Adaptive immunity in murine *Bartonella* infection

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Statement of my thesis

This work has been performed in the groups of Prof. Christoph Dehio, focal area infection biology at the Biozentrum, University of Basel, and Prof. Daniel Pinschewer, experimental virology at the Department for Biomedicine, University of Basel.

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This thesis is written in a cumulative format. It consists of a synopsis of a variety of aspects related to the project and a results section, including the manuscript, which covers most of the work performed during this project, and a collection of other, additional experiments. Finally, the major findings are summarized in the concluding remarks, giving also an outlook related to the findings of this thesis.

Abstract

Bartonella is a genus of facultative-intracellular bacteria causing a long-lasting intra-erythrocytic bacteremia in their mammalian reservoir hosts. Clearance of the bacteremia has been described to be mediated by antibodies. The exact clearance mechanism, however, has not been investigated so far. In this work, several aspects related to the immune response against *Bartonella* have been studied in more detail, with an emphasis on the host's protective antibody response.

For the manuscript "Neutralizing antibodies protect against murine *Bartonella* infection by interfering with erythrocyte adhesion", infection of mice with *B. taylorii* served as a model for the infection of the natural reservoir host by *Bartonella*. Bacteremia clearance kinetics and antibody responses were investigated in different murine knock-out models. The clearance was observed to be independent of the presence of the complement or Fc-receptors. An *in-vitro* erythrocyte adhesion inhibition assay was established, which correlated with protection by an immune serum or antibody *in vivo* and lead to the conclusion that neutralizing antibodies protect by interfering with the red blood cell attachment of the bacteria. We identified a so far understudied virulence factor, a predicted autotransporter on the bacterial surface, as a target of the murine antibody response and suggest that it plays a role in erythrocyte adhesion.
Further experiments investigating the role of T-cells in clearing the bacteremia showed that although the cytolytic response is negligible, T-help is essential for mounting a protective antibody response and thus clearance.
Some experiments concerning both, the antibody and the T-cell response upon infection, were additionally performed using *B. birtlesii* as a mouse model for *Bartonella* infection and indicate that the observations made with *B. taylorii* are not species specific but seem to be common for lineage 4, if not for all *Bartonella* species.

In addition, the vertical transmission of *B. taylorii* was investigated. The bacterium could be transmitted from mother to embryo before birth, however, only if the mother had a deficiency in the adaptive immune system. Immunocompetent offspring cleared the bacteremia after birth, indicating that there is no state of immunological tolerance induced by transplacental transmission of *Bartonella*.

Contents

1 Introduction

1.1 The innate immune system

The mammalian immune system is a crucial defense mechanism allowing the host to protect itself against intruding pathogens of different kind.
When a pathogen passes the natural barriers such as the skin or mucous membranes, it is sensed by the innate immune system, which can trigger the first inflammatory response. The adaptive immune system can then provide a specific response in order to clear the infection.
The cells of the innate immune system react to stimuli provided by the pathogen, but not the host, in a non-antigen specific manner [1]. These stimuli are called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) from bacterial cell walls or CpG [2]. Those PAMPs are sensed by germline-encoded pattern-recognition receptors (PRRs). PRRs are expressed by e.g. macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils and natural killer (NK) cells [3, 4] and can be present on cell membranes, intracellularly or be secreted into different body fluids [5]. One of the most famous PRRs is the Toll-like receptor 4 (TLR4), which is able to sense LPS [6].
Examples for secreted PRRs are serum amyloid protein or C-reactive protein, which can activate the complement cascade via C1q [7]. The complement is a complex protein cascade triggering phagocytosis via the opsonizing activity of C3b, further recruitment of macrophages and neutrophils and rupture of bacterial cell walls by formation of the membrane attack complex [8] (see also 1.2.2.2).
Besides pathogen related molecules, PRRs can also sense molecules that are released by the host's own cells upon damage, unscheduled cell death or pathogen invasion. Those molecules are called damage-associated molecular patterns (DAMPs) [4, 9].
Upon stimulation via PRRs, innate immune cells usually react with a fast pro-inflammatory cytokine response. Subsequently antigen-presenting cells such as DCs upregulate costimulatory molecules in order to activate the adaptive immune system [3].

1.2 The adaptive immune system

The adaptive immune system does not sense general PAMPs, but rather relies on recognizing specific antigens. It can thus provide a specific response against a pathogen.
Lymphocytes differentiate during haemaotpoiesis from a common lymphoid progenitor. Lymphocytes include natural killer cells, which belong to the innate immune system, B- (bone marrow- or bursa- derived cells) and T-cells (thymus derived cells). B- and T-cells are the main players in the adaptive immunity. T-cells can be either cytolytic T-cells, which are responsible for the cell-mediated immune response, or T-helper cells, which can provide signals to B-cells. B-cells produce protective antibodies upon activation,

forming the humoral immune response. Upon activation, both, B- and T-cells, can undergo expansion and can differentiate into effector or memory cells. Effector cells provide the immediate function of the cell type in order to eliminate the pathogen. Memory cells patrol peripheral tissues in order to provide a faster response upon re-encounter of the pathogen and can lead to up to life-long protection from reinfections.

1.2.1 T-cell mediated immunity

As mentioned, T-cells are divided into two major classes: cytotoxic and helper cells. CD8+ T-cells usually differentiate into cytotoxic T-cells and their T-cell receptor (TCR) binds to peptides presented on major histocompatibiliy complex (MHC) I by nucleated cells. CD4+ T-cells, usually serving as T-helper cells, are restricted by MHCII on professional antigen presenting cells (APCs). Both groups of T-cells undergo clonal expansion upon activation and are able to form memory cells. Cytotoxic T-cells may induce programmed cell death in infected or cancerogeneous cells of the host. They do so by expressing Fas ligand or by releasing granules. Fas ligand can directly interact with the Fas receptor on the target cell, inducing apoptosis. The granules contain perforin, which forms pores in the target membranes, and granzymes, which are serin proteases that can enter through those pores and activate caspases in order to induce apoptosis. Cytotoxic T-cells are mostly involved in eliminating virus infected host cells e.g. during influenza or vaccinia virus infection. In order to maintain a long lasting memory response, cytotoxic T-cells need to interact with CD4+ T-helper cells [10].
Besides providing help to cytotoxic T-cells, T-helper cells can also interact with B-cells in order to promote the antibody response against a pathogen and induce class-switch to other antibody types. CD4+ T-helper (Th) cells can be classified into different lineages, depending on their morphology, the expression pattern of certain transcription factors, the cytokines they produce and the immunological program they promote (reviewed in [10, 11]). Often a particular subset of Th effector cells can help to eliminate certain types of pathogens but deregulation is often involved in severe unwanted inflammatory diseases.
Th1 cells are characterized by their expression of the transcription factor T-bet. They produce mostly IFNγ, TNFα and TNFβ and promote cytolytic immune responses. Thus, a Th1 response is mostly involved in infections with obligatory intracellular pathogens such as *Leishmania* [12], *Salmonella* [13], *Listeria* [14], influenza A [15] or vaccinia virus [16]. Th1 cells may also activate infected macrophages and provide help to B-cells, promoting the production of IgG2a or IgG2c in mice.
Th2 cells on the other hand promote antibody class switch towards IgG1 and IgE, express the transcription factor GATA-3 and produce IL-4. Th2 cells promote an immune response against extracellular pathogens and infections of mucosal tissues by supporting high antibody titers in order to neutralize the intruders. Pathogens causing a classical Th2 response are helminthes and nematodes.
Th17 cells, as the name indicates, produce IL-17, but also IL-21 and IL-22. They express ROR-γt and share features with both Th1 and Th2. It is believed that a Th17 response is triggered by certain infections caused by fungi, *Klebsiella* [17], *Citrobacter rodentium* [18], *Borrelia* and *Mycobacterium tuberculosis* [19]. Th17 cells are reportedly necessary for their clearance. Th17 cells enhance the response of neutrophils and promote integrity of the natural barriers such as skin or intestine.
T follicular helper cells (Tfh) are present in the B-cell zones and promote high-affinity antibody production and the formation of memory B-cells. Tfh express high levels of CXCR5 and the cytokines IL-10 and IL-21.

Apart from to the so far mentioned T-helper cells, there is another category of CD4 T-cells: regulatory T-cells (Treg). Tregs do not promote a pro-inflammatory response but rather secrete anti-inflammatory molecules such as IL-10 and TGF-β and mediate immune homeostasis and self-tolerance. Treg cells may suppress the action of other T-cell subsets.

The T-cell groups described so far are termed "classical T-cells". They recognize peptide antigens presented on MHCI or II. Sensing of presented antigen is the first signal for the T-cell to become activated. Signal two is provided by costimulatory molecules, namely CD80 and CD86, on the surface of the APC interacting with CD28 on the T-cell. The third signal are cytokines sensed by the T-cell. The third signal is a crucial factor determining into which lineage the cell will differentiate.
There are however T-cells that do not fit the classical picture and are often called "non-classical" or "unconventional T-cells", mostly because they do not sense protein antigens presented on MHC molecules. However, the more is known about those non-classical cells, the more they seem to play the same roles as conventional ones [20].
One group of non-peptide restricted T-cells are CD1 restricted T-cells. The antigen presenting molecules of the CD1 family (in mice there is only one isoform CD1d) can present lipids. Amongst CD1d-restricted T-cells are invariant natural killer T (iNKT) cells reacting towards α-galactosylceramide, which is produced by a large group of bacteria. Similar to PRRs, they recognize conserved molecules produced by pathogens which is why they are also called innate-like T-cells. Whereas classical T-cells express highly variable TCRs, iNKT-cells express an invariant TCR. They harbor a conserved TCR Vα chain and use only a limited number of Vβ chains to form their TCR. However, more adaptive-like CD1d-restricted T-cells have been described. iNKT cells may secrete cytokines mimicking classical T-helper subsets as IL-4 or IFNγ [21, 22]. Since mice lacking iNKT-cells are more susceptible to microbial infections, an important role for CD1d-restricted T-cells in those infections has been suggested (CD1d antigen presentation and the biology of CD1-restricted T-cells have been reviewed in [20, 23, 24]).
Mucosal-associated invariant T (MAIT) cells are a second group of non-peptide reactive T-cells. MAIT cells recognize bacterial metabolites from riboflavin synthesis presented on the MHCI-related molecule 1 (MR1) [25]. In humans, there is a third group of cells expressing a fixed γδTCR and reacting towards metabolites from the mevalonate pathway. Similar to iNKT-cells, MAIT cells express a semi-variant TCR. Amongst other implications, MR1-restricted T-cells have been reported to have anti-microbial effects during infection in humans and mice [26, 27, 28] (the immunbiology of MR1-restricted T-cells has been reviewed in [20, 23, 24]). MAIT cells are believed to act in an innate manner by secreting pro-inflammatory cytokines [24] and are by that able to provide help to B-cells and promote antibody production [29].

1.2.2 Antibody-mediated immunity

The first proof of the importance of protection by antibodies was provided in Behring's famous experiment. A passive serum transfer from tetanus infected to healthy animals resulted in protection of the recipients [30]. Today even more is known about the role of antibodies in protecting from infections and there are more and more monoclonal antibodies used in clinics as therapeutics.
Antibodies or immunoglobulins (Ig) are proteins produced by activated B-cells. They can exist as membrane

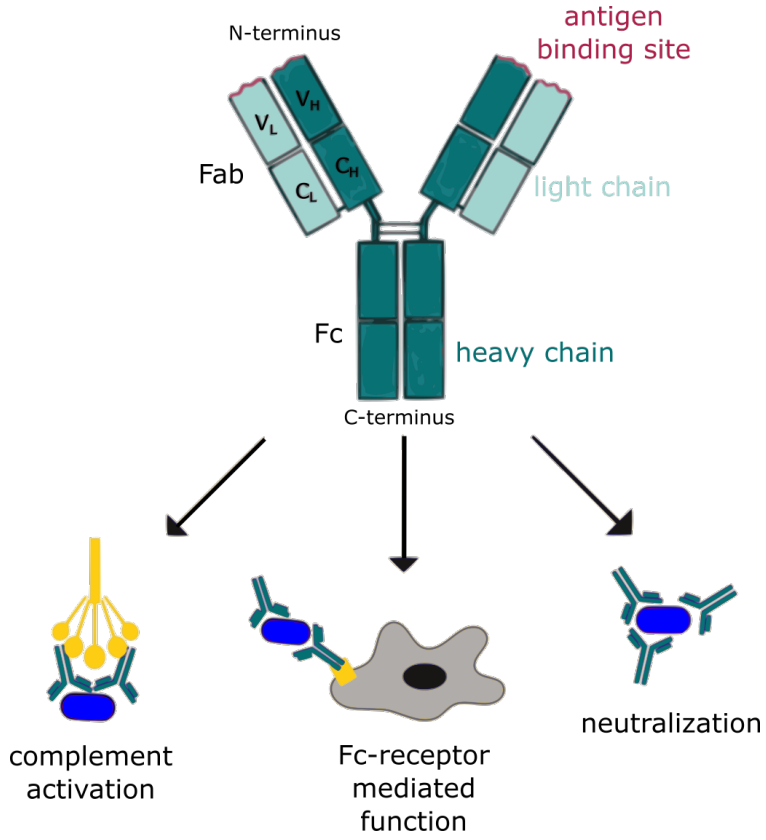

Figure 1.1: **Schematic overview of antibody structure and function.** An antibody consists of two heavy (dark green) and light chains (light green) forming its characteristic "Y"-shape. The variable (V) and constant (C) regions of heavy (H) and light (L) chain, aswell as Fab- and Fc-fragments are labeled. The antigen binding site (red) is located at the N-terminus. Sulfide bridges are marked as black lines. Upon binding of the antigen, antibodies can mediate effector functions in order to eliminate the pathogen (blue). They can cause activation of the complement via C1q (left) or bind Fc-receptors (middle) and mediate functions such as antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis. Antibodies can also inactivate the pathogen by neutralization (right). Antibody effector mechanisms will be discussed in more detail in 1.2.2.2.

bound isoforms on the cell surface or they can be secreted. Antibodies are present in the serum, body fluids and tissues. Depending on the isotype, antibodies can exist as monomers or multimers.

1.2.2.1 Antibody structure and isotypes

Antibodies consist of four peptide chains, two heavy and two light chains, forming a characteristic "Y"-shape (see figure 1.1). Two domains called antigen-binding fragment (Fab) form the "arms" and one so-called crystallizable fragment (Fc) or constant (C) region constitutes the "stem". The Fab region confers the binding to the antigen and the Fc domain may drive antibody effector functions. For both, heavy and light chain, the N-terminal variable (V) region is localized on the tip of the Fab. The V region contains hypervariable complementary-determining regions (CDRs), giving the actual antigen binding capacity. The hypervariable regions are flanked by less variable framework regions (FRs). During development, B-cells undergo the so-called V(D)J recombination for heavy and light chains in order to produce an antibody with

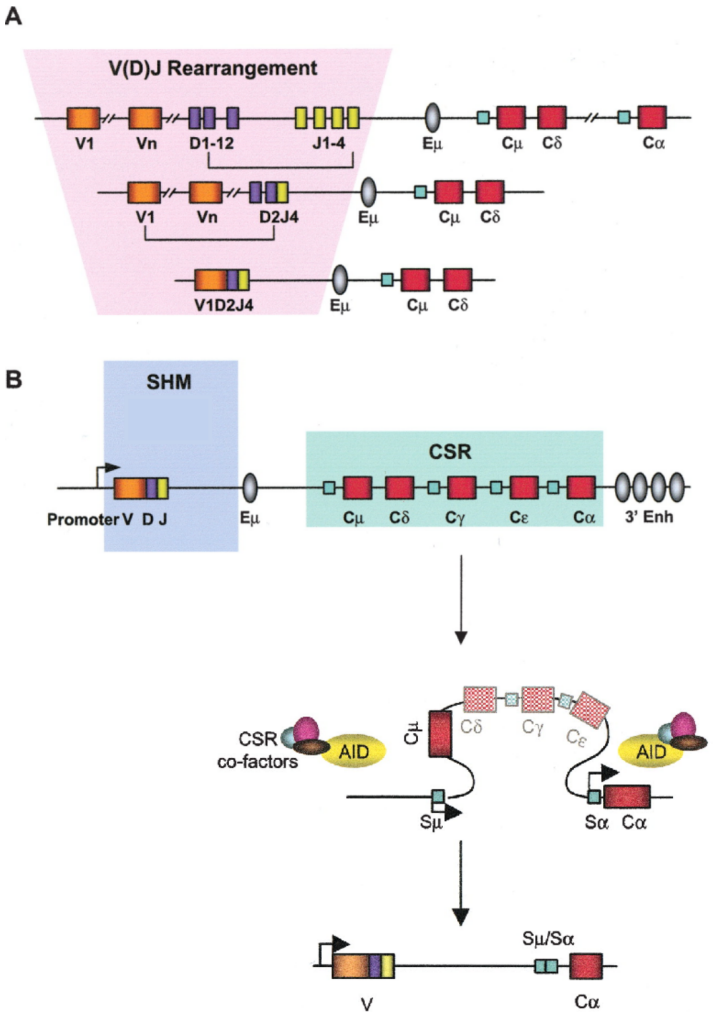

Figure 1.2: **V(D)J recombination, class-switch reaction (CSR) and somatic hyper mutation (SHM):** A) Schematic overview of the genetic organization of the murine Ig heavy chain locus. One of the 12 D segments (purple) arranges with one of the 4 J (yellow) segments in order to then recombine with one of the V segments (orange). The transcriptional regulator Eμ is shown in gray. B) After V(D)J recombination the heavy chain can undergo SHM in the blue highlighted region or CSR in the green highlighted region. The first constant (C in red) segment present is expressed. The first one is naturally C_μ encoding for IgM. In an AID dependent reaction, this or more segments can be removed in order to allow expression of one of the other C fragments, α encoding for IgA, γ for IgG, ϵ for IgE and δ for IgD. Segments between two S (switch) regions (green) are removed. In this example CSR is mediating a switch from IgM to IgA. Scheme adapted from [31].

a unique variable region for antigen binding. On the DNA level one of the existing D (diversity) segments binds one of the J (joint) regions to then recombine with one of the V segments in intrachromosomal recombination reactions (figure 1.2). Binding and cleavage of these specific DNA segments is mediated by the enzymes RAG1 and RAG2. Imprecise end joining between the segments guarantees further variability. There are two gene families which can do a V(D)J reombination for the light chain: the κ and the λ family. This again increases the variability. (V(D)J recombination has been reviewed in [31, 32, 33]).
Within the germinal center, the genes encoding for an antibody can undergo further modification. During somatic hypermutation (SHM), there is massive accumulation of point mutations within the V region, allowing to explore new binding sites with improved affinity. The first antibody expressed by a B-cell is IgM. The heavy chain locus can then undergo class-switch recombination (CSR) resulting in a switch to a different antibody isotype such as IgA or IgG. Different C segments encoding for different Ig isotypes are arranged in a row with the first one being expressed. In an intrachromosomal recombination event, regions between the switch regions are removed resulting in the expression of a new C segment (see figure 1.2). The CSR can be triggered by direct T-help via the interaction of CD40 on the B-cell with its ligand CD40L on the T-cell [34] or in a T-cell independent manner via the CD40L-related factors BAFF and APRIL, which are secreted by APCs and can bind receptors on the B-cell [35]. The CSR changes the isotype of the antibody without changing its specificity (SHM and CSR have been reviewed in [31]). Both reactions, SHM and CSR, are dependent on the enzyme activation-induced cytidine deaminase (AID) [36].
IgM, the first antibody class expressed, is a pentamer. This allows enhanced avidity due to multi-site binding even when the binding affinity is weak [37]. IgM is further able to activate the complement [38, 39]. IgD can be expressed by alternative splicing from the same mRNA as IgM [40] but there is also evidence that there can be a CSR towards IgD expression [41]. Membrane bound IgM and IgD (mIgM and mIgD) are part of the B-cell receptor. In the bone marrow after successful V(D)J recombination B-cells acquire mIgM and upon leaving the bone marrow also mIgD. Mature B-cells downregulate mIgD [42]. IgD exists in a secreted form but was until recently believed to be irrelevant for the humoral immune response [43]. However, IgD seems to be able to replace IgM function in an IgM -/- mouse model [44], is suggested to play an important role in bacterial control in mucosal tissues and can bind to basophils [45, 46]. IgA can be present in the serum as a monomer or secreted into mucosal tissues as a dimer. IgA is reported to have important function in the maintenance of commensal bacteria but also has been correlated with protective functions in local infections [47]. IgE is protective in helminth infection and interacts with mast cells via its own Fc-receptor FcϵRI [48]. IgG is the most abundant isotype in the serum and has a well described function in anti-pathogen control. IgG can be divided into further subclasses, in mice namely IgG1, IgG2a or c, IgG2b and IgG3, which have different properties in binding receptors, activating complement or in general in their effector functions.

1.2.2.2 Mechanisms of antibody effector functions

Binding of the antigen by an antibody can have several outcomes. As shown in figure 1.1, antibodies can either neutralize the pathogen or mediate downstream effector mechanisms. The Fc domain can activate the complement cascade or bind receptors and mediate phagocytosis, cellular toxicity or modulate the immune response. The quality and magnitude of the effector mechanism can be influenced by the stoichiometry

between antigen and Fab domain [49], the size of the immune complex [50] and antigen:antibody ratio [49, 51].

One example of receptors bound by antigen-antibody complexes are Fc-receptors (FcRs). Four different classes of FcRs binding IgG, called FcγRI - FcγRIV, have been described in mice. FcγRI, III and IV are activating receptors with only FcγRI being a high affinity receptor. The others show low to medium affinity for the ligand. FcγRI is believed to be activated by receptor crosslinking upon binding of immunocomplexes. Low affinity receptors avoid binding of always present non-antigen-bound antibodies in the surroundings. Binding of FcγRIIB results in inhibitory signaling. FcγRs are commonly expressed by cells of the hematopoetic system. Innate immune cells such as macrophages, monocytes, DCs and others express activating and inhibitory receptors. NK cells are reported to express only activating FcγRs. B-cells only express FcγRIIB. FcγRs differ in their ability to bind IgG subtypes. FcγRI binds IgG2, FcγRII and III bind IgG1 and 2. FcγRIV only binds IgG2. Mouse IgG3, however, has been reported to interact only weakly with FcγRs (FcγR-antibody interaction have been reviewed in [52, 53, 54]).

Besides FcγRs, there are other receptors binding antibodies such as FcRn and the polymeric Ig receptor, which are reported to transport Igs across membranes or the placenta [55, 56]. Intracellular sensing of antigen bound antibodies is mediated by the FcR TRIM21 [57]. C-type lectin receptors such as the complement initiators C1q and mannose-binding lectin (MBL) are also binding the Fc domain.

The following effector functions can be mediated by receptor binding of an antigen-antibody complex: antibody-dependent cellular cytotoxicity, antibody-dependent cellular phagocytosis and complement activation. Other important functions of antibodies independent of antibody receptors are neutralization and viral release inhibition. During the latter, viral particles are trapped on the cell surface by protective antibodies, which prevents budding of the newly formed viruses. This release inhibition has been shown for example for influenza virus [58, 59], Marburg virus [60] and LCMV (Mehmet Sahin, manuscript in preparation).

Antibody-dependent cellular cytotoxicity (ADCC)

ADCC describes the killing of a target cell, e.g. an infected cell or a tumor cell, by NK cells via the release of perforin and granzymes. Macrophages, DCs, neutrophils and others have also been described to act as effector cells in ADCC. In general, the process is triggered by crosslinking of FcγRIII on the cell surface (reviewed in [61, 62]).

ADCC has been reported to be an important mechanism for the control of different pathogens by the immune system such as HIV [63, 64, 65], influenza [66], *Plasmodium* [67, 68], *Chlamydia* [69] and tuberculosis [70].

Antibody-dependent cellular phagocytosis (ADCP)

ADCP or opsonophagocytosis can be triggered by FcR or complement receptor binding and is mostly performed by macrophages, monocytes and DCs but also by granulocytes. Antibody-opsonized targets are taken up by the endocytic pathway followed by rapid trafficking to lysosomes and degradation. ADCP allows directed antigen presentation after degradation and may be accompanied with reactive oxygen species production, the secretion cytokines or antimicrobial peptides (reviewed in [61]). Antibody-controlled trafficking of bacteria via opsonophagocytosis has been shown to be protective against the pathogens *Legionella* and *Salmonella* [61, 71, 72]. However, this uptake mechanism can also be hijacked by intruding pathogens. For example *Leishmania* opsonization enhances infectivity *in vitro* (personal communication with Fabienne

Tacchini-Cottier) and it could be shown that this organism uses ADCP to modulate the host's immune system in its favour [73, 74, 75].

Antibody-mediated complement activation

The complement consists of several proteins present in the blood and is part of the innate immune system. The complement cascade can be activated via C1q (classical pathway), MBL (lectin pathway) and the alternative pathway that is independent of those two. It is important to note that the complement can also be activated in a non-antibody dependent manner, for example by the sensing of PAMPs. All complement activation pathways are integrated in the same cascade lading to cleavage of C3. A schematic overview of the complement fighting a microbe is given in figure 1.3.

IgM as a pentamer can activate C1q after undergoing a conformational change upon antigen binding. The Fc-regions of IgG promote hexamer formation, which allows interaction with C1q [76]. Murine IgG1 is reported to activate the complement exclusively via the alternative pathway whereas IgG2 activates both the classical and the alternative one [38]. Upon binding, C1q becomes active and cleaves C2 and C4 which then form a C3 processing enzyme, the C3 convertase, in close proximity to the C1q complex. C3b, cleavage product of C3, binds the C3 convertase forming the C5 convertase. This allows cleavage of C5 into C5a and C5b. C5b recruits the other components of the membrane attack complex (MAC), named C6 through C9. The MAC is able to destroy target cells of eukaryotic or Gram-negative bacterial origin. During the cascade, several side-product peptides are produced that can recruit immune cells or generally mediate the immune response. Further, complement components bound to pathogens can have opsonizing function. Binding of immune complexes coated with complement to antigen-specific B-cells increases B-cell receptor signaling and with that promotes survival of the cell (reviewed in [77, 78]).

The importance of complement activation by antibody-antigen complexes has been observed in several infectious diseases. Complement-dependent effector mechanisms are crucial for the protective function of antibodies against influenza [79], West Nile Virus [80], vaccinia virus [81] or *Plasmodium* [82]. In patients who receive a therapeutic monoclonal antibody targeting the final stages of complement activation, elevated risks of bacterial infections are a reported side effect [83]. Deficiencies in the classical complement pathway are associated with increased infections by *Streptococcus*, *Neisseria*, *Haemophilus* and other encapsulated bacteria [84].

