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„Multimodal investigation of immune cell changes after tick bite and during tick-borne diseases "

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Table of contents

List of Figures and Tables

Introduction

Changes in the immune cell infiltrate after tick bite

Tick species and emergence

Ticks are blood feeding arthropod ectoparasites, that belong to the order *Ixodida* and are distributed worldwide. They consist of 3 families: *Ixodidae* (hard ticks), *Argasidae* (soft ticks), and *Nuttallielidae*. Here we focus on hard ticks, which feed for several days or even weeks on their mammal vectors (1, 9). Ticks belong to the most important arthropod vectors of human and livestock diseases and transmit many different pathogens. The most prevalent tick-borne diseases are Lyme borreliosis caused by *Borrelia burgdorferi* species, tick-borne encephalitis (TBE) caused by the TBE virus, tick-borne spotted fever caused by *Rickettsia* species, anaplasmosis caused by *Anaplasma* species, and babesiosis, caused by *Babesia* species (9, 10) .The tick *Ixodes ricinus* is the most common vector of TBE and Lyme disease in Europe (11).

Ticks and tick-borne diseases are on the rise. Climate change and in particular milder winters and extended growing seasons have led to the expansion of the distribution range of the Ixodes species towards northern latitudes and higher altitudes (Figure 1). Models predicted that with further temperature rise, a habitat expansion of Ixodes ticks will occur in all of Europe (12-14).

Figure 1. The approximate distribution of the Ixodes ricinus species complex.

Grey represents geographical distribution of *Ixodes* species. The area in black represents an overlap in the distribution of *I. persiculatus* and *I. ricinus*. (5)

Immune responses to tick bite and tick saliva induced suppression of immune responses

Ticks obtain their blood meal by attaching to the skin of vertebrate hosts, tearing the skin and feeding from the pool of blood formed at the bite site. After several hours of tick attachment, the ticks secrete an adhesive cement to stay stably attached to the host and to provide a physical barrier to the tick mouthparts (Figure 2) (15). Ticks stimulate a wide range of host innate and acquired immune responses (16). On the other hand, however, ticks need to suppress and evade host immune and hemostatic responses in order to take up blood. Ticks developed several measures to circumvent host defense mechanisms. When mouthparts are inserted into the skin, ticks create a feeding lesion into which saliva is secreted at continuous intervals over the course of several days. The secreted tick saliva contains hundreds of immunomodulatory molecules which can exert strong immunosuppressive effects (1). Tick saliva comprises an impressive mixture of proteins, peptides and non-peptide molecules, which act in a cytolytic, anti-coagulant, anti-inflammatory (Salp-15) (17), anti-chemokine (evasins) (18), anti-pain or vasodilating (prostaglandin E2) (19) manner (20). The first proteomic studies that addressed tick saliva were performed in the first decade of the 21st century (21). Since then, protein profiles have been annotated to different developmental stages, sexes and feeding stages of several tick species (22). However, only a small part of the salivary proteins has a known function (23).

Upon repeated tick infestation non-natural hosts acquire tick resistance, a phenomenon wherein the host elicits an immune response against tick salivary compounds, which can lead to tick rejection (24) . The tick-host molecular interaction can be viewed as a competition between host defense responses and evasion strategies developed by ticks (20) .

The skin represents the main interface of host-tick interactions and it possesses an abundant population of cells and molecular mediators of innate and adaptive immunity (25, 26). The first line of defense against inserted mouthparts are resident leukocytes of the epidermis and dermis, such as T cells, mast cells, eosinophils, dendritic cells, macrophages, innate lymphoid cells and keratinocytes. Some of these cells release chemokines and recruit inflammatory cells including neutrophils. Subsequent infestation may lead to adaptive immune responses involving T and B cells, which secrete cytokines and produce specific antibodies that target tick salivary or mouthpart-derived antigens in an effort to reject the tick (27, 28).

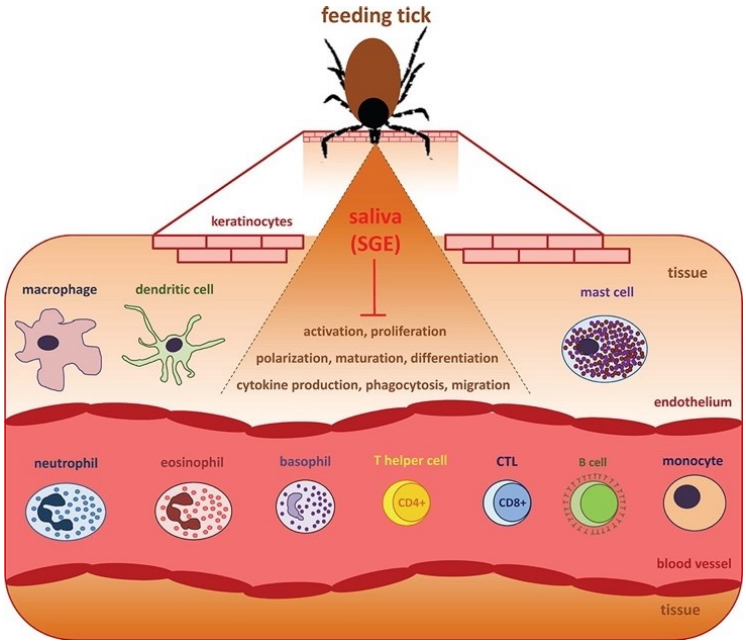


Figure 2. Modulation of host responses by tick saliva.

Tick salivary compounds modulate immune cell activation, proliferation, polarization, differentiation and functional capacities. (1)

The role of mast cells in tick infestation

Mast cells are sentinel cells present beneath epithelial surfaces of the skin (26). Activated mast cells degranulate and release a wide range of mediators, including serine proteases, vasoactive compounds, histamine and cytokines (29). The immunological importance of mast cells in tick-host interactions remains to be elucidated. Increased numbers of mast cells during secondary and tertiary infestation but not in primary infestation were observed in *Dermacentor variabilis* infested BALB/c mice and in *I.ricinus* infested rabbits (30, 31). Whether mast cells play a role in tick resistance is still debated. One study showed that mast cell-deficient mice are still able to develop a *D. variabilis* resistance, like wild type mice (30). However, another study found that mast cells played an important role for the manifestation of resistance to the *Ixodidae* tick *Haemaphysalis longicornis*. Mast cell-depleted mice were not resistant to *H. longicornis* and tick resistance was re-established after mast cell injection (32). Species-specific host responses might be the reason for the observed differences.

The release of histamine by mast cells and basophils can be detrimental to tick attachment, since it is promoting pain and itch responses at the bite site that eventually lead to host grooming the tick off (24). Multiple tick salivary proteins have been identified that interfere with histamine release that sequester histamine or modulate mast cell functionalities and thereby alleviate itch and pain (20, 33, 34).

The role of eosinophils in tick infestation

Eosinophils are present in the skin and are a source of many cytokines, chemokines and mediators upon certain pathologic processes. Furthermore, they release cytotoxic granules and play a role in allergic inflammation (27). High numbers of eosinophils were observed in the tick attachment site upon repeated hard tick infestations in different animal models (35-38). The

role of eosinophils in tick resistance remains unclear. The ablation of eosinophils in the tick feeding cavity led to a partially impaired tick resistance in guinea pigs (39). On the other hand, the susceptibility to ticks in cattle was negatively correlated with the number of eosinophils at the tick feeding cavity. Cattle breeds with more eosinophils were found to be more resistant to *R. microplus* feeding than breeds with fewer eosinophils (40). Ticks interfere with the chemokine-mediated attraction of eosinophils to the tick feeding cavity. The salivary gland extract (SGE) from different tick species inhibited CCL3, CCL5 and CCL11 eosinophil chemoattractant activity (41-43).

The role of basophils in tick infestation

Basophils are IgE-activated granulocytes, which play an essential role in inflammation. They have many similarities with mast cells, but unlike mast cells, they circulate in the blood (44). Basophils are recruited to the tick attachment site and expand in host skin during secondary but not primary tick infestation, where they play an important role in tick resistance (45). The role of basophils in tick rejection was described in mice and guinea pigs in several studies (46-48). The selective basophil depletion by diphtheria toxin led to the loss of resistance to *H. longicornis* feeding in subsequent infestations, suggesting that basophils play a non-redundant role in acquired immunity against ticks. Some studies showed that tick bites can cause cutaneous basophilia, which is characterized by the infiltration of large numbers of basophils to tick bite lesions, and contributes to tick resistance (48). The mechanism is not yet completely clear. Possible explanations are that the basophilic components released upon degranulation impairs tick feeding due to host responses such as vasodilation, pain and itching, or that the basophilic components enter the tick gut and cause damage to the organ (24).

The role of natural killer cells in tick infestation

Natural killer cells are lymphocytes of the innate immune system, which play a role in the control of several types of tumors and microbial infections (49). SGE from *Dermatocentor reticulatus* ticks that fed for several days on mice, lowered human NK cell activity, while SGE from unfed or 1 day-fed ticks had no effect (50). A similar but less pronounced anti-NK cell activity was observed for SGE from *Amblyomma variegatu*m and *Haemaphysalis inermis* ticks (51). In addition, it was reported that SGE from *Ioxides ricinus* suppressed the cytotoxicity of mouse NK cells in vitro (52).

The role of mononuclear phagocytes in tick infestation

Mononuclear phagocytes are phagocytosing antigen-presenting cells and produce a wide range of cytokines and chemokines (1). There are two major subpopulations: bone marrow-derived hematopoietic mononuclear phagocytes, which are either monocytes or pro-inflammatory macrophages, and tissue-resident macrophages, which specialized to tissues such as the skin (53). A slight increase in the presence of macrophages and monocytes was observed at the site of tick attachment in an animal model (54). Furthermore, in a recent study that characterized the early immune reaction (>24 hrs.) in human tick bite skin, a predominance of macrophages was observed at the site of tick bite (55). Many interactions between SGE or tick saliva and macrophages have been identified, suggesting that tissue-resident macrophages play an important role in the host defense against ticks. On the other hand, several macrophage functions were found to be inhibited by tick saliva or SGE, such as nitric oxide and superoxide

production, proinflammatory cytokine secretion, the expression of co-stimulatory proteins and phagocytosis (56-59).

The role of neutrophils in tick infestation

Neutrophils are granulocytes, short-lived and highly motile cells, that have both phagocytosis and degranulation roles. They represent the first line of defense of the innate immune system and play an essential role in acute inflammation by recruiting other leukocytes (60). Studies with different animal models infested by hard ticks showed that neutrophils are the most abundant cells in the inflammatory infiltrate at the tick attachment site during primary infestation but not in subsequent infestations (61-64). The expression of the neutrophil specific chemokines CXCL1 and CXCL5 was detected already 12 hrs. after tick attachment to the host (65). Interestingly, it was suggested that the tissue damage at the site of tick attachment is caused by neutrophils, as collagen destruction beneath the tick mouthparts of *R. sanguineus*-infested dogs could be prevented by neutrophil depletion (66). Several studies show that tick saliva inhibits proinflammatory neutrophil properties, including neutrophil reactive oxygen species production and phagocytosis. However, the formation of neutrophil extracellular traps, which are formed by extrusion of DNA, was not inhibited by SGE (67, 68).

The role of dendritic cells in tick infestation

Dendritic cells (DC) are professional antigen-presenting cells that induce the adaptive immune response. There are two distinct DC functional states: immature DC, which are located among others in skin and primarily take up and present antigens; and mature DC, which are potent stimulators of naive T-cell responses. Langerhans cells (LC) are a specialized immature DC population of the vertebrate skin and share some similarities with macrophages. Several chemokines are involved in the migration of immature DC from bone marrow and blood to peripheral tissues
(69).

Interactions between DC populations and tick saliva have been reported in several studies (70). An *in vitro* study demonstrated that LC stimulated with tick salivary antigens primed lymphocytes from tick-sensitized guinea pigs, indicating that LC play a role in stimulating an immune response during tick infestation (71). Another study in guinea pigs demonstrated that the infestation with *Dermacentor andersoni* led to a decrease in the frequency of LC around the sites of tick attachment. This may indicate LC migration to lymph nodes after contact with tick salivary components (72). However, a study in mice showed that *I. ricinus* saliva inhibited DC migration to draining lymph nodes (73). Furthermore, Oliveira et al. showed reduced migration of immature DC that have been stimulated with *R. sanguineus*-SGE, in response to MIP-1α, MIP-1β and RANTES chemokines. The reduced migration upon SGE stimulation could be explained by the downregulation of the surface receptor CCR5 on DC. In addition, they also observed that the DC turnover in skin of mice injected with SGE was reduced, suggesting that saliva has an effect on the DC recruitment to the skin (74). A possible reason for impaired DC migration may be the chemokine neutralizing properties of tick saliva (70). The recruitment of immune cells to the site of tick feeding is impaired by several tick salivary components, such as evasins. Evasins are produced by several hard tick species and bind to host chemokines, such as CCL3, CXCL8, CXCL1 or CCL5 to inhibit the activation of chemokine receptors and thereby suppress the host inflammatory response (18). In addition, some tick salivary

immunoregulatory peptides overcome host inflammation by modulating cytokine expression. For example, the non-proteinaceous substance prostaglandin E2 (PGE2), present in the saliva of *R. sanguineus* impairs the production of pro-inflammatory IL-12p40 and tumor necrosis factor alpha (TNF-α) and stimulates the release of anti-inflammatory IL-10 by murine DC (75). Conversely to the study of Oliveira et al., in another study it was observed, that dendritic cells were abundant in early human tick bite lesions (>24 hrs) (55). Furthermore, tick saliva can modulate diverse aspects of DC functionality, such as phagocytosis and the expression of co-stimulatory molecules and it can alter antigen presentation and processing and thereby prevent the initiation of the adaptive immune response (73, 76, 77). For example, RHS2 from *R. haemaphysaloides* can suppress the differentiation of bone marrow-derived cells into DC *in vitro* (78).

The role of T cells in tick infestation

T cells represent the main sensory arm of the adaptive immune system. Mature T cells are distributed throughout the whole body in peripheral lymphoid tissues and in the circulation. There exist two major T cell subpopulations: $CD4^+$ and $CD8^+$ T cells, described according to the receptor expressed on their surface. $CD8^+$ T cells can mature into cytotoxic T-cells capable of lysing target cells. $CD4^+$ T cells, also known as T helper (Th) cells, mature by recognizing peptides presented by antigen-presenting cells via MHC class II receptors (Figure 3). There are several $CD4^+$ Th cell subpopulations that have different roles in immune responses (79). Th1 populations, which are associated with host cellular and inflammatory responses and Th2 populations, which are associated with host humoral responses, are the best studied populations in tick-host interactions (80). T cells are important regulators and effectors of host immune responses to ticks (16) and they have been implicated in acquired tick resistance. In fact, tick resistance could be inhibited by depleting T cells in guinea pigs (81). Moreover, CD4+ tissue resident memory T cells, which were found to accumulate in the tick bite lesion, may play an important role in tick resistance by promoting skin infiltration of basophils (82).

Several studies showed that tick saliva and SGE affect T cell proliferation and functionality in multiple ways (1). SGE derived from *I. ricinus, Amblyomma variegatum* and *R. appendiculatus* affected human lymphocyte proliferation with species and sex-specific differences *in vitro*. It was observed both stimulatory and inhibitory effects on proliferation (83). In Concanavalin A (ConA)-activated murine T cells, *I. ricinus*- derived SGE treatment reduced the expression of early activation marker CD69 (84). However, soluble salivary gland antigens derived from *I. ricinu*s ticks stimulated the proliferation of lymph node T cells from mice infested with *I. ricinus* (85). In addition, a recent study that characterized the early local immune reaction after *I. ricinus* tick bites in human skin, showed that lymphocyte numbers increased at the site of tick bite within 24 hrs after the tick bite (55). Interestingly, feeding of *Haemaphysalis bispinosa* and *Hyalomma anatolicum anatolicum* ticks on sheep even led to reduced circulating T cell numbers in the peripheral blood (86).

Different studies have shown that tick saliva or SGE polarizes the immune response towards a Th2 response by the downregulation of Th1 and the upregulation of Th2 cytokines (87). Lymphocytes isolated from murine lymph nodes draining the infestation site of *I. ricinus* nymphs produced high levels of the Th2-cytokine interleukin (IL)-4 and low levels of the Th1 cytokine interferon-gamma (IFN-γ) after *in vitro* stimulation with ConA (88). In another study, tick-mediated T cell cytokine production was analyzed using human peripheral blood

mononuclear cells (PBMCs) stimulated with ConA or lipopolysaccharide (LPS). In line with the results in mice, SGE from *I. ricinus* ticks significantly inhibited the *in vitro* production of IL-2 and IFNγ and stimulated IL-4, IL-6 and IL-10 production by human PBMCs significantly (89). A potential mechanism involves tick saliva-pulsed DC, which stimulated sensitized $CD4^+$ T cell proliferation and the differentiation of naive $CD4^+$ T cells into Th2-type cells *in vitro*. Corresponding *in vivo* experiments, where saliva-pulsed DC were injected subcutaneously into BALB/c mice also led to a Th2 immune response (90). Many tick salivary compounds have been identified to inhibit T cell functions. For example, Sialostatin L inhibits T cell proliferation (91). In addition, Salp15 specifically suppresses $CD4^+$ T cell activity by binding to the CD4 co-receptor (92).

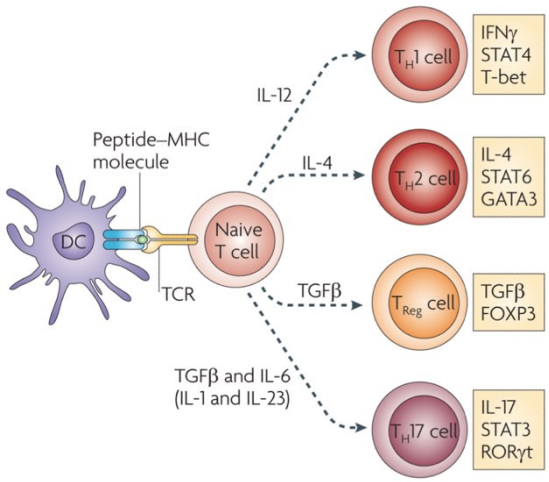


Figure 3. Differentiation of helper T cell subclasses.

After activation by antigen-presenting cells, naïve $CD4^+$ T cells can be polarized into Th1, Th2, Treg and Th17 cells depending on the local cytokine mileu. The key cytokines of Th1, Th2, Treg and Th17 cells are IFN-γ, IL-4, TGFβ and IL-17, respectively. (6)

The role of B cells in tick infestation

B cells secrete immunoglobulins upon activation and play an important role in adaptive humoral immunity. After they have been stimulated by follicular helper T cells, expanded and selected in germinal centers, B cells produce mono-specific high-affinity antibodies (93). Although initial histological studies were not able to identify B cells in healthy human skin, more recent studies in sheep and humans demonstrated the existence of skin-recirculating B cells (94). B cells may secrete specific antibodies against tick salivary and gut antigens. The role of antibodies in tick-resistance is uncertain. However, B cell-depletion with cyclophosphamide has been shown to partially block guinea pig resistance to *D. andersoni* (95).

Several studies showed that tick infestation can suppress total IgG and IgM production *in vitro* and *in vivo* (96, 97). It was suggested that tick infestation impairs the local differentiation of mature B cells into plasma cells, but does not alter the formation of memory B cells (98). Furthermore, SGE inhibited LPS-induced murine B cell proliferation *in vitro* (84). In contrast, B cell activity in the lymph nodes in response to LPS was increased in mice that have been bitten by a tick compared to naive mice (99). In addition, both primary and secondary infestations of sheep with *H. anatolicum anatolicum* ticks led to an increase in blood circulating B lymphocytes over several days (86). Many tick salivary compounds suppress B cell responses.

