The power of bioluminescence imaging in understanding host-pathogen interactions

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Abstract

Infectious diseases are one of the leading causes of death worldwide. Modelling and understanding human infection is imperative to developing treatments to reduce the global burden of infectious disease. Bioluminescence imaging is a highly sensitive, non-invasive technique based on the detection of light, produced by luciferase-catalysed reactions. In the study of infectious disease, bioluminescence imaging is a well-established technique; it can be used to detect, localize and quantify specific immune cells, pathogens or immunological processes. This enables longitudinal studies in which the spectrum of the disease process and its response to therapies can be monitored. Light producing transgenic rodents are emerging as key tools in the study of host response to infection. Here, we review the strategies for identifying biological processes *in vivo*, including the technology of bioluminescence imaging and illustrate how this technique is shedding light on the host-pathogen relationship.

Keywords

Bioluminescence, Biosensor, Host-pathogen relationship, Gene transfer, Transcription factor

1 Introduction

Bioluminescence imaging (BLI) is a powerful technology for studying microbial pathogenesis, immune response to infection and the effectiveness of anti-infective therapy. It has gained popularity, because unlike conventional methods which require the analysis of multiple cohorts of animals at different time points, BLI allows for continual analysis in the same cohort. Continual BLI for pathogen colonization and treatment response is a well-established tool but its use in determining how animals respond to infection is an emerging technology. Reporter genes are invaluable for studying cellular immune responses *in vivo*. They can be used, for example, to monitor specific inflammatory signalling pathways and immune cell recruitment to areas of infection. This review explores the numerous strategies for identifying biological processes *in vivo* and how the use of bioluminescent pathogens and the luciferase enzyme reporter is being applied to the study of host response to infection.

2 Models of infectious disease

Infectious diseases are one of the leading causes of death worldwide, accounting for over 15% of global mortality, according to the WHO [1]. Therefore, modelling human infection is imperative to understanding and developing treatments to reduce the global burden of infectious disease. Disease models of infection include *in vitro* modelling in cultured cells, *in vivo* animal models as well as *ex vivo* models of human organs or organoids [2–5]. Today, rodents account for the majority of models used in infectious disease research and they model a wide range of infectious disease agents [6].

To determine infection and the host response to infection using traditional disease models of infection, conventional markers such as blood samples or swabs are taken. The infected animals may then be sacrificed at defined time points and infected tissues harvested. An example of subsequent analysis is serial plating and colony counting to estimate pathogen numbers and to determine localisation. This whole process is highly invasive, end-point analysis intensive and expensive. To address this invasive and time-consuming process, imaging and reporter genes have been employed for use in *in situ* detection of specific infectious pathogens and inflammation as well as gene activities responsible for the immune response. The use of reporter genes provides a means of expedient, simple and highly-sensitive endpoint analysis compared to conventional infection markers.

3 Pre-clinical imaging modalities in infectious disease research

Pre-clinical imaging modalities are integral to translational research and they constitute a means of assessing biological structures and processes using non-invasive techniques. They enable longitudinal studies in individual animals. A main advantage is the reduction of biological variability as each animal can function as its own control. These imaging modalities also support two of the 3Rs of animal research; reduction and refinement, by minimising numbers of animals sacrificed and the intrinsic non-invasive nature of imaging which help to improve animal welfare [7]. Numerous non-invasive imaging modalities have been used for infectious disease research as discussed below.

Positron emission tomography (PET) labels biologically active molecules with positron-emitting radioisotopes to image *in vivo* pathophysiological processes. Using the clinically-established ^{18}F-FDG as a surrogate marker for infection-induced inflammation it was possible to monitor response to *Staphylococcus aureus* vascular graft infection [8]. Following tuberculous meningitis infection in a young rabbit model, activated immune cells in the brain were detected non-invasively using the ^{124}I-DPA-713 radio-isotope tracer [9].

Single photon emission computed tomography (SPECT), is a similar imaging modality to PET, whereby administered radioisotopes emit gamma radiation. SPECT combines multiple images to create a 3-

dimensional image. It has an established role in the imaging of myocardial and cerebral perfusion but its role in infection is evolving. For example, the radioisotope, [99mTc]annexin V-128, is an *in vivo* marker of cellular stress and apoptosis, and can be used to detect and trace bacterial infection and response to treatment by SPECT imaging [10].

