University of South Bohemia in České Budějovice
Faculty of Science

The effect of *Ixodes ricinus* tick serpin on the cytotoxic function of natural killer cells

MASTER THESIS

Bc. Simona Fišerová, BSc.

Supervisor**:** Mgr. Jaroslava Lieskovská, Ph.D.
Department of Medical Biology

České Budějovice 2020

Fišerová, S. 2020. The effect of *Ixodes ricinus* tick serpin on the cytotoxic function of natural killer cells. Faculty of Science. University of South Bohemia in České Budějovice. p. 43.

Annotation

The aim of my thesis was the functional characterization of IRS-4 from *Ixodes Ricinus*. Particularly the effect of IRS-4 on cytotoxic activity of NK cells was analysed. First, the optimization of calcein-AM release method, which was used for this analysis, was performed. Afterwards, the method was used for the measurement of the cytotoxic activity of NK cells. Moreover, the effect of tick serpin on the activation of NK cells by measuring the cytokine production IFN-γ was examined.

Sworn declaration

I hereby declare that I have worked on the submitted diploma thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my master thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the IS STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice 21/5/2020 Simona Fišerová

……...

Acknowledgement

Firstly, I would like to kindly thank my supervisor Mgr. Jaroslava Lieskovská, Ph.D. for constant and patient guidance through this project. Without her help, the thesis would not have been formed. I would also like to thank Ing. Eva Výletová not only for the help with cell lines but also for the ease of work in the laboratory. However, my thanks go to all those who participated in my work in any way, namely Mgr. Jan Kotál, Mgr. Adéla Chlastáková, Prof. RNDr. Jan Kopecký, CSc., Mgr. Zuzana Beránková and RNDr. Helena Langhansová, Ph.D.

Secondly, I would like to mention and thank Prof. RNDr. Libor Grubhoffer, CSc. and Univ.-Prof. Dr. Norbert Müller for the establishment and management of the Biological Chemistry program which I have been following from Bachelor studies on.

At last, I would like to thank and express my huge gratitude to my family throughout my whole studies. I do thank you.

Abstract

The main topic of my thesis was to test whether the tick serpin from *Ixodes ricinus*, IRS-4, affects the cytotoxic function of natural killer cells, hereinafter NK cells. Serpins are serine protease inhibitors present in tick saliva. It has been shown for other tick serpins that they can modulate the host immune system. NK cells are cells representing the main part of the immune system. They act as cytotoxic effector cells meaning they can kill tumor cells and virus-infected cells which is implemented by releasing of lytic granules containing granzymes and perforins. It was hypothesized that the tick serpin IRS-4 could inhibit the activity of NK cells since it strongly inhibits granzyme B activity *in vitro* and through this inhibitory activity, it could affect the enhancement of spreading of tick-borne encephalitis virus. However, our result showed that IRS-4 does not influence the cytotoxic activity of NK cells. A significant part of my thesis dealt with the optimization of calcein-AM release method, used for the testing of the cytotoxic activity of NK cells. Several parameters which could affect the method were tested.

CONTENT

1 INTRODUCTION

1.1 *Ixodes Ricinus*

Ixodes Ricinus, also known, besides others, under the quite misleading name the Sheep Thick, is an ectoparasite parasitizing on the variety of hosts such as mammals, birds and surprisingly also lizards. Developed adaptability is hugely contributing to its worldwide abundance (1). When mentioning the variability of tick pathogens, they stand in the first place, being the second in pathogen transmission after mosquitos (2; 3).

Physical description of the body is typically ovoid or pear-like with a red or brownish color. After engorgement, female abdomen turn light grey in color and such size reduction helps to delve closer to the host´s skin. Females are larger in size, usually, 3 – 4 mm, males reach the size of 2.5 – 3 mm, both when unfed. Morphologically, the tick body is divided into three main sections: the head, the body and the legs (4).

Nowadays ticks are taxonomically classified as *Arthropoda*, class *Arachnida*, subclass *Acari*, superorder *Parasitiformers*, order *Ixodida*, superfamily *Ixodoidea* (5) which is further split into three families namely *Ixodidae*, *Argasidae* and *Nuttallielidae*. The *Ixodidae* are family characterized by the presence of scutum or hard shield, so-called hard ticks. The lack of this feature in the other family, *Argasidae*, results in the naming soft ticks. *Ixodidae* are divided into two groups, *Prostriata* with one genus *Ixodes* and *Metastriata* with 11 remaining genera. Family *Nuttallielidae* contains one species only with features of hard as well as soft ticks, plus some characteristics typical for the mentioned family (6).

Formerly mentioned differences in soft and hard ticks are not only in the body constitution but also in the way of feeding. The *Ixodidae* are firmly attached to the host and stay in the position for days or weeks till the feeding is complete. Fixed attachment is supported by the formation of so-called cement which helps with the anchoring of the suction system in the wound. The weight of such feeding ticks could be enlarged by 100 times. On the other hand, the *Argasidae* stay attached to the host for a quite short time compared to the *Ixodidae*, feeding process is completed within hours and the bodyweight could be enlarged only 5 to 10 times (2).

In order to initiate the feeding and to complete it successfully, salivary materials containing coagulants, painkillers, and substances suppressing the host immune system are injected into the host´s bloodstream (7). Within such salivary fluid mixture, pathogens like Thogoto virus (8), TBEV (9; 10), *Borrelia burgdorferi* (11; 12; 13; 14), Powassan virus (15), African swine fever virus (16), *Francisella tularensis* (17) and *Rickettsia conorii* (18) are transmitted to the

host during feeding. Nevertheless, it usually takes several hours before the salivary fluid is released to the host bloodstream and before the blood is taken (1).

1.2 Tick salivary glands and saliva

As blood-feeders, ticks have developed innumerable amount of strategies on how to stay closely and firmly attached to the host´s skin. The main role in the duration of feeding plays tick saliva, representing the biological material suppressing a host´s immune system and thus allowing successful blood meal. Suppression of the host´s immune system is implemented by cytolytic, anti-inflammatory and vasodilator activities. Saliva is secreted by salivary glands and when salivary glands are invaded by tick-borne pathogens, transmission via saliva occurs. Thus, salivary glands play a critical role in the transmission of pathogenic microorganisms to vertebrate hosts, pathogenic microorganisms exploiting tick salivary molecules to reach the host (19).

Structure of salivary glands

In both families, *Ixodidae* and *Argasidae*, female salivary glands consist of a large number of 3 different types of alveoli (I, II and III). All types are present in the *Ixodidae*, type I and II are present in the *Argasidae* and type IV is additionally present in the *Ixodidae* males. Alveoli of type I are agranular, being part of the anterior part of the main salivary duct. Alveoli of type II and III are associated with secondary and tertiary ducts, respectively (2). Such a brief description is for the purpose of my thesis efficient, detailed structure description can be found in literature sources (2).

Function of salivary glands

Tick salivary glands do not play a key role while feeding period only, but also during off-host periods. Such diverse off-host functions include absorption of the moisture from the unsaturated atmosphere, concentration of already consumed blood meal into nutrients by the elimination of excess fluid, and most importantly production of the cement that ensures fine attachment of tick hypostome into the host skin (19). The moisture absorbance from surroundings is crucial to avoid death due to desiccation, especially in already mentioned off-host periods. The process is associated with actively involved type I acini and includes the formation of Na^+, K^+ and Cl^- rich hygroscopic solution on the surface of mouthparts which is afterwards swallowed with absorbed moisture (2). Moreover, the accumulation absorptive property of type I acini was documented by an experiment using a fluorescent dye Rhodamide 123 in *I. scapularis* females (20).

1.2.1 The role of tick saliva in the transmission

Ticks, as already mentioned, are hematophagous arthropods that could act as vectors of pathogens such as viruses, parasites and bacteria (1; 21; 22). Their longevity, high-reproductive potential and a large host variety promote the role of successful disease vectors. For most tick-borne pathogens (TBP), salivary glands and saliva play the key role in the transmission process (19). In addition, resolution of composition of tick saliva with its active compounds present would be of extreme pharmacological importance.

While feeding, ticks incorporate the same canal which is used for saliva transmission, and since they are considered pool feeders, they use up all the fluids exuded into the haemorrhagic pool produced by the bite. This way pathogens are ingested by ticks while feeding on the infected hosts ①. From the midgut ②, through the digestive epithelium ③ and invading haemocoel ④, salivary glands ⑤ are invaded through their epithelium. Following this pathway, TBP can be transmitted to a new host throughout salivary injection ⑥ while next feeding. The whole process is depicted in the scheme below in Figure 1 (19).

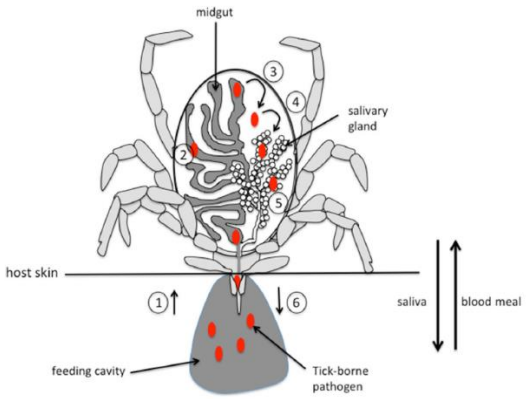

Figure 1: Schematic representation of pathogen acquisition, taken from (19)

After a tick bite, vertebrates respond with a usual 5-step skin healing process: 1) haemostatic plug formation, 2) vasoconstriction, 3) inflammation, 4) tissue remodeling and 5) healing itself. However, to assure that tick is not being rejected by the host and its long-lasting feeding, ticks have developed an opposing strategy to block the process of healing. Such complex strategy consists of blocking of pain and itch, it inhibits haemostasis and also modulates innate and adaptive immune responses together with angiogenesis and the host wound healing itself (23; 24; 25; 26; 27; 28; 29; 30; 19). It has been proven that molecules that are part of the so-called “ticks blocking-strategy” help to create an environment suitable for TBP spread (31; 25).

1.2.2 Pathogen transmission – SAT

SAT (saliva-assisted transmission) is a phenomenon of the enhanced transmission of pathogens in the presence of tick saliva which is observed when pathogens are transmitted from the vector to its host. The very first documented case of SAT effect was described for transmission of Thogoto virus. The experiment was based on the comparison of a number of nymphs that got infected when feeding on the guinea pig host. Hosts were then injected with Thogoto virus at particular feeding spots – with either virus alone or with the addition of salivary gland extract from *Rhipicephalus appendiculatus*. The presence of the extract resulted in ten times higher number of infected nymphs (8).

Host anti-tick (tick blocking strategy) immunity influences the transmission as well as an acquisition of TBP. For instance, repeated exposure of hosts with pathogen-free *Ixodes scapularis* nymphs resulted in protection against *Borrelia burgdorferi*, which lead to an assumption that immunity against tick salivary antigens can disrupt *Borrelia* transmission (32; 33). In addition, immunization of guinea pigs with salivary gland proteins from *I. scapularis* produced during the first 24 hours of tick feeding interfered with *Borrelia* transmission as well (34). Many other pathogens transmitted by ticks are mentioned in the introduction part, chapter 1.1, including TBEV.

Today, a relatively few tick molecules that take part in the transmission of pathogens to host has been characterized. For instance, SAT factors for *Borrelia burgdorferi* are Salp15 Iscap, Salp 15 Iric-1, TSLPI, TSLPI Iric and Sialostatin L2 (35). SAT factors for TBEV were not identified yet. The only tick salivary molecule, which increased replication of TBEV in dendritic cells *ex vivo* is Sialostation L2 from *Ixodes scapularis* (36). However, no related molecule from *I. ricinus* was found yet.

