Anti-bacterial mast cell activities and their role during *Mycobacterium tuberculosis* infection

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ABBREVIATIONS

5-HT	5-hydroxythryptamine
Ag85	Antigen 85
AMPs	Antimicrobial peptides
AMφ	Alveolar macrophages
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette-Guerin
BCG-PSN	Polysaccharide nucleic acid obtained from BCG
BMMCs	Bone marrow-derived mast cells
CBA	Capture bead array
CFU	Colony-forming units
CRAMP	Cathelicidin-related antimicrobial peptide
CSU	Chronic spontaneous urticaria
CTMC	Connective tissue mast cells
DAPI	4´,6-diamidino-2-phenylindole
DC	Dendritic cells
DC-SIGN	DC-specific intracellular adhesion molecule-3 grabbing non-integrin
DHR 123	Dihydorhodamine 123
DPI	Diphenyliodonium
ESAT-6	Early secretory antigenic target
ETs	Extracellular traps

FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF-2	Fibroblast growth factor 2
FMO	Fluorescence minus one
FSC	Forwards scatter
HBHA	Heparin binding hemagglutinin
HE	Haematoxylin and eosin
HMC-1	Human mast cell line-1
hMCs	Human mast cells
HRP	Horseradish peroxidase
HSP70	70 kilodalton heat shock protein
ICAM-1	Intercellular adhesion molecule 1
IGRAs	Interferon gamma reléase assays
IMDM	Iscove Modifiied Dulbecco medium
LAM	Lipoarabinomannan
LAMPs	Lysosomal associated membrane glycoproteins
LM	Lipomannan
LPS	Lipopolysaccharide
LTC_4	Leukotriene C_4
ManLAM	Mannosylated lipoarabinomannan
MC_C	Mast cell chymase
Mce	Mammalian cell entry

MCETs	Mast-cell extracellular traps
MCp	Mast cell progenitors
MCs	Mast cells
MC_T	Mast cell tryptase
MC_{TC}	Mast cell tryptase and chymase
MDC	Macrophage-derived chemokine
MDR	Multi-drug resistant
MHC-II	Major histocompatibility complex class II
MIP-1α	Macrophage inflammatory protein-1α
MMC	Mucosal mast cells
MOI	Multiplicity of infection
MRGPRX2	G-protein-coupled receptor X2
Mtb	*Mycobacterium tuberculosis*
MTBC	Mycobacterium tuberculosis complex
MyD88	Myeloid differentiation primary response protein 88
Mφ	Macrophages
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor-kappa B
NK	Natural killer cells
NO	Nitric oxide
OADC	Oleic albumin dextrose catalase
OVA	Ovalbumin

PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PGD_2	Prostaglandin D_2
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
RBL-2H3	Rat basophilic leukaemia cells
RD1	Region of diference-1
RD2	Region of diference-2
RLUs	Relative light units
ROS	Reactive oxigen species
rPMCs	Rat peritoneal mast cells
SCF	Stem cell factor
SP	Substance P
SRA	Scavenger receptor A
SSC	Side scatter
TB	Tuberculosis
TLR	Toll-like receptors
TLR-1	Toll like receptor 1
TLR-2	Toll like receptor 2
TMA	Tissue microarray
TST	Tuberculin skin test
VCAM-1	Vascular cell adhesion protein 1

WHO	World health organization
WLL	White light laser

ABSTRACT

Tuberculosis (TB) is still considered part of the top 10 mortal diseases caused by a single infectious agent. Thus, understanding the immune mechanisms during TB infection is essential to find new therapeutic and prophylactic approaches. Different immune cells have been widely studied to understand TB pathology. However, mast cells (MCs), which are important innate immune cells, have been little explored. Their strategic location and ability to secrete a wide repertoire of pro and anti-inflammatory molecules make MCs relevant against infections. To better define general strategies used by MCs against bacterial threats, using an *in vitro* model of human MCs (hMCs), we first characterized MC functions by means of degranulation, cytokine secretion, MC extracellular traps (MCETs) formation and production of reactive oxygen species (ROS) upon *Escherichia coli, Listeria monocytogenes*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* stimulation. Our data showed that while *L. monocytogenes* induce degranulation, MCET formation and cytokine release in the absence of ROS in MCs, *S. pneumoniae* promote ROS production without inducing MCET and degranulation and while *E. coli* promote MC degranulation in the absence of MCET and ROS, *S. aureus* induce a selective release of prostaglandin D_2 without degranulation. Thus, MCs display an individualized pattern of response depending on the bacterial type. Next, we characterized MC location, numbers and predominant phenotype in human lung post-mortem samples of patients affected by TB. Using fluorescent staining to identify MCs in lung infected tissue, we found MCs located at inflammatory sites, close to periphery of granulomas and predominantly abundant at fibrotic sites. We also found a co-localization of MCs with IL-17 and TGF-□ which are relevant for granuloma formation and fibrogenesis respectively. Thus, we proposed MCs as contributors to the early inflammatory stage and the late fibrotic phase of TB pathology. Finally, to better define the means of interaction between MCs and mycobacteria, we investigated the outcome of direct crosstalk between MCs and BCG, which is an attenuated strain of *Mycobacterium bovis* that fails to protect the adult infection. We found that MCs only respond to BCG when exposed to IL-33 pre-treatment by the release of IL-8 and MCP-1. Furthermore IL-33 enhanced both CD48-MC expression and number of MC-BCG interactions. Thus, IL-33 may serve to pre-activate MCs and potentiate inflammatory responses against BCG. Altogether the current study proposed MCs as important mediators during TB and as potential targets for vaccine boosters.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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Publications

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- **Garcia-Rodriguez, KM**, Bahri, R, Sattentau, C, Roberts, IS, Goenka, A, Bulfone-Paus, S. Human mast cells exhibit an individualized pattern of antimicrobial responses. *Immun Inflamm Dis*. 2020; 1– 13. doi: 10.1002/iid3.295.

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CHAPTER 1: Thesis outline: Journal format

The current thesis is written in an alternative format i.e. “journal format”. The introduction is described in Chapter 2 containing an initial general introduction followed by a detail introduction on the role of mast cells in tuberculosis (Paper 1). Chapter 3 consists of a detail description of the generation of human mast cells, which is the experimental model of the current study and is written in a journal-type structure. Results obtained from this work are shown as individual papers and listed below. All the experiments and work performed by the current author (KMGR) are listed in “author contributions” in the present chapter. It is worth to mention that Paper 1, which is part of the introduction (Chapter 2), is a published review while Paper 2 (Chapter 4) is an original published article, Paper 3 (Chapter 5) is already submitted and under revision at the time this thesis was delivered and Paper 4 (Chapter 6) is a draft manuscript. Each paper is written according to journal guidelines, however, numbers in tables and figures are formatted to allow consistency to the current thesis. Furthermore, all result chapters are ordered with an individual introduction, material and methods, results, discussion, conclusion, references and supplementary figures and material. A final chapter with the main conclusions, future work and study limitations were placed at the end in Chapter 7.

Paper 1:

Tittle: The Role of Mast Cells in Tuberculosis: Orchestrating Innate Immune Crosstalk?
Authors: K. M. Garcia-Rodriguez, A. Goenka, T. Alonso-Rasgado, R-Hernández-Pando, S. Bulfone-Paus
Journal: Frontiers in immunology
Category: Review
Status: Published; doi: https://doi.org/10.3389/fimmu.2017.01290
Author Contributions: KMGR and SBP conceived, wrote, designed, and coordinated the manuscript. AG, MAR, and RHP provided helpful discussions and edited the manuscript. All the authors read and approved the final manuscript.

Paper 2:

Tittle: Human mast cells exhibit an individualized pattern of anti-microbial responses
Authors: K. M. Garcia-Rodriguez, R. Bahri, C. Sattentau, I. Roberts, A. Goenka, S. Bulfone-Paus
Journal: Immunity, Inflammation and Disease

Category: Original article
Status: Published; doi: https://doi.org/10.1002/iid3.295

Author contributions: KMGR, CS, RB, AG, and SBP participated in the research design. KMGR, CS, RB conducted the experiments. KMGR, CS, RB, AG, and SBP performed data analysis. ISR contributed with the purification and isolation of bacterial strains. All authors contributed to the writing of the manuscript and reviewed the final version.

Paper 3:

Tittle: Differential mast cell numbers and characteristics in human tuberculosis pulmonary lesions

Authors: K. M. Garcia-Rodriguez, E. Bini, A. Gamboa-Domínguez, C. Espitia-Pinzón, S. Huerta-Yepez, S. Bulfone-Paus, R. Hernández-Pando

Journal: European Journal of Pathology

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Status: Submitted/in revision

Author contributions: KMGR, RHP and SBP participated in the research design. Material preparation, data collection and analysis were performed by KMGR. KMGR and EIB conducted experiments. SHY produced micro-array tissues. KMGR and RHP performed data analysis. The first draft of the manuscript was written by KMGR and RHP and SBP commented on previous versions of the manuscript. All authors revised and approved final version.

Paper 4:

Tittle: IL-33 primes human mast cells to BCG-induced activation

Authors: K. M. Garcia Rodriguez, A. Goenka, D. Thomson, R. Bahri, R. Hernandez-Pando and S. Bulfone-Paus

Category: Original article

Status: In preparation/Draft manuscript

Author contributions: SBP, RB, AG and KMGR participated in the research design. KMGR conducted experiments, collected data, performed data analysis and wrote the first draft of the manuscript. SBP revised and commented on previous versions of the manuscript. DT supervised confocal microscopy acquisitions.

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CHAPTER 2: Introduction

SECTION I: General introduction

2.1 TUBERCULOSIS

2.1.1 Clinical features

Tuberculosis (TB) is caused by a group of mycobacterial strains forming the *Mycobacterium tuberculosis* complex (MTBC) that include *Mycobacterium tuberculosis (Mtb)*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium caprae,* and *Mycobacterium pinnipeddi*, being *Mtb* the main causal agent in humans [1]. The clinical symptoms of pulmonary TB include chronic cough, weight loss, general weakness, fever, sputum production, and night sweats [2]. However, symptoms are less specific during extrapulmonary or disseminated TB which includes tubercular lymphadenitis, pleural, abdominal, central nervous system, bone and joint, genito-urinary, and military TB [3].

2.1.2 Epidemiology

Tuberculosis is still in the top 10 diseases causing death from a single infectious agent. According to the world health organization (WHO) in 2019, 1.4 million people died from TB and an estimated 10 million new TB cases were reported worldwide. Among these reported cases, 52% are men, 32% are women and 12% are children [4]. Furthermore, although a 9% reduction of TB incidence was notable between 2015 and 2019, TB is still considered a major threat, and efforts in research are still necessary to fight the infection.

2.1.3 Diagnosis

The current diagnostic procedures to detect *Mtb* include 1) bacterial culture, to distinguish *Mtb* colonies in agar plates, 2) microscopical analysis, using different staining techniques to identify *Mtb,* and 3) molecular techniques, to identify *Mtb*-related genes [1, 3]. Additionally, the tuberculin skin test (TST) and the interferon-gamma release assays (IGRAs) are immunological diagnostic tools based on T cell and innate response against *Mtb* [1]. Clinically, common signs and symptoms including cough that last 3 weeks, hemoptysis, and

chest pain which are the more evident signs. Furthermore, chest radiography can provide evidence of TB by lung abnormalities observed in the lung parenchyma [5].

2.1.4 Mycobacterium tuberculosis

Mycobacterium tuberculosis (*Mtb)*, is an aerobic, non-motile, and rod-shaped pathogenic mycobacteria forming rough colonies with a generational time for up to 20h that belongs to the *Mycobacteriaceae* family and the *Mycobacterium* genus [1]. The main difference between *Mycobacterium* genus from other bacteria is the cell wall content which is high in lipids and carbohydrates [6]. *Mtb* is characterized by an hydrophobic cell wall composed of mycolic acids, arabinogalactan, and polysaccharides which allows *Mtb* survival within the host and helps the bacilli to avoid drug penetration [7]. *Mtb* wall lipids include lipomannan, lipoarabinomannan, mannosylated lipoarabinomannan, and different lipoproteins [8] (**Figure 2.1**). Due to the high lipid content, *Mtb* is not decolorized by acid-alcohol therefore considered an acid-fast bacteria stained by the Ziehl-Neelsen [6].

2.1.5 Mycobacterial antigens

Mycobacterial surface antigens allow the bacilli to survive inside the host and to induce immune responses. Virulent antigens exhibited in the cell wall surface include *Fbp* (fibronectin-binding protein) also known as antigen 85 (Ag85) including 3 main proteins: FbpA (Ag85A), FbpB (Ag85B), and FbpC2 (Ag85C). These proteins are essential for mycobacterial uptake since they attach to fibronectin in mucosal surfaces. *Mce* (mammalian cell entry) proteins are also important for mycobacterial entrance into host cells including *mce1, mce2, mce3,* and *mce4*. HBHA (heparin-binding hemagglutinin adhesin) is also a surface protein necessary for mycobacterial dissemination (**Figure 2.1**). This adhesin attaches to glycosaminoglycan of non-phagocytic cells such as epithelial cells and fibroblasts promoting extra-pulmonary dissemination and mycobacterial aggregation [9, 10].

Secretory molecules are also necessary for mycobacterial survival within the host. ESX secretion systems are involved in mycobacterial virulence, specifically ESX-1 and ESX-5 [8, 9]. ESX-1 secretion system releases ESAT-6 (early secretory antigenic target, ESXA) and CFP-10 (culture filtrate protein, ESXB). ESAT-6 and CFP-10 are the most recognized antigens necessary for *Mtb* survival and replication within the host [11]. These antigens are recognized by T cells and are targets of mycobacterial attenuation. [9-11]. The HspX protein is also a virulent factor secreted by *Mtb* involved in the latent phase found in old stationary

cultures [9, 10]. Thus, different mycobacterial molecules presented at the cell surface or secreted at early times are necessary for bacilli survival within the host and are targets of drug development and host-pathogen studies.

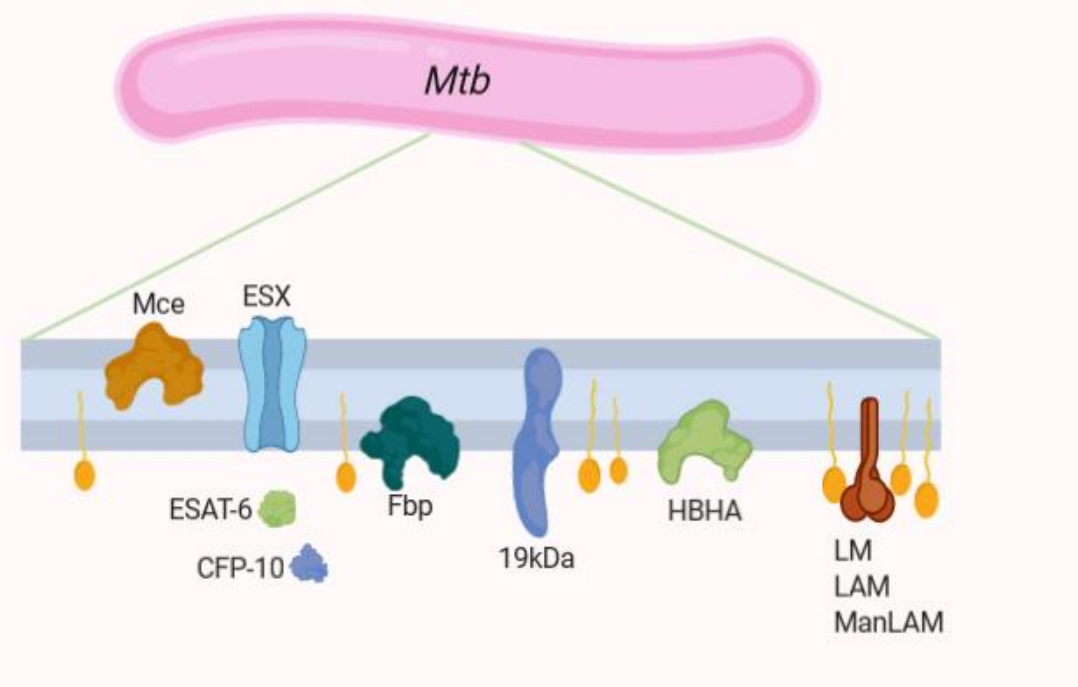

Figure 2.1. Mtb antigens. Different mycobacterial immunogenic antigens are exhibited at *Mtb* cell wall including: Lipomannan (LM), lipoarabinomannan (LAM), mannosylated lipoarabinomannan (ManLAM), Heparin Binding Hemagglutinin (HBHA), Fibronectin binding protein (Fbp), Mammalian cell entry (Mce) and 19 kilodalton lipoprotein (19kDa). The ESX secretion system release pathogenic antigens including ESAT-6 and CFP-10. Figure was created using *BioRender.com*.

2.1.6 Immune cell receptors recognizing mycobacterial antigens

Toll-like receptors (TLR), are the main receptors that recognize *Mtb,* [8, 12]. TLRs induce the MyD88 (myeloid differentiation primary response protein 88) which activates the transcription factor NFκB resulting in the release of pro-inflammatory mediators including TNF-α, IL-12, and IL-1β [13]. Among TLRs, TLR2, TLR4, and TLR9 are capable to recognize the bacilli, being TLR2 the most dominant receptor. TLR2 recognizes *Mtb* cell wall components including Lipomannan, lipoarabinomannan, mannosylated lipoarabinomannan,

and the secretory molecule ESAT-6. Furthermore, different receptors have been shown to recognize different *Mtb* wall components [8]. For instance, C-type lectin receptors recognize carbohydrate components from *Mtb* whereas DC-SIGN (DC-specific intracellular adhesion molecule-3 grabbing non-integrin) recognize Dectin-1 and lipoarabinomannan. Scavenger receptors including CD36, MARCO, and scavenger receptor A (SRA) [8] as well as the GPI-anchored protein CD48 also recognize *Mtb* molecules [14]. Besides, the mannose-binding lectin, which recognizes the mannosylated lipoarabinomannan, and the mannose receptor have shown to be necessary for the mycobacterial encounter, although more than one receptor is necessary for mycobacterial recognition [15].

2.1.7 Tuberculosis pathogenesis

TB infection starts with inhalation of saliva droplets containing *Mtb*, that reach the alveoli where alveolar macrophages (AMφ), dendritic cells (DCs), neutrophils, and mast cells (MCs) reside [16, 17]. Here, innate phagocytic cells, especially macrophages (Mφ), recognize pathogen-associated molecular patterns (PAMPs) on *Mtb* and phagocytose free bacilli [18]. Activated Mφ release of pro-inflammatory molecules capable to recruit different immune cells to the infection site [19]. To control the infection, non-infected Mφ surround infected Mφ, which allows mycobacterial survival by enabling *Mtb* to infect new cells [20]. In both, mice and humans, IFN-γ and IL-12 are necessary to activate AMφ and the lack of both cytokines results in increased susceptibility to *Mtb* infection [15]. Once activated, AMφ can differentiate into epithelioid cells, and the fusion of infected AMφ results in the formation of multinucleated giant cells [19, 21]. Furthermore, DCs internalize *Mtb* and migrate to the lymph nodes to activate naïve T cells that become effector T cells able to migrate to the infection site [22]. This cellular recruitment together with neutrophils, natural killer (NK) cells, DCs, and fibroblasts create a cellular barrier around infected AMφ resulting in the common TB granulomatous lesion [19, 23]. TNF-α, IL-17, IFN-γ, and IL-12 are essential for granuloma formation [23]. This “physical” barrier is beneficial for the pathogen allowing *Mtb* to survive in the lung in a latent state, avoiding antibiotics and immune mediator penetration [19]. Most patients (estimated to be one-third of the world’s population) carry a latent infection without developing the progressive disease [24]. The mechanism of TB reactivation is unclear yet, however, it is suggested that a failure of granuloma maintenance may be the cause [25]. Thus, the granuloma is the result of a non-efficient immune control that can eventually promote mycobacterial dissemination and chronic infection.

2.1.8 Mouse and human infection

C57BL/6 and BALB/c mice are common mouse strains used to study immune pathways and specific cell responses that are involved in the cross-talk between *Mtb* and the host [26]. However, mouse models exhibit differences in disease development compared to humans. One of the main limitations is that mice lack the latency stage observed in humans. Infected mice develop progressive disease leading to death without the development of critical immune responses that occur in humans [26]. Furthermore, resident immune cells are different between human and mouse lungs, for instance, MCs are highly represented in human lungs compared to mice [27]. The understanding of human infection is limited due to a lack of appropriate tools and the majority of our knowledge is based on data from bronchoalveolar lavage (BAL) and biopsies from post-mortem infected patients [19]. During human TB infection, cytokine and chemokine levels are observed abnormal in BAL concentrations compare to mice including, IL-1β, IL-6, TNF-α, CXCL10, CXCL8 and NF-κB (nuclear factor-kappa B) [19]. However, in response to *Mtb*, human Mφ produce IFN-γ, IL-1β, MIP-1α (macrophage inflammatory protein-1α), MDC (macrophage-derived chemokine), nitric oxide (NO) [15] and TNF-α [28], which is comparable to data obtained from mouse models [26]. Thus, although mouse models provide evidence that is comparable to human biology, differences between both species need to be considered.

2.1.9 Treatment

TB treatment depends on the stage of infection. The WHO recommends a combination of different antibiotics including isoniazid, rifampicin, pyrazinamide, ethambutol, and streptomycin. In new TB patients, the treatment includes two phases: An intensive phase for 2 months administrating isoniazid, rifampicin, pyrazinamide, and ethambutol followed by a continuation phase of 4 months administrating isoniazid and rifampicin for a total of 6 months. In multi-drug resistant (MDR)-patients or co-infected patients, the treatment includes higher chemotherapy dosage and the use of second-line antibiotics. Since the long-period time of treatment, most patients abandon the treatment before completing the 6-month chemotherapy which results in the formation of new resistant strains. Furthermore, second-line antibiotics are more toxic for patients and need a longer treatment [29]. Although chemotherapy is effective to control the infection, current studies are carrying out to enhance the existing treatment to reduce the administration time and avoid the use of second-line antibiotics.

2.1.10 Tuberculosis vaccine

The current vaccine to prevent TB is the BCG (Bacillus Calmette-Guerin) vaccine which is an attenuated strain of *Mycobacterium bovis*. BCG was first attenuated at Pasteur Institute by Calmette and Guerin through multiple passages from 1908 to 1921 [30]. After its attenuation, the strain lost the Region of Difference-1 (RD1) which encodes the ESX-1 secretion system. The RD1 locus comprises 9 genes that encode important virulent antigens including CFP-10 and ESAT-6. The lack of these secretory proteins in virulent *Mtb* inhibits intra-macrophage growth and phagolysosomal fusion [11, 31]. BCG was further distributed around the world in uncontrolled dissemination. This distribution induced different laboratories to produce BCG sub-strains with variable attenuation levels resulting in variable protection depending on each attenuated strain. In this second attenuation stage, BCG lost the RD2 locus encoding virulent antigens such as MPB64 and MPB70 [31].

Overall, BCG protects children from pulmonary and disseminated disease; however, the vaccine presents limited protection in the pulmonary infection in adults with high variability among populations ranging from 0 to 80% [32]. Different hypotheses are suggested trying to explain this variable protection offered by BCG which includes: 1) previous exposure with environmental mycobacteria, 2) genetic background among populations, 3) *Mtb* virulence, 4) BCG sub-strain and 5) BCG vaccination route [33, 34].

2.2 MAST CELLS: PROPERTIES AND ACTIVITIES

2.2.1 Mast cell characterization

In 1878 Paul Ehrlich first coined the name Mast Cell (MC) as “granular cells of the connective tissue” presenting an “undetermined chemical substance bound in the granular storages” [35]. More than a century after their discovery, interesting findings on MCs functions, granular contents, and localization within body sites continue to accumulate. MCs are mainly located at the skin and mucosal tissue including the airways and gastrointestinal and urogenital tract and are involved in physiological and pathological immune responses [36]. Furthermore, they exhibit a large repertoire of pre-synthesized, pre-stored and *de novo* synthesized mediators including cytokines, chemokines, proteases, histamine, proteoglycans, serotonin, and growth factors [37]. MCs are well known for their contribution

to allergic diseases, however, they were recently found to be involved in pathogenic defence [38].

Although MC progenitors are located at tissue sites, MCs principally migrate from bone marrow, as pluripotent CD34+ and CD117+ (receptor tyrosine kinase *c-kit*) hematopoietic stem cells to different tissues where they mature influenced by the local environment [36, 39]. Stem cell factor (SCF) is the main chemotactic factor for MCs which promotes their differentiation, survival, and maturation [40]. Cytokines such as IL-3, IL-4, IL-6, IL-9, and IL-10 also influence MC maturation [37].

2.2.2 Mast cell receptors

MCs display different receptors that can be activated by different stimuli [27] (**Figure 2.2**). This includes the complement C5a receptor (CD88) [41], the high-affinity IgE receptor that is involved in allergic disorders [42], TLRs that binds a variety of pathogens [43], CD48 which binds *Mtb* [44], and the major histocompatibility class II (MHC-II) which is low express in resting cells but inducible after LPS and IFN-γ stimulation [45]. Besides, MC express the G-protein-coupled receptor X2 (MRGPRX2), which is activated by peptides including substance P (SP) and compounds capable to induce MC degranulation including compound 48-80 [46].

2.2.3 Mast cell secreted products

Once stimulated MCs can release a great variety of pro and anti-inflammatory pre-stored and de novo synthesized mediators (**Figure 2.2**) such as histamine, heparin, tryptase, chymase, prostaglandin D_2 (PGD_2), Leukotriene C_4 and D_4 (LTC_4/D_4), chemokines as CCL1, CCL2, CCL4, CCL7, CCL12, CCL17, CXCL5 and CXCL8, and cytokines as IL-4, IL-3, IL-5, GM-CSF, IL-6, IL-13, and TNF-α [47]. In fact, MCs are the only immune cells to pre-store TNF-α [48]. MCs are found to be highly involved in the secretion and long-distance delivery of TNF-α from the periphery to the lymph nodes [49]. IL-17 is also released by MCs, a cytokine involved in the induction of antimicrobial peptides (AMP), and interestingly MCs and not T cells are the major IL-17-expressing cell in normal skin [50]. MCs can modulate different Th subtypes. For instance, MCs releasing IL-12, IFN-γ, CCL3, CCL4, CXCL9, and CXCL10 are involved in the Th1 subset differentiation while MC-dependent IL-4, CCL5, and CCL11 contribute to the Th2 differentiation subset and MC-mediated IL-6, TGFβ1, and CCL20 influence the Th17 subset [37]. This wide variety of products and their rapid release

(minutes/seconds) make MCs important mediators and coordinators in pro- and anti-inflammatory responses.

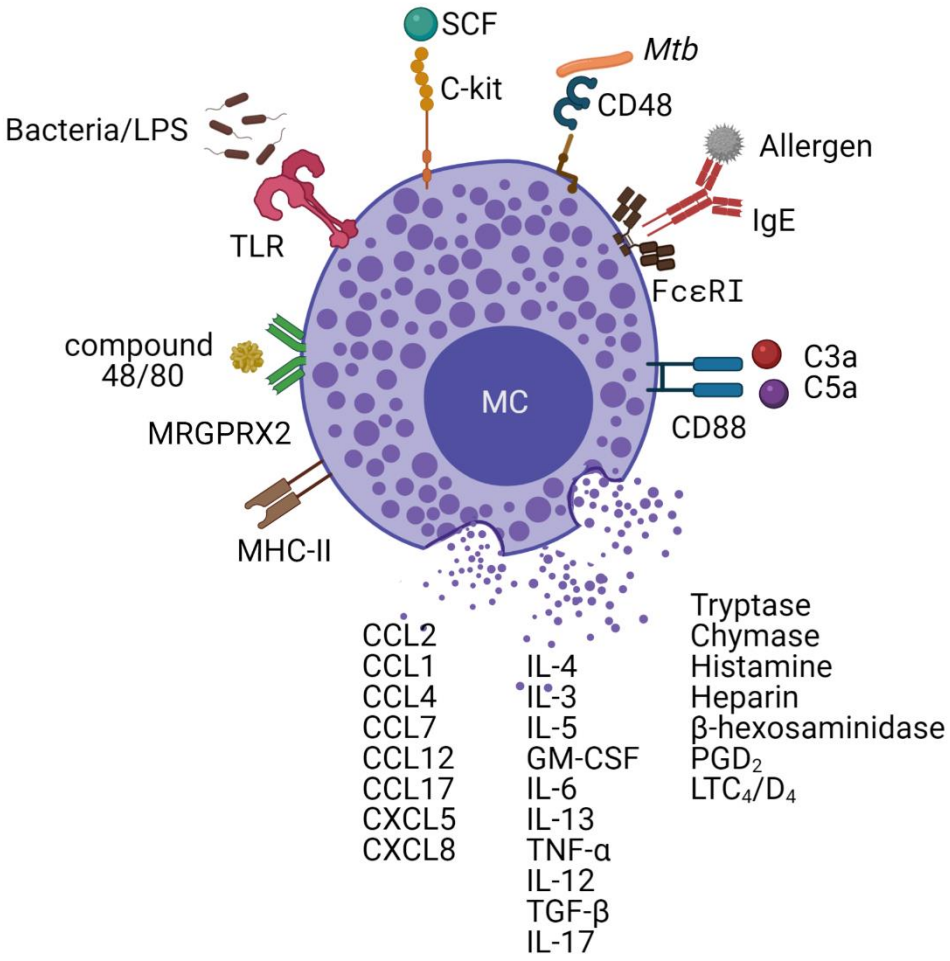

Figure 2.2. Mast cell properties and produced mediators. Mast cells are activated by different constitutive receptors including: 1) CD88 activated by complement molecules C3a and C5a, 2) FcεRI (high affinity IgE receptor), 3) CD48 activated by Mycobacterium tuberculosis, 4) c-Kit which links SCF (stem cell factor), 5) toll like receptors (TLRs) that are activated by pathogens and their products including LPS, 6) MRGPRX2 activated by different ligands including compound 48/80 and inflammatory peptides and 7) the major histocompatibility complex (MHC) class II. Once Activated MCs release a variety of products including cytokines, chemokines and degranulation products including CCL2, CCL1, CCL7, CCL12, CCL17, CXCL5, CXCL8, IL4, IL-3, IL-5, GM-CSF, IL-6, IL-13, TNF-α, IL-12, IL-17, tryptase, chymase, histamine, heparin, β-hexosaminidase, prostaglandin D2 (PGD_2), and leukotriene C_4 and D_4 (LTC_4/D_4). Figure was created using *BioRender.com*.

2.2.4 Mast cell classification

Human MCs are classically divided into two subtypes based on their protease content: tryptase (MC_T), chymase (MC_C), or tryptase and chymase (MC_{TC}) [51] whereas Mouse MCs are classified into convective tissue (CTMC) and mucosal MCs (MMC). The protease profile in mice includes MMCP-1 and MMCP-2 proteases and CTMCs express MMCP-4, MMCP-5, and MMCP-6 proteases [52]. In human lungs, most of the MCs in the alveoli and bronchial lamina propia are of the MC_T subtype (approximately 90%) while MC_{TC} are found in the smooth muscle of the airways [27]. In health, mouse MCs occur in low numbers in the lung and are localized around larger airways and blood vessels. Nevertheless, in pathology MC numbers increase in both human and mouse lungs [27].

REFERENCES: Chapter 2, section I

1. Delogu, G., M. Sali, and G. Fadda, *The biology of mycobacterium tuberculosis infection.* Mediterranean journal of hematology and infectious diseases, 2013. **5**(1): p. e2013070-e2013070.
2. Loddenkemper, R., M. Lipman, and A. Zumla, *Clinical Aspects of Adult Tuberculosis.* Cold Spring Harbor perspectives in medicine, 2015. **6**(1): p. a017848-a017848.
3. Natarajan, A., et al., *A systemic review on tuberculosis.* Indian Journal of Tuberculosis, 2020. **67**(3): p. 295-311.
4. Harding, E., *WHO global progress report on tuberculosis elimination.* The Lancet Respiratory Medicine, 2020. **8**(1): p. 19.
5. Sanyaolu, A., et al., *Tuberculosis: A Review of Current Trends.* Epidemiology International Journal, 2019. **3**: p. 000123.
6. Pfyffer, G.E., *Mycobacterium: general characteristics, laboratory detection, and staining procedures*, in *Manual of Clinical Microbiology, Eleventh Edition*. 2015, American Society of Microbiology. p. 536-569.
7. Jankute, M., et al., *Assembly of the mycobacterial cell wall.* Annual review of microbiology, 2015. **69**: p. 405-423.
8. Stamm, C.E., A.C. Collins, and M.U. Shiloh, *Sensing of Mycobacterium tuberculosis and consequences to both host and bacillus.* Immunological reviews, 2015. **264**(1): p. 204-219.
9. Forrellad, M.A., et al., *Virulence factors of the Mycobacterium tuberculosis complex.* Virulence, 2013. **4**(1): p. 3-66.
10. Smith, I., *Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence.* Clinical microbiology reviews, 2003. **16**(3): p. 463-496.
11. Brodin, P., et al., *ESAT-6 proteins: protective antigens and virulence factors?* Trends in microbiology, 2004. **12**(11): p. 500-508.
12. Killick, K.E., et al., *Receptor-mediated recognition of mycobacterial pathogens.* Cellular microbiology, 2013. **15**(9): p. 1484-1495.
13. Saraav, I., S. Singh, and S. Sharma, *Outcome of Mycobacterium tuberculosis and Toll-like receptor interaction: immune response or immune evasion?* Immunology and cell biology, 2014. **92**(9): p. 741-746.
14. Muñoz, S., B. Rivas-Santiago, and J.A. Enciso, *Mycobacterium tuberculosis Entry into Mast Cells Through Cholesterol-rich Membrane Microdomains.* Scandinavian Journal of Immunology, 2009. **70**(3): p. 256-263.

15. Sia, J.K., M. Georgieva, and J. Rengarajan, *Innate immune defenses in human tuberculosis: an overview of the interactions between Mycobacterium tuberculosis and innate immune cells.* Journal of immunology research, 2015. **2015**.
16. Ehlers, S. and U.E. Schaible, *The granuloma in tuberculosis: dynamics of a host-pathogen collusion.* Frontiers in immunology, 2013. **3**: p. 411-411.
17. Pai, M., et al., *Tuberculosis.* Nature Reviews Disease Primers, 2016. **2**(1): p. 16076.
18. Sanyaolu, A., et al., *Tuberculosis: A Review of Current Trends.* 2019. **3**: p. 000123.
19. Scriba, T., A. Coussens, and H. Fletcher, *Human Immunology of Tuberculosis.* Microbiology spectrum, 2016. **4**(4).
20. Philips, J.A. and J.D. Ernst, *Tuberculosis pathogenesis and immunity.* Annual Review of Pathology: Mechanisms of Disease, 2012. **7**: p. 353-384.
21. Orme, I.M., R.T. Robinson, and A.M. Cooper, *The balance between protective and pathogenic immune responses in the TB-infected lung.* Nature immunology, 2015. **16**(1): p. 57-63.
22. Nunes-Alves, C., et al., *In search of a new paradigm for protective immunity to TB.* Nature Reviews Microbiology, 2014. **12**(4): p. 289-299.
23. Ramakrishnan, L., *Revisiting the role of the granuloma in tuberculosis.* Nature Reviews Immunology, 2012. **12**(5): p. 352-366.
24. Dorhoi, A. and S.H. Kaufmann, *Versatile myeloid cell subsets contribute to tuberculosis-associated inflammation.* European journal of immunology, 2015. **45**(8): p. 2191-2202.
25. Carlos, D., et al., *TLR2-dependent mast cell activation contributes to the control of Mycobacterium tuberculosis infection.* Microbes and Infection, 2009. **11**(8): p. 770-778.
26. O'Garra, A., et al., *The immune response in tuberculosis.* Annual review of immunology, 2013. **31**: p. 475-527.
27. Reber, L.L., et al., *Potential effector and immunoregulatory functions of mast cells in mucosal immunity.* Mucosal immunology, 2015. **8**(3): p. 444-463.
28. Engele, M., et al., *Induction of TNF in human alveolar macrophages as a potential evasion mechanism of virulent Mycobacterium tuberculosis.* The journal of Immunology, 2002. **168**(3): p. 1328-1337.
29. Organization, W.H. and S.T. Initiative, *Treatment of tuberculosis: guidelines*. 2010: World Health Organization.
30. Abdallah, A.M. and M.A. Behr, *Evolution and strain variation in BCG*, in *Strain Variation in the Mycobacterium tuberculosis Complex: Its Role in Biology, Epidemiology and Control*. 2017, Springer. p. 155-169.
31. Liu, J., et al., *BCG vaccines: their mechanisms of attenuation and impact on safety and protective efficacy.* Human vaccines, 2009. **5**(2): p. 70-78.
32. McShane, H., *Tuberculosis vaccines: beyond bacille Calmette–Guérin.* Philosophical Transactions of the Royal Society of London B: Biological Sciences, 2011. **366**(1579): p. 2782-2789.
33. Pitt, J.M., et al., *Vaccination against tuberculosis: how can we better BCG?* Microbial pathogenesis, 2013. **58**: p. 2-16.
34. Moliva, J.I., J. Turner, and J.B. Torrelles, *Prospects in Mycobacterium bovis Bacille Calmette et Guerin (BCG) vaccine diversity and delivery: why does BCG fail to protect against tuberculosis?* Vaccine, 2015. **33**(39): p. 5035-5041.
35. Crivellato, E., et al., *Paul Ehrlich's doctoral thesis: a milestone in the study of mast cells.* British journal of haematology, 2003. **123**(1): p. 19-21.
36. Bradding, P., *Human mast cell cytokines.* Clinical & Experimental Allergy, 1996. **26**(1): p. 13-19.
37. Cardamone, C., et al., *Mast cells as effector cells of innate immunity and regulators of adaptive immunity.* Immunology Letters, 2016. **178**: p. 10-14.
38. Abraham, S.N. and A.L. St. John, *Mast cell-orchestrated immunity to pathogens.* Nature Reviews Immunology, 2010. **10**(6): p. 440-452.
39. Virk, H., G. Arthur, and P. Bradding, *Mast cells and their activation in lung disease.* Translational Research, 2016. **174**: p. 60-76.
40. Ribatti, D., *The development of human mast cells. An historical reappraisal.* Experimental Cell Research, 2016. **342**(2): p. 210-215.

41. Oskeritzian, C.A., et al., *Surface CD88 functionally distinguishes the MCTC from the MCT type of human lung mast cell.* The Journal of allergy and clinical immunology, 2005. **115**(6): p. 1162-1168.
42. Rios, E.J. and J. Kalesnikoff, *FcεRI expression and dynamics on mast cells.* Methods Mol Biol, 2015. **1220**: p. 239-55.
43. Sandig, H. and S. Bulfone-Paus, *TLR signaling in mast cells: common and unique features.* Frontiers in immunology, 2012. **3**: p. 185-185.
44. Muñoz, S., et al., *Mast Cell Activation by Mycobacterium tuberculosis: Mediator Release and Role of CD48.* The Journal of Immunology, 2003. **170**(11): p. 5590.
45. Kambayashi, T., et al., *Inducible MHC class II expression by mast cells supports effector and regulatory T cell activation.* Journal of immunology (Baltimore, Md. : 1950), 2009. **182**(8): p. 4686-4695.
46. Porebski, G., et al., *Mas-Related G Protein-Coupled Receptor-X2 (MRGPRX2) in Drug Hypersensitivity Reactions.* Frontiers in Immunology, 2018. **9**(3027).
47. Moiseeva, E.P. and P. Bradding, *Mast Cells in Lung Inflammation*, in *Mast Cell Biology: Contemporary and Emerging Topics*, A.M. Gilfillan and D.D. Metcalfe, Editors. 2011, Springer US: Boston, MA. p. 235-269.
48. von Köckritz-Blickwede, M., et al., *Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation.* Blood, 2008. **111**(6): p. 3070-3080.
49. Kunder, C.A., et al., *Mast cell–derived particles deliver peripheral signals to remote lymph nodes.* The Journal of experimental medicine, 2009. **206**(11): p. 2455-2467.
50. Lin, A.M., et al., *Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis.* Journal of immunology (Baltimore, Md. : 1950), 2011. **187**(1): p. 490-500.
51. Stone, K.D., C. Prussin, and D.D. Metcalfe, *IgE, mast cells, basophils, and eosinophils.* Journal of Allergy and Clinical Immunology, 2010. **125**(2): p. S73-S80.
52. Gri, G., et al., *Mast Cell: An Emerging Partner in Immune Interaction.* Frontiers in Immunology, 2012. **3**(120).