Computed tomography (CT) uses X-rays to measure and compare differences in tissue densities. It is helpful in detecting tissue or organ morphological changes caused by infection and inflammation, such as pulmonary fibrosis [11].

Magnetic resonance imaging (MRI) is a non-ionizing 3D imaging modality that uses the magnetic properties of tissues and their interactions with external magnetic fields. MRI not only provides anatomical information but it can also provide physiological data such as organ perfusion, molecule diffusion and tissue chemical composition [12]. Unsurprisingly MRI is an important tool in infectious disease research and has been used in multiple ways including the monitoring of inflammation[13], the quantification of blood flow to infected sites [8] and the imaging of host abscess formation [14].

Photoacoustic tomography works on the natural property of tissues to thermoelastically expand when stimulated with pulsed laser. This leads to ultrasound waves being emitted from the tissues which can be detected using an ultrasound transducer. It produces real-time high resolution scans and 3D reconstructions [15]. Its use in infectious disease research is limited but recently a new photoacoustic contrast agent that is highly specific for detecting certain bacteria *in vivo* has been described [16].

Optical imaging includes a variety of imaging techniques that rely on light production in the visible, ultraviolet or infrared electromagnetic spectrum. These optical imaging modalities usually require suitable reporter genes to be tagged in cells or pathogens of interest. The most relevant to infectious disease research are BLI, fluorescence imaging and two-photon intravital microscopy. Two-photon intravital microscopy, for example, contributed to detection of a pathway involved in the intravascular coagulation process which occurs during sepsis [17].

4 Conventional reporter gene methods

Monitoring biological processes *in vivo* is challenging therefore reporter genes may be used as surrogate markers to localise and quantify molecular signals. This technology relies upon the control of reporter genes by selected regulatory sequences; this confers the organism with a marker that can be easily detected and quantified, such as luminescence or fluorescence.

Numerous reporters have been developed. The first to be exploited were the bacterial enzymes chloramphenicol acetyltransferase (CAT) and beta-galactosidase (β-gal). CAT catalyses the transfer of the acetyl group from acetyl-coenzyme A to chloramphenicol [18]. Its popularity is limited by the need for radioisotopes and relatively elaborate purification and enzymatic assays to detect CAT reporter expression. β-gal, which recognises and cleaves X-gal to generate an intense blue stain, was first described by Jacob and Monod [19]. β-gal, the enzyme encoded by the LacZ gene in *E.coli*, became one of the most commonly used reporter genes for quantifying gene promoter activity [20].

These reporters have now been superseded by fluorescent markers. This began with the cloning of green fluorescent protein (GFP) in the 80s and the development of enhanced mutants of GFP [21–23]. The two best characterised GFPs are from the marine invertebrates; *Aeqourea victoria* and *Renilla reniformis*. Other GFP-like green, yellow and red proteins have been subsequently cloned [24]. The great advantage of these GFP-like proteins is their ability to form internal fluorophores without requiring accessory enzymes or substrates. They are also highly stable and are non-toxic in most cases.

They are widely used to visualise transcriptional activities of promoters and to locate proteins in live cells [25].

4.1 Disadvantages of conventional reporter gene methods

Conventional assays of host–pathogen interactions require that experimental animals be euthanized at multiple time points to analyse the response to infection. While this approach has been, and remains invaluable in defining key aspects of microbial pathogenesis, there are limitations to these methodologies. Analysing individual animals at single time points precludes real-time monitoring of spatial and temporal progression of infection in the same animal, which may reveal biologically relevant variations. Furthermore, host response at an unexpected site of infection may be missed because the tissue is not harvested and analysed (the "Streetlight effect"). Finally, conventional assays of pathogenesis typically require large numbers of animals to obtain statistically meaningful data at multiple time points.

