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Cellular basis of host immune response to experimental *Francisella tularensis* infection

Rigorous thesis

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Buněčné základy imunitní odpovědi hostitele na experimentální infekci *Francisella tularensis*

Rigorózní práce

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Abstract

Francisella tularensis (F. tularensis) is a highly virulent intracellular pathogen that causes tularemia, an infection which can manifest itself in various forms depending on the route of inoculation. Like other intracellular pathogens, *F. tularensis* infection is controlled primarily by T cells, which can provide protective immunity. In addition, an important role of B lymphocytes in early stages of infection has previously been uncovered. There is evidence that B cells as well as antibodies are necessary for a fully effective immune response to primary and secondary *F. tularensis* infections in mice. This thesis is focused on investigating an *F. tularensis*–B cell interaction model which can provide more information about direct contact between pathogens and B cells, the final fate of both the microbe and the host cell, and the role of cytokines resp. circulating antibodies in protecting against experimental infection by *Francisella* spp. The data presented here entitle us to believe that B cells have all the attributes necessary to participate actively in the induction and regulation of adaptive immune responses during early stages of *F. tularensis* infection. Moreover, the important role of circulating antibodies and selected cytokines in protecting against *Francisellae* in both immunocompetent as well as immunocompromised individuals was elucidated using a murine infection model. Understanding the role of the B cells and humoral part of the immune system in cooperation with T cell-mediated immunity is important for the effort to develop effective and safe prophylactic procedures for protecting against infection with *F. tularensis* strains, and it can help to elucidate the basic issues concerning expression of protective immunity against intracellular bacterial pathogens generally.

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Abstrakt

Francisella tularensis (*F. tularensis*) je vysoce virulentní intracelulární pathogen způsobující onemocnění zvané tularemia. Manifestace tohoto onemocnění se vyskytuje v různých formách v závislosti na místě infekce. Infekce *F. tularensis* je, stejně jako u dalších intracelulárních organizmů, primárně kontrolována T lymfocyty, které poskytují protektivní imunitu. Avšak role B lymfocytů v raných fázích infekce dosud nebyla plně objasněna. Nicméně je známo, že B lymfocyty, spolu s protilátkami, poskytují plný protektivní efekt proti primární a také sekundární infekci *F. tularensis* v myším modelu. Tato rigorózní práce je zaměřena na studium vzájemné přímé interakce *F. tularensis* s B lymfocyty, na studium konečného osudu mikroba a hostitelské buňky a také na roli vybraných cytokinů a protilátek v protektivní imunitní odpovědi proti *Francisella* spp. Výsledky prezentované v této práci nás opravňují k domněnce, že B lymfocyty jsou schopné se aktivně podílet na indukci a regulaci adaptivní imunitní odpovědi v časných fázích infekce *F. tularensis*. Navíc byla osvětlena role cirkulujících protilátek a vybraných cytokinů jak u imunokompetentních tak u imunosuprimovaných jedinců v rámci myšího infekčního modelu. Pochopení role B lymfocytů a protilátek v kooperaci s T-lymfocyty zprostředkovanou imunitou vede k efektivním a bezpečným preventivním postupům při ochraně proti infekci *F. tularensis*. Navíc lze získané výsledky všeobecně začlenit do objasnění základních otázek týkajících se protektivní imunitní odpovědi proti intracelulárním patogenům.

Klíčová slova	*Francisella tularensis*; B lymfocyty; buněčná biologie
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Prohlašuji:

Tuto práci jsem vypracovala samostatně. Veškeré literární prameny a informace, které jsem v práci využila, jsou uvedeny v seznamu použité literatury.

V Olomouci dne 4. května 2015

Klára Kubelková

SUMMARY

Francisella tularensis (F. tularensis) is a highly virulent intracellular pathogen that causes tularemia, an infection which can manifest itself in various forms depending on the route of inoculation. Like other intracellular pathogens, *F. tularensis* infection is controlled primarily by T cells, which can provide protective immunity. In addition, an important role of B lymphocytes in early stages of infection has previously been uncovered. There is evidence that B cells as well as antibodies are necessary for a fully effective immune response to primary and secondary *F. tularensis* infections in mice.

This thesis is focused on investigating an *F. tularensis*–B cell interaction model which can provide more information about direct contact between pathogens and B cells, the final fate of both the microbe and the host cell, and the role of cytokines resp. circulating antibodies in protecting against experimental infection by *Francisella* spp. The data presented here entitle us to believe that B cells have all the attributes necessary to participate actively in the induction and regulation of adaptive immune responses during early stages of *F. tularensis* infection. Moreover, the important role of circulating antibodies and selected cytokines in protecting against *Francisellae* in both immunocompetent as well as immunocompromised individuals was elucidated using a murine infection model.

Understanding the role of the B cells and humoral part of the immune system in cooperation with T cell-mediated immunity is important for the effort to develop effective and safe prophylactic procedures for protecting against infection with *F. tularensis* strains, and it can help to elucidate the basic issues concerning expression of protective immunity against intracellular bacterial pathogens generally.

SOUHRN

Francisella tularensis (*F. tularensis*) je vysoce virulentní intracelulární pathogen způsobující onemocnění zvané tularemia. Manifestace tohoto onemocnění se vyskytuje v různých formách v závislosti na místě infekce. Infekce *F. tularensis* je, stejně jako u dalších intracelulárních organizmů, primárně kontrolována T lymfocyty, které poskytují protektivní imunitu. Avšak role B lymfocytů v raných fázích infekce dosud nebyla plně objasněna. Nicméně je známo, že B lymfocyty, spolu s protilátkami, poskytují plný protektivní efekt proti primární a také sekundární infekci *F. tularensis* v myším modelu.

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I would never have been able to finish this thesis without the guidance of help from friends and support from my family…

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Plzakova L[*], **Kubelkova K**[*], Krocova Z, Zarybnicka L, Sinkorova Z, Macela A: B cell subsets are activated and produce cytokines during early phases of *Francisella tularensis* LVS infection. Microbial Pathogenesis, 2014, 75, 49 – 58.

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* these authors contributed equally to the work

1 INTRODUCTION

Protective immune responses against facultative intracellular bacterial pathogens require the concerted action of activated immunocompetent cells. In this respect, the dominant role has been attributed to activated T lymphocyte subpopulations and/or activated macrophages. Recently, it has become clear that the B cell subsets are the equivalent partner of T cells during the induction, regulation and expression phases of immune response against intracellular bacteria. They express their functions through both antibody-dependent and antibody-independent mechanisms. B cells are able to internalize bacteria through the B cell receptor (BCR), extract antigens from non-internalizable particles, and, due to the cross-presentation process, activate $CD4^+$ as well as $CD8^+$ T cells. Through the dichotomy of the recognition of bacterial targets by the BCR or a Toll-like receptor (TLR) and by the production of sets of cytokines, B cells regulate the expression of individual branches of acquired immune response. B cells also influence the development of T cell memory. Further, B cells play a dominant role as antibody-producing cells in influencing the course and final resolution of intracellular bacterial infections. Direct interaction of B cell subpopulations with bacteria results in autonomous B cell differentiation and rapid secretion of bacterium-specific antibodies that influence the pathogen's distribution to vital organs. Moreover, specific antibodies against intracellular bacteria have been demonstrated to have protective potential. On the other hand, intracellular bacteria survive intracellularly in B cells mainly in a non-replicative state and, as such, constitute a source of systemic infection and/or reservoir for reinfection. B cells thus constitute one of the cell types responsible for the final fate of the interaction of intracellular bacterial pathogens with their hosts. This thesis summarizes studies that have been conducted on a *Francisella tularensis* infection model and that document both the beneficial as well as the relatively deleterious role B cells play in the course of intracellular bacterial infections.

2 THEORETICAL

2.1. Lessons from history

The history of research on tularemia as well as of its causative agent's taxonomic classification goes back more than 100 years. The origin of the tularemia story is generally dated to the year 1911, when George W. McCoy, Director of the US Public Health Service Plague Laboratory, undertook bacteriological investigations of bubonic plague in ground squirrels and reported a plague-like disease of rodents in California (McCoy 1911). In parallel, physician R. A. Pearse reported six cases of fever caused by the bites of deerflies and named this "deerfly fever" (Pearse 1911). One year later, McCoy and Chapin successfully isolated a novel organism, which was named *Bacterium tularense (B. tularense).* The site of the original discovery was Tulare County in central California, (McCoy and Chapin 1912). Edward Francis studied deerfly fever in Utah during the 1920s. Because of the pathological changes of the disease observed in animals and humans and due to isolation of the bacterium from human blood, he renamed the disease tularemia (Francis 1925). Almost concurrently, Hachiro Ohara was studying wild hare disease (Yato-byo) in Japan and recognized that disease's similarity to tularemia. His observation was confirmed by Francis, who isolated *B. tularense* from specimens he received from Ohara (Ohara 1925). Soon thereafter, tularemia was recognized in the USSR, Norway, Canada, Sweden, and Austria (Suvarov et al. 1928; Zarkai 1930; Francis 1937). Thus, the names deerfly fever, rabbit fever, Pahvant Valley plague, lemming fever, Yato-byo, Ohara's disease, water-rat trapper's disease, hare meat poisoning, and probably also other historical names are synonyms for tularemia.

From a historical point of view, tularemia was probably first described on September 19, 1907. At the Annual Meeting of the Medical Library Association, Portland, Oregon, on June 25, 1940, Dr. William Levin cited a letter of Dr. Ancil Martin which had been sent to F. G. Novy at the University of Michigan. In this letter, Martin had stated that he had under observation and treatment five cases of an infection caused by the skinning and dressing of wild rabbits (Levin 1940). For the sake of completeness, it should be noted that there is a hypothesis that the biblical plague of the Philistines and a virulent epidemic, similar to the bubonic plague or typhus, in Ancient Egypt (around 1715 BC) may have been tularemia (Trevisanato 2004, 2007).

The original name and taxonomy of the etiological agent of tularemia changed several times over the years. In the literature can be found the name *Pasteurella tularensis* and in older literature the name *Brucella tularensis* (due to serological cross-reactions with the *Brucella* sp. antigens). Finally, to honor the achievements of Edward Francis in the field of tularemia research, the bacterium was renamed to *Francisella tularensis (F. tularensis)* (Dorofiev 1947; Rockwood 1983).

