UNIVERSITY OF UDINE

DEPARTMENT OF MEDICAL AND BIOLOGICAL SCIENCES

PHD COURSE IN BIOMEDICAL AND BIOTECHNOLOGICAL SCIENCES

XXV CYCLE

Network at a glance:
looking at Mast Cells' range of contact modes

PhD Student:

Federica D'Incà

Supervisor:

Prof. Carlo Ennio Michele Pucillo, MD

Tutor:

Dr. Barbara Frossi, PhD

ACADEMIC YEAR 2012-2013

To Stephen Stills, who wrote "Helplessly Hoping"
To Rolf de Heer, who invented a time machine
To Elliott Erwitt, who barks at dogs

The mast cell has earned a bad name,

because for wheezing it's partly to blame.

But it also keep us all healthy,

despite pathogen stealthy,

by helping us win in the host defense game.

S.J.Galli

Table of Contents

Abstract

Abstract

Over the past decades, a new picture of the function of MCs is gradually emerging; these cells have currently gained recognition as true immune effector cells in a variety of settings during innate and adaptive immune responses, but have also the potential to modulate many aspects of immune cells.
Besides paracrine communication, it is now clear that the onset and the regulation of a specific immune response is highly complex and requires immune cells to be specially adapted to interact directly with other cells. Such communication can result in different modes of interaction (with respect to morphology and duration) and leads to different outcome. The type and amount of information that is exchanged is determined by the duration of interaction, physical dynamics, identity of receptors and signaling molecules that are engage. Moreover they rely on signals that are transmitted, depending on the activation state of both the cell types, as well as the type of the environment in which the interaction takes place.
As MCs are traditionally thought of as a secretory cell type, an intriguing and not yet fully clarified aspect of MC biology concerns their physical interaction with other cellular partners.
This work aims to shed light on MC range of contact modes, starting to define a molecular code, in which the differences in timing, spacing and molecular composition of the signaling platform determine the outcome of "MAST CELLular interactions".
Distinct aspects of the interactions with different immune cells have been addressed.
Firstly the morphological features and functional profile of MC-regulatory T cell (Treg) synapses have been extensively described: it has been demonstrated that this cross-talk is regulated on a single cell level also providing the first morphological evidence for a role of the OX40-OX40L axis in Treg inhibition of MC function. A more detailed analysis obtained with electron microscopy indicated that MCs interacting with Treg probably underwent selective mediator secretion throughout piecemeal

degranulation (PMD). Few additional information were given on the ability of MCs to collect and add up signals to dictate their stringency of binding to Treg cells. Moreover, to explain the inhibitory mechanism exerted by Tregs on MC FcεRI-dependent extracellular calcium (Ca^{2+}) flux, the possibility that STIM1 may function as a switch governing in the fate of Ca^{2+} flux in BMMCs interacting with Tregs was explored.

Secondly, an overview of the regulation of MC-B cell adhesiveness under different stimulation conditions was provided. The capacity of naïve B cells and LPS- or CD40-stimulated B lymphocytes to form stable conjugate with MCs was assessed in the absence or in the presence of antigen stimulation, also demonstrating a role of CD40-CD40L axis in the dynamics and functional outcome of this interaction.

Finally, a fresh picture of the functional dynamics of MC-Neutrophil interactions has been drawn, showing the existence of a direct cell-contact communication leading to an endocytic process likely corresponding to phagocytosis.

Section I

Theoretical Notes

Chapter 1

Mast cells

"Oh they are the vicious cells that cause allergy and make us sneeze and wheeze". That was the standard answer received when an immunologist met a question about the identity and role of mast cell (MC) [1].

It is now clear that the physiological role of MCs extends far beyond allergic response, the initiation and propagation of which was the primary and detrimental role ascribed to these cells ever since the discovery in 1878 by Paul Ehrlich. Following Ehrlich's discovery, research on MC biology developed at an extremely slow pace; it is only in the past decade or so that we have been able to unscramble some of the various biological roles of these cells. This slow progress has been attributed to the nature of MCs themselves [2].

Since MCs were first observed in the connective tissue, Ehrlich had proposed that these cells differentiate from fibroblasts. Later Kitamura and colleagues unequivocally established the hematopoietic origin of MCs [1]. The currently accepted MC ontogeny describes the origin and development of these cells as arising from bone marrow-derived hemopoietic precursors, characterized as $CD34^+$ $ckit^+$ cells, that circulate in the blood and become differentiated after entering vascularized tissues or serosal cavity in which they ultimately will reside [3-4].

Environmental and genetic factors can finely control or "tune" precursor terminal differentiation, giving rise to phenotypically distinct subsets of MCs in different anatomical sites, as well as in different animal species. On the basis of diverse staining properties, it was quickly recognized that rodent MCs fall into two broad categories: mucosal and connective tissue MCs types. These distinct MCs subpopulations can now be further distinguished by several features including function, organ of residence and granule composition, differing degranulation responses to pharmacological stimulation and ability to proliferate in response to parasitic challenge [5-6]. In humans two analogues subsets of MCs have been described that differ on whether their granules contain the protease tryptase alone (MC^T), predominantly found at mucosal sites, or tryptase along with chymase (MC^{TC}) within connective tissue.

However tissue distribution is not as clearly demarcated as in rodents and most human tissues have a mixed population of MC types [7].

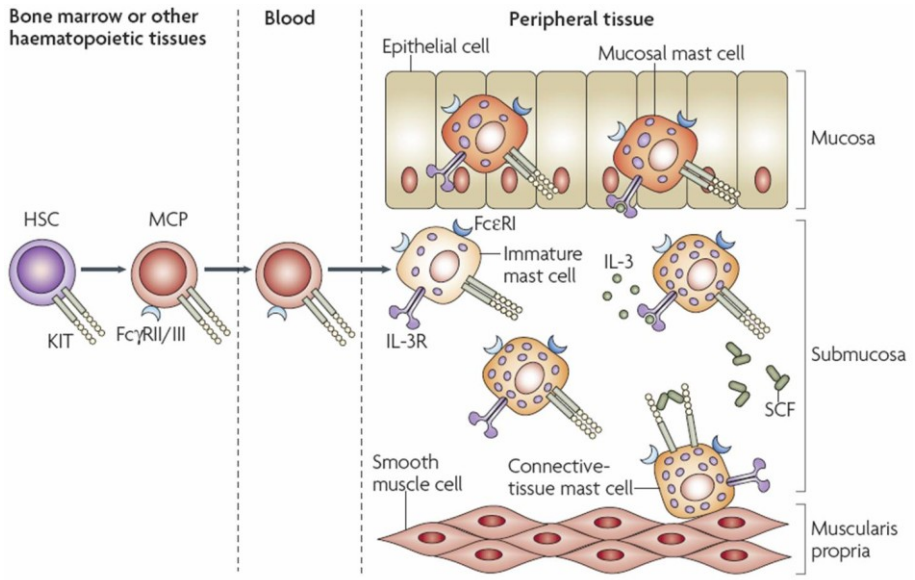

Figure 1.2. MCs differentiation. *Tissue MCs are derived from hemapoietic stem cells (HSCs), which ultimately give rise to MC-progenitors (MCPs). MCPs circulate in the blood and enter the tissues, where they undergo differentiation and maturation. Stem-cell factor (SCF; also known as KIT ligand) is normally required to ensure MCs survival in tissues, but the phenotype of mature MCs can vary depending on the growth-factor milieu and other microenvironmental factors* [8].

1.1 Exocytosis in mast cells

Biochemical and functional heterogeneity probably enables MCs to meet with flexibility the requirements of physiological, immunological, inflammatory or other biological responses that they can encounter at their respective location [9].
A huge repertoire of cell-surface receptors allow MCs to engage in a biphasic inflammatory response and to differentially or selectively respond to environmental danger signals by the secretion of distinct patterns of biologically active mediators (Table 1.1) [10].

Table 1 | MC membrane-bound receptors.

Receptor family	Members	Reference
FcR		
FcεR	FcεRI	Kinet (1999)
FcγR	FcγRI[a], FcγRII, FcγRIII[b]	Malbec and Daëron (2007)
TLR	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10[a]	Marshal et al. (2009)
MHC	MHC class I, MHC class II	Svensson et al. (1997)
Complement receptor	CR1, CR2, CR3, CR4, CR5, C3aR, C5aR	Füreder et al. (1995)
Cytokine receptor	CD117, IL-1R, IL-3R, IL-10R, IL-12R, INFγR, TGFβR	Edling and Hallberg (2007), Moritz et al. (1998), Frossi et al. (2004)
Chemokine receptor	CCR1, CCR3, CCR4, CCR5,CCR7, CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CX3CR1	Juremalm and Nilsson, (2005)
RECEPTOR FOR ENDOGENOUS MOLECULES		
Histamine receptor	H1/H2/H3/H4 receptor	Sander et al. (2006)
Others	Endothelin-1, neurotensin, substance P, PGE2, adenosine	Galli et al. (2005b)
Adhesion molecules	ICAM-1, VCAM, VLA4, CD226 (DNAM-1), Siglec8, CD47, CD300a, CD72	Hudson et al. (2011), Collington et al. (2011), Sick et al. (2009), Bachelet et al. (2006)
CO-STIMULATORY MOLECULES		
TNF/TNFR family members	CD40L, OX40L, 4-1BB, GITR, CD153, Fas, TRAIL-R	Juremalm and Nilsson (2005), Nakae et al. (2006), Nakano et al. (2009)
B7 family member	CD28, ICOSL, PD-L1, PD-L2	
TIM family members	TIM1, TIM3	
Notch family members	Notch1, Notch2	

Table 1.1. MC membrane-bound receptors. *Some molecules have been detected only in studies on human[a] or murine[b] MCs; where not indicated, receptors are expressed in both species* [10]

As Paul Ehrilich noted, "the cell is chiefly of a chemical nature" armed with granules, which are formed and maintained as discrete insoluble structures in the cytoplasm by virtue of charge interactions, containing pre-packaged compounds including proteases, histamine, proteoglycans and cytokines such as tumor necrosis factor (TNF-α) [11].
The earliest reaction of MCs involves the rapid (within seconds to minute) release of above-cited granule contents, a strategy that gives MC-derived products a temporal advantage over those produce by other immune surveillance cells [11]. Such immediate (early phase) release of preformed mediators, referred to as compound exocytosis or anaphylactic degranulation, consists of a rapid and massive secretory process characterized by extensive granule coalescence and extrusion through the plasma membrane that precedes granule secretion in a focused manner (single fusion pore) [12]. Following degranulation, some granule-associated mediators become soluble immediately, whereas most of the structure remains in an insoluble, particulate form. These exocytosed nanoparticles have been physiologically tailored for long-distance delivery of inflammatory mediators which can be released slowly and for a prolonged time [11].

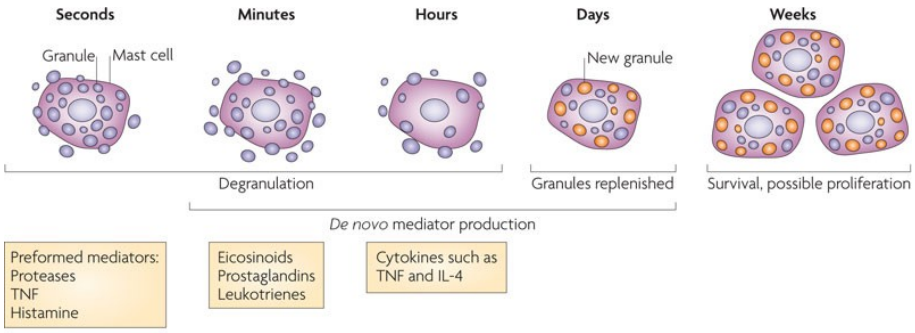

Figure 1.2. Timing of MC responses. MCs can respond quickly to antigen owing to the presence of preformed mediators in cytoplasmic granules that can can be immediately released through the process of degranulation. MCs also begin to produce lipid-derived eicosanoid mediators in the initial minutes of activation. In a second wave of the response MCs release the novo synthetized mediators, including a large number of cytokines. They can also replenish their granules, possibly with altered content, in response to inflammatory signals. MCs are unique in their ability to survive for prolonged period after activation compared with other immune cell types and can survive in tissues and might proliferate in response to appropriate stimuli [11].

An alternative degranulation phenotype, defined by precise ultrastructural criteria, has been frequently observed in MCs infiltrating areas of chronic inflammation or tumours [13]. Termed piecemeal degranulation (PMD) because bit by bit discharge of granule contents, without membrane fusion events and granule opening to the cell exterior, this particulate and selective pattern of cell secretion has been associated with cytokine mobilization and extrusion in MCs, basophils and eosinophils [14]. According to the "shuttling vesicles" hypothesis, during PMD secretory event an outward flow of small cytoplasmic vesicles loaded with "piece" of granule content bud from the perigranule membrane, move through the cytoplasm and fuse with the plasma membrane leading to content discharge [15]. This secretory model received experimental support in a series of elegant ultrastructural studies which conceptualized PMD as a general mechanism for the slow and regulated release of bioactive stored materials [16].
Depending on the mechanism of activation and the strength of the signal, the wide range of possible mediators produced by MCs allows them to follow a degranulation response with the production of factors that are suited to the activating stimulus [17].
The late-phase response involves *de novo* synthesis of adenosine, eicosanoids, and a broad panel of multifunctional cytokines, chemokines and growth factors which can be generated and quickly released after MC stimulation. Few functions (vascular permeability, chemotaxis and local activation of various cells) have been ascribed to each MC-derived factor but more work is needed to determine the individual contribution to host defense as well as any synergistic effect they may have when produce in unique combination [11,18-19].

1.2 Uncovering the journey of information into mast cells

MC exocytosis is a composite process that requires a signal input and a sophisticated molecular machine for output. A molecular architecture is also required for coordination of both early and late signaling events. Not only must the exocytotic machinery be mobilized, but signals must co-ordinately promote the activation of multiple enzymes, generate second messengers, activate specific molecular targets, cause cytoskeletal changes and promote movement and fusion of granules. This coordination is provided by specific protein-protein interactions regulated by the action of kinases, phosphatases, calcium and lipid signals. It is not surprising that this complex and energy demanding process requires fine-tuning by both positive and negative regulators that control the occurrence and the extent of the response [20].

There is an increasing realization that several receptors for different ligands – such as adenosine, complement component, chemokine, cytokines, pathogen-associated molecular patterns (PAMPs), sphingosine 1-phosphate (S1P) and stem cell factor (SCF) - might markedly influence MC activation in a physiological setting (Figure 1.2) potentiating antigen-mediated activation or stimulating, by themselves, the release of MC mediators [21].

Although various triggers can elicit MC exocytotic response [22], studies of MC-signal transduction have mainly be driven by the acknowledged central role of these cells in allergic inflammatory responses following antigen-induced aggregation of IgE-bound high affinity receptors for IgE (FcεRIs) expressed on MC surface.

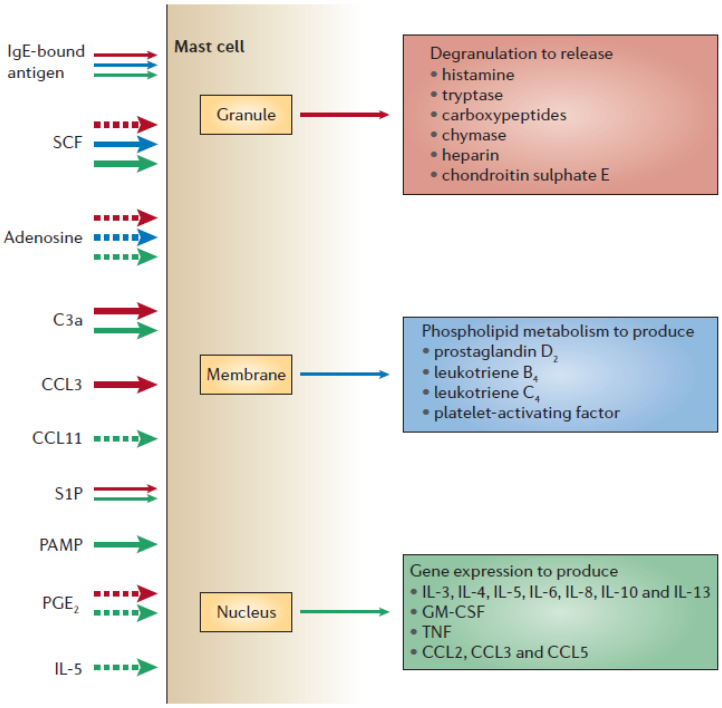

Figure 1.3. Influence of environmental and/or physiological stimuli on the release of pro-inflammatory mediators by mast cells. The colour of the arrows denotes the category of mediator that is released. The solid arrows signify that the agent induces mediator release on its own, whereas the dashed arrows signify that the agent can induce mediator release only in the presence of antigen. The thick arrows signify that the agent increases antigen-mediated responses. C3a, complement component 3a; CCL, CC-chemokine ligand; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; PAMP, pathogen-associated molecular pattern; PGE2, prostaglandin E2; S1P, sphingosine 1-phosphate; SCF, stem-cell factor; TNF, tumour-necrosis factor [21].

1.2.1 A general sketch of FcεRI signaling

The FcεRI is a heterotetrameric receptor with a ligand-binding α subunit, a signal-amplifying membrane-tetra-spanning β subunit and a homodimeric disulfide-linked γ subunit that provides the signaling ability of this receptor [20].

The lack of intrinsic kinase activity of the multimeric FcεRI makes its association with a kinase a prerequisite for transducing signals that elicits MC response [23]. Such interaction relies on the biophysical properties of the surrounding lipid milieu [23-26]. Thus, initial signaling events involve coalescence of the aggregated receptors with specialized microdomains of the plasma membrane

known as lipid rafts [21]. The termed "lipid rafts" conceptually defines highly ordered cholesterol- and sphingolipids-enriched assemblies which appear to act as driving platforms that enable a more effective and specific control of signaling components and may facilitate exocytosis [24,27].
Ligand-induced FcεRI clustering is proposed to stabilize rafts, making easier activation of raftophilic SRC-family kinase Lyn and, subsequentely, tyrosine phosphorylation of the receptor subunits [28-29]. Once phosphorylated, novel binding sites are created where other signaling proteins can bind and propagate signals required for MC effects. Lyn regulates the activation of Syk and the adaptor protein LAT which organizes and coordinates further signal, such as the activation of phospholipase Cγ (PLCγ) and calcium (Ca^{2+}) mobilization [30].
The linear signaling model, initiated by Lyn kinase, has emerged as the paradigm for upstream events after FcεRI engagements [31], yet recent evidence revealed an increasing complexity underling the presumed simplicity of MC response.

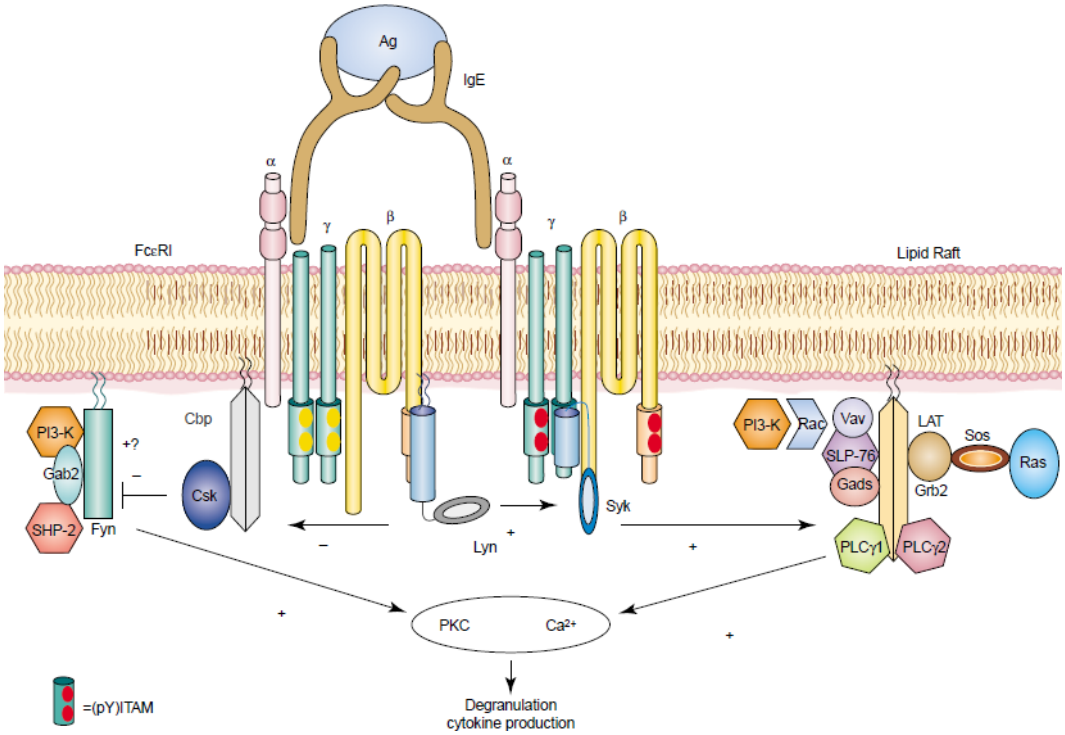

Figure 1.4. Scheme depicting major molecules and events in MC activation *A model of structure and functional coupling of the FcεRI to early signaling events (Blank 2004).*

The first direct evidence for the existence of a complementary signaling pathway for MC activation came from the laboratory of Juan Rivera. His group showed that FcεRI aggregation triggers a second Src family kinase, Fyn, which is an essential element for the positive control of FcεRI-induced MC degranulation through its role in regulating activation of phosphatidylinositol 3-OH kinase (PI3K) downstream the phosphorylation of the adaptor molecule GAB2 [32].
Fyn and Lyn kinases synergize in late events phosphorylating numerous targets and activating several signaling pathways, including the PI3K, PLC/Ca^{2+}, and several MAPK pathways, promoting and controlling the rate and the extent of MC degranulation and cytokine production [33].
These processes depend on a biphasic increase in intracellular Ca^{2+} concentration owing to an initial transient release from endoplasmatic reticulum (ER) stores, mediated by the PLCγ signaling pathway and followed by a more sustained Ca^{2+} entry across the plasma membrane through store-operated Ca^{2+} channels (SOCs) [34-35]. The Ca^{2+} influx mechanism was originally shown by Putney and colleagues to depend critically on the empty of luminal Ca^{2+} from the ER [36]. Chief component in such interplay is STIM1, the Ca^{2+} sensor responsible for communicating the depleted state of intracellular compartments to SOCs [37-38]. In quiescent cells STIM1 is distributed homogenously throughout the ER, but it relocates

upon release of Ca^{2+} from ER stores to distinct puncta on the ER in close proximity to the plasma membrane where it activates members of the Orai family of SOCs promoting the opening of Ca^{2+} channels [39].

Additional complexity in the Ca^{2+} apparatus has been recognized by the demonstration that SOCs appear to work synergistically and associate with canonical transient receptor potential channels (TRPCs) for optimal FcεRI-induced Ca^{2+} influx and degranulation [40-42].

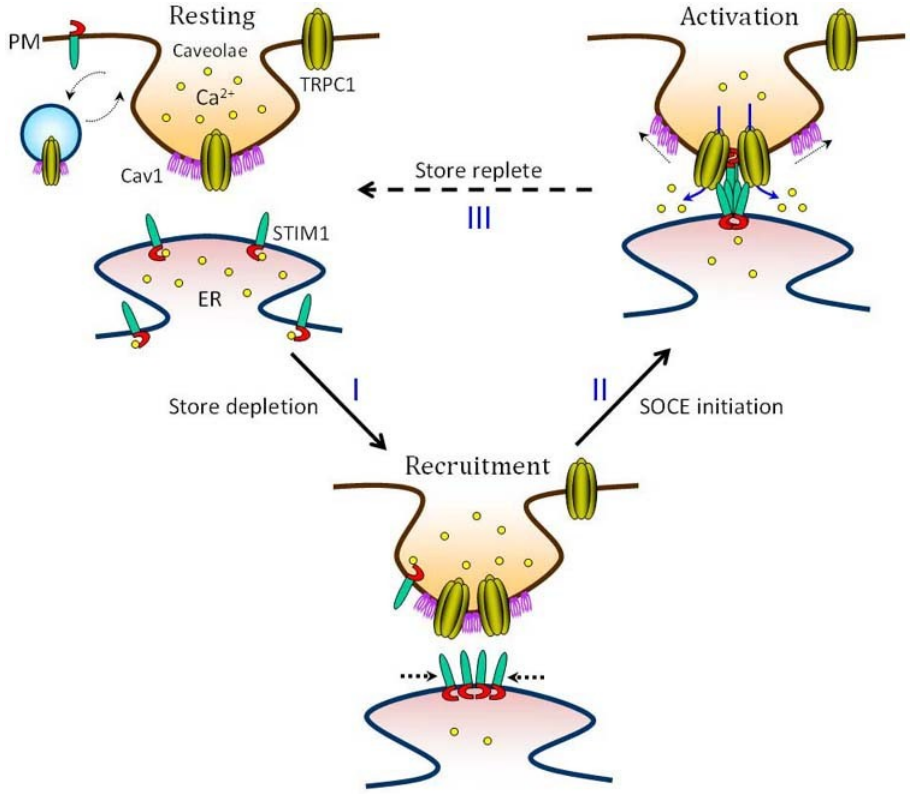

Figure 1.5. Steps in Ca^{2+} channel activation. *This model shows the compartmentalization of TRPC1-mediated Ca^{2+} influx at ER-raft juxtaposed microdomains (indicated as Caveolin1 (Cav1)-enriched domains). In "resting" state (ER-stores filled) STIM1, predominantly in the ER, is bound to Ca^{2+} and displays a diffused localization pattern; between resting and activation state, there is an intermediate step of channel "recruitment", wherein, following ER Ca^{2+} store-depletion (step-I), in which STIM1 unbinds Ca^{2+} and engages in oligomeric cluster (puncta) formation. The channel "Activation" is marked by SOC initiation (step II) where STIM1 interacts with TRPC1 and activates the channel resulting in the increase of Ca^{2+} influx. Ca^{2+} is then sequestered back to the ER to refill the ER-stores as indicated by store replete (step III), following which STIM1 binds Ca^{2+} and dissociates from TRPC1 getting back to the resting state. As the filled status of ER controls the STIM1-puncta kinetics it also determines the dynamic and reversible STIM1-TRPC1 associations. The molecular events ensuing each step (step I through step III) outlined in this model may not necessarily reflect their exact physiological sequence and further investigation in this aspect is necessary*[43].

