# GENE THERAPY FOR AN EXPERIMENTAL AUTOIMMUNE POLYGLANDULAR SYNDROME 1 MODEL CAUSED BY MUTATIONS IN AIRE

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October 2017

# ABSTRACT

Autoimmune Polyglandular Syndrome type-1 (APS-1) is a monogenic recessive disorder characterized by multiple endocrine abnormalities, chronic mucocutaneous candidiasis (CMC), and high titres of serum autoantibodies. To date, no effective treatment is available and current treatments, that manage the symptoms rather than treating the cause, are considered challenging and requires continuous follow-up. APS-1 caused by mutation in the Autoimmune Regulator gene (*AIRE*). AIRE mediates central tolerance by directing the ectopic expression of tissue-specific antigens (TSAs) in medullary thymic epithelial cells (mTEC), causing the deletion of self-reactive thymocytes. Therefore, loss-of-function mutation in *AIRE* produces a truncated protein resulting in a multisystem autoimmune disease.

Due to the monogenic aetiology of APS-1 and availability of APS-1 mouse model, gene therapy approach was proposed to restores AIRE function using Adeno-Associated Virus serotype 9 (AAV9). In this study, the efficacy of AAV9-AIRE gene therapy was assessed in APS-1 mouse model.

*In vivo* proof-of-concept studies using AAV9-AIRE in APS-1 mouse model (B6.129S2-*Aire*$^{tm1.1Doi}$/J) demonstrated high transduction efficiency and restoration of AIRE expression in the thymus. This led to a significant increase of TSAs expression and significant reduction of serum autoantibodies in treated *Aire*$^{-/-}$ mice to become almost undetectable levels 4-weeks post injection. Besides, histological analysis showed normal tissues morphology with no lymphocytic infiltrations, a hallmark in *Aire* deficient mice. This study has demonstrated the potential use of AAV9-AIRE as a vehicle for gene therapy of APS-1.

# ACKNOWLEDGMENTS

First and foremost, I would like to thank the Almighty Allah, the most gracious, the most merciful, for blessing, protecting me and giving me this chance to make a dream into reality.

My deepest gratitude goes to my role models, my parents. They inspired and motivated me, they believed in me and encouraged me with their endless support and precious prayers that made me fulfil my dream. Special thanks and appreciation to my dear husband, Bandar, who provided endless support and patience, thank you for standing beside me throughout my studies and career, your support means the world to me. My wonderful kids, Khalid and Sirin, thank you for always making me smile. You were my comfort zone in the toughest moments.

Secondly, my endless appreciation goes to my supervisors Dr. Rachid Tazi-Ahnini and Prof. Mimoun Azzouz for their invaluable support and scientific input throughout my time at the University of Sheffield. Thank you for your constructive guidance, encouragement, and support. The knowledge I gained from you throughout the study contributed immensely to my scientific, academic and personal development.

Thank you to the Dermatology lab group for their support, you have been a pleasure to work with and I have greatly enjoyed being part of the group. Special thanks to Dr. Thomas Lovewell for his help in the lab. I also wish to thanks everyone in Azzouz's lab group, SITraN. Special thanks go to Ioannis Tsagakis and Evangelia Karyka for their help in the lab, Dr. Katherine Lewis and Ian Coldicott for their help in the field labs. I also wish to thank everyone in the department of Infection, Immunity and Cardiovascular Disease for their help during my project. I also appreciate the help and support from the Histology core facility, especially Fiona Wright for her help in processing all the tissue samples collected during my project.

I have made many friends during my 4 years at the University of Sheffield and I appreciate all of their encouraging words, which got me through the days of lab work and thesis writing. However, special thanks have to go to Fatma Hamed who over the years has become very special friend to me. I hope to treasure our friendships for many years to come.

*This thesis is dedicated to my beloved parents,*

*Abdullah and Eman*

# LIST OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **AAV** | Adeno-associated virus |
| **AIRE** | Autoimmune Regulator |
| **APECED** | Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy |
| **APS-1** | Autoimmune polyglandular syndrome type 1 |
| **β-actin** | Beta actin |
| **CARD** | Caspase Recruitment Domain |
| **CaSR** | Calcium-sensing receptor |
| **CD80** | Cluster of designation 80 |
| **CMC** | Chronic mucocutaneous candidiasis |
| **CMV** | Cytomegalovirus |
| **DNA-PK** | DNA-dependent protein kinase |
| **Fezf2** | Family Zinc Finger 2 |
| **FOXP3** | Forkhead box P3 |
| **GFP** | Green Fluorescence Protein |
| **IF** | Immunofluorescence |
| **IFN** | Interferons |
| **IT** | Intrathymic injection |
| **LCH** | Leydig cells hyperplasia |
| **LV** | Lentivirus |
| **MHC** | Histocompatibility complex class II |
| **mTEC** | medullary Thymic Epithelial Cells |
| **NLRP5** | NOD-like receptor family pyrin domain containing 5 |
| **NLS** | Nuclear Localization Signal |
| **PBS** | Phosphate buffered saline |
| **PHD** | Plant Homeodomain |
| **PIAS1** | Protein inhibitor of activated STAT1 |
| **PID** | Primary immunodeficiency disease |

| | |
|---|---|
| **PRR** | Proline Rich Region |
| **RIPA** | Radio Immunoprecipitation Assay buffer |
| **RNA Pol II** | RNA polymerase II |
| **Srgn** | Serglycin |
| **T1D** | Type 1 diabetes |
| **TG** | Thyroglobulin |
| **Th17** | T helper-17 |
| **TPO** | Thyroid peroxidase |
| **$T_{reg}$** | Regulatory T cells |
| **TSAs** | Tissue-specific antigens |
| **TSS** | Transcription start sites |

# *CHAPTER ONE*

# INTRODUCTION

# 1. INTRODUCTION

## 1.1 Autoimmune Polyglandular Syndrome type-1

Autoimmune polyglandular syndrome type 1 (APS-1) also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (OMIM: 240300) is a monogenic autosomal recessive disorder (Neufeld *et al.* 1980). It is a rare devastating primary immunodeficiency disease (PID). Although it is rare, a high prevalence found among some geographical areas in certain populations including the Iranian Jews (1:9000) (Zlotogora *et al.* 1992), Sardinians (1:14,400) (Rosatelli *et al.* 1998), Finns (1:25,000) (Ahonen *et al.* 1990), Norwegians (1:80,000) (Myhre *et al.* 2001), and Irish (1:130,000) (Dominguez *et al.* 2006).

### 1.1.1 Clinical features of APS-1

APS-1 is an autoimmune syndrome characterised by the manifestation of endocrine and non-endocrine organs destructions (Neufeld *et al.* 1980, Ahonen *et al.* 1990). The onset usually occurs during childhood, although a minority of patients developed symptoms after the first decade of their lives (Ahonen *et al.* 1990). Typically, the first sign of the syndrome is chronic mucocutaneous candidiasis (CMC), followed by hypoparathyroidism and then Addison's disease (Neufeld *et al.* 1980, Ahonen *et al.* 1990). Diagnosis is classically defined by the presence of two of these three major disease symptoms (Neufeld *et al.* 1980).

In addition to the main pathognomonic triad, other patterns of destruction mediated by autoantibodies against endocrine glands have been reported (Ahonen *et al.* 1990, Betterle *et al.* 1998). For instance, patients with APS-1 develop hypergonadotropic hypogonadism (24-60%), autoimmune thyroid disease (4-36%), in addition to the very rarely type 1 diabetes (T1D) (1-18%) and pituitary failure (7%) (Ahonen *et al.* 1990, Betterle *et al.* 1998, Betterle *et al.* 2003). Secondary non-endocrine autoimmune disorders including autoimmune hepatitis, renal, gastrointestinal, and dermatologic diseases are also reported in APS-1 patients (Collins *et al.* 2006). The latter includes vitiligo and alopecia areata, which were observed in 30-20% of APS-1 patients (Betterle *et al.* 1998, Tazi-Ahnini *et al.* 2002). Ectodermal dystrophy manifestations which include; dental enamel hypoplasia, punctate nail dystrophy and keratoconjunctivitis (Ahonen *et al.* 1990) *[Figure 1.1].*

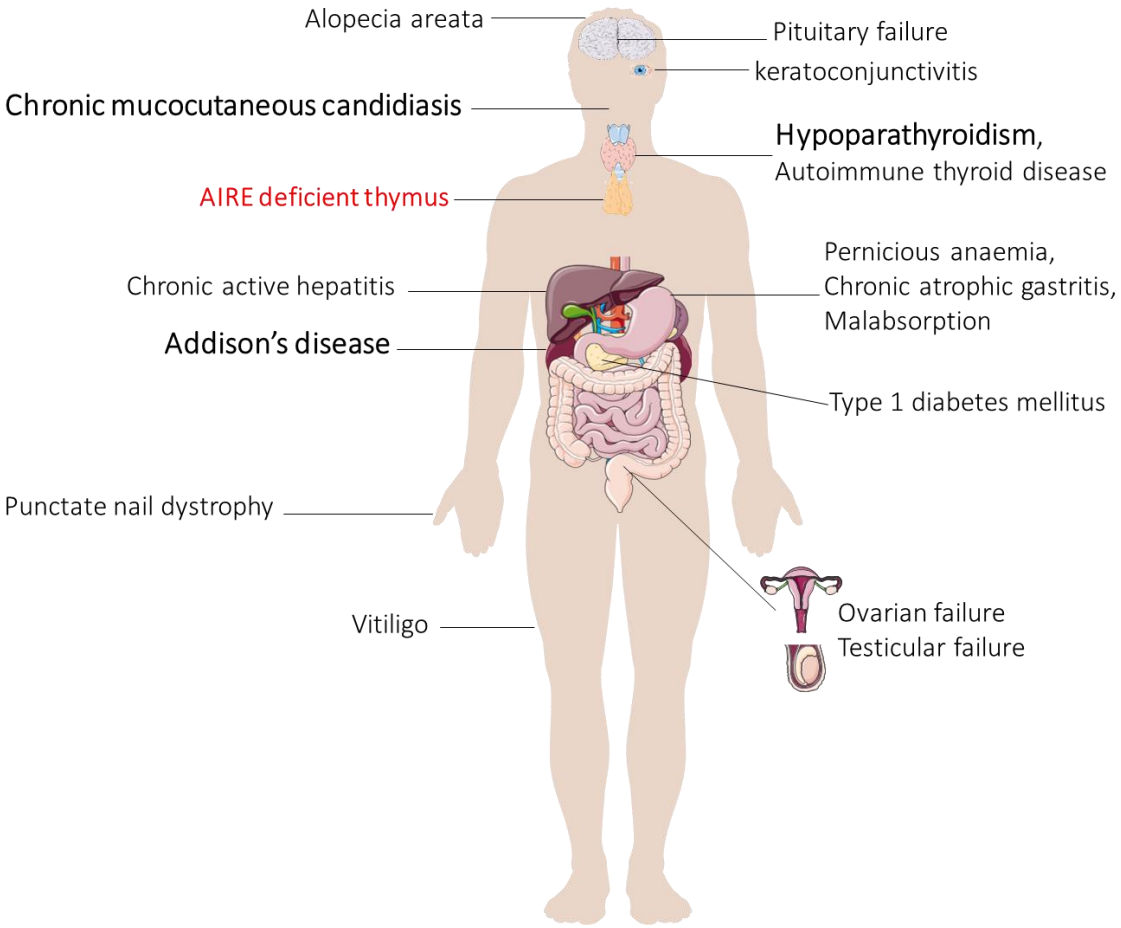



**Figure 1.1: Clinical manifestations of APS-1.** Representative diagram of some of the endocrine and non-endocrine tissues involved in APS-1. Common clinical manifestation (represented in **bold** font), less common clinical manifestation (represented in normal font). Figure created using Servier Medical Art.

### 1.1.2 Immunological features of APS-1

Serum from APS-1 patients was found to have high titres of autoantibodies against more than 20 tissue-specific antigens (TSAs) (Kisand *et al.* 2015) *[Table 1.1]*. For instance, in APS-1 with hypoparathyroidism, NLR family pyrin domain containing 5 (NLRP5) and calcium-sensing receptor (CaSR) have been identified as parathyroid autoantigens (Alimohammadi *et al.* 2008). NLRP5 is a cytoplasmic receptor expressed most specifically in the chief cells of the parathyroid gland and in oocytes (McDaniel *et al.* 2009). Autoantibodies against NLRP5 were detected in almost 50% of APS-1 patients with hypoparathyroidism but were absent in all APS-1 patients without hypoparathyroidism (Alimohammadi *et al.* 2008). Furthermore, autoantibodies against steroid 21-hydroxylase (CYP21A2), steroid 17α-hydroxylase (CYP17A1) and cytochrome P450 family 11 subfamily A member 1 (CYP11A1) were detected in many APS-1 patients with Addison's disease years before the adrenal insufficiency becomes clinically apparent (Winqvist *et al.* 1993, Cihakova *et al.* 2001). The aforementioned autoantigens have also reported to be involved in hypogonadism in APS-1 patients (Alimohammadi *et al.* 2008, Brozzetti *et al.* 2015). In addition to the autoantibodies against CYP molecules, autoantibodies against the prostate antigen transglutaminase 4 (TGM4), a major regulator of semen viscosity and promotes semen coagulation, can be found in adult male patients with APS-1 (Landegren *et al.* 2015). T1D considered a rare manifestation in APS-1, occurs only in 1%-18% in APS-1 patients. However, high titres of autoantibodies were detected in 30%-66% of APS-1 patients against diabetes-related autoantigens, such as glutamic acid decarboxylase isoform 65 (GAD65), glutamic acid

decarboxylase isoform 67 (GAD67), aromatic L-amino acid decarboxylase (AADC) and islet antigen 2 (IA-2) (Bjork *et al.* 1994, Fierabracci 2016). In addition, APS-1 patients reported to have autoantibodies correlate with intestinal malabsorption against tryptophan hydroxylase (TPH) (Ekwall *et al.* 1999). Moreover, interstitial lung disease manifestation and autoantibodies to potassium channel-regulating protein (KCNRG) and bactericidal/permeability-increasing fold-containing B1 (BPIFB1) has been described in 6%-10% of APS-1 respectively (Alimohammadi *et al.* 2009, Shum *et al.* 2013). APS-1 with hypothyroid were seropositive to thyroglobulin (TG) and thyroid peroxidase (TPO) (Perniola *et al.* 2000). Besides, 38 out of 80 APS-1 patients had autoantibodies against Tryptophan hydroxylase (TPH), an endogenous intestinal autoantigen (Ekwall *et al.* 1998). Furthermore, the transcription factors SRY-box 9 (SOX9) and SRY-Box 10 (SOX10) were reported to be vitiligo autoantigens in APS-1 patients, as autoantibodies were found in 15% and 22% of the 91 APS-1 sera respectively (Hedstrand *et al.* 2001). In a study by Hedstrand *et al.* (2000), autoantigen related to APS-1, tyrosine hydroxylase (TH), was identified in sera from patients with alopecia areata. Immunoreactivity was found in 44% of the 94 APS-1 patients studied and this reactivity associated with the presence of alopecia areata.

On the other hand, some APS-1 autoantibodies, such as autoantibodies specific for the Tudor domain containing protein 6 (TDRD6) or testis-specific gene 10 protein (TSGA10), are not related to pituitary failure or infertility (Bensing *et al.* 2007, Reimand *et al.* 2008). Hence, some autoantibodies do not associated with the clinical manifestations in APS-1 (Kisand *et al.* 2015). In fact, Meyer *et al.* (2016) found that APS-1 patients with

T1D-associated autoantibodies failed to develop T1D so long as they harboured high titres of neutralizing antibodies to type 1 interferons (IFNs). As a result, these unique disease-ameliorating autoantibodies could be protective against autoimmunity towards specific tissues and this could explain to some extend the rarity of some clinical manifestations in ASP-1 patients.

**Table 1.1: Tissue-specific autoantibodies identified in patients with APS-1**

| Tissue | Antigen | Disease | References |
|---|---|---|---|
| **Adrenal, testis, ovary** | NLRP5<br>CYP21A1<br>CYP17A1<br>CYP11A1 | Addison's disease<br>Gonadal failure | (Winqvist *et al.* 1993, Cihakova *et al.* 2001, Alimohammadi *et al.* 2008) |
| **Gastric** | IF | Pernicious anaemia, Gastritis | |
| **Hepatic** | CYP1A2<br>CYP2A6<br>AADC | Autoimmune hepatitis | (Bjork *et al.* 1994) |
| **Intestinal** | TPH<br>DEFA5 | Enteropathy<br>Loss of Paneth cells | (Ekwall *et al.* 1999) |
| **Pancreas** | GAD65<br>GAD67<br>IA-2 | Type 1 Diabetes | (Fierabracci 2016) |
| **Parathyroid** | NLRP5<br>CaSR | Hypoparathyroidism | (Alimohammadi *et al.* 2008) |
| **Pituitary** | GH<br>TDRD6 | Hypopituitarism, Gonadal failure<br>None | (Bensing *et al.* 2007) |
| **Prostate** | TGM4 | Infertility | (Landegren *et al.* 2015) |
| **Testis** | TSGA 10 | None | (Smith *et al.* 2011) |
| **Pulmonary** | KCNRG<br>BPIFB1 | Interstitial lung disease | (Alimohammadi *et al.* 2009, Shum *et al.* 2013) |
| **Renal** | proximal tubular | Tubulo-interstitial nephritis | (Landegren *et al.* 2016) |
| **Skin** | TH<br>SOX9/SOX10 | Alopecia areata<br>Vitiligo | (Hedstrand *et al.* 2000, Hedstrand *et al.* 2001) |
| **Thyroid** | TG<br>TPO | Hypothyroidism | (Perniola *et al.* 2000) |

In addition to tissue-specific autoimmunity, another immunological feature in APS-1 is the presence of circulating cytokines-neutralizing antibodies. The highest reported titres of autoantibodies were found to be against type I interferons (IFNs) and have become diagnostic markers for APS-1 as they are detectable as early as in few-months old children (Meager *et al.* 2006). These autoantibodies inhibit IFN-dependent gene expression and recently were associated with protection against T1D in APS-1 patients (Kisand *et al.* 2008, Meyer *et al.* 2016). A second group of APS-1 associated autoantibodies targets T helper-17 (Th17) cytokines, IL-17A, IL-17F and IL-22; they appear early in disease course and correlated with CMC in APS-1 (Kisand *et al.* 2010, Puel *et al.* 2010). The combination of high-titre autoantibodies to type I IFNs and IL-22 is certainly diagnostic for APS-1 as their prevalence is 100% for type I IFNs and 91% for IL-22 in serum of APS-1 patients as early as few months old before any clinical symptoms of APS-1 (Kisand *et al.* 2015). Recent study reported autoantibodies to IL-6 in 8 out of 41 patients with APS-1, suggesting that autoantibodies against Th17 cytokines might be generated from autoimmunity against upstream cytokines responsible for generating and maintaining Th17 cells (Karner *et al.* 2016). Meanwhile, the reason why APS-1 patients produce autoantibodies against cytokines remains to be elucidated. Further, the phenotype of APS-1, including the autoantibody repertoire and distinct organ involvement, varies among patients and still undetermined.

Meyer *et al.* (2016) conducted a large-scale study involved 81 APS-1 patients and demonstrated that each patient has a private repertoire of autoantibodies, which collectively, were against more than 3,700 proteins.

Strong immunoreactivity was found towards 100 proteins, 10 of them were recognised in almost all APS-1 patients including the diagnostic type I IFNs and IL-22 cytokines (Meyer *et al.* 2016).

In fact, the clinical picture is highly variable with great phenotypic heterogeneity, even among siblings (Ishii *et al.* 2000). This suggests a weak genotype-phenotype correlation, indicating the possible influence of other modifier genes and environmental factors. This notion was further supported by studies in APS-1 mouse models that demonstrated differing patterns of tissue-specific autoantibodies depending on the background mouse strain, suggesting that individual major histocompatibility complex might influence the disease phenotype (Jiang *et al.* 2005).

### 1.1.3 Prognosis and mortality of APS-1

The prevalence of APS-1 manifestations increases with age (Perheentupa 2006). The risk of developing new, possibly life-threatening, disease components can be a source of continuous distress. Moreover, the ectodermal components may cause great psychosocial burden (Perheentupa 2006, Eyerich *et al.* 2010).

Life expectancy and mortality of patients with APS-1 varies widely based on the clinical spectrum and severity of the disease (Perheentupa 2006). The most critical autoimmune manifestations are severe hepatitis and malabsorption (Michele *et al.* 1994, Padeh *et al.* 1997, Perheentupa 2002). Inadequate hormonal substitution or poor management of Addisonian crisis may also increase the mortality risk (Arlt *et al.* 2003). Furthermore, patients with long-lasting oral candidiasis are at increased risk of oesophageal

squamous cell carcinoma (Rautemaa *et al.* 2007). In Finnish APS-1 patients, the average life expectancy has been estimated to be 31.8 years (2.8–60.5 years) while the mean age at death has been 32.0 years (2.8–63 years) (Rautemaa *et al.* 2007). Medical history of APS-1 patients during lifetime is critical for better understanding of the disease morbidity, and appropriate treatment appears to prolong life of APS-1 patients.

### 1.1.4 Genetics of APS-1

APS-1 is caused by loss-of-function mutations in the Autoimmune Regulator *(AIRE)* gene (Aaltonen *et al.* 1994, Aaltonen *et al.* 1997, Nagamine *et al.* 1997), resulting in a dysfunctional AIRE protein. *AIRE* represents the first single gene defect resulting in a multisystem autoimmune disease (Ward *et al.* 1999). *AIRE* gene was identified by positional cloning of the genetic locus on chromosome 21q22.3; It codes for a translated product of 545 amino acids with a molecular mass of 57.5 kDa (Aaltonen *et al.* 1997, Nagamine *et al.* 1997). To date, more than 100 APS-1 causing mutations have been identified which vary from substitutions, insertions and deletions to splice site mutations (Zhang *et al.* 2013, Jin *et al.* 2014, Bruserud *et al.* 2016). Some of the most common mutations in specific ethnic groups are; R257X, Y85C, R139X, R203X, W78R, and the 13-base pair deletion in PHD1 domain (Bjorses *et al.* 2000, Peterson *et al.* 2004, Valenzise *et al.* 2012, Scarpa *et al.* 2013). These bi-allelic disease-causing AIRE mutations developed classic early-onset APS-1 phenotypes; however, several reported mutations including G228W, C311Y, C302Y and V301M were found to be inherited in a dominant manner (Ilmarinen *et al.* 2005, Abramson *et al.* 2010, Oftedal *et al.* 2015). The mono-allelic

dominant effect was found to cause milder phenotype with common organ-specific autoimmune diseases of varying severity ranging from late-onset classical APS-1 to isolated organ-specific autoimmunity (Oftedal *et al.* 2015).

## 1.2 Autoimmune regulator

*AIRE* is a single-gene that causes a systemic autoimmune disease when its function is abrupt. Linkage studies have indicated that *AIRE* gene is responsible for the pathogenesis of APS-1 (Aaltonen *et al.* 1997, Nagamine *et al.* 1997). Researchers rapidly cloned the mouse *Aire*, the murine *AIRE* orthologous, to develop an experimental model of APS-1 (Anderson *et al.* 2002, Ramsey *et al.* 2002). Studies using *in vivo* APS-1 mouse models have significantly elucidated and increased in the knowledge of APS-1 autoimmunity (Pereira *et al.* 2005).

Several studies have demonstrated that AIRE/Aire is expressed mainly by the thymus, in a subpopulation of medullary Thymic Epithelial Cells (mTECs), $AIRE^{+}$ mTECs (Nagamine *et al.* 1997, Blechschmidt *et al.* 1999, Mittaz *et al.* 1999, Kumar *et al.* 2001) *[Figure 1.2]*. AIRE/Aire is expressed largely in mTECs that express high levels of cluster of designation 80 $(CD80)^{Hi}$ and major histocompatibility complex class II $(MHC\text{-}II)^{Hi}$, thus termed as $mTEC^{Hi}$ (Gray *et al.* 2007, Peterson *et al.* 2008, Venanzi *et al.* 2008). These two markers are important in T cells activation. Naïve T cells activated by two-signal process, in which the T cell receptor (TCR) binds to the processed antigen presented by MHC-II complex; the second costimulatory signal is via CD28 expressed on T cells binding to CD80 expressed by antigen presenting cells (Podojil *et al.* 2009).

The high thymic expression of AIRE/Aire strongly suggests an association of AIRE/Aire protein with the development and maturation of T cells, therefore, central tolerance induction. One of the pivotal findings is that AIRE/Aire plays a crucial role in the expression of Tissue Specific Antigens (TSAs) within the thymus. AIRE promotes self-tolerance in the thymus by regulating the promiscuous expression of a wide array of TSAs that have the commonality of being tissue-restricted in their expression pattern in the periphery (Anderson *et al.* 2002). Autoreactive thymocytes (T cells) that recognise these TSAs with high affinity undergo negative selection through their apoptosis or, alternatively, Forkhead box P3 $(FOXP3)^{+}$ regulatory T cells ($T_{reg}$) are generated in order to prevent autoimmunity (Malchow *et al.* 2016). On the other hand, T cells that recognise TSAs with moderate avidity survive and further differentiate to single-positive CD4 or CD8 subsets (Passos *et al.* 2018). This finding highlights the importance of central tolerance in controlling autoimmunity.

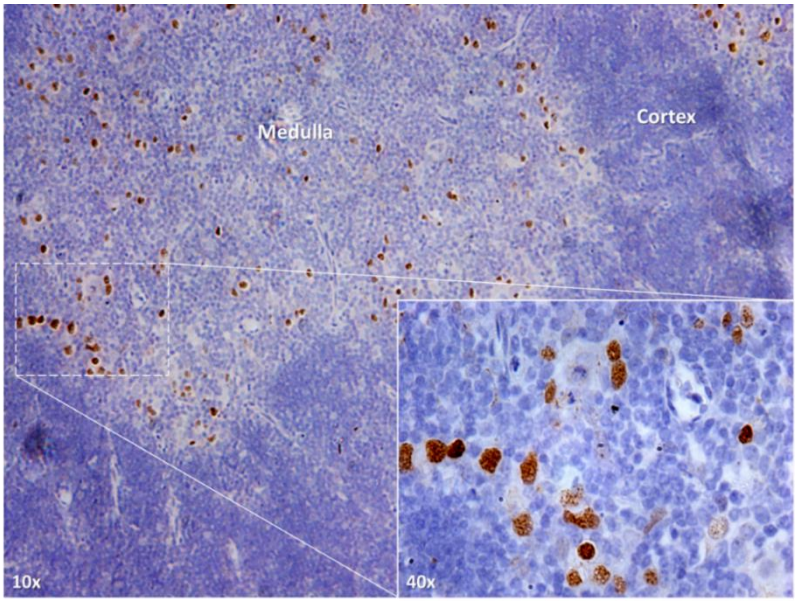



**Figure 1.2: Expression of Aire in thymus medulla.** IHC staining of Aire in wild type mouse thymic section shows the punctate nuclear staining of the Aire protein in a subset of thymus medullary cells.

### 1.2.1 Structure of Human *AIRE* and mouse *Aire*

The human *AIRE* gene was identified as the causative gene for APS-1 by positional cloning of the genetic locus on chromosome 21q22.3. It consists of 14 exons coding for a translated product of 545 amino acids with a molecular mass of 57.5 kDa (Aaltonen *et al.* 1997, Nagamine *et al.* 1997). On the other hand, the orthologous murine gene, *Aire*, was mapped to chromosome 10**,** consisting of 14 exons coding for a polypeptide of 552 amino acids with a molecular mass of 59 kDa (Blechschmidt *et al.* 1999, Mittaz *et al.* 1999). Both human *AIRE* and murine *Aire* share 71% amino acid identity and 75% nucleic acid identity (Blechschmidt *et al.* 1999, Mittaz *et al.* 1999). Furthermore, all functional domains are mostly conserved in both species except for some variations in the PRR region (Blechschmidt *et al.* 1999, Mittaz *et al.* 1999) *[Figure 1.3]*. The isolation and characterization of the orthologous murine gene has provided valuable tools to elucidate the mechanisms underlying APS-1. One important achievement was the generation of *Aire* knockout (*Aire*$^{-/-}$) mice, which helped in clarifying the molecular pathways involved in promiscuous gene expression and immunity in general.

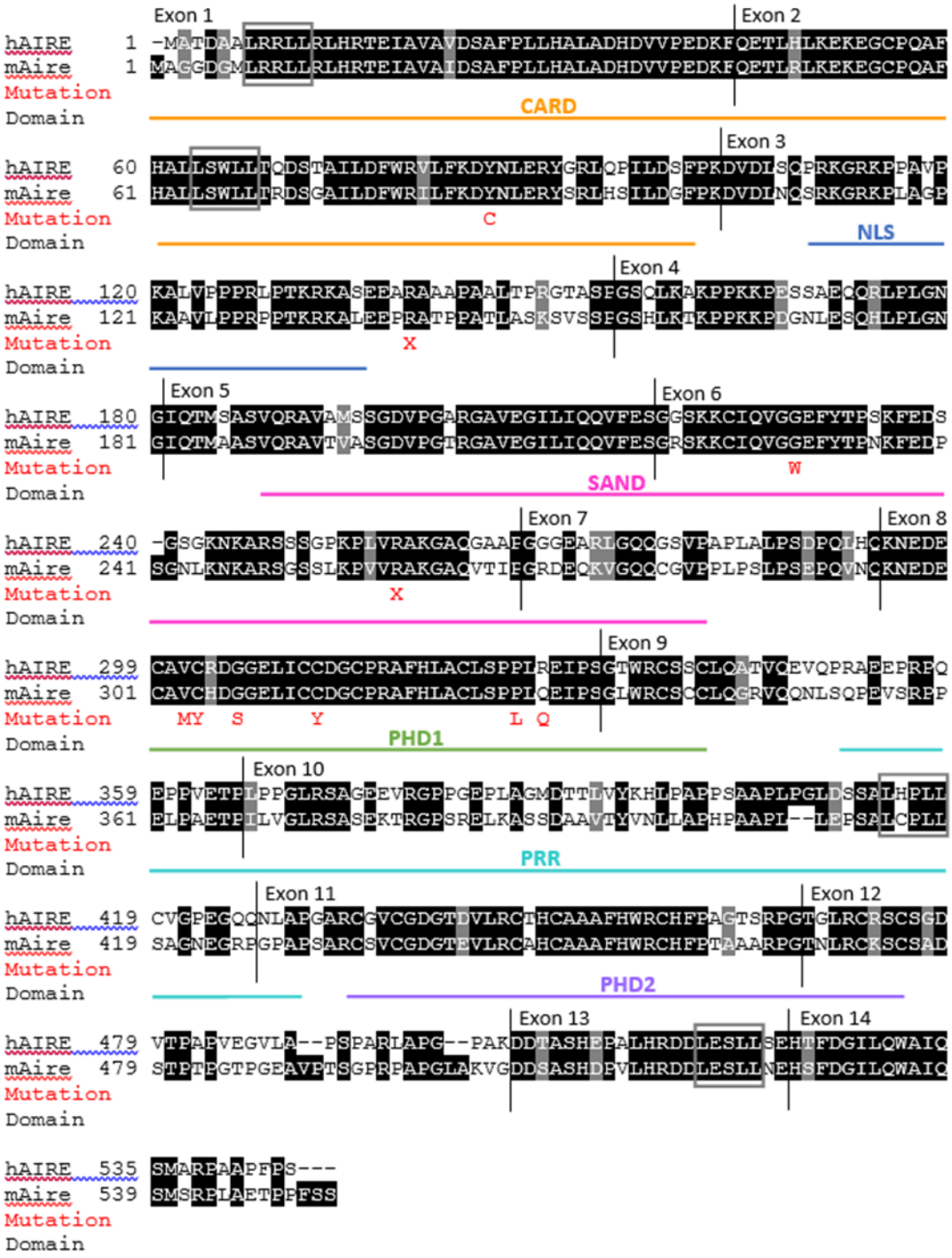



**Figure 1.3 Human AIRE and mouse Aire conserved domains.** A comparison of the human and mouse amino acids sequences. Common mutations shown beneath the sequences (red). Domains are underlined and named. CARD; Caspase Recruitment Domain, NLS; Nuclear Localization Signal, SAND; (Sp100, AIRE-1, NucP41/75, DEAF-1), PHD1-PHD2; Plant Homeodomain-type zinc fingers, PRR; Proline Rich Region and the four LXXLL motifs (grey box). Boundaries shows alternating exons.

### *1.2.1.1 AIRE/Aire at a molecular level*

As mentioned earlier, AIRE/Aire affects key aspects of central tolerance: the ectopic expression of TSA in the thymus, their presentation by mTECs, and the selection of $T_{reg}$. AIRE's/Aire's expression pattern, nuclear punctate, and domain structure are indicative of its role as a transcriptional regulator. However, at molecular level, several features influence AIRE/Aire mode of action on gene expression defining it as not classical, sequence-specific, transcription factor. Murine *Aire*$^{-/-}$ microarray data sets have shown that Aire regulates thousands of gene expression (Venanzi *et al.* 2008). Our group using overexpression of AIRE in a novel recombinant human cell line has confirmed these data; the meta-analysis identified 512 conserved AIRE/Aire regulated genes expression (*manuscript submitted*). This large range of TSA genes expressed by AIRE/Aire are challenging to be controlled by sequence-specific transcription factor, as these binding sites should occurs in thousands of silenced genes promoters (Mathis *et al.* 2009). Another aspect of AIRE/Aire role in gene expression as an atypical transcriptional regulator shows a biased program. A recent study reported that sex steroids such as oestrogen and androgen modulate Aire and Aire-dependent TSA genes expression levels; androgen upregulates Aire-mediated tolerance while oestrogen downregulates Aire-mediated tolerance (Bakhru *et al.* 2016, Zhu *et al.* 2016). However, a model that combines and explains all the details of AIRE/Aire and its role in the regulation of immunological tolerance still needs more elucidation.

### 1.2.2 Structure of AIRE/Aire protein

AIRE/Aire is a multi-domain protein. It harbours Caspase Recruitment Domain (CARD), Nuclear Localization Signal (NLS), SAND domain (Sp100, AIRE-1, NucP41/75, DEAF-1), two Plant Homeodomain-type zinc fingers (PHD), a Proline-rich region (PRR), and four nuclear receptor binding LXXLL motifs *[Figure 1.4]*.

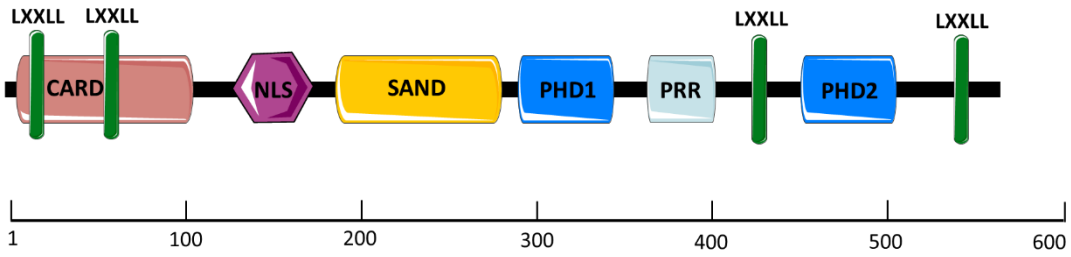



**Figure 1.4: The domains and functional elements of AIRE.** Domains are indicated in the coloured boxes and named. CARD; Caspase Recruitment Domain, NLS; Nuclear Localization Signal, SAND; (Sp100, AIRE-1, NucP41/75, DEAF-1), PHD1-PHD2; Plant Homeodomain-type zinc fingers, PRR; Proline Rich Region and the four LXXLL motifs.

CARD was reported to be involved in the process of AIRE homomultimerisation (Pitkanen *et al.* 2000, Halonen *et al.* 2004, Ferguson *et al.* 2008). Pitkanen *et al.* (2000) have also demonstrated an extra function of CARD, alterations in this domain reduced AIRE effectiveness in the activation of the gene transcription. NLS was found to mediate the nuclear import of AIRE (Ilmarinen *et al.* 2006). The AIRE's SAND does not have a distinct DNA-binding motif (Gibson *et al.* 1998). However, it is involved in protein-protein interaction with ATF7ip (Activating Transcription Factor 7–interacting protein)

which links Aire to other epigenetic processes (Waterfield *et al.* 2014). The PHD zinc finger 1 (PHD1) of AIRE interacts with amino-terminal tail of un-methylated histone H3 molecules at lysine position 4 (H3K4), and with DNA-dependent protein kinase (DNA-PK), which influences AIRE transcriptional activity (Liiv *et al.* 2008, Org *et al.* 2008). Moreover, *Aire* PHD2 knockout studies suggested an involvement of Aire in controlling mTECs gene transcription (Yang *et al.* 2013). These PHD zinc fingers can putatively bind two different DNA sequence motifs (ATTGGTTA), via amino acid residues 299–355 (PHD1) and 434–475 (PHD2) (Purohit *et al.* 2005). LXXLL and PRR motifs reported to facilitate the interaction of proteins with nuclear receptors, resulting in promoting gene transcription (Plevin *et al.* 2005, Meloni *et al.* 2008) *[Table 1.2]*.

### *1.2.2.1 AIRE as a part of protein complex*

AIRE/Aire is associated with a wide set of proteins to initiate the transcription of TSAs in mTECs (Yang *et al.* 2013). These proteins are classified into four groups according to their function: proteins involved in nuclear transport, proteins that interact directly with chromatin, proteins that participate in mRNA transcription and proteins that participate in mRNA processing (Yang *et al.* 2013).

**Table 1.2 Summary of the functional domains in AIRE protein.**

| Structural Domain | Location (a.a) | Function | References |
|---|---|---|---|
| **HSR/CARD** | 1-100 | • mediates homomultimerisation, Activate gene transcription | (Pitkanen *et al.* 2000, Halonen *et al.* 2004) |
| **NLS** | 159-167 | • Translocation into the nucleus | (Ilmarinen *et al.* 2006) |
| **SAND domain** | 189-280 | • Mediates protein-protein interaction, Important in oligomer formation, Nuclear transport mechanism | (Gibson *et al.* 1998, Waterfield *et al.* 2014) |
| **PHD 1** | 299-340 | • Regulate gene transcription, Binds to the DNA-dependent protein kinase (DNA-PK), E3 ubiquitin ligase. | (Liiv *et al.* 2008, Org *et al.* 2008) |
| **PRR** | 350-407 | • Mediates protein-protein interactions. | (Meloni *et al.* 2008) |
| **PHD 2** | 434-475 | • Regulates gene transcription. | (Yang *et al.* 2013) |
| **LXXLL** | 7-11 | • Promotes gene transcription | (Plevin *et al.* 2005) |
| **LXXLL** | 63-67 | | |
| **LXXLL** | 414-418 | | |
| **LXXLL** | 516-520 | | |

AIRE/Aire protein resides inside the nucleus and exhibits a punctate pattern; the location suggests that AIRE/Aire might function by directly regulating gene expression within the thymus (Heino *et al.* 1999). Although the biological relevance of this transcription factor is well documented, its pathway involvement remains elusive. However, *in vitro* and *in vivo* studies postulated that AIRE/Aire activates promiscuous gene expression of TSAs by releasing

blocked RNA polymerase II (RNA Pol II), suggesting its involvement in transcriptional elongation rather than transcriptional initiation.

Briefly, RNA Pol II is recruited to transcription start sites (TSS) and initiates transcription. However, due to the action of negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF), the transcriptional process is aborted. To release RNA Pol II, AIRE/Aire is recruited into the TSS of silenced genes. Once joining stalled RNA Pol II and through histone modifications and deacetylation mediated by Sirtuin-1 (SIRT-1), the complex allows DNA topoisomerase 2-alpha (TOP2α) to generate disruptions in the regions of transcription initiation that induce epigenetic changes and chromatin relaxation (Bansal *et al.* 2017). AIRE/Aire interacts with transcriptional co-activator CREB-binding protein (CBP), binds to local histone marks H3K4, recruits positive transcription elongation factor b (P-TEFb) by interacting with C-type cyclin (CycT1) (Pitkanen *et al.* 2000, Oven *et al.* 2007, Org *et al.* 2008). P-TEFb phosphorylates NELF and DSIF causing dislodging of the former and converting into an elongation factor of the latter. This change enables the phosphorylated RNA Pol II to productive elongation, results in the activation of gene expression which proceeds until termination (Giraud *et al.* 2012).

Moreover, DNA-dependent protein kinase (DNA-PK) phosphorylates AIRE/Aire and influences its transactivation function (Liiv *et al.* 2008). Therefore, sufficient levels of RNA Pol II, unmodified H3K4, and DNA-PK proteins ensure that AIRE/Aire is recruited to the genes sites and leads to the expression of TSAs and their presentation to T cells by MHC class II determinants. In addition, protein inhibitor of activated STAT1 (PIAS1) was

found to interact with AIRE/Aire to regulate the activities of the target genes (Ilmarinen *et al.* 2008) *[Figure 1.5]*.

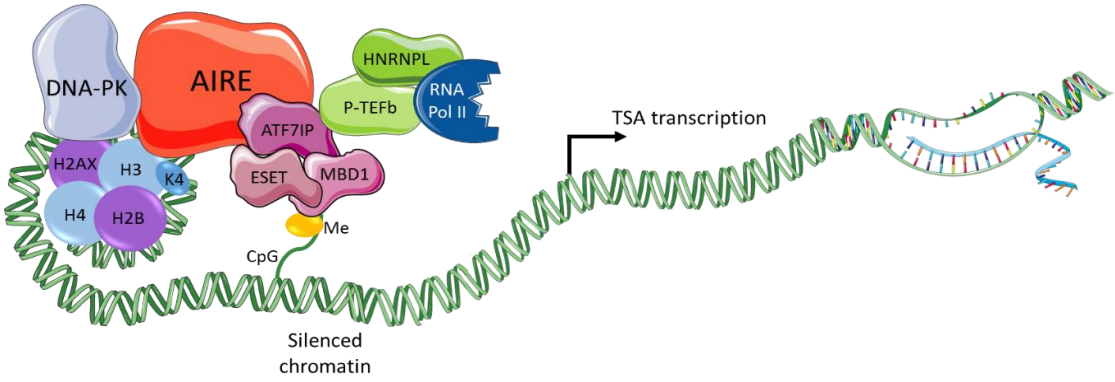



**Figure 1.5 Proposed mechanism of AIRE-mediated gene activation.** Interactions between AIRE and DNA-PK recruit AIRE to a TSA promoter. An AIRE interaction with CBP and unmodified H3K4 recruits P-TEFb to phosphorylate the RNAPII, this results in transcription elongation, mRNA processing, and TSA gene expression. Diagram recreated from (Anderson *et al.* 2016) using Servier Medical Art.

### 1.2.3 Role of AIRE/Aire in the thymus

Several epigenetics factors including methylation affect AIRE/Aire expression, such as the induction of the NF-κB pathway through RANKL-RANK-mediated signalling, and the LTβ- mediated pathway that define the fate of the differential expression of AIRE/Aire in a subset of mTEC cells (Zhu *et al.* 2006, Zhu *et al.* 2010). Populations of $CD80^{lo}MHC\text{-}II^{lo}$ mTECs, which do not express AIRE/Aire, display a significant reduction in the expression of TSAs (Anderson *et al.* 2002). The negative selection of T cells in the thymus

is an incomplete process, and autoreactive T cells are detectable in the serum of clinically healthy individuals (Danke *et al.* 2004). Therefore, mechanisms of tolerance within the thymus could also applied in the periphery to control autoimmunity and shape T cell repertoire.

### *1.2.3.1 Role of AIRE/Aire in negative selection and central tolerance*

The thymus is the main organ where central tolerance is established; two mechanisms are required to establish central-tolerance, positive and negative selection of thymocytes (Kyewski *et al.* 2006). Positive selection for native T cells occurs in the cortex of the thymus. Maturing T-cell recognises a MHC-II antigen in the thymus survives the programmed cell death; cells failing to recognise MHC-II on thymic epithelial cells will undergo apoptosis (Kyewski *et al.* 2006). Negative selection is the process where mTECs function as antigen presenting cells and display TSAs to maturing T-cells and those reactive T-cells undergo apoptosis (Liston *et al.* 2003). This process is highly dependent on the promiscuous expression of TSA, which is regulated by AIRE/Aire (Anderson *et al.* 2002, Derbinski *et al.* 2005).

AIRE/Aire is a crucial regulator of thousands of TSAs genes expression in mTECs and affects their transcription in a stochastic but ordered manner (Meredith *et al.* 2015). Indeed, AIRE/Aire is selectively expressed in approximately 50% of MHC class $II^{hi}$ mTECs, and a small subset (1–3%) of the total number of mTECs expresses a specific TSA (Derbinski *et al.* 2008, Anderson *et al.* 2016). Moreover, the ordered TSAs expression is the increased possibility that a particular set of TSAs genes will be co-expressed

in one mTEC cell. In addition, AIRE/Aire promotes negative selection by regulating thymic dendritic cells and inducing apoptosis of mTECs (Gray *et al.* 2007, Lei *et al.* 2011). It has been reported that over-expression of AIRE/Aire *in vitro* results in the induction of apoptosis in several cell lines; thus it has been proposed that the absence of AIRE/Aire-induced apoptosis is responsible for the increased numbers of mTECs observed in Aire-deficient mice (Liiv *et al.* 2012).

Recently, a study identified FEZ Family Zinc Finger 2 (Fezf2), a zinc finger transcription factor, as an additional regulator of promiscuous gene expression in mTECs (Takaba *et al.* 2015). The range of genes regulated by Fezf2 and Aire seems different. While Aire expression in mTECs is predominantly promoted by RANK-mediated signals, Fezf2 expression in mTECs is primarily regulated by LTβR-mediated signals (Takaba *et al.* 2015). Furthermore, like Aire, the lack of Fezf2 causes autoimmune disease; in Ltβr$^{-/-}$ mice, Fezf2-dependent TSA expression was decreased, but not Aire-dependent TSA expression (Takaba *et al.* 2015). Thus, RANK and LTβR regulate the induction of distinct TSA genes through Aire and Fezf2, respectively (Takaba *et al.* 2015).

Although most autoreactive T cells are negatively eliminated in the medulla, a portion of them differentiates into $T_{reg}$ cells, which are required for maintaining immune tolerance (Malchow *et al.* 2016). This based on the agonist selection process, where T cells interacts with TSAs with high affinity, but escape deletion and go to periphery in order to maintain immune tolerance (Sakaguchi 2004).

### *1.2.3.2 AIRE/Aire promotes thymic selection of regulatory T cells*

The fundamental pathogenic mechanisms in APS-1 appear to be T cells mediated. In addition to its role in negative selection, AIRE/Aire promotes the development of FOXP3$^+$ $T_{reg}$ cells (Nomura *et al.* 2007, Malchow *et al.* 2016). AIRE/Aire plays a role in the $T_{reg}$ cell development in the mouse thymus at the neonatal stage that is important for suppression of autoimmune responses (Guerau-de-Arellano *et al.* 2009). The influence of AIRE/Aire on agonist selection of FOXP3$^+$ $T_{reg}$ cells has been more debated. Ectopic TSA expression in AIRE$^+$ mTECs promoted the development of thymic antigen-specific $T_{reg}$ cells, this indicates that AIRE/Aire promotes thymic $T_{reg}$ cell development (Aschenbrenner *et al.* 2007, Lei *et al.* 2011, Malchow *et al.* 2013, Perry *et al.* 2014, Yang *et al.* 2015). In addition, $T_{reg}$ are defective in APS-1 patients, with deficient number and impaired function of $T_{reg}$ has been reported by several studies (Kekalainen *et al.* 2007, Wolff *et al.* 2010). Therefore, when *AIRE/Aire* is mutated, autoreactive T cells will target AIRE-dependent self-antigens. Thus, AIRE/Aire may enforce immune tolerance by both clonal deletion and ensuring that autoreactive T cells differentiate into $T_{reg}$ cells. This suggests that autoimmunity associated with AIRE/Aire deficiency results from two failed tolerance mechanisms.

## 1.2.4 Role of AIRE in the periphery

The negative selection of T cells in the thymus is an incomplete process, and autoreactive T cells are detectable in the serum of clinically healthy individuals (Danke *et al.* 2004). Therefore, mechanisms of tolerance

within the thymus could also be applied in the periphery to control autoimmunity and shape T cell repertoire.

Increasing evidence suggests low-level *AIRE/Aire* mRNA expression as well as AIRE/Aire protein in extra-thymic tissues; these findings suggest that AIRE/Aire might also have a function outside the immune system. AIRE/Aire was found to be expressed by thymic dendritic cells (DCs) and peripheral lymphoid organs such as spleen and lymph nodes (Nagamine *et al.* 1997, Blechschmidt *et al.* 1999, Heino *et al.* 1999, Poliani *et al.* 2010). In addition to spleen and lymph nodes, human *AIRE* expression has been reported in foetal liver and bone marrow (Nagamine *et al.* 1997, Heino *et al.* 1999). In murine tissues, extra-thymic *Aire* mRNA and its protein have been detected in liver, Kidney, pancreas, ovary and testis (Blechschmidt *et al.* 1999, Halonen *et al.* 2001, Adamson *et al.* 2004). Other groups reported that Aire is expressed in both the haematopoietic and stromal cells lineages (Gardner *et al.* 2008, Fletcher *et al.* 2010, Poliani *et al.* 2010). These cells are a unique MHC II$^{hi}$, CD80$^{lo}$, CD86$^{lo}$, epithelial cell adhesion molecule (EpCAM)$^{hi}$, CD45$^{lo}$ bone marrow-derived peripheral antigen presenting cells (Gardner *et al.* 2013). However, despite this accumulating evidence of peripheral expression of AIRE/Aire, not all studies have been in agreement, particularly those in mice. Several studies reported different expression levels of Aire in same peripheral tissues varying from weak to strong (Halonen *et al.* 2001, Hubert *et al.* 2008).