Neutralization

Neutralizing antibodies (nAbs) can target the entry or replication of pathogens but also directly target their released toxins or virulence factors. Prevention of cell entry may happen by interfering with the binding of the pathogen to its corresponding receptor. This functionality is usually quantified using an *in-vitro* neutralization assay. In viral infections, the presence of nAbs is in many cases a good - if not the best - correlate for protection. In HIV infections, many patients develop neutralizing antibody responses, however viral escape mutants emerge under the selective pressure of this response [86]. A smaller fraction of HIV patients mount so-called broadly neutralizing antibodies (bnAbs). bnAbs target conserved regions of several virus subtypes and are explored for the development of vaccine strategies [87]. Using the macaque model, infusion of HIV nAbs [88, 89, 90] and bnAbs [91, 92] have been shown to protect against the infection. It has further been suggested that bnAbs may act in a purely neutralizing and Fc-receptor- and complement-independent man-

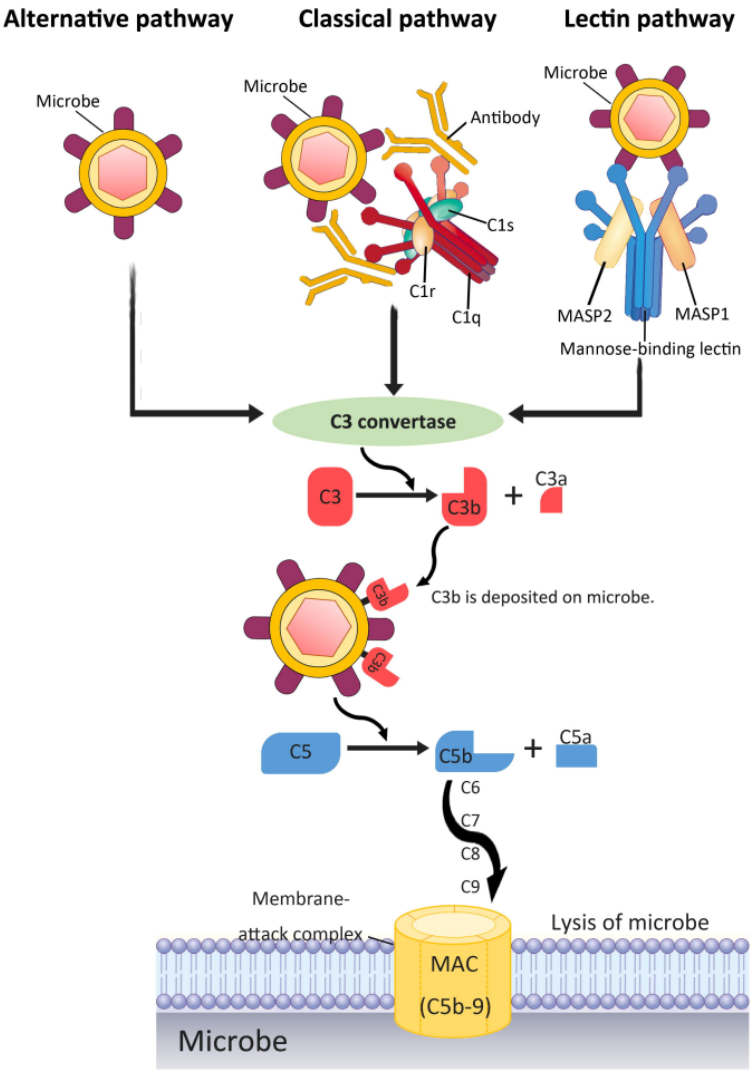

Figure 1.3: **Overview over the antimicrobial action by the complement cascade.** Three pathways can activate the C3 convertase: the alternative pathway, the classical pathway with an antigen-antibody complex activating C1q and the lectin pathway. Upon C3 cleavage by the C3 convertase, C3b is deposited on the pathogen membrane and the C5 convertase is formed. Upon C5 cleavage, C5b recruits C6-C9 in order to from the membrane attack complex (MAC) leading to lysis of the pathogen (scheme adapted from [85]).

ner against HIV [93]. The protective function of bnAbs has also been described for other viruses such as hepatitis C [94] and Zika Virus [95]. nAbs preventing host cell entry or attachment have been also described for non-viral pathogens such as *Plasmodium* [96, 97] and *Streptococcus* [98]. However, nAbs can also be protective by intra-cellular effector mechanisms such as inhibiting adenoviral uncoating [99], inhibition of viral transcription [100], replication [101] or the transport of viral DNA into the nucleus [102]. For *Listera* and *Anaplasma*, nAbs have been reported to inhibit intracellular replication [103, 104]. nAbs have been further described to be able to prevent staphylococcal biofilm formation [105]. In fungal infections, nAbs can inhibit cell budding and with that fungal growth [106].
As already mentioned, nAbs do not have to bind an antigen inside or on the surface of pathogens but can also neutralize secreted toxins or virulence factors. A neutralizing antibody against Shiga toxin was shown to prevent the toxin from reaching the cytosol and to promote its release from the cell [107]. Neutralizing antibodies targeting the anthrax toxin are used in clinics [108, 109].

Even though for many pathogens there appears to be one antibody effector mechanism which is the most important one for clearing this specific infection, one can imagine that in a normal immunocompetent host an antibody bound to its antigen will activate the complement and FcR mediated function. Neutralization is probably the only mechanism in which simple binding to the pathogen might not be sufficient but the right antigen needs to be targeted in order to provide protection. Often a combination of antibody effector mechanisms might be necessary in order to clear the infection. In other cases, different antibody effector mechanisms might be redundant.

1.3 The genus *Bartonella*

1.3.1 The life cycle of *Bartonella* and their host specificity

Bartonellae are Gram-negative, facultative-intracellular α-proteobacteria with at least 45 different species described so far [110]. The phylogeny of *Bartonella* can be divided into four distinct lineages with the human pathogens *B. ancashensis* and *B. bacilliformis* forming lineage 1 and a group of ruminant infecting species forming lineage 2. Lineage 3 and 4 in parallel underwent adaptive radiation, which lead to them becoming the lineages with the most species. Lineage 4 species, as *B. birtlesii*, *B. quintana* and *B. taylorii* used in this study, harbor a VirB/VirD4 and a Trw type 4 secretion system (T4SS) (virulence factors will be discussed in 1.3.3) [111, 112]. One hallmark of *Bartonella* species is their host specificity. Each species infects only one or a group of closely related mammalian reservoir host species in which they establish a persistent intra-erythrocytic bacteremia [113].
Bartonellae are transmitted between mammals via blood-sucking arthropod vectors, most dominantly fleas, but also sandflies, body lice and potentially ticks [113, 114, 115]. Since *Bartonella* species have been repeatedly detected in the feces or shown to replicate within the midgut of their vector [116, 117, 118, 119, 120, 121] it is believed that they are deposited on the host's skin with the contaminated feces and then enter the dermis through scratching [113, 117]. However, also direct transmission via oral shedding into the salivary and bites has been reported [122, 123].
After entering the dermis, it is believed that *Bartonella* travels within the lymphatic vessels to reach a so-

called primary niche where the bacteria replicate before seeding into the blood stream. First evidence for this was an *intra-venous* rat infection model using *B. tribocorum* where bacteremia appeared only 3 days post infection leading to the hypothesis that a primary replication niche must be present before erythrocyte infection may occur [124]. Because of their tropism for endothelial cells, those were suggested to be the primary site of replication [124, 125]. Later, *in-vitro* infection of DCs and an importance of migratory cells during the early stage of infection was shown [126] leading to the hypothesis that upon entry into the dermis, the bacteria are brought to their replication site by hijacking migratory DCs [113, 126]. This is further supported by the fact that *Bartonella* are often found in lymph nodes of infected patients [127] and that they can be found there 6 h after experimental mouse infection [128]. A schematic overview of this suggested life cycle is shown in figure 1.4. However, also an extra-cellular route within the lymphatic vessels and subsequent drainage into the blood stream were proposed since bacteria could be detected extra-cellularily in the lymph of experimentally infected rats and appeared to inhibit their own phagocytosis [129]. More experiments will be necessary in order to clarify how *Bartonella* travels from the dermis to the blood stream and of which nature the primary niche or first replication site is.

More is known about the whereabouts of *Bartonella* in the blood stream. Once in the blood, the bacteria usually cause a long-lasting intra-erythrocytic bacteremia and can be taken up by a new arthropod vector during a blood meal. It is assumed that *Bartonella* species of lineage 4, being non-haemolytic, cannot spread from one erythrocyte to another [124]. In experimental conditions, the bacteremia lasts approximately 10 weeks, depending on the strain and the host species [124, 130, 131, 132, 133, 134].

The high host specificity of Bartonellae cannot be explained by the feeding restrictions of the arthropod vector. An intra-erythrocytic bacteremia could only be experimentally achieved when infecting the natural reservoir host [111, 124, 127, 130, 135, 136, 137, 138, 139]. The high host specificity might however be explainable by the multi step infection cycle where several interactions with the host need to be fine tuned. Improper immune modulation has been proposed as a reason for failed infection of non-reservoir hosts [127]. Additionally, the adhesion towards and invasion of erythrocytes has been reported to be species-specific. The molecules on the bacterial surface facilitating the adhesion towards red blood cells, namely the Trw type 4 secretion system (discussed in 1.3.3), determine the ability to colonize erythrocyte of the right reservoir host and seem to restrict the bacteria to the corresponding host [140].

1.3.2 Epidemiology and clinical picture

The prevalence of *Bartonella* species in wild animals and pets is very high all over the planet. In one study 100 % of wild deers tested in California and France were infected with *Bartonella* [141]. The overall prevalence in wild rodents in central Europe was reported to be 64.8 % [142]. Also human pets such as cats and dogs have been reported to be important reservoirs, especially for *B. henselase*, the agent of cat scratch disease (CSD) [143]. Recently 11 out of 12 cats in Southern Germany were shown to be PCR positive for *Bartonella* even though only a small fraction of those cats were actually bacteremic [144]. In stray cats the prevalence of bacteremia was reported to be 53 % [145]. However, besides wild life and domesticated animals, *Bartonella* species have been also described in bats [146], a beluga whale [147], bees [148] and others. Also co-infections with several *Bartonella* species at the same time have been reported [149].

When looking at *Bartonella* infections in humans, they can appear as a zoonosis, with the patient being an

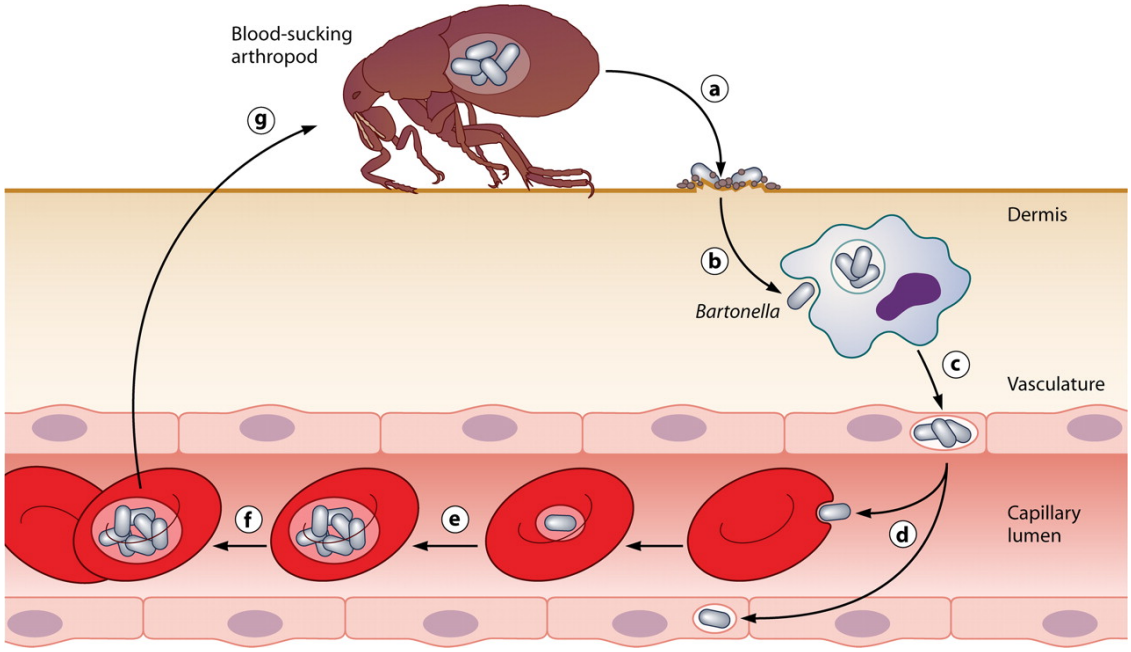

Figure 1.4: **Suggested life cycle of *Bartonella*.** Bacteria are transmitted via the flea feces onto the host's skin (a) and enter the dermis by scratching. Afterward they most likely travel with the lymph, hijacking DCs or other migratory cells (b) or passively in an extra-cellular state. After suggested replication within endothelial cells (c) they are able to infect erythrocytes (d) where they replicate (e), persist (f) and can be taken up during the next blood meal of a new arthropod vector. Graphic taken from [127].

incidental host, or can be caused by species infecting humans as their natural reservoir host. *B. bacilliformis* belongs to the latter group and causes Oroya fever and Carrion's Disease, an emerging health problem in Peru [150, 151, 152] that has been reported to spread towards Columbia and Ecuador [153, 154]. About 0.5 % of people in endemic regions show asymptomatic infection with an incidence of 12.7 % [155]. However, an even more widespread subclinical infection rate has been suggested [152, 156]. Oroya fever presents the acute phase of the infection. It can be mild but up to fatal and presents itself with anemia or more unspecific symptoms such as fever, malaise or headache. The chronic phase may occur without previous experience of Oroya fever. It is known as the Perruvian wart, a nodular skin lesion, or Carrion's Disease, a sudden profound heamolytic anemia [152].

B. quintana of lineage 4 is also infecting humans as its natural host. It is the agent of trench fever, which had historical implications for Napoleon's army and among the French army during World War I [157, 158]. More recently, it reemerged as urban trench fever associated with immune suppression, e.g. HIV infection, or poor hygiene conditions, e.g. homelessness [159, 160]. Up to 14 % of homeless people in Marseille, France, have been reported to be bacteremic [161]. Trench fever or 5-day fever in immunocompetent patients is usually accompanied with long-lasting bacteremia, periodic peaks of fever, headache or bone pain. However, the symptoms might vary with the patient [158, 162, 163]. In immunocompromised individuals *B. quintana* can further cause bacillary angiomatosis, a vasoproliferative tumor that can be found mostly in skin or liver [164, 165]. The skin lesion seems to be very similar if not the same as the Perruvian wart described for *B. bacilliformis* [166, 167].

The most common zoonotic pathogen amongst the genus *Bartonella* is *B. henselae*, the agent of catch scratch disease (CSD). However, other species have been associated with human pathology such as culture negative endocarditis or CSD, for example *B. clarridgeiae*, *B. grahamii*, *B. vinsonii* and *B. elizabethae* [168, 169, 170]. In immunocompromised patients, infection with *B. henselae* can also cause the described bacillary angiomatosis like in the case of *B. quintana*. However in immunocompetent patient the associated pathology is CSD, a usually benign, self-limiting lymphadenitis, which may be accompanied with mild fever, headache and malaise [171]. CSD may however progress to present itself with more systemic and severe symptoms such as arthritis, osteomyelitis, and numerous other, often atypical, manifestations [152, 172]. About 40.000 cases of CSD, mostly in children and young adults, are reported in the U.S. annually with approximately 2.000 hospitalizations [173], making CSD the most common disease associated with *Bartonella* infection [127].

1.3.3 Virulence factors in the mammalian host

Bartonellae evolved several important virulence factors allowing them to successfully colonize their specific host but also to avoid or even modulate its immune response. One example for their ability to avoid immune recognition is their LPS. Instead of activating TLR-4, the PRR which would normally sense LPS and then lead to immune activation and cytokine secretion, *B. quintana* LPS acts as a potent TLR-4 antagonist, suppressing this proinflammatory pathway [174, 175]. *B. henselae* LPS was also shown to lack TLR-4-activating properties because of its chemical modifications [176]. Further, no antibodies against the LPS of *B. henselae* could be detected in its natural host, the cat [177].
One group of genes found to be essential for erythrocyte infection are hemin binding and hemin uptake proteins [178, 179], which allow the bacteria to process hemin from their environment, namely the flea gut and the blood stream. Bartonellae are not able to produce hemin themselves [180]. A more recent study suggests that hemin binding proteins can also help to protect from hemin-induced toxicity [181]. Other very important protein complexes of lineage 4, which lacks flagella as usually important virulence factors in bacteria [127], are bacterial autotransporters, the VirB/D4 and the Trw type 4 secretion system (T4SS) which will be discussed in more detail.

1.3.3.1 Trimeric autotransporter adhesins - host cell adhesion and antigenic variation

Amongst Gram-negative bacteria, trimeric autotransporter adhesins (TAAs) are common virulence factors mediating adhesion to host proteins on cell surfaces or in the extracellular matrix (ECM) [182]. The best described TAA is YadA from *Yersinia*, which has been shown to be essential for infection [183] because of its ability to bind to ECM proteins [183, 184, 185]. Another prominent TAA is NadA of *Neisseria*, which is crucial for the adhesion to and the invasion of epithelial cells [186].
In *Bartonella*, two main TAAs have been studied in detail: *Bartonella* adhesin A (BadA) of *B. henselae* [187] and and the variably expressed outer membrane proteins (Vomp) family of *B. quintana* [188]. However, TAA genes have been found in most *Bartonella* species [189, 190, 191]. Both, BadA and Vomps, have been shown to mediate attachment to the ECM and endothelial cells under conditions mimicking the bloodstream [192].

BadA of *B. henselae* is a huge protein with over 3000 amino acids, which is present in the outer membrane of the bacterium and shares homologous domains with YadA. Besides being responsible for binding collagen, fibronectin and laminin of the ECM and endothelial cells, most likely via β1-integrins, BadA was also reported to prevent phagocytosis in macrophages. Further, BadA mediates the induction of proangiogenic host factors such as hypoxia-induced factor HIF-1 [187]. Whereas the neck-stalk region of BadA is suggested to bind to fibronectin, the head domain of BadA is responsible for cell adhesion and the angiogenic reprogramming, namely the induction of HIF-1, vascular endothelial growth factor VEGF and IL-8 [193]. HIF-1 induction was also found in biopsies from *B. henselae* lesions in patients. The BadA dependent HIF-1 induction was suggested to be upstream of the VEGF upregulation and to be responsible for increased oxygen consumption, cellular hypoxia and decreased ATP levels in the host cell [194]. Infection on its own may cause hypoxia due to higher consumption of oxygen. It is speculated that in addition the increased levels of HIF-1 caused by BadA together with inhibition of apoptosis in endothelial cells (see 1.3.3.3) explains the proangiogenic effect of *Bartonella*, which may in the end lead to bacillary angiomatosis [191]. This proangiogenic surrounding might promote *Bartonella* colonization of the host, since they strongly depend on hemin as a nutrient [113]. Recent findings suggest a strong role of BadA expression levels on the infectivity of the murine lineage 4 strain *B. taylorii* during experimental *in-vitro* infection (unpublished results by Katja Fromm).

The Vomp proteins of *B. quintana* are closely related to BadA and also mediate collagen binding. In a rhesus macaque infection model a genomic rearrangement of the *vomp* locus, containing tandemly-arranged copies of closely related *vomp* genes, has been observed during infection. This rearrangement was also observed in direct isolates from human patients [188]. The deletion of the entire *vomp* locus in *B. quintana* causes a loss of infectivity [195]. Also TAA defficient mutants of other *Bartonella* strains were no longer infectious *in vivo* [140, 179]. Several findings indicate that TAAs such as BadA and Vomps are highly immunogenic in the mammalian host [187, 190, 196, 197]. This may suggest that this rearrangement of the *vomp* locus and in general the modular, repetitive arrangement of TAA genes in *Bartonella*, allowing intragenic recombinations and phase variations, are immune evasion strategies [182, 188].

1.3.3.2 Other autotransporters - potential virulence factors in adhesion and beyond

Besides TAAs, *Bartonella* species also harbor other autotransporters belonging to the type V secretion systems. Some of them have been found to be essential for the infection of the natural host [140, 179]. One clade of these autotransporters was shown to be upregulated during *in-vitro* infection and thus termed inducible *Bartonella* autotransporters (Iba) [198, 199].
Little is known about the role of Ibas or similar autotransporters and their role during *Bartonella* pathogenicity. In general, autotransporters of Gram-negative bacteria harbor a N-terminal passenger domain and a C-terminal β-barrel domain. While the C-terminal domain is the anchor in the outer membrane, the N-terminal region plays a role in the bacterial virulence (reviewed in [200, 201]). Autotransporters have been described as important virulence factors amongst various bacterial pathogens, however, their role in infection remains often enigmatic. Suggested roles for autotransporters are mediation of adhesion to host cells [201], such as YapE of *Yersinia* [202], cleavage of host antibodies bound to the pathogen [203] and the degradation of

hemoglobin to allow heme uptake [204].
Within the genus *Bartonella*, two Ibas have been studied in some detail. *B. henselae* harbors a CAMP-like factor autotransporter (Cfa) which was identified as a cohemolysin [205]. The acidic repeat protein (Arp) of the same species has been discovered because of its high immunogenicity in CSD patients while showing no cross-reactivity with antibodies against other pathogens. However, no function was so far assigned to this protein [206]. Preliminary data suggests that Ibas are also upregulated during the infection of the natural host and that a mutant lacking the Arp homolog in *B. tribocorum* fails to establish bacteremia (unpublished results by Anja Seubert).
Bartonella autotransporters including Ibas are important virulence factors. Exploring their functions in the future will give more insight into the virulence strategy of this genus.

1.3.3.3 The VirB/D4 T4SS and secreted *Bartonella* effector proteins

Protein transfer into host cells via a type 4 secretion system evolved from DNA conjugation machineries [207]. T4SSs are common virulence factors found in many bacterial species, for example in *Helicobacter pylori* [208]. Bartonellae of lineages 3 and 4 harbor a VirB/D4 T4SS, which is homologous to the ones from *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) [111]. This protein complex contains 12 proteins (nomenclated according to their homologous T4SS, VirB1-11 and and VirD4) and spans the inner and outer membrane. It consists of a scaffold forming the translocation channel, a core complex and a pilus responsible for the actual substrate translocation. VirD4 serves as the coupling protein (reviewed in [209]). However, the main mechanism of substrate translocation remains unclear [127]. The putative tip of the *Bartonella* VirB/D4 T4SS, VirB5, has been described as highly immunogenic [210].
The VirB/D4 T4SS is essential for successful colonization of the host [140, 179]. Using *virB4*- and *virD4*-deficient mutants of *B. tribocorum* in a rat infection model lead to the complete loss of infectivity. Thus, the VirB/D4 T4SS is indeed necessary for the establishment of bacteremia. *Trans*-complemented mutants regained infectivity but the plasmids were not maintained in later stages of the infection, suggesting that the T4SS is needed before the erythrocyte infection stage - most likely during trafficking from the dermal niche or in order to establish a primary replication niche [211].
The VirB/D4 T4SS in *Bartonella* serves as a translocation machinery for *Bartonella* effector proteins (Beps) [212]. The so far best understood Beps are the ones of *B. henselae*. Its effectors named BepA-G show a modular domain architecture that is shared amongst all *Bartonella* species harboring Beps: An N-terminal host interaction domain that is often a so-called FIC (filamentation induced by cyclic AMP) domain or a tyrosin phosphorylation motif that is always followed by one or more *Bartonella* intracellular delivery (BID) domains and a positive charged C-terminus [179, 212]. The FIC domain, if present, is believed to mediate enzymatic effector function. It was shown that FIC domains in bacterial effectors can modify their target with AMP, AMPylate it, and thus manipulate its function in the targeted cell [127, 213]. A prominent example of a bacterial effector protein with an AMPylating FIC domain is VopS from *Vibrio parahaemolyticus* [214]. Tyrosine phosphorylation motifs are also common in bacterial effectors [215]. Indeed those domains within Beps have been shown to be phosphorylated upon translocation [212] and those of BepD-F were shown to interact with host SH2 domains which are key elements in host signaling [216]. The BID domain together with the positively charged C-terminus are the secretion signal for the

translocation machinery [212]. However, for BepA, the BID domain has been shown to also take part in the host interaction and the effector function [217, 218]. More and more BID domains are found to play an important role in mediating host cell manipulation indicating that their function goes beyond being a secretion signal [126, 219] (and unpublished results of Sabrina Siamer and Yun-yueh Lu).
BepA, the ancestral Bep of lineage 4 [111], prevents apoptosis in the target cell by elevating cAMP levels and by that protects infected HUVECs (human umbilical vein endothelial cells) from being cleared by cytolytic T-cells *in vitro* [220]. BepA further has strong proangiogenic effects that are counteracted by BepG [217]. It is believed that BepG alone and BepC together with BepF can inhibit endocytosis of bacteria in order to allow an uptake of a bigger bacterial aggregate termed the invasome [219, 221]. BepE counteracts the effects of BepC, which may otherwise lead to cell fragmentation and thus allows DCs to migrate. This finding strongly supports the idea of bacteria hijacking DCs in order to get to their replication niche [126]. BepD via its interaction with the transcription factor STAT-3 inhibits proinflammatory cytokine secretion by macrophages and DCs and promotes anti-inflammatory cytokine secretion (unpublished results by Christoph Schmutz, Yun-Yueh Lu and Isabel Sorg).
One study investigating BepE of *B. quintana* showed that host cells countermeasure the effector by targeting it for degradation by autophagosomes [222].

1.3.3.4 The Trw T4SS and other factors involved in erythrocyte infection

Most studies concerning the erythrocyte infection of Bartonellae were performed using *B. bacilliformis*, followed by *B. henselae*. In general, the infection of red blood cells is described to happen in four steps: adhesion, deformation, entry and replication within the erythrocyte [223].
A glycolipid was suggested as the target of *B. bacilliformis* on the erythrocyte surface [224]. Also protein targets on the erythrocyte surface have been reported for this strain, namely spectrin, band 3 and glycophorins A and B. Also in this case glycosylation was important [225]. Even though *B. henselae* does not bind human erythrocytes, it is able to bind the same target proteins as *B. bacilliformis* [226], indicating that the target proteins bound by *Bartonella* might be conserved across species. However, neuraminidase or trypsin had no effect on the infection of cat erythrocytes by *B. henselae* [227]. Indeed it was shown later that the species of lineage 4, containing *B. henselae*, have a unique strategy for attaching to erythrocytes, which also seems to provide an explanation for the strict host specificity of different species [140]. Members of lineage 4 acquired a second T4SS - the Trw T4SS - highly homologous to the conjugation machinery of the broad-host-range antibiotic resistance plasmid R388. This Trw T4SS was shown to be expressed *in vitro* during mammalian cell infection and to be important for successful host colonization *in vivo* [228]. The Trw T4SS was confirmed to be essential for the infection of the mammalian host in different transposon screens [140, 179]. The Trw T4SS mediates attachment to the host erythrocyte *in vitro* and seems to be causing the strict host specificity. [140]. Later it was shown that the tip of the Trw T4SS pilus TrwJ1 and 2 bind band 3 on the surface of erythrocytes [229]. The Trw system of *Bartonella* lacks the coupling protein necessary for translocation of a substrate and is thus believed to have lost this function [230, 231]. Most of the Trw components in *Bartonella* exist in tandem gene duplications which was postulated to allow binding to variations in the erythrocyte surface proteins [232].
Upon attachment, *B. bacilliformis* was reported to cause severe deformations of the erythrocyte that allow

the uptake of the bacteria. The uptake itself was suggested to happen by forced endocytosis [233]. Supernatants of *B. bacilliformis* and *B. henselae* cultures were shown to cause red blood cell deformation [226], however the nature for this so called "deformin" remains unknown. It was first suggested to be a secreted protein [234, 235] but later shown to be a hydrophobic small molecule able to bind do serum proteins such as albumin [236]. Invasion into the erythrocyte was shown to follow deformation [234], however the exact mechanism of how the deformin is secreted or its molecular action remain elusive.
The invasion of erythrocytes by *Bartonella* is also not fully understood. It has been speculated that since their ability to bind band 3 on the red blood cell surface [225, 229], the bacteria might be able to disconnect the actin skeleton from the membrane. This would allow them to destabilize the integrity of the erythrocytes in a similar manner to *Plasmodium*, the agent of malaria [127]. Further, the invasion associated locus (Ial) present in the membrane of Bartonellae was shown to mediate erythrocyte invasion [140, 237]. The locus encodes for two proteins, IalA and B, which are both necessary to mediate this function [238]. Insertion mutants in IalA, B and the ABC transporter livG both still showed erythrocyte adhesion but failed to invade [140]. Even though IalA has been described to be a nucleoside hydrolase [239, 240] and a structure for IalB has been published (Protein Data Bank no. 3DTD), their function during the entry process is unknown. It has been recently proposed, that IalAB could also help regulating the level of stress-induced nucleotides during erythrocyte invasion, maybe due to the nucleoside hydrolase activity [223].
In a rat model using *B. tribocorum* of lineage 4, bacteria persist mostly if not exclusively inside erythrocytes. It was observed that about one or two bacteria enter a red blood cell and then replicate inside a membrane-bound compartment until they reach about 8 bacteria per erythrocyte. Interestingly, only about 1 % of the total amount of erythrocytes were infected. It was further shown that *B. tribocorum* infects mature erythrocytes, not precursors, and is not haemolytic [124]. This goes in hand with observations from naturally infected cats showing exclusively intraerythrocytic bacteria with approximately 5 % of the erythrocytes being infected with about 1 bacterium per red blood cell [241, 242]. However, in an experimental cat infection, even though about 1 % of red blood cells were infected, only about 12 % of the detected bacteremia was observed to be intra-cellular [227]. Studies with *B. quintana* infected human patients also showed no signs of anemia or haemolysis but a very low amount of infected erythrocytes. 0.001 - 0.005 % infected red blood cells were reported per patient [243]. The only exception from this seems to be *B. bacilliformis*, which can lead to an average of 60 % infected erythrocytes in patients and haemolytic anemia, most likely caused by the host's immune response [151, 152, 233, 244].

