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NEUTROPHILs AND MAST CELLs ON THE LINE BETWEEN INNATE AND ADAPTIVE IMMUNITY

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Abstract

Mast cells (MCs) are the key effector cells in immunoglobulin E (IgE) -mediated allergic disease such as atopic asthma, allergic rhinitis and atopic dermatitis, but also participate in a variety of IgE-independent biologic responses. MCs can produce and secrete mediators influencing various aspects of the biology of dendritic cells, T cells, and B cells thus acting like regulatory cells (Merluzzi et al., 2010). With the advent of the MC deficient mice and selective engraftment with *in-vitro*-generated bone marrow derived mast cells (BMMC), it was shown that these cells are critical for the initiation of acute inflammatory responses. In several models for acute inflammation, the recruitment of neutrophils turned out to be initiated by MCs, which are localized at site of infection (Doener et al., 2013). In this study we showed that neutrophils and MCs interact through cell-cell contacts and via soluble factors. This cross-talk is bidirectional resulting in neutrophils activation and down-regulation of some of MC functions. Since the two cells are known to be involved in regulation of B cells activation, proliferation and immunoglobulin secretion we further investigated the role of their crosstalk in regulation of B cell response. We demonstrated an increased B cells survival in presence of conditioned media of neutrophils-MC co-cultures probably due to an increased presence of soluble BAFF. To extend the study to effect of this cross-talk on adaptive immune response we performed an in vivo T-independent immunization in Wild Type mice, in mast cell-deficient Kit^{W-sh}/Kit^{W-sh} mice and Kit^{W-sh}/Kit^{W-sh} mice engrafted with bone marrow derived MC. In parallel T-independent immunization was performed in WT, Kit^{W-sh}/Kit^{W-sh} mice and in reconstituted Kit^{W-sh}/Kit^{W-sh} mice depleted for neutrophils and immunoglobulin production among mice groups was compared. We discovered

that the MCs and neutrophils together seem to regulate Ig production after T-independent immunization in a reciprocal way.

Ab Antibody

Ag Antigen

ANCA Anti-Neutrophil cytoplasmic antibody

AND Anaphylactic degranulation

APC Antigen-Presenting Cell

APRIL A Proliferation-Inducing Ligand

BAFF B cell Activating Factor of the TNF family

BCR B-Cell Receptor

BMMCs Bone Marrow derived Mast Cells

B reg Regulatory B cell

CD Cluster of Differentiation

CM Conditioned Medium

CR complement receptor

CTMC Connctive tissue MCs

DC Dendritic Cell

DLL1 Delta-like ligand 1

DNP Dinitrophenol

FBS Fetal Bovine Serum

Fc Fragment crystallizable region

FGF Fibroblast Growth factor

FO Follicular

Foxp3 Forkhead box 3

G-CSF Granulocyte colony-stimulating factor

Grb2 Growth factor receptor-bound protein 2

HSC Hematopoietic Stem cell

ICAM Intercellular Adhesion Molecule

IFN Interferon

Ig Immunoglobulin

IL Interleukin

LFA Lymphocyte function-associated antigen 1

LPS Lipopolysaccharide

mAb Monoclonal Antibody

MAC1 Macrophage-1 Antigen

MAPK Mitogen-Activated Protein Kinase

MC Mast cell

MHC Major Histocompatibility Complex

MMC Mucosal MC

mMCP mouse MC protease

Myd88 Myeloid differentiation primary response gene 88

NADPH Nicotinamide Adenine Dinucleotide Phosphate

NETS Neutrophils Extracellular Traps

NOX NADPH Oxidase

NK Natural Killer cell

PAD Peptidyl arginine deiminase

PLCγ1 Phospholipase C, gamma 1

PMD piecemeal Degranulation

PMO Myeloperoxidase

PSGL 1 P selectin glycoprotein ligand 1

MZ Marginal Zone

NGF Nerve Growth Factor

NK Natural Killer

PAMPs Pathogen-Associated Molecular Patterns

PBS Phosphate Buffer

PMN Polymorphonuclear Cells

PPR Pattern Recognition Receptor

RNI Reactive Nitrogen Intermediates

ROI Reactive Oxygen Intermediates

ROS Reactive Oxygen Species

sIg Surface Immunoglobulin

SCF Stem Cell Factor

SLP76 Lymphocyte cytosolic protein 2

STAT3 Signal Transducer and Activator of Transcription 3

Tc cytotoxic T cell

TCR T Cell receptor

TD T dependent

Teff T effector

TGF Transforming Growth Factor

Th T helper

TI T independent

TIR Toll-Interleukin Receptor

TIRAP TIR adapter

TLR Toll Like Receptor

TNF Tumor Necrosis Factor

Treg T regulatory

TRIF TIR domain containing adapter-inducing interferonβ

TW Trans-well

VEGF Vascular Endothelial Growth Factor

WT Wild Type

List of abbreviations

1. INTRODUCTION

1.1. Innate and adaptive immune system crosstalk

The innate immune system constitutes the first line of host defense during infection and therefore plays a crucial role in the early recognition and subsequent triggering of pro-inflammatory response to invading pathogens (Medzhitov & Janeway, 2000). The adaptive immune system, on the other hand, is responsible for elimination of pathogens in the late phase of infection and generation of immunological memory. Whereas the adaptive immune response is characterized by specificity developed by clonal gene rearrangements from a broad repertoire of antigen-specific receptors on lymphocytes, the innate response is mediated primarily by phagocytic cells, antigen-presenting cells (APCs), such as granulocytes, macrophages, dendritic cells (DCs) and MCs and has been regarded as relatively nonspecific. However, the innate immune system is no longer regarded as a primitive, nonspecific system involved only in destroying and presenting antigen to cells of the adaptive immune system. In fact, it now appears that innate and adaptive immune responses are much more intimately connected than initially believed, and several important findings support the idea that the innate immune system, besides being essential for early pathogen recognition, is also involved in the activation and shaping adaptive immunity (Iwasaki & Medzhitov, 2004). Thus, the non clonogenic germ-line-encoded pattern recognition receptors (PPR), as the toll-like receptors (TLR), sense microbial compounds and bridge innate and adaptive immunity by activation of APCs (Werling, Jungi, & Bern, 2003). TLR-

induced signaling via intracellular adaptor molecules, including MyD88 and TRIF, drives transcriptional activation of genes including pro-inflammatory cytokines cell activation markers (Kawasaki & Kawai, 2014) (Pasare & Medzhitov, 2005), and co-stimulatory molecules, which subsequently control the activation of antigen-specific adaptive immune response (Hivroz, Chemin, Tourret, & Bohineust, 2012).

A critical element, in the interaction between the two arms of immune response, is the role played by dendritic cells (DCs), which represent “professional APC”. DCs endocytose and process antigen to peptide presented on the cell surface in association with Major Histocompatibility Complex (MHC) molecules (Hivroz et al., 2012).

This presentation results in interaction with and stimulation of helper T (Th) cells or cytotoxic T (Tc) cells, which recognize peptide in association with either MHC class II or MHC class I respectively (Bousso, 2008) (Hivroz et al., 2012). Stimulation of Th lymphocytes produces the growth and differentiation factors (cytokines) essential for the B lymphocytes that have responded to a more intact form of the antigen and that differentiate into antibody-producing cells. The precise interaction between the cells depends on cognate ligand-receptor recognition between the B and Th lymphocytes. DCs also play a direct role with the stimulation of the B cells. Additionally, DCs show a reciprocal influence in the process of activation with other adaptive immune cells, as Th17, regulatory T (Treg) cells, and regulatory B (B reg) cells (Qian et al., 2012), or innate immune cells, such as mast cells (MCs) (Gri et al., 2012), natural killer (NK) cells and NKT cells.

In the secondary lymphoid organs, the professional APCs, the DCs stimulate T cell to respond to antigen loading endocytosed antigenic peptides on both MHC

class I and MHC class II molecules, combined with secondary signals delivered by co-stimulatory molecules such ad CD80/86 (Guermonprez et al., 2003). Co-stimulation can simply promote more efficient engagement of T cell receptor (TCR) molecules to enhance the initial activation, or can provide additional biochemically distinct signals to regulate cells division, increase cell survival or induce T cell effector functions, such as cytokines secretion or cytotoxicity (Zingoni et al., 2005).

NK cells promote adaptive immune response through their production of type 1 and type 2 cytokines or chemokines. For example, following viral infections, the NK cells production of INFγ plays a key role in the initial antiviral response, activating macrophages to secrete IL-12, crucial for T cell differentiation into Th1 effectors and for the development of CD8+ cytotoxic cells (Zingoni et al., 2005). MCs and basophils express a number of B cell modulating molecules. MCs were shown to affect B cell survival, proliferation and to trigger IgE synthesis and differentiation into IgA secreting plasma cells. Basophils support B cell proliferation and promote plasma cells survival and Ig production (Merluzzi et al., 2014).

Neutrophils have long been recognized for their protein-destroying and bactericidal properties. Neutrophils can secrete products that stimulate monocytes and macrophages; these secretions increase phagocytosis and the formation of reactive oxygen compounds involved in intracellular killing. Moreover, activated neutrophils release large amounts of B cell stimulating factors, such as B cell activating factor (BAFF) and proliferation-inducing ligand (APRIL). Recent findings have found neutrophils around marginal zone (MZ) of the spleen, a B cell area specialized in T-independent Ig responses to circulating antigen. Neutrophils colonized peri-MZ areas after post-natal

mucosal colonization by microbes and enhanced their B-helper function upon receiving reprogramming signals from splenic sinusoidal endothelial cells, including interleukin 10 (IL10). Splenic neutrophils induced Ig class switching, somatic hypermutation and antibody production by activating MZ B cells through a mechanism involving the cytokines BAFF, APRIL and IL 21 (Cerutti, Puga, & Cols, 2012).

Dialogue between the innate and adaptive immune system is not one sided. Adaptive response, in fact, can support the function of the innate immune system. Antibodies secreted by B cell, for example, bind macrophages and other phagocytes by interaction with Fc receptors on these cells. Phagocytes recognize, internalize and destroy pathogens using these borrowed antibodies. This selective internalization enhances the ability of these cells to internalize and present these antigens to T cells increasing acquired responses (Clark & Kupper, 2005).

Appropriate innate and acquired immune system interactions lead to highly efficient recognition and clearance of pathogens, but maladaptive interaction between these two system can result in harmful immunologic responses including allergy, autoimmunity and allograft rejection (Clark & Kupper, 2005).

1.1.1. T– Independent B cell response

Mouse B cells are the effectors of humoral immunity and they are subdivided into two distinct lineages, B-1 and B-2, which differ from functional characteristics and anatomical localization. B-1 B cells have been considered to be part of the innate immune system, whereas B-2 B cells function primarily in

adaptive immune responses (Montecino-Rodriguez & Dorshkind, 2006). B-1 is a self-renewing population which is enriched in peritoneal and pleural cavities and constitute the first B cell population that appears early in life (Vitale, Mion, & Pucillo, 2010). B1 B cells start response to infection by T-independent mechanisms thanks to their capacity to produce antibodies that are specific for self-antigens and for pathogen expressed-molecules like Gram-positive bacteria, Lipopolysaccharide (LPS) of Gram negative bacteria and various viral and parasite-expressed antigens (Baumgarth, 2011). For this reason, B1 cells play an important role in innate immunity by secreting large amounts of natural antibodies of the Immunoglobulin M (IgM) class, which can be produced without exposure to any environmental antigens or immunization. They, also, express a reduced number of B-cell receptor (BCR) specificities, compared to B-2 follicular B cells that confirm their status of innate-like immune cells.

Two subset of B1 cells can be distinguished: B1a deriving from fetal liver B cells and identify as IgM^{Hi} IgD^{Lo} $CD11b^{+}CD5^{+}CD220^{Lo/+}$ and the similar $CD5^{-}$ B1b originating from BM-derived stem cells.

B1a cells represent a unique lineage distinguished by specific ontologic, phenotypic and functional characteristic. It has been shown that B1-a cells spontaneously and constitutively generate “natural” immunoglobulin, which constitutes the vast majority of resting serum IgM and about half of resting IgA (Holodick, Vizconde, & Rothstein, 2014).

While B1a B cells constitute a source of natural antibody (Ab) during early phases of infection, in contrast, B1b cells appear to be the primary source of dynamic T cell independent (TI) antibody production and long-term protection after bacterial infection such as Borrelia hermsii and Streptococcus pneumoniae.

B2 B cells, also known as “conventional” B cells, are produced from hematopoietic stem cells (HSCs) in the bone marrow during postnatal life and they constitute the predominant population of B lymphocytes found in spleen and lymph nodes. Their differentiation culminates in the generation of surface immunoglobulin M (sIgM) -expressing cells which then migrate to spleen, where they undergo further maturation into follicular (FO) or MZ B cells (Montecino-Rodriguez & Dorshkind, 2006). This diverse progeny of distinct subpopulation respond to antigens both by T dependent and T independent mechanisms (Vitale et al., 2010).

FO B cells, which are the dominating B-cell population in secondary lymphoid organs play an important role in adaptive immune responses. In contrast, the pre-activated phenotype of splenic MZ B cells qualifies them to participate in T-independent immune responses via rapid antibody secretion after antigenic stimulation (Düber et al., 2009). Similar to B-1 B cells, in fact, MZ B cells express polyspecific antibodies that recognize T-cell independent antigens and highly respond to BAFF and to APRIL released by innate immune cells after the encounter with pathogens. Both cells are characterized by a state of active readiness, which involves elevated TLR expression, and ‘innate’ Ig receptors with poorly diversified antigen-binding variable regions that permit them to recognize multiple highly conserved microbial products (Cerutti, Puga, & Cols, 2011).

B cells responses, in fact, are classified as T-dependent (TD) or T-independent (TI) based on their necessity for T cells help in antibody production (Nussenzweig, 2006).

In contrast to the large body of knowledge about cellular and molecular events in TD responses, little is known about cellular and molecular requirements of a

TI immune response (Martin, Zhou, Kearney, & B, 2002).

T cell-dependent immune responses generally involve conventional (B2) B cells. By contrast, the other subset of B cells, B1 cells, produce antibodies in a T-independent manner.

In T-cell dependent response, B cell activation by protein antigens requires binding of the antigen to the B cell surface immunoglobulin (Ig) and the co-stimulation by antigen-specific T cells through CD40-CD40 ligand (CD40L) interaction and the secretion of cytokines (Fagarasan, 2000).

T independent antigens, on the other hand, are able to initiate a serological response in absence of T-cell help. There are two type of TI antigens: type I and type II. The first one are mitogenic stimuli like LPS, CpG that elicit polyclonal B cell activation via TLRs, the other one are polysaccharides, glycolipids and nucleic acid that engage the B cell receptor (BCR) and induce antigen-specific B cell responses.

These antigens are not processed and presented along with MHC proteins so they cannot be recognized by helper T cells and so TI- type I antigens can directly stimulate B cells without requirement of any other cell (Nussenzweig, 2006). Type II TI antigens possess a highly organized, repeating structures that are able to activate naïve B cells in absence of $CD4^+$ T cell help, by cross linking multiple BCRs on their surface. Additional signal is required by activated B cell to stimulate antibody production, via TLR stimulation or complement activation and CD21 stimulation (Grant et al., 2012).

Moreover, also microenviromental signals from BAFF and APRIL receptors would integrate with those from Ig receptors and TLRs to optimize B cell survival, Ig production, Ig diversification and plasma cells differentiation (Cerutti et al., 2011).

1.2. Neutrophils

Neutrophils, also known as neutrophilic granulocytes or polymorphonuclear leukocytes (PMN), are the most abundant white blood cells in the human circulation.

They are one of the principal effector cells of the immune system and are the first leukocytes recruited in sites of microbial infection and tissue invasion (Nathan, 2006).

Neutrophils are inherently short-lived cells with a half-life of only 6-10 hours in the circulation and rapidly undergo spontaneous apoptosis. In infected tissues their apoptosis can be delayed both by microbial constituents and pro-inflammatory stimuli (Esmann et al., 2010).

The role played by neutrophils in immunity has long been viewed as restricted to the acute phase of inflammation and to resistance against extracellular pathogens. Several studies have recently challenged this dogma and placed neutrophils as key effector cells in the regulation of adaptive immunity and in the resolution of inflammatory response (Jaillon et al., 2013).

Recent findings, indeed, show that in addition to antimicrobial agents, neutrophils can also produce numerous cytokines, chemokines, and growth factors (Mantovani, Cassatella, Costantini, & Jaillon, 2011) and can direct the adaptive immune response by communicating with various cell types, including B cell, dendritic cells, T cells and mast cells (Scapini & Cassatella, 2014).

1.2.1. Neutrophil biology

Neutrophils that leave the bone marrow and enter the blood-stream are terminally differentiated cells with short lifespan. They originate from hematopoietic precursor under the instruction of growth factors (G-CSF) and cytokines (IL3). In the bone marrow, hematopoietic cells differentiate into myeloblasts, which have round nucleus, then into promyelocytes, which accumulate peroxidase-positive azurophil granules (primary granules). The next differentiation step is in myelocytes, with a prominent accumulation of peroxidase-negative granules (secondary granules), and less abundant azurophilic granules (Lieschke et al.,1994). In the final stages of this process, myelocytes differentiate in metemyelocytes, band cells, and finally, granulocytes with the presence of tertiary granules (Borregaard, 2010) Fig 1.2.1.

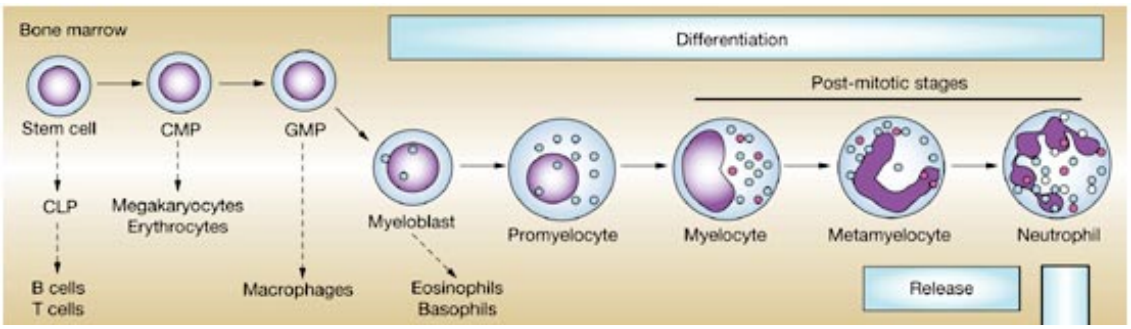


Fig 1.2.1 Neutrophil differentiation. Neutrophils differentiate in the bone marrow and mature neutrophils are released into peripheral blood. Mature neutrophils are unable to proliferate and undergo constitutive apoptosis (Eyles, Roberts, Metcalf, & Wicks, 2006).

Granules formed continuously during differentiation process are classified into four groups: primary or azurophilic, secondary or specific, tertiary or gelatinase, and secretory vesicles. Azurophilic granules contain myeloperoxidase (MPO),

an enzyme critical to the oxidative burst (Nusse & Lindau, 1988) hydrolitic and bactericidal enzymes. Secondary granules contain lactoferrin and lysozyme, as well as chemoattractant receptors. Tertiary granules contain few antimicrobial agents but store numerous metalloproteases, such gelatinase and leukolysin. Granules are not only repository organelles for dangerous substances but also indispensable for nearly all neutrophilic activities during inflammation (Borregaard, Sørensen, & Theilgaard-Mönch, 2007).

The union of antimicrobial proteins, receptors, and other membrane proteins in granules represent a potent mechanism to eliminate bacteria and provide a fast activation/degranulation mechanism during inflammation.

1.2.2. Neutrophil activation

A wide variety of stimuli induce neutrophil degranulation, including C5a, formyl-methionyl-leucyl-phenylalanine, LPS, platelet-activating factor, and tumor necrosis factor (TNF). Neutrophils also express Toll-like receptors TLR1 to TLR10, with the exception of TLR3, enabling them to initiate various potentially important immune responses upon recognition of pathogen-associated molecular patterns. (Borregaard et al., 2007)(Borregaard, 2010)(Prince, Whyte, Sabroe, & Parker, 2011).

The mechanism underlying neutrophil recruitment to the tissues is divided in three key steps: rolling, firm adhesion (endothelium) and diapedesis Fig 1.2.2.

The stimulants described above prompt endothelial cells to produce adhesion molecules on their luminal side: the P-selectins, E-selectins and several members of the integrin superfamily, the ICAMs (Borregaard, 2010).

On neutrophils surface, two constitutively expressed proteins are important for recognition of the endothelial inflammatory signals: the glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin (Hidari, Weyrich, Zimmerman, & sEver, 1997). After contact with endothelium, these molecules engage the P-E selectins of endothelial cells, resulting in selectin-mediated tethering of neutrophils to the vessel wall. This is followed by a characteristic rolling of neutrophils along the endothelium (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012). After selectin-mediated rolling, neutrophils enter a “firm adhesion” state mediated by the β2 integrin family of proteins (LFA-1, Mac1). This state is characterized by the arrest of neutrophil rolls along the endothelium, interaction with selectins, chemoattractants, cytokines and bacterial products results in activation and clustering of β2 integrins on the surface of neutrophils (Constantin et al., 2000) (Liu et al., 2002). After crawling along the vessel and reaching the preferred site of transmigration they begin to migrate through the endothelial junctions in the site of inflammation. Here neutrophils release cytokines that recruit other immune cells, and it begins to implement its antimicrobial potential. Among the process employed there are engulfment of microbes via receptor-mediated phagocytosis, release of granular antimicrobial molecules through degranulation, and formation of neutrophils extracellular traps (NETs) (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012).

