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Review

Imaging of the spleen in malaria

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ABSTRACT

Splenomegaly, *albeit* variably, is a hallmark of malaria; yet, the role of the spleen in *Plasmodium* infections remains vastly unknown. The implementation of imaging to study the spleen is rapidly advancing our knowledge of this so-called "blackbox" of the abdominal cavity. Not only has ex vivo imaging revealed the complex functional compartmentalization of the organ and immune effector cells, but it has also allowed the observation of major structural remodeling during infections. In vivo imaging, on the other hand, has allowed quantitative measurements of the dynamic passage of the parasite at spatial and temporal resolution. Here, we review imaging techniques used for studying the malarious spleen, from optical microscopy to in vivo imaging, and discuss the bright perspectives of evolving technologies in our present understanding of the role of this organ in infections caused by *Plasmodium*.

Contents

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1. Introduction

The spleen is a secondary lymphoid organ perfectly adapted to induce innate and adaptive immune responses, and to phagocytose both senescent and damaged red blood cells (RBCs) and blood-borne pathogens including the malaria parasite, *Plasmodium* spp. [1,2]. The capacity to perform these functions is closely linked to the complex structure of the spleen formed by (i) the white pulp, a lymphoid area composed of T and B cell zones where acquired immune responses develop; (ii) the red pulp, an important area for blood-filtering and iron recycling; and (iii) the marginal zone, a transit area harboring a large number of cells with unique properties that enable the efficient surveillance of circulating blood (Fig. 1).

Due to ethical and technical constraints for its study, the spleen was for long considered an 'orphan' organ, or a so-called 'black box' in the abdominal cavity [1]. In the past, studies of spleen pathology were mostly limited to post-mortem examinations and to the observation of surgically removed spleens [3]. It was not until the early 1930s that the advent of ex vivo imaging of fixed splenic structures, and of spleen cell dynamics in vitro provided invaluable information about the ultrastructure of cells and tissues of this organ in healthy and pathological states [4,5]. In the last two decades, the emergence of advanced dynamic live imaging modalities has enhanced our understanding of immune cell function and cell–cell communication in healthy and diseased states, as well as promising developments in immunoimaging in situ in organs including the spleen [6,7].

2. The spleen in malaria

Infections by malaria parasites induce a dramatic, albeit variable splenic response mostly characterized by splenomegaly. In fact, spleen size has been used as a tool to determine the intensity of malaria transmission in endemic regions [8–10]. Of note, during the erythrocytic stages of malaria infection, the spleen is the main organ involved in the development of the immune response and in elimination of pRBCs [2,11]. However, parasites retaliate by establishing chronic infections through evasion and modulation of immune responses and by remodeling the spleen, sometimes leading to immunopathology and severe disease [12]. In addition, several *Plasmodium* species escape spleen clearance through cytoadherence to endothelial cells in inner organs causing end-organ dysfunction [13,14]. The dual role of the spleen in protection and pathology, for instance in cerebral malaria and severe malarial anemia, highlights the need for further research on this organ.

The relevance of the spleen for control of malaria infection comes from direct evaluation of splenectomized human patients and rodent models. Several cases of *Plasmodium falciparum*-infected patients with various states of immunological competence, suffering splenectomy, have been reported [15]. In immune individuals, for instance, the

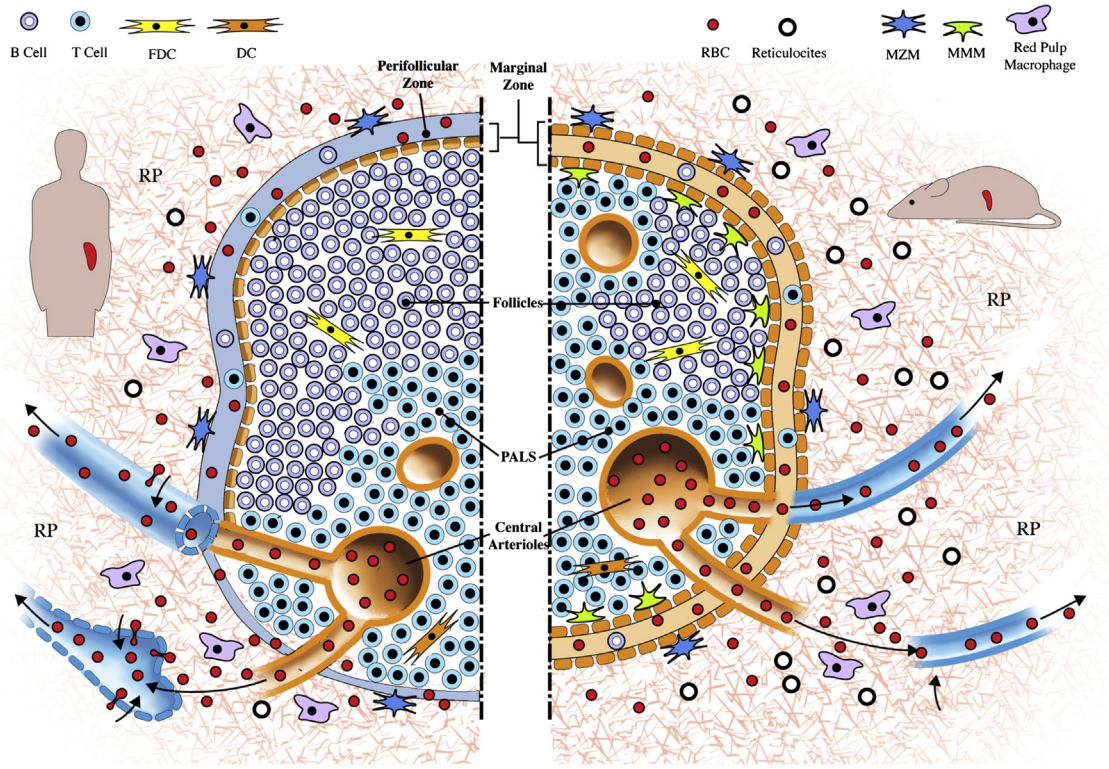

Fig. 1. Comparison of the human and mouse spleens. Three main differences exist between mouse and human spleens. (i) The marginal zone in human spleen lacks a clearly delimited marginal sinus and it is surrounded by an additional perifollicular zone, where some blood vessels terminate in capillaries that bypass the filtration cords as fast 'closed' microcirculation. (ii) The human spleen is sinusoidal whereas the mouse spleen is non-sinusoidal. The former consists of a honeycombed reticular meshwork of venous sinuses, in which endothelial cells align longitudinally in a barrel-like shape sustained by a fenestrated basement membrane and adventitial reticular cells. This forces blood to squeeze through the interendothelial slits before entering the lumen of venous sinuses. Differently, in non-sinusoidal spleens, blood flows through open-ended pulp venules provided by flat endothelium, a conventional basement membrane and adventitial reticular cells, thus offering little impedance to entrance of red blood cells. (iii) There is intense erythropoiesis elicited in the mouse spleen yet unreported in the human spleen. FDC, follicular dendritic cell; MMM, marginal metallophilic macrophages; MZM, marginal zone macrophages. Figure originally published in [11].

