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Identification of secreted and membrane-bound bat immunoglobulin using a Microchiropteran-specific mouse monoclonal antibody

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A B S T R A C T
Bat immunity has received increasing attention because some bat species are being decimated by the fungal disease, White Nose Syndrome, while other species are potential reservoirs of zoonotic viruses. Identifying specific immune processes requires new specific tools and reagents. In this study, we describe a new mouse monoclonal antibody (mAb) reactive with Eptesicus fuscus immunoglobulins. The epitope recognized by mAb BT1-4F10 was localized to immunoglobulin light (lambda) chains; hence, the mAb recognized serum immunoglobulins and B lymphocytes. The BT1-4F10 epitope appeared to be restricted to Microchiropteran immunoglobulins and absent from Megachiropteran immunoglobulins. Analyses of sera and other E. fuscus fluids showed that most, if not all, secreted immunoglobulins utilized lambda light chains. Finally, mAb BT1-4F10 permitted the identification of B cell follicles in splenic white pulp. This Microchiropteran-specific mAb has potential utility in seroassays; hence, this reagent may have both basic and practical applications for studying immune process.

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1. Introduction

There are over 1100 species of bats, and these comprise over 20% of all mammalian species (Calisher et al., 2006; Schountz, 2014). Genomic, anatomic, and behavioral data support the hypothesis that bats have a unique role in hosting infectious agents (Reviewed in (Baker et al., 2013; Schountz, 2014)). Bats (Chiroptera) have traditionally been divided into two major suborders: Megachiroptera (“megabats”) and Microchiroptera (“microbats”). However, based on recent genetic data, chiropterans are now classified as Yinpterochiroptera (Pteropodidae, Rhinolophidae, Hipposideridae, Craseonycteridae, Megadermatidae, Rhinopomatidae) and Yangochiroptera (all other families, including microbats) (Tsagkogeorga et al., 2013). The two orders of bats have a monophyletic origin. Bats are members of the superorder Laurasiatheria, which includes carnivores and ungulates (Peng et al., 1991; Zhou et al., 2012).

The importance of bats as animal reservoirs for emerging pathogens is now widely appreciated. Over a hundred viruses have been detected in bats, including many viruses discovered to be important to human or veterinary disease. Among these are Hendra and Nipah virus, SARS-like coronaviruses, Marburg virus and Ebola virus (Baker et al., 2013; Calisher et al., 2006). Despite hosting numerous viruses, bats do not usually exhibit disease, with the notable exception of rabies virus infection. The presence of persistent viral infections without pathology suggests a co-adaptation, thus, how viruses modulate bat-specific immune responses is an important question with respect to zoonotic disease transmission. Aside from their potential role as reservoirs, North American bats are also of interest because several species are threatened by the fungus Pseudogymnoascus destructans, which causes the disease known as White Nose Syndrome (Lorch et al., 2011; Blehert et al., 2009; Chaturvedi et al., 2010; Field et al., 2015). Millions of bats have succumbed to the fungus, which infects hibernating bats. However, some bat species exhibit greater resistance or susceptibility to disease, implying that qualitatively or quantitatively different immune responses are generated in response to infection.

Little is known about the immune system of bats, although there
is intense recent focus in this area, given the importance of bat reservoirs in emerging infections (Baker et al., 2013; Schountz, 2014). While bat immune responses to infectious agents have been studied in both Megachiroptera and Microchiroptera (e.g., Field et al., 2015), most investigations on components of the bat immune system have been characterized in Megachiroptera. Recent studies, including those that have focused on the immunoglobulin gene locus (Baker et al., 2010; Bratsch et al., 2011; Butter et al., 2011), have provided evidence that there is genetic diversity among the two bat suborders, hence potential unique features of Microchiroptera remain to be identified. Unfortunately, Microchiropteran immunity has been difficult to study for a number of reasons, including limited numbers of animals generally available for study, limited numbers of institutions with facilities for bat research, and, unlike for the mouse, there are no inbred bat strains. A major barrier to studying immune processes is the lack of specific immunological reagents and in vitro experimental models. For example, there are no cell lines of lymphoid origin, and there are few specific antibody reagents for detecting or characterizing bat cells or proteins. Thus, it has been difficult, if not impossible, to identify and/or isolate immune cells, such as lymphocytes, or to monitor cellular or humoral immune responses to infection or immunization.

In the present study we describe the development of bat-specific reagents that will be used to characterize bat immune components and processes. We have focused on Microchiroptera, and have used *Eptesicus fuscus* as our model bat species. We have produced monoclonal antibodies (mAbs), directed against bat lymphocytes, and herein, we characterize a mAb which recognizes both B cells and serum immunoglobulins. We have used this mAb to identify bat antibodies in serum and other body fluids, thereby extending prior genetic analyses of *E. fuscus* immunoglobulins. We have also used the mAb to identify immunoglobulin-producing B lymphocytes and we demonstrate how B cells are distributed in the bat splenic white pulp. These data underscore the potential for bat-specific antibodies as tools for unraveling unique facets of bat immune responses.

