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Impact of intrauterine growth retardation induced by maternal exposition to low-protein isocaloric diet during gestation on the structural and functional development of the offspring thymus in a murine model

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Abstract

Introduction: IUGR severely affects the development of organs and functions during prenatal life. This has remarkable consequences later in life as an increased incidence of non-communicable diseases. Immunological abnormalities in malnourished individuals are largely described, notably an increased susceptibility to early severe infections. IUGR is also associated with a higher risk of atopy, asthma or allergies. Yet, the association of IUGR exposition and later autoimmune disorders is poorly reported. Our work aims at studying the molecular modifications associated with IUGR exposition and predictive of a risk of later autoimmune disorders in a rat model.

Methods: Sprague-Dawley rat dams were exposed to normal chow (control, CTRL) or to a low protein diet (LPD, isocaloric 9% casein) during whole pregnancy to induce IUGR. At 180 days of life specimen were sacrificed and thymus were collected and analysed. We assessed basic morphologic thymic parameters (thymus on body weight ratio and thymic cellularity, namely the total number of mixed cells after mashing reported on thymus weight). To assess the thymic primitive functions, we sought for modifications in protein expression of selected markers of thymic function as Lymphotoxin-beta receptor (Ltßr) for the control of entrance and migration of thymocytes progenitors, AutoImmune Regulator (AIRe) for the acquisition of central tolerance, Forkhead box protein 3 (FoxP3) for the presence of regulatory T-cells. We also assessed the protein expression of Sirtuin-1 (Sirt-1) and Forkhead box protein O1 (FOXO-1) as markers of senescence.

Results: IUGR-exposed offspring was differently affected along a gender-manner. In male, the thymus weight / body weight ratio was significantly decreased in LPD group (0.56 vs. 0.4; p = 0.04) and in females, this ratio was significantly increased in LPD group (0.8 vs. 0.65; p = 0.03). In males, the thymic cellularity was significantly decreased in LPD group (71.5 vs. 144.7; p=0.02). The intra-thymic protein expression of the following markers was significantly decreased in males of the LPD group when compared to CTRL group: LtßR (0.22 vs. 0.47; p=0.01), AIRe (0.25 vs. 0.77; p=0.02) and FoxP3 (0.46 vs. 1.15; p=0.01). The intra-thymic protein expression of LtßR and AIRe was not significantly modified by IUGR condition in females (respectively: 0.69 vs. 0.63; p=0.20 and 0.63 vs. 0.81; p=0.39). The expression of FoxP3 was significantly decreased in females of LPD group when compared to CTRL (0.49 vs. 0.75; p=0.04).

Conclusion: Our murine model of IUGR brings evidence of significant changes in protein expression of selected markers of thymic function implicated in the prevention of autoimmunity. Our findings suggest early-programmed modifications of the primitive function of the thymus, associated with marks of intra-thymic immunosenescence. Whether these findings are associated with a significantly increased risk of autoimmune disorders later in life remains to be demonstrated. Interestingly, IUGR-exposed female offspring demonstrated better-conserved thymic development and function. These gender-mannered differences in the impact of IUGR deserve further experimental investigations.

Key words: IUGR, thymus, gender effect, immune system, autoimmunity

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Abbreviation

AIRe: Autoimmune regulator
APC: Antigen-presenting cell
APECED: Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS-1: Autoimmune polyendocrine syndrome type-1
cTEC: cortex thymic epithelial cell
DN: Double negative (CD4-/CD8-)
DOHaD: Developmental Origin of Health and Disease
DP: Double positive (CD4+/CD8+)
Fezf2: FEZ family finger protein 2
FOXO-1: Forkhead box protein O1
FoxP3: Forkhead box P3
IPEX: Immune dysregulation polyendocrinopathy, enteropathy, X-linked
IUGR: Intra-uterine growth retardation
Ltß: Lymphotoxin beta
LtßR: Lymphotoxin beta receptor
mTEC: medullar thymic epithelial cell
NCD: Non-communicable disease
Prmd1: PR domain zinc finger protein 1
Sirt-1: Sirtuin-1
TCR: T-cells receptor
TEC: Thymic epithelial cell
Treg: Regulatory T-cell
TSA: Tissue-specific antigen

Introduction

The concept of developmental origins of health and disease (DOHaD)

The concept of Developmental Origins of Health and Disease (DOHaD) suggests that chronic diseases have their origin in early life during developmental windows of susceptibility. Epidemiologic studies suggested that genes do not explain the whole pathologic process; further, than phenotypic variability, epigenetic mechanisms seem to take better into account environmental imprinting on the developmental programming of an organism (1). Epigenetic refers to heritable and reversible marks on DNA, regulating its transcription without changing the sequence (2). Large cohort studies indicate a critical time window, within the 1000 first days of life, that is sensitive to environmental imprinting via epigenetic modifications that have long lasting effect related to occurrence of non-communicable diseases (NCDs) later in life. The persistence of such epigenetic marks explains that an event early in life can have an aftermath much later (3). Classically, a first hit occurs during a particular time window of vulnerability; after a silent period, the exposition to a second hit later in life lets the disease appear. Another characteristic of epigenetic changes is their

reversibility if promptly detected during this period of vulnerability or even later in life. The mechanism behind this specificity is not fully understood yet (4).

Intra-uterine growth retardation as the first hit of an abnormal developmental programming in non-immunologic diseases

A small birth weight referred to gestational age defines the intrauterine retardation of growth (IUGR). IUGR is usually related to an energetic stress during pregnancy, as maternal malnutrition, fetal infection or placental abnormalities. It represents the inability for the fetus to grow along with its genetic heritage (5). This event has an impact on the development of the infant, it programs alterations of function (i.e. disease) in later life. More, this inability to adapt to poor conditions in early life exemplifies later in life when exposed to better energetic conditions. This phenomenon is called the “thrifty phenotype”. IUGR is followed by a rapid catch up growth. In this context of catch up growth, the child is exposed to a higher risk of metabolic and cardiovascular diseases such as hypertension, diabetes and coronary artery disease (1). IUGR affects most organ either by impairing a developmental process as apoptosis or inducing lasting changes in levels of key developmental genes (2). The influences of IUGR on the developing immune system has been largely reported in human studies and animal models. The classical anomaly is an impaired response of both innate and adaptive immune cells to infection, exposing the children to a greater mortality after more severe or earlier infection (6). Nonetheless, the consequences of IUGR on the thymic ontogeny and developing functions are poorly described.

Thymic ontogeny

In vertebrates, the immune system has developed to provide one of the main barriers against infections and tumors. If its development from very early fetal life is critical to allow the further maturation of the adaptive immune system, the first step is the acquisition of central tolerance to prevent autoimmunity, i.e. the activation of immune cells against one's self-antigens (7). The thymus is the typical site of acquisition of central tolerance: thymocytes develop through thymic cortex and medulla to undergo different step throughout life. It is, among other roles, responsible for central tolerance.

Thymus life: from creation to involution

The thymus origins from the endoderm at week 5 of gestational age in humans and grows until puberty to reach its maximum size. The T-cell progenitors colonize the thymus from the bone marrow at the gestational age of week 7 to 14 during which also begins the maturation and selection

of the thymocytes. It is characterized by a rapid expansion of thymic epithelial cells (TEC) and thymocytes (4,8,9).