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absence of a spleen aggravated the course of the disease, while antibody mediated clearance of pRBCs was undertaken by other organs though less efficiently. The spleen has also been found to play a role in chronifying infection, since removal of the spleen in a chronic carrier led to a significant increase in peripheral parasite loads and acute attack [16]. In addition, clearance of pRBCs in splenectomized patients undergoing anti-malarial treatment showed a marked delay in comparison to patients with functional spleens. On the other hand, splenectomy of both *P. falciparum* and *Plasmodium vivax* non-immune patients [17,18] has been related to aggravated severity and increased risk of death due to malaria. Overall, while the spleen may not be essential for parasite clearance in partially immune, splenectomized patients, it exerts a major protective role in non-immune patients, via macrophage responses or mechanical retention/filtering of parasites. In addition to pathological complications, higher numbers of parasitemia and of circulating mature forms were found in splenectomized patients. Similar to these observations in humans, in *Plasmodium chabaudi*- or *Plasmodium yoelii*-infected mice [19,20], prolonged waves of parasitemias and impaired parasite clearance have also been linked to splenectomy. Of interest, *P. yoelii* 17X infection in mice from distinct backgrounds responded differently to splenectomy: DBA/2 mice were not adversely affected by removal of the spleen, while C57BL/6 and Balb/c mice failed to resolve infection. The lower parasitemia levels in splenectomized mice were attributed to the lack of spleen-derived reticulocytes for parasitation [19]. Thereafter, host genotype may influence splenic response to infection, in which alternative mechanisms, such as pRBC phagocytosis in the liver, as well as bone marrow hematopoiesis, may help to overcome the lack of spleen.

A second line of evidence for the importance of the spleen in malaria comes from architectural remodeling which is so extensive, that in the past it led to the hypothesis that the spleen structure had evolved as a response to the presence of the parasite [21]. In fact, the complex anatomy of the spleen brings together adaptive and innate immune responses, ensuring filtration and careful surveillance of pRBCs in specialized splenic compartments. In the red pulp, macrophages uptake circulating and opsonized pRBCs, and mechanical filtering occurs [22,23]. In the white pulp and marginal zone, parasite capture and antigen processing by specialized dendritic cells leads to antigen-specific T cell priming, DC modulation, and B cell modulation as well as their repositioning to deliver antibodies into the circulation [2,8]. Given this link between structure and function, compromise of splenic compartments leads to alterations in organ-specific and systemic immune responses in ways that are not yet fully understood.

3. Conserved and differential features of human and murine spleen morphologies

Imaging methods have enabled us not only to characterize the morphology, structure, and function of the spleen in health and disease states, but also to study comparative morphology of this organ in various animal species. This has been key for determining the validity of experimental animal models to study events in the spleen, including malaria infections. A certain uniqueness between the spleens of different animals, such as dogs, horses, cats, monkeys, rats, and mice, has been documented. Banks et al. first proposed the classification of spleens into 'storage', and 'defense' spleens, based on the morphology of capsular and trabecular smooth muscle fibers [24]. More recently, Dellman and Brown proposed the classification of mammalian spleens into sinusal and non-sinusal, based on the type of post-capillary vessels present in the organ [25]. Based on this latter classification, the human and rat spleens are sinusal, while the murine spleen has no arteriovenous shunts, and is non-sinusal; this is considered a key difference for our understanding of splenic function in malaria research. While spleen remodeling during malaria infections has been observed in all animals studied, whether specific structural differences between humans and experimental animals – for instance, mice – invalidate them as models to study malaria remains a matter of controversy (Table 1). Specific differences in splenic responses during malaria between humans, rodents, and monkeys has been reviewed elsewhere [11]. However, in this section we will focus on overall morphological differences between murine and human spleens imaged over the last few decades.

Although important anatomical differences between humans and rodents exist, multiple morphological features are conserved [26]. This fact, together with observations on malaria responses in animal models gives rise to the question on whether spleen-dependent mechanisms of immunity against malaria might in fact, be redundant. Nevertheless, various authors have commented on the need to observe care when extrapolating results from one species to another.

Balogh et al. recently reviewed in great detail the reported observations on differences existing between human and mice spleens, gathered through the use of a variety of immunohistochemistry, confocal, and advanced fluorescence microscopy techniques [26]. A key splenic component that shows significant differences between human and mice is the marginal zone (MZ). Human spleens possess a perifollicular zone lined by MAdCAM-1 + cells, and populated by specialized cell types; this structure is altogether absent in mice and rats. Nevertheless, mice possess a fibroblastic stromal microdomain reminiscent of the perifollicular zone in humans [27]. Reportedly, the presence or absence of the perifollicular zone and its components impacts on the vascular structure and branching of the arterioles in the different species, as well as possibly influencing the morphology of efferent lymphatic vessels in the white pulp. Whether these differences have an impact on leukocyte circulation patterns, as well as response to infection, remains undetermined [27]. For malaria research, this is of relevance, as the marginal zone has been shown to be a key compartment differentially remodeled by the *Plasmodium* parasite in lethal and non-lethal infections. While various significant differences between species have been reported with regard to the microarchitecture of the spleen, organogenesis, and anlage, these topics are outside the scope of the present review.

As previously stated, various features of the splenic morphology are conserved among species. Similarities between murine and human spleens include the scaffolding structure surrounding the lymphoid architecture of the organ, and its composition (namely, vasculature and fibroblastic meshwork); presence of a conduit system; the compartmentalization of the spleen into white pulp, red pulp, and marginal zone; the overall composition and structure of the white pulp; the composition of the terminal segments of arteriolar capillaries within the red pulp and their lining by ellipsoid sheaths; the presence of fibroblastic reticular cells, and the conserved role they play in enabling ordered lymphocyte migration. The differences with regard to the splenic red pulp remain poorly studied and incompletely understood.

