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The utilisation of mast cells for exploration of immunomodulatory effects of tick salivary proteins

Master thesis

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Annotation

The aim of this thesis was to functionally compare mast cells derived from the bone marrow and MC/9 mast cell line. Activation of mast cells by ionomycin and IgE receptor crosslink were compared. Influence of tick saliva, propolis and a cysteine protease inhibitor on the degranulation and mast cell cytokine production *in vitro* was tested.

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Bc. Šárka Koudelková

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Contents

1 Introduction

Mast cells are cells of hematopoietic origin. They are situated in mucosal and connective tissues surviving there for a long time. Their abnormal activation results in asthma, allergy or anaphylaxis. The interest in mast cells has been justified by the raise of asthma and allergy occurrence in past 50 years. Extensive research in this field revealed that mast cells do not only cause adverse effects on the organism, but also play and important role in modulation of innate and adaptive immunity.

In the thesis the function of the mast cells, their biology, modes of activation and their role during tick infestation will be discussed. Then the experiments with mast cells of distinct origins after different ways of stimulation and influence of various proteins will be presented. Finally, the results will be discussed.

2 Literature research

2.1 Classification

Mast cells (MCs) are mainly residing at sites to be among the first cells encountering external antigen. Nevertheless, the population of mast cells is rather diverse (Table 1). Murine MCs are classified according to their phenotype and granule content into two categories. Connective tissue mast cells (CTMC) resided in skin and peritoneum are bigger in size, their main proteoglycan is heparin. Mucosal mast cells (MMC), on the other hand, are located in the intestine contain chondroitin sulphate as the main proteoglycan and ten times less histamine than CTMCs. Despite this classification there appear MCs that do not correspond to either of these groups. Subepidermal MCs, for example, share some properties with MMCs and some with CTMCs (Metcalfe et al., 1997).

In analogy to murine MCs, human MCs differ in protease content. Subpopulation of MCs in lungs and intestine corresponding to MMCs is labeled as MC_T. MC_Ts contain only tryptase. MC_{TC}s residing in skin contain chymase, carboxypeptidase and cathepsin G together with tryptase. Despite this variety it was found out that all mast cells originate from the same progenitor and their phenotypic differentiation is influenced by the microenvironment upon the settlement of immature MC (Metcalfe et al., 1997).

		Location	Tryptase	Chymase	MC-CPA
Human	MC_T	mucosa (e.g. intestine, lungs)	+	-	-
	MC_{TC}	skin	+	+	+
Mouse	MMC	mucosa (e.g. intestine, lungs)	-	+	+
	CTMC	connective tissue (skin, peritoneum)	+	+	+

Table 1. Classification of MCs. (Metcalfe et al., 1997; Pejler et al. 2010).

2.2 Mast cell growth and development

Mast cell $CD13^+$ $CD34^+$ $CD117^+$ progenitors originate in bone marrow and are liberated into the circulation. The number of mast cell progenitors in mouse bone marrow was determined to 10 or 20 precursors per 10^5 bone marrow cells (Sonoda et al., 1982; Jamur et al., 2005 respectively). The concentration of the precursors in the circulation was estimated at 90 mast cells per 10^6 blood cells (Sonoda et al., 1982). Committed progenitors lack granules. Their maturation and differentiation take place during their journey to the target tissues under the control of cytokines.

The draft of mast cells to different tissues is induced by activation of different pathways depending on the target tissue (Gilfillan et al., 2011). The population of mast cells in tissues is maintained by stem cell factor (SCF) - the KIT ligand. In case of inhibition of SCF, DNA damage and other intracellular signals, apoptosis takes place. SCF together with interleukin-4 (IL-4) ensure the endurance and proliferation of human mast cells (Bischoff, 2007). However, the need of SCF is not necessary for some types of mast cells. The most important factor for the growth and differentiation of murine bone marrow mast cells (BMMCs) is IL-3. For complete maturation additional factors such as SCF and IL-4 are needed. A cell culture similar to CTMCs originates after the synergy of all these factors (Metcalfe et al., 1997).

High affinity $Fc_\varepsilon RI$ receptors begin to be expressed after 1 week of culture even before the granulation occurs. By week 3 majority of cells express number of $Fc_\varepsilon RI$ and also show dense granulation (Rottem et al., 1993). $Fc_\varepsilon RI^+$ cells have lower ability to proliferate than the cells that do not express $Fc_\varepsilon RI$. It is therefore assumed that the $Fc_\varepsilon RI$ expression is a marker of fully differentiated cells. Cells that express $Fc_\varepsilon RI$ early are able to respond to IgE stimulus, hence directing maturation of the rest of the cells by secretion of cytokines (Metcalfe et al., 1997).

2.3 Mast cell mediators

The effect of mast cells is determined by the amount and type of released mediators. The set of released mediators depend on the character and magnitude of the activating stimuli. Immediately after stimulation of mast cells, pre-synthesized mediators stored in granules are released. Among these belong histamine, proteoglycans, and neutral proteases. Leukotrienes, prostaglandins, and cytokines are synthesized just after the activation of mast cells.

2.3.1 Histamine

Histamine interacts with H_1 receptors on target cells causing vasodilatation and bronchoconstriction. Through H_1 receptors histamine promotes T_{H1} activation. On the other hand it suppresses both T_{H1} and T_{H2} immune response through H_2 receptor (Jutel et al., 2006). It is rapidly metabolized therefore it is a mediator for effect in proximity of release site (Metcalfe et al., 1997).

2.3.2 Proteoglycans

Proteoglycans heparin and chondroitin sulfate E influence mast cell proteases and activity of enzymes. In addition, heparin is efficient anticoagulant. They form a matrix of the granule stabilizing neutral proteases (Metcalfe et al., 1997).

2.3.3 Neutral proteases

Neutral protease content is variable depending on maturation status and type of mast cell (Table 1). They are the most ample mediators in MCs. The mRNA for the proteases is even more abundant than that of housekeeping genes. They are expressed massively – about a quarter of total protein content is made up of proteases (Pejler et al., 2010). There are three classes of MC proteases – chymase, tryptase, and MC carboxypeptidase A (MC-CPA). Prevalence of individual classes in different species of MCs is given by the demand of different tissues. Since they are released in active form, they have immediate effect on the tissue at the site of degranulation. Variety of proteins to be cleaved implies positive as well as negative pleotropic effects of the proteases.

All human mast cells express tryptase which is stored and released in tetrameric form conjugated with heparin. Heparin – tryptase bond can be destabilized by antithrombin III. As was said above, heparin acts as anti-coagulant whereas tryptase cleaves fibrinogen and activates collagenase (Metcalfe et al., 1997). Tryptase is not necessary for the direct combat with parasitic or bacterial infection. It may be, however, a key agent influencing severity of the inflammation during arthritis (Pejler et al., 2010).

Chymase, another serine protease, is present in human MC_T is capable of a conversion of angiotensin I to II, vitiates the basement membrane between dermis and

epidermis, and initiates mucus secretion (Metcalfe et al., 1997). In contrast to tryptase, it is released in monomeric form, and it cleaves preferably sites after aromatic residue. It is important for pro-inflammatory response when bacterial or parasitic infection occurs. For example, chymase directly helps to expel nematodes from the body (McDermott et al., 2003). On the other hand, it is responsible for adverse effects in arthritis, atopic dermatitis, and airway allergy inflammation (Pejler et al., 2010).

MC-CPA is zinc-dependent metallo-protease cleaving peptides form the C-terminal end. It is essential for toxin degradation including snake or honeybee venom toxins (Pejler et al., 2010; Metz et al., 2006).

2.3.4 Lipid derived mediators

Leukotrienes and prostaglandins are metabolites of arachidonic acid synthesized *de novo* by lipoxygenase and cyclooxygenase pathways respectively, after the stimulation of mast cells. Leukotrienes LTC_4 and LTD_4 are responsible for the wheal-and-flare reaction, bronchoconstriction, bronchial mucus secretion, and constriction of smooth muscles of arteries and intestines (Lewis and Austen, 1984). Leukotriene B_4 not only cause influx of effector T cells (Ott et al., 2003), but also helps to increase mast cell population by draft of mast cell progenitors (Weller et al., 2005).

Prostaglandins prevent platelet aggregation and play as chemotactic factor for neutrophils (Lewis, 1984). PGE_2 after secretion by mast cells switch the immune response towards T_{H2} by inhibiting production of T_{H1} cytokines in autocrine or paracrine fashion (Gomi et al., 2000).

Platelet-activating factor is lipid-derived mediator that aggregates and degranulates platelets. It also causes wheal-and-flare reaction and may be responsible for anaphylaxis by inducing systemic hypotension (Ferenčík et al., 2000).

2.3.5 Chemokines and Cytokines

Cytokines and chemokines are mediators synthesized *de novo* after activation of mast cells. They are released hours after the activation. Spectrum of the chemokines depends on the strength and nature of the signal and also on the tissue microenvironment.

Granulocyte macrophage stem cell factor (GM-SCF) regulates differentiation of eosinophils, neutrophils and macrophages from myeloid progenitor (Clark and Kamen, 1987).

Tumor necrosis factor (TNF-α) is a chemotactic factor for neutrophils. Being pre-formed, it is instantly secreted to cause early influx of neutrophils to site of inflammation contributing to the clearance of pathogens (Galli, 2000; Metz and Maurer, 2007).

IL-1 is an inflammatory cytokine, pyrogenic factor, and chemotactic factor for phagocytes and lymphocytes. IL-2 is a growth factor for T and B cells. Additionally it activates Tc cells and NK cells. IL-3 shares a receptor with GM-CSF, therefore IL-3 works as a growth factor for myeloid blood corpuscles including mast cells. Except from being a growth factor for mast cells, IL-4 is also responsible for differentiation of B cells and their production of IgM, IgG_1 and IgE antibodies. Generally IL-4 belongs to anti-inflammatory cytokines. IL-5, similarly to IL-4 stimulates antibody switch from IgM to IgA by B cells. In synergy with IL-3 and GM-SCF they control the development of eosinophils. IL-6 is an archetypal inflammatory cytokine with pyrogenic effects. IL-6 assists the differentiation of B cells into plasma cells. IL-8 is another inflammatory chemokine. Directly influencing neutrophils, IL-8 causes their migration to the site of inflammation. IL-9 is an auxiliary factor influencing development of mast cells and also other hematopoietic cells. IL-10 inhibits the inflammatory response and also has immunosuppressive properties. IL-13 is another anti-inflammatory cytokine suppressing the production of pro-inflammatory cytokines in monocytes and macrophages. Like IL-4, it stimulates proliferation and differentiation of B cells. IL-16 is a pro-inflammatory cytokine, chemotactic factor for T cells, macrophages and eosinophils (Ferenčík et al., 2000). Vascular permeability factor (VPF), and vascular endothelial cell growth factor (VEGF) regulate angiogenesis (Boesiger et al., 1998).