2.2 The etiological agent – *Francisella tularensis*

F. tularensis is a highly virulent, non-sporulating, pleomorphic, facultative intracellular, Gram-negative coccobacillus that is capable of causing the zoonotic disease tularemia in a large number of mammals. *F. tularensis* strains grow slowly in CO_2 supplemented air and almost all strains of this fastidious organism have specific requirements for iron and cysteine or cystine. After 24 h of incubation, small, white and gray, smooth or shiny-surfaced colonies can be seen on an appropriate solid media.

Francisella is the only genus within the family *Francisellaceae* of the γ-subclass of *Proteobacteria* (McLendon et al. 2006). The *Francisellaceae* family is distinguished by a set of phenotypic characteristics unique for their morphology, capability for degrading only a limited number of carbohydrates, growth requirement for cysteine, and unique fatty acid composition (Tärnvik and Berglund 2003). The taxonomy of the genus *Francisella* is still subject to debate. A recent study of the phylogenetic relationships of all known *Francisella* species divided the genus *Francisella* into two genetic clades. One is represented by *F. tularensis*, *F. novicida*, *F. hispaniensis*, and the close neighbor *Wolbachia persica*; the second by *F. philomiragia* and the fish pathogen *F. noatunensis* (Sjödin et al. 2012). The majority of publications divide the species *F. tularensis* into four closely related subspecies that are highly conserved in their genomic content but differ in their virulence, biochemistry, and epidemiology: *F. tularensis* subsp. *tularensis* (also known as Type A), which is endemic in North America and known in Europe in only one isolate that possibly represents a laboratory escape (Gurycova 1998); *F. tularensis* subsp. *holarctica* (also known as Type B), widespread throughout the Northern Hemisphere; *F. tularensis* subsp. *mediasiatica*, endemic in Central Asia; and *F. tularensis* subsp. *novicida.* Most human cases of tularemia are caused by the *F. tularensis* subsp. *tularensis* Type A and *F. tularensis* subsp. *holarctica* Type B strains. Type A strains are now newly divided into 3

genotypes (clades) A1a, A1b, and A2, all of which have been shown to be epidemiologically important (Staples et al. 2006; Kugeler et al. 2009). Strains of *F. tularensis* subsp. *tularensis* are considered the most virulent for humans, with an infectious dose of less than 10 colony forming units (CFU). The lethality of Type A strains is up to 24% in untreated cases depending on the Type A genotype (Kugeler et al. 2009). *F. tularensis* subsp. *holarctica* also causes debilitating diseases, albeit with a milder course. The fatality rate barely reaches 1%. *F. tularensis* subsp. *mediasiatica* rarely causes human illness and is less virulent than *F. tularensis* subsp. *holarctica* (Ol'sufev and Meshcheriakova 1981). *F. tularensis* subsp. *novicida* is an opportunistic pathogen in humans, and it is significantly less pathogenic than are the other subspecies. It can cause the disease mainly in immunocompromised people (Tärnvik 1989). Fully virulent strains must be handled in labs under containment meeting the requirements of Biosafety Level 3 (Titball et al. 2007). The Centers for Disease Control and Prevention (USA) classify *F. tularensis* as a Category A bioterrorism agent (http://emergency.cdc.gov/agent/agentlist-category.asp).

2.3 Tularemia epidemiology

The name tularemia has been applied to all manifestations of infection caused by the *F. tularensis* bacterium (Evans 1985). While it is generally known that there are zones where tularemia has been occurring for decades, the overall ecology of *F. tularensis* is not well understood, and particularly the transmission cycle, ecological requirements of the different subspecies, and true natural reservoir hosts (Mörner 1992; Gyuranecz et al. 2011). More than 250 species of mammals, birds, amphibians, invertebrates, and protozoans have already been identified as hosts for *F. tularensis*, and that fact complicates understanding the transmission cycle. In general, human cases of tularemia are most often associated with exposure to lagomorphs, rodents, and blood-feeding arthropods; inhalation of contaminated dust particles; or drinking of contaminated water. Two cycles, terrestrial and aquatic, have been described for the disease caused by *F. tularensis*. Hares and rabbits are prototypical hosts for the terrestrial cycle while ticks, mites, and biting flies represent arthropod vectors. Beavers, muskrats, and voles are mammalian hosts that can contaminate water within the aquatic cycle (Bell and Stewart 1975; Bell and Stewart 1983]. The aquatic

cycle is associated with rivers, streams, and flooded landscapes, and it can be promoted by the persistence of the bacterium within protozoans (Parker et al. 1951; Lysy et al. 1979; Mörner 1992; Abd et al. 2003).

Outbreaks of tularemia in humans are typically associated with outbreaks of tularemia in animal populations. Thus, rural populations, and especially those individuals who spend some time in endemic areas, such as farmers, hunters, forest workers, and tourists, are most at risk of tularemia (Harris 1956; Levesque et al. 1995; Levesque et al. 2007). For example, two outbreaks of pneumonic tularemia at Martha's Vineyard, Massachusetts were associated with the use of lawn mowers or brush cutters while people were working around the houses (Teutsch et al. 1979; Feldman et al. 2001). In northern European countries, such as Sweden, Finland, and the northern part of Russia, mosquitoes are the dominant vector transmitting tularemia to humans (Tärnvik et al. 2004; Petersen et al. 2009). By contrast, mosquitoes in Central Europe probably do not carry *F. tularensis* in natural foci of tularemia; contact with infected animals, ingestion of contaminated food or water, along with possible infection caused by tick vectors are the dominant modes of transmission in this region (Libich 1981; Hubalek et al. 1997). The seasonality of reported tularemia cases corresponds well with these transmission modes. The spread of the pathogen *F. tularensis* and frequency of tularemia infection may be underestimated because many infections of humans with *F. tularensis* appear to be asymptomatic.

2.4 Disease manifestation

The majority of information on tularemia symptoms in animals has originated from laboratory experiments using mice, rabbits, guinea pigs, and monkeys as animal models of natural infection. In these cases, however, most studies have utilized the *F. tularensis* Live Vaccine Strain or *F. novicida*, which in murine models induce symptoms similar to the infection in humans caused by wild virulent strains (Conlan et al. 2011). The clinical manifestations of tularemia depend on the route of infection and the susceptibility of any particular animal species to tularemia.

Similarly to other bacterial zoonoses, tularemia is transmitted to humans by direct contact with infected animals, tissues or fluids from infectious animals or by bites from infected arthropod vectors. Inhalation of aerosol or ingestion of contaminated food and water are other sources of infection. Thus, the gateways for *F. tularensis* into the body

include the skin, mucosal membranes, lungs, and gastrointestinal tract. In general, infection is characterized by common symptoms including fever, sweats, headache, body ache, nausea, vomiting, and diarrhea. Pulse–temperature dissociation is seen in less than half of patients. The incubation time, which delineates the period of delay between infection and the outbreak of symptoms, varies around 6 days. Moreover, the delay between the onset of symptoms and the seeking of treatment ranges around 7 days (Koskela and Herva 1980; Koskela and Salminen 1985). Disease onset is abrupt, usually within 3 to 5 days, but it can be as rapid as 1 day or as prolonged as 14 days post-exposure.

A study by Dr. Francis had delineated four major clinical manifestations of tularemia (ulceroglandular, glandular, oculoglandular, and typhoidal) while additional manifestations were observed to include oropharyngeal, gastrointestinal, pneumonic, and other rare forms (Francis 1928). The most common form of human tularemia is the ulceroglandular form that results from contact with infectious material or from vector-borne transmission.

To summarize, the clinical forms of tularemia can be divided into various syndromes that complicate diagnosis. The incubation period is usually 3 to 5 days after inoculation. Clinical manifestation of illness begins with the rapid onset of fever, chills, headache, malaise, fatigue, and myalgia. Some patients suffer from coughing, nausea, and vomiting. Other findings may include skin ulcers, sore throat, pleural effusion, primary or secondary pneumonia, acute respiratory distress syndrome, and pericarditis. General signs and symptoms of different clinical types of tularemia are included in Table I.

Table I: Common clinical signs and manifestation of various type of tularemia

	Characteristics	Means of spread	Portal of entry
Ulceroglandular type	Skin papule followed by persistent ulcer Enlargement of regional lymph node Chronic granulomatous inflammation Fever	Vector-borne transmission Direct contact Indirect contact (tools)	Skin
Glandular type	Tender lymphadenopathy (usually axillary/epitrochlear) Absence of visible skin lesions Fever	Vector-borne transmission Direct contact Indirect contact (tools)	Unknown (probably skin)
Oropharyngeal type	Severe pharyngitis Tonsillitis Regional neck lymphadentis Cervical adenitis Persistent fever	Ingest of contamined food/water	Oropharyngeal mucosa
Oculoglandular type	Unilateral conjunctivis Swelling of eyelids Photophobia Mucopurulent discharge Enlargement of regional lymph node Parinaud´s syndrome	Touch eye with contamined fingers Infective dust	Conjunctiva
Typhoidal type	Myalgia Headache Fever of unknown origin Skin or mucous membrane lesions Lymph node enlargement	Unknown (probably oral/respiratory)	Oropharyngeal mucosa Respiratory tract
Pneumonic /respiratory Type A	Sudden onset of symptoms Pneumonia Bronchopneumonia Chest pain Dry or productive cough Dyspnoea Fever Profuse sweating Mental deterioration Septicemia Erythema nodosum	Inhaling contaminated dust Laboratory-acquired infection	Respiratory tract
Pneumonic /respiratory Type B	Hilar adenopathy Pneumonic infiltration Erythema nodosum Pneumonia (rarely)	Inhaling contaminated dust Laboratory-acquired infection	Respiratory tract

2.5 Prophylaxis and treatment

The first attempts at constructing a vaccine based on attenuated bacteria were made in the 1930s by Francis, Kudo, and Gotschlich, but without satisfactory results. Later, Elbert and Gaiskii prepared other attenuated strains, among which especially strains 155 and 15 were extensively tested and recommended for the preparation of a commercially safe and effective vaccine (Elbert and Gaiski 1945; Elbert 1946; Tigertt 1962). In 1956, a vial of the Soviet commercial live vaccine was transferred from the Gamaleia Institute in Moscow to the US Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland. The isolation of one selected *F. tularensis* colony from this ampoule gave rise to the *F. tularensis* Live Vaccine Strain (LVS). After protective efficacy testing on animal models, LVS was tested for safety and efficacy in humans and used for vaccination of at-risk personnel (Eigelsbach and Downs 1961; Saslaw et al. 1961a, 1961b). To complete the information on the prophylaxis of tularemia, mention should be made of attempts at passive transfer of immunity by immune sera or antibodies against *F. tularensis*. Classical experiments on animals have already demonstrated that injection of immune serum before virulent challenge only prolongs survival but cannot ensure protection against even low doses of virulent bacteria (Francis and Felton 1942; Bell and Kahn 1945). These results nevertheless clearly demonstrated that immune sera contain protective antibodies and their protective effect correlated well with precipitating antibody content (Foshay et al. 1947). Moreover, antibodies also can be used in therapeutic mode, especially when administered early after infection (Kirimanjeswara et al. 2007; Savitt et al. 2009).