1.3 TBEV

TBEV is classified as a member of clinically and epidemiologically most important tick-borne diseases. TBEV is a member of arbovirus (arthropod-borne virus) group, which is a group referring to any virus transmitted by arthropod vectors. TBEV is a spherical lipid-enveloped RNA virus (37), belonging to the genus *Flavivirus* (family *Flaviviridae*) which includes over 70 viruses. Considerable arbovirus group is responsible for human pathogens such as dengue virus, Japanese encephalitis virus, St. Lous encephalitis virus and yellow fever (38). Typical vectors are ticks belonging to Ixodidae family, namely *I. ricinus* (European subtype of TBEV) and *I. persulcatus* (Far Eastern subtype) that transmit the virus on the target mammalian hosts (39; 40; 41).

TBEV is the virus associated with tick-borne encephalitis and even though humans are not primary hosts and do not play any role in the maintenance of TBEV in nature (37), TBEV is still a serious illness affecting the nervous system, with thousands of cases reported in humans every year across Europe (about 3000 cases) and Asia (42; 41).

Majority of cases of TBEV infection occur while feeding (after attachment to the skin) of the infected tick on the host. Nevertheless, there are also known cases of infection after consumption of infectious unpasteurized milk, especially goat milk (43). The incubation period of TBE is in average 7 to 14 days, nevertheless, after alimentary transmission of the virus, the incubation period is shortened to 3 – 4 days. Documented case in Slovenia described a case of TBE outbreak in 4 out of 4 individuals after drinking of infected raw goat milk (37).

After TBEV infection, first, the multiplication of the virus occurs (at the site of the tick infestation) from where it then reaches the lymph nodes via Langerhans cells. This is followed by further replication of the virus which is transported to the blood (viremia) which enables spreading of the virus to organs – spleen, liver, also bone marrow – releasing the virus and maintaining viremia for several days (41). For long-lasting viremia, invasion into CNS can occur and there the virus replication results in damage of infected cells (44).

1.4 Tick serpins

Protease inhibitors and their function

Serpins are inhibitors of serine proteases. Proteases are enzymes cleaving proteins to peptides and amino acids. They take part in extracellular digestion, protein degradation and tissue development. Serpins superfamily, omnipresent in nature, has over 1500 members and the most studied protease inhibitors (45).

Serine proteases are known as being part of proteolytic cascades. Serine proteases are involved not only in blood coagulation but also in complement activation. Also, neutrophils, mast cells, natural killer cells and cytotoxic T cells produce serine proteases, which take part not only in microbe killing but also in extracellular matrix remodelling, cytokine activation, signalling via protease-activated receptors and chemoattraction of leukocytes (46). Examples of serine proteases present in immune cells are neutrophil proteases, cathepsin G, elastase and protease 3. Mast cells serine proteases, chymases and tryptases, are associated with the pathogenesis of abdominal aortic aneurysm and atherosclerosis (46).

The role of arthropod protease inhibitors was documented, as shown by transcriptomic data by their expression in *I. scapularis* nymphs, which was reduced upon infection with *Anaplasma phagocytophilum.* Moreover, some serpins in the experiment showed dual functions – inhibitory and also up-regulatory – due to the difference in expression of protease inhibitors in salivary glands and midguts in adult females of mentioned species (47).

Arthropod serpins dispose mostly of immunoregulatory and anti-hemostatic functions as can be seen in Figure 2. In *I.ricinus*, about 36 serpins have been detected (48). Two of them, IRS-2 and Iris, have been functionally characterized and it has been shown that they affect the function of immune cells (Figure 2).

Serpin IRS-2 inhibits mast cell chymase and cathepsin G. It has an anti-inflammatory effect on paw edema induced by decreased paw swelling and neutrophil recruitment in infected animals after treatment (49). Furthermore, it has been shown that IRS-2 can influence also adaptive immunity. IRS-2 inhibits the production of IL-6 in *Borrelia burgdorferi* stimulated dendritic cells which subsequently affects, via inhibition of the IL-6/STAT-3 signaling pathway, Th17 differentiation (50). Th17 cells play a role in host defence strategies against extracellular bacteria and fungi. Th17 cells are associated with the pathogenesis of various

inflammatory and autoimmune disorders. The matter of examination of the study was to analyse whether IRS-2 affects dendritic cells and possible consequences regarding the development of pro-inflammatory cells like Th17. It was confirmed that IRS-2 shows an inhibitory effect on Th17 differentiation (50).

Iris was the first serpin in *I. ricinus* characterized by its immunosuppressive function. It showed ant-inflammatory behaviour such as suppression of coagulation and fibrinolysis (51). It also exhibits suppressive function in secretion of TNF (52). It inhibits proliferation of T cells and splenocytes. It also regulates peripheral blood mononuclear cell-derived levels (53).

IRS-4, subject of our study can inhibit granzyme B *in vitro* (unpublished, Chmelař). The effect of tick serpin on the cytotoxic effect of NK cells has not been documented yet.

Immunomodulatory functions are contributed also to cystatins, cysteine proteases. Serpins and cystatins are the two main super-families of inhibitors that are part of intracellular and extracellular processes. Those processes include cytokine activation, phagocytosis, intracellular signalling and antigen processing. Serpins and cystatins are well represented in parasites, playing an important role in interaction with their hosts (54; 55; 56).

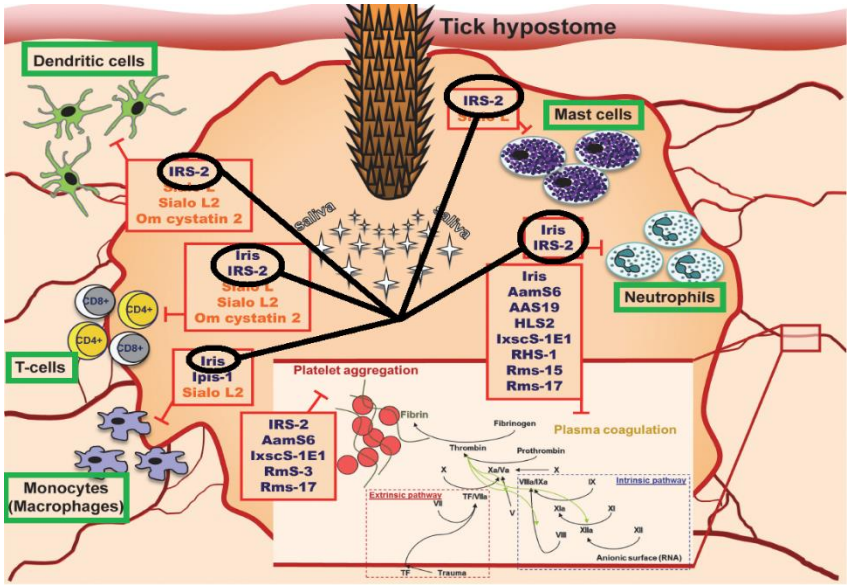

*Figure 2: Schematic representation of I.ricinus tick serpins (**highlighted**) and their targets after tick attachment, taken and adapted from (46)*

Tick serpins are important components of saliva which are released to the host during feeding. The other constituents secreted via saliva to the host are Kunitz inhibitors, Kazal domain inhibitors and trypsin inhibitor-like cysteine-rich domain (TIL) inhibitors, all disposing of anti-hemostatic features. Moreover, Kunitz inhibitors block host coagulation/platelet aggregation, and serpins and cystatins are modulating pluripotency when seen from a translational perspective (46).

1.5 NK cells

Natural killer cells are despite their lymphoid origin part of the innate immunity of an organism. They target and kill cells exhibiting infection or tumorigenic features. It was shown that NK cells recognize and kill cells infected by TBEV (57). The killing is implemented by lytic granules, specialized cytotoxic protein organelles present in the secretory lysosomes (29; 58). The recognition of endangering target cell activates the polarized exocytosis of secretory lysosomes which release the cytotoxic molecules to kill the target cell, forming a lytic immunological synapse between NK and target cells (58).

NK and CTL

Natural killer cells are together with cytotoxic T lymphocytes (CTL) part of the complementary immune mechanism of an organism related to tumors and viruses. CTLs are antigen-specific cells recognizing peptides derived from tumor and virus antigens presented by MHC I (major histocompatibility complex) (59; 60; 58). The MHC I cell surface expression is in many cases down-regulated by tumorous and virus-infected cells, causing pathway for such cells to escape CTL killing (61). Nevertheless, even such cells could be recognized by NK cells, the MHC class I molecules are recognized by cell inhibitory receptors which inhibit the cell activation on NK cells. On the other hand, deficiency of receptors activity can activate NK cytotoxicity (62; 63; 64).

Secretory lysosome exocytosis

Two main cytotoxic proteins present within secretory lysosomes are the granzymes and perforins utilizing the two major functions of secretory lysosome organelle – degradative and regulated exocytosis (65; 58). Target cell recognition activates secretory lysosome exocytosis releasing perforins, which enables the entry of granzymes into the target cell cytoplasm, where targets like caspases are cleaved which results in the cell death (66; 67).

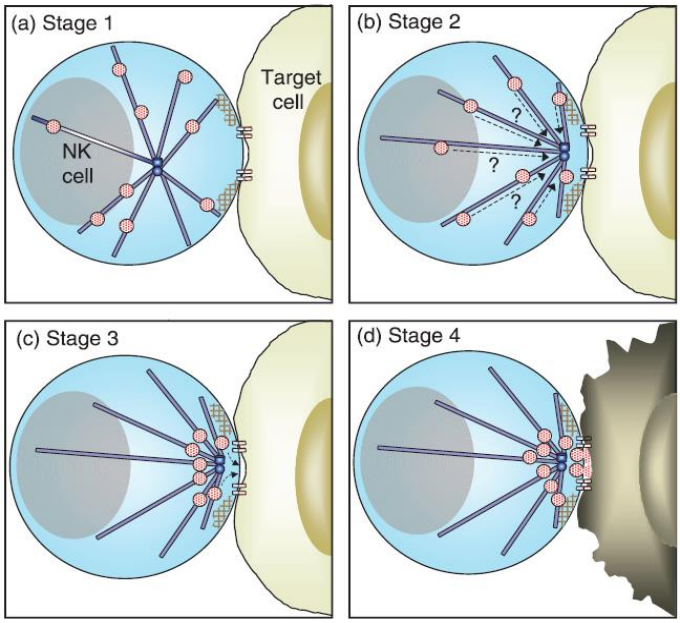

Figure 3: Secretory lysosome exocytosis depicted in 4 stages; taken from (58)

Secretory lysosome exocytosis is a highly ordered and regulated process to ensure a proper run of killing performed by NK cells. Such process could be simplified and divided into 4 stages shown in Figure 3 (58).

In a first stage, an activating lytic immunological synapse is formed at the point of attachment with the target cell and rearrangement of the actin cytoskeleton follows. During the second stage, the microtubule-organizing center (MTOC) of the NK cell are polarized and reorganized towards the lytic synapse. The third stage represents "docking" with the plasma membrane at the synapse. In the final fourth stage fusing with the plasma membrane occurs, subsequently followed by releasing of the cytotoxic content (58).

2 AIMS

1. Optimization of the calcein-AM release method for measurement of cytotoxic activity of NK cells
2. Testing of the effect of tick serpin on cytotoxic activity of NK cells
3. Testing of the effect of tick serpin on activation of NK cells (by measuring the cytokine production – IFN-γ)

3 MATERIALS

3.1 MICE

Female mice of the C57BL/6N inbred strain with the age of 6 to 8 weeks were used for the experiments. Mice were bred in the Animal Facility at the Department of Medical Biology in České Budějovice under pathogen-free conditions.

