The Koala and its Retroviruses:
Implications for Sustainability and Survival

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Immunization with Envelope Proteins of the KoRV as a Basis for a Preventive Vaccine

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Abstract. The rapid spread of the koala retrovirus (KoRV) in Australia and in international zoos calls for effective counter measures. As is the case with the human immunodeficiency virus (HIV) epidemic, a preventive vaccine is urgently needed. Vaccines inducing neutralizing antibodies are a good way to prevent retrovirus infections. Although for HIV there is still no effective vaccine available, commercial vaccines protecting cats from disease caused by the feline leukemia virus (FeLV) already exist and have been proven effective. KoRV is a retrovirus more closely related to FeLV than to HIV. Immunizing different species (rats, goats, hamsters, guinea pigs, mice, cats) with the transmembrane (TM) and surface (SU) envelope proteins of FeLV, as well as of the porcine endogenous virus (PERV) we always obtained neutralizing antibodies. PERV is also closely related to the KoRV. Based on the immunization studies with the envelope proteins of FeLV and PERV, we cloned and expressed the corresponding envelope proteins of the KoRV and immunized goats and rats. In all cases we obtained antibodies neutralizing the KoRV. However this does not mean that neutralizing antibodies will be obtained when immunizing koalas *(Phascolarctos cinereus)* with the envelope proteins of the KoRV or immunizing pigs with the envelope proteins of PERV. Therefore, koalas should be immunized with KoRV envelope antigens to determine whether neutralizing antibodies are induced and if so, whether such antibodies are able to protect healthy animals from infection. Furthermore, whether immunization with these antigens has a therapeutic effect on animals already infected with KoRV should be investigated. If *Chlamydia* infection of koalas is an opportunistic infection made possible by KoRV-induced immunodeficiency, immunization against KoRV will also protect animals from *Chlamydia* infection.

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Infection of koalas with the KoRV, and infection of humans with HIV-1 leading to AIDS. Retroviruses have long been known to be capable of infecting new host species by transspecies transmission. Interest in this subject has been boosted by the finding that the human immunodeficiency viruses (HIV-1 and HIV-2) are the product of such a transspecies transmission (Gao 1994,1999) and by recent concerns over the potential transmission of PERVs after xenotransplantation of pig organs into humans (Denner & Tönjes, 2012). The koala retrovirus (KoRV) is the result of such a transspecies transmission which is even associated with endogenization of the virus into the germ line of the animals (Hanger *et al*., 2000; Denner & Young, 2013). The KoRV is closely related to the gibbon ape leukemia virus (GaLV), which however remained exogenous in gibbons (Hanger *et al*., 2000). Both are related to endogenous retroviruses of South Eastern Asian mice, (Martin *et al*., 1999) and bats, (Cui *et al*., 2012a,b) however the origin and the transmission routes are still unknown.

Retroviruses are known to induce tumors and immunodeficiencies and HIV is the most prominent retrovirus inducing an acquired immunodeficiency syndrome. Although HIV, a lenti(retro)virus, and the KoRV, a gammaretrovirus, are not closely related, the clinical picture of the syndrome

