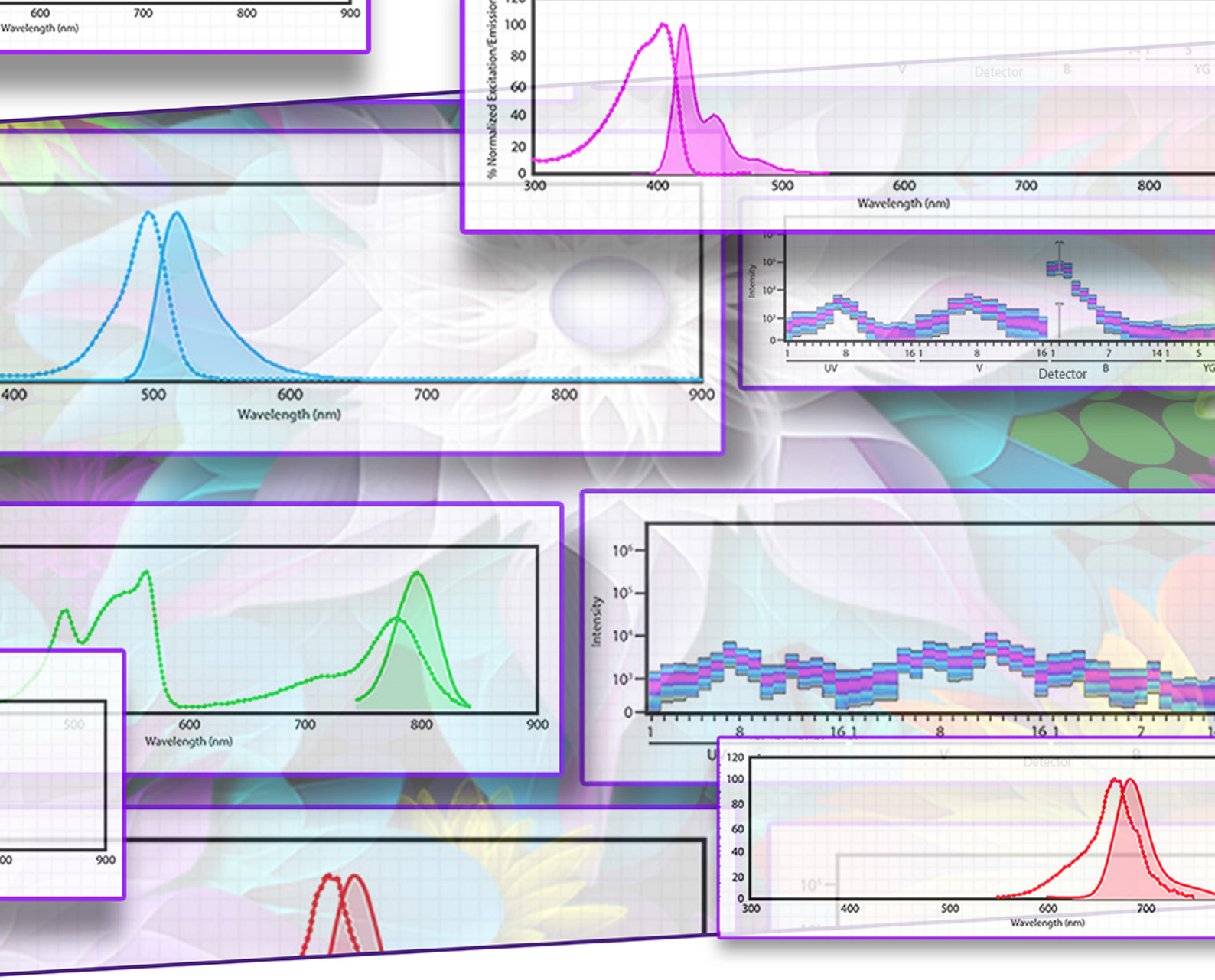

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REVIEW ARTICLE

CYTOMETRY
Journal of Quantitative Cell Science PART A

From the Cradle to the Grave of an Infection: Host-Pathogen Interaction Visualized by Intravital Microscopy

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Received 31 May 2019; Revised 12 September 2019; Accepted 6 November 2019

Grant sponsor: Deutsche Forschungsgemeinschaft, Grant number MU3744/4-1, Grant number SFB854-B31, Grant number SFB854-Z01; Grant sponsor: H2020 European Research Council, Grant number 714233; Grant sponsor: State of Saxony-Anhalt and European Regional Development Fund, Grant number NeutrEat

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Published online 27 November 2019 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23938

Abstract

During infections, interactions between host immune cells and the pathogen occur in distinct anatomical locations and along defined time scales. This can best be assessed in the physiological context of an infection in the living tissue. Consequently, intravital imaging has enabled us to dissect the critical phases and events throughout an infection in real time in living tissues. Specifically, advances in visualizing specific cell types and individual pathogens permitted tracking the early events of tissue invasion of the pathogen, cellular interactions involved in the induction of the immune response as well the events implicated in clearance of the infection. In this respect, two vantage points have evolved since the initial employment of this technique in the field of infection biology. On the one hand, strategies acquired by the pathogen to establish within the host and circumvent or evade the immune defenses have been elucidated. On the other hand, analyzing infections from the immune system's perspective has led to insights into the dynamic cellular interactions that are involved in the initial recognition of the pathogen, immune induction as well as effector function delivery and immunopathology. Furthermore, an increasing interest in probing functional parameters *in vivo* has emerged, such as the analysis of pathogen reactivity to stress conditions imposed by the host organism in order to mediate clearance upon pathogen encounter. Here, we give an overview on recent intravital microscopy findings of host-pathogen interactions along the course of an infection, from both the immune system's and pathogen's perspectives. We also discuss recent developments and future perspectives in extracting intravital information beyond the localization of pathogens and their interaction with immune cells. Such reporter systems on the pathogen's physiological state and immune cell functions may prove useful in dissecting the functional dynamics of host-pathogen interactions. © 2019 The Authors. *Cytometry Part A* published by Wiley Periodicals, Inc. on behalf of International Society for Advancement of Cytometry.

Key terms

intravital microscopy; immunodynamics; host-pathogen interaction; multiphoton; infection

Investigating the interplay between pathogens and cells of the immune system imposes a number of challenges: First, infections are mostly spatially confined to a distinct tissue compartment with very specialized properties. Therefore, any immune response launched against an infection is not only the result of pathogen signals, which trigger specific reactivities but is also adapted to the affected tissue. Therefore, host-pathogen interaction is ideally analyzed locally in the infected organ. Second, the constituents of the immune system are continuously recruited to the site of infection and exhibit a mode of operation that is mainly defined by highly dynamic networks of interaction and communication. This demands an analysis approach that offers the possibility to delineate the course of events at the site of infection with a temporal resolution of seconds to minutes. It is therefore not surprising that intravital microscopy of immunodynamic processes has become an integral part of the toolset of immunologists and has also entered the field of host-pathogen interaction.

