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Research paper

Development of an anti-ferret CD4 monoclonal antibody for the characterisation of ferret T lymphocytes

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ABSTRACT

The ferret is an established animal model for a number of human respiratory viral infections, such as influenza virus and more recently, Ebola virus. However, a paucity of immunological reagents has hampered the study of cellular immune responses. Here we describe the development and characterisation of a novel monoclonal antibody (mAb) against the ferret CD4 antigen and the characterisation of ferret CD4 T lymphocytes. Recombinant production and purification of the ferret CD4 ectodomain soluble protein allowed hybridoma generation and the generation of a mAb (FeCD4) showing strong binding to ferret CD4 protein and lymphoid cells by flow cytometry. FeCD4 bound to its cognate antigen post-fixation with paraformaldehyde (PFA) which is routinely used to inactivate highly pathogenic viruses. We have also used FeCD4 in conjunction with other immune cell markers to characterise ferret T cells in both primary and secondary lymphoid organs. In summary, we have developed an important reagent for the study of cellular immunological responses in the ferret model of infectious disease.

1. Introduction

The ferret is a well-established small animal model for the study of the infectivity, pathogenesis and transmission of both human and avian influenza viruses (Belser et al., 2011). In addition, the recent emergence of a number of novel zoonotic viral pathogens with the potential for causing fatal disease in human hosts has extended the biomedical significance of this species. Infection of ferrets with severe acute respiratory coronavirus (SARS-CoV) recapitulates a number of the clinical and pathophysiological signs observed in human infections, including viral replication in the upper and lower respiratory tract and significant lung damage (Chu et al., 2008; See et al., 2008; Martina et al., 2003). The related paramyxoviruses, Hendra and Nipah, exhibit a broad host range and cause acute respiratory infection and severe encephalitis associated with high mortality in humans (Vigant and Lee, 2011). Ferrets infected with either virus exhibit a disease pathology very similar to that observed in humans, characterised by widespread vasculitis, and severe respiratory and neurological disease (Bossart et al., 2009; Pallister et al., 2011). More recently, the ferret has been shown to recapitulate signs of human disease signs when infected with different species of ebolavirus (Cross et al., 2016), further demonstrating that the ferret provides a robust model for the evaluation of preventative and passive immune protection from infection.

Despite the importance of the ferret as a model for human viral diseases, research using the ferret has been hampered by a lack of ferret-specific immunological reagents to explore the cellular immune system. To date, the identification of monoclonal antibodies (mAbs) that recognise ferret immune cell markers and cytokines has relied largely on screens of antibodies in other species that cross-react with the ferret counterpart (Martel and Aasted, 2009; Rutigliano et al., 2008). While this approach has been successful in identifying antibodies that recognise, for example, CD8 (Rutigliano et al., 2008), gaps remain in the repertoire of available reagents. A number of studies have been published that describe the use of ferret-specific CD4 mAbs to monitor changes in the level of CD4 T lymphocytes in ferrets in response to different viral infections (Cheng et al., 2013; Music et al., 2016; Music et al., 2014; Xu et al., 2013). Most recently, DiPiazza et al. (2016) used both CD4 and CD8 specific reagents to look at changes in the antigen-specific T cell repertoire following influenza infection in ferrets. As the ferret is becoming a commonly used model for highly pathogenic virus studies such as Ebola virus, further characterisation with regards to phenotypic and functional aspects of the ferret CD4 T cell population are needed. Furthermore, reagents that can detect the antigen following virus

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inactivation are critical. In the current study, we describe the derivation and characterisation of a ferret CD4 specific mAb that is well-suited for studies involving infectious material.

2. Materials and methods

2.1. Animals and ethics

All animal studies were approved by the CSIRO Australian Animal Health Laboratory Animal Ethics Committee and conducted following the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes guidelines for housing and care of laboratory animals. Ferrets aged 12–18 months were euthanized and organs removed aseptically.

2.2. Protein structure and homology analysis

CLC Main Workbench Version 7.0.2 was used to compare protein and nucleic acid sequences derived from National Centre for Biotechnology Information (NCBI), UniprotKB and Ensembl. The sequences for mouse (AAC36010.1), human (AAB51309.1), ferret (ABS50090.1), dog (AAB02295.1), pig (ANE20437.1) and chicken (ABA55042.1) were converted to FASTA format before analysing the data. The three-dimensional (3D) CD4 models were constructed by using the automated comparative protein modelling server SWISS-MODEL (http://swiismodel.expasy.org).

2.3. Vector assembly

A DNA template encoding the signal peptide and extracellular domain of ferret CD4 (amino acids 1–400; NCBI RefSeq EF492055.1), together with a C-terminal Flag tag (DYKDDDDK), was optimised for expression in human cell lines and synthesised by GeneArt AG (Life Technologies). The coding region insert was isolated and sub-cloned into the mammalian expression vector, pEE6.4 (Lonza).

