INTRODUCTION

Bats have been known as reservoirs of various zoonotic viruses such as strains of coronavirus that cause severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS), Filovirus that causes Ebola and Marburg diseases, rabies and rabies-related lyssavirus that cause rabies, and several paramyxoviruses, including rubula, Nipah and Hendra viruses (Briand et al., 2014; Calisher, Childs, Field, Holmes, & Schountz, 2006; Hayman et al., 2012; Kuzmin et al., 2011; Looi & Chua, 2007; Peel et al., 2018). As many as 4,167 viruses belonging to 23 virus families have been isolated from 196 bat species in 69 countries worldwide (Chen, Liu, Yang, & Jin, 2014). Bats can be persistently and latently infected with the virus without showing any clinical symptoms (Field et al., 2011; Peel et al., 2018).

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Abstract
Spleen is one of the important lymphoid organs with wide variations of morphological and physiological functions according to species. Morphology and function of the spleen in bats, which are hosts to several viral strains without exhibiting clinical symptoms, remain to be fully elucidated. This study aims to examine the spleen morphology of fruit bats associated with their physiological functions. Spleen histological observations were performed in three fruit bats species: Cynopterus titthaecheilus (n = 9), Rousettus leschenaultii (n = 3) and Pteropus vampyrus (n = 3). The spleens of these fruit bats were surrounded by a thin capsule. Red pulp consisted of splenic cord and wide vascular space filled with blood. Ellipsoids in all three studied species were found numerous and adjacent to one another forming macrophages aggregates. White pulp consisted of periarteriolar lymphoid sheaths (PALS), lymphoid follicles and marginal zone. The lymphoid follicle contained a germinal centre and a tingible body macrophage that might reflect an active immune system. The marginal zone was prominent and well developed. This study reports some differences in spleen structure of fruit bats compared to other bat species previously reported and discusses possible physiological implications of the spleen based on its morphology.

KEYWORDS
flying foxes, histology, immunology, morphology, physiology, viral reservoir

1 | INTRODUCTION

Bats have been known as reservoirs of various zoonotic viruses such as strains of coronavirus that cause severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS), Filovirus that causes Ebola and Marburg diseases, rabies and rabies-related lyssavirus that cause rabies, and several paramyxoviruses, including rubula, Nipah and Hendra viruses (Briand et al., 2014; Calisher, Childs, Field, Holmes, & Schountz, 2006; Hayman et al., 2012; Kuzmin et al., 2011; Looi & Chua, 2007; Mohd, Al-Tawfiq, & Memish, 2016; Sasaki et al., 2012; Sendow et al., 2010,2013; M. Wang & Hu, 2013). Although there is twice the number of rodent species as there are bat species, bats are reported to be natural hosts to greater varieties of zoonotic viruses per species than rodents (Luis et al., 2013). As many as 4,167 viruses belonging to 23 virus families have been isolated from 196 bat species in 69 countries worldwide (Chen, Liu, Yang, & Jin, 2014). Bats can be persistently and latently infected with the virus without showing any clinical symptoms (Field et al., 2011; Peel et al., 2018).

Bats belong to the order Chiroptera, the second largest order of mammals after rodents. Studies have thus far reported 1,240 species of bats in the world, which represent more than 20% of mammalian species (Calisher et al., 2006; Wang, Walker, & Poon, 2011). The order Chiroptera is divided into two suborders: Yinpterochiroptera and Yangochiroptera (Lei & Dong, 2016; Teeling et al., 2005). Fruit bats, included in the suborder Yinpterochiroptera, are often found near residential areas (Basri et al., 2017). The decline of tree and fruit populations in forests, forest fires and extreme climate change
often lead to the foray of fruit bats into human farming areas, especially during the fruiting season (Basri et al., 2017; Páez, Restif, Eby, & Plowright, 2018). Large-sized fruit bats, such as flying foxes, are nomadic foragers capable of flying up to 100 km from the roosting site in one night (Hengjan et al., 2018). Fruit bats, being social beings, are reported to engage in conflicts over food with other species such as monkeys (Hengjan et al., 2017). Fruit-eating behaviour is also an important factor aiding in spreading of viruses between species. Because bats cannot swallow large amounts of food, they chew the fruit and spit out the fibre (Dobson, 2005). The ability of fruit bats to fly and interact with other species, including domesticated animals and humans, increases the potential for zoonotic outbreaks (Basri et al., 2017). Some zoonotic virus that have been serologically detected in fruit bats include paramyxovirus, herpesvirus, parvovirus, poliovirus, coronavirus and filovirus (Anindita et al., 2015; Calisher et al., 2006; Hayman et al., 2012; Kobayashi et al., 2015; Sasaki et al., 2016, 2014, 2012; Sendow et al., 2010, 2013).