The year 2002 marked a cornerstone of immunoimaging, when the research groups of Ellen Robey, Michael Cahalan, and Ronald Germain simultaneously published three multiphoton microscopy studies on the dynamic interactions of immune cells, providing the first glimpse into how T cells behave in their native environment (1–3). This work, together with a number of publications that followed, has changed our perception of immune responses: The processes and mechanisms that had been extrapolated from *ex vivo* and *in vitro* studies could now finally be validated and further characterized, generating an integrated view of the functions and behaviors of the different constituents of immunity (4).

It was only shortly after the first visualization of immune cell dynamics that multiphoton intravital microscopy (MP-IVM) was shown to be an invaluable tool for infection research (5). The use of fluorescence protein-encoding viruses (6,7), gram-positive (8) and gram-negative (9,10) bacterial pathogens as well as parasites (8,11) permitted for the first time an *in vivo* visualization of the early steps of infection, interaction with the immune system, and host cell tropism of pathogens in their living infection environment.

This has opened the way to new discoveries of how virulence mechanisms, but also immune defenses, are put into effect *in vivo* (12). On the one hand, the strategies by which pathogens breach the barriers imposed by the host in order to establish an infection and spread within the host could be delineated (9,13,14). Vice versa, analyzing infections from the immune system's perspective has resulted in an improved understanding of how immune cells recognize pathogens and induce effector mechanisms (15,16).

Often referred to as an explorative approach, MP-IVM has however contributed to the better understanding of host-pathogen interactions in a variety of ways. In some cases, this contribution consisted of critical initial observations of specific states or interactions of pathogens and immune cells in the ongoing infection, which were then further evaluated using other techniques like flow cytometry or histological stainings and subsequent confocal or widefield microscopy (17,18). Other studies relied almost exclusively on MP-IVM in order to identify and characterize a dynamic behavior that marks distinct critical steps for example in the establishment of the infection or the activation of the immune response (8,11,16). Finally, MP-IVM has proven to be an important tool for hypothesis-driven research by offering the possibility to validate concepts that rely on *in vitro* and *ex vivo* findings within a truly natural environment *in vivo* (19).

Here, we will give an overview on MP-IVM observations from the last decade that have made critical contributions to studies on host-pathogen interactions. These findings span the characterization of all stages of an infection, that is, from its cradle to its grave: Starting from the early steps after inoculation of the pathogen into the tissue through the execution of the immune effector response, which may result in successful control or detrimental immunopathology (Table 1). We will also discuss the possibilities of intravital microscopy to provide insights beyond the mere localization and motility of pathogens and immune cells. By offering the possibility of extracting functional information on immune cell signaling or metabolism, new tools for MP-IVM might permit to analyze side-by-side the molecular signaling events and distinct physiological states, which underlie the dynamic interplay of the pathogen with the host.

Invasion, Barrier Function, and Virulence Strategies

From the perspective of the pathogen, the host can be represented as a system of barriers and defenses, some of which can be broken, mitigated, or evaded in order to establish infection. Therefore, many pathogens have evolved systems to hijack physiological functions of the host for their purpose, for example the colonization of suitable niches or the deviation of defense mechanisms of the host immune system.

Invasion strategies strongly depend on the specific tissues that impose a barrier to the pathogen. As such, the rapid recruitment of polymorphonuclear neutrophils (PMNs) upon tissue damage can be regarded as an immune-induced barrier against pathogens that invade the body through damaged physical borders. Being rapidly recruited to tissues upon sensing of pathogens by tissue-resident cells, PMNs employ a wide array of antimicrobial effector mechanisms and are therefore essential for the clearance of many bacterial pathogens (20,21).

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterial pathogen causing a wide range of pyogenic infections (22). Once in the host, it can be shown by MP-IVM to be phagocytosed and killed by PMNs and macrophages, wherein a certain percentage of the bacteria can survive (Fig. 1A). This survival may in turn contribute to dissemination of the pathogen, possibly leading to chronic infections (23). For *S. aureus* infection, the visualization of PMN recruitment has brought forward mechanisms by which the bacteria can inhibit the deployment of this first line of defense. Specifically, *S. aureus* invasion into the parenchymal space of ear skin was accompanied by an inability of PMNs to emigrate from the blood vasculature in large numbers. Abtin et al. observed that tissue-resident macrophages (perivascular macrophages, PVMs), a source of PMN chemoattractants, cause PMN extravasation into the perivascular space within "hotspots" of PVM accumulation during *S. aureus* infection (24). Furthermore, the authors demonstrated that the pathogen is able to evade this series of events via the toxin hemolysin α (HIa) (25–27), which enabled the specific lysis of PVMs, eventually retaining PMNs within capillaries. Likewise, the *S. aureus* immune evasion factor Ecb, a potent complement inhibitor (28), blocked PMN accumulation at the site of infection (29). Similarly, Harding et al. observed an increased

Table 1. The different stages of host-pathogen interaction from infection through clearance investigated by MP-IVM since 2010

STAGE OF HOST-PATHOGEN INTERACTION	INVESTIGATED MECHANISM	PATHOGEN	REFERENCE
Invasion, barrier function, and virulence strategies	Breach of tissue compartment barriers	*B. burgdorferi* *S. Typhimurium* *T. gondii*	(38,42,43,45)
	Inhibition of leukocyte recruitment	*S. aureus* HIV	(24,29,32,57)
	Dissemination, cell-to-cell transfer	*L. major* HIV Murine leukemia virus	(48,49,55,56,59,61)
Initial recognition and innate immune response	Neutrophil recruitment and activation	*S. aureus* *P. aeruginosa* *A. fumigatus*	(24,29,32,34,64,65,68,71)
	Invariant natural killer cell recruitment and activation	*B. burgdorferi* *S. pneumoniae*	(74,75)
	Antigen capture and transfer into lymph nodes	*S. aureus* *P. berghei* *L. donovani* *L. monocytogenes* Modified vaccinia ankara virus	(34,67,72,73,76,77)
Antigen presentation and recognition	Antigen recognition by B cells	*Mycobacterium* BCG	(82)
	Antigen recognition by $CD4^+$ T cells	*L. donovani* *L. major* *T. brucei* *T. gondii* Herpes simplex virus LCMV	(17,83) (90–92,95)
	Sequestration of antigen	*L. mexicana*	(87)
	Antigen recognition by $CD8^+$ T cells	*L. donovani* *P. berghei* *P. yoelii* Herpes Simplex Virus Modified Vaccinia Ankara Virus LCMV	(18,67,88,89,92–95)
Effector function delivery and impact on the pathogen	Pathogen killing	*B. burgdorferi* *P. yoelii*	(74,88,98)
	Pathogen proliferation	*S. aureus* *L. major*	(48,96,97,99,100)
Immunopathology		*P. berghei* *S. aureus*	(32,101–106)

number of PMNs within blood capillaries besides the ones being able to transmigrate outside of the blood vasculature. Some of the capillary PMNs formed sausage-like structures, crawling up and down the walls without emigrating, causing capillary occlusion and increased cell death in skin. This effect was due to β_2 – and α_4 – integrins (30,31), and blocking them shortly after infection improved neutrophil extravasation and reduced cell death as well as lesion size (32).

