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CD26/DPPIV inhibition alters the expression of immune response-related genes in the thymi of NOD mice

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ABSTRACT

The transmembrane glycoprotein CD26 or dipeptidyl peptidase IV (DPPIV) is a multifunctional protein. In immune system, CD26 plays a role in T-cell function and is also involved in thymic maturation and emigration patterns. In preclinical studies, treatment with DPPIV inhibitors reduces insulitis and delays or even reverses the new -onset of type 1 diabetes (T1D) in *non-obese diabetic* (NOD) mice. However, the specific mechanisms involved in these effects remain unknown. The aim of the present study was to investigate how DPPIV inhibition modifies the expression of genes in the thymus of NOD mice by microarray analysis. Changes in the gene expression of β-cell autoantigens and *Aire* in thymic epithelial cells (TECs) were also evaluated by using qRT-PCR. A DPPIV inhibitor, MK626, was orally administered in the diet for 4 and 6 weeks starting at 6–8 weeks of age. Thymic glands from treated and control mice were obtained for each study checkpoint. Thymus transcriptome analysis revealed that 58 genes were significantly over-expressed in MK626-treated mice after 6 weeks of treatment. Changes in gene expression in the thymus were confined mainly to the immune system, including innate immunity, chemotaxis, antigen presentation and immunoregulation. Most of the genes are implicated in central tolerance mechanisms through several pathways. No differences were observed in the expression of *Aire* and β-cell autoantigens in TECs. In the current study, we demonstrate that treatment with the DPPIV inhibitor MK626 in NOD mice alters the expression of the immune response-related genes in the thymus, especially those related to immunological central tolerance, and may contribute to the prevention of T1D.

1. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease caused by the selective destruction of pancreatic β cells (Atkinson and Eisenbarth, 2001). The breakdown of immune self-tolerance homeostasis to pancreatic islet β cells is now recognized as the essential cause for the development of the diabetogenic autoimmune response (Geenen, 2012). Therefore, the reestablishment of autoimmune tolerance state toward self-antigens (Ags) is one of the primary objectives for the prevention of autoimmune diseases, including T1D. During the last decade, immunotherapeutic innovative strategies have focused on maintaining and restoring self-tolerance to pancreatic β cells in T1D (Staeva et al., 2013).

CD26, also known as dipeptidyl peptidase IV (CD26/DPPIV), is a multifunctional cell surface glycoprotein expressed on a variety of cell types, including immune cells. This protein is a proteolytic enzyme, receptor and co-stimulatory protein and is involved in

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adhesion and apoptosis (Boonacker and Van Noorden, 2003). CD26/DPPIV's proteolytic activity is capable of cleaving N-terminal dipeptides from polypeptides with either proline or alanine residues in the penultimate position, modulating the activity of biologically relevant peptides such as cytokines, chemokines and incretins, among others. In addition, several studies have highlighted the important role of CD26/DPPIV in T cell activation and its involvement in immune responses (Morimoto and Schlossman, 1998). CD26/DPPIV interacts with molecules such as adenosin deaminase and CD45 and is able to modulate the co-stimulation and proliferation of activated T cells (Ohnuma et al., 2008).

Inhibition of CD26/DPPIV suppresses antigen-stimulated T cell proliferation and cytokine production, thus suggesting a potential application for DPPIV inhibitors as immunomodulatory drugs in autoimmune diseases (Biton et al., 2011). The effect of treatment with a CD26/DPPIV inhibitor on the immune system has been recently evaluated in several animal models of inflammatory human diseases (Steinbrecher et al., 2011). In relation to T1D, treatment with CD26/DPPIV inhibitors has been shown to delay the onset of the disease as well as even to reverse new-onset diabetes in *non-obese diabetic* (NOD) mice, in both cases with an associated reduction in the islet lymphocyte infiltration (Ding et al., 2014; Jelsing et al., 2012; Tian et al., 2010) although the exact mechanism is unknown. Treatment with a CD26/DPPIV inhibitor was also described to modify T lymphocyte subsets with an increase in the percentage of regulatory T cells (Tregs) in the peripheral and thymic compartments (Tian et al., 2010). Moreover, in the NOD model, treatment with the CD26/DPPIV inhibitor sitagliptin has been reported to preserve islet transplants through a pathway involving modulation of $CD4^+$ T cell migration (Kim et al., 2009). We recently demonstrated that treatment with the CD26/DPPIV inhibitor MK626 decreases the incidence of type 1 diabetes (T1D) by 31% and reduces insulitis in the pre-diabetic NOD mouse model. No differences were observed in the percentage of T cell subsets from peripheral and central compartments between treated and control mice. However, MK626 treatment significantly increased the expression of CD26 in $CD8^+$ T effector memory (T_{EM}) T cells as well as their proliferative capacity and cytokine secretion. *In vitro* assays suggested an immunosuppressive role for $CD8^+$ T_{EM} cell subset that may be involved in the protection against autoimmunity to β pancreatic islets associated to CD26/DPPIV inhibitor treatment (Alonso et al., 2015).

