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The immune system of the ferret (*Mustela putorius furo*) – A review

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Abstract: The basic information dealing with the anatomy of the ferret's immune system, cross-reactivity of the ferret leukocytes with polyclonal and monoclonal antibodies *in vitro* and immune response to the mitogens and various infections are presented. The leukocyte numbers in the peripheral blood in the ferrets are lower compared to other species and only one subclass of IgG has been identified in ferrets so far. Lymphocytes make up 12–67% of all the leukocytes in the peripheral blood of the healthy adult ferrets. Lymphocyte subpopulations are similar to other mammals and include T- and B-lymphocytes. T-lymphocytes differentiate into helper (Th) lymphocytes and cytotoxic (Tc) lymphocytes. Currently, ferret granulocytes (CD11), B-lymphocytes (CD79α), T-lymphocytes (CD3), Th-lymphocytes (CD3, CD4), Tc-lymphocytes (CD3, CD8), and CD30, CD45 subpopulations are detected with the use of a number of polyclonal as well as with monoclonal antibodies. In a lymphocyte transformation assay, the mitogen response of the peripheral blood mononuclear cells to concanavalin A (ConA), phytohaemagglutinin (PHA), and pokeweed mitogen (PWM) is the greatest at day 2, 2 and 3, respectively. Serious lymphopenia is observed in ferrets during a distemper infection. A significant decrease in the lymphocyte transformation activity is observed on day 5 and reaches a maximal decrease on days 8–11, with full recovery on days 23–30 after the inoculation of laboratory ferrets with the distemper virus. Ferrets have also been used in studies related to the function of the immune system in *Helicobacter pylori* infections, Crohn's disease and bronchial asthma.

Keywords: cross-reactive reagents; immunology; immune response; *Mustelidae*

Introduction

Ferrets are used worldwide as pets and models for medical and pharmaceutical research (Fox 1999a; Mayer et al. 2015). Nowadays, ferrets are indispensable for studying infectious diseases, non-infectious diseases, the development of vaccines, and testing drug effects. Ferrets have found a place in almost all medicine specialty research where the use of different animal species is limited; such as *Helicobacter mustelae* infections that are similar to human *Helicobacter pylori* infections (Fox et al. 1990; Clyne et al. 2000; Swennes and Fox 2014), cardiology research (Schumacher et al. 1996; Morgan and Travers 1999; Liang et al. 2000), molecular medicine, ophthalmology and neurosci-

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ence (Kawasaki et al. 2004; Lu et al. 2018; Schwerin et al. 2018; Fujishiro et al. 2020).

Recently, their use in biomedical research has shown a remarkable increase (Belser et al. 2011; Cameron et al. 2012; Mayer et al. 2015; Otte et al. 2016; Patterson and Fox 2017; Albrecht et al. 2018; Gollakner and Capua 2020; Kim et al. 2020). Despite the increased interest in ferrets, there is still little information on the immune system of ferrets and its response to stimuli *in vitro* and the immune response to infection *in vivo*. The aim of this review is the presentation of the current information concerning the basic anatomy of the ferret's immune system, cross-reactivity of the ferret's leukocytes with polyclonal and monoclonal antibodies *in vitro* and the immune response to mitogens and various infections.

Ferret's immune system

The immune system protects the body against pathogens, fights off infections, gets rid of non-functional cells, and together with the endocrine and nervous system creates homeostasis (Trebichavsky et al. 2000). The structure of the immune system of ferrets is similar to that of other mammals and consists of a thymus, a spleen, lymph nodes, tonsils, bone marrow and leukocytes in the peripheral blood.

THYMUS

The ferret's thymus is located within the thoracic inlet in the cranial mediastinum. A branch of the brachiocephalic artery at the level of 3rd rib supplies blood to the thymus (Fox 2014). Wu et al. (2012) performed a study involving the ferret's thoracic anatomy using computer tomography (CT) and positron emission tomography (PET) scans as forms of *in vivo* imaging methods. A respectably-sized thymus was identified on the CT scans in the 4–6 month-old ferrets, but no exact measurements were given. The PET scans showed the thymus to be the second most metabolically active organ in the ferrets' thoracic cavity in this age group (Wu et al. 2012). Published pathologies of the thymus in veterinary medicine of ferrets are scarce to date (Taylor and Carpenter 1995; Mayer et al. 2014; Parry 2018).

