Calves Persistently Infected by Bovine Viral Diarrhea Virus and Human Autoimmune Polyglandular Syndrome Type-1: What to learn from two natural models of impaired immunological tolerance? A review.

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1. Abstract.

Human clinicians know about autoimmune polyglandular syndrome type 1 (APS-1) but do not know about bovine viral diarrhea virus (BVDV) persistently infected (PI) calves. These two clinical entities have as a common factor that they represent two natural models of immune tolerance failure occurring during thymocyte maturation in fetal life. In APS-1, mutations of the autoimmune regulatory gene (AIRE gene) are responsible for the reduction of promiscuous expression of tissue-specific antigens (TSA) by medullary thymus epithelial cells (mTECs) during presentation of self-antigens to single negative (SN) T cells. Such reduction results in the generation of autoreactive T cell clones that colonize secondary lymphoid tissues and cause APS-1 in postnatal life. APS-1 patients are the evidence of the effects of these mutations and support the importance of PGE during the generation of immune tolerance. Heifers or pregnant cows infected with a non-cytopathic strain of BVDV during the period of intrathymic maturation of fetal T lymphocytes generate PI calves. Although the molecular mechanism of PI calf generation is still unsolved, viral antigens presented as self-antigens during the developing T-cells' intrathymic maturation appear to be responsible. Although there is no published work on AIRE gene expression during T cell maturation in bovine fetuses, the high homology in nucleotide and amino acid sequence of the AIRE gene and protein between humans and bovines, and high conservation of the gene across the species, supports that the bovine AIRE gene functions similarly as in humans. In this paper, we discuss similarities between APS-1 patients and PI calves' clinical signs. We propose that there must be processes related to reduced PGE in bovine fetal mTECs caused by the virus, resulting in autoreactive T cells responsible for clinical signs of PI calf cases. In the absence of experimental evidence on the generation of PI animals, the knowledge achieved in APS-1 allows us to propose experimental models to fill these knowledge gaps. Accordingly, knowledge gained about AIRE gene mutations could contribute to understanding the

mechanisms of tolerance against viral infections in cattle and other animal species and their effect on postnatal life, to investigate prevention or treatment alternatives.

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Abbreviations

AIRE/AIRE: autoimmune regulator transcription gene/protein

APECED: Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy

APS-1: Autoimmune polyglandular syndrome type-1

BVDV-1/BVDV-2: Bovine Viral Diarrhea Virus species 1 and 2

CARD: Caspase-recruitment domain

Cp: Cytopathic Bovine Viral Diarrhea Virus

cTECs: cortical thymic epithelial cells

DCs: Dendritic cells

DP: $CD4^+/CD8^+$ double-positive T-cell

eTACs: extra thymus AIRE-expressing cells

IGR: Intrauterine Growth Restriction

MD: Mucosal Disease (Calves)

mTECs: medullary thymic epithelial cell

Ncp: Non-cytopathic Bovine Viral Diarrhea Virus

NS: Negative selection

PGE: Promiscuous gene expression

PI: Persistently infected

PS: Positive selection

PTA: Peripheral tissue antigens

SAND: complex in **S**P100, **A**IRE1, **N**ucP41/P75, and **D**EAF1 proteins

SP: single positive $CD4^+/CD8^-$or $CD4^-/CD8^+$ T-cell.

g/d T-cells: T-cells harboring a g/d T-cell receptor rearrangement.

T-reg: T regulatory cells

Th-17: IL-17 producing T helper cells.

2. Introduction

Most physicians know that the autoimmune polyglandular syndrome type 1 (APS-1) (1) is caused by mutations of the autoimmune regulatory (*AIRE)* gene and the resulting autoimmune diseases (2, 3). Still, all have never heard about the infection by bovine viral diarrhea virus (BVDV) and persistently infected (PI) calves. Otherwise, most veterinarians know the impact of PI calves in spreading BVDV infection in cattle herds (4) but have never heard about APS-1. We focused on these entities (APS-1 and PI-calves) because both represent natural models of impaired immune tolerance. Several mutations in the *AIRE* gene give rise to APS-1 and the Autoimmune Polyglandular Candidiasis Ectodermal Dystrophy (APECED) syndrome (5), characterized by a triad of mucocutaneous candidiasis, polyendocrinopathy, and Addison's disease (6-8).

In patients suffering from APS-1, mutations in the *AIRE* gene result in impaired central immune tolerance during thymocyte development in fetal life by generating autoreactive T lymphocytes against proteins of the central nervous, endocrine, and mucocutaneous tissues. The results are the clinical signs typical of APS-1 due to autoimmune responses. On the other hand, PI calves refer to young animals infected *in utero* by the bovine viral diarrhea virus (BVDV) due to infection of pregnant cows with non-cytopathic strains of the virus. Interestingly, authors reported several injuries in CNS, endocrine, and cutaneous tissues in PI-calves (9). When BVDV infects pregnant cows during fetal thymus development, viral particles circulate into the developing fetal thymus, causing the viral proteins to be recognized as self-antigens. Consequently, the fetus can survive until term, exhibiting no clinical signs or a variable range of clinical signs in postnatal life. Whatever the clinical condition, PI-calves eliminate viral particles throughout their lives. Interestingly, PI calves exhibit variable degrees of clinical signs due to the impaired immune response against BVDV. Some of the PI-calves' clinical signs are like those exhibited by APS-1 patients, suggesting the existence of common pathogenic mechanisms between APS-1 and PI-calves.

Given the molecular mechanisms of AIRE-dependent PGE of TSA (Reviewed in 10), which are impaired in patients suffering from mutations of the *AIRE* gene (11), and the lack of knowledge on the precise mechanisms of bovine T-cell development and bovine *AIRE* gene expression in the developing bovine thymus, it should be possible to define the morphological development, transcriptome, and proteome of the bovine developing fetus, as recently reported for human thymus development (12-16). The most advanced technological approaches must be applied to research focused on timing bovine intrathymic development and studying the molecular mechanisms of PI fetuses. In this paper, we will focus on the mechanisms of acquisition of central tolerance during intra-thymus maturation of Thymocytes, the description of the structure and function of AIRE gene and protein, the most relevant characteristics of APS type 1, and the generation of PI calves. Finally, we will discuss the similarities and differences between these two entities as models of immune tolerance and provide some cues on the existence of similarities to APS-1 syndrome in BVDV PI calves. The possibility of readdressing research activities in the context of modulating the generation of central tolerance for avoiding the generation of PI calves is also discussed.

2.1 Information search and analysis

A narrative review was conducted based on a paired literature search, involving common search terms for both syndromes (e.g., AIRE gen AND APS-1 vs. AIRE gen AND BVDV). The process of identifying relevant articles considered a specific research interest: What are the particularities and similarities of and between APS-1 and BVDV? Four search platforms (e.g., PubMed, Embase, ScienceDirect, SciELO) were searched. The inclusion criteria considered only those articles published in peer-reviewed journals, available in English, French, Portuguese, or Spanish. No institutional approval was required for the development of the present review.

2.2 Literature search.

The literature search was conducted in Pubmed and ScienceDirect databases from March 2017 to August 2023. The words used for the APS-1 search included: APS-1, APECED, Autoimmune, Polyglandular, Thymic development, Central Tolerance, and Intrathymic development. For PI calves the search included Bovine Viral Diarrhea Virus, BVDV, PI-calves, persistently infected calves, Immune response, or combinations. The search included original articles, systematic reviews, clinical cases, reviews, and case reports in both cases.

2.3 Papers analysis.

A total of 5659 papers were found for APS-1, APECED, BVDV, and PI calves (Table 1). Because no comparison can be made for clinical studies between APS-1 and PI calves, we selected original articles, Books and Documents, Clinical Trials, meta-analyses, Randomized Controlled Trials, Reviews, and Systematic Reviews, that help us to construct our hypothesis on the similitudes and differences between these two entities. There were no papers related to molecular mechanisms of the generation of PI calves. We finally used 146 articles for our review.

Table 1. Summary of papers related to the subject topic available in the literature.

Word	Full text	Books and Documents	Clinical Trial	Meta-Analysis	Randomized Controlled Trial	Review	Systematic Review	Total
APS-1	243	3	4	2	0	48	1	58
APECED	1755							
BVDV	3399	1	92	9	59	231	12	404
PI infected calves	262	0	12	0	7	17	1	37
Total	5659	4	108	11	66	296	14	499

3. Human thymocyte development.

During thymus development, early T-cell precursors (ETP) ($CD44^{hi}CD117^{hi}CD25^{-}$) migrate from the bone marrow and enter the developing thymus for thymocyte maturation. In the cortex, pre-T-cells ($CD3+/TCR^{low}/CD4^{-}/CD8^{-}$) interact with highly specialized cortical thymic epithelial cells (cTECs) (12, 17, 18), and mTECs to start their development toward the

DN, DP, and then SN steps of thymocyte development (Figure 1) in a tightly coordinated crosstalk between thymic epithelial cells, bone marrow-derived APC, and thymocytes (Reviewed in 16). The most recent phenotypic profile of developing T-cells is depicted in Table 2.

