

— Report —

Monoclonal antibodies against leukocytes in the beluga whale, *Delphinapterus leucas*

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To better understand cetacean immunology, it is important to develop markers that identify specific leukocyte populations. We created a monoclonal antibody (mAb) library against leukocytes of the beluga whale (*Delphinapterus leucas*), and established five hybridoma clones that produce mAbs. Three of these mAbs (ID: BW-3C3, BW-2G4, and BW-2G6) react with mononuclear leukocytes including lymphocytes and monocytes. mAb BW-3C3 react to a fraction of the lymphocytes. The mAb-positive cells were identical to cells that also stained with polyclonal anti-whale IgM antibodies, indicating that the mAb BW-3C3 specifically reacts to B lymphocytes. mAb BW-2G4 specifically binds to monocytes that possess a reniformed nucleus. mAb BW-2G6 was found to bind to heterogeneous lymphocytes, namely, anti-whale IgM antibody-positive and -negative lymphocyte populations. This indicates that this mAb reacts with B and non-B lymphocyte fractions. The other two mAbs (ID: BW-4B10 and BW-4G12) react with polymorphonuclear granulocytes. Double staining with Giemsa-eosin showed that mAb BW-4B10 and mAb BW-4G12 specifically identify neutrophils and eosinophils, respectively. This panel of mAbs will be a useful tool for classifying leukocytes and for determining their localization in different tissues, which in turn would contribute to our understanding of cetacean immunology and allow evaluation of leukocyte function in infectious diseases.

Keywords : leukocyte, monoclonal antibody, beluga whale, cetacean, top predator

Received 6 February 2018 ; Revised 29 May 2018 ; Accepted 6 June 2018

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1. Introduction

Marine mammals are attracting attention from various fields of biology, such as evolutionary biology and physiology from the view point of shifting from land to marine environments, and from ecology as the top or important predators. Some species, however, are endangered and their conservation is becoming a serious worldwide concern. Reports of threats to marine mammals from infectious diseases are accumulating. For example, morbillivirus has caused a number of epidemics with high mortality in cetaceans (Van Bressem et al., 2014). The virus is known to induce lymphoid depletion and immunosuppression in infected animals (McChesney and Oldstone, 1987). Dolphins with positive morbillivirus antibodies have lower lymphocyte proliferation rates and a decreased number of lymphocytes (Bossart et al., 2011). A better understanding of the immune system and how it combats infectious diseases may therefore contribute to the conservation of marine mammals.

Despite the fact that cetaceans share anatomically common organs and tissues with land mammals, they possess unique lymphoid tissues and circulatory systems (Cowan and Smith, 1999). The two arms of immunity, innate and acquired, protect mammals against pathogens, and leukocytes play an important role in both. Polymorphonuclear granulocytes and monocytes/macrophages are involved in innate immunity, whilst lymphocytes play a major role in acquired immunity. These leukocytes can be identified morphologically in the peripheral blood of cetaceans (Bossart et al., 2001); however, specific markers for the different cell types are not available. Some cross-reactive land mammal-specific antibodies have been shown to be effective for phenotyping cetacean leukocytes (Kumar and Cowan 1994, De Guise et al., 1997, Jaber et al., 2003, Nouri-Shirazi et al., 2017). However, access to specific monoclonal antibodies (mAbs) against cetacean leukocytes would allow researchers to better understand cetacean immunology. Some monoclonal antibodies against leukocytes in the beluga whale (*Delphinapterus leucas*) and bottlenose dolphin (*Tursiops truncatus*) have been reported (De Guise et al., 2002, Kato et al., 2009). To begin to establish a systematic panel of monoclonal antibodies for cetaceans, we created a monoclonal antibody library using leukocytes from the beluga whale. In the present study, we report

five novel monoclonal antibodies that specifically bind to B lymphocytes, monocytes, neutrophils, and eosinophils. All these mAbs are available from the Japan Agency for Marine-Earth Science and Technology (JAMSTEC).