2. Materials and methods

2.1. Animals

Experimental design and animal care were performed in compliance with the USDA animal care and welfare act (AWA) and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The use of animals in this study was approved and conducted in accordance with the Wadsworth Center IACUC.

Female BALB/c ByJ mice (6–8 weeks) were used in this study. These mice were bred and maintained at the Wadsworth Center. Bat tissues and blood were obtained from wild-caught *Eptesicus fuscus* and other species bats that had been obtained by our institution as part of the NYSDOH research and rabies testing/surveillance program. Both adult and juvenile bats were used in this study; since multiple species of bats were used, there was a wide range of bat sizes. Once the bats had been euthanized and tested to ensure that they were rabies-free, specimens were collected for experimentation. After euthanasia, bat spleens were removed aseptically and blood was obtained by cardiac puncture. Additional tissues and blood were obtained from frozen stocks of wild-caught bat samples from prior studies or testing. In these instances, the source of blood was heart tissue. Bat hearts had been homogenized using ceramic beads and 100 µl of tissue culture media.

2.2. Antibodies and reagents

Sera from several distinct Megachiropteran bat species were purchased through cooperation with the Lubee Bat Conservancy (Gainesville, FL). Purified IgG derived from dog, cat, guinea pig, swine, hamster, human were purchased (Rockland Immunocytobiologicals). Laboratory stocks of mouse and rat mAbs were used as unlabeled IgG or conjugated with EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific); horse serum was obtained from the Wadsworth Center Veterinary Sciences facility. For ELISAs, unlabeled and horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig, goat anti-mouse IgG1, goat anti-mouse IgG2a, and goat anti-mouse IgM, as well as HRP-streptavidin were used (Southern Biotech). In addition, polyclonal HRP-conjugated goat anti-bat Ig was purchased (Bethyl Laboratories). For flow cytometry, rabbit anti-mouse Ig (Ramig) (Jackson Research, West Grove, PA) was purchased and labeled with FITC (Sigma-Aldrich) or Cy-5 (GE Healthcare).

2.3. Immunizations, hybridoma fusion, and mAb screening

Five-to-six-week-old BALB/c ByJ mice were immunized with three i.p. injections, spaced 4–6 weeks apart, using *Eptesicus fuscus* spleen cells (2 × 10^7 cells per injection). Five days after the last injection, the spleen from one mouse was removed and a single cell suspension was prepared. To generate hybridomas that secreted bat-specific mAbs, a ClonCell-HY Hybridoma Kit (Stem cell Technologies, Vancouver, BC, CA) was used. Briefly, after lysis of red blood cells, the immune mouse lymphocytes were fused to Sp2/0 myeloma cells (Shulman et al., 1978) using polyethylene glycol. The resulting hybridomas were plated and cultured in methyl cellulose containing selection medium. Fourteen days after the fusion, individual hybridoma clones were picked from the methylcellulose plate and transferred into individual wells of a 96-well tissue culture plate. Two days later, culture supernatants were collected and screened using flow cytometry to identify mAbs which bound to *E. fuscus* splenocytes. For some hybridoma supernatants, assessment of reactivity with serum proteins was performed using surface plasmon resonance analysis; these analyses were performed by the Wadsworth Center Immunology Core, using a BiACore 3000 analyzer (GE Healthcare); the sensor chip was coated with a 50% ammonium sulfate (SAS) precipitant of *E. fuscus* serum. Over 60 unique hybridomas were obtained that bound to *E. fuscus* splenocytes (all cells or subpopulations of cells).

2.4. Immunoprecipitation, SDS-PAGE, and immunoblotting

For immunoprecipitation, serum SAS precipitant samples were mixed with purchased magnetic microbeads (Protein A and Protein G, GE Healthcare; Protein L, Biovision, Inc). Alternatively, mAbs were conjugated to NHS-MAG particles (GE Healthcare), according to the manufacturer’s instructions. Adsorbed samples were eluted either with 0.1M glycine buffer (pH 2.3), or with SDS-containing sample buffer (Life Technologies). Proteins were separated using SDS-PAGE on 4–12% NuPage Tris-Bis gels (Life Technologies) and either stained with SimplyBlue stain (Life Technologies) or Silver stain (Fisher-Thermo Pierce) or they were transferred to PVDF membranes, blocked with 4% fish gelatin (Sigma-Aldrich) and probed with specific mAb, followed by HRP-goat anti-mouse Ig. Probing with biotinylated mAb and HRP-Streptavidin produced similar results. Immunoblotts were developed using an ECL substrate (Fisher-Thermo Pierce) and either autoradiographed on ECL Hyperfilm (Fisher) or developed using a Chemidoc MP imager (BioRad). Where indicated, immunoblotts were developed using a colorimetric substrate (TrueBlue, KPL, Inc.). To identify immunoprecipitated proteins, stained bands were excised from the SDS-
PAGE gels and analyzed by LC-MS/MS using a QSTAR XL Mass Spectrometer (Ab Sciex) at the Center for Functional Genomics (University at Albany). Tandem mass spectra evaluation was assisted by MASCOT 2.2 (Matrix Science).

