Mammalia: Chiroptera: Immunology of Bats

Michelle L. Baker and Tony Schountz

Introduction

Bats (order Chiroptera) are a diverse group of nocturnal mammals comprising approximately 20% of all mammalian taxa and consisting of more than 1300 species across 21 families (Simmons 2005). Phylogenetic analysis places bats within the superorder Laurasiatheria, sister to carnivores (e.g., cats, dogs), ungulates (e.g., horses, cows), and cetaceans (e.g., dolphins) (Fig. 1) (Tsagkogeorga et al. 2013). Bats are believed to have diverged from other eutherian mammals approximately 88 million years ago (mya) (Lei and Dong 2016). The traditional classification system divided bats into two suborders: Microchiroptera (microbats) and Megachiroptera (megabats). Microbats are defined by their smaller size (4–16 cm), the use of echolocation, and the use of hibernation during the winter for many species. Megabats consist of the flying foxes (also called fruit bats) and are larger nonecholocating bats (up to 1.6 kg with wingspans of 1.7 m) belonging to the Pteropodidae family. However, more recent phylogenetic analyses based on molecular data have led to a reclassification of bats into the suborders Yinpterochiroptera and Yangochiroptera. The Yinpterochiroptera suborder includes the nonecholocating Pteropodidae family (flying foxes) and the echolocating Rhinolophoidea family, while the Yangochiroptera suborder consists of the remaining echolocating microbats (Teeling et al. 2005, 2016). The two suborders of bats are estimated to have diverged approximately 63 mya (Lei and Dong 2016). Although the new classification has strong statistical

M. L. Baker (✉)
CSIRO Health and Biosecurity Business Unit, Australian Animal Health Laboratory, Geelong, VIC, Australia
e-mail: Michelle.Baker@csiro.au

T. Schountz
Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

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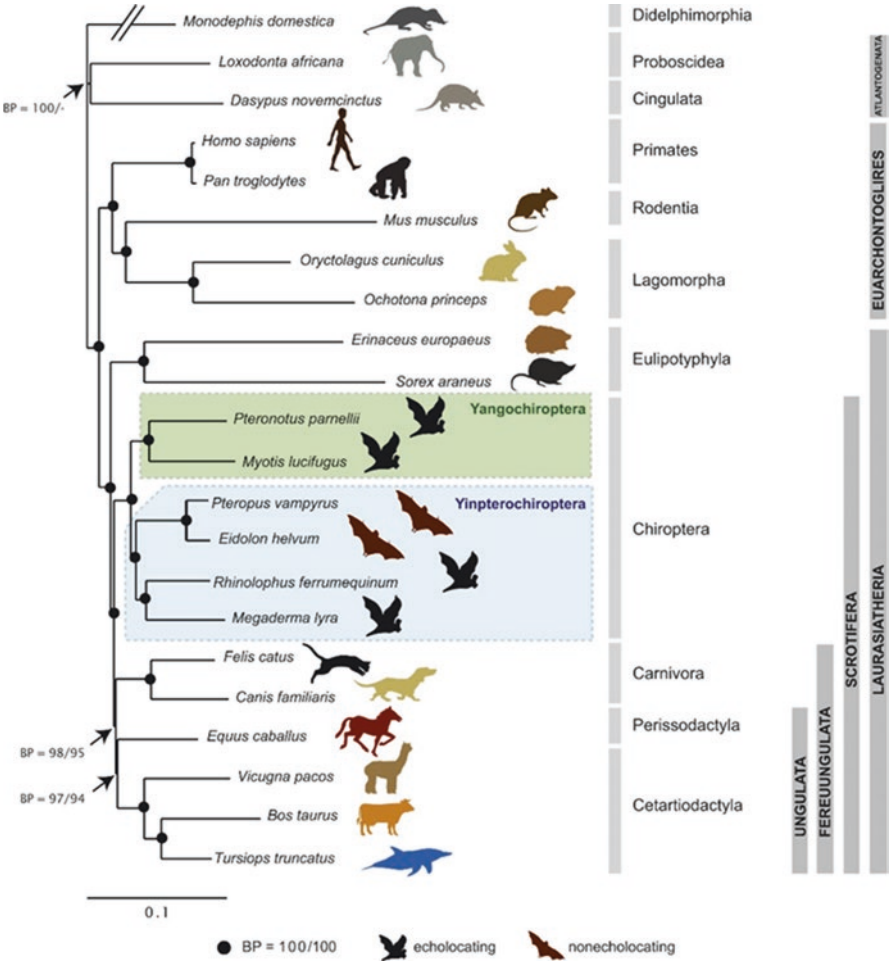

Fig. 1 Phylogenetic relationship of bats to other species. (From Tsagkogeorga et al. 2013 with permission)

support, it remains controversial as it suggests that laryngeal echolocation evolved twice in Chiroptera, once in Yangochiroptera and once in the rhinolophoids (Teeling et al. 2000).

Of all the mammals, bats are the most ecologically diverse. They are the only mammals that have evolved powered flight and have adapted to a variety of environments across all continents with the exception of the polar regions. Their diets are equally diverse, including fruits, pollen, insects, small vertebrates, and even blood, and they play important roles in the ecosystem through seed dispersal, pollination, and insect control. Bats have longer lifespans relative to other mammals, typically living 3.5 times longer than mammals of similar size (Austad 2010). Maternal investment is generally high, with most species giving birth to a single pup per year

and pups averaging approximately 23% of maternal body weight at birth (Barclay and Harder 2003). Curiously, despite their longer lifespans, there is anecdotal evidence that bats are resistant to tumors (Wang et al. 2011). The characteristic that has drawn the most attention in recent decades is their role as natural reservoirs for a variety of viruses that are highly pathogenic in other species yet rarely cause clinical disease in bats. This characteristic in particular has led to renewed interest in the immune systems of bats.

Bats are highly gregarious mammals, with most species living in high-density colonies, providing ideal environments for transmission and maintenance of pathogens within populations. Combined with their frequent movement between roosts, transmission of viruses, bacteria, parasites, and fungi could potentially occur readily between individuals and populations, resulting in a situation of constant pathogen exposure. Approximately 200 viruses have been detected across different bat species, and many of the viruses identified in bats are highly pathogenic in other species, including humans (Moratelli and Calisher 2015); however, they likely host many more (Anthony et al. 2013). Examples include high-profile viruses such as the severe acute respiratory syndrome coronavirus (SARS-CoV), Marburg virus, and Hendra and Nipah paramyxoviruses. These viruses occasionally spill over to other susceptible hosts, causing severe disease and mortality yet causing no disease in bats. The long coevolutionary history of bats and viruses has likely resulted in the establishment of a state of equilibrium, allowing both the viruses and their host to coexist in a disease-free state typical of natural reservoirs.

