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The mast cell transcriptome and the evolution of granule proteins and Fc receptors

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Abstract

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Protection against disease-causing pathogens, known as immunity, involves numerous cells organs, tissues and their products. To able to understand the biology of immune cells (hematopoietic cells) and their role in an immune system, we have used several different methods, including transcriptome analyses, bioinformatics, production of recombinant proteins and analyses of some of them, focusing on the granule proteases by substrate phage display.

Hematopoietic cells express surface receptors interacting with the constant region of immunoglobulins (Igs) known as Fc receptors (FcRs). These receptors play major roles in the immune system, including enhancing phagocytosis, activating antibody dependent cellular cytotoxicity and cell activation. A detailed bioinformatics analysis of FcRs reveals that the poly-Ig receptors (PIGR), FcR-like molecules and common signalling γ chain all appeared very early with the appearance of the bony fishes, and thereby represent the first major evolutionary step in FcR evolution. The Fc μ R, Fc α μ R, Fc γ R and Fc ϵ R receptors most likely appeared in reptiles or early mammals, representing the second major step in FcR evolution.

Cells of several of the hematopoietic cell lineages contain large numbers of cytoplasmic granules, and serine proteases constitute the major protein content of these granules. In mammals, these proteases are encoded from four different loci: the chymase, the met-ase, the granzyme (A/K) and the mast cell tryptase loci. The granzyme (A/K) locus was the first to appear and came with the cartilaginous fishes. This locus is also the most conserved of the three. The second most conserved locus is the met-ase locus, which is found in bony fishes. The chymase locus appeared relatively late, and we find the first traces in frogs, indicating it appeared in early tetrapods.

To study the early events in the diversification of these hematopoietic serine proteases we have analyzed key characteristics of a protease expressed by an NK-like cell in the channel catfish, catfish granzyme-like I. We have used phage display and further validated the results using a panel of recombinant substrates. This protease showed a strict preference for Met at the P1 (cleavage) position, which indicates met-ase specificity. From the screening of potential in vivo substrates, we found an interesting potential target caspase 6, which indicates that caspase-dependent apoptosis mechanisms have been conserved from fishes to mammals.

A larger quantitative transcriptome analysis of purified mouse peritoneal mast cells, cultured mast cells (BMMCs), and mast cells isolated from mouse ear and lung tissue identified the major tissue specific transcripts in these mast cells as the granule proteases. Mast cell specific receptors and processing enzymes were expressed at approximately 2 orders of magnitude lower levels. The levels of a few proteases were quite different at various anatomical sites between in vivo and cultured BMMCs. These studies have given us a new insights into mast cells in different tissues, as well as key evolutionary aspects concerning the origins of a number of granule proteases and FcRs.

Keywords: Mast cell, Fc receptors, Granule serine protease, Evolution and transcriptome.

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కర్మణ్యైవాధికారస్త మా ఫలేషు కదాచన!
మా కర్మఫల హేతుర్భూః మా తే సంగోఽ స్త్వ కర్మణి

Do your duty, but do not concern yourself with the results.

భగవద్గీత (Bhagavad Gita)

To my family, my friends and my Teachers

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Akula, S.**, Mohammadamin, S., and Hellman, L., 2014. Fc receptors for immunoglobulins and their appearance during vertebrate evolution. *PloS one*, 9(5), p.e96903.
- II **Akula, S.**, Thorpe, M., Boinapally, V., and Hellman, L., 2015. Granule associated serine proteases of hematopoietic cells—an analysis of their appearance and diversification during vertebrate evolution. *PloS one*, 10(11), p.e0143091.
- III Thorpe, M., **Akula, S.**, and Hellman, L., 2016. Channel catfish granzyme-like I is a highly specific serine protease with metase activity that is expressed by fish NK-like cells. *Developmental & Comparative Immunology*, 63, pp.84-95.
- IV **Akula, S.**, Paivandy, A., Fu, Z., Thorpe, M., Pejler, G, and Hellman, L., 2019 . The mouse mast cell transcriptome. *Manuscript*.

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Related papers (not included in this thesis)

- I Fu, Z., Thorpe, M., **Akula, S.**, and Hellman, L., 2016. Asp-ase activity of the opossum granzyme B supports the role of granzyme B as part of anti-viral immunity already during early mammalian evolution. *PloS one*, 11(5), p.e0154886.
- II **Akula, S.**, and Hellman, L., 2017. The appearance and diversification of receptors for IgM during vertebrate evolution. In *IgM and Its Receptors and Binding Proteins* (pp. 1-23). Springer, Cham.
- III Hellman, L.T., **Akula, S.**, Thorpe, M., and Fu, Z., 2017. Tracing the origins of IgE, mast cells, and allergies by studies of wild animals. *Frontiers in immunology*, 8, p.1749.
- IV Thorpe, M., Fu, Z., Chahal, G., **Akula, S.**, Kervinen, J., de Garavilla, L., and Hellman, L., 2018. Extended cleavage specificity of human neutrophil cathepsin G: A low activity protease with dual chymase and tryptase-type specificities. *PloS one*, 13(4), p.e0195077.
- V Fu, Z., Thorpe, M., **Akula, S.**, Chahal, G., and Hellman, L.T., 2018. Extended cleavage specificity of human neutrophil elastase, human proteinase 3 and their distant orthologue clawed frog PR3-three elastases with similar primary but different extended specificities and stability. *Frontiers in immunology*, 9, p.2387.
- VI Thorpe, M., Fu, Z., Albat, E., **Akula, S.**, de Garavilla, L., Kervinen, J., and Hellman, L., 2018. Extended cleavage specificities of mast cell proteases 1 and 2 from golden hamster: Classical chymase and an elastolytic protease comparable to rat and mouse MCP-5. *PloS one*, 13(12), p.e0207826.
- VII Fu, Z., **Akula, S.**, Thorpe, M., Chahal, G., de Garavilla, L., Kervinen, J., and Hellman, L., 2019. Extended cleavage specificity of sheep mast cell protease-2: A classical chymase with preference to aromatic P1 substrate residues. *Developmental & Comparative Immunology*, 92, pp.160-169.

Contents

Introduction	11
The Immune system.....	11
B lymphocytes	13
T lymphocytes	13
NK cells	14
Neutrophils	14
Mast cells.....	15
Basophils	16
Eosinophils	18
Macrophage	18
Fc receptors.....	19
Evolution of the immune system	20
Hematopoietic serine proteases	23
Present investigation	27
Results and Discussion.....	28
Paper I.....	28
Paper II	30
Paper III	35
Paper IV	37
Concluding remarks and future prospects.....	40
Populärvetenskaplig sammanfattning	43
Acknowledgments.....	46
References	48

Abbreviations

Ala	alanine
AMP	antimicrobial peptide
Arg	arginine
Asp	aspartic acid
BMMC	bone marrow-derived mast cell
CTL	cytotoxic T lymphocyte
Cys	cysteine
CFD	complement factor D
CPA3	carboxypeptidase A3
CTMC	connective tissue mast cell
FCRLs	Fc receptors like molecules
FcRs	Fc receptors
Fc α R	Fc alpha-receptor
Fc α μ R	Fc alpha mu receptor
Fc γ R	Fc gamma receptor
Fc ϵ R	Fc epsilon receptor
Fc μ R	Fc mu receptor
GM-CSF	granulocyte macrophage-colony stimulating factor
Glu	glutamic acid
Gly	glycine
GZM	granzyme
His	histidine
Ig	immunoglobulin
IGSF	Immunoglobulins superfamily
IL	interleukin
Ile	isoleucine
KIRs	killer-cell immunoglobulin-like receptors
LAIRs	leukocyte associated immunoglobulin like receptor
Leu	leucine
LILRs	leukocyte Ig-like receptors
LPS	lipopolysaccharide
LRC	leukocyte receptor complex
LTC4	leukotriene C4
Lys	lysine
MC	mast cell
MC-CPA	mast cell-carboxypeptidase A

Met	methionine
MHC	major histocompatibility complex
MMC	mucosal mast cell
mMCP	mouse mast cell protease
NCC	non-specific cytotoxic cell
NK	natural killer
Phe	phenylalanine
pNA	p-nitroanilide
Pro	proline
SCF	stem cell factor
Ser	serine
SMC	smooth muscle cell
Thr	threonine
TLR	toll-like receptor
TNF- α	tumour necrosis factor- α
Trx	thioredoxin
Tyr	tyrosine
Val	valine

Introduction

The Immune system

All living organisms have defense mechanisms to combat and eradicate the foreign disease-causing pathogens including viruses, bacteria, fungi and eukaryotic parasites as well as toxins and other potentially harmful macromolecules. Numerous cells, tissues, organs and their products participate in this defense. This complex network of actions known as the immune system often provides us immunity to infection. The immune system has two major arms; the first line of defense against microbes is a non-adaptive system named innate immunity. The innate immune system has no memory and has developed specificity over millions of years. The second arm named adaptive immunity, which usually comes in later during an immune response, has memory and develops specificity in the individual during an immune response. The innate immune system consists of several layers; firstly, the physical barriers like the skin, gastrointestinal tract, respiratory tract, cilia, eyelashes and other body hair. The second layer consists of different chemical barriers such as secretions, mucous, gastric acid, saliva, tears, and sweat, and as a third layer, the leukocytes, including phagocytes primarily represented by neutrophils and macrophages and by NK cells, dendritic cells, mast cells, basophils and eosinophils. Additionally, the complement system can act directly against disease-causing agents. The adaptive system consists of several types of cells and soluble molecules. The Ig (antibody) producing B lymphocytes are primarily involved in humoral immunity (acting by soluble factors), which act against extracellular bacteria and neutralization of toxins. T lymphocytes are involved in both humoral and cellular immunity by helping other lymphocytes to become activated and in the direct defense against intracellular microbes (viruses) and other intracellular eukaryotic and prokaryotic parasites (Chaplin, 2010; Murphy and Weaver, 2016; Parkin and Cohen, 2001; Pennock et al., 2013). The cells involved in both innate and adaptive immunity are hematopoietic cells derived from multipotent hematopoietic stem cells (HSCs) (Rieger and Schroeder, 2012), which in adults, are located in the bone marrow (figure 1) (Doulatov et al., 2012). The HSCs develop into myeloid and lymphoid progenitors (Huston, 1997; Orkin, 2000). These cells function either directly or indirectly by proteins produced by them including cytokines, chemokines, granule proteases and their receptors including toll-like receptors (TLRs), cytokine receptors and FcRs. All of

them are involved in the major functions of the immune system. In this thesis, we have studied the function, diversification and evolution of both the hematopoietic granule proteases, which are stored in their active form in cytoplasmic granules of mast cells, neutrophils, NK cells and cytotoxic T cells (CTLs), and to a lower extent also basophils, as well as the cell surface receptors for Igs, the FcRs that are expressed by the majority of immune cells and some epithelial cells. We have also focused on one particular cell type that expresses and stores large amounts of proteases in its cytoplasmic granules and which is activated by FcRs for IgE on its surface, namely the mast cell. We have performed a quantitative analysis of the transcriptome of mouse peritoneal mast cells and *in vitro* differentiated mast cells as well as studied the type and frequency of mast cells in two organs, the skin and lungs.