SPLEEN

The ferret's spleen has shape of a half-moon (Evans and An 1999). It is consistent in colour and smooth on the surface. It is located in the left hypogastrium copying the great curvature of the stomach and is attached to the liver and stomach by the *ligamentum gastrosplenicum* as a part of the *omentum* (Powers and Brown 2012). It can slightly change its localisation according to the position of the stomach and its content. The normal size of a spleen in adult ferrets is 5.1 cm × 1.8 cm × 0.8 cm in length, width, and thickness. Splenomegaly is a common finding in ferrets with inflammatory conditions, especially of the gastrointestinal tract (Williams and Wire 2020), with cardiomyopathy, especially congestive heart failure (Ellis 2006), and when extramedullary haematopoiesis (EMH) is present. Although it is unclear why, some ferrets possess extramedullary haematopoiesis in the spleen throughout their lifetime (Lennox 2012). It possibly represents a compensatory mechanism for the decreased bone marrow function or increased need for blood cells. Some authors believe that EMH might be an adaptation mechanism for oestrogens' bone marrow suppression and chronic inflammation (Sherill and Gorham 1985; Williams 2010; Mayer et al. 2014). Evaluation of the splenic size should be undertaken prior to anaesthesia as isoflurane causes sequestration of red blood cells (RBCs) in the spleen (Ko and Marini 2011). When fully enlarged, it extends diagonally from the upper left to the lower right abdomen while crossing the midline (Powers and Brown 2012). Microscopically, the spleen is divided into white pulp (immune response) and red pulp (lymphopoiesis, red blood cell apoptosis). In this species, red cell production in the spleen is thought to occur throughout the animal's life (Lennox 2012). The spleen is vascularised by the splenic artery that is branch of the celiac artery. Blood drainage is managed by the splenic vein that drains into the gastro splenic vein and then into the portal vein. Its innervation comes from the celiac plexus (Evans and An 1999).

LYMPH NODES AND TONSILS

Ferrets possess a vigorous system of lymph nodes. Major peripheral lymph nodes include a pair of submandibular lymph nodes, a medial retropha-

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ryngeal lymph node, a pair of axial lymph nodes, a pair of femoral lymph nodes, and a pair of popliteal lymph nodes. The submandibular lymph nodes are located just in front of the mandibular salivary gland which might make it confusing to recognise these structures (Powers and Brown 2012). None of these nodes should be palpable in healthy ferrets (Antinoff and Hahn 2004). In ferrets, the mediastinum is believed to be complete (Powers and Brown 2012) and the mediastinal lymph nodes include the anterior mediastinal lymph node (Lewington 2007), paratracheal lymph node and subcarinal lymph node (Wu et al. 2012). The abdominal cavity has multiple small lymph nodes. There is a single palpable lymph node (known as the jejunal lymph node or cranial mesenteric lymph node) (Suran et al. 2017) in the mesentery that has been studied (Paul-Murphy et al. 1999) and is consistent with the one found in minks. It is located at the junction of the cranial and caudal mesenteric veins and embedded in fat at the root of the small intestine mesentery, with the size in young adult ferrets being 12.4 ± 2.4 mm in length by 6.9 ± 2.0 mm in width (Paul-Murphy et al. 1999). In a later study (Garcia et al. 2011), the pancreaticoduodenal (5.29 ± 1.32 mm in length), splenic (5.93 ± 1.59 mm in length), gastric (7.7 ± 2.6 mm in length) and hepatic lymph nodes were identified in ferrets with average age of 3 years. Additional lymph nodes were later identified using ultrasound; specifically, the caudal mesenteric, medial iliac and lumbar aortic nodes (Suran et al. 2017). Abdominal lymph node enlargement is common in ferrets with gastrointestinal disease, lymphosarcoma or chronic exposure to enteric coronavirus referred to as reactive lymphadenopathy, especially in older ferrets (Paul-Murphy et al. 1999; Mayer et al. 2014; Suran et al. 2017). The retropharyngeal and jejunal lymph nodes are found to be main sites for lesions in animals infected with tuberculosis (Cross et al. 2000).

