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The effect of tick salivary proteins on innate immunity cells

Ph.D. Thesis

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ANNOTATION

Saliva of Ixodid ticks contains a whole array of pharmacologically active molecules with vasodilatory, antihemostatic, and immunomodulatory activities.
This thesis focuses on two types of salivary proteins, serpins and cystatins, and their role in immunomodulation. These protease inhibitors are known to affect many biological functions. To better understand their role in tick saliva we examined their effect on dendritic cells and their ability to modulate the immune response after pathogen infection. As model pathogens, *Borrelia* spirochetes and tick-borne encephalitis virus were used.

DECLARATION [IN CZECH]

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List of papers and author's contribution

The thesis is based on the following papers:

I.

Páleníková, J., Lieskovská, J., Langhansová, H., Kotsyfakis, M., Chmelař, J., Kopecký, J. (2015): *Ixodes ricinus* salivary serpin IRS-2 affects Th17 differentiation via inhibition of the interleukin-6/STAT-3 signaling pathway; *Infection and Immunity* 83: 1949-1956.DOI: 10.1128/IAI.03065-14 (IF= 4.156)

The study was designed by JP, JK, JL and HL also contributed to the study design. JP also performed all the experiments, analyzed data, and prepared the whole manuscript including writing and figures preparation.

II.

Lieskovská, J., **Páleníková, J.**, Langhansová, H., Chagas, A.C., Calvo, E., Kotsyfakis, M., Kopecký J. (2015): Tick sialostatins L and L2 differentially influence dendritic cell responses to *Borrelia* spirochetes; *Parasites & Vectors* 8: 275.DOI: 10.1186/s13071-015-0887-1 (IF= 3.25)

The study was designed by JL. JP performed the analysis of chemokines and interferon-β. Including the isolation and activation of dendritic cells, measurements of their concentration by ELISA assays and data analysis followed by figures preparation.

III.

Lieskovská, J., **Páleníková, J.**, Širmarová, J., Elsterová, J., Kotsyfakis, M., Campos-Chagas, A., Calvo, E., Růžek, D., Kopecký, J. (2015): Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells; *Parasite Immunology* 37: 70-78.DOI: 10.1111/pim.12162 (IF= 2.143)

The study was designed by JL. JP performed the IP-10 and IFN-β ELISA assays including the isolation and activation of dendritic cells. She also helped to prepare the figures.

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1 AIMS AND OBJECTIVES

Tick saliva is an amazing cocktail of pharmacologically active substances, which comprises of proteins and non-protein molecules. In the last decade many of these molecules and their properties were described in detail. Discovering and further investigation of these substances helps to understand the properties of tick saliva which were described many years ago. So today, we know salivary proteins which are responsible (or at least partly responsible) for inhibition of variety of immune cells (neutrophils, macrophages, dendritic cells, T cells) functions, for binding of cytokines or for complement inhibition. But still little is known about the mechanism of this strong immunomodulatory effect of tick saliva on molecular level. The understanding of how tick saliva influences transduction of the signals activated upon interactions of PAMPs (pathogen associated molecular patterns) with PRRs (pattern recognition receptors) is especially important because of the ability of tick saliva to facilitate the transmission of tick-borne pathogens. Moreover, the detailed characterization of particular tick salivary proteins and their role in modulation of immune response can be exploited for the development of new immunosuppressive drugs.

This thesis is a part of research project of the Department of medical biology at the University of South Bohemia and follows up the previous results obtained in our laboratoty. We observed the effect of particular salivary proteins (serpins, cystatins) on the activation of innate immune cells, especially dendritic cells.

Our goal was to further clarify the observed effects of these salivary proteins on the level of cellular signaling in innate immune cells activated with Toll like receptor ligands like lipopolysaccharide, *Borrelia* or tick-borne encephalitis virus.

Specific aims:

- **To test the effect of tick salivary serpin IRS-2 on dendritic cells ability to drive the adaptive immune response.**
- **To test the effect of recombinant tick salivary proteins (cystatins) on the activation of dendritic cells upon stimulation with TLR ligands and *Borrelia* spirochetes, including analysis of signaling pathways.**
- **To test the mechanism of tick cystatin-induced suppression of type I interferon signaling; analysis of the effect of saliva-derived proteins on activation of JAK/ STAT pathways.**

2 GENERAL INTRODUCTION

2.1. TICKS AS DISEASE VECTORS

2.1.1. CHARACTERISTIC

Ticks are highly specialized, obligate bloodsucking ectoparasites which suck on a broad spectrum of hosts including mammals, birds, reptiles, and amphibians through the world (Anderson and Magnarelli 2008). This fact makes ticks second most common vectors of human pathogens after the mosquitoes (Parola and Raoult 2001).

Based on taxonomy, ticks can be classified in phylum Arthropoda, subphylum Chelicerata, class Arachnida, subclass Acarina and suborder Ixodida. The subclass Ixodida can be further divided into four families, the main of them are Ixodidae or „hard ticks“ which are characterized by the presence of dorsal plate and Argasidae called also „soft ticks“ for their flexible cuticle (Oschmann et al. 1999, Gray et al. 2002). Another two families Nuttalliedae a Laelaptidae are from the medical and veterinary point of view non-significant (Anderson and Magnarelli 2008).

Ticks from the families Ixodidae and Argasidae can be, apart from the dissimilar morphology, distinguished also based on the feeding strategy. Ixodidae need to their full engorgement several days and in the whole course of blood sucking they remain tightly attached to their host. On the other hand Argasidae need just a few minutes or hours of repeated sucking and they are not firmly attached to their hosts (Bergman 1996).

The most important representative of argasids is *Ornithodoros moubata* which is a vector of *Borrelia duttoni*, the causative agent of relapsing fever in humans. Ixodidae are further divided into Metastriata, with genus *Dermacentor* and *Rhipicephalus* and Prostriata, with genus *Ixodes* (Francischetti et al. 2009). Concerning human pathogen transmission, the genus Ixodes is the most prominent. Ticks from this genus, mainly *Ixodes ricinus, I. scapularis, I. persulcatus,* and *I. pacificus* significantly participate in the transmission of severe human pathogens. These pathogens include bacteria such as *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, or *Francisella tularensis;* protozoa, for example *Babesia microti* and *Babesia divergens* or different tick-borne viruses (Gray et al. 2002). In Czech Republic, the most prominent tick-borne pathogens are *Borrelia burgdorferi* sensu lato group and tick-borne encephalitis virus, causing increasing number of severe disorders every year.

2.1.2. LIFE CYCLE

With the respect to pathogen transmission on humans, the main research is focused on the American deer tick *Ixodes scapularis* and the European castor bean tick *Ixodes ricinus*. *I. scapularis* is widespread mainly on the east part of USA and southeast part of Canada, whereas *I. ricinus* can be found nearly in the whole Europe. In the last few years, the shift of *I. ricinus* incidence into higher altitudes was noticed together with the higher occurrence of certain human pathogens in these regions (Danielová et al. 2006). The life cycle of these two ticks consists of three developmental stages. The first stage, six legged larvae, hatches from the eggs and after feeding molts into a nymph which after next blood meal molts into an adult stage. In this stage, the males and females can be distinguished. *Ixodes* ticks belong among the so called three-host species which means that each developmental stage sucks on different host.

The larvae suck mainly on small animals like rodents or reptiles and their blood-meal uptake lasts two to three days. Nymphs prefer middle sized animals like rabbits and suck also for longer time - three to five days. Adult females usually feed on big animals and they uptake the blood for seven to nine days. Adult males suck blood just rarely, but their presence is necessary for female's fertilization. Only the fertilized female can successfully complete the feeding and lay eggs.

The whole tick development can be under convenient conditions completed in two or three years (Oschmann et al. 1999, Anderson and Magnarelli 2008).

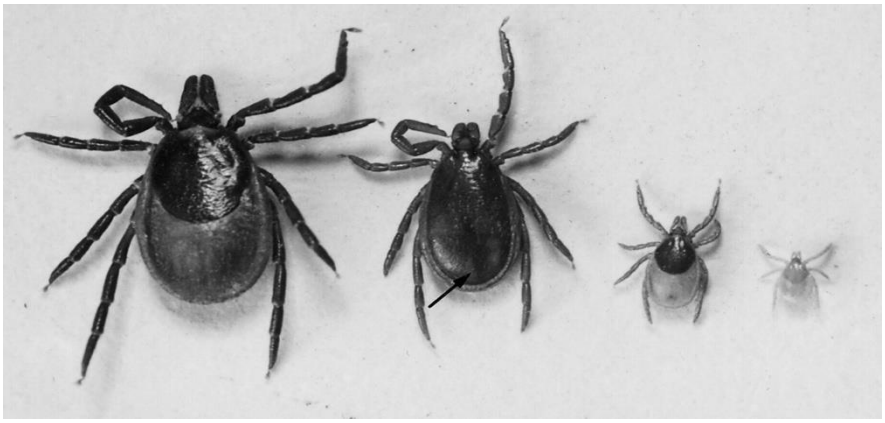


Figure 1: Developmental stages of *Ixodes ricinus* (Parola and Raoult 2001)

Figure 2: Developmental stages of *Ixodes scapularis* (www.aldf.com)

2.2. HOST RESPONSE - INNATE IMMUNITY

All Vertebrates are during their lives threatened by a broad variety of invading microorganism and others, for the body foreign, substances or by an injury. Therefore they have evolved system of immune defence in order to eliminate infective pathogens and undesirable substances entering the body. This defensive, immune, system is in mammalians comprised of two branches, so called innate and adaptive immunity. The innate immune system represents the first line of host defence. Acquired immunity is involved in elimination of pathogens in the late phase of infection as well as in the generation of immunological memory. Acquired immunity is characterized by its specificity (Akira et al. 2006).

Innate immune response, challenged after local injury caused by penetrating ticks mouthparts, represents the first line of hosts immune defence against feeding tick and invading pathogens. It involves components of complement system and acute phase proteins as well as resident leukocytes present in the epidermis and dermis such as dendritic cells (DCs), neutrophils, macrophages, mast cells, basophils, eosinophils, and natural killers (NKs). All these cells release pre-formed mediators such as cytokines or chemokines which recruit, or help to the recruit, additional inflammatory cells to the attachment site. Subsequent tick infestation triggers adaptive response which involves T cells and B cells activation and leads to the sensitization of mast cells and basophils that are predominant cells in the tick attachment site. In the generation of the acquired immunity which, in turn, leads to resistance against the tick, dendritic cells play crucial role.

2.2.1. ROLE OF DENDRITIC CELLS IN INNATE IMMUNITY

Dendritic cells are a special type of leukocytes arising from CD34 (cluster of differentiation) hematopoetic precursors in bone marrow. These precursors are carried by the

blood flow to various organs, where they transform into immature dendritic cells. These organs are mainly tissues bordering with external environment, i.e. skin, respiratory and gastrointestinal tract (Granucci et al. 2005, Lipscomb and Masten 2002). Here the immature dendritic cells act as the so called guardians of immune system and capture here the invading pathogens entering to the body. After the antigen processing, dendritic cells migrate into the draining lymph node, where the already mature dendritic cells present antigens to the T lymphocytes and start thus adaptive immune response (Banchereau and Steinman 1998, Granucci et al. 2005). Thus dendritic cells play an exceptional role among other immune system cells - they link the innate and the adaptive immunity.

Pathogen recognition is based on their interaction with so called pattern-recognition receptors on the membrane of dendritic cells. These receptors recognize specific pathogen structures so called pathogen-associated molecular patterns. These structures include for instance lipopolysaccharides (LPS) from Gramnegative bacteria, lipoproteins, peptidoglycans, microbial DNA (deoxyribonucleic acid) rich in CpG motive, single- and double-stranded viral RNA (ribonucleic acid) or flagellin. All these structures are highly conserved among pathogens and cannot be found in mammals (Granucci et al. 2005). In addition to microbial impulse, immature dendritic cells can be activated also by substances produced by tissue injury (DAMPs – damage associated molecular patterns) or by inflammatory cytokines.

The recognition of various pathogen structures by pattern recognition receptors (PRR) results in absorption of pathogens by means of phagocytosis or endocytosis (Banchereau and Steinman 1998, Wilson et al. 2004). Internalization of foreign pathogens triggers maturation of dendritic cells and their migration from peripheral tissues to lymphoid organs. During this transformation of immature cells into mature dendritic cells comes to a range of phenotypical and functional changes which include MHC (major histocompatibility complex) molecules redistribution from the inside cell compartments to the cell surface, the repression of ability to internalize pathogens, the enhancement of co-stimulatory molecules expression (mainly CD80 [B7-1] and CD86 [B7-2]) and the increase in chemokines, cytokines and adhesive molecules secretion (Banchereau and Steinman 1998).

Mature dendritic cells settle down in T-cell region of draining lymph nodes, where they interact with antigen specific T-lymphocytes. This interaction includes binding of T- cell receptor on the surface of T lymphocytes on MHC-antigen complex on the surface of dendritic cell. Under the presence of other co-stimulatory molecules it comes to clonal proliferation of T and B lymphocytes, cytokines production and thus to the development of

adaptive immune response. The development of immune reaction towards Th1, Th2, Th9 or Th17 type of response depends on the nature of antigen stimulus, repertoire of produced cytokines and current cytokine microenvironment. For instant antigens causing interleukin 12 (IL-12) secretion lead to the Th1 polarization of immune system, while IL-4 and IL-10 production or suppression of IL-12 production leads to Th2 polarization. The increased production of IL-6 and transforming growth factor β (TGF-β) leads then to the Th17 cells differentiation (Banchereau and Steinman 1998, Cella et al. 1996, Miller et al. 2007).

It should be mentioned that dendritic cells constitute a heterogeneous population of cells. Based on different properties, two main populations of dendritic cells are usually distinguished. First are the conventional dendritic cells, also called myeloid. They express myeloid markers, intercept invading pathogens in the periphery, and then migrate to the secondary lymphoid tissue where they present pathogen-derived peptides to antigen-specific T cells. The mDCs secrete big amounts of IL-12 when stimulated with tumour necrosis factor-α or CD40 ligand and drive a potent Th1-polarized immune response. They display the Toll-like receptors TLR2 and TLR4 (Cella et al. 1996, Macatonia et al. 1995). Second subtype is represented by plasmacytoid dendritic cells which are characteristic for robust production of type I IFN (interferon) after stimulation with various ligands and for the expression of TLR7 and TLR9 (Ito et al. 2005).

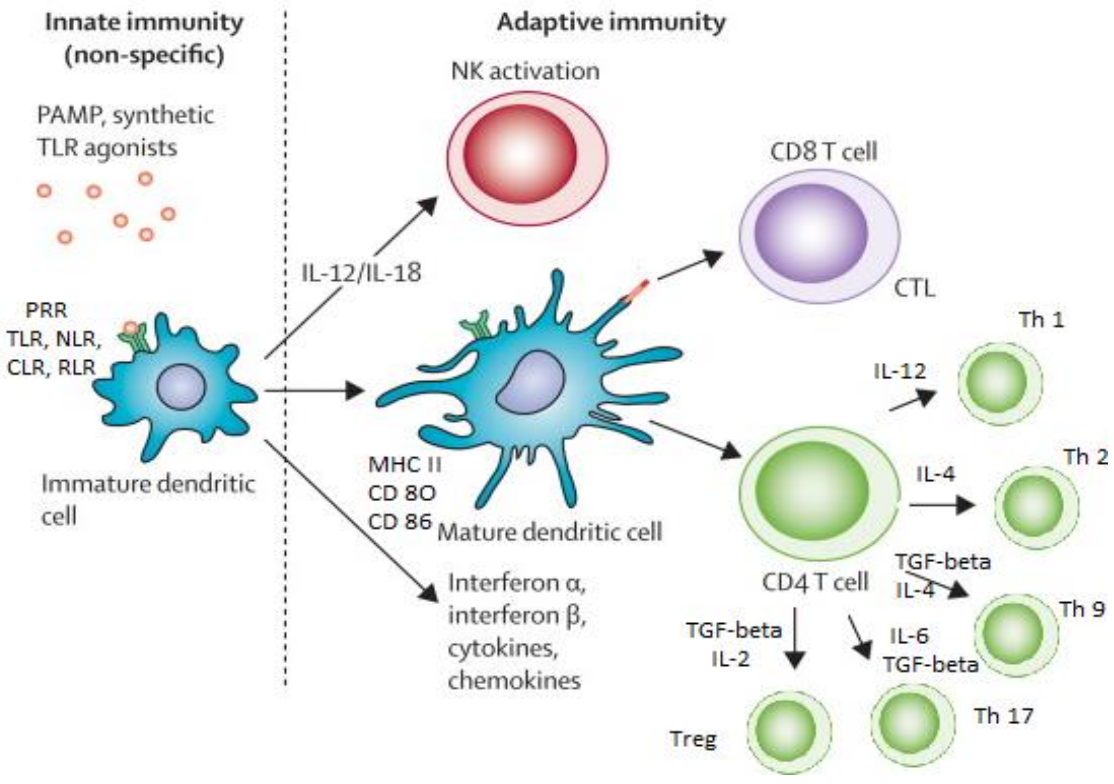


Figure 3: Overview of dendritic cells functions (Thomas and Hassan 2012 – adjusted)

2.3. CELL SIGNALING

Every time when vertebrate immune system recognizes invading pathogens it leads to the initiation of intracellular signaling pathways which results in the activation of transcription factors and consequently production of various components of immune system such as cytokines, chemokines, cell adhesion molecules or immune-receptors (Akira et al. 2006). The innate immune system recognizes microorganisms via a limited number of germ line-encoded pattern recognition receptors. This is in contrast to the large repertoire of rearranged receptors utilized by the acquired system.

2.3.1. CELL SIGNALING IN INNATE IMMUNITY

As already implied, the innate immunity response relies on recognition of evolutionary conserved structures on pathogens, so called pathogen associated molecular patterns through a limited number of germ line-encoded pattern-recognition receptors. PAMPs can be structures of bacterial, viral, fungal or parasitic origin, are invariant among whole classes of pathogens and are mostly essential for their survival (Mogensen 2009). Important feature of PAMPs is that they are distinguishable from self-antigens.

Upon recognition of PAMPs, pathogen recognition receptors activate intracellular signaling pathways, which include various adaptor molecules, kinases and transcription factors (Akira and Takeda 2004). Recruitment of adaptor molecules to given PRR is followed by activation of downstream signal transduction pathways which is driven by phosporylation, ubiquitination or protein-protein interaction and leads to activation of transcription factors that regulate the gene expression and synthesis of a broad range of molecules. These molecules then together orchestrate the early response to infection and represent an important connection to the adaptive immunity (Mogensen 2009).

Today we know several classes of pattern recognition receptors. The most common and best characterized family of PRRs are Toll-like receptors which I will mention further. Another membrane bound receptors which act as PRRs are C-type lectin receptors (CLRs). The most popular C-type lectin is DC-SIGN on dendritic cells. DC-SIGN is unique in that it regulates adhesion processes, such as DC trafficking and T-cell synapse formation, as well as antigen capture (van Vliet et al. 2008, Geitjenbeek et al. 2002). Besides these receptors there are also known two families of cytosolic receptors which are named retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Kanneganti et al. 2007, Yoneyama et al. 2004). These

receptors recognize intracellular cytosolic pathogens and their derivatives, such as viral ssRNA, dsRNA, and DNA, as well as components of internalized or intracellular bacteria. The two best characterized members of the NLR family are NOD1 and NOD2, which sense bacterial molecules derived from the synthesis or degradation of peptidoglycan (Kanneganti et al. 2007).

2.3.1.1. Toll-like receptors

The family of Toll-like receptors is the major and most extensively studied class of PRRs. TLRs obtained their name based on homology to the *Drosophila melanogaster* Toll protein, which plays a role in dorso-ventral patterning during embryogenesis as well as in the antifungal response in *Drosophila* (Lemaitre et al. 1996, Medzhitov et al. 1997).

Concerning structure, Toll-like receptors are type I transmembrane glycoproteins characterized by an extracellular-ligand binding domain which contains leucin-rich repeats and by a cytoplasmatic signaling Toll/interleukin-1 receptor homology (TIR) domain which is required for downstream signal transduction (O´Neill and Bowie 2007). They are expressed differentially among immune cells and appear to respond to different stimuli. Besides immune cells, TLRs expression was also observed in a variety of other cells, for example vascular endotelial cells, adipocytes or cardiac myocytes.

To date, 10 functional TLRs in humans and 12 functional TLRs in mice have been identified. Moreover TLR1-TLR9 are conserved between both species. Studies of mice deficient in each TLR have demonstrated that each TLR has a distinct function in terms of PAMP recognition and immune response. Particular TLRs can recognize a broad range of PAMPs including lipids, lipoproteins, proteins and nucleic acids derived from bacteria, viruses, parasites and fungi. The recognition of PAMPs by TLRs occurs in various cellular compartments, including the plasma membrane, endosomes, lysosomes and endolysosomes (Akira et al. 2006).

TLRs can be divided into two subgroups based on their cellular localization and respective PAMP ligands. One group is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, which are expressed on cell surfaces and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins. The other group is composed of TLR3, TLR7, TLR8, and TLR9 which are expressed exclusively in intracellular vesicles where they recognize microbial nucleic acids (Kawai and Akira 2010). Exceptions are TLR3 which can be localized both on the cell surface and in intracellular vesicles depending on the cell type and also TLR 1/2 and TLR4 (Vercammen et al.2008, Marre et al. 2010).

TLR2 plays the main role in recognition of bacteria. It forms heterodimers with TLR1 and TLR6 and recognizes diacyl- (TLR2/TLR6) or triacyl- (TLR2/TLR1) lipopeptides. TLR2 itself can recognize for example peptidoglykans or lipoteichoic acid from Grampozitive bacteria. TLR1 is involved in recognition of outer surface proteins from *Borrelia burgdorferi.* To date, there is a lot of evidence that TLR2 alone or in heterodimer with TLR1, in addition to being localized at the plasma membrane, can be found in the endozome where it triggers signals, different from those driven by plasma membrane TLR2/TLR1, leading to induction of inflammatory mediators (Marre et al. 2010). For example TLR2 mediates signaling from endosomal vesicles in response to *B. burgdorferi* and TLR2 synthetic ligands. Inhibition of endosomal acidification upon *B. burgdorferi* stimulation results in a decrease in type I IFN (interferon) and pro-inflammatory cytokine (f.e.IL-6) production (Marre et al. 2010, Cervantes et al. 2011). The molecule which was shown to mediate the recognition of *Bb* by TLR1/2 heterodimer within the endosome is integrin α3β1 (Marre et al. 2010). TLR2 within endosome cooperates with other endosomal TLRs to generate a *B. burgdorferi* specific inflammatory response. These are TLRs 7, 8, and 9. TLR7 is predominantly expressed in plasmacytoid DCs (pDCs) and identifies guanosin and uridin rich ss RNA viruses like HIV. TLR8, which was upon recently thought to be non-functional in mice, probably cooperates with TLR7, and TLR9 discriminates CpG motives of bacterial DNA (Akira et al. 2006, Takeda and Akira 2005, Cervantes et al. 2012). All these endozomal receptors were shown to be activated by *Borrelia* spirochetes which resulted in induction of pro-inflammatory cytokines and type I interferons (Shin et al. 2008, Petzke et al. 2009, Cervantes et al. 2011, 2013). Additional TLRs are TLR3, 4 and 5. TLR3 is responsible for the recognition of double stranded DNA (Alexopoulou et al. 2001). TLR4, which recognizes LPS from Gramnegative bacteria, has been also shown to signal from two different cellular locations as TLR 2. In the model of TLR4 signaling, signaling pathways from the cell surface vs. the endosome are clearly distinguished. TLR4 mediates signaling from the plasma membrane via TIRAP/MyD88 for MAP kinase and NF-kB activation resulting in pro-inflammatory cytokine activation. From the endosome TLR4 utilizes TRAM and TRIF, which results in the induction of type I IFNs, although TRAM/TRIF also mediate a delayed wave of NF-kB and pro-inflammatory cytokine activation (Kawai and Akira 2011). Toll-like receptor 5 reacts with bacterial flagellin. It is worthy of notice, that even though *Borrelia* spirochetes possess flagellin, this is not recognized by TLR5, since flagellas are localized under the outer membrane, where they are inaccessible for immune system.

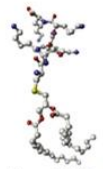
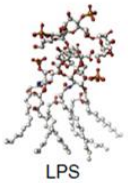
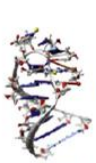
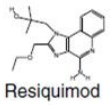
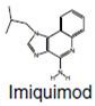
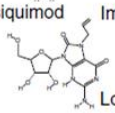
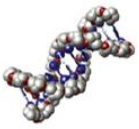
Receptor	TLR2/1 or 2/6	TLR3	TLR4	TLR5	TLR7	TLR9
Ligands	Lipopeptides	Poly I:C, dsRNA	LPS	Flagellin	ssRNA, resiquimod, imiquimod, loxoribine	Unmethylated DNA, CpG-DNA
Source	Gram-positive bacteria, fungi	Viruses	Gram-negative bacteria	Bacterial flagellum	Viruses	Bacteria
Examples	 Pam_2CSK_4	 dsRNA	 LPS	 Flagellin	 ssRNA  Resiquimod  Imiquimod  Loxoribine	 CpG-DNA

Figure 4: Toll-like receptor ligands (Moresco et al. 2011)

As already indicated, particular TLRs can activate specific immune responses. For example, TLR3 and TLR4 generate both type I interferon and inflammatory cytokine responses, whereas TLR1/TLR2 or TLR2/TLR6 heterodimers and TLR5 induce mainly inflammatory cytokines production. These differences can be explained by the existence of TIR domain–containing adaptor molecules, including MyD88, TIRAP, TRIF and TRAM, which are recruited by distinct TLRs and activate distinct signaling pathways (Akira and Takeda 2004, Fitzgerald et al. 2001, 2003, Horng et al. 2002). The first identified member of TIR family was MyD88 which is universally used by all TLRs except TLR3. MyD88 activates the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) (p38, Erk ½, Jnk) to induce inflammatory cytokines. MAP kinases trigger the cytokine production through the activation of AP-1 (Moresco et al. 2011). On the other hand another adaptor molecule, TRIF, which is used mainly by TLR3 and TLR4, induces alternative pathways that lead to activation of the transcription factors IRF3 and NF-κB and the consequent induction of type I interferon and inflammatory cytokines. TRAM and TIRAP function as sorting adaptors that recruit TRIF to TLR4 and MyD88 to TLR2 and TLR4, respectively. Taken together, TLR signaling pathways can be mainly divided into either MyD88-dependent pathways, which drive the induction of inflammatory cytokines, or TRIF-dependent pathways, which are responsible for the induction of type I interferon as well as inflammatory cytokines. But apparently there is a big liberty in using these adaptor molecules by particular Toll-like receptors. For example MyD88-dependent pathway which is triggered by TLR7 and 9 can induce type I IFN production in pDCs and endozomal TLR2 can signal through TRIF adaptor molecule (Mogensen et al. 2009, Kawai and Akira 2010, Petnicki-Ocwieja 2013).