In human thymic development, ETP enters the thymus around eight weeks post conception (WPC), where they consecutively develop to DN steps (DN1 to DN4), then to the DP at the cortico-medullary junction and the SP steps in the thymus medulla resulting in conventional alpha/beta (α/β, and gamma/delta (γ/δ) TCR rearrangements, and non-conventional T-cells (20) (Table 2). After completing their DN phenotype through the thymic cortex, cTECs provide the developing T-cells with specific signals for their positive selection consisting of transformation into T-cells (CD3+/TCR^{low}/$CD4^+$/$CD8^+$) (12). cTECs produce the cytokines T-cells require to become DP and increase their surface expression of TCR, becoming $CD3^+$/TCR^{high}/$CD4^+$/$CD8^+$. At this point of T-cell development, the DP T-cells must undergo a negative selection process consisting of DN and SP steps (7, 17, 21). At this stage, it is generated the γ/δ T-cell repertoire (Table 2). The exposition of DP T-cells to MHC-coupled self-antigens mainly presented by mTECs, DCs, and intrathymic B cells, results in the generation of SN ($CD3^+$/TCR^{high}/$CD4^+$ or $CD3^+$/TCR^{high}/$CD8^+$) T-cells, which represent the two major subsets of circulating T-cells in the post-natal life, being responsible for the adaptive cellular and humoral immune responses. Besides, other sets of mature T-cells comprise T-reg cells, unconventional $T_{(agonist)}$, and CD8αα$^+$(I), CD8αα$^+$(II), CD8αα or CD8γ/δ$^+$*EOMES*$^+$ subsets (Table 2) are generated.

Mature conventional T-cells leave the thymus to harbor the capability to recognize foreign antigens by their α/β or γ/δ TCR conformation. In peripheral lymphoid tissues, CD4+ T-cells interact with Møs, dendritic cells, B cells -the antigen-presenting cells or APC-, and some epithelial cells (Figure 1). Naïve T-cells refer to mature T-cells that have not encountered its specific antigen capable of binding their TCR with high affinity to elicit the cascade of intracellular signaling responsible for antigen-specific T-cell activation, differentiation, and proliferation when encountering their specific non-self-antigens. Three recent works elegantly depict intrathymic T-cell development (15, 16, 22). A detailed summary of intrathymic T-cell development is presented in Table 2. The myriad of ETP entering the thymus medulla and undergoing negative selection and apoptosis represents up to 75% of the overall population of hematopoietic precursors entering the thymus, all of which undergo apoptosis (23). The resulting "educated" T-cells can recognize self-antigens and not become activated against them, a fundamental mechanism to prevent the generation of autoimmune diseases.

3.1 An overview of the development of immune self-tolerance

Immune tolerance refers to the capability of the immune system to recognize the universe of self-antigens and not react against them, avoiding generating adaptive immune responses against self-antigens and tissues, resulting in autoimmunity (17). For this purpose, two mechanisms have been intensively studied: central tolerance and peripheral tolerance. Magrone and Jirillo (2019) summarize self-tolerance as follows: (i) During negative selection, autoreactive T cell clones (those that recognize self-antigens and undergo activation) are eliminated through Fas-mediated induction of apoptosis by signals from thymic DCs. (ii) Activated thymic Treg in conjunction with mTECs and DC suppress autoreactive T cell clones. (iii) In the periphery, Treg cells cooperating with tolerogenic DCs, eliminate clones of autoreactive T-cells that have escaped negative selection (24).

Molecular mechanisms of tolerance induction. During intrathymic maturation, expression of the *AIRE* gene results in the transduction of the AIRE protein by mTECs, and to a lesser extent by DCs, macrophages (Møs), and B cells. The AIRE protein functions as a multi-functional transcription promoter for the expression of genes encoding tissue-specific antigens (TSA) required to generate self-tolerance during the transition of DP to SP cells. This step is critical for the SP thymocyte to recognize the universe of self-peptides generated due to the PGE of genes encoding TSA and the corresponding protein processing through MHC-I and MHC-II self-peptide coupling (25). Consequently, SP T-cells recognize the self-peptides coupled to MHC-I or MHC-II antigens expressed on mTECs. SP T-cells that can recognize self-peptides not reacting (or become activated) against self-antigens are selected as the $CD4^+$ or $CD8^+$ T cells that finally leave the thymus as the T-cell repertoire able to colonize T-cell areas on secondary lymphoid tissues. T-cells not able to recognize MHC-restricted self-peptides or those able to do it and become activated against self-antigens are eliminated from the mature repertoire by apoptosis. Apoptosis is critical for generating a T-cell repertoire that can recognize conventional antigens in postnatal life, avoiding the generation of self-reactive T-cells (7, 26).

3.2 Human *AIRE* gene structure and function and its role in T-cell tolerance acquisition

The human *AIRE* gene is in the q22.3 region of human chromosome 21 (*chr21:45,705,721-45,718,531*). During prenatal life, the gene is expressed by thymic mTECs, DCs, and Møs through DP to SP steps of intra-thymic development. It is expressed also in several tissues during postnatal life (see below). The AIRE gene one contains fourteen exons; two additional variants, AIRE 2 and AIRE 3, contain only eight. AIRE 1 Exons encode a 1635 nucleotide-long mRNA. mRNA transcripts have been reported in immune (bone marrow, thymus, lymph nodes), internal cells and organs (adipocyte, bladder, colon esophagus, kidney, liver, lung, spleen, stomach), muscle (artery, heart, skeletal muscle), nervous (brain, cerebellum, cortex, retina, spinal cord, tibial nerve), reproductive (ovary, placenta, prostate, testis, placenta), and secretory (adrenal gland, breast, pancreas, pituitary, salivary gland, skin, thyroid) tissues (Gene Cards, Human AIRE gene, 2022). Its expression results in a 545 aa protein. The human gene and protein share 76.9 and 79.6% sequence homology with their bovine analogous, respectively (Figure 2) (27).

3.3 Structure and function of the human AIRE protein.

AIRE consists of a 54.5 kDa protein that controls thousands of genes encoding tissue-restricted antigens (TRA) in medullary thymic epithelial cells (mTECs). It is expressed also in intra-thymic dendritic cells and Møs, particularly during the negative selection of thymocytes (at the stage of SP T-cells) (25). Besides, it is expressed in postnatal life in APC present in peripheral lymphoid tissues (28, and reviewed in 29) and other tissues such as lymph nodes (28), which modulates the acquisition of B cell-dependent peripheral tolerance (30). It has also been implicated in the first wave of spermatocyte apoptosis during spermatogenesis where the AIRE protein is expressed sporadically in spermatogonia and spermatocytes (31).

Table 2. Summary of human intrathymic T-cell development.

Stage	PCW	Location	T-cell Phenotype	Functional/Molecular process	AIRE expression
ETP	< 9	Transiting the blood	$CD44^{hi}CD117^{hi}CD25^{-}$	None	Negative
DN1	9-12	Thymus cortex	$CD44^{h}CD25^{-}CD4^{-}CD8^{-}$ $TCR\beta^{+}$	Starts TCRβ recombination. Can differentiate into B or NK cells. $ST18^{+}$	Negative
DN2a		Thymus cortex	$CD44^{hi}CD25^{+}CD4^{-}CD8^{-}$ $TCR\beta^{+}$	Pre-commitment. TCRβ recombination continues. Can differentiate into B or NK cells	Negative
DN2b			$CD44^{hi}CD25^{+}CD117^{low}CD4^{-}CD8^{-}TCR\beta^{+}$	Post-commitment. V(D)J recombination of TCRb, TCRg, and TCRd loci start. Only differentiate into DN3 cells	Negative
DN3a	10-14	Thymus cortex	$CD44^{-}CD25^{hi}CD117^{low}$ $CD27^{-}CD28^{-}CD4^{-}CD8^{-}$	V(D)J recombination of TCRb, TCRg, and TCRd continues, giving rise to γδT-cells or DN3b (β-selection) by successful V(D)J recombination	Negative
DN3b			$CD44^{-}D25^{int}CD27^{+}CD28^{+}$ $CD4^{-}CD8^{-}$	β-selection	Negative
DN4			$CD44^{-}D25^{-}CD27^{+}$ $CD28^{+}CD4^{-}CD8^{-}$	Progressive CD25 loss	Negative
DN4			$CD4^{-}D8^{+}TCR\alpha/\beta^{low/neg}$ $CD69^{-}CD5^{-}$	Pre-selection DP thymocytes	Negative
DP		Cortico-medullary junction	$CD4^{+}CD8^{+}TCR\alpha/\beta^{hi/hi}$ $CD69^{+}CD5^{+}$	Post-selection DP thymocytes. $AQP3^{+}$	Negative
SP Conv.		Medulla	$CD4^{+}CD8^{-}TCR\alpha/\beta^{hi/hi}$ $CD62L^{+}MHC\text{-}I^{+}$	CD4+ SP is conventional or unconventional. *TOX*2 for DN to DP transition	Positive
SP Conv.		Medulla	$CD4^{-}CD8^{+}TCR\alpha/\beta^{hi/hi}$ $CD62L^{+}MHC\text{-}I^{+}$	CD8+ SP conventionnel (α/β) or non-conventionnel (γ/δ)	Positive
SP Unconv.	12-15	Medulla	$TCR\alpha/\beta FOXP3^{low}CTLA4^{low}$ $IKZF4^{hi}GNG8^{hi}PTGIR^{hi}$	Differentiating Treg or $Treg_{(diff)}$	Positive

SP Unconv.	Medulla	TCRα/β CD25^{+}*FOXP*3^{hi}*CTLA4*^{low}*IKZF4*^{hi}*GNG8*^{hi}*PTGIR*^{hi}	Treg
SP Unconv.	Medulla	MIR155HG—as/IL2RA^{+}/*FOXP3*^{low}	$T_{(agonist)}$
SP Unconv.	Medulla	CD8α/α^{+}(I), CD8α/α^{+}(II), CD8αα or CD8γ/δ^{+}*EOMES*$^{+}$	I: GNG4^{+}. II: ZNF683^{+} sharing mixed αβ and γ/δ signatures. CD8γ/δ^{+}*EOMES*$^{+}$: CD8α/α^{+} NKT-like.

