MAST CELLS IN CHRONIC INFLAMMATORY DISEASES

New insights in mast cell function and phenotype

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Stockholm 2008
Não sou nada. 
Nunca serei nada. 
Não posso querer ser nada. 
À parte isso, tenho em mim todos os sonhos do mundo.

I’m not nothing. 
I’ll never be nothing 
I can’t want to be anything. 
Apart from that, I have in me all the dreams in the world.

Fernando Pessoa
ABSTRACT

Mast cells are multifunctional cells that play important roles in health and disease. They are long lived cells with the capacity to be activated several times. Upon activation mast cells release a huge array of mediators. The nature of the mediators released is dependent on the stimuli. The microenvironment in which the mast cell resides determines its phenotype and modulates its function. The aims of this thesis were to study the phenotype and role of mast cells in different chronic inflammatory diseases, particularly the importance of activation via CD30L/CD153 and the effects of hypoxia on mast cells.

It has previously been shown that cells expressing CD30L can be activated by CD30 and that CD30 is associated with different inflammatory diseases, such as Hodgkin’s lymphoma. Here we show that mast cells can be activated via CD30L with a CD30-Fc fusion protein, secreting chemokines in a degranulation-independent mechanism. We also provide evidence for an involvement of the MAPK and PI3K pathways in the signalling downstream of CD30L. Furthermore, we show that mast cells are the predominant CD30L expressing cells in atopic dermatitis and psoriasis and that IL-8 expressing mast cells are upregulated in lesional skin from both diseases. IL-8 secretion by mast cells was induced in CD30-treated healthy skin cultures.

We next extended these findings to another chronic inflammatory disease, rheumatoid arthritis. In some studies mast cells have been implicated in the pathogenesis of the disease. The soluble form of CD30 is increased in the serum and synovial fluid of rheumatic patients, however there are no reports regarding the soluble form of CD30L in the serum or synovial fluid or the expression of CD30L in the synovium. We found a strong correlation between elevated levels of soluble CD30L in the serum and synovial fluid of patients suggesting a common origin for the molecule. A CD30L+ cell population was found in biopsies of the rheumatic synovium, but in contrast to what we observed in the first study, mast cells were not the CD30L predominant expressing cells.

Hypoxic areas are found in different inflammatory conditions. In situations of low oxygen tension some cells die while others change their metabolism and adapt to the hypoxic condition. In the previous studies we discussed the involvement of mast cells in different chronic inflammatory diseases. In this study we show that mast cells regulate their survival to hypoxia in an autocrine way by secreting IL-6. In response to hypoxia, mast cells also secrete the proinflammatory cytokine TNF-α. Furthermore, we show that hypoxia does not affect degranulation and that the response to different activators is still sustained, although attenuated, under hypoxia when compared to normoxic conditions. These results suggest that mast cells are capable of resisting hypoxia and still respond to different stimuli even under hypoxic conditions, which can have important consequences in health and disease.

In the last study presented in this thesis we validated the method used in previous studies and in the projects included in this thesis to identify human mast cells in vivo. Tryptase is the most abundant protein in mast cell granules and is frequently used as a mast cell marker in vivo. Here we show that different Hodgkin lymphoma cell lines also express tryptase in vitro. In vivo the tryptase expression is almost exclusively restricted to mast cells and the rare tryptase positive Hodgkin and Reed Stenberg cells are easily distinguished from mast cells by their characteristic morphology.

In conclusion, this thesis elucidates some aspects of the function of mast cells in different chronic inflammatory diseases.
LIST OF PUBLICATIONS
This thesis is based on the following articles


*These authors contributed equally to the work

Publications by the author not included in this thesis:

1. Rose-Marie Amini, Kirsimari Aaltonen, Heli Nevanlinna, **Ricardo Carvalho**, Laura Salonen, Päivi Heikkilä, Carl Blomqvist
   Mast cells and eosinophils in invasive breast carcinomas, *BMC Cancer*. 2007; 7: 165-169
2. **Ricardo F.S. Carvalho**, Gunnar Nilsson, Ilkka Harvima
   Increased expression of PAR-2 in mast cells in skin inflammatory diseases and release of IL-8 after PAR-2 activation, *Manuscript*
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Atopic dermatitis</td>
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<tr>
<td>Anti-CP</td>
<td>Anti-citrulline antibody</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>CBMC</td>
<td>Cord blood-derived mast cells</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CD30L</td>
<td>CD30 ligand</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FceRI</td>
<td>High affinity receptor for IgE</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating protein</td>
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<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
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<tr>
<td>HL</td>
<td>Hodgkin lymphoma</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HRS</td>
<td>Hodgkin and Reed-Sternberg</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<tr>
<td>MC&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Mucosal mast cells</td>
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<td>MC&lt;sub&gt;TC&lt;/sub&gt;</td>
<td>Connective-tissue mast cells</td>
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<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerisation domain</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>sCD30</td>
<td>Soluble CD30</td>
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<tr>
<td>sCD30L/CD153</td>
<td>Soluble CD30L/CD153</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>TGF</td>
<td>Tumour growth factor</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>TNFR</td>
<td>TNF receptor</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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1 INTRODUCTION

Mast cells are fascinating inflammatory cells that are traditionally recognised for their role in the context of hypersensitivity reactions. However, it is now recognised that they also have a very important physiological role in the regulation of inflammation, host defence and innate immunity. In fact, their localisation in the body and the capacity to react to different stimuli, releasing a huge panoply of mediators cannot be regarded as just a coincidence.

Besides their physiological role, mast cells have also been implicated in a number of different pathologies, including different types of cancers and autoimmune diseases. Mast cell numbers are normally elevated at sites of inflammation, which associated with their potential to recruit other cells and modulate the local microenvironment by release of different molecules, has to be taken into close consideration. The mechanisms regulating mast cell recruitment and their role in the context of chronic inflammatory diseases are still far from being understood. In this thesis we try to unravel some information regarding the different activation mechanisms of mast cells in the context of chronic inflammatory diseases and the response of mast cells to hypoxic conditions, processes which can occur during an inflammatory response and during cancer development.
1.1 THE IMMUNE SYSTEM

Over the course of evolution, living organisms had to develop mechanisms that conferred protection from external, as well as, internal aggression. In all forms of organisms, from unicellular to complex vertebrates, mechanisms are found that confer protection against diseases. These mechanisms are referred to as the immune system.

The word immunity derives from the Latin *immunis* and relates to the legal concept of exemption: Initially, in Rome, it was used to exempt an individual from service or duty and later, in the Middle Ages, it was used to refer to the exemption from the church and its properties and personnel from civil control [1]. The first written records of the concept of “Immunity” may date back to 430 BC when the Athenian Thucydides described the plague that hit Athens: “the sick and the dying were tended by the pitying care of those who had recovered, because they knew the course of the disease and were themselves free from apprehensions. For no one was ever attacked a second time, or not with a fatal result” [2]. But it was not until the 19th century that the term “Immunity” became a normal currency, with the rapid spreading of Edward Jenner’s smallpox vaccination [1].

In fact there is not one immune system, but two: the innate immune system and the adaptive immune system. These are tightly regulated and cooperate with each other. The innate immune system is evolutionarily ancient and can be found in most organisms acting as the first line of defence against external aggression. Before the onset of an infection, a series of barriers need to be breeched by the invading pathogens: skin and mucosal membranes, stomach acidity, some enzymes present in fluids such as tears and saliva [3]. Besides these primary barriers, different cells and molecules are involved in the recognition and neutralisation of invaders, based on the recognition of certain markers that are called pathogen-associated molecular patterns (PAMPs). The innate immune system cells work by recognition of PAMPs by molecules present at the cell membrane surface, in the cytosol or that are secreted into the circulation and tissues; these molecules are called pattern recognition receptors (PRRs), one example being the family of Toll-like receptors (TLR). Another way to recognize and combat pathogens is via secretion of molecules that facilitate the immune response; some examples of such molecules are the complement system, interferon or lysozyme. If both the physical and chemical barriers fail to prevent infection, cells of the host will recognize the pathogen via PRRs, taken up by phagocytic cells and destroyed in the phagosome. Phagocytes can also release molecules, cytokines, which provoke a stronger inflammatory response, by inducing other cells to differentiate and/or proliferate and/or produce other molecules. A special type of these molecules, the chemokines, are capable of inducing recruitment of other cells to sites of inflammation, which, among others, can induce the recruitment of antigen presenting cells (APCs) which have the capacity to initiate an adaptive immune response [4].

The adaptive immune response is a characteristic of vertebrates alone. It is unique among individuals and develops over the course of the lifetime. This system has the ability to distinguish subtle differences between antigens. Another of its features is the capacity of memory which is what makes the basis for the development of vaccines [5]. Once a foreign antigen is recognised, there is a clonal expansion of cells expressing the antibody that recognizes the antigen such that if there is a second encounter with the antigen, the adaptive immune response will immediately set in and, instead of taking a few days, will take place in just a few hours. Another important feature of the adaptive
Mast cells in chronic inflammatory diseases

immune system is its capacity to recognize self antigens from non-self antigens. The cells involved in recognizing the antigens presented by APCs are B and T lymphocytes. Upon formation in the bone marrow, B lymphocytes express a unique antibody on its surface. When these so-called naïve B cells encounter their specific antigen, they rapidly differentiate into memory B cells – which, as the name indicates, will be responsible for a fast response upon a second encounter with the antigen – and effector B cells (plasma cells), which release huge amounts of the soluble form of the antibody. T lymphocytes also originate in the bone-marrow, but then migrate to the thymus where they mature. They have the capacity to recognise peptide fragments of pathogens presented on the surface of APCs. The different types of T cells have the capacity to kill infected cells (cytotoxic T cells), activate macrophages and B cells (helper T cells) or inhibit the immune response (regulatory T cells) [3, 4].

