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Symbiotic gut commensal bacteria act as host cathepsin S activity regulators

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ABSTRACT

Cathepsin S (CTSS) is a lysosomal protease whose activity regulation is important for MHC-II signaling and subsequent activation of $CD4^+$ T cell mediated immune responses. Dysregulation of its enzymatic activity or enhanced secretion into extracellular environments is associated with the induction or progression of several autoimmune diseases. Here we demonstrate that commensal intestinal bacteria influence secretion rates and intracellular activity of host CTSS and that symbiotic bacteria, i.e. *Bacteroides vulgatus* mpk, may actively regulate this process and help to maintain physiological levels of CTSS activities in order to prevent from induction of pathological inflammation. The symbiont-controlled regulation of CTSS activity is mediated by anticipating reactive oxygen species induction in dendritic cells which, in turn, maintains cystatin C (CysC) monomer binding to CTSS. CysC monomers are potent endogenous CTSS inhibitors. This *Bacteroides vulgatus* caused and CysC dependent CTSS activity regulation is involved in the generation of tolerant intestinal dendritic cells contributing to prevention of T-cell mediated induction of colonic inflammation. Taken together, we demonstrate that symbionts of the intestinal microbiota regulate host CTSS activity and secretion and might therefore be an attractive approach to deal with CTSS associated autoimmune diseases.

1. Introduction

Cathepsin S (CTSS) is a lysosomal single-chain protease that is expressed in professional antigen-presenting cells (APC) like macrophages, dendritic cells (DC) [1] and B cells as well as in non-professional APC like intestinal epithelial cells [2]. It belongs to the papain-like cysteine protease family that comprises eleven members in humans [3]. It is mainly involved in the stepwise degradation of the MHC-II associated chaperone invariant chain (Ii) [4]. These proteolytic events are crucial for the loading of antigen-derived peptides on MHC-II and the subsequent transport of this MHC-II-peptide complex to the APC surface where it promotes $CD4^+$ T cell activation [5]. CTSS activity can be influenced by transcriptional and post-translational regulation or by protein-protein-interaction with endogenous inhibitors, mainly cystatins [6]. However, there are contradictory reports on cystatin-mediated CTSS activity regulation [7–10]. Though, adequate regulation of CTSS is necessary to retain the steady state of $CD4^+$ T cell dependent immune responses. In fact, dysregulation of CTSS activity, expression or secretion into the extracellular space is associated with the pathogenesis of various autoimmune diseases (AID), i.e. arthritis [11,12], type-I-diabetes [13], Sjögren's syndrome [14,15], multiple sclerosis [8,16] and atherosclerosis [17,18]. Furthermore, CTSS secretion contributes to pain induction during inflammatory bowel disease (IBD) [19], another example of AID.

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Due to the implications of CTSS dysregulation for the pathogenesis of AID, there is an intense search ongoing for the development of drugs that are able to specifically inhibit CTSS proteolytic activity [20]. Eventually, we demonstrate that an alteration of the gut microbiota composition can contribute to host CTSS regulation and that the microbiota composition itself could be decisive to control host CTSS secretion and activity, hence chemical treatment might not be necessary. As most autoimmune diseases are associated with dysbiosis of the intestinal microbiota [21], we compared effects of symbiotic or pathobiotic commensals on host CTSS activities and protein levels *in vivo* and *in vitro*. *Bacteroides vulgatus* mpk could already be demonstrated to be a symbiotic bacteria, mediating anti-inflammatory effects [22–24] which are directly associated with the induction of a tolerant DC phenotype termed semi-mature [23–25].

Here we demonstrate that in an experimental mouse model for $CD4^+$ T cell mediated colitis, *B. vulgatus* mpk promotes maintenance of the immune equilibrium via regulation of CTSS activity and secretion. We further provide evidence, that this regulation is mediated by altering cystatin C binding behaviour to CTSS through controlling intracellular levels of reactive oxygen species. This intestinal microbiota triggered CTSS regulation might deliver new possibilities for the treatment of other CTSS dysregulation-associated AID in general.

2. Materials and methods

2.1. T cell mediated induction of chronic colitis in $Rag1^{-/-}$ mice

Germfree (GF) or conventionally colonized (CV) C57BL/6J-$Rag1^{tm1Mom}$ ($Rag1^{-/-}$) mice were transplanted with 5×10^5 splenic $CD4^+CD62L^+CD45RB^{hi}$ *wt* T cells at 8–10 weeks of age. The conventional microbiota (CV) was free of Norovirus, Rotavirus and *Helicobacter hepaticus*. $Rag1^{-/-}$ mice were kept under SPF conditions and analyzed after 4–8 weeks after T cell transplantation as indicated in the results section.

