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Balancing tissue homeostasis and inflammatory responses against *Candida albicans* infections: is it a matter of mast cells' immunological memory?

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Personalmente penso che le qualità dell'uomo vengono esaltate dalla sua capacità di elevarsi nella conoscenza, più che nel suo talento di coltivare antiche illusioni. Per far questo, purtroppo, è però necessario rinunciare a un certo mondo incantato; è necessario cioè uscire dalla stanza delle fiabe e dei racconti fantastici. Ciò può essere scomodo e anche deludente. Personalmente non lo penso. Penso che l'avventura dell'intelligenza sia molto più stimolante di quella della credulità. E che il desiderio di scoprire sia più eccitante di quello di rimanere nella stanza delle fiabe. E credo che, comunque, sia difficile rimanerci quando la porta è ormai aperta.

Piero Angela - *Viaggio nel mondo del paranormale*, 1978

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1 ABSTRACT

Mast cells (MC) are long lived immune cells widely distributed at mucosal surfaces among the first immune cell type that can get in contact with the external environment. This study aims to unravel the mechanisms of reciprocal influence between mucosal mast cells, the human commensal *Candida albicans* and microbial bioproducts. Stimulation of bone marrow-derived mast cells (BMMC) with live forms of *C. albicans* induced the release of TNF-α, IL-6, IL-13 and IL-4. Quite interestingly, BMMC were able to engulf *C. albicans* hyphae, rearranging their α-tubulin cytoskeleton accumulating LAMP1$^+$ vesicles at the phagocytic synapse with the fungus. Infected MCs failed to promote macrophage chemotaxis but instead increased their crawling ability. On the other side, resting MCs inhibited macrophage phagocytosis of *C. albicans* in a contact-dependent manner.

Several microbial ligands are known to induce immune memory on macrophages and natural killer cells. To determine whether this is true also for MCs and if their training could afford protection against fungal infections, BMMCs were stimulated for 24 hour with microbial ligands, washed and allowed to rest for 6 days and then restimulated with LPS or *C. albicans*. LPS-primed cells showed a decreased release of TNF-α and IL-6 upon restimulation with LPS (a condition known as endotoxin tolerance) but an increased release of TNF-α during fungal challenge. The impaired response during endotoxin tolerance relied on the impaired activation of the PI3K-AKT-p38 pathway, which led to a decreased NF-κB activation and TNF-α expression. On the other side, the training effect of LPS was mediated by the enhanced activation of NF-κB and the sustained basal transcription of TNF-α.

These results indicate that MCs are important gatekeeper of tissue homeostasis. Signals received from the microbiota can shape their immune memory, dampening the inflammation in response to bacterial LPS but affording protection during fungal infections.

Part I

THEORETICAL BACKGROUND

2 INTRODUCTION

2.1 WHY DO WE HAVE MAST CELLS?

THE FIRST THING that comes to mind when trying to describe mast cells (MCs) is *"Those cells responsible for your itching, sneezing and allergies"*. The reason for such a bad reputation needs to be searched in history: these neglected cells have been described as principal effectors in anaphylaxis (and, later, allergy) since the beginning of the twentieth century.

MCs are granulated, tissue resident cells that belong to the innate arm of the immune system. These cells were firstly observed in 1863 by Friedrich von Recklinghausen which described them as granular cells found in frog mesentery[1]. 14 years later, MCs were identified and characterized also in human tissues by the medical student Paul Ehrlich in his thesis on the use of aniline dyes on microscopic specimens. He described MCs as aniline-positive cells localized around blood vessels in connective tissues, which granules content stained metachromatically with aniline dyes[2]. These cells were named *"Mastzellen"* after the german word *"mast"* (i.e. "fattening"), by virtue of their granules which were thought to have a nutritional function. Paul Ehrlich studies on MCs led him to be awarded in 1908, together with Ilya Ilyich Mechnikov, of the Nobel Prize in Physiology or Medicine. Later in 1902, Paul Portier and Charles Richet introduced the concept of anaphylaxis (even though analogous observations were made earlier in 1873 by Charles Blakely) as the symptoms of anaphylactic shock caused by the extract of the jellyfish *Phycalia* tentacles on dogs that were already challenged with the same extract several days earlier[3,4]. Further studies on the mechanisms of anaphylaxis led Richet to be awarded with the Nobel Prize in Physiology or Medicine five years after Ehrlich. Only four years later, in 1906, a similar concept was introduced by Clemens von Pirquet during his studies on smallpox vaccine administration as he noted that some patients showed worse adverse effects following the re-administration of the vaccine[5]. These observations led him to coin the word allergy (after the greek *allos*, diverse, and *ergia*, energy) to describe the phenomenon. Starting form 1910, when Dale and Laidlaw described for the first time histamine, different studies were performed to unravel the nature of anaphylaxis[6]. Albeit being supposed for many years, it was only in dur-

Figure 2.1.1: German 200 Deutsche Mark banknote. The banknote, depicting Paul Ehrlich and the chemical structure of Arsphenamine, was issued by the Deutsche Bundesbank in 1989. From the Paul-Ehrlich-Institut website. `http:\www.pei.de`

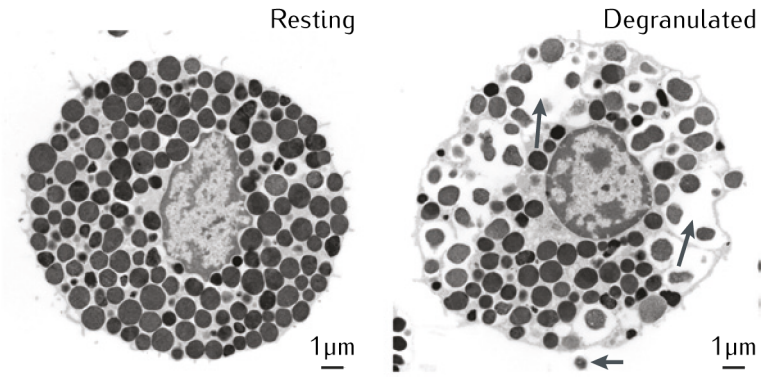

Figure 2.1.2: Transmission electron microscopy image of mast cells. Resting and degranulated rat peritoneal MCs. The resting cell show an homogenous distribution of granules which contain electron-dense material. During the degranulation process, granules are fused together and released from the cell. Long arrows indicate two regions where multiple granules have extensively fused; the short arrow indicates an exocytosed membrane-free granule remnant. Adapted from Wernersson and Pejler[15].

ing the 50's that the presence of histamine in MCs granules was confirmed[7]. In the same years, different studies determined that MCs were the predominant source of this molecule and, moreover, that different compounds (e.g. stilbamidine, compound 48/80) were able to induce the release of histamine by MCs eventually inducing anaphylactic shock[8,9]. Finally, a connection between MCs, histamine and anaphylaxis was defined.

A key point was still missing: which mechanism was responsible for MCs activation during allergy? Albeit primitive answers were already given during the 20's, it was only in 1959 that Ishizaka and Campbell determined how antigen-antibody complexes were able to induce passive cutaneous anaphylaxis in guinea pigs[10]. In a follow-up study, Ishizaka and Ishizaka finally identified the specific reaginic antibody as γE-antibodies (IgE)[11]. From this day on, many different studies have been performed to identify and characterize the putative IgE receptor on MCs surface. Finally, in 1989, the high affinity IgE receptor FcϵRI and its subunits were cloned and characterized[14].

***IgE** is an independent class of immunoglobulin characterized by the ϵ heavy chain. IgE antibodies are monomeric soluble proteins of ~180kDa and are a minor component of the Ig pool under normal conditions (<0.01% in human serum)[12,13].*

2.1.1 A peculiar cell in our bodies. MCs origin, development and biology.

MCs are tissue-resident cells widely distributed through the body and are particularly abundant in the skin, gut and airways. They are generally localized close to epithelial cell, venules, nerves and smooth muscle cells, and thus are one of the first immune cell type that can get in contact with signals from the external environment. The ability to detect and respond to external signals is ensured by the wide plethora of receptors expressed by these cells and by their ability to release many different soluble mediators. MCs possess a high number of cytoplasmic granules which contain a great number of preformed immunomodulatory compounds (Fig. 2.1.2). Various signals can trigger MCs activation and degranulation, i.e. a rapid release of granule content, but can also induce the synthesis of many biologically active molecules.

Evolutionary aspects.

It is believed that MCs may derive from a leukocyte ancestor which shared functional characteristics similar to those of actual invertebrate granular haemocytes. These cells were presumably involved in the protection of the host by encapsulation, phagocytosis and killing of pathogens[16]. A putative MC-basophil ancestor has been described in the `ascidian` *Styela plicata* (Fig. 2.1.3). These organism possess circulating granular haemocytes which ultrastructurally resemble vertebrate MCs and contain both histamine and heparin. In the same organism were also reported accessory cells termed "test cells" which contain histamine and tryptase, and can be activated by the vertebrate MCs secretagogue Compound 48/80[18,19]. MC-like cells can be found in many `Perciformes` and have been described to contain histamine[20]. Also zebrafish (*Danio rerio* Hamilton, order Cypriniformes) possess granulated cells mainly localized in the gills and in the intestine. These cells are very similar to murine MCs, can be stained metachromatically with toluidine blue and stain positively for tryptase and KIT, the stem cell factor (SCF) receptor. Strikingly, these cells express a FcϵRI-like receptor[22,23]. Albeit being described in zebrafish MCs, FcϵRI is believed to be a recent acquisition in MCs evolution seen that IgE (and IgG) originated in mammals probably from an IgY-like ancestor[16,24,25]. Moreover, there is still no evidence of a specific IgY or IgM receptor on avian, reptile or amphibian MCs, nor about the possible role of these antibodies in MCs activation. During mammal evolution, and particularly during primate speciation, a great number of mutations occurred in the chymase and tryptase gene locus, leading to an enormous gene diversification in different mammalian species[26,27]. The fact that these ancient cells persisted trough 500 million years of evolution indicates that they still play a role in the maintenance of host immunity. Their role clearly changed from a mere effector against pathogens to a more complex player in the regulation of both the innate and the adaptive arms of immunity, as well as many different non-immunological processes.

Ascidian *(class Ascidiacea, subphylum Tunicata, phylum Chordata) are a group of marine invertebrate organism which are closely related to vertebrates and have probably arisen ≈550 million years ago during the "Cambrian explosion"[17].*

Perciformes *(class Actinopterygii, phylum Chordata) are the largest order of vertebrates and represent the most evolutionarily advanced clade of teleost fish[21].*

Mast cell development.

MCs originate in the bone marrow from an hematopoietic progenitor through a myeloid lineage but, unlike other myeloid-derived cells, MCs progenitors leave the bone marrow at an early stage of differentiation to enter the circulation. Once in the blood stream, they rapidly migrate to peripheral tissues (so rapidly that they are barely detectable in peripheral blood) and complete their differentiation into mature MCs with tissue-specific phenotypes[29]. This uncommon developmental process is one of the peculiar trait of these unique cells.

MCs lineage development has been studied mostly in mice but is still rather debated. Early studies in the 70s by Kitamura et al. indicated that MCs were originated in the bone marrow (BM) but only in recent years it was demonstrated that MCs develop through the myeloid lineage from a Kit^+ $Sca1^+$ hematopoietic stem cell (HSC), to a Kit^+ $Sca1^{lo}$ FcγRII/III^{lo} common myeloid progenitor (CMP), and finally to a Kit^+ $Sca1^-$ FcγRII/III^{hi} granulocyte monocyte progenitor (GMP) (Fig. 2.1.4)[30,31]. This GMP is supposed to be shared by MCs, basophils, eosinophils, neutrophils and mono-

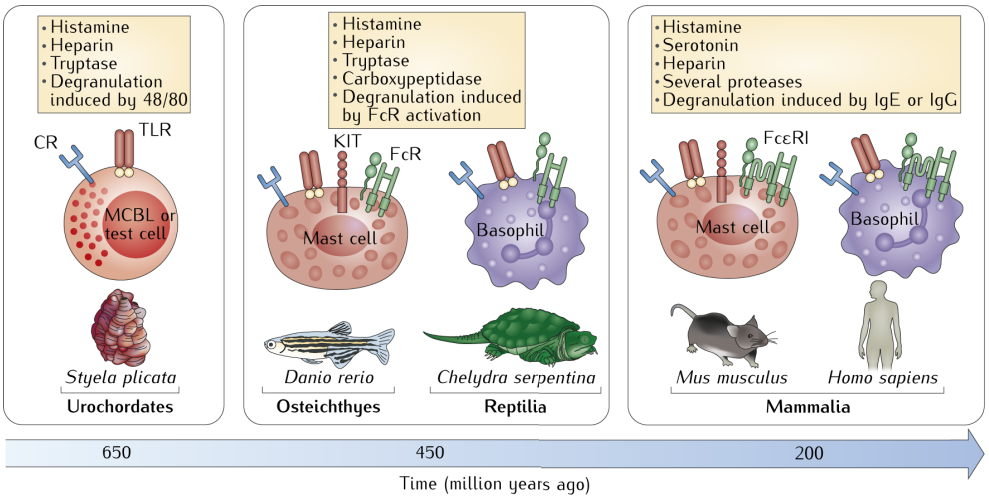

Figure 2.1.3: Evolution of mast cells and basophils. The first putative ancestor for MCs and basophil was found in the ascidian *Styela plicata*. This urochordate possess mast cell/basophil like (MCBL) granular cells and test cells, both containing histamine and heparin. With the divergence of teleost fish and reptiles, also MCs and basophil lineages separated. Both cells express activating FcR-like receptors and reptile basophils can degranulate in response to FcR triggering. With the comparison of mammals and IgE these cells acquired the complete Fcϵ receptor while the protease gene family underwent a great diversification. Adapted from Voehringer[28].

cytes. In addition, it appears that the commitment of this GMP to MCs or basophils is tightly regulated by the expression levels of the transcription factors GATA-binding protein 2 (GATA2) and CCAAT/enhancer-binding protein α (C/EBPα)[32]. On the contrary, two recent works demonstrated that mast cell progenitors (MCP) may derive directly from a CMP. The authors identified a rare population of Kit^{+} Sca1^{-} β7 integrin^{+} FcγRII/III^{lo} MCP derived from a Kit^{+} Sca1^{lo} CMP that sorted with the megakaryocite-erithroid lineage[33,34]. Increasing the complexity of this picture, a LIN^{-} CD34^{+} Kit^{+} FcϵRIα^{-} β7 integrin^{hi} FcγRII/III^{hi} bipotent basophil/mast cell progenitor (BMCP) was identified in the spleen of adult C57BL/6 mice and appeared to fill an intermediate step between GMP and MCP. Again, GATA2 and C/EBPα regulate the commitment of this progenitor at least *in vitro*, as the complete deletion of C/EBPα favors the development of MCs while the overexpression of the same transcription factor give rise to a basophil population[35]. The role of GATA2 and C/EBPα transcription factor in the decision of MCs fate was confirmed also by two works recently published[36,37]. Ohmori et al. showed that deletion of GATA2 in differentiated bone marrow-derived mast cells (BMMC) resulted in a loss of MCs phenotype and to the upregulation of C/EBPα; likewise, the re-expression of C/EBPα in mature MCs led to an equivalent loss of phenotype[36]. Similarly, the overexpression of C/EBPα in differentiated BMMC suppressed granule formation and downregulated the expression levels of different mast cell proteases (e.g. Mcpt-1, Mcpt-2, Mcpt-4)[37].

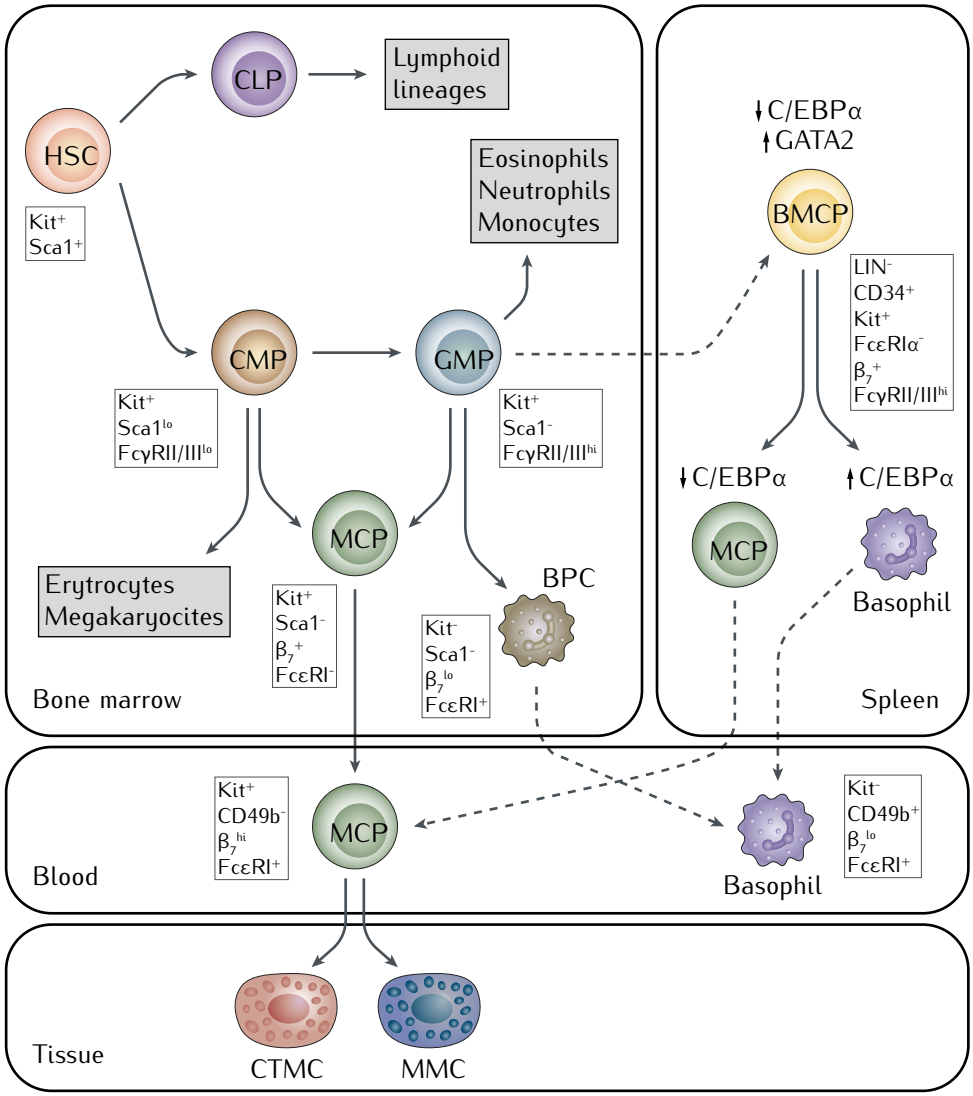

Figure 2.1.4: Model of murine MC lineage development. MC progenitors (MCP) originate in the bone marrow from hematopoietic stem cells (HSC), to a common myeloid progenitor (CMP) and a granulocyte monocyte progenitor (GMP) - which also gives rise to neutrophils, eosinophils, monocytes and basophils. It is also proposed that MCP originate directly from a CMP that sorts with the megakaryocite-erithroid lineage. Moreover, a bipotent basophil/mast cell progenitor (BMCP) which can further differentiate *in vitro* to MCs or basophils, was identified in the spleen. Once released in the blood stream, MCs quickly migrate to peripheral tissues where they complete their differentiation.

After entering the blood stream, MCP quickly leave it to enter into tissues by transendothelial migration. Nowadays it is known that many integrins and chemokines are involved in the recruitment of MCs at peripheral site. For instance, $\alpha 4\beta 7$ integrin, its ligands mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) and vascular cell adhesion molecule 1 (VCAM-1), and the CXC chemokine receptor 2 (CXCR2) are fundamental for the homing of MCP to the intestine[38]. Moreover, mice lacking integrin $\alpha M\beta 2$ and $\alpha IIb\beta 3$ have an impaired number of MCs in the peritoneum while injection of the chemokine CCL2 recruited MCP to the skin[39–41]. Recent data show that peritoneal MCs and -to a lower extent- skin MCs stained positive for the proliferation marker Ki67 suggesting that this cells might also locally proliferate[42].

Contrarily from most hematopoietic lineages, cKit expression is not lost with MCs differentiation. As a general rule, MCs development, survival and proliferation is dependent on stem cell factor (SCF), which binds cKit, and IL-3 signaling. However, *in vitro* stimulation with IL-3 is sufficient for murine BMMC development and addition of SCF synergizes for optimal MCs proliferation[43]. Surprisingly, *Il-3*-deficient mice are able to develop MCs despite their impaired IL-3 signaling[44]. Recent studies determined that also human $CD34^+$ mast cell progenitors could functionally develop *in vitro* in the absence of IL-3 or in the absence of SCF and KIT signaling[45,46]. This last observation might somehow reflect the fact that in MC-deficient $Kit^{W/W\text{-}v}$ mice (which harbor a mutated version of cKit, see Tab. 2.1.2) MCs deficiency can be partially reverted by chronic inflammatory stimuli as idiopathic chronic dermatitis[47,48].

Differential stimulation of MCs during their maturation leads to the development of MCs with peculiar phenotypes. This concept is of fundamental importance because *in vivo* MCs terminal differentiation occurs at peripheral sites where the microenvironment signals (cytokines, growth factors, cell-cell contact) are specific for all the distinct anatomical localizations, thus giving rise to MCs with tissue-specific phenotypes[29]. Moreover, MCs can dynamically change their phenotype also after being fully matured as the microenvironmental condition change - a process which is likely to occur since MCs are long lived cell and can survive in tissues for up to 12 weeks[49]. This concept of plasticity will be thoroughly discussed in the next section.

Mast cell biology.

Albeit their plasticity, murine mature MCs can be divided into two main categories on the basis of their localization and granule content: connective-tissue mast cells (CTMCs) and mucosal mast cells (MMCs). CTMCs are constitutively present in tissues and are mainly localized in serosal cavities, around venules and nerves. On the other side, MMCs are predominantly present in the mucosal tissues of the gut and in the respiratory tract mucosa[29,50]. MMCs are lower in number and expand during infection in a T-cell dependent manner seen that athymic mice fail to induce MMCs expansion during *Trichinella spiralis* infections[51]. At "baseline", MCs subpopulations can be distinguished on the basis of their protease and proteoglycan content (Tab. 2.1.1). MMCs express transcripts for the chymases MCPT1 and MCPT2,

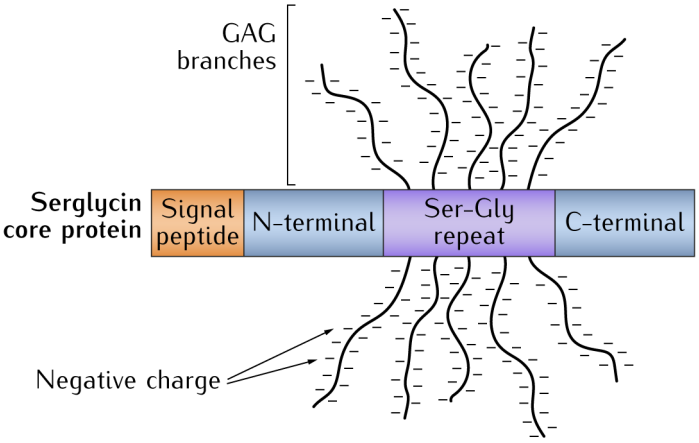

Figure 2.1.5: Serglycin structure. Serglycin core protein is characterized by a ~20 aa Ser-Gly repeat region, in which every repeat constitute a potential attachment site for GAG chains (heparin or chondroitin sulphate). GAG: glycosaminoglycan. Adapted from Wernersson and Pejler[15].

and contain serotonin but no (or little) histamine. On the contrary, CTMCs express the chymase MCPT4, the elastase MCPT5, the tryptases MCPT6 and MCPT7 (except for C57BL/6 mice which present a point mutation on the *Mcpt7* gene[52]) and Carboxypeptidase A4 (CPA3)[53–56]. CTMCs also contain serotonin and high levels of histamine[57]. Most importantly, the protease content of both MCs subsets can vary with the different anatomical localization of the cells. For instance, MMCs in the intestine of *T. spiralis* infected mice expressed only MCPT1, while CTMCs expressed both MCPT4, MCPT5, MCPT6, MCPT7 and CPA3[55,56]. On the other side, in an allergic inflammation model both tracheal MMC and CTMC expressed all the six proteases, while in the proximal bronchi MMC expressed only MCPT1, MCPT6 and MCPT7 while CTMCs were still positive for all the six proteases[58]. A recent work by Dwyer et al. compared different subsets of murine MCs purified from different anatomical compartments and demonstrated that these cells had considerable tissue-specific gene expression: among the most differentially expressed genes the authors found proteases, G protein-coupled receptors and homing markers as integrins[42].

A better marker which can be used to distinguish among MMCs and CTMCs, is the proteoglycan content of their granules. These heavily glycosylated proteins are fundamental for MCs granule development, homeostasis and for the storage of several MCs proteases[59]. Serglycin is a peculiar proteoglycan core protein highly expressed by MCs, which present an extended region of Ser-Gly repeats (~20 aa in mouse and human) with every Ser-Gly repeat constituting a potential attachment site for glycosaminoglycans (GAG) chains[60]. The high anionic charge of GAG is believed to interact electrostatically with the net positive charge of several MCs proteases such as MCTP4, MCPT5, MCPT6 and CPA3 (Fig. 2.1.5)[61]. MCs contain two different GAG: heparin and chondroitin sulphates (di-B, A and E). In particular, MMCs contain mainly chondroitin sulphate di-B, A and E, while CTMCs contain both chondroitin sulphate E and high amounts of heparin (which allow them to be stained with Safranin)[62–64].

Taken together this data increase the complexity of the picture and indicates that MCs maturation in peripheral tissues can give rise to a great number of MCs-subset with small or moderate differences in their phenotype, thus making it harder to make generalizations about MCs role independently of their localization. This concept of plasticity is further confirmed by *in vitro* studies. BMMC are considered partially immature MCs (if compared with mature *in vivo* isolated MCs) and their phenotype is thought to be an intermediate form between MMCs and CTMCs: they express MCPT5, MCPT6 and CPA3 (which is a CTMC-like phenotype) but the proteoglycan content is more similar to those of MMCs (low levels of histamine and heparin)[59,65]. Anyway, culturing BMMC on a monolayer of fibroblast and in presence of IL-3, increased the granule content of heparin and histamine and led to the development of MCs more similar to CTMCs[66]. This mechanism seems to be mediated by the release of SCF by fibroblast and culturing BMMC with IL3 and SCF phenocopied MCs obtained by the culture with fibroblast but without a full maturation of granules, suggesting that cell-cell contact is indeed fundamental for the maturation process[67]. In terms of protease content, BMMC obtained with IL-3 stimulation alone expressed MCPT5, MCPT6 and CPA3. Interestingly, these cells could be induced to express also MCPT4, MCPT1, MCPT2 or higher levels of MCPT6 by the addition of SCF, IL-9, IL-10 or IL-33 respectively[68–70].

Human MCs can also be divided into three main categories on the basis of the proteases stored in their granules: tryptase-positive MCs (MC_T), tryptase- and chymase-positive MCs (MC_{CT}), and chymase-positive MCs (MC_C) (see Tab. 2.1.1). MC_{CT} can be considered the human counterpart of murine CTMCs and are mainly localized in the skin, gastro-intestinal submucosae and esophageal subepithelium, while MC_T are more similar to murine MMC and are particularly abundant in the lung and in the gastro-intestinal mucosae[71,72]. On the contrary, MC_C are quite infrequent and have no peculiar localization[72]. Also human MC_T are thought to require T-cell for their expansion seen that patients with acquired or congenital T-cell deficiencies also show reduced number of MC_T[73]. Both MC_T and MC_{CT} subpopulations contain heparin and chondroitin sulphate (A ad E)[74].

Approaches to study mast cells in vivo

Over the past decades several animal models of MCs-deficiency have been developed in order to study MCs roles *in vivo* (Tab. 2.1.2). The first MCs deficient mice described, harbored naturally occurring loss-of-function mutations at the KIT-encoding locus (known as white spotting locus W). The two main models used are $Kit^{W/W\text{-}v}$ and $Kit^{W\text{-}sh/W\text{-}sh}$. $Kit^{W/W\text{-}v}$ mice carry one allele (W) with a point mutation which encodes a truncated version of KIT, and one allele (W-v) with a point mutation in the tyrosine kinase domain of KIT. This mutations causes the depletion of all MCs populations but also cause many important defects due to the loss of KIT: mice are sterile due to the lack of germ cells, are profoundly anemic and neutropenic, lack interstitial cells of Cajal in the small intestine and have an impaired $\gamma\delta$-T cells development in the gut[78]. The other model, $Kit^{W\text{-}sh/W\text{-}sh}$, present a 3 Mb inversion in chromosome 5 ~70 kb upstream of the KIT gene, causing abnormal KIT ex-

MC subtypes	Murine		Human	
	MMC	CTMC	MC_T	MC_{TC}
Anatomical localization	Respiratory tract; gastric, small intestinal and colonic mucosae[50,75–77]	Skin; tongue; trachea; large airways; serosal cavities[50,75–77]	Lung alveoli and bronchi; gastric, small intestinal and colonic mucosae[71,72]	Skin; breast parenchima and skin; axillary lymph nodes; esophageal subepithelium; gastric, small intestinal and colonic mucosae[71,72]
Proteases	MCPT1, 2[53–56]	MCPT4, 5, 6, 7 and CPA3[53–56]	Tryptase, CPA3 and cathepsin G	Tryptase, chymase, CPA3 and cathepsin G
Proteoglycans	Chondroitin sulphate di-B, A, E[59]	Heparin, Chondroitin sulphate E[59]	Heparin, Chondroitin sulphate A, E[59,74]	Heparin, Chondroitin sulphate A, E[59,74]
Biogenic amines	Histamine (low), serotonin[57]	Histamine (high), serotonin[57]	Histamine	Histamine, serotonin (low)
Histochemical staining	Alcian blue$^+$, toluidine blue$^+$ [57,76]	Alcian blue$^+$, safranin$^+$, toluidine blue$^+$ [57,76]	Tryptase$^+$, toluidine blue$^+$ [72]	Tryptase$^+$, chymase$^+$, toluidine blue$^+$ [72]

Table 2.1.1: Murine and human MC subtypes. This table summarize the main phenotypic characteristics of murine and human MCs at "baseline". Murine MCs can be classified in two main subpopulations based on the granule content. While the protease content can vary during infections or with the anatomical localization, the proteoglycan content remains almost fixed. Connective tissue MCs (CTMC) are rich in heparin (which positively stain with Safranin red) while mucosal MCs (MMC) contain mainly chondroitin sulphates. Human MCs can also be dived in two categories, based on the protease content: tryptase-positive MCs (MC_T), tryptase- and chymase-positive MCs (MC_{CT}). MC_T can be considered the human counterpart for murine MMC (and are both T cell-dependent) while MC_{CT} are more similar to CTMC. All the references are indicated in the table.

pression. Despite the fact that these mice present less hematological and non-hematological abnormalities, the large inversion in Chr5 contains 27 genes which are thought to be responsible for aberrant myelopoiesis, splenomegaly, neutrophilia, megakaryocytosis and thrombocytosis. Moreover, these mice suffer mild hypertension and cardiac hypertrophy due to the lack of the cardiac protease corin[79,80]. To overcome this multitude of non-MCs-related abnormalities, and thus confirming that a specific *in vivo* reaction is only due to the lack of MCs, BMMC are engrafted in *Kit* mutant mice in order to restore MCs population (the so-called "MCs knock-in mouse")[81]. Albeit this approach have been considered the gold standard to study MC function *in vivo*, it is clear now that the reconstitution of *Kit* mutant mice can lead to non-physiological accumulation and distribution of MCs in the animal, thus limiting the interpretation of experimental results[82]. In recent years, several groups generated new strains of MC-deficient mice independent on *Kit* mutations. Most of them were able to deplete MCs population by generating transgenic mice which harbor a Cre recombinase under the control of specific MCs genes (i.e. *Mcpt5*, *Cpa3* and *FcεRIβ*)[83–86]. These mice can be constitutively depleted of MCs (as in the case of $Cpa3^{Cre}$, *Cpa3*-Cre × $Mcl1^{fl/fl}$ and *Mcpt5*-Cre × R-DTA) or induced by the injection of diphtheria toxin (*Mcpt5*-Cre × iDTR and RMB mice). Differently from *Kit* mutant mice, these animals show far less hematological abnormalities and can thus be considered better models to study MCs roles *in vivo*, opening the scenario to a more rigorous evaluation of specific MCs gene functions[87].

The analysis of MCs role in human health is made more challenging by the fact that, apart from patients with T-cell deficiencies which show reduced number of MC_T, no MCs deficiency in humans have been reported[73,87]. The more likely explanation is that the lack of MCs does not cause any immunodeficiency or deviation of the immune response, so that these individuals might be unconscious of their status[87].

2.1.2 Mast cells: tissue-resident "rabbit ears".

The ability of MCs to respond to different pathogens rely on their ability to detect the presence of "non-self" antigens and other danger signals. According to their localization and their role of tissue-resident sentinels, MCs express a wide plethora of receptors aimed to interact with and detect pathogens such as toll-like receptors (TLRs), C-type lectin receptors (CLRs), complement receptors and several cytokine- and chemokine-receptors[89]. Activation of MCs can lead to a two-phased response in which a fast release of granule-stored mediators (the so-called degranulation process) is followed by the *de novo* synthesis and exocytosis of a variety of compounds (Fig. 2.1.6)[90]. Mast cells can release their granule content by means of three distinct mechanism: the classical exocytosis, where secretory granules are singularly extruded by fusion with the plasma membrane; the compound exocytosis, where multiple granules are fused together and then released as a single unit; and finally through the piecemeal degranulation, a peculiar secretion pattern (common also to basophils and eosinophils) in which granule containers are not fused together nor with the plasma membrane

Mouse strain	MCs deletion mechanism	Strain characteristics	Ref.
Kit$^{W/W\text{-}v}$ (W/Wv)	*Kit*^{W} allele: point mutation produces a truncated cKit protein which cannot be expressed on the cell surface; *Kit*$^{W\text{-}v}$ allele: encodes for cKit with impaired kinase activity due to a point mutation in the Tyr-kinase domain.	Depletion of 99% MCs and 75%-90% basophils. Mice are sterile, present macrocytic anaemia, impaired $\gamma\delta^{+}$-T cells, neutropenia, lack of interstitial cells of Cajal and melanocytes. MC-deficiency can be partially rescued by chronic inflammation.	48,78
Kit$^{W\text{-}sh/W\text{-}sh}$ (W-sash)	Reduced expression of cKit due to a large inversion of chromosome 5, ~75 kb upstream the cKit locus.	Depletion of 99% MCs. Mice present aberrant myelopoiesis, splenomegaly, neutrophilia, megakaryocytosis and thrombocytosis.	79,80,86,88
Mcpt5-Cre	Diphtheria toxin-induced deletion	Mice lack >90% CTMC. MMC and basophils are not deleted.	83
Cpa3^{Cre} (Cre-master)	Cre-mediated cytotoxicity	Depletion of both CTMC and MMC and 60% deletion of splenic basophils.	84
Cpa3-Cre (Hello *Kit*ty)	Cross to *Mcl1*$^{fl/fl}$, knock out of the intracellular anti-apoptotic factor *Mcl1*	Depletion of >90% CTMC and MMC, and 60-80% of basophils. Mice present macrocytic anaemia and splenic neutrophilia.	85
RMB	Diphtheria toxin-induced deletion. The 3′-UTR of the *Ms4a2* gene encoding the FcϵRIβ includes a red td-Tomato (tdT) cassette and the human diphtheria toxin receptor (hDTR).	95% MCs and basophils are depleted within 2 days. Basophil population is restored 12 days after DT injection while MCs levels remains low even after 6 months.	86

Table 2.1.2: Murine models to study mast cells *in vivo*. This table summarize the key features of the most used mice models of MCs deficiency. *Kit* mutant mice have been so far the most used models but show also many hematological and non-hematological abnormalities unrelated with MCs deficiency which can limit the interpretation of experimental results. In recent years, different new models of *Kit*-independent MCs deficiency have been generated. These new breed present far less phenotypic abnormalities and represent better alternatives to study MCs functions *in vivo*. All the references are indicated in the table.

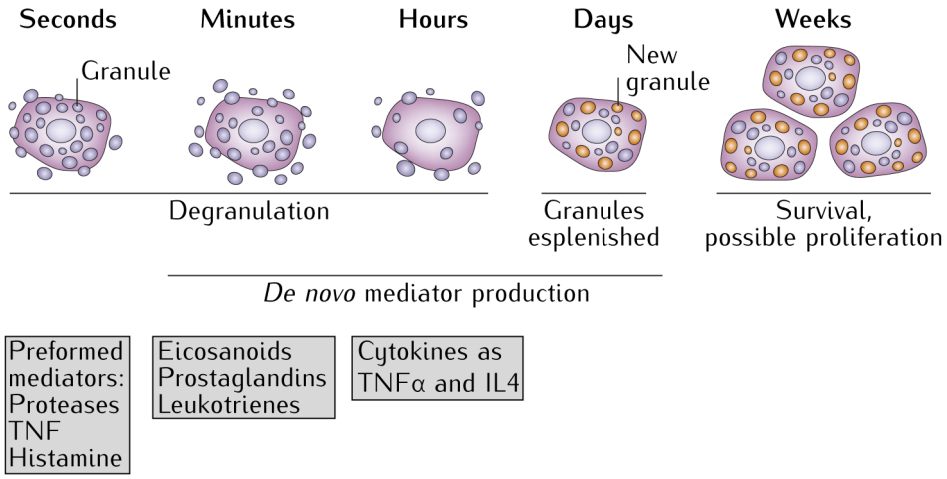

Figure 2.1.6: Timing of mast cell response to challenges. MCs earliest response begins with the release – within seconds – of granule-stored preformed mediators. Soon after, MCs are able to synthesize and release arachidonic acid-derived lipid mediators as leukotrienes and prostaglandins. A second phase of response is dependent on the release of *de novo* synthesized mediators comprising a great number of cytokines and chemokines. By virtue of being long-lived cells, MCs can survive after the stimulation and replenish their granules, thus allowing their response to further challenges. Adapted from Abraham and St John [90].

but are emptied and kept intact in the cytoplasm, allowing also a partial release of granule content[91,92]. A recent elegant report by Gaudenzio et al. demonstrated that MCs employ distinct degranulation strategies upon stimulation through G-protein coupled receptors (GPCR) or by the anaphylactic IgE/antigen (Ag) triggering. While the first stimulation induced MCs to quickly and briefly release small and spherical granules, the latter induced a partially retarded but sustained secretion of larger and more heterogeneous particles[93]. Interestingly, the larger granules released following IgE/Ag stimulation were found to be transported to draining lymph nodes, causing their enlarging. Such results are consistent with prior observations by Kunder et al. and suggest that transport of granules released by compound exocytosis might enable the diffusion of their content, thus promoting or sustaining the adaptive immune response even at distant loci[93,94].

FcεRI activation

__Lipid rafts__ are specialized plasma membrane microdomains enriched in cholesterol, sphingolipids and several membrane-bound signaling proteins. These microdomains serve as "docking stations" which allow the interaction among kinases and adaptor proteins and are believed to be crucial for the initiation of receptor signaling cascades.