1.2 MAST CELLS

In 1878 the brilliant German pathologist, Paul Ehrlich, described the mast cell – mastzellen – for the first time [6]. During his doctoral defence he described mast cells as “granular cells of the connective tissue” that stained reddish-purple with aniline dyes with a tendency to accumulate around preformed connective tissue structures [6]. The term “mast” might come from the greek mastos that means breast and might have to do with the notion that the characteristic granules were thought to be food reservoirs for neighbouring cells [6, 7].

1.2.1 Mast cell biology

The origin of mast cells remained a mystery for one hundred years. They were first thought to be a component of the connective tissue, derived from undifferentiated mesenchymal cells [8]. It is now well defined that mast cells differentiate from pluripotent stem cells that originate in the bone marrow [9]. In humans mast cells are derived from CD34+CD117+CD13-FcαR-CD14-progenitor cells [10-12]. These committed progenitor cells circulate in the blood until they reach peripheral tissues where the final differentiation and maturation process takes place. As a result of the local cytokine milieu tissue specific mast cells develop. Stem cell factor (SCF), the ligand for KIT tyrosine kinase receptor which is constitutively expressed on mast cell membranes, is the most important cytokine in the process of mast cell growth, differentiation and survival [13]. In humans, mutations in c-kit (the gene encoding KIT) give rise to a condition called mastocytosis, characterised by abnormal mast cell infiltration in several tissues, particularly in the skin [14]. Depletion of SCF in vitro leads to mast cell death [15], and in mice with mutations in the gene coding for SCF, the population of mast cells is reduced to 1% of the normal phenotype [9, 16]. Th2 derived cytokines, as IL-3, IL-4, IL-5, IL-6 or IL-9, chemokines and retinoids, in combination with SCF, have been shown to regulate the proliferation and differentiation of mast cells and to influence their protease content [17-23].

Mast cells are normally only found in tissues. They are distributed over vascularised tissues, especially near surfaces that are in close contact with the environment, like skin, airways, and gastrointestinal tract [24, 25]. The numbers of mast cells within the connective tissue are normally constant, whereas in mucosal surfaces they can change [24]. The connective tissue mast cells are long lived cells that retain their proliferative capacity even as mature cells [26, 27]. Due to their properties,
Mast cells play important roles both in innate immunity and adaptive immune responses [28].

### 1.2.2 Mast cells are heterogenic cells

The tissue microenvironment where mast cell progenitor cells migrate to and differentiate determines its final phenotype. The local cytokine pool and interactions with resident cells are determinant for the observed tissue mast cell phenotypical and functional heterogeneity [24, 25, 29, 30].

Traditionally two sub-sets of human mast cells are acknowledged based on their protease granular content, MC_T and MC_TC. MC_T mast cells only express tryptase in their granules and are found in the mucosal membranes, like the alveolar wall, often in the vicinity of T cells, mostly of Th2 type. They appear to be T cell dependent since patients with compromised T cells, like in AIDS, have decreased MC_T numbers [31]. The connective tissue mast cells – MC_TC – are predominant in for example the skin, and besides tryptases, also express chymase, carboxypeptidase A and cathepsin G [32]. Although one of the sub-sets is normally predominant in a tissue, both are almost always found in every tissue.

### 1.2.3 Mast cell activators and mediators

Mast cells can be activated in many different ways (figure 1). The activation stimuli determine the mast cell response. Depending on the stimuli they have the capacity to release a specific set of mediators (figure 1). The best described activation mechanism of mast cells is cross-linking of the high affinity receptor for IgE, the FceRI, leading to degranulation and secretion of newly synthesised mediators. Other activators of mast cells are opiates, components of the complement cascade, neuropeptides, cytokines, chemokines, parasites, pathogenic bacteria, among others [33]. Mast cell activation is not always accompanied by degranulation. Newly formed mediators can also be released upon activation, with or without degranulation [34].

![Figure 1](image_url)  
**Figure 1** – The type of mediators released by mast cells is dependent on the activator.
As mentioned, mast cells store in their granules a huge array of pre-formed mediators that can promote or suppress the development, survival, proliferation, migration, maturation or function of immune cells [35]. Some of these include histamine, proteoglycans, serotonin, proteases, cytokines and growth factors. Lipid derived mediators, like prostaglandins and leukotrienes, are de novo synthesised and secreted after activation. Mast cells also have the capacity of producing many different chemokines, cytokines and growth factors, including IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, MCP-1, TNF-α, SCG, VEGF and NGF [36]. All the different mediators play an important role not only in a context of innate immunity, but also in adaptive immunity and in the regulation of the inflammatory response that is a part of the pathogenesis of diseases in which mast cells have been shown to play an important part.

### 1.2.4 Mast cells in innate immunity

Mast cells are strategically located at sites that interface the external environment, like the gastrointestinal tract, the skin and the alveolar membranes and in close proximity to blood vessels and nerves [37]. They act as sentinels and are probably the first cells (together with tissue macrophages if present) to respond to pathogens and to initiate the inflammatory response [38].

It is now well defined that mast cells play an important role in the host immune defence against bacteria and parasites [39]. The role of mast cells in the host defence against several nematode parasites has been known for years [40, 41]. IL-9 has been shown to be important in the clearance of nematodes from intestines, by inducing both intestinal mastocytosis and production of IgE and IgG1 [42]. Furthermore, mouse mast cells proteases have been implicated to be directly responsible for increasing the epithelial paracellular permeability in the intestine, which ultimately facilitates expulsion of the nematodes [43]. In addition, mast cells have also been implicated in the host response against different bacteria. In a model of acute septic peritonitis, mast cell deficient mice (W/Wv mice) showed an increased mortality compared to normal littermates (+/+). Reconstitution of W/Wv mice with normal mast cells, prevented mouse death, possibly via release of TNF-α [44]. In a different study published in the same issue of Nature, normal littermates cleared enterobacteria 20-fold more efficiently than W/Wv mice, due to deficiencies in neutrophil recruitment by W/Wv mice, probably as result of lack of TNF-α secreted by mast cells in response to infection [45]. Mast cell-derived leukotrienes, LTB₄ and LTC₄, have also been shown to be involved in FimH⁺ bacterial clearance in a model of infectious peritonitis [46].

In order to respond to harmful microorganisms, mast cells need to recognise them. One of the best described families of PRRs is the TLR family. Mast cells express Toll-like receptors 1, 2, 3, 4, 6 and 9 [39] and by so, are capable of responding to products of bacterial, fungal or viral origin. Formation of multimeric-receptor complexes is often required for TLR immune activation. Two co-receptors that might play a role in TLR-signalling in mast cells are CD14 and dectin-1 [47, 48]. The cytosolic proteins NOD-1 and -2 are also expressed by mast cells and can recognise products derived from bacterial peptidoglycan (Enoksson et al unpublished observations). CD48 is a glycosylphosphatidylinositol-linked protein that binds the FimH protein, expressed by Gram- bacteria. Binding of FimH by mast cell CD48, results in degranulation and leukotrienes and TNF production [49]. The complement
system plays an important role in the recognition and clearance of pathogens and in the recognition of tissue injury. The complement components C3a and C5a have been shown to induce chemotaxis of human mast cells \textit{in vitro} [50]. Mast cells are rapidly activated by immunoglobulin-binding protein through membrane bound Fc receptors, which induces degranulation and release of several pro-inflammatory mediators, like histamine, proteases, leukotrienes and TNF-\(\alpha\). Recently it was shown that mast cells can kill bacteria by entrapping them in extracellular structures named mast cell extracellular traps, which are composed of DNA, histones, tryptases and the antimicrobial peptide LL-37 [51]. Finally, the local microenvironment has a huge influence on the mast cell response against pathogens. As an example, mast cell priming with IL-4 increases the expression of LTC\(_4\) synthase [52] and increases intestinal mast cell production of Th2 type cytokines [53].

TNF-\(\alpha\) has been shown to play important roles in inflammation, sepsis, haematopoiesis, angiogenesis, host resistance to parasites and cancer. Mast cells are the only cells that store pre-formed TNF-\(\alpha\) which places them upfront in the inflammatory network [54].

1.2.5 Mast cells in adaptive immunity

Not much is known about the role of mast cells in the generation of adaptive immunity. While some \textit{in vitro} studies have demonstrated that mast cells can process and present antigens via major histocompatibility complex (MHC) class I and II [45, 55, 56], this remains to be proven \textit{in vivo}. A recent study demonstrated that mast cells act as antigen reservoir rather than antigen presenting cells [57].

Mast cells can play an important role in the process of antigen presentation by dendritic cells (DCs) to T-cells by recruiting DCs to sites of inflammation and activating them. Some mast cell mediators are known to have the potential to induce DC maturation as histamine, IL-1\(\beta\), TNF-\(\alpha\) or GM-CSF [39]. Furthermore, it has been shown that mast cell derived exosomes are important in the immunological signalling network having the capacity to transfer functional mRNA and microRNAs to other cells and to stimulate B and T cells as well as DCs [58-62].