Fluorescence-based optical imaging is impeded by light absorption in tissues. Haemoglobin absorbs light with a peak absorbance of 575nm whilst water absorbs light with a peak absorbance of 977nm, leaving an optimal optical window between these two wavelengths [26]. GFP is not an ideal fluorophore for *in vivo* imaging since its excitation and emission peak wavelengths are 488nm and 509nm, respectively [27]. Autofluorescence from the tissue is also often problematic as it interferes with fluorescent signals in the green spectrum leading to high background fluorescent levels.

Transgenic fluorophores may also elicit immune responses in immune competent organisms. GFP has been shown to induce T-cell mediated immunogenicity in numerous mouse strains, thus limiting its application as a surrogate marker for immunological processes [28–30].

5 BLI and luciferase enzymes as reporter genes

Light is emitted when luciferase enzymes catalyse their substrates *in vivo.* BLI detects this light as a surrogate marker for protein expression using comparatively inexpensive charge-coupled device (CCD) cameras. Luciferase reporter genes have gained popularity in recent years because of their ability to quantify promoter activation with greater fidelity compared with conventional reporter genes. This is supported by the dramatic increase in luciferase-associated citations over the past twenty years (Figure 1). It can be used to detect, localize and quantify specific immune cells, pathogens or immunological processes.

The luciferase enzyme from the firefly, *Photinus pyralis*, was the first to be cloned and characterised [31]. Firefly luciferase catalyses an oxidative reaction in the presence of its substrate D-Luciferin and adenosine triphosphate, magnesium and oxygen. The production of oxyluciferin releases a photon of light. Firefly luciferase is a good biomarker reporter as it requires no post-translational modifications, it has a half-life of approximately 3 hours and it has a relatively red-shifted emission spectrum [32,33].

Subsequently, luciferase enzymes have been cloned from other organisms including from the sea pansy *Renilla reniformis*, the click beetle *Pyrophorus plagiophthalamus*, the copepod *Gaussia princeps* and the jellyfish *Aequorea victoria* amongst others. Coelenterazine is the substrate for *Renilla* and *Gaussia* luciferases in a reaction which leads to flash kinetics *in vivo* with rapid onset and significant diminution of bioluminescence after 10 seconds [34]. The blue-green light emission penetrates mammalian tissue poorly, compared with firefly luciferase, although a red-shifted *Renilla* luciferase mutant has subsequently been developed [35,36].

Other luciferase enzymes that are particularly important in animal infection models are those from the *luxCDABE* operon in bacteria such as *Photorhabdus luminescens* and *Xenorhabdus luminescens.*

The bacterial lux operon encodes enzymes that oxidise a long-chain aldehyde and a reduced flavin mononucleotide to produce oxidised flavin which emits light at 490nm [37]. This reaction is catalysed by a bacterial luciferase which is encoded by the *luxA* and *luxB* genes. The *luxC*, *luxD* and *luxE* genes encode a multienzyme complex which is responsible for the regeneration of the aldehyde substrate from the fatty acid produced by the initial reaction. The main advantage of the lux operon system is the ability to express the enzymes that synthesise the substrate, therefore addition of exogenous substrate is unnecessary. The lux operon has been transferred to other bacterial strains to confer them with bioluminescence.

BLI has several advantages over the conventional reporter gene methods described above. While *in vivo* imaging has been successfully performed with different fluorescent proteins [38], the signal to background ratio is notably greater with luciferase BLI [39]. It is also an extremely sensitive assay and it can be performed multiple times on a single animal [40]. This helps to reduce variation between animals and it eliminates the costly and time-consuming task of gathering data through sequential sacrificing of animals at different time points. Compared with other imaging strategies it is also quick and easy to use, it is less expensive and it has minimal post-image analysis.

6 BLI and its use in monitoring host response to infection

Luciferase BLI is now well-established in the field of infectious disease research. It has been commonly used to study colonization of pathogens, to monitor microbial gene expression, for example genes involved in virulence, as well as to test treatment and vaccine efficacy [41]. BLI's role in examining the host response to infection is an emerging field and there are three main ways that it can be used to examine host response to infection:

1. **Bioluminescent pathogens:** Using bioluminescent pathogens to infect animals with an altered immune system.
2. **Light-producing transgenic reporter animal**: Delivering a luciferase reporter gene under the control of a promoter or a transcription factor binding sequence of interest.
3. **Light-producing immune cells**: Tracking luciferase-reporter immune cells.