2.2. Administration of cathepsin S inhibitor into $Rag1^{-/-}$ mice

Cathepsin S Inhibitor LY3000328 was purchased from ApexBio. The lyophilized powder was reconstituted in 83% (v/v) NaCl (0.9%), 12% (v/v) DMSO and 5% (v/v) Tween-20 to obtain a concentration of 0.5 mg mL^{-1}200 μL suspension was administered daily by intraperitoneal injection in order to obtain an amount of 100 μg Cathepsin S inhibitor per dose. The inhibitor was started to be administered three days before T cell transplantation and was continued to be administered for 21 days after T cell transplantation.

2.3. Determination of cathepsin S activities in BMDC lysates

Cathepsin S activity assays of differentially stimulated bone marrow derived dendritic cells were performed as described previously [26,27]. using the fluorogenic substrate Mca-GRWPPMGLPWEK (Dnp)-D-Arg-NH_2 (abbreviated as PMGLP). Determined activities in stimulated samples were normalized to the activities detected in unstimulated control samples generated from the same individual.

2.4. Cultivation of bone marrow derived dendritic cells (BMDCs)

Bone marrow cells were isolated and cultivated as described previously [28]. At day 7 after isolation, resulting CD11c positive bone marrow derived dendritic cells (BMDCs) were used for *in vitro* experiments.

2.5. Stimulation of bone marrow derived dendritic cells

2×10^6 BMDCs were stimulated with either PBS or *B. vulgatus* mpk or *E. coli* mpk at a MOI of 1. Cells were stimulated for a maximum of 24 h. If a second challenge was used, cells were stimulated with bacteria followed by exchanging used for fresh medium and by challenging the cells with a second stimulus for a maximum of 16 h. PBS was used as mock stimulation control.

2.6. Cytokine analysis by ELISA

For analysis of cathepsin S concentrations in cell culture supernatants or blood serum of mice, the CTSS ELISA Kit purchased from Cusabio Biotech was used according to the manufacturer's instructions.

2.7. Flow cytometrical analysis

Multi-color FCM analyses were performed on a FACS Calibur or FACS LSRII (BD Biosciences). All fluorochrome-coupled antibodies were purchased from BD Biosciences. Data were analyzed using the FlowJo software (Tree Star Inc., USA).

2.8. Western blotting

For western blot analysis of MHC class II bound invariant chain and, cystatin C, cathepsin S and the MHC-II bound CLIP fragment, cells were lysed using 10 μL lysis buffer (50 mM Tris/HCl pH 7.6, 150 mM NaCl, 0.5% (v/v) NP40, 1× Roche Complete Protease Inhibitor, 0.4 mM DTT) per 10^6 cells. Non lysed cell components were removed by centrifugation at 12'000*g*. Concentration of solubilized proteins in the resulting supernatant was measured using a commercial BCA Kit (ThermoScientific). A total of 50 μg was transferred on polyacrylamide gels containing 8%–12% polyacrylamide depending on the protein to be detected. Since MHC-II-invariant chain heterotrimers are stable in SDS solutions but sensitive towards heating, samples were usually not heated before electrophoretic separation. Proteins were separated via SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked subsequently for 1 h at room temperature in a BSA containing blocking buffer (LiCor) followed by incubation with primary Abs diluted 1:1000 in blocking buffer over night at 4 °C. After incubation the membranes were washed three times in PBS/T (PBS pH 7.4 + 0.1% Tween-20) and were subsequently incubated with fluorochrome coupled secondary antibodies (LiCor) according to the manufacturer's instruction for 2 h at room temperature. After repeating the washing step with PBS/T the membranes were washed twice with PBS to remove detergent from the membranes. Proteins were detected using the LiCor visualization system. Before using β-actin (mouse anti-mouse β-actin; Sigma) as a control for protein loading, the blots were stripped for 20 min with 10 mL stripping solution (10 mM NaOH, 250 mM guanidinium chloride).

2.9. Purification of RNA and quantitative real-time PCR

Purification of RNA from BMDCs lysates was performed using Qiagen's RNeasy Mini Kit according to the manufacturer's instructions. Additional DNA digestion was conducted by using 4 U rDNase I and 40 U rRNasin for a RNA solution of 0.1 μg μL^{-1}. After 30 min of incubation at RT, DNase was inactivated using Ambion DNase inactivation reagent which was later removed by centrifugation for 1 min at 10'000*g*. SybrGreen based quantitative RT-PCR was performed on a Roche LightCycler480 using QiagenSybrGreen RT-PCR Kit. Primer annealing occured at 60 °C. 10–100 ng