SECTION II: Paper 1

The Role of Mast Cells in Tuberculosis: Orchestrating Innate Immune Crosstalk?

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ABSTRACT

Tuberculosis causes more annual deaths globally than any other infectious disease. However, progress in developing novel vaccines, diagnostics, and therapies has been hampered by an incomplete understanding of the immune response to Mycobacterium tuberculosis (*Mtb*). While the role of many immune cells has been extensively explored, mast cells (MCs) have been relatively ignored. MCs are tissue-resident cells involved in defence against bacterial infections playing an important Tyrole mediating immune cell crosstalk. This review discusses specific interactions between MCs and *Mtb*, their contribution to both immunity and disease pathogenesis, and explores their role in orchestrating other immune cells against infections.

INTRODUCTION

Tuberculosis (TB) is the world's major infectious disease killer, accounting for 1.4 million deaths in 2015 (1). Progress in developing vaccines, diagnostics, and therapies has been hampered by an incomplete understanding of the immune response to the causative pathogen, Mycobacterium tuberculosis (*Mtb*).

Following entry of *Mtb*-containing droplets into the airways, bacilli are initially phagocytosed by alveolar macrophages (AMφ), providing a comfortable niche in which *Mtb* can reside, replicate, and evade immune cell detection (2). Mycobacterial pathogen-associated molecular patterns engage pattern recognition receptors (PRRs) to trigger signalling pathways, resulting in the release of various chemokines and cytokines and the recruitment and activation of immune cells (3). This process results in the internalization of mycobacteria by dendritic cells (DCs), which migrate to lymph nodes where they polarize naïve T cells to antigen-specific Th1 effector cells in an IL-12-dependent manner (4, 5). IFN-γ produced by Th1-polarized T cells activates mycobactericidal mechanisms in AMφ (6). Various immune cells are sequentially recruited to the sites of infection; neutrophils in the earliest stages as well as T cells, NK cells, and fibroblasts. These surround the infected AMφ to form a mycobacterial granuloma (2, 7), which acts as a "physical barrier" limiting bacillary dissemination. However, chronic granulomas also promote *Mtb*'s intracellular survival and impair elimination, resulting clinically, in latent TB disease (2). One-third of the world's population is latently infected with *Mtb* and between 1 and 10% will develop progressive TB disease following "reactivation" of infection in later life (8). The mechanism of TB reactivation is unclear as yet, however, it is suggested that a failure of granuloma maintenance may be the cause (9). Thus, the granuloma is the result of a non-efficient immune control that will eventually progress to chronic infection, rather than mycobacterial clearance.

Mast cells (MCs) are tissue-resident cells strategically located in mucosal tissues (10) and are among the first cells to come in contact with pathogens (11). MCs contribute to bacterial immunity through multiple mechanisms such as bacterial recognition, activation, recruitment of immune cells to the site of infection, the release of inflammatory mediators, and direct bacterial killing by extracellular traps (ETs). However, their main role may be orchestrating other immune cells against infections (11–14).

Since little is known about the MC contribution to TB pathogenesis, this review summarizes the MC strategies used in bacterial defence as well as potential and reported interactions occurring between *Mtb* and MCs (**Figure 2.3**).

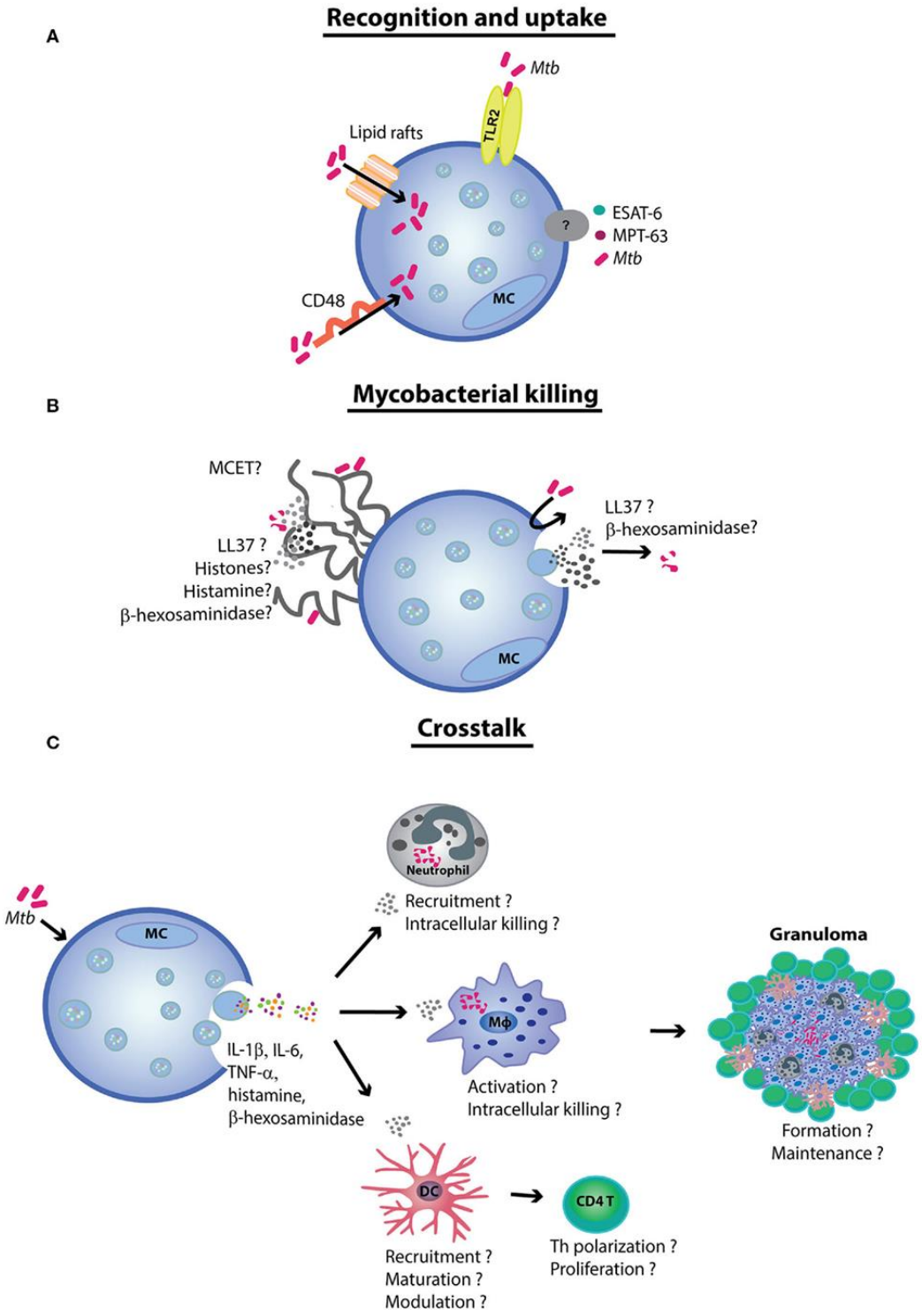

Figure 2.3. The role of mast cells (MCs) in tuberculosis. (A) MCs recognize Mycobacterium tuberculosis (*Mtb*) via the TLR2 and CD48 receptors. The latter also contributes to *Mtb* uptake. Although the uptake process remains yet unclear, mycobacteria have been demonstrated to be internalized by lipid rafts. (B) *Mtb* and the mycobacterial antigens early secretory antigenic target 6 (ESAT-6) and MPT-63 induce MC degranulation and cytokine release. It is likely that *Mtb* exposure induces antimicrobial peptide secretion and mast cell extracellular trap (MCET) formation. MCETs possibly contain antimycobacterial mediators, such as β-hexosaminidase and LL-37. (C) Finally, the MCs crosstalk with other immune cells [e.g., neutrophils, dendritic cells (DCs), and macrophages (Mφ)] contribute to antimycobacterial immunity. Although further experimental evidence is needed to prove the hypothesis, MCs seem to play a role in orchestrating tuberculosis granuloma formation and maintenance.

MC ontogeny and functions

Mast cells originate from pluripotent CD34+ and CD117+ hematopoietic stem cells and migrate as progenitors to various tissues where they mature influenced by the local microenvironment (15, 16). MCs are mainly located in the skin (~12,000/mm3) and mucosa including the lungs (~500–4,000/mm3) (10).

Mast cells express various PRRs (e.g., TLRs and CD48), complement receptors, and Fc receptors which upon engagement induce cell activation, degranulation, or both (17). Thereby, MCs release a great variety of pro- and anti-inflammatory pre-stored and de novo synthesized mediators such as histamine, heparin, tryptase, chymase, PGD2, LTC4/D4, chemokines as CCL1, CCL2, CCL4, CCL7, CCL12, CCL17, CXCL5, and CXCL8, and cytokines including IL-4, IL-3, IL-5, GM-CSF, IL-6, IL-13, IL-12, IFN-γ, TGFβ1, and TNF-α (18). This wide variety of products and their rapid release (in min/s) make MCs important modulators of inflammatory responses.

MC involvement in early mycobacterial infection

Although interactions between MCs and *Mtb* have been reported (**Figure 2.3**), the role of MCs in TB pathogenesis remains unclear. MC involvement in mycobacterial immunity was first observed in guinea pigs using electron microscopy. Shortly, after intratracheal infection with *Mtb*, a significant increase in MCs was detected in guinea pig lungs (19). A later study demonstrated that the number of MCs in mice lungs increases by ~23% after 15 days of *Mtb* exposure (20).

Bacillary recognition

Rat peritoneal MCs (rPMCs) recognize *Mtb* via CD48 that is a glycosyl phosphoinositol-anchored cell surface protein (**Figure 2.3A**). Incubation with increasing concentrations of anti-CD48 antibodies together with *Mtb* correlates with a proportional decrease in histamine release (21). Previous work has shown that CD48 recognizes FimH protein expressed by fimbriated bacteria (such as *Escherichia coli* and *Staphylococcus aureus*) resulting in MC degranulation (12, 22, 23), raising the question of how precisely CD48 recognizes *Mtb*, which is not known to be fimbriated (24).

TLRs are a key receptor family implicated in pathogen recognition (22). Carlos and colleagues found MC TLR2 to be relevant in *Mtb* recognition (**Figure 2.3A**) (9). The transfer of TLR2+/+ MCs into TLR−/− *Mtb*-infected mice showed an increase of cytokine release and cell recruitment, suggesting MC TLR2 as a key receptor upon mycobacterial challenge (9). MCs also express TLR4 that serves as a mannose receptor in complex with soluble CD14. In the absence of CD14, high concentrations of TLR4 ligands are required for MC–TLR4 activation (25–27). Presently, it is unclear if TLR4 (or other TLRs) are involved in *Mtb* recognition and if the TLR4–CD14 complex is necessary to trigger MC functions. Besides the TLRs and CD48, the CR3, CR4, C3aR, and C5aR complement receptors and the FcεRI, FcγRI, FcγRII, and FcγRIII receptors mediate MC responses to other bacteria, but it is as yet unknown if they are involved in MC–*Mtb* interactions (12, 22, 28).

Bacillary binding and uptake

Muñoz and colleagues suggested that *Mtb* is internalized by MCs via lipid rafts (**Figure 2.3A**). Cholesterol depletion of the rat basophilic leukaemia cells (RBL-2H3) reduced internalization of mycobacteria. Interestingly, once internalized, mycobacteria survived intracellularly for 4 days after which most of the infected MCs (70%) had undergone lysis. These findings implicate MCs as a reservoir for *Mtb* (29). However, still unclear are the requirements and dynamics of *Mtb* internalization by MCs since few studies have demonstrated it experimentally and all were performed in animal models (21, 29). It is therefore important that the *Mtb* internalization is demonstrated in human MCs, together with the underlying mechanisms of internalization (aside from lipid rafts) and the intracellular compartments in which *Mtb* may reside. Published data describing MC interactions with other bacteria may guide the design of these investigations. For example, *Salmonella typhi* is taken up by complement receptors while *E. coli* is attached and internalized by FimH to the MC surface (30). Furthermore, preincubation of human MCs with IFN-γ increases the

membrane attachment of *S. aureus* (31). Recently, the MC phagosome was demonstrated to interact with NOD-like receptors thus modulating cytokine production (32), and indicating that MC phagocytosis acts as an inducer of other MC activities.

Bacterial-Induced Mediator Release

Few studies have characterized the *Mtb*-induced MC production of soluble mediators of inflammation. Muñoz et al. reported that after stimulation with *Mtb*, rPMCs released de novo synthesized TNF-α and IL-6, followed by secretion of histamine and β-hexosaminidase. Besides, specific *Mtb* antigens [MPT-63 and early secretory antigenic target 6 (ESAT-6)] have also been shown to induce rPMCs to release TNF-α, IL-6, histamine, and β-hexosaminidase (**Figure 2.3**) (21).

Cytokines and Chemokines

Cytokines and chemokines released by MCs contribute to protective immunity in the context of bacterial infections. For example, it has been shown that MC depletion reduces TNF-α (important in mycobacterial granuloma maintenance) concentrations in bronchoalveolar lavage (BAL) of *Bordetella pertussis*-infected mice (33). Furthermore, following infection with *Streptococcus pneumoniae*, increased TNF-α concentrations in the BAL and MC numbers in the lung correlated with protection (34). Similarly, MC-derived IL-6 produced during *Klebsiella pneumoniae* challenge was observed to improve mouse survival by promoting neutrophil recruitment and intra-neutrophil killing (35), the importance of which in mycobacterial immunity is being increasingly recognized (36). MCs also produce a wide variety of soluble mediators potentially relevant to mycobacterial immunity, including IL-13, IL-12, IL-6, IL-4, TNF-α, CCL5, CXCL2, CCL7, and CCL2, following infection with *Streptococcus equi* (37). Since the cocktail of soluble mediators produced by MCs appears dependent on the specific pathogen, a comprehensive proteomic assessment of mediators produced by MCs in response to *Mtb* is needed.

Antimicrobial Peptides (AMPs)

Antimicrobial peptides kill pathogens by forming pores in cytoplasmic membranes; defensins and cathelicidins are the most studied (38). Early in infection, cathelicidins promote phagocytosis, upregulate the expression of costimulatory molecules in DCs and stimulate Th1 cytokine production, while later in the course of the disease they inhibit the production

of pro-inflammatory molecules (39). Although little is known regarding the AMP repertoire that MCs may release, MC supernatants reduce bacterial burden (40, 41). Cathelicidin LL-37 is expressed in human dermal skin MCs while the respective murine homolog cathelicidin-related AMP (CRAMP) is produced by bone marrow-derived MCs (BMMCs). Upregulation of CRAMP expression by LPS reduces group A streptococcus extracellular titters (42), while MC-derived LL-37 promotes clearance of *Enterococcus faecalis* (43). Finally, β-hexosaminidase, which is released by MCs after degranulation, was observed to exhibit antimicrobial activities upon intracellular *Listeria monocytogenes* infection (39) and mMCP-6, a mouse tryptase, was essential for *K. pneumoniae* clearance in mMCP-6−/− mice (44). Thus, antimycobacterial molecules are likely to be secreted by MCs upon *Mtb* exposure (**Figure 2.3B**).

MC Degranulation and Histamine Release

Mast cell degranulation in *Mtb*-infected mice is associated with a decrease in leukocytes, neutrophils, mononuclear cells, IL-1β, TNF-α, MIP-2, IL-12, IFN-γ, and MCP-1 (20). Histamine is released during MC degranulation. Carlos and colleagues used histamine-deficient C57BL/6 mice to investigate the role of histamine in *Mtb* infection, which is detectable in high concentrations 28 days after *Mtb* infection. Mice lacking histamine showed decreased neutrophils numbers, as well as TNF-α and IL-6 levels in lung tissue, while IL-12 and IFN-γ concentrations were increased. Furthermore, the histamine-deficient lungs showed lymphocytic infiltration with an increase in the number of CD4+ T cells that correlated with reduced bacterial growth (45). Taken together, these findings suggest that MC degranulation may have a complex role in modulating the inflammatory response to *Mtb*. It would be important to investigate the redundancy of these pathways in *Mtb* infection using in vivo models, as well as determine whether MCs are the source of histamine in this context, which may indicate novel therapeutic avenues.

MC Extracellular Traps

The formation of ETs, named ETosis, is a type of cell death characterized by the release of DNA (46). ETosis differs from apoptosis and necrosis since it lacks DNA fragmentation, disruption of the nuclear envelope, absence of phosphatidylserine in the outer membrane and caspase-independent activation (47). The formation of MCETs upon cell stimulation relies on ROS production by MCs that in turn promotes nuclear envelope disruption and

release of DNA together with granular components with antimicrobial properties. The DNA backbone in combination with histones, proteases, and AMPs (39, 48) forms physical traps that catch and expose pathogens to high concentrations of antimicrobial molecules (**Figure 2.3B**) (48, 49).

A human mast cell line (HMC-1), showed ET formation upon *L. monocytogenes* infection. This Gram-positive bacterium was shown to promote disruption of the nuclear envelope followed by an increase in ROS production. Interestingly, the presence of β-hexosaminidase in the traps was observed to have an antimicrobial activity to intracellular *L. monocytogenes* (39). *E. faecalis* was also found to induce MCET after 3 h of incubation with BMMCs. However, the level of MCET observed was lower compared with the one promoted by other bacteria (43). This was possibly due to the low multiplicity of infection (MOI) used in this study (MOI 1:1). *Streptococcus pyogenes* induced MCET after infection (MOI 25:1) in HMC-1 cells exhibiting cathelicidin LL-37, histones, and tryptase in the traps and in murine BMMCs displaying tryptase and histones (50). This suggests that a high bacterial burden promotes MCET activation. Interestingly, besides bacterial stimulation, IL-12 and IL-1β were found to induce MCET containing IL-17 after the stimulation of MCs from skin explants of patients with psoriasis (51).

Mycobacterium–MCET

Mycobacterium tuberculosis induces neutrophil ETs (NETs). However, Ramos-Kichik et al. have reported that although mycobacteria induce the formation of NETs, which include elastase and histones, the AMPs contained in the NETs are unable to kill mycobacteria (52). Three-hour incubation of human neutrophils with the virulent *Mtb* and the less virulent *Mycobacterium canettii* showed that both mycobacteria were entrapped in NETs; however, neither *Mtb* nor *M. canettii* were killed. In fact, mycobacteria were not eliminated even at low bacterial concentrations (MOI 0.1:1), nor did NETs restrict ongoing mycobacterial replication.

Virulent *Mtb* secretes ESAT-6 and CFP-10 (10-kDa culture filtrate protein) through the ESX-1 secretory system. Both factors are important for the pathogenic intracellular pore-forming activities and phagosomal subversion observed in the early phase of TB (53). Interestingly, ESAT-6 has been shown to induce extracellular NETs by Ca+ influx (54). In addition, *Mtb* can induce ETs in human macrophages (Mφ) via the ESX-1 system, which is enhanced by IFN-γ (55). By contrast, in highly infected Mφ, it has been reported that after IFN-γ initiates

necrosis without NETosis (56). This information suggests that virulent mycobacteria may actively promote NET formation to achieve their own ends of persistence, raising the hypothesis that MCETs may also be involved (**Figure 2.3B**). Although mycobacteria–MCETs have not been demonstrated, MCs produce a large repertoire of immunomodulatory mediators that are known to be contained in traps. Therefore, it is important that future studies investigate the mycobacteria induction of MCETs and the inclusion of antimycobacterial mediators.

MC Immune Crosstalk and the Mycobacterial Granuloma

Studies involving a broad array of bacterial pathogens have demonstrated the important role of MCs in promoting the recruitment, maturation, and bactericidal activity of Mφ, DCs, and neutrophils (**Table 2.1**). However, the potential roles of MCs in modulating the delicate orchestration of immune crosstalk in mycobacterial immunity have not yet been described (**Figure 2.3C**). MCs could easily coordinate granuloma formation and maintenance. In support of this notion, Taweevisit and Poumsuk reported a correlation between MC numbers and granuloma formation (57). Briefly, 45 lymph nodes from patients with TB lymphadenitis were analysed to determine the frequency of MCs in the granulomatous region. The authors observed that the number of MCs positively correlated with the number of granulomas. A similar correlation was found between multinucleated giant cells and MCs in the lymph nodes (57). Similar studies have been performed using skin biopsies of patients suffering from leprosy (*Mycobacterium leprae*) (58). Lepromatous leprosy (a disseminated disease with high bacillary load) was associated with the lowest dermal MC density compared with paucibacillary and localized tuberculous leprosy. This suggests that MC functions may have a role in driving a differential susceptibility to these polar forms of leprosy, and as yet poorly explained clinical phenomenon (59). Furthermore, the higher MC numbers located around granulomas in the tuberculous group were considered to be indirect evidence of the role of MCs in activating the immune response to *M. leprae* infection. Interestingly, numerous MCs were found in the highly fibrotic dermal area and in the epineural layer of lepromatous leprosy lesions, suggesting that MCs could be involved in the induction of fibrosis, including fibrotic leprosy neuritis (60).

Cell target	MC function	Mediator	MC type (mouse)	Bacteria/model	Technique	Reference	Open questions
Mφ	Inhibition of internalization and intracellular growth Trogocytosis, caspase I expression in Mφ and FceRI, and Ox40L upregulation in MCs	IL-4 FceRI-encompassed vesicles	BMMCs BMMCs	*Francisella tularensis* LVS *F. tularensis* LVS	*In vitro* coculture *In vitro* coculture	(41) (61)	Do MCs promote intracellular *Mtb* killing in Mφ?
Neutrophil	Recruitment and activation Recruitment Killing Recruitment Recruitment	TNF-α ? IL-6 ? MC TLR2	Peritoneal Intestinal BMMCs Skin BMMCs	*Listeria monocytogenes* *Clostridium difficile* toxin A *Klebsiella pneumoniae* *Pseudomonas aeruginosa* *Mycobacterium tuberculosis*	MC-depleted BALB/c mice $MC^{-/-}$ mice/reconstitution IL-6-deficient mice $MC^{-/-}$ mice/reconstitution $TLR2^{-/-}$ mice/reconstitution with $TLR^{+/+}$ MCs	(62) (63) (35) (64) (9)	Do MCs contribute to neutrophil recruitment? Do MCs promote intracellular Mtb killing in neutrophils? Is MC-derived TNF-α relevant in granuloma maintenance?

DC	Recruitment to the site of infection and migration to DLNs Maturation and Th polarization	TNF-α E-selectin IL-12 IFN-γ IL-6 TGF-β	BMMCs Peritoneal	Escherichia coli (urinary tract infection) LPS	$MC^{-/-}$ mice/reconstitution *In vitro* coculture	(65) (66)	Do MCs contribute to DC recruitment? Do MCs modulate DCs-induced Th1 polarization? Do MCs enhance DC functions upon *Mtb* challenge?
MC modulation upon bacterial stimulation, *LVS, live vaccine strain; MCs, mast cells; Mφ, macrophages; DCs, dendritic cells; BMMCs, bone marrow-derived MCs; DLNs, draining lymph nodes; LPS, lipopolysaccharide; FceRI, high-affinity receptor for IgE.*							

Table 2.1. MCs: immune cell crosstalk in antibacterial immunity.

MC-Derived Soluble Mediators and Mycobacterial Granuloma Maintenance

Mast cell-derived LL-37 and CRAMP are bactericidal for *Mtb* (67). Ramos-Espinosa and colleagues reported that the administration of adenovirus encoding the human cathelicidin LL-37 (AdLL37) and TNF-α (AdTNFα) had a protective role in inducing granuloma maintenance, and thus TB disease reactivation (68). These observations suggest that MCs and MC mediators in particular are involved in granuloma maintenance (**Figure 2.3C**). Similarly, Von Stebut and colleagues reported that upon encounter with pathogens MCs release pre-stored TNF-α that induces neutrophil recruitment to the site of granulomatous inflammation. This was followed by the release of neutrophil-derived MIP-1α/β and MIP-2 chemokines both responsible for Mφ recruitment. Lack of this immediate pre-stored TNF-α release delayed Mφ recruitment and granuloma formation (69).

The study by Carlos et al. discussed earlier also described that during *Mtb* infection, TLR2 engagement induces cytokine release in the lung; as observed in a reconstitution murine model after 60 days of infection (9). The transfer of TLR2+/+ MCs into TLR−/− *Mtb*-infected mice showed diminished lung bacterial growth and an increase of TNF, IL-6, IL-1β concentrations, and neutrophil and mononuclear cell recruitment resulting in the restoration of granuloma formation (9). Therefore, MCs may be involved not only in the early but also in the late phase of infection. Furthermore, MC-derived IL-6 and TNF-α in this phase of infection may contribute to granuloma maintenance (9). The precise contribution of MCs in mycobacterial granuloma maintenance remains an important open question, and *in-vivo Mtb* infection models combined with MC reconstitution experiments may yield critical insights into this area.

CONCLUSION

Tuberculosis is a highly contagious infectious disease caused by *Mtb* which infects billions and kills millions of people worldwide. Although many efforts have been made to reduce TB mortality, the infection remains one the most important threats to human health. MCs are key lung resident immune sentinels that contribute to antibacterial immunity and are likely to play a key role in TB pathogenesis. A potentially important and unique function of MCs is the crosstalk with other immune cells to orchestrate multiple effector functions, which may contribute to granuloma formation and maintenance. We have highlighted the potential roles

MCs may play during TB that once addressed could inform the design of novel therapeutic strategies.

REFERENCES: Chapter 2, section II

1. World Health Organization. Global Tuberculosis Report 2016. Geneva: World Health Organization (2016).
2. Scriba TJ, Coussens AK, Fletcher HA. Human immunology of tuberculosis. Microbiol Spectr (2016) 4(5):1–24. doi:10.1128/microbiolspec.TBTB2-0016-2016
3. Stamm CE, Collins AC, Shiloh MU. Sensing of Mycobacterium tuberculosis and consequences to both host and bacillus. Immunol Rev (2015) 264(1):204–19. doi:10.1111/imr.12263
4. Nunes-Alves C, Booty MG, Carpenter SM, Jayaraman P, Rothchild AC, Behar SM. In search of a new paradigm for protective immunity to TB. Nat Rev Microbiol (2014) 12(4):289–99. doi:10.1038/nrmicro3230
5. Sia JK, Georgieva M, Rengarajan J. Innate immune defenses in human tuberculosis: an overview of the interactions between Mycobacterium tuberculosis and innate immune cells. J Immunol Res (2015):12. doi:10.1155/2015/747543
6. Sasindran SJ, Torrelles JB. Mycobacterium tuberculosis infection and inflammation: what is beneficial for the host and for the bacterium? Front Microbiol (2011) 2:2. doi:10.3389/fmicb.2011.00002
7. Ramakrishnan L. Revisiting the role of the granuloma in tuberculosis. Nat Rev Immunol (2012) 12(5):352–66. doi:10.1038/nri3211
8. Dorhoi A, Kaufmann SH. Versatile myeloid cell subsets contribute to tuberculosis-associated inflammation. Eur J Immunol (2015) 45(8):2191–202. doi:10.1002/eji.201545493
9. Carlos D, Frantz FG, Souza-Júnior DA, Jamur MC, Oliver C, Ramos SG, et al. TLR2-dependent mast cell activation contributes to the control of Mycobacterium tuberculosis infection. Microbes Infect (2009) 11(8):770–8. doi:10.1016/j.micinf.2009.04.025
10. Kunder CA, St John AL, Abraham SN. Mast cell modulation of the vascular and lymphatic endothelium. Blood (2011) 118(20):5383–93. doi:10.1182/blood-2011-07-358432
11. Abraham SN, John ALS. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol (2010) 10(6):440–52. doi:10.1038/nri2782
12. Trivedi NH, Guentzel MN, Rodriguez AR, Yu J-J, Forsthuber TG, Arulanandam BP. Mast cells: multitalented facilitators of protection against bacterial pathogens. Expert Rev Clin Immunol (2013) 9(2):129–38. doi:10.1586/eci.12.95
13. Johnzon C-F, Rönnberg E, Pejler G. The role of mast cells in bacterial infection. Am J Pathol (2016) 186(1):4–14. doi:10.1016/j.ajpath.2015.06.024
14. Urb M, Sheppard DC. The role of mast cells in the defence against pathogens. PLoS Pathog (2012) 8(4):e1002619. doi:10.1371/journal.ppat.1002619
15. Virk H, Arthur G, Bradding P. Mast cells and their activation in lung disease. Transl Res (2016) 174:60–76. doi:10.1016/j.trsl.2016.01.005
16. Bradding P. Human mast cell cytokines. Clin Exp Allergy (1996) 26(1):13–9. doi:10.1111/j.1365-2222.1996.tb00051.x
17. John ALS, Abraham SN. Innate immunity and its regulation by mast cells. J Immunol (2013) 190(9):4458–63. doi:10.4049/jimmunol.1203420
18. Moiseeva EP, Bradding P. Mast cells in lung inflammation. In: Gilfillan AM, Metcalfe DD, editors. Mast Cell Biology: Contemporary and Emerging Topics. Boston, MA: Springer (2011). p. 235–69.

19. Ratnam S, Ratnam S, Puri B, Chandrasekhar S. Mast cell response during the early phase of tuberculosis: an electron-microscopic study. Can J Microbiol (1977) 23(9):1245–51. doi:10.1139/m77-186
20. Carlos D, de Souza Júnior DA, de Paula L, Jamur MC, Oliver C, Ramos SG, et al. Mast cells modulate pulmonary acute inflammation and host defense in a murine model. J Infect Dis (2007) 196(9):1361–8. doi:10.1086/521830
21. Muñoz S, Hernández-Pando R, Abraham SN, Enciso JA. Mast cell activation by Mycobacterium tuberculosis: mediator release and role of CD48. J Immunol (2003) 170(11):5590–6. doi:10.4049/jimmunol.170.11.5590
22. Marshall JS. Mast-cell responses to pathogens. Nat Rev Immunol (2004) 4(10):787–99. doi:10.1038/nri1460
23. Malaviya R, Gao Z, Thankavel K, van der Merwe PA, Abraham SN. The mast cell tumor necrosis factor α response to FimH-expressing Escherichia coli is mediated by the glycosylphosphatidylinositol-anchored molecule CD48. Proc Natl Acad Sci U S A (1999) 96(14):8110–5. doi:10.1073/pnas.96.14.8110
24. Ramsugit S, Pillay M. Identification of Mycobacterium tuberculosis adherence-mediating components: a review of key methods to confirm adhesin function. Iran J Basic Med Sci (2016) 19(6):579–84. doi:10.22038/IJBMS.2016.7124
25. Marshall JS, Jawdat DM. Mast cells in innate immunity. J Allergy Clin Immunol (2004) 114(1):21–7. doi:10.1016/j.jaci.2004.04.045
26. McCurdy JD, Olynych TJ, Maher LH, Marshall JS. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. J Immunol (2003) 170(4):1625–9. doi:10.4049/jimmunol.170.4.1625
27. Varadaradjalou S, Féger F, Thieblemont N, Hamouda NB, Pleau JM, Dy M, et al. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. Eur J Immunol (2003) 33(4):899–906. doi:10.1002/eji.200323830
28. Gri G, Frossi B, D'Inca F, Danelli L, Betto E, Mion F, et al. Mast cell: an emerging partner in immune interaction. Front Immunol (2012) 3:120. doi:10.3389/fimmu.2012.00120
29. Munoz S, Rivas-Santiago B, Enciso J. Mycobacterium tuberculosis entry into mast cells through cholesterol-rich membrane microdomains. Scand J Immunol (2009) 70(3):256–63. doi:10.1111/j.1365-3083.2009.02295.x
30. Malaviya R, Abraham SN. Mast cell modulation of immune responses to bacteria. Immunol Rev (2001) 179(1):16–24. doi:10.1034/j.1600-065X.2001.790102.x
31. Swindle EJ, Brown JM, Rådinger M, DeLeo FR, Metcalfe DD. Interferon-γ enhances both the anti-bacterial and the pro-inflammatory response of human mast cells to Staphylococcus aureus. Immunology (2015) 146(3):470–85. doi:10.1111/imm.12524
32. Zabucchi G, Trevisan E, Vita F, Soranzo MR, Borelli V. NOD1 and NOD2 interact with the phagosome cargo in mast cells: a detailed morphological evidence. Inflammation (2015) 38(3):1113–25. doi:10.1007/s10753-014-0077-x
33. Mielcarek N, Hörnquist EH, Johansson BR, Locht C, Abraham SN, Holmgren J. Interaction of Bordetella pertussis with mast cells, modulation of cytokine secretion by pertussis toxin. Cell Microbiol (2001) 3(3):181–8. doi:10.1046/j.1462-5822.2001.00106.x
34. Kerr AR, Irvine JJ, Gingles NA, Kadioglu A, Andrew PW, McPheat WL, et al. Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection. Infect Immun (2002) 70(3):1547–57. doi:10.1128/IAI.70.3.1547-1557.2002
35. Sutherland RE, Olsen JS, McKinstry A, Villalta SA, Wolters PJ. Mast cell IL-6 improves survival from Klebsiella pneumonia and sepsis by enhancing neutrophil killing. J Immunol (2008) 181(8):5598–605. doi:10.4049/jimmunol.181.8.5598
36. Lowe DM, Redford PS, Wilkinson RJ, O'Garra A, Martineau AR. Neutrophils in tuberculosis: friend or foe? Trends Immunol (2012) 33(1):14–25. doi:10.1016/j.it.2011.10.003

37. Rönnberg E, Guss B, Pejler G. Infection of mast cells with live streptococci causes a toll-like receptor 2-and cell-cell contact-dependent cytokine and chemokine response. Infect Immun (2010) 78(2):854–64. doi:10.1128/IAI.01004-09
38. Harder J, Bartels J, Christophers E, Schroder J. A peptide antibiotic from human skin. Nature (1997) 387(6636):861. doi:10.1038/43088
39. Campillo-Navarro M, Leyva-Paredes K, Donis-Maturano L, González-Jiménez M, Paredes-Vivas Y, Cerbulo-Vázquez A, et al. Listeria monocytogenes induces mast cell extracellular traps. Immunobiology (2017) 222(2):432–9. doi:10.1016/j.imbio.2016.08.006
40. Wei OL, Hilliard A, Kalman D, Sherman M. Mast cells limit systemic bacterial dissemination but not colitis in response to Citrobacter rodentium. Infect Immun (2005) 73(4):1978–85. doi:10.1128/IAI.73.4.1978-1985.2005
41. Ketavarapu JM, Rodriguez AR, Yu J-J, Cong Y, Murthy AK, Forsthuber TG, et al. Mast cells inhibit intramacrophage Francisella tularensis replication via contact and secreted products including IL-4. Proc Natl Acad Sci U S A (2008) 105(27):9313–8. doi:10.1073/pnas.0707636105
42. Di Nardo A, Vitiello A, Gallo RL. Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide. J Immunol (2003) 170(5):2274–8. doi:10.4049/jimmunol.170.5.2274
43. Scheb-Wetzel M, Rohde M, Bravo A, Goldmann O. New insights into the antimicrobial effect of mast cells against Enterococcus faecalis. Infect Immun (2014) 82(11):4496–507. doi:10.1128/IAI.02114-14
44. Thakurdas SM, Melicoff E, Sansores-Garcia L, Moreira DC, Petrova Y, Stevens RL, et al. The mast cell-restricted tryptase mMCP-6 has a critical immunoprotective role in bacterial infections. J Biol Chem (2007) 282(29):20809–15. doi:10.1074/jbc.M611842200
45. Carlos D, Fremond C, Samarina A, Vasseur V, Maillet I, Ramos S, et al. Histamine plays an essential regulatory role in lung inflammation and protective immunity in the acute phase of Mycobacterium tuberculosis infection. Infect Immun (2009) 77(12):5359–68. doi:10.1128/IAI.01497-08
46. Wartha F, Henriques-Normark B. ETosis: a novel cell death pathway. Sci Signal (2008) 1(21):e25. doi:10.1126/stke.121pe25
47. Guimaraes-Costa AB, Nascimento MT, Wardini AB, Pinto-da-Silva LH, Saraiva EM. ETosis: a microbicidal mechanism beyond cell death. J Parasitol Res (2012):11. doi:10.1155/2012/929743
48. Möllerherm H, von Köckritz-Blickwede M, Branitzki-Heinemann K. Antimicrobial activity of mast cells: role and relevance of extracellular DNA traps. Front Immunol (2016) 7:265. doi:10.3389/fimmu.2016.00265
49. Goldmann O, Medina E. The expanding world of extracellular traps: not only neutrophils but much more. Front Immunol (2013) 3:420. doi:10.3389/fimmu.2012.00420
50. von Köckritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, et al. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. Blood (2008) 111(6):3070–80. doi:10.1182/blood-2007-07-104018
51. Lin AM, Rubin CJ, Khandpur R, Wang JY, Riblett M, Yalavarthi S, et al. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. J Immunol (2011) 187(1):490–500. doi:10.4049/jimmunol.1100123
52. Ramos-Kichik V, Mondragón-Flores R, Mondragón-Castelán M, Gonzalez-Pozos S, Muñiz-Hernandez S, Rojas-Espinosa O, et al. Neutrophil extracellular traps are induced by Mycobacterium tuberculosis. Tuberculosis (2009) 89(1):29–37. doi:10.1016/j.tube.2008.09.009
53. Yu X, Xie J. Roles and underlying mechanisms of ESAT-6 in the context of Mycobacterium tuberculosis–host interaction from a systems biology perspective. Cell Signal (2012) 24(9):1841–6. doi:10.1016/j.cellsig.2012.05.014
54. Francis R, Butler R, Stewart G. Mycobacterium tuberculosis ESAT-6 is a leukocidin causing Ca2+ influx, necrosis and neutrophil extracellular trap formation. Cell Death Dis (2014) 5(10):e1474. doi:10.1038/cddis.2014.394

55. Wong K-W, Jacobs WR. Mycobacterium tuberculosis exploits human interferon γ to stimulate macrophage extracellular trap formation and necrosis. J Infect Dis (2013) 208(1):109–19. doi:10.1093/infdis/jit097
56. Lee J, Kornfeld H. Interferon-γ regulates the death of M. tuberculosis-infected macrophages. J Cell Death (2010) 3:1–11. doi:10.4137/JCD.S2822
57. Taweevisit M, Poumsuk U. High mast cell density associated with granulomatous formation in tuberculous lymphadenitis. Southeast Asian J Trop Med Public Health (2007) 38(1):115–9.
58. de Oliveira Magalhães G, da Costa Valentim V, dos Santos Pereira MJ, da Costa Nery JA, Illarramendi X, Antunes SLG. A quantitative and morphometric study of tryptase-positive mast cells in cutaneous leprosy lesions. Acta Trop (2008) 105(1):62–6. doi:10.1016/j.actatropica.2007.10.001
59. Talhari C, Talhari S, Penna GO. Clinical aspects of leprosy. Clin Dermatol (2015) 33(1):26–37. doi:10.1016/j.clindermatol.2014.07.002
60. Montagna NA, de Oliveira ML, Mandarim-de-Lacerda CA, Chimelli L. Leprosy: contribution of mast cells to epineurial collagenization. Clin Neuropathol (2005) 24(6):284–90.
61. Rodriguez AR, Yu J-J, Navara C, Chambers JP, Guentzel MN, Arulanandam BP. Contribution of FcεRI-associated vesicles to mast cell-macrophage communication following Francisella tularensis infection. Innate Immun (2016) 22(7):567. doi:10.1177/1753425916663639
62. Gekara NO, Weiss S. Mast cells initiate early anti-Listeria host defences. Cell Microbiol (2008) 10(1):225–36. doi:10.1111/j.1462-5822.2007.01033.x
63. Wershil BK, Castagliuolo I, Pothoulakis C. Direct evidence of mast cell involvement in Clostridium difficile toxin A-induced enteritis in mice. Gastroenterology (1998) 114(5):956–64. doi:10.1016/S0016-5085(98)70315-4
64. Siebenhaar F, Syska W, Weller K, Magerl M, Zuberbier T, Metz M, et al. Control of Pseudomonas aeruginosa skin infections in mice is mast cell-dependent. Am J Pathol (2007) 170(6):1910–6. doi:10.2353/ajpath.2007.060770
65. Shelburne CP, Nakano H, John ALS, Chan C, McLachlan JB, Gunn MD, et al. Mast cells augment adaptive immunity by orchestrating dendritic cell trafficking through infected tissues. Cell Host Microbe (2009) 6(4):331–42. doi:10.1016/j.chom.2009.09.004
66. Dudeck A, Suender CA, Kostka SL, von Stebut E, Maurer M. Mast cells promote Th1 and Th17 responses by modulating dendritic cell maturation and function. Eur J Immunol (2011) 41(7):1883–93. doi:10.1002/eji.201040994
67. Rivas-Santiago B, Santiago CER, Castañeda-Delgado JE, León-Contreras JC, Hancock RE, Hernandez-Pando R. Activity of LL-37, CRAMP and antimicrobial peptide-derived compounds E2, E6 and CP26 against Mycobacterium tuberculosis. Int J Antimicrob Agents (2013) 41(2):143–8. doi:10.1016/j.ijantimicag.2012.09.015
68. Ramos-Espinosa O, Hernández-Bazán S, Francisco-Cruz A, Mata-Espinosa D, Barrios-Payán J, Marquina-Castillo B, et al. Gene therapy based in antimicrobial peptides and proinflammatory cytokine prevents reactivation of experimental latent tuberculosis. Pathog Dis (2016) 74(7):ftw075. doi:10.1093/femspd/ftw075
69. von Stebut E, Metz M, Milon G, Knop J, Maurer M. Early macrophage influx to sites of cutaneous granuloma formation is dependent on MIP-1α/β released from neutrophils recruited by mast cell-derived TNFα. Blood (2003) 101(1):210–5. doi:10.1182/blood-2002-03-0921

AIMS

The overall aim of the present thesis is to investigate the role of MCs in TB infection using an *in vitro* model of primary MCs. The particular aims of this PhD thesis are to:

- Generate human primary mast cells derived from haematopoietic blood progenitors and evaluate their purity and maturity to be used for further *in vitro* experiments (mast cell model)

- Characterize MC functional activities that include degranulation, cytokine and chemokine release, MCET formation and ROS production upon *Listeria monocytogenes*, *Escherichia coli*, *Streptococcus aureus* and *Staphylococcus pneumoniae* stimulation (paper 2).