Since the description of *Francisella* spp. as an emerging pathogen in 2001, many molecular tools for diagnosis have been developed to rapidly confirm tularemia-positive patients and type the strain in order to recommend therapeutic treatment and predict patient prognosis. Tularemia Type A, as well as the less virulent Type B, is often associated with various complications that involve substantial periods of convalescence. Due to tularemia's long incubation period, it is imperative to treat patients immediately after the onset of symptoms. Nevertheless, only a few of the antibiotics currently at our disposal are effective against tularemia and for this reason we need new therapeutic strategies for tularemia, including to develop new antibiotics or new ways of using existing antibiotics (summarized in Maurin 2014). The choice of antibiotic type for treating tularemia is

dependent on the clinical manifestation of the illness, the subspecies of the infectious agent, and the immune status of the host.

In addition to testing new antibiotics, attempts are being made to use chemotherapy and immunotherapy against tularemia. The specific enzymes that *F. tularensis* needs for its survival and proliferation are the targets of enzyme inhibitors in the form of proteins or small molecules. These include, for example, recombinant cystatin 9 (Eaves-Pyles et al. 2013) and small compounds prepared by ligand- and structure-based drug design (Chaudhury et al. 2013). Modestly promising therapies against tularemia today consist in utilizing the immunosuppressive and/or immunomodulatory effects of antimicrobial peptides, such as human cathelicidin LL-37 peptide, novel synthetic hybrids designed from cecropin A, magainin II and granulysin peptides, or specific fly antimicrobial peptides such as attacin, cecropin, drosocin and drosomycin from *Drosophila melanogaster* (Endsley et al. 2009; Fox et al. 2012; Flick-Smith et al. 2013; Vonkavaara et al. 2013). These positively charged antimicrobial peptides are capable of disrupting the negatively charged bacterial membrane and limiting the proliferation of microbes. Such antimicrobial peptides and enzyme inhibitors are effective in cell-based *in vitro* and in animal *in vivo* systems. They display significant growth inhibition of *F. tularensis* or reduce organ bacterial burden, and they improve survival of experimental mice. In the future, alternatives to antibiotic therapy may be offered in cases of natural or intentionally generated *F. tularensis* (multi)resistance.

2.6 *F. tularensis* – an intracellular pathogen

To establish infection, *F. tularensis*, as an intracellular pathogen, needs to enter cells, find a target place to survive, and then grow inside host cells. Its ability to survive and multiply intracellularly has been well described in both professional and nonprofessional phagocytes via *in vitro* and *in vivo* models (Anthony et al. 1991; Conlan and North 1992; Pechous et al. 2009). The molecular mechanisms *F. tularensis* uses to mediate its uptake into the host cell are mostly unknown, however. In general, serum-opsonized *F. tularensis* can enter host cells using a process dependent on the presence of complement factor C3 in the serum and complement receptors on the surface of the host cell, which are engaged in forming pseudopod loops in the host cell's surface membrane (Clemens et al. 2005). Under this condition, Class A scavenger receptors, lung surfactant

protein A, nucleolin, as well as the Fcγ receptors are involved, to various degrees, in mammalian cells' internalization of *F. tularensis* (Balagopal et al. 2006; Pierini 2006; Barel et al. 2008). In the non-opsonic uptake of *F. tularensis* by macrophages, a significant role is played by the mannose receptor (Balagopal et al. 2006) and possibly other cell surface receptors that have not yet been defined. The mode of entry may influence the fate of *Francisella* inside the host cell by triggering different signaling pathways that control the expression of intracellular defense mechanisms and, in parallel, influence the survival of intracellularly localized bacteria (Geier and Celli 2011).

Inside the host cell (and mainly macrophages and dendritic cells have been studied), *F. tularensis* resides in an initial vacuolar compartment along the general endocytic degradative pathway, recently termed the *Francisella*-containing phagosome (FCP). The FCP sequentially acquires early and late endosomal markers, such as EEA-1, Lamp-1, and Rab-7, but not the marker cathepsin D, which is an indicator of phagosome–lysosome fusion (Clemens et al. 2004; Santic et al. 2005). The next step in intracellular trafficking of the bacterium consists in active FCP membrane disruption followed by escape into the cytosol, where it then replicates (Golovlion et al. 2003; Clemens et al. 2004; Santic et al. 2005). There is still debate as to whether the FCP is acidified before disruption of the membrane by *F. tularensis*. There are experimental data demonstrating progressive acidification of the vacuole by acquiring vacuolar ATPase before phagosomal disruption (Chong et al. 2008; Santic et al. 2008) as well as contradictory data that exclude FCP acidification (Clemens et al. 2004; Bönquist et al. 2008; Rajaram et al. 2009). Whether this discrepancy is due to different experimental conditions or different infectious agents is not yet clear. Phagosomal escape is followed by extensive cytosolic replication and, finally, the programmed cell death of the host macrophage (Lai et al. 2001; Hrstka et al. 2005). During later intervals of infection, some *F. tularensis* have been observed in the multi-membrane vacuolar compartment of the endocytic pathway that has the characteristics of an autophagosome (Checroun et al. 2006; Hrstka et al. 2007). Still unclear, however, are why *F. tularensis* reenters the membranous compartment and the consequences for further dissemination of infection and induction of immune response (see Fig. 1 for the scheme of the entire process).

Also still debated is whether the reentering of *Francisellae* into a multilamellar compartment is induced by *Francisella* itself or is the result of a defense mechanism based on eliminating the intracellular threat. During its history, *F. tularensis* has developed

molecular tools to avoid the intracellular defense mechanisms of the host cell. More than 300 genes considered to be virulence factors have been identified to date. Among these are genes involved in adhesion to host cells; genes associated with capsule biosynthesis contributing to serum resistance; and genes, including those from the *Francisella* pathogenicity island coding probably of the type VI secretion system, enabling "neutralization" of intraphagosomal milieu, escape into the cytosol, and proliferation inside the host cell (Nano et al. 2004; Bakshi et al. 2006; Lindgren et al. 2007; Charkaborty et al. 2008; Mohapatra et al. 2008; Schulert et al. 2009; Dai et al. 2010; Ma et al. 2014). *F. tularensis* is also able to delay cell death to increase its survival and replication through activating Ras by the SOS2/GrB2/PKCα/PKCβI quaternary complex, which stimulates cell survival through downregulation of caspase-3 activation (Al-Khodor et al. 2010). Moreover, *Francisella* spp. actively manipulate the timing of autophagy onset, interferon signaling, Toll-like receptor signaling, and phagocytosis (Butchar et al. 2008; Cremer et al. 2009), thus manipulating one of the early cell defense mechanisms against infection (Deretic and Levine 2009). Equally important for *Francisella* is its adaptation to the nutritional limitations inside a host cell (Barel and Charbit 2013).

Despite numerous studies dedicated to virulence factors, *F. tularensis* virulence in its complexity has not yet been sufficiently elucidated (Jones et al. 2014). Altogether, the ability of intracellular pathogens to evade clearance inside host cells and disseminate to other areas of the body is essential to the pathogen's virulence and pathogenicity. It remains unclear, however, as to how *Francisella* spp. actually kills their hosts. Deactivations of immune cells, uncontrolled cytokine response, and toxin production have all been implicated as mechanisms by which bacterial pathogens induce death during systemic infection.
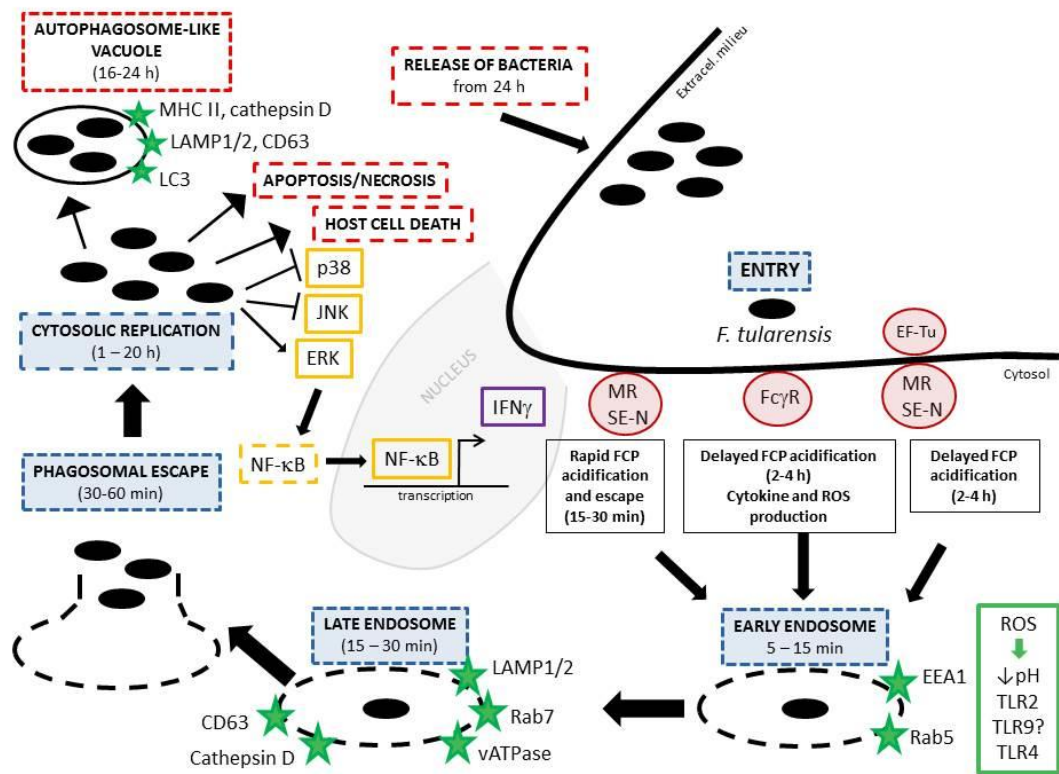
Fig. 1: Intracellular trafficking of F. tularensis after uptake by various macrophage receptors: Francisella uses multiple mechanisms to evade host defense. Infection eventually leads to apoptosis of the infected cells. See text for more details.

