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DAMPs and PAMPs have distinct roles in neutrophil recruitment during cutaneous microbial infections

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Alla mia famiglia

lontana,

ma sempre al mio fianco.

Table of contents

Chapter 1. General introduction 7

1.1 The skin barrier: a first layer of defence against infections 8

1.2 Innate Immunity 11

1.2.1 Granulocytes 14

1.2.1.1 Neutrophils 14

1.2.2 Monocytes 21

1.3 Pattern Recognition Receptors (PRRs) 23

1.3.1 Toll-like receptors (TLRs) 25

1.3.1.1 The MyD88-dependent signalling pathway 29

1.3.2 C-type lectin receptors (CLRs) 32

1.4 Damage Associated Molecular Patterns (DAMPs) 35

1.4.1 Alarmins 37

1.5 IL-1 cytokines and receptors family 40

1.5.1 Interleukin (IL)-1α and interleukin (IL)-1β 44

1.6 *Candida albicans* 49

1.7 *Staphylococcus aureus* 54

1.8 *Pseudomonas aeruginosa* 56

Scope of the thesis 58

References to chapter 1 60

Chapter 2. DAMPs and PAMPs have distinct roles in neutrophil recruitment during cutaneous microbial infections 78

2.1 The MyD88 deficiency impaired neutrophil recruitment after *Candida albicans* skin infection 79

2.2 MyD88 is involved in neutrophil recruitment in bacterial skin infections, through a PRR-independent mechanisms . 82

2.3 IL-1α and IL-1β are both involved in neutrophil recruitment after *Candida albicans* skin infection 84

2.4 IL1R1 KO mice are not able to recruit neutrophils after different microbial skin infections..... 87

2.5 DAMPs and PAMPs have distinct roles in neutrophil recruitment..... 90

Materials and methods 95

References to chapter 2 100

Chapter 3. Final considerations.....102

3.1 Summary..... 103

3.2 Conclusions and future perspectives..... 106

Chapter 1

General Introduction

1.1 The skin barrier: a first layer of defence against infections

Living microorganisms, from plantae and bacteria, to humans, developed evolutionary advantage against infections. Human beings have two different ways to protect the organisms form external injuries. Physical barriers, skin and mucosa, are the first line of defence against pathogens and provides a barrier against external environment, preventing the organisms from entry the body.

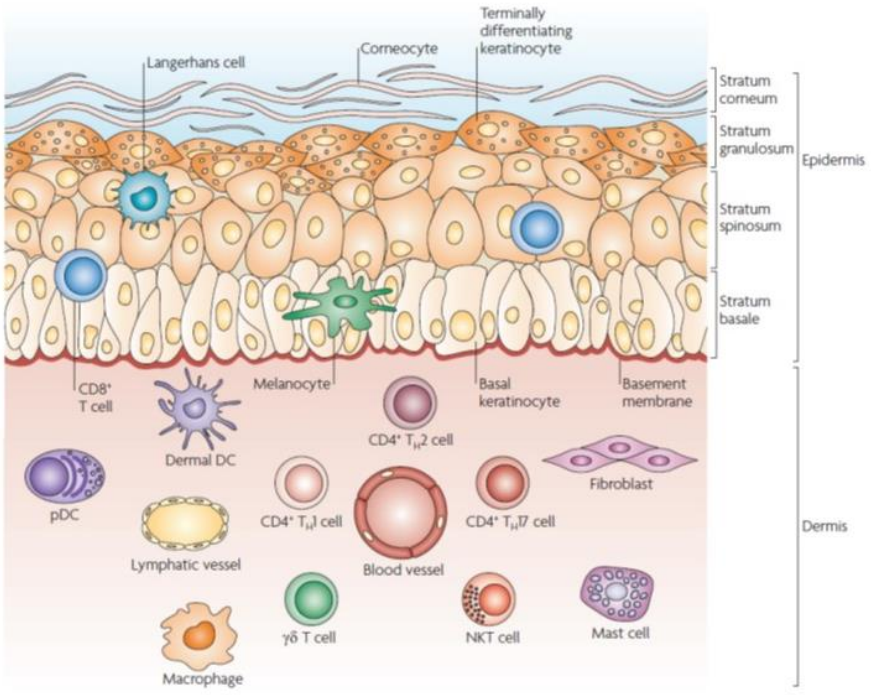

Figure 1 | Skin anatomy and cellular effectors.

Healthy human skin is characterized by a very complex architecture, which reflects the importance of its functions as a vital protective barrier. The epidermis contains the stratum basale, the stratum spinosum, the stratum granulosum and the outermost layer, the stratum corneum. Several specialized cells reside in the

epidermis, such as melanocytes and Langherans cells. However, $CD8^+$ T cells can be found in the two inner layers of epidermis, stratum spinosum and stratum basale, rarely. The dermis is composed of collagen, elastic tissue and reticular fibres and contains specialized cells, such as dendritic cells (DCs), T cells, and other cells, such as macrophages, mast cells and fibroblasts.

Skin is the outermost layer of the body and it provides a mechanical as well as immunologic barrier against pathogenic microorganisms. Healthy human skin is composed by three layers: epidermis, dermis and hypodermis (Figure 1). The epidermis on the top is a thin layer of stratified squamous epithelium, composed of four strata of keratinocytes, the most abundant cell type in this compartment, in different stages of differentiation: the stratum spinosum, an undifferentiated basal layer, the differentiated stratum granulosum and the stratum corneum with dead corneocytes. During their maturation process, keratinocytes move from the basal to the uppermost layer and orchestrate immune responses if microbes and their molecules penetrate the stratum corneum upon mechanical or pathological barrier defects. The intermediate layer, the dermis, consists of extracellular matrix, such as collagen, made by fibroblasts, and several structures such as blood vessels, lymphatics, sweat glands and nerves. The hypodermis is the innermost layer and is rich of fat and connective tissue and contains blood and lymphatic vessels (Chambers E. et al., 2019). Keratinocytes are most abundant in epidermis layer and are essential for the mainteinance of mechanical and barrier function of the skin (Figure 2). They contribute to the pathophysiology of infectious and inflammatory processes, expressing several pattern recognition receptor (PRRs) which contribute to the initial sensing of microorganism. Several Toll-like receptors (TLRs) are expressed in keratinocytes and are located on

the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) as well as in endosomes (TLR3 and TLR9), functioning as receptors of the pathogen associated molecular patterns (PAMPs), whose recognition triggered the production of inflammatory cytokines and the initiation of the immune response (Janeway CA, 1989; Quaresma J., 2019). Keratinocytes are able to produce several antimicrobial peptides (AMPs), called defensins and cathelicidins, which are highly conserved eukatioric cell defense mechanism. Expression of AMPs on the injured epithelium is a key antimicrobial event against a broad range of pathogens, including fungi, bacteria and enveloped viruses. Keratinocytes have also the ability to sense wound damage, producing inflammatory cytokines like interleukin-1β (IL-1β), IL-6, IL-10, IL-17, IL18, IL-22 and tumor necrosis factor α (TNF-α) (Albanesi C., 2005; Zepter K. et al., 1997). The expression of CXCL1 and CXCL8 by keratinocytes is important to attract neutrophils to the epidermis following skin reactions to inflammation (Quaresma J., 2019).

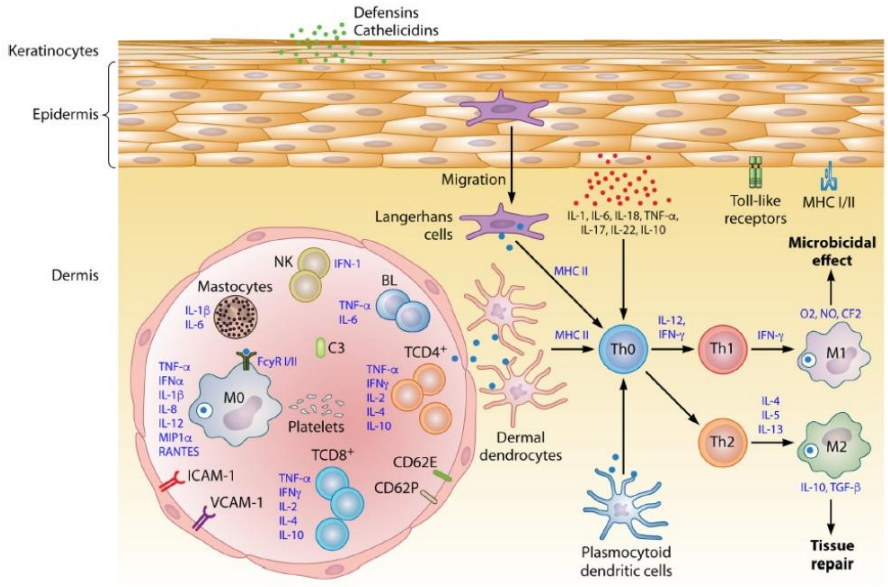

Figure 2 | Keratinocytes in epidermis layer are essential for the mainteinance of mechanical and barrier function of the skin.

Skin responses to infections are complex and involves several cellular and humoral factors. Altough Langherans cells are the main type of immune cells in the epidermis, keratinocytes are essential for the pathophysiology of infectious and inflammatory processes, expressing several pattern recognition receptor (PRRs) which contribute to the initial sensing of microorganism. Keratinocytes release antimicrobial peptides and partecipate in innate or adaptive immunity by releasing cytokines, like IL-1, that control the cascade of proteins in the immune response to microorganisms.

1.2 Innate Immunity

The mammalian immune system consists of two arms: the evolutionary ancient and fast innate immune system, and the highly specialized, but slower, adaptive immune system (Figure 3). The former is induced early, when an external injury occurred, and is non-specific, whereas the latter is triggered when the pathogen is able to overcome this initial control; its responses can specifically recognize pathogens and build

memory able to protect organism against reinfection. However, in the last decade, it has been proposed the existance of an “innate memory”, called trained immunity (Netea MG, 2011). This type of memory occurs in organism lacking adaptive immune responses such as invertebrates, plants and mamals lacking functional T and B cells. However, trained immunity is supposed to be maintained only for weeks or months, in contrast to the years-protection conferred by the adaptive immunity. The innate and the adaptive immune system use two different strategies to detect invading microbes. The innate system is able to recognize common molecular structures of infectious microbes, called pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), while the adaptive immune system uses clonally expressed, highly specific receptors (Farber DL et al., 2016). The innate immune system is able to directly eliminate pathogens through the prodution of pro-inflammatory cytokines and phagocytosis. Moreover, innate immunity is also required for the activation of the pathogen-specific adaptive immunity.

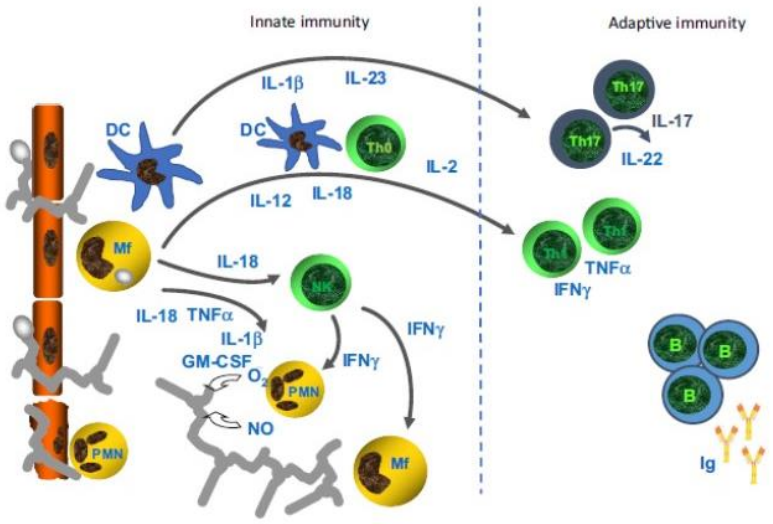

Figure 3 | Innate immunity and adaptive immunity.

Innate immunity is the first line of defence against pathogens and involves several cell types, such as macrophages, monocytes, NK-cells and humoral factors (the complement system). In the cases in which innate immunity is not sufficient to fight and eliminate the infection, pathogens are processed by antigen presenting cells, mediating the activation of T- and B-lymphocytes. These events involve the adaptive immune system, that is characterized by clonal expansion and activation of effector mechanisms, including the release of immunoglobulins (Ig) and cytokines.

The innate response includes soluble factors, such as complement proteins, and other immune activating proteins/peptides released as a result of degranulation, cell injury or death, called “alarmins”, cellular effectors, including granulocytes, mast cells, macrophages, dendritic cells (DCs) and innate lymphoid cells (ILCs) (Oppenheim JJ and Yang D., 2005). Among these types, granulocytes are the first line of defence, due to their ability to directly phagocyte pathogens through phagocytosis, or to release extracellular mediators. Macrophages and

dendritic cells have phagocytic activity too. Moreover, they serve as bridge between innate and adaptive immunity. Lastly, innate lymphoid cells (ILCs) are the most recently discovered. In 2008 and 2009, several groups reported the identification in mammals of new type of non-T, non-B lymphocytes, that were called innate lymphoid cells. Currently, ILCs include different subsets of cells: ILC1, ILC2, ILC3, NK and lymphoid tissue inducer (LTi) cells (Mebius RE, 2015).

1.2.1 Granulocytes

Granulopoiesis is a process that occurs in the bone marrow that give rise to the production of neutrophils (which represent the 70% of all leukocytes), eosinophils and basophils. These type of white blood cells, named polymorfonuclear lymphocytes (PMNs), are characterized by a multi-lobed nucleus and cytoplasmic granules within the cell. This observation was done by Paul Ehrlich between 1879 and 1880. He used acidic and basic dyes to recognized eosinophil and basophil granules, respectively and neutral dyes to identified granules in neutrophils (Kay AB, 2016).

1.2.1.1 Neutrophils

Neutrophils represent the predominant myeloid leukocytes subset in most mammals, and the most abundant cell type in human blood, approximately 10^{11} cell are produced in the bone marrow each day (Dancey JT et al., 1976). They originate from hematopoietic stem cells

(HSCs) in the bone marrow and differentiate into multipotent progenitor (MPP) cells that cannot self-renew themselves. Next, they switch into lymphoid-primed multipotent progenitors (LMPPs), which differentiate in granulocyte-monocyte progenitors (GMPs). This cells need granulocyte colony-stimulating factor (G-CSF) to follow next steps of maturation, from promyelocyte to finally differentiate into mature neutrophils (Von Vietinhoff and Ley, 2008). This last step of maturation is accompanied by the formation of granules, containing antimicrobial enzymes useful to eliminate pathogens, and secretory vescicles, containing proteins essential for neutrophils functions, into mature neutrophils (Figure 4 and Figure 5).

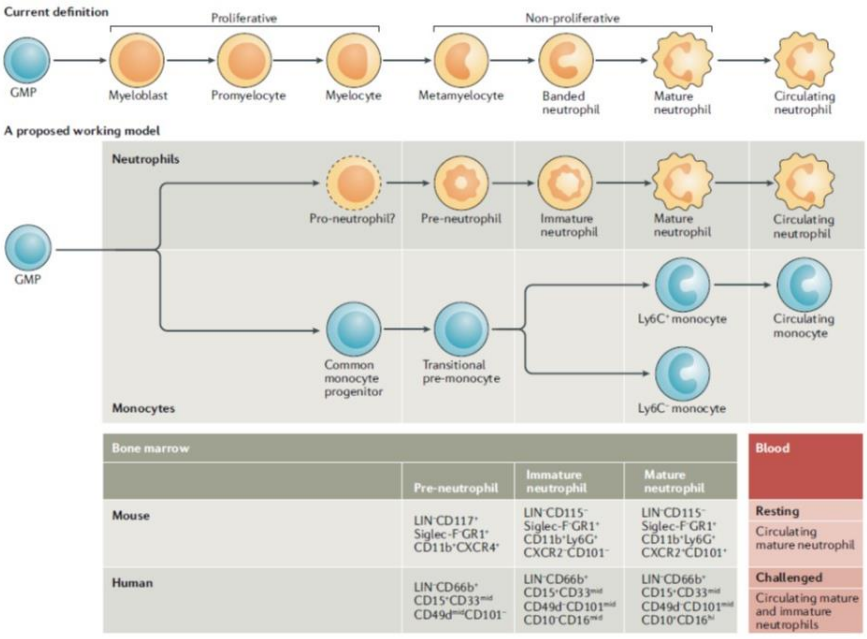

Figure 4 | The neutrophil differentiation pathway.

Neutrophils derive from a common progenitor, the granulocyte-monocyte progenitor (GMP). During the differentiation, there are two phases, the proliferative phase, in which GMP differentiate into myeloblasts, promyelocytes and myelocytes; and the non-proliferative phase, where myelocytes differentiate in metamyelocytes, banded neutrophils and finally mature neutrophils. A recent have proposed this model in which bone marrow neutrophils can be divided into three groups: a committed proliferative pre-neutrophil subset that sequentially differentiates into non-proliferative immature neutrophils and mature neutrophils. Comparing this pathway to the developmental hierarchy of monocytes, pre-neutrophils have the functional attributes of transitional pre-monocytes, suggesting that there could be a "common neutrophil progenitor", that is equivalent to the common monocyte progenitor. In the steady state, only mature neutrophils are detected in the circulation. In response to inflammatory stimuli, immature neutrophils are also released into the circulation work (Lai Guan Ng, Ostuni R. and Hidalgo A., 2019).