Most of the knowledge of mast cells in adaptive immunity has come from the study of IgE associated allergic responses. Mast cell activation in allergies and atopic diseases is dependent on B cell isotype switch, resulting in production of IgE which then binds to mast cells via FceRI resulting in mast cell activation [63, 64]. In allergic rhinitis, it was shown that nasal mast cells can induce IgE synthesis by B cells and show increased expression of FceRI, CD40L, IL-4 and IL-3 [65]. Although mast cell activation by IgE has been implicated in different inflammatory diseases, it is also known that IgE is important in the contexts of resistance and clearance from parasitic infections [66, 67].

An important role of mast cells in adaptive immunity appears to be the recruitment and regulation of different immune cells. In a mouse study, it was shown that interactions between mast cells, activated by IgE cross-linking, and T cells, lead to T cell activation, as result of the effect of mast cell secreted TNF and direct cell-cell contact via mast cell OX40L and T cell OX40 [68]. In a different study, migration of cytotoxic T cells was shown to depend on leukotriene B4 originating from activated mast cells [69]. Furthermore, in the context of allograft rejection, IL-9 derived from
activated regulatory T cells is important in the recruitment and activation of mast cells which regulate the immune suppression [70].

Thus, mast cells can regulate the migration and function of DCs, different types of T cells and B cells, contributing to the generation of an adaptive immune response.

1.3 INFLAMMATION

The hallmarks of the inflammatory response were first described in the first century AD by the Roman Celsus: rubor, tumor, calor and dolor (redness, swelling, heat and pain). Functio laesa (loss of function) was later added to this list by Virchow in 1793 [71]. Inflammation can be defined as a series of complex events between soluble factors and cells that can occur in any tissue in response to traumatic, infectious, post-ischemic, toxic or autoimmune injury [38]. The main goal of the inflammatory response is to contribute for the clearance of the primary source of injury and its consequences and to prepare the site of injury for repair of the damaged tissue. Although the inflammatory process is extremely important in response to trauma, it also has the potential of being extremely harmful for the organism. Inflammatory reactions in response to hypersensitivity reactions to allergens or toxins and chronic inflammation can be life threatening or have great repercussions in the quality of life.

Different cells, soluble plasma factors and tissues are involved in the inflammatory response. Shortly after aggression, vascular permeability increases at the site of injury, followed by increase in blood flow, causing heating of the place and redness. This leads to an increase in vascular permeability and to leakage of fluid from blood vessels, resulting in fluid accumulation – edema – and consequent tissue swelling. Leukocytes start extravasating and accumulating at the lesion site within a few hours after the beginning of the inflammatory response, which is followed by phagocytosis of the invading pathogens or necrotic cells and release of soluble mediators that will further induce the inflammatory response and recruit and activate effector cells [71].

Inflammation is a multifaceted reaction, where a complex set of communication between different cells and tissues determines the beginning and the end. This process

![Figure 2 – Different roles of mast cells in health and disease.](image-url)
is tightly regulated and whenever there is failure in one of the regulatory processes the risks for starting the inflammatory response or for a deregulated inflammatory response, are potentiated. When infection is accompanied by tissue damage, three different signals can be released by the damaged tissue: pain induces the release of bioactive peptides by neurons; broken cells release intracellular proteins, like HMGB1, heat-shock proteins or N-glycosilated-mitochondrial peptides, triggering cytokine production; and finally, the invading pathogens and molecules produced by them are recognized by immune system cells [38]. Although much importance has been given to circulating leukocytes, it is the cells resident in the connective tissue – mast cells and fibroblasts and, occasionally, macrophages and lymphocytes – that are responsible for the recognition of such signals and for the beginning of the inflammatory response [38]. Mast cells are particularly important as they act as sentinels ready at the first signals of trauma or infection to release a huge panoply of pre-formed or newly formed mediators, like histamine, eicosanoids, tryptases, TNF, cytokines, proteases and chemokines. These mediators have the potential to cause vasodilation and fluid extravasation (histamine, eicosanoids and tryptases) and also induce release of other mediators by neighbouring cells (different cytokines) and recruitment of inflammatory cells to the site of injury (chemokines). Furthermore, mast cells have been shown to regulate homeostasis by degrading peptides such as endothelin-1 and neurotensin, which have been shown to play important roles in the induction of the biological responses associated to sepsis [72, 73].

Inflammation can be classified as either acute or chronic. Acute inflammation is the organism’s response to infection or trauma. It has a rapid onset and a short life-span and it is characterised by oedema and emigration of leukocytes to sites of injury. In some situations, the inflammatory response can persist for a long period, which is known as chronic inflammation. This is associated with the presence of macrophages and lymphocytes and with proliferation of blood vessels and connective tissue.

1.3.1 Acute inflammation

As mentioned above acute inflammation is the primary response to an injurious agent. The acute inflammatory response starts with an increase in blood flow due to vasodilation causing heat and redness. Soon after, there is an increase in the vasculature’s permeability, which results in leakage of protein rich fluid, plasma, into the extracellular matrix with concomitant increase in blood viscosity. The complement system becomes activated and the anaphylatoxins C3a and C5a induce mast cell degranulation that results in release of histamine and serotonin which causes vasodilation and smooth muscle contraction. Other products of mast cell activation are prostaglandins and leukotrienes which cause vasodilation and increase vascular permeability [3, 71].

A few hours after the beginning of inflammation leukocytes start infiltrating at the inflammatory site. This is accomplished by a redistribution of adhesion molecules (E- and P-selectin) to the surface of the endothelial cells. Histamine, thrombin and PAF induce the mobilisation of P-selectin, whereas E-selectin expression is stimulated by such cytokines as TNF and IL-1. Neutrophils are the first leukocytes that adhere and extravasate the endothelial membrane. While the neutrophils adhere to the endothelium, chemoattractants such as IL-8, act on them, activating them. The
expression of Fc receptors for antibodies and complement receptors is increased in the activated neutrophils, increasing the phagocytosis of opsonised pathogens [71].

Activated neutrophils at sites of inflammation not only phagocytose pathogens, but also secrete a number of inflammatory mediators. MIP-1α and MIP-1β are chemokines that attract macrophages to the site of inflammation, further increasing phagocytosis and mediator and cytokine release, which will induce recruitment of more leukocytes to the inflammatory site (more neutrophils and macrophages, as well as lymphocytes, basophils and mast cells) [71].

During an inflammatory response, tissue is destroyed and toxic products are formed and released by the recruited inflammatory cells, so it is particularly important to suppress the inflammatory process once the initiating stimuli is gone. This is accomplished by e.g. regulatory T cells which act by recognising an antigen and secreting the anti-inflammatory cytokines IL-10 and TGF-β [5, 74, 75].

1.3.2 Chronic inflammation

In certain conditions, the inflammatory response persists for a longer period than needed for the healing of the trauma, leading to a status of chronic inflammation. In such conditions, mechanisms of inflammation, tissue destruction and tissue repair occur simultaneously. This is the case of a persistent infection or continued exposure to toxic agents. However, in certain conditions, the organism begins reacting against its own tissues and cells, leading to autoimmune disorders. Chronic inflammation also contributes to the tissue damage associated with some types of cancers [71].

Instead of neutrophil infiltration, chronic inflammation is characterised by mononuclear cells – macrophages, lymphocytes, plasma cells, NK cells and mast cells – infiltration. Macrophages play an important role in the process of chronic inflammation. Besides having the capacity to release substances like reactive oxygen species, nitric oxides and proteases that induce tissue damage, they can also induce remodelling of the tissue by stimulating fibrosis, via release of growth factors, fibrogenic cytokines and remodelling collagenases. Another characteristic of chronic inflammation is a response whiling to repair damaged tissue that is accomplished by connective tissue proliferation, caused by angiogenesis and fibrosis [71].

IFN-γ and TNF-α play an important role in chronic inflammation. IFN-γ is expressed by T and NK cells. It has the capacity to activate macrophages which in a chronic inflammatory condition release toxic substances as reactive oxygen species and nitrogen intermediates as well as different hydrolytic enzymes that contribute to the tissue damage observed in such state [3, 71]. Activated macrophages also secrete large amounts of TNF-α which acts synergistically with IFN-γ in modulating the infiltration of the inflammatory site with leukocytes, perpetuating the inflammatory response [3].

1.4 INFLAMMATORY DISEASES

In the western world there is an increasing number of cases of chronic inflammatory diseases. As mentioned before, the causes of these diseases are not yet fully understood. Some are just a result of deregulation of the inflammatory process – also called autoimmune disorders –, as is the case in atopic dermatitis, multiple sclerosis, psoriasis, rheumatoid arthritis, among many others. In other conditions, inflammation and microbial toxicity equally contribute to the perpetuation of the inflammatory condition. Some examples are cystic fibrosis pneumonitis, *Helicobacter pylori* gastritis, Hepatitis
C or tuberculosis. In other cases, it is the post-inflammatory fibrosis that is responsible for the pathologic conditions of the disease: chronic allograft rejection, hepatic cirrhosis and radiation induced pulmonary fibrosis are just a few examples [38].

1.4.1 Autoimmunity

One of the most important functions of the immune system is to recognize self from non self. When this fails, an inappropriate response against self components occurs in what is defined as autoimmunity. Autoimmune diseases can cause serious damage to tissues and organs, leading to severe incapacity and in some cases death [3]. B and T lymphocytes are the cells involved in the recognition of the self antigens. In the process of maturation T and B cells that recognise the individuals’ antigens are deleted. Nevertheless, some escape this regulatory process and are found in the circulation. In order to maintain tolerance, the activity of these cells is regulated by regulatory T cells, by inducing their death or limiting their activity [76].