2.3.6 Antimicrobial peptides

Mast cells can contribute to the fight with bacteria by expression of antimicrobial peptides. After stimulus from LPS mast cells express two antimicrobial peptides, β-defensin-4 and cathelicidin. It seems though, that cathelicidin plays a major role in microbes' direct extermination (Di Nardo et al., 2003). Curiously, it has been discovered that mast cells can phagocyte bacteria themselves (Malaviya et al., 1996). It is questionable, though, to what extend the amount and effect of phagocyting mast cells is important in

in vivo antibacterial combat, especially when taking in account mast cells' rather reduced abilities to move within tissues.

2.4 Activation of mast cells

2.4.1 Activation via $Fc_{\varepsilon}RI$ receptor

The most prominent activation mode is via the high-affinity $Fc_{\varepsilon}RI$ receptor. The process of activation includes the binding of IgE by the Fc region to the extracellular domain of the α-subunit of the receptor in ration 1:1 (Mendoza and Metzger, 1976). This bond however does not cause any conformational changes to the $Fc_{\varepsilon}RI$ or the actual activation. The activation follows after the cross-link of bounded IgE by multivalent antigen and subsequent dimerization of the receptor.

$Fc_{\varepsilon}RI$ comprise of four subunits ($\alpha\beta\gamma_2$). The α subunit bind IgE, β subunit amplifies the signal and stabilizes the receptor. The γ subunits are the main signal transducers (Kinet, 1999). Crosslink of IgE on sensitized receptors with a multivalent antigen or with an anti-IgE antibody results in receptor aggregation. Subsequent fusion of aggregated receptor with membrane lipid rafts is followed by an activation of Lyn, a Src family protein tyrosine kinase (PTK) which is associated with β subunit of the $Fc_{\varepsilon}RI$. Phosphorylated Lyn then phosphorylates tyrosine residues on immunoreceptor tyrosine activation motifs (ITAMs) which are located on cytoplasmic regions of β- and γ- subunits of the $Fc_{\varepsilon}RI$. ITAMs' phosphorylation promotes recruitment of more Lyn and Syk kinase molecules resulting in amplification of the signal (Kawakami and Kitaura, 2005). Alternative pathway involves activation of Fyn, another Src family PTK associated with $Fc_{\varepsilon}RI$. The cascade continues with activation of PI_3K. Whereas both these pathways are important for cytokine production, degranulation is achieved in greater extend by the activation of the Lyn cascade (Gilfillan and Tkaczyk, 2006).

Binding of IgE without specific antigen to FcεRI does not result only in sensitization of MC, but also expression of surface receptors (mainly FcεRI) is augmented, survival of MC is prolonged, histamine content increased, and DNA synthesis enhanced. However, for inducement of these effects 100-1000times higher concentration of IgE is needed than in case of presence of specific antigen (Kawakami and Kitaura, 2005).

2.4.2 $Fc_{\varepsilon}RI$ independent activation

Mast cells, however, may be activated by different mechanisms independent of high affinity IgE receptor. Alternative means of activation resulting in degranulation include activation of various G protein coupled receptor (GPCR) by their specific ligands, such as adenosine, sphingosine-1-phosphate or an anaphylatoxin C3a (Gilfillan and Tkaczyk, 2006). Activation of various cytokine receptors by different cytokines leads in inhibition or enhancement of cytokine production. KIT receptor after ligation of SCF supports growth and differentiation of mast cells (Metcalfe et al., 1997).

Bacteria, bacterial products, and viral nucleic acids activate mast cells through ligation of their PAMPs (pathogen-associated molecular pattern) with TLR (Toll-like receptor) (Metz and Maurer, 2007). TLR2, for example, recognize Gram+ bacterial peptidoglycan, TLR4 ligate Gram- bacterial lipopolysaccharide (LPS) followed by production of pro-inflammatory cytokines (Frossi et al., 2004). Antigens of *Escherichia coli* and *Mycobacterium tuberculosis* are recognized by MCs' CD48. Upon activation through CD48 mast cells degranulate, phagocyte the bacteria and produce cytokines (Muñoz et al., 2003).

Activation can be achieved also via $Fc_{\gamma}RI$ which is a high affinity receptor for IgG. The activation pathway is similar to that of $Fc_{\varepsilon}RI$ including receptor aggregation and Src kinase phosphorylation and PI_3K activation resulting in degranulation and cytokine production. This mode of activation though, is possible only in the presence of IFN-γ which increase the expression of $Fc_{\gamma}RI$. By this way IgG contributes to mast cell inflammatory response (Tkaczyk et al., 2002).

2.5 Immunomodulation

Next to the direct effect of mast cells on surrounding cells and tissues, they are able to modulate immune response of other immunocompetent cells such as natural killer cells (NK cells), dendritic cells, T cells, B cells, monocytes, granulocytes, vascular cells, endothelial cells, epithelial cells, and smooth muscle cells (Galli et al., 2008). The immunomodulation happens through virtually all mediators synthesized by mast cells.

Positive immunomodulatory effects promote the start and extent of immune response. IL-4, IL-13 and CD40L are involved in the increase of IgE production by B cells. TNFα is responsible for the recruitment of immune cells (Galli et al., 2008). T_{H2} response is enhanced by the effects of prostaglandin D_2 on maturation of dendritic cells. *In vitro*,

mast cells can present antigen on their main histocompatibility complexes MHC I or MHC II to T cells (Mekori et Metcalfe, 1999). They can also trap IgE-bound antigen via their $Fc_{\varepsilon}RI$, subsequently undergo apoptosis and present the antigen to T cells (Kambayashi et al., 2008).

Negative immunomodulatory effects result in immunosuppression and counteract inflammatory response by means of IL-10. In vitro studies explored that mast cell derived IL-10 can enhance the production of IL-10 by T cells, suppress production of pro-inflammatory cytokines by T cells, monocytes, and keratinocytes. By the down-regulation of an expression of adhesion molecules it inhibits influx of circulating effector cells (Galli et al., 2008). In vivo it has been found out that IL-10 is important for the reduction of leukocyte infiltration and tissue damage during contact dermatitis (Grimbaldeston et al., 2007), or prevention of the allograft rejection (Lu et al., 2006).

2.6 The role of mast cells during tick infestation

Ixodid ticks are ectoparasites feeding for several days on their hosts. They can suck up to 100 times of their body weight. They would not cause any major problem to the host unless they are not infected by a pathogen which is transmitted to the host body during the infestation. Tick-borne encephalitis virus causing tick-borne encephalitis and *Borrelia burgdorferi* causing Lyme disease belong among the notorious pathogens transmitted by ticks in Europe (Singh and Girschick, 2003).

Tick saliva with its versatile functions is truly ticks' "elixir of life". The saliva is important for water homeostasis, help the tick to attach to the host by cement secretion, provide lubrication for copulation, and also contain a spectrum of pharmacologically active substances to overcome host's defense response and prolong the feeding time (Mathias et al., 2011). The bioactive substances present in tick saliva block blood coagulation by inhibition of thrombin or coagulation factors and inhibition of platelet aggregation (reviewed in Francischetti, 2010). Salivary proteins also influence vasodilation, impair the chemotaxis of phagocyting cells, suppress inflammation and modulate host's immune response (reviewed in Kovář, 2004).

After a rupture of the skin by tick, the immune response is initiated by neutrophil influx to the injury site. During the primary infestation, neutrophils are predominant cell type combating the infection. To lesser extent, eosinophils, macrophages and mast cells are also present and contribute to the inflammation (Francischetti et al., 2010). Dendritic cells, potent antigen presenting cells, migrate to draining lymph nodes where they introduce tick antigens

to T cells (Brossard and Wikel, 2004). B cells are activated and differentiate into secretory cells. Released antibodies, together with cell mediated immune response eliminate the tick antigen. Simultaneously, memory T and B cells are established (Wikel et al., 1994).

When secondary infestation occurs, neutrophils are not the most abundant cells that influx the bite site. On the contrary, basophils prevail and macrophages, eosinophils and mast cells are elevated compared to the primary infestation. Also the reaction is faster and more vigorous mainly due to the homocytotropic antibodies that bind to Fc receptors of mast cells and basophils. After crosslink with tick antigen, degranulation of mast cells and basophils take place resulting in release of histamine, leukotrienes and prostaglandins. This leads to a lesion formation which impedes the tick feeding. Anaphylatoxins C3a and C5a additionally increase the amount of released mediators (Wikel et al., 1994).

Mast cells close to the infestation site degranulate. At the beginning of the secondary infestation, mast cells degranulate even more due to previous sensitization by homocytotropic IgE. Histamine release could cause a discomfort to the tick which can result in a tick withdrawal (Brossard and Fivaz, 1982). In order to overcome histamine effects ticks developed histamine-binding lipocalins that block histamine function (Mans et al., 2008). Tick engorgement and increased viability is also supported by histamine release factor (HRF) homolog which is also secreted in tick saliva. The HRF increase secretion of histamine by basophils to increase vascular permeability ensuring sufficient blood supply of the bite site (Mulenga et al., 2003; Mulenga and Azad, 2005).

Mast cells were considered to be responsible for the acquisition of tick resistance. Several studies on mast cell deficient mouse strains proved that those mice are unable to acquire resistance to ticks (Matsuda et al., 1985; Wada et al., 2010). Their fundamental role in tick resistance was confirmed when BMMCs were injected to MC deficient mice and those mice were able to reduce tick repletion during second infestation (Matsuda et al., 1987; Wada et al., 2010). Nevertheless, their exclusive role in tick resistance was disproved after the experiments on basophil-deficient mouse strain. It was found out that basophils are crucial for Ab/IgFc mediated acquisition of tick resistance. Both types of cells though, are still essential for expression of tick resistance (Wada et al., 2010).

2.7 Diseases associated with mast cells

To complete the image of mast cells it is appropriate to mention a variety of pathological conditions associated with mast cells. Weak but chronic activation of mast cells

leads to activation of fibroblasts resulting in tissue fibrosis. Few examples of the many, such as fibrosis of skin in patients with atopic dermatitis, pulmonary fibrosis in asthma patients, or even cirrhosis after chronic inflammation of the liver can be mentioned (Choi and Claman, 1987).

Mast cells also take part in several autoimmune diseases. They are crucial for development of rheumatoid arthritis by production of IL-1 (Nigrovic et al., 2007). Mast cells are also important for the outbreak and the course of experimental allergic encephalomyelitis (EAE) which is a model for human multiple sclerosis (Secor et al., 2000). On the contrary, mast cells have protective effect on the organism in case of anti-glomerular basement membrane glomerulonephritis (Hochegger et al., 2005).