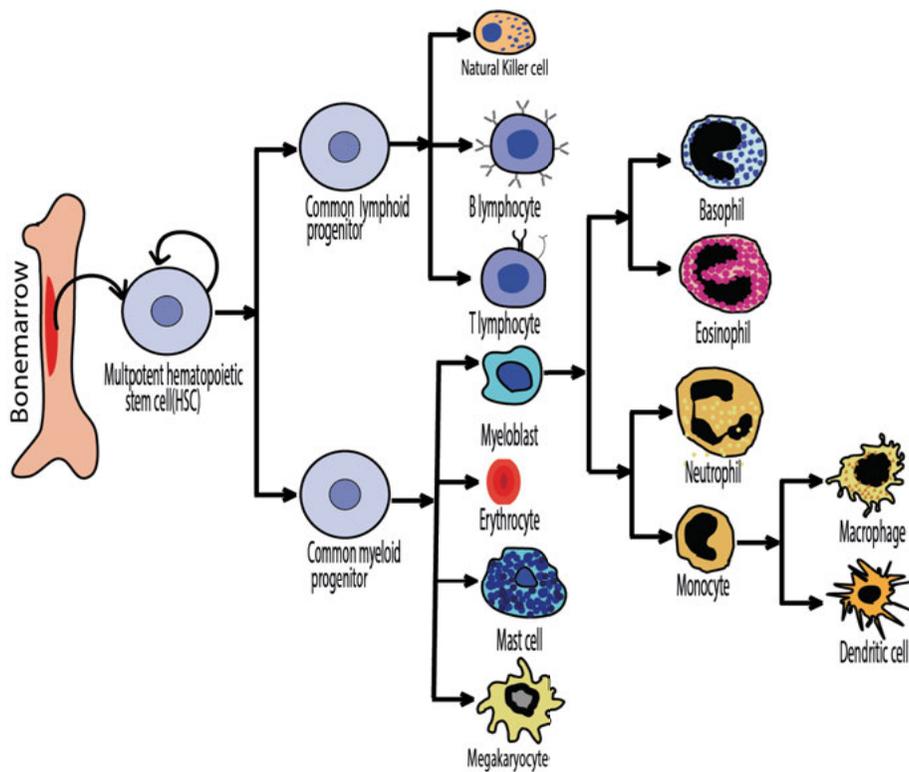


Figure 1. Hematopoiesis. The figure shows the development of the different cell lineages that originate from the hematopoietic stem cells (HSCs) in the bone marrow. The HSCs first differentiate into common lymphoid and myeloid progenitors. The common lymphoid progenitor later developed into three lineages, B lymphocytes, T lymphocytes and NK cells. The common myeloid progenitor develops into myeloblasts, erythrocytes, mast cells and megakaryocytes. The myeloblast further differentiates into basophils, eosinophils, neutrophils and monocytes. The monocyte develops into macrophages or dendritic cells when reaching peripheral tissues.

B lymphocytes

B lymphocytes (B cells) are generated from common lymphoid progenitors in the bone marrow (figure 1) in adults and from the fetal liver during the embryonic stage. In the bone marrow, the B cell first develops into a pro-B cell then to pre-B cell and finally into an immature but fully functional B cell that then leaves the bone marrow to the circulation and for peripheral immune organs (Hoffman et al., 2016; LeBien and Tedder, 2008). After activation, by antigen interaction, B cells proliferate and differentiate into antibody-producing plasma cells and memory cells. The antibodies or immunoglobulins (Igs) are soluble proteins that recognize the antigen and bind to them, which results in targeting by complement for easier uptake by phagocytes. This process of Ig binding to the surface of an antigen molecule, bacteria or virus is called opsonization and is very important for the clearance of the antigen. There are five Ig classes in humans: IgM, IgD, IgG, IgA, and IgE where the number of isotypes varies from species to species but in humans there are nine. Naive B cells express only IgM and IgD. The activated B cells goes through several processes, including Ig class switch recombination, affinity maturation and differentiation into plasma cells that are cells producing the bulk of secretory Ig that circulates in the blood (Murphy and Weaver, 2016; Pieper et al., 2013). Depending on the type of antigen and site of antigen contact, the B cell produces primarily one or a few Ig isotypes, the ones most suitable for the antigen and its organ location. Immunoglobulins are Y-shaped proteins that consist of two heavy chains and two light chains. Proteolytic cleavage can separate the antigen binding from the constant part of the antibody. These are named the antigen binding (Fab) region and the fragment crystalline (Fc) region. (Hoffman et al., 2016; Porter, 1959) The Fc part interacts with surface receptors (FcRs) of several immune cells and they are involved in diverse immune regulatory functions (Ravetch and Kinet, 1991) (figure 3).

T lymphocytes

T lymphocytes are morphologically similar but functionally quite different from the B lymphocytes. These also arise from common lymphoid progenitor in the bone marrow and mature in the thymus (Boehm and Bleul, 2006; Doulatov et al., 2012) (figure 1). The mature T cell primarily differentiates into two types of T cells, one is CD4⁺ expressing (T-helper cell), which helps other lymphoid cells to expand in numbers and mature in fully immune-competent cells (Koch and Radtke, 2011; Vallejo et al., 2004). They are mainly involved in humoral immunity, helping B cells, and in helping the other major T-cell population, the CD8⁺ expressing CTLs. These are involved in the killing of tumor or virus-infected cells by inducing apoptosis

mechanisms (Chowdhury and Lieberman, 2008; Groscurth and Filgueira, 1998). The CTLs thereby play an important role in cellular immunity. Cytotoxic T cells store cytolytic proteins in their cytoplasmic granules. The proteins stored in these granules are primarily perforin that forms membrane holes in the target cell, and a number of chymotrypsin-related serine proteases named granzymes. Human CTLs express the granzymes A, B, H and K (Bots and Medema, 2006; Grossman et al., 2003; Jenne et al., 1988; Jenne and Tschopp, 1988; Trapani, 2001), which are encoded from the granzyme A/K locus and from the chymase locus (Akula et al., 2015; Hellman and Thorpe, 2014). Perforin and the granzymes are involved in the induction of target cell death by various apoptosis mechanisms (Groscurth and Filgueira, 1998; Voskoboinik et al., 2015).

NK cells

NK cells are similar in function to the CTLs and they also develop from the HSCs in the bone marrow (figure 1) to reside in multiple lymphoid and non-lymphoid tissues including the bone marrow, lymph nodes, skin, gut, tonsils, liver, and lungs. NK cells constitute about 20–30% of total hepatic lymphocytes and 10% of lymphocytes in healthy human liver and lung, respectively (Carrega and Ferlazzo, 2012; Geiger and Sun, 2016; Sun and Lanier, 2011). NK cells are among the first cells that contact virus-infected cells and they are also involved in the killing of virus-infected or tumor cells by inducing the cell death of cells lacking MHC class I expression or by antibody-dependent cellular cytotoxicity (ADCC) (Borregaard, 2010; Caligiuri, 2008; Geiger and Sun, 2016; Sun and Lanier, 2011). Like CTLs, NK cells contain large numbers of granules containing perforin and granzymes (Jenne and Tschopp, 1988). Human NK cells express granzymes A, K, M and possibly H (Bots and Medema, 2006; Grossman et al., 2003; Jenne and Tschopp, 1988; Trapani, 2001). The genes for these granzymes are located in the chymase, met-ase and the granzyme (A/K) loci (Akula et al., 2015; Hellman and Thorpe, 2014) NK cells also express a large number of surface receptors, killer-cell Ig-like receptors (KIRs) (Natarajan et al., 2002), complement receptors (Biassoni, 2008), FcRs (Perussia, 1998) and cytokine receptors, which are involved in various immune regulatory functions (Mandal and Viswanathan, 2015).

Neutrophils

Neutrophils are the most abundant leucocyte in human blood making up around 55-70% of all white blood cells (Cowland and Borregaard, 2016; Fu et al., 2018). They are derived from the pluripotent HSC in the bone marrow

and their life span is generally short, only 3-4 days (figure 1) (Punt, 2018; Simon and Kim, 2010). Neutrophils are among the first cells to respond during bacterial infections and they act by a combination of oxidative and non-oxidative mechanisms and use phagocytosis to ingest and thereby remove the pathogen from the circulation (Amulic et al., 2012; Brinkmann et al., 2004; Rosales, 2018). Neutrophils have a lobular polymorph nucleus and a cytoplasm filled with at least four types of granules: azurophil, specific, gelatinase and secretory (Lacy, 2006). These granules are generated sequentially during the development of neutrophils and they are classified based on their protein content. Azurophil granules contain myeloperoxidase (MPO), bacterial permeability-increasing protein (BPI), defensins and a number of serine proteases or protease homologues including proteinase 3 (PRNT3), neutrophil elastase (NE), cathepsin G (CG) and neutrophil serine proteinase 4 (NSP4) as well as an inactive structurally related protein that is a potent antibacterial protein called azurocidin (AZU) (Borregaard and Cowland, 1997; Kessenbrock et al., 2011; Kolaczkowska and Kubes, 2013; Perera et al., 2012; Phillipson and Kubes, 2011). In addition to pathogen killing, the neutrophil proteases may also have a role in regulating inflammation by cleavage of cytokines (Clancy et al., 2018). These are similar in structure to mast cell tryptase, mast cell chymase and CTL granzymes. The neutrophil proteases are organized into two loci on two different chromosomes in humans. PRNT3, NE, AZU and NSP4 are, together with granzyme M, located within the met-ase locus and cathepsin G is located within the chymase locus together with the mast cell chymase genes (Akula et al., 2015; Hellman and Thorpe, 2014). The neutrophil proteases are involved in different protective mechanisms against microorganisms (Pham, 2006). The antimicrobial function of neutrophils also depends on neutrophil expressed receptors, which are involved in the recognition of the pathogen and the activation of the neutrophil to eliminate the pathogen (Brinkmann et al., 2004; Kolaczkowska and Kubes, 2013). There are different classes of surface expressed receptors involved in various functions including G-protein coupled receptors, integrins, selectins, cytokine receptors, C-type lectins, innate immune receptors (TLRs) and FcRs. Neutrophils express various FcRs, where the most important ones on human neutrophils are the low-affinity receptors for IgG: Fc γ RIIA, Fc γ RIIIB, and in mice the Fc γ RIII and Fc γ RIV. These FcRs are used during the activation of the immune response to opsonized bacteria or immune complexes (Futosi et al., 2013; Ravetch and Kinet, 1991). Resting neutrophils primarily express Fc γ RI and Fc α RIA (Daeron, 1991).