Furthermore, PMNs were indicated to be recruited to the draining lymph node (dLN), but not further, following the escape of *S. aureus* from the infection site within the footpad. Recruitment occurred via blood vessels and required L-selectin (33). Once in the dLN, PMNs were able to efficiently phagocytose escaped *S. aureus* bacteria (34).

In regards to the virulence strategy of gram-positive bacteria and subsequent sepsis within an infected organism, Boldock et al. reported that distinct constituents of the native commensal skin flora, referred to as proinfectious agents, augmented virulence of *S. aureus*. Kupffer cells were shown to be the key mediators of this augmentation, as they capture and internalize the virulent pathogen together with co-inoculated proinfectious agent. This concomitant uptake leads to reduced

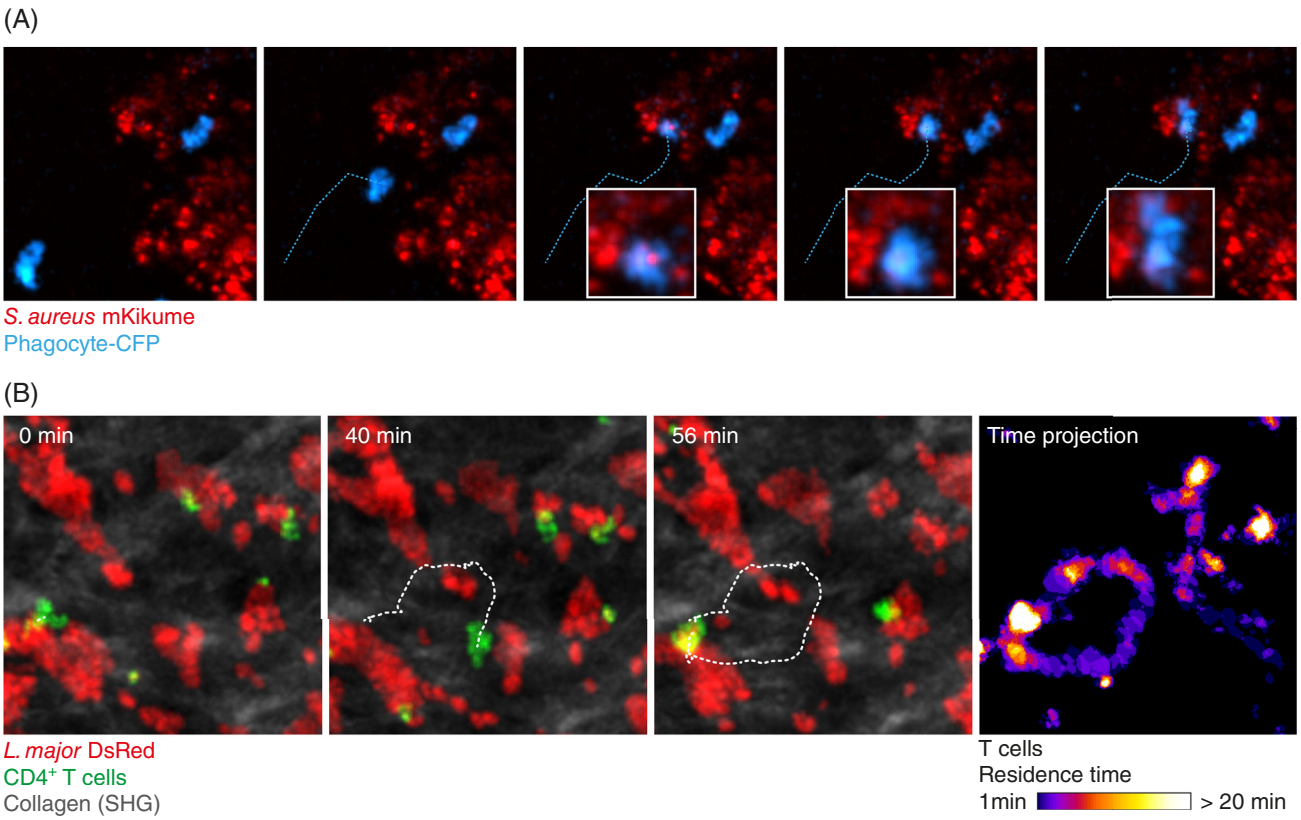

Figure 1. Host-pathogen interactions visualized on a single cellular level by MP-IVM. A., Migration and arrest of a recruited phagocyte before the lysis of a *S. aureus* bacterium. Microscopy of a bone marrow chimeric mouse with 10% CFP-expressing hematopoetic cells (blue) infected with *S. aureus* expressing the fluorescence protein mKikume (red). Note the fluorescence loss in an individual bacterium after arrival of the phagocyte, as well as the arrest of the previously highly motile phagocyte. Image acquired using a Zeiss LSM 700 with a W Plan-Apochromat 20x/1,0 DIC VIS-IR objective (Zeiss), Mai Tai DeepSee laser (Spectra-Physics) tuned at 840 nm for CFP and an Insight X3 laser (Spectra-Physics) tuned at 980 nm for mKikume were used in alternating line scanning mode for excitation. Images were shift-corrected and processed using the Imaris software (Bitplane). Z projections of five images spanning 10 μm are shown. B, Hotspots of adoptively transferred T cells (green) interacting with *L. major* (red)-infected cells in the ear dermis three weeks postinfection. Note one T cell returning to the site of the original stable interaction (motion path: dotted line). Image acquired as described above using a Mai Tai DeepSee laser (Spectra-Physics) tuned at 920 nm for excitation. Images were shift-corrected and processed using the Imaris software (Bitplane). Z projections of 13 images spanning 39 μm are shown. The right panel shows a time-projected overlay of the T cell channel, revealing motion paths (low residence times) and hotspots of interactions of T cells with the pathogen. Both experiments were conducted under approval by the Ethics Committee of the Office for Veterinary Affairs of the State of Saxony-Anhalt, Germany (permit license number 42502–2-1314 Uni MD) in accordance with legislation of both the European Union (Council Directive 4,992,010/63/EU) and the Federal Republic of Germany (according to § 8, Section 1 TierSchG, and TierSchVersV).

reactive oxygen species (ROS) production, in turn permitting increased *S. aureus* virulence and eventually resulting in liver sepsis (35).