Downstream of the early FcεRI-induced signaling events, the final stages of MC activation require membrane fusion events. Essential to granules exocytosis are SNARE (soluble-*N*-ethyl-maleimide-sensitive factor-attachment protein receptors) proteins that lie on opposing cellular membranes to form a stable multimeric complex that catalyzes fusion in response to elevated cytosolic Ca^{2+} concentration [27,44]. Several accessory proteins, such as NSF ATPase, Rab GTPases and Munc family members, are involved in different exocytotic trafficking steps, regulating the fusion-competent state of SNAREs [20]. The fusion efficiency also rely on the interaction of fusion machinery with the cytoskeleton inasmuch granules dynamics is totally dependent on microtubule network changes [45].

Several studies with MC knock-out mice have suggested that Fyn and its downstream substrates play an essential role in FcεRI-mediated MC degranulation [46-47]. Conversely, Lyn-deficient mice show hyperresponsive phenotype correlated with hyperactivation of Fyn kinase. Therefore, a model can be envisioned in which Lyn-mediated signals set a threshold for MC functions, whereas Fyn-mediated signals are necessarily required for degranulation [47].

The integration and regulation of the aforementioned signaling pathways is essential toward determining the type, duration and extent of MC response. Multiple molecular checks and balances provide an intrinsic regulatory network in which molecules act coordinately to achieve the desired response and limit the possible injurious effect of a persistent or excessive response [23,48].

1.3 The riddle of the mast cell

MCs have been slowly but definitely shed out their image of allergy-causing troublemakers and have emerged as versatile cells with the ability to orchestrate several biological processes [49].
Actually, genetic approaches probably represent the more definitive way to identify and characterize MC functions. Multiple model systems offer a wealth of opportunities to enhance progress in defining MC immunological and non-immunological roles. Despite several caveats with their use, researches with such models have suggested physiological roles of MCs in epithelial, endothelial and nervous system as well as in the regulation of immunity. These functions of MCs can also contribute to the pathology associated with many different diseases [50].

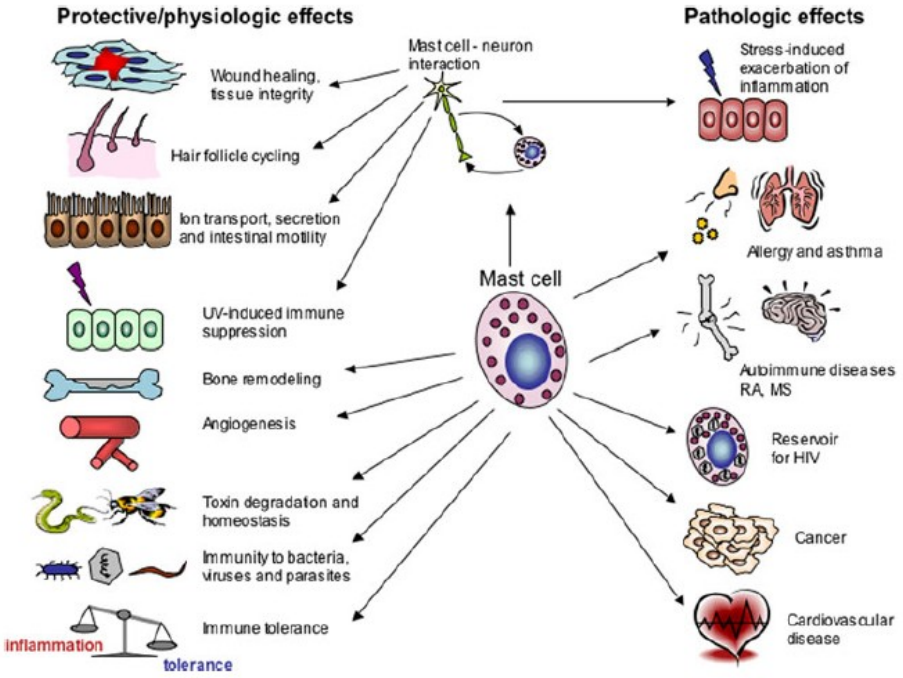

Figure 1.6. MCs in health and disease. *MCs are involved in physiological processes and maintenance of homeostasis, in addition to playing a critical role in host defense. However, these cells are also one of the main culprits involved in the pathogenesis of autoimmune and cardiovascular diseases and cancer* [1].

1.3.1 "Buddy" mast cells

The strategic position of MCs at many sites allows them a role in physiological homeostasis. Given their association with blood and lymphatic vessels, epithelial surfaces and smooth muscle, MCs are critical for the maintenance of tissue integrity and function, and it is not surprising that their mediators influence flow, permeability and contraction in many sites [51]. MC-secreted products are known to be involved in all phases of wound healing, including the initial inflammatory response followed by reepithelialisation and revascularization of the damaged tissue, and finally the deposition of collagen and remodelling of the matrix. In certain situations of wound healing and stress response MCs work cooperatively with neurons as a functional unit to affect various physiological conditions [7].
MCs are also important in hair follicle cycling and bone remodelling. MC-derived histamine, TNF and substance P are implicated in regulating development and regression of hair follicle between periods

of growth and rest. It has been speculated that MC products could also influence osteoclast recruitment and development. Recently MCs were found to be source of osteopontin, a glycoprotein component of bone matrix that contributes to bone resorption and calcification, one potential molecular mechanism for MC activities in bone metabolism [1].

1.3.2 "Watchdogs" mast cells

Beyond their physiologic functions, MCs are widely distributed at the host's interfaces with the environment as capable "watchdogs" to detect and respond to pathogens or to shape immune responses [1]. They have the ability not only to affect immediate innate processes for pathogen containment and/or clearance, but also to influence long term host programmes of defence. MCs have a kinetic advantage over other sentinel cells in initiating both innate and adaptive immune responses through their ability to store preformed mediators and release them nearly instantaneously into a site of infection. Even so, exocytosed granules seem to function as long distance delivery devices for their cargo, including inflammatory mediators [11]. The first strong evidence that MCs function in a protective capacity against infectious disease came from studies of host-parasite infections, and an increasing amount of work supports their essential contribution to control a wide range of pathogenic infections, including those by parasites, bacteria and viruses [49]. Direct pathogen recognition by MCs occurs both in response to factors that are common to classes of pathogen (such as TLRs) and those that are specific to only certain infectious challenge (such as through binding of antibodies specific for pathogen associated epitopes) [52]. Several MC functions have been implicated in host defence. The ability of MCs to orchestrate complex cellular migration within a tissue is a key mechanism by which they are essential to limit the pathology associated with infections. What is more, phagocytosis-dependent bactericidal activities, extracellular trap formations, and proteolytic degradation of toxic substances, such as the endogenous peptide endothelin-1 or snake- and honeybee-produced venom toxins, have also been proposed as potential tricks by which MCs exert protective functions [11].
MCs' peculiarities endow them with a superior ability to fine-tune themselves to various environmental cues, to react immediately by releasing a wide spectrum of mediators and to alert other components of the immune system. The same features also place them in a wobbly position where improper regulation of their functions can wreak havoc to the host [1].

1.3.3 "Cantankerous" mast cells

Many of MC products are best known for their association with detrimental conditions such as asthma, allergy and anaphylaxis, in which aberrant, chronic or systemic activation of MCs promotes harmful inflammatory sequelae and damage of host tissues [50].

Ag- and IgE-dependent MC activation is widely regarded to be a, if not the, major initiator of the clinical signs and symptoms that are induced rapidly after the exposure of sensitized, allergic individuals to small amounts of specific antigen. The immediate release of MC mediators causes most of the pathology associated with allergy including vascular permeabilization, smooth muscle contraction and induction of mucus secretion [50]. The extent of allergic symptoms is highlighted in anaphylaxis, a catastrophic and sometimes fatal systemic reaction to an otherwise innocuous antigen, that arguably represents the most striking imbalance between the cost and the benefit of an immune response. Allergic diseases do not consist only of "early responses", but also of subsequent events summarized as late-phase reactions that facultatively occur following the immediate acute phase, and that are thought to cause the recurrent and chronic symptoms of allergic individuals. Findings from human studies and work in MC-knock in mice indicate that MCs can also contribute to later consequences of allergen exposure, by promoting local inflammation and by directly or indirectly enhancing certain aspects of tissue remodeling [49].

Dysregulated activation of MCs through mechanism independent of IgE (such as IgG or IgM autoantibodies, immune complexes, and TLRs) has been implicated in several autoimmune disorders,

such as bullous pemphigoid, rheumatoid arthritis, multiple sclerosis and its experimental counterpart. The correlation seen in MC hyperplasia and increased MC products at sites of tissue injury, although circumstantial, suggests MC contribution to these disease. The exact mechanism by which MCs mediate the pathogenesis is unknown, anyhow it has become clear that MC dysfunction not only aids initially in breaking self-tolerance, but also in perpetuating the inflammation [1-2,7].

There is an increase in our awareness of the role plaied by MCs in the development of a variety of chronic inflammatory disorders, including cardiovascular disease. MCs reside in heart, where they are strategically located in close association with blood vessels and nerves, prompting the speculation that they can affect cardiac functions. Despite a lot of correlative evidence, there have been few studies directly implicating MCs in cardiac dysfunction. MCs have been shown to mediate cardiac hypertrophy and fibrosis in aortic constriction and abnormal aortic aneurysm. Other evidence also suggest MC contribution to the pathogenesis of atherogenesis, by the release of proinflammatory cytokines (IL-6 and interferon-γ) that augment the expression of matrix-degrading proteases [1].

Among the specific observation of Ehrlich that still have relevance today was the abundance of MCs in tumours, particularly carcinomas, where MCs are typically found to accumulate at the periphery of nodules but also within tumour lesions. Later studies provide ample evidence for increased populations of MCs in such tumours as mammary adenocarcinomas, basal cell carcinoma, melanomas, neurofibromatosis, and Hodgkin's lymphoma [52]. One focus of interest is the possible involvement of MCs in the inflammation associated with malignancy. Depending on the type of tumour, inflammation may exist before malignant changes or be induced with the onset of malignancy but in either case inflammation may be conducive to tumour growth. There is evidence for MCs both in promoting, but also in protecting against tumour growth. Up to now, much of the data linking tumour growth, or regression to specific MCs factors is circumstantial and controversial. These discordant findings have been attributed to variable contributions of MCs to different phases of tumour growth and the types of experimental model used [7,52].

Chapter 2

The Mast cells' Social Network

A correct communication is the essential component of the functional integration in the immune system [53].
The onset and regulation of a specific immune response result from a complex, multistep process largely shaped by direct or soluble factors-mediated interplay [53].
Besides paracrine communication, which is by its nature never informative or precise [54], the immune cells specialize in establishing reliable, wideband direct interaction between themselves. The stable, flattened interface between cells which are in process of recognizing is defined immunological synapse. This term fits the activation of immune cells in the context of highly organized and dynamic structure that can act as a platform for bidirectional and cell-specific flow of information, and that might offer additional tool for modulation of a cell' response [55-56]. Key aspect to the regulation of intercellular contacts is the extent to which surface receptors are made accessible to their binding partners which may be influenced by the dynamic membrane morphology at immunological synapses. Indeed, it is certain that intercellular contacts do not merely comprise two facing flat membranes. Filopodial contacts and membrane ruffles may greatly augment ligand-receptor interaction across the synaptic cleft and promote conjugation [57]. Thus, to get efficient cell-cell interaction, a migrating cell undergoes morphological changes, recruits cell-surface receptors and signaling components to the region of cell-cell contact and receives signals that are transduced intracellularly leading to the activation of nuclear factors and the transcription of specific genes. The type and amount of information exchanged is determined by the duration and physical dynamics of the interaction, the identity of the receptors and signaling molecules that are engaged and recruited, the strength of the signals transmitted and presence or absence of secretion. These functions depend

on the activation state of both the cell types, as well as the type of the environment in which the interaction takes place [55].

2.1 MAST CELLular partners

Over the past decades, a new picture of MCs function is gradually emerging; these cells have currently gained recognition as true immune effector cells in a variety of setting during innate and adaptive immune responses, but have also the potential to positively or negatively modulate many aspects of immune cells, including granulocytes, monocytes/macrophages, dendritic cells, T cells, B cells, natural killer cells (Figure 2.1) [8,10]. MCs are traditionally thought of as a secretory cell type, influencing recruitment, survival, development, phenotype or function of immune cells through the release of soluble mediators or the delivery of their cargo through binding and uptake of membrane-bound vesicles (exosomes) [8,58]. An intriguing and not yet fully clarified aspect of MC biology concerns their physical interaction with other cellular partners. MCs express a plethora of membrane molecules that can potentially empower these crosstalk directly. Several classes of costimulatory pathways have been identified and characterized for MCs, each able to operate in a specific physiological condition or disease, setting an array of thresholds to ensure a highly regulated response (Figure 2.2) [54].

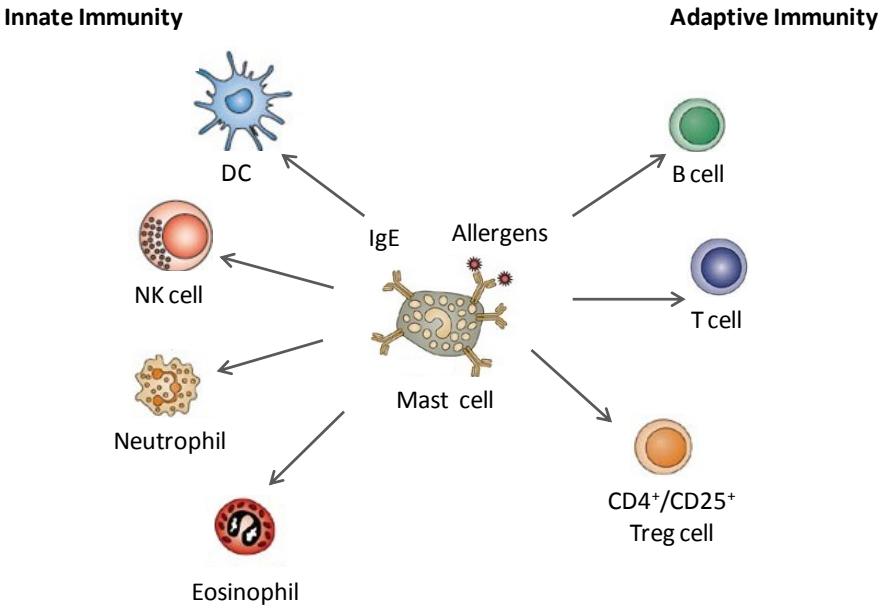

Figure 2.1. The Mast cells' social network. Sketch of MC interactions with cells of both innate and adaptive immune response.

2.1.1MCs and innate immune cells

2.1.1.1 MCs and Dendritic Cells

One of the key immunoregulatory facets of MC function is their capacity to control dendritic cells (DCs) behavior.

Up to now, a considerable degree of heterogeneity in DC population has complicated decoding the attempts to shed light on the network of interaction with MCs subtypes. MC-secreted histamine, TNF-α and lipid mediators have been widely implicated in the processes of DC mobilization from tissue to secondary lymphoid organs [59-61], DC maturation [62-63] and DC capacity to promote T cell responses [63-65]. On the other hand, only some clues on direct MC-DC interplay have been uncovered.

In an *in vitro* cultured human system the direct binding of cord blood derived-MCs with human monocyte-derived DCs is required for the optimal induction of Th2-promoting DCs [63]. Moreover, it has

been shown that resting murine peritoneal MCs (PCMCs) can prime DCs, in a contact-dependent fashion, to promote T cell proliferation and polarization toward Th1 and Th17 responses [64]. It also appears that murine bone marrow MCs (BMMCs) further the maturation and chemotactic activity of bone marrow DCs during the sensitization phase of contact hypersensitivity response. BMMC-DC interactions take place through intracellular adhesion molecule (ICAM)-1 and lymphocyte function-associated antigen (LFA-1) as well as membrane bound TNF-α-dependent pathways. An influx of Ca^{2+} in BMMCs is induced upon direct interaction with activated DCs that express high level of ICAM-1, while, on the other hand, this physical interaction enhances DC expression of CD40, CD80, CD86 and CCR7 co-stimulatory molecules, thus promoting their maturation and chemotaxis [66].

2.1.1.2 MCs and Natural Killer cells

Natural Killer (NK) cells are granular cytotoxic and circulating lymphocytes that participate in the early control of microbial infections and cancer. In the context of innate response, immune surveillance exerted by MCs is important for the selective chemotaxis of NK cells in different disease models [67-69]. Several MC-mediators, such as adenosine, IL-4, IL-12 and TNF-α, are also capable to modulate NK cell activation. Only recently, a cell-proximity and TLR-dependent mechanism through which MCs can directly induce NK cell cytotoxicity has been described. This functional interaction is partly mediated by OX40 ligand on MCs [70].

2.1.1.3 MCs and Eosinophils

Two main effector cells recognized for their role in orchestrating the acute and chronic phases of allergic reaction are MCs and eosinophils (Eos). Nevertheless, a clear interplay between MCs and eosinophils has been proven not only in allergic inflammatory tissues [71-72], but also in gastric carcinoma [73], chronic gastritis [74], Crohn's disease and Ascaris infection [75].
Eosinophils and MCs may mutually influence each other functions in a combined paracrine and physical manner. This interaction is stable, tight and physiologically relevant, as demonstrated by the high rates of MC-Eos coupling detected in human and murine systems [71,76-77]. Several ligand/receptors pairs are implicated in contact-dependent communication. Examples of such interface are the relationship between CD48 and 2B4 [76], LFA1 and ICAM1[76], CD226 and CD112 [54] which convey costimulatory signaling switches for reciprocal cell activation .

2.1.1.4 MCs and Neutrophils

Neutrophils or polymorphonuclear lymphocytes (PMNs) are essential innate immune cells which determine the host's resistance against infection or inflammation acting for containment and clearance of infectious particles [78]. Nevertheless, in contrast to the traditional view of these cells as first line of defense, there is increasing evidence for their effector and regulatory functions in different steps of immune responses [79-80].
Several studies pointed out that MC-regulated PMNs recruitment is essential for mounting a rapid and efficient innate immune response [81-84]. Till now, MC-PMN interaction has been merely described as a soluble factors-mediated interplay and possible receptor/ligand pairs that might physically mediate this crosstalk have not yet been described.

2.1.2 MC and adaptive immune cells

2.1.2.1 MCs and B cells

Certain MC population produce several mediators, such as IL-4, IL-5, IL-6 and IL-13, that are known to regulate B cell development and function. The first evidence of an effective direct MC-B cells crosstalk

was reported by Gauchat and coworkers. Since then, several studies demonstrated the importance of physical contact and in particular of CD40/CD40L axis, in modulating MC-mediated B cell-specific antibody responses in different settings [85-87];
A more recently studied mechanism, by which MCs exhibit their regulatory effect on B cells, is dependent on the release of exosomes. Heterogeneous in size and shape and stored in cytoplasmic granules, mast cell-derived exosomes contain a pool of MHC class II-, costimulatory- and adhesion-related molecules and are released during the process of exocytosis. Those antigen-containing exosomes interact with B cells in an leukocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1)-dependent way [58].
Only recently the study of the specific role of MCs in B-cell growth and differentiation has been investigated more in detail. Merluzzi and coworkers proved that both resting and activated MCs are able to induce a significant inhibition of cell death and an increase in proliferation of naïve and activated B cells. This effect relies both on cell-cell contact and MC-soluble factors. CD40-CD40L signaling, together with MC-derived IL-6, are also involved in the differentiation of B cells into $CD138^+$ plasma cells and in selective IgA secretion [88].

2.1.2.2 MCs and effector T cells

The functional interplay between MCs and T cells has been suggested by several studies documenting their close physical apposition during T cell-mediated inflammatory processes [89], as observed in cutaneous delayed-type hypersensitivity [90-91], graft-versus-host reactions, sarcoidosis [92] and in chronic inflammatory processes associated with the pathology of inflammatory bowel disease and rheumatoid arthritis [93-94]. Additional morphological studies have revealed that MCs and T cells co-localize in inflamed allergic tissues and at sites of parasitic infections [95-96].
Much of the influences between MCs and T cells have been attributed to the biological effects of a wide range of soluble mediators; however, increasing amount of literature documents recognizes the importance of intercellular communication involving the coupling of cell surface molecules.
Early studies demonstrated that intercellular contact between MCs and T cell lines is able to activate MC transcription machinery [97]. Adhesion of activated T lymphocytes, T cell membranes or microparticles also results in the release of several inflammatory factors by human MCs as well as MCs adhesion to endothelial cell receptors or extracellular matrix ligands [98-100].
The first membrane bound pathway involved in MC-T cell crosstalk to be described was the adhesion pathway mediated by LFA-1 and its ligand ICAM-1, which induced FcεRI-dependent murine BMMCs degranulation after heterotypic aggregation with activated T cells [101]. Only in 2004, the lymphotoxin-β receptor (LTβR) expressed on murine BMMCs was described to be triggered by LTβR ligands expressed by T cell lines and to convey a costimulatory signal leading to the release of cytokines (IL-4, IL-6, TNF-α) and chemokines (CXCL-2 and CCL5) from ionomycin-activated BMMCs [102].
Various mechanisms underlying MC ability to interact with T cells, yet the engagement of OX40 on activated $CD4^+$ T cells by OX40L-expressing MCs is still the most studied.
OX40 triggering, together with secretion of soluble MC-derived TNF-α, co-stimulate proliferation and cytokine production from activated $CD4^+$ T cells [103]. Similar results were also established in a culture system of human tonsillar MCs and human T cells [104].
One satisfying description of functional MC-T cells interaction arises from the observation the MCs can serve as unconventional antigen presenting cells for T lymphocytes [105]. A recent work provides experimental morphological evidence of direct antigen presentation by murine peritoneal cell-derived MCs and freshly isolated peritoneal MCs at a single cell level, eliciting functional responses in effector T cells, but not in their naïve counterparts [106]. MHC-II-dependent antigen presentation to $CD4^+$ T cells by MCs was also demonstrated in rat and human cell systems [107-108]. More recently, the possibility that MCs can be primed to acquire APC phenotype has been proposed: inducible expression of MHC-II molecules, MHC-II associated molecules as well as OX40L and PD-L1, by murine BMMCs, spleen-derived MCs and peritoneal MCs has been reported to occur in response to various *in vitro* treatments

[106,109-110]. MHC-II expression granted MCs the ability to effectively support T cell proliferation and effector functions and cause expansion of T regulatory cells (Tregs) [109].
As for $CD4^+$ T cells, MCs are capable of inducing antigen-specific $CD8^+$ T cell responses *in vitro* and *in vivo*. Murine BMMCs can process antigen from phagocytosed bacteria for presentation via MHC class I molecules to T cells [111], giving rise to $CD8^+$ T cell proliferation, cytotoxic potential and degranulation. In turn, $CD8^+$ T cells induce MHC class I and 4-1BB expression on BMMCs as well as the secretion of osteopontin [112].

2.1.2.3 MCs and regulatory T cells

One should keep in mind that a significant proportion of T cells specialize in regulation and suppression of the inflammatory process. This sub-population, known as regulatory T cells (Tregs), may appear in several forms, mostly characterized by the expression of CD4, CD25 and the transcription factor Foxp3.
The collaboration between Tregs and MCs in suppressing inflammatory responses has been suggested by several reports. These two cell-types reside in close proximity in secondary lymphoid tissues as well as in mucosal and tolerant tissues influencing each others' function [113-115].
Studies of several disease models revealed that Tregs can attract MCs by producing considerable amounts of IL-9, a MC growth and activation factor [115-118]. In a mouse model of chronic allergic dermatitis, MC-derived IL-2 contributes to Tregs proliferation and suppressive function at the site of inflammation [119]. However, MCs may also act to for counteraction of Tregs suppressive activity. Change in the suppressive phenotype of Tregs has been suggested in mouse models of hereditary colon cancer and autoimmune encephalomyelitis. In these inflammatory settings MCs, in presence of conventional T cells, break peripheral tolerance promoting Th17 skewing of Treg cells with loss of both Foxp3 expression and T cell suppressive properties [120-121] by contact-dependent mechanism and production of soluble factors [121]. Another mechanism for counteraction of Treg suppressive activity has also been suggested by the binding of MC-derived histamine to H1 receptors on Tregs [122].
Conversely, the paradigm of MCs as targets of Treg-mediated suppression has been rigorously investigated in the setting of immediate hypersensitivity-allergic responses. Tregs were shown to have the capacity to inhibit FcεRI expression on MCs *in vitro*, requiring contact between the two cell types [123]. Along this line, co-culture of Treg cells with murine BMMC suppresses degranulation, but primes MCs for production of IL-6 via contact-dependent surface-bound TGF-β mechanism [124]. Interestingly, in a model of colorectal cancer, highly suppressive Tregs lose the ability to suppress human LAD2 MC degranulation [125], suggesting that a complex MC-Treg interaction within tumor microenvironment exist, although the mechanism behind these events has not been yet discovered.
The physiological outcome of MC suppression was confirmed by a recent study documenting Treg-abrogation of BMMCs degranulation and immediate hypersensitivity response. This effect was achieved by Treg-MC contact leading to OX40-OX40L engagement, respectively. Suppression was associated with increased cyclic adenosine monophosphate (cAMP) and decreased Ca^{2+} influx, unrelated to PLC-γ2 or intracellular Ca^{2+} stores. These observations have led to the finding that soluble OX40 is sufficient to mimic the inhibitory effects of Tregs, providing a potentially novel therapeutic approach in allergic disease [126]. The engagement of OX40L selectively suppresses Fyn-initiated signals underling the dampening of MC response. In the proposed model OX40L triggering acts to "fine tune" FcεRI-stimulated MC degranulation, modifying lipid rafts organization and causing a considerable inactivation of downstream Fyn-dependent proteins Gab2, PI3K, Akt and RhoA [127].