While some of these autoimmune diseases have been linked to genetic factors that favour its appearance, it is hard to explain why in western societies the percentage of cases has been increasing. One of the most feasible explanations arose from the so called hygiene hypothesis, postulated for the first time by David P. Strachan in 1989 [77]. Briefly, Strachan hypothesised that the immune system needs to be educated from early childhood, by exposure to different environmental factors and pathogens. Failure to do so can result in allergies and autoimmune disorders. With the improvement of hygiene conditions in modern society, the human body is not exposed to pathogens that coexisted with the human evolution which results in a deregulated immune system.

1.4.2 Atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated to a hyper-reactivity to environmental factors that are normally harmless to non-atopic individuals [78]. It is a disease of the so called western civilization since its prevalence has increased two to three fold during the past three decades in industrialised countries while it remains low in rural areas [79]. AD normally arises during early infancy and childhood but it can persist into adulthood [80] affecting 10-20% of children and 1-3% of adults [81]. AD has a complex aetiology, with activation of multiple immunologic and inflammatory pathways [82, 83]. Interactions between susceptibility genes, the host’s environment, pharmacological abnormalities, skin barrier defects and systemic and local immunological responses contribute to the pathogenesis of AD [78]. It is characterised by severe itch – pruritus –, red and dry skin. During infancy the disease is often more acute and affects the face, scalp and the extensor surfaces of the extremities. In older and chronic patients, lichenification of the skin develops and the rash moves to the extremities [78]. Around 80% of the AD patients will develop rhinitis and asthma, giving support to the idea of an atopic march, from AD to the development of rhinitis and asthma. This suggests that allergen sensitization through the skin predisposes people to respiratory diseases [84-86]. These patients also show increased levels of serum IgE and are sensitive to air and food allergens and are so referred to as being of the extrinsic type. The remaining patients are said to be of the intrinsic type.

In most AD patients peripheral blood eosinophilia is observed. Memory T cells express the Th2 stimulating cytokines, IL-4 and IL-13, known to induce IgE synthesis and expression of vascular adhesion molecules, such as VCAM-1, as well as IL-5
which is important for eosinophil development and survival. The expression of IFN-\(\gamma\), a Th1 cytokine that inhibits Th2 cell function, is downregulated in memory T cells [87].

### 1.4.3 Psoriasis

Psoriasis is a chronic inflammatory disease of genetic origin which affects between 1-3% of the world population [88]. It is characterised by the manifestation of thick, erythematous plaques in the skin as a result of a hyperkeratinisation due to an increased keratinocyte proliferation [89]. In later stages of the disease, many of the patients develop a form of arthritis known as psoriatic arthritis. The prevalence of this disease in the world population is not well defined due to errors in the diagnosis of patients, but it is assumed that 6-39% of the psoriatic patients develop a form of psoriatic arthritis [88].

Psoriasis has been classified a Th1 type of disease [90-92], although unconventional T cells, expressing NK cell receptors (NK-T cells), have also been shown to play an important role in the immunopathogenesis of the disease [93-95]. Common cytokines that are found overexpressed in psoriatic lesions are TNF-\(\alpha\), INF-\(\gamma\), IL-2, all cytokines of the Th1 pathway [90].

In recent years, however, a lot more importance has been given to the role of the innate immune system in the pathogenesis of psoriasis [96, 97]. In the proposed model, the innate immune system overreacts to a still unknown stimulus, by production of TNF-\(\alpha\), IL-1\(\alpha\), IL-6 and IL-12, leading to the recruitment of Th1 cells which produce cytokines that induce the proliferation of keratinocytes. So far, the only cytokine that has been shown to have a role in the formation of the psoriatic lesions is IFN-\(\gamma\) [98]. Mast cells have been shown to produce IFN-\(\gamma\) in vitro and in psoriasis the number of mast cells expressing IFN-\(\gamma\) is significantly increased in lesion areas [99].

### 1.4.4 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease which does not have a cure and is highly incapacitating. It is a common disease, with a higher incidence among middle-aged woman. It is characterised by chronic inflammation of the joints, but the respiratory, cardiovascular and hematologic systems are also affected [3].

The aetiology of RA is not yet fully understood. It is believed that different variables, including genetic, environmental and immunological, contribute to the onset, propagation and perpetuation of the disease [100]. It has long been known that mutations in the human leukocyte antigen (HLA)-DR genes, which are a part of the MHC, are associated with an increased risk of developing RA [101, 102]. Furthermore, many additional gene polymorphisms have also been associated with RA [103]. The association between the three variables mentioned above and the development of RA, became evident in patients with increased levels of serum anti-citrulline antibody (anti-CP\(^+\)), where smoking associated with mutations in the HLA-DR gene were shown to contribute to the development of the disease [104-106]. Other factors, such as dietary patterns, occupational exposure to allergens, intake of sex hormones, among others, have also been reported as risk factors in the development of RA [107].

The synovial membrane of RA patients is characterised by hyperplasia, increased vascularisation and infiltration of inflammatory cells, mainly CD4\(^{+}\) T cells of the Th1 type. In the early stages of RA, cartilage and bone erosion is accompanied by formation of a proliferating pannus [108]. It is to the interface between the pannus and the
cartilage that activated macrophages and synovial fibroblasts expressing matrix metalloproteinases (MMPs) and cathepsins will migrate [109]. IL-1 and TNF-α can induce expression of adhesion molecules by the endothelial cells, inducing the neutrophil infiltration to the joints. Elastase and proteases are released by the infiltrating neutrophils, degrading proteoglycan in the superficial layer of the cartilage. This leads to the exposure of chondrocytes, which together with synovial cells release MMPs (such as stromelysin and collagenase) upon activation by IL-1, TNF-α or CD4⁺ T cells. The CD4⁺ cells stimulate monocytes, macrophages, synovial fibroblasts and mast cells to produce IL-1, IL-6 and TNF-α and to secrete MMPs [110]. MMPs are enzymes with the capacity to degrade connective-tissue. CD4⁺ T cells can also stimulate B cells to produce immunoglobulins, such as the rheumatoid factor, that is reactive towards the Fc part of IgG [111]. The resulting complex has the capacity to activate the complement cascade and cause chronic inflammation of the joint [112].

1.5 MAST CELLS IN CHRONIC INFLAMMATORY DISEASES

Besides playing a very important role as a part of the immune system, mast cells have also been implicated as main actors in some chronic inflammatory disorders. The wide array of mediators mast cells can release and produce can give rise to inflammation or sustain a persistent inflammatory condition. The accumulation of mast cells at sites of chronic inflammation was already noticed by Ehrlich in the late 19th century [6].

Allergy and asthma are the chronic inflammatory diseases where the role of mast cells has been more thoroughly studied. Allergies are normally considered as IgE-associated diseases. Sensitised individuals produce allergen-specific IgE that binds specific protein epitopes in allergens. Mast cells and basophils express the high affinity form of the IgE receptor, FcεRI. In allergic individuals, IgE is normally bound to its receptor and, upon cross-linking of two IgE molecules by an allergen, mast cells become activated, releasing their granular contents and causing an immediate hypersensitivity reaction [64]. The late phase reaction can be induced by continuous secretion of pro-inflammatory mediators, like IL-4, IL-5, IL-6, IL-13 and TNF-α, and expression of CD40L which can induce recruitment of B cells and stimulate them to produce IgE, and by so, prolonging and strengthening the inflammatory response [65, 113]. Tissue swelling, increased vascular permeability, interstitial clotting and neutrophils recruitment in both immediate and late phases of a cutaneous hypersensitivity reaction have been proven to be mast cell dependent [114-116]. Although it is now obvious that mast cells play an important role in the different steps of the allergic response, other cells have the capacity to produce the same mediators and effects as mast cells. The reactions observed in a normal allergic response should then be considered as the results of a complex signalling network between cells, in which mast cells play a central role.

I will now describe what is known about the role played by mast cells in three chronic inflammatory diseases and one lymphoma that are relevant in the context of this thesis: atopic dermatitis, psoriasis, rheumatoid arthritis and Hodgkin lymphoma.

1.5.1 Atopic dermatitis

Mast cells are key effector cells in AD. The high levels of circulating IgE observed in a large proportion of the AD patients [117] have the potential of, upon encounter with an antigen, activate mast cells to degranulate. Although the mast cell population remains
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stable in acute AD lesions compared to normal skin, they appear degranulated [118]. In contrast, areas of lymphocytic infiltration in the papillary dermis have a significant increase in mast cell numbers [118, 119]. Elevated levels of histamine, IL-4 and IL-13 have been found in the skin of AD patients [120, 121]. IL-4 and IL-13 are key cytokines for the development of a Th2 phenotype. 66% and 20% of the mast cells in AD lesions express IL-4 and IL-13, respectively, and can contribute to the Th2 polarisation observed in AD [122]. Furthermore, mast cells have the potential to release different angiogenic molecules, including VEGF, which can increase vascular proliferation, and thus, indirectly promote inflammation [123]. TNF-α, which is confined to mast cells in the human skin [124], has also been shown to be increased in the plasma of AD patients and positively correlate to the circulating histamine levels [125]. The number of TNF-α expressing mast cells is elevated in AD skin and mast cell-derived TNF-α can induce the expression of adhesion molecules by keratinocytes [126]. Chymase, a protease that is stored in mast cells granules, has also been found to be elevated in the AD skin [127].

