

SPECIAL ISSUE – BATS

Antiviral Immune Responses of Bats: A ReviewM. L. Baker¹, T. Schountz² and L.-F. Wang¹¹ CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Vic., Australia² School of Biological Sciences, University of Northern Colorado, Greeley, CO, USA**Impacts**

- This manuscript represents the first review of bat immunology published in almost four decades.
- We summarize the current literature on bat innate and adaptive immune responses to viral infections, highlighting the need for further research.
- Understanding how bats coexist with viruses has important implications for predicting spillover events from bats to other susceptible species and has the potential to lead to the development of therapeutics to treat viral infections in other mammals including humans.

Keywords:

Bats; Chiroptera; viral infection; innate immunity; adaptive immunity

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Summary

Despite being the second most species-rich and abundant group of mammals, bats are also among the least studied, with a particular paucity of information in the area of bat immunology. Although bats have a long history of association with rabies, the emergence and re-emergence of a number of viruses from bats that impact human and animal health has resulted in a resurgence of interest in bat immunology. Understanding how bats coexist with viruses in the absence of disease is essential if we are to begin to develop therapeutics to target viruses in humans and susceptible livestock and companion animals. Here, we review the current status of knowledge in the field of bat antiviral immunology including both adaptive and innate mechanisms of immune defence and highlight the need for further investigations in this area. Because data in this field are so limited, our discussion is based on both scientific discoveries and theoretical predictions. It is hoped that by provoking original, speculative or even controversial ideas or theories, this review may stimulate further research in this important field. Efforts to understand the immune systems of bats have been greatly facilitated in recent years by the availability of partial genome sequences from two species of bats, a megabat, *Pteropus vampyrus*, and a microbat, *Myotis lucifugus*, allowing the rapid identification of immune genes. Although bats appear to share most features of the immune system with other mammals, several studies have reported qualitative and quantitative differences in the immune responses of bats. These observations warrant further investigation to determine whether such differences are associated with the asymptomatic nature of viral infections in bats.

Introduction

More than a fifth of the nearly 5000 known species of mammals are bats (Teeling et al., 2005), second only to rodents. The order Chiroptera is divided into two suborders: the Megachiroptera and Microchiroptera. These two lineages are estimated to have diverged approximately 58 million years ago (Teeling, 2009). Megachiroptera consists

of a single family, the old world fruit bats, while Microchiroptera includes 17 families of echo-locating bats. Much is known about the ecology and physiology of bats; however, virtually nothing is known about their immunology (Kunz and Fenton, 2003; Calisher et al., 2006). This is principally because of a historical lack of interest in bats from the infectious diseases community and funding agencies because they were thought to be of

little importance as vectors or reservoirs. Much of our knowledge of bats and viruses is from studies of rabies virus and other lyssaviruses (Calisher et al., 2006). However, in recent years, many novel viruses of human and veterinary importance have been discovered that are hosted, or suspected to be hosted, by bats. In the 1990s, novel paramyxoviruses, Hendra and Nipah viruses, caused outbreaks of fatal disease in Australia and Malaysia (Murray et al., 1995; Chua et al., 2000). Both viruses are hosted by species of pteropid bats. Severe acute respiratory syndrome, caused by a coronavirus, was identified during an outbreak in China and Hong Kong in the early 2000s. Subsequent research has indicated its ancestor is a bat-borne virus (Lau et al., 2005; Li et al., 2005). Also in the 2000s, Marburg virus was demonstrated to be hosted by fruit bats, and there is compelling evidence that ebolaviruses are also hosted by fruit bats (Leroy et al., 2005; Towner et al., 2009). In addition, Melaka and Kampar viruses and related bat reoviruses from Malaysia are associated with respiratory disease in humans (Chua et al., 2007, 2008, 2011). Currently, more than 100 viruses have been isolated from or detected in bats. The diversity of viruses found in bats is matched only by rodents that are the most abundant and diverse group of mammals and are reservoir hosts to a large number of viruses that also cause disease in humans and other species (Meerburg et al., 2009).

It is now clear that bats have been substantially underappreciated as reservoirs of viruses important to human and veterinary health (Calisher et al., 2006; Wang et al., 2011). The peridomestic nature of some bat species and the encroachment of humans upon bat habitats make it likely that new human pathogens from bats will be discovered after spillover events. Bats have often been vilified by much of the public and have been frequently targeted for extermination, despite their critical roles in many ecosystems. Because of this, many bat biologists, whose help laboratory scientists will need to answer many questions about bats, are often reluctant to engage or assist in biomedical research. In addition, obtaining bats for biomedical research is extremely challenging. Most captive colonies are maintained by zoos, which are unwilling or unable to donate their excess animals for such research because of long-standing policies. The establishment of colonies from wild bats is also problematic because they require specialized housing and diets. Moreover, the animals must be evaluated for possible pathogens, including rabies virus. Therefore, most research is conducted on wild bats in their natural environments, or captured animals in laboratory settings, which introduces additional problems with housing, diet and occupational health (e.g. animal stress and rabies immunization for personnel). This practice severely limits experimental examination of viruses hosted by bats.

