Intravital Imaging of Skin Infections

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Abstract:

Intravital imaging of cutaneous immune responses has revealed intricate links between the skin's structural properties, the immune cells that reside therein, and the carefully orchestrated migratory dynamics that enable rapid sensing and subsequent elimination of skin pathogens. In particular, the development of 2-photon intravital microscopy (2P-IVM), which enables the excitation of fluorescent molecules within deep tissue with minimal light scattering and tissue damage, has proven an invaluable tool in the characterization of different cell subset's roles in skin infection. The ability to visualize cells, tissue structures, pathogens and track migratory dynamics at designated times following infection, or during inflammatory responses has been crucial in defining how immune responses in the skin are coordinated, either locally or in concert with the circulation. Skin pathogens affect millions of people worldwide, and skin infections leading to cutaneous pathology have a considerable impact on the quality of life and longevity of people affected. In contrast, pathogens that infect the skin to later cause systemic illness, such as malaria parasites and a variety of arthropod borne viruses, or infection in distant anatomical sites are a significant cause of morbidity and mortality worldwide. Here, we review recent advances and seminal studies that employed intravital imaging to characterize key immune response mechanisms in the context of viral, bacterial and parasitic skin infections, and provide insights on skin pathogens of global significance that would benefit from such investigative approaches.

Introduction:

Higher vertebrate species such as mammals and reptiles owe much of their evolution and survival to the maintenance of several distinct traits, but perhaps most importantly those that confer physical protection from the external environment. Over the course of 200 million years, the skin has proven to be an exquisite barrier against the multitude of physical threats mammals have endured, and arguably played a significant role in their survival on Earth[1]. Our current understanding of the skin's structure and function is the fruit of decades of investigations that have shed light on how outside organisms interact with the skin - and its various anatomical components - and affect its normal function. The skin, the body's largest organ, is a complex mucosal surface home to a multitude of organisms that span all kingdoms of life, and acts as a nurturing ecosystem to a plethora of commensals [2,3]. Indeed, without being breached the skin provides shelter to bacteria, eukaryotes, viruses, fungi and mites with its array of fine hairs, constantly shedding dead keratinocytes and deep hair follicle shafts [2,4,5]. The skin is a highly compartmentalized organ, with each compartment providing varying degrees of protection against invading pathogens. The epidermis constitutes the upper compartment of the skin and comprises of specialized cells known as keratinocytes. Keratinocytes are organized as a stratified layer, and are covered by an outermost layer of dead cornified keratinocytes, or corneocytes, which can secrete antimicrobial compounds, while basal keratinocytes are anchored to a collagen-rich basement membrane which delineates the border with the dermis beneath [6]. The epidermis is populated by few immune cells, such as Langerhans' cells, an innate immune cells with migratory properties and capable of presenting antigen, and long-lived resident memory CD8⁺ T cells that display specificity to previously encountered skin pathogens, as well as dendritic epidermal T cells, which are present in murine, but not human epidermis [7,8]. The dermis is a larger compartment replete with various anatomical structures,

such as blood microvessels and lymphatic vessels, peripheral nerves, as well as muscle fibers adjacent to hair follicles, and a variety of immune cells including dendritic cells (DC), macrophages, neutrophils and mast cells[9,10].

This knowledge, though the fruit of several decades of investigations and the object of seminal studies, has greatly benefited from a relatively new technique that enables the visualization of these processes *in situ*, and in real time. From an experimental perspective, identifying the mechanisms by which skin pathogen cause cutaneous inflammation and disease, or conversely how pathogens enter the skin to cause non-cutaneous illness requires the development of adequate animal models to study the variety of immune cells most implicated in host-pathogen interactions in the skin. In addition, visualizing these mechanisms, with the aim of refining our understanding of how immune responses against skin pathogens can be improved so as to abrogate illness, requires careful optimization and fine-tuning of intravital imaging techniques, as well as creating an “immunological toolkit” (e.g., transgenic (Tg) fluorescent reporter mice, T cell receptor (TCR) Tg strains, and fluorescently-tagged recombinant pathogens (viruses, bacteria, parasites)) to visualize key events of the immune response that dictate disease outcome.

Imaging the skin: what lurks beneath

As pathogens enter the skin, an array of skin-resident immune cells migrate towards the site of infection, while circulatory myeloid cells extravasate from dermal post-capillary vasculature as they respond to local chemotactic signals. The skin is populated with both resident myeloid and lymphoid cells, which are poised to respond to entry of foreign material and to tissue injury. In the skin, it is known that the relative expression of a specific set of cell surface markers

dictate the retention of cells, whether myeloid or lymphoid in the tissues [11]. Following infection, the capture of antigen by local dendritic cells (DC) enables the priming of antigen-specific T cell responses in the draining lymph nodes, and much work has been dedicated on the spatio-temporal dynamics of the “capture-migration-presentation-priming” sequence following infection [7,12].