In the periphery, lymph node stromal cells express TSAs to T cells that is sufficient to induce peripheral tolerance among T cells (Lee *et al.* 2007). In

addition, haematopoietic and stromal AIRE/Aire+ cells express unique Aire-regulated TSAs distinct from those driven by thymic AIRE/Aire, and support immune tolerance by inducing apoptosis of autoreactive T cells that escaped thymic negative selection (Gardner *et al.* 2008). However, it remains unclear whether AIRE/Aire expression in the periphery does definitely induce post-thymic T-cell tolerance. Therefore, AIRE's/Aire's role in peripheral tolerance needs more elucidation. One approach would be targeting peripheral tissues of *AIRE/Aire* expression in extra-thymic tissues and observe the effect of this blocking.

## 1.3 APS-1 mouse models

APS-1 syndrome characterised by the complex interaction of immune cells with different tissues in the body. To date no *in vitro* system has been developed to model APS-1 disease. As a monogenic disorder caused by loss-of-function mutations in *AIRE*, researchers rapidly cloned the mouse *Aire*, equivalent of the *AIRE* gene, to develop an experimental model of APS-1 (Anderson *et al.* 2002, Ramsey *et al.* 2002). *In vivo* murine models studies have led to significant increase in the knowledge of the mechanisms involved in the autoimmunity in APS-1 patients (Pereira *et al.* 2005, Liston 2006).

Several Aire deficient mice on different backgrounds have been developed separately, seven published strains by four groups are summarised in Table 1.3 (Anderson *et al.* 2002, Ramsey *et al.* 2002, Jiang *et al.* 2005, Kuroda *et al.* 2005, Hubert *et al.* 2009). In 2002, two groups separately

engineered Aire knockout mice on C57BL/6 background. A major Finnish mutation of *AIRE*, R257X, was represented in Peltonen's group Aire knockout mouse to mimic human APS-1 disease (Ramsey *et al.* 2002). Targeted disruption of the murine Aire by homologous recombination resulted in the insertion of the *Neo*-cassette in exon 6; this caused an early termination of all synthesised Aire polypeptides. On the other hand, Mathis's group has engineered Aire knockout mouse by deleting exon 2 (Anderson *et al.* 2002). Although both mice show normal Aire gene transcripts level, sequence analysis confirmed that these transcripts do not result into functional protein because of the frame shifts in both Aire copies. Later, a third Aire knockout mouse was developed by Matsumoto's group, gene targeting vector was constructed by deleting exons 5 to 12 with the neomycin resistance gene (*Neo*) (Kuroda *et al.* 2005). This deletion of the large proportion resulted in the absence of important domains: SAND, PHD-1 and PHD-2. Furthermore, Scott's group Aire knockout mouse has a disrupted PHD-1 domain at exon 8 to reflect the 13-bp deletion of human APS-1 patients. Expression of functional Aire protein in each mouse model was aborted, either no expression or truncated expression of non-functional premature Aire protein was reported (Anderson *et al.* 2002, Ramsey *et al.* 2002, Kuroda *et al.* 2005, Hubert *et al.* 2009). However, normal morphology of the thymic compartments was detected in all Aire knockout mice, suggesting that the absence of Aire does not alter the morphology of the thymus.

Phenotypically, all strains look grossly normal and resemble wild type littermates. Ramsey *et al.* (2002) reported that 42% of these mice revealed atrophy of adrenals and ovaries while 50% showed lymphocytic infiltration in

liver sections. Moreover, these mice were considered to be a spontaneous model for autoimmune uveitis (Chen *et al.* 2015). They all live to the expected age of their wild type littermates, except for the Aire knockout mice on the NOD background, the disease progress severely and rapidly and they live only up to 15 weeks (Jiang *et al.* 2005). Moreover, these investigators have reported spontaneous lymphocytic infiltration towards multiple tissues, circulating autoantibodies and infertility that increase with age, except for the mice generated by Matsumoto's group; they are fertile but produce offspring only occasionally. *[Table 1.4]*. This autoimmunity towards multiple organs equivalents to the human APS-1 phenotype, although these infiltrations never progress into any of the diagnostic classical triad: Chronic Mucocutaneous Candidiasis, Hypoparathyroidism and Addison's disease.

**Table 1.3: APS-1 mouse models**

| **Background** | **Peltonen group** | **Mathis group** | | | | **Matsumoto group** | | **Scott group** |
|---|---|---|---|---|---|---|---|---|
| | C57BL/6 | C57BL/6 | NOD/LtJ | BALB/cJ | SJL/J | C57BL/6 | NOD/Shi Jic | C57BL/6 |
| **Design** | Neo cassette insertion in exon 6 | Cre-lox mediated deletion of exon 2 | Backcrossed with *Aire*$^{-/-}$ C57BL/6 | Backcrossed with *Aire*$^{-/-}$ C57BL/6 | Backcrossed with *Aire*$^{-/-}$ C57BL/6 | Neo cassette insertion replacing exons 5-12 | Backcrossed with *Aire*$^{-/-}$ C57BL/6 | Cre-lox mediated deletion of exon 8 |
| **Predicted mRNA** | Exons 1-5 | Exon 1 | | | | Exons 1-4 | | Exons 1-7 |
| **Predicted protein domains** | CARD, NLS, premature SAND | Premature CARD | | | | HSR, NLS | | HSR, NLS, SAND, premature PHD-1 |
| **Translation terminate** | SAND | CARD | | | | NLS | | PHD-1 |
| **Major human equivalent mutation** | R257X | NA | | | | NA | | d1094-1106 del13 |
| **References** | (Ramsey *et al.* 2002) | (Anderson *et al.* 2002, Jiang *et al.* 2005) | | | | (Kuroda *et al.* 2005, Niki *et al.* 2006) | | (Hubert *et al.* 2009) |

The clinical manifestations among APS-1 patients show a notable difference between them, which suggests that other genetic or environmental factors are affecting the disease phenotype. In order to investigate the genetic variations effect on the variability between human APS-1 patients, Jiang and colleagues backcrossed C57BL/6J, NOD/LtJ, BALB/cJ, and SJL/J backgrounds mice to *Aire*$^{-/-}$ generated previously in Anderson *et al* (2002) (Jiang *et al.* 2005). In agreement with their previous study, Aire-deficient mice exhibited lymphocytic infiltrates and autoimmune endocrine destruction with slight differences in the set of pathologies developed between the different strains. Furthermore, their study confirmed that the genetic background variation affects the overall intensity of the disease; which ranges from mild in C57BL/6J background to infiltration that is more aggressive; pneumonitis, and higher autoantibodies production in Aire NOD/LtJ background mice (Jiang *et al.* 2005). For instance, the tissue infiltration of the stomach as a target organ on the NOD (87%) and BALB/c (100%) backgrounds was higher than the C57BL/6 (9%) background, and the pancreas tissue as a target on the NOD (100%) and SJL/J (71%) backgrounds (Jiang *et al.* 2005). These data suggest that NOD genetic background altered the mild autoimmunity reported on the C57BL/6 background into a fatal disease by 15-weeks of age (Jiang *et al.* 2005). Collectively, these data indicate the existence of disease modifier loci in mice. Indeed, this applied to the differences in the phenotype of APS-1 patients, disease modifier loci could alter the tissues infiltration involved in each APS-1 patient.

**Table 1.4: Summary of *Aire*$^{-/-}$ mice affected systems**

| Background | Peltonen group (Ramsey *et al.* 2002) | Mathis group (Anderson *et al.* 2002, Jiang *et al.* 2005) | | | | Matsumoto group (Kuroda *et al.* 2005) | | Scott group (Hubert *et al.* 2009) |
|---|---|---|---|---|---|---|---|---|
| | **C57BL/6** | **C57BL/6** | **NOD/LtJ** | **BALB/cJ** | **SJL/J** | **C57BL/6** | **BALB/c** | **C57BL/6** |
| **Digestive system** | o | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| **Respiratory system** | o | ✓ | ✓ | ✓ | ✓ | o | - | - |
| **Eye/ vision** | o | ✓ | ✓ | ✓ | - | o | - | ✓ |
| **Endocrine/exocrine glands** | ✓ | ✓ | ✓ | ✓ | - | ✓ | ✓ | ✓ |
| **Liver/biliary system** | ✓ | o | ✓ | ✓ | ✓ | - | ✓ | - |
| **Reproductive system** | ✓ | ✓ | ✓ | ✓ | ✓ | o | o | ✓ |
| **Mortality** | - | - | ✓ | - | - | - | - | - |

Symbols: (✓ = present), (- = investigated but not found), (o = not reported)

The general role of Aire in murine model, with a loss-of-function mutations and similar pathologies to the APS-1 human disease, is expected to be directly related to the role of AIRE in humans. However, as a model of APS-1, these Aire deficient mice are unable to reproduce the development of hypoparathyroidism, Addison's disease and candidiasis infection, the classical triad symptoms of APS-1. Conflicting evidence has been debated whether *Aire*$^{-/-}$ mouse is a relevant model for the human APS-1 disease (Pontynen *et al.* 2006, Kekalainen *et al.* 2007). In the Pontynen *et al.* (2006) study, they have concluded that the *Aire*$^{-/-}$ mouse model failed to develop the same autoantibodies profile as APS-1 patients. These observations were made in the genetic background C57BL/6 that generates milder immune phenotype than other strains such as NOD mice. While both Jiang *et al.* (2005) and Kuroda *et al.* (2005) experiments on different mice strains showed that the genetic background strongly affects the disease manifestations. A possible explanation for this difference is that Aire deficient mice represent the same vital defects in self-tolerance, but the clinical manifestations are against different targets (Liston 2006). Moreover, Aire deficient mice do not develop candidiasis, this could be due to the maintenance of all mice under sterile conditions. Ramsey *et al.* (2002) findings suggest an important role of environmental non-genetic factors, such as acquirement of infections, which may exaggerate the immune responses of *Aire*$^{-/-}$ mice.

Notably, a recent study demonstrated that the gene expression profile was not the same in a two genetically identical wild-type mice whose mTECs were analysed by microarray profiling data, this has important implications for the inter-individual variability in tolerance within species (Meredith *et al.* 2015).

Similar observation was reported by an earlier study, were gene expression profiling of wild-type mTECs from single thymic lobe, or from the two thymic lobes of the same mouse, revealed significantly greater variability in Aire-dependent gene expression than in Aire-independent transcripts (Venanzi *et al.* 2008). Although this diversity could complicate the interpretation of some experimental data, it could help in elucidating the cause of the different clinical manifestations amongst APS-1 patients, as similar to the different genetic backgrounds in mice, the clinical picture can vary among human APS-1 patients, even between siblings (Ahonen *et al.* 1990, Ishii *et al.* 2000). Immunologically, the *Aire*$^{-/-}$ phenotype is characterised by the presence of multiple autoantibodies against endocrine and non-endocrine organs similar to APS-1 patients. For this very reason, *Aire*$^{-/-}$ can mimic APS-1 phenotype, and can be considered as an adequate model for this disease.

## 1.4 APS-1 current treatments

Due to the rare incidence of APS-1 and the wide variability of its symptoms, the treatment is considered challenging and requires continuous follow-up. Treatment of APS-1 syndrome is mainly dependent on multiple therapeutic agents that care of each individual components such as hormone replacement therapy, anti-fungal agents and immunosuppressive therapy in severe forms including autoimmune hepatitis, interstitial lung disease, severe malabsorption with exocrine pancreatic insufficiency, and severe enteropathy (Lankisch *et al.* 2009, Kisand *et al.* 2015). Depletion of B cells with Rituximab has shown promising effects in APS-1 for the treatment of interstitial lung

disease, raising the hope of applying it to all patients with APS-1 (Popler *et al.* 2012).

Due to the complexity of the disease, it is important to be aware of the complication of some drugs when treating the clinical manifestations. For instance, vitamin D treatments to compensate Parathyroid hormone deficiency could increase the risks of hypercalciuria, while correction of gastrointestinal malabsorption could lead to hypocalcaemia (Proust-Lemoine *et al.* 2012). Some clinical studies used immunosuppressive therapy, cyclosporine A or azathioprine, to overcome the complications of using many treatments (Ward *et al.* 1999, Lankisch *et al.* 2005). However, a clinical study using immunosuppressive therapy on APS-1 patients influenced a systemic spread of candidiasis to a severe form, which lead to the death of a patient (Proust-Lemoine *et al.* 2007).

*Aire*$^{-/-}$ mice have been used to elucidate the function of Aire, which will help in modulating new treatments for APS-1, one approach is gene therapy. Due to the monogenic aetiology of APS-1, it is postulated that thymic compartment can be targeted to modulate immune tolerance by a gene therapy approach to restore a functional copy of the *AIRE* gene. Using viral vectors as means of delivery will provide advantages over conventional treatments.

## 1.5 Gene therapy strategy for APS-1

### 1.5.1 Principle of gene therapy using viral vectors

More than 300 rare gene defects have been identified as causative factors in primary immunodeficiency syndromes (PID). The only definitive correction is bone marrow transplantation; however, toxicity and infections are a major concern in transplantation even in fully matched allografts. Moreover, a mismatched donor may lead to fatal outcomes in some patients (Amrolia *et al.* 2001).

Another way of early investigated PID therapeutic approaches is gene therapy. Gene therapy includes the replacement of a defective gene with a functional one, nucleic acid transfer to cure or prevent disease. It provides the advantage of the selective treatment for specific cell-types or tissues and long-term treatment after one application over conventional treatments. Different gene therapy systems have been introduced including viral vectors such as lentivirus (LV) and adeno-associated virus (AAV), and hybrid viral/bacteriophage vectors such as AAV/phage (AAVP) (Hajitou *et al.* 2006). Non-viral vectors including naked DNA and the newly emerged CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR) associated nuclease 9) (Li *et al.* 2000, Mali *et al.* 2013), and cell-based therapies such as *ex vivo* gene transfer of autologous hematopoietic stem cells (Naldini 2011, Larochelle *et al.* 2013) were used as gene therapy vehicles.

Viral gene therapies have been extensively used in the past two decades. The crucial step for a successful viral gene therapy is the ability to

transfer the desired therapeutic gene to the target organ and efficiently transduce that organ. To date, several viral vectors have been developed and each have advantages and limitations *[Table 1.5]*.

**Table 1.5 Properties of the common viral vectors.**

| Vector | Envelope | Capacity | Integration | Limitations | Advantages | References |
|---|---|---|---|---|---|---|
| **Adenovirus** | Non-enveloped | 36 kb | Non-integrated | Inflammatory immune responses | Wide tropism | (Kay *et al.* 2001, Yamamoto *et al.* 2017) |
| **AAV** | Non-enveloped | <5 kb | Episomal | Small packaging capacity, | Non-inflammatory, non-pathogenic, | (Verma *et al.* 1997, Thomas *et al.* 2003) |
| **Lentivirus** | Enveloped | ~10 kb | Integrated | Random integration could activate oncogenes | Persistent gene transfer in dividing and non-dividing cells | (Verma *et al.* 1997, Kay *et al.* 2001) |
| **Retrovirus** | Enveloped | 8 kb | Integrated | could activates oncogenes | Transduces dividing cells only | (Thomas *et al.* 2003) |

## 1.5.2 Recent advances in clinical trials of monogenic diseases

Viral vectors represent a powerful new tool for long-term expression of therapeutic transgene *in vivo* due to their ability to infect host cells and stably integrate into the genome. Rosenberg and colleagues (1990) conducted the first approved clinical gene therapy protocol to treat Adenosine Deaminase-Severe Combined Immunodeficiency (ADA-SCID) and initial results reported

after four years (Blaese *et al.* 1995). Since then more than 2400 approved clinical trials have been carried out worldwide, with the number of monogenic clinical gene therapy trials (*n*=259) coming second after cancer trials (Wiley - J Gene Med, 2017). Since the beginning of the clinical trials, insights into the use of viral vectors and safety concerns were highlighted. For instance, Cavazzana-Calvo (2000) conducted the first successful gene-based therapy on Severe Combined Immunodeficiency-X1 (SCID-X1), an X-linked inherited disorder caused by mutations in the gene encoding the gamma-c cytokine receptor subunit of interleukin-2, -4, -7, -9, -15 and -21 receptors (Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008)(Leonard *et al.* 2008). Nevertheless, during the follow up of the study, four patients had developed leukaemia, this was due to the random integration of the viral vector near LIM Domain Only 2 (LMO2) oncogene (Hacein-Bey-Abina *et al.* 2003, Hacein-Bey-Abina *et al.* 2008). Despite these issues, the clinical trials continued with encouraging results, with immunodeficiency corrected in 17 out of 20 patients (Fischer *et al.* 2010).

Moreover, several trials have obtained approval for gene therapy products for clinical use, including Gendicine™ (p53 expression), Oncorine™ (conditionally replicative adenoviruses) and Cerepro® (thymidine kinase-ganciclovir therapy) (Wirth *et al.* 2013). Furthermore, in 2012, Glybera® was the first gene therapy product for an inherited monogenic disease approved by European Medicines Agency (EMA). It is a viral vector engineered to

express lipoprotein lipase as the transgene, for the treatment of severe Lipoprotein Lipase Deficiency (LPLD) (Bryant *et al.* 2013). A similar approach was carried out by Azzouz and colleagues, “Resagen” a potential gene therapy product for patients with Spinal Muscular Atrophy (SMA), has received orphan drug status from EMA. The viral vector expresses the missing Survival Motor Neuron (SMN) gene with the ultimate aim of restoration of SMN protein levels in SMA patients (Azzouz *et al.* 2004, Valori *et al.* 2010).

The most common vectors used for gene delivery in clinical trials include adenoviruses, retroviruses and adeno-associated viruses (AAV) ranking the third *[Table 1.6]* (Wiley - J Gene Med, 2017).

**Table 1.6: most common gene transfer viral clinical trials.**

| Viral vector | Gene therapy clinical trials | |
|---|---|---|
| | **Number of trials** | **Percentage** |
| **Adenovirus** | 509 | 20.7% |
| **Retrovirus** | 458 | 18.6% |
| **Adeno-associated virus** | 183 | 7.4% |
| **Lentivirus** | 158 | 6.4% |
| **Vaccinia virus** | 125 | 5.1% |
| **Herpes simplex virus** | 90 | 3.7% |
| **Others** | 24 | 0.8% |

Data obtained from the Gene Therapy Clinical Trials Worldwide online database and were provided by the Journal of Gene Medicine (Wiley - J Gene Med, 2017).

### 1.5.3 Adeno-associated viral vectors

Arguably, which viral vector is the most efficient and the safest for gene-based therapy is difficult to identify. Several pre-clinical studies revealed that

AAV and LV vectors are the most promising tools for efficient and long-term *in vivo* gene delivery.

Adeno-associated virus (AAV) is one of the most actively investigated gene therapy vehicle during the past five decades (Rose *et al.* 1966, Hastie *et al.* 2015, Weinmann *et al.* 2017). It represents one of the most potent, most versatile, and most promising vehicle available for gene delivery into cells (Naso *et al.* 2017, Weinmann *et al.* 2017). It is a non-pathogenic single-stranded DNA virus with a size of approximately 4.8 kilobases (kb), surrounded by a protein shell. AAV belongs to the parvovirus family and is dependent on co-infection mainly with adenoviruses, in order to replicate (Lusby *et al.* 1980, Srivastava *et al.* 1983, Grieger *et al.* 2005).

AAV-based vectors have been extensively used due to many advantages including wide tropism, ability to infect non-dividing cells and neuronal cells, and ability for stable gene expression (Kaplitt *et al.* 1994, Le Bec *et al.* 2006, Valori *et al.* 2010, Naso *et al.* 2017). Moreover, it does not always integrate into the host genome, thus the safety concerns regarding random genome integration is diminished (Kaplitt *et al.* 1994, Hastie *et al.* 2015). Out of hundreds of serotypes, only few have been investigated. The major capsid protein (VP3) is responsible for the diversity between serotypes, which in turn gives rise to diversity in antigenicity, tissue tropism and host specificity (Xie *et al.* 2002, Wu *et al.* 2006). However, the chosen delivery route plays an important role in tropism as well, for example intravenous administration of various serotypes showed high efficiency transduction of heart and liver by serotypes 6, 7, 8 and 9 (Wang *et al.* 2010). Furthermore, the

use of target-specific promoters can restrict transgene expression to specific cellular sub-populations, or enhance the transduction efficiency to target cells (Shevtsova *et al.* 2005).

It has extensively reported that AAV9 transduce widespread of targets including crossing the blood-brain barrier. Stable transgene expression was seen in the rat brain for more than 6 months (Klein *et al.* 2002). In addition, AAV9 vectors transduce rodent muscle, liver, and lung about 100-fold more efficiently than AAV2. Studies of AAV9 in large scale animal studies, no side effects or evidence of tumorigenesis has been reported yet (Bessis *et al.* 2004, Bell *et al.* 2005, Kay 2007).

## 1.6 Intrathymic delivery of viral vector

To date, several studies have targeted the thymus in order to manipulate immunologic tolerance by conducting intrathymic injections (IT) as a method for transduction. For instance, *in vivo* gene delivery targeting the thymus using LV vectors resulted in a re-establishment of T cell development, suggesting an efficient stable expression of the corrected gene in murine thymus (Marodon *et al.* 2004, Adjali *et al.* 2005). However, the level of gene transfer into thymus did not exceed 0.3% (Adjali *et al.* 2005). In order to overcome this issue, the same group had conducted another study using AAV8 vectors to mediate gene delivery (Moreau *et al.* 2009). Their data confirmed a high *in vivo* transduction of both murine and macaques thymus (Moreau *et al.* 2009). These studies suggest that IT injection of a transgene-expressing viral vector is a promising therapeutic tool aimed at thymocytes development and the induction of immune tolerance.

The process of IT gene delivery can be categorised into three methods: invasive surgical injection, direct injection and high-resolution imaging system-assisted injections, such as the use of an ultrasound system. Both the direct and the invasive surgery protocols have been widely used to inject the viral vectors into the thymus. While the invasive surgery requires the thoracic cavity to be exposed, the direct injections do not expose the thoracic cavity, it needs precise positioning of the thymus to blindly perform the IT delivery (Marodon *et al.* 2004, Marodon *et al.* 2006, Chu *et al.* 2010, Gottrand *et al.* 2012). Nevertheless, data showing liver and pulmonary infected cells as well as mRNA expression levels following intrathymic injection suggest leakiness of IT-injections. This low success rate could be due to the poor recovery and postsurgical complications in the case of major surgical procedure or because the thymus position was not correctly identified before direct injections (Marodon *et al.* 2004, Chu *et al.* 2010, Gottrand *et al.* 2012). However, Blair-Handon *et al.* (2010) had simplified and improved the IT injection technique by the use of an ultrasound probe for real time image guidance. This method had greatly reduced pain and distress to mice while maintained a high administration accuracy (Blair-Handon *et al.* 2010, Tuckett *et al.* 2014).

## 1.7 Hypothesis

The hypothesis of this study is to correct defective *Aire* gene in *Aire* deficient mice using a modified virus as a vehicle for delivery. The *AIRE* gene will be delivered using viral vectors to mediate self-tolerance in experimental models of APS-1. Over-expression of target gene in the transgenic mice will be accomplished by an adeno-associated viral vector system by intrathymic injections.

## 1.8 Aims of the study

The first stage will characterize APS-1 mouse model and assess the expression pattern of Aire. Once this has been established, an adeno-associated viral vector will be used to overexpress AIRE *in vivo* in the thymus of APS-1 mice. The objective will be to determine whether AIRE replacement can improve and rescue the disease phenotype, with the aim of taking this strategy to the clinic as therapeutic approach for APS-1.

The project specific objectives can be summarised as follows:

1. Assess the pattern of expression of Aire in wild type and mutant APS-1 mouse model.
2. Evaluate the transduction efficiency of AAV9-AIRE *in vitro*
3. Conduct a pilot study using AAV9-AIRE in APS-1 mouse model
4. Generate a pre-clinical proof-of-concept efficacy study using AAV9-AIRE in APS-1 mice.

# *CHAPTER TWO*

# MATERIALS & METHODS

# 2. MATERIALS & METHODS

## 2.1 Nucleic Acid Techniques

### 2.1.1 Polymerase chain reaction

PCR reactions were performed on cDNA to amplify the desired gene. Amplification was performed using Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies) according to the manufacturer's instructions. Standard PCR conditions were, depending on the template, 50-200ng of DNA in a 25-50 µl reaction mixture containing 0.5 units of *Taq* DNA polymerase High Fidelity (0.1 µl), 10x High Fidelity PCR buffer (5 µl), 10mM of dNTP mix (1 µl), 10mM of each primer (1 µl; see table 2.1), and distilled water to the final volume. The reaction was gently mixed and aliquot into 0.2ml thin-walled PCR reaction tubes. The PCR mixture was then run in the Thermal Cycler according to the program listed below.

- 94°C 2 minutes
- 94°C 20 seconds
- 58°C 15 seconds
- 68°C 10 seconds

35 cycles

- 72°C 15 seconds
- 4°C forever

**Table 2.1: Primers used for Human AIRE cloning**

| Primer | Sequence (5'-3') |
|---|---|
| **AIRE-NheI-FOR-1** | GATGCCGCTAGCGCCGCCACCATGGCGA |
| **AIRE-HindIII-REV-1** | CCGGCCAAGCTTGGGCCCTCAATGATG |

The human *AIRE* cDNA was cloned from pcDNA5/FRT plasmid constructed previously in our lab from an original plasmid (pET31/AIRE) that was a gift from Prof. J. She (Medical College of Georgia, Augusta University) *[Figure 2.1]*.

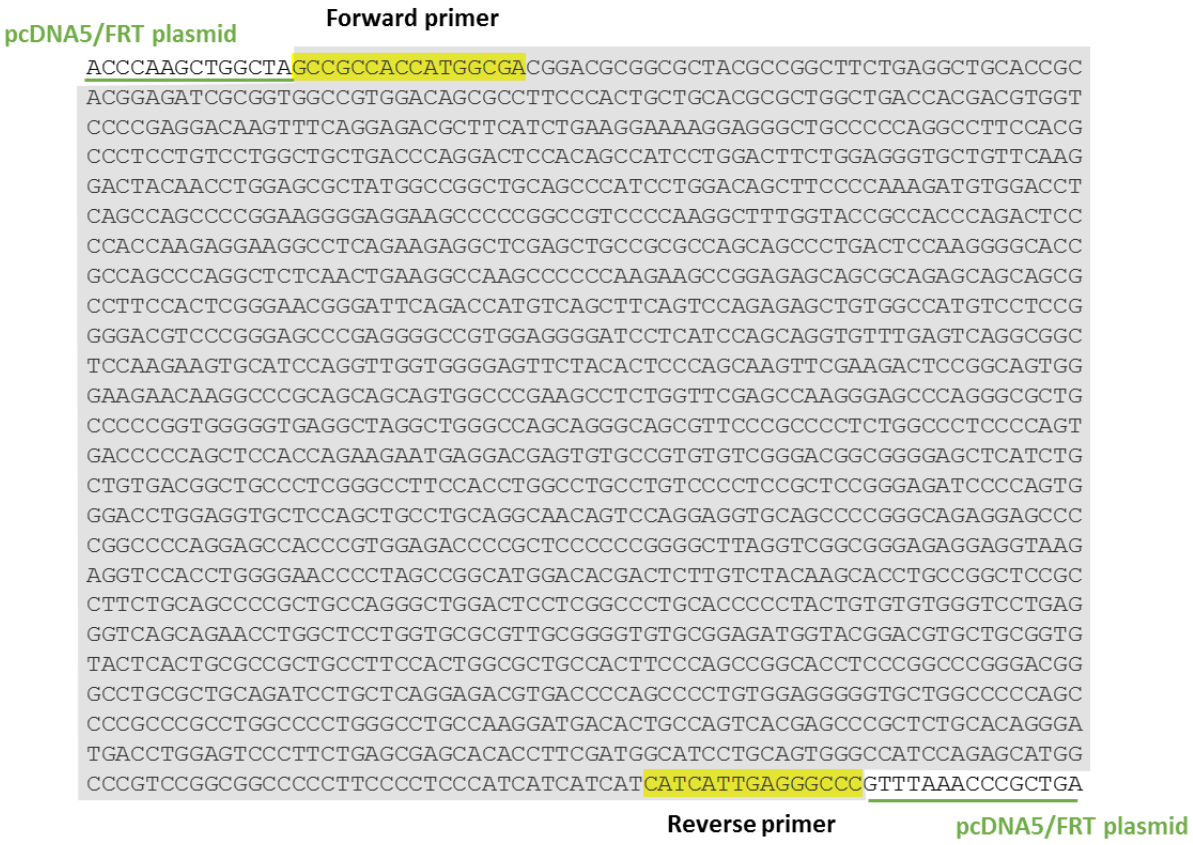



**Figure 2.1: Cloning of human AIRE from pcDNA5/FRT_hAIRE plasmid.** Human AIRE (NM_000383.3) sequence shaded (gray). Forward and reverse primers highlighted (yellow), showing only the hybridise sequences without the restriction sites.

### 2.1.2 Gel electrophoresis

DNA was separated on a 1-3 % (w/v) agarose gel (Bioline) using 1 X TAE buffer. Ethidium bromide (Sigma-Aldrich) 3 µl of 10 mg/ml concentration per 100 ml of gel was added for DNA bands visualisation. A 2-log DNA ladder (New England BioLabs) was run in the same time to provide size markers. Samples were loaded using 6X Loading dye (New England BioLabs). Gels were run using the voltage and duration required for the separation of the required DNA bands. Gels were photographed on ultraviolet transilluminator, UGENIUS system (Syngene).

### 2.1.3 Purification of DNA from Agarose gels

DNA was excised from agarose gels and were extracted using QIAquick™ Gel Extraction Kits (QIAGEN) according to manufacturer's protocol. Briefly, 3x volumes of Buffer QG 1 were added to 1x volume of the gel. The gel slice dissolved at 50°C after 10 minutes incubation. After that, 1 volume gel of Isopropanol (Sigma-Aldrich) was added to the dissolved gel. The solution was applied into 2 ml collection tube, washed with Buffer PE and eluted from the column using 30-50 µl of distilled water.

### 2.1.4 DNA quantification by spectrophotometry

The concentration of eluted samples was determined using the NanoDrop1000 (Labtech), (ND-1000 v3.2.1 software). Samples with ratio of 260/280= 1.8-2.0 were generally accepted as pure (Sambrook *et al.* 1989).

### 2.1.5 Digestion of DNA with restriction endonucleases

Restriction digestion reactions of all PCR products, expression vectors and extracted plasmids were performed as needed. Briefly, DNA was incubated with the appropriate endonucleases and 10X CutSmart™ buffer (New England BioLabs) at 37°C for two hours *[Table 2.2]*.

**Table 2.2: Restriction digestion protocol**

| | |
|---|---|
| **Restriction enzyme** | 10 units (1 µl) |
| **DNA** | 1 µg |
| **CutSmart™ buffer** | 3 µl |
| **Distilled water** | Up to 30 µl |

### 2.1.6 Total RNA extraction and cDNA synthesis

Total RNA was extracted using the TRI Reagent® (Sigma-Aldrich). Tissues were homogenised using mortar and pestle with liquid nitrogen. TRI reagent was added on the powdered tissues and transferred into 1.5 ml Eppendorf tubes for subsequent phase separation. The aqueous phase containing the RNA was separated and transferred to a fresh tube, and then the RNA was precipitated by adding 0.5 ml of isopropanol per 1 ml of TRI reagent. Pelleted RNA was then washed with 75% ethanol and re-suspended in 30 µl of RNase free water. RNA concentration was measured by NanoDrop (*see section 2.1.4*). RNA was reverse-transcribed, and cDNA was synthesized using SuperScript® IV First-Strand Synthesis System kit by random hexamer (18091050, Invitrogen) according the manufacturer's protocol.

### 2.1.7 Real time quantitative PCR

Samples were used for quantitative RT-PCR (qPCR). QPCR reaction consisted of 10 µl *power* SYBR™ Green PCR Master Mix (4367659, Applied Biosystems), cDNA, and 600nM forward and reverse primers *[Table 2.3]* to a total volume of 20 µl, was run in triplicate. The PCR reaction was carried out in 384-well plate using 7900HT Real-time PCR system (Applied Biosystems) following the program:

- 95°C 10 minutes
- 95°C 30 seconds
- 60°C 1 minute

} 40 cycles

- 95°C 1 minute
- 60°C 30 seconds
- 95°C 30 seconds

} Dissociation curve

**Table 2.3: Q-PCR primer sequences**

| cDNA | UCSC code | Primer sequence | Location within cDNA | Size (bp) |
|---|---|---|---|---|
| **Aire** | uc007fww.2 | For 5′CAGCAACTCTGGCCTCAAAG 3′<br>Rev 5′CTTCGAACTTGTTGGGTGTATAA 3′ | 518-802 | 285 |
| **AIRE** | uc062arj.1 | For 5′AGGCAACAGTCCAGGAGGTG 3′<br>Rev 5′TAGGGGTTCCCCAGGTGGAC 3′ | 1755-1885 | 131 |
| **Ccl1** | uc007kmu.1 | For 5′GGCTGCCGTGTGGATACAG 3′<br>Rev 5′AGGTGATTTTGAACCCACGTTT 3′ | 107-325 | 219 |
| **Fabp2** | uc008rex.2 | For 5′GTGGAAAGTAGACCGGAACGA 3′<br>Rev 5′CCATCCTGTGTGATTGTCAGTT 3′ | 359-475 | 117 |
| **IL-3** | uc007ixn.1 | For 5′GGGATACCCACCGTTTAACCA 3′<br>Rev 5′AGGTTTACTCTCCGAAAGCTCTT 3′ | 124-262 | 139 |
| **Ins2** | uc009kog.3 | For 5′GCTTCTTCTACACACCCATGTC 3′<br>Rev 5′AGCACTGATCTACAATGCCAC 3′ | 231-377 | 147 |
| **Spt1** | uc029svn.1 | For 5′CTGGTGAAAATACTGGCTCTGAA 3′<br>Rev 5′AGCAGTGTTGGTATCATCAGTG 3′ | 178-293 | 116 |
| **Csnα** | uc029viy.2 | For 5′ACCTTACTCCCAAAGCTGTCCTTA 3′<br>Rev 5′GAGGGTCCAGTCACATCAAATGT 3′ | 870-1005 | 136 |
| **Apoa1** | uc009phb.3 | For 5′ GGCACGTATGGCAGCAAGAT 3′<br>Rev 5′ CCAAGGAGGAGGATTCAAACTG 3′ | 182-310 | 129 |
| **Fam25c** | uc007tat.1 | For 5′GAGCAGTTCACGCAGTGGAA 3′<br>Rev 5′GCATGGGTAACAGCATCAGTG 3′ | 103-274 | 172 |
| **Ctrb1** | uc009nms.2 | For 5′ATGGCATTCCTTTGGCTTGTG 3′<br>Rev 5′GGATAGCATCCTCTCCGTTGAC 3′ | 19-142 | 124 |
| **Maoa** | uc009ssa.2 | For 5′GCCCAGTATCACAGGCCAC 3′<br>Rev 5′CGGGCTTCCAGAACCAAGA 3′ | 161-277 | 117 |
| **Pitpnc1** | uc011ygq.1 | For 5′CAACCCATCATGTGCTCCTAC 3′<br>Rev 5′CCCGAACATCATCCATTGTCAT 3′ | 1310-1484 | 175 |
| **Riok2** | uc008ape.2 | For 5′TAAGCTGTTCAACAATCCCTCC 3′<br>Rev 5′GCTGCTTGGTAAACACATTGG 3′ | 1730-1855 | 126 |
| **Tmem241** | uc008ebx.2 | For 5′TCTGCACCTGTTACCTGGCT 3′<br>Rev 5′AATGTCTGCCACCCTTGGAAT 3′ | 120-216 | 97 |
| **Cnnm2** | uc008hue.1 | For 5′AAGTGGCCCACCGTGAAAG 3′<br>Rev 5′CGCTTCTACTTCTGTTGCTAGG 3′ | 1951-2078 | 128 |
| **Cpox** | uc007zoa.2 | For 5′ACGGGCGTGTGTTTGAAAAG 3′<br>Rev 5′CACAGAACTTACACCCATAGCAG 3′ | 774-925 | 152 |
| **Mllt11** | uc033hxa.1 | For 5′TAAGTAGCCAGTACAGCTCCTT 3′<br>Rev 5′CGTAGGTAGGTGTATCTGACAGG 3′ | 14-115 | 102 |
| **Ncoa6** | uc008nkm.2 | For 5′GAATGTGCCCAACTTGTTACAC 3′<br>Rev 5′CCCTTCAATCTGAACGGAGAGAA 3′ | 353-536 | 184 |
| **Ppib** | uc009qei.1 | For 5′GGCTCCGTCGTCTTCCTTTT 3′<br>Rev 5′ACTCGTCCTACAGATTCATCTCC 3′ | 155-276 | 122 |
| **Fezf2** | uc007sfs.2 | For 5′ACTCGGCCTTGACAGCTGAACG 3′<br>Rev 5′TGAGCATTGAACACCTTGCCGCAC 3′ | 1063-1183 | 121 |
| **Foxn1** | uc007kjc.2 | For 5′TTCCATCAGTACTCCCCGGGTGG 3′<br>Rev 5′GCGTTGGCCTGGGGTGCAAT 3′ | 831-925 | 95 |
| **β-actin** | uc009ajk.2 | For 5′GGCTGTATTCCCCTCCATCG 3′<br>Rev 5′CCAGTTGGTAACAATGCCATGT 3′ | 193-346 | 154 |

# 2.2 Cloning of DNA Fragments

## 2.2.1 Gateway recombination cloning system

Gateway recombination cloning technology is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda (Landy 1989). This system was used to generate expression plasmids of both Aire and GFP each under either the conventional promoters (pCMV, pPGK, pCAG) or the tissue-specific promoters (pAire, pCsn2, pSrgn). Standard PCR conditions were used to generate *att*B PCR products (Platinum® *Taq* DNA Polymerase High Fidelity, Life Technologies), following the manufacturer's instructions. The cytomegalovirus promoter (CMV) was amplified from pcDNA5™/FRT plasmid (Life Technologies). The Green Fluorescence Protein (GFP), CMV immediate enhancer/β-actin (CAG) promoter (pCAG) and phosphoglycerate kinase promoter (pPGK) plasmids were provided by Prof. Mimoun Azzouz group, SITraN. All constitutive and mammalian tissue-specific promoters were directly PCR amplified from plasmids and the isolated genomic DNA respectively, using the *att*B flanked primers *[Table 2.4]*.

**Table 2.4: List of attB primers to amplify cDNAs and promoters for Gateway® Cloning System**

| Element 1 entry clone | *att*B-flanking site | PCR primers with flanking *att* sites (5'-3') |
|---|---|---|
| **pCMV** | *att*B1 For | GGGGACAAGTTTGTACAAAAAAGCAGGCTTAAGCTACAACAAGGCAAGGCT |
| | *att*B5r Rev | GGGGACAACTTTTGTATACAAAGTTGTACGCTAGCCAGCTTGGGTC |
| **pPGK** | *att*B1 For | GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGTACCGGGTAGGGGAGGCGC |
| | *att*B5r Rev | GGGGACAACTTTTGTATACAAAGTTGTCTCCGGAGGACCTTCGGGCG |
| **pCAG** | *att*B1 For | GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGACATTGATTATTGACTA |
| | *att*B5r Rev | GGGGACAACTTTTGTATACAAAGTTGTAATTCCGCCCGCCGCGCGC |
| **pAire** | *att*B1 For | GGGGACAAGTTTGTACAAAAAAGCAGGCTTATTCTGTCTGTGTAGCCCTGG |
| | *att*B5r Rev | GGGGACAACTTTTGTATACAAAGTTGTTCGGCGCCCCCTGCT |
| **pSrgn** | *att*B1 For | GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCTAGCAGACTCTGGACGTT |
| | *att*B5r Rev | GGGGACAACTTTTGTATACAAAGTTGTCTCCACTCAGCCTCTGGATTA |
| **pCsn2** | *att*B1 For | GGGGACAAGTTTGTACAAAAAAGCAGGCTTATGCTGCCTTGTTTAATGTACCC |
| | *att*B5r Rev | GGGGACAACTTTTGTATACAAAGTTGTAGGTGAAGCTGAAAGGATGATGT |
| **Element 2 entry clone** | ***att*B-flanking site** | **PCR primers with flanking *att* sites** |
| **Aire** | *att*B5 For | GGGGACAAGTTTGTACAAAAAAGCAGGCTTATTCTGTCTGTGTAGCCCTGG |
| | *att*B2 Rev | GGGGACAACTTTTGTATACAAAGTTGTTCGGCGCCCCCTGCT |
| **GFP** | *att*B5 For | GGGGACAACTTTGTATACAAAAGTTGTAGGATCCACCGGTCGCC |
| | *att*B2 Rev | GGGGACCACTTTGTACAAGAAAGCTGGGTTGCATCGAGTCAGGTCAGCTT |

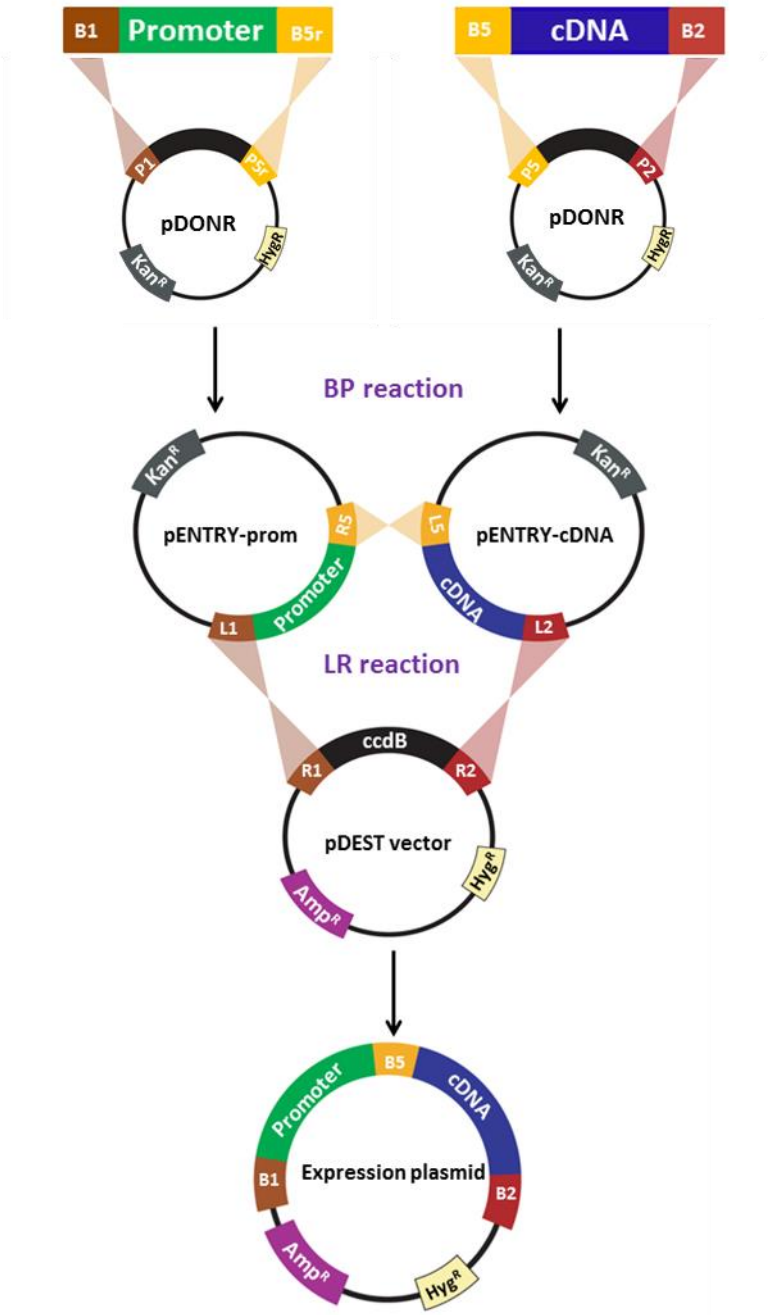



**Figure 2.2: Gateway recombination cloning technology overview.** A schematic diagram describing the Gateway® cloning system used to generate expression vectors. Twelve expression vectors were constructed encoding a promoter upstream of each cDNA. Image recreated from (Boulin, T. *et al*. 2006).

To clean up *att*B flanked PCR products prior to cloning, 30% PEG 8000/30 mM $MgCl_2$ (Life Technologies) purification method was performed on all cDNAs and promoters *att*B-PCR products, according to manufacturer's instructions. A gel electrophoresis was then performed to confirm the presence of the product after purification as described in standard procedures. After purification, a BP reaction was performed to transfer the cDNA/promoter into the appropriate *att*P-containing MultiSite Gateway® Pro Donor vector (Life Technologies) to create entry clones; pDONR™ P1-P5r was used for the promoters, while pDONR™ P5-P2 was used for the cDNAs, following manufacturer's instructions. After BP recombination reaction incubation, the plasmids were then transformed, individual colonies expanded and plasmid extracted (for more information see section 2.2.4). After the generation of the entry clones containing the promoters as elements one and the cDNA as element two, the MultiSite Gateway® Pro LR recombination reaction (Life Technologies) is performed to transfer both elements one and two into the *pJTI™ Fast DEST* vector (Life Technologies) in order to create 12 expression vectors. Two LR recombination reactions were performed for each promoter, one with each Aire or EGFP cDNAs *[Figure 2.1]*. The reaction tubes were incubated overnight under the action of LR Clonase® II Plus enzyme mix (Life Technologies), following manufacturer's instructions. The plasmids were then transformed, individual colonies expanded, and plasmid was extracted (*see section 2.2.4*). Success was determined by restriction enzyme followed by sequencing reaction.

## 2.2.2 Transformation of competent cells

For transformation, competent cells *[Table 2.3]* were thawed on ice. A 100 µl aliquot was incubated with the plasmid DNA for 30 minutes on ice before heat

shock at 42°C in water for 40 seconds, followed by 2 minutes on ice. 250 µl of SOC medium (Thermo Fisher) was added to the mix and incubated for 60 minutes at 37°C in a shaker incubator (225 rpm) before spreading onto a pre-warmed LB agar plate containing 100 µg/ml ampicillin or 50 µg/ml kanamycin. Plates were incubated overnight at 37°C to allow the growth of antibiotic-resistant colonies.

**Table 2.5: Competent cells used for transformation**

| Competent cells | Company |
|---|---|
| **One Shot® Mach1™ T1R** | ThermoFisher Scientific |
| **NEB® 5-alpha Competent E. coli** | New England BioLabs |

## 2.2.3 Isolation of plasmid DNA

The plasmids DNA were extracted using QIAprep Spin Miniprep (QIAGEN, 27106) and QIAGEN Plasmid Plus Mega (QIAGEN, 12981) kits, according to manufacturer's protocol. Briefly, bacterial cells were pelleted and resuspended in 250 µl buffer P1. Buffer P2 was added at 250 µl volume and mixed thoroughly. Buffer N3 was added at 350 µl volume and mixed immediately by inverting the tube 4-6 times. The tube was centrifuged for 10 minutes. The supernatant was then applied into the provided spin columns and centrifuged for 1 minute at 13000 rpm, flow discarded. Washing steps were followed using Buffer PB twice. The Plasmids were then eluted in distilled water. The plasmid DNA concentrations were measured using the Nanodrop1000 (Labtech), (ND-1000 v3.2.1 software). The plasmid purity and insert size were detected after restriction digestion reactions and electrophoresed on 1% agarose gel. The gels were then visualized using the ultraviolet transilluminator.

### 2.2.4 DNA sequence analysis

All isolated cDNA was sequenced to ensure that the fragments are integrated and have no mutations. All sequencing reactions were carried out on 100 ng/µl of each purified DNA using Applied Biosystems 3730 DNA analyser. Core Genomic Facility, University of Sheffield, provided the sequencing service. After confirmation with sequencing reactions, isolated plasmids were stored at -70°C in glycerol (Sigma-Aldrich) with a ratio 1:1.

### 2.2.5 Transfection

The transfection efficiency of the Gateway plasmids was evaluated in HEK293 cells (Human Embryonic Kidney Cell Line). In 6-well plates, 4-6 $x10^5$ cells were seeded and after 24 hours, the cells were transfected with 1 µg of DNA using FuGENE 6® (Promega) at a ratio of 3:1 (3 µl of FuGENE6: 1 µg of DNA), according to manufacturer's instructions. 48 hours post transfection cells were washed with PBS and observed under fluorescence microscope (Leica AF6000LX inverted microscope) for GFP expression. For protein analysis, cells were harvested and incubated in RIPA buffer for protein extraction (*See section 2.4.1*).