3.2 CELL LINES

3.2.1 L929

L929 cells are cells from mouse fibroma cell line. They were used for experiments before YAC-1 cells, to ensure a proper run of the experiments.

L929 cells were cultivated in DMEM medium containing high glucose enriched with 10 % bovine fetal serum (BOFES), 1 % antibiotics (ATB), 1 % L-glutamine (GL) and 0,1 % 2-mercaptoethanol (ME) at 37°C in an incubator with 5 % CO_2.

Cells were maintained for extended periods of time by cell passaging. After disposal of the old DMEM medium and two washing steps with PBS, 1 ml of the Versene-Trypsine (VT) enzyme solution was used to loosen cells from the wall of the cultivation flask. To ensure complete action of VT, the morphology of cells was being observed under the microscope. 10 ml of pre-warmed complete DMEM medium was then added and 6 ml of this cellular suspension was left in a cultivation flask and placed into an incubator with 5 % CO_2. Passaging was repeated every second day and a cultivation flask was replaced with a new one every 5 passages.

3.2.2 YAC-1

YAC-1 cells are cells of a mouse lymphoma, obtained by inoculation of Moloney Leukemia Virus (MLV) to new-born mice of an A/Sn strain. YAC-1 cells were purchased from LGC, European partner of ATCC.

YAC-cells are sensitive to the cytotoxic effect of NK (19).

YAC-1 cell line were cultivated in RPMI 1640 medium enriched with 10 % bovine fetal serum (BOFES), 1 % antibiotics (ATB), 1 % L-glutamine (GL) and 0,1 % 2-mercaptoethanol (ME) at 37°C.

Cells were maintained for extended periods of time by cell passaging, as for the L929 cells. After disposal of the old cultural medium, 10 ml of a new pre-warmed complete RPMI

medium were added and the flask was placed into the incubator with 5 % CO_2. Passaging was repeated every second day and a cultivation bottle was replaced with a new one every 5 passages.

3.3 IRS-4

IRS-4 is the serpin from *I. ricinus* (*Ixodes ricinus* serpin - 4). Briefly, this serpin was prepared as a recombinant protein and kindly provided by Dr. Chmelař. IRS-4 was cloned in pET-SUMO vector and transformed to Rosetta™ 2 Competent Cells. Autoinduction medium was inoculated with an overnight culture of bacteria (10 ml/l) and incubated at 25°C for ca 24h. Bacteria were harvested and disrupted using a cell disruptor. The soluble fraction was then purified using a HisTrap column. After the first purification, His and SUMO tags were cleaved using a SUMO protease overnight. Samples were then applied to the HisTrap column again to separate tags from the native serpin. This step was followed by Ion Exchange Chromatography and by Size Exclusion Chromatography. Recombinant IRS-4 was produced to a final concentration of 26.56 uM. The amount of LPS contamination in IRS-4 preparation was determined by LAL assay and reached 35 ng/ml.

3.4 LPS

Due to lipopolysaccharides (LPS) presence in IRS-4 preparation, controls containing the equivalent amount of LPS was prepared. LPS (L2018; 5 mg) was purchased from Sigma-Aldrich and an adequate volume of 35 ng/ml LPS was used in experiments.

4 METHODS

4.1 Calcein-AM release method

To perform the testing of the possible effect of IRS-4 on the cytotoxic activity of NK cells, a calcein-AM release method was utilized. The principle of the method is based on the measurement of the specific release of fluorescent calcein from so-called target cells (YAC-1) upon an attack of effector cells (activated NK). First target cells (YAC-1) are stained with calcein-AM. After calcein-AM is taken up into the cell, it loses its AM group due to esterases action and is converted to calcein (Figure 4). Calcein itself is

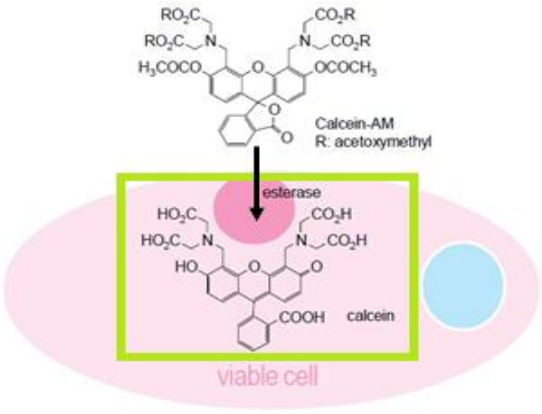

Figure 4: Conversion of calcein-AM to calcein in stained cells (adapted from Cell Counting Kit-F, catalogue no.: CK06-10)

fluorescent and cannot pass through an intact cellular membrane. Only upon attack by effector cells, the fluorescent calcein is released from target cells. The amount of released calcein is proportional to the cytotoxic activity of effector cells.

To determine 100 % release of calcein from cells, a mild detergent Triton X-100 was used for cell membrane permeabilization.

The experimental design

The experimental design is illustrated in scheme below (Figure 5). The method can be divided into several steps: 1. Staining of target cells with calcein-AM, 2. Isolation of effector cells, 3. Co-incubation of target and effector cells, 4. Detection of released calcein in cultural medium, 5. Calculation of cytotoxic activity of NK cells.

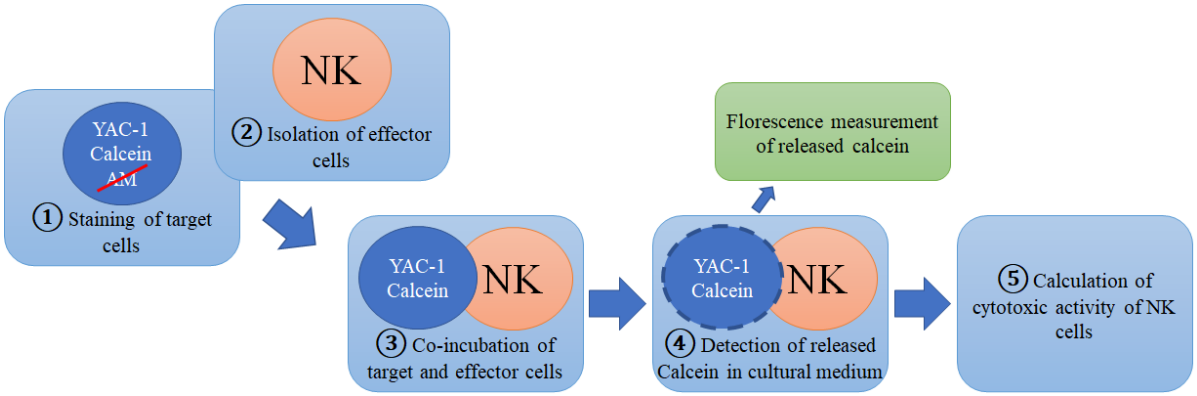

Figure 5: Experiment design of the calcein-AM release method; target cells: YAC-1 or L929, effector cells: spleen cells from Poly-IC activated mouse (further noted as NK cells)

4.1.1 Staining of target cells with calcein-AM

First, it was necessary to determine a suitable concentration of calcein-AM for staining of YAC-1 and L929 cells. All samples were prepared and treated in triplicates.

Cells with the concentration of $1x10^6$/ml were washed 2 times with PBS and resuspended in the equivalent volume of serum-free DMEM (L929)/ serum-free RPMI (YAC-1) to ensure an unchanged cells concentration. The suspension was placed into Eppendorf tubes, 1 ml each. 2 mM calcein-AM stock solution was used for the staining. 2.5 ul, 0.5 ul, 2.5 ul of 10 times diluted stock solution, 0.5 ul of 10 times diluted stock solution and 2.5 ul of 100 were taken respectively to result in aliquots with final calcein-AM concentration of 5 uM, 1 uM, 0.5 uM, 0.1 uM and 2.5 uM. Eppendorf tubes were then incubated for 15 mins at 37°C in dark. Tubes were afterwards quickly spun down, washed with PBS and resuspended in an equivalent volume of DMEM + 5 % serum (L929)/ RPMI + 5 % serum (YAC-1) to ensure an unchanged

cells concentration. Cells in solution were placed into the 96-well plate 100 ul/well. In 3 wells 100 ul of DMEM+0.1 % Triton X-100 (L929)/ RPMI+0.1 % Triton X-100 (YAC-1) were added respectively, which should result in complete release of calcein-AM, meaning its loss of AM group. Into the other 3 wells, 100 ul of an essential DMEM/RPMI without Triton X-100 were added respectively. The plate was then spun down for 3 mins at 110 x g, the supernatant was transferred into a clean 96-well plate and the fluorescence was read on Synergy H1 reader (serial number 1401161B) at excitation and emission wavelength of 485 and 520 nm. The reader set-up stayed unchanged through all measurements.

4.1.2 Spleen cells isolation (isolation of effector cells)

First, a mouse was injected intraperitoneally with Poly I:C (100 ul in 0.5 ml PBS) to stimulate proliferation of NK cells. Spleen cells from Poly I:C primed mouse serve as a source of activated NK cells. After 20 hours was the mouse put to death by cervical dislocation and spleen was taken out.

Spleen cells isolation was a procedure for obtaining effectors (NK cells). The mouse was put to death by the ligament fracture. The spleen was placed into 2 ml of the essential RPMI medium into the Petri dish.

The spleen was cut into small pieces and by using the Pasteur pipette placed throughout 70 um a strainer into 50 ml centrifugal tube. The strainer was spilt with serum-free RPMI into a final volume of 10 ml. The solution was afterwards centrifuged for 10 min at 1500 RPM at 4°C.

The supernatant was discarded, and the pellet was resuspended in 1 ml of 0.84 % NH_4Cl warmed to 37°C to lyse erythrocytes. After 2 minutes the lysis was stopped with the use of 10 ml RPMI addition. From such prepared suspension, the number of cells was calculated, and it was centrifuged again for 10 mins at 1500 RPM. Pellet was resuspended in RPMI with 5 % serum and placed into 96-well plate, 100 ul of $5x10^4$ cells per well. Such NK-enriched spleen cell population was labelled as NK cells in all following steps of the procedure.

3. Co-incubation of target and effector cells followed by (4.) detection of released calcein in cultural medium

Effectors and targets were placed into the 96-well plate with the “U” shaped bottom in the 50:1 ratio.

All samples prepared are described in the following Table 1. All samples were prepared in triplicates. The table contains the naming of all samples, concentrations of used components and ratios in which were used.

SAMPLES	LPS	IRS-4	[NK (effector cells)/100 ul]	[YAC-1(target cells)/100 ul]	E:T ratio
YAC-1 stained no Triton X-100				$5x10^4$	
YAC-1 stained + Triton X-100				$5x10^4$	
YAC-1 + NK			$2.5x10^6$	$5x10^4$	50:1
YAC-1 + NK + IRS-4		4 uM	$2.5x10^6$	$5x10^4$	50:1
YAC-1 + NK + LPS	5.25 ng/ml		$2.5x10^6$	$5x10^4$	50:1

Table 1: Samples with components concentrations and ratios; E = effector cells (NK), T = target cells (YAC-1)

All YAC-1 cells were stained as stated in chapter 4.1.1, with 5 uM calcein-AM.

Samples were prepared as shown in Table 1. 100 ul of YAC-1 cells were put together with 100 ul of NK cells (spleen cells). 30 ul of IRS-4 (at final concentration 4 uM) was added into YAC-1/NK samples, respectively. The same volume of Tris/NaCl (a solution in which IRS-4 was kept) was added into each sample. Due to LPS contamination present in IRS-4 stock (35 ng/ml), a control containing the same concentration of LPS was used as well. The final concentration of LPS then was 5.25 ng/ml.