2.5. ELISAs

In all experiments coating antigens were diluted in a carbonate buffer (pH 9.5) and wells were blocked using 4% fish gelatin (Sigma Chemicals) diluted in wash buffer (PBS/0.05% Tween-20). For determining the isotypes of mAbs, wells were coated with goat anti-mouse Ig and bound mAbs were detected using isotype-specific HRP-goat anti-mouse Ig's. For determining specificity of mAbs, wells were coated with a 1:100 dilution of serum or heart extracts or with 40 µg/ml of purified IgG, as indicated. Purified mAb (0.1 or 0.01 µg/ml, as indicated) was used either unconjugated or conjugated to biotin and detected using a 1:10000 dilution of HRP-goat anti-mouse Ig or HRP-streptavidin, respectively. Results using either means of detection were comparable. For some experiments, the coating antigen was detected using a 1:10,000 dilution of HRP-goat anti-bat Ig. ELISAs were developed using a single-solution TMB substrate and stop solution (SureBlue, KPL, Inc.).

2.6. Microscopy

Spleens harvested from E. fuscus bats or BALB/c mice were embedded in OCT compound (Sakura, Torrence, CA). The tissues were frozen in slurry of dry ice and isopentane, and 8 µm cryosections were generated. For immunohistochemical analysis, cryosections were stained with biotin-conjugated mAb BT1-4F10 (bats) plus HRP-Streptavidin or biotin-conjugated anti-mouse K chain (mAb 187.1 (Yelton et al., 1981)) plus HRP-Streptavidin. Immunohistochemistry was performed by the Wadsworth Center Histopathology Core. For fluorescence microscopy the cryosections were fixed in 100% ice-cold ethanol, blocked in Fc blocking solution (for mouse sections) or rabbit IgG (for bats), and stained with either Alexa 488-conjugated mAb BT1-4F10 (bats) or Alexa 488-conjugated anti-CD19 (mAb ID3; BD Biosciences. Stained sections were mounted with ProLong Gold with DAPI (Life Technologies). Images were captured with a 20 × objective on a Nikon TE2000 equipped with a CoolSNAP HQ charge-coupled device camera (Roper Scientific, Martinsried, Germany), and ImagePro software (Media Cybernetics, Rockville, MD), and Adobe Photoshop was used to process the images for display.

3. Results

3.1. Generation of a bat-specific monoclonal antibody

To develop novel monoclonal antibodies reactive with Yango-chiropteran/Microchiropteran immune cells, we obtained whole spleens from wild-captured E. fuscus for multiple immunizations of BALB/c mice. From a fusion of a single immune mouse, a number of cell-specific hybridomas were generated; the characterization and utility of one hybridoma, BT1-4F10, is described in this report. The initial hybridoma screen was accomplished using flow cytometry to identify hybridomas that secreted mAbs reactive with E. fuscus splenocytes. Hence, all of the selected bat-reactive hybridomas bound to bat cells or to subpopulations of splenocytes (data not shown). Among these hybridomas, BT1-4F10 was unique in that the target antigen was detected not only on a subpopulation (~36%) of spleen cells (Fig. 1A), but also as a soluble component in bat serum. The latter reactivity was initially determined using surface plasmon resonance analysis. A BIACore chip was coated with a reconstituted 50% saturated ammonium sulfate (SAS) precipitate of E. fuscus serum and individual hybridoma supernatants were passaged over the chip. The BT1-4F10 supernatant exhibited detectable binding to the serum-coated chip (Fig. 1B), but not to a control mouse (or human) serum-coated chip (data not shown). Given that BT1-4F10 recognized a lymphocyte surface molecule and secreted protein, we hypothesized that the target antigen was immunoglobulin and that the subpopulation of cells recognized by the mAb was slg-bearing B lymphocytes. Serum immunoglobulin was expected to be enriched in the 50% SAS precipitant used for the BIACore assay; however, other contaminating serum proteins may have been present on the chip. Hence, we next proceeded to directly identify the target antigen in the SAS precipitate.