Mast cells

Mast cells are innate-type hematopoietic cells derived from the HSCs in the bone marrow, as the other hematopoietic cells (Galli et al., 2005; Gurish and

Austen, 2012; Kitamura et al., 1981) (figure 1). They leave the bone marrow as relatively immature cells and migrate and develop into mature mast cells in the tissues under the influence of different cytokines and cell to cell contacts including the c-kit ligand, also named stem cell factor, and IL-3 (Dahlin and Hallgren, 2015; Stone et al., 2010; Wernersson and Pejler, 2014). Mast cells are present all over the body, however they are more abundant in tissues in contact with the external environment such as the skin, lungs, and mucosa (St John and Abraham, 2013). Their phenotype may vary depending on the local environment and factors present in that tissue. In the cytoplasm, they store a large number of the lysosomal-like granules, which contain an array of inflammatory mediators such as histamine, serotonin, proteoglycans, including heparin and chondroitin sulfate, cytokines and proteases (tryptase, chymase and CPA3) (da Silva et al., 2014; Pejler et al., 2007). Upon triggering of the cell cross-linking of IgE sitting on these receptors by antigen, results in activation and granule release, they also produce, and secrete other potent inflammatory mediators like leukotriene C4 and prostaglandin D2 (Benoist and Howard, 2000). The mast cell expresses a number of receptors including cytokine, chemokine, innate type receptors (TLR), adhesion molecules and FcRs including FcεRI, FcγRI, FcγRII, FcαμR and FcαR. FcεRI is the main receptor on mast cell (figure 2) and the most important receptor for its role in allergy (Buchmann, 2014; Daeron, 1991; Galli and Tsai, 2012; Hellman, 2007; Kinet, 1999; Migalovich-Sheikhet et al., 2012; Rivera et al., 2008). Mast cell plays important roles under both physiological and pathological conditions. They are involved in protection against bacterial and parasite infections neutralizing toxins like snake venom, tissue homeostasis but also in the activation of other immune cells like B cells, T cells and dendritic cells (Galli et al., 1999; Galli et al., 2005; Urb and Sheppard, 2012; Voehringer, 2013; Wernersson and Pejler, 2014).

To obtain a more detailed picture of the biology of mast cells we have analyzed the transcriptome of mast cells from different organs by RNA-seq and PCR based transcriptome analyses (unpublished data), which is discussed in more detail in paper IV.

Basophils

In comparison to mast cells, basophils primarily reside in the circulation and they show a number of similarities to mast cells, for example, the expression of FcεRI (Schroeder, 2009; Wedemeyer et al., 2000), as well as the content of their cytoplasmic granules such as histamine, heparin and proteases (Hellman, 2007; Hellman et al., 2017; Siracusa et al., 2011) (figure 1). However, they have fewer and much lower amounts of the proteases and some other mediators compared to the mast cell. Basophils are involved in host

defense against helminth parasites and have a pathological role in allergy (Hellman, 2007; Ishizaka et al., 1972; Sokol and Medzhitov, 2010; Voehringer, 2013). Mouse basophils express a specific protease mMMC-8, which is not expressed by mouse mast cells (Lutzelschwab et al., 1998; Poorafshar et al., 2000). The mMCP-8 gene is located within the chymase locus (Akula et al., 2015; Gallwitz and Hellman, 2006; Tsutsui et al., 2017)

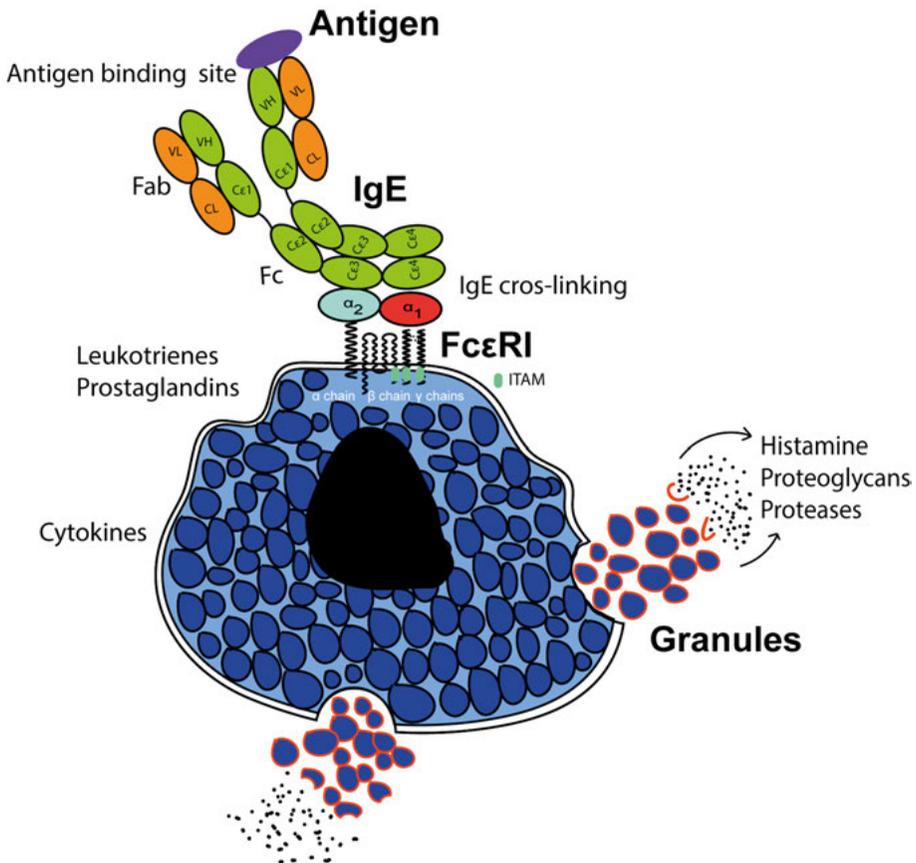


Figure 2. Mast cell activation. Mast cells express Fc receptors for IgE (FcεR). Cross-linking of IgE sitting on these receptors by antigen, results in activation and release of granule, histamine, proteoglycans, cytokines and proteases. For illustration purposes only a single IgE antibody is shown. Abbreviations: ITAM, immunoreceptor tyrosine-based activation motif. V_L, variable-light. V_H, variable-heavy. C_L, constant-light. C_H, constant-heavy, C_ε constant-epsilon, Fab fragment of antigen binding, Fc fragment of constant. Adapted from (Hellman, 2017).

Eosinophils

Eosinophils belong together with neutrophils and basophils to the polymorph nuclear granulocytes (figure 1). They are present in the circulation and have the capacity to quickly move into various tissues such as in the gastrointestinal tract, as well as the genitourinary tract upon infection or inflammation in response to cytokines, chemokines and other chemotactic substances (Blanchard and Rothenberg, 2009; Rothenberg and Hogan, 2006; Stone et al., 2010). The granule content of human eosinophils consists primarily of four different cationic proteins: the major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (Hamann et al., 1991). Eosinophils are primarily thought to be of importance in our defense against large parasites including helminthic worm infections (Fabre et al., 2009; Weltman, 2000). They are also found in large numbers in tissues that are inflamed due to allergic reactions (Acharya and Ackerman, 2014; Humbles et al., 2004; Lee et al., 2004). Eosinophils express several FcRs including Fc μ R, Fc δ R, Fc α R, Fc γ RII, Fc γ RI and Fc γ RIII. Some studies show they can express Fc ϵ RI but this function remains elusive, as the levels of expression are at least two orders of magnitude lower than the level seen on basophils (McBrien and Menzies-Gow, 2017; Muraki et al., 2011; Stone et al., 2010).

Macrophage

Macrophages are found in almost all tissues. They are evolutionary very old phagocytic cells, probably the first immune cell that appeared during early multicellular evolution (Ginhoux and Jung, 2014; Hirayama et al., 2017; Wynn et al., 2013). They are anatomically and functionally a very diverse set of cells (Wynn et al., 2013). The major function of macrophages is in eliminating microorganisms, antibody-coated viruses, dead cells and cell fragments, and immune complexes by phagocytosis (Luo et al., 2010). The professional phagocytic (mononuclear phagocytes) cells that in addition to phagocytosis these are involved in tissue homeostasis and inflammation (Davies et al., 2013; Wynn et al., 2013). These cells express several receptors involved in both innate (TLRs and cytokine receptors) and adaptive immunity (FcRs) (Lennartz and Drake, 2018). The cross-linking of FcRs and Igs triggers complicated signal transduction pathways that promote phagocytosis and inflammatory immune responses (Luo et al., 2010).

Fc receptors

The constant part of Igs, the Fc region, binds to FcRs and these FcRs are expressed on almost all hematopoietic cells and also on some epithelial cells (Daeron, 1991; Dickler, 1974; Ravetch and Kinetic, 1991; Sakamoto et al., 2001). Fc receptors play important roles in adaptive immunity, both humoral and cellular immunity, where they facilitate phagocytosis by opsonization, are key components in antibody-dependent cellular cytotoxicity as well as activating cells to release granules (Akula et al., 2014; Ravetch and Kinetic, 1991; Ravetch et al., 1986) (figure 3). The FcRs have three structural parts; the ligand binding extracellular region contains Ig-like domains, a transmembrane region and a cytoplasmic tail for signal transduction which consists of an immunoreceptor tyrosine-based activation motif (ITAM) and/or an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Bruhns et al., 2009; Daeron et al., 1995; Davis, 2007; Pincetic et al., 2014; Reth, 2014) (figure 2). The biological function of the FcRs is dependent on these cytoplasmic tyrosines leading to either activation or inhibition of the target cell (Hogarth et al., 1992). Each antibody has its respective FcR: Fc γ for IgG, Fc ϵ for IgE, Fc α for IgA, Fc μ for IgM and Fc δ for IgD. These all Fc receptors are members of immunoglobulin superfamily (IGSF) receptors (figure 2) (Nimmerjahn and Ravetch, 2007; Raghavan and Bjorkman, 1996; Williams and Barclay, 1988). Based on the structure, function and binding specificity of the different Ig isotypes, FcRs are distinguished into three types: The classical FcRs (Fc γ R, Fc ϵ R, Fc μ R, Fc δ R, and Fc α R), FcR-like molecules (FcRL1-FcRL6) (Davis et al., 2002; Davis et al., 2005) and the intracellular receptor-like proteins FcRLA and FcRLB (Ehrhardt et al., 2007). The function of FcRL molecules and intracellular receptor-like proteins is still unknown. The classical receptors have a large number of heterogenic forms, each form has subtypes and the affinity to antibody binding also varies between different types of receptors (Davis, 2007; Fridman, 1991). Fc γ R is classified into Fc γ RI, Fc γ RII, Fc γ RIII and Fc γ RIV. Human Fc γ RI has three homologous genes (IA, IB and IC), of which Fc γ RIB and Fc γ RIC are pseudogenes. Human Fc γ RII is also encoded from three genes (IIA, IIB and IIC), and Fc γ RIII by two genes (IIIA and IIIB) (Miller and Aladjem, 1975). Fc γ RI bind to antibodies with high affinity whereas the other binds with low affinity (Nimmerjahn and Ravetch, 2008; van der Poel et al., 2011). Fc α R binds to IgA with a medium affinity. There are two receptors for IgE, one is the high-affinity Fc ϵ RI (Blank et al., 1989), and one with low-affinity Fc ϵ RII (Lawrence et al., 1975). Fc μ R binds to IgM (Shima et al., 2010) whereas Fc $\alpha\mu$ R binds both IgA and IgM (Kubagawa et al., 2009). Poly-Ig receptor is a receptor for IgA (pIgA) and IgM (pIgM) positioned at mucosal surfaces facilitating the transport of IgA and IgM into the intestinal lumen, saliva and tears (figure 2) (Akula and Hellman, 2017). Fc δ Rs (Coico et al., 1985), the receptors for IgD, are not yet sufficiently characterized and is therefore not

included in our studies. FcRn is a neonatal receptor (Daeron, 1997) of IgG. This receptor is a member of the large family of non-varying MHC class I molecules (Story et al., 1994). The Fc δ R and Fc ϵ RII are not related in structure to the other receptors, as they do not contain Ig-like domains and instead belong to the lectin family. As previously mentioned all the other FcRs belong to the Ig superfamily of receptors (IGSF receptors) (Akula et al., 2014; Chenoweth et al., 2015; Daeron, 1997) In paper I of this thesis we have studied the genomic organization and the appearance during vertebrate evolution of the IGSF.