After sample preparation, the plate was spun down for 3 mins at 110 x g and cells were co-incubated at 37°C and 5 % CO_2 for 3 hours. After incubation, the plate was spun down again for 3 mins at 110 x g, 100 ul supernatant was transferred into a clean 96- well plate and the fluorescence read at excitation/emission 485/520.

4.1.3 Specific calcein leakage calculation

The resulting specific percentual calcein leakage was calculated using the following formula:

$$\text{Specific percentual calcein leakage} = \frac{(NK+Y)-Y}{(Y+T)-Y} \times 100\ [\%]$$

Where:

- NK + Y = RFV of the effector and target cells when put together
- Y = RFV of the target cells
- Y+ T = RFV of the target cells with Triton-X 100 addition representing a complete calcein leakage

4.2 Determination of NK activation upon IL-2 stimulation

The activation of NK cells was carried out by stimulation with IL-2 (1 ng/ul) followed by measuring cytokine production (IFN-γ) using a so-called sandwich Enzyme-linked immunosorbent assay method.

4.2.1 Sample preparation including IL-2 spleen cells stimulation

Spleen cells were isolated as described in chapter 4.1.2. 170 ul of spleen cells ($2x10^5$) were plated into each well of 96-well plate. Samples were analysed in triplicates and stimulated with IL-2 to final concentration 10 ng/ml in the presence or absence of 4 uM IRS-4 (30 ul of IRS-4 stock solution was added into one well). 30 ul Tris/NaCl were added to each well of unstimulated and stimulated cells to ensure the same conditions. Besides, a sample containing 35 ng/ml of LPS was included. Cells we incubated for 24 hours and 72 hours. Afterwards, supernatants were collected and stored at -20°C freezer.

4.3 Analysis of IFN-γ production

To determine the amount of IFN-γ in our samples, DuoSet ELISA Development System – Mouse IFN-γ kit was used. The protocol provided by the manufacturer was followed with a few modifications.

First, coating of a 96-well flat-bottom was performed using Rat Anti-Mouse IFN-γ Capture Antibody diluted in ELISA Plate-Coating Buffer (from 480 ug/ml to a final concentration of 4 ug/ml). 50 ul of the diluted antibody was pipetted into each well, placed into a wet box and left overnight at RT.

The next day wells were emptied and washed as follows: 360 ul wash buffer (PBS + 0.05 % Tween 20) was put in. Wash buffer was left in wells for 1 minute and afterwards completely removed using wash-machine. This was repeated 3 times and after the last washing, the well content was removed by a quick motion. Washing was performed in the same way each time. Afterwards blocking step was performed by adding 300 ul of Reagent Diluent. The plate was closed with a lid in the wet box and left to incubate for 1.5 hours at RT. The washing step was repeated. Mouse IFN-γ (2 000 pg/ml) was prepared and diluted in a 2-dilution series in Reagent Diluent. All calibration points were prepared in duplicates. After washing, 50 ul of standards, samples and blanks were put into the wells. The plate was sealed with a foil, closed with a lid and let to incubate in a wet box at RT overnight.

After washing, 50 ul Biotinylated Goat Anti-Mouse IFN-γ Detection Antibody followed. Were added and incubated in a wet box for 2 hours at RT. Washing was followed by the addition of 50 ul conjugate Streptavidin-HRP and incubation for 20 minutes at RT at dark. Next thorough washing was performed, 4 times with 2 minutes waiting time in between. The substrate solution was prepared with Colour Reagent A and B in 1:1 ratio. 100 ul of substrate solution was put into each well, followed by incubation for 20 minutes at dark.

The reaction was stopped with the addition of 100 ul 2M H_2SO_4 to each well. The plate was gently knocked to mix the content of the wells.

Absorbance was then measured at 450 nm and 540 nm for correction.

4.4 LAL assay

The amount of LPS in IRS-4 stock solution was determined with the LAL assay.

50 ul of sample and standard were put into Eppendorf tubes in a 37°C ± 1°C block. Blank (50 ul of LAL Reagent Water) and four LPS standards (100 pg/ml, 250 pg/ml, 500 pg/ml, 1000 pg/ml) were run in duplicates. 50 ul of LAL were added to the reaction vessel. After 10 minutes, 100 ul of the substrate solution, pre-warmed to 37°C ± 1°C, were added. After 6 minutes, 100 ul stop reagent was added. The absorbance was read at 410 nm.

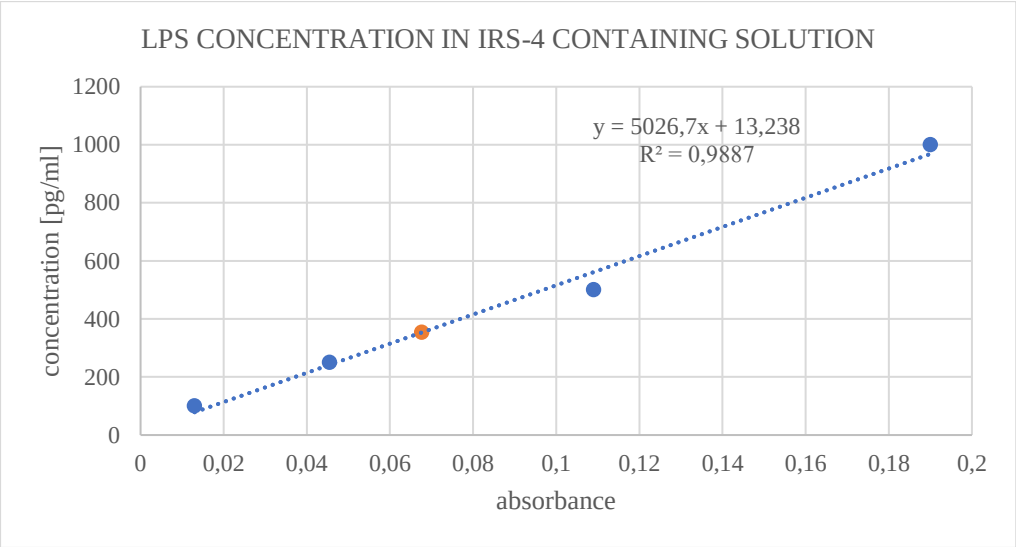

Figure 6: Calibration curve of the standard with depicted sample concentration (orange)

Determination of LPS concentration in IRS-4 stock solution was necessary so the proper controls could be included in further experiments. As shown in Figure 6, with the usage of calibration curve of the standard the LPS concentration in the IRS-4 stock solution was calculated to be 35 ng/ml (350 pg/ml, the sample was diluted 100x).

The equivalent amount of LPS, as determined by LAL assay, was added into a Tris/NaCl solution and used as a control in the main experiment.

4.5 Statistical evaluation

Statistical evaluation of the data was performed in the programme GraphPad PRISM using one-way ANOVA with unpaired t.test (chapter 5.4), Tukey´s Multiple Comparison test (chapter 5.5) and Tukey´s Post-hoc test (chapter 5.6). Statistically significant differences between groups (* $p < 0.05$ and **** $p < 0.0001$) are depicted in graphs using stars.

5 RESULTS

At first, the staining of target cells with calcein-AM had to be tested to find a suitable calcein-AM concentration. Possible side effects of phenol red, the dye present in the mediums, and the presence of LPS on calcein´s fluorescence were tested as well.

5.1 The optimal calcein-AM concentration for staining of target cells

This experiment aimed to find out which calcein-AM concentration was the most suitable for staining of target cells. L929 cells were used and the experiment was performed as stated in the methods part (chapter 4.1.1). Fluorescence was read at excitation/emission 485/520.

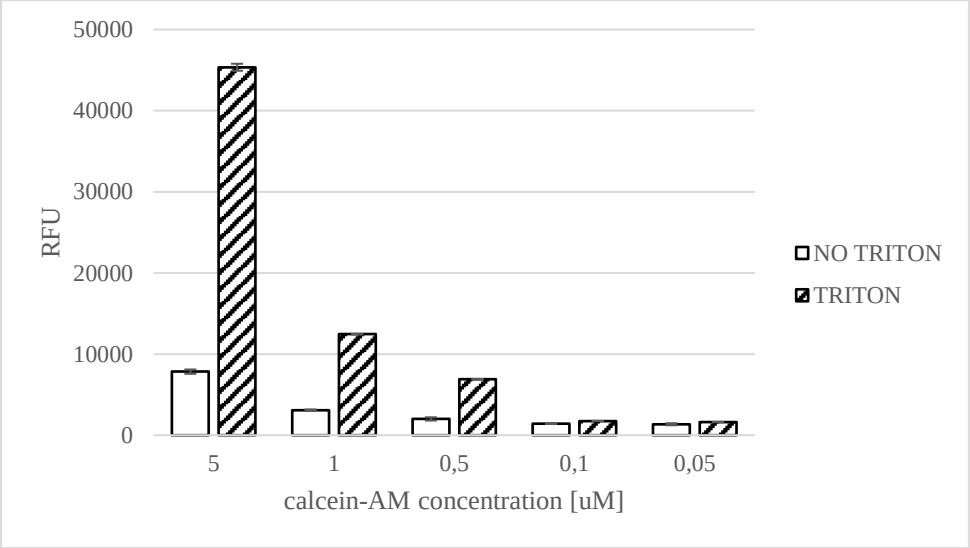

Figure 7: The optimal calcein-AM concentration for staining of L929 target cells using 5 different concentrations

As shown in Figure 7, it was found out that the optimal calcein-AM concentration was 5 uM since at this concentration the biggest difference in RF values between target cells without Triton X-100 (white) and with Triton X-100 (striped) was observed. Values of Triton X-100 treated cells represent a complete (100 %) release of calcein.

5.1.1 Kinetics of spontaneous calcein leakage from L929 and YAC-1 target cells

The kinetics of the spontaneous calcein leakage from stained L929 and stained YAC-1 cells was examined. Staining procedure described in chapter 4.1.1 was performed with 5 uM calcein-AM concentration. Stained cells were then incubated at 37°C and 5 % CO_2 for 0, 1, 2 and 3 hours. At each time point interval, the complete release was determined as well. Therefore, the first measurement was performed after 5 minutes, the time required for complete release of calcein caused by Triton X-100. Fluorescence was read at excitation/emission 485/520.