6.1 Bioluminescent pathogens

Two different strategies have been applied for engineering bioluminescent pathogens. The first strategy is using the luxCDABE operon from bacteria such as *Photorhabdus luminescens* or *Xenorhabdus luminescens* which is transferred to other bacteria to confer them with constitutive bioluminescence. The second strategy, which is mainly employed to create bioluminescent viruses and fungi, uses recombinant pathogens engineered to express a luciferase reporter gene, for example herpes simplex-1 luciferase reporter virus [42].

Bioluminescent pathogens can be used to infect animals that have an altered immune system to give insight into how the hosts immune system responds to infection. Neonates and young children are known to have an increased susceptibility to infection owing to a lack of protective immune responses by their immature immune system. To study the mechanisms behind neonatal susceptibility to infection, a bioluminescent *Salmonella typhmurium* strain was used to infect mice at different ages to monitor infectivity and systemic infection [43]. As expected, younger mice, with a less mature immune system, were more vulnerable to severe systemic infection; this susceptibility decreased with age. Similarly, a bioluminescent *Staphylococcus epidermidis* strain was used to infect mice to model indwelling medical device-related infections. Immune compromised mice were more susceptible to these device-related nosocomial biofilm infections than wild type mice [44].

Bioluminescent bacteria have also been used to study specific aspects of the host immune system by infecting mice in which immune-related genes have been genetically disrupted. Toll-like receptors (TLRs), an integral part of the innate immune system, recognise microbes by binding to specific pathogen-associated molecular patterns and in response they activate appropriate innate immune defences. TLR 2 and 4 knock-out mice (*Tlr2/4-/-* mice) were found to be particularly susceptible to lung infection by a bioluminescent strain of flagellin-deficient *Pseudomonas aeruginosa* [45]. This demonstrates the important role of the TLRs in mounting a sufficient host innate immune response to control bacterial infectivity.

Myeloid differentiation primary response protein (MyD88), another important innate immune system protein, acts as a signal transducing adaptor protein. Upon activation of TLRs by their specific ligand, MyD88 is recruited and leads to a propagation of downstream signalling pathways that results in activation of numerous pro-inflammatory transcription factors including NF-κB. *MyD88-/-* mice exhibited increased susceptibility to lethal colitis when infected with a bioluminescent strain of *Citrobacter rodentium* [46]. This highlights the key role of MyD88 and NF-κB in mediating host defence against enteric pathogens.

Interferons(IFN) have a significant role in mediating the host innate immune response to pathogens, in particular to viruses [47,48]. Mice lacking different IFN receptors were infected with herpes simplex virus-1 (HSV-1) carrying the firefly luciferase reporter virus. BLI revealed widespread and, ultimately lethal, dissemination in Type 1 and Type 2 IFN receptor knock-out mice; this illustrated the importance of these receptors in limiting HSV-1 infectivity and dissemination [42].

6.2 Light-producing transgenic (LPT) reporter rodents

Infection elicits numerous signalling pathways which initiate and control the inflammatory response including local cytokine production and recruitment of immune cells to the site of infection. Many of these responses are controlled at the transcriptional level and so reporter mice have been generated using a promoter or a transcription factor binding site sequence for the gene of interest controlling luciferase transgene expression.

6.2.1 Germline LPT reporter rodents

Light producing transgenic rodents have been generated, conventionally, using germline transgenic technology. This is useful in that every cell in the mouse containing the specific genetic biosensor which enables whole body transcriptional activity to be monitored by continual BLI. Germline LPT reporter mice that have been used in the study of immune response to infection are described below.

LPT interferon-β reporter mouse

Type 1 interferons are important mediators of the host immune response to infection [49]. They are also believed to have a role in the non-infected state by priming immune cells to enable them to respond to infections in a timely manner [48]. Interferon-β, part of the large type 1 interferon family, is considered the master regulator of the type-1 interferon signalling pathway [50].