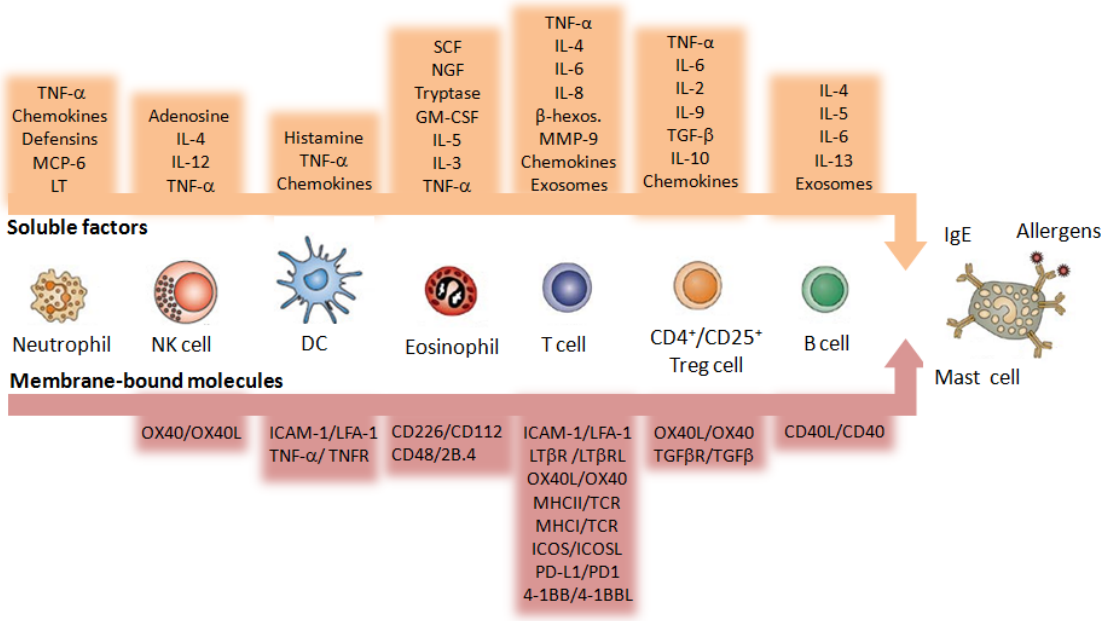

Figure 2.2. A schematic drawing of soluble factors and membrane-bound molecules involved in MC interaction with other immune cells. *MC-derived soluble mediators and ligand/receptor pairs identified for influencing recruitment, survival, development, phenotype or function of immune cells.*

Section II

Aim of the study

Chapter 3

Aim of the study

Over the past decades, a new picture of MCs function is gradually emerging; these cells have currently gained recognition as true immune effector cells in a variety of settings during innate and adaptive immune responses, but have also the potential to positively or negatively modulate many aspects of immune cells [10].

Besides paracrine communication, it is now clear that onset and regulation of a specific immune response is highly complex and requires immune cells to be specially adapted to interact directly with other cells during development and priming, as well as to function where they are needed [57]. Communication between cells can result in different modes of interaction (with respect to morphology and duration) and leads to different outcome. The type and amount of information that is exchanged is determined by the duration of interaction, physical dynamics, identity of receptors and signaling molecules that are engaged and signals that are transmitted, depending on the activation state of both the cell types, as well as the type of the environment in which the interaction takes place [55].

As MCs are traditionally thought of as a secretory cell type, an intriguing and not yet fully clarified aspect of MC biology concerns their physical interaction with other cellular partners.

This work aims to shed light on MC range of contact modes, starting to define a molecular code, in which differences in timing, spacing and molecular composition of the signaling platform determine the outcome of "MAST CELLular interactions". Distinct aspects of the interactions with different immune cells have been addressed.

Firstly the morphological features and functional profile of MC-Treg synapses have been extensively described, given few additional information on the modes of tuning such crosstalk and on the molecular mechanisms governing the fate of Ca^{2+} flux in MC interacting with Tregs. Secondly, an overview of the regulation of MC-B cell contacts under different stimulation conditions was provided.

Finally, a fresh picture of the functional dynamics of MC-PMN interactions has been drawn.

Section III

Results and Discussion

Chapter 4

MC-Treg interaction

4.1 Dynamics of MC-Treg interaction

Beyond the paracrine communication exerted by cytokines, MCs express a plethora of membrane molecules that can potentially empower their interaction with other cellular partners in a specific physiological condition or disease, setting an array of thresholds to ensure a highly regulated response[10]. Considerable progress in understanding the importance of physical contact and cell surface receptor was yielded by the discovery that bone marrow MCs (BMMCs) can functionally interact with $CD4^+/CD25^+$ Tregs by a contact-dependent mechanism requiring OX40-OX40L axis [113]. Although a great deal is known about the functional outcome of such communication, there is scant information regarding the single cell dynamics of this process.

4.1.1. Imaging MC-Treg interactions

To describe cell demeanor and interactions, real time imaging was performed. By time lapse bright field video microscopy the formation of conjugates between IgE-pre-sensitized murine BMMCs and $CD4^+/CD25^+$ Tregs was analyzed. The time series started after antigen (Ag)-addition and cell behavior was observed every minute for a total of 30 minutes. Each cell type was distinguished by its unique morphological characteristics. BMMCs were large (about 15-20 μm) and round, while Tregs were smaller (8-10 μm) with tiny cytoplasm. Numerous cells-cell contacts were observed (Figure 4.1A).
Under resting conditions both BMMCs and T cells were typically rounded, while during cell-cell contact both cell types became elongated and flattened (Figure 4.1B). Early BMMC tethering failed to result in firm adhesion; BMMC moved across the T cell, forming a mobile junction with a dynamic contact

plane (not shown). Individual interactions showed sequential phases of adhesion, slow later movement and dynamic crawling in different proportions and duration.

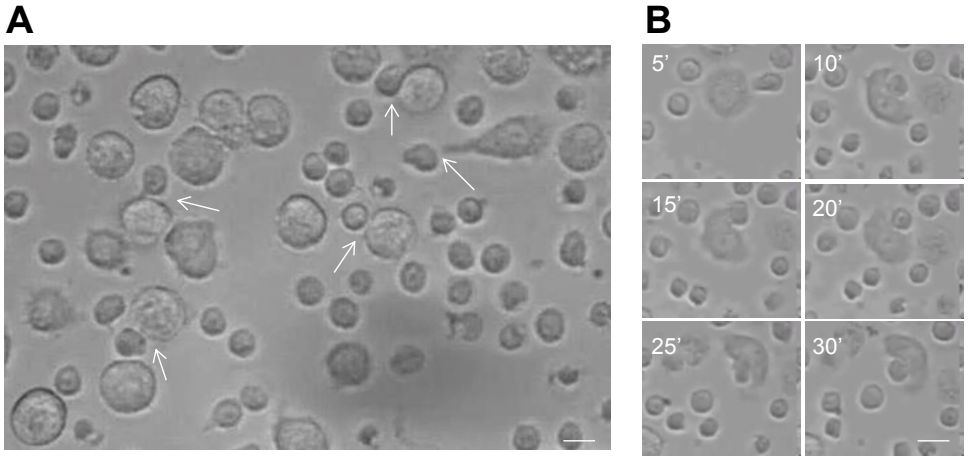

Figure 4.1. Imaging of MC-Treg interaction. (A) Equal number of pre-sensitized BMMCs and Tregs were seeded onto glass bottom Petri dishes and MCs were challenged with Ag (DNP-HSA). Arrows show contacts between MCs and Tregs. (B) Representative images of conjugate formation between BMMCs and Tregs monitored by time lapse video microscopy for 30 minutes. Bars = 10 μm

4.1.2 Similar interaction between different types of MCs and $CD4^+/CD25^+$ Tregs

As MCs heterogeneity has emerged as a fundamental principle for the understanding of the possible role of MCs in health and disease, the functional crosstalk between Tregs and different types of MCs has been characterized. Both in rodent and human, mucosal and connective tissue types of MCs are documented, based on different phenotypical, biochemical and functional properties [128-129].

4.1.2.1 Kinetics of conjugate formation by different MC types

BMMCs are often considered an *in vitro* equivalent of immature or mucosal MC phenotype; likewise, peritoneal MCs (PMCs) are currently recognized as a model of mature tissue resident MCs with features of connective MCs.

To further support the crucial role of Tregs in limiting the MC degranulation response, PMCs were purified and the conjugate formation in the presence of Tregs was evaluated. Moreover, the study was extended to human samples performing experiments using human $CD4^+/CD25^+$ Treg and the human LAD2 MC line.

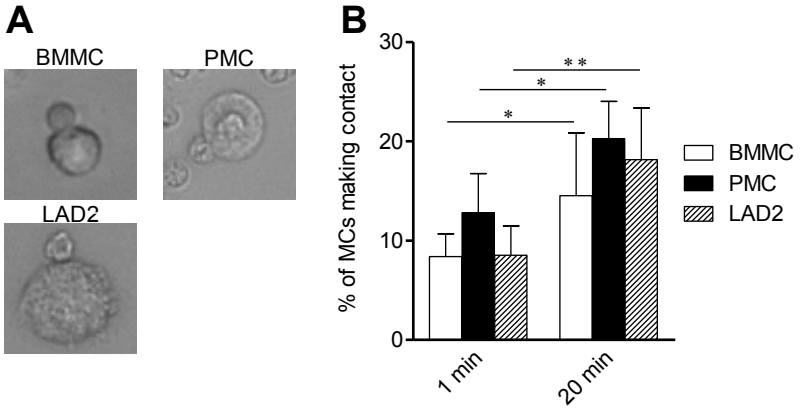

Figure 4.2. Kinetics of conjugate formation between $CD4^+/CD25^+$ Tregs and different MC types. (A) Representative images of conjugate formation between BMMC or PMC with $CD4^+/CD25^+$ Treg and between LAD2 with human $CD4^+/CD25^+$ Treg. (B) Percentages of MCs making contact with Tregs at 1 and 20 minutes after Ag (100 ng/ml) addition. Data from 2 independent experiments, were collected: n=337 ± 20.5, n=341 ± 0.7, n=286 ± 17.7 for BMMCs, PMCs and LAD2 cells, respectively. Shown are the means ± SD of 3 independent experiments, each performed in duplicate. * $p<0.05$ and ** $p<0.005$.

As depicted in figure 4.2A the $CD4^+/CD25^+$ T cell population efficiently made contact with both BMMCs and PMCs and, interestingly, similar conjugate formations were observed using human MCs and $CD4^+/CD25^+$ T cells. Percentages of MC-Treg contacts early after Ag addition were similar in both murine and human cell co-cultures (8% ± 2.3, 12% ± 3.9 and 8% ± 2.9 for BMMCs, PMCs and LAD2 respectively) and increased 20 minutes after FcεRI triggering (14% ± 6.3, 20% ± 3.8 and 18% ± 5.2 for BMMCs, PMCs and LAD2 respectively) (Figure 4.2B).

4.1.2.2. Inhibitory role of $CD4^+/CD25^+$ Treg cells on MC degranulation

Degranulation was evaluated by measuring the release of the MC granule-associated enzyme β-hexosaminidase. In agreement with previous findings, as shown in figure 4.3, $CD4^+/CD25^+$ Tregs co-cultured with both murine and human MCs showed similar ability to inhibit the MC compound exocytic response. The unchanged Treg suppressive function provides unequivocal proof that this regulatory mechanism is conserved across species

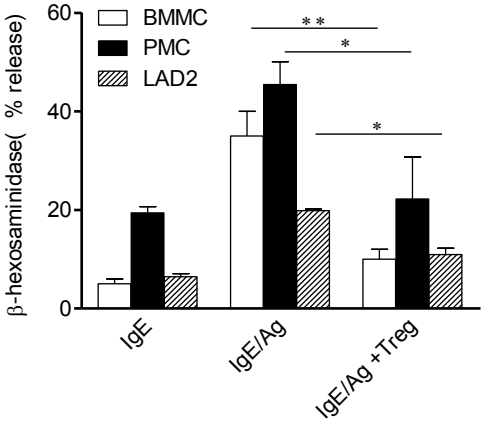

Figure 4.3. Inhibitory role of $CD4^+/CD25^+$ Tregs on MC degranulation. *Activated MCs alone or in presence of Tregs were examined for release of β-hexosaminidase expressed as percentage of the cells' total mediator content. Shown are the means ± SD of 3 independent experiments, each performed in duplicate. * p<0.05 and ** p<0.005.*

4.1.3 OX40 role in the dynamics of MC-Treg interaction

The physiological outcome of MC suppression was extensively described by a recent study documenting Treg-abrogation of BMMCs degranulation and immediate hypersensitivity response. This effect was achieved by Treg-MC contact leading to OX40-OX40L engagement, respectively [113].

4.1.3.1 OX40 and its receptor mediate MC-Treg physical interface

As MC-Treg functional interactions exist, the hypothesis that this contact could be facilitated by the binding of the surface molecule OX40 to its ligand was put forward.
To determine whether this axis could influence conjugation formation between BMMCs and Tregs, the percentages of BMMCs making contacts with WT or OX40-deficient (OX40-/-) Tregs over total BMMCs were quantified. As shown in figure 4.4A after Ag addition, the capacity of BMMCs to interact with WT, but not with OX40-/- Tregs, increased both at 5 and 20 minutes of incubation.
MC-Treg conjugates were monitored for 20 minutes and classified into three categories depending on their extent. The majority of MC-Treg interactions were short-lived, but some cell-cell contacts lasted more than 15 minutes and thus, considered long-lasting interactions (Figure 4.4B). In the presence of WT Tregs, BMMCs made 30% of short, 48% of intermediate and 22% of long lasting interactions. In co-cultured with OX40-/- Tregs, short contacts increased up to 42%, intermediate conjugates dropped to 30%, while the amount of long-lasting interactions remained almost similar to WT Tregs (28%).

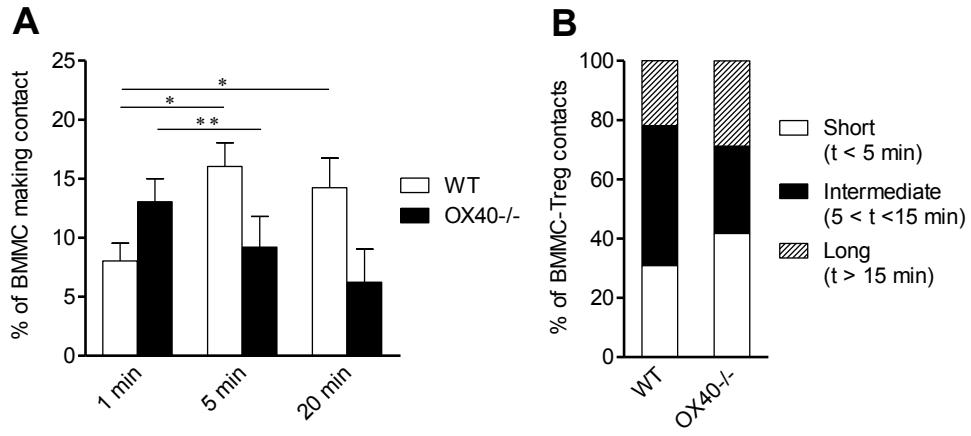

Figure 4.4. OX40-OX40L axis mediates MC-Treg interaction. *(A) Percentages of BMMCs interacting with WT or OX40-deficient Tregs (OX40-/- Tregs) over total BMMCs at 1, 5 or 20 minutes after Ag addition. Data from 2 independent experiments, each performed in duplicate are shown. Numbers of BMMCs analyzed each time point are as follow: n=139 ± 12.5 and n=102 ± 9.5 for WT and OX40-/-, respectively. * p<0.05 and ** p<0.005. (B) Percentages of short (<5 minutes), medium (5-15 minutes) and long-lasting (>15 minutes) contacts between BMMCs and WT or OX40-/- Tregs. Data from 3 independent experiments performed in duplicate are shown. Number of MC-Treg interactions analyzed: n=64 and n=56 for WT and OX40-/- respectively.*

4.1.3.2 OX40-drived inhibition of MC classical exocytosis

FcεRI aggregation following Ag challenge induced dramatic morphological changes within BMMCs, that displayed membrane ruffling and lamellipodial extension typical of a rapid and massive exocytotic process (Figure 4.5A, upper panel). Membrane ruffling was very dynamic and new ruffles continuously forming and collapsing were observed for at least 30 minutes.
Interestingly, BMMCs in contact with WT Tregs exhibited a smooth plasma membrane morphology with minimal membrane ruffling (Figure 4.5A, intermediate panel), likely corresponding to the absence of MCs degranulation. Conversely, when BMMCs were conjugated with OX40-deficient Tregs the ruffling response was not reduced (Figure 4.5A, lower panel). The morphological evidences of the inhibition of BMMCs degranulation response mediated by Tregs through OX40-OX40L axis were validated by reduced amount of released β-hexosaminidase (Figure 4.5B). The same effect was also observed using PMCs (Figure 4.5B).

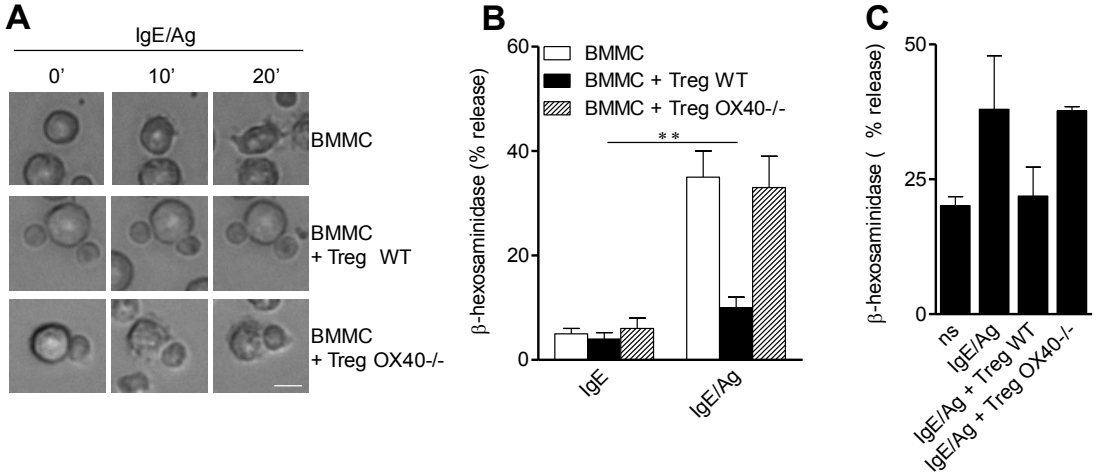

Figure 4.5. OX40-drived inhibition of MC classical exocytosis. *(A) Representative images of morphological changes of activated BMMC alone or in presence of WT and OX40-deficient Tregs are shown at different time frames of the video-recorded movies. (B) Activated BMMCs alone or in presence of WT and OX40-/- Tregs were examined for release of β-hexosaminidase expressed as percentage of the cells' total mediator content. Shown are the means ± SD of 4 independent experiments, each performed in duplicate. (C) IgE pre-sensitized PMCs were challenged with 100 ng/ml DNP alone or in presence of WT and OX40-deficient Tregs. Cell supernatants were examined for content of β-hexosaminidase. Shown are the means ± SD of three independent experiments. Bars = 10 μm (D). ** p<0.005.*

Together these results provide the first morphological evidence for the role of OX40-OX40L axis in Treg-mediated inhibition of MCs degranulation, but the evidence of conjugates between MCs and OX40 deficient Tregs does not exclude the involvement of other receptor-ligand counterparts in the MC-Treg connections.

4.1.4 Ultrastructural sketch of MC-Treg synapses

A key aspect to the regulation of intercellular contacts is the extent to which proteins are made accessible to their binding partners, which may be influenced by the membranes morphology at the contact site [57]. Thus, to gain insight into MC morphological changes occurring while interacting with a Treg, cells' couples were analyzed by transmission electron microscopy.
Ten minutes after Ag-stimulation MCs and Tregs formed numerous cell conjugates. Examined at low magnification, BMMCs appeared as activated cells endowed with numerous surface filopodia and lamellopodia which, in some instance, seemed to embrace and envelope Tregs (Figures 4.6A and 4.6B). Contact areas between BMMC and Treg plasma membranes were either contact point or extended surface area. Viewed at higher magnification, the latter exhibited the composite profile of true immunological synapses (Figures 4.6 C and 4.6D). They were arranged as alternating sites of tight membrane-to-membrane appositions and wider intermembrane spaces that corresponded to the synaptic clefts. Here, the distance between the pre- and post-synaptic membranes was 100-150 nm (Figure 4.6D).

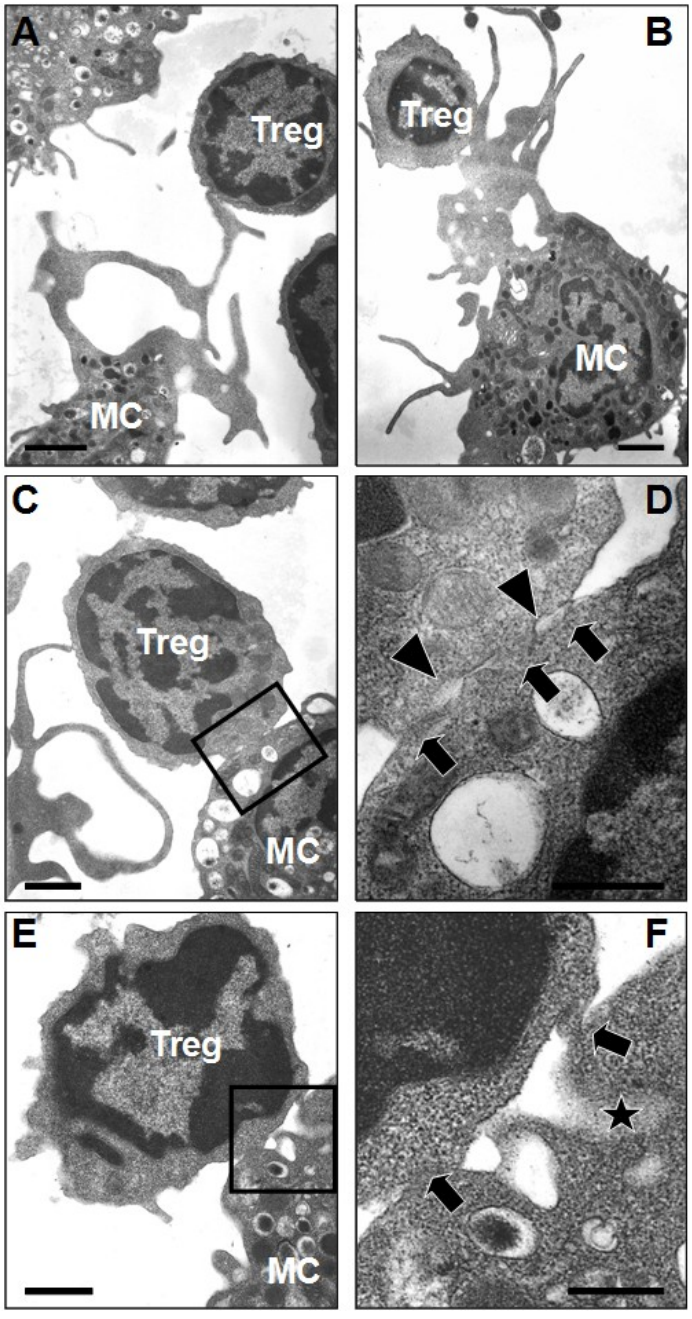

Figure 4.6. Transmission electron microscopy of MC-Treg connections. *(A)(B) Low-magnification images of BMMCs projecting extended cytoplasmic elongations towards Tregs. BMMC projections run for several microns and establish tight membrane appositions with Tregs. Whilst the contact area in (A) is spatially restricted, MC-Treg coupling in (B) covers a linear surface length of about 3 μm. In both images, BMMCs show no sign of exocytosis. (C)(D) Ultrastructural details of a MC-Treg interaction. The image in (D) is an enlargement of the boxed area in (C) and reveals that the synapsis is composed of two clefts (arrowheads) which are sealed apart by apposition areas. The membranes are in tight contact at these points (arrows). (E)(F) Paired micrographs – (F) is an enlargement of the boxed area in (E) – which illustrate a BMMC releasing the content of a secretory granule (asterisk) into the synaptic clefs sealed by tight intermembrane appositions (arrows). Bars = 2.5 μm (A)(B), 1 μm (C)(E), 0.5 μm (D)(F).*

The close intermembrane appositions presented an intermembrane thickness ~15 nm which sealed the synaptic clefts apart. In a few instances, the synaptic cleft formed a kind of pocket where the Treg-coupled MC released the content of one or two secretory granules in a process of limited exocytosis (Figures 4.6E and 4.6F)
The close intermembrane appositions presented an intermembrane thickness ~15 nm which sealed the synaptic clefts apart. In a few instances, the synaptic cleft formed a kind of pocket where the Treg-coupled MC released the content of one or two secretory granules in a process of limited exocytosis (Figures 4.6E and 4.6F).