One of the more intensely studied factors is the B cell inhibitory protein (BIP), which is derived from *I. ricinus* and inhibits B cell proliferation induced by *B. burgdorferi* molecules (100).

Overall, a complex immune cell network in human skin is involved in the response to blood-feeding ticks, and arthropod salivary compounds influence the local milieu in multi-facetted ways (Figure 4).

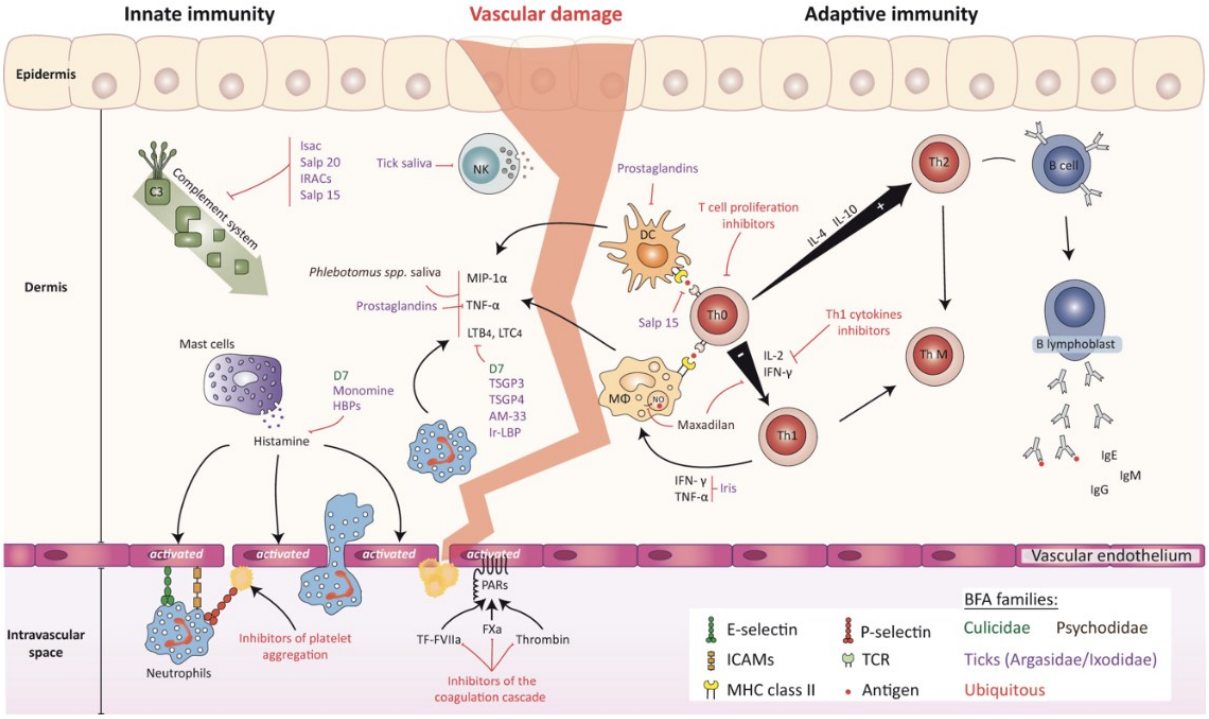


Figure 4. Graphical representation of innate and adaptive immune cells involved in immunity against blood feeding arthropods (BFA) and immunomodulation by salivary proteins.

Cells involved in the innate response (for example neutrophils, natural killers cells (NK), mast cells and macrophages (MΦ)) represent the first line of defence against ticks. Antigen-presenting cells, such as dendritic cells (DC) migrate to the lymph nodes where they interact with naïve $CD4^+$ Th lymphocytes. Tick saliva in general downregulates the expression of Th1 cells and upregulates the expression of Th2 cells. The effect of saliva or salivary proteins as well as their corresponding BFA organism's family are indicated in the bottom right corner legend, tick salivery proteins labelled in purple. (101)

Changes in the immune cell infiltrate during early Lyme disease

Lyme disease incidence

Lyme disease, which is caused by *Borrelia burgdorferi* sensu lato species and transmitted by *Ixodes* ticks, is the most common tick-borne disease in the USA and Europe. It is a multisystemic, multistage, inflammatory infection that may result in patients experiencing cardiac, neurological and arthritic complications, when not treated with antibiotics shortly after exposure. Each year, thousands of patients suffer from Lyme borreliosis and there is still a significant gap in effective diagnosis, targeted treatment, or sufficient preventative measures (102). In western Europe the incidence rate of Lyme borreliosis was estimated to be 22.05 cases per 100 000 people per year (103). Austria is one of the countries with the highest incidence of Lyme disease in Europe, along with Germany, Sweden and Slovenia. Furthermore, as a consequence of global temperature rise, *B. burgdorferi*-infestation of ticks and Lyme disease

are continually emerging (Figure 5). A study has predicted that the number of Lyme disease cases in the United States will increase by over 20 % in the coming decades (104).

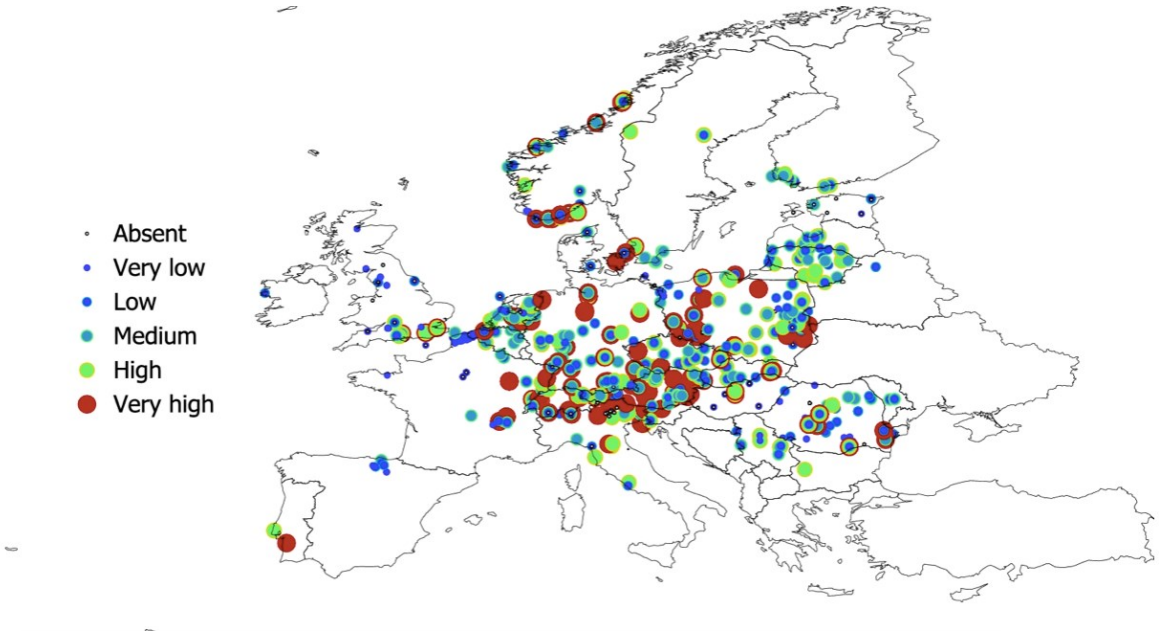


Figure 5. Reported prevalence and distribution of B. burgdorferi s.l. in nymphs of I. ricinus in Europe.

The size and the color of dots define the prevalence, location on map presents coordinates of sampling sites. (2)

Borrelia genospecies

B. burgdorferi sensu lato includes 20 different genospecies. The most common species of Lyme Borrelia are *B. afzelii*, *B. garnii* and *B. burgdorferi* sensu stricto. The genospecies are responsible for Lyme disease in different geographical regions. For example, *B. afzelii* and *B. garnii* are primarily responsible for Lyme borreliosis in Europe, whereas *B. burgdorferi* s.s. causes Lyme disease in the USA. It was suggested that the heterogeneity of *Borrelia burgdorferi* sensu lato spieces is causing the regional differences in clinical manifestations of Lyme disease. For example, *B. burdorferi* s.s. is causing arthritis, *B. afzelii* particularly causes skin infections and *B. garnii* is primarily neurotrophic (105).

Lyme *Borrelia* transmission and dissemination

Lyme borrelia species live in the midgut of unfed *Ixodes* ticks and move transiently through the tick salivary glands upon initiation of tick feeding. *B. burgdorferi* spirochaetes can persist in the tick's gut by binding the tick midgut protein (TROSPA)(106). When infected ticks take a bloodmeal, the carried *Borrelia* receive signals from tick engorgement. *Borrelia* increase in numbers and undergo a transformation from a state adapted to tick colonization to a state primed for infection of the vertebrate host (107). During feeding, the infected tick deposits *Borrelia* into the skin of hosts. Subsequently, spirochaetes first multiply locally and then disseminate to other locations (108). Intravital microscopy showed, that GFP-expressing *B. burgdorferi* immediately become motile within mouse skin after inoculation (109). After 48 hours, spirochaetes begin dissemination from the inoculation site through the dermis, especially in collagen-rich areas (110, 111).While some spirochaetes were found to migrate through the

blood stream, the majority appears to migrate through the dermis or the lymph vessels (8, 112, 113). *B. burgdorferi* can transiently inhabit almost any host tissue, even the meninges (8, 114).

The role of ticks in facilitating *B.burgdorferi* transmission and dissemination

The establishment of *B. burgdorferi* infection is substantially supported by the tick in two ways: First, the penetration of the host skin by the tick enables the delivery of *Borrelia* deep into the dermis (Figure 6). Secondly, immunomodulary tick salivary compounds aid the establishment of an infection by modulating host activities (115). Co-inoculation of *Borrelia burgdorferi* species with *I. scapularis* or *I.ricinus* salivary gland extract (SGE) into mice increased the pathogen burden in multiple organs compared to needle inoculation (116). In addition, when tick saliva was present together with *Borrelia* or just before the inoculation, a longer persistence of the bacteria in the skin was observed (117). This underlines the importance of studying natural tick-borne *Borrelia* infection.

The tick salivary components that facilitate *B. burgdorferi* transmission and dissemination are only partially defined. The early effect of tick saliva on the spirochaete survival is suggested to be mediated by the inhibition of cutaneous innate immune mechanisms. Factors that delay inflammation at the site of tick bite are important for the survival of *B. burgdorferi* in the skin. For example, the inoculation of sialostatin L2 with *B. burgdorferi* into mouse skin significantly increased the spirochaete burden by inhibiting the *Borrelia*-induced chemokine response and Toll-like-receptor signaling pathways (118).

Since *B. burgdorferi* is an extracellular bacterium, factors that interfere with the activity of phagocytes, the complement system or the production of antibodies are essential for its survival (8). For example, *I.ricinus* salivary gland extract suppresses macrophage production of reactive oxygen species and killing of *B. afzelii* by murine macrophages *in vitro* (56). Furthermore, it was reported that tick salivary compounds modulate adaptive immunity by skewing T cell differentiation towards a Th2 phenotype. This may aid *B. burgdorferi* infection by reducing the pro-inflammatory effect of Th1-associated cytokines on phagocytes (8). In line with this, repeated tick infestation of C3H/HeN mice led to a reduced production of IL-2 and IFN-γ and an increased production of the anti-inflammatory cytokines, IL-4 and IL-10 after ConA stimulation, potentially suppressing phagocyte activation and clearance of *B. burgdorferi*. In the same study, treatment of C3H/HeN mice with TNF-α, IL-2 or IFN-γ for 10 days after *B. burgdorferi*-infected tick feeding, led to partial protection from infection (119).

Interestingly, *B. burgdorferi* uses the tick salivary protein, Salp15, to colonize the bloodmeal host. The expression of Salp15 is increased in infected tick salivary glands and it binds to Outer surface protein C (OSPC) on *B. burgdorferi* as they enter the host. The binding of Salp15 to OSPC can protect *B. burgdorferi* from antibody-mediated killing *in vitro*. The injection of recombinant Salp15 with *B. burgdorferi* increased their survival in mice and the Salp15-depletion in infected ticks significantly decreased spirochaete infectivity (120). In addition, Salp15 might also reduce stimulation of host innate and adaptive immune responses by *B. burgdorferi*, such as $CD4^+$ T cell activation (121, 122).

Lastly, also the complement system is affected by tick feeding. For example, the tick salivary lectin pathway inhibitor (TSLPI) interacts with lectin pathway recognition molecules and thereby inhibits complement activation reducing complement-mediated lysis of *B. burgdorferi* (123). In addition, the expression of complement regulator-acquiring surface proteins (CRASPS) and antigenic variations of *B. burgdorferi* surface lipoproteins prevent complement-mediated killing (124, 125).

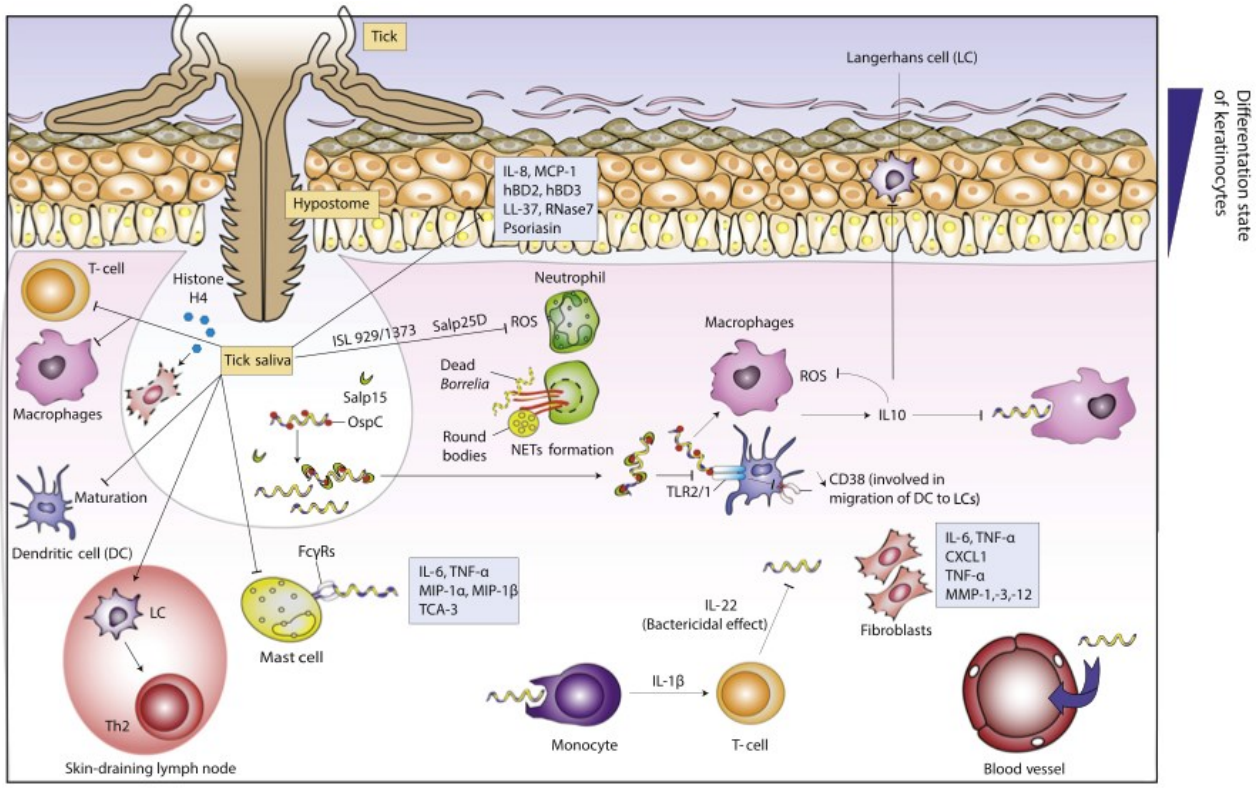


Figure 6. Tick saliva aids B.burgdorferi transmission.

Penetration of the host skin by the tick hypostome enables the delivery of *Borrelia* deep into the dermis. Tick saliva contains anti-inflammatory molecules which may aid *B. burgdorferi* transmission. For example, Salp-15 binds to OSPC on *B. burgdorferi* to prevent bacterial recognition by immune cells. (3)

Immune responses to *B. burgdorferi*

Despite intricate immune evasion mechanisms, *B. burgdorferi* is still recognized by both innate and adaptive immune responses (105). However, studies in disease-resistant mice indicate that the disease associated with *B. burgdorferi* infection is not a result of the pathogen causing damage, but rather a manifestation of the inflammatory response to the pathogen (126). Furthermore, Lyme borrelia species are not known to produce toxins and therefore most of the tissue damage seems to be caused by host inflammation (105).

Skin resident immune cells are especially important when *B. burgdorferi* enters the host, replicates and disseminates (8). The first resident immune cells that come into contact with the disseminating spirochaetes are epidermal LC and dermal DC(25). Electron microscopy studies showed that LC were associated with *B. burgdorferi* in the epidermis of patients affected by erythema migrans, an early manifestation of Lyme borreliosis. In addition, an *ex vivo* model using full thickness human skin tissues showed that *B. burgdorferi*-injection led to LC activation and increased tissue emigration (127). However, an in vitro study found that in contrast to dDC, LC were not able to phagocytose *B. burgdorferi* (128). Nevertheless, the

adoptive transfer of *B. burgdoferi*-pulsed LCs and DC into mice induced a protective immune response against *B. burgdorferi* and stimulated the production of specific antibodies *in vivo* (129). However, studies suggested that this response is less robust than that of other LPS-bearing bacteria, such as *E.coli* and the response is further impaired by the immunomodulatory mechanisms of tick saliva (71, 130).

The spirochaetes encounter cells of the myeloid lineage as they migrate through the dermis (8). Several *in vitro* studies showed that these cells are able to capture and phagocytose a part of the spirochaetes even though they travel much slower than *B. burgdorferi* (Figure 7) (131-133). *B. burgdorferi* phagocytosis leads to a potent inflammatory response evoking a wide range of pro-inflammatory mediators, such as cytokines, chemokines, leukotrienes and eicosanoids. On one hand, these mediators promote the clearance of the spirochaete. On the other hand, they also promote the inflammatory pathology typical for Lyme disease (8, 134, 135). In summary, the response of LC, DC and macrophage subsets to *B. burgdorferi* inoculation seems to only partially protect from infection and its detrimental effect on prolonged pathogen-independent inflammation remains to be defined.