As mentioned earlier, AD results from complex interactions between the environment and particular genetic factors. It has been revealed that several polymorphisms in mast cell-related genes are related to AD, like in the β chain of FceRI and in chymase [128, 129].

Using mouse models, it has been reported that mast cells contribute to AD by regulating IFN-γ in the skin and the levels of circulating IgE [130]. A mast cell chymase inhibitor improved the dermatitis observed in a commonly used dermatitis mouse model [131].

1.5.2 Psoriasis

Although mast cells are rarely found in the epidermis of healthy skin, that is observed in the psoriatic lesions [132]. Mast cell numbers are also found elevated in lesional skin of psoriatic patients comparing to healthy skin or to the skin of healthy donors [133]. As in AD, the number of degranulating mast cells in psoriatic lesions is increased compared to normal skin [134, 135]. SCF is a preponderant cytokine for mast cell survival and development [136, 137]. In psoriatic skin the number of SCF-expressing cells is increased in both the dermis and epidermis when compared to healthy looking skin [138]. The levels of circulating SCF are also increased in psoriatic patients [139]. Mast cells express the receptor for SCF, Kit, and in the psoriatic lesion they stain strongly when compared to normal skin [138], which is an indication for immunomodulation of the mast cell by the local microenvironment and a possible explanation for the increased mast cell numbers in psoriatic skin.

The MC<sub>TC</sub> sub-type is the predominant mast cell phenotype in psoriatic skin. Mast cells are immunoreactive for both tryptase and chymase in biopsies of lesional skin however, only tryptase retains its activity in the psoriatic skin [140]. The pathophysiological role for β-tryptase in psoriasis has not yet been confirmed, but different observations suggest its implication in disease development. Tryptase is capable of inducing endothelial cells vascular organisation<sup>in vitro</sup> [141] and increasing the expression of MCP-1 and IL-8 by endothelial cells [142, 143]. Chymase, another constituent of the granules of MC<sub>TC</sub> cells, has been shown to induce the recruitment of neutrophils and eosinophils to the skin of guinea pigs when injected intradermally [144].
Mast cells are the predominant source of TNF-α in the skin [124]. TNF-α positive mast cells have been shown to be increased in psoriatic skin and *ex vivo* experiments using skin punches showed that inhibition of a mast cell extract with an anti-TNF-α antibody prevented the increased expression of adhesion molecules by keratinocytes [126]. TNF-α has proven to be important in the development of the psoriatic disease, since recent therapies targeting TNF-α are effective in the suppression of the clinical symptoms of the disease [145-147].

### 1.5.3 Rheumatoid arthritis

Mast cells constitute up to 3% of the normal synovial cell population [148]. In the rheumatoid synovium the mast cell population expands up to 5% of the total synovial cells [149] and the concentration of mast cell associated mediators, like histamine and tryptase, found in the synovial fluid increases accordingly [150-152]. Another feature of synovial mast cells in rheumatoid arthritis patients is the increase in the number of MC_T mast cells [153, 154]. The MC_T subset predominantly secretes IL-5 and IL-6, which can have stimulatory effects on T and B lymphocytes, whereas the MC_TC subset preferentially secretes IL-4 which has profibrotic effects [155]. MC_T cells are predominantly found in association with lymphoid aggregates, while the MC_TC cells are mostly observed in fibrotic areas of the synovium [153].

As mentioned earlier, the macrophage population in the synovium of RA patients is increased as a result of monocyte recruitment to the synovium and not due to local expansion of the resident macrophages [156]. Mast cells have the potential to release chemokines that can attract and activate monocytes to differentiate into macrophages, by the secretion of IL-8, MCP-1, MIP-1α and RANTES and cytokines like IFN-γ and IL-6. The huge amount of mediators that can be released by mast cells can further induce the recruitment of other cell types characteristic of the inflamed rheumatic synovium, and contribute to the joint destruction [149].

The K/BxN serum transfer mice model is a model of inflammatory arthritis. Mast cell deficient mice, both Sl/Sld and W/Wv, are resistant to the antibody induction of arthritis observed in normal animals [157]. Reconstitution of these animals with mast cell precursors restores the sensitivity to induction of RA. Mast cell degranulation is observed early after administration of K/BxN serum, which is an indication for the involvement of mast cells in the early events of RA [157]. This appears to be a result of mast cell secretion of the cytokine IL-1 after activation via FcγRIII binding to IgG autoantibodies [158]. In a different study, restoration of W/Wv mice with wild type mast cells stabilised with cromolyn or salbutamol (degranulation inhibitors) prevented joint destruction and angiogenesis [159]. In another model of inflammatory arthritis, the mBSA/IL-1β model, the complexes between heparin and mMCP-6 and -7, the murine equivalents to human β-tryptase, were shown to be crucial for the induction of the disease [160]. These studies imply mast cells as initiators of inflammatory arthritis in mouse models of the disease.

### 1.5.4 Hodgkin Lymphoma

Hodgkin lymphoma (HL) is a rare type of lymphoid malignancy comprising less than 11% of all lymphomas in western countries. Over the years, since it was first described in 1832 by Thomas Hodgkin, HL has drawn researchers’ attention not only due to its peculiar inflammatory phenotype but also because it was one of the first malignancies
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to respond to chemotherapy. Among the symptoms associated with HL are recurrent
cycles of fever, night sweats and lymphadenopathy [161]. The most peculiar
characteristic of HL is the massive infiltration of inflammatory cells observed in the
affected lymph nodes, which greatly outstands the numbers of resident malignant cells:
Hodgkin and Reed-Sternberg cells (HRS), the malignant cells in HL, comprise only
0.1-10% of the cell population in the tumour tissue. In the HL tumour, the HRS cells
are surrounded by a reactive infiltrate composed mostly of T-cells, mixed by
histiocytes, plasma cells, neutrophils, eosinophils and mast cells [162]. HRS cells are
derived from germinal centre or post-germinal centre B cells which have lost their B
cell characteristic phenotype [163].

Due to the characteristic phenotype of HL, it is believed that the local
microenvironment has great importance in the development of the disease. Previously,
we have shown that the presence of a high number of mast cells correlates with a worse
prognosis in HL [164] and that mast cells not only express the ligand for CD30
(CD30L), but are also the predominant CD30L expressing cells in tumour biopsies
[165]. CD30 is expressed by HRS cells [166] and thus, interactions between CD30 and
mast cell CD30L can occur and contribute to the inflammatory phenotype observed in
the disease [167].

1.6 TNF AND TNF RECEPTOR FAMILY

The TNF and TNF receptor (TNFR) family members are important regulators in such
processes as organogenesis and homeostasis of the immune system. Furthermore,
interactions between the ligand and the receptor have been shown to play significant
roles in host defence and in the control of the inflammatory process. Deregulation of
these processes can result in a non-responsive or malfunctioning immune system and in
the generation of conditions like sepsis or chronic inflammation [168].

The TNFR superfamily consists primarily of type I transmembrane (extracellular
N-terminal and intracellular C-terminal) proteins, with some exceptions. Twenty nine
different receptors have been identified in humans to date. Soluble forms of the
transmembrane receptors can be shed by proteolytic cleavage. However, these soluble
forms are still capable of binding their ligand and appear to have a role in the
development of some chronic inflammatory diseases [169, 170].

The TNF superfamily consists of type II transmembrane proteins bearing an
intracellular N-terminus and a conserved C-terminal domain designated TNF homology
domain, that are biologically active as self assembling, non-covalently bound trimers.
So far, nineteen different ligands have been identified [170]. Despite the fact the
ligands are coined membrane bound, soluble forms are commonly found, as a result of
shedding by different enzymes [170].

Mast cells have been shown to express different members of the TNF and TNFR
superfamilies, such as OX40L, CD30L, CD40L, Fas or 4-1BB [113, 165, 171, 172].

1.6.1 CD30 and CD30L/CD153

CD30 is a 120 kD type I transmembrane protein that is a member of the TNFR
superfamily [173]. It was originally observed in HRS cells and is still used as a marker
for these cells due to its ubiquitous expression, although it has also been identified in
other non-Hodgkin lymphomas, Th2, Th1 and Th0 cells [166, 174-178]. The
extracellular domain of the protein can be proteolytically cleaved by a zinc
metalloprotease, releasing a soluble form known as soluble CD30 (sCD30) [179]. The levels of sCD30 are normally quite low or undetectable in healthy individuals, but in HL [180, 181] and other chronic inflammatory disorders, like juvenile and adult AD [182, 183], atopic asthma [184], ulcerative colitis [185] and RA [186], the serum levels of sCD30 are elevated compared to controls. Negative prognosis has been associated to elevated sCD30 levels in HL [180, 181, 187], systemic lupus erythematosus [188], Wegner’s granulomatosis [189] and AIDS [190, 191].

The ligand for CD30, CD30L or CD153 as it is also called, is a 40 kD type II transmembrane glycoprotein with a C-terminal extracellular domain and a cytosolic N-terminal domain, 37 amino acids long [192]. CD30L is expressed on activated T cells and macrophages as well as on B cells, mast cells, eosinophils and several cancer cells [193-196]. CD30 activation of different CD30+ lymphoma cell lines results in increased proliferation and cytokine production or apoptosis induction [192, 197, 198]. CD30 cross-linking in T cells leads to activation of the NFkB pathway [199, 200], which is mediated by TRAF proteins that interact with the cytosolic tail of the CD30 protein [201-204]. In chronically infected T cells, HIV production is induced by CD30 cross-linking [199].