2. Materials and Methods

2.1 Collection of whale leukocytes

Beluga whales were reared at the Port of Nagoya Public Aquarium, in a pool with a close-circulatory system kept at 15–16°C throughout the year. Peripheral blood was collected from presented flukes using a syringe (50 ml, Terumo Co. Ltd., Tokyo) and butterfly needle (18G, Terumo Co. Ltd., Tokyo). To morphologically identify different blood cell types, heparinized whole blood samples were smeared onto glass slides, stained using the May-Grunwald Giemsa method, and observed under a Nikon microscope (Eclipse E5600, Nikon Co. Ltd., Tokyo, Japan). Whole blood diluted with Hanks' balanced salt solution (Invitrogen Co. Ltd., Auckland, New Zealand) was overlaid onto Histopaque1119 (Sigma-Aldrich Co. Ltd., St. Louis, MO), and centrifuged at $180 \times g$ for 5 min at 4°C. Harvested leukocytes were used as antigens to inject into mice, or for detection of mAbs by immunofluorescence staining.

2.2 Construction of the monoclonal antibody library

Leukocytes collected from an adult male beluga whale (DL-001) were used as antigens to be inoculated into mice. mAbs were generated using a conventional polyethylene glycol (PEG) method (Galfre and Milstein, 1981) as previously described (Nakamura et al., 2013). Briefly, beluga leukocytes (1×10^6 cells) were injected into BALB/c mice, three times at 2-week intervals. On day 4, after the third injection, splenic lymphocytes were collected from the mice and fused with cells from a murine myeloma cell line, NS-1. The fused hybridoma cells were incubated in hypoxanthine-aminopterin-thymidine selective medium (GIT medium containing 100 μ M hypoxanthine, 0.4 μ M aminopterin, and 16 μ M thymidine; Wako Pure Chemical Industries, Osaka, Japan) for 10 days. Antibody production in the supernatants of the hybridoma cell cultures was examined by immunofluorescence staining using 2% paraformaldehyde-fixed leukocytes from beluga whales. Cloning was performed three times for the immunofluorescence-positive hybridoma cells using either a

limited dilution or methyl cellulose method.

2.3 Characterization of mAbs by immunofluorescence staining

Leukocytes were fixed with 2% paraformaldehyde in PBS for 30 minutes, then were applied to glass slides coated with 0.01% poly-L-Lysine (Sigma-Aldrich Co. Ltd, St. Louis, MO). The cells were then incubated with supernatants from the hybridoma cell cultures at room temperature for 2 hr. After washing three times with phosphate-buffered saline (PBS), the cells were incubated with an anti-mouse IgG goat antibody conjugated to Alexa Fluor 488 or 594 (Life Technologies Co. Ltd., Wyman, MA) at a 1000-fold dilution at room temperature for 1 hr. After washing three times with PBS, nuclear DNA was stained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich Co. Ltd, St. Louis, MO). For typing of polymorphonuclear granulocytes, the immunofluorescence-stained cells were subjected to Giemsa-eosin based staining using Hemacolor (Merck Co. Ltd., Darmstadt, Germany). A Keyence fluorescence microscope (BZ-9000, Keyence Co. Ltd., Osaka, Japan) and a Nikon fluorescence microscope (Optiphot, Nikon Co. Ltd., Tokyo, Japan) were used for observation.

2.4 Construction of anti-whale IgM polyclonal antibody

The polyclonal antibody was constructed by Nippon Bio-test Laboratories Inc. (Tokyo, Japan). Briefly, whale immunoglobulin proteins were obtained from the sera of Antarctic minke whales (*Balaenoptera bonaerensis*), collected using an ammonium sulfate precipitation method. After confirmation via SDS-polyacrylamide gel electrophoresis, the IgM fraction was obtained using gel filtration chromatography. Rabbits were inoculated twice

with IgM proteins together with complete Freund's adjuvant. Rabbit sera were collected and purified by ion exchange resin. The polyclonal antibody was diluted 200-fold and added to the whale leukocytes to identify B lymphocytes, according to the same protocol as for immunofluorescence staining for mAbs. Anti-rabbit IgG goat antibody conjugated to Alexa Fluor 488 (Life Technologies Co. Ltd., Wyman, MA) was used as a secondary antibody.

