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Research paper

Expression and *in vitro* upregulation of MHCII in koala lymphocytes

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ABSTRACT

Understanding and measuring immune activity of the koala (*Phascolarctos cinereus*), is important to studies of the epidemiology and impact of the widespread chlamydial and koala retroviral (KoRV) infections that occur in this iconic but increasingly threatened species. To explore the interaction of disease and immunity, and to assess the potential for use of class II major histocompatibility complex (MHCII) upregulation as an indicator of lymphocyte activation in *in vitro* immune assays, we have investigated the expression of MHCII in koala lymphocytes by flow cytometry. MHCII expression was upregulated in mitogen stimulated B lymphocytes *in vitro* but no such increase was detected *in vivo* in free-living koalas with active inflammation. In assessing phenotypic baseline data of captive koalas, we have identified that MHCII is expressed predominantly on circulating B lymphocytes ($85.7 \pm 2.4\%$) but on very few T lymphocytes ($3.4 \pm 1.9\%$), even following activation, and suggest that the latter finding might be compensated by the greater absolute numbers of peripheral blood B lymphocytes in this species relative to many eutherian species.

1. Introduction

Study of the immune system of koalas (*Phascolarctos cinereus*) is of high priority for species management. *Chlamydophila pecorum* and *Chlamydophila pneumoniae* have long been recognised to cause significant death and infertility of koalas (Obendorf and Handasyde, 1990). More recently, Koala Retrovirus (KoRV) has been proposed, but not yet proven, to cause lymphosarcoma, aplastic anaemia, myelodysplasia and immunodeficiency (Hanger et al., 2000; Tarlinton et al., 2005), which are reported commonly in koalas. Elucidating the significance and predisposing factors of these diseases requires an understanding of the role of the koala immune system in pathogenesis and the ability to measure immune function within koala populations. Although immunophenotypic analysis of peripheral blood has been performed in many eutherian species (Brown and Greaves, 1974; Stone et al., 1995; Byrne et al., 2000; Schwartz et al., 2005), in the koala, only absolute number of leukocytes and lymphocytes have been reported (Canfield et al., 1989). Understanding the potential impact of environmental factors, genetics, and pathogens on the adaptive immune response requires greater understanding of key immune pathways.

The limited available evidence points to koalas having an adaptive immune response with similar components to that of other species. Lymphoid tissues have structure similar to that of eutheria (Hemsley and Canfield, 1997), koala peripheral blood leukocytes proliferate or produce interferon gamma (IFNγ) when cultured with mitogens such as ConA, PHA or PMA/ionomycin (Wilkinson et al., 1992; Higgins et al., 2004), and koalas produce cellular and humoral immune responses in response to vaccination (Carey et al., 2010; Kollipara et al., 2012) and infection (Girjes et al., 1993; Higgins et al., 2005). However, koalas show delayed isotype class switching from IgM to IgG production (Wilkinson et al., 1994) and absence of anti-chlamydial hsp60 IgG titres in the presence of florid inflammation (Higgins et al., 2005) and it has been proposed that these might indicate diminished reliance on the adaptive immune response.

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The cell surface concentration of major histocompatibility complex class II (MHCII) molecules directly influences the magnitude and nature of the adaptive immune response (Fukui et al., 1997). MHCII molecules are cell-surface glycoproteins found on professional antigen presenting cells (APCs), which include B lymphocytes, macrophages, dendritic cells and activated mucosal epithelial cells and, in most species, activated T lymphocytes (Ishii et al., 1992; Hammond et al., 1998; Holling et al., 2002). MHCII molecules bind and present exogenous antigenic fragments, such as those of chlamydiae, to the T-cell receptor of $CD4^+$ T helper lymphocytes, which activates these cells to direct cellular and humoral immunity (Balakrishnan and Adams, 1995; Kalish, 1995).

Changes in MHCII expression in response to immunological and physiological perturbations, including contaminant exposure and disease, have potential application in evaluation of health of individuals or populations, as with other general indicators of immune activation such as blood acute phase proteins (Murata et al., 2004). For example, cattle infected with bovine leukaemia virus (BLV) and mink exposed to crude oil both show increased MHCII expression (Stone et al., 1995; Schwartz et al., 2004). If MHCII expression in koalas responded similarly, its study would facilitate investigations of chlamydial disease, KoRV-induced immunomodulation or exposure to agricultural or plant toxins.