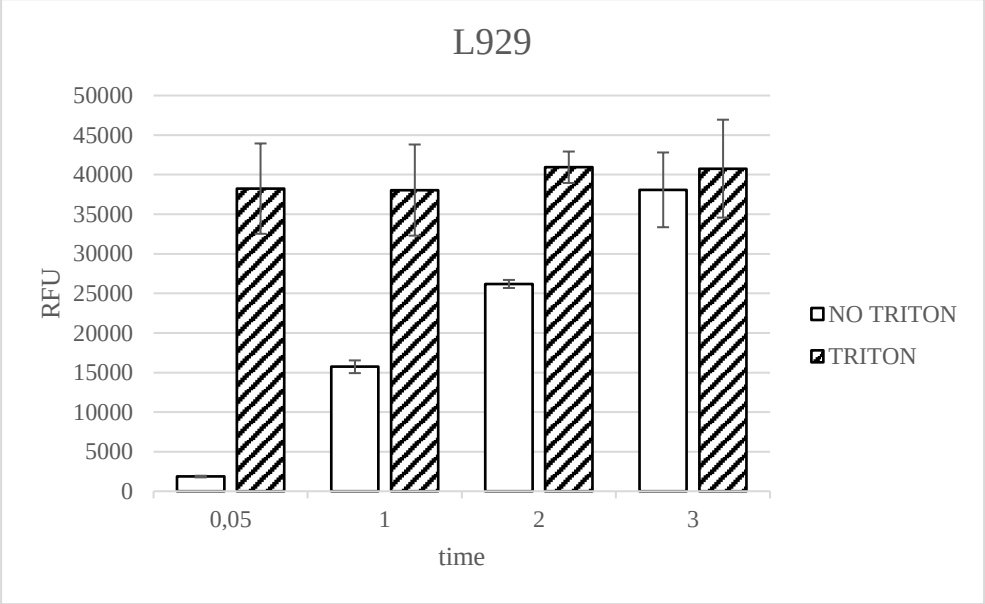

Figure 8: Kinetics of spontaneous calcein leakage from stained L929 cells (5 um calcein-AM)

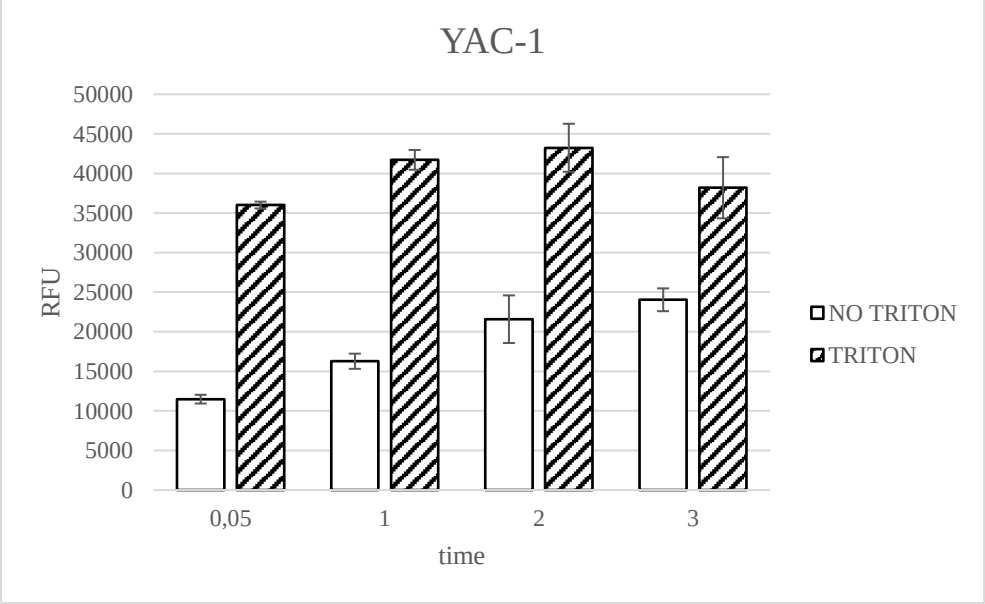

Figure 9: Kinetics of spontaneous calcein leakage from stained YAC-1 cells (5 um calcein-AM)

Data presented in Figure 8 and 9 states that the calcein leakage steadily increased with time in both cases. After 3 hours of incubation time, stained YAC-1 cells showed smaller calcein leakage compared to stained L929 cells.

5.2 The effect of phenol red on the sensitivity of YAC-1 assay

Phenol red is a dye commonly present in both DMEM and RPMI culture mediums used for culturing of L929 and YAC-1 cells. Phenol red is used as a pH indicator, so the pH of the medium is constantly indicated by its colour. However, phenol red could interfere with the sensitivity of the assay, as stated in the calcein-AM Cell Viability Assay Kit catalogue no. 4892-010-K especially with measured fluorescence signal. The effect of its presence in the medium had to be determined to ensure the best sensitivity of the assay.

The formerly mentioned staining procedure in chapter 4.1.1 was performed with 5 uM calcein-AM concentration for L929 and YAC-1 target cells, with the exchange of medium in both cases for phenol red-free.

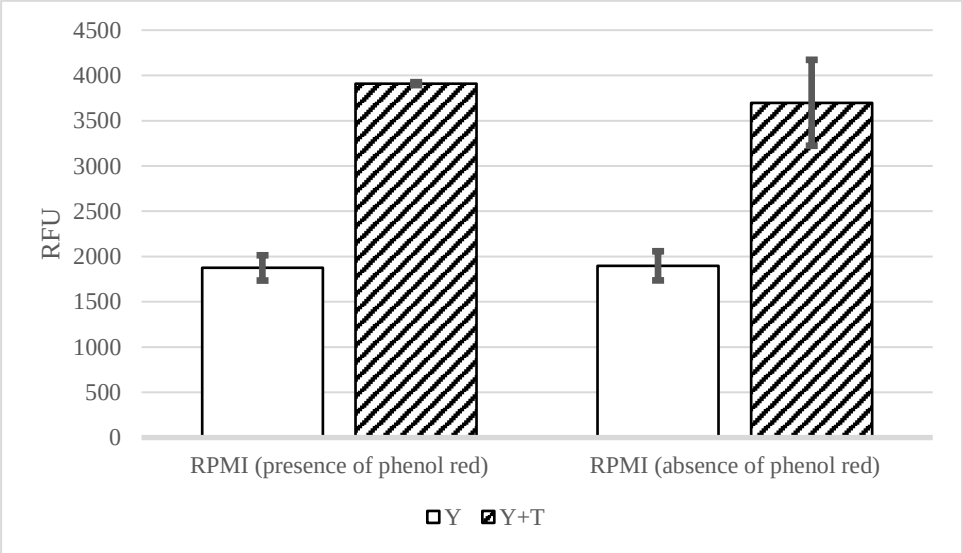

Figure 10: The effect of phenol red on staining of YAC-1

From the results presented in Figure 10, it could be concluded that the presence of phenol red in mediums did not affect the fluorescence signal of stained YAC-1 cells since no significant change in RF values was detected. Similar results were obtained for L929 assay with the usage of DMEM medium with and without phenol red (data not shown).

5.3 The effect of LPS on the sensitivity of staining method

Recombinant IRS-4 was produced in a bacterial expression system, therefore LPS contamination occurred. LPS could increase the fluorescence signal by activation of cells followed by influencing the number of calcium ions. The possible effect of LPS on the staining method was therefore examined.

It was necessary to determine the concentration in which could be LPS present in the sample devoid of any effect. Staining was performed as in chapter 4.1.1 with 5 uM calcein-AM concentration. LPS stock solution (10 ng/ul) was diluted 10 times and 1 ul was added to each sample - to stained YAC-1 samples without Triton X-100 addition and to stained YAC-1 samples with Triton X-100 addition. Measurement was performed after 3 hours incubation time.

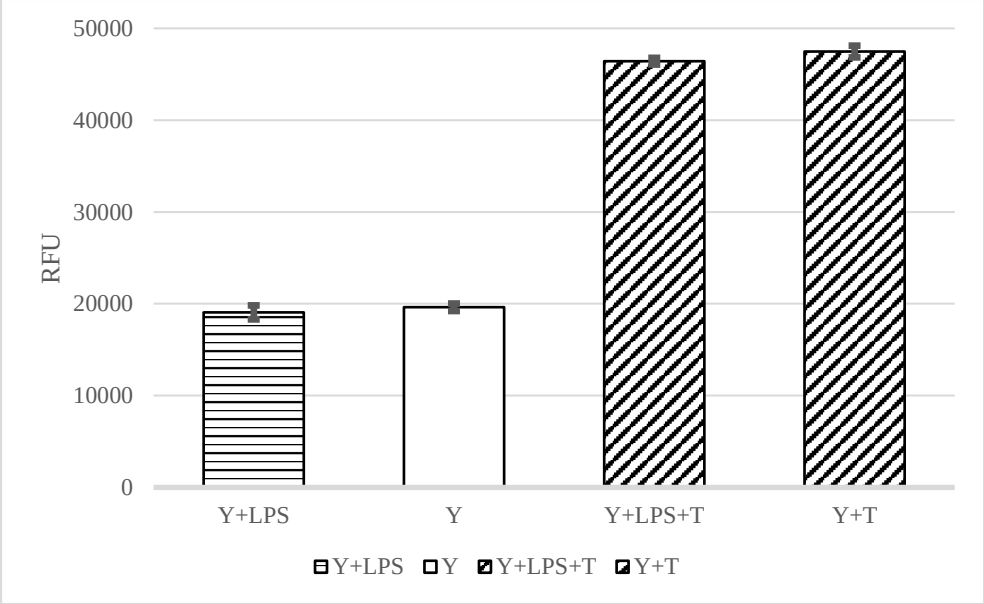

Figure 11: The effect of LPS on calcein fluorescence

The diagram in Figure 11 represents no significant change in RF values with and without LPS presence concluding that LPS did not affect the calcein fluorescence and thus did not interfere with the staining method.

5.4 The cytotoxic activity of NK cells

The cytotoxic activity of NK cells was analysed by optimized calcein-AM release method in which were mice spleen cells (NK) used as effector cells and stained YAC-1 cells as target cells. The fluorescence value reflects the amount of released calcein and corresponds to the cytotoxic activity of NK cells.

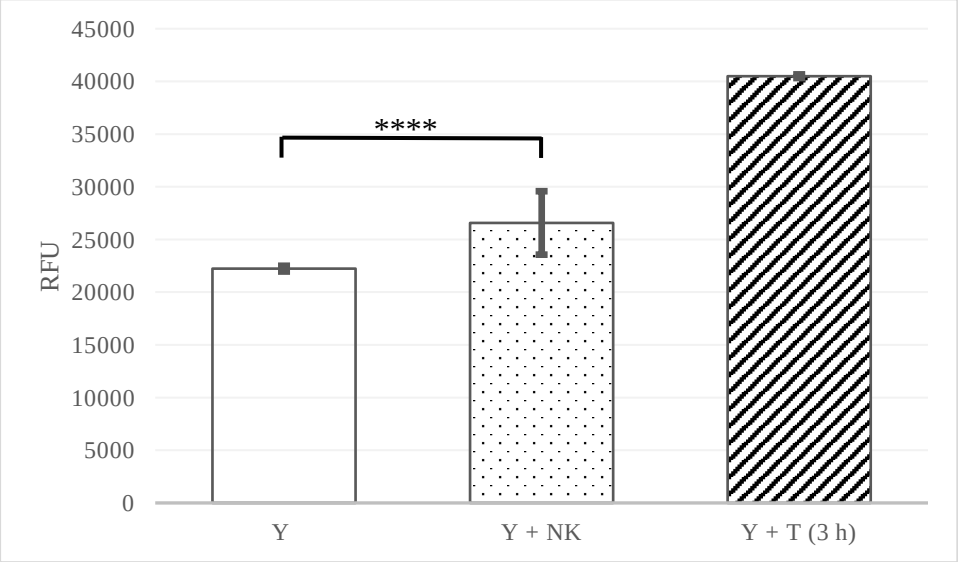

*Figure 12: RF values for YAC-1, YAC-1 with Triton X-100 addition and YAC-1/NK mixture; **** $p < 0.0001$*

Data in Figure 12 show the difference in the amount of released calcein from YAC-1 cells (0 % calcein release), Triton X- 100 treated YAC-1 cells (100 % calcein release) and YAC-1 cells co-incubated with NK cells. Co-incubation of YAC-1 cells with NK cells resulted in a statistically significant increase of calcein release (an increase of fluorescence value).

Specific cytotoxic activity of NK cells was calculated to be 23.8 %.

To ensure that calcein release observed in the presence of spleen cells was specific to activated NK cells, both stimulated and unstimulated NK cells (spleen cells from Poly I:C activated and non-activated mouse) were used.