Although bats may be persistently infected with many viruses, evidence from experimental and naturally infected bats has demonstrated that they rarely display clinical symptoms (Sulkin et al., 1966; Swanepoel et al., 1996; Williamson et al., 1998, 2000; Leroy et al., 2005, 2009; Middleton et al., 2007; Towner et al., 2009). Experimental infection of bats has included viruses such as Hendra and Nipah viruses that are known to result in high disease mortality in other mammals including humans. These studies have confirmed the virulence of the viruses used for experimental infections using conventional laboratory mammals such as guinea pigs that succumb to the same dose of virus infection that bats respond to in the absence of disease (Williamson et al., 2000; Middleton et al., 2007). The only viruses that have been demonstrated to cause clinical signs of disease in bats are rabies virus and the closely related Australian bat lyssavirus (Field et al., 1999; McColl et al., 2002). However, results of experimental infections are inconsistent, with only a small proportion of bats succumbing to infection (McColl et al., 2002). In addition, very few viruses have been shown to have negative impacts on natural bat populations. One exception is Tacaribe virus, an arenavirus closely related to the South American haemorrhagic fever viruses, which caused the deaths of many artibeus bats in Trinidad in the 1950s (Downs et al., 1963) and in experimental infections (Cogswell-Hawkinson et al., 2012). It is unknown whether this virus is still circulating or what impact it may have on artibeus bats today. Overall, these results demonstrate that for the most part, bats are able to coexist with viruses and may have evolved mechanisms to control viral replication more effectively than most other mammals. A similar situation exists in rodents that also show limited or no signs of disease in response to the viruses they harbour (Fulhorst et al., 1999; Botten et al., 2000).

All viruses must evade the immune response for a sufficient period of time to allow transmission to other susceptible hosts, and many viruses possess immunomodulating genes that provide a competitive advantage over the immune response. Much of what has been learned about immune responses has been based upon pathology models; however, many zoonotic viruses have coadapted with their vertebrate hosts to cause persistent, apathogenic infections. Because bats have not been examined in great detail, and because there are so many species of bats, virtually nothing is known about the role of their immune responses in control of viral infections, nor how viruses manipulate the immune responses of bats. Fortunately, much of what is known about other natural zoonotic virus-reservoir relationships, particularly rodent hosts (Fulhorst et al., 1999; Easterbrook et al., 2007; Schountz et al., 2007), and the availability of novel and increasingly less-expensive deep sequencing methods

(Glenn, 2011) makes the study of specific bat reservoirs and viruses highly tractable. Furthermore, the recent availability of partial genome sequences has provided important resources to study various aspects of bat biology, including genes associated with the immune system. Two bat genomes have been sequenced as part of the US National Institutes of Health funded Mammalian Genome Project, one from the megabat *Pteropus vampyrus* and a second from the microbat *Myotis lucifugus*. Although both bat genomes are low coverage (2.6× for *P. vampyrus* and 1.7× for *M. lucifugus*), these projects have an important role to play in revealing the mechanisms that have evolved to allow bats to remain asymptomatic when infected by so many viruses.

Immune Cell Populations

Although few bat-specific reagents exist to identify specific cell types in bats, a variety of cells have been described based on morphological and physiochemical characteristics, demonstrating the presence of similar populations of cells in bats to other mammals. Macrophages, B cells and T cells have been identified in the spleen and lymph nodes from the Indian fruit bat (*Pteropus giganteus*) using scanning electron microscopy and cellular adherence properties. These cells displayed similar characteristics to those from other mammals including humans and mice. In *P. giganteus*, the ratio of macrophages/B cells/T cells was 1 : 2 : 9, similar to that of mice in which the ratio was approximately 1 : 1 : 8 (Sarkar and Chakravarty, 1991). A variety of immune cells including lymphocytes, neutrophils, eosinophils, basophils and macrophages have also been identified by morphology in histological sections from the Brazilian free tailed bat (*Tadarida brasiliensis*) following injection of the T-cell mitogen, phytohaemagglutinin (PHA) (Turmelle et al., 2010a).

Cells resembling follicular dendritic cells (FDCs) have also been described in *P. giganteus* (Sarkar and Chakravarty, 1991). Follicular dendritic cells are capable of capturing and retaining antigen in the form of immune complexes that can persist for months or even years and are important for the induction and maintenance of memory immune responses (Mandels et al., 1980; Tew et al., 1980). Evidence for the ability of some viruses to retain infectivity when complexed within FDCs has been demonstrated (Keele et al., 2008). However, whether FDCs play a role in the persistence of viral infections in bats awaits further investigation.

Innate Immunity

One hypothesis for the ability of bats to remain asymptomatic to viral infection is that they are able to control

viral replication very early in the immune response through innate antiviral mechanisms. The recent description of a variety of innate immune genes in bats provides the first step in understanding the role of the innate immune system in antiviral immunity in bats.