It has been found out that mast cells may participate in tumorgenesis of basal cell carcinoma (Diaconu et al., 2007) and Hodgkin's lymphoma (Molin et al., 2001). Mast cells may promote also the development of induced intestinal tumors (Wedemeyer and Galli, 2005).

2.8 Mast cells versus basophils

Mast cells and basophils are both important for initiation of immune response. They both release pro-inflammatory mediators after the crosslink of IgE on $Fc_{\varepsilon}RI$ by multivalent antigen. By the release of histamine, proteases and other mediator from their granules, and synthesis of leukotrienes, prostaglandins and cytokines they contribute to pathologic symptoms in asthma and other allergic disorders.

In contrast to mast cells, basophils migrate from the bone marrow after complete diferentiation and circulate in peripheral blood until they are recruited to the site of inflammation. That is why they belong among granulocytes. Mature mast cells, on the other hand, cannot be found in the circulation under physiological conditions.

Quantity of basophils can be regulated by IL-3, although it is not clear if basophils can change their phenotype under the influence of IL-3 (Galli, 2000). Phenotype diversities of mast cells can be modified by IL-3, together with SCF, IL-4, -9, -10, and other micro-environmental factors (Galli, 2000; Metcalfe, 1997).

Apart from activation through IgE, mast cells and basophils can be activated independently of $Fc_{\varepsilon}RI$ directly by bacteria and parasites or by activation of complement system. Another way of mast cell activation is through IgG and FcγRIII (Daëron, 1997). Degranulation of basophils can be alternatively induced by secretory IgA, though this

possibility is conditioned by previous priming of basophils with IL-3 (Galli, 2000).

Following activation, both types of cells produce chemokines and cytokines. Basophils mainly produce IL-4 and IL-13 being better source of these cytokines than mast cells. Nevertheless, mast cells produce broad range of these mediators, such as IL-1, -2, -3, -4, -5, -6, -8, -13, -16, GM-SCF, TNF-α, VPF, and VEGF. Variety of secreted mediators might be the reason for mast cells' relevant role in adaptive immunity (Frossi et al., 2004).

3 Aims

- Functional comparison of mast cells derived from the bone marrow and mast cell line

- Functional comparison of activation of mast cells by ionomycin and IgE receptor crosslink

- Influence of tick saliva, propolis and cysteine protease inhibitor on the degranulation and mast cell cytokine production *in vitro*

4 Materials and methods

4.1 Mice

Inbred female mice C57BL/6 (AnLab) were kept under standard conditions in a menagerie at the Institute of Parasitology of Academy of Science in České Budějovice. They were fed with commercial rodent fodder and water *ad libitum.* Mice at the age of 8-12 weeks were used.

4.2 Mast cells

4.2.1 MC/9 mast cells

MC/9 (ATCC) are growth factor dependent mast cells. They were grown in modified D-MEM medium at 37ºC, 5% CO_2 in 50 ml flask with half-open lid. Half of the medium was replaced three times a week.

MC/9 medium	
D-MEM	PAA
10% bovine fetal serum	PAA
1% L-glutamine	PAA
1% ATB (Penicillin, Streptomycin Sulphate, Amphotericin B)	PAA (P11-002)
Mercapto-ethanol (1:10000)	Sigma-Aldrich
10% Rat T-Stim	BD Biosciences

4.2.2 Bone marrow derived mast cells

4.2.2.1 Isolation of BMMCs from bone marrow

C57BL/6 mouse was sacrificed by cervical dislocation. Femur and tibia were dissected from both legs. After cutting of the joints, bone marrow was flushed with MEM, homogenated and passed through a 70 μm cell strainer. Following the centrifugation (10 min, 140 g, 4ºC), erythrocytes were lysed by 1 ml of 0,84 % NH_4Cl water solution

at 37 ºC. After 2 minutes 10 ml of MEM was added and the cells were spun down again. Finally, the cells were re-suspended in modified IMDM medium and transferred to a 24-well culture plate at concentration $3x10^6$ cells/ ml (well).

BMMC medium	
IMDM	PAA
10% bovine fetal serum	PAA
1% L-glutamine	PAA
1% ATB (Penicillin, Streptomycin Sulphate, Amphotericin B)	PAA (P11-002)
1% Na pyruvate	Sigma-Aldrich
1% IL-3	Kindly provided by prof. Edgar Schmitt from Meinz, Germany
0.05% IL-4	eBioscience
0.025% SCF	Kindly provided by prof. Edgar Schmitt from Meinz, Germany

4.2.2.2 BMMC cultivation

BMMCs were cultivated in 24-well culture plates at 37ºC, 5% CO_2. Three times a week the cells were transferred to fresh culture plates to separate them from adherent cells. Half of the medium was replaced at the same time. After four weeks of cultivation the cells were not transferred anymore and half of the medium was exchanged twice a week. Cells older than four weeks and younger than eight weeks were used for the experiments.

4.3 Mast cell activation

Mast cells were activated either by incubation cells with Ionomycin (Sigma-Aldrich) at final concentration of 1μM. The second mode of activation demanded stimulation of the cells with IgE-anti-DNP at concentration 1μg/ml for three days. After washing, activation was achieved by anti-IgE at concentration 2 μg/ml. Both these antibodies were kindly provided by prof. Edgar Schmitt from the Institute of Immunology in Meinz, Germany.

4.4 Tested proteins and antigens

4.4.1 Propolis

In this project, propolis served as a model substance with previously reported immunomodulatory properties (Sforcin, 2007). Propolis was kindly provided by Dr. Michalis Kotsyfakis (Institute of Parasitology of Academy of Science in České Budějovice) from an unknown source as 60% solution of ethanol. The solution was tested for the presence of LPS (lipopolysaccharide). The concentration of propolis used in the experiments was 60 μg/ml.

4.4.2 Tick saliva

Tick saliva was obtained from wild females of *Ixodes ricinus* tick. After seven days of infestation the ticks were taped to a slide and 10 μl glass capillaries (Drummond Scientific Company) were placed on their hypostomes. Then 2 μl of 5 % pilokarpin (Sigma-Aldrich) in 96% ethanol were applied on their dorsal side to stimulate saliva production. Ticks were placed to 37 ºC for 3 hours and checked every 30 minutes and the saliva collected to a microtube. Saliva was passed through a filter (pore size of 0.22 μm) to remove potential microbes and stored at -70 ºC. Protein concentration was estimated by a test according to Bradford.

4.4.3 Cystein protease inhibitor

Cystein protease inhibitor (CPI) derived from salivary glands of *I. ricinus* was prepared recombinant in E. coli expression system in the lab of Dr. Michalis Kotsyfakis at the Institute of Parasitology. Purified protein was LPS-decontaminated in the Arvys Proteins company.

4.5 Cytokine secretion analysis

Activated BMMCs and MC/9 of respective concentrations of 1×10^6 and 0.5×10^6 cells/ml were incubated with antigens (see table) for up to 48 hours at 96-well culture plate in a volume of 250 μl. Supernatants were collected after 4, 24, and 48 hours.

Antigen	Effective concentration
Propolis	60 μg/ml
Tick saliva	10 μg/ml
CPI	6 μM 3 μM 1.5 μM

4.6 Enzyme Linked Imunosorbent Assay (ELISA)

Various cytokines were quantified by a kit Ready set Go ELISA (eBioscience) according to a manual of the manufacturer. Detection limit of the kit is 4 pg/ml.

Supernatant collection	Cytokines tested
4 hours	TNF- α
24 hours	IL-6
48 hours	IL-4 IL-10 IL-9

For quantification of IL-9 different ELISA procedure was used. Antibodies and standards were kindly provided by prof. Edgar Schmitt from the Institute of Immunology in Meinz, Germany.

IL-9 ELISA procedure		
Coating	50µl/well of purified Ab (clone 229.4) diluted 1:1000 in coating buffer (Ready set Go ELISA, eBiosciences)	Overnight, 4ºC
Washing	3x PBS/0.1% Tween	
Blocking	50µl/well of 1x assay diluent (Ready set Go ELISA, eBiosciences)	30 min, 37 ºC
Samples	50µl/well of samples, 6 two-fold dilutions	60 min, 37 ºC
Washing	3x PBS/0.1% Tween	
Detection	50µl/well of biotinyl Ab (clone C12) diluted 1:1000 in 1x assay diluent	60 min, 37 ºC
Washing	3x PBS/0.1% Tween	
Enzyme	50µl/well of SA-HPO diluted 1:1000 in 1x assay diluent	25 min, 37 ºC
Washing	3x PBS/0.1% Tween	
Substrate	50µl/well of freshly prepared substrate solution: 5ml phosphor-citrate buffer, 2 mg OPD (Sigma-Aldrich), 2µl 30% H_2O_2	10 min, dark
Stop	50µl/well of 2M H_2SO_4	
	Absorbance measured at 490 nm by ELx800 Fluorescence microplate reader (BioTek)	

Due to the direct proportion of the absorbance and concentration of cytokines, the cytokine concentration was calculated from a standard curve.

4.7 β-hexosaminidase release

Cells were spun and diluted in Tyrode's buffer to a concentration of 1×10^6 cells/ml. Cells activated by Ionomycin (final concentration of 2 µM) and anti-IgE (2 µg/ml) were incubated with antigens (see table of effective concentrations in section *4.5*) for 30 minutes at 37 °C at 96-well culture plate in a volume of 200 µl. After the incubation the samples were transferred to microtubes and spun (5 min, 1400 g). Supernatant were collected and the pellets were resuspended in 200 µl of 0.5 % Triton X (Serva) water solution. 20 µl of supernatants and resuspended pellets were pipetted into vacant wells and

50 µl of substrate solution (1,3 mg/ml of 4-nitrophenyl-N-acetyl-β-D-glucosaminid (Sigma-Aldrich) in citrate-citric acid buffer, pH 4,5) were added. After 90 minutes of incubation at 37 ºC the reaction was stopped by 150 µl of glycine buffer, pH 10,7. Absorbance measured at 450 nm by ELx800 Fluorescence microplate reader (BioTek). The percentage of β-hexosaminidase release was calculated by following formula:

$$\beta\ hexosaminidase\ release = \frac{A_S}{A_S + A_P} \cdot 100$$

Tyrode's Buffer		**Glycine Buffer**	
$CaCl_2 \cdot 2H_2O$	0.265 g	Glycine	15 g
$MgCl_2 \cdot 6H_2O$	0.214 g	NaCl	11.7 g
KCl	0.2 g	Water	1 l
$NaHCO_3$	1.0 g		
NaCl	8.0 g		
$NaH_2PO_4 \cdot H_2O$	0.05 g		
D-Glucose	1.0 g		
Water	1 l		

4.8 Flow cytometry

4.8.1 Sample preparation

Cells were pre-incubated with IgE anti-DNP for two days. Cells were washed in PBS (10 min, 140 g, 4 ºC). Afterwards the cells were re-suspended in 100 µl of a buffer solution. Addition of 1 µl of Annexin-V-FITC (50 µg/ml in tris-HCl) (Sigma-Aldrich) and 1 µl of propidium iodide (PI, 100 µg/ml in PBS) to the samples was followed by 10 minutes of incubation in dark at 4 ºC. Finally 300 µl of buffer solution was added to the samples.