Consistent with their well known role in allergy and anaphylaxis, the signaling cascades initiated by the cross-linking of IgE/Ag complexes on MCs' surface FcεRI are by far the most studied. FcεRI is a tetrameric receptor which comprises the IgE-binding α-chain, a membrane-tetraspanning β-chain and a disulphite-linked homodimer of the γ-chain[95]. Antigen recognition by FcεRIα-bound IgE induces receptor aggregation on `lipid rafts`, activation of SRC family kinases (SFKs) and tyrosine phosphorylation of the receptor subunits[96–98]. Four main SRC kinases have been described in FcεRI activation: LYN, SYK, FYN and HRC. These SFKs simultaneously initiate independent signaling cascades which interconnect and give rise to a complex

signalosome that regulate MC activation. Albeit being extremely complex and tightly regulated, two mayor signaling axis can be described, the first one being initiated by LYN and SYK, and other one relying uniquely on FYN[95,99].

THE LYN–FYN AXIS. After receptor triggering, aggregated FcϵRI associate with LYN in lipid rafts which phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAM) on the receptor subunits β and γ (Fig. 2.1.7A). Phosphorylated ITAMs provide high-affinity docking sites for LYN and the tyrosine kinase SYK, which is now trans- and auto-phosphorylated[100]. Both LYN and SYK promote the phosphorylation of the transmembrane adaptor molecule LAT, which in turn recruits many different signal molecules serving as a scaffold for protein-protein interactions[101]. Among the protein recruited to LAT, phospholipase-γ (PLCγ) and the guanine-nucleotide-exchange factors SOS and VAV are the most important for MC degranulation and cytokine production in response to Ag stimuli. Active PLCγ catalyzes the hydrolysis of phosphatidylinositol diphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is responsible for a "first wave" of Ca^{++} mobilization from endoplasmic reticulum stores, and this in turn induces a "second wave" of intracellular calcium influx[99]. On the other side, DAG is responsible for the activation of the protein kinase C (PKC). Both the signals from calcium influx and PKC activation are fundamental for the complex process of degranulation[95].

The induction of cytokine transcription requires the activity of guanine-exchange-factors SOS and VAV to shift the activation state of RAS. RAS then activates the RAF-dependent pathway which leads to the activation of the mitogen-activated protein kinases (MAPK) extracellular-signal-regulated kinase 1 (ERK1), ERK2, JUN aminoterminal kinase (JNK) and p38[95,102]. Together with the calcium influx induced by IP_3 (and the subsequent nuclear translocation of nuclear factor of activated T cells (NFAT) due to calcineurin-dependent dephosphorylation), these pathways lead to the activation of NF-κB, the activator protein 1 (AP1) members JUN and FOS and thus to cytokine expression[95,99,103].

THE COMPLEMENTARY FYN–GAB2–PI3K AXIS. The existence of a complimentary signaling pathway for IgE/Ag activation was inferred after a series of studies which demonstrated that a novel SFK, FYN, was required for MC activation independently of LAT and PLCγ (Fig. 2.1.7B)[104]. Instead, this pathway leads to the activation of phosphatidylinositol 3-kinase (PI3K) through the phosphorylation of the adaptor molecule GAB2[105]. PI3K phosphorylates membrane-bound PIP_2 to phosphatidylinositol 3,4,5-triphosphate (PIP_3) which serves as docking site (through pleckstrin homology domains) for several signaling molecules such as PLCγ, VAV, the Burton's tyrosine kinase (BTK) and AKT. While PLCγ phosphorylation leads to Ca^{++} influx and PKC activation, as described earlier, AKT mediates the activation of the mammalian target of rapamycin (mTOR) pathway by inactivating the negative regulator of mTOR tuberin[106]. Active mTOR forms two macromolecular complexes: the rapamycin-sensitive mTOR complex 1 (mTORC1) and the rapamycin-insensitive mTORC2, both sensitive to inhibition by the tuber-

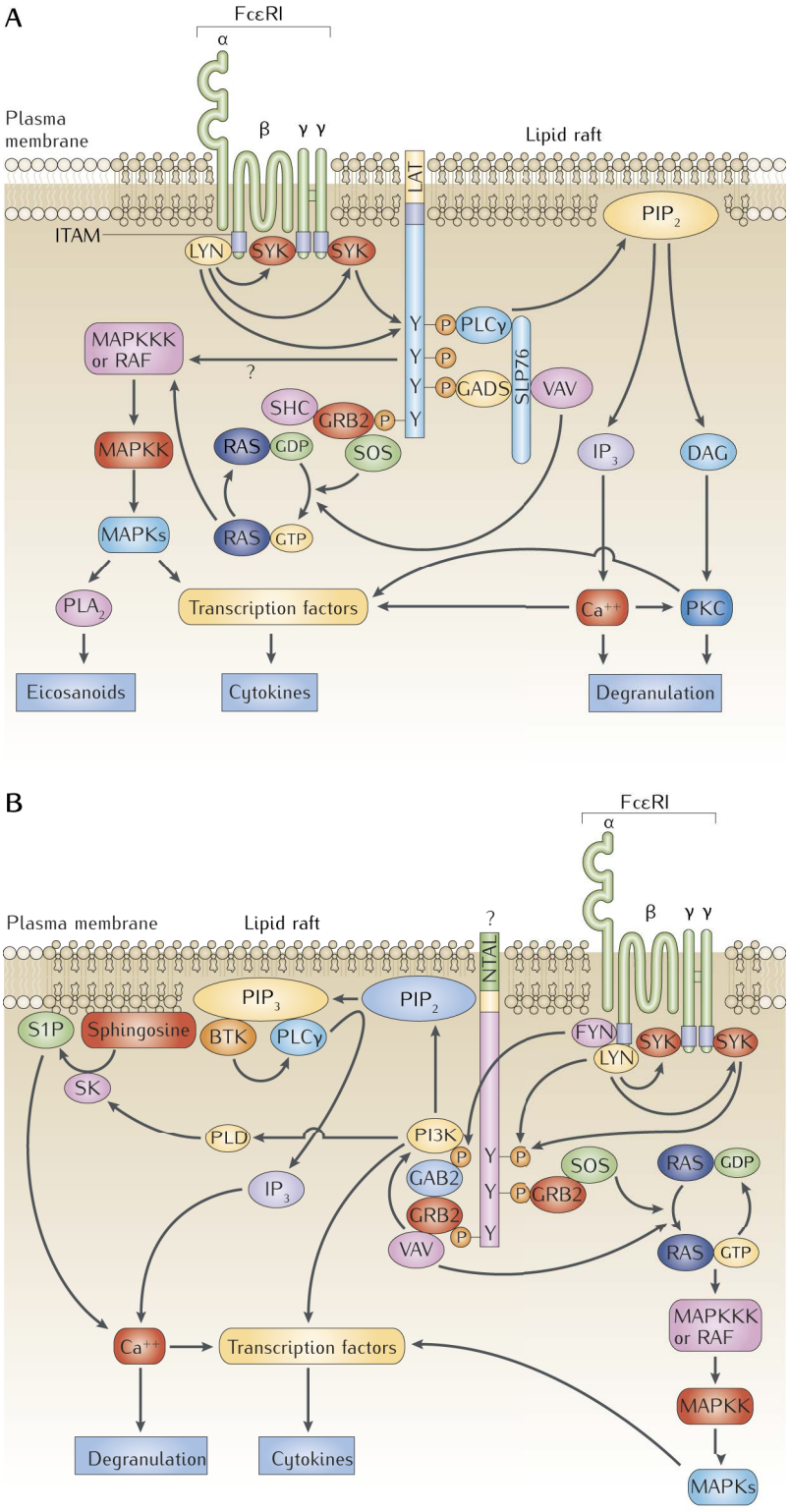

Figure 2.1.7: Signaling in FcεRI-induced activation. A. The "fundamental" LYN-SYK signaling cascade. After FcεRI aggregation (not shown) LYN associates with receptor ITAM and activates SYK. Both LYN and SYK promote the phosphorylation of LAT which serves as a scaffold for several signaling proteins. Here, PLCγ induces the activation of PCK and the influx of intracellular calcium, while SOS and VAV promote RAS activation and RAF-dependent activation of the MAPK pathway. **B. The "complimentary" FYN-GAB2-PI3K signaling cascade.** FYN- and GAB2-dependent phosphorylation of PI3K catalyzes a further Ca^{++} influx which sustains the initial PLCγ-dependent Ca^{++} influx and the activation of the AKT/mTOR pathway which leads to cytokine production. Adapted from Gilfillan and Tkaczyk[95].

ous sclerosis complex (TSC). It was demonstrated that during MC activation, both mTORC1 and mTORC2 are activated and finely regulated by TSC1 and TSC2, and that TSC deficiency led to increased cytokine production[107]. Interestingly, the activation of PI3K is not fundamental for the initiation of MC degranulation as it was demonstrated that its role is to maintain the signals induced by the "canonical" PLCγ activation[108]. This effect could be due to the activation of nonselective Ca^{++} channels such as TRPC-1[104]. Seen that FYN-mediated PI3K activation is independent on the presence of LAT, it has been proposed that a similar transmembrane protein, NTAL (also know as LAT2), might be the key adaptor molecule for the assembly of the macromolecular signaling complex necessary for FYN signaling[109].

Toll-like receptors.

TLRs are a family of evolutionarily conserved receptors profoundly involved in early host defense against pathogens. Since 1997 (when the first TLR was defined to be a pathogen recognition receptor - PRR) up to 13 different TLRs have been described in mammals[110]. TLRs are membrane-spanning receptors composed by an extracellular, a transmembrane and a cytoplasmic domain. The extracellular domain (responsible for pathogen recognition) contains blocks of 24-29aa repetitions called leucine-rich repeats (LRR) which fold in a horseshoe structure (Fig. 2.1.8A). The cytoplasmic tail present a conserved region known as the Toll/IL-1R (TIR) domain, which interacts with adaptor molecules for downstream signaling. The specificity of binding with different TIR-containing adaptor proteins is mediated by the exposed side chains close to the BB loop. The main TIR-domain-containing adaptor molecules involved in TLR signaling are: Myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). After ligand biding, TLRs undergo dimerization and a subsequent conformational change required for the binding of adaptor and signaling molecules.

TLR signaling comprises a complex network of signaling molecules but the MyD88-dependent and MyD88-independent pathways are of particular relevance.

MYD88-DEPENDENT PATHWAY The signaling cascade begins with ligand binding, the dimerization of TLRs and the association of MyD88 through the TIR domain (Fig. 2.1.8B). MyD88 present a N-terminal death domain (DD) which interacts with the same residue on the N-terminus of the kinase IRAK4. This leads to the recruitment and phosphorylation of the kinase IRAK1 which in turn induces further auto-phosphorylation of IRAK1 and the recruitment of TRAF6. At this point, the IRAK1-TRAF6 complex disengages the receptor and interact with the plasma membrane through the proteins TAK1, TAB1, TAB2 and TAB3. Phosphorylation of TAK1 and TABs induces IRAK1 degradation and subsequent translocation of the remaining complex to the cytosol. Here the complex is bound by the ubiquitin ligases UBC13 and UEV1A, leading to K63-polyubiquitination of TRAF6 and activation of the MAPKKK TAK1. TAK1 in turn is able to phosphorylate and

activate both MAP kinases and the IKK complex. The IKK complex then phosphorylates IκB, leading to its ubiquitination and degradation allowing the transcription factor complex NF-κB to translocate to the nucleus. On the other side, activation of the MAP kinase signaling pathway leads to phosphorylation of p38, JNK and ERK1/2, finally resulting in the activation of the transcription factor complex AP-1[111,112]. Such pathway of activation is shared by most TLRs (namely, TLR2, TLR4, TLR5, TLR7 and TLR9) and induces the expression of pro-inflammatory cytokines such as TNF-α and IL-6[111].

MYD88-INDEPENDENT, TRIF-DEPENDENT PATHWAY The existence of a TLR signaling independent on MyD88 was determined on the basis that MyD88-deficient macrophages fail to produce inflammatory cytokines in response to TLR4 triggering by lipopolysaccharide (LPS) but appear to activate both NF-κB and MAPK with delayed dynamics. On the other side, the same cells showed unaltered IRF3 activation and release of type-I IFN after TLR4 and TLR3 triggering[113,114]. The MyD88-independent activation of TLR signaling is dependent on the TIR-domain-containing adaptor protein TRIF. This protein is known to induce IRF3 and NF-κB activation via three mechanisms (Fig. 2.1.8C). At first, TRIF is able to bind the kinase TBK1 which in turn interacts with the IKK kinase IKKϵ and phosphorylate IRF3. Upon activation, IRF3 quickly migrates in the nucleus and recruits the co-activators p300 and CBP, inducing the expression of type-I IFNs. On the other side TRIF is able to activate the NF-κB pathway by the interaction with TRAF6 (at its N-terminus) and RIP1 (at its C-terminus). TRAF6 and RIP1 are both able to induce NF-κB activation and to promote IFN-β expression[111].

TLR4 TLR4 mediates response mainly against bacterial LPS and, among others, against VSV glycoprotein G, *Candida albicans* mannan and *Cryptococcus neoformans* glucuronoxylomannan[116]. TLR4 is expressed on the cellular surface and LPS-mediated triggering of this receptor induces the activation of both MyD88-dependent and MyD88-independent, TRIF-dependent pathways (Fig. 2.1.9). The "classical" MyD88-dependent activation is mediated by MyD88 but also by the TIR-domain-containing protein TIRAP, which was shown to interact with MyD88 and to be required for the early production of inflammatory cytokines in response to LPS[117,118]. On the other side, the MyD88-independent, TRIF-dependent activation requires the presence of the TIR-domain-containing adaptor molecule TRAM which associates with TRIF and TLR4[119]. TRIF-deficient mice showed impaired activation of IRF3 and reduced expression of type-I IFNs in response to TLR4 ligands[120]. Notably, *in vivo* studies on TRIF- and TRAM-deficient mice demonstrated that both TRAM and TRIF are required for the production of inflammatory cytokines despite showing normal levels of IRAK1 and early-phase NF-κB activation[119].

TLR4 also requires the cooperation of two co-receptors in order to fully activate downstream signals: MD2 and CD14. MD2 is associated with the cytoplasmic tail of TLR4 and it is fundamental for LPS recognition and activation since TLR4 alone fail to induce NF-κB activation after LPS challenge[121]. CD14, on the contrary, is not fundamental for TLR4 activation but

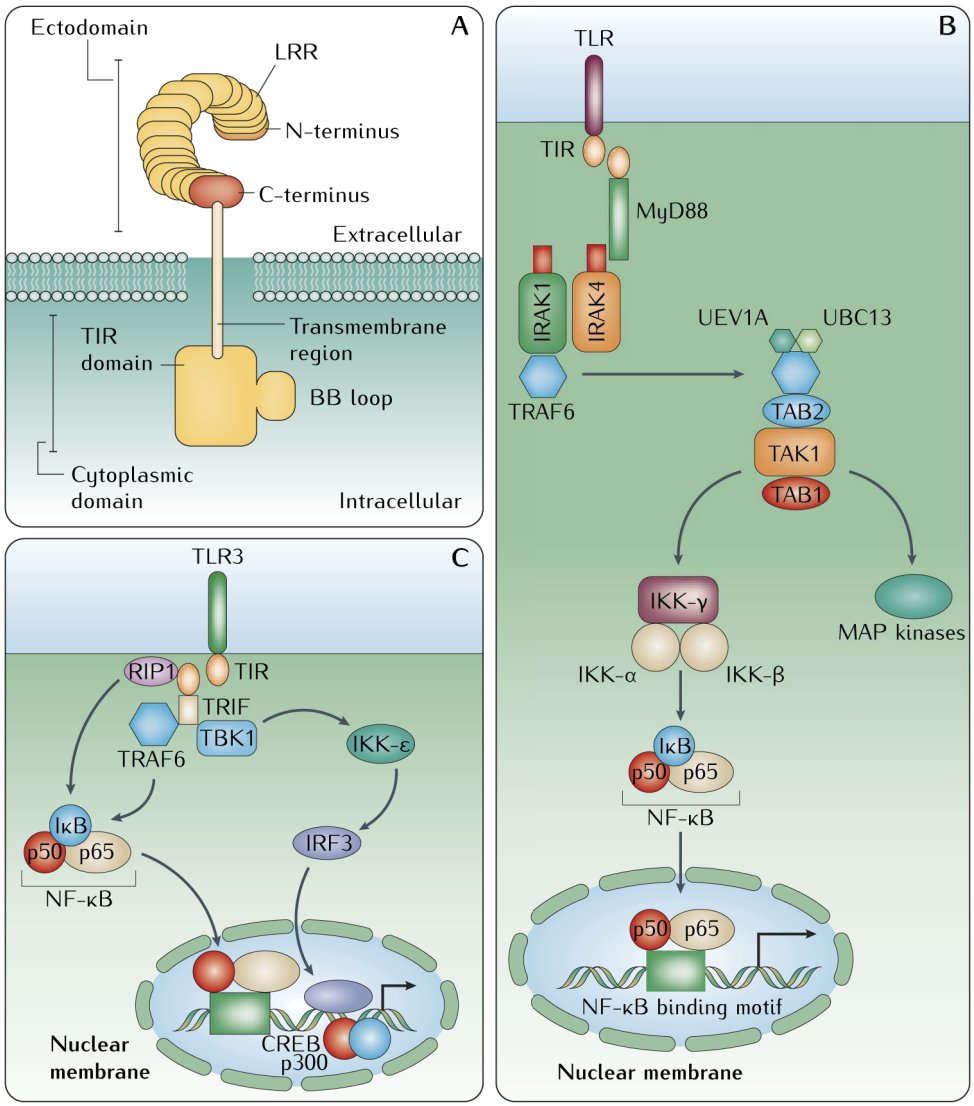

Figure 2.1.8: TLRs structure and activation pathways. A. TLR structure. TLR extracellular domain is deputed to the recognition of non-self products via its horseshoe structure composed by repeated LRR. The cytoplasmic tail present the TIR domain, which interacts with adaptor molecules (as MyD88) for downstream signaling. **B. Myd88-dependent activation pathway.** After ligand binding, TLRs dimerize and the adaptor protein MyD88 associates to the TIR domain. This leads to the recruitment of the kinases IRAK1 and IRAK4 which became activated and in turn recruit TRAF6. At this point the IRAK1-TRAF6 complex disengages the receptor and interact with TAK1 and TAB proteins, finally leading to IRAK1 degradation (not shown). The complex now is free to move to the cytoplasm where TRAF6 undergoes polyubiquitination and activates TAK1 which in turn promotes MAPK and canonical NF-κB activation. **C. MyD88-independent activation pathway.** After the activation of TLR3, TRIF is recruited and activated. Three kinases are then recruited at the C-terminus and N-terminus of TRIF: TBK1 activates IKKϵ and phosphorylate IRF3, which quickly migrates into the nucleus and promotes type-I IFNs expression; TRAF6 and RIP1 are able to induce late phase activation of NF-κB which promotes IFN-β expression. Adapted from Akira and Takeda [111] and Gay et al. [115].

its presence can increase the responsiveness of cells to TLR4 ligands by several mechanisms. For instance, LPS is known to bind the soluble protein LBP (LPS-binding protein), which is normally present in serum, and this complex is then able to bind CD14 and to activate TLR4/MD2[122]. CD14 can also activate TLR4 in a LBP-independent manner by favoring the endocytosis of TLR4, by enlarging the spectrum of LPS chemotypes recognized by the receptor, incrementing the sensitivity to LPS (i.e. being responsive to lower doses of the ligand) and allowing the MyD88-independent activation of TLR4[123–125]. Moreover, it was proposed that the main function of CD14 might be to catalyze the transfer of LPS from the extracellular environment to the cellular membrane where it interacts with a plethora of receptors involved in LPS binding (i.e. TLR4, HSP70, HSP90 and CXCR4)[126].

As discussed in the above paragraph, TLR triggering leads to the activation of both the NF-κB and the MAP kinase pathways by the action of TAK1 kinase. Notably, TLR4 can also interact with PI3K through a non-canonical PI3K-binding site on the C-terminus of MyD88[127]. The TLR4-induced activation of PI3K leads to the activation of NF-κB and production of IL-1β, TNF-α and MIP-2 by murine macrophages[128]. Stimulation with LPS can also result in the activation of the calcineurin/NFAT pathway in dendritic cells (DCs) (Fig. 2.1.9). After LPS binding, CD14 is supposed to cluster in lipid rafts, leading to the activation of SFKs which in turn phosphorylate PLCγ2. Once active, the phospholipase PLCγ2 catalyzes the hydrolysis of PIP_2 to IP_3 and DAG. IP_3 is then able to induce the opening of IP_3-dependent Ca^{++}-channels on the cell membrane, triggering the influx of extracellular Ca^{++}. Ca^{++} influx induces calcineurin activation and subsequent NFAT dephosphorylation and nuclear translocation[129,130]. This process relies exclusively on CD14 seen that TLR4-, MD2-deficient DCs, as well as MyD88/TRIF double deficient DCs show normal NFATc activation upon LPS stimulation[131].

Mast cells express TLR4 and are able to respond to LPS in a TLR4/MD2-dependent manner[132]. LPS stimulation induces the release of IL-1β, TNF-α, IL-6 and IL-13 but does not induce MC degranulation[133–135]. In addition, BMMC stimulation with LPS also induced the release of IL-10, IL-5, and the chemokines CCL3 and CXCL2[136,137]. The expression of CD14 on MC is quite controversial since different authors demonstrated the expression mCD14 transcripts but none succeeded to demonstrate the presence of the protein on MCs' surface[133,138–140]. Huber et al. demonstrated that MCs activation by LPS is dependent on the molecular structure of LPS[141]. Smooth-LPS (or S-form LPS) is composed by lipid A, a core oligosaccharide region and a region composed of repeated O-polysaccharide units, whilst rough-LPS (or R-form LPS) lacks the O-oligosaccharide chain. While R-form LPS induced the release of TNF-α and IL-6 from BMMCs in a TLR4/MD2-dependent manner, S-form LPS failed to induce cellular activation[141]. Further analyses on macrophages and DCs confirmed that S-form LPS requires CD14 for the activation of both MyD88-dependent and MyD88-independent signaling cascades at least at low doses, while R-form LPS leads to the activation of MyD88-dependent signals also in the absence of CD14[141,142]. In addition, MCs lack a complete TRIF-TRAF signaling cascade and thus can respond to LPS triggering by producing pro-inflammatory cytokines under the control of the MyD88-dependent pathway but not producing Type-I IFN[143,144]. This

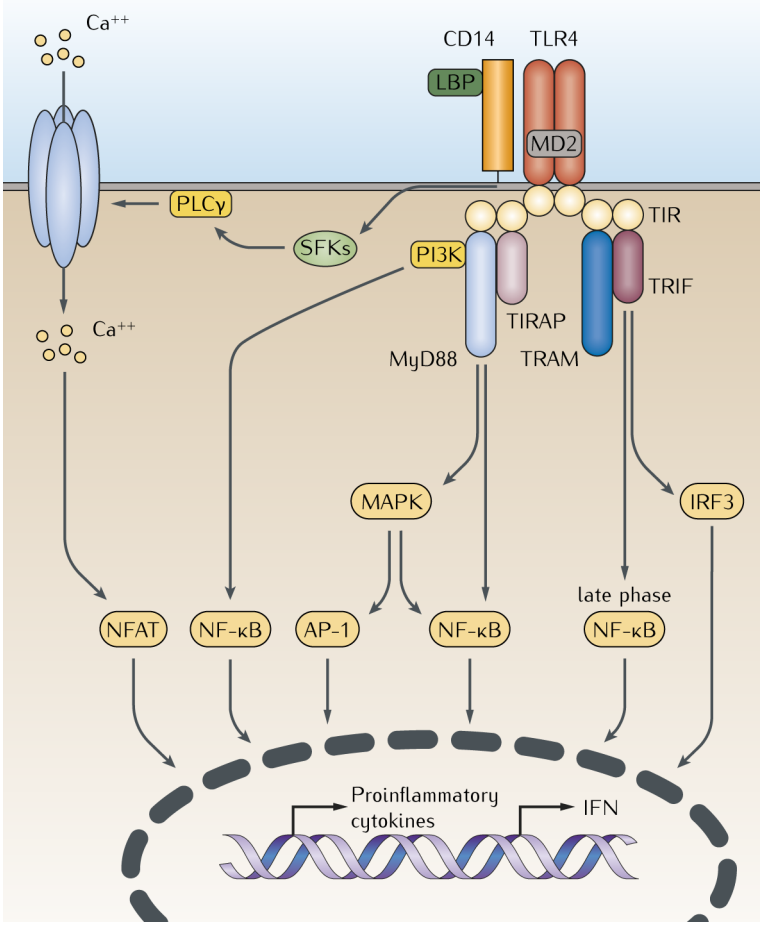

Figure 2.1.9: TLR4 signaling. TLR4 triggering can activate both the MyD88 dependent and the MyD88-independent, TRIF-dependent signaling pathways. TRIF requires the adaptor molecule TRAM in order to induce the activation of IRF3 and NF-κB. On the other side, MyD88 requires a further adaptor, TIRAP, to associate with the TLR4 TIR domain in order to start downstream signaling. PI3K can promote further activation of NF-κB by interacting with MyD88 through a non-canonical binding site. Moreover, LPS activation of DCs can lead to a CD14-dependent activation of NFAT through the action of the phospholipase PLCγ2 which catalyzes the hydrolysis of PIP_2 to IP_3 which in turn triggers the influx of extracellular Ca^{++}.

could be explained by the quite low expression of TRAM by MCs or by the fact that MCs fail to internalize TLR4 after LPS challenging, probably due to the lack of membrane-bound CD14[123,143,144].

TLR2 TLR2 is a peculiar TLR since can oligomerize with TLR1 or TLR6 for the recognition of triacetylated or diacetylated lipopeptides respectively. In addition, TLR2-TLR6 oligomerization can occur after binging to lipoteichoic acid (LTA) and zymosan (an insoluble glucan composed of β-1,3 glucose chains)[116]. TLR2 triggering activates the MyD88-dependent signaling pathway and, as TLR4, requires the collaboration of the adaptor protein TIRAP[118]. It is worth of note that TLR2 signaling often cooperates with C-type lectin receptors (CLRs) for the recognition of fungal pathogen-associated molecular patterns (PAMP).

Murine and human mast cells express TLR2, TLR1 and TLR6[137,145–147]. Even though it is supposed that BMMC express a truncated version of TLR2 which lacks the intracellular TIR domain, stimulation with TLR2 ligands induces MCs activation and the production of a greater range of mediators compared to TLR4 stimulation[134,148].

Stimulation of BMMC with bacterial peptidoglycan (PGN), a TLR2-TLR6 ligand, induced the release of TNF-α, IL-4, IL-5, IL-6, and IL-13 in a TLR2–dependent manner[132,134]. In addition, peritoneal cell-derived mast cells (PDMCs) stimulated with LTA and MALP-2 (a synthetic ligand of TLR2-TLR6) released a broader spectrum of cytokines: TNF-α, IL-1, IL-6, IL-10, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFN-γ[148]. PDMCs are considered to be more mature than BMMCs and this could reflect their increased ability to respond to TLR2 stimulation. Human MCs also behave similarly: stimulation of cord blood-derived mast cells (CBMC) with PGN or zymosan resulted in the production of TNF-α, IL-1β, IL-5, GM-CSF and cysteinyl leukotrienes[149].

MCs degranulation in response to TLR2 ligands is still matter of debate. Two studies reported that both BMMCs and human CBMCs were able to degranulate in response to PGN while a third one reported that zymosan and Pam_3CSK_4 (a synthetic TLR1-TLR2 ligand) but not PGN were able to induce human CBMCs degranulation[134,135,146]. On the other side, other groups were unable to demonstrate BMMCs and PCMCs degranulation in response to TLR2 stimuli such as PGN and LTA[137,140,148].

INTRACELLULAR TLRS Intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) are involved in the response to virus and certain bacteria by the recognition of foreign nucleic acids. Triggering of these receptors with their cognate ligands results in the production of pro-inflammatory cytokines and type-I IFNs[150]. With the exception of TLR3, the activation of downstream signaling after ligand binding is, at least in part, dependent on MyD88. Activation of the MyD88-dependent pathway leads to the nuclear translocation of AP-1 and NF-κB and thus to the transcription of pro-inflammatory cytokines as TNF-α. On the other side, TLR3 signals through the MyD88-independent, TRIF-dependent pathway to promote IRF3 activation and type-I IFN production (Fig. 2.1.8C)[111,150]. Furthermore, activation of TLR7 and TLR9 leads to

the MyD88-dependent activation of IRF and IRF7 which contributes to the expression of TNF-α, IL-12 and type-I IFNs[150].

MCs are able to recognize nucleic acids via intracellular TLRs as TLR3 (dsRNA), TLR7 (ssRNA) and TLR9 (CpG-DNA). BMMCs, PCMSs and fetal skin-derived MCs (FSMC) respond to polyI:C stimulation by releasing IFN-α, IFN-β, TNF-α, IL-6, and the chemokines CCL2, CXCL2 and CCL5 in a TLR3-dependent manner[137,145]. Matsushima et al. demonstrated that stimulation with the synthetic TLR7 agonist R848 induced the secretion of IL-6, TNF-α, CCL2, and CXCL2 from FSMCs but not BMMCs. Also synthetic oligonucleotides rich in CpG regions (which are recognized by TLR9) induced murine MCs to release pro-inflammatory cytokines[137]. In most of the cases, FSMCs responded more robustly than BMMCs, suggesting again that BMMCs may represent an immature phenotype of mast cells[137,151].

C-type lectin receptors.

C-type lectin receptors (CLRs) comprise a superfamily of proteins which are responsible for the recognition of both self- and non-self-ligands. Of peculiar interest for the purposes of this thesis, some CLRs are involved in the recognition of several carbohydrates such as mannose, *N*-acetylglucosamine and β-glucan. Among this family of receptors, much attention has been given to the mannose receptor (CD206), the DC-specific ICAM3-grabbing non-integrin (DC-SIGN), dectin-1, and dectin-2[152,153].

DECTIN-1 Dectin-1 has a single extracellular carbohydrate-recognition domain (CRD) which specifically recognize particulate β-1,3- and β-1,6-linked glucans that are found mainly in the cell walls of fungi and certain bacteria[154]. The CRD is kept separated from the cellular membrane by a stalk region and is connected to a cytoplasmic tail through a transmembrane domain. Dectin-1 activation can mediate a signaling cascade independently on the cooperation of adaptor molecules thanks to the presence of an ITAM-like motif on its cytoplasmic tail (Fig. 2.1.10)[155]. Upon binding to β-glucans, the YxxL motif (where "x" identifies any amino acid) became phosphorylated by the action of SRC kinases and two phosphorylated receptors cooperate for the recruitment and activation of the kinase SYK[156,157]. SYK phosphorylation leads to the formation of a complex comprising CARD9, Bcl-10, and MALT1, which then activate IKKβ and subsequently the canonical NF-κB pathway (Fig. 2.1.11)[158]. The CARD9–Bcl-10–MALT1 complex can also be activated by PKCθ through the SYK-dependent phosphorylation of PLCγ2. Phospholipase PLCγ2 activation also leads to IP_3–dependent Ca^{++} influx and subsequent NFAT nuclear translocation[152,159]. In addition to the canonical NF-κB pathway, dectin-1 triggering can also lead to the activation of the non-

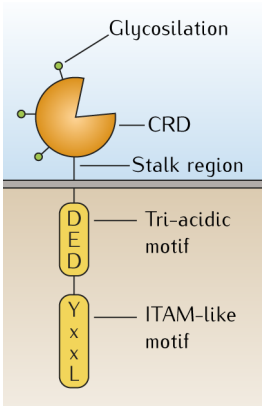

Figure 2.1.10: Dectin-1 structure. The receptor is composed by a single extracellular CDR, a stalk region and an intracellular ITAM-like motif which mediates dectin-1 signaling.

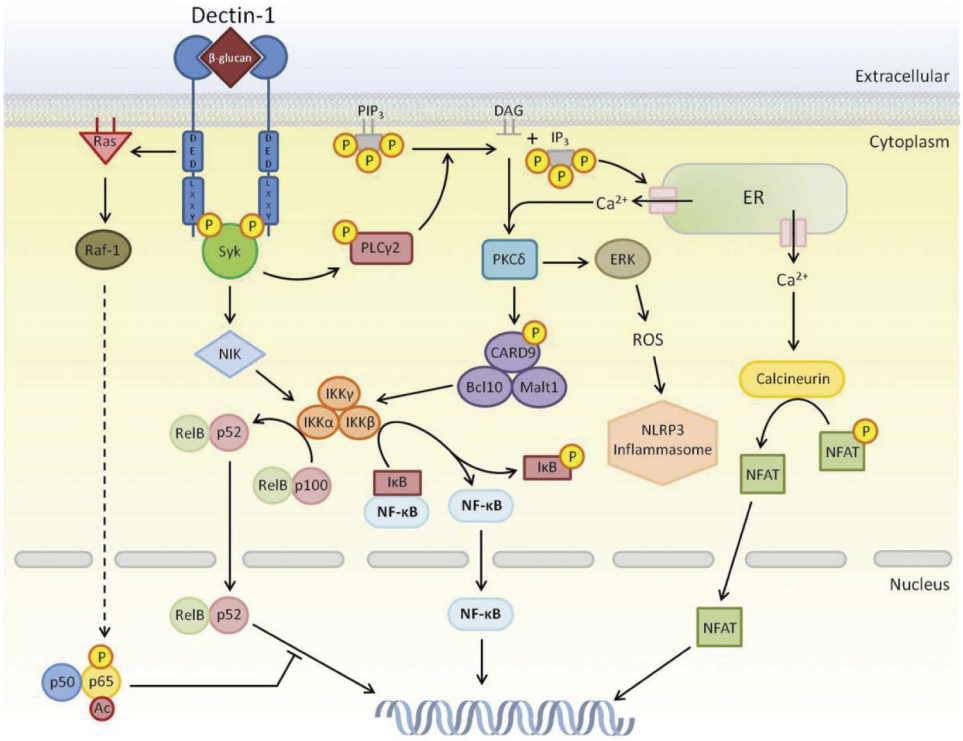

Figure 2.1.11: Dectin-1 signaling network. Dectin-1 engagement induces the phosphorylation of its cytoplasmic ITAM. Two activated receptors can cluster together causing the recruitment and activation of the key kinase SYK which mediates downstream signaling. SYK phosphorylates PLCγ which in turn activates PKCθ and leads to the formation of a CARD9–Bcl-10–MALT1 complex and the activation of ERK. The CARD9–Bcl-10–MALT1 complex mediates the activation of the canonical NF-κB pathway, while ERK induces the activation of the NLRP3 inflammasome. The hydrolysis of PIP_2 to IP_3 and DAG due to PLCγ2 activity, causes an influx of intracellular Ca^{++} and subsequent NFAT nuclear translocation. SYK-dependent activation of the kinase NIK promotes also the processing of the NF-κB subunit p100 to p52, leading to the nuclear translocation of the non-canonical p52-RelB. Finally, NF-κB transcription factor activity is modulated by the SYK-independent, Ras-dependent control of p65 S276 phosphorylation. Adapted from Plato et al.[152].

canonical NF-κB pathway. IKKα activation by the NIK kinase, promotes the processing of the NF-κB subunit p100 to NF-κB p52 and the consequent formation of a p52-RelB complex which translocates into the nucleus and initiate gene transcription[160].

In addition to the CARD9- and NIK-dependent NF-κB activation, dectin-1 engaging induces the phosphorylation of the kinase RAF1 through the action of RAS proteins which then induces NF-κB phosphorylation at S276. This modification fine-tunes NF-κB activity by altering the balance between the different subunits and their DNA-binding affinity, resulting in increased or reduced TLR-induced cytokine expression[160]. This mechanism is of particular interest since dectin-1 is known to cooperate with TLRs for the recognition of distinct microbial components. For example, dectin-1 activation in DCs stimulated with zymosan particles enhances TLR2-, CD14- mediated

NF-κB activation and production of cytokines such as TNF-α and IL-12[161]. Other studies on macrophages demonstrated that the collaboration between dectin-1 and TLR2 during β-glucan recognition is dependent on Syk and MyD88 as the deficiency in either one of the two proteins completely abolished synergistic TNF-α, CXCL2 and CCL3 release[162].

SYK-CARD9 activation in response to *C. albicans* and *A. fumigatus* also leads to the activation of the NLR family, pyrin domain containing (NLRP3) inflammasome. NLRP3 is able to catalyze the cleavage of pro-IL-1β to its active form IL-1β through the action of caspase I[163,164]. Recently, it has been proposed that dectin-1–dependent SYK activation induces the formation of a complex composed of CARD9, RAS-GRF1 and H-RAS which leads to downstream activation of ERK and subsequent release of TNF-α, IL-1β, IL-6 and IL-12[165].

Albeit being only poorly described, mast cells express dectin-1 and can respond to fungal antigens. For example, murine BMMC express dectin-1 on cell surface and stimulation with zymosan up-regulates dectin-1 levels and induce reactive oxygen species (ROS) production in a TLR2–independent manner[166]. A more recent work by Nieto-Patlan et al. showed that MCs stimulation with *C. albicans* induced BMMC and PMC degranulation, and production of TNF-α, IL-6, IL-10, CCL3, and CCL4[167]. Blockade of dectin-1, but not TLR2, partially reduced the release of TNF-α during *C. albicans* challenge, indicating that this receptor plays an important role in fungal recognition[167]. Human MCs also express functional dectin-1. Stimulation of CBMC with both PGN and zymosan resulted in the release of leukotrienes LTB_4 and LTC_4 while the addition of the dectin-1 inhibitor laminarin resulted in reduced release of leukotrienes during zymosan but not PGN stimulation[149].

Other receptors

COMPLEMENT RECEPTORS. The complement system is composed by a great number of plasmatic proteins which interact with pathogens in order to opsonize and kill it. Mast cells are known to interact with the complement system proteins with the complement receptor 3 (CR3; also known as CD11b-CD18), the CR4 (CD11c-CD18), and the receptors for C3a (C3aR) and C5a (C5aR; also known as CD88)[168]. MCs are generally activated to degranulate in response to complement peptides, and the C3a protein can induce the production of the chemokines CCL2 and CCL5 in human MCs[169,170]. In addition, both C3a and C5a are chemotactic factors for MCs[171]. Interestingly, CR3 recognize also non-opsonized ligands including β-glucans, but this ability has not been demonstrated in MCs yet.

2.1.3 MCs (social) networks: crosstalk with other immune system cells.

The fact that mast cells are able to modulate their phenotype depending on their physiological context together with their ability to release a wide plethora of mediators and to express many membrane-bound costimulatory molecules, suggest that these cells are deeply involved in cell-cell interactions[172]. In fact, MCs have been described to interact with members of both the innate and the adaptive arm of immunity. As can be expected, often the

interaction through MCs' costimulatory molecules is bi-directional, meaning that both MC and the interacting cell can be influenced [173].

MCS AND DENDRITIC CELLS. Soluble mediators released by MCs (as histamine and TNF-α) have been described to modulate DCs behaviour by favoring their recruitment at the site of infection, enhancing their maturation and migration into draining lymph nodes and by promoting their ability to activate T-cells [174–177]. On the other side, little is known about the direct interaction between MCs and DCs. *In vitro* co-culture of MCs with DCs induced the upregulation of maturation markers on DCs, such as CD80, CD86 and CD40 [83]. Similar results were obtained in a subsequent work, which demonstrated that DCs maturation was induced by interaction between lymphocyte function-associated antigen (LFA-1) and intracellular adhesion molecule (ICAM-1) on MCs [178].