Pattern recognition receptors

The recognition of pathogens by pattern recognition receptors (PRRs) including toll-like receptors (TLRs) and retinoic acid inducible gene-like helicases (RLHs) provides the first line of defence against infection (Xiao, 2009). Toll-like receptors have been described in two species of fruit bats, *Pteropus alecto* and *Rousettus leschenaultia* (Iha et al., 2009; Cowled et al., 2011). Not surprisingly given the role of TLRs in the recognition of conserved molecular patterns, the bat TLRs were highly conserved between bats and other mammals (Iha et al., 2010; Cowled et al., 2011). Evidence from *P. alecto* for the presence of transcripts corresponding to TLRs 1–10 and 13 provides evidence that bats are capable of recognizing a range of pathogens including viruses, bacteria and fungi. However, the *P. alecto* TLR13 transcript contained stop codons within its open reading frame and may represent a transcribed pseudogene (Cowled et al., 2011). To date, the only other mammals in which TLR13 has been identified are rodents. Although the ligand for TLR13 is unknown, knockdown of TLR13 in mouse cells results in greater susceptibility to vesicular stomatitis virus (VSV), indicating it likely has a role in viral recognition (Shi et al., 2011). The transcription of a TLR13 pseudogene in pteropid bats may indicate that this gene has only recently undergone inactivation and at one time may have encoded a functional protein involved in viral sensing and may still be intact and functional in other bat species. The cytoplasmic RLHs, retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (mda5) and laboratory of genetics and physiology 2 (LGP2) have also been described in *P. alecto*, providing evidence for a similar repertoire of RLHs in megabats to other mammals (Cowled et al., 2012). Overall, these reports provide evidence for the presence of the two major families of virus sensing PRRs in bats consistent with recognition of a similar range of pathogens to other species of mammals.

Interferon and signalling molecules

The interferon (IFN) response represents a potent first line of defence against viral infection conferring cells with an 'antiviral state' and preventing the spread of viral infection (Randall and Goodbourn, 2008). The IFN signalling and production pathway is therefore a logical

starting point in understanding the asymptomatic nature of viral infection in bats. Three classes of IFN have been identified, designated types I, II and III, which differ in their amino acid sequences and the receptor complex they signal through (Pestka et al., 2004; Schroder et al., 2004). Type I (including α and β) and III (λ) IFNs are induced directly in response to viral infection and thus play an important role in innate immunity. Although they differ in the receptor complex they signal through, type I and III IFNs result in the induction of an overlapping set of IFN stimulated genes (ISGs) that are responsible for the antiviral activity of IFNs (Sadler and Williams, 2008). Type I IFNs have been described in three species of fruit bats, *Rousettus aegyptiacus*, the Malaysian flying fox, *P. vampyrus*, and the Greenish Naked-backed fruit bat, *Dobsonia viridis*, and from the microbat, *M. lucifugus* (Omatsu et al., 2008; He et al., 2010; Kepler et al., 2010). In humans and mice, there are 13 and 14 IFNA genes, respectively, but in bats, only seven IFNA genes have been identified in the *P. vampyrus* genome and only IFNA pseudogenes have been identified in the *M. lucifugus* genome sequence (van Pesch et al., 2004; Kepler et al., 2010). However, as both currently available bat genomes are low coverage genome sequences, it is possible that some members of the type I IFN family are absent, despite the sequences being inferred from the unassembled trace archives that should contain a broader representation of the genome than the assembly. He et al. (2010) described the cloning of seven IFNA subtypes and one pseudogene from *D. viridis* with evidence for positive selection among this gene family. Both *M. lucifugus* and *P. vampyrus* also appear to have expanded the IFNW family of genes, with up to a dozen IFNW members in each species of bat. Humans have only a single functional IFNW family member and at least two pseudogenes, and mice have a single IFNW pseudogene (Hardy et al., 2004; Kepler et al., 2010). However, this family has expanded in cats that have 13 IFNW subtypes and in cattle that have 24 potentially functional IFNW genes (Yang et al., 2007; Walker and Roberts, 2009). Furthermore, cat (*Felis catus*) IFNW has been implicated in protection against parvovirus infection (Paltrinieri et al., 2007). Thus, the expansion of the IFNW family in bats may also have implications for antiviral immunity. Type III IFNs have also been identified in the *M. lucifugus* genome with the identification of a single full-length IFNL locus (Fox et al., 2009). In the pteropid bat *P. alecto*, two IFNL genes (IL28a and IL29) and the two chains of the type III IFN receptor complex (IL10R2 and IFN λ R1) have been characterized, and IFN λ R1 has been demonstrated to act as a functional receptor (Zhou et al., 2011a,b). Furthermore, unlike the type III IFN receptor of mammals such as mice and humans, in *P. alecto*, the type III IFN receptor displays a

wide tissue distribution consistent with a more significant role for the type III IFNs in antiviral immunity in bats (Sommereyans et al., 2008; Witte et al., 2010; Zhou et al., 2011a).