3.2. Characterization of the binding specificity of mAb BT1-4F10

We first determined that mAb BT1-4F10 was a mouse IgG1, using an ELISA (data not shown), and we affinity-purified the mAb from the hybridoma supernatant by passage over Protein G Sepharose. To determine the specific antigen recognized by mAb BT1-4F10, the mAb was directly conjugated to magnetic beads and used to immunoprecipitate its target antigen from an SAS precipitate of E. fuscus serum from a different batch of the bat SAS precipitate was passaged over a protein G-magnetic bead matrix and both the protein-G-binding and unbound fractions were also immunoprecipitated using BT1-4F10-magnetic beads. Analysis using SDS-PAGE indicated that several proteins were bound by mAb BT1-4F10 (Fig. 2A). A primary protein had a molecular weight of ~150 kDa, which, upon reduction, resulted in two protein bands of ~50 kDa and ~27 kDa, which was consistent with the electrophoretic migration of serum IgG in numerous animal species. This same protein also bound to Protein G, further suggesting that it was bat IgG. The 50 kDa band, and an additional non-protein G-binding band (70 kDa), were excised from the reduced gel and analyzed by mass spectrometry. These studies identified the precipitated proteins as being the E. fuscus immunoglobulin gamma chain and mu chain, respectively. In a subsequent experiment, the band of approximately 27 kDa was excised and analyzed by mass spectrometry; comparison to mammalian databases found its closest match to be the bat Ig lambda7 chain C region of Myotis davidii. Hence, mAb BT1-4F10 binds to E. fuscus serum immunoglobulins (IgG and IgM).

Given that mAb BT1-4F10 bound to at least two different bat Ig isotypes, we considered that the recognized epitope might be either a determinant common to all Ig heavy chains, or one that is present on an Ig light chain that can pair with several different heavy chains. In order to more precisely identify the antigen, we used mAb BT1-4F10 as a probe in an immunoblot assay. Using HRP-BT1-4F10 to immunoblot an unreduced E. fuscus SAS precipitate, we observed a number of high molecular weight bands. The most prominent bands were of high molecular weight, two ~150 kDa proteins, and ~25 kDa. However, upon reduction, only a single band of ~25 kDa was observed (Fig. 2B). Given that the mAb binds to serum Ig, it is likely that the unreduced bands were IgM (and/or IgA) and IgG, and the reduced band was the Ig light chain. Thus, we concluded that the antigenic target of mAb BT1-4F10 is E. fuscus IgL chain, and, therefore, the mAb would bind any whole Ig molecule, regardless of the utilized heavy chain.

To further characterize mAb BT1-4F10, we determined whether the epitope recognized by the mAb was unique to bats. Purified Ig or serum was obtained from a number of different animal species. ELISA showed that mAb BT1-4F10 bound only to E. fuscus serum and swine IgG, but not to any of the other tested immunoglobulins (Table 1). The epitope recognized on swine IgG was also found to be on the light chain, as reduction of the intact IgG showed only the Ig...
light chain in the both bat and swine samples (Fig. 3). In contrast, a commercial polyclonal goat anti-bat Ig serum that had been raised against a mixture of chiropteran IgG that included *E. fuscus* IgG, exhibited substantially more cross-reactivity with Ig from different species. Indeed, of all the various types of Ig that were tested, only mouse and rat IgG were not bound by the polyclonal preparation (Table 1). We next examined whether mAb BT1-4F7 recognized Ig from bat species other than *E. fuscus*. We obtained sera from a number of different megabat and microbat species to use as a coating antigens in ELISAs. As expected, the commercial polyclonal anti-bat Ig bound strongly to each bat serum and, less so, to *E. fuscus* serum (Table 2). In contrast, mAb BT1-4F10 did not bind to any of the Megachiropteran sera that were tested. The mAb did, however, recognize Ig from other Microchiroptera (Table 2). As a serum Ab source for microbats, we used saline extracts of heart tissue as the coating antigen and we examined representative cave-dwelling (*E. fuscus* (Big Brown), *Myotis lucifugus* (Little Brown)) and tree-dwelling (*Lasiorus cinereus* (Hoary), *Lasionycteris noctivagans* (Silver-haired), *Lasiorus borealis* (Eastern Red)) bats. mAb BT1-4F10 bound to each of the microbat sera. This assay is not quantitative as the amounts of Ig attached to the wells in each sera may vary. However, when we assessed multiple individual sera, we consistently found a relative binding strength of Big Brown > Little Brown > Hoary > Silverhair > Red bat. Hence, other than its cross-reactivity with swine Ig, mAb BT1-4F10 is highly specific for microbat immunoglobulin.

