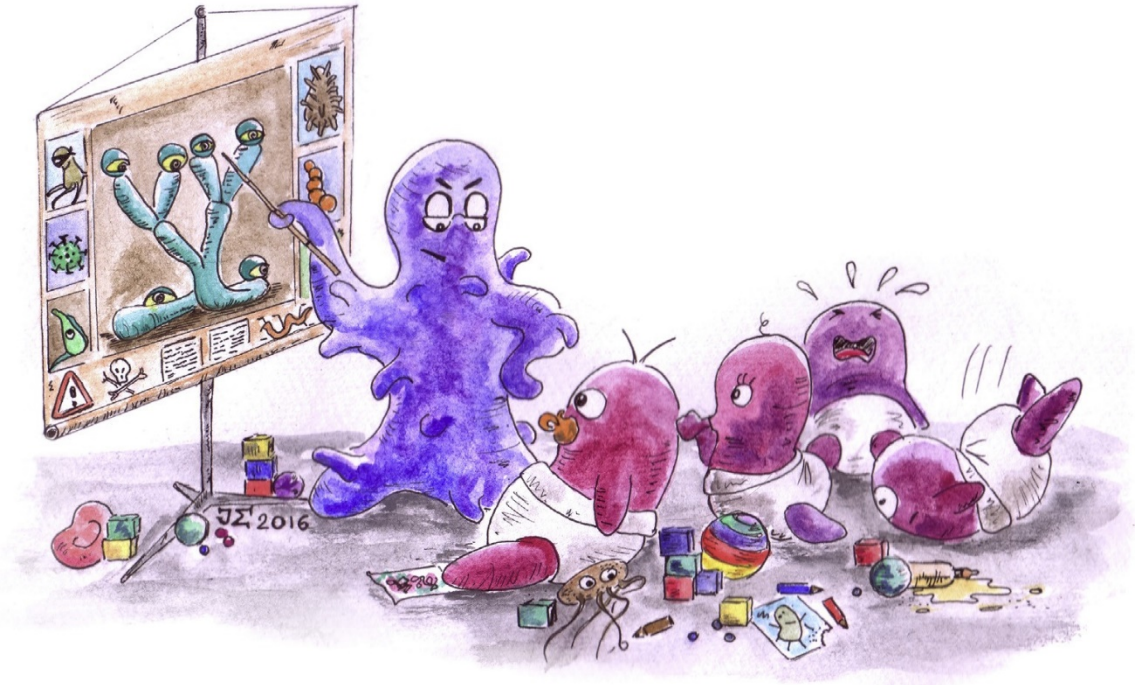
4. *C. albicans* systemic infection associated with low fungal burden generates *ex vivo* HSPC-derived macrophages with a trained phenotype, whereas high fungal burden gives rise to tolerized macrophages. On the other hand, a short *in vivo* TLR2 agonist treatment leads to the generation of *ex vivo* HSPC-derived macrophages with a tolerized phenotype, while an extended TLR2 agonist treatment gives rise to trained macrophages.

5. Extended *in vivo* TLR2 agonist treatment increases spleen HSPCs and myeloid cells, and protects mice from systemic *C. albicans* infection. Immunodepletion of HSPCs abrogates protection against the fungal infection.

6. HSPCs produce cytokines and chemokines *in vitro* in response to a TLR2 ligand or *C. albicans* yeasts, and these "secretomes" can induce myeloid differentiation of HSPCs and a tolerized phenotype in peritoneal macrophages.

7. *In vitro* exposure of HSPCs to a TLR2 or a Dectin-1 agonist determines the phenotype of the antigen presenting cells they produce, as they exhibit altered expression of MHCII, costimulatory molecules (CD40, CD80 and CD86) and altered cytokine production (TNF-α, IL-6, IL-12 p40 and IL-2) in response to PAMPs or *C. albicans* yeasts.

8. Antigen presenting cells derived from TLR2/Dectin-1-programed HSPCs prime enhanced Th1 and Th17 responses in $CD4^+$ T cell cocultures in response to PAMPs or *C. albicans* yeasts.



Highlight prime time, Microbes and Infection 18 (2016) 523-526.

Our paper Megías *et al.*, (2016) was chosen month-highlight by Microbes and Infection and consequently, the above cited paper was published

RESEARCH PUBLICATIONS

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

1. Megías J.*, Martínez A.*, Yáñez A., Goodridge H.S., Gozalbo D., and Gil M.L. (2016). TLR2, TLR4 and Dectin-1 mediated signalling in hematopoietic stem and progenitor cells determines the antifungal phenotype of the macrophages they produce. *Microbes and Infection*. 18, 354-363. *: Both authors contribute equally. doi: 10.1016/j.micinf.2016.01.005.

2. Martínez A., Bono C., Megías J., Yáñez A., Gozalbo D., and Gil M.L. (2017). PRR signaling during *in vitro* macrophage differentiation from progenitors modulates their subsequent response to inflammatory stimuli. *European Cytokine Network*. 28, 102-110. doi: 10.1684/ecn.2017.0398.

3. Martínez A., Bono C., Megías J., Yáñez A., Gozalbo D., and Gil M.L. (2018). Systemic Candidiasis and TLR2 Agonist Exposure Impact the Antifungal Response of Hematopoietic Stem and Progenitor Cells. *Frontiers in Cellular and Infection Microbiology*. 8, 309. doi: 10.3389/fcimb.2018.00309.