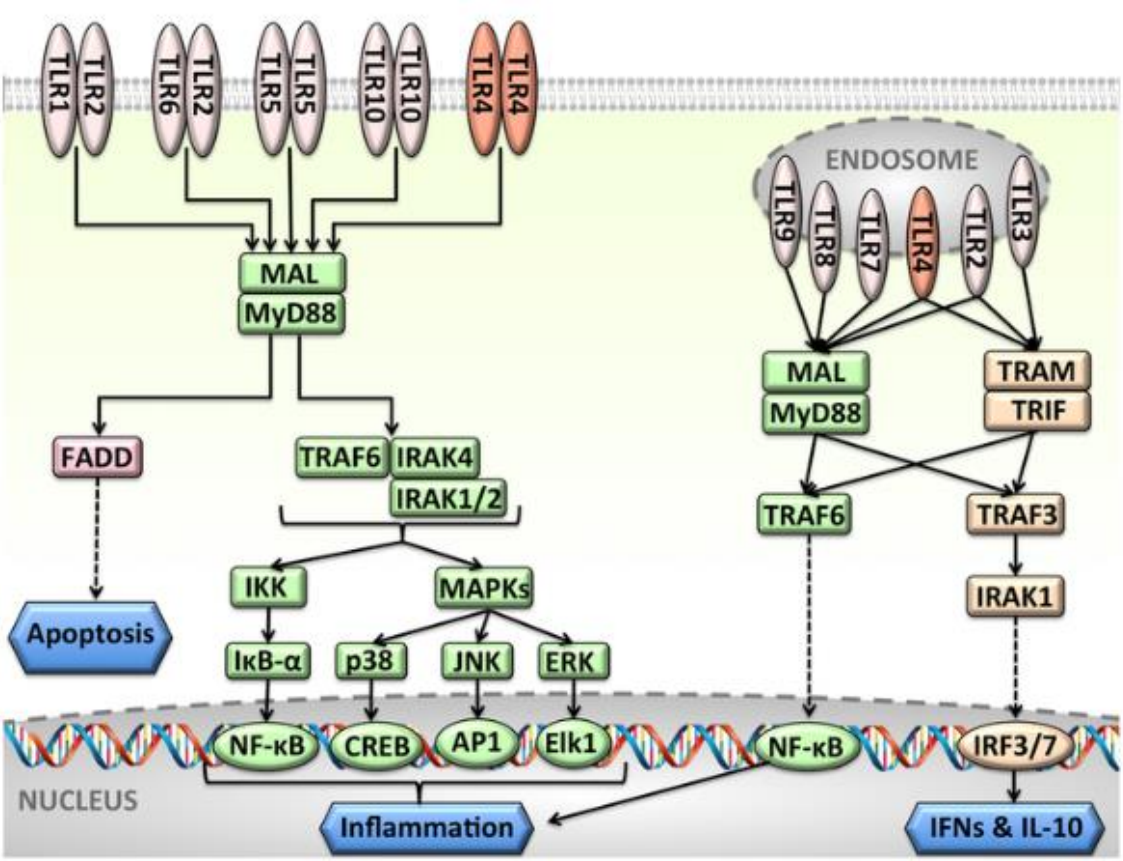


Figure 5: Scheme of complex TLR signaling (Guven-Maiorov et al. 2015)

1.1. TICK - HOST INTERACTIONS

Tick feeding is a complex process that can, by Ixodidae ticks, exceeds up to ten days. During this period, ticks have to break up the epidermis layer and establish so called feeding pool, a wound which is full of blood. In the whole course of blood meal ticks release from their salivary glands a broad range of molecules, which allow them to stay firmly attached in blood feeding site, facilitate the feeding itself and protect them from host immune system. In artificial hosts the strong attack on host defence system results in strong haemostatic and inflammatory immune response, which leads to the deterioration of tick feeding and subsequently to the falling off. Feeding ticks can provoke both innate and adaptive immunity (Bergman 1996).

As previously mentioned, the first contact with feeding tick mouthparts and saliva is initiated by cells permanently present in dermis and epidermis, eg. Langerhans cells, mast cells, and macrophages resulting in release of pre-fabricated mediators mounting the immune reaction and attracting other immune cells to the tick feeding site (Francischetti et al. 2009). In the feeding site originate thus local inflammatory response which is in naive host driven mainly by neutrophils (Brossard and Wikel 2004, Kovář 2004). They present the first line of

innate immune system defence. Except for invading pathogens killing and inflammatory cytokines production, neutrophils can also produce many chemoattractants playing a role in influx of other immune cells during the early phase of infection (Francischetti et al. 2009).

Adaptive immune response is mediated by contact of dermal Langerhans cells with tick immunogens. After pathogen recognition Langerhans cells migrate into the lymph nodes where they transform into mature dendritic cells. These cells function then as antigen presenting cells (APC) for T-lymphocytes, they stimulate T-ly activation and proliferation (Brossard and Wikel 2004).

After repeated tick feeding on the host the ratio of infiltrating cells in the early phase of infection changes and the basophils and eosinophils begin to predominate. These cells cause the so called cutaneous basophil hypersenstivity, the type of delayed hypersensitivity which is mediated by Th1 lymphocytes (Allen 1973, Brossard a Wikel 2004). This immune reaction leads then to the damage of tick and can be manifested by decreased weight of engorged tick or by the tick death (Ribeiro et al. 1995).

However, as was already mentioned, tick have evolved bunch of effective molecules, influencing the host immune system and enabling ticks to feed successfully. These molecules developed during the host-parasite co-evolution and possess mainly antihaemostatic, vasodilatatory, antiinflamatory and immunomodulatory properties (Wikel and Bergman 1997).

The effect of tick salivary molecules on host defence system is very comprehensive and in many cases also redundant, which reflects the complexity and redundancy of host defence responses. In recent years big effort was made to identify the bioactive molecules present in tick saliva. Thanks to that, many of the tick salivary compounds have been characterized and their functions identified, but still the function of most salivary molecules remains unknown (Francischetti et al. 2009).

2.4.1. THE EFFECT OF TICK SALIVA ON HOST DEFENCE SYSTEM

2.4.1.1. Molecules with antihaemostatic properties

When tick feeds, the penetrating mouthpart causes the damage of vascular endotelium and blood freely flows out of the wound where can be sucked be the tick. Under regular conditions, the bleeding would be stopped by host haemostatic process. Haemostasis include series of physiological events leading to termination of bleeding - vasoconstriction, platelet aggregation, formation of fibrin clot and subsequently fibrinolysis, when the fibrin clot is no

longer needed. Nevertheless, this process would negatively influence the tick ability to suck blood and therefore tick saliva contains molecules influencing hosts clotting cascade (Francischetti et al. 2009).

These molecules can act on each step of haemostasis. So we can find in saliva of different tick species inhibitors of vasodilatation, such as prostacyklins and prostaglandins (Bowman et al. 1995, Ribeiro et al. 1988). Platelet aggregation, which represents the initial and most immediate stage of haemostasis, can be also directly or indirectly inhibited by compounds contained in tick saliva. Following vascular injury, platelets adhere to the subendothelial tissue and become activated by various agonists such as collagen, thrombin, adenosine diphosphate, platelet-activating factor, or tromboxane A2. For example enzyme apyrase inhibits platelet aggregation by hydrolysis of adenosine diphospate and adenosine triphosphate on adenosine monophosphate (Ribeiro et al. 1991). Other platelet inhibitors are for example integrin antagonists Variabilin from *Dermacentor variabilis* (Wang et al. 1996) and Ixodegrin from *Ixodes* ticks (Francischetti et al. 2005). Further proteins such as Moubatin from *Ornithodoros moubata* (Waxman and Conolly 1993, Keller et al. 1993) or prostacyclins and disintegrins prevent binding of other ligands to the platelet receptors (Ribeiro et al. 1988). Another protein which inhibits chymase-G and thrombin induced platelet aggregation is serpin from *Ixodes ricinus* IRS-2 (Chmelař et al. 2011).

Salivary compounds can also affect coagulation. Tick salivary anticoagulants can be divided into four groups depending on the target and mechanism of action. These groups represent thrombin inhibitors, inhibitors of activated factor X, inhibitors of the extrinsic tenase complex, and contact phase protein inhibitors (Koh and Kini 2009). First group, thrombin inhibitors, comprises mainly the Kunitz-type proteinase inhibitors, for example Savignin (Nienaber et al. 1999) or the serpin from *Ixodes ricinus* Iris (Prevot et al. 2006). From Factor Xa inhibitors the tick anticoagulant peptide (TAP) from *Ornithodoros moubata*, Salp 14 from *I. scapularis* or the above mentioned Iris which also partly inhibits FXa driven coagulation can be mentioned (Waxman et al. 1990, Narashiman et al. 2002, Prevot et al. 2006). Ixolaris and Pentalaris from tick *Ixodes scapularis*, inhibiting coagulation induced by TF/FVIIa, belong into the third group (Francischetti et al. 2002, Francischetti et al. 2004). As a most representative member of the last group I would mention Ir-CPI protein from *I. ricinus* which is a Kunitz domain-containing inhibitor of FXIIa, FXIa and kallikrein (Decrem et al. 2009).

On this place it is of note, that many components of hemostasis play role in both the blood coagulation and inflammation. Activated platelets additionally secrete a number of pro-inflammatory molecules and may thus contribute to local inflammatory processes. Among these pro-inflammatory molecules are chemokines such as TGF-β, which display pro-apoptotic properties, and IL-1β, which activates white blood cells, induces tissue factor expression, and promotes adherence of neutrophils and monocytes (Francischetti et al. 2009). On the surface of different cell types can be found protease-activated receptors. These receptors are activated by the serine proteases thrombin, factor VIIa or Xa and their activation triggers the production of pro-inflammatory cytokines, the expression of adhesion molecules and the activation of inflammatory cells. Therefore it is not surprising that tick saliva contains proteins, such as serpins, which are able to affect both haemostasis and immunity. The first tick serpin which was reported to interfere with both the hosts haemostasis and immune response is Iris from *Ixodes ricinus*. This protein was shown not only to possess anti-haemostatic, anti-fibrinolytic and anticoagulant activity but also to suppress T cell proliferation, induce a Th2 type immune response and inhibit the production of pro-inflammatory cytokines IL-6 and TNF-α (tumor necrosis factor alpha) (Prevot et al. 2006). The second serpin with this dual effect is IRS-2. Recently described immunomodulatory effect of this protein on dendritic cells forms the basis of this thesis and is described in Chapter 5.1. (Páleníková et al. 2015).

2.4.1.2. Molecules with immunomodulatory properties

Molecules contained in tick saliva have, apart from haemostasis, strong effect on most of the cellular and noncellular components of the immune system. This enables ticks to successfully feed for the period of several days without being injured by the host defence.

Important role in inhibiting the host defensive reactions play the inhibitors of communication molecules. The ability of immune system to recognize foreign antigens and response to them depends just on the production and release of mediators which consequently activate other parts of immune system. Chemokines which control cell migration belong to these molecules. Chemokine binding proteins, which are called evasins, were discovered in *Rhipicephalus sanguineus* saliva. These proteins bind for instance CCL3 (MIP-1α-macrophage inflammatory protein 1), CCL4, CCL5 (RANTES), CCL11 (eotaxin) and CXCL8 (IL-8) (Déruaz et al. 2008). Marchal and colleagues also confirmed inhibitory activity of saliva on IL-8 and MCP-1 (monocyte chemoattractant protein 1). Moreover,

another inhibitory activity of tick saliva on alarmins is mentioned in this work. Alarmins are mediators released by keratinocytes and their main role is to attract and activate antigen presenting cells, chiefly dendritic cells (Marchal et al. 2011). Oliveira and colleagues also showed that *R. sanguineus* tick saliva selectively inhibits chemotaxis of immature DCs by down-regulating CCR5, and reduces the chemotactic function of MIP-1α. In addition, DCs cultured with tick saliva were revealed to be poor stimulators of cytokine production by antigen-specific T cells (Oliveira et al. 2008). Recently, the inhibition of MCP-1, TCA-3 (thymus-derived chemotactic agent 3) and MIP-2 by *Ixodes ricinus* saliva was also described (Langhansová et al. 2015). It is also well known that the whole saliva, salivary gland extract as well as particular salivary molecules can modulate cytokine production in many cell types, preventing thus their communication and further driving of immune response. Generally tick saliva polarizes the immune response towards Th2 phenotype (Cavassani et al. 2005, Ferreira and Silva 1999, Gwakisa et al. 2001, Konik et al. 2006, Páleníková et al. 2015, Ramachandra et al. 1992, Sá-Nunes et al. 2007, Skallová et al. 2008, Zeidner et al. 1997).

Other inhibited mediators are histamin and serotonin. These molecules are released during the degranulation of mast cells and basophils and participate in inflammatory response. In *Rhipicephalus appendiculatus* saliva lipocalins binding only histamine can be found, while in *Demacentor reticulatus* saliva is a homologous protein which binds both histamine and serotonine (Paesen et al. 1999). On the other hand, histamine releasing factor tHRF (tick histamine release factor), which binds to basophiles and stimulates histamine release, occurs in the saliva of *Ixodes scapularis*. This results in higher vessels permeability and higher blood influx into the wound, thus facilitating tick feeding (Dai et al. 2010).

The complement system also possesses an important role in defence against pathogens and is a key player in tick rejection reactions of the host. Several complement inhibitors from saliva of different tick species have been described. Among others Isac and its *I. ricinus* homolog Salp20 (Valenzuela et al. 2000, Tyson et al. 2007) and the two paralogous proteins IRAC I and II can be mentioned (Schroeder et al. 2007). These proteins inhibit formation of C3 convertase of the alternative pathway by blocking binding of factor B to C3b complement component (Daix et al. 2007, Courveur 2008). OmC1, an *Ornithodoros moubata* salivary protein belonging to lipocalins family, binds the C5 component of complement and prevents its cleavage by the C5 convertase (Nunn et al. 2005).

Many of tick salivary molecules influence directly function of immune cells. They can specifically influence function of only one cell type, but more often they directly or indirectly influence more cell subtypes.

First and the most abundant cells present at the site of inflammation, induced upon primary tick infestation, are neutrophils. They engulf and degrade invading pathogens via phagocytosis and subsequent release of lytic enzymes from their granules or by the respiratory burst. They produce many mediators such as prostaglandins, chemokines or major pro-inflammatory cytokines (Mantovani et al. 2011, Scapini et al. 2000). Although neutrophils represent very important part of immune system and play an irreplaceable role in acute inflammation, there are just few publications concerning the effect of tick saliva on neutrophils.

One of the first proofs, that tick saliva can influence neutrophils functions was brought by Ribeiro and colleagues, who showed that saliva from *I. dammini* (*I. scapularis*), can inhibit main neutrophil killing functions. The inhibitory effect ranged between 40 and 80 % (Ribeiro et al. 1990). It was also established that saliva of many hard tick species possess anti-CXCL8 activity, thus preventing neutrophil migration into the site of inflammation and inhibiting indirectly role of neutrophils in inflammation (Hajnická et al. 2001, 2005, Vancová et al. 2010). Other proteins preventing neutrophil migration are evasins, which were mentioned before. Evasin-3 is probably the most potent tick-produced inhibitor of neutrophil recruitment (Déruaz et al. 2008). The saliva of *Ixodes scapularis* also reduces polymorphonuclear leukocyte adhesion via down regulation of β2-integrins and decreases the efficiency of neutrophils in the uptake and killing of *Borrelia burgdorferi* spirochetes (Montgomery et al. 2004).

Two proteins ISL 929 and ISL 1373 from *I. scapularis* were shown to suppress superoxide radical production by neutrophils and thus inhibit their functions (Guo et al. 2009). Another protein, already mentioned, which influences neutrophils is IRS-2. This protein belongs to serine proteases inhibitors. The target enzymes of this protein are Cathepsin G and chymase. Both these enzymes are a part of acute inflammatory response and are produced by activated neutrophils and mast cells. Moreover, IRS-2 is able to inhibit swelling and migration of neutrophils into the inflamed tissue (Chmelař et al. 2011) This observation can be, at least partially, explained by the newly described inhibitory effect of IRS-2 on Th17 differentiation, since Th17 cells are known to play role in neutrophil recruitment (Pelletier et al. 2010, Páleníková et al. 2015). On the other hand saliva from *Ixodes ricinus* tick does not affect neutrophil extracellular traps (NET) formation or their stability (Menten-Dedoyart et al. 2012).

Dendritic cells belong among antigen presenting cells and thus play a key role in the development of immune reaction against invading pathogens. With their ability to recognize,

process and present antigens on their surface and thus activate T lymphocytes, DCs form a unique link between innate and acquired immunity and thus represent a great target for immunomodulation (Banchereau and Steinman 1998, Granucci et al. 2005).

Exposure of dendritic cells to *Ixodes ricinus* saliva *in vitro* leads to the inhibition of their maturation after stimulation with CD40 or TLR9, 3 a 7 ligands and to the decrease of antigen presenting ability. *In vivo* it comes to the inhibition of maturation and early migration of dendritic cells into draining lymph nodes and to the decreased ability of mature dendritic cells to present antigen to specific T-lymphocytes. Moreover, dendritic cells treated with tick saliva are not able to induce Th1 and Th17 immune response successfully and are skewing the immune reaction towards Th2. The same Th2 shift of immune reaction was described also by Mejri and Brossard (Skallová et al. 2008, Mejri and Brossard 2007).

Tick saliva induces also the development of regulatory dendritic cells which are characterized by increased production of IL-10 and decreased amount of IL-12 and TNF-α after TLR ligands stimulation. These regulatory dendritic cells express in greater extent TLR2 and inhibit ERK and p38 kinases which support the IL-10 production and thus the modulation of immune response (Oliveira et al. 2010). Cystatin OmC2 from the soft tick *Ornithodoros moubata*, can also suppress the host adaptive immune response by reducing TNF-α and IL-12 production by LPS activated DCs and by suppressing the proliferation of antigen-specific CD4+ T cells (Salát et al. 2010).

On the other hand Slámová and all observed both the decrease of TNF-α and IL-6 production in the presence of *Ixodes ricinus* saliva after DCs stimulation with *Borrelia*, which means Th1 cytokines, and the decrease of IL-10 production by DCs, which means Th2 cytokines. In addition the exposure of *Borrelia afzelii* activated dendritic cells to *I. ricinus* saliva lead to the decrease of the percentage of phagocytic dendritic cells. It also reduced the ability of dendritic cells to activate B*orrelia* specific T-lymphocytes (Slámová et al. 2011).

Another protein affecting DCs functions by altering their maturation towards the tolerogenic phenotype is Japanin. This lipocalin from *Rhipicephalus appendiculatus* specifically reprograms response of DCs to a wide variety of stimuli *in vitro,* altering their expression of co-stimulatory and co-inhibitory trans-membrane molecules and secretion of pro-inflammatory, anti-inflammatory and T cell polarizing cytokines (blocks LPS-induced secretion of Th17 and Th1 promoting cytokines), it also inhibits the differentiation of DCs from monocytes (Preston et al. 2013).

Inhibition of dendritic cell maturation and differentiation from bone marrow was described also after exposure of GM-CSF (granulocyte-macrophage colony-stimulating

factor) and IL-4 or LPS activated cells to the *Rhipicephalus sanguineus* saliva (Cavassani et al. 2005). Saliva of *I. scapularis* and *R. sanguineus* ticks contains non-protein molecules prostaglandin E2 (PGE2) and purin nucleosid adenosin (ADO), which seems to be responsible for most of the tick saliva inhibitory activity. Both molecules inhibit the IL-12p40 and TNF-α production by the inhibition of cAMP-PKA signaling pathway and increase the IL-10 production after cells stimulation with TLR ligands. Besides, they inhibit CD40 expression by mature dendritic cells and PGE2 also suppresses dendritic cells differentiation from the blood precursors (Sá-Nunes et al. 2007, Oliveira et al. 2011).

Dendritic cells functions can be influenced also by protein Salp15 from *I. scapularis*. This protein interacts with DC-SIGN lectin, which results in activation of Raf1/MEK cascade and subsequently in the reduction of IL-6 and TNF-α mRNA stability. It also leads to the deterioration of nucleosome remodelling on the IL-12p35 promoter. This cytokine inhibition can be observed after both the activation of cells with LPS and LTA (lipoteichoic acid) and after the activation with *Borrelia* spirochetes (Hovius et al. 2008). Additionally, Salp15 inhibits keratinocyte inflammation induced by *Borrelia in vitro* via the downregulation of alarmins, essential mediators that mobilize and activate antigen presenting cells (Marchal et al. 2011).

Another *I. scapularis* protein which interacts with dendritic cells is sialostatin L. Treatment of LPS activated dendritic cells with this protein leads to the decrease in IL-12p70 and TNF-α production and to the reduction of DCs maturation by decreasing co-stimulatory molecules CD80 and CD86 expression. Moreover, sialostatin L binds catepsin S inside dendritic cells and influences MHCII invariant chain processing. This results in worsened DCs maturation and thus in inhibition of antigen specific T-lymphocytes proliferation caused by mechanism dependent on cathepsin S (Sá-Nunes et al. 2009).

Speaking about sialostatin L, this protein was recently shown to affect other innate immune cells – mast cells. The role of mast cells in immunity against ticks is still unclear, but the histamine released during mast cells degranulation can cause pruritus and lead to the hosts itching resulting even in the dislodging of the tick from the feeding site. Sialostatin L restrains IL-9 production by mast cells through the down-regulation of IL-1β and IRF4 (interferon regulatory factor) which binds to IL-9 and also IL-1 promoter. The production of IL-6 and degranulation of mast cells were not affected. Moreover, mast cell-specific deficiency in IRF4 or administration of sialostatin L results in a strong reduction in asthma symptoms, which is in agreement with previous results obtained with this protein which are discussed later (Klein et al. 2015, Horká et al. 2011).

Macrophages are the other innate immune cells present in tick bite site. These resident cells produce cytokines and chemokines that attract inflammatory cells to the tick-bite site. They play role mainly in secondary tick infestations. Activated macrophages turn into potent killers able to engulf pathogens by phagocytosis and kill them by oxygen- and nitrogen-release (Rittig et al. 1992, Modolell et al. 1994). There are many reports about the impact of tick saliva or salivary gland extract (SGE) on macrophage functions. It was reported that salivary gland extract from *I. ricinus* impaired the production of superoxide and nitric oxide by macrophages (Kuthejlová et al. 2001). Interestingly this decrease of nitrid oxide production by macrophages was not observed in macrophages treated with tick saliva where the opposite effect, increase of production, could be seen (Kýčková and Kopecký 2006). *I. ricinus* SGE also decreases the number of activated macrophages capable to phagocytize *Borrelia afzelii* and the number of engulfed bacteria. Both SGE and saliva from *I. ricinus* also repress the production of pro-inflammatory cytokine TNF-α (Kýčková and Kopecký 2006). This effect could be caused by the presence of serpin Iris in *Ixodes ricinus* saliva, which is known to affect TNF-α secretion by the direct binding to monocytes or marcophages. This inhibition is not related to the anti-protease activity of this protein (Prevot et al. 2009). The non-proteinaceous molecule of tick saliva, PGE-2, was also shown to decrease the production of TNF-α and TNF-α receptor in macrophage cell line, moreover, it also regulates macrophages migration (Poole et al. 2013). Salivary gland extract from *Rhipicephalus microplus* induces different expression of co-stimulatory molecule CD86 in dose dependent manner; this probably results in the support of Th2 polarization of immune response (Brake et al. 2010).

Another way how to affect macrophage functions is represented by tick macrophage migration inhibitor factor (MIF) which had been identified in the salivary glands of *Amblyoma americanum*. This peptide inhibits the migration of macrophages and protects the tick from macrophage attack (Jaworski et al. 2001). Recently, the inhibitory impact of *Dermacentor variabilis* saliva on the phagocytosis of zymozan beads by IC-21 macrophage cell line was also described. Moreover, incubation of these cells together with saliva boosts their migration and increases the secretion of Th2 cytokine IL-10. Also the gene expression of some molecules is influenced in the presence of *D. variabilis* saliva (Kramer et al. 2011).

Furthermore, the ability of *I. scapularis* cystatin sialostatin L2 to inhibit inflamasome formation in macrophages during *Anaplasma phagocytophilum* infection was described two years ago (Chen et al. 2014). Recently, it was proven that this effect of sialostatin L2 is caused by the inhibition of NLRC4 inflammasome activity which occurs because sialostatin

caused by the inhibition of NLRC4 inflammasome activity which occurs because sialostatin L2 tethers to the phospholipid binding protein Annexin A2, hence blocking NLRC4 inflammasome oligomerization, caspase-1 activation, and IL-1β and IL-18 secretion (Wang et al. 2016).

Proteins contained in tick saliva influence also NK cell activation (Kopecký and Kuthejlová 1998, Kubeš et al. 1994).