There is a paired palatine tonsil (accumulation of lymphatic tissue) located in the tonsillar fossa near the ventral sulcus of the soft palate. It is an ovoid structure measuring 6.5 cm in length, 0.25 cm in width, and 0.01 cm in thickness. Histologically, ferret lymph nodes are equivalent to those of dogs and cats; structured as an outer layer being a rich cellular cortex with follicles and a perifollicular cortex. The predominant cell in both areas seems to be a small lymphocyte; however, lymphoblasts are present in some areas (Paul-Murphy et al. 1999). The medulla is a thin inner layer consisting of cords primarily made up of small lymphocytes. Histiocytic cells have been seen in the sinuses. A significantly higher number of eosinophils (no increase in the peripheral blood was observed) have been found in ferret lymph nodes compared to those of dogs and cats (Duncan 1989; Paul-Murphy et al. 1999).

BONE MARROW

The bone marrow is the main site of postnatal haematopoiesis where cells of the immune system, especially B-lymphocytes, differentiate from the progenitor pro-B-cell and mature ones. Matured lymphocytes leave the bone marrow and migrate to the secondary organs of the immune system (spleen and lymph nodes).

An immune-histochemical analysis of bone marrow was carried out using a CD3 marker for T-cells and a CD20 marker for B-cells. It revealed that most of the lymphoid cells in the ferret's bone marrow were mature T-cells (Vidana et al. 2014).

CELLS OF IMMUNE SYSTEM AND THEIR SURFACE MARKERS

Generally, the leukocyte values in the peripheral blood are usually lower in ferrets compared to other species (Siperstein 2008; Quesenberry and Orcutt 2012). Ferret neutrophils appear similar to that of dogs and cats (Campbell and Ellis 2007). Eosinophils are easy to differentiate from neutrophils as they are the only granulocytes that have eosinophilic cytoplasmic granules. Basophils tend to have lobed nuclei with basophilic cytoplasmic granules; they do not differ from those of dogs and cats. Monocytes are the largest of leukocytes with round to lobed nuclei and a moderately abundant light blue cytoplasm. Lymphocytes may vary in size, cytoplasmic colour, and degree of chromatin condensation; with small lymphocytes being inactive. B-cell lymphocytes have a more abundant basophilic cytoplasm and irregular nuclei. Large lymphocytes with an abundant light blue cytoplasm and azurophilic granules are considered to be T-cell lymphocytes or natural killer cells (Campbell and Ellis 2007). Lymphocytes make up 12–67% of all the leukocytes in the peripheral blood of healthy adult ferrets (Table 1).

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Table 1. Reference leukocyte values in ferrets

Values	Knotkova (2017)	Smith et al. (2015)		
	adult animals of mixed sex	adult males	adult females	young ferrets (2–3 months old)
Leukocytes	2.5–10.8 10^9/l	4.4–15.4 10^9/l	4–18.2 10^9/l	5.3–12.6 10^9/l
Neutrophils	0.6–7.0 10^9/l	24–76%	43–78%	46.1–76.6%
Lymphocytes	1.7–4.7 10^9/l	12–66.6%	12–67%	42.2–68.2%
Eosinophils	0.1–0.8 10^9/l	0–8.5%	0–8.5%	2.1–6.9%
Basophils	0–0.2 10^9/l	0–3%	0–2.9%	0–1.3%
Monocytes	0–0.4 10^9/l	0–8.2%	1–6.3%	0.7–4.7%