4. Martínez A., Bono C., Gozalbo D., Goodridge H.S., Gil M.L. and Yáñez A (2020). TLR2 and Dectin-1 signaling in hematopoietic stem and progenitor cells impacts CD4 T cell responses by changing the phenotype of the antigen presenting cells they produce. *Cells. 9*, 1317; doi:10.3390/cells9051317.

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RESUMEN

INTRODUCCIÓN

Las células mieloides (neutrófilos y macrófagos) forman parte de la primera línea de actuación del sistema inmunitario frente a los microorganismos patógenos, ya que son capaces de eliminarlos por fagocitosis y de secretar mediadores que reclutan y activan a otros leucocitos (Lionakis, 2014; Pappas *et al*., 2018). Durante una infección, los fagocitos se consumen rápidamente en la lucha frente al patógeno, por lo que su reaprovisionamiento resulta esencial para que la respuesta inmune sea efectiva. Esta pronta producción de células mieloides en situaciones de estrés se denomina mielopoiesis de emergencia, proceso mediante el cual la hematopoyesis favorece la producción de células mieloides frente a la generación de otras poblaciones celulares (Boettcher y Manz*,* 2017; Kobayashi *et al.*, 2016). Los mecanismos que provocan este cambio en la hematopoyesis están siendo objeto de numerosos estudios, entre los cuales existen diversas publicaciones que demuestran la interacción directa de patógenos o patrones moleculares asociados a patógenos (PAMP) con las células madre y progenitores hematopoyéticos (HSPC) (Yáñez *et al.*, 2013a; Zhao y Baltimore, 2015).

En este contexto, nuestro grupo ha demostrado que el hongo *Candida albicans* induce la proliferación y diferenciación de HSPC de ratón hacia linaje mieloide tanto *in vitro* como *in vivo*. Esta interacción directa de las HSPC con los microorganismos requiere la señalización a través del receptor tipo Toll 2 (TLR2) y del receptor tipo lectina Dectina-1, ambos pertenecientes a la familia de receptores de reconocimiento de patrones (PRR) y genera fagocitos funcionales capaces de internalizar levaduras y secretar citocinas inflamatorias (Megías *et al*., 2013; Yáñez *et al.*, 2010; Yáñez *et al.*, 2011). Estos resultados sugieren que los patógenos pueden ser directamente reconocidos por las HSPC a través de los PRR, promoviendo así la capacidad de reaprovisionamiento del sistema inmunitario innato durante una infección.

En una infección sistémica, el encuentro entre los microorganismos o moléculas derivadas de estos con las HSPC podría darse en la médula ósea, órgano hematopoyético principal. Además, las HSPC pueden movilizarse desde la médula ósea hacia los tejidos periféricos infectados o hacia órganos linfoides secundarios, como el bazo; así, los microorganismos podrían inducir la diferenciación de estas células madre migratorias por hematopoyesis extramedular, generándose las células maduras directamente en su lugar de actuación (Massberg *et al.*, 2007). Asimismo, se ha descrito que la activación de TLR en las HSPC induce la secreción de citocinas y quimiocinas, reforzando de este modo la respuesta

inflamatoria llevada a cabo por las células maduras (Zhao *et al.*, 2014, Granick *et al.*,2013; Buechler *et al.*, 2016).

El planteamiento de que los microorganismos puedan estimular directamente a las HSPC para generar rápidamente células mieloides y hacer frente a la infección resulta muy atractivo y permite especular sobre si este mecanismo podría generar células funcionalmente mejor preparadas para hacer frente a la infección. En este sentido, numerosos estudios demuestran que la respuesta de las células mieloides a microorganismos patógenos puede ser modulada por encuentros previos con los microorganismos o sus PAMP, propiedad que ha sido generalmente denominada "memoria de la inmunidad innata" (Netea *et al.*, 2020; Netea *et al.*, 2016). El primer contacto con el microorganismo o PAMP provoca una reprogramación funcional de las células mieloides, que puede dar lugar a una mayor respuesta (inmunidad entrenada) o una menor respuesta (tolerancia) frente al microorganismo en siguientes encuentros, en términos de producción de citocinas inflamatorias. Sin embargo, es importante destacar que durante el fenómeno de tolerancia también hay que genes cuya expresión aumenta, principalmente aquellos relacionados con el reconocimiento de los patógenos y la respuesta antimicrobiana (Foster *et al.*, 2007). Así pues, de una forma u otra, ambos efectos tendrían como objetivo beneficiar la resistencia del hospedador frente al patógeno en posteriores infecciones. Por esta razón, la memoria de la inmunidad innata adquiere especial relevancia en el contexto de la vacunación, mediando en parte los efectos heterólogos (no debidos al antígeno utilizado como vacuna) que se han descrito en ciertas vacunas vivas atenuadas (Goodridge *et al.*, 2016).

Sin embargo, la interacción directa de las HSPC con los microorganismos también podría ser aprovechada por algunos patógenos para desarrollar mecanismos de evasión inmune modulando la respuesta de las HSPC. Por lo tanto, resulta fundamental estudiar las propiedades funcionales de las células mieloides generadas a partir de las HSPC en respuesta a la señalización vía PRR, tales como la actividad antifúngica, la capacidad para activar otras células mediante secreción de citocinas o la capacidad de presentación antigénica. En este sentido, nuestro grupo ha demostrado que los macrófagos generados a partir de HSPC expuestas a Pam_3CSK_4 (ligando de TLR2) producen menos citocinas inflamatorias y menos radicales tóxicos del oxígeno, comparando con la producción por parte de macrófagos obtenidos a partir de HSPC no estimuladas (Yáñez, *et al.*, 2013b).