## 2.3 Generation of Viral Vectors

### 2.3.1 Viral vector construction

The cDNA encoding for AIRE gene was sub-cloned using appropriate sites in AAV vector backbone (pAAV-CMV-MSC) with ampicillin resistance property [*Figure 2.2*]. Firstly, AIRE was PCR amplified using primers listed in table 2.1. Then, the PCR product was treated with restriction enzymes *Hind*III

and *Nhe*I and subcloned into *Hind*III-*Xba*I digested pAAV-CMV-MSC vector. After ligation, the mixture was transformed into NEB 5- alpha competent E. coli as described in section (2.2.2). The isolated clones were analysed by restriction analysis and the purified plasmid DNA was sequenced as described previously. The bacterial cell stocks which express the AAV-AIRE vector were kept in 30% glycerol at -80°C. They were incubated in LB Broth (LB) (Merck Millipore, 1102850500) with the suitable antibiotic (Carbenicillin, 50µg/ml) (Sigma-Aldrich, C3416). The construct generated was sequenced using the university's Core Genomic Facility.

### 2.3.2 AAV9 viral Production

The AAV9-AIRE viral vector was prepared in-house with help from Ioannis Tsagakis (*see section 2.3.1*). Before large-scale production, a small scale of vector production for AAV9-AIRE vector was performed to test whether the *AIRE* cassette has successfully packaged into the AAV backbone.

Large-scale viral vector stocks of AAV9-AIRE were generated by seeding HEK293T cells (human embryonic kidney cell line immortalized by the adenoviral E1A/E1B protein and expressing the SV40 large T antigen) at a density of $4 \times 10^6$ cells in 20 ml medium per 15 cm petri dish and left to grow overnight. Viral vector stocks were generated using the cationic polymer Polyethylenimine (PEI) 3-plasmid co-transfection method, with 26 µg of the helper plasmid, 13 µg of the packaging plasmid (AAV2/9), and 13 µg of vector plasmid (AAV-AIRE), with a ratio of 2:1:1 respectively. The packaging plasmid AAV2/9 includes the replicase proteins from AAV2 and the capsid serotype of AAV9. Five-days post transfection, the supernatant was collected and treated with Benzonase, 12.5 unit/ml (Sigma), at 37°C for 2-3 hours, with mixing every 30 minutes. Cell debris

were centrifuged at 3850 x g for 3-5 minutes (Sigma 3-16PK, rotor 11180) in 50ml Falcon tubes.

The supernatant was then transferred to the Nalgene filtering unit (0.22 µm vacuum filter – Thermo Scientific Nalgene Rapid-Flow filter unit), and vacuum was applied. Then the clarified viral supernatant was concentrated in Amicon Ultra-15 Centrifugal 100K Filters (Millipore, UFC910024). Viral Supernatant was centrifuged 3800 x g at 4°C and the final concentrated viral supernatant volume achieved was 27-28 ml. To purify the viral particles Iodixanol gradient ultracentrifugation Quick-Seal 39 ml tubes (Beckman Coulter #344326) were used. Different percentage concentrations of Iodixanol solutions (Sigma Aldrich, D1556-250ML), (stock is 60% w/v) were prepared in PBS with 2.5mM KCl (PBS-MK) and 1mM MgCl2 *[Table 2.4]*.

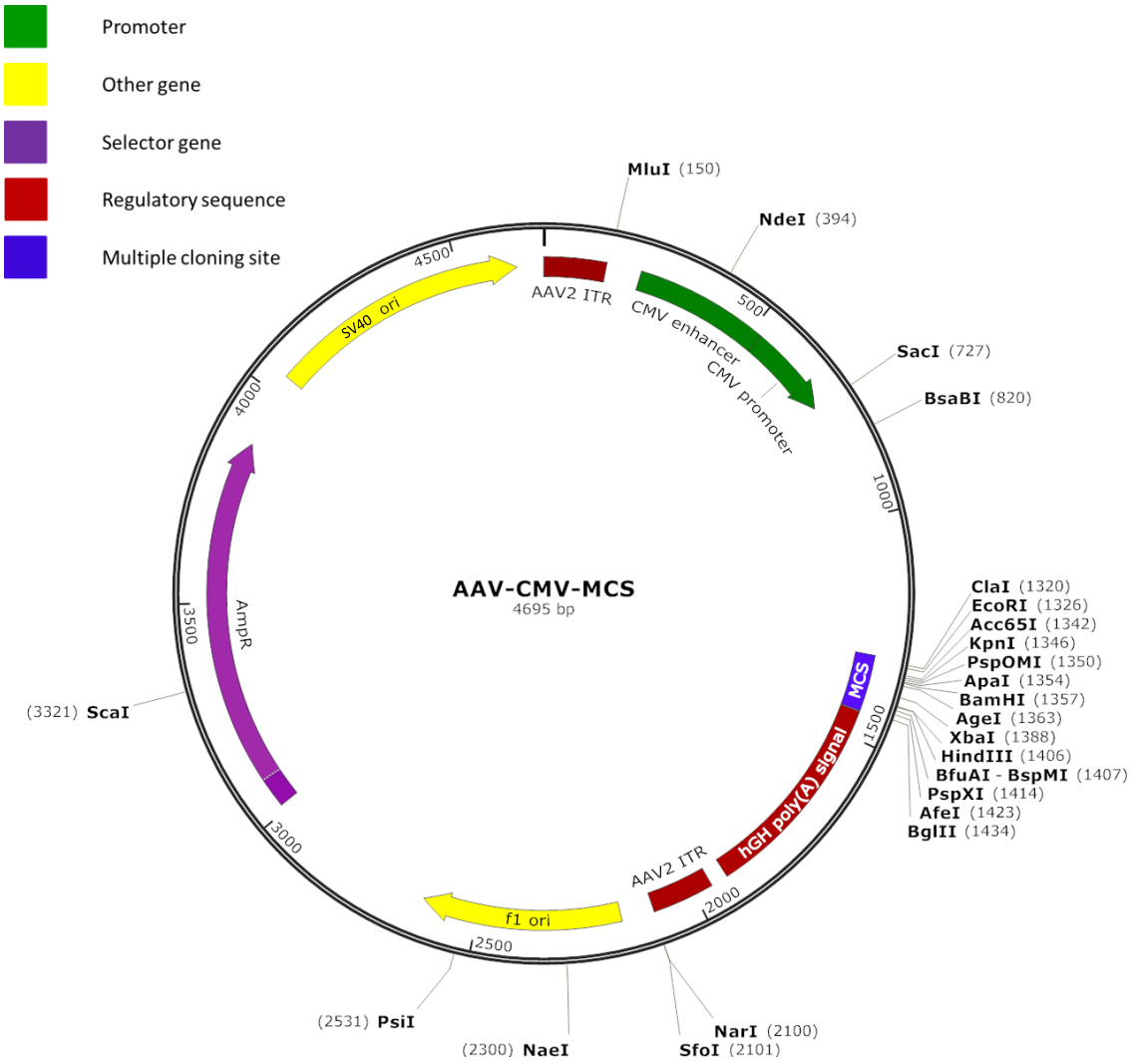



**Figure 2.3: AAV backbone expression viral vector used to generate AAV-AIRE.** The AAV vector was used for all the in vivo experimental work. Abbreviations: AAV – adeno-associated virus, CMV – cytomegalovirus, MCS – multiple cloning site, ITR – inverted terminal repeat, Ori – origin of replication, AmpR – Ampicillin resistance gene.

All the solutions were prepared as follows in 50ml falcon tube:

**Table 2.6: Preparation of Iodixanol solutions**

| Percentage Iodixanol | Iodixanol 60% stock | 5M NaCl | 5X PBS-MK | Water | Phenol Red |
|---|---|---|---|---|---|
| 15% | 12.5 ml | 10 ml | 10 ml | 17.5 ml | - |
| 25% | 20.8 ml | - | 10 ml | 19.2 ml | 100 µl |
| 40% | 33.3 ml | - | 10 ml | 6.7 ml | - |
| 54% | 45 ml | - | - | 5 ml | 100 µl |

Falcon tubes were prepared with the following: one tube with sterile PBS (for topping up the virus layer), another tube with 70% IMS and a tube with water. Using disposable syringes, the Iodixanol solutions were layered cautiously. The tubes were centrifuged in 70Ti rotor (Beckman Coulter) at 69,000 rpm for 80 minutes at 18°C. After centrifugation, the clear fraction of the 40% layer contains the virus. For fractions isolation, the tubes were fixed in a retort stand and a 19-gauge needle was inserted into the top of the tube to introduce air. Another 19-gauge syringe needle was inserted approximately 1 cm from the bottom of the tube with the bevel pointing upwards, and pushed towards the upper portion of the 60% Iodixanol layer. This caused slow outflow of the solution through the needle in a drop-wise fashion. The fractions were collected from the 40% layer in several 1500 µl Eppendorf tubes of approximately 250-500 µl per tube.

To evaluate the purity of the viral preparation, viral samples were run in SDS Polyacrylamide gel electrophoresis (PAGE) gel and stained with SYBRO RUBY (Lonza, 50562). From each fraction 5 µl was diluted with water in a 1:1 ratio, then 4x Laemmli buffer was added and the mixture was heated at 95°C for 5 minutes. The viral samples were loaded onto a 10% SDS-PAGE gel and was run at 120V for 60 minutes. Then the gel was fixed for 20 minutes with 50% methanol / 7% acetic acid prior to staining. Then the gel placed into a plate

containing the SYBRO RUBY stain and wrapped in aluminium foil to protect from light during the staining process. The gel was gently agitated at room temperature for 3 hours. The gel was then washed with a 10% methanol / 7% acetic acid solution for 30 minutes. Before visualisation, the gel was rinsed with water twice for 5 minutes each. The stained gel was visualized using the ultraviolet transilluminator, GENi (Syngene). In addition, to detect the AAV viral proteins (VP), western blot analysis was also performed by using an antibody against the capsid proteins VP1, VP2 and VP3. Fractions containing pure virus were pooled together and were considered as high-quality virus preparation, the other fractions that contains higher bands above the VP1 band were considered as low-quality virus preparations and stored for *in vitro* analysis. The pooled AAV fractions were centrifuging through Amicon Ultra centrifugal filter device, concentrated and desalted in the final buffer PBS/35mM NaCl.

Due to time constraints, AAV9-GFP control viral vector were prepared by CHOP (Philadelphia, USA). The titre of AAV9-AIRE viral vector solutions was determined by qPCR (*see section 2.1.7*) with serial dilutions of the vector plasmid DNA.

### 2.3.3 Transduction of HEK293 with AAV9-AIRE

HEK239T cells contain large T antigen, vectors with SV40 origin of replication initiated by binding of large T antigen to the origin region of the genome to propagate plasmids at high copy number (Ahuja *et al.* 2005). HEK239T cells were plated at a density of $1.5 \times 10^5$ -$2.5 \times 10^5$ cells per well in 2 ml DMEM medium on a 6-well plate and incubated overnight at 37°C in $CO_2$ incubator. The virus volume required for transduction was calculated using the following equation:

$$Volume\ from\ virus\ stock\ (ml) = \frac{multiplicity\ of\ infection\ (MOI) \times no.of\ cells}{viral\ titre}$$

Before adding the viral solution was added into each well, 1 ml of the medium from each well was discarded. After 6 hours incubation, the cells were topped up with 1 ml of DMEM medium and incubated for 5 days. For transduction efficiency analysis, protein was extracted, quantified and analysed as described in section 2.4.

# 2.4 Protein Analysis

## 2.4.1 Preparation of Cell Lysates

Cells were washed with cold PBS and pelleted in 1.5 Eppendorf tubes before adding the Radio Immunoprecipitation Assay (RIPA) buffer which is composed of 50 mM Tris-HCl pH 7.4 (Sigma-Aldrich), 150 mM NaCl (Sigma-Aldrich), 2 mM (ethylenediaminetetraacetic acid) EDTA, 1% Triton x-100 (Sigma-Aldrich), 0.5% Sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS) (Sigma-Aldrich), supplemented with 1% Protease Inhibitor Cocktail (Sigma-Aldrich). Briefly, the cell pellet was suspended in 200-500 µl of RIPA and incubated for 20 minutes on ice to lyse the cells. The lysates were centrifuged at 13,000g 4°C for 10 minutes, and then the supernatant containing the protein was separated from cells debris and collected into a labelled 1.5 Eppendorf tube.

## 2.4.2 Protein Quantification Assay

Bicinchoninic Acid Protein (BCA) assay kit (Thermo Fisher) was used to determine the protein concentration in each sample. As per the manufacturer's guidelines, nine serially diluted protein standards in addition to 10 µl of 1:3 diluted

samples were added separately and in duplicates to the aliquot BCA working reagent. The working reagent was made by adding reagent A to reagent B in a 50:1 ratio, then aliquot into a 96-well plate in a quantity of 100 µl per well. The plate was incubated for 30 minutes at 37 °C. A spectrophotometer plate reader detected the absorbed light at 570 nm. The absorbance values are related to the protein quantity. Based on the equation of the standard curve generated by plotting the absorbance readings, the unknown protein concentrations were determined using the Excel programme (Microsoft).

### 2.4.3 Western Blotting

Before loading the protein, samples were denatured by heating at 95°C for 5 minutes with Laemmli buffer (Sigma-Aldrich), and then loaded onto a 10% SDS-PAGE gel. The gels were electrophoresed in Tris/Glycine/SDS running buffer at 60V for 20 minutes then 60-90 minutes at 120V. For immunodetection, proteins were transferred onto a PVDF membrane (Millipore). The PVDF membrane was soaked in 100% methanol before being immersed in cold transfer buffer. The proteins were transferred onto the PVDF membrane in transfer buffer at 250 mA for 1 hour. Membranes were then blocked in 5% milk TBS-T blocking buffer for 60 minutes at room temperature in a roller (Milton). Primary and secondary antibody solutions were prepared in 5% milk TBS-T blocking buffer. Goat primary antibody against AIRE (sc-17986, SantaCruz Biotechnology) was diluted at 1:2500 and Rabbit primary antibodies against β-actin (ab49900, abcam) and GAPDH (ab181602, abcam) were diluted at 1:3000 and 1:10,000 respectively. All primary antibodies were incubated at 4°C overnight with agitation. HRP (horseradish peroxidase) secondary antibodies, anti-goat-HRP antibody (P0160, DAKO) and anti-rabbit-HRP antibody (7074S, Cell signaling) were used at a

dilution of 1:5000 and 1:10,000 respectively, incubated for 1 hour at room temperature in a roller (Milton). Between the primary and secondary antibodies incubations, the membranes were washed three times for 10 minutes in TBST at room temperature in the roller (Milton) to remove any un-bound antibodies. The proteins were visualized using the ECL Plus chemiluminescence detection kit (GE Healthcare) for HRP. The chemiluminescence was visualized using the G-BOX Image Capture System (G-BOX, Syngene).

### 2.4.4 Cell Lines and Cell Culture

HEK293 cells were used for transfection and transduction assays. HEK293T cells were used for AAV viral production. Dulbecco's Minimum Essential Medium (DMEM with 4.5 g/L Glucose, L-glutamine, Lonza) supplemented with 10% Foetal Bovine Serum (FBS, Lonza) was used to grow the cells. In transduction assays, the medium was supplemented with 100 U/ml of penicillin and 100 µg/ml streptomycin (penicillin G sodium and streptomycin sulphate in 0.85% saline, Invitrogen).

Cells were grown in 37°C incubators in a 5% $CO_2$ atmosphere. Cells were passaged when reaching 70-90% confluence. First, the cells were washed with Dulbecco's phosphate buffered saline (PBS) twice, and then incubated with Trypsin/EDTA for 2-5 minutes. Medium were added in double amount of Trypsin/EDTA used to inhibit its activity. Then, the cells were washed by centrifugation for 5 minutes at 1000 rpm. After discarding the supernatants, the cells were diluted 1:3 up to 1:10 in fresh complete culture media and transferred to new tissue culture flasks.

# 2.5 In-vivo Experimental Methods

## 2.5.1 Breeding and Genotyping of Transgenic Mice

For all *in vivo* studies, B6.129S2-*Aire*$^{tm1.1Doi}$/J (The Jackson Laboratory stock 004743) were used. The homozygote mice lack the murine *Aire* gene. All mice were maintained in a controlled facility in a 12-hour dark-light photocycle with free access to food and water. All *in vivo* experimental work was approved by local ethic committee and performed in accordance to the UK Home Office Animals (Scientific Procedures) Act 1986.

Heterozygotes were used for breeding, and the mice litters were genotyped 2-weeks after birth by PCR. Genotyping was performed using KAPA Mouse Genotyping Kit. (KR0385, KAPA Biosystems). Briefly, DNA extraction was achieved on ear clip in 100 µl solution containing 10X KAPA Express Extract Buffer, 1 U/µl KAPA Express Extract Enzyme and PCR-grade water for 10 minutes at 75°C and inactivated at 95°C for 5 minutes. Three primers were used recognising *Aire* with two different reverse primers specific for either the wild-type gene, or the mutant gene *[Table 2.7]*.

**Table 2.7: Primers used for genotyping**

| Primer | Sequence 5´- 3´ | Concentration |
|---|---|---|
| **Aire wild type reverse** | GGAGACTTGCCTATTCCTGTC | 0.5 uM |
| **Aire Mutant reverse** | CCGGCGGATTTGTCCTAC | 0.5 uM |
| **Aire Forward** | AGACTAGGTGTTCCCTCCCAACCTCAG | 0.5 uM |

The PCR reaction consisted of 1µl of DNA template, 0.5 µM of each of forward primer, wild-type and mutant reverse primers, 12.5 µl of 2X KAPA2G Fast

(HotStart) Genotyping Mix with dye and water to a final volume of 25µl. The PCR program was as follows:

94°C 2 minutes
94°C 20 seconds
65°C 15 seconds -0.5 per cycle decrease } 10 cycles (Touchdown)
68°C 10 seconds
94°C 15 seconds
60°C 15 seconds } 28 cycles
72°C 10 seconds
72°C 2 minutes
10°C forever

The PCR product was electrophoresed on a 3% agarose gel with 0.5µg/µL ethidium bromide at 120V for 60 minutes. Wild-type animals were expected to have one band at 195 bp. Homozygous transgenic animals were expected to have one band at 140 bp and hemizygous carriers were expected to have two bands, one at 195 bp and one at 140 bp. Bands were visualised on.

## 2.5.2 Experimental mice

For the pilot study (Chapter 5), Aire strain (C57BL/6 background) *Aire*$^{+/-}$ littermates were used to assess the transduction efficiency of AAV9-GFP virus, and *Aire*$^{-/-}$ littermates were used for dose-dependent experiment of AAV9-AIRE. For the efficacy study (Chapter 6), Aire strain (C57BL/6 background) *Aire*$^{+/+}$, and *Aire*$^{-/-}$ littermates were used.

### 2.5.3 Viral vector delivery

At 4-week-old the *Aire*$^{-/-}$ mice were injected in the thymus with 20µl of viral vector solution using a 25µl 28-Guage Hamilton syringe (ESS Lab) *[Table 2.8]*. Intrathymic injections were performed under gas anaesthesia (IsoFlo, 100% w/w isoflurane inhalation vapour, liquid, Abbott) using anaesthesia apparatus (Burtons) and they were kept for at least 10 minutes in a 33ºC incubator until they recovered. After recovery, the mice were returned to their cages. Sham control groups (*Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice) were injected with PBS using the same parameters of the viral vector solution injected group.

**Table 2.8: viral vector solutions and PBS used for intrathymic injections**

| Injectate | Titre | Dose |
|---|---|---|
| **AAV9-AIRE** | 4.3 x $10^{13}$ | 1.6 x $10^{10}$ in 20 µl |
| **AAV9-GFP** | 1 x $10^{13}$ | 1 x $10^{10}$ in 20 µl |
| **PBS** | - | 20 µl |

Mice were anaesthetized, placed in a stereotaxic frame (Stoelting), and fur over the ventral thorax was shaved and then skin exposed by applying depilatory cream (Nair) resulting in midline epilation from the base of the neck to mid-chest. Viral vectors were injected intrathymically, each thymic lobe with 10 µl of viral solution performed at a speed of 5 µl/minute.

## 2.6 Histological Analysis

### 2.6.1 Tissue Collection

Mice were euthanized by intraperitoneal injection of 500 mg/kg of sodium pentobarbital (sodium pentobarbital, 20% w/v solution for injections,

JML). Tissues were collected and immediately fixed by 4% Paraformaldehyde (4% PFA) overnight at 4°C. After 24 hours the tissues were transferred to PBS. Tissues were then paraffin embedded and sectioned at 5 µm. To analyse AAV9-GFP transduction efficiency (chapter 5), thymi were embed in OCT and frozen at -80°C. After sectioning, 1 in 10 slides were taken throughout the thymus for analysis. This equated to 15 thymic sections analysed per animal.

### 2.6.2 Immunofluorescence

For Aire staining in APS-1 mouse model thymus, sections were incubated in xylene twice for 10 minutes before being hydrated through 100%, 95% and 70% ethanol. Sections were then washed in water before being incubated in 3% hydrogen peroxide in methanol for 20 minutes to block endogenous peroxidase. Heat induced antigen retrieval was performed on all slides using the pressure cooker method with pH 6.0 Citrate buffer solution. Sections were then washed in water for 1 minute followed by PBS for 5 minutes. Sections were then incubated with protein block serum-free buffer (DAKO) for 10 minutes at room temperature to block any non-specific binding followed by 10 minutes incubation with 0.3% Triton x-100 in PBS for permeabilization. Sections were then incubated with a polyclonal goat antibody against Aire AIRE D-17 (sc-17986, SantaCruz Biotechnology) at a dilution of 1:50 in 0.15% Triton x-100 in PBS for 1 hour at room temperature or overnight at 4°C. Sections were then washed three times 10 minutes each with PBS before secondary antibody was added. Secondary antibody used was Alexa Fluor 488 donkey anti-goat IgG antibody (Invitrogen), was diluted 1:500 in PBS and incubated at room temperature for 1 hour before being washed in PBS three times for 10 minutes. For GFP visualisation in AAV9-

GFP thymic sections, slides were fixed with acetone for 20 minutes. All slides were mounted using VECTASHIELD Anti-fade Mounting Medium with DAPI (Vector Labs) and allowed to be cured overnight at room temperature. Images were taken with a fluorescence microscope (Leica AF6000) and were analysed using ImageJ software. Confocal microscope images were taken using Nikon A1 confocal microscope system.

*CHAPTER THREE*

# APS-1 MOUSE MODEL CHARACTERISATION

# 3. APS-1 MOUSE MODEL CHARACTERISATION

## 3.1 Outline

The aim of this chapter is to assess the expression of Aire in APS-1 mouse model (Anderson *et al.* 2002). Several studies showed that Aire regulation of TSAs is dose-dependent and decreased expression of Aire predispose to autoimmunity (Liston *et al.* 2004, Kont *et al.* 2008, Su *et al.* 2008). Thus, the relationship between gender, age and Aire expression level was investigated in this part of the study. In addition, characterising the development of autoimmunity in APS-1 mouse model up to 12-week-old using molecular and histological techniques was performed. This will determine the optimal conditions for *in vivo* evaluation of APS-1 mouse model before commencing the efficacy study.

Male and female mice of both $Aire^{-/-}$ and $Aire^{+/+}$ genotypes were examined at two different ages, pre-symptomatic (4-week-old, $n$=8) and at first signs of disease appearance (12-week-old, $n$=8). Using histological and molecular techniques, the results in this part of the study will identify potential readouts for *in vivo* gene therapy proof-of-concept efficacy studies (*see chapter 6*).

## 3.2 Choice of APS-1 mouse model

B6.129S2-*Aire*$^{tm1.1Doi}$/J mice, a well stablished APS-1 model (Anderson et al. 2002), were purchased from the Jackson Laboratory (USA). Benoist and Mathis group engineered this line of APS-1 mouse model carrying a defective Aire gene that represents the most common Finnish APS-1 mutation, R257X, by introducing lox/Cre-mediated recombination in embryonic stem (ES) cells. These mice carry in homozygous state a mutant allele bearing a deletion of exon 2 and portions of the upstream and downstream introns (Anderson *et al.* 2002).

Two breeding pairs of heterozygous Aire (*Aire*$^{+/-}$) mice were purchased from Jackson laboratory (USA); genotyping was performed on them in order to establish genotyping protocol in our lab and to confirm the genotyping results of the breeding pairs *[Figure 3.1A]*. The first litter of Aire mice were genotyped, and mice expressed both bands at sizes 140 bp and 195 bp similar to the breeding pairs were considered as heterozygous (*Aire*$^{+/-}$). Mice expressed only the larger band at size 195 bp were referred to as wild types (*Aire*$^{+/+}$), while the mouse expressed the mutant smaller band at size 140 bp was considered as homozygous (*Aire*$^{-/-}$) *[Figure 3.1B]*. Faint bands were discounted when one band is more intense than the other in the same sample, this common contamination products were observed in several occasions.

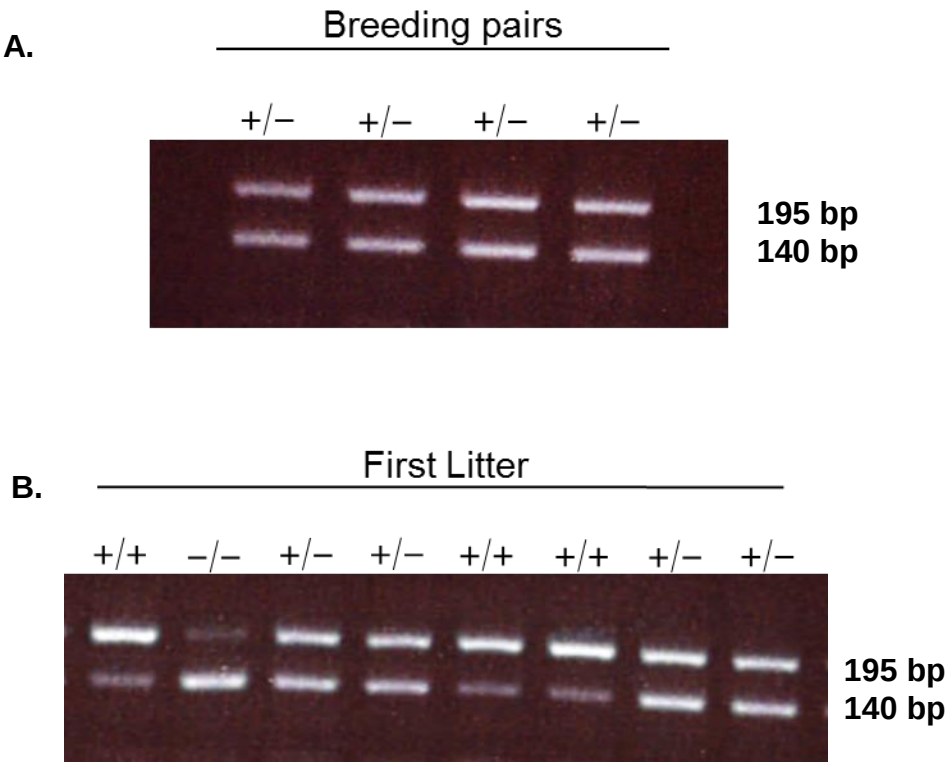



**Figure 3.1 Genotyping of Aire mice**. DNA was extracted from ear clips of 3-week-old mice. Genotyping was performed by Hot Start PCR amplification. (**A**) Genotype of the heterozygous breeding pairs was used as a control for genotyping the litters. The Heterozygous genotyping shows both two bands sizes at 140 bp and 195 bp. (**B**) Genotype of the first litter shows four heterozygous (+/-) two bands sizes at 140 bp and 195 bp (lanes 3,4,7 and 8), one Aire knockouts (-/-) band at size 140 bp (lane 2) and three wild types (+/+) bands at size 195 bp (lanes 1,5 and 6).

# 3.3 Aire expression pattern in APS-1 mouse model

## 3.3.1 Aire expression in the thymus

To confirm the expression pattern of Aire in the thymus of *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice, thymi were collected from 4-week-old and 12-week-old mice for histological analysis. Using immunofluorescence (IF) staining on 4-week-old *Aire*$^{+/+}$ (*n* = 3) and *Aire*$^{-/-}$ (*n* = 3) thymic sections, a subset of medullary cells were found to be positive for Aire in *Aire*$^{+/+}$ but not *Aire*$^{-/-}$ *[Figure 3.2]*. For subcellular location, Aire is localised to the nucleus in distinct punctate nuclear dot structures within a subset of thymic cells confirming previous data (Blechschmidt *et al.* 1999, Heino *et al.* 1999, Halonen *et al.* 2001) *[Figure 3.3]*.

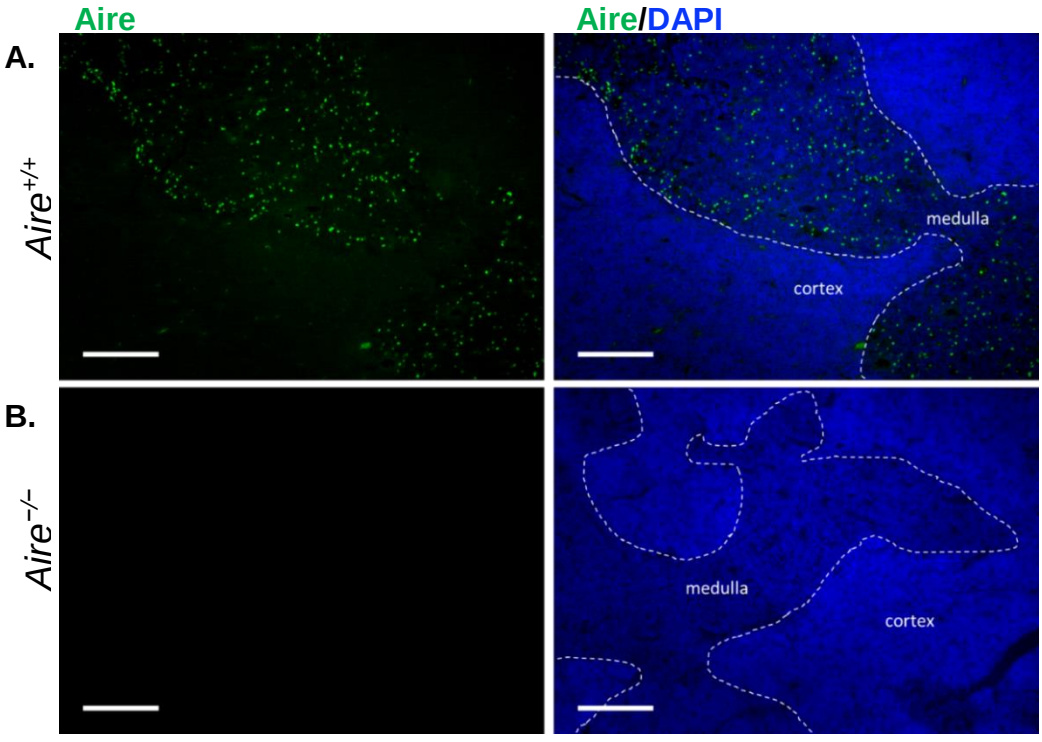



**Figure 3.2: Aire expression in the thymus.** Thymic sections from Aire mice. The sections labelled with Aire antibody coupled to Alexa Fluor 488 (green) and nuclei counterstained with DAPI (blue) (A, B). (A) Thymic section from wild type Aire mouse (*Aire*$^{+/+}$). (B) Thymic section from homozygote Aire mouse (*Aire*$^{-/-}$). Scale bar 200μm.

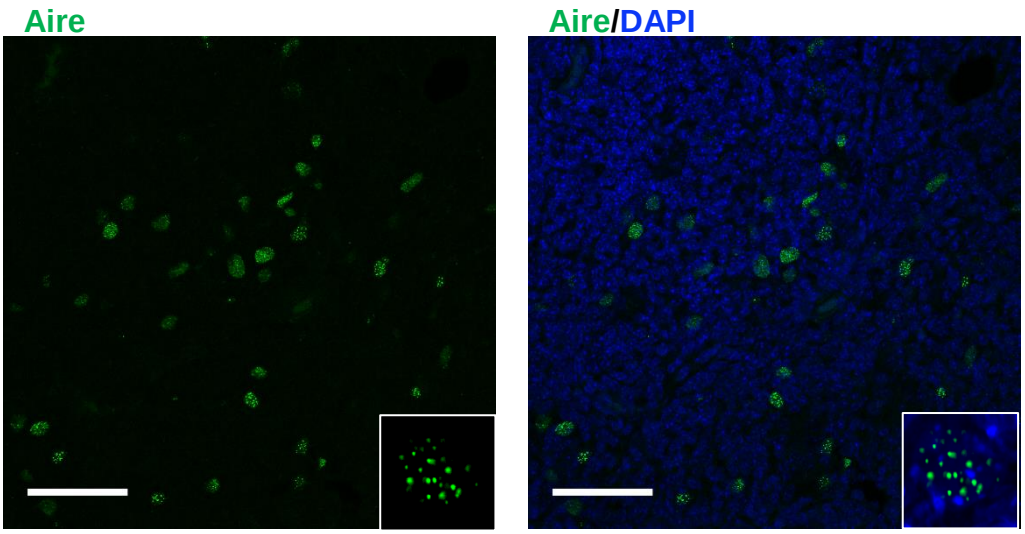



**Figure 3.3: Subcellular localisation of Aire.** Thymic sections from *Aire*$^{+/+}$ mice. Aire is expressed in the nucleus in punctate pattern within thymic cells. The sections were labelled with Aire antibody and nuclei counterstained with DAPI. Scale bar 50μm.

To assess Aire protein expression, we performed immunofluorescence staining of thymic sections from APS-1 mice. Aire positive cells (Aire+) in the thymic sections of APS-1 mice were counted to compare the expression levels of Aire between males and females, and to assess the expression level in two age groups, 4-week-old (*n*=4) and 12-week-old mice (*n*=4). Randomly chosen, non-overlapping 5 fields per thymic section per mouse were counted for Aire+ cells at x200 magnification. On average, the number of Aire+ cells in male thymic sections was 163 cells, while the average number of Aire+ cells in female thymic sections was 95 cells, at 4-week-old *Aire*$^{+/+}$ mice *[Figure 3.4]*. Thus, male thymic sections showed a significant increase in Aire+ cells compared to females, *P*= 0.0022. In 12-week-old *Aire*$^{+/+}$ mice, thymic sections showed a slight reduction in Aire+ cells in both male and female. The average number of Aire+ cells in 12-week-old *Aire*$^{+/+}$ male thymic sections was 127

cells, while the average number of Aire+ cells in 12-week-old *Aire*$^{+/+}$ female thymic sections was 60 cells. Although 12-week-old *Aire*$^{+/+}$ male thymic sections exhibited lower numbers of Aire+ cells than 4-week-old *Aire*$^{+/+}$ male thymic sections, this was not significant. On the other hand, the number of Aire+ cells in 12-week-old *Aire*$^{+/+}$ female thymic sections was significantly lower when compared to 4-week-old *Aire*$^{+/+}$ female thymic sections, *P*= 0.0438 *[Figure 3.4A]*.

At mRNA level, *Aire* expression was determined by qPCR and normalized to beta actin (β-actin), a housekeeping gene. In male thymi, *Aire* mRNA expressed at higher levels compared with *Aire* mRNA expression levels in female thymi in both age groups *[Figure 3.4B]*. At 4-week-old *Aire*$^{+/+}$ mice, female *Aire* was lower than male *Aire* but not significant. However, at 12-week-old female *Aire*$^{+/+}$ mice, *Aire* levels were significantly lower in females compared to males, *P*= 0.0049. Moreover, 12-week-old *Aire*$^{+/+}$ females displayed significantly lower Aire mRNA levels when compared to *Aire*$^{+/+}$ 4-week-old females, *P*= 0.0121. In *Aire*$^{+/+}$ males, however, *Aire* expression levels did not significantly change over these two age groups.

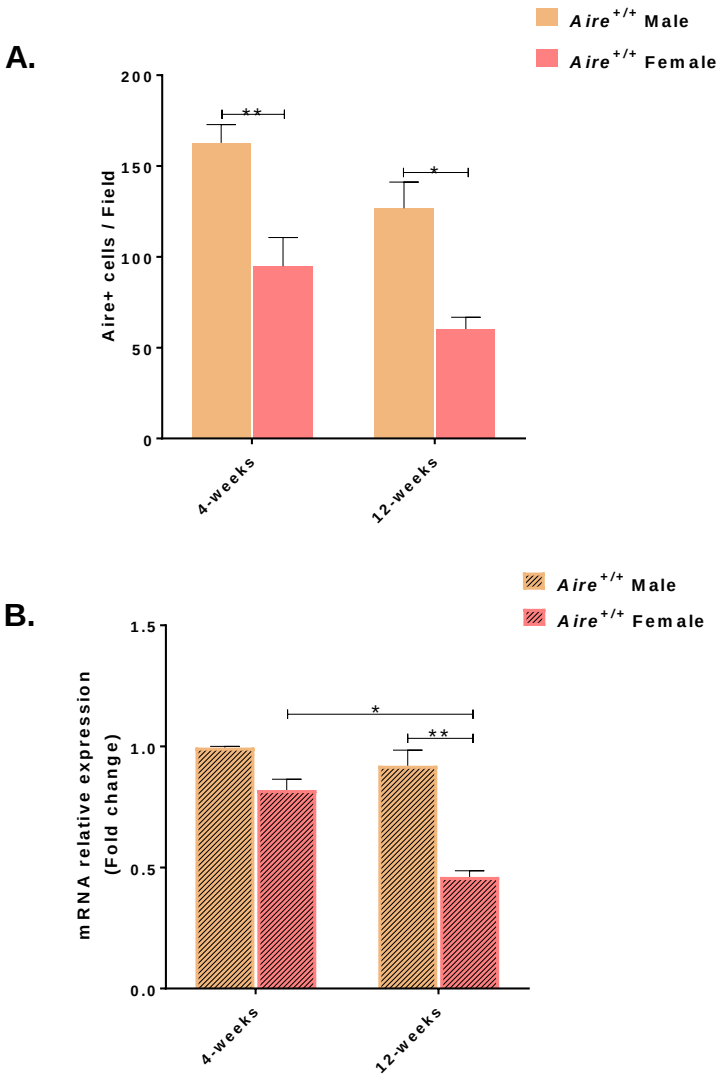



**Figure 3.4: Aire expression in *Aire*$^{+/+}$ thymi in males and females mice.** (A) Semi-quantification of Aire+ cells in thymic sections from 4-weeks and 12-weeks old *Aire*$^{+/+}$ mice. Random fields per thymic section were counted, $n = 5$ per thymic section microscopic field (x20) for males and females, $n = 2$ mice per group. (B) *Aire* gene expression in thymi from 4-weeks and 12-weeks old males and females. Data analysed with two-way ANOVA, Tukey's multiple comparisons test, $p < 0.05$. Error bars represent ±SEM.

# 3.4 Tissue-specific antigen expression in the thymus

## 3.4.1 Expression profile of Aire-dependent and Aire-independent genes

To explore the effect of Aire on downstream genes, a subset of Aire-dependent and Aire-independent genes, thymi were collected from *Aire*$^{+/+}$ (*n* = 2) and *Aire*$^{-/-}$ (*n* = 2) littermates for thymic *ex vivo* mRNA measurements. Three groups of TSAs genes were selected, Aire-dependent upregulated genes, Aire-dependent downregulated genes and Aire-independent genes *[Table 3.1]*. These TSAs were selected based on their expression levels in C57BL/6 mice at 3-4 weeks old, Affymetrix Mouse Genome 430 2.0 data (NCBI GEO accession no. GSE8564.) (Venanzi *et al.* 2008).

In this part of the study, qPCR results showed that 9 TSAs exhibited higher expression levels in *Aire*$^{+/+}$ mice, Aire-dependent upregulated TSAs. On the other hand 6 TSAs exhibited higher expression levels in *Aire*$^{-/-}$ mice, Aire-dependent downregulated TSAs. Thus, absence of Aire does has an effect on Aire-dependent groups with a coordinated pattern in each group.

In Aire-dependent upregulated genes, all nine genes tested demonstrated a substantial decreased expression levels in absence of *Aire* in 4-week-old *Aire*$^{-/-}$ compared to *Aire*$^{+/+}$ mice ranged from 3-14 decreased fold change. *Fam25c* displayed a 14 folds reduction in *Aire*$^{-/-}$ when compared to its level of expression in *Aire*$^{+/+}$ mice.

**Table 3.1: List of Aire-dependent and Aire-independent genes**

| Pattern of expression | Symbol | Full name | References |
|---|---|---|---|
| **Aire-dependent upregulated** | CCL1 | Chemokine (C-C motif) ligand 1 | (Venanzi *et al.* 2008) |
| | Fabp2 | Fatty acid-binding protein 2 | |
| | IL-3 | Interleukin 3 | |
| | Ins2 | Insulin II | |
| | Spt1 | Salivary protein 1 | |
| | Csnα | Casein α | |
| | Apoa1 | Apolipoprotein A-I | |
| | Fam25c | Family with sequence similarity 25 C | |
| | Ctrb1 | Chymotrypsinogen B1 | |
| **Aire-dependent downregulated** | Dnmt3l | DNA methyltransfer-3-like | (Venanzi *et al.* 2008) |
| | Maoa | Monoamine oxidase A | |
| | Pitpnc1 | Phosphatidylinositol transfer protein cytoplasmic 1 | |
| | Riok2 | RIO kinase 2 | |
| | Tmem241 | Transmembrane Protein 241 | |
| | Cnnm2 | Cyclin M2 | |
| **Aire-independent** | Cpox | Coproporphyrinogen oxidase | (Venanzi *et al.* 2008) |
| | Ddx21 | DExD-box helicase 21 | |
| | Mllt11 | Mixed-lineage leukemia translocated to 11 | |
| | Morf4l1 | Mortality factor 4 like 1 | |
| | Ncoa6 | Nuclear Receptor Coactivator 6 | |
| | Ppib | Peptidylprolyl Isomerase B | |
| | Stx6 | Syntaxin 6 | |
| | Wasf2 | WAS Protein Family Member 2 | |

*CCL1*, a chemokine secreted by T cells that plays a role in lymphocyte recruitment in bronchial asthma, showed a decline by almost 4 folds In *Aire*$^{-/-}$ compared to its expression level in *Aire*$^{+/+}$ mice. *Ins2* and *Spt1* expression levels decreased by 5 folds, *IL3* exhibited the same pattern. Moreover, *Csnα*, *Ctrb1* and *Fabp2* expression levels dropped by 7 folds because of Aire depletion. *Apoa1*, a principal protein component of high-density lipoprotein (HDL), was reduced by almost 3 folds in *Aire*$^{-/-}$ mice.

In Aire-dependent downregulated TSAs genes, *Dnmt3l*, an epigenetic factor that contributes to the establishment of DNA methylation and histone

modifications (Chédin *et al.* 2002), showed a high expression levels in *Aire*$^{-/-}$ mice with almost 12 fold change when compared to its expression levels in *Aire*$^{+/+}$ mice. *Maoa*, a key regulator for normal brain function (Brunner *et al.* 1993, Caspi *et al.* 2002), and *Cnnm2*, an important player in magnesium homeostasis (Stuiver *et al.* 2011), were highly expressed in *Aire*$^{-/-}$ with 2.5 fold increase and 2 folds increase, respectively. *Pitpnc1*, *Riok2* and *Tmem241* expression levels in *Aire*$^{-/-}$ were increased almost up to 1.3 folds when compared to their expression levels in *Aire*$^{+/+}$ mice.

Aire-independent TSAs genes analysis revealed that in presence or absence of Aire, their level of expression in the thymus behave in an Aire-independent manner (Venanzi *et al.* 2008). *Cpox*, *Mllt11*, *Ncoa6* and *Ppib* expression levels ranges from 0.85-1.38 fold change in *Aire*$^{-/-}$ mice when compared to their expression levels in *Aire*$^{+/+}$ mice with no significant differences.

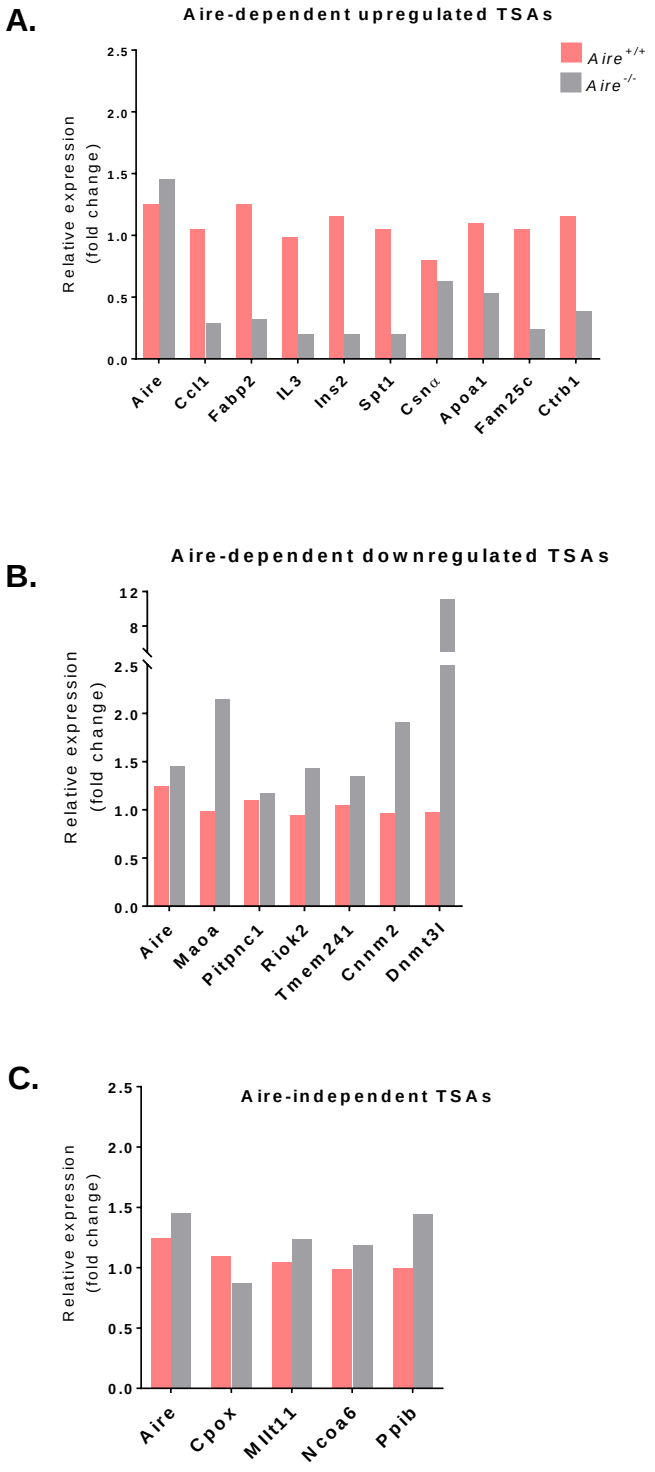



**Figure 3.5: TSAs expression pattern in *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice.** Quantitative PCR analysis of expression of 17 Aire-dependent TSAs and 5 Aire-independent TSAs in thymic cells obtained from 4 week old *Aire*$^{+/+}$ mice ($n$ = 2), *Aire*$^{-/-}$ mice ($n$ = 2). Results are normalized to the expression of *β-actin* and are presented relative to the expression values in *Aire*$^{-/-}$ thymic cells. TSAs full names listed in table (3.1).

## 3.5 Pathology in Aire-deficient mice

Phenotypically, the *Aire*$^{-/-}$ mice did not differ from the *Aire*$^{+/+}$ littermates. Therefore, tissue histology was investigated in 4- and 12-week-old *Aire*$^{-/-}$ and their *Aire*$^{+/+}$ littermates with special attention to eye, lung, liver, stomach and reproductive organs based on previous studies (Anderson *et al.* 2002, Ramsey *et al.* 2002, Gavanescu *et al.* 2007). In 4-week-old mice, no observable infiltrations were detected in the tissues *[Figure 3.6 and 3.7]*. Thus, consistent with previous studies, there was not any difference between *Aire*$^{-/-}$ and their *Aire*$^{+/+}$ littermates at 4-week-old (Anderson *et al.* 2002, Ramsey *et al.* 2002).

In the 12-week-old *Aire*$^{+/+}$ mice, no observable infiltrations were detected in their tissues. However, in 12-week-old *Aire*$^{-/-}$ mice, all 4 *Aire*$^{-/-}$ exhibited mild infiltrates in certain, but not all, investigated organs. For instance, moderate infiltration was detected in the lung of *Aire*$^{-/-}$ *[Figure 3.6]*. In the liver and stomach, mild infiltrates were detected in *Aire*$^{-/-}$ *[Figure 3.7 and 3.8].* Moreover, the testis of 12-week-old *Aire*$^{-/-}$ mouse exhibited a Leydig cells hyperplasia (LCH) morphology suggestive of autoimmune gonadal atrophy.