### 3.3. Immunoglobulins recognized by mAb BT1-4F10

Given that mAb BT1-4F10 binds the Ig light chain, we used the mAb to identify isotypes of circulating immunoglobulins in several microbat species. Heart extracts from the cave-dwelling and tree-dwelling bats examined above were assessed using SDS-PAGE and immunoblotting with mAb BT1-4F10. For each of the bat...
species, we generally observed a similar pattern of bands: high molecular weight proteins, some of which did not enter the polyacrylamide gel; a distinct doublet at approximately 150 kDa, likely IgG subclasses, and the Ig light chain (Fig. 4). As would be expected, the putative IgG bands were the most prominent in all of the serum/heart samples. Based upon previous studies, it is likely that the higher molecular weight proteins are serum IgM/IgA, and possibly IgE. Similar patterns were observed in all of the bat species tested. Most of the bands appeared to be of the same size in the different species, except that the putative IgG of Red bats appeared to be of slightly higher molecular weight. We also observed that with greater sample concentrations or longer exposure times, additional lower molecular weight bands could be seen. The identity of these bands is unclear; they may be various forms of the different intact Ig molecules or multimers of the Light chain. Examination of a similar amount of mouse serum showed that free light chains, as well as additional proteins, were observed, although at much lower abundance than was observed in bat samples (Fig. 5A). Further, the bands largely appeared to arise during the electrophoresis procedure, as passage of the samples through a 100 kDa membrane prior to electrophoresis showed that the lower molecular weight bands were largely retained in the high molecular weight fraction (Fig. 5B). Mixing increasing amounts of bat sera and microbat heart extracts were diluted to 1/100 for coating ELISA plates. Bound BT1-4F10 was detected with HRP-goat anti-mouse Ig (1:10000). Puriﬁed BT1-4F10 was diluted to 0.1 mg/ml or, in parentheses, 0.01 mg/ml for use. HRP-goat anti-bat Ig (1:10000) was used. Data are representative of 3 independent experiments.

Table 1
Corrected OD_{450} values for ELISAs using either mAb BT1-4F10 or polyclonal goat anti-bat Ig for detection.

| Species       | BT1-4F10 | Goat anti-bat Ig |
|---------------|----------|-----------------|
| Bat (E. fuscus) | 3.583 (3.104) | 1.970 |
| Alpaca        | 0.020    | 0.885           |
| Cat           | 0.118    | 3.079           |
| Dog           | 0.046    | 3.074           |
| Guinea Pig    | 0.047    | 2.369           |
| Hamster       | 0.037    | 2.546           |
| Human         | 0.220    | 2.978           |
| Mouse         | 0.015    | 0.379           |
| Rabbit        | 0.163    | 2.284           |
| Rat           | 0.044    | 0.244           |
| Swine         | 3.460 (0.730) | 2.529 |

E. fuscus (1/100 dilution) and alpaca (1/100 dilution) sera or puriﬁed IgG (40 µg/ml) were used to coat ELISA plates. Bound BT1-4F10 was detected with HRP-goat anti-mouse Ig (1:10000). Purified BT1-4F10 was diluted to 0.1 µg/ml or, in parentheses, 0.01 µg/ml for use. HRP-goat anti-bat Ig (1:10000) was used. Data are representative of 5 independent experiments.

Table 2
Corrected OD_{450} values for ELISAs using either mAb BT1-4F10 or polyclonal goat anti-bat Ig for detection.

| Type                  | Species                  | BT1-4F10 | Goat anti-bat Ig |
|-----------------------|--------------------------|----------|-----------------|
| Microchiropteran      | Eptesicus fuscus          | 3.411    | 0.916           |
| Megachiropteran       | Pteropus poliocephalus    | 0.004    | 2.233           |
| Megachiropteran       | Pteropus vampyrus         | 0.039    | 1.409           |
| Megachiropteran       | Pteropus giganteus        | 0.013    | 1.564           |
| Megachiropteran       | Pteropus rodricensis      | 0.012    | 1.577           |
| Megachiropteran       | Pteropus pumilus          | 0.088    | 1.854           |
| Megachiropteran       | Pteropus hypomelanus      | 0.025    | 0.926           |
| Megachiropteran       | Rousettus aegyptiacus     | 0.039    | 2.578           |
| Megachiropteran       | Eidolon helvum           | 0.030    | 1.456           |
| Microchiropteran      | Eptesicus fuscus          | 3.334    | N.D.            |
| Microchiropteran      | Myotis lucifugus         | 2.240    | N.D.            |
| Microchiropteran      | Lasius cinereus          | 1.000    | N.D.            |
| Microchiropteran      | Lasiomycteris noctivagus  | 0.724    | N.D.            |
| Microchiropteran      | Lasius borealis          | 0.503    | N.D.            |

Megabat sera and microbat heart extracts were diluted to 1/100 for coating ELISA plates. Bound BT1-4F10 was detected with HRP-goat anti-mouse Ig (1:10000). Purified BT1-4F10 was diluted to 0.1 µg/ml for use. HRP-goat anti-bat Ig (1:10000) was used. Data are representative of 3 independent experiments.