Experimental animal models of skin infection rely principally on the ease of mimicry of their human counterparts. Generally speaking, intravital imaging of healthy, uninfected skin allows the visualization of key cutaneous anatomical structures, such as dermal microvessels, the stratus corneum and hair follicles. The development of 2P-IVM and access to an increasingly diverse range of fluorescent reporter mouse strains has enabled investigators to assess the contribution and role of specific structures (such as the epidermis, hair follicles, peripheral dermal nerves, or lymphatic vessels) during skin immune responses. An added advantage of 2P-IVM is the ability to discern dermal collagen using second harmonic generation, which is important in delineating anatomical compartments in which different cells and pathogens circulate. Likewise, investigators have access to a variety of compounds visualise blood flow, (with blood tracers such as Evan’ s Blue, fluorescent dextrans an QTracker QDots), while *in vivo* via intradermal injection of fluorescently-labelled anti-LyVE-1 antibody may enable the visualization of lymphatic vessels, and painting the skin using fluorescent membrane permeant dyes (e.g. CMTMR, CMTPX) helps delineate the stratum corneum.

While dorsal ear skin is arguably the most commonly used skin region in intravital imaging studies, flank skin and footpad imaging techniques have been developed to suit specific experimental infection models. As such, investigators must bear in mind that the composition and distribution of cutaneous immune cells can differ between different regions of skin[10],

and the difference in tissue architecture (blood and lymphatic vasculature, hair follicle density, etc.) may impact local responses and recruitment of circulatory cells to the site of inflammation [13]. Imaging of ear pinnae dispenses the need for surgery and is highly adaptable to experimental settings, such as infections (viral, bacterial or parasites), sterile injury models, allergy and hypersensitivity [14]. Lymphatic drainage to the cervical lymph nodes provides a well-controlled system when investigating mechanisms of antigen presentation and T cell priming, where the tracking of migratory dermal antigen-presenting cells to the draining lymph node is required. In some cases, however, the route of infection requires surgical preparations adapted to the experimental model, as is the case of Herpes Virus Simplex-1 (HSV-1) epicutaneous infections. HSV-1 infection is a viral recrudescence model that results in a skin lesion, or zoster, and much work has aimed at on understanding how the adaptive immune system acts to clear viral infection, and in recent years, to better understand how it can maintain lifelong antiviral immunity [15]. Because HSV-1 is an epicutaneous infection, access to the epidermis and upper dermal layers is essential, and the band-shaped zoster lesion requires a larger skin surface area than the ear pinna offers. Hence, the skin flank imaging method was developed to suit both the physiological properties of the viral lesion, and the migratory dynamics of antiviral responses within the upper layers of the skin. To this end, a surgical preparation was developed (described in Figure 1A) to isolate the region of skin where the zoster lesion is located - while maintaining normal blood and lymphatic flow - to study T cell responses to HSV-1 infection, and the formation of HSV-1-specific epidermal tissue-resident memory T cells (T_{RM}). This method, while requiring simple surgery in the form of lateral cutaneous incisions, is highly reproducible, does not require cauterization and allows direct access to the lesion site from above. Given the somewhat motile nature of skin tissue - especially during acute inflammation where localized swelling is pronounced - and the requirement of a stable water drop for water-immersion microscope objectives, the upright

stage preparation owes its effectiveness to the use of veterinary glue (to attach the dermal underlayer to a stainless steel plate), vacuum grease and a fixed coverslip, which altogether help stabilize the tissue for several hours (>8) of continuous imaging (Figure 1A). It must be noted that while one study that investigated T_{RM} formation in the epidermis following flank skin epicutaneous infection with HSV-1 was performed on an inverted microscope [16] and thus did not require surgery, the bulk of published intravital imaging studies of HSV-1 responses in the skin were performed on an upright stage. Flank skin offers some advantages compared to ear skin: it allows for a greater region to be imaged, and the relative thickness of the tissue, lack of subdermal cartilage and distance from the mouth and paws altogether facilitate *in situ* delivery of compounds (e.g., inhibitors, peptide), cells and antibodies [17] used in intravital imaging experiments. Other techniques such as dorsal window and footpad are used more sparingly when imaging skin infections, and are generally opted for when invasiveness is a concern (ie. surgery) or when longitudinal imaging is required. In particular, intravital imaging of skin and microvascular regeneration in wound healing or tumour models have successfully exploited dorsal skin chambers for intravital imaging [18,19], which most often relies on stereoscopic imaging or confocal imaging, rather than multi-photon imaging. Dorsal skin fold chamber offers the advantage of non-invasive (no surgery required) and longitudinal imaging, which would certainly suit a range of skin infection models currently in use (Figure 1B). Where intravital imaging studies focus on the dynamics of T cell responses against a specific antigen, essential tools are required, such as T cell receptor (TCR) transgenic (Tg) T cell strains crossed to fluorescent transgenic reporter mice, to allow the visualization of pathogen-specific $CD8^+$ or $CD4^+$ T cells. For example, the visualization of T cell responses following HSV-1 infection in the skin was greatly facilitated by the development of HSV-specific $CD8^+$ and $CD4^+$ T cell TCR Tg mice, known as gBT-I [20] and gDT-II cells [21], respectively. Likewise, the use of MHC-I and MHC-II-restricted ovalbumin (OVA) epitopes,

and their respective OT-I [22] and OT-II [23] TCR Tg mouse strains, have been used extensively to study T cell responses in viral, bacterial and parasite infection models, though this required the development of recombinant, OVA epitope-expressing pathogens.