Bats are known to have a stronger immune component than other mammals (Pavlovich et al., 2018; Schountz, Baker, Butler, & Munster, 2017). Various components of their immunity have adapted to the existence of the virus, resulting in a symbiotic relationship between them. This suggests that bats have a strong immune organ structure to prepare for viral infection that might damage their system. However, details of the bat immune system remain to be elucidated completely. One important immune organ that performs several functions related to differences in structure between species is the spleen (Brendolan, Rosado, Carsetti, Selleri, & Dear, 2007; Cesta, 2006; Haley, 2003). This secondary lymphoid organ plays a major role in innate and adaptive immunity to blood-borne pathogens, including viruses (Den Haan & Kraal, 2012; Mebius & Kraal, 2005; Zhao, Liu, Guo, & Zhu, 2015). The organ also plays a role in extramedullary haematopoiesis, haemostasis, metabolism of red blood cells and blood storing. Role dominance of the spleen varies between species and is dependent on the organ structure and its constituent cell components (Bowdler, 2002; Brendolan et al., 2007; Udroiu, 2006). The spleen parenchyma is wrapped and infiltrated by capsules and trabeculae composed of collagen fibres and smooth muscles. The thickness of the capsule and the spread of smooth muscles affect contractility, which determines the speed of blood ejection from the spleen. The characteristics of the spleen sinuses also vary between species: some species have sinuses (sinusoidal type) and some do not have sinuses (non-sinusoidal type; Bowdler, 2002; Press & Landsverk, 2006; Udroiu, 2006).

It is interesting to explore the morphology of bat spleen because of the high structural variations of the organ between species affect its physiological function. The flying ability of bats makes them have a daily high metabolic rate; therefore, they require rapid oxygen supply (O’Shea et al., 2014; Thomas, 1975). The fulfilment of oxygen requirements in aerobic mammals activities can be done by increasing the circulating blood volume, which is in resting state, the blood stored in the spleen as reported in racehorses, hunter dogs and diving seals (Dane et al., 2006; Poole & Erickson, 2015; Thornton et al., 2001). Blood ejection from the spleen requires strong splenic contraction which is must be supported by the numerous smooth muscle among collagen that builds capsule and trabeculae structure. Chiroptera order is also known to have hibernation and torpor abilities. Hibernating mammals store blood and platelets in the spleen to prevent blood thickening and clotting (Cooper et al., 2012; Lidicker & Davis, 1955; Mann & Drips, 1916). Therefore, the spleen structure of the bats may support blood storing, blood generation and blood ejection capability. Bats as the reservoir of various viruses also demand strong defense structure of immune organ to prepare for viral infections. The morphophysiological aspect of bat spleen has not been widely described previously; therefore, the aim of this study was to examine different compartment of morphological structure of fruit bats spleen and to describe it physiological function based from the morphology findings.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

The fruit bat species analysed in this study were the *Cynopterus titthaechelius* (*n* = 9), *Rousettus leschenaultii* (*n* = 3) and *Pteropus vampyrus* (*n* = 3). *Cynopterus titthaechelius* and *Rousettus leschenaultii* were caught in Bogor, West Java Province, Java Island, Indonesia, whereas the spleens of *Pteropus vampyrus* were obtained from fixed specimens (NBF 10%). The capture process of fruit bats has been approved by the Ministry of Environment and Forestry, Department of Natural Resource and Ecosystem Conservation, Indonesia, with license numbers SK.197/KSDAE/SET/KSA.2/5/2017. Fruit bats were captured at night using mist nets and were transported in cloth bags to the laboratory for further studies. Captured bats had been confirmed to be sexually mature and not pregnant or breastfeeding. In the laboratory, bats were acclimatized for 24 hr and provided with fruit juice and drinking water ad libitum. Bats were identified by their morphological characteristics according to the field key described by Suyanto (2001) for these species in Indonesia.