Bats also host a variety of other pathogens, including parasites, bacteria, and fungi. Unlike viral infections, there are examples of these pathogens causing disease among bats. The fungus that causes white nose syndrome (WNS), *Pseudogymnoascus destructans*, has resulted in mass mortalities among a number of North American microbat populations, with some species now threatened with extinction (Blehert et al. 2009). Evidence for lower fungal loads consistent with the development of resistance to the fungus have been observed in some bat populations, providing hope that selection on immune genes may lead to the development of resistance or tolerance mechanisms (Langwig et al. 2017). However, it is unlikely that this will occur rapidly enough for many affected populations. Several bacterial infections, including tick-borne spirochaete bacteria, *Borrelia* spp., and some enteric bacteria, have also been associated with pathology in bats (reviewed in Brook and Dobson, 2015). Brooks and Dobson (2015) presented evidence that bats may have evolved mechanisms to eliminate intracellular pathogens such as viruses at the expense of their ability to eliminate extracellular pathogens (bacteria, parasites, and fungi) and hypothesize that mitochondrial adaptations may play a role.

In light of the increasing emergence of infectious diseases and the impacts of pathogens such as WNS, deciphering the immune systems of bats has never been more critical and offers potential for identifying novel antiviral therapies and approaches to the conservation of bats threatened by diseases such as WNS. Fortunately, progress in the area of bat immunology is rapidly advancing as new groups enter the field and advances in technology provide opportunities for more rapid discovery. Several reviews that have appeared over the last 5 years have

described the various aspects of the immune systems of bats (Baker et al. 2013; Butler et al. 2014; Schountz 2014; Baker and Zhou 2015; Schountz et al. 2017). In this chapter we provide a broad overview, with a focus on recent highlights in bat immunology and areas for future research.

Immune Tissues and Cells

Although few studies have examined the histology of bat lymphoid tissues, from an anatomical perspective, bats appear to have the majority of primary and secondary lymphoid organs present in other mammals, including thymus, bone marrow, spleen, and lymph nodes (Papenfuss et al. 2012; Zhou et al. 2016b). Bone marrow has been isolated from long bones, including humerus and radius, and from the ribs but appears to be absent in the distal wing bones (Papadimitriou et al. 1996; Zhou et al. 2016b). Notably absent, at least in the species that have been examined to date, are Peyer's patches, which are generally located in the submucosa and lamina propria of the small intestine. No Peyer's patches were present in the horseshoe bat, *Rhinolophus hildebrandtii*, or the common pipistrelle bat, *Pipistrellus pipistrellus* (Strobel et al. 2015; Makanya and John 1994). The submucosa of the intestine of the horseshoe bat was devoid of lymphoid tissue, with the exception of a few aggregations of lymphoid nodules in the rectal submucosa (Makanya and John 1994).

A range of immune cell types also appear to be present in bats. Morphological characteristics have been used to identify lymphocytes, neutrophils, eosinophils, basophils, and macrophages in the Brazilian free-tailed bat, *Tadarida bra*silensis (Turmelle et al. 2010a). Macrophages and T- and B-cell populations have also been identified in the Indian flying fox, *Pteropus giganteus*, based on cellular adherence and scanning electron microscopy (Sarkar and Chakravarty 1991). More recently, the phenotype, morphology, and function of dendritic cells and macrophages have been characterized from bone marrow from the black flying fox, *Pteropus alecto* (Zhou et al. 2016b). Cells resembling follicular dendritic cells (FDCs) have also been described in the Indian flying fox (Sarkar and Chakravarty 1991). Unlike dendritic cells that originate in the bone marrow, FDCs are of mesenchymal origin and are found in primary and secondary lymphoid follicles in B-cell areas of lymphoid tissue. FDCs are essential for high-affinity antibody production and for the development of B-cell memory. They also have the ability to maintain intact antigen for extended periods (van Nierop and de Groot 2002; Heesters et al. 2014). Whether they play the same role in bats remains to be determined but presents an interesting possibility for the maintenance of persistent viral infections.

Genetics and Genomics of Immune System

The lack of species-specific reagents has often been a hindrance to comparative immunologists. However, bat immunology made a resurgence in an age of rapid advances in species-independent approaches such as next-generation sequencing,

proteomics, and gene editing technologies such as CRISPR/Cas9. RNAseq studies on tissues and cells from a variety of different species of bats have provided evidence that bats have nearly all of the major components of the immune system that are present in other mammals, including receptors and molecules associated with innate and adaptive immunity and microRNAs (Papenfuss et al. 2012; Shaw et al. 2012; Cowled et al. 2014). RNAseq data from virus-infected bat cells and WNS-infected bat tissues have also offered insights into the genes associated with host–pathogen responses (Wynne et al. 2014, 2017; Field et al. 2015).

Bat Genomes

To date, partial genome sequences of 14 bat species are available in the NCBI database, providing valuable insights into the evolution of immune genes and essential sequence information for the design of primers and the development of reagents essential for studies of the immune responses of bats. The Bat1K project, which aims to sequence the genomes of the approximately 1300 species of bats, will no doubt provide a valuable resource for comparing the immune repertoire of different species of bats (Teeling et al. 2018).

The genomes of bats are condensed compared to other mammals, ranging from 1.6–3.54 Gb. Smaller genome sizes in both bats and birds have been hypothesized to be associated with the metabolic requirements of flight (Kapusta et al. 2017).

Genomic Characterization of Immune Regions

A number of genomic regions associated with immunity have been analyzed in detail, in particular in the black flying fox (*P. alecto*), using a combination of whole-genome data and additional sequencing. These include regions associated with innate, for example, type I interferon (IFN), and adaptive immunity, for example, major histocompatibility class I (MHC-I) and MHC-II. Consistent with the smaller size of the genomes of bats, these regions are also condensed and contain fewer genes compared with the corresponding region from other mammals (Ng et al. 2016, 2017; Zhou et al. 2016a). For example, the type I IFN locus of the black flying fox is highly condensed and contains fewer IFN genes than any other species sequenced to date (Fig. 2).