4.1.5 Evidence of Piecemeal Degranulation in MCs interacting with Tregs

MC challenged with the Ag underwent classical compound exocytosis and an extensive membrane ruffling was observed: granules and plasma membranes fused, membrane pores were formed and membrane-free granule contents were released *in toto* outside the cells (Figure 4.7A).

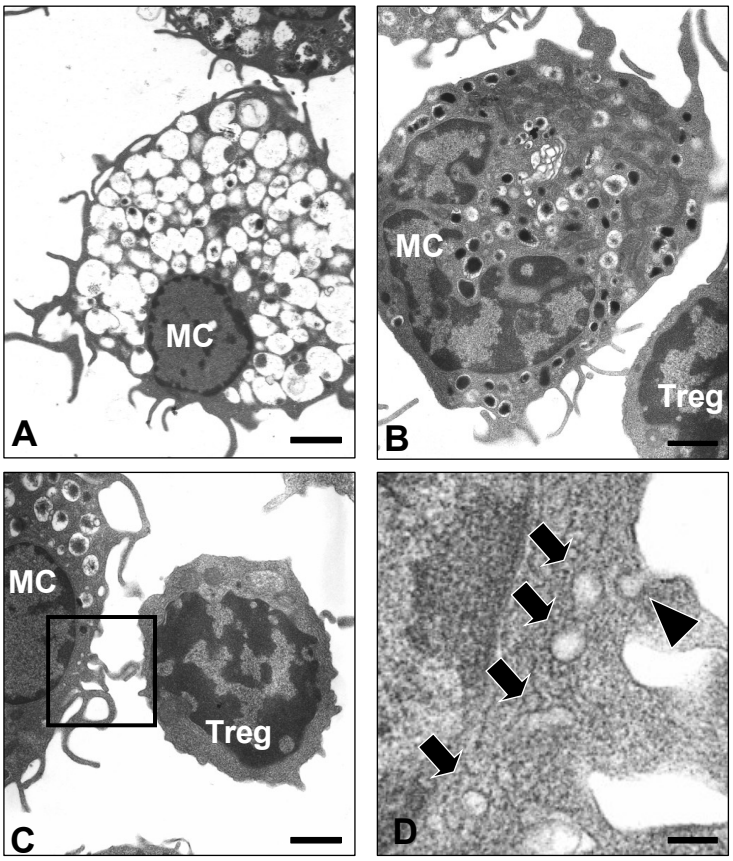

Figure 4.7. Distinctive ultrastructural features of MCs alone or incubated with Tregs. *(A) Pre-sensitized BMMCs challenged with the Ag exhibit the classical ultrastructural features of massive exocytosis. (B) By contrast, Treg-coupled BMMCs display granules with different loss of content material in the absence of granule-to-granule or granule-to-plasma membrane fusion events, the ultrastructural morphology which suggests an ongoing process of piecemeal degranulation (PMD). (C)(D) The MC-Treg connection is a focal point for endocytosis and exocytosis. The image in (D) is an enlargement of the boxed area in (C) and shows a series of vesicles free in the intergranular cytosol (arrows) close to the contact site. The arrowhead points to a coated vesicle. Bar = 3 μm (A), 1.5 μm (B), 2 μm (C), 50 nm (D)*

Interestingly, activated BMMC interacting with Treg exhibited cytoplasmic secretory granules with various degrees of content loss, i.e., granules with lucent areas in their cores, reduced electron density, disassembled matrices, residual cores, and membrane empty containers (Figure 4.7B).
Empty or partially empty secretory containers could be recognized intermingled with granules, whose shape, size, and density fell within normal range (Figure 4.7B). The dilated granule containers

maintained their limiting membranes, as no fusion events with the plasma membrane or with neighboring granule membranes occurred. In a small proportion of Treg-contacting MCs, 30-60 nm diameter lucent vesicles could be identified in the peripheral cytoplasm, next to granules or close to the plasma membrane (Figures 4.5C and 4.5D).
These ultrastructural features are characteristic of piecemeal degranulation (PMD), a morphological evidence of MCs activation, with "bit by bit" and selective pattern of cell secretion which has been associated with cytokine mobilization and extrusion in MCs, basophils and eosinophils [15,130].

4.1.6 Choosy Tregs give rise to timed release of MC mediators

The evidence of PMD in MC interacting with Treg could be in agreement with the earlier observation that Tregs impair FcεRI-mediated degranulation without affecting IL-6 and TNF-α production [113]. To further confirm the selective effect of Tregs on classical exocytosis, the release of different MC-granule associated mediators has been measured.
As shown in figure 4.8A, Tregs significantly inhibited secretion of mediators such as histamine and leukotrienes that are usually released immediately after activation and peaked their maximum within few minutes. On the other hand, the amount of several cytokines, chemokines and growth factors released by MCs 24 hours from Ag challenge is not significantly modified by the presence of WT or OX40-deficient Tregs. As expected, the loss of OX40 expression on Tregs impairs selectively their ability to inhibit the secretion of early-released MC mediators.

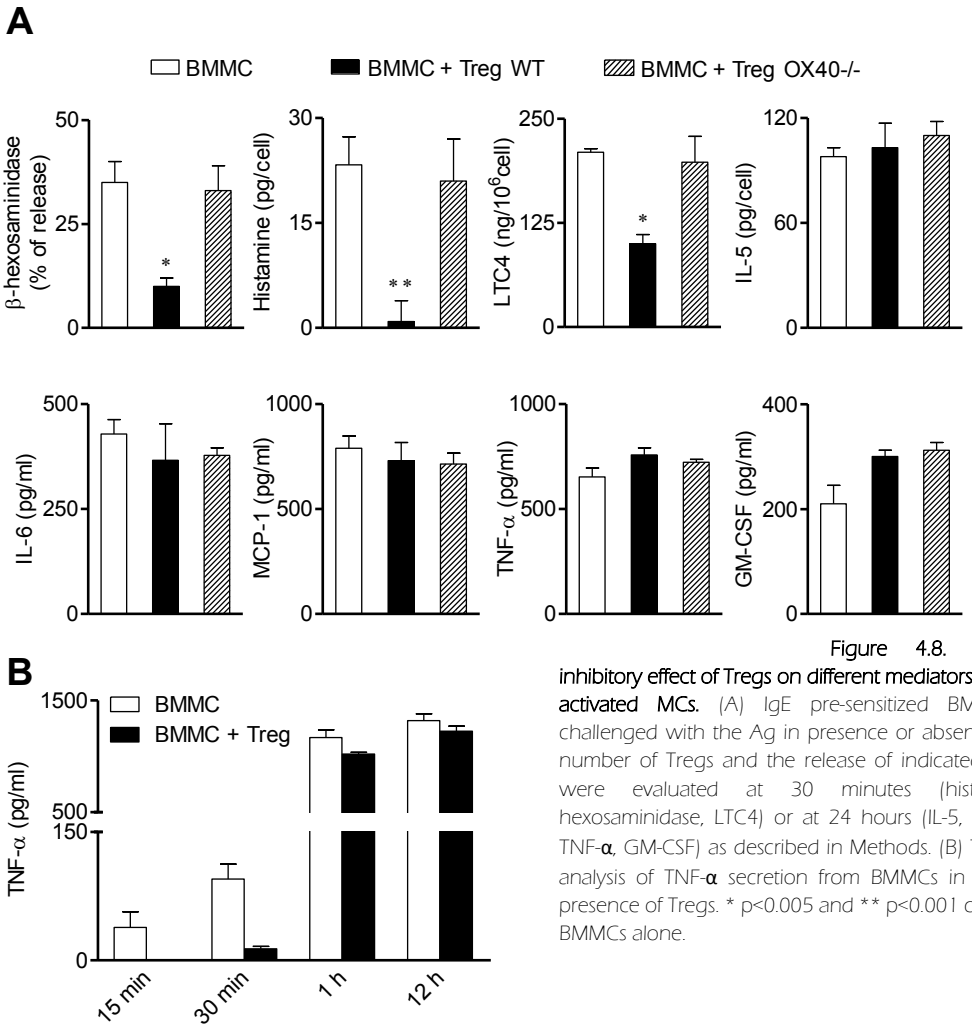

Figure 4.8. Selective inhibitory effect of Tregs on different mediators released by activated MCs. *(A) IgE pre-sensitized BMMCs were challenged with the Ag in presence or absence of equal number of Tregs and the release of indicated mediators were evaluated at 30 minutes (histamine, β-hexosaminidase, LTC4) or at 24 hours (IL-5, IL-6, MCP-1, TNF-α, GM-CSF) as described in Methods. (B) Time course analysis of TNF-α secretion from BMMCs in absence or presence of Tregs. * p<0.005 and ** p<0.001 compared to BMMCs alone.*

To assess the timing of Treg-mediated inhibition, we looked at the kinetics of TNF-α release, as this cytokine is rapidly released from preformed stores (within few minutes) followed by the subsequent release of large quantities of newly synthesized form upon IgE-dependent MC activation [131]. As shown in figure 4.8B, the amount of released TNF-α after 15 and 30 minutes from Ag addition was reduced when MCs are incubated with Tregs, but no differences were detected at 1 hour and 12 hours, indicating that the less degree of detected TNF-α in early time points could be due to a delay in secretion rather than an effective inhibition. This suggests a time-dependent effect of Treg inhibition.

4.2. Tuning MC-Treg synapses

Synapse formation elicits a "situation adapted" for signal transduction and activation response that can vary depending not only on duration of interaction, physical dynamics, identity of receptors that are engaged but also on the strength of signals that are transmitted [55]. The view arising from several studies is that the type and the overall potency of the stimulus are critical in determining which cellular outcomes are observed. Thus, the ability of MCs to collect and add up signals during a definite time period and achieve specific levels of activation was evaluated on MC stringency of binding to Treg cells.

4.2.1 FcεRI aggregation mediates MC-Treg joining

A homogeneous population of MCs can respond differentially to the same allergen depending on its concentration, on the extent of receptor occupancy (number of receptor aggregates) or on the persistence of the allergen (continued receptor aggregation) [132].

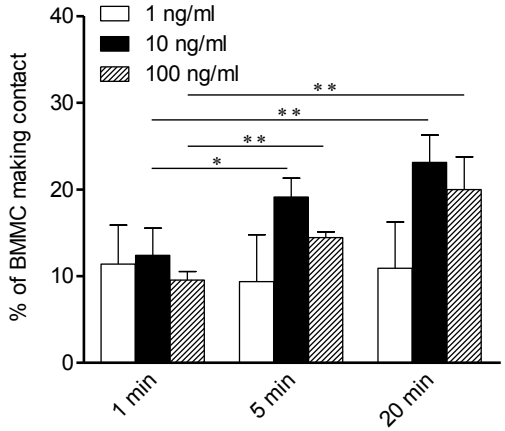

Figure 4.9. FcεRI aggregation mediates MC-Treg conjugation. Histograms represent percentages of BMMCs interacting with Tregs over total MCs at 1, 5 or 20 minutes after addition of increasing amounts of Ag. Data from 3 independent experiments were collected. The average numbers of BMMCs analyzed each time point are as follow: n=257 ± 51.9, n=228 ± 21.5, n=220 ± 15.1 for 1, 10 and 100 ng/ml of Ag condition, respectively.

As shown in figure 4.9, the frequency of BMMC-Treg conjugates varied depending on the amount and duration of FcεRI aggregation; 1 minute upon Ag addition an average of 11% ± 1.4 of BMMCs interacted with Tregs. Low dose of Ag (1 ng/ml) did not significantly change number of conjugates over time, while at higher Ag concentrations (10 ng/ml and 100 ng/ml) the percentages of BMMCs making contacts with Tregs steadily increased from 12% ± 3.1 to 23% ± 3.2 with 10 ng/ml at 1 and 20 minutes respectively and from 9% ± 0.9 to 18% ± 3.8 with 100 ng/ml at 1 and 20 minutes respectively.

4.2.2 Induced-APC phenotype does not prompt MCs for conjugation

Increasing number of reports contributed to put forth the idea that MCs interact with and present antigen to different lymphocytes subsets, including Tregs.
Various treatments have been described to prime MCs to acquire an APC phenotype inducing the expression of MHC class II and selected accessory molecules. The up-regulation of such molecules varies depending on the MC model employed and the stimulus applied [105].

In line with these observations, it has been recently shown that IFN-γ plus LPS stimulation allows MCs to present antigen in the context of MHC class II molecules and to interact with Tregs [109].
To test whether LPS/IFN-γ priming furthers MCs ability of binding to Treg cells, the formation of conjugates between primed-BMMCs and $CD4^+/CD25^+$ Tregs was analyzed.
As expected, treatment with LPS/IFN-γ for 72 hours did not alter the threshold of BMMCs activation but increased the frequency of MHC class II expression, resulting in approximately 45% of MHC class II^+ BMMCs (data not shown).
Under these conditions, BMMC capacity to interact with Tregs was observed by time-lapse video-microscopy. As depicted in figure 4.10A, early after Ag addition percentages of contacts were similar in the co-cultures with both primed and unprimed BMMCs (8% ± 2.1 and 7% ± 2.3 respectively); nevertheless at 5 and 20 minutes after FcεRI triggering, LPS/IFN-γ treatment did not significantly alter percentages of conjugates over time, on the contrary to what happened for unprimed BMMCs, (7% ±3.2, 8% ± 2.7 and 16% ± 3, 14% ± 3.2 for primed and unprimed BMMCs respectively) (Figure 4.10A).

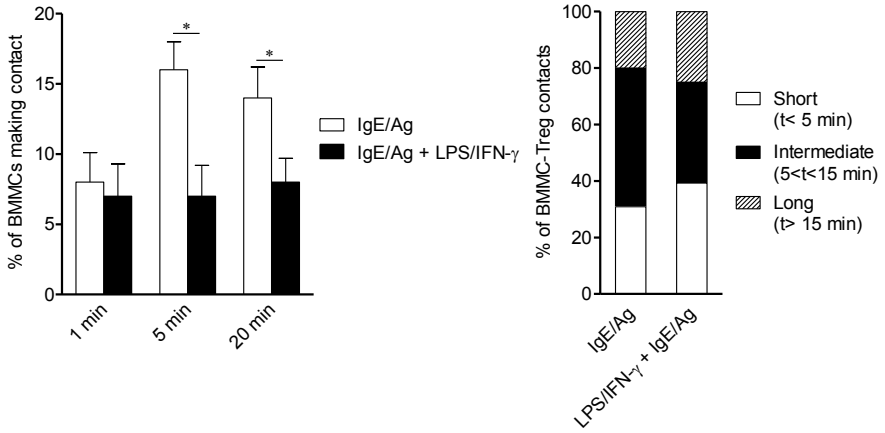

Figure 4.10. Induced-APC phenotype does not prompt MCs for conjugation. *(A) Percentages of LPS/IFN-γ treated or untreated BMMCs interacting with Tregs over total BMMCs at 1, 5 or 20 minutes after Ag addition. Data from 2 independent experiments, each performed in duplicate are shown. Numbers of BMMCs analyzed each time point are as follow: n=72 ± 12.5 and n=112 ± 9.5 for treated and untreated BMMCs, respectively. * p<0.05 (B) Percentages of short (<5 minutes), medium (5-15 minutes) and long-lasting (>15 minutes) contacts between LPS/IFN-γ treated or untreated BMMCs and Tregs. Data from 2 independent experiments performed in duplicate are shown. Number of MC-Treg interactions analyzed: n=11 and n=24 for treated and untreated, respectively*

BMMC-Treg conjugates were also monitored for 20 minutes and classified into three categories depending on their extend (Figure 4.10B). After Ag addition, BMMCs made 30.9% of short, 49.1% of intermediate and 20% of long lasting interactions. In co-cultured with LPS/IFN-γ treated BMMCs, short contacts increased up to 39%, intermediate conjugates dropped to 36%, while the amount of long-lasting interactions remained almost similar to unprimed BMMCs (20%).
The time-dependent process of aggregating synapse are certain to vary with circumstances and could directly dictate specific functional consequences of BMMC-Treg interaction; however, the links between stimulation, duration and cell function remain to be questioned.

4.3. OX40L-STIMulated inhibition of MC extracellular Ca^{2+} flux

Among the mysteries in the field of MC-Treg interaction is the nature of the inhibitory mechanism exerted by Tregs on MC FcεRI-dependent extracellular Ca^{2+} flux. This process occurs on a single cell level through the OX40-OX40L axis without modifying Ca^{2+} mobilization from intracellular stores [113].
Chief mechanism of the Ca^{2+} signaling pathway is the relocation at raft domains of STIM1, the molecular sensor that couples Ca^{2+} store depletion and external Ca^{2+} entry [43]. Hence, the possibility that STIM1 may function as a switch governing in the fate of Ca^{2+} flux in BMMCs interacting with Tregs was explored.

4.3.1 Treg contact inhibits STIM1 translocation into lipid rafts

Using primary BMMCs co-transfected with GFP-GPI and Cherry-STIM1, STIM1 recruitment to LR was tested upon Ag addition during cognate interaction with Tregs. Under control conditions, without T cell subset, the raft-anchored protein was distributed throughout the cell surface, whereas the majority of STIM1 was found in an ER pattern of localization (4.11A, panel A). After triggering, the aggregation and polarization of rafts component was evident, STIM1 formed *puncta* and became partially co-localized with rafts (Figure 4.11A, panels C and E).
After contact between BMMC and Treg, a pronounced polarization of LR can be observed and, in some instances, overexpressed GFP-GPI accumulated at high density within the contact zone, in addition to the remaining localization along the rest of the MC surface (Figure 4.11A, panels D and F). More importantly, Treg contact determined a lack of STIM1 association with LR after Ag addition (Figure 4.11A, panels D and F). This finding was confirmed by a statistical data evaluation and documented with the histogram of the percentages of BMMCs exhibiting STIM1-LR co-localization (Figure 4.11B).

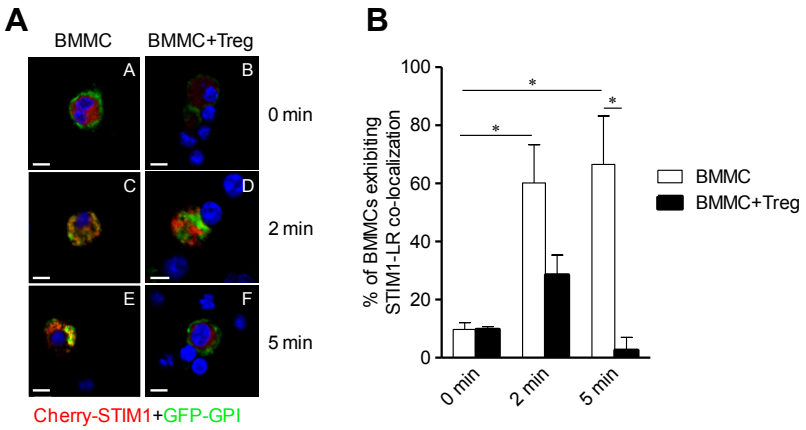

Figure 4.11. Inhibition of STIM1 dynamics in MC-Treg interaction. (A) Representative images of BMMCs and BMMC-Treg contacts showing the co-localization of Cherry-STIM1 (*red*) with a raft-specific marker (GFP-GPI, *green*) in resting (0 min) and activated cells at the indicated time points after Ag triggering. Bars = 10 μm. (B) Percentages of BMMCs alone or in contact with Tregs, showing STIM1-LR co.localization at 0, 2, 5 minutes after Ag addition. Data from 3 independent experiments. Number of MCs alone analyzed: n=71, n=243 and n=190 for 0, 2 and 5 minutes, respectively; number of MC interacting with Treg analyzed: n=82, n=45 and n=28 for 0, 2 and 5 minutes, respectively. * p<0.05.

4.3.2 OX40L engagement inhibits STIM1 enrichment into raft fractions

Given that Tregs have been reported to inhibit directly the FcεRI-dependent Ca^{2+} influx through cell-cell contact involving OX40-OX40L interaction, the combined stimulation of OX40L and FcεRI was investigated on STIM1 changes in the molecular content of LR. For this purpose, the recently described soluble form of OX40 molecule (sOX40), consisting on OX40 extracellular domain [126] which mimics the Treg suppressive effect on MC function was used. Following FcεRI stimulation (5 minutes), a great amount of STIM1 was detected in raft fractions; whereas a decrease of STIM1 in raft portions was observed following treatment with sOX40 (Figure 4.12A and histogram).

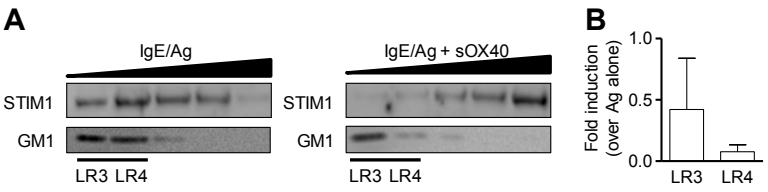

Figure 4.12. OX40 caused a reduction of STIM1 translocation in LR after Ag triggering. (A) LR were obtained from density sucrose gradient of BMMCs lysed 5 minutes after Ag addition in the presence or absence of sOX40. The rafts fractions (LR2 and LR3) were identified with the raft marker GM1 and analyzed for STIM1. (B) Fold induction of GM1-normalized proteins from IgE/Ag + sOX40-activated BMMCs (5 minutes) over IgE/Ag-activated BMMCs (5 minutes) in indicated raft fractions (LR3 and LR4).

4.3.3 Impaired STIM1 translocation in TRPC-deficient MCs

The engagement of OX40L selectively suppresses Fyn-initiated signals required for MC degranulation and serves to limit immediate hypersensitivity [127]. Additionally, a recent report argued that the major flaw in Fyn null MCs is the inability, linked to a defect in TRPC1 expression, to influx normally the Ca^{2+} required for key steps which regulate MC degranulation [42]. Although their role in MC biology is not well understood, TRPC channels have been reported to contribute to Ca^{2+} influx, and their activity is coupled to intracellular stores via STIM1 [43].
To determine if TRPCs are required for the normal dynamics of STIM1, its portioning into rafts was assessed by comparing preparations isolated from TRPC1/C4/C5/C6 quadruple *knock out* (TRPC(-/-)^4) BMMCs in resting condition or following FcεRI stimulation (5 minutes). As shown in figure 4.13, no STIM1 translocation into LR was observed in both conditions.

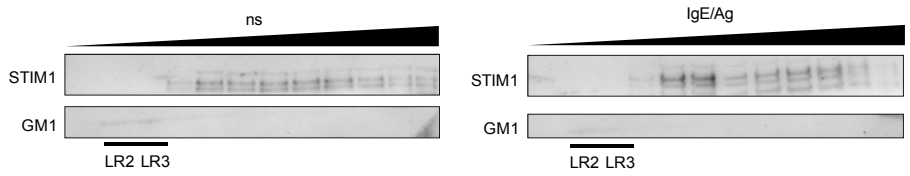

Figure 4.13. TRPC channels have a role in STIM1 translocation. (A). Density sucrose gradient distribution of STIM1 obtained from TRPC(-/-)^4 BMMCs at 0 or 5 minutes after Ag addition. The rafts fractions (LR2 and LR3) were identified with the raft marker GM1 and analyzed for STIM1.

4.3.4 Ag-stimulated OX40L association at LR in TRPCs-deficient BMMCs

It has been demonstrated that OX40L triggering modifies LR organization also causing a marked enrichment into raft fractions of OX40L itself [127]. Thus, these findings suggest a possible relocation of OX40L as an underlying mechanism for the STIM1 defect observed in TRPC-deficient BMMCs. To explore this possibility further, OX40L association at LR was assessed. As expected, an increase in OX40L into raft portions was observed following FcεRI stimulation (5 minutes) (Figure 4.14).

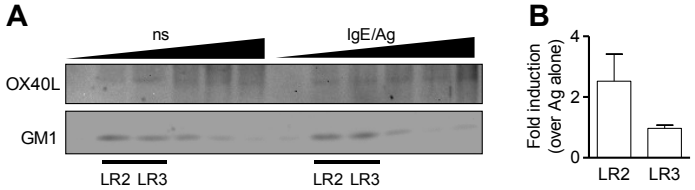

Figure 4.14. FcεRI-dependent OX40L portioning into rafts in TRPC-deficient BMMCs. (A). Density sucrose gradient distribution of OX40L obtained from TRPC(-/-)^4 BMMCs at 0 or 5 minutes after Ag addition. The rafts fractions (LR2 and LR3) were identified with the raft marker GM1 and analyzed for OX40L. (B) Fold induction of GM1-normalized proteins from IgE/Ag-activated BMMCs (5 minutes) over resting BMMCs in indicated raft fractions (LR2 and LR3).