3. Results

We picked 503 hybridoma clones into selection medium, and confirmed reactivity to beluga whale leukocytes for 179 of the clones by immunofluorescence staining. From these, we identified five clones that reacted to specific leukocyte populations (Table 1).

Smears of whole peripheral blood from beluga whales revealed many erythrocytes and heterogeneous leukocyte populations: mononuclear leukocytes (lymphocytes and monocytes), and polymorphonuclear granulocytes (neutrophils and eosinophils) (Fig. 1). Basophils were rarely found in the peripheral blood. Leukocytes were separated by density gradient centrifugation, and mononuclear cells (approximately 30%) and polymorphonuclear cells (approximately 70%) could be clearly recognized after DAPI staining (Fig. 2b, 3b and 4b). The mononuclear leukocytes contained lymphocytes (approximately 80%) and larger-sized monocytes with reniform nucleus (approximately 20%).

Signals from mAb BW-3C3 were seen in a fraction of lymphocytes (approximately 30%, Fig. 2a, b) that were detected on the cell membrane (Fig. 2c). The mAb-positive cells were identical to cells that stained positively with

Table 1. Reactivity of mAbs to leukocytes in beluga whales.

mAb ID No.*	Leukocytes reacted with mAb**				
	Monocyte	Mononuclear leukocyte		Polymorphonuclear granulocyte	
		Lymphocyte		Neutrophil	Eosinophil
		B lymphocyte	non-B lymphocyte		
BW-3C3	–	+	–	–	–
BW-2G4	+	–	–	–	–
BW-2G6	–	H	H	–	–
BW-4B10	–	–	–	+	–
BW-4G12	–	–	–	–	+

H indicates heterogeneity, i.e., mAb reacts to heterogeneous populations.

*, mAb and hybridoma cell line available from Japan Agency for Marine-Earth Science and Technology (JAMSTEC).

**, reactivity confirmed in leukocytes from three individuals.

polyclonal anti-whale IgM antibodies (Fig. 2d), indicating that mAb BW-3C3 reacts to B lymphocytes. Signals from mAb BW-2G4 were detected in the cytoplasm of a fraction of mononuclear cells, which had the reniform nuclei but did not react with the anti-whale IgM polyclonal antibody (Fig. 3). These results indicate that mAb BW-2G4 recognizes monocytes. Signal from mAb BW-2G6 was observed in a small fraction of anti-whale IgM antibody-positive lymphocytes (approximately 6%), as well as in a small fraction of anti-whale IgM antibody-negative lymphocytes (approximately 12%, Fig. 4). This indicates that mAb BW-2G6 recognizes antigens that are shared in small fractions of B lymphocytes and non-B lymphocytes, possibly T lymphocytes. The signal was present in the cytoplasm of the cells.

Signal from mAb BW-4B10 was found in a large fraction of granulocytes (approximately 85%), and that from mAb BW-4G12 was observed in a small fraction of granulocytes (approximately 15%) (Fig. 5a, c). Cells that reacted with the mAbs were further stained with conventional Giemsa-eosin to identify granulocytes. We confirmed that mAb BW-4B10 and mAb BW-4G12 bind to neutrophils and eosinophils, respectively (Fig. 5b, d). The mAb BW-4B10 signal was present in granules, while that of mAb BW-4G12 was in the cytoplasm.