PCW: Post-conception week. ETP: Early T-cell progenitor. DN1-4: Double negative 1 to 4. DP: Double positive. DCs: dendritic cells. mTECs: medullary thymic epithelial cells. *AQP*3: water channel protein aquaporin three. *TOX*2: TOX high mobility group box family member 2 (adapted from 14, 15, 19).

Extra thymic AIRE-expressing cells (eTACs) present in peripheral lymphoid tissues and antigen-presenting cells (APCs), particularly DCs and Mos, could play critical roles in inducing peripheral tolerance in adult life by inducing PGE when interacting with newly formed T-cells, a postnatal mechanism created to prevent the generation of autoreactive T-cells (29). Then, the AIRE protein induces tissue-specific PGE providing the developing thymocytes with the repertoire of self-antigen required for the acquisition of tolerance to self-antigen.

3.4 AIRE is a transcription regulator protein.

The mature AIRE protein contains 545 amino acid residues and contains six structural domains from the N- to the C-terminus (Review in 32): (i) the Caspase-recruitment domain (CARD) that functions as AIRE' homo-multimerization; (ii) Nuclear localization signal (NLS) that couple to factors related to nuclear traffic and signaling; (iii) two Plant-homeodomain (PHD) fingers (PHD1 and PHD2) that functions as the structural framework for promoting the transcription of *AIRE* gene (33); and (iv) the SAND complex consisting of **S**P100, **A**IRE1, **N**ucP41/P75, and **D**EAF1 (Table 3) that appears to interact with a transcriptional suppressive complex (for regulating *AIRE* overexpression) (33, 34) (Figure 2).

AIRE functions. The AIRE protein is responsible for inducing PGE to generate transcripts codifying for self-proteins representing 45-to-55% of self–antigens capable of being processed and coupled to MHC molecules to establish the immunopeptidome (35) during the intra-thymic T-cell development. mTECs (36) and intrathymic DCs in low proportion (37) express the *AIRE* gene. The peptides resulting from the Golgi processing of PGE are coupled to MHC-I and MHC-II molecules for self-antigens presentation. mTECs expressing MHC molecules harboring self-antigens interact with SN $CD4^+$ or $CD8^+$ thymocytes (Figure 1). If these cells react with high affinity with the self-peptides expressed into the antigen-binding groove of class I or class II MHC+ mTECs, then these "self-reactive" T-cells undergo negative selection and are eliminated by apoptosis (21). On the contrary, T-cells recognizing self-antigens and not reacting to them with high affinity undergo selection to become the pool of non-self-reacting T-cells. This is considered the central process for the generation of central immune tolerance, a critical process to avoid the generation of self-reactive T-cells capable of inducing autoimmune diseases (36, 38-41). Then at the intrathymic (36) and extrathymic milieus (42) AIRE gene expression plays a pivotal role in the induction of central and peripheral tolerance by contributing to the transcription of TSA required for priming the developing T-cells for its negative selection. In fact, due to their extrathymic expression authors proposed it is also implicated in the generation of peripheral self-tolerance (43, 44). The mechanisms of action of AIRE include the promotion of promiscuous transcription of self-proteins that, once translated, enter the Golgi apparatus and suffer proteolytic processing and coupling of the generated self-peptides onto the antigen-binding groove of Class I and Class II MHC expressed by mETCs, DCs and, in less proportion, medullary B cells (Reviewed in 33). In that process, selected clones of $CD4^+$ or $CD8^+$ SN T-cells are challenged to recognize the universe of self-antigens with two possible outcomes (Figure 1): T-cells recognizing self-antigens with high affinity and overcoming activated suffer apoptosis.

Table 3. Specific functions of AIRE protein domains

Domain	Function	Reference
Caspase-recruitment domain (CARD)	Homo-multimerization of AIRE protein	32
Nuclear localization signal (NLS)	Mediates AIRE nuclear import and export	45
SAND complex SP100		33, 34
SAND complex AIRE1	Binding AIRE with DNA/Interaction with other transcription factors	
SAND complex NucP41/P75		
SAND complex DEAF1		
Plant-homeodomain fingers-1 (PHD1)	Interaction with DNA-dependent protein kinases, and histones	46, 32
Proline-rich sequence (PRR)	Activation by signaling pathways	
Plant-homeodomain fingers-2 (PHD2)	negatively charged putative Zn-binding domain	25
Forty-four amino acid C-terminus	Interaction with elongation factor b (P-TEFb)	47

T-cells recognizing self-antigen with low affinity and overcoming non-activated are selected as non-self-recognizing T-cells, which are finally selected to become the “educated” T-cell tolerant against self-antigen (36). Low or high affinity refers to the intensity of TCR activation resulting in low or high intracellular calcium generation, respectively, and the activation (or not) of intracellular cascades responsible for T-cell proliferation (Reviewed in 48, 49).

T-cells recognizing self-antigen with low affinity and overcoming non-activated are selected as non-self-recognizing T-cells, which are finally selected to become the "educated" T-cell tolerant against self-antigen (36). Low or high affinity refers to the intensity of TCR activation resulting in low or high intracellular calcium generation, respectively, and the activation (or not) of intracellular cascades responsible for T-cell proliferation (Reviewed in 48, 49).

AIRE expression is responsible for the generation of self-peptide recognition by the developing T-cells (Table 4) (38). The remaining proportion is under the control of PGE induced by transcription factors analogs to AIRE (32, 50) (See section 3.5 below). As evidenced in the mouse model, AIRE-independent PGE also plays a critical role in the generation of cell tolerance (51). Anderson et al. (2005) evidenced the existence of post-thymic tolerogenic mechanisms responsible for generating peripheral tolerance (38). Accordingly, there occurs a suppression of *AIRE* expression by post-AIRE mTECs, resulting in reduced PGE, and MHC-class II expression in the post-AIRE stage (e.g., after the time of intra-thymic AIRE expression). For a more detailed description please see the review by Perniola, 2018 and therein references (33). In brief: secondary and tertiary lymphoid tissue contains lymphoid and mesenchymal $AIRE^{+}$ cells expressing low quantities of AIRE protein enabling these cells to participate in postnatal acquisition of tolerance (peripheral tolerance) (33). Whether these mechanisms occur also in bovine and particularly in PI-infected calves is still to be investigated. In cultured thymic cells, *AIRE* gene expression results in AIRE protein interaction with components of transcription complexes specific to certain regions of the genome through its interaction with the nuclear matrix (52).

Table 4. The proportion of genes and percentage of proteins expressed as results of AIRE activation.

Cell type	Expression of protein-coding genes* (n)	Proteins represented (%)	Reference
$mTECs^{low}$	16,151	76	32
$mTECs^{high}$	19,283	87	32
cTECs	15,198	68	32
Other tissues	7,500	31,2	53

*Based on approximately 24,000 genes. Reviewed in Shevyrev et al., 2022 (32).

Kaiser et al., (2022) summarize the functions AIRE protein as follows (25):

i. **Acts as a lineage-specific transcription factor**, a function exerted on thousands of genes encoding TSA in mTECs, through recognition of tissue-specific cis-regulatory elements independently of the native chromatin conformation (54).
ii. **Binds to promoter-distal regulatory elements**, exerted by directly binding to tens of thousands of genomic loci in mTECs.
iii. **Acts on a previously arranged chromatin environment**, exerted through promoting chromatic accessibility at promoter-distal sites surrounding tissue-specific genes.
iv. **Triggers RNA elongation**, exerted particularly in non-coding genome regions prompting activation and regulation of several transcription factors and its interaction with RNA-polymerase II (Pol II).
v. **Recruits the positive transcription elongation factor b (P-TEFb)**, which promotes the expression of multiple tissue-specific genes. This function is exerted to promote the expression of enhancer RNA (eRNA) through P-TEFb- Bromodomain-containing

protein 4 (BRD4)-mediated activation. BRD4 is responsible for AIRE's CARD domain phosphorylation and acetylation required for eRNA production.

vi. **Stimulates topoisomerase recruitment and improves DNA-damage response**, a critical step to promote transcriptional elongation and TEFb-BRD4 interaction and function.

vii. **Improves enhancer-promoter interaction**, required for promoting tissue-specific gene ectopic expression at gene-distal regulatory elements, to generate excess enhancer activity that increases the frequency of transcriptional bursts at the target promoters.

viii. **Acts as linear-specific chromatin effector**, acting as co-activator/effector protein at the end of the transcription cycle by enhancing the action of transcription factors and triggering transcriptional elongation.

ix. **Reads unmodified H3 tails**, exerted through its high-affinity PHD1 binding to H3 tails.

x. **Represses chromatin accessibility**, particularly at dense clusters of chromatin enhancers, resulting in increased gene expression (55).

xi. **Functions as a multivalent scaffold**, action exerted through CARD by homotypic multimerization, a process critical for protein kinases activation. Besides, AIRE becomes multimerized to promote nucleation sites responsible for concentrating its associated proteins at target genomic regions.