OBJETIVOS

En base a los antecedentes previamente comentados, los **objetivos generales** desarrollados en este proyecto de tesis han sido los siguientes:

1. **Estudiar los efectos de la exposición de HSPC a PAMP en el fenotipo antifúngico de los macrófagos generados tras su diferenciación.**

   1.1. Analizar las consecuencias de la señalización vía PRR en HSPC *in vitro* para los macrófagos generados mediante cultivo con M-CSF ("*Macrophage-Colony Stimulating Factor")* o GM-CSF ("*Granulocyte and Macrophage-Colony Stimulating Factor")*.

   1.2. Analizar las consecuencias de señalización vía PRR en HSPC *in vivo*, mediante un modelo de candidiasis sistémica o de tratamiento con Pam_3CSK_4 (ligando de TLR2), para los macrófagos generados *ex vivo* a partir de las HSPC purificadas.

2. **Estudiar los efectos de la exposición de HSPC a PAMP en la respuesta antifúngica de las propias HSPC.**

   2.1 Analizar *in vivo* la función que ejercen las HSPC frente a la infección por *C. albicans* en un modelo de exposición a varias dosis de Pam_3CSK_4.

   2.2 Analizar los mecanismos efectores de las HSPC en respuesta a PAMP.

3. **Estudiar los efectos de la exposición de HSPC a PAMP en la maduración de las células presentadoras de antígenos (APC; "Antigen presenting cells") generadas tras su diferenciación, así como en su capacidad para activar y polarizar linfocitos T $CD4^+$.**

RESULTADOS

<u>Capítulo 1.</u> Fenotipo funcional de macrófagos diferenciados a partir de HSPC expuestas *in vitro* o *in vivo* a PAMP

La exposición in vitro de las HSPC a PAMP altera el fenotipo antifúngico de los macrófagos que se generan en cultivos con M-CSF

Las HSPC, purificadas como células "linaje negativo" (Lin^-), se diferenciaron hacia macrófagos en presencia o ausencia (células control) de distintos agonistas de los PRR: Pam_3CSK_4 (agonista del TLR2), lipopolisacárido (LPS) (agonista del TLR4), zimosán deplecionado (ligando de Dectina-1) y células inactivadas de *C. albicans* (que señalizan principalmente vía TLR2 y Dectina-1). En concreto, se utilizaron levaduras inactivadas de la cepa ATCC 26555. La estimulación con los ligandos solubles Pam_3CSK_4 o LPS se mantuvo durante todo el periodo de diferenciación (exposición continua) o durante únicamente las primeras 24 h únicamente (exposición transitoria). En todos los casos, tras siete días de cultivo, se recogieron las células adherentes y se analizó (i) la expresión de los marcadores de superficie Ly-6C y de la molécula de histocompatibilidad de clase II (MHCII), (ii) la producción de citocinas inflamatorias en respuesta a Pam_3CSK_4 o LPS, y (iii) la capacidad fagocítica y microbicida de los macrófagos frente a levaduras de *C. albicans*.

Cuando la diferenciación hacia macrófago se llevaba a cabo mediante cultivo con el factor de crecimiento M-CSF, tras estimular de forma continua las HSPC con Pam_3CSK_4 o LPS, la mayoría de los macrófagos (células $CD11b^+$ $F4/80^+$) adquiría un fenotipo inflamatorio ($Ly6C^+$ $MHCII^-$); en exposición transitoria únicamente se veía dicho efecto al estimular las HSPC con Pam_3CSK_4. La estimulación de las HSPC con zimosán deplecionado o *C. albicans* no inducía cambios en los marcadores de superficie analizados, pero aumentaba la producción de las citocinas inflamatorias TNF-α (Factor de necrosis tumoral α) e IL-6 (Interleucina 6) por parte de los macrófagos obtenidos (fenotipo entrenado). Sin embargo, las HSPC estimuladas de forma continua o transitoria con Pam_3CSK_4 o LPS generaban macrófagos con menor capacidad para producir TNF-α e IL-6 (fenotipo tolerizado). Además, los macrófagos obtenidos a partir de HSPC estimuladas con Pam_3CSK_4 o *C. albicans* presentaban mayor actividad fungicida que los macrófagos producidos a partir de HSPC sin

estimular (macrófagos control), ya que eran capaces de eliminar una mayor cantidad de levaduras de *C. albicans*. Este aumento de la capacidad fungicida no está relacionado con una alteración de la capacidad fagocítica, ya que en ambos casos los macrófagos eran capaces de fagocitar un porcentaje de levaduras similar al fagocitado por macrófagos control.

Por otra parte, si la diferenciación hacia macrófago se llevaba a cabo mediante cultivo con el factor de crecimiento GM-CSF, tras estimular las HSPC con cualquiera de los PAMP utilizados, la mayoría de los macrófagos adquiría un fenotipo inflamatorio ($Ly6C^+$ $MHCII^-$), mientras que en exposición transitoria únicamente se veía dicho efecto al estimular con Pam_3CSK_4. La producción de citocinas inflamatorias TNF-α e IL-6, tanto en exposición continua como transitoria, solo se vio alterada en los macrófagos obtenidos a partir de HSPCs en presencia de Pam_3CSK_4, observándose, al igual que en cultivos con M-CSF, un fenotipo tolerizado. En cuanto a la actividad fungicida, ninguno de los PAMPs utilizados para estimular las HSPC daba lugar a macrófagos con una mayor capacidad para eliminar levaduras de *C. albicans*.