After reaching its maximum size, the thymus begins to involve. The functional tissue is replaced by fat. This is the visible sign of a larger process named immunosenescence, which main consequence is the decreasing production of naïve T-cells. The subsequent impairment of adaptive immune function progressively exposes older individuals to a higher risk of severe infections, a decrease in post-vaccinal antibodies response and a possible explanation of higher sensitivity to developing tumors (10). The involution of the thymus is accelerated by androgen exposition creating a gender dimorphism with smaller thymuses observed in males than in females at a comparable age. Nonetheless, female thymuses also involve with age (9).

Senescence is a mechanism related to organs aging and is regulated by specific proteins such as Sirtuin-1 (Sirt-1) or Forkhead box protein O 1 (FOXO-1). Decreased expression of these markers is associated with increased oxidative damage and aging (11). Such decreased expression has already been demonstrated in other organs, such as the kidney or the vascular systems, to be equally nefarious. Sirt-1 and FOXO-1 have anti-inflammatory effects and do protect against senescence and aging, as inflammatory process leads to premature functional impairment such as renal insufficiency or atherosclerosis (12,13). In IUGR, the process of senescence has been demonstrated to be more important than in control. Various studies in animal model demonstrated a decrease in the expression of Sirt-1 and FOXO-1 in the context of IUGR (14).

Thymic structure and thymopoiesis

The thymus is a tiny mediastinal organ formed of two lobes; inside each of them, stand the peripheral cortex and the inner medulla separated by a vascular cortico-medullary zone. Each of them has specific roles in the production, selection, and maturation of thymocytes. Thymocytes travel through different intra-thymic and extra-thymic compartment to follow a succession of translational stages of maturation, which some are unique to fetal life (4,7). During the whole process of maturation, the T-cell progenitors migrate from the bone marrow to the thymic cortex where they differentiate into thymocytes. Mature thymocytes then migrate to the thymic medulla (*figure 1*) under the influence of chemokines that are regulated by lymphotoxin beta (Ltß) pathway (15,16).

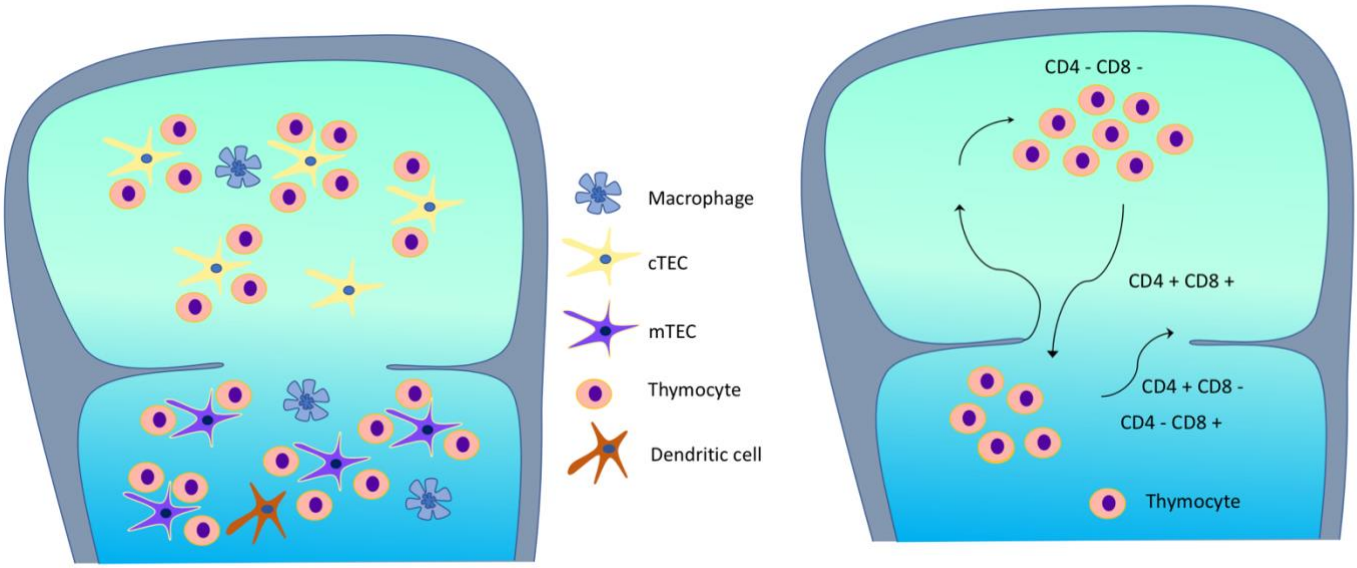

Figure 1 – **Thymic microscopic structure (left) and thymocyte migration and maturation in the thymus (right)**

Thymic primitive function: the acquisition of central tolerance and the prevention of autoimmunity

Tolerance is inherent in the development of the fetus: even before the acquisition of self-tolerance, the fetus has to tolerate his maternal womb to allow the course and termination of pregnancy (7). The central tolerance process is determined and regulated in the thymus: Tissue-specific self-antigen (TSA) are presented by antigens presenting cells (APCs) to the thymocytes via their T-cells receptor (TCR) to induce negative selection of autoreactive T-cells and the induction of regulatory T cell (Treg) that regulate the immune response as described below. TSA are peptides expressed by medullar thymic epithelial cells (mTECs). They represent a map of all organs of one's organism (the 'Self Map'), in order to educate thymocytes to tolerate them and to avoid autoimmune defense reactions (17). mTECs select $CD4^+$ and $CD8^+$ single positive thymocytes that are self-tolerant and induce an apoptotic process on thymocytes that are self-reactive; if reacting weakly to the TSA, thymocyte would be selected to become regulatory T-cells (Treg) by induction of the Forkhead box P3 (FoxP3) regulating factor, as discussed below (18,19). The expression of the TSA is mainly regulated by another major gene expression regulating factor, the Autoimmune regulator (AIRe) but also by many AIRe independent paths as shown below (20–22).

Thymopoiesis

Thymocytes precursors travel primarily from bone marrow to the thymus. They are initially characterized by a double negative phenotype CD4- CD8-. They, then, acquire CD4+ and CD8+;

consecutively, the rearrangement of the T-cell receptor (TCR) allows the creation of a unique repertoire for the recognition of self-antigens and acquisition of central tolerance. To be fully functional, thymocytes undergo an intra-thymic selection to ensure self-tolerance and to be programmed for later adaptive immune response against pathogens and neoplasms (4). This process will further allow the peripheral adaptive immune response to recognize and eliminate numbers of pathogens. Without an efficient TCR on the surface of maturing thymocytes to recognize a foreign peptide, cells are eliminated by apoptosis. This process is named the positive selection and take place in the cortex. Then thymocytes migrate to the medulla to be negatively selected. The medullar epithelium is characterized by the expression of self-antigen by mTECs that allow the negative selection by testing the auto-reactivity of mature thymocyte. This represents a major checkpoint and a large amount of thymocytes die by apoptotic mechanisms induced by mTECs towards highly autoreactive cells (8,20,23). Then the thymocytes migrate to bloodstream and peripheral secondary lymphoid organs. Environmental insult, such as early undernutrition, exposition to toxic substances, especially if during a critical developmental window may cause changes in the structure and/or function of sensitive cells populations, organs systems or metabolic pathways.