Pteropid bat cells and cell lines readily secrete IFN in response to stimulation with synthetic TLR ligands including polyinosine-polycytidylic acid (polyIC) and lipopolysaccharide (LPS), demonstrating that IFN production pathways are functional in bat cells (Stewart et al., 1969a; Cramer et al., 2009; Kepler et al., 2010; Zhou et al., 2011b). Bats also demonstrate an IFN response following viral infection *in vivo* and *in vitro*. The earliest work on IFN production in bats described the detection of IFN in spleen and brain tissues from microbats (*T. brasiliensis*) infected with Japanese B encephalitis (JE) virus. Although IFN was detected in both tissues during the first week of infection, it was detected only in brain tissue during the second week of infection despite the presence of virus in both tissues (Stewart et al., 1969a,b). The persistence of the virus in certain populations of cells even in the presence of IFN has been speculated to reflect the presence of populations of cells that may be insensitive to the action of IFN (Sulkin and Allen, 1974). *In vitro* studies have demonstrated the induction of IFNB in VSV-infected peripheral blood mononuclear cells (PBMCs) from *P. vampyrus*, demonstrating a delay in the IFN response in comparison with stimulation with the TLR ligands, polyIC or LPS, a result that is consistent with the mechanisms of IFN signalling of other mammals (Kepler et al., 2010). Recently, evidence for differences in the IFN responses of bats and the ability of viruses to evade the IFN response of bat cells have also been described. In *P. alecto* splenocytes, type I and III IFNs appear to be differentially induced following infection with the bat-borne paramyxovirus Tioman virus with type I IFNs downregulated and type III IFNs upregulated following viral infection (Zhou et al., 2011b). In contrast, henipavirus infection antagonized both type I and III IFN production in human cell lines and IFN production and signalling in pteropid bat cell lines (Virtue et al., 2011a,b; Zhou et al., 2011b). The ability of viruses to antagonize both the IFN signalling and production pathways in bat cells is intriguing and may indicate that factors other than IFN play a key role in antiviral immunity in bats. Thus, these results demonstrate not only differences in the IFN response following infection with different viruses but also differences between bats and humans which may be significant in terms of the ability of bats to control viral replication.

Few studies have examined the IFN signalling pathway following IFN production to determine the ability of IFNs to induce an 'antiviral state' in bat cells. IFNs exert their antiviral actions through binding to cell surface

receptors, which in turn activate the JAK-STAT pathway. Activation of the receptor-associated Janus family of tyrosine kinase enzymes results in the phosphorylation of latent cytoplasmic signal transduction and activator of transcription (STAT) family of transcription factors. The phosphorylated STAT1 and STAT2 dimerize to interact with IFN regulatory factor 9 and translocate to the nucleus resulting in ISG production and the induction of an antiviral state (Samuel, 2001). The STAT1 protein is the only component of this signalling pathway that has been characterized in bats. STAT1 is present in the Egyptian fruit bat, *R. aegyptiacus*, and is phosphorylated and translocated to the nucleus following stimulation with human IFNA consistent with its activation in a similar manner to other mammals. Rabies virus infection of human and bat cells antagonizes STAT1 function, resulting in failure of STAT1 to be translocated to the nucleus (Brzózka et al., 2006; Fujii et al., 2010). Overall, this study demonstrated that the STAT1 signalling pathway in *R. aegyptiacus* cells is similar to that of other mammals. Further characterization of other signalling molecules involved in the IFN response will play an important role in understanding the nature of innate antiviral immunity in bats.

The ability of IFN to induce an antiviral state through the induction of ISGs is the hallmark of the IFN response (Sadler and Williams, 2008). Stewart et al. (1969a) used IFN containing supernatant prepared from polyIC stimulated *T. brasiliensis* embryo cells to compare the antiviral activity of bat IFN with IFNs prepared from cells from other species. This study demonstrated each species of IFN has a characteristic spectrum of antiviral activity. Although bat IFN displayed antiviral activity within a similar range to other species, this study did not examine the effect of bat IFN on any bat-borne viruses. More recently, recombinant *P. alecto* type III IFN demonstrated antiviral activity against the bat-borne orthoreovirus, Pulau virus (Zhou et al., 2011b). The induction of ISGs has also been demonstrated in bats with bat type III IFN, resulting in the induction of ISG56 and RIG-I production in bat cell lines (Zhou et al., 2011a). Pteropid bat cell lines also produce ISG54 and ISG56 following stimulation with universal type I IFN that is an IFNA hybrid constructed from recombinant human IFNA A/D (Virtue et al., 2011a). The induction of 2',5'-oligoadenylate-synthetase 2 (OAS2) has also been detected in *P. vampyrus* PBMCs following infection with VSV or stimulation with PolyIC or LPS. The results of this study demonstrated a higher induction of OAS2 by VSV compared with either PolyIC or LPS (Kepler et al., 2010). These results provide evidence that the signalling molecules downstream of the IFN response are likely similar in bats to other mammals.