### 2.2 | Conservation status

*C. titthaechelius*, *R. leschenaultii* and *P. vampyrus* used in this study do not appear on any conservation list in Indonesia. According to IUCN red list of threatened species, *C. titthaechelius* and *R. leschenaultii* were categorized as least concerned and *P. vampyrus* as near threatened.

### 2.3 | Biological samples

The procedures of this study were conducted according to the ethical requirements and concerns about the welfare of wild animals. This study obtained ethical approval from the Animal Ethics Commission of Research Institution and Community Service (LPPM) Bogor Agricultural University (IPB) with approval number 74-2017 IPB. Animals were anesthetized using a combination of ketamine...
(10 mg/kg) and xylazine (2 mg/kg) (Sohayati et al., 2008). After losing consciousness, animals were killed with ketamine overdose. The spleen sample collection comprised two methods of fixation: perfusion and non-perfusion fixation. Perfusion procedure was performed by puncturing the left heart ventricle with a needle and flowing a saline solution into the bloodstream. The right atria were then cut to allow for blood to flow out and replaced by saline solution. The saline solution was then replaced by a 4% paraformaldehyde (PFA) until the entire bat organs, including the spleens, were perfectly fixed. The spleens were then collected and post-fixed using 4% PFA. Non-perfused spleens were collected after opening the abdominal cavity followed by fixing them immediately in 4% PFA or Bouin solution.

3 | HISTOLOGY

Perfused and non-perfused spleens were dehydrated through a graded series of ethanol, cleared in xylene, and then embedded in paraffin. Sections were cut within 3 μm, mounted on glass slides, and left overnight in a hot plate at 40°C. Before staining, the paraffin sections were dewaxed in xylene and rehydrated in graded ethanol. The tissue sections were stained with haematoxylin and eosin (HE), Masson’s trichrome Goldner’s Modification and periodic acid Schiff (PAS). The sections were observed under light microscope and analysed descriptively.

4 | RESULTS

The splenic parenchyma of fruit bats typically consisted of red and white pulps, surrounded by thin capsules (Figure 1). The capsule thickness in C. titthaecheilus, R. leschenaultii and P. vampyrus was 19 ± 8, 27 ± 9 and 16 ± 6 μm, respectively. Capsules consisted of collagen with scattered smooth muscle fibres (Figure 2). Capsule infiltrated the parenchyme of the spleen into primary trabeculae with a wide variation of size, about 35 ± 18 μm (C. titthaecheilus), 33 ± 13 μm (R. leschenaultii) and 28 ± 13 μm (P. vampyrus), respectively. Primary trabeculae branched out collagen fibres that continued to become secondary trabeculae or splenic cord. Red pulp generally consisted of cord, capillaries and a wide vascular space contains blood (Figure 3). Splenic cords divided red pulp into small compartments that contained red blood cells and macrophages (Figure 3). A few myeloid and erythroid cells were found in cords, whereas megakaryocytes were not observed. Haemosiderin, a brown pigment produced by macrophage phagocytes against erythrocytes, was only
found slightly. However, in *P. vampyrus*, several melanin pigments were observed (Figure 3c).

The white pulp consisted of PALS, follicles and marginal zones. In all three species, PALS consisted of lymphocytes that surrounded the central artery. In HE stain, the follicles were divided into light zones called germinal centres and dark zones called corona or mantle zones. The marginal zone clearly demarcated the outer area of the white pulp with the red pulp (Figure 5). The PALS and the marginal zone separated by a marginal sinus which is the branching of the central artery (Figure 5).

The extensions of the central arteries later become arterial capillaries. These capillaries were surrounded by one to two lines of macrophages called ellipsoids (Figure 6). Ellipsoids in all three species were numerously found in the marginal zone and splenic cord.