The description of the genomes of two divergent bat species, the Australian black flying fox (*P. alecto*) and David's myotis (*Mytois davidii*), provided the first glimpse into unique genetic signatures within immune pathways of bats, lending support to the idea of inadvertent changes in the immune system associated with the evolution of flight (Zhang et al. 2013). These include changes in the genes associated with DNA response/DNA repair pathways that are tightly linked with innate immune pathways (Fig. 3). The DNA damage sensor, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which is also part of the cytoplasmic microbial nucleic acid sensing complex, was among the genes that have undergone selection in bats

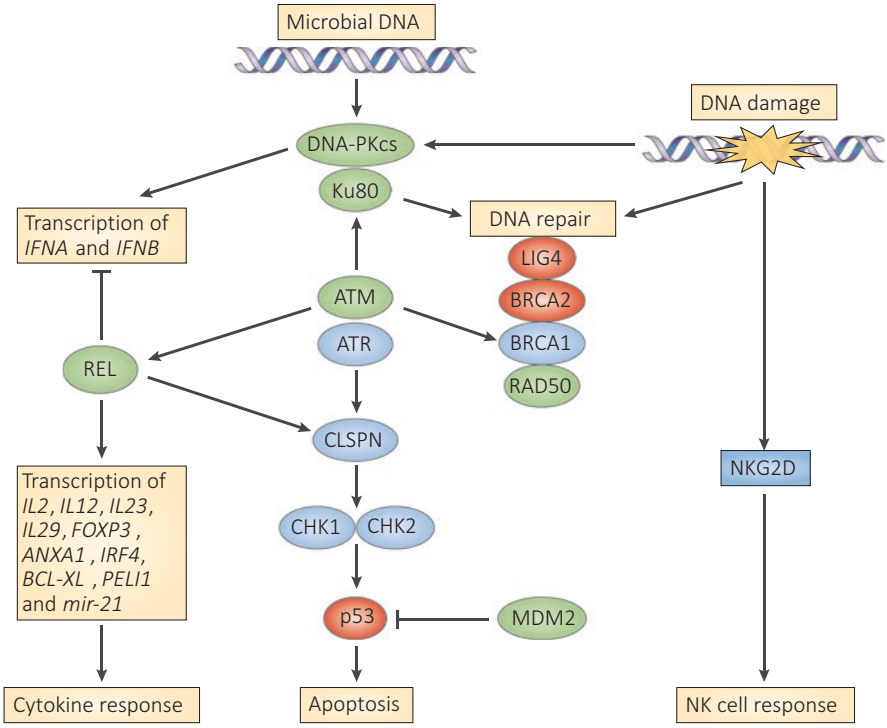

Fig. 2 DNA repair/immune pathway. Whole-genome analysis of two bat species (*Pteropus alecto* and *Myotis davidii*) showed that a high number of genes encoding components of these pathways are positively selected. Many of these genes are positively selected in both species (highlighted in green), whereas others have been positively selected in only one of the species (these encode proteins highlighted in red). (From Bean et al. 2013 with permission)

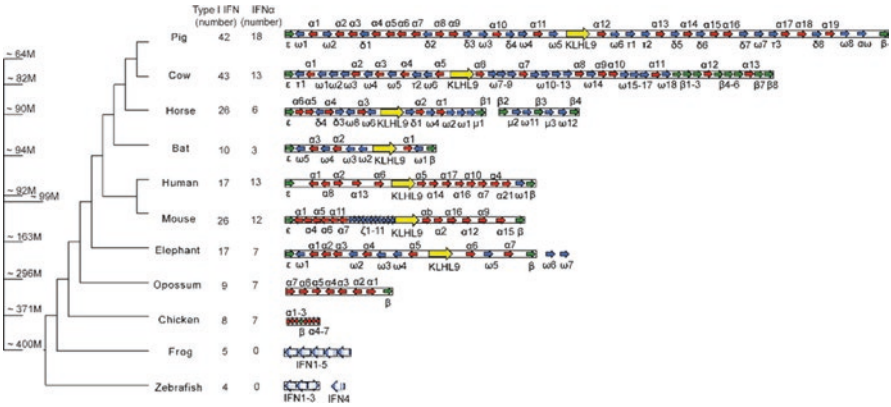

Fig. 3 The type I IFN locus of the black flying fox is highly contracted and contains fewer genes than other vertebrates. (From Zhou et al. 2016a with permission)

(Ferguson et al. 2012). Accelerated evolution of innate immune genes including nuclear factor-kB (NF-kB) family member REL, IFNAR1, Toll-like receptor 7 (TLR7), IFN stimulated gene 15 (ISG15), interleukin-18 (IL-18), and nucleotide-binding oligomerization domain-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) were also observed in the genomes of the two bats, an observation that may be a consequence of the coevolution of bats with viruses (Zhang et al. 2013).

Notably absent from the black flying fox and David's myotis genomes is the PYRIN and HIN domain (PYHIN) gene family, which are involved in the recognition of foreign DNA (Zhang et al. 2013). This finding was recently confirmed in eight additional bat species across both suborders (Ahn et al. 2016). The family member, absent in melanoma 2 (AIM2), is a cytosolic DNA sensor and also part of the inflammasome complex that results in the activation of inflammatory cytokines, including IL1β and IL18. A second component of the inflammasome, NLRP3, has undergone positive selection in the black flying fox and David's myotis, consistent with the possibility that the formation of inflammasomes is impaired in bats, which may in turn dampen the inflammatory response against pathogens (Zhang et al. 2013).