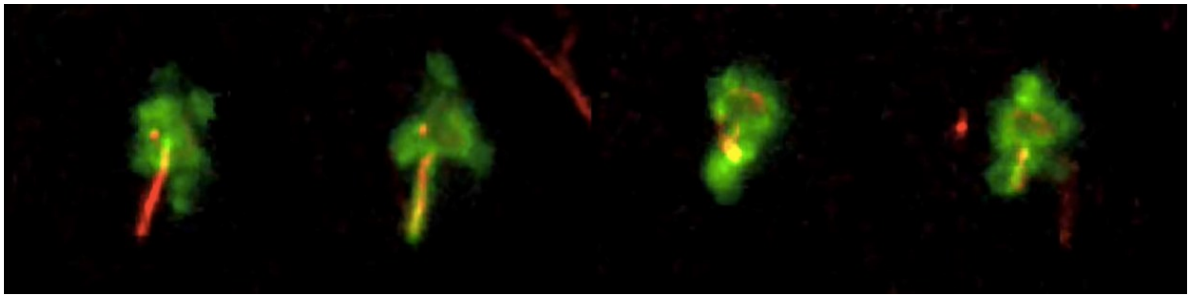


Figure 7. Borrelia phagocytosis by dermal APCs

Transgenic mice (I-Aβ-GFP expressing), displaying green fluorescently labelled MHC class II-expression, were infected with DsRed-expressing *B. burgdorferi* intradermally into the ear. Ear tissues were visualized using intravital confocal microscopy on living mice at 24 hrs post-infection. Image shows phagocytosis process of *B. burgdorferi* by a MHC class II-expressing APC (8)

Since *B. burgdorferi* is an extracellular pathogen, neutrophils might play an important role in controlling the spirochaete. The injection of *B. burgdorferi* or their lipoproteins into murine skin led to a rapid neutrophil infiltration (136, 137). However, while there is a significant neutrophil influx within 6 hrs post infection, neutrophil numbers rapidly drop 12 hrs after infection. Almost no neutrophils are found in the site of *Borrelia*-infection by day 7 post infection, although there are still spirochaetes present. It was suggested that this downregulation of the neutrophil influx may aid the dissemination of the spirochaete (138). Interestingly, in tick-borne *B. burgdorferi* infection, inflammatory cell numbers, like neutrophils were elevated in the tick bite site during the first 3 days of tick attachment, but nearly absent in the area immediately adjacent to the tick hypostome, were *B. burgdorferi* is deposited (28). Several in vitro studies demonstrated that neutrophils can efficiently phagocytose *B. burgdorferi* (139, 140). When *B. burgdorferi* was modified to express the neutrophil chemoattractant KC constitutively, there was substantial neutrophil infiltration at the inoculation site, and as a result the host was much more capable of controlling spirochaete infection. This shows that neutrophils can be deleterious to *B. burgdorferi* survival (138). Overall, the findings suggest

that neutrophils are important to control *B. burgdorferi* initially, but there is a lack of continuous neutrophil activation and recruitment to the site of infection.

Literature describing the importance of NK cells during acute *B. burgdorferi* infection is limited (8). Isolates of NK cells from mice became activated and produced IFN-γ *in vitro* after their exposure to *B. burgdorferi* or its surface proteins OSPA and OSPC. However, depletion of NK cells in mice did not appear to change *B. burgdorferi* numbers in multiple tissues (141). Previous studies suggest that NK cells primarily play a role during persistent Lyme disease and Lyme arthritis (142, 143). Thus far, the role of other innate lymphoid cell (ILC) subsets in acute *B. burgdorferi* infection has not been investigated.

Since *B. burgdorferi* is introduced into and persists within skin tissues, it is possible that skin-resident mast cells play a role in the control of the spirochaete infection and *B. burgdorferi*-induced inflammation (8). In fact, *B. burgdorferi* infection of gerbils led to increased numbers of mast cells in most tissues, including skin (144). An *in vitro* study determined that *B. burgdorferi* induce degranulation and the release of TNF-α by rodent and murine mast cells (145). However, a study with mast cell-deficient mice (Kitwsh$^{-/-}$ mice) revealed no significant involvement of mast cells in *B. burgdorferi* multiplication in the skin and its dissemination, although *B. burgdorferi* was detected in the joints slightly earlier than in wild type mice (146). Overall, there is limited data suggesting that mast cells play an important role in defense of disease (8).

Mice with severe combined immunodeficiency (SCID), which lack T and B cells, harbored similar numbers of *B. burgdorferi* in blood and tissues than in wild type mice early in infection but significantly greater numbers at 8 weeks of infection, indicating that the adaptive immune response is involved rather in later stages of infection (147, 148). Several studies indicate that B cells play a major role in the control of *B. burgdorferi* infection (149, 150). Mice depleted of B cells (Igh-6$^{-/-}$ mice) demonstrated significantly increased pathogen burdens in various tissues. In addition, B cells are essential to prevent or resolve Lyme-arthritis, -carditis and -myocarditis (150). During *B. burgdorferi* infection, B cells are activated, and lymph nodes significantly increase in size and cellularity, with much of the increase contributed by the proliferation of B cells (112, 151). The activation of B cells is affected by extrinsic and intrinsic inflammatory signals that act together with *Borrelia* antigens to stimulate strong B cell responses in the lymph nodes of infected mice. These responses led to significantly increased IgM- and IgG-concentration throughout the infection (110).The transfer of serum from previously infected mice or humans and the transfer of B and T cells, but not T cells alone into SCID or B/T cell-deficient Rag1$^{-/-}$ mice diminished the pathogen burden and resolved arthritis, indicating that antibodies may play role in the control of *B. burgdorferi* infection (147, 150, 152). Antibodies induced by *B. burgdorferi* infection were shown to kill spirochaetes by both complement-dependent and complement-independent spirochaete lysis (149). Antibodies can also opsonize the spirochaetes for uptake and degradation by macrophages and neutrophils (153, 154). However, produced antibodies are unable to fully clear *B. burgdorferi* infection (8). In fact, functional germinal center responses do not evolve after *B. burgdorferi* infection in mice, leading to a failure to sustain antibody affinity maturation and a failure to induce long lived plasma cells and memory B cells (155-157).

Similar to NK cells, human invariant NKT cells are activated in response to *B. burgdorferi* infection and produce IFN-γ. Thereby they may increase macrophage-mediated phagocytosis of *B. burgdorferi* and thus could reduce the *B. burgdorferi* burden. In addition, they could

stimulate the recruitment of macrophages and neutrophils by driving the upregulation of CD1d and changing the chemokine milieu (158-160).

The effect of T cells on host defense during *B. burgdorferi* infection is not completely understood. Studies with mice depleted of T cells (TCRβ/$\delta^{-/-}$) or with mice lacking $CD4^+$ T cells, showed surprisingly modest effects on the course of *B. burgdorferi* infection. These effects include an increase in the spirochaete tissue burden and enhanced arthritis severity (150, 156, 161-163). In contrast, $CD8^+$ T cells appear to promote the disease, possibly by interfering with the development of protective immunity, as the depletion of this subset *in vivo* led to a reduction in arthritis and a decrease in the spirochaete burden (161). The apparently small effects of T cells on *B. burgdorferi* infection are surprising, given the relevance of T cells as crucial effectors and regulators of anti-bacterial immune responses (8).

$CD4^+$ T cells polarized to express the transcriptional regulator RORγt and secreting the pro-inflammatory cytokine IL-17 (Th-17 cells) are thought to control extracellular pathogens, like *B. burgdorferi*. Through cytokine secretion, these cells recruit neutrophils to the site of infection, which then kill the pathogen (164). Surprisingly, in acute *B. burgdorferi* infection, IL-17 depletion did not affect the spirochete burden, the course of disease or the magnitude of the antibody response (165). However, interpretation of this data is complicated since IL-17 depletion can stimulate an increase in T-bet expression and IFN-γ production, which may mask some of the observed effects by stimulating Th-1 polarization (8).

A dominance of Th-1 cytokine IFN-γ was observed in several tissues affected by Lyme disease (166). Early studies using adoptive T cell transfer suggested, that the stimulation of a Th1-polarization of $CD4^+$ T cells could drive the inflammatory pathology seen in mice infected with *B. burgdorferi* (150) and that a Th2 response attenuated these effects (167). The Th-2 cytokine IL-4 seems to play a protective role in murine disease. Spleen cells from Borrelia-susceptible C3H mice produced significant lower levels of IL-4 compared to Borrelia-resistant Balb/c mice and the depletion of IL-4 led to more severe arthritis and higher spirochaete burden (167, 168). Borrelia-resistant Balb/c mice initially had high levels of IFN-γ, but then switched to the production of IL-4 (169). Overall, the T cell response in Lyme disease is a double-edged sword, as it both promotes the reduction of the spirochaete burden, but also supports inflammation and tissue destruction (8).

Translating research in animal models to human Lyme disease

There are significant differences between the immune responses that are engaged by *B. burgdorferi* in mice and in humans. Laboratory mice can show inflammatory lesions in hearts and joints and develop chronic inflammation in tissues. However, these pathologic changes do not replicate the clinical manifestations seen in human Lyme borreliosis (126, 170). Interestingly, lymphocytes are prevalent in human tissues, whereas innate immune cells are dominant in inflammatory lesions in mice. This may explain why mice exhibit an immune program more permissive to silent persistent infection than the immune reactions that arise in non-reservoir hosts, such as humans (8).

Without antibiotic treatment, *B. burgdorferi* can persist in humans despite the wide range of immune responses. It leads to a broad spectrum of tissue pathology, most often clinically visible in skin as erythema migrans, lymphocytoma or Acrodermatitis chronica atrophicans (ACA), the heart as conduction system abnormalities and myocarditis, the nervous system (cranial nerve abnormalities, meningitis, meningo-encephalities and radioculoneuropathologies) and

the joints (proliferative synovitis). While clinical manifestations in the skin (except for ACA), heart and the nervous system manifest itself in the first few weeks and months, ACA and arthritis may occur much later (Figure 8) (105).

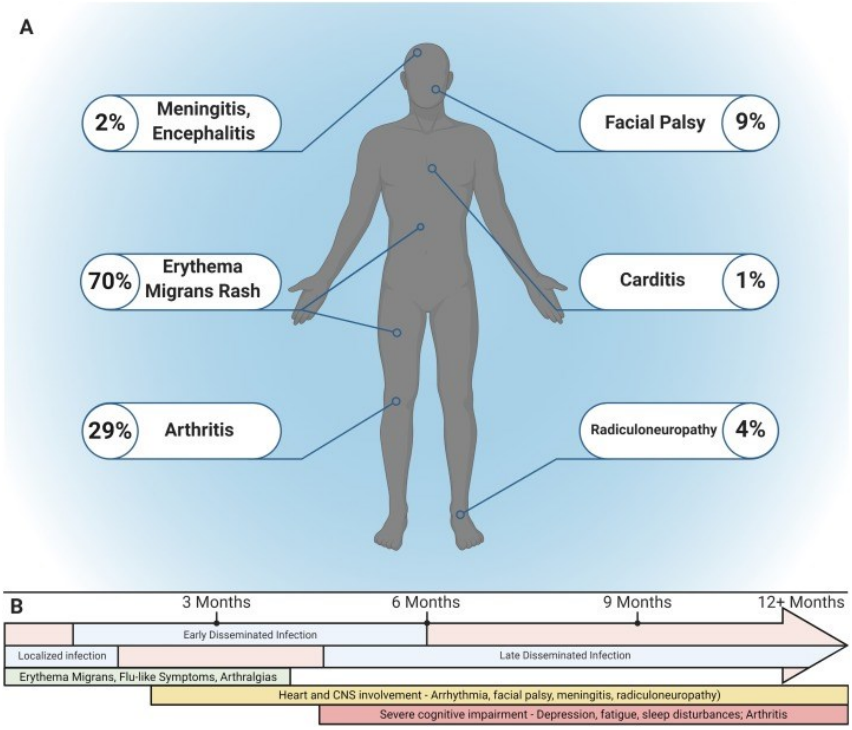


Figure 8. Summary of human Lyme disease symptoms and disease timeline.

The most common symptom of human Lyme disease is the erythema migrans (EM) rash occurring within first weeks of infection. Arthritis is typically seen in 29 % of patients, while neurological and cardiac manifestations are less common and typically occur in late infection stages. (7)

Erythema migrans

Localized early infection normally manifests as an expanding rash, known as erythema migrans (EM, Figure 9). Erythema migrans is the most common manifestation of Lyme borreliosis, and appears in around 70% of borrelia infections during the first 3 weeks after inoculation, shown in a case series in the United States (171). It manifests itself as red oval or round patch with centrifugal expansion that may show a central clearing. Systemic symptoms, such as malaise, lymphadenopathy and fever, may follow EM. The EM lesions may persist for weeks to months without treatment (105).

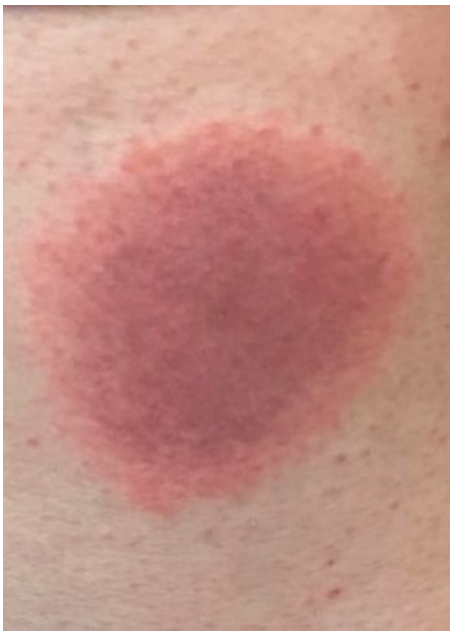


Figure 9. Erythema migrans

Clinical image of erythema migrans rash on the tigh.

Several studies showed that EM lesions are characterized by an immune cell infiltrate comprised of T cells, DC, macrophages, and rare plasma cells and lower proportions of neutrophils, with T cells dominating the pathology (166, 172, 173). Some studies also report an increased number of eosinophils in the lesions (172, 174). In addition, a recent study found that B cells, bearing plasma cell and memory B cell signatures were more abundant in EM lesions compared to autologous uninvolved skin (175). The characterization of EM lesions by immunohistochemistry demonstrated diverse patterns perivascular and interstitial immune cell infiltrates. EM perivascular infiltrates consisted dominantly of T lymphocytes, whereas interstitial infiltrates were dominated by $CD68^+$ macrophages (173, 174).

The size of the EM lesion increases with time, and smaller lesions harbor more spirochaetes than larger ones, indicating that the immune response is effective in reducing the bacterial burden in human skin (176). The observed cutaneous immune responses were reflected, at least in part, in peripheral blood (166).

A broad analysis of cytokines and chemokines in patients with EM, revealed that Th1- and Th-17-associated cytokines and chemokines were increased in EM lesions (177). Furthermore, it was observed that IL-6 and IFN-γ were the dominant cytokines in EM lesions (166). It was assumed that interactions between these two cytokines may promote *B. burgdorferi* clearance and relieve tissue damage. While IFN-γ has macrophage activating effects, which can increase their capacity to clear spirochaetes (132, 178), IL-6 promotes the recruitment of monocytes to the site of infection and their subsequent differentiation into macrophages in the skin (179). In addition, the number of chemokines that attract neutrophils (CXCL1), macrophages (CCL3, CCL4) and T cells (CXCL9, CXCL10, CXCL11) were elevated in EM lesions (180). In fact, a gene array study revealed that CXCL9, CXCL10 and CXCL11were the most upregulated genes in EM lesions. This is consistent with the dominance of IFNγ-producing $CD4^+$ Th1 cells in EM lesions, since these chemokines are important for the migration of $CD4^+$ Th1 cells and $CD8^+$ T

cells. In addition, CXCL9 and CXCL10 induce the polarization of $CD4^+$ T cells into Th1 and Th17 cells, whereas CXCL11 promotes T cell polarization into Treg cells. Furthermore, signatures for innate immune responses, cell migration and chemotaxis and IFN responsive genes were prominent among genes upregulated in EM lesions. Interestingly, they found that there is a significant increase in enzymes controlling the tryptophan metabolism, like IDO 1 (Idoleamine 2,3-dioxygenase 1). Tryptophan depletion inhibits CD8+ T-cell priming and stimulates the development of Treg cells (181). Overall, several studies showed that the immune response to *B. burgdorferi* is both complex and nuanced and requires many cell types, soluble mediators and varies based on the organ system affected by infection with this spirochaete (8).

Aims

Aim 1: Identify changes in the immune cell infiltrate after tick bite

As a consequence of climate change, ticks and tick-borne diseases including Lyme disease, are on the rise and the expansion of the distribution range of *Ixodes* species towards northern latitudes and higher altitudes is predicted in all of Europe (12-14). Since ticks constitute a growing threat for human health, it is important to study tick-host-pathogen interactions in detail (182).

The *in vitro* immunomodulatory effects of tick salivary compounds have been studied thoroughly. In addition, several studies have assessed tick feeding lesions by high throughput and histological methods, contributing to our knowledge of the dynamic processes taking place at the tick host interface (54, 55, 61, 65). These studies found that nearly all local immune cell types are affected by tick feeding and while some immune cell responses are induced by tick salivary compounds, others are suppressed, providing an advantage for the transmission of tick-borne pathogens (1). However, many of the consequences of natural tick bites on the local and circulating immune system of the human host are unknown due to a lack of model systems and studies in human skin. The investigation of natural tick bites in humans in contrast to animal models is especially important, as it was shown that the tick salivary composition is modulated by host skin components and differs between hosts, potentially contributing to differences in host immune responses (183).

The first aim of the study was to perform a systematic investigation of immune cells at human tick feeding sites and in blood samples of affected individuals, providing insights to the immune reaction to tick bites and to the immunomodulary capacity of tick saliva. According to the literature, we had hypothesized that some immune cells of first defense including neutrophils, accumulate at the site of tick bite. In contrast, we expected antigen-presenting cells to locally decrease after tick bite. In addition, we had hypothesized that T cell and ILC responses are impaired after tick bite, leading to an advantage for the transmission of tick-borne pathogens. To address this aim, we quantified immune cells in human natural tick bite lesions and in blood by flow cytometry. Furthermore, we investigated Th1, Th2 and Th17 responses as well as type-1, -2 and -3 ILC responses in cells isolated from human hosts using cytokine assays.

Aim 2: Identify changes in the immune cell infiltrate during early Lyme disease

Early Lyme borreliosis and erythema migrans have been characterized thoroughly from the standpoints of epidemiology, clinical characteristics, microbiologic and serologic diagnosis and therapeutic outcomes (105, 176, 184, 185). However, there are only few studies characterizing the immune cell infiltrate and response during early active and resolved Lyme disease in humans (166, 174, 175, 181). Furthermore, much of our knowledge about the immune response to *B. burgdorferi* has been derived from *in vitro* studies of the interactions of cultured *B. burgdorferi* with cells and from studies in inbred mice. It is important to study natural tick-borne *B. burgdorferi* infection in humans, because *B. burgdorferi* rapidly change its gene expression depending on its environment and there is a difference between inbred mice, which are surrogate reservoir hosts and non-reservoir hosts, like humans (8).

Hence, the second aim of this study was to characterize the immune cell infiltrate of EM lesions and peripheral blood in humans suffering from early Lyme disease. We had hypothesized that there is a pronounced immune reaction taking place in the EM lesions, which is in part still visible in resolved EM lesions. Furthermore, we hypothesized, that there is a difference in the immune cell infiltrate between the border and the center of the rash, which leads to the phenotypic differences. Lastly, we wondered whether the immune cell response is still attenuated in EM lesions due to long lasting immunosuppressive effects of tick saliva. To determine cellular players at the tick bite site, in rash expansion, and in disease remission, we investigated differences in the cell infiltrate between the center and the border of the annular rash and between active and resolved EM by flow cytometry. In addition, we also performed immunofluorescene staining's of *B. burgdorferi* with blood and lymph vessels in tissue biopsies of EM lesions, to determine via which routes *B. burgdorferi* is spreading to distant sites.

Results

Part 1 - Changes in the immune cell infiltrate after tick bite

Analysis of tick feeding sites reveals a pattern of immunomodulation in human skin

To provide insights to the early cutaneous immune reaction to tick bites in human skin, we identified changes in key immune cell populations in human skin samples taken after tick bite. For this purpose, healthy individuals were recruited after tick bite (Table 1). 19 skin biopsy samples of natural tick bites and 19 intra-individual control samples were obtained and processed (Figure 10A). One of the biopsy samples was infected with *B. afzelii* as determined by PCR and was therefore excluded from further analysis. One healthy individual had suffered from Lyme borreliosis in the past and was therefore also excluded. The remaining 17 biopsy samples were collected from 17 otherwise healthy individuals (mean 47,5 years, range 23-65 years). The tick bite sites in these patients were abdomen (n= 3), chest/ neck (n=3), leg (n=7) and upper extremities (n= 2). None of the individuals experienced Lyme borreliosis or other signs of infection at the site of tick bite.