Basic data are available concerning cross-reactive reagents with ferret lymphocytes (Albrecht et al. 2018; Wong et al. 2019). Currently, there is only one commercially available IgG antibody that is specific to ferrets (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). It is usable for studies with samples of fresh lymphocytes, thus limited in applicability for clinical practice (Coleman 1997). The surface markers of ferret lymphocytes have been defined using the heterologous antibodies (Table 2). Lymphocytes include T-cells and B-cells. T-lymphocytes differentiate into helper (Th) lymphocytes and cytotoxic (Tc) lymphocytes. The first study identifying antibodies valuable for the detection of ferret lymphocytes was undertaken in 1997 – the recognition of T-lymphocytes and B-lymphocytes was successful using the polyclonal rabbit anti-human CD3 antibody and monoclonal mouse anti-

Table 2. Antibodies cross-reacting with ferret lymphocytes

Antibody	Clone	Source	Dilution	Reference
CD3	Rabbit anti-human Pab** A0452	Dako, USA	N/A***	Ammersbach et al. (2008); Gupta et al. (2010); Ingrao et al. (2014)
CD3	Rabbit anti-human Pab**	Dako, Denmark	1 : 100	Hammer et al. (2007); Coleman (1997)
CD3	Mouse anti-human Mab*	Dako, Japan/USA	1 : 25	Onuma et al. (2008); Blomme et al. (1999)
CD3	Mouse anti-human Mab* F7.2.38	Dako, Denmark	1 : 100	Hammer et al. (2007)
CD3	Mouse anti-human Mab* PS1	Novocastra, UK	1 : 100	Hammer et al. (2007)
CD79α	Mouse anti-human Mab* HM57	Dako, USA/Denmark	1 : 100	Ammersbach et al. (2008); Hammer et al. (2007); Coleman (1997); Gupta et al. (2010)
CD79α	Anti-human	Dako, Japan	1 : 25	Onuma et al. (2008)
BLA.36	A27-42	BioGenex, USA	N/A***	Ammersbach et al. (2008)
BLA.36	N/A***	Dako, USA	1 : 10	Blomme et al. (1999)
CD45RO	Mouse anti-human Mab* UCHL1	Dako, Denmark	1 : 20	Hammer et al. (2007)
CD45	Mouse anti-human Mab* 2B11	Dako, Denmark	1 : 20	Hammer et al. (2007)
CD30	Mouse anti-human Mab* Ber-H2	Dako, Denmark	1 : 20	Hammer et al. (2007)
Bcl2	Mouse anti-human Mab* 124	Dako, Denmark	1 : 80	Hammer et al. (2007)
Bcl10	Mouse anti-human Mab* 151	Dako, Denmark	1 : 20	Hammer et al. (2007)
MUM-1	Mouse anti-human Mab* MUM1	Dako, Denmark	N/A***	Hammer et al. (2007)
Vimentin	Mouse anti-swine Mab* V9	Dako, Denmark	1 : 1 000	Hammer et al. (2007)
Ki-67	Mouse anti-human Mab* M1B-1	Dako, Denmark	1 : 40	Hammer et al. (2007)
P53	Rabbit Pab** CM1	Novocastra, UK	1 : 80	Hammer et al. (2007)
λ light chain	Rabbit anti-human Pab** A0193	Dako, USA	N/A***	Gupta et al. (2010)