Thymic epithelial cells (TEC)

TEC are present in the thymus, in the cortex as cTEC and medulla as mTEC. Their roles are to maintain thymocytes, produce the extracellular matrix and guide the thymocytes into and onto the thymus via the expression of chemokine depending on their localization. cTEC and mTEC have the same progenitor; mTEC seems to derivate from a cTEC. In the final stage of maturation, mTECs are characterized by the expression of AIRe (Auto Immune Regulator) and the expression of TSA at the surface. (23)

Role of AIRe and Ltßr

AIRe is an atypical transcriptional regulator of many genes using many mechanisms; direct and indirect to induce the expression of many genes, such as TSA, chemokine and other surface markers or protein responsible of cells adhesion and interaction (24). A major function of AIRe is to induce expression of thousands of genes encoding the TSA in mTECs to promote tolerance of self (20). The mechanisms behind this expression are plural. AIRe is able to target TSA gene by linking repressed chromatin state and, by various mechanism, is able to change the epigenetics marks to allow the expression of those; it generates promiscuous genes expression. Indirectly, it increases the expression of TSA by releasing RNA polymerase II that allows the elongation of polypeptide rather

than initiate the transcription or by expression of other transcription factor or even via miRNA; another mechanism is the activation of a DNA phosphoryl-kinase that restores the damaged DNA to allow transcription and cell cycle. More than just targeting the TSA, AIRe is also inducing chemokines that are necessary to thymocyte migration and to dendritic cells arrival in the thymus. (18,25–27)

It is important to notice that TSA are not exclusively expressed through AIRe but other pathways induce AIRe-independent TSA such as lymphotoxin ß receptor (LTßR) and others pathway. Also, Sirt-1 is a key in the expression of AIRe-dependant TSA (13,21).

Furthermore, LTßR pathway is a key factor in the migration of thymocyte from the bone to the thymus and in the thymus as well. LTßR pathway is a key regulator of chemokines expressed the medulla that allows the thymocytes to undergo thymic selection by migrating into the medulla. It also regulates the terminal differentiation of mTEC and determines the size of population of mTEC in the thymus. Thus, LTßR has a key role on thymopoiesis and thymic ontogeny (16,28,29).

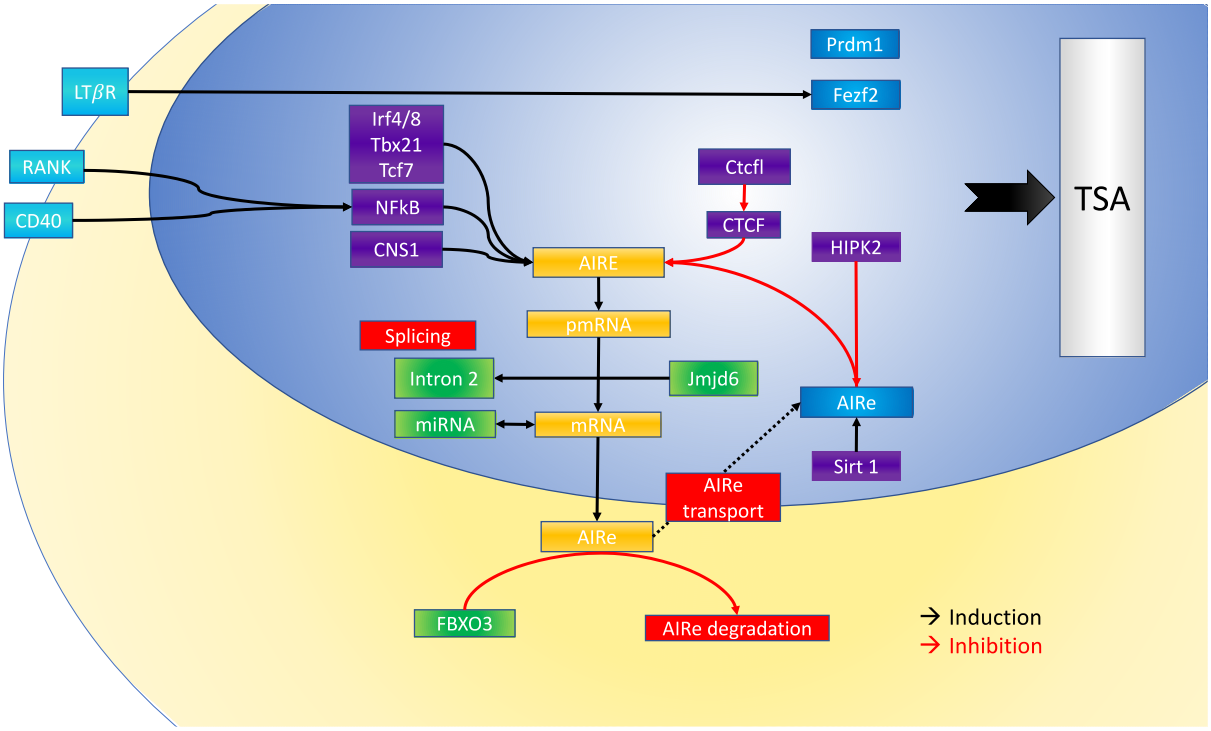

Figure 2 – **Expression of TSA in mTEC**

The interaction between RANKL-RANK et CD40-CD40L activate the pathways to the transcription of AIRe via NFkB, CD40 and RANK have overlapping function and are both sufficient to the expression of AIRe. This does not apply in prenatal timing where RANK signaling is essential(20). NFkB seems to be essential but is not sufficient to the expression of AIRe. Irf4/8, Tbx 21 and Tcf7 are other transcription factors that are implicated in the transcription of AIRe. CTCF isolate the AIRe locus in the cell that does not express AIRe, it represses its transcription. CTCFL inhibits CTCF and allows the transcription of the protein. Jmjd6 regulate the formation of the intron 2 that is essential to the maturation and the function of AIRe. Sirt1 activate AIRe mediated transcription by acetylation of AIRe.

A recent study showed that the AIRe expressional level is sufficient to perturb the interaction mRNA-miRNA in mTEC. These interactions are important for the transcriptional and post-transcriptional control of the TSA. (26)
Pmrd1 and Fezf2 induce AIRe independent-TSA. Fezf2 is activated via LTßR pathway. (21,22,27,30,31,31–33)

The criticalness function of AIRe is demonstrated in the AIRe mutations associated human diseases or animal models. They lead to autoimmune diseases, as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or autoimmune polyendocrine syndrome type-1 (APS-1) (25,34). APECED is genetically-characterized by a deficient expression of AIRE protein causing chronic mucocutaneous candidiasis, primary adrenal insufficiency and hypoparathyroidism. In addition, other organ-specific manifestations regularly appear such as vitiligo, pernicious anemia or primary ovarian deficiency (34,35).

Regulatory T-cells' production

Regulatory T-cells (Treg) are also fundamental for the homeostasis of tolerance. Tregs regulate the formation of some T-cells; they regulate the post-thymic homeostasis of self-tolerance. They downregulate the activation of T-cells, limit the propagations of inflammation and autoimmunity using different mechanism such as anti-inflammatory cytokine, cells interactions, and induction of anti-inflammatory cells, among others (36).

They are induced by the expression of FoxP3 and other epigenetic markers in $CD4^+$ (19). A mutation in this gene induces an auto-immune syndrome: IPEX that leads to severe autoimmune reaction and is lethal (37). It is important to note that there are two types of Treg, the thymic Tregs (tTreg) and the peripheral Tregs (pTregs). The first are produced in the thymus and are usually one with intermediate affinity with TSA, too low to be negatively selected. Several studies have suggested that AIRe produced a subpopulation of these tTregs in the perinatal periods; those tTregs seem to be more stable than the pTreg. The pTreg are induced by several mechanisms through conversion of naïve T cells in periphery such as $CD4^+$ and the mechanism of immunomodulation of inflammatory processes (19,38,39).