1.3.4 The immune response against *Bartonella*

As a stealth pathogen, *Bartonella* evades or even modulates the host's immune system to its own favour. Its LPS is a TLR-4 antagonist and it can dampen the pro-inflammatory cytokine response while promoting an anti-inflammtory one as discussed above. However, since experimental infections (for example in [124, 188]) and CSD in immunocompetent patients are usually self-limiting, it is clear that the immune response of the host must be able to eliminate the infection. In addition, complications such as bacillary angiomatosis described in immunosuppressed patients underline the importance of the host's immune system [245]. In the macaque model, only immunosuppressed animals showed this disease phenotype upon infection with *B. quintana* (personal communication with Prof. Jane E. Koehler). However, different, partially

contradicting data has been reported about the immune response against *Bartonella*. The findings seem to depend on the particular strain - host combination observed, for example when comparing the reservoir host with an incidental host. Differences between natural and experimental infection have also been reported.
The early stage of *B. bacilliformis* infection in patients is described to show transient immunosuppresion with elevated IL-10 levels, CD4+ T-cell lymphopenia and a Th1 response [151, 246, 247, 248, 249]. It is believed that the humoral immune response against *B. bacilliformis*, giving rise to neutralizing antibodies, provides protection [245]. This hypothesis is based on the high seroprevalence in endemic regions and increased susceptibility of foreigners to the infection [166], the fact that outer membrane proteins (OMPs) of *B. bacilliformis* appear to be highly immunogenic [250] and the finding that bacteria of this species exposed to anti-flagellin antiserum showed reduced invasion of human erythrocytes [251]. However, the appearance of the Peruvian wart despite a present antibody response and the presence of asymptomatic carrier's suggests that a protective antibody response is not sufficient to eliminate the bacteria from their replication site in endothelial cells. Thus, an important role for the cellular immune response has been postulated [245]. Also for *B. quintana* infection, antibody responses are observed in patients and even used for diagnostics [169, 252]. Amongst the most immunogenic OMPs of *B. quintana* are the Vomp family and hemin-binding proteins [196].
Infection of human patients with *B. henselae* is an incidental infection. The lymphoadenopathy and granulomas observed in CSD patients together with the lack of bacteremia are believed to be caused by the lack of adaptation to the incidental host [113] and/or because in immunocompetent patients the adaptive immune system can prevent a systemic infection [245]. A specific cellular immune response against *B. henselae* was detected in patients [253] and a high antibody response was reported. The latter is used for diagnostics similar to *B. quintana* [254]. The IgG response targets a main antigen of 83 kDa that seems to be shared by *B. henselae* and *B. quintana* [255] and a smaller 17 kDa protein [210]. Also BadA seems to be a major antigen of *B. henselae* [187]. When bound to an antibody, *B. henselae* uptake and killing by macrophages is increased. Further, the complement was reported to kill this bacterium very efficiently [256]. Interestingly, B-cells from CSD granulomas were shown to express the transcription factor T-bet, suggesting a T-independent class-switch reaction [257].
An often used model for the immune response against *Bartonella* during CSD is the inoculation of mice, an artificial, experimental host, with *B. henselae*. The granulomas from patients were described to be rich in B-cells, neutrophils [257] and macrophages undergoing apoptosis [258]. Similar findings with granulomas rich in proliferating and recruited lymphocytes, mostly B-cells, could be observed after subcutaneous infection of Balb/c and C57BL/6 mice. Further, infection with *B. henselae* caused a lower type I interferon response than infection with a strain naturally infecting mice [259]. After intraperitoneal injection, *B. henselae* caused granulomas in the liver and a Th2 signature in C57BL/6 mice [260]. In a Balb/c infection model a Th1 response was reported [128, 261] and IgA production was observed [128]. Clearance of the infection with *B. henselae* was also observed in nude mice, indicating that there might be a T-cell-independent clearance mechanism [262].
However, the natural reservoir of *B. henselae* is the cat. A specific antibody response, which in a few cases failed to prevent relapses, and a Th2 type response were reported for naturally infected cats [197, 263]. In experimentally infected cats a Th1 response was observed and even though an antibody response was detected, IFNγ and TNFα production were suggested to be more important for clearance of the bacteremia

[264]. The antibody response caused by immunization with killed *B. henselae* did prevent the occurrence of lesions but not bacteremia upon infection [265]. In a neonatal cat infection study, cats cleared the infection simultaneously, no matter if they produced antibodies against *B. henselae* or not. This lead to the suggestion that IgG is not necessary for clearance [266].
Other models have been used to study the immune response against *Bartonella* in its natural host. Dogs have been experimentally infected with *B. vinsonii* subspecies *berkhoffii* and showed signs of immunosuppression such as reduced and impaired CD8+ counts and reduced MHCII molecules on B-cells [130]. Using *B. birtlesii*, naturally infecting mice, in a Balb/c model showed a higher and prolonged bacteremia for CD4-/- mice whereas CD8-/- were comparable to the wildtype (WT) infection. The double knock-out CD4 x CD8 -/- showed an intermediate phenotype. This data set suggested that T-helper cells are necessary in order to mount a protective antibody response which can then mediate clearance [267]. Notably, splenectomized mice as well as splenectomized cats show a higher bacteremia at the peak of infection but clear the infection only slightly delayed compared to non-splenectomized control animals [268, 269]. In order to investigate the importance of the adaptive immune system and especially the antibody response, C57BL/6 mice lacking B- and T-cells (Rag1-/-) or B-cells alone (JHT) were infected with *B. grahamii*, which can be naturally found in mice. Both models did not clear the infection. Antiserum from WT mice that show elevated anti-*Bartonella* antibodies transferred into JHT mice resulted in clearance of the bacteremia. Thus, it was concluded that the adaptive immune system and especially B-cells producing antibodies are necessary for clearance [134]. It was speculated that in the natural host neutralizing antibodies can prevent the entry into the erythrocyte, but that intra-erythrocytic bacteria are protected since red blood cells cannot present antigen via MHCI [124, 134]. Even though the proof for this hypothesis is still missing, there is evidence for *B. bacilliformis* that entry into red blood cells can be prevented when treating the bacteria with an anti-flagellin serum [251]. Similarly, an antiserum against IalB prevented entry of *B. birtlesii* into murine erythrocytes [270].

As in cats, also in rodents there seems to be a discrepancy between the experimental model and natural infection. No antibody response could be detected in infected rodents captured from the wild [271, 272]. Also the high prevalence, for example 64.8 % of rodents in Europe being infected [142], suggests either longer infection duration than in experimental infections or often occurring reinfections. Experimentally infected cats cleared the infection between 1 and 8 months after infection and were protected against re-infection whereas naturally infected cats had reoccurring bacteremic cycles during a year of observation [273]. Kosoy *et. al* did not only show a lack of antibody response but could also detect *Bartonella* in embryos and neonates of captured rodents and explained the high prevalence of Bartonellae in the wild with transplacental transmission and induced immunological tolerance in the offspring [271]. An experimental mouse model using *B. birtlesii* confirmed transplacental transmission going in hand with placental lesions and fetal resorption [274]. Also one case of potential transplacental transmission of *Bartonella* in a patient has been reported [275]. However, no transplacental transmission was observed in a feline model of *B. henselae* [273] or for *B. bovis* in cattle [276].
Neonatally infected mice clear the infection, which speaks against the induction of immunological tolerance towards *Bartonella* after vertical transmission (unpublished observations by Yun-yueh Lu). A study performed with neonatally infected cats did not only show the clearance of the bacteremia in the infected

animals but also that they produce antibodies [266]. Another explanation for the high prevalence could be the usually high diversity of *Bartonella* species and strains within one species that co-infect single animals [149, 277]. Genomic variation could allow relapses and avoidance of the immune response [278]. Also reinfections by different strains have been suggested to prolong bacteremia [133]. Variation of surface proteins within a species during infection have been reported [188], as well as mosaic-like structures of genetic variants within primary isolates [279] and potential horizontal gene transfer during infection [280]. All those mechanisms could be explored by the bacteria in order to evade the antibody response by changing the antigen on the cell surface. Also an effect by the natural transmission by the arthropod vector on the course of bacteremia cannot be excluded.

It remains enigmatic what causes the differences between the experimental model and natural infection, a failure of the immune response, an effective avoidance mechanism of *Bartonella* or both. However, without a detailed clearance mechanism known, it is difficult to speculate about potential evasion mechanisms explored by *Bartonella* in order to prolong the infection or to allow reinfections.

2 Aim of the thesis

The aim of this study was to investigate the adaptive immune response against *Bartonella* in the murine model. The focus was on the clearance of the bacteremia by the protective antibody response, but other aspects were also explored.

The strain *B. taylorii*, naturally infecting rodents including mice, was so far only scarcely used for experimental infections despite its several advantages compared to previously used strains, such as its robustness in growth, the possibility of experimental mouse infection and existing protocols for genetic manipulation. Thus, while implementing the *B. taylorii* model, the pilot experiments were additionally performed with *B. birtlesii* in mice.

The first goal was to determine factors of the host's immune system which are essential for clearing the infection. Since the primary and replication niche of *Bartonella* are so far not well described, the focus was on the clearance of the bacteremia, which is easily accessible by determination of the blood cfu count. By infection of several murine knock out models and analyzing their capacity to clear the infection, different antibody effector functions were explored, as well as the role of T-cells. The second goal was to describe the mechanism of antibody mediated clearance by finding a suitable *in-vitro* correlate for protection. A flow cytometry based *in-vitro* erythrocyte adhesion inhibition assay also allowed the screening for functional monoclonal antibodies against *B. taylorii* and with that the exploration of the third goal, the determination of possible targets of protective antibodies on the surface of *Bartonella*.

Transplacental transmission of *Bartonella* causing life-long persistent bacteremia and immunological tolerance in the offspring was suggested in the literature. Thus, we also set out to investigate the capability of *B. taylorii* to be transmitted from mother to offspring via the placenta and the resulting bacterial burden in the offspring.

3 Results and discussion

3.1 Antibody mediated clearance of *Bartonella*

3.1.1 Manuscript in preparation

3.1.1.1 Statement of contribution

This draft of the manuscript "Neutralizing antibodies protect against murine *Bartonella* infection by interfering with erythrocyte adhesion" covers most of the work prepared in the course of this thesis. Katja Fromm and Claudia Mistl were involved in some of the animal experimentation, Jarosław Sędzicki provided aid for the microscopy and the plasmid for constant GFP expression in bacteria.

3.1.1.2 Manuscript: Neutralizing antibodies protect against murine *Bartonella* infection by interfering with erythrocyte adhesion

Neutralizing antibodies protect against murine *Bartonella* infection by interfering with erythrocyte adhesion.

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keywords: *Bartonella*, erythrocyte infection, neutralizing antibodies, induced *Bartonella* autotransporter

list of abbreviations

cfu	colony forming unit
dpi	days post infection
EAI	erythrocyte adhesion interference
i.d.	*intra-dermally*
Ial	invasion associated locus
Iba	induced *Bartonella* autotransporter
moi	multiplicity of infection
n.d.	not detected
RBC	red blood cell
Vomp	variable outer membrane protein
WT	wildtype

Abstract

Bartonella is a genus of facultative-intracellular bacteria causing a long-lasting intra-erythrocytic bacteremia in their mammalian reservoir host. Clearance of the bacteremia has been described to be mediated by antibodies, the exact clearance mechanism has, however, not been investigated so far.

Murine *B. taylorii* infection served as a model for the infection of the natural host. Exploring clearance kinetics and antibody responses in different murine knock-out models revealed that clearance of the bacteremia is independent of the complement or Fcγ receptors. Further, we could show that whereas the specificity of an antibody is important for its functionality *in vivo*, the antibody type - IgM or IgG - is redundant. An *in-vitro* erythrocyte adhesion inhibition assay correlated with protection of an immune serum or antibody *in vivo* and might explain the clearance mechanism of neutralizing antibodies by interfering with red blood cell adhesion. A neutralizing and highly specific monoclonal antibody against *B. taylorii*, targeting a putative autotransporter on the bacterial surface, was sufficient to provide protection *in vivo*, revealing the importance of a so far understudied virulence factor during infection but also for protection by the host's immune system.

Investigating the role of T-cells in clearing the bacteremia showed that, whereas the cytolytic response can be neglected, direct T-help via CD40L is essential for clearance. Our data suggests that there is a high redundancy amongst T-cell subtypes which are able to provide this help to B-cells in order to clear the infection.

This study describes in detail how neutralizing antibodies protect against *Bartonella* infection by preventing erythrocyte adhesion of the bacteria and suggests a role for a so far scarcely studied autotransporter in RBC attachment.

Introduction

Bartonellae are fastidious, facultative-intracellular Gram-negative bacteria. Their hallmark is the establishment of a long-lasting intra-erythrocytic bacteremia in the species specific mammalian host. In clinics, *Bartonella* infections are caused by human-specific species such as *B. quintana*, causing trench fever, or zoonotic pathogens such as *B. henselae*, the agent of cat scratch disease. In immunocompetent patients, *Bartonella* infections usually present themselves as self-limiting but long-lasting with mild to life-threatening symptoms [Maguiña and Gotuzzo, 2000, Maguiña et al., 2009].

After transmission by an arthropod vector, the bacteria enter the dermis. It is believed that they travel within the lymphatic vessels to reach their so far unknown primary niche where they can replicate and seed into the blood stream. *Bartonella* then infects red blood cells (RBCs) allowing the uptake by a new arthropod vector during a blood meal [Seubert et al., 2001, Chomel et al., 2009, Minnick and Battisti, 2009, Harms and Dehio, 2012, Hong et al., 2017].

Using *B. tribocorum* in the rat as a model for the infection of the natural reservoir host, it was shown that the bacteremia appears around day 7 post infection after *intra-dermal* (*i.d.*) inoculation and lasts for several weeks [Okujava et al., 2014]. This abacteremic window after infection is suggested to be necessary in order for the bacteria to travel to, infect and replicate within the primary niche. After infection of the RBCs, the bacteria replicate and reside within erythrocytes until the end of their life span. Being non-haemolytic, it is assumed that they do not spread from RBC to RBC [Schülein et al., 2001]. The infection of erythrocytes by *Bartonella* is reported to be a three step-mechanism: attachment, deformation and invasion [Deng et al., 2018]. Several virulence factors involved in this process have been identified, such as the Trw type-IV secretion system [Vayssier-Taussat et al., 2010] or the invasion associated locus (Ial) encoding for IalA and IalB [Mitchell and Minnick, 1995, Coleman and Minnick, 2001, Vayssier-Taussat et al., 2010]. The erythrocyte adhesion step was reported to be crucial for the strict host-specificity [Vayssier-Taussat et al., 2010] and the virulence factors involved in erythrocyte infection were identified to be essential for successful host colonization of *Bartonella* in two transposon screens [Saenz et al., 2007, Vayssier-Taussat et al., 2010].

A study using *B. grahamii* in mice showed that antibodies mediate the spontaneous clearance of the bacteremia [Koesling et al., 2001]. This goes in hand with reports of high species-specific IgG titers in rats after infection, which protect from reinfections with the same strain [Kosoy et al., 1999]. An induced antibody response has also been reported for *B. henselae*-infected cats [Kabeya et al., 2006, Kabeya et al., 2009, Vigil et al., 2010]. However, neonatally infected cats seem to fail to mount an antibody response while still clearing the infection [Guptill et al., 1999]. An importance for the cellular immune response in the natural host has also been suggested [Karem, 2000]. Infection of mice lacking CD4 with a murine *Bartonella* strain resulted in an increased and prolonged bacteremia, indicating a role of T-help in the establishment of the protective antibody response. Mice lacking CD8 did not show a phenotype [Marignac et al., 2010]. Even though the exact protective mechanism of the antibody response is unknown, it has been speculated that neutralizing antibodies might interfere with the infection of the host's erythrocytes [Karem, 2000, Schülein et al., 2001, Koesling et al., 2001]. Indeed, antisera raised against the flagellum of *B. bacilliformis* and ialB of *B. birtlesii* were shown to prevent erythrocyte infection by the respective species *in vitro* [Scherer et al., 1993, Deng et al., 2016]. However, it remained unknown, if those neutralizing antibodies are produced *in vivo* and if, which virulence factors are targeted by the mammalian host.

In this study we explore the murine immune response against *B. taylorii*, a species naturally infecting rodents, including mice. We find evidence confirming that antibodies interfering with erythrocyte adhesion are produced *in vivo* and mediate clearance of the bacteremia. Fc-receptor mediated functions or the complement are dispensable. A highly specific neutralizing monoclonal antibody isolated from an infected mouse against a putative *Bartonella* autotransporter is sufficient to provide clearance of the bacteremia. We also describe that while the cytolytic immune response is negligible, CD40L-mediated T-help is indeed important for the protective antibody response against *Bartonella*. However, there seems to be a high redundancy in which cell types are able to provide this help. This work confirms a long existing hypothesis about how *Bartonella* bacteremia is cleared and stresses the importance of so far not well understood autotransporters in infection. In addition, it gives rise to a new target for possible vaccine strategies and introduces a new mouse model for studying *Bartonella* infection of the natural reservoir host.

Results

Fcγ-receptor mediated function and complement are dispensable for clearing *Bartonella* bacteremia.

B. taylorii, a species naturally found in mice, was used as an experimental model in this study. We compared the course of the bacteremia in wildtype (WT) C57Bl/6 mice to animals lacking the adaptive immune system or only B-cells. Both, Rag1-/- and JHT mice, have been described to develop life-long persistent bacteremia after infection with *B. grahamii* [Koesling et al., 2001]. Upon *i.d.* inoculation, similar to observations previously made with *B. tribocorum* in the rat [Okujava et al., 2014], the mice remained abacteremic for a period of about 5-7 days. In WT mice, the infection peaked around day 12-14 post infection with about 10^5 bacteria per ml of blood. The bacteremia was then cleared within 50 days post infection (dpi) (Figure 1A). No relapses were ever observed for WT mice. Infected Rag1-/- and JHT mice showed a persistent bacteremia which remained on a plateau of about 10^6 cfu/ml for JHT mice and 10^7 cfu/ml for Rag1-/- for at least a year (Figure 1A, data shown for 200 dpi).

The lack of clearance in Rag1-/- and JHT mice indicated the importance of the adaptive immune system, especially antibodies, for the clearance of the bacteremia also for *B. taylorii*. The differences in bacterial titers between the WT and the mutant animals might be explained by a protective antibody response beginning to clear the bacteremia before the bacteria can reach their maximum titers.

In order to focus more on the antibody effector mechanism leading to clearance of the bacteremia, we next infected mice lacking all α chains of Fcγ-receptors and the major protein of the complement cascade C3 (FcγRnull x C3-/-). In this model, antibodies can no longer activate the complement or mediate effector functions such as antibody-mediated cellular cytotoxicity or antibody-mediated phagocytosis. However, there was no difference in the course of the bacteremia between FcγRnull x C3-/- and WT mice (Figure 1B), indicating that those effector mechanisms are dispensable for the clearance of the bacteremia.

This was further supported by the fact that immune serum from *B. taylorii* infected WT mice transferred into WT or FcγRnull x C3-/- recipients on day 3 post infection (Figure 1C-E) was protective. On day 3 the mice are still abacteremic and should not yet have produced antibodies of their own. Administration of protective serum on this day did prevent the occurrence of the bacteremia - most likely by neutralizing the bacteria before entering the blood stream as this was independent on the presence of complement or Fcγ receptors.

Protective antibodies against *Bartonella* interfere with the adhesion towards erythrocytes.

Interference with erythrocyte infection was one suggested effector mechanism for protection by antibodies [Karem, 2000, Schülein et al., 2001, Koesling et al., 2001]. In order to be able to quantify this protective, neutralizing capacity of antibodies, we modified an *in-vitro Bartonella* RBC infection assay previously established in our group [Vayssier-Taussat et al., 2010]. Murine erythrocytes were purified and infected with GFP-expressing *B. taylorii*. The infection rate was quantified using flow cytometry. In order to observe a potential interference of protective antibodies with the infectivity, the bacteria were preincubated with naïve or immune serum before the infection (Figure 2A). Immune serum prevented the occurrence of GFP+ RBCs in a concentration-dependent manner whereas naïve serum had no effect (Figure 2B and C). Heat-inactivation

of the immune serum in order to remove complement components had no effect on its suppression of RBC interaction (Figure S2A). Only a negligible bactericidal effect of the immune serum on *B. taylorii* was observed when performing cfu counts after 24 h of growth in the presence of serum (Figure S2B), indicating that the effect cannot be explained by killing of the bacteria.

In order to investigate if the immune serum interferes with the adhesion to or the entry into the erythrocytes, we performed confocal microscopy (Figure 2D) and gentamycin protection assays (Figure 2E) with the infected RBCs. Gentamycin does not enter eukaryotic cells and is only able to kill extra-cellular bacteria. As has been described previously for a 24 h infection time point *in vitro* [Vayssier-Taussat et al., 2010], most, if not all, bacteria observed were extra-cellular. Microscopy showed exclusively bacteria attached to the surface of RBCs (Figure 2D). *B. taylorii* remained gentamycin-sensitive during the assay. In comparison, erythrocytes obtained from mice infected with *B. taylorii* contained gentamycin-insensitive bacteria (Figure 2E), as expected for an intra-erythrocytic pathogen. This erythrocyte adhesion interference (EAI) assay does not entirely reflect the situation in the animal because of the discrepancy in localization of the bacteria. However, it allows the quantification of the quality of an antibody or immune serum. As the EAI assay correlates very well with the protective function *in vivo*, we hypothesize that is still serves as a model for what could happen in the animal since prevention of bacterial attachment also prevents the infection of the cell.

We infected WT mice with *B. taylorii* and observed the development of the EAI titer over time (Figure 2F). Between day 7 and 14 post infection the EAI titer sharply rose and was maintained during the course of the bacteremia, reaching top titers of 1:1024. This time course reflects the expected kinetic of an antibody response after inoculation with a pathogen. When comparing the course of the bacteremia with the EAI titers, one can observe that peaking EAI titers appeared when the blood cfu count started to drop and were maintained on similar levels until the end of the bacteremia.

To observe the detection limit of the established EAI assay, we transferred immune serum into mice lacking a B-cell response and observed the EAI titer over time (see Figure 3A and B). μMT mice, which lack B-cells similar to the JHT model, also showed life-long persistent bacteremia. We injected immune serum into infected μMT mice >100 dpi. This lead to clearance of the bacteremia within 30 days after transfer. The EAI titers, however, could only be observed until 14 days post transfer, when the mice were still bacteremic (Figure 3B). This could indicate that the EAI assay, even though its functional readout correlates with protection, has only limited sensitivity. In uninfected μMT mice, the drop of the EAI titer after serum transfer followed a similar kinetic (Figure S3A), showing that EAI titers are not masked by antibodies bound to free bacteria. The observations from Figure 3B also support the notion that the intra-erythrocytic state is a dead-end for *Bartonella* and that from there they cannot reseed into RBCs or other cells even when the protective antibody response disappears. Interestingly, immune serum transfer also depletes potential blood seeding replication sites since the mice became sterile after immune serum treatment and cleared the bacteremia after the disappearance of the protective immune serum.

EAI titers correlate with protection and reveal the independence of the protective antibody response of the Ig type.

Next, we infected AID-/- mice, lacking antibody class switch, and sIgM-/- mice, no longer able to secrete IgM, to investigate the importance of the antibody class. So far, anti-*Bartonella* IgG titers have been reported for infected mice [Koesling et al., 2001], but the importance of the class switch is unknown. As a control, we also crossed the double knock-out AID-/- x sIgM-/- which is no longer able to secrete antibodies.
AID-/- and sIgM-/- mice had similar bacteremia kinetics as WT mice, whereas AID-/- x sIgM-/- showed persistent bacteremia like μMT mice (Figure 3C). Clearance could be clearly correlated with the presence of an EAI titer during the infection. Comparable titers could be observed for WT, AID-/- and sIgM-/- mice 2 and 6 weeks post infection, whereas the double knockout and μMT mice failed to produce protective antibodies (Figure 3D, complete EAI titer kinetics are shown in Figure S3B-F). Even though the functional EAI titers are similar, measuring IgM and IgG titers against outer membrane proteins of *B. taylorii* revealed that as expected AID-/- mice only produce IgM and sIgM-/- mice only IgG (Figure 3E). Thus, as long as functional antibodies can be produced - independent whether they are IgM or switched - the infection is cleared.
To exclude redundant effects, we crossed the strain AID-/- x C3-/-. In this model only IgM can be produced, however this IgM response can no longer activate the complement cascade. The clearance of the bacteremia and production of functional antibodies in AID-/- x C3-/- was similar to WT and AID-/- mice (Figure S3G and H).

A specific antibody response is necessary to clear *Bartonella* bacteremia.

So far, we have shown that antibodies from immune animals clear the bacteremia in a complement- and Fc-receptor-independent manner, most likely by neutralization. We could correlate the specificity and functionality of those antibodies with *in-vitro* methods, namely ELISA (Figure 3E) and the EAI assay (Figures 2F and 3D). To proove that specificity of the neutralizing antibodies confirms protection *in vivo*, we used mouse models with restricted antibody repertoires. T11μMT mice express a transgenic heavy chain for an anti-VSV antibody in the μMT background, lacking endogenous heavy chains. Thus, T11μMT mice can only recombine light chains in order to produce specific antibodies as their heavy chains are fixed to bind an antigen which is irrelevant for *Bartonella* infection. Upon infection, T11μMT mice showed an increased titer at the peak of the bacteremia and cleared with several weeks delay compared to WT animals. In fact, within the first 4 weeks the bacteremia of T11μMT resembled the kinetic of the μMT mice before clearing the infection (Figure 4A). This delayed clearance could be explained with a delayed antibody response, which was only measurable 11 weeks post infections (Figure 4B).
To confirm the findings from the T11μMT model, we also infected MD4 mice, which express a transgene for an antibody against hen-egg lysozyme. Allelic exclusion should prevent those mice from producing a normal antibody response. The phenotype of the MD4 mice was milder than for the T11μMT model, with a modestly increased bacterial titer at the peak of the infection and a delay of the bacterial clearance of 1 week. 2 out of 4 MD4 mice became again bacteremic for 2 weeks before finally clearing the infection (Figure 4C). The milder phenotype of the MD4 mice could be explained by the fact that they started to produce a protective antibody response already 2 weeks post infection and reached similar titers as WT mice around 6 weeks post infection. MD4 mice failed to maintain titers as high as WT mice 11 weeks post infection,

which could explain the rebound infection in 50 % of the animals (Figure 4D). Complete EAI titer kinetics for T11μMT and MD4 mice are shown in Figure S4.