*Mab = monoclonal antibody; **Pab = polyclonal antibody; ***N/A = not known

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human CD79α antibody (Coleman 1997). Since that time, various clones of antibodies have been used: for CD79α clone CB3-1 (Kirchenbaum and Ross 2017) and clone HM47 –PerCP-Cy5.5, eBioscience, San Diego, CA, USA (Music et al. 2016) were used, and for CD3 clone PC3/188A –FITC and AlexaFluor 647, Santa Cruz Biotechnology, Santa Cruz, CA, USA were used (Music et al. 2016). They recognise the intracellular epitope on the conserved region of a cell, therefore, they are considered to be analogous. This epitope is present on cells from early development to late maturation, which makes it possible to mark all the stages of the T- and B-lymphocytes (Fox 1999b). B-lymphocytes can be further recognised by polyclonal *kappa* and *lambda* light chain immunoglobulin antibodies (Kirchenbaum and Ross 2017). Ferret T-lymphocytes can also be marked with the anti-human CD8 (clone OKT8 – eFluor 450; eBioscience, San Diego, CA, USA) along with Thy 1.1 (clone OX-7) antibodies (Rutigliano et al. 2008; Music et al. 2016). Cheng et al. (2013) produced monoclonal ferret-specific antibodies for their study of cellular and humoral immune responses concerning influenza vaccines. In this study, the ferret specific monoclonal antibodies CD8 and CD5 were created by immunisation of BALB/c mice with ferret thymocytes. CD4 was produced from hybridoma cells from mice splenocytes that were immunised with a recombinant ferret CD4 protein. The CD4 antibody was additionally created for its study purpose from female BALB/c mice (DiPiazza et al. 2016). In a different study, a monoclonal antibody that recognised ferret CD4 (clone 02 – PE; Sino Biological Inc., Beijing, P.R. China) was used (Music et al. 2016). Recently, a specific anti-ferret monoclonal CD4 (FeCD4) antibody has been developed (Layton et al. 2017). The first study combining the use of CD4 and CD8 antibodies in order to differentiate between the subset of T-lymphocytes was carried out in 2016 (DiPiazza et al. 2016). It was found that, in ferret tissues (except the spleen), the CD4 to CD8 ratio was approximately 2 : 1. In the same study, B-cells were identified using a polyclonal antibody, recognising ferret IgA, IgM and IgG isotypes as well as CD20 and CD79α.

LYMPHOCYTE TRANSFORMATION ASSAY

Lymphocytes play a role in the adaptive immune response, which emerges when triggered by an antigenic stimulus. One of the crucial functions of lymphocytes is to respond to antigenic stimulation with proliferation. In physiological situations, lymphocytes start to synthesise the DNA after cross-linking their antigen receptor followed by recognition of the antigen. The same response can be evoked *in vitro* by polyclonal activators – mitogens (Crevel 2005). Little information about ferret lymphocyte reactivity to mitogens is available due to the scarcity of their cross-reactive reagents. In ferrets, concanavalin A (ConA), phytohaemagglutinin (PHA), and pokeweed mitogen (PWM) can trigger the proliferation of lymphocytes (Kauffman et al. 1978) with PHA being the least toxic for ferret lymphocytes (von Messling et al. 2003).

For the lymphocyte transformation assay (LTA) in ferrets, foetal bovine serum (FBS) is used. Peripheral blood mononuclear cells are isolated and incubated for 2–5 days for mitogen response and 4–8 days for specific antigens. The mitogen response is the greatest on day 2 for ConA and PHA, and on day 3 for PWM. The proliferative responses are maximal on day 5. It was suggested that the response in the lymphocytes in the spleen is the same as in the peripheral ones, but they are rather T-lymphocytes than B-lymphocytes (Kauffman et al. 1978). Another study showed poor mitogen response of splenic lymphocytes (McLaren and Butchko 1978). Later on, variables of the lymphocyte transformation assay (LTA) on ferrets were used to study innate and adaptive immune responses. As an example, the lymphocyte transformation response was studied for *Mycobacterium bovis* and *M. avium* infections with an increase in the response for *M. bovis* infection only (Cross et al. 2000). Another LTA for ferret mononuclear leukocytes was performed by von Messling et al. (2003), where a different response was seen by comparing the innate and acquired immunity.

IMMUNOGLOBULINS

Immunoglobulins (Igs) or antibodies are proteins used by the immune system to recognise and destruct pathogens. These are part of the humoral immune response as they detect part of the microbe called an antigen. In ferrets, five immunoglobulin isotypes were identified (IgM, IgG, IgE, IgA, and IgD). All but IgD were found to be homologous in sequence to human and canine isotypes (Wong

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et al. 2020). So far, only one subclass of IgG has been identified in ferrets (Wong et al. 2019) when compared to four IgG subclasses in minks and other carnivores (Tabel and Ingram 1972). The estimated molecular weights of the immunoglobulin gamma, alpha, and mu heavy chains are 54 kDa, 69 kDa and 83 kDa, respectively (Martel and Aasted 2009). For ferret IgG, IgM, and IgA, cross-reacting polyclonal antibodies were identified; a rabbit antibody prepared to mink IgG (Aasted 1989), a rabbit anti-human IgM (Dako, Glostrup, Denmark; A0425), and a goat antibody prepared to canine IgA (AbD Serotec, Oxford, UK; AAI31) (Martel and Aasted 2009).