Since many of the TNF family members have cytosolic tails that are conserved across species, this lead to the hypothesis that the cytosolic domains could in fact have biological functions, such as signal transduction [205]. Wiley et al., demonstrated that CD30 binding to CD30L expressed on activated T cells induced metabolic activity, cell proliferation and IL-6 production whereas cross-linking of CD30L on neutrophils resulted in IL-8 production [206]. CD30-CD30L interactions can then have important biological consequences. CD30+ T regulatory cells induce apoptosis in CD8+ T cells expressing CD30L [207]. In HL, HRS cells inhibit proliferation, IL-2 production and expression of the IL-2 receptor and CD26 in T cells, which can explain the reduced anti-tumour responses and favour tumour growth [167]. It seems evident that CD30L reverse signalling can play an important role in biological processes. Nevertheless, its mechanism remains to be elucidated.

1.7 HYPOXIA

In healthy tissues the oxygen tension median changes between 40 and 60 mm Hg and never goes below 10 mm Hg [208]. However, in certain conditions like inflamed, proliferating and tumour tissues, the oxygen tension is decreased. This reduced oxygen availability is defined as hypoxia and all organisms have developed strategies to overcome it.

In inflamed and injured tissues the microenvironmental conditions are characterised by low levels of glucose and oxygen and by increased concentrations of inflammatory mediators [209]. Although many cells die under such conditions, others adapt to the hypoxic conditions. Common features of the response to hypoxia is an increase in the production of red blood cells and an induction of angiogenesis (generation of new blood vessels) in places where the oxygen supply has been compromised [210]. The expression of glycolytic enzymes and of glucose transporters is also increased, which leads to a metabolic change from oxidative phosphorylation to glycolysis in the process of ATP production [211].

Hypoxia inducible factor 1 (HIF-1) is a αβ-heterodimer that works as the key regulator in the responses to hypoxia [212, 213]. The HIF-1 complex is capable of
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initiating the transcription of different genes that promote cellular adaptation to hypoxia. Among such genes are glycolytic enzymes, erythropoietin (important for the transport of oxygen) and growth factors, such as VEGF (important in the process of angiogenesis) [214]. The HIF pathway is not only activated via low oxygen levels but different inflammatory mediators have also been shown to increase the expression of HIF-1α. In the inflamed joints of RA patients, the levels of HIF-1α are elevated in macrophages [215]. In healing wounds, induction of HIF-1α in inflammatory cells is partly induced by local cytokines, like TNF-α [216]. Non-hypoxic HIF activation in tumours can also modulate the HIF pathway in inflammatory cells leading to an increase in vascular leakage, tumour vascularisation, or metastasis [217].

Another mechanism of regulating cellular responses to hypoxia is via the adenosine receptors A2AR and A2BR. Cellular metabolism is changed by hypoxia causing the accumulation of intracellular cAMP, which is further degraded to adenosine. The decreased cellular metabolism as a consequence of hypoxia also causes the accumulation of extracellular adenosine. Signalling via adenosine receptors leads to a downregulation of the immune system, preventing tissues from excessive damage [218]. This regulatory system is fast and does not require energy for protein synthesis, resulting in a rapid response to hypoxia.

Hypoxic areas are present in different inflammatory conditions, including malignant tumours, atherosclerosis, synovial joints in RA, healing wounds or in bacterial infections [219]. Although the hypoxic environment results in a decreased activity of inflammatory cells, it can be a response to minimize the tissue damage caused by the inflammatory response [220]. It is known that inflammatory cells can adapt to hypoxic conditions [221], which further supports the previous observation, otherwise, hypoxia resistant pathogens would be in advantage towards the immune system. As previously referred, hypoxia induces a switch from phosphorylative oxidation to glycolysis. Although this normally shuts off many cell functions, it is known that leukocytes depend almost entirely on glycolysis for energy production which provides an adapting advantage to hypoxic conditions [222-225].

Mast cells are frequently found in tissues where low oxygen tensions occur, such as infections, inflammatory diseases or cancer. It is known that mast cells are very resistant to certain environmental changes [226, 227]. In a model of desferrioxamine-induced hypoxia, HMC-1.2 cells have been shown to secrete different pro-inflammatory cytokines and chemokines, whereas in another study, direct contact between HMC-1.2 and keloid fibroblasts resulted in the accumulation of HIF-1α and in the upregulation of VEGF in keloid fibroblasts [228, 229]. Furthermore, mast cells have been suggested to play an important role in the angiogenesis process in different tumours and inflammatory diseases implicating them in the process of disease progression [159, 230-233].
2 AIMS

The present work is based on previous findings on the role of mast cells in HL. Since mast cells were found to be the predominant CD30L expressing cells in HL and it is described that TNF family members, including CD30L, are capable of reverse signalling via a still unknown mechanism, we stimulated mast cells in vitro by cross-linking CD30L. It was also known that psoriasis and AD were CD30 diseases, so we decided to study mast cell activation in vivo in these diseases. Later we expanded the knowledge about CD30L to RA. Finally, due to the low oxygen tensions observed at sites of inflammation, we investigated the effect of hypoxia on survival and reactivity of mast cells. Therefore, the overall aim of this thesis was to study mast cells in the context of chronic inflammatory diseases, specifically by studying their activation via CD30L and to study mast cell responsiveness to culture in hypoxic conditions. The specific objectives for each project were:

**Paper I:** To determine whether mast cells can be activated via CD30L and its significance in the chronic inflammatory skin diseases psoriasis and atopic dermatitis.

**Paper II:** To investigate whether a soluble form of CD30L could be found in the serum and synovial fluid of RA patients and to determine its expression by mast cells in the rheumatic synovium.

**Paper III:** To study mast cell survival in hypoxic conditions and investigate mast cell reactivity to known mast cell activators when stimulated during or after hypoxic culture.

**Paper IV:** To validate the use of tryptase as a mast cell marker in HL.
3 METHODS

Methods used in the works presented in this thesis are described in detail in the respective “Materials and Methods” section of papers I-IV. The following methods were used in this thesis:

- Cell culturing (Paper I-IV)
- Preparation of Cord Blood derived Mast Cells (CBMCs) (Paper I, III and IV)
- In vitro CBMC stimulation (Paper I and III)
- Enzyme-linked immunosorbent assay (ELISA) (Paper I-III)
- Total tryptase quantification by the ImmunoCap™ assay (Paper III and IV)
- Western blotting (Paper I and IV)
- Antibody array (Paper III)
- Cytometric bead array (CBA) (Paper I and III)
- Flow cytometry analysis (Paper III)
- Cell death detection (Paper III)
- Total RNA isolation (Paper I and IV)
- Generation of cDNA from total RNA (Paper IV)
- Reverse transcriptase PCR (Paper IV)
- RNAse protection assay (RPA) (Paper I)
- Punched skin biopsies (Paper I)
- Skin organ culture (Paper I)
- Synovial fluid collection (Paper II)
- Synovial surgical and arthroscopic biopsies collection (Paper II)
- Sequential double staining of tryptase positive cells (Paper I)
- Immunohistochemistry (Paper I, II and IV)
- Immunofluorescence (Paper II)
- Immunocytospin (Paper IV)
- β-Tryptase activity (Paper I, III and IV)
- Whole blood serum extraction (Paper II)
- Statistical analysis (Paper II and III)
4 RESULTS AND DISCUSSION

Paper I: Mast cell CD30 ligand is upregulated in cutaneous inflammation and mediates degranulation-independent chemokine secretion

In this study we show that activation of mast cell CD30L with a CD30 fusion protein results in a degranulation-independent production of chemokines.

In vitro derived mast cells were treated with immobilised CD30-Fc fusion protein. After 30 min it was not possible to detect release of preformed mediators (histamine, tryptase or cytokines), or newly synthesised leukotrienes, which indicates that CD30 activation of mast cells does not result in degranulation or leukotriene synthesis. On the other hand, 3h after stimulation, the mRNA levels of different chemokines and cytokines were upregulated and after 24h it was possible to detect secretion of different chemokines, in what was due to a de novo synthesis of proteins. IL-8 mRNA expression was strongly upregulated after 3h and protein secretion increased with time and in a dose-dependent manner.

Since the mechanism of downstream signalling via CD30L is unknown, we investigated the involvement of the MAPK and PI3K pathways in the CD30 activation of CBMCs. ERK 1/2 is a member of the MAPK pathway. Treatment of CBMCs with CD30-Fc results in phosphorylation of ERK 1/2 suggesting an activation of the MAPK pathway. Furthermore, we investigated whether this pathway and/or others were involved in the upregulation of IL-8 secretion upon CD30-Fc treatment of CBMCs. Inhibition of MEK, localized upstream of ERK 1/2 in the MAPK pathway, by PD98059 resulted in a reduction in IL-8 secretion by 40%. Wortmannin and LY294002 (both PI3K inhibitors) inhibited the IL-8 secretion by approximately 50% and 90% respectively. This is to our knowledge the first time the MAPK and PI3K pathways are described as being involved in the CD30L reverse signalling and consequent IL-8 secretion, however further studies need to be conducted to further elucidate the downstream events.

Mast cells are capable of producing a wide array of different mediators upon activation [36]. The activation of mast cells can result in degranulation and release of preformed mediators or it can be degranulation-independent with release of de novo synthesised mediators [34]. In this study we observed a degranulation-independent activation of mast cells. This implies that this mechanism of activation, being a slow mechanism, does not play a role in adaptive immunity, but it can rather be of importance in the late recruitment of leukocytes to sites of inflammation, thus contributing to chronic disease development. This is further supported by the commonly observed upregulation of CD30 expression in different tumours and chronic inflammatory diseases [234] and by observations that mast cells play a role in the development of chronic autoimmune diseases [235].