Effect of malnutrition on the thymic development

Malnutrition is known to be the leading cause of acquired immunodeficiency worldwide and its impact on the immune development is undeniable. This impact is shown, for instance, in the thymic atrophy of undernourished infants and their susceptibility to infection. Their thymus shows an increase of thymocytes apoptosis, that affect particularly the immature double positive (DP) CD4+CD8+ thymocytes. This effect is thought to be due to an excess of glucocorticoid that induces

a massive apoptosis for the very immature cells (6,40,41). The specific pathways of this mechanism remain to be further described.

Early malnutrition has a long-term effect, as the function of the thymus remains altered many years later (42). This suggests a developmental altered programming, but few studies report the long-lasting effect of malnutrition on immune tolerance.

Hypothesis

IUGR has a major impact on thymus structure and expression of selected functional markers

IUGR induces a change in the expression of numerous proteins and has consequences throughout life. The susceptibility to infections in malnourished individuals has largely dominated the clinical spectrum of reported immunological abnormalities. In human birth cohorts, the association between IUGR and a higher risk of atopy, asthma or allergies is largely reported (43). But so far, IUGR has never been demonstrated a risk of later autoimmunity. Our work aims at bringing evidence on the molecular mechanisms underlying this association in a rat model. The point of this experiment is to show if there is an impact on thymic structure (cellularity and weight) and a modification of protein expression in relevant determinants of thymic function as the entrance and migration of thymocytes progenitors (Ltßr), the acquisition of central tolerance (AIRe), the control of autoimmunity (FoxP3) and markers of senescence (Sirt-1; FOXO-1).

This study will allow us to describe the impact of IUGR on protein expression pattern of various markers of thymic development, namely in its primary function of prevention of autoimmunity. There is, for now, no literature on this subject.

Methods:

Model

A murine model of IUGR has been developed in the DOHaD laboratory at CHUV, in Lausanne (veterinary authorization reference: VD 3050). During gestation, Sprague-Dawley rat dams were exposed to normal chow (control) or to a low protein diet (LPD) (isocaloric 9% casein) restriction to induce IUGR. Animals were anesthetized and exsanguinated at age of 180 days. Organs of interest (including the thymus) were fixed and embedded in paraffin for histological analysis or frozen for molecular analyses. Thymuses were freshly prepared along a local standardized protocol for cellularity analysis (see annex).

Morphology

Each rat and each thymus were weighted during the harvest. The ratio between the weight of the thymus and the total body weight was then calculated.

Protein extraction

Thymuses were collected and frozen at -80°C. Thymuses were milled using a cryogenic mortar on dry ice then homogenized in a lysis buffer (HEPES pH 7.9; 1.5 mM MgCl2; 10 mM KCl; 1mM ethylenediaminetetraacetic acid; 10% glycerol; 1% NP-40) in which a protease inhibitor tablet was added (Roche Diagnostics, Indianapolis, IN, USA). After homogenization of the samples, those were centrifuged for 25 minutes at 4°C at 14'000 RPM to discard insoluble material. The supernatant was then extracted to be analyzed for protein quantification and western blot analysis.

Microplate procedure

Total proteins were quantified using BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). The supernatant was diluted in pure water at 1:100 and put on the array as well as the known BSA concentration (standard). Working reagent was then added in the well. Those were incubated at 37°C for 30 minutes and the absorbance was measured at 562 nm.

I	I	A	A	97	97	88	88
H	H	White	White	77	77	93	93
G	G	96	96	90	90		
F	F	78	78	71	71		
E	E	94	94	85	85		
D	D	91	91	95	95		
C	C	92	92	70	70		
B	B	84	84	89	89		

Table 1 – **Example of array**
I to A are standards concentration of BSA (in yellow) "White" is water. Numbers are samples of different animals (in blue). All samples are in duplicate.

Analysis the proteins dosage of each sample was calculated in function of the standard samples by reporting the curve of absorbance on the concentration of each sample to be able to extract exactly 30 µg of protein from the supernatant.

Western blot

Denaturated proteins of the thymus from each group were separated on the same gradient gel (NuPAGE 4–12% Bis-Tris gel, Thermo Scientific). The same amount of protein (30 µg) was taken for each animal and homogenized with lysis buffer, NUPAGE (2µl) and Laemli (4µl) to have 20µl of total volume.

Animal	**condition**	**sex**	**masse**	**[]*100 µg/mL**	**µg/ µl**	**30 µg**	**10X-1X**	**4X-1X**	**Add up**	Volume
78	Ctrl	F	55,13	3968,18	3,97	**7,6**	**2,0**	5	**5,4**	20
84	LPD	M	72,63	5422,73	5,42	**5,5**	**2,0**	5	**7,5**	20
71	Ctrl	M	55,86	3763,64	3,76	**8,0**	**2,0**	5	**5,0**	20
70	Ctrl	M	51,34	3895,45	3,90	**7,7**	**2,0**	5	**5,3**	20
89	LPD	F	49,69	6068,18	6,07	**4,9**	**2,0**	5	**8,1**	20

Table 2 – **Example of a sample**

Migration was performed at room temperature for two hours at 110 V; followed by transfer on an activated nitrocellulose paper overnight at 4°C at 30V. A Ponceau was then realized to assure that the proteins have actually been transferred. The membrane incubated in casein for two hours to block the membrane.

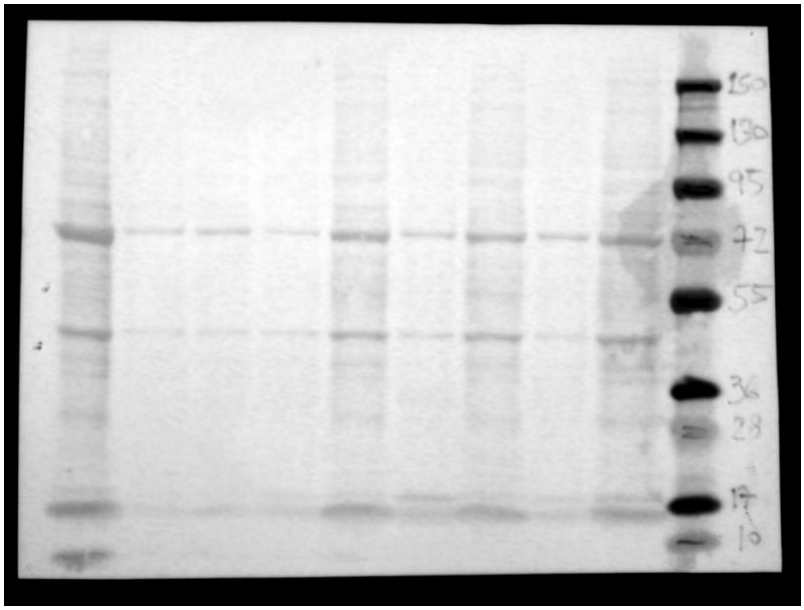

Photo 1 – **Example of Ponceau**

The primary antibodies were then incubated in the correspondent membrane at 4°C overnight and then washed by three baths of TBST 5%. Then they were incubated in the secondary antibodies for two hours at room temperature, who were correspondent of the animal's primary antibodies. Revelation was performed after 3 washing baths and adding Revelation solution (Western Femto, Thermo Scientific) for 2 to 5 minutes in the dark.