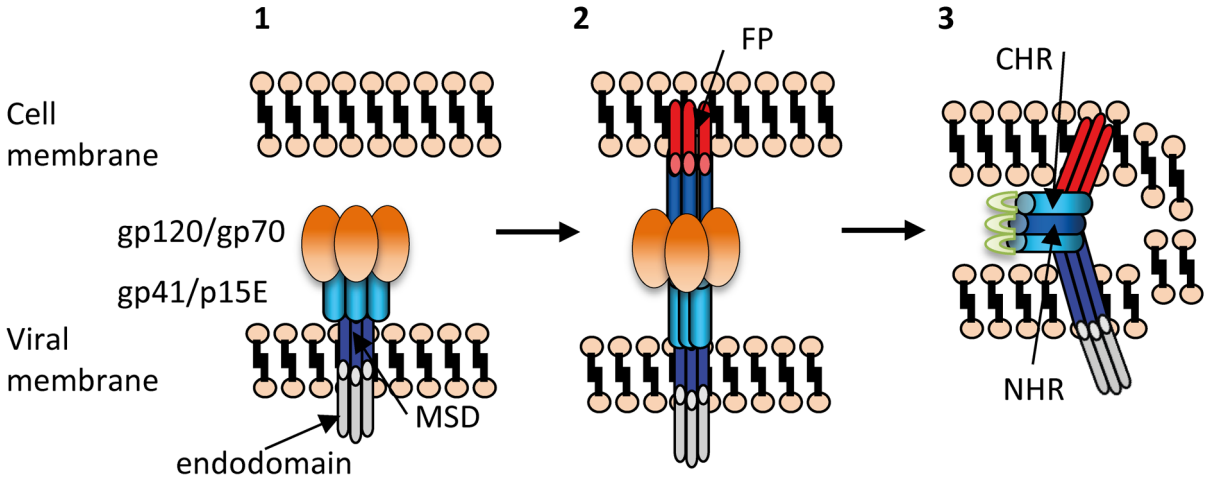

Figure 1. Schematic presentation of retroviral infection. Step 1: Interaction of the SU protein (orange, gp120, molecular weight 120,000 Dalton in the case of HIV-1; gp70 in the case of KoRV) with the cellular receptor (not shown). The TM protein (light blue, gp41, molecular weight 41,000 Dalton in the case of HIV-1, p15E, molecular weight 15,000 Dalton, E stands for envelope, in the case of the KoRV) is partially hidden in the SU protein, MSD, membrane spanning domain of the TM protein, dark blue. Step 2: Conformational changes in the TM protein, its fusion peptide (red, FP) penetrates the target cell membrane. Step 3: Interaction of the N-helical region (blue, NHR) and the C-helical region (light blue, CHR) of the TM protein and fusion of the viral and cellular membranes leading to subsequent internalization of the virus. Between the helical regions a hinge is shown composed of a Cys-Cys-loop (light green).

induced by HIV in humans and that induced by the KoRV in koalas *(Phascolarctos cinereus)* is similar concerning the immunodeficiency. HIV infections are usually accompanied by opportunistic infections among them *Chlamydia* infections (Contini, 2003). The major opportunistic infection in the case of the KoRV infection represents *Chlamydia* infection (Brown *et al*., 1987). *Chlamydia* infections are also commonly associated with FIV (feline immunodeficiency virus) infections (O'Dair *et al*., 1994). In addition, koalas infected with KoRV suffer from leukemia (Booth & Blanshard, 1999). Leukemia, lymphoma and immunodeficiency were also induced by FeLV which is closely related to the KoRV (Hardy 1985, 1993). Whereas only 5 to 10% of FeLV-infected cats suffer from leukemia and lymphoma, more than 65% of them die from opportunistic infection based on the immunodeficiency (Hardy 1985,1993). FeLV-infected cats as well as HIV-1-infected humans are characterized by a decrease in the number of $CD4^+$ cells (Hofmann-Lehmann *et al*., 1997). To summarize, a comparison of the KoRV infection with the infection with HIV-1 leading to AIDS may help to understand the immunopathogenesis.

Is vaccination more effective and economical than treatment?

Taking into account the costs of highly active antiretroviral therapy (HAART) used for the treatment of individuals infected with HIV-1 and the overall socio-economic impact of the AIDS pandemic on mankind, a vaccine protecting from HIV-1 infection would be the most efficient and cost effective of solutions. Unfortunately, such a vaccine is not yet available and until it is, the development costs for a vaccine depend on numerous factors. These include the selection of the best immunization strategy, the correct antigen and the most efficient adjuvant as well as the time and expense of preclinical and clinical trials. In the case of gammaretroviruses (to which KoRV belongs), the situation is quite different. For example, vaccines that protect from FeLV-induced disease in cats are commercially available and are being used successfully. In addition there are numerous publications demonstrating the efficacy of envelope antigens inducing neutralizing antibodies specific for other gammaretroviruses such as the FeLV, the PERV and different murine leukemia viruses (MuLV) (see below).

Neutralizing antibodies versus T cell immunity

There are two arms of the immune system, the humoral immunity based on B cells producing specific antibodies and the cellular immunity based on cytotoxic T cells (CTL). Most of the commercial vaccines protect humans from viral infection by inducing neutralizing antibodies. However, it is still unclear whether protection from retrovirus infections requires antibodies or CTL, or both. Retroviruses copy their genetic information, which is a single stranded RNA, into a double stranded DNA using the viral enzyme reverse transcriptase and later integrate this copy into the genome of the target cell. The DNA copy is the basis for the production of viral genomic and mRNA, of proteins and viral particles. On the other hand, the virus can persist undetected from the immune system if it does not express viral proteins. Therefore, neutralizing antibodies preventing infection in the first place represent the protection of choice. Neutralizing antibodies are usually directed against the envelope proteins which play an important role during infection (Fig. 1).