3.5 Other genes analogous to AIRE responsible for PGE.

Authors proposed that almost 60% of AIRE-independent tissue-restricted antigens are expressed in the thymus during T-cell development. This expression is mediated by the Forebrain Embryonic Zinc Finger-Like Protein 2 (Fezf2) gene (50), Deformed Epidermal Autoregulatory Factor 1 (DEAF1) (13), and chromodomain helicase DNA binding protein 4 CHD4 helicase (Ch4d) (Reviewed in 41). According to Shevyrev et al., (2022) AIRE, Fezf2, AIRE + Fezf2, Fezf2 + DEAF1, DEAF1, and other unknown factor(s) contributes to 29, 21, 12, 5.4, 4.6, and 28% of TSA expression by mTECs in the human thymus (32).

3.6 How do mutations in the *AIRE* gene result in impaired immune tolerance? The Autoimmune Polyglandular Syndrome type 1 (APS-1).

As mentioned above, clinical cases of APCED or APS-1 are the results of mutations affecting several AIRE domains, highlighting the pivotal role this complex protein plays in immunological tolerance. APS-1 is a group of concomitant autoimmune disorders generated by mutations in the *AIRE* gene (56) (Table 4). More than sixty mutations have been reported, consisting of an arginine substitution in position 257 of the gene and a thirteen base pair deletion in exon 8 (57, 58). Bruserud et al., (2016) depicted in their figure 2 a complete description of genes mutated and their structure-to-function relationship and summarized the reported AIRE gene mutations as follows: 14, 19, 5, 6, 1, 10, 3, 18, 5, 15, 12, 3, 2, and 3 mutations (including several deletions and substitutions) in exons 1 to 14, respectively (58). New mutations in affected ASP-1 settings have been reported in China (59, 60), Sweden (61), and Brazil (62). The most recent report on mutations of the *AIRE* gene indicates the existence of 167 mutations comprising 54 Missense/non-sense, fourteen splicing, two insertions, twenty small deletions, 15 Small insertions, 6 Gross deletions, and 5 Gross insertions (1). The AIRE protein interacts with at least 28 proteins, including 9 proteins interacting at the DNA level responsible for the regulation of AIRE' function (21). The mutated aire protein carries a reduced PGE in mTECs during the intra-thymic step of negative selection, which results in a reduction of the expression of PGA and the generation of self-reactive T-cell clones (21). The resulting clinical outcomes of AIRE mutations are presented in Table 5.

1 **Table 5**. Comparative assessment of clinical signs between APS-1 human patients and BVDV PI-calves.

Typical APS-1 clinical signs*				**Comparable clinical signs occurring in PI-Calves**			
Clinical signs[&]	**Age/Frequency (% of APS-1)**	**Pathogenesis**	**Ref.**	**Clinical signs**	**Frequency/ type of study**	**Pathogenesis**	**References**
1. Candidiasis in the oral mucous membrane, nails, and esophagus. **	1.7-3 years/ 77-100%	Autoantibodies against IL-22, IL17F, Myosin 9.	63	Susceptibility to secondary infections	n.r.	Lymphocyte depletion	64
2. Adrenal insufficiency: fatigue, muscle weakness, abdominal pain, diarrhea, nausea, vomiting.	3-5 years/ 33-77% [a]	Autoantibodies against CYPC17, CYP21, CYPSSC, CYP11A1	65, 66	Mild adrenalitis	n.r.	Virus-induced inflammation	67
3. Intestinal dysfunction	31% [a]		68	Overall gastrointestinal tract alterations	30 to 80%	Virus-induced apoptosis	67, 69
				Upper digestive tract ulcerative lesions	PI calves		70
4. Gonadal failure (hypogonadotropic hypogonadism)	62.5% [a]	Autoantibodies against: CYPC17, CYP11A1	62	Reduced follicular dynamics, ovarian structures	n.r.	Decreased number of tertiary follicles, Graafian follicles, and atretic follicles	71
5. Alopecia	23% [a]		9, 62	Alopecia, dermatitis Generalized alopecia	n.r.	Virus-induced inflammation	72

6. Pernicious anemia	38.5% [a]		(62)	Hemorrhagic syndrome-like disease	n.r.		(73)
7. Hypoparathyroidism: dry hair and skin, heart failure, low blood pressure, muscle cramping, seizures, spasms, tetany.	63-100%[a]	Autoantibodies against: NACHT, NALP5, CaSR	(62, 74)	Neurological abnormalities	Natural or experimental PI calves	Cerebellar hypoplasia hydranencephaly, hydrocephalus, microencephaly, porencephaly	(75, 76)
8. Hypothyroidism: dry skin, fatigue, myxedema, swelling.	15.5% [a]	Autoantibodies against: TPO, TGAb, TSHR	(62)	Reduce Thyroid hormone circulating levels	n.r.	Direct effect on the thyroid gland	(72)
9. Autoimmune hepatitis: cirrhosis, itching, jaundice	7.5% [a]	Autoantibodies against: CYP-1A2, TPH, CYP-2A6, AADC.	(62, 77)	Early fetal hepatic immune responses	Experimental in utero infection at 75 days of gestation	Early expression of MHC-II in fetal Kupffer cells inducing inflammation	(78)
10. Vitiligo	15.5% [a]		(62)	Non-reported			
11. Asplenia	Familiar cases		(79)	Lymphocytic depletion of lymphoid tissues	n.r.	BVDV-induced apoptosis	(67, 80)
12. Growth hormone deficiency	15.5% [a]		(62)	Reduced growth rate, unthrifty calves	n.r.	Impaired intestinal function.	(81)
13. Type 1 diabetes	23% [a]	Autoantibodies against: IA-2, GAD65, ICA512, ZNT8, Insulin,	(62)	n.r.			

PI calves and APS-1: natural mistakes of immune tolerance.

14. Dental enamel hypoplasia/Nail dystrophy	38.5%/ 38.5% [a]		(33, 62)	Focal crusty and ulcerative lesions affecting the mucocutaneous and skin-horn junctions' interdigital clefts, pastern, and areas surrounding the dewclaws	PI cattle	Ballooning degeneration and spongiosis in the epidermis	(70)
15. Keratopathy	7.5% [a]		(62)				
16. Cerebellar hypoplasia	Case report	Acute disseminated encephalomyelitis	(82)	Cerebellar hypoplasia Incoordination, nystagmus	n.r.	Depletion of the external germinal layer Neuraxial hypomyelination	(75, 76, 80, 83)
17. Red cell aplasia	Case report	Immune-mediated pathogenesis	(82)	Neutrophil abnormalities			(84)
18. Severe chronic intermittent neutropenia	Familial mutations investigation	Autoimmune-mediated	(68)	Immune deficiencies		Epigenetic DNA modifications	(85)
19. Bone abnormalities	A study of several patients	abnormal mineralization decreased trabecular thickness and increased osteoid	(86)	Impaired long bone trabecular modeling	Experimentally-infected PI fetuses	Increased calcified cartilage core and bone, reduced mineralizing surface. Epigenetic DNA modifications	(88) (85)
Osteoporosis, vertebral fractures, multiple non-spinal fractures	Adult APS-1 patients	Bone structural alterations and risks of	(86, 87)				

20. Upper respiratory tract infections	APS-1 patients	Mutations of the PHD1 domain	(68)	Respiratory abnormalities	PI calves	Moderate to strong presence of BVDV antigens in alveolar macrophages and alveolar epithelial cells	(72, 89)

*Adapted from Guo et al., 2018. Non-Classical APS-1 includes autoantibodies against IFN-ω. **Humbert et al., 2018 (63). a: this value includes data from Weiler et al., 2018 (62). N.r.: not reported. [&]Several clinical signs were shared by a Chinese study in which four novel mutations were reported (90)

The self-reactive T-cell clones circulate and interact with B cells to generate autoantibodies against self-proteins in several organs, particularly in tissues with endocrine function, and renders the patient more susceptible to mucocutaneous candidiasis (63). The resulting APS type-1/APECED syndrome is caused by organ-specific autoimmunity in most affected patients (57), characterized by a triad of clinical signs comprising mucocutaneous candidiasis, adrenal insufficiency, and hypothyroidism (1). Up to twenty clinical signs have been described (62) (Table 5).

Interestingly, APS-1 is evidence of failure in the expression of self-antigen during intra-thymic negative selection of T-lymphocytes, providing a natural model for explaining central tolerance (21, 62). The most frequent clinical signs of APS type 1 are presented in Table 5. The APS-1 occurs by mutations of the *AIRE* gene failing to generate immune tolerance by impairing the intra-thymus and extra-thymus PGE expression of self-peptides by mTECs and DCs resulting in the generation of self-reactive T-cells that scape the negative selection step during central or peripheral T-cell development (21). The number of mutations causing APS-1 reported by Perniola and Musco (2014) increased from 32 (34) to 100 mutations in the work by Bruserud et al. (2016) (58). Besides, these authors summarize APS-1 by type of mutations as follows: fifty-four missense/nonsense, fourteen splicing, two regulatory, twenty small deletions, fifteen small insertions, six small indels, five gross deletions, and one gross insertion, which affects exons, 1, 2, 6, 8, 10 and 11 (58). The most common mutations affecting the AIRE gene are reviewed in the article by Weiler et al. (2018) (62). Most mutations consist of simple nucleotide substation (almost 85%), whereas in low proportion, there occur deletions and translocations. Furthermore, several authors report new mutations yearly (59-62). Although authors proposed that APS-1 is an autosomal-recessive disease, Oftedal et al., proposed two genetic forms of the disease: (i) classical APS-1, which presents a recessive inheritance pattern showing two of three main components and the presence of anti-IFN-omega autoantibodies. (ii) non-classical APS-1, which presents dominant heterozygous mutations affecting the PHD1 domain, and a middle-less penetrant autoimmune phenotype (57). In Table 6 we summarize the clinical outcome of mutations according to physiological processes affected.