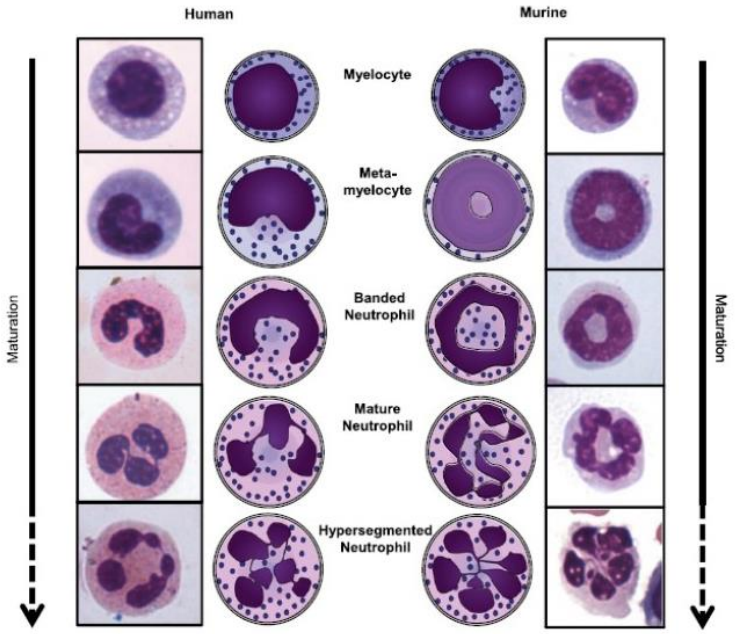

Figure 5 | Human and murine neutrophils morphology during the development.

At the end of the proliferative differentiation stage, myelocytes mature into metamyelocytes, banded neutrophils, and finally into mature neutrophils. This stage of differentiation is characterized by the formation of granules (segmentation). Moreover, neutrophils may become hypersegmented, with more than 4 nuclear lobes (human neutrophils) or a cloverleaf shape (mouse neutrophils).

For many years, neutrophils have been considered as short-lived cells, with an half-life of about 1 day. However, a recent study in human considered a lifespan of up to 5.4 days, providing the possibility to consider neutrophils as cells involved not only in the first phases of inflammation, but also able to respond to specific stimuli after infections (Pillay et al., 2010; Lai Guan NG et al., 2019).

In homeostatic conditions, neutrophils migrate from the bone marrow into the blood where they circulate until they leave into tissues. At the end of their lifespan, they are cleared by macrophages through phagocitosys (Bratton and Henson, 2011). The release of neutrophils is tighly controlled since only 1 or 2% of all neutrophils in the body are found in the blood under normal homeostatic condition. Mature neutrophils are kept in the bone marrow through the balanced activity of two chemokine receptors: CXCR2 and CXCR4 (Rosales C, 2018). Bone marrow stromal cells produce CXCL12 and keep CXCR4-expressing neutrophils in the bone marrow. G-CSF interfere with the CXCR4-CXCL12 interaction, favoring neutrophil exit from the bone marrow (Summers C. et al., 2010). Meanwhile, CXCL1, CXCL2, CXCL5 and CXCL8 (in human), ligands for CXCR2, promote neutrophils exit from the bone marrow into the blood.

Neutrophils released from the bone marrow highly express CD62L on their surface, but they gradually lose it during the day, increasing C-X-C chemokine receptor type 4 (CXCR4) prior to their exit from blood (Casanova-Acebes et al. 2013). The mobilization of neutrophils from the blood to inflamed tissues in known as leukocyte adhesion cascade (Ley K et al., 2007). Phenotypical changes of neutrophils that occurs during the day is known as “neutrophil ageing” is extrinsecally regulated by the gut microbiota and is considered beneficial to control vascular inflammation (Zhang D. et al., 2015) (Figure 6). The ability of neutrophils to fight pathogens and also to be involved in severe damage to tissues, suggest the existence of a thiny balance of their activity. Recently, it has been reported that during the day, phenotypical changes of circulating neutrophils occurs in order to coordinate immune defence

and vascular protection (Casanova-Acebes et al. 2013). Notably, the diurnal ageing of neutrophils is regulated by the circadian-related protein Bmal1 in coordination with Toll-like receptor and CXCR2 signalling, which favors aging, and CXCR4, which antagonizes it. According to this model, fresh neutrophils released from the bone marrow into the blood are most prone to enter tissues in case of inflammation, and aged neutrophils, present during daytime, are most prone to damage the vasculature in a mouse model of sickle cell disease (Zhang D. et al., 2015). However, they gradually lose their ability to enter inflammatory sites and left the circulation becoming aged neutrophils, ready to be cleared at the end of their resting stage. This "neutrophil timer" could be useful to anticipate infections and, at the same time, it turn off when the risk of infection is low to prevent damage to the vasculature. However, how neutrophil ageing is regulated by trascriptional properties of Bmal1 or by the circadiam rhythm remains to be defined (Adrover JM et al., 2019).

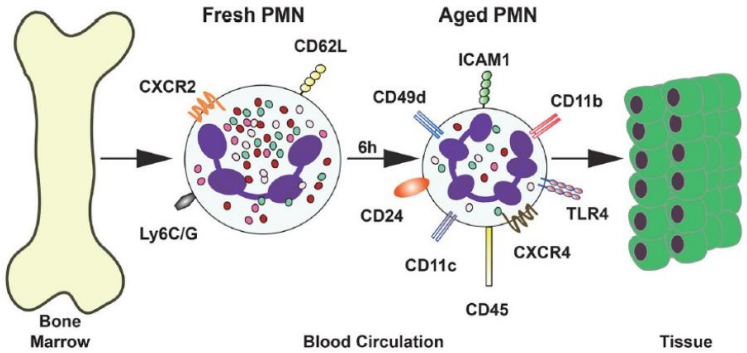

Figure 6 | Fresh and aged neutrophils.

Neutrophils released from the bone marrow, highly express CD62L on their surface, and are known as "fresh" neutrophils. After about 6 hours in the circulation, neutrophils gradually lose CD62L, changing the expression of surface molecules. The increase expression of CXCR4 is correlated with their aging. These "aged" neutrophils are cleared from the blood by migration into tissues or by returning to the bone marrow.

Aged-neutrophils have enhanced capacity to release neutrophil extracellulatr traps (NETs), structures composed of nuclear component (DNA and histones) and granule proteins, interconnected in a scaffold of decondensed chromatin, after the activation of a cell death program called NETosis (Brinkmann V. et al., 2004). After the permeabilization of the neutrophil's plasma membrane, NETs are released in the extracellular space, neutralizing and killing bacteria, fungi like *Candida albicans*, viruses and parasites. Neutrophil elastase, myeloperoxidase and other antimicrobial peptides like defensins are released during NETosis. Moreover, NETosis is influenced by pathogen size, when a microbe is too large to be phagocytosed, such as some parasites and fungal hyphae, NETosis is the alternative pathway that occurred to eliminate it (Branzk N. et al., 2014). However, if not regulated, NETs can cause several immune-related diseases (Wong SL et al., 2015; Garmia-Romo GS et al. 2011; Villanueva E. et al., 2011). Neutrophils express several PRRs of different families, such as almost all TLRs, except for TLR3, and various CLRs and NLRs (Thomas CJ et al., 2013). Neutrophils are the first cell type recruited to sites of inflammation and they can interact directly, or through cytokines and chemokines, with other kind of immune cells to modulate both innate and adaptive immune responses. Therefore, neutrophils are the most important effector cells in fungal killing. Adaptive immunity against *Candida* infection is mediated by interleukin-17 (IL-17)-producing

lymphocytes, whereas cells of the innate immunity are important for recognize and contain local infections as well as for preventing systemic candidiasis (Puel A. et al., 2011)

1.2.2 Monocytes

Monocytes are innate immune cells originated in the bone marrow from two distinct precursor: granulocyte-monocyte progenitor (GMP) or a monocyte-dendritic cell progenitor (MDP) (Yanez A. et al., 2017) (Figure 7).

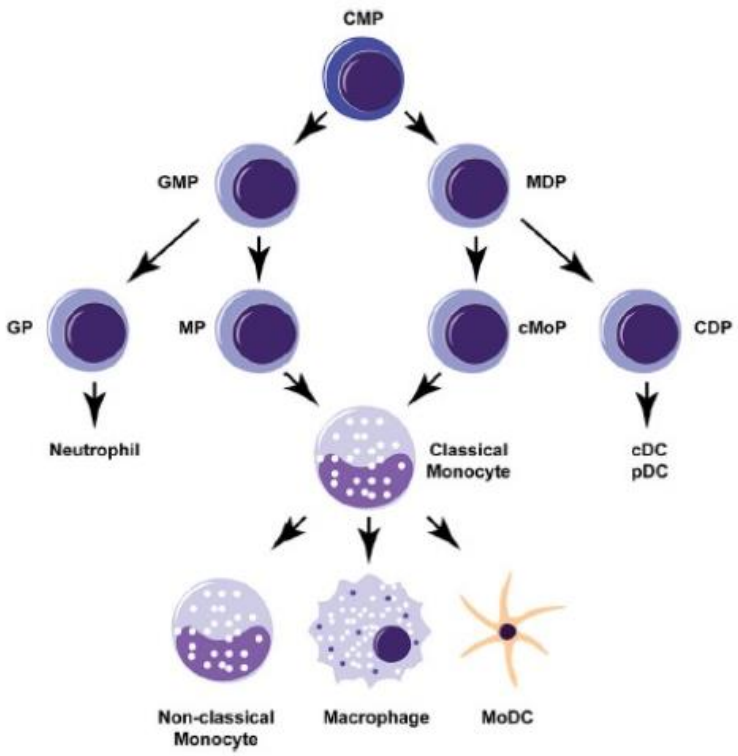

Figure 7 | Monocyte development and differentiation.

Monocytes differentiate from either a granulocyte-monocyte progenitor (GMP) or a monocyte-dendritic cell progenitor (MDP). Classical "inflammatory" monocytes give rise to non-classical monocytes or further differentiate into macrophages or monocyte-derived dendritic cells (MoDCs).

In mice, two main types of monocytes can derive from these precursors. The classical inflammatory monocytes, $CCR2^{+}Ly6C^{high}$ and the non-classical monocytes $CCR2^{low}Ly6C^{low}$, developed from classical monocytes, that have a role during homeostatic conditions in the surveillance of the circulation (Yona S. et al., 2013; Auffray C. et al., 2007). During inflammation, classical monocytes are mobilized from bone marrow in response to chemokines like CCL2 and CCL7, which bind the CCR2 receptor, and they can further differentiate in monocyte derived dendritic cells (MoDCs) and macrophages. Notably, macrophages differentiated from monocytes in the bone marrow are different from tissue resident macrophages derived from yolk sac progenitor cells or monocytes from the fetal liver (Shi and Pamer, 2011; Hoeffel and Ginhoux, 2018). Moreover, MoDC have also distinct nature from conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs), derived from a common dendritic cell progenitor (CDP). Microenvironment, signals sensed by HSPCs such as cytokines and microbial components, can influence monocyte expression and production, in homeostatic condition but also in inflammatory one. IRF8 is considered a key regulator of monocyte differentiation in both conditions. It is dispensable for monocyte lineage specification, but necessary for monocytes production in steady state (Yanez A. et al., 2015). IRF8 regulates the induction of the transcription factor Klf4, inducing the formation of enhances to guide the expression of monocytes genes. It also interacts with the transcription factor c/EBPa

to inhibit granulocytes commitment (Kurotaki D. et al., 2013; Kurotaki D. et al., 2018; Kurotaki D., et at., 2014). Interestingly, the inflammatory environment generated by monocyte-derived cytokines contributes of both innate and adaptive immune response to fungal pathogens. Monocytes express several PRRs that gave them the ability to recognize different morphological forms of fungi, like *Candida albicans,* contributing to the immune system response to fungal infections. CLRs, TLRs and NLRs can detect PAMPs like β-glucan, chitin and mannose in the fungal cell wall, triggering downstream signalling pathways to activate the innate immune response. Dectin-1, Dectin-2 and Mincle collaborate to host defence against *Candida albicans* infection, regulating monocyte production and phagocytosis of the pathogen (Thompson A. et al., 2019). Moreover, monocyte-deficient mice are more susceptible to infections with fungi like *Aspergillus fumigatus* and *Candida albicans* (Espinosa V. et al., 2014; Domingues-Andres et al., 2017). However, the specific mechanisms by which monocytes mediates the host response to fungal pathogens is still under investigation.

1.3 Pattern recognition receptors (PRRs)

The innate immune system is the first line of host defence against pathogens. Microorganisms detection occurs via germline-encoded pattern recognition receptors (PRRs), that recognize microbial structures, known as pathogen associated molecular patterns (PAMPs), essential for the maintenance of microbial structures and functions (Janeway CA, 1989). Another important feature of PRRs is that they

are expressed constitutively by the host and detect the pathogens independently of their life-cycle status. PRRs are germline encoded, nonclonal and expressed not only in macrophages and DCs, but also in nonprofessional cells, such as epithelial and endothelial cells (Takeuchi and Akira, 2010). Under physiologic conditions, PRRs are also strictly involved in the discrimination of self- versus non self- microbial component, preventing autoimmunity (Marongiu L. et al., 2019). PAMPs are ideal targets for innate immune recognition because they are produced by microbes and not by the host and are invariant between microorganisms of a given class, allowing to a limited number of germ-line encoded PRRs to recognize microbial infection.

According to their function, PRRs may be divided into endosomal PRRs, that promote the attachment, engulfment and phagocytosis of microorganisms, without releasing on intracellular signal, or intracellular PRRs, that trigger specific transduction pathway involved in innate cell activation and anti-microbial mediator production. PRRs are classified into five families: Toll like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and AIM2-like receptors (ALRs) (Table 1). In detail, TLRs and CLRs can be localized on the cell surface and in the endocytic compartments, detecting microbes extracellularly but also within endosomes. NLRs, RLRs and ALRs are in the cytoplasm and are activated only when a microbe accesses the host cytosol. Among these receptors, TLRs are the best characterized.

Family	Members	Shared domains	Receptor locations
TLR	1–10 in humans, 1–9 and 11–13 mice	LRR, TIR	Cell surface, endosomal compartments
CLR	Dectin-1, Dectin-2, … etc. (reviewed in Reference 4)	C-type lectin	Cell surface
NLR	NOD1 (NLRC1), NOD2 (NLRC2), NLRC3–5, NLRP1–9 and 11–14, NAIP1, -2, -5, -6	Nucleotide binding, LRR	Cytoplasm, plasma, and endosomal membrane associated
RLR	RIG-I, MDA5, LGP2	DExD/H helicase	Cytoplasm
ALR	AIM2, IFI16	PYRIN, HIN-200	Cytoplasm, nucleus (IFI16)

Abbreviations: AIM, absent in melanoma; ALR, AIM2-like receptor; CARD, caspase recruitment domain; CLR, C-type lectin receptor; IFI, interferon, γ-inducible; LGP, laboratory of genetics and physiology; LRR, leucine-rich repeat; MDA, melanoma differentiation gene; NAIP, NLR family, apoptosis inhibitory protein; NLR, nucleotide-binding oligomerization domain receptor; NLRC, NLR family CARD domain containing; NLRP, NLR family PYD domain containing; NOD, nucleotide-binding oligomerization domain; RIG-I, retinoic acid–inducible gene I; RLR, RIG-I-like receptor; TIR, Toll/IL-1 receptor/resistance; TLR, Toll-like receptor.

Table 1 | Pattern-recognition receptor families.

Toll like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and AIM2-like receptors (ALRs).

1.3.1 Toll-like receptors (TLRs)

The first identification of the evolutionary conserved NF-kB family transcription factors as regulators of anti-microbial responses in *Drosophila melanogaster*, led to the idea that similar mechanisms might be present also in mammals. Toll, the first member of the TLR family, was identified as an essential factor for the development of embryonic dormoventral polarity in *Drosophila* and later it was associated to the antifungal response of these flies (Hashimoto C. et al., 1988; Lemaitre B., 1996). The human homolog of Toll protein is known today as TLR4. It was identified as a receptor able to guide antigene-presenting cell responses to promote inflammation and adaptive immunity (Medzitov R., et al., 1997). Currently, 12 members of the TLR family have been identified in mammals. All the TLRs are type I integral membrane glycoproteins, characterized by an extracellular leucine-rich-repeat (LRR)-rich motif and a cytoplasmic signalling domain, homologous to that of the interleukin 1 receptor (IL-1R), the

Toll/IL-1R homology (TIR) domain. Intracellular TLRs (TLR3, -7, -8, -9, -11, -12, -13) are expressed in the endoplasmic reticulum (ER), endosomes, multivesicular bodies and lysosomes, where self-DNA is rarely present, in order to prevent autoimmunity (Marongiu L., et al., 2019). On the contrary, the plasma-membrane associated TLRs (TLR1, -2, -4, -5, -6) recognize structural components present on the external surface of microorganisms (Figure 8).

TLRs are expressed in cells that act as sentinels of the immune system such as DCs and macrophages. However, its expression is observed also in vascular endothelial cells, adipocytes, cardiac monocytes and intestinal epithelial cells.

All mammalian TLRs directly interact with their PAMP ligands through their leucine-rich-repeat-containing ectodomains and cognate microbial products. Ligand binding occurs through interactions with a dimer of TLR ectodomains that could be homodimers, for TLR3, -4, -5, -7, -8 and -9, or heterodimers for TLR1, -2 and -6. The PAMP-mediated dimerization of TLRs ectodomains is necessary for the signalling, as ectodomain dimerization results in the coordinate dimerization of the cytosolic TIR domains of these PRRs. PRRs cannot function as signalling molecules alone, but they require several signalling adaptors to engage downstream signalling pathways. The adaptor molecules the TLRs utilize are the TIR-containing adaptor protein (TIRAP) and the protein myeloid differentiation primary response 88 (MyD88), that constitute a functional adaptor set; the TIR domain–containing adaptor-inducing IFN-β (TRIF) and the TRIF-

related adaptor molecule (TRAM), that constitute an other functional adaptor sets (Brubaker SW et al., 2015).