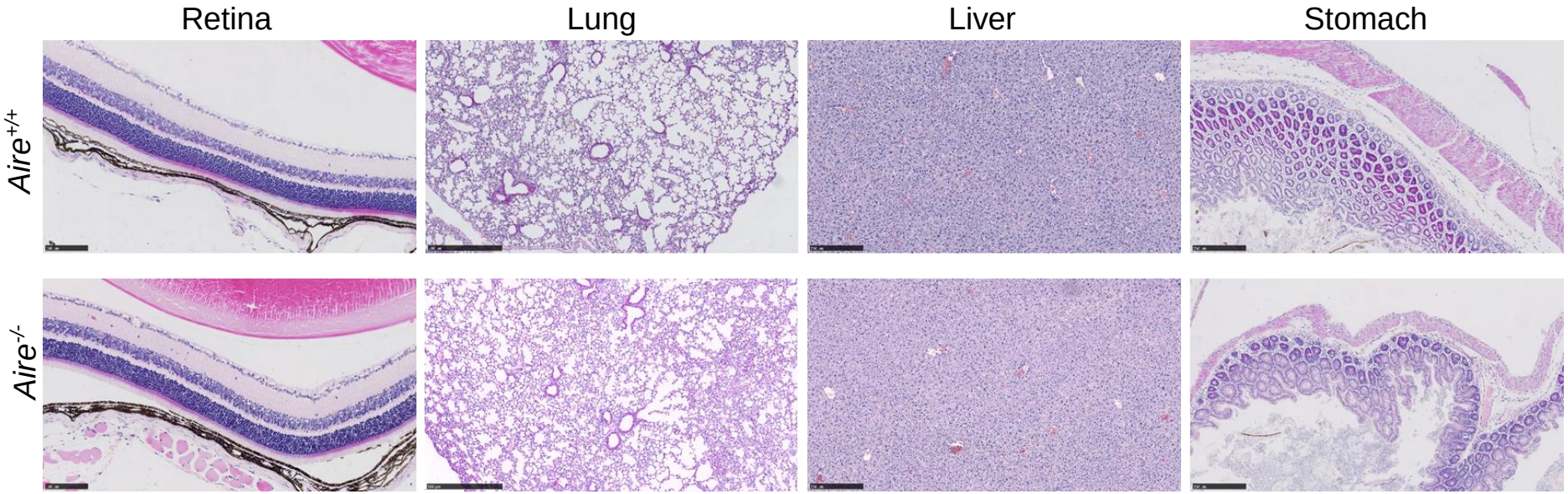



**Figure 3.6: Representative tissue sections from 4-week-old *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice.** Retina, lung, liver and stomach sections from *Aire*$^{+/+}$ (upper lane) and *Aire*$^{-/-}$ (lower lane) stained with haematoxylin and eosin (H&E). Scale bar: retina 100 μm, lung 500 μm, liver and stomach 250 μm.

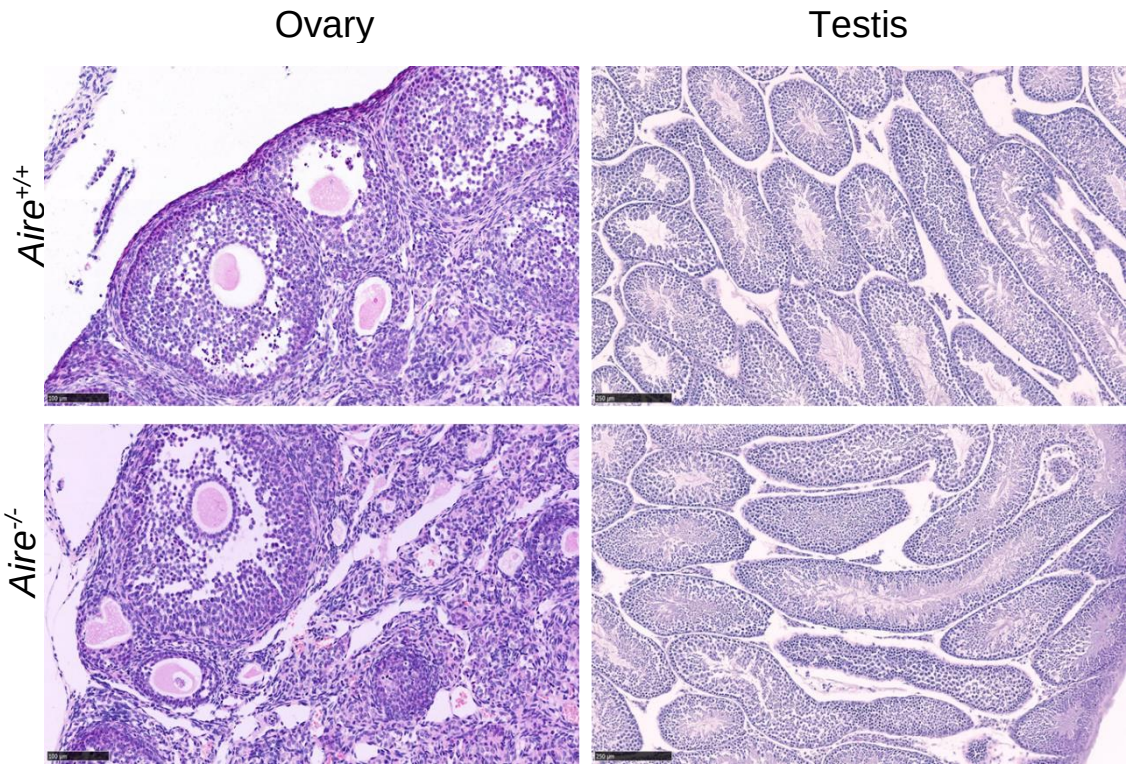



**Figure 3.7: Representative tissue sections from 4-week-old *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice.** Ovary and testis sections from *Aire*$^{+/+}$ (upper lane) and *Aire*$^{-/-}$ (lower lane) stained with haematoxylin and eosin (H&E). Scale bar: ovary 100 μm and testis 250 μm.

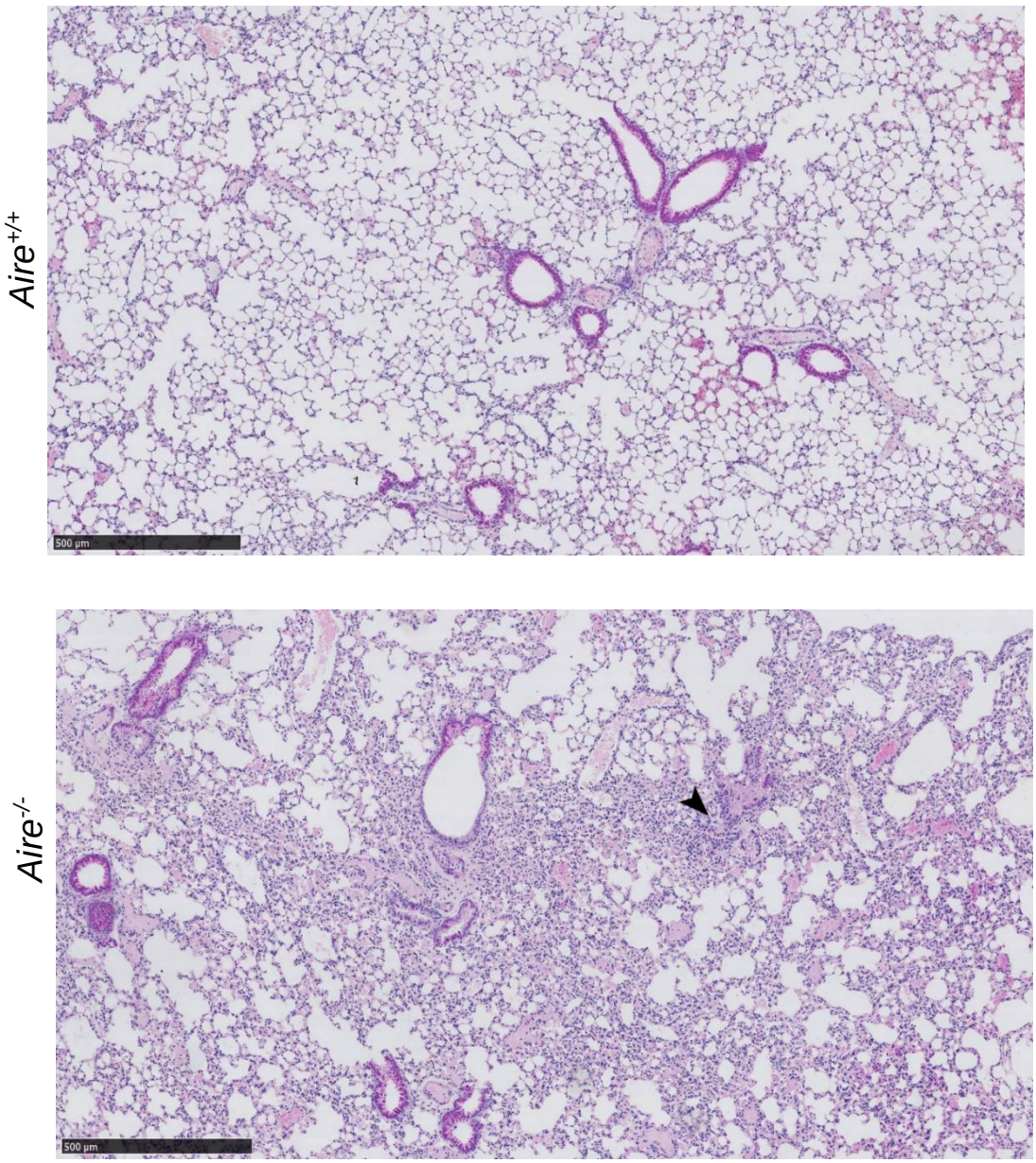



**Figure 3.8: Representative tissue sections from the lung of 12-week-old *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice.** Lung sections from *Aire*$^{+/+}$ (upper lane) and *Aire*$^{-/-}$ (lower lane) stained with haematoxylin and eosin (H&E). Infiltration in *Aire*$^{-/-}$ lung (arrowhead). Scale bar 500 µm.

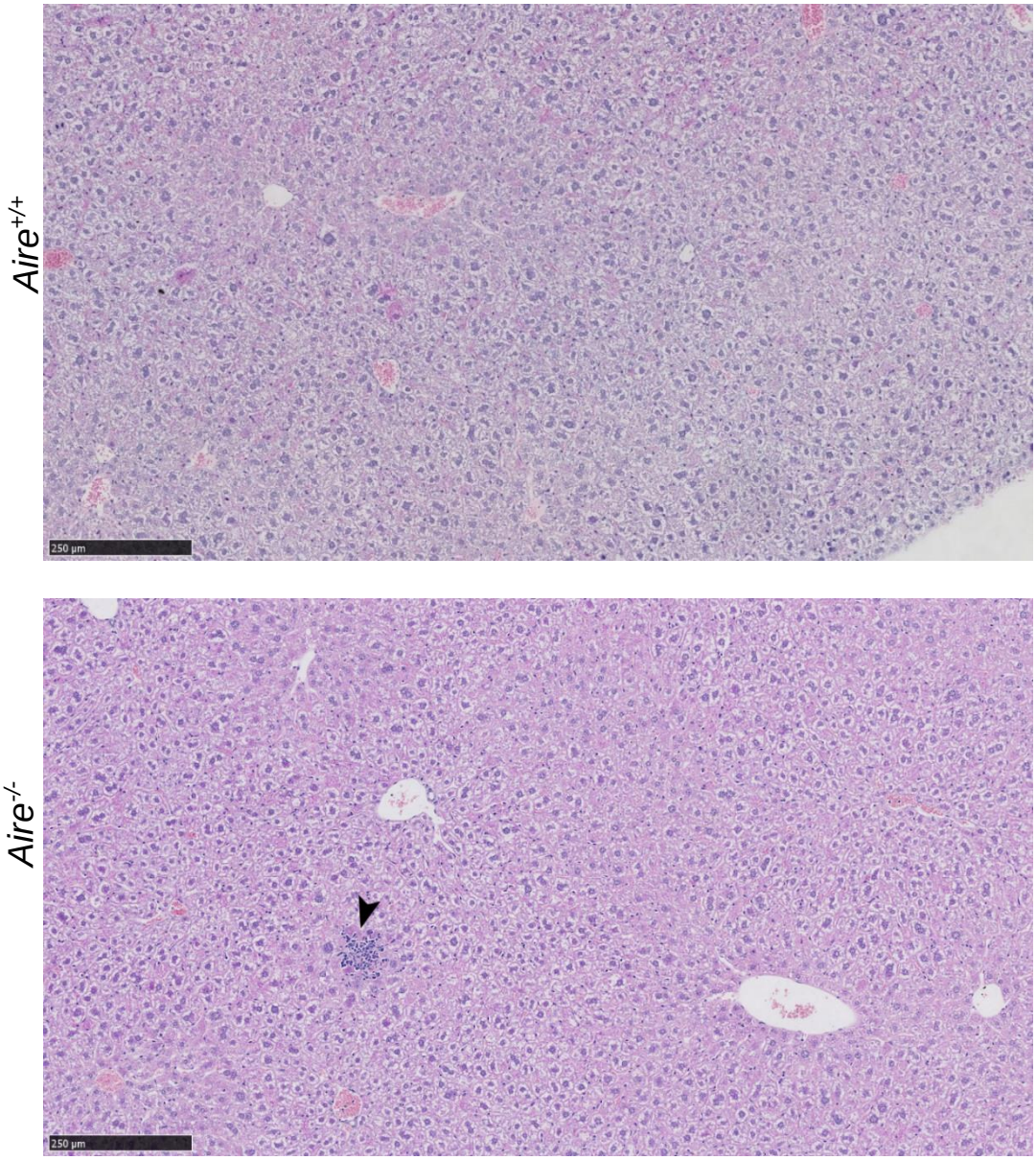



**Figure 3.9: Representative tissue sections from the liver of 12-week-old *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice.** Liver sections from *Aire*$^{+/+}$ (upper lane) and *Aire*$^{-/-}$ (lower lane) stained with haematoxylin and eosin (H&E). Infiltration in *Aire*$^{-/-}$ liver (arrowhead). Scale bar 250 µm.

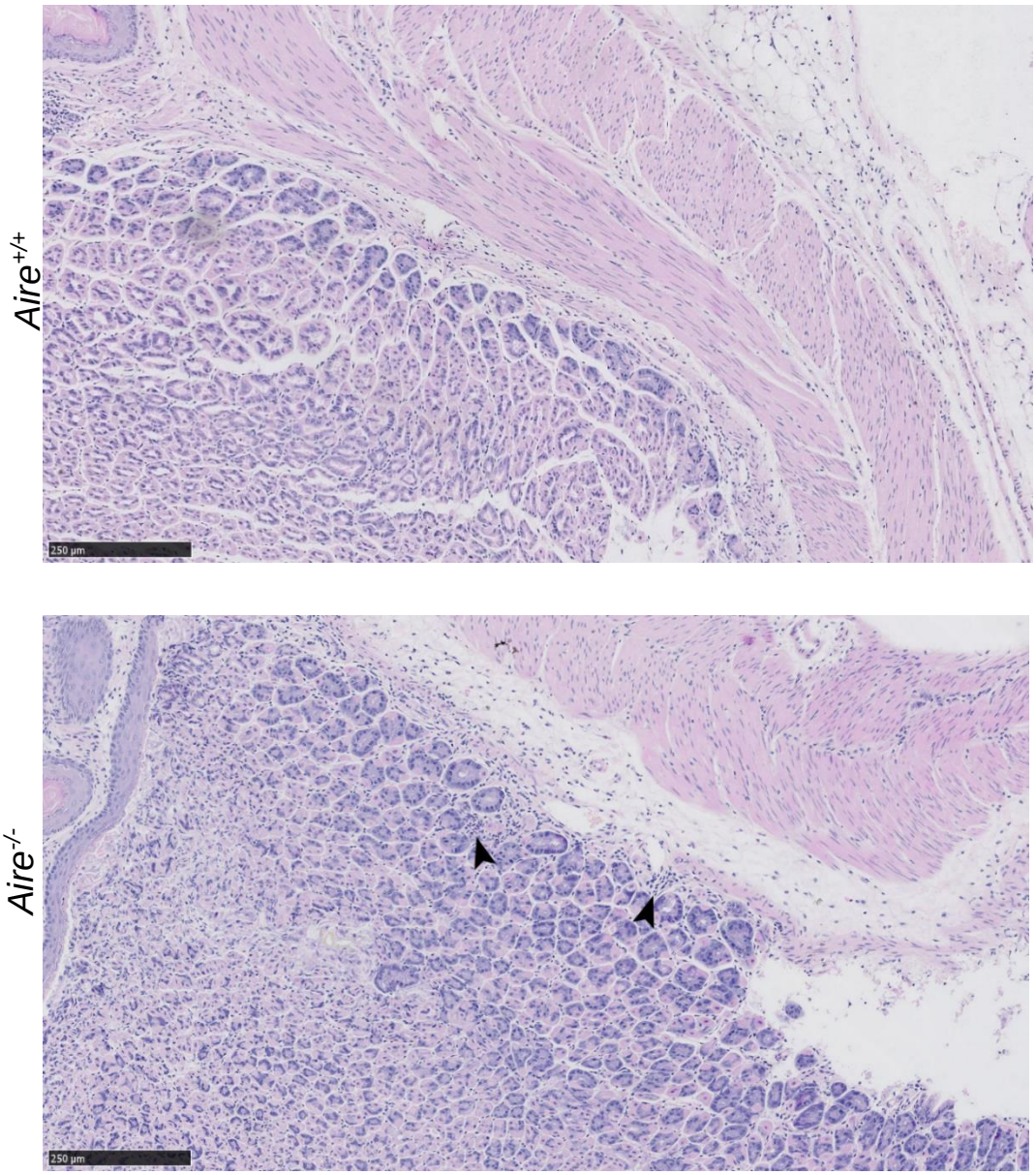



**Figure 3.10: Representative tissue sections from the stomach of 12-week-old *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice.** Stomach sections from *Aire*$^{+/+}$ (upper lane) and *Aire*$^{-/-}$ (lower lane) stained with haematoxylin and eosin (H&E). Infiltration in *Aire*$^{-/-}$ stomach (arrowhead). Scale bar 250 µm.

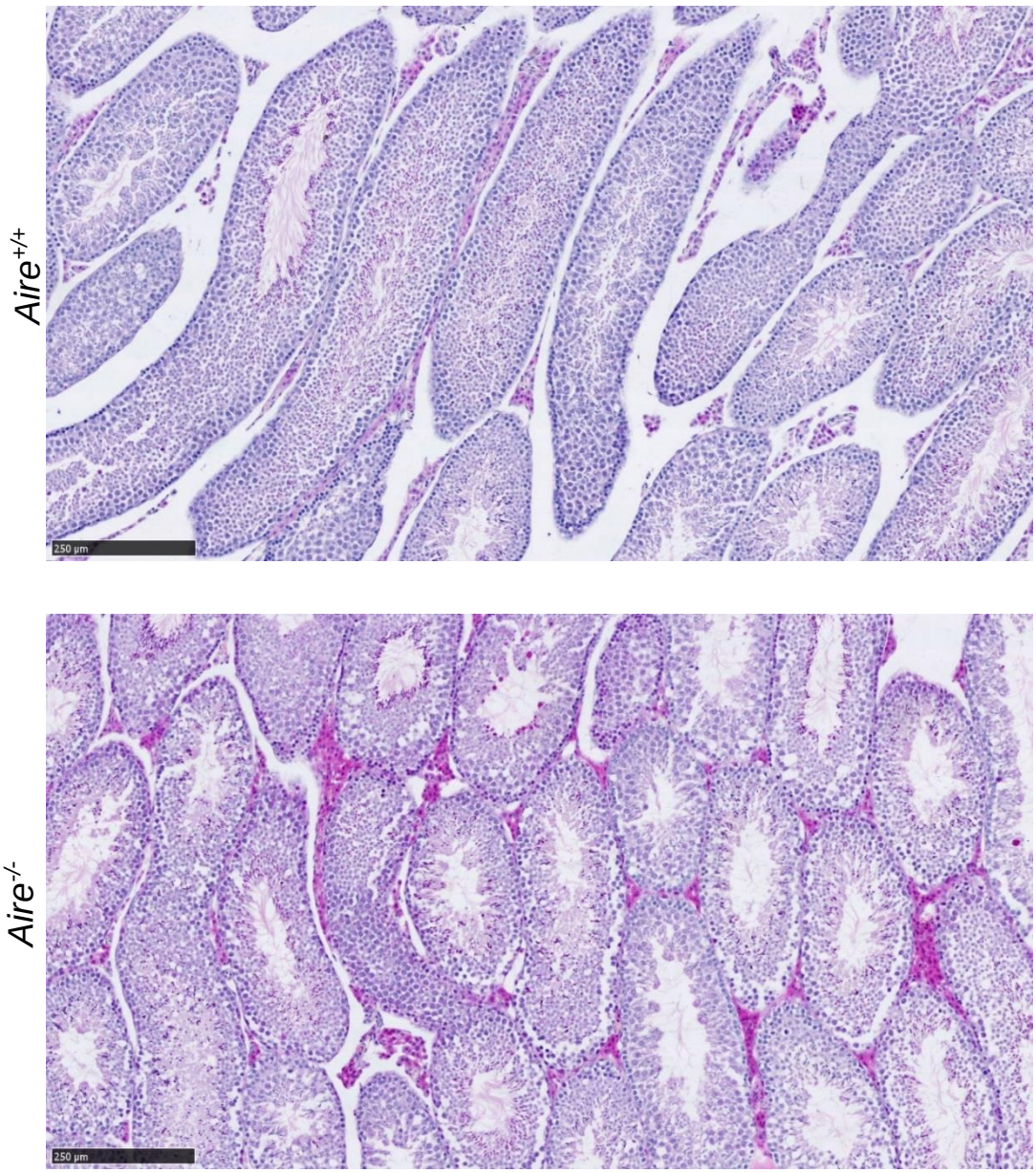



**Figure 3.11: Representative tissue sections from the testis of 12-week-old *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice.** Testis sections from *Aire*$^{+/+}$ (upper lane) and *Aire*$^{-/-}$ (lower lane) stained with haematoxylin and eosin (H&E). Leydig cells hyperplasia (LCH) like morphology in *Aire*$^{-/-}$ testis. Scale bar 250 µm

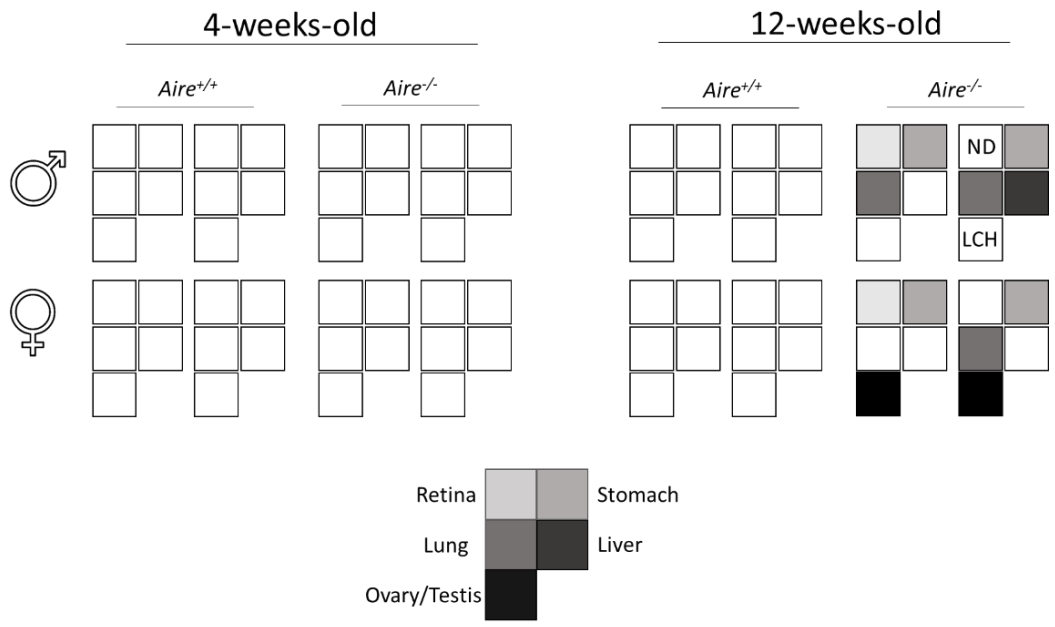



**Figure 3.12: Summary of tissue infiltrate in *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice**. In 4-week-old mice, no tissues infiltrations were observed in both *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice. In 12-week-old mice, tissues with infiltration highlighted for each *Aire*$^{-/-}$ mouse.

## 3.6 Discussion

In this chapter, it was demonstrated that Aire display a sexual dimorphic expression levels, with more expression in males than females. This difference in expression was detected at both protein and mRNA levels. A very recent study has confirmed this finding (Zhu *et al.* 2016). They conducted flow cytometry analysis to compare the frequency of mTEC$^{Hi}$ cells, and no differences were reported in frequency of mTEC$^{Hi}$ between males and females (Zhu *et al.* 2016). Another recent study demonstrated that oestrogen in females upregulates the number of methylated CpG sites in *Aire* promoter, leading to reduced Aire expression in mTEC (Dragin *et al.* 2016). This

association between Aire and oestrogen may partially increases female susceptibility to autoimmune diseases.

Furthermore, it was shown here that Aire expression levels of both protein and mRNA levels decreases with age, with significant reduction in 12-week-old *Aire*$^{+/+}$ females. Recent study has found that this gradual age-dependent decline in Aire expression has been found to be due to the reduction in the frequency of Aire+ mTECs, but not due to the reduction of total mTECs (Coder *et al.* 2015). Thus, the level of Aire expression per mTECs and Aire+ mTECs frequency are both critical in maintaining self-tolerance.

Aire regulates the transcription of a large set of TSAs genes in thymic mTECs (Anderson *et al.* 2002). To confirm the Aire-dependent TSAs genes expression profile in *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice, qPCR analysis was performed on thymic extracts to measure and compare TSAs mRNA levels. In total, 18 TSAs were screened to determine their level of expression in both *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice at 4-week-old. They were divided into three groups, Aire-dependent upregulated TSAs, Aire-dependent downregulated TSAs and Aire-independent TSAs. The data confirm that there was a consistent pattern of expression in each group. In absence of Aire, Aire-dependent upregulated TSAs were dramatically reduced, whereas, Aire-dependent downregulated TSAs were moderately induced. On the other hand, Aire-independent TSAs exhibited neutral expression pattern. This screening will give an idea on the most affected genes in the absence of a functional Aire, and provides a baseline of the TSAs genes expressions in 4-week-old *Aire*$^{-/-}$ mice before administration of therapeutic *AIRE* copy into their thymi (*see chapter 6*).

Another hallmark of Aire deficient mice that was investigated in this study is the presence of multiple tissue infiltration. In this part of the study, various tissues were investigated including, skin, brain, eye, heart, lung, liver, stomach, spleen, pancreas, kidney, testis and ovary. Infiltrates were observed in some of these tissues in *Aire*$^{-/-}$ mice on C57BL/6 background at 12-week-old, when the first signs of disease appear (Ramsey *et al.* 2002). For instance, infiltrates were seen in the lung, liver, and stomach of 12-week-old *Aire*$^{-/-}$ mice, common infiltrations reported in Aire deficient mice on C57BL/6 background (Anderson *et al.* 2002, Ramsey *et al.* 2002). Moreover, LCH morphology has been observed in one *Aire*$^{-/-}$ 12-week-male mouse, this could be an effect of manifestation by hypogonadism which is associated with spermatogenic failure. A study reported that Leydig cell hyperplasia manifests in the adult by signs of hypogonadism (Tazi *et al.* 2008). This finding suggests a gonadal atrophy comparable to APS-1 patients. There were no obvious pathologic changes in other organs from 12-week-old *Aire*$^{-/-}$ mice examined in this study. In contrast, no such changes were observed in 12-week-old *Aire*$^{+/+}$ mice. In agreement with previous studies, the infiltrates showed a clear age-dependence pattern, as tissue infiltrates in 4-week-old *Aire*$^{-/-}$ mice were undetectable (Anderson *et al.* 2002, Ramsey *et al.* 2002).

Together, this part of the thesis established useful readouts to be used for *in vivo* pilot and efficacy gene therapy approaches in the APS-1 mouse model (*see chapters 5 and 6*).

*CHAPTER FOUR*

# AAV9-MEDIATED DELIVERY OF AIRE GENE *IN-VITRO*

# 4. AAV9 DELIVERY OF AIRE GENE *IN-VITRO*

## 4.1 Outline

The aim of this project was to produce an efficient gene transfer approach using AAV9 viral vector system to mediate expresses of human *AIRE* under an optimal promoter *in vitro*.

The transcriptional activity of several promoters was analyzed and verified, since promoter activity and specificity play an important role in regulating transgenes. Transcriptional activity of selected promoters was monitored *in vitro* after sub-cloning of either *GFP* or *Aire* in the Gateway® expression plasmids. In order to limit Aire expression to the thymus, expression plasmids were constructed carrying murine cell-type specific promoters including Aire, Serglycin (Srgn) and Casein beta (Csn2). To ensure high transgene expression, another set of expression plasmids were constructed carrying constitutive virus-derived promoters such as Cytomegalovirus immediate-early promoter (CMV) and mammalian origin promoters such as phosphoglycerate kinase 1 promoter (PGK) and chicken β-Actin promoter coupled with CMV early enhancer (CAG). The outcome of transfecting each plasmid in HEK293 cells were compared and analyzed.

AAV9 expressing AIRE under the control of CMV promoter was generated, titred and its transduction efficiency was assessed *in vitro*. The latter is a validation step prior AAV9-AIRE, which would then be applied to the APS-1 mouse model for both *in vivo* pilot and efficacy studies (*see chapters 5 and 6*).

## 4.2 In vitro promoter activity assay

### 4.2.1 Choice of constitutive promoters

The promoter plays an important role in the initiation and regulation of gene expression. Constitutive promoters are used frequently in gene therapy research in order to obtain high-level gene expression. Some of the most used constitutive promoters comprising viral promoters are CMV, PGK and CAG *[Figure 4.1]*.

Constitutively active promoters are required for efficient viral propagation as they induce much higher levels of transgene expression. The CMV promoter is regularly utilised in gene transfer vectors because of its ability to drive very high levels of transgene expression in a broad range of cellular systems (Watakabe *et al.* 2017). PGK is another promoter commonly used for gene expression in mammalian system. PGK promoter resembles those of other constitutive promoters being GC rich, having no TATA box, but having a CAAT box motif at -108bp (Pfeifer *et al.* 1990). It is effectively used for constitutive transgene expression during long-term culture cells (Schiedner *et al.* 2002, Liu *et al.* 2009, Norrman *et al.* 2010). The CAG promoter, which contains an enhancer element from CMV promoter coupled with elements of the chicken β-actin promoter and rabbit β-globin gene, is a powerful synthetic promoter. It drives high levels of transgene expression in various mammalian cells (Miyazaki *et al.* 1989, Alexopoulou *et al.* 2008).

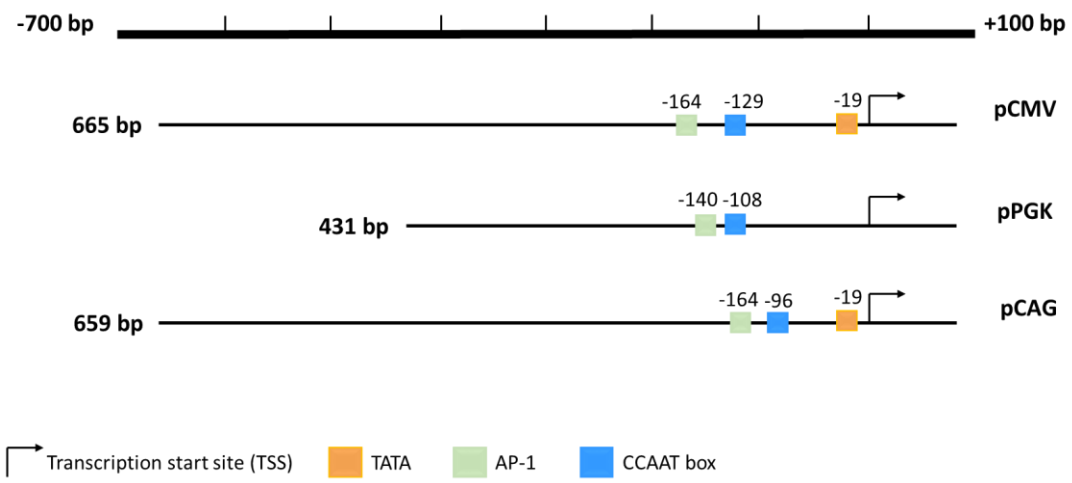



**Figure 4.1: Schematic representation of CMV promoter (665 bp), PGK promoter (431 bp) and CAG promoter (659 bp).** The transcription start site (TSS) shown by an arrow at +1. Key motifs are highlighted including the TATA box ( ) at -19 in CMV and CAG promoters. CCAAT box ( ) at -129, -108 and -96 in CMV, PGK and CAG respectively. AP-1 ( ) at -164 in CMV and CAG, -140 in PGK.

### 4.2.2 Choice of tissue-specific promoters

Tissue specificity of transgene expression is essential to ensure efficacy of gene therapy approach. A transcriptome sequencing data of neonatal thymic epithelial cells used to select tissue-specific promoters (St-Pierre *et al.* 2013). According to the data, mTECs expressed genes more than other cell populations, 2736 genes out of 14,523 genes were classified as mTECs lineage-specific genes. Three highly-expressed genes in mTECs were chosen; *Aire*, *Srgn* and *Csn2* and their promoters were amplified and cloned into expression plasmids *[Figure 4.2]*. Analysis of mouse *Aire* promoter sequence showed that the promoter contains conserved binding sites for essential components of the basal transcriptional complex such as AP-4, an

inverted CCAAT box and a typical TATA box *[Figure 4.2]* (Mittaz *et al.* 1999). *Casein* promoter has a strong *Footprint* (FP 4) in the regions -80 to -106 of the lower strand and -81 to -104 of the upper strand. This *Footprint* contains a sequence TTCTTGGAATT between positions -85 and -98, which is highly conserved among mouse *β*-; rat *α*- *β*- *y*-; and bovine $\alpha_{s1}$- and *β-casein* genes (Schmitt-Ney *et al.* 1991). *Serglycin* (*Srgn*) is found in all nucleated hematopoietic cells and platelets, blood vessels, various reproductive and developmental tissues. Studies using a mouse model lacking *Srgn* demonstrated important functional roles for Srgn in immune system processes and inflammation, which may explain its high concentration in the thymus (Schick 2010).

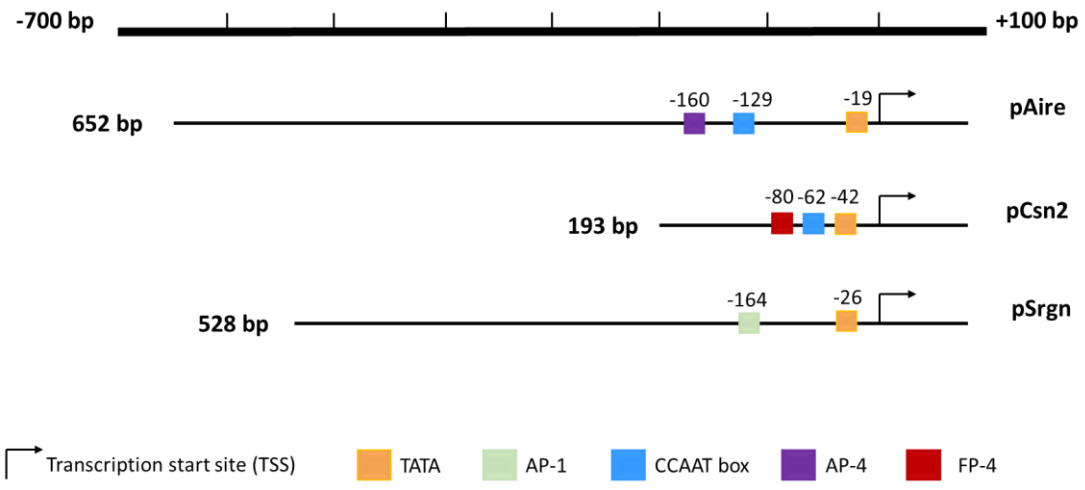



**Figure 4.2: Schematic representation of Aire promoter (652 bp), Csnβ promoter (193 bp), and Srgn promoter (528 bp).** The transcription start site (TSS) shown by an arrow at +1. Key motifs are highlighted including the TATA box ( ) at -19, -42 and -26 in Aire, Csn2 and Srgn promoters, respectively. CCAAT box ( ) at -129 and -62 in Aire and Csn2 promoters, respectively. AP-4 ( ) at -160 in Aire promoter, FP-4 ( ) at -80 in Csn2 promoter and AP-1 ( ) at -164 in Srgn.

### 4.2.3 *In vitro* characterization of Gateway® GFP expression plasmids

Green Fluorescent Protein (GFP) reporter gene allows easy visualization and verification of gene expression level in target tissues upon transduction with a given viral vector. In this project, GFP not only comprises an invaluable tool in defining transcriptional activity of a promoter, but it will also guide us in defining the optimum viral vector dosage for *in vivo* transduction (*see chapter 5*). It should be mentioned here that the reporter gene does not have any therapeutic effect on the cells (Jensen 2012).

Initially, the GFP expression plasmids were developed as control plasmids *(See chapter 2 for details).* The visualization of HEK293 transfected cells under a fluorescent microscope served as a verification of the functionality of the gateway expression plasmid, as well as a tool for evaluating transfection efficiency. HEK293 cells were transiently transfected with the six different expression plasmids: pCMV-GFP, pPGK-GFP, pCAG-GFP, pAire-GFP, pCsn2-GFP and pSrgn-GFP. All three constitutive promoters expressed GFP transiently in HEK293 cells 48 hours post transfection, with fluorescence microscopy used as a semi-qualitative evaluation. CMV promoter exhibited a strong and intense GFP expression, whereas PGK and CAG promoters expressed GFP moderately *[Figure 4.3]*.

Semi-quantitative analysis demonstrated that GFP expression under the CMV promoter had a transfection efficiency of almost 30%, which is significantly higher than PGK and CAG promoters with $P = 0.0007$ and $P = 0.0001$ respectively *[Figure 4.4]*. PGK promoter had a transfection efficiency

of 12.2% of total HEK293 cells, while CAG promoter showed the least percentage of GFP expression with just 6% of the total HEK293 cells.

On the other hand, weak or not detectable transcriptional activity of the eukaryotic mTEC-specific promoters: pAire, pCsn2 and pSrgn in HEK293 cells has been observed. These data suggest that successful GFP expression was driven by the constitutive promoters; pCMV, pPGK and pCAG in HEK 293 cell lines when compared to mammalian genomic tissue-specific promoters.

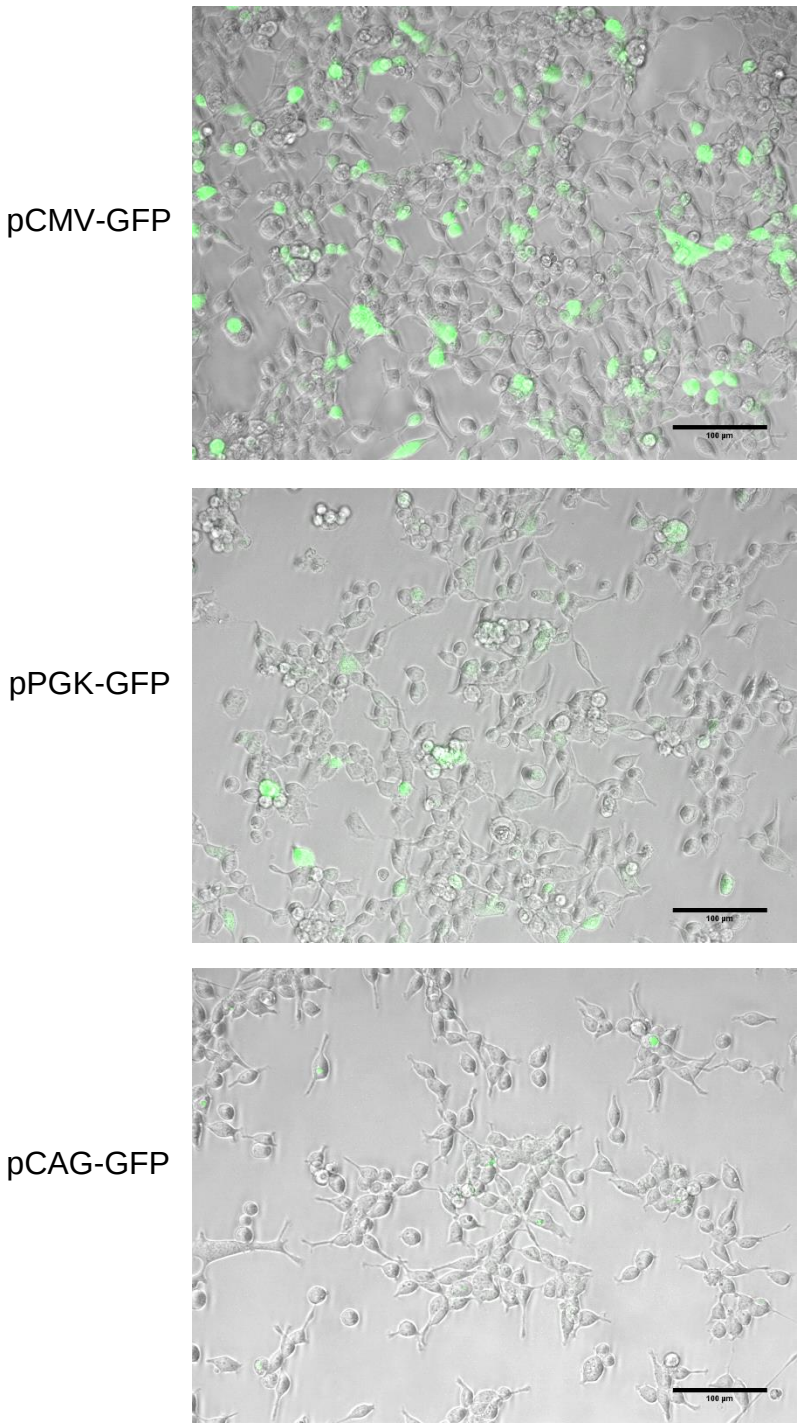



**Figure 4.3: Constitutive promoter's GFP expression plasmids.** HEK293 cells were seeded on 6-well plate, transfected with GFP expression plasmids the following day. 2-days post transfection; the cells were visualized under a fluorescent microscope. GFP – Green Fluorescent Protein; pCMV – Cytomegalovirus promoter; pPGK – Phosphoglycerate Kinase 1 promoter; pCAG – Chicken β-actin promoter coupled with CMV early enhancer.

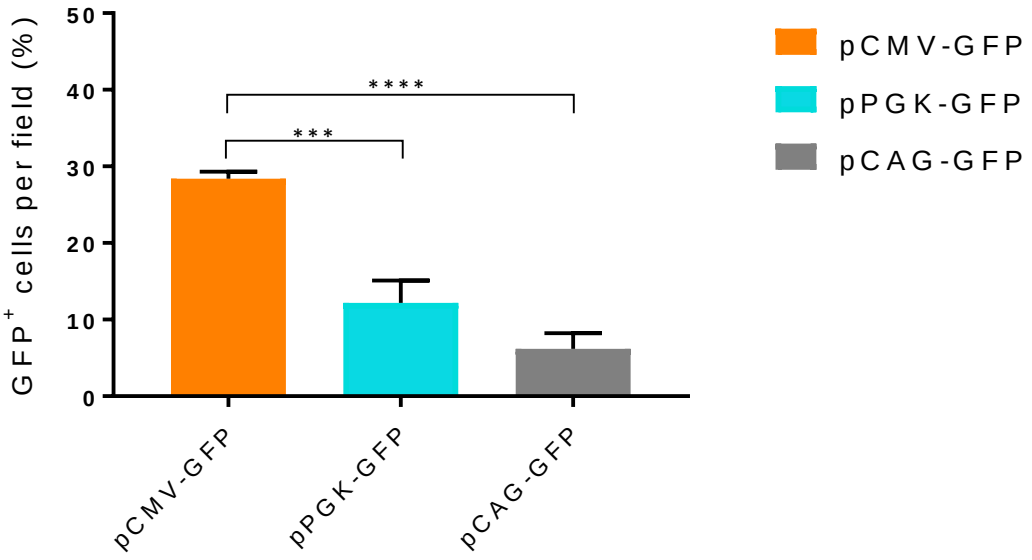



**Figure 4.4: In-vitro transfection efficiency of GFP constructs measured as a proportion of GFP positive cells/total cells.** Percentage of GFP+ cells in HEK293 cells seeded in 6-well plate and transfected with pCMV-GFP, pPGK-GFP and pCAG-GFP. Data analysed with one-way ANOVA, Dunnett's multiple comparisons test, *** $P \leq 0.001$, **** $P \leq 0.0001$. Error bars represent ±SEM

### 4.2.4 Transient transfection of Gateway® Aire expression plasmids

After the transduction efficiency of the GFP under constitutive promoters compared to tissue-specific promoters, Aire Gateway™ expression plasmids were developed under the control of the constitutive promoters: pCMV, pPGK and pCAG (*See chapter 2 for details*). As a first approach to determine the activity of the promoters used in Aire expression plasmids, HEK293 cells were transiently transfected with the different promoters driving expression of *Aire [Figure 4.5]*. Transfected cells were collected after 48 hours, lysed and proteins were loaded on SDS-PAGE for western blot analysis.

These data demonstrated that CMV promoter resulted in moderate to high expression of Aire in HEK293 cells *[Figure 4.6]*. On the other hand, no Aire expression under both PGK and CAG promoters was detectable using western blot analysis. These data suggest that successful Aire expression was driven by the constitutive promoter; pCMV, but not pPGK or pCAG, in HEK293 cell line.

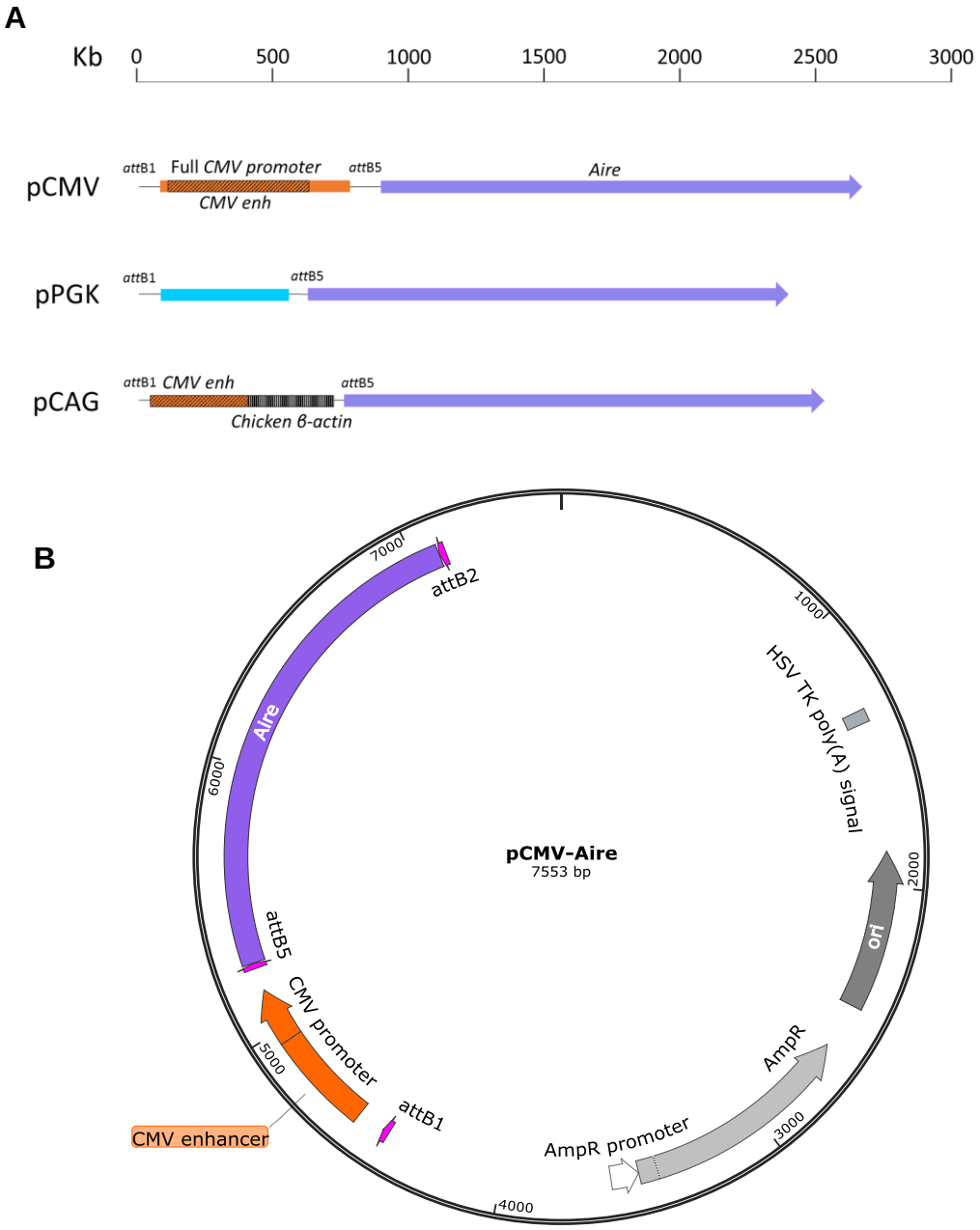



**Figure 4.5: Construction of gateway Aire expression plasmids with constitutive promoter/ enhancer elements.** (A) Schematic representation of the gateway Aire expression plasmids utilized in this study. Promoter elements were polymerase chain reaction amplified from corresponding plasmids. The entire promoter fragments were cloned between *att*B sites in destination (DEST) vectors. (B) A representative vector containing full CMV promoter. Enh; enhancer, AmpR; Ampicillin resistance gene, orf; open reading frame.