Even pathogens, which reach the blood stream, may have to overcome barriers within the body. MP-IVM has been instrumental to uncover several measures of pathogens to do so, for example for the tick-borne, gram-negative spirochete bacterium *Borrelia burgdorferi*, the etiologic agent of Lyme disease that disseminates through the blood stream and eventually establishes an infection in distal tissue sites (36,37). *B. burgdorferi* starts transmigrating through the skin vasculature at 24 h postinfection and was shown to require the adhesin p66 for efficient invasion of tissues (38–40).

The intestinal epithelium represents another important barrier to infectious agents such as *Salmonella* Typhimurium (*S.* Typhimurium), which was further characterized by *in vivo* visualization in the last years. This enteric, intracellular bacterium causes an array of infections, which may be acute or chronic and can be limited to the intestine or distributed systemically. *S.* Typhimurium actively invades and survives within virtually all nucleated cells, including phagocytes (e.g. macrophages and dendritic cells), which may be hijacked by the bacteria to translocate to systemic sites of the body, such as the liver, spleen, and bone marrow (41). *S.* Typhimurium was demonstrated to be able to extracellularly adhere to epithelia and transgress intracellularly through infected intestinal epithelial cells. This process was dependent of a functional Type III secretion system, which enables the bacteria to modulate host intracellular trafficking (42). Moreover, Sellin et al. even observed the intraepithelial proliferation of *S.* Typhimurium (43). The ability to not only proliferate within, but also to transfer into new host cells is central to the lifestyle of intracellular pathogens, and could be shown by

MP-IVM for *S.* Typhimurium, which exits from infected intestinal epithelia on the basolateral side and is rapidly taken up by phagocytes of the underlying lamina propria (42).

Likewise, the apicomplexan parasite *Toxoplasma gondii* is also confronted with the challenge of crossing tissue compartments in the body (44): In order to be able to enter the brain from the vasculature, *T. gondii* needs to replicate in and lyse endothelial cells of the blood brain barrier (45).

In contrast to actively invading *S.* Typhimurium or *T. gondii*, the intracellular parasite *Leishmania major* (*L. major*), the causative agent of cutaneous Leishmaniasis and an important model pathogen for visualizing $CD4^+$ T cell-mediated immune responses (Fig. 1B), relies mainly on the uptake by phagocytes in order to reach its intracellular niche (46,47). In the course of the visualization of cell-to-cell transmission *in vivo*, it could be demonstrated that *L. major* only exhibited minimal extracellular residence time and was phagocytosed right after release from the previous host phagocyte by monocytes that had been newly recruited to the site of skin infection (48,49).

The virus life cycle, in contrast to prokaryotic and eukaryotic pathogens, relies solely on the metabolism of a host cell. Until recently, the visualization and exploration of the virus life cycle has been largely implemented using *in vitro* approaches and ex vivo organ cultures (50–53). However, in recent years, several studies have begun to employ MP-IVM, which contributed to the understanding of viral infections *in vivo*. For the human immunodeficiency virus (HIV), which targets $CD4^+$ T cells within lymph nodes, it was demonstrated that dissemination and virulence relies on motile infected donor $CD4^+$ T cells resembling the central memory T cell phenotype. These infected cells displayed enlarged, thin, and elongated morphologies and formed syncytia with each other. Moreover, long-lived cell–cell contacts with target cells that often clustered at anchor sites at the uropod of donor cells were shown to be involved in the dissemination of viral particles. The elongated phenotype as well as virus dissemination was dependent on the presence of viral envelope (Env) protein (54–56). Furthermore, T cells expressing HIV-1 negative factor (Nef) were observed to be drastically impaired to home in peripheral lymph nodes by negatively affecting extravasation through high endothelial venules (HEV) and reduced subsequent parenchymal motility (57). Nef-mediated obstruction of the recirculation of T cells *in vivo* may therefore be a mechanism of interfering with T cell help, representing an instrument of HIV pathogenicity.

The model system of murine leukemia virus (MuLV) dissemination (58), which affects B- as well $CD4^+$ T cells *in vivo*, also depends on Env protein and the formation of virological synapses (VS) via polarization of the capsid protein Gag. The *in vivo* presence of the VS has been proven for the first time employing MP-IVM (59,60). Likewise, MuLV-burdened macrophages were able to form long-lived synaptic contacts in order to trans-infect B cells, which then migrated to lymph nodes in order to spread infection through VS (61).

Initial Recognition and Innate Immune Response

Upon recognition of pathogen- and danger-associated molecular signatures, innate immune cells are recruited in order to promote pathogen clearance and eventually trigger the adaptive immune system via the presentation of antigen. Although it had been a long standing concept that PMNs are among the first cells that are recruited to an infected tissue (62,63), MP-IVM extensively contributed to elucidation of the cascade of events for PMN recruitment (20). Recently, PMNs were shown to extravasate at hotspots of the perivascular space harboring clusters of tissue-resident macrophages (24) and to consequently reduce their velocity upon interaction with bacteria in the parenchymal space and within abscesses following *S. aureus* infection (29). Extravasation during *S. aureus* infection was impeded by β_2 – and α_4-integrins on the PMN surface, which caused them to crawl along vasculature walls displaying sausage-like shapes. This effect could be reversed by blocking these integrins with antibodies (32). Ecb, a complement inhibitor produced by *S. aureus*, was shown to diminish PMN recruitment to the site of infection. Conversely, the leukocyte factor LTB4 is accountable for neutrophil swarming and the formation of abscesses in infections with *S. aureus*, as well as *Pseudomonas aeruginosa*, an opportunistic gram-negative bacterium causing acute and chronic infection of lung and soft tissues in predisposed individuals (64–66).

In contrast, migratory *Plasmodium berghei* sporozoites localized to the dLN early on, where they are taken up by lymph node-resident dendritic cells (DCs) (67). This parasite infection is a model system for human malaria, which is marked by a complex pathogen life cycle in the host involving intracellular liver and erythrocytic stages and is contained by $CD8^+$ T cell and humoral responses. A notable hallmark-study bringing forward the advantages of MP-IVM is the first direct *in vivo* visualization and phenotypic description of neutrophil extracellular traps (NETs) (68). Previous *in vitro* studies reported that PMNs undergo cell-death as they produce NETs, a structure containing granule proteins and chromatin, forming extracellular fibers that bind and kill bacteria (69,70). Likewise, NET formation by recruited PMNs was shown in lung explants infected by the filamentous fungus *Aspergillus fumigatus* (71). Notably, the study of Yipp et al. additionally demonstrated that PMNs remain viable and functioning during *S. aureus* infection-induced NETosis, which is characterized by nuclear breakdown and chromatin decondensation. Furthermore, PMNs undergoing NETosis display a unique crawling phenotype related to their nuclear structure (68).