Concerning acquired immunity, tick salivary compounds can also affect B and T cell mediated immune response. Best explored immunomodulatory protein from *I. scapularis*, Salp15, influences activation and proliferation of CD4+ T-lymphocytes. Effects of Salp15 are based on his ability to bind to CD4 co-receptor on T cells resulting in inhibition of signal leading through T-cell receptor and subsequently decrease in IL-2 production, which stimulates T cell proliferation. Homologous protein influencing CD4+ T lymphocyte proliferation was found also in *Ixodes ricinus* (Anguita et al. 2002, Garg et al. 2006). Another protein from tick saliva suppressing T- lymphocyte function is Iris from *I. ricinus* saliva, which inhibits T cells proliferation and suppresses production of IFN-γ, IL-6 and TNF-α production by T cells and macrophages (Leboulle et al. 2002). Saliva of feeding *D. andersoni* includes 36 kDa protein suppressing T cell proliferation (Bergman et al., 2000). Moreover, an IL-2 binding protein was described in *I. scapularis* saliva, which decreases the availability of this cytokine and affects T-cell proliferation and activity of immune effector cells responsive to IL-2 stimulation (Gillespie et al., 2001).

Lately, an inhibitory effect of sialostatin L from *I. scapularis* on the production of interleukin 9 by Th9 lymphocytes was described. This well-marked effect led to suppression of mouse asthma symptoms. Sialostatin L has also anti inflammatory properties and is an inhibitor of cysteine proteases, mainly cathepsin L and C. Inhibition of serine proteases which are contained in granules of cytotoxic lymphocytes (CTL), NK cells, mast cells and neutrophils was also documented. Sialostatin L also inhibits proliferation of CTLs, neutrophils migration and influences dendritic cell function (Horká et al. 2011, Kotsyfakis et al. 2006, Sá-Nunes et al. 2009).

As it was already implied the abrogation of T cells proliferation and function can also be the consequence of impaired dendritic cell function and thus their inability to polarize the immune response correctly.

Among molecules influencing cells of acquired immunity B-cell inhibitory protein from *I. ricinus* should be mentioned. This protein decreases B-lymphocyte activation during

the initial phase of infection (Hannier et al. 2004). Humoral immunity can be influenced also be the Ig-binding proteins which were found in *Rhipicephalus appendiculatus* saliva. These proteins bind ingested hosts IgG and excrete them by salivation (Wang and Nuttal 1995).

There are only few publications concerning the effect of tick saliva on cell signaling. The most comprehensive information exists about the effect of *Ixodes scapularis* salivary protein Salp15. Besides other properties, Salp15 inhibits the activation of T cells through its interaction with the co-receptor CD4, thus preventing activation of protein tyrosine kinase Lck upon TCR engagement and the formation of lipid rafts. Salp15 affects also Toll-like receptor-induced activation of dendritic cells through interaction with C-type lectin molecule DC-SIGN. Ligation of Salp15 with DC-SIGN leads to activation of the kinases Raf-1 and mitogen-activated protein kinase (MEK) and results in decreased IL-6 and TNF-α mRNA stability and impaired nucleosome remodelling at the IL-12p35 promoter (Hovius et al. 2008). Recently, it was also published, that the observed inhibition of pro-inflammatory cytokine production by dendritic cells is caused by induction of cAMP-PKA signaling by two salivary non-protein molecules ADO and PGE2 (Oliveira et al. 2011). Moreover, saliva from *Riphicephalus sanguineus* suppresses the phosphorylation of p38 and Erk 1/2 kinases in bone marrow derived dendritic cells and increases the expression of TLR2 on their surface (Oliveira et al. 2010). Lieskovská and Kopecký also published that saliva from *Ixodes ricinus* suppresses activation of STAT-1 (signal transducer and activator of transcription) signaling molecule in splenic dendritic cells and thus affects IFN signaling. Additionally, *I. ricinus* saliva inhibits the activation of NF-κB p65, Erk 1/2 and PI3K/Akt pathways in splenic dendritic cells stimulated with TLR-2 ligand and *Borrelia afzelii* which is accompanied by decrease of TNF-α production. Moreover, tick saliva up-regulated the amount of secreted IL-10 and this effect could be completely blocked by the presence of PKA inhibitor H-89, suggesting that the enhancement of IL-10 is dependent on PKA pathway (Lieskovská and Kopecký 2012 a, b).

Recently we also showed, that sialostatin L2 interferes with IFN-triggered signal transduction by decreasing phosphorylation of STAT1/2 proteins, negatively affects IFN-β mediated induction of IFN-sensitive genes in LPS stimulated cells, and promotes TBE virus replication in dendritic cells *in vitro*. Moreover, we proved that sialostatin L2 inhibits the production of chemokines MIP-1α and IP-10 in response to *Borrelia* spirochetes and attenuates the activation of Erk1/2, PI3K/Akt, and NF-κB pathways in response to TLR-2 ligation. On the other hand the related cystatin, sialostatin L, suppresses the production of

IFN-β and attenuates the maturation and differentiation of plasmacytoid DC. Details are described in Chapters 5.2. and 5.3. (Lieskovská et al. 2015 a, b).

2.5. SALIVA ASSISTED TRANSMISSION

Today it is well known that tick saliva molecules can facilitate transmission, survival and reproduction of *Borrelia* and other pathogens in the host (Nuttall et al. 1994, Macháčková et al. 2006). This phenomenon when the vector-borne pathogen transmission and activity is facilitated and promoted by saliva of blood-feeding vectors was termed SAT or saliva-activated transmission of pathogens, this term was lately revised to saliva assisted transmission (Jones et al. 1987, Nuttal and Labuda 2008). SAT phenomenon was experimentally described as a facilitated transmission of pathogens when uninfected ticks fed together (co-feeding) with the infected one. For this way of pathogens transmission, the high level of host viremia or bacteraemia was not required (Jones et al. 1987, Jones et al. 1989, Labuda et al. 1993a, 1993b, Ogden et al. 1997). This non-viraemic transmission of pathogens from infected to non-infected ticks co-feeding on the same host is considered to be an indirect evidence of the SAT. Increased infection of ticks feeding on animals experimentally inoculated with both pathogens and tick saliva or SGE, compared with ticks feeding on hosts inoculated with pathogen alone, represents direct evidences of SAT (Labuda et al. 1993b, Nuttall and Labuda 2008). This direct evidence was also reproduced experimentally when the pathogens infectivity for the host was increased by syringe inoculation of the pathogen in the combination with SGE or saliva (Macháčková et al. 2006).

SAT phenomenon was for the first time described by Thogotho virus whose vector is *Rhipicephalus appendiculatus* (Jones et al. 1987, Jones et al. 1989). Today SAT was directly and indirectly demonstrated for several other viral or bacterial pathogens and their vectors. For example for vector-borne viruses, such as tick-borne encephalitis virus (Labuda et al. 1993a, Labuda et al. 1993b), Crimean-Congo hemorrhagic fever virus (Gordon et al. 1983), Bhanja virus, Palma virus (Labuda et al. 1997), West Nile virus (Lawrie et al. 2004) or Powassan virus (Hermance and Thangamani 2015). Within the *Bbsl* complex SAT was noticed in several *Borrelia* species (Jones et al. 1987, Labuda et al. 1993a, b, Kročová et al. 2003, Gern and Rais 1996, Sato and Nakao 1997, Zeidner et al. 2002, Pechová et al. 2002, Macháčková et al. 2006, Horká 2009).

Despite the intensive research in this area, so far only few salivary proteins have been directly described as SAT factors. The first molecularly characterized SAT factor was protein

Salp 15 from *I. scapularis* and later his homologue from *I. ricinus* (Anguita et al. 2002, Ramamoorthi et al. 2005). Production of Salp15 is selectively enhanced in salivary glands infected with *Borrelia* spirochetes. Salp15 binds to the spirochetal outer surface protein C thus protecting spirochetes from antibody mediated killing and enhancing their proliferation in target organs. Moreover, the number of spirochetes in host skin, joints and bladder is increased in the presence of Salp15. RNA interference-mediated repression of Salp15 in *I. scapularis* significantly reduced the capacity of tick-borne spirochetes to infect mice (Ramamoorthi et al. 2005). Dai and colleagues also showed that antibodies against Salp15 significantly protected the mice against infection with tick-transmitted *Borrelia burgdorferi* (Dai et al. 2009). The Salp15 and its homolog from *I. ricinus* protect a serum-sensitive isolate of *B. burgdorferi* against complement-mediated killing (Schuijt et al. 2008).

Another salivary protein which appears to assist *Borrelia* spirochete transmission is sialostatin L2. This cysteine protease inhibitor from *I. scapularis* salivary glands dramatically affects tick feeding success, most likely by influencing the activity of cathepsins involved in host inflammation, tissue remodelling and angiogenesis. Co-administration of *Borrelia* spirochetes with sialostatin L2 exacerbates skin infections in a murine model. Unlike Salp 15, sialostatin L2 does not influence growth of *Borrelia* spirochetes *in vitro* and the stimulatory effect, the mechanism of which remains still unclear, seems not to be due to direct interaction of the protein with spirochetes (Kotsyfakis et al. 2007, 2010).

2.6. CYSTEINE AND SERINE PROTEASES AND THEIR INHIBITORS

Proteases play an important role in many physiological processes; their role is not restricted only to digestive purposes and remodelling of extracellular matrix and tissues but they are also key factors in the initiation of physiological immune responses. This initiation can be either direct, through the degradation of pathogens within phagolysosomes, or indirect, through the activation of pattern recognition receptors (Manoury et al. 2011).

Cysteine proteases, also known as thiol proteases - for the cysteine thiol as a part of their catalytic site, take place in antigen presentation and in the induction of immunity. Cathepsins also belong among these papain-like proteases. To date cathepsins B, C, F, H, K, L, S, V, and W have been described. They are constitutively expressed in most cell types including dendritic cells. Like many other enzymes, cysteine proteases require acidic pH for their proper function. Thus the activity of these proteins is localized in the lysosomal compartments. Here the cysteine proteases participate on the protease-mediated degradation

of antigen for the presentation with MHC II, and they are involved in processing of invariant chain, a polypeptide blocking peptide-binding cleft of MHC II. This Ii proteolysis is driven mainly by cathepsins L and S (Manoury et al. 2011, Chapman 2006).

Serine proteases, which represent one third of all known enzymes, play an irreplaceable role in many physiological processes like blood coagulation, hemostasis, haemodynamic, inflammation, wound healing or fertilization. They are characterised by the presence of catalytic triad of aminoacids Ser, Asp and His in their active site. The nucleophilic serine also gave the name to the whole group. However, recently serine proteases with novel catalytic triads or diads were discovered. Serine proteases belong among endoproteases, catalyzing the bond hydrolysis in the middle of polypeptide chain. Based on substrate specificity, they can be divided into several groups with trypsin-like, chymostrypsin-like, elastase-like and subtilisin-like being the main (Hedstrom 2002, Di Cera 2009).

Both classes of enzymes have their inhibitors, serving as regulators of cascades involving these enzymes, namely cystatins for cysteine proteases and serpins for serine proteases; these inhibitors will be discussed below.

2.6.1. CYSTATINS

Cystatins form a large family of reversible, tight binding inhibitors of C1 cysteine proteases, which are also known as papain-like proteases. They can be found in many live organisms including invertebrates, vertebrates, plants, and protozoa (Abrahamson et al. 2003). Here they control essential biological processes where cysteine proteases are involved. This means antigen presentation, immune system development, apoptosis, or cell and tissue homeostasis (Honey and Rudensky 2003, Lombardi et al. 2005, Wille 2004).

Based on molecular organization, the cystatins superfamily can be divided into three groups. The type I cystatins, also called stefins, possess unglycosylated, intracellular proteins which lack signal peptide and disulfide bonds. Type II cystatins represent glycosylated, secreted proteins which can be found in body fluids. They contain signal peptide and two disulfide molecules in their molecule (Ochieng and Chauduri 2010). The cystatins of third type, also known as kininogens, are glycosylated, secreted, multidomain proteins possessing eight disulfide bridges (Müller-Esterl et al. 1986).

Cystatins contain three conserved motifs which form a wedge-shaped structure that blocks the active site of C1 cysteine proteases. These motifs are an N-terminal glycine, a glutamine-valine-glycine (Q-x-V-x-G) loop and a second c-terminus hairpin loop consisting of proline-typtophan (P-x-W) residues (Bode et al. 1988).

In ticks, number of cystatins I and II type can be found. One of the type II, secreted cystatins, which was found in the hard tick *I. scapularis* thanks to the sialotranscriptome study, is sialostatin L (Valenzuela et al. 2002). A few months later second cystatin sialostatin L2 was described. It shares 75% homology at the amino acid level with sialostatin L and has also the same molecular weight (Valenzuela et al. 2002, Ribeiro et al. 2006). However, BLAST analysis revealed that these two proteins are encoded by separated genes (Kotsyfakis et al. 2007).

2.6.1.1. Sialostatin L and Sialostatin L2

Detail information about the effect of these two proteins on innate immune cells are described in the chapter „Molecules with immunomodulatory properties“, thus in this subchapter I will focus on the brief summarization of known information.

Sialostatin L is inhibitor of cathepsins C, L, V, S, and X, with the highest affinity to cathepsin L and S. In agreement with this, sialostatin L was shown to inhibit proliferation of CTLL-2 cell line, which is known to be affected by cathepsins. Moreover it also inhibits antigen presentation, more accurately it affects processing of the invariant chain of MHC class II, where cathepsin S is involved (Kotsyfakis et al. 2007, Sá-Nunes et al. 2009). In addition to these effects, sialostatin L increases the production of IL-9 by Th9 cells and mast cells and thus prevents experimental asthma (Horká et al. 2011, Klein et al. 2015).

Sialostatin L2 strongly inhibits cathepsin L. Expression of sialostatin L2 gene is strongly induced in feeding tick and it’s silencing, together with silencing of sialostatin L, by RNA interference resulted in impaired tick feeding, poor growth and increased mortality. Moreover sialostatin L2 was shown to facilitate the growth of *Borrelia burgdorferi* spirochetes in murine skin, thus being one of the known SAT factors (Kotsyfakis et al. 2010). It also affects inflamasome formation in macrophages infected wit *Anaplasma phagocytophilum* (Chen et al. 2014).

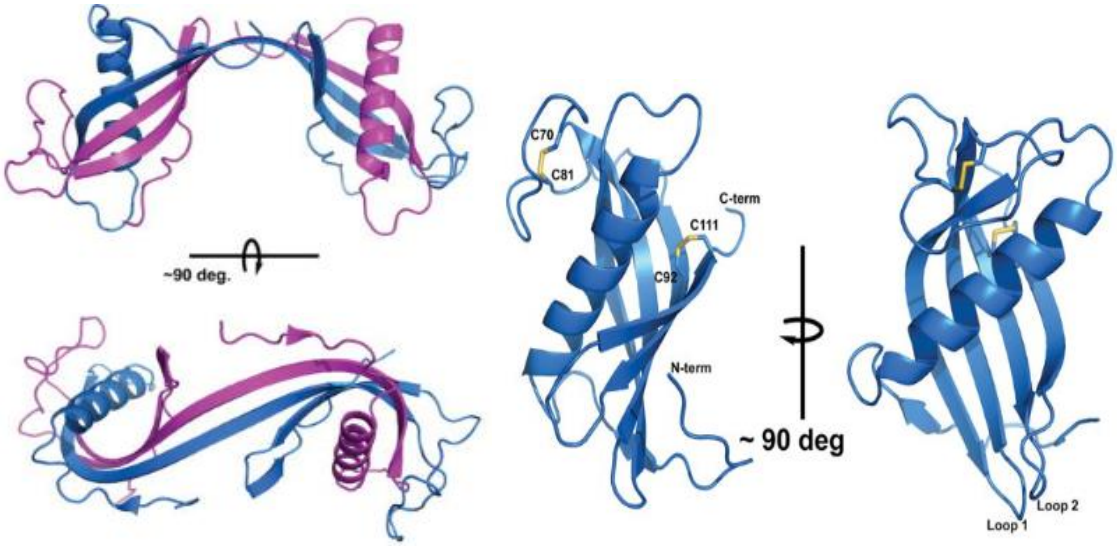


Figure 6: Crystal structure of sialostatin L (left) and sialostatin L2 (right) (Kotsyfakis et al. 2010)

2.6.2. SERPINS

Serpins represent a broad family of structurally related but functionally diverse proteins. Like cystatins, they can be found in the whole range of organisms including viruses, vertebrates or plants where they play an important role in a broad variety of biological processes. Seprins participate for example in the blood coagulation, fibrinolysis, programmed cell death, and in the development of the inflammation. In other words, they regulate physiological processes that are dependent on proteolytic activity. They can either trigger proteases activating other enzymes in proteolytic cascade or proteases which activate signaling molecules. Serpins also play an important role during tick feeding (Potempa et al. 1994, Carrell et al. 1987, Silverman et al. 2001). Majority of serpins belong among inhibitors of serine proteases, which also gave them the name serpins (serine proteases inhibitors), however, serpins can also inhibit cysteine proteases or don´t even have the inhibitory activity at all (Gettins 2002).

Serpins are single-stranded proteins with the size 350-400 aminoacids with the typical structure consisting of three β sheets (A, B, C) and seven to nine α helices (Gettins 2002, Khan et al. 2011). Characteristic is also the reactive central loop (RCL) , which is a protein motive, composed of twenty amino acids located in the proximity of protein's C-end. This motif represents the most variable part of protein and includes easily cleavable bond between so called P1 and P1´residues. Just this bond is cleaved by the target protease. Once the protease cleaves this bond, it comes to the structural changes which result in the formation of irreversible complexes and in the inactivation of both partners (Prevot et al. 2006).

Within the Ixodes ticks, the whole set of serpins was identified at the level of DNA sequence, however very few of them have been functionally characterized. So far, there are only two functionally characterized *Ixodes ricinus* serpins Iris and IRS-2, the latter will be described in the following subchapter as a part of this thesis (Leboulle et al. 2002, Chmelař et al. 2011).

2.6.2.1. IRS-2

Ixodes ricinus serpin-2 (IRS-2), as the name indicates, is the second recently characterized serpin from *I. ricinus*. Target enzymes of this protein are chymotrypisne like serine proteases cathepsin G and chymase. Both these enzymes are involved in acute inflammatory response and are produced by activated neutrophils (cathepsin G) and mast cells (chymase). Cathepsin G is also involved in the cell signaling leading to the activation and aggregation of trombocytes. IRS-2, in higher concentrations, was also shown to inhibit thrombin and thus suppress thrombin-induced platelets aggregation. Moreover, IRS-2 also inhibits swelling and migration of neutrophils into the inflamed tissue in mice with artificially provoked inflammation.

From mentioned effects of IRS-2, it is apparent that this protein plays an important role during tick feeding, since it is able to influence both the development of inflammation in tick feeding site and the heeling of the arisen wound. This also corresponds with the expression of IRS-2 mRNA which increases during the tick feeding.

It is also interesting that IRS-2 evinces considerable structural homology with α-1-antichymotrypsin, which is a natural regulator of cathepsin G and chymase (Chmelař et al. 2011).

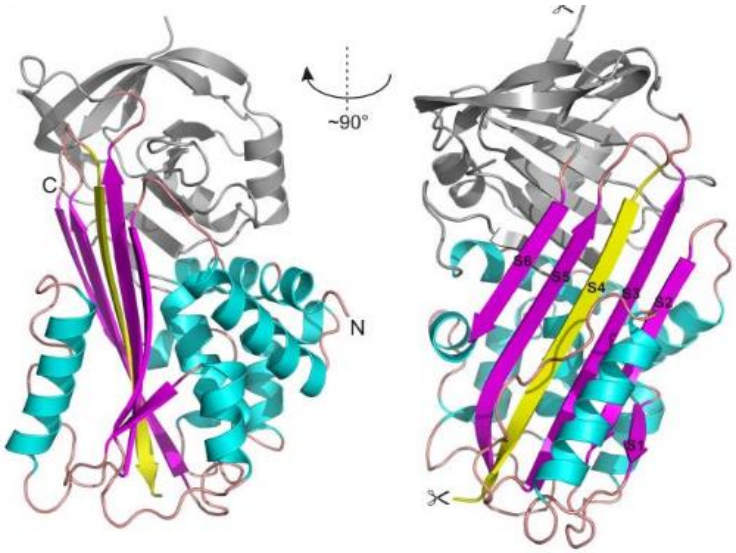


Figure 7: Crystal structure of IRS-2 (Chmelař et al. 2011)

3 SUMMARY OF RESULTS

This thesis is based on three research papers, with the first being the main of them. Here the papers are referred as **paper I**, **paper II** and **paper III**. The common subject of this thesis is the characterization of the effect of tick salivary proteins on various functions of dendritic cells. This includes mainly modulation of cytokine and chemokine production and subsequent impairment of other processes which are tightly regulated, or dependent on particular cytokines. Besides the modulation by itself, we also concentrated on the molecular basis of these effects, e.g. impairment of involved signaling pathways.

In **paper I** we described novel findings about the ability of the *I. ricinus* salivary serpin IRS-2 to inhibit Th17 differentiation upon *B. burgdorferi* exposure via inhibition of the IL-6/STAT-3 signaling pathway. IRS-2 was shown to strongly inhibit the production of pleiotropic cytokine IL-6 by splenic dendritic cells, whereas the production of cytokines IL-1β, IL-10, and TNF-α was not affected. This result was obtained in various types of mice and also in various types of cells (results not included in publication). Based on this observation, we decided to further focus on IL-6 and its function in driving immune response. In attempt to find out the mechanism standing behind the IL-6 production impairment, we first measured IL-6 mRNA level. We found out that expression of IL-6 mRNA is severely decreased in the presence of IRS-2 and that this decrease is not caused by the impaired stability of IL-6 mRNA. Next the monitoring of signaling pathways important for induction of IL-6 did not show any defect and that led us to the conclusion that gene induction is not impaired. Since there is a positive-feedback loop in IL-6/STAT-3 signaling (IL-6 binds to IL-6R on a cell and activates phosphorylation of the STAT-3 molecule, which in turn boosts the production of autocrine IL-6), we tested also the hypothesis that IRS-2 directly inhibits STAT-3 phosphorylation thus inhibiting IL-6 expression and production. However, this option was also excluded (data not shown). Recently, it was shown that endocytosis of *Borrelia* spirochetes and subsequent acidification of the endosome is crucial for the induction of IL-6 and IFN-α (Marre et al. 2010). Our unpublished data indicated that IRS-2 could interfere with phagosome maturation, so we hypothesized that IRS-2 decreases IL-6 production by inhibiting acidification of phagosome. Unfortunately neither this option turned to be right (data not shown). Thus, the precise mechanism of the IRS-2 effect still remains to be clarified.

It is known that IL-6, together with TGF-β, drives the differentiation of Th lymphocytes towards the Th17 subpopulation. In response to invading pathogens, IL-6 binds to IL-6

receptors on T cells and activates the signaling pathway, leading to phosphorylation of the transcription factor STAT-3, an essential molecule for Th17 differentiation (Kimura and Kishimoto 2010, Heinrich et al. 2003). Since the production of IL-6 in DCs was strongly inhibited by IRS-2, we expected that the phosphorylation of the STAT-3 signaling molecule would be decreased. And indeed, the phosphorylation of STAT-3 was significantly decreased in both dendritic cells and CD4 T lymphocytes. We also showed, after addition of exogenous IL-6 to sDCs in the presence or absence of IRS-2, that this observed effect is due to the impact of IRS-2 on IL-6 production rather than the intrinsic ability of IRS-2 to inhibit IL-6-induced signals.

Finally we proved that the impairment of IL-6/STAT-3 signaling pathway leads to impairment of Th17 development. This was demonstrated by a decreased amount of produced IL-17 and by flow cytometry assessment of intracellular IL-17 in CD4 T lymphocytes co-cultured with activated DCs. Similar results, showing that tick saliva inhibits the Th17 subset, were reported by Skallova and colleagues, who showed that saliva-exposed DCs failed to induce efficient Th1 and Th17 polarization and promoted development of Th2 responses (Skallova et al. 2008). Since Th17 subpopulation is known to be involved in recruitment of neutrophils, obtained data also correspond with previous *in vivo* observation that shows that this protein inhibits the influx of neutrophils into the inflamed tissue (Chmelar et al. 2011).

Papers II and **III** are focused on the effect of two *I. scapularis* cystatins sialostatin L and L2 on dendritic cell functions. **Paper II** shows that sialostatin L2 affects dendritic cell functions which are connected with inflammation whereas sialostatin L affects more the ability of DCs to drive the adaptive immune response.

Using the chemokines array, we demonstrated that *Borrelia* spirochetes induce in bone marrow-derived dendritic cells (BMDCs) production of 13 chemokines. Except for two of them, all the chemokines were inhibited by sialostatin L2. To confirm this observation we chose five of these chemokines and performed ELISA. From obtained results we confirmed that sialostatin L2 significantly decreases the production of MIP-1α and IP-10 (interferon gamma-induced protein) by *Borrelia* stimulated BMDCs. MIP-1α is a chemotactic factor for mononuclear cells, T cells, and mast cells and plays a role in differentiation of Th1 lymphocytes. IP-10 is a CXC chemokine and attracts, in addition to monocytes and Th1 cells, also NK cells. Inhibitory effect of sialostatin L2 on the production of chemokines attracting inflammatory cells can lead to reduced inflammation in tick feeding site. Reduced influx of inflammatory cells can also explain the previous observation that sialostatin L2 facilitates

establishment and proliferation of spirochetes in the murine skin, since tick feeding site free of inflammatory cells, provides *Borrelia* spirochetes favourable conditions to spread (Kotsyfakis et al. 2010).

To explain the mechanism of sialostatin L2 mediated decrease of chemokine production, we analyzed the activation of chosen signaling molecules (Er1/2, p38, NF-κB and PI3K/Akt) after TLR2 ligation with lipoteichoic acid or *Borrelia* respectively. TLR2 signaling pathway was chosen because of its importance for mediating signals in response to *Borrelia*. We showed that sialostatin L2 affects the phosphorylation of Erk1/2, NF-κB, and Akt where even the basal level of phosphorylated Akt kinase was decreased. However, after *Borrelia* stimulation, only the decrease in phosphorylation of Er1/2 was observed. Since dendritic cells sense *Borrelia* by several PRR, the effect of sialostatin L2 on signals triggered through TLR-2 could be masked by signals triggered through other receptors.

