Interplay Between JSRV, an Oncogenic Retrovirus, and the Pulmonary Epithelium

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Abstract: Jaagsiekte sheep retrovirus (JSRV) is a retrovirus which infects small ruminants and is responsible for a natural lung cancer. This virus bears an oncogenic envelope inducing epithelial cell transformation from the deep lung. The cells, main target of the infection and constitutive of the tumors, are alveolar type II cells in the alveoli and Clara cells in the bronchioli. The immune response to JSRV infection is poorly understood. The specific humoral response is limited and the cell-mediated response is marked by CD4+ lymphocytopenia, neutrophilia, and macrophage invasion of the tumor. The mechanisms of viral immune evasion could be explained by the direct and indirect immunosuppressive effects of the virus, and by the immune tolerance of the infected hosts due to the presence in the sheep genome of endogenous viral forms (enJSRV).

Keywords: Adenocarcinoma, alveolar type II cells, immune response, JSRV, lung.

The airway epithelium being strategically positioned at the interface with the environment, it plays a key role in the host defence system. A large volume of air is inhaled every day and the extensive surface area of the lung makes the respiratory system especially vulnerable to airborne-infectious agents such as *influenza* viruses, respiratory syncitial virus, rhinovirus, coronavirus and retroviruses. We will focus here on JSRV, a retrovirus infecting the lung parenchyma and inducing an adenocarcinoma in sheep.

VIRAL IMMUNE RESPONSE IN THE LUNG

The innate immune response is the first line of defense against viral pathogens. It requires their early detection through pathogen-specific recognition receptors and recruitment of efficient antiviral effectors (extensively reviewed in [1-4]). Upon pathogen infection, the lung epithelium responds by releasing antimicrobial peptides into the airway lumen, chemokines and cytokines into the submucosa that initiate an inflammatory response and induce the recruitment of phagocytes, dendritic cells and lymphocytes. Cells of the innate immune system express PRRs (Pattern Recognition Receptors) able to regulate the synthesis of antiviral type I and III interferons (IFNs) and pro-inflammatory cytokines [5, 6]. PRRs that participate in viral sensing include toll-like receptors (TLR), RNA helicases and cytosolic DNA sensors [4, 7]. Toll like receptors located on cell surfaces and/or in cytoplasm act as initiators of the innate immune response by providing the ability for the host to recognize PAMPS (Pathogen-Associated Molecular Patterns). Intracellular TLRs and other PRRs such as RNA helicase molecules (i.e. retinoic-acid inducible protein 1 [RIG-1] and melanoma differentiation-associated gene-5 [mda-5]) expressed in the cytosol of sentinel and epithelial cells, are able to detect intracellular dsRNA (double strand RNA) and ssRNA (single strand RNA) to initiate type I IFN and pro-inflammatory cytokine production *via* activation of specific-transcription factors including IRFs (Interferon Regulatory Factors) and NF-κB (Nuclear Factor-kappaB).

In recent years, it has become clear that airway epithelial cells not only constitute a mechanical barrier, but also actively contribute to the innate immune system [7]. Although there have been numerous studies on viral infection of the conducting airways (reviewed in [8]) relatively few have focused on the distal gas-exchange unit of the deep lung, site of severe clinical infection such as SARS-CoV (Severe Acute Respiratory Syndrome-Coronavirus) or avian *influenza* virus. The epithelium where gas exchanges occur is constituted of two main cell types. Alveolar type I cells are flat terminally-differentiated cells that cover about 95% of the alveolar surface area and are mainly involved in gas exchange. Cuboidal alveolar type II cells are metabolically active, produce pulmonary surfactant and transport fluid and sodium to the basolateral surface to keep the alveolar surface suitable for gas exchange [9]. Alveolar type II cells are self-renewing and are able to transdifferentiate into alveolar type I cells when they need to be replaced. Both alveolar type I and type II cells participate in the innate immune response by producing interferons and cytokines in response to virus infection [4, 10, 11]. They also produce antimicrobial substances such as surfactant protein A (SP-A), surfactant protein-D (SP-D), beta-defensins or lysozyme in response to microbial infection [7, 12, 13]. SP-A and SP-D interact with the glycoconjugates and lipids on the virus surface inducing aggregation of complexes, enhancement of phagocytosis and release of cytokines and chemokines at the site of infection [14]. Beta-defensins have a dual role in antiviral activity: they may block viral infection by directly acting on virions or they may affect the target cell and thereby indirectly interfere with viral infection [15]. Taken together, all these mechanisms take place in