3.7 BVDV- infection in cattle.

BVD is a disease caused by the BVD virus (BVDV), a positive single-stranded RNA virus that belongs to the genus *Pestivirus* of the *Flaviviridae* family and affects domestic and wild ruminants (4, 91, 92). Bovine viral diarrhea virus is a morbillivirus affecting cattle and wild ruminants worldwide with prevalence ranging from 0.44, 0.71, 0.03, and 0.07 for individual antibody levels, herd antibody levels, antigen, and nucleic acid detection, respectively (93). In countries like Colombia, the prevalence in animals and farms is 0.7 and 0.22, respectively (94). The main route of infection is respiratory, resulting in infection of the respiratory tract and digestive tract with a high compromise on dairy cattle (95).

1 **Table 6**. Genetic to the functional relationship of mutations affecting the AIRE gene.

Domain	Mutation	Function affected	Clinical outcome	Reference
CARD	p.His14Pro p.Leu323fs	Homo multimerization	Hypoparathyroidism, and at least two diseases out of the triad of candidiasis, hypoparathyroidism, and hypoadrenalism in 45% of patients	(74)
HSR	L28P, K243/245Q, D312A	Impaired DNA binding Homodimerization resulting in decreased transcriptional activity	Several, APS-1 signs	(96)
	c.232T>A (p.W78R), p.V22_D23del		Juvenile rheumatoid arthritis	(97)
	c.994+5G>T and a novel mutation, c.230 T>C (p.F77S)		Chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal failure.	(97)
SAND	G228W	Protein-interaction DNA-binding	APS-1 APS-1	(98) (68, 99)
PHD1*	c.901G>A (p.V301M), c.916G>A (p.G306R), c.926T>C (p.I309T), c.977C>T (p.P326L), and c.982C>T (p.328W)	Histone code readers Autoantibodies against INF-w Others	Ranged from no autoimmune manifestation to severe autoimmune disease and autoantibodies. Multiples gastrointestinal disorders. Upper respiratory tract infections and skin infections. Severe chronic intermittent neutropenia. Refractory status epilepticus, type 1 diabetes. Recurrent sinus infection, oropharyngeal candidiasis. Vitiligo	(68)
Inter PHD*	.1102C>G (p.368A) and		Headache, chronic constipation, poor appetite, recurrent	(68)

	c.1235 C>T (S412L)			
PHD2*	.1399G>C (p.G467R)	Downregulation of IFN I gene.	Multiple granulomas and abscess formation	(68)
Forty-four aa C-terminus	Impaired binding to transcription elongation factor b (P-TEFb)		APECED	(47)

*Please see a detailed description in Table 4 from Oftedal et al., 2023 (68)

3.7.1 Etiology of BVDV.

BVDV is an encapsulated plus RNA virus of the genus Pestivirus (*Flaviviridae* family) (100-102). Infection by BVDV affects livestock worldwide leading to important economic losses in the industry (103-105). Based on genetic and antigenic characteristics, the virus has two species: BVDV-1 and BVDV-2, standing for 88.2% and 11.8% of virus isolated worldwide (Reviewed in 106). Each one of these two species is segregated in twenty-one (nominated 1a to 1u for BVDV-1); and four (nominated 2a to 2d for BVDV-2) sub-genotypes (106). Two more viruses formed this group including border disease virus and classical swine fever virus (Reviewed in 107).

Functionally the virus is subdivided into cytopathic (cp) and non-cytopathic (ncp) biotypes according to its capability to destroy cell culture in vitro (108-111). In vivo, cp biotypes are more related to digestive symptoms and Mucosal Disease (MD), while ncp is more related to respiratory symptoms. Similarly, cp biotypes are implicated in the pathogenesis of abortion and fetal death in pregnant cows and heifers (112). The virus has been isolated in domestic and wild ungulates including buffalo, goats, sheep, pigs, deer, and elk (92).

3.7.2 Pathogenesis.

The main route of infection is the nasal and respiratory route from which the BVDV infects the central nervous system, digestive, respiratory, and reproductive tract. The virus has two genotypes (BVDV-1 and BVDV-2) (113) and two biotypes according to their ability to cause cytotoxicity in cell cultures *in vitro*: the cytopathic (cp) and the non-cytopathic (ncp) biotypes (114, 115). Besides, infection by BVDV causes reproductive abnormalities, resulting in abortion, intrauterine growth restriction (IGR), stillbirths, impaired reproductive efficiency, reduced conception rates, and generation of PI calves (69, 71, 112, 116, 117). Cytopathic variants of the virus are the most pathogenic and cause mucosal disease (118) and abortion in affecting pregnant goats (119) cows and heifers (112). Animals infected with ncp variants usually present respiratory symptoms, while pregnant cows could give birth to normal calves or calves with IGR (120).

3.7.2.1 Infection of pregnant cows and heifers and generation of PI calves.

Cows and heifers infected at any gestational age with by cp biotype of the BVDV will always suffer abortions, whilst calves often suffer from digestive and respiratory diseases, mostly related to virus-related alteration of the IFN responses (Reviewed in 113) resulting in immunosuppression and impaired immune response. PI calves infected post-nataly with a cp biotype suffer from a lethal form of mucosal disease (121). In a herd, a single BVDV genotype can generate multiple variants by insertion in the NS2/3 coding region of the virus (122). If pregnant cows or heifers become infected by a ncp biotype of the virus during the first trimester of gestation, particularly between days 45 and 120 of gestation when the thymus (123) and immune tolerance develops (124), the virus enters the fetus into maternal-fetal circulation through the placenta, infects the developing fetus and proliferates in a pan epithelial infection pattern (125). Viral antigens entering the fetal thymus during negative selection (NS) of CD4+ or CD8+ T-cells (SP) are incorporated as part of the organism's self-antigens (126-128). This process, although not completely evidenced by scientific research, is considered the basic mechanism for generating PI calves: the PI fetus generates circulating T-cells not recognize and do not react against viral proteins.

Consequently, PI calves are born "tolerant" to BVDV antigens, although virus antigens can be detected in the epithelium of almost all organs (129, 130) and the PI calf excretes infecting viruses throughout their lives, becoming the main source of infection into the herd (64, 94, 120, 131, 132). Interestingly, PI calves exhibit some clinical signs related to the pathogenesis caused by the "tolerated" viral particles present in the endocrine, digestive, and respiratory systems, and the kin, being generalized alopecia and hypothyroidism within the most critical signs (72). Although viral particles have been described in the affected organs and tissues, authors have always favored the infectious nature of these lesions. However, the possibility of an autoimmune reaction has not been considered, even though several immune processes related to the acquisition of immunological tolerance against the virus have been described as summarized in Table 7. We propose these signs are similar to several of the clinical signs described for APS-1 patients (62, 63, 66) and we wonder if some of these signs could also be related to an impaired immune tolerance of PI calves. Accordingly, in Table 5 we propose a comparative description of clinical signs exhibited by APS-1 patients and PI calves.

3.7.2.2 Persistence of BVDV in cattle herds through the generation of PI calves.

Some authors reported a severe depletion of thymocytes and other lymphoid tissue damage in PI fetuses (133, 134), which could provide insights into the mechanism responsible for clinical signs developed by PI calves. However, other authors reported no compromised CD4+, CD8+, or B-cell repertoires in PI calves experimentally infected with traditional virulent BVDV strains but not with enhanced virulence strains (135) or in gnotobiotic calves during postnatal infection (136). Likewise, how BVDV circumvents the generation of reactive clones against viral antigens has not been elucidated, providing the opportunity to develop a field of knowledge for studying therapeutic alternatives for preventing the generation of PI-calves. The reproductive outcome in a pregnant cow after viral infection would depend on the time of pregnancy when infection occurs. If the cow is infected in the first third of pregnancy with an ncp virus, the virus passes the placenta and infects the fetus (137). When occurring between 40 days of pregnancy, the earlier time when thymus development starts in the growing fetus (138) to 140 days of pregnancy, the most probable resulting scenario would be the virus entering the thymus during the maturation of thymocytes (139). Even though there are no specific studies demonstrating the exact mechanisms for the acquisition of virus tolerance, it could be speculated that it is the incorporation of viral proteins and peptides into the pool of "self" antigens being presented to the developing T-cells during negative selection accounts for the acquisition of such tolerance. Accordingly, virus-derived peptides are codified as "self" peptides by the developing thymocytes resulting in tolerance against the virus and generation of multiple sites of virus replication and establishment, with no generation of cellular or humoral immune responses against the virus. It was suggested that in that way it is established the fetal and postnatal PI calves (78). On the contrary, if the fetus is infected after 135 days of pregnancy when the fetus has developed its immunological competence to recognize foreign antigens, an adaptive immune response against the virus is elicited resulting in fetal death or intrauterine growth restriction depending on the cytopathogenic strain of the virus (80), or the infected fetuses efficiently controlling viral RNA replication (140).