In addition to chemokines, type I interferons play an important role in dendritic cell development and functions. They are not only able to induce the antiviral state in cells but they are also involved in Th differentiation and maturation of DCs. Thus we decided to investigate whether, apart from inhibition of chemokine production, sialostatins also affect interferon production.

For this type of experiment we chose plasmacytoid dendritic cells which express high levels of TLR7 and TLR9 and produce high amount of type I interferon In contrast to chemokines, the production of IFN-β was highly inhibited in the presence of sialostatin L in cells stimulated with *Borrelia* or imiquimod (TLR7 ligand) and moderate decrease was observed also after stimulation with CpG (TLR9 ligand). Cathepsin L has been implicated in processing of TLR-9, and sialostatins L and L2 are strong inhibitors of this protease, thus we could speculate that the decline of IFN-β is the result of impaired TLR-9 processing. Moreover, in Paper III we showed that the amount of endogenously produced IFN-β was not affected by sialostatins in splenic DCs stimulated with TLR-4 agonist, where no processing had occurred.

Since IFN-β is involved in dendritic cell maturation, we predicted that the observed decrease of IFN production will result in impaired cell maturation. And indeed, maturation measured as level of expression of CD86 co-stimulatory molecule was significantly decreased in the presence of sialostatin L after stimulation with imiquimod and CpG. As poorly maturated dendritic cells, cannot properly trigger T cells differentiation and proliferation, this result matches with the observed inhibition of CTL lymphocyte proliferation in the presence of sialostatin L (Kotsyfakis et al. 2007). Moreover, the same

effect was observed also in splenic dendritic cells stimulated with LPS, where sialostatin L negatively affected the expression of both CD80 and CD 86 (Sá-Nunes et al. 2009).

Finally we examined the effect of tick cystatins on the differentiation or derivation of dendritic cells from bone marrow and found that sialostatin L negatively affects the number of differentiated dendritic cells, measured as MHC class II and CD11c positive cells. MHC class II molecule is necessary for the presentation of antigen to naive T-cells. As cathepsin S is implicated in the processing of the invariant chain within MHC class II antigens and sialostatin L strongly inhibits this protease, it seems likely that the decrease in MHC class II expression is mediated through inhibition of cathepsin S (Sá-Nunes et al. 2009, Riese et al. 1996). This finding (decrease of differentiated DC) can also contribute to the observed inhibition of CTL proliferation in the presence of sialostatin L.

In **paper III** we tested the hypothesis that sialostatins can modulate immune response throught the interference with IFN I action.

It is well established that interferons α and β act through the binding to heterodimeric receptor consisting of two subunits IFNAR (interferon α/ β receptor) 1 and 2 which in turn leads to phosphorylation of STAT molecules which dimerize and bind to IRF9 and together translocate to the nucleus where they bind IFN-stimulated response elements (ISREs) in DNA and initiate transcription of interferon stimulated genes (ISG) which set the antiviral state in cells (Platanias 2005). The western blot analyzis revealed that both cystatins markedly decreased the phosphorylation of STAT-1 and 2 in splenic DCs activated with recombinant IFN-β. As we excluded that the STATs phosphorylation is decreased due to protein degradation, we tested whether the cystatins can interfere with IFN signaling upstream of STATs, at the receptor level. The internalization rate of IFNAR1 receptor, which is crutial for further downstream signaling, was tested on flow cytometer and it was unaffected by either cystatin.

Although the exact mechanism how cystatins interfere with STATs phosphorylation remains unclear, we wondered if this impairment in IFN signaling observed in the presence of cystatins can result in altered induction of ISG. To test this hypothesis the induction of two IFN inducible genes, transcription factor IRF7 and chemokine IP-10, were tested in dendritic cells stimulated with LPS. Sialostatin L2 almost completely abolished induction of both genes, as we expected. Interestingly, sialostatin L had no effect on the expression of both molecules. Thus it seems that other pathway, except for IFN signaling, may be also critically involved in the induction of these two genes.

Lastly we checked whether all the above mentioned effects of sialostatin L2 on IFN signaling can impact the replication of tick-borne encephalitis virus in dendritic cells. And indeed, we proved that the replication of Neudoerfl virus (strain of TBE virus) in bone marrow DCs was enhanced in the presence of sialostatin L2. Moreover it was shown that sialostatin L2 completely canceled antiviral effect of exogenouslly added IFN-β. The splenic dendritic cells were replaced in this experiment with bone marrow derived DCs because BMDC produce less IFN-β than spleen dendritic cells and are therefore more convenient for showing the effect of exogenously added IFN-β.

This third paper showed that not only *Borrelia* spirochetes, but also TBE virus can take advantage of sialostatin L2 properties and employ them to the establishment of infection. Interestingly, it seems that sialostatin L2 does not facilitate growth of another tick borne pathogen *Anaplasma phagocytophilum* in mouse skin (Chen et al. 2014). Finally, the inhibitory effect of tick cystatin on interferon responses described herein can represent a novel mechanism by which tick saliva can facilitate tick borne pathogen transmission.

4 CONCLUSION AND FUTURE PROSPECTIVES

The data presented in this thesis helped to better uderstand the comprehensive activity of tick saliva and broadened the knowledge about the effect of three tick salivary proteins. Obtained results are valuable source of information and data which can be used in further research concerning cystatins and serpins.

At the beginning of my PhD. there was only the pivotal paper of Dr. Chmelař describing the *I. ricinus* salivary serpin IRS-2 and showing that IRS-2 inhibits cathepsin G and mast cell chymase and displays anti-inflammatory and anti-hemostatic activities.The data which are described in this thesis prove that IRS-2 affects also the adaptive branch of host's immunity. Together these studies indicate that IRS-2 is promising protein with multiple effects on host immune systeme, therefore it was proposed as a candidate for so called "murinization", an approach when the mouse structural part of a serpin is combined with a tick serpin RCL. The newly created protein is then non-immunogenic but preserves original tick specificity and function. This can be exploited in further testing of IRS-2 as a therapeutic agent. As it was shown that another tick salivary protein, sialostatin L, is able to cure experimental asthma in mice (by inhibiting Th9 responses), similarly the ability of newly created non-imunogenic IRS-2 to treat some Th17 based immunopathology (f.e. rheumatoid arthritis, multiple sclerosis) can be tested.

Concerning cystatins, all data collected together denote that both sialostatin L and L2 are interesting and promising proteins with broad range of effects. Especially impact of sialostatin L2 on immune cells seems to be benefitial for the transmission of tick borne pathogens. Although in our work the virus was used only as a model pathogen to show the inhibition of antiviral effect of IFN, it would be very interesting to prove the beneficial effect of sialostatin L2 on virus replication *in vivo*. However, we have to be aware of the fact that sialostatin L2 is a protein from *Ixodes scapularis*, but this tick is not natural vector of the TBE virus, which is transmited by *Ixodes ricinus* or *I. persulcatus*. Thus, the virus and protein are not completely compatible. For this reason we now proceeded to work with cystatins from *Ixodes ricinus* which are similar to sialostatin L and L2 but are more appropriate for further testing in experiments with TBE virus.

5 RESEARCH PAPERS

5.1. Paper I

Ixodes ricinus salivary serpin IRS-2 affects Th17 differentiation via inhibition of IL-6/STAT-2 signaling pathway

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Ixodes ricinus Salivary Serpin IRS-2 Affects Th17 Differentiation via Inhibition of the Interleukin-6/STAT-3 Signaling Pathway

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Th17 cells constitute a subset of CD4$^+$ T lymphocytes that play a crucial role in protection against extracellular bacteria and fungi. They are also associated with tissue injury in autoimmune and inflammatory diseases. Here, we report that serpin from the tick *Ixodes ricinus*, IRS-2, inhibits Th17 differentiation by impairment of the interleukin-6 (IL-6)/STAT-3 signaling pathway. Following activation, mature dendritic cells produce an array of cytokines, including the pleiotropic cytokine IL-6, which triggers the IL-6 signaling pathway. The major transcription factor activated by IL-6 is STAT-3. We show that IRS-2 selectively inhibits production of IL-6 in dendritic cells stimulated with *Borrelia* spirochetes, which leads to attenuated STAT-3 phosphorylation and finally to impaired Th17 differentiation. The results presented extend the knowledge about the effect of tick salivary serpins on innate immunity cells and their function in driving adaptive immune responses.

Ticks are bloodsucking arthropods, major vectors of human pathogens like *Borrelia burgdorferi* and tick-borne encephalitis virus. Ticks from the family Ixodidae (hard ticks) require several days to fully engorge. During feeding, ixodid ticks remain tightly attached to their host (1, 2). To avoid attack from the host immune system during the feeding period, tick saliva contains two groups of molecules, the first with antihemostatic and the second with immunomodulatory properties. These groups include both proteinaceous and nonprotein molecules (3). One group of immunomodulatory proteins is represented by serine proteinase inhibitors (serpins), a large superfamily of structurally related, but functionally diverse, proteins that control essential proteolytic pathways (4, 5). Recently, three serine protease inhibitors, namely, purified human urinary trypsin inhibitor (UTI) and two synthetic serpins, gabextate mesilate (FOY) and nafamostat mesilate (FUT), which are widely used in treatment of acute inflammatory disorders, such as disseminated intravascular coagulation (DIC), have been shown to attenuate allergic airway inflammation and remodeling in a murine model of chronic asthma. These effects were associated with inhibition of Th2 cytokines (interleukin-4 [IL-4], IL-5, IL-6, and IL-13) and Th17 cell functions. These serpins also inhibited NF-κB activation in lung tissues (6).

Until now, more than 60 serpins have been identified at the sequence level in ixodid ticks, but only two serpins from *Ixodes ricinus* have been further functionally characterized (7–9). The first known *I. ricinus* serpin, Iris (*I. ricinus* immunosuppressor), is known to preferentially target leukocyte elastase. It also interferes with the contact phase coagulation pathway, fibrinolysis, and disrupts platelet adhesion. Moreover, Iris has the ability to modulate both innate and adaptive immunity. It affects T lymphocyte and macrophage responsiveness, and it induces a Th2-type response and inhibits the production of proinflammatory cytokines. Interestingly, it was shown that the anti-inflammatory properties of the protein are independent of its proteolytic activity and are mediated through its exosite domain (10–13).

IRS-2, the second described serpin from *I. ricinus*, targets cathepsin G and chymase. Both enzymes are part of the acute inflammatory response and are produced by activated neutrophils (cathepsin G) and mast cells (chymase). Moreover, IRS-2 is able to inhibit swelling and the migration of neutrophils into the inflamed tissue (14). The effects of IRS-2 on other cells of innate and acquired immunity have not been described so far.

Dendritic cells (DCs) are known as antigen-presenting cells and play a critical role in initiating and modulating the immune response. With their ability to recognize, process, and present antigens on their surfaces and thus activate T lymphocytes, DCs form a unique link between innate and acquired immunity (15, 16). Depending upon the recognized pathogens and other stimuli produced by activated DCs, such as cytokines and chemokines, T lymphocytes differentiate into cytotoxic CD8$^+$ or helper CD4$^+$ cells, which can further differentiate into various subsets (17). The IL-6/STAT-3 signaling pathway leads to differentiation of CD4$^+$ T lymphocytes into the Th17 subset. IL-6, a pleiotropic cytokine produced by dendritic cells in response to invading pathogens, binds to IL-6 receptors on T cells and activates the signaling pathway, leading to phosphorylation of the transcription factor STAT-3, an essential molecule for Th17 differentiation (18, 19). Th17 cells participate in host defense against extracellular bacteria and fungi by mediating the recruitment of neutrophils and macrophages into infected tissues. It is also known that regulation of Th17 cells plays a significant role in the pathogenesis of various inflammatory and autoimmune disorders (20–22). Moreover, it was shown that Th17 cells are involved in

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the development of severe destructive arthritis caused by the Lyme disease spirochete *B. burgdorferi* (23).

The objective of this study was to analyze the effect of a tick salivary serpin on dendritic cells and its consequences for the development of proinflammatory cells, like Th17 lymphocytes.

MATERIALS AND METHODS

Experimental animals. Specific-pathogen-free C57BL/6 mice (6- to 10-week-old females) were purchased from Charles River Laboratories. The animals were maintained under standard conditions in the animal house facility of the Institute of Parasitology, Biology Centre AS CR, České Budějovice. All experiments were performed with permission of the Czech animal ethics committee.

Recombinant IRS-2. Recombinant serpin from *I. ricinus*, IRS-2, was overexpressed in *Escherichia coli* BL21(DE3) pLysS cells. The expressed protein accumulated in inclusion bodies, which were separated. Refolded and concentrated IRS-2 was purified using a standard chromatographic method (fast protein liquid chromatography [FPLC]) (14, 24). Lipopolysaccharide (LPS) contamination was removed by Arvys Proteins Company using the detergent-based method.

Bacteria. *B. burgdorferi sensu stricto* ATCC 35211 isolated from *I. ricinus* was grown in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma-Aldrich) supplemented with 6% rabbit serum at 34°C. The number of spirochetes was calculated by dark-field microscopy according to the method of Magnuson et al. (25). The fourth to sixth passages were used in the experiments.

Splenic DC isolation. Isolated mouse spleens were minced with scissors, digested in RPMI containing 0.25 mg/ml Liberase DL (Roche) and 0.2 mg/ml DNase I (Roche) at 37°C for 30 min, and passed through a 70-μm nylon cell strainer (BD Falcon). The dendritic cells were isolated using magnetic beads conjugated with anti-CD11c antibody (Ab) and magnetically activated cell sorting (MACS) column separation following the manufacturer's instructions (Miltenyi Biotec). The purified dendritic cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 μg/ml penicillin, and 100 U/ml streptomycin (all from Sigma-Aldrich). The purity of the isolated dendritic cells (~90% $CD11c^+$ cells) was determined by subsequent fluorescence-activated cell sorter (FACS) analysis.

$CD4^+$ T cell isolation. The fourth day after subcutaneous infection of mice with 1×10^5 *Borrelia* spirochetes, isolated mouse spleens were passed through a 70-μm nylon cell strainer (BD Falcon), and $CD4^+$ T cells were isolated using magnetic beads conjugated with anti-CD4 Ab and MACS column separation following the manufacturer's instructions (Miltenyi Biotec). Purified $CD4^+$ T cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 μg/ml penicillin, and 100 U/ml streptomycin (all from Sigma-Aldrich). The purity of the isolated $CD4^+$ T cells (≥90% $CD4^+$ $CD62L^{high}$) was determined by FACS analysis.

Specific activation of $CD4^+$ T lymphocytes. Purified splenic DCs were seeded at 5×10^4 cells per well in 96-well plates and stimulated with *Borrelia* spirochetes (5×10^5 per well) and IRS-2 (6 μM). After 24 h incubation, the medium was removed, and 3×10^5 freshly isolated *Borrelia*-primed $CD4^+$ T lymphocytes in 200 μl of culture medium were added to each well. The T cells were incubated with DCs for 3 days before restimulation with phorbol myristate acetate (PMA) (20 ng/ml) and ionomycin (1 μM) (both Sigma-Aldrich). Cell-free culture supernatants for IL-17 and IL-9 assessment were harvested at 2, 6, 12, 24, and 48 h after restimulation.

To determine the number of IL-17-producing Th cells, *Borrelia*-exposed DCs and *Borrelia*-primed $CD4^+$ T cells were cocultured as described above. On day 5 of coculture, the cells were restimulated with PMA and ionomycin and, after an additional 2 h, treated with monensin (2 μM; eBiosciences). The cells were then incubated for 4 h before staining was performed with anti-IL-17 antibody conjugated with phycoerythrin (PE) (eBioscience).

Cytokine measurement. Freshly isolated dendritic cells were seeded at 2×10^5 cells per well on 96-well plates. The next day, the DCs were stimulated with *B. burgdorferi* spirochetes at a multiplicity of infection (MOI) of 10 (2×10^6 per well) in the presence or absence of IRS-2 (6 μM). Cell-free culture supernatants were harvested 2, 9, 12, 24, or 48 h after stimulation and used for detection of IL-1β, IL-6, IL-10, and tumor necrosis factor alpha (TNF-α) with Ready-Set-Go! enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) following the manufacturer's instructions. Cell-free culture supernatants for IL-17 and IL-9 assessment were prepared as described in "Specific activation of $CD4^+$ T lymphocytes" above, and the amount of cytokines was measured with a Ready-Set-Go! ELISA kit (eBioscience) following the manufacturer's instructions. All reactions were performed in triplicate.

RNA extraction, quantitative real-time PCR, and mRNA half-life determination. To assess relative mRNA expression, DCs were seeded at 2×10^6 cells per well in 24-well plates. The next day, the DCs were stimulated with *Borrelia* spirochetes at an MOI of 10 (2×10^7 per well) in the presence or absence of IRS-2 (6 μM) and incubated for 6 or 12 h. RNA was then isolated with the Nucleospin RNA II kit (Macherey-Nagel) following the manufacturer's instructions. The quality and concentration of the isolated RNA were assessed by measurement on a Nanophotometer P-330 (Implen). cDNA was synthesized with the High-Capacity RNA-to-cDNA kit (Applied Biosystems). Real-time PCR analysis was performed with a TaqMan gene expression set (Applied Biosystems) containing primers and probe specific for IL-6 and β-actin using a Rotor Gene 3000 and Rotor-Gene 6.0.19 software (Corbett Research). The relative expression of IL-6 mRNA was determined by the comparative threshold cycle (C_T) method (26), where the mouse β-actin gene was used as a housekeeping gene (Applied Biosystems). All reactions were performed in triplicate.

Immunoblotting. Freshly isolated dendritic cells were seeded at 1×10^6 cells per well in 24-well plates. The next day, the DCs were stimulated with *Borrelia* spirochetes at an MOI of 10 (1×10^7 per well) in the presence or absence of IRS-2 (6 μM). Following stimulation (15, 30, and 60 min for C/EBP, phosphorylated NF-κB [p-NF-κB], p-CREB, p-p-38, and p-ERK1/2 and 6 and 16 h for p-STAT-3), the cells were lysed in a modified RIPA buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5) in the presence of protease and phosphatase inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg/ml pepstatin, 25 mM NaF, and 2 mM $NaVO_3$). The protein extracts, mixed with Laemmli sample buffer, were separated by SDS-PAGE and transferred to Immobilon-P membranes. Following blocking in Tris-buffered saline (TBS)-containing 5% fat-free milk, the blots were incubated overnight with the antibodies against C/EBP, phospho-STAT-3 (Tyr^{705}), phospho-NF-κB (Ser^{536}), phospho-CREB (Ser^{133}), phospho-p38 (Thr^{180}), and phospho-ERK1/2 (Thr^{202}) (all from Cell Signaling) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-actin (Santa Cruz Biotechnology). The proteins were visualized using enhanced chemiluminescence (Pierce), and their abundances were analyzed using a charge-coupled-device (CCD) imaging system (ChemiDoc MP Imaging System) and Image Lab software v. 4.1 (Bio-Rad).

To assess the level of phosphorylated STAT-3 in T lymphocytes, freshly isolated dendritic cells were seeded at 1. 5×10^5 per well in a 96-well plate. After 6 h, the DCs were stimulated with *Borrelia* spirochetes (1×10^7 per well) in the presence or absence of IRS-2 (6 μM). The next day, cell-free culture supernatants were harvested and added to freshly isolated *Borrelia*-primed $CD4^+$ T lymphocytes (9×10^5 per well). Following stimulation (15 and 30 min), the cells were lysed, and Western blotting was performed as described above with phospho-STAT-3 (Tyr^{705}) and β-actin antibodies.

Flow cytometry. $CD4^+$ cells were prepared and stimulated as described in "Specific activation of $CD4^+$ T lymphocytes" above. After 4 h of restimulation with PMA, ionomycin, and monensin, the cells were harvested (using cold 5 mM EDTA in PBS) and stained with anti-CD4 antibody (conjugated with allophycocyanin [APC]; eBioscience). After wash-

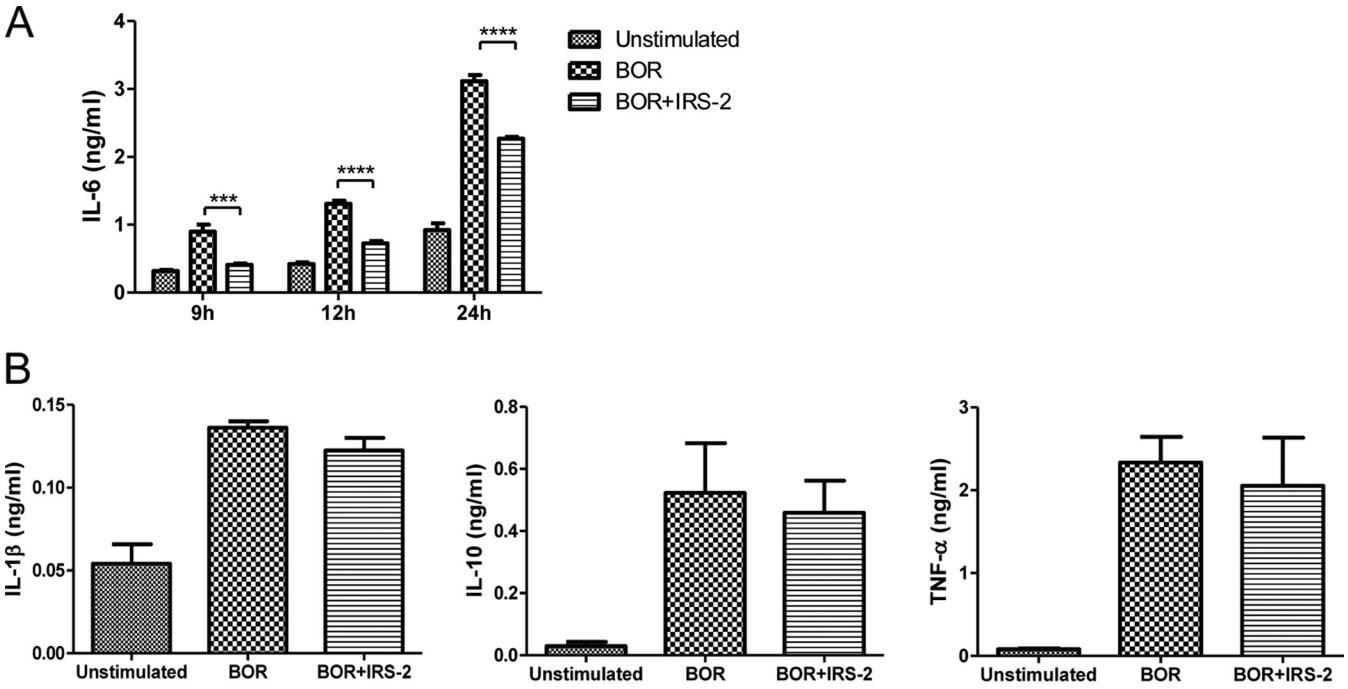

FIG 1 IRS-2 selectively inhibits IL-6 production by DCs. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (BOR) (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). (A) Culture supernatants were harvested 9, 12, and 24 h after stimulation, and the amount of IL-6 was determined by ELISA. Two independent experiments were performed, and data from a representative experiment are shown. The data are expressed as the mean cytokine concentrations from three wells plus standard errors of the mean (SEM). *** and ****, effects of IRS-2 on IL-6 production were significant at P values of <0.001 and <0.0001, respectively. (B) Culture supernatants were harvested 24 h (TNF-α) or 48 h (IL-1β and IL-10) after stimulation, and the presence of cytokines was detected by ELISA. Three independent experiments were performed, and the data were pooled. The data are expressed as the mean cytokine concentrations from nine wells plus SEM.

ing, the cells were fixed and permeabilized with a Foxp3/transcription factor staining buffer set (eBioscience) and labeled with anti-IL-17A antibody (conjugated with PE; eBioscience). The prepared cells were resuspended in cold PBS with 1% FCS. Flow cytometry was performed on a FACSCanto II cytometer using FACS Diva software v. 5.0 (BD Biosciences).

Statistical analysis. One-way analysis of variance (ANOVA) followed by a Bonferroni test in GraphPad Prism, version 5.0, was used to compare the differences between control and treated groups. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

IRS-2 selectively inhibits IL-6 production by DCs upon stimulation with *Borrelia* spirochetes. Cytokines produced by activated DCs play a key role in shifting the immune response toward particular Th subsets. To investigate the effects of IRS-2 on the production of different pro- and anti-inflammatory cytokines by DCs, immature DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2 for 9, 12, 24, or 48 h, and the production of IL-1β, IL-6, IL-10, and TNF-α was measured.

Serpin significantly inhibited the production of IL-6 in DCs (Fig. 1A), whereas the production of other cytokines remained unaltered (Fig. 1B). The same inhibitory effect of IRS-2 on the production of IL-6 was also observed in the PMJ2-R cell line (macrophages) and primary neutrophils (data not shown).

IRS-2 inhibits IL-6 production at the level of mRNA. Gene expression can be regulated by many mechanisms at many stages, including chromatin accessibility, transcription activation, mRNA nuclear export, mRNA decay, and translation. To understand the mechanism of IL-6 decline caused by IRS-2, the expression of the IL-6 gene was measured in DCs activated with *Borrelia* spirochetes in the presence or absence of IRS-2. mRNA specific for IL-6 was determined by quantitative RT-PCR. As shown in Fig. 2, the IL-6 transcript level was slightly increased after as little as 6 h (no significant effect of IRS-2 was observed). However, the mRNA of IL-6 was severely suppressed by IRS-2 at a later time point (12 h). We concluded that a decline in IL-6 production is the result of impaired

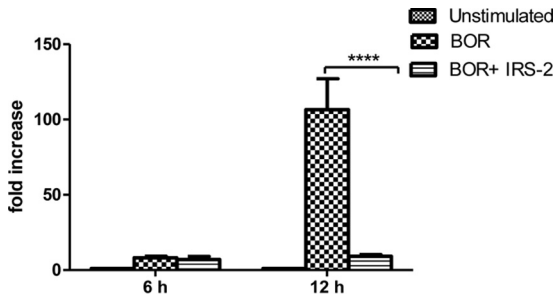

FIG 2 IRS-2 inhibits IL-6 production at the level of mRNA expression. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). The transcript level of IL-6 was determined by quantitative PCR (qPCR) using specific primers for IL-6. The gene expression of IL-6 was normalized to the β-actin transcript. Two independent experiments were performed, and the data were pooled. The data are expressed as the average fold IL-6 mRNA increase (plus SEM) from six wells compared with the control. ****, the effect of IRS-2 on the relative expression of IL-6 mRNA was significant at a P value of <0.0001.