- Investigate location, numbers and predominate phenotype of MC in TB-infected lungs and cytokine expression from MCs located at granulomas and fibrotic sites (Paper 3).

- Examine MC responses upon BCG infection by analysing degranulation, bacterial uptake, intracellular growth and cytokine release. Here we also aimed to identify the effect of IL-33 priming in hMCs to react to BCG (Paper 4).

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CHAPTER 3: Mast cell model

In vitro generation of human mast cells

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ABSTRACT

MCs are resident immune cells involved in homeostatic and pathological conditions and their contribution to immune defence against pathogens has become evident. Different models are used to study MC activities including cell lines, primary cells, *ex vivo* cells isolated from tissue and *in vivo* models. However, cell lines including HMC-1, LUVA and LAD2 lack for essential receptors expressed in resident cells and *ex vivo* cells are limited to low yields. Furthermore, *in vivo* models are not fully representative for human MC behaviours. To study the role of MCs in bacterial infections, the present study used human primary MCs differentiated from peripheral blood progenitors and cultured for 8 weeks in the presence of IL-3, SCF and IL-6. MC purity was evaluated by expression of the MC markers CD117 and FcεRI and maturity was measured by inducing MC degranulation and chemokines release upon IgE activation. Our generated hMCs express FcεRI and CD117 in >90% of the total cell population. Furthermore, FcεRI-IgE crosslinking induced degranulation with >80% $CD63^+$ and $CD107a^+$ cells and correlating with hMC β-hexosaminidase release. Finally, IgE-activated MCs secreted IL-8, MCP-1 and GM-CSF chemokines. This data suggests that *in vitro* human primary MCs used for this study actively degranulate and secrete mediators mimicking MC functions that occur in human resident MCs.

INTRODUCTION

Mast cells (MCs) are involved in different homeostatic and pathological conditions including inflammatory processes, skin barrier homeostasis, wound-healing, tumour development and pathogenic defence [1] and are present in almost all organs of the body and located in mucosal and epithelial tissue, near blood and lymphatic vessels and nerves [1-9]. They are classified depending on their protease content and localization [10]. Human MCs (hMCs) are divided in tryptase positive (MC_T), chymase positive (MC_C) and tryptase and chymase (MC_{TC}) positive MCs [11], whereas mouse MCs are classified into convective tissue MCs (CTMC) and mucosal MCs (MMC) [12]. Human and rodent MCs express the surface receptors FcεRI and KIT, which allow their identification and tissue isolation [1]. Moreover, after IgE activation, mature MCs release a wide repertoire of mediators including histamine, β-hexosaminidase, tryptase PGD2 and MCP-1, IL-8 and GM-CSF, TNF, IL-6, IL-17 and IL-10 cytokines and chemokines [13]

MCs originate from MC progenitors (MCp), which are pluripotent $CD34^+CD13^+Fc\varepsilon RI^-CD117^+$ hematopoietic stem cells more abundant in bone marrow but also located in, spleen, intestine, lungs, foetal liver, yolk sac, white adipose tissue (WAT) and peripheral blood [1-9, 14]. MCp migrates and differentiates in peripheral tissue to become mature MCs ($Fc\varepsilon RI^+CD117^+$) [15]. Stem cell factor (SCF) is essential for MCs recruitment, differentiation, survival and maturation [15, 16]. However, different cytokines from the local tissue environment including IL-3, IL-4, IL-6, IL-9 and IL-10 influence MC differentiation [15, 16]. For instance, IL-9 in combination with SCF induce MMC differentiation in mouse bone-marrow derived MCs (BMMC) [16] whereas IL-4 induce the differentiation of mouse CTMCs [16, 17]. Moreover, MC maturation is regulated by local cytokines, as observed by the growth suppression of human BMMC induced by IFN-γ [18]. Besides, MC maturation was shown to be dependent on tissue localization as observed with IL-4 in combination with SCF that enhance intestine but not lung hMC proliferation [16]. This specificity on MC maturation influenced by the local environment represents a challenge in generating MCs for research purposes [19].

Different approaches are currently used to investigate MC functions including *ex vivo* MC models, MC lines, differentiated MCs and *in vivo* models. To date, research still lacks a mouse model with a complete absence of MC populations [20, 21]. The most commonly used *in vivo* models are $WBB6F_1$-$Kit^{W/W\text{-}v}$ and C57BL/6-$Kit^{W\text{-}sh/W\text{-}sh}$ mice, that have mutations

affecting KIT resulting in a MC deficiency [21]. However, these models carry abnormalities related to the KIT mutation, for instance, WBB6F$_1$-*Kit*$^{W/W\text{-}v}$ mice have lower numbers of neutrophils and basophils and are usually anaemic compared to WT mice [20, 21]. The adoptive transfer of primary MCs in these mice represent another *in vivo* approach called MC knock-in mice, which serve to compare WT functions after restoring MCs [22]. Nevertheless, MC restoration depends on the MC inoculation site and obtained data has to be taken carefully. MC-protease depletion is also used as a MC-deficient *in vivo* model including the Mcpt5-Cre-iDTR, however, MC depletion is not homogenous in all tissues as observed when comparing skin and peritoneal MC numbers in Mcpt5-Cre-iDTR mice [21, 23]. Accordingly, different MC-deficient mice with KIT -dependent and -independent mutations are available and their individual limitations need to be considered when studying MCs [24].

In vitro MC techniques including *ex vivo* MCs, MC lines, and differentiated MCs, are more insightful than *in vivo* models to study unique aspects of MC activation upon specific stimuli [25]. *Ex vivo* MCs, which are isolated from human or rodent tissues, are relevant to investigate the MC biology of skin, lung and intestine tissue-resident cells [25]. However, upon isolation, the cellular yield is poor that is insufficient for experimental procedures [25-27]. Conversely, MC lines and differentiated MCs are more convenient to generate larger MC numbers that can be cultured for long periods. Human cell lines such the HMC-1, LAD1/2 and very recently LUVA, ROSA and MCPV-1 are available [28, 29], whereas, in rodents, the mouse line P815, MC-9 and FMA-3 and a rat basophilic leukaemia line (RBL) [30] are commonly used. However, cellular lines present limitations [28], for instance, HMC-1 and MCPV-1 lack the expression of FcεRI [31], whereas LUVA and ROSA cells loose expression of FcεRI after long-term periods thus being unstable for FceRI-mediated activation [29, 32]. Moreover, although LUVA cells express the KIT receptor they are SCF independent [33] therefore not reflecting the biology of primary MCs. Finally, LAD2 cells are considered immature as shown by the lower level of protease content (chymase and tryptase) compared to human skin MCs [34]. Therefore, mature MCs differentiated from MCp are a better experimental tool for mimicking MC activities taking place in the tissue [28].

Although mouse MCs have provided key insights for understanding the biology of human MCs [4], physiological differences in location and activity are remarkable between these species, especially in the lung [35], which is an organ with more interest for the current

project. Anatomically, human lungs have 2 left and 3 right lobes whereas mouse lungs are composed of 1 left and 4 right lobes [36]. In mice lungs, MCs are mainly located the trachea and larger airways and absent in the central parenchyma and the alveoli, whereas in human lungs, MCs are present in the parenchyma and throughout the airways [37, 38]. In response to IgE-anti-IgE stimulation, hMCs secrete histamine inducing a direct contraction in the smooth muscle whereas mouse MCs, instead, secrete 5-hydroxythryptamine (5-HT), which promotes acetylcholine release to induce smooth muscle contraction after muscarinic receptor engagement [37]. Furthermore, proteases content from human and mouse MCs differ between species. While hMCs secrete tryptases (α, β and γ) and a chymase protease, murine MCs exhibit a variety of tryptases and chymases (MCP1-MCP14) [39], which are specific for MCs phenotypes. For instance, MMCs contain MMCP-1 and MMCP-2 proteases while CTMCs express MMCP-4, MMCP-5 and MMCP-6 proteases [12]. In addition, cytokine content also differs between human and murine cells [39]. While mouse MCs produce great amounts of TNF, which is very relevant to induce inflammation during bacterial infections including tuberculosis [40], this cytokine is less released from hMCs [39]. Similarly, murine MCs respond greater to lipopolysaccharide (LPS) as they express CD14, which is a co-receptor for toll-like receptors (TLRs) that is rarely expressed in hMCs. In fact, hMCs usually require soluble CD14 to respond upon LPS activation [39]. Therefore, a human model using primary MCs may provide a more accurate tool to understand MC functions in humans after a bacterial encounter.

The present investigation used an *in vitro* approach to differentiate human MCs derived from blood hematopoietic progenitors. Stem cell progenitors (CD117+) were isolated from peripheral blood mononuclear cells (PBMCs) and differentiated for 8-10 weeks in the presence of SCF, IL-6 and IL-3. The purity and maturity of *In vitro* generated MCs was tested by evaluating FcεRI and KIT receptors expression that was found to be >90% in the total population. Furthermore, the generated hMCs were tested for their functionality. FcεRI crosslinking with IgE showed a high level of responsiveness with degranulation and cytokine and chemokine secretion. Thus, the generated hMCs used in the current study revealed to be a relevant model to study hMC activities.

METHODOLOGY

Human primary mast cell generation

hMCs were generated as previously described [41]. Briefly, $CD117^{+}$ haematopoietic progenitors were isolated from blood mononuclear cells using immunomagnetic cell sorting (Miltenyl Biotec, Bergisch Gladbach, Germany). hMC precursors were cultured for 4 weeks at 37C 5% CO_2 in Iscove Modified Dulbecco´s medium with GlutaMAX-I supplemented with penicillin (100 U/ml) (Invitrogen), streptomycin (100 µg/ml) (Invitrogen), human IL-3 (10 ng/ml) (Peprotech), human IL-6 (50 ng/ml) (Peprotech), human stem cell factor (SCF) (100 ng/ml) (Peprotech), and β2-mercaptoethanol (50µmol/L). Cells were transferred progressively to an Iscove's modified Dulbecco's medium containing GlutaMAX-I (Life Technologies) and supplemented with 2β-mercaptoethanol (50 µM) (Sigma-Aldrich), Insulin-Transferrin-Selenium (1%) (Life Technologies), penicillin (100 U/ml) (Invitrogen), streptomycin (100 µg/ml) (Invitrogen), human IL-6 (50 ng/ml) (Peprotech) and human SCF (100 ng/ml) (Peprotech) and cultured for 4 weeks.

Flow cytometry

In vitro cultured hMCs were analysed for purity and maturity using flow cytometry. To evaluate the purity, cells at a concentration of $5x10^5$ cells/ml were incubated for 30 minutes with anti-CD117 (BioLegend, clone A3C6E2) and anti-FcεRI (BioLegend, clone AER-37) antibodies conjugated with PerCp/Cy5.5 and FITC fluorochromes respectively. Maturity was measured by evaluating cell degranulation., Eight-weeks cultured MCs were pre-sensitized with IgE antibody overnight and stimulated using anti-IgE antibody for 1h. After stimulation cells were incubated with anti-CD63 (BioLegend, clone H5C6) and anti-CD107a (BioLegend, clone, H4A3) anti-human antibodies conjugated with PacificBlue™ and PE/Cy7 fluorochromes. After immunostaining cells were washed, stained with DAPI to discriminate live cells, fixed with 4% paraformaldehyde and analysed using a BD LSR-II flow cytometer. Fluorescence minus one (FMO) was used as gating control and compensation controls were prepared using compensation beads (OneComp eBeads, Thermofisher Scientific). FlowJo (Treestar version 10.4.2) was used to analysed acquired data.

Cytokine and chemokine release

Generated MCs ($5x10^5$ cells/ml) were pre-sensitized with IgE antibody and incubated with anti-IgE antibody. After 8h incubation, supernatants were collected and analysed to investigate IL-8, MCP-1 and GM-CSF release using a multiplex capture bead array (CBA) (BD biosciences) assay following the manufacturer's instructions. FACSVerse cytometer was used to acquire capture beads and data analysis was performed using FCAP Array v3.0 software (BD biosciences).

β-hexosaminidase assay

To induce degranulation via the IgE-FcεRI crosslinking, MCs at a concentration of $5x10^5$ cells/ml were pre-sensitized with human IgE antibody and stimulated for 1h with anti-IgE antibody. After incubation, cells and supernatants were separated by centrifugation. Cells were lysate with 50uL of Triton X-100 (1%) (Sigma-Aldrich) and both cell lysates and supernatants were incubated with p-nitrophenyl N-acetyl-beta-D-glucosamine (1nM) (Sigma-Aldrich) in citrate buffer (0.05M) for 2h at 37°C. After incubation, samples were treated with sodium carbonate (0.05M, pH 10.0) to stop the reaction and OD was analysed at 405 nm. Data were obtained by the percentage of total β-hexosaminidase release

Statistical analysis

Student's *t*-test was used to test significance using Graph Pad Prism 7.02 (GraphPad Software).

RESULTS

Human mast cell purity

hMCs were differentiated from haematopoietic $CD117^+$ progenitors and cultured for 8-10 weeks in the presence of SCF and IL-6. Cellular purity was evaluated using flow cytometry to measure the expression of CD117 and FcεRI markers of mature MCs [10]. Upon flow cytometry acquisition, live cells were selected and gated as shown in **Figure 3.1**. As observed in **Figure 3.2a**, compared to unstained control, 90% (SD±3.1) of the total cell population was $CD117^+$ whereas 94.3% was $Fc\varepsilon RI^+$ (SD±1) (**Figure 3.2b**). Thus, our differentiated MCs actively express SCF receptor, necessary for MC survival, and FcεRI, necessary to engage IgE [10].

Human mast cell maturity

hMC maturity was evaluated by cell activation inducing degranulation and mediator production. Cells were pre-sensitized with human IgE antibody and stimulated with anti-IgE antibody for 8h to induce cytokine and chemokine release and for 1h to induce degranulation measured by surface markers and β-hexosaminidase release. **Figure 3.3a** shows a representative dot plot of degranulated MCs expressing CD107a and CD63 degranulation markers, which are intracellular lysosomal associated membrane glycoproteins (LAMPs) expressed at the cellular membrane on degranulated cells [42, 43]. As observed in **Figure 3.3b**, 83.47% (SD±3) of activated hMCs were $CD63^+$ and 81.40% (SD±0.87) were $CD107a^+$ compared to unstimulated controls. Furthermore, FcεRI engagement in mature hMCs induced 54.5% (SD±2.3) of β-hexosaminidase release (**Figure 3.3c**). Thus, generated hMCs are functionally active.

Moreover, as shown in **Figure 3.3d**, secretion of IL-8 (12,914 pg/ml, SD±2,492), MCP-1 (8,623 pg/ml, SD±741) and GM-CSF (1,123 pg/ml, SD±397) was detected in hMC supernatants after 8h IgE stimulation, thus indicating the functionality of the *in vitro* differentiated hMCs

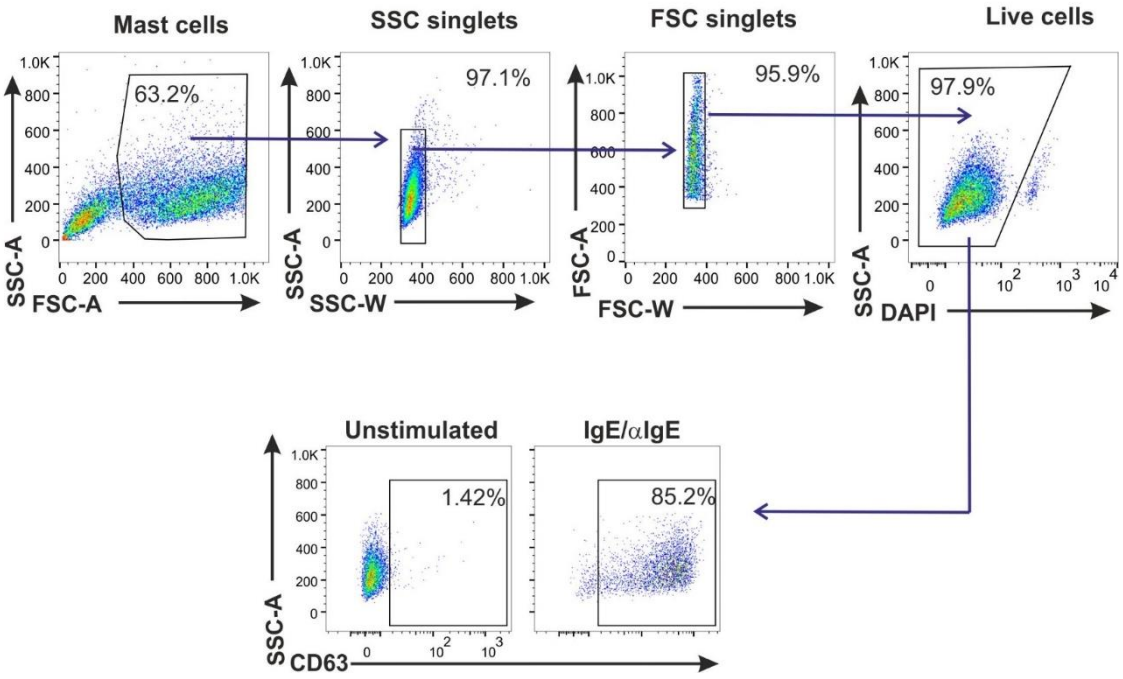

Figure 3.1. Gating strategy. Hematopoietic MC progenitors were isolated from PBMCs from different donors (n=3) and cells were differentiated in the presence of cytokines into mature MCs. After 8 weeks of differentiation and maturation hMCs were analyzed to revise purity and maturity before being used for further experiments. Mature hMCs were analyzed by flow cytometry and selected using side scatter (SSC-A) and forward scatter (FSC-A). Single cells were selected by SSC and FSC singlets and live cells were discriminated using DAPI. Live cells were used for further analysis including degranulation and cell surface markers. Lower panel shows a representative example of cellular gating on live cells. Dot plot is representative of 3 independent experiments.

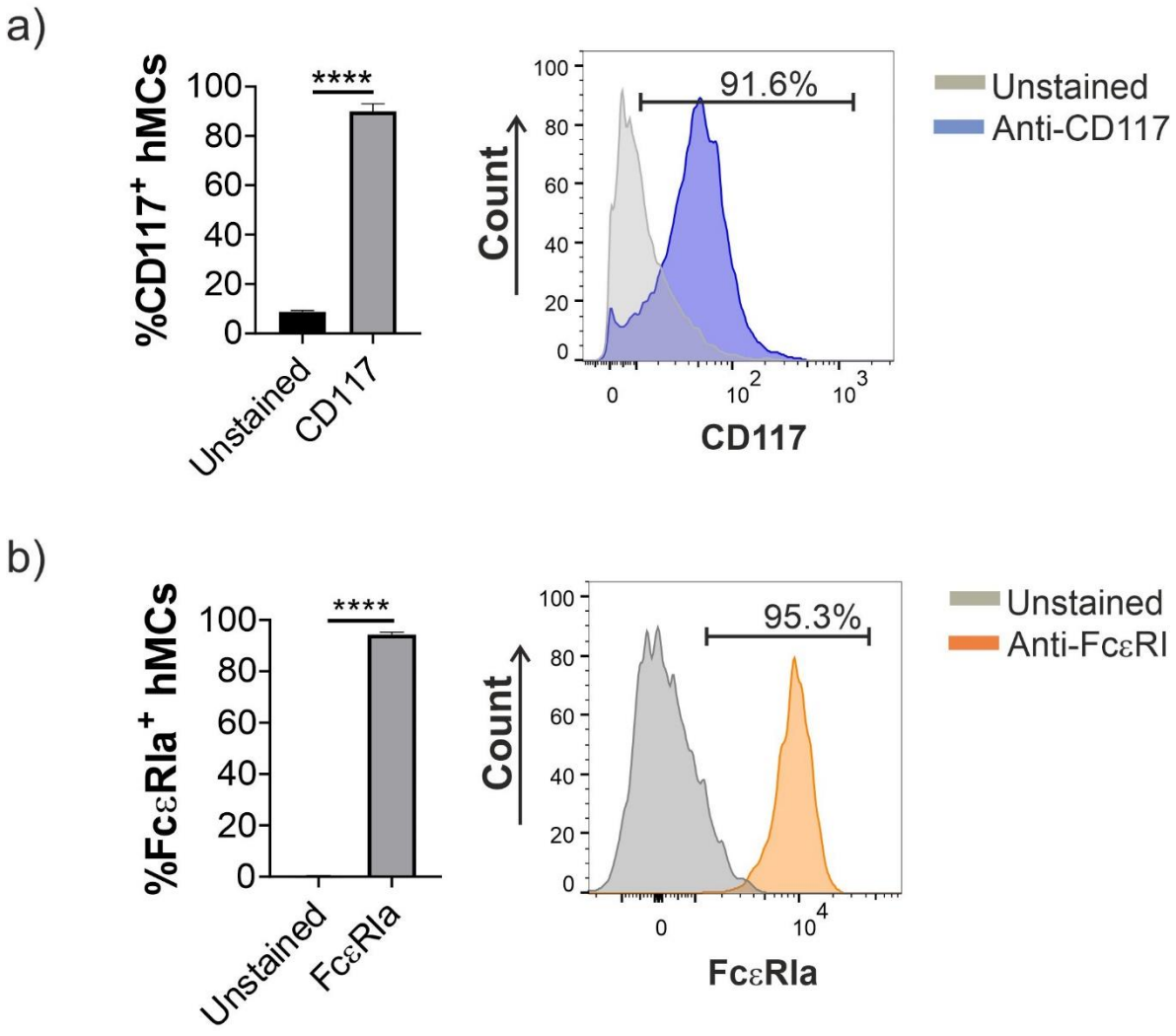

Figure 3.2. Human mast cell purity. After 8 weeks of cellular culture, hMCs were stained with anti-human anti-CD117 and anti-FceRIa antibodies conjugated with Percp-Cy7 and FITC fluorochromes respectively to investigate purity of cellular population by identifying surface markers expressed on mature MCs. Analysis was performed using flow cytometry and FMO (fluorescence minus one) was used as gating control. **(a)** shows CD117 (c-kit) and **(b)** shows FceRIa expression in pure naïve MCs. Graphs shows 3 staining replicates of a representative single donor and histograms shows a representative replicate of one donor. Statistical analysis was done using *t*-test *p*= ****<0.00001.

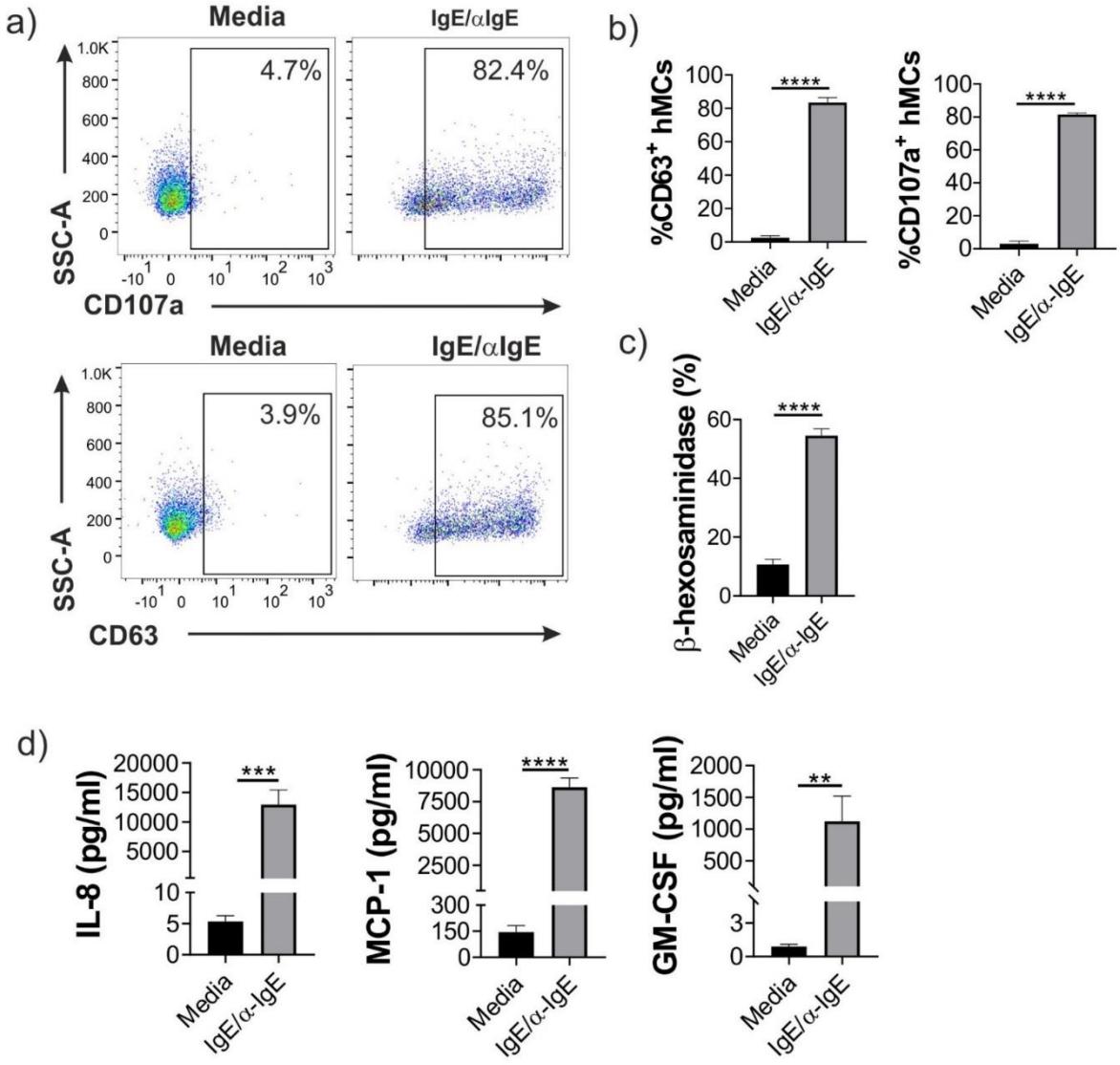

Figure 3.3. Human mast cell maturity. hMCs ($0.5x10^6$ cells/mL) were sensitised overnight with IgE antibody, washed (1µg/mL) and stimulated with anti-IgE antibody (1µg/mL) for either 1 or 8h to investigate hMC activation by means of degranulation and cytokine secretion. After 1h incubation cells were stained with anti-CD107a and anti-CD63 antibodies conjugated with fluorescent fluorochromes and analysed by flow cytometry. **(a)** a representative dot plot and **(b)** graphs from 3 replicates from a representative donor show hMC degranulation by percentage of $CD107a^+$ and $CD63^+$ hMCs. **(c)** cells and supernatants were used to investigate percentage of β-hexosaminidase release. **(d)** after 8 incubation, supernatants were collected and IL-8, MCP-1 and GM-CSF release was measured by cytometric bead array assay. Results are expressed as the mean values (±SD) of 3 replicates and are representative of 3 independent experiments using 3 different donors. Statistical analysis was done using student's *t* test (*p*= *<0.05, **<0.01, ***<0.0001).

DISCUSSION

In the present study, human primary MCs were differentiated from peripheral blood precursors and cultured for 8 weeks to obtain mature MCs as previously described [41]. Cellular purity was identified by the percentage of $CD117^+$ and $Fc\varepsilon RI^+$ cells showing a pure MC population with >90% of MCs actively expressing KIT and IgE receptors. Furthermore, produced MCs degranulated upon FcεRI engagement and released IL-8, GM-CSF and MCP-1 chemokines. Thus, the model used in the current study mimics functional MCs localized at human body sites.

Since hMCs are exclusively tissue residents, their isolation is limited to low yields and tissue access. Furthermore, their differentiation and maturation are conditioned by the specific organ/tissue microenvironment, which represents a challenge in the laboratory to mimic cellular differentiation happening in tissue [7]. However, current MCs models have provided accurate data to understand MC functions. Available MCs and MC models include human cell-lines, human primary MCs, ex *vivo* MCs and mouse *in vivo* models [15]. Each of these models presents individual characteristics to be considered when trying to approach specific scientific questions [28]. For instance, expression of pathogen-associated receptors including TLRs and CD14 are lower expressed in human compare to murine MCs [39]. Therefore, this can result in a greater activation after bacterial challenge using mouse models, which poorly represents MC activities in human infections [28, 44]. The current project selected an *in vitro* approach of differentiated hMCs to investigate MC functions after bacterial interactions.

Mature MCs are differentiated from different sources [28]. In rodents, mouse and rat bone marrow-derived MCs (BMMC) and mouse peritoneal cell-derived MCs (PCMC) are commonly differentiated [25]. In mouse-derived MCs, a committed MCp population was identified in bone marrow from adult mice as Lin^- $c\text{-}kit^+$ $Sca\text{-}1^-$ $Ly6c^-$ $Fc\varepsilon RI^-$ $CD27^-$ $integrin\beta7^+$ $T1/ST2^+$ [45] and since, these identification markers have been used to isolate mouse MCp. Meanwhile, human primary MCs are derived from MCp usually differentiated from $CD34^+/CD117^+$ or $CD133^+$, $CD13^+$, $Fc\varepsilon RI^-$ precursors that are obtained from bone-marrow, cord-blood, peripheral blood [46-49] and recently from WAT and peripheral tissue [14, 19, 50, 51]. Since KIT (CD117) receptor remains expressed from MCp to mature MCs [52], CD117 serve as an isolation target of MCp. Thus, in this study, primary hMCs were differentiated from $CD117^+$ progenitors from peripheral blood obtained from buffy coats.

The protocol used in the present study to generate human primary MCs complies with repeated evidence generating MCs by culturing stem cell progenitors in the presence of SCF, necessary for MC survival, proliferation and maturation [28, 51, 53], and IL-6 necessary for MC differentiation [1, 51]. A study by Hirohisa Saito demonstrated that medium supplemented with IL-6 and SCF, increases 10-fold MC numbers obtained from CD34+ from cord blood compared to media supplemented only with SCF [28, 54]. Similar to this, the combination of IL-3 and SCF have shown to increase MC proliferation and maturation in MC *in vitro* cultures derived from CD34+ progenitors [55, 56]. Furthermore, equivalent to our generated MCs obtained after 8-10 weeks, Mete Holm reported a 7-week protocol of MC differentiation using media supplemented with IL-3 only during the first stage of proliferation (3 weeks) followed by a supplemented media with SCF and IL-6 during a second maturation stage [46]. The method used here using SCF, IL-6 and IL-3 (in the first stage) to generate MCs is comparable to this protocol.

Our generated MCs positively express the SCF receptor (c-kit, CD117) and the IgE receptor (FcεRI), which are essential for MC functions and serve as identification markers to distinguish MC pure populations [10, 51, 57]. This is in line with Mete Holm and others producing mature MCs ($Fc\varepsilon RI^{+}$ and $CD117^{+}$) after 7-10 weeks that express both receptors [46, 58-60]. Furthermore, Andersen *et. al.* showed that compared to human cord-blood derived MCs, human peripheral blood-derived MCs present a higher expression of FcεRI and CD117 [47]. Thus, primary MCs obtained from blood are more efficient to constitutively express SCF and IgE receptors as observed in our differentiated cells.

Upon activation, MCs rapidly release several mediators including cytokines, chemokines, proteases, prostaglandins and antimicrobial components [61]. For instance, after FcεRI-IgE crosslinking MCs rapidly degranulate and secrete histamine, tryptase and β-hexosaminidase [62]. Since MC precursors are $Fc\varepsilon RI^{-}$, whereas mature MCs are $Fc\varepsilon RI^{+}$ [7, 10], activation of MCs via this receptor serves as an indicator of cell maturity. Here we demonstrated MC degranulation upon IgE activation by the expression of CD107a and CD63 surface markers. These highly glycosylated proteins are contained intracellularly in granules and are exposed at the cell surface upon degranulation [43]. Moreover, CD63 and CD107a expression in degranulated MCs was found to correlate with the β-hexosaminidase release. Additionally, FcεRI engagement promotes cytokine and chemokine secretion that includes MCP-1, IL-8, GM-CSF, TNF and IL-6 secretion [63], similar to Mete Holm generated MCs that actively release IL-8, MCP-1, MIP1α−β and histamine upon IgE activation [46]. As

reported, we could demonstrate, the generated hMCs release of IL-8, MCP-1 and GM-CSF in response to IgE activation.

To date, different laboratories generate hMCs from CD34+, CD133+ and/or CD117+ precursors using similar methodologies with minor adaptations among laboratory groups. For instance, Dean D. Metcalfe group has developed a method to differentiate hMCs from CD34+ precursors by culturing cells in StemPro-34 medium supplemented with human recombinant SCF at 100ng/ml, IL-6 at 100ng/ml and IL-3 only for the first week at 30ng/ml [49, 64]. This is similar to the group from René Toes [65, 66] in the Netherlands and Robert Schleimer [67] in the U.S.A. culturing CD34+ blood progenitors in StemPro-34 with the same IL-6, IL-3 and SCF conditions [65-67]. This protocol slightly differs to Nicolas-Gaudenzio and Stephen-Galli research groups, who instead, use StemSpan medium supplemented with IL-6 at 50ng/ml, IL-3 at 10ng/ml (only for one week) and 3% of the supernatant of Chinese hamster ovary (CHO) transfectants secreting murine SCF for one week followed by 8-week culture in Iscove´s Modified Dulbecco's with GlutaMax-1 with IL-6 (50ng/ml) and murine SCF (~50ng/ml) [68]. In fact, this group demonstrated equivalence between murine and human SCF [69]. This protocol is similar to ours in terms of cytokine concentrations with a difference in IL-3 exposure. However, other laboratories use IL-3 (1ng/ml) for 3 weeks, similar to our settings, from a total of 7-8 week culture [70].

Nevertheless, although different research groups adapted their own standardization to differentiate hMCs, all of the above groups have specified pure hMCs populations with <90% CD117+ and FcεRI+ cells and with functional degranulation upon FcεRI crosslinking after 6-8 week culture [64, 66-68, 70-72]. Thus, our differentiated hMCs complies with general characteristics necessary to identify hMCs.

CONCLUSION

Primary hMCs differentiated from peripheral blood progenitors constitutively express KIT and FcεRI receptors. Furthermore, FcεRI engagement activates MCs to degranulate and to release proinflammatory mediators. Thus, generated MCs used for the present study are a valuable tool to study human MC activities *in vitro*.

REFERENCES: Chapter 3

1. Varricchi, G., et al., *Physiological Roles of Mast Cells: Collegium Internationale Allergologicum Update 2019.* International Archives of Allergy and Immunology, 2019. **179**(4): p. 247-261.
2. Virk, H., G. Arthur, and P. Bradding, *Mast cells and their activation in lung disease.* Translational Research, 2016. **174**: p. 60-76.
3. Bradding, P., *Human mast cell cytokines.* Clinical & Experimental Allergy, 1996. **26**(1): p. 13-19.
4. Galli, S.J., et al., *Mast cells as "tunable" effector and immunoregulatory cells: recent advances.* Annu. Rev. Immunol., 2005. **23**: p. 749-786.
5. Li, Z., et al., *Adult Connective Tissue-Resident Mast Cells Originate from Late Erythro-Myeloid Progenitors.* Immunity, 2018. **49**(4): p. 640-653.e5.
6. Caslin, H.L., et al., *Controlling Mast Cell Activation and Homeostasis: Work Influenced by Bill Paul That Continues Today.* Frontiers in Immunology, 2018. **9**(868).
7. Jamur, M.C. and C. Oliver, *Origin, maturation and recruitment of mast cell precursors.* Front Biosci (Schol Ed), 2011. **3**: p. 1390-406.
8. Kirshenbaum, A.S., et al., *Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells.* The Journal of Immunology, 1991. **146**(5): p. 1410-1415.
9. Méndez-Enríquez, E. and J. Hallgren, *Mast Cells and Their Progenitors in Allergic Asthma.* Frontiers in Immunology, 2019. **10**(821).
10. Nigrovic, P.A., *Chapter 15 - Mast Cells*, in *Kelley and Firestein's Textbook of Rheumatology (Tenth Edition)*, G.S. Firestein, et al., Editors. 2017, Elsevier. p. 250-263.e4.
11. Stone, K.D., C. Prussin, and D.D. Metcalfe, *IgE, mast cells, basophils, and eosinophils.* Journal of Allergy and Clinical Immunology, 2010. **125**(2): p. S73-S80.
12. Gri, G., et al., *Mast Cell: An Emerging Partner in Immune Interaction.* Frontiers in Immunology, 2012. **3**(120).
13. Moiseeva, E.P. and P. Bradding, *Mast Cells in Lung Inflammation*, in *Mast Cell Biology: Contemporary and Emerging Topics*, A.M. Gilfillan and D.D. Metcalfe, Editors. 2011, Springer US: Boston, MA. p. 235-269.
14. Paupert, J., et al., *Rapid and Efficient Production of Human Functional Mast Cells through a Three-Dimensional Culture of Adipose Tissue-Derived Stromal Vascular Cells.* J Immunol, 2018. **201**(12): p. 3815-3821.
15. Ribatti, D., *The development of human mast cells. An historical reappraisal.* Experimental Cell Research, 2016. **342**(2): p. 210-215.
16. Okayama, Y. and T. Kawakami, *Development, migration, and survival of mast cells.* Immunologic Research, 2006. **34**(2): p. 97-115.
17. Hamaguchi, Y., et al., *Interleukin 4 as an essential factor for in vitro clonal growth of murine connective tissue-type mast cells.* J Exp Med, 1987. **165**(1): p. 268-73.
18. Kirshenbaum, A.S., et al., *Inhibition of human mast cell growth and differentiation by interferon gamma-1b.* Exp Hematol, 1998. **26**(3): p. 245-51.
19. *Human Mast Cell Development from Hematopoietic Stem Cells in a Connective Tissue-Equivalent Model.* Tissue Engineering Part A, 2019. **25**(21-22): p. 1564-1574.