Complement activity

The complement cascade kills foreign microbes by disrupting the microbial plasma membrane following activation through the binding of complement to antibodies that have attached to microbial surfaces (Prodinger et al., 2003). A variety of assays have been used to measure complement activity in bats to assess immunity under various environmental conditions. A comparison of complement activity in three microbats (*Eptesicus fuscus*, *M. lucifugus* and *T. brasiliensis*) and one megabat (*P. vampyrus*) demonstrated higher levels of complement activity in the microbats by immune haemolysis but not by immune adherence. Furthermore, complement activity in *Eptesicus* microbats was relatively insensitive to changes in temperature above or below 37°C, whereas activities of guinea pig and pteropid bat complements decreased at temperatures above or below 37°C (Hatten et al., 1973). The ability to maintain complement activity may be a biological necessity in hibernating animals such as microbats where body temperatures can vary extensively and over prolonged periods of time. Allen et al. (2009) used complement activity as a measure of bactericidal activity in *T. brasiliensis* bats, demonstrating variation in activity with roosting ecology, providing evidence for the influence of environmental factors on immune function.

Adaptive Immunity

Studies of bat adaptive immunity have provided evidence for the presence of both antibody and cell-mediated immunity in bats. However, several reports have demonstrated qualitative and quantitative differences in adaptive immune responses and in the generation and maintenance of immunological memory. These findings warrant further investigation to determine the relevance of these findings to the maintenance of viruses in bats.

Immunoglobulins

Butler et al. (2011) demonstrated that four species of bats, including one megabat (*Cynopterus sphinx*) and three microbats (*Carollia perspicillata*, *M. lucifugus* and *E. fuscus*) transcribe IgM, IgE, IgA and multiple IgG classes, the latter of which appears to have diversified after speciation as in other mammals. Serum fractionation using normal serum from the neotropical species, *Artibeus lituratus* and from *P. giganteus* has confirmed that bat IgM, IgG and IgA are homologous to corresponding human immunoglobulins (McMurray et al., 1982; Chakravarty and Sarkar, 1994). However, evidence so far indicates that IgD may be unique to microbats, with IgD present at the genomic and transcriptional level in *M. lucifugus* but not in the

megabats. IgD is an apparently ancient isotype and has a spotty distribution among vertebrates (Ohta and Flajnik, 2006). Among mammals, IgD was once believed to be present only in humans and rodents until its more recent identification in various classes of animals including artiodactyls and monotremes (Zhao et al., 2002, 2009). Although a broader survey of megabats will be required to rule out the presence of IgD in this group, given that IgD is not present in all mammals, it may not be surprising to find that megabats have lost this immunoglobulin isotype.

Immunoglobulin genes are assembled by recombination of germline-encoded gene segments: variable (V), diversity (D) and joining (J) for heavy (H) chains and V and J for light (L) chains. Variation in the amino acid residues at the N terminal ends that are encoded by the V regions of both H and L chains contribute to antibody diversity and establishes antibody specificity (Max, 2003). To obtain insight into the antigen-binding capability and specificity of bat antibodies, several studies have examined the diversity of the VH regions of bat immunoglobulin genes. Evidence from both megabats and microbats has demonstrated a highly diverse antibody repertoire, exceeding that of most of species and on par only with humans and mice (Baker et al., 2010; Bratsch et al., 2011). In the pteropid bat, *P. alecto*, the amino acid sequence composition of the antigen-binding site of the expressed VH region is enriched in arginine and alanine residues and has a lower proportion of tyrosines compared to other mammals (Baker et al., 2010). Tyrosines are directly involved in antigen binding and confer structural diversity, while arginines have been reported to be detrimental to antigen binding and may contribute to self-reactivity (Radic et al., 1993; Birtalan et al., 2008). Whether these characteristics are associated with differences in antigen-antibody interactions in bats awaits further functional characterization. However, differences in antigen binding may help to explain previous observations of the simultaneous presence of virus and antibody in bats (Sulkin et al., 1966).

Comparison of the germline and expressed VH repertoire of *M. lucifugus* has revealed a very low mutation rate consistent with the possibility that this species relies on combinatorial and junctional diversity rather than somatic hypermutation (Bratsch et al., 2011). All other mammals studied thus far use post-combinatorial mechanisms to fine tune their antibody repertoire resulting in antibodies that recognize fewer epitopes per antigen but do so with greater specificity and affinity. This result may provide evidence that bats rely solely on combinatorial mechanisms. However, as this study focused only on VH sequences expressed with IgG in a single *M. lucifugus*, further work is required to confirm this

result across multiple individuals and species using all of the immunoglobulin subclasses.