4.3.5 Defective STIM1 association at LR in OX40L-deficient BMMCs

To determine if the physical presence of OX40L could be related to normal STIM1 dynamics, STIM1 portioning into LR was evaluated in OX40L-/- BMMCs. Unexpectedly, a defect in STIM1 translocation was observed upon Ag addition (5 minutes; Figure 4.15A). The same outcome was obtained visualizing STIM1 fate comparing WT and OX40L-/- BMMCs co-transfected with GFP-GPI and Cherry-STIM1 (Figure 4.15B). This finding was documented with the histogram of the percentages of BMMCs exhibiting STIM1-LR co-localization (Figure 4.15C).

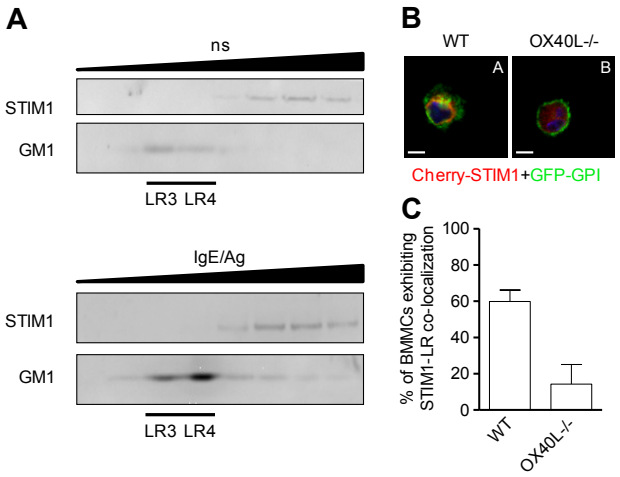

Figure 4.15. Defective STIM1 association at LR in OX40L-deficient BMMCs. *(A). Density sucrose gradient distribution of STIM1 obtained from OX40L-/- BMMCs at 0 or 5 minutes after Ag addition. The rafts fractions (LR2 and LR3) were identified with the raft marker GM1 and analyzed for STIM1. (B) Representative images of WT and OX40L-/- BMMCs showing the co-localization of Cherry-STIM1 (red) with a raft-specific marker (GFP-GPI, green) after Ag triggering. Bars = 10 μm. (C) Percentages of WT and OX40L-/- BMMCs showing STIM1-LR co-localization 5 min after Ag addition. Data from 2 independent experiments. Number of BMMC analyzed: n=388 and n=144 for WT and OX40L-/-, respectively;*

Chapter 5

MC-B cell interaction

B cells are a vital part of the humoral immunity branch of the adaptive immune system. Nevertheless, the last decades have witnessed a continuum of unfolding complexities in B -cell development, subsets and functions which contributed to expand the traditional view of these cells. Evidence now indicates that B lymphocytes are required to regulate immune homeostasis with direct or indirect communication with other cell populations [133].

The major compelling element strengthening the need for a deep understanding of MC-B cell interactions is the long-standing evidence of MCs abundance at sites of leukemic/lymphomatous infiltration [134-135]. MCs have been able to directly interact with neoplastic B cell, influencing their growth and survival [136-137].

Despite the role of physical contact and costimulatory molecules for MC-driven B cell proliferation and activation was substantiated by several works [58,85-86], only recently the specific role of MCs in B cell growth and differentiation has been investigated more in detail. Merluzzi and coworkers proved that MCs promote both survival and activation of naïve B cell, as well as proliferation and further plasma cell differentiation through cell-cell contact requiring CD40-CD40L axis [88].

On this basis, to investigate if conjugates between BMMCs and B cells could be established, the kinetics and functional profile of cell-cell interaction was examined under different stimulation condition.

5.1 Imaging BMMC-B cell interactions

To describe cell behavior and interaction, the conjugates formation between resting or IgE-pre-sensitized murine BMMCs and naïve B cells or LPS-/CD40-stimulated B lymphocytes was analyzed by time lapse bright field video microscopy.

Cell demeanor was observed every minute for a total of 30 minutes and cell type was distinguished by its unique morphological characteristic. As depicted in figure 5.1 the B cell population efficiently made contact with BMMCs (Figure 5.1).

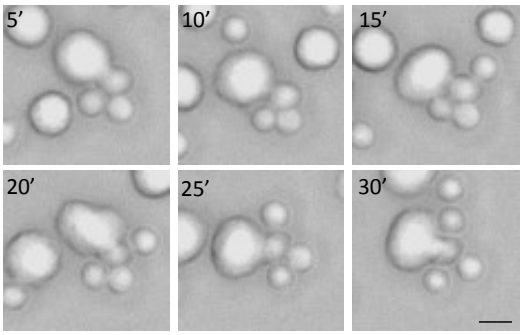

Figure 5.1. Imaging of MC-Treg interaction. (A) Equal number of pre-sensitized BMMCs and B cells were seeded onto Glass bottom Petri dishes and was observed every minutes for a total of 30 minutes. (B) Representative images of conjugate formation between BMMCs and Tregs monitored by time lapse video microscopy for 30 minutes. Bars = 10 μm

5.2 Kinetics of conjugate formation between BMMCs and B cells

Cell activation can increase synaptic adhesion or, by contrast, contribute to immune cell detachment. To determine whether the dynamic regulation of BMMC-B cell adhesiveness could be the result of different stimulation condition, the capacity of naïve B cells and LPS- or CD40-stimulated B lymphocytes to form stable conjugate with BMMCs was assessed in the absence or in the presence of IgE/Ag stimulation.
It is known that B cell activation results in enhanced CD40 expression [138] and that the interaction of this molecule with its receptor on MCs results in costimulatory signals leading to productive survival, activation and expansion of B cells. To test if CD40-CD40L axis breakdown would destabilize synapse formation, conjugates formation was also assessed in the presence of CD40-blocking antibody.

5.2.1 Naïve B cells

In the presence of naïve B cells, percentages of contacts were similar in both cell co-culture settings (9% ± 1,2% and 8% ± 3,2% for resting and activated BMMCs respectively) and the percentages of conjugates over time did not significantly change (7% ± 0.2 and 9% ± 5.3 respectively for resting BMMCs; 2% ± 1.2 and 7% ± 2.5 for activated BMMCs). With a CD40 neutralizing antibody, the capacity of BMMCs to form stable interaction with B cells was lower at all times of incubation tested (1% ± 1.8, 1% ± 1.6, 2% ± 2.6 for resting BMMCs; 1% ±0.5, 1% ± 0.6, 1% ± 0.4 for activated BMMCs) (Figure 5.2).

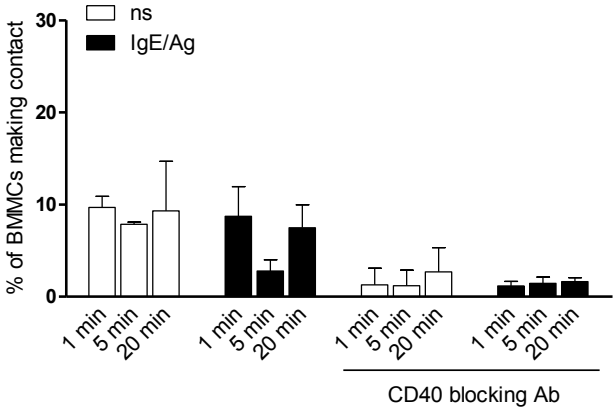

Figure 5.2. Kinetics of BMMC-B cell interactions. Percentages of BMMCs interacting with naïve B cells over total BMMCs at 1, 5 or 20 minutes. Data from 2 independent experiments, each performed in duplicate are shown. Numbers of interacting BMMCs analyzed each time point are as follow: n=12 ± 1 and n=12 ± 9.5 for ns and IgE/Ag activated BMMCs, respectively n=3 ± 1 and n= 3± 0.6 for ns and IgE/Ag activated BMMCs in the presence of CD40 neutralizing Ab, respectively.

5.2.2 CD40-stimulated B cells

As BMMC-B lymphocyte interactions exist, the hypothesis that this contact could be facilitated by CD40-triggering of B cells was put forward.
Conjugation formation between the two cell types was quantified (Figure 5.3); compared with the first time of incubation tested, at 5 minutes and 20 minutes BMMCs showed an enhanced capability to forme stable interaction with CD40-triggered B cells (6% ± 2.3% *vs* 10% ± 2.3% and 12% ± 10.1%, respectively). Furthermore, FcεRI aggregation following Ag challenge furthers an increase in the percentages of conjugates (10% ± 1.3%, 10% ± 3.5% and 15% ± 16.2%). Interestingly, likewise for naïve B cells, neutralization of CD40 results in a lower proportion of contacts (3% ± 1%, 3% ± 1.1% and 4% ± 2.6% for resting BMMCs; 4% ± 0.1%, 4% ± 0.3% and 4% ± 1.5% for activated BMMCs).

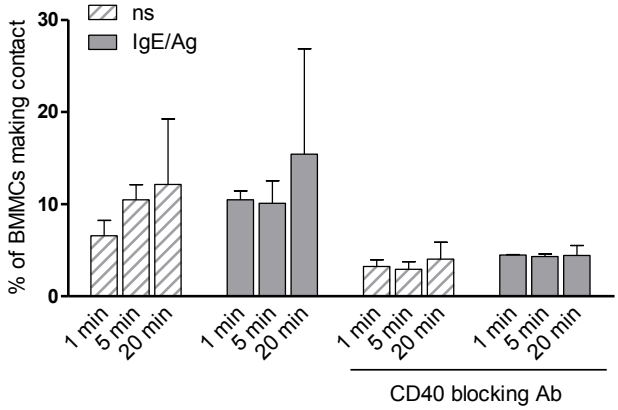

Figure 5.3. Kinetics of BMMCs interactions with CD40-stimulated B cells. Percentages of BMMCs interacting with CD40-stimulated B cells over total BMMCs at 1, 5 or 20 minutes. Data from 2 independent experiments, each performed in duplicate are shown. Numbers of interacting BMMCs analyzed each time point are as follow: n=19 ± 5.6 and n=16 ± 4.7 for ns and IgE/Ag activated BMMCs, respectively and n=8 ± 1.2 and n= 5± 0.6 for ns and IgE/Ag activated BMMCs in the presence of CD40 neutralizing Ab, respectively.

5.2.3 LPS-stimulated B cells

As depicted in figure 5.4, the capacity of BMMCs to form stable conjugates with LPS-stimulated cells was similar in the absence or presence of Ag (5% ± 0,8% *vs* 7 % ± 7,1 % at 1 minute of co-culture) and the percentages of conjugates over time did not significantly change (4% ± 0,5% *vs* 1% ± 0,7 % at 5 minutes; 5% ± 1,6% *vs* 3 % ± 1,2 % at 20 minutes). Unexpectedly, CD40 neutralizing antibody-treatment of B cells, did not alter the threshold of BMMC-capacity to form stable interaction with B cells at 3 times of incubation tested (1% ± 1.8, 1% ± 1.6, 2% ± 2.6 for resting BMMCs; 1% ±0.5, 1% ± 0.6, 1% ± 0.4 for activated BMMCs).

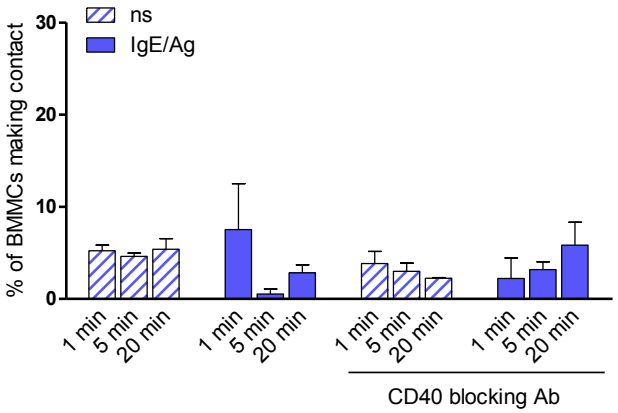

Figure 5.4. Kinetics of BMMCs interactions with LPS-stimulated B cells. Percentages of BMMCs interacting with LPS-stimulated B cells over total BMMCs at 1, 5 or 20 minutes. Data from 2 independent experiments, each performed in duplicate are shown. Numbers of interacting BMMCs analyzed each time point are as follow: n=16 ± 2 and n=6 ± 0.6 for ns and IgE/Ag activated BMMCs, respectively and n=5 ± 1.5 and n= 5± 1 for ns and IgE/Ag activated BMMCs in the presence of CD40 neutralizing Ab, respectively.

5.3 CD40-CD40L axis in BMMC-B cell interactions

The evidence of reduced synapse formation in the presence of CD40-neutralizing antibody, even if in not all the stimulation conditions tested, put forth the idea that CD40-CD40L axis could influence the dynamics and functional outcome of this interaction. To further test this hypothesis, B cells interaction with WT and CD40L-deficient BMMCs was extensively characterized.

5.3.1 Naïve B cells

The percentages of WT or CD40L-deficient BMMCs making contacts with naïve B cells, were quantified over the total number of BMMCs. As shown in figure 5.5, in resting condition CD40L-/- BMMCs showed a lower capacity of conjugate formation with B cells compared to WT BMMCs (1% ± 0.9% *vs* 4% ± 2.2%, 1% ± 1.2% *vs* 4% ± 3.1%, 1% ± 1.3% *vs* 2% ± 0.4% at 1, 5 and 20 minutes respectively). On Ag treatment, CD40L-deficient BMMCs continued to express lower ability to contact B lymphocytes than WT BMMCs (0% ± 0.6% *vs* 4% ± 2.1%, 1% ± 0.6% *vs* 3% ± 1.4%, 0% ± 0.6% *vs* 4% ± 2.4% at 1, 5 and 20 minutes respectively).

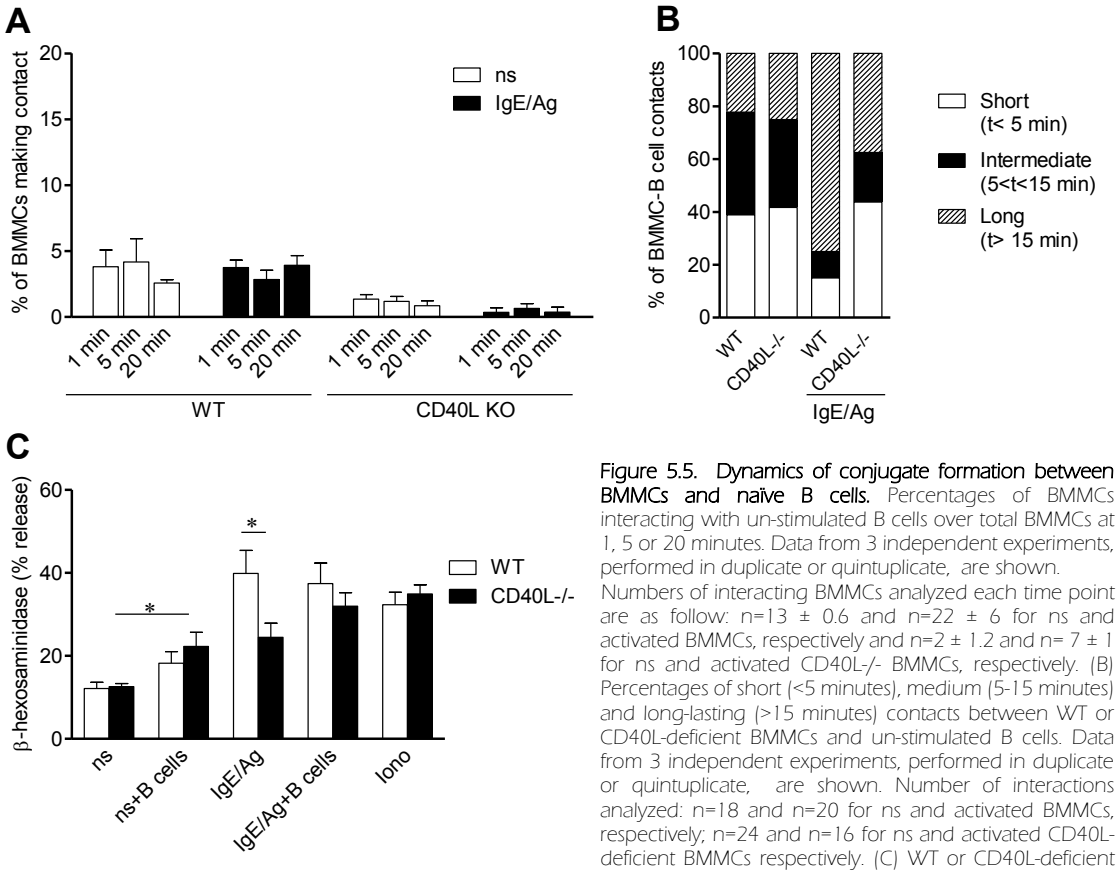

Figure 5.5. Dynamics of conjugate formation between BMMCs and naïve B cells. Percentages of BMMCs interacting with un-stimulated B cells over total BMMCs at 1, 5 or 20 minutes. Data from 3 independent experiments, performed in duplicate or quintuplicate, are shown. Numbers of interacting BMMCs analyzed each time point are as follow: n=13 ± 0.6 and n=22 ± 6 for ns and activated BMMCs, respectively and n=2 ± 1.2 and n= 7 ± 1 for ns and activated CD40L-/- BMMCs, respectively. (B) Percentages of short (<5 minutes), medium (5-15 minutes) and long-lasting (>15 minutes) contacts between WT or CD40L-deficient BMMCs and un-stimulated B cells. Data from 3 independent experiments, performed in duplicate or quintuplicate, are shown. Number of interactions analyzed: n=18 and n=20 for ns and activated BMMCs, respectively; n=24 and n=16 for ns and activated CD40L-deficient BMMCs respectively. (C) WT or CD40L-deficient BMMCs alone or in presence of B cells were examined for release of β-hexosaminidase expressed as percentage of the cells' total mediator content. Shown are the means ± SD of 3 independent experiments, each performed in duplicate. * p<0.05.

BMMC-B cell conjugates were monitored for 20 minutes and classified into three categories depending on the duration of their extend (Figure 5.5B). In resting condition, the majority of B cell interactions with WT BMMCs were short-lived and medium-lived (39% for both). In contrast after Ag addition, WT BMMCs showed a significant higher proportion of long-lived contacts (75%) at the expense of both short and intermediate interactions (15% and 10% respectively). CD40L-deficient

BMMCs displayed a similar pattern of contact durations: un-sensitized cells showed a major proportion of short- and medium-lived contacts (41% and 33%, respectively). Even if the rate of short interactions remained similar, once again FcεRI aggregation induced an increase in the percentage of long-lived conjugate (37%), but in a smaller proportion compared to WT BMMCs.

To explore the functional consequence of B cells crosstalk, FcεRI-initiated degranulation of WT and CD40L-deficient BMMCs was measured. As shown in figure 5.5C, BMMCs from CD40L *knockout* mice showed a significant decrease in IgE/Ag-dependent degranulation (24% ± 3.4% *vs* 40% ± 5.5%); interestingly, in both cell co-cultures B lymphocytes supported the release of granule contents by un-stimulated BMMCs, with WT cells alone releasing 12% ± 1.5 of their pre-stored mediators compared with 18% ± 2.8% for co-incubated cells and with CD40L-deficient BMMCs alone releasing 12% ± 0.7% compared to 22% ± 3.4% of co-cultured CD40L-deficient BMMCs. The same trend was observed in activated CD40L-deficient BMMCs (24% ± 3.4% alone *vs* 31% ± 3.2% with B cells), but not activated WT BMMCs (40% ± 5.5 alone *vs* 37% ± 4,9% with B cells), indicating that CD40-CD40L axis is not required for this functional outcome.

5.3.2 CD40-stimulated B cells

The percentages of WT or CD40L-deficient BMMCs making contacts with CD40-stimulated B cells, was quantified over the total number of BMMCs. As shown in figure 5.6A, compared with the first time of incubation tested, at 5 minutes and 20 minutes WT BMMCs showed an enhanced capability to form stable interaction with CD40-triggered B cells (2% ± 0.4% *vs* 3% ± 0.4% and 4% ± 0.8%, respectively).

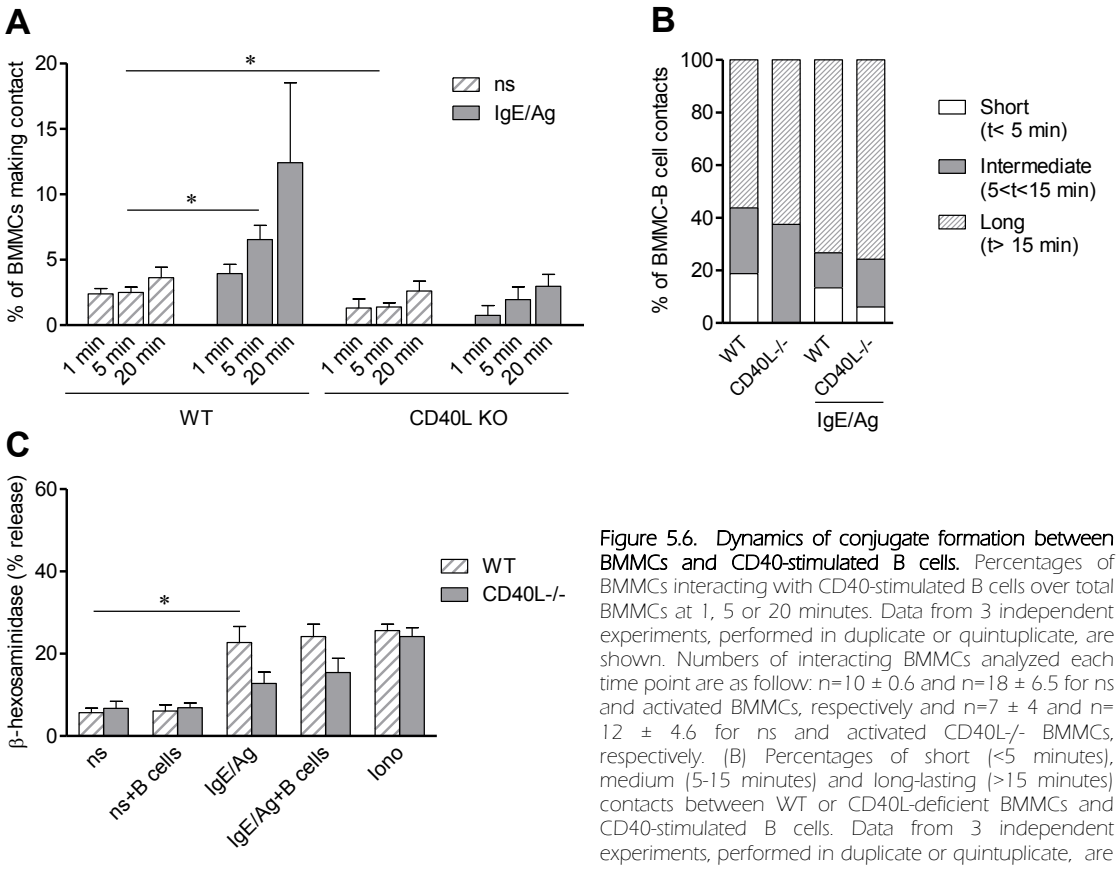

Figure 5.6. Dynamics of conjugate formation between BMMCs and CD40-stimulated B cells. Percentages of BMMCs interacting with CD40-stimulated B cells over total BMMCs at 1, 5 or 20 minutes. Data from 3 independent experiments, performed in duplicate or quintuplicate, are shown. Numbers of interacting BMMCs analyzed each time point are as follow: n=10 ± 0.6 and n=18 ± 6.5 for ns and activated BMMCs, respectively and n=7 ± 4 and n= 12 ± 4.6 for ns and activated CD40L-/- BMMCs, respectively. (B) Percentages of short (<5 minutes), medium (5-15 minutes) and long-lasting (>15 minutes) contacts between WT or CD40L-deficient BMMCs and CD40-stimulated B cells. Data from 3 independent experiments, performed in duplicate or quintuplicate, are shown. Number of interactions analyzed: n=16 and n=15 for ns and activated BMMCs, respectively; n=15 and n=20 for ns and activated CD40L-deficient BMMCs respectively. (C) WT or CD40L-deficient BMMCs alone or in presence of CD40-stimulated B cells were examined for release of β-hexosaminidase expressed as percentage of the cells' total mediator content. Shown are the means ± SD of 3 independent experiments, each performed in duplicate. * p<0.05.