20. Elieh Ali Komi, D., S. Wöhrl, and L. Bielory, *Mast Cell Biology at Molecular Level: a Comprehensive Review.* Clinical Reviews in Allergy & Immunology, 2020. **58**(3): p. 342-365.
21. Reber, L.L., T. Marichal, and S.J. Galli, *New models for analyzing mast cell functions in vivo.* Trends Immunol, 2012. **33**(12): p. 613-25.
22. Nakano, T., et al., *Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/Wv mice. Evidence that cultured mast cells can give rise to both connective tissue type and mucosal mast cells.* The Journal of experimental medicine, 1985. **162**(3): p. 1025-1043.
23. Katz, Howard R. and K.F. Austen, *Mast Cell Deficiency, A Game of Kit and Mouse.* Immunity, 2011. **35**(5): p. 668-670.
24. Galli, S.J., N. Gaudenzio, and M. Tsai, *Mast Cells in Inflammation and Disease: Recent Progress and Ongoing Concerns.* Annual Review of Immunology, 2020. **38**(1): p. 49-77.
25. Arock, M., et al., *Ex vivo and in vitro primary mast cells.* Methods Mol Biol, 2008. **415**: p. 241-54.
26. Sellge, G. and S.C. Bischoff, *Isolation, culture, and characterization of intestinal mast cells*, in *Mast Cells*. 2006, Springer. p. 123-138.
27. Patella, V., et al., *Human heart mast cells. Isolation, purification, ultrastructure, and immunologic characterization.* The Journal of Immunology, 1995. **154**(6): p. 2855-2865.
28. Yu, T., et al., *The development of methods for primary mast cells in vitro and ex vivo: An historical review.* Experimental Cell Research, 2018. **369**(2): p. 179-186.
29. Arock, M., et al., *Preclinical human models and emerging therapeutics for advanced systemic mastocytosis.* Haematologica, 2018. **103**(11): p. 1760-1771.
30. Moon, T.C., et al., *Advances in mast cell biology: new understanding of heterogeneity and function.* Mucosal Immunology, 2010. **3**(2): p. 111-128.
31. Sundström, M., et al., *Functional and phenotypic studies of two variants of a human mast cell line with a distinct set of mutations in the c-kit proto-oncogene.* Immunology, 2003. **108**(1): p. 89-97.
32. Michel, A., et al., *Preclinical human models and emerging therapeutics for advanced systemic mastocytosis.* Haematologica, 2018. **103**(11): p. 1760-1771.
33. Laidlaw, T.M., et al., *Characterization of a novel human mast cell line that responds to stem cell factor and expresses functional FcεRI.* J Allergy Clin Immunol, 2011. **127**(3): p. 815-22.e1-5.
34. Guhl, S., et al., *Mast cell lines HMC-1 and LAD2 in comparison with mature human skin mast cells – drastically reduced levels of tryptase and chymase in mast cell lines.* Experimental Dermatology, 2010. **19**(9): p. 845-847.
35. Reber, L.L., et al., *Potential effector and immunoregulatory functions of mast cells in mucosal immunity.* Mucosal immunology, 2015. **8**(3): p. 444-463.
36. Pan, H., et al., *Comprehensive anatomic ontologies for lung development: A comparison of alveolar formation and maturation within mouse and human lung.* Journal of Biomedical Semantics, 2019. **10**(1): p. 18.
37. Lei, Y., et al., *Insights into mast cell functions in asthma using mouse models.* Pulmonary Pharmacology & Therapeutics, 2013. **26**(5): p. 532-539.
38. Andersson, C.K., et al., *Novel site-specific mast cell subpopulations in the human lung.* Thorax, 2009. **64**(4): p. 297-305.
39. Bischoff, S.C., *Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data.* Nature Reviews Immunology, 2007. **7**(2): p. 93-104.
40. Lin, P.L., et al., *Tumor Necrosis Factor and Tuberculosis.* Journal of Investigative Dermatology Symposium Proceedings, 2007. **12**(1): p. 22-25.
41. Bahri, R., et al., *Mast cell activation test in the diagnosis of allergic disease and anaphylaxis.* The Journal of allergy and clinical immunology, 2018. **142**(2): p. 485-496.e16.
42. Alter, G., J.M. Malenfant, and M. Altfeld, *CD107a as a functional marker for the identification of natural killer cell activity.* Journal of immunological methods, 2004. **294**(1): p. 15-22.

43. Betts, M.R., et al., *Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation.* Journal of immunological methods, 2003. **281**(1): p. 65-78.
44. Marshall, J.S., *Mast-cell responses to pathogens.* Nature Reviews Immunology, 2004. **4**(10): p. 787-799.
45. Chen, C.-C., et al., *Identification of mast cell progenitors in adult mice.* Proceedings of the National Academy of Sciences, 2005. **102**(32): p. 11408-11413.
46. Holm, M., et al., *Seven week culture of functional human mast cells from buffy coat preparations.* Journal of Immunological Methods, 2008. **336**(2): p. 213-221.
47. Andersen, H.B., et al., *Comparison of short term in vitro cultured human mast cells from different progenitors — Peripheral blood-derived progenitors generate highly mature and functional mast cells.* Journal of Immunological Methods, 2008. **336**(2): p. 166-174.
48. Schmetzer, O., et al., *A novel method to generate and culture human mast cells: Peripheral CD34+ stem cell-derived mast cells (PSCMCs).* Journal of Immunological Methods, 2014. **413**: p. 62-68.
49. Kirshenbaum, A.S., et al., *Demonstration That Human Mast Cells Arise From a Progenitor Cell Population That Is CD34+, c-kit+, and Expresses Aminopeptidase N (CD13).* Blood, 1999. **94**(7): p. 2333-2342.
50. Durand, B., et al., *Long-term generation of human mast cells in serum-free cultures of CD34+ cord blood cells stimulated with stem cell factor and interleukin-3.* Blood, 1994. **84**(11): p. 3667-74.
51. Rådinger, M., et al., *Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood.* Current protocols in immunology, 2010. **Chapter 7**: p. Unit-7.37.
52. Schernthaner, G.H., et al., *Detection of differentiation- and activation-linked cell surface antigens on cultured mast cell progenitors.* Allergy, 2005. **60**(10): p. 1248-55.
53. Bankova, L.G., et al., *Maturation of mast cell progenitors to mucosal mast cells during allergic pulmonary inflammation in mice.* Mucosal Immunology, 2015. **8**(3): p. 596-606.
54. Saito, H., et al., *Selective growth of human mast cells induced by Steel factor, IL-6, and prostaglandin E2 from cord blood mononuclear cells.* The Journal of Immunology, 1996. **157**(1): p. 343-350.
55. Tsuji, K., et al., *Effects of interleukin-3 and interleukin-4 on the development of" connective tissue-type" mast cells: interleukin-3 supports their survival and interleukin-4 triggers and supports their proliferation synergistically with interleukin-3.* 1990.
56. Kirshenbaum, A., et al., *Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from CD34+ pluripotent progenitor cells.* The Journal of Immunology, 1992. **148**(3): p. 772-777.
57. Cruse, G. and P. Bradding, *Mast cells in airway diseases and interstitial lung disease.* Eur J Pharmacol, 2016. **778**: p. 125-38.
58. Gaudenzio, N., et al., *Different activation signals induce distinct mast cell degranulation strategies.* The Journal of Clinical Investigation, 2016. **126**(10): p. 3981-3998.
59. Joulia, R., et al., *Mast cells form antibody-dependent degranulatory synapse for dedicated secretion and defence.* Nat Commun, 2015. **6**: p. 6174.
60. Folkerts, J., et al., *Rapid identification of human mast cell degranulation regulators using functional genomics coupled to high-resolution confocal microscopy.* Nature Protocols, 2020. **15**(3): p. 1285-1310.
61. Gri, G., et al., *Mast cell: an emerging partner in immune interaction.* Front Immunol, 2012. **3**: p. 120.
62. Krystel-Whittemore, M., K.N. Dileepan, and J.G. Wood, *Mast Cell: A Multi-Functional Master Cell.* Frontiers in Immunology, 2016. **6**(620).
63. Erjefält, J.S., *Mast cells in human airways: the culprit?* European Respiratory Review, 2014. **23**(133): p. 299-307.
64. Yin, Y., et al., *An optimized protocol for the generation and functional analysis of human mast cells from CD34+ enriched cell populations.* Journal of Immunological Methods, 2017. **448**: p. 105-111.

65. Suurmond, J., et al., *Communication between human mast cells and CD4+ T cells through antigen-dependent interactions.* European Journal of Immunology, 2013. **43**(7): p. 1758-1768.
66. van Schaarenburg, R.A., et al., *The production and secretion of complement component C1q by human mast cells.* Molecular Immunology, 2016. **78**: p. 164-170.
67. Kulka, M., et al., *Neuropeptides activate human mast cell degranulation and chemokine production.* Immunology, 2008. **123**(3): p. 398-410.
68. Joulia, R., et al., *Mast cells form antibody-dependent degranulatory synapse for dedicated secretion and defence.* Nature Communications, 2015. **6**(1): p. 6174.
69. Gaudenzio, N., et al., *Human mast cells drive memory CD4+ T cells toward an inflammatory IL-22+ phenotype.* J Allergy Clin Immunol, 2013. **131**(5): p. 1400-7.e11.
70. Parveen, S., et al., *IL-33 mediated amplification of allergic response in human mast cells.* Journal of Receptors and Signal Transduction, 2019. **39**(4): p. 359-367.
71. Al-Afif, A., et al., *Respiratory syncytial virus infection of primary human mast cells induces the selective production of type I interferons, CXCL10, and CCL4.* Journal of Allergy and Clinical Immunology, 2015. **136**(5): p. 1346-1354.e1.
72. Matsuda, K., et al., *Monomeric IgE enhances human mast cell chemokine production: IL-4 augments and dexamethasone suppresses the response.* Journal of Allergy and Clinical Immunology, 2005. **116**(6): p. 1357-1363.

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CHAPTER 4: Paper 2

Human mast cells exhibit an individualized pattern of anti-microbial responses

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ABSTRACT

INTRODUCTION: Mast cells (MCs) are tissue-resident immune cells implicated in antibacterial responses. These include chemokine secretion, degranulation, and the release of MC extracellular traps (MCETs) which are primarily dependent on reactive oxygen species (ROS) production. Our study investigated whether human MCs (hMCs) develop individual response patterns to bacteria located at different tissue sites: *Escherichia coli* (gut commensal), *Listeria monocytogenes* (food borne intracellular pathogen), *Staphylococcus aureus* (skin commensal and opportunistic pathogen) and *Streptococcus pneumoniae* (upper respiratory tract commensal and lung pathogen).

METHODS: After live bacteria exposure, hMCs were analysed by a combined flow cytometry assay for degranulation, ROS production, DNA externalization and for β-hexosaminidase, chemokine and prostaglandin release.

RESULTS: *L. monocytogenes* induced hMC degranulation, IL-8 and MCP-1 release coupled with DNA externalization in a novel hMC ROS-independent manner. In contrast, *S. pneumoniae* caused ROS production without DNA release and degranulation. *E. coli* induced low levels of hMC degranulation combined with IL-8 and MCP-1 secretion and in the absence of ROS and DNA externalization. Finally, *S. aureus* induced hMCs prostaglandin D2 release and DNA release selectively. Our findings demonstrate a novel hMC phenomenon of DNA externalization independent on ROS production. We also showed that ROS production, degranulation, DNA externalization and mediator secretion occur as independent immune reactions in hMCs upon bacterial encounter and that hMCs contribute to bacterial clearance.

CONCLUSIONS: Thus, hMCs exhibit a highly individualized pattern of immune response possibly to meet tissue requirements and regulate bacteria co-existence versus defence.

INTRODUCTION

Mast cells (MCs) are immune cells that primarily reside at mucosal surfaces and the skin [1]. MC progenitors either egress from the bone marrow and yolk sac to become mature MCs at tissue sites or differentiate locally under the influence of the tissue microenvironment [2-4]. Recent studies have identified that MCs perform essential functions in response to pathogenic threats [1, 5, 6]. MCs are strategically positioned to be among the first cells to interact with microbes, such as bacteria, and trigger specific immune responses against pathogens [5]. Furthermore, the ability of MCs to rapidly release antimicrobial mediators and pro-inflammatory molecules has raised interest in the possibility of their therapeutic modulation for infectious diseases [7].

MCs exhibit a range of defence mechanisms against bacteria that include the secretion of pro- and anti-inflammatory mediators which shape both innate and adaptive immunity [1, 5]. Such mediators can be released upon degranulation or following the engagement of the classical secretory pathway [8]. For instance, *Mycobacterium tuberculosis* and *Enterococcus faecalis* are associated with MC cytokine release coupled with degranulation, whereas commensal bacteria including *Bifidobacterium bifidum*, do not induce MC degranulation [9-11]. MC extracellular trap (MCET) formation is a defence mechanism that exposes microbes to inflammatory mediators within a mesh of DNA [12, 13]. MCETs have shown to contribute to antibacterial immunity, including infections caused by *Listeria monocytogenes*, *Streptococcus pyogenes* and *Enterococcus faecalis* [13].

Neutrophils form extracellular traps by two mechanisms [14, 15]. The first involves cell death and occurs through decondensation of the nuclear envelope that is triggered by the presence of reactive oxygen species (ROS) [14, 16, 17]. The second involves DNA release without compromising cell viability and independently of ROS production [18]. However, in MCs, only ROS-dependent extracellular traps have been described [19, 20] with the majority of studies performed in animal models or human cell lines [19]. Furthermore, MCET formation seems to depend on specific bacterial stimulation. For instance, DNA release is observed after *L. monocytogenes* or *E. faecalis* encounter, whereas release of DNA is inhibited after *M. tuberculosis* exposure [19, 21].

We hypothesized that different microbes induce individual patterns of functional response by MCs, including degranulation, chemokine secretion, and DNA release in the presence or absence of ROS production. We therefore assessed human MC (hMC) responses after their encounter with bacteria typically localized at tissue interfaces: *E. coli* (gut commensal), *L.*

monocytogenes (food borne intracellular pathogen), *S. aureus* (skin commensal and opportunistic pathogen) and *S. pneumoniae* (upper respiratory tract commensal and lung pathogen). We found that *L. monocytogenes* induced hMC degranulation and DNA release in the absence of ROS production while *S. pneumoniae* promoted ROS production without inducing DNA release and degranulation. Furthermore, *E. coli* promoted hMC degranulation in the absence of DNA or ROS release, whereas *S. aureus* did not induce hMCs to degranulate or release DNA but selectively induced MC prostaglandin D_2 secretion. These data suggest that hMCs display specific patterns of response according to the individual pathogen, involving degranulation, DNA release, ROS production and chemokine release. These activities occur as independent reactions. Therefore, our findings suggest that hMCs respond strategically to individual threats orchestrating unique immune responses in bacterial defence.

MATERIAL AND METHODS

Bacterial strains

Bacterial strains were kept as stocks at -80°C in 15% glycerol. Before bacteria were used, growth curves were generated to correlate optical density (O.D) with colony-forming units (CFU). *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were cultured in LB broth (Sigma-Aldrich) and *Listeria monocytogenes* (inlA) in TSB broth (Sigma-Aldrich), at 37°C under shaking incubator (200rpm). *Streptococcus pneumoniae* strain D39 (R6) was cultured in Todd Hewitt Broth and yeast extract (0.5%) at 37°C without shaking. Bacterial strains were cultured and harvested at a midpoint of log-growth phase (O.D 0.3-0.6) for cell stimulations.

Human primary mast cell culture

hMCs were generated as previously described [22]. Briefly, cells were obtained by a positive selection of $CD117^+$ haematopoietic progenitors obtained from buffy coat blood mononuclear cells by immunomagnetic sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured for 4 weeks in Iscove Modifiied Dulbecco medium with GlutaMAX-I supplemented with 50µmol/L β2-mercaptoethanol, 0.5% BSA, 1% Insulin-Transferrin-Selenium, 100 U/mL penicillin, 100µg/mL streptomycin (Invitrogen, Carlsbad, Calif), ciprofloxacin (bio-world), human IL-6 (50ng/mL; PeproTech, Rocky Hill, NJ), human IL-3 (10ng/mL; PeproTech), human stem cell factor (SCF) (100ng/mL; PeproTech) and StemRegenin (1µM; Cayman). Cells were progressively transferred to culture media containing Iscove Modified Dulbecco Medium with GlutaMAX-I supplemented with 50µmol/L β2-mercaptoethanol, 0.5% BSA, 1% Insulin-Transferrin-Selenium, 100U/mL penicillin, 100µg/mL streptomycin, human IL-6 (50ng/mL) and human stem cell factor (SCF) (100ng/mL) for 4 weeks. After 8-10 weeks of culture, cells were tested for purity and maturity, measuring CD117 and FcεRIa expression.

Human mast cell infection

hMCs were washed three times to remove antibiotics and plated at a density of $5x10^5$ cells/mL in supplemented medium without antibiotics. Cells were rested at 37°C in 5% CO_2

for 1h before infections. Bacteria were cultured and prepared at a multiplicity of infection (MOI) of 25:1 in supplemented medium without antibiotics. MOI (25:1) was selected by testing different bacterial concentrations and choosing the minimum MOI able to induce hMC degranulation. Each bacterial species was incubated with plated hMCs for 2h at 37°C, 5% CO_2. Supplemented media without antibiotics was used to incubate un-stimulated controls

Flow cytometry

For the measurement of ROS, hMCs were pre-treated with dihydorhodamine 123 (DHR 123) (Thermo Fisher Scientific) at 1.33µg/mL for 15 minutes before stimulation. To inhibit ROS, 10µM diphenyliodonium (DPI) (Sigma-Aldrich) was added 30 minutes before stimulation. Once stimulated with the four different bacteria, hMCs were washed several times with PBS before staining. Cells were incubated with Fc-block and stained with fixable Live/Dead Zombie NIR (BioLegend) according to the manufacturer's protocol to exclude dead cells with compromised cell membranes.

Cells were stained with anti-human antibodies CD107a (LAMP-1) (H4A3 BD BioLegend) and analysed by flow cytometry as above. Degranulation was measured as percentage of $CD107a^+$ cells.

TO-PRO-3 (Invitrogen) was added to cells in the final 15 minutes of staining at 1µM. All dyes and antibodies were diluted in FACS buffer and incubated for 30 minutes at 4°C before washing and fixing cells with 4% paraformaldehyde. Flow cytometric data were acquired using the BD LSR-II. Single stain controls were prepared using compensation beads (OneComp eBeads, Thermofisher Scientific). The post-acquisition data analysis was performed using FlowJo software (Treestar version 10.4.2).

Confocal microscopy

hMCs were washed several times to remove antibiotics and seeded on a glass slide pre-coated with poly-D-Lysine at a concentration of $5x10^4$ hMCs/ml. Cells were left untreated (control) or incubated with *L. monocytogenes* for 16h at a MOI 25:1. After stimulation, cells were washed three times and fixed with formaldehyde 4% for 20 min and permeabilised with triton (0.2%) (Sigma-Aldrich). After three washes cells were incubated first with blocking buffer (10% goat serum in PBS) for 1h and then with mouse anti-human tryptase antibody (ab2378 Abcam) for 1h followed by 1h incubation with goat anti-mouse antibody conjugated

to Alexa 647 fluorophore (ab150115 Abcam). Cells were washed three times and mounted with fluoroshield mounting media with DAPI (Abcam) to be analysed by confocal microscopy. Images were collected at a zoom factor of 2.58 using a Leica TCS SP8x inverted confocal microscope equipped with a tuneable white light laser (WLL), and a diode 405nm laser, a 40X/0.85 dry objective and HyD hybrid detectors. AL647 fluorescence was excited at 647 nm with WLL laser and detected at 655 to 718 nm on a HyD point detector. DAPI fluorescence was excited at 405 nm using the UV laser and collected on a HyD detector at 410 to 470nm. Confocal 3D stacks were acquired with a depth of 8µm using Leica LASX software. Images stacks were then processed, sum projected and analysed using *Fiji* [23].

β-hexosaminidase assay

hMCs were stimulated with *L. monocytogenes* (100µL, $5x10^5$ cells/mL) for 2 hours. After incubation, supernatants were harvested and cell pellets were lysed in 1% Triton X-100. β-hexosaminidase activity was measured in supernatants as well as in cell pellets by adding the substrate *p*-nitrophenyl N-acetyl-beta-D-glucosamine at 1 mmol/L (Sigma-Aldrich, St Louis, Mo) in 0.05 mol/L citrate buffer (pH 4.5) for 2 hours at 37°C in a 5% CO2 atmosphere [22]. The reaction was stopped by using 0.05 mol/L sodium carbonate buffer (pH 10). OD was measured at 405 nm. Degranulation was assessed as percentage release of total β-hexosaminidase.

Measurement of IL-8, MCP-1, prostaglandin D2

After the incubation of hMCs with the different bacterial strains, cells were centrifuged, and supernatants were collected to measure mediator content. Supernatants were either used immediately after collection or frozen at -80°C before being analysed. Prostaglandin D2 (PGD2) levels were measured according to the manufacturer´s protocol using ELISA´s chemical Cayman kit (cat 412012). IL-8 and MCP-1 were quantified by a BD cytometric bead array (CBA) multiplex kit following manufacturer´s protocol using a FACSVerse flow cytometer. The analysis was performed using FCAP Array Software v3.0.

Colony forming unit assay

hMCs were stimulated for 2 hours with *L. monocytogenes* at a MOI 25:1 in 50µL of cell media. 50µL of initial stocks of *L. monocytogenes* (corresponding to MOI of 25:1) were incubated alone for 2 hours. After incubation, 100µL of cold-sterile ultra-pure water was added on the top of cell-bacterial suspensions to lyse hMCs, and incubated for 10 minutes at 4°C. Then, serial dilutions 1:10 to $1:10^8$ were prepared in PBS, and 10µL of each dilution was plated on TSB agar (Sigma-Aldrich) in five replicates (50µL in total). CFUs were counted after 1 to 3 days (3 days final count).

Statistical analysis

Data were analysed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). All samples were processed in triplicates. Comparison between more than 2 groups was achieved using one-way ANOVA correcting for Tukey´s multiple comparisons and comparisons between 2 groups were performed by unpaired t-test. Correlations were calculated using Spearman R (R^2). *P* values of 0.05 or less were considered to be statistically significant.

RESULTS

Listeria monocytogenes and Escherichia coli induce hMC degranulation

Several bacteria are known to trigger MC degranulation, whereas non-pathogenic gut resident strains, including *Lactobacillus* spp. appear to inhibit this process [24]**.** We sought to characterise further and compare MC degranulation as a defence mechanism against bacteria encountered at different tissue sites. hMCs derived from peripheral blood precursors that had the phenotypic and functional properties of mature hMCs (**Figure 4.1a and Supplemental Figure 4.A**) were stimulated with an intracellular intestinal pathogen *L. monocytogenes*, an intestinal commensal *E. coli*, a skin commensal *S. aureus*, or a lung commensal *S. pneumoniae* intending to detect differences in induced hMC degranulation.

To assess hMC degranulation following incubation with bacteria at a multiplicity of infection (MOI) of 25:1, we measured: i) surface expression of CD107a (**Figure 4.1b and c**) [25] using flow cytometry; and ii) granule compound release by means of β-hexosaminidase secretion (**Figure 4.1d**). Unstimulated hMCs were used here as negative control.

The level of degranulation ($CD107a^+$ hMCs; β-hexosaminidase release) was highest in hMCs incubated with *L. monocytogenes* (45.8%; 36.1%), compared to *E. coli* (9.8%; 26.1%), *S. pneumoniae* (3.7%; 2.9%) and *S. aureus* (3.7%; 6.4%) exposure (**Figure 4.1b-d**). Both *S. aureus* and *S. pneumoniae* showed no significant effect on hMC degranulation (1.9%) (**Figure 4.1b-d**).

Furthermore, the percentage of β-hexosaminidase release was found to correlate (r_s=0.82) with the CD107a hMC expression (**Supplemental Figure 4.B**). Thus, hMCs exhibit a diverse degranulation response to different bacterial strains, and obligate pathogens invading gut, such as *L. monocytogenes*, may promote higher levels of MCs degranulation compared with opportunistic pathogens resident in skin or lung.

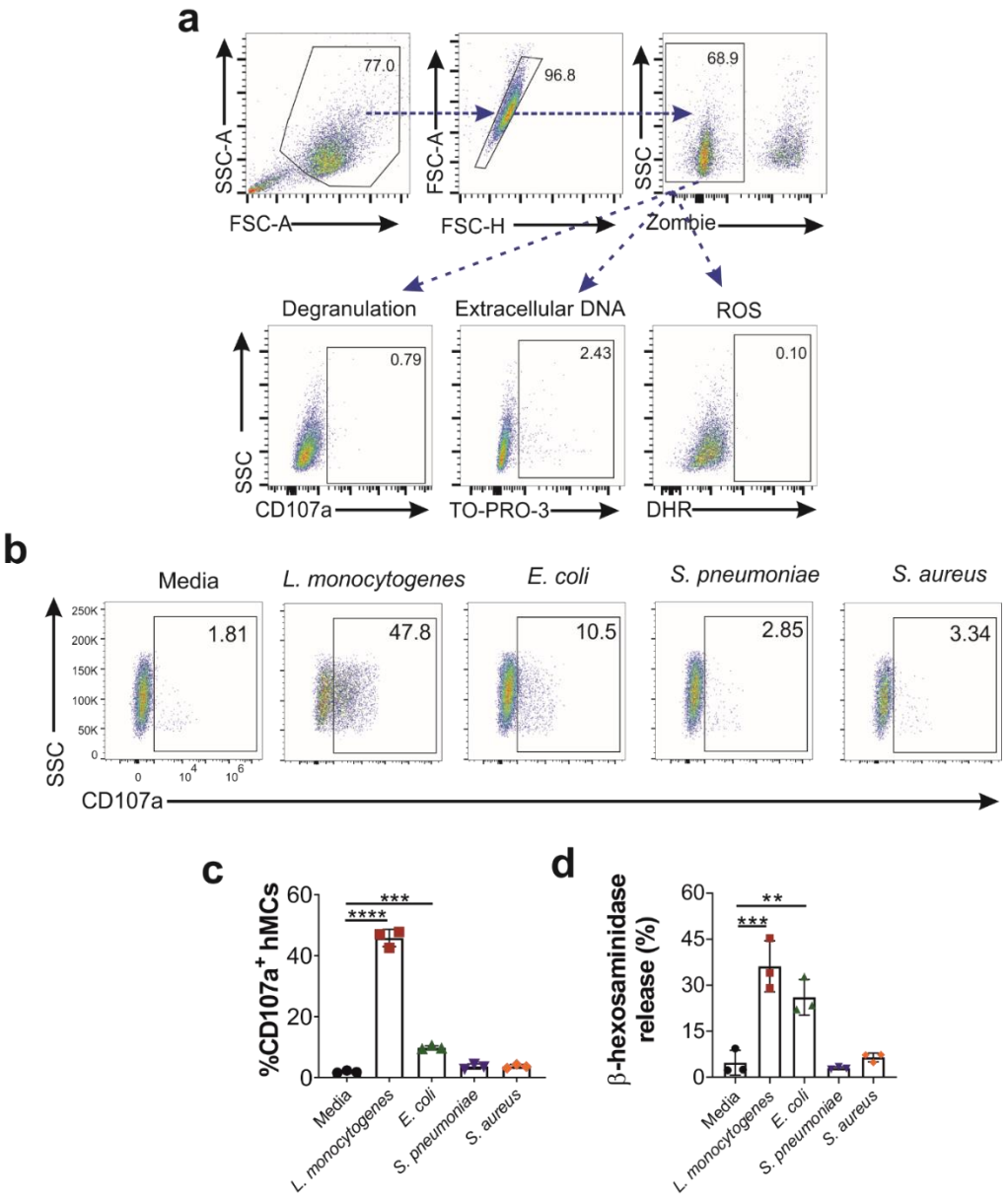


Figure 4.1 Human MCs show different patterns of degranulation in response to different bacterial strains. hMCs were stimulated with either *L. monocytogenes*, *E. coli*, *S. pneumoniae* or *S. aureus* at a MOI of 25:1 or media for 2h. **(a)** Single live cells were selected by the side scatter (SSC-A), forward scatter (FSC-A). Live versus dead cells were discriminated by Zombie NIR staining. CD107a antibody and TO-PRO-3 and DHR dyes were used to investigate degranulation, DNA secretion and ROS production, respectively. **(b)** CD107a flow cytometry representative of four independent experiments shows MC degranulation upon bacterial stimulation. **(c)** Percentage of CD107a expressing cells **(d)** and % of β-hexosaminidase release upon bacterial stimulation. Graphs (c) and (d) are a representative of four independent experiments performed each with three replicates.

Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparisons test (****p<0.0001, ***p<0.001, **p<0.01).

Selective bacterial stimulation induces MCs to release IL-8, MCP-1, and prostaglandin D_2

Among the studied bacteria, only *L. monocytogenes* and *E. coli* induced hMC degranulation. However, hMCs also release a wide variety of pro-inflammatory mediators independently of degranulation in a stimuli-specific manner [8]. Such pro-inflammatory molecules allow hMCs to orchestrate the response of other immune cells to bacteria [26]. We hypothesized that bacteria encountered at different tissue sites induce a specific pattern of cytokine production independently of degranulation. To test this hypothesis, hMC mediators (MCP-1, IL-8 and Prostaglandin D_2) were quantified in culture supernatants after stimulation with bacteria.
L. monocytogenes, which induced the strongest degranulation among the studied bacteria (**Figure 4.1b-d**) significantly promoted the release of pro-inflammatory mediators (mean chemokine release) including IL-8 (314.2 pg/mL) and MCP-1 (1663 pg/mL) secretion compared with unstimulated cells (**Figure 4.2a**). *E. coli*, induced a relatively low degranulation (**Figure 4.1b-d**) and IL-8 (61.2 pg/mL) and MCP-1 (553.9 pg/mL) secretion (**Figure 4.2a**) compared to *L. monocytogenes*. The levels of hMC mediator secretion upon *S. aureus* and *S. pneumoniae* exposure were similar to the unstimulated controls. The release of GM-CSF, IL-10 and IL-1β was tested but found undetectable (data not shown).
L. monocytogenes, *S. aureus* and *E. coli* significantly induced prostaglandin D_2 secretion with mean levels of 65.9 ng/mL, 27.4 ng/ml and 62.1 ng/mL, respectively. No significant prostaglandin D_2 release compared with unstimulated controls (0.6 ng/ml) was observed upon *S. pneumoniae* stimulation (9 ng/ml) (**Figure 4.2b**). Taken together, our findings suggest that mediator release is both bacterial specific and independent of degranulation.

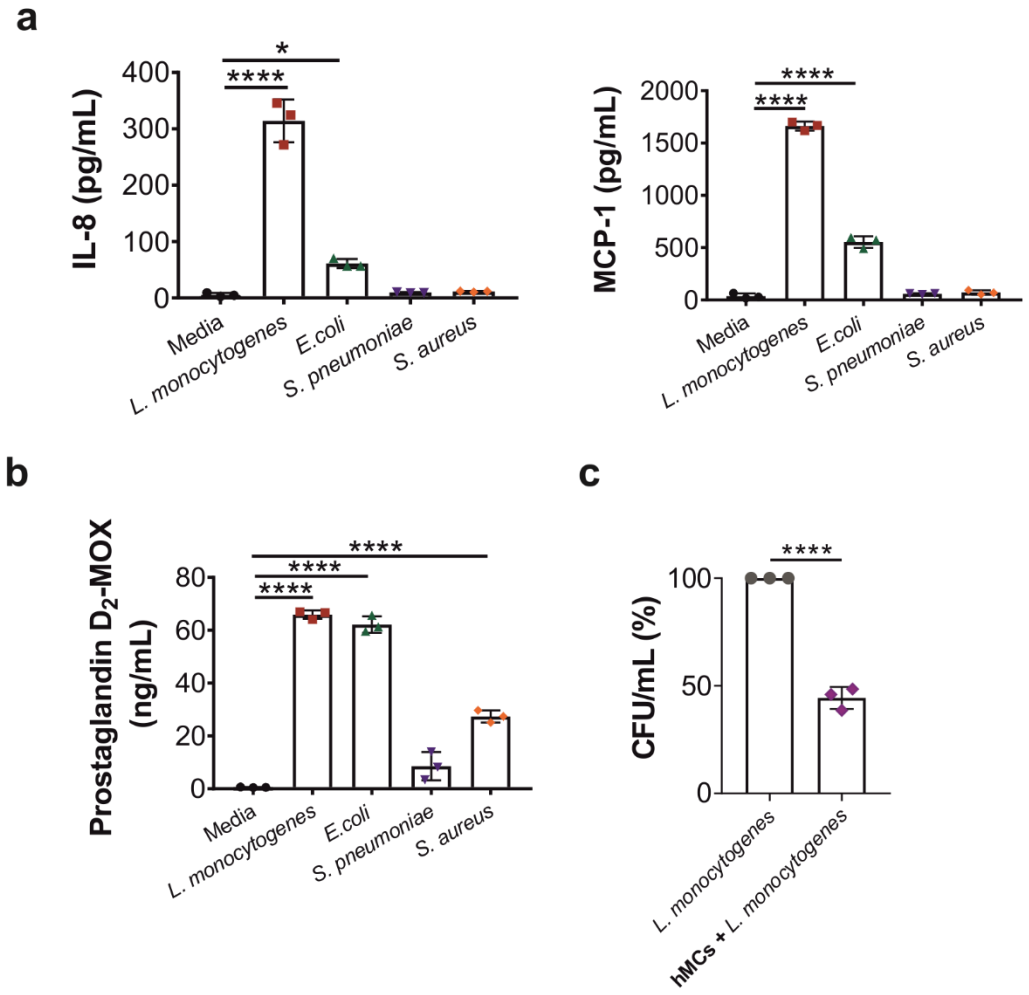


Figure 4.2. Bacterial stimulation induces selective IL-8, MCP-1 and prostaglandin D2 secretion and hMC-mediated bacterial clearance. hMCs were stimulated with either *L. monocytogenes*, *E. coli*, *S. pneumoniae* or *S. aureus* at a MOI of 25:1 or media control for 2h. Supernatants were collected for measurement of **(a)** IL-8 and MCP-1 and **(b)** prostaglandin D_2-MOX concentrations by a multiplex cytometric bead array (CBA). **(c)** To analyse bacterial survival, hMCs were stimulated with *L. monocytogenes* at a MOI of 25:1 for 2 hours. Bacterial inoculums were used as control bacterial concentration (100%) and treated equal to stimulated hMCs. After incubation, samples were lysed, serial dilutions were prepared, cultured in agar plates, and CFUs were counted. Data shown are the mean of three replicates of a representative experiment out of four independent experiments performed. Statistical analysis was performed using un-paired t-test and two-way ANOVA and Tukey's multiple comparisons test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.1).

Human MCs contribute to *Listeria monocytogenes* killing

Human MCs produce high levels of extracellular DNA, IL-8, MCP-1 and prostaglandin D_2 upon *L. monocytogenes* stimulation. Different studies have shown that cytoplasmic content exposed in DNA traps is able to kill pathogens [12, 13, 27]. Therefore, we aimed to investigate whether the hMC mediator secretion induced by *L. monocytogenes* infection affects bacterial survival. After hMCs incubation with *L. monocytogenes,* bacterial numbers were determined by counting colony forming-units (CFUs). As observed in **Figure 4.2c**, 55% less CFUs were observed in the cultures containing hMCs compared with the ones of bacteria alone. These findings suggest that hMC responses to *L. monocytogenes* contribute to bacterial clearance.

Human MCs display a distinctive pattern of degranulation, DNA secretion and ROS production upon bacterial encounter

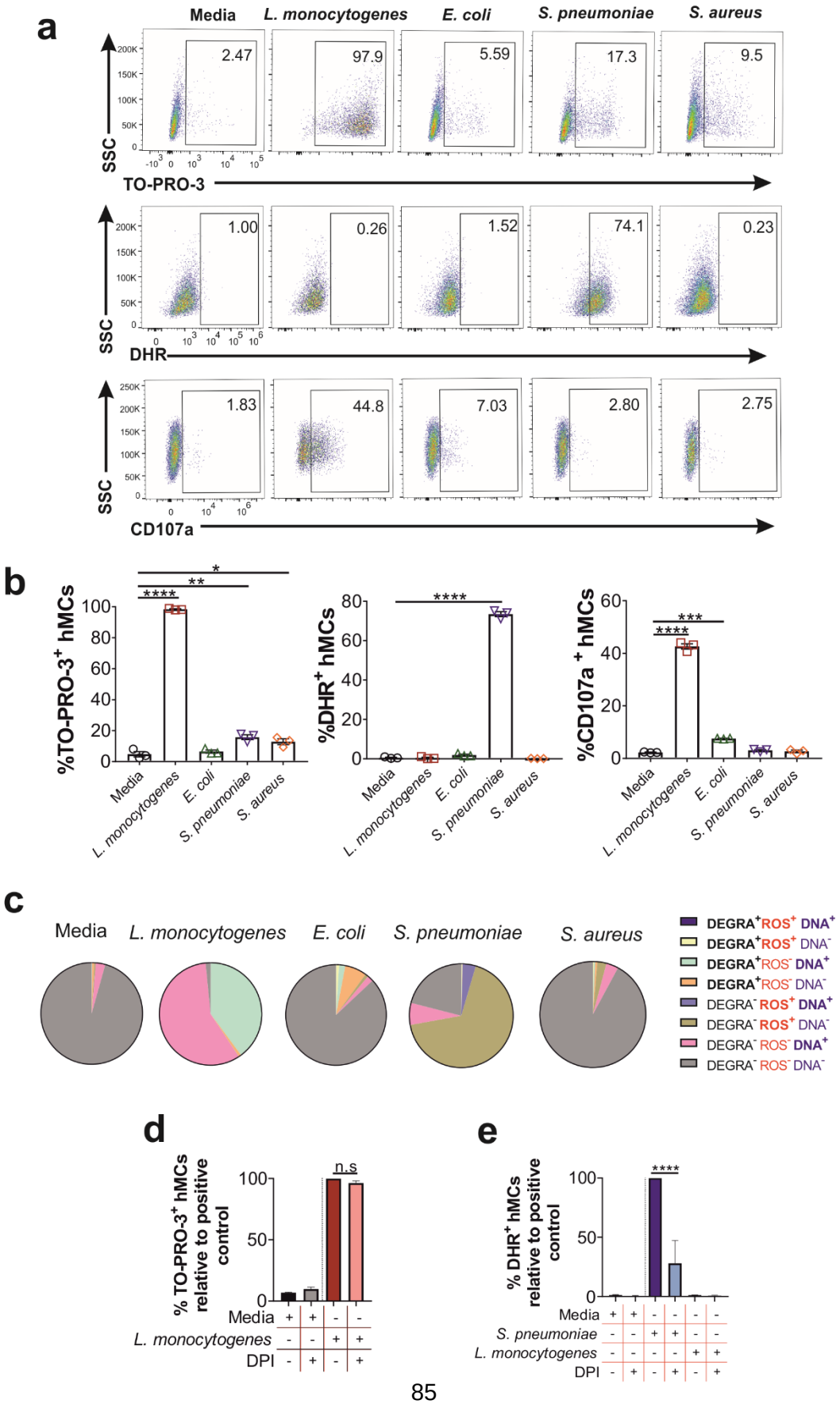
hMCs contribute to bacterial containment through the formation of extracellular traps. Current literature reports MCET formation upon MC exposure to *L. monocytogenes*, *S. aureus*, *S. pyogenes* and *M. tuberculosis* [19, 20]. However, DNA secretion, reflective of MCET formation, has been reported as dependent on ROS production. We have shown that the complex nature of hMC responses allows degranulation and mediator secretion to be discrete and independent processes. Thus, we hypothesized that bacterial-induced DNA release might occur in a bacterial specific and ROS independent manner, as in neutrophils [18]. To investigate this idea, hMCs were incubated with *L. monocytogenes*, *E. coli*, *S. pneumoniae* or *S. aureus* for 2 hours and stained with CD107a antibodies, TO-PRO-3 and DHR dyes to assess degranulation, DNA release and ROS production respectively.
Different levels of DNA externalization (mean %TO-PRO-3^+ cells), ROS release (mean %DHR^+ cells) and degranulation (mean %$CD107a^+$ cells) were observed among stimulated cells (**Figure 4.3a-c**). *L. monocytogenes* induced in hMCs a significantly higher DNA externalization (98.4%) compared to unstimulated controls (4.9%), (**Figure 4.3b**). *L. monocytogenes*-dependent degranulation and DNA secretion occurred in the absence of ROS production (**Figure 4.3a-b**). *E. coli* induced lower degranulation compared to *L. monocytogenes* (**Figure 4.1**) occurring in the absence of DNA secretion and ROS

production (**Figure 4.3a-b**). In contrast, ROS production upon *S. pneumoniae* stimulation (73.5%) was significantly higher compared to unstimulated controls (0.6%). Interestingly, despite the high numbers of hMCs producing ROS after *S. pneumoniae* stimulation, only 16% of hMCs released DNA and in the absence of degranulation (**Figure 4.3a-b**). Additionally, *S. aureus* significantly induced DNA externalization (12.9%) in hMCs compared to unstimulated controls occurring in the absence of ROS production and degranulation (**Figure 4.3a-b**).