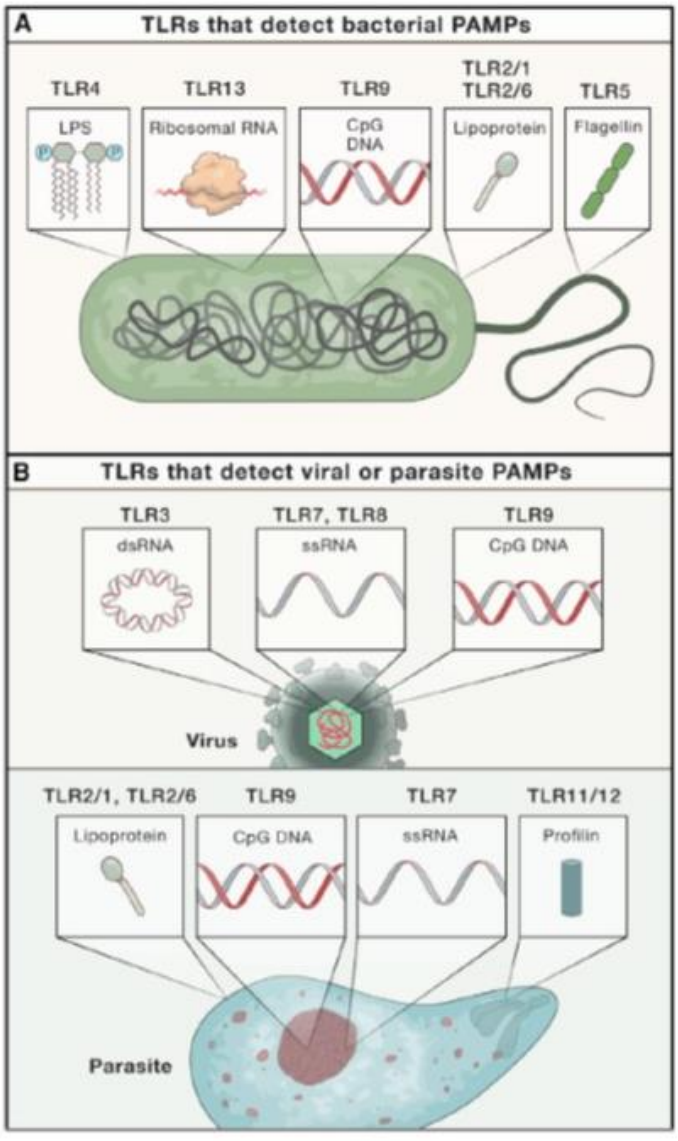

Figure 8 | Toll Like Receptors (TLRs) family members.

Twelve members of the TLRs family have been identified in mammals. TLR4, TLR13, TLR9, TLR2/1; TLR2/6, TLR5 detect bacterial PAMPs. TLR3, TLR7, TLR8, TLR9, TLR2/1, TLR2/6, TLR11/12 detect viral or parasite PAMPs.

After microbial binding, dimerized receptor TIR domains are detected by the receptor-proximal membrane proteins TIRAP and TRAM (Fitzgerald KA et al., 2001; Horng T., et al. 2001; Fitzgerald KA et al., 2003b; Yamamoto M. et al., 2003b), stimulating the assembly of a large oligomeric scaffold of cytosolic proteins known as a supramolecular organizing centres (SMOCs) (Kagan JC et al., 2014) (Figure 9). SMOCs are not present in resting cells, but they are constituted after TLR dimerization. In the context of TLRs signalling, SMOCs are the myddosome and the triffosome. Following the assembly of these two complexes, several transcription factors are activated to enhance the expression of inflammatory and IFN genes. Among them, NF-kB, AP-1, and members of the IRF family.

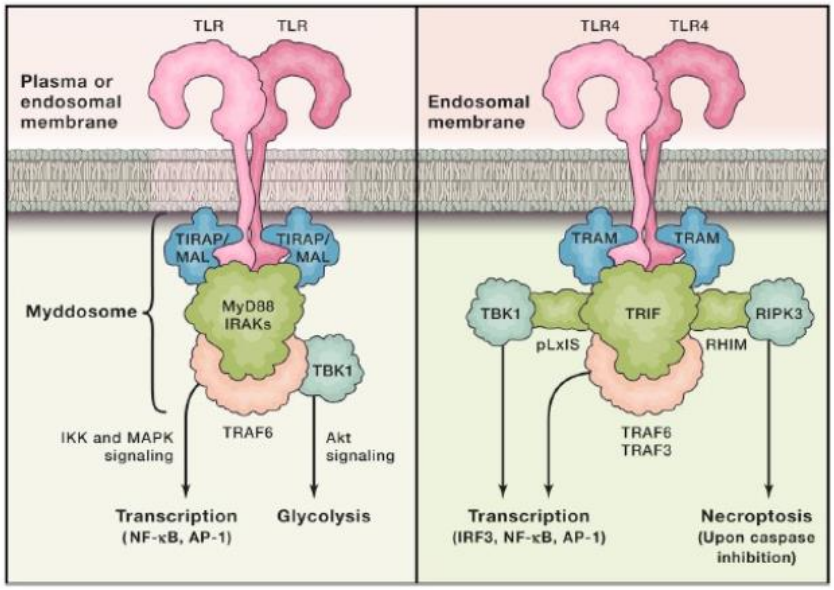

Figure 9 | TLR could signal through either Myddosome or Triffosome.

TLRs, except TLR3, could mediate their signalling through supramolecular organizing centres (SMOCs), called Myddosome, if included the protein myeloid differentiation primary response 88 (MyD88), and Triffosome, if mediated by TIR-domain-containing adaptor protein inducing interferon TRIF.

1.3.1.1 The MyD88-dependent signalling pathway

TLRs signalling is mediated by two distinct pathways depending on the usage of the adaptor molecules MyD88 and TRIF. MyD88 is essential for the downstream signalling of several TLRs, except for TLR3. MyD88 was isolated originally as a gene induced during IL-6 stimulated differentiation of M1 myeloleukaemic cells into macrophages (Lord KA et al., 1990). The encoded protein has an amino (N)-terminal death domain (DD), which is separated from its carboxy (C)-terminal TIR domain by a short linker sequence. MyD88 is

subsequently cloned as an adaptor molecule that functions to recruit IRAK to the IL-1R complex following stimulation with IL-1 complex (Muzio M. et al., 1997; Wesche H. et al., 1997; Burns K. et al., 1998). The association between MyD88 and IRAK is mediated through a DD-DD interaction. MyD88 forms homodimers interactions and exists as a dimer when recruited to the receptor complex (Dunne A. et al., 2003). Therefore, MyD88 functions as adaptor linking TLRs/IL-1Rs with downstream signalling molecules that have DDs. TLR2 and TLR4 signalling utilizes TIRAP to link TLR and MyD88. MyD88 interacts with IL-1R associated kinase (IRAK)-4 that activates other IRAK family members, IRAK-1 and IRAK-2 (Kawagoe T. et al., 2008). Then IRAKs dissociate from MyD88 and in turn recruit TNF-receptor-associated factor 6 (TRAF6), which activates the transforming growth factor β (TGF-β)-activated kinase 1 (TAK1). A complex of TGF-β - activated kinase 1 (TAK1), TAK-1 binding protein 1 (TAB1), TAB2 and TAB3 phosphorylates IkB kinase (IKK)-b and MAP kinase 6. Then, the IKK complex, composed by IKK-a, IKK-b, and NF-kB essential modulator (NEMO) phosphorylates IkBa, and NF-kB inhibitory protein (Mitchell S. et al., 2016; Wang C. et al., 2001). Phosphorylated IkB undergoes degradation by the ubiquitin-proteasome system, thereby NF-kB can translocate into the nucleus, activating the transcription of pro-inflammatory genes, including tumour necrosis factor alpha (TNFα) and IL-6. Mutations affecting the MyD88 pathway in humans are associated with recurrent pyrogenic bacterial infections in childhood. Like mice lacking MyD88, the patients with MyD88 deficiency are susceptible to invasive diseases caused by *Streptococcus pneumoniae,* such as meningitis and

septicaemia and in some cases to infection with *Staphylococcus* or *Pseudomonas* (Maglione PJ et al. 2014; Picard C. et al., 2010; Von Bernuth H. et al., 2008). Further, these patients have defective responses to IL-1β, IL-18, and IL-33, all which signal via MyD88-IRAK4 in all hematopoietic and non-hematopoietic cells.

TLR4 is the best-characterized member of TLR family. It binds lypopolysaccaride (LPS), a complex glycolipid and major constituent of the gram-negative bacterial cell wall and its binding requires a multi-receptor complex consisting of LPS-binding protein (LBP), CD14 and MD-2, which act to sequestrate LPS from bacterial membrane to promote TLR4 signalling (Gioannini TL et al., 2004; Zanoni I. et al., 2011). TLR4 signals through both the MyD88- and the MyD88-independent pathway. The MyD88-independent pathway, engaged by TLR3 and TLR4, relies on TIR-domain-containing adaptor protein inducing interferon (TRIF). This adaptor recruits TRAF3 and the protein kinase TBK1 and IKKi, which catalyse the phosphorylation of IRF3, leading to the expression of type I IFNs. TRIF also recruit TRAF6 and TAK1 to mediate late-phase of NF-kB and MAP kinases. TLR2 and TLR4 use TIRAP as an additional adaptor to recruit MyD88. TRAM acts as a bridge between TLR4 and TRIF.

The site of signalling is important to direct signalling outcomes. TLR-4-induced MyD88- dependent pathway originates from the cell surface, whereas TRIF-mediated signalling occurs from endosomes (Figure 9).

The ancient observation that Toll-deficient Drosophila are highly susceptible to fungal infection, led to the hypothesis that mammalian TLRs also be involved in antifungal immunity. Fungal PAMPs, such as mannans and glucans, are recognized by TLR2 or TLR4. In vivo infection using mutant mice suggest differential roles of TLR2 and TLR4 in fungal infection. $TLR4^{-/-}$ mice showed increased susceptibility to disseminated *Candida* infection, whereas $TLR2^{-/-}$ mice showed increased resistance (Netea MG et al., 2004).

1.3.2 C-type lectin receptors (CLRs)

The CLRs are a superfamily of proteins with one or more structurally related C-type lectin-like domains (CTLDs) (Figure 10). They were originally named for the presence of a carbohydrate-binding domain able to bind to carbohydrates in a Ca^{2+}-dependent manner through conserved residues within the CTLD. However, the CTLDs of many C-type lectins lack the components required for Ca^{2+}-dependent carbohydrate recognition and can recognize a broader repertoire of ligands including proteins, lipids and even inorganic molecules (Zalensky AN and Gready JE, 2005; Weis WL et al., 1998). Furthermore, recognition of pathogens through CLRs causes in DCs and macrophages an increase in intracellular Ca^{2+} concentration, leading to the activation of NFAT family members and release of inflammatory cytokines such as IL-2 and IL-12 (Goodridge HS et al., 2007). CLRs have been subdivided into 17 groups of receptors, which classification depends on their domain organization and phylogeny.

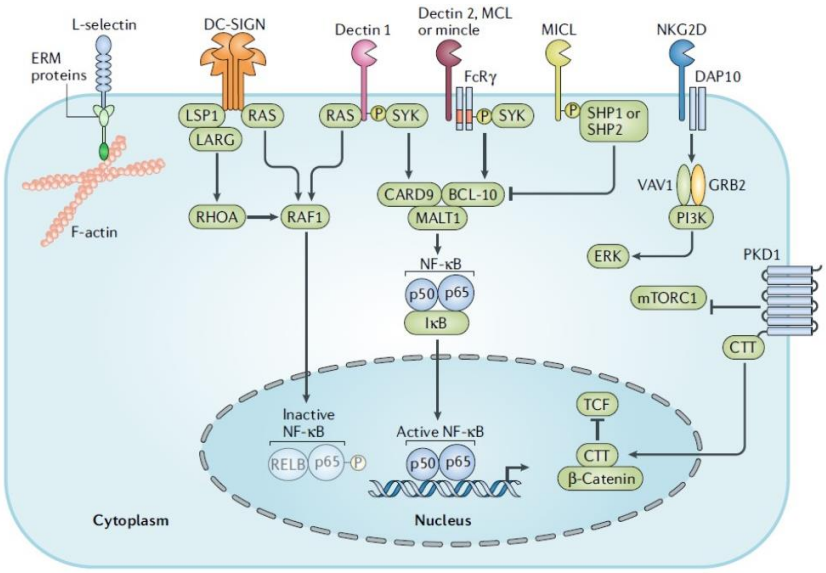

Figure 10 | The signalling pathways mediated by C-type lectin receptors (CLRs).

The best studies CLRs induce activation pathways through immunoreceptor tyrosine-based activation motifs (ITAMs), that can be an integral component of the CLR cytoplasmic tail, or can require the use of signalling adaptors, such as the Fc receptor γ-chain (FcRγ) or DAP10. In myeloid cells, these ITAMs facilitate the recruitment of SYK kinase, inducing a downstream signalling pathway involving the caspase-recruitment domain protein 9 (CARD9)-B cell lymphoma/leukaemia 10 (BCL-10)-mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) complex. These signalling pathways result primarily in the induction of nuclear factor-kB (NF-kB)-dependent pro-inflammatory responses.

In mammals, CLRs can be either soluble or membrane bound. Most of the CLRs act as opsonins, without activation of an NF-kB signalling cascade. However, other CLRs participate to a pro-inflammatory response, promoting T cell immune responses. The signalling cascade of these groups of receptors is initialized by CARD9/Bcl-10/MALT-1

proteins. Among them, Dectin-1 and Dectin-2 are the best-characterized CLRs.

The ability to recognize carbohydrate-rich cell walls of fungi is well known for CLRs. Dectin-1 is s type II transmembrane protein, expressed by DCs, macrophages, neutrophils and monocytes. Upon ligand binding, Dectin-1 promotes ligand uptake by phagocytosis and the initiation of a signalling cascade that regulates gene expression and cytokine production. The major ligands for Dectin-1 are fungal β-1,3-glucans, and both mice and human studies confirmed a crucial role for this receptor in antifungal defence (Taylor PR et al., 2007; Ferwerda B et al., 2009). Signalling of Dectin-1 activates cells via a Syk dependent pathway, leading to the formation of the CARD9-BCL10-MALT1 trimer and following activation of NF-kB leading to transcription of pro-inflammatory cytokines such as IL-1β, IL-6 and IL-12.

Dectin-2 is the prototype receptor of the Dectin-2 family of CLRs that consists of Mincle, DCAR, BDCA-2; Dectin-3 and DCIR. All the members of this family, exception for DCIR, contain a short intracellular tail with no signal transduction capabilities. Therefore, Dectin-2 associate with the ITAM-bearing molecule FcRγ for signal transduction to occur. Dectin-2 binds α-mannans of *Candida albicans* cell wall in association with Dectin-3, with which forms heterodimers, conferring higher sensitivity to fungal detection (Zhu LL et al., 2013). However, the responses mediated by Dectin-1 and Dectin-2 may overlap, although signalling pathways activated by Dectin-2 are less investigated compared to Dectin-1.

Certainly, CLRs are involved in antifungal immunity, considering the increased susceptibility to infection resulting from polymorphisms in these receptors and downstream signalling components, such as the deficiency of the signalling adaptor CARD9, which is characterized by severe mucosal and systemic fungal infections in the central nervous system (Drummond RR and Lionakis MS, 2016). However, Dectin-1 or CARD9 deficiencies link to mucocutaneous candidiasis is controversial, because a causative connection for Dectin-1 is not fully defined (Lionakis MS et al., 2014; Borriello F et al., 2020).

1.4 Damage associated molecular patterns (DAMPs)

Although very important, the Janeway's theory of PAMPs does not explains several immune reactions such as tolerance to the commensal microbiota, immunity against tumour, responses throughout trauma or injuries, and autoimmunity. In 1994 Polly Matzinger tried to answer to some of these open questions, proposing her Danger theory, in which she proposed that the immune system can recognize damage signals, independent of the recognition of foreign pathogen itself (Matzinger P., 1994). The Damage theory proposed that damage associated molecular patterns (DAMPs) are conserved, abundant and ubiquitously expressed self-molecules, released by distressed, injured or necrotic cells. DAMPs would be recognized by antigen presenting cells (APCs) via conventional receptors, mediating the activation of APCs to produce costimulatory signals to start an appropriate response to damage. Therefore, the activation of DCs can be either induced by endogenous danger molecules, released by stressed, damaged or dying tissues, or by

exogenous danger signals derived from pathogens. These exogenous danger molecules are cytolytic proteins and peptide toxins of several bacterial pathogens that, when released, are able to disrupt epithelial cell barrier, activating host immune responses. Recently, it has been identified the first fungal cytolytic peptide toxin in *Candida albicans.* This toxin, called "Candidalysin", is a pore-forming peptide toxin secreted by fungal hyphae that directly damages epithelial membranes, stimulating a danger response and the activation of epithelial immunity (Moyes DL et al., 2016). However, how fungal hyphae induce epithelial inflammatory responses and cell damage during mucosal infections remains to be clarified.

After infection or injury, neutrophils are the first kind of cells recruited in inflamed tissues. However, during sterile inflammation, when no microbial threats are present, neutrophils recruitment is mediated by DAMPs release from damaged cells. Moreover, DAMPs and PAMPs have some similarities. They bind the same PRRs despite their structural differences. For example, TLR4 can bind the DAMPs high mobility group box-1 (HMGB1) and heat shock protein (HSP), and also the PAMP lipopolysaccharide (LPS) present in Gram-negative bacterial cell wall (Chen GY et al., 2010).

Among DAMPs, high mobility group box 1 (HMGB1) is one of the best characterized. It is a highly conserved non-histone protein involved in the stabilization of nucleosomes. HMGB1 has been shown to play as both DAMP and a cytokine, with a pro-inflammatory role. Necrotic cells, for example, release high amount of HMGB1 following membrane disruption (Scaffidi P. et al., 2002; Rovere-Querini P. et al.,

2004). HMGB1 releasing can directly stimulate innate cells via PRRs, be a co-receptor for TLRs, acting in collaboration with PAMPs.