Table 7. Mechanisms of immune tolerance induction and PI generation during ncp BVDV infection.

Process	Molecular basis	Reference
Autophagy	BVDV induces the activation of apoptotic cascades mediated through	(115)
Self and non-self-modulation of interferon-mediated defense	Ncp BVDV biotype induces tolerance of infected cells by inhibiting the effect of IFN-omega against them, but not the IFN response against another type of viruses	(141)
Resistance state to IFN α/β effector functions	Ncp BVDV can modulate the IFN-depending response of the infected cells, with no effect on the response against other unrelated viruses *in vitro*.	(142)
Resistance state to IFN α/β effector functions	Ncp BVDV can modulate the IFN-dependent response of the fetuses and placenta after experimental infection during the first trimester of pregnancy *in vivo*. IFN-stimulated genes in endometrial cells were affected by the addition of IFN-tau in vitro	(124, 142)
Attenuated innate and adaptive immune responses	Ncp BVDV inoculated in utero at 75 days of pregnancy in heifers, induces IFN-γ production and significant upregulation of Type I IFN-stimulated genes (ISGs) in and reduces BVDV viremia of PI fetuses. The attenuated IFN-γ production was not associated with the elimination of all viruses.	(143, 144)

3.7 Structure of the bovine *AIRE* gene and AIRE protein

The bovine AIRE gene is located on *Bos taurus* chromosome one and contains fourteen exons codifying a predicted 1665 RNA template responsible for translating a protein containing 554 to 561 amino acids, with a calculated molecular weight of 58,492 Daltons. The sequence of three referenced transcripts is deposited in XM_024996717.1, XR_003036248.1, and XR_003036250.1, and one referenced sequence protein in XR_003036250.1 (NCBI Orthologs, September 2023). The HSR, SAND, PHD1, H3-binding site, Zinc binding site, BHA, and PHD2 domains are spanned thorough regions 22 to 154, 206 to 270, 306 to 348, (306, 312-to-318,320, 328, 338-to-343), (307, 310, 319, 322, 327, 330, 345, 348), 326-to-372, 441-to-483 and (441, 450-to-454, 458, 478) of the amino acid sequence, respectively (NCBI Orthologs, September 2023). Also, the deposited *AIRE Bos taurus* gene in the Bovine Genome Database has 13364 nucleotides, twenty-four exons, and four coding sequences and transcripts (Bovine Genome Database, September 2023). Besides, the bovine AIRE gene shares high homology, structure, and genomic organization with the human counterpart. However, no mutations in the bovine AIRE gene have been described until now. Access to the available genetic resources related to the bovine *AIRE* gene and protein is presented in Table 8. Consequently, we provide elements to be considered for further research in areas such as bovine thymocyte maturation and acquisition of central and peripheral tolerance. The lack of scientific evidence on this subject in cattle allowed us to assume the bovine AIRE gene functions like the human *AIRE* gene and protein in addition to sequence homology (Figure 2). Unfortunately, research for depicting the mechanisms of AIRE expression in bovines has not been performed until now.

4. Discussion

4.1 BVDV in PI fetuses: A trojan horse into and outside the thymus?

The common factor that appears to occur in PI calves and patients affected by APS-1 is impaired immunological tolerance: in PI calves, viral antigens are incorporated as part of the universe of fetal antigens, in such a way that naïve T-cell clones are not capable of recognizing the virus antigenic repertoire, even though there is an important modulation of the innate immune response mediated by IFN-g signaling-mediated mechanisms (113, 124, 141, 145, 146). In APS-1 patients, mutations of the AIRE gene cause impaired PGE and TSA expression, generating autoreactive T-cell clones recognizing those self-antigens that were not taught by mTECs during T-cell intra thymic maturation (Reviewed in 25).

However, because there is no scientific evidence on the association between BVDV replication, impaired *AIRE* gene expression, and autoimmunity, we wonder if BVDV would also cause fetal and postnatal pathogenesis related to impaired immune tolerance and cross-reaction between tolerance and the immune response against the virus. Let us review the evidence on virus tissue localization and its relationship to impaired immune responses.

Table 8. Genetic resources to the bovine AIRE gene and protein.

Sequence	Locus	Accession	Database source	Reference
RNA	XP_024852485	XP_024852485	RefSeq: accession XM_024996717.1	https://www.ncbi.nlm.nih.gov/protein/XP_024852485.1
RNA	XR_003036248	XR_003036248	BioProject: PRJNA450837	https://www.ncbi.nlm.nih.gov/nucleotide/XR_003036248.1
RNA	XR_003036250	XR_003036250	BioProject: PRJNA450837	https://www.ncbi.nlm.nih.gov/nucleotide/XR_003036250.1
Protein	XP_024852485	REFSEQ: accession XM_024996717.1	BioProject: PRJNA450837	https://www.ncbi.nlm.nih.gov/protein/XP_024852485.1
Gene ID	1:144284837-144298200		Gene Source: Ensembl95	http://bovinemine-v16.rnet.missouri.edu/bovinemine/report.do?id=37920673
Bovine genome				
RNA	ENSBTAG00000023393	ENSBTAT00000083211, ENSBTAT00000074633, ENSBTAT00000031852, and ENSBTAT00000071863		http://bovinemine-v16.rnet.missouri.edu/bovinemine/report.do?id=37920673
mRNA DB identifier	ENSBTAT00000031852			http://bovinemine-v16.rnet.missouri.edu/bovinemine/report.do?id=37920673
Polypeptide DB identifier	ENSBTAP00000031798			http://bovinemine-v16.rnet.missouri.edu/bovinemine/report.do?id=37920673

4.1.1 Are tissue distribution of BVDV in fetuses and PI calves related to its pathogenesis and clinical signs?

Once into the fetus the ncp BVDV biotype is widespread after the establishment of immune tolerance in central nervous and endocrine tissues during fetal development (120) and persists beyond postnatal life in PI calves (131). In these tissues, the virus is located in pericytes (neuronal-related endothelial cells), microglia, and neurons. In most PI calves viral particles persist in the anterior and caudal cerebrum, basal nuclei, septal nuclei, piriform lobe, thalamus (Anterior, middle, and caudal), hypothalamus, hippocampal gyrus, dentate hippocampus, entorhinal cortex, mesencephalon, cerebellar cortex, and medulla (Anterior, middle, obex and caudal) (131). Similarly, other neural and non-neural tissues contain viral particles in experimentally induced PI fetuses (120), in both cases with variable degrees of intensity immunostaining. Curiously, these central nervous system regions could be related to impaired neuro-endocrine functions that could result affected during innate immune responses against the virus, particularly those mediated by Møs (See a complete description provided in Table 5 by 131).

In the pioneering work by Ohmann (1982), this author evaluated four PI fetuses produced under experimental conditions by inoculating ncp viruses *in utero* between 120-165 days of gestation. He reported hypoplasia of thymuses in all fetuses due to morphological immaturity rather than a pathological response as evidenced by light and electron microscopy. Similarly, he found necrosis and depletion of the external germ layer of the cerebellum and in the skin and mucous membranes. Besides, viral antigens were detected in mononuclear cells of lymphoid tissues and cerebellum (80). Accordingly, Straver et al. (1983) reported PI calves born exhibiting variable degrees of neurological abnormalities: whereas a percentage of calves survived and recovered from clinical signs, others died shortly after calving due to irreversible neurological abnormalities (75). Similarly, Binkhorst et al. (1983) reported PI calves suffering from incoordination, oscillating nystagmus, negative blinking reflex, and tremors (75) in the same group of calves of the report by Straver et al. (1983) in four years of study comprising eleven affected calves (76). Neurological finding was also reported in the study by Otter et al., (2009) who found neuraxial hypomyelination in 23 British farms during a 16-year follow-up herds of PI calves born with neurological abnormalities (83). Besides, the study by Gallina et al. (2021) found hypomyelination as the predominant finding in a cohort of PI calves suffering from neurological signs in Italy (147). In these studies, the BVDV was also reported by molecular diagnosis. Agerholm et al., (2015) reported the following neurological signs in calves affected by BVDV infection: Cerebellar hypoplasia, hydranencephaly, hydrocephalus, microencephaly, and porencephaly (See Table 1 and Figure 1 in 148). Similar lesions were reported in the study by Golchin et al., (2023) in BVDV PI calves (149). Interestingly, one of the neurological signs reported in patients affected by APS-1 cerebellar hypoplasia (11) suggests that BVDV PI calves and APS-1 patients share a common pathogenesis in neurological abnormalities.

Besides, Georges et al., (2022) evidenced the hypermethylation of 1951 DNA regions in spleens of PI calves in regions related to bone development, cardiac, immune system, and neural tissues (150). We wonder if these regions when hypermethylated in the thymus from PI fetuses would account for reduced PGE. Besides, this group provided evidence on the generation of immune tolerance of PI fetuses against BVDV between 97 to 190 days of gestation by attenuation of lymphocyte activation (150), suggesting a mechanism of immune tolerance occurring beyond the acquisition of thymus central tolerance. The evidence provided by Smirnova et al. (2012) (124) and Hansen et al. (2015) (144) suggests that ncp BVDV can elicit innate and adaptive immune responses, in agreement with case reports

discussed by Ring et al., 2019. In the same work, the authors provide evidence suggesting that acquisition of the BVDV PI status could result from a combination of genetic selection, particularities of the virus, and the dam and fetus' immune response (117).