Reference	Antibody	Dilution	Animal
Abcam	AIRE	1/2000	Goat
SigmaAldrich	LtßR	1/2000	Goat
NovusBio	FoxP3	1/2000	Rabbit
CellVigly	Sirt1	1/2000	Rabbit
CellVigly	Actin	1/2000	Rabbit

Table 3 – **Antibody reference**

The images were taken by a G-Box with different time exposure depending on the protein. To reuse the membrane on which, there were different proteins tested; the membranes were cleaned up in Stripping Buffer. They were then re-incubated in primary antibodies.

Actin was assessed in every membrane to control the protein amount by normalization via division of the intensity of each band to the intensity of the actin's animal. The images were analyzed via ImageJ ® software to assess the intensity of each band. Potential imprecisions were therefore normalized.

Thymic cellularity

At PND 180, the offspring thymuses were isolated and cellularity assessed using local protocol (*annex 1*). Data were analyzed using a FlowJo Software.

Statistical analysis

All data were presented as mean ± SEM. Experimental data were compared using non-parametric Mann-Whitney U test if $n < 5$ and Student t test if $n > 5$ in groups.

ZD has done the western blot for AIRe, Ltßr, FoxP3, Sirt-1 and FOXO-1 prepared the sample for analysis and analyzed the results with ImageJ ® software.

Results

Thymic morphology

In males, the thymus weight / body weight ratio was significantly decreased in LPD group (0.56 vs. 0.4; $p = 0.04$). In females, the thymus weight / body weight ratio was significantly increased in LPD group (0.8 vs. 0.65; $p = 0.03$) (*figure 3*).

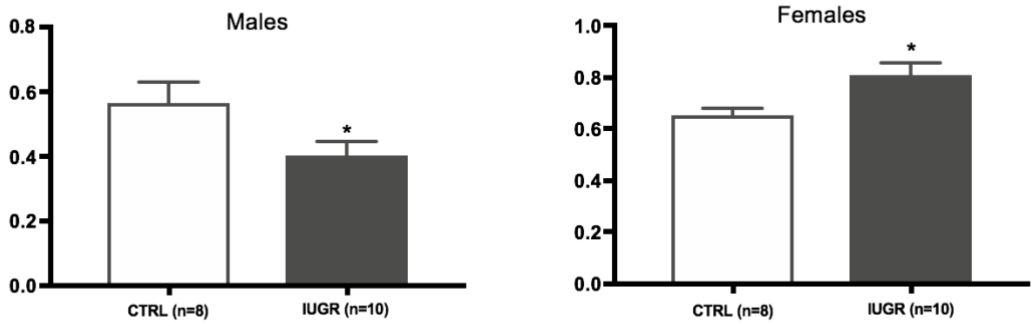

Figure 3 – **Thymus weight/body weight ratio in CTRL vs IUGR groups: a gender effect is observed**

Thymic cellularity

In males, the thymic cellularity (total number of cells after thymic cell suspension preparation/thymus weight, expressed in millions of cells/gram of thymus) was significantly decreased in LPD group (71.5 vs. 144.7; p=0.02) (*figure 4*). In females, the very small number of samples did not allow to compare the thymic cellularity between CTRL and LPD groups.

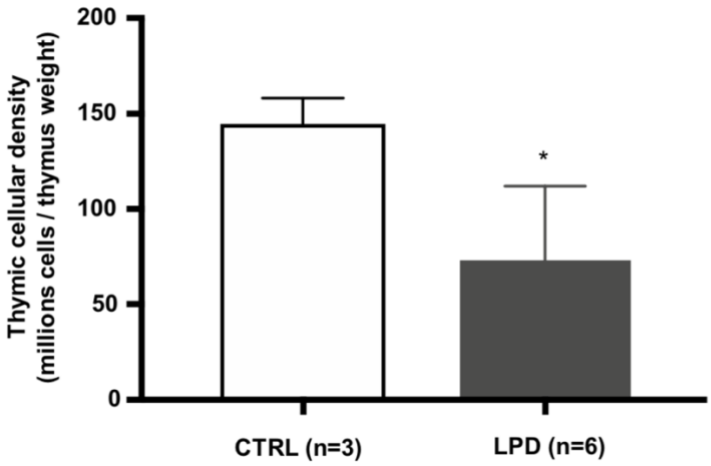

Figure 4 – **Thymic cellular density compared to body weight in 6-month-old male offspring**

Molecular changes

The analyzed proteins were reported to the amount of actin to be able to assess the ratio of the expression of the protein (*figure 5*).

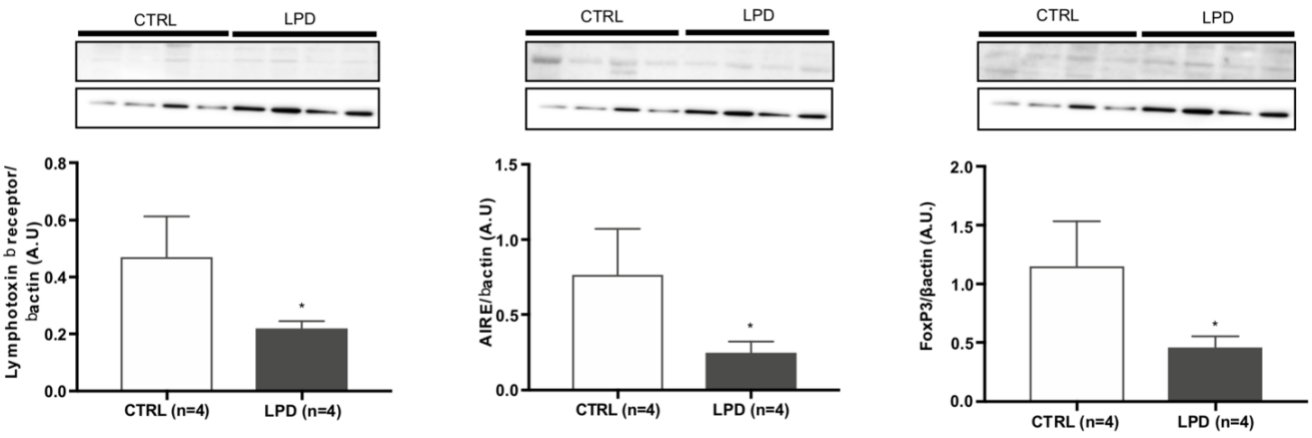

Figure 5 – **Expression of selected markers of thymic function in 6-month-old male offspring**

LtßR

In males, the intra-thymic protein expression of LtßR (expressed in arbitrary units) was significantly decreased in the LPD group (0.22 vs. 0.47; p=0.01) (*figure 5*).

In females, the intra-thymic protein expression of LtßR (expressed in arbitrary units) was not significantly different between CTRL and LPD groups (0.69 vs. 0.63; p=0.20)

AIRe

In males, the intra-thymic protein expression of AIRe (expressed in arbitrary units) was significantly decreased in the LPD group (0.25 vs. 0.77; p=0.02) (*figure 5*).

In females, the intra-thymic protein expression of AIRe (expressed in arbitrary units) was not significantly different between CTRL and LPD groups (0.63 vs. 0.81; p=0.39).

