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Aire-dependent peripheral tissue antigen mRNAs in mTEC cells feature networking refractoriness to microRNA interaction

Claudia Macedo[a], Ernna H. Oliveira[a], Renata S. Almeida[a], Paula B. Donate[a], Thaís A. Fornari[a], Nicole Pezzi[a], Elza T. Sakamoto-Hojo[b], Eduardo A. Donadi[c], Geraldo A. Passos[a,d,*]

[a] Molecular Immunogenetics Group, Department of Genetics, School of Medicine of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, SP, Brazil
[b] Department of Biology, Faculty of Philosophy, Sciences and Letters, USP, Ribeirão Preto, SP, Brazil
[c] Department of Clinical Medicine, School of Medicine of Ribeirão Preto, USP, Ribeirão Preto, SP, Brazil
[d] Department of Morphology, Physiology and Basic Pathology, Disciplines of Genetics and Molecular Biology, School of Dentistry of Ribeirão Preto, USP, Ribeirão Preto, SP, Brazil

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ABSTRACT

The downregulation of PTA genes in mTECs is associated with the loss of self-tolerance, and the role of miRNAs in this process is not fully understood. Therefore, we studied the expression of mRNAs and miRNAs in mTECs from autoimmune NOD mice during the period when loss of self-tolerance occurs in parallel with non-autoimmune BALB/c mice. Although the expression of the transcriptional regulator Aire was unchanged, we observed downregulation of a set of PTA mRNAs. A set of miRNAs was also differentially expressed in these mice. The reconstruction of miRNA–mRNA interaction networks identified the controller miRNAs and predicted the PTA mRNA targets. Interestingly, the known Aire-dependent PTAs exhibited pronounced refractoriness in the networking interaction with miRNAs. This study reveals the existence of a new mechanism in mTECs, and this mechanism may have importance in the control of self-tolerance.

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Introduction

The discovery that PTAs are ectopically expressed in the thymic medulla by mTECs that contribute to the elimination of nascent autoreactive thymocytes throughout negative selection has improved our understanding of the molecular and genetic mechanisms controlling central tolerance (Kyewski et al. 2002).

mTECs express virtually all PTAs, and this guarantees the representation of autoantigens from all tissues and organs. Due to the great diversity of autoantigens, this phenomenon was termed PGE, which is the key to central tolerance. Thus, mTECs are essentially self-antigen-presenting cells (Klein and Kyewski 2000; Derbinski et al. 2001; Mathis and Benoist 2004; Magalhães et al. 2006).

Developing thymocytes are presented with the major histocompatibility complex (MHC)-containing peptides coded by the PTA genes of mTECs. Dendritic cells also participate in the negative selection process after acquiring PTA peptides from mTECs (Klein and Kyewski 2000; Derbinski et al. 2001; Kyewski and Derbinski 2004; Gotter et al. 2004; Kyewski and Klein 2006; Takahama 2006; Hollander 2007; Irla et al. 2008; Villaseñor et al. 2008; Danso-Abeam et al., 2011; Klein et al. 2014).

Abbreviations: AID, autoimmune disease; Amelx, amelogenin X chromosome; Amy1, amylase 1, salivary; ANOVA, analysis of variance; Aire, autoimmune regulator; Alb, albumin; CD, cluster of differentiation; cDNA, complementary DNA; CIA, collagen-induced arthritis; Col1a2, collagen type 1 alpha 2; Cryaa, crystallyn alpha A; cTECs, cortical thymic epithelial cells; Cy3, cyanine 3; Cyp1a2, cytochrome P450, family 1, subfamily a, polypeptide 2; DNAase 1, desoxyribonuclease 1; Gad2, glutamic acid decarboxylase 2; Ghr, growth hormone receptor; Gabrb2, gamma-aminobutyric acid A – GABA – receptor, subunit beta 2; Hbb-y, hemoglobin Y, beta like embryonic chain; hsa, Homo sapiens; Ins1, insulin I; Ins2, insulin II; Igf2, insulin-like growth factor 2; K, thousand; KO, knockout; Lalba, lactoalbumin, alpha; MHC, major histocompatibility complex; mRNA, messenger RNA; miRNA, microRNA; mer, Merrifield; mmu, *Mus musculus*; Mir, microRNA; Mog, myelin oligodendrocyte glycoprotein; Muc6, mucin 6, gastric; Mucl1, mucin-like 1; Mup1, major urinary protein 1; Mup4, major urinary protein 4; mTEC, medullary thymic epithelial cell; ng, nanogram; NB, newborn; NCBI, National Center for Biotechnology Information; NOD, non-obese diabetic; PGE, promiscuous gene expression; Oxt, oxytocin; PTA, peripheral tissue antigen; Plp1, proteolipid protein (myelin) 1; qRT-PCR, quantitative real-time polymerase chain reaction; RIN, RNA integrity number; RNA Pol II, RNA polymerase II; Sag, retinal S antigen; SPF, specific pathogen-free; Spt1, salivary protein 1; Sst, somatostatin; S100b, S100 protein, beta polypeptide, neural; T1D, type 1 diabetes mellitus; 5WE, five-week old.