Buffer solution	
Deionized water	
HEPES	10 mM
NaCl	140 mM
$CaCl_2$	2,5 mM
pH 7,2-7,4	

4.8.2 Measuring of apoptosis

Anexin-V-FITC (green fluorescence) binds to a membrane phospholipid phosphatidyl serine. Under normal conditions phosphatidyl serine occurs on the inner side of the cell membrane. During the apoptosis it is translocated on the outer side of the membrane. PI (red fluorescence) penetrates into a cell with damaged membrane therefore it was used to distinguish necrotic cells. Cells that are alive are not stained neither by Anexin-V-FITC or PI.

Samples were analyzed by a flow cytometer BD FACS Canto II (BD). Minimum of 10 000 events were measured for each sample. The data were analyzed by BD FACSDiva Software.

4.9 LAL test

For estimation of LPS in propolis sample, the Limulus Amebocyte Lysate (LAL) test (Lonza) was used. Sample was incubated with LAL for 10 minutes at 37 ºC. After addition of a substrate, the mixture was incubated for another 6 minutes at 37 ºC. The reaction was stopped by 25 % acetic acid and the absorbance was measured spectrophotometrically at 405 nm.

Due to the direct proportion of the absorbance and concentration of LPS, the LPS concentration was calculated from a standard curve. The final concentration of LPS in the experiment was 2 pg/ml. This amount of LPS in the propolis sample was considered irrelevant, without any substantial effect on the function of mast cells.

4.10 Statistics

The data are expressed as mean ± standard error of mean (SEM). They were analyzed by two way analysis of variance (two-way ANOVA). Statistical difference between groups was estimated using a Bonferroni multiple comparison test. $P < 0,05$ was considered significant.

5 Results

5.1 Cytokine production

5.1.1 Comparison of cell types

BMMCs produced more TNF-α than MC/9 cells, in case of activation via FcεRI even four times more with statistical significance (Fig. 1A). Ionomycin activation led to slightly higher IL-6 production by MC/9 cells. In contrast, IL-6 was not detected when the MC/9s were activated by IgE cross-link, although BMMCs produced some IL-6 with the same mode of activation (Fig. 1B). MC/9 produced no IL-4, whereas BMMCs did (Fig. 1C). BMMCs produced significantly 13times less IL-10 than MC/9 cells when activated by Ionomycin. When activated by IgE both types of cells produced nearly the same amount of IL-10 (Fig. 1D).

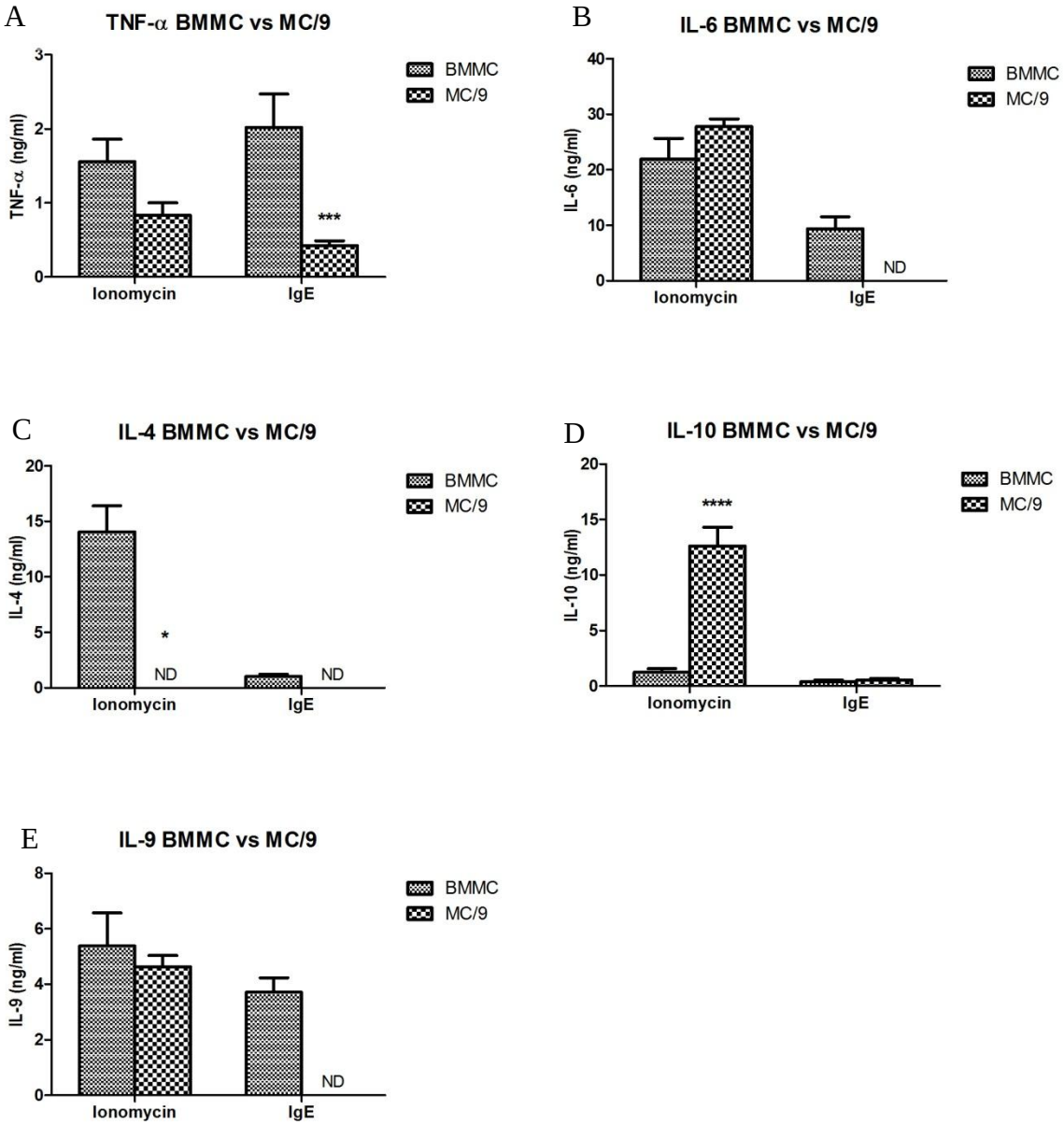


Figure 1. Production of various cytokines by BMMCs and MC/9: Comparison of mast cell types.

5.1.2 Comparison of modes of activation

After activation with Ionomycin, BMMCs produced moderately less TNF-α than after activation with IgE. On the contrary, MC/9s produced little bit more TNF-α after Ionomycin activation than after activation via IgE crosslink (Fig. 2A). There was a significant reduction in IL-6 production by BMMCs after IgE activation. MC/9s also produced significantly less IL-6 when IgE-activated (Fig. 2B). IL-4 production was 13times higher when the BMMCs were activated by Ionomycin. MC/9s did not produce any IL-4 at

all (Fig. 2C). Similar trend could be observed in IL-10 production, although not so pronounced in case of BMMCs and much more evident in case of MC/9s (Fig. 2D). IL-9 production in both types of cells had a decreasing trend in IgE activation, in case of of MC/9s, the decrease was statistically significant (Fig. 2E).