More interestingly, FcεRI aggregation following Ag challenge furthers an increase in the percentages of conjugates (4% ± 0.7%, 7% ± 1.1% and 12% ± 6.1%). Once again, the lack of CD40L results in a lower proportion of contacts (1% ± 0.7%, 1% ± 0.3% and 3% ± 0.7% for resting BMMCs; 1% ± 0.8%, 2% ± 0.9% and 3% ± 0.9% for Ag-triggered BMMCs) underscoring the importance of CD40-CD40L axis in supporting MC-B cell synapse formation.
Upon CD40L-triggering, the majority of B cell interactions with WT BMMCs were medium-lived and long-lived (25% and 56% respectively). After Ag addition, WT BMMCs showed a significant higher proportion of long-lived (74%) at the expense of short-lived contacts (13%) and intermediate interactions (13%).
FcεRI-activated CD40L-deficient BMMCs displayed a similar pattern of contact durations compared to WT cells (Figure 5.6B): co-cultured cells showed a major proportion of long-lived contacts (76%) and similar amount of short- and intermediate-contacts (6% and 18%, respectively). When CD40L-/- BMMCs were used in resting conditions, medium and long-lasting conjugates increased up to 38% and 62% respectively, while the amount of short contacts dropped to 0% suggesting that, even if CD40L may influence several cellular behaviors such as the amount of intercellular contacts, its expression is not directly related to the duration of MC-B lymphocytes crosstalks after B cell activation and does not exclude the involvement of other receptor-ligand counterpart in this connection.
To explore the functional consequence of CD40L-engagement after CD40-triggering of B cells, FcεRI-initiated degranulation of WT and CD40L-deficient BMMCs was measured. As shown in figure 5.6C, CD40L-deficient BMMCs showed a decrease in IgE/Ag-dependent degranulation (13% ± 2.7% *vs* 24% ± 3%); contrary to what happened for naïve B cells, CD40-triggered lymphocytes did not support BMMCs degranulation, with WT cells alone releasing 6% ± 1.1 of their pre-stored mediators compared with 6% ± 1.5% for co-incubated cells and with CD40L-deficient BMMCs alone releasing 7% ± 1.7% compared to 7% ± 1.1% of co-cultured CD40L-deficient BMMCs. More or less, the same trend was observed in activated WT and CD40L-deficient BMMCs (23% ± 3.9% alone *vs* 24% ± 3% with B cells and 13% ± 2.7% alone *vs* 15% ± 3.4% with B cells, respectively). Thus, CD40L-engagement after CD40-triggering of B cells appeared not to influence the functional outcome of MC-early response.

5.3.2 LPS-stimulated B cells

The percentages of WT or CD40L-deficient BMMCs making contacts with LPS-triggered B cells was quantified over the total number of BMMCs (Figure 5.7A). In resting condition BMMCs capacity of conjugate formation with LPS-stimulated B cells decreased over time (5% ± 1.7%, 3% ± 1.1% and 3% ± 1.2% at 1, 5 and 20 minutes respectively). FcεRI aggregation following Ag challenge furthers an increase in the percentages of conjugates which, likewise for resting cells, got off over time (7% ± 2.4%, 7% ± 2.4% and 3% ± 1% 1, 5 and 20 minutes respectively). Again, the lack of CD40L results in a lower proportion of contacts (1% ± 0.3%, 1% ± 0.5% and 1% ± 0.6% for resting BMMCs; 2% ± 1.1%, 2%± 1.1% and 2% ± 0.8% for Ag-triggered BMMCs) underlining the importance of CD40-CD40L axis in supporting MC-B cell synapse formation in all the stimulation conditions tested. As depicted in figure 5.7B, upon LPS-triggering, the majority of B cell interactions with WT BMMCs were short- and long-lived (38% for both). After Ag addition, WT BMMCs showed a significant higher proportion of medium- and long-lasting contacts (39% and 46%) at the expense of short interactions (15%). When CD40L-/- BMMCs were used in resting contidions, short-lived conjugates increased up to 53% at the expence of medium- and long-lasting contacts (20% and 27%). FcεRI-activated CD40L-deficient BMMCs displayed a similar pattern of contact durations compared to WT cells: 44%, 28% and 28% of short-, medium-, long-contacts respectively. Thus, while LPS triggering of B cells seemed to not influence the duration of intercellular contacts, FcεRI triggering promoted long-lasting interactions. In the latter case, CD40L expression favored the persistence of MC-B lymphocytes crosstalks not excluding the involvement of other receptor-ligand pairs in this connection.
To explore the functional outcome of CD40L-engagement after LPS-triggering of B cells, the early response of WT and CD40L-deficient BMMCs was assessed (Figure 5.7C). Likewise for CD40-triggered B cells, LPS stimulation of B cells did not support BMMCs degranulation, with WT cells alone releasing

5% ± 1.1 of their pre-stored mediators compared with 6% ± 0.7% for co-incubated cells and with CD40L-deficient BMMCs alone releasing 7% ± 1.7% compared to 7% ± 1% of co-cultured CD40L-deficient BMMCs. Interestingly, in both cell co-cultures B lymphocytes supported the release of granule contents by Ag-triggerd BMMCs, with WT cells alone releasing 23% ± 3.9 of their pre-stored mediators compared with 28% ± 5.1% for co-incubated cells and with CD40L-deficient BMMCs alone releasing 13%± 2.7% compared to 15% ± 3% of co-cultured CD40L-deficient BMMCs, confirming that CD40L-engagement appeared to not influence the functional outcome of MC-early response (Fig. 5.C).

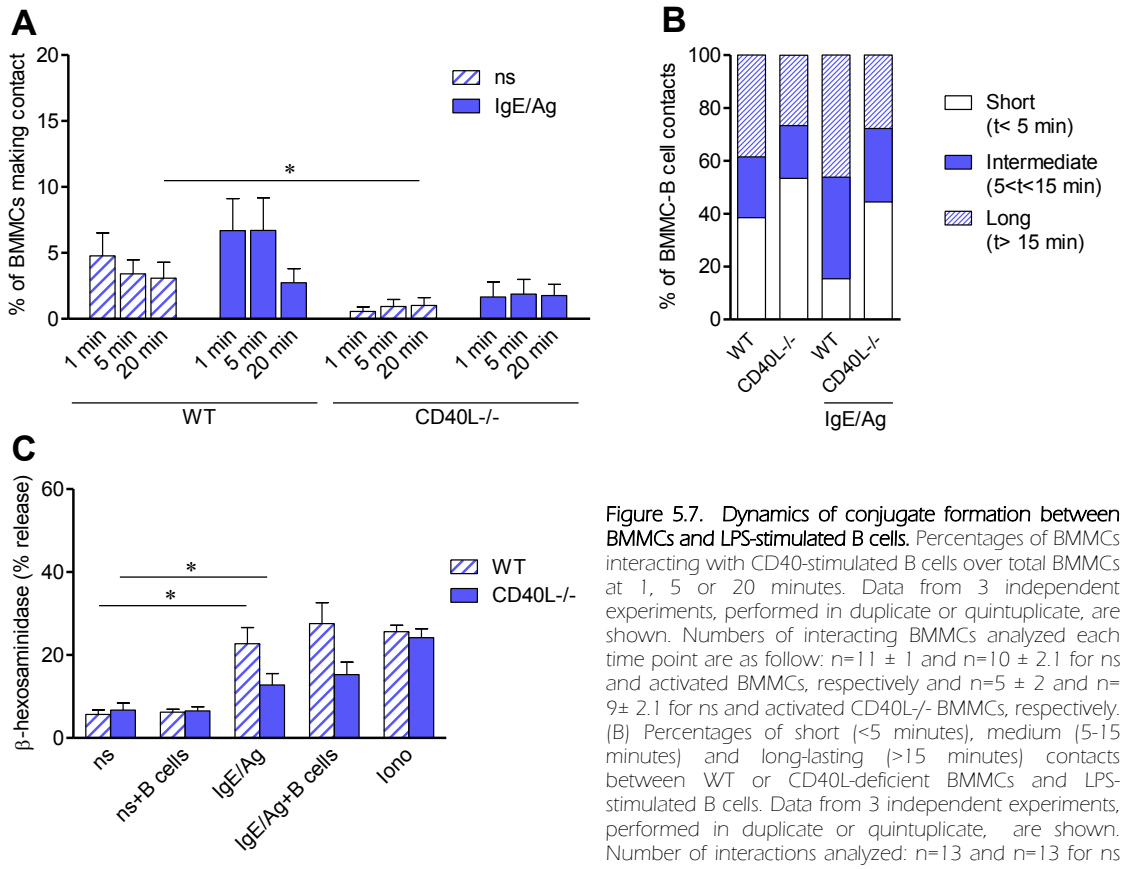

Figure 5.7. Dynamics of conjugate formation between BMMCs and LPS-stimulated B cells. Percentages of BMMCs interacting with CD40-stimulated B cells over total BMMCs at 1, 5 or 20 minutes. Data from 3 independent experiments, performed in duplicate or quintuplicate, are shown. Numbers of interacting BMMCs analyzed each time point are as follow: n=11 ± 1 and n=10 ± 2.1 for ns and activated BMMCs, respectively and n=5 ± 2 and n= 9± 2.1 for ns and activated CD40L-/- BMMCs, respectively. (B) Percentages of short (<5 minutes), medium (5-15 minutes) and long-lasting (>15 minutes) contacts between WT or CD40L-deficient BMMCs and LPS-stimulated B cells. Data from 3 independent experiments, performed in duplicate or quintuplicate, are shown. Number of interactions analyzed: n=13 and n=13 for ns and activated BMMCs, respectively; n=16 and n=33 for ns and activated CD40L-deficient BMMCs respectively. (C) WT or CD40L-deficient BMMCs alone or in presence of CD40-stimulated B cells were examined for release of β-hexosaminidase expressed as percentage of the cells' total mediator content. Shown are the means ± SD of 3 independent experiments, each performed in duplicate. * $p<0.05$.

Chapter 6

MC-PMN interaction

PMNs have been viewed for long time as the final effector cells of an acute inflammatory response, with a primary role in the clearance of extracellular pathogens. More recent findings provided new insight on their functions. Depending on the context, PMNs can produce key inflammatory mediators, throw extracellular traps and engage in bi-directional interactions with diverse components of both the innate and adaptive immune systems, differentially influencing their response [80].
With the advent of MC-deficient mice it has been possibly to show the critical role of MC-derived molecules for PMN recruitment and activation. Despite a clear functional interaction between MCs and PMN has been established, the physical crosstalk between these two cell populations has not yet been described [10].

6.1 Dynamics of BMMC-PMN interactions

To verify whether a cell-cell contact interaction between BMMCs and PMN exists, real time imaging was performed. By time lapse bright field video microscopy, the formation of conjugates between resting or IgE-pre-sensitized murine BMMCs and DiI-labelled PMNs was analyzed; cell behavior was observed every minute for a total of 30 minutes. As depicted in figure 6.1A, the leukocyte population efficiently made contact with BMMCs and, at the earlier time points, similar percentages of contacts were observed in both cell co-culture settings (2% ± 0.2% and 3% ± 1.2% at 1 minute, 3% ± 0.8% and 4% ± 1.1% at 5 minutes for resting and activated BMMCs, respectively). Interestingly, 20 minutes after FcεRI aggregation, the capacity of BMMCs to form stable interaction with PMNs increased compared to the resting cells (12% ± 1.3% *vs* 4% ± 1.1%, respectively) (Figure 6.1B).

BMMC-PMN conjugates were classified into three categories depending on their extend (Figure 6.1C). In resting conditions, the majority of interactions were intermediate-lived (48%), but some cell-cell contacts lasted more than 15 minutes (26%). After Ag addition, intermediate contacts increased up to 65%, short conjugates dropped to 14%, while the amount of long-lasting interactions remained almost similar to resting BMMCs (21%).

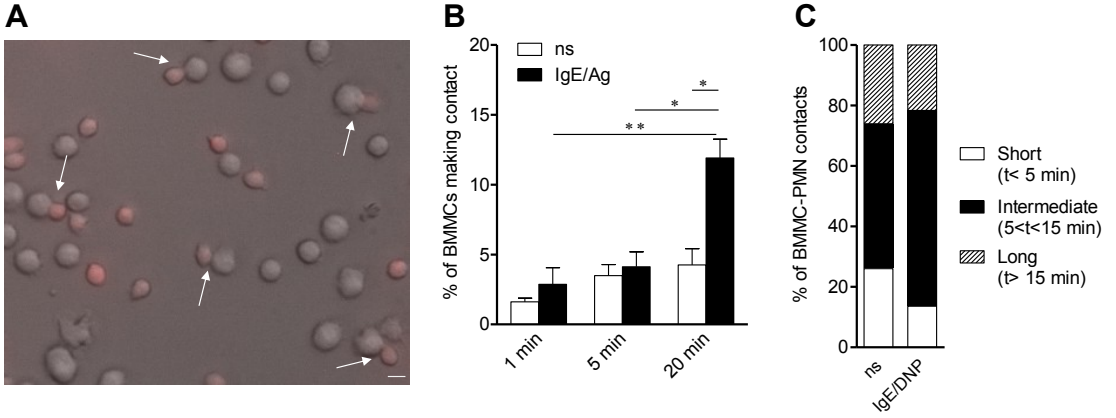

Figure 6.1. Dynamics of BMMC-PMN interaction. (A) Equal number of pre-sensitized BMMCs and PMNs were seeded onto Glass bottom Petri dishes. Representative images of conjugate formation between BMMCs and PMNs monitored by time lapse video microscopy for 30 minutes. Bars = 10 μm. (B) Percentages of BMMCs making contact with PMNs at 1, 5 and 20 minutes without or after Ag (100 ng/ml) addition. Numbers of interacting BMMCs analyzed each time point are as follow: n=31± 17.3 and n=70 ± 63.9 for resting and activated BMMCs, respectively. Shown are the means ± SD of 3 independent experiments, each performed in quintuplicate. * $p<0.05$ and ** $p<0.01$. (C) Percentages of short (<5 minutes), medium (5-15 minutes) and long-lasting (>15 minutes) contacts between PMNs and resting or Ag-activated BMMCs. Data from 3 independent experiments performed in quintuplicate are shown. Number of BMMC-PMN interactions analyzed: n=73 and n=88 for resting and Ag-activated BMMCs respectively

6.2 Think of MCs as cell-*eating* machines

As described above (Figure 4.5A), FcεRI aggregation following Ag challenge induced dramatic morphological changes within BMMCs, that displayed membrane ruffling and lamellipodial extension typical of a rapid and massive exocytosis. Interestingly, BMMC-dynamic membrane ruffling, concomitant to the release of preformed mediators, induced the rapid recruitment of neutrophils in the proximity of activated MC, promoting cellular adhesion. MC-membrane extensions, as cytoplasmic arms, surrounded, came in contact with and engulfed PMNs with an endocytic process likely corresponding to phagocytosis (Figure 6.2).

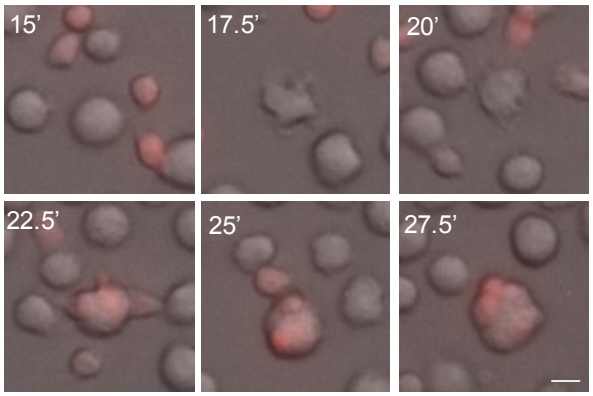

Figure 6.2. PMNs engulfment by BMMCs. Representative images of conjugate formation between BMMCs and neutrophils monitored by time lapse video microscopy for 30 minutes. Bars = 10 μm.

The new perspective opened by this finding raises new questions and calls for a reappraisal of MCs role in the regulatory circuits of the immune system. These results provide the first morphological evidence for the direct cell-contact communication between BMMCs and neutrophils but a more detailed analysis is necessary to understand the functional outcome and the molecular composition of this interaction.

Chapter 7

Discussion

High motility and the ability to make transient interactions are prominent features of immune cells; as a matter of fact, a complex orchestration of communication across intercellular contacts is a key correlate to establishing appropriate immune responses [53].
Immune cells' interaction results in different modes (as regards morphology and duration) and leads to different outcome. To achieve efficient cell-cell interactions, a migrating cell undergoes dramatic morphological changes, recruits cell-surface receptors and signaling molecules to the contact region, and integrate signals eliciting a "situation adapted" activation response that determines specific effector functions [57]. The type and amount of information exchanged may be influenced by the duration of interaction, the identity of the receptors and signaling components that are engaged and recruited, and the signals that are transmitted. These functions depend on the maturation or activation state of each cell type, as well as the type of environment in which the interaction takes place [55].
Even though MCs are traditionally considered a secretory cell type, they express a broad array of cell surface receptors, which mediate the specific delivery of co-stimulatory signals that empower these cells to interact with different targets [10].
It is, therefore, possible to ascribe MC phenotypic and functional plasticity not only to their capacity to respond to a wide range of signals with the production of distinct patterns of mediators, but also to their potential ability in establishing reliable, wideband direct intercellular interactions.
The present work starts to define a molecular code for MC range of contact modes, in which the differences in timing, spacing and molecular arrangements determine the outcome of "MAST CELLular interactions".
Distinct aspects of the physical and functional crosstalk between MCs and different cells of both innate and adaptive immune system have been addressed.

On the basis of previous reports [10], it has been postulated the capacity of MCs to form stable contacts with Tregs; however as MC heterogeneity is widely documented [9] this variability should be considered in the investigation of such interaction. Thus, the functional crosstalk between different types of MCs and $CD4^+/CD25^+$ Tregs has been characterized throughout multiple microscopy imaging approaches. The direct communication between different MC types, such as BMMC, mature PMC and human MC, and $CD4^+/CD25^+$ Tregs, has been demonstrated. In addition, the finding that both murine and human MCs can be subject to Treg-mediated suppression warrants the deeper investigation of how MC-Treg functional interplay takes place on a single cell level.

The time-dependent processes of aggregating synapse are certain to vary with circumstances (as regards to different stimuli and specific levels of activation) and could directly dictate specific functional consequences of BMMC-Treg interaction; however, the links between stimulation, duration and cell function remain to be questioned.

Substantial differences were found between WT Treg and OX40-deficient Treg to make conjugates with both BMMC and PMC. While MCs made sporadic contacts in presence of OX40-deficient Tregs, accompanied by Ag-induced degranulation, MCs incubated with WT Tregs showed an increase in numbers of contacts and displayed lack of evident, classical signs of exocytosis. Thus, OX40-OX40L axis increases the ability of cells to interact each other and contributes to support a long-lasting interaction. Nevertheless, the reduced but still evident ability of MCs to make long-lived contacts with Tregs lacking OX40 molecules suggests that other receptor-ligand counterparts could be involved into the initial formation of this synaptic contact, likely through PD1-PDL1 [139-141] and Notch ligands-Notch1 [110,142] expressed on Tregs and MCs, respectively.

It has been previously demonstrated that FcεRI-dependent Ca^{2+} mobilization in MCs is impaired in the presence of WT but not OX40-deficient Tregs [113]. The Treg-mediated effect does not affect PLC-γ2 activation nor the emptying of intracellular Ca^{2+} stores, but prevents the uptake of extracellular Ca^{2+}. Thus, this inhibition is likely to result from the absolute requirement of the MC secretory granule fusion machinery for Ca^{2+} influx, as the release of Ca^{2+} from intracellular stores alone is not sufficient to properly activate secretory fusion proteins [20]. Here, preliminary results demonstrate that the physical interaction with a single Treg leads to the inhibition of the relocation at raft domains of STIM1, the molecular sensor that couples Ca^{2+} store depletion and external Ca^{2+} entry [43]. Additional findings suggest the engagement of OX40L as an underlying mechanism for the STIM1 defect observed. This is in agreement with previous studies demonstrating that the triggering of OX40L selectively suppresses Fyn-initiated signals determining the dampening of MC response. In the proposed model OX40L engagement acts to "fine tune" FcεRI-stimulated MC degranulation, modifying lipid raft organization and causing a considerable inactivation of downstream Fyn-dependent proteins Gab2, PI3K, Akt and RhoA [127]. A recent report argued that the major flaw in Fyn null MCs is the inability, linked to a defect in TRPC1 expression, to influx normally the Ca^{2+} required for key steps which regulate MC degranulation [42]. Although their role in MC biology is not well understood, TRPC channels have been reported to contribute to Ca^{2+} influx [43]. Here, preliminary results show their coupling to STIM1 dynamics and to OX40L relocation in rafts domain.

In presence of WT but not OX40-/- Treg the reduced Ca^{2+} uptake was accompanied by the inhibition of early-preformed mediators release from IgE/Ag activated MCs while later events of MC activation are not affected. Additionally, a more detailed analysis obtained with electron microscopy confirmed that "classical" degranulation was inhibited when MCs were in close contact with Tregs, but it also indicated that MCs probably underwent to a selective mediators secretion throughout piecemeal degranulation (PMD), rather than classical exocytosis. PMD refers to a particulate pattern of cell degranulation, which was formerly described in basophils, MCs and eosinophils [143-144]. This ultrastructurally defined secretory model implies a discrete release of granule particles from storage granules without granule fusion with the plasma membrane. Secretion occurs by translocation of loaded vesicles, or by means of vesiculotubular structures. In the former case, small vesicles filled with secretory cargoes bud from granules, move to the cell periphery and fuse with the plasma membrane, thus releasing small quanta of secretory material [143]. In the latter case, the secretory mechanism involves intragranular compartments organized as tubular vesicles or tubular networks, which bud from donor granules and relocate specific granule products in response to stimulation [130].

Consequently, PMD would accomplish discharge of secretory constituents from storage granules without granule-to-granule and granule-to-plasma membrane fusion events and without direct granule opening to the cell exterior, as we have observed in our experiment.
PMD has been demonstrated to occur in case of cytokine secretion [15,130], but molecular mechanisms underlying PMD are largely unknown. In particular, very little is known about what governs the cell decision to opt for either release of entire granules or PMD, and the precise molecular mechanisms that regulate mobilization of vesicle-associated secretory aliquots in a PMD fashion.
In light of these results, it can be speculated that the less availability of cytosolic Ca^{2+} in activated MCs interacting with Tregs could be responsible for un-successful exocytosis, but could be enough for promoting PMD. This can explain the selective inhibitory effect of Treg on the secretion of pre-stored and usually early-released mediators and the delay of TNF-α release observed at early time point.
In conclusion, this study describes the dynamic and functional profile of MC-Treg interactions. These crosstalks are not restricted to BMMCs but are a common feature of mature MCs and human MCs. Importantly, we found that this crosstalk is regulated on a single cell level, also providing the first morphological evidence for a role of OX40-OX40L axis in Tregs inhibition of MCs function. However, the dynamics of Treg-MC conjugates reflects a complex synaptic structure and a more detailed analysis is necessary to understand the molecular composition of this interaction. Moreover the evidence of PMD in MCs interacting with Tregs underlines the necessity of understanding of all events and mechanisms governing differential sorting, packing and secretion of granule-stored mediators in order to identify selectively inhibitory pathway of MC degranulation without modifying the innate immune functions.

The need for a deep understanding of MC-B cell interactions has been strengthened by the long-standing evidence of the abundance of MCs at sites of leukemic/lymphomatous infiltration [134-135].
The first proof of an effective direct MC-B cells crosstalk was reported by Gauchat and coworkers. Since then, several studies demonstrated the importance of physical contact and, in particular, of CD40-CD40L axis, in modulating MC-mediated B cell-specific antibody responses in different settings [85-87].
It is known that B cell activation results in enhanced CD40 expression [138] and that the interaction of this molecule with its receptor on MCs results in costimulatory signals leading to productive survival, activation and expansion of B cells. Both resting and activated MCs are able to induce a significant inhibition of cell death and an increase in proliferation of naïve and activated B cells. This effect relies on cell-cell contact and MC-soluble factors. CD40-CD40L signaling, together with MC-derived IL-6, are also involved in the differentiation of B cells into $CD138^{+}$ plasma cells and in selective IgA secretion [88].
Cell activation can increase synaptic adhesion or, by contrast, contribute to immune cell detachment.
On this basis, to investigate whether the dynamic regulation of BMMC-B cell adhesiveness could be the results of different stimulation condition, the capacity of naïve B cells and LPS- or CD40-stimulated B lymphocytes to form stable conjugate with BMMCs was assessed in the absence or in the presence of antigen stimulation.
Interestingly activation status of MCs seems not to influence the frequency of conjugate formation, except for CD40-stimulated B cells. Nevertheless, FcεRI aggregation induces a great increase in the percentage of long-standing interactions.
On the contrary, activation of B cells greatly influences the dynamics of conjugates. CD40-triggering, not only determines an increase in the frequency of contacts but also determines a persistence of such interactions.
It is known that the frequency and the duration of contacts between immune cells varies considerable and depends on the maturation or activation state of each cell type [57]. It has been suggested that the principle of kinetic proofreading can operate at the level of intercellular contacts. That is, for effector function to be induced, the duration of adhesion must persist long enough to allow each step in the cellular process to be completed. Thus, depending on the stimulus, MCs and B cells may be induced to express different pattern of activating or inhibitory receptors (adhesion and co-stimulatory molecules) which can increase synaptic adhesion or, by contrast, favor cell migration and destabilize the interaction to obtain specific functional outcome.

CD40-CD40L axis can influence many B cell effector functions and can potentially exert key effects on the duration and the frequency of intercellular contacts.
In all the stimulation condition tested, even if the frequency of conjugates decreased in the absence of CD40L, that seems not to influence the duration of cellular adhesion, except for CD40-stimulated B cells. In the latter case, the recognition of CD40 ligand could trigger outside-in signaling, increase the affinity of integrins and influence several cell behaviors destabilizing synaptic contact. However, the link between stimulation, duration and expression of adhesion molecules remain to be questioned.
Despite the differences observed in the dynamics of intercellular contacts, no significant diverse functional outcome (as regards MC early response) were observed. Noteworthy, naïve B cells support the release of granule contents by un-stimulated BMMCs. This effect does not require CD40-CD40L axis.
However, the dynamics of BMMC-B cells conjugates reflects a complex synaptic structure and a more detailed analysis is necessary to explore their functional consequences.