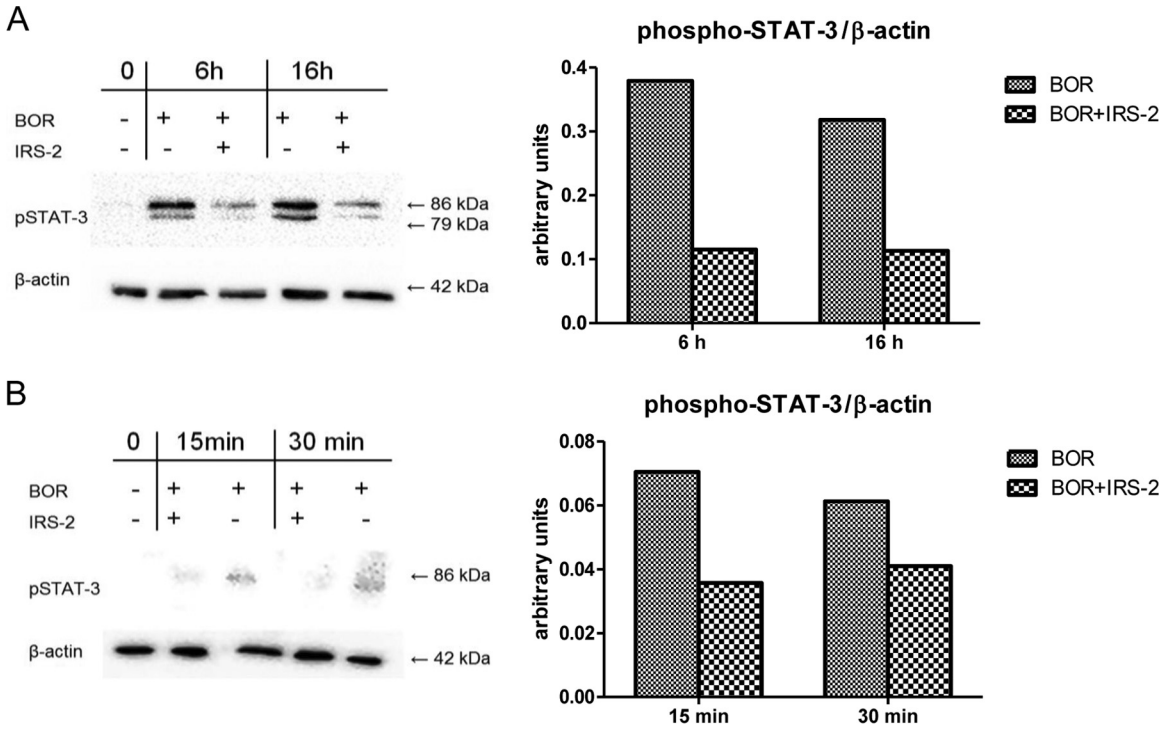

FIG 3 IRS-2 inhibits STAT-3 molecule phosphorylation. (A) Splenic dendritic cells (sDCs) were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). Six and 16 h after stimulation, cell lysates were prepared and analyzed by immunoblotting with anti-phospho-STAT-3. The membranes were reprobed with antibody against β-actin. The phosphorylation of STAT-3 was quantified using chemiluminescence and normalized to the β-actin protein level. The two bands represent different isoforms (α and β) of pSTAT-3 that are present in DCs. (B) Freshly isolated *Borrelia*-primed $CD4^+$ T cells were stimulated with 24-h supernatants from sDCs stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). Fifteen and 30 min after stimulation, cell lysates were prepared and analyzed by immunoblotting with anti-phospho-STAT-3. The membranes were reprobed with antibody against β-actin. The phosphorylation of STAT-3 was quantified using chemiluminescence and normalized to the β-actin protein level.

gene expression in IRS-2-exposed cells after activation with *Borrelia* spirochetes (Fig. 2).

Decreased stability of IL-6 mRNA is often responsible for a decline in IL-6 production. Moreover, inhibition of IL-6 production due to increased IL-6 mRNA decay was observed with another tick salivary protein (27). Therefore, we investigated whether the same mechanism could be responsible for the IRS-2-induced effect. Splenic DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2. After 9 h, actinomycin D was added to block mRNA synthesis, cells were harvested (after 1 and 2 h), and mRNA decay was determined. The IL-6 mRNA half-life observed in the presence of IRS-2 was comparable to that of control cells stimulated only with *Borrelia* spirochetes (data not shown). This result suggests that the impaired gene expression of IL-6 is not due to impaired stability of IL-6 mRNA.

In our effort to reveal the mechanism of the IRS-2 effect on IL-6, we further tested whether signaling pathways leading to induction of IL-6 are affected by IRS-2.

Gene expression of IL-6 is controlled by several transcription factors and signaling molecules, including NF-κB, C/EBP, CREB, and kinases p38 and ERK1/2 (28–32); therefore, the phosphorylation of these molecules was tested. DCs were stimulated with *Borrelia* in the presence or absence of IRS-2 for 15, 30, and 60 min. After stimulation, cell lysates were prepared and analyzed by immunoblotting. The phosphorylation of none of these signaling molecules was inhibited by IRS-2, so we concluded that induction of the IL-6 gene is intact and does not seem to be responsible for decreased IL-6 transcript expression (data not shown).

IRS-2 impairs Th17 differentiation via inhibition of the IL-6/STAT-3 signaling pathway. It is well known that the major transcription factor activated by IL-6 is STAT-3. STAT-3 phosphorylation is mediated through the association of IL-6 with the IL-6 receptor (IL-6R) and the signal transducer glycoprotein 130 (gp130), followed by subsequent activation of Janus kinases (19). Since the production of IL-6 in DCs was strongly inhibited by IRS-2, we expected that the phosphorylation of the STAT-3 signaling molecule would be decreased. DCs were activated with *Borrelia* spirochetes in the presence or absence of IRS-2, and the level of phosphorylated STAT-3 molecules was determined 6 and 16 h after activation. Indeed, a marked decrease of phospho-STAT-3 was observed (Fig. 3A). *Borrelia*-primed T lymphocytes were activated with supernatants from DCs (stimulated for 24 h with *Borrelia* spirochetes in the presence or absence of IRS-2), and the phosphorylation of STAT-3 in T lymphocytes was also decreased, likely due to diminished production of IL-6 by DCs (Fig. 3B).

The IL-6/STAT-3 signaling pathway is known to be crucial for development of the Th17 subset (18, 20). The main effector cytokines produced by Th17 cells are IL-17 (IL-17A), which is a hallmark of the subpopulation; IL-21; IL-22; and IL-9 (22, 33, 34). We predicted that the inhibition of IL-6/STAT-3 signaling by IRS-2 could lead to impaired Th17 differentiation, and therefore, the number of Th17-producing cells and the amounts of IL-17 and

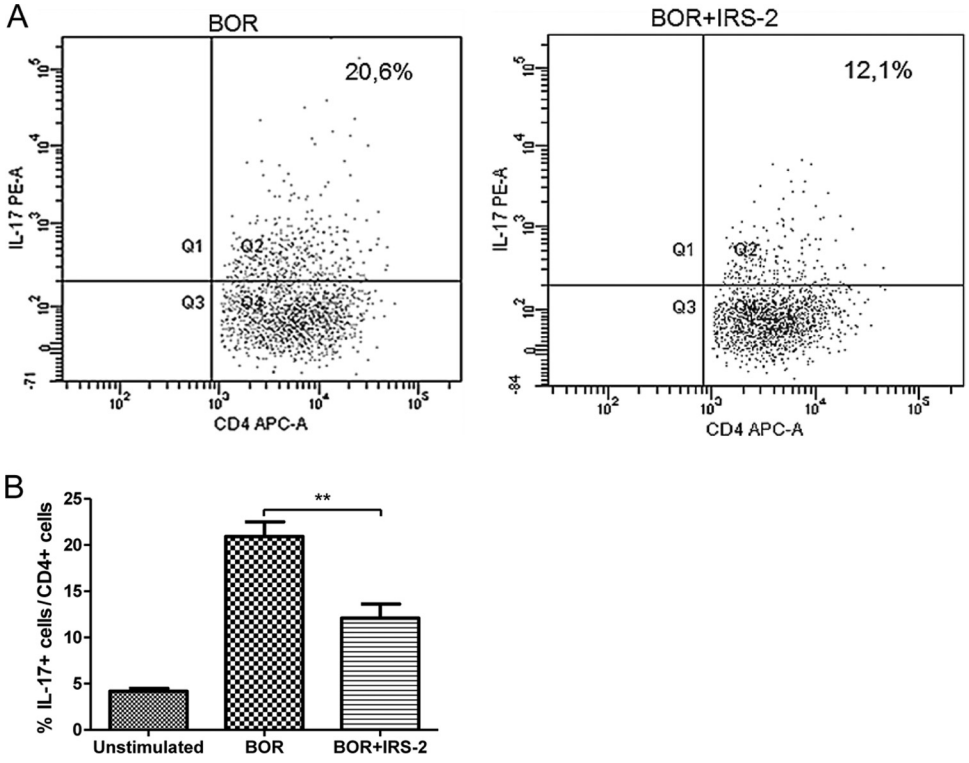

FIG 4 IRS-2 reduces the number of IL-17-producing $CD4^+$ T cells. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). After 24 h, freshly isolated *Borrelia*-primed $CD4^+$ T cells were added to the cultured DCs, and the cells were cocultured for 5 days. Then, T lymphocytes were restimulated with PMA and ionomycin, treated with monensin, and stained for IL-17. (A) Flow cytometry dot plots of T lymphocytes treated with DCs stimulated with *Borrelia* in the presence or absence of IRS-2. Quadrant 2 (Q2) shows $CD4^+$ $IL\text{-}17^+$ cells. (B) The percentage of IL-17-producing cells was determined in live $CD4^+$ cells. The data are expressed as the mean percentages of $CD4^+$ $IL\text{-}17^+$ cells from triplicate wells plus SEM. **, the effect of IRS-2 on the presence of IL-17-producing cells was significant at a *P* value of <0.01.

IL-9 were determined. Splenic DCs were stimulated with *Borrelia* spirochetes in the presence or absence of IRS-2. After 24 h, freshly isolated *Borrelia*-primed $CD4^+$ T cells were added to the cultured DCs, and the cells were cocultured for the next 5 days. Afterward, the cells were restimulated with PMA and ionomycin. To detect the Th17 subset, intracellular staining for IL-17A was performed, and the cells were analyzed by flow cytometry. To block cellular transport, monensin was added to the restimulated cells. As seen in Fig. 4A and B, the number of IL-17-producing $CD4^+$ T cells was significantly decreased by IRS-2.

To measure the production of IL-17 and IL-9 cytokines, the coculture of DCs and *Borrelia*-primed $CD4^+$ T cells lasted 3 days before restimulation with PMA and ionomycin. The supernatants were then collected at various time points and analyzed. The levels of both IL-17 and IL-9 were significantly decreased in the presence of IRS-2 (Fig. 5A and B). The reduced levels of the measured cytokines, together with the decreased number of IL-17-producing $CD4^+$ T cells, in the presence of IRS-2 clearly indicate that IRS-2 inhibits Th17 differentiation.

DISCUSSION

During coevolution with their hosts, ticks evolved various mechanisms enabling them to avoid the hosts' hemostatic and immune systems and successfully finish their blood meals.

In recent years, attention has been focused on identification and functional characterization of particular tick salivary proteins that are responsible for antihemostatic and immunomodulatory effects (3).

Thanks to this intensive research, many tick salivary substances that have immunomodulatory effects on various immune cell populations have been identified. Among these substances, molecules that can affect DC functions seem to play important roles, since DCs are among the first cells present at the site of inflammation and can further modulate or shift the immune response by driving T cell differentiation.

Here, we describe specific and extensive inhibitory effects of the tick salivary serpin IRS-2 from the hard tick *I. ricinus* on Th17 differentiation mediated by impairment of the IL-6/STAT-3 signaling pathway.

Proteins from the serpin superfamily are involved in fundamental biological processes, such as blood coagulation, complement activation, fibrinolysis, angiogenesis, inflammation, and tumor suppression (35, 36). From this enumeration, it is apparent that tick serpins can be expected to play a role in tick feeding, suppressing both the antihemostatic and immune responses of the host. To date, only two *I. ricinus* serpins have been functionally characterized (12, 14).

We showed that IRS-2 decreased IL-6 at the protein and mRNA levels in spleen dendritic cells activated by *B. burgdorferi*. A decrease by Sapl15, the best-studied tick salivary protein, in the IL-6 level in response to *B. burgdorferi* was also observed. Salp15 binds to DCs via the DC-SIGN receptor, which results in activa-

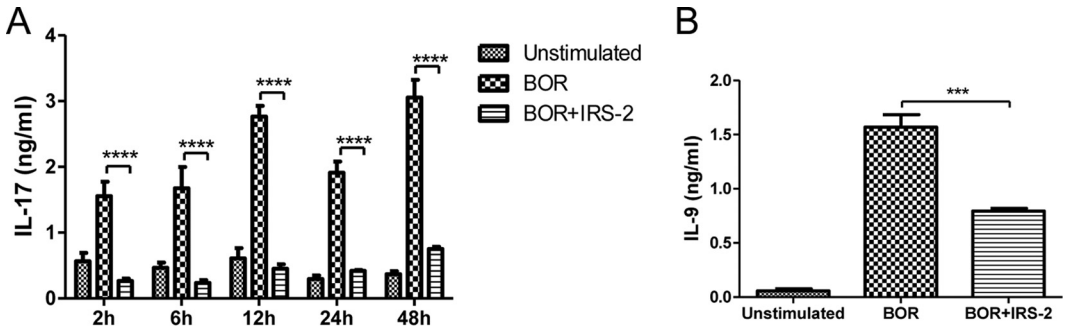

FIG 5 IRS-2 reduces levels of Th17 cytokines. Splenic dendritic cells were stimulated with *Borrelia* spirochetes (10 spirochetes per cell) in the presence or absence of IRS-2 (6 μM). After 24 h, freshly isolated *Borrelia*-primed $CD4^+$ T cells were added to the cultured DCs, and the cells were cocultured for the next 3 days. Afterward, the cells were restimulated with PMA and ionomycin, and the production of cytokines was analyzed at various time points. (A) Cell supernatants for IL-17 assessment were harvested 2, 6, 12, 24, and 48 h after restimulation and analyzed by ELISA. The data are expressed as the mean cytokine concentrations from triplicate wells plus SEM. Two independent experiments were performed, and data from a representative experiment are shown. ****, the effect of IRS-2 on the IL-17 level was significant at a *P* value of <0.0001. (B) IL-9 production was assessed by ELISA 24 h after restimulation. The data are expressed as the mean cytokine concentrations from triplicate wells plus SEM. ***, the effect of IRS-2 on IL-9 production was significant at a *P* value of <0.001.

tion of the serine/threonine kinase Raf-1/mitogen-activated protein kinase (MEK)-dependent signaling pathway and subsequently in a decrease of IL-6 and TNF-α mRNA stability and impaired nucleosome remodeling at the IL-12p35 promoter in human DCs activated with *B. burgdorferi* (27). However, the authors point to the fact that the addition of rabbit polyclonal anti-Salp15 antibodies abrogates the capacity of *I. ricinus* saliva to inhibit IL-12, but not IL-6 and TNF-α, which might be due to the presence of other molecules in tick saliva that are able to block IL-6 and TNF-α. In our study, we proved that one of these molecules, which can be responsible for IL-6 inhibition, is the salivary serpin IRS-2. To reveal the possible mechanism of IRS-2 effects, mRNA for IL-6 was assessed. However, it turned out that, in contrast to Salp15, IRS-2 does not act through impaired stability of IL-6 mRNA. In addition, monitoring of signaling pathways important for induction of IL-6 did not show any defect that led us to the conclusion that gene induction is not impaired. There is a positive-feedback loop in IL-6/STAT-3 signaling (IL-6 binds to IL-6R on a cell and activates phosphorylation of the STAT-3 molecule, which in turn boosts the production of autocrine IL-6), so direct inhibition of STAT-3 phosphorylation by IRS-2 could explain the observed decrease in IL-6 mRNA expression and, subsequently, IL-6 production (37). However, this option was also excluded (data not shown). Thus, we did not reveal the precise mechanism of the IRS-2 effect.

It has been shown that tick saliva and tick salivary proteins, like Salp15, Japanin, and sialostatin L, can modulate the T cell response by modulating DC accessory functions or directly by interaction with $CD4^+$ T cells. It was well demonstrated that tick saliva or salivary gland extract (SGE) diminishes the production of Th1-related cytokines and increases the production of Th2-related cytokines. Salp15 specifically binds to CD4 molecules on the surfaces of $CD4^+$ T (helper) cells, which results in inhibition of T cell receptor-mediated signaling, leading to reduced IL-2 production and impaired T cell proliferation (38). Japanin, a lipocalin from *Rhipicephalus appendiculatus*, specifically reprograms the response of DCs to a wide variety of stimuli *in vitro*, altering their expression of costimulatory and coinhibitory transmembrane molecules and secretion of proinflammatory, anti-inflammatory, and T cell-polarizing cytokines (it blocks LPS-induced secretion of Th17- and Th1-promoting cytokines); it also inhibits the differentiation of DCs from monocytes (39). Recently, Horka et al. showed that cystatin from *I. scapularis*, sialostatin L, which also inhibits several dendritic cell functions, can inhibit IL-9 production by Th9 cells, thus preventing the development of experimental asthma (40). Another cystatin, OmC2 from the soft tick *Ornithodoros moubata*, can also suppress the host adaptive immune response by reducing TNF-α and IL-12 production and the proliferation of antigen-specific $CD4^+$ T cells (41).

In line with these reports, our data show that the serpin IRS-2 is another tick salivary protein that is able to modulate T cell differentiation. We demonstrated that inhibition of *Borrelia*-induced IL-6 production in the presence of IRS-2 in DCs was accompanied by decreased phosphorylation of the STAT-3 signaling molecule, which is essential for the development of Th17 cells. Indeed, the impairment by IRS-2 of Th17 development was observed and was demonstrated by a decreased amount of IL-17 produced and by flow cytometry assessment of intracellular IL-17 in $CD4^+$ T lymphocytes cocultured with activated DCs. Similar results, showing that tick saliva inhibits the Th17 subset, were reported by Skallova and colleagues, who showed that saliva-exposed DCs failed to induce efficient Th1 and Th17 polarization and promoted development of Th2 responses (42). Interestingly, treatment with Salp15, which also inhibits IL-6 production in dendritic cells, was shown to increase the differentiation of Th17 cells *in vivo*, as evidenced by higher IL-17 production from PLP139-151-specific $CD4^+$ T cells isolated from the central nervous system and the periphery (43).

Th17 cells, a quite recently described subpopulation of $CD4^+$ T lymphocytes, can be characterized by production of the hallmark cytokine IL-17. Overproliferation of Th17 cells is connected with many severe autoimmune diseases, like human psoriasis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, asthma, and some bacterial and fungal infections. However, it is well established that Th17 cells not only play an important role in autoimmunity, but also function in the clearance of specific types of pathogens that require a massive inflammatory response and are not adequately dealt with by Th1 or Th2 immunity. Thus, the Th17 response can be triggered by many bacteria, including *Borrelia* spirochetes (22). Infante-Duarte showed that *B. burgdorferi*

lysate is able to induce massive amounts of IL-17 in T cell cultures and that microbe-induced IL-17 production can mediate infection-induced immunopathology in Lyme disease (44). Involvement of the Th17 subset in the development of severe destructive arthritis in patients with Lyme disease was also demonstrated by Burchill et al. (23). A causative protein, neutrophil-activating protein A (NapA) from *B. burgdorferi*, which is able to stimulate IL-17 production in synovial-fluid-derived T cells and could thus be crucial for the induction and maintenance of Lyme arthritis, was identified (45). Moreover, it is well described that synthetic or human-derived serpins, which are commonly used in the treatment of many autoimmune diseases, are able to decrease Th17 differentiation (6).

All these findings highlight the importance and potential of the *I. ricinus* serpin IRS-2 described here as a prospective molecule in many pharmaceutical applications.

In conclusion, here, we present a newly described ability of the *I. ricinus* salivary serpin IRS-2 to inhibit Th17 differentiation upon *B. burgdorferi* exposure via inhibition of the IL-6/STAT-3 signaling pathway, thus extending the knowledge about the effect of tick salivary serpins on innate immunity cells and their function in driving the adaptive immune response. This paper contributes to the understanding of tick saliva-mediated modulation of the host immune system.

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5.2. Paper II

Tick sialostatins L and L2 differentially influence dendritic cells responses to *Borrelia* spirochetes

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RESEARCH Open Access

Tick sialostatins L and L2 differentially influence dendritic cell responses to *Borrelia* spirochetes

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Abstract

Background: Transmission of pathogens by ticks is greatly supported by tick saliva released during feeding. Dendritic cells (DC) act as immunological sentinels and interconnect the innate and adaptive immune system. They control polarization of the immune response towards Th1 or Th2 phenotype. We investigated whether salivary cystatins from the hard tick *Ixodes scapularis*, sialostatin L (Sialo L) and sialostatin L2 (Sialo L2), influence mouse dendritic cells exposed to *Borrelia burgdorferi* and relevant Toll-like receptor ligands.

Methods: DCs derived from bone-marrow by GM-CSF or Flt-3 ligand, were activated with *Borrelia* spirochetes or TLR ligands in the presence of 3 μM Sialo L and 3 μM Sialo L2. Produced chemokines and IFN-β were measured by ELISA test. The activation of signalling pathways was tested by western blotting using specific antibodies. The maturation of DC was determined by measuring the surface expression of CD86 by flow cytometry.

Results: We determined the effect of cystatins on the production of chemokines in *Borrelia*-infected bone-marrow derived DC. The production of MIP-1α was severely suppressed by both cystatins, while IP-10 was selectively inhibited only by Sialo L2. As TLR-2 is a major receptor activated by *Borrelia* spirochetes, we tested whether cystatins influence signalling pathways activated by TLR-2 ligand, lipoteichoic acid (LTA). Sialo L2 and weakly Sialo L attenuated the extracellular matrix-regulated kinase (Erk1/2) pathway. The activation of phosphatidylinositol-3 kinase (PI3K)/Akt pathway and nuclear factor-κB (NF-κB) was decreased only by Sialo L2. In response to *Borrelia burgdorferi*, the activation of Erk1/2 was impaired by Sialo L2. Production of IFN-β was analysed in plasmacytoid DC exposed to *Borrelia*, TLR-7, and TLR-9 ligands. Sialo L, in contrast to Sialo L2, decreased the production of IFN-β in pDC and also impaired the maturation of these cells.

Conclusions: This study shows that DC responses to *Borrelia* spirochetes are affected by tick cystatins. Sialo L influences the maturation of DC thus having impact on adaptive immune response. Sialo L2 affects the production of chemokines potentially engaged in the development of inflammatory response. The impact of cystatins on *Borrelia* growth *in vivo* is discussed.

Keywords: Dendritic cells, *Borrelia burgdorferi*, Tick cystatin, Signalling

Background

Borrelia burgdorferi, the causative agent of Lyme disease, is transmitted to mammals through the bite of infected *Ixodes* ticks. In the skin, dendritic cells (DC) are among the first immune cells to come into contact with *B. burgdorferi* [1]. *B. burgdorferi* elicits a potent cytokine/chemokine response through activation of multiple pattern recognition receptors on innate immune cells, including Toll-like receptor (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) [2]. TLRs have an essential role in the control of *B. burgdorferi* burden, because mice deficient in the common TLR signaling molecule myeloid differentiation primary response 88 (MyD88), have up to 250-fold more spirochetes than the wild-type controls [3, 4]. Among Toll-like receptors (TLRs), TLR-2 has been found to be the most important receptor for induction of pro-

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inflammatory mediators, whereas endosomal receptors TLR-7 and TLR-9 mediate type I interferon production [5–9]. All these TLRs utilize MyD88 as adaptor molecule, however, TLR-2 dependent inflammatory responses to *B. burgdorferi* can also be mediated by Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) [10]. *Borrelia* spirochetes activate multiple signalling pathways through these adaptors, including nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPK) (extracellular matrix-regulated kinase (Erk) 1/2, p38, Janus N-terminal kinase (JNK)) [11–13], phosphatidylinositol-3 kinase (PI3K) [14], and Protein kinase C (PKC) pathways [15]. The p38 MAPK and NF-κB are critically involved in the expression of pro-inflammatory cytokines [12, 16], whereas PI3K pathway is fundamental for optimal phagocytosis [14]. *Borrelia* also strongly induces anti-inflammatory cytokine IL-10, which has overall suppressive effect on induction of pro-inflammatory mediators [17, 18].

Dendritic cells, as a part of innate immune system, produce several cytokines and chemokines which in autocrine and paracrine manner regulate the establishment of an innate immune response, including the recruitment of monocytes, macrophages, and neutrophils [19]. In addition, DC upon sensing pathogens undergo the maturation process, characterized by increased expression of co-stimulatory molecules, which is necessary for proper presentation of antigen to naïve T-cells. *In vitro*, dendritic cells can be obtained by culturing of bone- marrow cells in the presence of two cytokines, granulocyte-macrophage colony-stimulated factor (GM-CSF) or Fms-like tyrosine kinase 3 ligand (Flt-3L), respectively. By GM-CSF, the myeloid subset of dendritic cells can be generated, while with the Flt-3 ligand, lymphoid- type of plasmacytoid dendritic cells (pDC) can be obtained [20, 21]. The pDC are characterised by robust production of type I IFN [22]. These subsets of DC differ in the cytokine profiles they induce in T cells *in vivo* [23].