Para evaluar la capacidad de los ligandos fúngicos de revertir la tolerancia al Pam_3CSK_4 en HSPC, las HSPC fueron expuestas a Pam_3CSK_4 durante 24 h y, tras eliminar el ligando mediante lavado, los progenitores se cultivaron con estímulos fúngicos (zimosán deplecionado o levaduras inactivadas de *C. albicans*) y M-CSF o GM-CSF durante siete días. Tras este periodo, se recogieron las células adherentes y se reestimularon con Pam_3CSK_4 o LPS. Los resultados obtenidos mostraron que, en cultivos con M-CSF, la presencia de levaduras de *C. albicans* o zimosán deplecionado durante la diferenciación hacia macrófago a partir de HSPC revertía parcialmente el fenotipo de tolerancia inducido por Pam_3CSK_4. Los macrófagos generados producían más TNF-α que los macrófagos expuestos únicamente a Pam_3CSK_4, aunque estos niveles continuaban siendo significativamente menores que los de macrófagos control. Por el contrario, respecto a la IL-6, la disminución de su producción por parte de macrófagos derivados de HSPC expuestas a Pam_3CSK_4 no se veía alterada por la presencia ni de levaduras ni de zimosán deplecionado durante la diferenciación. De la misma manera, el efecto de tolerancia inducido por Pam_3CSK_4 en HSPC en condiciones inflamatorias no se veía revertido por la presencia ni de levaduras ni de zimosán deplecionado durante la diferenciación hacia macrófago; incluso sorprendentemente, la producción de citocinas inflamatorias en respuesta al Pam_3CSK_4 era menor en comparación a la de células tolerizadas.

La infección sistémica por C. albicans y la exposición a Pam_3CSK_4 in vivo altera el fenotipo antifúngico de los macrófagos producidos ex vivo a partir de las HSPC

Utilizando un modelo murino de candidiasis sistémica (mediante infección intraperitoneal con la cepa ATCC26555), observamos que en las primeras etapas de la infección (a bajos niveles de carga fúngica en órganos internos) las HSPC generaban macrófagos con un fenotipo entrenado: mayor producción de TNF-α en respuesta a Pam_3CSK_4 y aumento de la actividad fungicida, comparando con macrófagos control obtenidos a partir de HSPC de ratones no infectados. Sin embargo, cuando la infección avanzaba y se alcanzaban altos niveles de carga fúngica en los órganos internos, los macrófagos producidos presentaban un fenotipo tolerizado en cuanto a la producción de citocinas inflamatorias, mientras que su capacidad microbicida era similar a la de macrófagos control.

Para determinar el efecto de la activación *in vivo* de HSPC por Pam_3CSK_4 se utilizaron dos pautas de tratamiento: una exposición corta al ligando (mediante una única dosis vía intravenosa) o una exposición continuada (mediante tres dosis vía intraperitoneal a días alternos). A continuación, se purificaron las HSPC de los ratones tratados y de ratones sin tratar (control) y se procedió a su diferenciación mediante cultivo con M-CSF. Con una única dosis de ligando, los macrófagos generados presentaban un fenotipo entrenado en respuesta a ligandos solubles de los TLR, al comparar con la respuesta de macrófagos control. Tras una exposición continua al agonista de TLR2, por el contrario, los macrófagos generados *ex vivo* presentaban un fenotipo entrenado en respuesta a ligandos solubles de los TLRs. Además, como previamente había descrito Herman y colaboradores (Hernan *et al.*, 2016), comprobamos que los ratones tratados con esta pauta de inyección de tres dosis de Pam_3CSK_4 sufrían esplenomegalia, observándose un aumento en la cantidad total de HSPCs (células Lin^- c-Kit^+) en bazo. Purificamos las HSPC del bazo de ratones tratados y comprobamos que, al igual que a partir de HSPC de médula, los macrófagos generados presentaban un fenotipo entrenado en cuanto a la producción de citocinas. Sin embargo, estos macrófagos eran prácticamente incapaces de eliminar levaduras de *C. albicans* en comparación con los macrófagos control. En este caso, debido a la poca cantidad de células Lin^- en los bazos de los ratones sin tratar, se utilizaron como macrófagos control los producidos a partir de HSPC de médula.

<u>Capítulo 2.</u> Respuesta antifúngica de las HSPC expuestas a PAMP

Una exposición continuada a Pam_3CSK_4 protege frente a la infección por C. albicans, efecto mediado en parte por las HSPC

Los bazos de los ratones tratados con varias dosis de Pam_3CSK_4, además de presentar un aumento en HSPC, también presentaban un aumento de las células mieloides maduras, ya que el número total de células que expresaban el marcador mieloide CD11b aumentaba con respecto a los bazos de ratones sin tratar. Se observaba un incremento de neutrófilos, células dendríticas clásicas, monocitos y macrófagos ($CD11b^+$, $MHCII^-$, $Ly6C^-$ y $CD11c^-$), siendo esta última población la que sufría una expansión más significativa. Dado el importante efecto del tratamiento continuado con Pam_3CSK_4, tanto en el fenotipo de los macrófagos producidos *ex vivo* a partir de HSPC, como en la cantidad de HSPC y células mieloides maduras presentes en el bazo, decidimos evaluar la influencia del tratamiento en la susceptibilidad a la infección sistémica por *C. albicans*. Los ratones tratados con varias dosis de Pam_3CSK_4 mostraron una carga fúngica en los órganos internos significativamente disminuida con respecto a ratones infectados sin tratar. Este resultado nos indica que un tratamiento extendido con Pam_3CSK_4 protege a los ratones frente a la infección por *C. albicans*.