Neutralizing antibodies specific for the surface envelope protein gp120 and the TM protein gp41 of HIV-1 were found in HIV-1 infected individuals, however normally they cannot stop progression to AIDS (Kwong & Mascolla, 2012). Furthermore, some of these neutralizing antibodies were isolated, and generated as monoclonal antibodies. The localization of the epitopes recognized by these antibodies neutralizing HIV-1 is shown in Fig. 2.

Such monoclonal antibodies were shown to be broadly neutralizing, they inhibit infection with up to 90% of the HIV-1 strains (Muster *et al*., 1993; Zwick *et al*., 2001). Application of these human neutralizing antibodies to monkeys prevented an infection of the animals when they were challenged with infectious hybrid virus composed of the core of the simian immunodeficiency virus (SIV) and the envelope of HIV-1 (Mascola *et al.,* 1999; Ruprecht, 2009). Application of these broadly neutralizing antibodies to HIV-infected humans significantly decreased the virus load (Stiegler *et al*., 2002; Trkola *et al*., 2005). These data demonstrate that neutralizing antibodies are able to prevent a retrovirus infection *in vivo* and to inhibit progression to AIDS. However, until now such antibodies broadly neutralizing HIV-1 could not be induced in sufficient amounts after immunization with different envelope-derived antigens.

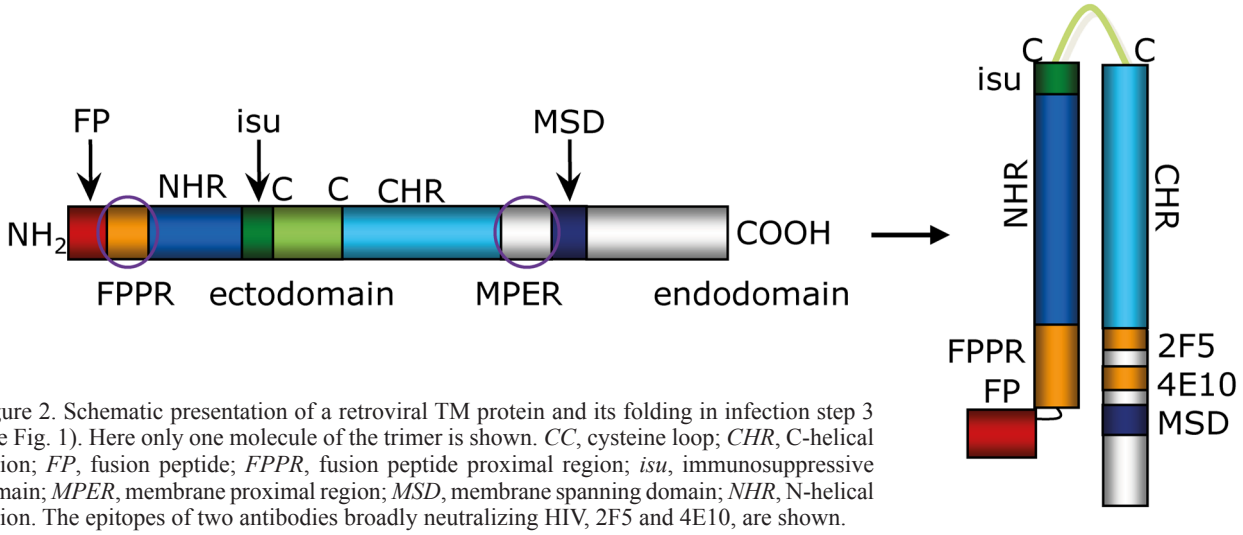

Figure 2. Schematic presentation of a retroviral TM protein and its folding in infection step 3 (see Fig. 1). Here only one molecule of the trimer is shown. *CC*, cysteine loop; *CHR*, C-helical region; *FP*, fusion peptide; *FPPR*, fusion peptide proximal region; *isu*, immunosuppressive domain; *MPER*, membrane proximal region; *MSD*, membrane spanning domain; *NHR*, N-helical region. The epitopes of two antibodies broadly neutralizing HIV, 2F5 and 4E10, are shown.