There is now evidence that a failure in thymus-dependent central tolerance to pancreatic β cells plays a primary role in T1D pathogenesis (Geenen, 2012). The thymus is the organ responsible for the establishment of immunological central tolerance by the deletion of self-reactive T cells through positive and negative selection mechanisms. Defects in the negative selection of self-reactive T cells in the NOD thymus have been reported (Kishimoto and Sprent, 2001). On the other hand, medullary thymic epithelial cells (mTEC) can express a broad range of tissue-restricted Ags (TRAs) (Derbinski et al., 2001; Fornari et al., 2010; Gillard and Farr, 2006; Kyewski et al., 2002; Oliveira et al., 2013; Sospedra et al., 1998; Tykocinski et al., 2010), also known as "promiscuous gene expression", that imposes T cell tolerance and protects from autoimmune disease (Sospedra et al., 1998).

In the thymus, CD26/DPPIV has been shown to play a role in the differentiation and maturation of thymocytes, whose impairment has remarkable effects on lymphocyte subsets and thymic architecture (Klemann et al., 2009). Moreover, CD26/DPPIV has been proposed as a mediator of intrathymic lymphocyte migration and may play a role in thymic deletion of emerging clones (Ruiz et al., 1996) thus implying a possible role for CD26/DPPIV in the establishment of central tolerance.

To our knowledge, this is the first report that describes the effect of treatment with a CD26/DPPIV inhibitor on the thymus transcriptome in the NOD mice and hypothesizes its possible involvement in the modification of the expression of genes related to central tolerance mechanisms. Here, we investigated the impact of treatment with the CD26/DPPIV inhibitor MK626 on the thymic gene expression profile of pre-diabetic NOD mice by DNA microarray technique, with particular emphasis on those genes involved in the immune response. We also evaluated the effects of MK626 treatment on islet autoantigens and *Aire* gene expression in thymic epithelial cells by qRT-PCR.

2. Materials and methods

2.1. Mice

Wild-type NOD mice were obtained from our colony established with mice from the Jackson Laboratory (Bar Harbor, ME, USA). Only females were used for this study. Mice were kept under specific pathogen-free conditions and monitored daily for diabetes onset. At the end of the study, mice were sacrificed by cervical dislocation. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Generalitat de Catalunya, Catalan Government. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Germans Trias i Pujol Research Institute (Permit number: DAAM 5928).

2.2. Treatment with MK626

Female NOD/Ltj mice were placed on either a normal chow diet (Research Diets, Inc, New Brunswick, NJ) or the same diet containing the CD26/DPPIV inhibitor MK626 (21 mg/kg of diet), kindly donated by Dr. James Mu (Merck Research Laboratories, New Jersey, USA), for 4 and 6 weeks starting at 6–8 weeks of age (pre-diabetic stage). The CD26/DPPIV inhibitor used in this study, MK626, was a des-fluoroanalog of sitagliptin (Kim et al., 2005). The treatment protocol is based on previous studies published in the literature using DPPIV inhibitors in experimental diabetes (Jelsing J et al., 2012; Kim D et al., 2005; Tian L et al., 2010), and data reported by Merck Research Laboratories. Also, the chosen concentration of the drug was the one able to maximize plasma DPPIV inhibition in order to get full effect. Mice were monitored daily for urine glucose using Glucocard strips during the whole study (Menarini, Barcelona, Spain). Thymic glands from NOD mice were obtained after 4 weeks (at approximately 10–12 weeks of age, $n = 5$) and 6 weeks (at approximately 12–14 weeks of age, $n = 5$) of treatment with MK626. Thymic glands were also obtained from a NOD mouse control group for each time point ($n = 10$). Samples were snap-frozen in an isopentane/cold acetone bath and were kept at −80 °C until RNA extraction.

2.3. Microarray experiments

RNA was obtained from the thymi of pre-diabetic treated mice at 4 and 6 weeks of treatment, using RNeasy Micro (QIAGEN, Hilden, Germany). Thymic glands were also obtained from a NOD mouse control group for each time point. RNA quality (2100 Bioanalyzer, Agilent Technologies Inc., Santa Clara, CA) was optimal for microarray experiments (RIN between 6 and 8). cDNA was synthesized with 50–100 ng of total RNA using the WT expression kit (Ambion, Applied Biosystems, CA, USA), fragmented and labeled with the Terminal labeling kit (Affymetrix, Inc. Santa Clara, CA), purified (GeneChip® Sample Cleanup Module, Affymetrix), fragmented and checked to verify its integrity. Mouse Gene1.1 ST 16 array plates (28.853 genes) were hybridized and scanned by an Affymetrix G3000 Gene Array Scanner.