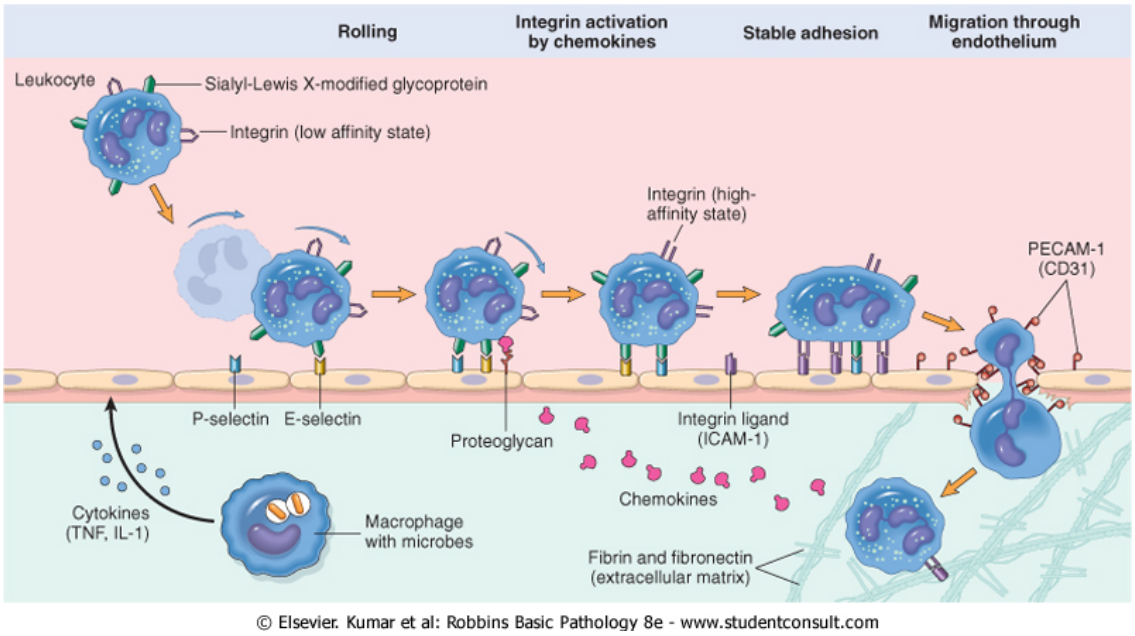


Figure 1.2.2 Migration of neutrophils to the sites of infection. This is a multi-step process in which chemoattractants are produced in response to an infection and neutrophils are recruited from blood by binding to adhesion molecules on luminal surface of endothelial cells. The process involves rolling, adhesion and migration of these cells through the endothelial cells (Robbins & Cotran 2009).

1.2.2.1. Neutrophil Phagocytosis

Neutrophils are efficient phagocytes and engulf microbes into phagosomes that rapidly fuse with the granules, exposing microorganisms to proteases, phospholipases and cationic peptides (Nordenfelt & Tapper, 2011).

The first step in this process is the recognition of microbes. Neutrophils recognize pathogen-derived molecules including peptidoglycan, lipoproteins, lipoteichoic acid, LPS and flagellin.

Like macrophages, neutrophils can internalize both opsonized and non-opsonized particles. Two separate classes of receptors are especially important during this mechanism: Fcγ-receptors (CD32, CD1, CD64 and CD89) (Bredius et al., 1994) and the complement receptors (CR1 and CR3).

While complement-opsonized particles are internalized by gently “sinking” into the cell, Fcγ receptor ligation initiates the vigorous extension of pseudopods that surround and ultimately entrap the particle (Greenberg & Grinstein, 2002). Once formed, the vacuole undergoes a rapid series of remodeling events that alter its composition, conferring onto it the ability to kill pathogens and dispose of debris. Fusion of the phagosome with the neutrophil lysosomes leads to the formation of phagolysosome (Lee, Harrison, & Grinstein, 2003).

1.2.2.2. Neutrophils ROS production

Activation of primed neutrophils, by phagocytosis of opsonized bacteria or by frustrated phagocytosis, generates rapid production of ROS via the action of NADPH oxidase. This enzyme is a multi-component enzyme, assembled at the plasma membrane during priming, so resting neutrophils have very little capacity to produce ROS (Wright, Moots, Bucknall, & Edwards, 2010). Neutrophils, macrophages, endothelial produce two types of free radicals. The first type is represented by reactive oxygen intermediates that are formed in neutrophils by the activity of NADPH oxidase. The second type includes reactive nitrogen intermediates, the first member of them, nitric oxide being produced by nitric oxide synthase (Carter R. Kettlerworth, 2007).

Reactive oxygen species most often cause damage of DNA, oxidations of amino acids in proteins, oxidations of polyunsaturated fatty acids in lipids (lipid peroxidation), and inactivation of specific enzymes by oxidation of co-factors.

1.2.2.3. Neutrophil NETS

In addition to the well-known capacity of neutrophils to phagocytose and kill invading microorganisms neutrophils can capture and kill pathogens extra-cellularly through the release of neutrophil extracellular traps (NETs) (Kirchner et al., 2012). These traps are networks of extracellular fibres, primarily composed of DNA and various bactericidal proteins (neutrophil elastase, histones), which bind and disarm pathogens (Kaplan, 2013).

There are three mechanisms of NETs release. Fig 1.2.2.3

NETs can be released through a vesicular mechanism. Initially, neutrophils become rounded with uniformly condensed chromatin and then undergo nuclear envelope breakdown. Within these cells, small vesicles containing DNA can be seen in the cytoplasm near the plasma membrane. The DNA-containing vesicles eventually fuse with the plasma membrane, and NETs are released to trap bacteria. NETs can also be released through cell lysis, and this typically takes longer than the vesicular-mediated mechanism. The nuclear envelope is degraded, and chromatin decondensation occurs because of peptidyl arginine deiminase 4 (PAD4)-mediated citrullination of histones. NET release by mitochondria has also been observed, in one study, although the steps of this process remain poorly characterized (Phillipson & Kubes, 2011) .

NET formation require NADPH oxidase (NOX) activity, disintegration of the nuclear envelope and most granule membranes, in concert with the citrullinating activity of PAD 4 on histones, they promote chromatin decondensation (Casanova, Abel, & Quintana-Murci, 2011) (Wang et al., 2009) (Li et al., 2010)(Hemmers, Teijaro, Arandjelovic, & Mowen, 2011).

During NET formation, neutrophils may die through a distinct cell death program

termed NETosis (Brinkmann et al., 2004).

A variety of stimuli can trigger NETosis, including pathogens, pro-inflammatory cytokines interleukin 8 (IL-8), TNFα, activated platelets and endothelial cells, nitric oxide, monosodium urate crystals, anti-neutrophils cytoplasmic antibodies (ANCAs), and immune complexes (Yu & Su, 2013).

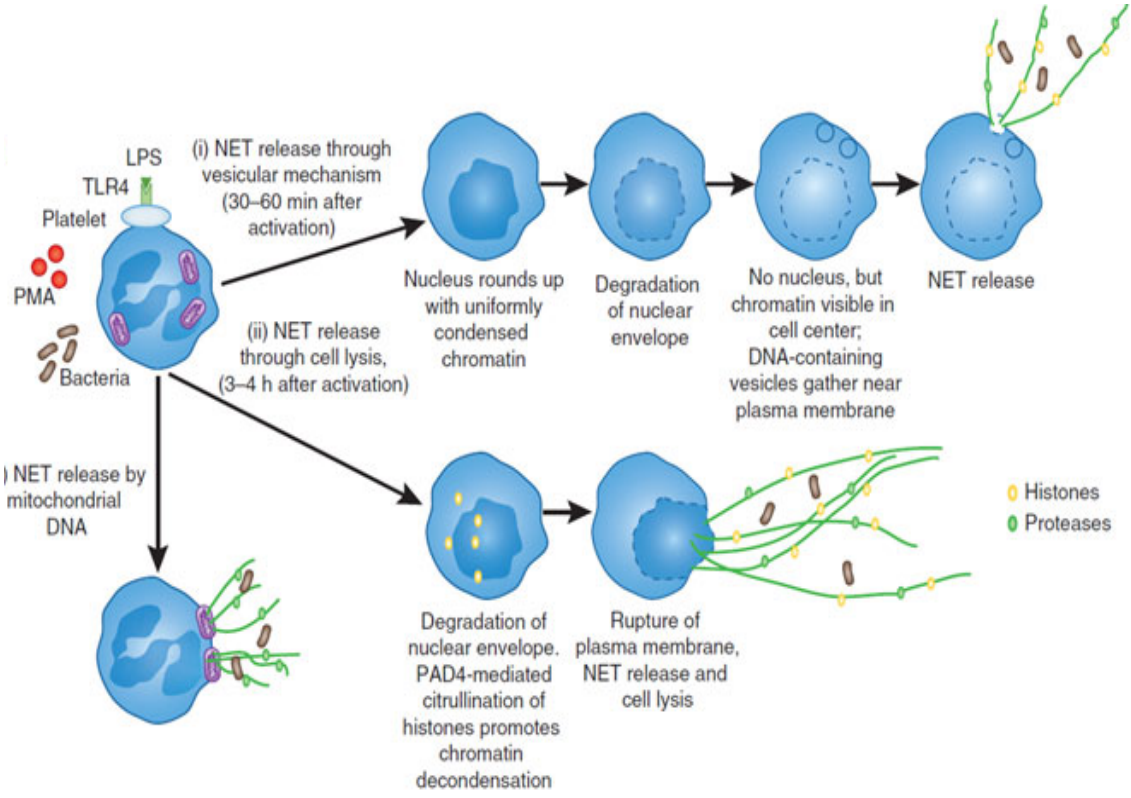


Fig 1.2.2.3 Various mechanism of NETS NETs can be released through: a vescicular mechanism, trough cell lysis, by mitochondria (Phillipson & Kubes, 2011).

1.2.3. Neutrophil clearance

Circulating numbers of neutrophils are determined by the rate of neutrophil production, storage, release, and clearance (Rankin, 2010).

Maintenance of circulating, functional neutrophils and their robust recruitment to tissues in response to injury and/or microbial infection are critical for host

defense. Equally important are the processes for removal of these short-lived cells (Bratton & Henson, 2011). Because uncontrolled release of toxic substances from neutrophils can propagate the inflammatory response leading to tissue destruction, recognition of dying inflammatory neutrophils has a critical function for the resolution of inflammatory response (Esmann et al., 2010).

Clearance of circulating neutrophils that do not enter the site of infection is mediated by macrophages in the liver, spleen and bone marrow.

Neutrophils removal from either the circulating pool, or from tissues, depends on signals resulting from neutrophil death, activation or aging (Bratton & Henson, 2011).

Macrophages present in the liver and spleen are part of the reticulo endothelial system and are in direct contact with blood. These macrophages recognize, bind and phagocytose circulating and at sites of inflammation apoptotic neutrophils (Rankin, 2010).

A number of changes on the dying cell surface lead either to binding molecules which give rise to “eat me” signals or to direct engagement of macrophage receptors. These “eat me” signals include externalized phosatidylserine, altered lipids, changes in surface charges likely attributed to amino sugars, and exposure of intracellular molecules such as calreticulin, mitochondrial and nuclear constituents (Bratton & Henson, 2011).

Clearance via bone marrow takes place in an age dependent manner.

The chemokine CXCL12 is produced in the bone marrow and binds to CXCR4 receptors on neutrophils. Circulating senescent neutrophils up-regulate expression of CXCR4 that binds chemokine CXCL12 highly produced in the bone marrow. Via the CXCR4/CXCL12 axis, these aging and pre-apoptotic neutrophils go back to the bone marrow where they are phagocytized by

macrophages (Rankin, 2010).

The recognition of dying cells results in profound alterations in macrophages responses. TGFβ and/or IL10 production by macrophages exposed to neutrophils undergoing apoptosis suppresses pro-inflammatory cytokine, chemokine and eicosanoid production. Failure of neutrophil removal results in the so called "nuclear dust" and it is present in many autoimmune disease (Bratton & Henson, 2011).

1.2.4. Neutrophils and B cells

Recent studies have shown that neutrophils can release a broad array of preformed and newly synthesized mediators, including chemokines and cytokines. The long held view that neutrophils function exclusively in the innate phase of immune response has been challenged, in fact these molecules regulate mobilization and function of neutrophils, monocytes, DCs, NK cells, but also the recruitment and activation T cells and B cells (Cerutti, Puga, & Magri, 2013). Neutrophils crosstalk with B cells derive by their binding to B cell-derived IgG and IgA on opsonized microbes and by their production of BAFF and APRIL, which are two TLR-inducible B cell-stimulating factors related to the ligand for the T cell molecule CD40 (CD40L) (Puga et al., 2012). Recent findings show, also, that neutrophils occupy peri-MZ areas of the spleen in the absence of infection. The splenic microenvironment stimulates conventional neutrophils to become B cell helper neutrophils (N_{HB}) through a process that involves the delivery of neutrophils reprogramming signals from splenic sinusoidal endothelial cells and other cells (Cerutti et al., 2012).

N_{HB} induce IgM secretion, as well as IgG and IgA, by stimulating MZ B cells via BAFF, APRIL, IL21 and CD40L (Cerutti et al., 2012). On their surface they present cell suppressive factors that suppress T cell proliferation in a contact dependent manner. By exerting this dual B cell helper and T cell suppressor function, N_{HB} can maximize extra-follicular B-cell responses to TI antigens while minimizing follicular B cell responses to T dependent antigens and inflammation (Cerutti et al., 2012).

1.3. Mast Cells

Mast cells (MCs) play an important role in the initiation and regulation of immune response. They were first described by Paul Enrlich in 1878, on the basis of their staining characteristics and large granules content. He named them "*Mastzellen*" which means “well-fed cells” in belief that their granules contain deposits of nutrients (Ehrlich P. 1978). They have long been known to be central to the initiation of allergic disorders but they have also important role in host defense, in directing and/or mediating a protective immune response and also in inducing tolerance (Alvarez-Errico, Lessmann, & Rivera, 2009).

1.3.1. Mast cell biology

MCs are derived from hematopoietic progenitor cells but not ordinarily circulate in mature form: after migration of their precursors to the vascularized tissues or serosal cavities in which they will reside they locally differentiate and mature (Marone, Galli, & Kitamura, 2002).

Maturation of the MC precursors is dependent on stem cell factor (SCF) expressed on the surface of fibroblasts, stromal cells, and endothelial cells (Arinobu et al., 2005) and interleukin 3 (IL3).

MCs are strategically located along the microvasculature in tissues in close contact with the external environment, such as the skin, lung and intestine.

There are two main MC populations in mice, based on their secretory granules: the connective tissue MCs (CTMC) and mucosal MCs (MMC). Fig1.3.1 Safranin postive granules of CTMC contain heparin proteoglycans and mouse mast cells protease 4 (mMCP4), 5 and 6. Safranin negative granules of MMC express mMCP 1 or 2 (Godfraind et al., 1998). Human MCs are divided in two population in relation of neutral protease content. They contain good levels of tryptase, chymase a cathepsin G-like protease and carboxypeptidase: MC_{TC} contain tryptase and chymase MC_T contain only tryptase (Metcalfe, Baram, & Mekori, 1997).

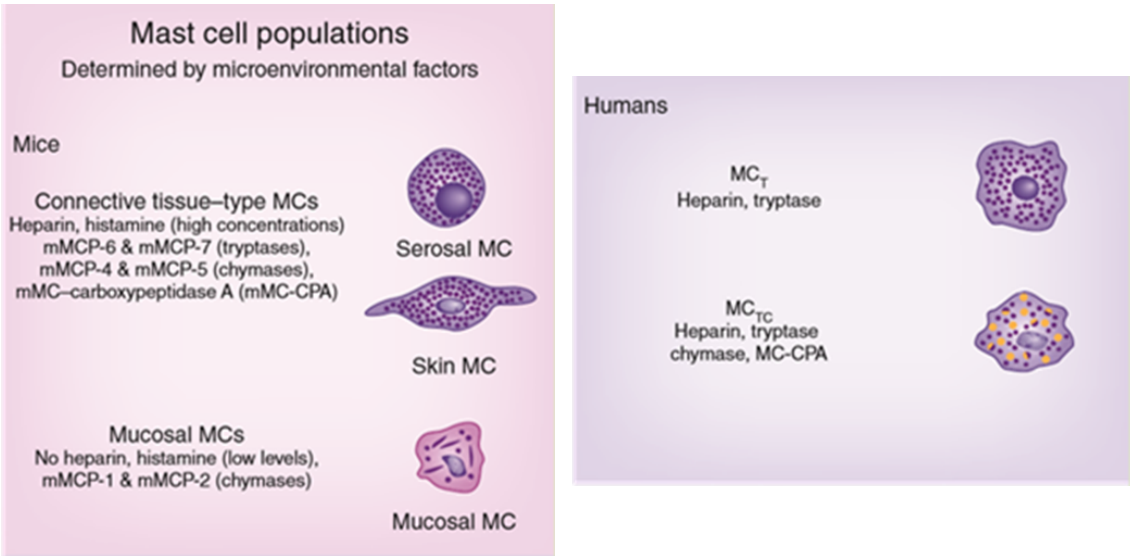


Fig 1.3.1 MCs in mice or humans can be divided into populations dependent by anatomical location, secretory granules and neural proteases content (Galli, Borregaard, & Wynn, 2011)

MCs are capable of reacting, both within minutes and over hours, to a variety of physical, biological and chemical stimuli with either local or systemic effects Fig 1.3.1.2.

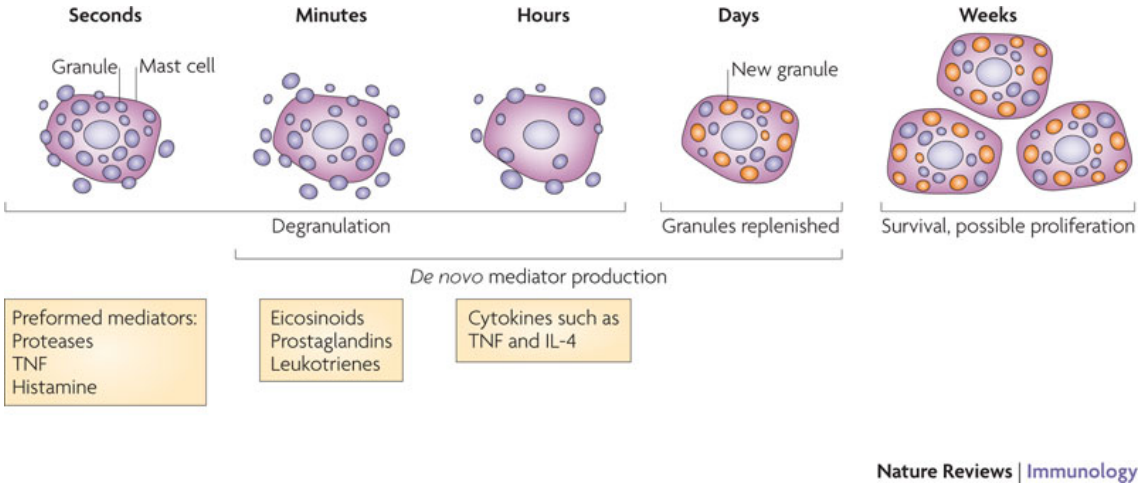


Fig 1.3.1.2 MC timing reaction MCs can respond to different stimuli in different time point. In seconds by releasing preformed mediators, in minutes producing eicosanoid mediators. In a second wave of response, MCs begin to release *de novo* synthesized mediators (Abraham & St John, 2010).

They participate in the innate and acquired immune response and play a key

role in many immunological and inflammatory reactions. A beneficial role for these cells has been describe in defense against bacteria, virus and parasites (Galli, Nakae, & Tsai, 2005).

Degranulation of activated cells results in the secretion of preformed mediators that are stored in the cells' cytoplasmic granules, growth factors, cytokines and chemokines. In contrast, proinflammatory lipid mediators are newly synthesized. These MC-derived mediators generate the early initiation phase (vascular reaction and exudation) and contribute to the late phase (leukocyte accumulation and wound healing) of allergic inflammation (Mekori & Metcalfe, 2000).

The best studied mechanism by which MCs accomplish immunologically specific function is through antigen and IgE-dependent aggregation of the high affinity IgE receptor FcεRI (Kalesnikoff & Galli, 2008). However, MCs can be activated to perform important effector and immunomodulatory functions by many mechanisms that are independent of IgE.

1.3.2. Mast cell receptors

MCs present on their surface an array of immune-response receptors and other surface molecules that confer the advanced capability to react to many nonspecific and specific stimuli.

The most important stimulatory receptors of MCs include the FcεRI, c-Kit (receptor for stem cell factor), IgG receptors like FcγRI (or CD64) and FcγRIII (or CD16), complement proteins and TLRs. Many of these receptors contain immuno-receptor tyrosine-based activation motifs (ITAMs) that are essential for

activating signal.

The multivalent binding of Ag to receptor-bound IgE and subsequent aggregation of the FcεRI provide triggers for MC activation. Ag aggregation of IgE-occupied FcεRI results in phosphorylation of immunoreceptor tyrosine based activation motifs by Lyn kinase (Lyn), receptor association and activation of Syk kinase (Syk), and inclusion of the FcεRI in lipid rafts (Frossi, De Carli, & Pucillo, 2004).

C-kit or CD117 is a receptor tyrosine kinase type III, which binds to stem cell factor (SCF). When this receptor binds SCF it forms a dimer that activates its intrinsic tyrosin kinase activity that activates signal transduction molecules that propagate signals in the cells.

The signaling pathways activated by triggering the IgG receptor generally resemble those observed following FcεRI aggregation that induces phosphorylation of Src kinases and p72 and subsequent activation of multiple substrates (Okayama, Tkaczyk, Metcalfe, & Gilfillan, 2003).