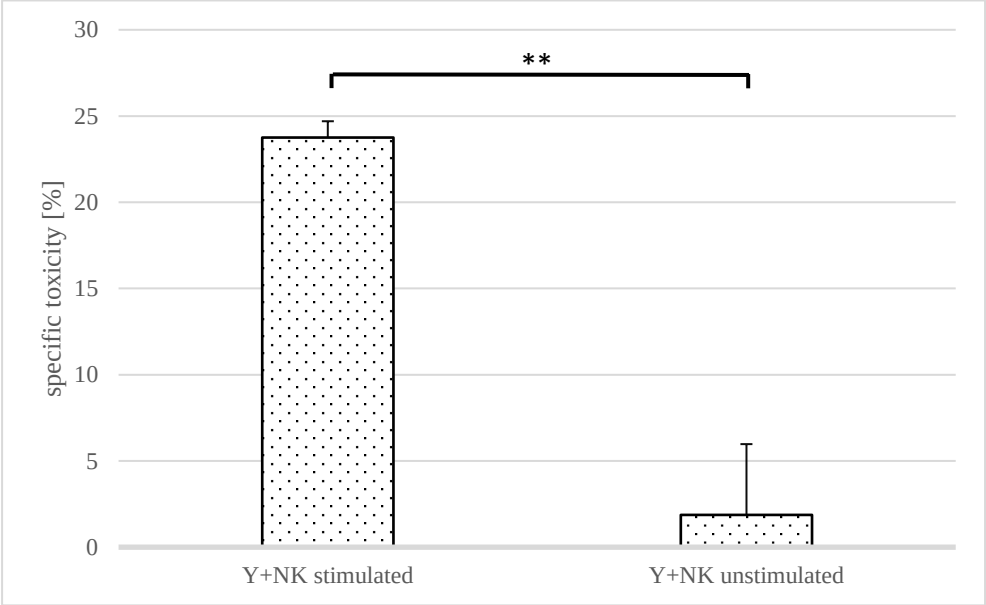

*Figure 13: Cytotoxic activity of stimulated and unstimulated NK cells expressed by specific calcein leakage [%]; ** $p < 0.01$*

The graph in Figure 13 depicts that unstimulated NK cells (1.9 %) seemed to be less active compared to activated/stimulated NK cells (23.8 %), which corresponded to our presumption.

5.5 The effect of IRS-4 on the cytotoxic activity of NK cells

The effect of IRS-4 on the cytotoxic activity of NK cells was tested. Mice spleen cells (NK) were used as effector cells and stained YAC-1 cells as target cells. To a mixture of YAC-1 and NK cells, 30 ul of IRS-4 with a final concentration of 4 uM and 30 ul of control containing an adequate amount of LPS were added. (LPS concentration in the IRS-4 stock solution was calculated to be 35 ng/ml**,** chapter 4.4).

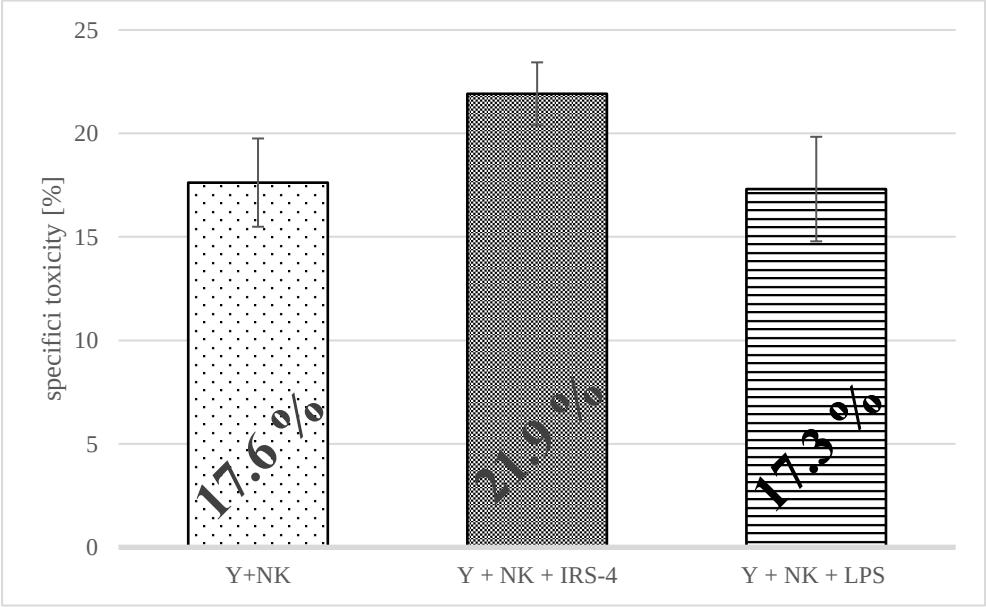

Figure 14: The effect of IRS-4 on the cytotoxic activity of NK cells expressed by specific calcein leakage [%]; NS p > 0.05

The data presented in Figure 14 are representative of results obtained from two independent experiments. Percentage of specific toxicity of NK cells is depicted in the graph, the cytotoxic activity of NK cells reached 17.6 % and was not negatively affected by IRS-4 as predicted. A slight non-significant increase of cytotoxic activity of NK cells from 17.6 to 21.9 % was observed in the presence of IRS-4.

A control containing LPS showed no difference in the percentage value of the cytotoxic activity of NK cells compared to YAC-1/NK sample. It could be therefore concluded that LPS did not affect the run of the assay.

5.6 The effect of IRS-4 on activation of NK cells by determination of INF-γ production

To test whether IRS-4 influence the activation of NK cells, we used murine spleen cells and activated them with IL-2 in the presence or absence of IRS-4. We presume that IL-2 will activate mainly NK cells. Production of IFN-γ is a feature of activated NK cells. Spleen cells were isolated, as described in chapter 4.1.2, and stimulated with 10 ng/ml IL-2, as described in chapter 4.2.1. Cells we incubated for 24 and 72 hours.

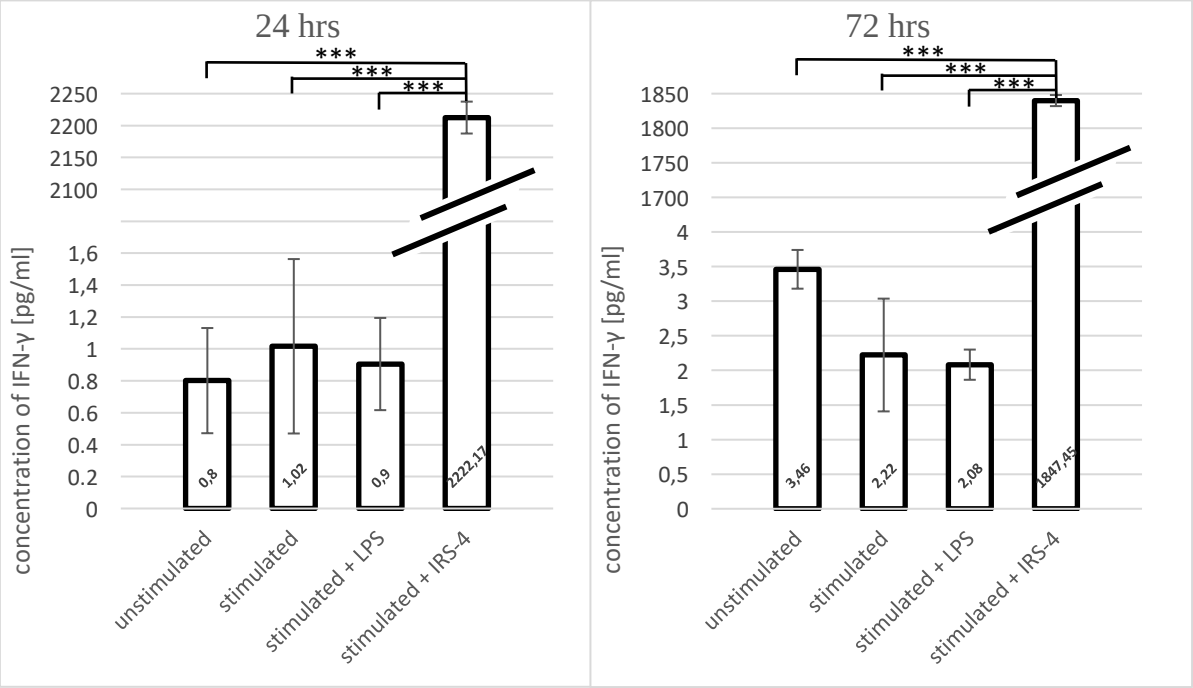

*Figure 15: Representation of corresponding concentrations to unstimulated NK cells, stimulated NK cells, stimulated NK cells with LPS and stimulated NK cells with IRS-4 (24 and 72 hrs incubation); *** p < 0.001*

According to a diagram in Figure 15 (left), after 24 hours the production of IFN-γ was not induced by IL-2, however presence of IRS-4 caused a huge increase in production of IFN-γ at both time point interval tested.

6 DISCUSSION

There are several tick molecules which are considered as transmission factors of tick-borne pathogens due to their interference with pathogens and host immune system. Such factors, so-called SAT factors, support pathogen spreading and establishment of bacterial or viral infection. The primary aim of the thesis was to functionally characterize tick serpin from *Ixodes ricinus* named IRS-4. It has been found by *in vitro* analysis that tick serpin strongly inhibits granzyme B, serine protease present in lytic granules of NK cells and cytotoxic T cells (unpublished, Chmelař). TBEV is one of many pathogens transmitted via SAT and the transmission occurs right after tick attachment (8; 9; 10). It was shown that NK cells recognize and kill cells infected by TBEV (57). Therefore, we hypothesized that if IRS-4 inhibits the cytotoxic function of these effector cells, it could potentially function as an SAT factor for TBEV.

First, we set to optimize the calcein-AM release method, a method used for assessment of the cytotoxicity of NK cells. The principle of the method lies in the measurement of the specific release of fluorescent calcein from stained target cells (YAC-1) upon an attack of effector cells (activated NK). It was necessary to determine the suitable concentration of calcein-AM which was found out to be 5 uM. The effect of phenol red and LPS presence on the staining procedure was tested as well. Phenol red is a dye commonly present in culture mediums used for culturing of target cells, LPS contamination occurred due to production of recombinant IRS-4 in a bacterial expression system. Both phenol red and LPS presence showed no negative effect on the procedure. Afterwards, the kinetics of spontaneous calcein leakage was examined in two types of target cells, L929 and YAC-1 cells. L929 fibroblasts resulted in a higher calcein leakage as shown in results chapter 5.1.1. Relatively high leakage of calcein occurred also when using K-562 cells as targets (68). Therefore, it could be stated that the leakage phenomenon is target cells dependent. And even though it was present as an unwanted side effect of the procedure, the time frame of 3 hours incubation time interval showed the satisfactory result.

After optimization of the calcein-AM release method, the specific cytotoxic activity of NK cells was determined, and it reached approximately 24 %. Nonetheless, there are other methods besides calcein-AM release method to measure cytotoxicity, the major defence mechanism against tumorous and virus-infected cells, as an evaluation feature of NK cells.

Radioactive ^{51}chromium release method (^{51}Cr) was in former times considered as the main method for valuation of NK cytolytic activity *in vitro*.