Germline LPT interferon-β mice were developed in which cre-recombinase was used to replace the interferon-β gene with a firefly luciferase reporter transgene. Therefore these mice expressed luciferase under the control of the endogenous interferon-β promoter. The luciferase surrogate of interferon-β expression was determined following influenza A and La Crosse viral infections. This mouse was also used to detect constitutional expression of interferon-β, and specifically found that the thymus is responsible for non-infective interferon-β expression [51].

LPT interleukin-1ϐ reporter mouse

Interleukin- 1β is a potent mediator of infection and the pro-inflammatory response. It is responsible for a wide range of infection-related reactions including fever, hypotension and the production of other pro-inflammatory cytokines. A germline transgenic interleukin-1β reporter mouse was developed which firefly luciferase was placed under the control of a 4.5kb fragment of the human interleukin-1β promoter. Luciferase activity was increased in this mouse following systemic LPS administration. This activity was found to be suppressed with the anti-inflammatory agent dexamethasone [52].

LPT CCL20 reporter mouse

Chemokines play an important role in the chemotactic movement of immune cells to areas of infection and inflammation. CCL20 is a major constitutive chemokine which is crucial in recruiting immune cells to mucosal lymphoid tissues such as Peyer's patches and tonsils [53]. It also has a significant role as an inducible chemokine to promote migration of immune cells to areas of infection and inflammation [54]. A LPT CCL20 reporter mouse was created by incorporating a mouse CCL20 promoter upstream of the firefly luciferase transgene [55]. The luciferase activity was used as a surrogate for CCL20 expression in the mouse after systemic administration of flagellin (a major constituent of bacterial flagella) and heat-inactivated salmonella. Luciferase activity was comparable to that seen with a germline LPT NF-κB reporter mouse. The results from these two strains led the authors to conclude that NF-κB was an important transcriptional activator of CCL20 expression [56].

LPT inducible nitric oxide synthase (iNOS) reporter mouse

iNOS is an inducible enzyme that catalyses the production of nitric oxide from L-arginine. It is produced by many different cell types and plays an important role in infection and inflammation [57]. iNOS-mediated release of nitric oxide is important in the pathogenesis of septic shock whereby it leads to systemic hypotension and myocardial depression [58]. A germline LPT iNOS mouse containing firefly luciferase under the control of murine iNOS promoter showed significantly increased liver luciferase activity upon interferon-ϒ and LPS induction of sepsis. In inflamed joints in a model of acute septic arthritis, elevated luciferase was significantly reduced by the administration of the anti-inflammatory steroid dexamethasone. This illustrates the utility of such models in validating new therapeutic compounds in a range of inflammatory models [59].

LPT glial fibrillary acidic protein (GFAP) reporter mouse

Astrocytes are a diverse sub-type of glial cells found in the central nervous system. They have numerous functions but their role in providing defence against brain insults appears to be an important one [60,61]. GFAP is a major intermediate filament protein used as a marker to distinguish astrocytes from other cells in the central nervous system [62].

Germline LPT GFAP reporter mice have been generated in which firefly luciferase is controlled by a fragment of the GFAP promoter. Kadurugamuwa et al. generated a mouse where firefly luciferase was transcriptionally regulated by a 12kb GFAP promoter fragment [63]. They used this in combination with *Streptococcus pnemoniae* containing the lux operon to study host and pathogen responses in a model of meningitis. Since firefly luciferase and lux operon have distinct emission spectra, they were able to correlate the progression of bacterial meningitis with astrocyte activation in the CNS. Importantly they found that early administration of antibiotics was sufficient to prevent neuronal injury. Cho and colleagues produced a transgenic mouse in which firefly luciferase was transcriptionally regulated by a 2.2kb fragment of the GFAP promoter [64]. They generated a second strain in which *Renilla* luciferase was placed under transcriptional control of a 0.5kb fragment of the promoter of the housekeeping gene Glyceraldhehyde 3-phosphate dehydrogenase. Crossing these strains produced a dual transgenic reporter mouse where neuroinflammation, induced by kainic acid

administration, could be monitored by firefly luciferase and in which the signal could be normalised against the *Renilla* luciferase.