Fig. 3. mAb BT1-4F10 cross-reacts with bat and swine Ig light chain. A 50% SAS precipitate of E. fuscus serum or purified swine IgG (40 µg/ml) were used to coat ELISA plates. Bound BT1-4F10 was detected with HRP-goat anti-mouse Ig (1:10000). Reduced and non-reduced samples were run on the same gel; lanes were cropped to remove irrelevant lanes.

Fig. 4. Serum Ig from different microchiropteran species is identiﬁed by Ab BT1-4F10. Unreduced heart extracts from (1) E. fuscus, (2) M. lucifugus, (3) L. cinereus, (4) L. noctivagus, and (5) L. borealis bats were resolved by SDS-PAGE followed by immunoblotting with mAb BT1-4F10. All samples were assessed in the same experiment; shown are lanes cropped from samples of different volumes and exposures to permit normalized display of similar IgG band intensities. Similar sample volumes showed staining intensities of the order indicated by Table 2. Data are representative of analysis of at least 5 individual bats per indicated species. All bats of a given species showed the same staining pattern.
serum with mouse serum did not result in the appearance of free mouse light chain, suggesting that bat serum did not contain a high amount of reducing agent that might break inter-chain disulfide bonds (data not shown). If the bat serum was first mixed with protein G-magnetic microbeads, the majority of the lower molecular weight material was in a form that was originally captured by protein G (Fig. 5C). A similar pattern was observed when using protein A-magnetic microbeads (data not shown). Taken together, these data suggest that in some bat Ig, heavy and light chain pairing is achieved via noncovalent interactions.

Given that mAb BT1-4F10 binds to a large proportion of B cells, we considered that the epitope might either be present on both kappa and lambda light chain classes, or that light chain usage was skewed to favor predominantly kappa or predominantly lambda expression. The ratio of kappa to lambda chain usage varies widely among different species from >90% kappa chain (e.g., mice) to >90% lambda chain (e.g., horse). To examine E. fuscus light chains, we mixed serum with Protein L–magnetic beads. Protein L binds to kappa chains in many, but not all, mammalian species (Nilson et al., 1992) (De Chateau et al., 1993). We also examined protein L reactivity with mouse (predominantly kappa), horse (predominantly lambda), and swine (60% kappa). Examination of both the bound and unbound factions showed that the majority of bat Ig was not bound to protein L (Fig. 6). When higher amounts of bat sera were assessed, a Protein L binding light chain was observed. At present, it is unclear if this band is kappa chain or non-specifically retained lambda chain. These data suggest that the majority, if not all, E. fuscus immunoglobulins use lambda light chains.

We next wished to determine if Ig isotype distribution varied, depending upon the sample source. Fig. 7A shows an immunoblot of Ig in serum/heart extracts as compared to fecal extracts and saliva. As with serum, we observed the presence of proteins with molecular weights less than IgG, with the most prominent being a ~45 kDa band found in the fecal extracts. The identity of this band is unclear. Examination of fecal material mixed with either protein G or protein A-magnetic microbeads prior to SDS-PAGE, showed that most of the ~45 kDa material did not bind. Two additional IgL-containing proteins of lower molecular weight than IgG were found in fecal samples. These proteins, which also we have not yet identified, bound to protein A but did not bind to protein G (Fig. 7A). In these gels multimeric IgA and IgM would not be distinguishable but we note that all samples contain proteins of high molecular weight. Finally, we note that fecal extracts appear to contain little IgG as compared to serum or saliva extracts.

### 3.4. Splenic B lymphocytes visualized by mAb BT1-4F10

In addition to identifying serum Ig, the anti-Light chain reactivity of mAb BT1-4F10 permitted us to target membrane Ig and visualize B cells. Spleens from E. fuscus and Myotis lucifugus were cryosectioned and examined by immunohistochemistry and microscopy to identify Ig-bearing cells. For comparison, mouse spleens were stained with either anti-Ig<sub>k</sub> or anti-CD19, to identify mouse B cells. For E. fuscus and M. lucifugus, a similar morphology with respect to Ig<sub>k</sub> B cells was observed (Fig. 8). Well-defined follicles were observed that were distinct from those identified in mice using an anti-mouse Igk mAb. In both bat species, but not in mice, we observed punctate staining of Ig outside of the follicles, which may reflect the presence of bat Ig bound to FcR. Follicle structure appeared to differ in the bats as compared to mouse, but not dissimilar to humans. In particular, the “horseshoe” shape observed for mouse follicles was absent, with bat follicles appearing as circular clusters of B cells. We observed a much denser staining in the center of the follicles. This could indicate the accumulation of secreted Ab or clusters of cells that are actively secreting Ab, or even immune complexes associated with follicular dendritic cells. A similar area of intense staining within the follicle was more clearly seen using fluorescent microscopy (Fig. 9). In these regions, the central areas of dense staining are surrounded by cells with a high level of surface staining and then B cells with more moderate levels of Ig expression.