The ^{51}chromium release method where was used previously in our laboratory when the influence of recombinant proteins from tick saliva on the activity of murine NK cells was examined. The method resulted in specific cytotoxicity of NK cells reaching 62 % (69). In comparison to our results, the difference in the level of measured cytotoxicity of NK cells could be seen (62 % versus 24 %). As conditions of experiments were otherwise similar it seems that calcein-AM release method is only suboptimal. Nevertheless, the ^{51}chromium release method faces its disadvantages associated with radioactivity of chromium. Therefore, there was a need for an alternative method. From this point of view, the advantage in handling of the calcein-AM release is a very valuable aspect.

Other alternative assays to ^{51}chromium release method were designed in time. Such assays were based on flow cytometry analysis and included 4 different fluorescent dyes: calcein acetoxymethyl ester (CAM), carboxyfluorescein succinimidyl ester (CFSE), Vybrant DiO (DiO) and MitoTracker Green (MTG). All experimental measurements within those assays, similar to ones performed within this thesis, included staining stability, spontaneous release of fluorochromes and also an examination of unstained cells. In the assays, purified NK cells were used as effector cells and were co-incubated with target cells K – 562 at different ratios at two-time intervals of 3 hours and 90 minutes. The Bland-Altman statistical method was used to determine the difference in all 4 methods compared to ^{51}Cr. It was once again concluded that all four methods cannot be stated equivalently effective in comparison to ^{51}Cr release method (68).

To confirm the accuracy of the obtained results, the experiment testing the viability of the used target cells could be conducted in the future.

The main aim of the thesis was to determine whether tick serpin IRS-4 from *I. ricinus* influences the activity of NK cells. NK cells represent due to their cytotoxic feature an essential part of the innate immunity. Both serpins and cystatins modulate immunity and immune cells represent an important part of immunity and especially constituents of an innate immunity play the key role in the defence mechanism in the first days after tick attachment. At the attachment spot the activation of a variety of innate immune cells such as neutrophils, dendritic cells, macrophages, T cells, mast cells and already mentioned NK cells occurs. Ticks are forced to resist the immune response of the host and have therefore developed many

defence mechanism strategies (29). The influence of tick saliva on the immune response of the host has been a focus of a few studies, a very few of them focuses on the influence of tick saliva on the activity of NK cells (70; 71; 69). Nevertheless, the effect of tick serpins on the cytotoxic effect of NK cells has not been documented at all which is another reason for this project.

To determine the cytotoxic activity of NK cells, targets, YAC-1 cells, and effectors, spleen cells from Poly I:C treated mouse, were co-incubated in ratio 1: 50. Measured cytotoxic activity of NK cells was specific to activated NK as shown in Figure 13, chapter 5.4. That is supported by the fact that the spleen cells (noted NK) from non-treated mouse resulted in specific toxicity of only 1.9 %. In contrary, the spleen cells (noted NK) from Poly I:C treated mouse resulted in specific toxicity of 23.8 % (chapter 5.4, Figure 12). We were considering the usage of sorted NK cells from spleen cell using antibodies however antibodies could interfere with the activity of NK cells – inhibitory vs. activating receptors. Moreover, it was a very small quantity of NK cells obtained by sorting.

The main aim of the thesis was to determine whether tick serpin IRS-4 from *I. ricinus* influences the activity of NK cells. Our results show that IRS-4 does not negatively affect NK activity as hypothesized. In contrast, a slight non-significant increase of NK activity was observed by IRS-4 – from 17.6 % to 21.9 % (chapter 5.5). The cause of the increase could be the synergistic cumulative effect of IRS-4 and LPS since IRS-4 was not available in a pure form. A few purification methods were performed to eliminate LPS presence however a huge loss of the IRS-4 accompanied decline of LPS in sample. Nevertheless, proper control containing LPS in an adequate concentration was used when the effect of IRS-4 was tested and LPS alone showed no effect on the assay (chapter 5.5).

The ability of IRS-4 to inhibit granzyme B seems to be unique. For instance, IRS-2 did not show any inhibitory activity towards this protease (49). In contrary, it has been shown that tick serpin IRS-2 has the effect on Th17 differentiation. The analysis revealed that IRS-2 inhibits IL-6 production in dendritic cells and consequently the development of pro-inflammatory cells like Th17. (50).

NK cells represent an important source of cytokines such as TNF-α and INF-γ. Some of those dispose of pro-inflammatory features or they activate components of immune systems which support inflammation formation. On the other hand, cytokines like IFN-γ are important for the development of specific (adaptive) immunity (72). Another and the last way in this thesis of

determination whether IRS-4 influences the activation of NK cells was performed via ELISA and subsequent IFN-γ production determination. Murine spleen cells were used and activated with IL-2 in the presence or absence of IRS-4. We presumed that IL-2 activates mainly NK cells and the production of IFN-γ is a feature of activated NK cells. However, the experiment did not result in a noticeable activation/stimulation of NK cells so it cannot be stated that the IFN-γ production was induced by IL-2. Nevertheless, a tremendous and unexpected increase in IFN-γ production occurred in the presence of IRS-4. We cannot explain what causes this increase though one possibility could be a synergistic effect of IRS-4 and LPS. Further experiments are required to clarify this result.

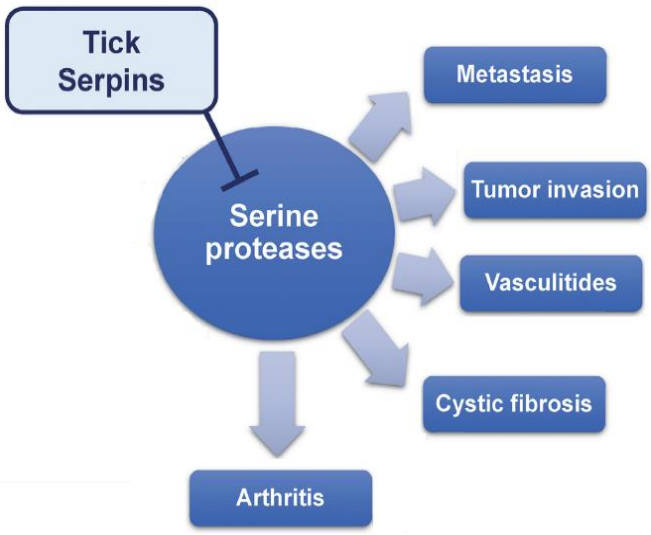

Figure 16: Therapeutic potential of serpins by inhibition serine proteases; adapted from (46)

Tick saliva contains numerous pharmacologically active compounds which attract the attention of scientist because of their potential to be used as drugs. From a pharmacological perspective, tick serpins are interesting thank to their possible therapeutic potential. As shown in the scheme (Figure 16) serine proteases are associated with a variety of diseases. Serpins as their inhibitors could be considered as potential novel drugs (46), therefore functional characterization of tick serpin is of importance. Tick proteins and the processes which they can affect could be at least to the certain degree predicted with the help of transcriptomic data (46). Additional study is a key step for the development of immunotherapeutic strategies adapted to the patient (73).

7 CONCLUSION

- The optimization of the calcein-AM release method for measurement of cytotoxic activity of NK cells was performed and the method was found suboptimal.
- Tick serpin IRS-4 does not negatively affect the cytotoxic activity of NK cells as was hypothesised.
- It seems that IRS-4 strongly upregulates the production of IFN-γ in IL-2 stimulated spleen cells. However, the activation of NK was not observed by IL-2 stimulation.

8 ABBREVIATIONS

ATB	antibiotics
BOFES	bovine fetal serum
CAM	calcein acetoxymethyl ester
CFSE	carboxyfluorescein succinimidyl ester
Cr	^{51}chromium release method
CTL	cytotoxic T lymphocytes
DiO	Vybrant DiO
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	enzyme-linked immunosorbent assay
GL	L-glutamine
IFN	interferon
IL	interleucine
IRS	Ixodes ricinus serpin
LAL	limulus amebocyte lysate
LPS	lipopolysaccharides
ME	2 – mercaptoethanol
MTG	MitoTracker Green
NK	natural killer cells (spleen cells)
PBS	phosphate buffered saline
Poly (I:C)	polyinosinic-polycytidylic acid
RFU	relative fluorescence unit

RFV	relative fluorescence value
RPMI 1640	Roswell Memorial Park Institute 1640 Medium
RT	room temperature
SAT	saliva-activated transmission
T	Triton X-100
TBEV	tick borne encephalitis virus
Y/YAC	YAC-1 cells

9 REFERENCES

1. **Walker, Mark.** The biology and ecology of the sheep tick *Ixodes ricinus*. *Antenna.* 2018, Vol. 42, 2, pp. 61-65.

2. **Sonenshine, Daniel E.** *Biology of ticks.* s.l. : Oxford University Press, 1991. Vol. 1.

3. **Dantas-Torres, Filipe, Chomel, Bruno B and Otranto, Domenico.** Ticks and tick-borne diseases: a One Health perspective. *Trends in Parasitology.* 2012, Vol. 28, 10, pp. 437-446.

4. **Beccaloni, Janet.** *Arachnids.* s.l. : Collingwood: CSIRO Publishing, 2009.

5. **Krantz G.W. and Walter D.E.** *A manual of acarology.* s.l. : Lubbock, Tex. : Texas Tech University Press, 2009.

6. **Nava, Santiago, Guglierlmone, Alberto A and Mangold, Atilio.** An overview of systematics and evolution of ticks. *Frontiers in Bioscience.* 2009, Vol. 14, pp. 2857-2877.

7. **Willadsen, P and Jongejan, F.** Immunology of the tick–host interaction and the control of ticks and tick-borne diseases. *Parasitology Today.* 1999, Vol. 15, 7, pp. 258-262.

8. **Jones, Linda D, Hodgson, Elizabeth and Nuttall, Patricia A.** Enhancement of virus transmission by tick salivary glands. *Journal of General Virology.* 1989, Vol. 70, 7, pp. 1895-1898.

9. **Alekseev , A N and Chunikhin , S P.** The exchange of the tick-borne encephalitis virus between ixodid ticks feeding jointly on animals with a subthreshold level of viremia. *Med Parazitol.* 1990, pp. 48-50.

10. **Labuda, Milan, et al.** Efficient transmission of tick-borne encephalitis virus between cofeeding ticks. *Journal of Medical Entomology.* 1993, Vol. 30, 1, pp. 295-299.

11. **Gern, Lise, Schaible, Ulrich E and Simon, Markus M.** Mode of inoculation of the Lyme disease agent *Borrelia burgdorferi* influences infection and immune responses in inbred strains of mice. *The Journal of Infectious Diseases.* 1993, Vol. 167, 4, pp. 971-975.

12. **Pechová, Jitka, et al.** Tick salivary gland extract-activated transmission of *Borrelia afzelii* spirochaetes. *Folia Parasitologica.* 2002, Vol. 49, 2, pp. 153-159.

13. **Zeidner, N S, et al.** Coinoculation of *Borrelia* spp. with tick salivary gland lysate enhances spirochete load in mice and is tick species-specific. *Journal of Parasitology.* 2002, Vol. 88, 6, pp. 1276-1278.

14. **Macháčková, Martina, Oborník, Miroslav and Kopecký, Jan.** Effect of salivary gland extract from *Ixodes ricinus* ticks on the proliferation of *Borrelia burgdorferi* sensu stricto *in vivo*. *Folia Parasitologica.* 2006, Vol. 53, 2, pp. 153-158.

15. **Hermance, Meghan and Thangamani, Saravanan.** Tick saliva enhances Powassan virus transmission to the host, influencing its dissemination and the course of disease. *Journal of Virology.* 2015, Vol. 89, 15, pp. 7852-7860.

16. **Bernard, Jennifer, et al.** Effect of *O. porcinus* tick salivary gland extract on the African swine fever virus Infection in domestic pig. *PLOS ONE.* 2016, Vol. 11, 2.