The absence of a number of natural killer (NK) cell receptors from bat RNAseq and genome data sets is also striking (Papenfuss et al. 2012; Shaw et al. 2012; Zhang et al. 2013). Genes that encode mammalian NK cell receptors are located within the leukocyte receptor complex (LRC) and the natural killer complex (NKC) of the genome. The two families have undergone convergent evolution to bind MHC-I molecules for the control of NK cell function. Genes within the LRC encode immunoglobulin (Ig)-like genes, including the killer cell Ig-like receptors (KIR), leukocyte Ig-like receptors (LILRs), and leukocyte-associated Ig-like receptors (LAIRs). Those within the NKC encode lectin-like receptors, including the Ly49 C-type lectin family. The composition of the LRC and NKC varies considerably among species. While most species have expanded either their LRC or NKC gene families, there are exceptions to this rule. In humans and nonhuman primates, the main NK cell receptors are encoded in the LRC and belong to the Ig superfamily. Rodents and horses have only expanded their Ly49 C-type lectin family of NK receptors (Kelley et al. 2005). In contrast, cattle appear to have diversified NK genes within both the NKC and LRC regions, whereas domestic dogs and four species of marine carnivores contain single copies of KIR and Ly49 genes (Hammond et al. 2009; Schwartz et al. 2017). In bats, KIRs and Ly49-like receptors appear to be absent from transcriptome and genome data sets from the black flying fox, and only a single pseudogene of Ly49 was identified in the genome of David's myotis bat (Papenfuss et al. 2012; Zhang et al. 2013). Two KIRs have been identified in the genome of the big brown bat, *Eptesicus fuscus*, but whether they are functional remains to be determined (Guethlein et al. 2015). Overall, evidence to date is consistent with the contraction of both KIR and Ly49 families of receptors in bats. Other NK cell coreceptors have been identified in bat genome and RNAseq data sets, hinting at some level of NK cell function in bats. These include the presence of CD94 and NKG2C, which form heterodimers to generate inhibitory signals. The

more divergent NKG2D, which binds MHC-I chain-related genes, MICA/B, and the UL16 binding proteins (ULBPs) in humans (Kelley et al. 2005), was also detected. Coreceptors, including CD16, CD56, and CD244, were also transcribed in the black flying fox (Papenfuss et al. 2012). The failure to identify a number of NK cell receptors in several bat species supports the hypothesis that bats may have atypical NK cell responses or use different subsets of receptors.

Characterization of Immune Genes

The availability of RNAseq and genomic data has also accelerated the characterization of a variety of immune genes and provided opportunities to examine transcription in various tissues and cells. Molecular information exists for a variety of mammalian cytokines that have been described in bats including interleukins (IL2, IL4, IL6, IL10, IL12), cytokines (TNFα, TGFβ), and IFNs (types I, II, and III) (Iha et al. 2009; He et al. 2010, 2014; Kepler et al. 2010; Zhou et al. 2011a, 2016a; Janardhana et al. 2012; Loria-Cervera et al. 2014). Detailed descriptions of pattern recognition receptors, TLRs, and RIG-I like helicases have also been reported (Iha et al. 2010; Cowled et al. 2011, 2012)

Although only a few studies have examined the nature of Ig genes in bats, a few unusual characteristics have already emerged that have been extensively reviewed elsewhere (Butler et al. 2014). The constant regions of bat Igs appear to correspond to the canonical structure and repertoire found in other eutherian mammals. Bats transcribe IgM, IgD, IgA, IgE, and multiple subclasses of IgG (Baker et al. 2010; Butler et al. 2011; Wynne et al. 2013), although some species do not have Ighδ genes and others have only a single Ighγ gene (Bratsch et al. 2011; Gerrard et al. 2017). Studies of the heavy chain variable (VH) region repertoires of black flying foxes and little brown bats (*Myotis lucifugus*) suggest bats may have the greatest number of VH gene segments among mammals (Baker et al. 2010; Bratsch et al. 2011). Furthermore, evidence from little brown bats indicates that bats may depend more on combinatorial diversity and less on somatic hypermutation (Bratsch et al. 2011). The antigen-binding region of black flying fox VH genes contains amino acids typically associated with lower antigen avidity but greater specificity (Baker et al. 2010). This, combined with the lack of evidence for somatic hypermutation, is consistent with the possibility that highly specific VH segments are encoded in the genomes of bats because of the long coevolutionary history of bats and viruses.

Functional Studies of Immune System of Bats

Innate Immune Activation of Bat Cells

The availability of cell lines from a range of different bat species has provided opportunities to study several aspects of the immune response of bat cells in vitro. This has been particularly useful for studying host–virus interactions. IFN responses

of bat cells and cell lines following stimulation with viruses and synthetic TLR ligands, including polyinosinic:polycytidylic acid (polyI:C) and bacterial lipopolysaccharide (LPS), have demonstrated that IFN production pathways are functional in bat cells and supernatant from stimulated cells has antiviral activity (Stewart et al. 1969; Omatsu et al. 2008; Crameri et al. 2009; Kepler et al. 2010; Zhou et al. 2011b). Significantly, IFNα and IFN signaling molecules, such as IFN regulatory factor 7 (IRF7), are constitutively expressed in unstimulated pteropid bat tissues and cells, consistent with the possibility that the innate immune systems of bats are at higher states of activation than other mammals, presumably allowing bats to rapidly respond to microbial infection (Zhou et al. 2014, 2016a). The constitutive expression of IFNα has been described in two species of pteropid bats (*P. alecto* and *Cynopterus brachyotis*) and is a first for any species. Curiously, fetal and kidney cell lines from a third pteropid bat species, the Egyptian rousette bat (*Rousettus aegyptiacus*), have low constitutive expression of IFNα, indicating that high baseline levels of IFNα may not be a feature of all bat species (Kuzmin et al. 2017).

The downstream signaling events triggered by IFN result in the induction of hundreds of IFN-stimulated genes (ISGs), which are responsible for the antiviral state induced by IFNs. The profile of ISGs in unstimulated bat cells and the kinetics of ISG induction following stimulation with IFN also differs from other species. Unstimulated cells from the black flying fox have higher levels of ISGs compared to human cells. The ISG profile of bat cells consists predominantly of a subset not associated with the acute inflammatory responses that often accompany elevated IFN activity (Cheon et al. 2013; Zhou et al. 2016a). Stimulation of cells from the black flying fox with IFNα also leads to the induction of novel subsets of ISGs, including ribonuclease L (RNaseL), that are not known to be induced by IFN to other species and the ISG response is elevated for a shorter period of time in bat compared to human cell lines (De La Cruz-Rivera et al. 2017; Zhang et al. 2017); RNaseL is also elevated in bats that die from experimental Tacaribe virus infection (Gerrard et al. 2017). Overall, these studies point to differences in the regulation and profile of bat ISGs as being central to the ability of bats to tolerate constitutive IFNα expression without pathology.