Previously we have shown that mast cells are the predominant CD30L expressing cells in HL [165]. Here we describe that mast cells are also the predominant CD30L expressing cells in lesional skin of two different chronic cutaneous inflammatory diseases, AD and psoriasis. We also observed that CD30 and CD30L positive cells were found in the same areas of the upper dermis in both diseases. Accordingly to CD30L, CD30 expressing cells were also increased in lesional skin compared to healthy skin in both diseases. CD30 is expressed in different types of T cells and these have been shown to induce IL-8 secretion by mast cells via an unknown mechanism.
In addition, the extracellular part of CD30 can be cleaved by metalloproteinases giving it the potential to bind and activate CD30L without direct cell-cell contact [179]. IL-8 has been found to be upregulated in AD and psoriasis [237, 238] and to correlate to disease prognosis in psoriasis [239]. Taken this information together we hypothesised whether mast cells could be activated via CD30L to produce IL-8, contributing to the recruitment of leukocytes to the inflamed tissue and so, supporting the chronic inflammation. In lesional skin from both AD and psoriatic patients, the percentage of mast cells expressing IL-8 is higher than in healthy skin. In fresh skin punch biopsies from three healthy donors cultured \textit{ex vivo} for 2 days in the presence CD30-Fc, IL-8 was upregulated and mast cells were the only IL-8 expressing cells.

In this study we demonstrate that mast cells can be activated via CD30 ligand in a degranulation-independent mechanism. This results in the release of pro-inflammatory chemokines (figure 3) that can play a role in the recruitment of leukocytes to sites of active inflammation in different chronic inflammatory diseases like AD and psoriasis.

**Paper II: CD153 in rheumatoid arthritis: detection of a soluble form in the serum and synovial fluid and expression by mast cells in the rheumatic synovium**

We have previously shown that mast cells are the predominant CD153 expressing cells in HL and in the chronic inflammatory cutaneous diseases AD and psoriasis [165, 240]. Although it is known that a specific set of CD30$^+$ T cells can be recruited to the synovium of RA patients [241] and that sCD30 is upregulated in the serum and synovial fluid of RA patients [186] there are no reports about its ligand expression in the rheumatic synovium or about sCD153 in any disease.

Here we confirmed that sCD30 is increased in the serum of RA patients and that it is also found in synovial fluid of RA patients [186, 241]. Furthermore we report for the first time the presence of increased levels of sCD153 in the serum of RA patients. More important, these levels were higher in synovial fluid from the same patients, and a very strong correlation between serum levels and synovial fluid levels was found. This suggests a common origin for sCD153, possibly arising from CD153 expressing cells in the inflamed synovium.

Mast cells have been shown to play an important role in the onset of inflammatory arthritis [157]. Besides this, the mast cell population resident in the synovium is increased in the inflamed rheumatic synovium [153, 242, 243]. 10-15% of the synovial mast cells appear to be degranulating [244] and common mast cell mediators are found in the synovial fluid [150, 245, 246]. Here we report that synovial mast cells can express CD153, but they are not the predominant CD153 expressing cells, as previously observed in other CD30 associated diseases [165, 240].

Mast cells are important effector cells with the capacity to release a huge array of pro-inflammatory mediators. Since they appear to be important in the onset of RA, it is important to study in which way they can contribute to the disease. Previously we reported that mast cell activation via CD153 results in the upregulation of different chemokines [240]. Here we show that synovial mast cells can express CD153 which gives them the potential to be activated by CD30 expressing cells or by sCD30 whose concentration is high in the synovial fluid. Further studies need to be conducted in order to fully understand the role of mast cell CD153 activation in the context of RA.
Mast cells in chronic inflammatory diseases

Paper III: Mast cell survival and mediator secretion in response to hypoxia

Mast cells are present in almost every tissue and in the connective tissues their numbers are relatively constant during an individual lifetime [24]. Many of the mast cells are long lived with the capacity to survive activation and be reactivated [247, 248]. However, in certain situations, such as infections, inflammatory diseases or tumours, mast cell numbers have been shown to increase locally [28]. In many of these situations the oxygen tension is reduced, due to a decrease in blood supply. Many cells are not able to survive low oxygen tensions and enter necrosis or apoptosis [249]. However, other cells are known to resist hypoxia, although their normal functions are affected [250, 251]. In the situations mentioned above, where mast cell numbers appear to be increased, it is also possible that hypoxic conditions occur. In such conditions it is important for the inflammatory cells to resist the decreased oxygen tension.

In this study we cultured CBMCs both in a normoxic (20% oxygen) and hypoxic atmosphere (1% oxygen) for different timepoints and then analysed cell death and spontaneous release of different known mast cell mediators. The viability of CBMCs was not influenced by hypoxia for up to three days culture in hypoxic conditions. After that cell viability decreased. Furthermore, hypoxia did not cause degranulation. Hypoxia is known to induce changes in cellular metabolism, mostly by shutting off a number of pathways which require energy from oxidative phosphorylation. Using a cytokine array, we compared the spontaneous release of different cytokines and chemokines after 24h culture in normoxia or hypoxia. Most proteins were downregulated, however we found that IL-6, which is known to be important for sustaining mast cell survival [23, 252] was strongly upregulated. The IL-6 upregulation was confirmed by ELISA and we also determined that the TNF-α secretion was increased when CBMCs were cultured under hypoxia. We then hypothesised whether the upregulation of IL-6 as a consequence of hypoxia would be an autocrine regulation of survival by mast cells. Addition of an anti-IL-6 neutralising antibody to CBMCs cultured in hypoxia, resulted in a decrease in viability, confirming our hypothesis for an autocrine regulation of mast cell survival in hypoxia.

These results suggest that mast cells are capable of regulating their own survival to hypoxic conditions by secreting IL-6. Hypoxia induces the transcriptional pathway governed by HIF-1, which is known to play important roles in the expression of genes involved in angiogenesis, vasodilation, glycolysis and erythropoiesis [253]. HIF-1 has also been shown to play a role in the control of inflammation, since it controls the expression of different inflammatory genes, such as IL-8 or adenosine 2B receptor [254, 255]. TNF-α can be released from monocytes stimulated by LPS in hypoxia [256]. Furthermore, hypoxia is known to activate NFκB, a transcription factor which plays roles in the promotion and progression of inflammation and in antiapoptotic events [257, 258]. In a model of induced hypoxia, IL-6 secretion by a human mast cell line, HMC-1, was increased in vitro via induction of the HIF-1 and NFκB pathways [228]. Taking this information together, we can then speculate that, in our system, hypoxia can induce IL-6 production by CBMCs via HIF-1 and/or NFκB, sustaining the cell survival. In addition, the increased secretion of TNF-α by hypoxia suggests that mast cells contribute to the recruitment of other cells to hypoxic sites of inflammation since TNF-α is a strong modulator of inflammation. TNF-α is also a strong regulator of the HIF-1 pathway and can activate HIF-1 even in normal oxygen conditions [259]. Secretion of TNF-α by hypoxic cells can then result in activation of the HIF-1 pathway both in hypoxic and normoxic cells, and contribute to the inflammatory response.
As previously referred, cellular metabolism is affected by hypoxia. Therefore, we determined whether the “shut off” observed in secretion of different mast cell mediators was permanent or if mast cells could still be activated under hypoxic conditions or after reoxygenation. In order to do so, mast cells were stimulated for 24h, in hypoxia or after 24h culture in hypoxia followed by restoration of the normal oxygen conditions. Mast cells can be activated via their FcεRI [64]. This induces degranulation which is particularly important in pathologies like asthma and allergies. Here we show that only A23187, an ionophore that induces mast cell degranulation, induced CBMC degranulation in all the conditions tested. Neither IgE receptor cross-linking, CD30 or LPS activation induced CBMCs degranulation. One possible explanation for the low response to IgE receptor cross-linking observed is probably due to the low expression of the FcεRI on CBMCs [260, 261]. On the other hand, CD30 and LPS, which are known to upregulate the secretion of different mast cell mediators, did induce IL-8 secretion in all the conditions tested, but when activation was performed in hypoxia or after reoxygenation, the CBMC response to activation was attenuated. IL-8 is a strong pro-inflammatory chemokine. We have previously demonstrated that upon activation by CD30 mast cells secrete IL-8 and that this mechanism can be important in cutaneous inflammatory diseases [240]. Furthermore, IL-8 secretion can be induced by a model of induced hypoxia in HMC-1 cells [228] and endothelial cells cultured in hypoxic conditions have also been shown to secrete IL-8 which induced neutrophil migration [262]. This information, together with our results suggests that mast cells can still respond to activation via CD30L in a hypoxic microenvironment and after reoxygenation, although the response is decreased. Mast cells recognise LPS via TLR-2 and respond by releasing different mediators, playing an important role in the host defence against bacterial infection [39]. These results suggest that mast cells can still respond to pathogens expressing LPS, in hypoxic conditions and that this response is sustained even after reoxygenation. However, the response to LPS is decreased by hypoxia, which can affect the host response against infection.

Overall this study shows that mast cells have the capacity to survive transient hypoxic conditions, possibly by secreting the pro survival cytokine IL-6 (figure 3) and so regulating survival in an autocrine way. Hypoxia also induces secretion of TNF-α (figure 3) which can be important in the promotion of inflammation. Upon activation during hypoxic culture and after reoxygenation, with different mast cell activators, mast cells are still able to respond, secreting IL-8, which can play important roles in the recruitment of neutrophils. This can be important in the context of inflammation and host response to infection.