More novel studies on spleen morphology using advanced fluorescence and electron microscopy techniques with potential relevance for the malaria field have been done in mice and humans. Among the most relevant observations in mouse spleens is the description of the conduit system in the white pulp, by Nolte et al. [28], which aside from playing a role in the separation of fluid extracellular spaces and parenchymal compartments, may also be implicated in the directional movement of lymphocytes via the display of specific chemokines. In humans, one of the most novel observations, done by Ogembo et al. [29], focused on unique markers of littoral cells, a potentially key, yet understudied population of the human splenic red pulp. This specialized, highly plastic cell population is thought to be unique to *Hominidae* [30]. The importance of littoral cells is believed to lie on their role as a determinant for cell recirculation following passage through the splenic red pulp. On one hand, it has been shown that prior to return to venous circulation, splenic constituents have an obligatory passage through the littoral cells, lining the venous sinuses. On the other hand, various blood components, including leukocytes, phagocytosed erythrocytes, hemosiderin, and debris, have been visualized within littoral cells. This has led to the suggestion that they may be key mediators of pathogen clearance and cellular filtration, although functional evidence remains

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Table 1
Main findings of the role of the spleen in human and experimental malaria infections.

	Human	Animal models
Protective role of the spleen in malaria infection	-Major protective role of the spleen in *P. falciparum* and *P. vivax* non-immune patients [18,107] -Not essential in immune individuals since antibody-mediated clearance of pRBCs was overtaken by other organs though with less efficiency (reviewed in [15]). -Spleen removal in chronically infected *P. falciparum* patients led to acute attacks [16]. -Reduced risk of severe complications, such as cerebral malaria, could be related to retention of *P. falciparum* ring forms in the spleen that reduces the parasite biomass that sequesters in vital organs [108].	-Role of the spleen in parasite killing and immune response during crisis in *P. chabaudi adami*-mouse and *P. berghei*-rat infected models [23,109] -Influence of host genotype in the splenic response to infection: removal of the spleen in *P. yoelii* 17X-BalB/c mice aggravated non-lethal malaria and ameliorated lethal malaria [21], while it had no effect in DBA/2 mice [19], in which alternative mechanisms, such as pRBC phagocytosis in the liver and/or bone marrow hematopoiesis, may supply its function.
Pathogenic features related to the presence of the spleen	-Role in chronifying infection [16]. -Splenomegaly is a hallmark of malaria disease [8–10]. Spleen rupture, anemia and thrombocytopenia [110] are complications associated with malaria.	-Spleen-derived reticulocytes provide a pool for parasitation in *P. yoelii*/DBA/2 mouse model [19] -Thrombocytopenia has been related to the presence of a spleen in *P. chabaudi* infected mice [111].. -Priming of CD4 and CD8 T cells in the spleen contributed to development of *P. berghei* experimental cerebral malaria, which was reversed by splenectomy or by T cell depletion [112].
Spleen effect on parasite variant genes	-Absence of surface antigens and lack of sequestration of parasites after splenectomy in immune patients infected with *P. falciparum* [16,113] suggest modulation of parasite population antigens on the surface of pRBC and the ability of pRBC to bind to endothelial receptors (or parasite sub-population lacking expression of surface antigens and cytoadherence, which upon splenectomy expands).	– -Absence of variant surface antigens and lack of sequestration of parasites after splenectomy in *P. knowlesi*-, *P. fragile*- and *P. falciparum*-infected monkey models [114–118]; and in *P. chabaudi chabaudi* AS-CBA/Ca mice [119]
Sequestration and cytoadherence of pRBCs in the spleen	-Cordal expansion and sequestration of altered RBCs [42] -Mechanical retention of the less deformable subset of ring forms of *P. falciparum* in an ex vivo model of the spleen [108] -High prevalence of *P. vivax*-infected reticulocytes in the ruptured spleen of an untreated *P. vivax* patient during active infection [50]. -*P. vivax* VIR-mediated cytoadherence to ICAM-1 and spleen cells in vitro under flow conditions [120]	-Intravital imaging studies of the dynamic passage of *P. yoelii* 17X through the spleen demonstrated accumulation and active cytoadherence of infected reticulocytes to a spleen–blood barrier of fibroblastic origin [52]. -*P. berghei* mutants expressing luciferase under the control of the ama1 promoter have been used to determine the distribution of sequestered schizonts in different organs including the spleen [13]
Structural remodeling and vascular changes	-*P. falciparum* and *P. vivax* parasites were reported to induce splenic infarction and capsule distension based on CT images [88–90] -Agglutination of parasitized cells in the splenic vasculature in cases leading to infarction or spontaneous spleen rupture. Vascular changes occurring during gradual progressive enlargement of the spleen [1]. -White pulp expansion and a diffuse hypercellularity in the splenic red pulp, reported from morphological studies of post-mortem or spleen-rupture cases. -In *P. falciparum* and acute malaria, reactive white pulp nodules were predominant, some displaying fibrinoid necrosis. In chronic malaria, red pulp expansion and abundant hemozoin accumulation were common, with less marked changes in the white pulp [3]. -Immunohistochemical studies of spleen sections from patients dying from severe *P. falciparum* malaria revealed disorganization characterized by MZ B cell loss, and eventually, MZ loss [49]. -Presence of large numbers of mitotic plasmablasts in peri-vascular spaces of an untreated ruptured spleen from a *P. vivax* patient [50] -The existence of barrier cells in the human spleen remains controversial in malaria even though their presence in the human spleen in other pathologies has been substantiated [1]. -Littoral cells may be key mediators of pathogen clearance and cellular filtration, with unique markers enabling them to perform surveillance of circulating cells, and discriminating between permitting entry back into circulation or blocking passage	-The red pulp expands, and in mice is the site of significant hematopoiesis [121–123] -Marginal zones are transiently lost [124], and T and B cell zones can become indistinct with extrafollicular development of plasma cells [123] -Non-lethal *P. yoelii* infections induced a general expansion of white and red pulps, and an increase in the number of reticular and plasma cells [46]. Conversely, lethal *P. yoelii* caused widespread degeneration of reticular cells, and overcrowding of the reticular meshwork with macrophages, some of which showed intracellular or pericellular lysis [47]. -In non-lethal malaria infection of BALB/c mice with the reticulocyte-preferring *P. yoelii* 17X, the "open" circulation of the spleen is suddenly and temporarily changed to a "closed" circulation, through the appearance of barrier cells [46]. Such cells putatively contain receptors for the specific cytoadherence of *P. yoelii* 17X infected reticulocytes allowing macrophage-clearance escape and establishment of chronic infections [52] -Identification of a fibroblastic barrier to which parasites sequestered and its changes throughout the course of infection with *P. yoelii* [52]
Immune response	-Red pulp macrophages uptake circulating and opsonized pRBCs [22,125] -An ex vivo human spleen model shows that more than 90% of artesunate pretreated-iRBCs were retained and processed in the red pulp [93] -In *P. falciparum*, removal of crisis forms or drug-treated pRBCs is achieved through "pitting" [18].	-Red pulp macrophages involved in removal of *Plasmodium* infected RBC [23] -Inflammatory monocytes, which migrate into the spleen during an acute *P. chabaudi* infection phagocytose iRBC in the spleen and contribute to the control of acute parasitemia [126,127] -Adoptive transfer of splenocytes to splenectomized mice confers protection to *P. chabaudi* infection [20,128]