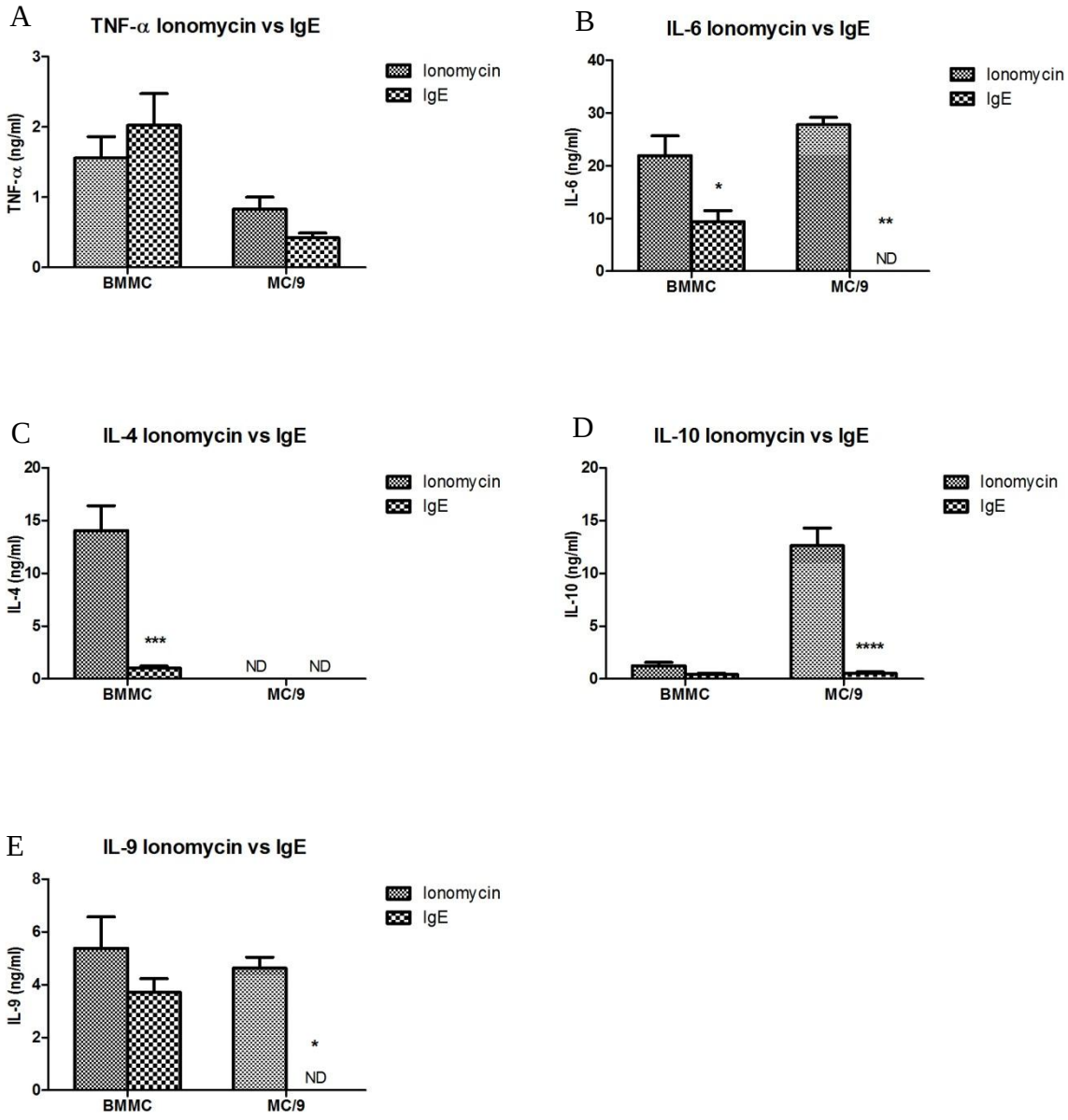
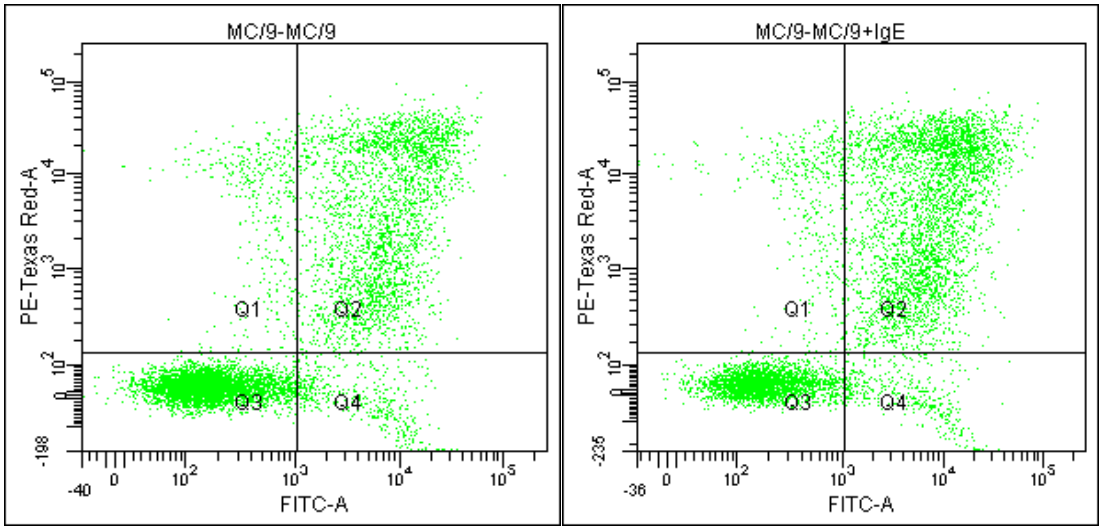


Figure 2. Comparison of the production of various cytokines by BMMC and MC/9 activated by Ionomycin/IgE.

5.1.3 Detection of viability of MC/9 by flow cytometry

It is obvious from Fig. 1 and 2 that MC/9 cells after IgE activation produced little or

no cytokines at all. A suspicion arose that IgE may cause MC/9s apoptosis. Staining of the cells with Annexin-V-FITC and propidium iodide (see chapter 4.8) showed that IgE does not have significant influence on MC/9 mortality (Fig. 3). The difference between the number of living MC/9s and MC/9 with IgE cannot be the reason for zero production of certain cytokines by IgE-activated MC/9 cells.



		MC/9 (%)	MC/9 + IgE (%)
$Q_1 + Q_2$	Late apoptotic and/or necrotic cells	40,5	55,1
Q_3	Living cells	53,9	39,4
Q_4	Early apoptotic cells	5,7	5,5

Figure 3. Comparison of MC/9 viability after co-culture with and without IgE.

5.1.4 Treatment of mast cells with antigens

5.1.4.1 Propolis

Propolis had no significant effect of TNF-α production by BMMCs, although a very little increase was seen independent on type of activation (Fig. 4A). The same trend was observed in IL-6 production when the BMMCs were activated through IgE cross-link. Activation of BMMCs by Ionomycin though, resulted in a significant decrease in IL-6 production compared to the control sample (Fig. 4B). The treatment of BMMCs with propolis induced a decent decrease in IL-4 secretion after both types of activation (Fig. 4C). No IL-10 detection in BMMCs after propolis-IgE exposure was counteracted by a significant noticeable increase of the IL-10 level in case of Ionomycin activation (Fig. 4D). Significant

increase was also observed in IL-9 production. Both ways of activation induced about 3times increase in BMMCs IL-9 secretion (Fig. 4E).

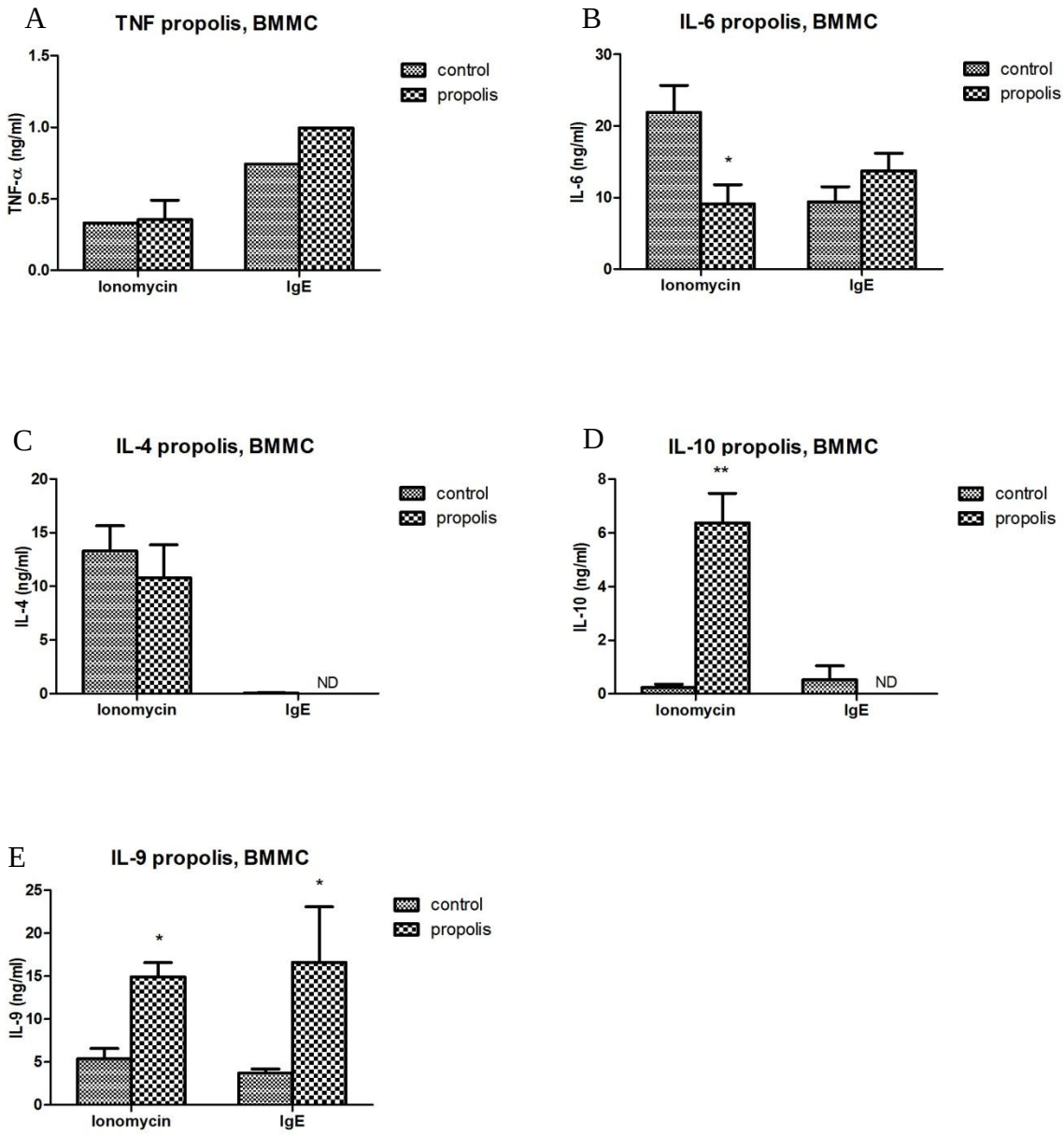


Figure 4. Effect of propolis on the production of various cytokines by BMMCs.

MC/9s after both types of activation produced comparable amount of TNF-α (Fig. 5A). MC/9s produced significantly more IL-6 when treated with Ionomycin. IgE induced no IL-6 production, even in the control samples (Fig. 5B). MC/9s' IL-4 was not detected. IL-10 was moderately decreased in both activation ways (Fig. 5C). IL-9 was

increased after Ionomycin-propolis 48 hours incubation, although the IgE activation did not result in IL-9 production by MC/9 (Fig. 5D).

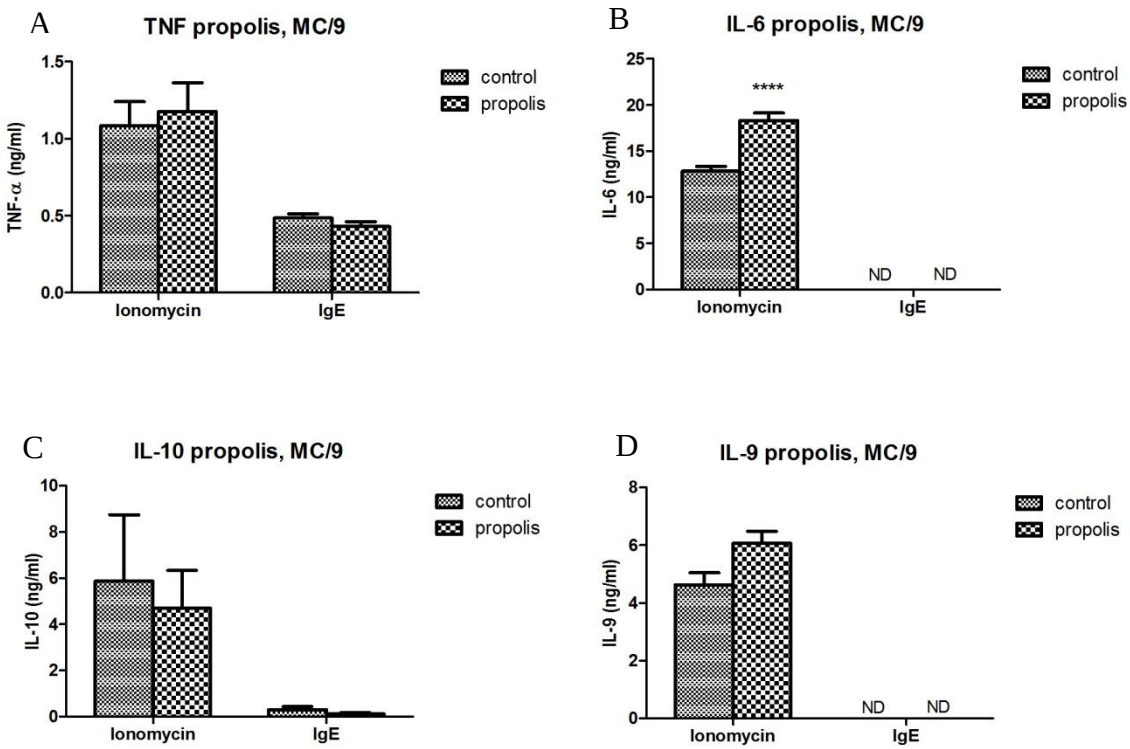


Figure 5. Effect of propolis on the production of various cytokines by MC/9s.