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order to eliminate viruses from the lung and maintain the integrity of the alveolar function. Some viruses have developed strategies to bypass or delude this defence in order to initiate cell deregulation or even tumours.

OVINE PULMONARY ADENOCARCINOMA: A RETROVIRUS-INDUCED LUNG CANCER

JSRV (Jaagsiekte Sheep RetroVirus) is a β-retrovirus, infecting small ruminants. This virus is responsible for a pulmonary adenocarcinoma associated with the transformation of epithelial cells from the lung parenchyma, i.e. alveolar type II cells in the alveoli and Clara cells in the bronchioli [16] (Fig. **1**). The disease is usually sporadic within flocks but can be epizootic in some specific conditions of farming, leading to the death of up to 5% of the animals [16]. This animal tumour is clinically, radiologically and histologically related to the pneumonic form of human bronchioloalveolar cancer (pBAC), a rare form of lung tumour in humans which represent less than 4% of all adenocarcinomas [16, 17]. These similarities have always intrigued pathologists and chest physicians and have been stressed as early as 1939 [18, 19]. Both the pneumonic form of human lung adenocarcinoma and the ovine pulmonary adenocarcinoma are described as mixed-type adenocarci-noma, associating a predominance of bronchioloalveolar lesions and papillary and/or acinar lesions. Ovine pulmonary adenocarcinoma has been considered as a model for human adenocarcinomas, and especially for the pneumonic-type bronchioloalveolar cancer [18]. Similarly to ovine pulmonary adenocarcinoma, bronchioloalveolar cancer is a slow-growing tumour with rare distant metastases. It is clinically associated with highly productive cough and progressive restrictive respiratory failure [18, 20].

Given the similarities between ovine pulmonary adenocarcinoma and human BAC, a viral cause to human bronchioloalveolar cancer has long been hypothesized; but up to now reports exploring the link between JSRV and BAC [21-23] remain controversial. Nevertheless, ovine pulmonary adenocarcinoma offers an invaluable animal model for studying the molecular mechanisms of lung epithelial transformation occurring in cancer.

JSRV AND ITS ONCOGENIC ENVELOPE

JSRV genome contains the typical retrovirus genes *gag*, *pro*, *pol* and *env* respectively encoding the capsid and nucleocapsid proteins, the protease, the enzymatic activities and the envelope glycoproteins. An additional small ORF (open reading frame), called "x" and encoding a putative protein of unknown function, overlaps the end of the *pol* gene (Fig. **2**). JSRV mainly infects and only transforms alveolar type II cells and Clara cells [24] leading to development of tumours in the deep lung. Using molecular detection of RNA and DNA, JSRV has been demonstrated to infect not only the respiratory tract but also several lymphoid tissues such as the mediastinal lymph nodes draining the lungs [25]. JSRV has been detected at low level in the spleen, the thymus, the bone marrow and the peripheral blood mononuclear cells [25]. Although very few cells of the immune systems are infected, the highest provirus load is in the monocytes/macrophages, followed by B and T cells. There is no evidence of virus transformation of any of these cell types [25, 26]. Hyal2 (Hyaluronidase type 2) is the cell receptor for JSRV [27] and is expressed at the surface of many cell types. Hyal2 expression is not restricted to lung epithelial cells, suggesting that the entry of the virus into the cells is not the sole determinant of pathogenesis.