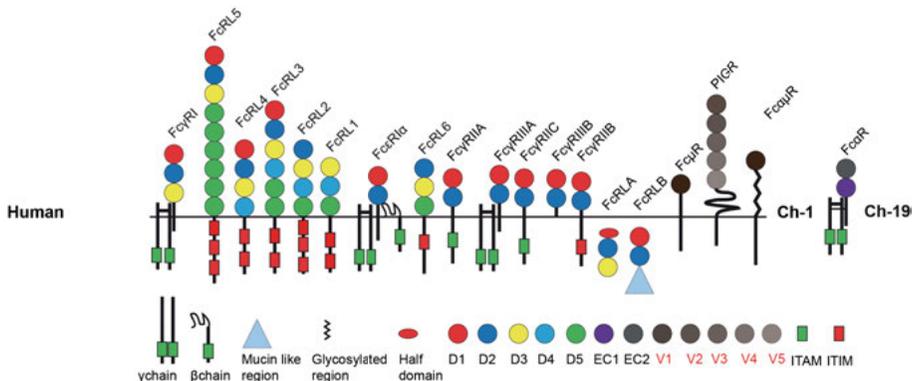


Figure 3. A summary figure of human Fc receptors. The figure is adopted from Paper I. The different regions of the human Fc receptors are visualized schematically with the extracellular Ig-like domains as filled circles and the cytoplasmic tail with the signaling motifs as boxes. The Ig-like domains are color-coded based on sequence similarity. The C2 type domains D1 (red), D2 (blue), D3 (yellow) D4, (light blue) and D5 (green), the V-type domains V1 to V5 in different grey shade color. Fc α R EC1 domain in purple and EC2 in light black color.

Evolution of the immune system

Life on the Earth began sometime between 3.5 and 4.2 billion years ago as a single cellular organism most likely resembling a primitive prokaryotic bacterium. Subsequently life has developed in several major directions, one to a fantastic array of different bacteria inhabiting all thinkable places on earth and the second into the archaea, a separate kingdom of prokaryotic single cellular organisms, which most likely became the early ancestor of all present eukaryotic life including the eukaryotic multicellular metazoan animals. These early metazoan eukaryotes later, through a long series of events like environmental changes, selection pressure, increases in oxygen levels, mutations, deletions and whole genome duplications were the organisms that also gave rise to mammals (Cooper and Alder, 2006; Cooper and Herrin, 2010; Yatim and Lakkis, 2015). All of these factors and probably many more

played an essential role in the evolution of life. All living organisms have a well functioning, often complicated metabolic system that may be interesting to use by other organisms which therefore needs to be protected (Buchmann, 2014; Cooper and Herrin, 2010; Du Pasquier, 1992; Muller et al., 1999). In all parts of the evolutionary tree of life, there is a need to protect themselves from intruders (Beutler, 2004). The prime function of such a defense mechanism is to recognize and discriminate self from non-self, develop the effector cells, receptors, and molecules and try to remove such potential intruders (Akira et al., 2006; Janeway and Medzhitov, 2002). The type of receptors, effector cell and mechanisms are different in each species and the complexity also increases from bacteria to mammals during evolution (Buchmann, 2014). Bacteria protect themselves from viruses by the use of restriction enzymes, decoy proteins, apoptosis-like mechanisms and also an adaptive immunity based on clustered regularly interspaced palindrome repeats (CRISPRs) to remember and degrade incoming bacterial virus (phage) DNA (Dunin-Horkawicz et al., 2014). Single cell amoeba uses phagocytic-like cell mechanisms to take up food by pinocytosis. Such phagocytic-like cells and phagocytic cells are present in all invertebrates and vertebrates with different names like amoebocytes, coelomocytes, granulocytes and macrophages, and are probably the first type of immune cells in multi-cellular organisms (Desjardins et al., 2005) (figure 4). The complexity of the receptors on cells to recognize non-self or foreign pathogens has also increased during evolution. Examples of such receptors are the nucleotide binding, primarily viral RNA binding, receptors, the NOD-like receptors (NLRs), as well as TLRs, and many additional related and unrelated receptors which all recognize molecular structures of different pathogens. These receptors are all part of innate immunity, which is the evolutionary oldest immune system present in all living organisms (Akira et al., 2006). Later, adaptive immunity appeared, where the individual developed new antigen-binding proteins within days for protection against a new pathogen (Cooper and Alder, 2006; Dzik, 2010; Flajnik and Kasahara, 2010) Such adaptive systems have recently been identified in most multi-cellular organisms (Zimmerman et al., 2010). However, a fully developed system with very large complexity seems first to have appeared with vertebrates. Two structurally very different but functionally very similar such systems have been identified in vertebrates (Muller et al., 2018). The most well known complex system, including memory, a large repertoire of cells and receptors are found in jawed vertebrates including cartilaginous fishes, bony fishes, amphibians, reptiles, birds, and mammals. This system probably appeared in the first jawed vertebrates around 450 million years ago. The major players of this type of adaptive immunity are lymphocytes including B cell and T cells and their receptors, the B-cell receptors (BCR) the Igs, the T-cell receptors (TCR), the MHC molecules for antigen presentation, and the generative organs primary lymphoid organs: the thymus and bone marrow (Cooper and Alder, 2006; Flajnik, 2002; Kishishita and

Nagawa, 2014; Zhang et al., 2014). This system is found in all vertebrate species from cartilaginous fishes to humans, however, the complexity has increased during evolution (Flajnik and Kasahara, 2010; Zhao et al., 2009). The evolution of Ig isotypes and their specific FcRs appearance during the vertebrate evolution is studied in (Paper I) this thesis. Interestingly, a functionally similar system also with high complexity and specificity is found the jawless fishes, lampreys and hagfish. The form of adaptive immunity consists not of Igs and TCRs but a structurally completely different set of molecules with leucine-rich repeats (LRRs), similar to the TLRs. These variable leukocytes receptors (VLR) used to recognize the pathogen are functionally very similar to the mammalian antigen receptors but have no structural similarity, which in our minds is one of the most beautiful examples of convergent (Alder et al., 2005) evolution (Pancer et al., 2004; Rogozin et al., 2007). This system probably developed during the early Cambrian period around 530 million years ago) (Cooper and Alder, 2006; Flajnik and Kasahara, 2010).

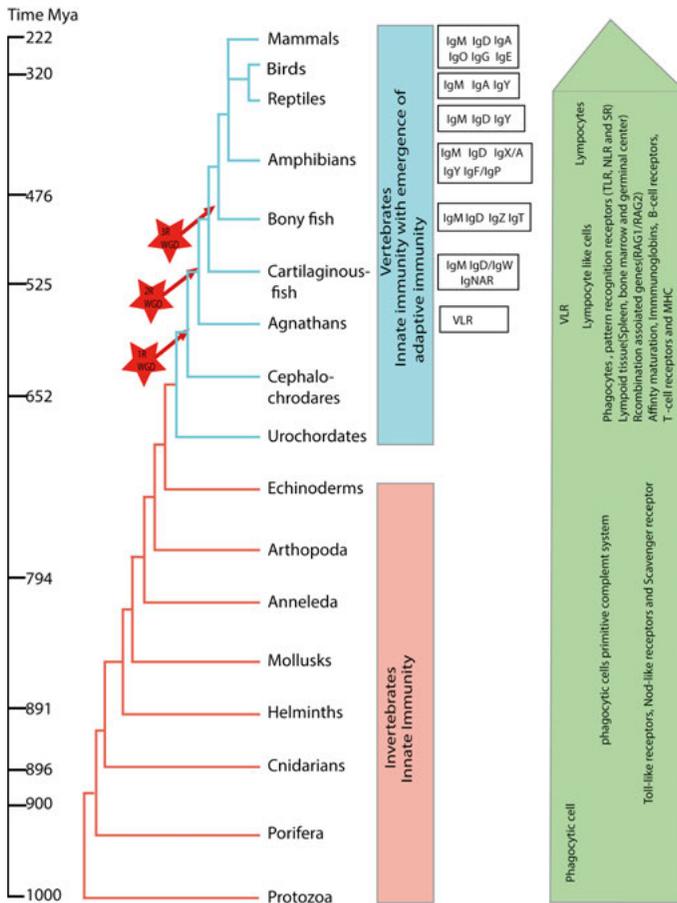


Figure 4. A summary figure of the evolution of the immune system. Phylogeny of the animal kingdom with an approximate time of appearance of the different major lineages. Invertebrate branches are shown in orange and vertebrate in blue. The emergence of the different immune cells, the tissues, the organs, the molecules and the receptors are shown within the green large arrow. Phagocytic cells, pathogen recognition receptors, and complement system appeared already in invertebrates. The lymphocyte-like receptors, the VLRs, are found in in agnathans, whereas the immunoglobulins, lymphocytes, bone marrow, thymus, spleen, BCR, TCR, MHC, germinal centres, and affinity maturation developed in jawed vertebrates. The red stars represent the timing of three whole genome duplications. The third is affecting only some of the bony fishes.

Hematopoietic serine proteases

The hydrolysis of peptide bonds is facilitated by catalytic proteins known as proteases. Based on structural and sequence similarity they can be classified

into serine, cysteine, metallo, aspartic, glutamic, and threonine protease according to the MEROPS classification (Rawlings et al., 2008; Rengel et al., 2007). Serine proteases constitute around one-third of all the proteases and among them, the chymotrypsin-like serine proteases are a major family. The catalytic mechanism in the chymotrypsin-related serine proteases is mediated by the active site, which uses three amino acids, His57, Asp102, and Ser195 (termed catalytic triad) (Carter and Wells, 1988; Dodson and Wlodawer, 1998; Greer, 1990; Hanson et al., 1990) to destabilize a peptide bond and later break this bond (figure 5). The numbering is based on chymotrypsinogen that has a similar structure, 245 amino acids in size. Four types of protease have similar catalytic triads and similar catalytic mechanisms. However, these four families are structurally very different and have therefore most likely evolved independently. These four serine protease families (catalytic triad order in brackets) are the chymotrypsin (His-Asp-Ser), subtilisin (Asp-His-Ser) (Yousef et al., 2003), carboxypeptidase Y (Ser-Asp-His) and Clp (Ser-His-Asp) and they are present in almost all living organisms including, bacteria, viruses, plants, and animals (Berg et al., 2002). Several of the major hematopoietic cell lineages including T cells, NK cells, neutrophils, basophils, eosinophils and mast cells often have large numbers of cytoplasmic granules filled with mediators (Caughey, 2006; Di Cera, 2009; Dodson and Wlodawer, 1998; Schwartz et al., 1987) (figure 1). In several of the cell types, the major proteins content in these granules are serine proteases (Caughey, 1994). These serine proteases all belong to the chymotrypsin-like serine proteases. Serine proteases of the trypsin/chymotrypsin family play important roles in a number of physiological processes including blood coagulation, clot resolution, complement activation, food digestion, fertilization, fibrinolysis, blood pressure regulation, tissue homeostasis, and immunity (Puente et al., 2003; Schmidt et al., 2008). The catalytic triad (His-Asp-Ser) of these proteases is initiated the hydrolysis of the peptide bond, but the preference for the substrate depends on amino acids of the S1 pocket (Hedstrom, 2002; Tsu et al., 1997) which often consist of the amino acids in positions 189, 216 and 226 (chymotrypsinogen numbering) (Perona and Craik, 1995; Tsu et al., 1997) (figure 5). These residues are important for substrate cleavage, where a negative Asp in position 189 of the S1 pocket accommodates positively charge Arg or Lys in the substrate making the protease trypsin-like. The chymotrypsin-like proteases have a non-polar S1 pocket, they, therefore, prefer aromatic acids such as tryptophan, phenylalanine, tyrosine or leucine in the P1 position of the substrate. The bulky amino acids valine or threonine in the S1 pocket results in a preference for small hydrophobic residues, such as alanine making the protease having elastase specificity (Akula et al., 2015; Di Cera, 2009; Hellman and Thorpe, 2014) (figure 6). The genes encoding proteases expressed by hematopoietic cells the hematopoietic serine proteases are in mammals organized in four different loci: the mast cell chymase locus, the mast cell tryp-

tase locus, the granzyme (A/K) and the met-ase locus. In this thesis, I have studied three of these four loci: the mast cell chymase, the granzyme (A/K) and met-ase loci and their appearance and diversification from sea urchins and tunicates to placental mammals using bioinformatics analyses (Paper II). From the analyses, we have identified several interesting proteases from non-mammalian species. The aim is now to study their specificity and their *in vivo* targets to understand the evolution of the key functions of these hematoopoietic serine proteases and their roles in the immune system. Here, the first step in such a task has been to analyze in more detail one member of one of the sub-branches of the chymase-locus related proteases, the catfish granzyme like-I (paper III).