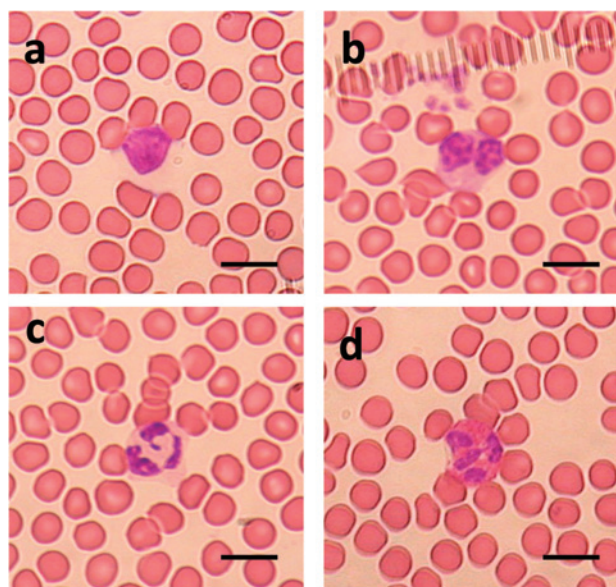


Fig. 1. Morphology of smeared leukocytes from beluga whale peripheral blood **a**, lymphocyte. **b**, monocyte. **c**, neutrophil, **d**, eosinophil. Scale bars indicate 10 μ m.

4. Discussion

In the present study, we report five monoclonal antibodies that recognize specific leukocyte populations in the beluga whale. mAb BW-3C3 and mAb BW-2G4 specifically recognize B lymphocytes and monocytes, respectively, while mAb BW-4B10 and mAb BW-4G12 are specific to neutrophils and eosinophils, respectively. Although we did not determine the molecules recognized by the respective mAbs, they do enable us to identify these specific leukocyte populations. The fifth mAb, BW-2G6, showed binding to small fractions of B lymphocyte and non-B lymphocyte populations, suggesting that the antigen it recognizes is commonly expressed in activated lymphocyte populations. Some immunological response proteins, such as CD69, an activation inducer molecule, and CD150, a signaling lymphocyte activating molecule, are expressed on activated human B and T lymphocytes (Sidorenko and Clark, 1993, Testi et al., 1994). The antigen recognized by mAb BW-2G6 may be a similar activated lymphocyte marker.

Our data show that the mAbs recognize their corresponding leukocytes in fixed tissue sections from beluga whales and bottlenose dolphins (Saito C., personal communication). Recent epidemiological studies showed that *Brucella* are prevalent among large cetaceans in the western North Pacific (Ohishi et al., 2003, 2016). The infected whales had marked granulomatous testes containing various types of infiltrated leukocytes. The mAbs we present here could therefore be useful tools for histopathological studies aimed at identifying the leukocytes involved in such inflammation. In addition to infectious diseases, ocean pollution is a threat to cetaceans, and some contaminants have been reported to impair immune function. High contaminant levels are associated with a decrease in leukocyte numbers, lymphocyte proliferation, and phagocytosis (Nakata et al., 2002, Levin et al., 2004, Schaefer et al., 2011). The mAbs reported here would be useful to identify which immune cells are specifically affected by the contaminants, as well as to analyze specific functions of the immune cells.

For future work, it will be important to establish mAbs that identify specific T lymphocyte subpopulations i.e., helper T cells, cytotoxic T cells, and $\gamma\delta$ T cells. It is known that the predominant fraction in the peripheral blood of ruminants is $\gamma\delta$ T cells, whereas they are rare in humans and mice (Hein and Mackay, 1991). Cetaceans