IL-1 is considered a danger mediator, for its role in the damage-induced inflammation. IL-1α is a well-known pro-inflammatory cytokine, which can promote on the endothelium for the upregulation of integrins. in order to trigger leucocyte recruitment and pro-inflammatory cytokines release (Chen and Nunez, 2010). IL-1 seems to be very important in the induction of acute inflammation in response to sterile injury, because mice deficient in components of the IL-1 pathway have reduced neutrophil infiltration (Chen C. et al, 2007). However, IL-1 role in inflammation will be discuss in the next paragraphs.

The Danger theory is interesting because proposed an evolutionary advantage of the immune system to quickly answer to endogenous or exogenous danger signals, preventing adverse events. In this context, neutrophils may not be able to discriminate between damaged tissue and infection, but they rapidly act to neutralize the inflammation, engulfing and removing cellular debris accumulated in a site of damage. Moreover, neutrophils and monocytes can act together to help tissue repair through the secretion of pro-angiogenic factors that promote vessel growth and tissue repair (Broggi A. and Granucci F., 2015).

1.4.1 Alarmins

Alarmins are endogenous, constitutively expressed and rapidly released proteins/peptides that are release in response to infection and non-programmed cell death (Figure 11). Therefore, they have been also

considered damage-associated molecular patterns (DAMPs) (Matzinger P, 1994; Oppenheim JJ et al, 2005). According to their capacities, alarmins/DAMPs are considered as “first responders”, because in response to microbial infection or sterile cell injury, they are released initiating innate immune responses. Currently, alarmins include several types of chromatin-binding moieties in addition to HMGB1, such as HMGN1, IL-1α, and IL-33, as well as heat shock proteins (HSPs), S100 proteins, ATP, and uric acid crystals, which can be classified based on their origin. Alarmins of nuclear origin (HMGB1, HMGN1, IL-1alpha) activate human and mouse conventional and plasmacytoid DCs (cDCs and pDCs) (Messmer D. et al, 2004; Rovere-Querini P. et al, 2012, Yang D. et al, 2012). Notably, it has been shown that alarmins induce the recruitment of granulocytes and macrophages in vivo, contributing to inflammatory response into danger sites (Yang D et al., 2013; Yang D. et al, 2008; Venereau E, 2012; Schiraldi M. et al, 2012) (Table 2).

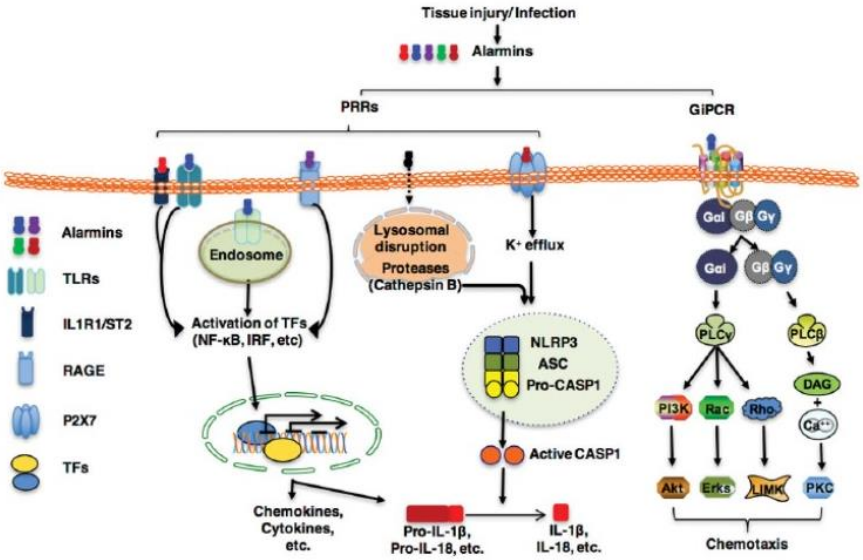

Figure 11 | The signalling pathways triggered by alarmins and DAMPs.

Alarmins are released in response to a danger, caused by injury or infection. They can trigger PRRs and GiPCRs to induce neutrophil activation and recruitment.

Table 2 | Major categories, receptors, and biological effects of alarmins.

Origin	Example	Receptor	Biological effects
Granule-derived	Defensins (α, β)	CCR2, CCR6, TLR4	Antimicrobial Leukocyte recruitment DC and Mϕ activation Promotion of immune responses
	Cathelicidin (LL37/CRAMP)	FPRL1, TLR7,8,9, P2X7, EGFR, MrgX2, CXCR2	
	EDN	TLR2	
	Granulysin	TLR4	
Nuclear	HMGB1	CXCR4, RAGE, TLR2,4,9	Regulating gene transcription Leukocyte recruitment DC and Mϕ activation Promotion of immune responses
	HMGN1	TLR4	
	IL-1α	IL-1R	
	IL-33	ST2	
Cytoplasmic	HSP60,70,90,96	TLR2,4, CD91	Cell homeostasis Leukocyte recruitment DC and Mϕ activation Promotion of immune responses
	S100 proteins	RAGE, TLR4	
	ATP	P2Y2,6,12, P2X1,3,7	
	Uric acid	P2X7	

Abbreviations used in the table: CRAMP, cathelin-related antimicrobial peptide; EDN, eosinophil-derived neurotoxin; HMGB1, high-mobility group box 1 protein; HMGN1, high-mobility group nucleosome-binding domain 1 protein; TLR, Toll-like receptor; CCR, CC chemokine receptor; FPRL1, formyl peptide receptor-like 1; EGFR, epidermal growth factor receptor; MrgX2, Mas-related gene X2; CXCR, CXC chemokine receptor; RAGE, receptor for advanced glycation end-products; Mϕ, macrophage.

1.5 IL-1 cytokines and receptors family

Interleukins were first identified as pyrogenic molecules produced by leukocytes after LPS-stimulation (Beeson P., 1948). Interleukin-1 (IL-1) term was born in 1979 comprehending features like fever, lymphocyte activation and haematopoiesis (Aarden et al., 1979). Although the term was referred to a single molecule, a previous work on these pyrogenic molecules shown the existence of two cytokines (Dinarello CA, 1974).

Later on, DNA sequencing allowed to better define these molecules, described as IL-1α and IL-1β. Nowadays, the IL-1 family is comprised of 11 soluble molecules and 10 receptors, primarily associated with damaging inflammation and innate immunity, although some of them are also involved in adaptive immunity (Figure 12).

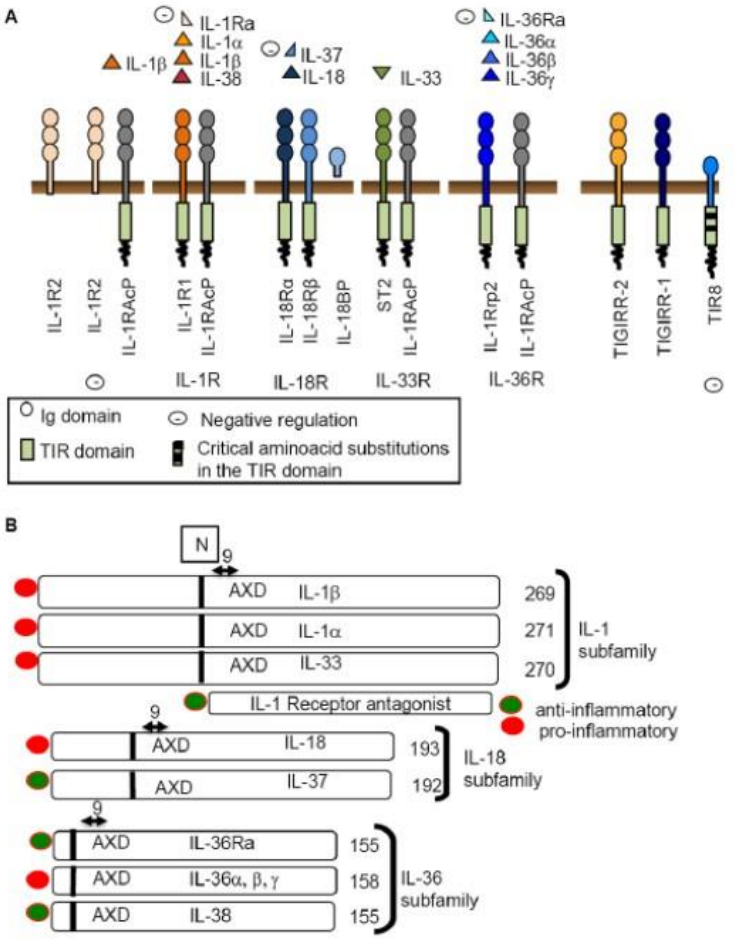

Figure 12 | IL-1 family members and their structural organization.

Ligands and receptor chains in IL-1 family (A). Subfamilies among IL-1 ligands, based on the length of the N-terminal pro-domain. Numbers refer to aminoacids. Il-37 has been identified only in humans.

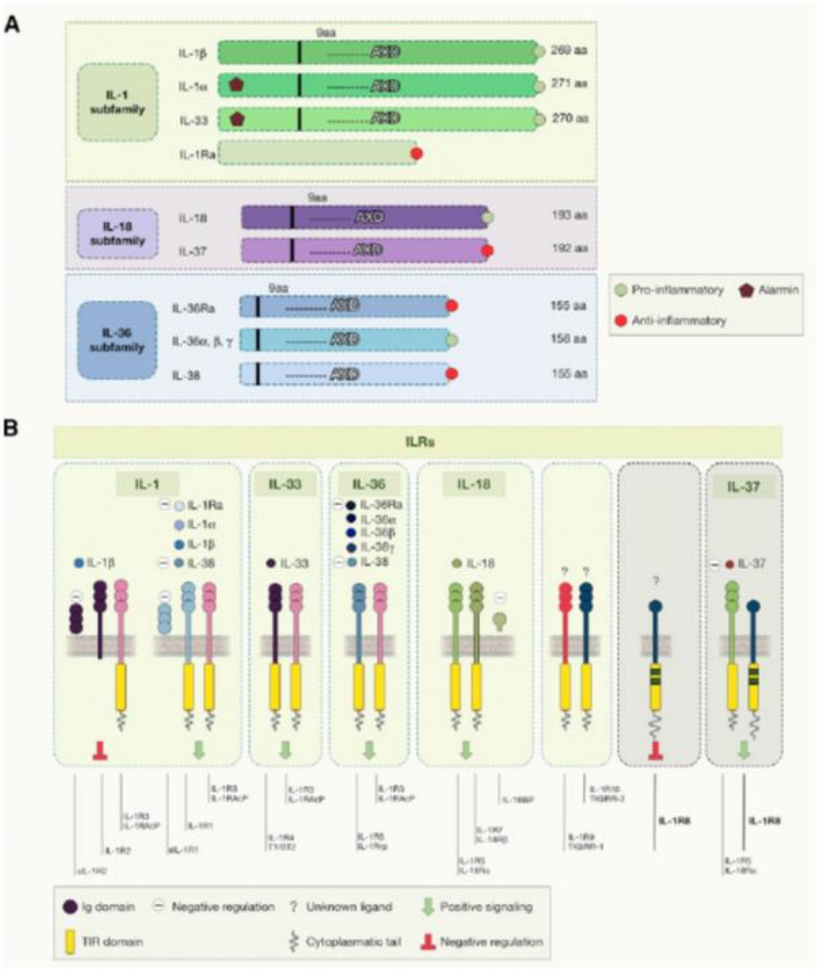

Figure 13 | IL-1 family members and their receptors

IL-1 ligands, grouped by structural characteristics and their ability to be pro- and anti-inflammatory or alarmins (A). Agonists and antagonists of IL-1 receptors (ILRs) (B).

IL-1 family cytokines are classified into three subfamilies, based on the IL-1 consensus sequence and the primary ligand binding receptor. Among them, IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ

are secreted and have agonistic activity; IL-1Ra, IL-36Ra and IL-38 are receptor antagonists, IL-37 is an anti-inflammatory cytokine (Figure 13) (Dinarello CA, 2018). Evolution analysis showed that agonists co-evolved with receptor antagonists and anti-inflammatory molecules and are conserved in all vertebrates, suggesting the evolutive relevance of an organised response mediated by IL-1 molecules.

Among the IL-1 family receptors, IL-1R1 binds IL-1α, IL-1β and IL-1Ra. IL-1R3 (IL-1R accessory protein) is the co-receptor for forming a trimeric signalling complex with IL-1α or IL-1β. In steady state, IL-1R and IL-1R3 are on cell membrane. Once IL-1α/β binds to IL-1R1, structural changes allow IL-1R3 to bind to IL-1R1. Ligands do not interact directly with IL-1R3 co-receptor, but the trimeric complex allows for the approximation of the TIR domains of each receptor chain. IL-1R3 also exists as a soluble form. IL-1R3 is also the co-receptor for IL-33, IL-36α, IL-36β, or IL-36γ (Jensen LE et al, 2003; Jensen LE et al, 2000). IL-R2 is a decoy receptor for IL-1β, it lacks a cytoplasmic domain, does not signal, but rather sequesters IL-1β (Colotta F. et al, 1993). IL-1R2 exists as an integral membrane protein, as well as a soluble form. In the extracellular space, neutralization of IL-1β activity is enhanced by a complex with soluble IL-1R3. Intracellularly, IL-1R2 binds the IL-1α precursor, preventing its release and processing. IL-R4 (also known as ST2) is the receptor for IL-33. IL-1R6 binds IL-36α, IL-36β, or IL-36γ but also IL-38.

IL-1 receptor family members share a common intracellular signalling domain with TLRs, the Toll-IL-1 resistance (TIR) domain, and an extracellular immunoglobulin (Ig)-like domain. Upon ligand binding,

they dimerize through their TIR domains, inducing the recruitment of the TIR domain-containing adaptor protein MyD88, which couples to downstream protein kinases (e.g. IRAKs and TRAF6). This signalling mediates the activation of several transcription factors, such as NF-kB, AP-1, c-Jun N-terminal kinase (JNK), p38 and mitogen-associated protein kinases (MAPKs), extracellular signal-regulated kinases (ERKs), and members of the interferon-regulatory factor (IRF) (Mantovani A. et al, 2019).

IL-1-related cytokines are not translated and secreted as bioactive molecules, but they are found in the cytoplasm as precursors. Each precursor contains a three-aminoacid conserved consensus sequence A-X-D (except for IL-1Ra), in which A may be any aliphatic amino acid, followed by any aminoacid (X) and the D for aspartic acid. Nine amino acids before the cleavage site is the N-terminal aminoacid, useful to reach the optimal bioactivity of the cytokine for the binding with the receptor (Afonina IS et al., 2015).

1.5.1 Interleukin (IL)-1α and interleukin (IL)-1β

Phylogenetic analysis shown that IL-1α had origin from a duplication event of the ancestral IL-1β gene, after which IL-1α underwent a different evolutionary pressure and finally a distinct function of the pro-domain of the precursors (Rivers-Auty J. et al, 2018). Both IL-1α and IL-1β signal via IL-1R. IL-1α is constitutively expressed in epithelial, endothelial, stromal cells and not constitutively also by myeloid cells. On the contrary, IL-1β is not constitutively expressed and is produced

only by myeloid cells. Endothelial cells contain IL-1α into the "apoptotic bodies", highly inflammatory vesicles comprising the endothelial membrane (Berda-Haddad Y. et al, 2011). Both IL-1α and IL-1β are translated into 31 kDa pro-forms. Unlike IL-1β, pro-IL-1α is not cleaved by caspase-1, but it is active as a precursor. Both pro-IL-1α and mature IL-1α bind to IL-1R with similar kinetics and have similar biological activity on epithelial and hematopoietic cells (Kim B., 2013). Therefore, the biological role of proteolytic processing of Il-1α is currently unknown. Pro-IL-1α can be cleaved by proteases to yield a 17kDa cytokine isoform. Calcium-activated membrane calpain, in vitro, can cleave pro-IL-1α, although it is unlikely that this takes place under physiological conditions (Kobayashi Y. et al, 1990). Caspase-1 activation by inflammasomes does facilitates secretion of IL-1α by inducing an inflammatory form of cell death termed pyroptosis (Gross O. et al, 2012). Chymase, elastase and granzyme B in the extracellular space are also able to cleave pro-IL-1α (Carruth LM et al, 1991; Afonina IS et al, 2011). Notably, it has been recently demonstrated that pro-IL-1α is cleaved by thrombin at a conserved consensus site in an 18 kDa isoform. Indeed, the coagulation system developed from an ancient innate immune system. Epidermal keratinocytes (KCs) constitutively express IL-1α and can secreted it after processing by thrombin. Cleavage of epidermal IL-1α after wounding could be a mechanism to rapidly alert the immune system against infection. Il-1α cleavage by thrombin suggested a functional link between coagulation and immune system, important for several diseases (Burzynski LC et al, 2019). IL-1α have a dual role. Extracellularly, IL-1α binds to the IL-1R1 on the surface of the cell, recruiting its co-receptor IL-1R3 and initiating a pro-

inflammatory signal, identical to that of IL-1β. Into the nucleus, the IL-1α precursor functions as a transcription factor (Dinarello CA, 2009; Wessendorf JH et al., 1993). Nuclear translocation of IL-1α between the cytosol and the nucleus happens in few nanoseconds (Cohen I. et al., 2010). During apoptosis, IL-1α leaves cytosol and rapidly migrates to the nucleus, where it binds to chromatin and fails to induce inflammation. On the contrary, when the cell is exposed to a necrotic signal, IL-1α migrates from nucleus to the cytosol, and the cell debris are highly inflammatory. Indeed, when pro-IL-1α is release from necrotic, it is a DAMP and functions as an alarmin into the extracellular space, because it mediates several inflammatory reactions via the IL-1R1 and the production of chemokines resulting in the infiltration of neutrophils (Figure 14) (Di Paolo NC et al., 2016). For example, IL-1α signalling is critical in pulmonary *Aspergillus fumigatus* challenge because it enhances the expression of CXCL1 and leukocyte recruitment (Caffrey AK et al., 2015). Therefore, sterile inflammation due to necrotic tissue appears to be IL-1α mediated, but independent of TLR4 (Chen CJ et al., 2007).