Dendritic cells are key players in host defense against tick-transmitted borreliae [1]. However, many functions of DC are negatively influenced by tick saliva [24–26]. In addition to prostaglandin E2 [27], purine nucleoside adenosine [28] and Salp15 [29], tick cystatins are also involved in the effect of tick saliva on dendritic cells [30].

Sialostatins L and L2 are cysteine protease inhibitors of the hard tick *Ixodes scapularis.* Both are strong inhibitors of cathepsin L [31, 32], but sialostatin L also inhibits cathepsin S. Immunosuppressive effects of Sialo L have been demonstrated in T cell line CTLL-2 [32] and lipopolysaccharide-activated DC [33]. Expression of Sialo L2 is greatly enhanced by feeding and is necessary for tick feeding success [34]. In addition to being able to enhance the growth of *Borrelia burgdorferi in vivo* [35], this sialostatin has been shown to inhibit the inflammasome formation during infection with *Anaplasma phagocytophilum* in macrophages through targeting caspase-1 activity [36].

In order to understand how Sialo L2, a tick salivary molecule, can support *Borrelia* establishment in the host, we studied the effect of tick cystatins on DC maturation and function. The effect on the production of chemokines, IFN-β and signalling pathways activated in dendritic cells by *Borrelia* spirochetes and relevant TLR ligands was analysed.

Methods

Animals

Female C57BL/6 mice (10 weeks of age) were obtained from Charles River Laboratories. All experiments were performed with permission from Local animal ethics committee of the Institute of Parasitology, Biology Centre ASCR České Budějovice, PID 167/2011.

Bacteria

The strain of *Borrelia burgdorferi* sensu stricto obtained from ATCC collection was grown in Barbour-Stoenner-Kelly-H (BSK-H) medium (Sigma) supplemented with 6 % rabbit serum at 34 °C. The fourth passage was used in the experiments.

Preparation of recombinant cystatins

Recombinant cystatins Sialo L and Sialo L2 were expressed in *Escherichia coli* followed by purification of active protein, as previously described [31, 35]. LPS contamination was removed by Arvys Proteins using the detergent-based extraction method. The presence of endotoxin was estimated with a sensitive fluorescent-based endotoxin assay (Lonza Biologics) and was $<3 \times 10^{-14}$ endotoxin g/μg protein for both cystatins. The endotoxin level did not exceed 2 pg/ml during testing the effect of cystatins on DC.

Generation of bone-marrow-derived dendritic cells

Bone-marrow derived conventional dendritic cells (DC) and plasmacytoid (pDC) dendritic cells were prepared as described before [20, 21], respectively, with minor modifications. Briefly, mice were sacrificed by cervical dislocation, intact femurs and tibias were removed, and bone marrow was harvested by repeated flushing with MEM. To derive conventional DC, bone marrow cells (10^6/ml) were cultured for 7 days in 6-well plate in RPMI 1640 medium supplemented with 10 % FCS, 50 mM HEPES, 2 mM glutamine, 50 μM mercaptoethanol, penicillin, streptomycin, amphotericin B, and 30 ng/ml of recombinant mouse GM-CSF (Sigma-Aldrich). Half of the medium was replaced with the fresh medium on day 3 and 5. On day 7, non-adherent cells were harvested and used as immature DC.

To analyse the effects of cystatins on DC differentiation, 10^5 bone-marrow cells were seeded in 96-well plate in the

same medium as described above (including GM-CSF) and the Sialo L or Sialo L2 were added to the culture on day 3 to final concentration 3 μM. Cells were fed on day 5 and 7, and harvested on day 9. Surface expression of MHC class II was determined by flow cytometry within CD11c-positive population.

To derive plasmacytoid cells, bone marrow cells (1.5×10^6 / ml) were cultured for 8 days in 6-well plate in RPMI 1640 medium supplemented with 10 % FCS, sodium pyruvate, glutamine, penicillin, streptomycin, amphotericin B (PAA) and 100 ng/ml of recombinant human Flt-3L (R&D Systems). Half of the medium was replaced once after 4 days of culture. On day 8, non-adherent cells were harvested, washed in fresh medium and used in subsequent experiments.

IFN-β measurement

Freshly derived pDC were seeded in 96-well plate at a concentration of 2×10^5 cells per well. Following 2 h incubation with Sialo L or Sialo L2 (each 3 μM) the cells were stimulated with spirochetes at MOI = 10 (10 spirochetes per 1 cell), imiquimod (R837, 2 μg/ml) (InvivoGen), or CpG (ODN1668, 50 nM) (Enzo Life Sciences). MOI = 10 was sufficient to activate DC as shown previously [37]. IFN-β was determined in cell-free culture supernatants harvested 5 and 16 h after stimulation using LEGEND MAX™ mouse IFN-β ELISA Kit (BioLegend) following the manufacturer's instructions.

Chemokine measurements

BMDC were seeded at concentration 0.5×10^6 or 2×10^5 cells per well in 24-well plate or 96-well plate, respectively. Next day DCs were incubated 2 h with Sialo L or Sialo L2 (both 3 μM) and then *B. burgdorferi* was added at MOI = 10. After 24 h, cell-free supernatants were collected and analysed in Proteome Profiler™ antibody array according the manufacturer's instructions (R&D). The chemokines were visualized by enhanced chemiluminescence and the abundance of signal was measured using CCD image system (ChemiDoc™ MP Imaging System) and Image Lab software, v. 4.1 (BIO-RAD). Alternatively, the amount of secreted chemokines (IP-10, MPC-1, MIP-1α, MIP-1β, and MIP-2) was determined in cell-free culture supernatants using ELISA kits (PeproTech) following the manufacturer's instructions.

Flow cytometry

Bone marrow-derived pDC were seeded on 96-well plate at the concentration of 1×10^6 cells per ml of complete culture medium with Flt-3L and pretreated with either Sialo L or Sialo L2 (both 3 μM). After 2 h, cells were activated either with imiquimod (2 μg/ml), CpG (ODN1668, 50 nM) or *B. burgdorferi* spirochetes (MOI = 10). After 24 h incubation, cells were washed once in PBS with 1 % FCS and stained for flow cytometry analysis with anti-CD11c-PE mAb, anti-MHCII-AlexaFluor700 mAb, anti-CD86-APC mAb (all from eBioscience), anti-CD11b-FITC mAb, and anti-B220-PE-Vio770 mAb (both from Miltenyi Biotech). Dead cells were excluded from analysis using propidium iodide. Flow cytometry was performed on FACS Canto II flow cytometer and data were analysed using FACS Diva software, v. 5.0 (BD Biosciences). Plasmacytoid DCs were gated from living single cells as CD11c+, CD11b- and B220+. Levels of expression of CD86 were measured as MFI of APC.

Immunoblotting

BMDC were seeded at 0.5×10^6 cells per well in 24-well plate. Next day DCs were incubated 2 h with tick cystatins (each 3 μM) prior to the addition of LTA (2 μg/ml) for 15, 30, and 60 min or *Borrelia* spirochetes (MOI = 10) for 15, 30, 60, and 120 min. Afterwards, cells were lysed in a RIPA buffer (1 % Nonidet P-40, 0.25 % sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5)) in the presence of protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin) and phosphatase inhibitors (25 mM sodium fluoride and 2 mM sodium orthovanadate). 20 μg of total proteins were separated by SDS-PAGE using an 8 % gel and then electrotransferred to Immobilon-P membranes. The blots were incubated overnight at 4 °C with the antibody recognizing phospho-NF-κB p65 (Ser^{536}), phospho-p44/42 MAPK (Erk1/2) (Thr^{202}/Tyr^{204}), phospho-p38 MAPK (Thr^{180}/Tyr^{182}), phospho-Akt (Ser^{473}), total NF-κB p65, p44/42 MAPK (Erk1/2), p38 MAPK, Akt, and β-actin (all from Cell Signalling) followed by incubation with secondary antibody conjungated with horse radish peroxidase. The proteins were visualized using enhanced chemiluminescence (Pierce), and their abundance was analysed using CCD image system (ChemiDoc™ MP Imaging System) and Image Lab software, v. 4.1 (BIO-RAD).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni test in GraphPad Prism, version 5.0 was used to compare the differences between control and treated groups. $P \leq 0.05$ was considered statistically significant and is marked by one star, $P \leq 0.01$ is marked by two stars.

Results

Sialostatin L2 decreases the MIP-α and IP-10 production by dendritic cells in response to *Borrelia burgdorferi*

Numbers of chemokines known to recruit leukocytes to the infection site are upregulated in DC during *Borrelia* infection [18, 38]. We aimed to determine the effect of cystatins on chemokine production by bone-marrow derived dendritic cells upon *Borrelia* stimulation. We utilised

proteome chemokine array to screen which chemokines are induced by *Borrelia* spirochetes, and which might be affected by sialostatins. Addition of borreliae resulted in a 3.5-fold increase of neutrophil-recruiting chemokine CXCL1 (KC), 4.5-fold rise of CXCL10 (IP-10) and 18.6-fold increase of monocyte/macrophage recruiting chemokine CCL3/CCL4 (MIP-1α/β). Two-fold rise and less was observed in case of CXCL2 (MIP-2), CCL5 (RANTES), CCL2 (MCP-1/JE), CXCL5 (LIX), and CXCL16 (Fig. 1a). The production of all tested chemokines was reduced by Sialo L2, except for KC and MIP-1γ which remained unchanged (MIP-1α/β by 23 %, MIP-2 by 18 %, RANTES by 15 %, JE by 29 %, LIX by 25 %, CXCL16 by 32 % and IP-10 by 44 %). Sialo L, in contrast to Sialo L2, did not influence either of these chemokines in the array. The effect of sialostatins on selected chemokines (MIP-1α, MIP-1β, IP-10, MIP-2, and JE) was further analysed by ELISA. The inhibitory effect of sialostatin L2 was confirmed for two chemokines; the production of MIP-1α and IP-10 was significantly decreased (Fig. 1b, c). However, we did not observe any effect of sialostatin L2 on other tested chemokines (data not shown). Interestingly, MIP-1α was inhibited also by sialostatin L. This was not seen in the proteome array likely because of using pan antibody not able to distinguish between MIP-1α and MIP-1β.

The effect of sialostatin L2 on the signalling pathways activated by LTA and *Borrelia burgdorferi* in dendritic cells

Induction of proinflammatory mediators by *B. burgdorferi* is mediated by multiple signalling pathways through ligation of several TLRs. Because TLR-2 is known to be strongly activated by *Borrelia* lipoproteins, we first tested the activation of signalling pathways upon addition of lipoteichoic acid (LTA), a ligand for TLR-2, in the presence or absence of both cystatins. The pathways important for induction of pro- inflammatory cytokines and chemokines were analysed: Erk1/2 and p38 MAP kinases, NF-κB, and PI3K/Akt pathways (Fig. 2a). Sialo L2 attenuated phosphorylation of Erk1/2 (decrease by 72 % at 60 min), while Sialo L decreased this signalling molecule by 37 % at 60 min (Fig. 2b). The activation of p38 MAP kinase remained unchanged in the presence of both cystatins (Additional file 1a). Interestingly, the activation of PI3K pathway, measured by the phosphorylation of its downstream target Akt, was decreased by 76 % in the presence of Sialo L2. No such effect was

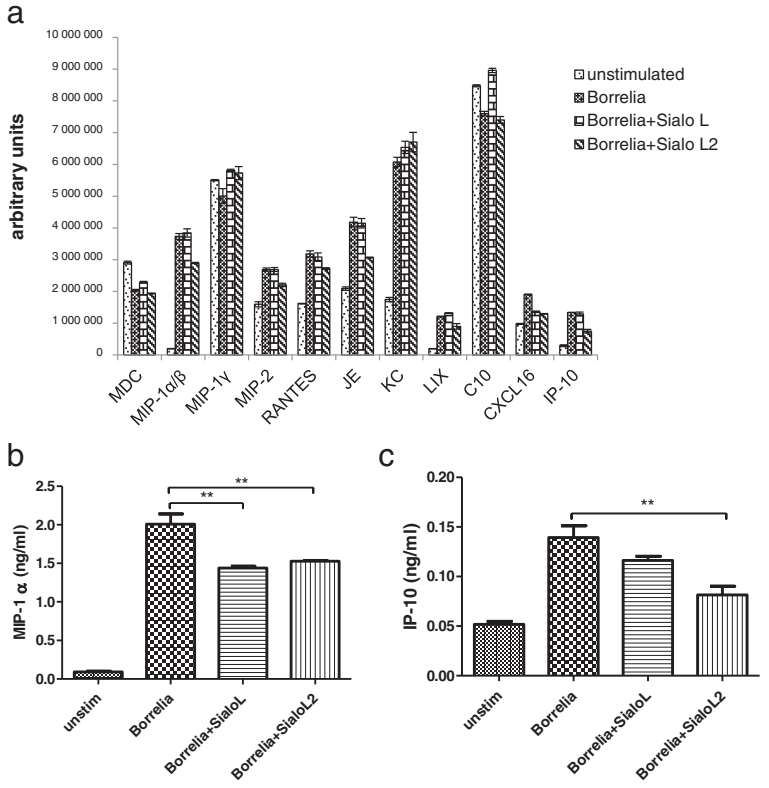

Fig. 1 Sialostatin L2 inhibits the MIP-α and IP-10 production by dendritic cells in response to *Borrelia burgdorferi*. Dendritic cells were exposed to *B. burgdorferi* at MOI = 10 in the presence or absence of sialostatin L (3 μM) or sialostatin L2 (3 μM). After 24 h, supernatants were collected and used in proteome chemokine array. The chemokines were visualized by enhanced chemiluminescence and their amount is expressed as arbitrary units (**a**). Similarly, supernatants from cells treated as described above were analysed in ELISA assay for the presence of MIP-1α (**b**) and IP-10 (**c**). Graphs (in **b**, **c**) show results of one representative experiment out of at least three independent experiments performed

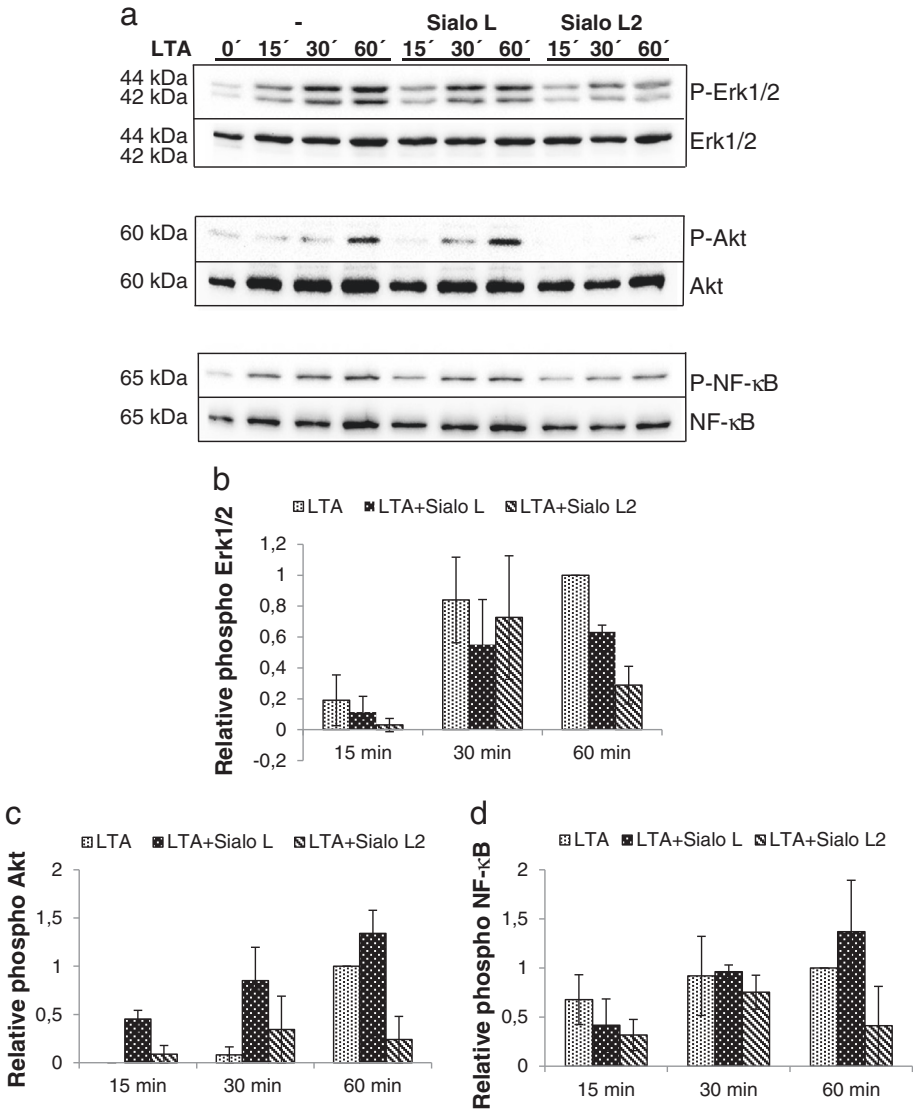

Fig. 2 Effect of sialostatins on the signalling pathways activated by LTA in dendritic cells. Dendritic cells were seeded in 24-well plate. Next day DCs were incubated 2 h with tick cystatins (both 3 μM) prior to the addition of LTA (2 μg/ml) and further incubated for indicated times. Afterwards, cells were lysed and obtained protein extract was further analysed by immunoblotting using antibodies recognizing phosphorylated form of tested kinases. Membranes were stripped and reprobed with antibodies against total kinase protein, which served as a control (**a**). Proteins were visualized by enhanced chemiluminescence. Bands were quantified using scanning densitometry and phosphorylation/activities of Erk1/2 (**b**), Akt (**c**), and NF-κB (**d**) kinases were normalized by total kinase protein level (relative activity = phospho kinase/total kinase). Three independent experiments were performed and representative blots are shown. Graphs represent the average ± SD from 2-3 experiments, the relative phosphorylation of kinase achieved at 60 min upon LTA stimulation was set up to 1 to allow pooling of data

observed in case of Sialo L (Fig. 2c). The phosphorylation of NF-κB was decreased by sialostatin L2 by 59 % at 60 min (Fig. 2d).

Similarly, dendritic cells were exposed to *B. burgdorferi* and the effect of Sialo L2 and Sialo L on signalling pathways was analysed. All tested pathways were activated with different kinetics compared to LTA. The phosphorylation of Erk1/2 kinase was impaired by Sialo L2 (decrease by 45 % at 30 min and by 22 % at 60 min) and by Sialo L by 29 % at 60 min (Fig. 3a, b). The phosphorylation of p38 MAPK, NF- κB and Akt remained unaffected in the presence of both Sialo L2 and Sialo L (Additional file 1b).

Sialostatin L decreases production of IFN-β in plasmacytoid dendritic cells activated by *Borrelia burgdorferi* and TLR-7 ligand

It has been shown that Borreliae are able to induce type I IFNs (IFN-α and IFN-β) in macrophages and dendritic cells and that this induction is mediated by endosomal TLR7/8 and TLR9 receptors [7–9]. Plasmacytoid (pDC)

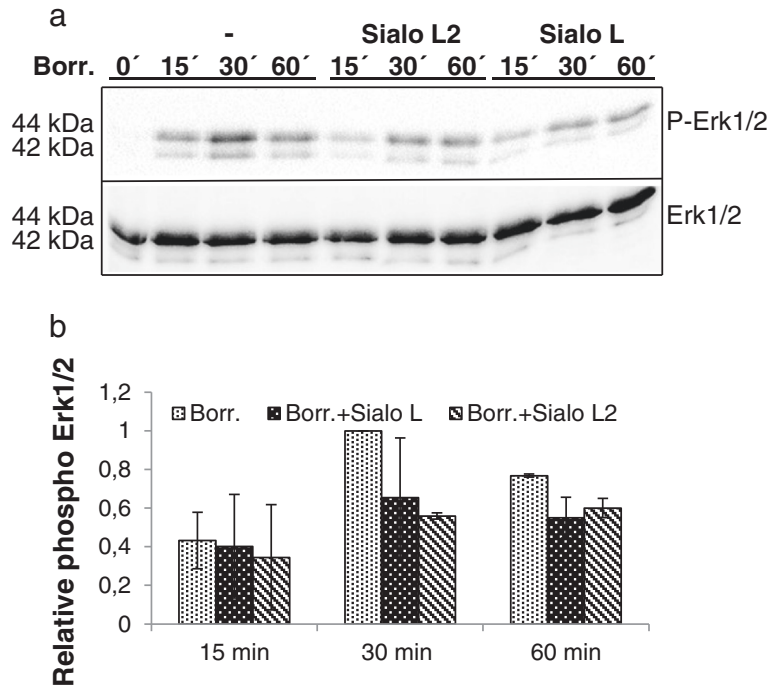

Fig. 3 Effect of sialostatin L2 and sialostatin L on the Erk1/2 signalling pathway activated by *Borrelia burgdorferi* in dendritic cells. Dendritic cells were seeded in 24-well plate. Next day DCs were incubated 2 h with sialostatin L2 and sialostatin L (both 3 μM) prior to the addition of Borreliae (MOI = 10) and further incubated for indicated times. Afterwards, cells were lysed and obtained protein extract was analysed by western blotting using antibodies against phosphorylated form of Erk1/2 and total Erk1/2 (**a**). Proteins were visualized by enhanced chemiluminescence. Relative phosphorylation/activity of Erk1/2 (**b**) kinase is shown; signal corresponding to phosphorylated kinase was adjusted to total kinase level. Three independent experiments were performed with sialostatin L2 and two with sialostatin L and representative blots are shown. Graph represent the average ± SD from 2 experiments, the phosphorylation of kinase achieved at 30 min upon *Borrelia* addition was set up to 1 to allow pooling of data

dendritic cells were chosen to examine the effect of tick cystatins on *Borrelia,* TLR-7, and TLR-9 - induced production of IFN-β. This subset of DC is known for great production of type I IFN and higher expression of TLR-7 and TLR-9 [39]. pDC were activated with *Borrelia* spirochetes, imiquimod (TLR-7 agonist), and CpG (TLR-9 agonist) in the presence or absence of cystatins and subsequently the amount of IFN-β was determined at indicated time points (chosen according to Petzke *et al.* [9] (Fig. 4). Upon addition of *Borrelia* spirochetes to cells we observed the induction of IFN-β mainly at later time point and this induction was significantly decreased by sialostatin L (Fig. 4a). Similarly, the amount of secreted IFN-β was significantly decreased by sialostatin L upon TLR-7 ligation at both tested time points (Fig. 4b). The presence of sialostatin L2 did not influence the amount of produced IFN-β. When pDC were stimulated with CpG, the production of IFN-β was more robust and increased with time. Sialostatin L decreased the IFN-β production by almost 50 % (without statistical significance), and sialostatin L2 remained without effect (Fig. 4c).

Sialostatin L negatively affects TLR-7 and TLR-9 mediated maturation of DCs but does not influence *Borrelia burgdorferi* induced maturation

Dendritic cells, upon sensing pathogen, undergo process of maturation, which is accompanied by an increase of expression of some co-stimulatory molecules, like CD86, CD40, and CD80. We wondered whether cystatins Sialo L and Sialo L2 can influence the maturation of plasmacytoid dendritic cells stimulated by *B. burgdorferi*, TLR-7 and TLR-9 ligands. The expression of co-stimulatory molecule CD86 was analysed by flow cytometry. The phenotype of pDC is shown in Fig. 5d, pDC were gated as CD11c+, CD11b-, and B220+ cells. As expected, addition of *Borrelia* spirochetes led to the increase of CD86 expression (Fig. 5a). However, the expression of CD86 increased to the comparable levels also in the presence of tested cystatins. Thus *Borrelia-* induced maturation was not affected by cystatins. On the contrary, in imiquimod-stimulated pDC, was observed small but significant decrease in CD86 surface expression in the presence of sialostatin L compared to control (Fig. 5b). Similarly, the increase of CD86 expression on DC, induced by ligation of TLR-9, was

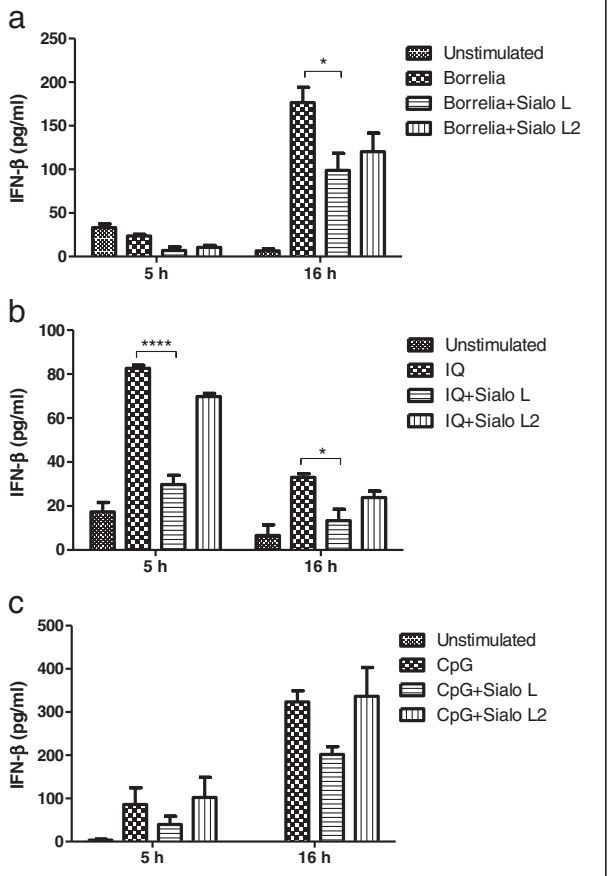

Fig. 4 The effect of tick sialostatins on the production of IFN-β in plasmacytoid dendritic cells activated by *Borrelia*, TLR-7, and TLR-9 ligands. Plasmacytoid dendritic cells were activated with *B. burgdorferi* at MOI = 10 (**a**), imiquimod (2 μg/ml) (**b**) or CpG (50 nM) (**c**) in the presence or absence of sialostatins (both 3μM). Supernatants were collected 6 and 16 h after stimulation and analysed by ELISA assay

inhibited by sialostatin L, but not sialostatin L2 (Fig. 5c). Thus sialostatin L negatively affects TLR-7, and TLR-9 mediated maturation of DC but does not significantly affect *Borrelia*-induced maturation.