Further, cellular changes of cells of the innate immune system within their natural habitat were studied. Beattie et al. described liver Kupffer cell shape changes as a read-out of membrane activity upon infection with the live causative agent of a visceral form of Leishmaniasis, *L. donovani*. Upon phagocytosis, rapid activation of both infected and uninfected Kupffer cells in the vicinity is initiated, as measured by a decrease in membrane velocity (72). For the gram-positive pathogen *Listeria monocytogenes* (*L. monocytogenes*), differential roles in the immune response induction could be shown for subcapsular red pulp (scDC) and myelomonocytic cells (MMC) in the spleen, which swarmed around non-motile scDC forming foci from which blood flow was excluded, thus contributing to control of *L. monocytogenes* prior to development of T cell immunity (73).

Moreover, novel behaviors of invariant natural killer T (iNKT) cells could be visualized *in vivo*. Lee et al. established the imaging of knee joint CXCR6$^+$-GFP iNKT cells via dual-laser multichannel spinning-disk intravital microscopy upon infection with *B. burgdorferi* spirochetes. The labeled iNKT were not found within the vasculature, but in the tissue, closely associated with blood vessels, where they rapidly and directly responded to the joint-homing pathogen. iNKT cells interacted with pathogens at vessel walls to disrupt their dissemination attempts into joints. In case of successful dissemination of the pathogen out of the vasculature into joint tissue, *B. burgdorferi* were hit by lethal attacks from extravascular iNKTs in a granzyme-dependent manner (74). In lung tissue, CXCR6$^+$-GFP iNKT cells were recruited from intra- to extravascular sites in a CD1d-dependent manner upon *Streptococcus pneumoniae* infection, where distinct iNKT cell behaviors were observed to be associated with different timepoints after infection (75).

Upon infection of peripheral tissues, viral particles can reach the lymph node and activate innate immune pathways required for the activation of antiviral defenses. Using MP-IVM, natural killer cells could be shown to accumulate and decelerate in the subcapsular sinus (SCS) upon skin inoculation with modified vaccinia Ankara (MVA) virus. SCS macrophages were required for this behavior, suggesting that they act as early sensors of local infection and may serve as mediators for viral-based vaccine strategies (76). GFP-tagged inflammasome proteins permitted *in vivo* visualization of the initial activation kinetics and propagation of the innate immune response upon MVA inoculation. Thus, the release of specks containing the inflammasome adaptor ASC from pyroptotic SCS macrophages resulted in robust innate and adaptive immune cell recruitment (77).

Antigen Presentation and Recognition

While the innate immune response largely contributes to the control of many bacterial infections like *S. aureus* (78,79), especially the clearance of pathogens specialized on an intracellular lifestyle relies heavily on a functional cellular adaptive immune system. *Leishmania* and *Mycobacterium*, including the causative agent for Tuberculosis (80), represent examples of such T cell-controlled infections.

Granuloma formation is a hallmark of infection with *Mycobacterium* spp., in which a specialized microenvironment fosters the interactions between innate and adaptive immune cells that contain the infection and maintain an asymptomatic state (81). Imaging of these interactions within *Mycobacterium* BCG granulomas revealed that pathogen-specific CD4$^+$ effector T cells had severely limited access to their cognate antigen. This resulted in only partial activation of the infected host's effector function potential (82).

Granuloma-like structures also form in the liver during visceral Leishmaniasis, in which B cells were recruited to and aggregated within *L. donovani*-induced hepatic granulomas in an antigen-dependent manner (83). Their migration speeds were similar to the free movement previously observed in lymphoid tissues (84–86). Furthermore, the same work showed that B cells were capable of forming long contacts with T cells within this environment. Another study presented the first *in vivo* evidence for Kupffer cells serving as MHCI-antigen-presenting cells, and for intra-granuloma antigen-recognition by CD8$^+$ T cells (18).

In a MP-IVM study of the very early events of infection, the recruitment of and *Leishmania mexicana* uptake by monocyte-derived DCs was shown to be reduced when the pathogen was efficiently taken up by PMNs in early stages of the infection (87). This finding was discussed to be a reason for the manifestation of a chronic infection, as activation of effector T cells may also be scaled down as a consequence. Stable interactions of CD4$^+$ T cells with antigen-presenting cells (APC) during cutaneous *L. major*-induced Leishmaniasis was observed to occur in hotspots within the infected tissue, which locally concentrated activation of effector CD4$^+$ T cells, but not effector function delivery (17).

In contrast to *Leishmania*, the infection with *Plasmodium* is mainly contained by CD8$^+$ T cells. During the liver-stage of Malaria, antigen-specific and nonspecific CD8$^+$ T cells clustered around *Plasmodium*-infected hepatocytes, a process which however relied on the presence of the antigen-specific CD8$^+$ T cell fraction. Recruitment was proven to be density dependent, as the entry rate of CD8$^+$ T cells into a given cluster positively correlated with the number of CD8$^+$ T cells already present in that cluster. Moreover, CD11c$^+$ APCs were present in these clusters in close proximity to antigen-specific CD8$^+$ T cells (88,89). Furthermore, sporozoite migration to the dLN appears to additionally be required for CD8$^+$ T cell priming, since CD8$^+$ T cell cluster formation occurred around CD11c$^+$ APCs in the dLN and their activation correlated with durable interactions with APCs (67).

T cell dynamics have also been studied in brain infections of *T. gondii* and *Trypanosoma brucei* (*T. brucei*), the causative organism of sleeping sickness. By revealing that effector CD4$^+$ T cells within the meninges of the brain were highly migratory, whereas regulatory T cells moved more slowly and were found in close association with CD11c$^+$ cells, an anatomical restriction of this T cell subset within the central nervous system could be demonstrated during the infection with *T. gondii* (90). *T. brucei* infection leads to increased T cell and DC numbers in the meninges, in which extravascular pathogens were observed to appear in the meninges, surrounded by collagen (91).

T cell priming during primary Herpes simplex virus (HSV) infection is a step-wise event. Specifically, CD4$^+$ T cells markedly clustered in T cell zones of the lymph node around migratory DCs, displaying reduced mean velocities in early time points post infection (12 h). In contrast, CD8$^+$ T cells displayed no such behavior. Only at later stages of the infection (40-48 h), both T cell types formed antigen dependent, dynamic clusters consisting of mostly one of the two T cell types. This observation indicated that migratory and resident DCs specifically interact with CD4$^+$ or CD8$^+$ T cells, respectively. There was, however, transient interaction between CD4$^+$ T cells that visited CD8$^+$ T cell clusters. Indeed, after their activation by migratory DCs, CD4$^+$ T cells

accessed $CD8^+$ T cell clusters, possibly to provide DC-licensing signals required for $CD8^+$ T cell priming (92). HSV was also found to be recognized by tissue-resident memory $CD8^+$ T cells continuously patrolling the skin (93).

