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Thymic CCL2 influences induction of T-cell tolerance

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ABSTRACT

Thymic epithelial cells (TEC) and dendritic cells (DC) play a role in T cell development by controlling the selection of the T cell receptor repertoire. DC have been described to take up antigens in the periphery and migrate into the thymus where they mediate tolerance via deletion of autoreactive T cells, or by induction of natural regulatory T cells. Migration of DC to thymus is driven by chemokine receptors. CCL2, a major ligand for the chemokine receptor CCR2, is an inflammation-associated chemokine that induces the recruitment of immune cells in tissues. CCL2 and CCR2 are implicated in promoting experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis. We here show that CCL2 is constitutively expressed by endothelial cells and TEC in the thymus. Transgenic mice overexpressing CCL2 in the thymus showed an increased number of thymic plasmacytoid DC and pronounced impairment of T cell development. Consequently, CCL2 transgenic mice were resistant to EAE. These findings demonstrate that expression of CCL2 in thymus regulates DC homeostasis and controls development of autoreactive T cells, thus preventing development of autoimmune diseases.

1. Introduction

T cell development in the thymus is a complex process. T cell selection and maturation are guided by cytokines and chemokines that thymocytes encounter in the thymic cortex and medulla [1–6]. Thymic selection is controlled by interactions with stromal cells, mainly thymic epithelial cells (TEC) and dendritic cells (DC). Double negative (DN) $CD4^{-}CD8^{-}$ thymocytes migrate through the thymic cortex where they acquire both CD4 and CD8 co-receptors. These $CD4^{+}CD8^{+}$ double positive (DP) thymocytes are then selected on the basis of the ability of the T cell receptor (TCR) to recognize major histocompatibility complex (MHC) molecules, a process called positive selection [5]. This selection event induces thymocyte migration into the medulla, via the chemokine receptor CCR7 [7,8] and their differentiation into either $CD4^{+}$ or $CD8^{+}$ single-positive (SP) thymocytes. SP thymocytes in the medulla encounter antigen presenting cells such as medullary TEC (mTEC) and DC, which display self-antigens [5,9–11]. mTEC have the ability to express a wide range of tissue-restricted antigens [12,13], whose expression is mainly regulated by the transcription factor AIRE (autoimmune regulator). This ectopic gene expression program is important for central T-cell tolerance, since Aire-deficient mice show a decrease in tissue-restricted antigen expression and develop multi-organ autoimmunity [14]. SP thymocytes whose TCR recognize tissue-restricted antigen expressed by mTEC are deleted (negative selection) or are induced to differentiate into natural regulatory T cells [15–18]. Thus these selection events lead to the generation of self-tolerant T-cells.

Three subsets of DC have been identified in the mouse thymus. The $CD11c^{+}B220^{+}$ plasmacytoid DC (pDC) and two conventional DC (cDC) subsets: $CD11c^{+}B220^{-}CD8\alpha^{+}$ signal regulatory protein α $(Sirp\alpha)^{lo/-}$ and $CD11c^{+}B220^{-}CD8\alpha^{-/lo}Sirp\alpha^{hi/+}$ cDC [19,20]. pDC and $Sirp\alpha^{+}$ cDC derive extrathymically and migrate to the thymus from the blood while the $Sirp\alpha^{-}$ cDC are generated in the thymus [21]. It has been shown that resident $Sirp\alpha^{-}$ cDC can cross-present self-antigens via the transfer of antigens from mTEC [22], and that circulating DC can take up antigens in the periphery and migrate into the thymus to delete autoreactive T cells or induce regulatory T cells [9,21,23–25]. DC express chemokine receptors [19,24] including CCR2, which is expressed by all three DC subsets [19].

Abbreviations: AIRE, autoimmune regulator; cDC, conventional DC; CNS, central nervous system; cTEC, cortical TEC; DN, double negative; DP, double positive; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MCP-1, monocyte chemoattractant protein 1; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; mTEC, medullary TEC; pDC, plasmacytoid DC; SP, single-positive; TCR, T cell receptor; TEC, thymic epithelial cells.

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CCR2 is a receptor for CCL2, also called monocyte chemoattractant protein 1 (MCP-1), a chemokine involved in regulation of migration of immune cells, mainly monocytes/macrophages [26]. Both CCL2 and CCR2 are implicated in induction of autoimmunity eg. virus-induced arthritis [27] or Guillain-Barré Syndrome [28]. CCL2 and CCR2 have also been shown to play an important role in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis [29]. CCR2 deficient mice or mice injected with CCR2 antagonist were resistant to EAE [26,30,31] or had mild EAE [32] and CCR2 has been shown to increase in spinal cord during disease attack [33]. CCL2 deficient mice also showed mild EAE and a delayed onset [34] as do mice that have central nervous system (CNS) cells deficient for CCL2 [35]. CCL2 has been shown to increase in CNS during disease onset and at peak of disease [30,36].

We therefore predicted that overexpression of CCL2 in CNS should lead to more severe EAE. However we observed that transgenic mice in which CCL2 is expressed under control of a myelin basic protein (MBP) promoter were resistant to EAE. We thus investigated the mechanism for EAE resistance of these MBP/CCL2 transgenic mice. We show here that CCL2 is constitutively expressed in thymus, by endothelial cells and TEC. Transgenic mice overexpressing CCL2 in the thymus showed an increased number of pDC, and deletion of autoreactive T cells that consequently led to protection against EAE. This identifies a novel role for CCL2 in thymic tolerance and prevention of autoimmunity.