We compared single leukocyte suspensions isolated from tick bite skin to those isolated from healthy skin of the same individual by flow cytometry. First, we quantified granulocytes and found that neutrophil numbers were significantly increased in tick bite skin compared to intraindividual control biopsies (Figure 10B). This indicates that tick feeding leads to an inflammatory response. However, no significant changes in eosinophil, basophil and mast cell frequencies were detected.

Next, we investigated cutaneous antigen-presenting cells. Interestingly, we observed that $CD11c^+$ dDC as well as $CD207^+$ LC frequencies were decreased after tick feeding. No significant changes were observed in $CD11c^+CD123^+$ plasmacytoid dendritic cell numbers, $CD14^+$ monocyte numbers and $CD11b^+$ $CD68^+$ macrophage numbers (Figure 10C).

Subsequently, we assessed frequencies of lymphocytes of the adaptive immune system. Previous studies found that early *Borrelia* skin infection is characterized by an increase in B cell numbers as well as plasma cell numbers (175). However, it remains to be elucidated, whether this effect is mediated by *Borrelia* or tick feeding and associated tick salivary products. We found that the B cell frequencies were significantly increased after tick bite. However, plasma cell numbers remained unchanged (Figure 10D). In addition, we found that T cell numbers were significantly increased after tick bite. Frequencies of NKT cells and activated $CD69^+$ T cells frequencies in the T cell compartment remained unchanged (Figure 10E).

With regard to innate lymphoid subsets, we did not observe changes in the numbers of ILCs subsets and NK cells (Figure 10F).

For a better understanding of the time dependence of the above-described events, we subdivided samples into 3 categories depending on the duration between tick bite and tissue sampling (<24 hrs, n=5; 2-4 days, n= 6; 5-9 days, n= 6). The cellular effects, including T cell / neutrophil infiltration and decrease in dDC and LC, seemed to occur early after tick bite (<24 hrs) (Figure 11A, 11B). However, only Langerhans cells were significantly decreased 24 hrs after tick bite (Figure 11B). Taken together, our results indicate that despite the absence of tick-borne pathogens, tick bites lead to rapid changes in the immune cell network of human skin, which includes neutrophil and lymphocyte-mediated inflammation and a decline in antigen-presenting cells.

Table 1. Age, Lyme borreliosis history and PCR results of the patient cohort.

Sample No.	Age	Lyme borreliosis history	PCR result	Culture result	Site of tick bite
1	57	Acute erythema migrans	negative	negative	unknown
2	47	none	negative	negative	leg
3	65	none	negative	negative	abdomen
4	58	none	negative	negative	leg
5	63	none	negative	negative	upper extremities
6	27	none	*Borrelia afzelii*+	negative	chest/neck
7	56	none	negative	negative	chest/neck
8	23	none	negative	negative	leg
9	54	none	negative	negative	unknown
10	51	none	negative	negative	abdomen
11	57	none	negative	negative	upper extremities
12	42	none	negative	negative	chest/neck
13	35	none	negative	negative	leg
14	30	none	negative	negative	abdomen
15	40	none	negative	negative	leg
16	30	Acute erythema migrans	negative	negative	unknown
17	59	none	negative	negative	leg
18	46	none	negative	negative	unknown
19	63	none	negative	negative	leg
Mean age	47,5				

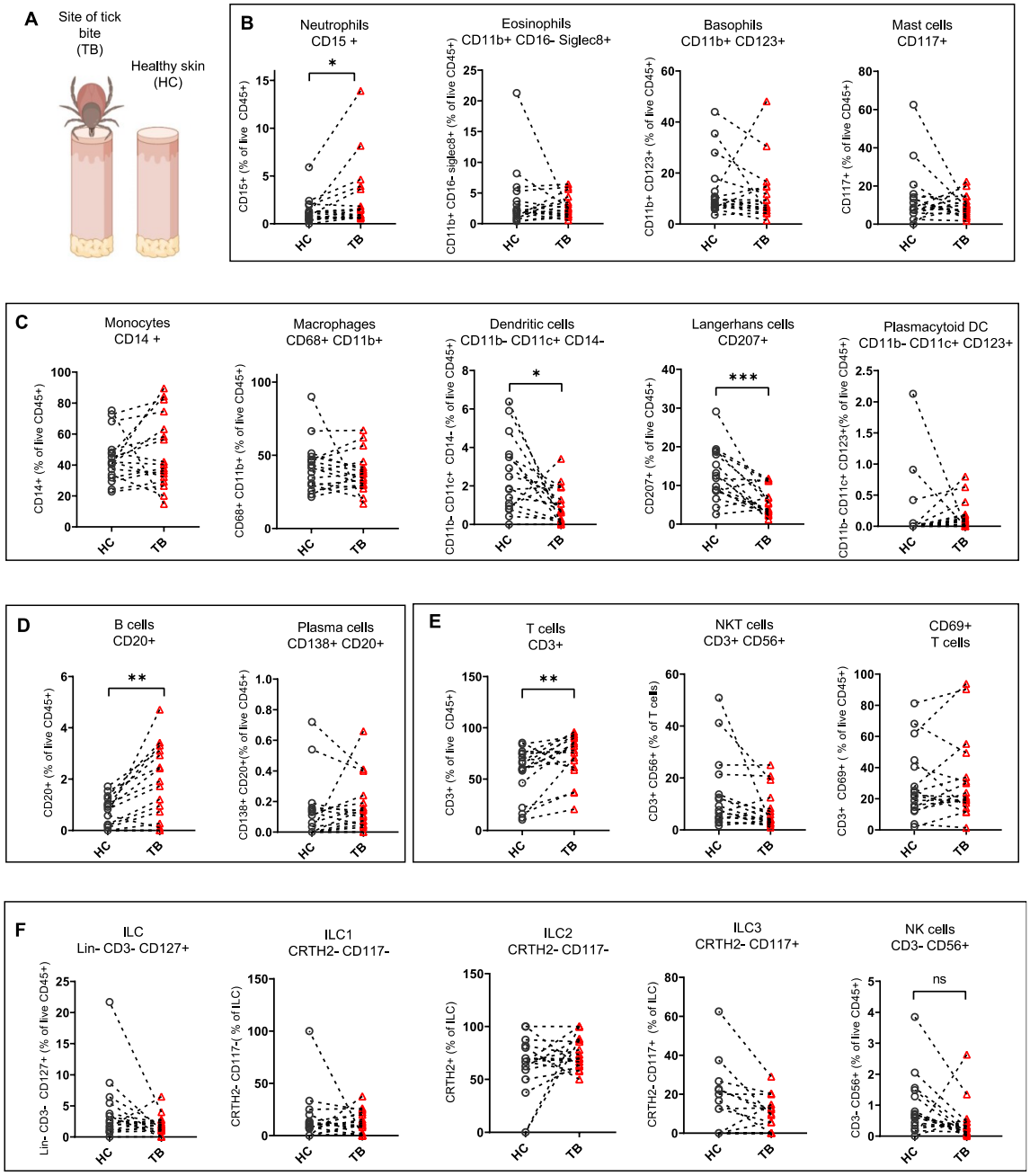


Figure 10. Skin biopsies of tick feeding sites show changes in the immune cell composition in human skin.

(A) Graphical representation of the sampling sites. (B-F) Percentage of neutrophils, eosinophils, basophils and mast cells (B), monocytes, macrophages, dendritic cells , Langerhans cells, pDC (C), B cells, plasma cells (D), T cells, CD69+ T cells (E), innate lymphoid cells and NK cells (F) among live cells in tick bite (n= 17) and healthy skin (n= 17) .Percentage of CD4+ T cells , CD8+ T cells and NKT cells (E) among T cells in tick bite (n=17) and healthy skin (n=17). Percentage of ILC1, ILC2 and ILC3 (F) among ILCs in tick bite (n=16) and healthy skin (n=17) . One dot represents one healthy individual. Dotted lines connect inter-individual samples. Statistical analysis was performed by paired Student's T-test. *p<0,05; **p<0,01; ***p<0,001. (4)

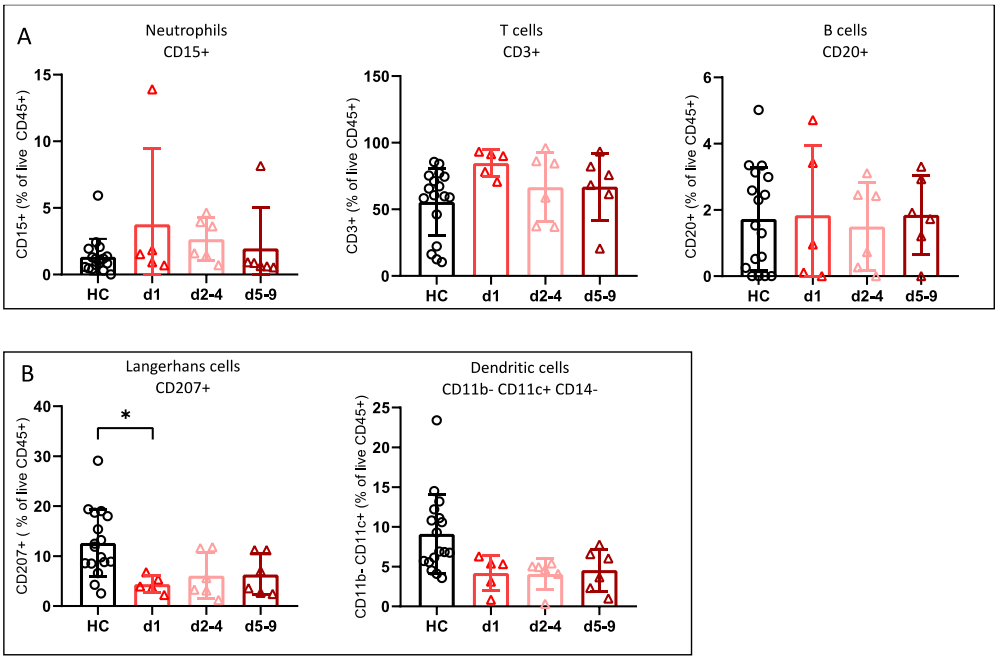


Figure 11. Cellular changes occur early after tick bite.

(A-B) Percentage of neutrophils, T cells, B cells (A), dendritic cells and Langerhans cells (B) among live CD45+ cells in healthy skin and at day 1 (n=5), day 2-4 (n= 6) and day 5-9 (n=6) after tick bite. One symbol represents one individual. Bars indicate the mean, error bars indicate the SEM. Statistical analysis was performed by unpaired Student's T-test. *p<0,05.(4)

Tick bite immunomodulation induces a systemic effect on peripheral blood leukocyte populations

To assess whether tick bites influence peripheral blood leukocyte frequencies, we sampled peripheral blood of the recruited individuals and compared single leukocyte suspensions isolated from blood to age-matched healthy, tick bite-naïve individuals. We did not observe changes in the granulocytes (Figure 12A), mononuclear phagocytes and antigen-presenting cells (Figure 12B) in peripheral blood, showing that the inflammation was locally restricted. However, in parallel to skin, neutrophils were increased in peripheral blood after tick bite.

We detected no changes in B cell and plasma cell frequencies in blood of individuals affected by tick bite (Figure 12C). Interestingly, we found a significant decrease in blood derived $CD3^+$ T cells, NK cells and NKT cell frequencies (Figure 12D, E). Surprisingly, frequencies of type-3 ILCs, which are important sentinels of barrier tissue homeostasis and respond rapidly to damage, inflammation and infection (186), were also decreased in peripheral blood.

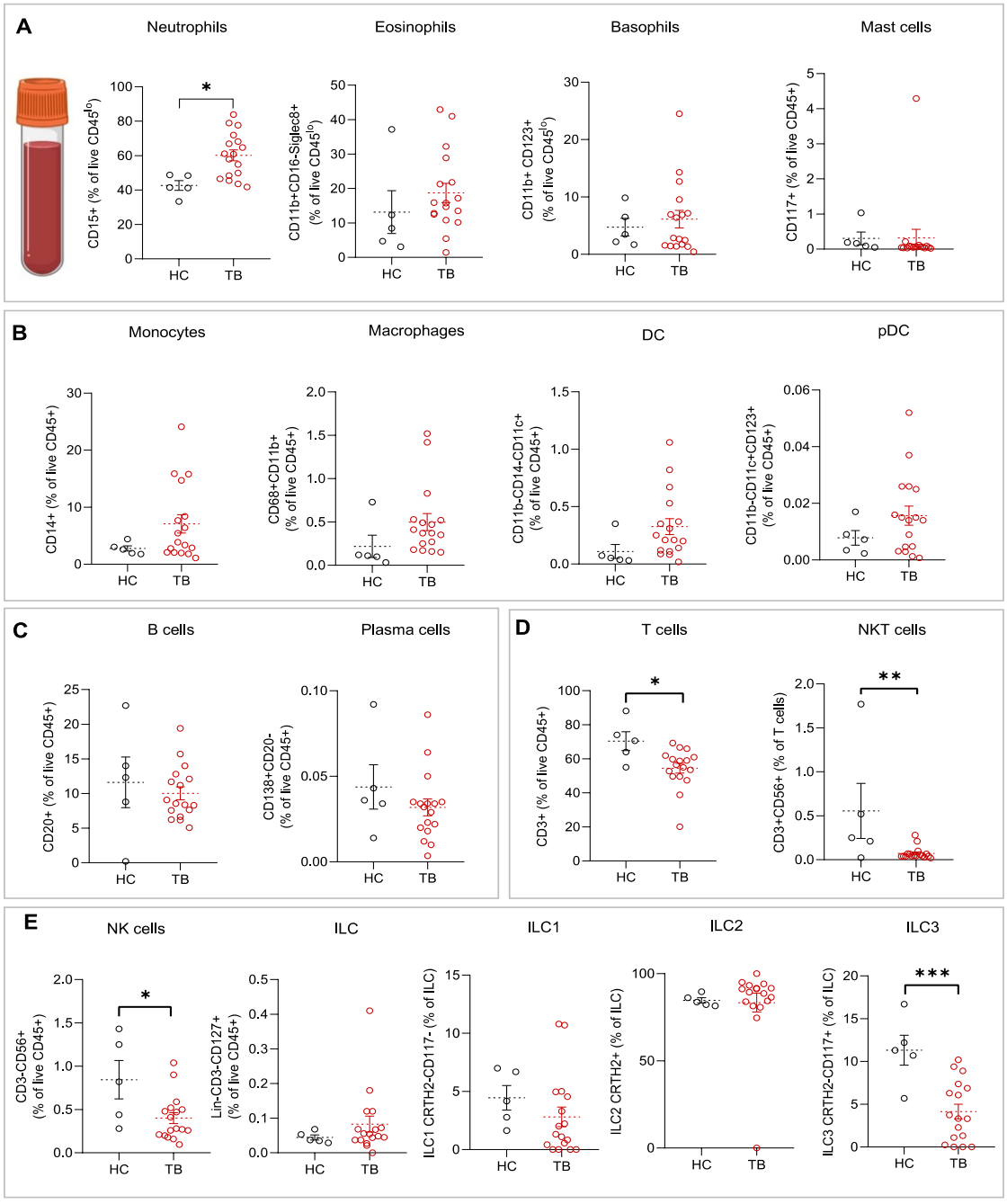


Figure 12. Peripheral blood leukocyte populations in individuals affected by tick bite differ from those of healthy individuals.

(A-E) Percentage of neutrophils, eosinophils, basophils, mast cells (A), monocytes, macrophages, dendritic cells, plasmacytoid dendritic cells (B), B cells, plasma cells (C), T cells, CD69 T cells (D), NK cells, innate lymphoid cells (E) among live CD45+ cells in blood from individuals affected by tick bite (n= 17) and blood from healthy individuals (n=5). Percentage of CD4+ T cells, CD8+ T cells and NKT cells (D) among T cells in blood from individuals affected by tick bite (n=17) and blood from healthy individuals (n=5). Percentage of ILC1, ILC2 and ILC3 among ILCs (E) in blood from individuals affected by tick bite (n=17) and blood from healthy individuals (n=5). One dot represents one individual. The bars indicate the mean. The error bars indicate the SEM. Statistical analysis was performed by unpaired Student's T-test. *p<0,05; **p<0,01; ***p<0,001. (4)

T cell and ILC responses are impaired after tick bite

Studies in both mice and humans have shown that tick saliva inhibits certain T cell functions while inducing others (1). To get more insights into the immunomodulation after tick bites, we assessed the functionality of T cells and ILC by determining frequencies of key cytokines in a cytokine secretion assay.

Although the frequency of $CD4^+$ T cells increased in blood of individuals affected by tick bite (Figure 13A), the type-1 cytokine IFN-γ significantly decreased, indicating that the Th-1 cell response was impaired. Frequencies of IL-4 and IL-17a-expressing T cells were decreased in some individuals, but this effect was not significant overall (Figure 13B). We next assessed the expression of cytokines by circulating ILCs. We observed that similar to T cells, frequencies of IFN-γ and IL-17a-producing ILCs were decreased in peripheral blood of individuals bitten by a tick (Figure 13C). In tick bite-affected skin, $CD4^+$ and $CD8^+$ T cells equally increased (Figure 10E, Figure 13D). Surprisingly, in tick bite skin, IL-4-expressing T cells were decreased and IFN-γ expressing T cell frequencies did not differ between tick bite skin and autologous healthy skin (Figure 13E). This is in contrast to findings in mice, which have shown that tick saliva polarizes the immune response towards a Th-2 response by the downregulation of type-1 and the upregulation of type-2 cytokines (87). In addition, we observed a significant effect of tick bites on ILC responses in human skin. Similar to blood, we observed a significant decrease of IFN-γ, IL-17a and IL-4-expressing ILCs in tick bite skin (Figure 13F).

Taken together, we observed impaired skin and peripheral blood T cell and ILC responses, which might have implications for cutaneous immunity and host defense to tick borne pathogens.