2.7 Host immune response

Immunity against *F. tularensis* has been studied for decades, but unanswered questions remain. Some kinds of bacteria have evolved mechanisms for survival inside host cells, and *Francisella* spp. are among these. The immune system is classically divided into innate and adaptive branches, and the innate branch is the most evolutionarily conserved part of the host defense. A substantial part of innate responses is based on such phagocytic cells as macrophages and neutrophils that represent the cellular component while the complement system constitutes the most important humoral component of the innate system. Only macrophages are considered to be primary host cells for *F. tularensis*, but several other cell types within the organism, such as neutrophils, dendritic cells, hepatocytes, and alveolar epithelial cells, also serve as hosts for *Francisella* spp. (Clemens and Horowitz 2007; Celli and Zahrt 2013).

2.7.1 Basic elements of innate and adaptive immune responses to *Francisella*

The initial interaction of *Francisella* with host cells, such as macrophages, dendritic cells, epithelial cells, and endothelial cells, stimulates production of pro-inflammatory cytokines and chemokines in a manner that is dependent upon MyD88, TLR2, and other, unidentified receptors that signal through MyD88 (Collazo et al. 2006; Cole et al. 2007). Bacterial DNA engagement of the NOD-like receptor (NLR) AIM2 may also be critical for inflammasome assembly and release of IL-1β (Fernandes-Alnemri et al. 2010). This, in the opinion of our research group, may occur in the later stages of *Francisella*–host cell interaction, when the bacterial DNA will be accessible for receptors in intracellular space, regardless of whether they are cytosolic or intracellular membrane-bound. Simultaneously, important innate immune cells recruited to the area of infection produce effector cytokines such as IL-12p40, TNF-α, IFN-γ, and IL-17A that influence T cell development and induce host cell production of antimicrobial molecules (Karttunen et al. 1991). In addition to the classic Th1-type cytokines, other mediators include mast cell production of IL-4, which can directly inhibit *Francisella* intramacrophage growth, and B-1a cell production of anti-LPS antibodies that limit intraperitoneal infection (Surcel et al. 1991, Dreisbach et al. 2000). Polymorphonuclear neutrophils are essential for survival of *Francisella* infections initiated via some routes, but, because these fail to eradicate intracellular organisms *in vitro*, their contribution to infection remains unclear (Berker et al. 2006). After several days, activation and expansion of *Francisella*-specific T cells and B cells occur. The αβ T cells are essential for clearance of primary infection, and they produce effector cytokines, such as IL-17A and IFN-γ, as well as the membrane-bound and soluble forms of TNF-α (Ericson et al. 2001). These factors presumably amplify and extend activation of infected mouse host cells to limit *Francisella* intracellular growth through production of reactive oxygen and nitrogen intermediates, as well as other unidentified antimicrobials.

2.7.2 T cell-mediated immune response to *F. tularensis*

Generally, the complement system's crucial activity is in inducing immune responses via the optimal contact of target antigens with macrophages, dendritic cells, and both T and B cells. However, the process of eliminating *Francisella* spp. from the body – similar to that for other intracellular pathogens – is controlled primarily by adaptive immunity and depends on the function of T cell subsets which finalize the expression of

protective immunity (Elkins et al. 2003; Elkins et al. 2007). Regarding intracellular bacteria, T cell-mediated immune responses are paramount for the control of both primary as well as secondary infections. The crucial role of T cells in controlling and eradicating *F. tularensis* infection has been predominantly demonstrated in experiments carried out on experimental animals (Conlan et al. 2011). In humans, an immunospecific T cell response can be demonstrated after the first 2 weeks from disease onset (Tärnvik 1989). Notwithstanding this fact, during the initial stages of tularemia the immune response is almost entirely independent of T cells. Meanwhile, the final resolution and clearance of bacteria from the cells and tissues is completely dependent on αβ T cells, which need to be activated (Elkins et al. 2003; Elkins et al. 2007). Furthermore, in contrast to the well-known role of CD4$^+$ and CD8$^+$ T cell subpopulations in the immune response against *F. tularensis*, the role of other T cell subpopulations is not well understood. For example, it seems likely that CD4$^-$CD8$^-$ double negative T cells play a substantial role during pulmonary *F. tularensis* infection by producing IL-17A and IFN-γ cytokines that additively contribute to control of the infection (Cowley and Elkins 2003; Cowley et al. 2010).

Considerable attention has been devoted also to understanding effector mechanisms provided by the γδ T cells. Although these cells appear to play a minimal role during primary infection of mice, they seem to have a larger role in the case of *F. tularensis* infection in humans, where their numbers remain elevated for as long as 1 year after infection (Poquet et al. 1998; Kroca et al. 2000). Their contribution to the protective response is still rather unclear.

In recent decades, research on anti-infection immunity has been focused on understanding the effects of important cytokines and chemokines during tularemia infection. Studies on murine models have clearly demonstrated that, as early as the initial stage of infection, cells of the innate immune system can produce IFN-γ that activates mononuclear phagocytes and thus controls the retardation of bacterial replication. It is known that CD4$^+$ T helper (Th) 1 cells produce interleukin 2 and IFN-γ and mediate macrophage activation. Th2 cells, on the other hand, are able to produce interleukin 4 and interleukin 5 and provide assistance to B cells. Macrophages secrete TNF-α and IL-12 that stimulate natural killer (NK) cells to produce IFN-γ as well (Lopez et al. 2004; Gosselin et al. 2005). Thus, one major mechanism of cytokine-mediated early host response is operated through the activation of immunocompetent cells, namely NK cells and T cells.

Both activated cell types produce IFN-γ and TNF-α that subsequently activate mononuclear phagocytes to escalate their bactericidal effect and eliminate bacteria from cells and tissues.

Other studies have also described the major role of IL-12 and IL-23. Both IL-12 and IL-23 have been found able to positively regulate IFN-γ production despite the fact that IL-23 still has an unidentified role in the clearance of bacteria during intradermal sublethal *F. tularensis* LVS infection (Butchar et al. 2007; Butchar et al. 2008; Kurtz et al. 2014). Moreover, IL-23 is also an important contributor to promotion of Th17 response, which is critical for host immunity to type A *F. tularensis* infection during primary immune response but is not required during secondary immune response (Skyberg et al. 2013; Roberts et al. 2014).

In contrast, Th2 cell cytokines production has not been studied in such detail as has that of Th1 cytokines. This is despite the fact that data have been accumulated confirming the role of B cells that in the classic model require the assistance of Th2. In looking for the role of Th-2 cytokines, moreover, one comes upon conflicting data from experiments utilizing the vaccine and the virulent *F. tularensis* strains. Recent studies have demonstrated, for example, that IL-6, one of the Th-2 cytokines, is essential for primary resistance to *F. tularensis* LVS (Kurtz et al. 2013), but it fails to exert any effect on the progression of virulent strain infections (Laws et al. 2013).

2.7.3 B cell-mediated immune response to *F. tularensis*

The immune system is highly dynamic and diverse, and this enables it to effectively protect an individual from numerous potentially pathogenic encounters. Protective immunity against *F. tularensis* infection is usually attributed to an effective T cell response. Indeed, there is evidence that B cells are necessary for mice to develop fully protective immunity to primary and secondary LVS infections (Culkin et al. 1997; Elkins et al. 1999). Recent research focusing on the function of B cells in the course of intracellular bacterial infections clearly demonstrates that B cells constitute one of those cell types needed for the expression of an effective protective response. Beyond their historically well-recognized role in mediating humoral immune responses, B cells respond directly to such bacterial surface components as peptidoglycans, capsular polysaccharides, outer membrane proteins or lipopolysaccharides and in a T-independent manner influence

the induction of immune response. Moreover, despite the classical conception of B cells as non-phagocytizing, the peripheral B cells have been shown to phagocytize inert particles or bacteria when these corpuscles are recognized by the BCR. Thus, in fact, B cells can be characterized as ligand-selective phagocytic cells (Souwer et al. 2009). B cells can recognize and respond to both soluble and membrane-associated antigen, although recent insights suggest that membrane-associated antigens are more important for B cell activation *in vivo* (reviewed by Batista and Harwood 2009). The internalization of antigenic particles, along with the ability to extract the antigens from non-internalizable surfaces (Batista and Neuberger 2000), is a prerequisite for the function of B cells as antigen-presenting cells, which is also a well-recognized function of B cells within the immune system.

In human beings and mice, there exist multiple B cell subsets, including follicular B cells and marginal zone (MZ) B cells, as well as transitional B cells (Fagarasan et al. 2000; Lopes-Carvalho and Kearney 2004; Weill et al. 2009; Baumgarth 2011; Vaughan et al. 2011; Garraud et al. 2012; Pieper et al. 2013). It seems probable that humans contain, along with these subsets, also other functionally distinct B cell subsets that have been significantly identified in mice (see below). Follicular and MZ B cells are known as B-2 cells. MZ B cells contain naïve and antigen-experienced B cells. Follicular B cells in both species express IgM and IgD and are CD21$^{int/lo}$ and CD23^{hi}. The level of diversity in BCR usage is greatest in follicular B cells and accounts for most of the enormous antibody diversity seen in B cells. Their productive engagement in immune responses shows a clear dependence on T cells. MZ B cells also share some similarities in mice and human beings in terms of phenotype, with both subsets being IgM^{+}, IgD$^{lo/-}$, B220^{hi}, CD21^{hi}, and CD23^{lo}.