Neutralizing antibodies against MuLV, FeLV and PERV

In contrast to the non-successful attempts to induce neutralizing antibodies against HIV, antibodies neutralizing gammaretroviruses were induced easily. Many experiments have been conducted with potential murine leukemia virus vaccines. The approaches have included killed virus (Fink & Rauscher, 1964), subunit vaccines (Fischinger *et al*., 1976; Hunsmann *et al*., 1975; Hunsmann *et al*., 1981; Hunsmann, 1985), recombinant vaccinia viruses expressing viral gene products (Earl *et al*., 1986; Morrison *et al*. 1987), peptide vaccines (Bayer & Hunsman, 1987), and live attenuated viruses. Attenuation was achieved by prolonged passage through tissue culture (Mayyasi & Moloney, 1967; Ruan & Lilly, 1992), or by the use of live pathogenic virus blocked by antiretroviral drugs such as azidothymidin (AZT) and interferon alpha from replicating (Ruprecht *et al*., 1990, 1996). When mice were immunized with the SU (gp70, molecular weight 70,000 Dalton) and TM (p15E, molecular weight 15,000 Dalton, E stands for envelope) antigens of the murine leukemia virus (MuLV) substrain Friend leukemia virus (FLV) neutralizing antibodies were induced and protection from disease was reported (Fischinger *et al*., 1976; Hunsmann *et al*., 1975; Hunsmann, 1985; Schäfer *et al*., 1977; Thiel *et al*., 1987). Most importantly, the immune response and the protection were more efficient when both envelope proteins, p15E and gp70, were used for immunization. This was also true, when an immunotherapy was performed (Thiel *et al*., 1987). In AKR mice the onset of spontaneous leukemia induced by endogenous retroviruses could be dramatically delayed and the overall incidence was significantly reduced following treatment with high-titer heterologous antibodies against the surface envelope protein gp70 and p15E (Schäfer *et al*., 1976, 1977; Schwarz *et al*., 1976; Thiel *et al*. 1987; de Vos *et al*., 1998).

The mechanism of protection when immunizing with the envelope proteins was studied in transfer experiments. In one of these experiments mice were immunized with attenuated Rauscher leukemia virus (RLV), another substrain of MuLV. Passive transfer of the immune serum into mice challenged subsequently with infectious RLV was protective only at a very high serum dose, whereas immune T cells alone were fully protective, suggesting that cellular immunity alone is protective (Ruprecht *et al*., 1990, 1996). On the other hand, an essential role for virus-neutralizing antibodies in sterilizing immunity was described for Friend virus infection (Messer *et al*., 2004). In these investigations B cell-deficient mice were poorly protected by vaccination and passive transfer of neutralizing antibodies completely compensated for the B cell deficiency.

Similar immunization experiments were performed with envelope proteins derived from FeLV and first commercial vaccines were developed based on these immunizations (Pedersen *et al*. 1979; Pedersen, 1993; Pedersen & Johnson, 1991; Torres *et al*., 2010; Legendre *et al*., 1991). One of these commercial vaccines contains the recombinant SU envelope protein (Marciani *et al*. 1991). The SU protein in the virus is glycosylated (gp70), however the recombinant protein used for immunization was produced in bacteria and is not glycosylated, therefore its molecular weight is 52 kDa (recombinant, rp52).

We were mainly interested in using the TM protein of retroviruses for immunization (Denner, 2011, 2012). This interest was based on publications demonstrating that antibodies against the membrane proximal external region (MPER) of the TM protein gp41 of HIV-1 such as 2F5 and 4E10 (Fig. 2) isolated from HIV-infected individuals were neutralizing up to 90% of all HIV-1 (Muster *et al*. 1993; Zwick *et al*., 2001). We started to immunize with the TM protein p15E of PERV. Effective neutralizing antibodies were induced and epitopes in the MPER as well as in the fusion peptide proximal region (FPPR) were identified. The epitopes in the MPER of p15E were similarly located and despite the evolutionary distance between PERV and HIV-1 a sequence homology was observed. The epitope in the MPER sequence of gp41 of HIV-1 had the sequence NWFN/DIT, in the MPER of p15E of PERV the sequence GWFEGWFNRSP was recognized (identical amino acids are underlined) (Fiebig *et al*., 2003). Antibodies neutralizing PERV and binding to the FPPR and MPER were induced in different species including goats, rats, guinea pigs, hamster, rabbits, and mice (Fiebig *et al*., 2003; Kaulitz *et al*., 2011; Waechter *et al*., 2013). Using affinity chromatography and recombinant proteins corresponding to the N- and C-terminal part of p15E as well as synthetic peptides corresponding to the FPPR and MPER, we were able to show that only the isolated antibodies specific for the MPER were neutralizing (Waechter *et al*., 2013). When we immunized with a combination of the TM protein p15E and the SU protein gp70 (rp52) of PERV, higher titers of neutralizing antibodies were induced (Denner *et al*., 2012).