Para comprobar el papel de las HSPC en la protección frente a la candidiasis sistémica que presentaban los ratones tratados con varias dosis de Pam_3CSK_4 decidimos ver el efecto que causaba la depleción de estas células. Para ello utilizamos el anticuerpo monoclonal ACK2, cuya administración *in vivo* provocaba una rápida depleción de las células madre hematopoyéticas (Czechowicz *et al.*, 2007). La inmunodepleción de las HSPC causó la pérdida de protección frente a la candidiasis sistémica conferida por el tratamiento con varias dosis de Pam_3CSK_4, por lo que podríamos afirmar que la protección de ratones tratados con Pam_3CSK_4 frente a la candidiasis está mediada, al menos en parte, por HSPC.

El "secretoma" producido por HSPC en respuesta a PAMP altera la diferenciación de HSPC de forma paracrina y *la producción de citocinas por parte de macrófagos maduros*

Células Lin^- fueron cultivadas en presencia de Pam_3CSK_4 o levaduras inactivadas de *C. albicans* durante tres días, en un medio sin suero para evitar su diferenciación inherente al ser cultivadas *in vitro*. Tras este periodo, se recogió el medio "condicionado" para evaluar sus efectos en HSPC y macrófagos peritoneales. El efecto del secretoma de células Lin^- activadas por Pam_3CSK_4 lo analizamos en macrófagos peritoneales y HSPC de ratones $TLR2^{-/-}$, para evitar la estimulación directa por Pam_3CSK_4. Nuestros datos mostraron que tanto Pam_3CSK_4 como *C. albicans* promovían la secreción de factores solubles que inducían la diferenciación de HSPC hacia linaje mieloide, medida como porcentaje de células $CD11b^+$. Además, el secretoma generado por las HSPC en respuesta a Pam_3CSK_4, pero no a *C. albicans*, tenía un efecto en la función de los macrófagos peritoneales disminuyendo su capacidad para producir TNF-α en respuesta a LPS.

<u>Capítulo 3.</u> Fenotipo de las APCs obtenidas tras la diferenciación de HSPC expuestas a PAMP y su impacto en la respuesta de los linfocitos T $CD4^+$

La activación de HSPC con PAMP promueve un cambio fenotípico en las APC obtenidas tras su diferenciación

Células Lin^- se cultivaron con GM-CSF y en presencia o ausencia (células control) de Pam_3CSK_4 o zimosán deplecionado durante 24 h. Tras este periodo, se llevó a cabo un cambio de medio con el objetivo de exponer las HSPC a los ligandos fúngicos únicamente de forma transitoria. A día tres se llevó a cabo un cambio de medio, de forma que el medio existente que contenía en suspensión las células no adherentes se redujo a una cuarta parte de su volumen y se añadieron las tres cuartas partes restantes de medio fresco. El sexto día de cultivo con GM-CSF se recogieron las células adherentes generadas, se procedió a su resiembra en placa y tras 24 h se estimularon con Pam_3CSK_4 o zimosán deplecionado durante 18–20 h. En este caso también se dispuso de células control sin estimular de cada una de nuestras condiciones de estudio (HSPC sin estimular, estimuladas con Pam_3CSK_4 o

estimuladas con zimosán deplecionado). Tras este período, se analizó la expresión de las moléculas de superficie y la producción de citocinas implicadas en la activación de linfocitos T $CD4^+$.

Mediante citometría de flujo analizamos la expresión de MHCII y de las moléculas coestimuladoras CD40, CD80 y CD86 en las células $CD11b^+$ $CD11c^+$, las cuales consideramos APC. Como era de esperar, la estimulación de las APCs con Pam_3CSK_4 o zimosán deplecionado aumentó la expresión de la mayoría de las moléculas de superficie analizadas en comparación con APC no estimuladas. Además, el tratamiento de las HSPC con Pam_3CSK_4 aumentó la expresión de MHCII, CD80 y CD86 en las APC estimuladas. A continuación, la producción de citocinas fue determinada en los sobrenadantes de las APC estimuladas. El tratamiento de las HSPC con Pam_3CSK_4 o zymosán deplecionado aumentó la producción de TNF-α, IL-6, IL-12 p40 e IL-2 en todas las condiciones, a excepción de las APC estimuladas con Pam_3CSK_4 producidas a partir de HSPC activadas con Pam_3CSK_4, que produjeron una menor cantidad de IL-6.

Para investigar las respuestas funcionales de las APC a microorganismos completos utilizamos levaduras inactivadas de una cepa no virulenta (PCA2) y una cepa virulenta (ATCC 26555) de *C. albicans* como estímulo. La expresión de MHCII, CD40, CD80 y CD86 en las células $CD11b^+$ $CD11c^+$ aumentó tras la estimulación con ambas cepas de *C. albicans,* y de acuerdo con nuestros resultados anteriores, la estimulación de HSPC con Pam_3CSK_4 aumentó la expresión de estas proteínas en las APC obtenidas tras su diferenciación. Por el contrario, la estimulación de HSPC con zimosán deplecionado no alteró la expresión de estas moléculas de superficie en las APC en respuesta a PCA2, pero en respuesta a ATCC26555 provocó una disminución de su expresión. La estimulación de HSPC con Pam_3CSK_4 también aumentó la producción de citocinas IL-6 e IL-2 por parte de las APC en respuesta tanto a la cepa virulenta como a la cepa no virulenta de *C. albicans*, pero no mostró ningún efecto en la producción de TNF-α. Por otra parte, la estimulación de HSPC con zimosán deplecionado aumentó la producción de IL-6 por parte de las APC estimuladas con ambas cepas, TNF-α e IL-12 p40 en respuesta a la cepa virulenta, pero no tuvo ningún efecto en la producción de IL-2.