To investigate the association of hMC degranulation with DNA secretion and ROS production after exposure to the four studied bacteria, we carried out a *part of a whole* analysis correlating degranulation ($CD107a^+$), ROS production (DHR^+) and extracellular DNA release (TO-PRO-3^+) in stimulated hMCs (**Figure 4.3c**). Upon *L. monocytogenes* exposure, 40% of hMCs were $CD107a^+$, DHR^- and TO-PRO-3^+ (**Figure 4.3c**, green) and 60% were $CD107a^-$, DHR^- and TO-PRO-3^+ (**Figure 4.3c**, pink). This indicates that although most of the *L. monocytogenes*-stimulated cells secreted DNA (98%), only 60% were associated with degranulation and none of the total population produced ROS. In contrast, degranulated cells stimulated by *E. coli* were not associated with DNA and ROS production. Furthermore, after *S. pneumoniae* encounter, 67% of cells produced ROS in the absence of DNA and degranulation. Additionally, only 4% of the total DNA released (17%) after *S. pneumoniae* exposure was associated with ROS. Finally, after *S. aureus* stimulation, DNA release and degranulation were not found to be linked.

To further prove that DNA release can occur in hMCs independently from ROS secretion, hMCs exposure to *L. monocytogenes* was preceded by incubation with diphenyleneiodonium (DPI), a ROS inhibitor [28]. TO-PRO-3 and DHR were measured by flow cytometry (**Figure 4.3d-e**). hMCs infection with *S. pneumoniae* was used as positive control for DPI-induced ROS inhibition (**Figure 4.3e**). As shown in **Figure 4.3d**, ROS inhibition did not affect DNA release.

Altogether, these findings suggest that in hMCs the release of DNA, ROS production and degranulation can occur independently and under the control of distinct bacterial-cell interaction.


a
Media
L. monocytogenes
E. coli
S. pneumoniae
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97.9
5.59
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SSC
TO-PRO-3
1.00
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1.83
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CD107a
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L. monocytogenes
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S. pneumoniae
L. monocytogenes
DPI

Figure 4.3. The MC pattern of degranulation, DNA secretion and ROS production is bacteria specific. hMCs were stimulated with either *L. monocytogenes*, *E. coli*, *S. pneumoniae* or *S. aureus* at a MOI of 25:1 or media controls for 2h. After stimulation, cells were stained with TO-PRO-3 (DNA, DNA secretion), DHR (ROS, ROS production) and CD107a (DEGRA, degranulation) and analysed by flow cytometry. **(a)** Flow cytometry data show one representative plot. **(b)** Percentage of TO-PRO-3^+, DHR^+ and $CD107a^+$ cells after bacterial stimulations. Each graph shows the mean of three replicates of a representative experiment out of four independent experiments performed. Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparisons test (*****p*<0.0001, ****p*<0.001, ***p*<0.01, **p*<0.1). **(c)** Frequency of $CD107a^{+/-}$ (DEGRA, black), $DHR^{+/-}$ (ROS, red) and TO-PRO-$3^{+/-}$ (DNA, blue) cells upon bacterial exposure. Data for each condition (*L. monocytogenes*, *E. coli*, *S. pneumoniae, S. aureus* and media control) was taken from the mean (n=3) of a representative experiment of four independent experiments. To investigate DNA release is ROS independent, hMCs were incubated with DPI to inhibit ROS production, and stimulated then with *L. monocytogenes* and stained with TO-PRO-3. **(a)** Values were normalized to the TO-PRO-3 positive control hMCs challenged with *L. monocytogenes* in the absence of DPI. **(b)** The inhibitory effect of DPI on ROS production was controlled in hMCs stimulated with *S. pneumonia,* and stained with DHR. Data show values normalized to hMC incubated with *S. pneumonia* without DPI.

L. monocytogenes infection affects hMC viability

We showed that *L. monocytogenes* induces an extensive release of DNA (assessed by TO-PRO-3) from hMCs (**Figure 4.3**). To investigate whether bacterial infection affects cell viability, hMCs were incubated with the four bacterial strains and cell viability (mean $Zombie^-$ cells) was assessed by flow cytometry. While *E. coli*, *S. pneumoniae*, *S. aureus*, did not affect hMC survival, *L. monocytogenes* reduced cell viability ($Zombie^-$ cells, 70.2%) (**Figure 4.4a** and **Supplemental Figure 4.C**). Furthermore, most of the live cells ($Zombie^-$) were TO-PRO-3^+ (66.8%). However, we observed that 28% of hMCs releasing DNA (TO-PRO-3^+) were positive for the cell death marker ($Zombie^+$) (**Figure 4.4b**). Thus, these findings indicate that DNA release induced by *L. monocytogenes* occurs in live hMCs, and that this phenomenon is associated with a low level of cell death.

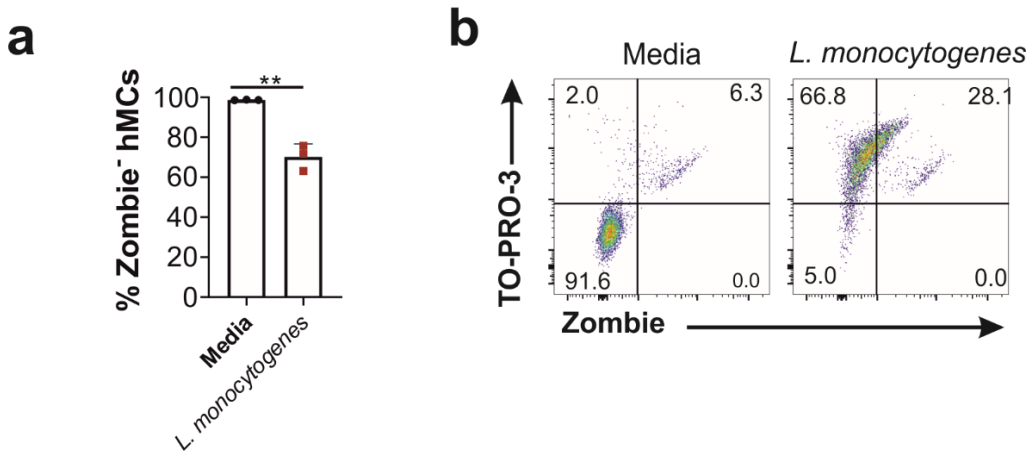


Figure 4.4. L. monocytogenes affects hMC viability. hMC viability upon bacterial infection was measured after *L. monocytogenes* stimulation, cells were stained with **(d)** Zombie NIR (live cells) and **(e)** TO-PRO-3 (DNA releasing cells). Flow cytometry plots show one replicate of a representative experiment out of three independent experiments. Graph shows the mean of three replicates of a representative experiment out of three independent experiments performed. Statistical analysis was performed using unpaired t-test (**p<0.01).

Infection of hMCs with *L. monocytogenes* induces the formation of extracellular traps

Mast cell lines and rodent MCs have been shown to release DNA using microscopy techniques [19-21, 29]. To investigate whether the release of extracellular DNA in hMCs was associated with the formation of extracellular traps, cells were incubated with *L. monocytogenes* for 16h and co-localization of extracellular DNA (DAPI staining) and MC granule content release (tryptase staining) was analysed by confocal microscopy. As shown in **Figure 4.5**, *L. monocytogenes*-stimulated hMCs showed externalized DNA (**Figure 4.5a**) that colocalized with tryptase compared to unstimulated controls (**Figure 4.5b**). These data indicate that hMCs exposed to *L. monocytogenes* form extracellular traps.

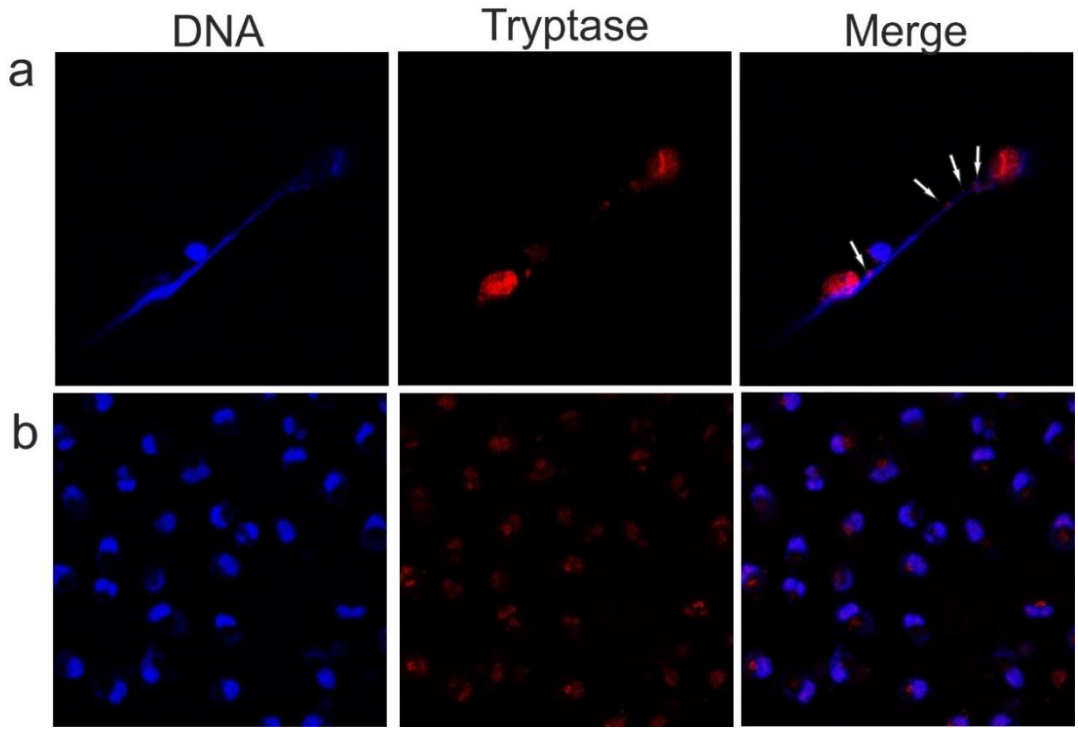


Figure 4. 5. L. monocytogenes induces extracellular trap formation. hMCs were seeded in a glass slide pre-coated with poly-D-Lysine. Cells were settled and stimulated with *L. monocytogenes* or media (unstimulated control) for 16h. After incubation cells were washed and stained for extracellular DNA using DAPI and for tryptase with anti-human tryptase antibody conjugated to Alexa 647 fluorophore. Slides were analysed by confocal microscopy. **(a)** *L. monocytogenes*-stimulated cells and **(b)** unstimulated controls were stained and merged to visualize extracellular and intracellular tryptase-DNA co-localization, respectively.

DISCUSSION

The present study demonstrates that hMCs employ distinctive patterns of responses to *L. monocytogenes*, *E. coli, S. pneumoniae* and *S. aureus*. The hMC immune reactions include DNA externalization, ROS production, degranulation and chemokine and prostaglandin D2 secretion.

Differences between hMCs and rodent MCs or MC lines have been described [30]. Furthermore, since hMCs are tissue resident cells, their location and relative low numbers makes it difficult to isolate and use them for complex experimental settings. Therefore, blood-differentiated mature hMCs were used in this study as an alternative useful tool to tissue cells.

The release of extracellular traps is well characterised in neutrophils as a mesh of histones, antimicrobial peptides and granular proteins to trap and kill bacteria [14]. Using a flow cytometry assay, we have found that *L. monocytogenes* and *S. aureus* induce DNA externalization without intracellular ROS production. Such mechanism is novel in hMCs, which have so far been described to form ETs while undergoing NADPH-dependent cell death [19-21]. In neutrophils, the latter type of ET production is termed “suicidal NETosis” as it leads to cell death [14]. However, in response to *S. aureus* Pilsczec et al. described a second ROS-independent mechanism in neutrophils called “vital NETosis” in which the release of DNA occurs by fusion of DNA-containing vesicles with the plasma membrane [18]. A similar mechanism of “vital MCETosis” might occur in ROS-negative, DNA positive hMCs exposed to *L. monocytogenes, S. aureus* and *E. coli* that would be in line with the long-lived, tissue-resident nature of hMCs [31]. Our study demonstrated that *L. monoctyogenes* induces MCETs and a low level of cell death. Therefore, we suggest that, upon *L. monoctyogenes* encounter, hMCs exhibit early “suicidal MCETosis” as well as early “vital-DNA release”, and following this, late (after 16hrs) DNA externalization and web-like structures formation, late MCETosis. A similar process was described in neutrophils [18], where a ROS-independent vital-NETosis induces rapid DNA release (5-60mins) without compromising the cell membrane [18]. In MCs, the rapid release of DNA may contribute for the release of inflammatory mediators and bacterial clearance.

The pore forming toxin, listeriolysin O, is a primary virulence factor of *L. monocytogenes* that conveys phagocytosis avoidance and allows bacteria to reside intracellularly [32]. Previous studies have shown that this toxin is necessary to trigger hMCs responses such as degranulation and mediator secretion [33]. Although this needs to be confirmed, this toxin

in addition to inducing tryptase secretion may be involved in the process of DNA release and MCET formation. In our study, *L. monocytogenes,* the only obligate pathogen tested, which also resides intracellularly, induced a robust MC response with active DNA release. In line with the extent of DNA release measured in our assay, Campillo-Navarro and colleagues observed a peak of DNA release in a human mast cell line after 3h exposure to *L. monocytogenes* by fluorescent microscopy [19]. Primary hMCs generally respond faster to external triggers [34, 35]. However, by microscopy, we demonstrated an *L. monocytogenes*-induced DNA externalization co-localized with granular content (tryptase) only after 16h incubation. In contrast, flow cytometry demonstrated an early DNA externalization after only 2hrs incubation. Thus, flow cytometry may provide a sensitive and more suitable detection technique for quantifying DNA release at early stages upon infection.

The rapid and robust release of DNA observed may serve MCs, which display low phagocytic capacity [36, 37], the ability to inhibit bacterial multiplication alongside MC degranulation together with chemokine and cytokine secretion which is vital in driving neutrophil recruitment during *L. monocytogenes* infection, as suggested by Gekara and Weiss [38]. In our study, we confirmed that *L. monocytogenes* is killed by hMCs, while promoting hMC degranulation and the release of IL-8, and MCP-1, thus supporting the role of MC in the inflammatory process of cellular recruitment and bacterial clearance.

In previous studies minimal degranulation was observed after exposing murine bone marrow derived mast cells (BMMC) for 2h to *L. monocytogenes* (MOI 10:1). Furthermore, a peak of MCP-1 secretion was shown at 2h using a MOI of 100:1 [39]. In contrast, our findings showed a stronger hMC degranulation after *L. monocytogenes* exposure and similar concentrations of secreted MCP-1 at a MOI of 25:1 This suggests that hMCs are more sensitive to pathogenic threats compared to mouse MCs.

MCs appear to exhibit a different range of functional responses toward commensal bacteria. Magerl and colleagues demonstrated *in vitro* that high doses ($1x10^9$ CFU/ml) of a probiotic *E. coli* strain could inhibit mouse MC degranulation [24]. Furthermore, gut resident probiotic bacterial strains such as *Lactobacillus rhamnosus* and *Bifidobacterium animalis* downregulate degranulation-inducing receptors [40]. While degranulation may be redundant in the MC response to *E. coli*, the release of pro-inflammatory mediators, including TNF, IL-8 [41-43] and leukotrienes appears pivotal for *E. coli* infection recovery [44]. In our study, *E. coli* strain ATCC 25922, originally a clinical strain, was not associated with an inhibitory effect on degranulation, perhaps reflecting differences in the *E. coli* strains used by

ourselves and Margerl [24]. However, it induced a lower level of degranulation compared to *L. monocytogenes*. IL-8 and MCP-1 (CCL2) and prostaglandin D2 are important chemo-attractants that recruit inflammatory cells in defence to pathogens [45]. We showed the release of IL-8, MCP-1 and prostaglandin D_2. This suggests hMCs may strategically contribute to cell recruitment during an acute infection against pathogenic bacteria by the selective release of chemokines while avoiding an enhanced inflammatory reaction caused by an uncontrolled hMC degranulation upon non-pathogenic bacterial encounter. Furthermore, *E. coli* virulence factors activate MC responses. These include the type 1 fimbriae, which induces the release of TNF, IL-6 and eicosanoids [46], and the pore-forming toxin α-hemolysin, which has been described to contribute to MC degranulation and IL-8 release [41]. Thus, these virulence factors may be involved in the chemokine secretion, degranulation and prostaglandin D2 release observed in our hMC cultures.

S. pneumoniae is a Gram-positive coccus that resides within the upper respiratory tract but can cause pneumonia and other mucosal infections by its outgrowth [47]. Pneumolysin is a crucial virulent factor of *S. pnaumoniae* [48]. Cruse et al. observed that pneumolysin together with H_2O_2 are virulent factors causing hMC cytotoxicity [49]. Our data demonstrated that after 2hrs of hMC stimulation, *S. pneumoniae* was capable of inducing significant amounts of ROS, while cell viability was not affected. However, we cannot exclude that hMC cytotoxicity occurs at later time points.

The un-encapsulated *S. pneumoniae* strain D39 (R6) used in the present study serves to understand dynamics of colonisation in mucosal tissue where hMC reside. Previous studies have shown that MC degranulation after encountering encapsulated *S. pneumoniae* only occurs with high bacterial concentrations [49-51]. For instance, Barbuti et al. observed MC degranulation using an MOI of 250:1 with a peak of histamine release after 4h [50]. Our study, demonstrated that *S. pneumoniae* does not causes either degranulation or chemokine secretion in primary hMCs at a MOI of 25:1. This lack of MC response against *S. pneumoniae* may reflect differences in the capsule status of the strains used, but interestingly this lack of MC response seen in our studies has also been observed in the *in vivo* infection [52] where van den Boogaard et al. showed prolonged survival of MC-deficient mice compared to WT mice during *S. pneumoniae* infection [52]. Furthermore, the inhibition of MC degranulation in WT mice did not change the disease outcome [52]. This suggests that during *S. pneumoniae* infection, MCs plays a detrimental role for the host, which is independent of degranulation.

Finally, we have investigated hMC responses against *S. aureus*, which is a Gram-positive coccus colonizing the skin and gut [53]. The *in vivo* activity of *S. aureus* on MCs appears diverse and remains controversial and in part may reflect that different *S. aureus* strains have been used in a number of different studies. *S. aureus* has been shown to induce bone marrow-derived murine MCs to release TNF-α and tryptase [54, 55]. The latter phenomenon also occurs in skin MCs during *in vivo* infection followed by bacteria internalization [54]. In a murine peritoneal *S. aureus* infection model, MCs have no impact on the outcome of the disease [56]. However, in a lung *S. aureus* infection model, MCs display a protective role [57]. In contrast, our data show a lack of hMC degranulation in response to *S. aureus*, but rather a selective release of a major pro-inflammatory mediator, prostaglandin D_2 [58]. Therefore, the *in vivo* mouse data cited above demonstrates that the nature of the tissue influences the type of MC response raised to commensals and pathogens, and our data indicates that hMCs cells exploit differential strategies in antibacterial responses and commensalism compared to their mouse counterparts.

In summary, hMCs control bacterial infections via different mechanisms which are stimulus-specific and include degranulation, cytokine and chemokine secretion and ET formation. Our data demonstrated that while *L. monocytogenes* robustly induces degranulation independent of ROS production, *S. pneumoniae* releases ROS with negligible DNA externalization. Furthermore, *E. coli* exhibited a relatively low level of degranulation with higher release of pro-inflammatory mediators, whereas *S. aureus* selectively released prostaglandin D_2 without promoting any degranulation. Thus, the present study not only underlines how versatile and plastic hMCs operate in antibacterial immune responses, but also how adaptable MCs are in the interaction with commensals.

REFERENCES: Chapter 4

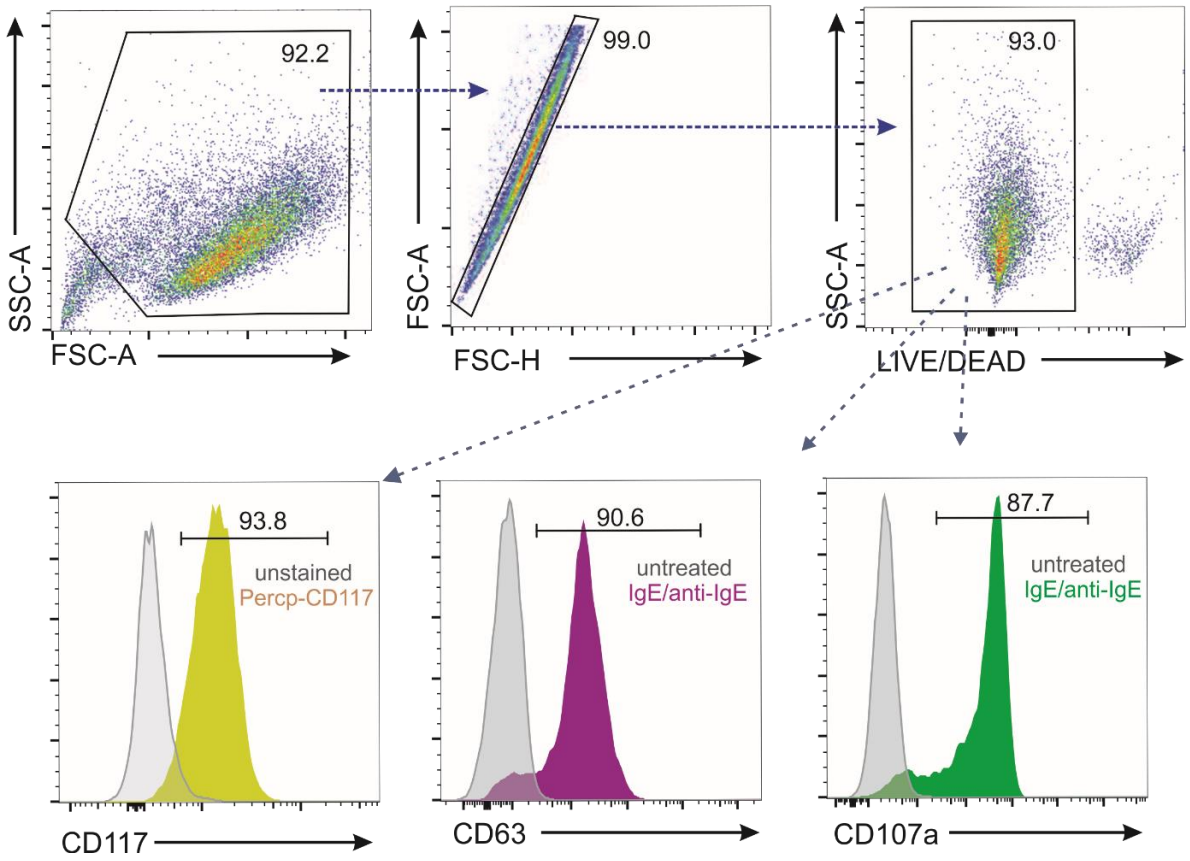
1. Johnzon, C.-F., E. Rönnberg, and G. Pejler, *The Role of Mast Cells in Bacterial Infection.* The American Journal of Pathology, 2016. **186**(1): p. 4-14.
2. Puxeddu, I., et al., *Mast cells in allergy and beyond.* The International Journal of Biochemistry & Cell Biology, 2003. **35**(12): p. 1601-1607.
3. Li, Z., et al., *Adult Connective Tissue-Resident Mast Cells Originate from Late Erythro-Myeloid Progenitors.* Immunity, 2018. **49**(4): p. 640-653.e5.
4. Kitamura, Y. and J. Fujita, *Regulation of mast cell differentiation.* BioEssays, 1989. **10**(6): p. 193-196.
5. Abraham, S.N. and A.L. St. John, *Mast cell-orchestrated immunity to pathogens.* Nature Reviews Immunology, 2010. **10**: p. 440.
6. Gri, G., et al., *Mast cell: an emerging partner in immune interaction.* Deciphering new molecular mechanisms of mast cell activation, 2014: p. 52.
7. Mukai, K., et al., *Mast cells as sources of cytokines, chemokines, and growth factors.* Immunological Reviews, 2018. **282**(1): p. 121-150.
8. Gaudenzio, N., et al., *Different activation signals induce distinct mast cell degranulation strategies.* The Journal of Clinical Investigation, 2016. **126**(10): p. 3981-3998.
9. Harata, G., et al., *Bifidobacterium suppresses IgE-mediated degranulation of rat basophilic leukemia (RBL-2H3) cells.* Microbiology and Immunology, 2010. **54**(1): p. 54-57.
10. Scheb-Wetzel, M., et al., *New insights into the antimicrobial effect of mast cells against Enterococcus faecalis.* Infection and immunity, 2014. **82**(11): p. 4496-4507.
11. Muñoz, S., et al., *Mast cell activation by Mycobacterium tuberculosis: mediator release and role of CD48.* The Journal of Immunology, 2003. **170**(11): p. 5590-5596.
12. Goldmann, O. and E. Medina, *The expanding world of extracellular traps: not only neutrophils but much more.* NETosis: At the Intersection of Cell Biology, Microbiology, and Immunology, 2012: p. 9.
13. Möllerherm, H., M. von Köckritz-Blickwede, and K. Branitzki-Heinemann, *Antimicrobial Activity of Mast Cells: Role and Relevance of Extracellular DNA Traps.* Frontiers in Immunology, 2016. **7**.
14. Papayannopoulos, V., *Neutrophil extracellular traps in immunity and disease.* Nature Reviews Immunology, 2017. **18**: p. 134.
15. Yipp, B.G. and P. Kubes, *NETosis: how vital is it?* Blood, 2013. **122**(16): p. 2784-2794.
16. Brinkmann, V., et al., *Neutrophil Extracellular Traps Kill Bacteria.* Science, 2004. **303**(5663): p. 1532-1535.
17. Fuchs, T.A., et al., *Novel cell death program leads to neutrophil extracellular traps.* The Journal of Cell Biology, 2007. **176**(2): p. 231-241.
18. Pilsczek, F.H., et al., *A Novel Mechanism of Rapid Nuclear Neutrophil Extracellular Trap Formation in Response to Staphylococcus aureus.* The Journal of Immunology, 2010. **185**(12): p. 7413-7425.
19. Campillo-Navarro, M., et al., *Listeria monocytogenes induces mast cell extracellular traps.* Immunobiology, 2016.
20. von Köckritz-Blickwede, M., et al., *Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation.* Blood, 2008. **111**(6): p. 3070-3080.
21. Campillo-Navarro, M., et al., *Mycobacterium tuberculosis Catalase Inhibits the Formation of Mast Cell Extracellular Traps.* Frontiers in Immunology, 2018. **9**(1161).
22. Bahri, R., et al., *Mast cell activation test in the diagnosis of allergic disease and anaphylaxis.* Journal of Allergy and Clinical Immunology, 2018. **142**(2): p. 485-496.e16.
23. Schindelin, J., et al., *Fiji: an open-source platform for biological-image analysis.* Nature methods, 2012. **9**(7): p. 676-682.
24. Magerl, M., et al., *Non-pathogenic commensal Escherichia coli bacteria can inhibit degranulation of mast cells.* Experimental Dermatology, 2008. **17**(5): p. 427-435.

25. Metcalfe, D.D., et al., *Biomarkers of the involvement of mast cells, basophils and eosinophils in asthma and allergic diseases.* World Allergy Organization Journal, 2016. **9**: p. 7.
26. Cardamone, C., et al., *Mast cells as effector cells of innate immunity and regulators of adaptive immunity.* Immunology Letters, 2016. **178**: p. 10-14.
27. Ramos-Kichik, V., et al., *Neutrophil extracellular traps are induced by Mycobacterium tuberculosis.* Tuberculosis, 2009. **89**(1): p. 29-37.
28. Li, Y. and M.A. Trush, *Diphenyleneiodonium, an NAD(P)H Oxidase Inhibitor, also Potently Inhibits Mitochondrial Reactive Oxygen Species Production.* Biochemical and Biophysical Research Communications, 1998. **253**(2): p. 295-299.
29. Lin, A.M., et al., *Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis.* Journal of immunology (Baltimore, Md. : 1950), 2011. **187**(1): p. 490-500.
30. Mestas, J. and C.C.W. Hughes, *Of Mice and Not Men: Differences between Mouse and Human Immunology.* The Journal of Immunology, 2004. **172**(5): p. 2731-2738.
31. Krystel-Whittemore, M., K.N. Dileepan, and J.G. Wood, *Mast Cell: A Multi-Functional Master Cell.* Frontiers in Immunology, 2016. **6**(620).
32. Wallecha, A., et al., *Listeria monocytogenes-derived listeriolysin O has pathogen-associated molecular pattern-like properties independent of its hemolytic ability.* Clinical and vaccine immunology : CVI, 2013. **20**(1): p. 77-84.
33. Jobbings, C.E., et al., *Listeria monocytogenes alters mast cell phenotype, mediator and osteopontin secretion in a listeriolysin-dependent manner.* PloS one, 2013. **8**(2): p. e57102-e57102.
34. Rådinger, M., et al., *Generation, Isolation, and Maintenance of Human Mast Cells and Mast Cell Lines Derived from Peripheral Blood or Cord Blood.* Current Protocols in Immunology, 2010. **90**(1): p. 7.37.1-7.37.12.
35. Michel, A. *Primary culture of mast cells and mast cell lines in rodent and humans*. European Mast Cell and Basophil Research Network 2018.; Available from: http://www.embrn.eu/wiki/wiki-start/primary-mast-cells-and-mast-cells-lines/.
36. Gekara, N.O. and S. Weiss, *Mast cells initiate early anti-Listeria host defences.* Cellular microbiology, 2008. **10**(1): p. 225-236.
37. David, D. and P. Cossart, *Recent advances in understanding Listeria monocytogenes infection: the importance of subcellular and physiological context [version 1; peer review: 3 approved].* F1000Research, 2017. **6**(1126).
38. Gekara, N.O. and S. Weiss, *Mast cells initiate early anti-Listeria host defences.* Cellular Microbiology, 2008. **10**(1): p. 225-236.
39. Jobbings, C.E., et al., *Listeria monocytogenes Alters Mast Cell Phenotype, Mediator and Osteopontin Secretion in a Listeriolysin-Dependent Manner.* PLOS ONE, 2013. **8**(2): p. e57102.
40. Oksaharju, A., et al., *Probiotic Lactobacillus rhamnosus downregulates FCER1 and HRH4 expression in human mast cells.* World J Gastroenterol, 2011. **17**(6): p. 750-9.
41. Krämer, S., et al., *Selective Activation of Human Intestinal Mast Cells by Escherichia coli Hemolysin.* The Journal of Immunology, 2008. **181**(2): p. 1438-1445.
42. Malaviya, R., et al., *Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α.* Nature, 1996. **381**(6577): p. 77-80.
43. Echtenacher, B., D.N. Männel, and L. Hültner, *Critical protective role of mast cells in a model of acute septic peritonitis.* Nature, 1996. **381**(6577): p. 75-77.
44. Malaviya, R. and S.N. Abraham, *Role of mast cell leukotrienes in neutrophil recruitment and bacterial clearance in infectious peritonitis.* Journal of Leukocyte Biology, 2000. **67**(6): p. 841-846.
45. Turner, M.D., et al., *Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2014. **1843**(11): p. 2563-2582.
46. Abraham, S.N., J.-S. Shin, and R. Malaviya, *Type 1 Fimbriated Escherichia coli–Mast Cell Interactions in Cystitis.* The Journal of Infectious Diseases, 2001. **183**(Supplement_1): p. S51-S55.

47. Weiser, J.N., D.M. Ferreira, and J.C. Paton, *Streptococcus pneumoniae: transmission, colonization and invasion.* Nature Reviews Microbiology, 2018. **16**(6): p. 355-367.
48. Kadioglu, A., et al., *The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease.* Nature Reviews Microbiology, 2008. **6**(4): p. 288-301.
49. Cruse, G., et al., *Human Lung Mast Cells Mediate Pneumococcal Cell Death in Response to Activation by Pneumolysin.* The Journal of Immunology, 2010. **184**(12): p. 7108-7115.
50. Barbuti, G., et al., *Streptococcus pneumoniae induces mast cell degranulation.* International Journal of Medical Microbiology, 2006. **296**(4): p. 325-329.
51. Fritscher, J., et al., *Mast Cells Are Activated by Streptococcus pneumoniae In Vitro but Dispensable for the Host Defense Against Pneumococcal Central Nervous System Infection In Vivo.* Frontiers in immunology, 2018. **9**: p. 550-550.
52. van den Boogaard, F.E., et al., *Mast Cells Impair Host Defense During Murine Streptococcus pneumoniae Pneumonia.* The Journal of Infectious Diseases, 2014. **210**(9): p. 1376-1384.
53. Thomer, L., O. Schneewind, and D. Missiakas, *Pathogenesis of Staphylococcus aureus Bloodstream Infections.* Annual Review of Pathology: Mechanisms of Disease, 2016. **11**(1): p. 343-364.
54. Abel, J., et al., *Staphylococcus aureus Evades the Extracellular Antimicrobial Activity of Mast Cells by Promoting Its Own Uptake.* Journal of Innate Immunity, 2011. **3**(5): p. 495-507.
55. Lei, Z., et al., *Activation of mast cells in skin abscess induced by Staphylococcus aureus (S. aureus) infection in mice.* Research in Veterinary Science, 2018. **118**: p. 66-71.
56. Rönnberg, E., et al., *Mast cells are activated by Staphylococcus aureus in vitro but do not influence the outcome of intraperitoneal S. aureus infection in vivo.* Immunology, 2014. **143**(2): p. 155-163.
57. Liu, C., et al., *Mast cells participate in regulation of lung-gut axis during Staphylococcus aureus pneumonia.* Cell Proliferation, 2019. **52**(2): p. e12565.
58. Scher, J.U. and M.H. Pillinger, *The Anti-Inflammatory Effects of Prostaglandins.* Journal of Investigative Medicine, 2009. **57**(6): p. 703-708.

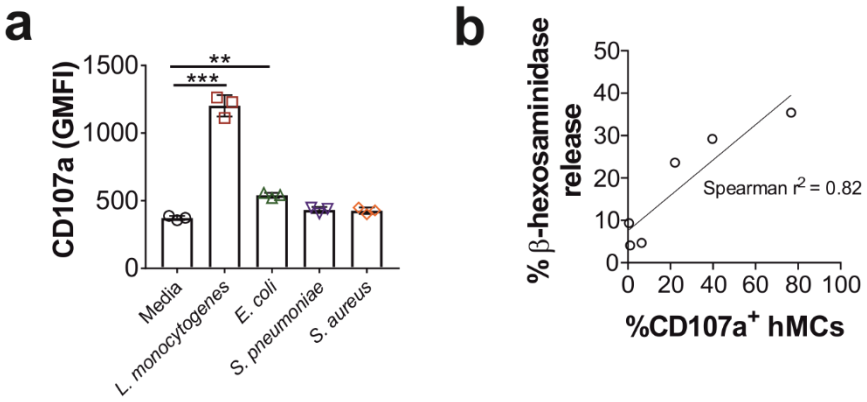
SUPPLEMENTARY MATERIAL

Supplemental Figure 4.A



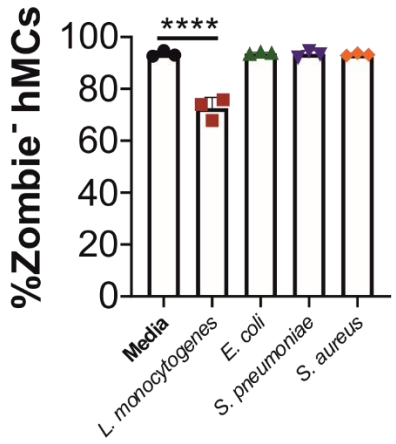
Supplemental Figure 4.A. Characterization of hMCs. hMCs isolated from haematopoietic progenitors were cultured for 8 to 10 weeks, and cellular maturity was analysed using flow cytometry. Cells were pre-sensitized with human IgE antibodies, stimulated for 1 hour with anti-IgE antibodies, stained with anti-CD107a, anti-CD63 and anti-CD117 antibodies and DAPI dye and analysed by flow cytometry. (a) Total cell population was selected by side and forward scatter (SSC-A and FSC-A) and single cells were discriminated by forward scatter A and H (FSC-A and FSC-H). Live cells (a) were selected used to gate (b) $CD117^{+}$ cells, (c) $CD107a^{+}$ and $CD63^{+}$ cells (degranulated cells). Fluorescence minus one values (FMO) for CD107a, CD63 and CD117 were used as a control of staining.

Supplemental Figure 4.B



Supplemental Figure 4.B. Correlation between percentage of β-hexosaminidase release and CD107a expressing cells. hMCs were stimulated with *L. monocytogenes*, *E. coli*, *S. pneumonia*, and *S. aureus,* and stained with anti-CD107a antibodies **(a)** bars show the mean of CD107a GMFI of three replicates from one representative experiment out of three independent experiments. Analysis was performed using one-way ANOVA and Tukey's multiple comparisons post-test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.1). **(b)** hMCs were stimulated with *L. monocytogenes* at a MOI of 25:1 for 2h. β-hexosaminidase release and CD107a expression was measured. The correlation between the two is shown Dots show the mean of three independent experiments. The Spearman correlation test was performed using GraphPad Prism, the value r represents the Spearman coefficient correlation.

Supplemental Figure 4.C



Supplemental Figure 4.C. Mast cell viability after bacterial stimulation. Cell viability was studied stimulating hMCs with *L. monocytogenes, E. coli, S. pneumonia* and *S. aureus* for 2hrs. After stimulation cells were stained with Zombie NIR to evaluate the percentage of live cells. (a) Live cells are represented as Zombie⁻ cells. Graph shows the mean of three replicates of a representative experiment out of three independent experiments performed. Analysis was calculated using one-way ANOVA and Tukey's multiple comparisons post-test (****p<0.0001).

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CHAPTER 5: Paper 3

Differential mast cell numbers and characteristics in human tuberculosis pulmonary lesions

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Journal: European Journal of Pathology

Status: Submitted

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Compliance with Ethical Standards: Authors declare that no conflict of interest happened during the present study. The present research involved the use of lung sections obtained from deceased individuals. Autopsies were selected from the Pathology Department files at the National Institute of Medical Sciences and Nutrition "Salvador Zubiran" in Mexico City. Tissue samples were obtained during legally authorized autopsies with signed permission by a relative who agreed with tissue sample donation for this and previous studies [1]. Additional tissue was obtained from the Pathology Department of the General Hospital of Mexico 'Eduardo Liceaga' at Mexico City, and the ethical statement was approved by the local ethic committee of the Hospital Infantil de Mexico, Federico Gomez (No. HIM/2008/015).

ABSTRACT

Tuberculosis (TB) is still a major worldwide health threat and primarily a lung disease. The innate immune response against *Mycobacterium tuberculosis* (*Mtb*) is orchestrated by dendritic cells, macrophages, neutrophils, natural killer cells and apparently mast cells (MCs). MCs are located at mucosal sites including the lungs and contribute in host-defence against pathogens, but little is known about their role during *Mtb* infection. This study investigates the location and characteristics of MCs in TB lesions to assess their contribution to TB pathology. To this purpose, number, location and phenotype of MCs were studied in 11 necropsies of pulmonary TB and 3 necropsies of non-TB infected lungs that were used as controls. MCs were localised at pneumonic areas, in the granuloma periphery and particularly abundant in fibrotic tissue. Furthermore, MCs displayed intracellular *Mtb* and IL-17A and TGF-β immunostaining. These findings were validated by analysing, post-mortem lung tissue microarrays from 44 individuals with pulmonary TB and 25 control subjects. In affected lungs, increased numbers of MCs expressing intracellularly both tryptase and chymase were found at fibrotic sites. Altogether, our data suggest that MCs are recruited at the inflammatory site and that actively produce immune mediators such as proteases and TGF-β that may be contributing to late fibrosis in TB lesions.