5.1.4.2 Tick saliva

Tick saliva decreased TNF-α production by BMMCs in both types of activation, although the decrease was not statistically significant (Fig. 6A). A little decreasing trend can be observed in IL-6 production in both types of activation modes (Fig. 6B). Tick saliva significantly reduced IL-4 secretion by BMMCs when activated by Ionomycin. On the other hand, a little increase occurred when the cells were activated via the IgE crosslink (Fig. 6C). IL-10 was significantly remarkably increased after activation with Ionomycin. IgE activation though did not cause IL-10 production by BMMCs (Fig. 6D). IL-9 production was increased in both activation modes but the increase was not statistically significant (Fig. 6E).

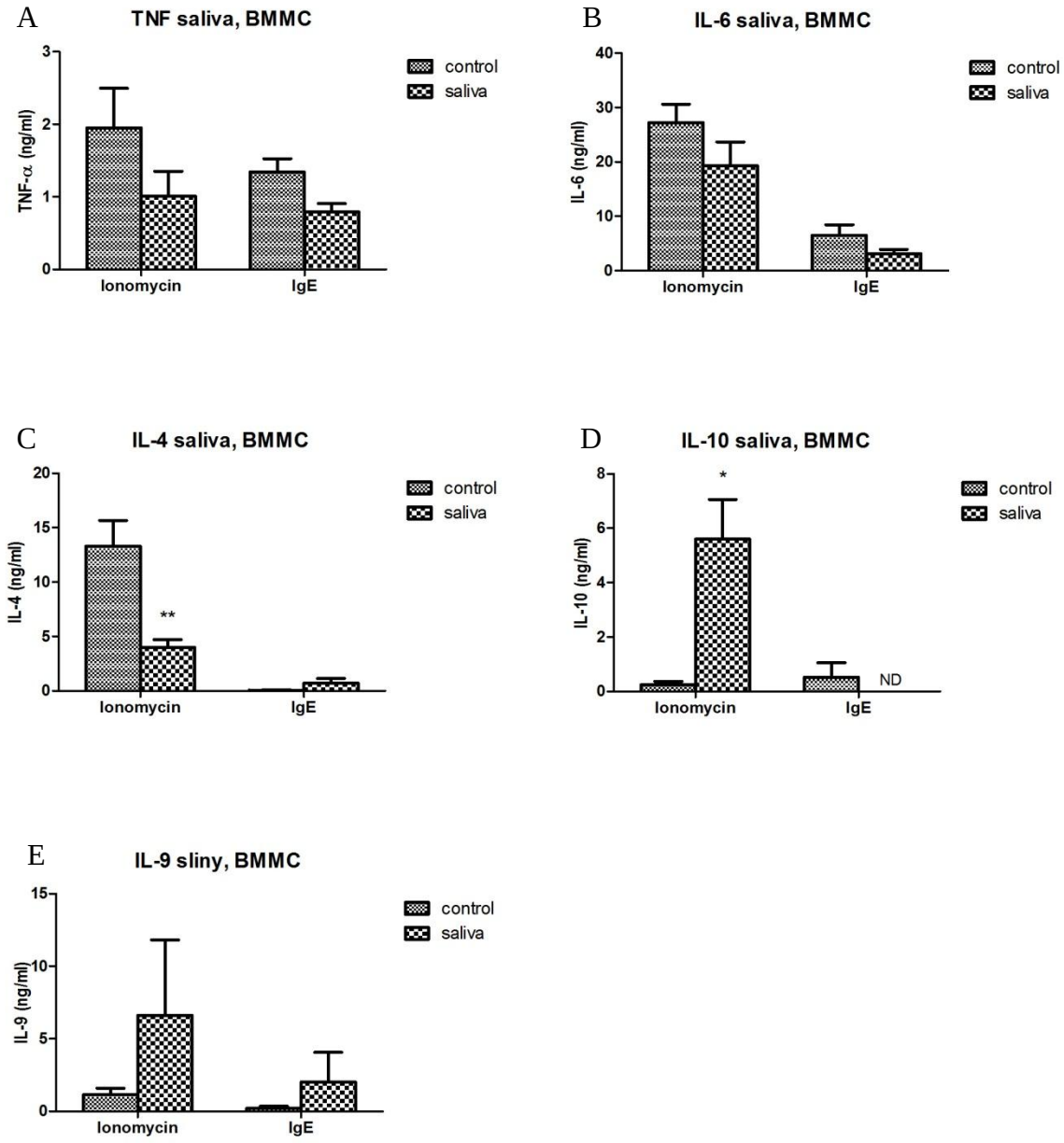


Figure 6. Effect of tick saliva on the production of various cytokines by BMMCs.

MC/9 produced less TNF-α when exposed to tick saliva. The 3fold decrease was significant in case of Ionomycin activation (Fig. 7A). A significant decrease was also observed in IL-6 production by Ionomycin-activated MC/9s. IgE-activated MC/9s did not produce any IL-6 (Fig. 7B). Also IL-4 was not produced by MC/9 cells. No effect of tick saliva was observed on IL-10 production (Fig. 7C). IL-9 was decreased after the activation with Ionomycin, but MC/9 cells apparently did not produce any IL-9 when activated by IgE (Fig. 7D).

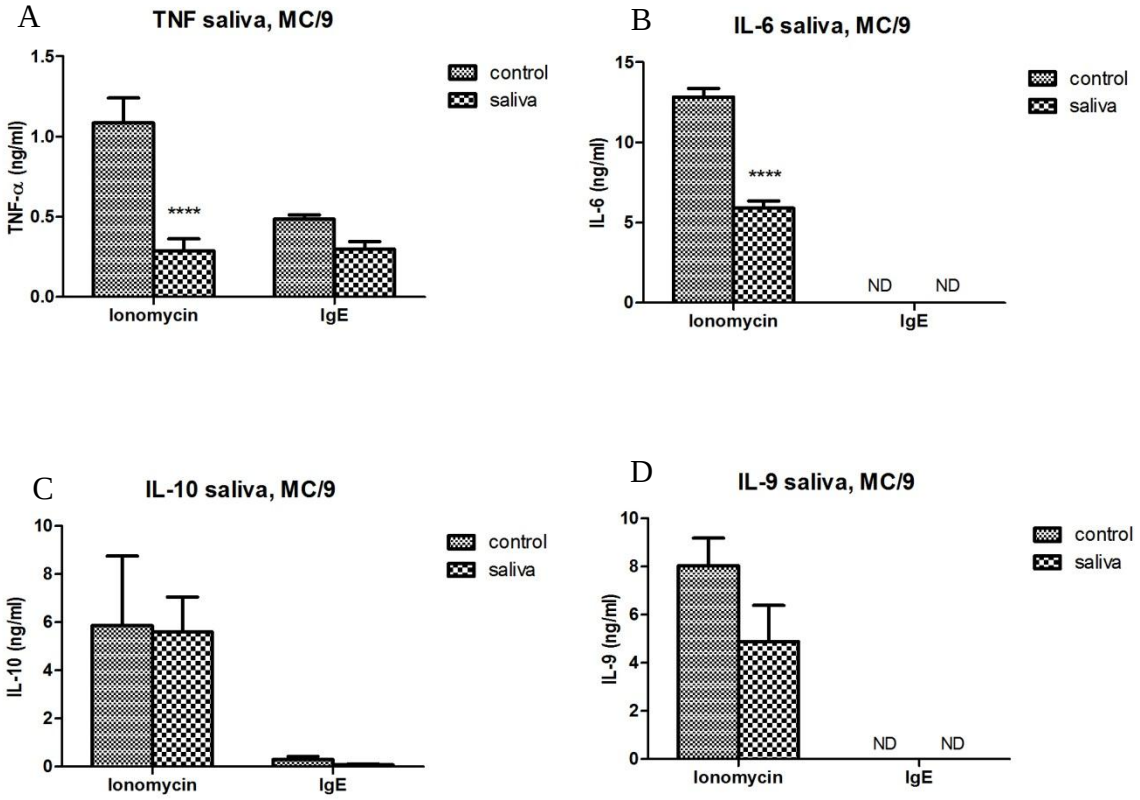


Figure 7. Effect of tick saliva on the production of various cytokines by MC/9s.

5.1.4.3 CPI

When the BMMCs were treated with CPI, no effect was observed in TNF-α production, only the 3 μM CPI caused a little decline in TNF-α. When the cells were activated by IgE, the production of TNF-α decreased by half. Nevertheless, the decrease was not statistically significant (Fig. 8A). Ionomycin activation and CPI treatment was followed by a decrease in IL-6 production by BMMCs. When the cells were activated by IgE, the CPI had no remarkable effect on IL-6 level (Fig. 8B). An increasing trend was also observed in IL-4 production by both Ionomycin- and IgE- activated BMMCs (Fig. 8C). IL-10 was decreased after activation by Ionomycin, the most striking decrease was induced by 6 μM CPI. Treatment of IgE activated BMMCs with 6 μM CPI led to no production of IL-10, whereas 3 μM and 1.5 μM CPI increased the production of IL-10 (Fig. 8D). IL-9 was also decreased following the Ionomycin activation and CPI exposure. 6 μM CPI did cause hardly any difference in IgE-activated BMMCs, but 3 μM and 1.5 μM CPI led to

an increased production of IL-9 (Fig. 8E).