2. Materials and methods

2.1. Mice

C57Bl/6 (B6) mice were purchased from Taconic Europe A/S and the CCL2-deficient mice from The Jackson Laboratory. MBP/CCL2 mice that express a mouse CCL2 transgene under control of an MBP promoter [37] were originally obtained from Bristol-Myers Squibb and maintained as a homozygous colony. 2D2 mice, which have $CD4^+$ T cells that express a transgenic Vα3.2β11 TCR specific for MOGp35-55 were obtained from Florian C. Kurschus (Max-Planck-Institute of Neurobiology, Martinsried, Germany, currently at Institute of Molecular Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany). OTII mice, which have $CD4^+$ T cells that express a transgenic Vα2β5 TCR specific for ovalbumin were obtained from Burkhard Becher (University Hospital Zürich, Switzerland). 2D2xCCL2 and OTIIxCCL2 mice were offspring from MBP/CCL2 mice crossed with 2D2 or OTII mice. Mice were maintained under pathogen-free conditions in our animal facility (Biomedical Laboratory, Institute of Molecular Medicine, University of Southern Denmark). Animal experiments, breeding and maintenance were approved by The Animal Experiments Inspectorate (Dyreforsøgstilsynet; number 2012-15-2934-00110) and performed according to Danish government guidelines on ethical use of animals.

2.2. Experimental autoimmune encephalomyelitis

Eight-week-old female mice were immunized with 100 μg of myelin oligodendrocyte glycoprotein (MOG)p35–55 (Department of Biochemistry and Molecular Biology, University of Southern Denmark) in complete Freund's adjuvant (CFA) (DIFCO) that included 400 μg of H37Ra Mycobacterium tuberculosis (DIFCO). At day 0 and day 2, 300 ng of Bordetella pertussis toxin (Sigma) was injected intra-peritoneally. The animals were monitored daily by weighing and clinical symptoms were scored using a scale of 0–6 with the following criteria: 0, no symptoms; 1, complete loss of tail tonus; 2, difficulty to right; 3, paresis in one or both hind legs; 4, paralysis in one or both hind legs; 5, front limb paresis; 6, moribund. Animals were sacrificed at the peak of the disease defined as 3 consecutive days at the same grade, or grade 5.

2.3. Proliferation assay

Lymph nodes were homogenized by forcing through a 70 μm nylon mesh (BD Falcon™ cell strainer). Cells were stained with 2 μM carboxyfluorescein succinimidylester (CFSE) (Sigma Aldrich) and then cultured in RPMI Glutamax supplemented with 10% fetal calf serum (FCS, Sigma–Aldrich), 100 U/ml penicillin-0.1 mg/ml streptomycin (Sigma–Aldrich) and maintained at 37 °C, 5% CO_2 for 4 days in presence of MOGp35-55 (10 μg/ml) (Department of Biochemistry and Molecular Biology, University of Southern Denmark). At day 4, cells were stained with anti-CD4 and anti-CD8 antibodies and analyzed on a LSRII or FACSCalibur cytometer (Becton Dickinson). Data analysis was done using FACSDiva™ version 6.1.2 software (BD Biosciences). Proliferation index was calculated on $CD4^+$ T cells.

2.4. Intrathecal injection

The injection was performed as described in Ref. [38]. Briefly CCL2-deficient or B6 mice were anaesthetized with an intraperitoneal injection of ketamine (ketaminol, 100 mg/kg of weight body; OUH Sygehusapotek) and xylazine (Rompum®, 10 mg/kg of weight body; OUH Sygehusapotek). Replication-defective adenovirus (10^8 PFU) in sterile PBS (8 μl in total) was injected into the cisterna magna using a 30G stainless-steel needle curved (40°) at 2.5 mm from the tip [39,40]. After the injection, mice received a subcutaneous injection of temgesic (Temgesic, 10 mg/kg of weight body; OUH Sygehusapotek).

Adenovirus encoding CCL2 was type 5 E1-E3-deleted encoding murine CCL2 gene (AdCCL2), driven by the CMV immediate-early promoter [41], kindly provided by Dr J. Gauldie (Pathology & Molecular Medicine, McMaster University, Hamilton, Ontario, Canada). Adenovirus encoding the β-*galactosidase* gene (AdLacZ) was provided by Dr J. Nalbantoglu (Montreal Neurological Institute, Montreal, Quebec, Canada).

2.5. Stromal cell isolation and $CD45^+$ and $CD45^-$ stromal cell sorting

Thymic stromal cells and thymocytes were isolated with a modified protocol as described in Ref. [42]. Thymuses of 4-5-week-old mice were finely minced and digested in a mixture of 0.5 mg/ml of Collagenase D (Roche) and 2 mg/ml of DNase I (Roche) in HBSS (Invitrogen) for 4 incubations of 20 min at 37 °C under agitation. Supernatant from each digestion step were pooled and supplemented with 10 mM EDTA (Invitrogen) then incubated for 5 min at 37 °C. Thymic stromal cells and thymocytes were then separated using Percoll (GE Healthcare; 1.130 g/ml) gradient (densities 1.065 and 1.115) centrifugation. Stromal cells were first incubated with anti-Fc receptor (Clone 24G2; 1 μg/ml; BD Pharmingen) and Syrian hamster IgG (50 μg/ml; Jackson Immuno Reasearch Laboratories Inc.) in PBS, 1% FCS and then stained with a biotin-anti-CD45 antibody (Biolegend; clone 30F-11) and streptavidin-microbeads (Miltenyi Biotec). Fractions were separated using MS columns (Miltenyi Biotec) on a VarioMACS separator (Miltenyi Biotec).

2.6. Cell sorting

To isolate mTEC, cTEC, fibroblasts and endothelial cells, stromal cells were isolated from thymus of 4-5-week-old mice as described