In mice, there are a number of additional B cell subsets that have been unambiguously recognized and these come under the umbrella term of B-1 cells (Fagarasan et al. 2000; Martin and Kearney 2001; Berland and Wortis 2002; Hardy 2006; Montecino-Rodriguez and Dorshkind 2006; Baumgarth 2011). A key feature that distinguishes B-1 cell subsets from B-2 cell subsets is their capacity to self-renew. In the steady state within the peritoneal cavity of a wild-type mouse, about 50% of B cells are B-1 cells, and the ratio of B-1a cells to B-1b cells is approximately 2:1 (Gil-Cruz et al. 2009; Marshall et al. 2012). A factor that complicates the study of B-1 cells is their more elusive phenotype. They express CD19 and low levels of IgD but relatively high levels of IgM. They can express CD43 and CD9, are negative or low expressers for other markers such as

CD21 and CD23, and are mostly intermediate for B220 (Wells et al. 1994; Won and Kearney 2002; Tung et al. 2004; Hardy 2006; Baumgarth 2011). At minimum, B-1 and MZ B cells participate in innate immune responses and may represent the functional link between innate and actively acquired immune responses (see Table II). Therefore, B cells, in their diversity and complexity, are integral to both natural as well as acquired immune system responses to infectious agents and can also contribute effectively to the phase of immunological memory.

Table II: Cell-surface molecules that are implicated in presenting antigen to B cells

Presenting cell	Receptor	Antigen presented
Marginal-zone B cell	CR1 and CR2	Complement-coated antigen
Follicular B cell	CR1 and CR2 FcγRIIB	Complement-coated antigen IgG-coated antigen
Macrophage	MAC1 DC-SIGN FcγRIIB	Complement-coated antigen IgG-coated antigen Carbohydrate-coated antigen
Dendritic cell	DC-SIGN FcγRIIB	IgG-coated antigen Carbohydrate-coated antigen
Follicular dendritic cell	CR1 and CR2 FcγRIIB	Complement-coated antigen IgG-coated antigen

CR, complement receptor; DC, dendritic cell; DC-SIGN, DC-specific ICAM3-grabbing non-integrin; FcγRIIB, low-affinity Fc receptor for IgG; ICAM3, intercellular adhesion molecule 3; MAC1, macrophage receptor 1.

The best characterized tool of B cells for regulating immune responses is the production of IL-10 and TGF-β (O'Garra et al. 1990; Suda et al. 1990) by the subset of B cells recently termed Breg. This B cell subset expressing CD5 has been shown to be the producer of IL-10 in response to lipopolysaccharide (LPS) (O'Garra and Howard 1992). B cells, similarly to T cells during the induction of acquired immunity, can be subdivided into at least two functional effector subsets producing distinct arrays of cytokines and chemokines. So-called B effector 1 cells (Be-1 cells) produce IFN-γ, TNF-α, IL-12/p40

and IL-10 while B effector 2 cells (Be-2) produce cytokines IL-2, IL-4, IL-6, IL-10, IL-13, and TNF-α. The original resolution to Be-1 or Be-2 cells is dependent on the cognate interaction with Th1 and Th2 cells, respectively. Interestingly, these subsets of B cells can interact with naïve CD4^{+} T cells and prime them into the Th1 or Th2 immunoregulatory branch of the acquired immune response.

F. tularensis has a significant extracellular phase in the host, which makes it accessible to humoral immune responses (Forestal et al. 2007). It has been demonstrated that antibody responses containing IgM, IgG, and IgA antibody isotypes are detectable at the end of the first week following natural infection of humans (Koskela and Salminen 1985). Studies on several animal tularemia models have shown that passive transfer of polyclonal as well as monoclonal antibodies can protect naïve hosts against attenuated *F. tularensis* LVS and less virulent strains of subspecies *holarctica*, but not against inhalation infection with highly virulent *F. tularensis* of subspecies *tularensis* (Khlebnikov et al. 1992; Drabick et al. 1994; Stenmark et al. 2003; Kubelkova et al. 2012). While many laboratories have demonstrated that serum antibodies are mainly directed against *F. tularensis* lipopolysaccharide, serum antibodies with reactivity to bacterial proteins have also been detected. Among these are antibodies oriented against some outer membrane proteins such as FopA, OmpA (Sundaresh et al. 2007; Savitt et al. 2009) and Tul4 (Havlasova et al. 2005); against other intracellular proteins such as GroEL, KatG (Sundaresh et al. 2007) and DnaK; and against several putative virulence markers such as nucleoside diphosphate kinase, isocitrate dehydrogenase, the RNA-binding protein Hfq, and the molecular chaperone ClpB (Havlasova et al. 2002; Havlasova et al. 2005). Thus, antibodies can potentially contribute to the protective response by eliminating *Francisella* virulence factors and, together with the antibody-independent functions of B cells, can demonstrate the potential of B cells to collaborate with T cells in the induction, regulation, and expression of protective immunity against *F. tularensis* infection. The construction of B cell-deficient mice renders studying the role of B cells in protective immune mechanisms against *F. tularensis* more feasible. These animals have no B cells and no detectable antibody levels, but they do have a fully functional T cell compartment (Elkins et al. 1999; Bosio and Elkins 2001).

3 AIMS OF THESIS

➢ To determine the final fate of B cells after mutual interaction with *F. tularensis*

➢ To determine the B cell participation in the induction and regulation of the adaptive immune response during early stages of *F. tularensis* infection

➢ To determine the B cell-mediated effector responses together with the induction of T cell-mediated immunity against *F. tularensis* infection

4 OVERVIEW OF METHODOLOGICAL APPROACHES

➢ To analyze the mutual interaction of B cells with intracellular pathogen the murine model of *F. tularensis* infection has been used. Mice were killed by cervical dislocation and the organs have been aseptically removed. Peritoneal cells were harvested using lavage technique. Bacterial burden has been determined by plating peritoneal and/or spleen suspensions onto McLeod solid plates and by counting the CFU. *In vivo* experiments on mice were conducted under supervision of the institution's Animal Unit and were approved by the Animal Care and Use Committee of the Faculty of Military Health Sciences, University of Defense, Hradec Kralove, Czech Republic. All cultivation and cocultivation experiments have been conducted under Biosafety level 2 lab safeties with ensuring of sterility of all samples.

➢ All bacterial lysates of *F. tularensis* have been prepared according the lab manual using Chamberlain growth medium and French Press technology for disruption of bacteria. Disrupted cultures were then tested for lack of viability by inoculation undiluted samples onto McLeod solid agar followed by incubation at 36.8 °C for 72 h.

➢ The influence of bacterial load and time of infection on B cell apoptosis was determined using following flow cytometry approaches:

a) determination of cell populations, infected cells, and activating markers using antibodies against cell surface components and specific markers,

b) depolarization of electrochemical mitochondrial membrane potencial, as an important index in the induction of cellular apoptosis using fluorescent cationic dye JC-1,

c) detection of apoptosis using Wildan´s propidium iodide solution staining of target infected of uninfected cells,

d) externalization of phosphatidylserine in apoptotic cells using recombinant annexin V conjugated to green-fluorescent FITC dye and dead cells using propidium iodide (while propidium iodide stains necrotic cells with red fluorescence),

e) cell sorting using anti-CD19-FITC staining

➢ Individual apoptotic pathways were detected using the Western blot analysis where increased expression of active forms of selected caspases has been observed. Moreover the activation of mitochondrial apoptotic pathway followed by a release of apoptogenic factors

from the mitochondrial space were determined by monitoring of expressed level of selected proapoptotic molecules.

➢ Several genes associated with apoptosis were also analyzed using Oligo DNA Microarray analyses resulting in the gene expression changes.

➢ Levels of selected cytokines and antibodies were determined using cytokine Quantibody Array technology or enzyme-linked immunosorbent assays.

➢ Cobalt-60 gamma irradiation source was used for sublethal whole-body irradiation of mice in the followed dose of 4 Gy.

Detailed information on the individual experimental procedures is given in publications related to this thesis.

5 RESULTS AND DISCUSSION

5.1 Activation of apoptotic processes in human cell (Paper I.)

Although *F. tularensis* replicates during infection primarily in macrophages, it also proliferates in other cell types. Various receptors mediate its entry into host cells. Complement-opsonized *F. tularensis* enters macrophages by looping phagocytosis. Uptake is mediated in part by Syk, which may activate actin rearrangement in the phagocytic cup and result in *F. tularensis* being engulfed in a lipid raft-rich phagosome (Parsa et al. 2008). Inside host cells, *F. tularensis* resides transiently in an acidified late endosome-like compartment prior to disruption of the phagosomal membrane and the escapes into the cytosol, where bacterial proliferation occurs. The *Francisella* pathogenicity island-encoded type VI-like secretion system mediates modulation of phagosome biogenesis and escape into the cytosol (Santic et al. 2005). During late infection stages in mouse macrophages, *F. tularensis* is taken up in an autophagy-like compartment (Checroun et al. 2006). This reentry of *F. tularensis* into the endosomal–lysosomal pathway through autophagy does not occur in human macrophages, however, and is therefore not relevant to infection of humans.

It recently has become generally clear that B cells can be an equal partner with T cells, dendritic cells, and macrophages in creating an effective immune response against *F. tularensis* infection. The precise mechanism and general fate and trafficking of B cells within *F. tularensis* infection has not yet been elucidated. This thesis provides more detailed information with regard to this unanswered question.

Interaction of human B cells with F. tularensis

F. tularensis interacts directly with the Ramos human B cell line (RA-1) and invades it. The first contact of *Francisella* with the B cell is characterized by the creation of a tight junction of bacterial and eukaryotic cell membranes. *F. tularensis* LVS bacteria adhere to the cell surface within 6 h. Within the next 24 h of co-cultivation, about 25% of cells in cultures of Ramos cells have intracellularly localized bacteria (see Fig. 2).