A neutralizing monoclonal antibody recognizing a *B. taylorii* autotransporter is protective *in vivo*.

We then wanted to find out more about the antigens targeted in a protective antibody response against *Bartonella*.

After producing hybridomas from *B. taylorii* immunized Balb/c mice, we obtained a monoclonal antibody, LS4G2, which interfered with erythrocyte adhesion of *B. taylorii in vitro* (Figure 5A). The purified antibody had no bactericidal effect (Figure S5A) and bound to live bacteria (Figure 5B, C). LS4G2 is highly specific for *B. taylorii* IBS296 used in this study, since it did not bind to other tested *B. taylorii* isolates or the rodent-borne species *B. birtlesii* and *B. tribocorum* (Figure S5B). Structured illumination microscopy revealed that the antibody LS4G2 binds to the surface of *B. taylorii*. A heterogeneous staining pattern was observed amongst the bacterial population. A smaller fraction of the bacteria remained unstained, however, most of them displayed a homogeneous fully surrounded staining. Some bacteria showed a possible intermediate phenotype, with surrounding stained foci on their surface (Figure 5B).

LS4G2 was selected for its *in-vitro* activity in the EAI assay. To observe its protective capacity *in vivo*, mice were infected with *B. taylorii* and obtained LS4G2 or an isotype on day 3 post infection (Figure 5D). To prove that LS4G2 is indeed protective because of its neutralizing function, we transferred the antibody into WT C57Bl/6 (Figure 5E) and into FcγRnull x C3-/- (Figure 5F) mice. LS4G2 had a similar activity to immune serum *in vivo* (Figure 1C-E). Independent on the presence of complement and Fcγ-receptors, the antibody suppressed the occurrence of bacteremia in infected mice.

In order to find the target bound by LS4G2, we performed immunoprecipitation from the solubilized membrane protein fraction of *B. taylorii* followed by mass spectrometry analysis. The analysis revealed a so far undescribed 75 kDa outer membrane protein (OPB34894.1) harboring a predicted autotransporter β-barrel domain. It was over 30-fold enriched compared to the isotype control and had a coverage of 68 % (see table S1). Homology analysis revealed that the protein is present in most, if not all, *Bartonella* species of lineage 4 with varying homology between species and isolates (data not shown). However, no function has been assigned to any of the homologs so far. Similar inducible *Bartonella* autotransporters (Ibas) have, however, been found to be essential for the infection of the natural host in different transposon screens [Saenz et al., 2007, Vayssier-Taussat et al., 2010].

Further analysis of the identified target of LS4G2 and its function in pathogenicity will also provide further insight in the immmune response against *Bartonella*.

T-help via CD40L from redundant sources is necessary for clearance of *B. taylorii*.

The almost 10-fold difference observed in bacterial titers between JHT and Rag1-/- animals (Figure 1A) lead us to explore also the importance of the T-cell response. The cellular immune response has been suggested to clear potential seeding niches [Karem, 2000].

In order to address this, we infected mice lacking all T-cell compartments (TCRβδ-/-) and compared the clearance kinetic with WT mice and mice lacking classical CD4 T-help (MHCII-/-) or CD8 killing (K^bD^b-/-).

In contrary to previous findings, in which CD4-/- mice showed increased bacterial burden and delayed clearance [Marignac et al., 2010], MHCII-/- mice cleared the infection as WT and K^bD^b-/- animals. TCRβδ-/- animals, however, showed a persistent bacteremia for at least 150 dpi (see Figure 6A). A role for CD8 killing can be excluded, since also β2M-/- mice, another model lacking cytotoxic T-cell responses, cleared the infection normally (Figure S6A and B). To investigate if T-cells restricted by other molecules than MHCII-/- are providing help to B-cells in order to produce antibodies clearing the *Bartonella* infection, we infected CD1d-/- and MR1-/- mice. CD1d on antigen presenting cells is a molecule, which is able to present lipids to lymphocytes; MR1 presents bacterial metabolites [Corbett et al., 2014, Mori et al., 2016, Lepore et al., 2018]. However, CD1d-/- and MR1-/- animals cleared similar to WT mice (Figure 6B), suggesting that T-help is either not required for clearance or that there is a high redundancy between cell types providing this help. Infection of CD40L-/- animals, in which direct help from T-cells do B-cells is not possible, revealed a persistent bacteremia in this model (Figure 6C). The course of the bacteremia was similar to WT mice until shortly before the clearance. At this point CD40L-/- animals stopped to control the bacteremia and *Bartonella* persisted in low to intermediate titers. As previously described for these mice [Whitmire et al., 1996], we found that CD40L-/- mice, despite producing a protective antibody response early after infection (week 2 and 6 post infection), failed to maintain the response in later time points (Figure 6D, complete antibody kinetics are shown in Figure S6C).

Since this finding supports the hypothesis, that a direct action of T-helper cells is important, which can be provided by T-cells restricted by different antigen molecules, we compared TCRβδ-/- to the single knock outs TCRβ-/- and TCRδ-/-. Invariant T-cells restricted by other molecules express exclusively αβ-TCRs. Indeed, TCRδ-/- mice produced protective antibodies and cleared the infection like WT animals, whereas TCRβ-/- mice showed the same phenotype as TCRβδ-/- animals. Both failed to produce a protective antibody response (Figure 6E and F, complete antibody kinetics are shown in Figure S6D-G).

This data supports the notion that T-help is important for clearance of the bacteremia, most likely by supporting the antibody response. There seems to be a high redundancy amongst T-cells being able to provide this help.

Discussion

Neutralizing antibodies are one of the main means of protection against pathogens by the adaptive immune system. Various functions have been described for neutralizing antibodies, such as prevention of host cell entry or attachment of viruses [Lu et al., 2018] and *Plasmodium* [Dutta et al., 2003, Irani et al., 2015] or inhibition of intra-cellular replication of *Anaplasma* [Wang et al., 2006] and *Listeria* [Edelson and Unanue, 2001]. For *Bartonella* infections, neutralizing antibodies were previously suggested to mediate clearance [Karem, 2000, Schülein et al., 2001, Koesling et al., 2001]. Antisera raised against purified proteins of different species could prevent RBC infection *in vitro* [Scherer et al., 1993, Deng et al., 2016]. However, the presence of neutralizing antibodies *in vivo* and their potential targets have not yet been investigated. In this work, we confirm that neutralizing antibodies are produced during *Bartonella* infection and mediate protection by preventing RBC attachment.

Our study introduced *B. taylorii* as a new mouse model for *Bartonella* infection. Its main advantages over previously used species such as *B. grahamii* [Koesling et al., 2001] or *B. tribocorum* [Schülein et al., 2001, Okujava et al., 2014] are the ability to use the murine infection model, its robustness and genetic accessibility. We have shown that *B. taylorii* infection follows kinetics similar to previously described rodent models of related *Bartonella* species [Schülein et al., 2001, Koesling et al., 2001, Marignac et al., 2010, Okujava et al., 2014]. We could confirm the intra-erythrocytic state of *Bartonella* in the natural host [Schülein et al., 2001] and the importance of antibodies in clearing the infection [Koesling et al., 2001].

Bacteremia kinetics and serum transfers indicate that antibodies clear *Bartonella* infection in a complement- and Fc-receptor independent fashion. Despite potential complement activation and antibody-mediated-phagocytosis or -cytotoxicity in WT animals, those mechanisms are dispensable for clearance. Interestingly, Fc-dependent functions were recently shown to be also redundant in a model for broadly neutralizing antibodies against HIV [Parsons et al., 2019].

To our knowledge, we are the first ones to report that infected animals indeed produce antibodies interfering with erythrocyte adhesion upon *Bartonella* infection. We could further correlate the occurrence of these antibodies with clearance of the bacteremia in several mouse models and show that different antibody classes are redundant for this function. The EAI assay, established in this study, gives a good correlate for the protective capacity of the antibody response and serves as a model for what happens *in vivo*. It has, however, its limitations. The hallmark of *Bartonella* infection *in vivo* is the intra-erythrocytic state. In our hands, *in-vitro* infection resulted in almost exclusively extra-cellular bacteria attached to erythrocytes, a finding that has been observed for similar time points before [Vayssier-Taussat et al., 2010]. Erythrocyte infection by *Bartonella* has been described as a strict three step mechanism: attachment, deformation and invasion [Deng et al., 2018]. Thus, antibodies interfering with the first step, adhesion to the RBC, would also prevent the following steps. A serum against IalB, a protein involved in entering the erythrocyte [Vayssier-Taussat et al., 2010] prevented RBC infection *in vitro* [Deng et al., 2016], suggesting that antibodies targeting later steps would also be functional.

We have shown that the neutralizing monoclonal antibody LS4G2, obtained from an infected mouse, is sufficient to provide protection *in vivo*. It was selected because of its *in-vitro* activity in the EAI assay and further confirms that this assay is a good correlate for *in vivo* protection. Interestingly, the target of LS4G2 is not one of the previously described molecules involved in RBC attachment or infection, such as the Trw-

type IV secretion system, IalA, IalB or LivG [Mitchell and Minnick, 1995, Coleman and Minnick, 2001, Vayssier-Taussat et al., 2010], but a predicted autotransporter. In other *Bartonella* species, this protein and other autotransporters have been found to be essential for the colonization of the natural reservoir host in two transposon screens [Vayssier-Taussat et al., 2010, Saenz et al., 2007], to be highly immunogenic [Riess et al., 2004, Gilmore et al., 2005, Litwin et al., 2007, Boonjakuakul et al., 2007, Vigil et al., 2010] and to be upregulated during *in-vitro* infection [Seubert et al., 2001, Quebatte et al., 2010]. Autotransporters have so far not been associated with RBC cell adhesion in *Bartonella* infection. However, in general, autotransporters have often been found to be important for adhesion towards host cells or the extra-cellular matrix [Henderson and Nataro, 2001]. Other functions assigned to this class of proteins have been cleavage of antibodies bound to the pathogen [Mistry and Stockley, 2006], involvement in heme uptake [Otto et al., 2005] or functioning as a cohemolysin [Litwin and Johnson, 2005]. The most studied ones in *Bartonella* are the family of variable outer membrane proteins (Vomps), that mediate collagen adhesion [Zhang et al., 2004], and *Bartonella* adhesin A (BadA), which mediates adhesion and proangiogenic reprogramming of the host [Riess et al., 2004, Kaiser et al., 2008].

The role of the Vomps of *B. quintana* has been studied in the experimental macaque model. They have been shown to be essential during infection [MacKichan et al., 2008] and rearrangement of the *vomp* locus has been observed during experimental infection and in isolates from human patients, which was suggested to be an immune evasion strategy [Zhang et al., 2004]. Interestingly, even though we found homologs of the target of LS4G2 in all related *Bartonella* species, they had varying homology on the amino acid sequence level. This might explain the high specificity of LS4G2, which exclusively binds the strain the mouse was immunized with. A high variation of this important but immunogenic protein between different *Bartonella* species or even different strains within one species could be an essential immune evasion mechanism within this genus.

In accordance to previous findings [Marignac et al., 2010], our results indicate the importance of direct T-help in clearing the bacteremia. Their main role seems to be the maintenance of the antibody production long enough to mediate clearance. At the same time, there seems to be high redundancy amongst T-cells providing this help. A common feature of those T-cells is the expression of an $\alpha\beta$TCR, but they can be restricted by different antigen presenting molecules. We could further confirm that the cytotoxic T-cell response does not play a major role in clearing *Bartonella* bacteremia, in accordance with previous findings [Marignac et al., 2010].

Our results also suggest that the important role of antibodies in clearing *Bartonella* infection goes beyond the interference with erythrocyte infection. Previous results suggest that the bacteria cannot reinfect other cells after entering RBCs, indicating that the pathogen does not spread from RBCs within the same host [Schülein et al., 2001]. Thus, all reinfection cycles into erythrocytes have to come from another replication site. If cytotoxic T-cells would control this replication site, once this niche is cleared, the bacteremia should spontaneously decrease independently of the presence of protective antibodies. However, the presented work and previously described data [Koesling et al., 2001] show that in mice without B-cells the bacteremia persists. A cytolytic T-cell response should be present in these mice. Only administration of immune serum lead to clearance. Thus, we hypothesize that antibodies are also responsible for the clearance of potential replication niches of *Bartonella* and that the cytolytic T-cell response is also dispensable for this step. A short-lived replication site from where bacteria would regularly seed into the blood stream as well as into new

replication sites would allow neutralizing antibodies to not only interfere with erythrocyte attachment but to also block the interaction with other cell types and prevent the seeding of a new replication niche. However, since there is no proof of an intra-cellular replication niche *in vivo*, neutralizing antibodies preventing the replication of extra-cellular bacteria could also be an explanation.

Taken together, our data for the first time shows the existence of neutralizing antibodies against *Bartonella* in the natural host. In addition, we indicate one target of this antibody response, a so-far poorly studied outer membrane protein. Our data suggests that this protein is a virulence factor playing a role in erythrocyte attachment. Further studies of this virulence factor and its function will allow deeper insight into the infection strategy of *Bartonella* and the immune response of the host. Importantly, the role of autotransporters might not be limited to RBC infection. They could be also involved in the attachment to other cell types and thus important for seeding of the replication niche. Besides their unknown function, their immunogenicity and the observation that a monoclonal antibody against one protein is sufficient for clearance makes autotransporters interesting targets for potential vaccines against *Bartonella*. However, more research is needed in order to be able to design a vaccine strategy that is able to circumvent the high variability of this protein amongst the bacteria of one species or the entire genus.

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Materials and Methods

Bacterial strains and plasmids

B. birtlesii IBS325 LSB002 [Bermond et al., 2000], *B. taylorii* IBS296 LSB001 [Harms et al., 2017b] and *B. tribocorum* IBS506 LSB009 [Heller et al., 1998] were grown at 35°C, 5 % CO_2 on Columbia blood agar (CBA) containing 5 % defibrinated sheep blood and appropriate antibiotics (streptomycin 100 µg/ml, gentamycin 20 µg/ml). Bacteria were streaked 5 and expanded 2 days prior to usage.
E. coli strains were cultivated in lysogeny broth or on solid agar plates containing the appropriate antibiotics (kanamycin 30 µg/ml, gentamycin 10 µg/ml) or diaminopimelic acid (DAP, 1 mM) at 37°C over night.
The plasmid pLS04 was generated by replacing the kanamycin resistance cassette of pJC43 (kind gift form Jean Celli [Celli et al., 2005]) with a gentamycin resistance cassette from pPG100 [Schülein et al., 2005]. The entire sequence of pJC43 excluding the kanamycin resistance cassette was amplified by PCR with primers prJS370 and prJS371. The 833 bp fragment of pPG100 constituting the gentamycin resistance gene and upstream promoter region was amplified by PCR using primers prJS372 and prJS373. Both fragments were combined using the In-Fusion HD cloning kit (Takara, Kyoto, Japan). The plasmid pLS04 was conjugated into *Bartonella* as previously described [Harms et al., 2017a] resulting in *B. tayorii* LSB115.

Primer sequences:
prJS370: 5' CTCATCCTGTCTCTTGATCAGATC
prJS371: 5' AGATCTGGGGTTCGAAATGACCG
prJS372: 5' TCAAGAGACAGGATGAGAAGCCCTGCAAAGTAAACTGG
prJS373: 5' TTTCGAACCCCAGATCTTTAGGTGGCGGTACTTGGG

Animal experimentation

Animal handling was performed in accordance with the Swiss Animal Protection law and local animal welfare bodies. The animal experiments in this work were approved by the Veterinary Office of the Canton Basel-Stadt (License number 1741).
All animals were kept at SPF (specific pathogen free) conditions. WT animals C57/BL6 and Balb/c were obtained from Janvier labs, Le Genest-Saint-Isle, France. The homozygous murine knock-out strains AID-/- [Muramatsu et al., 2000], β2M-/- [Zijlstra et al., 1989], C3-/- [Wessels et al., 1995], JHT [Chen et al., 1993], K^bD^b-/- [Vugmeyster et al., 1998], MHCII-/- [Koentgen et al., 1993], Rag1-/- [Mombaerts et al., 1992], sIgM-/- [Tsiantoulas et al., 2017], TCRβδ-/- [Mombaerts et al., 1993] and T11-µMT [Klein et al., 1997] were bread at the Laboratory Animal Science Center (LASC, University of Zurich, Switzerland). FcγR4alpha x C3 -/- (Mehmet Sahin, manuscript in preparation), were bred at the transgenic mouse core facility (TCMF, University of Basel, Switzerland). µMT mice [Kitamura et al., 1991] were obtained from Jackson Laboratories, Maine, USA. The strains CD1d-/- [Smiley et al., 1997] and MR1-/- [Treiner et al., 2003] were a kind gift from Prof. Genaro De Libero, University of Basel. All mice were in C57Bl/6 background. AID x C3-/- was obtained by crossing AID-/- and C3-/-. TCRβ-/- and TCRδ-/- single

knock outs were obtained by back-crossing TCRβδ-/- with WT C57/Bl6.

Animals were infected *i.d.* with 10^7 cfu bacteria in PBS. Blood was drawn in 3 % sodium citrate on several days post infection. For blood cfu count, whole blood was frozen at -80°C, thawed and plated in a limited dilution series on blood agar (see above). For serum analysis, the blood samples were centrifuged for 5 min at RT at 5000 x g in serum tubes (Sarstedt, Nümbrecht, Germany). Serum was frozen at -20°C until usage.
Immune serum was obtained 45 dpi from immunized C57Bl/6 mice and pooled from 20 animals. Naïve or immune serum were injected in 100 µl *i.v.*. Antibodies were given in a dose of 250 µg in PBS *i.v.*. If indicated, the serum was heat-inactivated at 60°C for 30 min before usage.

Erythrocyte infection

Murine Erythrocytes were isolated from Balb/c mice after blood collection in 3 % sodium citrate from the tail vein or by terminal bleeding. Erythrocytes were purified using a Ficoll-gradient and kept at 4°C in DMEM (Gibco) 10 % FCS for up to two weeks until usage.
For EAI assay, a serial dilution of sera or antibodies was performed in a U-bottom 96-well plate in DMEM 10% FCS. 10^5 bacteria were added per well and incubated for 2 h at 35°C and 5 % CO_2. 10^6 (MOI 0.1) erythrocytes were added in 100 µl DMEM 10% FCS. After 24 h the supernatant was removed and the cells were fixed in 1% PFA and 0.2 % GA in PBS for 10 min at 4°. FACS buffer (2 % FCS in PBS) was added and the plates were analyzed in flow cytometry (CantoII from BD, New Jersey, USA, using the HTS autosampler) or stained for Confocal microscopy.

Microscopy

Bacteria were grown as described above, collected in PBS and stained with purified antibody for 1 h at RT. Bacteria were then washed with PBS, centrifuged at 4000 x g for 5 min and stained with the secondary antibody (anti-mouse IgG-Alexa 568, Biolegend, California, USA) for 1 h at 4°C in the dark. After another wash, the bacteria were fixed in 3.7 % PFA for 10 min at 4° in the dark.
RBCs were infected as described above. Erythrocytes were then stained with anti-Ter119-Alexa 647 (Biolegend, California, USA) for 1 h at 4°C in the dark. After washing and centrifugation at 100 x g, 5min, the cells were fixed in 3.7 % PFA for 10 min at 4° in the dark.
Fixed erythrocyte samples were centrifuged at 100 x g for 5 min. Stained bacteria samples were centrifuged at 4000 x g for 5 min. For both types of samples, the supernatant was removed and the cell pellet was resuspended in ProLong Diamond Antifade mountant (Thermo Fisher Scientific, Waltham, MA). 15 µl of suspension was applied on 18 mm, #1.5 thickness coverslips (Thermo Fisher Scientific, Waltham, MA) and mounted onto glass slides (Thermo Fisher Scientific, Waltham, MA). After 24 h of curing at room temperature, the coverslips were sealed with nail polish.
Confocal images were acquired with a SP8 confocal microscope (Leica) equipped with 488 and 638 nm solid-state lasers and a Plan Apo CS2 63x, 1.40 NA oil objective.
3D-SIM was performed using a DeltaVision OMX-Blaze system (version 4; GE Healthcare) equipped with 488 and 568 nm solid-state lasers, Plan Apo N 63x, 1.42 NA oil objective and 4 liquid-cooled sCMOs cameras

(pco Edge, full frame 2560 x 2160; Photometrics). Optical z-sections were separated by 0.125 µm. Exposure times were between 3 and 10 ms, with three rotations of the illumination grid.
Multichannel imaging was achieved through sequential acquisition of wavelengths by separate cameras. First, the channels were aligned in the image plane and around the optical axis using predetermined shifts measured using a target lens and the SoftWoRx alignment tool. Afterwards, they were carefully aligned using alignment parameters from control measurements made with 0.5 µm diameter multi-spectral fluorescent beads (Invitrogen, Thermo Fisher Scientific). Raw 3D-SIM images were processed and reconstructed using the DeltaVision OMX SoftWoRx software package (GE Healthcare) (Gustafsson, 2000). The final voxel size was 40 nm x 40 nm x 125 nm.

Gentamycin protection assay

Blood from infected animals or over night *in-vitro* infected erythrocytes (see above) were incubated for 2 h at 35°C, 5 % CO_2 either in PBS alone or in PBS containing 40 µg/ml gentamycin. Cells were washed 3 times by adding at least 1 ml of PBS and centrifugation at 100 x g for 5 min. Cell pellets were frozen at -80°C for erythrocyte lysis and plated in serial dilutions on blood agar plates.

Antibody binding assay

A serial dilution series of monoclonal antibody or isotype was performed in a U-bottom 96-well plate. Bacteria were grown as for the EAI assay and added with 10^7 bacteria per well. After 1h at RT in the dark, bacteria were washed with FACS buffer (2 % FCS in PBS, centrifugation at 4000 x g, 5 min) The bacteria were fixed with 3.7 % PFA for 10 min at 4° in the dark and resuspended in FACS buffer before analysis via flow cytometry (CantoII from BD, New Jersey, USA, using the HTS autosampler).

ELISA

Purification of *Bartonella* outer membrane proteins (OMP) was performed as has been described previously [Otsuyama et al., 2016].
For antibody titers in serum, anti murine IgG-HRP or anti murine IgM-biotin and streptavidin-HRP (Biolegend, California, USA) were used.
In brief, ELISA plates (corning, New York, USA) were coated over night with purified *Bartonella* OMP (1 mg per 96-well plate) in coating buffer (Biolegend, California, USA). The plates were washed with 0.05 % Tween in PBS and blocked 1h at RT with 1x ELISA buffer (Biolegend, California, USA). After another wash, sera were added in dilution series for 3h at RT. After 3 washes, detection antibody was added in the recommended dilution for 1 h at RT. If necessary streptavidin-HRP was added after another 3 washes for 30 min at RT. After 5 washes 1x ELISA substrate (Genetech, California, USA) was added at RT for 5-10 min and stopped with 1 M phosphoric acid. Plates were read at 450 and 570 nm.

Hybridoma production

For hybridoma production the DiSH Kit from Enzo Life Sciences, New York, USA, was used. Balb/c mice were infected with 10^7 cfu *B. taylorii i.d.* 6 weeks before *i.v.* reinfection with 10^7 cfu. 2 weeks later, the

animals were boosed with 10^7 cfu heat inactivated bacteria (3 h at 60 °C) and the spleens were harvested 2 days later. A collagenase digest (3 mg/ml Collagenase IV, 2 % FCS, in RPMI) was performed for 30-60 min at 37°C. The digestion mixture was put through a cell strainer and approx. 10 ml of RPMI + 10 % FCS were added. The cells were centrifuged for 10 min at 300 x g at 4°C and a red blood cell lysis was performed by resuspending the pellet in ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 0,1 mM EDTA). After 1 min at RT, 10 ml of RPMI + 10 % FCS was added and the cells were centrifuged again and used according to the DiSH Kit protocol. In brief, the obtained lymphocytes were washed with WCM. Myeloma fusion partner SP2ab grown in PMC medium, washed once with WCM and added to the lymphocyte pellet in a ration of 1:5 Sp2ab : lymphocyte. After centrifugation for 10 min at 4°C, 300 x g, the cell pellet was warmed to 37°C in a water bath. While keeping the pellet in the water bath, PEG1000 was added drop wise to the cell mixture. After 60 sec at 37°C, 4 ml of prewarmed WCM were added drop wise over the course of 2 min while keeping the cells at 37°C. Another 5 ml were added over the course of 1 min. Another 6 ml were added over the course of 1 min. After 10 min at 37°C, 30 ml of FCM were added and the cells were centrifuged at 150 x g for 5 min. The cells were then incubated over night in FCM at 37°C, 5 % CO_2. The fused cells were plated in semisolid medium (AbeoClone from Enzo Life Sciences) until the appearance of single colonies, approx. after 8 days. Clones were then harvested in RCM, expanded in ECM and screened for specificity using the EAI assay. Hybridoma clones were then grown in RPMI (sigma) + 10 % FCS.

Purification of monoclonal antibody

Sequencing of the antibody expressed by a hybridoma cell line was performed by Absolute Antibody, UK.
signal peptide and VH of LS4G2:
ATGAAATGCAGCTGGGTCATCTTCTTCCTGATGGCAGTGGTTATAGGAATCAATTCAGAGGTTCA
GCTGCAGCAGTCTGGGGCAGAGCTTGTGAGGTCAGGGGCCTCAGTCAAGTTGTCCTGCACAGCTT
CTGGCTTCAACATTAAAGACTACTATATGGATTGGGTGAAGCAGAGGCCTGAACAGGGCCTGGAG
TGGATTGGATGGATTGATCCTGAGAATGGTGATAGTGAATATGCCCCGAAGTTCCAGGGCAAGGC
CACTATGACTGCAGACACATCCTCCAACACAGCCTACCTGCAGCTCAGCAGCCTGACATCTGAGG
ACACTGCCGTCTATTACTGTAATGCCGGACAGCTCGGGCTAGGAGCTTACTGGGGCCAAGGGACT
CTGGTCACTGTCTCTGCA

VL of LS4G2:
ATGTCAGTAGGACAGAAGGTCACTATGAGCTGCAAGTCCAGTCAGAGCCTTTTAAATAGTAGCAAT
CAAAAGAACTATTTGGCCTGGTACCAGCAGAAACCAGGACAGTCTCCTAAACTTCTGGTATACTT
TGCATCCACTAGGGAATCTGGGGTCCCTGATCGCTTCATAGGCAGTGGATCTGGGACAGATTTCA
CTCTTACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGATTACTTCTGTCAGCAACATTATAGC
ACTCCTCGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC

The antibody LS4G2 is of the IgG3 isotype with a κ light chain. The corresponding gene fragments were synthesized (Genscript, New Jersey, USA). For recombinant expression, rearranged V regions of LS4G2 were subcloned into the CMV-promoter-driven mammalian expression vector pXLG1.2, followed by the

Cγ2a constant domain (kindly provided by Prof. Shozo Izui, University of Geneva), corresponding to the Genbank sequences for mouse IgG2a (J00470.1). The procedure for the light chain was identical.
Antibodies were expressed by transient co-transfection of HEK and CHO cells (Protein Production and Structure Core Facility, EPFL, Lausanne, Switzerland) and purified on protein G columns (GE Healthcare, Illinois, USA) with ÄKTAprime plus (GE Healthcare, Illinois, USA) followed by PBS dialysis.
For conjugation of the purified antibody we used the lightning link labeling kit from Expedeon (Sygnis, Heidelberg, Germany) according to the manufacturer's protocol.