5 | DISCUSSION

The present study has shown that the spleen of three species of fruit bats had thin capsule (Figure 1) and trabeculae with various size composed of collagen and a few smooth muscles (Figure 2). These characteristics are similar to thus in humans and rodents (Press & Landsverk, 2006; Ward, Cherian, & Linden, 2018). Dogs, cats, pigs, horses, camels, ruminants and diving seals have thick spleen capsule that have 1–2 layers of smooth muscle (Bacha & Bacha, 2000; Gray, Canfield, & Rogers, 2006; Press & Landsverk, 2006; Zidan, Kassem, Dougbag, et al., 2000). The thickness of collagen and the presence of smooth muscle in the spleen are related to the spleen ability for contraction. The spleen is a blood storage site for animals with high activities and oxygen consumption such as racehorse (Persson, Ekman, Lydin, & Tufvesson, 1973; Poole & Erickson, 2015; Wagner et al., 1995), diving seal (Thornton et al., 2001) and hunter dog (Dane et al., 2006). Horses show an extreme increase of haematocrit during exercise whereas hunter dogs present an increase in reticulocyte count (Poole & Erickson, 2015).

Erythrocytes ejection from spleen is affected by sympathetic nervous systems and catecholamines (Bergman, Heidger, & Scott-Conner, 2000; Poole & Erickson, 2015).

Bats as the flying mammals experience a daily surge of oxygen consumption. Bats consume oxygen with a similar volume to other mammals with the same size while at rest, but in the first three seconds of flying, their oxygen consumption jumps up to 10–17 times (Neuweiler, 2000; Thomas, 1975). A thin capsule with a few smooth muscles in the bat spleen does not support rapid blood ejection from spleen in order to meet oxygen requirements in the first seconds of flying. The need for oxygen in bats during flight may be fulfilled by rapid oxygen supply from blood circulation which is supported by the specialized structure of cardiovascular (Maina, 2000; Neuweiler,
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2000), superior structure and large capacity of lungs (Maina, 2000; Maina & King, 1984), high respiratory rate (Neuweiler, 2000) and large capacity of blood to carrying oxygen (Jurgens, Bartels, & Bartels, 1981).

Erythroid and myeloid cells in the red pulp of the three species of fruit bats were very few indicating low extramedullary activities which are similar to other Chiropteran species, Vespertilio orientalis reported by Tanaka (1990). Extramedullary haematopoiesis in adults can generally occur in some conditions of infection, inflammation, severe anaemia and cancer (De Back, Kostova, Kraaij, Berg, & Bruggen, 2014; Boes & Durham, 2017; Bronte & Pittet, 2013). High extramedullary haematopoiesis is also normally found in Eulipotyphla and Rodentia spleen throughout their life which is characterized by the high number of erythroid and myeloid in the red pulp (Barthold, Griffey, & Percy, 2016; Cesta, 2006; Udroiu, 2017; Ward et al., 2018). This study found no megakaryocyte in the red pulp of the three bat species. This indicates that the bat’s spleen has little or no role in thrombopoiesis. Megakaryocyte is normally found in the high number in the spleen as reported in cats, dogs, rats, mice, camels and leopard seals (Gray et al., 2006; Rothermel, 1930; Ward et al., 2018; Zidan, Kassem, & Pabst, 2000). The absence of megakaryocyte is also observed in the Cape fur seal spleen (Stewardson, Hemsley, Meyer, Canfield, & Maindonald, 1999) and African palm squirrel (Ekele & Niebedum, 2014). Melanin pigment in P. vampyrus spleen (Figure 3) was commonly found in species with black hair like in several rat species (Ward et al., 2018).