1.3.3. Mast cell signaling via FcεRI

MC activation may be initiated upon interaction of a multivalent antigen (allergen) with specific IgE antibody attached to the cell membrane via the high affinity receptor, FcεRI (Mekori & Metcalfe, 2000).

FcεRI is a member of the Fc receptor family and it's the high affinity receptor for the crystalizable fragment (Fc) of IgE. It is a tetrameric receptor complex consisting of one alpha, one beta and two disulfide bridge connected gamma chains.

FcεRI binds monovalent IgE antibodies secreted by plasmacells. The most potent MC responses are seen when antigen-specific IgE bound to FcεRI encounters the specific antigen (Alvarez-Errico et al., 2009).

Ag aggregation of IgE-occupied FcεRI results in phosphorylation of immune receptor tyrosine based activation motifs (ITAM) by Lyn kinase since FcεRI lacks intrinsic tyrosine kinase activity. Once activated, Fyn, Lyn and Syk contribute to the formation of multi-molecular signaling complexes that are coordinated by adaptors, like LAT1 and 2, Gab2,Grb2,Gads, among others (Alvarez-Errico et al., 2009).

When modified by FcεRI-induced phosphorylation, LAT forms a molecular complex that includes the adapters SLP-76, Grb2, phospholipase Cγ1 (PLCγ1), and the guanine nucleotide exchange factor, Vav1.

IP3 release Ca2+ form intracellular stores as it binds to receptors in endoplasmic reticulum (ER) and activates store-operated calcium channels for the influx of Ca2+ allowing the regulation of later events of the signaling cascade (Frossi, De Carli, & Pucillo, 2004).

MC activation results in the release of a variety of allergic mediators that are stored in intracellular granules and initiate de novo synthesis and secretion of inflammatory lipid mediators and a wide range of cytokines and chemokines (Blank & Rivera, 2004).

1.3.4. Direct activation by pathogens

TLR are a family of pattern-recognition receptors that play an important role in host defense. MCs express a range of TLRs, including TLR1, 2, 3, 4, 6 and 9.

TLRs are involved in the specific recognition of different pathogens: Gram positive and -negative bacteria are recognized by TLR2 and TLR4, respectively, and flagellin is recognized by TLR5, dsRNA is recognized by TLR3, and unmethylated CpG DNA is recognized by TLR9 (Frossi et al., 2004). TLR signaling pathways arise from intra-cytoplasmic Toll-Interleukin receptors (TIR) domains, which are conserved among all TLRs. TIR domain-containing adaptors, such as MyD88, TIRAP, and TRIF, modulate TLR signaling pathways. MyD88 is essential for the induction of inflammatory cytokines triggered by all TLRs. TIRAP is specifically involved in the MyD88-dependent pathway via TLR2 and TLR4, whereas TRIF is implicated in the TLR3- and TLR4- MyD88-independent pathway (Takeda & Akira, 2004).

An additional mechanism of direct interaction of mast cells with pathogens that leads to their activation is mediated by the protein CD48, which binds to the fimbrial protein FimH on gram negative bacteria (Rao & Brown, 2008).

1.3.5. **Mast cell mediators**

Mature MCs possesses numerous granules that store a wide range of potent, biologically active mediators. Fig 1.3.5

Mediators can be release in two mode: in the classical anaphylactic degranulation (AND) mode, in which the entire contents of each granule are released by exocytosis immediately after cells activation; in a piecemeal degranulation (PMD), in which granules contents are released in a slow, progressive manner (Rao & Brown, 2008).

The first mechanism of degranulation is responsible of immediate Type I hypersensitivity and innate immune responses, the second one is thought to play a role in chronic inflammatory disorders and cancers.

MC mediators can be divided in three categories: a) preformed mediators such as proteoglycans, histamine, neutral proteases and certain cytokines, in particular TNFα which are stored within the cytoplasmic MC granules and instantaneously released after activation; b) de *novo* synthetized mediators such as cytokines, chemokines growth and angiogenic factors that start to be synthetized after MC activation and c) lipid mediators that are newly synthesized following activation like prostaglandins, leukotrienes or platelet – activating factor (Galli et al 2005).

These mediators are important for regulate both innate and acquired immunity in fact they are able to regulate immune-cell trafficking and activation. Among early released MC products, TNFα play a crucial role in inducing the early influx of neutrophils promoting the clearance of pathogens. Histamine is the most abundant vasoactive amine stored in MCs and it exerts many effect pertinent to the allergic response like vasodilation, increased vaso-permeability and it drive dendritic cells migration and activation.

MCs can influence the polarity of T cell responses by releasing Th2 polarizing cytokines like IL-4, IL-10 and IL-13. Among MC pre-synthesized mediators, protease group is the largest one. Serin proteases, chymase and tryptase are the major protein components in granules. They offer protection against parasites and venoms, and favor the expulsion of nematodes (Metz & Maurer, 2007). MCs are thought to be crucial for the maintenance of tissue integrity and function. They produce nerve growth factor (NGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth

factor 2 (FGF 2) that induce proliferation of epithelial cells and fibroblasts, so they can influence wound healing in every step. MCs are important in the homeostasis of organs that undergo continuous growth and remodeling such as hair follicles and bones. IL-1, transforming growth factor (TGF-β), IL-6, and histamine, in fact, could influence osteoclast recruitment and development (Rao & Brown, 2008).

MCs have also anti-inflammatory properties, MC-derived IL-10, TGF-β and histamine have been purposed as modulators of the severity of the illness. These data confirm the ability of MCs to suppress the development and magnitude of the adaptive immune response (Depinay, Hacini, Beghdadi, Peronet, & Mécheri, 2006).

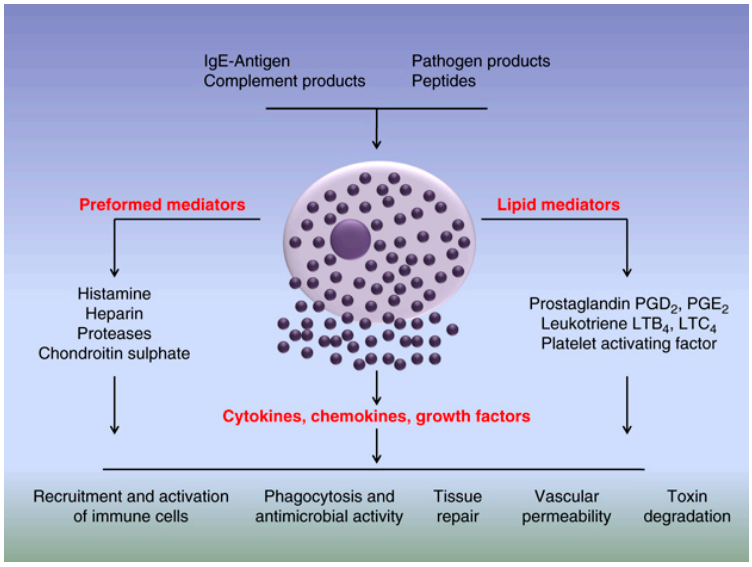


Fig 1.3.5 MC mediators: activated MCs release mediators: preformed granules; cytokines and chemokies, newly synthetized mediators. (Pundir & Kulka, 2010)

1.3.6. Mast cells and B cells

MCs have long been known for their participation in pathological allergic reactions characterized by dysregulated production of the inflammatory antibody isotype IgE, but also participate in a variety of IgE independent biologic responses (Cerutti et al., 2012). Several studies revealed the role of

mast cells in the development of adaptive immune responses, including antibody production B cells. MCs can influence B cell development and isotype class switching through the release of some cytokines, such as IL-4, IL-5, IL-6 and IL-13 and surface molecules such as CD40L (Gri et al., 2012). The interaction between CD40 and CD40L, together with MCs derived cytokines, were involved in the differentiation of B cells into CD138+ plasma cells and in selective IgA secretion and IgE production (Merluzzi et al., 2010). Moreover, un-stimulated MCs are able to induce resting B cells to proliferate and become IgM-producing cells.

2. AIM OF THE WORK

MCs are strategically located along the microvasculature within tissues in close contact with the external environment, such as the skin, lung and intestine and can rapidly respond to a variety of stimuli even in absence of IgE antibodies (R. Schramm & Thorlacius, 2004). By releasing a multi-faceted spectrum of pro-inflammatory mediators, such as cytokines and chemokines they could coordinate the trafficking and function of different circulating cells like neutrophils (Rene Schramm, Schaefer, Menger, & Thorlacius, 2002). Neutrophils are the most abundant white blood cells in humans. In addition to their crucial role in protecting against infections, recent evidence suggests that neutrophils are also key components of the effector and regulatory mechanisms of both innate and adaptive immune responses. Neutrophils are the first cells to migrate to sites of infection and exhibit a wide range of sophisticated functions such as phagocytosis, the release of anti-microbial substances, ROS. Under inflammatory conditions and in homeostasis, the activation and survival of neutrophils must be tightly controlled because the release of cytotoxic substances by neutrophils can easily cause collateral damage of adjacent healthy tissue. Since MCs could be a potential candidate in controlling neutrophils, the first aim of my study is to investigate and characterize the crosstalk between MCs and neutrophils.

As It has become increasingly evident that MCs and neutrophils have the potential to influence the generation and intensity of the adaptive immune response the second aim of the work is to focus on the effect of MC-neutrophils crosstalk in the regulation of the adaptive immune system, in particular of B cells, in a T-independent contest.

Aim of the work

3. RESULTS

3.1. MCs induce neutrophils chemotaxis

The recruitment of circulating neutrophils to specific sites plays a crucial role in a number of physiological and pathophysiological events so neutrophil recruitment is an important early step in controlling tissue infection or injury.

To demonstrate the ability of MCs to induce neutrophil migration, chemotaxis assays were performed using bone marrow purified neutrophils and conditioned media (CM) of *in vitro* differentiated MCs. As shown in figure 3.1 CM of IgE/DNP and LPS activated wild type (WT) MCs induce migration of neutrophils. Using CM obtained from TNFα-deficient MCs the amount of migrated neutrophils is diminished but not abrogated. TNFα is a cytokine implicated in neutrophils recruitment at inflammatory sites (De Filippo et al., 2013) but as shown in this experiment probably it's not the unique MC-derived mediator involved in neutrophil migration. Previous studies suggest positive and negative regulatory roles for TNFα signaling during neutrophil motility and chemotaxis, and much work suggest that TNFα induces inflammation indirectly via the induction of other inflammatory mediators (Lokuta & Huttenlocher, 2005). Moreover, other MC-derived mediators such as leukotrienes, chymases and tryptases are all reported to be important for neutrophil recruitment (De Filippo et al., 2013). Therefore, it is possible to speculate that MCs contribute to neutrophil recruitment through different pathways.

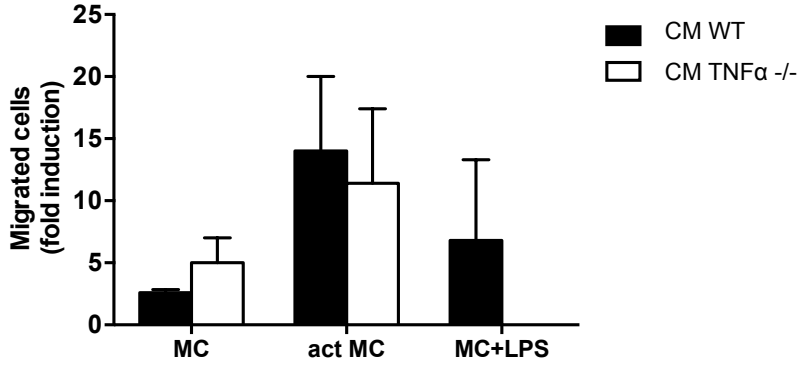


Fig. 3.1: Conditioned media of activated MCs induce neutrophils chemotaxis. Sensitized or not wild type (WT) MCs and TNFα$^{-/-}$ MCs were cultured for 24 hours with medium alone or containing different stimuli: DNP (100 ng/ml), LPS (1 µg/ml). Conditioned media (CM) were retrieved and used to induce neutrophil chemotaxis in a transwell filter diffusion assay. Migrated cells were counted and results (mean + standard deviation (s.d) of 3 separated experiments performed in duplicate) are shown as fold induction compared to cells migrated in response to non- conditioned media.

3.2. MCs activate neutrophils

To verify whether MCs can influence neutrophil activation, a co-culture system using bone marrow purified neutrophils and in vitro generated MCs has been set up. The expression levels of the surface activation markers CD11b and CD62L, the degranulation and the vitality of neutrophils were investigated by FACS analysis directly in the co-cultures gating on neutrophil population and were compared with those of neutrophils cultured alone.

3.2.1. **CD11b up-regulation**

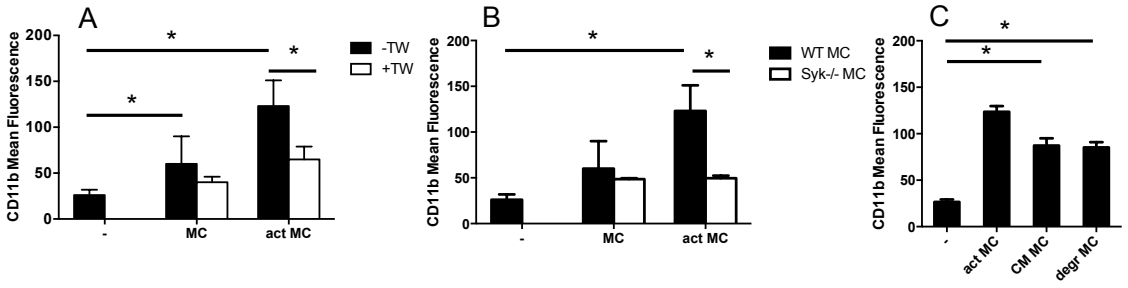


Fig 3.2.1 MCs enhance CD11b expression on neutrophils. A. MCs (not act and act) and neutrophils were co-cultured in presence and absence of transwell system (TW) **B.** MCs (not act and act) from WT and Syk$^{-/-}$ mice were co-cultured with neutrophils. **C.** Neutrophils were cultured with conditioned media (CM MC) of activated MCs or with MCs that have been previously activated for 30 minutes, washed and then added to the co-culture and re-stimulated with DNP (degr MC). The expression of CD11b on neutrophils (identified as FcεRI negative cells) was analyzed at 4 hours. Data (mean + s.d) are from at least 5 independent experiments. *p<0,05

CD11b (Mac-1) is a member of the β-integrin family of adhesion proteins that is expressed in very low levels on the surface of un-stimulated neutrophils but is primarily stored in secretory granules within the cell (Weirich et al., 1998). The activation of neutrophils is followed by the rapid translocation and accumulation of CD11b to the plasma membrane. To investigate CD11b expression, bone marrow purified neutrophils and *in vitro* differentiated MCs sensitized and not with monoclonal ant-DNP IgE were co-cultured in 1:1 ratio. To activate MCs, DNP (100ng/ml) was administered directly in the co-culture. After 4 hours, the expression of CD11b on FcεRI negative cells in the co-culture was evaluated by FACS analysis. As shown in the figure 3.2.1 MCs, especially once activated, induce the up-regulation of CD11b, thus induce neutrophil activation. To investigate whether cell contact is necessary for neutrophil activation, cells were co-cultured in contact or separated by trans-well membrane (TW). The

presence of the TW insert, that physically hinders the contacts between MCs and neutrophils, reduces significantly but not prevents the up-regulation of CD11b expression. Thus, both cell-cell contacts and soluble factors should be implicated in MC-driven activation of neutrophils. In fact, conditioned media of activated MCs (figure 3.2.1 C) induce a higher increase of CD11b expression respect resting MCs but not at the same level observed in neutrophils in contact with activated MCs. This data is further confirmed by the use of MCs lacking the Syk tyrosine and therefore MCs that don't degranulate. In this case in fact we didn't find neutrophil activation (figure 3.2.1 B).

The soluble factor responsible of activation is probably an early released mediator because when we put in co-culture neutrophils with MCs that were previously challenged with DNP for 30 minutes, washed and then re-stimulated with DNP, we found a decreased neutrophil activation respect the system with fully activated MCs (figure 3.2.1 C).

A possible candidate for CD11b up-regulation could be TNF-α noted in literature to be involved in the regulation of some functions of neutrophils (Montecucco et al., 2008). So we co-cultured neutrophils with $TNF^{-/-}\alpha$ activated MCs or with WT activated MCs in presence of mAb anti TNFα. As shown in figure 3.2.1.1 TNFα has a role in neutrophils activation since in presence of $TNF\alpha^{-/-}$ MCs and in presence of mAb anti TNFα we found a reduced increase in CD11b expression.

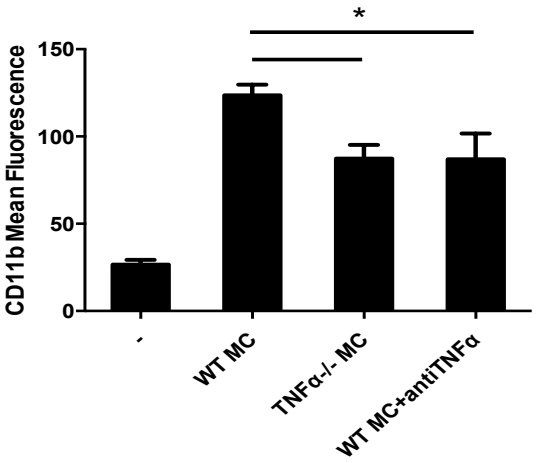


Fig 3.2.1.1 TNFα influences neutrophil activation. Neutrophils were incubated with WT or $TNF^{-/-}$ activated MCs in presence or absence of mAb anti TNFα at a concentration of 10μg/ml. The expression of CD11b marker was analyzed by FACS analysis after 4 hours of co-culture. Mean+ s.d of at least 3 independent experiments are shown. *p<0,05

Histamine release occurs during the early phase allergic reaction concomitantly with neutrophil infiltration. To see if histamine, besides TNFα, can be involved in neutrophils-MC crosstalk, two histamine antagonists diphenhydramine and thioperamide were added to the co-culture and CD11b expression was evaluated after 4 hours. No differences respect the un-treated samples have been found, indicating that histamine is not implicated in neutrophil CD11b up-regulation (figure 3.2.1.2)

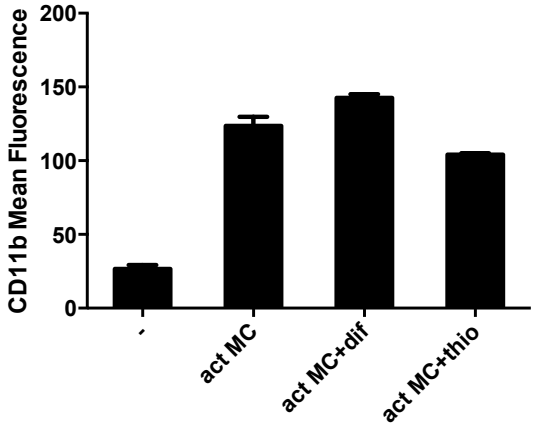


Fig 3.2.1.2 Histamine agonists don't change neutrophil CD11b expression. Neutrophils and activated MCs were co-cultured in presence of 10 μg/ml histamine H_1 receptor antagonists diphenhydramine (dif) and histamine H_4 receptor antagonist thioperamide (thio). Data represent the mean + s.d of 2 independent experiments.

Experiments with TW suggested a crucial role for cell-cell contact in CD11b up-regulation together with soluble factors. So we decided to carry on the study

using different combination of several blocking mAb against surface molecules that could be involved in MC-neutrophil interaction. CD18 has been implicated in the mediating the intercellular contacts that neutrophils may establish with other leukocytes or NK (Costantini et al., 2010). Since MCs express on their surface ICAM1 which is a binding receptor of CD18, we incubate neutrophils and MCs in presence of neutralizing monoclonal antibodies (mAb) against CD18 and ICAM1. We focused also on the SIRP1α/CD47 axis because these co-stimulatory molecules are constitutively expressed on MCs and neutrophils respectively and on the role of soluble TNFα. As shown in figure 3.2.1.3 the block of the CD18/ICAM axis weakly reduces the increase of CD11b expression while the block of the SIRP1α/CD47 axis has not effect. The concomitant addition of both the mAb against ICAM and the mAb against SIRPα didn't show a further effect. However the addition in the co-culture of both mAb against ICAM and mAb against soluble TNFα revealed a synergistic effect in inhibiting the up-regulation of CD11b as demonstrated by 20% reduction of the CD11b mean fluorescence (mean fluorescence 104±10 versus 80±8). A similar reduction was observed by the addition of TNFα blocking antibody in presence of TW.