MCS AND MONOCYTES/MACROPHAGES. Mast cell interaction with macrophages has been described in the context of bacterial infections. During the infection with *Francisella tularensis*, which replicates within macrophages, bacterial replication was inhibited in a contact-dependent manner and required MC-derived IL-4 [179]. Additional investigation showed that IL-4 is able to induce macrophage alternative activation, leading to the upregulation of the mannose receptor and to a more efficient bacterial phagocytosis. Moreover, IL-4 treatment resulted in an increased ATP production in macrophages, which promoted phagosomal acidification [180]. On the contrary, MC-derived IL-4 had a detrimental effect on bacterial phagocytosis by macrophages both *in vitro* and in a murine model of acute sepsis [86].

INTERACTIONS WITH THE ADAPTIVE ARM OF IMMUNITY. Interaction between MCs and T-cells have been described during many inflammatory processes such as inflammatory bowel disease (IBD), parasite infections and allergy [181–183]. MCs can interact with both $CD4^+$ and $CD8^+$ effector T-cells. Phagocytosis of different Gram-negative enterobacteria induced MCs to process bacterial antigens for presentation through MHC-I molecules to T-cells [184]. Interestingly, MC-dependent antigen presentation induced the activation and proliferation of $CD8^+$ T-cells *in vitro* and *in vivo* [185]. Moreover, MCs interaction with $Foxp3^+$ $CD4^+$ regulatory T-cells (T_{reg}) inhibit their suppressive activity and skew their development into IL-17–producing T-cells (T_H17) [186]. On the other side, T_{reg} reduce MC degranulation in a contact-dependent fashion [187]. Both these processes requires MC- or T-cell–derived cytokines (e.g. IL-6) and cell-cell interaction through the OX40-OX40L axis.

Mast cells are an important source of cytokines involved in the regulation of B-cell and express the ligand for CD40 (CD40L) on their surface. Interaction of MCs with B-cells through the CD40-CD40L axis, promoted B-cell survival and differentiation into IgA-secreting $CD138^+$ plasma cells [188]. An analogous mechanism which involved the CD40-CD40L axis was also described to be fundamental for the expansion of IL-10–competent B-cells by MCs [189].

2.1.4 Do we really need mast cells? Roles in health and disease.

Until recent years, MCs were kept in the shadow by their well known role in allergy and anaphylaxis but this circumstance changed completely as different new roles for this cells were reported[50,190]. Nowadays the scientific community agrees on the fact that they are involved in the regulation of both innate and adaptive immune response as well as some non-immunological process. For instance, MCs have an important role during parasitic infections of different pathogens such as helminths, bacteria and fungi. Gastrointestinal `helminth` infections are generally associated with the expansion of MCs at the site of infection[191,192]. In this context, MCs are traditionally known for their role during early- and late-stage induction of a T_H2 response, mainly mediated by interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP)[193]. Nevertheless, MCs have a direct effect on helminth survival and proliferation by secreting cytotoxic proteases such as chymase and tryptase, as well as an indirect role on round-worm expulsion by the action of secreted mast cell protease 1 (MCPT1) which disrupts intestinal epithelial barrier integrity[194,195]. A recent work published by Reitz et al. highlighted the fundamental role of MMC but not CTMC in the termination of *Strongyloides ratti* infection: $CPA3^{Cre}$ mice (which lack both MMC and CTMC) were unable to resolve the parasitic infection before 150 days, while $MCPT5^{Cre}$ × R-DTA mice (which lack only CTMC) terminated the infection already after 1 month[192].

*The term "**helminth**" is used to describe a group of unrelated organisms belonging to different phyla (Nematoda, Platyhelminthes and Annelida) which display similar characteristics.*

Mast cells have also been implied in bacterial infections. Several papers published in the last years show that MCs are able to phagocyte *in vitro* different bacteria such as *Escherichia coli* and $FimH^+$ Enterobacteria, *Streptococcus faecium*, *Pseudomonas aeruginosa* and *Francisella tularensis*[196–200]. After being recognized by different receptors, bacteria are engulfed in a plasma membrane-derived vesicle called phagosome. Phagosomes then undergo subsequent processes of fusion with lysosomes and secretory vesicles resulting in strong acidification and digestion of the engulfed bacteria into peptides[201]. Professional antigen-presenting cells (APCs) are then able to load those peptides on newly synthesized MHC class II complexes and to redirect them to the plasma membrane in order to be recognized by naïve $CD4^+$ T cells[202]. The ability of MCs to behave as antigen-presenting cells (APCs) is still controversial; however, a study by Kambayashi et al. showed that LPS and interferon (IFN)-γ stimulation lead to the expression of MHC class II on BMMCs and PDMCs and allowed them to present antigens directly to T cells[203]. Similar results were also recently observed in human primary MCs isolated from cytomegalovirus (CMV) seropositive patients, in which *ex vivo* IFN-γ stimulation induced the expression of human leukocytes antigen complexes (HLA-DR and HLA-DM), CD40 and CD80 by MCs and allowed them to uptake, process and present antigens to autologous $CD4^+$ T_H1 cells[204]. Bacterial challenge also induces MCs activation in term of degranulation and subsequent release of pre-stored and de-novo synthesized mediators. For instance, *in vitro* co-culture of MCs with *Mycobacterium tuberculosis* resulted in cell degranulation, while stimulation of MCs with *S. faecium* induced the release of TNF-α[198,205]. Both murine and human MCs have been described to respond to *in vitro* infections with *Staphylococcus au-*

reus: murine PMC released IL-3, IL-13 and TNF-α, while human CBMCs released TNF-α and IL-8[206,207]. It is worth of note that several commensal bacteria such as *Lactobacillus casei*, *Enterococcus fecalis* and non-pathogenic *E. coli* can instead decrease MCs degranulation induced by IgE/Ag or Ca^{++} ionophores stimulation, most likely by affecting signaling pathways downstream of FcϵRI[208–210]. It also appears that *in vitro* stimulation of MCs with *Streptococcus pyogenes* and *Listeria monocytogenes* can induce the release of so-called "MCs extracellular traps", namely extracellular structures composed of DNA, histones, tryptase, and the antimicrobial peptide LL-37[211,212].

The role of MCs in bacterial infections has been studied also *in vivo* with murine models of MC-deficiency. For example, MCs and MC-derived IL-6 were fund to be important in the survival during *Klebsiella pneumoniae* infection and in a murine model of cecal ligation and puncture: $Kit^{W\text{-}sh/W\text{-}sh}$ mice and $Kit^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with IL-$6^{-/-}$ BMMC showed diminished survival compared to WT mice and $Kit^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with IL-$6^{+/+}$ BMMC. Moreover, using a BALB/c model of MCs deficiency, it was demonstrated that MCs play an important role in the clearance of *L. monocytogenes* by releasing TNF-α and recruiting neutrophils in the peritoneal cavity during the initial phase of the infection[213]. On the other side, in a recent work by Rönnberg et al. MCs were completely dispensable for the resolution of inflammation during intraperitoneal *S. aureus* inflammation, since no difference in bacterial clearance, inflammation or cytokine production were described between WT and $MCPT5^{Cre}$ $\times$ R-DTA mice[206].

MCs are also able to recognize and respond to fungal infections. Even though the topic is still quite controversial (mostly because of the limited literature), a complete overview on this subject will be given in Section 2.2.4.

The concept that MCs could be involved in tumor biology goes back in time, as Ehrlich and his student Westphal noticed and described the accumulation of MCs at the periphery of tumors already at the end of the XIX century[2,214]. Nowadays it is known that MCs partecipate in the progression of different tumors but it is controversial whether they play protective rather than pro-tumorigenic roles. For example, heparin and histamine released by MCs interfered with the growth of human breast cancer cells and human primary melanoma cells[215,216]. On the other side, many MCs mediators (such as the matrix metalloprotease MMP-9, tryptase and VEGF) are known to be involved in extracellular matrix (ECM) remodeling and angiogenesis, and were found to favor tumor progression in different mouse models[217–219]. A protective role for MCs in tumor progression has been described also in a model of colon cancer: APC^{Min} mice backcrossed on a $Kit^{W\text{-}sh/W\text{-}sh}$ background showed increased tumor size compared to APC^{Min} littermates[220]. Conversely, the lack of MCs in the inducible model of $Mcpt5^{Cre}$/$iDTR^{+}$ mice resulted in lower tumor growth of transplanted EL4 T-cell lymphoma[221].

MCs are also involved in autoimmunity and have been described to have a detrimental role in experimental autoimmune encephalomyelitis (EAE) and rheumatoid arthritis, while being important in the induction of a tolerant microenvironment during skin grafting[222–224] . MCs are also supposed to be involved in the pathology of celiac disease (CD)[225,226], and were found to be expanded and activated in irritable bowel syndrome (IBS)[227,228] and Crohn's

disease[229]. Finally, mast cells are believed to be involved in many other non-immunological processes such as defense against venoms[230], tissue morphogenesis[231], wound healing[232], nociception and pain signaling[233,234], depression and axiety[235,236].

2.2 HOLD THE DOOR! IMMUNITY TO FUNGAL INFECTIONS.

A GARGANTUAN NUMBER of microorganisms resides in the human body and coexist with its host. Although the ratio between the number of bacterial and human cells is often believed to be around 10:1, a recent report suggests that this proportion is much closer to 1:1, estimating $\sim 3.9 \times 10^{13}$ bacterial cells in a human body[237]. As such, thousands of studies published in the last decade revealed that these tiny microbes have a huge impact on human health (Fig. 2.2.1)[238].

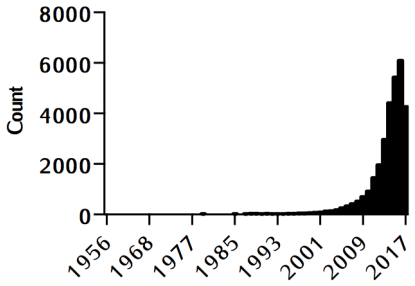

Figure 2.2.1: Number of published papers about "microbiota" from 1956 to 2017. In the last decade, a huge number of studies contributed to the understanding of the role of human microbiota. Bars indicate the number of annual publications containing the word *"microbiota"* from 1956 to 2017. Source: `https://www.ncbi.nlm.nih.gov/pubmed`

Sequencing of 16S-rRNA genes allowed the identification of the human intestinal microbiota composition, which mainly comprises bacteria belonging to the Bacteroidetes and Firmicutes phyla. The ratio of abundance between the two phyla is important for human health; for example, Firmicutes are reduced in patients suffering IBD, while are expanded in obese individuals[239,240]. These microorganism are restrained in the intestinal lumen by a thick and dense mucus layer which avoid bacteria to contact the epithelium but also provides a scaffold for the gut microbiota. However, certain bacteria (such as short filamentous bacteria (SFB), Proteobacteria and *Bacteroides fragilis*) are able to associate with the intestinal epithelium[241]. This close interaction is fundamental for the promotion of beneficial immunosuppression in the intestine and IgA production[242]. Lymphoid-tissue-resident commensal (LRC) bacteria are subset of microorganisms which typically colonize the Peyer's patches and the mesenteric lymph nodes of healthy individuals. Their interaction with DCs induce the release of cytokines which modulate type-3 innate lymphoid cells (ILC3) and T_H17 response promoting gut-associated-lymphoid tissue (GALT) maturation, IgA production and intestinal-tissue repair[238]. Together with direct contact-dependent modulation of the immune response, commensal bacteria can release several metabolites with immunomodulatory properties. Fermentation of non-digestible polysaccharides by anaerobic bacteria leads to the accumulation of short-chain fatty acids (SCFA), including butyric acid and propionic acid, which behave as potent anti-inflammatory and help in the maintenance of intestinal homeostasis[243]. In a similar way, metabolization of dietary tryptophan by commensal bacteria generate indole ligands of the Aryl-hydrocarbon receptor (AHR). For example, indole-3-aldehyde derived from *Lactobacillus reuterii* was shown induce the release of IL-22 from ILC3 in a AHR-dependent manner, resulting

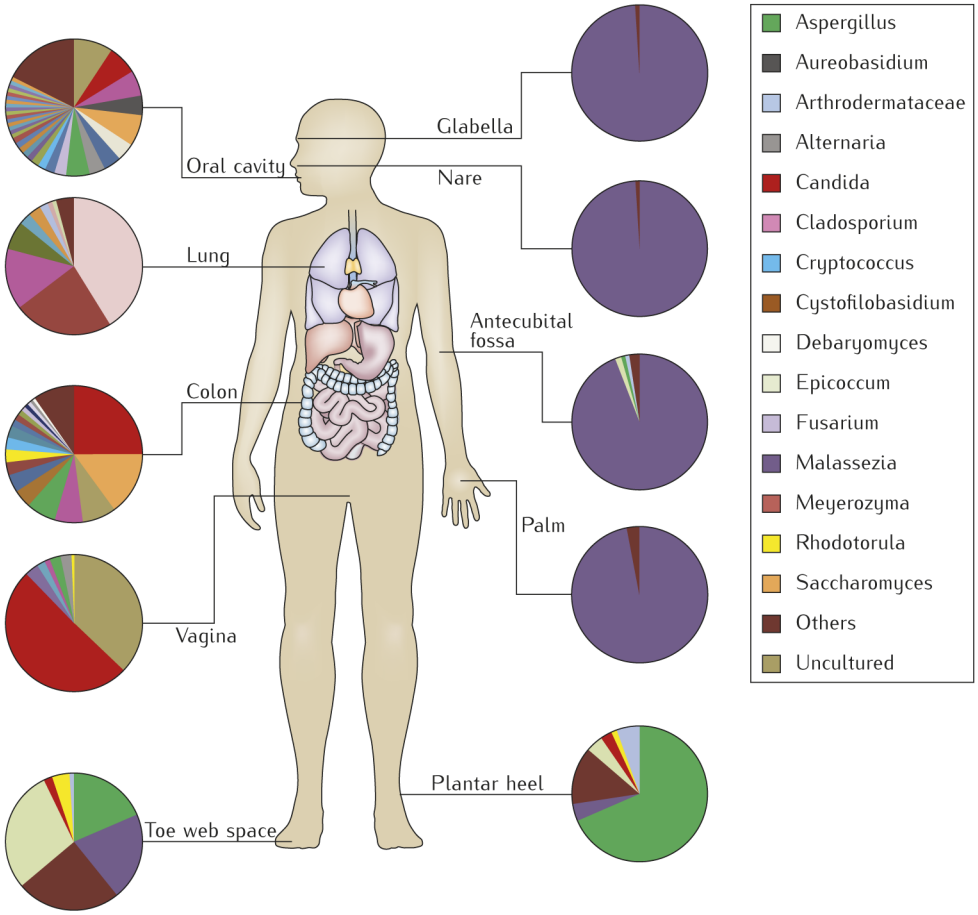

Figure 2.2.2: The human mycobiota. The pie charts indicate the proportion between different fungal genera identified in healthy humans. Noteworthy, mucosal populations are rather diversified compared to skin. Adapted from Underhill and Iliev[245].

in the expansion of *L. reuterii* and promoting resistance to *Candida albicans* colonization[244].

Albeit the majority of the investigations on human microbiota focused exclusively on the bacterial community, recent sequencing of the internal-transcribed-spacer regions ITS-1 and ITS-2 of the fungal ribosomal DNA uncovered the presence of a great community of fungi among mammalian microbiota – named the mycobiome. This community comprises several genera and change profoundly depending on the anatomical localization but *Candida*, *Saccharomyces* and *Cladosporium* are among the most common in the gut (Fig. 2.2.2)[245]. Fungal and bacterial communities interact and influence each other. Studies on germ-free mice and on mice undergoing prolonged antibiotic treatment, demonstrated that these animals are highly susceptible to *Candida* infections[246,247]. This is believed to be true also for humans, since administration of broad-spectrum antibiotics to adult cancer patients induced an increased gastrointestinal colonization by yeasts[248]. A recent paper by Sokol et al. showed that mycobiota composition was skewed in patients affected by IBD, with an expansion of Basidiomycota over Ascomycota, and a more prominent colonization by *C. albicans* compared to healthy individuals[249]. As reported above for *L. reuterii*, specific members

of the bacterial community can hinder fungal colonization. A further example is *Bacteroides thetaiotaomicron*, whose presence prevents *C. albicans* expansion by inducing the production of the antimicrobial peptide CRAMP[250]. On the other side, *C. albicans* can actively promote mucosal tolerance by skewing mucosal T-cell response and favoring the establishment of an anti-inflammatory/tolerogenic state[251]. In a similar way, *Malassezia* spp. can induce an anti-inflammatory state in normal skin through the induction of TGF-β1 and IL-10, but is also responsible for disease exacerbation in pathological skin[252]. Fungi are also used as probiotic, as in the case of *Saccharomyces boulardii*. This yeast inhibits the growth of different pathogens as *Salmonella typhimurium*, *Yersinia enterocolitica* and *C. albicans*, even under inflammatory conditions. Moreover, the administration of *S. boulardii* in patients with Crohn's disease and ulcerative colitis resulted in the amelioration of the pathology[253,254]. Taken together, these evidences suggest that the host-mycobiome interaction resides on a tight equilibrium between commensalism and pathogenesis.

Disturbance of this equilibrium by different means can lead to a pathological and uncontrolled proliferation of fungal species. Albeit being only poorly considered as a public health concern, over 800 million people worldwide suffer from life-threatening fungal-related diseases and 1.6 million of them die annually[255]. Moreover, it has been proposed that global warming will increase the prevalence of fungal infections in mammals, highlighting the need for novel therapies and vaccination strategies[256].

2.2.1 *Candida albicans.*

Candida spp. are commensal fungi that colonize mucous membranes and the skin of healthy individuals (Fig. 2.2.2) and among all the species, *C. albicans* is the most common in human mycobiota. However, they can cause severe invasive diseases in patients hospitalized in intensive care units, with solid tumors or hematological malignancy, undergoing surgery or being treated with broad-spectrum antibiotics[257]. It is estimated that *C. albicans* is responsible for more than one half of the cases of `candidaemia`, which mortality rates in Europe vary between 28% and 59%[257].

***Candidaemia** is a bloodstream infection caused by Candida spp. which can develop into invasive candidiasis when the fungus reaches different organs and tissues.*

CANDIDA ALBICANS BIOLOGY Among *Candida* species, *C. albicans* is the only one able to grow as a unicellular yeast and as a filamentous hyphal and pseudohyphal forms (Fig. 2.2.3A)[258]. Generally, the growth of hyphal forms requires a temperature of 37°C and is induced in response to the presence of CO_2 and serum[259]. This property is rather important as *C. albicans* hyphal growth is an important virulence factor and represent a key step for tissue-invasion processes[260].

An important feature for fungal biology and for their recognition by the host immune system is the fungal cell wall composition. *C. albicans* cell wall is composed of a strong structural core and a more plastic layer of proteins. The structural core is quite common to all fungi and is composed by β-(1,3)-glucans covalently linked with β-(1,6)-glucans and chitin (Fig. 2.2.3B). The outer part of the cell wall is mainly composed by highly-glycosylated pro-

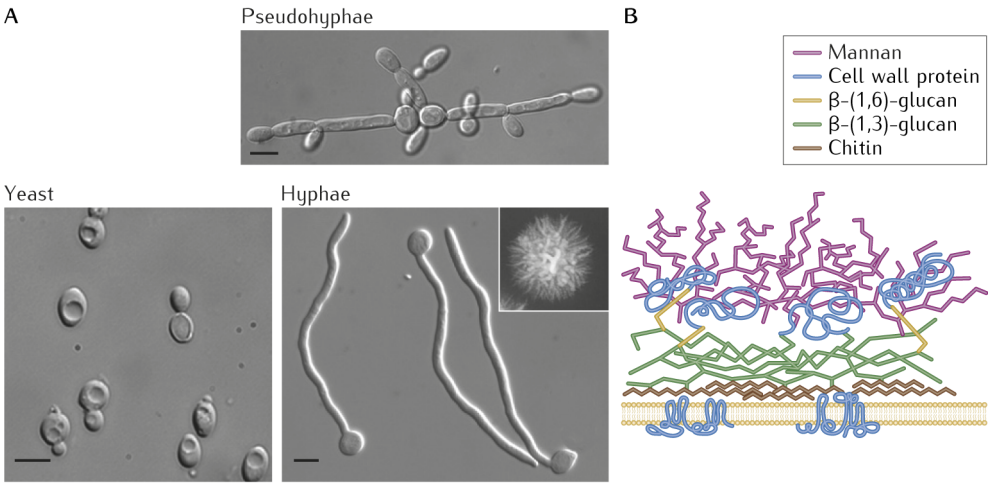

Figure 2.2.3: Morphology and structure of *Candida albicans*. A. Morphology of *C. albicans* biological forms. *C. albicans* can grow as a unicellular yeast or as filamentous hyphal and pseudohyphal forms. While hyphae form long, thin, and tube-like structures, pseudohyphae are wider and present constrictions at the sites of septation. Scale bars in the main panels are 5μm and 1mm in the inset. Adapted from Sudbery[258]. **B. Molecular structure of *C. albicans* cell wall.** *C. albicans* cell wall is composed of an inner core with structural functions composed of β-(1,3)- and β-(1,6)-glucans, and a thick outer layer mainly composed of mannoproteins. Adapted from Netea et al.[261].

teins anchored to glucans or chitins. These proteins contain long chains of carbohydrates and mannans (mannose-containing polysaccharides) further stabilized by *O*-linked mannan side chains[261]. Most importantly, this thick proteic layer masks perfectly the underlying β-glucans in both yeasts and hyphae, except for the "bud scars"(the scars left on the mother yeast cell after the separation of the budding yeast cell) preventing the recognition of these polysaccharides by the host's immune system[262]. Interestingly, small differences in the cell wall architecture (as the glycosylation status) can lead to a delayed migration and phagocytosis of *C. albicans* by macrophages[263].

2.2.2 Innate sensing

The immune response to *C. albicans* (and, more generally, to fungal pathogens) begins with the recognition of specific PAMPs by the innate arm of the immune system. The recognition of fungal PAMPs is mediated by several PRRs, including C-type lectin receptors (CLRs), Toll-like receptors (TLRs) and intracellular NOD-like receptors (NLRs)[252].

CLRs

CLRs are the most important receptors for fungal recognition. As deeply reviewed before (cfr. 2.1.2), dectin-1 is the best characterized CLR and is fundamental for the recognition of β-glucans and subsequent production of pro- and anti-inflammatory cytokines.

C. albicans hyphae β-glucans are completely shielded by the mannoproteins layer, preventing its recognition by dectin-1[262]. However, it has also been hypothesized that dectin-1 may be responsible for the recognition of hyphal β-glucans perhaps due to the thinning of mannan fibrils[264]. Moreover, structural differences between the β-glucans of *C. albicans* yeasts and hyphae induce different responses upon dectin-1 binding which, in some cases, can also fail to recognize specific strains of *C. albicans*[265,266].

Mannans and mannoproteins are recognized by several CLRs including the mannose receptor (CD206), the DC-specific ICAM3-grabbing non-integrin (DC-SIGN), MINCLE and dectin-2. The mannose receptor is primarily expressed on macrophages and recognize *N*-linked mannans and α-glucans. Its binding and activation induce a potent expression of IL-17, thus playing a fundamental role in the promotion of T_H-17 anti-fungal responses[267]. *N*-linked mannans are recognized also by DC-SIGN on DCs and macrophages. Similarly to the mannose receptor, activation of DC-SIGN promotes the release of cytokines involved in T_H cell activation[268]. Dectin-2 is expressed on DCs, macrophages and neutrophils and recognize α-mannan. Dectin-2 couples with the Fc receptor γ-chain FcRγ in order to activate downstream signaling pathways leading to the expression of pro-inflammatory cytokines and the production of ROS during *C. glabrata* phagocytosis[252,269].

TLRs

TLR2, TLR4 and TLR6 are the main TLRs that are involved in the recognition of the fungal cell wall mannoproteins. For example, *C. albicans O*-linked mannosyl residues are recognized by TLR4 and induce the expression of pro-inflammatory cytokines (TNF-α, IL-6) as well as the expression of the anti-inflammatory IL-10 in human mononuclear cells. Moreover, TLR2 cooperated with dectin-1 to boost cytokine expression in response to β-glucans[270]. In recent years it has been proposed that also the intracellular TLR3 and TLR9 are involved in the recognition of *C. albicans*. In a clinical study on patients affected by `chronic mucocutaneous candidiasis`, a mutation in the TLR3 gene (L412F) increased the susceptibility to *Candida* infections due to an impaired activation of NF-κB and IFN-γ production[271]. On the other side, TLR9 is able to recognize chitin and possibly fungal DNA, and induce the release of the anti-inflammatory IL-10 in both mice and humans[273,274].

Chronic mucocutaneous candidiasis *is characterized by persistent or chronic infections (typically occurring in the oral and genital mucosae, skin and nails) generally caused by C. albicans*[272].

NLRs

NOD-like receptors are a family of cytosolic proteins which ligation induces the activation of the inflammasome and the production of several pro-inflammatory cytokines. Activation of NOD-, LRR- and pyrin domain (PYD)-containing protein 3 (NLRP3) inflammasome in response to *C. albicans* hyphae, induces the activation of caspase-1 which in turn cleaves pro-IL-1β and pro-IL-18 to their bioactive forms[275]. As a result, mice lacking NLRP3 are more susceptible to candidiasis[163].

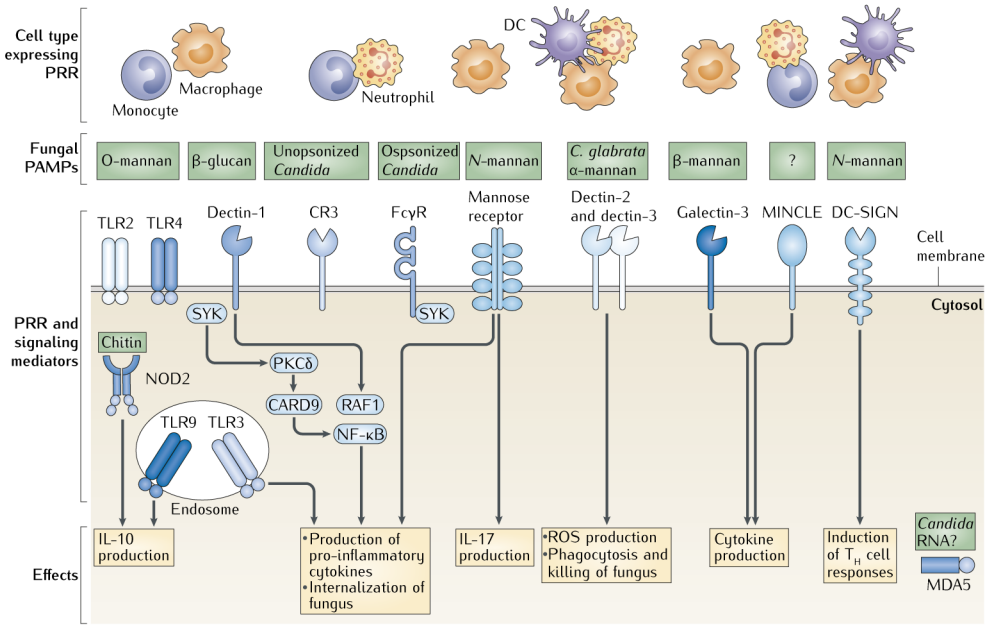

Figure 2.2.4: Recognition of *Candida* by innate immune cells. Engagement of extracellular TLRs induces the expression of pro-inflammatory cytokines, while binding of nucleic acids and chitin to intracellular TLRs induces the production of IFN-γ and IL-10. The dectin family of CLRs are involved in the recognition of β-glucans and mannans and play a fundamental role in the phagocytosis and killing of the fungus. CR3 is important for the recognition of unopsonized *Candida* while FcγR recognize opsonized fungi. The signaling cascade activated by the mannose receptor is still not clear but its engagement induces the production of pro-inflammatory cytokines and IL-17. Finally, galectin 3 and MINCLE are two poorly studied receptors but their activation by *Candida* leads to the release of different cytokines. Adapted from Netea et al.[268].

2.2.3 Innate immunity to fungal infections

After the recognition of fungal PAMPs, different immune and non-immune cells participate to the clearance of *Candida albicans*. Fundamental players of this process are the components of the innate arm of immunity and, more specifically, myeloid phagocytes (Fig. 2.2.4).

Immunity at the epithelial barrier

The first line of defense against fungi is the epithelial barrier, which serves as a mechanical barrier to contrast tissue invasion by fungi. As reported earlier, tissue invasion by *C. albicans* is strictly dependent on the morphological change from yeast to hyphae which allows the fungus to actively penetrate the epithelial barrier[260]. Besides, *Candida* can interact with epithelial cell proteins such as E-cadherin and human epidermal growth factor receptor 2 (Her2), triggering its endocytosis[276]. *Candida* hyphae overgrowth allows its recognition by epithelial cells and the activation of the MAPK partway leading to the c-Fos–dependent release of pro-inflammatory cytokines and of antimicrobial peptides such as β-defensins and cathelicidin[277]. Moreover, disruption of the epithelial barrier induces the release of alarmins (e.g. S100B),

matrix metalloproteases, chemokines and growth factors that concomitantly recruit effector cells as neutrophils and promotes tissue remodeling and barrier repair[278].

Neutrophils

Neutrophils are the most important effector cells in host defense against *Candida*. As a matter of fact, neutropenia is one of the major risk factors for invasive fungal infections in both mice and humans[279,280]. Neutrophils are recruited to the site of infection by the chemokines released by activated epithelial cells and tissue resident macrophages. Recognition of *Candida* by FcγR leads to the clearance of the fungus through the production of ROS. On the other side, triggering of CR3 induces the killing of *Candida* via a non-oxidative effector mechanism[281]. This latter mechanism rely on the production of antimicrobial factors as β-defensins, cathepsin-G, lactoferrin and lysozyme. Moreover, neutrophils are able to release neutrophil extracellular traps (NETs), which are composed of chromatin mesh associated with granule-derived antimicrobial peptides and enzymes such as elastase, cathepsin-G, and myeloperoxidase[282]. Importantly, human neutrophils are able to inhibit the germination of *C. albicans* yeasts to hyphae[268].

Monocytes and macrophages

Tissue-resident macrophages produce pro-inflammatory cytokines and chemokines with recruit other immune cells (among all, neutrophils) to the site of fungal infection[261]. Macrophages can phagocyte both *C. albicans* yeast and hyphae and thus exert potent candidacidal activity[283]. Similarly to neutropenia, lack of macrophages *in vivo* cause accelerated fungal proliferation and increased mortality[284]. Albeit being thought to be less effective than neutrophils in controlling bloodstream *Candida* infections, circulating monocytes are recruited to the site of infection in a CX3CR1-dependent fashion and participate in fungal clearance after developing into macrophages[285,286].

Dendritic cells and T-cell activation

DCs are able to phagocyte and kill *Candida* but their activity is less effective compared to neutrophils and macrophages[287]. However, DCs are fundamental players during fungal infections. For example, their production of IL-23p19 upon recognition of *C. albicans* is fundamental to enhance neutrophil activity through the release of GM-CSF by natural killer (NK) cells[288].

Most importantly, DCs are the main cells responsible for the processing and presentation of fungal antigens to T_H cells. Their wide expression of PRR together with their plasticity to activate distinct signaling pathways confer DCs the ability to affect the balance between $CD4^+$ effector T cells and T_{reg} cells, thus setting the threshold between commensalism or infection[252]. DCs recognition of fungi by TLR and CLR induce the activation of a T_H1 response and the subsequent release of INF-γ by activated $CD4^+$ T-cells[289]. Interestingly, INF-γ– and IL-18–deficient mice fail to induce an effective T_H1 response and thus are more susceptible to disseminated candidiasis[290,291].

T_H17 responses are predominantly activated in response to DCs activation through the mannose receptor[252]. The release of IL-17 and IL-22 is important for the recruitment and activation of neutrophils as well as the release of antimicrobial peptides by epithelial cells[292]. Moreover, patients harboring mutations in members of the IL-17 axis or with defects in T_H17 activation are more susceptible to mucosal candidiasis, while mice with IL-17 signaling deficiency are susceptible also to systemic candidiasis[293,294].

T_H2 commitment by IL-4 and IL-13 has detrimental roles during fungal infections by dampening T_H1 responses and favoring fungal survival and disease relapse[252,295]. In agreement, IL-4 depletion make mice more resistant to *C. albicans* infection but at the same time fail to promote the development of a protective T_H1 response during re-infection[296].

2.2.4 Are mast cells playing the game?

Despite their potential role during fungal infections, interactions between mast cells and fungi have been only poorly investigated and published data are often contradictory.

Gastrointestinal colonization with *C. albicans* induced mast cell infiltration and degranulation, and promoted the sensitization against food antigens by increasing the permeability of the gastrointestinal mucosa[297,298]. Two studies showed that rat peritoneal MCs as well as murine BMMCs were able to phagocytose heat killed and opsonized live *C. albicans* yeasts, in a mechanism involving both TLR2 and dectin-1[299,300]. Interestingly, BMMCs were not able to kill phagocytosed yeasts but instead efficiently killed the un-opsonized yeasts in the extracellular environment[299]. Phagocytosis of heat killed yeasts was found to be dependent on the recognition of fungal PAMPs by TLR2 and dectin-1. Moreover, triggering of these receptors by *Candida* or zymosan particles resulted in the production of nitric oxide (NO) but not ROS[300]. On the contrary, a recent study demonstrated that BMMCs released ROS in response to *in vitro* stimulation with *C. albicans* yeasts and hyphae. Fungal challenge also induced BMMCs and PDMCs degranulation, the activation of the NF-κB pathway and the secretion of TNF-α, IL-1β, IL-6, IL-10, CCL3 and CCL4. Interestingly, TNF-α release could be partially reduced after the neutralization of dectin-1 but not TLR2 signaling[167].

Human MCs also respond to *Candida* challenge. Lopes et al. demonstrated that *C. albicans* challenge of the human mast cell line HMC-1 induced degranulation (at high multiplicity of infection - MOI) and the release of pro- and anti-inflammatory cytokines. Interestingly, a two-phased response was observed, characterized by an early secretion of IL-8, and a later release of IL-16 and IL-1ra. Supernatants of infected MCs induced the chemoattraction of neutrophils but not monocytes, confirming the important role of IL-8 in neutrophil recruitment. Moreover, MCs were transiently able to release tryptase-containing MC-extracellular traps and to kill *Candida* in the early phase of infection. However, at the late stage of infection *C. albicans* potently triggered MCs death, also by the outgrowth of internalized yeasts[301].

As already discussed, mast cells can be also activated by dectin-1 ligands which mimic the fungal cell wall such as zymosan and curdlan. Stimula-

tion of BMMCs with zymosan resulted in the generation of ROS and the upregulation of dectin-1 expression[166]. A similar study on human CBMC reported that zymosan stimulation induced LTB_4 and LTC_4 production in a SYK-dependent manner[149]. Recently, it has been shown that curdlan stimulation was able to induce BMMCs degranulation but not the release of LTC_4, IL-6 nor CCL-2[302].

As a matter of fact, deciphering the interaction between MCs and *Candida* remains a challenging effort.

2.3 INNATE IMMUNE MEMORY: BRIDGING THE INNATE AND ADAPTIVE ARMS OF IMMUNITY.

IT IS COMMON KNOWLEDGE that the vertebrate immune system is composed of two arms: the innate and the adaptive immunity arms. Innate immune responses are mainly mediated by the recognition of non-self molecules by myeloid cells (as monocytes/macrophages, neutrophils, and DCs), which culminates with the eradication of the invading pathogens. Current opinion is that innate responses are primitive, nonspecific and unable to induce immunological memory. On the contrary, adaptive immunity rely on the slower activation of B and T lymphocytes which specifically recognize pathogens' antigens and give rise to antibody-mediated humoral responses. Upon secondary infections with the same specific pathogen, memory-B and T cells rapidly expand promoting faster and more effective humoral- and cellular-mediated responses[303].

Despite this widely accepted dichotomy between innate and adaptive responses, there is an increasing body of evidence suggesting that innate immune processes might be more sophisticated than what is currently believed. A first clue is given by the *Regnum Vegetabile*: plants that have been locally infected can develop an enhanced, systemic, long-lasting resistance to reinfections, a condition referred to as systemic acquired resistance (SAR)[304]. Moreover, several studies published in the last 40 years demonstrated that immune responses of several invertebrates such as cockroaches, shrimps, beetles, mosquitoes and clams (which all lack adaptive immune responses) could be enhanced upon reinfection[305–312]. This phenomenon has been termed "innate immune memory" or "trained immunity" and is complementary to the phenomenon of tolerance, in which an infectious episode can lead to a refractory responce to secondary infections (Fig. 2.3.1)[313].

Innate immune memory differs from the classical adaptive memory from several point of view. Fist of all, this process exclusively involves innate cells as myeloid cells, NK cells and ILCs, which are not involved in the classical immunological memory. Second, contrarily from adaptive immune mechanisms it does not depend on permanent genetic mutations or recombinations but rather on the epigenetic rewiring of the transcription programs. As such, the increased responsiveness upon reinfection is not specific for a

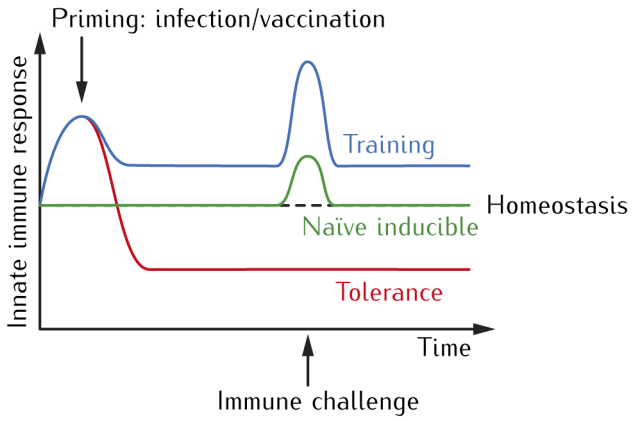

Figure 2.3.1: Proposed model of differential programming of innate immunity. Innate immune response to infections (priming) can induce immunological reprogramming, leading to an enhanced (training) or a refractory (tolerance) status. Adapted from Quintin et al.[314].

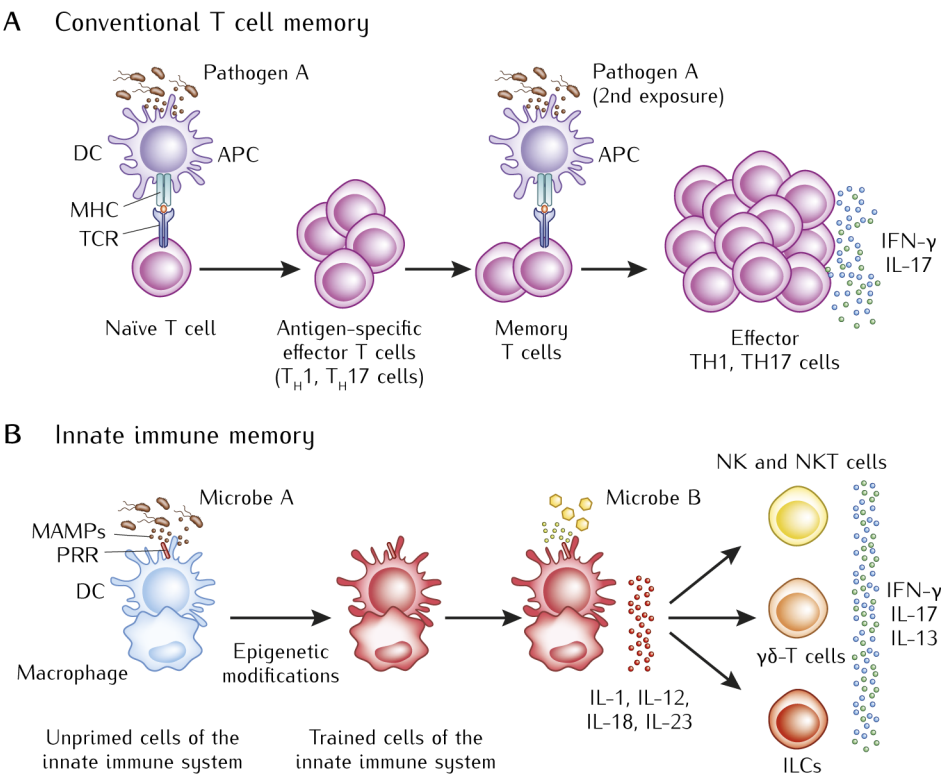

Figure 2.3.2: Classical immunological memory and innate immune memory. **A.** Classical immunological memory relies on the somatic recombination of the loci encoding for immunoglobulins and the T-cell receptor (TCR) after the first encounter with the pathogen. This process confers a long-term, pathogen-specific protection. **B.** The recognition of microbial-associated molecular patterns (MAMPs) by cells belonging to the innate immune system induces their epigenetic reprogramming. Trained cells display enhanced inflammatory and antimicrobial properties, and thus are able to mount a prompter non-specific response during reinfection. Adapted from Netea et al.[316].

specific pathogen or ligand (Fig. 2.3.2). Last but not the least, this enhanced or repressed status lasts for a shorter period of time compared to adaptive immunity[315].