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*), remains one of the deadliest bacterial infections worldwide [2]. During infection, *Mtb* reaches the lungs where different innate immune cells reside including mast cells (MCs) [3-5]. *Mtb* is phagocytosed by macrophages leading to the release of diverse cytokines, including TNFα and IL-6 that drive the inflammatory process [6]. To control *Mtb* spread, innate and adaptive immune cells surround infected phagocytic cells promoting granuloma formation [6, 7]. The cytokines IL-17A and TNF-α are known to contribute to the process [8]. When granuloma containment fails, different lung injuries including pneumonia, bronchitis, caseous necrosis and eventually fibrosis prevail [9, 10]. The fibrotic process in human lungs has been associated with the presence of TGF-β, and the proteases, tryptase and chymase [11, 12]. The final TB pathology phase culminates in irreversible lung tissue damage manifested by necrosis and fibrosis [9, 10]. Although many cells are involved in this process, little is known about the contribution of MCs in this pathology.

MCs are distributed in lungs and mucosal tissues and contribute to host-defence against bacterial infections [13]. In humans, MCs are classified as tryptase+ MCs (MC_T), chymase+ MCs (MC_C) or both tryptase+ and chymase+ MCs (MC_{TC}) [14]. Upon bacterial exposure, they release a wide variety of cytokines and chemokines, including IL-17, TNF-α, IL-8 and TGF-β, either by degranulation or canonical secretory pathways [15]. Additionally, during lung infections, MCs are altered in numbers, phenotype and localization [16, 17]. For instance, MC numbers are decreased in lungs of *Streptococcus pneumoniae* infected patients [18]. Furthermore, MCs are capable to phagocytose bacteria and present antigens [19]. Although hypothetically MCs have an important role in TB [4], little has been explored. For instance, it is unknown if MCs are localized at pulmonary tuberculous human lesions and what is the predominant MC phenotype. It is also unclear whether MC cytokines, e.g. IL-17A, and TGF-β, contribute to the inflammatory process, and formation and maintenance of granulomas and fibrogenesis.

In this study, we examined number, distribution and phenotype of MCs in human TB-infected lungs and MC cytokine expression at granulomas and fibrotic sites in TB-infected lung tissue. Our descriptive findings demonstrated that MCs are likely to participate in the early inflammatory phase, as well as to the late fibrosis formation during TB pathology.

MATERIALS AND METHODS

Ethical statement and tissue procurement

Lung tissue sections from 11 necropsies of deceased TB patients and 3 controls from non-TB infected necropsies were obtained from the Pathology Department files at the National Institute of Medical Sciences and Nutrition “Salvador Zubiran” in Mexico City. Tissue samples were obtained during legally authorized autopsies with signed permission by a relative who agreed with tissue sample donation for the present and previous studies [1]. The microarray tissue study from 44 individuals with pulmonary TB and 25 control subjects were taken from the Pathology Department of the General Hospital of Mexico ‘Eduardo Liceaga’ at Mexico City, and the ethical statement was approved by the local ethic committee of the Hospital Infantil de Mexico, Federico Gomez (No. HIM/2008/015).

TB tissue processing

Macroscopically, lung tissue from 11 necropsies of deceased TB patients showed extensive cavitary bilateral lung disease, surrounded by numerous white nodules with irregular shapes and size that alternated with pneumonia patches. Extensive sampling was performed, obtaining several tissue fragments from different lesions and were embedded in paraffin blocks sectioned at 3µm and mounted in glass slides. Additionally, we used post-mortem lung tissue, essentially granulomas, from 44 individuals with pulmonary TB and 25 control subjects (subjects who died as a result of any other cause without significant pulmonary disease). The lung tissues (TB and control tissue samples) were organized in a tissue microarray (TMA) as previously described [20]. One sample of each section (lung sections and TMA) were stained with haematoxylin and eosin (HE) to select lung lesions for study. Spare sections were left at room temperature before being used for immunoperoxidase and immunofluorescence staining.

Immunoperoxidase staining

Lung sections were deparaffinized and treated with antigen retriever (1X; Bio SB, Santa Barbara, California) for 5 minutes under microwave heating. Endogenous peroxidase was blocked incubating tissue with methanol-H2O2 (9:1) for 10 minutes. After three washes, unspecific sites were blocked using a background sniper (BIOCARE MEDICAL; Pacheco, California) for 30 minutes. Slides were washed and incubated with either a rabbit anti-human

chymase antibody (Ab186417, Abcam; Cambridge, United Kingdom) or a mouse anti-tryptase antibody (Ab2378, Abcam; Cambridge, United Kingdom) for 2 hours. After three washes, tissue was processed using a mouse/rabbit PolyDetector DAB (3-3´-diaminobenzidine)/HRP (horseradish peroxidase) brown detection system (BSB0219, Bio SB; Santa Barbara, California) following manufacturer´s instructions. Micrographs were acquired using a LEICA DMLS microscope with a 2.5X and 40X dry objectives equipped with a LEICA DFC295 camera and analysed using an automated image analyser (QWin Leica; Wetzlar, Germany).

Immunofluorescence staining

To visualize MC phenotypes, lung tissue sections were deparaffinized, treated with DNA retriever (1X; Bio SB; Santa Barbara, California) for 5 minutes under heating and incubated with blocking buffer (goat serum 1:10 in PBS + tween 0.1%) for 30 minutes. After three washes, tissue was incubated with a rabbit anti-human chymase antibody (Ab186417, Abcam; Cambridge, United Kingdom), and a mouse anti-tryptase antibody (Ab2378, Abcam; Cambridge, United Kingdom) for 1h followed by 1h incubation with a goat anti-mouse antibody conjugated to Alexa 488 fluorophore (Ab150117, Abcam; Cambridge, United Kingdom) and a goat anti-rabbit antibody conjugated to Alexa 647 fluorophore (Ab150083, Abcam; Cambridge, United Kingdom). After three washes, tissue was mounted using a fluoroshield mounting media containing 4´,6-diamidino-2-phenylindole (DAPI, Abcam; Cambridge, United Kingdom). Slides were analysed using a fluorescent microscope OlympusBX41 with either 40x and 10x dry objectives. Images were acquired using a Zen 2.6 blue and analysed using Fiji.

To analyse cytokine expression and *Mtb* internalization, lung sections were deparaffinized, treated with DNA retriever (1X; Bio SB; Santa Barbara, California) for 5 minutes under heating and incubated with blocking buffer (goat serum 1:10 in PBS + tween 0.1%) for 30 minutes. After three washes, tissues were incubated with a mouse anti-tryptase antibody (Ab2378, Abcam, Cambridge, United Kingdom) and either a rabbit polyclonal anti-*Mtb* (CP140C, BioCare Medical), capable to recognize diverse *Mtb*-cell wall and secreted antigens, rabbit anti-TGF-β (Jackson Immunoresearch, Cambridge, United Kingdom) or rabbit anti-IL-17 (SC7927 Santa Cruz Lab, USA) antibodies for 1h followed by 1h incubation with a goat anti-mouse antibody conjugated to either Alexa 488 (Ab150117, Abcam; Cambridge, United Kingdom) or Alexa 647 fluorophores (Ab150115, Abcam; Cambridge,

United Kingdom) and a goat anti-rabbit antibody conjugated to either Alexa 647 (Ab150083, Abcam; Cambridge, United Kingdom) or Alexa 488 fluorophores (Ab150081, Abcam; Cambridge, United Kingdom). After three washes, tissue was mounted using a fluoroshield mounting media containing 4´,6-diamidino-2-phenylindole (DAPI, Abcam; Cambridge, United Kingdom). Slides were analysed using a confocal microscope LSM 710 DUO, Carl Zeiss.

MC quantification

Forty-four TMAs from TB-infected lung sections from autopsy cases and 22 non-TB infected controls contained in 4 different slides were stained with HE and analysed. Five TMAs were selected as control lung tissue and 10 TMAs presenting fibrosis were selected as representative fibrotic tissue. Selected TMAs were immunostained with tryptase and chymase (as described above) and studied at 10x magnification using a fluorescent microscope OlympusBX41 and acquired using Zen 2.6 blue software system. One high power field was taken for each TMA and all single positive (MC_C or MC_T) and double-positive (MC_{TC}) cells were counted per field using Fiji. MC numbers were graphed using GraphPrism.

Statistical analysis

A Shapiro-Wilk test was performed to determine normality during phenotype quantification. Statistical analysis was achieved using the Kruskal-Wallis test and a Dunn´s multiple comparison post-test (adjusted $p \leq 0.01$) using GraphPad Prism 8th edition.

RESULTS

Tryptase positive mast cells are the most abundant phenotype in non-TB infected human lungs

In physiological conditions, MCs expressing either tryptase or chymase or both proteases reside in alveolar parenchyma. However, in pulmonary infections, MC numbers and phenotype are altered [21]. To investigate lung MC distribution and their characteristics we studied their number, location and phenotype in autopsies from control lungs (non-TB infected). Control cases had heart attacks as death cause with lungs showing overall a normal structure with some focal patches of centrilobular emphysema **(Figure 5.1A)**. These tissues showed MC_T (**Figure 5.1B)** and lesser MC_C (**Figure 5.1C)** at alveolar walls. Both MC_T and MC_C were preferentially located in blood vessels adventitia. Moreover, MC_T were the most abundant phenotype (median=5 cells per field) (**Figure 5.1D)** followed by MC_{TC} (median=2 cells per field), whereas MC_C were not detected. In fact, all MC_C observed were also tryptase+ therefore MC_{TC} (**Figure 5.1E)**. Thus, MCs expressing only chymase were rare whereas tryptase positive MCs were predominant in the human lung parenchyma.

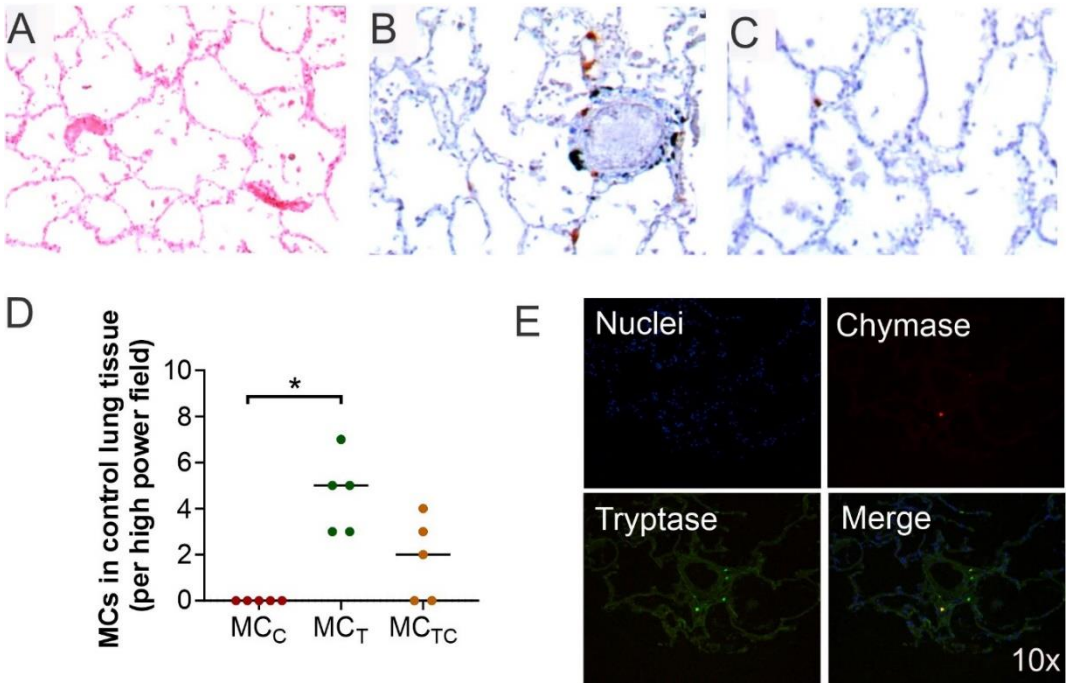

Figure 5.1. Tryptase positive mast cells are predominant in non-tuberculosis infected human lung tissue. (A) Representative micrograph HE staining of lung section from non-TB infected lung autopsy (cause of death heart attack) showing preserved alveolar structure. **(B)** Tryptase Immunostaining shows several positive MCs in the alveolar-capillary interstitium and in the adventitia layer of blood vessels. **(C)** Chymase immunostaining shows occasional positive cells. **(D)** Predominance of tryptase+ MCs in non-tuberculous lung tissue was analysed by morphometry study performed in five microarrays. **(E)** Representative immunofluorescence microarray studied with fluorescent microscopy shows MC_T (green), MC_C (red) and MC_{TC} (merge) surrounding a blood vessel. Shapiro-Wilk test was done to determine normality. Statistical comparison was performed using the Kruskal-Wallis test and Dunn´s multiple comparison post-test (adjusted $p \leq 0.01$).

Mast cells are located at active inflammatory sites of TB-infected lungs and show intracellular mycobacterial antigens

MC numbers, location and phenotype found in non-infected lungs were used as controls to investigate MC characteristics in TB lung lesions. Both MC phenotypes (MC_T and MC_C) were abundant at inflammatory (granulomas, pneumonia, vascular and airways walls) and fibrotic areas **(Figure 5.2)** but absent in the vicinity of necrotic sites (**Supplemental Figure 5.A**). At pneumonic areas **(Figure 5.2A),** numerous MCs were seen in alveolar walls and alveolar lumen (**Figure 5.2B-C**), while in blood vessels **(Figure 5.2D)** MCs were found in the adventitia and bronchial airways **(Figure 5.2E-F)**. Both phenotypes were positioned below the epithelium, in the submucosa and the muscular wall between smooth muscle cells. Furthermore, as shown in **Figure 5.2G**, MC_T stationed in inflammatory regions of TB-infected lung sections showed vacuoles containing *Mtb* antigens. Thus, both MC_T and MC_C reside in TB lung lesions and store *Mtb*.

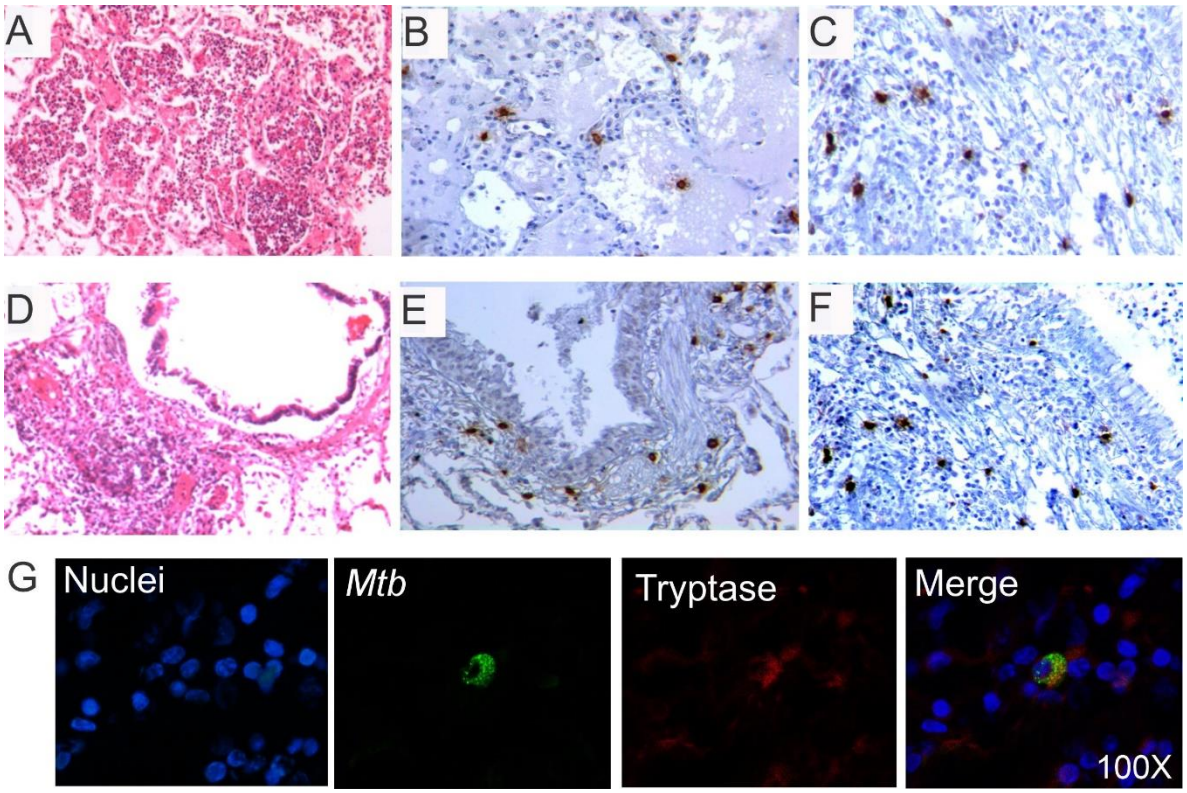

Figure 5.2. Tryptase and chymase mast cells are located at active inflammatory lesions and show intracellular mycobacterial antigens. (A) Representative HE micrograph showing areas of tuberculous pneumonia. **(B)** Tryptase+ MCs are present in alveolar walls and lumen. **(C)** Chymase+ MCs show similar numbers and distribution as tryptase+ MCs in the pneumonic areas. **(D)** Extensive inflammatory infiltrate is detected in bronchial walls. **(E)** Numerous tryptase+ MCs are located in the bronchial submucosa. **(F)** Numerous chymase+ MCs are in bronchial wall and neighbour alveoli. Micrographs are representative of 11 necropsies of TB patients. **(G)** A representative section with extensive inflammation in pneumonic areas was incubated with polyclonal anti-*Mtb* (Alexa 488 label) and anti-tryptase antibodies (Alexa 647 label). High power micrograph shows a MC expressing tryptase that colocalizes with *Mtb* antigens.

Mast cells are located at the periphery of granulomas and express IL-17

Granulomas are characterized by a necrotic core containing *Mtb* surrounded by macrophages and lymphocytes and a fibrotic external layer [20]. As shown in **Figure 5.3**, granulomas at different stages were analysed. In early or incipient granulomas **(Figure 5.3A)**, characterized by small nodular congregates of inflammatory cells, occasional MCs were seen intermixed with lymphocytes and macrophages (**Figure 5.3B-C**). However, MCs were not observed to infiltrate typical or mature well-organized granulomas **(Figure 5.3D)** but were located at their periphery (**Figure 5.3E-F**). Indeed, MCs were abundant at the fibrotic outer layer of necrotic granulomas (**Figure 5.3F**). Representative TB-infected tissue containing typical granulomas or incipient granulomas was incubated with anti-IL-17A and anti-tryptase antibodies followed by fluorescent staining. As shown in **Figure 5.3G**, IL-17 positive MCs were observed at neighbour inflammatory tissue near to typical granulomas or mixed with inflammatory cells in incipient granulomas. Thus, MCs are virtually absent inside mature granulomas but located at their periphery and expressing IL-17.

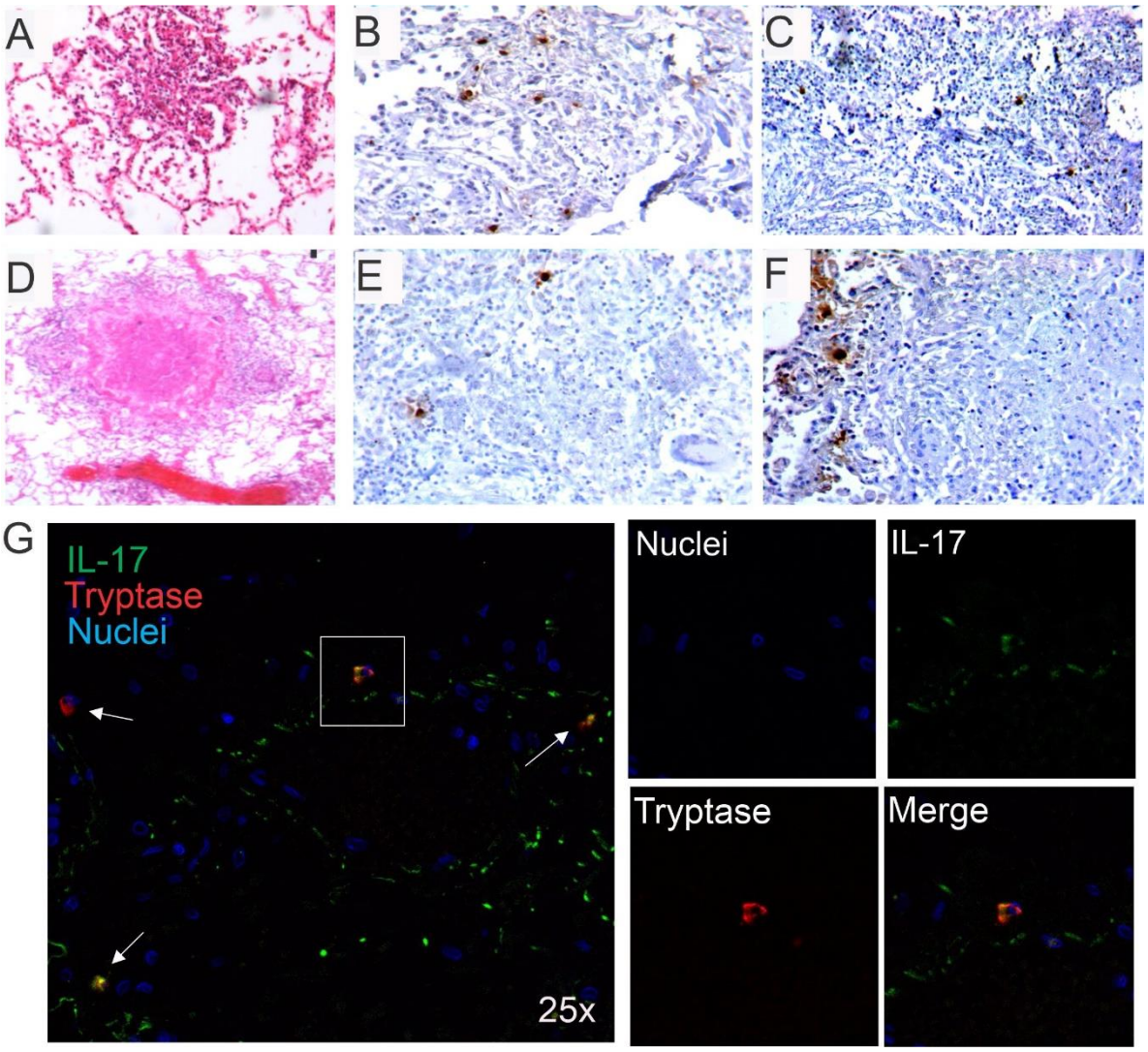

Figure 5.3. Mast cells are observed in granulomas and express IL-17A. Representative micrographs of different types of granulomatous lesions (incipient, mature or necrotic) stained with HE or with anti-human anti-tryptase or anti-chymase immunoperoxidase antibodies to identify MC_T or MC_C. **(A)** Small incipient granuloma. Both MC phenotypes **(B)** MC_T and **(C)** MC_C are detected within the inflammatory area of incipient granulomas. **(D)** Mature granuloma with central necrosis. **(E)** MC_T and MC_C **(F)** are not infiltrating mature granulomas but both subtypes are observed in the periphery. Micrographs are representative of 11 TB necropsies. Representative section of incipient granuloma was incubated with anti-IL-17A (Alexa 488 label) and anti-tryptase antibodies (Alexa 647 label). **(G)** Low power micrograph shows numerous IL-17+ cells and MC_T. High power micrograph of the inset shows a MC_T staining positive for IL-17A.

Mast cells are in high numbers in fibrotic tissue that surrounds granulomas and cavitary lesions

MCs were constantly detected at fibrotic areas around granulomas or wall cavities supporting a number of studies that have shown MCs participate in the fibrotic process [22, 23]. To characterize the nature of the MCs residing at fibrotic areas of TB infected tissues we determined their proteases expression. Although MC_T, MC_C and MC_{TC} phenotypes were all seen in fibrotic areas (**Figure 5.4A**), MC_{TC} were the most abundant (median=8.5 cells per field), followed by MC_T (median=2 cells per field) (**Figure 5.4B**). Since non-TB infected lung controls are colonised by MC_T (**Figure 5.1D**), our data suggest a switch in proteases expression with an increase of chymase at TB-induced healing and fibrotic sites. Furthermore, by a double immunofluorescence labelling for TGF-β and tryptase, we could demonstrate that some MCs in fibrotic tissue not only express TGF-β (**Figure 5.4D)** but exhibit a conserved but disorganized granular content suggesting their activation or partial degranulation (**Figure 5.4C)**. Thus, MCs at TB-induced lung lesions show an increased expression of chymase and pro-fibrogenic TGF-β and both may contribute to the fibrotic process.

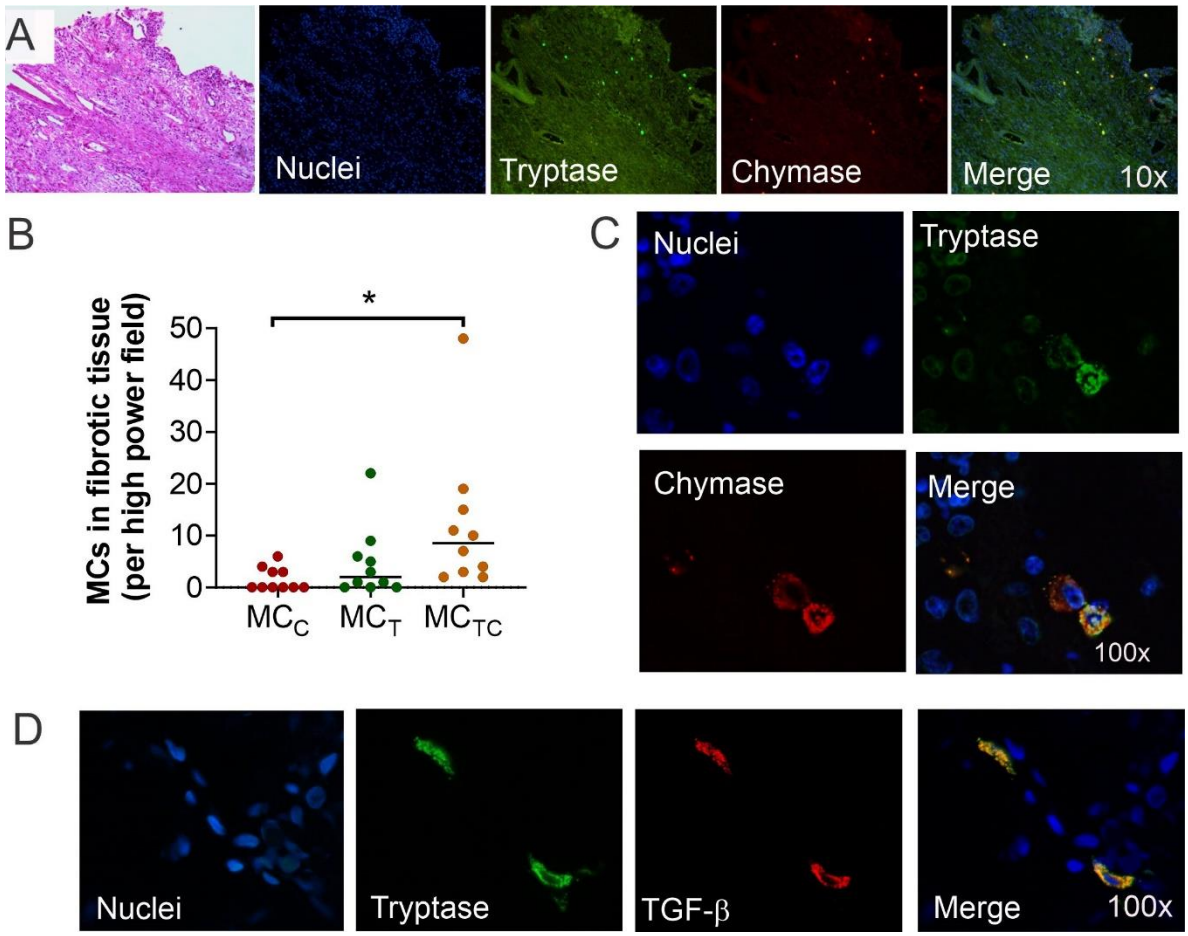

Figure 5.4. Tryptase and chymase positive mast cells are numerous in fibrotic lesions of tuberculous lungs. (A) Representative low power micrograph of fibrotic tuberculous nodule stained with HE and immunofluorescence micrographs from the same lesion showing MC_T (green), MC_C (red) and MC_{TC} (merge). **(B)** MCs subtypes counted in ten different high-power fields (microarrays) (40x) from 44 autopsy cases confirm that MC_{TC} were the most abundant phenotype at fibrotic areas. **(C)** High power micrograph at fibrotic areas shows MCs that express tryptase and chymase cytoplasmic granules which are partially degranulated. Shapiro-Wilk test was done to determine normality. Representative section of a fibrotic area was incubated with anti-TGF-β (Alexa 647 label) and anti-tryptase antibodies (Alexa 488 label). **(D)** High power micrograph shows TGF-β colocalization with MC_T. Statistical comparison was performed using the Kruskal-Wallis test and Dunn´s multiple comparison post-test (adjusted *$p \leq 0.01$).

DISCUSSION

Although MCs may play a role in TB infection [4], little has been explored and the available studies were performed in rodent models. Considering the differences in MC numbers and phenotypes between mouse and human lungs it is unclear whether the described findings are relevant for human pathology [21]. Our study demonstrates that in TB-infected human lungs MCs are located at pneumonic areas, in proximity to granulomas and particularly abundant at fibrotic sites. Furthermore, we observed a MC colocalization with *Mtb* antigens indicating an intracellular localisation of the pathogen. In addition, MCs located in proximity to granulomatous lesions were found to express IL-17, while TGF-β positive MCs were found embedded in the fibrotic tissue.

Our observations in lung controls showing MCs surrounding blood vessels and throughout the alveolar-capillary interstitial tissue with MC_T as the most abundant population are in line with current evidence that shows MC_T as predominant phenotype in large airways including bronchial and alveolar, followed by MC_{TC} which are more common at blood vessels, whereas MC_C are rare or sporadically observed in human lungs [24, 25]. Although it is not clearly understood yet, this suggests that MC_T reside in the alveolar parenchyma prone to activation or differentiation upon environmental stimulus [17].

MCs contribute to inflammatory processes [26] by secreting diverse chemoattractant molecules including IL-8, TNF and IL-6 [27] and are abundant at inflammatory sites as seen during idiopathic interstitial pneumonia [28]. In addition, in lung infections caused by *Chlamydia pneumoniae,* cellular infiltration in the lung airways is MC dependent [13, 29-31]. Our findings showing MCs located in inflammatory areas of TB human lungs suggest their potential involvement in this process. MC contribution in inflammation is likely to occur by MC-inflammatory mediator release as seen by MC secretion of TNF-α and IL-6 upon *Mtb* stimulation [32-34]. In our study, we observe that human-lung resident MCs display intracellular *Mtb* fragments. Since MCs have shown to phagocytose and kill bacteria via acidified vacuoles [35], our findings suggest that MCs uptake *Mtb* and could eliminate bacteria through phagocytosis.

Mtb persistence promotes ongoing cellular recruitment [36] that results in the formation of early granulomas [7, 36] that mature under the influence of cytokines such as IL-17A and TNF-α [37]. The association between MCs and TB-induced granulomas is still controversial. A positive correlation between MCs and granuloma formation was observed in tuberculous

lymphadenitis tissue [38] but not in tuberculous liver tissue [39]. Our data demonstrate that MCs infiltrate incipient granulomas and locate at the periphery or close proximity in mature or necrotic granulomas. Thus, this suggests a MC contribution in orchestrating granuloma formation, maturation or maintenance. This concept is supported by evidence showing that MC cytokines including IL-6 and TNF-α are necessary for granuloma maintenance in mouse TB infection [33]. Although MC-TNF-α association was not observed (data not shown), we showed IL-17A positive MCs in the periphery of granulomas. Using an IL-17A gene-knockout mouse model, Okamoto-Yoshida *et al.* reported that IL-17A is necessary for granuloma maturation with $\gamma\delta$ T cells as the major IL-17 source [40]. Also, granuloma formation was impaired in an IL-17A-deficient mouse model of sarcoidosis [41]. Therefore, we would like to propose the concept that MCs contribute to the initial step of cellular recruitment at the infection site, remain outside the inflammatory core during the adaptive immune stage, and orchestrate granuloma maturation via IL-17 expression.

In severe and chronic TB infection, fibrosis is the result of excessive inflammation [12]. MCs are abundant in fibrotic sites in non-infectious lung diseases [42], including idiopathic pulmonary fibrosis [28] and cystic fibrosis [43]. In addition, MCs products such as the fibroblast growth factor 2 (FGF-2) [44], prostaglandin E2 (PGE2) [45], TGF-β [11, 46, 47], tryptase [22] [23, 48, 49] and chymase [28, 50, 51] are known to contribute to fibrogenesis [45, 52, 53]. Our study reproduces findings described in idiopathic pulmonary fibrosis where MCs are increased in numbers and are partially degranulated at fibrotic sites [42, 54]. Furthermore, we found a switch in MC phenotype from MC_T to MC_{TC} in fibrotic areas. In line with this, Andersson *et al*, correlated high MC_{TC} numbers with lung function, tissue remodelling and TGF-β1 expression [43], suggesting MC_{TC} as important fibrosis mediators. Besides, mucosal MCs (MC_T) in coculture with fibroblast lead to differentiation of MC_T into connective tissue MCs (MC_{TC}). This process is coupled with an increase in fibroblast proliferation and an enhanced collagen synthesis necessary for fibrosis generation [55]. Thus, phenotypic change from MC_T to MC_{TC} with an increase in chymase expression would initiate or promote fibrosis together with the release of additional fibrogenesis-specific molecules.

CONCLUSIONS

In conclusion, our results demonstrate that MCs expressing IL-17 are localizing in TB-induced human lung injuries at inflammatory sites while TGF-β positive and chymase rich MCs are stationed in the proximity of mature granulomas and embedded in fibrotic tissue. Although this is a descriptive study, our data suggest that MCs probably contribute to both, early immune cellular recruitment via IL-17A release and late fibrosis formation with differentiated MC_T into MT_{TC}.

REFERENCES: Chapter 5

1. Bini, E.I., et al., *The implication of pro-inflammatory cytokines in the impaired production of gonadal androgens by patients with pulmonary tuberculosis.* Tuberculosis (Edinb), 2015. **95**(6): p. 701-706.
2. Pezzella, A.T., *History of pulmonary tuberculosis.* Thoracic surgery clinics, 2019. **29**(1): p. 1-17.
3. Sanyaolu, A., et al., *Tuberculosis: A Review of Current Trends.* 2019. **3**: p. 000123.
4. Garcia-Rodriguez, K.M., et al., *The Role of Mast Cells in Tuberculosis: Orchestrating Innate Immune Crosstalk?* Front Immunol, 2017. **8**: p. 1290.
5. de Martino, M., et al., *Immune Response to Mycobacterium tuberculosis: A Narrative Review.* Front Pediatr, 2019. **7**: p. 350.
6. Ramakrishnan, L., *Revisiting the role of the granuloma in tuberculosis.* Nat Rev Immunol, 2012. **12**(5): p. 352-66.
7. Pagán, A.J. and L. Ramakrishnan, *The Formation and Function of Granulomas.* Annu Rev Immunol, 2018. **36**: p. 639-665.
8. Shen, H. and Z.W. Chen, *The crucial roles of Th17-related cytokines/signal pathways in M. tuberculosis infection.* Cell Mol Immunol, 2018. **15**(3): p. 216-225.
9. Im, J.G., et al., *CT-pathology correlation of pulmonary tuberculosis.* Crit Rev Diagn Imaging, 1995. **36**(3): p. 227-85.
10. Subbian, S., et al., *Lesion-Specific Immune Response in Granulomas of Patients with Pulmonary Tuberculosis: A Pilot Study.* PLoS One, 2015. **10**(7): p. e0132249.
11. Nakayama, S., et al., *Transforming growth factor β- and interleukin 13-producing mast cells are associated with fibrosis in bone marrow.* Hum Pathol, 2017. **62**: p. 180-186.
12. Wilson, M.S. and T.A. Wynn, *Pulmonary fibrosis: pathogenesis, etiology and regulation.* Mucosal Immunol, 2009. **2**(2): p. 103-21.
13. Johnzon, C.-F., E. Rönnberg, and G. Pejler, *The Role of Mast Cells in Bacterial Infection.* The American Journal of Pathology, 2016. **186**(1): p. 4-14.
14. Gri, G., et al., *Mast cell: an emerging partner in immune interaction.* Front Immunol, 2012. **3**: p. 120.
15. Krystel-Whittemore, M., K.N. Dileepan, and J.G. Wood, *Mast Cell: A Multi-Functional Master Cell.* Frontiers in Immunology, 2016. **6**(620).
16. Hsieh, F.H., et al., *Human airway epithelial cell determinants of survival and functional phenotype for primary human mast cells.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(40): p. 14380-14385.
17. Cruse, G. and P. Bradding, *Mast cells in airway diseases and interstitial lung disease.* Eur J Pharmacol, 2016. **778**: p. 125-38.
18. van den Boogaard, F.E., et al., *Mast Cells Impair Host Defense During Murine Streptococcus pneumoniae Pneumonia.* The Journal of Infectious Diseases, 2014. **210**(9): p. 1376-1384.
19. Urb, M. and D.C. Sheppard, *The Role of Mast Cells in the Defence against Pathogens.* PLOS Pathogens, 2012. **8**(4): p. e1002619.
20. Seligson, D., et al., *Expression of transcription factor Yin Yang 1 in prostate cancer.* Int J Oncol, 2005. **27**(1): p. 131-41.
21. Erjefält, J.S., *Mast cells in human airways: the culprit?* European Respiratory Review, 2014. **23**(133): p. 299-307.
22. Abe, M., et al., *Effect of mast cell–derived mediators and mast cell–related neutral proteases on human dermal fibroblast proliferation and type I collagen production.* Journal of Allergy and Clinical Immunology, 2000. **106**(1): p. S78-S84.
23. Cairns, J.A. and A.F. Walls, *Mast cell tryptase stimulates the synthesis of type I collagen in human lung fibroblasts.* The Journal of clinical investigation, 1997. **99**(6): p. 1313-1321.
24. Andersson, C.K., et al., *Novel site-specific mast cell subpopulations in the human lung.* Thorax, 2009. **64**(4): p. 297-305.
25. Bradding, P., *Human lung mast cell heterogeneity.* Thorax, 2009. **64**(4): p. 278-280.