VIRAL-INDUCED IMMUNOSUPPRESSION

The ferret's place in virology research has mostly been seen in vaccine development (Pearson and Gorham 1999; Shoji et al. 2009; Pillet et al. 2011; Cameron et al. 2012; Oh and Hurt 2016; Stittelaar et al. 2016; Chan et al. 2018) and in studies of various viral diseases (Parker et al. 2013), especially SARS infections, where transmission among animal groups (Martina et al. 2003), the clinical progression and evaluation of treatments (Chu et al. 2008) have been evaluated. The most recent focus is being seen in COVID-19 (Gollakner and Capua 2020; Kim et al. 2020; Shi et al. 2020). It became obvious in the early 1900s that ferrets can suffer from diseases similar to those of humans; specifically, the first research studies involving ferrets were focused on the influenza virus (Pyle 1940; Maher and DeStefano 2004; Jin et al. 2007; Matsuoka et al. 2009; Belser et al. 2011; Otte et al. 2016; Albrecht et al. 2018).

Influenza virus infections in ferrets have been widely studied and it is implied that the infection does not induce changes in the lymphocyte transformation (Kauffman et al. 1978). In bronchoalveolar lavage (BAL) of ferrets infected with influenza, a positive staining of anti-mouse CD44 clone IM7 (trait of T-cell activation) appeared. The same study showed positive staining of anti-mouse CD11b (clone M1/70-FITC; eBioscience, San Diego, CA, USA). Lymphocytes (marked with CD8 and Thy 1.1) followed by monocytes (marked with CD11b) (Rutigliano et al. 2008) were the predominant cells in inflammatory BAL.

Ferrets are susceptible to canine distemper virus. In ferrets, 100% mortality rates have been reported with acute onset (Perpinan et al. 2008). Prevalence depends on the area and rate of the vaccinated animals (Perpinan et al. 2008; Wyllie et al. 2016). It is known that the distemper virus causes the fatal suppression of the cell mediated immune response (von Messling et al. 2003). The initial infection occurs in the epithelial cells and primary replication takes place in the lymphatic tissue of the respiratory tract (von Messling et al. 2003). A study was carried out to monitor changes in the cell-mediated immune response caused by distemper in ferrets (Kauffman et al. 1982). A significant decrease in the lymphocyte transformation activity in LTA was observed starting on day 5 and reaching the maximal decrease on days 8–11 followed by a steady function increase with full recovery on days 23–30 after the inoculation. Serious lymphopenia was observed in all the ferrets during infection (Kauffman et al. 1982).

IMMUNOSUPPRESSION AND OTHER INFECTIOUS DISEASES IN FERRETS

Studies of the ferret's immune system function regarding various infectious diseases, especially *Helicobacter pylori* infections (Fox et al. 1990; Clyne et al. 2000; Swennes and Fox 2014), Crohn's disease together with the still unclear pathogenesis of inflammatory bowel disease (Osborne et al. 1993; Warren and Watkins 1994) and the inflammatory reaction of the ferret's immune system in bronchial asthma (Szelenyi 2000) have been published.

Conclusion

Despite the fact that a large amount of new information about the organs and cells of the ferret immune system can be accessed, certain important facts are still absent.

Moreover, the lack of verified and time-proved immunological assays hinders the understanding of immune responses in ferrets. Recent years have brought a decent number of various reagents applicable for laboratory experiments with ferret lymphocytes and for new polyclonal antibodies cross-reacting with ferret immunoglobulins. The practical use of those reagents opens new horizons for basic research as well as for practical use in clinical immunology.

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Conflict of interest

The authors declare no conflict of interest.

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