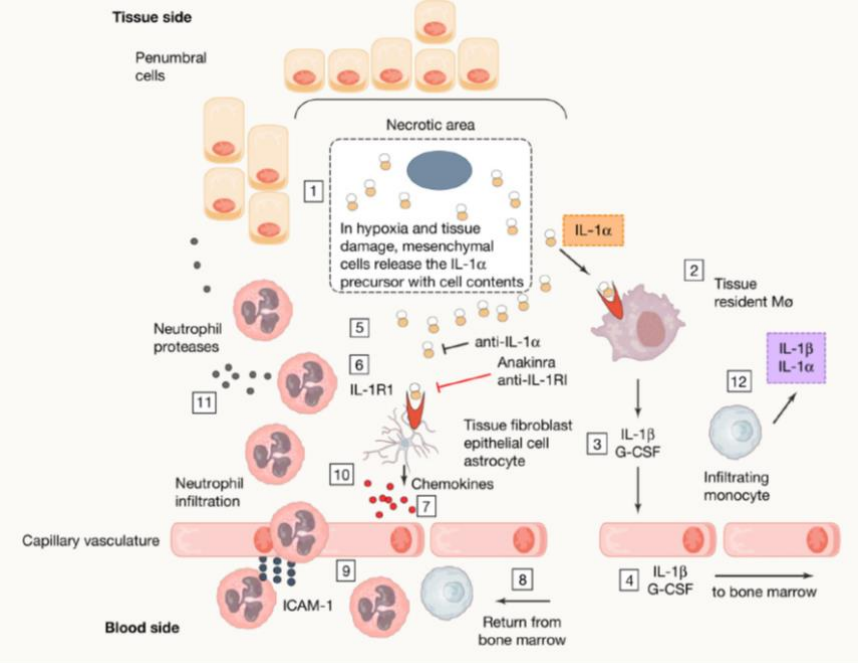

Figure 14 | IL-1α as an alarmin in tissue damage.

In hypoxia and tissue damage, mesenchymal cells release the IL-1α precursor with cell contents. IL-1α binds to the IL-1R1 on tissue resident macrophages. IL-1β, on the contrary, is released via NLRP3 inflammasome activation. IL-1β and G-CSF, released by hypoxic cells, enter the venous circulation and into the right ventricle, finally inducing the release of neutrophils and monocytes into the venous drainage. The IL-1α precursor accumulates in the extracellular space of the ischemic tissue, and IL-1α binds to IL-1R1 on tissue fibroblasts or epithelial cells. These events lead to the production of chemokines and neutrophils recruitment in the damaged tissue.

IL-1β was first purified to homogeneity in 1977 with a specific activity of producing a monophasic fever in rabbits (Dinarello CA et al., 1977). However, the cDNA cloning of human IL-1β was done in 1984 (Auron PE et al., 1984). In the following years, multiple biological properties of IL-1β were studied. IL-1β processing and secretion takes place with activation of the NOD-, LRR -and pyrin domain-containing protein 3

(NLRP3) inflammasome. NLRP3, also termed cryopyrin, was initially discovered in patients with ‘familial cold autoinflammatory syndrome’, genetic disease characterized by constitutional symptoms, fevers, and elevated acute phase proteins following exposure to cold (Hoffman HM et al., 2001). NLRP3 is an intracellular sensor that detects several microbial motifs and endogenous danger signals, resulting in the formation and activation of the NLRP3 inflammasome. These events lead to caspase 1-dependent release of the pro-inflammatory cytokines IL-1β and IL-18, as well as to gasdermin D-mediated pyroptotic cell death (Swanson KV et al., 2019). Assembly of the NLRP3 inflammasome with inactive procaspase-1 occurs after a fall in intracellular potassium. ATP activation of the P2X7 receptor opens the potassium channel and caspase-1 is activated by the inflammasome (Perregaux DG et al., 2000). Pro-IL-1β cleavage by active caspase-1 take place in specialized secretory lysosomes or in the cytoplasm. Therefore, other pathways are involved in IL-1β released from the cell, such as exocytosis of the secretory lysosomes, shedding of plasma membrane microvesicles, direct release via transporters and pyroptosis (Andrei C. et al., 1999; Andrei C. et al., 2004; Gardella S. et al., 2000; Qu Y. et al., 2007; Bergsbaken T. et al., 2009). Notably, other proteases such as elastase, matrix metalloprotease 9, and granzyme A process the IL-1β precursor extracellularly. Neither mice deficient in IL-1α nor IL-1β have any kind of defect. However, its deficiency in some pathologic conditions, such as microbial infection, will be discuss in the next chapters.

1.6 *Candida albicans*

Mucosal surfaces of human body are daily challenged by different microbial species, such as Candida species (spp.), a major component of the mycobiome of 70% of the healthy population (Schulze J. and Sonnenborn U., 2009; Witherden EA et al., 2017). Candida spp. are pathobionts, meaning that when the host is safe, they are symbiotic, but when in pathologic conditions, they can become opportunistic. Among these species, *C. albicans* is the most relevant, causing both colonization and infection events. In healthy patients, *C. albicans* can causes mild mucosal infection, such as oral rush or vulvovaginal candidiasis (highly prevalent and recurrent in women). However, in hospitalized or weaker patients, this infection could degenerate, and in such cases, lead to death. Therefore, Candida disseminated infections are considered the fourth most common nosocomial bloodstream infections, with high associated mortality of 45-75% (Brown GD et al., 2012). Maintenance of homeostatic balance of immune responses at mucosal surfaces is critical for preserve host health. Epithelial cells are the first player in the recognition of external microbe's attack, and physical contact and adherence of *C. albicans* to epithelial cells is mediated by fungal adhesins, like the agglutinin-like sequence (Asl) protein family, interacting with different host receptors, such as epidermal growth factor receptor (EGFR) and E-cadherin, improving fungal cell adhesion (Moyes DL et al., 2015; Nikou SA et al., 2019).

Candida multi-layered cell wall is approximately 0.5 mm thick and is formed by two layers composed of different polysaccharides. The innermost layer is composed by a network of polysaccharides

comprising around 5% N-acetylglucosamine polymer chitin, β-1,3 glucans for the 40% associated with β-1,6 glucans (20%). The outer layer is composed by proteins with N- or O-linked mannosyl residues. Several classes of PRRs recognize Candida's PAMPs, of which TLRs and CLRs are the most important. However, their relevance in the mucosal infection rather than the systemic one is quite different. In fact, Dectin-1, for example, is involved in the recognition of β-glucans and is critical during systemic infections but its role during oropharyngeal candidiasis is minor (Taylor PR et al., 2007; Verma AH et al., 2017). Dectin-2, DC-SIGN, and Mincle recognized branched mannan component of the *C. albicans* cell wall (Lionakis MS et al., 2013). Chitins are not recognized by a specific receptor; however, they appear to have a role in inhibiting the pro-inflammatory effects according to their structure, although their interaction with immune cells is not well understood and is actually under investigation. Recognition of mannans is mediated also by TLR4 binding to O-linked mannosyl residues, phospholipomannans of *C. albicans* are recognized by TLR2, whereas β-glucans were recognized by a complex of TLR2 and Dectin-1 (Gantner BN et al., 2003; Brown GD et al., 2003). Among other PRRs, NLRs are important for the formation of the inflammasome, in particular NLRP3 inflammasome, important in the response to *C. albicans* hyphal infection (Figure 15).

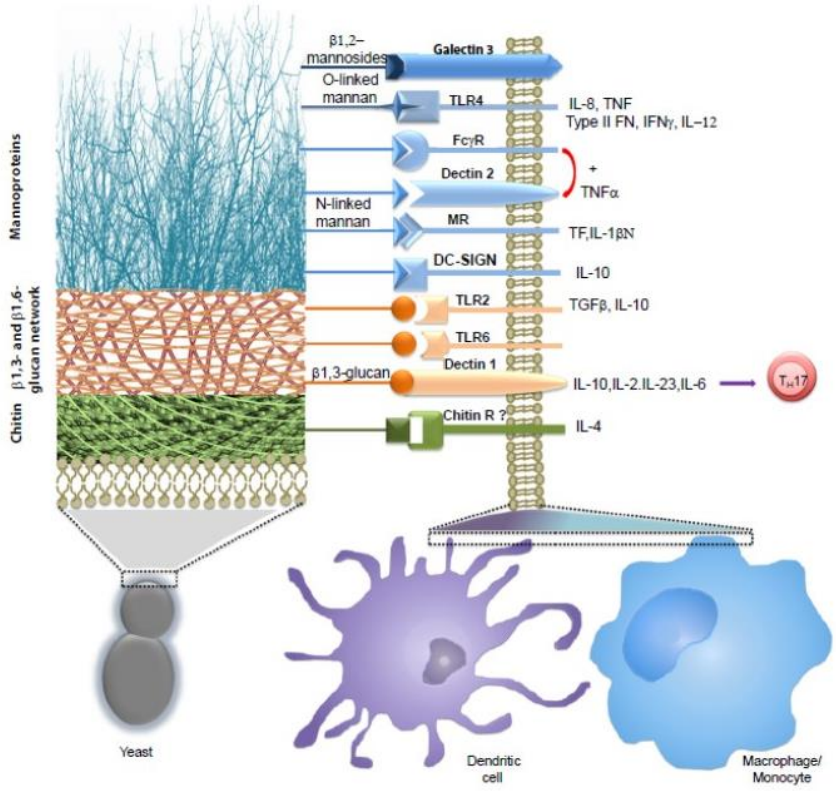

Figure 15 | *Candida albicans* cell wall.

C. albicans cell wall is composed by different types of PAMPs, which interact with PRRs present on immune cells, such as monocytes, macrophages, and DCs. The innermost layer is composed by N-acetylglucosamine polymer chitin, and β-1,3 glucans associated with β-1,6 glucans. The outer layer is composed by N- or O-linked mannosyl proteins. Among the PRRs that recognize *C. albicans*, Dectin-1 is involved in the recognition of β-glucans; Dectin-2, DC-SIGN, and Mincle recognized mannans; chitins are not recognized by a specific receptor. Mannans recognition is also mediated by TLR4, which binds O-linked mannosyl residues, and TLR2, which binds phospholipomannans. Moreover, β-glucans were recognized also by a complex of TLR2 and Dectin-1.

After the adhesion to the epithelial surface, Candida undergoes a transition to hyphal form, that is associated with the following invasion of the epithelium. Candida entrance occurs via two steps. Firstly,

endocytosis induced by the engagement of the fungal proteins Ssa1p and Als3p to cadherins and EGFR/Her2 (Phan QT et al., 2007; Sun JN et al., 2010; Zhu W et al., 2012). During endocytosis, *C. albicans* forms an "invasion pocket" in the epithelial cells into which secretes the cytotoxic peptide candidalysin (Moyes DL et al., 2016). Endocytosis is followed by active penetration that occurs when the growing hyphal tip pushes epithelial cell membrane, eventually leading to cell damage. Neutrophils are considered the most important innate immune cells involved in the mucosal fungal infections control and its regulation in response to *C. albicans* is linked to IL-1 signalling (Cheng SC et al., 2012; Altmeier S., 2016). However, when neutrophils detect higher microbial size, such as long hyphae, they undergo a specialized form of cell death, called NETosis, mediated by the formation of neutrophil extracellular traps (NETs). Notably, *C. albicans* can evade NETosis by releasing DNAse to the extracellular medium or by forming biofilms, rendering fungal cells less accessible to immune cells (Zhang X. et al., 2017; Kernien J et al., 2017). Neutrophil recruitment in the site of infection depends on rapid chemokine induction. In mice, blocking or genetic deficiency of the chemokine receptor CXCR2 (which binds CXCL1/KC) results in delayed neutrophil airway recruitment in a kind of *Aspergillus fumigatus* infection. *Aspergillus fumigatus* and *Candida albicans* infection induce IL-1α and IL-1β production (Vonk AG et al., 2006). Lung-resident myeloid cells represent a potential source of IL-1α/β at early time point post-infection. In condition of tissue damage or injury, lung epithelial cells can release IL-1α, which rapidly induce chemotactic mediators and neutrophil recruitment to sites of inflammation (Jhingran A. et al., 2015). In a mouse model of

oropharyngeal candidiasis (OPC), IL-1R signalling is essential for *C. albicans* infection control. Therefore, neutrophils recruitment to the site of infection and the mobilization of new neutrophils from the bone marrow depend on IL-1R signalling, in particular it depends on IL-1α (Altmeier S. et al., 2016). In the last years, other cytokines have been proposed to have a role in antifungal response, such as IL-17. Its role it has been investigated in oropharyngeal candidiasis (OPC) and seems to be triggered by candidalysin release by *C. albicans* hyphae during oral epithelium colonization (Conti HR et al., 2016; Verma et al., 2017) The source of IL-17 during OPC seems to be natural Th17 (nTh17) IL-17^{+}TCRab^{+} cells, present in the oral mucosa. However, the role of γδ-Tcells and innate lymphoid cells type 3 (ILC3) is still under debate (Sparber F et al., 2018; Gladiator A. et al., 2013; Conti HR et al., 2014). Recently, in the laboratory it has been demonstrated that intradermal *C. albicans* infection leads to abscess formation in which the pyogenic membrane is formed in a TGF-β dependent manner. The elimination abscess required IFNγ that hampers fibroblast's differentiation into myofibroblasts and promotes plasmin formation, useful to eliminate abscess. In this context, IFNγ is produced by NK cells through an NFATc2-mediated production of IL-2 by DCs (Santus W. et al., 2017).

Although *C. albicans* is one of the most prevalent fungal species of healthy human microbiota, in pathologic conditions caused by antibiotic use, disease, immunosuppressive therapies it can lead to severe infections. In hospitalized patients, *C. albicans* could be dangerous for its ability to form biofilms on medical devices, such as catheters, pacemakers, heart valves and others. Microbial biofilms are communities of cells that adhere to surfaces, such as tissues and

implanted medical devices. Cells within biofilms are often resistant to drugs and to physical perturbations and, for these reasons; its removal can require invasive surgical procedures, worsening patient condition (Lohse MB et al., 2018).

1.7 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium responsible for a wide spectrum of human skin and soft tissues inflammatory diseases. Although 30% of healthy people are asymptomatically infected by this bacterium, when the skin barrier is altered or patients are immunocompromised, *S. aureus* can cause acute bacterial skin and skin structure infections (ABSSSIs) and several infections from minor skin infection, to lethal bacteraemia and sepsis (Miller LG and Kaplan LS, 2009). Treatment of these infections is complicated by the antibiotic resistance, like methicillin-resistant *S. aureus* (MRSA) strains. MRSA infections are endemic in hospital worldwide, resulting in among 18,500 annual deaths in United States of America (Klevens RM et al., 2007). A hallmark of *S. aureus* infections is neutrophil abscess formation, which is necessary for bacterial clearance (Molne L. et al., 2000). *S. aureus* recognition is mediated by TLRs and intracellular NLRs which are expressed by keratinocytes, the first responders to bacterial challenges, that can detect the presence of *S. aureus*. The *S. aureus* cell wall contains two potential TLR activators: lipoteichoic acid (LTA) and peptidoglycan (PGN), typically recognized by TLR2 (Akira S. et al., 2006). Furthermore, *S. aureus* produces several proteins known to damage skin cells and skin

integrity, such as phenol-soluble modulin alpha (PSMα), that induces the release of keratinocyte IL-1α and IL-36α (Nakagawa S. et al., 2017).

Antimicrobial peptides released by keratinocytes play an important role in cutaneous innate defence against *S. aureus*. Neutrophil recruitment and clearance of subcutaneous bacteria appear to be TLR2 independent in mice and dependent upon IL-1β and IL-1R type I (IL-1RI) (Miller LS et al., 2006). IL-1α and IL-1β increased mRNA production and protein secretion of the neutrophil chemotactic CXCL1, CXCL2 and IL-8 in keratinocytes after *S. aureus* skin infection. Notably, expression of IL-1α is significantly higher than that of IL-1β (Olaru and Jenses, 2010). Patients who suffer from recurrent and severe *S. aureus* cutaneous infections have also an impairment in IL-1R and/or TLR signalling, as a result of MyD88 or IRAK4 deficiency (Picard C. et al., 2003; Von Bernuth H. et al., 2008; Picard C. et al., 2010) (Figure 16).