Antibody-mediated immune responses

The effector functions mediated by antibodies include neutralization, precipitation, agglutination, opsonization, antibody-dependent cellular cytotoxicity and the activation of the classical complement pathway. Neutralizing antibodies to viruses including Hendra virus, ebolaviruses and SARS-like CoV have been detected in wild-caught bats, demonstrating that bats are capable of mounting an antibody response (Halpin et al., 2000; Lau et al., 2005; Leroy et al., 2005). Some of the earliest experiments performed on bat immune systems were measuring antibody responses. Early studies of antibody responses in bats were consistent with differences in both the kinetics and magnitude of antibody responses compared with other mammals. Several studies have used model antigens such as sheep red blood cells (SRBCs), ϕ X174 bacteriophage and 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) to compare the nature of the antibody response of bats with that of conventional laboratory animals (Hatten et al., 1968, 1970; Chakraborty and Chakravarty, 1984; Wellehan et al., 2009). Hatten et al. (1968) reported that the magnitude and duration of the neutralizing antibody response of big brown bats (*Eptesicus fuscus fuscus*) maintained at 24 and 37°C to immunization with ϕ X174 bacteriophage was lower than that of guinea pigs and rabbits. A delay in attaining a peak in the primary antibody response was also reported in pteropid bats immunized with SRBCs (Chakraborty and Chakravarty, 1984). Secondary responses also appeared to be slower or non-existent. A more pronounced IgM response was observed in *E. fuscus*, and the appearance of IgG appeared to be slower supporting poor isotype switching (Hatten et al., 1968). Secondary immunization with ϕ X174 bacteriophage has demonstrated an anamnestic response only in bats housed at 24°C but not at 37°C (Hatten et al., 1968, 1970). However, evidence for an increase in the affinity of antibodies for ϕ X174 has been reported in *E. fuscus* (Hatten et al., 1970). Clearly, further work is needed to understand the nature of antibody responses in bats. However, overall these studies demonstrate differences in both primary and secondary antibody responses in bats compared to conventional laboratory mammals.

Experimental infections and vaccinations have also been performed in bats to provide information on the kinetics and nature of antibody responses to viruses. Consistent with the results obtained from bats immunized with ϕ X174 or SRBC antigens, vaccination and experimental viral infections have provided evidence for

quantitative and qualitative differences in antibody responses in bats compared with other mammals. In addition, results from experimental infections appear to vary between species and viral infections. Big brown bats (*E. fuscus*) experimentally infected with JE virus generally demonstrate a neutralizing antibody response within 20 days of infection. However, these studies have failed to detect evidence of complement fixation (CF) or haemagglutination (HI) by JE virus antigen (Sulkin et al., 1966; Leonard et al., 1968). As CF and HI responses were demonstrated in guinea pigs and rabbits during these experiments, the failure to detect a response in bats was considered to reflect a difference in the host antibody response rather than the assay. Experimental infection of neotropical bats with Venezuelan equine encephalitis virus resulted in a high HI and neutralizing antibody response in artibeus fruit bats but low or undetectable response in *Phyllostomus discolor* (Seymour et al., 1978). Furthermore, bats exposed to prolonged periods of cold (8°C) likely to be encountered during hibernation failed to develop an antibody response to JE virus despite the persistence of the virus in various tissues but developed detectable antibody within 1 week following transfer from 8 to 24°C (Sulkin et al., 1966). These results are consistent with the ability of bats to maintain viruses, which are likely biochemically inert for long periods of time under states of immunosuppression.

The ability of antibody to provide long-lasting protection is one of the hallmarks of the adaptive immune response. Vaccination of bats against rabies virus appears to confer resistance to challenge compared to unvaccinated bats that succumb to disease. However, several studies have demonstrated that vaccinated bats are capable of clearing viral infection even in the absence of detectable neutralizing antibody (Seymour et al., 1978; Sétien et al., 1998; Aguilar-Setien et al., 2002; Turmelle et al., 2010b). Although these studies provide evidence that bats develop protective immunity following vaccination, the failure to detect an antibody response in some bats is striking and may indicate that the nature of protective immunity in bats differs from other mammals. Evidence for viral recrudescence has also been reported in a captive *P. vampyrus*, which displayed changes in neutralizing antibody to Nipah virus, providing evidence of the maintenance of virus in bats in a manner that does not sustain an antibody response. One individual was initially seropositive, became seronegative within 1–2 months and remained seronegative for 11 months before displaying a gradual increase in neutralizing antibody and viral excretion (Sohayati et al., 2011). These results demonstrate that failure to detect specific antibodies may be insufficient evidence for excluding prior exposure. However, as this event was observed in only

one individual, the significance of viral recrudescence in bat populations remains to be investigated and will require long-term studies of captive individuals of known history of viral exposure.

Differences in the numbers of cells expressing surface immunoglobulin (sIg) have also been observed in *P. giganteus* with a higher number of sIg-positive cells in peripheral blood (~82%) compared to humans and mice (~15–30%) (Chakravarty and Sarkar, 1994). As no bat-specific reagents existed to further characterize the nature of this population of cells, the significance of this result remains unknown. Further studies to characterize the nature of B cells in bats may assist in resolving whether differences in the numbers of B cells are a general characteristic of bats and whether this plays a role in the observed differences in antibody responses of bats.