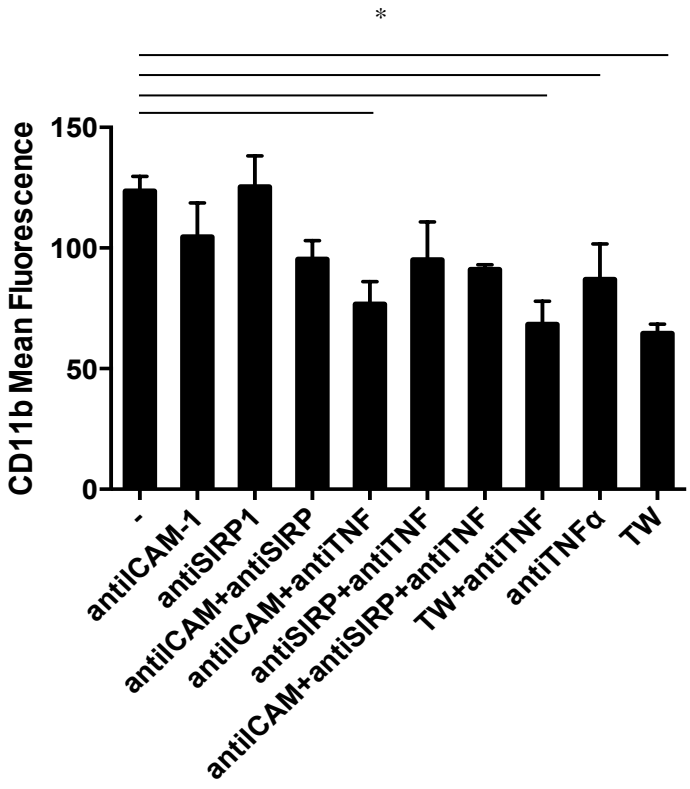


Fig 3.2.1.3 Effect of different monoclonal blocking antibody in neutrophil activation. Neutrophils and activated MCs were co-cultured in presence of different neutralizing monoclonal antibody (mAb) at a concentration of 10µg/ml. Mean + s.d of at least 3 independent experiments are shown. *p<0,05

Neutrophils recruited at the site of inflammation or infection are probably already activated and therefore an important point is to understand if MCs can further influence the activation of already activated neutrophils. So we incubated neutrophils with suboptimal doses of LPS in presence of WT MCs and Myd88$^{-/-}$ MCs. The latter ones lack of the adapter protein used by almost all TLRs (including TLR4), so they can't respond to LPS stimulus. As shown in figure 3.2.1.4 the expression of CD11b on neutrophils activated with suboptimal amounts of LPS is enhanced by the presence of MCs. This experiment, once again, emphasizes the role of MCs in neutrophil activation because it sheds on light that MCs can lower the LPS activation threshold of neutrophils making them susceptible to respond to suboptimal concentration of LPS.

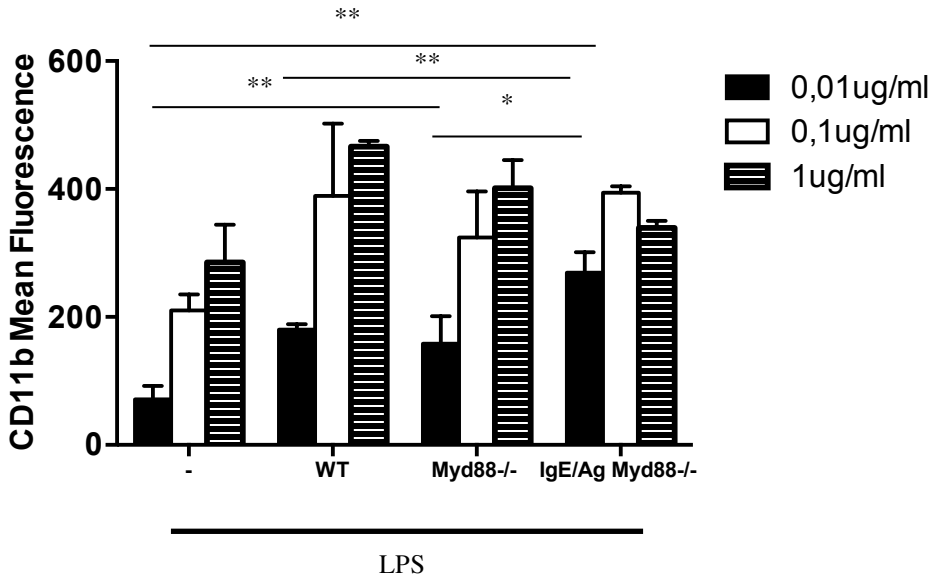


Fig 3.2.1.4 MCs further enhance CD11b expression of LPS-activated neutrophils. Myd88$^{-/-}$ and WT MCs activated with IgE/Ag or not were cultured with neutrophils in presence of different concentrations of LPS. CD11b expression marker was analyzed at 4 hours by FACS. Data (mean + s.d) are from at least 3 independent experiments. *p<0,05: **p<0,005

3.2.2. CD62L down regulation

Another activation marker of neutrophils is the adhesion molecule L-selectin (CD62L). Resting neutrophils constitutively express high levels of CD62L on their surface and their activation is accompanied by a down-regulation of this marker by shedding. It interacts with carbohydrate ligands mediating the initial rolling of leukocytes on the endothelium (Sengstake, Boneberg, & Illges, 2006). Similarly to CD11b analysis, neutrophils were incubated with not activated and DNP-activated MCs in presence or absence or TW and CD62L in neutrophils has been detected after 30 minutes.

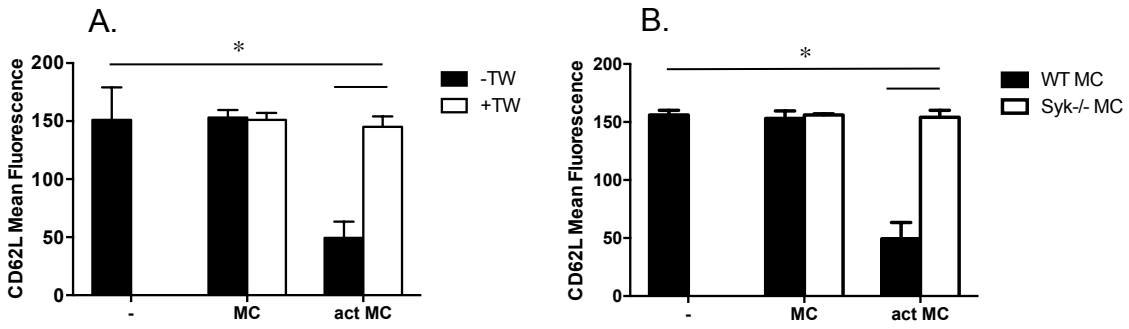


Fig 3.2.2 Neutrophil CD62L down-regulation in presence of MCs. A MCs activated or not and neutrophils were co-cultured in presence and absence of transwell system (TW). **B**. WT and Syk$^{-/-}$ MCs, activated or not, were co-cultured with neutrophils. Neutrophils surface expression of CD62L was analyzed at 30 minutes by FACS gating on GR1 positive cells. Mean + s.d of at least 3 independent experiments are shown. *p<0,05

As shown in figure 3.2.2 the presence of activated MCs induces an evident decrement of CD62L that is completely prevented when cells are separated by TW. The null effect on CD62L expression shown in presence of Syk$^{-/-}$ MCs confirm the hypothesis that besides a contact-dependent mechanism, also a contribution of early released soluble factors could be involved in MC-induced neutrophils activation.

3.2.3. Activated MCs induce an increase of ROS production and lactoferrin degranulation

Next, the ability of neutrophils to mediate an oxidative burst in presence of MCs was investigated. A resting neutrophil has very little capacity to produce ROS, as NADPH oxidase is a multi-component enzyme that is assembled at the plasma membrane during priming. To test ROS production neutrophils were previously pulsed with CM-H_2DCFDA, then incubated with activated and non

activated MCs, in presence or absence of TW and the levels of ROS were monitored after 30 minutes by FACS. As shown in the figure 3.2.3 panel A, the production of ROS in neutrophils is enhanced in the presence of activated MCs but nor when cells are separated by TW or when cell-cell contact is prevented by the addition of the blocking mAb against CD18 (figure 3.2.3 panel B), suggesting once again the importance of the physical interaction in MC-driven activation of neutrophils.

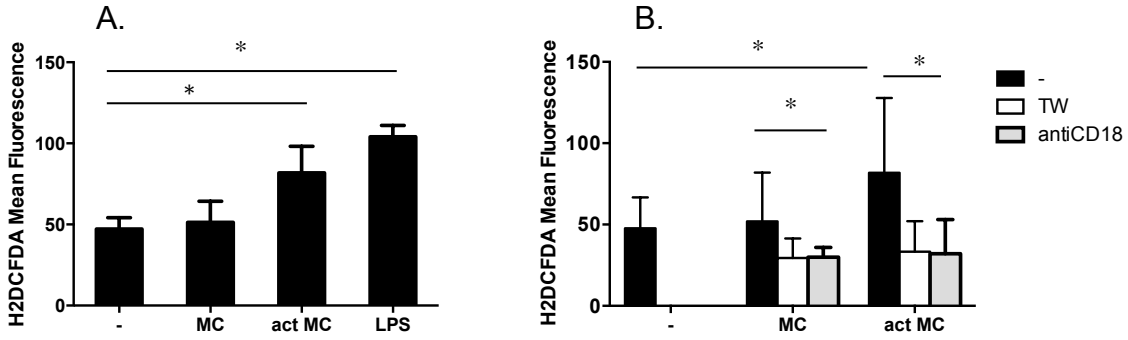


Fig 3.2.3 MCs promote neutrophil ROS production. Neutrophils were co-cultured with MCs activated or not (**A**) in presence or absence of TW system or mAb anti-CD18 at 10µg/ml (**B**). ROS production was examined at 30 minutes using H_2DC-FDA as described in methods. Data are the mean + s.d of 3 independent experiments. *p<0,05

Lactoferrin is an iron-binding protein that is released from activated neutrophils at sites of inflammation and has anti-microbial as well as anti-inflammatory properties. (Wong et al., 2009) The levels of lactoferrin in the supernatants of the co-culture were evaluated by ELISA assay and were found higher when neutrophils were cultured with activated MCs (figure 3.2.3.1). The pg/ml released, however, were not so abundant suggesting that MCs can promote but not induce a strong neutrophil degranulation. Again, the addition of mAb anti

CD18 seems to prevent neutrophil activation, as shown by the reduced levels of lactoferrin (figure 3.2.3.1) in the supernatants.

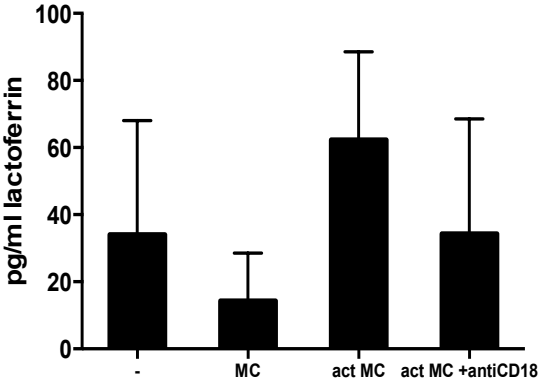


Fig 3.2.3.1 MCs promote neutrophil degranulation. $5x10^6$ /ml neutrophils were co-cultured for 1 hour with equal number of activated or not activated MCs in presence or absence of mAb anti CD18 (10µg/ml). Supernatants were harvest and lactoferrin levels were measured by ELISA. Data are the mean + s.d of 2 independent experiments.

3.3. Increase of soluble BAFF in neutrophil-MC co-culture

Neutrophils have been "re-discovered" as very versatile cells, contrary to their traditional description as terminally differentiated effectors of inflammation. The newly discover effector functions of neutrophils are also marked by the fact that they represent an important source of several cytokines including BAFF/BLyS. BAFF is a member of TNF superfamily of cytokines that exist as a membrane protein or as a soluble protein. It's express mainly, but not exclusively, by cells of myeloid origin and plays a fundamental role in B-cell differentiation, proliferation and immunoglobulin production.

Remarkably, the production of neutrophil-derived cytokines can be controlled by sophisticated regulatory mechanisms, and the pool of intracellular BAFF accumulated in neutrophils can be release in extracellular milieu when neutrophils are exposed to pro-inflammatory stimuli like chemokines CXCL1, or

eicosanoid (LTB_4), cytokines (TNFα) or bacterial product (LPS) that act like secretory –like molecules (Scapini, Bazzoni, & Cassatella, 2008).

As activated MCs can secrete a large array of mediators including those responsible of BAFF release, we tested if MCs can control and influence neutrophil BAFF production. Supernatant of neutrophil-MC co-culture were harvest at 4 and 24 hours and BAFF presence was measured by ELISA. As shown in figure 3.3 MCs seems to enhance BAFF production and it seems also to be related to the contact between the two cells as in presence of TW system there is a lower presence of soluble BAFF.

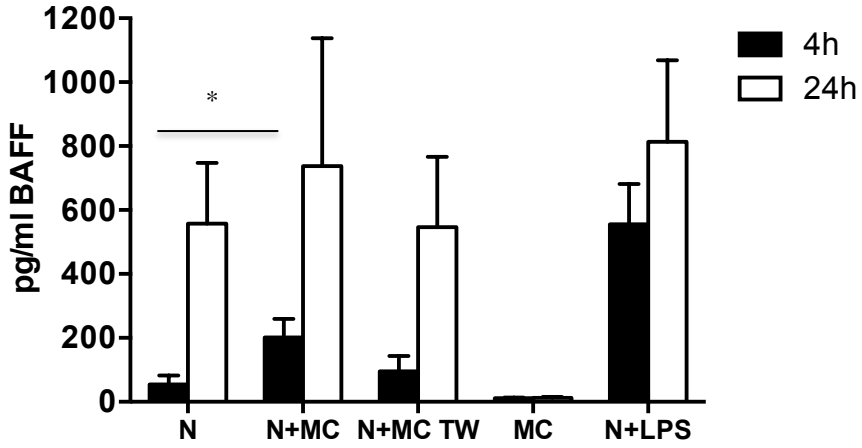


Fig 3.3 MCs can enhance neutrophil BAFF release. Neutrophils (N) and activated MCs were co-cultured at ratio 1:1 for 4 and 24 hours with medium containing different stimuli: DNP (100 ng/ml), LPS (5μg/ml) and with transwell system (TW). Supernatants were retrieved and used to assess ELISA assay. Mean +s.d of 3 independent experiments are shown. *p<0,05.

3.4. MCs induce neutrophil death

Neutrophils are normally short living cells, but recent studies demonstrate that in presence of some cells, like for example bone marrow derived mesenchimal stromal cells, they can display a lower tendency to undergo apoptosis (Cassatella et al., 2011). To investigate the influence of MCs on neutrophil survival, purified neutrophils were plated with MCs either in direct contact or in TW condition. Viability of GR-1 positive cells was investigated at 20 hours of incubation. As shown in figure 3.4 neutrophil survival was affected by the presence of MCs. In fact MCs decrease neutrophil survival by a contact dependent mechanism as demonstrated by the employment of TW system: when cells are separated neutrophils viability was restored to the resting condition.

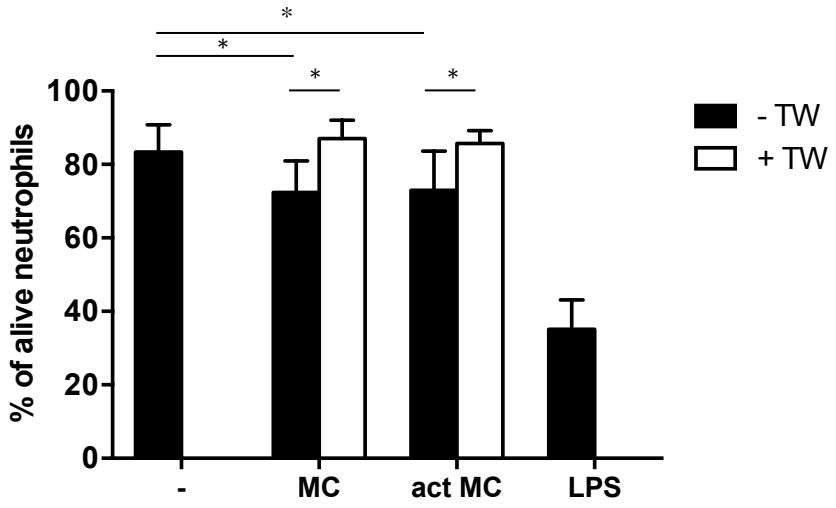


Fig 3.4 Neutrophil survival is affected by MC presence. Neutrophils were co-cultured with MCs (activated or not) in presence or absence of TW for 20h. LPS was used as positive control. The vitality of cells was measured by annexin V/ propidum assay by FACS analysis gating on GR-1 positive cells. Results (mean + s.d) of at least 3 independent experiments are shown. * $p<0,05$.

Since CD18 has been implicated in MC-induced neutrophil CD11b up-regulation and ROS production, we evaluated also its role in mediating neutrophil survival in presence of MCs. However, the presence of neutralizing monoclonal antibody (mAb) against CD18 and ICAM1 did not seem to modify neutrophil survival as shown in figure 3.4.1 suggesting that the axis CD18/ICAM could not be involved.

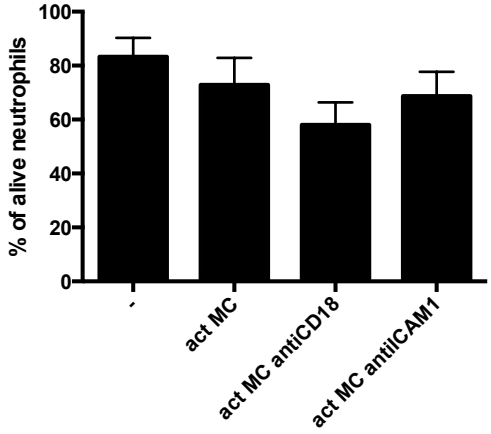


Fig 3.4.1 Effect of CD18/ICAM axis in neutrophil viability Neutrophils were co-cultured with activated MCs in presence of 10µg/m of mAb anti CD18 and anti ICAM1. Mean + s.d of 3 independent experiments are shown.

3.5. MC and neutrophil phenotype doesn't change

In addition to the capacity to produce and release soluble factors, MCs can express several co-stimulatory molecules. Neutrophils, furthermore, have been shown to contain many different types of receptors molecule preformed that can rapidly translocate to the cell surface providing an immediate change in cells phenotype (Sandilands, McCrae, Hill, Perry, & Baxter, 2006). The co-stimulatory molecules expressed by MCs and neutrophils that could be involved in MC-neutrophil interaction were analyzed in co-culture system at 3 and 24 hours. We focused our attention in some molecules note to have a role in the

interaction between immune cells and that are constitutively express on the cells of our interest like for example BAFF, CD200R (Gorczynski, 2012), DNAM-1 or CD226 (Bachelet,Munitz,Mankutad&Levi-Schaffer,2006), GITR (Ronchetti et al., 2004), OX40, OX40L (Sibilano et al., 2011) and finally CD40-CD40L (Merluzzi et al., 2010). We didn't find any differences in the expression of these molecules in co-culture system neither in neutrophils nor in MCs. Fig 3.5

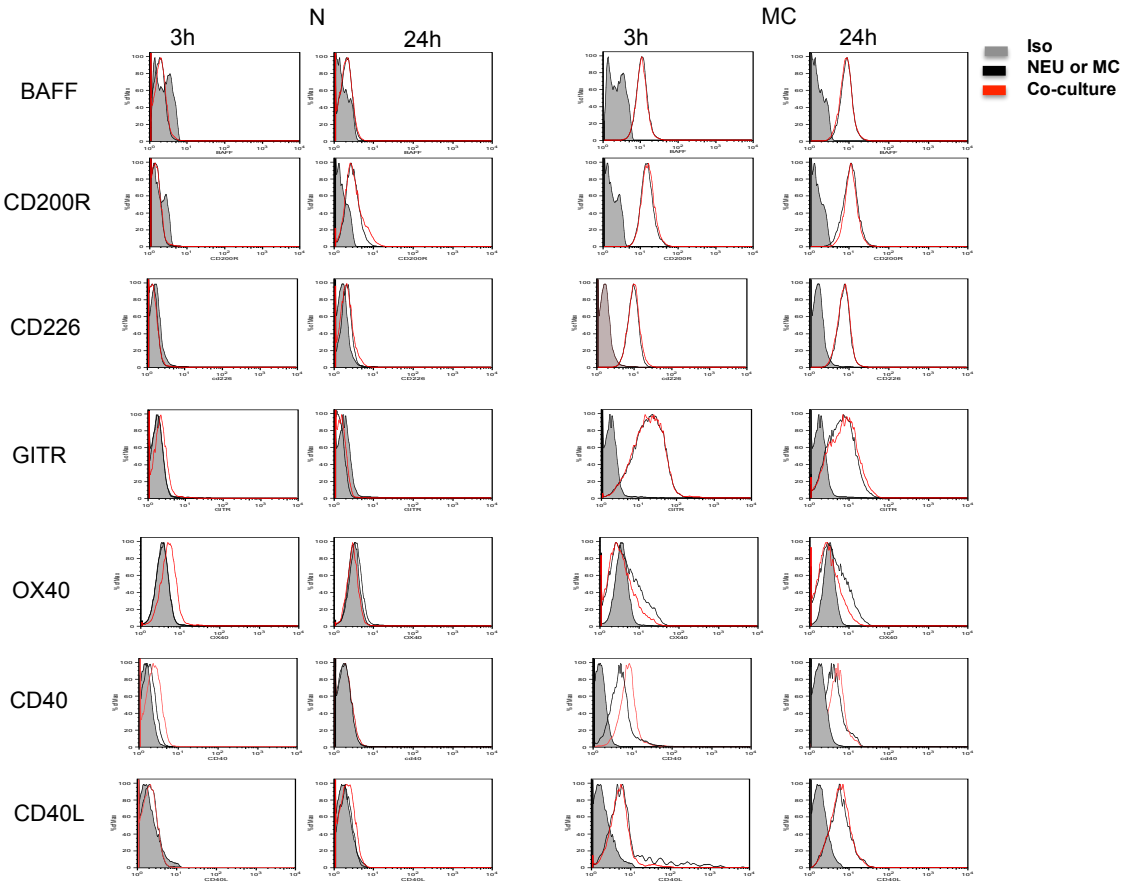


Fig 3.5 No differences in co-stimulatory molecules expression in co-cultures. The presence of different receptors in neutrophils (N) and MCs (MC) alone (Black line) or in co-culture (red line) were analyzed at 3 hour and 24 hours by FACS analysis gating on GR1 and FcERI positive cells. Results from one representative experiment are shown.