Several studies pointed out that MC-regulated PMNs recruitment is essential for mounting a rapid and efficient innate immune response [81-84]. Till now, MC-PMN interaction has been merely described as a soluble factors-mediated interplay and possible receptor/ligand pairs that might physically mediate this crosstalk have not yet been described.
The present study demonstrates the physical direct cell-cell communication between BMMCs and PMNs.
More interestingly, BMMC-dynamic membrane ruffling, concomitant to the release of preformed mediators induced the rapid recruitment of PMNs in the proximity of activated MC, promoting cellular adhesion. Thus, for the first time, the morphological evidence of PMNs recruitment induced by MC-derived molecules has been provided. The unique ability of MCs to store and immediately release TNF-α on demand is essential for the rapid onset and for sustaining inflammatory reactions. It has been demonstrated that MCs and MCs-derived TNF-α initiate the life-saving inflammatory response, promoting PMNs influx in different mouse models. Thus, it can be speculated that MCs, through the release of TNF-α, approach and physically engage the leukocyte population; however, this possibility needs to be confirmed.
Notably, FcεRI-induced MC-membrane extensions, as cytoplasmic arms, surrounded, came in contact with and engulfed PMNs with an endocytic process likely corresponding to phagocytosis.
It is thought that phagocytes only eat dead cells or cells doomed to die; however, it is now clear that phagocytosis can execute death of viable cells, a process named phagoptosis. It is provoked by exposure of "eat me" signals and/or loss of "don't-eat me" signals by viable cells, causing their engulfment. This process mediates the normal turnover of several cell types including erythrocytes and PMNs but can also contribute to pathology [145]. In light of these considerations, the new perspective opened by these findings raises new questions and calls for a reappraisal of MCs role in the regulatory circuits of the immune system.

Section IV

Concluding Remarks

Chapter 8

Concluding remarks

It is clear that MCs are versatile migratory cells that integrate signals derived from a range of interactions – from short-lived and pulsed interactions to long-lived and continuous interactions – which span a broad tuning range for cell-cell communication. Signal intensity and quality are determined by contact duration and frequency, as well as by the signaling codes provided by the immunological synapse, and the timing and identity of co-engaged receptors and released cytokines. Therefore, specific molecular arrangements of the immunological synapse probably have different functions, such as enabling controlled activation, effector function or tolerance. The different modes of MC-communication *in vitro* are important for establishing concepts about signaling requirements and for establishing the kinetics of signaling. The understanding of variation in these cell-cell interactions will provide a molecular understanding of regulated and de-regulated cell-cell communication and could result in novel targeted strategies for immune intervention.

Section V

Experimental procedures

Chapter 9

Materials and Methods

9.1 Mice

C57BL/6 mice were purchased from Harlan Laboratories (Harlan Italy); femurs from C57BL/6 OX40-deficient mice were kindly provided by M. Colombo (Istituto Nazionale Tumori, Milan); femurs from C57BL/6 OX40L-deficient mice were from A. McDonald (University of California, San Francisco); femurs from C57BL/6 CD40L-defiecient mice were gently provided by E. Lutgen (University of Amsterdam, Amsterdam); femurs from C57BL/6 TRPC1/C4/C5/C6-deficient mice were kindly provided from M. Freichel (Universität Heidelberg, Heidelberg).

9.2 Cell preparation and culture conditions

Bone marrow MCs (BMMCs) were obtained from 6–8-week-old mice by *in vitro* differentiation of bone marrow cells taken from mouse femur. Precursor cells were cultivated in RPMI 1640 medium (Euroclone) supplemented with 20% FBS (Euroclone), 100 U/ml penicillin (Euroclone), 100 mg/ml Streptomycin (Euroclone), 2 mM Glutammine (Euroclone), 20 mM Hepes buffer (Euroclone), 1X non essential amminoacid (from 100X mix, Euroclone), 1 mM Sodium Pyruvate (Euroclone), 50 mM β-mercaptoethanol (Sigma Aldrich) and 20 ng/ml IL-3 (Peprotech). After 5 weeks, BMMCs purification was confirmed by flow cytometry with anti-c-kit and anti-FcεRI fluorescence-conjugated antibodies (eBioscences). Purity was usually more than 97% (data acquired with FACScan Cytofluorimeter, Becton Dickinson; data analyzed with FlowJo software).

Peritoneal MCs (PMC) were obtained from 6–8-week-old mice through *ex vivo* peritoneal lavage with 10 ml of HBSS (Euroclone). The mixed peritoneal cells were sedimented by centrifugation at 400 x *g* for 5 minutes and resuspended in 8 ml of 70% isotonic Percoll solution (GE-Healthcare, England). DMEM (Euroclone) was laid on the top and the cells were centrifuged at 580 x *g* for 15 minutes. MCs were recovered at the bottom of the gradient, washed and cultured overnight in complete DMEM supplemented with IL-3 (20 ng/ml, Peprotech). Purification was confirmed by toluidine blue staining and by flow cytometry with anti c-kit and anti FcεRI antibodies (eBioscences). Purity was usually more that 98% (data acquired with FACScan Cytofluorimeter, Becton Dickinson; data analyzed with FlowJo software).

The human LAD2 MC line was kindly provided by A. Kirshenbaum (NIH, Bethesda, MD). The cell line was established from bone marrow aspirates of patient with MC sarcoma leukaemia and is closely related to human MCs [146]. LAD2 cells were grown in serum-free medium StemPro-34 (Invitrogen, Carlsbad, CA) containing 2mM glutamine (Euroclone) and 100 ng/ml human SCF (Peprotech) and were periodically tested for c-kit and FcεRI expression on the cell surface by flow cytometry (anti-human c-kit and anti-human FcεRI conjugated antibody were purchased from Biolegend and Miltenyi Biotec, respectively). Data were acquired with FACScan Cytofluorimeter, Becton Dickinson and analyzed with FlowJo software.

$CD4^+/CD25^+$ cells were purified from 6–8-week-old mice using the $CD25^+$ T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. To determine the percentage of $CD4^+/CD25^+$ purity, cytofluorimetric analysis was performed and Treg population was acquired for its positivity to PE (FL-2 channel).

Human $CD3^+/CD4^+$ T cell were selected from peripheral blood mononuclear cells by immunomagnetic cell sorting (Miltenyi Biotech). The $CD4^+/CD25^{high}$ cells were then purified from the $CD3^+/CD4^+$ T-cell fraction by FACSAria sorter (Becton Dickinson).

Purified splenic B cells were obtained from 6–12-week-old mice as follows: splenocyte cell suspension were depleted of red blood cells by hypotonic lysis with red blood cell lysing buffer (Sigma-Aldrich) and of T cells by complement-mediated cytotoxic lysis using anti-Thy 1.2 mAb (a gift from K. Hathcock, Experimental Immunology Branch, National Cancer Institute/National Institutes of Health, Bethesda, MD) in conjunction with rabbit complement (Low-Tox M; Cedar Lane).

Neutrophils (PMNs) were purified from 6–8-week-old mice femurs using Anti-Ly-6G MicroBead Kit (Miltenyi Biotec) according to the manufacturer's instructions. Cells were fluorescently stained with anti-Gr1-PE (Miltenyi Biotec) and CD11b-FITC (Miltenyi Biotec).

9.3 *In vitro* cells activation

Before experiments, 1 x 10^6/ml murine MCs (BMMCs and PMC) were sensitized in medium without IL-3 for 4 h with 1 µg/ml of DNP-specific IgE and washed twice with Tyrode's buffer (10 mM HEPES buffer [pH 7.4], 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA – Sigma Aldrich). Equal number of murine MCs and $CD4^+/CD25^+$ Tregs (ratio 1:1) were challenged with DNP-HSA (Sigma Aldrich) in Tyrode's buffer/0.05% BSA.
In some experiments, before co-culture with B cells, BMMCs were treated with 50 µg/ml of anti-CD40L blocking antibody (a gift from M. Colombo, Istituto Nazionale Tumori, Milan)

LAD2 cells were overnight pre-sensitized with 1 µg/ml of human myeloma IgE (Chemicon, Millipore) and challenged in Tyrode's buffer/0.05% BSA with 2 µg/ml of anti human IgE (Sigma-Aldrich) in presence or absence of equal number of human $CD4^+/CD25^+$ T cells (ratio 1:1).

Purified B cells were plated at 0.6 x 10^6/well in a 24-well flat bottom plat (Corning Star) at the final concentration of 10^6 cells/ml and cultured for 48 h in the presence or absence of anti-mouse CD40 monoclonal antibody HM40-3 (1 μg/ml, BD Pharmigen) or LPS (20 ng/ml, Sigma Aldrich).

9.4 BMMCs transfection

For transient gene transfection, nucleofection (Amaxa, Lonza) of BMMCs was performed according to the manufacturer's instructions with minor modification. After BMMCs were washed twice in PBS, 4 × 10^6 cells were resuspended in 100 μl mouse macrophage nucleofection solution (Amaxa, Lonza). 4 μg of GFP-GPI and 2 μg of Cherry-STIM1 were added, and the samples were transferred into certified cuvettes (Amaxa, Lonza) and transfected by using the nucleofector program Y-001.
Gene expression was verified at 48 h by cytofluorimetric analysis.

9.5 Synapses Fixation and Confocal Microscopy

5 x 10^4 pre-sensitized MCs were mixed with equal amount of Tregs on microscope slides coated with 0.05 mg/ml poly-L-lysine, and incubated with DNP-HSA (Sigma Aldrich) for 0, 2 or 5 minutes at 37° C. Cells were fixed with 4% paraformaldehyde for 20 minutes, washed and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). For acquisitions, slides were mounted on an inverted confocal microscope (Nikon Confocal Microscopy C1, Nikon), equipped with a 63 x 1.40 oil objective.

9.6 Real time video microscopy and conjugate formation evaluation

The formation of MC-Treg/B cell/PMN conjugates in real time was analyzed by time–lapse epiluminescent microscopy using the Leica AF6000LX system (microscope, DMI6000 B; camera, DFC350FX; software: LAS AF). 0.5 x 10^6 pre-sensitized MCs and 0.5 x 10^6 Tregs/B cells/PMNs (ratio 1:1) were plated on glass bottom Petri dishes (Nunc). The chamber was placed on heating plate pre-warmed at 37°C and DNP was added. Phase contrast images were recorded at indicated time points and resulting video-recorded movies were processed with Photoshop CS3 software. At different time points (1, 5, 20 minutes) the number of MCs in conjugation were counted and the percentage of conjugated-MCs over total MCs per field was calculated. 45±10 MCs were analyzed per condition at indicated time points. For the experiments with Tregs, the mean of percentages ± SD and total number of counted MCs are shown in figures. For the experiments with B cells and PMNs, the mean of percentages ± SEM and total number of counted MCs are shown in figures. Each contact was also monitored for its duration and interactions were classified into 3 categories based on their extend: short (< 5 minutes), medium (5-15 minutes) and long (> 15 minutes). Data represent the proportion of each category.
Before experiments, PMNs were labelled with Fast-DiI (Invitrogen) according to manufacturer's instructions.

For experiment in Fig. 4.2, 5 x 10^4 pre-sensitized MCs were mixed with equal amount of Tregs on microscope slides coated with 0.05 mg/ml poly-L-lysine, and incubated with DNP-HSA (Sigma Aldrich) for 1 or 20 minutes at 37°. Cells were fixed with 4% paraformaldehyde for 20 minutes, washed and mounted with Mowiol. Phase contrast images were acquired and scoring of the slides was performed in a blinded fashion by evaluating for each condition at least 270 MCs in randomly selected fields from 3 independent experiments

9.7 Transmission electron microscopy

4 x 10^6 pre-sensitized BMMCs and 4 x 10^6 Tregs (ratio 1:1) were challenged with 100 ng/ml DNP-HSA (Sigma Aldrich) in Tyrode's buffer/0.05% BSA. After 10 minutes cells were fixed by addition of glutharaldeide to reach 3% final concentration. Cells were kept for 3 hours in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, postfixed in phosphate buffered 1% osmium tetroxide for 1.5 hours, dehydrated in graded ethanol series and embedded in Epon 812. Thin sections were counterstained with uranyl acetate and lead citrate and examined in a Philips CM 12 electron microscope at 80 kV.

9.8 Histamine, β-hexosaminidase, leukotrienes and cytokine release assays

1 x 10^6 pre-sensitized BMMCs and 1 x 10^6 Tregs/B cells (ratio 1:1) were challenged with 100 ng/ml DNP-HSA (Sigma Aldrich) in Tyrode's buffer/0.05% BSA for 30 minutes.
LTC4 was measured by specific immunoassay (GE-Healthcare) while histamine concentration was determined by ELISA according to the producer instruction (DRG Instruments GmbH).
β-hexosaminidase was determined by the cleavage of its synthetic substrate (p-nitrophenyl N-acetyl-glucosamide, Sigma Aldrich) in p-nitrophenol, reaction product with the absorption spectrum between 310 and 500 nm. The amount of chromogenic substrate was measured with an ELISA reader at 405 nm wavelength. The percentage of release was calculated with the following formula:

100 x β-hexosaminidase released /(β-hexosaminidase released + β-hexosaminidase in BMMC lysates)

For cytokine analysis, equal number of IgE-sensitized BMMCs and Tregs were cultured alone or together for 24 h in presence of 100 ng/ml DNP-HSA (Sigma Aldrich). Concentrations of TNF-α, IL-6 and MCP-1 were determined in supernatants using Mouse Inflammation Kit (BD Biosciences). IL-5 and GM-CSF were determined in supernatants using specific ELISA Kit assays (eBiosciences).

9.10 Lipid Rafts Isolation and Western Blot analysis

BMMCs (20 x 10^6) were lysed with 1% Triton X-100 in TNE/P buffer (25 mM Tris/HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin – Sigma Aldrich) for 20 mins at 4°C. Lysates were mixed with ice-cold 80% sucrose in TNE/P buffer and then overlaid onto ice-cold 35% and 5% sucrose in TNE/P buffer and centrifuged at 180000xg for 18 hrs at 4°C. After centrifugation, ~300μl aliquots were harvested and diluted in equal volume of SDS sample buffer and loaded on SDS 10% polyacrilamide gels. After 1 hour and 30 minutes running gels were transferred on a nitrocellulose membrane (Invitrogen) and blotted at 300 V, 90 mA, 4°C for 3 hours and 30 minutes. All antibodies were used by diluting as indicated on datasheet and the membrane was probed at 4°C with gentle shaking O/N for primary antibodies, 1 hour at RT for secondary antibodies. For signal detection, ECL Plus liquids (Thermo Scientific) were used. Antibody to OX40L was provided by R&D Systems, antibody to STIM1 was purchased from Santa Cruz Biotechnology, HRP-conjugated Cholera toxin Subunit B (used for GM1 detection) was from Sigma Aldrich.
WB densitometric analysis was performed using ImageJ software (NIH). Protein content was normalized to GM1. In figure 4.1.2, fold induction of IgE/Ag+sOX40-stimulated BMMCs over IgE/Ag-stimulated BMMCs was calculated. In figure 4.1.4, fold induction of IgE/Ag-stimulated BMMCs over resting BMMCs was calculated.

9.11Statistical analysis

Results are expressed as the means ± SD or mean ± SEM were indicated. Data were analyzed using Student's *t* test (Prism GraphPad Software).

Section VI

References

Chapter 10

References

1 Rao, K. N. & Brown, M. A. Mast cells: multifaceted immune cells with diverse roles in health and disease. *Ann N Y Acad Sci* **1143**, 83-104, doi:NYAS1143023 [pii] 10.1196/annals.1443.023 (2008).

2 Weller, C. L., Collington, S. J., Williams, T. & Lamb, J. R. Mast cells in health and disease. *Clin Sci (Lond)* **120**, 473-484, doi:CS20100459 [pii] 10.1042/CS20100459 (2011).

3 Gurish, M. F. & Boyce, J. A. Mast cells: ontogeny, homing, and recruitment of a unique innate effector cell. *J Allergy Clin Immunol* **117**, 1285-1291, doi:S0091-6749(06)00867-0 [pii] 10.1016/j.jaci.2006.04.017 (2006).

4 Hallgren, J. & Gurish, M. F. Pathways of murine mast cell development and trafficking: tracking the roots and routes of the mast cell. *Immunol Rev* **217**, 8-18, doi:IMR502 [pii] 10.1111/j.1600-065X.2007.00502.x (2007).

5 Welle, M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *J Leukoc Biol* **61**, 233-245 (1997).

6 Wasiuk, A., de Vries, V. C., Hartmann, K., Roers, A. & Noelle, R. J. Mast cells as regulators of adaptive immunity to tumours. *Clin Exp Immunol* **155**, 140-146, doi:CEI3840 [pii] 10.1111/j.1365-2249.2008.03840.x (2009).

7 Moon, T. C. *et al.* Advances in mast cell biology: new understanding of heterogeneity and function. *Mucosal Immunol* **3**, 111-128, doi:mi2009136 [pii] 10.1038/mi.2009.136 (2010).

8 Galli, S. J., Grimbaldeston, M. & Tsai, M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat Rev Immunol* **8**, 478-486, doi:nri2327 [pii] 10.1038/nri2327 (2008).

9 Galli, S. J. *et al.* Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annual review of immunology* **23**, 749-786 (2005).

10 Gri, G. *et al.* Mast cell: an emerging partner in immune interaction. *Front Immunol* **3**, 120, doi:10.3389/fimmu.2012.00120 (2012).

11 Abraham, S. N. & St John, A. L. Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol* **10**, 440-452, doi:nri2782 [pii]

10.1038/nri2782 (2010).
12 Logan, M. R., Odemuyiwa, S. O. & Moqbel, R. Understanding exocytosis in immune and inflammatory cells: the molecular basis of mediator secretion. *J Allergy Clin Immunol* **111**, 923-932; quiz 933, doi:S0091674903014295 [pii] (2003).
13 Crivellato, E., Beltrami, C. A., Mallardi, F. & Ribatti, D. The mast cell: an active participant or an innocent bystander? *Histol Histopathol* **19**, 259-270 (2004).
14 Melo, R. C. & Weller, P. F. Piecemeal degranulation in human eosinophils: a distinct secretion mechanism underlying inflammatory responses. *Histol Histopathol* **25**, 1341-1354 (2010).
15 Dvorak, A. M. Basophils and mast cells: piecemeal degranulation in situ and ex vivo: a possible mechanism for cytokine-induced function in disease. *Immunol Ser* **57**, 169-271 (1992).
16 Dvorak, A. M. *et al.* Ultrastructural evidence for piecemeal and anaphylactic degranulation of human gut mucosal mast cells in vivo. *Int Arch Allergy Immunol* **99**, 74-83 (1992).
17 Theoharides, T. C., Kempuraj, D., Tagen, M., Conti, P. & Kalogeromitros, D. Differential release of mast cell mediators and the pathogenesis of inflammation. *Immunol Rev* **217**, 65-78, doi:IMR519 [pii]
10.1111/j.1600-065X.2007.00519.x (2007).
18 Hines, C. The diverse effects of mast cell mediators. *Clin Rev Allergy Immunol* **22**, 149-160, doi:CRIAI:22:2:149 [pii]
10.1385/CRIAI:22:2:149 (2002).
19 Metz, M., Siebenhaar, F. & Maurer, M. Mast cell functions in the innate skin immune system. *Immunobiology* **213**, 251-260, doi:S0171-2985(07)00136-2 [pii]
10.1016/j.imbio.2007.10.017 (2008).
20 Blank, U. & Rivera, J. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol* **25**, 266-273, doi:10.1016/j.it.2004.03.005
S1471490604000973 [pii] (2004).
21 Gilfillan, A. M. & Tkaczyk, C. Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol* **6**, 218-230, doi:nri1782 [pii]
10.1038/nri1782 (2006).
22 Stassen, M., Hultner, L. & Schmitt, E. Classical and alternative pathways of mast cell activation. *Crit Rev Immunol* **22**, 115-140 (2002).
23 Gilfillan, A. M. & Rivera, J. The tyrosine kinase network regulating mast cell activation. *Immunol Rev* **228**, 149-169, doi:IMR742 [pii]
10.1111/j.1600-065X.2008.00742.x (2009).
24 Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* **1**, 31-39, doi:10.1038/35036052
35036052 [pii] (2000).
25 Kovarova, M. *et al.* Structure-function analysis of Lyn kinase association with lipid rafts and initiation of early signaling events after Fcepsilon receptor I aggregation. *Mol Cell Biol* **21**, 8318-8328, doi:10.1128/MCB.21.24.8318-8328.2001 (2001).
26 Kovarova, M. *et al.* Cholesterol deficiency in a mouse model of Smith-Lemli-Opitz syndrome reveals increased mast cell responsiveness. *J Exp Med* **203**, 1161-1171, doi:jem.20051701 [pii]
10.1084/jem.20051701 (2006).
27 Puri, N. & Roche, P. A. Ternary SNARE complexes are enriched in lipid rafts during mast cell exocytosis. *Traffic* **7**, 1482-1494, doi:TRA490 [pii]
10.1111/j.1600-0854.2006.00490.x (2006).
28 Brown, D. A. Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology (Bethesda)* **21**, 430-439, doi:21/6/430 [pii]
10.1152/physiol.00032.2006 (2006).
29 Holowka, D. & Baird, B. Fc(epsilon)RI as a paradigm for a lipid raft-dependent receptor in hematopoietic cells. *Semin Immunol* **13**, 99-105, doi:10.1006/smim.2000.0301
S1044532300903014 [pii] (2001).
30 Kalesnikoff, J. & Galli, S. J. New developments in mast cell biology. *Nat Immunol* **9**, 1215-1223, doi:ni.f.216 [pii]
10.1038/ni.f.216 (2008).
31 Nadler, M. J. & Kinet, J. P. Uncovering new complexities in mast cell signaling. *Nat Immunol* **3**, 707-708, doi:10.1038/ni0802-707
ni0802-707 [pii] (2002).
32 Parravicini, V. *et al.* Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat Immunol* **3**, 741-748, doi:10.1038/ni817
ni817 [pii] (2002).

33 Rivera, J. *et al.* Macromolecular protein signaling complexes and mast cell responses: a view of the organization of IgE-dependent mast cell signaling. *Mol Immunol* **38**, 1253-1258, doi:S016158900200072X [pii] (2002).

34 Vig, M. *et al.* Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. *Nat Immunol* **9**, 89-96, doi:ni1550 [pii]
10.1038/ni1550 (2008).

35 Vig, M. & Kinet, J. P. Calcium signaling in immune cells. *Nat Immunol* **10**, 21-27, doi:ni.f.220 [pii]
10.1038/ni.f.220 (2009).

36 Putney, J. W., Jr. A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1-12 (1986).

37 Liou, J. *et al.* STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol* **15**, 1235-1241, doi:S0960-9822(05)00570-1 [pii]
10.1016/j.cub.2005.05.055 (2005).

38 Baba, Y. & Kurosaki, T. [Regulation of store-operated calcium entry by STIM1]. *Seikagaku* **80**, 1123-1128 (2008).

39 Barr, V. A., Bernot, K. M., Shaffer, M. H., Burkhardt, J. K. & Samelson, L. E. Formation of STIM and Orai complexes: puncta and distal caps. *Immunol Rev* **231**, 148-159, doi:IMR812 [pii]
10.1111/j.1600-065X.2009.00812.x (2009).

40 Yuan, J. P., Zeng, W., Huang, G. N., Worley, P. F. & Muallem, S. STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat Cell Biol* **9**, 636-645, doi:ncb1590 [pii]
10.1038/ncb1590 (2007).

41 Ma, H. T. & Beaven, M. A. Regulation of Ca2+ signaling with particular focus on mast cells. *Crit Rev Immunol* **29**, 155-186, doi:0772bd76689c273e,6c775c40425e272d [pii] (2009).

42 Suzuki, R. *et al.* Loss of TRPC1-mediated Ca2+ influx contributes to impaired degranulation in Fyn-deficient mouse bone marrow-derived mast cells. *J Leukoc Biol* **88**, 863-875, doi:jlb.0510253 [pii]
10.1189/jlb.0510253 (2010).

43 Pani, B., Bollimuntha, S. & Singh, B. B. The TR (i)P to Ca(2)(+) signaling just got STIMy: an update on STIM1 activated TRPC channels. *Front Biosci* **17**, 805-823, doi:3958 [pii] (2012).

44 Castle, J. D., Guo, Z. & Liu, L. Function of the t-SNARE SNAP-23 and secretory carrier membrane proteins (SCAMPs) in exocytosis in mast cells. *Mol Immunol* **38**, 1337-1340, doi:S0161589002000846 [pii] (2002).

45 Nishida, K. *et al.* Fc{epsilon}RI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. *J Cell Biol* **170**, 115-126, doi:jcb.200501111 [pii]
10.1083/jcb.200501111 (2005).

46 Gomez, G. *et al.* Impaired FcepsilonRI-dependent gene expression and defective eicosanoid and cytokine production as a consequence of Fyn deficiency in mast cells. *J Immunol* **175**, 7602-7610, doi:175/11/7602 [pii] (2005).

47 Hernandez-Hansen, V. *et al.* Dysregulated FcepsilonRI signaling and altered Fyn and SHIP activities in Lyn-deficient mast cells. *J Immunol* **173**, 100-112 (2004).

48 Rivera, J. & Gilfillan, A. M. Molecular regulation of mast cell activation. *J Allergy Clin Immunol* **117**, 1214-1225; quiz 1226, doi:S0091-6749(06)00863-3 [pii]
10.1016/j.jaci.2006.04.015 (2006).