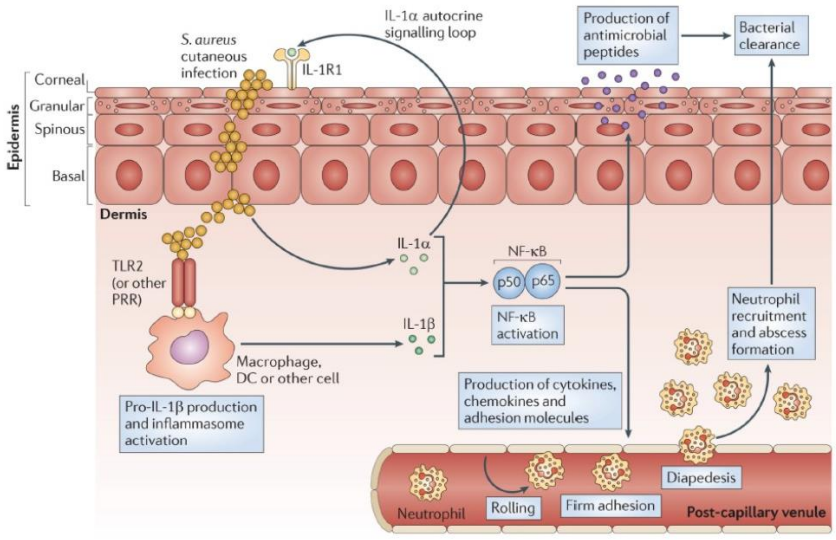

Figure 16 | The IL-1 mediated cutaneous immune response against *Staphylococcus aureus.*

S. aureus recognition is mainly mediated by TLR2. IL-1α is produced and released by keratinocytes, IL-1β is produced by resident and recruited cells, such as macrophages and DCs. Both IL-1α and IL-1β activate the nuclear factor-kB (NF-kB), leading to the production of anti-mibrobial peptides. IL-1-mediated responses result in the production of pro-inflammatory cytokines, chemokines and adhesion molecules, promoting the recruitment of neutrophils from the circulation to the site of *S. aureu* infection in the skin.

1.8 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative bacterium, member of the skin microflora of both animals and human, with a large genome that enables its adaptation to diverse growth conditions and infections in several species, from nematodes to vertebrates, including humans (Silby MW et al., 2011; Picard C: et al. 2010). *P. aeruginosa* can be found in the environment in two different growth forms, as planktonic bacteria and biofilms. As well as for *C. albicans*, healthy humans are protected against *P. aeruginosa* and do not typically suffer from infections caused by this bacterium. However, it poses serious health risk for immunocompromised patients, because it is responsible for about 10% of in-hospital infections in patients with cancer, diabetes, transplantation, implants and haematological disorders and its pathogenicity is associated with antibiotic-resistance, such as in human infections by *S. aureus*. Therefore, it is the second most frequent agent causing skin infections in burn human patients (Andonova and Urumova, 2013) and it causes both acute respiratory infections and chronic infections, such as those seen in patients with cystic fibrosis (Sadikot RT et al., 2005). The cutaneous manifestation of *P. aeruginosa* are high variable, ranging from mild and localized

benign infections to life-threatening systemic infections with skin lesions characterized by precise morphologies and can occur in both immunocompromised and healthy individuals. Among factors that contribute to virulence of *P. aeruginosa*, there are various proteases, such as elastase, that facilitates its dissemination by allowing the bacterium to broke membrane integrity (Beiarano PA et al., 1989). Other factors that contribute to pathogenicity include phospholipase C activity, lipopolysaccharide (LPS) and the exoproducts secreted by the type III secretion system (Ostroff RM and Vasil ML, 1987; Pier GB and Goldberg JB, 1995; Nicas TI and Iglewski BH, 1985).

P. aeruginosa has a flagellum responsible for the motility and bacterial invasion potential and genetically engineered flagellum-negative strains injected to mice, are less virulent than flagellum positive strains (Arora SK et al., 2005; Montie TC et al., 1982). *P. aeruginosa* is recognized by TLR4, that plays a major role in the host response to lipopolysaccharide (LPS) and TLR5, which binds flagellin, the protein component of bacterial flagella. *P. aeruginosa* recognition activates downstream both MyD88-dependent and -independent signalling pathways in the lung, and IL-1R signalling by epithelial cells (Mijares LA et al., 2011). However, its role in *P. aeruginosa* skin infection remains to be clarified.

Scope of the thesis

Innate immunity represents the most conserved and fast defence mechanism activated upon adverse injury, and it is characterized by the involvement of unspecific effector functions with the aim to prevent dissemination of invading pathogens and to restore homeostatic conditions. The activation of inflammatory responses comprehends the release of cytokines and chemokines, adhesion molecules, proteins involved in the regulation of cell survival and so on.

In 1989, Charlie Janeway proposed the theory that the inflammation occurring post infections, was induced by pathogens associated molecular patterns (PAMPs) that were recognized by pattern recognition receptors (PRRs). Moreover, Janeway's theory explained how adaptive immunity is activated by innate immunity, assuming that, following interaction with PAMPs, PRRs initiate signal transduction pathways for the upregulation of costimulatory molecules (signal 2) necessary for the activation of T cells. Alongside Janeway's PRR theory, Polly Matzinger proposed the Danger theory, by which she supported the idea that not only PAMPs, but any danger signals released by stressed cells, called damage associated molecular patterns (DAMPs), could activate DCs. Nowadays, we know that PRRs can recognize some danger signals, but it has not been clarified if DAMPs are sufficient, or not, to activate immunity independently of PAMPs. In the inflammatory process, neutrophil recruitment is essential for the control of the infection. Intradermal infections with *C. albicans* are eliminated by neutrophils recruited at the site of infection with an

unknown mechanism. Moreover, it has been demonstrated that Gram-positive bacteria, such as *Staphylococcus aureus*, are able to recruit neutrophils in the tissue infected without PRR involvement, and through IL-1β release in the site of infection. Additionally, IL-1α represents a vasoactive inflammatory factor, responsible of neutrophils recruitment in a PRR-independent manner, especially at cutaneous level.

Considering these assumptions, the scope of this thesis is to dissect how neutrophils are recruited during cutaneous infection, by defining the specific roles of DAMPs and PAMPs during primary infections. To assess this hypothesis, we used animal models deficient for some PRRs and we evaluated the early immunological responses, in terms of cell recruitment, within 24 hours after intradermal infection with different type of pathogens, such as bacterial (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and fungal (*Candida albicans*) pathogens.

We demonstrated that MyD88 deficiency impaired neutrophil recruitment after pathogens skin infection, independently from PRRs and dependently from IL-1 signalling. Moreover, we evaluated the phenotype of neutrophils recruited after microbial skin infections, and in particular, the prevalence of “fresh” or “aged” neutrophils after a danger (DAMP) or PAMP stimulation.

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Chapter 2

DAMPs and PAMPs have distinct roles in neutrophil recruitment during cutaneous microbial infections

2.1 MyD88 deficiency impaired neutrophil recruitment after *Candida albicans* skin infection

In pathological conditions, *C. albicans* can cause severe mycoses once it reaches the bloodstream or pass the epithelial barriers. In its hyphal form, *C. albicans* is able to penetrate the epithelium, invading the skin. Once penetrated inside the organism, *C. albicans* is recognized by several immune cells due to the expression, on its surface, of different PAMPs recognized by different PRRs, as C-type lectin receptors (CLRs) and Toll-like Receptors (TLRs). In our laboratory, it has been recently demonstrated that wild type mice intradermally infected with *C. albicans,* form a capsulated abscess under the skin, which is resolved with ulcer formation (Santus W: et al., 2017). The elimination of the infection requires neutrophils recruitment, but the mechanism by which this occurs remains to be clarified.

Among TLRs, TLR2 recognizes phospolipomannans and TLR4 recognizes O-linked mannosyl residues. Mice deficient for one of these two receptors have an impairment in the activation of the immunity against the pathogen, demonstrating that they are involved in fungal infection (Netea MG et al., 2004). In addition, CLRs are involved in antifungal immunity because they recognize the carbohydrate-rich cell wall of fungi. Dectin-1 is involved in the recognition of β-glucans; Dectin-2 recognizes mannosylated residues. Moreover, deficiency of the signalling adaptor CARD9, through which Dectins activate downstream signalling, is related to systemic fungal infections (Drummond RA and Lionakis MS, 2016).

Considering these assumptions, we investigated the roles of these receptors, and of the adaptor molecules through which these receptors function. Firstly, we performed *C. albicans* skin infection on TLR4$^{-/-}$, MyD88$^{-/-}$, Dectin-1$^{-/-}$, and CARD9$^{-/-}$ mice. We intradermally injected hyphae, and we analysed immune cells recruitment after 6 hours and 24 hours post-infection. Since neutrophils are the first line of defence in host immune response, and they are rapidly recruited in response to microbial infections, we evaluated neutrophils recruitment, considering local expression of *Cxcl1* and *Ly6G* as a read-out. CXCL1 is a chemokine that plays a role in the recruitment and activation of neutrophils during infections and LY6G is highly expressed on circulating neutrophils (Sawant KV et al., 2016). Moreover, we evaluated *Ly6C* mRNA levels to evaluate monocytes recruitment. Although Ly6C is expressed by neutrophils in the bone marrow and in the circulation, the level of Ly6C expression is used to distinguish monocytes subsets. Ly6C^{hi} monocytes, also called inflammatory monocytes, express the chemokine receptor CCR2 and migrate from the bone marrow to the site of infection or inflammation (Yona S et al., 2013).

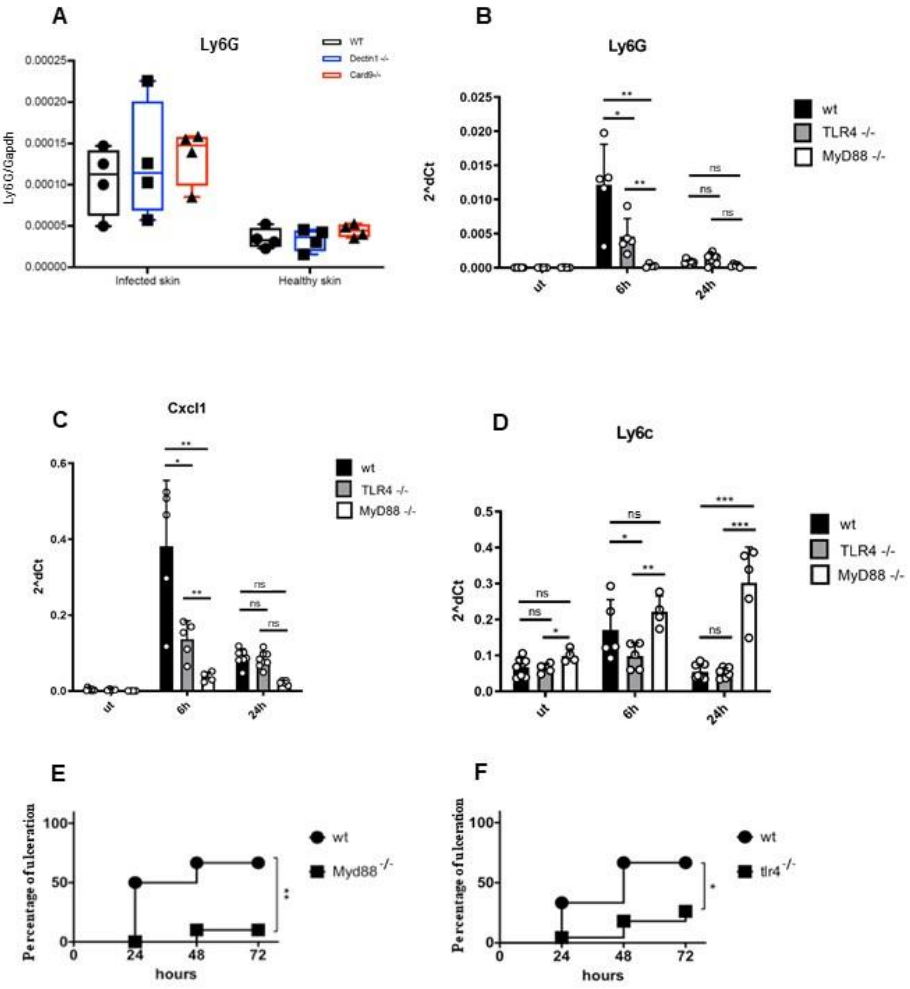


Figure 1 | MyD88 deficiency impaired neutrophil recruitment *in vivo* after *C. albicans* skin infection.

Real-time PCR analysis of *Ly6G* (A and B), *Cxcl1* (C), and *Ly6C* (D) mRNA induction 6 hours and 24 hours after *C. albicans* skin infection in the dorsal region of WT, Card9 $^{-/-}$, Dectin-1 $^{-/-}$, TLR4 $^{-/-}$, and MyD88 $^{-/-}$ mice. Data reported 1 representative experiment of 3 independent experiments, with WT (n=8), TLR4 $^{-/-}$ (n=4), MyD88 $^{-/-}$ (n=4) mice untreated; WT (n=5), TLR4 $^{-/-}$ (n=5), MyD88 $^{-/-}$ (n=4) mice sacrificed after 6h; WT (n=8), TLR4 $^{-/-}$ (n=8), MyD88 $^{-/-}$ (n=5) mice sacrificed after 24h. Two-way ANOVA statistical analysis. Percentages of ulceration of WT, MyD88$^{-/-}$ (E) and TLR4$^{-/-}$ (F) mice after 24- 48- and 72- hours post *C. albicans* skin infection.
Ly6G: lymphocyte antigen 6 complex locus G6D; *Ly6C*: lymphocyte antigen 6 complex, locus C1; *Cxcl*1: chemokine C-X-C motif ligand 1.

Dectin-1- and CARD9-deficient mice did not show any defect in Ly6G expression, compared to WT mice, demonstrating that the Dectin-1/CARD9 mediated pathway is not involved in the early neutrophil recruitment after *C. albicans* skin infection (Figure 1A). TLR4-deficient mice recruited less neutrophils compared to WT mice (Figure 1 B-C), but they were able to ulcerate (Figure 1F). Diversely, MyD88 deficiency impaired neutrophil recruitment after *C. albicans* skin infection (Figure 1 B-C) and affected ulceration, because capsulated abscess persisted at 72 hours post-infection (Figure 1E). No functional differences were observed in monocytes recruitment in TLR4- and MyD88-deficient mice (Figure 1D). All these data demonstrated that MyD88 is essential for neutrophil recruitment after *C. albicans* infection in a PRRs-independent mechanism.

2.2 MyD88 is involved in neutrophil recruitment in bacterial skin infections, through a PRR-independent mechanism

We then investigated whether MyD88 could have a role in neutrophil recruitment during other types of microbial skin infections, such as Gram-positive and Gram-negative bacteria, in a PRRs-independent manner. We performed skin infections using *Staphylococcus aureus* (a Gram-positive bacterium recognized by the host through TLR2) and *Pseudomonas aeruginosa* (a Gram-negative bacterium that activates host immunity via TLR4), respectively on $TLR2^{-/-}$ and $TLR4^{-/-}$ mice, and we sacrificed them at 6 hours post-infection.

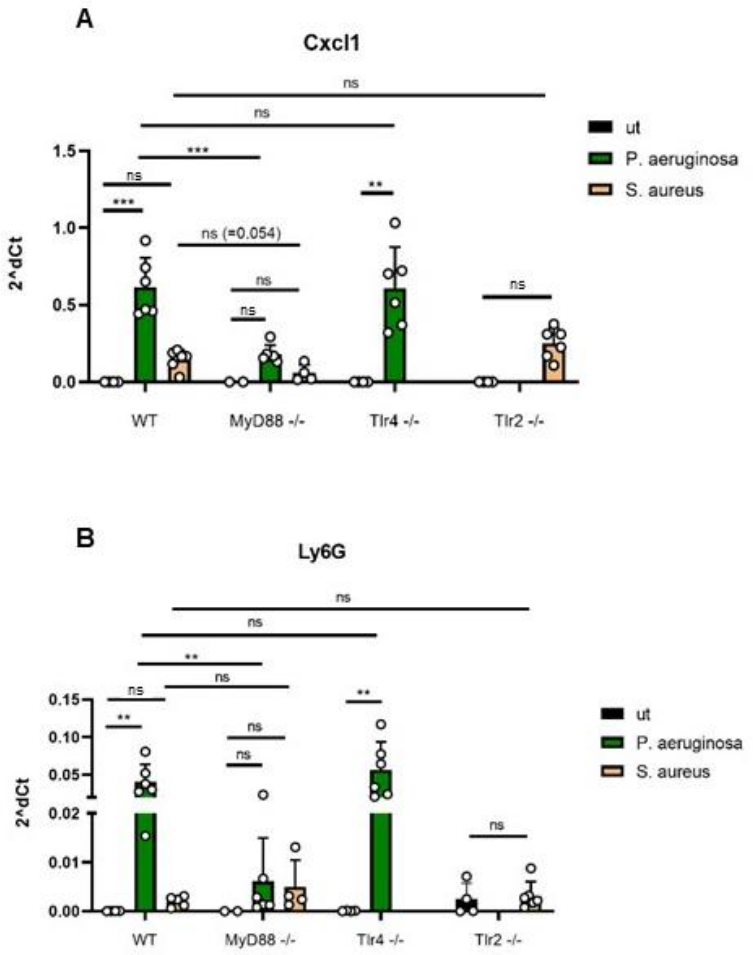


Figure 2 | MyD88 is also involved in neutrophil recruitment in bacterial skin infections, through a PRR-independent mechanism.

Real-time PCR analysis of *Cxcl1* (A), and *Ly6G* (B) mRNA induction 6 hours after *Pseudomonas aeruginosa* and/or *Staphylococcus aureus* intradermal infection. TLR4 $^{-/-}$ and TLR2 $^{-/-}$ mice did not show any defect in neutrophil recruitment after *P. aeruginosa* and *S. aureus* intradermal infection, respectively. On the contrary, MyD88 $^{-/-}$ mice had an impairment in neutrophil recruitment after *P. aeruginosa* infection, compared to WT mice. Diversely, after *S. aureus* infection there is a mild effect. Two-way ANOVA statistical analysis.