2.3.1 Innate immune memory in vertebrates.

The discovery of memory mechanisms in plants and invertebrates raised the question whether this phenomenon was confined to lower taxa or also functional in vertebrates. This hypothesis is supported by the great number of studies published in the last years, showing that also mice and humans posses innate immune memory *in vivo*.

Stimulation with the bacterial protein flagellin induces the cross-protection against *Streptococcus pneumoniae* and also against rotavirus infections, in a mechanism dependent on DCs activation[317,318]. Similarly, β-glucan stimulation afforded protection against infections with *S. aureus*[319,320]. Also ligands for intracellular PRRs are able to induce trained immunity in mice, as the treatment with CpG and muramyl dipeptide (MDP, a component of PGN

recognized by intracellular NOD receptors) confer resistance to *E. coli* sepsis and *Toxoplasma* infection, respectively[321,322]. It is interesting to note that also cytokines can be directly involved in the induction of innate immunological memory. For example, survival of mice infected with *P. aeruginosa* was improved in animals that were injected with recombinant IL-1β 3 days before the bacterial infection[323].

Further proofs of trained immunity in vertebrates come from *in vivo* studies on animals primed with pathogens rather than purified PAMPs. Mice that were infected with the attenuated strain PCA-2 of *C. albicans* (which is unable to germinate hyphae) were protected against the reinfection with the virulent *C. albicans* strain CA-6[324]. Importantly, this mechanism was found to be independent on B and T lymphocytes activation, as the protection was induced also in athymic and *Rag1*-deficient mice (which are unable to develop mature B and T lymphocytes)[325,326]. Infections with the helminth *Nippostrongylus brasiliensis* induced the expansion of neutrophils with a peculiar transcriptional profile, which in turn activated M2 macrophages, conferring a long-lasting protection from reinfection. Interestingly, adoptive transfer of primed macrophages from infected to naïve animals was sufficient to enhance protection against *N. brasiliensis*[327]. Viral infections also seem to have similar biological activity. Challenge with murine CMV induced the activation of Ly49H$^+$ NK cells which persisted within different organs. Upon CMV reinfection these "memory" NK cells were able to rapidly expand and degranulate, finally sustaining a strong protective immune response[328]. Moreover, it has been shown that herpesvirus latency increased the resistance to *L. monocytogenes* and *Yersinia pestis* by promoting the production of IFN-γ by activated macrophages[329].

Interestingly, immunization of mice with the tuberculosis vaccine bacillus Calmette-Guérin (BCG) induces a T cell-independent protection against secondary infections with *C. albicans* or the trematode *Schistosoma mansoni*[330,331]. This phenomenon could also be observed in humans, as immunization with BCG was found to alter the functional status of circulating monocytes, enhancing their response to *M. tuberculosis*, *C. albicans* and *S. aureus*. Macrophage response was still markedly enhanced after 3 months from the vaccination and returned only partially similar to the baseline 12 months after the immunization[332]. The hypothesis that BCG immunization might offer nonspecific beneficial effects was validated by epidemiological studies and clinical trials on children and newborns BCG vaccination in West Africa. These studies demonstrated a nonspecific protection of the vaccine against unrelated pathogens, resulting in a lower overall mortality[333,334]. Moreover, many other epidemiological studies suggested that live vaccines other than BCG (as measles and polio vaccines) protect against nonspecific pathogens[335]. It is thus tempting to say that these beneficial effects may rely on the induction of innate immune memory.

2.3.2 Endotoxin tolerance: the other side of the coin?

Although inflammation is crucial for a proper eradication of pathogens, uncontrolled inflammation can lead to excessive tissue damage and to the

development of pathological states as chronic autoimmune diseases, degenerative diseases and cancer. As such, a number of mechanisms evolved in order to restrain excessive activation of inflammatory processes[336].

Endotoxin tolerance (ET) is a well known (albeit not completely understood) mechanism to restrain inflammation and protect the host against endotoxin shock. ET is defined as a process in which cells already exposed to LPS enter a transient state of unresponsiveness against further challenges with LPS, making them tolerant to re-stimulation. These tolerant cells respond to LPS restimulation with a less robust release of pro-inflammatory mediators and a sustained production of anti-inflammatory mediators[338]. This phenomenon was initially described during rabbit vaccination with the typhoid vaccine (an attenuated strain of the Gram- *Salmonella enterica*), as it was noticed that the vaccine-induced fever was gradually reduced after recurring injections of the vaccine[339]. *In vivo* studies demonstrated that the injection of a sublethal dose of LPS in mice protected them against a subsequent lethal dose of LPS. This phenomenon was ascribed to monocytes and macrophages and particularly to their tolerant status which was responsible for the attenuated response to the second challenge of LPS[340,341]. Several *in vitro* studies on endotoxin-tolerant macrophages demonstrated a drastic reduction of the expression and release of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 together with the chemokines CCL2, CCL4 and CXCL10 both in mice and humans. On the contrary, these tolerant cells showed a marked upregulation of anti-inflammatory cytokines such as TGF-β, IL-10, IL-1ra together with cell surface receptor as the macrophage receptor with collagenous structure (MARCO) and CD64[342–346]. These macrophages phenotypically resemble the "alternatively-activated" M2 macrophages, also demonstrating an improved phagocytosis and an impaired ability for antigen presentation[345].

***Endotoxin shock** is a complex pathology that arise from a severe inflammatory response towards Gram- bacteria. Patients typically present fever and hypotension, which generally evolves in multiorgan failure and death[337].*

On the other side, it has been reported that the stimulation with a very low dose of endotoxin had a completely opposite effect, potentiating the response to subsequent LPS challenge. This effect was also true *in vivo*, as mice that received a super low dose of LPS showed higher mortality rates after a second injection of a high dose of LPS, compared to controls[347,348].

Taken together, it appears clear that also LPS priming can induce a strong immunological memory on monocytes and macrophages. Moreover, the initial stimulation with LPS is fundamental for the induction of a tolerant rather than a trained phenotype, depending on the dose. From this point of view, tolerance and training can be considered the two sides of the same coin.

2.3.3 Mechanisms of innate immune memory.

Studies in lower taxa.

The existence of a mechanism of innate immune memory was initially speculated in plants an invertebrates since both the taxa displayed characteristics of immune memory albeit lacking adaptive immune responses[315].

Upon infections with necrotizing agents, plants often develop an enhanced resistance to other pathogens also at sites distant from the first infection, a

phenomenon named "systemic acquired resistance". It was demonstrated that SAR induction is dependent on the production of salicylic acid at the site of the infection, which then act as a signaling molecule to induce resistance even in remote tissues[349]. Similarly, the colonization of plant roots with non-pathogenic fungi and bacteria induce an analogous phenomenon of systemic resistance which protect the plant against a broad number of pathogens in a salicylic acid-independent mechanism[350]. SAR can be induced also by treatment with chemicals: studies on the fungicide Pyraclostrobin demonstrated that this molecule is able to induce resistance of Tobacco plants to *Tobacco mosaic virus* and *Pseudomonas syringae* infections, as well as long-term protection of several crops to different pathogens[351–353].

Mechanisms responsible for SAR induction were only poorly understood until a few years ago. It is now hypothesized that this phenomenon is regulated at 4 levels. As described above, release of salicylic acid and pipecolic acid is fundamental for the induction of SAR and stimulation with salicylic acid or by its functional analog benzothiadiazole is sufficient to recapitulate SAR[349]. Studies on *Arabidopsis* demonstrated that benzothiadiazole-treated plants expressed higher levels of transcripts coding for MAP kinases (as MPK3 and 6). These MAPK represent the second step of regulation: albeit being normally found in a inactive state (which counted for their name "dormant MAPK"), they rapidly become phosphorylated upon pathogen challenge and mediate a signaling network that culminates with the expression of defense genes. As a consequence of their heightened expression, MAPK activation is much more prominent during SAR[354]. Together with increased MAPK expression, SAR plants also display a higher synthesis and secretion of several PRRs[355]. Finally, it has been reported that epigenetic modifications (mainly histone marks) are important regulators of defense genes upon restimulation. For example, it was described that SAR induced increased H3K4me3 and H3K4me2 on the transcription factors *WRKY6/29/53* promoters, and H3K9ac, H4K5ac, H4K8ac, and H4K12ac on *WRKY29* promoter (see Box 1 for clarifications)[356].

Two different mechanism of innate immune memory can be described in invertebrates. The first mechanism comprises several strategies responsible for the enhancement of the innate response upon reinfection. For example, in the European woodlouse *Porcellio scaber*, *in vitro* priming of haemocytes with heat killed bacteria resulted in the increased phagocytosis of the same live bacteria during reinfections[357]. Differential responses to reinfection could also be the consequence of phenotypic changes in the cellular population, as in the case of *Anopheles gambiae* in which training by gut microbiota induces the expansion of granulocytes increasing the protection agains *Plasmodium* reinfections[311].

On the the other hand, another mechanism involved in invertebrate innate immune memory is much closer to the specific adaptive memory of vertebrates and rely on RAG-independent somatic diversification. Two of the most prominent example are represented by the diversification at genomic level of fibrinogen-related proteins (FREPs) in the snail *Biomphalaria glabrata* and the Down Syndrome Cell Adhesion Molecule (Dscam) in *Drosophila*

melanogaster and *Anopheles gambiae* which confer adaptive and specific recognition of pathogens[358–360].

Molecular mechanisms of trained immunity in vertebrates.

It is supposed that induction of innate immune memory in vertebrates rely on the convergence of different regulatory networks. These includes chromatin remodeling, the modulation of upstream signaling pathway as well as the contribution of microRNAs (miRNA) and long non-coding RNAs (lncRNA). Since we are living in the epigenetics era, much attention has been given to the epigenetic reprogramming of monocytes/macrophages during stimulation and it is common belief that this mechanism accounts for the major features of innate immune memory[315].

Box 1 - Epigenetic markers

The basic unit of chromatin is the nucleosome, which is composed of 147bp of DNA coiled around an octamer of histone proteins (two of each H2A, H2B, H3 and H4). DNA bases and histone proteins can be covalently modified by several enzymes, finally affecting chromatin accessibility and gene transcription. Methylation of cytosine bases on DNA is linked with gene transcription repression, while the role of histone tails modification (acetylation, methylation, phosphorylation and ubiquitination) is less clear. Acetylation of histone lysine residues (e.g. H3K27ac) is believed to loose the interaction between DNA and histone core proteins, allowing the recruitment of the transcription machinery. On the other side, methylation of histone lysine residues can activate as well as repress gene transcription. H3K4 methylation (H3K4me), H3K9me, H3K27me and H3K4 trimethylation (H3K4me3) are linked with gene transcription, while H3K9 di-methylation (H3K9me2), H3K9me3, H3K27me2 and H3K27me3 are known markers of gene repression[361,362].

In resting conditions, chromatin regions encoding for inflammatory genes are in a repressed status, showing limited accessibility to transcription factors (TF), low level of histone acetylation and absence of RNA polymerase II (RNA pol II) activity (Fig. 2.3.3). Upon the first stimulation, the chromatin structure of these regions is deeply modified: histones gain activation marks (as acetylation and tri-methylation) thus increasing chromatin accessibility and the recruitment of TF and RNA pol II, finally leading to gene transcription. A growing body of evidence supports the hypothesis that after the removal of the stimulus, some of these chromatin modifications remain persistent on specific promoters and enhances of inflammatory genes, allowing a more efficient transcription during reinfection[363,364]. This process is particularly important in the case of latent or *de novo* enhancers, which are regulatory regions that gain histone modification typical of enhancers only upon stimulation[365]. For example, training of human monocytes with β-glucan or *C. albicans*, induced a stable and global increase in the levels of H3K4me3, especially at the level of the promoters of genes associated with immune signaling pathways (e.g. *Myd88*, *Raf1*, *Dectin-1*, *Tlr4*) and of pro-inflammatory cytokines (e.g. *Tnf-α*, *Il-6*, *Il-18*). Concordantly, total level of endogenous p38 MAPK were increased in trained monocytes, and its phosphorylation upon restimulation was quicker and more prominent than controls. On the contrary, trained monocytes did not show any differences in the global levels of H3K27me3[326].

Similarly, Yoshida et al. demonstrated that in normal conditions, the transcription factor ATF7 is able to repress LPS-induced gene expression by binding to promoters and driving the deposition of the repressive histone mark H3K9me2 by action of H3K9 dimethyltransferase G9a. Concomitantly, LPS treatment of murine peritoneal macrophages induced p38-dependent ATF7 phosphorylation, which led to the release of the TF from promoters and a marked decrease in repressive H3K9me2 marks. Interestingly, macrophage memory *in vivo* was maintained for at least 3 weeks, providing resistance to *S. aureus* reinfections[366].

Innate immune memory induced by vaccination is also supposed to rely on similar mechanisms. BCG vaccination of healthy volunteers induced a long lasting increase in the response against bacterial and fungal pathogens by circulating monocytes, which persisted for at least 3 months after injection. This training effect relied on the marked increase of H3K4me3 at cytokines and TLR4 promoters[332].

Another proposed mechanism of innate immune memory is based on the recent finding that monocyte training by β-glucan caused a prominent shift of the cellular metabolism from oxidative phosphorylation toward anaerobic glycolysis. This phenomenon was dependent on the activation of a dectin-1/AKT/mTOR/HIF-1α pathway, as treatment of mice with metformin (an mTOR inhibitor) prior to β-glucan training prevented the induction of a protective response against *C. albicans* infections[367]. Importantly, the availability of the Krebs cycle metabolites α-ketoglutarate and succinate is fundamental for the activity of the JMJ-family of lysine-demethylase and the TET-family of methylcytosine hydroxylases, two important groups of enzymes involved in the remodeling of the epigenetic landscape[368]. As a whole, this hypothesis is supported by the fact that also the two main phenotype of macrophages, M1 and the alternatively activated M2, rely on distinct metabolic pathways[369].

Molecular mechanisms of endotoxin tolerance.

Albeit endotoxin tolerance have been studied for over 60 years, many aspects of the molecular mechanisms governing this phenomenon remain enigmatic. As for immune training, the induction of ET is dependent on many factors such as the modulation of signaling networks, the reprograming of gene expression via chromatin remodeling and the effect of miRNA and autocrine signaling[370].

A great number of studies demonstrated that this phenomenon is not dependent of the expression level of any of the proteins involved in LPS recognition (e.g. TLR4, CD14, MD2 or LBP)[371]. On the contrary, ET macrophages (i.e. tolerant macrophages as a result of LPS exposure) showed an impaired formation of both the TLR4-MyD88 and MyD88-IRAK1 complexes together with the decreased kinase activity of IRAK1, suggesting that ET was rather characterized by an impaired intracellular signaling[372,373]. It is known that LPS stimulation induces the activation of the MAPK pathway, leading to the activation of the TF AP-1 and NF-κB[374]. Consistent with the hypothesis of an impaired signaling during ET, it was demonstrated that LPS-treated cells were refractory to a secondary LPS challenge due to their reduced levels of

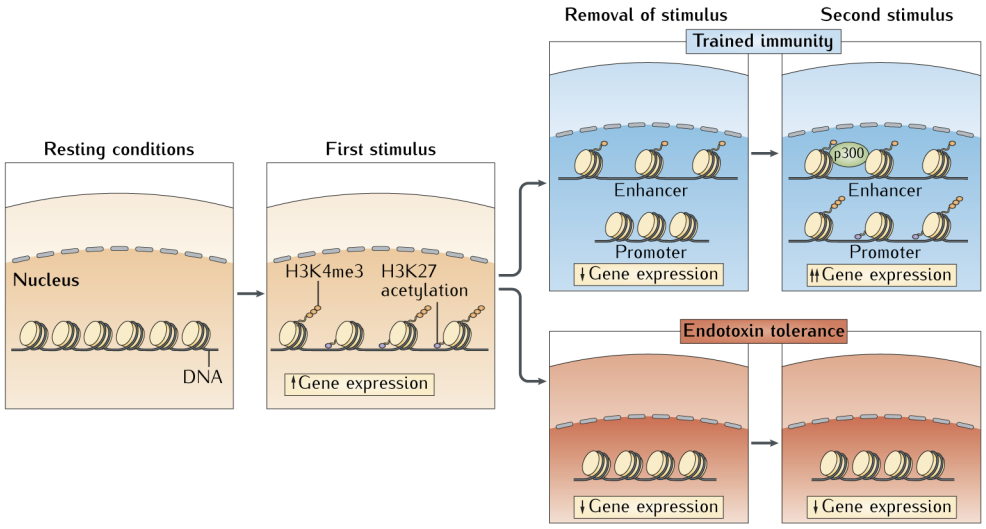

Figure 2.3.3: Epigenetic regulation of innate immune memory. Initial stimulation induces the acetylation of histone H4 and H3K4me3 on the promoters of pro-inflammatory genes. In the case of trained immunity, activation marks such as H3K4me are retained on the promoters and enhancers of pro-inflammatory genes which facilitate the recruitment of the transcription machinery and chromatin modifiers upon a second stimulation. On the contrary, during ET histone modifications are lost after the removal of the stimulus and chromatin become again poorly accessible. Upon restimulation, "tolerizeable" genes fail to gain histone activation marks and are not be transcribed. Adapted from Alvarez-Errico et al.[362].

ERK1/2, JNK and p38 phosphorylation during restimulation[371,375,376]. This impaired activation of MAPK signaling was also correlated with the defective activity of both AP-1 and NF-κB[346,374].

With regard to NF-κB activation, it is worth of note that LPS stimulation mostly induce the activation of the canonical NF-κB pathway, which culminates with the nuclear translocation of the p65/p50 heterodimer[377]. As previously described, a key step required for NF-κB activation is the proteasomal degradation of its inhibitor IκB[112]. Several authors reported that the impaired NF-κB activation during endotoxin tolerance was correlated with a reduced IκB-α and IκB-β degradation after LPS restimulation, possibly due to both an impaired proteasomal degradation and to a more rapid turnover of the inhibitory subunit[346,374,378,379]. Another interesting feature of NF-κB in ET macrophages is it plasticity to undergo homo- and heterodimerization. It has been shown that in LPS-treated cells the NF-κB subunit composition is shifted from the canonical p65/p50 heterodimer to the p50/p50 homodimer. Notably, p50 homodimers lack the transactivation domain and are unable to trigger the transcription upon binding to gene promoters: by competing with the p65/p50 heterodimers for DNA binding is then able to inhibit gene transcription[380,381]. In agreement, ET was not induced in macrophages derived from p50$^{-/-}$ mice[382].

As reported for trained immunity, macrophages are subjected to substantial gene-expression reprogramming during endotoxin tolerance (Fig. 2.3.3). A study by Foster et al. identified two classes of genes modulated in murine

macrophages after induction of ET[343]. The first class of genes (named 'tolerizeable") comprised mostly inflammatory genes which were drastically downregulated upon LPS restimulation (e.g. *Il-6*, *Il-1b*, *Mmp13*). On the contrary, the second class of genes included genes encoding for antimicrobial mediators that were upregulated or remained inducible after the second LPS challenge and were named "non-tolerizeable". Notably, albeit the first LPS stimulation induced H3K4 acetylation of the promoters for both the tolerizeable and non-tolerizeable genes, histones of non-tolerizeable genes failed to be re-acetylated upon a further LPS challenge. Also H3K4me3 was induced on the promoters of both classes during the initial stimulation: albeit being rapidly lost on tolerizeable promoters, it was stably maintained on non-tolerizeable promoters[343].

With regard to chromatin remodeling, a previous study reported that after LPS priming, the NF-κB subunit RelB was able to bind to the promoter regions of TNF-α and IL-1β, directing the di-methylation of H3K9 and silencing gene transcription[383].

2.3.4 MASTer memory: a changing concept in mast cell biology.

In recent years several studies reported that also mast cells show classical traits of immunological memory. Albeit being only poorly investigated, the topic is of relevant importance since mast cells are long lived cells and their priming might affect tissue homeostasis.

Initially it has been reported that LPS stimulation induced endotoxin tolerance in BMMCs. This phenomenon was due to the upregulation of the phosphatase SHIP (but not of SHIP2 nor PTEN) by the autocrine effect of LPS-induced release of TGF-β. In agreement, SHIP$^{-/-}$ MCs and Mφ did not display ET and SHIP$^{-/-}$ mice succumbed quickly after injection of low doses of LPS[384]. More recent studies further demonstrated the ability of MCs to respond to LPS by releasing pro-inflammatory TNF-α and IL-6 in a p38-dependent manner, and that the release of these cytokines was severely decreased upon a secondary stimulation with LPS or with TLR2 agonists, suggesting that endotoxin tolerance could induce also cross tolerance against different stimuli[385,386]. Interestingly, TLR4-dependent LPS stimulation amplified the release of TNF-α and IL-6 induced by IgE/Ag cross linking. This effect was also induced in tolerant BMMCs with a less marked effect due to the decreased activation of p38 and NF-κB and to the expression of SOCS1 and SOCS3 which are known to be negative regulators of LPS-induced TLR4 activation[386]. In a similar way, prolonged activation with TLR1/2, TLR2/6, TLR3 or TLR4 ligands amplified MCs response to IgE/Ag challenge[387]. On the other way around, MCs sensitization with IgE alone increased the response to LPS stimulation by enhancing IKK-IκB phosphorylation and NF-κB nuclear translocation. However, IgE sensitization was not sufficient to abolish ET establishment after LPS stimulation[388].

A recent report by Poplutz et al. demonstrated that during ET mast cells displayed a decreased nuclear translocation and DNA binding ability of the canonical NF-κB p65/p50 heterodimer. The latter phenomenon was due to the constitutive presence of the suppressive marker H3K9me3 on the pro-

moters of TNF-α and IL-6 which inhibited NF-κB binding to the promoters. These repression markers were transiently lost during the initial LPS stimulation (thus allowing NF-κB binding and gene transcription), but remained unchanged during the second LPS challenge. According to previous reports, ET tolerance did not affect TNF-α and IL-6 release during IgE/Ag cross-linking, possibly by the involvement of Ca^{++}-dependent NFAT activation. The whole process was demonstrated to be mediated by the IκB-family member BCL3, as $BCL3^{-/-}$ MCs failed to induce ET[389].

3 AIM OF THE WORK

Mast cells (MCs) are long lived, tissue resident cells belonging to the innate immune arm of immunity. They are localized in tissues prone to be colonized by bacteria and fungi and, as such, are among the first immune cell types which can get in contact with commensals and pathogens. Despite this potential role, interactions between MCs and the commensal microbiota have been poorly investigated.

The aim of this project is to unravel the mechanisms of reciprocal influence between mucosal mast cells and the human commensal *Candida albicans*. The first part of this work will focus on the physical interactions between murine mast cells and the fungus. Additionally, since MCs are known to promote or restrain the activity of other members of the immune system, the crosstalk with tissue-resident macrophages during fungal clearance will be evaluated. The second part of this project will try to depict mast cells' role in the wider picture of the mutual interplay with the intestinal microbiota. Known that tissue-specific stimuli are fundamental for the terminal differentiation of mast cell progenitors, we aimed to determine the long term effect of microbial ligands on MCs development and response to *C. albicans*.

Part II

EXPERIMENTAL STUDIES AND RESULTS

4 RESULTS

4.1 MAST CELL – CANDIDA INTERACTIONS.

4.1.1 MC - *Candida* immunological synapse.

To dissect the interaction between MCs and *C. albicans*, BMMCs were co-cultured with live *C. albicans* both in the yeast and hyphal forms. *Dectin-1*$^{-/-}$ BMMCs were obtained from *in vitro* differentiation of *dectin-1*$^{-/-}$ mice bone marrow precursors. As expected, the absence of the receptor did not impair BMMCs development since cellular yield as well as c-Kit and FcεRIa expression were comparable between WT and *dectin-1*$^{-/-}$ cells (Fig. 4.1.1 A). On the contrary, dectin-1 surface expression was lower in *dectin-1*$^{-/-}$ BMMCs compared to WT (C57BL/6) controls (Fig. 4.1.1 B).

Intriguingly, after a few hours of co-culture, MCs were found to tightly interact with the hyphal form of the fungus in a way that resembled phagocytosis. Time-lapse bright field microscopy experiments showed that MCs interacted with *C.albicans* as soon as it changed its morphology from yeasts to hyphae but not with yeasts alone, suggesting that this phenomenon specifically relies on the progression of *Candida* germination (Fig. 4.1.2 A).

Dectin-1 signaling is known to be activated only when the receptor binds to particulate β-glucans. This interaction induce the receptor to cluster in synapse-like structures (called "phagocytic synapses") to which signaling molecules are recruited[390]. Albeit *C. albicans* hyphae β-glucans are shielded by a layer of mannoproteins and thus fail to activate dectin-1, it has also been hypothesized that this receptor may be responsible for the recognition of hyphal β-glucans probably due to the presence of thinner mannan fibrils[264].

Interaction with *C. albicans* by *dectin-1*$^{-/-}$ BMMCs was comparable with WT BMMCs. Immunofluorescence staining showed that phagocytosis of *C. albicans* hyphae induced the rearrangement of the α-tubulin cytoskeleton in

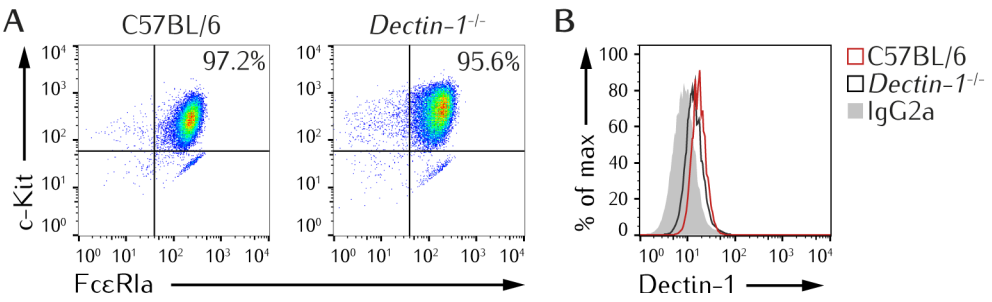

Figure 4.1.1: *Dectin-1*$^{-/-}$ BMMC differentiation. *Dectin-1*$^{-/-}$ BMMCs were obtained from *in vitro* differentiation of bone marrow progenitors of *dectin-1*$^{-/-}$ mice. BMMCs were checked for cKit and FcεRIa expression (A) and for dectin-1 expression (B). Representative results of two independent experiments.

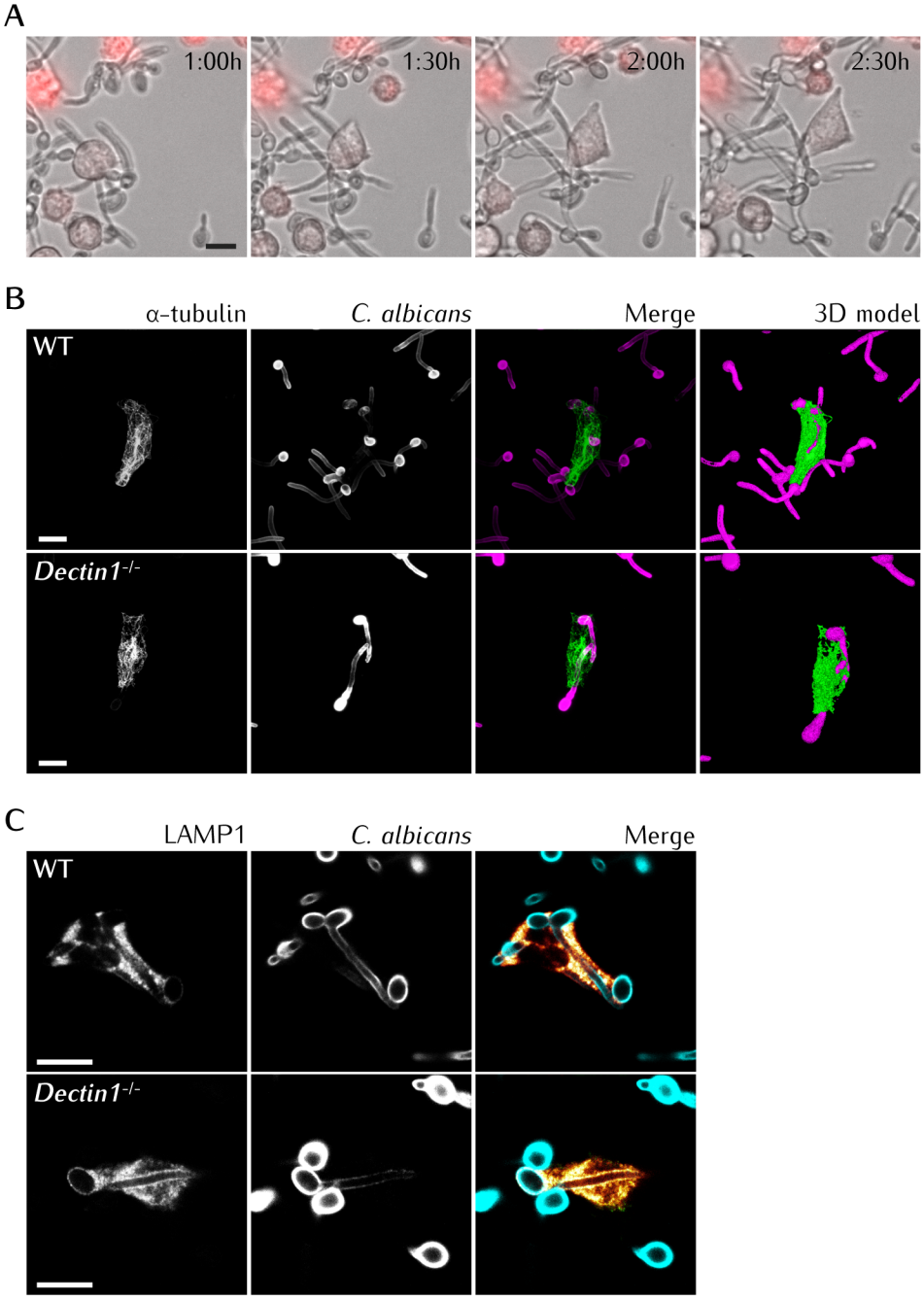

Figure 4.1.2: Mast cells can tightly interact with *C. albicans* hyphae. **A:** Time-lapse microscopy show MC (stained in red) intimately interacting with *C. albicans* hyphae after 90 minutes of co-culture, resembling the phenotype of "frustrated phagocytosis". On the contrary, phagocytosis of yeasts was not observed. **B, C:** Immunofluorescence images show that mast cells rearrange their α-tubulin cytoskeleton during the interaction and accumulate LAMP1^{+} vesicles at the interface with the fungus. *Dectin-1*$^{-/-}$ BMMCs display an identical ability to interact with *C. albicans* hyphae. Scale bars: 10μm.

both WT and *dectin-1*$^{-/-}$ BMMCs (Fig. 4.1.2 B). 3D-modeling of α-tubulin stained BMMCs, indicate that BMMCs are able to "wrap" around the fungal hypha (Fig. 4.1.2 B), resembling the phenotype of the so-called frustrated phagocytosis[391,392].

In order to define whether this behaviour could be ascribed as phagocytosis or not, BMMCs were stained for two markers of early- and late-endosomes. During phagosome maturation, phagosomes acquire different surface molecules (e.g. Rab GTPases) which play key roles in the process of maturation. The early-endosome antigen 1 (EEA1) is involved in the initial stages of the maturation process by binding to PIP_3 and mediating endosomes fusion. On the other side, the late phase marker lysosomal-associated membrane protein 1 (LAMP1) is acquired at the late stages of maturation, after the endosomes has fused with acidic lysosomes[393]. None of the cells stained for EEA1 (not shown) while most of them stained positively for LAMP1. Interestingly, both WT and *dectin-1*$^{-/-}$ BMMCs stained positively for LAMP1, suggesting that this receptor is not required for the accumulation of LAMP1$^+$ vesicles.

4.1.2 MCs degranulation in response to fungal challenge.

Seen that LAMP1 is also considered a marker of degranulation, MCs degranulation in response to *C. albicans* was evaluated. Fungal challenge was performed in the presence of 10% serum in order to allow *C. albicans* switch to the hyphal form, and the release of β-hexoseaminidase and leukotrienes C_4, D_4 and E_4 was determined after 30 minutes, 1 hour and 2 hours. The release of β-hexoseaminidase was minimally increased over the control only after 2 hours of stimulation (7.2% $\pm$ 4.4% *C. albicans* vs. 4.9% $\pm$ 1.9% control, p=0.5262), while leukotrienes levels remained constant at all the time points (Fig. 4.1.3 A). IgE/Ag stimulation was used as positive control of MCs degranulation.

Taken together, these data indicate that, in our setup, BMMCs do not degranulate upon the encounter of *C. albicans*. Alternatively, it is possible that the exocytosed cargo rapidly interacted with the fungus due to the tight interaction between the mast cell and *C. albicans*, thus making it barely detectable in culture supernatants.

4.1.3 Cytokine release in response to fungal challenge.

MCs can release a broad range of *de novo* synthesized mediators which play an important role in the modulation of the immune response to pathogens[190]. To understand the role of MC-derived mediators during fungal infections, mast cells were co-cultured with live *C. albicans* yeast and hyphae and culture supernatants assessed for different cytokines. After 3 hours of co-culture it was already possible to detect IL-6 and TNF-α (Fig. 4.1.3 B), while after 24 hours also IL-4 and IL-13 were detected in culture supernatants (Fig. 4.1.3 C). Interestingly, *C. albicans* yeasts were more effective than hyphae in inducing MCs release of TNF-α, IL-6, IL-4 and IL-13. These data were also confirmed by gene expression analyses that revealed a strong

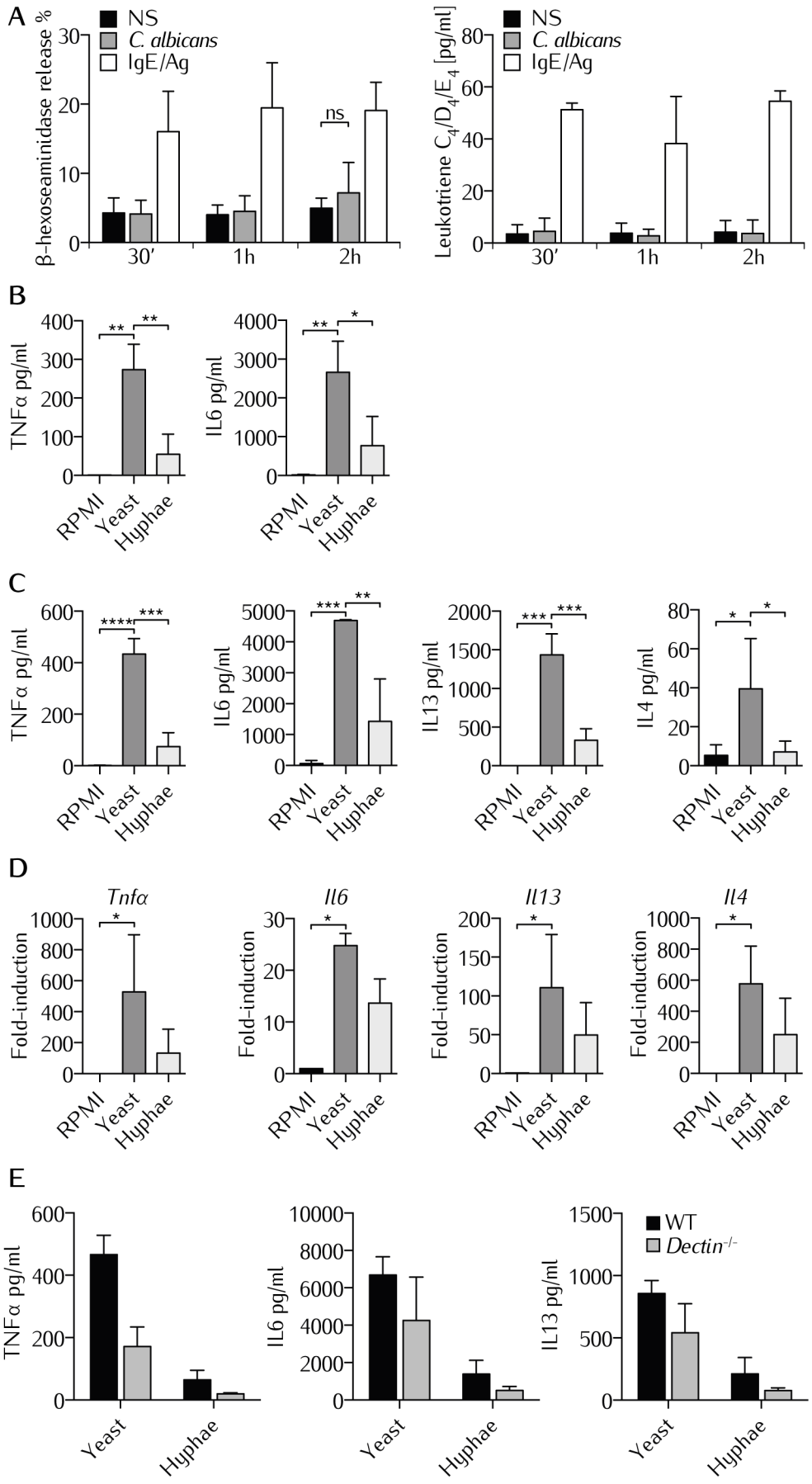

Figure 4.1.3: MCs degranulation and cytokine release in response to *C. albicans*. **A:** BMMCs degranulation in response to *C. albicans* was evaluated by the release of β-hexoseaminidase and the synthesis of leukotrienes. No degranulation was observed after 30 minutes, 1 or 2 hours. *Continues on next page...*

upregulation of *tnf-α*, *il6*, *il13* and *il4*. Again, stimulation with *C. albicans* yeasts rather than hyphae induced higher levels of cytokines expression (Fig. 4.1.3 D).

To assess whether dectin-1 plays a role in MCs activation by *C. albicans*, *dectin-1*$^{-/-}$ BMMCs were co-cultured with the fungus and cytokine levels were assessed after 24 hours. Preliminary results showed an impaired release of TNF-*α*, IL-6 and IL-13 by *Dectin-1*$^{-/-}$ BMMCs compared to WT controls, both during the stimulation with yeasts and hyphae (Fig. 4.1.3 E). Notably, cytokine release was only impaired and not completely abolished, in line with the hypothesis that dectin-1 is not the only receptor involved in *C. albicans* recognition.