Cutaneous viral infections: bridging adaptive and innate immune responses

Immune responses to viral infection are sequentially coordinated by two distinct types of immune cells. Myeloid cells, either tissue-resident or recruited innate immune cells, are responsible for the capture, processing and presentation of viral antigen to adaptive, cognate T cells in nearby draining lymph nodes. Few skin viruses have influenced modern immunology as much as Vaccinia Virus (VV), and the dynamics of skin immune responses to this pathogen of historical significance continues to garner attention. In 1797, Edward Jenner prepared an inoculum from the pus of handmaids' cowpox blisters, administered it to patients through a scratch on the skin. This resulted in mild illness, but ultimately conferred immunity to cowpox, thus heralding the dawn of immunology. Centuries later, a VV vaccine was used against smallpox, the eradication of which was arguably one of the greatest achievements of modern science [24]. Moreover, this example illustrates how the skin can be used as a prime antigen-delivery site, and to instruct the immune system to generate long-lasting, antigen-specific immunity. Yet despite significant advances, the mechanisms of skin immune response following VV infection and the factors underlying the success of the vaccine remained poorly understood for decades. Skin immune responses are highly dynamic and involve a variety of immune effectors, and little was known of how skin immunity and long-lasting protection was orchestrated after VV infection.

To address this question, Yewdell and colleagues exploited 2P-IVM to characterize the spatio-temporal dynamics of VV infection and antiviral responses in the skin by using recombinant fluorescent protein (FP)-tagged VV and fluorescent reporter TCR Tg mice [25]. After scarifying ear skin with recombinant VV, intravital imaging showed that inflammatory monocytes recruited to the site of epicutaneous infection became infected with VV. While standard confocal microscopy and flow cytometry enabled the identification of several infiltrating cell subsets with a certain degree of accuracy, 2P-IVM imaging showed a distinct difference in motile behavior between monocytes at the center of the lesion and those at the periphery [25]. Monocytes at the centre of the lesion were largely sessile, while those at the periphery were migrating through the dermis at higher velocity. Further, to understand the kinetics of CD8⁺ T cells responses in the skin (a key step in generating VV-specific immunity), the authors made use of a recombinant VV expressing the the MHC-I-restricted OVA epitope and fluorescent OT-I T cells, to track virus-specific CD8⁺ T cells in VV-infected skin in real time. CD8⁺ T cells migrated to the dermis 5 days following VV infection, thus after the initial response from inflammatory monocytes, and eliminated infected cells *in situ* – a process that could not be observed without 2P-IVM - indicating that innate and adaptive immunity exhibited complementary antiviral function in VV-infected skin [25]. Subsequent studies also used 2P-IVM to show that CD8⁺ T cell-mediated clearance of VV in the skin was dependent on chemokine signaling. In VV-infected skin, chemokines CXCL9 and CXCL10 were up-regulated, prompting the authors to examine the role for their receptor, CXCR3, in CD8⁺ T cell antiviral function [26]. While wild-type CD8⁺ T cells migrated normally within VV-infected areas, and displayed reduced motility in proximity to infected cells, CXCR3^{-/-} T cells exhibited reduced entry into infected regions, were significantly more motile than their wild-type counterparts, and were unable to control viral replication *in situ*. In this instance, 2P-IVM helped show that in VV-infected skin, CD8⁺ T cell-mediated antiviral function required

efficient T cell migration to the site, which was dependent on a distinct chemokine signaling pathway [26]. A similar approach using cytokine reporter transgenic mice was used to show that CD8⁺ T cells that expressed IL-10, a potent regulatory cytokine, were more efficient at controlling viral spread in VV-infected skin [27]. While IL-10-expressing CD8⁺ T cells migrated slowly in confined areas around the foci of infection, locally produced IL-10 also contributed to the recruitment of monocytes to the foci, again revealing a complex interplay between immune and adaptive immunity in skin viral infections (illustrated in Figure 2). These studies highlight the invaluable role intravital imaging has played in identifying mechanisms by which innate and adaptive components of the immune system coordinate antiviral response in the skin to mediate long-term protection.

Recurrent viral infections of the skin have also been the object of investigation. In particular, Herpes Simplex virus 1 (HSV-1), which enters the skin by infecting keratinocytes, most often on breached skin. HSV-1 has an enormous disease burden compared to other skin infections, with over 3 billion people infected with prevalent HSV-1, and the lack of successful vaccination strategies reinforce the need to understand how skin immune responses unfold, and how recrudescence is orchestrated [28]. HSV-1 exploits two anatomically distinct replication sites, the skin and the dorsal root ganglia, and similarly to VV skin infection, synergistic immune responses are key for the local clearance of the virus. Some of the first cells to be recruited to a site of infection in the skin (as is the case for sterile injury [29]) are neutrophils, which extravasate from dermal post-capillary venules in a chemokine-dependent manner. Mueller and colleagues used LysM-GFP reporter mice and 2P-IVM to visualize how neutrophils migrated towards HSV-infected keratinocytes on the primary site of infection, and whether, as first-responders, they were required to mediate antiviral CD8⁺ T cell responses [30]. LysM-GFP mice are a fluorescent reporter strain in which myelomonocytic cells express