LPT NF-κB reporter mice

Luciferase reporters are widely used for the study of promoter activity but the more recent design of synthetic transcription factor binding elements has enabled targeted interrogation of gene and transcriptional factor relationships. These consist of serial transcription factor binding consensus sequences upstream of a minimal polymerase initiating sequence which drive luciferase expression. For example, this technology has been developed for the NF-κB transcription factor whose canonical NF-κB genomic binding site has been defined as 5'-GGGACTTTC-3' [65].

NF-κB regulates the expression of genes responsible for many cellular processes including immune and inflammatory pathways. These proteins are ordinarily bound and inhibited by IκB. However, following infection or the release of pro-inflammatory cytokines, there is activation of IκB kinase which leads to the phosphorylation of IκB and subsequent ubiquination and proteasomal degradation [66]. Free NF-κB dimers are therefore able to translocate to the nucleus where they bind directly with the consensus DNA sequence in the promoter of NF-κB target genes to initiate an inflammatory response [67].

Germline LPT rodents have been produced in which luciferase expression is a surrogate marker for NF-κB transcriptional activity [68,69]. Yull et al. produced mice in which the NF-κB-dependent portion sites of the HIV-1 long terminal repeat was used to drive luciferase expression [69]. These mice showed NF-κB dependent luciferase activity in the lung, liver, spleen, kidneys and forebrain after intraperitoneal lipopolysaccharide (IP LPS). This technology was also used to assess potential anti-inflammatory therapeutic options including an IκB kinase inhibitor which successfully inhibited pro-inflammatory NF-κB dependent luciferase activity [68]. A similar transgenic model was developed to monitor NF-κB activity following systemic LPS administration as well as TNF-α and IL-1α cytokine administration[70]. In this case, the transgenic mouse contained luciferase under the transcriptional control of a triplet repeat of a consensus NF-κB.

Kielland et al. developed transgenic mice using a novel bidirectional promoter regulated by a sextuplicate repeat of consensus NF-κB binding elements driving both luciferase and enhanced GFP transgenes[71]. These mice were used to determine pro-inflammatory NF-κB transcriptional activity in the brains of newborn pups injected with intracerebral LPS.

6.2.2 Somatic LPT reporter rodents

An advantage of germline LPT rodents is that every cell in the body carries the genetic biosensor. However, it is very difficult to monitor activity in individual organs and tissues because of the confounding bioluminescence signal in surrounding tissues. The generation of germline transgenics is also time-consuming and colonies are both expensive to generate and to maintain. An alternative approach to the germline LPT reporters is using post-natal somatic gene transfer to produce somatic transgenic reporter animals.

Non-viral vector-mediated somatic LPT reporter mouse

Interleukin-8 is a pro-inflammatory cytokine produced in response to a variety of infective and inflammatory stimuli and is an important chemoattractant for immune cells such as neutrophils [72]. A mouse model was described in which the luciferase gene under the control of a bovine interleukin-8 promoter was transiently expressed [73]. Mice received a plasmid containing this expression cassette by intra-tracheal administration. Although mice lack an exact homolog of the interleukin-8 gene, the authors demonstrated that mice possess the transcriptional apparatus to activate the

bovine interleukin-8 promoter in response to LPS or TNF-α administration for up to 60 days after gene delivery. This technology was subsequently used in a cystic fibrosis mouse model to monitor the inflammatory response to *Pseudomonas aeruginosa* infection [74].

Viral vector-mediated somatic LPT reporter mice

In the early 1980s, gene transfer vectors based upon viruses were developed from adenovirus, retrovirus and adeno-associated virus [75–77]. Compared with non-viral approaches, viral vectors are much more efficient and it is this efficiency which has contributed to their widespread adoption in the burgeoning field of gene therapy. They are firmly established as gene-targeting tools in biological research.

Adenoviral vector-mediated generation of somatic LPT rodents

There have been several studies exploiting adenoviral vectors to make somatic LPT rodents. However, since adenoviral vectors often evoke potent immune responses, they have mainly been used in profoundly immune-deficient mouse strains which are often not ideal for the study of infection [78,79].