26. Katwa, P. and J. Brown, *Pulmonary Mast Cells.* Comparative Biology of the Normal Lung: Second Edition, 2015: p. 665-682.
27. Moiseeva, E.P. and P. Bradding, *Mast cells in lung inflammation.* Adv Exp Med Biol, 2011. **716**: p. 235-69.
28. Hirata, K., et al., *Enhanced mast cell chymase expression in human idiopathic interstitial pneumonia.* Int J Mol Med, 2007. **19**(4): p. 565-70.
29. Chiba, N., et al., *Mast Cells Play an Important Role in Chlamydia pneumoniae Lung Infection by Facilitating Immune Cell Recruitment into the Airway.* The Journal of Immunology, 2015. **194**(8): p. 3840-3851.
30. Abraham, S.N. and A.L. St. John, *Mast cell-orchestrated immunity to pathogens.* Nature Reviews Immunology, 2010. **10**(6): p. 440-452.
31. Carlos, D., et al., *Mast Cells Modulate Pulmonary Acute Inflammation and Host Defense in a Murine Model.* The Journal of Infectious Diseases, 2007. **196**(9): p. 1361-1368.
32. Muñoz, S., et al., *Mast Cell Activation by Mycobacterium tuberculosis: Mediator Release and Role of CD48.* The Journal of Immunology, 2003. **170**(11): p. 5590-5596.
33. Carlos, D., et al., *TLR2-dependent mast cell activation contributes to the control of Mycobacterium tuberculosis infection.* Microbes and Infection, 2009. **11**(8): p. 770-778.
34. Muñoz, S., et al., *Mast Cell Activation by Mycobacterium tuberculosis: Mediator Release and Role of CD48.* The Journal of Immunology, 2003. **170**(11): p. 5590.
35. Malaviya, R., et al., *Mast cell phagocytosis of FimH-expressing enterobacteria.* Journal of Immunology, 1994. **152**(4): p. 1907-1914.
36. Ehlers, S. and U.E. Schaible, *The granuloma in tuberculosis: dynamics of a host-pathogen collusion.* Frontiers in immunology, 2013. **3**: p. 411-411.
37. Curtis, M.M. and S.S. Way, *Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens.* Immunology, 2009. **126**(2): p. 177-185.
38. Taweevisit, M. and U. Poumsuk, *High mast cell density associated with granulomatous formation in tuberculous lymphadenitis.* Southeast Asian journal of tropical medicine and public health, 2007. **38**(1): p. 115.
39. Celasun, B., J. Crow, and P.J. Scheuer, *Mast cells in granulomatous liver disease.* Pathol Res Pract, 1992. **188**(1-2): p. 97-100.
40. Okamoto Yoshida, Y., et al., *Essential Role of IL-17A in the Formation of a Mycobacterial Infection-Induced Granuloma in the Lung.* The Journal of Immunology, 2010. **184**(8): p. 4414-4422.
41. Jiang, D., et al., *Inhibited IL-17A ameliorates sarcoid-like granulomatosis in mice.* European Respiratory Journal, 2015. **46**(suppl 59): p. PA3310.
42. Pesci, A., et al., *Mast Cells in Fibrotic Lung Disorders.* Chest, 1993. **103**(4): p. 989-996.
43. Andersson, C.K., et al., *Activated MCTC mast cells infiltrate diseased lung areas in cystic fibrosis and idiopathic pulmonary fibrosis.* Respiratory Research, 2011. **12**(1): p. 139.
44. Inoue, Y., et al., *Human mast cell basic fibroblast growth factor in pulmonary fibrotic disorders.* The American journal of pathology, 1996. **149**(6): p. 2037-2054.
45. Bradding, P. and G. Pejler, *The controversial role of mast cells in fibrosis.* Immunological Reviews, 2018. **282**(1): p. 198-231.
46. Yue, X., B. Shan, and J.A. Lasky, *TGF-β: Titan of Lung Fibrogenesis.* Current enzyme inhibition, 2010. **6**(2): p. 10.2174/10067.
47. Overed-Sayer, C., et al., *Are mast cells instrumental for fibrotic diseases?* Frontiers in Pharmacology, 2014. **4**(174).
48. Bagher, M., et al., *Mast cells and mast cell tryptase enhance migration of human lung fibroblasts through protease-activated receptor 2.* Cell communication and signaling : CCS, 2018. **16**(1): p. 59-59.
49. Wygrecka, M., et al., *Mast Cells and Fibroblasts Work in Concert to Aggravate Pulmonary Fibrosis: Role of Transmembrane SCF and the PAR-2/PKC-α/Raf-1/p44/42 Signaling Pathway.* The American Journal of Pathology, 2013. **182**(6): p. 2094-2108.
50. Tomimori, Y., et al., *Involvement of mast cell chymase in bleomycin-induced pulmonary fibrosis in mice.* European Journal of Pharmacology, 2003. **478**(2): p. 179-185.
51. Matsumoto, T., et al., *Chymase Inhibition Prevents Cardiac Fibrosis and Improves Diastolic Dysfunction in the Progression of Heart Failure.* Circulation, 2003. **107**(20): p. 2555-2558.

52. Hügle, T., *Beyond allergy: the role of mast cells in fibrosis.* Swiss Med Wkly, 2014. **144**: p. w13999.
53. Overed-Sayer, C., et al., *Are mast cells instrumental for fibrotic diseases?* Frontiers in pharmacology, 2014. **4**: p. 174-174.
54. Groot Kormelink, T., et al., *Immunoglobulin free light chains are increased in hypersensitivity pneumonitis and idiopathic pulmonary fibrosis.* PloS one, 2011. **6**(9): p. e25392-e25392.
55. Rubinchik, E. and F. Levi-Schaffer, *Mast cells and fibroblasts: two interacting cells.* Int J Clin Lab Res, 1994. **24**(3): p. 139-42.

SUPPLEMENTARY MATERIAL

Supplemental Figure 5.A

Supplemental Figure 5.A. Mast cells are not present at necrotic sites of TB infected lungs. 44 microarrays from TB-infected sections and 22 from non-TB infected controls were stained with HE to visualize lung morphology to select necrotic tissue. Microarray sections with necrosis were incubated with anti-tryptase and anti-chymase antibodies followed by their fluorescent staining. The figure shows 2 representative micrographs of necrotic tissue with no presence of MC_T (green), MC_C (red) and MC_{TC} (merge) at necrotic sites of TB-infected lungs.

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CHAPTER 6: Paper 4

IL-33 primes human mast cells to BCG-induced activation

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ABSTRACT

BCG is an attenuated strain of *Mycobacterium bovis* and, although it is the only approved vaccine against tuberculosis (TB), it provides children and adults with poor protection. However, BCG has been found to boost innate immunity and used successfully as a treatment for bladder cancer. Nevertheless, many questions regarding the BCG cell targets and mechanism of action remain unanswered. In this study, we aimed to characterize the response of human MCs (hMCs) to BCG. We found a lack of response from naïve hMCs to BCG stimulation through cytokine secretion, degranulation, intracellular growth and bacterial uptake. However, when we expose hMCs with to IL-33, but not to IFN-γ, we found an increase in IL-8, MCP-1 and IL-13 secretion and an increase in the number of MC-BCG interactions. Furthermore, we showed an IL-33-induced increase in expression in the mycobacteria-binding receptor CD48. An effect similar to the one obtained with IL-33 treatment of hMCs is the one caused by the virulent *Mycobacterium tuberculosis* (*Mtb*) antigen 19KDa, low express in BCG. Thus, we proposed that a tissue microenvironment rich in IL-33 sensitise hMCs to respond to BCG, mimicking a response given by TB antigens, and could contribute to boost the weak immunity provided by this vaccine.

INTRODUCTION

BCG is currently the only approved vaccine against tuberculosis (TB) [1] and although it has a protective effect in children, BCG provides limited effectiveness in preventing pulmonary infection in adults with variable protection among populations [2]. Additional to its original purpose to prevent TB, BCG has been widely used providing positive outcomes as a gold standard treatment or complementary therapy in multiple pathological conditions [3]. This includes: autoimmune disorders e.g. multiple sclerosis and type 1 diabetes [4], anaphylaxis [5], bladder cancer [6], cutaneous melanoma [7] and recently its use in training immunity has shown protective effects against parasitic, fungal, viral and bacterial infections [8]. In fact, training immunity using BCG has been proposed as an adjuvant therapy against the current COVID-19 pandemic [9]. Furthermore, evidence implies that BCG vaccination provides overall increased surveillance during childhood [4]. Contrary, BCG has shown detrimental effects in immunocompromised patients and causes Kawasaki and disseminated BCG diseases in children [3]. Although BCG is widely used and provides both beneficial and detrimental effects in different pathologies, the mechanisms occurring after its inoculation are not fully understood yet.

Upon BCG inoculation, the first host-immune interactions occur in the dermis where innate cells including neutrophils, dendritic cells (DC), macrophages (Mφ) [10] and mast cells (MCs) reside [1]. Mφ recognizes BCG via toll-like receptors 1 and 2 (TLR-1, TLR2), neutrophils via CD11b, CD18 and DCs by ICAM-3 grabbing non-integrin (DC-SIGN) in addition to CD11b and CD18 [10]. After binding, Mφ release pro-inflammatory cytokines including IL-6 and TNF whereas DCs migrate to the lymph nodes to trigger the followed adaptive immune responses [10].
However, little is known regarding the modalities and outcomes of BCG-MCs crosstalk. MCs are tissue-resident immune cells present at sites of host-environment interactions such as the skin or lung mucosa [11]. Upon activation, MCs release a wide variety of pro- and anti-inflammatory mediators including cytokines, chemokines, proteases and anti-microbial compounds [12].

Although no reports are available on the activities of MCs upon BCG vaccination to prevent TB, studies have indicated MCs as important regulators of immune responses upon BCG inoculation in non-TB pathologies. For instance, BCG has been used as gold standard

immunotherapy in bladder cancer for more than 25 years [6] and evidence has demonstrated that increased IL-17+ MCs are associated with the beneficial outcome followed by BCG treatment in patients with primary and concomitant carcinoma in situ [13]. A proposed mechanism to describe BCG therapeutic action in bladder cancer, suggests that IL-17 released from MCs upon BCG exposure induces an increase of IL-8 which promotes cellular recruitment and tumour suppression [14]. In addition, polysaccharide nucleic acid obtained from BCG (BCG-PSN) was recently proposed as a therapeutic treatment to chronic spontaneous urticaria (CSU) [15]. Here, BCG-PSN has shown to suppress β-hexosaminidase release upon IgE-FcεRI crosslinking in RBL-2H3 cells [15]. This correlates with *in vivo* data showing BCG extracts to inhibit ovalbumin (OVA)-induced anaphylaxis by suppressing IgE-mediated degranulation in MCs in mice [5]. Although direct MC-BCG interactions have not been demonstrated, evidence obtained in rat MCs infected with *Mycobacterium tuberculosis* (*Mtb*) has suggested CD48 as the main receptor for *Mtb*-MC binding [16]. However, the MC responses initiated by BCG infection remain unclear.

MC activation is enhanced by MC exposure to specific cytokines, including IL-33 [17]. IL-33 is an alarmin, that belongs to the IL-1 family cytokines, and is produced by endothelial, epithelial cells, and fibroblasts in the skin, lung and gastrointestinal tract [18]. IL-33 sustains a Th2-dominated microenvironment by enhancing both the production of IL-5 and IL-13 and the recruitment of Th2 cells [18]. Furthermore, hMCs stimulated with IL-33 increase the secretion of pro- and anti-inflammatory cytokines *in vitro* [19]. However, it is unclear whether IL-33 modulates MC activities upon bacterial infections including BCG vaccination.

Here we investigate whether IL-33 priming enables human mast cells (hMCs) to respond to BCG *in vitro* exposure. Our findings demonstrate that naïve hMCs are not induced by BCG to release cytokines and degranulate. Furthermore, BCG is not detectable intracellularly in hMCs. However, IL-33 but not IFN-γ treatment activates hMCs that respond to the interaction with BCG by releasing cytokines such as IL-8, MCP-1 and IL-13. IL-33 increases the expression of $CD48^+$ on hMCs and thus seems to be responsible for the increase interactions between bacteria and MCs. Thus, we suggest that an inflammatory microenvironment rich in IL-33 is key to promote a BCG-induced MC protective response.

MATERIALS AND METHODS

Cellular culture

hMCs were generated as previously described [20]. Briefly, $CD117^+$ haematopoietic progenitors were isolated by immunomagnetic sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) from peripheral blood mononuclear cells (PBMCs). Cells were cultured for 4 weeks in Iscove Modified Dulbecco medium (IMDM) with GlutaMAX-I supplemented with 50µmol/L β2-mercaptoethanol, 0.5% BSA, 1% Insulin-Transferrin-Selenium, 100 U/mL penicillin, 100µg/mL streptomycin (Invitrogen, Carlsbad, Calif), human IL-6 (50ng/mL; PeproTech, Rocky Hill, NJ), human IL-3 (10ng/mL; PeproTech), human stem cell factor (SCF) (100ng/mL; PeproTech) and StemRegenin (1µM; Cayman) and in IMDM with GlutaMAX-I supplemented with 50µmol/L β2-mercaptoethanol, 0.5% BSA, 1% Insulin-Transferrin-Selenium, 100U/mL penicillin, 100µg/mL streptomycin, human IL-6 (50ng/mL) and SCF (100ng/mL) thereafter. hMCs, cells were tested for purity and maturity after 8 weeks of culture (**Supplemental Figure 6.1**). THP-1 monocyte cellular line (ATCC) were cultured in RPMI (Sigma-Aldrich) medium containing L-glutamine supplemented with 10% foetal bovine serum (FBS, Invitrogen) at 37C and differentiated into macrophages at a concentration of $5x10^5$ cells/mL in a flat bottom plate using phorbol 12-myristate 13-acetate (PMA) (100µM; Thermo-Fisher) for 2h. Cells were then washed and rested in RPMI for at least 1h before being used.

Bacterial cultures

BCG Danish, BCG-LUX and BCG-mCherry were cultured in 7H9 broth (7H9, BD Diagnostics) supplemented with 0.05% Tween 80, 0.2% glycerol and 10% oleic albumin dextrose catalase (OADC; BD Microbiology Systems) for 2 weeks until reaching an optical density (O.D) of 0.5. Hygromycin (100ug/ml; Sigma Aldrich) was added to BCG-mCherry cultures and Kanamycin (50µg/ml, Sigma Aldrich) was added to BCG-LUX cultures. Bacterial suspensions were washed using PBS-tween (0.05%; Sigma Aldrich) and diluted in glycerol (15%; Sigma Aldrich) to prepare homogenous concentrated stocks. Bacterial stocks were frozen down with liquid nitrogen and kept at -80°C. After at least 24h, 3 stocks were randomly selected, thawed, and diluted in serial concentrations before being cultured in 7H11 agar plates (7H11, BD) for colony-forming units (CFU) and incubated for 15 days at

37°C. Colony counting was recorded after 7-15 days and the final count was taken at day 15. Corresponding calculations were done to obtain bacterial concentrations using the mean of 3 representative stocks. Before infections, a single stock was thawed at room temperature and diluted accordingly.

Cellular priming, stimulation and infection

hMCs at a density of $5x10^5$ cells/mL were incubated with recombinant IL-33 (50ng/ml) (Thermo-Fisher Scientific) or IFN-γ (50ng/ml) (Sigma-Aldrich) for 24h at 37C and 5% CO_2 and washed 3 times before further stimulation. For BCG infection, naïve or cytokine-treated hMCs or THP-1-derived macrophages were washed 3 times to remove antibiotics and plated at a density of $5x10^5$ cells/mL in supplemented IMDM without antibiotics. Bacteria were diluted from stocks at a final multiplicity of infection (MOI) of 10:1 in the same media without antibiotics. The bacterial suspension was added to plates containing cells and incubated at 37°C, 5% CO_2 for 1h for degranulation and 16h for cytokine secretion. For *Mtb*-antigen stimulation, hMCs at a density of $5x10^5$ cells/mL were incubated with 19kDa lipoprotein (10µg/ml) (Lionex, GmbH) and with HSP70 (10µg/ml) (Lionex, GmbH) for 16h to evaluate cytokine release. hMCs ($5x10^5$ cells/mL) and 19kDa (10µg/ml) (Lionex, GmbH) were incubated for 1h to investigate degranulation and receptor expression.

Flow cytometry

hMCs were washed and stained with anti-human CD63 (BioLegend, clone H5C6), CD107a (BioLegend, clone H4A3) CD48 (FITC, clone BJ40, BioLegend), TLR2 (AL547, clone QA16A01, BioLegend) and TLR4 (PE, clone HTA125, BioLegend) in FACS buffer for 30min at 4°C. Fluorescence minus one (FMO) was used as a staining control, compensation was performed using compensation beads (OneComp eBeads; Thermofisher Scientific) and the acquisition was taken using a BD LSR-II flow cytometer. Data were analysed using FlowJo software (Treestar version 10.4.2) and expressed as a percentage of positive cells.

Cytokine and chemokine secretion

Cellular supernatants were collected after 16h and used to determine IL-8, GM-CSF, MIP-1α, MCP-1, TNFα, IL-10, IL-13 and IL-1β. Cytokine and chemokine concentrations were measured using a multiplex capture bead array assay (CBA; BD biosciences) by flow

cytometry (FACSVerse) following the manufacturer's instructions. Data were analysed using FCAP Array v3.0 software (BD biosciences).

β-hexosaminidase assay

β-hexosaminidase release was measured as previously reported [21]. Briefly, hMCs ($0.5x10^6$cells/ml) were stimulated with BCG or pre-sensitized with anti-IgE and stimulated with anti-IgE antibodies for 1h. Supernatants were harvested and cells were treated with Triton X-100 (1%; Sigma-Aldrich). Both cell lysates and supernatants were incubated with p-nitrophenyl N-acetyl-beta-D-glucosamine (1mmol; Sigma-Aldrich) in citrate buffer (0.05M; pH 4.5) for 2h at 37°C. After incubation, samples were treated with sodium carbonate (0.05M, pH 10.0) and O.D was taken at 405 nm using spectrophotometric analysis. β-hexosaminidase was expressed as a percentage of total release.

BCG intracellular growth

hMCs or THP-1 cells (both at $0.5x10^6$ cells/ml) were washed to remove antibiotics and incubated with BCG-Lux using a multiplicity of infection of 10 (MOI 10:1) for 4h in supplemented media without antibiotics. Cells were then washed to remove extracellular bacteria and incubated for up to 6 days (150h). Luminescence was measured at different time points as relative light units (RLUs) using a microplate Luminometer (BMG LABTECH, LUMIstar Omega).

Confocal Microscopy

Naïve or cytokine-treated hMCs ($0.2x10^5$ cells) were placed in an 8 well chamber (Ibidi) for 30min at 37C and 5% CO^2. BCG mCherry was added at a MOI 10:1 for 2h and cells were fixed using paraformaldehyde 4% and stained using wheat germ agglutinin, conjugate with Alexa Fluor 488 (Invitrogen). Images were collected at a zoom factor of 1.58 using a Leica TCS SP8x inverted confocal microscope equipped with a tunable white light laser (WLL), and a diode 405nm laser, a 40X/0.85 dry objective and HyD hybrid detectors. BCG mCherry was excited at 590 nm with WLL laser and detected at 598 to 690 nm on a HyD point detector. FITC fluorescence was excited at 488 nm using the Argon laser and collected on a HyD detector at 492 to 550nm. Confocal 3D stacks were acquired with a depth of 8µm

using Leica LASX software. Videos were recorded using the same settings at 37C and 5% CO2 with 30-second interval acquisitions. Images stacks were then processed, sum projected and analysed using *Fiji*

RESULTS

BCG does not induce MC activation

Since MCs release mediators upon Mycobacteria, including *Mtb* encounter [12, 22] we aimed to investigate whether the BCG vaccine elicits hMC activation by inducing cell degranulation and cytokine secretion. We used blood-derived hMCs cultured for 8 weeks and examined for purity and maturity (**Supplemental Figure 6.A**). hMCs were exposed to BCG at a MOI 10:1 for 1h to investigate BCG-induced cell degranulation and for 16h to measure cytokine and chemokine release. As shown in **Figure 6.1**, BCG did not affect hMC degranulation measured by the expression of CD63 (% of $CD63^+$ cells) and by β-hexosaminidase release (% of total release) since no significant increase in CD63 expression or β-hexosaminidase release was found in BCG-infected hMCs compared to untreated cells **(Figure 6.1a)**. hMC degranulation induced by FcεRI-crosslinking was used here as control. Similarly, secretion of IL-8, MCP-1, GM-CSF and MIP-1α (**Figure 6.1b**) was not induced by BCG since values were found comparable to unstimulated controls while THP1 cells infected by BCG, used here as a positive control, released TNF and IL-1β in their supernatants (**Figure 6.1c**). Thus, hMCs do not display signs of activation, measured by induced-degranulation and cytokine secretion, when exposed to BCG.

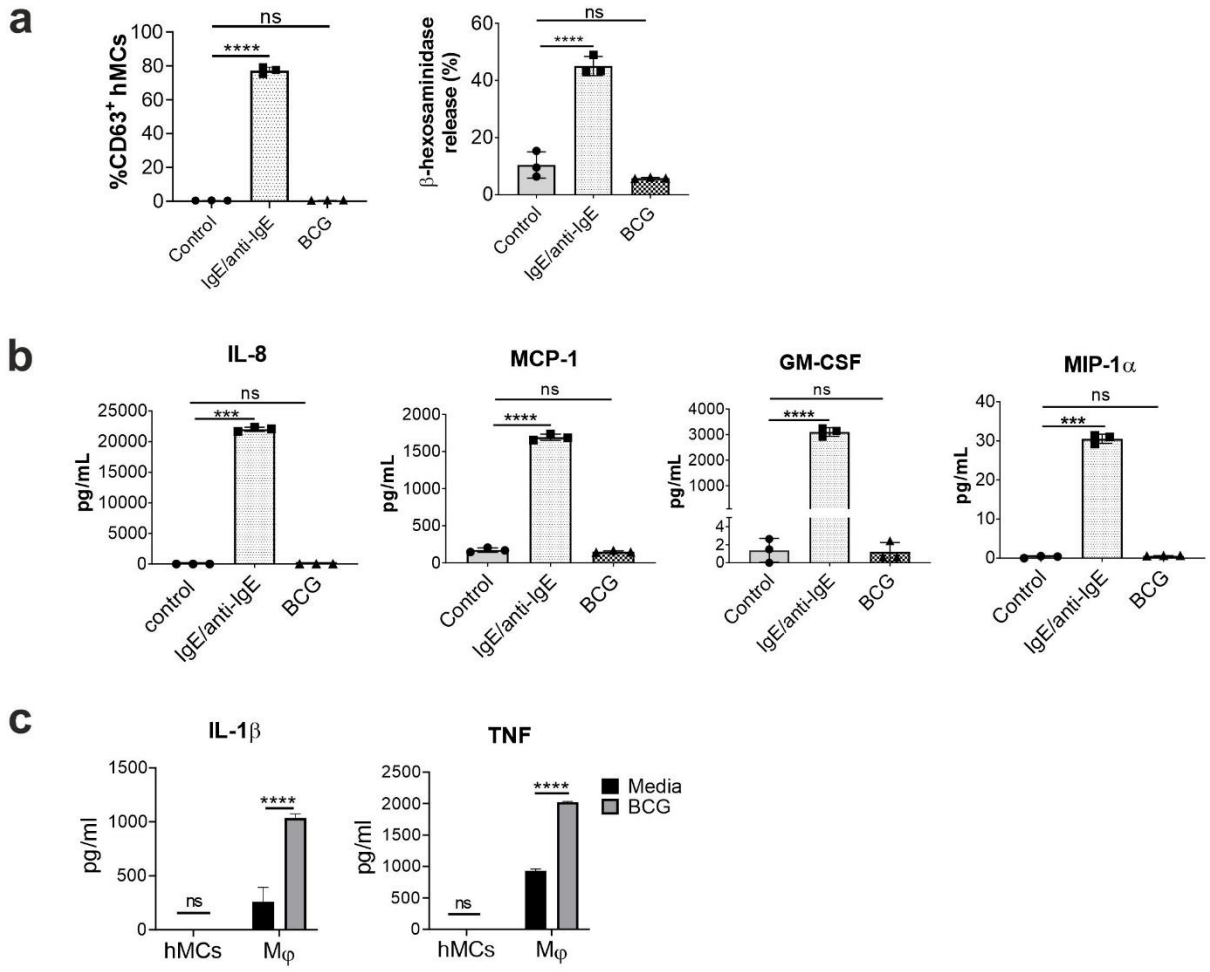


Figure 6.1. BCG does not induce MC activation. Human mast cells (hMCs, 0.5x10^6 cells/ml) were stimulated with BCG (MOI 10:1) for 1 or 16h. IgE-sensitized cells activated with anti-IgE antibodies (IgE; 1µg/ml, anti-IgE; 1µg/ml) were used as a positive control. **(a)** After 1h incubation, cell degranulation was measured by CD63 antibody staining (% of CD63+ cells) and β-hexosaminidase release. **(b)** Cytokine secretion was measured in supernatants collected after 16h incubation using CBA. **(c)** THP-1 cells (Mφ) were used as positive control for BCG-induced cytokine release. Each graph shows a representative experiment out of 3 independent experiments performed. 3 different donors were used for hMC experiments from 7 different cell cultures. Statistical analyses were performed using one-way ANOVA analysis followed by a Tukey's multiple comparison test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.1).

Human MCs are unable to internalize and promote BCG intracellular growth

To clarify the lack of BCG-induced MC activities, we investigated intracellular growth and BCG internalization in hMCs. To evaluate intracellular growth, hMCs or THP-1 cells were exposed to BCG-LUX (MOI 10:1), which is a BCG strain that emits luminescence, for 2h. After incubation, cells were washed to remove extracellular bacteria and relative light units (RLUs) were measured at different incubation times. No increase in RLUs was detected in hMCs (**Figure 6.2a**, left panel), while THP-1 cells showed a significant increase in RLUs, BCG intracellular growth, at all measured times (**Figure 6.2a**, right panel). Furthermore, hMCs were incubated with BCG mCherry (MOI 10:1) for 2h, fixed and analysed by confocal microscopy to investigate bacterial internalization. Although interactions between hMCs and BCG mCherry were observed (**Figure 6.2b**), BCG was not detected intracellularly. Thus, hMCs seem not to uptake, store or favour BCG growth.

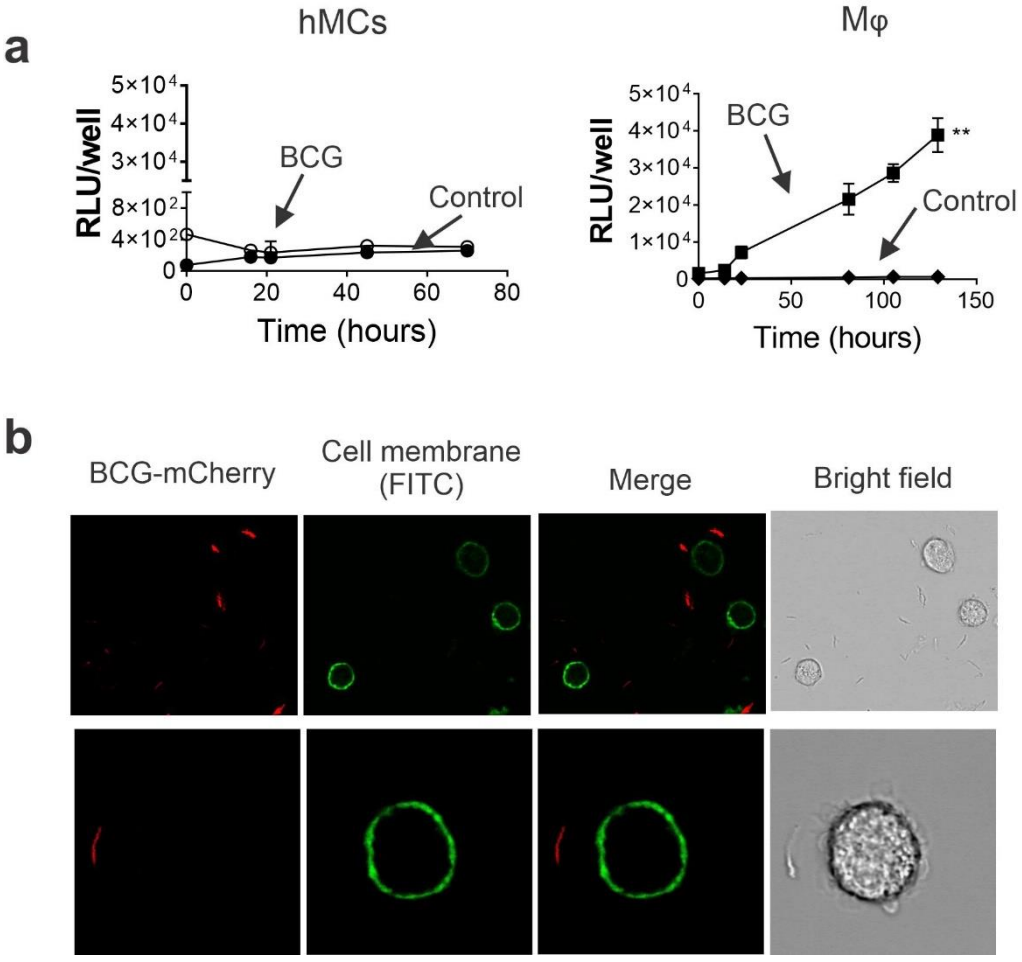


Figure 6.2. BCG does not show intracellular presence and growth in hMCs. (a) To investigate intracellular growth, hMCs (left) and THP-1 cells (Mφ) (right) (both at $0.5x10^6$ cells/ml) were incubated with BCG-LUX (MOI 10:1) for 2h, washed and intracellular growth was measured at different time points as increase of relative light units (RLU). Each graph displays a representative experiment out of 2 independent experiments performed. 3 different donors were used for hMC experiments from 7 different cell cultures. Statistical analysis was performed using Man-Withney test. **(b)** hMCs ($0.5x10^6$ cells/ml) were incubated for 2h with BCG-mCherry (MOI 10:1), fixed and stained with a FITC-fluorescent membrane dye. BCG uptake was analysed using confocal microscopy. Upper and lower panel show representative micrographs at 40X from a single and multiple cells (green) surrounded by BCG (red).

The 19Kda lipoprotein induces hMC to secrete cytokines and augments CD48 expression

Since BCG vaccine seems not to activate naïve hMCs, we aimed to investigate whether virulent *Mtb* antigens including the 19kDa lipoprotein and the 70 kilodalton heat shock protein (HSP70) which are low express in BCG, could stimulate hMC responses. hMCs were incubated with 19kDa and HSP70 for 16h and IL-8, MCP-1, GM-CSF and IL-13 (pg/ml±SD) concentrations were measured in supernatants. While supernatants from 19kDa-treated hMCs contained increased concentrations of IL-8 (363.2±7.7), MCP-1 (808.3±17.72), GM-CSF (96±1.6) and IL-13 (189.7±9.6) compared to untreated controls, HSP70 stimulation showed no significant effect on mediators' release (**Figure 6.3a**). Since 19kDa activate hMCs by mediator secretion, we investigated 19kDa-mediated degranulation and bacterial receptor expression. hMCs were incubated with 19kDa for 1h, stained with anti-CD63, anti-CD48, anti-TLR2 and anti-TLR4 fluorescent antibodies and analysed by flow cytometry to evaluate receptor expression (% +ve cells±SD). As shown in **Figure 6.3b**, degranulation was not induced by 19kDa as CD63 expression was comparable to unstimulated controls. However, 19kDa lipoprotein, increased the hMC expression of CD48 (22.8±3.2) and TLR-2 (7.8±0.23) but not TLR-4 (2.7±0.3). Nevertheless, one should notice that the level of TLR2 expression is low. Thus, the virulent 19kDa lipoprotein, which is low expressed in BCG, induces hMC cytokine secretion and increases expression of CD48, the *Mtb*-binding receptor.

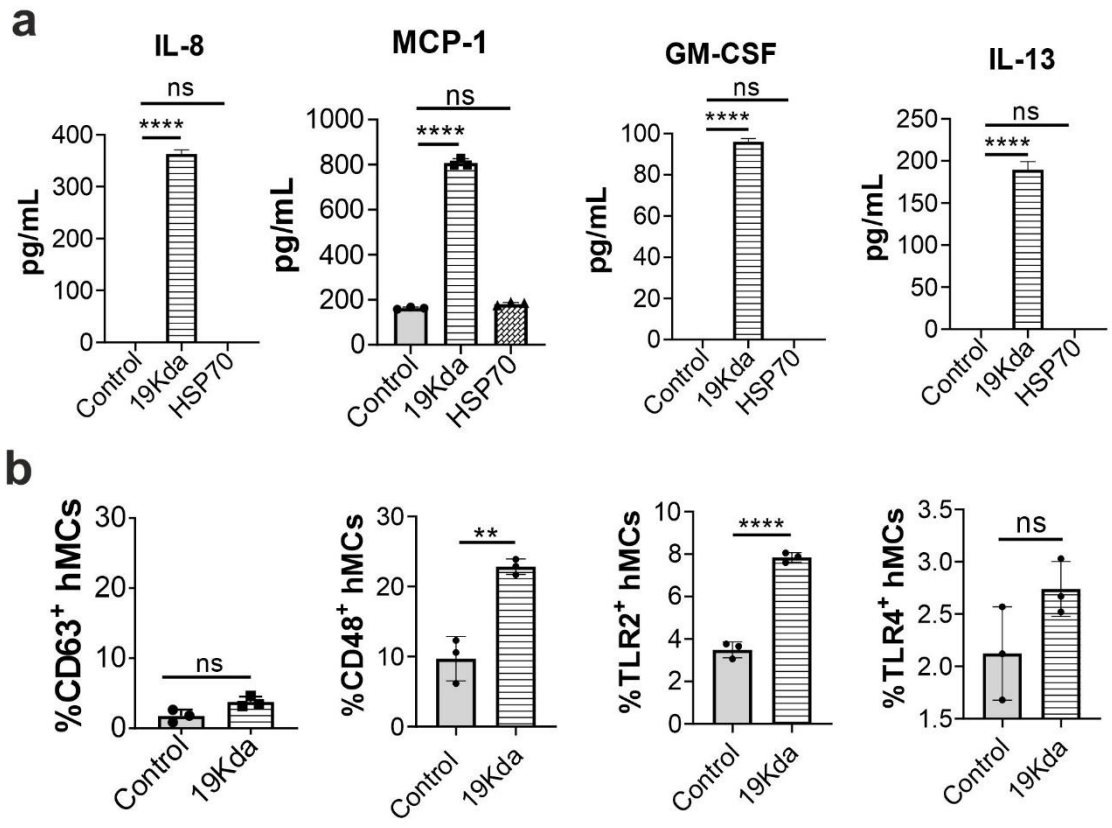


Figure 6.3. 19KDa lipoprotein induces cytokine and chemokine release and increases CD48 expression in hMCs. **(a)** hMCs were stimulated with 19KDa (10µg/ml) or HSP70 (10µg/ml) antigens for 16h. Supernatants were collected and mediators release was analysed using the CBA multiplex assay. To investigate receptor expression and degranulation, hMCs were incubated with 19kDa for 1h. **(b)** After incubation cells were washed, immunostained with CD63, CD48, TLR2 and TLR4 antibodies and analysed by flow cytometry. Each graph shows a representative experiment from 3 independent experiments. 3 different donors were used for hMC experiments from 7 different cell cultures. Statistical analyses were performed using **(a)** one-way ANOVA followed by a Tukey's multiple comparison test and **b)** student *t*-test (****p<0.0001, ***p<0.001, **p<0.01, *p<0.1).

IL-33 priming alters hMC responses upon BCG exposure

IL-33 is known to potentiate hMC mediators secretion upon histamine [23], whereas IFN-γ shown to boost hMC pro-inflammatory responses upon *Staphylococcus aureus* infection [24] and to LPS stimulation (**Supplemental Figure 6.B**) Due to the lack of response upon BCG stimulation, we investigated whether IL-33 or IFN-γ priming enhances the susceptibility of hMCs to BCG uptake and mediated activation by measuring cytokine secretion, degranulation and intracellular growth. To this purpose, hMCs were incubated for 24h in media containing either IL-33 or IFN-γ and then stimulated with BCG (MOI 10:1) for 1 or 16h to investigate degranulation (% $CD63^+$ cells) and cytokine secretion (pg/ml±SD) respectively. IgE/anti-IgE-mediated (100ng/ml) activation was used as a positive control for degranulation in IL-33 pre-treated cells (**Figure 6.4a**) and both LPS and FcεRI-IgE crosslinking were used to compare cytokine responses in IL-33 and IFN-γ primed cells (**Supplemental Figure 6.B**). As shown in **Figure 6.4b**, IL-33 priming increased hMC secretion of IL-8 (2090.6±495), MCP-1 (806.8±121.9) and IL-13 (79.5±18.9) whereas cytokine levels in IFN-γ primed cells were comparable to untreated controls. However, while IgE/anti-IgE degranulation was enhanced by IL-33 treatment (67.3±3), the latter was not sufficient to induce BCG-mediated degranulation in hMCs (**Figure 6.4a**). To investigate whether IL-33 or IFN-γ priming influence BCG intracellular growth, hMCs were incubated with BCG-LUX (MOI 10:1) for 2h. After incubation, cells were washed to remove extracellular bacteria and relative light units (RLUs) were measured at different incubation times. As shown in **Figure 6.4c**, IL-33 and IFN-γ pre-treatments did not induce any increase in RLUs in hMCs, therefore did not promote BCG intracellular growth. Thus, hMCs treated with IL-33 respond to BCG stimulation by secreting IL-8, MCP-1 and IL-13 in the absence of degranulation and intracellular bacterial growth.

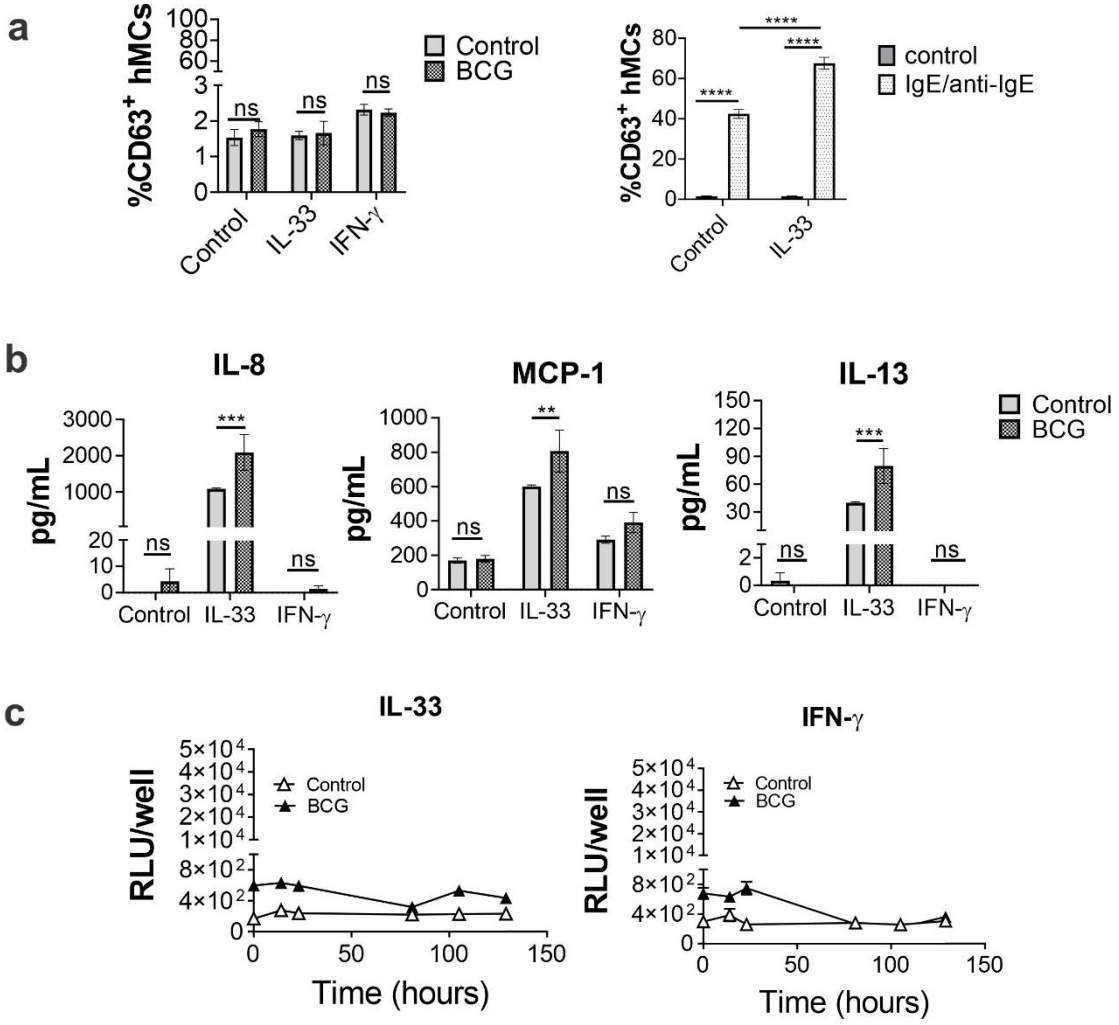


Figure 6.4. hMC cytokine secretion upon BCG infection is enhanced by IL-33. hMCs were treated either with IL-33 (50ng/ml) or IFN-g (50ng/ml) for 24h or left untreated (control). Cells were then washed and stimulated with BCG (MOI 10:1) for **(a)** 1h to measure degranulation (% CD63+ cells) or **(b)** 16h to evaluate cytokine secretion in supernatants using CBA multiplex assay. **(a)** IgE/anti-IgE stimulation (IgE; 100ng/ml, anti-IgE; 100ng/ml) was used as positive control of degranulation. **(c)** IL-33 (left) and IFN-g (right) treated hMCs were incubated with BCG-LUX (MOI 10:1) for 2h, washed and intracellular growth was measured at different time points as increase of relative light units (RLU). Each graph shows a representative experiment from 3 independent experiments. 3 different donors were used for hMC experiments from 7 different cell cultures. Statistical analyses were performed using **(a-b)** one-way ANOVA analysis followed by a Tukey's multiple comparison test and **(c)** Man-Withney test (****p<0.0001, ***p<0.001, **p<0.01).