Sialostatin L reduces differentiation of bone-marrow DC

As salivary molecules have an opportunity to enter bone marrow through the bloodstream, we decided to examine the influence of cystatins on the differentiation of dendritic cells from bone-marrow cells. The experiment was performed according to Sun *et al.* [40]. Bone-marrow cells were cultured (differentiated) in the presence of GM-CSF and on day 3 sialostatin L or sialostatin L2 were added to the cultures. After 8 days, cells were harvested and the expression of MHC class II molecules was determined. As shown in Fig. 6, among the CD11c positive cells, the number of MHCII positive cells reached 65.85 %. In the presence of sialostatin L the number of MHCII - positive cells decreased significantly to 43.91 %. Sialo L2 did not affect significantly the percentage of MHC class II positive cells.

Discussion

Sialo L2 and Sialo L are tick salivary cystatins, which are together with other salivary compounds released by the hard tick *I. scapularis* into the wound during tick feeding. During this process *B. burgdorferi* could be transmitted to the host. In response to *Borrelia* spirochetes, dendritic cells and other skin-resident immunocompetent cells become activated which leads to the production of proinflammatory mediators attracting further immune cells to the site of infection and activating them. These events can lead to clearing of most bacteria. It has been shown that Sialo L2, when injected intradermally into the mice, increased the burden of *Borrelia* spirochetes in the skin [35]. We hypothesized that observed effect could result from Sialo L2 evoked changes in dendritic cells function. Therefore we analysed the effect of Sialo L2 and related cystatin Sialo L on the immuno-modulatory function and signal transduction of mouse bone-marrow derived dendritic cells (DC) activated by *Borrelia* and relevant TLR ligands. We found that these two tick cystatins differentially modulate the function of DC. While Sialo L2 inhibited the production of chemokines MIP-1α and IP-10 in response to *Borrelia* spirochetes and attenuated the activation of Erk1/2, PI3K/Akt, and NF-κB pathways in response to TLR-2 ligation (the major receptor activated by spirochetal lipoproteins), the related cystatin Sialo L suppressed the production of IFN-β and attenuated the maturation and differentiation of DC.

In our *ex vivo* experiments, *Borrelia*-stimulated bone-marrow dendritic cells secreted several chemokines, including neutrophil-, monocyte/macrophage-, and T cell-recruiting chemokines, similarly as was reported by other studies [18, 38]. Sialo L2 suppressed significantly production of two chemokines, MIP-1α and IP-10. MIP-1α is a chemotactic factor for mononuclear cells, T cells, and mast cells and plays a role in differentiation of type 1 Th lymphocytes. IP-10 is a CXC chemokine and attracts, in addition to monocytes and Th1 cells, also NK cells [41]. We predict that the recruitment of these cells could be impaired by Sialo L2 *in vivo*.

Dendritic cells are among the first immune cells to come into contact with *Borrelia* in the skin [1]. Phagocytosis of *Borrelia* spirochetes leads to production of various proinflammatory cytokines [42] including chemokines. Inhibitory effect of sialostatin L2 on the production of chemokines attracting inflammatory cells into tick feeding site can lead to reduced inflammation due to tick saliva effect [43]. Reduced influx of inflammatory cells could facilitate establishment and proliferation of spirochetes in the skin [44].

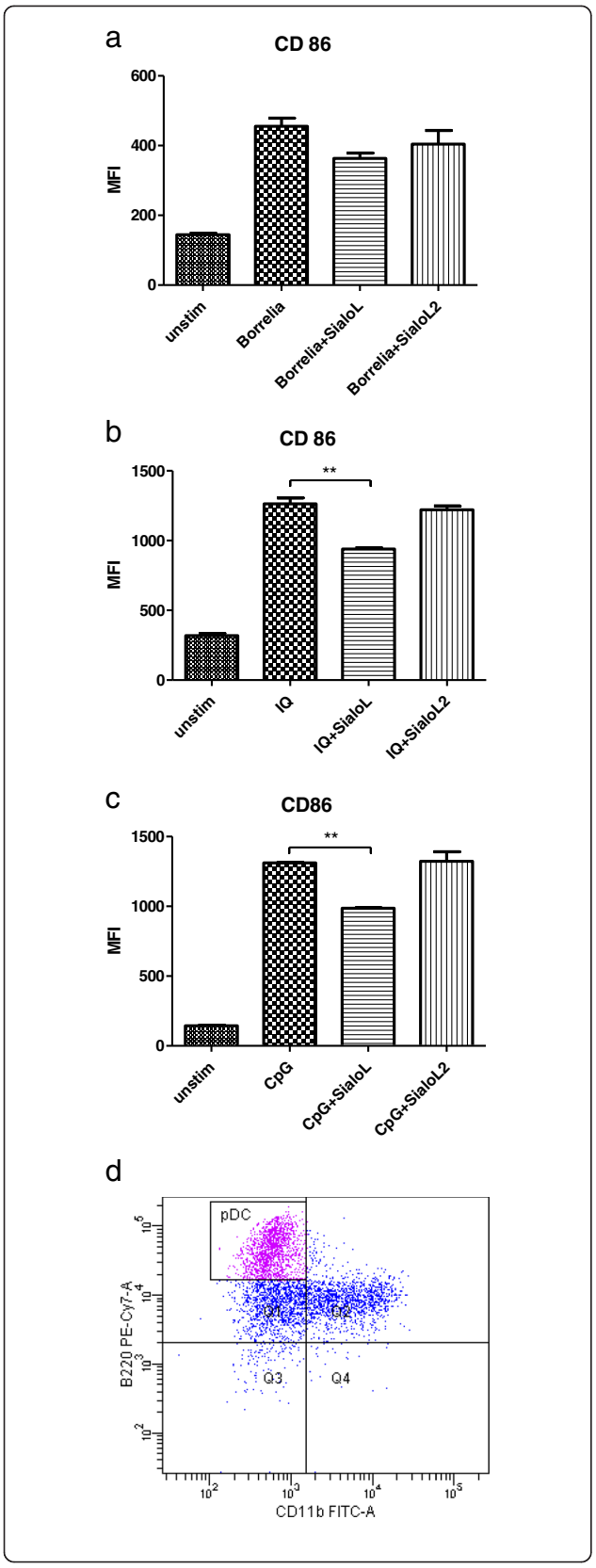

Fig. 5 Maturation of plasmacytoid dendritic cells induced by *Borrelia*, TLR-7, and TLR-9 ligation in the presence of sialostatins. Plasmacytoid dendritic cells were activated with *Borrelia* at MOI = 10 (**a**), imiquimod (2 μg/ml) (**b**), or CpG (50 nM) (**c**) in the presence or absence of sialostatins (both 3 μM). The expression of costimulatory molecule CD86 was analysed by flow cytometry among CD11c+, CD11b-, and B220+ cell population. Medium fluorescence intensity (MFI) is shown. The phenotype of plasmacytoid DC from CD11c population is shown (**d**)

Dendritic cells are equipped with several pattern recognition receptors (PRR), which sense Borreliae, including TLR, NLR, and LTR [2]. To reveal the mechanism of Sialo L2 effect on chemokine production by *Borrelia*-activated DC, we analysed the activation of chosen signalling molecules first upon TLR-2 ligation. TLR-2 is robustly activated by *Borrelia* lipoproteins [5] and critically involved in production of pro-inflammatory mediators, including chemokines. Moreover, TLR have an essential role in the control of *B. burgdorferi* burden [2, 4], which is enhanced by Sialo L2 *in vivo* [35]. The most pronounced effect of Sialo L2 on activation of tested signalling molecules in response to LTA was observed on phosphorylation of Akt, the downstream target of PI3K pathway. Interestingly, even the basal level of this kinase was decreased by Sialo L2. Consequences of PI3K pathway inhibition can be predicted. The inhibition of PI3K significantly impaired induction of chemokine and cytokine genes via TLR-2 in DC, including IP-10 [45]. Of note, PI3K pathway plays an important role in phagocytosis of *Borrelia* spirochetes by macrophages [14]. The inhibition of Akt phosphorylation was not observed by Sialo L2 in *Borrelia*-activated DC, possibly due to weak activation of this kinase.

The other pathway attenuated by Sialo L2 (in LTA and *Borrelia* activated DC) was Erk1/2 mediated cascade. Both, Erk1/2 and PI3K kinases are indispensable for induction of MIP-1α and MCP-1 in LTA stimulated murine macrophages [46]. IP-10 induction is mediated by IFNs (often produced in response to microbial products) and its upregulation is associated with the activation of JAK1, JAK2/STAT1 and MAPK pathways [47–49]. The decline of MIP-1α and IP-10 production in *Borrelia*-activated DCs by Sialo L2 could be thus mediated via inhibition of the Erk1/2 and PI3K signalling pathways. Recently, we have found that Sialo L2 attenuates IFN signalling triggered by IFN-β or LPS which leads to the suppression of interferon stimulated genes like IRF-7 and IP-10 [50]. The decrease of IP-10 production by Sialo L2 in response to *Borrelia* spirochetes could be in part also a consequence of impaired IFN/JAK/STAT signalling.

The third pathway influenced by sialostatin L2 upon LTA stimulation was NF-κB pathway. The involvement of NF-κB pathway in the induction of proinflammatory

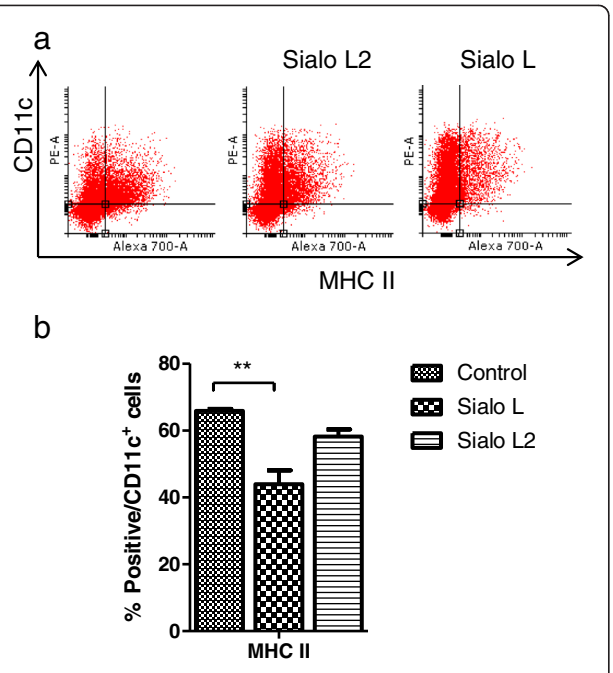

Fig. 6 Differentiation of dendritic cells from bone-marrow cells is inhibited by sialostatin L. Bone-marrow cells were cultured in the presence of GM-CSF and on day 3 the Sialo L or Sialo L2 (3 μM) were added to cultures. After 8 days, cells were harvested and analysed by flow cytometry for surface expression of MHC class II and CD11c (**a**). Differentiated cells (MHC II and CD11c positive) are shown as a percentage of total CD11c positive cells (**b**). The representative of three independent experiments is shown

mediators was documented; e.g. TLR-2/NF-κB/MAPK signalling plays a key role in IL-8 induction in macrophage cell line THP-1 exposed to *B. burgdorferi* [51]. We however did not detect any defect in the activation of this pathway in response to borreliae. Since dendritic cells sense borreliae by several PRR [2], the moderate effect of Sialo L2 on signals triggered through TLR-2 could be masked by signals triggered through other receptors.

In addition to chemokines, type I interferons are important cytokines modulating immune response to pathogens. *B. burgdorferi* is able to induce type I IFN and this induction is mediated through endosomal receptors TLR-7 and TLR-9 [6–9]. Plasmacytoid DC are major producers of type I IFN [52]. We found out that in plasmacytoid dendritic cells, the amount of produced IFN-β in response to *Borrelia* spirochetes and TLR-7 activation was decreased by sialostatin L and only weakly or not at all by sialostatin L2. IFN is pleotropic cytokine which recruits NK cells, has a direct antiviral effect on cells, and links the innate and adaptive immunity.

The down-regulation of IFN-β production by Sialo L in *Borrelia*/TLR-7/TLR-9 stimulated cells may have further consequences for the development of adaptive immune responses. In general, type I interferon directly influences the fate of CD4+ and CD8+ T cells during the initial phases of antigen recognition contributing to Th1 commitment and negatively regulating Th2 and Th17 differentiation [53]. Down-regulation of interferon can bring about an opposite effect. Moreover as sialostatin L inhibits production of IL-12 and TNF-α by DC as well as their differentiation [30], it probably leads to Th2 polarization of the immune response which is advantageous for *Borrelia* establishment in the skin [54]. In addition to modulation of the Th differentiation, type I IFNs also positively influence DC maturation [55, 56].

Indeed, we show that the maturation of plasmacytoid DC induced by TLR-7 or TLR-9 ligands was also decreased by Sialo L (judged by expression of co-stimulatory molecule CD86). When the maturation of DC was initiated by borreliae, only statistically not significant decline in CD86 expression was observed in the presence of Sialo L, presumably due to the fact that *Borrelia* spirochetes are weaker inducers of maturation then TLR ligands. In agreement, it was previously published that Sialo L inhibits the maturation of DC induced by LPS; it negatively affects the expression of the costimulatory molecules CD80 and CD86 [30]. Thus, Sialo L influenced function of dendritic cells in a different way in comparison to Sialo L2.

We did not investigate the mechanism which is behind the declined IFN-β production due to sialostatin L effect. However, since cathepsin L has been implicated in processing of TLR-9 [57], and sialostatins L and L2 are strong inhibitors of this protease [35], we could speculate that the decline of IFN-β is the result of impaired TLR-9 processing. Moreover, the amount of endogenously produced IFN-β was not affected by sialostatins in splenic DCs stimulated with TLR-4 agonist, where no processing had occurred [50].

Finally we examined the effect of tick cystatins on the differentiation/derivation of dendritic cells from bone marrow and found that Sialo L negatively affects the number of differentiated dendritic cells (MHC class II and CD11c positive cells). MHC class II molecule is necessary for the presentation of antigen to naive T-cells. As cathepsin S is implicated in the processing of the invariant chain within MHC class II antigens and sialostatin L strongly inhibits this protease [30], it seems likely that the decrease in MHC class II expression is mediated through inhibition of cathepsin S [58]. The inhibitory effect on differentiation of BMDC (measured by expression of MHC class II molecules) was also reported for cystatin rHp-CPI from murine nematode parasite *Heligmosomoides polygyrus* [40].

Conclusions

We show here that two related tick sialostatins affect different functions of dendritic cells. While sialostatin L influences the maturation of DC in part through the inhibition of IFN-β having thus an impact on adaptive immune response, sialostatin L2 affects, through attenuation of several signalling pathways, the production of chemokines engaged in the development of inflammation.

Additional file

Additional file 1: Effect of sialostatins on the signalling pathways activated by LTA and *Borrelia burgdorferi* in dendritic cells. Dendritic cells were seeded in 24-well plate. Next day DCs were incubated 2 h with tick cystatins (both 3 μM) prior to the addition of LTA (2 μg/ml) or Borreliae (MOI = 10) and further incubated for indicated times. Afterwards, cells were lysed and obtained protein extract was further analysed by immunoblotting using antibodies recognizing phosphorylated form of tested kinases. Afterwards, membranes were reprobed with antibodies against total kinase protein (a) or β-actin (b) which served as a control. Proteins were visualized by enhanced chemiluminescence.

Competing interests
We declare no financial competing interests. There are no non-financial competing interests.

Authors' contributions
JL carried out the signalling pathways experiments, participated in the design of the study and drafted the manuscript, JP performed the immunoassays, HL carried out flow cytometry experiments, AC, EC and MK contributed by design and preparation of recombinant cystatins and JK participated in the design of the study and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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5.3. Paper III

Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells

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Tick salivary cystatin sialostatin L2 suppresses IFN responses in mouse dendritic cells

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SUMMARY

Type I interferon (IFN), mainly produced by dendritic cells (DCs), is critical in the host defence against tick-transmitted pathogens. Here, we report that salivary cysteine protease inhibitor from the hard tick Ixodes scapularis, sialostatin L2, affects IFN-β mediated immune reactions in mouse dendritic cells. Following IFN receptor ligation, the Janus activated kinases/signal transducer and activator of transcription (JAK/STAT) pathway is activated. We show that sialostatin L2 attenuates phosphorylation of STATs in spleen dendritic cells upon addition of recombinant IFN-β. LPS-stimulated dendritic cells release IFN-β which in turn leads to the induction of IFN-stimulated genes (ISG) through JAK/STAT pathway activation. The induction of two ISG, interferon regulatory factor 7 (IRF-7) and IP-10, was suppressed by sialostatin L2 in LPS-stimulated dendritic cells. Finally, the interference of sialostatin L2 with IFN action led to the enhanced replication of tick-borne encephalitis virus in DC. In summary, we present here that tick salivary cystatin negatively affects IFN-β responses which may consequently increase the pathogen load after transmission via tick saliva.

Keywords *tick, dendritic cell, interferon, cystatin*

INTRODUCTION

Ticks are blood-feeding parasites that transmit many pathogens of medical importance (e.g. Lyme disease agent *Borrelia burgdorferi*, tick-borne encephalitis virus, etc.). Hard ticks feed on their host for several days and successful feeding depends on a cocktail of salivary proteins which are injected into the host (1). These proteins have antihaemostatic, anti-inflammatory and immunomodulatory properties and support transmission of pathogens (2).

Sialostatin L2 (Sialo L2) and sialostatin L (Sialo L) are inhibitors of cysteine peptidases, which have been characterized in the hard tick *Ixodes scapularis.* Both sialostatins are strong inhibitors of cathepsin L, while sialostatin L strongly inhibits also cathepsin S, a protease which plays an important role in the processing of antigens (3, 4). Sialostatin L2 is necessary for tick feeding success (5) and is one of the salivary molecules identified as saliva activated transmission factors; particularly, sialostatin L2 supports *Borrelia burgdorferi* transmission *in vivo* (6). The precise mechanism behind this effect is however not clear. In addition, it has been reported that sialostatin L2 inhibits caspase-1-mediated inflammation during *Anaplasma phagocytophilum* infection (7).

Numbers of immunosuppressive effects of sialostatin L have been demonstrated. Sialostatin L inhibited the inflammatory reaction induced by carrageenan and caused decline of the T-cell line CTLL-2 proliferation (4). Further, the asthma symptoms were severally suppressed by sialostatin L in the model of experimental asthma. The inhibition of host hypersensitivity was due to suppression of the asthma promoting cytokine IL-9 (8). With regard to dendritic cell function, sialostatin L can inhibit LPS-induced dendritic cell maturation and proliferation of Ag-specific CD4+ T lymphocytes (9).

Dendritic cells**,** known as professional antigen-presenting cells, play a critical role in initiating and modulating immune response elicited upon recognition of pathogens. Upon sensing pathogens by pattern recognition receptors, including Toll-like receptors (TLR), DCs produce several cytokines and chemokines which in turn regulate, in auto-

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crine and paracrine manner, the establishment of an innate immune response (10). DCs are producers but also key responders to IFN.

Type I IFNs, represented by IFN-α and IFN-β, play an important role in direct antiviral defence as well as linking the innate and adaptive immune responses. Type I IFNs bind to their heterodimeric IFNα/β receptor containing IFNAR-1 and IFNAR-2, both members of cytokine receptor superfamily (11). The binding of IFN to its receptor triggers the internalization of IFNAR-1 and IFNAR-2 and results in activation of associated tyrosine kinases Tyk2 and JAK1, which in turn phosphorylate signal transducer and activator of transcription (STAT)-1 and STAT-2. Upon activation, STATs bind to IFN-stimulated response elements in the promoter of IFN-stimulated genes (ISG) (11), for example IFN regulatory factor (IRF)-1 and IRF-7. Products of ISG confer to these cells an antiviral status. Type I interferons also regulate the production of CX chemokine ligand 10 (CXCL10/IP-10), as well as interleukin-12 (12).

In the previous report, we demonstrated that tick saliva suppresses IFN signalling in dendritic cells upon LPS and *Borrelia* infection (13). In this study, we analysed the possible effect of tick salivary cystatins on the host immune response through interfering with type I IFN action.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (10 weeks of age) were obtained from Charles River Laboratories. All experiments were performed with permission from local animal ethics committee.

Preparation of recombinant cystatins

Recombinant cystatins were expressed in *Escherichia coli* followed by purification of active protein, as previously described (3, 6). LPS contamination was removed by Arvys Proteins using the detergent extraction method. After this procedure, the presence of endotoxin was estimated with a sensitive fluorescent-based endotoxin assay (PyroGene recombinant factor C endotoxin detection system; Lonza Biologics); estimated presence of endotoxin was 4×10^{-5} endotoxin U/μg protein (approximately 3×10^{-14} endotoxin g/μg protein) for both cystatins. When testing the recombinants, the endotoxin level was <2 pg per ml.

Spleen dendritic cells isolation

Mouse spleens were minced with scissors, digested in RPMI containing Liberase DL (0·67 U/mL) and DNase I (0·2 mg/mL) (both from Roche) for 30 min at 37°C and passed through a 70-μm nylon cell strainer (BD Falcon). Spleen dendritic cells (sDCs) were isolated using magnetic beads conjugated with anti-CD11c (N418) Ab and MACS Column separation following the manufacturer's instructions (Miltenyi Biotec). Purified sDCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Sigma-Aldrich). Purity of isolated sDC (approximately 90% CD11c+ cells) was determined by subsequent FACS analysis.

Derivation of dendritic cells from bone marrow

Bone marrow-derived dendritic cells (bmDCs) were prepared as described before by Inaba *et al.* (14), with minor modifications. Briefly, mice were sacrificed by cervical dislocation, intact femurs and tibias were removed, and bone marrow was harvested by repeated flushing with MEM. Bone marrow cells (10^6/mL) were cultured for 7 days in 6-well plate in RPMI 1640 medium supplemented with 10% FCS, 50 mM HEPES, 2 mM glutamine, 50 μM 2-mercaptoethanol, penicillin, streptomycin, amphotericin B and 30 ng/mL of recombinant mouse GM-CSF (R&D). On day 3 and 5, nonadherent cells were depleted and 70% of medium was replaced with the fresh medium. On day 7, nonadherent cells were harvested, washed in fresh medium and used as immature DCs.

IP-10 and IFN-β measurement

Freshly isolated sDCs were cultured in 96-well plate at a concentration of 2×10^5 cells per well for 20 h. Following 2 h incubation with tick cystatins (3 μM), the cells were stimulated with LPS (100 ng/mL) (from *Escherichia coli* K-235, Sigma-Aldrich). Cell-free culture supernatants were harvested 6 h after stimulation and used for IFN-β determination by LEGEND MAX™ mouse IFN-β ELISA Kit (BioLegend) (detection limit for IFN-β was 1·8 pg/mL) or after 24 h for IP-10 determination using Murine IP-10 ELISA Development Kit (PeproTech), following the manufacturer's instructions.

Quantitative PCR – measurement of IRF-7 gene expression

Freshly isolated sDCs were cultured in 24-well plate at a concentration of 2×10^6 cells per well for 18 h. Following 2 h incubation with tick cystatins (3 μM), the cells were

stimulated with LPS (100 ng/mL) for 6 h. Cells were lysed and total RNA was isolated using Nucleospin RNA II kit (Macherey-Nagel, GmbH & Co. KG, Duren, Germany) according to the manufacturer's instructions. 1 μg of total RNA was used for transcription to cDNA by High capacity RNA-to-cDNA kit (Applied Biosystems). 1 of 10 of cDNA was added to qPCR reaction using a cycler Rotor-Gene 3000 and Rotor-Gene 6.0.19 software (Corbett Research). IRF-7-specific primers, forward 5′-CACTGAG TTCTGAACCTA-3′, reverse 5′-GTTGGTAACAGGTAG GAA-3′, and FAM-labelled probe 5′-ACCACAAGTTCTC AAACCTCATCTG-3′ with BHQ1 quencher (Sigma) were used. The relative expression of IRF-7 was determined by comparative CT method (15), where mouse-β-actin was used as a housekeeping gene (Applied Biosystems). All reactions were performed in triplicates.

Flow cytometry – surface expression of IFNAR-1

sDCs were cultured in 96-well plate at a concentration of 2×10^5 cells per well for 20 h. Following 2 h incubation with sialostatins (3 μM), the cells were stimulated with 10 U of IFN-β for 30 and 60 min. Cells were collected (using cold 5 mM EDTA in PBS) and stained with anti-mouse-IFNAR-1 antibody (clone MAR1-5A3) or with corresponding isotype control, both conjugated with phycoerythrin (BioLegend). CD11c positive cells (detected by anti-CD11c antibody conjugated with APC, eBioscience) were analysed for surface expression of IFNAR-1. Dead cells were excluded from analysis using propidium iodide. Flow cytometry was performed on FACS Canto II cytometer using FACS DIVA software, v. 5.0 (BD Biosciences) and FLOWING software 2.