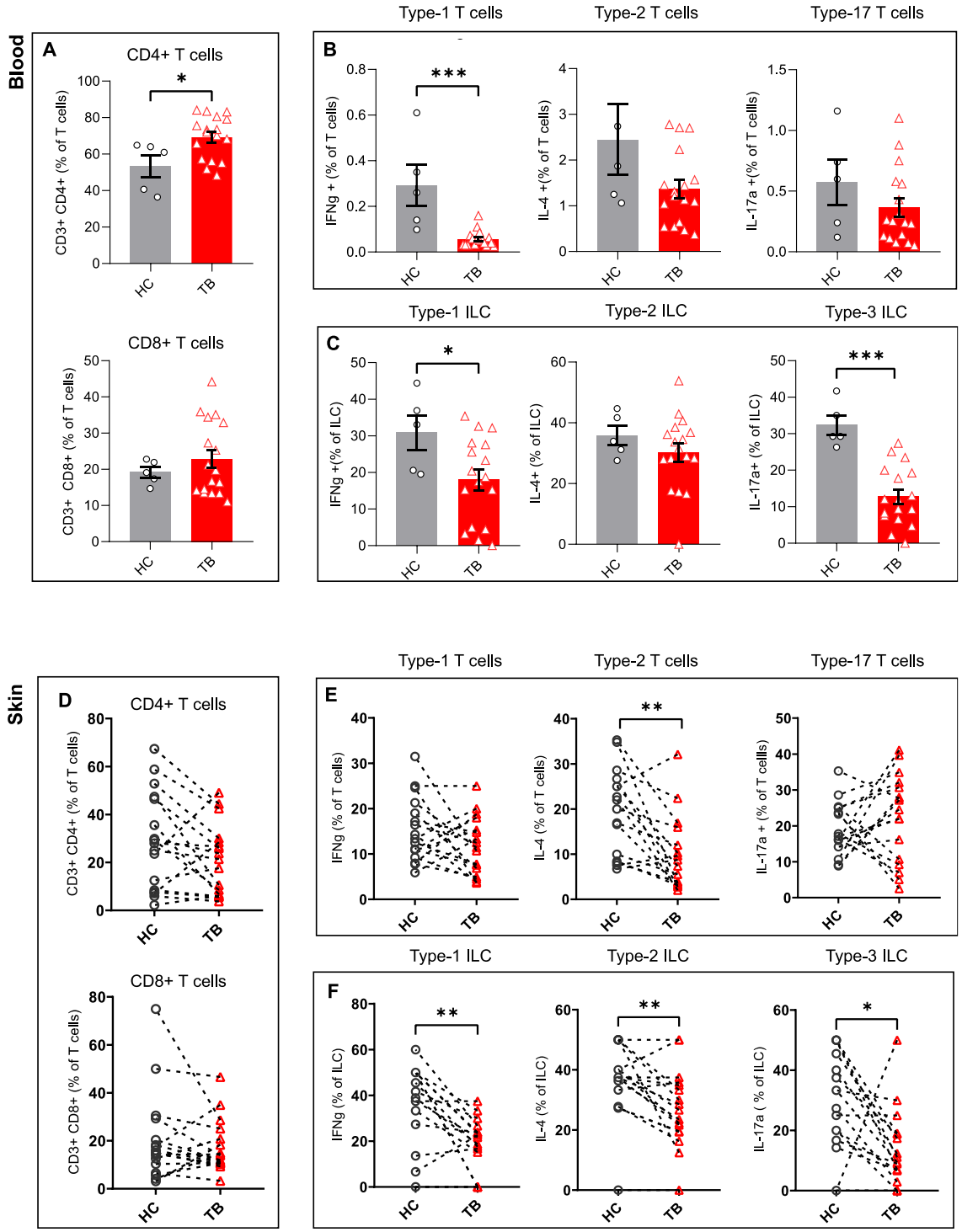


Figure 13. intracellular cytokine staining shows impaired T and ILC immune responses in skin and blood after tick bite.

(A) Percentage of CD4+ and CD8+ T cells among T cells in blood of individuals affected by tick bite (n=17) and of healthy individuals (n=5). (B-C) Frequencies of IFNγ, IL-4 and IL-17a expressing T cells (B) and innate lymphoid cells (C) in blood from individuals affected by tick bite (n=17) and of healthy individuals (n=5). (D) Percentage of $CD4^+$ and $CD8^+$ T cells among T cells in tick bite skin (n=17) and autologous healthy skin (n=17). (E-F) Frequencies of IFNγ, IL-4 and IL-17a expressing T cells (E) and innate lymphoid cells (F) in tick bite skin (n=17) and autologous healthy skin (n=17). One symbol represents one individual. The bars indicate the mean. The dotted lines connect inter-individual samples. The error bars indicate the SEM. Statistical analysis was performed with unpaired (A-C) and paired (D-F) Student's T-test. *p<0,05; **p<0,01; ***p<0,001. (4)

Tissue-resident memory T cells are induced by tick bite

Tissue-resident memory T cells (TRM) persist long term in the skin and provide a first line of adaptive cellular defense against pathogens in the skin (187). The role of TRM during tick feeding is not known. Therefore, we analyzed T cells and their expression of the TRM surface markers Integrin αE (CD103) and CD69 by immunofluorescent tissue staining's and fluorescence microscopy. An increased number of cells, as determined by DAPI nuclear staining, was observed on dermal, but not on epidermal tick bite-affected skin sections (Figure 14A). In line with our flow cytometry data, T cell frequencies were increased in tick bite affected epidermal as well as dermal skin sections (Figure 14B). Furthermore, T cells expressing the TRM markers CD69 and CD103, as well as CD69/CD103 double positive TRM were elevated in the epidermal compartment, but not in the dermal compartment (Figure 14C, D). Overall, our results indicate that T cells, and especially TRM, accumulate in the epidermis after tick bites.

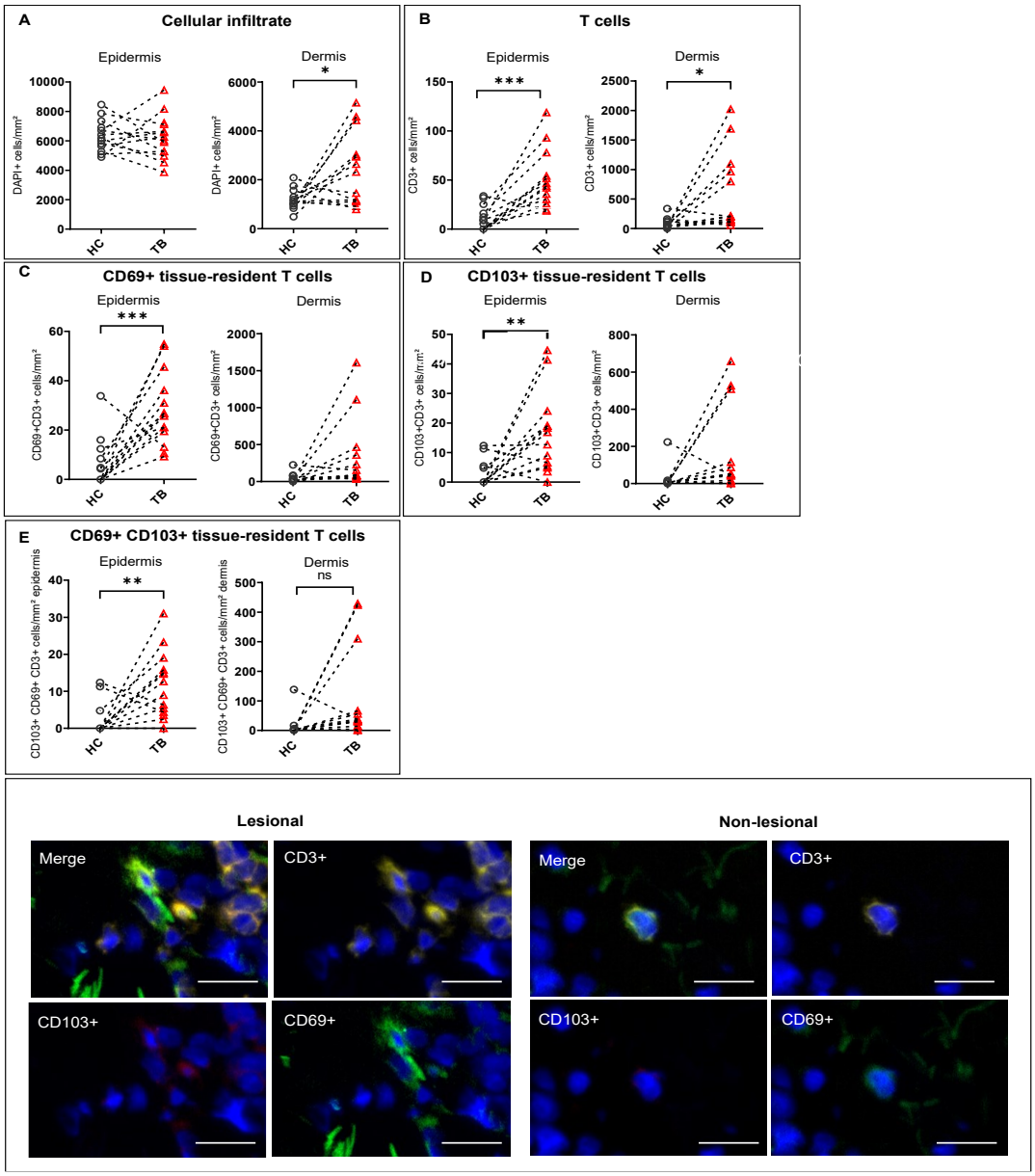


Figure 14. Skin sections of clinical tick bites show increased numbers of tissue resident T cells.

(A-E) The cellular infiltrate in tick bite (n=11) and intraindividual healthy control samples (n=11) in the epidermis and dermis as determined by fluorescent immunolabeling of DAPI+ cells (A), T cells (B), CD69+ cells (C), CD103+ cells (D) and CD69+ and CD103+ double positive cells (E). The data was shown as cell number per mm2. (F) Representative image of DAPI, CD3 , CD103 and CD69 immunolabeling in lesional and non-lesional skin. The scale bar indicates 20 µm. One symbol represents one individual. Dotted lines connect inter-individual samples. Statistical analysis was performed using a paired Student's T-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Part 2 - Changes in the immune cell infiltrate during early Lyme disease

Analysis of the erythema migrans rash reveals cutaneous immune responses to *B. burgdorferi* infection

To investigate the direct effect of *B. burgdorferi* infection on human immune cells in skin we recruited patients, which presented with EM skin rash (n=18, Table 2). Clinical EM diagnosis was confirmed by routine histopathological assessment by an experienced dermato-histopathologist. For the present study, we obtained and processed 18 skin biopsy samples of the center and the border of EM lesions and 18 intra-individual control samples. In 12 patients a further skin biopsy sample was obtained from the site of EM 6 weeks after the first diagnosis. Patients presenting with EM included in the study had a mean age of 55.3 years at diagnosis (range: 35-73). The EM sites in these patients were lower extremities/leg (n=10), upper extremities/arms (n=4), and trunk (n=4). *B. afzelii* subspecies infection was found in all but four samples from the center of the rash using PCR. Culture of *B. afzelii* spirochetes from skin samples was successful in four samples. The applied standard therapy was oral doxycycline 200 mg daily over 14 days. In some individual cases, therapy regimen was changed to include oral phenoxymethylpenicillin, oral amoxicillin or prolonged standard treatment.

We determined leukocyte frequencies in lesional skin and compared it to intraindividual healthy skin by flow cytometry. Furthermore, we investigated differences in the immune cell landscape between the border and the center of the rash and between active and resolved EM.

Our analysis revealed a distinct pattern of immunomodulation in the EM lesions. First, we determined the granulocyte frequencies. Surprisingly, in contrast to previous studies (166, 172, 174),we observed that mast cell numbers were decreased in the center and the border of the EM rash compared to intraindividual healthy skin and to resolved EM (Figure 15A). Neutrophils, eosinophils and basophils remained unchanged (Figure 15A). Furthermore, we investigated the frequencies of antigen-presenting cells. Interestingly, while macrophage, monocyte and $CD207^+$ LC numbers remained unchanged, $CD11c^+CD11b^-CD14^-$ dDC numbers were decreased in the border of the rash compared to control biopsies and resurfaced 6 weeks after the first sampling date (Figure 15B). In addition, plasmacytoid DC frequencies were also significantly decreased in the border and the center of the rash compared to autologous healthy skin (Figure 15B).

Next, we investigated changes in lymphocyte numbers. Although B cells are rare in skin (94), we observed a slight increase in B cells in the border of the erythema migrans rash, which remained elevated in resolved EM, 6 weeks after the first sampling date (Figure 15C). However, plasma cell frequencies did not differ between the active and the resolved EM rash and healthy skin (Figure 15C).

As expected from previous studies (166, 172, 174, 175), we observed that T cells were significantly increased in the border and the center of the EM rash and decreased in resolved EM, 6 weeks after the first sampling date (Figure 15D). This indicates that *B. burgdorferi* infection leads to a local, lymphocytic inflammatory response. There were no significant changes in the frequencies of NKT cells, $CD69^+$ T cells and in the distribution of CD4 and CD8 surface marker expression on T cells (Figure 15D). We next assessed frequencies of innate lymphocytes, ILC and NK cells. There were no significant changes detected in ILC frequencies and in the different ILC subsets (ILC1, ILC2, ILC3). Surprisingly, although we did not observe changes in the NK cell frequency at the first sampling date, we observed an increase in the NK

cell number in resolved EM lesions (Figure 15E). Although, clinically distinct zones, we did not observe any significant differences between the immune cell landscape of the border and the center of the EM rash.

Table 2. EM patient cohort.

Sample No.	Age (yrs.)	Therapy	Histopathology result	PCR result	Culture result	Location of EM
1	61	Doxycyclin 200 mg	EM	*B. afzelii*+	-	leg
2	73	Doxycyclin 200 mg	EM	*B. afzelii*+	-	leg
3	44	Amoxicillin 2.25g/d	EM	*B. afzelii*+	*B. afzelii*+	trunk
4	35	Doxycyclin 200 mg	EM	*B. afzelii*+	*B. afzelii*+	leg
5	58	Doxycyclin 200 mg	EM	*B. afzelii*+	*B. afzelii*+	trunk
6	56	Doxycxclin 200 mg	EM	*B. afzelii*+	-	arm
7	47	Phenoxymethylpenicillin 4.5g/d	EM	*B. afzelii*+	-	leg
8	60	Doxycyclin 200 mg	EM	*B. afzelii*+	-	arm
9	45	Doxycyclin 200 mg	EM	*B. afzelii*+	-	leg
10	62	Doxycyclin 200 mg	EM	-	-	leg
11	53	Doxycyclin 200 mg	EM	*B. afzelii*+	-	arm
12	65	Doxycyclin 200 mg	EM	-	-	leg
13	63	Doxycyclin 200 mg	EM	*B. afzelii*+	-	leg
14	61	Doxycyclin 200 mg, 21 days	EM	*B. afzelii*+	-	trunk
15	55	Doxycyclin 200 mg d	EM	*B. afzelii*+	-	trunk
16	52	Phenoxymethylpenicillin 4.5g/d	EM	*B. afzelii*+	*B. afzelii*+	leg
17	53	Doxycyclin 200 mg	EM	*B. afzelii*+	-	leg
18	53	Doxycyclin 200 mg	EM	-	-	arm
mean age	55.3			n=15	n=4	

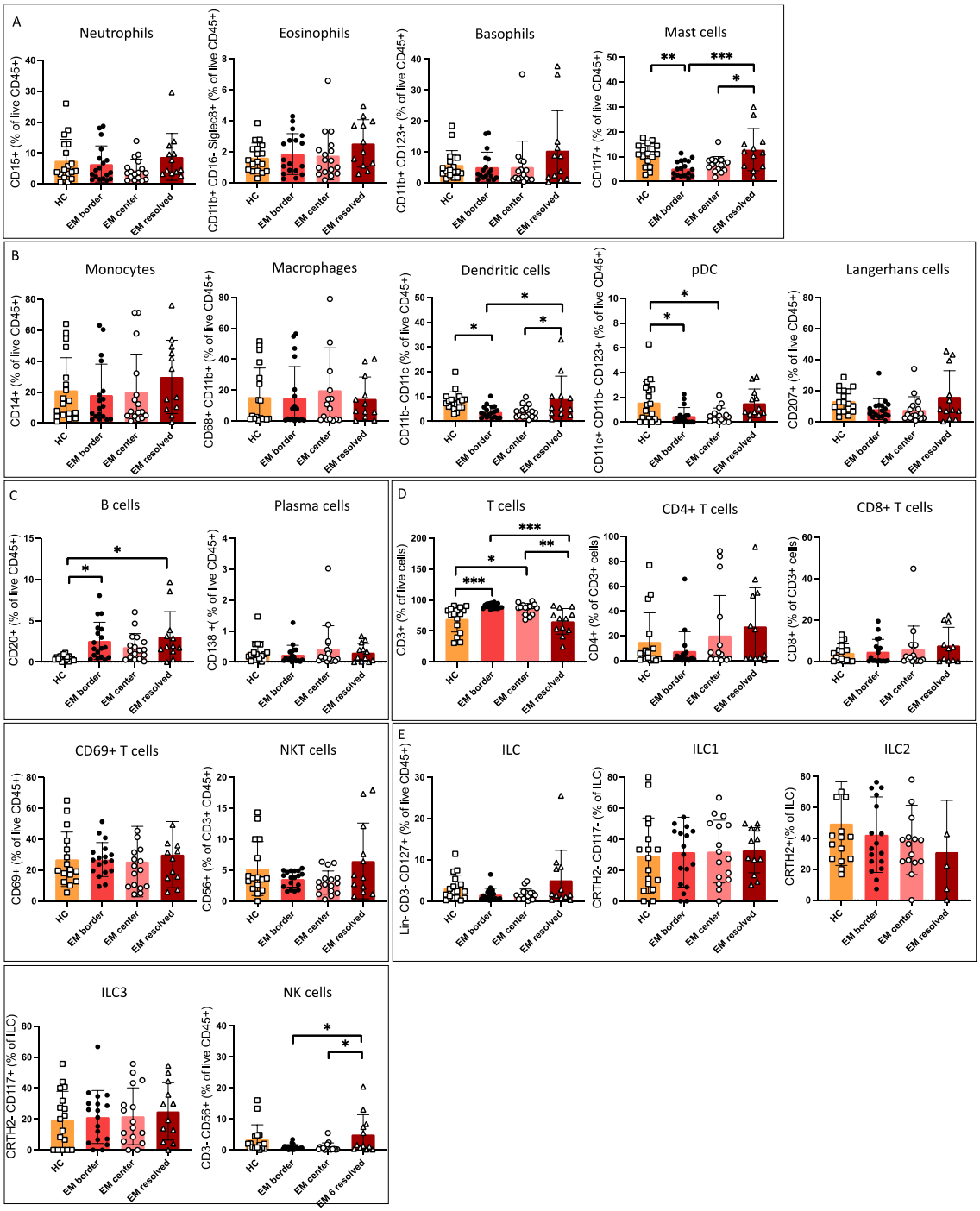


Figure 15. Skin biopsies at the center and at the border of the EM lesions and of resolved EM show changes in the immune cell composition.

(A-F) Percentages of neutrophils, eosinophils, basophils and mast cells (A), monocytes, macrophages, DC, LC, pDC (B), B cells, plasma cells (C), T cells, CD69+ T cells (D), ILC and NK cells (E) among live cells; Percentages of CD4+ T cells , CD8+ T cells and NKT cells among T cells (D) and percentages of ILC1, ILC2 and ILC3 among ILCs (E) in lesional skin at the border (n= 17) and the center (n=17) of the acute EM rash, in resolved EM (6 weeks after the first sampling date, n=12) and in autologous healthy skin, obtained at the acute EM sampling time point (n= 17). One symbol represents one individual. The bars indicate the mean. Error bars indicate the SEM. Statistical analysis was performed by One-way ANOVA. *p<0,05; **p<0,01; ***p<0,001. (4)

Lastly, we validated flow cytometry data in tissue. We performed immunofluorescent staining's of key immune cell types in cryosections of skin biopsies from the border of EM lesions and compared them to cryosections of intraindividual healthy skin. We observed a trend towards increased cellular infiltrate, as determined by DAPI$^+$ cells, in the border of the EM lesion compared to autologous healthy skin. As observed in our flow cytometric data, we detected no changes in the frequency of macrophages and CD1a$^+$ LCs. Furthermore, in line with our previous findings, we observed a trend towards decrease of DC in the border of the EM lesion compared to intraindividual control cryosections. Differences did not reach level of significance. In contrast to flow cytometric results, we observed no significant change in the frequency of mast cells between the border of the EM lesion and autologous healthy skin (Figure 16A).
Overall, our results indicate that B. burgdorferi infection leads to a pattern of immunomodulation in EM lesions, which includes the decline of dermal dendritic cell subsets and the stimulation of a local T- and B-lymphocyte mediated inflammation. Surprisingly, healed EM still harbored increased numbers of B and NK cells, arguing for a long-lasting immunological response at the infection site.

