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Collagen induced arthritis (CIA) in mice features regulatory transcriptional network connecting major histocompatibility complex (MHC *H2*) with autoantigen genes in the thymus

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ABSTRACT

Considering that imbalance of central tolerance in the thymus contributes to aggressive autoimmunity, we compared the expression of peripheral tissue autoantigens (PTA) genes, which are involved in self-representation in the thymic stroma, of two mouse strains; DBA-1/J (MHC-$H2^q$) susceptible and DBA-2/J (MHC-$H2^d$) resistant to collagen induced arthritis (CIA). We evaluate whether these strains differ in their thymic gene expression, allowing identification of genes that might play a role in susceptibility/resistance to CIA. Microarray profiling showed that 1093 PTA genes were differentially modulated between collagen immunized DBA-1/J and DBA-2/J mice. These genes were assigned to 17 different tissues/organs, including joints/bone, characterizing the promiscuous gene expression (PGE), which is implicated in self-representation. Hierarchical clustering of microarray data and quantitative RT-PCR analysis showed that *Aire* (autoimmune regulator), an important regulator of the PGE process, Aire-dependent (insulin), Aire-independent (*Col2A1* and *Gad67*), and other 22 joint/bone autoantigen genes were down-regulated in DBA-1/J compared with DBA-2/J in the thymus. Considering the importance of MHC-*H2* in peptide-self presentation and autoimmunity susceptibility, we reconstructed transcriptional networks of both strains based on actual microarray data. The networks clearly demonstrated different MHC-H2 transcriptional interactions with PTAs genes. DBA-1/J strain featured MHC-*H2* as a node influencing downstream genes. Differently, in DBA-2/J strain network MHC-*H2* was exclusively self-regulated and does not control other genes. These findings provide evidence that CIA susceptibility in mice may be a reflex of a cascade-like transcriptional control connecting different genes to MHC-*H2* in the thymus.

Introduction

The unique structural and functional thymic microenvironment, which allows for a quality control of the nascent T cell repertoire, is provided by stromal cells, which besides macrophages and dendritic cells, is mainly formed by the two major subsets of thymic epithelial cells (TEC), cortical (cTEC) and medullary (mTEC). These two last, also define the two major compartments of the thymus, the cortex and the medulla, respectively (Van Ewijk 1991; Bleul et al. 2006).

T cells pass through both compartments in a spatially and temporally ordered process. During the cortical phase, a highly diverse T cell repertoire is generated and subjected to positive selection for self-MHC restriction. The subsequent medullary phase imposes T cell tolerance on the nascent repertoire via negative selection of auto-reactive effectors cells (Anderson et al. 2006). Although much is known about the biology of thymocytes, our understanding of the thymic stroma, and all its implication during the development of the autoimmune process, is still poor.

A better understanding of the central tolerance mechanism emerged from evidence that peripheral tissue antigens (PTAs) are

Abbreviations: CII, type II collagen; CIA, collagen induced arthritis; CFA, complete Freud's adjuvant; HLA, human leukocyte antigen; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PGE, promiscuous gene expression; PTA, peripheral tissue autoantigens; TEC, thymic epithelial cell; cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell; RA, rheumatoid arthritis; SAM, significance analysis of microarrays.

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expressed in the thymus and contribute to the selection of the T cell repertoire (Kyewski et al. 2002). Expression of PTAs is a physiological property of TECs, in particular mTECs, a phenomenon known as promiscuous gene expression (PGE) due to the extensive self-representation of autoantigens, which mobilizes up to 5–10% of all known mouse genes (Derbinski et al. 2001; Gotter et al. 2004).

Autoimmune regulator (*Aire*) considered a master regulator, drives the self presentation in the thymus. Expression profiling of mTECs isolated from *Aire*-knockout mice allowed the identification of autoantigen-encoding genes, such as insulin (*Ins*), salivary protein 1 and fatty-acid-binding protein (*Fabp*), which are under control of this transcription factor (Anderson et al. 2002). Meanwhile, other PTA genes, such as C-reactive protein (*Crp*) and glutamic acid decarboxylase of 67 kDa (*Gad67*), appeared to be independent of *Aire*, which also regulates the transcription of genes that do not encode PTAs (Derbinski et al. 2005).

Recent evidence showed that *Aire* also controls PGE either in an indirect manner; initially controlling the transcription of *Gucy2d* gene, which is connected to downstream PTA genes through transcriptional network (Macedo et al. 2009).

Studies on the control of PGE and PTA expression in the thymus have gained priority among several research groups, including our own, allowing the identification of gene expression and demarcation of PGE emergence during thymus ontogeny (Magalhães et al. 2006; Sousa Cardoso et al. 2006; Fornari et al. 2010).

Immunologists realize that the main implication of the heterogeneous expression of PTAs in the thymic stroma is associated with maintenance of the immunological homeostasis in the body. Deregulation of PGE may be an early warning sign for aggressive autoimmunity, which may lead autoimmune diseases, as it guarantees PTA representation in the thymus.