GFP, and relative expression of the LysM reporter allows some discrimination between cell types, with neutrophils appearing as GFP^{hi}, whereas monocytes and macrophages are GFP^{dim}. The authors found that while neutrophils did migrate en masse towards infected keratinocytes as early as 2 hours post-infection, they did not migrate to the draining lymph nodes to help initiate CD8⁺ T cell responses. Much work has been done to show that HSV-1-specific CD8⁺ T cells are essential in controlling HSV-1 replication and were needed for clearance, and their absence from the skin prior to infection implies the local contribution of other cell subsets. It has been shown HSV-1 infection of the skin results in the formation of long-lived HSV-1-specific tissue-resident memory T cells (T_{RM}), which remain in the epidermis without recirculating but exhibit all the functional features of cognate memory T cells [31]. The use of 2P-IVM enabled, for the first time, the visualization of different migratory behavior and tissue localization of CD8⁺ T cells in the skin during the acute and memory phase of adaptive responses, and prompted further investigations on the potential of these T_{RM} as a means for pathogen-specific local protection in HSV-1 infection [32]. T_{RM} cells are found in a variety of tissues such as the lungs, brain, liver and female reproductive tract. Skin T_{RM} are confined to the epidermis, and 2P-IVM was used to characterize their slow, patrolling migratory behavior and their unique dendritic-like morphology, both of which are likely dictated by the physical environment of keratinocyte-rich epidermis; yet these skin T_{RM} are morphologically distinct from other immune cells that populate the epidermis, such as Langerhans cells and dendritic epithelial T cells (DETC) [16,32,33]. Mathematical modelling of long-term 2P-IVM imaging data revealed that T_{RM} patrolled the epidermis following a random diffusion migratory pattern, which is particularly important should tissue-specific T_{RM} be developed as a local protection mechanism against re-infection [33]. Parallel studies had previously shown that T_{RM} lodged in the epidermis – without antigen or virus input – offered complete protection against HSV-1 challenge where T_{RM} were present, but how epidermal T_{RM} – effectively HSV-1-specific ‘first

responders' – are recalled following HSV-1 skin challenge and mediate effective clearance was not known [34]. By combining an array of tools including fluorescent reporter HSV-1 constructs, as well as fluorescent and photoconvertible reporter gBT-I mice, 2P-IVM revealed that epidermal T_{RM} initially migrated towards and established contact with HSV-1-infected keratinocytes upon challenge with HSV-1 and were largely sessile, though interestingly without displaying any direct killing activity of HSV-1-infected cells [35]. This initial response was followed by a massive recruitment of circulatory memory (T_{CIRC}) $CD8^+$ T cells, which migrated towards HSV-1-infected cells and were highly motile – However, to distinguish epidermal T_{RM} from recruited T_{CIRC} in the skin, the authors used photoconvertible Kaede gBT-I reporter cells to convert epidermal T_{RM} from Kaede Green (the native emission wavelength of the Kaede protein) to Kaede Red, while circulatory T_{CIRC} remained unconverted. Intravital imaging of recall responses revealed that converted T_{RM} were first to migrate towards HSV-1-infected keratinocytes and maintained slow, constrained migration in the epidermis, whereas unconverted T_{CIRC} migrated throughout the underlying dermis at high velocity [35]. The study also revealed that T_{RM} were able to proliferate *in situ* during viral challenge, and that multiple successive T_{RM} seeding episodes were cumulative, rather than displacing, indicating that the skin could host a variety of antigen-specific T_{RM} that would be poised to respond upon infection with various pathogens. Altogether, these intravital imaging studies illustrate how vital the use of imaging techniques such as 2P-IVM are in dissecting the dynamics of immune responses, both adaptive and innate, following viral skin infection.

Bacterial infections: friends and foes under one lens

Bacterial infections remain a far greater burden on the human population than viral infections. As outlined earlier, the skin microbiome is composed primarily of commensal bacterial species,

which, in humans, are distributed in various skin regions on the body depending on a variety of factors, including sex, age, hygiene and relative humidity [2-4]. Commensal bacterial species, such as *Staphylococcus aureus*, are not always pathogenic, but have been shown to cause skin lesions and abscesses such as impetigo or ‘school sores’ in children, and can colonize other tissues such as the airways, bone and cardiac endothelium [36,37]. Skin infections with *S. aureus* are problematic, with an estimated 30% of the human population colonized with this bacterium, and a higher incidence in developing countries [37]. Epidemics are common in high density areas such as prisons, hospitals and schools, and the development of antibiotic-resistant strains is a major global public health concern [38]. A significant body of evidence indicates that in the skin, *S. aureus* infection requires efficient neutrophil responses for control and clearance [38,39].

Our understanding of the complex relationship between the skin microbiome and the immune system, both in health and disease is still in its infancy, yet some pathogenic bacteria of significant public health concern have been the focus of several studies. In a first attempt to visualize skin immune responses to *Staphylococcus aureus* (*S. aureus*) *in vivo*, Dustin and colleagues inoculated the flank skin of LysM-GFP mice intradermally with a fluorescent strain of *S. aureus*, and proceeded to imaging as early as 30 minutes post-infection using 2P-IVM[40]. In this study, the authors employed a skin flap intravital imaging model, in which the skin flank is surgically excised while maintaining essential blood flow, and fixed upon a coverslip to allow imaging of the epidermal side on an inverted microscope stage. The study showed that very early after infection with *S. aureus*, neutrophils migrated to the site of infection in the dermis at high velocity, but neutrophils in close proximity to *S. aureus* exhibited low motility and more confined displacement, suggesting that neutrophils arrest on contact with bacteria, in a similar fashion to CD8⁺ T cells that establish contact with virus-

infected cells [35,40,41]. The authors were able to investigate the role of chemokine receptor signaling in vivo by administering pertussis toxin (PTx), an inhibitor of $G\alpha_i$ protein-coupled receptor signaling, on which chemokine receptors rely. Mice treated with PTx and infected with *S. aureus* showed significant reductions in neutrophil infiltration to the site of infection, and this was associated with higher bacterial burden, indicating that neutrophils require chemokine signaling to migrate towards bacteria and control the infection in the skin.