FIGURE 5 White pulp structure. Central artery (CA) is surrounded by periarteriolar lymphoid sheaths (PALS). Follicle is located adjacent to the PALS which is consists of a dark zone (corona [Co]) and a bright zone (germinal centre [GC]). Some tingible body macrophage (circle) can be found in the centre of germinal centre. Marginal zone (MZ) is the boundary zone between red pulp and white pulp which consists of lymphoid cells, blood and PAMS (arrow). Marginal sinus (rectangle) become a barrier between white pulp and marginal zone. (d, e, f) Higher magnification of tingible body macrophage (circle). (g, h, i) Higher magnification of marginal sinus. (a, d, g) Cynopterus titthaechelus, (b, e, h) Rousettus leschenaultii, (c, f, i) Pteropus vampyrus. HE

FIGURE 6 Ellipsoids structure. Capillary artery (Cap) is surrounded by macrophage (arrow) of various sizes. (a) Cynopterus titthaechelus, (b) Rousettus leschenaultii and (c) Pteropus vampyrus. Masson Trichrome
This study found that the three species of fruit bats had numerous and wide vascular space containing blood (Figures 3 and 4). Tanaka (1990) described Vespertilio’s red pulp as having a wide splenic cord and less differentiated vein belonging to a non-sinusal type. This study found that fruit bats have a wider vascular space than splenic cords that resemble sinusal type character. However, further observations using electron microscopes need to be done to confirm this finding. The sinusal type of spleens was reported in rats, humans and dogs (Press & Landsverk, 2006; Udroui, 2006; Ward et al., 2018). Mice along with various mammals such as horses, cows, cats, pigs and armadillos were classified to have the non-sinusal type of spleen (Bacha & Bacha, 2000; Galindez, Codón, & Casanave, 2000; Press & Landsverk, 2006; Snook, 1950; Ward et al., 2018).

Evolutionary, sinusal spleens have a dominance of defensive function, whereas non-sinusal spleens have a dominant role as a blood reservoir (Udroiu, 2006). The sinuses of the spleen have a special function in the filtration of damaged erythrocytes and blood pathogens (Buffet et al., 2011). Although this study has not been able to confirm the type of spleen vascular space, numerous and wide vascular space in the three fruit bats species were filled with blood indicating there is a space to reserve blood (Figures 3 and 4). The ability of the spleen of fruit bats to store blood is also supported by spleen size, which has a mid-value between rats and mice that have a wide area of splenic cord to reserve blood. C. brachyotis and R. aegyptiacus were reported to have a spleen weight ratio compared to body weight of 0.4% (Attia, El-Desouki, Madkour, Sayed, & El-Refaiy, 2007; Hanadhita, Rahma, Prawira, Sismin Satyaningtias, & Agungpriyono, 2018), while rats were 0.2%–0.3% (Cesta, 2006) and mice 0.7% (Webster & Liljegren, 1977). Sinusal spleens with blood-storing ability are present in camels (Zidan, Kassem, Doubag, et al., 2000), hunter dogs (Dane et al., 2006) and humans from bajau tribe (Ilardo et al., 2018). Chiropteran order had been reported to store blood in the spleen during torpor and hibernation (Nuweller, 2000; Lidicker & Davis, 1955). Myotis sodalis is reported to have a six times larger spleen and less circulating erythrocyte counts in the inactive phase than when active (Lidicker & Davis, 1955). Bats are endotherms as the other mammals. Bats with small body mass need to consume a lot of food relative to their body mass to maintain their energy requirements. However, during extreme climate and lack of food supply, bats were able to regulate their temperature to conserve energy by undergoing torpor and hibernation (Altringham, 2011). Small pteropodids (body weight 16–18 g) in tropical and subtropical climate with warm ambient temperatures can also undergo daily torpor especially when the food source is scarce (Coburn & Geiser, 1998; Geiser & Stawski, 2011; Kelm & Von Helversen, 2007; McNab & Bonaccorso, 2001; Stawski, Willis, & Geiser, 2014). C. titthaeceillus and R. leschenaultii were medium size pteropodids and the observations done of this two genus showed that they did not undergo daily torpor (Barclay et al., 2017; McNab & Bonaccorso, 2001). Meanwhile, P. vampyrus is a large size pteropodid which is always at positive energy state; therefore, it does not require torpor (McNab & Bonaccorso, 2001); and instead, they fulfil their energy by foraging food all the time (Hengjan et al., 2018). However, with the capacity of the red pulp vascular space to store blood, all three species of bats might undergo torpor during extreme conditions or in condition when the food is lacking.