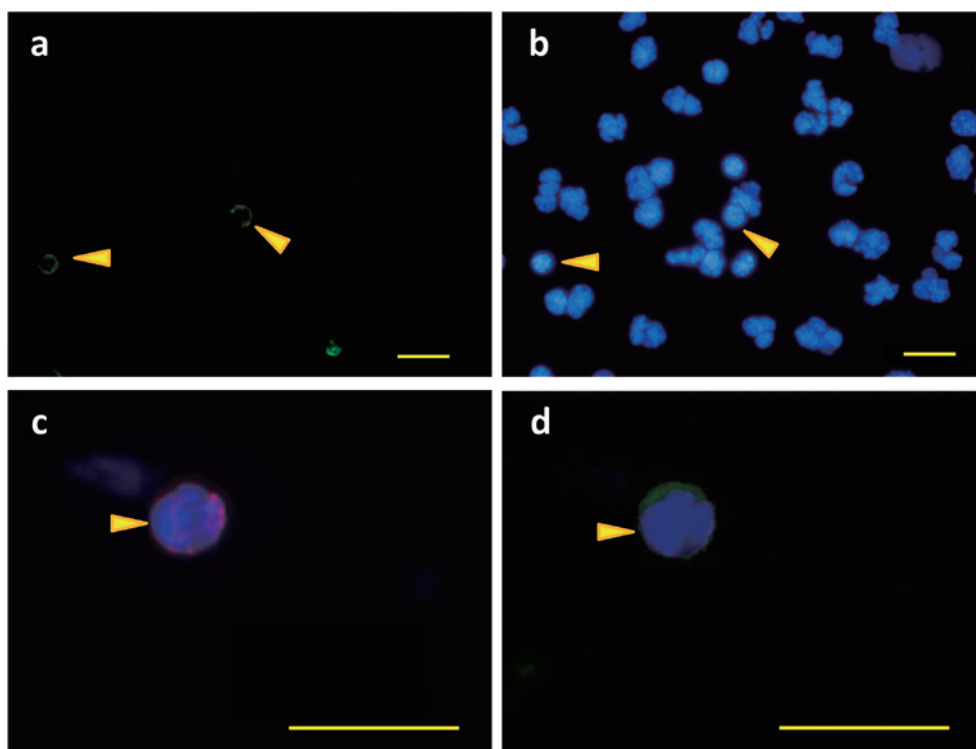


Fig. 2. Immunofluorescence micrographs of mAb BW-3C3 binding to B lymphocytes. **a** and **b**, Lower magnification fluorescent images of mAb BW-3C3 (**a**, green) and DAPI (**b**, blue). **c** and **d**, Higher magnification fluorescent images of mAb BW-3C3 (**c**, red) and anti-whale IgM polyclonal rabbit antibody (**d**, green). Scale bars indicate 20 μ m. Yellow arrowheads indicate cells stained with mAb BW-3C3.