For the production of somatic LPT rodents, Peterson et al. used adenoviral vectors to deliver NF-κB and AP-1 firefly luciferase reporter constructs into distinct central nervous system nuclei [80]. They were able to monitor the local NF-κB activity and AP-1 activity in response to systemic LPS administration.

Lentiviral reporter gene production for neonatal somatic LPT mice

Lentiviral vectors are a subclass of retrovirus, which naturally integrate into dividing and non-dividing cells as well as showing stable and long-term expression [81]. Systemic vector delivery in adult rodents can evoke an immune response which can not only complicate the transcriptional activity being studied but also lead to a loss of transduced cells [82]. Viral vector administration to neonatal rodents prior to the full maturation of the immune system may induce immune tolerance to the transgenic protein, permitting prolonged expression of the transgene [83,84].

We have generated liver-, brain- and lung- targeted NF-κB reporter mice using a combination of route administration and lentiviral pseudotyping in newborn rodents [85] (Figure 2). We observed that the NF-κB reporter was activated directly by TLR4 mediated-signalling since LPS-induced BLI signal was completely abolished in *Tlr4 -/-* mice. The following section describes the method of producing lentiviral transcription factor activated luciferase reporters (method 1) and how to use them for monitoring transcriptional activity *in vivo* (method 2). See appendix for materials and equipment.

Method 1 [85]*: Co*nstruction and insertion of synthetic transcription factor response element into lentiviral reporter gene cassette using Gateway® recombination cloning technology *(Figure 4)*

PCR amplification of firefly luciferase (Fluc) and 2A-eGFP(enhanced-GFP) sequences is performed followed by overlap extension PCR to anneal and create a Fluc-2A-eGFP insert. The LNT-Gateway®-Multiple cloning site plasmid vector (pLNT-GW-MCS) is digested using compatible ends to those generated for the insert. The Fluc-2A-eGFP insert is ligated into the digested pLNT-GW-MCS vector.

A minimal promoter (MP) sequence (the adenoviral E1A minimal promoter was used) flanked by XhoI sites is *de novo* synthesized (Aldevron, Fargo MD, USA). This is then cloned into unique XhoI sites in the pENTR-1A Gateway® cloning shuttle vector to produce pENTR-MP. Minimal consensus binding sequence are determined for the candidate transcription factor. 5'-GGGACTTTC-3' is used for the NF-κB response element [65]. A serial transcription factor binding sequence (TFBS) is designed by interspersing 4-10 binding sequences with 10 random nucleotides and then synthesized with

restriction enzyme sites at the 5'- and 3'- termini (Aldevron, Fargo MD, USA). This TFBS is then cloned into pENTR-MP to produce pENTR-TFBS-MP. The TFBS-MP sequences are then recombined into pLNT-GW-Fluc-2A-eGFP using Gateway® cloning technology.

In order to produce a high-titre second generation lentivirus a three plasmid system is used [86]. Envelope plasmid VSV-G (pMD.G2; Vesicular Somatitis Virus glycoprotein) and the packaging plasmid pCMVΔR8.74 containing the gag and pol genes is used. The packaging plasmid allows the lentivirus carrying the transgene to integrate into the host genome.

Method 2 [85]: Neonatal organ-targeted delivery of lentiviral vector

All *in vivo* neonatal injections are performed within 24 hours after birth. Neonatal pups are anaesthetised on ice. Pups are injected by the following routes and volumes: intracranially (5 µl), intravenously (20 µl), subcutaneously (10 µl), intranasally (20 µl), or ventral subcutaneously (5 µl). The luciferase reporter activity is monitored in living adult mice by anesthetizing mice with 4% isofluorane in 100% O2. 300 µl of D-luciferin solution at a concentration of 15 mg/ml (a dose of approximately 150 mg/kg) is injected into the intraperitoneal cavity approximately 5 minutes before imaging. The mice are imaged in a warmed, light-proof detection chamber in the CCD imager. Images are viewed and analysed using the Living Image software (Perkin Elmer, Coventry, UK).

6.3 Light producing immune cells

Trafficking of immune cells to areas of infection is an integral part of the host response to infection. Technology has been developed to allow for localisation and tracking of certain cells involved in the immune response using BLI.