4.1.4 MCs activation status affects macrophage behaviour.

Clearance of fungal pathogens rely mostly on the activity of phagocytic cells and especially on neutrophils and macrophages. Fungal clearance by macrophages is a fundamental step in the resolution of infections, and depletion of mononuclear phagocytes has been described to worsen fungal proliferation and overall survival[283]. Mast cells interact with many members of the innate and adaptive immune system (cfr. Section 2.1.3) and can affect monocyte/macrophage behaviour during infections[86,179]. Thus, we aimed to determine how mast cells could induce macrophage migration and affect their ability to phagocyte *C. albicans*.

Macrophage crawling is increased in the presence of activated MCs.

Monocyte/macrophage migration to the site of infection is a key step for an efficient clearance of pathogens. Since MC are known to release chemotactic factors, the ability of mast cells to induce macrophage chemotaxis was determined with the ibidi® *μ*-Slide Chemotaxis. This special microscope slide allows to observe the migration of adherent cells over a gradient of the putative chemoattractant. For each experiment, peritoneal macrophages were purified from C57BL/6 mice peritoneal lavages and checked for F4/80, CD11b and MHC-II expression by flow cytometry and immunofluorescence (Fig. 4.1.4). To determine the release of chemotactic factors during *C. albicans* infections, conditioned media (CM) of BMMCs- *C. albicans* co-cultures were collected after 3 hours and used to assess macrophage migration. Conditioned media from *C. albicans* alone and complete RPMI media were used as controls.

Figure 4.1.3 ***(previous page)*****: B, C, D:** Stimulation of BMMCs with *C. albicans* induced a quick release of TNF-*α* and IL6 (already detectable after 3 hours) and of IL-13 and IL-4. Interestingly, *C. albicans* yeasts induced a more prominent cytokine release compared to hyphae. These data were also confirmed by qPCR analyses. **E:** Challenge of *Dectin-1*$^{-/-}$ MCs with *C. albicans* resulted in an impaired release of TNF-*α*, IL-6 and IL-13 compared to WT controls. Mean (SD) of two independent experiments.

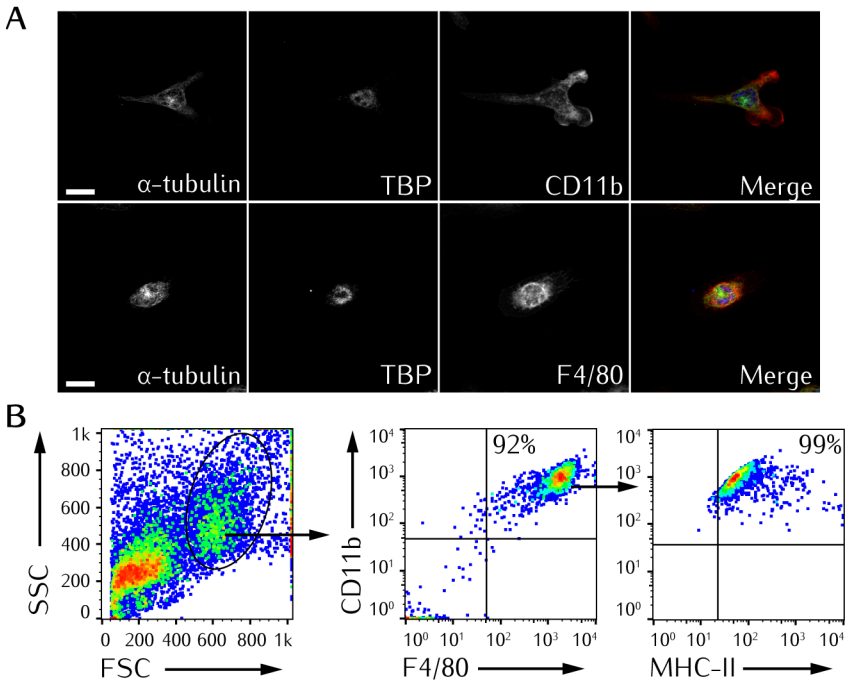

Figure 4.1.4: Peritoneal macrophages purification. Peritoneal Mφ were obtained from the peritoneal lavage of C57BL/6 mice and checked for purity. **A:** Z-stack averaging of confocal images. Scale bars: 10μm. **B:** Flow cytometry gating strategy to confirm Mφ purification.

Figure 4.1.5 A shows all the single cells trajectories during 24 hours incubation. Notably, conditioned media from BMMCs + *C. albicans* co-cultures induced a more evident movement of macrophages. Forward migration indexes (FMI, represent the efficiency of the migration of cells) were calculated and showed no significant differences ($FMI_{\parallel}$=0.0147 $\pm$ 0.0126 and $FMI_{\perp}$=-0.0008 $\pm$ 0.0228)[394]. Rayleigh test reported a p-value of 0.5327, thus indicating that cell endpoints are uniformly distributed. On the other side, macrophages incubated with BMMC + *C. albicans* culture supernatants moved with higher velocity which resulted in greater accumulated distance compared to controls (Fig. 4.1.5 B). Taken together, these data indicates that BMMCs do not release chemotactic factors for macrophages during *C. albicans* infections but release soluble factors that increase macrophage crawling. Albeit being unable to attract tissue resident macrophages, BMMCs might improve macrophage crawling, resulting in a better chance to encounter pathogens even at distant sites from the infection.

Resting MCs partially inhibit macrophage phagocytosis ability.

Mast cells are known to modulate macrophages' phagocytosis ability[86,180]. To establish whether MC phagocytosis of *C. albicans* could be responsible for a better fungal clearance by providing "eat-me" signals to tissue-resident macrophages, peritoneal Mφ were co-cultured with BMMCs and *C. albicans*, and their phagocytosis ability was determined.

BMMCs were stimulated with CPD-stained *C. albicans* for 3 hours, in order to allow phagocytosis of *Candida* hyphae (from now on, these cells

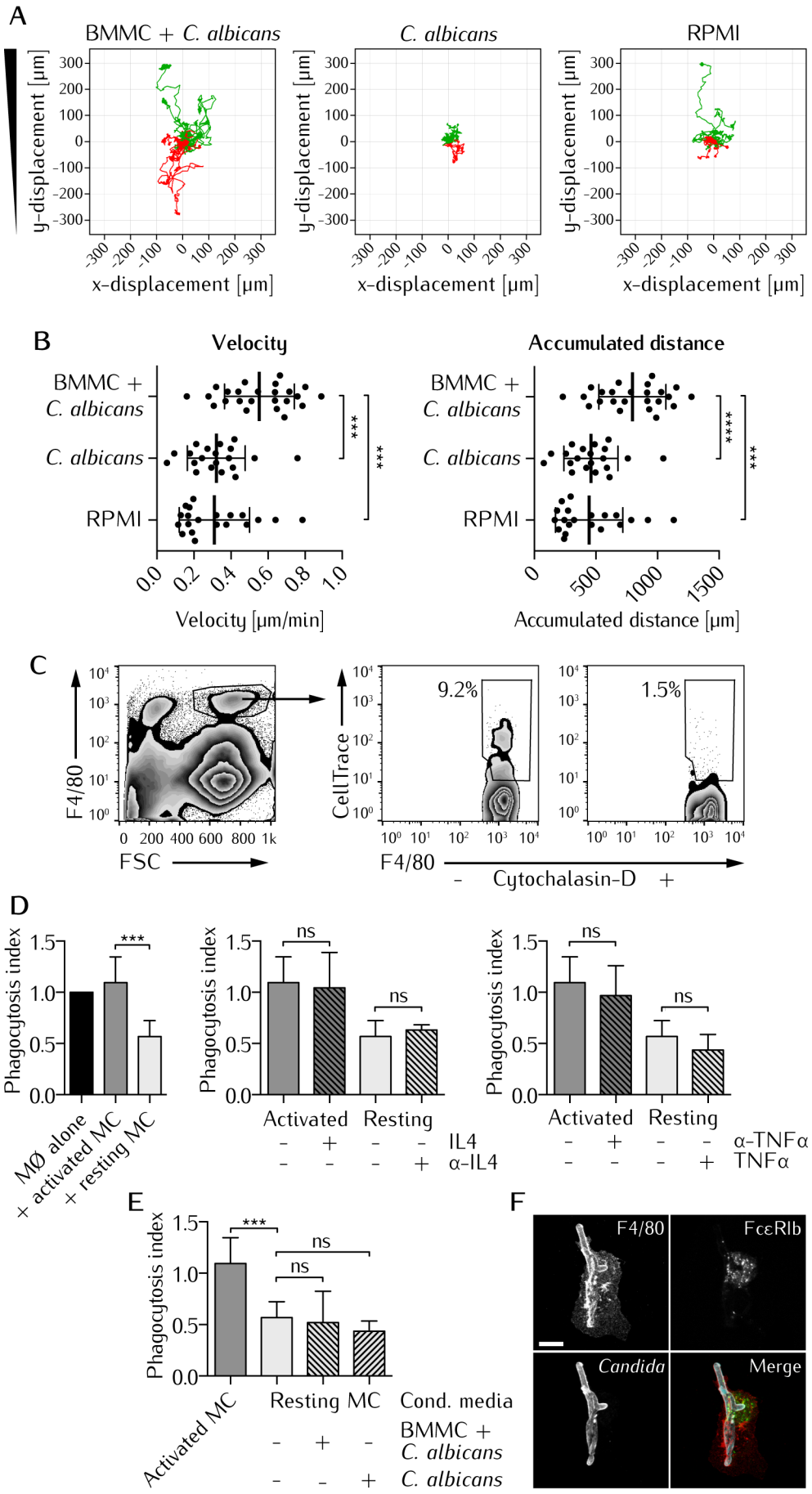

Figure 4.1.5: Mast cell activation influence peritoneal macrophage behaviour. **A:** Single cell tracks during 24 hours chemotaxis experiment. Green tracks indicate cells moving towards the conditioned media, red tracks indicate cells moving away from the chemoattractant. *Continues on next page...*

will be referred as "activated MCs"), scraped and seeded to peritoneal Mφ. Naïve BMMCs + CPD-stained *C. albicans* (referred as "resting MCs") or CPD-stained *C. albicans* alone were seeded to peritoneal Mφ as control. After 1 hour of co-culture, the percentage of phagocytosis was determined by flow cytometry (Fig. 4.1.5 C).

Albeit activated MCs had no effect on the phagocytosis of *C. albicans* by Mφ, resting MCs were able to inhibit Mφ phagocytosis (Fig. 4.1.5 D).

Impaired phagocytosis of Candida by macrophages is not dependent on MCs soluble mediators.

MC-dependent inhibition of phagocytosis has already been described during bacterial infections and appear to be mediated by a quick release of IL-4 from MCs upon bacterial encounter[86]. To determine whether naïve BMMCs inhibition of Mφ phagocytosis was dependent on quickly released IL-4, macrophages were stimulated in the presence of exogenous IL-4 or anti-IL-4 blocking antibody.

Addition of recombinant IL-4 to activated MCs or the neutralization of IL-4 activity on resting MCs did not affect Mφ phagocytosis of *C. albicans*, suggesting that IL-4 is not involved in the modulation of phagocytosis (Fig. 4.1.5 D). Seen that *C. albicans*-stimulated MCs release TNF-α already after 3 hours (cfr. Fig. 4.1.3 B), it was hypothesized that TNF-α may be responsible for the reversion of the phenotype by activated MCs. However, addition of recombinant TNF-α to resting MCs or neutralizing TNF-α activity in activated MCs did not revert the phenotype (Fig. 4.1.5 D).

To undoubtedly exclude a role of MC-derived soluble mediators in the modulation of Mφ phagocytosis, conditioned media were collected after 3 hours of BMMCs and *C. albicans* co-culture or from *Candida* alone, and added to Mφ together with resting MCs and *C. albicans*. Again, phagocytosis inhibition was not reverted, suggesting that this mechanism is soluble mediators-independent but rather contact-dependent (Fig. 4.1.5 E). This hypothesis is further sustained by the fact that *in vitro* Mφ and BMMCs interact during *Candida* phagocytosis (Fig. 4.1.5 F). Intriguingly, activated MCs lose the inhibitory activity, indicating that fungal-dependent BMMCs activa-

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Figure 4.1.5 ***(previous page)*****: B:** Albeit failing to attract Mφ, culture supernatants from BMMCs + *C. albicans* co-culture increase Mφ crawling ability. **C:** Gating strategy used for the evaluation of Mφ phagocytosis. **D:** *C. albicans* phagocytosis by Mφ is impaired by the presence of naïve MCs. Addition of exogenous IL-4 or TNF-α, nor their neutralization with monoclonal antibodies restored of inhibited Mφ activity. Phagocytosis was assessed by flow cytometry after 1 hour of co-culture and the phagocytosis index expressed as the fold-change over the phagocytosis percentage of Mφ stimulated with *C. albicans* alone. **E:** Similarly, the presence of conditioned media (CM) did not affect macrophage phagocytosis ability. **F:** Immunofluorescence analyses of Mφ-BMMC-*C. albicans* co-cultures indicate that MCs and Mφ interact during the process of phagocytosis. Scale bar: 10μm.

tion can somehow down-regulate the expression of putative co-stimulatory molecules.

4.2 MAST CELLS TRAINING BY MICROBIAL PAMPS.

Mast cells are particularly abundant at sites where it is likely their encounter with microbial ligands (e.g the gut mucosa) and their terminal differentiation is highly influenced by tissue-specific stimuli. For these reasons we decided to investigate the role of microbial ligands in the modulation of MCs differentiation and response to inflammatory stimuli[50]. Different microbial ligands such as β-glucan, and the PGN components Tri-DAP and MDP are able to induce trained immunity in monocytes *in vitro* as well as *in vivo*, while LPS and Pam3CSK4 promote tolerance[326,343,347]. Stimulation with LPS is known to induce a transient unresponsiveness in MCs while IgE sensitization was described to increase their responsiveness to LPS. Anyway, these phenomena have been only described in short-term experiments, with the cells being restimulated immediately after the washout of the first stimulus[384,388,389]. To assess whether long-lasting trained immunity or tolerance could be induced also in MCs, BMMCs were differentiated for 6 weeks in IL-3 media and then primed by stimulation for 24 hours with LPS, curdlan (a dectin-1 agonist consisting of β-(1,3)-linked glucose residues), IgE/Ag or left untreated (RPMI). After the stimulation, cells were washed and put back in culture in fresh IL-3 media for 6 days in order to allow their recovery. Each cell subset was then re-stimulated with a 10-fold lower dose of LPS, live *C. albicans* yeasts or IgE/Ag for 24 hours (Fig. 4.2.1).

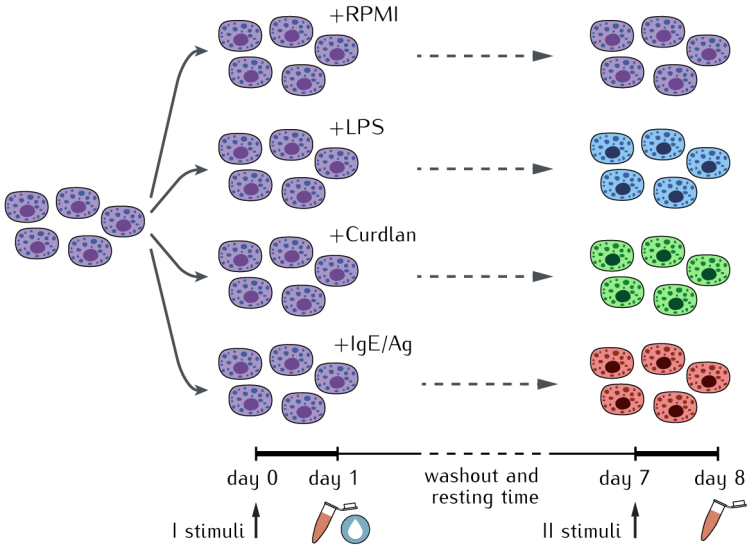

Figure 4.2.1: Schematic representation of the experimental protocol. After 6 weeks of *in vitro* differentiation, BMMCs were stimulated with either LPS (1 μg·ml^{-1}), curdlan (10 μg·ml^{-1}), IgE/Ag (100 ng·ml^{-1} Ag) or left untreated (RPMI) for 24 hours. Cells were then washed and put back in culture in RPMI complete medium with IL3. At the end of the "resting period", each BMMCs population was subsequently stimulated with low-dose LPS (100 ng·ml^{-1}), *C. albicans* or IgE/Ag.

4.2.1 MCs priming affect their response to secondary inflammatory stimuli.

In agreement with previously published data, LPS and IgE/Ag stimulation induced the release of both TNF-α and IL-6 while curdlan induced only a faint release of TNF-α (Fig. 4.2.2 A)[134,302,395]. During the 6 days of resting time, all the cells kept proliferating at a low rate, with a slightly more marked effect in the cells trained with LPS. Cell vitality (determined by trypan blue exclusion) was comparable for all the stimulations (Fig. 4.2.2 B).

As expected, priming with LPS impaired MCs response to a secondary challenge with LPS as demonstrated by the lower release of TNF-α and IL-6 which was already evident (although not statistically signficant) 3 hours after the restimulation. On the other side, priming with curdlan or IgE/Ag did not affect MCs' responsiveness to secondary LPS challenge (Fig. 4.2.2 C).

In order to determine if LPS priming could induce cross-tolerance also against non-TLR4 stimulations, BMMCs were restimulated either with live *C. albicans* yeasts or IgE/Ag. Strikingly, priming with LPS induced a stronger response to *Candida* compared to RPMI control, enhancing the release of TNF-α but not of IL-6. As for LPS, the response against *C. albicans* challenge by MCs primed with curdlan or IgE/Ag was comparable with the control (Fig. 4.2.2 D). By contrast, the response to a restimulation with the classical anaphylactoid stimulation IgE/Ag was comparable between all the four groups, in terms of release of both TNF-α and IL-6 (Fig. 4.2.2 E). Moreover, LPS priming did not affect BMMCs degranulation in response to IgE/Ag nor in response to the Ca^{++} ionophore ionomycin (Fig. 4.2.2 F).

Collectively, this data suggests that LPS priming induces long-term memory also in MCs. The fact that LPS priming was responsible for the development of both tolerance and training in the context of TNF-α release, suggests that the regulation of this cytokine may not rely exclusively on epigenetic mechanisms (as described for Mφ), but rather on a multi-layered process of regulation dependent on the secondary stimulus.

4.2.2 MCs training by microbial products does not affect MCs phenotype.

Histochemical comparison.

Tissue-specific stimuli are fundamental during the terminal differentiation of MCs precursors, driving the expression of surface markers and granule content of the differentiated cells[29]. *In vitro*, certain stimuli (as SCF, IL-9, IL-10 and IL-33) as well as pathogen challenge can induce MCs reprogramming in term of protease expression and granule content[68–70]. In order to define whether MCs priming was responsible for a strong phenotypic change in MCs, primed BMMCs were stained with two classical staining techniques: toluidine blue and alcian blue–safranin.

BMMCs granule content and metachromasia were comparable between the four conditions. Interestingly, BMMCs that degranulated after the first challenge with IgE/Ag, recovered most of their granules and stained metachromatically with toluidine blue (Fig. 4.2.3 A). Similarly, all the primed cells maintained a similar phenotype after the alcian blue–safranin staining:

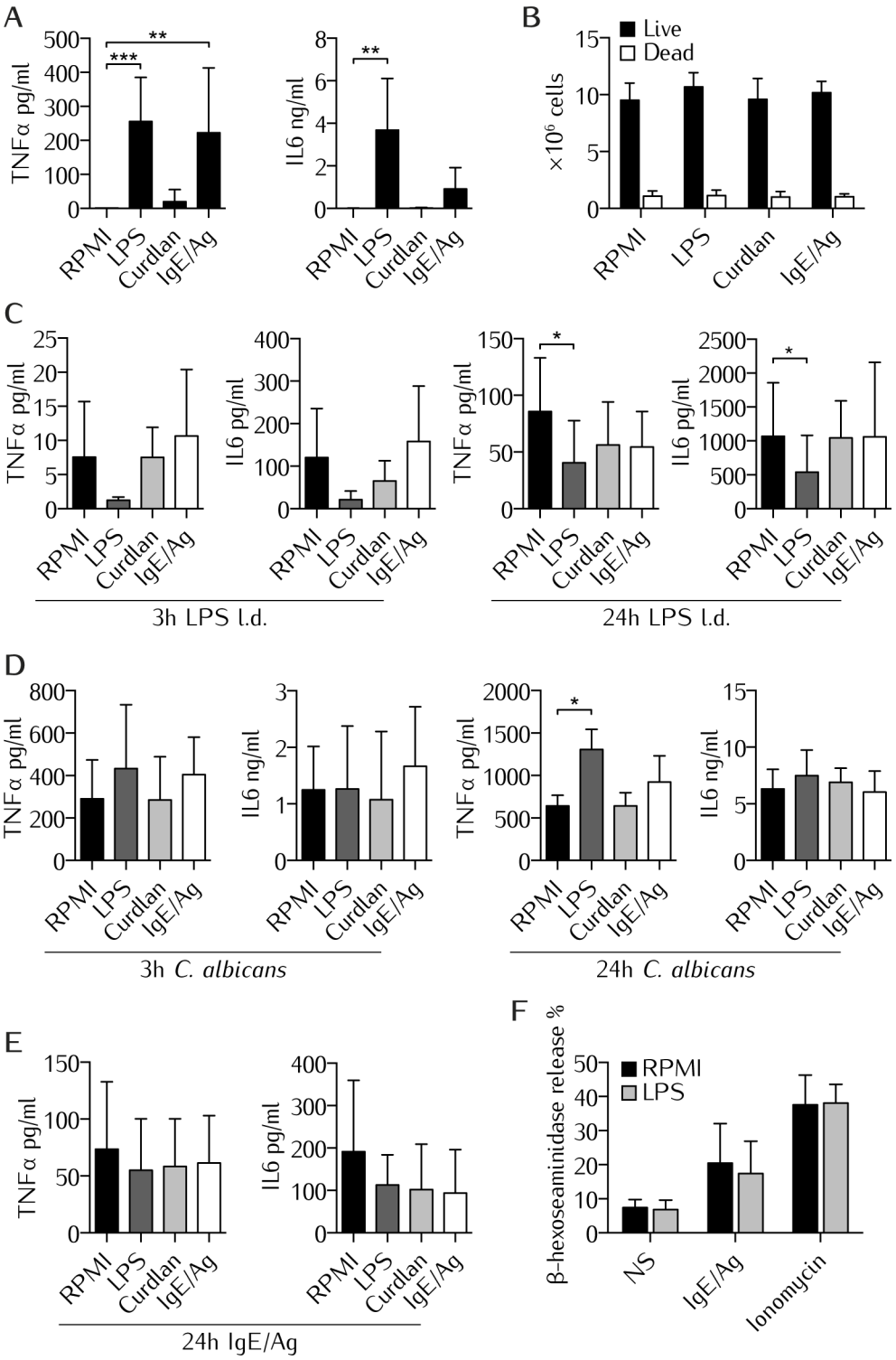

Figure 4.2.2: BMMCs priming modulate the response to secondary challenges. **A:** BMMCs were stimulated for 24h with LPS, curdlan, IgE/Ag or left untreated. Supernatants were collected and screened for TNF-α and IL-6 release. **B:** After 6 days of resting, primed-MCs growth and death was evaluated by means of Trypan blue exclusion. *Continues on next page...*

BMMCs were all alcian blue positive and showed only little staining for safranin (with LPS-primed BMMCs being slightly less positive), suggesting that their granules contained low levels of histamine (Fig. 4.2.3 B). These data suggest that BMMCs conserved their mucosal-like phenotype, showing toluidine blue metachromatic granules, and little or no safranin positive granules.

Surface markers expression.

It has been reported that one mechanism involved in innate immune memory is the regulation of receptors levels on the cellular surface. For example, ET macrophages showed ad upregulation of receptors such are CD64, MARCO and CLEC4A and a marked downregulation of MHC molecules[345]. Moreover, it was recently described that macrophages trained with the non-pathogenic yeast *Saccharomyces cerevisiae* upregulated dectin-1 levels[396]. In a similar report, Garcia-Valtanen et al. demonstrated that β-glucan trained splenic monocytes differentiated into macrophages but expressed lower levels of F4/80, CD11b and CD11c compared to LPS-trained Mφ and na$\acute{i}$ve Mφ[397]. Since LPS-primed BMMCs showed both an impaired response to LPS and an enhanced response during *C. albicans* challenge, it was supposed that this phenomenon could be due to a differential expression of the receptors involved in the recognition of LPS and *Candida*.

Surface expression levels of dectin-1, TLR2, TLR4 and CD14 were evaluated by flow cytometry after 6 days from the first stimulation. No differences in dectin-1, TLR2 and TLR4 protein expression were detected but LPS-primed BMMCs presented higher levels of surface CD14 although the difference was not statistically significant (Fig. 4.2.3 C). This is particularly interesting since many studies reported that MCs express mRNA transcripts for CD14 but no one succeeded in demonstrating the presence of a functional protein on MCs' surface[133,138–141]. In agreement, resting "naïve" BMMCs stained negatively for CD14 (FMI over isotype $\simeq$1). Intriguingly, CD14 upregulation did not enhance MCs responsiveness to LPS stimulation. This lack of effect could be explained by two mechanism that are not mutually exclusive. First, the slight expression of CD14 and the -presumed- activation of its signaling pathway could be too limited to compensate the process of tolerance activated by the cells. On the other side, endogenous CD14 activation could be masked by the effect of soluble CD14 (sCD14) commonly present in the serum[135,398–400].

Primed BMMCs were also checked for classical MCs markers. The size of the cells and their granularity were comparable, as indicated by the almost identical levels of forward scatter (FSC) and side scatter (SSC). FcϵRIa levels were slightly decreases in LPS-primed BMMCs, while cKit levels remained

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Figure 4.2.2 ***(previous page)*****: C-E:** Primed-MCs were restimulated with 100 ng·ml^{-1} LPS (**C**), live *C. albicans* yeasts (MOI=1, **D**) or IgE/Ag (**E**). TNF-α and IL-6 levels were detected in culture supernatants after 3 or 24 hours. **F:** Degranulation of LPS-primed BMMCs and RPMI control cells was determined by means of β-hexoseaminidase release after IgE/Ag or ionomycin stimulation.

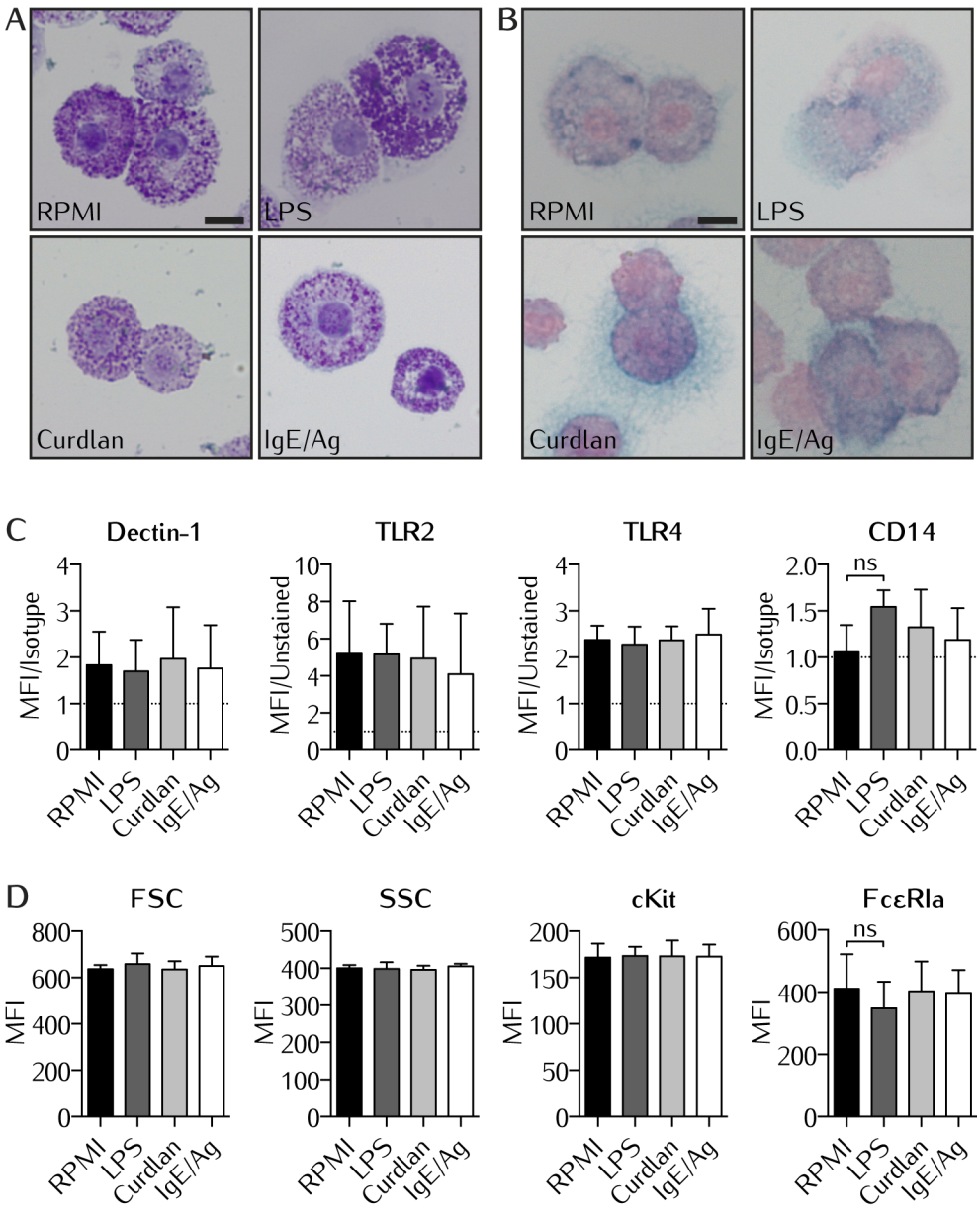

Figure 4.2.3: MCs phenotype after the conditioning period. After the resting period, before the second stimulation, BMMCs phenotype was determined by histochemical staining and flow cytometry. **A.** Toluidine blue staining of cytospin preparations. Granules were still present in all conditions and stained metachromatically. Scale bar: 10μm **B.** Alcian blue–safranin staining of cytospin preparations. All the cells stained positively for alcian blue but only weakly for safranin red, highlighting a "mucosal-like" phenotype. Scale bar: 10μm **C.** The level of expression of MCs' surface markers was analysed by flow cytometry. No differences in the expression levels of dectin-1, TLR2, TLR4 nor cKit were detected. On the contrary, LPS-primed BMMCs expressed slightly higher levels of CD14 and lower levels of FcεRIa.

constant after the resting time (Fig. 4.2.3 D). This indicates that BMMCs did not lost their cellular identity after the priming. Albeit the reduced expression of FcϵRIa was not sufficient to significantly reduce LPS-primed MCs degranulation and release of pro-inflammatory cytokines upon anaphylactic stimulation (cfr. Fig. 4.2.2 E and F), it is tempting to speculate that prolonged exposure of MCs to bacterial ligands such as LPS could be fundamental for the induction of a tolerant MCs phenotype in response to IgE/Ag stimulation and thus may avoid the occurrence of allergies. This phenomenon is corroborated by several studies demonstrating that bacterial species have immunoinhibitory properties on MCs, and together would nicely fit in the greater picture of the hygiene hypothesis[209,401–404].

4.2.3 LPS conditioning impairs the PI3K-AKT pathway.

The innate reprogramming by LPS is believed to be mediated by several factors as the rewiring of the signaling networks and the manipulation of the epigenetic landscape. Considered the dualistic expression of TNF-α in LPS-primed BMMCs upon the restimulation with LPS or *Candida*, we hypothesized that the first LPS stimulation could induce a remodulation of the signaling networks involved in the response to the two ligands. One proposed mechanism of ET is based in the modulation of the PI3K-AKT signaling pathway. It has been reported that this pathway played an important role in the limitation of TNF-α, IL-6 and IL-12 production. Chaurasia et al. demonstrated that primary macrophages isolated from mice lacking PDK1 displayed a higher release of TNF-α, IL-6 and CCL2 compared to WT mice. This phenomenon was correlated with the lack of AKT phosphorylation and with a sustained IKK/NF-κB activation due to enhanced TRAF6 ubiquitination[406]. Similarly, PI3K$^{-/-}$ DCs and WT DCs treated with the PI3K inhibitor wortmannin showed an increased production of IL-12 in response to LPS, due to the enhanced activation of p38 MAPK[407]. Moreover, AKT activation in macrophages was found to inhibit the activity of the transcription factor forkhead box O1 (FoxO1), which in turn triggered the expression of genes involved in the TLR4 signalling pathway[408].

On the contrary, PI3K is known to be activated after TLR4 engagement by directly binding to a non-canonical YXXM PI3K-binding site on the C-terminus of MyD88 and to cooperate with the p38 MAPK to properly activate the TLR4 signaling cascade[127,409]. In mast cells, activation of the PI3K pathway induced the upregulation of TNF-α and IL-6 during TLR-mediated stimulation[410]. Moreover, this pathway is of great importance for MCs development and for the activation of the complimentary FYN-GAB2-PI3K signaling cascade during IgE/Ag activation[105,411].

Seen that PI3K seems to play a dual role depending on the cellular context, the modulation of PI3K, AKT and p38 MAPK pathways in primed BMMCs was evaluated. Strikingly, LPS-primed MCs showed a decreased level of total PI3K p85 and lower levels of PI3K p85 and p55 phosphorylation. This phenomenon correlated with the expression of a putative isoform (or truncated version) of PI3K with an approximative molecular weight of $\simeq$65 kDa (Fig. 4.2.4 A). This putative isoform (from now on referred as PI3K p65) was

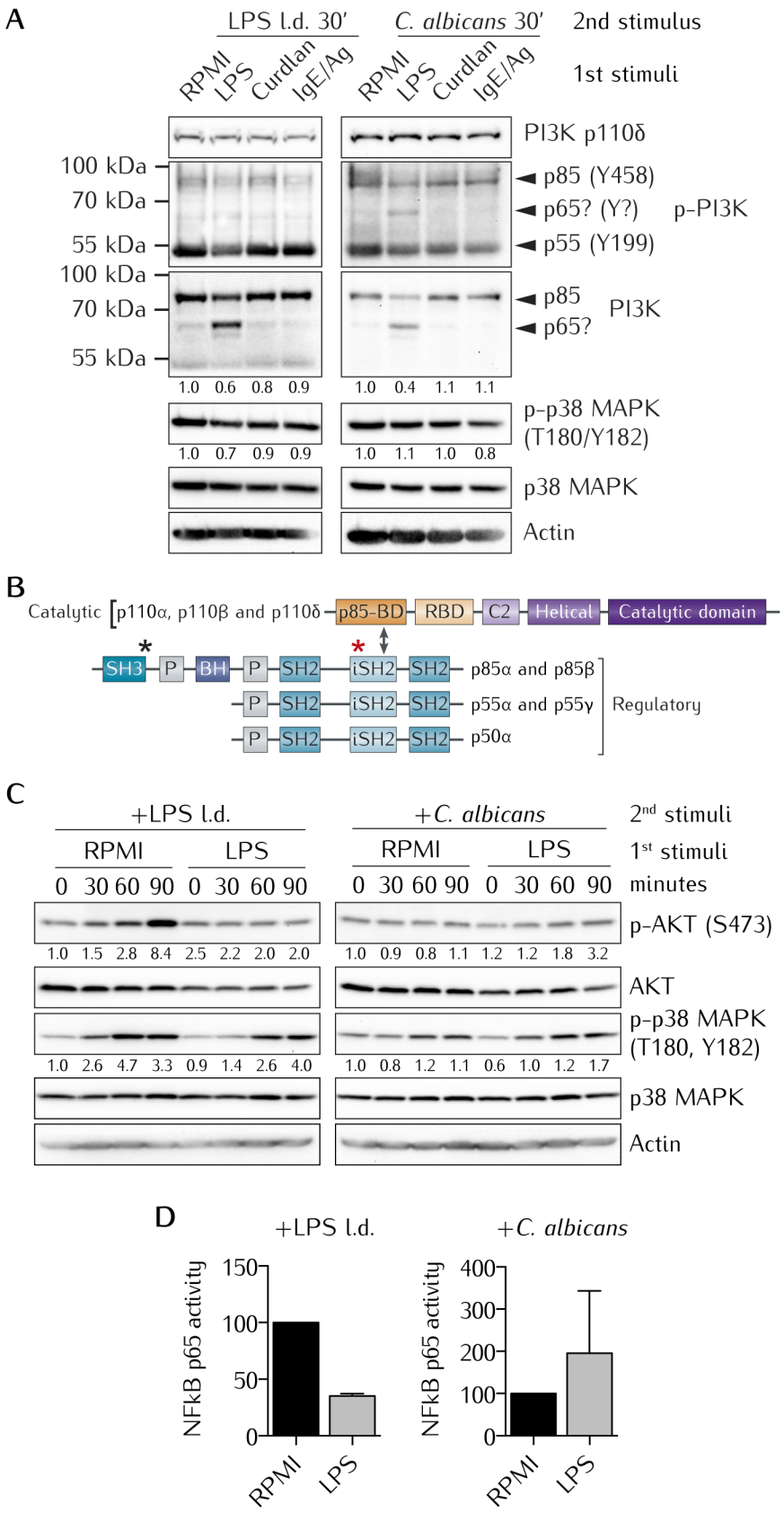

Figure 4.2.4: LPS-primed MCs differentially activate the PI3K-AKT pathway. **A:** WB analyses of primed-MCs restimulated with 100ng·ml^{-1} (LPS l.d.) or *C. albicans*. A ≃65 kDa PI3K isoform is expressed by LPS-primed MCs. **B:** Known PI3K splicing variants. The stars indicates the sites antibodies binding sites (black: endogenous p85; red: phospho Tyr458 and Tyr199). SH: SRC-homology domain; BH: BCR-homology domain; P: proline-rich region; p85-BD: p85 binding domain. Adapted from Thorpe et al.[405]. *Continues on next page...*

recognized both by the anti-p85 full length antibody (which is specific for the region surrounding aa 80 of human PI3K p85) and to a lesser extent by the anti-phospho PI3K antibody which recognize the phosphorylated Tyr458 of p85 and Tyr199 of p55 (Fig. 4.2.4 A). Seen that the protein is recognized by both the antibodies, it is possible that PI3K p65 conserved both the SH3 and iSH2 domains, the latter being responsible for binding to the catalytic subunit p110 (Fig. 4.2.4 B).

LPS-primed MCs displayed also a reduced phosphorylation of p38 MAPK after 30 minutes of low-dose LPS restimulation (Fig. 4.2.4 A). Conversely, the same cells showed no impairment of p38 phosphorylation after 30 minutes of *C. albicans* challenge. All the cell expressed comparable levels of the catalytic subunit PI3K p110δ (Fig. 4.2.4 A).

This phenomenon prompted us to compare AKT activation level during LPS- and *C. albicans*-induced activation of primed MCs. Control BMMCs (RPMI) showed a strong activation of AKT already after 60 minutes of stimulation with low-dose LPS, while it was completely abolished in LPS-primed MCs. AKT activation in control cells correlated with an earlier and more sustained activation of p38 MAPK (Fig. 4.2.4 C). On the other side, *C. albicans* failed to induce the activation of AKT in both the cell populations but LPS-primed MCs displayed an enhanced activation of p38 MAPK. Taken together, these data suggest that PI3K may be involved in AKT activation in response to TLR4 stimulation. However, LPS conditioning of MCs caused an impaired activation of the PI3K-AKT pathway finally leading to a weaker phosphorylation of p38 MAPK. This phenomenon could be possibly mediated by a novel PI3K isoform p65 which is expressed exclusively in LPS-primed MCs.