Since animal models are not available in which the efficacy of antibodies neutralizing PERV could be tested,

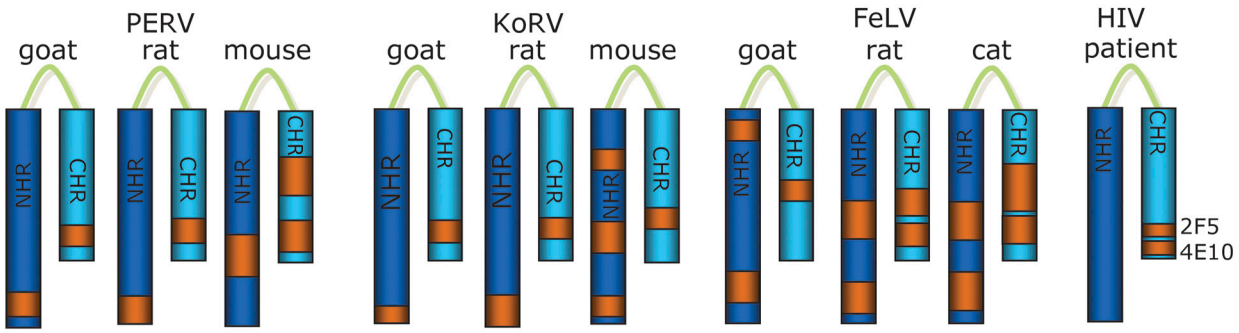

Figure 3. Schematic presentation of the epitopes recognized by neutralizing antisera induced in different species by immunization with the ectodomain of the transmembrane envelope proteins of PERV, KoRV, and FeLV. 2F5 and 4E10 were isolated from HIV-1 infected individuals and broadly neutralize HIV-1.

we used infections of cats with the related FeLV to study this topic. Immunization of cats (and several other species) with the TM protein p15E of FeLV resulted in neutralizing antibodies which recognized similar epitopes in the FPPR and MPER as described for PERV (FEGWFN in p15E of FeLV, HIV-1 and PERV see above, identical amino acids underlined) (Langhammer *et al*., 2005, 2006, 2011b). When we immunized with gp70 (rp52) of FeLV or a combination of both gp70 and p15E, the combination induced the highest titer of neutralizing antibodies (Langhammer *et al*., 2011a). When cats immunized with p15E, gp70 (rp52) and a combination of both were challenged with infectious FeLV, all animals immunized with gp70 (rp52) or the combination, and 50% of the animals immunized with p15E were protected from antigenemia and disease (Langhammer *et al*., 2011b). The absence of antigenemia indicates that the virus is not replicating and viral antigens cannot be detected in the serum. Thus, immunization with the envelope proteins protects the animals. However, even in the case of combination of both proteins, no sterilizing immunity was achieved (Langhammer *et al*., 2011b). Sterilizing immunity means complete protection from virus infection. In fact, protection from disease, but absence of sterilizing immunity was also reported for other commercial FeLV vaccines (Hofmann-Lehmann *et al*., 1997, 2007).