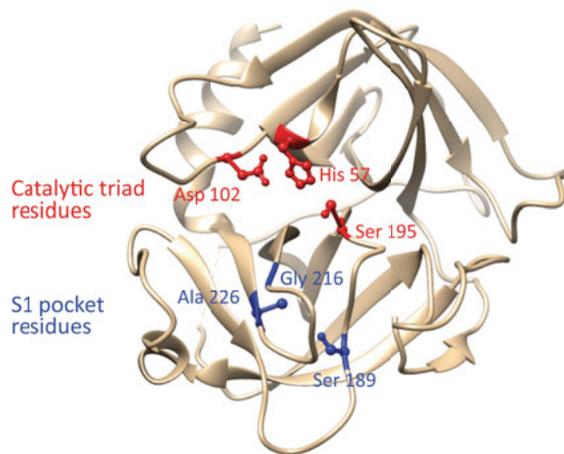


Figure 5. The crystal structure of the human mast cell chymase (PDB 3N70) showing the residues of the catalytic triad in red and the residues of the S1 pocket in blue. The image used with the permission from Dr. M. Thorpe.

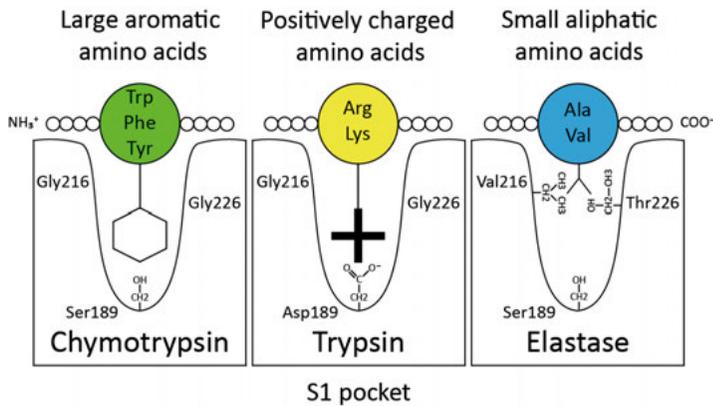


Figure 6. S1 pocket. The primary specificity defining S1 Pocket of chymotrypsin related serine proteases. The size and the characteristics, including hydrophobicity and charge of the different residues in the pocket determines the primary specificity of the protease. In the figure examples of chymotrypsin, trypsin, or elastase specificity are shown. The colour coding matches the phage display aligned amino acid sequences in paper III of this thesis. The image used with the permission from Dr. M. Thorpe.

Present investigation

Aim

The general aim of this thesis was to obtain a detailed picture of the mast cell by analysis of its transcriptome and an evolutionary analysis of granule proteins and Fc receptors expressed by mast cells and other hematopoietic cells.

Results and Discussion

Paper I

Fc Receptors for Immunoglobulins and Their Appearance during Vertebrate Evolution.

The aim of this article was to study the appearance and diversification of FcRs during vertebrate evolution.

The complexity of the immune system has increased gradually during vertebrate evolution, which resulted in an increase in both the number of classes and isotypes of Igs. In this paper, we have investigated how the receptors interacting with the constant domains of Igs, the FcRs, have evolved in relation to the increase in the number of Ig-isotypes. These receptors have a number of important functions, participating in the uptake of opsonized immune complexes, viruses and bacteria, they trigger cells to release granules and they can aid in antigen presentation by enhancing antigen uptake by antigen presenting cells. All the five mammalian Ig classes (IgM, IgD, IgG, IgE and IgA) have specific receptors interacting with their constant domains. Such receptors have been identified in a number of different mammals, however information concerning when these receptors appeared during vertebrate evolution and how they have diversified has been lacking, which was aim of this study.

Immunoglobulin M is most likely the first Ig isotype to appear during vertebrate evolution and is also the first Ig isotype to be expressed on B cells during an immune response. Three types of receptors binding to the Fc constant domain of IgM have been identified, the transporter receptors for IgM and IgA (PIGR), the dual receptor for IgA and IgM (Fc $\alpha\mu$ R) and a specific receptor for IgM (Fc μ R). Poly Ig R was the first to appear of these three receptors for IgM where it was found in all tetrapods and in bony fishes, but not in lampreys, hagfishes and sharks. Interestingly in zebrafish, 25 PIGR genes were located on two chromosomes, with 13 PIGR genes on chromosome 2, and 12 PIGR genes on chromosome 3. Strikingly only one copy of the PIGR was found in the gar, representing an early branch of the bony fishes, which is an indication for a massive expansion during bony fish radiation. In amphibians, reptiles and birds we also found the PIGR, however

often only as one gene. In contrast to the PIGR the other two receptors for IgM, Fc μ R and Fc $\alpha\mu$ R, seem to have appeared much later in vertebrate evolution. The IgM specific receptor most likely appeared in reptiles, as there was a copy of the Fc μ R gene in the American and Chinese alligators and in all mammals analyzed. However, it seems to have been lost in birds and some reptile lineages. The dual receptor for IgM and IgA, Fc $\alpha\mu$ R, was only found in mammals and thereby the youngest of the three IgM receptors. It was found in all three extant mammalian lineages, the egg-laying monotremes, the marsupials and the placental mammals indicating an appearance during early mammalian evolution.

IgD is probably the second isotype to appear during vertebrate evolution and was found in most species from bony fishes to mammals. This isotype was most likely secondarily lost in some species such as the chicken and the opossum. The specific receptors for IgD are not yet sufficiently characterized and were therefore not included in our bioinformatics analysis. Two new isotypes appeared with early tetrapods, IgA (IgX) and IgY, and these two Ig classes were found in almost all studied amphibians and reptiles. Interestingly, the specific receptor for IgA (Fc α R) was only found in placental mammals, despite the relatively early appearance of IgA/IgX. It was also the only FcR that was found on another chromosome (Ch-19) in humans together with the NK cell KIRs and LILRs, and not together with the other FcRs on chromosome 1 in humans.

IgG and IgE were the last two Ig classes to appear during vertebrate evolution and they both were present only in mammals. However, all three extant mammalian lineages have IgE and IgG and in placental mammals, four specific receptors for IgG have been identified: Fc γ RI, Fc γ RII, Fc γ RIII and Fc γ RIV, as well as one high-affinity receptor for IgE, the Fc ϵ RI. The organization of IgG and IgE receptors are similar in all placental mammals. However, some differences were seen in some species. In humans, a duplication of the high-affinity receptor for IgG (Fc γ RI) has occurred resulting in three genes (IA, IB and IC) where two of them IB and IC have become inactivated and are now present as pseudogenes. Duplications of the low-affinity IgG receptor Fc γ RII (IIA, IIA, IIC) and Fc γ RIII (IIIA and IIB) have also occurred in humans. In the mouse, we found an additional receptor Fc γ RIV that was closely related to the low-affinity receptors Fc γ RII and Fc γ RIII. Local duplications of FcR genes have been observed in the rabbit, platypus and clawed frog. However, the specificities of the receptors in the platypus and clawed frog are still not known. Receptors structurally similar to classical FcRs, the FcRL molecules (FcRL-1-5, FcRLA and FcRLB), have been identified in all studied species from fishes to mammals, following the analyses of the full human and mouse genome sequences, but the number FcRL molecules and the organization varies from species to species.

To obtain a more detailed picture of the evolution of FcRs, individual FcR domains were studied for their phylogenetic relationship using Maximum-

likelihood, Neighbor-joining and distance algorithms. These three methods gave very similar results indicating the robustness of the analyses. In the phylogenetic tree the IgM receptors (PIGR, Fc α μ R and Fc μ R) formed a separated branch, the most distantly related to the other receptors thereby forming an out-group and IgA receptor also formed a sub-branch outside the IgM receptors. The classical receptors for IgG and IgE formed one branch, the and the FcRL molecules form a branch more closely to classical receptors between the IgG, IgE and IgM receptors. This latter finding indicated that the classical receptors arose as a subfamily from the FcRLs. The individual domains of the FcRs clustered very stably into clearly defined branches based on their structural similarity. V-type domains from the IgM receptors clustered in one branch, IgA receptor domains formed a separate sub-branch outside of the IgM receptor domains, and all the IgG, IgE and FcRLs domains formed one large branch where each internal domain formed separate sub-branches. In the figures in paper I, all these domains were color-coded based on their structural similarity.

In conclusion, the complexity of the FcRs has increased in parallel with the complexity of the Ig isotypes during vertebrate evolution. This started with PIGR, FcRL molecules and common signaling γ chain, where all three appeared with the bony fishes. This was the first major evolutionary step in FcRs evolution. The Fc μ R, Fc α μ R, IgG and IgE receptors then appeared in reptiles or early mammals. This was the second major step in FcRs evolution. The receptor for IgA is only found in placental mammals and thereby the last to appear on the different FcRs. Structural relationships of domains support the conclusion that the FcRL molecules are the ancestor for both the IgG and the IgE receptors, and that the Fc α μ R and Fc μ R evolved from the PIGR by gene duplications involving only the first Ig-like domain of the PIGR, therefore the PIGR is the ancestor of all three FcRs for IgM.

Paper II

Granule Associated Serine Proteases of Hematopoietic Cells – An Analysis of Their Appearance and Diversification during Vertebrate Evolution.

The aim of this paper was to study the locus organization and evolution of hematopoietic serine proteases from sea urchins to mammals.