3.6. Neutrophil influence on MC degranulation

MCs are effector cells of immediate hypersensitivity and allergic diseases. Cross-linking the high-affinity IgE receptor, FcεRI, initiates the activation of MCs, which results in immediate release of a variety of preformed mediators including β-hexosaminidase, histamine and TNFα (Carlos et al., 2006).

As we have firstly investigated how MCs could directly activate neutrophils, the reverse effect, that is the effect of neutrophils on MC, was then investigated.

We co-cultured IgE/Ag activated MCs with alive, necrotic and apoptotic neutrophils and compared their activation status with IgE/Ag activated MCs cultured alone. Necrotic and apoptotic neutrophils were obtained respectively by incubating freshly purified neutrophils at 56°C for 30 minutes or by culturing them for 24 hours in medium without serum. Necrosis and apoptosis were monitored using Annexin V/ propidium assay. To test MC degranulation the expression of Lysosomal-associated membrane protein 1 (LAMP1) on FcεRI positive cells in the MC-neutrophil co-culture was analyzed. LAMP1 also known as CD107a, is a glycoprotein marker of degranulation on MCs, lymphocytes and NK. As degranulation occurs, secretory lysosome are released, and LAMP1 is transported to the surface of cells rendering accessible for antibody binding (Aktas, Kucuksezer, Bilgic, Erten, & Deniz, 2009). As shown in figure 3.6 apoptotic, necrotic, resting neutrophils or their conditioned media don't influence MC degranulation.

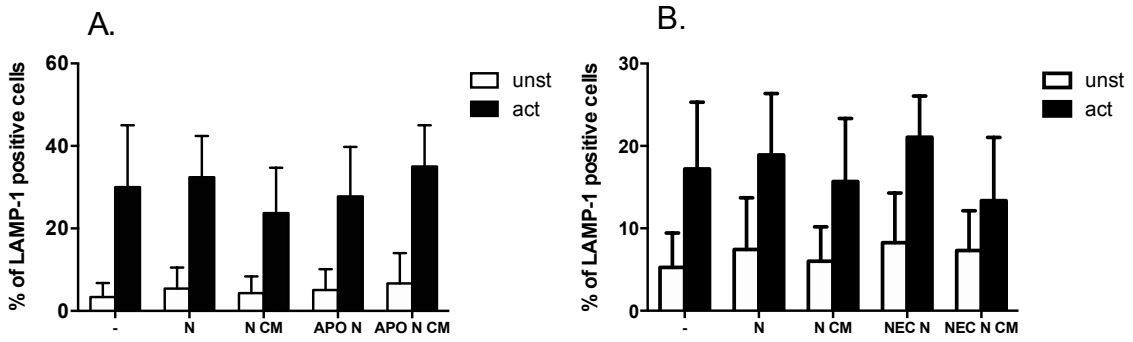


Fig 3.6 MC degranulation is not influenced by resting, apoptotic and necrotic neutrophil presence. MCs activated (act) or not (unst) were co cultured for 30 minutes with: **A** resting (N), apoptotic (APO N) neutrophils and their conditioned media (CM)**; B** resting (N) and necrotic (NEC N) neutrophils and their conditioned media. LAMP1 expression, on FcεRI positive cells, was analyzed by FACS. Data (mean +s.d) of at least 3 independent experiments are shown.

However, co-culturing activated MCs with LPS activated neutrophils, with conditioned media of LPS activated neutrophils and with neutrophils pre-incubated with LPS for 1 hour, the percentage of LAMP positive cells were reduced. We used Myd88$^{-/-}$ MC to be sure to exclude the effect of LPS on MCs (figure 3.6.1) Thus activated neutrophils, but not resting, apoptotic or necrotic, seem to down-regulate MC degranulation.

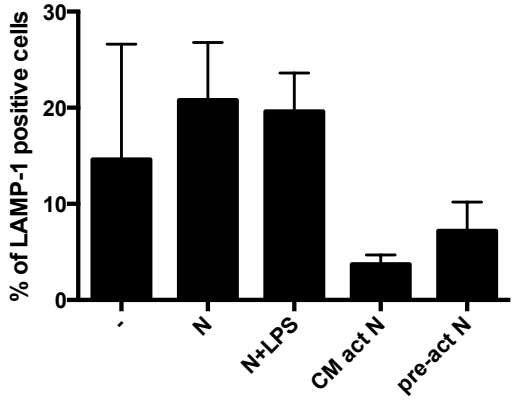


Fig 3.6.1 LPS-activated neutrophils decrease MC LAMP1 expression. IgE/Ag activated Myd88$^{-/-}$ MCs were co-cultured 30 minutes in presence of neutrophils, neutrophils activated with LPS 5µg/ml, 24 hours conditioned medium (CM) of neutrophils activated with LPS (act N) and with neutrophils pre-activated (pre-act) with LPS for 1 hour. LAMP 1 expression, on FcεRI positive cells, was analyzed by FACS. Mean + s.d of 2 independent experiments are shown.

3.7. Levels of cytokines in neutrophil-MC co-culture

MCs are known to secrete a large array of mediators in response to different stimuli. To evaluate the behavior of MCs in presence of neutrophils, supernatants of the co-cultures were harvested at different time points and the presence of some of the most important cytokines produced by MCs and implicated in neutrophils functions was measured by ELISA (figure 3.7 A, C, E). To better analyze the production of two of them, IL6 and TNFα, an intracellular staining was also preformed (figure 3.7 B, D). As shown in fig 3.7 A in MC-neutrophil co-culture there is a decrease of TNFα at 3 hours and 24 hours in the supernatants. It was not dependent on the contact between the two cells because using TW system or mAb anti CD18 we haven't found any differences. Deepening this data by intracellular staining (figure 3.7 B) we found that there is no difference in TNFα production between MCs alone or in co-culture with neutrophils. Thus, the diminished levels of TNFα in the co-cultures don't depend on an inhibitory effect of neutrophils in MC production, but probably it suggests that neutrophils consume the TNFα produced and released by MCs.

Regarding IL6 and TGF-β secretion we haven't found any differences between the co-culture and single cells system (figure 3.7 C, D, E).

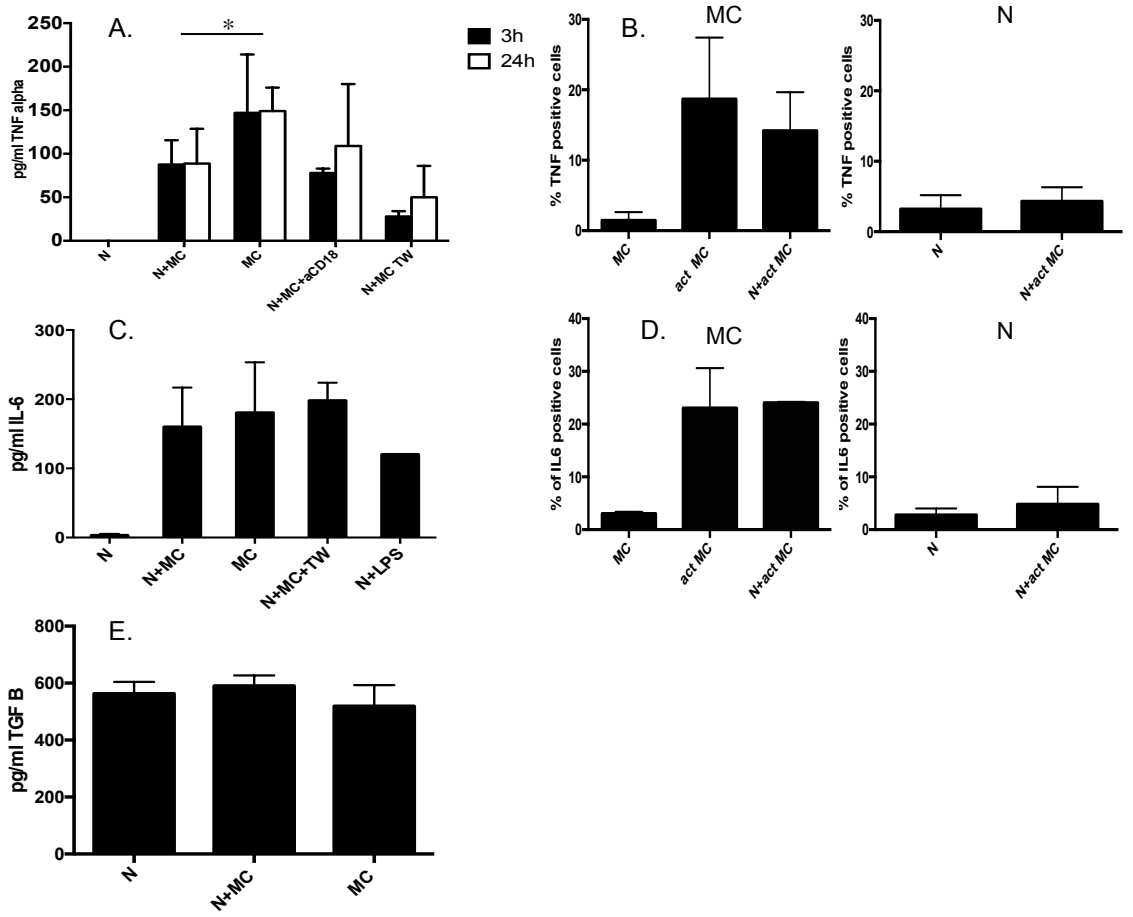


Fig 3.7 Cytokines production and release in MC-neutrophil co-cultures. A.C.E activated MCs and neutrophils (N) were co-cultured at ratio 1:1 for 24 and 3 hours (**A**) with medium containing different stimuli: DNP (100 ng/ml), LPS (5µg/ml), mAb anti CD18 (10µg/ml), and with transwell system (TW). Supernatants were retrieved and used to assess ELISA assay . **B.D** Intracellular staining: neutrophils (N) and MCs were co- cultured at ratio 1:1 in presence or not of DNP (100ng/ml). After 12h of incubation with brefeldin A intracellular staining assay was perform. Data were analyzed by FACS. Results (mean + s.d) of at least 5 independent experiments are shown. *p<0,0

3.8. Effect of MC-Neutrophil crosstalk on B cells

Recent studies shown that MCs promote both survival and activation of B cells as well as proliferation and further plasma cells differentiation of activated B cells through cell-cell contact and soluble factors (Merluzzi et al., 2010). Latterly also neutrophils role has been challenge. Numerous studies have shown that

neutrophils mediate also diverse immune functions by releasing preformed and newly synthetized mediators that can regulate recruitment and activation of other cells including B cells. On the basis of these evidences we decided to study the effect of MC-neutrophil interaction in the activation of adaptive immunity, namely B cells. The viability of freshly purified splenic B cells incubated with conditioned medium derived from MCs, neutrophils and MC-neutrophil co-culture was investigated. As shown in figure 3.8 conditioned media of neutrophils, of activated MCs and more clearly of neutrophil-MC co-culture enhanced B cell survival (figure 3.8).

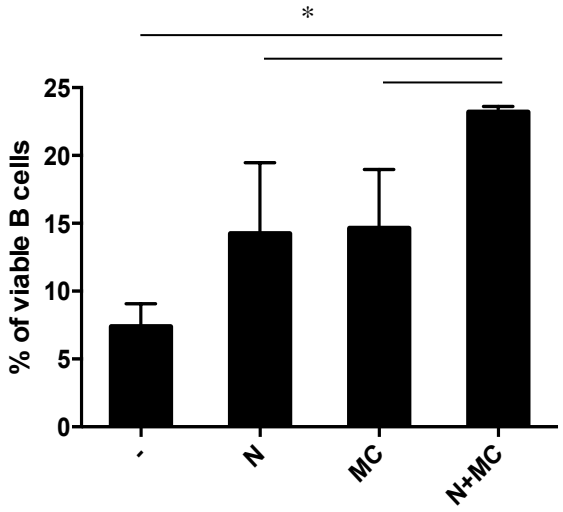


Fig 3.8 Conditioned media of neutrophils and activated MCs enhance B cell survival. IgE/DNP activated MCs and neutrophils alone or at 1:1 ratio were co-cultured for 24 hours. Supernatants were retrieved and used to assess B cells survival assay. Annexin V/propidium assay was used to test B cell vitality. Data were analyzed by FACS. The mean + s.d of 3 independent experiments are shown. *p<0,05

In the same supernatants the levels of cytokines known to be involved in B cells survival like BAFF, IL6 and TGFβ were also measured. Only BAFF was increased in supernatants (harvested at 4 and 24 hours) from activated MC-neutrophil co-culture (figure 3.8.1 A). This is a very important point because previous studies revealed that soluble BAFF released by neutrophils was found

to be biologically active and to prolong survival of murine splenocytes (Scapini et al., 2008).

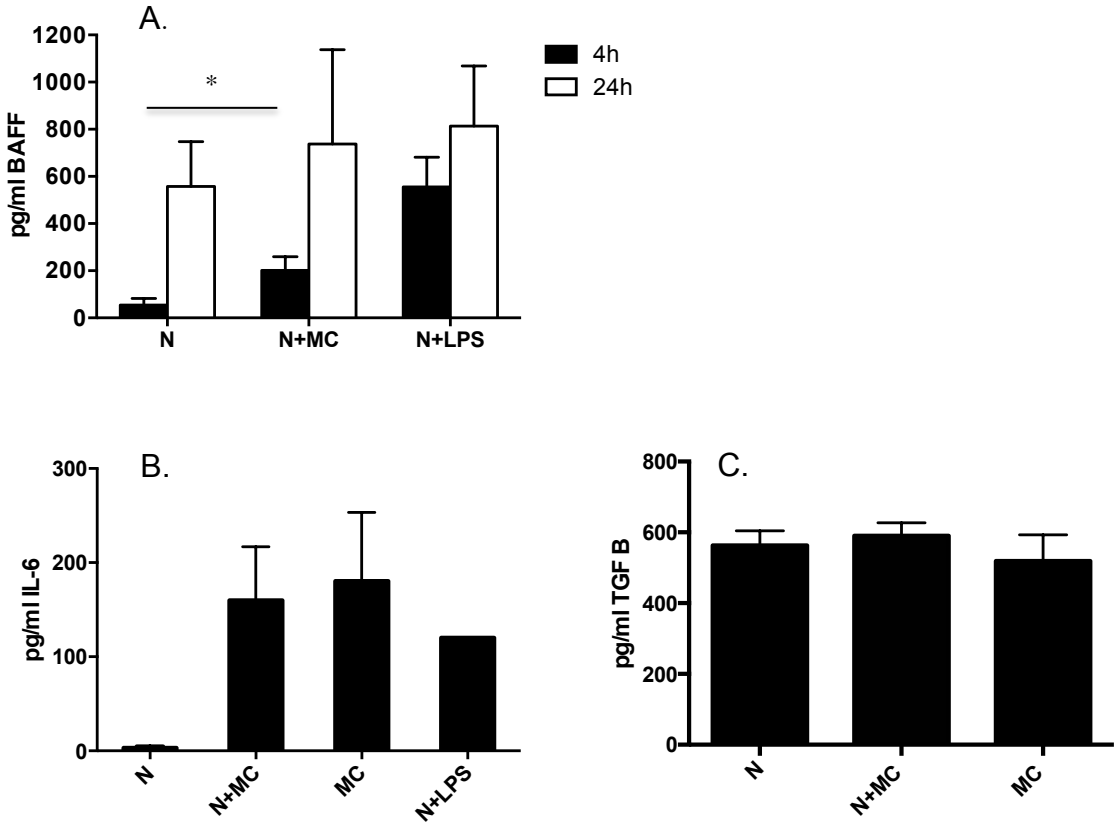


Fig 3.8.1 Increase of BAFF in supernatants of neutrophil-MC co-culture. A, B, C activated MCs and neutrophils were co-cultured at ratio 1:1 in medium containing different stimuli. Supernatants were retrieved and used to assess ELISA assay. **A** IgE/DNP activated MCs and neutrophils were co-cultured for 4 and 24 hours in medium containing different stimuli: DNP (100 ng/ml), LPS (5µg/ml). **B, C** IgE/Ag activated MCs and neutrophils were co-cultured for 24 hours in presence of different stimuli DNP (100 ng/ml), LPS (5µg/ml). Data (mean + s.d) of at least 5 independent experiments are shown. *p<0,05

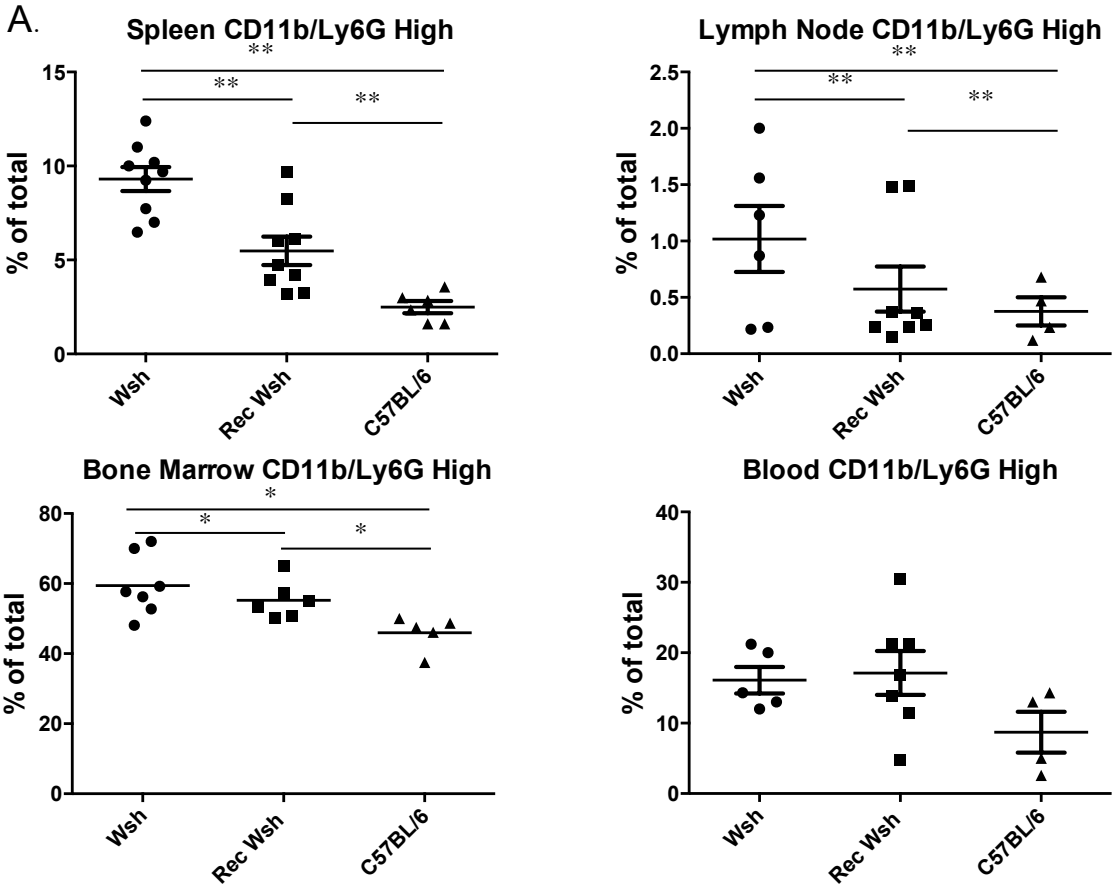
3.9. $Kit^{W\text{-}sh/W\text{-}sh}$ mice as a mouse model to investigate MC-neutrophil relationship

C57BL/6 $Kit^{W\text{-}sh/W\text{-}sh}$ mice, bearing the W-sash (w^{sh} or Wsh) inversion mutation, have MC deficiency in all tissues but normal levels of major classes of other differentiated hematopoietic and lymphoid cells. Compared to other MC-deficient mouse strain, $Kit^{W\text{-}sh/W\text{-}sh}$ mice are experimentally advantageous because of their background strain and fertility. In these mice, local reconstitution of MC population can be achieved by intra-peritoneal (i.p) injection of wild-type bone marrow-derived cultured MCs. For these reason, they represent a useful model in MC research, especially for analyzing MC function *in vivo.* (Grimbaldeston et al., 2005) However, $Kit^{W\text{-}sh/W\text{-}sh}$ exhibit splenomegaly with expanded myeloid and megakaryocyte populations. Moreover, hematopoietic abnormalities extend to the bone marrow and are reflected by neutrophilia and thrombocytosis (Nigrovic et al., 2008) but there are no data correlating the increase of neutrophils with the deficiency of MCs in $Kit^{W\text{-}sh/W\text{-}sh}$ mice.

In our hand $Kit^{W\text{-}sh/W\text{-}sh}$ represents a good animal model to investigate *in vivo* the role of MCs and neutrophils alone or in combination in the regulation of the immune system response. In fact $Kit^{W\text{-}sh/W\text{-}sh}$ is a mouse with high number of neutrophils but lacking MCs. The injection of bone marrow derived MC restablish normal levels of tissue MC and could shed in light the effect of MC on neutrophil levels. In addition, the depletion of neutrophils in these three types of mice could consent to understand the reciprocal role of MCs and neutrophils in influencing the immune response.

3.9.1. Characterization of neutrophils in Kit$^{W\text{-}sh/W\text{-}sh}$ mice

Spleen, lymph nodes, bone marrow and blood isolated from C57BL/6 mice, Kit$^{W\text{-}sh/W\text{-}sh}$ mice, and MC-reconstituted Kit$^{W\text{-}sh/W\text{-}sh}$ mice were stained for two markers of neutrophil identification, CD11b/Ly6G. As shown in figure 3.9.1 neutrophilia was strongly present in all secondary lymphoid organs of Kit$^{W\text{-}sh/W\text{-}sh}$ mice respect of C57BL/6 mice and only weakly in the bone marrow. However, surprisingly, in intra-peritoneally reconstituted Kit$^{W\text{-}sh/W\text{-}sh}$ mice neutrophilia is reduced. The effect was evident and statistically significant in secondary lymphoid organs, spleen and lymph nodes, while fewer differences were found in blood and bone marrow. This experiment indicates that the presence of MCs in peripheral tissues controls someway the basal levels of neutrophils.