Envelope proteins of the KoRV induce neutralizing antibodies: basis for a vaccine

We had isolated a KoRV from an animal in the Zoo of Duisburg, Germany, which we designated KoRV Duisburg-Berlin ($KoRV_{D-B}$) (Fiebig *et al*., 2006). Part of the virus including the envelope proteins was sequenced (GenBank DQ174772). Only three amino acid substitutions in the Env region compared with a previously reported sequence of KoRV isolated in Australia were found (Hanger *et al*., 2000). We investigated the host range of the virus showing that the virus infected cells from humans and rats, but not from mice (Fiebig *et al*., 2006). These data were confirmed recently (Shojima *et al*., 2013). We characterized the protein pattern of purified virus and immunized with the recombinant TM protein p15E (Fiebig *et al*., 2006). p15E was cloned, expressed in *E. coli*, purified and used for immunization of goats, mice, and rats. A novel neutralization assay using $KoRV_{D-B}$ and susceptible human 293 cells was generated and we showed that the induced antibodies were neutralizing. The assay measures provirus DNA in the infected human 293 cells using real-time PCR (Fiebig *et al*., 2006). Epitope mapping showed that the sera recognized epitopes in the FPPR and MPER, and the sequence WFN was found in the MPER epitope (unpublished data) (Fig. 3).

Meanwhile we had also immunized with the purified SU protein gp70 (rp52) and with DNA corresponding to the Env protein gp70 and to the Env precursor molecule gp85. In all cases neutralizing antibodies were induced. The titer of neutralizing antibodies was higher when we immunized with gp70 compared with immunization with p15E (unpublished data).

Retroviruses cause immunosuppression

Many retroviruses induce immunosuppression in the infected host (Denner 1998, 2014; Mangeney *et al*., 2001; Mangeney *et al*., 2007; Oostendorp *et al*., 1993). Immunosuppression has been shown *in vivo* for HIV-1, HIV-2, MuLV, and FeLV and is always associated with opportunistic infections. The high prevalence of an opportunistic *Chlamydia* infection suggests that KoRV also induces immunosuppression. Unfortunately this has not been well-studied with *Chlamydia*, and, in addition, it is not known whether other opportunistic infections such as herpes virus and trypanosoma infection are increased in KoRV-infected animals. The mechanism how retroviruses induce immunodeficiencies is still unclear, but there is accumulating evidence that the TM protein is involved. We recently demonstrated that the TM protein gp41 of HIV-1 (Denner *et al*., 1994, 2013; Morozov *et al*., 2012), the TM protein of the human endogenous retrovirus HERV-K (Morozov *et al*., 2013) and the TM protein p15E of PERV (Denner, 1998; Tacke *et al*., 2000) inhibited lymphocyte activation by mitogens and modulated cytokine expression in PBMCs. The interleukins IL-10 and IL-6 were shown elevated and molecules involved in innate immunity were down regulated. When we studied purified KoRV, we showed that the virus particles induced enhanced expression of IL-10 in human donor PBMCs (Fiebig *et al*., 2006). Using a cytokine array, elevated expression of IL-10, of the grow-related oncogene GRO, of IL-6 and the monocyte chemotactic protein-1 (MCP-1) was observed after 24 hrs, whereas 18 other cytokines remained unchanged at that time (Denner et al, unpublished data). It was shown that all TM proteins contain a highly conserved domain, the so-called immunosuppressive (isu) domain (Fig. 2), and synthetic peptides corresponding to these domains are also able to inhibit lymphocyte activation and to modulate gene expression (Cianciolo *et al*., 1985; Denner *et al*., 1994; Ruegg *et al*., 1989).

We recently showed that single mutations in the immunosuppressive domain of gp41 of HIV-1 abrogated the immunosuppressive activity of the molecule and immunization with the mutated gp41 resulted in better antibody responses when compared with immunization with the wild-type gp41 (Morozov *et al*., 2012). It would be interesting to analyze whether mutations in the immunosuppressive domain of p15E of the KoRV also improves the immune response.

Conclusion and outlook

Koalas should be immunized with KoRV envelope antigens to determine whether neutralizing antibodies are induced and if so, whether such antibodies are able to protect animals from infection. Furthermore, whether immunization with these antigens has a therapeutic effect on animals already infected with KoRV should be investigated. Mutations in the immunosuppressive domain of the TM protein may increase the antibody response. Immunizing with a subunit of the TM protein of PERV we recently found novel neutralizing antibodies directed against an epitope in the N-terminal helix of the molecule (Denner & Waechter, 2014). Broadly neutralizing antibodies directed against the N-terminal helix of gp41 of HIV-1 were also found in HIV-infected individuals. Therefore a mixture of envelope antigens may be used for immunization. Prevention of infection or decreasing the virus load will prevent or reduce the potential KoRV-induced immunodeficiency and hopefully also protect koalas from infection with *Chlamydia* and other opportunistic infections.

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