Several hematopoietic cell lineages like mast cells, neutrophils, NK cells, T cells and basophils contain vast numbers of cytoplasmic granules. A major fraction of the protein content of these granules is often proteases, and the absolute majority of them belong to the large family of chymotrypsin-related serine proteases. These proteases can have very diverse primary cleavage

specificities and cleave after many different types of amino acids such as aromatic, basic (positively charged), negatively charged, methionine and small aliphatic amino acids. They are therefore classified as chymases, trypsinases, aspartases, metases and elastases, respectively. The serine proteases expressed by hematopoietic cells are encoded from four different loci in mammals: the chymase, the metase, the granzyme (A/K) and the mast cell tryptase loci, where not much is known about the situation in non-mammalian species. To study their appearance and diversification during vertebrate evolution we have performed a bioinformatics analysis of their relatedness and also compared their chromosome organization in a large panel of vertebrate species. In this study, we have focused on three of these loci: the chymase, the metase and the granzyme (A/K).

From this analysis, we concluded that the granzyme A/K locus was the evolutionary oldest of the three loci. It appeared with the cartilaginous fishes and was relatively well conserved between fishes and humans. We have observed duplications of the GzmA/K genes in some species. In sheep, cattle and rabbits there were two gene copies of GzmA. In addition in some fish species, massive expansions were observed within the granzyme A/K locus, which most likely is a result of successive gene duplications. In different cichlid species we observed everything from five to thirteen GzmA/K genes, and in cartilaginous fishes five GzmA/K related genes were commonly found. There were no significant homologues GzmA/K genes found in lamprey, hagfish, tunicate and sea urchin genomes, indicating that GzmA/K or their ancestor emerged at the base of jawed vertebrates.

The second most conserved loci of these three loci was the metase locus. We found strong evidence for its appearance at the base of bony fishes. In placental mammals, this locus contained a number of distantly related serine proteases genes. When it comes to primates, GzmM was at one end of the locus, the PRSS57 gene that encodes the neutrophil protease NSP-4 in the middle of the locus, and three additional neutrophils expressed serine proteases or protease homologues (PRTN3, N-elastase and the inactive azurocidin (AZU1)) at the other end. A gene for complement factor D (CFD/Adipsin) was also located at this end. A number of non-serine protease-coding genes flanked the locus i.e., TPSG1, CD34, PALM MED16, R3HHDM4 KISS1R, SK11, MIDN, PLORMT, and FBGF22 or 10-like, BSD, RNF and FST-like, which made it relatively easy to trace related loci in distantly related species. The metase locus showed high similarity in all placental mammals that were analyzed. However, a duplication of the CFD gene in cattle and a loss of AZU1 gene in mice and rats were observed. The AZU1 gene was only found in placental mammals. A slightly different organization of this locus was seen in marsupials, as represented by the opossum, where the gene size was much larger than GzmM and PRSS57, most likely due to insertions in introns. The metase locus in the platypus was still incomplete and found on several contigs. In the chicken, we only found the genes for GzmM and

CFD, and in the Chinese alligator also PRSS57. However, no traces of PRTN3, N-elastase and azurocidin were observed. Interestingly in an amphibian, the clawed frog, we found two genes encoding PRSS57 and one gene for PRTN3, but no genes for N-elastase or azurocidin. In all fish species, only one gene from the mammalian met-ase locus was found, the gene for CFD, but this gene was in most fishes located on a different chromosome with different flanking non-protease genes. It was only in the spotted gar that a locus similar to the mammalian met-ase locus was seen. In the gar, we also saw duplication of the CFD gene where one was located in the same surrounding as the mammalian met-ase locus. In some fishes we observed chymase related genes (in cichlids) and GzmA/K genes in their met-ase loci, this indicated the granule serine proteases evolved in a convergent evolution rather than the divergent evolution. The origin of these genes is still a mystery.

The chymase locus was more complicated and appeared comparatively late. In primates, this locus had at one end the mast cell expressed α -chymase (Cma1) gene flanked by genes of non-serine proteases RIPK3, NYNRIN, CBLN3, and SDR39U1, and at the other end the gene for the T-cell expressed gene granzyme B (GzmB) along with the flanking non-serine protease gene STXBP6. In the middle of the locus were the genes for the neutrophil expressed serine protease cathepsin G (CTSG) and the T-cell expressed granzyme H (GzmH). This locus was very similar in all primates that were analyzed. In other placental mammals, sometimes quite dramatic changes in the size of the locus and in the number of serine protease genes were seen. In rodents, extensive duplications have occurred within this locus. Two new subfamilies of proteases were present: the β -chymases, that are closely related to (Cma1), and the mMCP-8 subfamily closely related to CTSG and granzymes. In addition, many new granzymes were present in both the rat and mouse chymase loci. A remarkable expansion of the locus has also occurred in rodents compared to humans. Humans have four serine protease genes: chymase, cathepsin G, granzyme H and B, whereas in mice there are ten active protease genes and in rats, we find as many as 28. The locus has also expanded in size, where the rat locus is fifteen times larger than the corresponding locus in dogs. Cats also have a β -chymase like a gene in their chymase locus, which indicates that the β -chymase appeared during early placental mammalian evolution but has been lost in some species and branches of the placental mammalian evolutionary tree.

A new subfamily of hematopoietic serine proteases has also been identified in ruminants, sheep and cattle, a subfamily closely related to the granzymes and cathepsin G. Interestingly, these proteases have changed tissue specificity and are now expressed in the duodenum and have therefore been termed duodenases. Related genes have also been found in pigs, indicating that they potentially are present in all ungulates (hooved mammals). The cattle locus now has three duodenases in addition to the genes described

for the primates. In sheep and cattle, duplications of both CTSG and the chymase gene were found. The duplicates showed 93-94 % identity, which indicates that the duplications occurred recently, possibly around 20-30 million years ago before the separation of sheep and cattle. In marsupials, represented by the opossum, only two chymase locus genes Cma1 and GzmB were found. The lack of expansion of the locus may have been blocked by an inversion based on the position of the flanking STXB6. In the platypus we found three chymase locus-related protease genes on two different contigs, one consisted of GZMB and DDN1, which are both similar to the chymases in placental animals, and another gene on the second contig, the granzyme BGH that shows structural similarities to GZMB. The chymase locus was quite similar in the overall organization in all the placental mammals and even in marsupials. However, in some species we saw changes in the flanking genes, indicating rearrangements close to the locus, and as previously mentioned in the opossum we saw an inversion involving the centre of the locus.

In birds, reptiles, amphibians and fishes only a few chymase locus related genes were found and they were often not located in the same chromosomal surroundings as in mammals. In birds, such as the chicken, there were genes named CTSG-like and GzmH-like on chromosome 28; the zebra finch had the genes GzmE-like and MCP1A on chromosome 28, and in the green anole lizard, genes CTSG-like and MCP1-like exist, all of them having similar neighbouring genes, GNA11 at one end NCLN at the other end. Not one of these matched the bordering genes of the chymase locus in mammals and therefore represents a completely different locus even though the genes showed some similarity to the mammalian chymase locus proteases. When we analyzed them for relatedness, we found that these genes formed a separate branch in the phylogenetic tree, indicating they are related but not directly corresponding to a classical chymase locus. Only in the reptile, the Chinese alligator, where 9 genes organized as four different contigs we found a few genes that may originate from a locus with high similarity to the mammalian chymase locus. The other genes clustered with the aforementioned novel bird and reptile chymase locus. Interestingly in an amphibian the clawed frog, *Xenopus tropicalis*, we found two genes CTSG and GZMH that were linked but with a huge gap between the genes 115 MB. The clawed frog CTSG clustered with the mammalian chymase locus similar to some of the alligator genes and the second the GZMH clustered with the novel bird and reptile chymase loci-like genes. Therefore in the frog and the alligator we have members that may be early representatives of the classical mammalian chymase locus.

In fishes, some genes related to the mammalian chymase locus genes were found. However, all of these genes formed a separate fish branch in the phylogenetic tree and they were found in at least two different loci. Some were actually found within the fish met-ase locus, whilst others were found

in a region with different neighbouring genes including *rab13*-like and CD276 or ERCC-1. No chymase locus homologues were detected in cartilaginous fishes, lampreys, hagfishes, sea urchins and tunicates, indicating that the classical mammalian-type chymase locus appeared with the tetrapods, as we found closely related genes in amphibians and some reptiles, where the related genes in fishes may have appeared by convergent evolution from genes within other related loci.

All the genes from the three loci of serine proteases used protein sequences (from the active protease form) were analyzed using Neighbor-joining, Maximum-likelihood and distance algorithms. In the phylogenetic tree, we saw five clearly separated branches, the *granzymeA/K* locus genes, the met-ase locus genes, the fish serine proteases, the new chymase locus-related reptile and bird genes, and the classical mammalian type chymase locus genes. In the phylogenetic tree, all the fish proteases except for the *GzmA/K* genes fell into the separate fish branch in the tree.

The primary cleavage specificity defined by S1 pocket amino acids positions (189, 212 and 216) gives us primary evidence of protease cleavage specificity. Generally, they are conserved in mammals: the tryptases often have the triplet Asp-Gly-Gly (DGG), the chymases SGA, the Asp-ase SGR, TGR or AGR and the elastases SVN, NVA and NVS. To predict the primary specificity of serine proteases from non-mammalian species, we aligned sequences and tried to identify the triplet and based on these amino acids make a prediction of its primary specificity. Within the *GzmA/K* locus, most enzymes had a DGG triplet. However, we saw some differences between a few members within cichlids and sharks. The met-ase locus proteases also showed a well-conserved triplet where all complement factor Ds had DGG, *GzmM* had ASP (met-ase), PRSS57 had GSD, PRTN3 and N-elastase had GVD or GID. The chymase locus genes were more complicated as this locus contained proteases with very different primary specificities. The classical chymases have, in general, an SGA triplet, the *GzmB* like proteases are aspartases with a general triplet of SGR, the elastases in rodents NVA or NVS. The new reptile chymase locus genes were most likely tryptases as they generally had a triplet of DGG. The fish proteases of the large fish branch were more difficult to assign a primary specificity based on this triplet as they have very varying triplets although the more dominating ones were GNN, GTH GSS, GAY and GSY. These triplets give a hint to the primary specificity. However, this needs to be verified by substrate assays or phage display. Interestingly, and as shown in paper III, initial studies of the fish proteases indicate that the triplet gives little guidance to the primary specificity and that many of the fish proteases show both a very strict primary and extended cleavage specificities.

In summary, our studies indicated that the *granzyme (A/K)* locus was the first to appear with the cartilaginous fishes and that it was the most conserved of the three. The second most conserved locus was the met-ase locus,

which we found in bony fishes but not cartilaginous fishes. Finally, the one that seems to have appeared relatively late was the chymase locus, which also showed the most complicated structure and evolution. Some of the chymase locus-related genes were located in a separate locus that we have identified in reptiles, birds and amphibians. It indicated that the traces of the classical chymase locus might have arisen with amphibians, the first tetrapods. In fishes chymase locus homologues were found in a third separate locus or in the fish met-ase locus or even in the granzyme A/K locus, indicating that these granule-associated serine proteases have evolved by both divergent evolution, as seen in the mammalian chymase locus, and by convergent evolution as seen with the fish proteases.

Paper III

Channel catfish granzyme-like I is a highly specific serine protease with met-ase activity that is expressed by fish NK-like cells.

The aim of this study was to determine the primary and extended specificities of the catfish granzyme-like I. This catfish enzyme is a member of a large branch in the phylogenetic tree (paper II) with fish proteases, which are related in primary structure to the mammalian hematopoietic serine proteases.