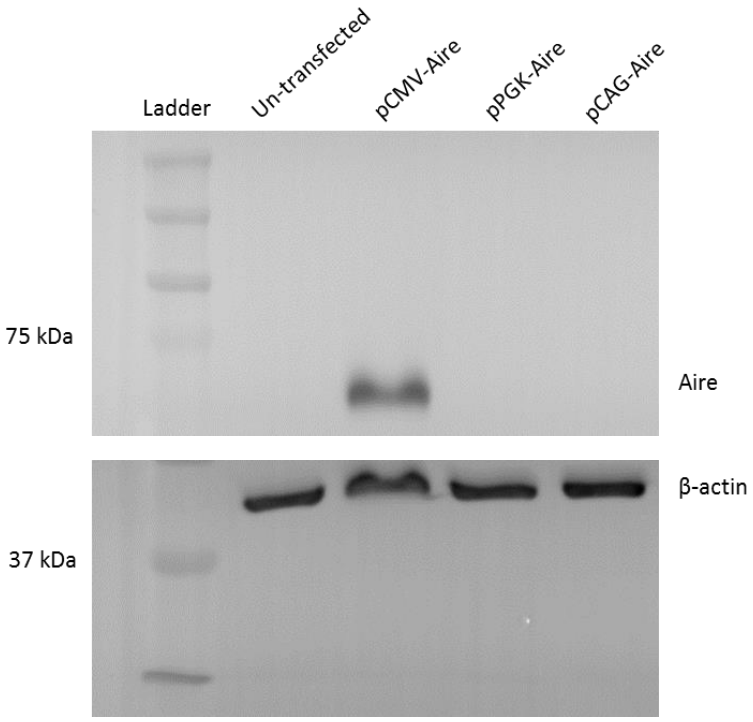



**Figure 4.6: *In vitro* transfection of gateway Aire expression plasmids *in-vitro*.** Representative western blot of the transfected cells. HEK293 cells were transfected with pCMV-Aire, pPGK-Aire and pCAG-Aire respectively. Transfection was evaluated after western blotting. The AIRE band is clearly visible (AIRE 58 kDa) as well as the loading control (β-actin 42 kDa). AIRE – Autoimmune Regulator; β-actin – Beta actin.

## 4.3 AAV9-AIRE viral production

### 4.3.1 Choice of AAV viral vector

AAV has many advantages as a vehicle of choice. It is a small non-pathogenic virus causes a very mild immune response without causing any disease. It transduces and integrates a wide cell tropism leading to a long-term expression (Kaplitt *et al.* 1994). A conventional single-stranded ssAAV serotype 9 was used under the CMV promoter in this project.

AAV9 transduces widespread of targets including crossing the blood-brain barrier. Stable transgene expression was seen in the rat brain for more than 6 months (Klein *et al.* 2002). In addition, AAV9 vectors transduce rodent muscle, liver, and lung about 100-fold more efficiently than AAV2. Studies of AAV9 in large-scale animal studies, showed no side effects or evidence of tumorigenesis has been reported yet (Bessis *et al.* 2004, Bell *et al.* 2005, Kay 2007). Considering the early administration of this therapeutic cassette into APS-1 patients, AAV9 demonstrated potential benefits in perinatal gene delivery for treating irreversible diseases *in utero* or at birth, making it suitable for this project (Foust *et al.* 2010, Mattar *et al.* 2011, Rahim *et al.* 2011).

Although the conventional ssAAV express transgenes in a slow onset due to the need for a cellular stress to produce the complementary strand, its large capacity to accommodate large inserts up to 4.8 kb is a great advantage (Davidson *et al.* 2003, Grieger *et al.* 2005). Whereas self-complementary AAV (scAAV) contains two complementary strands that allows a faster expression of the transgene; its packaging capacity of inserts is limited to 2.4 kb (McCarty *et al.* 2001, Davidson *et al.* 2003, McCarty *et al.* 2003, Wu *et al.* 2007). Therefore, it was preferred to use ssAAV as the size of AIRE insert is 1.8 kb and thus the conventional ssAAV could accommodate both CMV promoter and AIRE.

### 4.3.2 Cloning of the human *AIRE* cDNA into AAV9 backbone

In the perspective of clinical application, a therapeutic construct that could be used in future clinical studies was developed by replacing the mouse

*Aire* cDNA with a human *AIRE* cDNA (AIRE-201, ENST00000291582.5). A recent study by Nishijima *et al.* (2017) utilised human *AIRE* to produce transgenic mice showed that human *AIRE* was immunologically competent and physiologically relevant to the role of endogenous mouse Aire with no risk of immunity.

The protein coding *AIRE* transcript is 2257 bp with 14 exons providing a coding region of 1234 bp, while the mouse Aire transcript is 1938 bp with 14 exons providing a coding region of 1356 bp. The 545 amino acid human AIRE protein has a homology of 77% at nucleotide level with the 552 amino acid mouse Aire. Human AIRE and mouse Aire share 71% amino acid identity and 75% nucleotide identity over the coding region, indicating that the two proteins appear remarkably conserved across the species (Blechschmidt *et al.* 1999, Mittaz *et al.* 1999).

The human *AIRE* cDNA was cloned from pcDNA5/FRT plasmid constructed previously in our lab from an original plasmid (pET31/AIRE) that was a gift from Prof. J. She (Medical College of Georgia, Augusta University). Primers were used to amplify the entire coding region of *AIRE*. The 1698 bp PCR product was gel purified, digested and ligated into the ssAAV backbone to produce the therapeutic construct AAV-AIRE of 6277 bp *[Figure 4.8A]*. This construct was checked by restriction digests *[Figure 4.8B]* and direct sequencing of the plasmid confirmed that no spontaneous mutations have occurred.

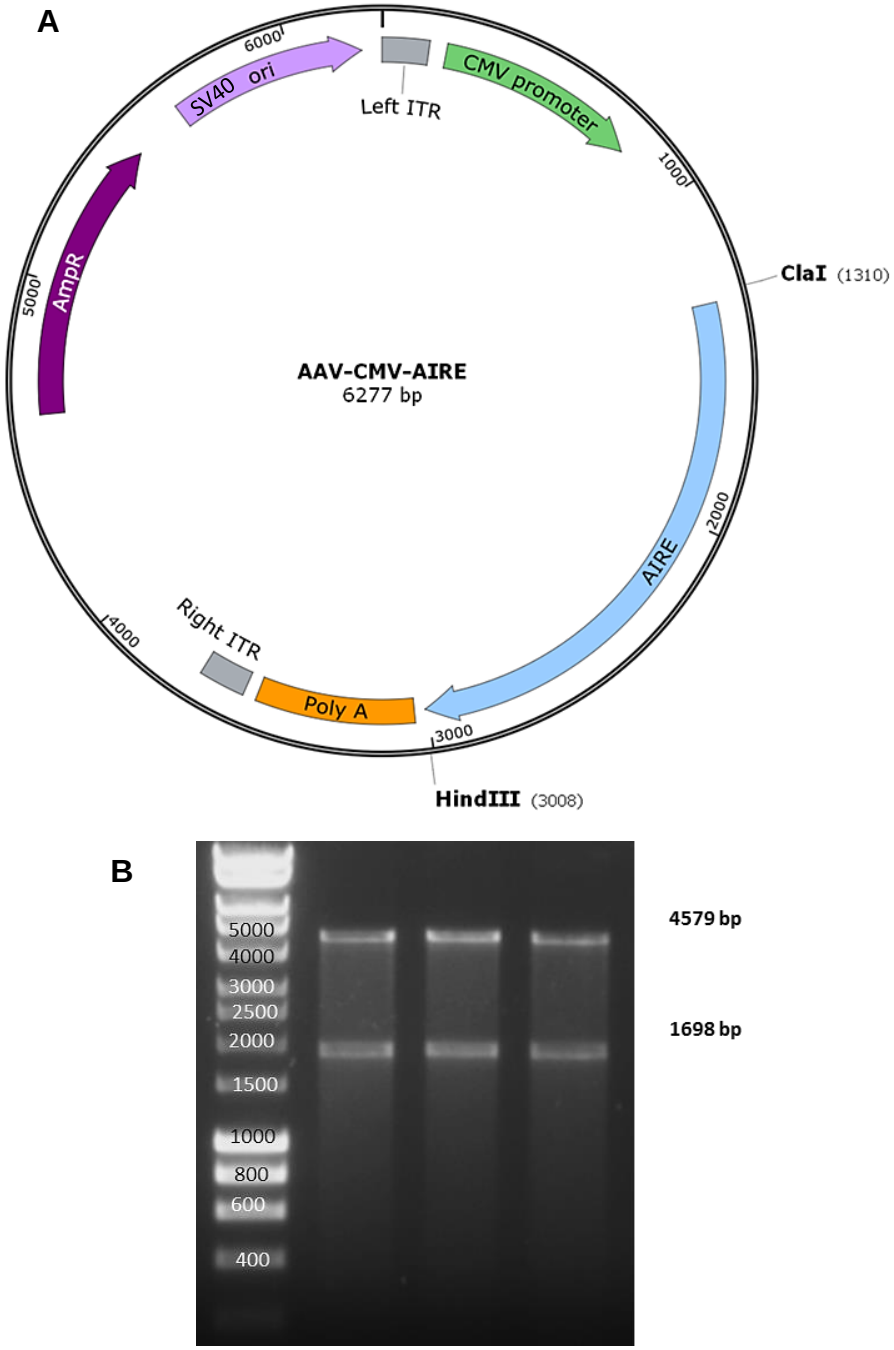
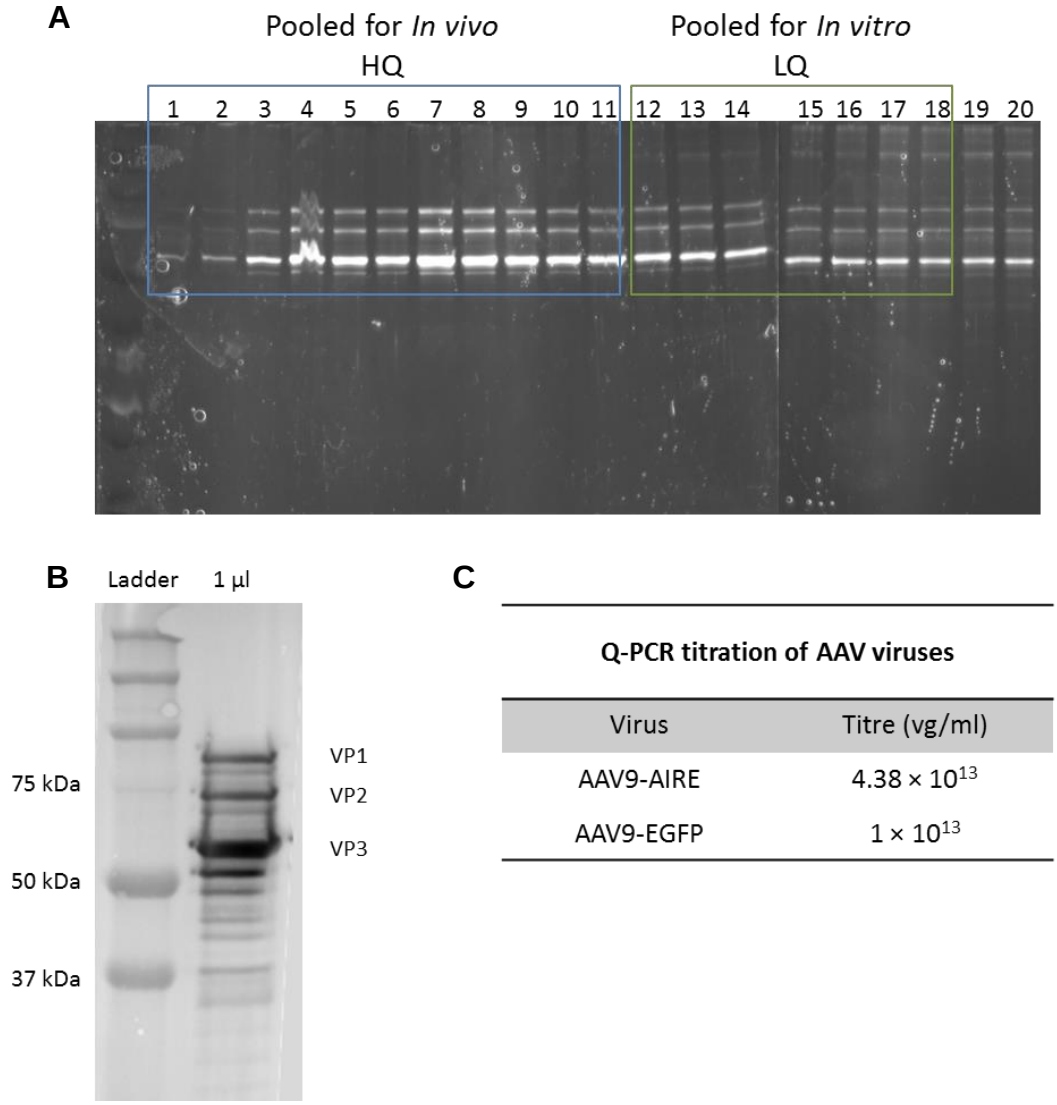



**Figure 4.7: Insertion of AIRE cDNA into ssAAV backbone.** (A) AIRE was inserted between the Inverted Terminal Repeats (ITRs) region of the AAV plasmid to form a recombinant AAV-AIRE plasmid (6277 bp). (B) Restriction digestion assay using *Cla*I and *Hind*III to confirm the size of the insert (1698 bp) and the plasmid (4579 bp).

### 4.3.3 Large Scale production of AAV9-AIRE virus

AAV9-AIRE production was achieved by using polyethyenimine (PEI) calcium phosphate-mediated triple transfection of HEK293T cells, and viral vector was harvested after 5 days. The virus was then purified by Iodixanol gradient ultracentrifugation (*see section 2.3.2*). In order to determine purity, a small sample from each fraction was loaded onto an SDS-PAGE gel and stained with the SYPRO RUBY dye to check for impurities. SYPRO Ruby dye is a ruthenium based fluorescent stains suitable for visualizing proteins on SDS-PAGE gel (Patton 2000). Pure fractions showing all three viral capsid proteins, VP1, VP2 and VP3 were considered as high quality fractions (HQ) and pooled for *in vivo* experiments, whereas fractions with some higher molecular weight bands were considered as low quality fractions (LQ) and pooled for *in vitro* transduction assays (Mulcahy *et al.* 2015) *[Figure 4.9A]*. Furthermore, western blot analysis using antibodies against the capsid proteins revealed all three VP proteins, suggesting proper packaging of the virus *[Figure 4.9B]*. In addition, the viral titre was determined by Q-PCR. Primers for determining the titre were designed to detect the transgene AIRE, and the measured titre was $4.38 \times 10^{13}$ vg/ml *[Figure 4.9C]*.




**Q-PCR titration of AAV viruses**

| Virus | Titre (vg/ml) |
|---|---|
| AAV9-AIRE | $4.38 \times 10^{13}$ |
| AAV9-EGFP | $1 \times 10^{13}$ |

**Figure 4.8: Purity and viral titre of AAV9-AIRE vector preparation.** (A) Representative SDS-PAGE gel of AAV9-AIRE preparation after SYPRO RUBY dye staining. SYPRO RUBY stains the proteins separated by SDS-PAGE representative of VP proteins. The first 11 lanes show the high quality preparations, whereas lanes 12-18 show lower quality preparations with faint larger bands. (B) The three viral bands are clearly visible (VP1 87 kDa, VP2 72 kDa, VP3 62 kDa). (C) List of the viral titres used in this project. Viral titres (viral particles per ml) were obtained through Q-PCR. HQ – High Quality; LQ – Low Quality; AAV9 – Adeno-associated virus serotype 9; AIRE – Autoimmune Regulator; EGFP – Enhanced Green Fluorescence Protein; VP – Viral protein

### 4.3.4 Transduction efficiency of AAV9-AIRE virus

Different concentrations of the AAV9-AIRE added to HEK293 cells, and 5-days post transduction the gene transfer efficiency calculated by harvesting the cells and measuring the yield of AIRE protein expressed by the cells. A concentration of $4.38 \times 10^{10}$ vg/µl of AAV9-AIRE yielded high AIRE expression level compared to un-transduced. In addition, higher expression of AIRE was observed at a concentration of $4.38 \times 10^{11}$ vg/µl *[Figure 4.10A]*. Densitometry analysis confirmed this dose-dependent pattern of AIRE expression, showing more than 2-fold increase in AIRE expression with the higher dose at 5-days after transduction as compared to transduced cells with $4.38 \times 10^{10}$ vg/µl *[Figure 4.10B]*.

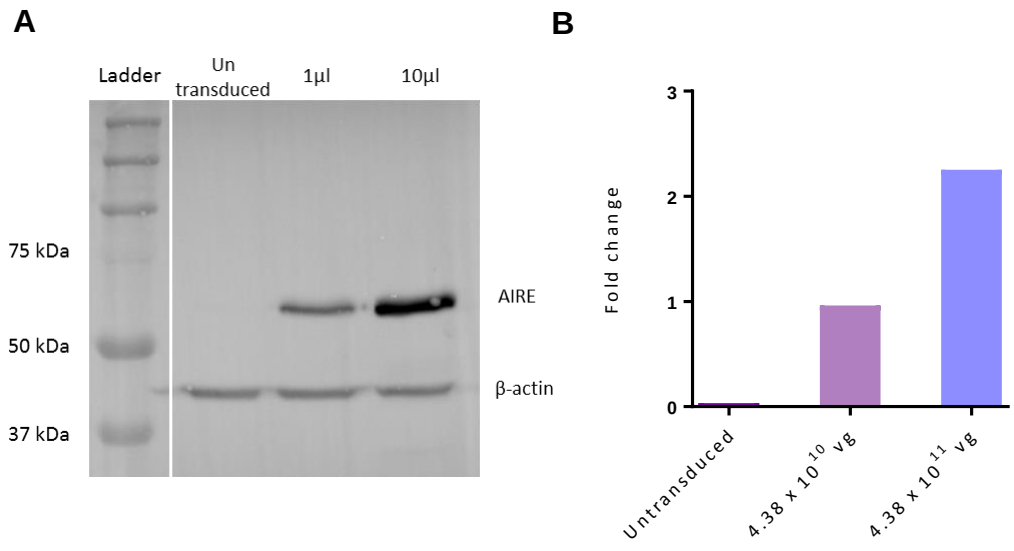



**Figure 4.9: Transduction efficiency of AAV9-AIRE virus *in-vitro*.** (A) Representative western blot of the transduced cells. HEK293 cells transduced with different volumes of AAV9-AIRE. The AIRE bands are clearly visible (AIRE 58 kDa) as well as the loading control (β-actin 42 kDa). (B) Densitometry analysis of transduced HEK293 cells was determined after western blotting by fold change normalized to β-actin and compared to un-transduced control. AIRE – Autoimmune Regulator; β-actin – Beta actin.

## 4.4 Discussion

The transcriptional activity of various promoters were analysed for effective transgene expression in cells. Gateway™ cloning system was used to construct expression plasmids under the control of six different promoters, including three cell-type specific promoters, *Aire*, *Csn2* and *Srgn*, and three viral constitutive promoters, CMV, PGK and CAG. *GFP* was used as a reporter gene to monitor transcriptional activity of the six promoters. Whereas *Aire* was constructed under the constitutive promoters for transgene expression comparison.

Cell-type specific promoters were chosen for transcriptional targeting approach. It was our first attempt to restrict our transgene expression specifically to the thymus, by using promoters that are either specifically-expressed or highly-expressed in mTECs. Three highly expressed genes have been chosen from the transcriptome data of the thymus of neonatal mice *Aire*, *Csn2* and *Srgn* (St-Pierre *et al.* 2013), their promoters were cloned due to their high expression activities in mTECs (St-Pierre et al. 2013). *GFP* expression plasmids allowed validation and visualisation of transfected HEK293 driven by these cell-type specific promoters. Transient transfection of these expression plasmids allowed the GFP to be visualised as early as two days post transfection. Transfection pattern of *GFP* expression plasmids under the mammalian promoters: *Aire*, *Csn2* and *Srgn* shows undetectable transcriptional activity of GFP in HEK293 cells. This can be explained as tissue specificity is often accompanied by lower transgene expression (Powell *et al.* 2015). This was expected, as thymic-specific promoters are not expressed in

HEK293 cell line. These promoters could be investigated using a thymic specific cell line but due to limited resources and time these promoters were not investigated further in *Aire* expression plasmids.

In contrast, *GFP* expression plasmids driven by constitutive promoters have shown consistent expression in HEK293, although the expression strength varied between these promoters. The CMV promoter resulted in a very high transgene expression, up to 30% of the transfected cells. This GFP expression level under the CMV promoter was expected as the transfection efficiency of CMV constructs ranges from 20-60% (Raup *et al.* 2016). On the other hand, the transfection efficiency of CAG promoter resulted in just 6.2% of GFP-expressing cells. *GFP* expression plasmid under the control of PGK promoter had a moderate transcriptional activity with 12% transfection efficiency. Aire expression plasmids were constructed under the same constitutive promoters, cells were harvested post transfection and protein was run on SDS-PAGE for western blot analysis. As expected, Aire was strongly expressed under the CMV promoter when compared to the PGK and CAG promoters. Taken together, high transgene expression levels are needed to meet efficacy requirements for viral gene therapy. Thus, out of the three constitutive promoters tested so far, the CMV promoter appears to be the optimal choice for *AIRE* expression *in vivo* using viral vectors.

On the basis of these findings, we constructed our AAV9-AIRE virus under the constitutive CMV promoter. *In vitro* transduction efficiency assay was successfully achieved in HEK293 cells. HEK293 cells transduced with $4 \times 10^{10}$ vg/µl of AAV9-AIRE expressed AIRE at higher level compared to un-

transduced. Furthermore, HEK293 cells transduced with a higher concentration of AAV9-AIRE $4 \times 10^{11}$ vg/μl yielded a higher AIRE expression, almost 2-fold of protein over-expression compared to the cells transduced with $4 \times 10^{10}$ vg/μl of AAV9-AIRE. This persistent dose-dependent high expression of AIRE *in vitro* mediated by AAV9 under CMV promoter is a promising tool that could restore Aire function in APS-1 mouse model.

Overall, in this part of the study, it has been demonstrated that AAV9-AIRE under the control of the CMV promoter, successfully transduced HEK293 cells leading to high transgene expression levels. The therapeutic AAV9-AIRE virus has been applied for the *in vivo* optimisation and efficacy studies *(see chapters 5 and 6)*.

*CHAPTER FIVE*

# OPTIMISATION OF AAV9 DELIVERY: PILOT STUDY

# 5. OPTIMISATION OF AAV9 DELIVERY: PILOT STUDY

## 5.1 Outline

In this optimisation study, a detailed *in vivo* AAV9-AIRE intrathymic gene transfer approach into APS-1 mouse model will be discussed. Determining the optimal intrathymic injection method, the optimal AAV9-AIRE dose and the method of detection of AIRE transgene are critical parameters for subsequent efficacy studies.

Intrathymic injections has been central to some studies of thymic biology, hence mouse thymic anatomy is similar to humans, for that researchers had extensively studied this organ in mice. Intrathymic injection has traditionally been done using surgical procedures consisting of opening the thoracic cavity for direct visualization and administration into the thymus. However, the surgical approach subjects the mouse to substantial pain and distress as well as altering the T-cell development (Hogan et al. 2011). Moreover, it also increases the risk of infection and potentially increases mortality rate. Recently, a non-invasive ultrasound-guided intrathymic injection in mice was introduced as an alternate, less stressful approach (Blair-Handon *et al.* 2010, Tuckett *et al.* 2014). Adopting this approach in this study, intrathymic delivery would directly distribute the viral particles into the thymic epithelial cells with minimal distress to APS-1 mouse model.

In this study, a small pilot study using two experimental viral vectors, AAV9-GFP and AAV9-AIRE with five different concentrations were administered to pre-symptomatic *Aire*$^{-/-}$ mice. Histological analysis has been

performed, 4 weeks post-viral delivery, to evaluate transduction efficiency in the thymus. This pilot study consisted of three objectives, (1) to evaluate the intrathymic injection delivery method, (2) to test the transduction efficiency of AAV serotype 9 in the thymus using GFP as a transgene, (3) to assess the optimal concentration of the AAV9-AIRE that could restores the thymus function with no signs of cytotoxicity.

After evaluating the transduction efficiency of this method with different doses of the experimental AAV9-AIRE in a small number of mice, the optimal dose was selected for the pre-clinical proof-of-concept efficacy study.

## 5.2 Intrathymic injection as a route of administration

### 5.2.1 Ultrasound imaging of the thymus

To avoid using procedures that require opening the thorax and to improve success rates with non-invasive procedures, ultrasound-imaging technique was adopted for accurately locating the thymus in anesthetized 4-week-old mice.

To verify this technique, ten 4-week-old Aire mice were used. Before intrathymic injection and while under anaesthesia, fur over the ventral thorax of the mice was removed, resulting in midline epilation from the base of the neck to mid-chest *[Figure 5.1]*. With the Vevo 770 (VisualSonics, Toronto, Canada) preclinical scanner, the thymus of 4-week Aire mice could easily be visualized. A bi-lobed structure in the anterior mediastinum interposed between the sternum and heart/great vessels, with the mean distance between the skin and the thymus was calculated to be 2.0 mm *[Figure 5.2]*.

Syringe within the thymus was detected by the ultrasound imaging system *[Figure 5.3]*. Mice were terminally anesthetized after injections with Trypan blue, and thoracotomies carried out to verify the accuracy of the injections. Post-mortem examination of the 10 mice administered with Trypan blue by allowed us to confirm that the dye had been successfully delivered to one thymic lobe of 7 mice (70%) *[Figure 5.4]*.

The use of ultrasound guidance to perform free-hand injections of individual thymic lobes in 4-week-old Aire was possible. However, regardless that this method facilitates locating the thymus and individual thymic lobes performing the injections under the probe within the thick layer of the aqueous gel was challenging. Furthermore, holding the needle very steadily and injecting the material slowly was difficult. Therefore, the parameters gained by this approach were helpful to be able to accurately locate the thymus blindly. Hence, the use of the stereotaxic instrument as a method for steady and slow injections was considered for subsequent intrathymic administration.

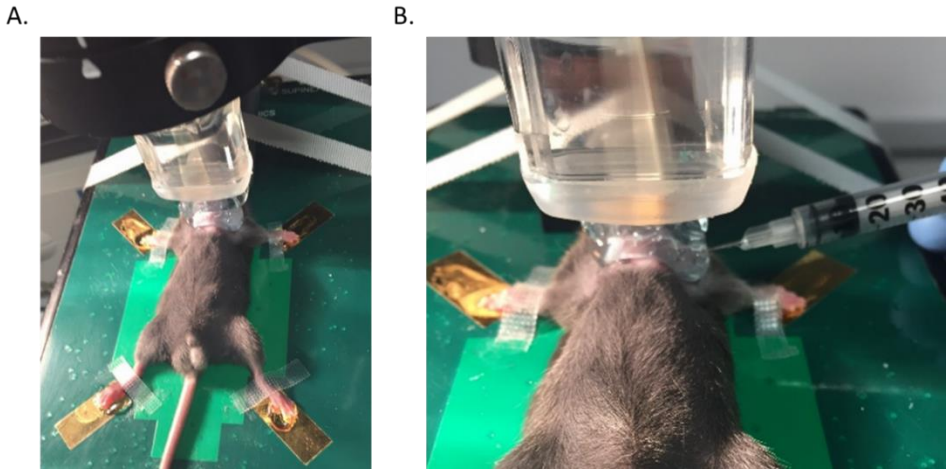



**Figure 5.1: Ultrasound-guided imaging system.** (A). Mouse was positioned on the platform with the ventral thorax exposed. (B). The syringe approaching the thymus within the sterile gel.

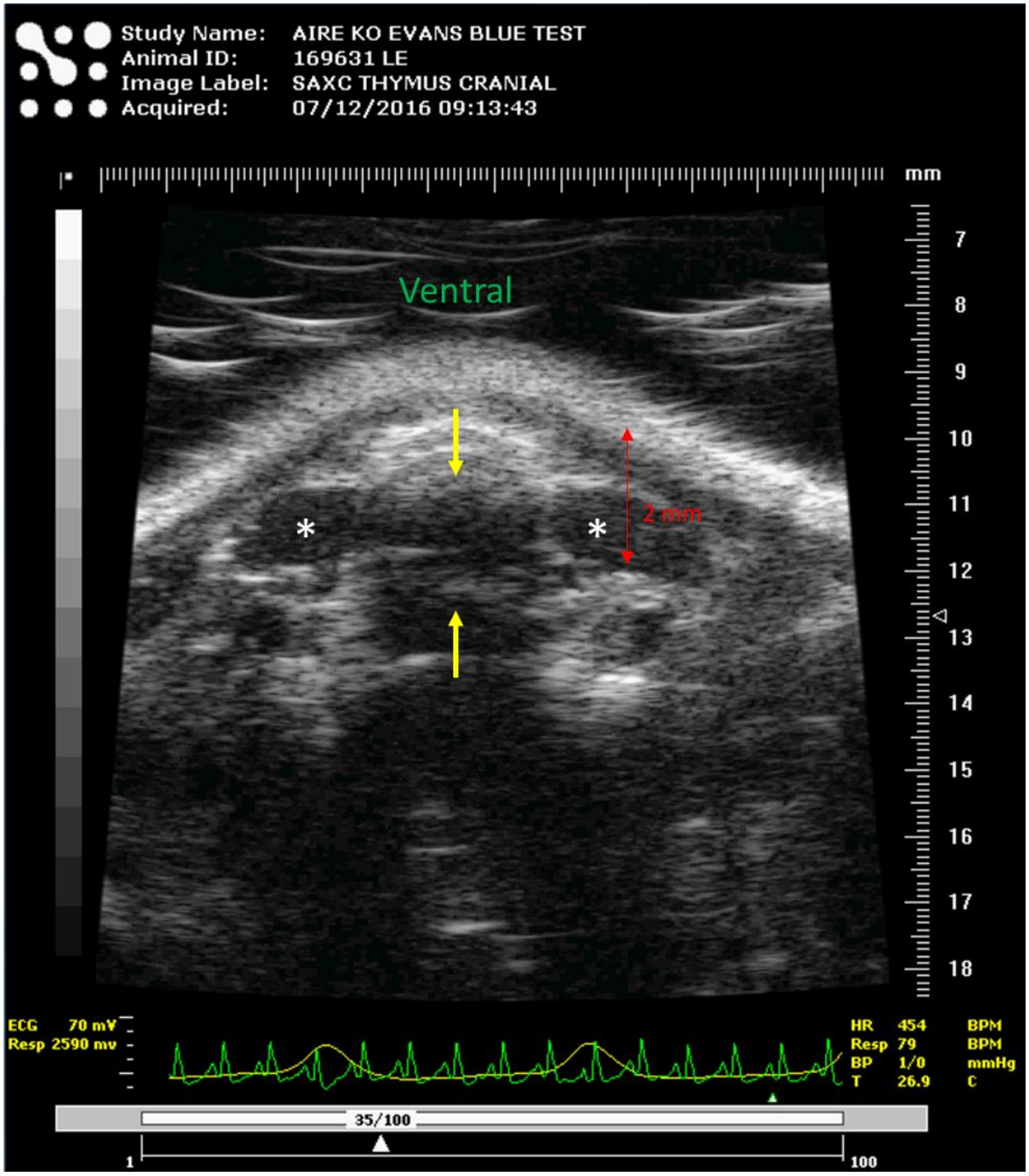



**Figure 5.2: Ultrasound imaging of thymus of Aire mouse.** Axial plane image revealing left and right thymic lobes (*asterisks*) between the sternum (*upper arrow*) and ascending aorta (*lower arrow*). The thymus in 4-week-old mice located 2 mm below the skin.

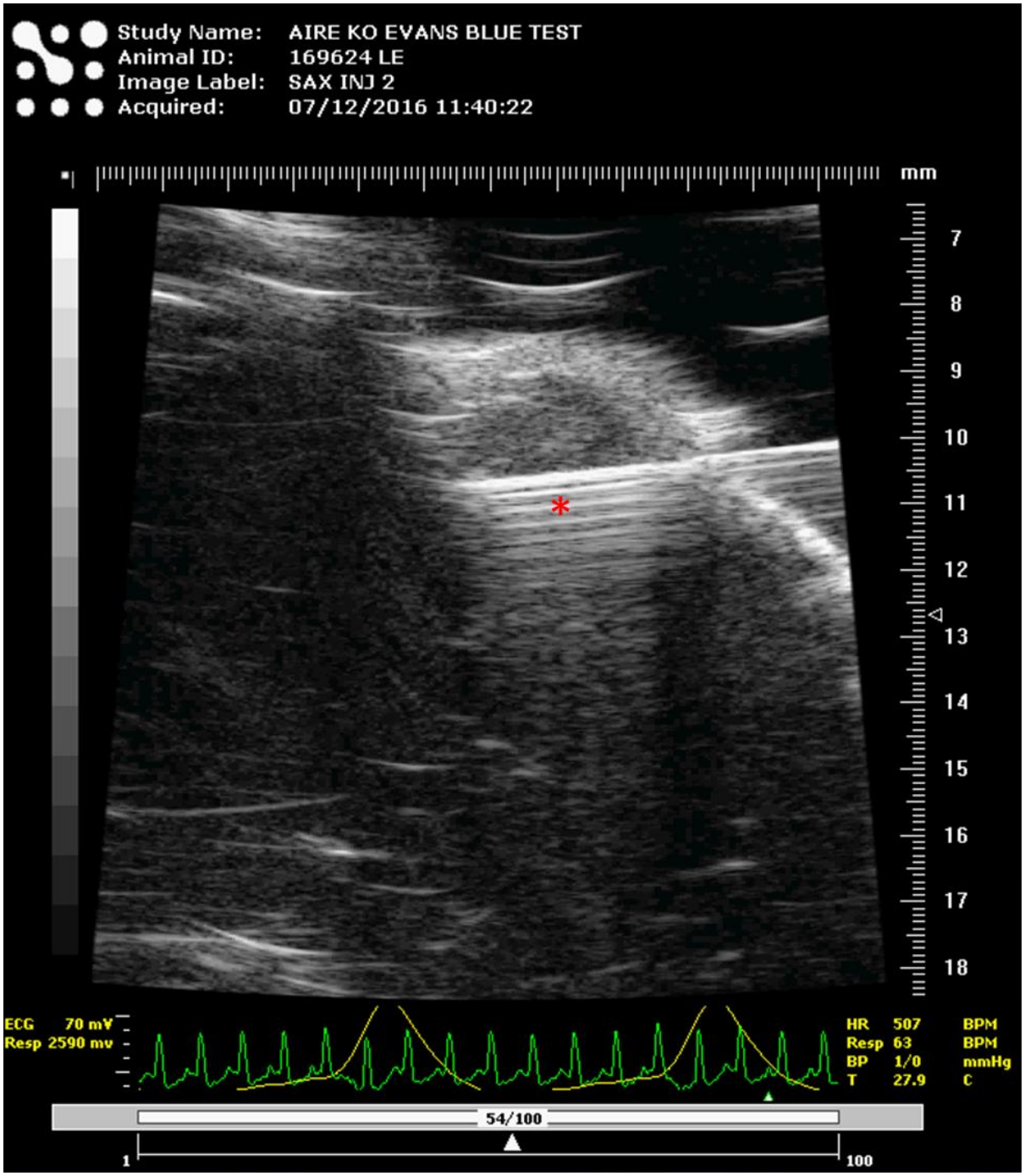



**Figure 5.3: Ultrasound imaging of intrathymic injection of Aire mouse.** The needle (*asterisk*) has been advanced from left to right across the midline septum for right thymic lobe injection.

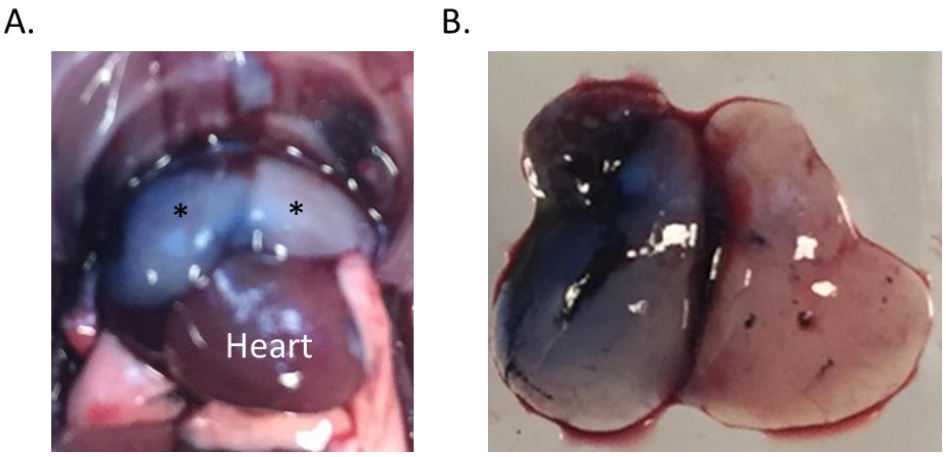



**Figure 5.4: Verification of accuracy of ultrasound-guided intrathymic injection.** Thymi of 4-week-old Aire mice were injected with trypan blue, and the accuracy of injections was confirmed by necropsy. (a) Trypan blue-stained the right thymic lobe (asterisks) *in-situ* after injection. (b) Trypan blue-stained thymus ex-situ after injection of a single (right) lobe.

## 5.2.2 Intrathymic injections using stereotaxic instrument

After successfully positioning the thymus in 4-week-old Aire mice using the Vevo 770 ultrasound imaging system, trypan blue dye injections were performed on Aire mice at 4-week-old using the stereotaxic instrument *[Figure 5.5]*. After injection of trypan blue dye, mice were euthanized and thoracotomies were carried out to verify the accuracy of the injections. Successfully, 35 out of 38 thymic lobes from 4-week-old mice were injected with trypan blue dye given a success rate of 92% *[Figure 5.6]*.

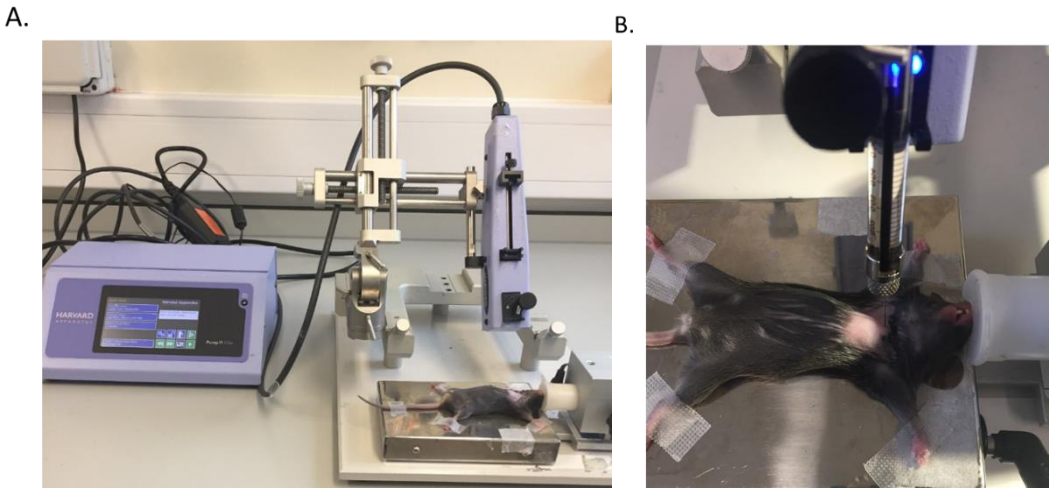



**Figure 5.5: Stereotaxic administration of intrathymic injection of Aire mouse.** The needle (*asterisk*) has been advanced from left to right across the midline septum for right thymic lobe injection.

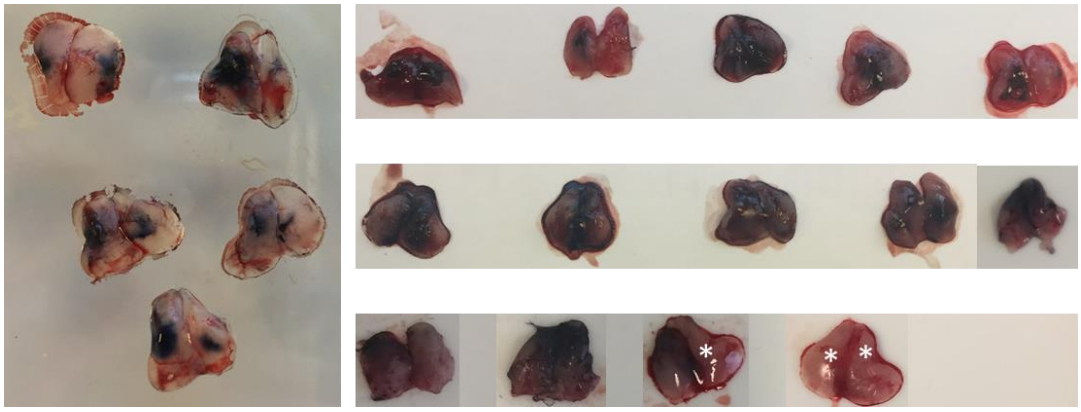


**Figure 5.6: Verification of accuracy of intrathymic injection using stereotaxic instrument.** Thymi of 4-week-old Aire mice were injected with trypan blue, and the accuracy of injections was confirmed by necropsy. Unstained thymic lobes (*asterisks*) ex-situ after injection.

## 5.3 Pilot study design and validation

In an attempt to test the transduction efficiency of the AAV9 in the thymus, we injected a group of *Aire*$^{-/-}$ mice with AAV9-GFP (*n*=5). This would allow the assessment of the viral vector transduction efficiency for thymic cells. Another approach before commencing the efficacy study is to determine the optimal concentration of AAV9-AIRE to be injected for therapeutic effects without toxicity by testing five different doses of both virus preps AAV9-GFP and AAV9-AIRE on *Aire*$^{-/-}$ mice (*n*=5 per group).

### 5.3.1 *In-vivo* dose dependent study

For determining the optimal dose of AAV9-AIRE to be used for our efficacy studies, a dose dependent experiment was carried out on Aire mice with the doses of the virus listed in table 5.1. Four groups of mice were recruited; *Aire*$^{+/+}$ mice were injected with PBS (*n*=5), *Aire*$^{-/-}$ were injected with PBS (*n*=5), *Aire*$^{-/-}$ mice injected with AAV9-GFP (*n*=5) and *Aire*$^{-/-}$ mice injected with AAV9-AIRE (*n*=5) each mouse with a different dose *[Table 5.1]*.

**Table 5.1: Concentrations of AAV9-AIRE and AAV9-GFP injected per mouse.**

| Dilution of the viral prep | Amount of virus injected per mouse |
|---|---|
| 1 | $8 \times 10^{11}$ vg |
| 1:2 | $4 \times 10^{11}$ vg |
| 1:10 | $8 \times 10^{10}$ vg |
| 1:50 | $1.6 \times 10^{10}$ vg |
| 1:100 | $8 \times 10^{9}$ vg |

Injected mice were monitored for up to 4 weeks. Two mice injected with the AAV9-AIRE at concentration of ($8 \times 10^{11}$ vg) and ($4 \times 10^{11}$ vg) exhibited toxicity effect. These mice had malaise, a piloerection fur coat and were

hunched walking pattern 11-days post injection, and humane welfare endpoint was applied. Aire mice injected with the same doses of AAV9-GFP virus showed normal appearance and normal body weight gain suggesting that the toxicity effect is from overexpression of AIRE rather than the AAV9 viral vector itself *[Figure 5.7A]*. Overall, the mice showed normal weight gain during the 4-week duration of the pilot study *[Figure 5.7]*. Except for the *Aire*$^{-/-}$ AAV9-AIRE mouse treated with the highest dose (8 × $10^{11}$ vg) which lost 5% of its weight 11 days post injection *[Figure 5.7B]*.

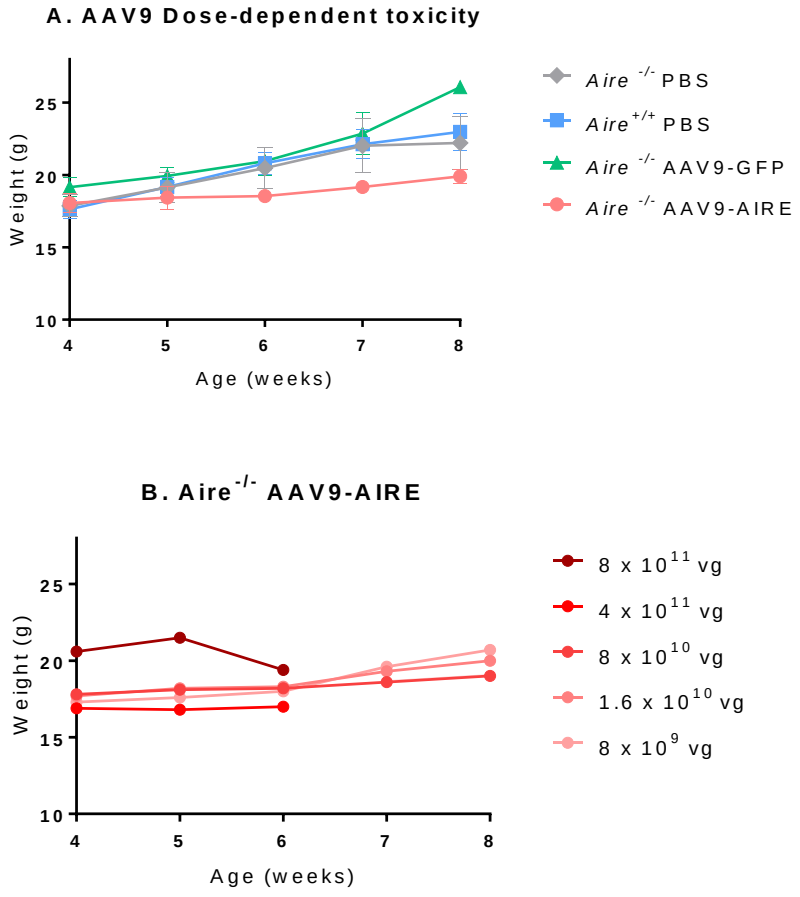



**Figure 5.7: Body weight of Aire mice from 4 to 8 weeks old.** [A] Mean weight of Aire$^{+/+}$ mice injected with PBS (*n*=5), *Aire*$^{-/-}$mice injected with PBS (*n*=5), *Aire*$^{-/-}$ mice injected with 5 different concentrations of AAV9-GFP (*n*=5) and *Aire*$^{-/-}$ mice injected with 5 different concentrations AAV9-AIRE. [B] Weight of individual *Aire*$^{-/-}$ mice injected with 5 different concentrations of AAV9-AIRE.

### 5.3.2 *In-vivo* thymic transduction efficiency of AAV9

Thymic section from mice injected with AAV9-GFP were fixed and cryopreserved in OCT (*see 2.6.1 for details*). Intrathymic injections of AAV9-GFP resulted in significant diffusion throughout the entire thymi of Aire mice (*n*=5). This finding demonstrates that intrathymic AAV9 delivery is highly efficient for targeting the thymus in young mice [*Figure 5.8*].

The levels of *AIRE* transgene expression following intrathymic injections of AAV9-AIRE into *Aire*$^{-/-}$ mice thymi was initially assessed by counting AIRE$^{+}$ cells per field in the thymi of *Aire*$^{+/+}$, *Aire*$^{-/-}$ and *Aire*$^{-/-}$ AAV9-AIRE mice at ×200 magnification. Intrathymic injections of AAV9-AIRE with five different doses successfully transduced the thymi of *Aire*$^{-/-}$ mice (*n*=5). Thymi were collected and fixed in 4% PFA, sectioned and histological analysis was undertaken by labelling thymic sections with AIRE D-17 antibody and the slides were analysed under a fluorescence microscope *[Figures 5.9, 5.10]*. AIRE D-17 antibody does cross-react with the mouse Aire protein. *Aire*$^{-/-}$ mice injected with high doses of AAV9-AIRE, 8 × $10^{11}$ vg and 4 × $10^{11}$ vg, were monitored only up to 11 days post injection as mentioned earlier in section 5.3.1. At 11-days post injections, AIRE$^{+}$ cells were counted in 5 random fields at ×200 magnification *[Figure 5.9]*. The proportion of AIRE$^{+}$ cells in the thymi of these mice were significantly high (48.4 ± 6.9 *P*=0.0001 and 25.8 ± 4.7 *P*=0.0006, respectively) when compared with AIRE$^{+}$ cells in untreated *Aire*$^{-/-}$ mouse *[Figure 5.11]*. At 30-days post injection, thymi were collected from *Aire*$^{-/-}$ mice that received intrathymic injections of AAV9-AIRE with the doses (8 x $10^{10}$, 1.6 x $10^{10}$, 8 x $10^{9}$) and from untreated *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice. AIRE$^{+}$

cells were counted at 5 random fields at ×200 magnification *[Figure 5.10]*. All three doses of AAV9-AIRE virus transduced the thymi of treated *Aire*$^{-/-}$ mice successfully and expressed AIRE when compared to untreated *Aire*$^{-/-}$ mice *[Figure 5.11]*.