Figure 3 – Mast cells are adaptable cells that respond differently to different stimuli.
Paper IV: Expression of Mast Cell Tryptases in Hodgkin and Reed-Sternberg (HRS) Cells

Tryptase is abundantly expressed in mast cells [263], but basophils and other cells of myeloid origin have also been shown to express it, though in low amounts [264-267]. Due to its abundance in mast cells and to its expression being almost exclusive to mast cells, tryptase is often used as a mast cell marker. Previously we showed that increased mast cell infiltration in HL correlates to a poorer prognosis [164]. Tryptase staining was used to assess mast cell numbers in HL, but also in the studies reported in paper I and II in this thesis.

In order to clarify whether HRS cells express tryptase, we screened different HL cell lines for the presence of tryptase. At the mRNA level all HL cell lines expressed tryptase, but in smaller amounts than mast cells. The HL cell line, L1236 was also found to express tryptase but in lower levels than CBMCs. When analysing in vivo expression of tryptase by HRS cells in HL biopsies, we found that this was quite sparse and HRS cells were easily distinguishable from other tryptase positive cells due to their characteristic morphology.

Taken together, these results show that, although HRS cells can express tryptase both in vitro and in vivo, they rarely do so in vivo and they can be easily distinguished from mast cells due to their characteristic morphology, so tryptase can still be used as a valid mast cell marker in HL.
5 CONCLUSIONS

I: In this study we identified CD30L activation as a novel mechanism for mast cell activation. Stimulation of CBMCs with a CD30-Fc fusion protein leads to a degranulation-independent secretion of chemokines. This activation mechanism can be important in different chronic inflammatory diseases. Here we also show that mast cells are the predominant CD30L expressing cells in lesional skin of AD and psoriasis patients. Furthermore, mast cells can be induced to release IL-8 by CD30-Fc fusion protein treatment of healthy skin cultured *ex vivo*, which supports the importance of this activation mechanism in chronic inflammatory cutaneous diseases.

II: In this work, we demonstrate that a soluble form of the TNF family member CD153 is upregulated in the serum of RA patients. Synovial fluid levels of sCD153 are higher than serum levels supporting a common origin for serum and synovial fluid sCD153, possibly arising from the CD153 positive population of cells observed in the synovium of the RA patients. We also found that in one third of the patients analysed, mast cells expressed CD153, but they were not the predominant CD153 expressing cells. To our knowledge, this is the first time that a soluble form of CD153 is described in the context of RA and chronic inflammatory diseases and that a CD153 positive synovial cell population is described in the context of RA.

III: In this study we show that CBMCs can transiently resist culture in hypoxic conditions by an autocrine secretion of the pro-survival cytokine IL-6. TNF-α secretion was also upregulated by hypoxia. Furthermore, CBMCs can be activated by CD30-Fc and LPS under hypoxia and after reoxygenation. This shows that mast cells can still react to different stimuli even when facing hypoxic conditions or after reoxygenation, although their response is decreased compared to cells grown in normal conditions. Mast cells can therefore play important roles in different inflammatory responses where hypoxic conditions occur.

IV: Here we show that HRS cells can express tryptase both at the mRNA and protein level *in vitro* and *in vivo*. Nevertheless, *in vivo* tryptase positive HRS cells are very rare and easily distinguishable from other tryptase expressing cells by their characteristic morphology. Therefore, tryptase staining is still a valid method for identifying mast cells in HL biopsies.

Overall, the different works presented in this thesis support the idea that mast cells are multifunctional cells, showing a multitude of different phenotypes dependent on their localisation and origin and are modulated by the microenvironment where they are present. Mast cells play a Dr Jekyll and Mr Hyde role in health since they can both have important roles in physiological and pathological events. In this work we give evidence for mast cell involvement in different chronic inflammatory diseases – Mr Hyde – but also to the capacity to still respond to different stimuli even after hypoxia – Dr Jekyll.
6 FUTURE PERSPECTIVES

It is common sense that in science an answer always brings up new questions. In the work presented in this thesis, most of the objectives proposed were fulfilled. Nevertheless, the answers found to our initial questions probably raised an even bigger number of questions than the ones initially proposed.

It is now clear that CD30L activation of mast cells plays a role in different chronic inflammatory diseases. We have shown, both in this work and in previous works that mast cells are the predominant CD30L expressing cells in HL [165], atopic dermatitis and psoriasis (paper I), and here we also show that CD30L expressing mast cells can be found in the inflamed synovium of RA patients (paper II). As mentioned in previous sections, activation downstream of CD30L is possible [206] and in this study we demonstrate that activation of mast cells via CD30L can induce the release of pro-inflammatory chemokines that can worsen the inflammatory condition in such diseases. Targeting CD30L might be a good therapeutic approach in the diseases mentioned above. It is also known that sCD30 is increased in the serum of different inflammatory diseases [268]. Here we report that sCD30L is increased in the serum of RA patients. Other TNF family members have been shown to exist as soluble active forms, being capable of exerting their functions without the need of direct cell-cell contact [170, 269, 270]. It would be interesting to extend the current study to other chronic inflammatory diseases and cancers, determine whether sCD30L can have a prognostic value or if it is important for therapy and try to understand whether mast cells can play a role in these diseases. It is also important to understand whether the sCD30L is biologically active and which effects does it have in CD30 expressing cells. If activation of mast cells via CD30L is found to be relevant in the promotion of disease, then blocking sCD30 and/or CD30$^+$ cells should be considered.

Previously there were no reports of a CD30L positive cell population in the rheumatic synovium. However, the presence of a CD30$^+$ cell population in the rheumatic synovium, capable of producing IFN-$\gamma$ and IL-4, two potent downregulators of inflammation, was already known [241]. The sCD30L upregulation in the rheumatic synovium can be an attempt to stimulate CD30$^+$ cells to downregulate the inflammatory response and can constitute an important prognostic factor. We know that mast cells are important in the onset of inflammatory arthritis [157], they store huge amounts of pre-formed TNF [54] and anti-TNF therapies have been shown to be effective in diminishing the disease symptoms [271]. Furthermore, upon anti-TNF treatment the serum levels of sCD30 increase in patients that respond to the treatment [272]. Manipulation of the CD30-CD30L pathway could improve the outcome of current treatments to RA. Anti-TNF treatment is also used in the treatment of other inflammatory diseases and some cancers. In psoriasis anti-TNF treatments have proven efficient in reducing the clinical symptoms [273]. Also here, targeting CD30-CD30L interactions in combination with TNF targeting could possibly result in more substantial improvement in the clinical symptoms.

We demonstrated that CBMCs can resist hypoxia and still be activated under hypoxic conditions. It is known that hypoxia develops in different inflammatory diseases and in cancer. Targeting cells that are able, not only to resist local hypoxic conditions, but also to sustain the chronic inflammation, could be an important therapeutic strategy. In such a context, mast cells appear as an obvious possible target
since they are able to regulate their survival in an autocrine way, and under hypoxic conditions they release the strong pro-inflammatory cytokine TNF-α. It is also known that mast cells have phenotypic tissue specificity. In this study we used CBMCs which resemble the connective tissue like mast cells. Since the pathologies indicated can occur in different tissues, it would be important to expand the study by using other types of mast cells, preferably derived from different tissues. As IL-6 can act as a pro-survival cytokine and since we showed that mast cells secrete it in response to hypoxia, investigating whether mast cells present in chronic inflammatory diseases known to be hypoxic are expressing IL-6 should be a next step. In such cases, blocking IL-6 would induce mast cell death and likely hinder the inflammation. Another interesting approach would be to co-culture mast cells with different types of cells under hypoxic conditions to determine possible interactions that may occur.

Mast cells also play important roles in the innate and acquired immune responses to bacteria, parasites and virus [39]. In this context, our finding that mast cells can still be activated by LPS in hypoxic conditions is extremely important. Testing if mast cells can in fact react against bacteria under hypoxic conditions in vitro would be interesting. Studying the effect of hypoxia in parasites and viral activation mechanism of mast cells should also be considered to determine whether the response to these pathogens is still sustained. Mast cells are also involved in the response against parasites so specific mast cell parasite response mechanisms should be tested in hypoxic conditions. In situations where respiration has been compromised, hypoxic situations can occur and mast cell reactivity can play an important role in the defence against pathogen invasion [251].

In the studies here reported we used CBMCs. Nevertheless, it was mentioned before that mast cells phenotype is dependent on the microenvironment where they are present. Future studies should take this into account and try to determine whether the results here reported are still observed when using primary cells isolated from tissues where hypoxic conditions can occur, such as RA or tumours. Recently it was shown that HIF-α accumulation in mast cells is dependent not only on the upregulation of HIF-1A gene transcription but also on the NFAT-calcineurin signalling pathway [274]. Biologically relevant activators of mast cells might be involved in the upregulation of the NFAT-calcineurin pathway and so contribute to the mast cell function under hypoxia, but these remain to be elucidated.

The field of mast cell research is growing fast. Much research remains to be done in order to understand their role in health and disease. Here we focused mostly on the mast cell function and phenotype in the context of chronic inflammation. However, as mentioned in the hypoxia study, mast cells cannot be disregarded in the context of host defence against infection. Research should focus in determining the role played by mast cells in each specific situation and from there design proper therapeutic approaches.
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