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Table 1 (*continued*)

	Human	Animal models
Immune response	-Preferential phagocytosis of immature parasite forms [43]. -Splenic dendritic cells are increased in malaria and there is a reduction of B lymphocytes and macrophages in the splenic cords [49] -In *P. vivax*, a large plasmablastic proliferation in vascular and peri-vascular spaces, as well as significant increase in B-cells and plasma cells, confirmed a robust humoral immune response during active non-treated infection [50]	-Two-photon microscopy has reported T-DC synapses in the marginal zone and red pulp of the spleen [72,73], respectively. -Both populations of classical DC can take up parasite material, process and present *Plasmodium* peptides to CD4 T cells [129,130] but with different kinetics. -Viable *P. berghei* parasites have been observed in plasmacytoid DC in vivo [131]. Whether this could result in parasite destruction and antigen presentation remains unknown. -Suggestions that splenic DCs during infection are unable to activate T cells effectively in vitro [132–136] -Prominent germinal center formation and generation of a long-lived memory B and CD4 T cell response as well as long-lived plasma cells and protective antibodies [137,138] -T cell recruitment to and migration within the white pulp were suggested to be directed by fibroblast channels originating in the marginal zone [70,71] -The conduit system in the white pulp [28] may be implicated in the directional movement of lymphocytes via the display of specific chemokines.

lacking [29]. Ogembo et al. recently focused on the relevance of unique markers expressed on littoral cells, which may enable them to perform surveillance of circulating cells, and discriminating, based on their content and overall status, between permitting entry into circulation or blocking passage. FHOD1 and SIRPα (CD172a) were the main markers addressed in this study [29]. On one hand, the highly polarized expression of SIRPα hints towards a role in mediation of RBC turnover, phagocytosis, and iron regulation [31,32] via its interaction with CD47. SIRP1α is believed to transduce negative intracellular signals that block phagocytosis, so that upon reduced CD47 (as is the case of senescent RBCs), signaling leading to cell destruction would ensue, while absence of such signal would enable passage into the sinus. On the other hand, the marker FHOD1, a member of the *Drosophila*-related formin family of actin regulator proteins, is believed to control various cellular phenomena including cytoskeletal regulation, and is believed to be related to the littoral cells' function in relation to antigens/pathogens, and cell invasion/internalization [33–35]. Other markers of interest in littoral cells include DARC, a chemokine receptor known for its relation to malaria; and CD8α/α among others [29]. The multi-lineage characteristics of, and the plethora of antigens expressed by littoral cells make them an attractive research target in general, and for future malaria research in particular.

4. Ex vivo imaging of the spleen

Over the last few decades, the transfer of imaging technology into the biology domain has improved our understanding of splenic architecture, and given clues on the immunological impact of splenic remodeling. It is expected that the rapid and continuous improvement of imaging techniques will provide better insight into the implications of host–pathogen interactions at the spleen and its role in clinical outcomes [36,37].

4.1. Light microscopy

Light microscopy has enabled the observation of phenomena such as congestion of the spleen with parasitized RBCs, accumulation of hemozoin and hemosiderin in splenic cords, and differential white pulp and red pulp remodeling upon infection with malarial parasites. The study of changes to splenic tissue and parasite accumulation, has been greatly aided by techniques including hematoxylin and eosin staining, and Giemsa staining visualized under polarized light. The former technique has led to characterization of changes in splenic compartments, most importantly the red pulp, periarteriolar lymphoid sheath, and marginal zones of infected hosts. The latter technique has enabled detection of, for instance, parasite localization in splenic compartments and their quantification due to the refringent properties of hemozoin. Additionally, immunohistochemical (IHC) histology has enabled the study of specific splenic lymphocyte populations throughout the course of infection. Cell populations whose nature and timing have been partially studied in fixed samples from humans and mice include neutrophils, red pulp macrophages, T cells (including CD4+, CD8+ and regulatory T cells), B cells, marginal zone and metalophillic macrophages, marginal zone B cells, and splenic dendritic cells, among others [38].

Aside from lymphocyte characterization, light microscopy has also enabled the observation of agglutination of parasitized cells in the splenic vasculature in cases leading to infarction or spontaneous spleen rupture, as well as vascular changes occurring during gradual progressive enlargement of the spleen [1]. The recent advent of other imaging techniques has enabled increased resolution, higher tissue penetration, and in vivo dynamic imaging. Nevertheless, the light microscope remains a valuable diagnostic tool for splenic pathology.

4.2. Electron microscopy

Electron microscopy (EM) observations of the normal spleen were published almost four decades ago. These include the complex vascular architecture and microcirculation in the spleen, and its implications for pathology [39]. They also include analyses of the ultrastructure of the red pulp in the human spleen, including the close relationship between splenic cords, splenic sinuses and macrophages, and their relevance for malaria [5,40]. Furthermore, transmission (TEM) studies enabled the recognition that reticular cells form a spongework which constitutes the filtration mechanism of the spleen, and that this filtration meshwork differs in different parts of the spleen [41]. Additionally, TEM and scanning (SEM) studies have provided insight of erythrocyte pliancy – deformability and elasticity – upon their transit through the spleen [42]. These include observations on the alterations in the cellular configuration of RBCs which leads to cordal expansion and sequestration.