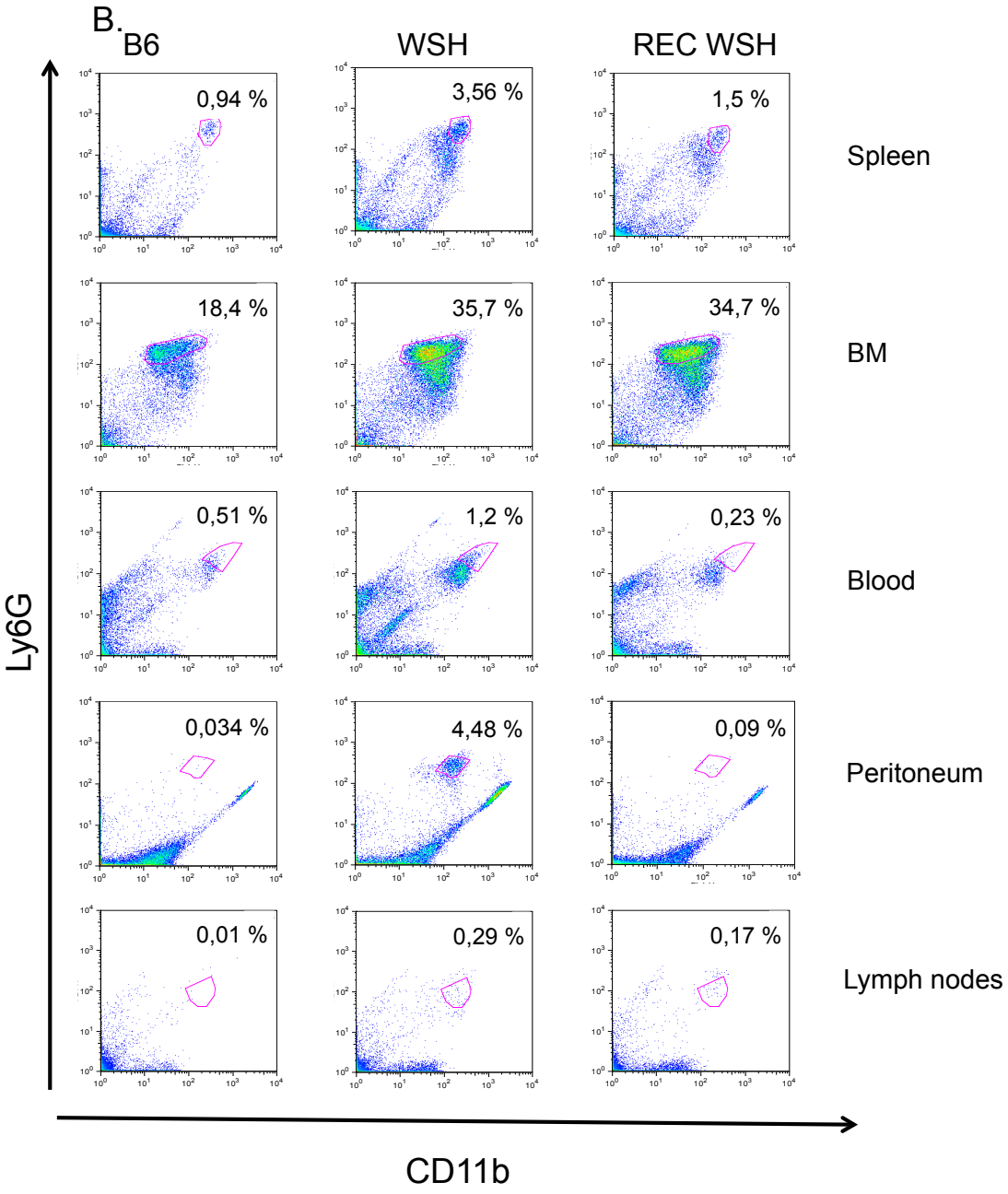


Fig 3.9.1 Neutrophils in C57BL/6, Kit$^{W\text{-}sh/W\text{-}sh}$ (wsh) and reconstituted Kit$^{W\text{-}sh/W\text{-}sh}$ (rec Wsh) mice. Cell suspension from spleen, lymph nodes, bone marrow (BM), blood, peritoneum, were isolated, stained for CD11b and antiLy6G and analyzed by FACS . **A:** Graph representing the percentage of total Ly6G high and CD11b positive cells of at least 3 independent experiments. **B** Representative dot plots of Ly6G high/CD11b positive cells in different organs. **p<0,005 *p<0,05

To test if neutrophils from Kit$^{W\text{-}sh/W\text{-}sh}$ and C57BL/6 mice have the same characteristic, we focused our attention on neutrophils size and granularity and

GR1-CD11b expression. We haven't found any differences between neutrophils of the two mice neither in morphology nor in GR1 CD11b expression (figure 3.9.1.1 A; B).

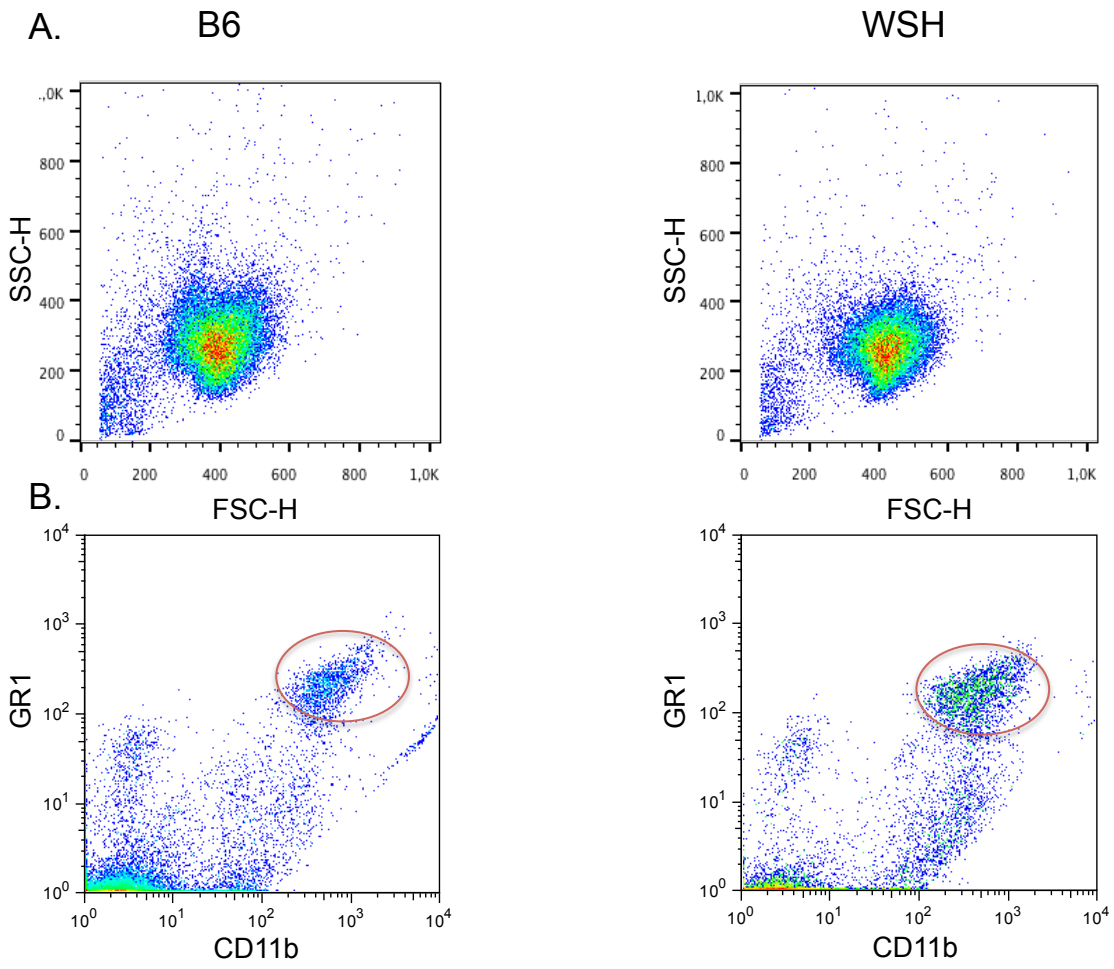


Fig 3.9.1.1 There are no morphological and CD11b/GR1 expression differences in C57BL/6 and Kit$^{W\text{-}sh/W\text{-}sh}$ mice neutrophils. A. Kit$^{W\text{-}sh/W\text{-}sh}$ (wsh) and C57BL/6 (B6) bone marrow neutrophils morphology. **B.** GR1-CD11b expression in Kit$^{W\text{-}sh/W\text{-}sh}$ (wsh) and C57BL/6 (B6) blood neutrophils. GR1-CD11b expression was analyzed using anti CD11b and anti GR1 antibodies. Representative data of one of at least 3 experiments are shown.

To exclude that kit mutation in $Kit^{W\text{-}sh/W\text{-}sh}$ mice could affect the life span of neutrophil we also compared the vitality of neutrophils isolated from C57BL/6 and $Kit^{W\text{-}sh/W\text{-}sh}$ mice. We cultured neutrophils in medium containing 10% of serum and in medium without serum. No differences in number of viable neutrophils was noted at indicated time points either in complete media or under pro-apoptotic condition. This suggests that cell survival in $Kit^{W\text{-}sh/W\text{-}sh}$ mice is not affected. (figure 3.9.1.2)

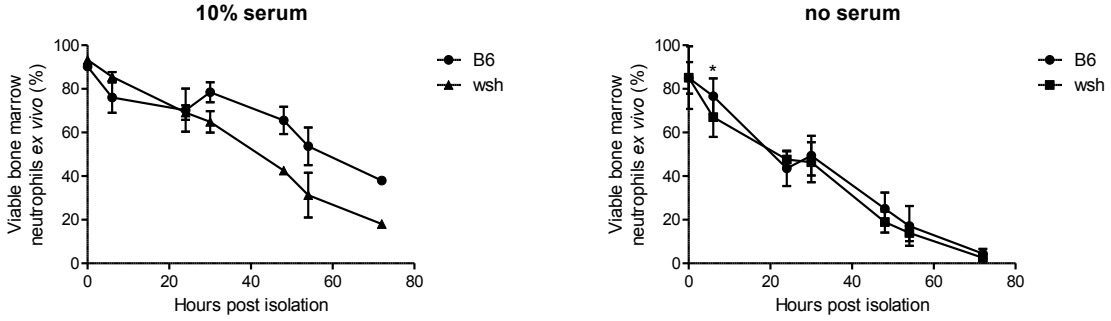


Fig 3.9.1.2 No differences in frequency of viable neutrophils between C57BL/6 and $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Frequency of viable neutrophils (PI-negative, annexin V negative) in C57BL/6 (B6) and $Kit^{W\text{-}sh/W\text{-}sh}$ (wsh) mice were obtained annexin V/propidium staining in media containing 10% of serum and in media without serum at different time point from their isolation. Data (mean + s.d) of at least 3 independent experiments are shown.

Finally, to verify whether the activation of neutrophils could be differently modulated in $Kit^{W\text{-}sh/W\text{-}sh}$ mice, we analyzed the expression levels of the surface antigens CD11b and CD62L, in presence or absence of MCs. As shown in figure 3.9.1.3 the expression of CD11b and CD62L was significantly up-modulated and down-modulated respectively under co-culture with MCs. Neutrophils from $Kit^{W\text{-}sh/W\text{-}sh}$ mice respond to MC activation following the same trend of C57BL/6 neutrophils.

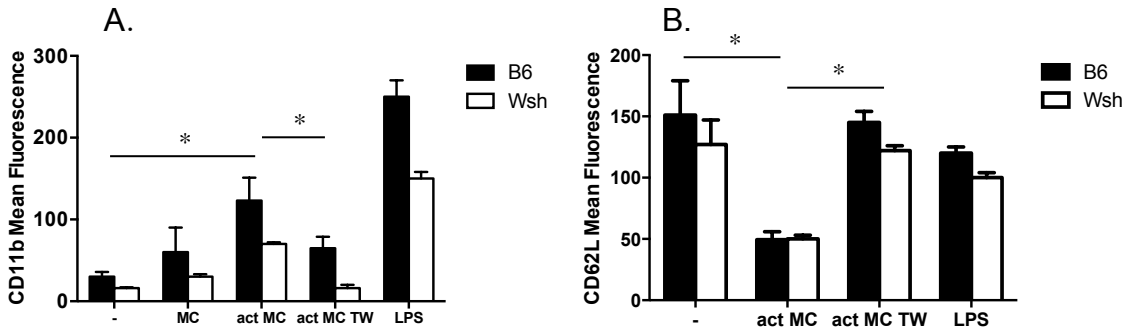


Fig 3.9.1.3 C57BL6 and Kit$^{W\text{-}sh/W\text{-}sh}$ mice neutrophils respond to MC activation following the same trend. Activated or not MCs and C57BL/6 (B6) or Kit$^{W\text{-}sh/W\text{-}sh}$ (Wsh) neutrophils were co-cultured in presence and absence of trans well system (TW) and with different stimuli DNP (100ng/ml), LPS (5µ/ml). Neutrophils surface expression of CD11b was analyzed at 4 hours using anti CD11b antibody (**A**) and CD62L was analyzed at 30 minutes using antiCD62L antibody (**B**) by FACS**.** Mean + s.d of at least 3 independent experiments are shown. *p<0,05

3.9.2. Characterization of B cell population of KitW-sh/W-sh mice

One of the aims of the study is to define the effect of neutrophils-MCs crosstalk in the activation of B cells. To do this, we wanted to take advantage of the Kit$^{W\text{-}sh/W\text{-}sh}$ mice model because these mice are mice lacking MCs and with a strong neutrophilia while reconstituted mice almost display the WT background. First of all we needed to verify if B cells levels were the same in the three different mice. As shown in figure 3.9.2 both Kit$^{W\text{-}sh/W\text{-}sh}$ mice and reconstituted Kit$^{W\text{-}sh/W\text{-}sh}$ mice displayed normal levels of CD19$^+$ cells in spleen and peritoneum in fact CD45$^+$CD19$^+$ cells population in the spleen and in the peritoneum was comparable in the three groups of mice (figure 3.9.2 A, B). The levels of naïve (CD45$^+$CD19$^+$CD23$^-$CD21$^-$), B2 (CD45$^+$CD19$^+$CD23$^+$CD21$^+$), MZ (CD45$^+$CD19$^+$CD23$^-$CD21$^+$) B cells and plasma-cells (CD138$^+$) in spleen and B1a (CD45$^+$CD19$^+$CD5$^+$CD11b$^+$), B1b (CD45$^+$CD19$^+$CD5$^-$CD11b$^+$) and B2

($CD45^+CD19^+CD5^-CD11b^-$) in peritoneum were the same in $CD19^+$ cells population of the three mice. This is a very important starting point that permits us to going on with the study focusing our attention only in the effect of neutrophil-MC-B cell crosstalk as the starting B cell population is the same in the different mice.

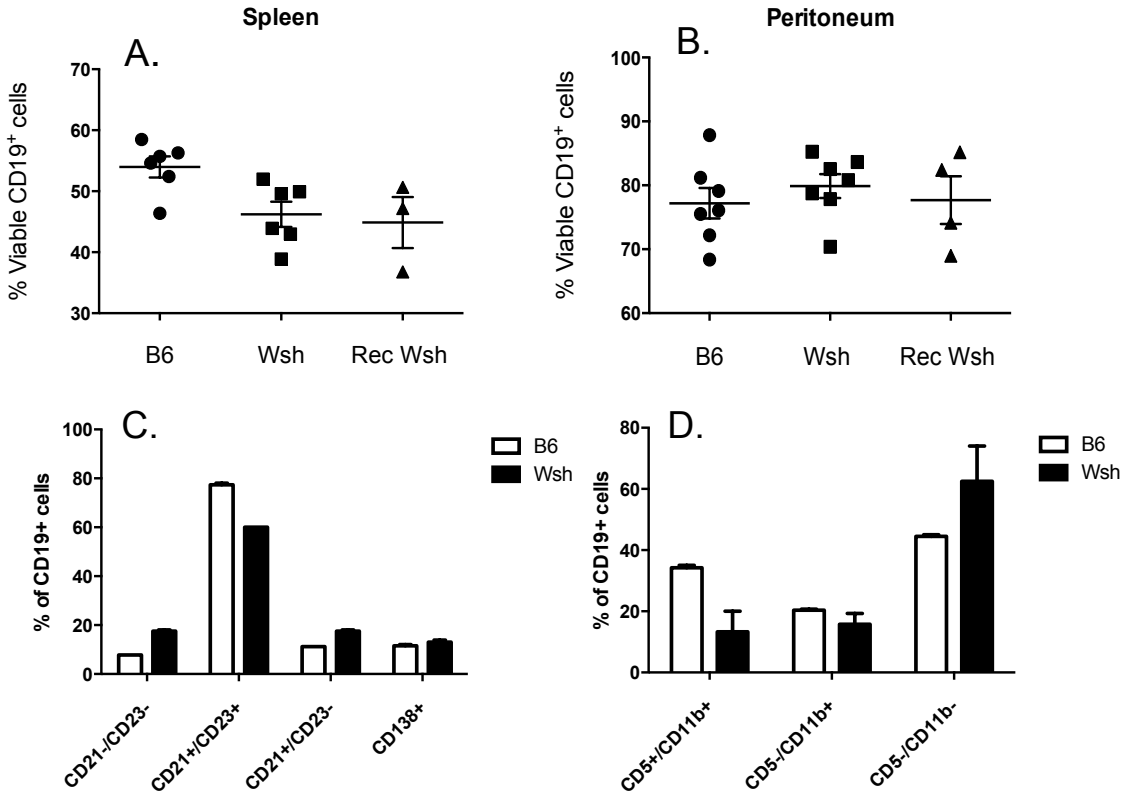


Fig 3.9.2 Characterization of B cell populations in $Kit^{W\text{-}sh/W\text{-}sh}$. A,B Levels of $CD19^+$ viable cells in C57BL/6 (B6), $Kit^{W\text{-}sh/W\text{-}sh}$ (Wsh) and $Kit^{W\text{-}sh/W\text{-}sh}$ reconstituted mice (rec Wsh) respectively in spleen and peritoneum of at least 3 independent experiments are shown. **C.** Mean + s.d of percentage of $CD19^+CD45^+$: Naïve ($CD23^-CD21^-$), B2 ($CD23^+CD21^+$), MZ ($CD23^-CD21^+$) B cells and plasma-cells ($CD138^+$) in spleen of at least 3 independent experiments are shown **D.** Mean + s.d of percentage of $CD19^+CD45^+$: B1a ($CD5^+CD11b^+$), B1b ($CD5^-CD11b^+$) and B2 ($CD5^-CD11b^-$) B cells population in peritoneum of at least 3 independent experiments are shown.

3.9.3. T-independent immunization

To elucidate the contribution of MCs and neutrophils in the humoral immunity *in vivo,* mice were immunized with TNP-AECM-FICOLL and the serum levels of specific anti-TNP immunoglobulin (anti-TNP IgM, IgG3 and IgA) were evaluated 8 days after. TNP-AECM-FICOLL is a high molecular weight polysaccharide useful as T-independent antigen for studying B cells activation. We performed the experiment using C57BL/6 WT mice, $Kit^{W\text{-}sh/W\text{-}sh}$, $Kit^{W\text{-}sh/W\text{-}sh}$ reconstituted via i.p with BMMCs, and the same groups of mice depleted for neutrophils. Depletion was carried on, as described in methods, by injecting i.p the anti-granulocyte receptor-1 (GR-1) mAb and (clone1A8) or the Isotype of control (clone 2A3) every other days figure 3.9.3.

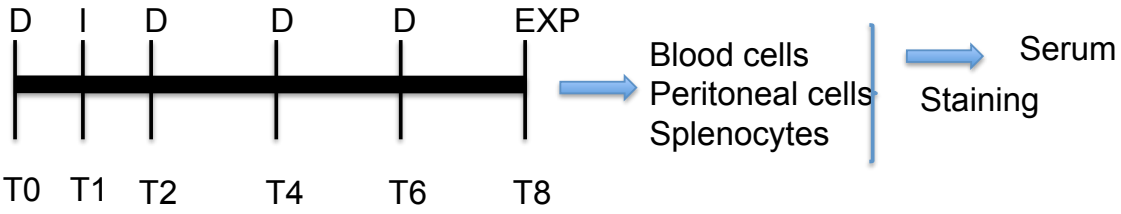


Fig 3.9.3 Representative scheme of depletion and immunization experiment. At T0 mice were depleted for neutrophils injecting i.p anti-GR1 mAb or isotype of control (D), the following day (T1) mice were immunized (I) with TNP-AECM Ficoll. Depletion (D) was carried on every other day for 8 days. At day 8 the experiments (EXP) were performed. Blood, peritoneal cells and splenocytes were collected and used for different staining. Blood was also use to obtain serum for Ig detection.

We first analyzed if B cells subsets in different mice change after immunization. We selected $CD45^+$ $CD19^+$ cells and then we analyzed the expression of CD5, CD11b, CD21, CD23. As shown in figure 3.9.3.1 the different B cell populations

(B1a, B1b and B2 in peritoneum and naïve MZ B2 in spleen) were comparable among different mice after immunization. Also the percentage of plasma-cells in blood and spleen was comparable among mice.