Serine proteases have a number of essential physiological functions in mammals. However, little is known about the roles of these serine proteases in non-mammalian vertebrates. To obtain a broader view of their physiological role in non-vertebrates, we have identified several interesting proteases from the non-mammalian vertebrates from the previously described bioinformatics analysis. In addition, further interest in studies of enzymes from reptiles, amphibians and fishes is coming from the fact that there has been an increase in non-rodent models to study various physiological processes. From the large bioinformatics analysis, we have identified several interesting proteases from non-mammalian vertebrates that may shed light on the appearance and diversification of the large family of hematopoietic serine proteases during early vertebrate evolution. The first step in such a task has been a more detailed analysis of one member of one of the sub-branches of the fish branch of chymase locus related proteases, the catfish granzyme like-I. In order to study the function of this protease we ordered the coding region, including purification (six histidine tag) and activation tags (enterokinase site) of the catfish granzyme like-I cDNA from Genscript as a designer gene and re-cloned it into mammalian expression vector pCEP-Pu2. The catfish granzyme like-I DNA was transfected into HEK293-EBNA

cells. The cells secreted an inactive protein with the purification tag, which was purified on Ni²⁺ chelating IMAC-columns. The protein was activated by enterokinase cleavage, which results in removal of the six-histidine tag and the cleavage site for enterokinase, leaving an active protease that is identical in sequence as the *in vivo* produced protease.

To determine the primary and extended specificities of this protease we used substrate phage display. The resulted sequence alignment obtained from the phage display showed a preference for Met at the P1 position, indicating met-ase activity. There were also other strong preferences for the extended specificity with a Gly in the P2, a Thr in the P3, a Val in the P4, a Met/Ser/Ala in the P1', a Leu P2' and a Val in P3' position, highlighting an extremely strict extended specificity. The phage display results were then validated by the use of a new type of recombinant substrate developed in our lab. This analysis confirmed that this enzyme preferred Met in the P1 position. Furthermore, we used mass spectrometry analysis of the cleavage products of a peptide designed based on the phage display consensus sequence (Arg-Val-Thr-Gly-Met-Ser-Leu-Val), which identified the precise cleavage site after the Met. The catfish granzyme-like I enzyme was highly active on the consensus substrate, where the determined V_{max} and the K_m were 69.74 μM/min and 62.68 μM, with also a very board range of activity at different pHs, from pH 5.0 to 9.5.

To obtain clues to the physiological role of the catfish granzyme I-like, we performed a bioinformatics screening for potential *in vivo* targets by using BLASTP search. The most interesting potential target identified in this screening was catfish caspase 6. The catfish granzyme I-like enzyme was later shown to efficiently cleave the region covering the target in catfish caspase 6, but a very minimal effect on the related zebrafish caspase 6. This may indicate that the enzyme is highly species specific in its target recognition. The position of the cleavage site in caspase 6 was very similar to the activation site of several mammalian caspases, indicating that the catfish enzyme may have caspase, and thereby apoptosis, activating/inducing function similar to mammalian granzyme B. Granzyme B of CTLs in mammals are central for the killing of intracellular parasite-infected cells by CTLs. There were some doubts about this potential function, as the enzyme did not efficiently cleave caspase 6 of another fish species the zebrafish, therefore we tried to identify the possible corresponding zebrafish enzyme from the phylogenetic analysis. One such enzyme did exist, termed the zebrafish arginine esterase-like enzyme, this enzyme is very closely related to catfish granzyme-like I. Recently we have performed an analysis of this protease on the zebrafish caspase 6 sequence and found that zebrafish arginine esterase-like also prefers Met at the P1 position and cleaved all of the preferred substrates of catfish granzyme I-like as well as the zebrafish caspase 6 sequences. This makes us more confident that caspase 6 is a bonafide important target for these enzymes and that this indicates that apoptosis induction by

caspace cleavage is a very old conserved mechanism of immunity in vertebrates maintained from fish to mammals, although with different primary and extended specificities of the initiator proteases.

Paper IV

The mouse mast cell transcriptome.

The aim of this study was to obtain a more detailed picture of the transcriptome of different mast cell populations from BALB/c mice and put them in relation to *in vitro* differentiated mast cells by the use of RNA-seq and PCR based transcriptome methods.

Mast cells are often found at the interphase between the body and environment, and can differ extensively from one tissue to another. We have two major subtypes of mast cells, the connective tissue mast cells found, as its name suggests are found in connective tissue throughout the entire body, and the mucosal mast cells, which primarily are found in the intestinal mucosa and the lungs. Mast cell-like cells can also be developed from bone marrow cells grown in the presence of IL-3 or SCF. These cultures contain 95-98 % mast cell-like cells after 3 weeks in culture and are named bone marrow derived mast cells (BMMCs). To obtain a more multifaceted view of mouse mast cells, we have performed transcriptome analyses of purified mouse peritoneal mast cells, cultured mast cells (BMMCs) plus LPS treated (4 hours of incubation) cells, as well as cells purified from mouse tissue like the ear and lungs. We have used two different methods to analyze the gene expression, Illumina RNA seq by GATC-Biotech and Ion Ampliseq from Thermo Fisher. We have analyzed the data from the two different methods, and primarily focused on the molecules of importance for the physiological function of mast cells, namely granule proteins, processing enzymes and surface receptors. This information could serve as a fundamental reference for all future work on mast cell biology.

As a first step we have analyzed mouse peritoneal mast cells as a representative of normal connective tissue mast cells. Here, we observed from the RNA-seq data that housekeeping genes represented approximately 60-80% of the total transcriptome. The most abundant mast cell specific genes were found to be the granule proteases, three serine proteases, mMCP-4, mMCP-5 and mMCP-6 as well as the mast cell-specific carboxypeptidase (CPA3). All other proteases expressed at least 2 orders of magnitude lower than the major protease transcripts. The level of all granzymes (A, K, C, D, E, F and G) were undetectable and the N-terminal protease processing enzyme cathepsin C, also named di-peptidyl peptidase (DPP), was relatively low. Looking at other processing enzymes and receptors, like the mast cell-specific high-

affinity receptor for IgE, the α -chain this expressed at approximately 3% of the levels for mMCP-5. The high affinity receptor for IgE on mast cells has three different subunits that are encoded from separate genes, the α , β , γ chains. The receptor for the stem cell factor (SCF), c-kit, was expressed only at half the level (1.5 %) of the Fc ϵ RI α -chain. The IL-3 receptor also showed very low levels (0.5%). The mRNA levels for the different cytokines were almost untraceable and only a very low level of IL-5 mRNA was observed.

Cultured bone marrow cells in the presence of IL-3 for three weeks results in almost 100 % mast cell-like cells, called BMMCs, as previously described. Interestingly, when we compared these cells to peritoneal mast cells, only high level expression of two granules proteases, mMCP-5 and CPA3 was detected. Very low, almost undetectable, levels of mMCP-4 and mMCP-6 were observed. The α -chain of the Fc ϵ RI was expressed at very high levels in BMMCs compared to peritoneal mast cells. LPS treatment of BMMCs (4 hours) resulted in very few changes in mRNA levels with the expression of a few proteases increasing: mMCP-4, mMCP-7, mMCP-2 and granzyme B by 4 -5 times, mMCP-1 by 10—12 times, and a 1000 times increase for GzmC. However, the GzmC levels were already very low in the beginning, therefore the biological significance of this increase can still be questioned.

Our main goal of this study was to obtain a more complete, general picture of the mast cell. For that we chose to look at mast cell populations from different mouse tissues and organs. We started with mouse ear and lung tissue with the focus on mast cell-specific transcripts. In the ears the most abundant transcripts were skin keratins (12 different) and we could also detect a relatively high level of α -actin and troponin C2. When looking for mast cell specific transcripts we observed only connective tissue mast cell (CTMC) specific proteases like mMCP-4, mMCP-5, mMCP-6 and CPA3, and no traces of mMCP-1 and mMCP-2, which are the mucosal mast cell (MMC) specific proteases. Expression of mast cell specific receptors and granzymes were very low. In the lungs the major transcripts were the surfactants and lysozyme. We detected very low levels of transcripts for both CTMC, MMCs and also for basophils. The characteristic transcripts for CTMC are mMCP-4, mMCP-5, mMCP-6 and CPA3, for MMC mMCP-1 and mMCP-2 and for basophils mMCP-8. Lungs thereby had a very heterogeneous population of mast cell and basophil-like cells, which was in marked contrast to the skin where the CTMCs were dominating.

In summary, the mouse mast cells transcriptome analysis resulted in a few important findings. The transcript levels of the granule proteases were, in general, almost two orders of magnitude higher than that for the receptors and processing enzymes. The level of few proteases was very different in peritoneal mast cells compared to BMMCs, indicating that BMMCs can be considered to be relatively immature mast cell-like cells. Only a few transcripts increased in BMMCs after incubation LPS. Finally, very different mast cell/basophil populations were detected in the ear and lung tissue. In

the ear, we only detected CTMCs, whereas in the lungs both CTMC, MMC and basophil transcripts were found, indicating a very heterogeneous population.

Concluding remarks and future prospects

The first two papers in this thesis present detailed bioinformatic analyses of hematopoietic granule serine proteases and the Ig-binding FcRs. These two studies have resulted in a relatively detailed picture of their appearance and diversification during vertebrate evolution. From paper I, we concluded that Fc α μ R and Fc μ R evolved from the PIGRs and that both Fc γ R and Fc ϵ R evolved as a subfamily from the FcRL receptors. The first major step of FcRs evolution occurred at the base of bony fishes with the appearance of the PIGRs, the FcRLs and the FcR common gamma chain. The second major step came during reptile or early mammalian evolution with the appearance of the IgG, the IgE, the IgM and the dual IgA/M receptors. The third step was the appearance of the receptor for IgA, which came with the placental mammals.

From paper II, we concluded that the granzyme (A/K) locus was the first locus to appear and that it is also the most conserved of the three. The met-ase and chymase loci might have appeared as a result of the two-genome doublings, the so-called tetraploidizations that most likely occurred during early vertebrate evolution. The initial locus then became four, of which one may have become the granzyme (A/K) locus, the second the met-ase, the third the chymase and the fourth the new chymase related locus seen in amphibians, reptiles and birds. This latter locus may have secondarily been lost in mammals. In fishes, some chymase and the granzyme A/K locus-related genes were found in the met-ase locus, which indicated that these genes appeared as the result of gene transfer by still unknown mechanisms, or that some of the genes possibly underwent convergent evolution.

Serine proteases have a number of essential physiological functions in mammals. However, little is known about the roles of these serine proteases in non-mammalian vertebrates. In order to trace the evolution of the different functions of the non-mammalian hematopoietic serine proteases, we have started an analysis of related proteases in marsupials, monotremes, reptiles, birds, amphibians and fishes. The first of these studies is the article presented as paper III in this thesis. Here we described a detailed analysis of the channel catfish granzyme-like I, a protease expressed by fish NK-like cells. This protease was found to display a remarkably high extended specificity and a primary specificity for methionine. A bioinformatics screening of a few fish genomes and cDNA banks identified caspase 6 as a potential *in vivo* substrate. The cleavage of this substrate sequence by the catfish enzyme

strongly favours the role of this enzyme in the induction of apoptosis in target cells similar to granzyme M and granzyme B in mammals. This indicates that this mechanism has been conserved for over 400 million years of vertebrate evolution, however with different extended specificities.