* Corresponding author at: Molecular Immunogenetics Group, Department of Genetics, School of Medicine of Ribeirão Preto, University of São Paulo, 3900 Bandeirantes Avenue, 14049-900 Ribeirão Preto, SP, Brazil. Fax: +55 16 3602 0222.
E-mail address: passos@usp.br (G.A. Passos).

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The observation that mutations in the Aire gene are associated with severe, multi-organ, tissue-specific autoimmunity in both humans and mice and the use of KO mouse model systems identified the role this gene plays in controlling central tolerance (Björses et al. 2000; Derbinski et al. 2005; Kont et al. 2008; Nagamine et al. 1997; Ramsey et al. 2002; Su and Anderson 2004; Su et al. 2008).

In addition to mutations, unbalanced levels of Aire expression in the thymic stroma is also associated with the aggressive autoimmunity observed in mouse models including NZB for systemic autoimmunity (Fletcher et al. 2009), NOD for T1D (Fornari et al. 2010) and DBA-1/J for collagen-induced arthritis (Donate et al. 2011). Lima et al. (2011) and Skogberg et al. (2014) have found that patients presenting with 21 trisomy (Down syndrome) feature thymic dysfunction and/or tolerance disturbances including altered expression of PTAs in the thymus due to the additional Aire gene in the genome, which is located at chromosome 21q22.3.

The AIRE protein coded for by this gene acts as a nuclear transcription controller that positively regulates the expression of many but not all PTAs considered being Aire dependent (Anderson et al. 2002; Anderson and Bluestone 2005).

Aire, PTA and MHC genes act in a pathway to control PGE (Danso-Abeam et al. 2011; Donate et al. 2011; Macedo et al. 2009), and additional proteins can operate in concert with AIRE to control PTA gene expression (Abramson et al. 2010). Recent evidence has shown that AIRE protein releases stalled RNA Pol II from blockage at the promoter region of its target genes (Giraud et al. 2012). This finding could explain the marked lack of specificity of AIRE and, consequently, the promiscuous gene expression.

The loss of self-tolerance may be due to the absence or deregulation of Aire and the consequent downregulation of Aire-dependent PTA genes in the thymus (Fletcher et al. 2009, 2011; Fornari et al. 2010; Donate et al. 2011).

Gene regulation is not only controlled by nuclear transcription factors but is also mediated by miRNAs, which are primarily active in the cytoplasm and mainly play a role as negative controllers of gene expression. Thus, miRNAs prevent mRNA translation into proteins and/or mediate its degradation. These small 19–24 nucleotide-long non-coding RNA molecules exhibit important biological activity by influencing the normal and pathological development of organs and systems including the immune system in general and the thymus, specifically (Lodish et al. 2008; Dai and Ahmed 2011; Khan et al. 2014).

We hypothesized that the loss of self-tolerance due to the downregulation of PTAs in the thymus could be due to miRNA activity.

Based on our previous studies (Fornari et al. 2010; Donate et al. 2011), we established an approach for determining the association between gene expression changes in the thymic stroma and aggressive autoimmunity using an experimental model system that reproduces an autoimmune disease. We have employed NOD mice, which develop autoimmune T1D during the aging process (Atkinson and Leiter 1999). Given the recent findings that Aire-dependent and Aire-independent PTA genes are downregulated in the thymus of NOD mice during the emergence of T1D (Fornari et al. 2010), this approach seemed ideal for measuring the interaction between miRNAs and PTA mRNAs and examining whether these interactions were associated to the development of autoimmunity.