T-cell-mediated immune responses

Cell-mediated responses are controlled by T lymphocytes, which include cytotoxic and helper functions. The different populations of T cells in bats have not been characterized to date, and only one T-cell coreceptor, CD4, has been characterized (Omatsu et al., 2006). However, a number of reports have described *in vitro* responses of lymphocytes to T-cell mitogens in pteropid bats and microbats (McMurray and Thomas, 1979; Chakravarty and Paul, 1987; Paul and Chakravarty, 1987). These studies have indicated that bats display evidence for delayed responses to T-cell mitogens, PHA and concanavalin A (ConA) with a peak at 120 h compared to 48 h in mice (McMurray and Thomas, 1979; Paul and Chakravarty, 1986, 1987). A delay in mixed lymphocyte responses (MLR) have also been observed with a peak at 7 days for *P. giganteus* in comparison with 5 days in mice, thus providing further evidence that cell-mediated immunity in bats is slower than that of other mammals (Chakravarty and Chakravarty, 1983). The presence of suppressor T cells has also been 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 recently, an IFN γ response was demonstrated following stimulation of pteropid bat lymphocytes with the T-cell mitogens PHA and ConA, demonstrating that bats are capable of a similar IFN γ response to other mammals (Janardhana et al., 2012).

In vivo cell-mediated responses in bats have been measured using delayed-type hypersensitivity (DTH) tests using the PHA skin test or skin sensitivity to 2–4 dinitrofluorobenzene (DNFB) (Christe et al., 2000; Allen et al., 2009; Turmelle et al., 2010a). Delayed-type hypersensitivity

of *P. giganteus* to DNFB resulted in a characteristic DTH response within 48 h, similar to other mammals. However, only three of twelve bats tested in this study responded to treatment with DNFB, suggesting that bats may not be sensitive to DNFB to the same extent as other mammals (Chakraborty and Chakravarty, 1983). A time series of histological skin sections taken from skin biopsies of *T. brasiliensis* following PHA or saline injection has also demonstrated substantial individual variation but overall has provided evidence for a strong cell-mediated immune response (Turmelle et al., 2010a).

Cell-mediated immunity has also provided evidence for changes in immunocompetence because of environmental and physiological factors. Differences in immunocompetence because of roost ecology were observed in *T. brasiliensis* using subcutaneous PHA injection as a measure of *in vivo* T-cell-mediated immunity, providing evidence for the effect of environment on immune responsiveness (Allen et al., 2009). Christe et al. (2000) used PHA skin tests to demonstrate that greater mouse-eared bats, *Myotis myotis*, mount weaker cell-mediated responses during pregnancy compared with that of non-reproductive and lactating females. Immunocompetence was also lower in early pregnancy than at later stages of gestation (Christe et al., 2000). This result is consistent with changes in immunocompetence reported in other mammals that undergo a shift in the immune response towards a humoral immune response and away from cell-mediated immunity during pregnancy (Szekeres-Bartho, 2002). Changes in immune function during pregnancy have been speculated to favour replication of viruses including Zaire ebolavirus in bats. High titres of virus present in birthing fluids, blood and placental tissues may then be a source of infection to terrestrial mammals including apes (Leroy et al., 2005). Further studies into the antiviral immune response during pregnancy may provide insights into whether changes in immune function influence viral infections in bats and/or correlate with spillover events from bats to other susceptible species.

Although cell-mediated responses both *in vivo* and *in vitro* have provided important information on the T-cell-mediated responses of bats, no reagents currently exist to identify different populations of T cells in bats. The ability to identify and sort different populations of T cells would provide valuable insight into the role of T cells in antiviral immunity in bats.

The major histocompatibility complex

The MHC plays an important role in resistance to infectious diseases, autoimmunity, transplantation and reproductive success (Kumánovics et al., 2003). Despite the importance of the MHC, no work has been reported

on the MHC class I genes of bats and only a few studies have provided information on MHC class II polymorphism in bats (Mayer and Brunner, 2007; Richman et al., 2010; Schad et al., 2011). The earliest evidence for the degree of MHC polymorphism in bats came from MLR assays. Mixed lymphocyte responses test the recognition and proliferation of T cells from different individuals, and this response is highly dependent on MHC class II polymorphism (Derks and Burlingham, 2005). *Pteropus giganteus* lymphocytes undergo delayed and lower levels of proliferation in MLR tests compared to their responses to mitogens such as ConA (Chakraborty and Chakravarty, 1983; Chakravarty and Paul, 1987). As the proliferation of cells in MLRs correlates with the degree of genetic difference in MHC loci between individuals, delayed and weaker MLR responses in bats may be evidence for low MHC polymorphism.