A family of related endogenous sequences, enJSRV closely related to the exogenous JSRV, is present in domestic and wild ungulates [28]. JSRV and enJSRV genomes are highly related with 90-98% homology in

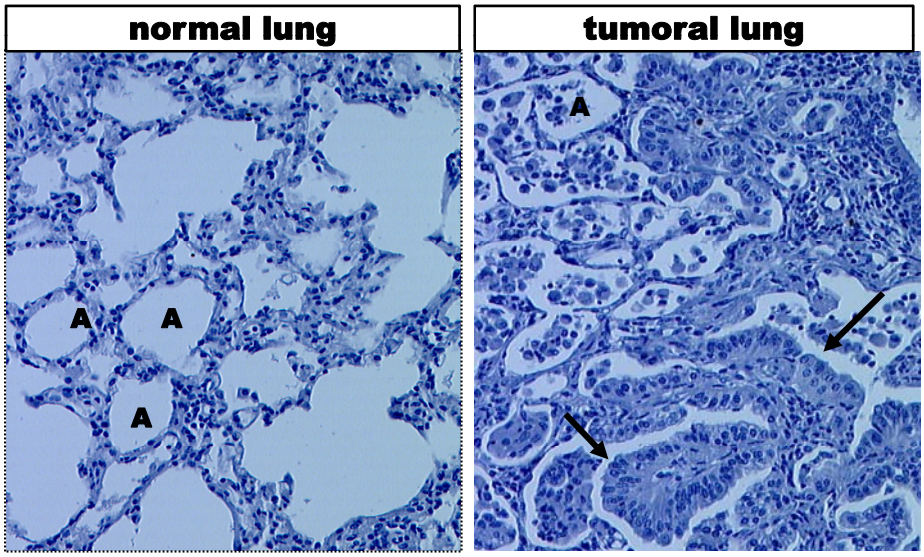


Fig. (1). Tumoral lesions observed during ovine pulmonary adenocarcinoma result from the transformation and accumulation of alveolar type II cells in the alveoli and Clara Cells in the bronchioli. While the normal lung presents large open alveoli (A), the architecture of the parenchyma is disturbed in the tumoral lung. Arrows point the accumulation of airway type II cells along the alveoli walls in a typical tumour.