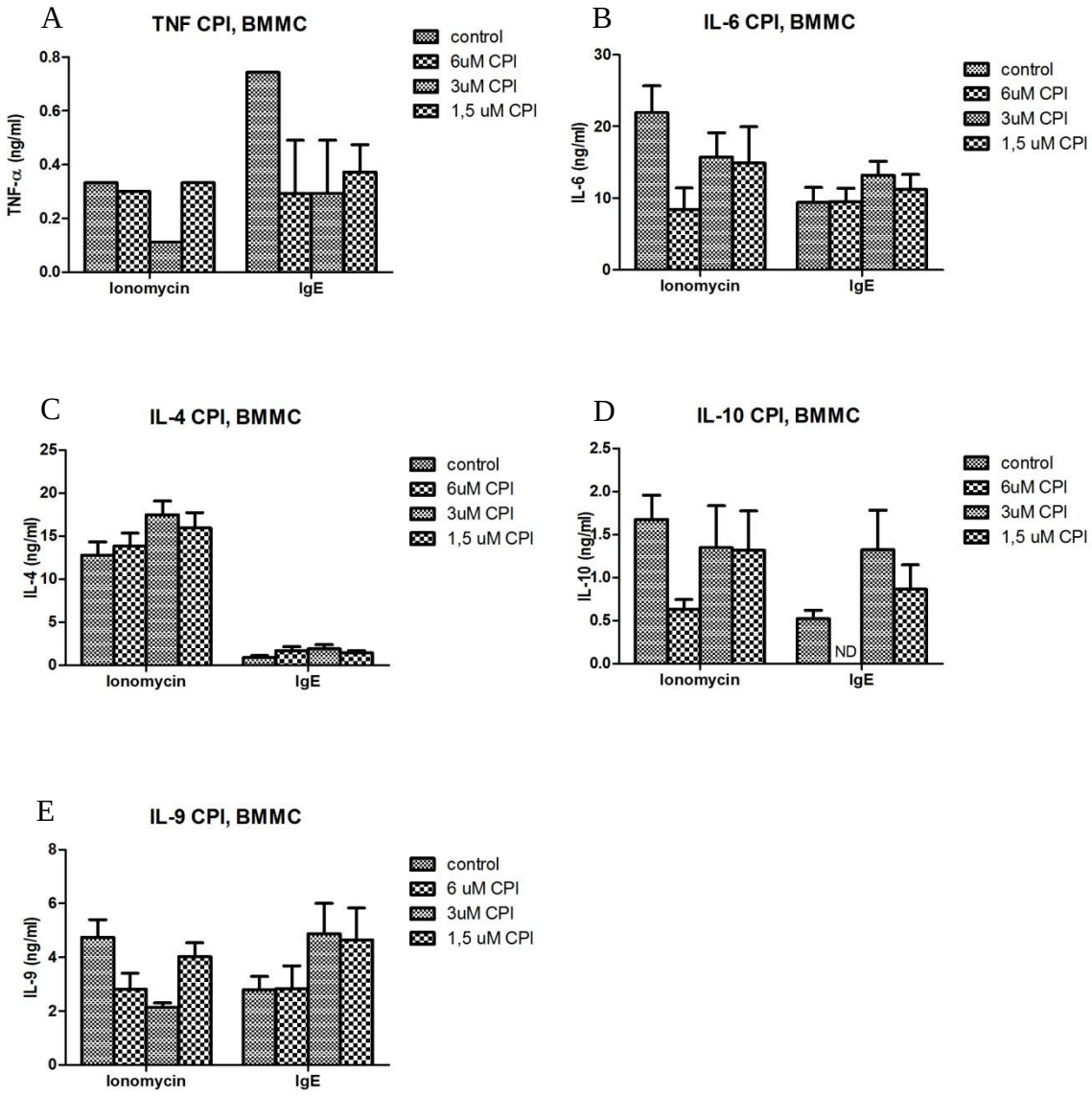


Figure 8. Effect of CPI on the production of various cytokines by BMMCs.

Ionomycin-activated MC/9s produced little bit more TNF-α after incubation with 3 μM CPI. Treatment of IgE-stimulated cells with CPI led to a modest decreasing trend in TNF-α production (Fig. 9A). Ionomycin/CPI treatment resulted in an increasing trend of the IL-6 secretion. IgE-stimulated MC/9 cells produced no IL-6 (Fig. 9B). IL-4 was also not produced by MC/9s. IL-10 was decreased in both types of activation, although in case of IgE crosslink activation, the decrease was subtle (Fig. 9C). IL-9 was not produced by IgE-stimulated MC/9s. 3 μM and 1.5 μM CPI, however induced a significant increase in IL-9 secretion (Fig. 9D).

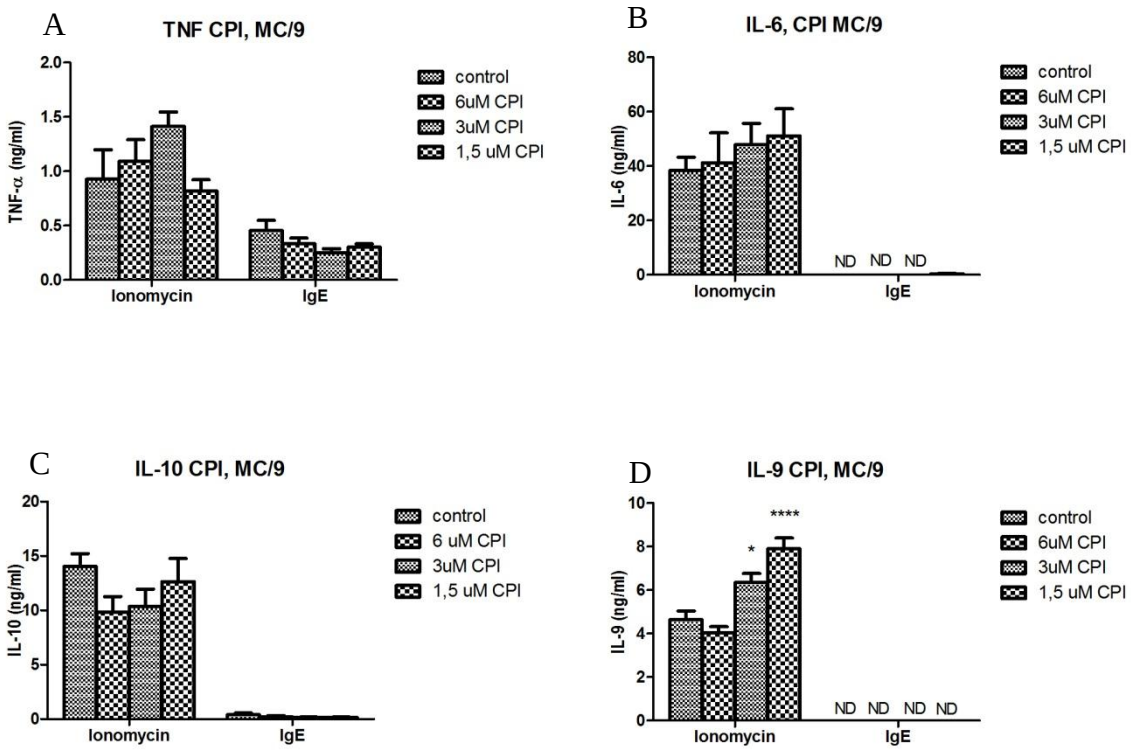


Figure 9. Effect of CPI on the production of various cytokines by MC/9s.

5.2 β-hexosaminidase release

5.2.1 Comparison of cell types and means of activation

Figure 10A shows that both BMMCs and MC/9s released approximately the same amount of β-hexosaminidase. IgE activation resulted in significantly higher β-hexosaminidase by BMMCs. MC/9s released also bigger amount of β-hexosaminidase after the IgE crosslink (Figure 10B).

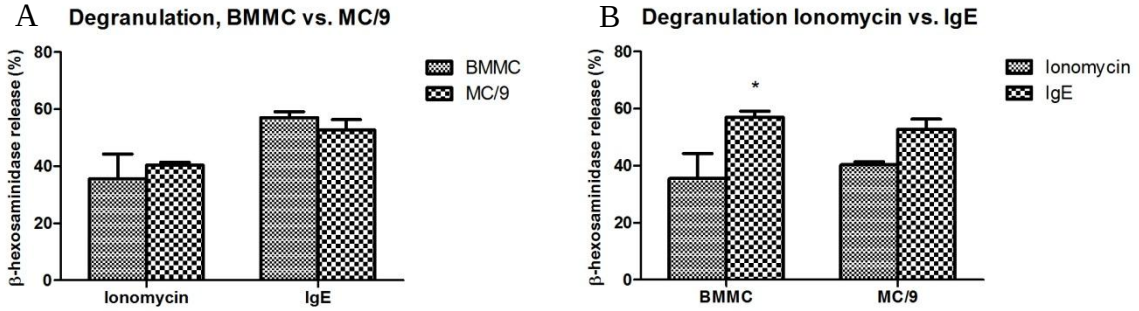


Figure 10. β-hexosaminidase release: A. BMMCs vs. MC/9. B. Comparison of Ionomycin and IgE activation.

5.2.2 Stimulation of mast cells with antigens

5.2.2.1 Propolis and tick saliva

Propolis had a little effect on β-hexosaminidase release by BMMCs, whereas tick saliva induced an increase in β-hexosaminidase liberation by BMMCs (Fig. 11A). Treatment of MC/9s with propolis had very little decreasing effect in β-hexosaminidase release. Similar response was observed after a treatment of IgE-stimulated MC/9s by tick saliva. After Ionomycin activation, tick saliva caused no change in β-hexosaminidase release compared to control sample (Fig. 11B).

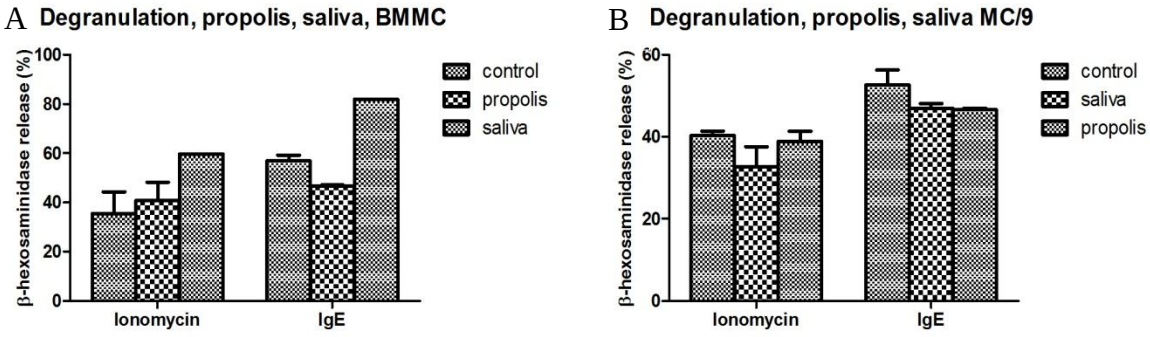


Figure 11. β-hexosaminidase release by BMMCs and MC/9 after stimulation with propolis and saliva.