Another focus of investigation in cutaneous *S. aureus* infection is the role of bacterial virulence factors, which are thought to drive bacterial pathogenicity and potentially impede the ability of immune cells to migrate to the site of infection [42]. One such virulence factor examined by Abtin *et al.*, is α -hemolysin (*Hla*), a cytotoxin responsible for pathogenic lung and skin *S. aureus* infections [43]. Here, Weninger and colleagues used 2P-IVM to visualize how *Hla* impacted neutrophil recruitment to ear skin after *S. aureus* infection, and found that *Hla* prevented efficient neutrophil extravasation from dermal microvessels to the dermal tissue. *S. aureus* strains lacking *Hla* enabled better vessel adhesion and extravasation, and a stronger induction of neutrophil chemoattractants CXCL1 and CXCL2. Upon closer inspection of their 2P-IVM time-lapse sequences, the team noticed that neutrophils formed distinct clusters, or 'hotspots' alongside dermal postcapillary venules, and hypothesized that these neutrophils were clustering to cellular, rather than vascular structures. This led to the identification of a subset of dermal macrophages termed perivascular macrophages (PVM), found on the postcapillary venule adventitia, and used a DPE-GFP mouse strain - in which PVM are positive for GFP [44] - to observe interactions between neutrophils and PVM during attachment and extravasation events in the context of *S. aureus* cutaneous infection. This was of particular relevance, as the ability of tissue macrophages - and incidentally, PVM - to produce large amounts of CXCL1 and CXCL2 pointed towards a distinct role for PVM in the recruitment

and extravasation of neutrophils in a bacterial infection setting. In addition, 2P-IVM time-lapses showed that neutrophils interacted with PVM during extravasation events, but more importantly, that *S. aureus*-derived *Hla* lysed PVM *in vivo*, providing evidence that neutrophil extravasation in response to skin bacterial infection required PVM as a tissue-resident intermediary, which would likely provide a suitable chemokine milieu (*i.e.*, CXCL1 and CXCL2) for neutrophils to exit the vasculature. This study is yet another example of how 2P-IVM can be utilized to identify key biological mechanisms implicated in skin infection from a host-pathogen interaction perspective.

Similar approaches investigating the role of neutrophils in cutaneous antibacterial responses were employed to better understand the dynamics of infection with *Streptococcus pyogenes*, or *Yersinia pestis*, the causative agent of the bubonic plague. *S. pyogenes* causes soft tissue infections which can, in some cases, lead to severe penetrative infections such as necrotizing fasciitis and myositis. In Lin *et al.*, a footpad model of *S. pyogenes* infection was used in LysM-GFP mice to characterize neutrophil responses, and the role of key virulence factors implicated in bacterial dissemination [45]. The authors investigated the role of streptolysin S (SLS) in skin infection, and their 2P-IVM imaging revealed that while wild-type *S. pyogenes* and SLS-deficient (SLS⁻) strains induced similar numbers of neutrophils to migrate to the skin, neutrophils in SLS⁻-infected skin extravasated faster from blood vessels than their counterparts in wild-type *S. pyogenes*-infected skin. Through the use of 2P-IVM, this study showed that SLS, rather than inhibiting neutrophil recruitment, temporarily impaired the dynamics of extravasation, thus delaying neutrophil accumulation to the site of infection, and lends further proof that intravital imaging can help unravel rather mechanisms host-pathogen interactions that may be key to the development of therapeutic intervention strategies [45]. Another bacterial pathogen of interest, *Y. pestis*, is transmitted through the skin by fleas, and although

the bubonic plague is longer the health concern it once was, virulence factors that may enable this pathogen to enhance infectivity and survival remain the focus of investigations [46]. The use of 2P-IVM techniques and LysM-GFP reporter mice showed for the first time that cutaneous infection with *Y. pestis* attracted neutrophils to the site in large numbers. Similar to findings with *S. aureus*, Shannon *et al.*, showed that selective modulation of *Y. pestis* virulence factors could affect the quality and efficacy of neutrophil responses [47]. Interestingly, by utilizing CD11c-YFP reporter mice, in which CD11c⁺ dendritic cells (DC) express the yellow fluorescence protein (YFP), the authors found that cutaneous CD11c⁺ DC did not engage in significant contact with *Y. pestis* in the dermis, and that neither neutrophils nor DCs were required for the dissemination of the bacterium to the draining lymph nodes, from which *Y. pestis* spreads systemically [47]. This initial study was followed up with a more comprehensive analysis of the relative contribution of different dermal immune cell subsets, and incorporate a live transmission model by allowing *Y. pestis* -infected fleas to feed on ear skin prior to 2P-IVM imaging [48]. While uninfected flea bites did induce neutrophil migration to the bite site, flea-transmitted *Y. pestis* resulted significantly higher accumulation of neutrophils to the bite site – and as was initially observed following needle transmission, skin DCs did not interact with *Y. pestis* derived from fleas but exhibited increased motility in the vicinity of the bite site [48].