Activation of death receptor-mediated (extrinsic) caspase pathway

The term 'apoptosis' was coined by Kerr, Wyllie and Currie in 1972 to describe a mode of cell death associated with fragmentation of genomic DNA (Kerr et al. 1972). Cysteine-aspartic proteases (caspases) comprise a family of cysteine proteases playing essential roles in apoptosis (programmed cell death), necrosis, and inflammation. The mechanisms and functions of caspase activation remain central to the apoptotic signaling network. Activation of apoptotic caspases results in inactivation or activation of substrates and the generation of a cascade of signaling events permitting the controlled demolition of cellular components.

Depending upon the inducers of apoptosis involved, apoptotic signals originate either extracellularly (extrinsic pathway) or intracellularly (intrinsic pathway). The extrinsic apoptotic pathway is activated by such death receptors on the plasma membrane as tumor necrosis factor receptor 1 (TNFR1) and Fas/CD95. As ligands bind to these receptors, the complex inducing death signaling is formed and leads to initiation of the caspase cascade through caspase 8.
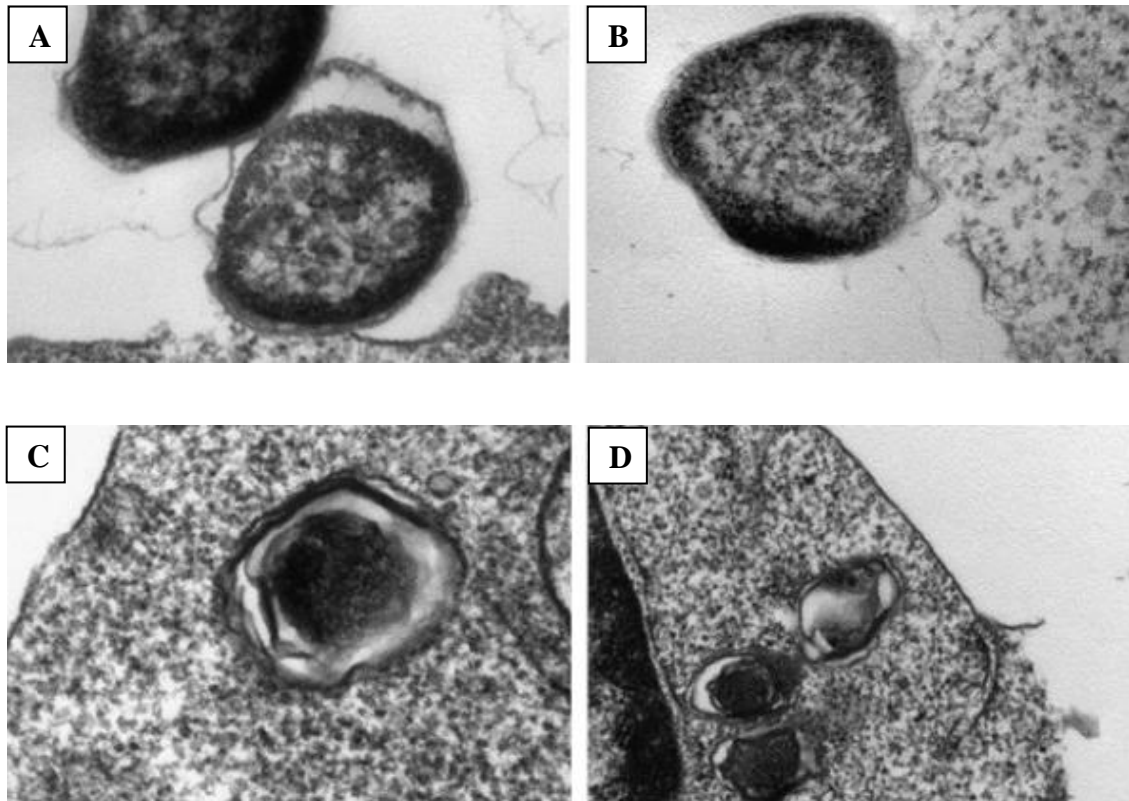
Fig. 2: Transmission electron microscopy of human Ramos cell line infected with *Francisella tularensis* LVS (MOI 500) after 6 h (**A–B**), 12 h (**C**), and 24 h (**D**). Magnification ×55,000 (**C**) and ×45,000 (**D**) (Krocova et al. 2008).

Increased expression of cleaved active forms of caspase 8 was detected by western blot from 9 h after B cell infection with *F. tularensis*. Active caspase 8 activates caspases 3 and 7 either directly or by cleavage of the Bcl-2 interacting protein.

Activation of mitochondrial-mediated (intrinsic) caspase pathway

Because it depends on factors released from the mitochondria, intrinsic apoptosis is also known as mitochondrial apoptosis. A vast array of cellular stresses activate this pathway, including growth factor deprivation, cytoskeletal disruption, DNA damage, accumulation of unfolded proteins, hypoxia, and many others. It can be activated, too, by developmental signals that instruct cells to die, such as hormones (Brenner and Mak 2009). Caspase 9 is the initiator caspase responsible for the intrinsic apoptosis pathway. It is activated by dimerization induced when the caspase-9 CARD domain binds to the adapter protein apoptotic protease-activating factor-1 (APAF1) (Shiozaki et al. 2002). The intrinsic

apoptotic pathway is characterized by permeabilization of the mitochondria and release of cytochrome c into the cytoplasm. Cytochrome c then forms a multi-protein complex known as the "apoptosome" and initiates activation of the caspase cascade through caspase 9, which acts as a focal point for multiple protein kinase signaling pathways regulating apoptosis (Shiozaki et al. 2002).

Changes in mitochondrial membrane potential were found 1 h after infection with *F. tularensis*. After 6 h, more than 60% of infected cells showed depolarized membrane potential. Moreover, disappearance of cytochrome c from the mitochondrial fraction was observed when compared with noninfected or killed counterparts. In contrast, increased levels of apoptosis-inducing factor (AIF) were detected at 12 and 24 h after infection of Ramos cells. This points to a caspase-independent apoptotic pathway, because AIF is a mitochondrial positive intrinsic regulator of apoptosis that triggers chromatin condensation and DNA fragmentation in a cell to induce programmed cell death. Enhanced expression of the active form of caspase 9 was observed at 9, 12 and 24 h post infection, and this was confirmed by the colorimetric method.

Activation of caspase 3

Caspase 3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. To prevent untimely activation of the executioner caspase 3, it is produced as an inactive procaspase dimer that must be cleaved by an initiator caspase. This cleavage between the large and small subunits allows a conformational change that joins the two active sites of the executioner caspase to create a functional mature protease (Riedl and Shi 2004). Once activated, a single executioner caspase can cleave and activate other executioner caspases, leading to an accelerated feedback loop of caspase activation. Caspase 3 may have the opposite effect, however, as B cells lacking caspase 3 have shown increased proliferation *in vivo* and hyperproliferation after mitogenic stimulation *in vitro* (Woo et al. 2003).

In our study, the activity of caspase 3 was significantly increased 24 h after infection of Ramos cells with live as well as with killed *F. tularensis* bacteria. Despite the limited multiplication of *Francisella* inside Ramos RA-1 cells, the cells in infected culture undergo the apoptotic process. Surprisingly, the number of apoptotic cells in culture is much higher than is the number of infected cells. An experiment to transfer apoptosis from

infected to noninfected cultures was unsuccessful, and this may suggest that only the contact of a bacterium with a B cell can activate one of the receptor-mediated apoptotic pathways.

In conclusion, flow cytometry, western blot, colorimetric assay and transmission electron microscopy data show that B cells are infected with *F. tularensis*, probably resulting in cell death through apoptosis. Taken together, the collective results indicate that live *F. tularensis* bacteria activate all of the tested apoptotic pathways in B cells within 24 h of interaction, including the extrinsic type I (caspase 8) and type II (tBID) pathways, the intrinsic (cytochrome *c*, caspase 9) pathways, and the caspase-independent (AIF) pathways. Clearly then, these events can play an important role in the host's innate immune response to *Francisella* infection.

5.2 Significant role of murine B cells in the course of *F. tularensis* infection (Paper II.)

Direct interaction of B cell subpopulations with bacteria results in autonomous B cell differentiation and rapid secretion of bacterium-specific antibodies that influence the pathogen's distribution to vital organs. Moreover, specific antibodies against intracellular bacteria have been demonstrated to have protective potential. On the other hand, intracellular bacteria survive intracellularly in B cells mainly in a non-replicative state, as such constituting a source of systemic infection and/or reservoir for reinfection. B cells thus comprise one of the cell types responsible for the final fate when intracellular bacterial pathogens interact with their hosts. Because the relevant data in the literature has been ambiguous, we have studied the role of B cells in the course of *F. tularensis* infection. Within the study, not only was a murine B cell line used, but spleen and peritoneal cells were also applied for analyzing various cell types during *F. tularensis* infection.