Consistent with the nature of the ISG response, additional evidence is also accumulating for differences in the activation of other components of the inflammatory immune response in bats. Comparison of the inflammatory cytokine production of polyI:C-stimulated cell lines from big brown bats (*E. fuscus*) and humans have demonstrated that the induction of high levels of proinflammatory cytokines, TNFα and IL8, occurs in human but not in bat cells (Banerjee et al. 2017). This result again demonstrates that bats may regulate their immune response more tightly compared to other species.

Innate Immune Responses of Bat Cells to Viruses

Experimental infections of bat cells and cell lines have also provided insight into the antiviral response of bats, revealing differences in the responses to different viruses

and between cell types. Infection of black flying fox splenocytes with the bat paramyxovirus, Tioman virus, resulted in the downregulation of type I IFNs and the upregulation of type III IFNs, indicating that type III IFNs may play an important role in the ability of bats to coexist with viruses (Zhou et al. 2011a). In contrast, henipavirus infection antagonizes type I and type III IFN production and signaling in black flying fox cells but only IFN production in human cells (Virtue et al. 2011a, b). The difference in the behavior of bat IFNs upon Tioman and henipavirus infection may reflect different IFN production mechanisms in splenocytes, which are professional immune cells, and cloned bat cells, which are predominantly fibroblast-like (Crameri et al. 2009). Infection of cells from the black flying fox with henipavirus and the Egyptian rousette bat with Ebola or Marburg results in the induction of IFNβ, but curiously no increase in IFNα has been observed, at least at the time points examined in these studies (Zhou et al. 2016a; Kuzmin et al. 2017). As described earlier, *P. alecto* has high constitutive IFNα, which may account for its low induction, but this does not appear to be the case for the rousette bat. Both Marburg and Ebola viruses, but particularly Marburg, induced a potent innate immune response in rousette cells, which was generally stronger than that in human cells. The timing of induction of IFNs and ISGs in Ebola-virus-infected cells was also delayed compared to cells infected with Marburg virus (Kuzmin et al. 2017). The natural reservoir for Marburg virus is known to be the rousette bat, but the reservoir for Ebola is unknown and believed to be another bat species. The differences in host response of rousette bat cells to the two filoviruses may therefore reflect adaptations associated with the role of this species as a natural reservoir for Marburg but not Ebola.

Although ISG responses have also been examined following viral infections in vitro, their ability to restrict viral replication has only been examined for a few ISGs (De La Cruz et al. 2017; Zhou et al. 2013). The best-characterized ISGs include Myxovirus resistance (Mx) genes and 20–50-oligoadenylate synthetase 1 (OAS1). Mx proteins are large GTPases that were initially described as inhibitors of influenza viruses and act by detecting viral replication and then trapping viral components. The OAS1 proteins are activated by dsRNA leading to the activation of Rnase L, which then degrades both cellular and viral RNA (Sadler and Williams 2008). Mx1 and OAS1 from the black flying fox have been demonstrated to be highly upregulated by pteropine orthoreovirus NB (PRV1NB) virus infection, an orthoreovirus carried by pteropid bats (Zhou et al. 2013). Furthermore, bat Mx1 proteins from Pteropidae, Phyllostomidae, and Vespertilionidae demonstrate antiviral activity against Ebola and bat influenza-like viruses. However, Thogoto virus, a tick-transmitted orthomyxovirus that is not known to infect bats, was not inhibited by bat Mx1 despite the ability of human Mx1 to inhibit Thogoto virus replication. Evidence for positive selection in two variable and surface-exposed regions of bat Mx1 genes were hypothesized to explain some of the species-specific antiviral activities of these proteins (Fuchs et al. 2017). However, antiviral activity of black flying fox RNaseL has been demonstrated against the yellow fever flavivirus, which is carried by mosquitoes, consistent with differences in specificity among different bat ISGs (De La Cruz et al. 2017).

Cell-Mediated Immunity In Vitro

Cell-mediated immune (CMI) responses are controlled by $CD8^+$ cytotoxic and $CD4^+$ helper T-lymphocyte populations and result in the killing of virus-infected cells or activation of the antibody and cytokine response. Fewer studies have examined CMI in bats. The single type II IFN, IFNγ, is produced by black flying fox bat cells stimulated with mitogens such as phytohaemagglutinin (PHA) and ConA, and recombinant bat IFNγ has antiviral activity against Semliki Forest virus and HeV in vitro (Janardhana et al. 2012). At least in vitro, IFNγ from the black flying fox appears to have activity similar to that of IFNγ from other mammals, consistent with its role in the CMI response. Curiously, in rousette bat cell lines, IFNγ is induced following infection with Marburg virus but not following infection with Ebola virus, indicating there may be differences in the CMI response induced by these two closely related viruses (Kuzmin et al. 2017). A number of earlier studies have described the in vitro responses of pteropid bats and microbats to T-cell mitogens and mixed lymphocyte responses in pteropid bats (McMurray and Thomas 1979; Chakraborty and Chakravarty 1983; Chakravarty and Paul 1987; Paul and Chakravarty 1987). Although these studies have been relatively crude due to the absence of specific reagents, they have all reported delayed responses compared with those of conventional laboratory animals. The presence of regulatory T cells was implicated in the delay in mitogenic responses of B cells in bats (Chakravarty and Paul 1987). Whether these cells are involved in the delay in T-cell-mediated immune responses observed in bats remains to be determined.

More recent studies have used proteomics to functionally characterize black flying fox MHC-I molecules and identify endogenous and viral peptide ligands. Peptides derived from bat MHC-I molecules display a relatively broad length distribution, consistent with earlier observations based on sequence information demonstrating relatively large peptide binding grooves in the bat class I molecules (Ng et al. 2016; Wynne et al. 2016). Furthermore, an unusual preference for a C-terminal proline residue was identified in endogenous and Hendra virus (HeV)-derived peptides presented by bat MHC-I molecules, consistent with the possibility that differences in antigen presentation or processing may exist in bats (Wynne et al. 2016).