Las APC obtenidas a partir de HSPC estimuladas con PAMP inducen una mayor polarización de linfocitos T CD4⁺ hacia Th17 y/o Th1

A continuación, examinamos si los cambios observados en el fenotipo de las APC podían alterar la proliferación y activación de los linfocitos T $CD4^+$. Para ello, se utilizaron dos aproximaciones dependiendo del estímulo que recibían las APC maduras: (i) si las APC eran estimuladas con Pam_3CSK_4 o zimosán deplecionado, se cocultivaban con linfocitos T $CD4^+$ de ratones transgénicos que expresan un TCR de especificidad conocida frente a un péptido de ovoalbúmina (cepa OT-II). Previamente, las APC habían estado expuestas a dicho péptido en concreto del antígeno ovoalbúmina para que tuviera lugar la presentación antigénica a los linfocitos T. O bien, (ii) si las APC eran estimuladas con levaduras de *C. albicans*, se cocultivaban con linfocitos T $CD4^+$ de ratones "silvestres". En estas condiciones, las células de *C. albicans* actuaban como una fuente de múltiples antígenos, así como estímulo de activación de las APC. Tras tres o cuatro días de cocultivo, cuantificamos la proliferación de los linfocitos T $CD4^+$ mediante la determinación de la dilución del éster de succinimidil-carboxifluoresceína (CFSE, "Carboxyfluorescein succidimidyl ester"). Además, mediante ELISA cuantificamos la producción de IL-17A e IFN-γ por parte de los linfocitos T $CD4^+$ en los sobrenadantes libres de células de los cocultivos.

Si las APC se estimulaban con PAMP, el tratamiento previo de las HSPC con Pam_3CSK_4 o zimosán deplecionado no inducía cambios en la proliferación o en el número de linfocitos T $CD4^+$. Sin embargo, la producción de IFN-γ aumentó con la activación por ambos ligandos, mientras que solo la estimulación de HSPCs con zimosán deplecionado aumentaba la producción de IL-17A por parte de los linfocitos T $CD4^+$. Por otra parte, si las APC se estimulaban con levaduras inactivadas de *C. albicans*, tampoco se observaban cambios importantes en la proliferación de los linfocitos T $CD4^+$ tras la activación de HSPC con los citados ligandos microbianos, pero sí en la producción de citocinas. El tratamiento de HSPC con Pam_3CSK_4 o zimosán deplecionado aumentó la producción de IFN-γ cuando las APC eran estimuladas con una cepa virulenta o una cepa no virulenta de *C. albicans*, mientras que en la IL-17A únicamente se observó dicho aumento cuando las APC eran estimuladas con la cepa virulenta de *C. albicans*.

DISCUSIÓN

La memoria de la inmunidad innata ha suscitado un gran interés en la comunidad científica desde que se descubrió que ciertos modelos de vacunación conferían una protección no específica de patógeno debido a un aumento en la activación de los macrófagos (Bistoni *et al.*, 1986; van 't Wout *et al.*, 1992). Además, en ese momento también se demostró el papel de los macrófagos en la inducción de la tolerancia a la endotoxina, proceso mediante el cual la exposición a LPS (también denominado endotoxina) u otros ligandos de los TLR protege frente a posteriores infecciones al disminuir la producción de citocinas inflamatorias y prevenir así el daño celular (Cavaillon y Adib-Conquy, 2006; Medvedev *et al.*, 2006). Un importante aspecto a tener en cuenta, referente a este fenómeno de memoria, es la vida media de las células de la inmunidad innata, particularmente de los monocitos y macrófagos. En los humanos, los monocitos entrenados se pueden detectar en circulación hasta tres meses tras la vacunación con Bacillus Calmette-Guérin (BCG) (Kleinnijenhuis *et al.*, 2014; Kleinnijenhuis *et al.*, 2012). Esta observación sugiere que la reprogramación fenotípica observada también tiene lugar a nivel de los progenitores hematopoyéticos que generan las células mieloides, lo cual podría explicar la persistencia de la memoria de la inmunidad innata en el tiempo. De hecho, los efectos de la estimulación de HSPC con ligandos microbianos se pueden transferir a las células maduras generadas a partir de estos progenitores, como demuestra un artículo publicado por nuestro grupo en el que HSPC expuestas a Pam_3CSK_4 generan macrófagos que producen menor cantidad de citocinas inflamatorias (Yáñez *et al.*, 2013b).