In infection models of vaccinia virus (VV) or vesicular stomatitis virus (VSV), infected cells displayed localization just beneath the SCS within the lymph node, where $CD8^+$ T cells redistributed via HEVs. This relocalization depended on individual antigen-presenting cells in the SCS as well as antigen specificity of $CD8^+$ T cells. Moreover, long-lasting contacts are formed between clustering $CD8^+$ T cells and VV- or VSV-antigen presenting DCs but not macrophages (6). Contrarily, during murine cytomegalovirus (MCMV) infection, $CD8^+$ T cells only slow down for cell–cell contacts with infected cells for a short time, forming kinapses but not synapses. Effector function delivered by cytotoxic $CD8^+$ T cells to MCMV- and MVA-infected cells is a cooperative effect, that is, several $CD8^+$ T cells are required to form contacts with infected cells in order for them to be sufficiently killed. This cooperative killing strategy leads to elongated calcium-signals in the infected cells and subsequent disintegration (94). Also, MP-IVM analysis of the brain established that microglia infected with lymphocytic choriomeningitis virus (LMCV) interact in an antigen-specific manner with $CD4^+$ and $CD8^+$ T cells, a process that induced interferon-gamma (IFNγ)-mediated signaling but did not initiate programmed cell death in the microglia (95).

Effector Function Delivery and Impact on the Pathogen

While the activation of effector cells has been intensely studied, *in vivo* data on the mode of action of antimicrobial activities of immune responses is relatively scarce. In general, effector functions of the immune system are difficult to visualize, due to a plethora of factors that may be secreted by cells, which is challenging to unravel using intravital microscopy. Intravital probing of the pathogen can be used as an indirect indicator for the output of the effector response. Specifically, the effector functions can mediate the eradication of the pathogen by lethally and irreversibly damaging it, or contain the pathogen burden by dampening its growth. Both modes of impact on the pathogen have been observed in different infection models using MP-IVM.

In the course of the innate immune response, direct bactericidal activity of iNKTs against *B. burgdorferi* was determined via the motility and shape changes of the bacteria, and shown to depend on granzyme B (74). Furthermore, the sequestration and reactive oxygen-mediated killing of systemic MRSA by intravascular Kupffer cells was demonstrated in the liver, with only a minority of the bacteria overcoming the antimicrobial defenses (96). Furthermore, *S. aureus* growth rate was demonstrated to decrease after the onset of the innate immune response and uptake into PMN (97), whereas from *in vitro* data, it would have been expected that the bacteria are efficiently killed extracellularly by NETs (69).

The impact on the pathogen by the adaptive immune response has been studied for $CD8^+$ T cells on liver stage *Plasmodium yoelii* as well as for nitric oxide (NO) production on cutaneous *L. major*. Specifically, during the imaging of liver infections with GFP-expressing *P. yoelii*, infected hepatocytes which were surrounded by clusters of $CD8^+$ T cells, were observed to lose GFP-fluorescence, indicating the death of the parasite. $CD8^+$ T cell-mediated killing of the pathogen relied on G-protein coupled receptor-signaling, since treatment of antigen-specific $CD8^+$ T cells with pertussis toxin reduced both cluster formation around infected hepatocytes and pathogen death significantly (88). Recently, using propidium iodide staining in combination with fluorescence protein expression, Aliprandini et al. demonstrated the cytotoxic effect of antibodies against the sporozoite form of *P. yoelii* (98). During cutaneous *L. major* infection, inhibition of the NO synthetase iNOS resulted in an increase of pathogen proliferation at the site of infection, suggesting that a chronic pressure on pathogen proliferation represents a sublethal mode of the control that is required for the eventual resolution of the infection (99). Furthermore, by longitudinal imaging in the ear tissue, Romano et al. could demonstrate that during primary infections with *L. major*, monocytes are permissive for intracellular pathogen proliferation (100).

Immunopathology

Malfunctions in either the innate or adaptive immune response against pathogens can provoke pathologies, which fail to resolve infection and unnecessarily damage to the host. For instance, cerebral malaria is a severe and potentially fatal complication of *Plasmodium* infection in humans that results in swelling and bleeding within the brain, with the underlying mechanisms being poorly understood.

MP-IVM has contributed greatly to unraveling the mechanisms that are responsible for the emergence of this condition, such as the first characterization of a mouse model experimental cerebral malaria (ECM) using *P. berghei* ANKA (PbA), which mirrors many of the pathological features observed in human cerebral malaria as opposed to other *Plasmodium* species. It was further demonstrated that PbA-GFP-infected red blood cells (iRBCs) carrying mature parasites pass slowly through capillaries, engaging in intimate contacts with the endothelium without arresting completely. Moreover, postcapillary venules exhibited platelet marginalization, extravascular fibrin deposition, CD14 expression along with extensive vascular leakage in ECM mice. Blocking cellular interactions mediated by the integrin LFA-1 prevented leukocyte adhesion, vascular leakage as well as neurological signs of and death from ECM (101). In a successive study, the blood flow in postcapillary venules of PbA-infected mice with neurological signs of ECM appeared to be altered, associated with the recruitment of $CD8^+$ Tcells, PMNs and macrophages to the cortical microvasculature. Furthermore, endothelial as well as leukocyte ICAM expression was elevated (102). Thus, these initial studies underlined that ECM pathology is due to the modulation of the blood brain barrier along with

recruitment of activated leukocytes that cause a severe restriction in the venous blood efflux from the brain, which in turn exacerbates edemas and increases the intracranial pressure.

Parasite-specific $CD8^+$ T cells also displayed similar activation status and recruitment, but more stable interactions with APCs specifically in infections with ECM inducing as compared to non-ECM inducing *Plasmodium* strains. This suggests that antigen availability in the tissue might be a major driver of $CD8^+$ T cell-induced fatal vascular breakdown and subsequent neuronal death during ECM (103). Approaches to counteract plasmodium-specific $CD8^+$ T cell accumulation in postcapillary venules include the interference with vascular adhesion. In this regard, $CD8^+$ T cell adhesion was observed to occur in a CXCR3/CCL10-dependent manner that regulates vascular pathology (104,105). Furthermore, also blockade of the integrins LFA-1 and VLA-4 displaced PbA-specific $CD8^+$ T cells from cerebral blood vessels and promoted survival (106).

Also, innate immune functions have the potential to exert immunopathological effects upon infection. As such, PMNs recruited to the site of *S. aureus* infection remained within capillaries to a large extent, which is caused by their CD18 and VLA-4 integrins, as it could be reversed by blocking these adhesion molecules. Thus, PMNs being retained within capillaries upon *S. aureus* infection may prevent the pathogen to quickly spread across the host, but however cause tissue damage by mediating ischemia (32).