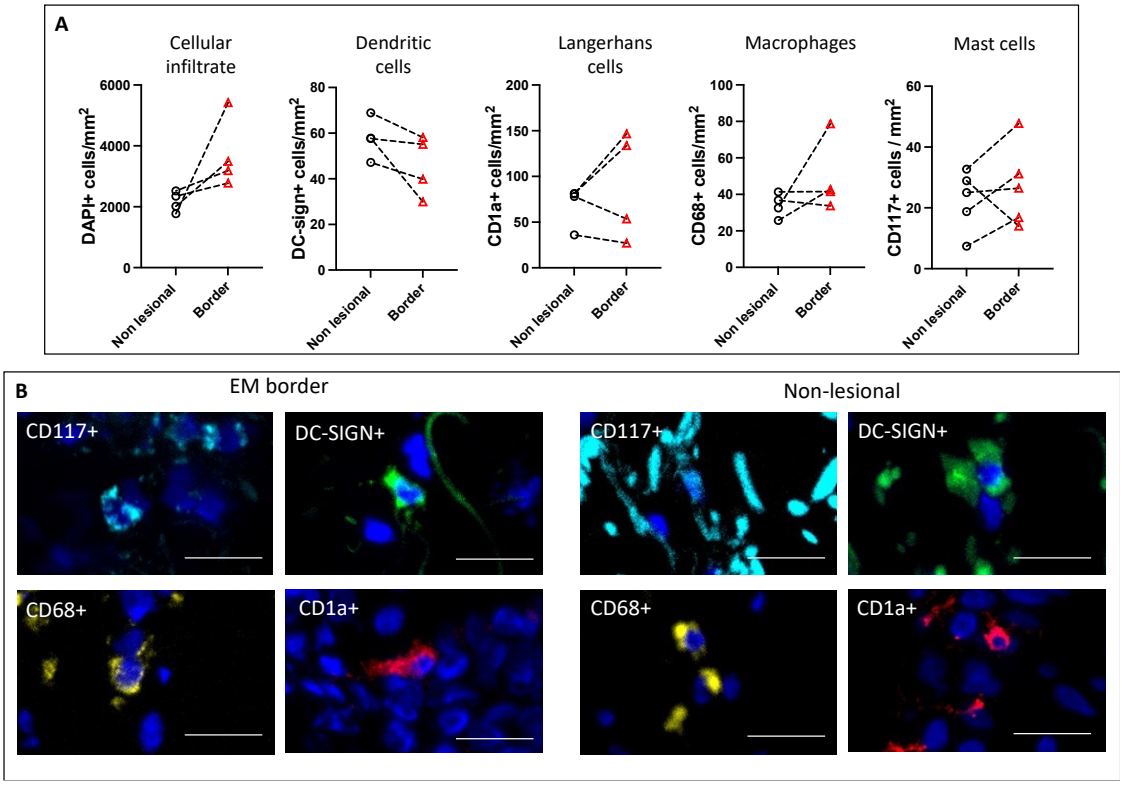


Figure 16. Skin sections of EM lesions harbour decreased numbers of DC.

(A) The cellular infiltrate in the border of the EM rash (n=5) and in intraindividual healthy control samples (n=5) as determined by fluorescent immunolabeling of DAPI$^+$ cells, DC (DC-SIGN$^+$), LC (CD1a$^+$), macrophages (CD68$^+$) and mast cells (CD117+). The data is shown as cell number per mm^2. (B) Representative images of DAPI, CD117, DC-SIGN, CD68 and CD1a immunolabeling in the border of the EM rash and in non-lesional skin. The scale bar indicates 20 µm. One symbol represents one individual. Dotted lines connect inter-individual samples. Statistical analysis was performed using a paired Student's T-test.

B. burgdorferi infection elicits a systemic effect on blood leukocyte populations

Since previous studies revealed that cutaneous changes during early Lyme borreliosis were reflected in part in the blood (166), we investigated if *B. burgdorferi* infection leads to changes in peripheral blood leukocyte frequencies in our patient cohort. For this purpose, we took peripheral blood of the recruited patients suffering from early Lyme borreliosis and from the same patients 6 weeks after the first sampling date. We compared single leukocyte suspensions isolated from blood to age-matched healthy tick bite- and Lyme-naïve individuals. We first assessed granulocyte frequencies and detected increased eosinophil numbers in peripheral blood 6 weeks after the first sampling date, although they remained comparable to healthy controls at the first sampling date. Neutrophils, basophils and mast cells remained unchanged (Figure 17A). Next, we investigated antigen-presenting cell subsets, including those that may have emigrated from the skin. We did not observe changes in monocyte/macrophage, pDC and dDC frequencies (Figure 17B). In addition, we did not detect any changes in the frequencies of lymphocytes of the adaptive immune system, including T cells, B cells and plasma cells (Figure 17C, Figure 17D). Surprisingly, we observed that, although the overall ILC number did not change, ILC1, which are particularly present under inflammatory conditions (188), increased, and ILC2, which are an important source of type-2 cytokines (189), decreased among ILCs (Figure 17E). ILC2 remained decreased 6 weeks after the first sampling date. Overall, we observed modest systemic changes on peripheral blood leukocytes frequencies, but the cutaneous changes during early Lyme borreliosis were not reflected in blood.

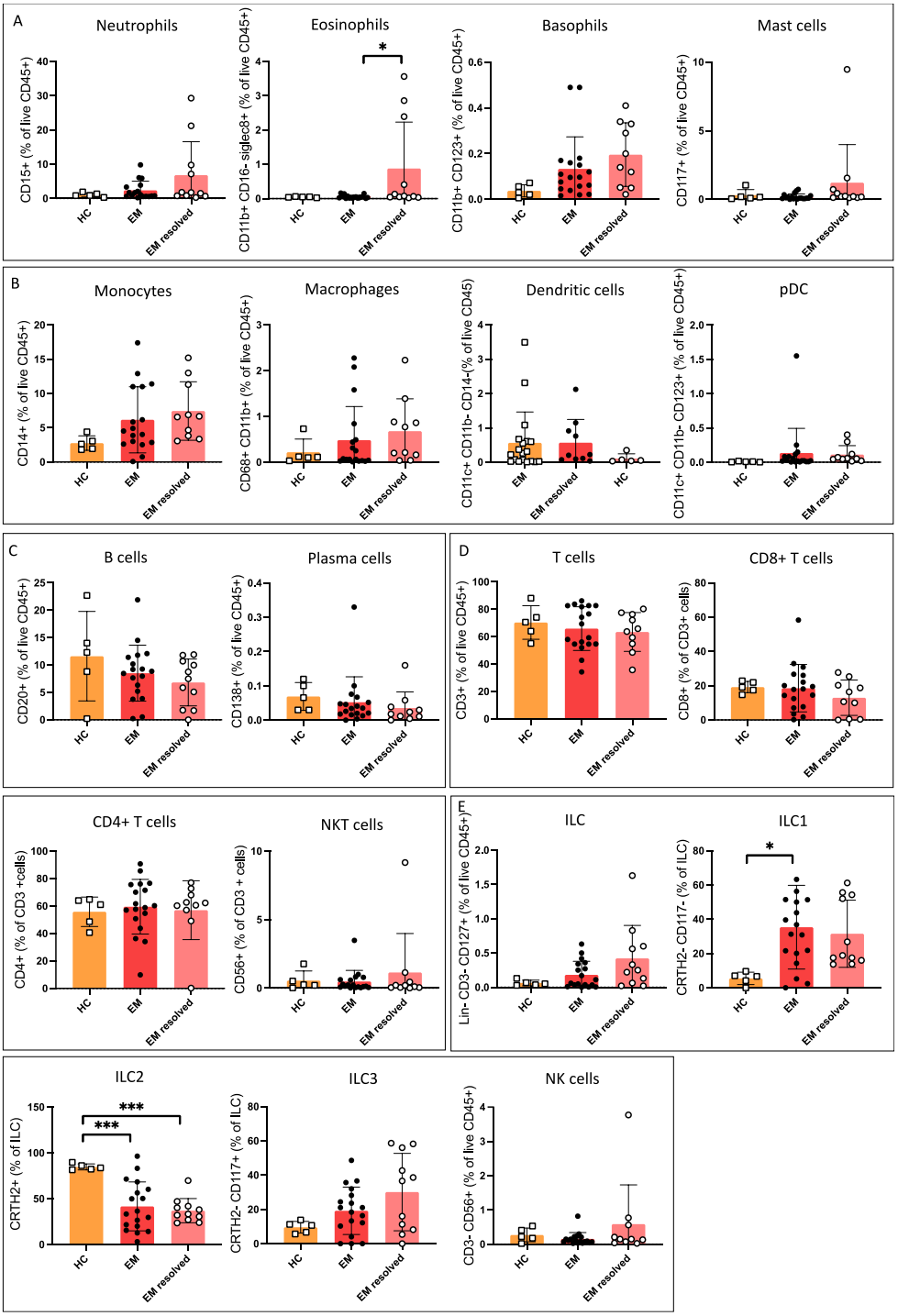


Figure 17. Peripheral blood leukocyte populations in patients with acute and resolved EM differ from those of healthy individuals.

(A-F) Percentages of neutrophils, eosinophils, basophils and mast cells (A), monocytes, macrophages, DC, pDC (B), B cells, plasma cells (C), T cells (D), ILC and NK cells (E) among live CD45+ cells; Percentages of CD4+ T cells, CD8+ T cells and NKT cells among T cells (D) and percentages of ILC1, ILC2 and ILC3 among ILCs (E) in blood of patients with acute EM (n=17), resolved EM, 6 weeks after the first sampling date (n=10) and in healthy individuals (n=5). One symbol represents one individual. The bars indicate the mean. Error bars indicate the SEM. Statistical analysis was performed by a one-way ANOVA. *p<0,05; **p<0,01; ***p<0,001.

B. burgdorferi infection leads to impaired ILC responses in peripheral blood

To get a better understanding of the immune responses mounted against *B. burgdorferi* infection locally in the skin and in peripheral blood, we investigated the functionality of T cells and innate lymphoid cells (ILC) by determining frequencies of key cytokines in a cytokine secretion assay. Th17- and Th1-responses have been reported to be increased in patients which suffer from early Lyme disease (177). In our patient cohort, a significant proportion of T cells and ILCs produced cytokines upon stimulation (Figure 18). However, we could not observe a difference in the frequencies of IFN-γ, IL-4 and IL-17a-expressing T cells between the blood of patients with EM and healthy individuals, or between lesional skin and autologous healthy skin (Figure 18A, 18C). Interestingly, IFN-γ and IL-17a expressing ILCs were decreased in blood of patients with EM and remained decreased in resolved EM (Figure 18B). However, we observed no changes in cytokine expression by ILCs in the skin (Figure 18D).

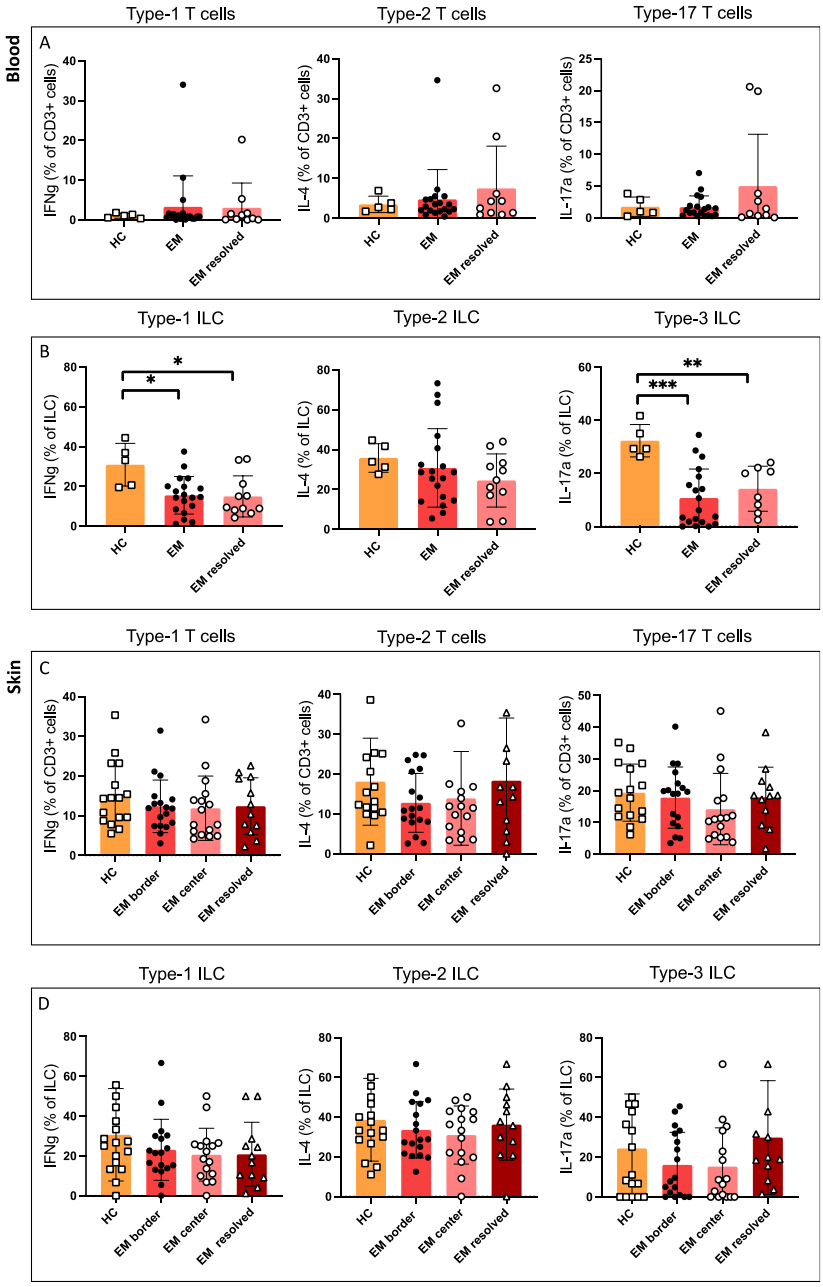


Figure 18. Impaired ILC responses in peripheral blood during acute Lyme disease.

(A-B) Frequencies of IFN-γ, IL-4 and IL-17a-expressing T cells (A) and ILC (B) in blood from patients with acute EM (n=17), resolved EM (n=10) and healthy individuals (n=5). (C-D) Frequencies of IFN-γ, IL-4 and IL-17a-expressing T cells (C) and ILC (D) in lesional skin at the border (n= 17) and the center (n=17) of the EM skin rash, in resolved EM 6 weeks after the first sampling date (n=10) and in autologous healthy skin (n= 17). One symbol represents one individual. The bars indicate the mean. The error bars indicate the SEM. Statistical analysis was performed by an one-way ANOVA. *p<0.05; **p<0.01; ***p<0001.

B. burgdorferi is spreading through lymph and blood vessels from the center of the erythema migrans rash

During the pathogenesis of Lyme borreliosis, *B. burgdorferi* spreads through the blood and lymph vessels from the site of tick bite to several tissues throughout the body (110, 113). We investigated the *B. burgdorferi* burden and the dissemination of the spirochaetes within the skin, to determine migration dynamics from the center and the border of the EM rash. For this purpose, we performed immunofluorescent staining's of *B. burgdorferi* and of blood and lymph vessels in cryosections of skin biopsies and quantified the abundance of *B. burgdorferi* in proximity to blood and lymph vessels. We observed a trend towards higher numbers of *B. burgdorferi* in the proximity of lymph vessels (Figure 19A) and blood vessels (Figure 19B) compared to inter-vascular skin in the center of the EM lesion, indicating that *B. burgdorferi* is spreading through lymph and blood vessels from the center of the EM rash. However, this effect was not significant overall and not found in in the border of the EM rash. In most cases the *B. burgdorferi* burden was higher in the center than in the border of the rash (Figure 19C). However, this effect was not significant overall. Furthermore, we did not observe any difference in the *B. burgdorferi* frequency in proximity of lymph and blood vessels between the center and the border of the EM lesion (Figure 19C).

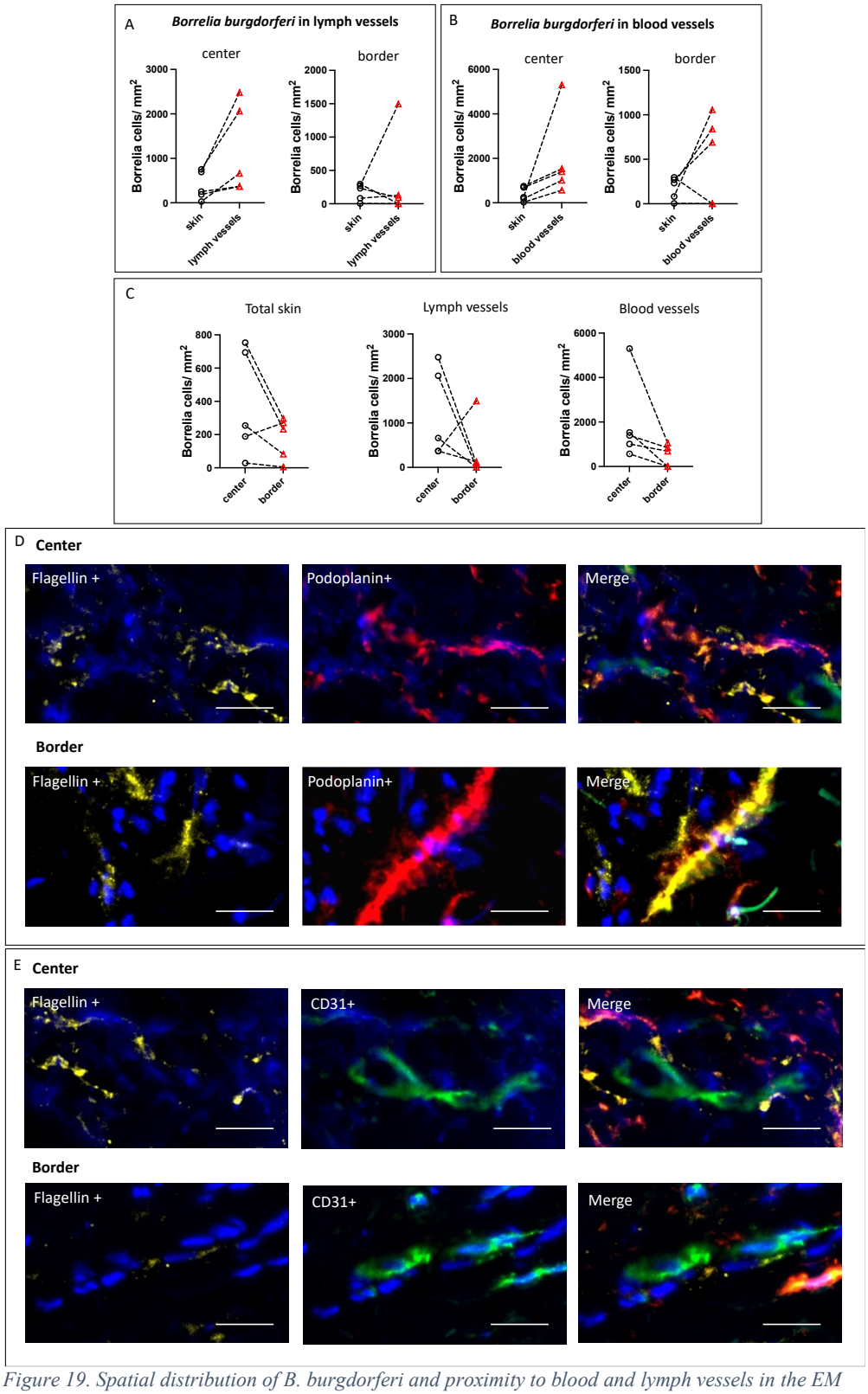


Figure 19. Spatial distribution of B. burgdorferi and proximity to blood and lymph vessels in the EM rash.