Interaction of mouse B cells with F. tularensis

Transmission electron microscopy was used to study the direct interaction of *F. tularensis* LVS with B cells. *F. tularensis* interacts directly with and invades the A-20 mouse B cell line. Approximately 6% of bacteria were adherent to A-20 cells after 6 h of infection (Figs. 3A and 3B). The number of bacteria localized on the outer side of the cytoplasmic membrane was similar for A-20 cells when compared with the Ramos cell line, and it decreased over the course of infection to approximately 3% after 12 h and 0.5% after 24 h. The first contact of *Francisella* with the B cell is characterized by the creation of a tight junction of bacterial and eukaryotic cell membranes. *F. tularensis* LVS bacteria adhere to the cell surface within 6 h. Within the next 24 h of co-cultivation, about 25% of cells in cultures of A-20 cells have intracellularly localized bacteria (see Figs. 3C and 3D).
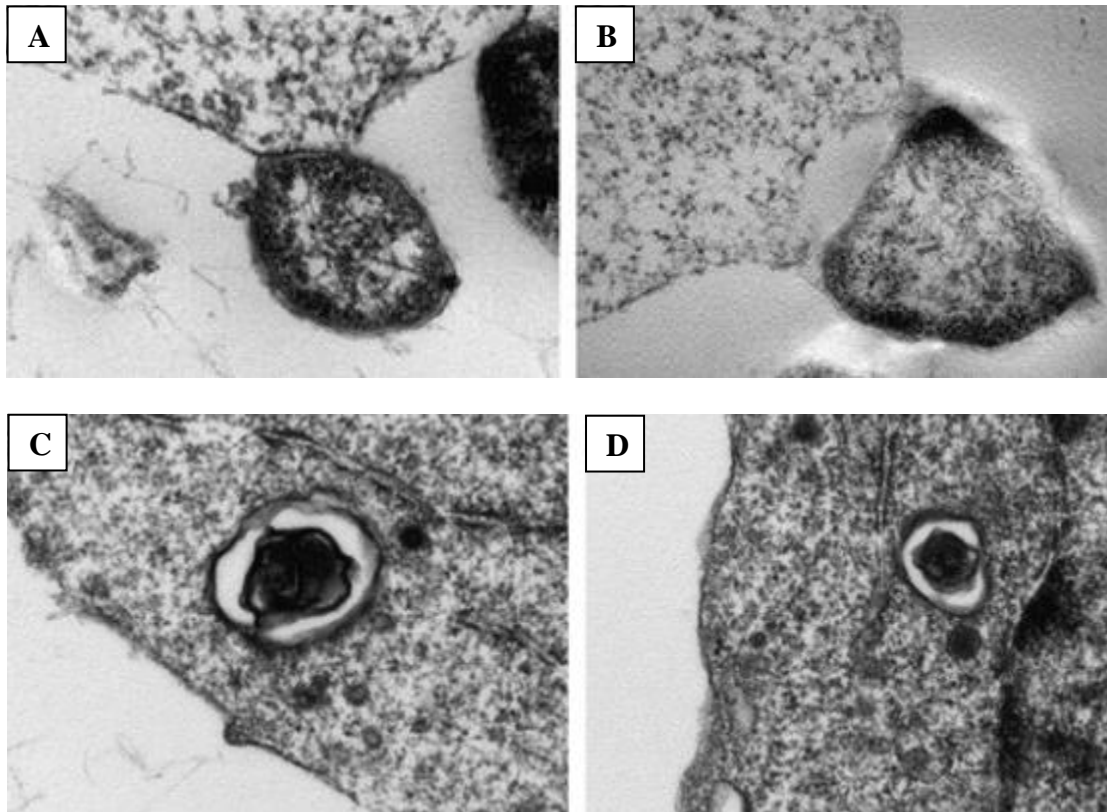
Fig. 3: Transmission electron microscopy of mouse A-20 cell line infected with *Francisella tularensis* LVS (MOI 500) after 6 h (**A–B**), 12 h (**C**), and 24 h (**D**). Magnification ×55,000 (**C**) and ×45,000 (**D**) (Kročová et al. 2008).

We found that bacteria were adherent to the surface of eukaryotic cells independently of the type of host cells (in this case A-20 cells). Together with bacteria adherent to the cell surface 6 h after infection, we also detected approximately 7% of A-20 cells with engulfed *F. tularensis* LVS. The number of cells containing bacteria inside increased to 25% after 12 h, which level persisted through 24 h. (Detailed information about the direct interaction of *F. tularensis* with A-20 cells can be found in Kročová et al. 2008.)

Cellular changes in the peritoneal cavity after F. tularensis infection

B-1 cells may have phagocytic and antigen-presenting functions, secrete cytokines to modulate the host, and produce antibodies, even as these properties may not necessarily be mutually exclusive within individual cells (Yanaba et al. 2008; Popi et al. 2009; Gao et al. 2012; Parra et al. 2012). B-1a cells express CD5 and, at least in the steady state, are the dominant B-1 cell subset in the peritoneal cavity. B-1 and B-2 cells are lymphocyte

populations that play a major role in maintaining host immunological tone and homeostasis, make striking contributions to the control of infections, and differ in development, surface marker expression, tissue localization, and function (Baumgarth et al. 1999, 2000; Baumgarth 2011).

It has been observed through the first three weeks of infection that the populations of individual cell types underwent significant changes as measured by their relative proportions in the peritoneal cavity. Already 30 min after infection, different infected cell types (T cells, B cells, macrophages, dendritic cells, NK cells, granulocytes) were detected in the spleen and peritoneum. The percentage of B-1a cells was growing until 10 d after infection, even as the percentage of B-2 cells was declining. The proportion of B-1b cells appeared to be relatively stable (see Table III).

Table III: Distribution of different cell populations in peritoneal cavity of F. tularensis LVS infected Balb/c mice

Time Population	Control	1 d	3 d	5 d	7 d	10 d	14 d	21 d
T cells[a]	19.77 ± 1.75	13.42 ± 2.99**	16.60 ± 1.23*	23.43 ± 1.41	21.91 ± 8.46	28.78 ± 3.8*	30.40 ± 3.40*	30.18 ± 1.41**
B cells[a]	62.07 ± 3.08	50.89 ± 8.48	59.13 ± 4.25	30.81 ± 10.41*	51.62 ± 3.22	51.45 ± 8.08	48.17 ± 0.38**	48.88 ± 2.12*
B-2[b]	37.62 ± 6.23	26.17 ± 6.39	22.44 ± 11.51	13.26 ±2.00**	11.45 ± 1.83**	16.96 ± 1.26*	38.08 ± 0.32	39.80 ± 5.75
B-1a[b]	18.51 ± 3.36	19.90 ± 0.05	44.31 ± 2.72**	47.64 ± 8.89*	50.69 ± 3.40**	53.55 ± 2.62**	29.54 ± 0.44	26.82 ± 6.43
B-1b[b]	43.93 ± 3.52	53.54 ± 6.63	34.06 ± 8.68	40.70 ± 7.68	39.85 ± 4.50	30.05 ± 1.80	32.51 ± 0.17	33.30 ± 0.90
Mf[a]	6.87 ± 1.33	16.42 ± 5.16*	21.35 ± 2.05**	22.41 ± 2.86**	13.86 ± 1.91*	12.31 ± 9.17	7.43 ± 0.13	4.86 ± 0.55
DC[a]	5.17 ± 2.06	6.12 ± 2.59	0.55 ± 0.06*	2.03 ± 0.63	1.30 ± 0.30	0.54 ± 0.92*	0.31 ± 0.31*	10.43 ± 1.94
Gr-1[a]	3.95 ± 2.95	7.22 ± 5.13	10.41 ± 3.0*	11.51 ± 0.99	8.32 ± 1.51	5.11 ± 4.13	6.07 ± 0.09	2.00 ± 0.68
NK1.1[a]	2.55 ± 0.72	5.75 ± 0.71*	2.79 ± 0.61	9.47 ± 1.60*	4.41 ± 1.03	3.19 ± 2.84	4.67 ± 0.04	3.73 ± 1.40

a) Results are expressed as percentage of all measured cells.
b) Results are expressed as percentage of all B lymphocytes.
c) Results shown are representatives of three independent experiments, each using three individual animals.
Results are expressed as means ± standard deviation. Asterisks indicate values that are statistically different in comparison with those from uninfected control mice.
(* $P \leq 0.05$, ** $P \leq 0.01$).

CD19^{+} cell subset express activation markers after F. tularensis infection

Kinetics studies of B-1a, B-1b and B-2 cells were performed on cells isolated from the peritoneum and spleen of infected Balb/c mice using flow cytometry. To establish whether the mutual interaction of *F. tularensis* and B cells activates the latter selected

activation markers, major histocompatibility complex II class molecules, CD25, CD54, CD69, CD80, and CD86 were used to monitor the activation process of B cell subsets in peritoneum and spleen of control and infected mice. The results demonstrated the activation of B-1a and B-1b peritoneal as well as spleen cells in response to *F. tularensis* infection. In general, it can be said that splenic B cells were activated during later intervals of infection when compared to their peritoneum counterparts. Nevertheless, the sole exceptions were the early activating marker CD69 and co-stimulatory molecule CD86, whose expression was significantly increased 24 h after infection on B-1 cell subsets overexpressed on the B-2 cell surface 5 d after *F. tularensis* infection.

CD69 is one of the cell surface antigens expressed earliest on B cells and also on a variety of other cell types, including macrophages, platelets, neutrophils, and NK cells. Shiow et al. (2006) reported that endogenous CD69 inhibits the function of the sphingosine-1-phosphate receptor in T cells and B cells, engagement of which is known to play an important role in lymphocyte activation and relocation. Moreover, the CD86 co-stimulatory molecule plays a prominent role in immune regulation. Regarding activation, development of the inflammatory process depends upon T cell activation, which requires engagement of the T cell receptor with the MHC-peptide complex presented on the cell surface of antigen-presenting cells. In addition to this antigen-specific stimulation, a second interaction involving a co-stimulatory molecule, CD28, on T cells and its ligands, CD80 and CD86, on antigen-presenting cells is required for optimal T cell activation. Changes in the expression of receptors and/or receptor ligands on the surface of cooperating immunocompetent cells are crucial for the functional cell–cell interactions. Such interactions are indispensable during the regulatory stage of immune response and during the induction of secondary response to pathogens.

CD19^{+} cells produce set of cytokines in response to F. tularensis infection

Interrelationships among B cells and other lymphoid cells during the induction of immune response against intracellular pathogens are controlled by the secretion of cytokines and expression of membrane-bound receptor ligands and their receptors. B cells are potent producers of a whole range of cytokines after infection. Depending, though, on the phase of infection, responding B cell subsets, and stage of induced immune response, the cytokines produced by B cells can positively and/or negatively regulate the expression

of defense mechanisms and bacterial load in the organs of the infected host. Activation of B cells is associated with those cells' increased production of cytokines.

To observe the cytokine response of peritoneal and spleen B cells to *F. tularensis* infection, a kinetic study of selected cytokine production was made using sorted and infected B cells from the peritoneum and spleen. Sorted, infected peritoneal CD19$^+$ cells produced significantly more of the cytokines IFN-γ, IL-12, IL-17, IL-1β, IL-23, IL-4, IL-6, and TNF-α than did control cells at least as early as 12 h after intraperitoneal (*i.p.*) infection. The levels of tested cytokines in supernatants of CD19$^-$ cells isolated from the peritoneum and spleen during the first 24 h after infection were comparable to those of CD19$^-$ cells isolated from uninfected mice. Significantly greater production was observed only for IL-2 and IL-17 by peritoneal CD19$^-$ cells in intervals 48 and 72 h after infection.