A manner to establish association between changes in the thymic stroma and aggressive autoimmunity is to make use of an experimental model-system, reproducing an autoimmune disease. Rheumatoid arthritis (RA) is a prototype of autoimmune disease, which in mice should be experimentally induced by immunization with collagen (collagen induced arthritis or CIA) of susceptible DBA-1/J strain (Wooley et al. 1981). Given that it is still unclear whether imbalance in the central tolerance influences CIA and/or RA, this approach seems adequate to the study.

Moreover, major histocompatibility complex (MHC) is a strong genetic determinant of susceptibility to CIA as determined by Vyacheslav et al. (2002) in a study showing that combining $H2^q$ and $H2^d$ alleles in mice resulted in codominant inheritance with a reduction of disease severity. MHC haplotypes as well as mutations along the *Aire* gene are associated to autoimmune diseases (Tiwari and Terasaki 1985; Anderson et al. 2002; Ramsey et al. 2002). However, the detailed mechanism(s) that trigger aggressive autoimmunity is still not totally understood despite our knowledge on the role of MHC molecules in autoantigen presentation and/or their role as genetic determinant of autoimune diseases.

For the reasons abovementioned and given the essential contribution of PTAs in the negative selection of autoreactive T cells in the thymus, we decided to compare both their expression and transcriptional interactions in mouse strains featuring CIA susceptibility (DBA-1/J) or resistance (DBA-2/J). MHC-H2 in these strains was highlighted due to its association with CIA development. The microarray method enabled us to explore the large scale expression of PTAs and quantitative RT-PCR to detect the expression of the *Aire* gene and genes that code for specific PTAs including *Col2a1*; the most important antigen associated with the RA development.

In view, *Aire* and genes that code joint/bone PTAs were down-regulated in DBA-1/J when comparing with the DBA-2/J mice. Interestingly, the reconstructed transcriptional networks based on the actual microarray data showed that while in DBA-1/J MHC-*H2* modulate downstream PTA genes, in DBA-2/J it was solely self-regulated and does not connect to any downstream genes. The differential PTA profiling and transcriptional networks observed between the two mouse strains, which include (or not) MHC-H2, may influence CIA susceptibility in mice.

Materials and methods

Animals and collagen-induced arthritis (CIA)

DBA/1J and DBA-2/J mice (12–14 weeks old) weighing 18–22 g each were housed in temperature-controlled rooms (22–25 °C) in the animal facility of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil, and received water and food *ad libitum*. DBA-1/J and DBA-2/J male mice were used in type II collagen immunizations.

For CIA, at day 0, DBA/1J and DBA-2/J males were intradermally injected at the base of the tail with 200 μg of bovine type II collagen (CII) (Sigma) in 100 μl of 0.05 M acetic acid emulsified with equal volume of complete Freund's adjuvant (Chondrex, Redmond, WA). On day 21, a second injection of CII (200 μg in acetic acid) was given intraperitoneally (i.p). A caliper was used to determine the paw diameter and swelling was determined as the increase in diameter compared to day 0 of immunization.

The severity of CIA was graded according to a score (CIA score) attributing the 0 (zero) value to normal paw with no swelling, erythema and no increase in joint diameter, 1 to slight swelling, erythema and 0.1–0.3 mm increase in joint diameter, 2 to swelling, erythema and 0.3–0.6 mm increase in joint diameter, 3 to extensive swelling, erythema and 0.6–0.9 mm increase in joint diameter and 4 to pronounced swelling and erythema with joint thickness of 0.9–1.2 mm increase or obvious joint destruction associated with visible joint deformity or ankylosis. Each limb was graded, resulting in a maximum clinical score of 16 per animal and expressed as the mean score on a given day. Disease onset was typically seen between days 25 and 35 after CII injection and was characterized by erythema and/or paw swelling. Only those animals presenting score 16 at day 35 were used for thymic stromal cell preparation and RNA extraction. For all experiments, we used six animals per group.

The experimental protocols were approved by the Commission for Ethics in Animal Research, Faculty of Medicine of Ribeirão Preto, USP, Brazil (Protocol # 244/2005).

Thymic stroma separation, mTEC 3.10 cell strain culture and total RNA preparation

The thymic stroma was separated from the whole thymus, as previously described (Gray et al. 2002). In brief, thymi were dissected and trimmed of fat and connective tissue. Tissue fragments were then 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 then transferred to 10 ml of fresh RPMI 1640 medium and remaining thymocytes were dispersed by successive pipetting. Medium was changed 2–3 times after agitations, with fragments recovered by settling each time. The thymic fragments were then incubated in 5 ml of 0.125% (w/v) collagenase type II with 0.1% DNAse I (both from Invitrogen, Carlsbad, CA, USA) in RPMI 1640 at 37 °C for 15 min, with gentle agitation every 5 min using a 1 ml pipettor. After 3–4 digestions, stromal cells were pooled and centrifuged at 450 × *g* for 5 min and finally resuspended in 200 μl PBS. These cells were then used for total RNA preparation.

The mTEC 3.10 medullary thymic epithelial cell line was established from C57BL/6 mice, and the original medullary phenotype was confirmed by immunostaining with Th-3 and Th-4 antibody, recognizing cortical and medullary phenotypes, respectively