In addition, MHCII expression might be used as an alternative to proliferation, to indicate lymphocyte activation in *in vitro* assays. Lymphocyte proliferation assays and flow cytometric detection of IFNγ show large variance in koalas (Wilkinson et al., 1992; Higgins et al., 2004) and would benefit from cross-validation with additional indicators. IFNγ is released from mitogen or antigen-stimulated T lymphocytes (Iking-Konert et al., 2008) and induces upregulation of MHCII (Steimle et al., 1994). Monocytes from tuberculosis-infected human patients showed greater upregulation of IFNγ-induced MHCII expression than those of healthy donors (Garibay-Escobar et al., 2003) and T lymphocytes from BLV-infected cows had significantly increased mitogen-induced MHCII expression (Stone et al., 1995).

In this study, we use dual labelled flow cytometric analysis of whole blood to characterise MHCII expression in koalas, in terms of cell distribution and intensity, across sex and seasons. We also evaluate the potential use of MHCII as a non-specific indicator of immune activation through investigation of *in vivo* inflammation in free-living koalas as well as *in vitro* mitogen-stimulated cellular assays.

2. Materials and methods

2.1. Sample collection from captive koalas

Blood (4–8 ml) was collected into EDTA tubes (BD Vacutainer®, Becton Dickinson and Co., NJ, USA) from the cephalic vein of manually restrained adult koalas (9 males, 4 females) from Taronga Zoo, Sydney, Australia, in July 2010 (winter). Additional blood samples were collected opportunistically from nine of these koalas and an additional fourteen individuals (4 males, 10 females) from Taronga Zoo and Sydney Wildlife World, Sydney, Australia; in spring ($n = 8$), summer ($n = 5$), and autumn ($n = 16$). The captive koalas showed no evidence of disease in their clinical history, or on physical examination and standard haematological analysis.

Blood samples were used, within 4 h of collection, for two procedures. All 42 samples (from 13 male and 14 female koalas) were subjected to immunophenotypic analysis to establish mean and variance of baseline MHCII expression and concentration of circulating koala B and T lymphocytes. Secondly, aliquots of blood collected from the 13 koalas in July 2010 were subjected to PBMC isolation and mitogen stimulation *in vitro*, to study the upregulation of MHCII expression and affects of these *in vitro* processes on lymphocyte subsets (Table 1).

2.2. Flow cytometry

An aliquot of fresh blood was mixed with 1× FACS lysing solution (BD Biosciences, San Jose, USA) at a ratio of 1:10, to lyse red blood cells and fix peripheral blood leukocytes. Fixed blood aliquots were stored at −80 °C for retrospective immunofluorescence staining and flow cytometric analysis, performed in one single assay. Absolute leukocyte and lymphocyte counts were also obtained from fresh blood using the Unopette system (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) and a haemocytometer, combined with a manual differential count.

Antibodies targeting conserved intracellular epitopes were similar to those used on koalas in immunohistochemical studies (Hemsley and Canfield, 1997), and included anti-human CD79b mAb conjugated to FITC or phycoerythrin (PE) (Clone AT107-2, Serotec Ltd., Oxford, UK); anti-human CD3 mAB (Clone CD3-12, Serotec Ltd., Oxford, UK) conjugated to PE using an antibody labelling kit (Innova Biosciences, Cambridge, UK); and anti-human HLA-DP, DQ, DR conjugated to FITC (Clone CR3/43, DakoCytomation, Denmark) which is a single antibody that reacts with the β-chain of the α β heterodimers of the DP, DQ and DR gene families. A rat anti-human IgG isotype control (Serotec Ltd., Oxford, UK) was used to control against non-specific staining when required. Antibodies that target intracellular epitopes were used as they have been shown to be cross-reactive in many species (Jones et al., 1993; Kreiss et al., 2009); this is expected because intracellular epitopes are more conserved than surface epitopes, which are often under pathogen-driven selection. Optimal antibody concentrations were determined by titration, and validated based on production of expected labelling patterns on immunohistological sections and flow cytometry.

Cells frozen in 1× FACS lysing solution were thawed in a 37 °C water bath, and centrifuged at 1000 × *g* for 15 min. The supernatant was then decanted and the cell pellet resuspended and transferred to triplicate wells of a 96-well U-bottomed tray (Corning, NJ, USA) such that each well had minimum of 2×10^5 cells. To allow penetration of the intracellular antibodies, cells were then treated with 200 μl of 1× BD FACS Permeabilizing Solution 2 (BD Biosciences, San Jose, USA) for 15 min in the dark. All subsequent wash steps were performed with 1× PBS containing 1% FCS. The cells were washed twice and incubated for 1 h at 4 °C in