5.2.2.2 CPI

CPI had no noticeable effect on the release of β-hexosaminidase on any of the cell type.

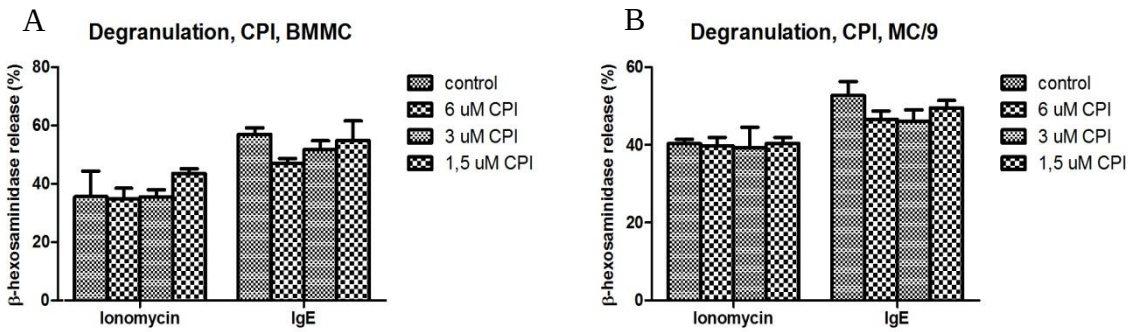


Figure 12. β-hexosaminidase release by BMMCs and MC/9 after stimulation with different concentrations of CPI.

6 Discussion

Mast cells are a cell type residing in mucosal and connective tissues. Their location makes them one of the first cells in contact with the environmental stimuli. Whether the stimuli are allergens, parasites or pathogens, mast cells can regulate the immunity in negative as well as positive manner. It is important to understand the mechanisms of mast cell immunoregulation and their contribution to the whole immune system. The immunoregulatory properties of mast cells, once they are understood, may be applied in therapeutical approaches either to promote mast cell function to support the immune response or to suppress those mechanisms that evolve into disease (Galli et al., 2008).

Bone marrow-derived mast cells serve as convenient *in vitro* model for screening of mast cell properties. Their isolation and cultivation is rather prolonged and the yield of isolated cells is limited. Moreover, the cultivation takes minimum of three weeks until the mast cells are differentiated. The long time increases the risk of contamination because of frequent manipulation with the cells during the change of media. Also the cytokines that are essential for the differentiation of bone-marrow cells are rather expensive. That is why our laboratory considered using MC/9 mast cell line for the sake of their undemanding cultivation. They can be stored in liquid nitrogen and after defrosting they are ready to use after few days.

Similar trend is applicable when considering whether to use Ionomycin or IgE-mediated activation. Ionomycin is cheap and easily available, whereas IgE is more expensive and also requires pre-incubation of the cells which prolongs the experiment. The advantage of IgE activation is that it corresponds to the *in vivo* situation.

MC/9 appeared to follow similar trends in production of some cytokines which is in accordance with previous studies (Burd et al., 1989; Ishizuka et al., 1998). Degranulation assays of BMMCs and MC/9 were comparable. Interestingly, MC/9 produce much more IL-10 than BMMCs. Having in mind that MC/9 originate from tumor cells, the increased IL-10 production may be associated with immunosuppressive properties of this cytokine. IL-10 production was observed in several carcinoma cell lines (Gastl et al., 1993). Another striking discovery was that MC/9 do not produce any IL-4. What is behind this deviation remains to be clarified, especially when the production of this cytokine by IgE-activated MC/9 has been reported before (Hsu et al., 2010).

Ionomycin activation of BMMCs resulted in higher production of cytokines. This result was according to our expectation and data from the literature

(Matsushima et al., 2004). Ionomycin is a ionophore increasing intracellular levels of Ca^{2+} and is therefore very efficient activator of the cells. IgE on the other hand requires aggregation of the FcRI receptors and the strength of the signal is limited by number of the receptors. Accordingly, the IgE mediated activation is weaker but corresponding with the natural *in vivo* activation process.

After the IgE stimulation IL-6 and IL-9 were not produced by MC/9 cells, even though these cytokines were produced when the cells were activated with ionomycin. This cannot be explained by the lack of FcRI receptor as the MC/9s express FcRI receptor in the same number as for example peritoneal mast cells (Hara et al., 1994). Also the IgE does not bind to the MC/9 FcRI receptor with lower affinity (Galli et al., 1982). On the other hand, when speaking about degranulation IgE mediated activation appeared to be more potent activation tool for both types of cells.

Generally BMMCs are convenient, however not ideal model for cytokine analysis. The main limitations of this model is that the cells are differentiated after four weeks, but in contrast to their *in vivo* maturation they are cultivated in the absence of antigens, therefore they are not fully mature. Consequently, their response to various stimuli can be different from that of cells isolated from mice.

Propolis is cheap and easily available substance with previously reported immunomodulatory properties (Sforcin, 2007). Our results have confirmed the anti-inflammatory influence of propolis that was described before (Hu et al., 2005; Khayyal et al., 2003). When propolis extract was administered to asthmatic patients their clinical symptoms ameliorated and their plasma TNF-α and IL-6 dropped. On the other hand IL-10 was elevated (Khayyal et al., 2003). In our investigation we observed similar trend: pro-inflammatory IL-6 was significantly decreased and anti-inflammatory cytokine IL-10 was substantially increased. Controversy arose with considerably increased IL-9. IL-9 serves as an auxiliary growth factor for mast cells. It can therefore serve as an autocrine agent promoting survival of mast cells during the immune response. Furthermore IL-9 enhances production of IL-13 by mast cells promoting the allergic reaction (Noelle and Nowak, 2010). On the other hand, it has been found out that IL-9 has a dual role in modulation of the immune cells. Having effect on regulatory T cells, IL-9 can mediate immunosuppression (Nowak et al., 2009).

Orsi and colleagues found out that propolis of a concentration of 300 μg/ml increased histamine release due to cytotoxic effects. Lower concentrations of propolis did not have any effect of mast cell degranulation (Orsi et al., 2005). We obtained analogous trends with

propolis of a concentration 60 μg/ml. These results complete the data from our cytokine assay confirming anti-inflammatory properties of propolis.

As described above in chapter 2.6, tick saliva contains many bioactive molecules helping the tick to overcome the host's immune response. Upon the saliva exposure, mast cells are supposed to degranulate to impede the tick engorgement and the length of infestation. In our experiments, BMMCs showed an increasing trend in the rate of degranulation in response to the exposure of tick saliva. The elevation however was not so pronounced and also previous experiments in our lab did not prove the effect of tick saliva on mast cell degranulation (Langhansova et al., 2012). Rather low rate of mast cell degranulation was reported before in an *in vivo* study. Mast cells showed little response upon the first tick infestation. Successive infestations however, resulted in greater mast cell degranulation (Gill, 1986).

Tick infestation leads to suppression of pro-inflammatory cytokines and polarization of the immune response towards T_{H2} (Ferreira and Silva, 1999; Macaluso and Wikel, 2001; Schoeller et al., 2000). In agreement with previous studies we also observed a down-regulation of pro-inflammatory cytokines. On the other hand, anti-inflammatory IL-10 was increased. In contrast with earlier observation we observed a decrease in IL-4 which is an important T_{H2} cytokine. Decrease in IL-4 production of BMMCs after stimulation with tick saliva was observed in our laboratory before (Langhansova et al., 2012). IL-4 is a cytokine that shifts the immune response towards T_{H2} by stimulation of the differentiation of T_{H2} lymphocytes from naïve precursors (O'Garra, 1998). Low production of IL-4 can lead into decreased ability of the hosts' antibody response (Wikel et al., 1994). It is questionable though if the mast cells' IL-4 contribution to total IL-4 production is relevant (Mejri and Brossard, 2007).

Characterization of components of tick saliva is important for comprehension of tick-host interactions. It can also contribute to anti-tick vaccine research. Our cysteine protease inhibitor (CPI) is newly derived protein from salivary gland of *I. ricinus*. Although little decrease in TNF-α was observed, the CPI did not have any remarkable effect on cytokine production by mast cells. This CPI probably does not influence mast cell-mediated immunomodulation on the host. Similarly no profound effect of the CPI was observed on mast cell degranulation. Most likely it does not have any effect on the effector properties of mast cells.

In conclusion we claim that BMMCs are good model for testing the effects of immunomodulatory effects of tick proteins. MC/9 cell line is also convenient for their

availability although the spectrum of cytokines produced by this cell line may be different. We suggest that results from preliminary tests on MC/9 are confirmed by the experiments on BMMCs. Analogously we assume that Ionomycin activation is suitable *in vitro* activation mode which can be advantageous to compare with IgE-mediated activation.

7 Summary

- MC/9s produce less TNF-α and IL-4 than BMMCs. On the other hand, they produce more IL-10.

- Activation through IgE receptor crosslink leads to lower production of all tested cytokines except TNF-α.

- Immunosuppressive effect of propolis has been confirmed (in terms of decreased IL-6 and increased IL-9 and IL-10).

- *Ixodes ricinus* tick saliva decreases the production of TNF-α, IL-6, IL-4, and increases IL-10 production.

- CPI has no significant effect on mast cells.

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9 List of abbreviations

CTMC	Connective Tissue Mast Cells
EAE	Experimental Allergic Encephalomyelitis
GM-SCF	Granulocyte Macrophage Stem Cell Factor
GPCR	G Protein Coupled Receptor
HRF	Histamine Release Factor
IL	Interleukine
ITAM	Immunoreceptor Tyrosine Activation Motif
LAL	Limulus Amebocyte Lysate
LPS	Lipopolysaccharide
LTC	Leukotriene
MC	Mast Cells
MC-CPA	Mast Cell Carboxypeptidase A
MHC	Main Histocompatibility Complex
MMC	Mucosal Mast Cells
NK cells	Natural Killer cells
PTK	Protein Tyrosine Kinase
SCF	Stem Cell Factor
TNF-α	Tumor Necrosis Factor α
TLR	Toll-Like Receptor
VEGF	Vascular Endothelial cell Growth Factor
VPF	Vascular Permeability Factor