In the context of malaria, studies using electron microscopy have described events such as the interaction of parasitized erythrocytes with heterogeneous populations of phagocytic cells, whereby preferential phagocytosis of immature forms of parasites was observed [43]. Other observations include phenomena such as splenic trapping, pitting [15,44], destruction of infected and uninfected erythrocytes, adherence to splenic walls, differential association of phagosomes, and rosetting [43,45].

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One of the major contributions of EM to our understanding of *Plasmodium* interactions in the spleen in rodent models came from Leon Weiss and collaborators. In 1986, Weiss et al. published the first detailed description of the architectural changes induced in the spleens of mice infected with lethal and non-lethal strains of *P. yoelii*. Non-lethal *P. yoelii* infections induced a general expansion of white and red pulps, and an increase in the number of reticular and plasma cells [46]. On the other hand, the lethal form of *P. yoelii* was mostly characterized by widespread degeneration of reticular cells, and overcrowding of the reticular meshwork with macrophages, which showed intracellular or pericellular lysis [47]. In this model, failure to control parasitemia led to failure to relief anemia, and to continued increase of parasitemia up to 60–85% and death. These observations are consistent with observations in human spleens. In *P. falciparum* and acute malaria, reactive white pulp nodules were shown to be predominant, and in some cases displayed fibrinoid necrosis. In chronic malaria, red pulp expansion and abundant hemozoin accumulation were common, with less marked changes in the white pulp [3].

4.3. Fluorescence microscopy

Fluorescence microscopy has enabled the study of biological specimens on a structural level, and is dependent on sample fluorescence either through the use of fluorescent stains, immunofluorescence, or genetic modification to introduce a fluorescent protein reporter [48]. Major advances in this field include the introduction of bright-field and dark-field microscopy, the advent of fluorescent dyes, and the introduction of labeled antibodies, as well as the surge of epifluorescence, confocal, and super-resolution microscopies which has enabled the study of specific splenic population changes in lethal and in non-lethal malaria in rodents and humans.

Urban et al. published one of the first immunohistochemical studies of spleen sections from patients dying from severe *P. falciparum* malaria [49]. By labeling with various leukocyte markers, including neutrophils, myeloid dendritic cells, T cells, and B cells, they were able to show differential patterns of splenic architectural disorganization in fatal malaria cases, mainly characterized by MZ B cell loss, and eventually, marginal zone loss. More recently, the first confocal fluorescence and IHC study of an untreated ruptured spleen from a *P. vivax* patient demonstrated the presence of large numbers of mitotic plasmablasts in peri-vascular spaces as well as a high prevalence of *P. vivax*-infected reticulocytes outside macrophages [50].

Detailed information on structural and functional changes in spleen cell populations in rodent models infected with *Plasmodium* strains of different levels of lethality has also been obtained through fluorescence imaging. Krucken et al. showed malaria-inducible spleen-inherent closing mechanisms controlling the uptake of parasitized and non-parasitized RBCs and explored the potential role of TNF/TNFR1 signaling in regulation of splenic closure, thereby challenging the idea of complex mechanisms of sequestration developed by the parasite to avoid passage through the spleen [51]. Yet, more recent work on lethal and non-lethal GFP-transgenic *P. yoelii* strains used immunofluorescence to confirm the identification of a fibroblastic barrier to which parasites sequestered and its changes throughout the course of infection with *P. yoelii* [52]. Additional to changes in splenic structure, fluorescence microscopy has enabled the study of various phenomena including pitting and spherocyte formation [53], red blood cell deformability and spleen-filtering functions [15,54–56].

5. In vivo imaging

The possibility to observe immune cells and infectious agents in real-time during their dynamic passage and interactions in living tissues is generating new and unsuspected insights into host–pathogen interactions, including malaria [57–59]. Noticeably, imaging of the spleen in malarial infections is challenging our present views on the role of this organ in infections and is opening new avenues for our understanding of immune cells and the mechanisms elicited by iRBC in their passage through this lymphoid organ.

Diverse imaging modes have been engineered based on energy–matter interactions. Their suitability to image live small animal models depends on their specific features and the spatiotemporal resolution achieved [60,61]. On one hand, intravital microscopy techniques (IVM) have been extensively used to image dynamic molecular and cellular events within tissues, achieving high spatiotemporal resolution (μm/s). As it is based on light interactions, it usually requires surgical exposure of the organ of study and imaging is restricted to short depths beyond the exposed surface. On the other hand, non-invasive imaging techniques, such as magnetic resonance imaging (MRI), radiotracer modalities (scintigraphy, positron emission tomography (PET) and single-photon emission computed tomography (SPECT)), X-ray computed tomography (CT), ultrasound and bioluminiscence systems, permit whole body imaging with repeated observations of the same subject over longitudinal studies. Though they can penetrate deep into the tissues, low spatiotemporal resolution is reached (50 μm-mm, min–s). Some applications of these techniques include biodistribution analyses, metabolite tracking, cell migration and imaging of tissue architecture and vascularity.

5.1. Intravital imaging

Intravital imaging using two-photon laser scanning microscopy was pioneered in 1990 and has since revolutionized our understanding of biological systems [62]. In malaria, implementation of intravital imaging has provided knowledge of the mechanisms of parasite invasion, dissemination and tissue distribution at different stages of the life cycle, including the mosquito and mammalian hosts [52,57,63–67]. *Plasmodium* transgenic lines for GFP or RFP derivatives, like tdTomato and mCherry, have been created to unveil dynamics of parasite behavior and to image parasite–host cell interactions in GFP-expressing cell subsets of transgenic mouse strains [68]. With the implementation of intravital microscopy to experimental models of malaria, the journey of malaria parasites to the blood is revealed much more complex than originally thought [66,69]. Moreover, in vivo imaging of parasite–host interactions in the context of immunity and disease paves the way for the understanding of malaria pathogenesis.

The spleen is a particularly difficult organ for imaging as it accounts for a three-dimensional branched vasculature, containing both closed/rapid and open/slow circulations, and a compartmentalized parenchyma (red pulp, white pulp and marginal zone) enclosed within a dense capsule [1]. Implementation of intravital imaging of the spleen was first accomplished by Grayson et al. to assess T cell recruitment in the white pulp [70], in which migration was later suggested to be directed by fibroblast channels originating in the marginal zone [70,71]. Other studies using two-photon microscopy have reported T-DC synapses in the marginal zone and red pulp of the spleen [72,73], respectively. As well, clustering of T cells in the white pulp was visualized ex vivo in *Listeria* experimental infections [74] and another study reported clusters of monocytes in the cords of subcapsular red pulp that functioned as storage for their rapid deployment to regulate inflammation [75].