To further characterize the nature of the ambivalent behaviour of LPS-primed MCs, we looked at the activation status of the NF-κB transcription factor p65. Although NF-κB role in TNF-α transcription is quite controversial, it is believed that this transcription factor is involved in the regulation of TNF-α[412]. The NF-κB p65-p50 complex is normally restrained in the cytoplasm by the interaction with its inhibitor IκB. Upon stimulation, the IKK kinases promote IκB ubiquitination and proteasomal degradation thus allowing NF-κB to freely translocate into the nucleus[413]. Preliminary results showed that already after 30 minutes there was a differential nuclear accumulation of NF-κB p65 in LPS-primed MCs compared to controls. More in detail, during LPS restimulation NF-κB p65 nuclear translocation was impaired in LPS-primed MCs while during *C. albicans* challenge LPS-primed cells displayed a more prominent nuclear accumulation of NF-κB p65 (Fig. 4.2.4 D).

Figure 4.2.4 ***(previous page)*****: C:** Time-course WB analyses of primed-MCs restimulated with 100ng·ml^{-1} (LPS l.d.) or *C. albicans*. Numbers indicates the results of densitometric analyses calculated over endogenous protein expression. **D:** NF-κB p65 activity in primed-MCs was determined after 30 minutes from restimulation. Results are expressed as fold percentage over control (RPMI) translocation. Mean (SD) of two independent experiments.

Taken together these data suggest that LPS conditioning is able to induce a profound remodeling of the signaling pathways involved in the response against TLR4 ligands and invading pathogens. The state of unresponsiveness to LPS triggering was mediated by the abrogation of the AKT pathway activation which resulted in a impaired p38 MAPK activation and downstream NF-κB translocation. This phenomenon was possibly mediated by the presence of a novel PI3K p65 isoform which may be unable to promptly activate AKT signaling in response to TLR4 triggering. On the other side, response to *C. albicans* was independent of the activation of the PI3K-AKT axis in naïve MCs. Notably, LPS priming allowed a slight activation of the AKT pathway, which resulted in a more prominent p38 MAPK activation and NF-κB nuclear translocation.

4.2.4 LPS conditioning modulates TNF-α transcription.

The modulation of the PI3K-AKT and p38 MAPK pathways induced by LPS priming, resulted in the differential nuclear translocation of NF-κB p65

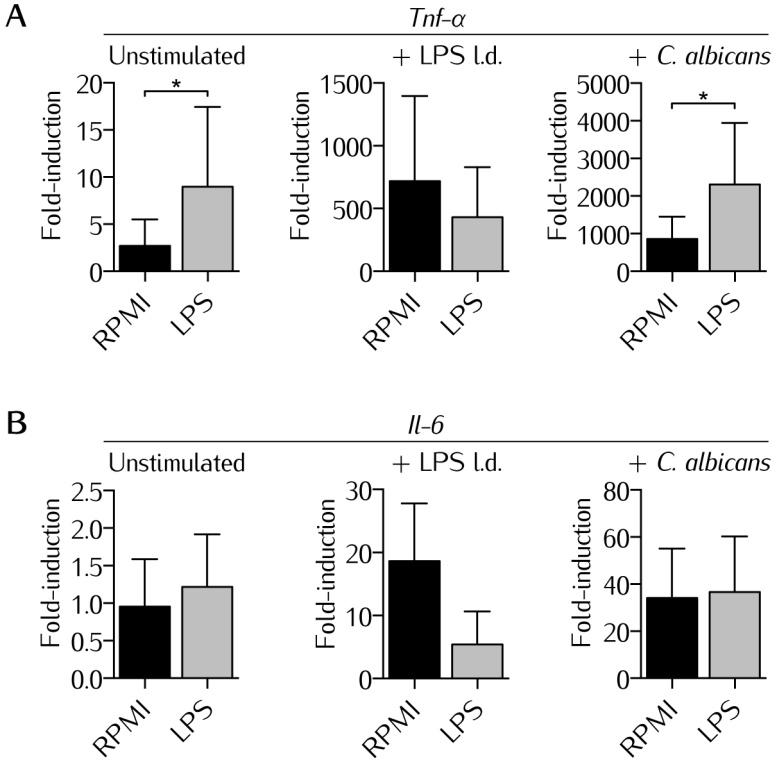

Figure 4.2.5: TNF-α expression is skewed after LPS priming. *Tnf-α* (A.) and *Il-6* (B.) expression were measured by qPCR on LPS-primed and control MCs after 3 hours of stimulation with low-dose LPS or *C. albicans*. Priming with LPS induced a higher basal transcription of *Tnf-α*, which was ≃3.3 times higher than control cells. The same ratio of *Tnf-α* expression was also induced after stimulation with *Candida*. On the contrary, *Tnf-α* and *Il-6* expression were markedly reduced in LPS-primed MCs receiving a second dose of LPS. Data expressed as mean (SD), n≥3 except for LPS l.d. (n=2). Statistical analyses were performed with a ratio t-test.

during LPS and *Candida* restimulation. Anyway, NF-κB p65 is not the unique transcription factor involved the control of TNF-α transcription and several processes can be involved in the regulation of cytokine secretion, independently from their transcription[412]. This is of particular importance in mast cell seen that the secretion of their granule content is a tightly-regulated process[91–93].

To assess whether NF-κB levels correlated with TNF-α and IL-6 transcription, qPCR analyses were performed 3 hours post-restimulation. Concordantly with the protein release detected after 3 and 24 hours, stimulation with low-dose LPS resulted in a impaired expression of the transcripts for both TNF-α and IL-6 in LPS-primed MCs (Fig. 4.2.5). Moreover, stimulation of LPS-primed MCs with *C. albicans* yeast caused an increased expression of TNF-α but not of IL-6, again in line with the protein release. Interestingly, unstimulated LPS-primed MCs showed an increased basal transcription of TNF-α, $\simeq$3.3 times higher than controls. Stimulation with *C. albicans* induced a $\simeq$300-fold increase in TNF-α transcription levels in both LPS-primed MCs and controls, which were found to be $\simeq$2.7 times higher in LPS-primed cells (Fig 4.2.5 A). Taken together, this results suggest that LPS-priming induce a higher basal transcription for TNF-α but not IL-6 and that *C. albicans* challenge likely has the same effect on both LPS-primed and control cells. The heightened release of TNF-α by LPS-primed MCs could be relying only on the enhanced basal transcription which in turn may be due to an improved chromatin accessibility on TNF-α regulation regions or due to the presence of active enhancers/co-activators. On the other side, LPS-primed cells may be able to override this enhanced TNF-α transcription by modulating upstream signaling networks involved in the response to LPS.

5 DISCUSSION

Every multicellular organism contain a rich and diverse microbiota and their interactions profoundly affect the fitness of both the host and the microbial community. The coexistence of these two entities is based on a fragile equilibrium between commensalism and pathogenesis which is maintained by proper mechanisms of activation and suppression of the immune system. Importantly, the disruption of this stable host-microbiota equilibrium can lead to pathological consequences[414,415]. A striking example is provided by numerous studies which reported a clear reduction in gut microbiota diversity on patients with autoimmune diseases[416].

Candida albicans is the most common member of the human and murine mycobiota and is found as a commensal especially in the colon and the vagina. When the equilibrium between the host and the fungus is perturbed (e.g. during broad-spectrum antibiotics treatment, or in conditions of pathological or pharmacologically-induced immunosuppression) *C. albicans* can overgrow and cause severe diseases as recurrent vulvovaginal candidiasis and invasive candidaemia[252,257].

The main effector cells involved in the control of fungal infections are neutrophils and macrophages but in recent years several studies reported that also mast cells might be involved in the outcome of pathological *Candida* overgrowth[167,268,299,300]. This should not be surprising as a growing body of evidence highlighted the concept that MCs are not mere effector of allergies and anaphylaxis but are rather involved in the maintenance of tissue homeostasis as well as in many pathological circumstances[50,190].

The present study provide novel proofs of the role of mast cells as tissue-resident sentinels involved in the recognition of fungal infections and in a wider cross-talk with the commensal microbiota.

Mast cells role in the control of C. albicans infections.

Previous studies demonstrated that MCs respond to fungal infections with *C. albicans* but often reported contradictory data. In order to provide additional elements of the interaction between MCs and *Candida albicans* we set up an *in vitro* co-culture system using murine WT and dectin-1$^{-/-}$ BMMCs. Time-lapse microscopy experiments showed that MCs tightly interacted with the fungus only after the switch to the hyphal form, in a way that resembled frustrated phagocytosis[391,392]. The formation of this phagocytic synapse was further characterized by immunofluorescence analyses which revealed that MCs were able to re-organize their α-tubulin cytoskeleton and to accumulate LAMP1^{+} vesicles at the interface with the hyphae. Interestingly, no differences were observed between WT and dectin-1$^{-/-}$ BMMCs suggesting that other receptor than dectin-1 might be involved in the recognition of *C. albicans*. This finding is in line with the current belief that *C. albicans* is able to

efficiently shield the β-glucan layer after the germination to the hyphal form, thus preventing its recognition by dectin-1[262].

LAMP1 is also considered a marker of degranulation in MCs but incubation of BMMCs with *C. albicans* even in the presence of serum (to allow the germination of hyphae) did not result in the release of β-hexoseaminidase nor leukotrienes. A possible explanation is that granules' cargo was released directly on the fungal surface due to their close interaction and thus preventing their detection in the supernatants. It has been demonstrated that MC-derived β-hexoseaminidase was able to disrupt *Staphylococcus epidermidis* cell wall, rendering mice more resistant to bacterial infections[417]. Moreover, a similar polarized degranulation was recently described and named "antibody-dependent degranulatory synapse". Opsonized *Toxoplasma gondii* tachyzoites induced FcγR-triggering on mast cells and the localized release of granule contents at the interface with the pathogen[418].

On the contrary, we observed the release of TNF-α, IL-6, IL-13 and IL-4 during the co-cultures, especially during the stimulation with the yeasts. This observation might reflect the fact that MCs recognize the morphological switch from yeasts to hyphae, possibly by discriminating the composition of the outer layer of the fungal cell wall or through the recognition of cell wall debris released during the germination.

It was demonstrated that the release of IL-4 during *C. albicans* infections *in vivo* was fundamental for the induction of a protective T_H1 response during reinfection. As such, IL-$4^{-/-}$ mice were more resistant then WT littermates during the first infection with *C. albicans* (probably due to the absence of a T_H2 skewing) but failed to survive a secondary infection[296]. The authors did not identify the source of IL-4 in this context but our data supports the idea that MCs may account for most of the release of this cytokine.

Several reports demonstrated that MCs are able to phagocytose bacteria and fungi, and that in particular conditions they can also present antigens to autologous T cells[185,196–200,299,300]. Anyway, their ability to kill phagocytosed pathogens is much more limited than "professional" phagocytes so we hypothesized that engulfment of *C. albicans* could be an early line of defense addressed to recruit tissue-resident macrophages and promote the clearance of the pathogen. Time lapse chemotaxis experiments revealed that the soluble factors released by MCs during *Candida* infection failed to induce peritoneal Mφ chemotaxis but instead markedly improved their crawling ability. This result is in agreement with a previous study by Lopes et al. which reported that *C. albicans*-infected human MCs were able to recruit neutrophils but not circulating monocytes[301]. To assess whether MCs engulfment could promote macrophage-mediated *C. albicans* clearance by providing "eat-me" signals, co-cultures between BMMCs, peritoneal Mφ and *C. albicans* were set up. Interestingly, we found that resting MCs were able to inhibit Mφ phagocytosis of the fungus. A similar phenomenon was described in a model of severe septic peritonitis, in which a very fast release of IL-4 by MCs upon bacterial encounter resulted in the inhibition of bacterial clearance by peritoneal macrophages[86]. Anyway, the neutralization of extracellular IL-4, nor the stimulation with infected MCs conditioned media reversed Mφ activation, suggesting that this phenomenon might rely on cell-cell contact. This should not be surprising since it was demonstrated that MCs modulated T

and B cells activation through the OX40-OX40L and the CD40-CD40L axes, respectively [186,187,189].

Taken together, this data demonstrate that MCs are able to respond to fungal infections by tightly interacting with *C. albicans* hyphae and releasing pro-inflammatory mediators as TNF-α and IL-6. Fungal challenge induced also the release of the T_H2 cytokines IL-4 and IL-13. While IL-4 has been correlated with a protective effect during fungal reinfection, IL-13 is known to promote intestinal goblet cell hyperplasia and increased mucin expression during parasitic helminth infections [419]. Thus, it is possible that a similar mechanism might be involved in the elimination of *C. albicans* hyphae. Moreover, we demonstrated that MCs-derived soluble mediators can increase tissue-resident macrophage crawling during the infection. Interestingly, resting MCs were found to limit Mφ phagocytosis of *C. albicans*: this might reflect their ability to restrain effector functions of myeloid cells in homeostatic conditions, highlighting once more that mast cells are important gatekeepers of tissue homeostasis.

Microbial products modulate mast cells response to inflammatory signals.

The fact that mast cells can survive in the tissues for up to 12 weeks and that their terminal differentiation is highly influenced by tissue-specific stimuli, render these cells possibly implicated in innate immune memory [29,49]. Several studies on the nature of endotoxin tolerance demonstrated memory-like traits also in mast cells [384,386,389]. These studies were based only on short-term experiments, with the cells being restimulated immediately after the washout of the first stimulus. Anyway, an important feature of innate immune memory is the long lasting effects induced by the first "priming" stimulation [315]. To verify whether microbial ligands could induce a long-lasting immune memory in MCs, and if their priming could affect the response also against fungal pathogens, we set up an experimental panel which allowed the cells to "rest" for 6 days between the fist stimulation and the subsequent restimulation.

As indicated by the impaired release of TNF-α and IL-6, mast cells primed with LPS became tolerant to a secondary dose of LPS, indicating that endotoxin tolerance could be maintained for at least 6 days. Unexpectedly, the restimulation of LPS-primed cells with *C. albicans* induced a higher release of TNF-α but not of IL-6, suggesting that LPS priming could induce both tolerance and training depending on the secondary stimuli. Moreover, the response to the classic anaphylactoid IgE/Ag stimulation was not affected by the priming with LPS, both in terms of TNF-α, IL-6 and β-hexoseaminidase release.

To exclude that this phenomenon was mediated by the differential expression of PRRs by "naïve" and primed cells, phenotype and surface markers expression were compared after the resting period (i.e. just before the restimulation). Interestingly, BMMCs phenotype (determined by toluidine blue and alcian blue–safranin staining) did not change after the priming simulation. Similarly, expression levels of TLR4, TLR2, and dectin-1 were comparable between primed and naïve cells. The unique exception was found in LPS-primed BMMCs which showed an increased expression of surface CD14 and

slight decreased level of FcεRIa. CD14 expression on MCs is still matter of debate since many authors reported the presence of a CD14-encoding mRNA transcript but no one was able to demonstrate the presence of a functional protein on the cell surface[133,138–141]. Moreover, LPS-mediated upregulation of CD14 levels was demonstrated only by two independent studies on the human monocytic cell line Mono-Mac-6 while many others failed to report any difference in CD14 expression during ET[371,420,421]. Notably, increased CD14 levels in LPS-primed MCs did not increase their responsiveness to LPS. This effect could be explained by: *i)* a masking effect of the sCD14 present in the serum; *ii)* an insufficient activation of the CD14-downstream signaling pathway; *iii)* MCs lack of the TRIF-TRAF signaling cascade that is activated (at least in DCs) after CD14-TLR4 endocytosis[123,143,144]. On the other side, the slight downregulation of the FcεRIa in LPS-primed cells is in line with previous reports demonstrating that bacterial ligands as LTA and PGN or the probiotic *Lactobacillus rhamnosus* induced FcεRIa downregulation on human MCs[402,422]. Anyway, this mild downregulation of the receptor did not affect MCs response to IgE/Ag challenge.

Innate immune memory is supposed to rely on different regulatory networks comprising the modulation of intracellular signaling cascades as well as epigenetic mechanisms of chromatin remodeling. As previously discussed, the modulation of the PI3K/AKT pathway plays a dual role during the response to TLR-triggering. On one hand, PI3K is activated in a MyD88-dependent manner upon TLR4 triggering and, in turn, participates to the proper phosphorylation of p38 MAPK[127,409]. On the other hand, PI3K-AKT activation dampened the expression of pro-inflammatory cytokines in response to LPS by inhibiting TRAF6 K63-polyubiquitination and the activity of FoxO1[406,408].

WB analyses revealed that LPS-primed BMMCs expressed lower amounts of PI3K p85 but presented a putative isoform (or truncated version) of PI3K with an approximative molecular weight of $\simeq$65 kDa. Based on the recognition by the antibodies directed against the endogenous and the phosphorylated forms, we hypothesize that this putative isoform conserved both the SH3 and iSH2 domains, the latter being responsible for binding to the catalytic subunit p110. To our knowledge only one PI3K variant with a similar molecular weight has been described in the literature. This protein was obtained from a transformed cell line (X-ray–induced murine lymphoma) and caused the constitutive activation of the PI3K pathway[423]. Anyway, this PI3K p65 variant did not exhibit an hyperactive phenotype since LPS-primed cells failed to activate AKT upon LPS restimulation. On the contrary, naïve MCs showed a marked AKT phosphorylation which correlated with a stronger p38 MAPK phosphorylation during LPS restimulation. Moreover, restimulation with *C. albicans* failed to promote AKT activation but induced similar levels of p38 phosphorylation in both the MCs population, with a slightly more marked effect in LPS-primed cells. These results indicate that the activation of the PI3K-AKT axis is fundamental for the expression of LPS-induced TNF-α and IL-6 in MCs. Moreover, the lack of AKT activation, possibly related to the impaired expression of PI3K, could represent a novel mechanism of ET tolerance in MCs. These evidences are in agreement with two reports by Hochdorfer et al. that demonstrated the important role of

PI3K and p38 in MCs response to LPS. Interestingly, the activation of the PI3K pathway increased the expression of TNF-α and IL-6 but reduced the expression of IL-1β upon TLR4 triggering[385,410].

Further efforts will be required in order to define the nature of this PI3K variant, its activity and binding affinity, and its involvement in the induction of ET. In this regard, PI3K impairment failed to modulate MCs degranulation and cytokine production during IgE/Ag challenge although its well established role in the complimentary FYN-GAB2-PI3K pathway[105].

Although NF-κB role in TNF-α transcription is quite controversial, it is known that ET tolerance relies also on the impairment of NF-κB p65/p50 dimerization and nuclear translocation[377,379,380,412]. Preliminary results indicate that already after 30 minutes from the re-stimulation, LPS-primed BMMCs differentially regulated NF-κB p65 nuclear translocation. The activation of NF-κB in primed cells was impaired during LPS restimulation but enhanced upon *Candida* challenge. Accordingly, *Tnf-α* mRNA expression in LPS-primed MCs was reduced upon LPS challenge but increased during *C. albicans* infection while *Il-6* levels were found to be impaired only during LPS restimulation. Moreover, *Tnf-α* basal expression in LPS-primed cells was $\simeq$3 times higher that in naïve MCs.

Taken together, these data suggest that LPS challenge induce long-term memory in MCs. Albeit being known that LPS can induce endotoxin tolerance or training depending on the dose used for cell priming, here we report an ambivalent role for LPS in the induction of both tolerance and training depending on the secondary challenge. This phenomenon is possibly the result of the combined rewiring of intracellular signaling cascades and of epigenetic changes in the regulating loci of TNF-α.

On one hand, LPS-priming induced long-term tolerance to endotoxin by impairing the PI3K-AKT pathway possibly through the modulation of PI3K p85 expression. The lack of AKT activation correlated with an impaired p38 phosphorylation and NF-κB p65 nuclear translocation, finally leading to a reduced expression of TNF-α and IL-6. On the other hand, LPS-priming induced a higher basal transcription of the *Tnf-α* gene. We believe that this phenomenon could be the result of improved chromatin accessibility on TNF-α regulating regions or due to the constitutive presence of active enhancers/co-activators. The higher release of TNF-α in LPS-primed cells during stimulation with *C. albicans* could rely mostly on the enhanced *Tnf-α* gene transcription. At the same time, these cells developed a profound rearrangement of the intracellular signaling network involved in TLR4 responses which could override the higher basal TNF-α transcription.

Further proof of this hypothesis is given by the pattern of IL-6 expression: its reduction during LPS-restimulation indicates that the impaired signaling pathway controlling TNF-α expression also affects other pro-inflammatory cytokines. On the other side, restimulation with *C. albicans* did not affect IL-6 expression in primed cells, suggesting that the control of TNF-α transcription might be governed by gene-specific chromatin modification rather than by the rewiring of the signaling cascades activated by the fungus.

Additional efforts will be required in order to elucidate the nature of this ambivalent phenomenon mediated by LPS. By expanding the signaling pathway involved in LPS and fungal sensing and characterizing the putative

epigenetic mechanisms controlling TNF-α expression it will be possible to describe the roles of mast cells priming in homeostatic and pathological conditions.

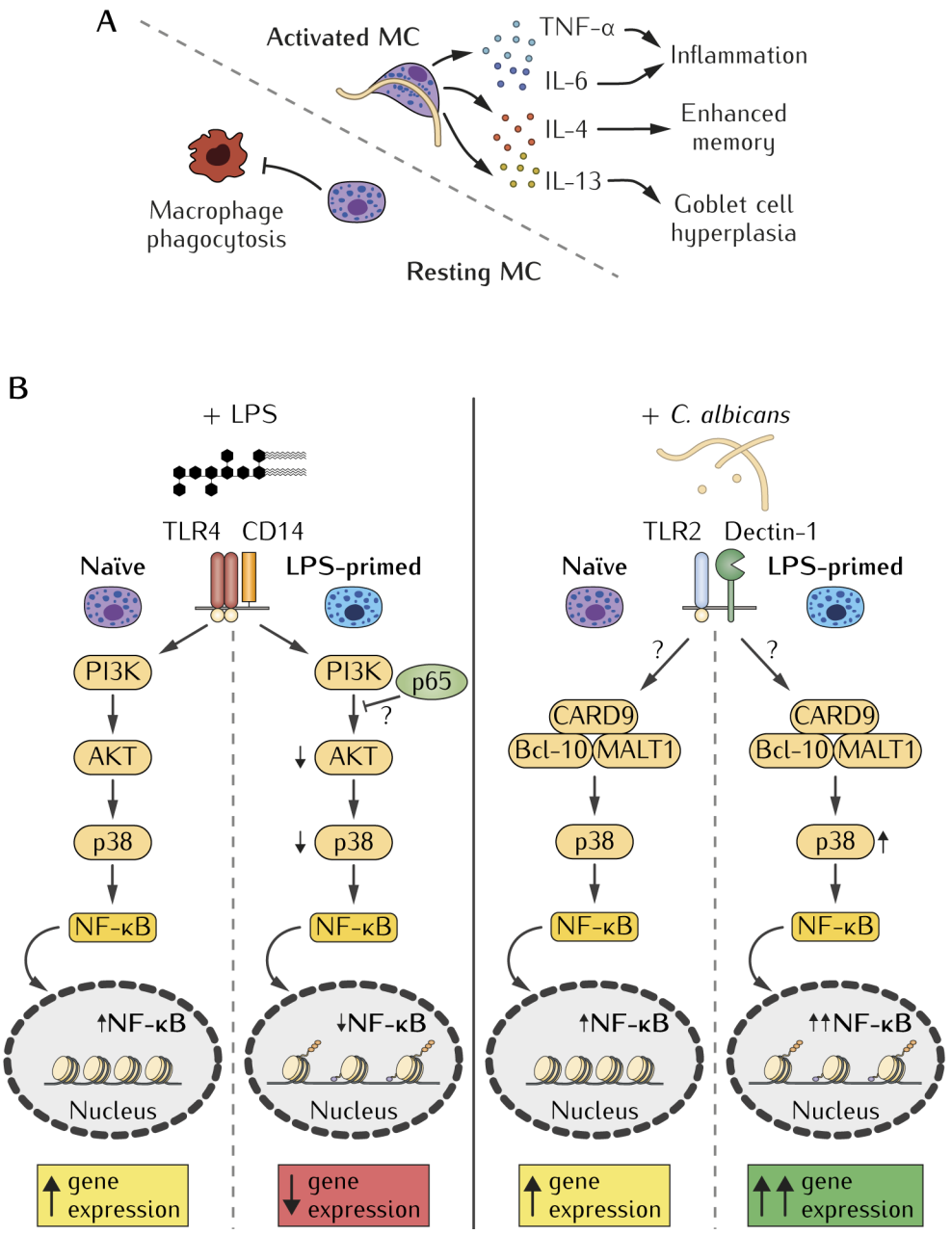

Figure 5.0.1: Proposed model of MCs activation during fungal infections. **A.** MCs recognize and tightly interact with *C. albicans* releasing pro-inflammatory and T_H2 cytokines involved in many physiological processes. On the other side, resting MCs inhibit Mφ phagocytosis, highlighting their role in the restriction of myeloid cell activation in homeostatic conditions. **B.** LPS induces ET in MCs by impairing the PI3K-AKT pathway and NF-κB nuclear translocation. At the same time, LPS-primed cells show a higher basal transcription of TNF-α, enhancing their response to *C. albicans* infections.

6 EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from Envigo (The Netherlands) and maintained at the animal facility of the Department of Medicine, University of Udine (Italy). *Dectin-1*$^{-/-}$ femurs and tibiae were kindly gifted by Prof. Gordon Brown, University of Aberdeen (Aberdeen, UK). All animal experiments were performed in accordance with the animal care and experiments were performed in accordance to institutional guidelines and national law (D.Lgs. 26/2014).

BMMCs generation

Bone marrow derived mast cells (BMMCs) were obtained from 6- to 8-week-old mice by *in vitro* differentiation of bone marrow derived progenitors obtained from mice femurs and tibiae. Precursor cells were cultivated in complete–IL-3 medium (RPMI 1640 medium (Euroclone) supplemented with 20% FBS (Sigma Aldrich), 100 U·ml^{-1} penicillin, 100 mg·ml^{-1} Streptomycin, 2 mM Glutammine, 20 mM Hepes, non essential amminoacid, 1 mM Sodium Pyruvate (Euroclone), 50 mM β-mercaptoethanol (Sigma Aldrich) and 20 ng·ml^{-1} IL-3 (Peprotech)) at 37°C in 5% CO_2 atmosphere. After 5 weeks, BMMCs differentiation was confirmed by flow cytometry by staining with anti-FcϵRIa–PE (MAR-1) and anti-cKit–PE-Cy7 (ACK2) conjugated antibodies (eBiosciences). Data was acquired with a FACScan cytofluorimeter (Becton Dikinson) and analyzed with FlowJo software (FlowJo LLC). BMMCs were usually $\geq$ 96% cKit$^+$ and FcϵRIa$^+$.

BMMCs activation

Before all *in vitro* experiments, BMMCs were starved for 1 hour in IL-3–free complete RPMI medium (RPMI 1640 medium (Euroclone) supplemented with 10% FBS (Sigma), 100 U·ml^{-1} penicillin, 100 mg·ml^{-1} Streptomycin, 2 mM Glutammine, 20 mM Hepes, 1X non essential amminoacid, 1 mM Sodium Pyruvate (Euroclone), and 50 mM β-mercaptoethanol (Sigma Aldrich)).

For IgE-dependent activation, BMMC were sensitized in IL-3–free complete RPMI medium for 3 hours with 1 μg·ml^{-1} of dinitrophenol (DNP)-specific IgE, washed twice and challenged with 100 ng·ml^{-1} DNP (Sigma-Aldrich).

For *C. albicans* infections, 10^6 BMMCs were stimulated with *C. albicans* yeast (1:1 ratio) or hyphae (1:10 ratio) at a final concentration of 2×10^6 cells·ml^{-1} in IL-3–free complete RPMI medium. In order to limit fungal growth, amphotericin-B (Sigma Aldrich) was added to each well at a final

concentration of 10 ng·ml^{-1}. RNA extraction was performed before the addition of amphotericin-B.

For the stimulation–restimulation panel, BMMCs were differentiated for 6 weeks in complete–IL-3 medium and checked for purity by flow cytometry. BMMCs were left untreated or stimulated for 24 hours with 1 μg·ml^{-1} LPS (LPS from *E. coli* O55:E5, Sigma Aldrich), 10 μg·ml^{-1} curdlan (Invivogen), IgE/Ag (100 ng·ml^{-1} DNP) in IL-3–free complete RPMI medium. After the stimulation, supernatants were collected and cells were washed twice with PBS and put back in culture in fresh complete–IL-3 for 6 days. Each cells subset was then re-stimulated with 100 ng·ml^{-1} LPS, live *C. albicans* yeasts (MOI=1) or IgE/Ag for 24 hours (Fig. 4.2.1).

BMMCs degranulation assay

BMMCs degranulation response was determined as the percentage of β-hexosaminidase released. 0.5×10^6 BMMCs were incubated in Tyrode's buffer (10 mM HEPES buffer [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA) with or without the addition of 10% FBS, and stimulated with the same number of *C. albicans* yeasts at 37° for the indicated time points. As positive control, 0.5×10^6 BMMCs were sensitized in complete RPMI medium for 3 hours with 1 μg·ml^{-1} of dinitrophenol (DNP)-specific IgE, then washed twice, resuspended in Tyrode's buffer and challenged with 100 ng·ml^{-1} DNP (Sigma-Aldrich). The enzymatic activity of the released β-hexosaminidase was assessed by the cleavage of its synthetic substrate (p-nitrophenyl N-acetyl- glucosamide, Sigma Aldrich) in p-nitrophenol and measuring the p-nitrophenol absorbance at 405nm with a plate spectrophotometer. Results are expressed as the percentage of β-hexosaminidase released over β-hexosaminidase retained in the cytoplasm.

Leukotrienes C_4, D_4 and E_4 were measured in the same samples using a specific detection kit (GE Healthcare) according to manufacturer's instructions.

Purification of peritoneal macrophages

The peritoneum of 8- to 12-week-old C57BL/6 mice was lavaged using a PBS solution containing 100 U·ml^{-1} penicillin and 100 mg·ml^{-1} Streptomycin (Euroclone). Following lavage, the cells were washed, resuspended in complete RPMI medium (RPMI 1640 medium (Euroclone) supplemented with 10% FBS (Sigma), 100 U·ml^{-1} penicillin, 100 mg·ml^{-1} Streptomycin, 2 mM Glutammine, 20 mM Hepes, non essential amminoacid, 1 mM Sodium Pyruvate (Euroclone), and 50 mM β-mercaptoethanol (Sigma Aldrich)), plated in 24-well plates at a concentration of 0.5×10^6 cells/well, and cultured for 6 h at 37°C in 5% CO_2 atmosphere. Non-adherent cells were removed by washing the cells twice with PBS, and adherent cells were cultured overnight in complete RPMI at 37°C in 5% CO_2 atmosphere. Peritoneal macrophages (Mφ) purity was confirmed by flow cytometry and immunofluorescence by

staining with anti-F4/80–APC (BM8), anti-CD11b–FITC (M1/70) and anti-MHC-II–PE (M5/114.15.2) conjugated antibodies (BioLegend).

Candida albicans cultures

Wild-type *Candida albicans* SC5314 strain was a kind gift of Prof. Luigina Romani, University of Perugia (Perugia, Italy). Yeast were seeded on Sabouraud agar supplemented with 50 $\mu g \cdot ml^{-1}$ chloramphenicol and incubated at 30°C for 24 hours. To generate *C. albicans* hyphae, 10^7 yeast cells were resuspended in complete RPMI medium, seeded into T-25 adhesion flasks and allowed to germinate for 3 hours at 37°C. Hyphae were harvested by scraping, centrifuged at 700 $\times$g for 10 minutes and washed with PBS.

Flow cytometry

0.5×10^6 cells were harvested, washed with PBS and stained for 30 minutes at 4°C in the dark with monoclonal conjugated antibodies. Cells were then washed twice with PBS and acquired with a FACSCalibur flow cytometer (Becton Dickinson).

Time-lapse bright-field microscopy

MC-*C. albicans* interaction was analysed by time–lapse epiluminescent microscopy using the Leica AF6000LX system (DMI6000-B microscope equipped with a DFC350FX camera) at a magnification of 40×. Before each experiment, BMMCs were labelled with *FAST* DiI (Invitrogen) according to manufacturer's instructions. 0.5×10^6 BMMCs and 0.5×10^6 *C. albicans* yeasts (ratio 1:1) were plated on 8-well Permanox® Chamber Slide (Lab-Tek, Nunc). The chamber was placed at 37°C in 5% CO_2 atmosphere. Phase contrast images were recorded every 10 minutes for a total of 12 hours and resulting video-recorded movies were processed with LAS AF (Leica) and Fiji (ImageJ) softwares[424].

Immunofluorescence

Cells were seeded onto sterile glass coverslip in a 24 well plate and stimulated. Non-adherent cells were gently removed by pipetting, coverslips washed with PBS and fixed for 20 minutes with 4% formaldehyde (Sigma Aldrich). Cells were permeabilized for 5 minutes with 0.1% Triton-X100 (Fluka) and incubated for 30 minutes with PBS + 10% FBS (Sigma Aldrich) to reduce unspecific binding. Coverslips were incubated with primary antibodies (diluted in PBS + 5% FBS) overnight at 4°C in a humidified chamber. AlexaFluor-488, -546 or -647 conjugated anti-mouse, anti-rabbit or anti-rat secondary antibodies (Invitrogen) were incubated for 1 hour at room temperature. Coverslips were washed and mounted on SuperFrost glass slides (Menzel) with Mowiol 40-88 mounting media containing 2.5% 1,4-diazobicyclo-[2,2,2]-octane (Sigma Aldrich). Images were acquired with a with a Leica DM IRBE microscope equipped with a TCS-SP confocal scanner

head (with 488 nm Ar and 543–633 nm HeNe lasers) at a magnification of 63× and processed with Fiji (ImageJ) software[424].

Antibodies (clones, vendors): α-tubulin (DM1A, Sigma Aldrich), Candida (Abnova), CD11b (M1/70, Thermo Fisher), F4/80 (BM8, BioLegend), FcϵR β chain (JRK b), LAMP-1 (1D4B, eBiosciences), TBP (SI-1, Santa Cruz).

Histochemical staining

Histochemical staining were performed based on the protocol reported by Kovarova[425].

10^4 BMMCs were cytospun onto SuperFrost glass slides (Menzel) at 300 ×g for 3 minutes and allowed to air-dry overnight. For toluidine blue staining, slides were incubated for 5 minutes with a solution of 1% toluidine blue [pH 5] (Sigma Aldrich), rinsed twice in PBS and once in water, than allowed to air-dry and mounted with Micromount (Diapath). For alcian blue–safranin staining, slides were incubated for 10 minutes with a solution of 0.5% alcian blue (Sigma Aldrich) in 0.3% acetic acid [pH 3], rinsed twice in PBS then incubated for 20 minutes with a solution of 0.1% safranin-O (Sigma Aldrich) in 0.1% acetic acid [pH 4]. Slides were then rinsed and mounted as described above.

Images were acquired with a Leica DM750 equipped with a Leica ICC50W camera at a magnification of 63× and processed with Fiji (ImageJ) software[424].

Cytokine ELISA assays

Supernatants for cytokine quantitation were collected 3 or 24 hours after BMMC stimulation. Supernatants were assessed for TNF-α, IL-6, IL-13 and IL-4 using specific ELISA kits (eBiosciences) according to manufacturer's instructions.

Macrophage chemotaxis and migration assay

Chemotaxis of peritoneal Mφ was evaluated using the ibidi® μ-Slide Chemotaxis kit according to manufacturer's instructions. ∼15000 peritoneal Mφ were seeded in the observation area and the slide incubated at 37°C in 5% CO_2 atmosphere. After cell attachment, non-adherent cells were removed by washing three times with PBS. 24 hours after cell seeding, reservoirs were filled with either complete RPMI medium or conditioned media and the slider were immediately placed at 37°C in 5% CO_2 atmosphere. DIC images were recorded at 10× magnification every 10 minutes for a total of 24 hours and resulting video-recorded movies were processed with LAS AF software (Leica). At least 25 cells per condition were manually tracked with Fiji Software (ImageJ) and resulting data were analysed with the Chemotaxis and Migration Tool software (ibidi)[424].

Macrophage phagocytosis assay

C. albicans yeasts were resuspended at 2×10^6 cells·ml^{-1} in PBS and labelled for 20 min with 5 μM Cell Proliferation Dye eFluo 670 (CPD, eBioscience) at 37°C in 5% CO_2 atmosphere. Extracellular stain was quenched by the addition of 10 ml complete RPMI media for 5 minutes, then the cells were pelleted at 700 ×g for 5 minutes and washed twice with PBS. CPD-labeled *C. albicans* was seeded to BMMCs at a 1:1 ratio or plated alone on 24-well plates, incubated for 3 hours at 37°C in 5% CO_2 atmosphere and harvested by scraping. Peritoneal Mφ received BMMCs co-cultured with *C. albicans*, na′ive BMMCs and CPD-labled *C. albicans* or CPD-labeled *C. albicans* alone at a 1:1:1 ratio. As negative control of phagocytosis, some Mφ were pretreated with 1 μM cytochalasin-D for 1 hour. After 1 hour, cells were harvested by scraping, washed with PBS and stained with anti-F4/80–PE (BM8, Biolegend). Flow cytometry was used to quantify the number of F4/80$^+$ cells that had engulfed CPD-labeled *C. albicans*. Percentage of phagocytosis was calculated by subtracting the percentage of double positive cells in presence of 10 μM cytochalasin-D to the percentage of double positive cells in non-treated Mφ. Phagocytosis index was further determined as fold-change over the phagocytosis percentage of Mφ stimulated with CPD-labeled *C. albicans* alone.

In some experiment, 50 pg·ml^{-1} recombinant IL-4 (Peprotech), 100 pg·ml^{-1} recombinant TNF-α (Immunotools), 10 μg·ml^{-1} anti-IL-4 neutralizing antibody (11B11, eBioscience), 10 μg·ml^{-1} anti-TNF-α neutralizing antibody (MP6-XT22, Miltenyi Biotec), or conditioned media were used to stimulate peritoneal Mφ together with BMMCs and *C. albicans*.

RNA extraction and real-time PCR analyses

Cells were lysed with EUROGOLD TriFast (Euroclone) and total RNA extracted with the phenol-chloroform protocol according to manufacturer's instructions. Total RNA was quantified using a NanoDrop™ spectrophotometer (ThermoFischer) and retro-transcribed with the SensiFAST™ cDNA Synthesis kit (Bioline). Quantitative qPCR analyses were performed with SYBR Green chemistry (BioRad) using a BioRad iQ5 real-time PCR detection systems. Target genes expression were quantified with the $\Delta\Delta C_t$ method using G3PDH (glyceraldehyde 3-phosphate dehydrogenase) as normalizer gene.

Cell lysis and western blot analyses

5×10^6 BMMCs were stimulated with an equal number of *C. albicans* yeasts or with the indicated stimuli or left untreated for the indicated times. Cells were then incubated for 5 minutes on ice to stop the reaction and centrifuged 300 ×g, 5 minutes at 4°C. Before the lysis, BMMCs were washed twice with PBS + phosphatase inhibitors (Active Motif). Cell pellet were lysed in 50 μl NP-40 buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, 1 mM Na_3VO_4, 50 mM NaF (Sigma Aldrich), and COmplete Mini protease inhibitor cocktail (Roche, usage according to

manufacturer)) for 10 minutes on ice. Lysates were then centrifuged at 12000 $\times$g, 4°C for 10 minutes and supernatants were collected and stored at -80°C.