4.1.2 How does BVDV persist in the host? The molecular complexity of inducing immune tolerance.

Whereas the cp biotype of BVDV is the most pathogenic and results in the abortion of pregnant heifers and cows and death of calves affected by mucosal disease (MD), the ncp BVDV biotype evolved to evade the immune response by inducing the PI status by several mechanisms (Reviewed in 139). As proposed by Amaya-Uribe et al. (2019) who discuss the interrelationships between primary immunodeficiency and autoimmune diseases in humans (151), it could also happen in PI calves where the innate and adaptive immune responses are impaired (reviewed in 139).

4.2 Mechanisms of innate immune tolerance in PI calves.

Interferon-mediated responses. In the report by Hong et al., (2023) the authors proposed the most recent review on IFN-mediated responses modulated by BVDV in PI calves (152). IFNs play a critical role in reducing pestivirus replication in pestivirus-infected cells and initiate antiviral responses to resist pestivirus invasion after viruses are recognized by the host's pattern recognition receptors (PRRs). Interferon regulatory factors (IRFs) are activated after Pestivirus recognition by PRRs. The N^{pro} protein of pestiviruses modulates IRF3 and IRF7 as key molecules in the IFN-I production pathway, reducing IFN-I and IFN-III production. Also, BVDV E^{rns} structural protein exerts RNase activity essential for blocking or suppressing IFN-I production (152, 153). The inclusion of the viral genome by the E^{rns} protein through degradation of the immunostimulatory viral RNA prevents inappropriate activation of IFN by host nucleic acids. Lussi and Schweizer (2016) proposed that this mechanism supports the role of extracellular RNases in the sustained prevention of the organism's immunostimulatory RNA from acting as a molecular template associated with danger when cells are infected by viruses such as BVDV. Similarly, they proposed innate tolerance as a complementary strategy to adaptive tolerance for inducing and maintaining the PI status of calves (153).

Macrophages and dendritic cells. Most macrophages in mucosae surface and specific organs are the main sources of viral persistence, at least as evidenced by the presence of viral antigens (71, 130, 154). On the contrary, these cells become not infected when cultured in vitro with BVDV, and in infected animals are responsible for clearing lymphocytes infected with the virus (155). Accordingly, no significant differences were found in leukocyte counts and phagocytic profiles between PI-infected and non-PI animals (156). Otherwise, authors support that during the persistence of BVDV the fetal liver, and particularly Kupffer cells, play critical roles in driving the developing fetal T-cells toward specific BVDV-tolerant status (155). Schweizer et al (2001) provided evidence on the effect of ncp BVDV inhibiting apoptosis induction and interferon alpha/beta by poly(IC). Interference of BVDV ncp in the innate antiviral response of bovine macrophages cultured in vitro with the virus could explain the successful establishment of persistent infection in fetuses in the early stages of development (157).

T-cells. Lopez et al. (1993) evidenced that BVDV established productive PI status in monocytes $CD4^+$, $CD8^+$, and gamma-delta T cells (158). The reports by Brewoo et al. (2007) (156) and Falkenberg et al. (2019) (132) support the concept of the existence of not statistically significant differences in lymphocyte subpopulations between PI and non-PI

animals. Outstandingly, the presence of the virus is critical for maintaining the tolerance against BVDV infection because reduced viral loads are not compatible with the survival of PI calves (132).

4.3 What is the role of the innate immune response against the "self-viral" antigens during PI development?

Cohen and Efroni (2019) proposed a model of immune tolerance functioning beyond clonal selection as an integrated system comprising the integration of experience-based training repertoires of autoreactive T-cells functioning in cooperation with the developing T-cell repertoires in adult life for modulating the generation of inflammatory immune responses (in this case in autoimmunity) (159). This would be the situation of T-cells present in PI calves in damaged tissues where viral particles are present. As mentioned above, researchers have been focused on the infectious rather than the auto-immune mechanisms in these scenarios. This area of research could be a matter of further research.

4.4 Two trojan horses into the thymus.

The common factor observed in PI calves and patients affected by APS-1 is impaired immunological tolerance: In APS-1, patients are affected by mutations of the AIRE gene that result in impaired PGE and TSA expression, resulting in the generation of clones of T-cells recognizing self-antigens that were not presented to them by mTECs during intrathymic maturation (Reviewed in 25). In PI calves, viral antigens are incorporated as part of the universe of fetal antigens, in such a way that naïve T-cell clones are not capable of recognizing the virus' antigenic repertoire, even though there is an important modulation of the innate immune response mediated by the production of IFN-γ (113, 114, 141).

4.5 Is there an option to address impaired immune tolerance searching for therapeutic alternatives?

From clinical and social points of view, the scientific community must consider the possibility of searching for effective preventive or treatment options for patients affected by APS-1. This must be considered in two scenarios: first, what options could be offered to couples that are carriers of the recessive allele responsible for the mutation of the AIRE gene causing APS-1 who wish to conceive children? And second; what option could be offered to children affected by APS-1? The elegant work by Provin et al., (2022) provided the first concluding evidence on the generation of thymic organoids as one of the first steps for providing therapeutic strategies for the treatment of APS-1/APECED patients (160). Accordingly, fetuses carrying AIRE gene mutations in cases associated with autosomal recessive inheritance could be identified through isolation of fetal cells or free fetal DNA circulating in maternal peripheral blood, chorionic villus biopsy. Once fetal material is separated and mutation carrier diagnosis performed, one treatment would be to transfer the normal gene: umbilical cord stem cells could be isolated from the fetus, differentiated to the thymus medullary epithelial precursor cell phenotype, and transfected with a vector carrying the normal AIRE gene. The cells would then be inoculated back into the fetus, which could be done through umbilical cord puncture using the cord veins.

Using maternal stem cells carrying the normal gene: stem cells would be differentiated toward the mTECs phenotype and then transferred to the fetus as indicated above. Transfection must be performed before fetal thymus development, at gestational age when mTECs precursors migrate into the thymus at post-conception weeks 7-17 (14), or 9 to 19 of amenorrhea. We wonder whether technological advances would provide the means to transfer mTECs

precursors carrying the normal AIRE gene transfected to autologous cells by gene transfer or gene editing. The work by Provin et al., (2022) is a cornerstone toward this purpose (160). Hopefully, transferred cells carrying the normal *AIRE* gene migrate and establish homing in the developing fetal thymus becoming AIRE-expressing mTECs. Maternal bone marrow precursor cells could be isolated to obtain stem cells, differentiate into mTECs, and transfer to the fetus. In this scenario, the maternal cells must circulate in peripheral blood and migrate into the developing thymus, performing as mTECs and starting AIRE expression to overcome the problem of the defective gene in the embryonic thymus.

This therapeutic approach could provide the developing fetus with maternal cells establishing micro chimerism in the thymus and induce TSA expression and self-peptide presentation to the developing thymocytes. This process must provide a mixed phenotype of maternal cells expressing the normal gene and abnormal fetal cells expressing the defective gene. The outcome must be a postnatal APS-1 phenotype suffering from reduced clinical signs. Treatment alternatives for typical APS-1 patients diagnosed postnatally must be focused on transfection of maternal cells carrying the normal AIRE gene and colonizing the thymus and sites of extra-thymic maturation of T lymphocytes, for inducing TSA and PGE improving the generation of no self-reactive T-cells.

4.6 Should the PI BVDV model provide insights on APS-1 treatment?

In this case, a vector could be designed to inoculate the fetus susceptible to developing APS-1 with a vector carrying the normal *AIRE* gene, to be incorporated into the thymus of the embryo during its intrathymic development and allow the expression of the gene, its translation, and the expression of the protein. In this situation, the vector would be expected to be incorporated as the fetus's genetic material, with the AIRE gene incorporated, so it can begin to be expressed. Whether to consider offering experimental approaches for the prevention or immunologically based treatment for APS-1 patients is an extremely difficult but not impossible scientific challenge. The strategy should be focused on early diagnosis of fetuses carrying AIRE mutations, which are subjected to cell therapy strategies including transfection of the AIRE gene into stem cells of the affected fetus, transfer of maternal mTECs precursor to colonizing the fetus during intrathymic maturation of thymocytes weeks 8-14 post conception (WPC) and expressing the transfected *AIRE* gene to achieving development of immune tolerance. In addition, studies could be conducted for developing treatment alternatives to APS-1 neonates, performing the transfer of their mTECs transfected with the gene so that they colonize sites of extrathymic maturation of T-cells. This step must result in reconstituted immunological tolerance through eliciting peripheral tolerance. These approaches must be based on recent advances in technologies related to single-cell mRNA expression according to spatiotemporal dynamics of intrathymic development in human fetuses; micro chimerism detection and expression monitoring of transfected proteins co-expressed with green fluorescent protein; co-culture of tissue explants with immune cells, and nucleic acid expression. What could be the experimental protocols for these therapeutic approaches?