(A-B) *B. burgdorferi* burden in proximity to podoplanin+ lymph vessels (A) and CD31+ blood vessels (B) compared to inter-vascular skin in the center and the border of the EM rash as determined by fluorescent immunolabeling of *B. burgdorferi* flagellar antigen (n=5). (C) The *B. burgdorferi* burden in the center of the EM rash (n=5) compared to the border of the EM rash (n=5) in total skin, in proximity to lymph vessels and in proximity blood vessels. Data is shown as cell number per mm^2. Dotted line connects intra-individual samples. Statistical analysis was performed using a paired Student's T-test. (D-E) Representative images of DAPI, borrelia flagellar antigen, $CD31^+$ blood vessels (D) and $podoplanin^+$ lymph vessels (E) immunolabeling in the border and in the center of the EM rash. The scale bar indicates 20µm.

Materials and Methods

Human tissue samples

In the first cohort, healthy individuals, who were seen in the department of Dermatology at the AKH Vienna for recent tick bites (≤9 days) were recruited to the study after fully informed written consent (n=19). The healthy individuals were bitten by non-*B. burgdorferi* infected ticks. From each individual, a 6mm punch biopsy specimen was obtained from the site of the tick bite and from healthy appearing skin of the opposite limb or distant body site. In addition, peripheral blood was obtained from the same healthy individuals.

For the second sampling cohort, patients suffering from early Lyme borreliosis presenting with erythema migrans and were seen in the department of Dermatology at the AKH Vienna were recruited to the study (n=17). From each patient, three 6mm punch biopsy specimens were taken: from the center and the border of the annular rash and from healthy appearing skin of the opposite limb or distant body site. From 12 patients an additional forth 6mm punch biopsy was taken 6 weeks after the first diagnosis. In addition, peripheral blood was obtained for Borrelia serology and flow cytometry work-up. The study was approved by the Ethics Committee of the Medical University of Vienna, Austria, and was performed in accordance with the guidelines of the Declaration of Helsinki.

Sample processing

A quarter of the fresh biopsies was embedded in Tissue-Tek optimal cutting temperature (OCT) compound and subsequently deep-frozen in liquid nitrogen. Tissue specimens were stored at -80°C until cryosectioned in 5µm sections. After 30 minutes of air-drying, the cryosections were fixed in ice-cold acetone for 10 minutes and stored at -20°C. The remainder of the fresh biopsies was digested following an enzymatic collagenase IV skin digestion protocol. Subcutaneous fat was removed and the remaining tissue was minced with a scalpel and digested over night at 37°C with type IV collagenase and deoxyribonuclease 1, dissolved in RPMI. Subsequently, the cell suspension was filtered through a 70 µm cell strainer. The isolation of live PBMCs from fresh blood samples was carried out under sterile conditions by density gradient centrifugation on Ficoll-Paque reagent (Sigma-Aldrich) according to the manufacturer's protocol.

Flow cytometry

Isolated skin cells and peripheral blood mononuclear cells (PBMC) were stained in single-cell suspensions with surface and intracellular antibodies. For the surface staining, single cell suspensions were incubated on ice for 20 minutes with conjugated monoclonal antibodies against surface antigens and subsequently washed once with PBS (Table 3). For the intracellular staining, surface antibody-labelled cells were fixed by incubation in fixation buffer (Biolegend) for 20 minutes at room temperature. Subsequently, cells were permeabilized by washing with intracellular staining/perm wash buffer according to the manufacturer's protocol (Biolegend), incubated with conjugated monoclonal antibodies against intracellular antigens for 20 minutes at room temperature and washed twice with PBS.

Cytokine secretion assay

Cytokine secretion assays were carried out with peripheral blood mononuclear cells and isolated skin cell suspensions. Freshly isolated cells were stimulated in a 96 well plate using a cell

activation cocktail containing PMA (phorbol 12-myristate 13-acetate), ionomycin and brefeldin A (Biolegend) for 4 hours at 37°C. Stimulated cells were labeled using surface antibodies followed by fixation, permeabilization, and intracellular cytokine staining as described above (Table 3).

Table 3. Antibodies for flow cytometry.

Antibody	Conjugate	Isotype	Clone	Manufacturer
CD45	PE/TR	Mouse IGg1	HI30	BD Biosciences
CD11b	BV421	Mouse IgG1	ICRF44	Biolegend
CD11c	BV510	Mouse IgG1	3.9	Biolegend
CD207	PE	Mouse IgG1	10E2	Biolegend
CD123	FITC	Mouse IgG1	6H6	Biolegend
CD14	PE-Cy7	Mouse IgG1	HCD14	Biolegend
CD68	APC	Mouse IgG2b	Y1/82A	Biolegend
CD20	BV510	Mouse IgG2b	2H7	Biolegend
CD138	PE	Mouse IgG1	Ml15	Biolegend
CD15	FITC	Mouse IgG1	W6D3	Biolegend
CD16	APC-Cy7	Mouse IgG1	3G8	Biolegend
Siglec-8	APC	Mouse IgG1	7C9	Biolegend
CD69	BV421	Mouse IgG1	FN50	Biolegend
CD3	BV510	Mouse IgG1	UCHT1	Biolegend
CD56	PerCp/Cy5	Mouse IgG1	HCD56	Biolegend
CD4	PE-CY7	Mouse IgG2b	OKT4	Biolegend
CD8	APC	Mouse IgG1	SK1	Biolegend
IFN-γ	BV785	Mouse IgG1	4S.B3	Biolegend
IL-4	PE	Mouse IgG1	8D4-8	BD Biosciences
IL-17a	FITC	Rat IgG2a	eBio17B7	eBioscience
CD117	BV711	Mouse IgG1	104D2	Biolegend
Lineage (CD3/14/16/19/20/56)	FITC	Mouse IgG1 Mouse IgG2b	UCHT1;HCD14; 3G8;HIB19;2H7; HCD56	Biolegend
CD127	PE-Cy7	Mouse IgG1	A019D5	Biolegend
CRTH2	APC	Rat IgG21	BM16	eBiosciences
IL-17a	BV421	Mouse IgG1	BL168	Biolegend

Tissue immunofluorescence

Multi-color immunofluorescence staining's were established on acetone-fixed skin cryosections. After defrosting cryosections in PBS, the sections were blocked with 3% mouse serum in 2% Bovine serum albumin/PBS. The directly labeled antibodies were diluted in 2% BSA/PBS, applied to cryosections and incubated in a humid chamber for 1-2 hours at room temperature or overnight at 4 °C (Table 4). After washing with PBS, the cryosections were counterstained with 4,6-Diamidino-2-phenylindol (DAPI). Subsequently, the sections were washed with PBS and mounted in PermaFluorTM aqueous mounting medium (Thermo scientific). Negative controls were attained in all staining experiments by substituting the primary antibodies with isotype-matched conjugate antibodies.

Table 4. Antibodies for tissue immunofluorescence.

Cell type	Specificity	labeling	Clone	Isotype	Dilution	Manufacturer
T cell	CD3	APC	SK7	Mouse IgG1	1:50	BD Biosciences
TRM	CD3	APC	SK7	Mouse IgG1	1:50	BD Biosciences
	CD103	PE	Ber-ACT8	Mouse IgG1	1:50	Biolegend
	CD69	FITC	FN50	Mouse IgG1	1:30	BD Biosciences
DC	DC-SIGN	FITC	9E9A8	Mouse IgG2a	1:50	Biolegend
LC	CD1a	PE			1:200	Immunotech
Mast cell	CD117	BV421	104D2	Mouse IgG1	1:50	Biolegend
Macrophage	CD68	APC	Y1/82A	Mouse IgG2b	1:100	Biolegend
Lymph vessel	Podoplanin	PE	NC-08	Rat IgG2a	1:200	Biolegend

Data Acquisition and Analysis

Flow cytometry data were acquired on a FACSAria III cell sorter using FACSDiva software (BD Biosciences) and all staining's were adapted to isotype matching controls. FACS data were analyzed using FlowJo software, exported using Microsoft Excel, and visualized in Prism software (Version 9, GraphPad). Gating of cell types was standardized for all samples (Table 5).

Table 5 Gating for flow cytometry.

Cell type	Gating
Leukocytes	$CD45^+$
Dendritic cells	$CD11b^-$ $CD11c^+$ $CD14^-$
Plasmacytoid dendritic cells	$CD11b^-$ $CD11c^+CD123^+$
Basophils	$CD11b^+$ $CD123^+$
Langerhans cells	$CD207^+$
Monocytes	$CD14^+$
Macrophages	$CD68^+$ $CD11b^+$
Neutrophils	$CD15^+$
B cells	$CD15^-$ $CD14^-$ $CD20^+$
Plasma cells	$CD15^-$ $CD14^-$ $CD138^+$
Eosinophils	$CD11b^+$ $CD16^-$ siglec-8 $^+$
T cells	$CD3^+$
NKT cells	$CD3^+$ $CD56^+$
NK cells	$CD3^-$ $CD56^+$
$CD4^+$ T cells	$CD3^+$ $CD4^+$
$CD8^+$ T cells	$CD3^+$ $CD8^+$
ILC	$CD3^-$ Lin^- $CD127^+$
ILC1	$CD3^-$ Lin^- $CD127^+CRTH2^-$ $CD117^-$
ILC2	$CD3^-$ Lin^- $CD127^+CRTH2^+$
ILC3	$CD3^-$ Lin^- $CD127^+CRTH2^-$ $CD117^+$
Mast cells	$CD117^+$

Immunofluorescence images were acquired using a Z1 Axio Observer microscope equipped with a LD Plan-Neofluar 20x/0.4 objective (Zeiss) and TissueFAXS imaging system. Subsequently, the cryosections were analyzed using TissueQuest software (TissueGnostics GmbH) using automated surface marker detection by DAPI ring mask. Gating was performed as described in Table 4. Two subsequent sections were analyzed as replicates. Data was

exported and analyzed using Microsoft Excel followed by visualization in Prism (Version 9, GraphPad).
All statistical analyses were performed in Prism (Version 9, GraphPad). For each experiment, mean, standard deviation (SD) and standard error of the mean (SEM) were calculated. For comparison of two groups, paired or unpaired Student's T-tests were applied. For comparison of multiple groups, One-way ANOVA and Turkey's multiple comparison post-test were used. P-values equal to and below 0.05 were considered statistically significant.

Discussion

Previous studies have shown that tick bites, tick saliva or its components interact with and modulate innate and adaptive immunity of the host. However, most of the studies used *in vitro* or *ex vivo* models, which may not reflect the events of natural tick infestation in human skin, in which a multitude of factors, such as the saliva components, change over time (16). Only a few studies characterize the immune response to natural tick bites in animal or human skin *in vivo*. In *Amblyomma americanum* tick feeding cavities in guinea pigs the predominant cell types were neutrophils, followed by eosinophils, macrophages, lymphocytes and mast cells (54). In human skin, the early immune cell infiltrate (< 24 hrs. after tick bite) was characterized by DC and macrophages. After prolonged feeding(>24 hrs. after tick bite) DC and Macrophages declined again (55).
In line with these studies, we found elevated frequencies of neutrophils and lymphocytes and decreased innate immune cell numbers after tick bite in human skin. However, we did not observe an increase in eosinophils, macrophages and mast cells. This discrepancy could be due to species-specific differences between rodents and humans and different times of sampling in human tick bites. Glatz and colleagues observed an accumulation of macrophages during very early tick feeding (<24 hrs) (53), whereas we conducted tissue sampling starting at 24 hrs. after tick bite, to allow transmission of tick salivary compounds.
We observed a decline in LC upon tick bite. This is in accordance with a previous study in guinea pigs, which demonstrated that the infestation with *Dermacentor andersoni* led to a decrease in the frequency of LC around the sites of tick attachment (72). A possible explanation for the observed decrease in LC might be the migration of LC to lymph nodes after contact with tick salivary components, but further investigation is needed to elucidate this in detail.
Interestingly, in contrast to the early infiltrates studied by Glatz et al. (55), we detected a declined cutaneous DC frequency in the days after tick bite. We speculate, that this may be due to i) rapid emigration of locally residing DC to lymph nodes and ii) hampered migration of DC to the skin site. This is supported by a study that found that immature DC, which have been stimulated with *R. sanguineus* SGE, showed a reduced migration from bone marrow and blood to the peripheral tissues in response to several chemokines. In addition, DC turnover was reduced in skin injected with SGE, indicating that the recruitment of DC to skin was decreased (74)(72). However, to ultimately determine whether the observed decrease in DC at the site of tick bite is also caused by a reduced migration of DC from the bone marrow to the human skin, further investigations are needed.

Interestingly, saliva-primed DC failed to promote an efficient Th1 and Th17-polarization and stimulated the development of Th2-responses (73). Hence, a potential method for vaccine development is to stimulate an immunogenic DC-response to induce a robust effector T cell immunity (185).

We next looked into lymphocyte subsets and observed an increase in cutaneous B cells in our patient cohort. An *in vitro* study with murine B cells, showed that tick SGE inhibited LPS-induced B cell proliferation (84). This shows that in vitro studies may not reflect events of natural tick feeding.

While B cell numbers increased, plasma cell numbers, unlike in *B. burgdorferi* infection (175), remained unchanged upon tick bite. This indicates, that although B cells play a role in immunosurveillance after tissue disruption by the tick mouthparts, there is a lack of T helper cell signals for trans-differentiation to plasma cells after the bite of uninfected ticks.

Consistent with this, we observed a significant impairment of T cell and ILC responses after tick bite. Previous studies suggested that the immune response to tick bites is skewed towards a Th2 response (87, 89, 190). In contrast, we found that both IL-4-producing T cells and ILCs were decreased at the site of tick attachment, indicating that the type-2 response is impaired. In addition, frequencies of IFN-γ-producing T cells in tick bite-affected skin did not differ from autologous, uninvolved healthy skin. However, IFN-γ-producing ILCs were decreased. The fact that previous studies did not investigate T cell responses in the skin but in draining lymph nodes or in blood (89, 90), might explain the divergent results. In fact, the tick salivary compound iristatin has been found to inhibit IL-4 production *in vitro* (191). In line with previous studies (89, 90), we observed a decrease in IFN-γ-expressing T cells and ILCs in peripheral blood after tick bite. In addition, we observed a decrease in IL17a-expressing ILCs in peripheral blood upon tick bite. Interestingly, the 19ISP mRNA vaccine, which induced tick resistance, promoted IL-17 signaling pathways in guinea pigs(192), indicating that it is possible to reverse this tick-induced immunosuppression.

Notably, our results on the cutaneous immune cell infiltrate are in part reflected in peripheral blood. As in the skin, neutrophils are increased in peripheral blood after tick bite. On the other hand, in contrast to the skin, T cell frequencies were decreased in peripheral blood. This is in line with a study, that found that feeding of the tick *B. microplus* on cattle decreased the T cell frequency in peripheral blood (193). Furthermore, NK, NKT and ILC3 cell frequencies were decreased in peripheral blood after tick bite. The decreased frequency of ILC3 might explain the reduced expression of IL-17a, since ILC3 produce a significant amount of IL-17a (186).

The characterization of the immune cell response after tick bite is important for a successful development of preventive measures, including so-called “tick vaccines”, which are promising in the control of tick infestation and tick-borne disease transmission (194). An mRNA vaccine that encodes 19 salivary proteins of *Ixodes ricinus*, (19ISP) was able to induce tick resistance and prevent *B. burgdorferi* infection in guinea pigs (192). Several studies found that tick resistance, which results in decreased weights and numbers of engorged ticks or the death of ticks, is in part mediated by mast cells and was characterized by an accumulation of basophils in the tick feeding site in guinea pigs (39, 195). In addition, $CD4^+$ TRM were found to play a pivotal role in tick resistance of rodents by promoting skin infiltration of basophils (82). We could not observe an accumulation of basophils and mast cells in tick bite lesions and our patients did not report any signs of tick resistance. However, we found that TRM were increased

in tick bite lesions, indicating that TRM are recruited also to tick bite lesions in non-resistant individuals.
Overall, the observed immunomodulation in human skin upon tick feeding is characterized by a neutrophil and lymphocyte mediated inflammation, a decrease in DC and LC and an impaired T and ILC response. These profound changes on the skin immune cell network, might interfere with the primary response against tick-borne pathogens, such as *B. burgdorferi*.

In addition to our study on the immunomodulation after tick feeding, we therefore also conducted a comprehensive analysis of leukocyte subsets and cytokines in patients with early Lyme disease associated with EM. There are only a few studies that investigate immunocytological changes in EM lesions, and none characterize differences between the center and the border of the lesions. It is important to study EM lesions, because rising disease incidence and the lack of natural protection from re-infection cumulate in a high clinical need for further understanding of the pathology. In addition, the immune-pathological processes promoted by *B. burgdorferi* within skin can grant valuable insights regarding disease development in other organ systems (166). Previous studies found that the cellular infiltrates of EM lesions comprise activated macrophages, monocytoid and plasmacytoid DC memory and effector T cells, occasional plasma cells and, less commonly eosinophils and neutrophils (166, 172, 174, 196). In addition, a recent study found that B cells were increased in EM lesions (173). In line with this, we observed an increased frequency of B cells and T cells in EM lesions compared to autologous healthy skin, indicating that a lymphocyte-mediated inflammation is taking place in EM lesions. While T cell frequencies decreased again 6 weeks after the first sampling date, B cells remained elevated, indicating that an antibody-mediated immune response is important against early *B. burgdorferi* infection, but also later in resolvement of infection. This is in accordance with previous findings, that suggested that antibodies play a critical role in the control of *B. burgdorferi* infection and help to resolve carditis and arthritis (147, 150, 152).
In contrast to other studies (174, 196), we observed that plasma cells were not increased in EM lesions compared to autologous healthy skin. This indicates that similar to the tick feeding-response there is a lack of T helper cell signals for trans-differentiation of B cells to plasma cells.
We found that dDC and plasmacytoid DC were decreased in EM lesions, indicating that the cells migrate to lymph nodes after contact with *B. burgdorferi*. In accordance with this, a study in an *ex vivo* skin model found a significant increase in the migration of dDC cells in response to *B. burgdorferi* (127). Alternatively, a decreased recruitment of DC to the skin might be another explanation for this effect, since this has been observed in skin that has been injected with tick SGE (74) and DC were already decreased in our tick bite cohort. Also, in immunofluorescent staining of skin cryosections we detected a trend towards decline of DC in EM lesions. However, the effect is not significant overall. A larger sample number might have been needed to improve statistical power and potentially obtain significant results.
Interestingly, we detected a decreased frequency of mast cells in EM lesions with flow cytometry, which is in contrast to previous studies (166, 172, 174). However, we could not observe the decrease in the mast cell frequency with tissue immunofluorescence staining. This underlines the importance to validate the results with different methods.

In line with previous findings (166) we observed that, early *B. burgdorferi* infection elicited systemic effects on peripheral blood lymphocytes. However, the observed cutaneous immune responses were not reflected in peripheral blood. Interestingly, although eosinophils normally account for only a small minority of peripheral blood leukocytes (197), we observed an increase in eosinophils in peripheral blood in patients with resolved EM lesions 6 weeks after the first sampling date. This is in accordance with previous studies that observed an eosinophilia during Lyme disease (198, 199). Surprisingly, ILC1, which are particularly present under inflammatory conditions (200), were increased in peripheral blood of patients with early Lyme disease, while ILC2 were decreased.

While most immune cells return to normal frequencies in resolved EM lesions, we found NK cells and B cells increased in resolved EM lesions and eosinophils increased in blood of patients with resolved EM. Inflammation and immune reactions are probably not completely resolved in normal-appearing skin at the site of resolved EM, because *B. burgdorferi* organisms can persist in the resolved rash (201). Previous studies implicated NK cells primarily with persistent Lyme disease and Lyme arthritis (142, 143). Our findings indicate that NK cells play also a role during uncomplicated local infection in the skin.

