The Koala and its Retroviruses:
Implications for Sustainability and Survival

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Koala Immunology and the Koala Retrovirus (KoRV)

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Abstract. Although koala retrovirus (KoRV) is widely termed a pathogen, direct evidence for causation of disease impacts in koalas *(Phascolarctos cinereus)* remains elusive. Examination of the immune system of koalas could provide a sharper tool to investigate this but progress has been slow due to a paucity of immunological reagents in this species, and historical contradictions in research findings in this area. Our work using cross reactive antibodies to examine behaviour of resting and stimulated koala T cells (anti-human CD3); B cells (anti-human CD79b); MHCII (anti-human HLA-DP, DQ, DR) and interferon gamma (anti-bovine IFNg) by flow cytometry have revealed some features consistent with a skew to a Th2 (B cell) immune focus. Assessing the role of KoRV in immunomodulation in koalas clearly requires more in-depth research. We have used recent advances in genomics of other marsupials to develop tools necessary to assess KoRV's effects on koala immune function in free-ranging, captive and in-vitro systems.

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Immunosuppression in koalas: is it clear-cut?

Early researchers working on koala immunology formed the belief that koalas were immunologically "lazy", and this has coloured perceptions of koala immunology in the broader community ever since. This idea was originally put forward based on apparently slow seroconversion to chlamydial infection, and a limited cellular response to overwhelming sarcoptic mange in a small number of koalas (Brown, 1988). It sparked a series of studies that pioneered marsupial immunology but also set the scene for two decades of intriguingly disparate findings: lymphoid tissues of koalas are generally more sparsely populated than those of many species (Wilkinson *et al*., 1992a), yet the arrangement of these tissues is consistent with those of eutheria, with similar distribution of T and B cells (Hemsley *et al.,* 1995, 1996a, b); initial experiments indicated slow and weak local cutaneous delayed type hypersensitivity reactions (Wilkinson *et al*., 1994), yet koalas are clearly capable of mounting prolific lymphoplasmacytic responses, with their inflammatory infiltrates and distribution of B and T lymphocytes in chlamydial disease being very similar to the non-protective, deleterious response to conserved chlamydial heat shock proteins that induces pelvic inflammatory disease in humans (Hemsley & Canfield, 1997; Morrison, 1991). Similarly, in contrast to poor antibody responses described initially (Wilkinson *et al*., 1992b; Wilkinson *et al*., 1994), recent vaccine trials induced strong humoral and cellular responses (Carey *et al*., 2010; Kollipara *et al*., 2012). Also, in response to natural *Chlamydia pecorum* infection, koalas develop neutralizing anti-MOMP antibodies (Girges *et al*., 1993), and also develop high anti-hsp60 and hsp10 antibody titres in association with chlamydial reproductive tract fibrosis (Higgins *et al*., 2005), as do women similarly affected by *C. trachomatis* (Domeika *et al*., 1998; LaVerda *et al*., 2000).

Clearly, we have evidence of outcomes of a functional adaptive immune response in the koala. However, in terms of its strengths and weaknesses, and the evolutionary forces that have shaped it, we are just beginning to scrape the surface. KoRV as a potential immunosuppressive agent needs to be considered in the context of a range of forces

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and trade-offs shaping a co-evolutionary relationship between a host, its environment and a range of potential pathogens. Many diseases of koalas are shared across other marsupials, as might be expected, based on the shared environments in which they evolved and now inhabit; *Cryptococcus gatti* invasion occurs in immunocompetent hosts of many species (Krockenberger *et al*., 2003); a range of marsupial species show tendency to disseminated mycobacterial infection (as do badgers and ferrets) (Buddle & Young, 2000); and wombats are also highly susceptible to sarcoptic mange (Skerratt, 2005). At the same time, it would not be surprising to find species- or even habitat-specific weaknesses or strengths in the koala immune response. In theory, if *Chlamydia pecorum* were a European introduction, a relatively non-gregarious species on a lean energy and nutrient budget, such as the koala, might (or might not) have evolved a more limited investment in adaptive immunity than that needed to protect against contagious pathogens in a more social species. As another hypothetical example on a finer scale, those koala populations needing serological defence against the paralysis tick *(Ixodes holocyclus)* toxin (i.e. in coastal regions of Queensland and New South Wales, where chlamydial disease is also generally most common) might benefit from a strong antibody-based (Th2) immunity, in a trade-off, at the expense of the cellular (Th1) response considered critical to elimination of *Chlamydia*. When laid over the impact of a history of hunting, regional translocation and habitat fragmentation on immune gene (MHCII) diversity (Lau *et al*., 2013, 2014); the likelihood of some MHCII variants being associated with survival and chlamydial disease (Lau, 2013); and diversity of chlamydial strains among and within koala populations (Higgins *et al*., 2012; Marsh *et al*., 2011); it becomes evident that we are dealing with a complex system. This highlights the need for integrated studies including both eco-immunological and epidemiological studies in free-ranging animals in a variety of populations and disease states, and exploration of pathogenic mechanisms in more controllable captive or *in vitro* systems.