Upon western blot analyses, lysates were diluted with 4$\times$ Laemmli buffer and denatured 7 minutes at 95°C. Lysates were then separated on SDS 10% polyacrilamide gels and blotted on a nitrocellulose membrane (Amersham) at 300 V, 250 mA, 4°C for 3 hours. Membranes for phosphoproteins were blocked in TBS-T (20 mM Tris-base [pH 7.6], 150 mM NaCl, 0.05% Tween-20) + 5% BSA while membranes for total proteins were blocked in TBS-T + 5% non-fat dry milk for 1 hour at room temperature. Primary antibodies for phosphoproteins were diluted 1:1000 in TBS-T + 5% BSA and membranes probed overnight at 4°C with gentle agitation. HRP-conjugated secondary antibodies (Pierce) were incubated for 1 hour at room temperature with gentle agitation. Signals were detected using ECL substrates Luminata forte (Millipore) or SuperSignal West Femto (Thermo Scientific) and acquired with a ChemiDoc imaging system (BioRad). Densitometric analyses were performed with the Image Lab 5.2 software (BioRad).

Probed membranes were stripped twice for 10 minutes at room temperature in mild stripping buffer (0.2 M glycine, 0.1% SDS (Sigma Aldrich), 1% Tween20, pH 2.2) than blocked in TBS-T + 5% non-fat dry milk for 1 hour at room temperature and reprobed overnight at 4°C with gentle agitation.

Antibodies (clones, vendors): actin (C4, BD biosciences), AKT (BD biosciences), phospho-AKT S473 (D9E, CST), PI3K p85 (CST), phospho-PI3K p85 p55 Y459 Y199 (CST), p38 MAPK (CST), phospho-p38 MAPK T180 Y182 (CST), PI3K p110δ (A-8, Santa Cruz).

Nuclear-cytoplasmic fractionation

10^7 BMMCs were were stimulated as indicated, harvested and washed twice with PBS + phosphatase inhibitors (Active Motif). Cytoplasmic fractions were obtained by lysing the cell pellet with EDG lysis buffer + 0.1% Triton-X 100 (10 mM Hepes-KOH [pH 8.0], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4 (Sigma Aldrich) and COmplete Mini protease inhibitor cocktail (Roche, usage according to manufacturer)) for 5 minutes on ice. Nuclei were pelleted at 500 $\times$g for 10 minutes at 4°C and washed with EDG lysis buffer. Nuclear pellet were lysed with complete lysis buffer (Active Motif) on ice for 30 minutes and cleared by centrifugation at 12000 $\times$g, 10 minutes, 4°C. Both fractions were immediately used for downstream analyses or kept at -80°C.

NF-κB activity

Nuclear protein fractions were obtained as described above and protein concentration was measured with the Pierce™ BCA Protein Assay Kit (Thermo Scientific). 20 μg of protein extracts were used for the quantification of NF-κB p65 activity using the TransAM™ NF-κB kit (Active Motif) according to manufacturer's instructions.

Statistical analyses

Unless otherwise indicated, results are expressed as mean (SD) of at least three independent experiments. Data were analysed using paired on unpaired Student's *t* test, ratio t test, paired or ordinary one-way ANOVA, paired two-way ANOVA, or Kruskal-Wallis tests (GraphPad Prism v6). Rayleigh tests were performed with the Chemotaxis and Migration Tool software (ibidi). A confidence level of 95% was used. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

Part III

BACK MATTER

LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
BMMC	Bone marrow derived mast cells
BCG	Bacillus Calmette-Guérin
CBMC	Cord blood-derived mast cells
CM	Conditioned media
CPA3	Carboxypeptidase A3
CTMC	Connective-tissue mast cells
cysLT	Cysteinyl leukotriene
DAG	Diacylglycerol
DCs	Dendritic cells
ECM	Extracellular matrix
ET	Endotoxin tolerance
FcϵRI	High affinity receptor for IgE
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IFN-γ	Interferon-γ
IgE	Immunoglobulin-E
IL	Interleukin
IP_3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Mφ	Macrophages
MOI	Multiplicity of infection
MC	Mast cell
MCPT	Mast cell protease
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MMC	Mucosal mast cell
MyD88	Myeloid differentiation primary-response protein 88
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK	Natural killer cells
NLRP3	NOD-, LRR-, and pyrin domain (PYD)-containing protein 3
PAMP	Pathogen-associated molecular pattern
PGN	Peptidoglycan
PIP_2	Phosphatidylinositol 4,5-diphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PMC	Peritoneal mast cell
SAR	Systemic acquired resistance
SCF	Stem cell factor
SFK	SRC family kinase

TCR	T-cell receptor
TF	Transcription factor
T_H	T-helper cell
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor

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A mast cell-ILC2-Th9 pathway promotes lung inflammation in cystic fibrosis

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T helper 9 (Th9) cells contribute to lung inflammation and allergy as sources of interleukin-9 (IL-9). However, the mechanisms by which IL-9/Th9 mediate immunopathology in the lung are unknown. Here we report an IL-9-driven positive feedback loop that reinforces allergic inflammation. We show that IL-9 increases IL-2 production by mast cells, which leads to expansion of CD25$^+$ type 2 innate lymphoid cells (ILC2) and subsequent activation of Th9 cells. Blocking IL-9 or inhibiting CD117 (c-Kit) signalling counteracts the pathogenic effect of the described IL-9-mast cell-IL-2 signalling axis. Overproduction of IL-9 is observed in expectorates from cystic fibrosis (CF) patients, and a sex-specific variant of IL-9 is predictive of allergic reactions in female patients. Our results suggest that blocking IL-9 may be a therapeutic strategy to ameliorate inflammation associated with microbial colonization in the lung, and offers a plausible explanation for gender differences in clinical outcomes of patients with CF.

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Innate lymphoid cells (ILCs) perform a variety of immune functions at barrier surfaces[1]. Three types of ILCs have been reported, which differ on the basis of the cytokines produced. ILC1 encompass natural killer cells and interferons (IFN)-γ-releasing cells; ILC2 release IL-5, IL-9 and IL-13, and ILC3 release IL-17A and IL-22. ILC2 preferentially localize to the interface between the host and the environment (lung, intestine and skin) and perform a variety of biological functions in mice[2] and humans[3]. In the lung, ILC2 and their cytokines play pro-inflammatory roles in allergic inflammation[2,4,5], but also protective roles in airway epithelial cell repair and control of tissue inflammation linked to pathogens[6,7]. Thus, ILC2 may affect the course of airways diseases, resulting in either pathological or protective outcomes. Lung ILC2 rapidly produces IL-5 and IL-13 on exposure to IL-33 (ref. 5), an effect potentiated by IL-25 and thymic stromal lymphopoietin (TSLP)[5], and IL-9 on the exposure to IL-2 (ref. 8). By promoting ILC2 survival[8], IL-9 provides a positive feedback loop that amplifies ILC2 cytokine production and the ensuing allergic airway inflammation[9]. However, IL-9 also dampens the pathogenic activities of Th17 cells[10] and mediates tolerance imparted by regulatory T cells (Treg) via mast cells (MC)[11]. Produced by MC, in addition to ILC2 and Th9, IL-9 in turn affects the expansion[12] and function[13] of MC, which are known to have positive, as well as negative, immunomodulatory roles *in vivo*[13–16]. Thus, IL-9, like ILC2, may have different roles in lung immune homeostasis.

In patients with cystic fibrosis (CF), the primary source of morbidity and mortality is due to a vicious cycle of airway infection and inflammation eventually resulting in lung damage. The inflammatory response in CF is dysregulated at several levels, resulting in inefficient microbial clearance and contributing to lung damage[17]. This is supported by several studies that have documented an altered balance of inflammatory/anti-inflammatory cytokines in CF (ref. 17), providing evidence that targeting specific inflammatory/anti-inflammatory pathways is a valid therapeutic strategy in CF (ref. 18). This balance is essential for the efficient control of *Aspergillus fumigatus* diseases in CF (ref. 18), where the colonization by the fungus is common and may lead to fungal sensitization, bronchitis and allergic broncho-pulmonary aspergillosis (ABPA)[19] as well as worse forced expiratory volume in the first second (FEV1) (ref. 20). In CF patients, the expression of IL-9 and IL-9R is increased and is associated with mucus overproduction, but whether and how IL-9 contributes to immunity and pathology in response to the fungal infection in CF is not known.

In the present study, we determine the contribution of IL-9 to *Aspergillus* infection and allergy in murine and human CF, and assess the therapeutic effectiveness of targeting IL-9-dependent pathways and the diagnostic potential of this approach. We find that IL-9-driven IL-2 production by MC expands $CD25^+$ ILC2, which in turn activate Th9 cells, leading to an amplified allergic inflammation. Overproduction of IL-9 is observed in expectorates from CF patients and a genetic variant of IL-9 shows a sex-specific association with IgE levels in female patients. Blocking IL-9 or inhibiting CD117 (c-Kit) signalling counteracts the pathogenic potential of the IL-9-MC-IL-2 axis, thus providing a therapeutic angle to ameliorate the pathological consequences of microbial colonization in CF.

Results

IL-9 production and ILC2-Th9 activation during aspergillosis. We infected C57BL/6 or $Cftr^{-/-}$ mice intranasally with *A. fumigatus* and measured IL-9 production, ILC2 and Th9 cell activation in infection. We have already shown that $Cftr^{-/-}$ mice are susceptible to *Aspergillus* infection (from 2.5 ± 0.7 to 3.9 ± 1.0 log colony forming unit (cfu) ± s.d. per lung, C57BL/6 versus $Cftr^{-/-}$ mice, respectively) and allergy (from 9.2 ± 0.7 to 22.4 ± 1.3 ng ml^{-1} total serum IgE in C57BL/6 and $Cftr^{-/-}$ mice, respectively). A peak production of IL-9 occurred during the first week of the infection in C57BL/6 mice to decline thereafter as opposed to $Cftr^{-/-}$ mice in which levels of IL-9 were sustained throughout the infection (Fig. 1a). Peak production of IL-9 was also observed in $Rag1^{-/-}$, and less in $Rag1^{-/-}/Il9R^{-/-}$, mice early but not late in infection (Fig. 1a), a finding suggesting that early IL-9 production is IL-9R-dependent and late is T-cell-dependent. We looked therefore for the presence of $IL\text{-}9^+$ ILC2 and Th9 cells in infection by characterizing IL-9-producing Lin^- and $CD4^+$ T cells in the lung. ILC2 are marked by expression of the IL-33R as well as the common γ chain (γc) cytokine receptors for IL-2 and IL-7 (ref. 2). Flow cytometry analysis revealed that $CD90.2^+$ ILC2 expressing IL33R or CD25 were present in the lung of naive C57BL/6 (4.5 and 3.3% for $CD25^+$ and $ST2^+$ cells, respectively) and $Cftr^{-/-}$ mice (5.8 and 4.0% for $CD25^+$ and $ST2^+$ cells, respectively; Fig. 1b,c). In C57BL/6 mice, and similarly in $Rag1^{-/-}$ mice (Supplementary Fig. 1a), $ST2^+$ ILC2 cells decreased early in infection to return to baseline level 10 days later while $CD25^+$ ILC2 stably decreased (Fig. 1b,c). In contrast, in $Cftr^{-/-}$ mice, both types of ILC2 steadily increased throughout the infection (Fig. 1b) along with the expression of the ILC2 transcription factors, *Rora*, and *Gata3* (Fig. 1d) and the production of ILC2 effector cytokines, IL-5 and IL-13 (Fig. 1e). IL-9-producing $CD90.2^+$ ILC2 were also expanded in $Cftr^{-/-}$ mice but not in C57BL/6 (Fig. 1b,c) and $Rag1^{-/-}$ mice (Supplementary Fig. 1a), as revealed by flow cytometry. In terms of Th9 cell activation, $CD4^+$ $IL\text{-}9^+$ T cells appeared in C57BL/6 mice a week after the infection to decline thereafter (Fig. 1h), consistent with the short retention of Th9 at the inflammatory sites[21]. The expansion was instead sustained in $Cftr^{-/-}$ mice (Fig. 1h) along with the expression of *Il9*, *Pu.1* (purine-rich box 1) and *Irf4* (interferon regulatory factor 4) transcription factors (Fig. 1g). These data indicate that $IL\text{-}9^+$ ILC2 and Th9 cells are all increased in $Cftr^{-/-}$ mice during *A. fumigatus* infection.

Given that ILC1 through IFN-γ (ref. 22) and ILC3 through IL-22 (ref. 23) may affect ILC2 expansion, the differential expansion of ILC2 could reflect the ILCs dynamics in the lung. However, $NKp46^+NK1.1^+$ ILC1 cells producing IFN-γ did not expand and ILC1-promoting cytokines IL-15 and IL-18 were not produced in $Cftr^{-/-}$ as opposed to C57BL/6 mice (Supplementary Fig. 2a,b). Similarly, despite expanded in $Cftr^{-/-}$ mice, $CCR6^+ROR\gamma t^+$ ILC3 produced IL-17A more than IL-22 (Supplementary Fig. 2c,d). Thus, while confirming the defective production of IFN-γ and IL-22 in $Cftr^{-/-}$ mice[18], these findings suggest that the expansion of ILC2 in $Cftr^{-/-}$ mice is not dependent on ILCs dynamics in the lung but rather on the production of ILC2 promoting cytokines. This appeared to be the case, as the levels of cytokines promoting $ST2^+$ ILC2, IL-33 and $CD25^+$ ILC2, IL-2, were constantly elevated in $Cftr^{-/-}$ mice whereas a peak production was only observed at an early time point in C57BL/6 mice (Fig. 1f).

IL-9 contributes to inflammatory pathology in infection. To assess the role of IL-9 in *A. fumigatus* infection and allergy, we resorted to $Il9R^{-/-}$ mice that, given the crucial role of the IL-9R, a member of the γc receptor family, for the survival of lung ILC2 (ref. 8), also have a decreased ILC2 (ref. 8). Mice were either acutely infected with *Aspergillus* conidia intranasally or subjected to fungal allergy (ABPA) by repeated sensitization with *Aspergillus* culture filtrate extracts (5.5 ± 0.7 ng ml^{-1} versus 11 ± 1.0, total serum IgE in $Il9R^{-/-}$ versus C57BL/6 mice,

respectively). We found that the absence of IL-9R signalling conferred resistance to both infection and allergy, as indicated by the reduced fungal load (Fig. 2a) and decreased inflammatory lung pathology in infection as well as in ABPA (Fig. 2b). The numbers of lung CD25$^+$ and ST2$^+$ILC2 were decreased in these mice as revealed by immunofluorescence staining (Fig. 2c). Concomitantly, Th9 and, partially, Th2 cells—revealed by *Stat6* expression and STAT5 phosphorylation (Supplementary Fig. 1b,c)—were decreased in both infection and allergy (Fig. 2d,e), while Th17 and Treg cells were unaffected and Th1 cells increased (Supplementary Fig. 3a,b). Corroborating these findings, IL-9 neutralization in C57BL/6 or *Cftr*$^{-/-}$ mice greatly ameliorated lung pathology in response to the fungus, both in terms of inflammatory cell recruitment (Fig. 2f) and fibrosis as shown by Masson's trichrome staining (insets of Fig. 2f) and production of TGF-β (Fig. 2g), a mediator of pulmonary fibrosis[24]. Together, these results indicate that the IL-9/IL-9R signalling pathway is required for the expansion of pathogenic ILC2 and Th9 cells in response to the fungus. However, whether ILC2 promote Th9 cell activation via IL-9R signalling is not known. To directly assess this, we did criss-cross experiments in which CD4$^+$ T cells from either C57BL/6 or *Cftr*$^{-/-}$ mice were assessed for IL-9 production and Th9 transcription factor expression on co-cultivation in a transwell permeable support with lung Lin$^-$ cells exposed to *A. fumigatus* and either IL-2 or IL-33. We found that Th9 cell activation was observed upon

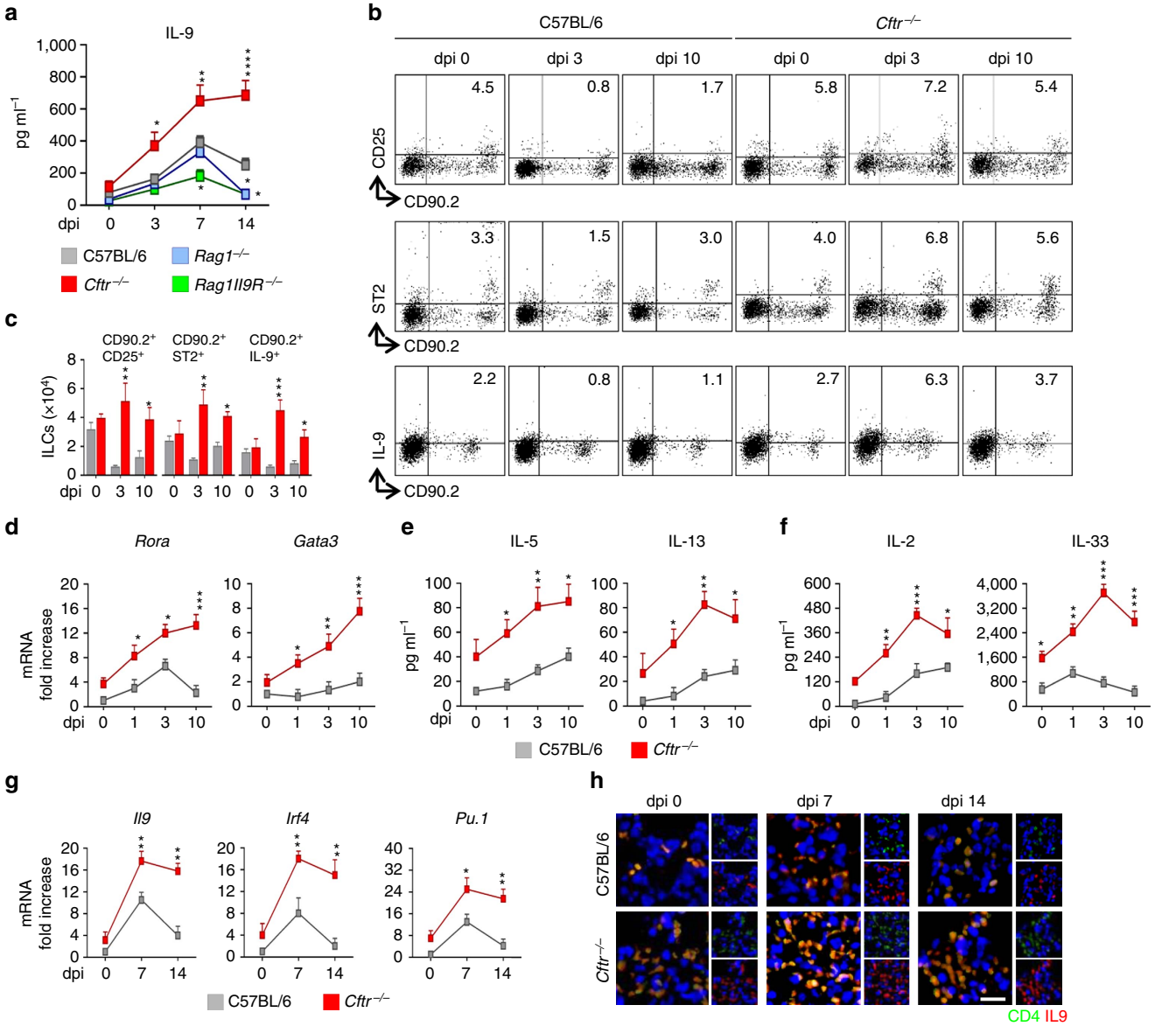

Figure 1 | IL-9 production and ILC2-Th9 cells activation in *Aspergillus fumigatus* infection. (**a**) Time course of IL-9 production at various days post infection (dpi) in mice (six per group) infected intranasally with live *A. fumigatus* conidia. (**b**) Detection of CD90.2$^+$CD25$^+$, CD90.2$^+$ST2$^+$ and CD90.2$^+$IL-9$^+$ lung type 2 ILCs by flow cytometry (numbers refer to percentages of positive cells) and immunofluorescence staining. (**c**) Absolute number of lung ILC2; (**d**) ILC2-specific transcript on lineage negative lung cells; (**e**,**f**) ILC2 effector and activating cytokines; (**g**) *Il9* and Th9-cell specific transcripts on lung CD4$^+$ T cells and (**h**) immunofluorescence staining of lung CD4$^+$ IL-9$^+$ T cells. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×40 objective; scale bar, 100 μm. Mean values ± s.d. cytokines were determined on lung homogenates by ELISA, *Il9* and transcripts assessed by PCR with reverse transcription. 0, uninfected mice. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, knockout versus C57BL/6 mice (data represent pooled results or representative images from three experiments, Two-way ANOVA, Bonferroni post test). *Gata3*, GATA binding protein 3; *Irf4*, interferon regulatory factor 4; *Pu.1*, purine-rich box 1; *Rora*, RAR-related orphan receptor alpha.

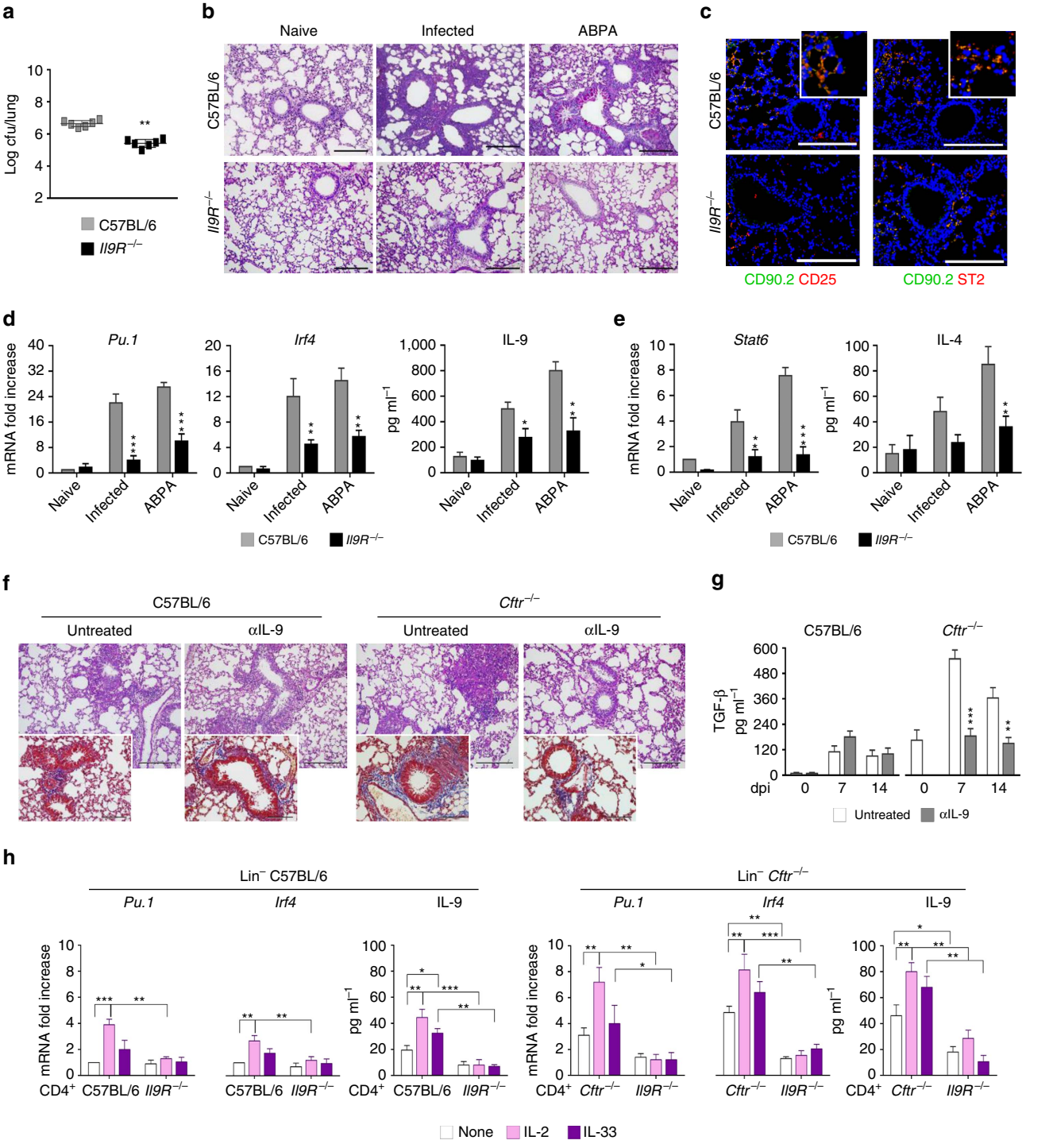

Figure 2 | IL-9R signaling contributes to inflammation and allergy. C57BL/6 and *Il9R*$^{-/-}$ mice (six per group) were intranasally infected with live *Aspergillus fumigatus* conidia or subjected to ABPA and assessed for (**a**) lung fungal growth (log_{10} cfu, mean ± s.d.); (**b**) lung histology (periodic acid – Schiff staining); (**c**) expression of CD90.2^{+}CD25^{+}, CD90.2^{+}ST2^{+} lung ILC2 by immunofluorescence; (**d**,**e**) Th-cell specific transcripts and cytokine production. (**f**) Lung histology (periodic acid–Schiff and, in the inset, Masson's trichrome staining) and (**g**) TGF-β production in C57BL/6 or *Cftr*$^{-/-}$ mice infected as above and treated with IL-9 neutralizing antibody for a week. Days post infection (dpi). (**h**) Th-cell specific transcripts and IL-9 production of lung CD4^{+} T cells from naive mice co-cultured with lung lineage negative (Lin^{-}) cells in the presence of *A. fumigatus* conidia, IL-2 or IL-33. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×20 objective; scale bars, 200 μm and a ×40 objective (insets of **f**, scale bars, 100 μm). Results are mean values ± s.d., ELISA was done on lung homogenates and culture supernatants for cytokines and PCR with reverse transcription on CD4^{+} lung cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$, *Il9R*$^{-/-}$, *Cftr*$^{-/-}$ versus C57BL/6 mice; IL-9-treated versus control isotype-treated mice; stimulated versus unstimulated (none) cells and *Il9R*$^{-/-}$ versus C57BL/6 or *Cftr*$^{-/-}$ CD4^{+} T cells. Naive, uninfected mice. Data represent pooled results or representative images from three experiments, Two-tailed Student's *t*-test (**a**) or Two-way ANOVA (**d**,**e**) Bonferroni post test. *Gata3*, GATA binding protein 3; *Irf4*, interferon regulatory factor 4; *Pu.1*, purine-rich box 1.

co-cultivation of CD4$^+$ T cells with Lin$^-$ cells in the presence of IL-2 more than IL-33, an effect magnified in *Cftr*$^{-/-}$ as compared to C57BL/6 mice (Fig. 2h), and requiring the presence of IL-9R on responder CD4$^+$ T cells being significantly negated with *Il9R*$^{-/-}$ responder cells (Fig. 2h and Supplementary Fig. 4). These results indicate that ILC2, and particularly CD25$^+$ILC2, may account for the sustained IL-9 production and Th9 activation responsible for pathology in CF.

IL-9 activates mast cells to produce IL-2. The sustained production of IL-33 and IL-2 in *Cftr*$^{-/-}$ mice prompted us to investigate mechanisms behind this production. IL-33 is constitutively expressed at epithelial barrier surfaces where it is rapidly released from cells during tissue injury[25]. However, tight regulation of IL-33 following its release to dampen ST2-dependent inflammation to fungi has also been described[26]. This likely occurred in C57BL/6 but not in *Cftr*$^{-/-}$ mice in which the high levels of epithelial damage observed upon the infection (Fig. 3a) likely accounted for the sustained IL-33-dependent ST2$^+$ILC2 expansion. For IL-2, predictably high in CF, given the sustained NFAT activity[27], in addition to CD4$^+$ T cells[8] and dendritic cells[28], MC are known to produce it in the lung[7]. We looked therefore for MC presence and activity in the lung of C57BL/6 and *Cftr*$^{-/-}$ mice after the infection. MC were much expanded in *Cftr*$^{-/-}$ mice, as seen by flow cytometry (Fig. 3b) and toluidine staining (Fig. 3c). MC are distinguished by their granule content whose expression is tissue-dependent[29]. In the lung, chymase-positive MC numbers positively correlated with better lung function[30], whereas chymase- and tryptase-positive MC were expanded in areas of fibrosis in CF lungs and positively correlated with the degree of fibrosis and lung function[31]. Immunohistochemistry revealed that while tryptase-positive cells could not be detected, chymase-positive MC were present in C57BL/6 mice (insets of Fig. 3c). In contrast, chymase-positive and tryptase-positive MC were observed in *Cftr*$^{-/-}$ mice (insets of Fig. 3c).

As MC are known to produce cytokines through different receptor mechanisms[32], we evaluated cytokine production on magnetically purified c-Kit$^+$ MC (as characterized by morphometry and MC specific transcripts, Fig. 3d) upon stimulation with IgE, IL-9 or IL-33. We found that IL-2 production was induced by IL-33 and, more, by IL-9 and not by IgE, mostly in MC from *Cftr*$^{-/-}$ mice (Fig. 3e). As IL-9 also induced IL-9 production (Fig. 3e) and IL-9$^+$MC could also be detected *in vivo* (Fig. 3b), this suggests that an autocrine loop appears to mediate the IL-9-dependent IL-2 release by MC. As a matter of fact, IL-2 production (39 ± 6 ng/ml versus 127 ± 22, IL-2 in lung homogenates at 3 dpi in *Il9R*$^{-/-}$ versus C57BL/6 mice, respectively) and IL-2$^+$MC (Fig. 3f) were greatly reduced in *Il9R*$^{-/-}$ mice, thus contributing to the defective expansion of CD25$^+$ILC2 in these mice. Of interest, IL-9 stimulation also induced TGF-β in MC from *Cftr*$^{-/-}$ mice but not IL-6 (Fig. 3e), a finding suggesting that the autocrine IL-9 stimulation appears to be specific for IL-2 and TGF-β (Fig. 3e). These data indicate that MC may contribute to IL-2 production eventually leading to CD25$^+$ILC2 expansion in *Cftr*$^{-/-}$ mice. This appears to be the case, as IL-2$^+$MC, more than IL-2$^+$CD90.2 or IL-2$^+$CD4$^+$ T cells, were expanded *in vivo*, early in infection, particularly in *Cftr*$^{-/-}$ mice (Fig. 3f).

To directly prove this, we assessed susceptibility to inflammatory allergy of MC-deficient C57BL/6-*Kit*$^{W/W\text{-}v}$ mice or *Cftr*$^{-/-}$ mice treated with the tyrosine kinase inhibitor imatinib known to inhibit IL-9-driven mastocytosis in the lung[12]. Airway mastocytosis was reduced in MC-deficient *Kit*$^{W/W\text{-}v}$ mice (Fig. 4a) along with reduced levels of IgE, IL-2, IL-9 and TGF-β (Fig. 4b). Concomitantly, the number of CD25$^+$ILC2 were also decreased in the lung but promptly restored upon MC engraftment or exogenous IL-2 administration (Fig. 4a). Thus, MC appear to be able to control CD25$^+$ILC2 expansion in the lung during the infection via IL-2. Similar results were obtained upon treatment of *Cftr*$^{-/-}$ mice with imatinib. Both inflammation (Fig. 4c), collagen deposition (insets of Fig. 4c), IL-2, IL-9 and TGF-β production (Fig. 4d) and Th9 cell activation (Fig. 4e) were attenuated. Interestingly, imatinib apparently increased early inflammation in C57BL/6 mice (Supplementary Fig. 5), a finding suggesting that c-Kit$^+$ cells could contribute to pathogen resistance early in infection. As a matter of fact, MC-deficient *Kit*$^{W/W\text{-}v}$ mice displayed increased susceptibility to the infection as compared with C57BL/6 mice, as indicated by the increased fungal load (Supplementary Fig. 6a), neutrophils recruitment (Supplementary Fig. 6b) and a degree of lung inflammation (Supplementary Fig. 6c).

CFTR deficiency contributes to inflammation. The above results suggest that a circuit involving IL-33, IL-2 and IL-9 and different types of cells is pathogenically amplified in *Cftr*$^{-/-}$ mice. The finding that IL-33, IL-2 and IL-9 are also elevated in CF patients[33,34] prompted us to evaluate the contribution of cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction on the activation of the inflammatory circuit. Given that CFTR dysfunction on both epithelial and myeloid cells impacts on lung inflammation[35], we assessed chimeric mice with CFTR unresponsive myeloid or epithelial cells for lung damage and inflammation, production of IL-33, IL-2 and IL-9 and MC/ILC2 activation upon *Aspergillus* infection. We found that epithelial cell damage and lung inflammation (Fig. 5a), levels of cytokines and IgE (Fig. 5b), MC and ILC2 expansion (Fig. 5c) were all attenuated or decreased in condition of CFTR deficiency in epithelial cells but CFTR sufficient myeloid cells, a finding suggesting that myeloid, and perhaps lymphoid, deficiency could contribute to the activation of the inflammatory circuit in CF. This seems to be the case as the opposite findings were observed in recipient C57BL/6 mice receiving CFTR deficient myeloid cells (Fig. 5a,c) These mice, however, showed an intermediate inflammatory phenotype as compared to *Cftr*$^{-/-}$ mice, a finding suggesting that, although to a different extent, CFTR deficiency on epithelial and myeloid cells may predispose to lung inflammation in response to microbial and non-microbial stimuli.

The *IL9* rs2069885 SNP correlates with high IgE levels in CF females. To assess whether IL-9 may contribute to allergy in CF patients, we determined the effect of the non-synonymous *IL9* p.Thr117Met (c.350C>T, rs2069885) polymorphism, known to be associated with lung function and sensitization[36,37] on total and *Aspergillus*-specific IgE levels in CF patients (Supplementary Table 1). Previous association studies demonstrated the existence of sex dimorphism linked to this polymorphism[36,37]. Therefore, we carried out association testing separately in males and females. The distribution of the total IgE was skewed but after natural logarithmic transformation, the distribution adequately approximated a normal distribution. *IL9* rs2069885 genotype distribution did not deviate from Hardy–Weinberg equilibrium (χ^2 test, $P=0.776$) and it displayed a minor allele frequency of 0.129, comparable to the European population from 1000 Genome Consortium, minor allele frequency = 0.128 (ref. 38). *IL9* rs2069885 genotype distribution did not differ between males and females (χ^2 test, $P=0.243$), but a significant rs2069885-sex interaction on total IgE levels was found (general linear model, $P=0.004$), where female T allele carriers showed high IgE levels (linear regression: females, $\beta=0.624$, $P=0.043$; males,

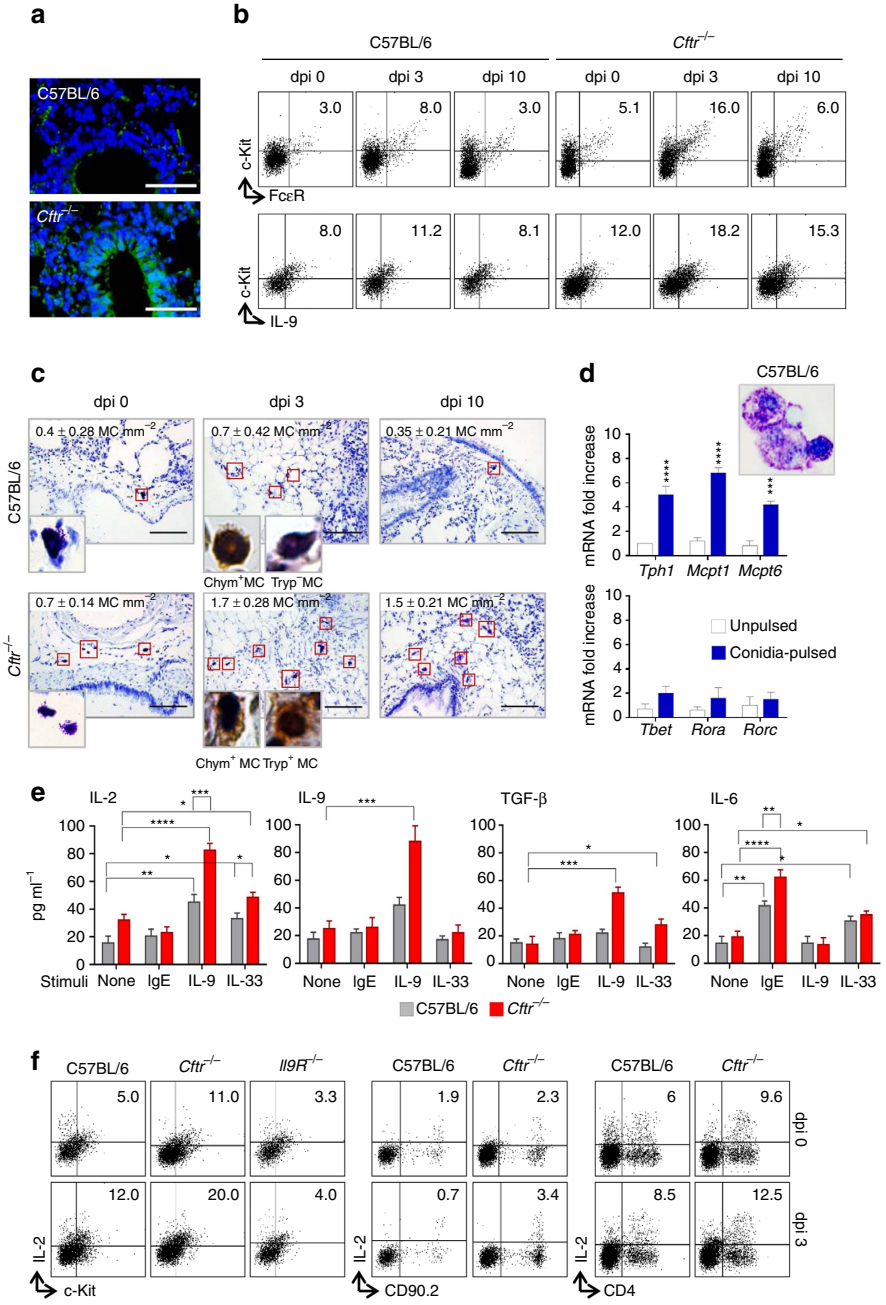

Figure 3 | IL-9 activates mast cells to produce IL-2. C57BL/6 or *Cftr*$^{-/-}$ mice (six per group) infected intranasally with live *A. fumigatus* conidia were evaluated at different days after infection (dpi) for (**a**) deposition of DNA on lung epithelial cells by TUNEL, resulting in bright DNA staining; (**b**) detection of c-Kit^{+}FcεR^{+} and c-Kit^{+}IL-9^{+} lung mast cells (MC) by flow cytometry (numbers refer to percentages of positive cells); (**c**) toluidine blue, relative MC number mm^{-2} and, in the inset, immunohistochemical staining for chymase- and tryptase-positive MC in lung section. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×40 objective and (in the inset) a ×100 objective and with EVOS FL Color Imaging System with a ×60 objective (immunohistochemical staining). (**d**) Toluidine blue stain and transcription factors expression (PCR with reverse transcription) of c-Kit^{+} cells magnetically isolated from lung of uninfected C57BL/6 mice and pulsed with live *A. fumigatus* conidia. (**e**) Cytokine production (mean values ± s.d., ELISA on culture supernatants) by purified lung c-Kit^{+} cells, pulsed with *A. fumigatus* and stimulated with IgE, IL-9 and IL-33; (**f**) detection of c-Kit^{+}IL-2^{+}, CD90.2^{+}IL-2^{+} and CD4^{+}IL-2^{+} lung cells by flow cytometry (numbers refer to percentages of positive cells). *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, conidia-pulsed versus unpulsed c-Kit^{+} cells, stimulated versus unstimulated c-Kit^{+} cells and *Cftr*$^{-/-}$ versus C57BL/6 c-Kit^{+} cells (data represent pooled results or representative images from three experiments, Two-way ANOVA, Bonferroni post test). *Mcpt1*, mast cell protease 1; *Mcpt6*, tryptase beta 2; *Rorc*, retinoic acid receptor-related orphan receptor C; *Rora*, RAR-related orphan receptor alpha; *Tbet*, T box expressed in T cells; *Tph1*, tryptophan hydroxylase 1.