The implementation of intravital microscopy of the spleen in malaria used the rodent Balb/c-*P. yoelii* 17X-GFP model [52] and opened the possibility of investigating the dynamic passage of parasites through this organ. In this pioneering study, the dynamics of parasite–spleen interplay were assessed through intravital microscopy to the spleen in Balb/c mice infected with two GFP-expressing *P. yoelii* strains (17XL) or (17X) infections. Parasites were imaged passing through the spleen at day 3 p.i. using laser scanning confocal microscopy and characterized off-line. As control, uninfected mice were injected with FITC-labeled RBCs. Of interest, erythrocyte reflection and intravascular dyes were used to identify the microcirculation and to measure blood flow in this organ, which is an important factor to control when studying cell

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dynamics [76]. Moreover, it allowed sufficient penetration to visualize events in the subcapsular zone, composed mainly of red pulp [75], and permitted high scanning velocities when used in resonance mode. In vivo images of the passage of the non-lethal strain showed an adhesive, rolling-circle behavior of pRBCs in real time.

5.2. Quantitative imaging of parasite–spleen interactions

Intravital imaging of GFP parasites in the spleen revealed differences in mobility between the two strains of parasites [52]. In order to quantify and compare pRBC mobility, specific procedures were developed to track individual particles in space and time [77], where automatic software failed to follow fast moving particles over time. Comparative analysis between *P. yoelii* 17XL and17X strains and between uninfected mice injected with FITC-labeled RBCs revealed significant differences in the accumulation and dynamic behaviors of parasite populations. To avoid any confounding result, parasites were imaged passing through the spleen at day 3 post-infection, when hematocrit, reticulocytemia, parasitemia and host cell invasion preference are comparable in both strains. Quantitative analysis of mobility parameters of single parasites indicated reduced velocity, lack of directionality and augmented residence time of parasites of mice infected with 17X strain overall suggesting adhesion to spleen cells. Of note, the parameters used to describe parasite mobility have been previously described in other studies to report lymphocyte recruitment and adhesion in vivo [70,78]. Thus, this methodology and parameters should be considered a new tool to the in vivo studies of adherence in malaria. Whether the adherent population in non-lethal infected mice corresponded to infected reticulocytes and the developmental stage of the parasites remains to be determined. Of interest, blood flow in this organ was calculated from the streaks resulting from moving RBCs when imaged with high line average in vessels with different diameters and over different phases of the cardiac cycle. In the future, this technology may be used to gain insight into the immunobiology and parasite–spleen cell interactions by imaging infection in transgenic mice expressing fluorescent reporter genes in different cells. Moreover, the generation of transgenic parasites expressing fluorescent markers other than GFP may be used in combination to image dual infections in this model (Fig. 1).

5.3. Bioluminiscence

Bioluminiscence imaging (BLI) refers to the process of visible light emission in living organisms as initially shown after cloning and expression of the first luciferase gene in bacteria [79–81]. Its power as an imaging tool lies on its non-invasiveness, easy use, deep penetration, and reduction of the number of animals required for experimentation. Bioluminiscence imaging systems allow relative quantification of luciferase activity in the whole body of live small animals, or in their dissected organs, by detection of the luminescent signal resulting from the enzymatic degradation of an exogenous substrate [81].

Since its first application in vivo in a rodent model of *Salmonella* infection [82], different pathogens and cells have been genetically modified to express luciferase enzymes from bacteria, insects, and the sea pansy [83]. *Plasmodium berghei* transgenic parasites expressing the firefly luciferase reporter protein at different developmental stages [84] have been created to study the distribution and sequestration patterns of infected red blood cells (pRBCs) in rodent host tissues [63]. Moreover, the development of *Plasmodium* preerythrocytic stages in the liver has been studied in detail by means of intravital microscopy using transgenic parasites that express fluorescent reporter proteins [66]. The possibility to visualize individual liver schizonts from bioluminiscence signals [85] may offer the opportunity to further analyze the process of merosome formation, release and migration to the lungs. To evaluate the use of bioluminiscence systems in investigating both preerythrocytic and erythrocytic stages of the parasite for vaccine and drug discovery, Mwakingwe et al. performed imaging of mice infected with sporozoites of a *P. yoelii* YM-Luciferase transgenic line [86].

Plasmodium parasites have been engineered to enable the simultaneous expression of fluorescent and luciferase protein reporters [85], which makes them jump to the micron scale for further studies of the role of the spleen in infections. To illustrate its usage in imaging the spleen, Franke-Fayard and collaborators have recently used *P. berghei* mutants expressing luciferase under the control of the strong ama1 promoter to determine the distribution of sequestered schizonts in different organs including the spleen [13]. Their results have elegantly shown that in this model of sequestration analogous to *P. falciparum*, nonsequestering parasites have reduced growth indicating the importance of spleen clearance in this model (Fig. 2). More recently, we have used *P. yoelii* expressing GFP/luciferase to study the distribution of the parasite after intraperitoneal injection of mice with infected RBCs. Real-time monitoring of infection revealed the accumulation of parasite on day 3 p.i. mainly in the spleen and also in the lungs. As the infection progresses, the accumulation of parasites in the lung was larger and appears also in other organs (heart, kidneys, liver) (not shown). Bioluminescence is rapid and sensitive and requires little manipulation, allowing real-time monitoring of individual animals. It is thus foreseen that this technology will rapidly advance our knowledge of the role of the spleen in different models.

5.4. Magnetic resonance imaging

Magnetic resonance imaging offers a variety of tools to obtain functional, anatomical and molecular information with high contrast in soft tissues [60]. Implementation of microMRI to investigate changes in the murine spleen upon malaria infection has only recently been achieved [52]. Differences in splenic remodeling induced by lethal and non-lethal *P. yoelii* strains were assessed on abdominal T2-weighted scans. Quantitative measures obtained on red and white pulp areas showed prolonged T2 relaxation times in the white pulp of infected mice, likely revealing edema, hyperplasia and/or proliferation of the lymphoid compartment. Moreover, T2 variances were accentuated in spleens of mice infected with non-lethal strain, in which signal heterogeneity has been attributed elsewhere to fibrosis, necrosis and hemorrhage, thus reinforcing evidence of structural reorganization in the malarial spleen [52] (Fig. 3).