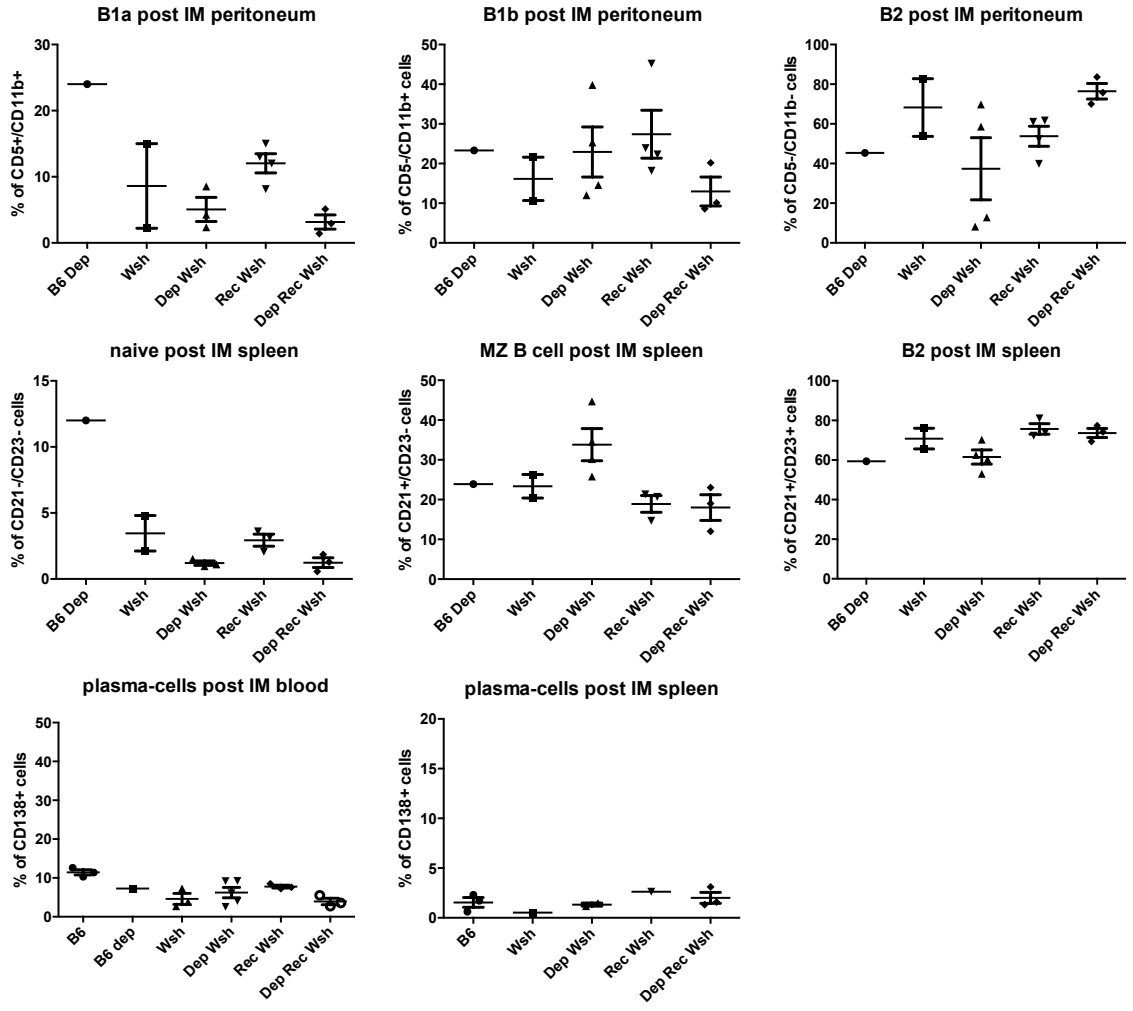


Fig 3.9.3.1 Characterization of B cell populations after T-independent immunization. Eight days after immunization $CD45^{+}CD19^{+}$ viable cells of peritoneum and spleen in C57BL/6 (B6), $Kit^{W\text{-}sh/W\text{-}sh}$ (Wsh), $Kit^{W\text{-}sh/W\text{-}sh}$ reconstituted mice (Wsh Rec) and the same group of mice depleted for neutrophils (Dep) were selected. Cells were then analyzed for their expression for CD5, CD11b, CD21, CD23 and data reported in graph are of two independent experiments. Percentage of $CD138^{+}$ cells in blood and spleen analyzed from 2 independent experiments.

Then, the levels of specific anti TNP-Ficoll immunoglobulins (Ig) IgM, IgG3 and IgA in the serum of mice collected 8 days after immunization were analyzed (figure 3.9.3.2).

TI-immunization resulted in increased production of IgM and IgG3 in $Kit^{W\text{-}sh/W\text{-}sh}$ mice, both reconstituted and not reconstituted respect to C57BL/6 mice, and Ig levels were also higher in all mice that have been depleted of the neutrophil pool.

Regarding IgA production, there are no significant differences between C57BL/6 WT mice, $Kit^{W\text{-}sh/W\text{-}sh}$ mice, and MC-reconstituted $Kit^{W\text{-}sh/W\text{-}sh}$ mice. However, depletion of neutrophils clearly increases IgA production especially in $Kit^{W\text{-}sh/W\text{-}sh}$ mice.

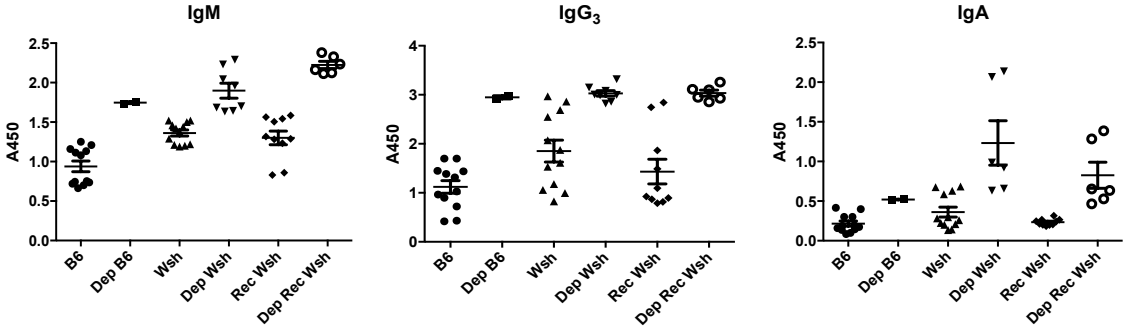


Fig 3.9.3.2 Ig presence in sera of T-independent immunized mice. Sera were separate from blood after 8 days after of immunization and specific Igs levels were analyzed by ELISA assay. Data reported are from 2 independent experiments.

4. DISCUSSION and CONCLUSION

MCs are pivotal in innate immunity and play important role in amplifying adaptive immunity (Alvarez-Errico et al., 2009). They are strategically localized along the microvasculature in tissues in close contact with external environment, such as skin, lung and intestine. By releasing their pro-inflammatory mediators, they can influence various aspects of the biology of dendritic cells, T cells and B cells thus acting like regulatory cells (Merluzzi et al., 2010). MCs have a critical role also in the initiation of acute inflammatory responses where they are able to induce neutrophils recruitment. Neutrophils are phagocytic cells that act as the first line of defense against infectious pathogens in inflammatory response. In addition to their crucial role in protecting against infections, recent evidence suggests that neutrophils are also key components of the effector and regulatory mechanisms of both innate and adaptive immune responses. Their potency and ability to interact with other immune cells involve neutrophils in immune defense and also in several immune-mediated disease, such as anaphylaxis, metabolic disease, autoimmunity and tumor biology (Mócsai, 2013). The control of neutrophil activation is therefore highly relevant for all inflammatory conditions and tissue resident MCs, known to be involved in the regulation of all phases of the inflammatory response, could be good candidates in controlling neutrophil functions. The idea that MCs could have a role not only in the recruitment but also in the regulation of neutrophil function has been only recently formulated. In a paper published in 2013, Doener and colleagues investigated for the first time the effect of the presence of MCs on the in vitro activation of freshly isolated neutrophils, demonstrating the capacity of MCs to induce up-regulation

of neutrophil surface markers, production of ROS and enhancement of phagocytosis (Doener et al 2013). In that work the authors incubated neutrophils with MCs or with conditioned media of IgE/DNP activated MCs and found the same impact on neutrophil effector functions. These results led the authors to speculate that the MC-dependent activation of neutrophils relies on soluble factors released by MCs. The work of this PhD thesis, instead, revealed that, in addition to soluble factors, the contact between the two cells is fundamental. By incubating neutrophils with CM of activated MCs we found an increase of neutrophil activation, confirming that soluble factors are involved in the effect, but CD11b up-regulation was less marked respect the increased expression found when neutrophils were co-cultured with activated MCs. The use of tans-well in the co-culture system almost reported neutrophil activation (studied on their up regulation of CD11b and down regulation of CD62L membrane marker, ROS production, degranulation and vitality) at levels of neutrophils cultured alone, suggesting the importance of cell-cell contact in MC-induced activation. Based on of these evidences we focused our attention on the possible molecules involved in the crosstalk and we found an important role for the CD18/ICAM axis in ROS production and less obviously in lactoferrin degranulation. Furthermore, incubating neutrophils with activated MCs but deprived of their early released mediators neutrophil activation was found less evident indicating a crucial role for soluble factors immediately released by MC. Using BMMCs from TNFα deficient mice we confirmed a crucial role for pre-stored and early released TNFα in up-regulation of neutrophil CD11b expression. Moreover, interestingly, we found that the presence of MCs can also lower the activation threshold of neutrophils making them susceptible to respond to suboptimal concentration of LPS.

On the other hand we described for the first time that neutrophils can affect some of the MC activities. Resting, apoptotic or necrotic neutrophils don't have any effect on MC degranulation but, interestingly, LPS-activated neutrophils reduce MC IgE/Ag dependent degranulation of MCs. As MCs are known to secrete a large array of mediators in response to different stimuli we tested if neutrophils can influence cytokine production. Intracellular staining and ELISA assays for TNFα, IL6 and TGFβ demonstrated that the presence of neutrophils doesn't affect MC cytokine production. However, the levels of TNFα in the co-culture system are reduced. Since TNFα is crucial for some neutrophils activities (Lokuta & Huttenlocher, 2005) we can speculate that neutrophils consume the TNFα that is produced and released by MCs diminishing the amount of the TNFα present in the co-culture. All these findings indicate that MCs and neutrophils interact each other and that their crosstalk is bidirectional and influence mutually the response of both partners.

MCs and neutrophils are involved in physiological and pathological immune responses acting at the interface between the innate and adaptive branches of the immune system in particular of B cells. As note in literature MCs can induce both survival and proliferation of primary naïve B cells and proliferation and further differentiation of activated B cells into IgA-secreting plasma cells by cell-cell contact and through soluble factors (Merluzzi et al., 2010). On the other hand activated neutrophils express the B cell activating factor cytokine BAFF and are a major source of the BAFF-related B cell-stimulating cytokine APRIL that strongly modulate B cell function. In mice splenic neutrophils are demonstrated to be able to activate marginal zone B cells, inducing B cell survival, antibody production, IgG and IgA class switching and somatic

hypermutation (Puga et al., 2012). Taken together these studies have led us to further investigate if MCs and neutrophils together had an additional role or collaborated in the activation of B response. We reported that supernatants of neutrophils and activated MCs co-culture further enhance B cells vitality respect to supernatants from neutrophils or MCs cultured alone. This augmented vitality correlates positively with increased levels of BAFF in the co-culture supernatants. Thus the presence of activated-MCs induces neutrophils to increase BAFF production that consequently could enhance B cell survival.

To investigate the role of neutrophil and MC crosstalk on B cell response in vivo we have decided to perform T-independent immunization experiments using the Kit $^{W\text{-}sh/W\text{-}sh}$ mice. We take advantage of this mouse strain because it exhibits profound MC deficiency concomitantly to a strong neutrophilia (Nigrovic et al., 2008) and because MC population can be restored after i.p injection of mature BMMCs. The staining of neutrophils (identified as $CD11b^{High}/Ly6G^{High}$ cells) in primary and secondary lymphoid organs of Kit $^{W\text{-}sh/W\text{-}sh}$ and reconstituted Kit $^{W\text{-}sh/W\text{-}sh}$ mice confirmed the neutrophilia in all secondary lymphoid organs of Kit $^{W\text{-}sh/W\text{-}sh}$ mice and, surprisingly, pointed out a reduced neutrophilia when mice were reconstituted with *in vitro* differentiated MCs. There aren't apparently differences between neutrophils from C57BL/6 and Kit $^{W\text{-}sh/W\text{-}sh}$ mice because they show the same vitality and the same ability to respond to MC-induced activation. However, the reduction of neutrophilia in reconstituted Kit $^{W\text{-}sh/W\text{-}sh}$ mice suggests that MCs someway can control the levels of neutrophils in peripheral lymphoid organs.

The experiments of T-independent immunization performed in the three different groups of mice (C57BL6, Kit $^{W\text{-}sh/W\text{-}sh}$, and Kit $^{W\text{-}sh/W\text{-}sh}$ reconstituted with

in vitro differentiated MCs) depleted or not for neutrophils, indicated important role for neutrophils and MCs in Ig production from B cells. The depletion of neutrophils in C57BL/6 depleted mice correlates with increased level Ig in serum suggesting a suppressive role of neutrophil in B cell immunoglobulin production. These results contrast with Cerutti's findings, because he demonstrated that a splenic population of neutrophils defined B helper (N_{BH}), phenotypically and functionally distinct from circulating neutrophils, gives to MZ B cells signals to produce IgM as well as class-switched IgG and IgA antibodies (Cerutti et al., 2013). In his work, the group identified innate lymphoid cells (ILCs) in MZ and peri-follicular zone of the spleen. They found that these cells, in addition to stimulating MZ B cells and plasma cells via BAFF, APRIL, CD40L and Notch2 ligand Delta-like 1 (DLL1), communicate with MZ B cells-helper neutrophils (N_{BH}) via the cytokine GM-CSF. They found that incubating neutrophils with ILCs conditioned medium, they up-regulated APRIL expression and stimulated IgA production in MZ B cells *in vitro*. In vivo they saw that in ILCs-deficient mice, impaired production of IgG3 was associated with fewer N_{BH} cells. Furthermore, our results are interesting in light of recent report from Jee that demonstrated a suppressive role of neutrophils in IgA production. *In vitro,* co-culturing B cells with neutrophils, Jee and colleagues found a significantly reduced IgA heavy chain transcript in B cells and a reduced secretion of IgA. This was also confirmed by in vivo experiments where they demonstrated that depletion of Ly6G+ cells improves the development of IgA responses after sublingual immunization with Bacillus anthracis edema toxin. Thus both our data and data from Jee' s group suggest a suppressive role for neutrophil in *in vivo* Ig production.

However Kit $^{W\text{-}sh/W\text{-}sh}$ mice displayed increased levels of Ig despite their neutrophilia and lack of MCs. The lack of both neutrophils and MCs in depleted Kit $^{W\text{-}sh/W\text{-}sh}$ mice results in a further augment of Ig production that are lowered following MC reconstitution. The explanation of these results should kept in account the recent findings from Michael and colleagues who demonstrated that the deregulation of c-Kit expression in Kit $^{W\text{-}sh/W\text{-}sh}$ mice causes accumulation in the spleen of $CD11b^{+}/Ly6G^{int\text{-}high}$ $Ly6C^{+}$ cells which resemble granulocytic myeloid derived suppressor cells. So to understand the effect of MC deficiency and neutrophilia further experiments must be performed to better characterize and comparing $GR1^{+}$ cells subpopulation in C57BL6 and Kit $^{W\text{-}sh/W\text{-}sh}$ mice to verify if different subpopulations can differently influence B cell response.

Taken together results from the thesis underline that neutrophils and MCs physically interact and this interaction brings to neutrophils activation and down-regulation of some MC functions. Since IgA, IgM and IgG3 production by B cells, MC degranulation and the presence of some of MC soluble factors seem to be impaired by the presence of neutrophils It can be supposed that probably neutrophils could limit MC-B cell interaction (figure 4). Hence, a regulating role of neutrophils in B cell Ig secretion was put on light but it must be further investigated.

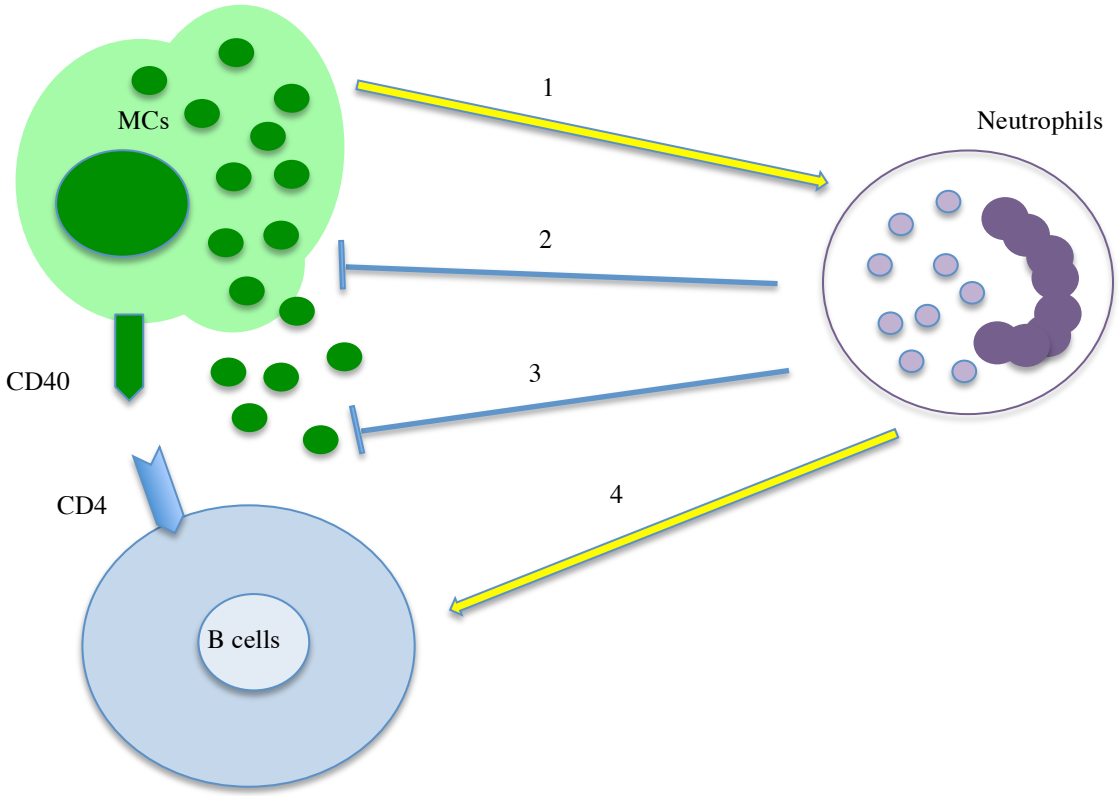


Fig.4 Schematic representation of neutrophil-MC interaction in B cell activation. It was possible to speculate that: MCs can activate neutrophils (1), activated neutrophils can reduce MC response (2) so they can limit MC-B cell interaction (3), and can interact directly with B cells (4) (like suppressor cells for example).

5. METHODS

5.1. Mice

C57BL/6 mice were purchased from Harlan Laboratories (Harlan Italy); C57BL/6 TNFα, and MC-deficient C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$ mice (*wsh*) were kindly provided by Dr. M. Colombo (Fondazione IRCCS Istituto Nazionale dei Tumori di Milano). C57BL/6 Myd88-deficient mice and C57BL/6 Syk-deficient mice were kindly provided by Dr Cassatella (University of Verona).

5.2. Isolation of murine Neutrophils

Neutrophils were isolated from bone marrow by positive selection magnetic cell separation (MACS) using the mouse Anti LY6G MicroBead Kit (Miltenyi Biotec). The purity of the isolated cell was at least 97% according to the expression of Gr-1 and CD11b (figure 5.2). Neutrophils receptors expression was determined by flow cytometry using fluorochrome-conjugated monoclonal antibodies.

After the dissection of femur and tibia, the tissue was cleaned and the ends of bone were cut as marrow could be visible. With 23G needle and 10 ml syringe cells were flush out in 50 ml falcon tube, until bones appear white. After centrifugation at RT at 1500 rpm, 5 minutes 6 ml of 0,2% of NaCl solution for 30 seconds and then 14 ml of 1,2% of NaCl solution were added to allow osmotic lysis of red blood cells. Cells were passed through 70 μm strainer and collected at 1500 rpm 5 minutes at RT. After centrifugation the obtained cellular

suspension was re-suspended in MIN Buffer (a solution containing PBS, 0,5% BSA, 2mM EDTA) and spinned at 1300 rpm 10 minutes.

Then the pellet was re-suspended in 250µL of MIN buffer per 10^8 cells containing Anti-Ly-6G Biotin (50µL for 10 minutes at 4°C). After 10 minutes 150 µl of buffer per 10^8cells and anti Biotin micro Beads (100 µl for 15 minutes at 4°C) were added. Washed cells were re-suspended in 500µL of buffer. Anti-Ly6G-Biotin and anti-Biotin Micro Beads magnetic labeled Gr-1^{high} Ly-$6G^+$ PMN were positive selected through magnetic separation. Magnetic purification was performed in a Miltenyi Biotech LS column, placed in the magnetic field of a MACS separator. Cell suspension was applied onto the column and the flow-through containing unlabeled cells, that represent the pre-enriched Gr-1^{dim} Ly-$6G^-$ fraction, was collected. After removing from separator, the magnetically labeled PMN were flushed out by pushing the plunger into the column. After a second passage in column of the eluted fraction to increase the purity of LY-$6G^+$ cells, PMN were re-suspend in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 200 U penicillin, and 200 mg/ml streptomycin.