$\beta = -0.666$, $P = 0.034$; Fig. 6a). In a subset of CF patients ($N = 114$; 57 males and 57 females), *Aspergillus*-specific IgE were also significantly higher in *IL9* rs2069885-T carriers (general linear model, $P = 0.002$; Fig. 6b). Notably, in line with the results obtained on total IgE, the association of *IL9* rs2069885-T allele with higher *Aspergillus*-specific IgE levels was observed in females (linear regression, $\beta = 1.417$, $P = 0.002$) more than males (linear regression, $\beta = 0.489$, $P = 0.279$). We also tested other

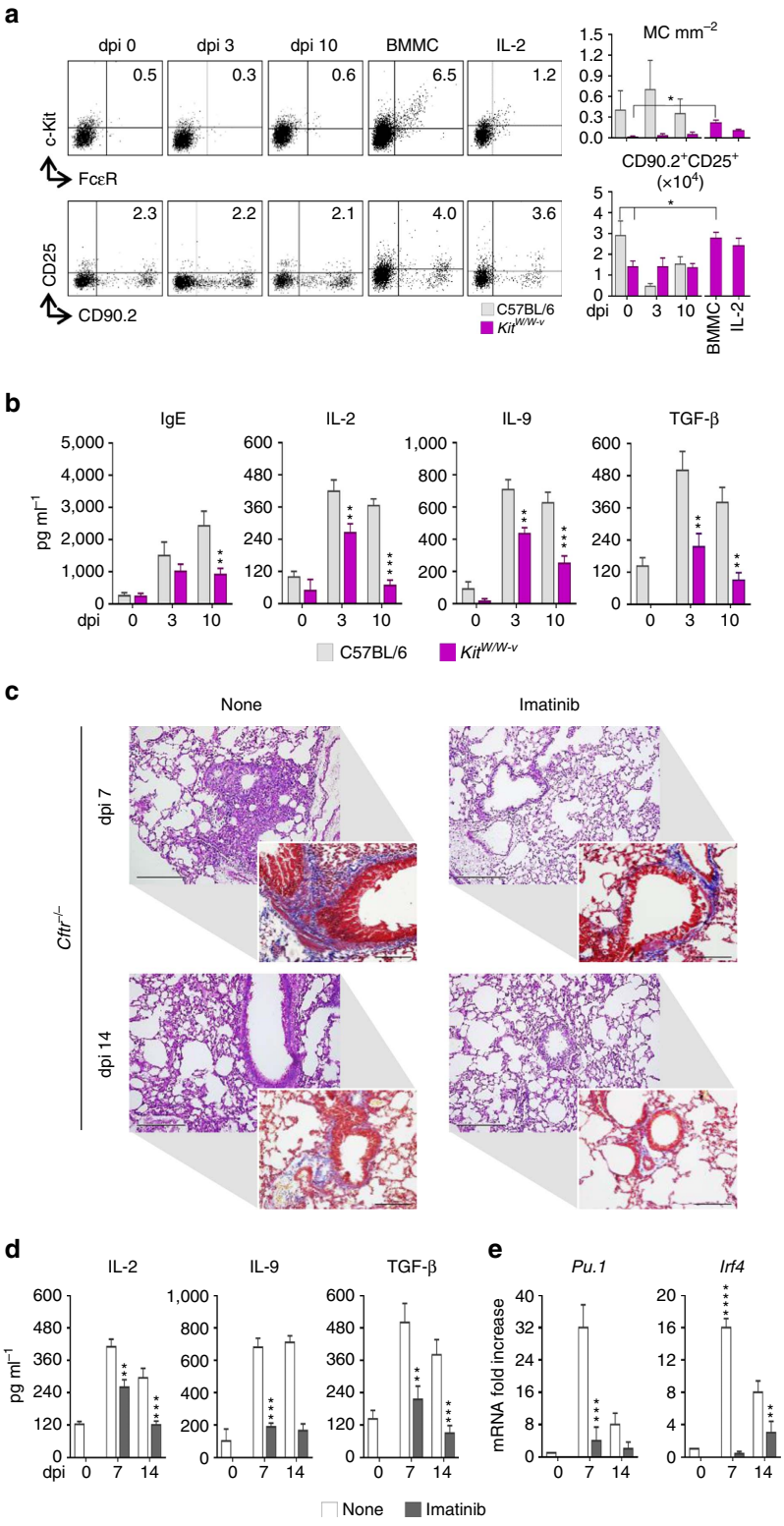

Figure 4 | IL-9 activates mast cells to produce IL-2. MC-deficient C57BL6-*Kit*$^{W/W\text{-}v}$ mice (six per group) were infected intranasally with live *A. fumigatus* conidia, engrafted intravenously with wild-type bone marrow-cultured mast cells (BMMC) or treated intraperitoneally with IL-2 for a week and assessed for (**a**) c-Kit$^+$FcεR$^+$ lung mast cells (MC) and CD90.2$^+$CD25$^+$ lung ILC2 by flow cytometry (numbers refer to percentages of positive cells) with relative cell number and (**b**) IgE and cytokine production. (**c**) Lung histology (periodic acid–Schiff and Masson's trichrome staining, in the insets); (**d**) cytokine production and (**e**) Th9-cell specific transcripts expression in *Cftr*$^{-/-}$ mice infected as above and treated with imatinib intraperitoneally for a week. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×20 objective, scale bars, 200 μm and a ×40 objective (insets of **c**, scale bars, 100 μm). Results are mean values ± s.d., ELISA on lung homogenates for cytokines and PCR with reverse transcription on lung CD4$^+$T cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, MC-deficient C57BL6-*Kit*$^{W/W\text{-}v}$ versus C57BL/6 mice or imatinib-treated versus untreated (none) mice (data represent pooled results or representative images from three experiments, Two-way ANOVA, Bonferroni post test). *Irf4* = interferon regulatory factor 4; *Pu.1* = purine-rich box 1.

IL9 tagSNPs (rs2069882, rs31564, rs1859430, rs1799962; Supplementary Tables 2 and 3), encompassing the whole *IL9* gene region, but none associated with IgE levels. Linkage disequilibrium (LD) analyses of the five SNPs in *IL9* failed to reveal the presence of significant LD blocks (Supplementary Fig. 7). Haplotype analysis yielded no significant result

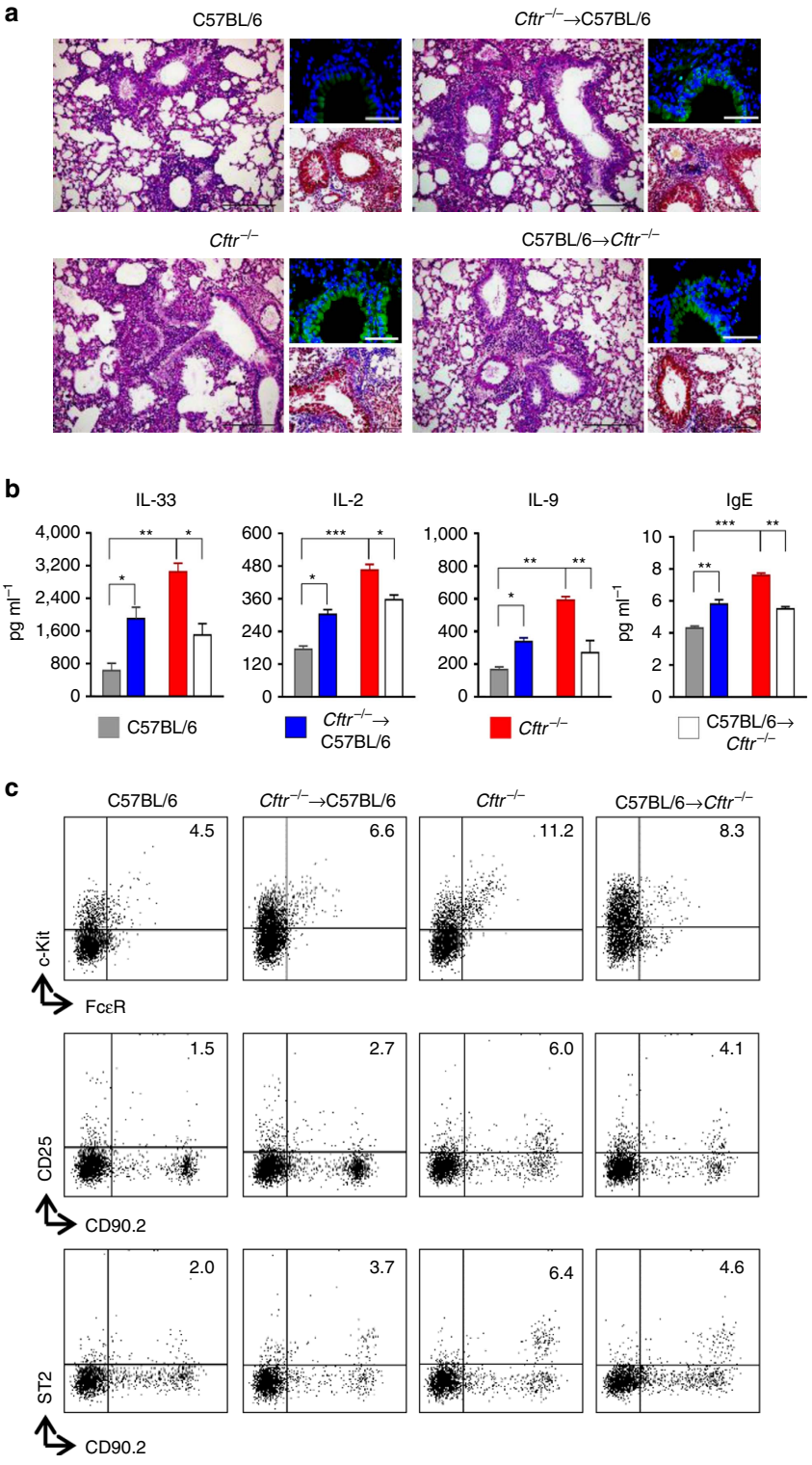

Figure 5 | Epithelial and myeloid CFTR deficiency contribute to the inflammatory phenotype. C57BL/6, *Cftr*$^{-/-}$ and chimeric C57BL/6 and *Cftr*$^{-/-}$ mice (10 per group) received 10×10^6 viable bone marrow cells 4 weeks before the intranasal infection with *A. fumigatus*. Chimeric mice were evaluated 7 days after the infection for (**a**) lung histology (periodic acid–Schiff and, in the insets, Masson's trichrome and TUNEL staining); (**b**) cytokines and IgE levels (mean values ± s.d., ELISA on lung homogenates); (**c**) detection of c-Kit$^+$FcεR$^+$ mast cells, CD90.2$^+$CD25$^+$ and CD90.2$^+$ST2$^+$ type 2 ILCs by flow cytometry (numbers refer to percentages of positive cells in the lung). Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×20 objective; scale bars, 200 μm and a ×40 objective (insets of panel **a**, scale bars, 100 μm). *$P<0.05$, **$P<0.01$, ***$P<0.001$, *Cftr*$^{-/-}$ versus C57BL/6, chimeric C57BL/6 versus C57BL/6, chimeric *Cftr*$^{-/-}$ versus *Cftr*$^{-/-}$ mice, Two-way ANOVA, Bonferroni post test.

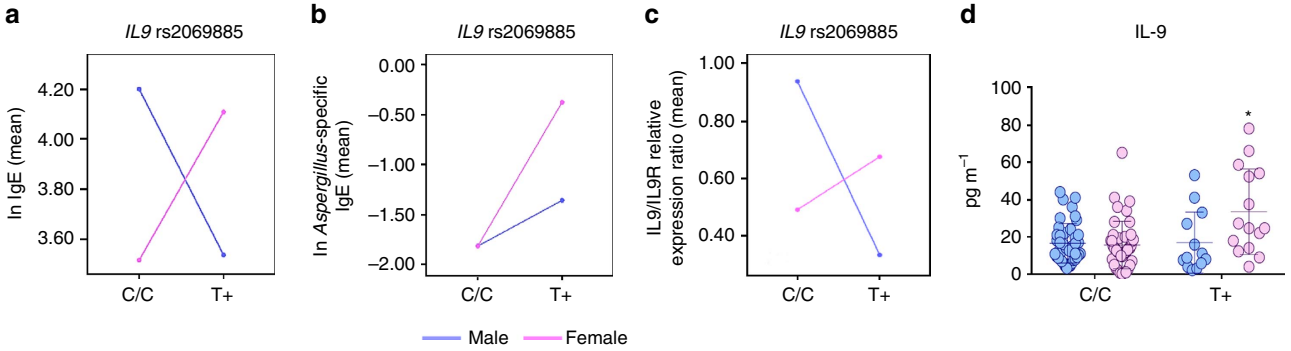

Figure 6 | The *IL9* rs2069885 polymorphism correlates with high IgE levels in CF females. (**a**,**b**) *IL9* rs2069885 sex interaction on total or *Aspergillus*-specific IgE levels and (**c**) *IL9/IL9R* expression ratio measured on CF patients. (**d**) Determination IL-9 (mean ± s.d., ELISA) in expectorates from CF patients carrying diverse genotypes at rs2069885.

(Supplementary Table 4), although the most common haplotype C–A–A–C–A (frequency 43.2%) turned out to be associated with higher IgE levels in males (linear regression, $\beta = 0.567$, $P = 0.022$).

We next determined whether the reported sex-specific associations could be attributable to a differential expression of *IL9* and its receptor, which is located on the pseudoautosomal region Xq/Yq, between males and females. A significant *IL9* rs2069885 by sex interaction was found when analysing the *IL9/IL9R* expression ratio (general linear model, $P = 0.025$) where T allele carriers showed opposite effects according to sex (linear regression, females, $\beta = 0.375$, $P = 0.116$; males, $\beta = -0.426$, $P = 0.375$; Fig. 6c) paralleling the finding on specific IgE (Fig. 6a) and on IL-9 levels in expectorates (Fig. 6d). No significant difference was noticed between males and females when *IL9* or *IL9R* gene expression levels were analysed independently.

Although these results are to be considered with caution given the small sample size–despite the sufficient power ($\beta = 0.20$, Power = 0.8; Supplementary Table 6)—which demands for a definitive validation using a larger new cohort of CF patients, the present findings confirm the existence of a sex dimorphism at *IL9* and *IL9R* loci, as already reported in several respiratory-related human phenotypes[36,37].

Discussion

We have shown that IL-9 may expand pro-inflammatory CD25$^+$ ILC2/Th9 cell in CF, an activity involving the production of IL-2 by MC. Human MC co-localize near ILC2 in the human lung and could directly promote ILC2 responses *in vitro*[39]. We found here that, in addition to CD4$^+$ T cells, known to contribute to CD25$^+$ILC2 expansion via IL-2 (ref. 8), lung MC may also affect the expansion of CD25$^+$ILC2 thought to contribute to chronic inflammation through multiple mechanisms[3]. Failure to expand CD25$^+$ILC2 occurred indeed in MC-deficient mice or mice treated with imatinib.

MC hyperplasia during chronic allergen challenge is associated with remodeling of airways[40]. In a mouse model of ovalbumin-induced airway inflammation, the influx of MC into lung peaks early after allergen challenge to mature over 14 days into cells expressing lower level of c-Kit, FcεRI and integrins[41]. MC hyperplasia was observed in the lung of *Cftr*$^{-/-}$ mice along with the detection of tryptase-positive and chymase-positive MC, known to be expanded in asthmatic patients[42] and in disease areas of CF lung[31]. Tryptases and chymases contribute to inflammation and tissue remodeling through the selective proteolysis of matrix proteins and the activation of protease-activated receptors and matrix metalloproteinases[29]. Consistent with the finding that MC from CF patients are not high in FcεR1 expressing[43], we found that lung MC from these mice poorly responded to IgE in terms of IL-6 production but released IL-2, in addition to TGF-β, in response to IL-9.

IL-9 is a pleiotropic cytokine that has multiple effects on structural as well as numerous hematopoietic cells, which are central to the pathogenesis of asthma[44,45]. The important role for the IL-9-MC axis in the pathology associated with chronic allergic inflammation has been already described[46]. IL-9 not only stimulates MC growth and expansion but also stimulates changes in gene expression that might alter responsiveness to other stimuli[47]. *In vivo*, IL-9 governed allergen-induced MC numbers in the lung, and anti-IL-9 antibody-treatment protected from airway remodeling, decreased expression of the profibrotic mediators TGF-β and improved lung function[48]. The correlation between a reduction in MC numbers and decreased airway remodeling, after IL-9 inhibition, is consistent with reports that MC-deficient mice demonstrate significantly attenuated fibrosis and inflammation after silica[49], ozone[50], or bleomycin injury[51].

We found that not only were IL-9 production and MC expansion significantly increased in CF mice but that the IL-9-MC axis contributed to the expansion of CD25$^+$ILC2 leading to Th9 cell activation that further contributed to the allergic inflammatory pathology (Fig. 7). IL-9 promoted IL-2 production by lung MC from *Cftr*$^{-/-}$ mice, a finding that may explain the increased and persistent expansion of CD25$^+$ILC2 in these mice. CD25$^+$ILC2 were indeed not expanded in condition of IL-9 ablation or MC-deficiency, a finding suggesting that the IL-9/MC/IL-2 axis drives the expansion of CD25$^+$ILC2. In addition, as IL-2 stimulated lung CD25$^+$ILC2 to produce IL-9 (ref. 9), this may have a positive feedback effect on ILCs, since lung ILCs cultured with IL-9 increased the production of type 2 cytokines[9] and up-regulated the anti-apoptotic protein BCL-3, thereby promoting ILC2 survival[8]. Of great interest, Lin$^-$ cells from CF lung also promoted Th9 cell activation *in vitro*, an activity that required IL-9R expression on responding CD4$^+$ T cells. Thus, the pathogenic role of IL-9 in promoting allergic inflammation may go beyond CD25$^+$ILC2 expansion to include the activation of Th9 cells. That Th9 cells are a major source of IL-9 in models of allergic inflammation and play an important role in MC accumulation and activation has been reported[52]. By producing IL-9, Th9 cells may in turn serve as a positive loop amplifying the IL-9/MC/ILC2 axis, promoting a deleterious vicious circle in which the production of profibrotic TGF-β by IL-9-stimulated MC plays a plausible important role. The presence of TGF-β dependent signalling in areas of prominent fibrosis in CF has been already documented[24] along with its inhibition of chloride channel activities[53] and the association of the TGF-β genetic variants with more severe lung disease[54].

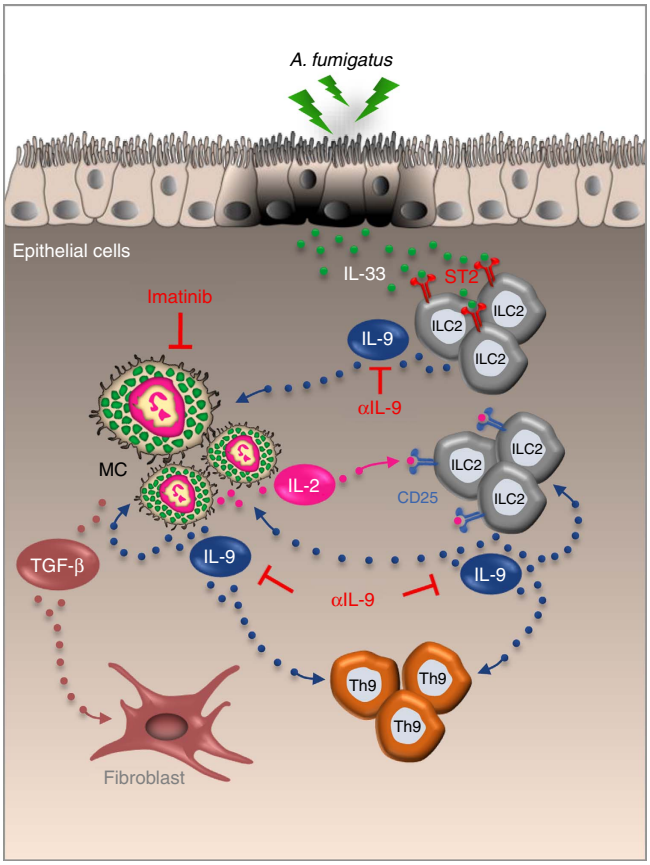

Figure 7 | Proposed model for the role of IL-9 in promoting a mast cells/ILC2/Th9 fibrotic pathway in CF. IL-9, produced by IL-33-expanded ILC2, activates MC for IL-2 production leading to the expansion of CD25$^+$ ILC2 that promote Th9 cell activation. The resulting increased production of IL-9 further amplifies the inflammatory loop by promoting ILC2 survival and type 2 cytokines production and by activating MC for the production of fibrotic TGF-β. IL-9 ablation or MC inhibition (imatinib) are potential drugable pathways through which inflammation and allergy could be restrained in CF. EC, epithelial cells. αIL-9, IL-9 neutralizing antibody.

Of note, together with IL-4, is able to promote Th9 cell development *in vitro*[55], a finding highlighting the potential role of TGF-β in reinforcing Th9 activity *in vivo*. However, TGF-β production by MC in response to IL-9 may also serve a regulatory, anti-inflammatory role[11,56].

In addition to IL-9, IL-33 is also a crucial regulator of MC functions and both IL-33 and MC have been influentially associated to the pathophysiology of allergic diseases and inflammation[57]. IL-33 is expressed in epithelia from patients with CF and potentiates neutrophil recruitment[58] as well as in type-2 pneumocytes on allergic lung inflammation[59]. In this study, IL-33 was increased in CF mice and likely correlated with the expansion of ST2$^+$ ILC2. At variance with IL-2, IL-33 did not stimulate MC for IL-9/TGF-β production, a finding indicating a minor contribution of the IL-33/MC axis in promoting inflammatory allergy and pathology in response to the fungus. As a matter of fact, the IL-33/MC/IL-2 axis was found to suppress, rather than promote, papain-induced allergic inflammation by promoting Treg[7].

Airway inflammation and recurrent pulmonary infections play a central role in the progression of CF lung disease. It is still an open question whether CFTR deficiency *per se* may enhance the inflammatory response to the different environmental cues[60]. Our data would suggest that CFTR dysfunction on both epithelial and myeloid cells may impact on the inflammatory circuit leading to the activation of the inflammatory IL-9/Th9 pathway: namely, among others[35], on both epithelial damage eventually leading to overproduction of IL-33 and on the MC propensity to respond to IL-9 with IL-2. However, PU.1 translocation into the nucleus has also been shown to be significantly higher in CF monocytes than in controls[61]. It seems that defective CFTR impacts on the mechanisms of communication between innate and adaptive immune response.

The potential contribution of IL-9 to CF pathogenesis in humans is unknown. Elevated levels of IL-9 were observed in the expectorates from CF patients, likely accounting for the expansion of TGF-β-producing MC in diseased lung areas[31]. It is of interest that in human asthmatic lung tissue, MC were the main IL-9R expressing population[46]. It seems therefore that the IL-9/MC/IL-2 axis may have a pathogenic role in CF patients and that its targeting could lead to a reduction in chronic inflammation and improved lung function of these patients. Studies have indeed highlighted the importance of Th9 cells in allergic lung inflammation by promoting epithelial alterations, goblet-cell hyperplasia, mucus production and infiltration of MC and eosinophils[46]. Considering that the costimulatory signal OX40 is required for Th9 activation[62], it is intriguing that OX40 ligand was critical in driving Th2-allergic responses to *A. fumigatus* in peripheral CD4$^+$ T cells isolated from CF patients with ABPA (ref. 63). It is clear that the inflammatory response in the lung involves different Th cell types whose specific role in CF remain unclear. Intriguingly, there are conditions where promoting IL-9 might be therapeutically beneficial[11] and ILC2 and MC could be exploited for pathogen immunity and tissue repair[64]. In this regard, the fact that imatinib apparently exacerbated signs of inflammation during infection points to a some beneficial role c-Kit$^+$ cells may have in the control of *Aspergillus* infection. MC indeed exhibited conidiocidal activity (Supplementary Fig. 6d), a finding suggesting that MC could serve as tissue sentinels modulating antifungal immune responses, as suggested[65].

In conclusion, IL-9 and MC may have an important role in the pathogenesis of lung disease and inflammation in CF. Considering the inherent resistance to steroids of MC in asthmatic patients[66], a better understanding of cellular and molecular pathways leading to inflammation and impaired lung functions may inspire new treatment avenues in patients with CF. Our study would suggest that imatinib, known to inhibit lung fibrosis[67], could be therapeutically exploited in CF patients with an exalted IL-9/Th9 responses. In addition, it is of great interest that the *IL9* rs2069885 polymorphism, linked to high IgE levels, was associated with females more than males with CF, a finding offering an explanation for the, as yet unexplained, 'gender gap' in mortality between females and males in CF[68] and fostering gender medicine in CF.

Methods

General experimental approaches. Mice were randomized and assigned to group allocation at the time of purchase to minimize any potential bias. No blinding was applied on harvesting cells after the treatments.

Mice. C57BL/6 (wild-type, WT), *Rag1*$^{-/-}$ and MC-deficient C57BL/6-*Kit*$^{W/W\text{-}v}$ mice, 6–8 week old, of both sexes, were purchased from Charles River (Calco, Italy). Genetically engineered homozygous *Cftr*$^{-/-}$ mice[69] were bred at the CF core animal facility at San Raffaele Hospital, Milan, Italy. *Il9R*$^{-/-}$ and *RagIl9R*$^{-/-}$ mice were from the Ludwing Institute for Cancer Research, Brussells.

Fungal infection allergy and treatments. Anaesthetized (by inhalation of 3% isoflurane (Forane Abbot) in oxygen)) mice were infected by the intranasal instillation of 2×10^7 resting conidia/20 μl saline. For allergic broncho-pulmonary aspergillosis, *A. fumigatus* culture filtrate extract in incomplete Freund's adjuvant

(Sigma-Aldrich) was given (100 µg) to intact mice intraperitoneally (i.p.), subcutaneously and then intransally (20 µg), twice a week apart. A week after the last intranasal challenge, mice received 10^7 *Aspergillus* resting conidia and evaluated a week later. Murine monoclonal anti-IL-9 antibody (MM9CI from BioXcell), or control isotype IgG, were administered i.p. at the dose of 500 µg kg^{-1} for a week starting the day of the infection. The levels of IL-9 after antibody treatment were 65 ± 15 versus 295 ± 16 pg mg^{-1} for C57BL/6 and 103 ± 27 versus 719 ± 14 pg mg^{-1} for *Cftr*$^{-/-}$ mice, treated versus untreated mice. IL-2 at the dose of 1 µg per mouse was given i.p. for a week. Imatinib mesylate (Glivec, ST1571 Novartis, Basel) were administered i.p. at the dose of 1 mg kg^{-1} for a week starting the day of the infection.

Mast cell engraftment. Selective engraftment of MC in MC-deficient C57BL/6-*Kit*$^{W/W\text{-}v}$ mice was performed as follow. Briefly, bone marrow cells derived from 6-week-old female C57BL/6 mice were cultured in WEHI-3–conditioned medium (ATCC number TIB-68), as a source of IL-3, for 4–5 weeks to obtain MC populations (BMCMC) which purity was higher than 95%. Via the tail vein, 5×10^6 BMCMC were injected into each mouse, and the recipients were used for experiments 4 weeks later.

Generation of bone marrow chimeras. Femurs and tibias were removed aseptically from donor C57BL/6 and *Cftr*$^{-/-}$ euthanized mice. Bone marrow was retrieved by flushing with cold Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum and 2 mM L-glutamine (Invitrogen). Cells were washed twice with PBS without calcium and magnesium supplemented with 1% foetal calf serum. Recipient C57BL/6 and *Cftr*$^{-/-}$ mice were irradiated with 9 Gy and reconstituted no later than 6 h after the last irradiation with 10×10^6 T cells by intravenous injection. Mice were given sulfamethoxazole (150 mg ml^{-1}) and trimethoprim (30 mg ml^{-1}) in drinking water for the first 3 weeks of reconstitution. Mice were used no earlier than 4 weeks after transplantation. Before use in experiments, all mice were bled from the retro-orbital plexus, and the peripheral blood lymphocytes were analysed for the stable donor-type chimerism by reverse transcription-PCR of *Cftr*.

***In vivo* staining analysis.** For histology, paraffin-embedded tissues were stained with Periodic acid-Schiff, Masson's trichrome or Toluidine Blue staining to investigate inflammation, collagen deposition and MC infiltration, respectively. For immunofluorescence, lungs were incubated at 4 °C with phycoerythrin-conjugated (PE) anti-CD25 (Miltenyi Biotec clone 7D4, 1:60), anti-T1-ST2 (BioLegend clone DIH9, 1:400), anti-IL-9 (Milenyi Biotec clone RM9A4, 1:60) and fluorescein isothiocyanate-conjugated (FITC) anti-mouse CD90.2 (Miltenyi Biotecclone 30-H12, 1:60) and anti-CD4 (BioLegend clone GK1.5, 1:1,000). Nuclei were counterstained with 4,6-diamidino-2-phenylindole. Immunostaining with appropriate irrelevant antibodies did not give positive staining of the lung. For immunohistochemistry, the lung sections were incubated overnight with polyclonal anti-chymase (Bioss, 1:100) or monoclonal anti-tryptase (Abcam clone EPR8476, 1:500) followed by the secondary biotinylated antibodies. Cells were counterstained with haematoxylin. Photographs were taken using a high-resolution Olympus DP71 microscope with a × 20 and × 40 objective or EVOS FL Color Imaging System with a × 60 objective. For immunoblotting, blots of lung lysates were incubated with polyclonal antibodies against STAT5 and phospho-STAT5 (both from Cell Signaling, 1:1,000) and normalized on β-actin (clone AC-15 from Sigma). The ChemiDocTM XRS + Imaging system (Bio-Rad) was used to detect chemiluminescence on the addition of the LiteAblotPlus chemiluminescence substrate (Euroclone S.p.A). Quantification was done by densitometry image analysis using Image Lab 5.1 software (Bio-Rad). The uncut blot is shown in Supplementary Fig. 8.

TUNEL assay of lung sections. Sections of lungs fixed in 4% buffered paraformaldehyde, pH 7.3, for 36 h and embedded in paraffin, were deparaffinized, rehydrated, treated with 0.1 M citrate buffer, pH 6.0, washed and blocked in 0.1 M Tris-HCl buffer, pH 7.5, supplemented with 3% bovine serum albumin and 20% foetal calf serum. The slides were then incubated with fluorescein-coupled dUTP and terminal deoxynucleotidyltransferase–mediated deoxyuridine triphosphate nick-end labelling (TUNEL) enzyme (Roche Diagnostics) in the presence of terminal deoxynucleotidyltransferase. Unspecific binding was removed by washing with phosphate-buffered saline for 10 min at 70 °C. The sections were mounted and analysed by fluorescence microscopy, using a × 40 objective.

Cell isolation and culture. Lungs were finely minced, digested in 16 mg ml^{-1} Collagenase P (Roche) for 30 min and meshed through a 70-µm cell strainer. ILCs were isolated from total lung cells by magnetic depletion of Lineage Positive cells (Miltenyi Biotec). CD4$^+$ T cells and c-Kit$^+$ cells were purified from total lung cells after incubation of CD4 microbeads and with PE-labelled anti-c-Kit followed by anti-PE MicroBeads respectively (both from Miltenyi Biotec). FcεRIα-APC and c-Kit-PE (Miltenyi Biotec) staining and morphological examination after toluidine blue staining on the cytospin slides were used fir c-Kit$^+$ cell phenotyping. For Lin$^-$-CD4$^+$ T cell co-culture, 2×10^6 CD4$^+$ T cells were co-cultured with 1×10^6 Lin$^-$ cells with or without 50 ng ml^{-1} recombinant IL-33, 40 ng ml^{-1} recombinant IL-2 and pulsed with *A. fumigatus* conidia. Three days later, IL-9 levels in culture supernatants were analysed by ELISA and Th9 transcription factors by real-time PCR. To separate Lin$^-$ and CD4$^+$ T cell, we used the Transwell culture system (Costar, 0.4 µm pore size; Corning) with CD4$^+$ T cells in the lower wells and Lin$^-$ in the upper wells[7]. For c-Kit$^+$ cells culture, 5×10^5 cells were cultured overnight in RPMI medium and pulsed with *A. fumigatus* conidia with or without 10 µg ml^{-1} IgE, 100 ng ml^{-1} IL-33 and 100 ng ml^{-1} IL-9.

Flow cytometry. Flow cytometry on enriched Lin$^-$ cells was performed with a combination of the following fluorescence-conjugated mAbs (all from Miltenyi Biotec unless specified otherwise): APC-conjugated anti-NKp46 (29A1.4.9), anti-CD90.2 (30-H12), anti-Rorγ (t; REA278), anti-FcRIa (MAR-1), anti-CD4 (GK1.5); PE-conjugated anti-NK1.1 (PK136), anti-CD25 (7D4), anti-T1-ST2 (DIH9, from Biolegend), anti-CD117 (3C11), anti-IL-9 (RM9A4) and anti-IL-2 (JES6-5H4). For intracellular staining, phorbol 12-myristate 13-acetate (PMA)/ionomycin-stimulated cells were added of brefeldin, and then permeabilized with the CytoFix/CytoPerm kit (BD Biosciences) for intra-cytoplasmic detection of IL-9 and IL-2. Flow cytometry was done at 4 °C on cells first exposed to Fc receptor mAb (2.4G2). Cells were analysed with a BD LSRFortessa flow cytometer equipped with BD FACSDiva 7.0 software.

ELISA and real-time PCR. The levels of cytokines and IgE in lung homogenates, culture supernatants or expectorates were determined by ELISA kits (R&D Systems) following manufacturer's instructions. Real-time PCR with reverse transcription was performed using CFX96 Touch Real-Time PCR Detection System and SYBR Green chemistry (Bio-Rad) on total RNA reverse transcribed with the cDNA Synthesis Kit (Bio-Rad). The PCR primers were as listed in Supplementary Table 5. Amplification efficiencies were validated and normalized against *Gapdh*. The thermal profile for SYBR Green real-time PCR was at 95 °C for 3 min, followed by 40 cycles of denaturation for 30 s at 95 °C and an annealing/extension step of 30 s at 60 °C. The messenger RNA-normalized data were expressed as relative gene messenger RNA in treated versus untreated groups or cells.

Human study. A cohort of 347 patients of Caucasian origin with a proven diagnosis of CF (CFTR genotyping, sweat testing and clinical phenotype) was enroled in a prospective multicenter longitudinal genetic association study, See Supplementary Table 1 for clinical data including age, gender, lung function testing, measures of nutrition, microbiological findings and vital status of the patients' cohort.

SNPs selection and genotyping. DNA was isolated from blood with the QIAamp DNA Mini (Qiagen, Milan, Italy) system and stored at −20 °C. *IL9* SNPs were selected based on literature review[37] and their ability to tag surrounding variants in the HapMap-CEU population of the International HapMap project, NCBI build B36 assembly HapMap phase III (http://www.hapmap.org). Haplotype-based tagging SNPs were selected by assessing LD blocks from the genes of interest with a pairwise correlation coefficient r^2 of at least 0.80 and a minor allele frequency higher than 5% in the HapMap-CEU population. Five *IL9* SNPs complied with the selection criteria: rs2069885, rs2069882, rs31564, rs1859430 and rs1799962. The applied Biosystems 7500 Fast qPCR system (Life Technologies) was used for SNP genotyping by KASPar assays (KBiosciences, Hertfordshire, UK). Each genotyping set comprised randomly selected replicates of sequenced samples and negative controls. Agreement between original and duplicate samples was ≥99% for all SNPs. Laboratory personnel were blind to the sample status.

Statistical analysis. Data are expressed as mean ± s.d. Horizontal bars indicate the means. Statistical significance was calculated by two-way ANOVA (Bonferroni's *post hoc* test) for multiple comparisons and by a two tailed Student's *t*-test for single comparison. The distribution of levels tested by Kolmogorov–Smirnov normality test turned out to be non-significant. Values of P not >0.05 were considered significant. The data reported are either representative from two or three experiments (FACS data, histology, immunofluoresce and TUNEL assay) or pooled otherwise. The *in vivo* groups consisted of 6 mice per group. Data were analysed by GraphPad Prism 4.03 programme (GraphPad Software). No statistical method was used to predetermine sample size. Genetic association testing was carried out considering additive and dominant models by linear regression implemented in Plink v1.07 (ref. 70), adjusting for age at sampling. Haplotype-based association tests were performed by general linear model using Plink v1.07 (ref. 70). LD analysis was performed using Haploview, and defining LD blocks based on the solid spine of LD algorithm. Analyses were conducted stratifying the study population according to sex since previous evidence highlighted the existence of sex dimorphism at *IL9* locus[36,37]. *IL9* rs2069885 by sex interaction was tested by general linear model using SPSS v.21. Two-tail P values are reported. Bonferroni's correction for multiple testing was not performed since we are assessing specific questions on a candidate gene and we are not searching for associations without a priori hypotheses. Power calculation (using QUANTO v1.2.4) was performed to determine whether the sample study had sufficient power to detect a significant

association between *IL9* rs2069885 and total IgE levels. Considering sample size ($N = 237$), allele frequency (13%), dominant genetic model, mean level of IgE and its standard deviation ($131.7 \pm 248.2\ U\ ml^{-1}$) and $\alpha = 0.05$ two-sided, we could establish that our sample have sufficient power ($\beta = 0.20$, Power = 0.8) to detect an association explaining more than 3% of total IgE levels variance (Supplementary Table 6).

Study approval. All animal experiments were approved by local government authorities and were in agreement with the Italian Approved Animal Welfare Authorization 360/2015-PR and Legislative decree 26/2014 obtained from the Italian Ministry of Health lasting and lasting for five years (2015–2020). Infections were performed under anaesthesia and all efforts were made to minimize suffering. Human studies approval was obtained from institutional review boards the Bambino Gesù Children's Hospital (Rome, Italy), Ospedale Maggiore Policlinico, University of Milan, (Milan, Italy), Innsbruck Medical University, (Innsbruck, Austria) and Servizio di Supporto Fibrosi Cistica, (Cerignola, Foggia, Italy). Written informed consent was obtained from the participants, or, in case of minors, from parents or guardian.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions

G.R. and V.O. performed *in vivo* murine experiments. C.G. and V.N. performed human genotyping. M.P., M.P. (Puccetti), R.G.I. and M.B. performed immunohistochemistry, immunofluorescence and histopathology; M.D.Z., G.P., T.Z. and C.E.P. contributed to mast cell experiments; J.-C.R. provided scientific advice; O.B. and P.S. provided reagents and contributed to experimental design; V.L., C.C., M.C.R., E.F., C.L.-F., F.M., G.R., H.E. and L.R. (Ratclif) provided the clinical samples. S.M., V.N.T., V.N. and L.R. designed the experiments, analysed data and wrote the paper.

Additional information

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