Since it has been observed previously that IFN-γ is the dominant cytokine in EM lesions (166) and is protective for the host (178), it would be suggestive that we also observe an increase in IFN-γ. However, we did not detect differences in the frequency of IFN-γ-expressing T cells and ILCs in EM lesions compared to autologous healthy skin. Surprisingly, we observed even a decreased frequency of IFN-γ and IL17a-expressing ILC in peripheral blood in EM patients, as we did in individuals after tick bite. Since IL-17 cytokines play a role in the control of diverse infectious diseases (164), the lack of IL-17a-expressing ILC might also play a role in promoting the survival of the spirochaete. However, a previous study showed that the depletion of IL-17 did not affect the *B. burgdorferi* burden, the course of disease or the magnitude of the antibody response (165). Further investigations are required to determine whether the failure of the patients to mount a robust ILC cytokine response is due to *B. burgdorferi* immune evasion, the immunosuppressant activities of tick salivary proteins or both.

Furthermore, since all our patients acquired their disease via tick bite, our results seem to contradict the contention that immunomodulators in tick saliva can skew the cutaneous immune response towards the Th2-profile (202, 203). As we defined Th2-cells solely by IL-4 production, additional investigations of cytokines and expression of Th-2 transcription factors would be needed to conclusively determine presence of Th2 cells in our samples.

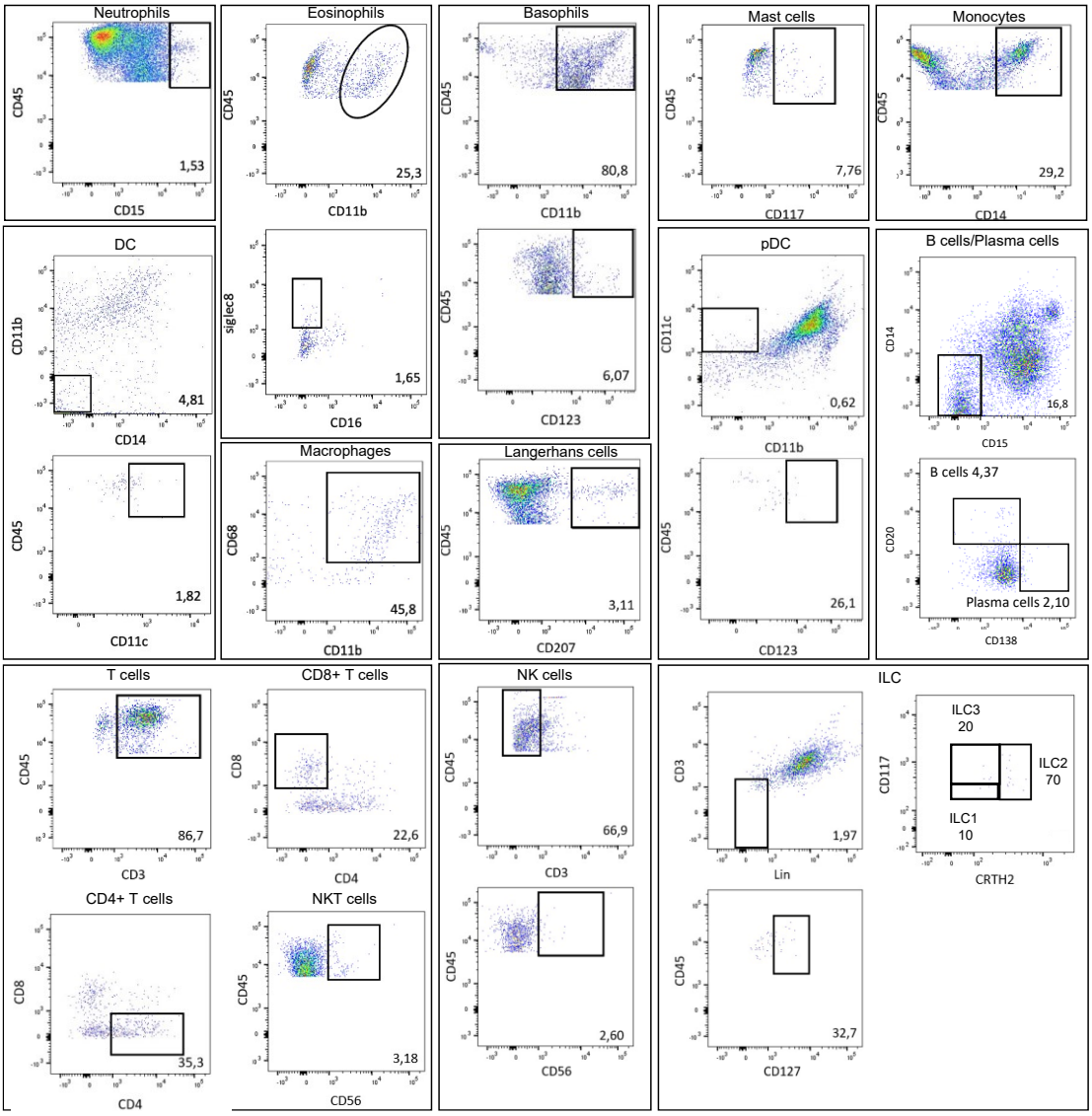
In general, the establishment of the role of specific early cellular immunity may have ramifications for successful vaccine development in tick-induced infection.

Previous findings suggest that spirochaetes may migrate through the dermis or the lymph vessels (8, 110, 113). However, the exact routes remain unclear. To investigate, if spirochete migration might underlie the phenotypic differences between the center and the border of the rash, we investigated immune cells and the *B. burgdorferi* burden in the center and the border of the rash and the dissemination of *B. burgdorferi* to the lymph and blood system. Our findings indicate that the spirochaetes disseminate from the center of the EM rash through blood and lymph vessels, since we observed an accumulation of *B. burgdorferi* in proximity to blood and lymph vessels in the center of the rash, but not in the border. In addition, it also seemed that the *B. burgdorferi* burden is higher in the center compared to the border of the rash. However, the effects were not significant overall, and more samples are probably needed to get meaningful

results. The immune cell infiltrate did not differ between the center and the border of the rash, which may indicate that accumulation of immune cells occurs more rapidly than spirochete migration.

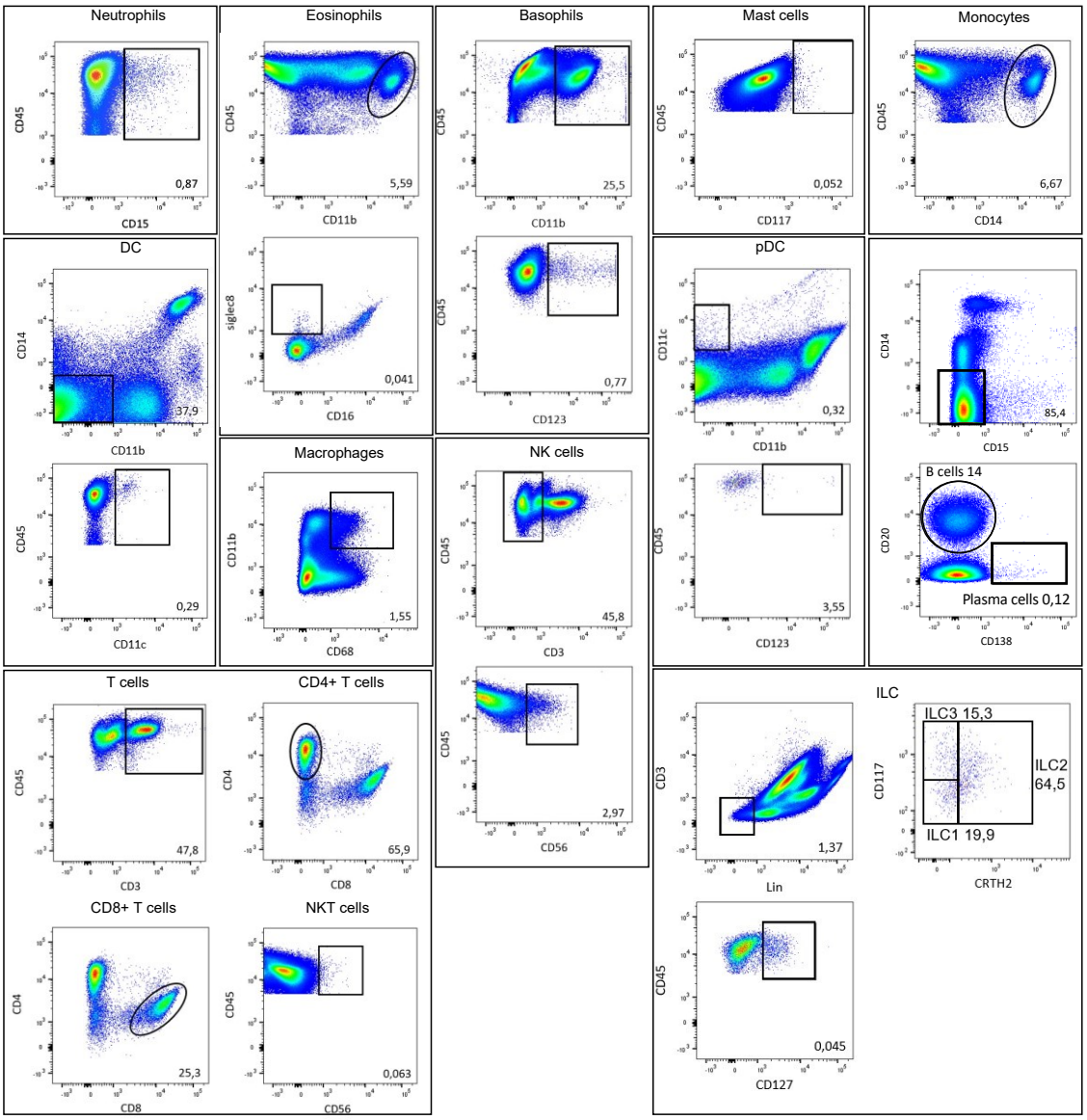
Taken together, we observed profound changes of the skin and blood immune cell network upon tick bites and during early Lyme disease. The cellular infiltrate consisted of cells of the innate and the adaptive immune system. Notably, DC subsets were decreased in tick bite and EM lesions and the ILC immune response was impaired in peripheral blood. Indeed, the immunosuppressive effects of tick saliva are likely responsible for the immune favorable microenvironment in EM lesions. In resolved EM, we detected a lasting immune reaction with accumulation of NK and B cells despite lack of clinical symptoms. Overall, our detailed description of cell-specific changes sheds further light on the complex immune reaction after tick feeding and in Lyme disease.

Appendix



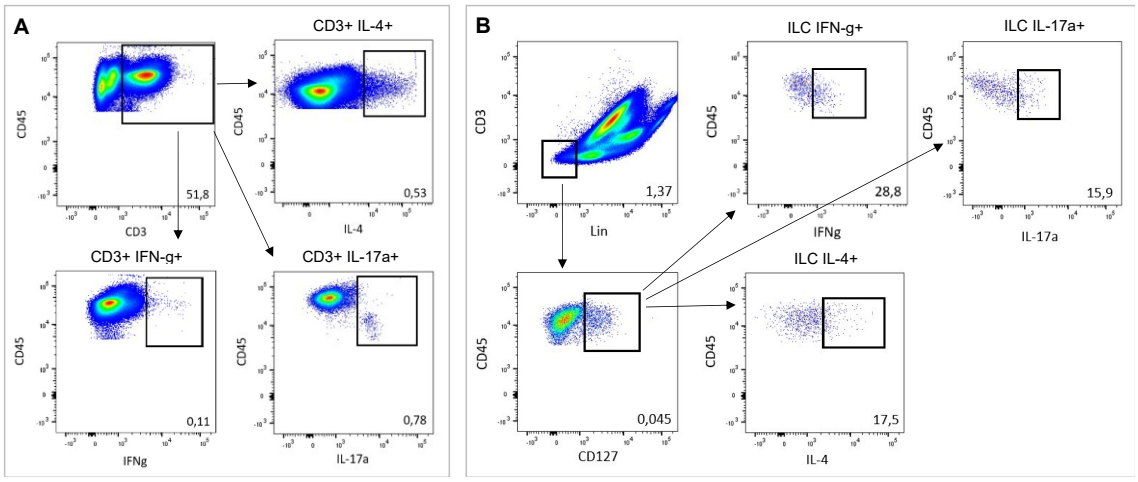
Supplementary Figure 1.Gating strategy for tick bite-affected and EM skin.

Graphs show representative scatter plots and gates for isolated skin cells, created in Flowjo software. All subsets are gated on live, $CD45^{+}$ singlets.(4)



Supplementary Figure 2. Gating strategy for PBMCs from tick bite-affected individuals and EM patients.

Graphs show representative scatterplots and gates for isolated blood cells, created in Flowjo software. All subsets are gated on live $CD45^{+}$ singlets.(4)



Supplementary Figure 3. Gating strategy for cytokines from skin and PBMCs of tick bite affected individuals and EM patients.

Graphs show representative scatterplots and gates for isolated skin and blood cells, created in Flowjo software. All subsets are gated on live singlets.(4)

Abstract

As a consequence of climate change, ticks (*Ixodida*) and tick-borne diseases are emerging. During tick attachment to human skin, ticks secrete immunomodulary salivary compounds into skin, which can exert strong immunosuppressive effects. The suppressed local immune cell activation may be advantageous for tick-borne pathogens. The most common human tick-borne infection is Lyme disease, a multisystemic, multistage infection, caused by strains of *Borrelia burgdorferi* sensu lato. It first manifests as an expanding skin rash, known as erythema migrans. Here we identify changes in key immune cell populations in human blood and skin samples taken after natural tick bites and in active and resolved human Lyme disease, providing insights to the immune reaction to tick feeding and tick-borne pathogens.

Leukocytes were isolated from blood and skin biopsies obtained from individuals after tick bite or with *Borrelia* infection. Isolated cells were analyzed by flow cytometry and lymphocyte subsets were assessed for cytokine production upon stimulation. In addition, immunofluorescent staining of key immune cells including tissue-resident memory T cells (TRM) was performed on skin sections.

The skin infiltrate at tick feeding sites was characterized by an increase in T cells, TRM, B cells and neutrophils, indicating that an initial inflammatory response is taking place independently of pathogen transmission. In skin samples obtained from patients with early Lyme disease, we similarly observed that B cells and T cells were increased, suggesting a mainly lymphocyte-driven inflammatory response. We detected a decrease in dermal dendritic cells and plasmacytoid dendritic cells in erythema migrans, which was already present in tick bite biopsies, indicating a long lasting effect of tick feeding on dendritic cell subsets.

Interestingly, cytokine production by T cells and innate lymphoid cells was impaired in tick bite skin and tick feeding also induced discreet systemic immune effects on peripheral blood leukocytes. In active erythema migrans, skin cytokine responses were largely conserved, however, production of interferon-gamma and interleukin-17 by peripheral blood innate lymphoid cells was significantly reduced. Between the center and the border of the EM lesion, we detected differences in *B. burgdorferi* bacterial load and the association of spirochaetes with blood and lymph vessels. However, there were no significant differences in the immune cell infiltrate between the rash center and the rash border.

Overall, we observed that tick feeding leads to significant changes on the skin and blood immune cell network, which might intrude with the primary response against tick-borne pathogens, such as *B. burgdorferi* and are still partially found in early Lyme disease. This detailed analysis of cell-specific changes in human skin may help to design potent prevention and treatment strategies for tick-borne diseases in the future.

Zusammenfassung

Als Folge des Klimawandels treten Zecken (*Ixodida*) und von Zecken übertragene Krankheiten immer häufiger auf. Während des Bisses sezernieren Zecken hunderte immunomodulatorische Speichelkomponenten in die menschliche Haut, die eine starke immunsuppressive Wirkung haben können. Von Zecken übertragene Krankheitserreger, wie *Borrelia burgdorferi sensu lato*, können von der Unterdrückung der lokalen Immunzellaktivation profitieren und den Wirt vermehrt infizieren. Die häufigste durch Zecken übertragene Infektion ist die Lyme-Borreliose, eine mehrstufige Multisystem-Erkrankung, die durch *Borrelia burgdorferi sensu lato* Stämme verursacht wird. Lyme Borreliose manifestiert sich als ein expandierender, geröteter Hautausschlag im Bereich des Zeckenbisses, bekannt als Erythema migrans.

In der vorliegenden Studie untersuchten wir Veränderungen in Immunzellpopulationen in menschlichen Blut- und Hautproben, die nach einem Zeckenstich und während aktiver und abgeklungener Lyme-Borreliose entnommen wurden. Wir isolierten Leukozyten aus Hautbiopsien und Blut, stimulierten die isolierten Zellen mit einem Zellaktivierungscocktail und analysierten sie mittels Durchflusszytometrie. Darüber hinaus wurden Immunfluoreszenz-Färbungen von wichtigen hautresidenten Immunzellen, einschließlich gewebeansässiger Gedächtnis-T-Zellen auf Hautschnitten durchgeführt.

Das Hautinfiltrat an der Zeckenbissstelle war durch eine Zunahme von T-Zellen, gewebeansässiger Gedächtnis-T-Zellen, B-Zellen und neutrophilen Granulozyten gekennzeichnet, was darauf hindeutet, dass eine erste Entzündungsreaktion unabhängig von der Krankeitserregerübertragung stattfindet. In Hautproben von Patienten mit Lyme-Borreliose beobachteten wir ebenso eine vermehrte Anzahl an B- und T-Zellen im Bereich des Erythems, was auf eine Lymphozyten dominierte Entzündungsreaktion hindeutet. Wir beobachteten einen Rückgang an dermalen dendritischen Zellen und plasmatytoiden dendritischen Zellen in EM Läsionen, welcher schon in Zeckenbiss-Biopsien vorhanden war. Dies deutet auf langanhaltende immunsuppressive Wirkungen der Zeckenspeichelkomponenten auf Dendritische Zellen hin. Interessanterweise, zeigte sich die Zytokinproduktion von T-Zellen und innate lymphoiden Zellen (ILCs) nach einem Zeckenbiss beeinträchtigt und die Zeckenbisse lösten auch diskrete systemische Effekte auf Leukozyten des peripheren Blutes aus. In aktivem Erythema migrans-Hautproben blieb die Zytokinproduktion weitgehend erhalten, jedoch war die Produktion von Interferon-gamma und Interleukin-17 durch innate lymphoide Zellen des peripheren Blutes signifikant reduziert. Zwischen dem Zentrum und dem Rand der Erythema migrans Läsion stellten wir Unterschiede in der *B. burgdorferi*-Bakterienlast und der Assoziation von Spirochäten mit Blut- und Lymphgefäßen fest. Es gab jedoch keine signifikanten Unterschiede im Immunzellinfiltrat zwischen dem Zentrum und dem inflammierten Randsaum des Erythems.

Insgesamt beobachteten wir, dass Zeckenbisse zu erheblichen Veränderungen im Immunzellnetzwerk der Haut und des Blutes führen, die die Abwehrreaktion gegen *B. burgdorferi* beeinträchtigen könnten. Teilweise sind diese Effekte auch in der frühen Lyme-Borreliose noch nachweisbar, wo sie die protektive Immunantwort gegen Erreger negativ beeinflussen könnten. In Zukunft könnte diese detaillierte, multi-modale Analyse zellspezifischer Veränderungen nach Zeckenbiss und im Frühstadium der Lyme-Borreliose zur Entwicklung wirksamer Präventions- und Behandlungsstrategien für durch Zecken übertragene Krankheiten beitragen.

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