Though we are far from studying the dynamics of infection in this organ in humans, the advent of powerful non invasive imaging techniques and the development of specific probes offer promising development for studying infection at adequate spatiotemporal resolution, not restricted to endpoint or snapshots of histopathological studies.

6. The human spleen

With the advent of non-invasive imaging techniques in the medical field, a consistent bridge is being built that helps to extrapolate research from animal models to human disease.

6.1. Computed tomography: findings in human malarial spleen

During malaria infection, changes in splenic architecture and cellularity result in tissue enlargement, infarction and/or ultimately rupture. Arterial phase CT scans revealed an abnormal enhancement of malarial splenic parenchyma that progressively regressed to a normal pattern — where differences in blood flow between red and white pulp become apparent after antimalarial treatment [87]. Both *P. falciparum* and *P. vivax* malaria cases have been reported to induce splenic infarction based on CT images showing hypodense heterogeneous multifocal areas [88,89]. This pathology seems to be increasing among non-immune travelers despite antimalarial prophylaxis and may eventually trigger splenic rupture, which is a life-threatening complication in malaria disease. Analysis of 55 cases of malarial splenic rupture revealed

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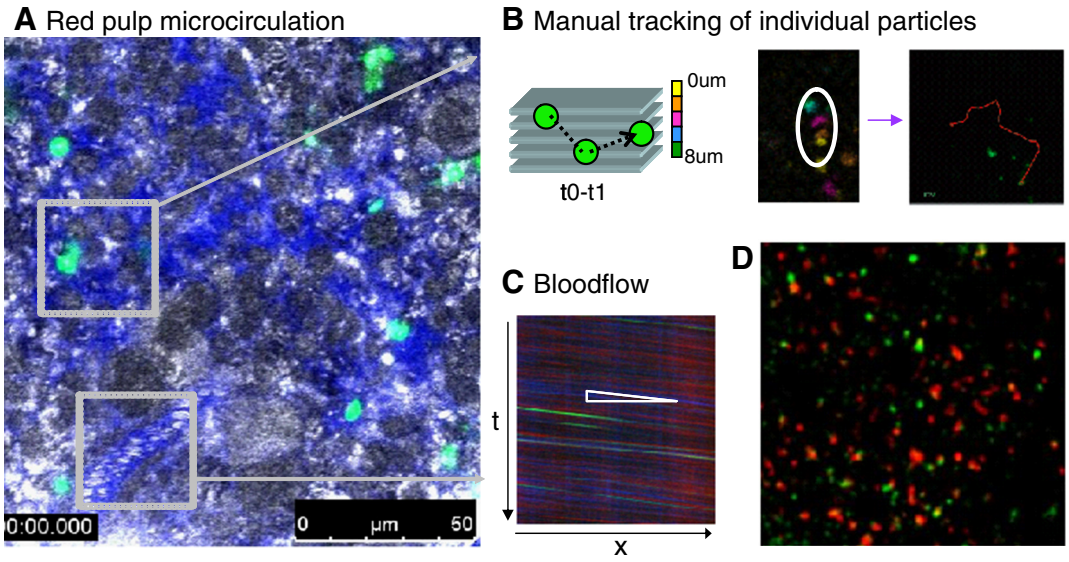

Fig. 2. Quantitative imaging of parasite mobility in the spleen. A. Image of a representative area of the spleen of a non-infected animal injected with FITC-labeled RBCs and 70 kDa Dextran-Texas Red to visualize the vasculature. Reflection (white), Dextran (blue) and FITC-RBCs (green) are shown. Boxes represent open-circulation (up) and close-circulation (down) areas. B. Quantitative analysis of particle movement in the four dimensions (4D) is facilitated by using the depth information from Z-coded color images, represented using maximum intensity projection of five different depths. White circle represents the same particle at different Z in one time point. Different positions are due to time lapses between the acquisition of different Z images. Z-colored image processing. Manual tracking of single particles was performed in ImageJ software and (x, y, z, t) values were used to obtain mobility parameters such as velocity, directionality and residence time. Depth code: yellow (0 µm), orange (2 µm), pink (4 µm), blue (6 µm), green (8 µm). C. Spleen blood flow was measured by performing a line-scan of the central lumen of spleen vessels imaging plasma stained with 70 kDa dextran (red), pRBC (green) and erythrocyte reflection (blue). The slope resulting from the streaks of moving erythrocytes in the xt image was calculated (white line). D. Imaging of the spleen in dual infections by *P. yoelii* 17X-mCherry and 17XL-GFP parasites at day 3 post infection. Images were obtained in a Leica TCS-SP5 confocal microscope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

several features associated with this lesion, namely splenic enlargement and capsule distension, regardless of the causative *Plasmodium* species [15]. Of note, splenomegaly and risk of splenic rupture were still exacerbated in *P. vivax* malaria and non-immune patients [90]. Though splenectomy has long been the treatment of choice for this pathology, new interventions are committed to conservative management that takes advantage of CT, ultrasonography and/or MR scans for diagnosis and daily monitoring of tissue recovery [91].

6.2. Ultrasonography

Ultrasound imaging has higher spatial and temporal resolution than CT and MR and allows for the dynamic detection of tissue flow of both macro and microvasculatures [92]. The circulatory pattern of the human spleen has been recently investigated in vivo by means of contrast-enhanced ultrasonography using microbubbles [15]. In this study, the authors showed a dual microcirculatory organization with