IL-33 enhances hMC CD48 expression

Since IL-33 treatment promotes cytokine release upon BCG infection, and since CD48 has shown to be involved in murine MC activation by mycobacteria [16], we hypothesized that IL-33 similarly to 19kDa could modulate CD48 expression. To prove this hypothesis, hMCs were incubated for 24h with either IL-33 or IFN-γ , washed, incubated with anti-CD48, anti-TLR2 and anti-TLR4 antibodies conjugated to fluorescent fluorochromes and the expression of these receptors (% +ve cells±SD) was analysed by flow cytometry As observed in **Figure 6.5a**, IL-33 increased the expression of CD48 (65.3±3.6) whereas IFN-γ upregulated TLR-2 (92.37±1.9) and TLR-4 (8.3±0.7). Furthermore, inhibition of TLR2 was observed upon IL-33 treatment as shown by the decreased receptor expression compared to control (8.9±3.9) (**Figure 6.5a**). The increased expression of CD48 upon IL-33 treatment of hMCs was confirmed by confocal microscopy (**Figure 6.5b**).IL-33 pre-treated cells incubated with anti-CD48 antibody conjugated with a FITC fluorochrome showed detectable expression of CD48 compared to controls. Thus, while IFN-γ induces an increase in TLR2 expression, IL-33 upregulates the expression of the mycobacterial receptor CD48 while reducing TLR2 in hMCs.

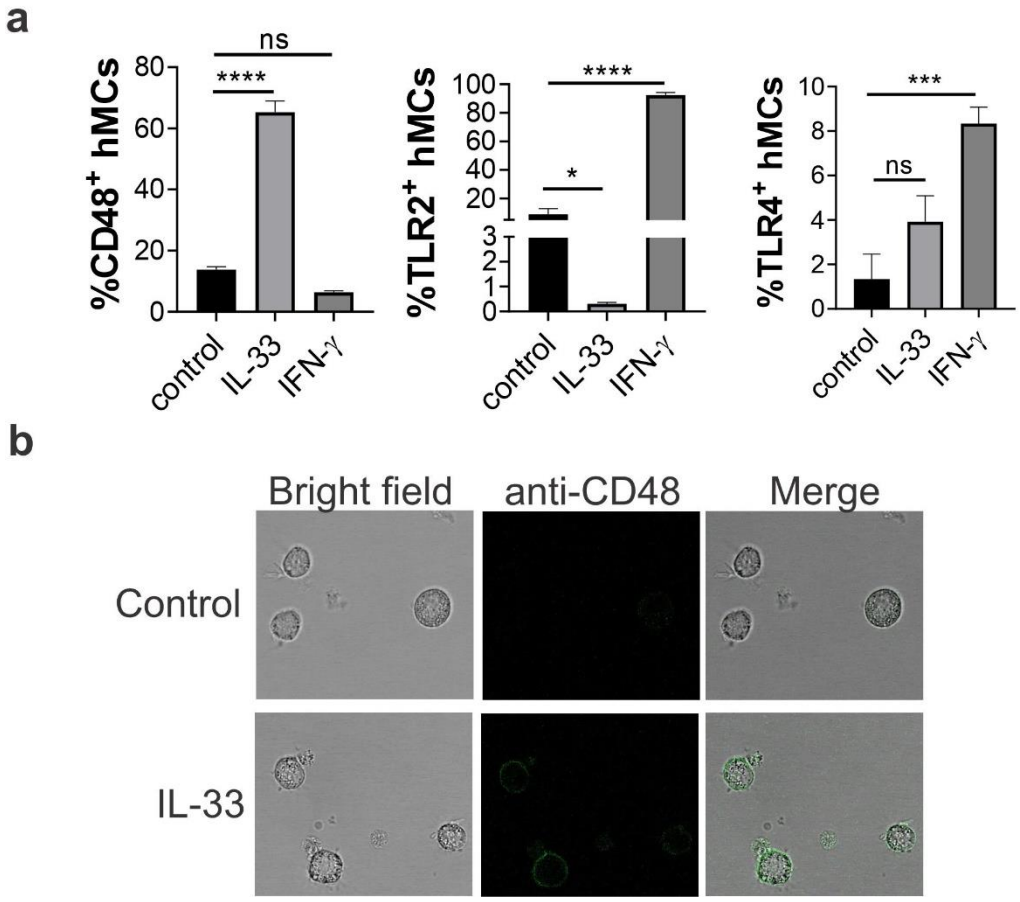


Figure 6.5. IL-33 treatment increases CD48 expression in hMCs. (a) hMCs were incubated 24h with IL-33 (50ng/ml), IFN-g (50ng/ml) or left untreated (control), washed, stained with CD48, TLR2 and TLR4 antibodies conjugated to fluorescent fluorochromes (FITC, CD647 and PE respectively) and analysed by flow cytometry. Each graph shows a representative experiment from 3 independent experiments. Statistical analysis was performed using one-way ANOVA followed by a Tukey's multiple comparison test (****p<0.0001, *p<0.1). Each graph shows a representative experiment from 3 independent experiments. 3 different donors were used for hMC experiments from 7 different cell cultures. **(b)** CD48 expression was confirmed by microscopy staining in IL-33 treated hMCs incubated for 24h with a CD48 fluorescent antibody (FITC; green). Upper panel micrographs are representative of untreated while the lower panel shows IL-33-treated hMCs. Micrographs are representative of 1 experiment using a single donor from 3 independent experiments.

IL-33 priming increases BCG interactions to hMCs

Next, we investigated whether IL-33 pre-treatment and the resulting increase in CD48 receptor expression would affect the interaction between BCG and hMCs. To this purpose, hMCs were incubated with BCG mCherry (MOI 10:1) and recorded for 8h using confocal microscopy at 37C and 5%CO_2. The number of hMC-BCG interactions was counted manually from 2 independent videos (included as **Supplementary Material Video 1 and 2**). As shown in **Figure 6.6a**, BCG-hMC interactions were considered as touches over the membrane or at cellular protrusions. As shown in **Figure 6.6b**, IL-33 pre-treatment favoured a higher number of brief cell-bacteria interactions (number of interactions±SD) that lasted less than 1min (36.8±8.7) compared untreated hMCs (15.4±7.3). Although interactions lasting less than 10min were not significantly different between groups, IL-33 pre-treated cells exhibited altogether a higher number of interactions (4±1.8) compared to untreated control (0.8±0.9). Thus, IL-33 seem to boost the number of hMC-BCG interactions that are more likely to activate hMC responses.

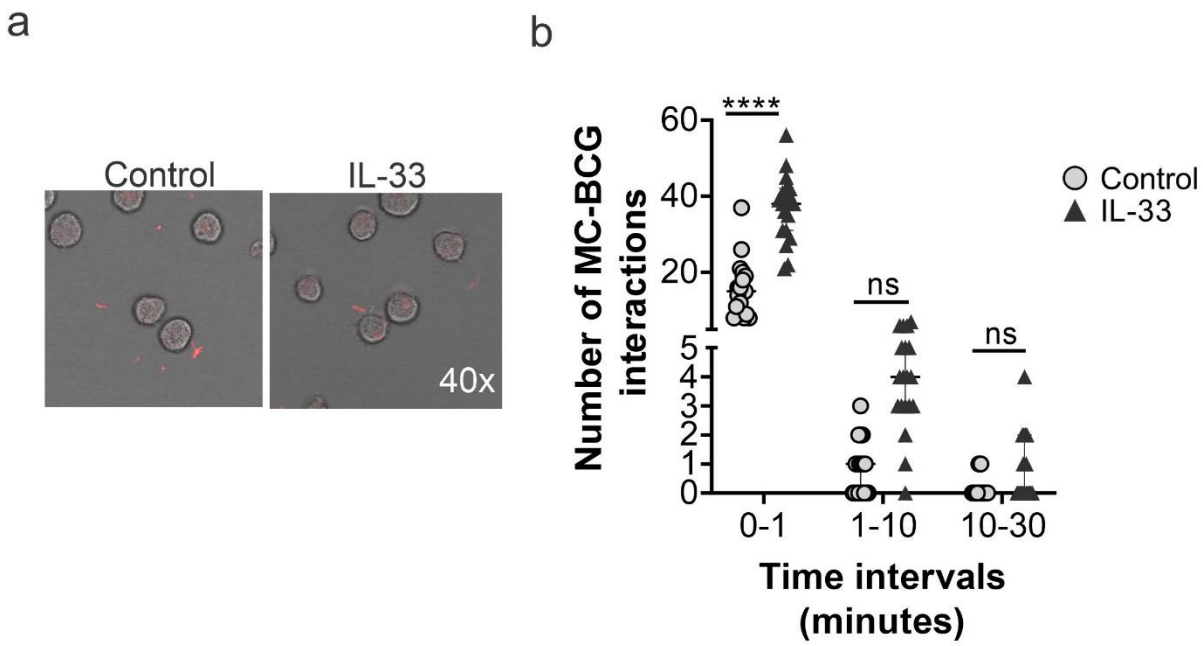


Figure 6.6. IL-33 treatment increases BCG-hMCs interactions. hMCs were treated for 24h with IL-33 (50ng/ml) or left unstimulated (control). After incubation cells were washed and left 30min in a 8 well chamber at 37C. BCG mCherry at a MOI 10:1 was added to wells and a video was recorded for 8h using confocal microscopy at 37C and 5% CO2. Videos were analysed using FIJI and BCG-hMCs short interactions (less than 1min) were counted manually. A total of 19 cells per power field were counted in each condition (IL-33 treated and untreated) and interactions were considered as single BCG touches at MC membrane or at their protrusions **(a)** a representative image from an 8h video shows BCG-hMCs interactions in IL-33 treated and control cells. **(b)** each dot represents a cell and graph shows the number of interactions in IL-33 treated and control cells. Full videos are shown in Supplementary material (video 1 and 2). Graph shows a representative experiment from 2 independent experiments. Statistical analysis was performed using one-way ANOVA followed by a Tukey's multiple comparison test (****p<0.0001, *p<0.1).

DISCUSSION

In the present study, we demonstrated a lack of hMC activation upon BCG stimulation. However, hMCs exposed to IL-33 show an acquired susceptibility to BCG stimulation, an increase in the expression of CD48 receptor, and an augmented interaction with bacteria. Thus, our data suggest that an inflammatory microenvironment rich in IL-33 is key to promote a BCG-induced MC protective response.

It is known that in children and adults, BCG promotes a Th1-type immune response after vaccination [25]. Furthermore, in infants, the formation of a scar and erythema at the inoculation site upon BCG vaccination is a common tool to prognosticate protection and good prognosis against TB [26], which suggest that a strong innate reaction is necessary to develop immunity. At this point, local innate cells including DC, Mφ, neutrophils and MCs are necessary to develop the initial inflammatory response [27]. Here we showed a lack of response from naïve hMCs against BCG stimulation *in vitro*. In this context, this lack of rapid response may serve to either avoid an excessive inflammation at the inoculation site or may explain the weak prophylaxis given by BCG.

MCs are activated upon a variety of virulent pathogens [28]. However, evidence has shown that pathogen-associated molecular patters (PAMPs) from virulent bacteria are necessary to activate hMCs [21, 28]. For instance, after *Streptocccus equi* exposure, MCs release a wide repertory of cytokines and chemokines which are not secreted upon the heat-inactivated bacteria [29]. Similarly, the 19kDa lipoprotein, which is low expressed in BCG, mediates the release of pro-inflammatory cytokines and chemokines by hMCs. 19kDa is a highly immunogenic cell-wall component of *Mtb* [30], is recognized by TLR2, promotes Mφ apoptosis and secretion of L-1β, IL-12 and TNF-α [31] and induce proliferation of T lymphocytes [32]. It has been shown that prolonged TLR2 activation (16h exposure) by 19kDa induce an immunosuppressive effect as observed in Mφ by a decreased response to IFN-γ [33, 34]. However, as shown in our controls and published data [35], MCs lack or exhibit a minimal expression of TLR2. Our data demonstrated that hMCs, upon prolonged exposure to19kDa (16h) upregulate CD48 and not TLR2 and secrete inflammatory mediators. This is in line with the findings showing that *Mtb* activates rat MCs through CD48 receptor binding [36]. Thus, it seems that MCs, differently than Mφ engage virulent antigens, including 19kDa, via CD48 but not TLR2 receptor.

Since attenuated strains fail to activate MCs, molecules that potentiate MC activation have attracted interest [17]. These molecules include compound 48/80, IFN-γ and IL-33, which are used as powerful vaccine adjuvants [17]. For instance, upon *Staphylococcus aureus* infection, IFN-γ priming boosted hMC pro-inflammatory molecules including GM-CSF and IL-8 *in vitro* [24]. Furthermore, the MC activator, compound 48/80, enhances humoral responses, increases MC-derived TNF-α and promotes dendritic cell and lymphocyte migration to the lymph nodes in a MC-dependent manner when used as a vaccine adjuvant against *Bacillus anthracis* [37]. Similar to this, an *in vivo* study demonstrated that IL-33, used as a vaccine adjuvant against influenza virus, potentiated MC-dependent IFN-γ and increased mice survival (in 80%) in a MC-dependent manner [38]. In TB, IL-33 mediates $CD4^+$T and $CD8^+$T cellular recruitment when used *in vivo* as a vaccine adjuvant [39]. Here we showed that upon BCG stimulation, treatment of hMCs with IL-33 but not IFN-γ enhanced IL-8 and MCP-1 secretion, chemokines involved in neutrophil and monocyte recruitment. This is in accordance with *in vitro* and *in vivo* data showing IL-33 to promote a MC-dependent neutrophil recruitment [40]. Thus, in a vaccination context, IL-33 may serve to potentiate a MC-mediated strong innate immune response at the inoculation site necessary to trigger TB immunity.

Both IL-33 and IFN-γ treatments did not promote BCG uptake and intracellular growth. Although evidence has shown MCs capable to phagocytose bacteria [41-43], this lack of internalization may be explained by the BCG deficiency in adhesins that are necessary for phagocytosis [27, 44, 45]. Moreover, IL-33 is known to potentiate MC adhesion to fibronectin and increase the intercellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion protein 1 (VCAM-1) expression thus enhancing MC adhesion to endothelial cells [46]. These findings would support our observations of an increased number of hMC-BCG interactions in IL-33 pre-treated cells. Thus, IL-33 is a potent MC activator that boosts BCG-hMC interactions and promotes mediator release from MCs.

CONCLUSION

Although hMCs are not activated by BCG, an inflammatory microenvironment rich in IL-33 increases the MC responsiveness to BCG likely by upregulating the expression of the CD48 receptor. Thus, this cytokine can be used to potentiate BCG vaccination to trigger TB immunity and to enhance BCG therapeutic activities against diverse non-TB pathologies.

REFERENCES: Chapter 6

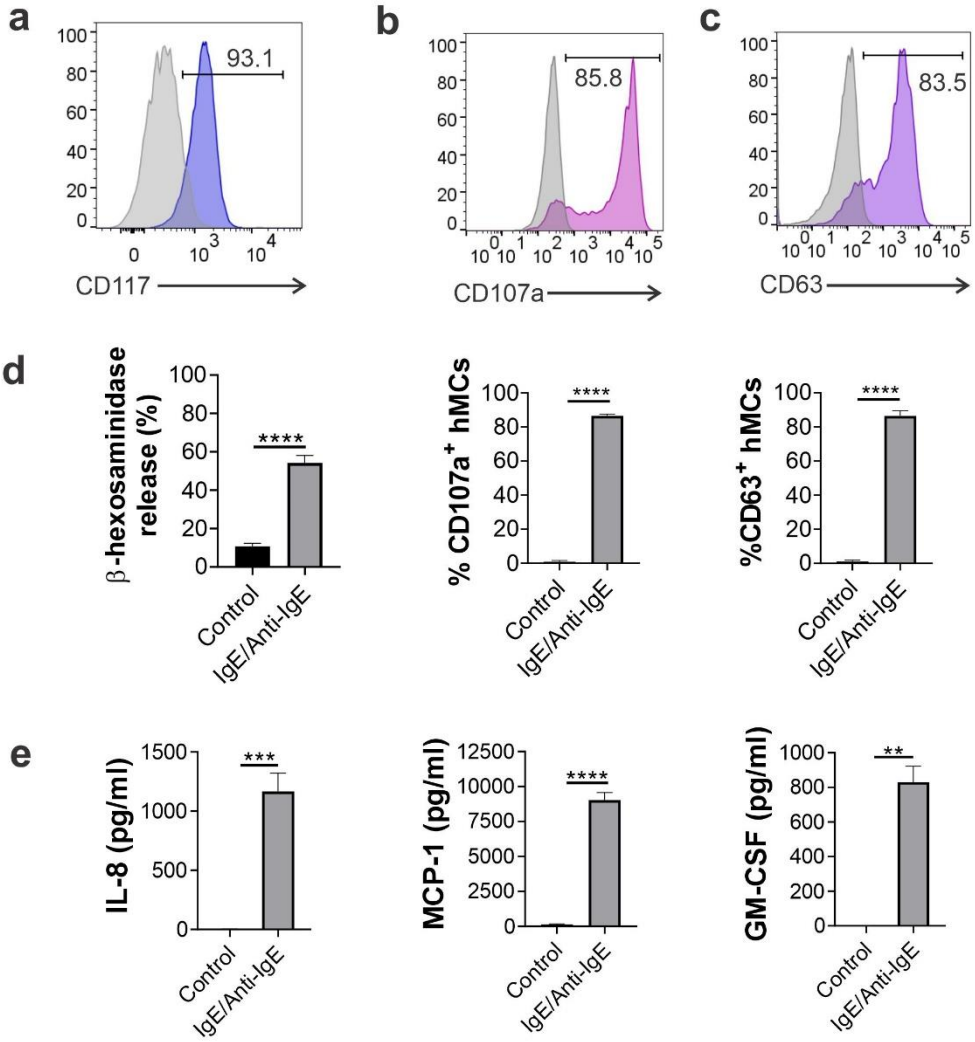
1. Dockrell, H.M. and S.G. Smith, *What Have We Learnt about BCG Vaccination in the Last 20 Years?* Frontiers in immunology, 2017. **8**: p. 1134-1134.
2. Davenne, T. and H. McShane, *Why don't we have an effective tuberculosis vaccine yet?* Expert Review of Vaccines, 2016. **15**(8): p. 1009-1013.
3. Yamazaki-Nakashimada, M.A., et al., *BCG: a vaccine with multiple faces.* Human Vaccines & Immunotherapeutics, 2020. **16**(8): p. 1841-1850.
4. Kowalewicz-Kulbat, M. and C. Locht, *BCG and protection against inflammatory and auto-immune diseases.* Expert Review of Vaccines, 2017. **16**(7): p. 699-708.
5. Sun, M., et al., *Anti-allergic action of bacillus Calmette-Guerin extract in experimental mast cell-mediated anaphylactic models.* Mol Med Rep, 2017. **16**(5): p. 6248-6254.
6. Joseph, M. and D. Enting, *Immune Responses in Bladder Cancer-Role of Immune Cell Populations, Prognostic Factors and Therapeutic Implications.* Frontiers in Oncology, 2019. **9**(1270).
7. Kremenovic, M., M. Schenk, and D.J. Lee, *Clinical and molecular insights into BCG immunotherapy for melanoma.* Journal of Internal Medicine. **n/a**(n/a).
8. Arts, R.J.W., et al., *BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity.* Cell Host & Microbe, 2018. **23**(1): p. 89-100.e5.
9. O'Neill, L.A.J. and M.G. Netea, *BCG-induced trained immunity: can it offer protection against COVID-19?* Nature Reviews Immunology, 2020. **20**(6): p. 335-337.
10. Moliva, J.I., J. Turner, and J.B. Torrelles, *Immune Responses to Bacillus Calmette-Guérin Vaccination: Why Do They Fail to Protect against Mycobacterium tuberculosis?* Frontiers in immunology, 2017. **8**: p. 407-407.
11. Piliponsky, A.M. and L. Romani, *The contribution of mast cells to bacterial and fungal infection immunity.* Immunological Reviews, 2018. **282**(1): p. 188-197.
12. Garcia-Rodriguez, K.M., et al., *The Role of Mast Cells in Tuberculosis: Orchestrating Innate Immune Crosstalk?* Front Immunol, 2017. **8**: p. 1290.
13. Dowell, A.C., et al., *Interleukin-17-positive mast cells influence outcomes from BCG for patients with CIS: Data from a comprehensive characterisation of the immune microenvironment of urothelial bladder cancer.* PloS one, 2017. **12**(9): p. e0184841-e0184841.
14. Stone, L., *Mastering the immune microenvironment.* Nature Reviews Urology, 2017. **14**(11): p. 639-639.
15. Yan, S., et al., *Therapeutic effect of Bacillus Calmette-Guerin polysaccharide nucleic acid on mast cell at the transcriptional level.* PeerJ, 2019. **7**: p. e7404-e7404.
16. Muñoz, S., et al., *Mast Cell Activation by Mycobacterium tuberculosis: Mediator Release and Role of CD48.* The Journal of Immunology, 2003. **170**(11): p. 5590-5596.
17. Johnson-Weaver, B., et al., *Mast cell activators as novel immune regulators.* Current opinion in pharmacology, 2018. **41**: p. 89-95.

18. Chan, B.C.L., et al., *IL33: Roles in Allergic Inflammation and Therapeutic Perspectives.* Frontiers in Immunology, 2019. **10**(364).
19. Parveen, S., et al., *IL-33 mediated amplification of allergic response in human mast cells.* Journal of Receptors and Signal Transduction, 2019. **39**(4): p. 359-367.
20. Garcia-Rodriguez, K.M., et al., *Human mast cells exhibit an individualized pattern of antimicrobial responses.* Immunity, Inflammation and Disease. **n/a**(n/a).
21. Garcia-Rodriguez, K.M., et al., *Human mast cells exhibit an individualized pattern of antimicrobial responses.* Immunity, Inflammation and Disease, 2020. **8**(2): p. 198-210.
22. Johnzon, C.-F., E. Rönnberg, and G. Pejler, *The Role of Mast Cells in Bacterial Infection.* The American Journal of Pathology, 2016. **186**(1): p. 4-14.
23. Iikura, M., et al., *IL-33 can promote survival, adhesion and cytokine production in human mast cells.* Laboratory investigation, 2007. **87**(10): p. 971-978.
24. Swindle, E.J., et al., *Interferon-γ enhances both the anti-bacterial and the pro-inflammatory response of human mast cells to Staphylococcus aureus.* Immunology, 2015. **146**(3): p. 470-85.
25. Marchant, A., et al., *Newborns Develop a Th1-Type Immune Response to Mycobacterium bovis Bacillus Calmette-Guérin Vaccination.* The Journal of Immunology, 1999. **163**(4): p. 2249-2255.
26. Jason, J., et al., *Clinical and Immune Impact of Mycobacterium bovis BCG Vaccination Scarring.* Infection and Immunity, 2002. **70**(11): p. 6188-6195.
27. Dockrell, H.M. and S.G. Smith, *What Have We Learnt about BCG Vaccination in the Last 20 Years?* Frontiers in Immunology, 2017. **8**(1134).
28. Abraham, S.N. and A.L. St. John, *Mast cell-orchestrated immunity to pathogens.* Nature Reviews Immunology, 2010. **10**(6): p. 440-452.
29. Rönnberg, E., B. Guss, and G. Pejler, *Infection of Mast Cells with Live Streptococci Causes a Toll-Like Receptor 2- and Cell-Cell Contact-Dependent Cytokine and Chemokine Response.* Infection and Immunity, 2010. **78**(2): p. 854-864.
30. Liu, L., et al., *Mycobacterium tuberculosis 19-kDa lipoprotein induces Toll-like receptor 2-dependent peroxisome proliferator-activated receptor γ expression and promotes inflammatory responses in human macrophages.* Mol Med Rep, 2015. **11**(4): p. 2921-2926.
31. Stewart, G.R., et al., *Effect of Deletion or Overexpression of the 19-Kilodalton Lipoprotein Rv3763 on the Innate Response to Mycobacterium tuberculosis.* Infection and Immunity, 2005. **73**(10): p. 6831-6837.
32. Becker, K. and P. Sander, *Mycobacterium tuberculosis lipoproteins in virulence and immunity – fighting with a double-edged sword.* FEBS Letters, 2016. **590**(21): p. 3800-3819.
33. Pai, R.K., et al., *Prolonged toll-like receptor signaling by Mycobacterium tuberculosis and its 19-kilodalton lipoprotein inhibits gamma interferon-induced regulation of selected genes in macrophages.* Infection and immunity, 2004. **72**(11): p. 6603-6614.
34. Pennini, M.E., et al., *Mycobacterium tuberculosis 19-kDa Lipoprotein Inhibits IFN-γ-Induced Chromatin Remodeling of MHC2TA by TLR2 and MAPK Signaling.* The Journal of Immunology, 2006. **176**(7): p. 4323-4330.
35. Bischoff, S.C., *Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data.* Nature Reviews Immunology, 2007. **7**(2): p. 93-104.
36. Muñoz, S., et al., *Mast cell activation by Mycobacterium tuberculosis: mediator release and role of CD48.* J Immunol, 2003. **170**(11): p. 5590-6.
37. McLachlan, J.B., et al., *Mast cell activators: a new class of highly effective vaccine adjuvants.* Nature Medicine, 2008. **14**(5): p. 536-541.
38. Kayamuro, H., et al., *Interleukin-1 family cytokines as mucosal vaccine adjuvants for induction of protective immunity against influenza virus.* Journal of virology, 2010. **84**(24): p. 12703-12712.
39. Villarreal, D.O., R.J. Siefert, and D.B. Weiner, *Alarmin IL-33 elicits potent TB-specific cell-mediated responses.* Hum Vaccin Immunother, 2015. **11**(8): p. 1954-60.
40. Enoksson, M., et al., *Intraperitoneal influx of neutrophils in response to IL-33 is mast cell–dependent.* Blood, 2013. **121**(3): p. 530-536.

41. Malaviya, R., et al., *Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells.* The Journal of Immunology, 1996. **156**(4): p. 1490-1496.
42. Féger, F., et al., *The role of mast cells in host defense and their subversion by bacterial pathogens.* Trends in Immunology, 2002. **23**(3): p. 151-158.
43. Pinke, K.H., et al., *Mast cells phagocyte Candida albicans and produce nitric oxide by mechanisms involving TLR2 and Dectin-1.* Immunobiology, 2016. **221**(2): p. 220-227.
44. Espinosa-Cueto, P., A. Magallanes-Puebla, and R. Mancilla, *Phosphate starvation enhances phagocytosis of Mycobacterium bovis/BCG by macrophages.* BMC Immunology, 2020. **21**(1): p. 34.
45. Vinod, V., et al., *The cell surface adhesins of Mycobacterium tuberculosis.* Microbiological Research, 2020. **232**: p. 126392.
46. Chan, B.C.L., et al., *IL33: Roles in Allergic Inflammation and Therapeutic Perspectives.* Frontiers in immunology, 2019. **10**: p. 364-364.

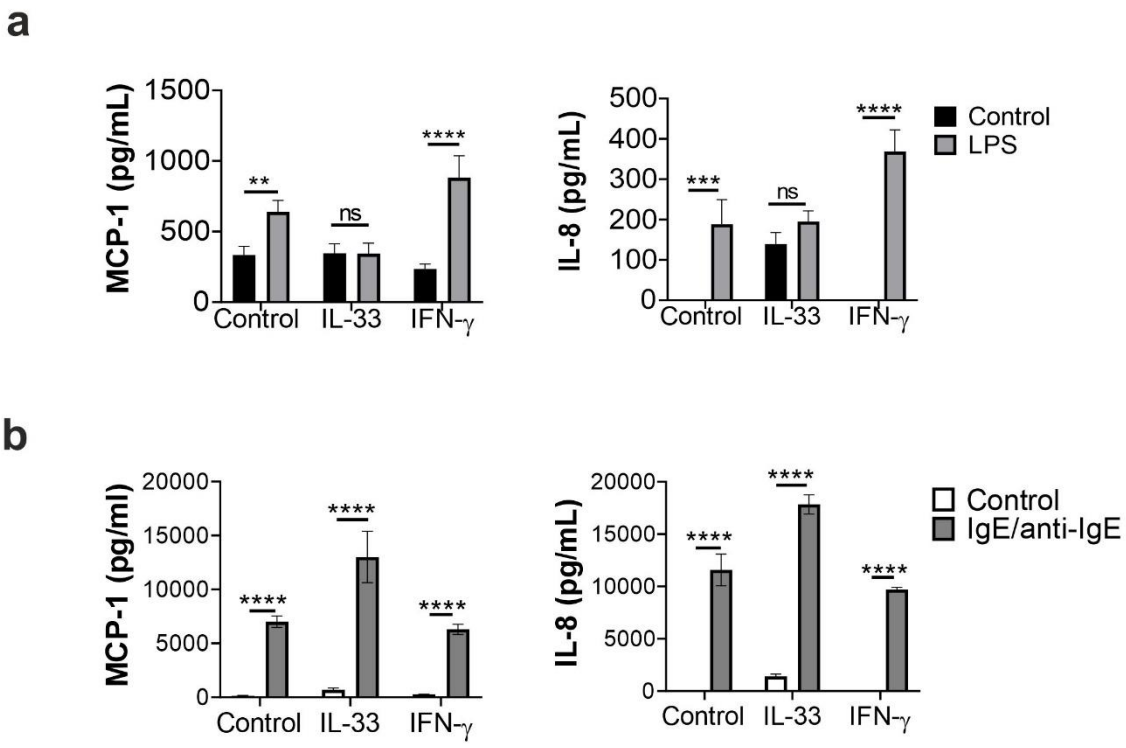
SUPPLEMENTARY MATERIAL

Supplemental Figure 6.A



Supplemental Figure 6.A. Blood progenitors-derived human mast cells: purity and maturity. After 8 weeks of cellular culture, hMCs were stained with anti- CD117 antibodies to investigate the purity of the cellular population and analysed by flow cytometry **(a)**. To evaluate cellular maturity **hMCs** ($0.5x10^6$ cells/mL) were sensitised overnight with IgE (1µg/mL), washed and either left untreated (control) or stimulated with anti-IgE antibodies (1µg/mL) for 1h to investigate degranulation or 8 hours to analyse cytokine secretion. After 1hr IgE incubation cells were stained with anti-CD63 or anti-CD107a antibodies and analysed by flow cytometry. Graphs show hMC degranulation as percentage of **(b)** $CD107a^+$ and **(c)** $CD63^+$ hMCs. Degranulation was confirmed with β-hexosaminidase release assay **(d)**. After 8hrs IgE incubation, supernatants were collected and IL-8, MCP-1 and GM-CSF release was measured by CBA multiplex assay **(e)**. Each graph shows a representative experiment from 3 independent experiments. 3 different donors were used for hMC experiments from 7 different cell cultures. Statistical analysis was done using *t*-test (*p*= **<0.01, ***<0.0001, , ****<0.00001).

Supplemental Figure 6.B



Supplemental Figure 6.B. hMCs mediator secretion upon LPS stimulation and IgE-FceRI crosslinking is enhanced by IL-33. hMCs were treated with IL-33 (50ng/ml) or IFN-g (50ng/ml) for 24h or left untreated (control). **(a)** Cells were washed and stimulated with LPS for 16 h or **(b)** sensitized overnight with IgE (100µg/ml) and stimulated with anti-IgE (100µg/ml) antibodies for 16h. After incubation supernatants were collected and MCP-1 and IL-8 were measured using the CBA multiplex assay. Each graph shows a representative experiment from 3 independent experiments. Statistical analyses were performed using one-way ANOVA analysis followed by a Tukey's multiple comparison test (****p<0.0001, ***p<0.001, **p<0.01).

Supplemental material video 1 and 2

Videos are available at:

https://data.mendeley.com/datasets/679j32jt45/draft?a=f2bcb428-39a2-4109-a205-c1f95e4d8b09

Supplemental video 6.C. BCG-hMCs interactions are increased by IL-33 pre-treatment. hMCs were treated for 24h with IL-33 (50ng/ml) or left unstimulated (control). After incubation cells were washed and left 30min in an 8 well chamber at 37C. BCG mCherry at a MOI 10:1 was added to wells and a video was recorded for 8h using confocal microscopy at 37C and 5% CO2 with 30-second interval acquisitions. Videos were analysed using FIJI and compressed to 4 minutes to allow visualization. Video 1 shows naïve MCs and Video 2 shows IL-33 pre-treated MCs. Videos are representative of 2 independent experiments

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CHAPTER 7: Main conclusions, future work and limitations

MAIN CONCLUSIONS AND FUTURE WORK

The present thesis characterized human mast cell (hMC) activities upon pathogenic bacteria showing that hMCs display specific responses depending on the bacterial type. We demonstrated that while *Listeria monocytogenes* induced a ROS-independent DNA externalization together with degranulation and IL-8 and MCP-1 release, *Streptococcus pnaumoniae* promoted ROS release without DNA externalization and degranulation, and while *Escherichia coli* induce degranulation without promoting ROS, *Staphylococcus aureus* exclusively releases prostaglandin D_2 in the absence of degranulation. This implies that rather than promoting a general response, MCs are capable to differentiate specific bacteria and display individual activities. However, is still unclear how these responses are triggered and which receptors are involved. In future, it will be important to investigate if these activities are more beneficial to the host than to the pathogen. Furthermore, a future study needs to investigate receptors that are upregulated after these bacterial encounters including TLR2, TLR4 and CD48. Once receptors are characterized, an additional study to investigate molecular pathways happening after bacterial activation would be important. Furthermore, as we showed that live cells externalize DNA, a future experiment would need to comprehend the mechanistic pathways that are involved in this phenomenon and for how long MCs remain alive after DNA is secreted or if MCs are recovered after externalizing DNA. Besides, as we showed that MC reduced *L. monocytogenes* numbers, an additional study would need to investigate which molecules are involved in this bacterial killing. This would provide a greater understanding of how MCs discriminate pathogens and probably these molecules or receptors can be used as therapeutic targets.

In addition, the present study investigated location, numbers and phenotype of MCs in human tissue obtained from TB necropsies. In fact, this was the first time MCs were studied in TB-infected human lungs. We showed MCs located near pneumonic areas and granulomas but more abundant in fibrotic sites. We also showed MCs capable to internalize and digest *Mtb*. Moreover, MCs were shown to expressed IL-17, necessary for granuloma maintenance, and TGF-β, which contributes to fibrosis formation [1]. Therefore, we proposed MCs as contributors to both, the initial and late stages of TB pathology. To confirm the role of IL-17 and TGF-β during TB infection, a future approach would be to inhibit MC-mediated IL-17 and TGF-β in knock out mice and evaluate pneumonia and fibrosis formation. Furthermore, key future research would be to perform *in vitro* experiments using hMCs and the virulent *Mtb* strain to confirm the release of IL-17 and TGF-β. As this study was mainly descriptive, deeper research to understand MC mechanisms is needed. For instance, it would be important to investigate if MCs secrete IL-17 and TGF-β by a direct activation from *Mtb* or by other molecules displayed at the infection site. Additionally, as we found MCs located at inflammatory and fibrotic sites but absent within granuloma structures, it is still unclear how MCs are modulated, and which molecules are involved on their migration, differentiation and activation in a TB pathology context. Furthermore, as MC_C were more abundant at fibrotic sites, a future study would need to investigate what triggers MC differentiation and what is the role of chymase in TB-induced fibrosis. Besides, as MCs were observed to internalize and phagocytose *Mtb*, a future approach would be to understand if MCs are involved in the antigen presentation and if these cells may serve as *Mtb* reservoir during the latent infection. Altogether, this would provide a deeper understanding and potentially propose MCs as therapeutic targets to prevent TB-induced fibrosis and to diminish MC-associated inflammation.

Moreover, we studied MC interactions with BCG, which is the TB vaccine [2]. We demonstrated that although naive MCs are unable to respond to BCG, IL-33 priming pre-activates MCs to release MCP-1 and IL-8 upon BCG infection. IL-33 promoted MC expression of CD48 and increased the number of BCG-MC interactions. This suggests that IL-33 is necessary for MCs to respond to BCG stimulation. However, to complete this study more experiments are needed. For instance, to understand the role of CD48, it would be necessary to block CD48 using an anti-CD48 antibody and measure cytokine secretion and the number of BCG-MC interactions. Furthermore, as IL-33 enhance MC-adhesion molecules including ICAM-1 and VCAM-1 [3], an important future work would be to evaluate the expression of these receptors and to block them to understand their involvement in BCG-MC interactions. In addition, it would be relevant to understand the term of IL-33 effect on MCs. To evaluate this, MCs can be pre-treated with IL-33 and stimulated with BCG few days after priming to measure mediator release. Besides, it would be important to investigate whether IL-33 promote MCs to form extracellular traps (MCETs) after BCG encounter and if such MCETs are ROS dependent. Finally, the present study used BCG Danish strain, however, using multiple BCG strains to compare their pattern of MC activation would be necessary to confirm the lack of MC response upon BCG stimulation.

LIMITATIONS

Research design limitations

The present study allowed us to understand direct responses and functional activities of hMCs upon bacteria but lacks to describe molecular routes to explain the mechanisms of the observed events. Besides, our generated cells are considered naïve as they lack pre-activation from the tissue environment, thus they are possibly different from tissue MCs. Furthermore, the present study limits the understanding of MC heterogeneity during MC infection. Although we obtained relevant data on MC activities upon bacterial encounter, it is important to evaluate the role of MCs in a disease context where more immune cells and molecules are involved. For instance, here we showed that naive MCs are unable to uptake BCG, however, we also reported MCs capable to internalize *Mtb* in human infected lungs. Thus, it is still unclear if this lack of internalization is due to the need of MC pre-activation or due to adhesion molecules that are absent in BCG but present in *Mtb*. Thus, the findings shown in the present study would be enriched from additional studies using different approaches that include molecular mechanisms in MCs and *in vivo* models proving MCs effects in a disease context.

Furthermore, in this model, cell progenitors were taken from anonymous patients which limits the understanding of any background related to the donors. It is currently known that BCG vaccination promotes epigenetic transformations (trained immunity) in haematopoietic stem cells [4]. Thus, knowing the vaccination status of received donors would have provided a better understanding of MC activities not only after BCG but after any bacterial infection.

Technical limitations

During the present study, time restrictions limited the conclusion of additional experiments. Besides, our *in vitro* model of hMCs are generated in a period of 8 weeks, which required a

well-planned organization and time management. Furthermore, as one section of this study was performed in Mexico City, this was limited to the short time that was available during the planned visits to Mexico. In addition, infected tissue was limited to the number of lung tissue samples and control tissues. Finally, this work was limited to the bacterial strains including BCG sub-strains available in our laboratory.

REFERENCES: Chapter 7

1. Ehlers, S. and U.E. Schaible, *The granuloma in tuberculosis: dynamics of a host-pathogen collusion.* Frontiers in immunology, 2013. **3**: p. 411-411.
2. Abdallah, A.M. and M.A. Behr, *Evolution and strain variation in BCG*, in *Strain Variation in the Mycobacterium tuberculosis Complex: Its Role in Biology, Epidemiology and Control*. 2017, Springer. p. 155-169.
3. Chan, B.C.L., et al., *IL33: Roles in Allergic Inflammation and Therapeutic Perspectives.* Frontiers in Immunology, 2019. **10**(364).
4. Cirovic, B., et al., *BCG Vaccination in Humans Elicits Trained Immunity via the Hematopoietic Progenitor Compartment.* Cell host & microbe, 2020. **28**(2): p. 322-334.e5.

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