Functional Reporter Approaches for Visualizing Pathogen Physiology

Fluorescent-based reporters for probing host immune cells have been used for some time in a variety of approaches both *in vivo* and *in vitro*: localization of cells within tissues, localization of proteins within cells, interaction studies of proteins within cells (107–109), metabolism (110–112), cellular activation (e.g. calcium signaling reporters; (113,114)), behavior such as movement of cells or proliferation (88,115).

In contrast to immune cells, the pathogen has been assigned a somewhat passive role in the intravital analysis of host-pathogen interactions. Although used extensively for localization in the tissue, fluorescently labeled bacteria and parasites have been used merely as "markers" for infected tissue sites and cells (17,29,42,88). Their abilities to react to extrinsic stress factors and signals imposed by the immune system were much less well studied.

Promoter-based reporters have been utilized to determine the onset of virulence gene expression in the ongoing infection. In *S. aureus*, such a transcriptional fluorescence reporter was established in order to evaluate expression of the virulence-related agr operon in the context of pathogen density. In contrast to a predicted quorum sensing-mediated upregulation, *S. aureus* that were mainly located in the periphery of the bacterial focus exhibited agr-dependent GFP fluorescence (29). Yet another promoter-based reporter was employed to quantify *S. Typhimurium* invasion events into the intestinal epithelium *in vivo*. By coupling the expression of GFP to the intracellularly active ssaG promoter (116), intracellular transmigration through the epithelium could be unambiguously identified (42,43).

Recently, the impact of the immune system and its effector functions on the pathogen's life cycle has been approached. Some fluorescence-based systems have successfully been characterized and used for the investigation of pathogen killing in MP-IVM studies. Exploiting the loss or decrease of GFP signal within *P. yoelii* in infected mice, distinct death phenotypes of intracellular pathogens during liver stage malaria were characterized in the ongoing infection (88). Likewise, bactericidal activity of iNKTs against *B. burgdorferi* was determined via the quantification of bacterial motility and shape in the tissue (74).

Pathogen proliferation, on the other hand, has profound implications for persistence, treatment strategies, and recognition by the immune response: First, rapidly proliferating pathogens are a source of large amounts of antigen and pathogen-associated molecular patterns (PAMPs). Second, pathogen proliferation is often inversely correlated with resistance against both immune defense mechanisms and antimicrobial treatment. Thus, pathogens with very low proliferation rates can constitute a reservoir for chronic or relapsing infections, while high proliferating pathogens can be more easily cleared (117–119). Third, pathogens often do not uniformly proliferate, which might contribute to the establishment of persistent subpopulations (120,121).

In vivo proliferation of *L. major* could be examined via adapting the green fluorescent protein mKikume, which can be photoconverted to red fluorescence upon UV light exposure. *L. major* resides within relatively immobile monocyte-derived phagocytes and can therefore be traced over a period of several days at the site of infection. The recovery from photoconversion back to green fluorescence is strictly correlated with proliferative activity, as pathogens displaying high proliferation rates present high amounts of newly-synthesized green mKikume, whereas the photoconverted red mKikume will be progressively diluted. As observed by MP-IVM, proliferation during peak phases of the infection was chronically dampened by NO, which represents a new sublethal mode of control of the pathogen required for ultimately resolving the infection (99). Measuring proliferation *in vivo* also helped to detect the niche for high proliferation of the pathogen during the peak of infection, the monocyte-derived $Ly6C^+$ $CCR2^+$ phagocytes expressing CD11c. Furthermore, it was demonstrated that high proliferating parasites preferentially underwent cell-to-cell spread (48). Photoconvertible mKikume was also implemented for characterizing *in vivo* proliferation of *S. aureus*, which helped to unravel a NADPH-oxidase dependent dampening of bacterial proliferation after initiation of the innate immune response (97).

Outlook

Beyond the fluorescence protein-based approaches already employed for MP-IVM, many interesting experimental systems have been developed *in vitro*, *ex vivo* or in non-vertebrate models. As such, a variety of new genetically encoded fluorescent