Another skin pathogen, tick-borne *Borrelia burgdorferi*, the causative agent of Lyme disease, was the focus of a study using 2P-IVM to study the mechanism of tick transmission, however the authors did not investigate the dynamics of cutaneous immune responses [49,50]. These and other similar studies using 2P-IVM to examine transmission mechanisms of other arthropod-borne bacterial pathogens point toward the need for a better understanding of host-pathogen interaction in the context of live transmission in the skin. This is particularly

important as a substantial proportion of human and animal pathogens are transmitted by arthropods through the skin, and therapeutic strategies aimed at enhancing cutaneous immune responses to limit dissemination and disease warrant further investigation.

Parasitic infections: transiting through the skin

Besides viral and bacterial infections of the skin, parasitic organisms - some of which are transmitted by arthropods - are a major cause of illness worldwide. From mosquito-borne *Plasmodium* spp., which causes malaria and is responsible for over 500,000 deaths worldwide [51], to *Leishmania* spp., transmitted by sandflies and the cause of leishmaniasis, of which the cutaneous form is the most common in humans with up to 12 million worldwide cases annually [52]. As is the case for parasitic infections in general, the biology of *Plasmodium* transmission and malaria disease involves successive stages in the lifecycle of the organism, which requires any intervention strategies to be specific to a particular stage of the lifecycle. After taking a blood meal, mosquitoes transmit *Plasmodium* spp. parasites to the skin as sporozoites, which travel to the liver to develop as merozoites, and subsequently enter the circulation to infect red blood cells, reproducing to high numbers and lysing the cells, leading to typical symptoms of malaria infection, such as high fever, anemia and cerebral malaria. [51].

Much of the work to date has been carried out to understand how the immune system responds to liver and blood-stage parasitic stages, including experimental models that used 2P-LSM to identify host-pathogen interactions and mechanisms of memory immunity that could be exploited in future therapeutic approaches [53,54]. Seminal work from Ménard and colleagues helped establish a picture of the early events following the delivery of *Plasmodium* to the skin by mosquitoes. The team infected ear skin with *P. berghei* sporozoites following single mosquito bite, and showed for the first time that parasites moved in the dermis at a higher

velocity than what they had observed *in vitro*, and in a migratory pattern that was distinct from that observed in mosquito salivary ducts [55,56]. The team was not only able to capture the dynamics of blood vessel invasion, showing how sporozoites migrated robustly towards dermal blood vessels before attaching to the walls and entering the bloodstream, but also showed that sporozoites could invade lymphatic vessels, and drain to the lymph nodes [55]. These observations now form the basis of our understanding the early events following *Plasmodium* skin infection that could lead to dissemination, and while other studies carried out additional works to visualise the fate of sporozoites in the dermis [57], and the finer details of the molecular mechanisms that guide sporozoites' interactions with dermal or vascular structures[58], few studies focused on visualizing cutaneous host immune responses to sporozoite infection. A study from Ménard's group sought to characterize the dynamics of local skin responses following mosquito-transmitted *P. berghei*, and used GFP-tagged sporozoites to visualize invaded dermal cells *in vivo*. Without resorting to the ubiquitous fluorescent transgenic reporter systems, the team was able to show that skin infection with sporozoites resulted in a myeloid cell infiltrate composed mostly of CD11b⁺ inflammatory monocytes and neutrophils [59]. Interestingly, neutrophils were found to be largely dispensable for the initiation of local immune responses, and did not contribute to the dissemination of sporozoites [59]. These findings are important as they provide evidence that parasites like *Plasmodium* spp. may not rely on immune cell recruitment to facilitate dissemination, thus relying instead on motility and adhesion mechanisms to invade blood vessels and dermal lymphatics. It is clear that more work is needed to understand precisely how different immune cell subsets found in the skin, and those that are recruited to sites of infection contribute to local responses following sporozoite infection. In addition, the role of compounds found in mosquito saliva - which have been shown in several studies to directly impact neutrophil responses in the skin following

arthropod-borne viral infection [60-62] - on the migratory and effector function of cutaneous immune cells deserves more attention from an intravital imaging perspective.