En este contexto, el objetivo principal de esta tesis ha sido estudiar si la estimulación de HSPCs con otros ligandos de PRR o con el hongo *C. albicans* podía alterar el fenotipo funcional de las células mieloides derivadas de estos progenitores mediante diferenciación *in vitro*. Nuestros resultados demuestran que la estimulación *in vitro* mediante ligandos solubles de TLR2 y TLR4 da lugar a macrófagos con menos capacidad para producir citocinas inflamatorias (fenotipo tolerizado), mientras que los macrófagos que se generan a partir de HSPC estimuladas con levaduras inactivadas de *C. albicans* producen más cantidad de citocinas y tienen mayor actividad antifúngica (fenotipo entrenado). Así pues, estos resultados indican que el concepto de memoria de la inmunidad innata podría aplicarse también a las HSPC. Apoyando esta hipótesis, recientemente se ha demostrado que la vacunación intravenosa con BCG (que es una cepa de *Mycobacterium bovis*) en un modelo murino educa a células madre hematopoyéticas para generar macrófagos y monocitos

entrenados que protegen frente a la tuberculosis. Este efecto está mediado por cambios epigenéticos que reprograman a los macrófagos generados a partir de las HSPCs para que respondan de forma más eficiente a la infección por *Mycobacterium tuberculosis* (Kaufmann *et al.*, 2018).

Además, hemos comprobado que la capacidad de los macrófagos para producir citocinas depende en gran medida de cómo las HSPC a partir de las cuales se generan reciben e integran múltiples señales de su entorno (PAMP o factores de crecimiento como M-CSF o GM-CSF), así como del orden temporal en el que son activadas por los distintos estímulos durante el proceso de diferenciación. Utilizando un modelo de diferenciación *ex vivo* también hemos comprobado que una exposición breve al agonista de TLR2 *in vivo* provoca que los macrófagos generados *ex vivo* a partir de las HSPC estimuladas presenten también un fenotipo tolerizado. Así pues, en nuestro modelo *ex vivo* se reproducen los resultados obtenidos previamente con el modelo *in vitro* de activación de HSPC. Sin embargo, si sometemos a los ratones a una estimulación con varias dosis de Pam_3CSK_4, los macrófagos que se obtienen producen más cantidad de citocinas inflamatorias. Por tanto, podemos concluir que el fenotipo tolerizado o entrenado de los macrófagos generados *ex vivo*, en cuanto a la producción de citocinas, depende de la dosis y de la pauta de las señales que reciben las HSPC *in vivo*.

La relación entre el fenotipo de los macrófagos generados *ex vivo* y el papel que puedan tener durante una infección no es obvia, por lo que predecir la influencia de la exposición a Pam_3CSK_4 en la susceptibilidad a la infección resulta complicado. Nosotros hipotetizamos que el aumento de HSPC y la importante acumulación de células mieloides maduras en el bazo de ratones tratados con varias dosis de Pam_3CSK_4 podrían contribuir a la protección frente a las candidiasis, en parte por la generación de nuevas células efectoras directamente en el sitio de infección. Los resultados obtenidos indican que, en nuestro modelo, el tratamiento con Pam_3CSK_4 protege a los ratones de la infección por *C. albicans* y dicho efecto protector se debe, al menos en parte, a las HSPC. En este contexto, Granick y colaboradores (2013) describen que la proliferación de HSPC en heridas cutáneas en respuesta a *Staphylococcus aureus* está mediada por TLR2 y contribuye a la producción de neutrófilos y a la eliminación de la infección local, dándole un papel a la señalización por TLR2 en la hematopoyesis extramedular. Otros autores han descrito también que una infección sistémica en ratón con *Escherichia coli* induce la movilización de HSPC al bazo, las cuales generan monocitos y neutrófilos que contribuyen a limitar una segunda infección (Burberry *et al.*, 2014).

La inmunodepleción de las HSPC en los ratones tratados con Pam_3CSK_4 podría provocar alteraciones en la cantidad de células maduras, que explicarían en parte la eliminación de la protección en estos ratones. Sin embargo, hemos comprobado que en el momento de la infección (48 h tras la inyección del anticuerpo para deplecionar las HSPC) tanto los ratones deplecionados de células madre como sus respectivos controles presentaban la misma cantidad de células mieloides en el bazo. Aun así, no podemos descartar que las células mieloides maduras tengan un papel en la protección frente a la infección. Apoyando esta hipótesis, Wang y colaboradores (2002) han demostrado que la tolerancia inducida por una única dosis de un ligando de TLR2 en ratones reduce su susceptibilidad a la infección por *S. aureus* y *Salmonella typhimurium*. Además, sugieren que la protección en estos ratones viene mediada por un aumento del reconocimiento y la capacidad microbicida de neutrófilos y macrófagos.

Numerosos estudios recientes apoyan la hipótesis de que las HSPC tienen un papel relevante en la lucha frente a la infección, aunque los mecanismos que subyacen al proceso de detección y respuesta frente a microorganismos patógenos no se conocen con exactitud (Boettcher y Manz, 2017; Zhao y Baltimore, 2015). Zhao y coaboradores (2014) describen que las HSPC son capaces de responder a la estimulación por los ligandos solubles de TLR2 y TLR4 produciendo una amplia variedad de citocinas. En este sentido, nuestros resultados demuestran que las HSPC secretan mediadores en respuesta a Pam_3CSK_4 y a *C. albicans*, que son capaces de inducir la diferenciación de HSPC de forma paracrina y alteran la producción de citocinas por parte de macrófagos peritoneales residentes. Entre estos mediadores, destaca la presencia de quimiocinas que podrían facilitar el reclutamiento de las células de la inmunidad innata a los sitios de infección, proceso crucial para el control local de las infecciones fúngicas.