2.4. Cell culture, transient transfection and purification of soluble CD4

Suspension-adapted cultures of FreeStyle-293 cells (Life Technologies) were grown in Freestyle 293 Expression Medium (Life Technologies) supplemented with Glutamax-I. Scale-up transient transfections were performed on a 1 L culture of cells (2×10^6 cells/mL) in a 3 L flask using linear polyethylenimine (PEI, Polysciences Inc.) according to a published protocol (Tom et al., 2008). The culture was harvested after seven days (cell viability > 50%), clarified by centrifugation at 400*g* and filtered. Soluble, recombinant CD4 was purified from 1.1 L of conditioned medium by immunoaffinity chromatography followed by size exclusion chromatography through Superdex™ S200 (GE Healthcare) as previously described (Elleman et al., 2001). The resulting peak fractions were concentrated and sterile-filtered.

2.5. Derivation of hybridomas

The production of mAbs was undertaken at the Monash Antibody Technology Facility, Clayton, Australia. Briefly, CD1 mice were immunised intraperitoneally with ferret CD4 protein (10 μg/injection) in a water-in-oil emulsion (Sigma) containing CpG, three times at two week intervals. Two weeks after the third immunisation a serum sample was taken and the antibody titre assessed using a conventional ELISA format using antigen-coated wells. Once a serum titre of three-fold higher than background (pre-bleed) was reached, a final boost immunisation was given followed four days prior to euthanasia and splenectomy. The spleen cells were homogenised into a single cell suspension between two frosted glass slides and 100×10^6 spleen cells were mixed with 25×10^6 SP2/0 cells. These cells were washed in DMEM and 1 mL of polyethylene glycol was added drop-wise over a one minute period follow by additional washes in DMEM. The cells were plated into 20 × 96 well plates and allowed to divide for two weeks. At this point the supernatant from each well was tested for reactivity against the immunogen by protein microarray using antigen-coated slides and further confirmed by ELISA, and positive binders were expanded, subcloned until clonal and frozen. The production and purification of the FeCD4 was undertaken in pleated-surface roller bottles in Hybridoma-serum free media (Gibco) supplemented with 1% heat-inactivated FBS at 37 °C for 10 days. The resultant medium was filtered through a 0.22 μm filter and applied to a Protein A Sepharose column (Fast Flow 4; GE) and antibody fraction eluted with 50 mM glycine, pH 3.0, into tubes containing 0.2 elution volumes of 1 M Tris-HCl, pH 8.0. Antibody fractions were exchanged into phosphate-buffered saline (PBS) and concentrated using Amicon Ultra units (Millipore).

2.6. Fluorescence-activated cell scanning

Spleen, thymus and lymph node were excised from euthanized ferrets, cleaned of any connective tissue and mechanically digested in cold FACS buffer (2% (v/v) FCS, 0.02% (v/v) NaN_3 in PBS) to produce a single suspension. Mechanical digestion was achieved by pressing the tissue through a 70 μm sieve (BD Biosciences) using the plunger of a 10 mL syringe. Red blood cells were removed from the spleen by Lymphoprep density gradient (Stemcell Technologies) centrifugation at 1000*g* for 20 min with no brake. One million cells were stained with 0.1 μg of anti-ferret CD4, washed in FACS buffer and resuspended in 150 μL of FACS buffer. Additional antibodies used for phenotyping included CD8 (OKT8), CD81 (JS081), CD11b (M1/70) (BD Biosciences), MHC class II (CAT82a) (Kingfisher) and anti-ferret Ig (H + L) (Rockland). Where required an anti-IgG2a or anti-IgG1 secondaries were used (Invitrogen). For fixation cells were resuspended in 200 μL of 4% paraformaldehyde (PFA) in PBS. Data was acquired using a BD FACS LSRII flow cytometer (BD Biosciences) equipped with 405, 488, and 633 nm excitation lasers in conjunction with FACS Diva acquisition software (BD Biosciences). Data analysis was performed using FlowLogic FCS analysis software (Inivai Technologies).

2.7. ELISpot

Lymph node (LN) cells (1.5×10^8 cells) from three separate ferrets were stained with FeCD4 for 30 min at 4 °C. The cells were washed with 10 mL cold PBS + 2% FCS and centrifuged at 400*g* for 5 min. The supernatant was decanted and cells were resuspended in 2 mL PBS/FCS and 150 μL of MACS anti-mouse IgG beads added and incubated at 4 °C for 15 min. The cells were washed again and passed over a MACS LS column (Miltenyi) according to manufacturer's instructions. The CD4 cells were collected and remaining cells stained with CD8 (clone OKT8) and previous steps repeated. The cells were then plated in a 96 well Ferret IFNγ ELISpot plate (Mabtech) at a density of 2.5×10^4 cells/well in 100 μL with or without ConA (10 μg/mL) and the cells cultured for 48 h. The ELISpots were then developed and counted as per the manufacturer's instructions.

3. Results and discussion

3.1. Ferret CD4 homology and predicted structure

Analysis of CD4 protein sequences shows that of the proteins compared, the ferret CD4 protein is most closely related to dog followed by human and pig (Fig. 1a). As the majority of CD4-specific antibodies are more commonly available for human and mouse we investigated the conservation of ferret with these two species. Nucleic acid level analysis showed the human and ferret CD4 genes were 59% identical, whereas mouse and ferret were 50%. Intriguingly, at the amino acid level, a more important measure for cross-reactive antibody binding, mouse and ferret displayed 58% identity compared to 37% between