FoxP3

The intra-thymic protein expression of FoxP3 (expressed in arbitrary units) was significantly decreased in LPD group in males (0.46 vs. 1.15; p=0.01) and in females (0.49 vs. 0.75; p=0.04) (*figure 5*).

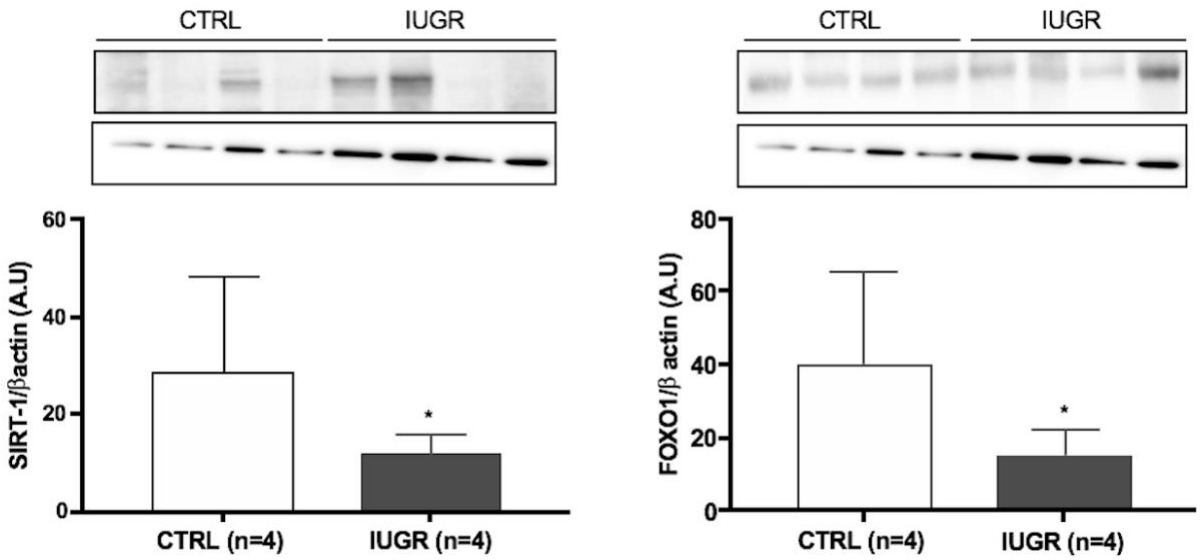

Figure 6 – **Selected markers of intra-thymic senescence in 6-month-old male offspring**

Sirt-1

In males, the intra-thymic protein expression of Sirt-1 (expressed in arbitrary units) was significantly decreased in the LPD group (12.07 vs. 38.99; p=0.03) (*figure 6*).

In females, the intra-thymic protein expression of Sirt-1 (expressed in arbitrary units) was not significantly different between CTRL and LPD groups (0.37 vs. 0.38; p=0.85).

FOXO-1

In males, the intra-thymic protein expression of FOXO-1 (expressed in arbitrary units) was significantly decreased in the LPD group (14.44 vs. 52.99; p=0.05) (*figure 6*).

In females, the intra-thymic protein expression of FOXO-1 (expressed in arbitrary units) was not significantly different between CTRL and LPD groups (6.36 vs. 6.79; p=0.59).

Discussion

Morphologic changes

The significantly diminished weight of the male thymus (*figure 2*) relatively to total body weight indicates that early life exposition to a low protein diet definitely programs structural changes in the developing thymus; these alterations may program thymic functional abnormalities in the long run without any compensatory mechanism. Thymuses being studied at 6 months of life, many mechanisms could explain such as a structural change in the thymuses exposed to early low protein diet: diminution of cellularity due to decreased recruitment of thymocytes progenitors, premature and/or accelerated apoptosis or a faster immunosenescence resulting as a smaller thymus by involution or an association of several of them (6,44,45). Interestingly in female offsprings at the

same age, the thymus/body weight ratio was significantly higher in the IUGR exposed group than in the CTRL group (*figure 2*). A gender dimorphism is known in the involution of the thymus but the significant differences we observed between CTRL and IUGR were unexpected. It could be a compensatory mechanism that is not present in males, possibly associated with a protective effect of female sexual hormones.

We quantified Sirt-1 and FOXO-1 to assess senescence in the thymus in a context of early malnutrition. The significant diminution of both markers (*figure 6*) in IUGR-exposed adult male offsprings indicates that senescence process is greater in this context and thus it could lead to a faster thymic involution. Such decrease of these markers is known to cause an accelerated aging of many organs affected by the diminution. We quantified Sirt-1 and FOXO-1 in the female thymus and there were no significant differences in the expression of these selected senescent markers (data not shown). The lack of difference in female could be due to the gender dimorphism in thymus involution. As the female thymuses involve later, it is thought that either androgens cause a faster involution or estrogens have a protective effect against immunosenescence, or both combined (45). We could hypothesize that the consequences of IUGR in senescence are not shown due to the physiological retardation of immunosenescence in female. To isolate proper mechanisms, the assessment of the same markers at earlier and later time points would be of great interest.

We also measured the thymic cellularity in our male specimens. We observed a decreased cellularity in IUGR male offspring at adulthood (*figure 4*). It indicates a lasting change in morphology and probably in functionality. Unfortunately, we did not have enough sample/specimens to assess it for females.

Intra-thymic functional markers

There is a significant decreased in the production of all selected markers of thymic function in IUGR-male offsprings at adulthood (*figure 5*). The ratio protein-actin allowed us to confront the data and analyze the expression of the protein without the bias of lack of substrate due to LPD.

Interestingly the production is not significantly modified in female for most of the protein except for FoxP3 that is significantly diminished in IUGR female (data not shown).

A decrease of AIRe expression has phenotypical consequence in TSA expression leading to their downregulation and to a putative risk of later autoimmunity (46). The diminution of AIRe in IUGR-male offsprings could support our hypothesis of an increased autoimmune risk in later life after exposition to IUGR. Interestingly the expression of AIRe does not falter in IUGR female compared to CTRL. Surprisingly, studies have shown that there is no gender effect in control condition on the

expression of AIRe despite the dimorphism on thymus involution. Though it is known that there is a gender effect on the interaction of AIRe with his local partners (regarding regulating factor of expression); in female, the interactions are decreased compared to male. This change in interaction could explain the epidemiologic augmented risk of immune disease in female (47). IUGR condition in female does not implicate a change in AIRe expression, thus a compensatory mechanism could help recover from the initial insult. It could be a protector effect of female hormone or a decrease in androgen hormone since it is linked with a faster involution.

Sirt-1 is partly responsible for the production of several AIRe-dependant TSA. Furthermore, the LtßR is also decreased in IUGR male (*figure 5*). It could lead to a lesser production of functional thymocytes by a decrease maturation through a decrease of migration intra-thymic and extra-thymic or a decreased interaction of thymocytes with mTEC. It could be linked to a greater impairment in antigen presentation inside the thymic medulla and thus, having a consequence in negative selection. The diminution of those proteins could slow a compensatory effect and amplify the effect of the diminution of AIRe. It could be linked to a greater risk of immune disease in male, which is not observed.

The decrease of FoxP3 (*figure 5*) could be associated with a lesser production of TSA and thus less interaction leading to the production of central Treg resulting in an increased risk of autoimmune and inflammatory disease.