Además de la reprogramación de monocitos/macrófagos y de los progenitores hematopoyéticos que los generan, la protección conferida por la memoria de la inmunidad innata también podría ser debida a una modificación en el potencial de las células mieloides para presentar antígenos y activar la respuesta de los linfocitos T $CD4^+$. Algunos estudios previos avalan esta hipótesis, describiendo que las APC diferenciadas a HSPC de la médula ósea de ratones expuestos a una glicoproteína presente en nematodos producen una respuesta antiinflamatoria respecto a macrófagos control (Goodridge *et al*., 2004). Para contrastar esta hipótesis, tras su estimulación con Pam_3CSK_4 o zimosán deplecionado, las HSPCs fueron cultivadas en presencia de GM-CSF para obtener células con una elevada capacidad de presentación antigénica (generalmente denominadas células dendríticas). De la misma forma que en la diferenciación hacia macrófago, el fenotipo de las células

dendríticas generadas se ve alterado tras la estimulación de HSPC con PAMP. La estimulación de HSPC con Pam_3CSK_4 genera APC que aumentan las tres señales que estas células envían a los linfocitos T $CD4^+$ para inducir su activación: la expresión de MHCII, la expresión de moléculas coestimuladoras (CD80, CD86, CD40) y la producción de citocinas. Además, estos cambios se traducen en un aumento en la polarización de los linfocitos T $CD4^+$ hacia Th1 y Th17. Sin embargo, futuros experimentos serán necesarios para definir mejor (i) los factores que contribuyen a la polarización hacia Th1 y Th17, (ii) el efecto de este cambio fenotípico de las células dendríticas sobre su capacidad para activar en los linfocitos T $CD8^+$, y (iii) el posible papel protector frente a la infección de las APC generadas a partir de HSPC estimuladas con PAMP.

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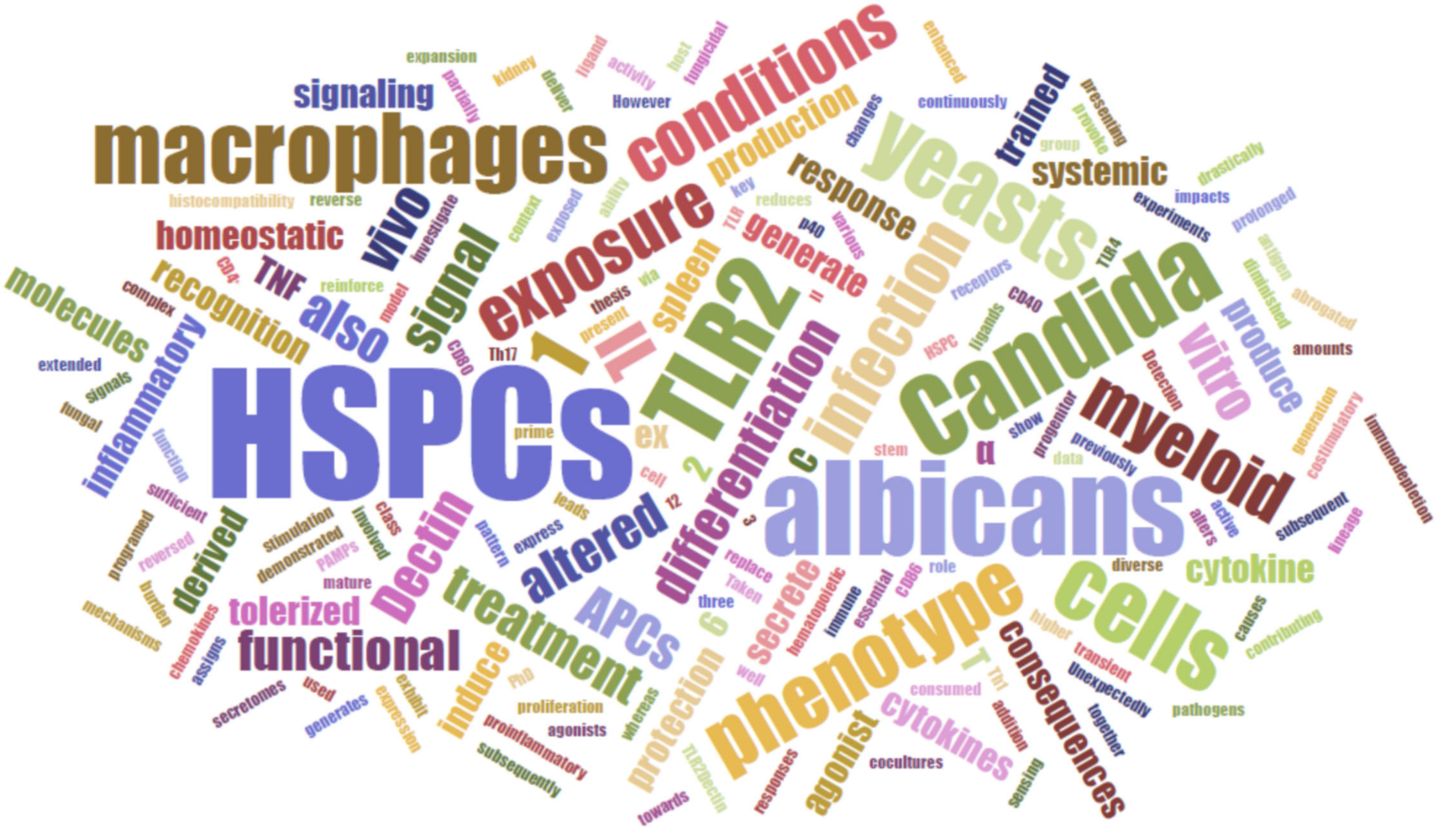

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