Immunoprecipitation

Bartonella taylorii was grown as described before. Bacteria were collected in wash buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 5% glycerol) containing benzoase and protease inhibitors and lyzed using French Press and centrifuged at 10.000 x g for 10 min at 4°C. The supernatant was centrifuged at 100.000 x g at 4°C to obtain the membrane protein fraction. The pellet was solubilized over night at 4°C with 1 % DDM in wash buffer and again centrifuged at 100.000 x g at 4°C. The supernatant was incubated for 3h at 4°C with antibody or isotype. 100 µl of UltraLink Resin (Thermo Fisher Scientific, Waltham, MA) was added for 2h at RT. The mixture was centrifuged at 6000 x g and the beads were washed 3 times with excess wash buffer before elution with 20 ul of Laemmli buffer.
After TCA precipitation, the samples were prepared for LC-MS/MS. Sample preparation, mass spectrometry and data analysis was performed as previously described for *B. henselae* [Québatte et al., 2013].

Data analysis

Statistical analysis of the obtained data was performed using GraphPad Prism Software. Unpaired student's t-test were used for the analysis of the gentamycin protection assay and growth analysis and two-way ANOVA was used for the comparison of bacteremia curves.

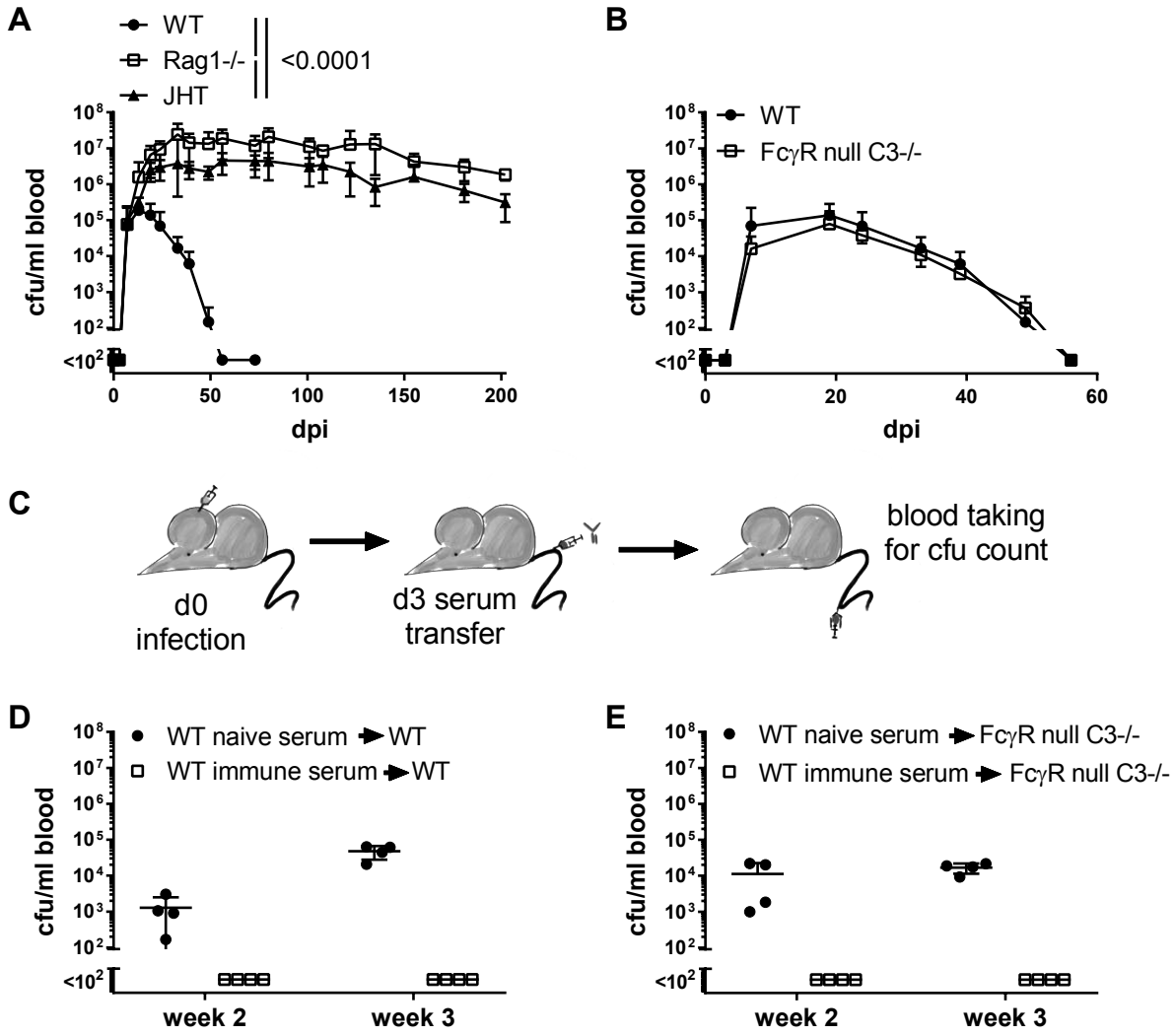

Figure 1: **Antibodies mediate clearance of *Bartonella* bacteremia.** Mice were infected with 10^7 cfu *B. taylorii i.d.*. The blood cfu count after infection is shown for A) C57Bl/6 wildtype (WT) vs. Rag1-/- and JHT and B) WT vs. FcγRnull C3-/- mice. C) Schematic overview of the experimental setup used in D and E: mice were infected on day 0, 100 µl naïve or immune serum raised against *B. taylorii* was transferred on day 3 and blood was drawn in the following days to determine the bacteremia. The blood cfu count after serum transfer is shown for WT (D) and FcγRnull C3-/- (E) recipients. Data was collected from at least 4 mice per group. The figures show representative data from at least two independent experiments. Statistical analysis was performed using two-way ANOVA and P-values are given for significant findings.

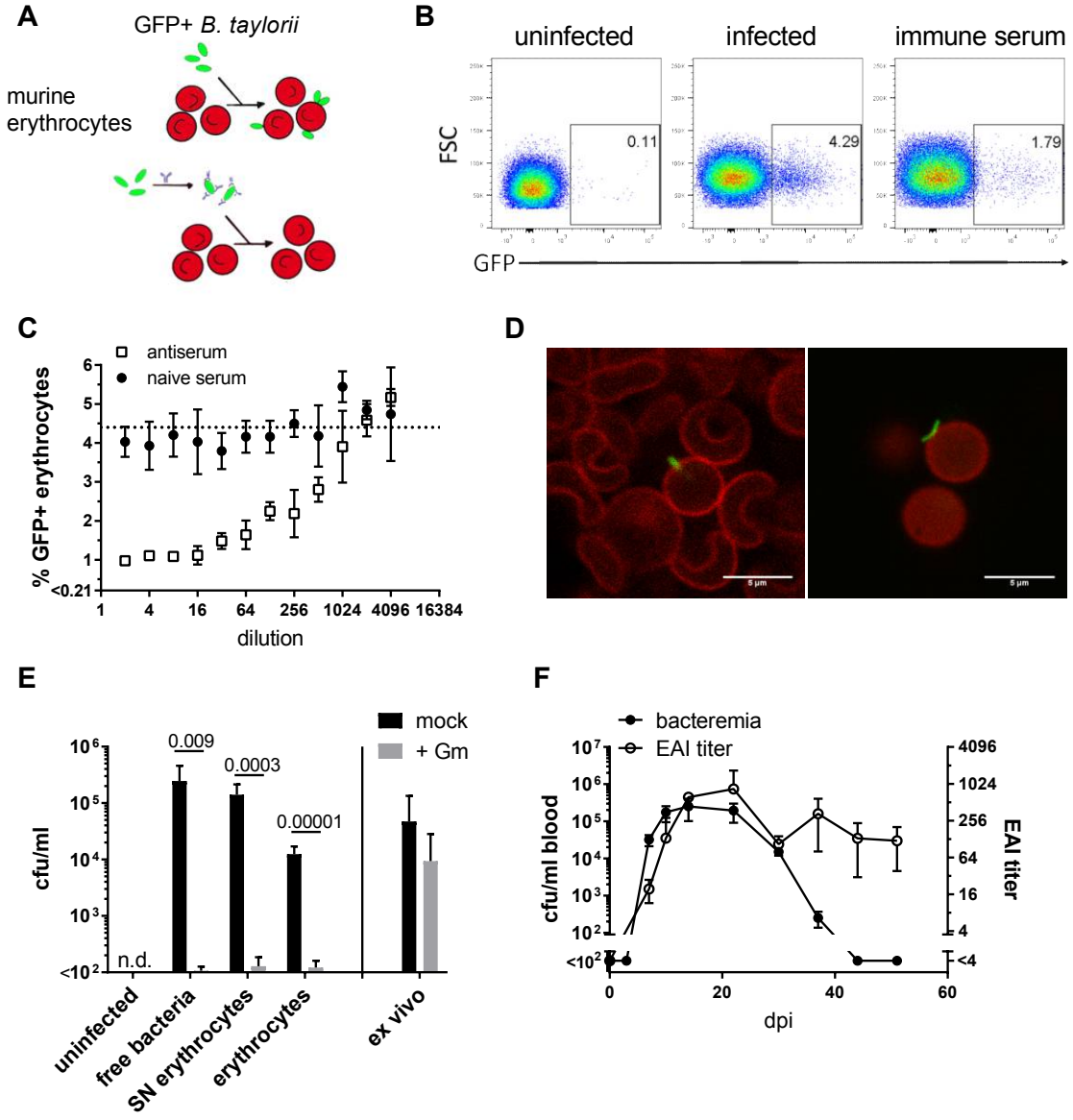

Figure 2: **Antibodies clear the bacteremia by RBC adhesion inhibition.** A) Schematic overview of the EAI assay. RBCs were infected with GFP+ *B. taylorii* (moi 0.1) preincubated with immune or naïve serum. The infection rate was quantified using flow cytometry. B) Exemplary FACS plots of infected erythrocytes from the EAI assay: uninfected and RBCs infected with free bacteria or those that have been preincubated with immune serum. C) EAI assay titration curve of naïve and immune serum. The mean of the no serum control is given as a dashed line. D) Confocal images of murine RBCs (stained with anti-Ter119) infected with GFP+ *B. taylorii* (moi 0.1). E) Gentamycin (Gm) protection assay of *in-vitro* infected RBCs (moi 0.1) compared to RBCs obtained from mice infected with 10^7 cfu *B. taylorii* 14 dpi (*ex vivo*). SN = RBC supernatant. F) Comparison between blood cfu count of WT C57Bl/6 mice after infection with 10^7 cfu *B. taylorii i.d.* with their measured EAI titer (right Y-axis). *In-vitro* data was collected in technical triplicates. *In vivo* experiments were performed using at least 3 mice per group. The figures show representative data from at least two independent experiments. Statistical analysis was performed using unpaired t-test and P-values are given for significant findings.

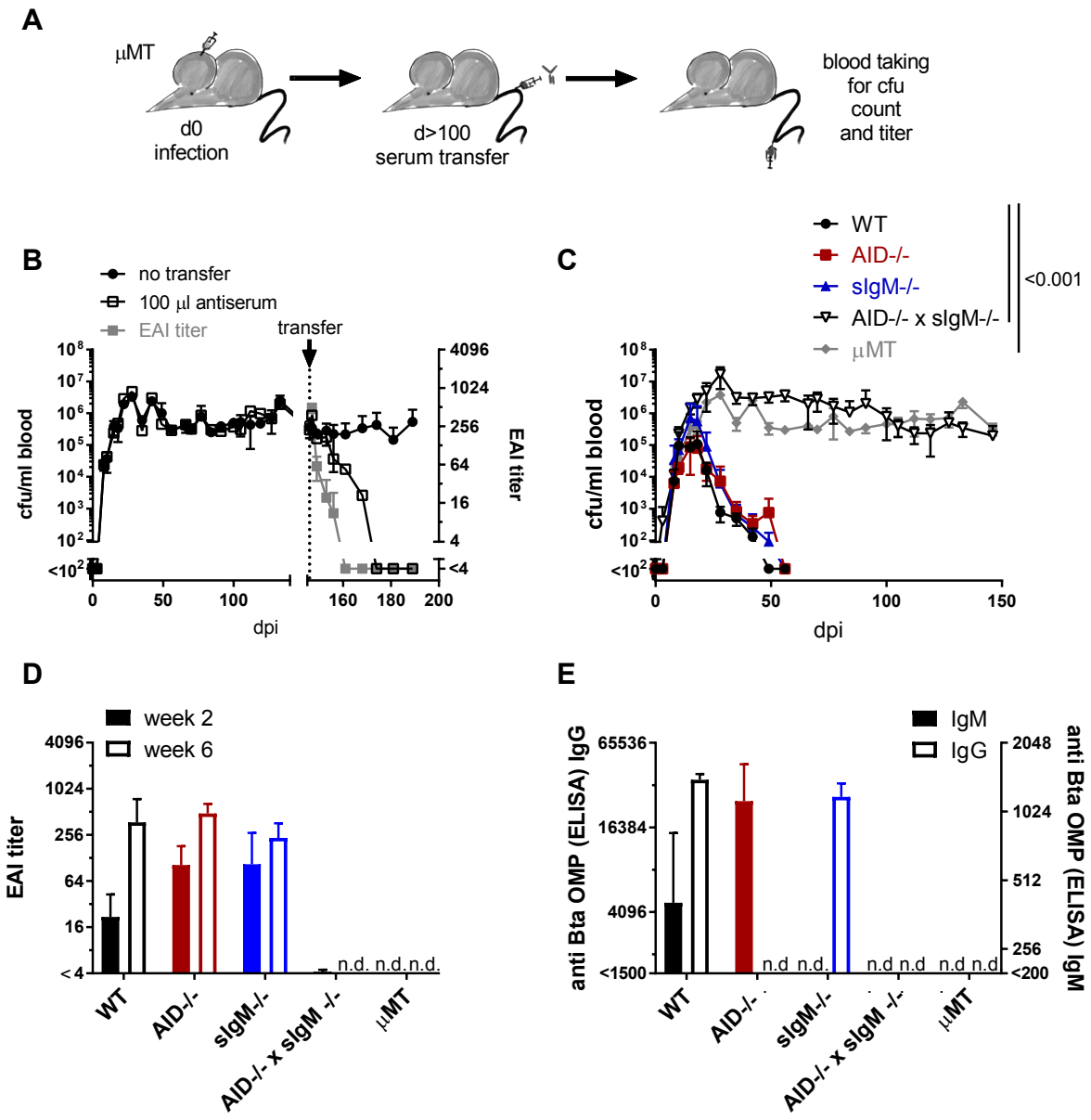

Figure 3: **Functionality of protective antibodies against *Bartonella* is Ig-type independent.** A) Schematic overview of the serum transfer experiment shown in B: µMT mice were infected with 10^7 cfu *B. taylorii i.d.*. After establishment of persistent bacteremia 100 µl of immune serum were injected *i.v.*. B) Blood cfu count (left Y-axis) of µMT mice before and after serum transfer on day 146 post infection. EAI titers (right Y-axis) after transfer are shown for mice which obtained serum. C) C57Bl/6 WT mice were compared with AID-/-, sIgM-/-, AID x sIgM-/- and µMT mice for blood cfu counts after infection with 10^7 cfu *B. taylorii i.d.* D) EAI titers weeks 2 and 6 post infection for A. E) IgM (right Y-axis) and IgG (left Y-axis) titers against *B. taylorii* outer membrane proteins (OMPs) measured by ELISA 6 weeks post infection for A. Data was collected from at least 4 mice per group. The figures show representative data from at least two independent experiments. Statistical analysis was performed using two-way ANOVA and P-values are given for significant findings.

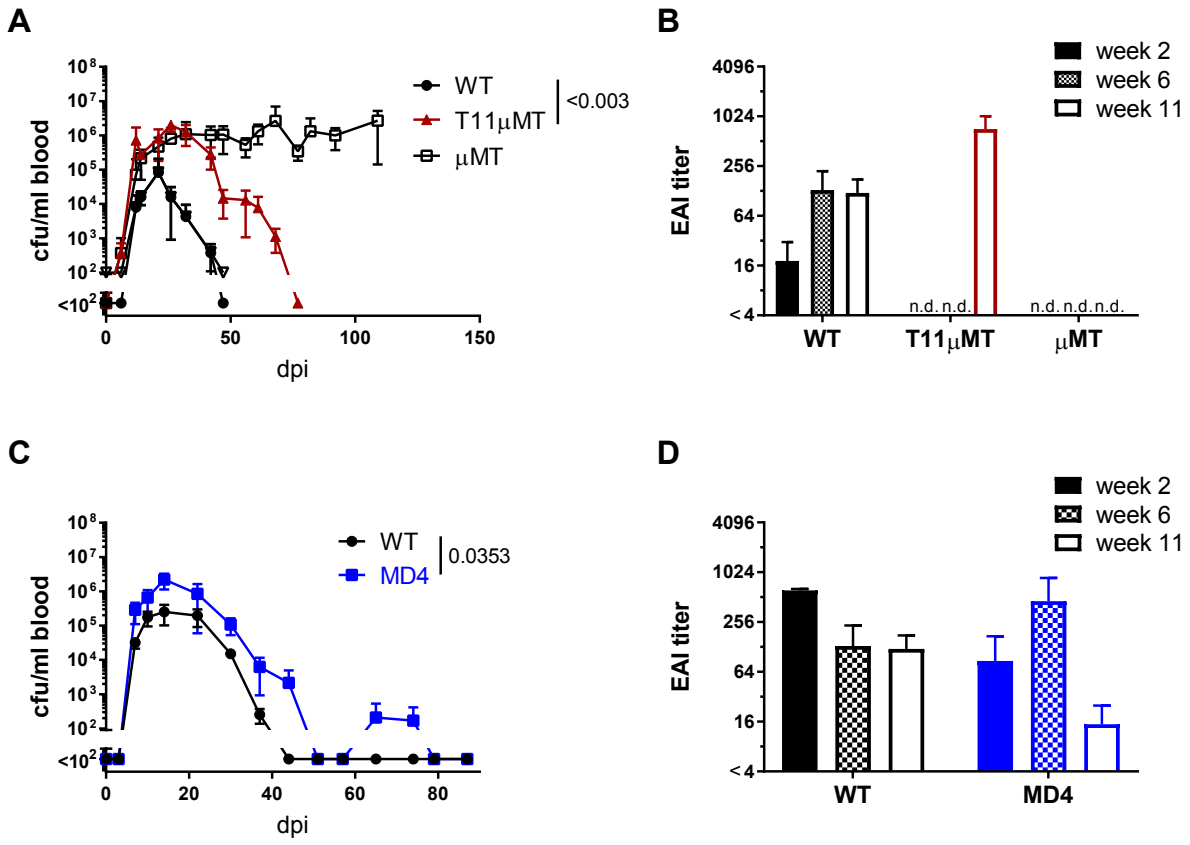

Figure 4: **Functionality of protective antibodies against *Bartonella* is specificity dependent.** Mice were infected with 10^7 cfu *B. taylorii i.d.* A and C) Blood cfu count for WT mice compared to T11μMT and μMT (A) or MD4 (B) mice. EAI titers for weeks 2, 6 and 11 post infection are shown respectively in C and D. Data was collected from at least 4 mice per group. The figures show representative data from at least two independent experiments. Statistical analysis was performed using two-way ANOVA and P-values are given for significant findings.

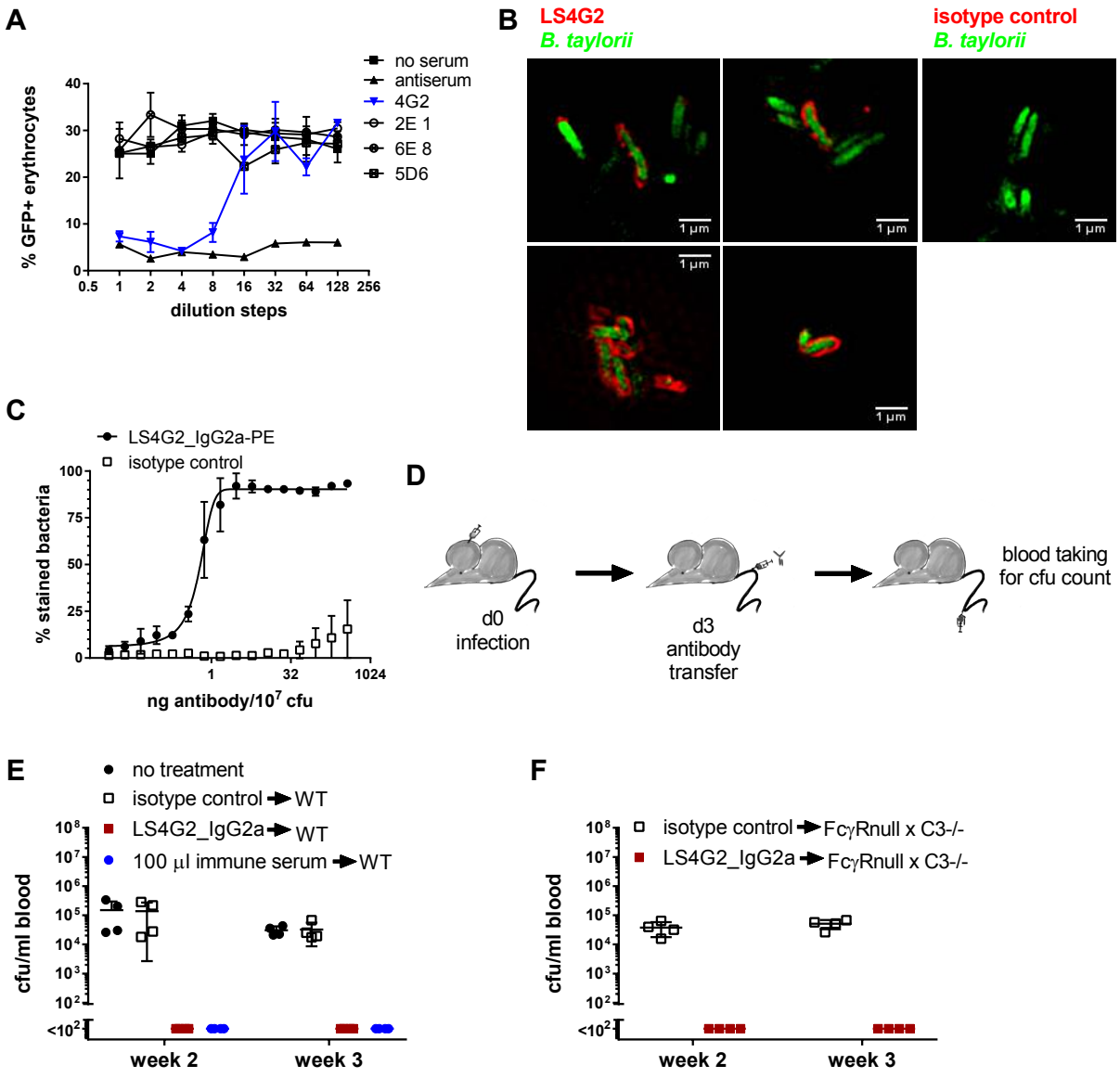

Figure 5: **A monoclonal neutralizing antibody is protective *in vivo*.** A) Titration curve of the hybridoma supernatant LS4G2 in EAI assay compared to other - negative - hybridoma clones. B) Structured illumination microscopy of GFP expressing *B. taylorii* (green) stained with LS4G2 (left pictures, red) compared to isotype control (right picture). C) Titration curve of binding of purified LS4G2 (IgG2a) conjugated to PE to live *B. taylorii* compared to isotype control, quantified by flow cytometry. D) Schematic overview of the experiments shown in E and F: Mice were infected with 10^7 cfu *B. taylorii i.d.* on d0. 250 µg purified LS4G2 or an isotype control was transferred on day 3 post infection. E) Blood cfu count after transfer of 250 µg purified LS4G2 or an isotype control compared to 100 µl immune serum into WT C57Bl/6 mice. F) Blood cfu count after transfer of 250 µg purified LS4G2 or an isotype control into FcγR null x C3-/- mice. *In-vitro* data was collected in technical triplicates. *In vivo* data was collected from at least 4 mice per group. The figures show representative data from at least two independent experiments.

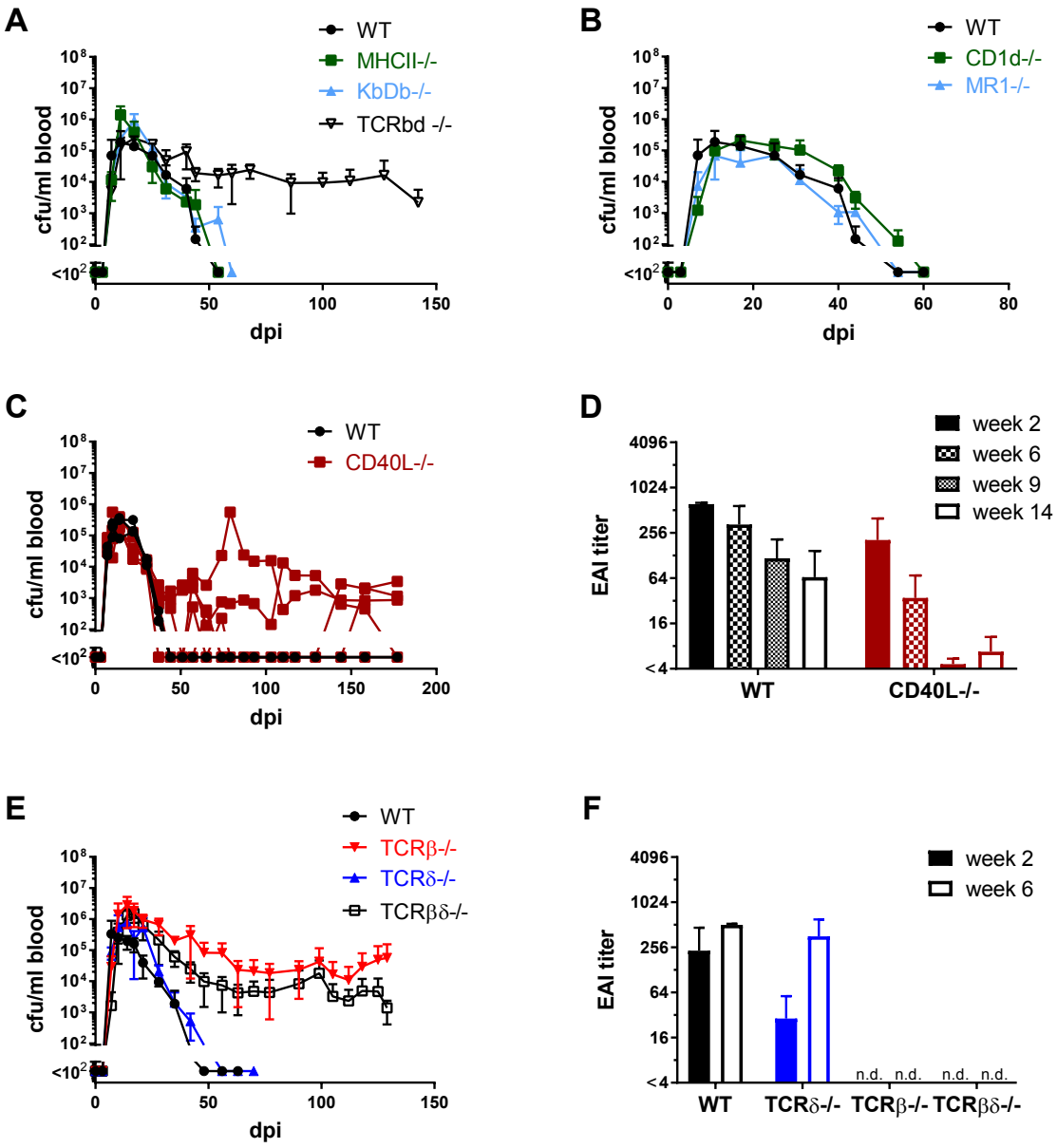

Figure 6: **T-help is important for the formation of protective antibodies clearance of *Bartonella* bacteremia but there is high redundancy in the involved cell types.** Mice were infected with 10^7 cfu *B. taylorii i.d.*. Blood cfu counts are shown for C57Bl/6 (WT) mice compared to MHCII-/-, K^bD^b-/- and TCRβδ (A), C57Bl/6 mice compared to CD1d-/- and MR1-/- (B), C57Bl/6 mice compared to CD40L-/- (C) and C57Bl/6 mice compared to TCRβ, TCRδ and TCRβδ (E). EAI titers on different time points post infection are shown for C57Bl/6 mice compared to CD40L-/- (D) and C57Bl/6 mice compared to TCRβ-/-, TCRδ-/-, and TCRβδ-/- (F). Data was collected from at least 4 mice per group. The figures show representative data from at least two independent experiments. Statistical analysis was performed using two-way ANOVA and P-values are given for significant findings. C shows data for single mice.