Immunoblotting

Freshly isolated dendritic cells were seeded at 1×10^6 cells per well in 24-well plate. Next day, DCs were incubated 2 h with indicated concentration of tick cystatins prior to the addition of 5 U/mL of recombinant mouse IFN-β (PBL interferon source). Following stimulation, the cells were lysed in a modified RIPA buffer (1% Nonidet P-40, 0·25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl and 50 mM Tris-HCl (pH 7·5)) in the presence of protease and phosphatase inhibitors (10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM PMSF, 1 μg/mL pepstatin, 25 mM NaF and 2 mM $NaVO_3$). The protein extracts, mixed with Laemmli sample buffer, were separated by SDS-PAGE and transferred to Immobilon-P membranes. Following blocking in TBS-containing 5% fat-free milk, the blots were incubated overnight with the antibodies against phospho-STAT-1 (Tyr^{701}), phospho-STAT-2 (Tyr^{689}), STAT-1 protein, STAT-2 protein, (all from cell signalling except phospho-STAT-2 antibody, which was from Millipore) and GAPDH (Santa Cruz Biotechnology). The proteins were visualized using enhanced chemiluminescence (Pierce), and their abundance was analysed using CCD image system (Chemi-Doc™ MP Imaging System) and IMAGE LAB software, v. 4.1 (Bio-Rad).

Virus and infection

Low-passage TBE virus strain Neudoerfl (a generous gift from Prof. F. X. Heinz, Medical University of Vienna), a prototype strain of the European subtype, was used in the experiments. Bone marrow-derived DCs were seeded to 96-well plate at concentration 2×10^5 per well. 24 h later, the cells were infected by the virus at multiplicity of infection (MOI) of 10 (5 μL of virus suspension in RPMI 1640 medium with 10% FCS was added into each well). After virus adsorption for 1 h at 37°C and 5% CO_2, the cells were washed with PBS, and complete medium without or with sialostatin L2 (3 μM) was applied. After another two hours post-infection, recombinant mouse IFN-β (PBL interferon source) was added to the final concentration of 100 U per ml. At 48 h post-infection, supernatant medium from the wells was collected and frozen at −70°C. Virus titres were determined by plaque assay.

Plaque assay

The porcine kidney stable cells (PS) were used to determine virus titre according to protocol described previously with minor modifications (16). PS were grown at 37°C in L-15 medium supplemented with 3% newborn calf serum and mixture of penicillin and streptomycin (Sigma-Aldrich). Tenfold dilutions of the virus samples were placed in 24-well tissue culture plates, and PS cells were added in suspension (10^5 of PS cells per well). After incubation for 4 h, the suspension was overlayed with carboxymethylcellulose (1·5% in L-15 medium). After incubation for 5 days at 37°C, the plates were washed with PBS and the cells were stained with naphthalene black (Sigma-Aldrich). Virus titre was expressed as PFU/mL.

Statistical analysis

Student's *t*-test in Medcalc 11.2.0.0 program or one-way analysis of variance (ANOVA) followed by Bonferroni test in GRAPHPAD PRISM, version 5.0, was used to compare the differences between control and treated groups. $P \leq 0{\cdot}05$ was considered statistically significant.

RESULTS

Tick salivary cystatins attenuate IFN-β-triggered JAK/STAT signalling pathway

Here, we tested tick salivary cystatins for their potential to interfere with IFN signalling. Major signalling pathway activated by IFN is JAK/STAT pathway (11). Both, STAT-1 (consisting of isoforms α and β) and STAT-2 proteins are phosphorylated in response to IFN. Therefore, the phosphorylation of these STATs was measured in spleen dendritic cells activated by recombinant IFN-β (5 U/mL) in the presence or absence of sialostatins for indicated times. Three independent experiments were performed with sialostatin L and two with sialostatin L2. Both cystatins attenuated the phosphorylation of STAT-1 induced by IFN-β (Fig. 1a). While the most prominent decrease by sialostatin L was seen at the time point 30 min and 60 min upon IFN-β addition (76·32% decrease at 30 min (±9·74%, $n = 3$, $P = 0{\cdot}01$) and 83·44% decrease at 60 min (±18·56%, $n = 3$, $P = 0{\cdot}03$), respectively), the effect of sialostatin L2 at the same time points was 46·68% decrease ±19·24% ($n = 2$) and 54·28% decrease ±32·68% ($n = 2$), respectively.

The phosphorylation of STAT-2 was induced with similar kinetic as STAT-1, and the inhibitory effect of both sialostatin L and sialostatin L2 was observed (Fig. 1b). Sialostatin L decreased the phosphorylation of STAT-2 by 63·44% at 30 min (±12·45%, $n = 3$, $P = 0{\cdot}05$), by 59·64% at 60 min (±0·36%, $n = 3$, $P = 0{\cdot}02$) and by 42·77% at 120 min (±3·38, $n = 3$, $P = 0{\cdot}1$). The phosphorylation of STAT-2 was decreased by sialostatin L2 at 60 min by 19·9% (±4·51%, $n = 2$) and at 120 min by 21·2% (±25·67%, $n = 2$). The effect of sialostatins on IFN signalling was also tested in peritoneal macrophages; however, no differences in STAT-1 activation were observed (data not shown). The inhibitory effects of sialostatins on IFN signalling thus seem to be restricted to dendritic cells.

Tick cystatins do not influence internalization rate of IFN receptor

To understand how sialostatins attenuate STATs phosphorylation, we first tested at which step sialostatins interfere with IFN signal transduction pathway. IFN receptor is the most upstream molecule, which could be targeted by sialostatins. IFN receptor consists of two chains, IFNAR-1 and IFNAR-2. Upon ligation, IFNAR-1 is internalized and rate of its internalization influences downstream signalling [reviewed in references (17, 18)]. We determined the kinetics of receptor internalization by measuring the amount of IFNAR-1 on the cellular surface by flow cytometry. Dendritic cells were preincubated with sialostatin L or sialostatin L2 for 2 h and stimulated with recombinant IFN-β for 30 min. The amount

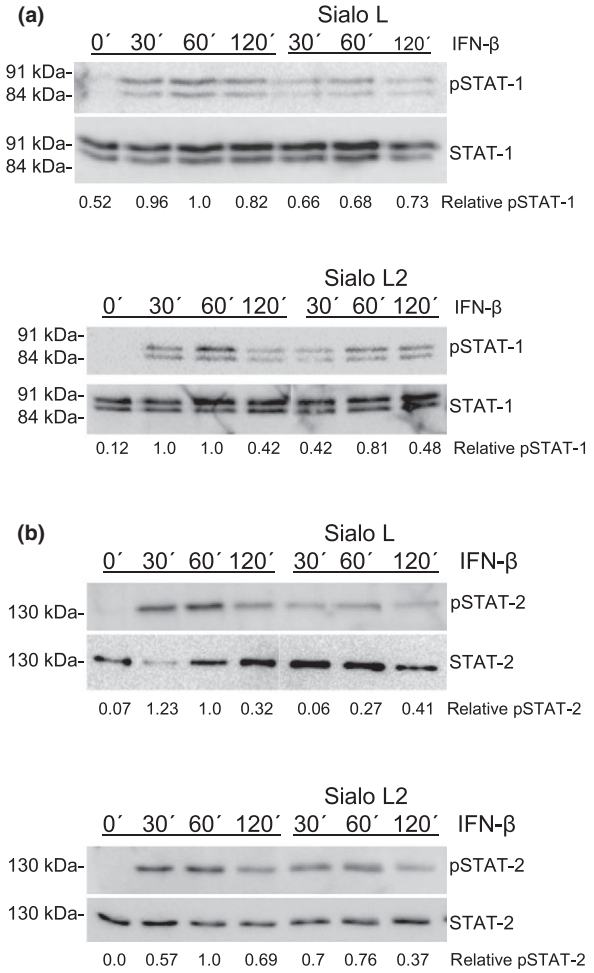


Figure 1 Tick cystatins attenuate IFN-β signalling at the level of JAK/STAT activation in dendritic cells. Spleen dendritic cells were stimulated with five units of IFN-β for indicated times in the presence or absence of 3 μM sialostatin L and sialostatin L2. Cell lysates were analysed by immunoblotting with anti-phospho-STAT-1 antibody (a) or anti-phospho-STAT-2 antibody (b). The membranes were after stripping reprobed with antibody against STAT-1 protein (a) or STAT-2 protein (b), what served as a control. Bands corresponding to the phosphorylated forms of STAT-1 (pSTAT-1) or STAT-2 (pSTAT-2) and nonphosphorylated forms of STATs were quantified using scanning densitometry. Relative phosphorylations/activities of STATs were normalized by the STAT-1 or STAT-2 protein levels, respectively (relative activity = pSTAT/STAT; densitometric measurements of both STAT-1 isoforms were pooled together). Relative activities of STAT-1 and STAT-2 achieved 60 min after IFN stimulation were set up to 1 for better illustration. Three independent experiments were performed with sialostatin L and two with sialostatin L2. Representative blots are shown.

of IFNAR-1 decreased upon IFN-β addition as expected. In the presence of either cystatins, the clearance of IFNAR-1 from cellular surface was however comparable to the control (Fig. 2a). The preincubation of cells with sialostatins for even longer time did not affect the basal surface level of IFNAR-1 (Fig. 2b, data shown for sialostatin L). This result suggests that sialostatins do not interfere with IFN signalling at IFNAR-1 level but rather influence transduction downstream of IFN receptor.

Induction of interferon-stimulated gene IRF-7 and production of IP-10 is suppressed by sialostatin L2 in LPS-activated dendritic cells

Stimulation of cells with TLR-4 agonist causes the release of IFN-β which in turn activates IFN-receptor-mediated signalling cascade and results in the induction of IFN-stimulated genes, such as the transcription factor IRF-7 and the chemokine IP-10 (19–21). We wondered whether the impairment of IFN signalling, observed in the presence of cystatins, would result in the failure to induce IRF-7 and IP-10 in LPS-stimulated spleen DC. We determined the level of IRF-7 gene expression in dendritic cells incubated with LPS for 6 h in the presence or absence of sialostatin L and sialosatin L2 (Fig. 3a). LPS induced 1·89-fold increase of IRF-7 expression as compared to nonstimulated control. While sialostatin L did not affect gene expression of IRF-7, the induction of IRF-7 was completely inhibited by sialostatin L2.

The amount of chemokine IP-10 produced by LPS-stimulated dendritic cells was measured by ELISA (Fig. 3b). LPS stimulation induced comparable level of IP-10 as 10 U of IFN-β, and the significant decrease was observed in the presence of sialostatin L2 but not sialostatin L. IP-10 thus represents another ISG, together with IRF-7, which is affected by sialostatin L2.

To exclude the possibility that observed impairment in ISG by sialostatin L2 is due to the decline of IFN-ß production, we measured the amount of IFN-β after LPS stimulation. Dendritic cells were stimulated by LPS for 6 h, and the secreted IFN-β was measured in culture

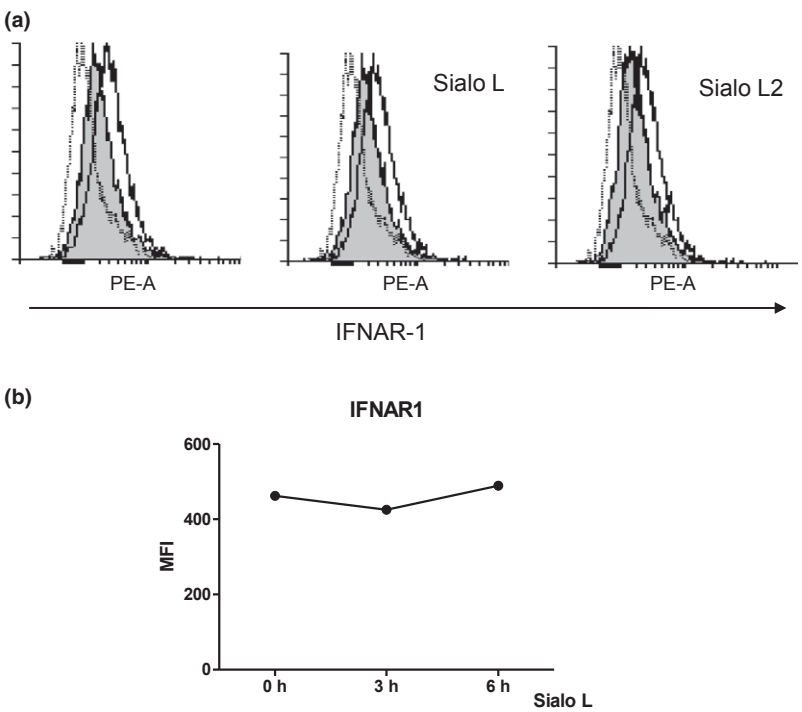
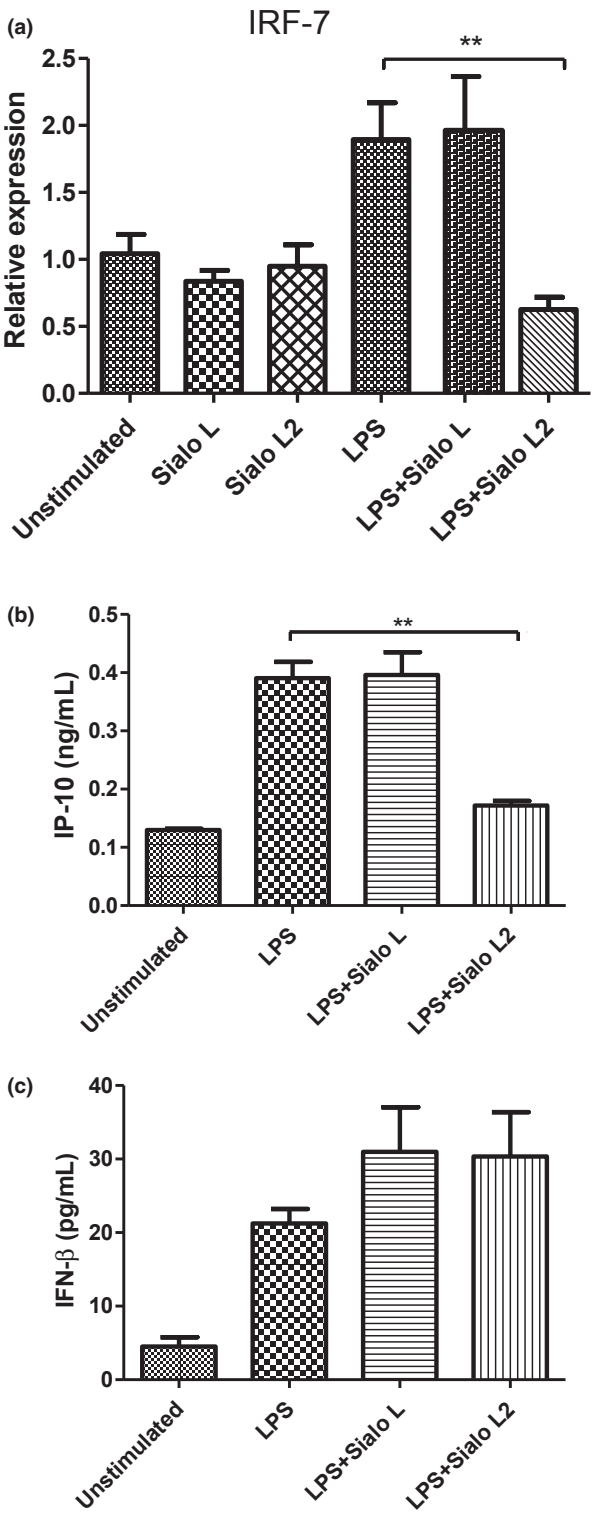


Figure 2 Sialostatins do not impact the internalization rate of the IFN receptor (IFNAR-1). (a) Dendritic cells were preincubated with 3 μM sialostatins L or L2 for 2 h and stimulated with IFN-β (10 U/mL) for 30 min. Cells were collected, stained with anti-IFNAR-1 antibody and analysed by flow cytometry. The surface expression of IFNAR-1 was determined in CD11c positive cell population. Representative histograms are shown, empty histograms with solid line represent unstimulated cells, grey-filled histograms represent IFN-β-stimulated cells, and dotted line histograms are isotype control. (b) Dendritic cells were incubated with 3 μM sialostatin L for the times as indicated. Cells were collected, stained and analysed as in A. Mean fluorescence intensity (MFI) values of IFNAR-1 are shown.



supernatant. As shown in Fig. 3c, none of tested sialostatins influenced IFN-β production in LPS-activated spleen DC.

Figure 3 Sialostatin L2 impairs induction of interferon-stimulated genes, IRF-7 and IP-10 in LPS-stimulated DC and does not affect the level of secreted IFN-β. (a) Dendritic cells were activated by 100 ng/mL of LPS for 6 h in the presence or absence of sialostatins L and L2 (both 3 μM). Transcript level of IRF-7 was determined by qPCR using specific primers for IRF-7. Expression of IRF-7 was normalized to β-actin transcript. Data from three independent experiments were pooled together and relative expression of IRF-7 is shown. (b) Dendritic cells were activated by 100 ng/mL of LPS in the presence or absence of sialostatin L and sialostatin L2 (both 3 μM). Culture supernatants were collected 24 h after ligand addition and analysed for the presence of IP-10. (c) Dendritic cells were treated as in (a). Culture supernatants were then collected and analysed by ELISA for IFN-β. Data are expressed as the mean cytokine concentration from triplicate wells ± SD. Two independent experiments were performed (for b and c), and data are shown from the representative one. **Indicates the effect of cystatin on IRF-7 induction/IP-10 production significant at $P < 0{\cdot}01$.

Sialostatin L2 promotes replication of TBE virus in dendritic cells and compromises the antiviral action of IFN-β

The above-described findings prompted us to investigate whether sialostatin L2 would impact the replication of tick-borne encephalitis virus in dendritic cells. We used Neudoerfl strain to infect dendritic cells derived from bone marrow by GM-CSF. Spleen cells were substituted by bone marrow DC because they produce less IFN-β than spleen dendritic cells and therefore are more appropriate for showing the effect of exogenously added IFN-β. Following virus adsorption, sialostatin L2 was added to cell cultures and virus titre was measured 48 h later as described in 'Materials and Methods'. As shown in Fig. 4, the replication of TBE virus was significantly enhanced by sialostatin L2. The effect of IFN-β on virus replication was determined by adding 100 U/mL of recombinant IFN-β 2 h after virus adsorption in the presence or absence of sialostatin L2. The antiviral effect of IFN-β was significantly compromised by sialostatin L2.

DISCUSSION

The effective inhibition of the host immune response is essential for feeding success of ticks and as a side effect it supports transmission and dissemination of tick-transmitted pathogens (2). Intact type I IFN signalling pathway is required for protection against viral infection. We report in this paper that tick salivary cystatin, sialostatin L2, attenuates IFN signalling. More specifically, it (i) interferes with IFN-triggered signal transduction by decreasing phosphorylation of STAT-1/2 proteins, (ii) negatively

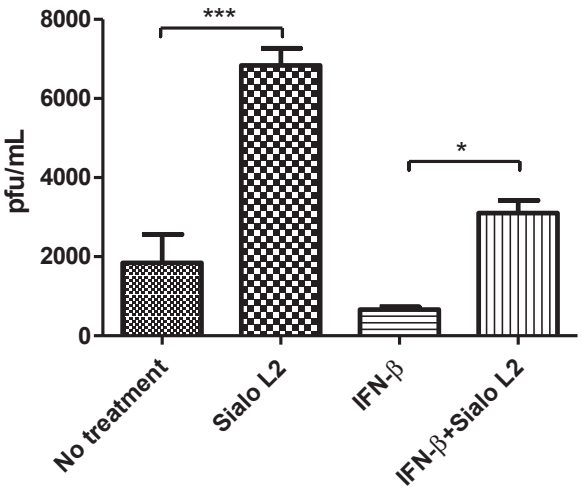


Figure 4 Sialostatin L2 promotes replication of TBE virus in dendritic cells and compromises the antiviral action of IFN-β. Bone marrow-derived dendritic cells were infected with Neudoerfl strain of TBE virus at MOI 10. After adsorption of the virus, recombinant IFN-β (100 U/mL) and/or sialostatin L2 (3 μM) were added to the cells. Following 48 h lasted incubation, the virus titre was determined as described under 'Materials and Methods'. ***Indicates the difference in virus production between control and sialostatin L2-treated cells significant at $P < 0{\cdot}001$; indicates the difference in virus production between IFN-β-treated and IFN-β+ Sialo L2-treated cells significant at $P < 0{\cdot}05$.

affects IFN-β-mediated induction of IFN-sensitive genes in LPS-stimulated cells and (iii) promotes TBE virus replication in dendritic cells *in vitro*.

Type I interferons, represented by IFN-α and IFN-β, are ones of the earliest cytokines secreted upon viral or bacterial infection, and dendritic cells are able to secret IFNs and react to them (22, 23). Dendritic cells play a key role in the recognition of pathogens, and as professional antigen-presenting cells, they determine the development of adaptive immunity. By releasing soluble mediators, including type I IFN, they influence the innate type of immunity as well.

Binding of IFN to its cognate receptor activates JAK/STAT signalling pathway and leads to the induction of IFN-stimulated genes (11). Interference of tick cystatins with IFN-receptor-triggered signal transduction was detected at the level of STATs phosphorylation. We found that STAT-1 and STAT-2 phosphorylation was decreased by both sialostatin L and sialostatin L2. Several ways how cystatins can affect the phosphorylation of STATs can be considered. The phosphorylation of STAT proteins is controlled by proteolytic processing in addition to dephosphorylation (17, 24). The degradation of STAT proteins was not observed by sialostatin L or sialostatin L2, so it is apparently degree of phosphorylation/dephosphorylation which is influenced by these polypeptides. Whether cystatins interfere directly with STATs phosphorylation or affect some upstream molecule is not clear. At least, the possibility that cystatins affect IFN signalling at the receptor level can be excluded as the internalization rate of interferon receptor (IFNAR-1) was unaffected by either cystatin. Type I IFN, when secreted from DC, not only establishes an antiviral state in themselves and other cells, but negatively effects their phenotypic and functional activation (25). In agreement, LPS-induced maturation of bone marrow DC was impaired by sialostatin L, so defective IFN signalling, observed by us could contribute to this effect (9).

Immunomodulatory effects of several cystatins from various parasites were reported pointing to the importance of these inhibitors in parasite–host interaction [reviewed by (26)]. The interference of parasitic cystatin with the IFN action is a novel finding. In fact, there is only one reported case showing the inhibition of IFN signalling by a cystatin, particulary cellular cystatin B (27, 28). Cystatin B is co-expressed with STAT-1 in human macrophages, inhibits IFN-β response by preventing phosphorylation of STAT-1 and is associated with increased HIV-1 replication in human macrophages. Interestingly, these effects are likely mediated by cystatin interaction with proteins lacking any proteolytic function (28). Thus, cystatins, although primarily recognized as inhibitors of cysteine proteases, can function as regulatory proteins.

In DC, the stimulation with TLR-4 agonist leads to the production of IFN-β. Released IFN-β binds to IFN receptor and induces the expression of IFN-stimulated genes, such as the transcription factor IRF-7 and the chemokine IP-10 (20, 21, 29). We show that the induction of both, IRF-7 and IP-10, was impaired by sialostatin L2 in LPS-stimulated dendritic cells. IRF-7 is an important transcription factor involved in positive regulation of TLR signalling and is also required for robust IFN induction (30). Furthermore, it has been shown that IRF-7 is critical for the induction of antiviral IFN-α response during West Nile virus (another member of Flaviviridae family) infection (31). We can speculate that sialostatin L2 could similarly interfere with antiviral host response through downregulation of IRF-7.

The consequences of impaired IFN signalling for pathogen transmission/growth can be expected. And indeed, the replication of TBE virus in bone marrow dendritic cells was enhanced in the presence of sialostatin L2, possibly as a consequence of impaired IFN signalling. Moreover, the antiviral action of exogenously added recombinant IFN-β was also compromised by Si-

alo L2. To show this effect, we had to substitute spleen DC by bone marrow DC. It was due to the lack of antiviral effect of exogenous IFN-β on TBE virus replication in spleen DC (presumably because of high endogenous level of IFN-β). Thus, for mechanistic part of the study, we utilized spleen DC and the biological effect of sialostatin L2 was shown in bone marrow DC. Of note, the promotion of *Borrelia burgdorferi* 'growth' by sialostatin L2 was observed *in vivo* when intradermally injected into mice (6).

In summary, we present here a novel finding that tick salivary cystatin sialostatin L2 attenuates the interferon responses in dendritic cells. The suppression of IFN-stimulated genes induced in LPS-stimulated dendritic cells and the enhancement of the TBE virus replication in DC by sialostatin L2 have been demonstrated. The inhibitory effect of tick cystatin on interferon responses in host dendritic cells is a newly described mechanism elucidating the role of tick saliva in the transmission of tick-borne pathogens.

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6 LIST OF ABBREVIATIONS

ADO	adenosine
APC	antigen presenting cell
BLAST	basic local alignment search tool
BMDC	bone marrow dendritic cells
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CLR	C-type lectin receptor
CTL	cytotoxic T lymphocytes
CXCL	chemokine (C-X-C motif) ligand
DAMP	danger associated molecular pattern
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	deoxyribonucleic acid
HRF	histamine releasing factor
IFN	interferon
IFNAR	interferon-α/β receptor
IL	interleukin
IP-10	IFN-γ-inducible protein 10
IRAC	*Ixodes ricinus* anti-complement
ISG	interferon-stimulated genes
ISRE	interferon-sensitive response element
Ir-CPI	*Ixodes ricinus* contact phase inhibitor
IRF	interferon regulatory factor
IRS-2	*Ixodes ricinus* serpin
LPS	lipopolysacharide
LTA	lipoteichoic acid
MAPK	mitogen activated protein kinase
MCP	monocyte chemoatractant protein
MHC	major histocompatibility complex
MIF	migration inhibitory factor

MIP	macrophage inflammatory protein
MEK	MAPK/ERK Kinase
NET	neutrophil extracellular traps
NK	natural killer
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen associated molecular pattern
PGE	prostaglandin E
PKA	protein kinase A
PRR	pattern recognition receptor
RAF/MEK	rapidly accelerated fibrosarcoma/ mitogen/extracellular signal-regulated kinase
RANTES	regulated on activation, normal T cell expressed and secreted
RCL	reactive center loop
RIG	retinoic acid-inducible gene
RLR	RIG-like receptor
RNA	ribonucleic acid
SAT	saliva assisted transmission
SGE	salivary gland extract
STAT	signal transducer and activator of transcription
TCA	thymus-derived chemotactic agent
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper cell
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β

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