In paper IV we analyzed the transcriptome of peritoneal mast cells, cultured BMMCs, and mast cells or basophils in the ear and lung tissue of mice in order to understand the biology and heterogeneity of mast cells in different tissues. The transcriptome of mast cells showed that the levels of the major granule proteins of mast cells were very high, and that the levels of the most specific receptors for mast were close to two orders of magnitude lower than the granule proteases. The processing enzymes of proteases like DPP, histamine, serotonin and heparin syntheses were also relatively low. We also observed that the majority of the transcriptome were housekeeping (60-80 %) genes involved in general metabolism.

The major aim of my thesis project has been to try to determine when the mast cell connected to adaptive immunity during vertebrate evolution by the presence of Fc-specific receptors for Igs and thereby became involved in allergy. Data from the bioinformatics analyses of FcRs, granule proteases and mast cell transcriptomes provided detailed information on the evolution and their appearance in during vertebrate evolution and general mast cell biology. The next step will be to study the physiology of mast cells in more detail, to determine functional properties of receptors and serine proteases in mammalian species as well as in non-mammalian species. We have selected a few key, and thereby informative, receptors and proteases from different species, which gives us important clues to the evolution of the immune system. Future planned studies include the production of recombinant proteases and analyzing cleavage specificity using substrate phage display, followed by studies of tissue specificity and potential *in vivo* function. The main focus here is to see if non-mammalian species (like fishes, amphibians, reptiles, and birds) by convergent evolution have obtained enzymes with similar function as their potential mammalian counterparts. Similarly for the FcR project, the aim is to produce the recombinant FcR protein and study their isotype specificity of a few key species, with primary focus on platypus and *Xenopus* FcRs, as they represent important steps in evolution.

To obtain a more complete picture of the mast cell and its biology we are scheduling to isolate additional tissues from mice including the heart, tongue, liver and intestines, uterus and pancreas to study by transcriptome analyses mast cells in these tissues. The continuation of this project would involve isolating mast cells from a number of non-mammalian species, like fishes, amphibians, reptiles and birds, and send mRNA from these cells for transcriptome analysis. There we can look for the expression of mast cell related receptors and granule proteases to determine the different steps as to when during vertebrate evolution the mast cell became the cell of such importance in allergy. These studies will hopefully give us information of when

the mast cells started to interact with adaptive immunity by expression of FcRs and the key physiological functions of the various mast cell proteases and their role in allergy and general immunity.

Populärvetenskaplig sammanfattning

Alla levande organismer behöver skydda sig mot infektioner av bakterier, virus eller parasiter men även mot toxiner och andra potentiellt farliga ämnen. Detta skydd kallar vi immunitet. Ett antal celler och organ och deras produkter deltar i vårt immunförsvar, som fått namnet vårt immunsystem. Immunsystemet kan delas upp i två huvudsakliga grenar, det icke adaptiva, som också kallas det naturliga immuniteten och det adaptiva immunförsvaret. Den naturliga immuniteten finns alltid där och förändras inte vid upprepade kontakter med den infektiösa organismen och har därför inget minne. Det adaptiva försvaret däremot har minne och det är därför vi kan komma ihåg att vi sett ett virus eller bakterie och att det därför går att vaccinera sig. Nästan alla celler som deltar i vårt immunförsvar tillhör de så kallade hematopoietiska cellerna, eller i dagligt tal de vita blodkropparna. Dessa utvecklas från stamceller i vår benmärg. En av dessa vita blodkroppar är en cell som kallas B-lymfocyt, eller B-cell. Det är dessa celler som producerar våra antikroppar. Dessa antikroppar har som viktig uppgift att neutralisera sjukdomsalstrande mikroorganismer och toxiner. Antikropparna har formen av ett Y och består av fyra så kallade polypeptider, två identiska lätta kedjor och två identiska tunga kedjor. Antikroppen har två huvudsakliga funktionella delar, en som binder antigenet, d.v.s. bakterien viruset eller toxinet och en som kan binda till receptorer på ytan av immunceller. Den antigenbindande delen kallas den variabla delen, Fab delen, då den ser olika ut från en antikropp till en annan medan den andra delen, den konstanta delen, Fc delen, ser lika dann ut inom en viss klass av antikropp. Däggdjur har fem olika huvudtyper, klasser, av antikroppar s.k. immunoglobuliner (Igs) IgM, IgD, IgA IgG and IgE.

Nästan alla hematopoietiska celler har receptorer för dessa antikroppar på sin yta som binder till den konstanta delen av antikroppen. Dessa receptorer kallas därför Fc-receptorer då de binder Fc delen av antikroppen. Dessa receptorer har en mängd viktiga funktioner. De kan hjälpa till att ta upp immunkomplex, d.v.s. hopklumpade toxiner, bakterier eller virus. De kan stimulera celler att släppa ut immunproteiner och kan även delta i att presentera antigener för immunförsvarets andra celler. Dessa Fc-receptorer har tre strukturella delar, en som binder antikroppen, en som går igenom cellmembranet och en del som sitter inne i cellen. Den intracellulära delen har till uppgift att skicka signaler in i cellen och tala om att något bundit till ytan. De finns korta motiv i denna del, vissa som är stimulerande har motivet

Immune tyrosine-based activation motif (ITAM) och andra som skickar en inhiberande dämpande signal Immune tyrosine-based inhibitory motif (ITIM). Varje klass av antikropp har sina specifika receptorer, Fc γ för IgG, Fc α för IgA, Fc ϵ för IgE, Fc μ för IgM och Fc δ för IgD. Receptorerna för IgG, IgE och IgA uppvisar stora strukturella likheter och tillhör alla en stor proteinfamilj kallad super immunoglobulinfamiljen (IgSF). Nyligen har man även hittat strukturellt liknande receptorer i både vårt och musens genom, som fått namnet Fc liknande receptorer eller FcRL. Genom att använda bioinformatik har vi studerat när dessa receptorer dyker upp under ryggradsdjurens evolution samt hur de har diversifierats. Flera av dem dyker upp med benfiskarna någon gång kring 450 miljoner år sedan bl.a. poly Ig receptorn (PIGR), FcRL receptorerna och en signalkomponent den så kallade gamma kedjan. Detta representerar det första steget i dessa receptorers utveckling. Andra receptorer dyker först upp i samband med de fyrfotade djuren, de s.k. tetrapoderna, till vilka vi räknar groddjur, kräldjur, fåglar och däggdjur, någon gång kring 380-400 miljoner år sedan. I reptiler och däggdjur hittar vi receptorerna för IgM och IgM+IgA (Fc μ R, Fc $\alpha\mu$ R) och med däggdjuren kommer de specifika receptorerna för IgG och IgE. Den sista av receptorerna att se dagens ljus är den specifika receptorn för IgA som endast hittas hos placentala däggdjur. Receptorerna för IgM och IgA+M kan direkt härledas från en genduplikation av PIGR-receptorn, som därför är ursprunget till alla tre dessa receptorer.

Flera av de hematopoietiska cellgrupperna som mastcellerna, de neutrofila granulocyterna, de naturliga mördarcellerna (NK-celler), T-lymfocyter och de basofila granulocyterna lagrar stora mängder aktiva substanser i små membraninneslutna säckar inne i cellerna s.k. granula. De huvudsakliga proteininnehållet i dessa granula, i flera av dessa celltyper, består av proteinnedbrytande enzymer s.k. proteaser. Huvuddelen av dessa tillhör en stor proteasfamilj som kallas serinproteaser. Till denna familj hör proteaser som deltar i blodkoagulering, komplementaktivering, matsmältning, fertilisering, blodtrycksreglering, vävnadshomeostas, fibrinolys samt i immunitet. Generna för de medlemmar av dessa trypsin/chymotrypsinliknande proteaser som uttrycks i vita blodkroppar hos däggdjur kodas från fyra olika lokus, d.v.s. regioner på våra kromosomer, kymaslokuset, metaslokuset, T-cells-tryptas lokuset (även kallad granzyme (A/K) locus) och mastcellstryptaslokuset. För att ta reda på när dessa dyker upp under ryggradsdjurens evolution och hur de diversifieras har vi gjort en omfattande bioinformatisk analys av dessa gener från fiskar till däggdjur. Granzyme A/K locus är det första som dyker upp, detta lokus hittar vi redan i broskfiskar, vilket inkluderar hajar och rockor. Det andra lokus som dyker upp är metaslokuset som kommer med benfiskarna. Det klassiska kymaslokuset kommer sedan med tetrapoderna och hittas därför i groddjur, reptiler och däggdjur och mastcells-tryptaslokuset möjligen allra sist med tidiga däggdjur.

Serinproteaser har ett antal essentiella funktioner hos däggdjur. Vi vet dock mycket lite om dessa proteasers motsvarigheter hos andra vertebrater inkluderande fiskar, groddjur, reptiler och fåglar. Genom våra bioinformatiska studier har vi identifierat flera intressanta proteaser från andra ryggradsdjur bl.a. flera från fisk, grodor, reptiler och fåglar. Jag har valt ett av dessa proteaser att studera närmare, ett proteas från en mal, catfish granzyme like-I. Vi har producerat detta proteas rekombinant och studerat dess klyvningsspecificitet med s.k. phage display. Vi har kunnat visa att detta proteas är mycket specifikt och klyver efter aminosyran metionin. När vi letade i fiskens genom efter möjliga målmolekyler hittade vi en mycket intressant kandidat ett protein som inducerar självmord s.k. apoptos på virusinfekterade celler, ett s.k. kaspas närmare bestämt caspase 6 vilket indikerar att denna typ av mekanism i vårt virusförsvar är konserverat mellan fisk och människa.

Mastceller är en cell som främst är känd för sin roll i IgE medierade allergier, till vilka räknas pollen, pälsdjur, dammkvalster, mjölk och nötallergier samt de flesta former av astma. Denna cell uttrycker Fc receptorer för IgE och IgG på sin yta och är full med granula innehållande mastcellspecifika proteaser i stora mängder. Förutom att vara ansvarig för allergier har denna cell ett antal viktiga fysiologiska funktioner i vår kropp. Den är inblandad i försvaret mot parasiter, bakterier och i neutralisering, genom klyvning, av ormgifter men även i aktivering och reglering av andra immunceller inkluderande B, T och dendritiska celler. För att få en mer detaljerad bild av dessa celler har vi närmare studerat deras mRNA uttryck, deras s.k. transkriptom. Vi har renat mastceller från bukhålan, drivit fram mastcellslignande celler i kultur från musbenmärg samt renat mRNA från hud och lunga. Dessa preperationer av RNA har sedan analyserats med flera olika tekniker som alla ger en bild av det totala RNA uttrycket. Vi har därmed fått en bra kvantitativ bild av uttrycket av alla ca 21 000 gener i musens genom och fokuserat oss på proteiner vi vet uttrycks specifikt i mastceller. Detta har givit oss en mycket bra bild av musens mastcellers transkriptom och skillnader i genuttryck mellan olika mastcellspopulationer. Dessa data kommer att kunna fungera som en mycket bra databas för alla vidare studier av denna immunologiskt så viktiga cellgrupp.

Min avhandling har fokuserats kring studiet av mastcellens biologi med fokus på Fc-receptorer och granulaproteiner och dessa celler och molekylers roll i vårt immunsystem samt hur de har utvecklats under ryggradsdjurens evolution. För att uppnå detta mål har jag använt mig av en bred panel av olika tekniker, inkluderande bioinformatik, produktion och analys av rekombinanta proteiner, "Phage Display", och transkriptomanalys.

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