Another parasitic skin infection that has been the focus of several 2P-IVM studies is cutaneous leishmaniasis, and while our current understanding of *Leishmania* immunobiology indicates that phagocytosis of the parasite by macrophages is an important step in the invasion process [63], little is known of how such events unfolded in the skin, and how the diverse myeloid subsets found in the skin may be affected. An initial study by Sacks and colleagues used 2P-IVM to reveal that *Leishmania major* (*L. major*), when transmitted to the ear skin of mice by sand flies, was readily phagocytosed by dermal neutrophils, which migrated en masse to the bite site [64]. An interesting observation was that, in contrast to *Plasmodium* spp. sporozoites, which displayed high motility in the dermis, *L. major* parasites were largely sessile in the skin. As is commonly observed in arthropod transmission models, the variability of parasite delivery prompted the authors to perform intradermal inoculation to visualize phagocytosis and invasion events more accurately and more reproducibly. With *L. major*, neutrophils were seen to phagocytose parasites early after intradermal delivery, and internalized parasites were found to be viable, indicating that invaded neutrophils most likely contributed to dissemination of *L. major* [64]. A similar study by Weninger and colleagues showed that dermal DCs could also migrate towards *L. major* parasites delivered intradermally, and were able to internalize individual parasites [65]. As a result of this process, dermal DCs became more dendritic and displayed reduced motility, in contrast to epidermal Langerhans cells (LC), which were not affected by *L. major* infection. The team showed that dermal DCs actively sought *L. major* promastigotes in the skin and extended pseudopodia to capture individual parasites, and although chemokine receptor signaling was required for dermal DCs to migrate towards parasites, it was not essential for parasite uptake. This elegant study paved the way for

subsequent in-depth investigations of migratory and morphological profiles displayed by skin immune cells during infection, and 2P-IVM was instrumental in revealing these subtle functions. While innate immune responses in the skin are important, and form the focus of most intravital imaging studies of cutaneous infections, the role of the adaptive immune system in skin immune responses against parasite infection is less well defined. A 2009 study by Bousso and colleagues used 2P-IVM and *L. major*-specific WT15 CD4⁺ T cells to examine how the later stages of skin responses following *L. major* infection were orchestrated in the dermis [66]. The team found that following *L. major* infection in the skin, WT15 CD4⁺ T cells migrated preferentially to the site of parasite accumulation, and exhibited characteristic cell arrest behavior when in contact with infected cells. More importantly, the study showed that most of these interactions involved WT15 cells and infected phagocytes, adding further evidence of the complex interplay between innate and adaptive immune responses in the skin. Moreover, the use of IFN γ -YFP fluorescent reporter mice (also known as *Yeti* [67]), which allows *in vivo* and *in situ* detection of IFN γ -secreting cells, enabled the authors to show that as late as two weeks post-infection, IFN γ ⁺ CD4⁺ T cells were found in the vicinity of *L. major*-infected cells (most likely phagocytes), thus providing an unappreciated view of the ongoing immune response mechanisms that occur in the skin well beyond initial infection events [66]. One of the key debilitating outcomes of cutaneous leishmaniasis is the onset of vascular permeability in the dermis, and recent work by Scott and colleagues successfully utilised multiphoton ontravital imaging to define the signaling pathway that dictates vascular leakage during leishmania infection. The authors were able to observe how *L. major* infection stimulates angiogenesis and lymphangiogenesis in the dermis over extended periods (6 weeks post-infection), and by quantifying Evan's Blue dye (EBD) leakage into the extravascular space *in situ*, identified the VEGF-VEGFR pathway as a critical requirement for vascular remodeling in cutaneous leishmaniasis [68]. By incorporating traditional approaches to vascular leakage

assessment - such as EBD - into elaborate intravital imaging setups, this study unveiled a previously unappreciated mechanism by which *L. major* infection activates vascular remodeling through VEGFR-2 signaling, and established this pathways' critical role in limiting inflammation and promoting subsequent lesion repair.

Concluding Remarks and Future Perspectives

The development of advanced microscopy techniques that allow *in vivo* imaging of immune responses has greatly contributed to our understanding of how the immune system orchestrates pathogen recognition and clearance. While we are still appreciating how such processes require highly coordinated, sequential steps (cellular recruitment, vascular egress, specific migratory behavior, *in situ* internalization / killing), a wealth of questions remain unanswered. Skin infection are particularly important as they affect a highly complex, protective organ that functions as a shield against a range of physical threats. The ability to visualize how the immune system responds to a variety of pathogens, be they viral, bacterial or parasitic, combined with seminal studies that helped discover skin-resident immune subsets, helped highlight the heterogenous nature of skin immune responses to infection. While viral infections rely on a two-step coordinated response culminating in a T cell-dependent clearance process, bacterial and parasitic infections exploit the non-specific recruitment process to the site of infection to disseminate systemically. With an increasing array of immunological and molecular tools available, and the development of static, high-dimensional quantitative imaging techniques such as histocytometry[69], skin pathogens that have received little or no attention will likely be the focus of comprehensive studies that incorporate intravital imaging. Of particular relevance are arthropod-borne viruses (arboviruses), which in recent years have caused large outbreaks and continue to burden public health systems with ongoing epidemics:

arboviruses such as Dengue, Chikungunya and Zika viruses are the target of several worldwide vaccine development initiatives, but little work has been done to identify the mechanisms of immune responses following skin infection that lead to systemic dissemination and disease[70,71]. In cases like these, the use of intravital imaging would be of immense benefit, considering the substantial technological advances and the resources available to date to integrate functional, quantitative and qualitative analysis.