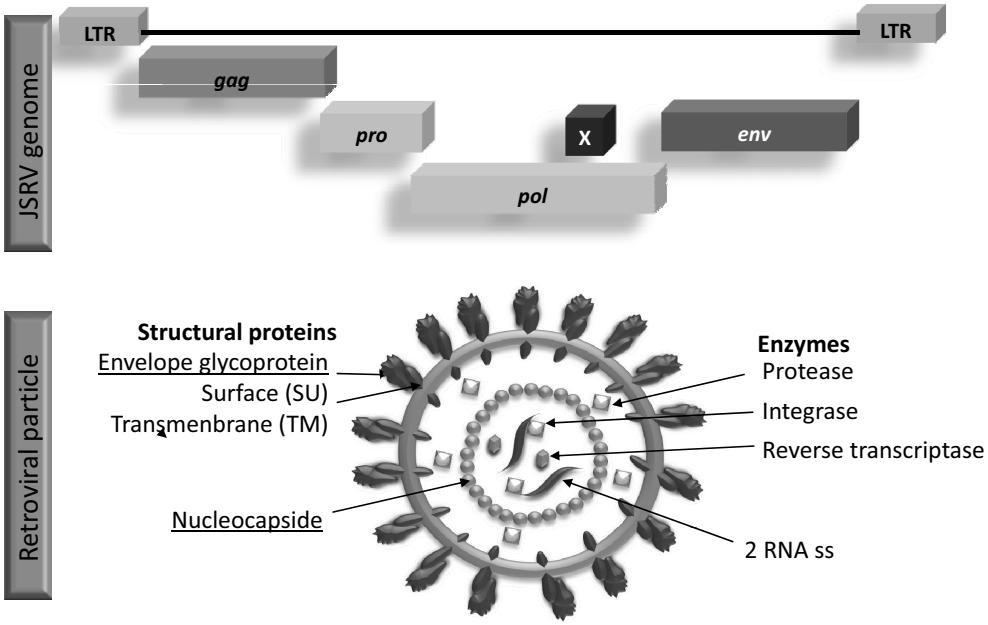


Fig. (2). JSRV viral genome is typically a dimmer of linear, positive-sense, single-stranded RNA with each strand being 7.5 kilobases in length. The ***gag*** gene encodes the matrix, the capsid and the nucleocapsid; the ***pro*** gene encodes a protease, the ***pol*** gene encodes the reverse transcriptase, RNaseH and integrase proteins; and the ***env*** gene encodes the glycoproteins needed for receptor recognition and envelope anchoring. An important feature of the retroviral genome is the **long terminal repeat (LTR)** regions found on both end of the provirus genome. The LTR plays an important role in initiating viral DNA synthesis and its integration as well as regulating transcription of the viral genes. The JSRV genome also contains an additional gene called "x" of unknown role.

deduced amino-acid sequences [29]. Endogenous JSRVs derive from ancestral integration of exogenous JSRV into the host genome. They are maintained as Mendelian genes and are vertically transferred to the offspring. They are defective for at least one viral gene as a result of mutations acquired during their integration into the germ-line. The involvement of enJSRV in the carcinogenesis remains unknown.

It is now clearly established that JSRV induces tumors *via* the oncogenic properties of its envelope, Env, which is both necessary and sufficient to induce transformation (reviewed in [30]). The oncogenic property of JSRV Env has been shown both *in vitro* in various cell types including murine NIH 3T3 fibroblasts [33], rat 208F fibroblasts [27], avian DF-1 fibroblasts [34, 35], bronchial human BEAS-2B epithelial cells [36], canine kidney MDCK epithelial cells [37], and rat kidney RK3E cells [38]; and *in vivo* in immune-deficient mice [31] and in sheep [32]. This oncogenic mechanism is unusual and JSRV is among the few retroviruses using one of its structural proteins to transform cells. Deletion experiments show that the TM (Trans-Membrane) region of the envelope is the main determinant for cell transformation [39-41]. The cytoplasmic tail (CT) of TM, composed of 43 amino acids, is essential for the transformation process in MDCK and NIH-3T3 cells [37, 42]. This region contains a peptidic YXXM motif, corresponding to a potential consensus site (phosphorylated on tyrosine Y) linked to the SH2 domain of the p85 subunit of PI3K (Phosphatidyl-Inositol-3 Kinase), a kinase that activates Akt (Fig. **3**). The mechanisms leading to JSRV-induced cell transformation are in fact much more complex than previously considered. Several experiments ruled out a direct role for the YXXM motif in Akt activation [35, 37, 43].

Although a number of points remain unanswered, the transformation steps are dependent on the nature and origin of the cells. Hence, understanding the key events in the transformation of ovine alveolar type II cells is now the challenge of ongoing work. Our group has developed primary cell cultures isolated from ovine tumoral lungs [44, 45]; those tumour-derived cells are alveolar type II cells as revealed by the presence of lamellar bodies in the cytoplasm, and the expression of surfactant protein C (SP-C) and A (SP-A). We have reported activation of telomerase in ovine pulmonary adenocarcinoma-derived alveolar type II cells and in tumoral lung tissues, suggesting that replicative senescence may be negatively regulated in this tumour. Moreover, the Akt pathway deregulation has been shown to be deregulated in the tumoral process [45] (Fig. **3**).