Cell-Mediated Immune Responses of Bats In Vivo

Bats are capable of mounting antibody responses to viruses and model antigens, and the appearance of antibodies appears to follow the same succession as that of other mammals with the early appearance of IgM followed by IgG (Hatten et al. 1968, 1970; Chakraborty and Chakravarty 1983; Wellehan Jr et al. 2009). Although all of the Ig isotypes have been detected at the mRNA level in a variety of bat tissues, IgA protein appears to be present at surprisingly low levels in tissues and secretions from the black flying fox, which may have implications for its role in mucosal immunity in bats (Wynne et al. 2013). There are also differences in the time course, quantity, and duration of antibody responses, and questions exist over the protective

nature of antibodies in bats (Hatten et al. 1968; McMurray et al. 1982; Chakraborty and Chakravarty 1984; Davis et al. 2007; Wellehan Jr et al. 2009; Turmelle et al. 2010b). Responses to antigens such as ϕX174 bacteriophage and sheep red blood cells have demonstrated that the generation of neutralizing antibodies is delayed in the big brown bat, the pteropid bat, and the Indian flying fox (*Pteropus giganteus*) (Hatten et al. 1968; Chakraborty and Chakravarty 1984). Isotype switching from IgM to IgG also appears to be delayed in the big brown bat (Hatten et al. 1968). Despite genetic evidence for limited somatic hypermutation in the little brown bat, an increase in antibody affinity as measured by the ability of antibodies to dissociate from ϕX174 increased following secondary immunization in the big brown bat (Hatten et al. 1970).

Measures of CMI in bats have been crude relative to studies in other species and are limited to studies demonstrating T-cell-mediated inflammation to protein antigens such as purified protein derivative (PPD), PHA, and bovine serum albumin (BSA). Such skin sensitivity tests in two bat species, the common vampire bat (*Desmodus rotundus*) and Seba's short-tailed bat (*Carollia perspicillata*), immunized with PPD or BSA revealed delayed responses in both species compared to similar reactions in mice (McMurray and Thomas 1979). Lack of inflammatory responses have also been reported in most Indian flying foxes subjected to skin sensitivity tests using the contact allergen 2–4 dinitrofluorobenzene (Chakraborty and Chakravarty 1983).

Immune Responses of Bats to Experimental Viral Infections

Unlike conventional laboratory animals, few "clean" captive colonies of bats exist, and experimental infections often rely on the use of wild caught individuals, which represent a mixed population of unknown age, susceptibility, and prior viral exposure. Experimental infections have been performed on a number of species of bats using rabies virus, Australian bat lyssavirus (ABLV), Marburg, HeV, Nipah virus (NiV), Japanese B encephalitis (JE) virus, and Tacaribe virus (TCRV) (Williamson et al. 1998, 1999; Almeida et al. 2005; Davis et al. 2007; Middleton et al. 2007; Turmelle et al. 2010b; Halpin et al. 2011; Cogswell-Hawkinson et al. 2012; Paweska et al. 2012). Although the only immune parameter measured during these studies has been antibody responses, these experiments have provided valuable information on the kinetics of viral infection, the timing and duration of antibody responses and the nature of protective immunity following reinfection. With the exception of rabies virus, ABLV and TCRV, bats generally show no clinical signs of disease following infection. Neutralizing antibodies to a variety of viruses have been detected in wild caught bats, demonstrating they are capable of mounting an antibody response to viruses (Halpin et al. 2000; Lau et al. 2005; Leroy et al. 2005). The transfer of maternal antibody to pups occurs in bats, and the decline of maternal antibodies has been examined in captive black flying, variable flying foxes (*Pteropus hypomelanus*), and straw-colored fruit bats (*Eidolon helvum*) (Epstein et al. 2013; Baker et al. 2014). However, whether bats transfer maternal antibody both pre- and

postpartum and the isotypes involved is unknown. The interpretation of antibody responses in bats is extremely challenging, and, as described earlier, the nature of antibody responses in bats often differs both qualitatively and quantitatively compared to other species.

Experimental Infection of Bats with Rabies and ABLV

Rabies and ABLV are among the only viruses known to result in clinical disease in naturally infected and experimentally infected bats. However, not all bats develop disease, and the mechanisms responsible for differences in disease outcome between individuals are not understood. Evidence from experimental infections has demonstrated that even the development of neutralizing antibodies does not always provide protection from reexposure. For example, a group of wild caught bats (12 big brown bats, *E. fuscus*, and 12 Mexican free tailed bats, *Tadarida brasiliensis*) challenged by oral-nasal inoculation with rabies virus all developed antirabies neutralizing antibodies within 3 months. Rechallenge by intramuscular inoculation 6 months later resulted in an amnestic response in 21 animals, including 9 that developed clinical rabies (Davis et al. 2007). Low seroconversion rates have also been reported in big brown bats inoculated with rabies by intramuscular challenge with only 15 of 43 inoculated animals developing antibodies. This study also reported clinical disease following secondary or tertiary infections in bats that had seroconverted following primary inoculation (Turmelle et al. 2010b). Similarly, Almeida et al. (2005) described the intramuscular challenge of 40 vampire bats (*D. rotundus*) with rabies virus, of which 30 bats survived. Once again, there was no correlation between the level of neutralizing antibody and survival. Many bats that developed low or undetectable antibodies, as well as those with high antibody titers, survived infection. Infection of gray-headed flying foxes, *Pteropus poliocephalus*, with rabies or ABLV results in similar rates of mortality and seroconversion. McColl et al. (2002) reported clinical signs of disease in three of ten ABLV-infected and two of four rabies-infected gray-headed flying foxes, none of which seroconverted prior to euthanasia. Five of the ABLV-infected survivors seroconverted by 23 dpi, with titers waning by 50 dpi. One of the rabies-infected survivors also seroconverted, but not until 70 dpi (McColl et al. 2002). These studies indicate that antibodies may not provide protection and support a role for other components of the immune response in those animals that survive infection.