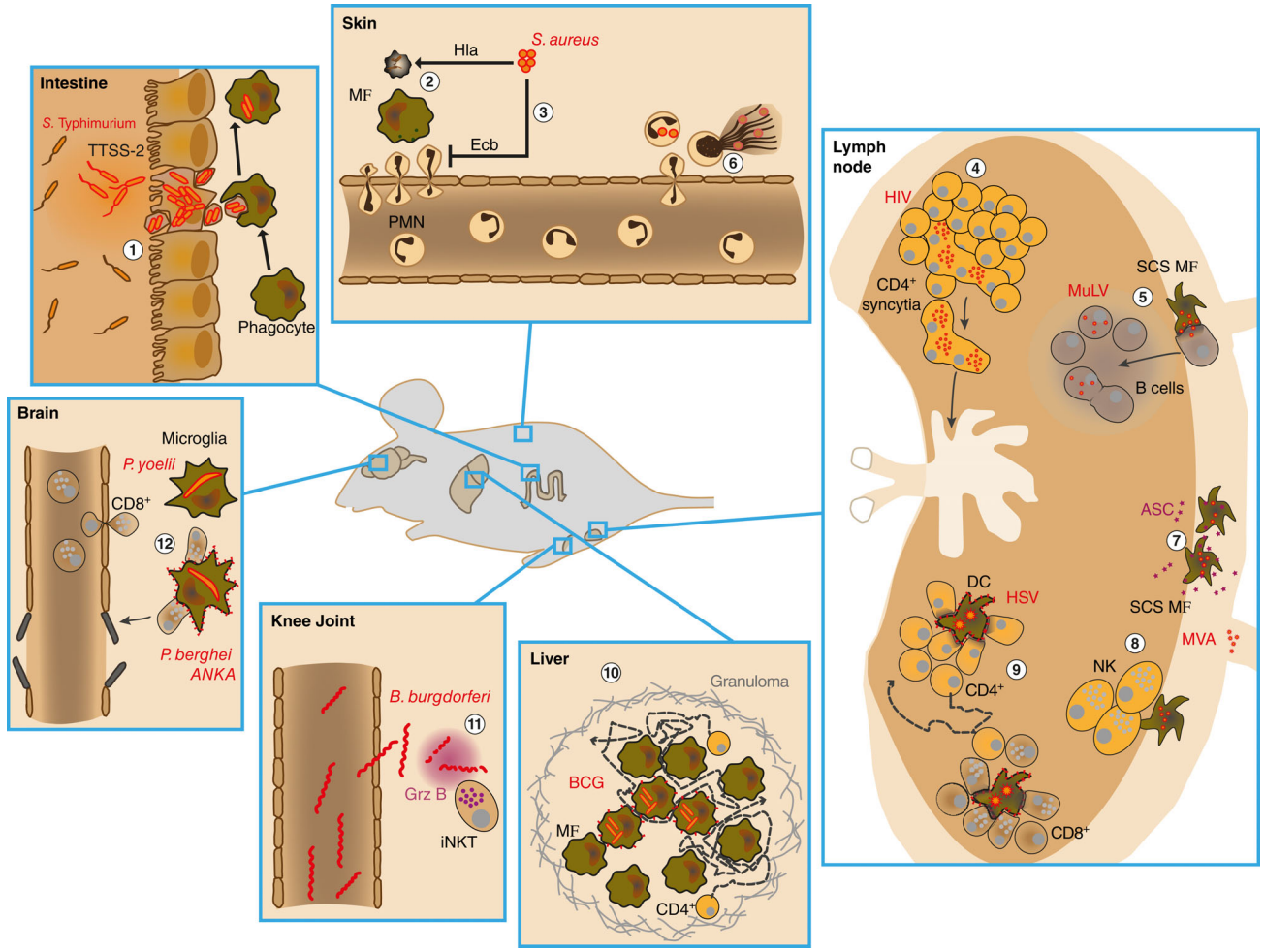

Figure 2. Examples of groundbreaking MP-IVM on host-pathogen interactions from barrier breach through clearance or immunopathology. Examples on MP-IVM findings on barrier breach and virulence strategies of pathogens (1–5), mechanisms of innate immune recognition and responses (6–8), antigen presentation and the initiation of adaptive immune responses (9,10), immune effector function impact on the pathogen (11), and dynamics of immunopathology induction (12). **(1)** Active epithelial invasion and TTSS-2 secretion system-dependent transgression *in vivo* by *S.* Typhimurium. Intracellular proliferation and shedding both back into the intestinal lumen as well as the lamina propria underlying the epithelium (42,43). **(2)** Neutrophil extravasation to the *S. aureus*-inoculated skin occurs at hotspots in direct vicinity of perivascular macrophages, which are killed by the *Staphylococcus* toxin hemolysin α (Hla) (24). **(3)** Inhibition of neutrophil extravasation by the extracellular complement binding protein (Ecb) secreted by *S. aureus* (28,29). **(4)** Dissemination of the human immunodeficiency virus (HIV) relies on motile infected donor $CD4^+$ T cell that display enlarged, thin and elongated morphologies and form syncytia with each other (57). **(5)** Dissemination of the murine leukemia virus (MuLV) depends on the formation of virological synapses, whose presence has been proven for the first time *in vivo* by MP-IVM. MuLV-burdened Macrophages form long-lived contacts with B cells, which, once trans-infected, migrate to the B cell follicle (61,62). **(6)** Neutrophils exhibit a distinct motility behavior after undergoing neutrophil extracellular trap (NET) formation, which is the first demonstration of a certain degree of viability in these cells after NETosis (68). **(7)** First *in vivo* demonstration of inflammasome formation and release of particles containing the inflammasome adaptor ASC following modified vaccinia Ankara virus (MVA) capture by subcapsular sinus (SCS) macrophages (77). **(8)** NK cell recruitment to SCS macrophages upon MVA infection (76). **(9)** Step-wise T cell priming during primary HSV infection is marked by early $CD4^+$ T cell interaction clusters with infected dendritic cells, followed by $CD8^+$ T cell interaction clusters, which are transiently accessed by $CD4^+$ (92). **(10)** Limited access to *Mycobacterium* BCG antigen in liver granulomas is responsible for only partial activation of antigen-specific $CD4^+$ T cell effector functions (82). **(11)** Invariant NK T cells (iNKT) kill blood vessel-extravasating *Borrellia* (*B. burgdorferi*) via the release of Granzyme B (Grz B) (74). **(12)** Parasite species-specific differences in T cell—microglia interaction dynamics define immunopathology *Plasmodium* infection: $CD8^+$ T cells display more stable interactions with microglia in infections with experimental cerebral malaria (ECM) inducing *P. berghei* ANKA than with non-ECM inducing *P. yoelii* (103).

biosensors are now available, which have the potential of tremendously contributing to an increased information depth for intravital imaging. Examples of such systems include timer proteins, which allow the time-resolved measurement of protein turnover via maturation-dependent fluorescence (122), or signaling molecules, which act as conformational biosensors for immune cell activation (123). Furthermore, redox- (124) and pH-sensitive fluorescence proteins (125), as well as metabolic sensors (111) offer the possibility of probing cellular physiology together with the dynamic behavior of both immune cells and pathogens.

While employing fluorescent proteins requires the use of transgenic animals and cells, label-free approaches might expand even further the applicability of MP-IVM. Examples for this are the noninvasive imaging based on multimodal nonlinear optical microscopy, which allows the detection of nonlabeled bacteria within the tissue using endogenous NADH two photon-excited fluorescence (NADH-TPEF) (126). Simultaneous label-free autofluorescence-multiharmonic microscopy (SLAM) represents another very promising possibility of label-free MP-IVM: By broad far-red excitation and spectrally resolved autofluorescence detection, You et al. enabled the tracking of intercellular and stromal–cell interactions in the non-perturbed living tumor microenvironment (127). A recent *in vitro* study established a MP-IVM-based FLIM approach that allows for the visualization of the dynamics of NADPH oxidase activation and its requirement in triggering NETosis (128). This concept is also applicable for MP-IVM, as has been shown for imaging of the small intestine (129), and has the potential to become a powerful tool for investigating the interactions between pathogens and the host immune system.

Conclusions

The possibility to study by MP-IVM the interaction of pathogens with their host in the appropriate tissue environment, and in real-time, has continued to reveal under-appreciated and even unknown aspects of pathogen virulence, immune induction, and clearance of infections (Fig. 2). This has greatly contributed to critical initial observations of phenomena that had previously been only extrapolated from *ex vivo* and *in vitro* works. Furthermore, completely new mechanisms by which pathogens establish infection within the host, and by which the immune system detects, combats, and clears the pathogen, have been discovered using MP-IVM. While within the last ten years many studies employed MP-IVM mainly for the investigation of pathogen and immune cell interaction and motility, first approaches have been taken that allow extracting functional information on pathogens and host cells directly from intravital imaging. The application of such tools for the measurement of dynamic changes in the pathogen's physiology and infection microenvironment doubtlessly has the potential to continue the success story of MP-IVM investigations on host-pathogen interactions.

Acknowledgments

A. J. M. was supported by funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (StG ImmProDynamics, grant agreement n° 714233), the German Research Foundation DFG (SFB854-Z01, SFB854-B31 and MU3744/4-1), and the federal state of Saxony-Anhalt and European Regional Development Fund (Project "NeutrEat").

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