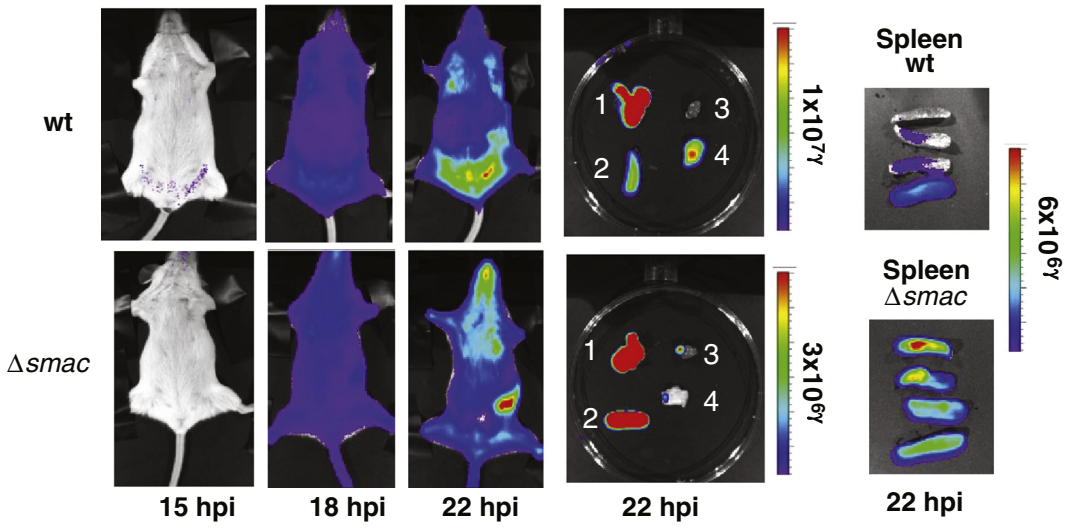

Fig. 3. Representative distribution of sequestered schizonts in mice (and extracted organs) with synchronized infections of parasites that express luciferase under the schizont-specific ama1 promoter as shown by measuring luciferase activity (RLU). WT-infected mice show the characteristic CD36-mediated schizont distribution in adipose tissue (belly), lungs, and spleen, whereas Δsmac-infected mice parasites show distribution throughout the body as shown by luciferase activity in the upper body (lungs and head), decreased sequestration in adipose tissue, and increased accumulation in the spleen (1, lungs; 2, spleen; 3, heart; 4, belly fat tissue). Original figure published in [13].

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approximately 10% of the blood input flowing through the slow compartment. In addition, the development of a system for ex vivo perfusion of intact human spleen enabled the study of *P. falciparum*-RBC clearance in an intact spleen, independent from parasite-priming and serum factors, where ring-iRBC retained in the slow compartment of the red pulp [93].

The portability and affordability of ultrasound equipment have permitted its application in endemic areas to study malaria-related alterations of microvasculature [94] and to monitor spleen size as a sign for asymptomatic malaria and chronicity, though it cannot be used as a first line diagnostic technique [95].

6.3. Other findings from radionuclide imaging of the spleen

Using scintillographic imaging, increased uptake of Tc-99m sulfur colloid was detected in the spleen, liver and lungs of malaria patients; with *P. falciparum* and *P. vivax* having severe cases exacerbating pulmonary clearance and splenic clearance, respectively. Interestingly, Karanikas et al. implemented this technology to assess the kinetics and biodistribution of autologous ^{111}In-labeled platelets in malaria patients with thrombocytopenia. Scintigraphic imaging indicated a diffuse pattern of sequestration, thus discarding the spleen and liver as major sites for platelet destruction and rather pointing to reduced platelet lifespan as the cause for thrombocytopenia [96].

7. Future perspectives

7.1. Novel imaging technologies

While major advances in our knowledge of spleen structure and function, and its role in malaria pathology have been achieved with the aid of imaging modalities as reviewed above, novel techniques currently unexplored, hold potential important contributions in the context of spleen–malaria interactions. They include, among others, optical projection tomography (OPT), atomic force microscopy (AFM), spinning disc microscopy, infrared microspectroscopy, 4D electron microscopy, and fluorescence microendoscopy. The potential of some of these techniques are discussed below.

Optical projection tomography (OPT) is a relatively new technique that enables 3D imaging of biological specimens ranging from 1 to 10 mm. Its main advantages include higher resolution imaging than MRI while enabling visualization of samples larger than those used for confocal-laser microscopy imaging; and the possibility of doing transmission and fluorescence imaging studies allowing for tissue- or gene-specific staining [97]. Recent work with OPT includes organogenesis and developmental studies, as well as lymph node vascular and HEV changes upon infection and inflammation [98]. Its use in spleen studies remains to be reported.

As opposed to OPT, which enables visualization at a macro-level, atomic force microscopy enables resolution in the order of fractions of a nanometer, thus allowing exploration of subcellular structures and biomolecules. This imaging technique has been used to study structures from whole cells to individual micromolecules, and their micromechanical properties. Moreover, AFM enables studying of biological specimens in a near physiological environment, and thus includes the possibility of real-time dynamic studies, and structure–function correlations at a molecular level [99]. In the context of malaria, it has mostly been used to visualize morphological changes occurring in infected erythrocytes [100,101], yet its use for host–pathogen interaction or organ-specific pathology remains unexplored.

A further imaging technique recently introduced to the field of biology, and which has gained attention due to its potential for real-time imaging, is 4D electron microscopy [102]. This technique enables nanosecond high-speed imaging while maintaining high spatial resolution, and its applications in biology include phenomena ranging from atomic motions in structures, to chemical bonding, phase transitions, and nanomechanical properties of complex systems. Its use for understanding nanometric and subcellular changes at an organ level in real time remains to be explored.

Finally, one of the main challenges ahead is to visualize deep tissue spleen structures. The recent usage of fluorescence microendoscopy to visualize deep brain areas [103], combined with constantly evolving better lasers, optics and detectors, seems a promising approach.

7.2. 3D model of the human spleen

The complex microcirculation comprising both closed and open circulation and the compartmentalization with different cells and functions limits our present 2D view of this organ. However, recent advances in bioengineering and microfluidics coupled to imaging, are paving the way to construct 3D organs-on-a-chip, including the spleen [104–106]. Although many challenges remain ahead, it is envisaged that a combination of 3D models of the human spleen, microfluidics and imaging along with clinical observations will soon give us an alternative, accurate and affordable dynamic view of the role of the spleen in normal and pathological conditions caused by malaria and other blood disorders.

8. Concluding remarks

The implementation of imaging into the studies of the spleen in normal and pathological conditions caused by malarial infections is rapidly changing its classical view of the “blackbox” in the abdominal cavity. In fact, ex vivo approaches were fundamental in the description of its compartments and cells; yet, these approaches are now being replaced by real-time in vivo studies in animal models allowing a comprehensive view at spatial and temporal resolution of the 3D complex structure and function of the spleen. Indeed, it is foreseen that advances in optics and fluorescence probes will soon allow cell/tissue resolution of the human spleen. Ultimately, imaging of the spleen in experimental and natural infections at this molecular dynamic level will make feasible the development of new diagnostics and the testing of the mechanisms and effectiveness of drugs and vaccines to treat the disease and to further elucidate the role of immune effector cells and mechanisms during malarial infection.

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