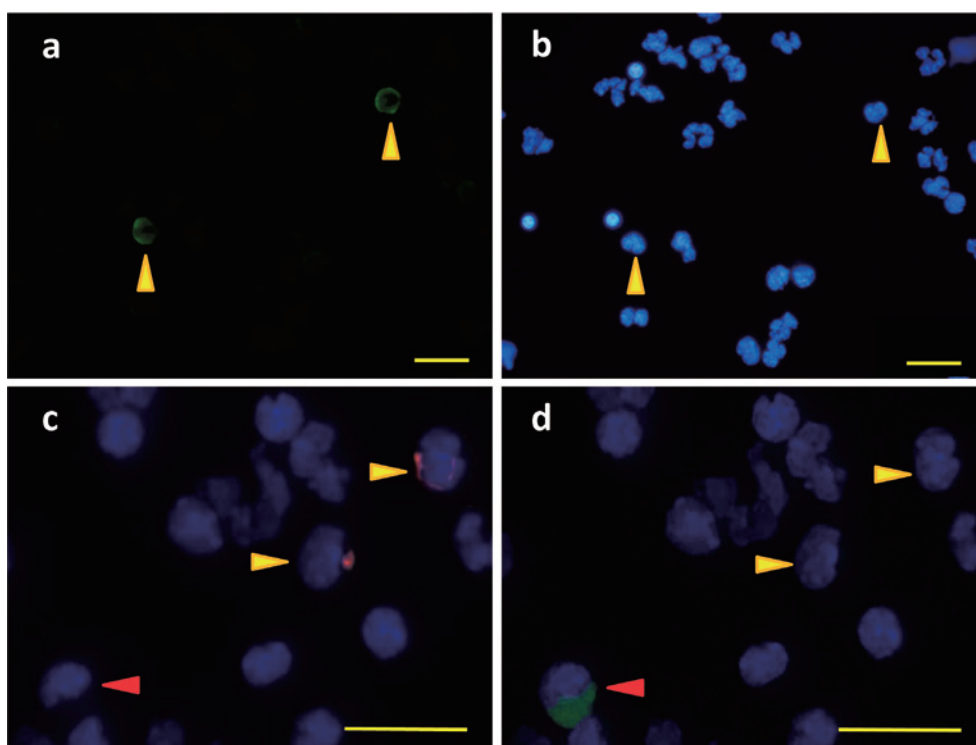


Fig. 3. Immunofluorescence micrographs of monocytes stained with mAb BW-2G4. **a** and **b**, Lower magnification fluorescent images of mAb BW-2G4 (**a**, green) and DAPI (**b**, blue). **c** and **d**, Higher magnification fluorescent images of mAb BW-2G4 (**c**, red) and anti-whale IgM polyclonal rabbit antibody (**d**, green). Yellow arrowheads indicate cells stained with mAb BW-2G4; red arrowheads indicate cell stained with anti-whale IgM polyclonal rabbit antibody. Scale bars indicate 20 μ m.

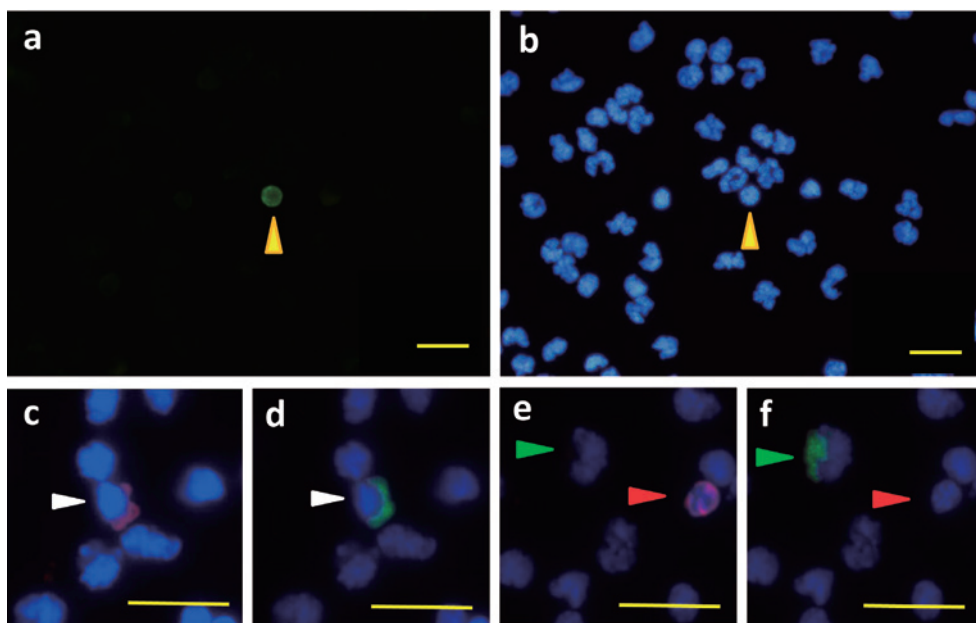


Fig. 4. Immunofluorescence micrographs of B and non-B lymphocytes stained with mAb BW-2G6. **a** and **b**, Lower magnification fluorescent images of mAb BW-2G6 (**a**, green) and DAPI (**b**, blue). Yellow arrowhead indicates mAb BW-2G6 (+) cell. **c-f**, Higher magnification fluorescent images of mAb BW-2G6 (**c** and **e**, red) and anti-whale IgM polyclonal rabbit antibody (**d** and **f**, green). **c** and **d**, White arrowheads indicate mAb BW-2G6 (+)/anti-IgM antibody (+) cell. **e** and **f**, Red arrowheads indicate mAb BW-2G6 (+)/anti-IgM antibody (-) cell. Green arrowheads indicate mAb BW-2G6 (-)/anti-IgM antibody (+) cell. Scale bars indicate 20 μm .