Gender dimorphism

We observed a gender effect on the consequence of IUGR in the thymus. Indeed, the morphology and the function of the thymuses were significantly decreased in male offspring (*figure 3,4*), whereas there was no significant difference in female offsprings. On the opposite, the thymus weight was significantly higher in IUGR-female offspring and the function remained unaltered (*figure 3*).

It is known that androgens cause an accelerated involution of the thymus. Interestingly the effect is opposite on male and female, it could be an effect of elevated androgen levels in IUGR-male offsprings at adulthood and much lower in IUGR-female offsprings (9). We could also hypothesize that female hormone would have a potential of recovering from some anomalies related to IUGR and thus that female hormone would have a protective effect against early environmental insult as IUGR.

Studies have shown that thymic cellularity was higher in female than in male (45). In this context, the gender may be supported by epigenetic change in the expression profile of sexual hormones genes after exposition in IUGR.

The clinical significance of such gender effect is not yet clear. Indeed, females are more prone to immune diseases with a female to male ratio of 9 to 1 (48). The supposedly augmented risk in male could be well hidden due to such a gender difference.

Limits of study

The small number of specimen per group could bias our findings and did not allow us to bring significance to many of our data, especially in female offsprings.

Our rat model does not represent the most appropriate tool for translational research to humans, especially when dealing with the developing immune system. Monkey and pig models have a more similar immune system to human than rats or rodents (49).

Perspectives

Further investigations must be made before we understand fully the implications of IUGR in the thymus. This study focused on the augmented risk of auto-immune disease at a precise time point but a complete study including different time point and immunodeficiency and inflammatory diseases should be conducted. The epigenetic consequence of early low protein diet is yet to be described and whether a reversibility is possible and how (50). Studies begin to interest themselves to a nutrient reversibility and susceptibility with different targets such as vitamin A, B, D, E, beta-carotene, acid folic, Magnesium, Zinc (4) and to assess the effect of such nutrient in the context of IUGR would be of great interest.

Even more, the phenotypical consequence of the decreased function is not yet known. To assess the consequences, it would be interesting to assess the most immune targeted tissues such as the eye, the kidney, the skin and more, and to analyze the ratio of auto-immune precursor in peripheral tissues such as the spleen or lymph nodes.

Another key point in understanding the implications of IUGR on the thymus would be to assess the immunosenescence and possibility of rejuvenation. It could lead to a better understanding of the decaying immunity in life and to evaluate possible therapy for rejuvenation.

Conclusion

In conclusion, IUGR in rats seems to have quite an impact on male thymuses with a decreased in function, cellularity and weight. The analyses indicate that the mechanisms could be due to an early

involution of the thymus or “premature aging” and an increased immunosenescence. This is the first description of programming abnormalities in the development of the thymus induced by malnutrition. The clinical significance of such a difference with IUGR is not yet known and could be the subject of another work.

On the opposite, female seems to be less impacted in thymus functions and weight. The most probable hypothesis is a protector effect of female hormone associated with a decreased in androgen hormone in IUGR. Again, clinical significance is not yet evaluated and could be insignificant due to the large difference in the ratio of immune disease in male and female.

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References

1. Barker DJ, Eriksson JG, Forsén T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. Int J Epidemiol. 2002;31(6):1235–1239.
2. Joss-Moore LA, Lane RH. The developmental origins of adult disease. Curr Opin Pediatr. 2009 Apr;21(2):230–4.
3. Gervin K, Andreassen BK, Hjorthaug HS, Carlsen KCL, Carlsen K-H, Undlien DE, et al. Intra-individual changes in DNA methylation not mediated by cell-type composition are correlated with aging during childhood. Clin Epigenetics [Internet]. 2016 Dec [cited 2017 Jun 11];8(1). Available from: http://clinicalepigeneticsjournal.biomedcentral.com/articles/10.1186/s13148-016-0277-3
4. Palmer AC. Nutritionally Mediated Programming of the Developing Immune System. Adv Nutr Int Rev J. 2011 Sep 1;2(5):377–95.
5. Yzydorczyk C, Armengaud JB, Peyter AC, Chehade H, Cachat F, Juvet C, et al. Endothelial dysfunction in individuals born after fetal growth restriction: cardiovascular and renal consequences and preventive approaches. J Dev Orig Health Dis. 2017 Aug;8(4):448–64.
6. Moore S, Prentice A, Wagatsuma Y, Fulford A, Collinson A, Raqib R, et al. Early-life nutritional and

environmental determinants of thymic size in infants born in rural Bangladesh. Acta Paediatr. 2009 Jul;98(7):1168–75.
7. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. Proc R Soc B Biol Sci. 2015 Dec 22;282(1821):20143085.
8. Pearse G. Normal Structure, Function and Histology of the Thymus. Toxicol Pathol. 2006 Aug;34(5):504–14.
9. Boehm T, Swann JB. Thymus involution and regeneration: two sides of the same coin? Nat Rev Immunol. 2013;13(11):831.
10. Weiskopf D, Weinberger B, Grubeck-Loebenstein B. The aging of the immune system. Transpl Int. 2009 Nov 1;22(11):1041–50.
11. Xiong S, Salazar G, Patrushev N, Alexander RW. FoxO1 mediates an autofeedback loop regulating SIRT1 expression. J Biol Chem. 2011 Feb 18;286(7):5289–99.
12. Guan Y, Hao C-M. SIRT1 and Kidney Function. Kidney Dis Basel Switz. 2016 Mar;1(4):258–65.
13. Zhang W, Huang Q, Zeng Z, Wu J, Zhang Y, Chen Z. Sirt1 Inhibits Oxidative Stress in Vascular Endothelial Cells. Oxid Med Cell Longev. 2017;2017:7543973.
14. Chriett S, Le Huërou-Luron I, Vidal H, Pirola L. Dysregulation of sirtuins and key metabolic genes in skeletal muscle of pigs with spontaneous intrauterine growth restriction is associated with alterations of circulating IGF-1. Gen Comp Endocrinol. 2016 Jun;232:76–85.
15. Savino W, Dardenne M. Neuroendocrine control of thymus physiology 1. Endocr Rev. 2000;21(4):412–443.
16. Boehm T, Scheu S, Pfeffer K, Bleul CC. Thymic Medullary Epithelial Cell Differentiation, Thymocyte Emigration, and the Control of Autoimmunity Require Lympho–Epithelial Cross Talk via LTβR. J Exp Med. 2003 Sep 1;198(5):757–69.
17. Anderson MS. Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein. Science. 2002 Nov 15;298(5597):1387–95.
18. Anderson MS, Su MA. AIRE expands: new roles in immune tolerance and beyond. Nat Rev Immunol. 2016 Mar 14;16(4):247–58.
19. Li X, Zheng Y. Regulatory T cell identity: formation and maintenance. Trends Immunol. 2015 Jun;36(6):344–53.
20. Anderson MS, Su MA. Aire and T cell development. Curr Opin Immunol. 2011 Apr;23(2):198–206.
21. Takaba H, Morishita Y, Tomofuji Y, Danks L, Nitta T, Komatsu N, et al. Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance. Cell. 2015 Nov;163(4):975–87.
22. Roberts NA, Adams BD, McCarthy NI, Tooze RM, Parnell SM, Anderson G, et al. Prdm1 Regulates Thymic Epithelial Function To Prevent Autoimmunity. J Immunol. 2017 Aug 15;199(4):1250–60.
23. Abramson J, Anderson G. Thymic epithelial cells. Annu Rev Immunol. 2017;35:85–118.
24. Pezzi N, Assis AF, Cotrim-Sousa LC, Lopes GS, Mosella MS, Lima DS, et al. Aire knockdown in medullary thymic epithelial cells affects Aire protein, deregulates cell adhesion genes and decreases thymocyte interaction. Mol Immunol. 2016 Sep;77:157–73.
25. Akiyama T, Shinzawa M, Qin J, Akiyama N. Regulations of Gene Expression in Medullary Thymic Epithelial Cells Required for Preventing the Onset of Autoimmune Diseases. Front Immunol [Internet]. 2013 [cited 2017 Jun 11];4. Available from: http://journal.frontiersin.org/article/10.3389/fimmu.2013.00249/abstract
26. Oliveira EH, Macedo C, Collares CV, Freitas AC, Donate PB, Sakamoto-Hojo ET, et al. Aire Downregulation Is Associated with Changes in the Posttranscriptional Control of Peripheral Tissue Antigens in Medullary Thymic Epithelial Cells. Front Immunol [Internet]. 2016 Nov 23 [cited 2017 Jun 11];7. Available from: http://journal.frontiersin.org/article/10.3389/fimmu.2016.00526/full
27. Herzig Y, Nevo S, Bornstein C, Brezis MR, Ben-Hur S, Shkedy A, et al. Transcriptional programs that control expression of the autoimmune regulator gene Aire. Nat Immunol. 2016 Dec 12;18(2):161–72.
28. Zhu M, Chin RK, Tumanov AV, Liu X, Fu Y-X. Lymphotoxin Receptor Is Required for the Migration and Selection of Autoreactive T Cells in Thymic Medulla. J Immunol. 2007 Dec 15;179(12):8069–75.
29. Wu W, Shi Y, Xia H, Chai Q, Jin C, Ren B, et al. Epithelial LTβR signaling controls the population size of the progenitors of medullary thymic epithelial cells in neonatal mice. Sci Rep [Internet]. 2017 Dec [cited