Supplementary information

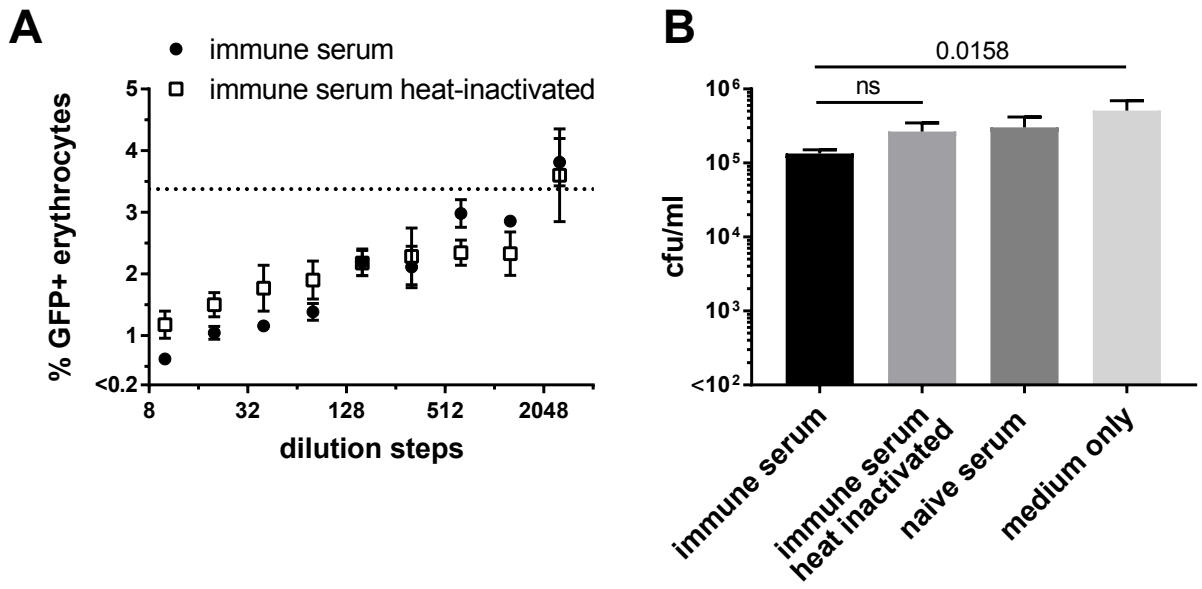

Figure S2: **Functionality of EAI assay is due to neutralization, not complement in serum or bactericidal effects.** A) Effect of immune serum before and after heat-inactivation on the inhibition of erythrocyte adhesion of GFP+ *B. taylorii*. The mean of the no serum control is given as a dashed line. B) Growth analysis of *B. taylorii* during the conditions of the EAI assay. Cfu count shown for bacteria grown in the presence of immune serum (normal and heat-inactivated), naive serum and in medium only. All data obtained in technical triplicates. The figures show representative data from at least three independent experiments. The results were analyzed using unpaired t-tests and P-values are given for significant findings.

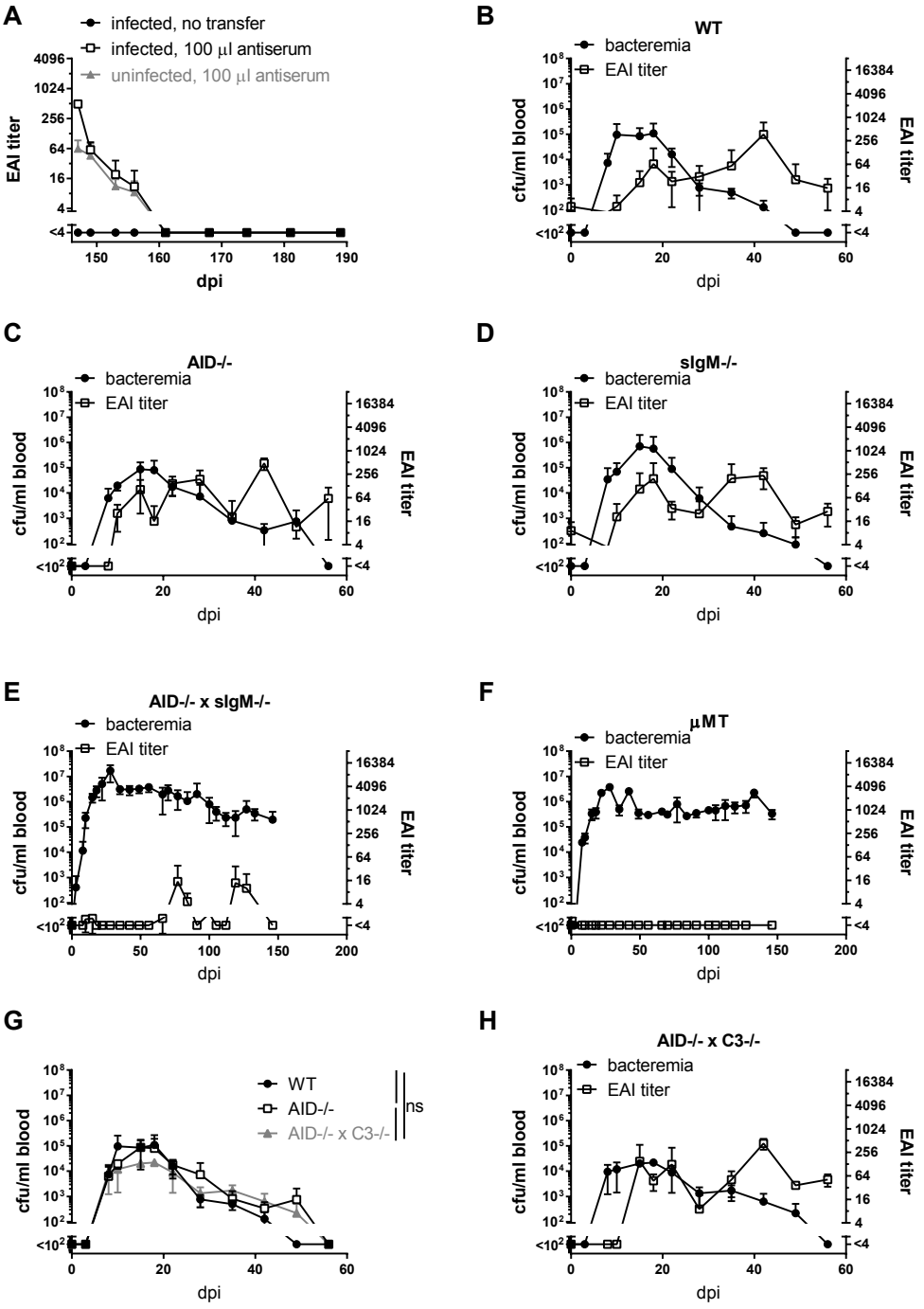

Figure S3: **Observation of the EAI titers in different mouse models over time.** A) Comparison of measured EAI titers after serum transfer into infected and uninfected µMT mice. B-F) For the experiments shown in figure 3B-D blood cfu counts (left Y-axis) compared to obtained EAI titers (right Y-axis) are shown for WT C57Bl/6 (B), AID-/- (C), sIgM-/- (D), AID x sIgM-/- (E), µMT and (F). G and H) AID x C3-/- were infected with 10^7 cfu *B. taylorii i.d.*. Blood cfu counts are shown in comparison to WT and AID-/- mice (G). Blood cfu counts (left Y-axis) in comparison to EAI titers (right Y-axis) are shown for AID -/- x C3 -/- in H. Data was collected from at least 4 mice per group. The figures show representative data from at least two independent experiments.

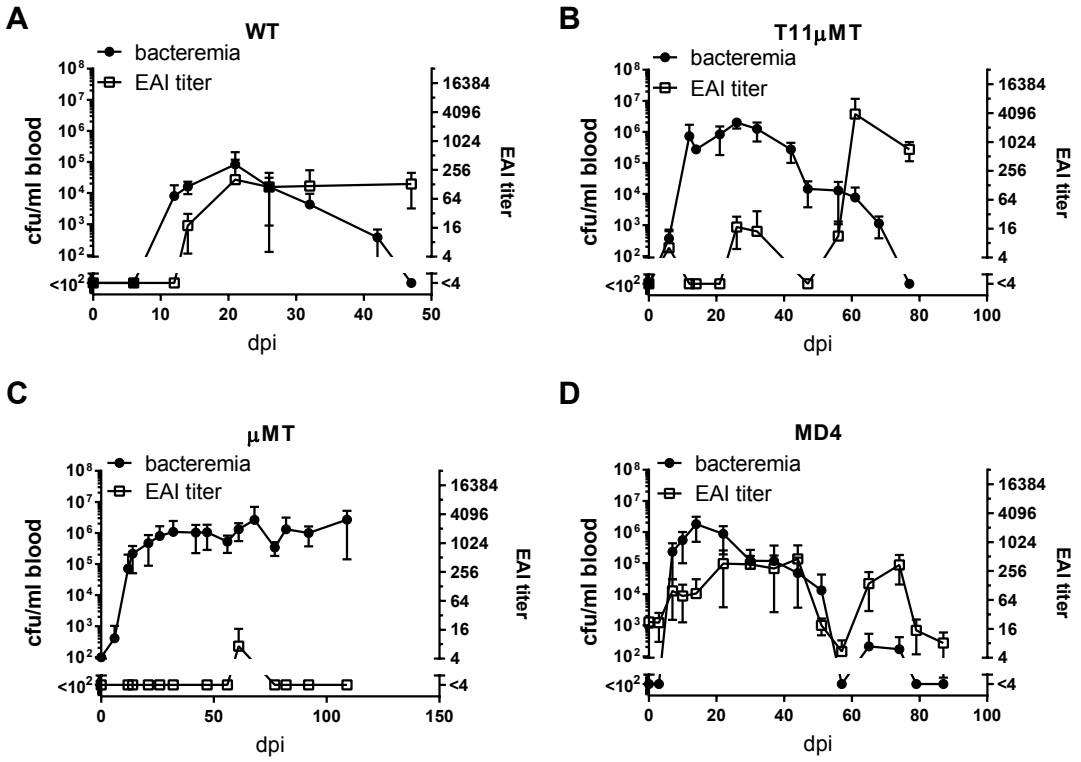

Figure S4: **Observation of EAI titers in mice with reduced antibody repertoire over time.** Blood cfu counts (left Y-axis) compared to measured EAI titers (right Y-axis) over time for the experiments shown in figure 4: WT (A), T11μMT (B), μMT (C) and MD4 (D) mice. Data was obtained from at least 4 mice per group. The figure shows representative data from at least two independent experiments.

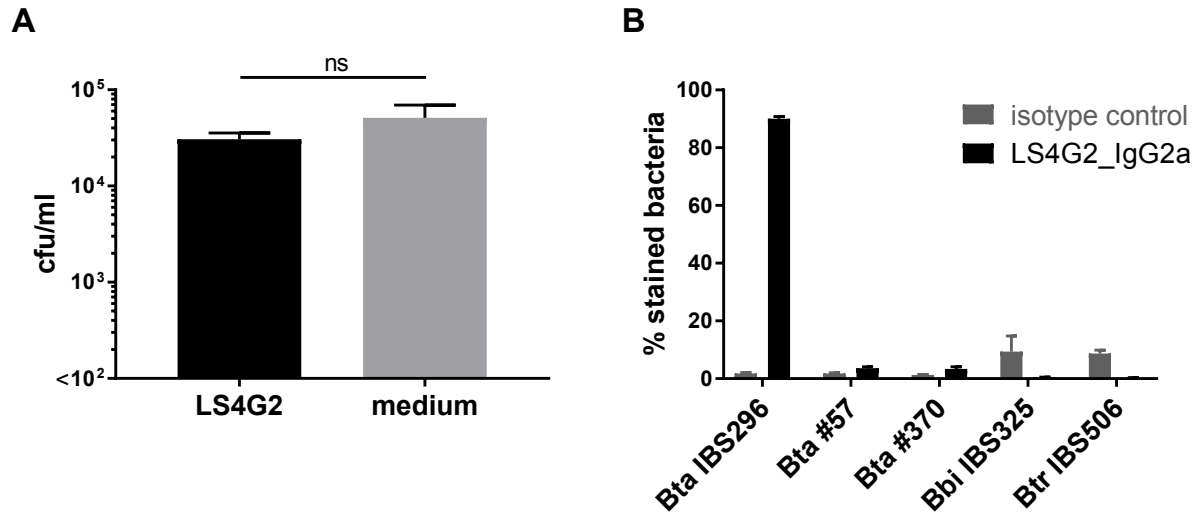

Figure S5: **Monoclonal neutralizing antibody LS4G2 is not bactericidal and highly specific.** A) Growth analysis of *B. taylorii* during the conditions of the EAI assay. Cfu count shown for bacteria incubated in the presence of LS4G2 or only in medium. B) Surface staining of *B. taylorii* (Bta) IBS296 compared to *B. taylorii* isolates #57 and #370 and to *B. birtlesii* (Bbi) IBS325 and *B. tribocorum* (Btrb) IBS506 by LS4G2-PE, quantified by Flow Cytometry. All data obtained in technical triplicates. The figure shows representative data from at least two independent experiments. The results were analyzed using unpaired t-tests and P-values are given for significant findings.

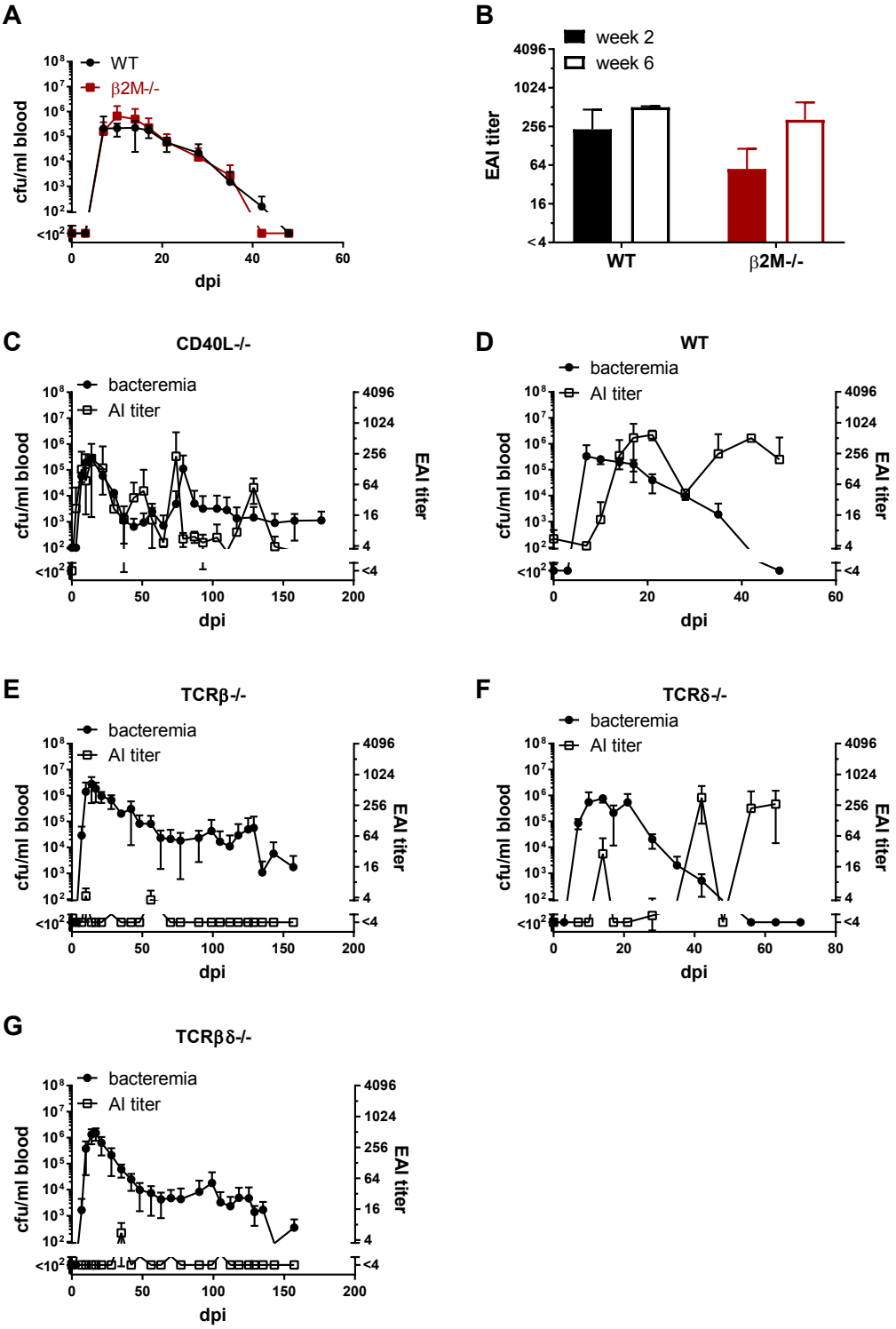

Figure S6: **Clearance of the bacteremia correlates with a measurable antibody titer in different T-cell deficient mice**. Mice were infected with 10^7 cfu *B. taylorii i.d.*. Blood cfu count (A) and EAI titer (B) is shown for WT C57Bl/6 compared to β2M-/- mice. Comparison between the course of bacteremia and obtained EAI titers for the experiments shown in figure 6 are given for CD40L-/- (C), WT C57Bl/6 (D), TCRβ-/- (E), TCRδ-/- (F) and TCRβδ-/- (G). Data was collected from at least 4 mice per group. The figures show representative data from at least two independent experiments.

Protein	Accession Number	Molecular Weight	coverage		# peptides	
			isotype	LS4G2	isotype	LS4G2
outer membrane autotransporter barrel domain-containing protein [Bartonella taylorii]	OPB34894.1	75 kDa	5,00%	68%	3	96
hypothetical protein Btaycd_012310, partial [Bartonella taylorii]	OPB34623.1	45 kDa		46%		24
membrane protease FtsH catalytic subunit [Bartonella taylorii]	OPB35138.1	79 kDa	2,60%	27%	2	19
outer membrane autotransporter barrel domain-containing protein [Bartonella taylorii]	OPB35004.1	84 kDa	0,00%	24%	0	14
serine protease Do [Bartonella taylorii]	OPB35116.1	54 kDa		15%		8
outer membrane autotransporter barrel domain-containing protein [Bartonella taylorii]	OPB34803.1	118 kDa		10%		9
signal peptide peptidase SppA [Bartonella taylorii]	OPB35302.1	24 kDa	4,60%	28%	1	7
Do/DeqQ family serine protease [Bartonella taylorii]	OPB35259.1	50 kDa		14%		6
amino acid ABC transporter ATP-binding protein, PAAT family [Bartonella taylorii]	OPB34936.1	28 kDa		31%		9
HemY protein [Bartonella taylorii]	OPB35802.1	58 kDa		14%		6
Outer membrane protein beta-barrel domain-containing protein [Bartonella taylorii]	OPB34763.1	34 kDa	3,50%	15%	1	4
Protein of unknown function (DUF1460) [Bartonella taylorii]	OPB34828.1	31 kDa	0,00%	7,40%	0	2
peptide/nickel transport system ATP-binding protein [Bartonella taylorii]	OPB35089.1	30 kDa	0,00%	13%	0	3
NADH dehydrogenase subunit G [Bartonella taylorii]	OPB35491.1	75 kDa	1,20%	2,50%	1	2
hypothetical protein Btaycd_006300 [Bartonella taylorii]	OPB35316.1	63 kDa		5,10%		2
signal recognition particle-docking protein FtsY [Bartonella taylorii]	OPB35683.1	45 kDa	2,00%	4,10%	1	2
signal peptidase I Serine peptidase, MEROPS family S26A [Bartonella taylorii]	OPB35541.1	32 kDa	0,00%	11%	0	3
Tetratricopeptide repeat-containing protein, partial [Bartonella taylorii]	OPB35140.1	40 kDa		5,80%		3
peroxiredoxin Q/BCP [Bartonella taylorii]	OPB35019.1	17 kDa		19%		3
acetyl-CoA carboxylase carboxyltransferase subunit alpha [Bartonella taylorii]	OPB35437.1	34 kDa		6,50%		2
hypothetical protein Btaycd_011900, partial [Bartonella taylorii]	OPB34758.1	19 kDa		27%		4
carbohydrate ABC transporter ATP-binding protein, CUT1 family [Bartonella taylorii]	OPB35621.1	40 kDa		16%		4
peptide/nickel transport system ATP-binding protein [Bartonella taylorii]	OPB35090.1	32 kDa		7,60%		2
protein translocase subunit secF /protein translocase subunit secD [Bartonella taylorii]	OPB35072.1	92 kDa	38%	37%		2
SSU ribosomal protein S4P [Bartonella taylorii]	OPB35501.1	24 kDa	63%	76%	1	2
Porin subfamily protein [Bartonella taylorii]	OPB35613.1	44 kDa	43%	51%	30	34
SSU ribosomal protein S7P [Bartonella taylorii]	OPB34930.1	18 kDa	57%	65%	14	26
LSU ribosomal protein L3P [Bartonella taylorii]	OPB35285.1	26 kDa	60%	63%	20	21
protein of unknown function (DUF3472) [Bartonella taylorii]	OPB34226.1	38 kDa	45%	42%	10	17
SSU ribosomal protein S19P [Bartonella taylorii]	OPB35281.1	10 kDa	59%	67%	15	17
protease FtsH subunit HflK [Bartonella taylorii]	OPB35737.1	42 kDa	35%	34%	15	14
SSU ribosomal protein S16P [Bartonella taylorii]	OPB35795.1	13 kDa	49%	59%	12	15
protease FtsH subunit HflC [Bartonella taylorii]	OPB35736.1	36 kDa	21%	27%	13	15
bacterial translation initiation factor 3 (bIF-3) [Bartonella taylorii]	OPB35206.1	15 kDa	39%	37%	11	18
LSU ribosomal protein L20P [Bartonella taylorii]	OPB35903.1	15 kDa	53%	53%	8	10
chaperonin GroEL [Bartonella taylorii]	OPB34236.1	58 kDa	18%	24%	7	15
SSU ribosomal protein S3P [Bartonella taylorii]	OPB35279.1	27 kDa	19%	43%	12	11
LSU ribosomal protein L4P [Bartonella taylorii]	OPB35284.1	23 kDa	31%	53%	9	12
LSU ribosomal protein L15P [Bartonella taylorii]	OPB35266.1	17 kDa	41%	46%	4	15
LSU ribosomal protein L17P [Bartonella taylorii]	OPB35260.1	16 kDa	51%	52%	7	11
SSU ribosomal protein S13P [Bartonella taylorii]	OPB35263.1	14 kDa	60%	60%	7	10
ETC complex I subunit conserved region [Bartonella taylorii]	OPB35559.1	12 kDa	43%	58%	8	11
LSU ribosomal protein L13P [Bartonella taylorii]	OPB34909.1	17 kDa	58%	58%	10	9
SSU ribosomal protein S9P [Bartonella taylorii]	OPB34910.1	18 kDa	43%	49%	3	13
F-type H+-transporting ATPase subunit beta [Bartonella taylorii]	OPB35101.1	57 kDa	8,40%	34%	10	9
Beta-barrel assembly machine subunit BamD [Bartonella taylorii]	OPB35754.1	30 kDa	20%	15%	6	8
SSU ribosomal protein S2P [Bartonella taylorii]	OPB35823.1	28 kDa	8,60%	26%	3	12
translation elongation factor 1A (EF-1A/EF-Tu) [Bartonella taylorii]	OPB35842.1	43 kDa		35%	5	4
outer membrane immunogenic protein [Bartonella taylorii]	OPB34085.1	30 kDa	18%	22%	2	8
SSU ribosomal protein S15P [Bartonella taylorii]	OPB35710.1	10 kDa	42%	42%		13
LSU ribosomal protein L19P [Bartonella taylorii]	OPB35776.1	16 kDa	34%	51%	6	7
ATP synthase F1 subcomplex alpha subunit [Bartonella taylorii]	OPB35103.1	56 kDa	7,80%	12%	4	6
SSU ribosomal protein S5P [Bartonella taylorii]	OPB35268.1	20 kDa	32%	24%	5	9
LSU ribosomal protein L28P [Bartonella taylorii]	OPB35786.1	11 kDa	51%	58%	4	6
hypothetical protein Btaycd_003160 [Bartonella taylorii]	OPB35594.1	30 kDa	15%	30%	7	6
membrane fusion protein, multidrug efflux system [Bartonella taylorii]	OPB35126.1	40 kDa	6,00%	12%	4	6
protein translocase subunit yajC [Bartonella taylorii]	OPB35047.1	16 kDa	16%	43%	3	7
Metal-dependent hydrolase, endonuclease/exonuclease/phosphatase family [Bartonella taylo	OPB35173.1	33 kDa	13%	14%	2	4
SH3 domain-containing protein [Bartonella taylorii]	OPB34921.1	23 kDa	22%	9,10%	3	7
LSU ribosomal protein L29P [Bartonella taylorii]	OPB35277.1	8 kDa	20%	20%	4	3
Beta-barrel assembly machine subunit BamA [Bartonella taylorii]	OPB34973.1	89 kDa	1,00%	7,50%	5	2
hypothetical protein Btaycd_010000 [Bartonella taylorii]	OPB34941.1	24 kDa	17%	21%	3	4
SSU ribosomal protein S17P [Bartonella taylorii]	OPB35276.1	9 kDa	42%	42%	1	6
sodium/proton antiporter, NhaA family [Bartonella taylorii]	OPB35317.1	49 kDa	4,20%	6,60%	5	4
SSU ribosomal protein S18P [Bartonella taylorii]	OPB35471.1	10 kDa	45%	45%	4	5
LSU ribosomal protein L24P [Bartonella taylorii]	OPB35274.1	11 kDa	32%	32%	2	5
LSU ribosomal protein L27P [Bartonella taylorii]	OPB35108.1	10 kDa	25%	25%	4	4
hypothetical protein Btaycd_010010 [Bartonella taylorii]	OPB34942.1	20 kDa	14%	18%	4	3
SSU ribosomal protein S20P [Bartonella taylorii]	OPB35872.1	10 kDa	13%	20%	3	3
bacterial translation initiation factor 2 (bIF-2) [Bartonella taylorii]	OPB35706.1	93 kDa	3,30%	2,60%	2	4
LSU ribosomal protein L14P [Bartonella taylorii]	OPB35275.1	13 kDa	26%	27%	1	2
SSU ribosomal protein S11P [Bartonella taylorii]	OPB35262.1	14 kDa	20%	37%	2	2
SSU ribosomal protein S21P [Bartonella taylorii]	OPB35242.1	9 kDa	22%	31%	3	4
LSU ribosomal protein L21P [Bartonella taylorii]	OPB35109.1	18 kDa	19%	15%	2	5
SSU ribosomal protein S12P [Bartonella taylorii]	OPB34931.1	14 kDa	22%	14%	2	3
3',5'-cyclic AMP phosphodiesterase CpdA [Bartonella taylorii]	OPB35238.1	34 kDa	22%	2,70%	4	3
transcription termination factor Rho [Bartonella taylorii]	OPB35692.1	47 kDa		9,00%	3	2
hypothetical protein Btaycd_011810 [Bartonella taylorii]	OPB34768.1	27 kDa	15%	15%	6	1
LSU ribosomal protein L16P [Bartonella taylorii]	OPB35278.1	16 kDa	26%	26%		4
LSU ribosomal protein L22P [Bartonella taylorii]	OPB35280.1	14 kDa	16%	22%	3	3
LSU ribosomal protein L35P [Bartonella taylorii]	OPB35904.1	8 kDa	33%	33%	3	3
Endonuclease YncB, thermonuclease family [Bartonella taylorii]	OPB33638.1	21 kDa	6,00%	27%	2	4

Multidrug resistance efflux pump [Bartonella taylorii]	OPB35098.1	37 kDa	9,00%	3,90%	3	2
protein translocase subunit secY/sec61 alpha [Bartonella taylorii]	OPB35265.1	49 kDa	3,80%	5,40%	1	2
hypothetical protein Btaycd_009990 [Bartonella taylorii]	OPB34940.1	20 kDa	15%	6,70%	3	1
LSU ribosomal protein L2P [Bartonella taylorii]	OPB35282.1	30 kDa	9,40%	12%	2	2
membrane fusion protein, macrolide-specific efflux system [Bartonella taylorii]	OPB35219.1	45 kDa	0,00%	10%	2	2
single-strand binding protein [Bartonella taylorii]	OPB35326.1	19 kDa	29%		0	3
Conjugal transfer protein TrbH [Bartonella taylorii]	OPB34753.1	15 kDa	14%	18%	4	
LSU ribosomal protein L6P [Bartonella taylorii]	OPB35270.1	20 kDa		18%	2	2
tRNA(Ile)-lysidine synthase [Bartonella taylorii]	OPB35139.1	57 kDa	3,40%		4	
protein translocase subunit secG [Bartonella taylorii]	OPB33915.1	16 kDa	17%	25%	1	2
LSU ribosomal protein L30P [Bartonella taylorii]	OPB35267.1	8 kDa	13%	31%	1	2
MFS transporter, MHS family, alpha-ketoglutaratepermease [Bartonella taylorii]	OPB35740.1	48 kDa	2,80%	4,40%	1	2
hypothetical protein Btaycd_009980 [Bartonella taylorii]	OPB34939.1	9 kDa	12%	23%	1	2
Beta-barrel assembly machine subunit BamE [Bartonella taylorii]	OPB35200.1	17 kDa	12%	6,50%	2	1
Global cell cycle regulator GcrA [Bartonella taylorii]	OPB35879.1	19 kDa	5,10%	13%	1	2
SSU ribosomal protein S1P [Bartonella taylorii]	OPB35893.1	63 kDa		3,90%		2
LSU ribosomal protein L25P [Bartonella taylorii]	OPB35164.1	23 kDa		14%		2

table S1: **mass spectrometry analysis of LS4G2 immunoprecipitation.**

Overview of all found proteins in eluted fraction from IP with LS4G2 or isotype control. FDR 0.1.