### 4. Discussion

Given the important role that bats may play as reservoirs for emerging infections, it is not surprising that there has been recent
emphasis on studying immune processes in bats. However, as reports note, such studies face several challenges, not the least of which is a lack of suitable bat-specific reagents (Baker et al., 2013; Schountz, 2014). Few, if any, commercially available Abs reactive with various species also cross-react with bats. Hence, we attempted to address this gap through the generation of monoclonal antibodies that can be used to identify important immune cells and proteins. Our focus has been on Microchiropteran bat species primarily because of their ecological importance in North America, their endangerment by the fungal pathogen *Pseudogymnoascus destructans*, and their ready availability to us for experimentation.

Immunization of mice with bat splenocytes provoked a vigorous immune response, which was not surprising considering their evolutionary distance (Tsagkogeorga et al., 2013). This response resulted in many hybridomas that produced mAbs reactive with bat cell surface antigens. As we were initially interested in generating mAbs which might differentiate cell types involved in adaptive immunity, when we performed our initial flow cytometry screen we focused on mAbs that showed heterogeneous binding that separated the splenocytes into two or more subpopulations. We found that, as compared to staining mouse cells, there was a larger amount of non-specific binding, which was somewhat reduced by pre-treatment of the cells with unlabeled rabbit IgG. It is likely that this binding occurs via Fc receptors (FcR). Because we also saw a higher amount of extracellular Ig staining by microscopy, we speculate that the overall levels of FcR may be higher in bats than mice. Others have noted that elevated FcR can occur with higher core body temperatures and that higher core body temperatures are associated with flight (Blatteis, 2003; O’Shea et al., 2014; van Bruggen et al., 1991). This may point to a prominent role for FcR-mediated effector functions, such as opsonization, pathogen clearance or retention of immune complexes for immune memory maintenance.

For this report, we have characterized the binding reactivity of one mAb BT1-4F10. We show that this mAb recognizes an epitope on microbat Ig light chains. Based upon the lack of binding to Protein L, in E. fuscus, the lambda chain appears to be utilized by most, if not all Ig molecules. We cannot, however, exclude the possibility that bat lambda chains do not bind to protein L (De Chateau et al., 1993). We do note that genomic sequencing of the closely related *M. lucifugus* provided no evidence for the presence of genes encoding kappa light chains (Das et al., 2008). We do observe that some *E. fuscus* light chain bind to Protein L; however, we also find that a similar low level of light chain is bound when assessing sera from *M. lucifugus* bats (unpublished observations). Hence, for both bat species, this bound material may be lambda light chains. In contrast, other reports, including a recent transcriptome analysis by Papenfuss et al. (Papenfuss et al., 2012), show that other (megabat) bat species do utilize kappa chains. Bats and ungulates are members of the superorder Laurasiatheria. Most ungulates, other than pigs, have a strong preferential usage of lambda chains (Arun et al., 1996; Butler et al., 2006; Hood et al., 1967). Because BT1-4F10 still binds to Ig after fixation (microscopy) or denaturation (western blotting), we suggest it binds to a
linear epitope. Much harsher treatment does alter the epitope, as staining is lost when whole tissues are embedded in paraffin and subjected to prolonged fixation (10% formalin overnight) for histology examination (unpublished observations). Among bats, the epitope bound by this mAb appears to be restricted to microbats. However, as we found a range of binding of mAb BT1-4F10 to Ig from different microbat species, it appears that there are some differences in the amino acid sequences which comprise the epitope. Other than an anomalous reactivity with the light chain from swine Ig, mAb BT1-4F10 is specific for Microchiropteran Ig. For a single epitope, the lack of cross-reactivity to Megachiropteran Ig may not be surprising and the degree of immunological difference between the two bat suborders has not yet been fully established.