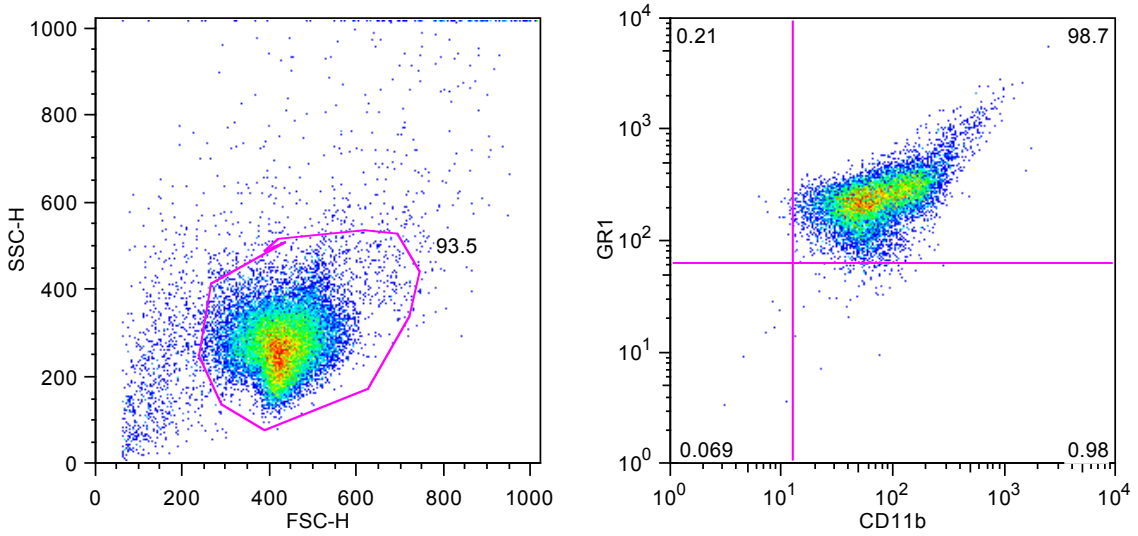


Fig 5.2 Purity of the bone marrow-derived neutrophils. Representative example of FACS analysis to evaluate neutrophils purity. Neutrophils gated on the basis of their size and granularity (FSC vs SSC plot, left side), are positively stained for both CD11b and GR1 (right panel).

5.3. Differentiation of BMMC in vitro

Bone marrow-derived MCs (BMMCs) were obtained by *in vitro* differentiation of bone marrow cells taken from murine femur. Bone marrow precursors were cultured in RPMI 1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin, nonessential amino acids, sodium pyruvate, HEPES buffer, 50mM β-mercaptoethanol and containing 20 ng/ml stem cell factor (SCF) and 20 ng/ml IL-3 for 4 to 8 wk. All cell cultures were grown at 37°C in a humidified atmosphere with 5% CO_2.

After 4-5 weeks, BMMCs were monitored for FcεRI (Biolegend) and ckit (Biolegend) expression by flow cytometry with purity usually more than 95% Figure 5.3).

For IgE dependent activation, BMMC were sensitized in medium (RPMI 10% FCS) without IL3 for 3 hours with 1µg/ml of dinitrophenol (DNP)-specific IgE washed and then challenged with 100 ng/ml DNP (Sigma-Aldrich)

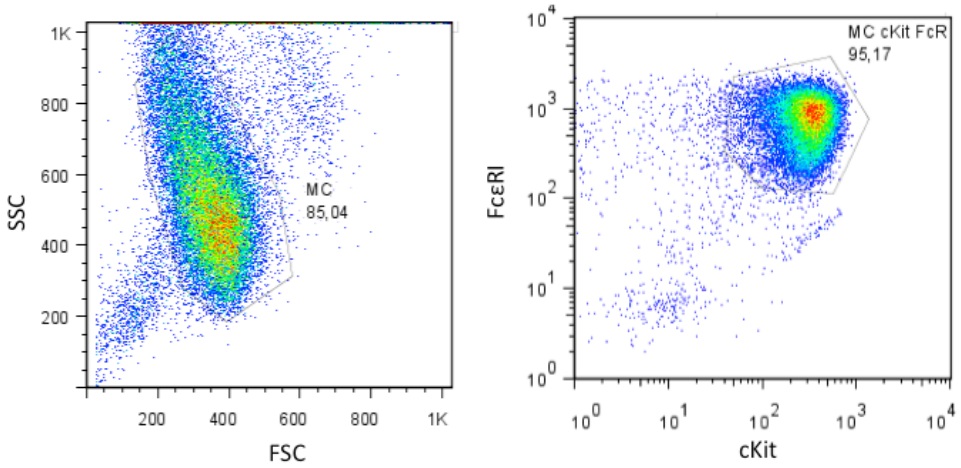


Fig 5.3 Purity of the bone marrow-derived mast cells (BMMC) population. Representative example of FACS analysis to evaluate BMMC maturation. Mature BMMCs, gated on the basis of their size and granularity (FSC vs SSC plot, left side), are positively stained for both FcεRI and c-Kit (right panel).

5.4. Purification of murine B cells

Splenic B cells were obtained from 6-12 week-old mice by negative depletion. Spleens were dissociated in PBS / sodium citrate (0,0192M) and pellet after centrifugation at 1800 rmp 5 minutes. Red blood cells were lysed using ACK lysis buffer (Sigma Aldrich) 2 minutes at room temperature. Splenocytes suspension was depleted of T cells by complement-mediated cytotoxic lysis using and anti-Thy 1.2 monoclonal antibody 40 minutes at room temperature (a gift from K.Hatcock, Experimental Immunology Branch NIC/NHI), in conjunction with rabbit complement (Cedar Lane) 30 minutes at 37°C. The cell suspension was then cultured for at least one hour at 37°C and 5% CO_2 to allow the

adhesion of mononuclear cells at the flask. Cells in suspension were collected and re-suspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin, nonessential amino acids, sodium pyruvate, HEPES buffer, 50mM β-mercaptoethanol at 10^6 cell/ml. The purity of the isolated cell was between 85-95% according to the expression CD19 (figure 5.4).

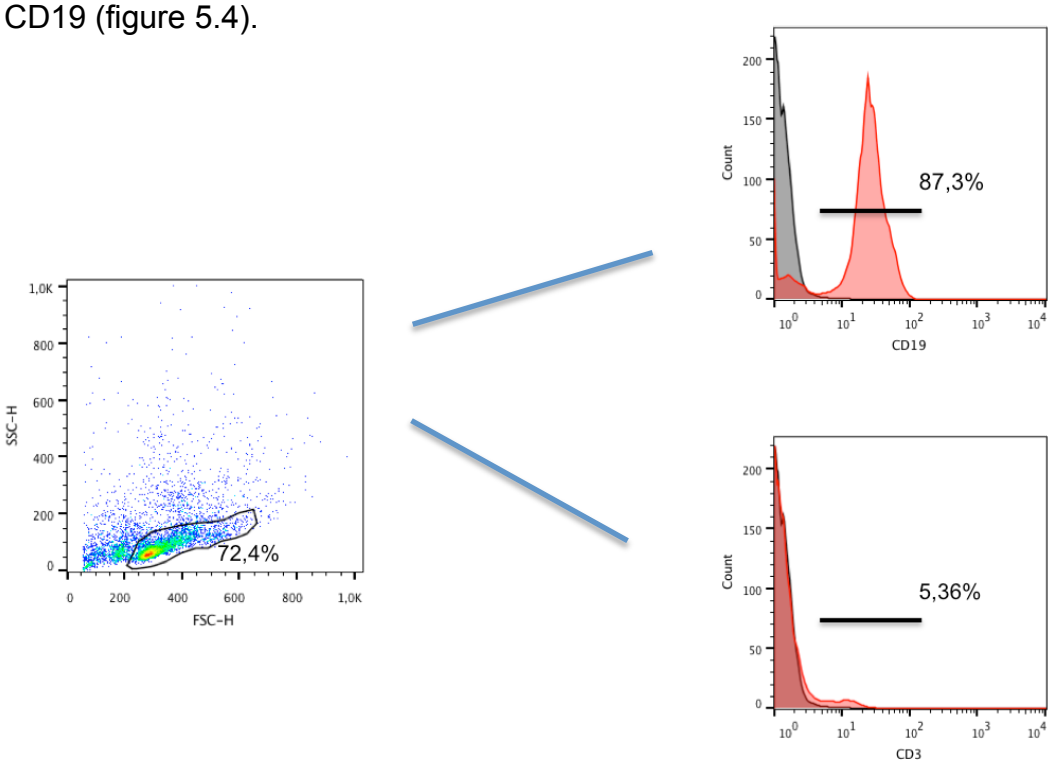


Fig. 5.4 Purity of B cell population isolated from mouse spleen. Representative example of FACS analysis to evaluate B cell purity. On the left side B cells are shown gated on the basis of their size and granularity (FSC vs SSC plot). The hystograms reported on the right side of the picture show how the great majority of the isolated lymphocytes (87,3%) are B cells and that T cell contamination is only 5,36% of total cells.

5.5. Co-Colture

Cells were co-coltured in a ratio of 1:1 ($2x10^6$ cells/ml), in complete RMPI medium without IL3. Different stimuli and blocking antiobodies were added directly to the co-culture. ICAM blocking antibody (Biolegend), CD18 blocking

antibody (Therno), SIRP1α blocking antibody (Biolegend), TNFα blocking antibody (Miltenyi) at a concentration of 10μg/ml.

In some co-culture experiments neutrophils and MCs were separated by a transwell polycarbonate membrane (Corning Costar) with pores of 0,4 μm that allow the passage of soluble factors but not of cells. Neutrophils (0,6x 10^6) were cultured in the bottom chamber in a final volume of 600 μl while 0,2 x 10^6 MC were placed in the top chamber in a final volume of 100 μl.

5.6. Flowcytometry

Anti-CD11b (M1/70 clone), anti-Gr-1 (RB6-8C5 clone), anti-CD117 (c-kit, ACK2 clone), anti-FcεRIα (MAR-1 clone), anti-CD40, anti -CD40L, anti-CD62L, anti-CD19, anti-CD226, anti-CD5 were purchased from Biolegend. Anti-OX40, anti-IL6 were purchased from e-Bioscience. Anti-TNFα, anti CD45, anti GITR, anti-CD200R3 were purchased by Miltenyi. Anti-CD21, anti-CD23, anti-CD138 were purchased by BD Pharmigen. Anti-BAFF (Buffy 2 clone) was purchased by Abcam.

Isotype control antibodies, Rat IgG2a, Rat IgG 2b, Rat IgG2bk purchased by Biolegend, Rat IgG2b, Rat IgG2a were purchased by e Bioscience.

To assess cell-surface expression of different co-stimulatory molecules and/or activation markers, 3-4 x 10^5 cells were collected into polystyrene tubes (Sarstedt), washed with PBS and then incubated in the dark for 30 minutes at 4°C with a fluorescent mAb. In case of multicolor staining, multiple fluorescent antibodies were added at the same time. After the incubation, the cells were washed with PBS, re-suspended in 0.3 ml of PBS and kept at 4°C until flow

cytometric analysis.

Flow cytometry data were acquired on a FACSCalibur (BD Biosciences) and analyzed with FlowJo.

5.7. Intracellular staining

Neutrophils and MCs were co-cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin. DNP (100ng/ml), LPS (10µg/ml), and brefeldin A (10 mg/ml; all from Sigma-Aldrich, St. Louis, MO) were added to the cells. After incubation for 3h or 12 hours cells were washed and incubated with FcR (50µl) blocking antibody, and Live/Dead (Invitrogen) for 15 minutes at 4°C. Washed cells were subsequently labeled with antibodies against surface molecules (FcεR, c-kit, GR1) at 4°C for 30 min and then labeled with the antibody of cytokines of interest (TNFα, IL6).

The fixation and permeabilization of cells were performed using BD perm Wash, and BD Cytofix Cytoperm, according to the manufacturer's instructions. To demonstrate the specificity of staining, isotype-matched control mAbs were also used. The stained cells were analyzed by flow cytometry.

5.8. Degranulation assay: LAMP1

IgE pre-sensitized BMMCs were challenged with DNP antigen (100ng/ml) in RPMI 10% FCS, 2 mM L-glutamine, 100U/ml penicillin and HEPES buffer. MCs, when indicated, were plated with equal number of neutrophils for 30 min concomitant to DNP challenge. After incubation samples were washed and

stained in the dark for 30 minutes at 4°C with the fluorescent marker LAMP1 (Biolegend)

5.9. Peritoneal cells

To obtain peritoneal cells, 5 to 10 ml of sterile PBS were injected into the peritoneal cavity of sacrificed mice using a sterile syringe with 23G needle. The abdomen was massaged and the cells and fluid were removed from the peritoneal cavity drawing with the syringe. The peritoneal cells were collected by centrifugation at 1800 rpm for 5 min and suspended to 1 x 10^6 to 2 x 10^6 cells/ml in PBS.

5.10. Blood cells

Blood cells were obtained by heart and tail. Heart: The animal was positioned on its back, the abdomen was opened and the heart visualized through the membranous part of the diaphragm. A 23G needle and a 1 ml syringe was used to obtain ~0,5 ml of blood which was immediately transferred to an eppendorf containing heparin. Part of blood was used for cell counting and flow cytomety after red blood cells lysis with a solution of ammonium chloride (1,5M NH_4Cl; 100mM $NaHCO_3$; 10mM Na_2EDTA; pH 7,4)

The other part of blood was used for serum collection.

Tail: The proximal part of the tail was incised and blood was collected in a tube containing sterile PBS. After red blood cells lysis cells were used in flow cytometry to monitoring neutrophils depletion.

5.11. Migration assay

Chemotaxis was performed in a 96-transwell insert (Corning) with 5 μm pore-size filters. 10^5 cells (in 75 μl) were plated in the upper layer of cell permeable membrane and solutions containing different stimuli were added in the lower compartment (for a total volume of 310 μl). After 4 hours, cells migrated in the lower chamber were counted and results expressed as fold induction compared to number of cells migrated in response of not-conditioned medium.

5.12. ROS Production

The amount of intracellular ROS was identified by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich). When applied on the cells, the non-ionic, non-polar, color- less DCFH-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF). Therefore, intra- cellular DCF fluorescence can be used as an index to quantify the overall ROS in cells. Neutrophils were stained in PBS with DCFH-DA for 10 minutes in dark at 37°C. After centrifugation the cells were incubated with MCs and different stimuli for 30 minutes in dark at 37°C. To stop the reaction the cells were put in ice and were immediately analyzed by flow cytometry.

5.13. Detection of necrotic and apoptotic cells

Necrotic and apoptotic cells were detected by labeling with annexin V and Propide Iodide using Annexin V-FITC Apoptosis detection Kit (eBioscience), as indicated. 2-4 x 10^5 cells were re-suspend in the provided Binding Buffer 1X and 2,5 µL of Annexin V-FITC were added to 197,5 µL of cell suspension for 10 minutes at room temperature. After a washing, cells were re-suspended in 200 µL Binding Buffer with 5 µL of Propidium Iodide before performing FACS analysis.

If required, cell surface staining was performed before Annexin staining.

5.14. Cytokine and chemokine ELISA assays

Supernatants from different experimental settings were collected at the indicate times for quantification of TNFα, TGFβ and IL-6 (e-Bioscience), BAFF (Boster Biological Technology, LTD), through ELISA test according to manufacturer's protocol.

5.15. T Independent Immunization

On day 0, mice were injected intra-peritoneally with 25 µg of TNP(65)-AECM-Ficoll (BIOSEARCH Technologies, Petaluma CA) in a total volume of 200µL of PBS. On day 8 after immunization, the blood was collect form each mouse and centrifuged at 1800 rmp for 15 minutes to separate serum.

5.16. IgM, IgG, IgA ELISA

ELISA plate was prepared coating it overnight at 4°C with 5µg/ml TNP(30)-BSA (BIOSEARCH Technologies, Petaluma CA) in carbonate buffer (pH9).

After snapping and washing the plate to remove the coating antigen the plate were incubated for 1-3 hours with PBS-BSA (0,5%) at 37°C. Each serum obtained from the immunization and from control mice was diluted in PBS-BSA (2µl of sera in 1ml of PBS). Several dilutions of sera were plate for 1 h at 37°C and then, after washing, HRP-conjugated anti mouse IgM, IgA or IgG3 (Biolegend) diluted in PBS-BSA were added for 1h at 37°C. The plate was developed by adding 100 µL of room temperature TMB Sure Blue reagent per well and the reaction was stopped adding 100 µL of TMB Stop Solution. Absorbance was read at 450 nm.

5.17. Neutrophils depletion

C57BL/6 and $Kit^{W\text{-}sh/W\text{-}sh}$ were injected intra-peritoneally (i.p) with 250 or 400 µg (for Whs mice) of anti Ly6G antibody (clone 1A8) or Isotype of control (clone2A3), purchased from BioXcell, the day before the immunization and every other days. Neutrophils depletion was monitored at different time point figure 5.17 controlling Ly6Ghigh/CD11b positive cells in mice tail blood. The depletion was carried on until the end of immunization.

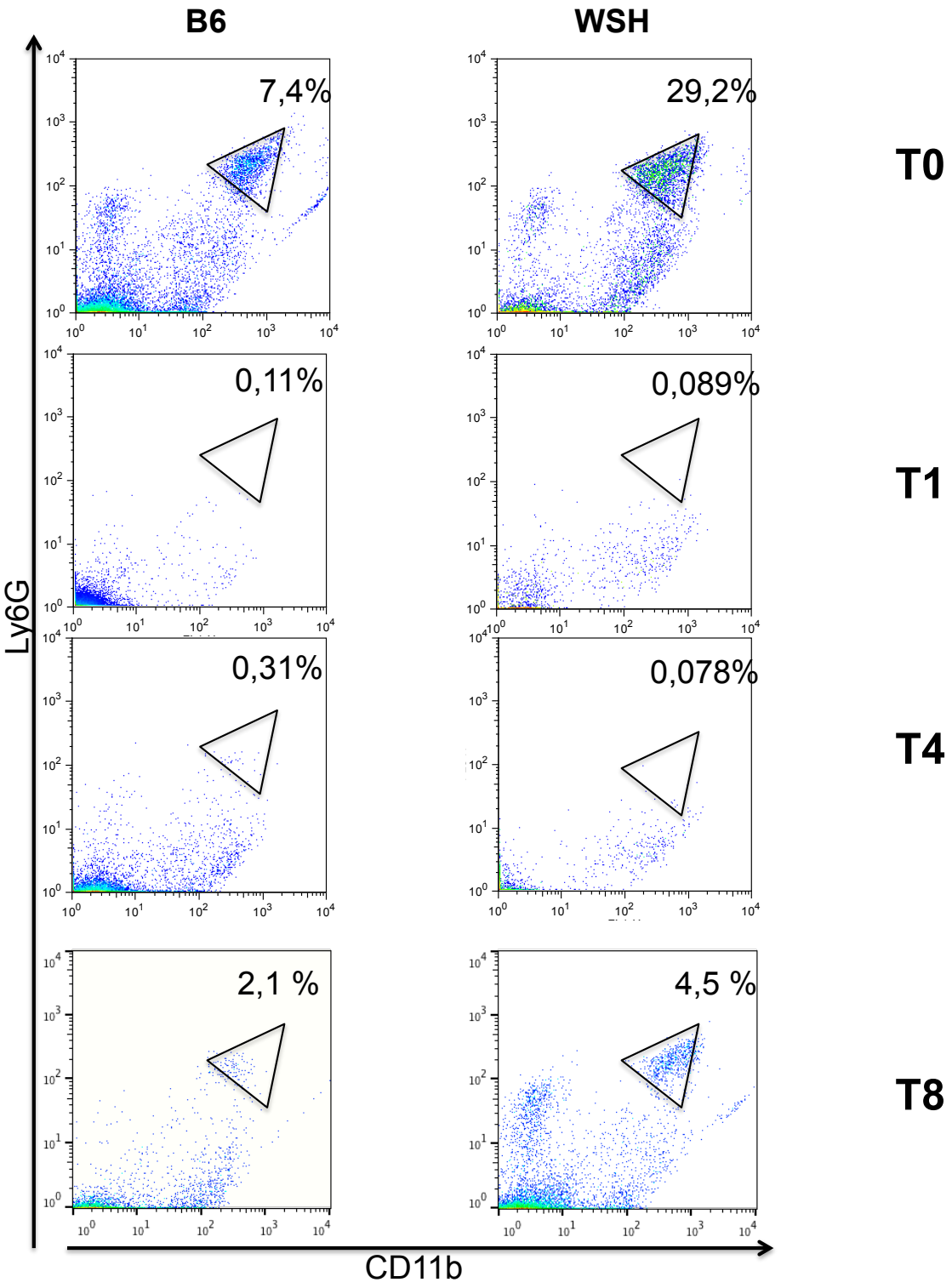


Fig. 5.17 Neutrophil depletion in C57BL/6 and Kit$^{W\text{-}sh/W\text{-}sh}$ mice after mAb anti-Ly6g administration. Representative example of FACS analysis to evaluate neutrophil depletion. T0 blood neutrophils in mice before depletion; T1 neutrophil presence in mice blood after the first i.p administration of mAb anti-Ly6g (clone 1A8); T4 neutrophil presence in mice blood two days after the second and before the thied administration of mAb anti-Ly6G; T8 neutrophil presence in mice blood the day of experiment.

5.18. Reconstitution of mast cell population in WSH mice

5-6 weeks aged C56BL/6 mice were reconstituted by intra-peritoneal injection of $3x10^6$ BMMC and used 5 weeks after reconstitution.

5.19. Statistical analysis

Results are expressed as the mean plus standard deviation (or standard error of the mean where specifically indicated). Data were analyzed using two-tailed Student t test.

$p < 0,05$ was considered statistically significant.

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