The white pulp of the three species of bats spleen consists of PALS, lymphoid follicles and MZ (Figure 5). Among PALS and marginal zone, there is marginal sinus as the entrance gate of blood into the marginal zone (Figure 5g–i). PALS is the area of lymphocyte aggregation that surrounds the central artery. PALS is mostly dominated by T-cell lymphocytes but dendritic cells can also be found (Steiniger & Barth, 2000). The group of lymphocyte cells adjacent to PALS which has a dark zone (corona) with a bright zone in the middle (germinal centre) is a lymphoid follicle. Corona is a zone of B cells that have not been activated by antigens. It has dense nuclei and smaller cytoplasm that form a dark zone. The germinal centre consists of B cells that have been activated by antigens (Steiniger & Barth, 2000). Follicle lymphoid in all three bat species appeared to show active immune activity because it has a germinal centre (Figure 5) which is commonly found in wild animals exposed to antigens from the outer environment. Germinal centre cells in bats that are kept in the laboratory under conventional condition are positive for anti-bat IgG antibody which indicates activated immunity (Omatsu, Watanabe, Akashi, & Yoshikawa, 2007). B cells that fail to produce immunoglobulins, experience apoptosis and engulfed by macrophages or called tingible body macrophages (Steiniger & Barth, 2000) can also be found in the centre of the germinal centre of the three bat species (Figure 5d–f).

Marginal zones in the three bat species are prominent. Rats are also reported to have wide and prominent marginal zones whereas mice have a thin marginal zone (Ward et al., 2018). Marginal zones are an important zone for recognition and phagocytes of bloodborne antigens which may induce innate immunity and also the production of antibodies in the marginal zone (Gatto, Ruedi, Odermatt, &
Although the role of vascular space in the spleen cannot be ascertained, the spleen is expected to have a large role in blood pathogen filtration. Marginal zone B cells play a role in the first recognition of viral infection and then produce local antibodies to opsonize pathogens that enter the spleen (Gatto et al., 2004). Marginal zone macrophages especially have surface receptors that can recognize major components of various enveloped bacteria and several types of viruses (Meibius & Kraal, 2005; Oehen et al., 2002).

Ellipsoids are the groups of macrophages that surround the arterial capillary of the spleen (Figure 6). Ellipsoids of the three bat species were found numerous and spread in splenic cord with the largest population near the marginal zone (Figures 3 and 7). The ellipsoid structure was not reported in other bat species, Vespertilio orientalis (Tanaka, 1990). Some ellipsoids gathered and closed together forming an aggregation of macrophages which form white pulp-like structure (Figure 3). Ellipsoids can be found in almost all vertebrates except rodents (Steiniger, 2015). Moles, pigs, cats and dogs have numerous and wide ellipsoids, while horses, cows and sheep have medium ellipsoids sizes (Bacha & Bacha, 2000; Blue & Weiss, 1981; Press & Landsverk, 2006; Snook, 1950). Ellipsoids have a strategic function as filtration of bloodborne pathogen as well as marginal zones (Blue & Weiss, 1981; Boes & Durham, 2017; Steiniger, 2015) and act as a major site of blood pathogen filtration in soft-shelled turtles and chickens that do not possess a marginal zone (Bao, Li, Wang, Qin, & Xu, 2009; Zhang et al., 2015). A large number of ellipsoids in the fruit bats indicate that the central arteries branch out many capillaries. A large number of capillaries with narrow lumen make a more thorough screening of the blood-borne pathogen by macrophages of ellipsoids.

6 | CONCLUSION

The thin collagen and few smooth muscles of splenic capsule and trabecula of the three species make the spleen of the fruit bats does not have a good contraction ability to rapid blood ejection. However, a lot of wide vascular space make the fruit bats spleen have a blood storage capacity that also allows storage functions. The spleen of the three bat species does not play a major role in extramedullary haematopoiesis and thrombopoiesis. As a viral reservoir, the bat spleen is expected to have a large role in blood pathogen filtration. Although the role of vascular space in the spleen cannot be ascertained, the existence of the marginal zone and numerous ellipsoid structures may support the role of splenic filtration in fruit bats.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest concerning the research, authorship and publication of this article.

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