The commercial goat anti-bat Ig, which has been used to previously investigate megabat and microbat immunoglobulins, binds to Ig of both suborders. However, the strength of binding is much greater with the megabat antigens. Since the immunogen used to raise the commercial antisera contained E. fuscus IgG, among 11 different bat species, it is not clear whether the binding is due to cross-reactive Abs or due to a mixture of megabat- and microbat-specific Abs in the antisera. It should be noted that Wellehan et al. have produced a mAb reactive with megabat IgG by immunization of mice with Pteropus hypomelanus IgG (Wellehan et al., 2009). By western blot analyses, that mAb bound to a number of different Pteropus species and had some cross-reactivity with the one microbat tested (Phyllostomus hastatus). Similarly, Omatsu et al., raised a polyclonal anti-IgG serum by immunization of rabbits with Rousettus aegyptiacus and showed substantial cross-reactivity, using an ELISA, with serum from a microchiropteran (an unidentified Philippine insectivorous) bat (Omatsu et al., 2003). In contrast, mAb BT1-4F10 showed no cross-reactivity with either Pteropus hypomelanus or Rousettus aegyptiacus Ig.

Genetic analyses has shown that bats possess the 5 major canonical Ig isotypes (Baker et al., 2010; Butler et al., 2011). However, the number of IgG subclasses varies between different species. For example, E. fuscus contains two IgG isotypes, whereas M. lucifugus has five (Butler et al., 2011). In the present study, regardless of the microbat species, we were able to readily detect one prominent and one lesser Ig species with the expected molecular weights of IgG. While this could suggest the preferential usage of two IgG isotypes, it also may be that two or more subclasses were not resolved in our gel system. If the former is true, however, this may indicate that there is not as much practical functional difference between the two Microchiropteran species as the number of Ig classes would suggest. These putative IgG classes were not only found in serum but they were the main Ig we detected in saliva. Little IgG was
found excreted in feces. Instead we saw evidence for high molecular weight and sub-IgG molecular weight species, with the former likely to be IgA and IgM, both isoforms known to be excreted.

In both serum and feces, the identities of most of the low molecular weight Ig light-containing moieties are unclear. The major protein is unassociated light chain itself. Secreted light chains have been observed in other species, including humans (Dul et al., 1996; Shapiro et al., 1966); an increase in free lambda chains can be associated with pathologies, such as myeloma (Edelman and Gally, 1962). However, most of our data is consistent with the release of light chains from intact immunoglobulins and the subsequent formation of light chain multimers and/or heavy/light chain dimers during the processing of the samples for SDS-PAGE. This, in turn, would suggest that in some portion of the intact Ig pool, there are no interchain disulfide bonds between the heavy and light chains.

In their analysis of heavy chain genes in microbats, Butler et al. showed that bat IgA is similar to human IgA2 and IgA in cattle and swine in that all the lack the CH1 Cys used in H-L disulfide bridging (pos 15) (Butler et al., 2011). The authors speculated that a different CH1 Cys (pos 77) was used instead to link the heavy and light chains, but left open the possibility for noncovalent associations. Although, cattle and swine appear to secrete only covalently linked IgA, human IgA2 can be secreted as a noncovalently linked molecule (Wood and Russell, 2011). Based on our studies here, we speculate that some portion of secreted bat Ig, perhaps a fraction of IgA, also exists as a noncovalently associated molecule. However, we note that, in serum, the low molecular weight moieties were originally complexed to a protein G-binding Ig. As protein G in most species binds primarily or exclusively to IgG classes, our data might suggest that some IgG contains non-covalently associated H-L chains. However, we do note that in some species, IgA can bind to protein G (Peng et al., 1991). Thus, the Igotype origin of the free light chain in bats remains unresolved. In addition to serum, proteins with a lower molecular weight than IgG were also identified in fecal samples, with the major protein being ~45 kDa. This protein is likely not derived from IgG as we found little IgG in fecal samples at all. Thus, it is not surprising that the ~45 kDa protein did not bind to either Protein G or Protein A. Other proteins were bound to protein G, however, they did not bind to protein A. This result was unexpected given that Protein G is generally considered to have a more restricted specificity than protein A.

Because mAb BT1-4F10 recognizes virtually all Ig molecules, we were able to identify B cells by virtue of their sIg. By flow cytometry, nearly 40% of the lymphocytes, chosen based upon forward and side light scatter properties, were B cells. This percentage was slightly lower than typical values (45–55%) obtained from mice (Pellegrini et al., 2007) and unpublished observations). For mice, B cell percentages differ in different strains and are influenced by factors such as age, antigen exposure, and inflammation (Pellegrini et al., 2007). As our sampling of bats is still small and, because we examined wild-caught bats with unknown infection history, more studies are required to determine the full range of the B cell contribution to the lymphocyte pool. Still, we report a much lower percentage of Ig- cells than previously reported by Chakravarty and Sarkar (Chakravarty and Sarkar, 1994). In their study, the authors found that two-thirds or more of Indian fruit bat splenic and porcine plasma cells. Zentralbl. Veterinarmed. A 43, 573–576.

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