A

B

C

Pathogen	Route of infection	Ref.
Herpes Simplex virus (HSV-1)	Epicutaneous (scarification)	17, 30, 32, 33, 35
	Epicutaneous (tattoo)	16
<i>B. burgdorferi</i>	Intradermal (nymph)	49, 50
<i>S. aureus</i>	Intradermal	40
Flank skin		

Pathogen	Route of infection	Ref.
Vaccinia virus (VV)	Epicutaneous (scarification)	25, 26, 27
<i>S. aureus</i>	Epicutaneous	43
<i>Y. pestis</i>	Intradermal; flea bite	47, 48
<i>P. berghei</i>	Intradermal; mosquito bite	55, 56, 57, 58
<i>L. major</i>	Intradermal; sandfly bite	63, 64, 65, 66, 68
Ear skin		

Pathogen	Route of infection	Ref.
<i>S. pyogenes</i>	Subcutaneous	45
Footpad skin

Figure 1: A summary of different skin imaging techniques and skin infection studies using intravital imaging. **A)** Flank skin imaging method, originally designed and optimized to visualize cellular immune responses to herpes virus zoster lesion. *Left:* photograph showing the imaging stage setup of a mouse following surgical incision of the left flank upon which viral lesions form. *Right:* diagram depicting the steps that follow the initial incision procedure: after fixing the dermal layer of the skin to the stainless plate using veterinary glue, saline-moistened gauzes are placed on the incised skin regions to prevent dehydration during imaging, and a small border of vacuum grease is placed on the edges of the region to be imaged. A coverslip is placed above the PBS-moistened lesion and secured using the vacuum grease border. A drop of water is placed on the coverslip, and a water immersion objective is lowered onto the drop. After a short stabilization period, time-lapse acquisition of cellular migration in epidermal and dermal layers can be performed. **B)** Dorsal skin imaging windows allow longitudinal imaging of a defined region of skin, and necessitate the use of caliper-style window frames with integrated coverslips. The procedure enables mice to develop without the need for endpoint imaging surgery (images *i* and *ii* adapted from [72,73]). **C)** Schematic summarizing studies of skin infections using intravital imaging. Imaging the dynamics of host-pathogen interactions and immune responses in the skin has helped dissect mechanisms by which viral, bacterial and parasitic infections unfold in the skin. The most commonly used methods for intravital imaging of the skin have focused on ear skin (pinna), flank skin and footpad skin as a site of infection and observation, and typically rely on the use of fluorescently tagged pathogens, and fluorescent reporter transgenic mice that allow the visualisation of specific immune cell subsets. The route of inoculation varies depending on the natural mode of infection and transmission of the pathogen, and may involve the use of live, natural vectors (e.g., arthropods such as fleas, mosquitoes or sand flies) to deliver arthropod-borne pathogens to the skin of mice.

Figure 2: Example of intravital imaging of antiviral immune responses in the skin using the vaccinia virus lesion model. Ear skin was infected by scarification with recombinant vaccinia virus (rVV^{OVA-BFP}, pseudocoloured in magenta), leading to the accumulation of T cells (IL10^{GFP} reporter T cells in green, top left panel; OVA-specific OT-I T cells in red, bottom left panel), and multiphoton imaging was used to acquire time-lapse 3D stacks. Displacement analysis of individual T cells can be visualized with individual cells tracks (middle panels), showing the relative position and directionality of cells towards virus-infected lesion. Individual T cell tracks can be normalized to the position of origin to help visualize differences in displacement (i.e., length of tracks from origin) and velocity using colour-coded tracks (right panels). This form of visual analysis is useful in determining cell subset-specific migratory behavior (such as displacement, directionality, velocity): here, IL-10^{GFP} T cells are seen closer to the VV lesion than OT-1 T cells (left and middle panels), move slower and display reduced displacement compared to OT-1 T cells (right panels), indicating that a large proportion of the low-velocity cells that remain near the lesion are IL-10 producers (Adapted from [27]).

Figure 3: Summary of example studies characterizing skin immune responses to pathogens and mechanisms of uptake using intravital imaging. **A)** In an intradermal inoculation model of *L. major* infection, Weninger and colleagues showed that CD11c⁺ dermal dendritic cells (dDC) migrated towards *L. major* promastigotes and by extending pseudopodia, the majority of dDC were able to internalize individual parasites and discriminate them from inert material (e.g. fluorescent beads). **B)** In an intradermal model of *S. aureus* infection, Dustin and colleagues showed that shortly after infection, neutrophils were recruited from the circulation and migrated towards bacteria in a G α_i protein-coupled receptor-dependent manner, and exhibited cellular arrest behavior similar to that seen in CD8⁺ T cells upon sensing of cognate antigen [40]. The authors confirmed this mechanism by inhibiting this pathway using pertussis toxin (PTx), highlighting the complex interplay between local cellular responses and finely-tuned molecular signaling pathways. **C)** Cutaneous responses to another arthropod-borne bacterial pathogen, *Y. pestis* were characterized using intravital imaging by Shannon *et al.* [47]. In this study, neutrophils, macrophages and CD11c⁺ DCs were observed migrating towards bacteria in the dermis and, despite a large and rapid accumulation of neutrophils to the site of infection, and some contact with bacteria by macrophages, the authors found no evidence of DC contact or uptake leading to dissemination of the bacteria to the draining lymph nodes. *Diagrams drawn using Biorender.*

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Highlights:

This review identifies the key studies that explored the dynamics of immune responses in the skin using intravital imaging techniques. Viral, bacterial and parasitic models of skin infection are discussed, and seminal findings that paved the way for further work are discussed. The significance of the outcomes of these studies, and the bearing thereof on our understanding of how cutaneous infections unfold, is also discussed.

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