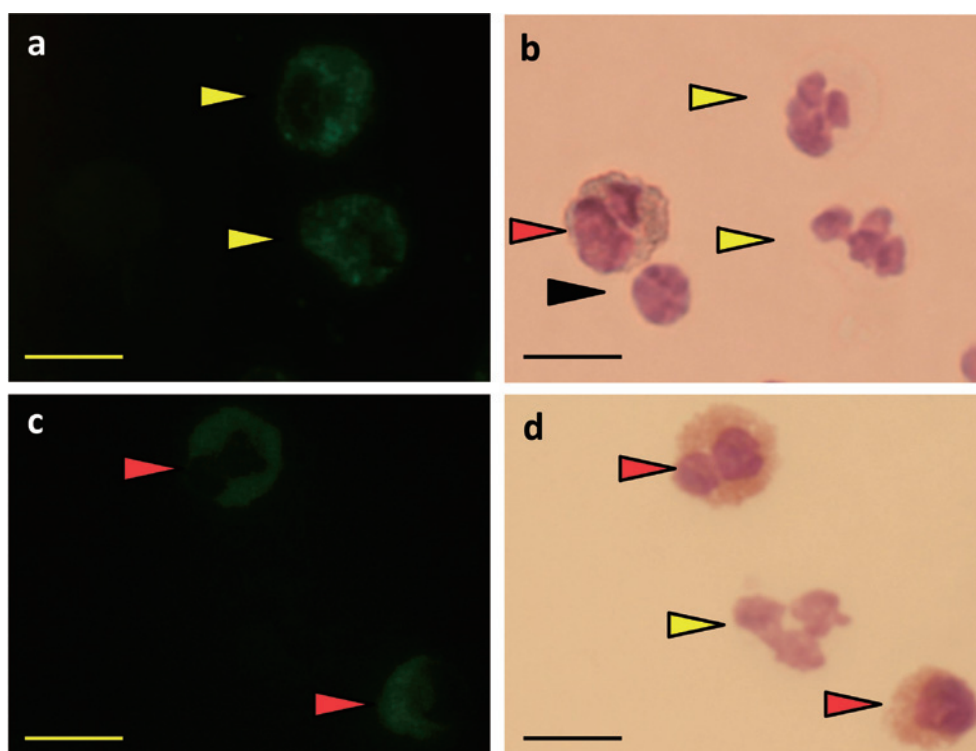


Fig. 5. Immunofluorescence and conventional Giemsa-eosin stained micrographs of neutrophils and eosinophils that react with mAb BW-4B10 or BW-4G12. **a** and **b**, fluorescent images of mAb BW-4B10 (**a**, green) followed by Giemsa-eosin staining (**b**). **c** and **d**, fluorescent images of mAb BW-4B12 (**c**, green) followed by Giemsa-eosin staining (**d**). Yellow, red and black arrowheads indicate neutrophil, eosinophil and lymphocyte, respectively. Scale bars indicate 10 μm .

that belong to the same order as ruminants, Cetartiodactyla, have been suggested to contain predominantly $\gamma\delta$ T cells in the peripheral blood (De Guise et al., 1997). Studies into their function and distribution in cetaceans using leukocyte-specific markers may contribute not only to improving the health of cetaceans but also to our understanding of the evolution of the mammalian immune system.

Acknowledgements

The authors thank the staff at the Port of Nagoya Public Aquarium for maintaining whales. We also thank Dr. A. Tame (Marine Works Japan Ltd.) and Mrs. C. Kusaka (JAMSTEC) for technical advice, and Prof. T. Tokai (Tokyo University of Marine Science and Technology) for his kind encouragement. We acknowledge Dr. Fujise (Institute for Cetacean Research) for permission to use Antarctic minke whale sera. We would like to thank Editage (ww.editag.jp) for English language editing.

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