4.7 What can we learn from APS-1 to improve our knowledge of BVDV PI calves?

All scientific advances in developing thymocytes in the human fetus and the role of AIRE expression in developing central and peripheral tolerance are still lacking in cattle. Besides, we wonder whether BVDV infection before the generation of PI fetuses is implicated in impaired RNA expression during intrathymic T-cell development in cattle, its consequences in the generation of the self-repertoire and the possible relationship with the pathogenesis of

PI fetuses and calves. Given the similarities of clinical signs we proposed between APS-1 and PI calves we presented in Table 5, gain in this field of knowledge would be of critical importance for the development of novel control, prevention, and treatment strategies for BVDV infection in the cattle industry. Also, this knowledge would be of critical importance for the study of other viral infections and other animal species suffering from PI diseases.

Conflict of interest

The authors declare there are no conflicts of interest. JOA's works for Ingredion a Company that has no relationship with the subject matter of this article.

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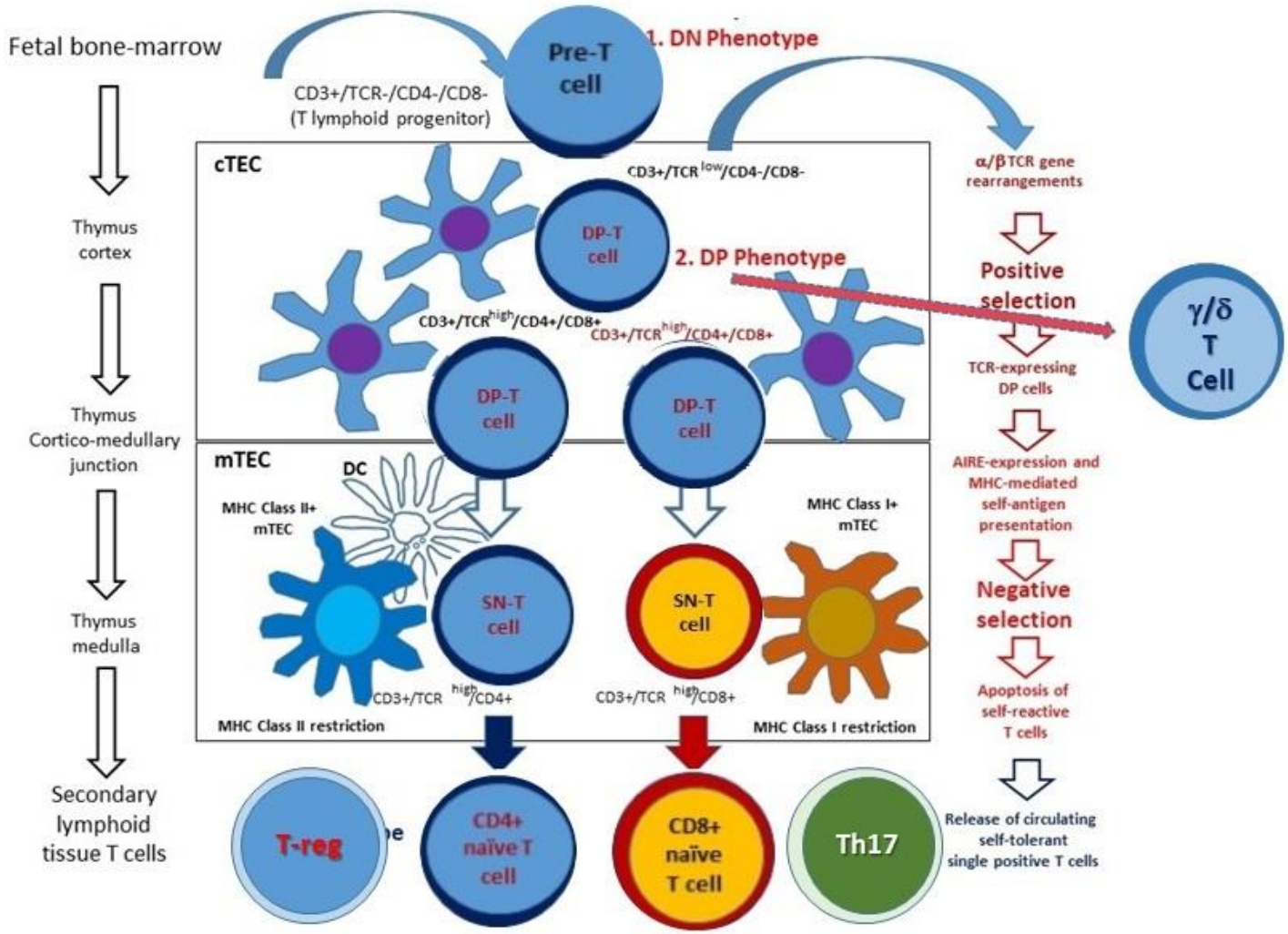
Figure legends

Figure 1. Overview of intra-thymic T-cell development. From left to right it is depicted the circulation of early T-lymphoid progenitors (TLP) from bone marrow through the thymus (left column), its exposition to thymic epithelial cells (central box), and the pattern of MHC and TCR expression according to CD8/CD4 expression phenotype. From the top to the bottom, depicted phenotyping changes from TLP to mature circulating T-cells: a passage from the blood to the thymic cortex and interaction with cTECs (Top), maturation toward the DP stage while in the cortico-medullary junction (Middle), interaction with mTECs and evolution toward the single negative stage (bottom), and outcome as circulating post thymic T-cells, including the early differentiation of g/d T-cells, and T-reg, CD4+, CD8+, and Th17 cells. (Adapted from various authors).

Figure 2. Comparative amino acid sequences between human (NP_000374.1) and predicted bovine (XP_002685182.2) AIRE protein. n, indicates the amino acid position. Hyphen means no amino acid concordance at the same position.

707 Figure 1.



708

709 Figure 2.

Protein accession	n	Sequence	n
NP_000374.1	1	- - - - - - MATDAALRRLLRLHRTEIAVAVDSAFPLLHALADHDVVPEDKFQ	44
XP_002685182.2	1	MAGETRAGGDAALRRLLRLHRTEIAVAVDSAFPLLHALADHDVVPEEKFQ	50
NP_000374.1	45	ETLHLKEKEGCPQAFHALLSWLLTQDSTAILDFWRVLFKDYNLERYGRLQ	94
XP_002685182.2	51	ETLRLKEKEGCPQAFHALLSWLLTQDTGAILDFWRVLFKDYNLERYARLQ	100
NP_000374.1	95	PILDSFPKDVDLSQPRKGRKPPAVPKALVPPPRLPTKRKASEEARAAAPA	144
XP_002685182.2	101	SILDTFPKDVDLSQPRKGRRSPAGPKATVLLPRPPTKRKALEEPRTVPPA	150
NP_000374.1	145	- ALTPRGTASPGSQLKAKPPKKPESSAEQQRLPLGNGIQTMSASVQRAVA	193
XP_002685182.2	151	- ALSPRGTSSPGSQTKTKPAKKPESNAEPQRLPLGNGIQTMSTSVQRAMA	199
NP_000374.1	194	MSSGDVPGARGAVEGILIQQVFESGGSKKCIQVGGEFYTPSKFEDSGSG-	242
XP_002685182.2	200	VSSGDVPGARGAVEGILIQQVFESGGSKKCIQVGGEFYTPNKFEDPAGG-	248
NP_000374.1	243	KNKARSSSGPKPLVRAKGAQGAAPGGGEARLGQQGSVPAPLALPSDPQLH	292
XP_002685182.2	249	KNKTRS-SSLKTLVRAKGTQAPAPGGGDSRAGPRDRAPAPPALPSEPQLH	297
NP_000374.1	293	- QKNEDECAVCRDGGELICCDGCPRAFHLACLSPPLREIPSGTWRCSSCL	341
XP_002685182.2	298	- QKNEDECAACRDGGELLCCDGCPRAFHLACLTPPLSEIPSGTWRCSNCV	346
NP_000374.1	342	QA - TVQEVQPRAEEPRPQEPPVETPLPPGLRSAGEEVRGPPGEPLAGMDT	390
XP_002685182.2	347	QGTTAQRDLPRAEQPRPQELPAETPAFLGLR-SGEEARALSTGLPPGTDA	395
NP_000374.1	391	TLVYKHLPAPPSAAPLPGLDSSALHPLLCVGPEGQQNLAPGAR - - CGVCG	438
XP_002685182.2	396	AVTYKHLLAPPSVAPLPVLDPSALRPLLCVGPEGQQGPVPGAR - - CGVCG	443
NP_000374.1	439	- DGTDVLRCTHCAAAFHWRCHFPAGTSRPGTGLRCRSCSGDVTPA - - - - -	482
XP_002685182.2	444	- DGADALRCAHCAAAFHWRCHFPGCATRPGAALRCRACSGDTAPELDVRN	492
NP_000374.1	483	- - PVEGVLAPS - - - - - - PARLAP - - - - - - - GPAK—DDTASHEPALHRDD	515
XP_002685182.2	493	QFPGSHLEKESCFCGDDPSRGVEVGLTSYLSGIQVGDDSAGHEPVLHRDD	542
NP_000374.1	516	LESLLSEHTFDGILQWAIQSMARP - - - AAPFPS	545
XP_002685182.2	543	LESLLSEHSFDGILQWAIQSMSRPLAEAPTFPS	575

Source: autoimmune regulator [Homo sapiens] NCBI Reference Sequence: NP_000374.1. PREDICTED: autoimmune regulator [Bos taurus] NCBI Reference Sequence: XP_002685182.2

710