Akimoto et al. used luciferase as a marker of bone-marrow derived cells to determine their ability to cross the blood-brain barrier and differentiate into microglia (the resident macrophages of the brain) following LPS administration to the hippocampus [87]. BLI showed increased luciferase activity in the brain and these luciferase-expressing cells were confirmed to be microglia. This study successfully distinguished between two different types of brain microglia; resident and bone-marrow derived, as well as providing a powerful tool for imaging the movement of bone-marrow derived cells.

An alternative way of using BLI imaging to detect immune cell response *in vivo* is with luminol bioluminescence. Luminol is a redox-sensitive compound that emits blue luminescence upon exposure to an appropriate oxidising agent. As luminol is cheap to produce and relatively simple to synthesis it is popular in a variety of fields including biochemistry, clinical diagnostics and forensic sciences for detecting reactive intermediates. Luminol luminescence has been used to study whole blood and phagocytes for many years [88]. Myeloperoxidase is activated during phagocytosis in the azurophilic granules of neutrophils and in the lysosomes of macrophages. It is released into phagosomes and catalyses the reaction which produces bactericidal hypochlorous acid. [88]. The produced hypochlorous acid can directly or indirectly oxidize luminol to produce light (Figure 3). Gross et al. developed a method to image myeloperoxidase activity in small laboratory animals in vivo [89]. In a focal arthritis model, generated by intra-articular injections of LPS, luminol bioluminescence was readily detected from LPS-treated joints allowing tracking of activated phagocytes to areas of infection [90].

7 Conclusions

The use of BLI is becoming widespread for infectious disease research as it avoids many of the problems associated with conventional research methods. BLI is becoming a useful technology for monitoring host response to infection. In the future, the use of optical imaging modalities in

combination with other imaging modalities may further help us to understand the complexities of the host pathogen relationship.

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10 Appendix

10.1 List of materials and Equipment

- Primers and template to amplify reporter gene
- High-fidelity polymerase
- DNTPs
- PCR cleanup kit
- 1.5% agarose gel
- Gel extraction kit
- Xho I, Mlu I and BamHI restriction enzymes
- Quick Ligase
- SOC outgrowth media
- ccdB resistant competent cells such as DB3.1 or One Shot® ccdB Survival™ 2 T1R Competent Cells
- Luria broth (LB)
- Ampicillin (100 µg/ml)
- Plasmid DNA mini-prep kit
- WPRE reverse primer for sequencing
- De novo synthesis of minimal promoter and synthetic promoter DNA (Aldevron, ND, USA)
- TOP10, DH5α or similar regular cloning competent cells
- LB containing kanamycin antibiotic (50 µg/ml)
- Plasmid DNA mini-prep kit
- pENTR-1A (Invitrogen, UK)
- Buffer TE (pH 8.0)
- LR clonase II ™ (Invitrogen, UK)
- Proteinase K
- Stbl3 competent cells
- Highly proliferating, mycoplasma-free HEK293T cells
- OptiMem® I reduced-serum medium (Gibco, UK)
- Phosphate buffered saline (PBS) containing calcium and magnesium
- Vesicular Stomatitis Virus glycoprotein (VSV-g) envelope plasmid (pMD2.G) (Aldevron, Fargo, USA)
- Packaging plasmid containing gag, pol, tat, rev viral genes (pCMVΔR8.74) (Aldevron, Fargo, USA)
- Polyethylenimine transfection reagent (10 mM): 10 ml branched PEI made up to 41.2 ml with dH2O (pH 7.0)
- 0.45 µM PVDF sterile filter cups
- Complete Media: Dulbecco's Modified Eagles Media, 10% FBS, 1% penicillin/streptomycin
- 33 gauge Hamilton needle and 100 µl syringe (Fisher Scientific)
- D-luciferin (Gold Biotechnology) reconstituted in sterile PBS to a working concentration of 15 mg/Ml
- 27 gauge needle
- Anaesthetic, 100% Isoflurane inhalation liquid vapor liquid (Abbott)
- Oxygen - 5.1%, flow rate 1.5 per min
- IVIS machine (Perkin-Elmer)