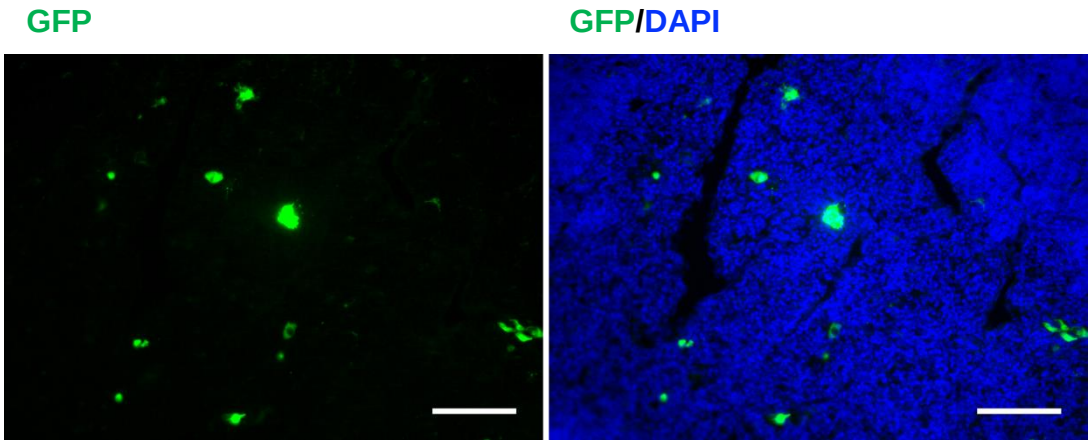



**Figure 5.8: Representative transduction of thymic cells in *Aire*$^{-/-}$ mice with AAV9-GFP (1 x 10$^{11}$ vg) after 30-days.** Thymic sections from *Aire*$^{-/-}$ mice were fixed and cryopreserved. Sections were immediately stained with DAPI (blue) for visualization of nuclei. Images were taken using x200 magnification. Scale bar 100 µm.

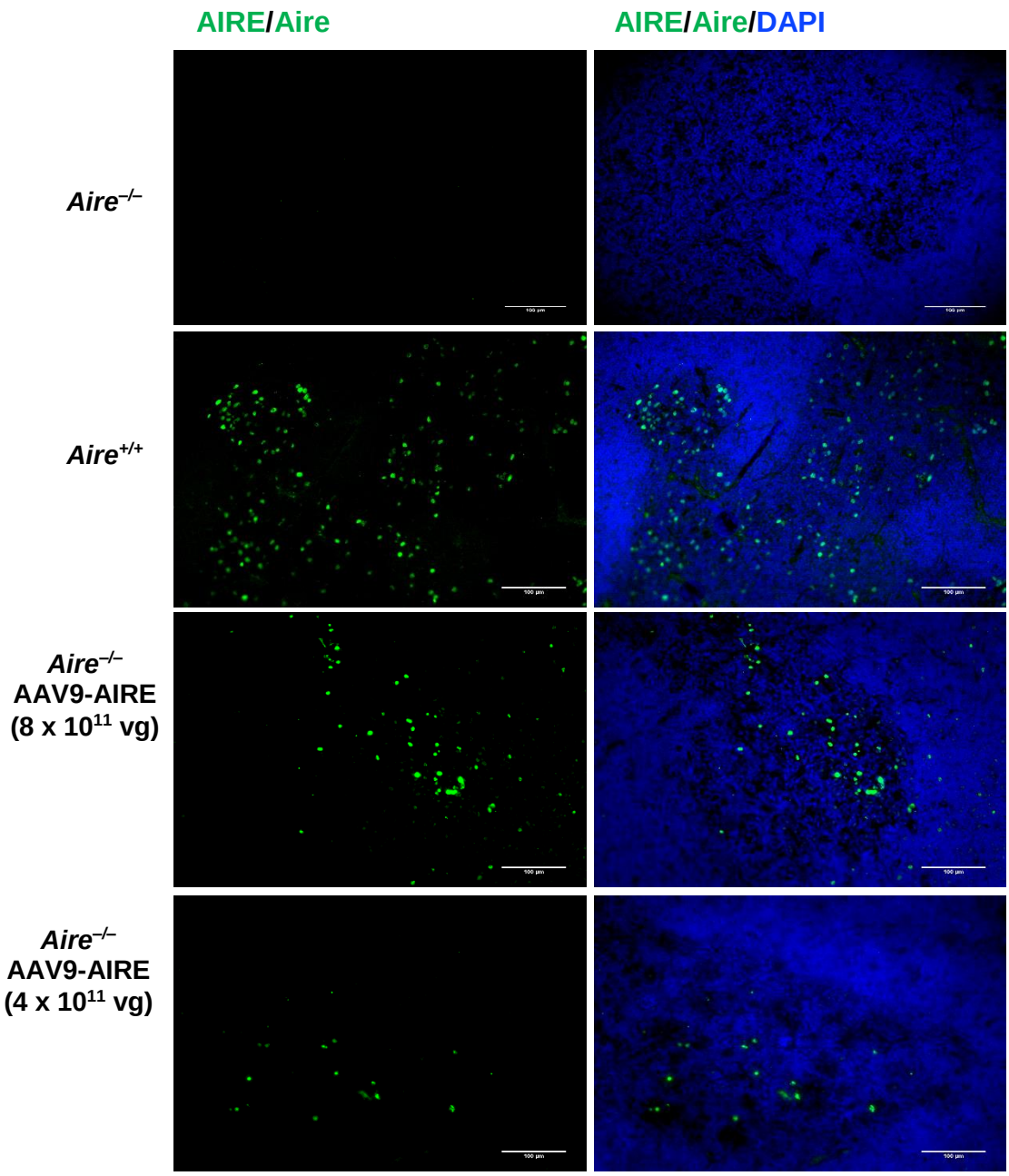



**Figure 5.9: Transduction of thymic cells in *Aire*$^{-/-}$ mice with AAV9-AIRE at $10^{11}$ vg up to 11 days.** Thymic sections from *Aire*$^{-/-}$ mice treated with AAV9-AIRE. The sections were fixed, labelled with AIRE D-17 antibody (green) and DAPI (blue) for visualization of nuclei. Images were taken using x200 magnification. Scale bar 100 µm.

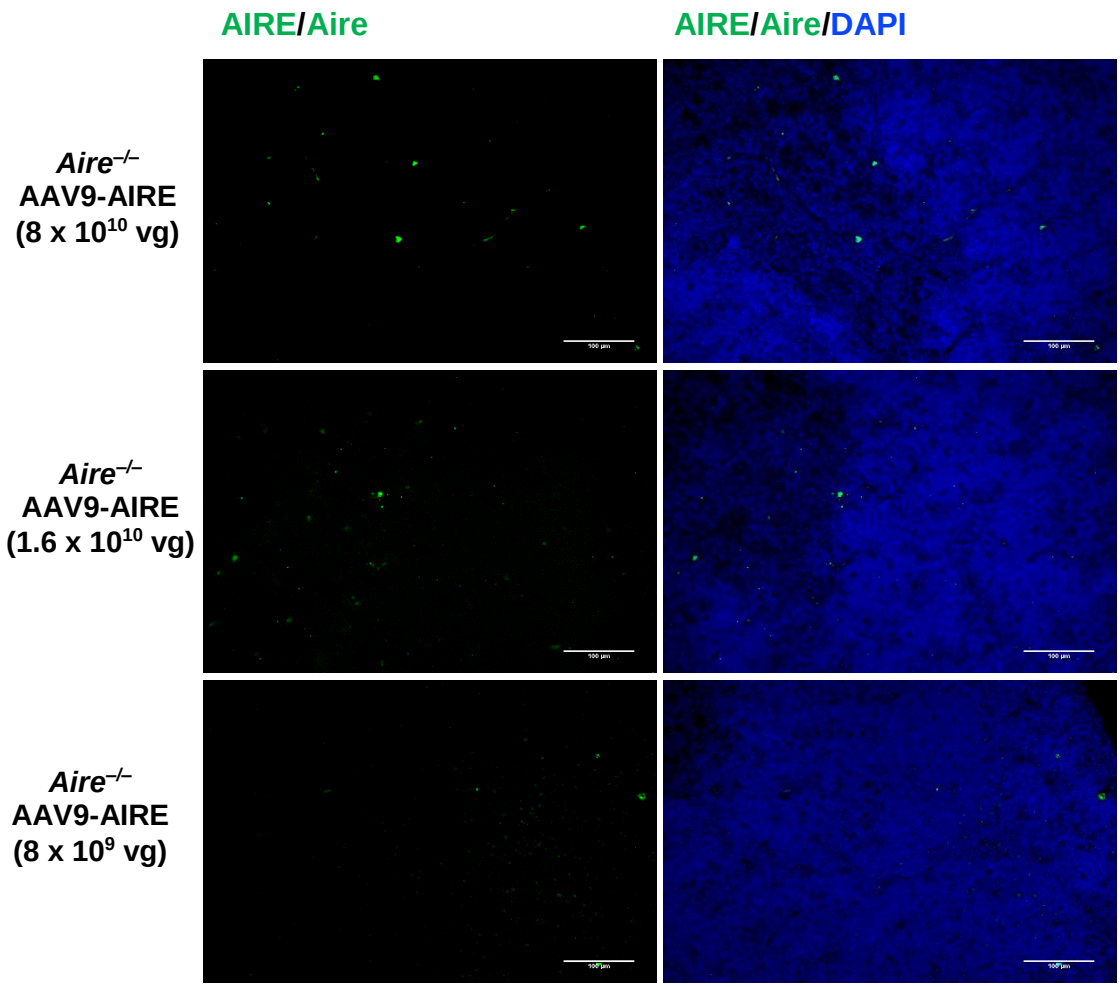



**Figure 5.10: Transduction of thymic cells in *Aire*$^{-/-}$ mice with AAV9-AIRE at $10^{10}$-$10^{9}$ vg up to 30 days.** Thymic sections from *Aire*$^{-/-}$ mice treated with AAV9-AIRE. The sections were fixed, labelled with as AIRE D-17 antibody (green) and DAPI (blue) for visualization of nuclei. Images were taken using x200 magnification. Scale bar 100 µm.

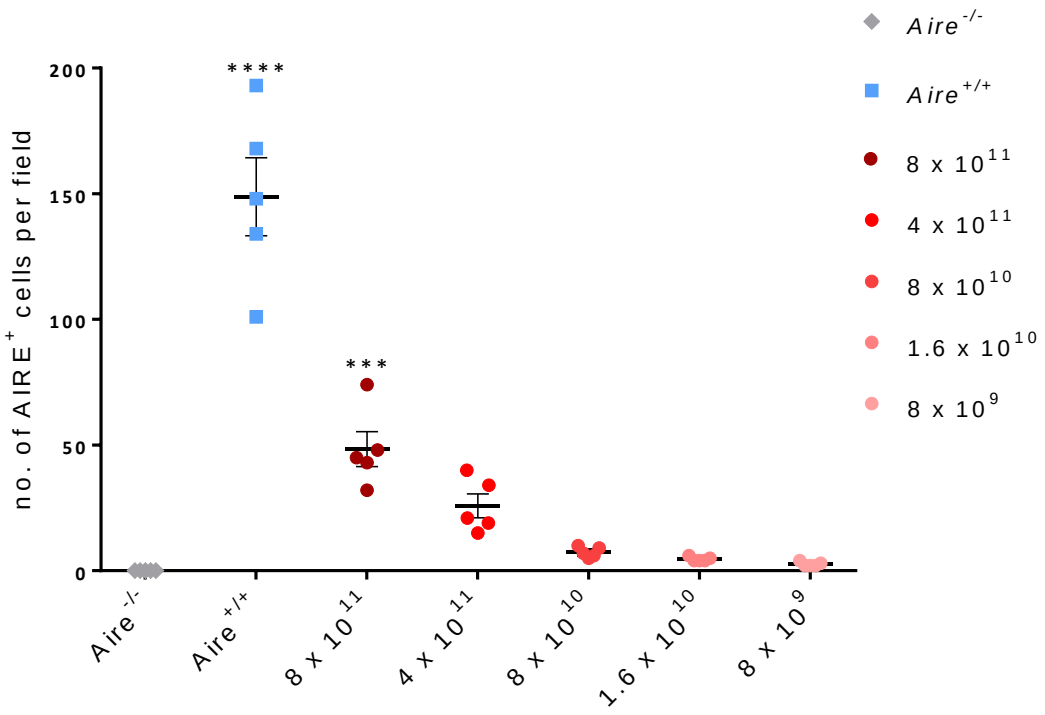



**Figure 5.11: Quantification of AIRE⁺ cells by counting across all groups.** All data represent the average number of AIRE⁺ cells per field of view per group (*n*=5) and were compared to AIRE⁺ cells of *Aire*$^{-/-}$ mice. Data analysed by one-way ANOVA, Dunnett's multiple comparisons test, *** $P \leq 0.001$, **** $P \leq 0.0001$, ± SEM.

### 5.3.3 Induction of expression of AIRE-dependent genes by AAV9-AIRE virus

Next, we wanted to determine whether the ectopic expression of any Aire-dependent genes could be induced after AAV9-mediated expression of AIRE in *Aire*$^{-/-}$ mice. Overall, AAV9-AIRE mediated significant over-expression of *AIRE* in *Aire*$^{-/-}$ mice in a dose-dependent manner.

The qPCR data demonstrate that 11-30 days post injection, AAV9-AIRE successfully upregulated the Aire-dependent TSA, insulin II (*Ins2*) *[Figure 5.12]*. Moreover, elevated expression of *AIRE* had neutral effect on Aire-independent gene: Forkhead box N1 (*Foxn1*). The two high doses of AAV9-AIRE at $10^{11}$ vg expressed *AIRE* significantly from 1000 folds up to almost 4000 folds relatively higher to its expression in untreated *Aire*$^{-/-}$ mice. The lower doses of AAV9-AIRE ranged from $10^{9}$-$10^{10}$ vg also significantly expressed *AIRE* at high levels ranging from 34-300 folds increase relative to its expression in untreated *Aire*$^{-/-}$ mice (***P* = 0.0036 and ****P* =0.0002). All five mice treated with AAV9-AIRE at different doses expressed *Ins2* up to 42 folds higher than its relative expression in untreated *Aire*$^{-/-}$ mice. The increased expression levels of *Ins2* in AAV9-AIRE treated mice are comparable to the expression levels of *Ins2* expressed in *Aire*$^{+/+}$ littermates, except for the dose 8 x $10^{10}$ where the level of expression was higher than in *Aire*$^{-/-}$ mice but lower than *Aire*$^{+/+}$ littermates.

The three mice that were injected with AAV9-AIRE for 30-days moderately expressed *Csnα* at a higher levels when compared to the *Aire*$^{-/-}$ mice. On the other hand, the two high doses of AAV9-AIRE at $10^{11}$ vg showed

lower level of *Csnα* compared to the three doses of AAV9-AIRE at $10^{10}$ vg and $10^{9}$; this could be due to the short duration of AIRE expression in these mice. Given that the AAV9 used in this study is conventional ssAAV9, where few days are often required for adequate transgene expression, and few weeks to achieve significant transgene expression after vector delivery (Wang *et al.* 2003). In addition, as mentioned in section 5.3.2, these mice were sacrificed at 11-days post injection, which means AIRE transgene was expressed only for few days.

These data suggest a clear correlation between the expression of AIRE and Ins2 and indicate that approaches to stimulate AIRE expression in *Aire*$^{-/-}$ mice could be considered to regulate self-tolerance induction to peripheral antigens.

### 5.3.4 Serum autoantibodies analysis in AAV9-AIRE and AAV9-GFP injected *Aire*$^{-/-}$ mice

To see whether the abrupt expression of AIRE had maintained the self-tolerance in *Aire*$^{-/-}$ mice, we analysed the presence of autoantibodies in *Aire*$^{-/-}$ mice sera using 4% PFA fixed tissue sections from healthy mice by indirect immunofluorescence. As the two mice treated with the high doses of AAV9-AIRE at $10^{11}$ vg were sacrificed 11-days post injection, positive reactions from their sera were found against: retina, lung and reproductive system: testis in the case of the male ($8 \times 10^{11}$ vg) and ovary in the case of the female ($4 \times 10^{11}$ vg) *[Figure 5.13]*.

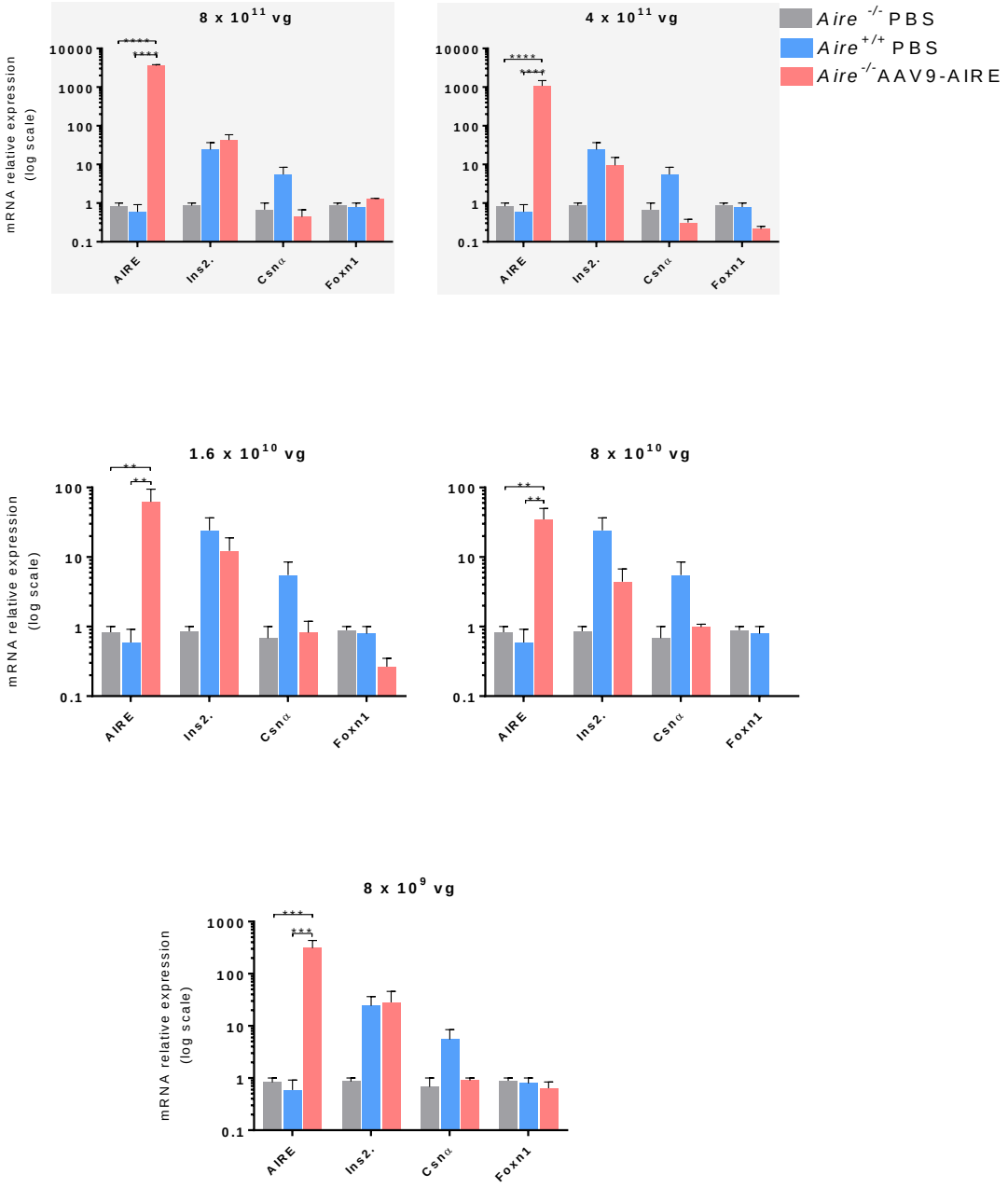



**Figure 5.12: Dose-response differential TSAs gene expression fold changes of selected genes.** Quantitative PCR analysis of expression of *Aire/AIRE*, Aire-dependent TSAs (*Ins2* and *Csnα*), and TECs transcriptional regulator (*Foxn1*) in thymic cells obtained from 8 week old *Aire*$^{+/+}$ mice (*n*=2), *Aire*$^{-/-}$ mice (*n*=2), and *Aire*$^{-/-}$ AAV9-AIRE injected mice (*n*=1 for each dose). Results are normalized to the expression of *β–actin* and are presented relative to the expression values in *Aire*$^{-/-}$ thymic cells. Data are representative of two independent experiments with similar results by using the 2^(-ΔΔCt) method. Data analysed by two-way ANOVA, Tukey's multiple comparisons test. **** *P* <0.0001, *** *P* < 0.0005, ** *P* < 0.005. ± SEM

Autoantibodies positive for the retina were found against the photoreceptor layer. In the lung, autoantibodies were found against the bronchial epithelium. In the ovary, serum was reactive against the cytoplasm of the oocyte, and in the testis serum was reactive against the Leydig cells. However, sera from mice treated with AAV9-AIRE for 30-days exhibited reduced to almost non-detectable levels of autoantibodies against tissue sections from *Aire*$^{+/+}$ littermates *[Figure 5.14]*. These data suggest that the clearance rate of serum autoantibody is the variable and not the dose of the injected AAV9-AIRE.

As a control group, five *Aire*$^{-/-}$ were injected with AAV9-GFP at different doses similar to AAV9-AIRE and monitored for up to 4 weeks post injection. Sera from these mice were reactive for most of the tissues: retina, lung, stomach and ovaries *[Figure 5.15]*. Unfortunately, the testis tissues were not performed in this group as the slides were mixed up with another tissue. Overall, neither the AAV9 vector nor the GFP transgene had an effect on the serum autoantibodies of *Aire*$^{-/-}$ mice, confirming that the reduced levels of serum autoantibodies in mice treated with AAV9-AIRE were due to the therapeutic effect of AIRE expression *[Figure 5.16]*.

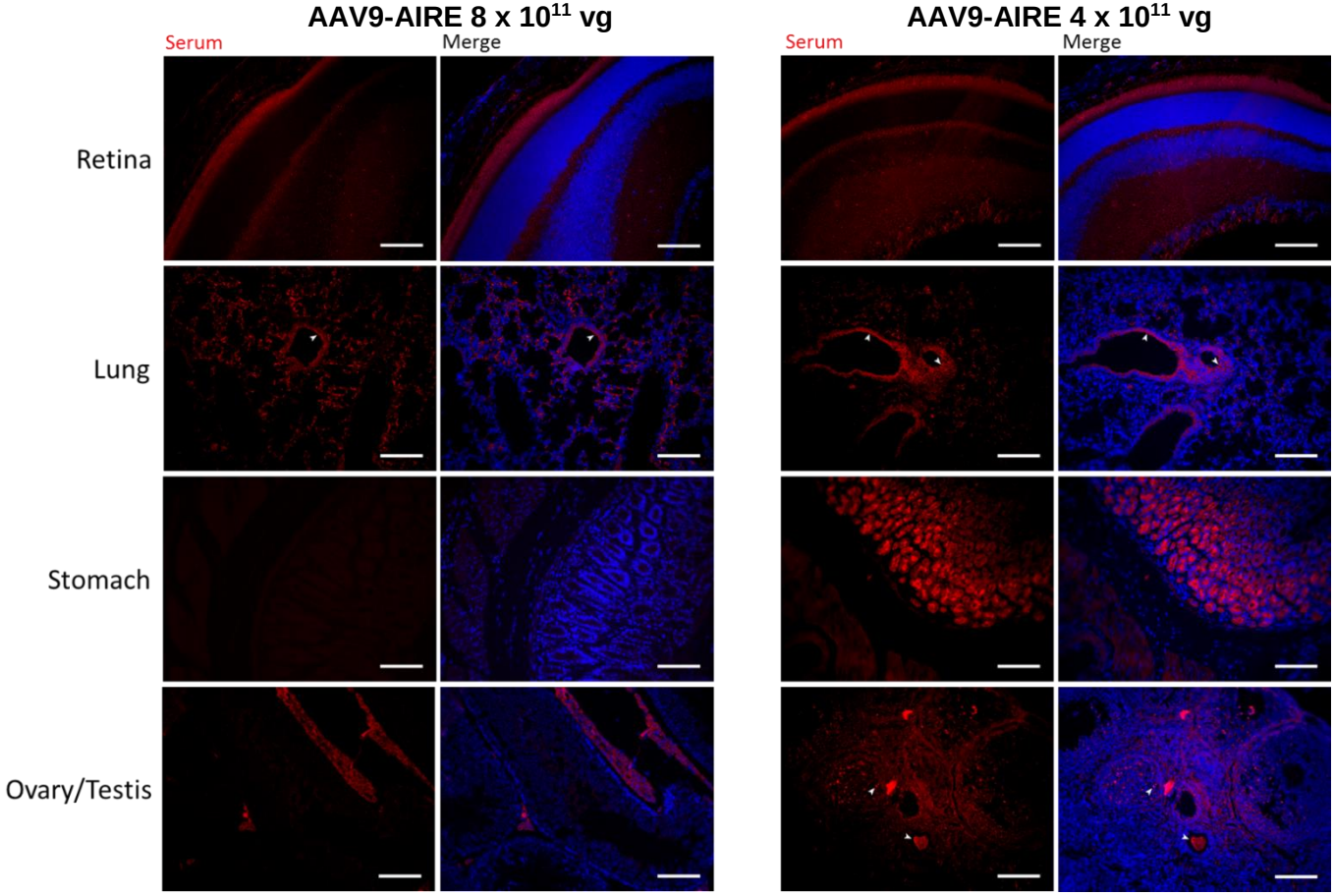



**Figure 5.13: Immunofluorescence analysis of serum autoantibodies in *Aire*$^{-/-}$ AAV9-AIRE (8x10$^{11}$ – 4x10$^{11}$vg) injected mice for 11 days.** Staining of healthy mouse organs with sera from *Aire*$^{-/-}$ AAV9-AIRE (8 x 10$^{11}$ vg, 4 x 10$^{11}$ vg) mice using indirect immunofluorescence. Autoantibodies were detected in serum from *Aire*$^{-/-}$ AAV9-AIRE (8 x 10$^{11}$ vg, 4 x 10$^{11}$ vg) reactive to retina; Photoreceptor layer, lung; Bronchial epithelium. Autoantibodies from *Aire*$^{-/-}$ AAV9-AIRE (4 x 10$^{11}$ vg) mouse reactive to stomach; Parietal cells. Autoantibodies from *Aire*$^{-/-}$ AAV9-AIRE (8 x 10$^{11}$ vg) mouse reactive to testis; Leydig cells. Autoantibodies from *Aire*$^{-/-}$ AAV9-AIRE (4 x 10$^{11}$ vg) mouse reactive to ovary; cytoplasm of the oocyte. Serum autoantibodies (red), the nucleus counterstained with DAPI Diamidino-2-phenylindole (blue). Scale bar 100 µm.

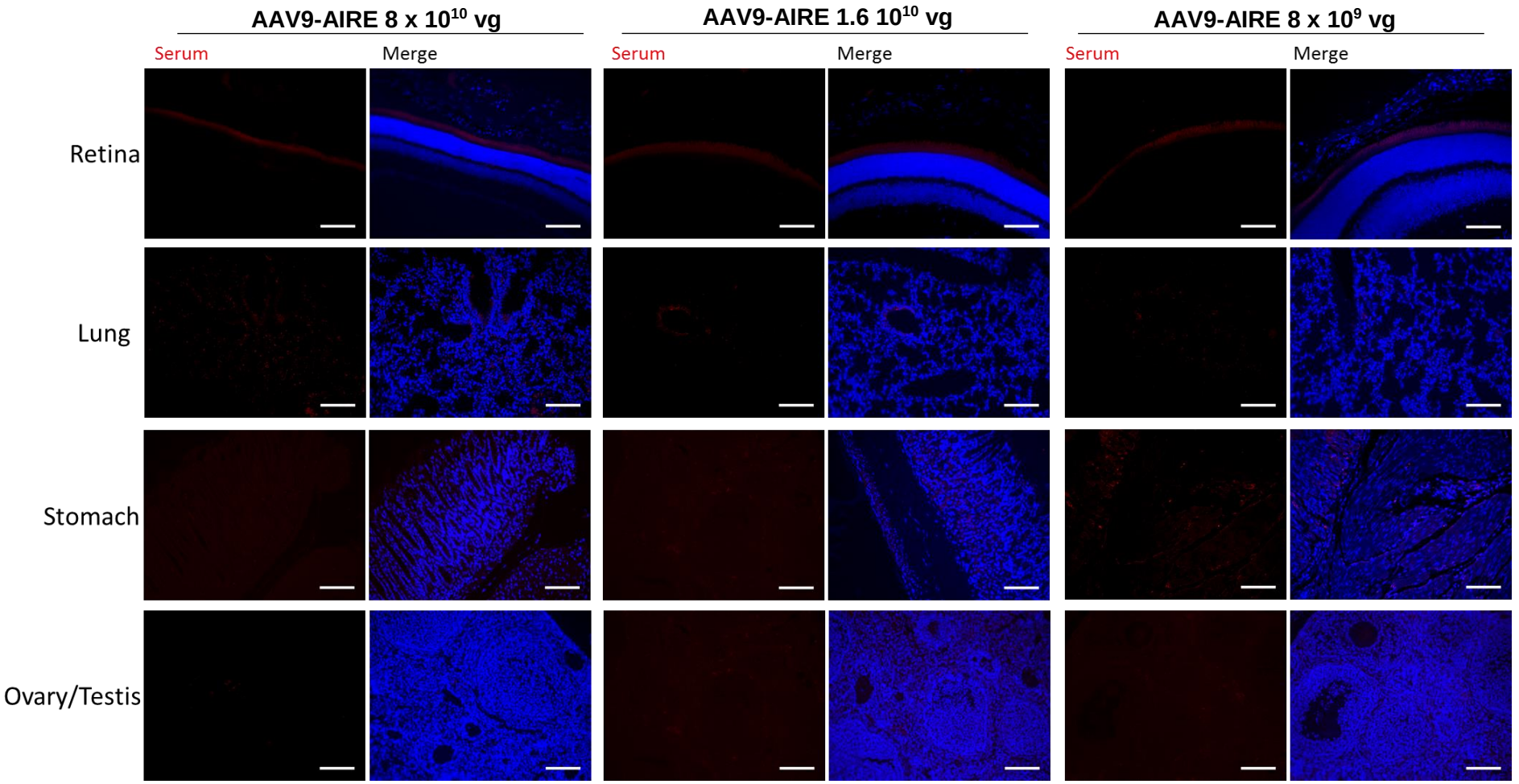



**Figure 5.14: Immunofluorescence analysis of serum autoantibodies in *Aire*$^{-/-}$ AAV9-AIRE (8x10$^{10}$ – 1.6x10$^{10}$ and 8x10$^{9}$ vg) injected mice up to 30 days.** Staining of healthy mouse organs with sera from *Aire*$^{-/-}$ AAV9-AIRE (8 x 10$^{10}$ vg, 1.6 x 10$^{10}$ vg, 8 x 10$^{9}$ vg) mice using indirect immunofluorescence. Autoantibodies levels were reduced to almost undetectable on these tissues: retina, lung, stomach and ovary. Serum autoantibodies (red), the nucleus counterstained with DAPI Diamidino-2-phenylindole (blue). Scale bar 100 µm.

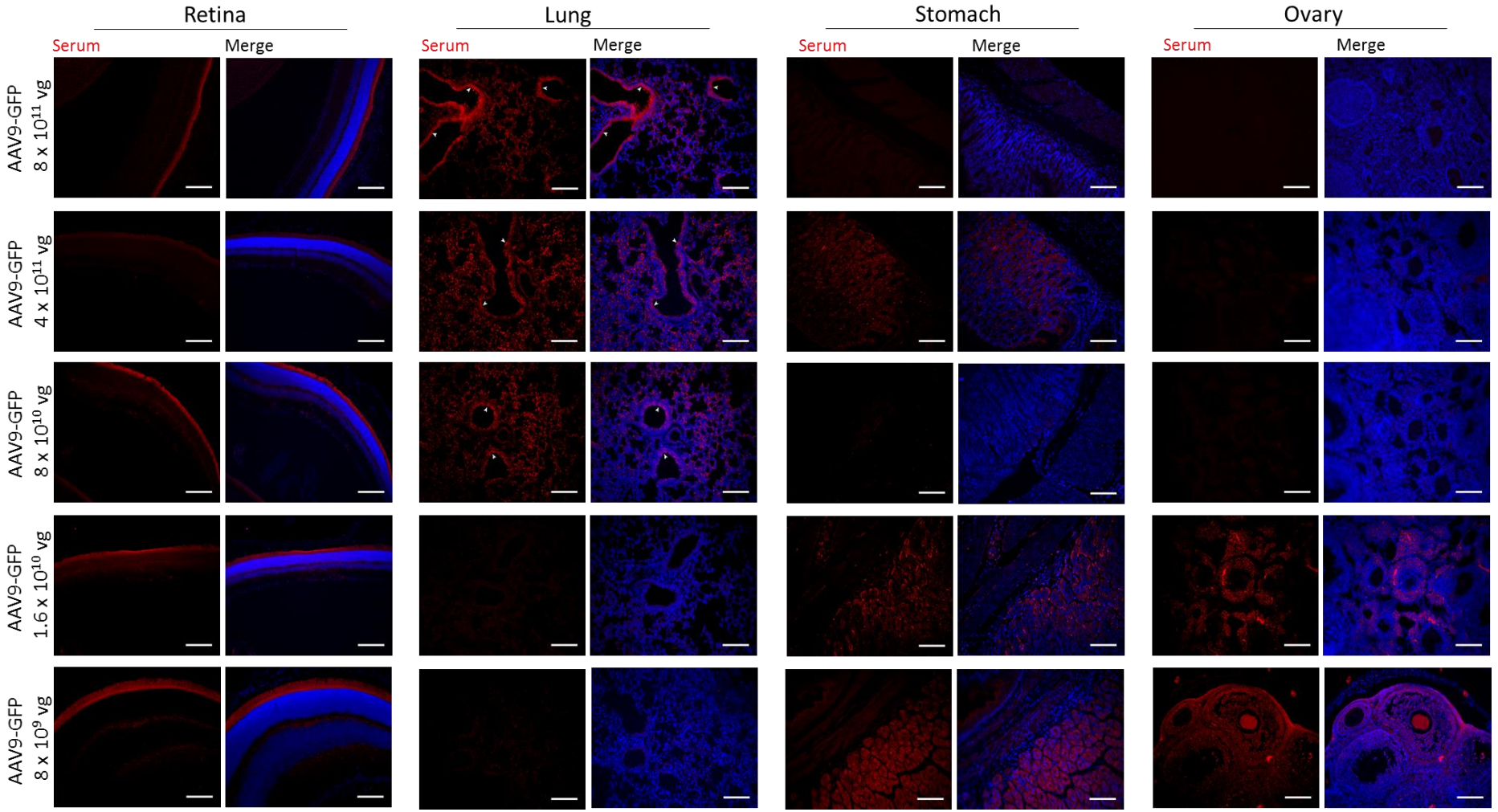



**Figure 5.15: Immunofluorescence analysis of serum autoantibodies in *Aire*$^{-/-}$ AAV9-GFP injected mice.**

**Figure 5.15: Immunofluorescence analysis of serum autoantibodies in *Aire*$^{-/-}$ AAV9-GFP injected mice.** Staining of healthy mouse organs with sera from Aire AAV9-GFP (8 x $10^{11}$ vg, 4 x $10^{11}$ vg, 8 x $10^{10}$ vg, 1.6 x $10^{10}$ vg, 8 x $10^{9}$ vg) mice using indirect immunofluorescence. Autoantibodies were detected in sera from Aire AAV9-GFP (8 x $10^{11}$ vg, 8 x $10^{10}$ vg, 1.6 x $10^{10}$ vg) mice reactive to retina; Photoreceptor layer. Autoantibodies from AAV9-GFP (8 x $10^{11}$ vg, 4 x $10^{11}$ vg, 8 x $10^{10}$ vg) mice reactive to lung; Bronchial epithelium. Autoantibodies from AAV9-GFP (4 x $10^{11}$ vg, 8 x $10^{10}$ vg) mice reactive to stomach; Parietal cells. Autoantibodies from AAV9-GFP (1.6 x $10^{10}$ vg, 8 x $10^{9}$ vg) mice reactive to ovary; cytoplasm of the oocyte. Serum autoantibodies (red), the nucleus stained with DAPI Diamidino-2-phenylindole (blue). Scale bar 100 µm.

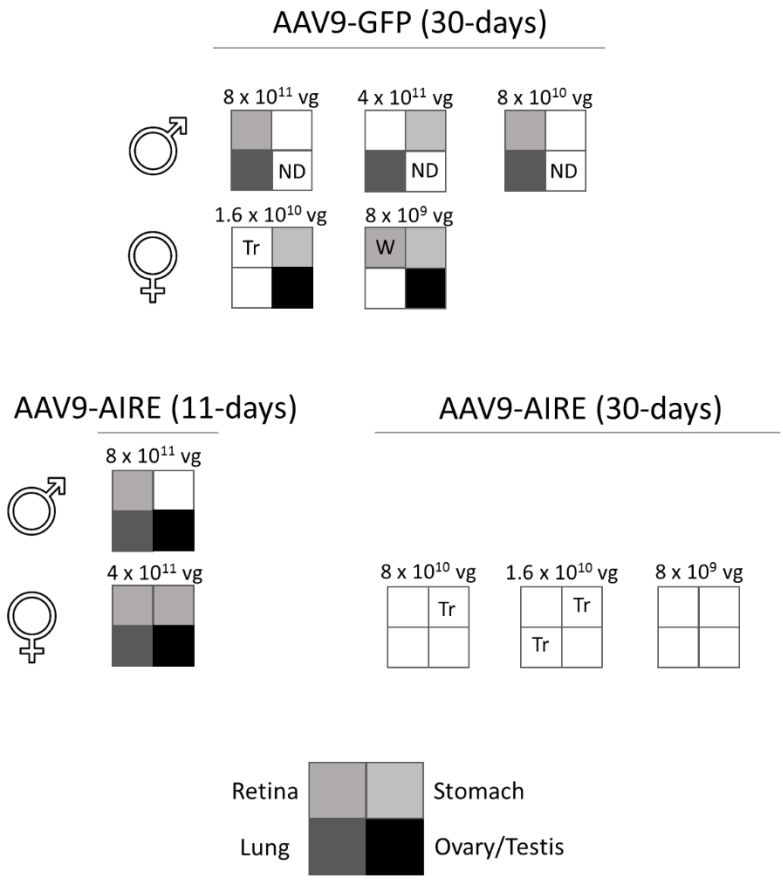



**Figure 5.16: Summary of serum autoantibodies against tissues in *Aire*$^{-/-}$ mice injected with AAV9.** For each mouse of the indicated AAV9 virus, gender and dose, presence (shaded) or absence of autoantibodies against: retina, lung, stomach and ovary/testis. Tr; trace autoantibodies, W; weak reaction, ND; not done.

## 5.4 Discussion

In this optimisation study, the route of delivery and the methods of gene transfer detection and doses were evaluated. Accurate intrathymic injections were confirmed in 92% of cases, with no sign of complications were observed in *Aire*$^{-/-}$ mice suggesting that this approach is non-invasive. Based on the results from this study, intrathymic injection was chosen as the route of delivery. Histological analysis was then used to assess transduction efficiency. The expression of *AIRE* in thymic cells of 5-8 weeks old *Aire*$^{-/-}$ mice after intrathymic injections of AAV9-AIRE was confirmed. The augmented expression of functional AIRE led to upregulated expression of the Aire-dependent TSA, *Ins2* in thymocytes and reduced the levels of serum autoantibodies against several tissues, which attenuates the severity of autoimmunity in APS-1 mouse model.

In APS-1 mouse model, intrathymic delivery appears to be most appropriate approach to restrict AIRE transgene expression within the thymus. *Aire*$^{-/-}$ mice were injected at 4-week-old, during the first 30 days of postnatal life in mice; the immune system has not fully matured. For instance, it has been shown that B-cells have an immature phenotype until four weeks of age (Landreth 2002). In addition, antibodies response to T-cell independent antigens occurs soon after birth and reach near-adult levels by 2 or 3 weeks. In contrast, T-cell dependent antibody responses begin after two weeks and mature around 6–8 weeks of age (Holladay *et al.* 2000). For these reasons, 4-weeks of age was ideal to introduce AAV9-AIRE into *Aire*$^{-/-}$ mice as the immune system still immature. At the same time, mice are considered infant

from birth (P0) to 21 days of age, at that age they are weaned. They moved into new cages, and during that fourth week of age, AAV9-AIRE injections were carried out directly into the thymus.

To define the dose range for the long-term efficacy study, a short-term toxicity study was conducted over a 4-week period. Five 4-week-old *Aire*$^{-/-}$ mice were individually injected with 5 different AAV9-AIRE doses, $8 \times 10^{11}$ vg, $4 \times 10^{11}$ vg, $8 \times 10^{10}$ vg, $1.6 \times 10^{10}$ vg and $8 \times 10^{9}$ vg per thymus. Eleven days after injection, the two mice that were injected with $8 \times 10^{11}$ or $4 \times 10^{11}$ vg exhibited toxicity phenotype including: weight loss, rough coat and hunched posterior. These mice had to be withdrawn from the experiment and humanly sacrificed. The same doses were used to inject AAV9-GFP into *Aire*$^{-/-}$ littermates as a control group. However, *Aire*$^{-/-}$ injected with the high doses of AAV9-GFP ($8 \times 10^{11}$ or $4 \times 10^{11}$ vg) did not exhibited any toxicity effects suggesting that high doses of GFP was well tolerated, and high expression of AIRE rather than AAV9 capsid is causing this effects. A possible explanation could be due to the fact that AIRE overexpression induced apoptosis in mouse thymic cell lines (Gray *et al.* 2007, Dooley *et al.* 2008, Abramson *et al.* 2010). In addition, AIRE-induced apoptotic signals may originate from genotoxic or oxidative stress resulting in GAPDH translocation into the nuclei (Liiv *et al.* 2012). Therefore, the doses of: $8 \times 10^{11}$, $4 \times 10^{11}$ vg as well as $8 \times 10^{10}$ vg per thymus were not included in the subsequent long-term efficacy study. As the toxicity observed at high vector doses, highlights the importance of careful dose optimization in future clinical experiments.

The *in-vivo* transduction efficiency was verified by immunofluorescence analysis of the vector-treated thymi. More AIRE$^{+}$ cells were observed in $8 \times$

$10^{11}$ vg and $4 \times 10^{11}$ vg per thymus when compared to the AIRE$^{+}$ cells in the other three doses of AAV9-AIRE vector. This observation was confirmed with the mRNA analysis, where *AIRE* expression was expressed up to two-fold change when compared to the endogenous *Aire* levels in wild type littermates. While the doses at $10^{10}$ vg and $10^{9}$ vg/thymus showed lower AIRE$^{+}$ cells than the high doses, but significantly high number of AIRE$^{+}$ thymic cells when compared to AIRE$^{+}$ thymic cells in *Aire*$^{-/-}$ mice.

Following the significantly high transduction levels of AIRE in *Aire*$^{-/-}$ mice thymi, the mRNA expression levels of TSAs were tested including two Aire-dependent genes and one Aire-independent gene. *Ins2* is Aire-dependent gene that is highly expressed in mTECs as well as pancreatic beta cells (Chentoufi *et al.* 2002, Derbinski *et al.* 2005). Furthermore, *Csnα*, a milk protein gene that is Aire-dependent and highly expressed in mammary glands (Liston *et al.* 2004, Derbinski *et al.* 2005). To make sure that augmented expression of AIRE will not alter the expression of other Aire-independent genes, the mRNA expression level of *Foxn1* gene was measured. Foxn1 is a transcriptional regulator that is highly expressed in the thymus and regulates TECs lineage development (Coffer *et al.* 2004). Real-time PCR showed that *Ins2* expression was elevated in all *Aire*$^{-/-}$ injected with AAV9-AIRE. This observation is similar to another study, where Aire was over-expressed in thymic medullary epithelial cell line using adenoviral vector has led to upregulation of four Aire-dependent TSAs *in-vitro* (Kont *et al.* 2008).

To further confirm that AIRE restored its function in self-tolerance in *Aire*$^{-/-}$ mice injected with AAV9-AIRE, serum autoantibodies presence/absence in these mice and *Aire*$^{-/-}$ mice injected with AAV9-GFP

were analysed. These data demonstrate that 30-days post injections, serum autoantibodies were reduced to almost non-detectable levels in *Aire*$^{-/-}$ mice treated with AAV9-AIRE. While the control group demonstrated serum autoantibodies reactive to at least two tissues from healthy age and gender-matched mice. On the other hand, the two Aire mice injected with two highest doses exhibited toxicity effect, they have been treated for only 11 days, and so, serum autoantibodies were still detectable at that time point.

In conclusion, all five different doses of AAV9-AIRE transduced the thymi of Aire mice successfully, and generally have the same effect on the three tested TSAs expression and serum autoantibodies reduction. Thus, the dose, $1.6 \times 10^{10}$ vg, was chosen for the next efficacy study as a lower safer dose but over-expresses *AIRE*.

# *CHAPTER SIX*

# PROOF-OF-CONCEPT EFFICACY STUDY:

# THERAPEUTIC EFFECTS OF AAV9-AIRE

# 6. PROOF-OF-CONCEPT EFFICACY STUDY: THERAPEUTIC EFFECTS OF AIRE

## 6.1 Outline

Chapter 5 demonstrated that *AIRE* over-expression in *Aire*$^{-/-}$ mice led to significant transgene expression and ameliorated autoimmunity phenotype; suggesting that restoration of AIRE may re-establish self-tolerance in *Aire*$^{-/-}$ mice. This *in vivo* proof-of-concept efficacy study will assess the therapeutic effects of Aire replacement on APS-1 mouse model following delivery of AAV9-AIRE intrathymically in *Aire*$^{-/-}$ mice.

In this part of the study, 40 mice were used based on a power analysis. A combination of 4-week-old male and female *Aire*$^{-/-}$ and *Aire*$^{+/+}$ mice were injected with either PBS (sham control group, $n$ = 20), AAV9-GFP (vehicle control group, $n$ = 10), or AAV9-AIRE (treatment group $n$ = 10). Following the intrathymic injections, the impact of AIRE replacement on restoring the central tolerance in APS-1 mouse model was assessed.

## 6.2 Efficacy study

Four weeks old *Aire*$^{-/-}$ mice were intrathymically injected with 20µl of AAV9-AIRE containing 1.6 x $10^{10}$ vg (*n* = 10). As a control, *Aire*$^{-/-}$ mice were intrathymically injected with AAV9-GFP containing 1 x $10^{10}$ vg (*n* = 10). Sham littermates were intrathymically injected with PBS, *Aire*$^{+/+}$ mice (*n* = 10) and *Aire*$^{-/-}$ mice (*n* = 10). To determine whether there was any effect on the mice growth, weekly body weight assessment was recorded up to 8-weeks (short-term treatment, *n* = 5 per group) or 16-weeks (long-term treatment, *n* = 5 per group).

## 6.3 Short-term effect of a single dose of AAV9-AIRE injection on APS-1 mouse model

### 6.3.1 Weight distribution

The short-term groups were monitored up to 8-week-old, 4-weeks post injections *[Figure 6.1]*. All mice gained weight normally and no statistical difference was found between groups, suggesting a normal body weight gain obtained by all *Aire*$^{-/-}$ mice.

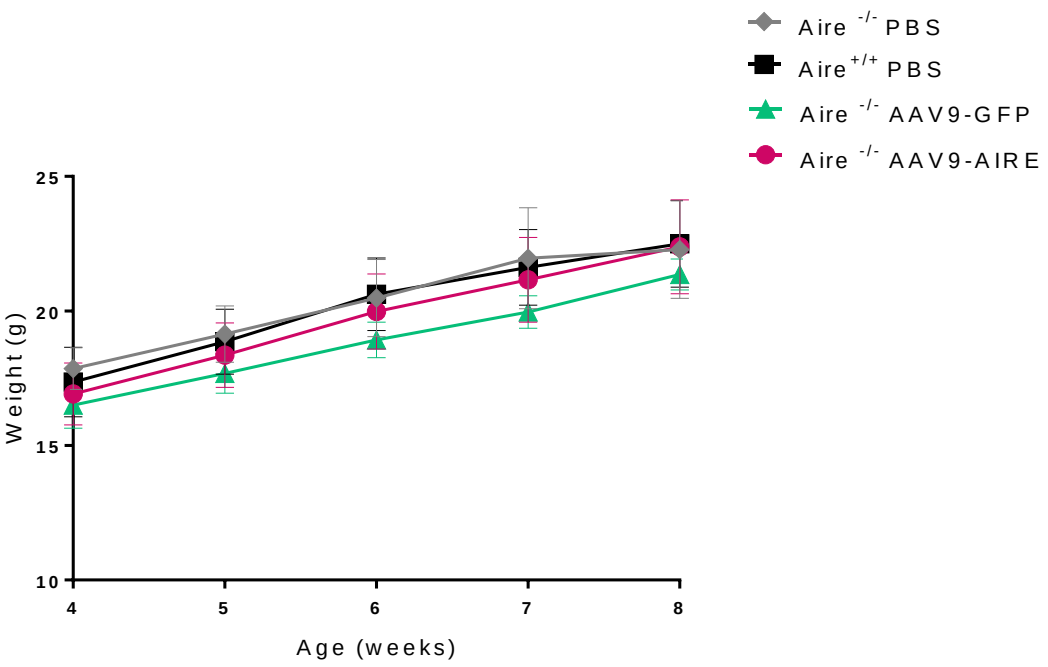



**Figure 6.1: Body weight assessment of *Aire* mice 4-weeks post injections.** Changes in body weight over time in all experimental mice, (*n* = *5* per group). All data points represent the average weight per group. AAV9 – Adeno-associated virus serotype 9; GFP – Green fluorescent protein; AIRE – Autoimmune regulator.