Recent evidence showing the expression and the role of miRNAs in the thymic epithelium (Papadopoulou et al. 2011; Zuklys et al. 2012; Ucar et al. 2013; Khan et al. 2014) and that Aire regulates expression of miRNAs in mTECs (Macedo et al. 2013) led us to ask if miRNAs are involved in post-transcriptional control of PTAs in mTECs. If there was post-transcriptional control of PTAs, then this mechanism could be associated with loss of immune tolerance. In addition, we formulated a second hypothesis that these regulatory molecules might represent important factors that negatively control PTA mRNAs and central tolerance.

The microarray analysis that we utilized enabled us to explore the transcriptome (mRNAs) and the mirnome (miRNAs). A bioinformatics algorithm was then used to reconstruct miRNA–mRNA interaction networks.

We analyzed the expression of these RNA species in mTECs isolated from the thymus of newborn and five-week-old pre-diabetic NOD mice because this is the period when the loss of self-tolerance occurs in the mice. However, to evaluate the extent of this phenomenon, we also analyzed the RNA species isolated from mTECs of non-autoimmune BALB/c mice during the same periods of development.

Aire expression was unchanged during this period in both mouse strains. However, the levels of Aire-dependent and Aire-independent PTA mRNAs including those that are involved with autoimmune T1D in NOD mice were downregulated. We also observed the expression of a set of modulated miRNAs during this time period, which suggests the possibility that they might interact with target PTA mRNAs.

Due to the essential contribution of PTAs to negative selection and central self-tolerance, we compared the expression and post-transcriptional interactions with miRNAs in mTECs from autoimmune non-obese diabetic (NOD) in parallel with non-autoimmune BALB/c mice.

Materials and methods

Mice, medullary thymic epithelial cell separation and total RNA preparation

Three-day-old NB and 5WE female pre-diabetic NOD mice were used for the experiments. The animals were born and housed in SPF conditions at the Isogenic Mice Facility of the School of Medicine of Ribeirão Preto, University of São Paulo, Brazil in a temperature-controlled room (22 °C) with 12-h dark/light cycles. The animals received sterile water and food *ad libitum*. This study used only the clinically healthy animals with blood glucose levels ≤250 mg/dL, and these animals were considered pre-diabetic. The experimental procedures performed followed ethical guidelines under strict guidance and approval from the University of São Paulo Ethics Committee for Animal Experimental Research (Permit # 117/2008).

The thymic stroma was separated from the whole thymi of the two groups of mice (NB and 5WE) with minor modifications from a protocol previously described by Gray et al. (2002). In brief, thymi were dissected and trimmed of fat and connective tissue. The tissue fragments were gently agitated in 50 ml of RPMI 1640 medium at 4 °C with a magnetic stirrer for 30 min to remove the majority of thymocytes. The resulting thymic fragments were subsequently transferred to 10 ml of fresh RPMI 1640 medium, and the remaining thymocytes were dispersed by successive pipetting. The medium was changed 2–3 times after agitation, and fragments were recovered by settling for each sample. The thymic fragments were incubated in 5 ml of 0.125% (w/v) collagenase type II and 0.1% DNAse I (Invitrogen, Carlsbad, CA, USA) in RPMI 1640 medium at 37 °C for 15 min. The fragments were gently agitated every 5 min with a 1-ml pipettor. After 3–digestions, whole stromal cells were pooled and centrifuged at 450 × *g* for 5 min. These cells are known as thymic stromal cells and were used for isolation of mTECs.

To isolate mTECs, we used a modified version of the protocol described by Kont et al. (2008). Briefly, whole stromal cells were obtained as described above and then resuspended at a density of 1×10^8/98 μl RPMI 1640 and incubated with 2 μl of rat anti-mouse IgG2a BP-1 antibody (e-Bioscience, San Diego, CA, USA) for 15 min. The cells were mixed with magnetic micro-beads coated with anti-rat IgG (MACs Miltenyi Biotec Inc., Auburn, CA, USA) to remove $CD45^-$ cTECs. The cell fraction containing mTECs was

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