Only recently has genetic evidence for the degree of MHC class II polymorphism in bats been reported. The class II DR beta (DRB) locus is the most extensively studied of the MHC loci in mammals because of its high diversity and has been the focus of all of the MHC class II analyses performed on bats to date (Mayer and Brunner, 2007; Richman et al., 2010; Schad et al., 2011). Richman et al. (2010) demonstrated extreme differences in polymorphism between bat species with extensive polymorphism at the MHC class II DRB locus in *Myotis velifer* compared to the extremely limited polymorphism in *Myotis vivesi*. *M. velifer* is a geographically widespread continental species compared to *M. vivesi* that is a narrowly distributed and endangered island endemic species. The lower population size of *M. vivesi* may have relaxed selection for the maintenance of many alternative alleles in the population, thus lowering MHC polymorphism. A single DRB locus has been described in the bulldog bat, *Noctilio albigentris* displaying moderate allelic variability within the range of other mammals. In addition, males displayed a significantly higher heterozygosity rate and genetic variability compared to female bats (Schad et al., 2011). The single DRB locus of the sac-winged bat, *Saccopteryx bilineata*, displayed low heterozygosity and evidence for diversifying selection. Substantial nucleotide sequence variation between the DRB alleles of *S. bilineata* was consistent with a history of balancing selection, but there was no evidence for ongoing balancing selection acting to maintain alternative alleles at intermediate frequency. In addition, unexpected homozygosity for a common allele was observed in this population of *S. bilineata*, consistent with pathogen-driven positive selection playing a role in the evolution of MHC genes in this species (Mayer and Brunner, 2007). DRB intron sequences from three species of bats (*R. aegyptiacus*, *C. perspicillata* and *Phyllostomus discolor*) have also been used to infer

phylogenetic relationships and demonstrate the monophyly of Chiroptera (Kupfermann et al., 1999). Overall, studies of DRB polymorphism in bats provide evidence for the influence of factors such as population size and pathogen pressure on the diversification of class II genes. The degree of variation in DRB polymorphism described above may be consistent with wide variation in the MHC variability in bats that may in turn influence the ability of different populations of bats to respond to infections.

Cytokines

A number of bat cytokine genes have now been characterized including cDNAs corresponding to interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p40 and tumour necrosis factor (TNF) from *Rousettus leschenaultii* (Iha et al., 2010). Partial cDNAs for IL-10, IL-23a, TNF and granulocyte macrophage colony-stimulating factor have been cloned from Seba's fruit bat (*C. perspicillata*) (Cogswell-Hawkinson et al., 2011). These cytokines appear to be highly conserved with those from other mammals. Kepler et al. (2010) described the *in silico* identification of IFN γ from *P. vampyrus* and *M. lucifugus*, confirming that both bats appear to have a single IFN γ locus similar to other mammals. More recently, *P. alecto* IFN γ has been described, including the characterization of its antiviral activity against Semliki forest virus and Hendra virus. This study included the generation of important bat-specific reagents for the detection of IFN γ , an important step for future studies of the role of IFN γ and T-cell-mediated immunity during viral infections in bats (Janardhana et al., 2012). Although only limited work has been performed on bat cytokines, these studies pave the way for examining the role of cytokines in antiviral immunity in bats.

Future Directions

As discussed above, functional and genome sequence analyses of bats have revealed some surprises, but overall, it appears that bats share many of the immunological features of other mammals. It is evident they have similar antibody and T-cell receptor genes, cytokines and chemokines, transcription factors, cluster of differentiation (CD) markers and activation pathways found in the immune responses of other mammalian species. Bats have molecules involved in cell self-defence against viruses, innate response mechanisms and adaptive responses. Defining these molecules will be relatively easy; understanding how the viruses and bat reservoirs have shaped one another, and the tempo and mode of immune responses will be more challenging. Such functional studies will likely result in significant insights into host-virus relationships, the implications of which will

have impacts on the development of novel therapeutics for other species and the ability to predict viral spillover events.

The variability in the results obtained from studies involving wild-caught bats emphasizes the need for captive colonies of bats of known age and history of infections for focused studies of bat immunology. Although challenging, information from such colonies would greatly assist in the interpretation of data obtained from wild-caught individuals. The development of cell lines also plays an important role in this regard. Although cell lines have now been generated from a number of tissues from the pteropid bat, *P. alecto* (Cramer et al., 2009), the development of additional cell lines from immune relevant cells will also assist in developing assays for studying various aspects of bat immune function. Future studies using expression tools, such as real-time PCR arrays, can be rapidly and inexpensively deployed to study particular species of bats and their viruses, and they can be highly informative regarding the genetic responses of bats during infection. However, transcription data are limited because many genes are post-transcriptionally regulated. Furthermore, post-translational modification events such as phosphorylation cannot be assessed by transcriptional analysis. Thus, it will be necessary to determine which antibodies currently available are cross-reactive with bat cells and to generate antibodies for those proteins that appear important based upon transcriptional analysis. Because most intracellular signalling proteins are often highly conserved between mammalian species, it is likely that some antibodies specific for those proteins in humans or mice will be useful for bat studies. However, most cytokines and CD molecules are often quite divergent between species and will likely require development of new reagents.

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