3.1.2 Additional experiments: The antibody response against *B. birtlesii*

3.1.2.1 Results and discussion

Some of the mouse infections with *B. taylorii* reported in the manuscript (3.1.1.2) have been additionally performed with a second strain of lineage 4, *B. birtlesii*. *B. birtlesii* is also naturally found in mice. The data is, however, less robust and the strain repeatedly showed growth problems. Thus, quantification of cfu was sometimes problematic. We decided to continue the experiments only with *B. taylorii*.
In general, the findings with *B. birtlesii* are very similar to the ones reported with *B. taylorii*, some phenotypes are, however, less pronounced. C57Bl/6 WT animals stayed sterile for 5-7 days after *intra-dermal* inoculation with *B. birtlesii* and cleared within 30-40 days (figure 3.1A). The peak of the bacteremia was with approximately 10^4 cfu per ml of blood lower than for *B. taylorii*. Mice lacking the adaptive immune system or only B-cells, namely Rag1-/- and JHT animals, showed a persistent bacteremia after infection with *B. birtlesii* (figure 3.1A), comparable to *B. taylorii* (see 3.1.1.2) or *B. grahamii* [134]. The 10-fold differences observed between the different models after *B. taylorii* infection were not observed with *B. birtlesii*. Infection of FcγRnull x C3-/- (figure 3.1B), AID-/- and sIgM-/- animals (figure 3.1C) with *B. birtlesii* revealed that also in this model, the mutant mouse strains cleared the bacteremia independently of the presence of complement or Fc-receptors and independently of the nature of the antibody type (IgM or IgG). However, for *B. birtlesii* there might have been a very mild prolongation of 1 week in the clearance of the bacteremia in FcγRnull x C3-/- (figure 3.1B) and AID-/- (figure 3.1C) mice compared to WT animals - a finding that has not been observed for *B. taylorii*. If this is biologically significant, however, is questionable.
In order to investigate the role of the T-cell response, MHCII-/-, K^bD^b-/- and TCR$\beta\delta$-/- mice were also infected with *B. birtlesii*. Again, similar findings were observed compared to the same mouse strains infected with *B. taylorii* (see 3.1.1.2). MHCII-/- and K^bD^b-/- animals cleared comparable to WT animals, whereas TCR$\beta\delta$-/- mice showed persistent bacteremia (figure 3.1D).
These findings observed from *B. birtlesii* infections lead to the same conclusion as the findings from *B. taylorii* infections. *Bartonella* bacteremia is cleared by neutralizing antibodies, independent of the dominant antibody Ig type and independent of the presence of complement or Fc-receptors. In mice without a T-cell compartment, the bacteremia persists, in mouse strains lacking either classical T-help or T-killing, the bacteremia is cleared. In general, these findings promote the idea that the observations for *B. taylorii* are not strain- or species-specific effects but show general mechanisms for the whole genus *Bartonella*.

3.1.2.2 Material and methods

Cultivation of bacteria *B. birtlesii* IBS325 LSB002 [281] was grown at 35°C, 5 % CO_2 on Tryptic soy agar (TSA) containing 5 % defibrinated sheep blood. Bacteria were streaked 5 and expanded 2 days prior to usage.

Animal experimentation
Animal handling was performed in accordance with the Swiss Animal Protection law and local animal welfare bodies. The animal experiments in this work were approved by the Veterinary Office of the Canton Basel-Stadt (License number 1741).

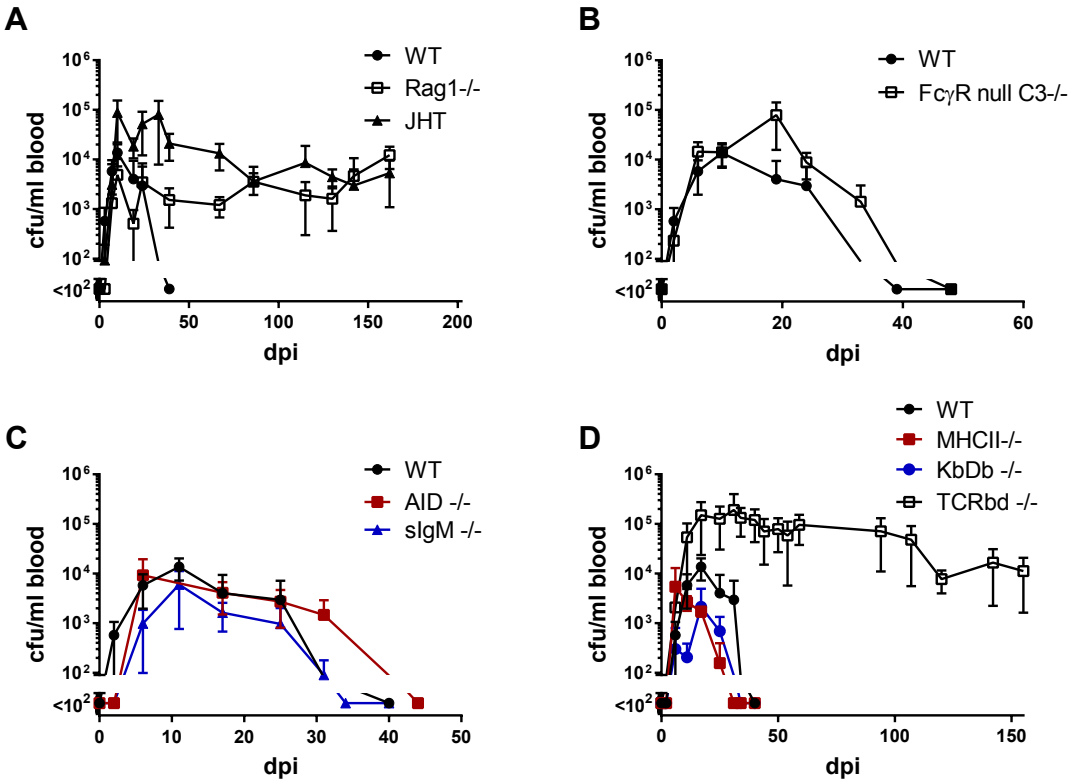

Figure 3.1: **Neutralizing antibodies also clear *B. birtlesii* bacteremia.** Mice were infected with 10^7 cfu *B. birtlesii i.d.*. Blood cfu counts are shown for A) WT C57Bl/6 vs. Rag1-/- and JHT , B) WT vs. FcγRnull C3-/- C) WT vs AID-/- and sIgM-/- and D) WT vs MHCII-/-, K^bD^b-/- and TCRβδ-/- mice. Data was collected with at least 3 mice per group. The figures show representative data from at least two independent experiments.

All animals were kept at SPF conditions. C57/BL6 WT animals were obtained from Janvier labs, Le Genest-Saint-Isle, France. The homozygous knock-out strains AID-/- [36], JHT [282], K^bD^b-/- [283], MHCII-/- [284], Rag1-/- [285] and TCR$\beta\delta$-/- [286] were bred at the Laboratory Animal Science Center (LASC, University of Zurich, Switzerland). FcγR4alpha x C3 -/- (Mehmet Sahin, manuscript in preparation) were bred at the transgenic mouse core facility (TCMF, University of Basel, Switzerland).
Animals were infected *i.d.* with 10^7 cfu bacteria in PBS. Blood was drawn in 3 $\%$ sodium citrate on several days post infection. For blood cfu count, whole blood was frozen at -80°C, thawed and plated in a limited dilution series on blood agar (see above).

3.2 Additional preliminary results concerning the *in-vitro* erythrocyte adhesion assay

3.2.1 Summary

The establishment of the erythrocyte adhesion inhibition assay allowed the exploration of additional questions. First we analyzed if the assay is specific for anti-*B. taylorii* immune serum or if there would be an effect of an irrelevant serum. Second, we wondered if the assay could potentially be transferred to a more clinical set up using *B. quintana* and human erythrocyte infection.
An immune serum raised against a different murine *Bartonella* strain did not have an effect on the erythrocyte adhesion by *B. taylorii*. *B. quintana* appeared to adhere to human erythrocytes in similar settings, however in lower frequencies than *B. taylorii*. In principle, the assay might be also used for *B. quintana* and seems to be highly specific for evaluating the functionality of a serum.
When we were still looking for the target of LS4G2, the monoclonal antibody neutralizing *B. taylorii*, we hypothesized that a protein of the Trw T4SS, described to be important for RBC adhesion [140], might be a potential target. Later findings contradicted this hypothesis revealing a putative autotransporter as the target (3.1.1.2). Nevertheless, the Trw expression during different growth conditions was investigated. Mutants lacking TrwK, an integral protein of the machinery, were constructed. They appeared, however, to still adhere to erythrocytes.
The results described in the following part are of preliminary nature.

3.2.2 Results and discussion

The erythrocyte adhesion inhibition assay is specific for the strain-immune serum combination used
Anti-*B. taylorii* immune serum, in opposite to naïve serum, was able to inhibit erythrocyte adhesion during *in-vitro* infection (3.1.1.2). In order to assess the specificity of the assay, we used an immune serum raised against the mouse-specific species *B. birtlesii*. Preincubation of GFP-expressing *B. taylorii* with specific immune serum lead to reduction in the occurrence of GFP+ erythrocytes while the serum against *B. birtlesii* had no effect (figure 3.2A). Thus, the established assay indeed measures only the effect of a specific antibody response.

Adhesion of *B. quintana* to human erythrocytes is assessable by flow cytometry
When establishing the EAI assay, we learned that we would obtain a patient isolate and serum collection from our collaborator, Prof. Jane E. Koehler. To investigate if our assay conditions would allow observation of attachment of *B. quintana* to human erythrocytes and maybe later to investigate if those patients produced neutralizing titers, a GFP expression plasmid was conjugated into *B. quintana*. We purified human erythrocytes and performed the erythrocyte adhesion assay with different moi (figure 3.2B). We could observe GFP+ erythrocytes, however, only with higher moi than used for *B. taylorii*. Thus, generally speaking, this assay could be used for the *B. quintana*-human system after moi adjustment.

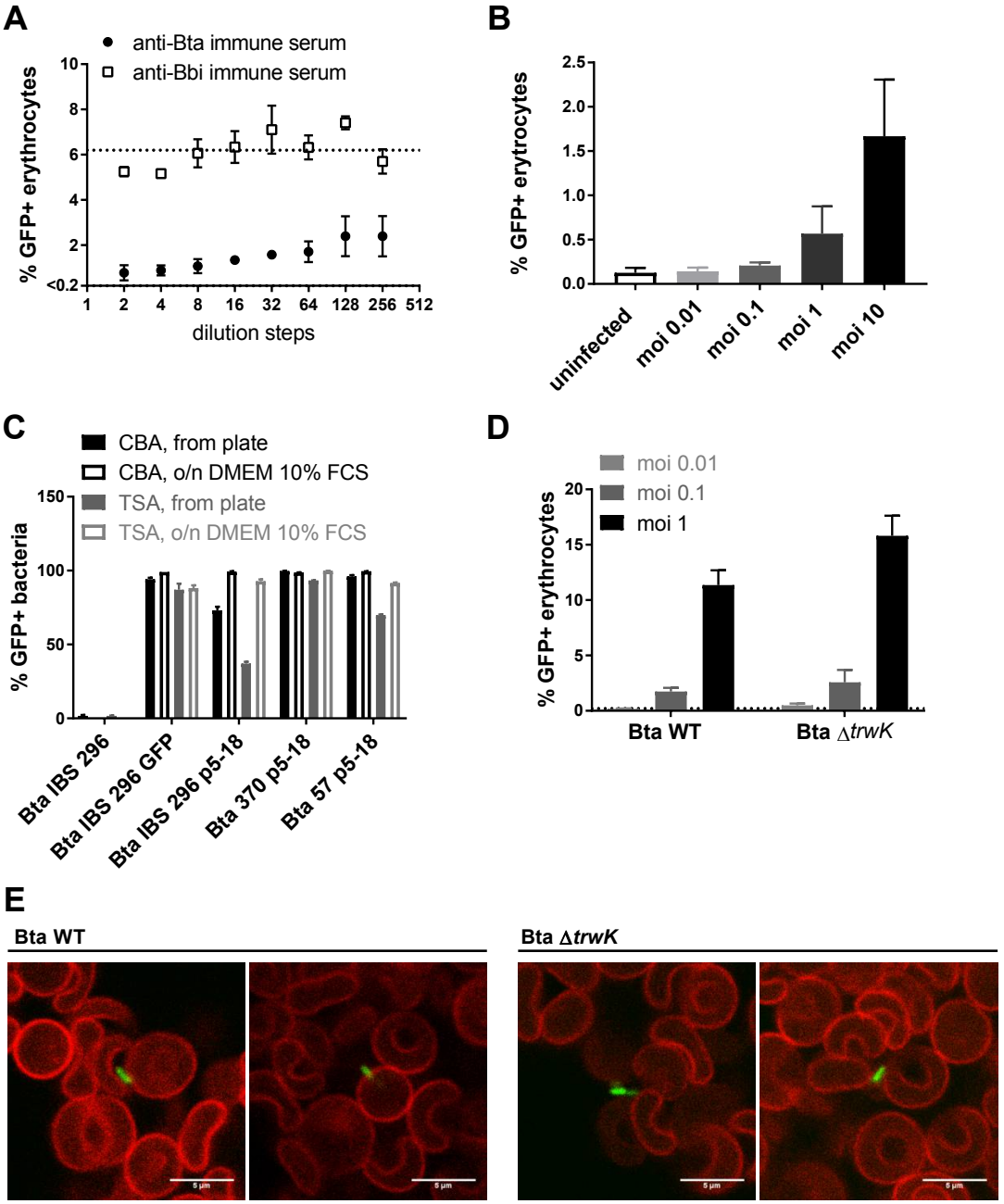

Figure 3.2: **Preliminary experiments show that the EAI assay is specific, that Δ*trwK* mutants might still be able to bind RBCs and that a neutralization assay would be possible using *B. quintana* and human erythrocytes.** A) EAI assay comparing the effect of immune serum raised against *B. taylorii* (anti-Bta) or against *B. birtlesii* (anti-Bbi) on the ability of GFP+ *B. taylorii* to bind murine RBCs. The dashed line gives the mean of the no serum control. B) moi titration of *B. quintana* expressing GFP for the infection of human erythrocytes quantified by flow cytometry. C) *B. taylorii* (Bta) isolates IBS296, #370 and #57 harboring the plasmid p5-18 (*B. henselae* Trw promotor fused to *gfp*) in order to assess their Trw T4SS expression levels. GFP+ bacteria were quantified via flow cytometry after being grown on CBA or TSA and with or without overnight (o/n) incubation in DMEM 10 % FCS. D) moi titration of *B. taylorii* WT vs Δ*trwK*, both expressing GFP, in erythrocyte adhesion, quantified by flow cytometry. E) Exemplary confocal images of (D) with moi 0.1, RBCs stained with anti-Ter119. All experiments shown in this figure were performed in technical triplicates but are preliminary results and have to be interpreted with caution.

Trw T4SS expression of *B. taylorii* in different growth conditions and observations made with the *ΔtrwK* mutant

After obtaining the monoclonal LS4G2, which binds and neutralizes specifically *B. taylorii* IBS296, we speculated about potential targets. One suggestion was a protein of the Trw T4SS, which has been described to mediate RBC adhesion [140]. Later, however, we identified the target to be a putative autotransporter. Plasmids carrying a *gfp* gene under the control of the Trw promotor of *B. henselae* had been constructed previously [228]. The plasmids were conjugated into the *B. taylorii* strains IBS296, #57 and #370. The GFP expression as a correlate for the Trw T4SS expression was quantified by flow cytometry. Different growth media (CBA and TSA) were compared, since they seem to promote different expression levels of BadA and the VirB/D4 T4SS (unpublished observations by Katja Fromm). Further, bacteria were taken from plate or incubated over night in DMEM 10 % FCS, the erythrocyte infection medium and which had been shown to induce VirB/D4 T4SS expression in *B. henselae* (personal communication with Dr. Maxime Québatte) (figure 3.2C). *B. taylorii* IBS296 with constitutive GFP expression served as a positive control. For IBS296, the expression was higher on CBA compared to growth on TSA plates and it could be in general improved by incubation in medium. #370 always showed elevated expression levels, the same is true for #57, besides coming directly from TSA plates. This data indicates that different isolates of the same species might have varying expression levels of virulence factors. In the conditions used in this study, directly from CBA plates or in medium, a vast majority of the bacteria expressed the Trw T4SS.

To further assess the role of the Trw T4SS in *B. taylorii* infection, we made use of the mutants lacking the TrwK protein provided by Jonas Körner. TrwK is an integral part of the machinery, the absence of this protein should prevent the assembly of the Trw T4SS. A similar mutant in *B. birtlesii* has been previously described to no longer bind to erythrocytes [140]. In our hands, however, GFP+ erythrocytes were observed in similar frequencies for WT *B. taylorii* or the Δ*trwK* mutant when infected with GFP-expressing bacteria (figure 3.2D). Also in confocal microscopy, we could observe the association of bacteria with erythrocytes for both cases (figure 3.2E). Several clones of Δ*trwK* mutants were tested, all showing similar results, all were previously validated by PCR (data not shown). This data sets suggests that either *B. taylorii* has a way to compensate for the lack or TrwK in its assembly of the Trw T4SS or in its way to attach to erythrocytes, suggesting that the Trw T4SS or TrwK alone might be dispensable for this species in order to adhere to RBCs. With later findings (see 3.1.1.2) showing that a predicted autotransporter is the target of the protective monoclonal antibody LS4G2 and suggesting a role for this virulence factor in RBC adhesion, the second hypothesis becomes more likely. The possible redundancy of the Trw T4SS and the autotransporter or other reasons for the attachment of the Δ*trwK* mutant of *B. taylorii* to RBCs will have to be examined in future experiments.

3.2.3 Material and methods

Bacterial strains and growth conditions *B. taylorii* IBS296 WT (LSB001), expressing GFP (LSB115), harboring p5-18 (LSB066), Δ*trwK* expressing GFP (LSB123), *B. taylorii* #57 and #370 harboring p5-18 (LSBLSB069, LSB072) and *B. quintana* with IPTG inducible GFP expression (LSB050) were grown on CBA (or TSA if indicated) containing the appropriate antibiotics (streptomycin 100 μg/ml, gentamycin 20 μg/ml, kanamycin 30 μg/ml) and 5 % defibrinated sheep blood.

If indicated, the bacteria were incubated over night in DMEM containing the appropriate anitbiotics and 10 % FCS.

Erythrocyte infection and microscopy

RBCs were purified, infected, analyzed and imaged as previously described (3.1.1.2).

3.3 Transplacental transmission of *Bartonella taylorii* in mice

3.3.1 Summary

One issue in *Bartonella* research are discrepancies in the course of infection between the experimental *in vivo* models and findings in animals captured from the wild. This concerns in particular the course of the bacteremia, which is described to be longer in wild animals than in experimental infections. Further, relapses have been observed in captured animals, whereas they are not present or if at all extremely rare in experimental animals [273]. A lack of antibodies in infected captured animals [271, 272] compared to a strong humoral immune response in experimental mouse infection [134], together with reported transplacental transmission of *Bartonella* [271, 274, 275] lead to the general hypothesis that those bacteria are transmitted from mother to offspring, inducing immunological tolerance and life-long persistence in the offspring.
In order to tackle questions related to vertical transmission, we used again the *B. taylorii* mouse model. Transplacental transmission took place only when the infected mother was immunosupressed, lacking the adaptive immune system (Rag1-/-). We were unable to observe transmission in immunocompetent WT animals. Bacteremic pups from Rag1-/- parents do show life-long persistent infection as their parents. However if infected Rag1-/- mothers were mated with WT males, resulting in Rag1+/- offspring, bacteremic pups cleared the infection within weeks. Thus, even though transplacental transmission is possible, immunocompetent offspring does not develop life-long bacteremia.

3.3.2 Results

A murine model using *B. birtlesii*-infected Balb/c mice showed transplacental transmission of the bacterium and resorption of potentially infected fetuses [274]. We similarly infected C57Bl/6 and Balb/c females with *B. taylorii* and performed matings on day 10 post infection (figure 3.3A). However, we failed to detect *B. taylorii* in embryos extracted on gestation day 18 or living offspring of WT mothers (figure 3.3B and data not shown). Also fetal resorptions were not observed.
We then infected immunosuppresed Rag1-/- females and again performed mating on day 10 post infection. In order to observe the effect of the immune system of the offspring, those Rag1-/- females were mated with either C57Bl/6 WT or Rag1-/- males (resulting in Rag1+/- and Rag1-/- offspring). In contrast to WT females, vertical transmission has been observed in infected Rag1-/- mothers (figure 3.3B) independent on the genotype of the male. 0 - 66.7 % of the offspring of Rag1-/- mothers were infected, with an average of 40 %. This made no difference when analyzing extracted embryos (25-62.5 % were *B. taylorii* positive) or living offspring, suggesting that the transmission is indeed transplacental and is happening before birth (figure 3.3C). We compared the bacteremic burden of immunocompromised (Rag1-/-) with immunocompetent (Rag1+/-) offspring. Rag1-/- animals displayed life-long persistent bacteremia with similar titers observed for infected adult Rag1-/- animals (figure 3.3D, see also 3.1.1.2). Rag1+/- offspring, however, cleared the infection between 50 and 86 days after birth (on average 76 days, see figure 3.3D) showing that transplacental transmission of *B. taylorii* does not lead to a life-long persistent bacteremia.

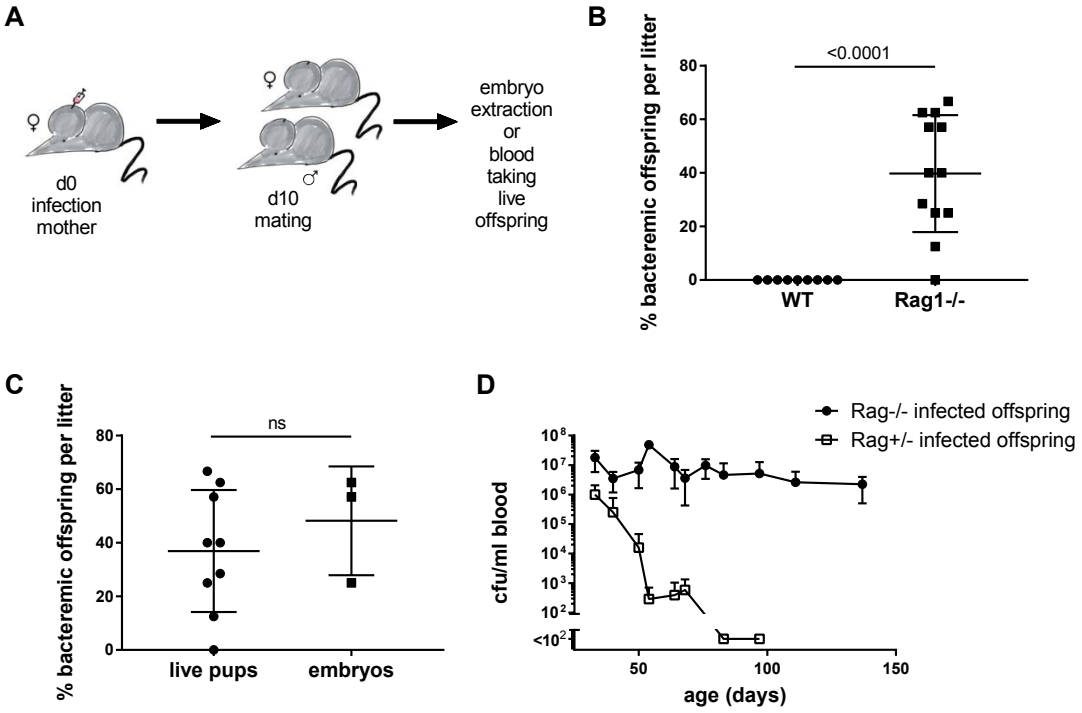

Figure 3.3: **Transplacental transmission of *B. taylorii* is dependent on the immunosuppressive state of the mother and does not induce life-long persistent bacteremia in the offspring.** A) Schematic overview of the experiments performed: WT C57Bl/6 or Rag1-/- females were infected with 10^7 cfu *B. taylorii i.d.* 10 days before mating with uninfected WT C57Bl/6 or Rag1-/- males. Either embryos were extracted 1 day before the calculated birth date or the bacteremia was analyzed in living offspring. B) Percentage of bacteremic offspring per litter. C) Comparison of bacteremic offspring of Rag1-/- mothers per litter before and after birth. D) Bacteremia of living offspring of Rag1-/- mothers mated with WT C57Bl/6 males (Rag1+/- offspring) and Rag1-/- males (Rag1-/- offspring). Statistical analysis was performed using unpaired t-test and P-values are given for significant findings.