ADAPTATIVE IMMUNE RESPONSE TO JSRV

The immune response to JSRV infection is poorly understood [46]. The cell-mediated response is narrow with CD4+ lymphocytopenia and neutrophilia being demonstrated in the peripheral blood of adult sheep during the terminal stages of the cancer [26, 47]. More recently, a study reports invading macrophages in the tumours and its surrounding, during naturally or experimentally induced ovine pulmonary adenocarcinoma [48]. Immature macrophages (CD14+, weak CD11b+) or monocytes expressing high levels of IFN-γ are present in the immediate vicinity of the tumour and within the alveolar lumina. Macrophage activity seems to be suppressed at the periphery of the tumour, suggesting a local immune suppressive response that may be mediated by the surfactant secretion [48]. No increase of dendritic, B and γδT cell populations has been evidenced. Older reports suggested

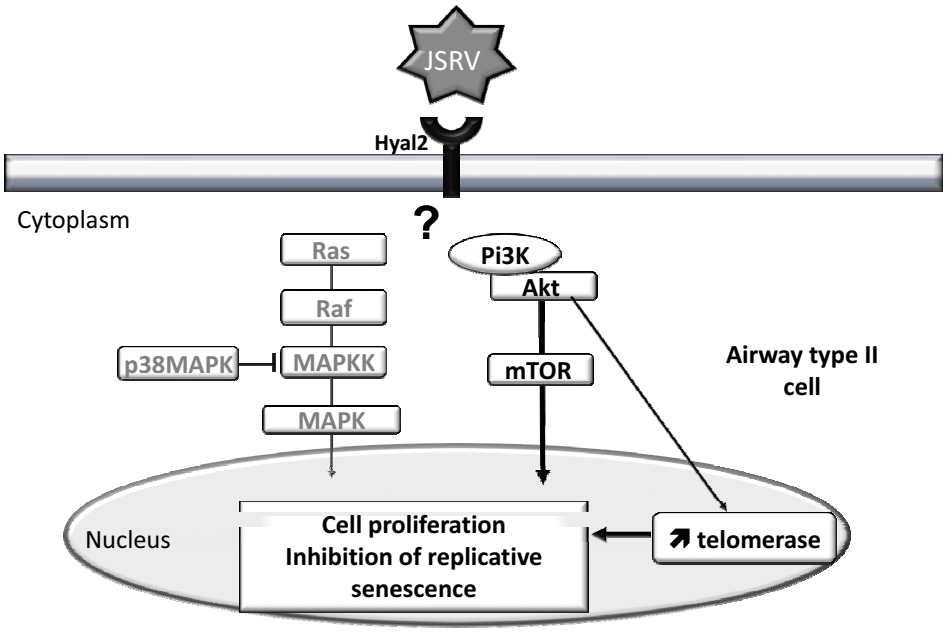


Fig. (3). Signalling pathways activated by JSRV in lung epithelial cells. Primary alveolar type II cells derived from ovine tumours display a proliferative advantage compared to normal cells, with a high telomerase activity leading to inhibition of the replicative senescence. Those cells also show a deregulation of the Pi3K/Akt pathway. These mechanisms are implicated in the tumour development and maintenance. Works done on various cell lines have shown that the MAPK pathways can also be implicated when cells are expressing the envelope protein of JSRV [30].

induction of a minor immunosuppression upon JSRV infection and its association with an increased susceptibility to other infectious agents such as *Pasteurella haemolityca* [49]. Reduced lymphoproliferative responses to mitogen stimulation such as concanavalin A has been shown in terminally ill adult sheep as well as in JSRV-inoculated lamb, prior to the onset of clinical disease [46] suggesting that JSRV may compromise the host cellular immune response.