Experimental Infection of Bats with Other Bat-Borne Viruses

Unlike rabies and ABLV infections, clinical disease has not been reported in any bat species either naturally or experimentally infected with a variety of other bat-borne viruses, including HeV, NiV, Marburg, Ebola, and JE viruses. However, similar to rabies infection, the role of the antibody response in providing protection remains unclear, and many animals survive infection but fail to seroconvert. The

henipaviruses HeV and NiV are carried by pteropid bats. In Australia, HeV antibodies have been identified in all four species of Australian flying foxes (*P. alecto*, *P. poliocephalus*, *P. scapulatus*, and *P. conspicillatus*) (Field et al. 2001). NiV antibodies have been identified in bats from Southeast Asia and Africa. In Malaysia, two pteropid species, small flying foxes (*P. hypomelanus*) and Malayan flying foxes (*P. vampyrus*), are considered to be the reservoir hosts (Yob et al. 2001). A number of experimental infections of pteropid bat species have been performed to understand the nature of viral infection in the natural reservoir of these viruses. NiV infection of 11 gray-headed flying foxes by subcutaneous injection resulted in the production of neutralizing antibody in all individuals inoculated, but in a separate study, only 4 of 8 Malayan flying foxes that were infected by the oral-nasal route produced a neutralizing antibody response (Middleton et al. 2007; Halpin et al. 2011). Both subcutaneous and oral-nasal routes of infection have also been used for HeV inoculation of pteropid bats. Neutralizing antibody responses were detected in 10 of 20 black flying foxes inoculated oral-nasally with HeV (Halpin et al. 2011). Similarly, in gray flying foxes challenged with HeV, neutralizing antibodies were detected in two of four bats inoculated by subcutaneous injection and three of the four bats inoculated by the oral-nasal route, with none of the bats displaying clinical signs of disease (Williamson et al. 1998). A study of four gray-headed flying foxes in late gestation infected subcutaneously with HeV also described the presence of neutralizing antibodies in all four bats, and no abnormalities were observed in the fetuses or adults at necropsy (Williamson et al. 1999). In other mammals, pregnancy results in a bias in the immune response toward humoral immunity and away from CMI, which could be harmful to a fetus (Szekeres-Bartho 2002). Whether the nature of the maternal immune response facilitates greater production of antibody in infected bats during pregnancy remains to be investigated.

A natural reservoir of Marburg virus are the Egyptian rousette bats (*R. aegyptiacus*) (Towner et al. 2009), and a number of experiments have been performed to study the nature of viral transmission and infection in this species (Paweska et al. 2012; Schuh et al. 2017a, b). Marburg virus is capable of horizontal transmission between inoculated and naïve *R. aegyptiacus*. All inoculated bats seroconverted, with IgG antibodies peaking between 14–28 dpi. Marburg virus antibody titers in both inoculated and in contact bats declined within 1 month following attainment of peak levels and were undetectable after 2 months (Schuh et al. 2017a). A subsequent study revealed that bats rechallenged with Marburg virus 17–24 months following primary experimental infection developed virus-specific secondary antibody, indicative of the development of long-term protective immunity (Schuh et al. 2017b).

Clearly, additional work is needed to understand the antibody responses of bats and the nature of antibody-mediated protection against various viruses. Given what we have learned about innate immunity, particularly in pteropid bats, it is possible that innate immune mechanisms, such as IFN, reduce viral replication to low levels, delaying the generation and magnitude of an antibody response. Evidence for a highly diverse germline repertoire of antibodies and the absence of somatic hypermutation could indicate that bats have evolved a repertoire of antibodies that are

highly pathogen specific. Such antibodies may provide some level of early protection without reaching the higher titers observed in other species. Although no studies have examined the CMI responses of bats to viral infections, the generation of an IFNγ reagent for pteropid bats has been described and will assist in future studies to examine CMI in bats (Janardhana et al. 2012).

Immune Responses of Bats to Fungal Infections

Immunity to *P. destructans*

WNS is caused by a cold-loving (pyrophilic) and keratinophilic fungus (*P. destructans*) first identified in North American bats in 2006 that infects the epidermis and dermis of the muzzle, ears, and wings. Since its discovery, it has been detected in six species of North American bats, and infected populations have undergone a decline of up to 90%, with several species threatened with regional extinction within the next decade. *P. destructans* infects bats during hibernation, causing them to arouse early, leading to depletion of energy reserves and ultimately leading to a severe inflammatory response and resulting histopathology. The fungus is widely distributed in North America and Europe and has recently been found in Asia (Hoyt et al. 2016). Although naturally infected European bats also develop histopathological lesions in response to *P. destructans*, no mass mortality is observed in European or Asian bats (Zukal et al. 2016). Similar to the situation with viruses, the long coevolutionary relationship of European and Asian bats with *P. destructans* has presumably led to an equilibrium between the host and pathogen. In the longer term, this may also evolve in North American bats, and evidence of some level of resistance has been reported in some populations (Langwig et al. 2017). However, the rate of mortality among some bat species is too high to ignore. Understanding the host–pathogen relationship and the genes and pathways associated with disease tolerance and resistance will be important for identifying viable treatments and assessing the immune responses of bats to drugs or vaccines.

Earlier reports describing the immune response of bats during hibernation indicate that, like other hibernating mammals, their immune responses are suppressed during torpor when they are initially infected with *P. destructans*. For example, hibernating *E. fuscus* bats maintained at 8 °C fail to generate antibodies in response to infection with JE virus (Sulkin et al. 1966). In addition, activation plasma complement against bacteria (*Escherichia coli*, *Staphylococcus aureus*) and fungi (*Candida albicans*) is lower in hibernating little brown bats compared to nonhibernating bats (Moore et al. 2011).

Several studies have now begun to examine the host response of bats to *P. destructans* to determine the level of immune activation that occurs during torpor and after arousal. *P. destructans* begins to colonize bat skin during hibernation, yet visible signs of inflammation are characteristically absent in torpid animals, and neutrophils and macrophages are absent from sites of pathogen invasion in hibernating bats with WNS. In little brown bats, overt skin damage does not occur until

2–3 weeks after bats have emerged from hibernation with intense neutrophilic inflammation associated with invasive *P. destructans* infection (Meteyer et al. 2012). Studies of bats from WNS-affected and unaffected sites have also demonstrated significantly higher circulating leukocyte counts in WNS-affected bats with elevated body temperatures (above 20 °C). The latter is consistent with the mobilization of cells associated with arousal from torpor and euthermia (Moore et al. 2013). The absence of neutrophil and T-cell infiltration has been confirmed through RNAseq analysis of WNS-infected little brown bat wing tissues during hibernation (Field et al. 2015). Despite the absence of neutrophil invasion, increases in gene expression for inflammatory cytokines have been detected in wing tissues from hibernating WNS infected bats compared to hibernating bats not affected by WNS. These include IL1β, IL6, IL17C, IL20, IL23A, IL24, and G-CSF and chemokines, such as Ccl2 and Ccl20. Hibernating little brown bats exhibiting visible fungal infections elevated levels of transcripts for proinflammatory cytokines, IL23 and TNFα, the anti-inflammatory cytokine IL10, and the antimicrobial peptide cathelicidin in lung tissue compared to hibernating uninfected bats (Rapin et al. 2014). Overall, these studies are consistent with the induction of an innate antifungal response in WNS-infected bats prior to emergence from hibernation followed by infiltration of immune cells and, presumably, activation of adaptive immune responses following arousal. Overactivation of the immune response following arousal from torpor, combined with a depletion of energy reserves, appears to be the main cause of mortality.