### 6.3.2 Induction of AIRE expression in thymic cells by AAV9-AIRE virus

At the end stage of the short-term treatment, thymi were collected from all groups. From each thymus, one lobe was fixed in 4% PFA for histological analysis and the other lobe was harvested for RNA extraction (*see section 2.6.1*). Thymic sections were stained with anti-AIRE antibody and the nuclear counterstain DAPI *[Figure 6.2]*. AIRE+ thymic cells were observed in thymic sections of 8-week-old *Aire*$^{+/+}$ (*n* = 5) and *Aire*$^{-/-}$ AAV9-AIRE mice (*n* =5). AIRE expression pattern in *Aire*$^{-/-}$ AAV9-AIRE treated mice was nuclear and resembles the punctate pattern of AIRE expression in *Aire*$^{+/+}$ littermates.

These data suggest that AAV9 mediates successful restoration of thymic AIRE protein expression 4 weeks post-vector delivery.

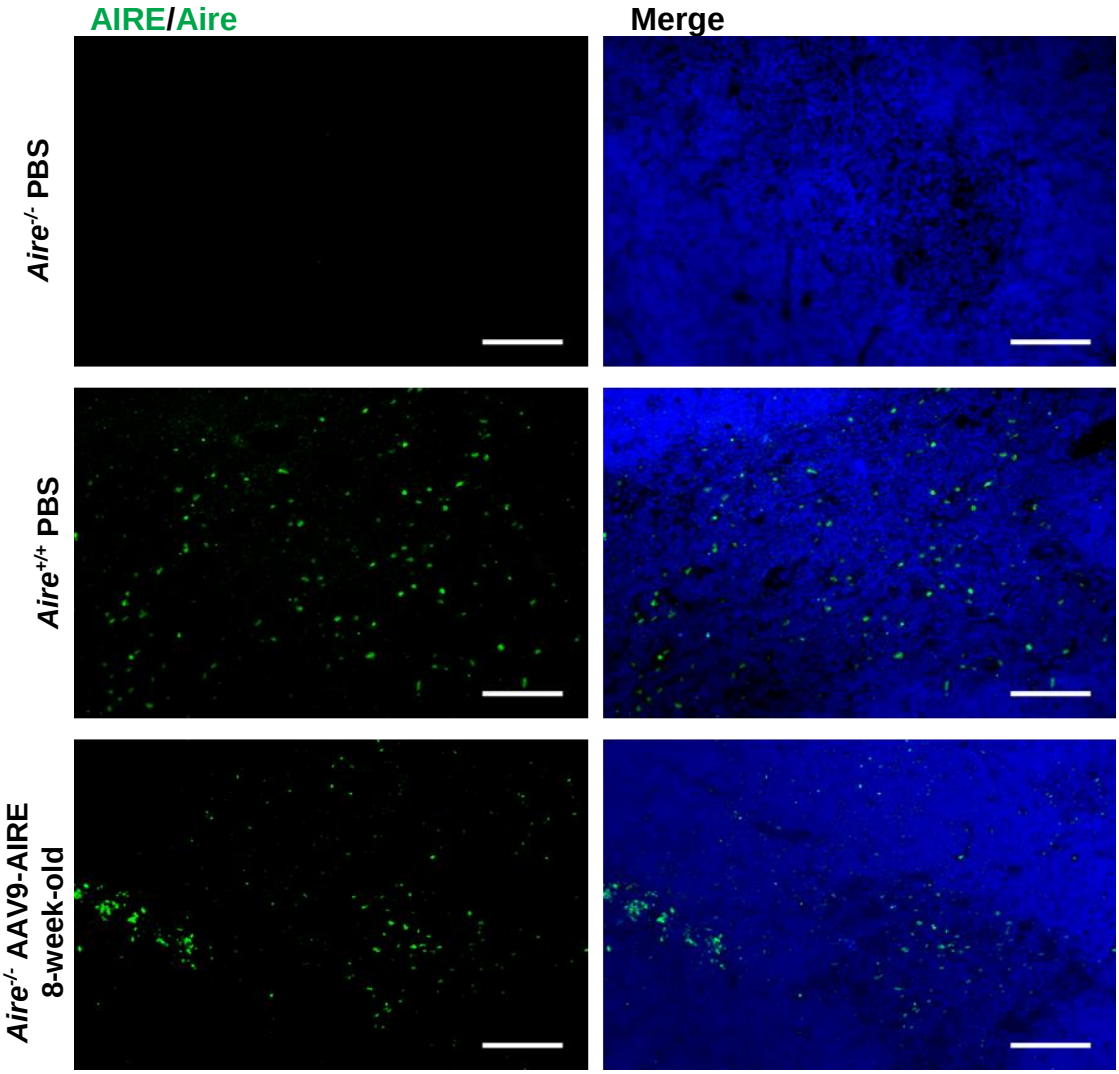



**Figure 6.2: Representative images of transduced thymi of 8-week-old APS-1 mice.** Mice were injected at 4-week-old: *Aire*$^{+/+}$ with PBS, *Aire*$^{-/-}$ with PBS or AAV9-AIRE. Thymi were extracted at 4-weeks post-injection. The thymic sections were labelled with anti-AIRE antibody, coupled to Alexa Fluor 488 secondary antibody and counterstained with DAPI for visualization of nuclei. AAV9 – Adeno-associated virus serotype 9; AIRE – Autoimmune regulator; DAPI- Diamidino-2-phenylindole (blue); PBS – Phosphate buffered saline. Scale bar 100 μm.

To quantify the restored *AIRE* at the mRNA level, qPCR analysis was performed on thymic lysates of all groups. 4-weeks post injection, AAV9-AIRE significantly expressed *AIRE* at very high levels, more than 2000 fold change ($p$ = 0.0183) when compared to endogenous mRNA levels of *Aire* in *Aire*$^{-/-}$ mice *[Figure 6.3]*. Moreover, the levels of *AIRE* expression in AAV9-AIRE injected mice were higher than the endogenous levels of *Aire* in *Aire*$^{+/+}$ mice. However, some *AIRE* mRNA was detected in *Aire*$^{+/+}$ mice. This could be due to the 75% homology between human *AIRE* and murine *Aire* at mRNA level, *AIRE* primers might detect some exons that are only present in the full length *Aire* in *Aire*$^{+/+}$ mice but not in the mutated *Aire* in *Aire*$^{-/-}$ mice. Nevertheless, *AIRE* expression level in AAV9-AIRE injected mice is significantly higher than its expression level in *Aire*$^{+/+}$ mice ($p$ = 0.0123).

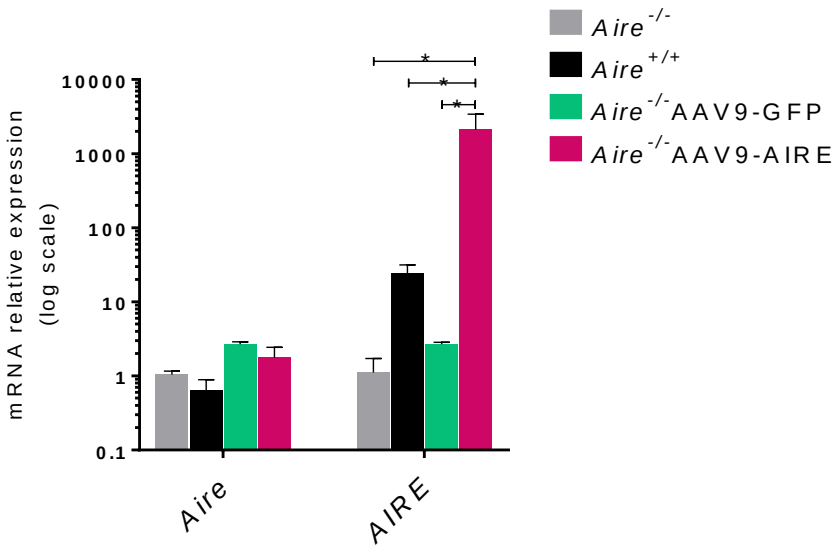



**Figure 6.3: *AIRE* over-expression after viral delivery of AIRE *in-vivo* in 8-week-old APS-1 mice**. Quantitative PCR analysis of expression of *Aire* and *AIRE* in thymic cells obtained 4-weeks post injection from *Aire*$^{+/+}$ mice ($n$ = 4), *Aire*$^{-/-}$ mice ($n$ = 4), *Aire*$^{-/-}$ AAV9-GFP mice ($n$ = 3), and *Aire*$^{-/-}$ AAV9-AIRE injected mice ($n$ = 3). Results are normalized to the expression of *β-actin* and are presented relative to the expression values in *Aire*$^{-/-}$ thymic cells. Data analysed with two-way ANOVA, Tukey's multiple comparisons test, $p \leq 0.05$. Error bars represent ±SEM. Aire – murine Autoimmune Regulator, AIRE – human Autoimmune Regulator.

### 6.3.3 TSAs following over-expression of *AIRE*

To address the question of whether elevated levels of *AIRE* resulted in an increase in TSAs expression within the thymus, several TSAs expression were measured at mRNA level. The investigated Aire-dependent TSAs, *Ccl1*, *IL3*, *Ins2*, *Spt1*, *Apoa1*, *Reg1* were all upregulated following the over-expression of *AIRE* 4-weeks post injections *[Figure 6.4A]*. Expression levels of both *Ins2* and *Spt1* were significantly increased in *Aire*$^{-/-}$ AAV9-AIRE mice, 1300 fold change ($p$ = <0.0001) and 700 fold change ($p$ = 0.0038) respectively, when compared to their expression levels in *Aire*$^{-/-}$ mice. Furthermore, *Ins2* and *Spt1* expression levels were significantly higher than their expression levels in *Aire*$^{+/+}$ mice. *IL3* expression levels in 8-week-old *Aire*$^{-/-}$ AAV9-AIRE mice were higher than their expression levels in *Aire*$^{-/-}$ mice and comparable to their expression levels in *Aire*$^{+/+}$ mice. *Ccl1*, *Apoa1* and *Reg1* were all expressed higher in *Aire*$^{-/-}$ AAV9-AIRE mice when compared to *Aire*$^{-/-}$ mice. *Aire*$^{-/-}$ AAV9-GFP mice seems to have high *Ins2* expression levels, this could be due to the overexpression of a foreign protein in the thymus that activates the transcription of some TSAs.

Two transcription factors expressed highly in mTECs were also included in the investigation to measure their expression levels following Over-expression of *AIRE*, *Fezf2* and *Foxn1 [Figure 6.4B]*. At 4-weeks post injections, *Fezf2* followed the over-expression of *AIRE* with significant high expression levels. *Fezf2* expression levels were over 35 fold change higher than in *Aire*$^{-/-}$ AAV9-AIRE mice when compared to its endogenous levels in *Aire*$^{-/-}$ mice ($P$ <0.0001). In addition, this high expression of *Fezf2* in *Aire*$^{-/-}$

AAV9-AIRE mice was significantly higher than its expression levels in *Aire*$^{+/+}$ mice, ($P$ <0.0001). On the other hand, the transcription factor *Foxn1* expression levels were comparable in all mice groups at 8-week-old. Thus, the augmented expression of *AIRE* had no effect on the expression levels of the transcription factor *Foxn1*.

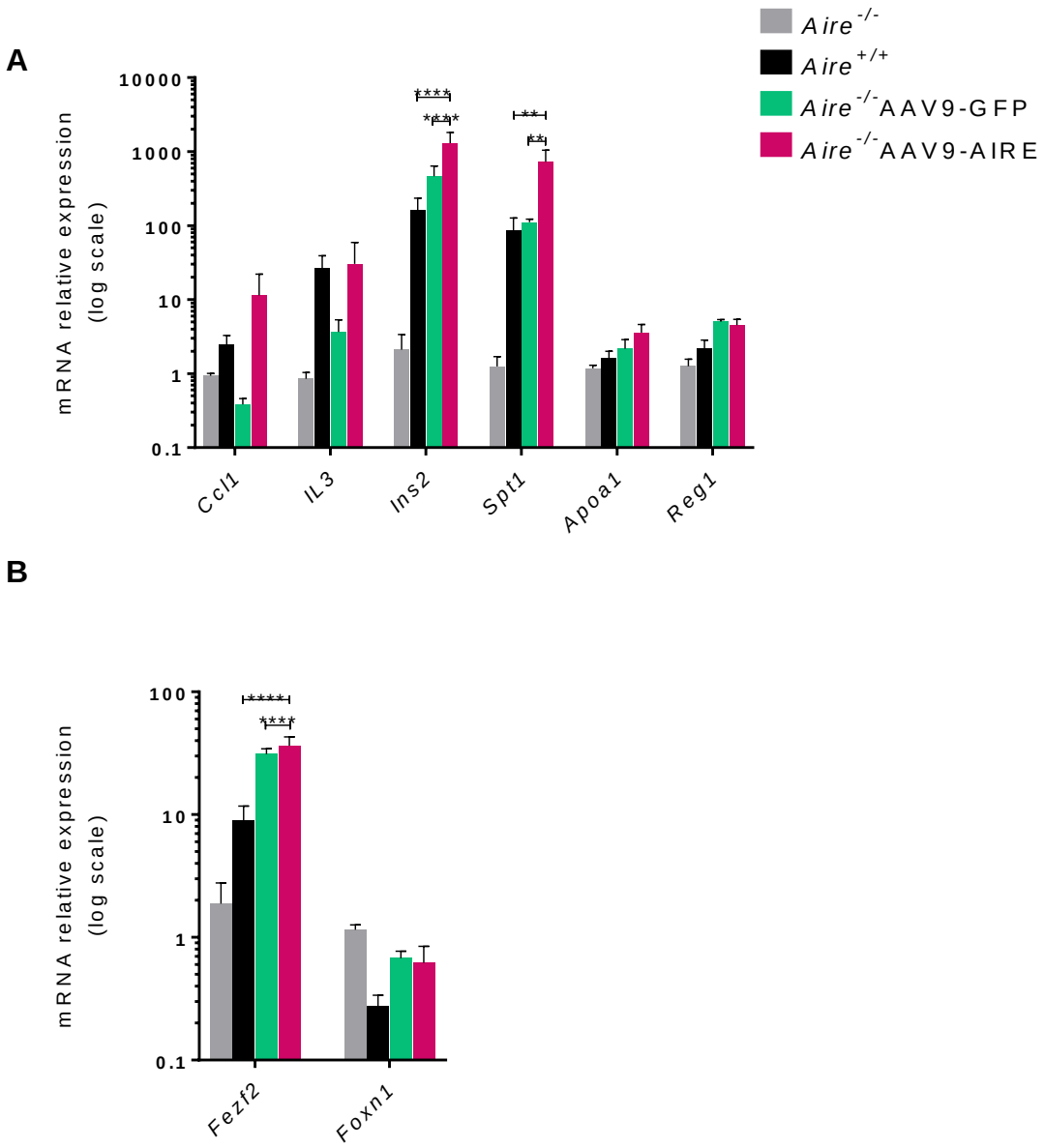



**Figure 6.4: TSAs expression following over-expression of *AIRE in-vivo* in 8-week-old APS-1 mouse model**. Quantitative PCR analysis of expression of (A) Aire-dependent and (B) Aire-independent TSAs in thymic cells obtained 4-weeks post injections from *Aire*$^{+/+}$ mice ($n$ = 4), *Aire*$^{-/-}$ mice ($n$ = 4), *Aire*$^{-/-}$ AAV9-GFP mice ($n$ = 3), and *Aire*$^{-/-}$ AAV9-AIRE injected mice ($n$ = 4). Results are normalized to the expression of *β-actin* and are presented relative to the expression values in *Aire*$^{-/-}$ thymic cells. Data analysed with two-way ANOVA, Tukey's multiple comparisons test, **** $P$ <0.0001, ** $P$ < 0.005. ± SEM. *Ccl1* – Chemokine (C-C motif) ligand 1, *IL3* –Interleukin 3, *Ins2* – Insulin II, *Spt1* – Salivary protein 1, *Apoa1* – Apolipoprotein A-I, *Reg1* – Regenerating islet-derived 1, *Dnmt3al* – DNA methyltransferase 3, *Fezf2* – Fez family zinc finger 2, *Foxn1* – Forkhead box N1.

### 6.3.4 Serum autoantibodies in AAV9 injected *Aire*$^{-/-}$ mice

In the pilot study, augmented expression of AIRE ameliorated autoimmunity phenotype in AAV9-AIRE injected *Aire*$^{-/-}$ mice by reducing the levels of serum autoantibodies towards several tissues. In this section, this effect was investigated on a larger number of mice using a single dose of AAV9-AIRE, 1.6 x $10^{10}$ vg per mouse. Indirect immunofluorescence technique was performed by incubating healthy mouse tissues sections with sera of mice from all groups. At 4-weeks post injections, circulating autoantibodies against retina, lung, stomach, testis and ovary were detected in *Aire*$^{-/-}$ mice injected with PBS and *Aire*$^{-/-}$ mice injected with AAV9-GFP *[Figure 6.5 and 6.6]*. On the other hand, weak or almost undetectable serum reactivity was observed in *Aire*$^{+/+}$ mice injected with PBS and *Aire*$^{-/-}$ mice injected with AAV9-AIRE *[Figure 6.5 and 6.6]*. The serum autoantibodies were found to be against 1 or 2 tissues per mouse in the investigated tissues *[Figure 6.7]*. In *Aire*$^{-/-}$ mice control groups (*n* = 10), 70% of mice exhibited autoimmunity against retina; ranging from weak to strong reaction. Autoantibodies against the reproductive tissues, testis and ovary, were present in 60% of *Aire*$^{-/-}$ mice. Furthermore, 30% of *Aire*$^{-/-}$ mice control groups showed autoreactivity against both lung and stomach. At 8-week-old none of the investigated *Aire*$^{-/-}$ mice exhibited autoantibodies against Islets of Langerhans in the pancreas. In AAV9-AIRE treated group (*n* = 5), one mouse showed autoreactivity towards retina, and another mouse showed weak reactivity towards retina and reactivity against lung tissues. These data suggest that AAV9-AIRE significantly decreased circulating autoantibodies from the sera of 8-week-old *Aire*$^{-/-}$ AAV9-AIRE mice.

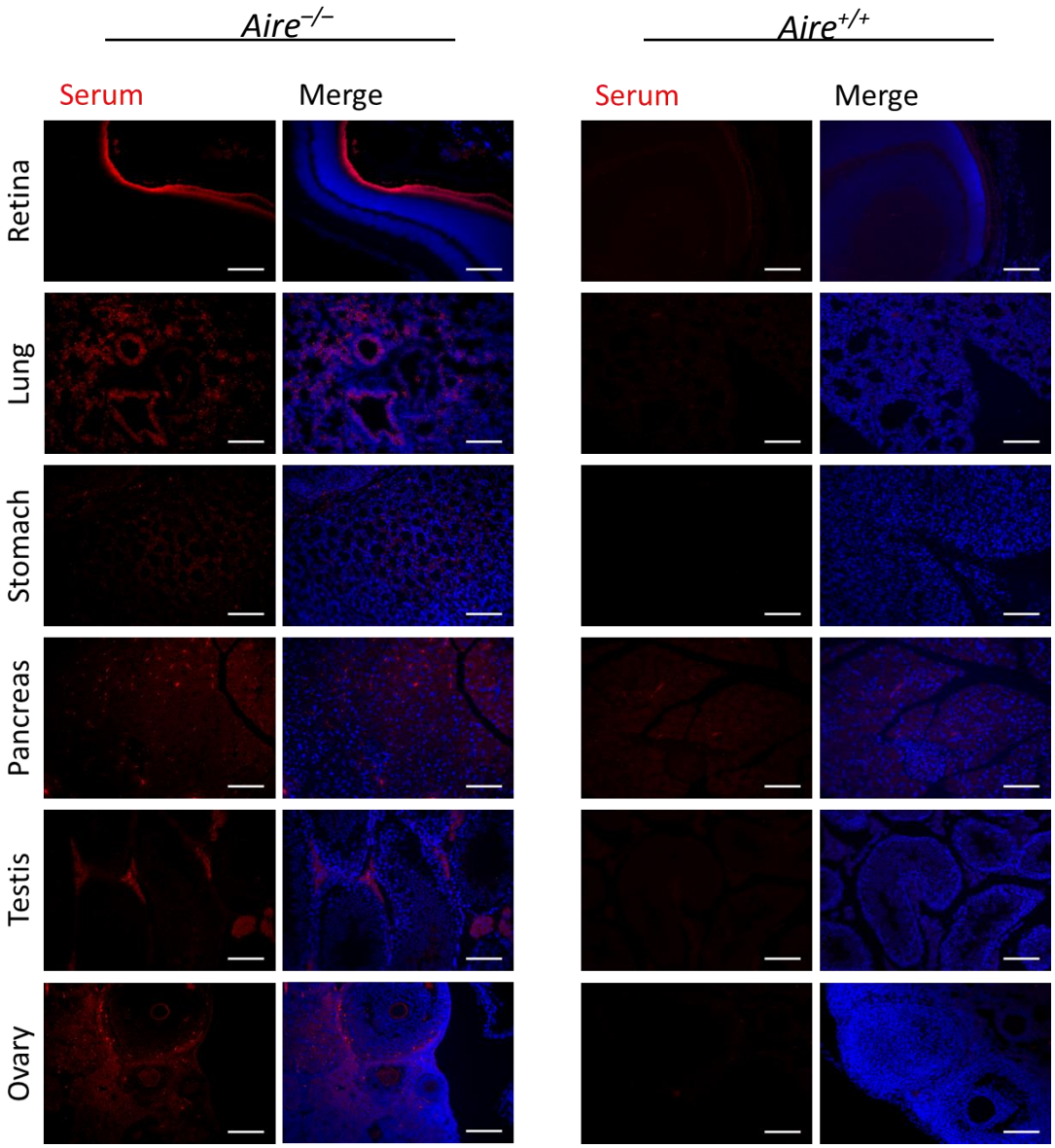



**Figure 6.5: Representative serum autoantibodies in sham control groups injected with PBS.** Serum was collected from *Aire*$^{-/-}$ mice (*n* = 10) and *Aire*$^{+/+}$ mice (*n* = 10) 4-weeks and 12-weeks post injections with PBS. Tissues including: retina, lung, stomach, pancreas, testis and ovary were obtained from healthy 8-week-old *Aire*$^{+/+}$ mice. Sections were incubated with individual mouse serum. Nuclei counterstained with DAPI. AIRE – Autoimmune regulator; PBS – Phosphate buffered saline; DAPI- Diamidino-2- phenylindole (blue). Scale bar 100 μm.

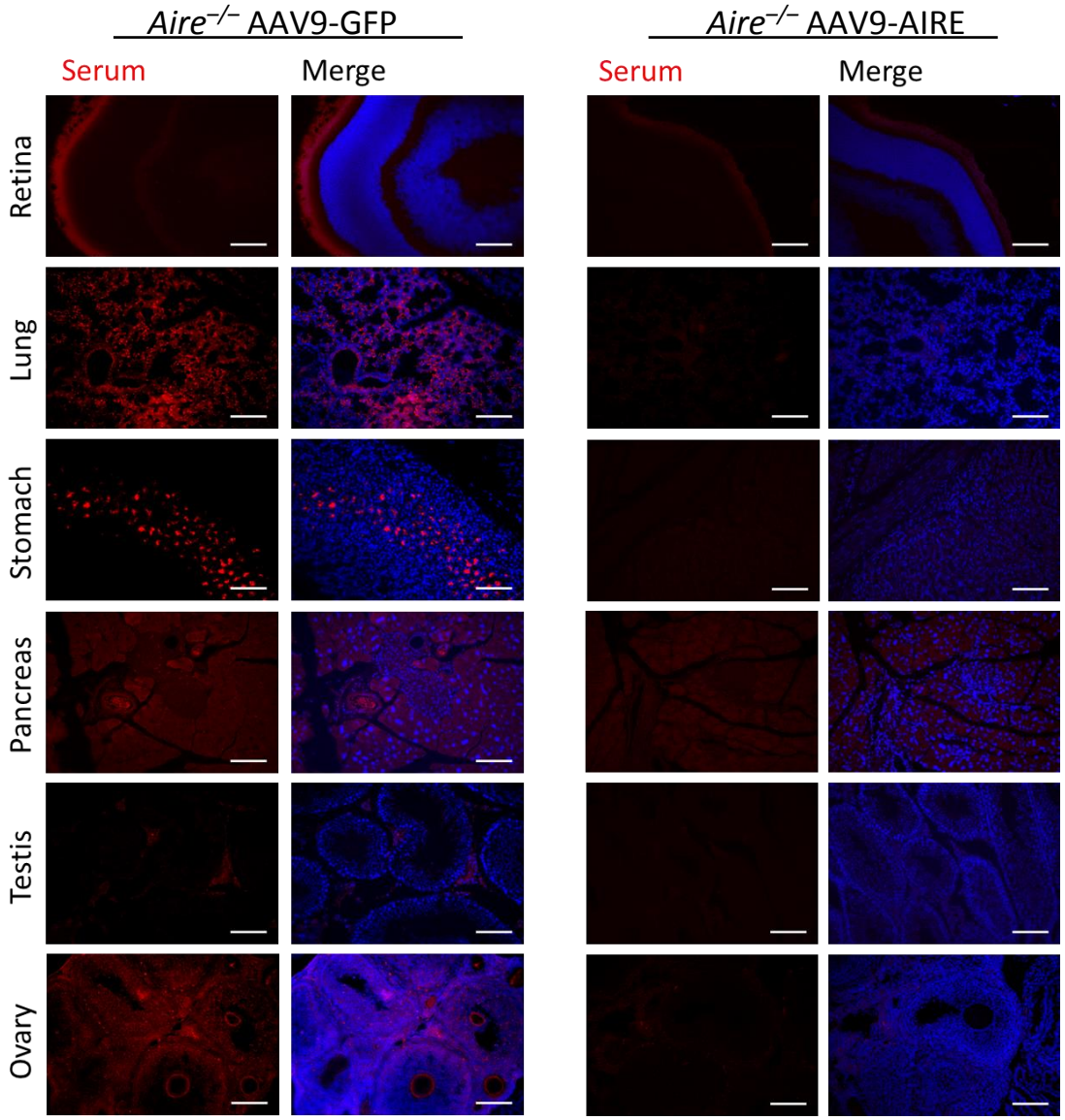



**Figure 6.6: Representative serum autoantibodies in AAV9-GFP and AAV9-AIRE injected *Aire*$^{-/-}$ mice (8-weeks-old).** Serum was collected from *Aire*$^{-/-}$ AAV9-GFP mice (*n* = 5) and *Aire*$^{-/-}$ AAV9-AIRE mice (*n* = 5) 4-weeks post injections with viruses. Tissues including: retina, lung, stomach, pancreas, testis and ovary were obtained from healthy 8-week-old *Aire*$^{+/+}$ mice. Sections were incubated with individual mouse serum. Nuclei counterstained with DAPI. AAV9 – Adeno-associated virus serotype 9; AIRE – Autoimmune regulator; GFP – Green fluorescent protein; DAPI- Diamidino-2- phenylindole (blue). Scale bar 100μm.

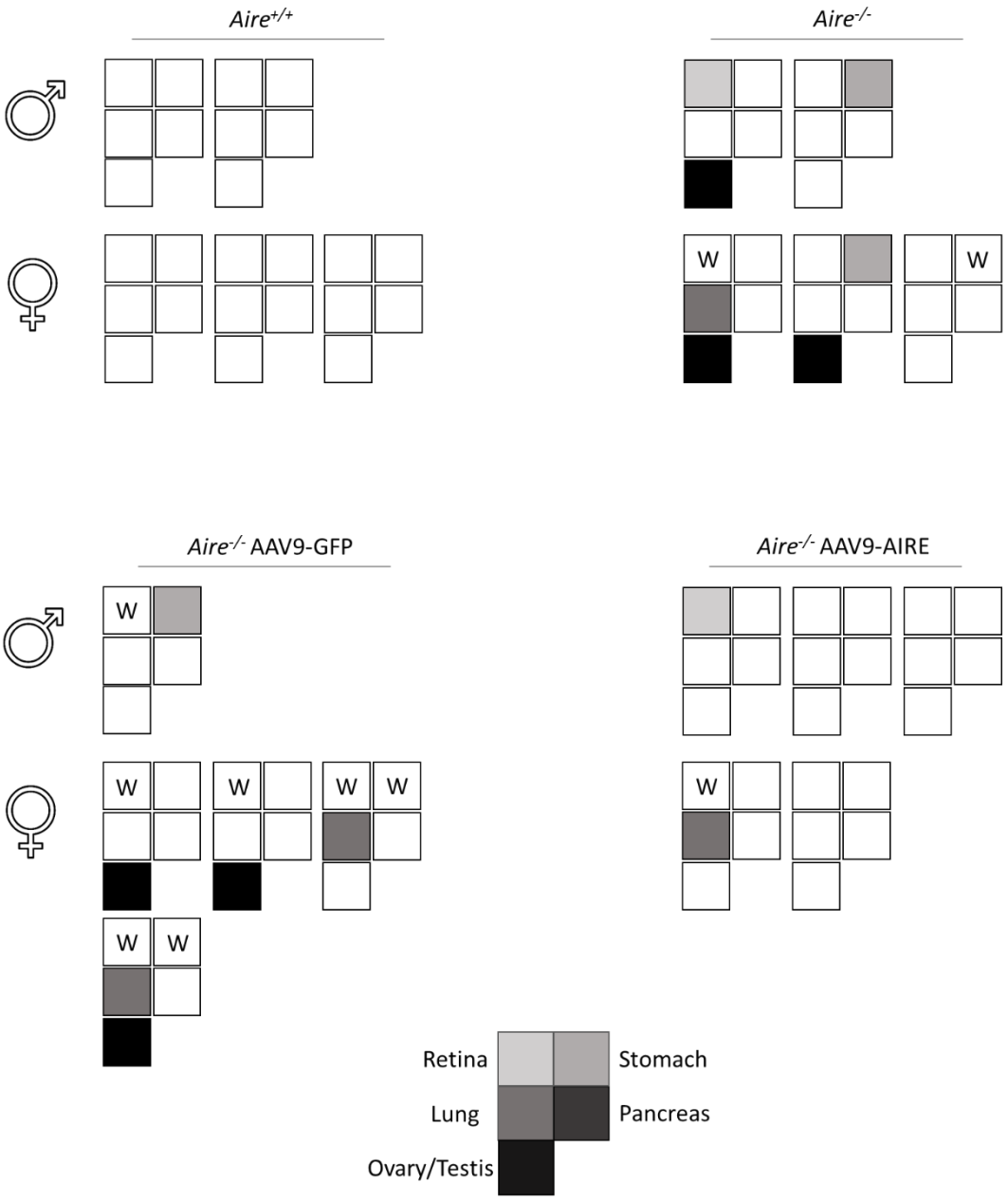



**Figure 6.7: Summary of serum autoantibodies in 8-week-old groups of APS-1 mouse model.** For each mouse of the indicated group AAV9 virus and gender, presence (shaded) or absence of autoantibodies against: retina, lung, stomach, pancreas and ovary/testis. W; weak reaction.

### 6.3.5 Pathology

As mentioned earlier in APS-1 mouse model characterisation section (*see section 3.5*), infiltrations shows an age-dependant pattern, ranging from none at 4-week to at least three or four at 12 weeks. At this part of the study, the mice in the short-term therapeutic effect group were 8-week-old.

At 8-week-old mice retinal degeneration, damaged oocytes, and LCH morphology were detected in *Aire*$^{-/-}$ mice and AAV9-GFP treated *Aire*$^{-/-}$ mice *[Figure 6.8]*. Furthermore, mild infiltrations were observed in the lung, stomach and liver of the same mice groups *[Figure 6.8]*. Moreover, some of the observed infiltrations do correlate with the serum autoantibodies present in the same mice *[Figure 6.9]*. In agreement with several studies, the majority of *Aire*$^{-/-}$ mice had tissues targeted by serum autoantibodies are also targeted by infiltrates (Anderson *et al.* 2002, Ramsey *et al.* 2002). All *Aire*$^{-/-}$ mice AAV9-AIRE (*n* = 5) mice do not show any tissue damage or infiltration in the investigated tissues *[Figure 6.8]*. Suggesting that these mice were protected from tissue-specific autoimmune reactions when injected with AAV9-AIRE for 4-weeks.

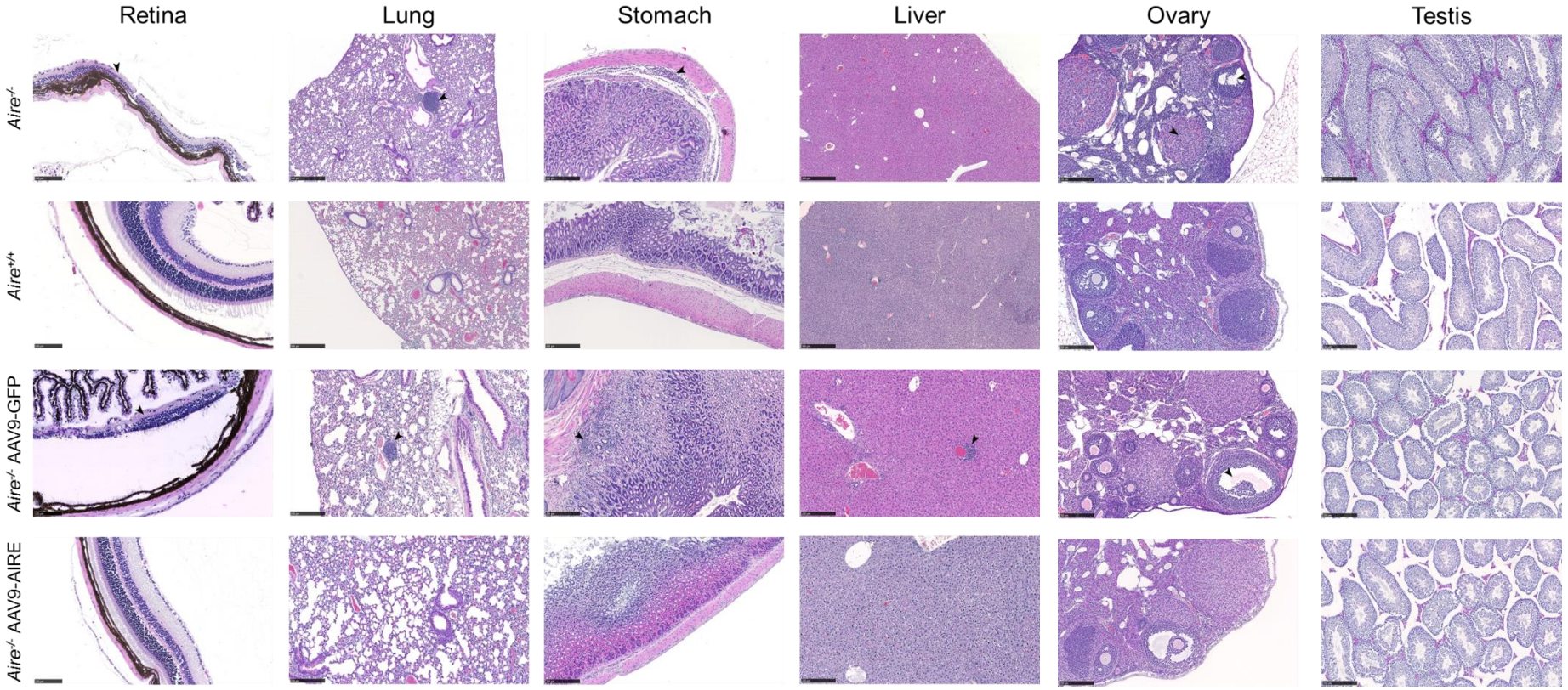



**Figure 6.8: Representative tissue sections from 8-week-old APS-1 mice.** Retina, lung, liver and stomach sections from *Aire*$^{+/+}$, *Aire*$^{-/-}$, *Aire*$^{-/-}$ AAV9-GFP, and *Aire*$^{-/-}$ AAV9-AIRE mice, stained with haematoxylin and eosin (H&E). Arrowhead indicates infiltration. Scale bar: retina 100 µm, liver 500 µm, lung, stomach, ovary and testis 250 µm.

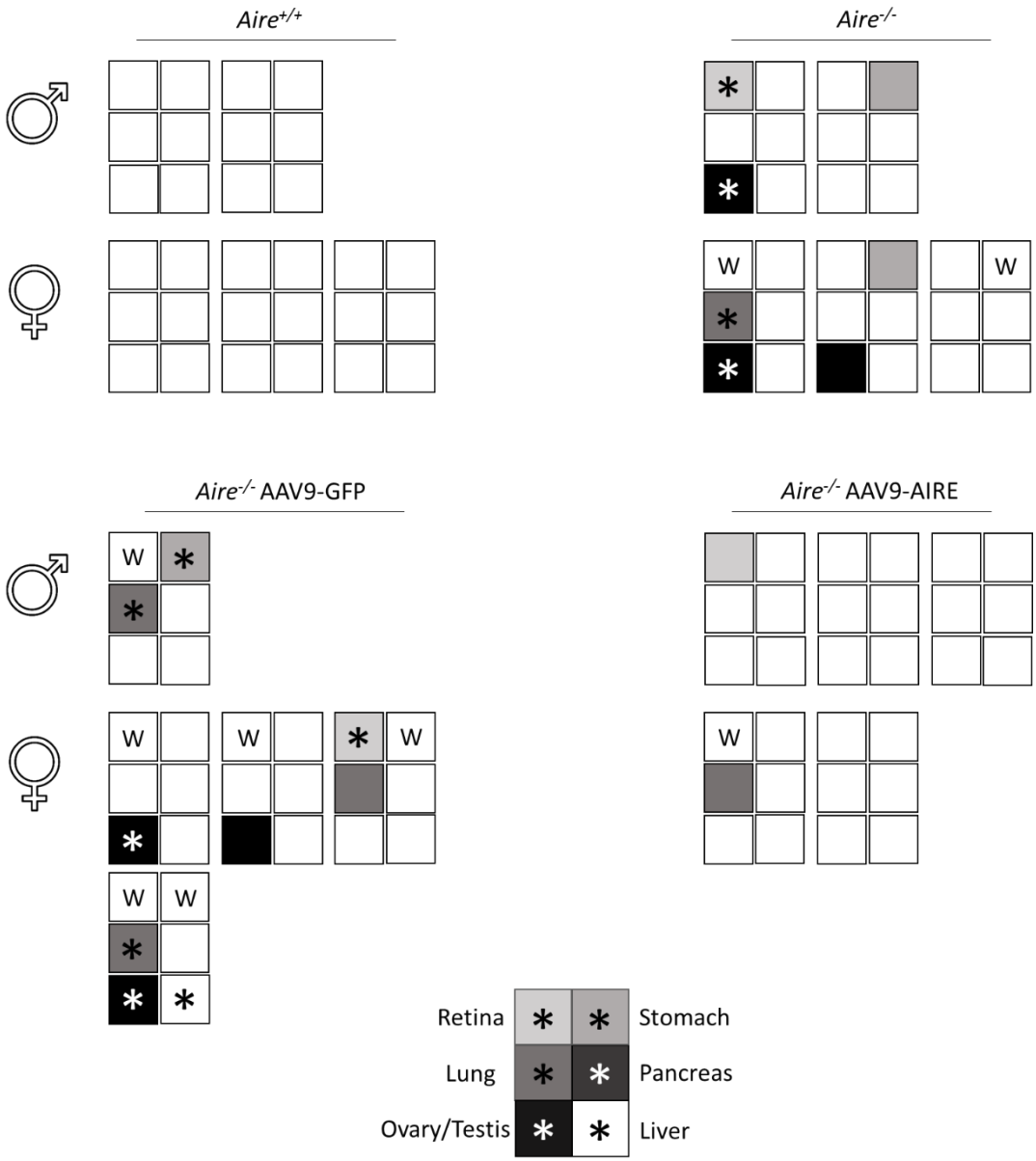



**Figure 6.9: Correlation of serum autoantibodies and tissue infiltrations in 8-week-old groups of APS-1 mouse model.** For each mouse of the indicated group AAV9 virus and gender, presence (shaded) or absence of autoantibodies against: retina, lung, stomach, pancreas and ovary/testis. Asterisk; tissue infiltration.

# 6.4 Long-term effect of a single dose of AAV9-AIRE injection on APS-1 mouse model

## 6.4.1 Weight distribution

The long-term groups were monitored up to 16-week-old, 12-weeks post injections *[Figure 6.10]*. Similar to the short-term treated groups, all mice gained weight normally and no statistical difference was found between groups, suggesting a normal body weight gain obtained by all *Aire*$^{-/-}$ mice.

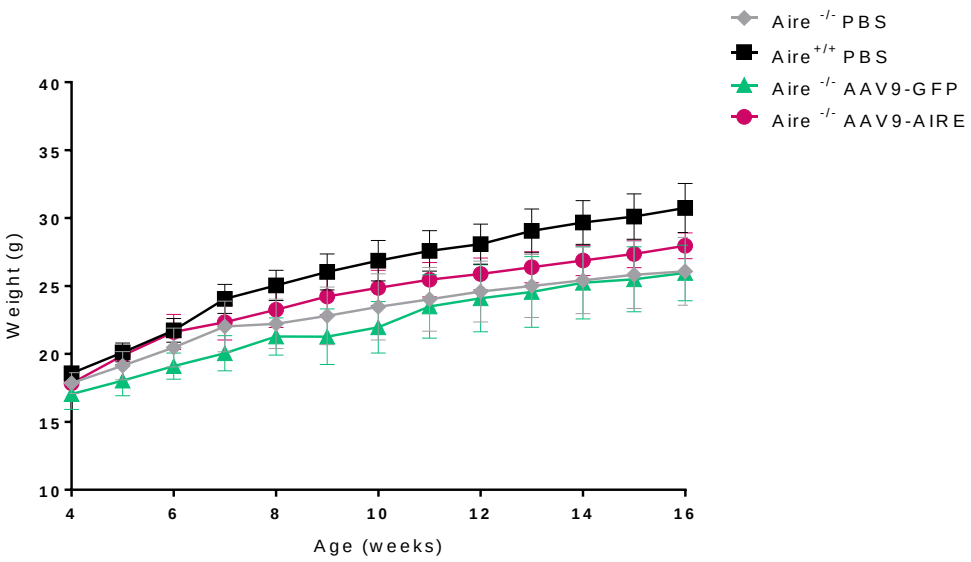



**Figure 6.10: Body weight assessment of *Aire* mice 12-weeks post injections.** Changes in body weight over time in all experimental mice (*n* =5 per group). Data represent average body weight per group. Mice were injected at 4-week-old with either PBS, AAV9- GFP or AAV9-AIRE and monitored up to 16-weeks of age. AAV9 – Adeno-associated virus serotype 9; GFP – Green fluorescent protein; AIRE – Autoimmune regulator.

## 6.4.2 Induction of AIRE expression in thymic cells by AAV9-AIRE virus

At the end stage of the long-term treatment, 12-weeks post injections, thymi were collected from all groups. Similar to the short-term group, from each thymus, one lobe was fixed in 4% PFA for histological analysis and the other lobe was harvested for RNA extraction (*see section 2.6.1*). In the thymic sections of *Aire*$^{-/-}$ AAV9-AIRE mice ($n$ = 5), a weak cytoplasmic staining of few thymic cells but no nuclear punctate AIRE+ thymic cells were observed 12-weeks post injections *[Figure 6.11]*.

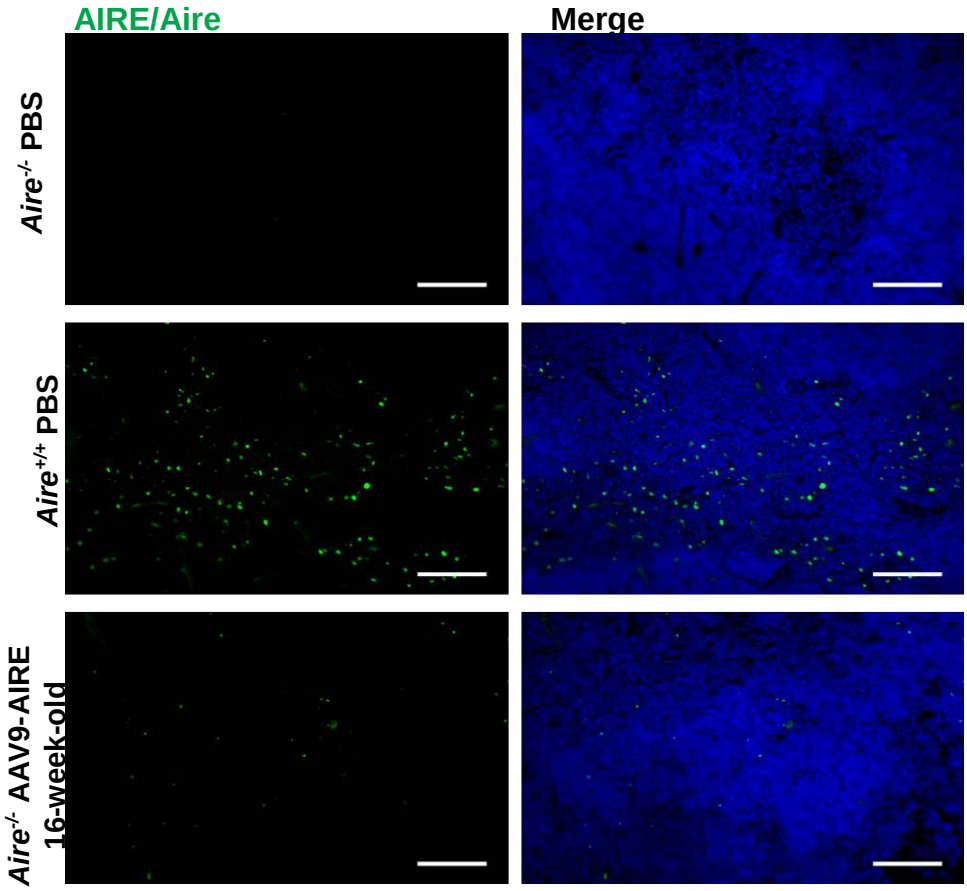



**Figure 6.11: Representative images of transduced thymi of 16-week-old APS-1 mice.** Mice were injected at 4-week-old: *Aire*$^{+/+}$ with PBS, *Aire*$^{-/-}$ with PBS or AAV9-AIRE. For 8-week-old mice, thymi were extracted at 12-weeks post-injection and for 16-week-old mice. The thymic sections were labelled with anti-AIRE antibody, and counterstained with DAPI for visualization of nuclei. AAV9 – Adeno-associated virus serotype 9; AIRE – Autoimmune regulator; DAPI- Diamidino-2- phenylindole (blue); PBS – Phosphate buffered saline. Scale bar 100 μm.

To quantify *AIRE* at the mRNA level, qPCR analysis was performed on thymic lysates of all groups. 12-weeks post injection, *AIRE* expression in *Aire*$^{-/-}$ AAV9-AIRE injected mice was 5 folds higher than the endogenous levels of *Aire* in *Aire*$^{-/-}$ mice *[Figure 6.12]*. This expression level was not considered statistically significant when compared to *Aire*$^{-/-}$ mice. Moreover, *AIRE* expression levels in 16-week-old *Aire*$^{-/-}$ AAV9-AIRE mice were much less than *AIRE* expression levels in 8-week-old *Aire*$^{-/-}$ AAV9-AIRE, 5 fold change and 2000 fold change respectively.

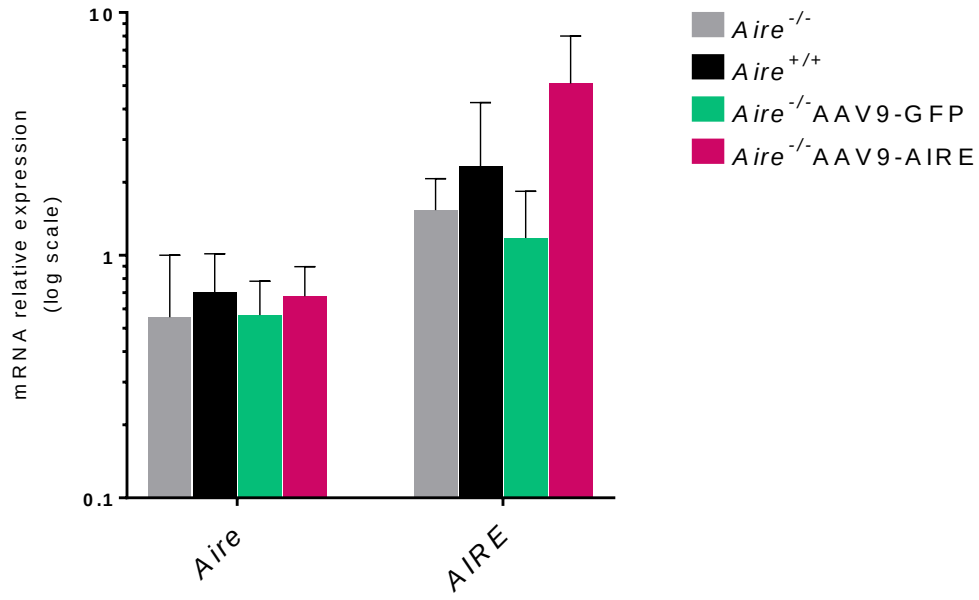



**Figure 6.12: *AIRE* over-expression after viral delivery of AIRE *in-vivo* in 16-week-old APS-1 mice**. Quantitative PCR analysis of expression of *Aire* and *AIRE* in thymic cells obtained from 16-week-old *Aire*$^{+/+}$ mice ($n$ = 4), *Aire*$^{-/-}$ mice ($n$ = 4), *Aire*$^{-/-}$ AAV9-GFP mice ($n$ = 3), and *Aire*$^{-/-}$ AAV9-AIRE injected mice ($n$ = 3). Results are normalized to the expression of *β-actin* and are presented relative to the expression values in *Aire*$^{-/-}$ thymic cells. Error bars represent ±SEM. Aire – murine Autoimmune Regulator, AIRE – human Autoimmune Regulator.