Interestingly, DC-LAMP/CD208 (Dendritic Cell-Lysosomal Associated Membrane Protein) is strongly expressed at the apical side of alveolar type II cells in lung tissues from JSRV-infected animals and human bronchioloalveolar cancer [50]. DC-LAMP/CD208 has been originally described as a molecule specifically expressed in mature dendritic cells at the limiting membrane of MHC Class II-containing intracellular compartments involved in the MHC class II peptide loading and transport to the cell surface [51, 52]. In mouse, DC-LAMP/CD208 colocalizes with MHC class II molecules in lamellar bodies, intracytoplasmic organelles specific of alveolar type II cells [50]. This may link DC-LAMP to the controversial role of alveolar type II cells in MHC class II restricted antigen presentation. Alveolar type II cells constitutively express MHC Class II molecules [53] increased by IFNγ in rats [54] or pathological conditions in humans [55, 56], cathepsin [57] or CD54 [57]. Alveolar type II cells strategically localized at the interface between the parenchyma and the outside environment, express molecules required for efficient antigen presentation to CD4+ T cells and may act as pulmonary antigen-presenting cells (APC) [58].

Evidence of a JSRV-specific humoral response remains a subject of discussion. Some studies showed a local IgA response, the formation of viral immune complexes, and a systemic antibody response that cross-react with recombinant antigens of highly related viruses [59-61]. On the other hand, accumulating data concluded to the absence of a JSRV-specific immune response [62-66]. This failure to detect JSRV specific antibodies in experimentally or naturally infected sheep remained puzzling. But more recently, it has been shown that antibodies could be detected in the serum or lung lavages of sheep immunized with recombinant JSRV capsid or surface glycoprotein inoculated with various adjuvants [67, 68]. These results indicate that sheep are not inherently unresponsive to JSRV antigens and that the apparent tolerance, perhaps as a consequence of endogenous sequence expressed *in utero* or as a direct consequence of infection by JSRV, can be broken.

The infection of the immune system by JSRV may be advantageous to the virus either by directly facilitating the infection and the subsequent transformation of alveolar type II cells or indirectly by the induction of an immunosuppressive state. MMTV (mouse mammary tumour virus) and JSRV share interesting features. Both are retrovirus that have their own endogenous counterparts and are associated with epithelial tumours. Both viruses replicate actively in transformed epithelial cells but are also maintained at a low-level of infection in host lymphoid cells. However, the infection route and the targeted organs are distinct. MMTV enters through the digestive tract and has to reach the mammary gland, meaning that the infection of B and T cells is an absolute requirement for the virus transfer to the target organs. In the case of JSRV, initial infection is mainly through the respiratory route and therefore alveolar type II cells and Clara cells are readily accessible. It is then conceivable that JSRV first infects lung epithelial cells and replicates. After few replications, surrounding lymphoid

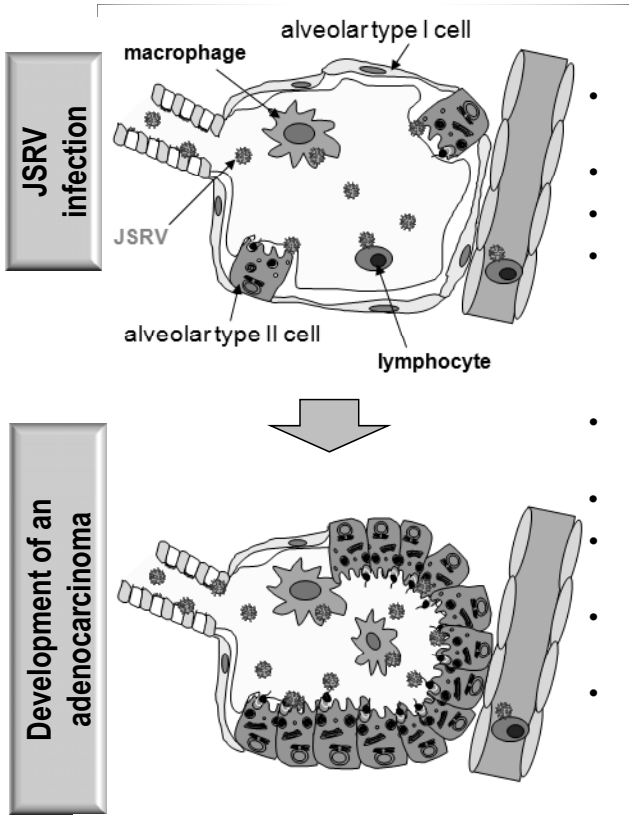
cells or phagocytic cells may carry the virus to the mediastinal lymph nodes. Holland and colleagues have shown that dissemination to the lymphoid tissues precedes tumour formation, as detection of JSRV proviral DNA is positive as early as 7 days post-inoculation and well before the onset of tumoral lesions [26]. Lymphocyte proliferation studies in response to mitogen stimulation have revealed an alteration in host cellular responses due to the presence of JSRV [46] (reduced response to ConA stimulation). This modification occurring early in the infection course is sustained through tumorigenesis up to the terminal stage of the disease. Lastly, despite low viral load in immune cells during the early steps post infection, the presence of viruses does alter the cell functions [46]. This may affect the function of lymphoid tissues and interfere or prevent the development of a specific immune response against the tumoral cells or the virus allowing the tumour progression (Fig. **4**). At present, the role of JSRV infection of lymphoid tissues in pathogenesis is still unknown.