Immunity to Other Fungal Pathogens

In contrast to *P. destructans*, bats are known to carry other fungal pathogens, such as *Histoplasma capsulatum*, without disease. *H. capsulatum* is a pathogenic fungus that causes pulmonary and systemic infections in humans. Bats are considered to be the main reservoir of this fungus, and it is commonly found in bat guano (Taylor et al. 2005). Although bats are susceptible to infection, mortality is rare in bats that are inoculated intranasally, which is the most likely route of natural infection. Higher mortality rates are observed in bats inoculated intraperitoneally (McMurray and Greer 1979; Greer and McMurray 1981). Great fruit eating bats (*Artibeus lituratus*) respond to infection with the generation of complement fixing antibodies by 3 weeks and precipitating antibodies by 5 weeks post infection (McMurray and Greer 1979). Natural infection rarely results in disease, indicating that, similarly to the situation with most viruses, bats have likely evolved mechanisms to control infection, at least under conditions where they are infected under nontorpid conditions.

Future Directions

The field of bat immunology is very much in its infancy, and significant opportunities exist for future research. Thanks to advances in technology, such as whole-genome sequencing and RNAseq, considerable progress has been made, in particular with regard to our understanding of the immune system of the black flying fox, *P. alecto*. However, as bats are a highly diverse group of mammals that have evolved independently for a long period of time, it is possible that different immune mechanisms exist between the two suborders and across species. There is likely much more to be learned from comparative studies across different bat species.

Comparative genomics of bats have provided important clues to the adaptations that may allow bats to coexist with viruses in the absence of disease. These include evidence for positive selection on a variety of immune genes and differences in the repertoires of NK cell receptors. Additional genomic data, including long read assemblies, will be required to resolve highly repetitive regions such as the LRC and NKC to confirm the absence of important receptor families and to resolve other repetitive regions of the bat immunome. A number of genomic regions also remain largely unexplored, partly owing to their repetitive nature. These include B- and T-cell receptor (BCR and TCR) regions. Examining the repertoire and diversity of these regions will provide opportunities to examine their functional activities and importance. For example, no information exists on the repertoire of TCRs in bats and the relative importance and roles of αβ and γδ T cells. Observations from genomic data sets pave the way to further addressing the role of different components of the immune system in the responses of bats to infection. The mechanisms involved in TCR and BCR diversification also remain unknown. The roles of terminal deoxynucleotidyl transferase (TdT), recombination activating gene (RAG), and activation-induced cytidine deaminase (AID) on recombination, somatic hypermutation, gene conversion, and class switching remain to be explored.

A number of important differences in the innate immune system have also been identified in bats that are at odds with the responses in humans and other species. In particular, the constitutive activation of IFNα in the black flying fox is striking. In other mammals, constitutive IFN expression can have implications for inflammation and autoimmunity. Identifying the mechanisms responsible for the ability of bats to tolerate high levels of IFN in the absence of inflammation has significant potential for identifying novel therapeutics to treat viral diseases in humans and other species. To this end, functional characterization of the different subsets of ISGs already identified in both unstimulated and stimulated cells would provide valuable insights into the mechanisms responsible for the control of viral infection in the absence of inflammation. Additionally, dissection of the signaling pathways responsible for the control of IFN response will contribute to our understanding of differences in the regulation of IFN in bats compared to other species.

As described earlier, a number of functional differences have been identified in the immune system of bats compared to other species. These include the nature of cell-mediated and antibody responses of bats. To advance our understanding of the nature of these responses, appropriate bat-specific reagents will be required. Some

commercially available human and mouse antibodies generated against highly conserved intracellular proteins are cross reactive with bat proteins and have already proven useful (Zhou et al. 2016b). A handful of bat-specific antibodies have also been generated (Janardhana et al. 2012; Wynne et al. 2013). Additional reagents will be necessary to advance the field, including monoclonal antibodies for use in flow cytometry, immunohistochemistry, and ELISAs, to dissect the roles of different cell types, including B and T cells, dendritic cells, and macrophages. Reagents will also be required to examine the responses of various cytokines to examine proinflammatory and anti-inflammatory pathways for comparison to other species and to answer specific questions, including the confirmation of cytokine expression at the protein level (e.g., IFNα to confirm its constitutive expression). Recombinant cytokines and growth factors will also be important for examining the responses of cells to cytokine stimulation and the expansion of specific subsets of antigen-specific lymphocytes. Lastly, the development of closed breeding colonies of bats will be essential in progressing research into immunity in bats, overcoming the issues associated with wild caught individuals of unknown age and history of infection.

Conclusions

Renewed interest in bat immunology emerged following the identification of bats as reservoirs for a number of viruses, including SARS-CoV and Ebola, that are highly pathogenic in other species. Prior to the emergence of these viruses, few studies had examined any aspect of bat immunology. A number of important observations have already been made through studies of the immune systems of bats, with evidence for adaptations not observed in any other species. Significant progress has now been made in the identification of genes and pathways associated with immunity, and one of the recurring themes that is emerging with regard to viral infections is the ability of bats to control inflammatory responses. Regulation of the immune system is likely an important mechanism for preventing pathology associated with infection. However, bats are an extraordinarily diverse group of mammals, and the adaptions identified to date may not apply across all bat species. In contrast to the apparent regulation of the immune response during viral infections, uncontrolled inflammatory responses due to infection with pathogens such as WNS clearly demonstrate that bats are capable of overactivating their immune system, causing immunopathology. As described earlier, there are still gaps in our understanding of the immune systems of bats, and significant opportunities exist. Studies of bat immunology provide opportunities to identify novel mechanisms that could be applied to redirecting the immune system of other species to prevent disease and to the conservation of bats affected by pathogens such as WNS.

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