3.3.3 Discussion

Transplacental transmission of different *Bartonella* species has been previously reported in rodents [271, 274] and a human case [275]. However, vertical transmission could not be demonstrated in cats and cattle [273, 276]. Taken together with our data for *B. taylorii* in mice, transplacental transmission of *Bartonella* is possible. However, it seems highly dependent on the immune status of the mother, since only immunosuppressed animals were able to transmit the infection to their offspring (figure 3.3B). We failed to observe vertical transmission or fetal resorptions, as have been previously reported for *B. birtlesii* in Balb/c mice [274]. Thus, it might also be strain dependent if *Bartonella* can cross the placenta in an immunocompetent host. As previously discussed, the resorption of an infected fetus might then be a result of the maternal immune system [274].

To our knowledge we were the first ones to experimentally investigate the course of the bacteremia in murine offspring which got infected by bacteria crossing the placenta. Previous experiments with neonatal infection of C57Bl/6 mice with *B. taylorii* did not lead to life-long persistent bacteremia (unpublished observations by Yun-yueh Lu). Similar observations have been made in cats using *B. henselae* [266]. Even though suggested by the lack of an antibody response in infected captured rodents from the wild [271, 272], we do not find evidence for a life-long persistent bacteremia in immunocompetent offspring. Immunsuppressed (Rag1-/-) offspring from infected Rag1-/- mothers showed persistent bacteremia whereas Rag1+/- offspring cleared the infection. We believe that there is no evidence for immunological tolerance in the offspring caused by transplacental transmission. The formal proof could be provided by analyzing the serum of Rag1+/- offspring with the methods developed in 3.1.1.2.

These experiments provide evidence that there is no life-long persistent bacteremia after transplacental transmission in the offspring. Thus, the differences between experimental infection and infected animals captured from the wild must be explained differently. As discussed in the introduction, alternative reasons could be the high rate of co-infections [149, 277], aswell as variations of surface proteins between different strains or even within subspecies [188, 278, 279, 280]. The high specificity of immune serum or a protective monoclonal risen against one isolate of a species (see 3.1.1.2) support that even with a pre-existing immunity against a different *Bartonella* species or a different substrain of the same species, reinfection could be possible.

Even though we find no evidence for immunological tolerance or life-long persistence of the bacteria in infected offspring after transplacental transmission, the vertical transmission itself is still a powerful alternative to transmission via the arthropod vector allowing the bacteria to spread amongst a population.

3.3.4 Material and methods

Cultivation of bacteria

Bartonella taylorii IBS 296 (LSB001) [287] was grown at 35°C, 5 % CO_2 on Columbia blood agar (CBA) containing 5 % defibrinated sheep blood and 100 µg/ml streptomycin (Sm). Bacteria were streaked 5 and expanded 2 days before usage.

Animal experimentation Animal handling was performed in accordance with the Swiss Animal Protection law and local animal welfare bodies. The animal experiments in this work were approved by the Veterinary

Office of the Canton Basel-Stadt (License number 1741).
All animals were kept at SPF conditions. WT animals C57/BL6 and Balb/c obtained from Janvier labs. Rag1-/- [285] were bred at the Laboratory Animal Science Center (LASC, University of Zurich, Switzerland).
Female adult mice were infected with 10^7 cfu *B. taylorii i.d.* under anesthesia with isofluorane. Blood takings followed by limited dilution plating on CBA with Sm confirmed infection. On day 10 pi, the mice were mated with uninfected adult males (C57/BL6 or Rag1-/-) for 7 days. Females were checked daily for the formation of vaginal plugs. Embryos were extracted via caesarian section on day 18 of gestation, homogenized and plated on CBA with Sm. Blood takings followed by limited dilution plating on CBA with Sm was performed on living offspring starting 5 weeks after birth.

4 Concluding remarks and outlook

The work reported in this thesis focuses on different aspects of the immune response against *Bartonella*.
The manuscript "Neutralizing antibodies protect against murine *Bartonella* infection by interfering with erythrocyte adhesion" in section 3.1.1.2 discusses the protective antibody response against the murine species *B. taylorii*, describes in detail by which mechanism antibodies can mediate clearance of the bacteremia and suggests a target bound by those antibodies. The potential role of this target and its interaction with protective antibodies will be discussed in more detail later in this chapter and are promising starting points for follow-up experiments.
The manuscript also reports observations made about the T-cell response against *B. taylorii*. We confirmed the cytolytic T-cell response to be dispensable for clearing *Bartonella* infection and direct T-help via CD40L to be crucial for the protective antibody response. However, we observed a high redundancy among cell types within the T-cell compartment that can mediate this function. Our data suggests that MHCII restricted CD4 T-cells might be redundant with T-cells restricted by other antigen presenting molecules such as CD1d or MR1 in mice. To dissect the role of those different T-cells in more detail, murine double- or even triple-knock out strains lacking combinations of MHCII, MR1 and CD1d would be necessary. This very fastidious approach could give final proof of the hypothesized redundancy and indicate if all three or just two of those are actually functionally redundant in this case.
Similar to the T-helper cell response, the different immunoglobulin types seem to be highly redundant during *Bartonella* infection. Both, IgM and IgG, were able to mediate clearance of the bacteremia. Both findings, the redundancy amongst T-helper cells and amongst antibody types, are reassuring, showing that the mammalian immune system often does not rely on one single mechanism but is able to circumvent the lack e.g. of MHCII-restricted T-cells or class switch.
The results presented in section 3.1.2 were obtained with *B. birtlesii*, a second species naturally found in mice. They allow drawing the same conclusions and support the notion that the findings observed with *B. taylorii* as a model organism can be translated to at least the rodent-borne, if not all *Bartonella* species of lineage 4.

As reported in section 3.3, we also briefly looked at potential immunological tolerance against *Bartonella* after vertical transmission. While we observed transplacental transmission in immunosuppressed mothers, we do not find evidence for immunological tolerance or life-long bacteremia in the offspring. These findings, however, might still have important implications when it comes to spreading of the bacteria amongst a population or in clinics in case of pregnant patients.
It could be interesting in the future to study the mechanism of how those bacteria interact with or even cross the placenta, in order to gain a deeper understanding of the infection strategy of *Bartonella*. Although it might be a passive, incidental process, it might also be a fine-tuned mechanism with specific virulence

factors involved.

4.1 Outlook for the manuscript: Following up on the antibody response against *B. taylorii* and the role of the identified autotransporter in infection

The manuscript in section 3.1.1.2 provides a detailed analysis of the humoral immune response in mice against the pathogen *B. taylorii*. It shows the presence and stresses the importance of a specific neutralizing antibody response for the clearance of the bacteremia. We also provide evidence that prevention of adhesion or in general interference with the erythrocyte infection is the key functionality for this protective immune response.
The most recent results obtained for the manuscript, being the target for the monoclonal LS4G2 and the antibodies' protective function during *in vivo* infection, lead to new questions: 1) What is the role of the identified autotransporter during infection? 2) How conserved is the target and its function within one or more *Bartonella* species, e.g. the human pathogenic ones? Could antigenic variation of this protein within species or strains within a species be an immune evasion strategy? 3) Can this autotransporter be used as a target to develop a vaccine strategy?

4.1.1 The role of the identified autotransporter during infection

The data presented in this thesis suggests that the target of LS4G2 is involved in erythrocyte attachment of *Bartonella*. However, the proof for this is still lacking and other functions of the identified putative autotransporter during *Bartonella* colonization of the mammalian host cannot be excluded. Autotransporters of other Gram-negative bacteria have been associated with host cell adhesion. Since this autotransporter can be found in a broad variety of related *Bartonella* species, its role during infection, its potential interaction partners from the host side and its regulation would be of general interest for the field.
The first steps would be the generation of a *B. taylorii* mutant lacking the identified target of LS4G2. Reintroduction of the protein from plasmid or in the chromosome would serve as an essential complementation control. Important validation experiments, also for the functionality of LS4G2 and the established erythrocyte adhesion interference assay, could be done with this setup of mutant strains. Binding of LS4G2 and a polyclonal immune serum to the mutant should be investigated. To observe if this autotransporter plays a role in RBC attachment, a comparison of *in-vitro* erythrocyte infection rates of the mutant and the complemented strain with WT bacteria should be performed. Similarly, attachment to other cell types such as macrophages or DCs of the mutant compared to WT bacteria should be investigated.
Several autotransporters of *Bartonella*, including the target of LS4G2, have been shown to be essential for and to be upregulated during infection. Previously unpublished data from our lab confirmed that a *B. tribocorum* mutant lacking a similar autotransporter loses infectivity *in vivo*. These findings encourage us to test the generated *B. taylorii* mutant strains in mice. Confirming the hypothesis that the target of LS4G2 is essential for the infection would be a crucial experiment for further studies of this virulence factor.
Subsequently, the role of the predicted autotransporter during the infection should be studied in more detail. Potential interaction partners on the host cell surface should be identified - for example by co-

immunoprecipitation from cell lysate followed by mass spectrometry. The abundance of these interaction partners on different cell types could further indicate if the autotransporter is limited to mediating attachment to RBCs or if it could be involved in the adhesion towards other cell types or have additional functions. Further, previous data suggests that autotransporters may be induced upon infection. By generating fluorescent reporter constructs, the regulation and presence of the autotransporter under different conditions could be studied.

4.1.2 The conservation of the identified autotransporter in *Bartonella* and potential immune escape by antigenic variation of this protein

The monoclonal antibody LS4G2 is highly specific in binding to the *B. taylorii* isolate it was raised against. BLAST analysis of the identified target revealed that related autotransporters are present in all species of lineage 4, showing, however, highly variable homology on the amino acid sequence level. A more detailed bioinformatic analysis comparing the sequence of this autotransporter amongst different *Bartonella* species could show which domains of the protein vary the most and if there are stretches that are highly conserved. However, the conservation of the target of LS4G2 is not only interesting when comparing different species but also when comparing different strains within a species. Variation of this potent antigen within a species could allow reinfection of an animal with a closely related sub-species.

In our lab, we have currently 3 *B. taylorii* isolates, also used in the manuscript for LS4G2 binding analysis. Further, we recently obtained a collection of sequential isolates after experimental macaque infection and primary patient isolates of the related species *B. quintana*, naturally infecting humans. Sequencing of the respective locus of those strains and analysis of their homology in this particular protein could give interesting information about which parts of the autotransporter are more conserved than others. Together with further analysis and experiments, this could give rise for ideas if there are immunodominant parts of this protein, which might be more variable amongst isolates, and parts which have to be more conserved in order to retain the function of the autotransporter, which would be less variable amongst the isolates. In the hands of our collaborators, the sequential isolates from experimental macaque infection were previously used to identify the *vomp* locus, which undergoes rearrangement during infection to evade the host's immune system by modifying its gene product, an outer membrane protein, on the surface of the bacterium. A similar study could investigate whether the locus encoding the target of LS4G2 also undergoes rearrangement in the host during the infection.

The library of human *B. quintana* isolates comes with a corresponding serum collection from the same patients. Similar studies for binding of the corresponding serum to the bacterium, as have been performed for LS4G2 and *B. taylorii*, could show if those patients produce antibodies binding to the corresponding isolate. If so, erythrocyte adhesion inhibition assays could be performed using an *in-vitro* infection of human RBCs by *B. quintana*. If EAI titers are measurable in human patients, our hypothesis that interference with erythrocyte attachment is a common theme in anti-*Bartonella* immunity would be validated.

Last but not least, even though the genetic manipulation of *B. quintana* is more fastidious than for *B. taylorii*, the respective mutant lacking the autotransporter could be constructed, followed by similar experiments as suggested for the *B. taylorii* mutant, in order to look for the conservation of the function of this protein.

4.1.3 Developing a vaccine strategy against *Bartonella*

As a proof of concept of LS4G2 being protective *in vivo* because of its neutralizing capacity upon binding of its target on the bacterial surface, mice immunized with this autotransporter should be able to mount an antibody response protecting them from infection.
A simple pilot experiment for this could be the purification of the whole autotransporter or a fraction of the protein lacking the transmembrane domain from an *E. coli* expression system. Mice could then be immunized with the protein and an adjuvant, the antibody response of the mice against the autotransporter observed by ELISA and successfully immunized mice could be infected with *B. taylorii*. Observing the occurrence or absence of the bacteremia could serve as a read-out for protection.
If our hypothesis holds true and immunization with this protein allows protection against infection and expecting the functional conservation of the autotransporter, this finding might allow the design of an anti-*Bartonella* vaccine. Even though, *Bartonella* is not a life-threatening or severe pathogen in most parts of the world, vaccination could be beneficial in some situations. Since cat-scratch disease is a threat to HIV-infected or other immunosuppressed patients, vaccinating their pets against *B. henselae* could protect them from this zoonotic infection. With climate change allowing the spread of the vectors and growing poverty on this planet, a vaccination against *B. bacilliformis* or *B. quintana* could be important measures in endemic areas or amongst people living in low-hygiene conditions. Due to the fact that *B. bacilliformis* is more distant to the other species, we can, however, at this point only speculate about vaccines against the other mentioned species. Further, a general vaccination approach would also be possible if there are enough conserved regions within the target that could be used for immunization. However, if the identified autotransporter turns out to be too variable within sub-strains of a species, designing a vaccine on this basis would become more fastidious and maybe impossible.

4.2 The life cycle of *Bartonella* and how the protective immune response of the host may interfere with it

Upon deposit of the flea feces on the host's skin, the bacteria are brought into the dermis by scratching. Different reports suggest that they then either travel free in the draining lymph or hijack dendritic cells in order to reach their primary replication niche. As a stealth pathogen, *Bartonella* evades the host's immune system or even modulates it to its own favor. For example, *Bartonella* LPS is a TLR4 antagonist and secreted effector proteins reprogram the host cell to switch on an anti-inflammatory cytokine response instead of a pro-inflammatory one. It is believed that the bacteria are so protected from the immune system until they enter their replicative niche. The nature of this niche is not well understood. Because of the bacteria's tropism for endothelial cells *in vitro* the general hypothesis is that they replicate within those cells before seeding into the blood stream. However, the nature of the replication niche might be blood or lymphatic endothelial cells, a different cell type or even extra-cellular. The identification of this replicative niche remains, one of the great so far unreached goals of the field.
Our mouse infection model using *B. taylorii* confirms previous observations with different *Bartonella* species-host combinations. The blood of the animals remains sterile after inoculation for several days before the occurrence of the bacteremia. We, as others before, used the *intra-dermal* inoculation route in order

to mimic the transmission by the arthropod vector. Similar observations have been also made with *intravenous* infection routes. It is generally accepted that this delay in bacteremia development is because the bacteria need go through their replication niche in order to be able to infect red blood cells. It was further previously hypothesized that this step is necessary in order to upregulate virulence factors involved in this process. After infection of the erythrocyte, *Bartonella* replicates within it and can be taken up by the next arthropod vector during a blood meal. The species of lineage 4 are described to be non-haemolytic. Thus, the RBC is considered to be a kind of "dead end" from which the bacteria do no longer re-enter the infection cycle within the mammalian host. The data presented in this thesis supports this hypothesis as *B. taylorii* was found to be intra-erythrocytic within the murine host and as in mice lacking B-cells the bacteremia continued to decline even after the transferred immune serum was no longer detectable. However, to our knowledge the final proof for this notion, for example blood transfer from an infected to a naïve animals in order to observe whether the recipients develop bacteremia, has not been performed yet. Previous experiments from the rat infection model do, however, show that regular infection waves seed from the replicative niche into the blood stream, respectively into the RBCs, until the bacteremia is finally cleared.
This study investigated how the host's immune system is able to break the infection cycle of *Bartonella* in order to fight the infection. Within erythrocytes, which are not able to present antigen via MHC molecules, the bacteria are well protected from the immune system. It was previously suggested that neutralizing antibodies interfere with RBC infection and that maybe cytolytic T-cells control the replicative niche within endothelial cells. Due to the inaccessibility of the replication site, we focused on the clearance of the bacteremia in this study. We could confirm that neutralizing antibodies can prevent the interaction of free bacteria with the erythrocyte. Thus, these antibodies break the infection cycle of *Bartonella* by stopping the seeding from the replication niche into the red blood cells. The infection cycle of *Bartonella* and the interference of antibodies are schematically summarized in figure 4.1, for simplicity reasons endothelial cells are shown as the site of replication. However, our findings suggest that antibodies can also interfere with at least one more step during this cycle. We could show that cytolytic T-cells are dispensable for clearance, suggesting that they are also not controlling the replication niche. Instead, serum transfer experiments into mice lacking B-cells suggest that antibodies also interfere with the maintenance or re-seeding of replication sites. This allows two different explanations. The first option would be that the replication niche is actually extracellular, thus cytolytic T-cells could not even target this niche but neutralizing antibodies might for example block replication. This hypothesis is supported by the fact that the bacteria are found extra cellularly in biopsies from CSD patients. The second explanation would be an intra-cellular replication niche which would be for some reason protected from cytolytic T-cells. BepA has been shown to be antiapoptotic and might prevent induced cell death in the infected cell or maybe *Bartonella* avoids the presentation of an epitope targeted by cytolytic T-cells. In both cases, neutralizing antibodies could prevent the re-seeding of new replication sites by interfering again with the host cell attachment.

As mentioned before, the identification of the replication site within the natural host should be one of the main priorities when studying the infection strategy of *Bartonella*. An approachable first step would be the investigation whether this niche is intra- or extra-cellular. Administration of gentamycin, an antibiotic that does not enter eukaryotic cells, during infection would eliminate extra-cellular replication sites. Giving the antibiotic in the early phase after inoculation before the bacteria are found in the blood stream would give

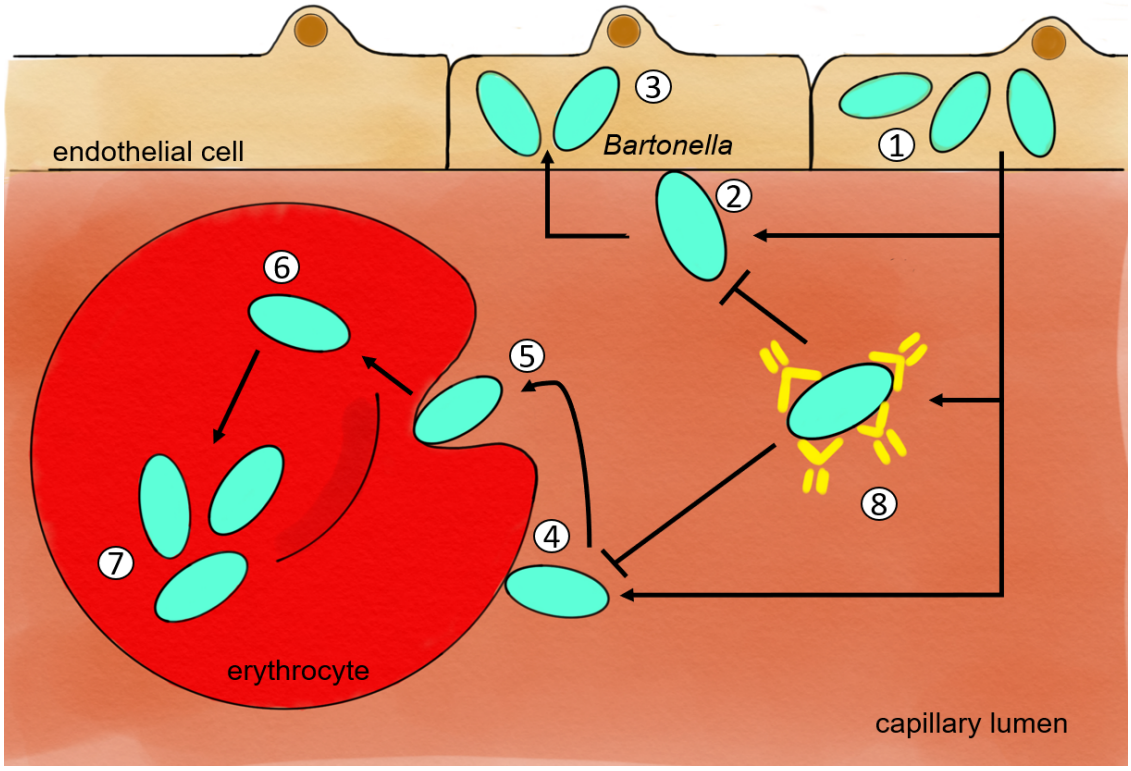

Figure 4.1: **How antibodies interfere with the life cycle of *Bartonella*:** After being transmitted from the arthropod vector, the bacteria (cyan) travel from the dermis to their replication niche, which has been hypothesized to be within endothelial cells (1). After replication, *Bartonella* seeds into the blood stream. They may interact with so far uninfected endothelial cells (2) in order to establish a new replication niche (3) or infect erythrocytes. For this, they need to first attach (4), then deform (5) and finally enter the red blood cell (6). They replicate within the erythrocyte (7) and remain within it until the end of the cells' life span or until the uptake by a new arthropod vector during a blood meal. Neutralizing antibodies (yellow) produced by the host's immune system can interfere with this infection strategy. By binding the antigen on the bacterial surface (8), the antibodies may prevent attachment to new endothelial cells and erythrocytes until the bacteremia is cleared.

insight whether they are mostly intra- or extra-celluar in this period. Giving the antibiotic in later bacteremic stages of the infection would eliminate replication sites if they are extra-cellular. However, those experiments would need to be performed in a host lacking antibodies in order to really only observe the effect of the antibiotic.

Tagging of the bacteria such as by fluorescent or luminescent markers followed by imaging approaches might be a way, even though a very fastidious one, to identify the whereabouts of the replication site. In general, solving the enigma of the primary or replicative niche would allow better *in-vitro* models for studying different aspects of the infection of *Bartonella*, such as effector functions. Once the replication niche is identified, it could also be of interested to repeat some of the experiments performed in this study in order to validate the clearance mechanism of the replication niche by the host's immune system.

With recently established *in-vitro* protocols for the infection of cell lines and primary cells with *B. taylorii* in our lab, this model might be the first in the history of *Bartonella* research that can be used as a robust model, both *in vitro* and *in vivo*, for the infection of the natural reservoir host. Further exploration of this model will shed light into many more aspects of the infection strategy of *Bartonella*.

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7 Appendix

7.1 LS strain and plasmid lists

name	strain	description	plasmid	growth conditions
LSE013	*E. coli* β2150	for conjugation into *Bartonella*	pCD353	Km
LSE020	*E. coli* β2150	for conjugation into *Bartonella*	p5-18	Km
LSE025	*E. coli* β2150	for conjugation into *Bartonella*	pLS04	Gm
LSE029	*E. coli* β2150	for conjugation into *Bartonella*	pKSC009	Km
LSE031	*E. coli* StrataPack®	LS4G2 light chain (murine kappa)	pLS09	Amp
LSE033	*E. coli* StrataPack®	LS4G2 heavy chain (murine IgG2a)	pLS06	Amp
LSE035	*E. coli* StrataPack®	LS4G2 heavy chain (murine IgG3)	pLS08	Amp

Table 7.1: ***E. coli* strain list.** Amp = ampiciline, Gm = gentamycine, Km = kanamycine

name	strain	abbreviation	plasmid	growth conditions	source
LSB001	*B. taylorii* IBS296	Bta WT		CBA Sm	[287]
LSB002	*B. birtlesii* IBS325	Bbi WT		TSA	[288]
LSB009	*B. tribocorum* IBS506	Btrb WT		CBA Sm	[289]
LSB040	*B. taylorii* IBS296	Bta GFP IPTG ind.	pCD353	CBA Sm Km	
LSB045	*B. quintana*	Bqu WT / JK31		CBA	Prof. Jane Koehler
LSB048	*B. taylorii* no. 370	Bta no. 370		HIA	Dr. Yves Piemont
LSB049	*B. taylorii* no. 57	Bta no. 57		HIA	R. Birtles
LSB050	*B. quintana*	Bqu GFP	pCD353	CBA Km	
LSB066	*B. taylorii* IBS296	Bta IBS296 p5-18	p5-18	CBA Km	
LSB069	*B. taylorii* no. 57	Bta no. 57 p5-18	p5-18	HIA Km	
LSB072	*B. taylorii* no. 370	Bta no. 370 p5-18	p5-18	HIA Km	
LSB115	*B. taylorii* IBS296	Bta GFP	pLS04	CBA Sm Gm	
LSB118	*B. taylorii* IBS296 ΔTrwK			CBA Sm Km	
LSB123	*B. taylorii* IBS296 ΔTrwK	Bta ΔTrwK	pLS04	CBA Sm Km Gm	

Table 7.2: ***Bartonella* strain list.** CBA = columbia blood agar, HIA = heart infusion agar, Gm = gentamycine, Km = kanamycine, Sm = streptomycine

name	description	resistance	source
p5-18	*B. henselae* Trw promoter - GFP	Km	[228]
pCD353	IPTG inducible GFP expression	Km	[290]
pKSC009	disruption of *B. taylorii* TrwK	Km	Jonas Körner
pLS03	constant dsRED expression	Gm	
pLS04	constant GFP expression	Gm	
pLS06	LS4G2 heavy chain (murine IgG2a) for mammalian cell expression	Amp	
pLS08	LS4G2 heavy chain (murine IgG3) for mammalian cell expression	Amp	
pLS09	LS4G2 light chain (murine kappa) for mammalian cell expression	Amp	

Table 7.3: **Plasmid list.** Amp = ampiciline, Gm = gentamycine, Km = kanamycine

7.2 Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AID	activation-induced cytidine deaminase
APC	antigen presenting cell
Arp	acidic repeat protein
BadA	*Bartonell* adhesin A
Bbi	*B. birtlesii*
Bep	*Bartonella* effector protein
Bhe	*B. henselae*
bnAb	broadly neutralizing antibody
Bta	*B. taylorii*
Bqu	*B. quintana*
C region	constant region
CBA	columbia blood agar
CDR	complementary-determining region
Cfa	CAMP-like factor autotransporter
CSD	cat scratch disease
CSR	class switch reaction
DAMP	danger-associated molecular pattern
DC	dendritic cell
EAI	erythrocyte adhesion inhibition
ECM	extracellular matrix
Fab	antigen-binding fragment
Fc	crystallizable fragment
FcR	Fc receptor
FIC	filamentation induced by cyclic AMP

FR	framework region
HIA	heart infusion agar
HIF	hypoxia induced factor
i.d.	intra dermally
Ial	invasion associated locus
Iba	inducible *Bartonella* autotransporters
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNKT	invariant natural killer T-cell
LPS	lipopolysaccharide
MAC	membrane attack complex
MAIT	mucosal-associated invatiant T-cell
MBL	mannose-binding lectin
MHC	major histocompatibiliy complex
mIgD/M	membrane bound IgD or IgM
MOI	multiplicity of infection
MR1	MHCI-related molecule 1
nAb	neutralizing antibody
NK	natural killer cell
OMP	outer membrane protein
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
RBC	red blood cell
RT	room temperature
SHM	somatic hypermutation
T4SS	type 4 secretion system

TAA	trimeric autotransporter adhesin
TCR	T-cell receptor
Th	T-helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T-cell
TSA	tryptic soy agar
V region	variable region
VEGF	vascular endothlial growth factor
Vomp	variably expressed outer membrane protein