The majority of cases of JSRV-induced adenocarcinomas presents with a peripheral CD4+ lymphocytopenia and neutrophilia [26]. Taken together with the lack of circulating JSRV antibodies and the increased susceptibility to secondary bacterial infection, this could participate to a mild immunosuppression of JSRV-infected sheep. However, it is difficult to believe that immunosuppression could explain this absence of immune response, as even the highly immunosuppressive HIV is unable to abolish the circulation of virus-specific antibodies.

enJSRV INTERFERENCE

The absence of a strong immune JSRV-specific response may be related to the presence of the closely related enJSRV sequences. During ontogeny, clonal deletion of JSRV-specific repertoire may lead to immunological tolerance. Indeed, retroelements form a large and diverse family of mobile elements that can be found in most eukaryotic organisms. They propagate by reverse transcription of RNA intermediates and integrate their genetic information into the genomic DNA of the host cells. Retroelements include retrovirus-like elements; the integrated forms or proviruses consist of two long terminal repeats (LTRs) flanking an internal region containing one to three major open reading frame coding for structural (Gag and sometimes Env) and enzymatic (Pol) functions necessary for their replication cycle. These elements, known as endogenous retroviruses (ERVs) are transmitted through the germline as stable Mendelian genes. It is assumed that ERVs derived from ancient integration events of exogenous retroviruses into the germline. Generally, ERVs are transcriptionally silent and are often defective, differing from the exogenous counterparts by deletions or point mutations that render them unable to produce infectious virus. However, several ERVs maintain at least some intact open reading frames that can be expressed. Particularly, it has been shown that several ERVs containing *gag* and *pol* genes also retain the ability to encode functional Env glycoproteins.

The enJSRVs are genetically highly related to JSRV [69], which indicates a recent evolutionary relationship [70]. Several studies suggest that the endogenization of enJSRV



EARLY STEPS

- Clara Cells and alveolar type II cells are infected by JSRV.
- Macrophages, B and T cells : JSRV+/-
- Detection of specific antibodies : low
- Reduced level of CD4+

LATE STAGE

- Accumulation of transformed infected type II alveolar cells and Clara cell
- Lack of circulating JSRV antibodies.
- CD4+ lymphocytopenia and neutrophilia. No increase on dendritic cells
- Increased susceptibility to secondary bacterial infection.
- Local immune suppressive response. Invading macrophages in the tumour and its surroundings but no macrophage activity at the periphery of the tumour .

Fig. (4). Maintenance of a reduced cellular and humoral immune response upon JSRV infection and during the course of the virally-induced disease. At the early step of infection, a reduced level of CD4+, the absence of dendritic cells and a low level of circulating specific antibody may be observed. Later, while tumour has developed with alveolar type II cells colonizing the alveolar walls, there is a noticeable influx of macrophages around and in the tumour area, without any other major changes.