### 6.4.3 TSAs following over-expression of *AIRE*

To investigate whether the moderate expression levels of *AIRE* resulted in an increase in TSAs expression within the thymi of 16-week-old *Aire*$^{-/-}$ AAV9-AIRE mice. The same TSAs profile from the short-term effect of AAV9-AIRE were investigated in the long-term treated groups at mRNA level. *IL3* has been excluded from the analysis of 16-week-old mice, as its expression levels were undetermined in most of them. The rest 5 TSAs expression levels in *Aire*$^{-/-}$ AAV9-AIRE mice were lower than their expression levels in *Aire*$^{-/-}$ mice *[Figure 6.13A]*. This suggests that although *AIRE* was moderately expressed in *Aire*$^{-/-}$ AAV9-AIRE mice with 5 folds higher than *Aire* in *Aire*$^{-/-}$ mice, this amount of expression was not sufficient to induce TSAs 12-weeks post injection.

Moreover, *Fezf2* and *Foxn1* expression levels in *Aire*$^{-/-}$ AAV9-AIRE mice were lower than their expression levels in *Aire*$^{-/-}$ mice *[Figure 6.13B]*. In the case of *Fezf2*, the reduced expression levels confirms the observation mentioned earlier in the short-term effect of AAV9-AIRE (*see section 6.3.3*). This suggest that augmented expression of *AIRE* affects *Fezf2* expression at mRNA level.

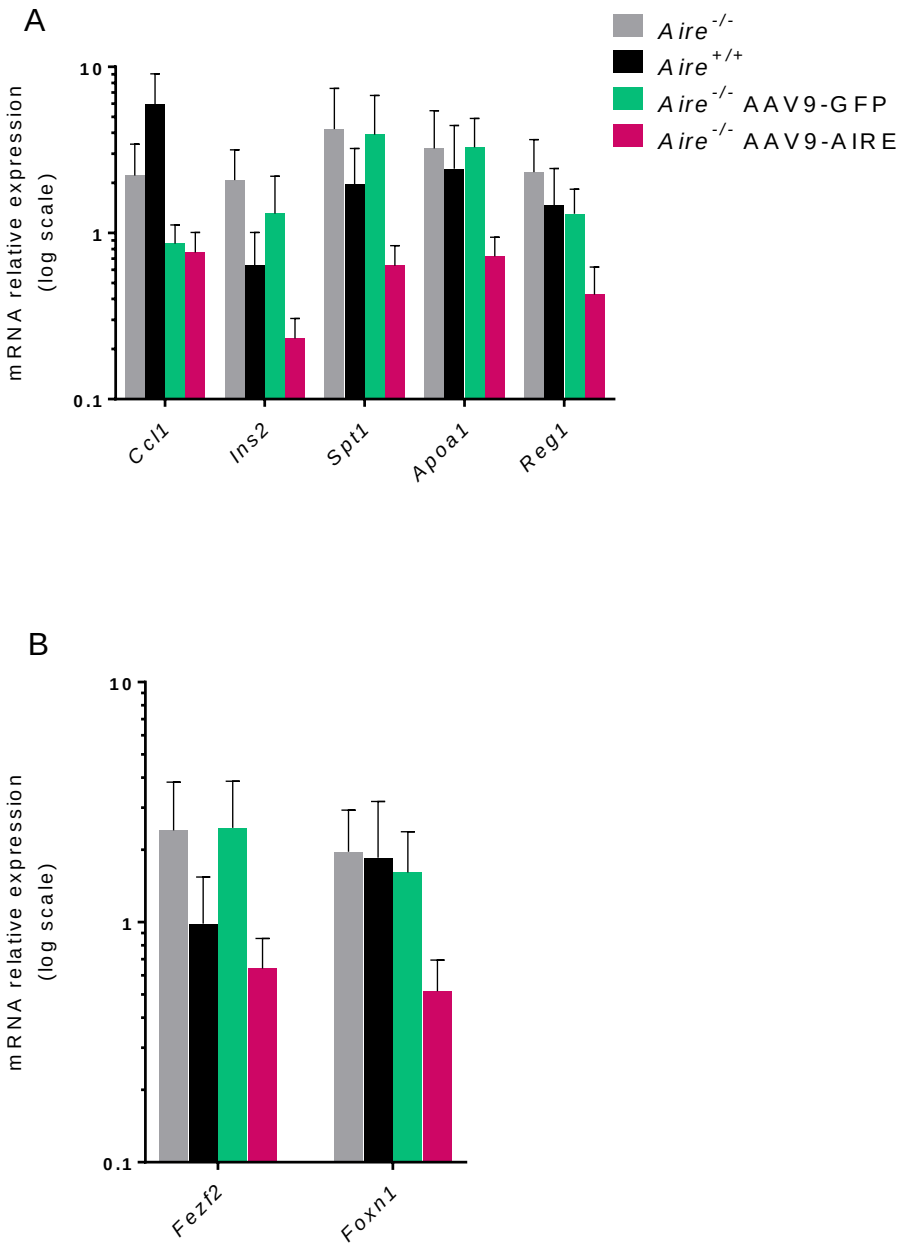



**Figure 6.13: TSAs expression following over-expression of *AIRE in-vivo* in APS-1 mouse model**. Quantitative PCR analysis of expression of (A) Aire-dependent and (B) Aire-independent TSAs in thymic cells obtained 12-weeks post injections from *Aire*$^{+/+}$ mice (*n* = 4), *Aire*$^{-/-}$ mice (*n* = 4), *Aire*$^{-/-}$ AAV9-GFP mice (*n* = 3), and *Aire*$^{-/-}$ AAV9-AIRE injected mice (*n* = 4). Results are normalized to the expression of *β-actin* and are presented relative to the expression values in *Aire*$^{-/-}$ thymic cells. Error bars. ± SEM. *Ccl1* – Chemokine (C-C motif) ligand 1, *IL3* –Interleukin 3, *Ins2* – Insulin II, *Spt1* – Salivary protein 1, *Apoa1* – Apolipoprotein A-I, *Reg1* – Regenerating islet-derived 1, *Dnmt3al* – DNA methyltransferase 3, *Fezf2* – Fez family zinc finger 2, *Foxn1* – Forkhead box N1.

### 6.4.4 Serum autoantibodies in AAV9 injected *Aire*$^{-/-}$ mice

At 12-weeks post injections, circulating autoantibodies were tested against the same tissues investigated in short-term group including retina, lung, stomach, pancreas, testis and ovary. Serum autoantibodies in *Aire*$^{-/-}$ AAV9-AIRE mice were detected towards the investigated tissues similar to those injected with AAV9-GFP *[Figure 6.14]*. At this age, all *Aire*$^{-/-}$ mice demonstrated autoimmunity towards 3 to 4 of the 5 investigated tissues *[Figure 6.15]*. Moreover, one *Aire*$^{-/-}$ mouse at 16-week-old showed cytoplasmic autoreactivity towards Islets of Langerhans in the pancreas. These data suggest that *Aire*$^{-/-}$ AAV9-AIRE mice exhibited the same autoimmune phenotype of the control untreated *Aire*$^{-/-}$ mice.

### 6.4.5 Pathology

All 16-week-old *Aire*$^{-/-}$ mice at the different groups ($n$ = 15) exhibited tissue damages and infiltrations towards the investigated tissues including retina, lung, stomach, liver, ovary and testis *[Figure 6.16]*. In addition, the earlier investigated *Aire*$^{-/-}$ mouse that showed cytoplasmic seroreactivity against the Islets of Langerhans exhibited pancreatic infiltration *[Figure 6.17]*. Similar to the short-term investigated groups, some of the observed infiltrations do correlate with the serum autoantibodies present in the each mice *[Figure 6.18]*. In agreement with the serum autoantibodies profile, *Aire*$^{-/-}$ AAV9-AIRE mice were not protected from autoimmunity 12-weeks post injections.

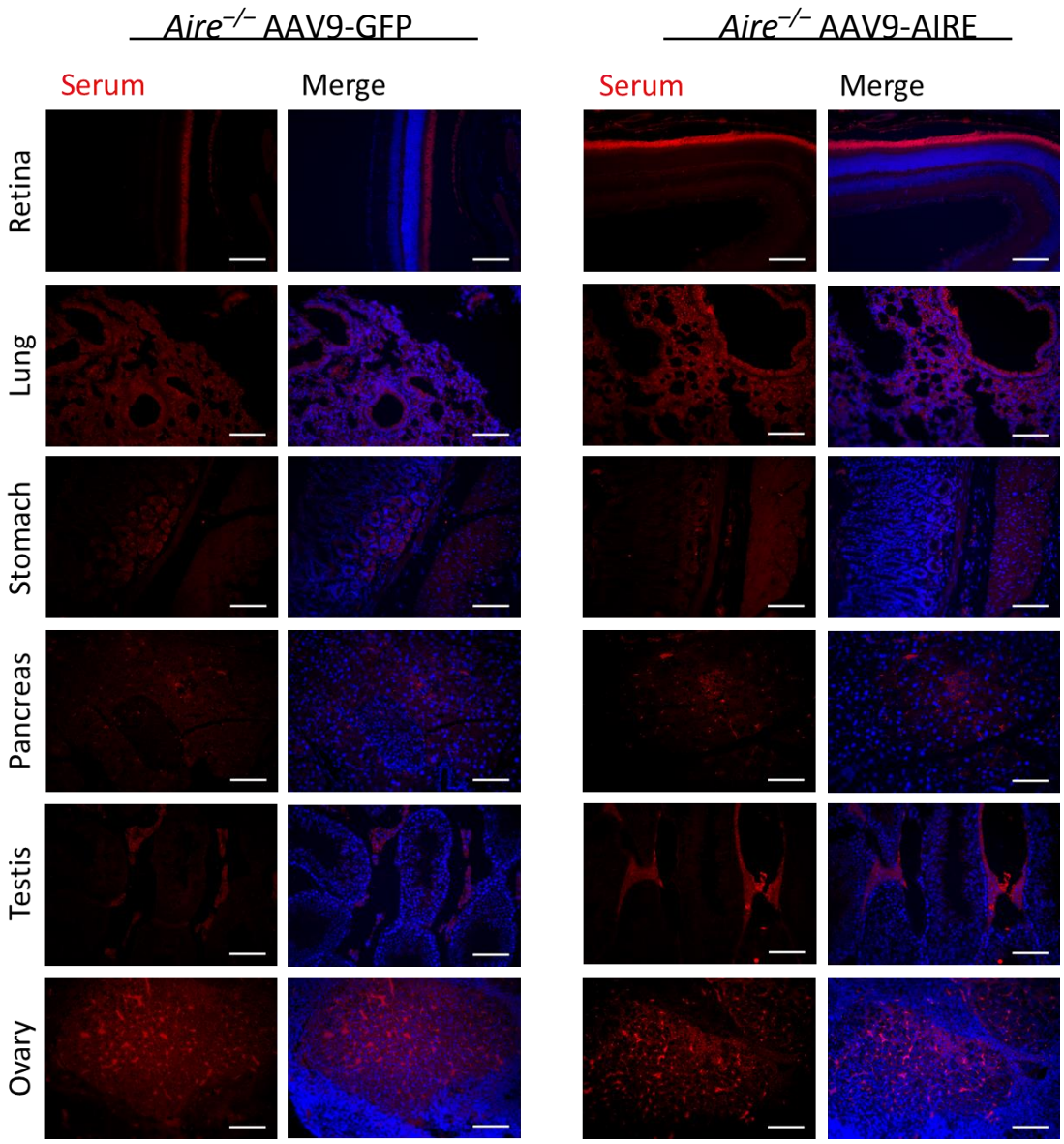



**Figure 6.14: Representative serum autoantibodies in AAV9-GFP and AAV9-AIRE injected *Aire*$^{-/-}$ mice (16-weeks-old).** Serum was collected from *Aire*$^{-/-}$ AAV9-GFP mice (*n* = 5) and *Aire*$^{-/-}$ AAV9-AIRE mice (*n* = 5) 12-weeks post injections with viruses. Tissues including: retina, lung, stomach, pancreas, testis and ovary were obtained from healthy 8-week-old *Aire*$^{+/+}$ mice. Sections were incubated with individual mouse serum. Nuclei counterstained with DAPI. AAV9 – Adeno-associated virus serotype 9; AIRE – Autoimmune regulator; GFP – Green fluorescent protein; DAPI- Diamidino-2- phenylindole (blue). Scale bar 100μm.

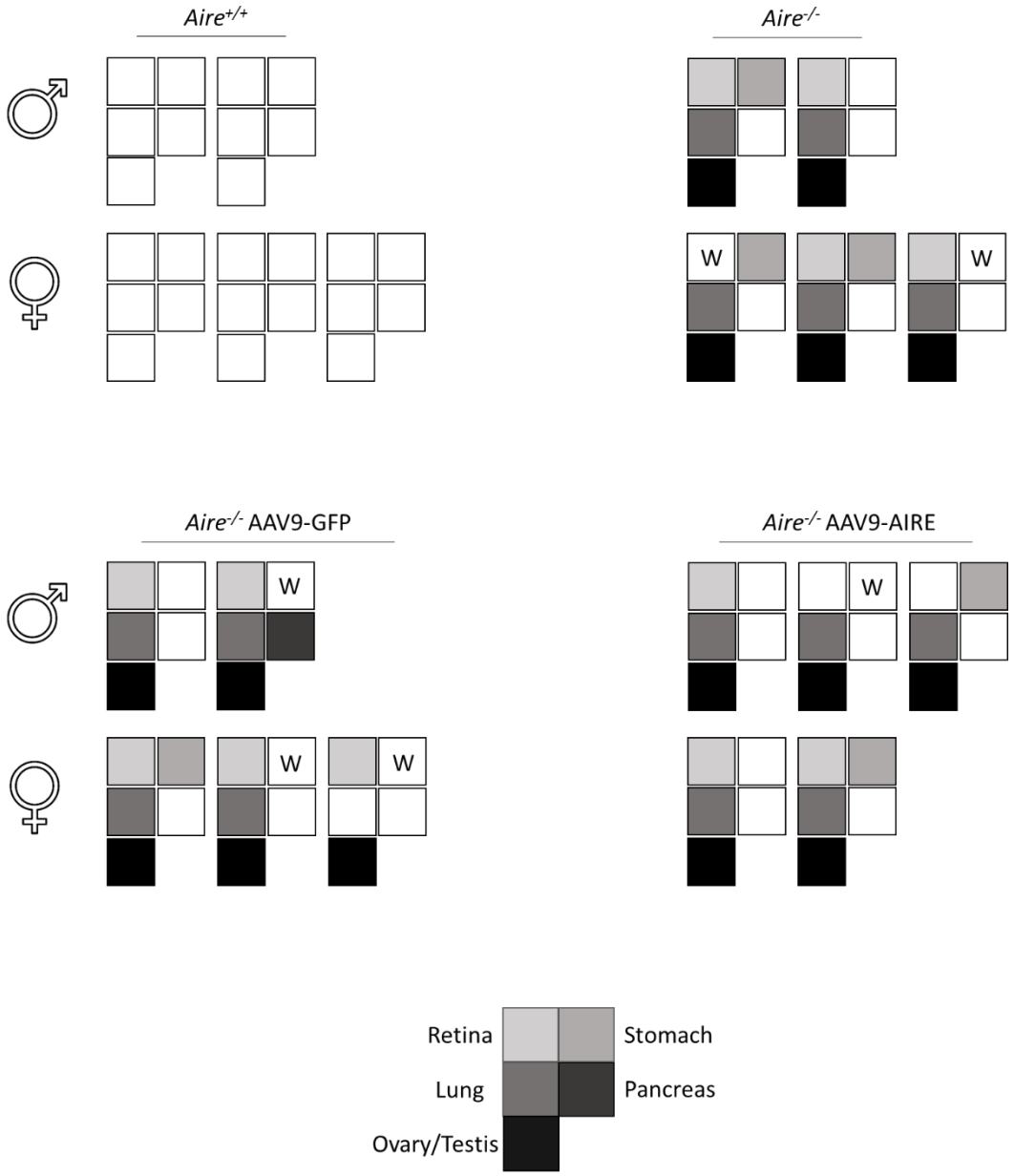



**Figure 6.15: Summary of serum autoantibodies in 16-week-old groups of APS-1 mouse model.** For each mouse of the indicated group AAV9 virus and gender, presence (shaded) or absence of autoantibodies against: retina, lung, stomach, pancreas and ovary/testis. W; weak reaction.

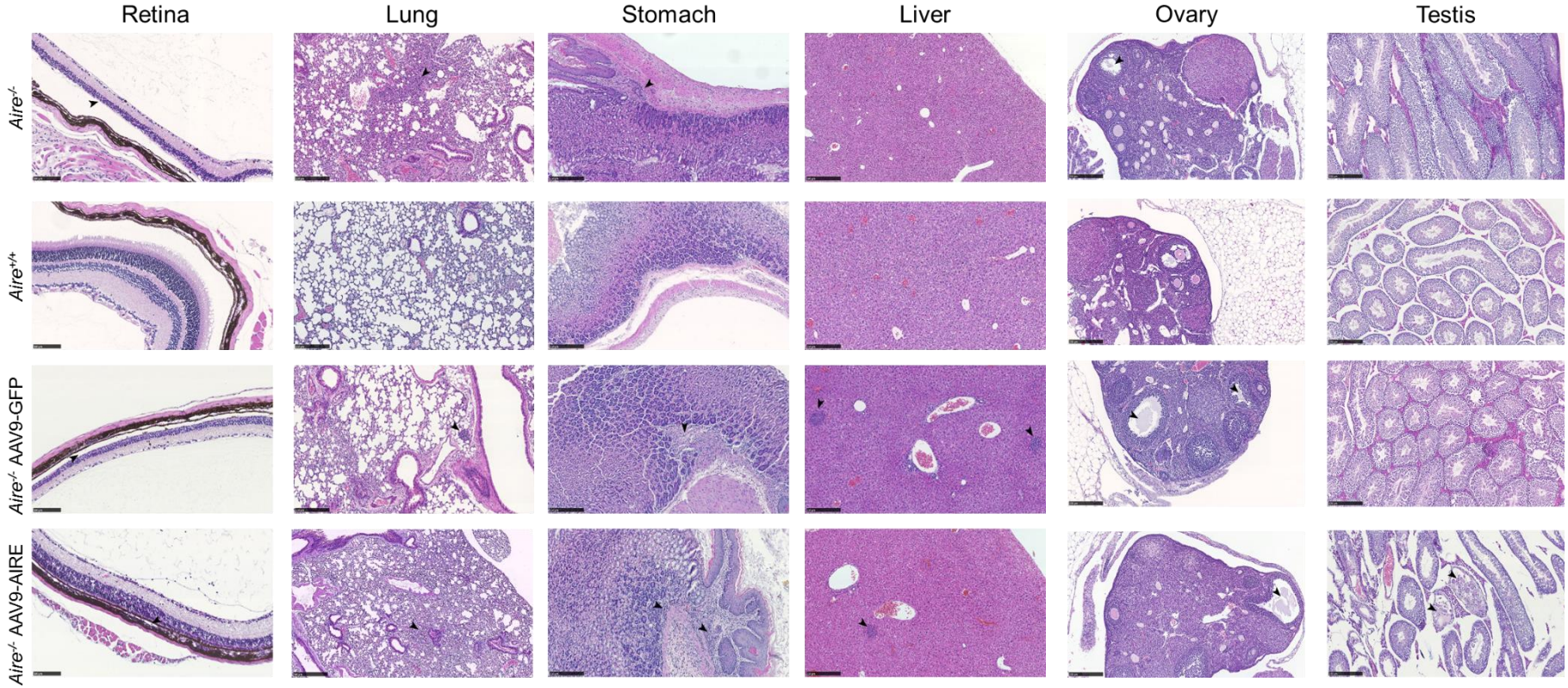



**Figure 6.16: Representative tissue sections from 16-week-old APS-1 mice.** Retina, lung, liver and stomach sections from *Aire*$^{+/+}$, *Aire*$^{-/-}$, *Aire*$^{-/-}$ AAV9-GFP, and *Aire*$^{-/-}$ AAV9-AIRE mice, stained with haematoxylin and eosin (H&E). Arrowhead indicates infiltration. Scale bar: retina 100 µm, liver 500 µm, lung, stomach, ovary and testis 250 µm.

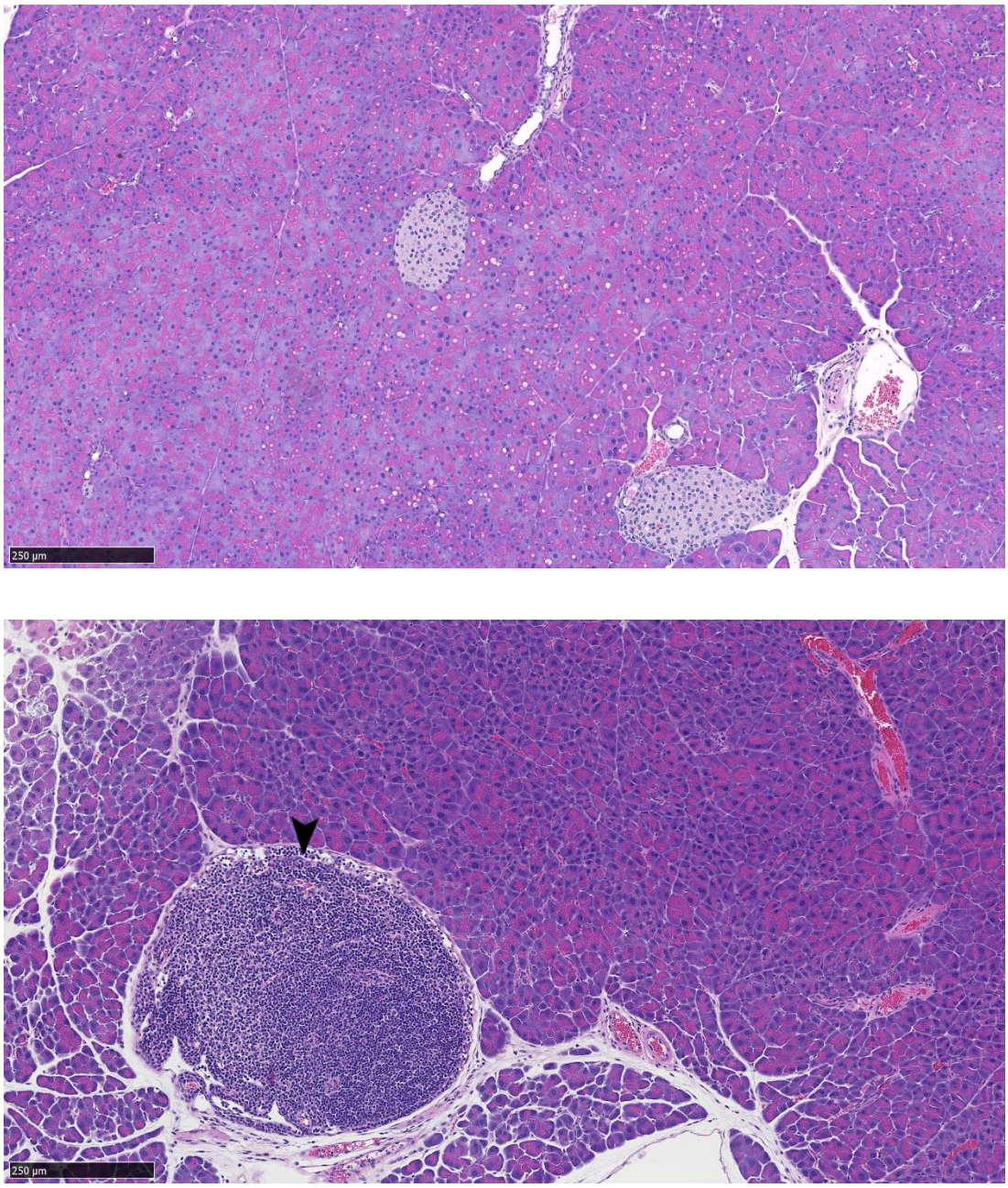



**Figure 6.17: Pancreatic infiltration in 16-week-old *Aire*$^{-/-}$ mouse.** Pancreatic sections from *Aire*$^{+/+}$ (upper lane) and *Aire*$^{-/-}$ (lower lane) stained with haematoxylin and eosin (H&E). Infiltration in *Aire*$^{-/-}$ pancreas (arrowhead). Scale bar 250 µm.

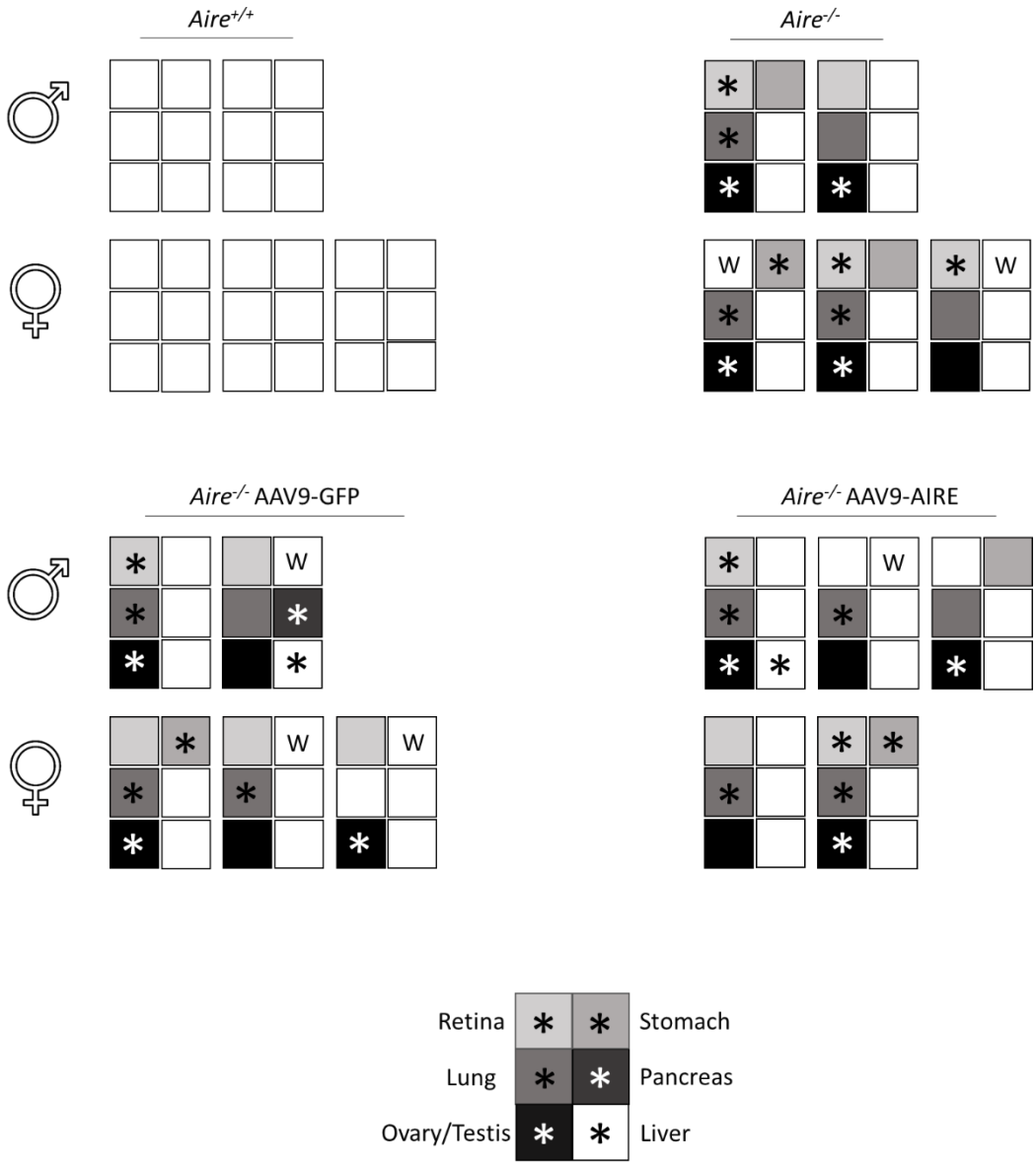



**Figure 6.18: Correlation of serum autoantibodies and tissue infiltrations in 16-week-old groups of APS-1 mouse model.** For each mouse of the indicated group AAV9 virus and gender, presence (shaded) or absence of autoantibodies against: retina, lung, stomach, pancreas and ovary/testis. Asterisk; tissue infiltration.

## 6.5 Discussion

This section detailed the *in-vivo* preclinical evaluation of AAV9-AIRE in the thymus of APS-1 mouse model. This proof-of-concept efficacy study demonstrated that 4-weeks post intrathymic injections of the therapeutic AAV9-AIRE in *Aire*$^{-/-}$ mice resulted in elevation of AIRE and the Aire-dependent TSAs. In addition, following AIRE expression, serum autoantibodies levels were reduced 4-weeks post injections. These novel data demonstrate an ameliorated autoimmunity phenotype in APS-1 mouse model following injections with AAV9-AIRE.

In this part of the study, an AAV9-AIRE dose of 1.6 x $10^{10}$ vg per mouse was enough to induce over-express AIRE at both protein and mRNA levels 4-weeks post injection. Immunostaining of thymic sections from AAV9-AIRE injected mice revealed nuclear punctate expression pattern of AIRE, similar to the endogenous Aire nuclear expression in *Aire*$^{+/+}$ mice. This observation suggests that AIRE is transcriptionally active and functioning as the wild type Aire. Indeed nuclear punctate structure is important for AIRE transcriptional activity, as diffused nuclear localisation results in loss of transcriptional activity (Bjorses *et al.* 2000, Halonen *et al.* 2004).

QPCR analysis showed that *AIRE* was over-expressed more than 2000 fold change when compared to the endogenous mRNA *Aire* levels in *Aire*$^{-/-}$ mice. This elevated expression resulted in an increase of expression of all 6 Aire-dependent TSAs studied. Significant increase was observed in both *Ins2* and *Spt1*; two well established Aire-dependent TSAs (Anderson *et al.* 2005, Peterson *et al.* 2008). These data indicate that modulation of AIRE *in-vivo* can

directly lead to alterations in TSA levels and may affect the maintenance of central tolerance. Another novel finding in this study is that *Fezf2* expression levels were significantly increased AAV9-AIRE gene transfer. This suggests that upregulation of *AIRE* might have an effect on the expression levels of *Fezf2*. Fezf2 is an mTEC transcription factor that regulates a set of TSAs independently from Aire (Takaba *et al.* 2015). To confirm that thymic expression of TSAs affect self-tolerance, serum autoantibodies from AAV9-AIRE injected mice were screened for autoreactivity against several tissues including: retina, lung, pancreas, stomach, ovary and testis (Anderson *et al.* 2002, Ramsey *et al.* 2002). Most of the investigated sera from these mice harboured no autoantibodies against the investigated tissues. Except two AAV9-AIRE injected *Aire*$^{-/-}$ mice exhibited autoimmunity and one of them had weak reactivity only against the outer layer of the retina. However, in this study, serum autoantibodies were qualitatively assessed, and measurement of specific antibody titres and isotypes could show more differences between the mice. On the other hand, histological analysis of 8-week-old *Aire*$^{-/-}$ AAV9-AIRE mice revealed no tissue infiltrations in all treated mice ($n$ = 5). This suggests that the detected serum autoantibodies in this group might not be pathogenic. Overall, this could be considered as an ameliorated phenotype when compared to the autoimmunity profile of *Aire*$^{-/-}$ mice and AAV9-GFP injected *Aire*$^{-/-}$ mice.

At 12-weeks post injections, AIRE expression was not detected at protein level within the thymic sections of AAV9-AIRE treated *Aire*$^{-/-}$mice. Moreover, *AIRE* expression levels at mRNA levels considered low after 12-weeks post injection when compared to its expression levels after 4-weeks

post injection. TSAs expression in thymic lysates of this group demonstrated lower expression levels than in *Aire*$^{-/-}$ mice, which led to the presence of serum autoantibodies against few of the investigated tissues. These poor expression patterns of AIRE at both protein and mRNA levels could be due to silencing effect on the CMV promoter. CMV promoter is prone to transcriptional silencing due to methylation effects at the CpG sites of the promoter (Brooks *et al.* 2004, Nuo *et al.* 2016). Methylation inhibitor treatments such as 5-azacytidine retrieved the CMV transcriptional activity and improved the transgene expression *in vivo* (Nuo *et al.* 2016). This could be considered for long-term transgene expression using CMV promoter. Another study found that using AAV8 vectors, transgene expression in the liver reduced after 2 to 5 months and this reduction was mainly due to loss of vector DNA (Wang *et al.* 2005). This could be resolved by considering a second injection of the viral vector to restore the transgene expression *in vivo*.

In conclusion, this study provides the first *in vivo* gene therapy proof-of-concept of AIRE immunomodulation in APS-1 mouse model, emphasizing the impact of AAV9-AIRE as a potential therapeutic vehicle in APS-1 therapy. In addition, repeated injections of AAV9-AIRE (i.e. every 4-weeks), is essential to maintain the high therapeutic expression levels of AIRE in APS-1 mouse model.

*CHAPTER SEVEN*

# GENERAL DISCUSSION

# 7. GENERAL DISCUSSION

APS-1 is a rare inherited PID, with complex and diverse endocrine and non-endocrine manifestations (Ahonen 1985, Ahonen *et al.* 1990). This childhood onset monogenic disease is caused by loss-of-function mutations in *AIRE* (Finnish-german Apeced 1997, Nagamine *et al.* 1997). APS-1 treatment options are narrow, limited to hormone replacement, anti-fungal agents, and immunosuppressive therapy with corticosteroids, which treat the symptoms rather than the cause (Gavanescu *et al.* 2008). In addition, treatments by immunosuppressive causes infections that could lead to major complications including spread of candidiasis which lead to the death of a patient (Proust-Lemoine *et al.* 2007). AAV9-AIRE gene therapy is considered to be appealing approach for AIRE replacement in the monogenic PID, APS-1.

In this study, a successful over-expression of AIRE in APS-1 mouse model was demonstrated. Human AIRE was used for further clinical application in mind, which successfully was able to compensate for the loss of endogenous mouse Aire; AAV9-AIRE promoted several TSAs expression and ameliorated APS-1 phenotype in *Aire*$^{-/-}$ mice 4-weeks post injection. AAV9 was chosen as AIRE transfer vehicle for its high transduction efficiency. Gene transfer in the thymus is a promising site for modulation of autoimmunity, as it is the organ responsible for central-tolerance induction. Thus, inducing tolerance to foreign antigens. Moreover, following prenatal diagnosis of inherited diseases, gene therapy of APS-1 is more likely to be performed at early stages of gestation; in utero foetal gene therapy. Several studies reported successful in utero gene therapy performed on mice and non-human

primates. In utero gene transfer of Atonal homologue 1 (Atoh1) produced functional auditory hair cells in mice (Gubbels *et al.* 2008). Recent study by Mattar *et al.* (2017) reported in utero gene therapy using AAV encoding human factor IX (hFIX) on macaques has mediated long-term transgene expression for over 6 years with no toxicity. This is a potential useful approach to treat hereditary genetic conditions.

The primary action of AIRE/Aire has been reported to promote promiscuous gene expression of TSAs within mTECs and clonal deletion of autoreactive T cells (Anderson *et al.* 2002). This role was investigated further by enforcing the expression of a single TSA including α-isoform of myosin heavy chain (α-MyHC) and interphotoreceptor retinoid-binding protein (IRBP), which are not expressed in mTECs of some transgenic mice, resulted in tolerance to those TSAs (DeVoss *et al.* 2006, Lv *et al.* 2011). This approach could be applied to ameliorate several isolated autoimmune diseases, by inducing their identified TSA gene expression within mTECs to maintain self-tolerance.

Another aspect that has been investigated with regard to promiscuous expression of TSAs is the expression level of AIRE/Aire. Several studies demonstrated that TSAs expression levels follow AIRE/Aire expression pattern in a dose-dependent manner (Liston *et al.* 2004, Giraud *et al.* 2007, Kont *et al.* 2008). In addition, G229W-knockin mouse model, that represents the autosomal dominant mutation in APS-1 patients, partially expressed Aire-dependent TSAs with disease spectrum different to *Aire*$^{-/-}$ mice (Su *et al.* 2008). Moreover, in APS-1 patients, dominant inheritance of heterozygous

missense mutations in *AIRE* characterised by milder phenotype (Oftedal *et al.* 2015). These findings suggest that quantity of AIRE/Aire is crucial for efficient induction of TSAs expression and thus negative selection of T cells. In an attempt to compensate for Aire expression in athymic mice, co-transfer of one thymic lobe from each *Aire*$^{+/+}$ and *Aire*$^{-/-}$ into athymic mice did not prevent autoimmunity (Anderson *et al.* 2005). However, when one thymic lobe from *Aire*$^{-/-}$ and 4 thymic lobes from *Aire*$^{+/+}$ were transferred into athymic mice, autoimmune infiltrates were reduced (Anderson *et al.* 2005). It should be noted that these athymic mice were 10-12 weeks old when thymic grafting was performed (Anderson *et al.* 2005). This study suggests that the over-expression of Aire at least 4 times could oppose the autoimmunity phenotype. Another study performed by the same group showed that endogenous levels of Aire were not sufficient to prevent autoimmunity in *Aire*$^{-/-}$ mice. After birth, endogenous Aire was introduced to *Aire*$^{-/-}$ mice on the NOD background in a time-related controlled manner using a doxycycline-regulated Aire expression (Guerau-de-Arellano *et al.* 2009). When *Aire* was turned on only one day after birth, the endogenous expression levels of Aire in these mice did not prevent autoimmunity (Guerau-de-Arellano *et al.* 2009).

Indeed, in this study it has been demonstrated that TSAs expression follows AIRE expression pattern in a dose-dependent manner *in vivo* (*Chapters 5 and 6*). Furthermore, after a pilot study aiming at optimising the dose of AAV9-AIRE injections, over-expression of *AIRE* is essential for proper induction of TSAs but expression levels for more than 4000 fold change could induce toxicity. The ability to modulate AIRE function *in-vivo* using AAV9-AIRE was observed in 8-week-old *Aire*$^{-/-}$ mice AAV9-AIRE, 4-weeks post injection,

but not in 16-week-old *Aire*$^{-/-}$ mice AAV9-AIRE, 12-weeks post injection. The low *AIRE* expression levels in the thymi of 16-week-old *Aire*$^{-/-}$ mice AAV9-AIRE was not sufficient to induce thymic TSAs expression, therefore these mice exhibited autoimmunity similar to the control *Aire*$^{-/-}$ mice. This part of the study needs further investigation, it is possible to deduce that CMV promoter was silenced by autoregulation mechanisms such as methylation of CpG sites, or loss of vector DNA occurred over the duration of the treatment (Brooks *et al.* 2004, Wang *et al.* 2005). Tissue-specific promoters that are highly expressed in mTECs could be utilised instead of constitutive promoters as mentioned in chapter 4, to avoid transgene silencing. Moreover, a second intrathymic injection of AAV9-AIRE could be administered to compensate for the loss of vector DNA in longer duration of treatments.

Another question to investigate is why when *AIRE* expression was depleted after 12-weeks in *Aire*$^{-/-}$ mice AAV9-AIRE, they were not protected from autoimmunity. A possible explanation for that is the generated repertoire of $T_{reg}$, following the augmented expression of thymic AIRE, have been changed or cleared. Several studies reported that Aire promotes the generation of a distinct repertoire of $T_{reg}$ during perinatal period that are stably persist in adult mice (Guerau-de-Arellano *et al.* 2009, Yang *et al.* 2015, Fujikado *et al.* 2016). In the same study mentioned earlier using the doxycycline-regulated Aire expression, when *Aire* was turned on during the neonatal period up to 3 weeks after birth and then turned off, these mice were protected from autoimmunity (Guerau-de-Arellano *et al.* 2009). Following this study, several investigations have led to the discovery of a distinct subset of $T_{reg}$ produced during the perinatal window; this population has a role in

maintaining self-tolerance unique from those of $T_{reg}$ repertoire produced during adulthood (Yang *et al.* 2015). This highlights the importance of Aire expression early in life for long-term self-tolerance. Thus, endogenous Aire expression during perinatal period is sufficient to generate a distinct $T_{reg}$ repertoire that is necessary to induce long-term self-tolerance and protect from autoimmunity (Guerau-de-Arellano *et al.* 2009, Yang *et al.* 2015, Fujikado *et al.* 2016). However, over-expression of AIRE/Aire after perinatal window and during adulthood is essential to produce short-term $T_{reg}$ repertoire that could ameliorate autoimmunity in *Aire*$^{-/-}$ mice during the phase of augmented expression of AIRE/Aire.

In conclusion, restoration of AIRE expression using AAV9 vector can ameliorates the autoimmunity phenotype in APS-1 mouse model. This was achieved by promoting Aire-dependent TSAs expression in a dose-dependent manner. This study revealed that AAV9-AIRE reduced serum autoantibodies in 8-week-old *Aire*$^{-/-}$ mice to almost undetectable levels and improved the phenotype of APS-1.

## 7.1 Future work:

- Further studies should be carried out with lower doses of AAV9-AIRE virus to assess whether lower expression of AIRE under the CMV promoter would last for longer periods without affecting transduction efficiency.
- If the depleted expression levels of AIRE after 12-weeks was due to loss of vector DNA, investigations to detect the VPs protein expression levels of AAV9 might give an idea on the number of vector DNA.

- Regarding the $T_{reg}$ repertoire, flow cytometry analysis to characterise the $T_{reg}$ subset that might have been produced following the augmented AIRE expression could explain the short-term presence of such repertoire.
- Another approach that could be investigated for APS-1 gene therapy is the use of CRISPR/Cas9 system in order to integrate *AIRE* into the mutated *Aire* locus. However, this approach would be advisable to be introduced during foetal stages to ensure that proper gain-of-function AIRE expression would generate the pivotal $T_{reg}$ repertoire during the perinatal period for long-term self-tolerance.

# *CHAPTER EIGHT*

# APPENDIX

# 8. APPENDIX

## 8.1 Inserts sequences

### 8.1.1 Aire insert

CGCAGCCCCTGTGAGGAAGATGGCAGGTGGGGATGGAATGCTACGCCGTCTGCTGAGGCTGCACCGCA
CCGAGATCGCGGTGGCCATAGACAGTGCCTTTCCGCTGCTGCATGCTCTAGCCGACCACGACGTGGTC
CCTGAGGACAAGTTCCAGGAGACGCTCCGTCTGAAGGAGAAGGAAGGCTGCCCCCAGGCCTTCCACGC
CCTGCTGTCCTGGCTCCTGACCCGGGACAGTGGGGCCATCCTGGATTTCTGGAGGATTCTCTTTAAGG
ACTACAATCTGGAGCGGTACAGCCGCCTGCATAGCATCCTGGACGGCTTCCCAAAAGATGTGGACCTA
AACCAGTCCCGGAAAGGGAGAAAGCCCCTTGCTGGTCCCAAGGCCGCGGTACTGCCACCCAGACCCCC
CACCAAGAGAAAAGCACTGGAGGAGCCTCGAGCCACCCCACCAGCAACTCTGGCCTCAAAGAGCGTCT
CCAGCCCAGGCTCCCACCTGAAGACTAAGCCCCCTAAGAAGCCAGATGGCAACTTGGAGTCACAGCAC
CTTCCTCTTGGAAACGGAATTCAGACCATGGCAGCTTCTGTCCAGAGAGCTGTGACCGTGGCCTCTGG
GGATGTTCCAGGAACCCGAGGGGCCGTGGAAGGGATCCTTATCCAGCAGGTGTTTGAGTCAGGAAGAT
CCAAGAAGTGCATTCAGGTTGGGGGAGAGTTTTATACACCCAACAAGTTCGAAGACCCCAGTGGCAAT
TTGAAGAACAAGGCCCGGAGTGGTAGCAGCCTAAAGCCAGTGGTCCGAGCCAAGGGAGCCCAGGTCAC
TATACCTGGTAGAGATGAGCAGAAAGTGGGCCAGCAGTGTGGGGTTCCTCCCCTTCCATCCCTCCCCA
GTGAGCCCCAGGTTAACCAGAAGAACGAGGATGAGTGTGCCGTGTGCCACGACGGAGGTGAGCTCATC
TGTTGTGACGGCTGTCCCCGGGCCTTCCACCTGGCTTGCCTGTCCCCACCTCTGCAGGAGATCCCCAG
TGGCCTCTGGAGATGCTCCTGCTGCCTCCAGGGCAGAGTCCAACAGAACCTGTCCCAGCCTGAGGTGT
CCAGGCCCCCGGAGCTACCTGCAGAGACCCCGATCCTCGTGGGACTGAGGTCAGCTTCAGAGAAAACC
AGGGGCCCATCCAGGGAGCTCAAAGCCAGCTCTGATGCTGCTGTCACATATGTGAACCTGCTGGCCCC
GCACCCTGCAGCTCCTCTGCTGGAGCCTTCAGCACTGTGCCCTCTACTGAGTGCTGGGAATGAGGGGC
GGCCAGGTCCAGCACCAAGCGCGCGATGCAGTGTGTGTGGCGATGGCACCGAGGTGTTGCGGTGTGCA
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CCGCTGCAAATCCTGCTCTGCAGACTCGACTCCCACGCCAGGCACACCGGGCGAAGCTGTACCCACCT
CTGGGCCCCGTCCAGCACCTGGGCTTGCCAAGGTAGGGGACGACTCTGCTAGTCACGACCCTGTTCTA
CATAGGGACGACCTGGAGTCCCTCCTCAATGAGCACTCATTTGACGGCATCCTGCAGTGGGCCATCCA
GAGCATGTCACGCCCGCTGGCCGAGACACCACCCTTCTCTTCCTGATGACAGGTGGCCCAGGAAGGGG
TGGGCAGCACAGCATTGGCTCCCTCCCCACCCAGCCCCATCGGATGAGGCACTCTGTTCTGAGAGGCC
TGGGCTGATTAGGACCAAGAGCTGGCAGGTTCTGGCCTGCTGGACTCAGCTTGCAGATGG

### 8.1.2 AIRE insert

CGCGGCGCTACGCCGGCTTCTGAGGCTGCACCGCACGGAGATCGCGGTGGCCGTGGACAGCGCCTTCC
CACTGCTGCACGCGCTGGCTGACCACGACGTGGTCCCCGAGGACAAGTTTCAGGAGACGCTTCATCTG
AAGGAAAAGGAGGGCTGCCCCCAGGCCTTCCACGCCCTCCTGTCCTGGCTGCTGACCCAGGACTCCAC
AGCCATCCTGGACTTCTGGAGGGTGCTGTTCAAGGACTACAACCTGGAGCGCTATGGCCGGCTGCAGC
CCATCCTGGACAGCTTCCCCAAAGATGTGGACCTCAGCCAGCCCCGGAAGGGGAGGAAGCCCCCGGCC
GTCCCCAAGGCTTTGGTACCGCCACCCAGACTCCCCACCAAGAGGAAGGCCTCAGAAGAGGCTCGAGC
TGCCGCGCCAGCAGCCCTGACTCCAAGGGGCACCGCCAGCCCAGGCTCTCAACTGAAGGCCAAGCCCC
CCAAGAAGCCGGAGAGCAGCGCAGAGCAGCAGCGCCTTCCACTCGGGAACGGGATTCAGACCATGTCA
GCTTCAGTCCAGAGAGCTGTGGCCATGTCCTCCGGGGACGTCCCGGGAGCCCGAGGGGCCGTGGAGGG
GATCCTCATCCAGCAGGTGTTTGAGTCAGGCGGCTCCAAGAAGTGCATCCAGGTTGGTGGGGAGTTCT
ACACTCCCAGCAAGTTCGAAGACTCCGGCAGTGGGAAGAACAAGGCCCGCAGCAGCAGTGGCCCGAAG
CCTCTGGTTCGAGCCAAGGGAGCCCAGGGCGCTGCCCCCGGTGGGGGTGAGGCTAGGCTGGGCCAGCA
GGGCAGCGTTCCCGCCCCTCTGGCCCTCCCCAGTGACCCCCAGCTCCACCAGAAGAATGAGGACGAGT
GTGCCGTGTGTCGGGACGGCGGGGAGCTCATCTGCTGTGACGGCTGCCCTCGGGCCTTCCACCTGGCC

TGCCTGTCCCCTCCGCTCCGGGAGATCCCCAGTGGGACCTGGAGGTGCTCCAGCTGCCTGCAGGCAAC
AGTCCAGGAGGTGCAGCCCCGGGCAGAGGAGCCCCGGCCCCAGGAGCCACCCGTGGAGACCCCGCTCC
CCCCGGGGCTTAGGTCGGCGGGAGAGGAGGTAAGAGGTCCACCTGGGGAACCCCTAGCCGGCATGGAC
ACGACTCTTGTCTACAAGCACCTGCCGGCTCCGCCTTCTGCAGCCCCGCTGCCAGGGCTGGACTCCTC
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### 8.1.3 GFP Insert

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## 8.2 Gateway® plasmids sequences

### 8.2.1 Aire DEST vectors

>pCMV-Aire

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## 8.2.2 GFP DEST vectors

>pCMV-GFP

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>pCAG-GFP

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# 8.3 ssAAV9 vectors

## 8.3.1 AAV9-CMV-AIRE

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## 8.3.2 AAV9-CMV-GFP

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# *CHAPTER NINE*

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# 9. BIBLIOGRAPHY

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