2018 Nov 22];7(1). Available from: http://www.nature.com/articles/srep44481
30. Yanagihara T, Tomino T, Uruno T, Fukui Y. Thymic epithelial cell–specific deletion of Jmjd6 reduces Aire protein expression and exacerbates disease development in a mouse model of autoimmune diabetes. Biochem Biophys Res Commun. 2017 Jul;489(1):8–13.
31. Rattay K, Claude J, Rezavandy E, Matt S, Hofmann TG, Kyewski B, et al. Homeodomain-Interacting Protein Kinase 2, a Novel Autoimmune Regulator Interaction Partner, Modulates Promiscuous Gene Expression in Medullary Thymic Epithelial Cells. J Immunol. 2015 Feb 1;194(3):921–8.
32. Chuprin A, Avin A, Goldfarb Y, Herzig Y, Levi B, Jacob A, et al. The deacetylase Sirt1 is an essential regulator of Aire-mediated induction of central immunological tolerance. Nat Immunol. 2015 May 25;16(7):737–45.
33. Shao W, Zumer K, Fujinaga K, Peterlin BM. FBXO3 Protein Promotes Ubiquitylation and Transcriptional Activity of AIRE (Autoimmune Regulator). J Biol Chem. 2016 Aug 19;291(34):17953–63.
34. Bruserud ?yvind, Oftedal BE, Wolff AB, Husebye ES. AIRE-mutations and autoimmune disease. Curr Opin Immunol. 2016 Dec;43:8–15.
35. Oftedal B, Hellesen A, Erichsen M, Bratland E, Vardi A, Perheentupa J, et al. Dominant Mutations in the Autoimmune Regulator AIRE Are Associated with Common Organ-Specific Autoimmune Diseases. Immunity. 2015 Jun;42(6):1185–96.
36. Piccioni M, Chen Z, Tsun A, Li B. Regulatory T-Cell Differentiation and Their Function in Immune Regulation. In: Sun B, editor. T Helper Cell Differentiation and Their Function [Internet]. Dordrecht: Springer Netherlands; 2014. p. 67–97. (Advances in Experimental Medicine and Biology). Available from: https://doi.org/10.1007/978-94-017-9487-9_4
37. Pesenacker AM, Cook L, Levings MK. The role of FOXP3 in autoimmunity. Curr Opin Immunol. 2016 Dec;43:16–23.
38. Yang S, Fujikado N, Kolodin D, Benoist C, Mathis D. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance. Science. 2015;348(6234):589–594.
39. Tao J-H, Cheng M, Tang J-P, Liu Q, Pan F, Li X-P. Foxp3, Regulatory T Cell, and Autoimmune Diseases. Inflammation. 2017 Feb;40(1):328–39.
40. Bourke CD, Berkley JA, Prendergast AJ. Immune Dysfunction as a Cause and Consequence of Malnutrition. Trends Immunol. 2016 Jun;37(6):386–98.
41. Savino W. The thymus gland is a target in malnutrition. Eur J Clin Nutr. 2002;56(S3):S46.
42. McDade TW, Beck MA, Kuzawa CW, Adair LS. Prenatal undernutrition and postnatal growth are associated with adolescent thymic function. J Nutr. 2001;131(4):1225–1231.
43. Liu X, Olsen J, Agerbo E, Yuan W, Cnattingius S, Gissler M, et al. Birth weight, gestational age, fetal growth and childhood asthma hospitalization. Allergy Asthma Clin Immunol. 2014;10(1):13.
44. Tarry-Adkins JL, Aiken CE, Ashmore TJ, Fernandez-Twinn DS, Chen J-H, Ozanne SE. A suboptimal maternal diet combined with accelerated postnatal growth results in an altered aging profile in the thymus of male rats. FASEB J. 2018 Jul 5;fj.201701350RR.
45. Min H, Montecino-Rodriguez E, Dorshkind K. Reassessing the role of growth hormone and sex steroids in thymic involution. Clin Immunol. 2006 Jan 1;118(1):117–23.
46. Oliveira EH, Macedo C, Donate PB, Almeida RS, Pezzi N, Nguyen C, et al. Expression profile of peripheral tissue antigen genes in medullary thymic epithelial cells (mTECs) is dependent on mRNA levels of autoimmune regulator (Aire). Immunobiology. 2013 Jan;218(1):96–104.
47. Moreira-Filho CA, Bando SY, Bertonha FB, Ferreira LR, Vinhas C de F, Oliveira LHB, et al. Minipuberty and Sexual Dimorphism in the Infant Human Thymus. Sci Rep [Internet]. 2018 Dec [cited 2018 Nov 4];8(1). Available from: http://www.nature.com/articles/s41598-018-31583-3
48. Rose NR. Prediction and Prevention of Autoimmune Disease in the 21st Century: A Review and Preview. Am J Epidemiol. 2016 Mar 1;183(5):403–6.
49. Calder PC, Krauss-Etschmann S, de Jong EC, Dupont C, Frick J-S, Frokiaer H, et al. Early nutrition and immunity–progress and perspectives. Br J Nutr. 2006;96(4):774–790.
50. Wu H, Zhao M, Yoshimura A, Chang C, Lu Q. Critical Link Between Epigenetics and Transcription Factors in the Induction of Autoimmunity: a Comprehensive Review. Clin Rev Allergy Immunol. 2016 Jun;50(3):333–44.

TABLES AND FIGURES

Annex

1. Cellularity methods