49 Galli, S. J. & Tsai, M. Mast cells in allergy and infection: versatile effector and regulatory cells in innate and adaptive immunity. *Eur J Immunol* **40**, 1843-1851, doi:10.1002/eji.201040559 (2010).

50 Rodewald, H. R. & Feyerabend, T. B. Widespread immunological functions of mast cells: fact or fiction? *Immunity* **37**, 13-24, doi:S1074-7613(12)00288-9 [pii]
10.1016/j.immuni.2012.07.007 (2012).

51 Maurer, M. *et al.* What is the physiological function of mast cells? *Exp Dermatol* **12**, 886-910 (2003).

52 Beaven, M. A. Our perception of the mast cell from Paul Ehrlich to now. *Eur J Immunol* **39**, 11-25, doi:10.1002/eji.200838899 (2009).

53 Vitale, M. Time for integration: communication in the immune system. *Acta Biomed* **78 Suppl 1**, 227-230 (2007).

54 Bachelet, I., Munitz, A., Mankutad, D. & Levi-Schaffer, F. Mast cell costimulation by CD226/CD112 (DNAM-1/Nectin-2): a novel interface in the allergic process. *J Biol Chem* **281**, 27190-27196, doi:M602359200 [pii]

10.1074/jbc.M602359200 (2006).
55 Friedl, P., den Boer, A. T. & Gunzer, M. Tuning immune responses: diversity and adaptation of the immunological synapse. *Nat Rev Immunol* **5**, 532-545, doi:nri1647 [pii] 10.1038/nri1647 (2005).
56 Dustin, M. L. The immunological synapse. *Arthritis Res* **4 Suppl 3**, S119-125 (2002).
57 Davis, D. M. Mechanisms and functions for the duration of intercellular contacts made by lymphocytes. *Nat Rev Immunol* **9**, 543-555, doi:nri2602 [pii] 10.1038/nri2602 (2009).
58 Skokos, D., Goubran-Botros, H., Roa, M. & Mecheri, S. Immunoregulatory properties of mast cell-derived exosomes. *Mol Immunol* **38**, 1359-1362, doi:S0161589002000883 [pii] (2002).
59 Dawicki, W., Jawdat, D. W., Xu, N. & Marshall, J. S. Mast cells, histamine, and IL-6 regulate the selective influx of dendritic cell subsets into an inflamed lymph node. *J Immunol* **184**, 2116-2123, doi:jimmunol.0803894 [pii] 10.4049/jimmunol.0803894 (2010).
60 Jawdat, D. M., Albert, E. J., Rowden, G., Haidl, I. D. & Marshall, J. S. IgE-mediated mast cell activation induces Langerhans cell migration in vivo. *J Immunol* **173**, 5275-5282, doi:173/8/5275 [pii] (2004).
61 Suto, H. *et al.* Mast cell-associated TNF promotes dendritic cell migration. *J Immunol* **176**, 4102-4112, doi:176/7/4102 [pii] (2006).
62 Skokos, D. *et al.* Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo. *J Immunol* **170**, 3037-3045 (2003).
63 Kitawaki, T. *et al.* IgE-activated mast cells in combination with pro-inflammatory factors induce Th2-promoting dendritic cells. *Int Immunol* **18**, 1789-1799, doi:dxl113 [pii] 10.1093/intimm/dxl113 (2006).
64 Dudeck, A., Suender, C. A., Kostka, S. L., von Stebut, E. & Maurer, M. Mast cells promote Th1 and Th17 responses by modulating dendritic cell maturation and function. *Eur J Immunol* **41**, 1883-1893, doi:10.1002/eji.201040994 (2011).
65 Mazzoni, A., Siraganian, R. P., Leifer, C. A. & Segal, D. M. Dendritic cell modulation by mast cells controls the Th1/Th2 balance in responding T cells. *J Immunol* **177**, 3577-3581, doi:177/6/3577 [pii] (2006).
66 Otsuka, A. *et al.* Requirement of interaction between mast cells and skin dendritic cells to establish contact hypersensitivity. *PLoS One* **6**, e25538, doi:10.1371/journal.pone.0025538 PONE-D-11-08365 [pii] (2011).
67 St John, A. L. *et al.* Immune surveillance by mast cells during dengue infection promotes natural killer (NK) and NKT-cell recruitment and viral clearance. *Proc Natl Acad Sci U S A* **108**, 9190-9195, doi:1105079108 [pii] 10.1073/pnas.1105079108 (2011).
68 Burke, S. M. *et al.* Human mast cell activation with virus-associated stimuli leads to the selective chemotaxis of natural killer cells by a CXCL8-dependent mechanism. *Blood* **111**, 5467-5476, doi:blood-2007-10-118547 [pii] 10.1182/blood-2007-10-118547 (2008).
69 Oldford, S. A. *et al.* A critical role for mast cells and mast cell-derived IL-6 in TLR2-mediated inhibition of tumor growth. *J Immunol* **185**, 7067-7076, doi:jimmunol.1001137 [pii] 10.4049/jimmunol.1001137 (2010).
70 Vosskuhl, K., Greten, T. F., Manns, M. P., Korangy, F. & Wedemeyer, J. Lipopolysaccharide-mediated mast cell activation induces IFN-gamma secretion by NK cells. *J Immunol* **185**, 119-125, doi:jimmunol.0902406 [pii] 10.4049/jimmunol.0902406 (2010).
71 Minai-Fleminger, Y. & Levi-Schaffer, F. Mast cells and eosinophils: the two key effector cells in allergic inflammation. *Inflamm Res* **58**, 631-638, doi:10.1007/s00011-009-0042-6 (2009).
72 Wong, C. K., Ng, S. S., Lun, S. W., Cao, J. & Lam, C. W. Signalling mechanisms regulating the activation of human eosinophils by mast-cell-derived chymase: implications for mast cell-eosinophil interaction in allergic inflammation. *Immunology* **126**, 579-587, doi:IMM2916 [pii] 10.1111/j.1365-2567.2008.02916.x (2009).
73 Caruso, R. A., Fedele, F., Zuccala, V., Fracassi, M. G. & Venuti, A. Mast cell and eosinophil interaction in gastric carcinomas: ultrastructural observations. *Anticancer Res* **27**, 391-394 (2007).
74 Piazuelo, M. B. *et al.* Eosinophils and mast cells in chronic gastritis: possible implications in carcinogenesis. *Hum Pathol* **39**, 1360-1369, doi:S0046-8177(08)00058-0 [pii] 10.1016/j.humpath.2008.01.012 (2008).

75 Beil, W. J. *et al.* Selective alterations in mast cell subsets and eosinophil infiltration in two complementary types of intestinal inflammation: ascariasis and Crohn's disease. *Pathobiology* **70**, 303-313, doi:71270 71270 [pii] (2002).

76 Minai-Fleminger, Y. *et al.* Ultrastructural evidence for human mast cell-eosinophil interactions in vitro. *Cell Tissue Res* **341**, 405-415, doi:10.1007/s00441-010-1010-8 (2010).

77 Elishmereni, M., Bachelet, I., Ben-Efraim, A. H., Mankuta, D. & Levi-Schaffer, F. Interacting mast cells and eosinophils acquire an enhanced activation state in vitro. *Allergy* **68**, 171-179, doi:10.1111/all.12059 (2013).

78 Kumar, V. & Sharma, A. Neutrophils: Cinderella of innate immune system. *Int Immunopharmacol* **10**, 1325-1334, doi:S1567-5769(10)00266-3 [pii] 10.1016/j.intimp.2010.08.012 (2010).

79 Fridlender, Z. G. *et al.* Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* **16**, 183-194, doi:S1535-6108(09)00215-3 [pii] 10.1016/j.ccr.2009.06.017 (2009).

80 Mantovani, A., Cassatella, M. A., Costantini, C. & Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* **11**, 519-531, doi:nri3024 [pii] 10.1038/nri3024 (2011).

81 Zhang, Y., Ramos, B. F. & Jakschik, B. A. Mast cells enhance the antibody-mediated injury of skin basement membrane in mice. *J Immunol* **149**, 2482-2487 (1992).

82 Ramos, B. F., Zhang, Y., Qureshi, R. & Jakschik, B. A. Mast cells are critical for the production of leukotrienes responsible for neutrophil recruitment in immune complex-induced peritonitis in mice. *J Immunol* **147**, 1636-1641 (1991).

83 Caughey, G. H. Mast cell tryptases and chymases in inflammation and host defense. *Immunol Rev* **217**, 141-154, doi:IMR509 [pii] 10.1111/j.1600-065X.2007.00509.x (2007).

84 Wang, Y. & Thorlacius, H. Mast cell-derived tumour necrosis factor-alpha mediates macrophage inflammatory protein-2-induced recruitment of neutrophils in mice. *Br J Pharmacol* **145**, 1062-1068, doi:0706274 [pii] 10.1038/sj.bjp.0706274 (2005).

85 Gauchat, J. F. *et al.* Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* **365**, 340-343, doi:10.1038/365340a0 (1993).

86 Pawankar, R., Okuda, M., Yssel, H., Okumura, K. & Ra, C. Nasal mast cells in perennial allergic rhinitics exhibit increased expression of the Fc epsilonRI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. *J Clin Invest* **99**, 1492-1499, doi:10.1172/JCI119311 (1997).

87 Tkaczyk, C. *et al.* Mouse bone marrow-derived mast cells and mast cell lines constitutively produce B cell growth and differentiation activities. *J Immunol* **157**, 1720-1728 (1996).

88 Merluzzi, S. *et al.* Mast cells enhance proliferation of B lymphocytes and drive their differentiation toward IgA-secreting plasma cells. *Blood* **115**, 2810-2817, doi:blood-2009-10-250126 [pii] 10.1182/blood-2009-10-250126 (2010).

89 Mekori, Y. A. & Metcalfe, D. D. Mast cell-T cell interactions. *J Allergy Clin Immunol* **104**, 517-523, doi:S0091674999005072 [pii] (1999).

90 Dvorak, A. M., Mihm, M. C., Jr. & Dvorak, H. F. Morphology of delayed-type hypersensitivity reactions in man. II. Ultrastructural alterations affecting the microvasculature and the tissue mast cells. *Lab Invest* **34**, 179-191 (1976).

91 Waldorf, H. A., Walsh, L. J., Schechter, N. M. & Murphy, G. F. Early cellular events in evolving cutaneous delayed hypersensitivity in humans. *Am J Pathol* **138**, 477-486 (1991).

92 Bjermer, L., Engstrom-Laurent, A., Thunell, M. & Hallgren, R. The mast cell and signs of pulmonary fibroblast activation in sarcoidosis. *Int Arch Allergy Appl Immunol* **82**, 298-301 (1987).

93 Malone, D. G., Irani, A. M., Schwartz, L. B., Barrett, K. E. & Metcalfe, D. D. Mast cell numbers and histamine levels in synovial fluids from patients with diverse arthritides. *Arthritis Rheum* **29**, 956-963 (1986).

94 Marsh, M. N. & Hinde, J. Inflammatory component of celiac sprue mucosa. I. Mast cells, basophils, and eosinophils. *Gastroenterology* **89**, 92-101, doi:S0016508585001962 [pii] (1985).

95 Friedman, M. M. & Kaliner, M. In situ degranulation of human nasal mucosal mast cells: ultrastructural features and cell-cell associations. *J Allergy Clin Immunol* **76**, 70-82, doi:0091-6749(85)90807-3 [pii] (1985).

96 Smith, C. A. & Rennick, D. M. Characterization of a murine lymphokine distinct from interleukin 2 and interleukin 3 (IL-3) possessing a T-cell growth factor activity and a mast-cell growth factor activity that synergizes with IL-3. *Proc Natl Acad Sci U S A* **83**, 1857-1861 (1986).

97 Oh, C. K. & Metcalfe, D. D. Activated lymphocytes induce promoter activity of the TCA3 gene in mast cells following cell-to-cell contact. *Biochem Biophys Res Commun* **221**, 510-514, doi:S0006-291X(96)90627-0 [pii]
10.1006/bbrc.1996.0627 (1996).

98 Baram, D., Mekori, Y. A. & Sagi-Eisenberg, R. Synaptotagmin regulates mast cell functions. *Immunol Rev* **179**, 25-34 (2001).

99 Brill, A. *et al.* Induction of mast cell interactions with blood vessel wall components by direct contact with intact T cells or T cell membranes in vitro. *Clin Exp Allergy* **34**, 1725-1731, doi:CEA2093 [pii]
10.1111/j.1365-2222.2004.02093.x (2004).

100 Shefler, I., Salamon, P., Reshef, T., Mor, A. & Mekori, Y. A. T cell-induced mast cell activation: a role for microparticles released from activated T cells. *J Immunol* **185**, 4206-4212, doi:jimmunol.1000409 [pii]
10.4049/jimmunol.1000409 (2010).

101 Inamura, N., Mekori, Y. A., Bhattacharyya, S. P., Bianchine, P. J. & Metcalfe, D. D. Induction and enhancement of Fc(epsilon)RI-dependent mast cell degranulation following coculture with activated T cells: dependency on ICAM-1- and leukocyte function-associated antigen (LFA)-1-mediated heterotypic aggregation. *J Immunol* **160**, 4026-4033 (1998).

102 Stopfer, P., Mannel, D. N. & Hehlgans, T. Lymphotoxin-beta receptor activation by activated T cells induces cytokine release from mouse bone marrow-derived mast cells. *J Immunol* **172**, 7459-7465, doi:172/12/7459 [pii] (2004).

103 Nakae, S. *et al.* Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. *J Immunol* **176**, 2238-2248, doi:176/4/2238 [pii] (2006).

104 Kashiwakura, J., Yokoi, H., Saito, H. & Okayama, Y. T cell proliferation by direct crosstalk between OX40 ligand on human mast cells and OX40 on human T cells: comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. *J Immunol* **173**, 5247-5257, doi:173/8/5247 [pii] (2004).

105 Valitutti, S. & Espinosa, E. Cognate interactions between mast cells and helper T lymphocytes. *Self Nonself* **1**, 114-122, doi:10.4161/self.1.2.11795 (2010).

106 Gaudenzio, N. *et al.* Cell-cell cooperation at the T helper cell/mast cell immunological synapse. *Blood* **114**, 4979-4988, doi:blood-2009-02-202648 [pii]
10.1182/blood-2009-02-202648 (2009).

107 Fox, C. C., Jewell, S. D. & Whitacre, C. C. Rat peritoneal mast cells present antigen to a PPD-specific T cell line. *Cell Immunol* **158**, 253-264, doi:S0008-8749(84)71272-X [pii]
10.1006/cimm.1994.1272 (1994).

108 Poncet, P., Arock, M. & David, B. MHC class II-dependent activation of CD4+ T cell hybridomas by human mast cells through superantigen presentation. *J Leukoc Biol* **66**, 105-112 (1999).

109 Kambayashi, T. *et al.* Inducible MHC class II expression by mast cells supports effector and regulatory T cell activation. *J Immunol* **182**, 4686-4695, doi:182/8/4686 [pii]
10.4049/jimmunol.0803180 (2009).

110 Nakano, N. *et al.* Notch signaling confers antigen-presenting cell functions on mast cells. *The Journal of allergy and clinical immunology* **123**, 74-81 e71 (2009).

111 Malaviya, R., Ikeda, T., Ross, E. & Abraham, S. N. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature* **381**, 77-80, doi:10.1038/381077a0 (1996).

112 Stelekati, E. *et al.* Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions. *Immunity* **31**, 665-676, doi:S1074-7613(09)00409-9 [pii]
10.1016/j.immuni.2009.08.022 (2009).

113 Gri, G. *et al.* CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity* **29**, 771-781 (2008).

114 Vliagoftis, H. & Befus, A. D. Rapidly changing perspectives about mast cells at mucosal surfaces. *Immunol Rev* **206**, 190-203, doi:IMR279 [pii]
10.1111/j.0105-2896.2005.00279.x (2005).

115 Lu, L. F. *et al.* Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* **442**, 997-1002, doi:nature05010 [pii]
10.1038/nature05010 (2006).

116 Eller, K. *et al.* IL-9 production by regulatory T cells recruits mast cells that are essential for regulatory T cell-induced immune suppression. *J Immunol* **186**, 83-91, doi:jimmunol.1001183 [pii]
10.4049/jimmunol.1001183 (2011).
117 Feng, L. L., Gao, J. M., Li, P. P. & Wang, X. IL-9 contributes to immunosuppression mediated by regulatory T cells and mast cells in B-cell non-hodgkin's lymphoma. *J Clin Immunol* **31**, 1084-1094, doi:10.1007/s10875-011-9584-9 (2011).
118 Yang, Z. *et al.* Mast cells mobilize myeloid-derived suppressor cells and Treg cells in tumor microenvironment via IL-17 pathway in murine hepatocarcinoma model. *PLoS One* **5**, e8922, doi:10.1371/journal.pone.0008922 (2010).
119 Hershko, A. Y. *et al.* Mast cell interleukin-2 production contributes to suppression of chronic allergic dermatitis. *Immunity* **35**, 562-571, doi:S1074-7613(11)00396-7 [pii]
10.1016/j.immuni.2011.07.013 (2011).
120 Gounaris, E. *et al.* T-regulatory cells shift from a protective anti-inflammatory to a cancer-promoting proinflammatory phenotype in polyposis. *Cancer Res* **69**, 5490-5497, doi:69/13/5490 [pii]
10.1158/0008-5472.CAN-09-0304 (2009).
121 Piconese, S. *et al.* Mast cells counteract regulatory T-cell suppression through interleukin-6 and OX40/OX40L axis toward Th17-cell differentiation. *Blood* **114**, 2639-2648, doi:blood-2009-05-220004 [pii]
10.1182/blood-2009-05-220004 (2009).
122 Forward, N. A., Furlong, S. J., Yang, Y., Lin, T. J. & Hoskin, D. W. Mast cells down-regulate CD4+CD25+ T regulatory cell suppressor function via histamine H1 receptor interaction. *J Immunol* **183**, 3014-3022, doi:jimmunol.0802509 [pii]
10.4049/jimmunol.0802509 (2009).
123 Kashyap, M. *et al.* Cutting edge: CD4 T cell-mast cell interactions alter IgE receptor expression and signaling. *J Immunol* **180**, 2039-2043, doi:180/4/2039 [pii] (2008).
124 Ganeshan, K. & Bryce, P. J. Regulatory T cells enhance mast cell production of IL-6 via surface-bound TGF-beta. *J Immunol* **188**, 594-603, doi:jimmunol.1102389 [pii]
10.4049/jimmunol.1102389 (2012).
125 Blatner, N. R. *et al.* In colorectal cancer mast cells contribute to systemic regulatory T-cell dysfunction. *Proc Natl Acad Sci U S A* **107**, 6430-6435, doi:0913683107 [pii]
10.1073/pnas.0913683107 (2010).
126 Sibilano, R. *et al.* Technical advance: soluble OX40 molecule mimics regulatory T cell modulatory activity on FcepsilonRI-dependent mast cell degranulation. *J Leukoc Biol* **90**, 831-838, doi:jlb.1210651 [pii]
10.1189/jlb.1210651 (2011).
127 Sibilano, R. *et al.* Modulation of FcepsilonRI-dependent mast cell response by OX40L via Fyn, PI3K, and RhoA. *J Allergy Clin Immunol* **130**, 751-760 e752, doi:S0091-6749(12)00537-4 [pii]
10.1016/j.jaci.2012.03.032 (2012).
128 Irani, A. M. & Schwartz, L. B. Mast cell heterogeneity. *Clin Exp Allergy* **19**, 143-155 (1989).
129 Irani, A. M. & Schwartz, L. B. Human mast cell heterogeneity. *Allergy Proc* **15**, 303-308 (1994).
130 Melo, R. C., Spencer, L. A., Dvorak, A. M. & Weller, P. F. Mechanisms of eosinophil secretion: large vesiculotubular carriers mediate transport and release of granule-derived cytokines and other proteins. *J Leukoc Biol* **83**, 229-236 (2008).
131 Gordon, J. R. & Galli, S. J. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *The Journal of experimental medicine* **174**, 103-107 (1991).
132 Gonzalez-Espinosa, C. *et al.* Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells. *J Exp Med* **197**, 1453-1465, doi:10.1084/jem.20021806
jem.20021806 [pii] (2003).
133 LeBien, T. W. & Tedder, T. F. B lymphocytes: how they develop and function. *Blood* **112**, 1570-1580, doi:112/5/1570 [pii]
10.1182/blood-2008-02-078071 (2008).
134 Satodate, R., Schwarze, E. W. & Lennert, K. [Tissue mast cell count in immunocytoma and chronic lymphocytic leukaemia (author's transl)]. *Virchows Arch A Pathol Anat Histol* **373**, 303-309 (1977).
135 Galinsky, D. S. & Nechushtan, H. Mast cells and cancer–no longer just basic science. *Crit Rev Oncol Hematol* **68**, 115-130, doi:S1040-8428(08)00114-5 [pii]

10.1016/j.critrevonc.2008.06.001 (2008).
136 Tournilhac, O. *et al.* Mast cells in Waldenstrom's macroglobulinemia support lymphoplasmacytic cell growth through CD154/CD40 signaling. *Ann Oncol* **17**, 1275-1282, doi:mdl109 [pii]
10.1093/annonc/mdl109 (2006).
137 Molin, D. *et al.* Mast cell infiltration correlates with poor prognosis in Hodgkin's lymphoma. *Br J Haematol* **119**, 122-124, doi:3768 [pii] (2002).
138 Lee, B. O. *et al.* CD40, but not CD154, expression on B cells is necessary for optimal primary B cell responses. *J Immunol* **171**, 5707-5717 (2003).
139 Franceschini, D. *et al.* PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. *The Journal of clinical investigation* **119**, 551-564 (2009).
140 Francisco, L. M. *et al.* PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *The Journal of experimental medicine* **206**, 3015-3029 (2009).
141 Keir, M. E., Butte, M. J., Freeman, G. J. & Sharpe, A. H. PD-1 and its ligands in tolerance and immunity. *Annual review of immunology* **26**, 677-704 (2008).
142 Asano, N., Watanabe, T., Kitani, A., Fuss, I. J. & Strober, W. Notch1 signaling and regulatory T cell function. *J Immunol* **180**, 2796-2804 (2008).
143 Dvorak, A. M. Piecemeal degranulation of basophils and mast cells is effected by vesicular transport of stored secretory granule contents. *Chem Immunol Allergy* **85**, 135-184 (2005).
144 Dvorak, A. M. Degranulation and recovery from degranulation of basophils and mast cells. *Chem Immunol Allergy* **85**, 205-251 (2005).
145 Brown, G. C. & Neher, J. J. Eaten alive! Cell death by primary phagocytosis: 'phagoptosis'. *Trends Biochem Sci* **37**, 325-332, doi:S0968-0004(12)00070-9 [pii]
10.1016/j.tibs.2012.05.002 (2012).
146 Kirshenbaum, A. S. *et al.* Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leukemia research* **27**, 677-682 (2003).

Section VII

Publications

Chapter 11

Publications

1. Frossi B*, **D'Incà F***, Crivellato E, Sibilano R, Gri G, Mongillo M, Danelli L, Maggi L, Pucillo CE. *Single-cell dynamics of mast cell-CD4+ CD25+ regulatory T cell interactions*. **Eur J Immunol**. 2011 Jul;41(7):1872-82.

2. Gri G, Frossi B, **D'Incà F**, Danelli L**,** Betto E, Mion F, Sibilano R, Pucillo C. *Mast cell: an emerging partner in immune interaction*. **Front Immunol**. 2012;3:120.

3. Sibilano R, Frossi B, Suzuki R, **D'Incà F**, Gri G, Piconese S, Colombo MP, Rivera J, Pucillo CE. *Modulation of FcεRI-dependent mast cell response by OX40L via Fyn, PI3K, and RhoA*. **J Allergy Clin Immunol**. 2012 Sep;130(3):751-760.

4. Mion F, **D'Incà F**, Danelli L, Toffoletto B, Gerbert N, Guarnotta C, Frossi B, Burocchi A, Lutgens E, Tripodo C, Colombo MP, Rivera J, Vitale G, Pucillo CE. *Mast cells contribute to the expansion and differentiation of IL-10^{+} regulatory B cells*. Manuscript in preparation.

* B.F. and D.F. contributed equally to this study.

Section VIII

Acknowledgments

Chapter 12

Acknowledgements

Firstly, I would like to thank my supervisor, Pr. Carlo Pucillo for accepting me as his PhD student. I consider myself extremely lucky to have had the chance to work with him.

The present work is the result of the collaboration with a number of researchers.

A very special thanks to Prof. Silvia Bulfone-Paus and Dr. Hilary Sanding; their hospitality, expertise and critical thinking added considerably to my graduate experience.
An acknowledgement to Prof. Francesca Levi-Shaffer for her advice during these years.

I am heartily grateful to Dr. Barbara Frossi; her scientific knowledge, kindness and scholarship were fundamental to my academic and personal growth.

A further thanks to Dr. Gaetano Vitale, Dr. Giorgia Gri and Dr. Elena Betto for the information exchanges that have been very relevant to improve my knowledge.

I would like to extend my deepest gratitude to Riccardo, Francesca, Luca, Raffaella and Alice, whose collaboration, help and friendship have been essential in my PhD experience. Thanks for making my working days very pleasant.

Last but not least, I would like to thank my families and friends for their constant and precious support, encouragement and patience during these years. Thanks for your smile. Thank you for making me smile.