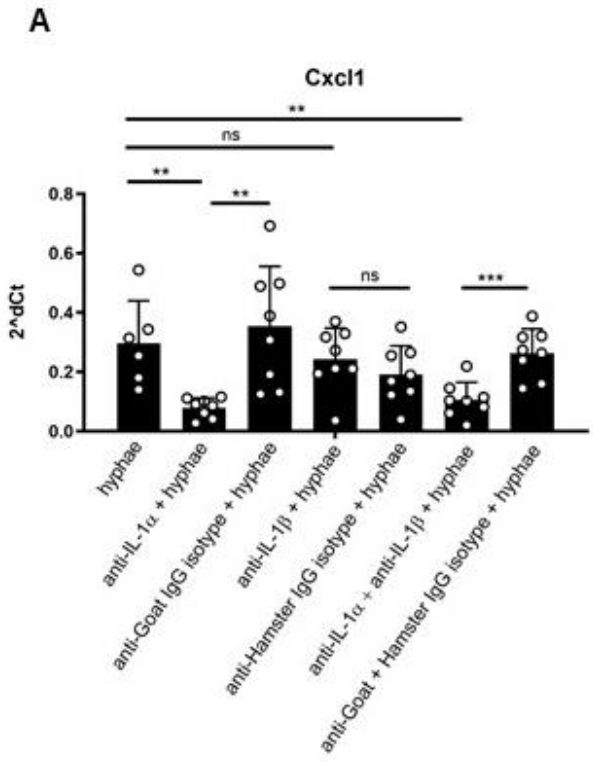
MyD88$^{-/-}$ mice showed a strong reduction in neutrophil recruitment after *P. aeruginosa* skin infection, while a mild effect was measured in *S. aureus* infection. However, TLR4$^{-/-}$ do not have any significant defect in neutrophil recruitment after *P. aeruginosa* skin infection, as well as TLR2$^{-/-}$ mice after *S. aureus* infection (Figure 2A and B). Although the *S. aureus* infection have a low effect in this model, TLR2$^{-/-}$ and WT mice have the same trend of neutrophil recruitment after the bacterial infection.

These results highlighted the evidence that, independently of the kind of microorganisms (fungal or bacterial) and the recognition of their PAMPs, the recruitment of neutrophils is a process mediated by a common mechanism, primarily mediated by MyD88.

2.3 IL-1α and IL-1β are both involved in neutrophil recruitment after *Candida albicans* skin infection, although the IL-1β role is less important

MyD88 is essential for the downstream signalling of several TLRs, but it functions also to recruit IRAK to the IL-1R complex, following stimulation with IL-1 (Wesche H. et al., 1997; Muzio M., et al., Science 1997). IL-1α and IL-1β are two vasoactive cytokines involved in the production of CXCL1 by stromal cells and keratinocytes (Caffrey AK et al., 2015). IL-1β production requires PRR activation, instead IL-1α is constitutively produced and released in a PRR-independent manner and, for this reason, it could be considered a DAMP signal (Kim B. et al., 2013).

In order to investigate the PRR-independent mechanism through which MyD88-deficiency affects neutrophil recruitment after *C. albicans* skin infection, we evaluated the role of IL-1α and IL-1β in our model. We firstly performed an *in vivo* experiment on WT mice, pre-treating their dorsal region with neutralizing antibodies for IL-1α and IL-1β, before *C. albicans* skin infection.



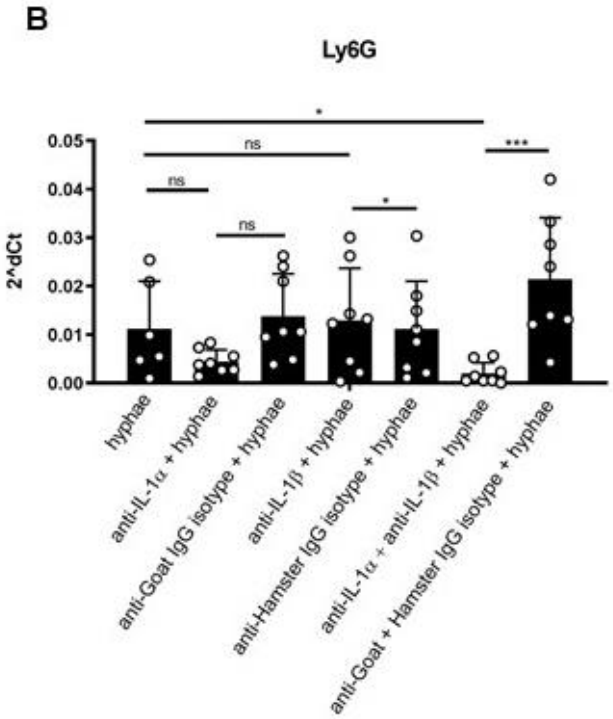


Figure 3 | IL-1α and IL-1β are both involved in neutrophil recruitment *in vivo*, after *Candida albicans* skin infection, although the IL-1β role is less important

Real-time PCR analysis of *Cxcl1* (A), and *Ly6G* (B) mRNA induction 6 hours after *C. albicans* skin infection in the dorsal region of WT mice. WT mice were treated with *C. albicans* hyphae (n=5) or pre-treated 30' before *C. albicans* hyphae intradermal infection with neutralizing antibodies for IL-1α (anti-IL-1α) or IL-1β (anti- IL-1β) or both, and their isotypes (n=8). Paired T-test statistical analysis.
Ly6G: lymphocyte antigen 6 complex locus G6D; *Ly6C*: lymphocyte antigen 6 complex, locus C1; *Cxcl1*: chemokine C-X-C motif ligand 1. Anti-IL-1α: IL-1α neutralizing antibody. Anti-Goat IgG isotype: isotype of IL-1α neutralizing antibody. Anti-IL-1β: IL-1β neutralizing antibody. Anti-Hamster IgG isotype: isotype of IL-1β neutralizing antibody. Anti-IL-1α + IL-1β: IL-1α neutralizing antibody + IL-1β neutralizing antibody. Anti-Goat + Hamster IgG isotype: isotype of IL-1α neutralizing antibody + isotype of IL-1β neutralizing antibody.

The decrease of *Cxcl1* production occurs strongly after 6 hours in mice co-treated with anti-IL-1α neutralizing antibody and hyphae compared to WT mice treated only with hyphae (Figure 3A). Accordingly, a trend of decrease in neutrophil recruitment is observed in this group of mice (Figure 3B). Treatment with anti-IL-1β neutralizing antibody, and then hyphae, causes a small reduction of *Cxcl1* production (Figure 3A) and does not affect *Ly6G* levels (Figure 3B). Notably, anti-IL-1α e anti-IL-1β neutralizing antibodies co-treatment with hyphae lead to a strong reduction of *Ly6G* and *Cxcl1* levels, compared to its expression in WT mice treated only with *C. albicans* hyphae (Figure 3 A and B).

These results demonstrated that early neutrophil recruitment, mediated by the chemoattractant activity of *Cxcl1*, depends both on IL-1α and IL-1β after *C. albicans* intradermal skin infection. Nevertheless, IL-1α seems to have a major role, probably because it is constitutively expressed and it is released immediately after the infection, functioning as an alarmin, in contrast to IL-1β, which needs to be cleaved and, only later, released.

2.4 IL-1R1 KO mice are not able to recruit neutrophils after different microbial skin infections

In our previous experiments, we demonstrated that early neutrophil recruitment (within 6 hours post-infection) after different types of microbial skin infections (fungal and bacterial) is mediated by a MyD88-dependent mechanism. Moreover, we investigated to role of IL-1, which act downstream MyD88, performing *in vivo* experiment on

WT mice, using anti-IL-1α e anti-IL-1β neutralizing antibodies before *C. albicans* skin infection. We demonstrated that neutrophil recruitment after fungal infection is strongly regulated by IL-1.

Considering these assumptions, we decided to test the role of IL-1 in early neutrophil recruitment *in vivo*, after different types of pathogens infection, using IL1R1$^{-/-}$ mice.

Among the IL-1 family receptors, IL-1R1 binds IL-1α, IL-1β and IL-1Ra. IL-1R3 is the co-receptor and forms a trimeric signalling complex with IL-1α or IL-1β. Unlike IL-1β, pro-IL-1α is active as a precursor and both pro-IL-1α and IL-1α bind to IL-1R with similar kinetics (Kim B. et al., 2013). Extracellularly, IL-1α binds to the IL-1R1 on the cell surface, recruiting the co-receptor IL-1R3 and initiating a pro-inflammatory signal.

We performed intradermal infection with *C. albicans*, *P. aeruginosa* and *S. aureus* in IL-1R1$^{-/-}$ mice. To distinguish the role of IL-1R1 in the early (6 hours) or late (24 hours) steps of neutrophil recruitment, we sacrificed the mice at these two time points.

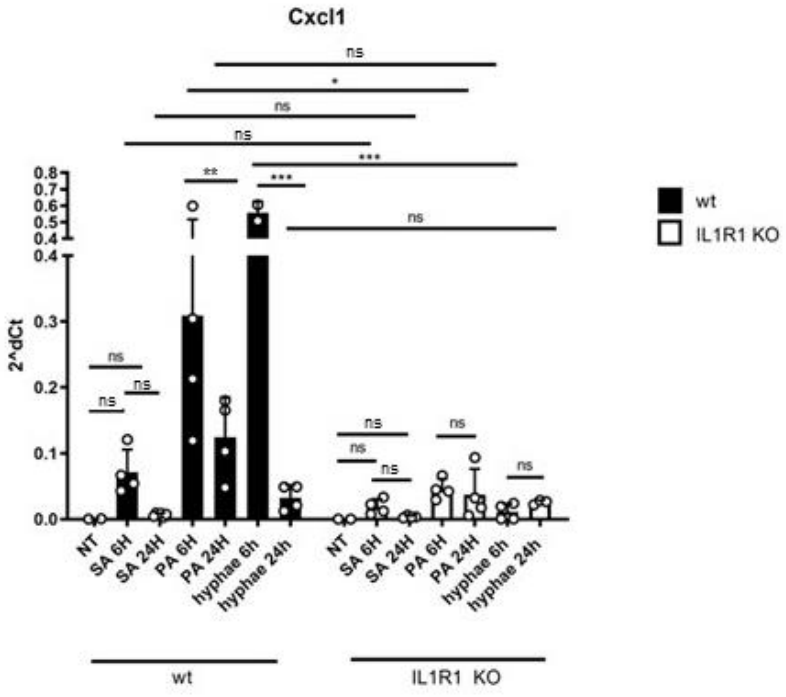


Figure 4 | IL-1R1-MyD88 signalling is involved in neutrophil recruitment after different repertoire of pathogens (fungal or bacterial) skin infection.

Real-time PCR analysis of *Cxcl1* mRNA induction 6 hours after *Pseudomonas aeruginosa*, *Staphylococcus aureus* intradermal infection, and *Candida albicans* on IL-1R1 KO and WT control mice. Two-way ANOVA statistical analysis.

We confirmed that, independently of the type of pathogens, the IL-1R1-MyD88-dependent neutrophil recruitment is conserved in the first stages of bacterial skin infections (Figure 4).
In detail, *Cxcl1* increased expression is more evident at 6 hours post-microbial infections in WT mice, decreasing at 24 hours. On the contrary, IL1R1 KO mice showed a strong impairment of *Cxcl1* production, after microbial infections, independently of the type of pathogen. All these results inspired the idea that the initiation of the

inflammatory process during primary infections could be due to unspecific release of alarmins (like IL-1α) by distressed cells, stimulating an immediate neutrophil recruitment at the site of infection mediated by IL-1 pathway and independent from PRRs involvement.

2.5 DAMPs and PAMPs have distinct roles in neutrophil recruitment

Although the microorganisms we have used express different PAMPs and have different pathological outcomes, we demonstrated that the early recruitment of neutrophils is mediated by a common IL1R1-MyD88 dependent mechanism.

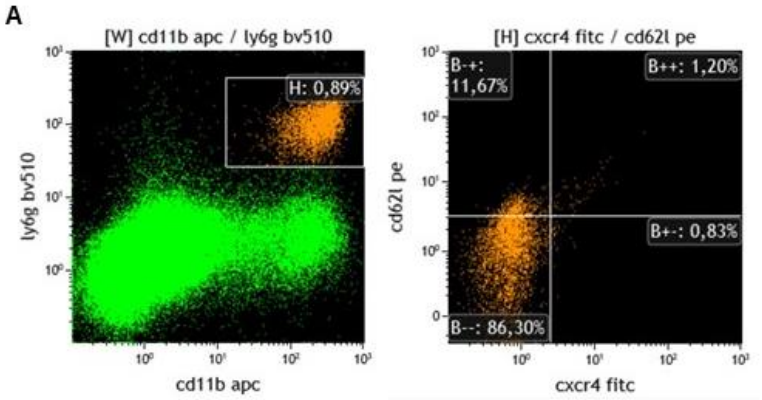
IL-1 is considered a danger mediator and have a role in the damage-induced inflammation, promoting the leucocyte recruitment and pro-inflammatory cytokine release (Chen and Nunez, 2010).

Given these results, we hypothesized that DAMPs (like IL-1 or, in general, a danger signal) and PAMPs could have a different role in neutrophil recruitment. The neutrophil ability to eliminate pathogens and its involvement in tissue damage, suggested the idea that a controlled balance of their activity is necessary. During the day, neutrophil phenotype changes in order to coordinate immune response (Casanova-Acebes M., et al., 2013). Interestingly, in the last years, the idea of "neutrophil aging" has been completely revaluated. In detail, neutrophils released from the bone marrow into the blood are considered "fresh neutrophils" ($CD62L^+$ $CXCR4^-$) and are able to enter tissues in case of inflammation (Zhang D. et al., Nature 2015).

Neutrophils present during daytime lose their ability to enter inflammatory sites and are considered “aged neutrophils” ($CD62L^-$ $CXCR4^+$) (Adrover JM et al., 2019).

However, the mechanisms that influence neutrophil aging during infections, remains to be clarified.

In order to investigate the role of DAMP (IL-1-mediated) or PAMP (witch signal through a PRR-dependent mechanism) in neutrophil recruitment, we decided to treat mice’s skin with LPS (PAMP) or to induce a damage through scratch and scarification (DAMP). We considered two time points, 6- and 48- hours, because it has been reported that neutrophils lose CD62L (L-selectin) expression and gain CXCR4 expression over about 6 hours after leaving the bone marrow, acquiring an aged-related phenotype (Casanova-Acebes M. et al., 2013; Adrover JM et al., 2019).



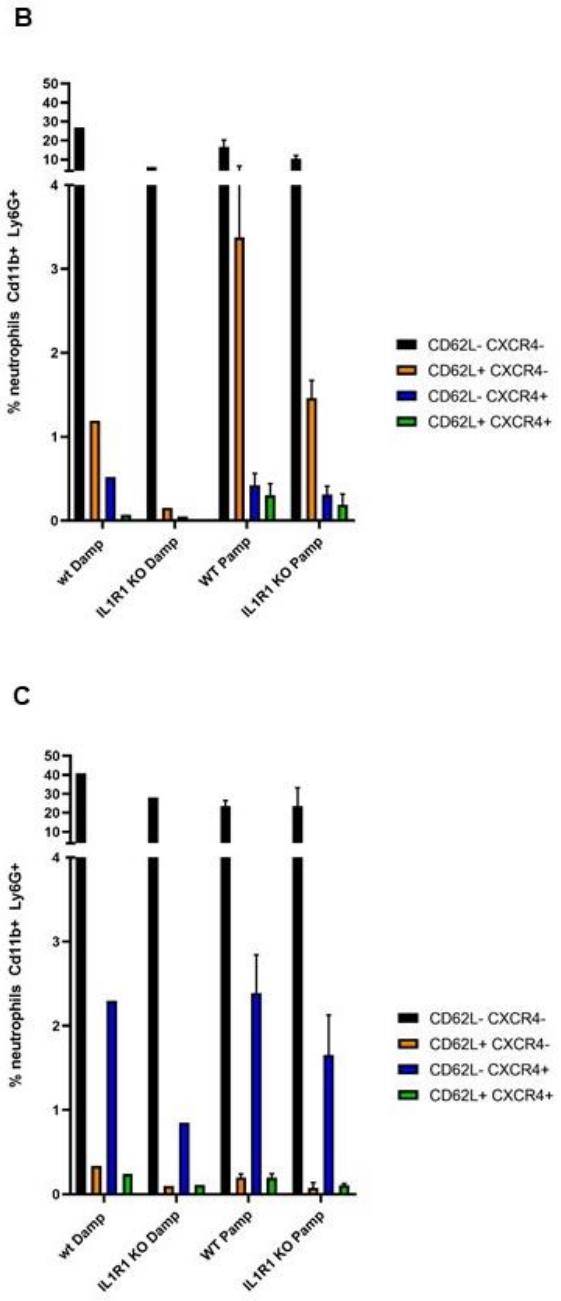


Figure 5 | DAMPs and PAMPs have distinct roles in neutrophil recruitment in WT vs. IL1R1$^{-/-}$ mice.

Gating strategy to distinguish fresh ($CD62L^+$ $CXCR4^-$) and aged ($CD62L^-$ $CXCR4^+$) neutrophils ($CD45.2^+$ $CD11b^+$ $Ly6G^{hi}$ cells) (Figure 5A). Flow cytometry analysis of fresh or aged neutrophil recruitment after 6- (Figure 5B) and 48- (Figure 5C) hours after DAMP (scratch and scarification) or PAMP (LPS) skin (ear) stimulation, on $IL1R1^{-/-}$ and WT mice. Fresh ($CD62L^+$ $CXCR4^-$) and aged ($CD62L^-$ $CXCR4^+$) neutrophils are normalized on $CD11b^+Ly6G^+$ total neutrophils.

Interestingly, there is a strong recruitment of fresh neutrophils ($CD62L^+$ $CXCR4^-$) rather than aged ones ($CD62L^-$ $CXCR4^+$), both in WT and IL1R1 KO mice, at 6 hours post-DAMP/PAMP stimulation (Figure 5A and B). Moreover, WT mice recruit more fresh neutrophils than IL1R1 KO mice, both after DAMP and PAMP stimulation. Overall, PAMP stimulation induce higher fresh neutrophils recruitment respect to DAMP, and WT mice recruit more $CD11b^+$ $Ly6G^+$ neutrophils than IL1R1 KO mice, probably because lack the IL1-mediated inflammatory response activation.