17. **Kročová, Z, et al.** Tick salivary gland extract accelerates proliferation of *Francisella tularensis* in the host. *Journal of Parasitology.* 2003, Vol. 89, 1, pp. 14-20.

18. **Milhano, N, et al.** The role of *Rhipicephalus sanguineus sensu lato* saliva in the dissemination of *Rickettsia conorii* in C3H/HeJ mice. *Medical and Veterinary Entomology.* 2015, Vol. 29, 3, pp. 225-229.

19. **Šimo, Ladisalv, et al.** The essential role of tick salivary glands and saliva in tick feeding and pathogen transmission. *Frontiers in Cellular and Infection Microbiology.* June 2017, Vol. 7, 281.

20. **Kim, Donghun , et al.** Multiple functions of Na/K-ATPase in dopamine-induced salivation of the Blacklegged tick, *Ixodes scapularis*. *Scientific Reports.* 2016, Vol. 6, 1.

21. **Fuente, de la Jose, et al.** Overview: Ticks as vectors of pathogens that cause disease in humans and animals. *Frontiers in Bioscience.* 2008, Vol. 13, pp. 6938–6946.

22. **Rizzoli, A, et al.** *Ixodes ricinus* and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health. *Frontiers in Public Health.* 2014, Vol. 2, 251.

23. **Francischetti, Ivo M B, et al.** Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, *Ixodes scapularis*: identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. *Blood.* 2002, Vol. 99, 10, pp. 3602–3612.

24. **Mans , Ben J.** Evolution of vertebrate hemostatic and inflammatory control mechanisms in blood-feeding arthropods. *Journal of Innate Immunity.* 2011, Vol. 3, 1, pp. 41-51.

25. **Kazimírová, Mária and Štibrániová, Iveta.** Tick salivary compounds: their role in modulation of host defences and pathogen transmission. *Frontiers in Cellular and Infection Microbiology.* 2013, Vol. 3.

26. **Štibrániová, Iveta, Lahová, M and Bartíková, P.** Immunomodulators in tick saliva and their benefits. *Acta Virologica.* 2013, Vol. 57, 2, pp. 200-216.

27. **Wikel, Stephen.** Ticks and tick-borne pathogens at the cutaneous interface: host defenses, tick countermeasures, and a suitable environment for pathogen establishment. *Frontiers in Microbiology.* 2013, Vol. 4.

28. **Valdés, James J.** Antihistamine response: a dynamically refined function at the host-tick interface. *Parasites&Vectors.* 2014.

29. **Kotál, Jan, et al.** Modulation of host immunity by tick saliva. *Journal of Proteomics.* 2015, Vol. 128, pp. 58-68.

30. **Chmelař, Jindřich, et al.** All for one and one for all on the tick–host battlefield. *Trend in Parasitology.* 2016, Vol. 32, 5, pp. 368-377.

31. **Wikel, Stephen K.** Tick modulation of host immunity: an important factor in pathogen transmission. *International Journal for Parasitology.* 1999, Vol. 29, pp. 851-859.

32. **Wikel, Stephen K, et al.** Infestation with pathogen-free nymphs of the tick *Ixodes scapularis* induces host resistance to transmission of *Borrelia burgdorferi* by ticks. *Infection and Immunity.* 1997, Vol. 65, 1, pp. 335-338.

33. **Nazario, Sylvette, et al.** Prevention of *Borrelia burgdorferi* transmission in guinea pigs by tick immunity. *The American Journal of Tropical Medicine and Hygiene.* 1998, Vol. 58, 6, pp. 780-785.

34. **Narasimhan, Sukanya, et al.** Immunity against *Ixodes scapularis* salivary proteins expressed within 24 hours of attachment thwarts tick feeding and impairs *Borrelia* transmission. *PLoS ONE.* 2007, Vol. 2, 5, p. e451.

35. **Nuttall, Patricia A.** Tick saliva and its role in pathogen transmission. *Wiener Klinische Wochenschrift.* 2019.

36. **Lieskovská, Jaroslava, et al.** Tick sialostatins L and L2 differentially influence dendritic cell responses to *Borrelia* spirochetes. *Parasites & Vectors.* 2015, Vol. 8, 1.

37. **Bogovic, Petra and Strle, Franc .** Tick-borne encephalitis: a review of epidemiology, clinical characteristics, and management. *World Journal of Clinical Cases.* 2015, Vol. 3, 5, pp. 430-441.

38. **Kuno, Goro, et al.** Phylogeny of the genus *Flavivirus*. *Journal of Virology.* 1998, Vol. 72, 1, pp. 73–83.

39. **Gritsun, T S, Lashkevich, V A and Gould , E A.** Tick-borne encephalitis. *Antiviral Research.* 2003, Vol. 57, 1-2, pp. 129–146.

40. **Ecker, Michael, et al.** Sequence analysis and genetic classification of tick-borne encephalitis viruses from Europe and Asia. *Journal of General Virology.* 1999, Vol. 80, 1, pp. 179–185.

41. **Haglund, Mats and Günther, Göran.** Tick-borne encephalitis - pathogenesis, clinical course and long-term follow-up. *Vaccine.* 2003, Vol. 21, pp. S11-S18.

42. **Mansfield, K L, et al.** Tick-borne encephalitis virus – a review of an emerging zoonosis. *Journal of General Virology.* Vol. 90, 8, pp. 1781–1794.

43. **Süss, Jochen.** Tick-borne encephalitis 2010: epidemiology, risk areas, and virus strains in Europe and Asia - an overview. *Ticks and Tick-borne Diseases.* 2011, Vol. 2, 1, pp. 2-15.

44. **Růžek, Daniel, Dobler, Gerhard and Mantke, Oliver Donoso.** Tick-borne encephalitis: pathogenesis and clinical implications. *Travel Medicine and Infectious Disease.* 2010, Vol. 8, 4, pp. 223-232.

45. **Law, Ruby H P, et al.** An overview of the serpin superfamily. *Genone Biology.* 2006, Vol. 7, 5.

46. **Chmelař, Jinřich, et al.** Protease inhibitors in tick saliva: the role of serpins and cystatins in tick-host-pathogen interaction. *Frontier in Cellular and Infection Microbiology.* 2017, Vol. 7.

47. **Ayllón, Nieves , et al.** Systems biology of tissue-specific response to *Anaplasma phagocytophilum* reveals differentiated apoptosis in the tick vector *Ixodes scapularis*. *PLOS Genetics.* 2015, Vol. 11, 3.

48. **Schwarz, Alexandra , et al.** *De novo Ixodes ricinus* salivary gland transcriptome analysis using two next-generation sequencing methodologies. *The FASEB Journal.* 2013, Vol. 27, 12.

49. **Chmelar, Jindrich, et al.** A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation. *Blood.* 2011, Vol. 117, 2, pp. 736-744.

50. **Páleníková, Jana, et al.** *Ixodes ricinus* salivary serpin IRS-2 affects Th17 differentiation via inhibition of the interleukin-6/STAT-3 signaling pathway. *Infection and Immunity.* 2015, Vol. 83, 5, pp. 1949-1956.

51. **Prevot, Pierre-Paul, et al.** Anti-hemostatic effects of a serpin from the saliva of the tick *Ixodes ricinus*. *Journal of Biological Chemistry.* 2006, Vol. 281, 36, pp. 26361–26369.

52. **Prevot, Pierre-Paul, et al.** Exosites mediate the anti-inflammatory effects of a multifunctional serpin from the saliva of the tick *Ixodes ricinus*. *FEBS Journal.* 2009, Vol. 276, 12, pp. 3235-3246.

53. **Leboulle, Gérard, et al.** Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. *Journal of Biological Chemistry.* 2002, Vol. 277, 12, pp. 10083–10089.

54. **Müller, Sabrina, Dennemärker, Julia and Reinheckel, Thomas .** Specific functions of lysosomal proteases in endocytic and autophagic pathways. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics.* 2012, Vol. 1824, 1, pp. 34-43.

55. **Schwarz, Alexandra, Valdés, James J and Kotsyfakis, Michalis.** The role of cystatins in tick physiology and blood feeding. *Ticks and Tick-borne Diseases.* 2012, Vol. 3, 3, pp. 117-127.

56. **Meekins, David A, Kanost, Michael R and Michel, Kristin.** Serpins in arthropod biology. *Seminars in Cell & Developmental Biology.* 2017, Vol. 62, pp. 105-119.

57. **Vargin, V V and Semenov, B F.** Changes of natural killer cell activity in different mouse lines by acute and asymptomatic *Flavivirus* infections. *Acta Virologica.* 1986, Vol. 30, 4, pp. 303-308.

58. **Topham, Nicola J and Hewitt, Eric W.** Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology.* 2009, Vol. 128, 1, pp. 7-15.

59. **Wong, Phillip and Pamer, Eric G.** CD8 T cell responses to infectious pathogens. *Annual Review of Immunology.* 2003, Vol. 21, 1.

60. **Boon, Thierry , et al.** Tumor antigens recognized by T lymphocytes. *Annual Review of Immunology.* 1994, Vol. 12, 1, pp. 337-365.

61. **Hewitt, Eric W.** The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology.* 2003, Vol. 110, 2, pp. 163-169.

62. **Moretta, Lorenzo, et al.** Human NK cells and their receptors. *Microbes and Infection.* 2002, Vol. 4, 15, pp. 1539–1544.

63. **Waldhauer, I and Steinle, A.** NK cells and cancer immunosurveillance. *Oncogene.* 2008, Vol. 27, pp. 5932–5943.

64. **Lanier, Lewis L.** NK cell recognition. *Annual Review of Immunology.* 2005, Vol. 23, 1, pp. 225-274.

65. **Lettau, Marcus , et al.** Secretory lysosomes and their cargo in T and NK cells. *Immunology Letters.* 2007, Vol. 108, 1, pp. 10-19.

66. **Lieberman, Judy.** The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nature Reviews Immunology.* 2003, Vol. 3, 5, pp. 361–370.

67. **Trapani, Joseph A and Bird, Phillip I.** A renaissance in understanding the multiple and diverse functions of granzymes? *Immunity.* 2008, Vol. 29, 5, pp. 665-667.

68. **Cholujova, Dana, et al.** Comparative study of four fluorescent probes for evaluation of natural killer cell cytotoxicity assays. *Immunobiology.* 2008, Vol. 213, 8, pp. 629-640.

69. **Beránková, Zuzana.** Vliv rekombinantních proteinů z klíštěcích slin na aktivitu myších NK buněk. *Jihočeská univerzita v Českých Budějovicích; Přírodovědecká fakulta*. České Budějovice : s.n., 2015. p. 42.

70. **Kopecký, Jan a Kuthejlová , M.** Suppressive effect of *Ixodes ricinus* salivary gland extract on mechanisms of natural immunity in vitro. *Parasite Immunology.* 1998, Sv. 20, 4, stránky 169-174.

71. **Kubes, M, et al.** Salivary gland extracts of partially fed *Dermacentor reticulatus* ticks decrease natural killer cell activity *in vitro*. *Immunology.* 1994, Vol. 82, 1, pp. 113-116.

72. **Vivier, Eric , et al.** Innate or adaptive immunity? The example of natural killer cells. *Science.* 2011, Vol. 331, 6013, pp. 44-49.

73. **Scherer, Hans U, Dörner, Thomas and Burmester, Gerd R.** Patient-tailored therapy in rheumatoid arthritis: an editorial review. *Current Opinion in Rheumatology.* 2010, Vol. 22, 3, pp. 237-245.