Is KoRV immunomodulatory in koalas? Do we have enough data?

Associations with infectious disease are equivocal (Simmons, 2011; Tarlinton *et al*., 2005), though this might be due to the multi-factorial nature of disease, especially in populations of free-ranging animals. The most direct evidence for immunomodulatory effects of KoRV comes from the effect of purified KoRV on cytokine expression by cultured human PBMCs, whereby it induced elevated expression of interleukin-6 (IL-6), IL-10, growth-related oncogene (GRO) and monocyte chemotactic protein-1 (MCP-1) (Fiebig *et al*., 2006). This is consistent with the highly conserved nature of the retroviral transmembrane envelope protein p15E, or immunosuppressive domain (ISD), across KoRV, GALV, MuLV and FeLV (Fiebig *et al*., 2006); and the wide range of its effects on cells of other species in vitro, including: inhibition of respiratory burst and chemotaxis of the human monocyte; inhibition of macrophage accumulation at inflammatory foci in mice; suppression of neutrophil function; inhibition of human natural killer cell activity; inhibition of lymphocyte proliferation and mitogenic cytokine production; and increased production of interleukin-10 (IL-10) (Denner, 1998). Whether these effects occur in koalas and whether these have significant downstream effects in this species has not yet been tested.

Due to constant pathogen-driven selection, immune molecules are among the least conserved between species. Our ability to examine koala immune profiles has, therefore, been limited to detection of antibodies, lymphocyte proliferation assays, immunophenotyping by flow cytometry with a limited number of cross-reactive antibodies to conserved (mostly intra-cytoplasmic) domains (T cell, anti-human CD3; B cell, anti-human CD79b; MHCII, anti-human HLA-DP, DQ, DR; IFNg) (Higgins *et al*., 2004; Lau *et al*., 2012) and, very recently, qPCR for IL10, IFNg and TNFa (Mathew *et al*., 2013a; 2013b). Our recent immunophenotypic studies on captive, KoRV-positive koalas (Lau *et al*., 2012) revealed some interesting features: elevated numbers of B cells relative to other species (1.0–4.9×10^6 cells/ml vs 0.17–0.56×10^6 cells/ml, respectively), and absence of MHCII expression on stimulated and non-stimulated T cells. Both Concavalin A (ConA) and Pokeweed Mitogen (PWM) induced MHCII up-regulation in koala B cells but not T cells; in contrast to the marked (e.g., 50–90%) MHCII expression on T cells of all other species studied to date, but mice (Byrne *et al*., 2000; Rideout *et al*., 1992; Schwartz *et al*., 2005; Holling *et al*., 2004). Ability of koala T cells to respond to mitogens was evident, in that PWM induced proliferation of T and B cells and ConA induced preferential proliferation of T cells in our study and, in our previous study, 14% of PMA-Ionomicin stimulated koala lymphocytes labelled strongly with cross-reactive anti-IFNg antibodies (Higgins *et al*., 2004). Increased B cell numbers and absence of T cell MHCII expression would be consistent with retrovirus-associated increased Th2 profile (Denner, 1998; Haraguchi, 2008). However, it could alternatively reflect an evolutionary adaptation within the koala's immune response and this phenomenon needs to be examined in more detail KoRV positive and negative koalas from a range of habitats and disease states.

Where to now: testing the effects of KoRV on koala immune function

By using available sequence from non-koala marsupial genomes (common opossum *Monodelphis domestica*, tammar wallaby *Macropus eugenii*, Tasmanian devil *Sarcophilus harrisii*, common brushtail possum *Trichosurus vulpecula*) (Morris *et al*., 2010) we have recently generated koala sequence and developed and validated a series of koala-specific qPCRs for immune genes CD4, CD8, IL-10, IL-4, IFNg, IL-6 and several reference genes (Maher *et al.,* 2014). We are applying these to our collections of samples from KoRV positive and negative free-ranging koalas, and cells of captive koalas in *in vitro* studies to better describe normal and abnormal immune function in these koalas.

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