On the contrary, at 48 hours post-DAMP/PAMP stimulation there is a stronger recruitment of aged neutrophils, compared to fresh, both in WT and IL1R1 KO mice (Figure 5C). However, there is no significant difference in DAMP- or PAMP-mediated aged neutrophils recruitment, probably because the DAMP contribution on the inflammatory response is more evident at 6 hours. Moreover, WT mice recruit more $CD11b^+$ $Ly6G^+$ neutrophils than IL1R1 KO mice, also at this time point.

Although the role of fresh and aged neutrophils during infections remains to be clarified, we hypothesized that fresh neutrophils could be involved in the confinement of the damage and the containment of the infection, events required in the first phases of the inflammatory response; instead aged neutrophils could have a major role in the

elimination of the pathogen. IL-1 seems not to be involved in a selective recruitment of fresh or aged neutrophils but, of course, it is required for $CD11b^+$ $Ly6G^+$ neutrophils recruitment after microbial skin infections.

Materials and methods

Mice. WT C57BL/6 were supplied by Envigo, Italy. Zanoni's laboratory (Boston Children Hospital, Harvard University, Massachusetts) provided Dectin-1$^{-/-}$, Dectin-2$^{-/-}$, and CARD9$^{-/-}$ mice. TLR4$^{-/-}$ mice and MyD88$^{-/-}$ mice were purchased by The Jackson Laboratory. TLR2$^{-/-}$ mice were provided by Greta Guarda (IRB, Bellinzona, Switzerland). IL-1R1$^{-/-}$ mice were provided from Cecilia Garlanda (Humanitas University, Milan, Italy). All mice were used at 7 to 12 weeks of age. All animals were housed under pathogen-free conditions, and all experiments were carried out in accordance with relevant laws and institutional guidelines.

Candida albicans. The *C. albicans* strain CAF3-1 (*ura3*D*::imm434/ura3*D*::imm434*), provided by W. A. Fonzi (Georgetown University), was routinely grown at 25°C in rich medium [YEPD (yeast extract, peptone, dextrose), 1% (w/v) yeast extract, 2% (w/v) Bacto Peptone, and 2% (w/v) glucose] supplemented with uridine (50 mg/liter) as described. In this growth condition, cells showed a typical yeast morphology, and growth was monitored by counting the cell number using a Coulter Counter-Particle Count and Size Analyser. Once cells reached a concentration of about 8 × 106 cells/ml, the total culture was harvested by centrifugation and resuspended in an equivalent volume of YEPDuridine medium buffered with Hepes (50 mM, pH 7.5). Cells were incubated at 37°C for hyphal induction. Formation of hyphae was evaluated under a microscope at different time points following induction until its amount was assessed at 95%.

C. albicans was gently provided by Marina Vai's laboratory of University of Milano Bicocca.

S. aureus. *S. aureus* ATCC6538P cells were grown in LB medium (Difco) at 37°C. For subcutaneous infections, stationary phase cultures were diluted to an optical density at 600 nm (OD600) of 0.05 and then grown until they reached an OD600 of 0.25 that corresponded approximately to 10^6 colony-forming units (CFU)/ml. Cells were washed in phosphate buffered saline, and appropriate dilutions were injected in mice (10^7 CFU in 50µl of sterile PBS). *S. aureus* was gently provided by Alessandra Polissi's laboratory of University of Milan.

P.aeruginosa. *P. aeruginosa* mutant PW8407 (*fliC*-B03::IS*phoA*/hah) and its isogenic parental strain mPAO1 were obtained from the Comprehensive *P. aeruginosa* Transposon Mutant Library at the University of Washington Genome Center (Jacobs et al. 2003. PNAS 100, 14339; Held et al 2012. J. Bacteriol., 194, 6387-6389). A mutation in the flagellin structural gene *fliC* results in a swimming defect that confers a non-motile phenotype to the strain (Rashid and Kornberg PNAS 2000). *P. aeruginosa* cells were grown in LB medium (Difco) at 37°C.

For subcutaneous infections, stationary phase cultures were diluted to an optical density at 600 nm (OD600) of 0.05 and then grown until they reached an OD600 of 0.20 that correspond approximately to 10^6 colony-forming units (CFU)/ml. Cells were washed in phosphate buffered saline, and appropriate dilutions (10^6 CFU in 50µl of sterile PBS)were injected in mice.

P. aeruginosa was gently provided by Alessandra Polissi's laboratory from University of Milan.

***In vivo* infections.** For *in vivo* treatment, mice were first anesthetized with a mixture of ketamine and xylazine (10 μg/kg body weight), shaved on back with electric clipper and chemically depilated in the flank (dorsal region) 24 hours before injection. The day after, mice were injected intradermally with *C. albicans* hyphae ($5x10^6$ in 50μl of sterile PBS) or *S. aureus* (10^7 CFU in 50μl of sterile PBS) or *P. aeruginosa* (10^6 CFU) in the shaved dorsal region or in the ear. Infected skin was collected at different time points (6-, 24-, and 48-hours) for biochemical and flow cytometry analysis.

DAMP and PAMP stimulation. Danger was stimulated with stratum corneum removal (scratch) using 15 strokes with 220 grit sandpaper (3M, St Paul) according to the method proposed by Botond Z. et al., followed by repeatedly puncture of the top epidermal-dermal sheets using a 28-gauge needle (scarification), according to Gerner MY et al. As PAMP we injected LPS (LPS from *E. Coli*, Serotype O55:B5, Enzo Life Sciences) subcutaneously in the mouse ear (10 μg/ear).

Neutralizing antibodies. Mice were treated with 10 μg/ml of anti-IL-1α (R&D system, Polyclonal goat IgG, cat no. AB-400-NA) and 10 μg/ml anti-IL-1β (Purified NA/LE Hamster Anti-Mouse IL-1β Clone B122, RUO, cat no. 557539) neutralizing antibodies or their isotype controls (R&D system, Polyclonal goat IgG, cat no. AB-108-C; Purified Armenian Hamster IgG Isotype, clone HTK888, cat no. 400901) intradermally, 30' before hyphal injection (same site).

Quantitative reverse transcription polymerase chain reaction. Dorsal regions of the skin were collected, gently washed in cold PBS, lysed in TRIzol reagent, and mechanically disrupted using a TissueLyser (20 shakes/second for 30 minutes). Total RNA was extracted using Direct-zol^{TM} RNA MiniPrep, Zymo Research (cat no. R2050/1/2/3), according to the manufacturer's instructions. A nanodrop spectrophotometer (Thermo Scientific) was used to quantify mRNA and to assess its purity. 600 ng of mRNA were retrotranscribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, cat no. 4368814). cDNA were amplified using the Luna Universal Probe qPCR Master Mix (M3004E, New England BioLabs) with the TaqMan Probes (Cxcl1, Mm04207460_m1, Ly6c, Mm03009946_m1; Gapdh, Mm99999915_m1) or a Luna Universal qPCR Master Mix (M3003E New England BioLabs) (Ly-6G: forward, 5′-TGGACTCTCACAGAAGCAAAG-3′ and reverse, 5′-GCAGAGGTCTTCCTTC- CAACA-3′; Gapdh: forward, 5′-CTGGCCAAGGTCATCCATG-3′ and reverse, 5′-GCCATGCCAGTGAGCTTCC-3′) in a 7500 Fast-Real-Time PCR System (Applied Biosystems), and finally relative mRNA expression was calculated using the ΔCt method, with *Gapdh* as a reference gene.

Isolation of skin cells from mouse ears. Skin was isolated and digested for 90 minutes at 37°C in complete medium (RPMI 5% FBS, L-glutamine and antibiotics) plus 0.25 mg/ml of Liberase (Roche) and 0.2 mg/ml DNase I (Sigma). After the digestion, cells were passed

through a 70 μm cell strainer and centrifuge. Then, FACS analysed were performed.

Flow cytometry. Single-cell suspensions of skin were centrifuged and resuspended with the appropriate amount of antibody in 200 ul of PBS and incubated for 20 minutes on ice in the dark. Cells were washed with 1 ml of PBS. Intracellular staining for cytokines or transcription factor were performed following manufacturer's instructions. For FACS analyses, the following anti-mouse antibodies were used: anti-CD45.2 (APC-Cy7, Biolegend), CD11b (APC, Biolegend), Ly6G (Brillian Violet 510, Biolegend), CXCR4 (FITC, BD Pharmingen), CD62L (PE, BD Pharmingen). Neutrophils were identified as $CD45.2^{+}$ $CD11b^{+}$ $Ly6G^{hi}$ cells. Fresh neutrophils were identified as $CD62L^{+}$ $CXCR4^{-}$ cells, and aged neutrophils were identified as $CD62L^{-}$ $CXCR4^{+}$ cells. Data were acquired using a Gallios flow cytometer (Beckman-Coulter) and analyzed with Kaluza Analysis software.

Statistical analysis. Means were compared by either paired T-test or two-way analysis of variance (ANOVA). Data are expressed and plotted as mean ± S.D. values. Sample sizes for each experimental condition are provided in the figures and the respective legends. Differences were considered significant if $P \leq 0.05$. ($P \leq 0.05$ *; $P \leq 0.01$ ** $P \leq 0.001$ ***). All P values were calculated using Prism (GraphPad).

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Chapter 3

Final considerations

3.1 Summary

Human skin surface is daily challenged by microbes and maintenance of homeostatic balance of immune responses, at mucosal surfaces, is critical for preserve host health.

C. albicans is usually present as commensal microorganisms in the intestinal tract, the skin and the genital mucosa. However, in pathological conditions, it can cause severe mycoses once it reaches the bloodstream or pass the epithelial barriers. *C. albicans* can grow in different morphological forms, such as yeast, pseudohyphae and hyphae. In its hyphal form, it is able to penetrate the epithelium, invading the skin. Once penetrated inside the cutaneous barrier, *C. albicans* is recognized by different PRRs, as CLRs and TLRs, due to the expression on its surface of different PAMPs. Santus et al. recently demonstrated that wild type mice intradermally infected with *C. albicans,* form a capsulated abscess under the skin, that is resolved with ulcer formation and the elimination of the infection requires neutrophils recruitment at the site of the infection. Nevertheless, other microbial skin infections, such as *Staphylococcus aureus,* required neutrophil recruitment for its resolution (Molne L.G. et al, 2000) although the mechanisms by which this occurs is unknown.

We investigated the role of PRRs in neutrophil recruitment in the site of infection, after microbial infections. We analysed innate immune responses in PRR-deficient mice or mice deficient for the adaptor molecules (MyD88- and CARD9) through which these receptors function, after fungal or bacterial skin infections.

We demonstrated that MyD88 is significantly involved in neutrophil recruitment after *C. albicans* skin infection, in a PRR-independent manner.

Considering that IL1-family cytokines signal through MyD88, we investigated the role of IL-1 in the initiation of inflammation and neutrophil recruitment.

Firstly, we evaluated the role of IL-1α and IL-1β in *C. albicans* skin infection, and we found that they are both involved in neutrophil recruitment, although IL-1α seems to have a major role, probably because it is constitutively expressed and it is released immediately after infection, in contrast to IL-1β that needs to be cleaved and, than, released. Furthermore, we confirmed the role of IL-1 *in vivo* using IL1R1 KO mice.

Moreover, we decided to investigate the role of MyD88 and IL-1 also in other types of infections, like Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria.

We confirmed that this IL-1-MyD88-dependent neutrophil recruitment is conserved in bacterial skin infections.

These results suggested us that the initiation of the inflammatory process and the neutrophil recruitment during primary infections, could be due to the unspecific release of alarmins (like IL-1α) by distressed cells. According to this hypothesis, PRRs were not involved in this first phase of inflammation, during microbial skin infections.

Finally, we started to investigate whether DAMPs and PAMPs could have distinct roles in neutrophil recruitment during microbial infections, and, in particular, if they could have a distinct role in recruiting “fresh” or “aged” neutrophils.

3.2 Conclusions and Future Perspectives

Innate immune cells represent the highly conserved defence mechanism that acts immediately upon insult, limiting the spreading of invading pathogens. Moreover, all vertebrates possess a complex immune system that provides them to acquire an evolutionary success against exogenous insults, due to the more specific and efficient adaptive immune system. Inflammation is a response of the innate immune system, activated by detrimental stimuli. It is an unspecific process, characterized by the release of cells and molecules from blood to the site of infection, with the aim to restore tissue homeostasis. Inflammatory mediators activate several downstream signalling that lead to the activation of transcription factors in innate immune cells, like macrophages and dendritic cells (DCs). These events are followed by the production and release of cytokines and chemokines, adhesion molecules, acute phase proteins and other inflammatory mediators.

In the last century, the activation of adaptive immune responses was explained by two theories.

In 1979, Charlie Janeway proposed the “Infectious non-self and non-infectious self” theory, which support the idea that distinct forms of immune recognition, innate and adaptive, played different roles in the immune system. He suggested that innate immunity is mediated by a group of non-clonal receptors, called Pattern Recognition Receptors (PRRs), which detect conserved component of microorganisms, or Pathogen-Associated-Molecular-Patterns (PAMPs). PRRs are expressed by cells of innate immunity, like dendritic cells (DCs) and,

once the pathogen has been recognised, they trigger DCs activation, upregulation of costimulatory molecules and, later, cytokines production. These events are propaedeutic to adaptive immunity and T cells activation (Janeway C.A., 1989).

In 1994, Polly Matzinger tried to explain several facts that Janeway's theory does not clarified, such as tolerance to the commensal microbiota, responses throughout trauma, and autoimmunity. She proposed her "Danger theory", according to which the immune system can recognize damage signals, independently of self/non-self discrimination. She supposed that Damage Associated Molecular Patterns (DAMPs) are conserved and expressed self-molecules, released by distressed or necrotic cells, and recognised by antigen presenting cells, activating an innate immune response (Matzinger P. et al, 1994). However, the activation of DCs can be induced also by exogenous danger signals derived from pathogens, such as peptide toxins, that, once they are released, disrupt epithelial barriers, activating host immune responses. Importantly, it has not been completely clarified whether danger signals are sufficient, or not, to initiate the activation of adaptive immunity.

Nevertheless, Charlie Janeway and Polly Matzinger theories do not explain the activation of the inflammatory process that occurs after infections. Interestingly, a combination of these two theories, seen as the coincidence of DAMPs, released after tissue damage before infections, and microorganisms PAMPs, could constitute the signals for the activation of innate immunity and the inflammatory process.

During inflammation and, in particular, during pathogen infections, the first immune cells recruited are neutrophils. Santus et al. recently demonstrated that neutrophils are recruited at the site of infection after intradermal infections with *C. albicans*. However, the mechanism by which this occurs remained to be clarified.

C. albicans is usually present in the healthy microbiota of individuals. However, under pathological conditions, when the immune system is compromised, it can lead to severe mycoses, causing infections. Several recent studies reveal that fungal infections severely compromised the health status of hospitalized people with severe comorbidities, or post-operative patients. One of the main issue of candidiasis is its diagnosis, because *C. albicans* infections mimic bacterial infections, and the diagnosis originates from the resistance of the infection to antibiotic therapy. Moreover, candidemia can affects patients with neutropenia, caused by pathological conditions, such as blood cancer or immunosuppressive therapies. Nevertheless, *C. albicans* can survive on medical devices, such as catheters and artificial heart valves, forming biofilms on these surfaces.

Moreover, other type of pathogen, such as *Staphylococcus aureus* (Gram-positive bacterium) and *Pseudomonas aeruginosa* (Gram-negative bacterium), can cause severe infections in immunocompromised patients, because of their antibiotic resistance.

This evidence highlighted the need to better understand the mechanisms that control microbial infections, in order to define new

therapeutic targets to polarize immune response, hindering the spreading of infections, and to prevent the worsening of the susceptible patient's health.

Nowadays, we know that PRRs, like TLR4, recognize also danger signals, such as the endogenous oxidized phospholipid oxPAPC (Zanoni, Tan et al. 2016). However, it has not been clarified if DAMPs are sufficient to activate adaptive responses independently from PAMPs recognition. Moreover, several evidences demonstrate that PRRs are dispensable for immune cells recruitment during microbial infections.

We defined how neutrophils are recruited at the site of infection, after different types of pathogen skin infection. We demonstrated that IL1R1-MyD88 dependent signalling pathway is important for neutrophils recruitment within 6-24 hours post *C. albicans, S. aureus* and *P. aeruginosa* intradermal infection. Although these microorganisms bear different PAMPs, we hypothesized that the mechanism of neutrophil recruitment in the first phase of infections is a stereotyped response, independent of the type of infection, necessary to confine the infection. We supposed that, after this first step of infection containment, PRRs act to specifically eliminate the pathogen through its PAMP recognition. This idea could represent an evolutionary advantage to quickly fight microbial infections. Of course, these data need to be validated also through other methodologies, e.g. immunohistochemistry for Ly6G expression on infected tissue sections,

and $CD45.2^+$ $CD11b^+$ $Ly6G^{hi}$ cells recruitment through FACS analysis of cutaneous samples after pathogens skin infection.

Moreover, in the last years it has been proposed that neutrophil aging have a functional role in the immune defence and vascular protection. For this reason, we were interested in the comprehension of whether DAMPs and PAMPs recognition could influence a different neutrophil recruitment.

We found that fresh neutrophils are preferentially recruited within 6 hours post DAMP/PAMP stimulation, whereas aged neutrophils are dominant later, about 2 days after infection. We hypothesized that fresh neutrophils are recruited earlier, because they could be necessary to avoid the spreading of the infection. Once the infection is contained, aged neutrophils recruitment could be important to eliminate the pathogen.

Interestingly, the idea that neutrophils aging could be a tool to better understand the quality of immune responses after infections is an attractive point of view and would be a novelty in the comprehension of the immune response activated after microbial infections.

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