The production of cytokines by CD19$^+$ cells at the initial phase of *i.p.* infection may be functionally important because at the early time intervals the CD19$^-$ cells do not respond significantly to infection by producing any of those cytokines tested. Their response, according to our data, began approximately 3 d after infection and can correspond to the onset of a T-dependent phase of the immune response. The spectrum of cytokines produced in response to *F. tularensis* infection corresponds to that produced by Th1, Th2, as well as Th17 cells. Although the exact contribution of CD19$^+$ cells to induction and regulation of the protective response is still to be defined, it is clear that peritoneal as well as spleen CD19$^+$ cells respond to *i.p.* infection by cytokine production earlier than do CD19$^-$ cells. This can suggest that the abilities of T and B cells to activate the second immunocompetent partner are reciprocal.

5.3. The role of humoral response is important for development of protective immunity against *F. tularensis* infection (Paper III)

Antibodies constitute one of the main components of humoral immunity, having their role in natural as well as acquired immune reactions. Both types of reactions mediated by antibodies play a role in the course of infections mediated by such bacterial intracellular pathogens. Antibodies provide a wall of protection against infection. In the course of infection by intracellular bacteria, the role of antibodies in the protective response has long been underestimated. Since antibodies against many antigens are not protective, it may be that antibodies derived from B-1 cells provide greater protection in relative terms than do those from B-2 cells and particularly those from follicular B cells. Natural antibodies produced by B-1 cells have been demonstrated to be protective (Shilova et al. 2011), which may suggest that a wide repertoire of antibody clones (or responses) is not always necessary to protect against intracellular bacterial infection.

Passive protection of Balb/c mice against subsequent F. tularensis challenge

F. tularensis has a significant extracellular phase in the host, thus making it accessible to humoral immune responses (Forestal et al. 2007). Antibody responses containing IgM, IgG, and IgA antibody isotypes have been shown to be detectable at the end of the first week following natural infection of humans (Koskela and Salminen 1985). The characteristic Th1 and Th2 antibody isotypes (IgM, IgG, IgG2a, IgG2b, IgG3, and IgA) can long persist in sera after infection of mice (Kirimanjeswara et al. 2007). In addition to LPS, the antibodies in immune murine and human convalescent sera are directed also against proteins, among which are actual or putative virulence factors (Havlasova et al. 2005; Janovska et al. 2007a, 2007b). Multiple experimental models of infection show that IgM is critical for much of the short- and long-term protection afforded after natural infection and that the functional roles of IgM and IgG are likely to be synergistic (Racine and Winslow 2009). Pentameric IgM's high avidity means that it is efficient in activating complement, whereas not all IgG isotypes are equally efficient in doing so (Dangl et al. 1988).

To examine the functioning of antibodies in protecting against tularemia, we have employed the experimental model of sub-lethally irradiated mice. Naïve Balb/c mice were

irradiated using ^{60}Co at a sublethal dose of 4 Gy to test the possibility for protecting an immunocompromised host by passive transfer of immune sera. The fact that sublethal γ-irradiation of mice resulted in a Th1/Th2 imbalance can help to elucidate in greater detail the importance of cytokines and specific circulating antibodies in the mouse model of *F. tularensis* infection (Park et al. 2011). One of the effects of ionizing radiation on the immune system is the resulting disbalance of cytokine production. Substantial lymphopenia, caused by an induction of lymphocyte apoptosis, starts within several hours after irradiation, and recovery requires at minimum 2 weeks (as demonstrated on the murine model). During this period, the resistance to infections is greatly impaired.

Doses of γ-irradiation greater than 3 Gy completely impair the resistance to *F. tularensis* in mice. Our results demonstrated that the passive transfer of immunity by sera from mice immunized with *F. tularensis* LVS protected sub-lethally irradiated mice against the challenge with otherwise lethal infection caused by *F. tularensis* subsp. *holarctica* as well as provided full protection against a subsequent challenge with a dose of the fully virulent *F. tularensis* strain SchuS4 that was lethal for unirradiated control counterparts. Some limits were nevertheless observed in the capability of transferred sera to protect irradiated mice against *F. tularensis* LVS infection. Within the model of irradiated mice overall, a significant effect was demonstrated from passive transfer of immunity through specific anti-*F. tularensis* antisera.

Irradiated and passively protected Balb/c mice – the interplay of cytokine production

Post-irradiation changes in the composition of immunocompetent cells strongly influence immune system functions. Within the context of our experimental model, changes in the production of cytokines that ensure intercellular communication among immune cells, fibroblasts and other cells are of key importance. Immediately after irradiation, the synthesis and secretion of pro-inflammatory and anti-inflammatory cytokines can be detected, and these can increase the robustness of the immune response. Such traditional pro-inflammatory cytokines as IL-1β, IL-4, IL-6 with TNF-α and INF-γ that are critical for resolving the primary infection were selected for monitoring cytokine responses to irradiation and/or infection. Due to the fact that γ-irradiation in general depresses cytokines' response to infection, we also monitored the levels of selected cytokines in immunocompromised infected mice.

Specifically, cytokine production following *i.d.* infection with *F. tularensis* LVS was studied. The first approach was to determine selected cytokines in sera from irradiated Balb/c mice and irradiated and passively protected Balb/c mice infected *i.d.* with 10^2 CFU of *F. tularensis* LVS on day 3 after irradiation. Because the levels of cytokines can vary in different tissue compartments, we decided to take also a second approach consisting of determining cytokines levels in selected organs (spleen, liver, and lungs). We analyzed the cytokine levels in homogenates of organs obtained from irradiated and irradiated and passively protected Balb/c mice infected *i.d.* with 10^2 CFU of *F. tularensis* LVS.

As seen similarly in the case of other intracellular pathogens, rapid production of pro-inflammatory and Th1-type cytokines is critical for initial control of *Francisella* infection in all settings examined to date. There nevertheless is a striking lack of crucial pro-inflammatory cytokines during the first 48 h of murine pulmonary *Francisella* infection. It is not until after the first 48–72 h of murine infection that key cytokines and chemokines become readily detectable. During virulent *F. tularensis* respiratory infection of mice, mRNA levels of such essential antimicrobial cytokines as IFN-γ and TNF-α have been shown to rise in the lungs between days 2 and 4 (Andersson et al. 2006b), and serum/distal organ levels of pro-inflammatory mediators such as RANTES, IL-6, and IL-1β have become detectable on days 3–4 (Conlan et al. 2008). After 2 d of unrestricted bacterial growth, however, key organs such as the lungs and liver harbor extremely high bacterial burdens. Thus, the relatively late upregulation of antimicrobial host immune mechanisms appears to be too late to prevent death.

6 CONCLUSION

As an experimental model, *Francisella* is unique in establishing productive infection in a huge variety of insects and mammals by many routes. In mice, basic immune parameters that operate in defense against other intracellular pathogen infections, such as IFN-γ, TNF-α, and reactive nitrogen intermediates, are central for control of *Francisella* infection. However, other important immune mediators have been newly revealed, including IL-17A, TLR2, and the inflammasome. Furthermore, a variety of cell types in addition to macrophages are now recognized as supporting *Francisella* growth, including epithelial cells and dendritic cells. CD4$^+$ and CD8$^+$ T cells are clearly important for control of primary infection and vaccine-induced protection, but new T cell subpopulations and the mechanisms employed by T cells are only beginning to be defined. Along with the classic spectrum of protective responses to *F. tularensis*, a significant role for B cells and specific antibodies has been established. Their contributions vary greatly, however, between bacterial strains of lower and higher virulence. Although B cells and their products have significant roles in protective immunity to *Francisella*, optimal protection against *Francisella* infection clearly requires T cell-mediated immunity.

The thesis presented here has thus clearly demonstrated that B cell subsets, namely during the early stages of immune response, play an essential role at the border of innate and acquired immune responses against an intracellular bacterium. The production of antibodies, production of cytokines, and mutual contacts with other immunocompetent cells are all attributes of B cell subsets during the induction and regulation of immune responses against *F. tularensis* infection.

The *Francisella* models appear to be particularly attractive for tackling some current themes regarding immunity to pathogens, such as comparing similarities and differences in systemic or mucosal immunity, understanding the tension between pathogen-induced immunosuppression and host immune responsiveness, and appreciating the ongoing interplay between rapid innate reactions and later specific immunity.

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8 LIST OF ABBREVIATIONS AND SYMBOL

^{60}Co	cobalt 60
µg/µL	microgram/microliter
α	alpha
β	beta
γ	gamma
Ab	antibody
AIF	apoptosis-inducing factor
AIM	absent in melanoma
BCR	B-cell receptor
CD	cluster of designation
CFU	colony forming unit
CO_2	carbon dioxide
ddH_2O	deionized water
EEA	early-endosome antigen
ELISA	enzyme-linked immunosorbent assay
F. tularensis	*Francisella tularensis*
FACS	fluorescence-activated cell staining
FCP	*Francisella*-containing phagosome
FITC	fluorescein isothiocyanate
Gy	Grey
HRP	horse raddish peroxidase
Ig	immunoglobulin
IL	interleukin
IMS	immune mouse serum
LPS	lipopolysaccharide

LVS	live vaccine strain
MyD	myeloid differentiation factor
MZ	marginal zone
NLR	nod-like receptor
NK cell	natural killer cell
NMS	normal mouse serum
OD	optical density
subsp.	subspecies
Th cell	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
USA	United States of America
UV	ultraviolet

9 OUTPUTS

Paper I.

Paper II.

Paper III.

OTHER PUBLICATIONS AND OUTPUTS

Papers related to the thesis

Plzakova L, Krocova Z, <u>Kubelkova K</u>[#], Macela A: Entry of *Francisella tularensis* into murine B cells: The role of BCRs and CRs. PLOS One, **2015**, Under revision (#correspondidng author).

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Other publications

Kmochova A., Pejchal J., Tichy A., Zarybnicka L., Sinkorova Z., Rehacek V., Durisova K., <u>Kubelkova K</u>., Kuca K., Vavrova J.: Modulation of ionizing radiation-induced effects by NU7441, KU55933 and VE821 in peripheral blood lymphocytes. Int. J. Mol. Sci. **2015**, 16, 1-x manuscripts; doi:10.3390/ijms160x000x.

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