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Short communication

The protease cathepsin L regulates Th17 cell differentiation

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ABSTRACT

Previously we reported that IL-17$^+$ T cells, primarily IL-17$^+$ γδ cells, are increased in mice lacking the protease inhibitor serpinB1 (*serpinb1*$^{-/-}$ mice). Here we show that serpinB1-deficient CD4 cells exhibit a cell-autonomous and selective deficiency in suppressing T helper 17 (Th17) cell differentiation. This suggested an opposing role for one or more protease in promoting Th17 differentiation. We found that several SerpinB1-inhibitable cysteine cathepsins are induced in Th17 cells, most prominently cathepsin L (catL); this was verified by peptidase assays, active site labeling and Western blots. Moreover, Th17 differentiation was suppressed by both broad cathepsin inhibitors and catL selective inhibitors. CatL is present in Th17 cells as single chain (SC)- and two-chain (TC)-forms. Inhibiting asparagine endopeptidase (AEP) blocked conversion of SC-catL to TC-catL and increased generation of *serpinb1*$^{-/-}$ Th17 cells, but not wild-type Th17 cells. These findings suggest that SC-catL is biologically active in promoting Th17 generation and is counter-regulated by serpinB1 and secondarily by AEP. Thus, in addition to regulation by cytokines and transcription factors, differentiation of CD4 cells to Th17 cells is actively regulated by a catL-serpinB1-AEP module. Targeting this protease regulatory module could be an approach to treating Th17 cell-driven autoimmune disorders.

1. Introduction

IL-17 producing T helper cells (Th17 cells) are critically important for protective immunity of mucosal surfaces against fungal infections and extracellular bacteria, but are also implicated in the pathology of severe inflammatory and autoimmune diseases including multiple sclerosis, psoriasis, rheumatoid arthritis, and inflammatory bowel disease. Since the discovery of Th17 cells as the third major T helper lineage distinct from Th1 and Th2 cells [1,2], much has been learned about the mechanisms that control Th17 cell generation, survival and pathogenesis, in particular about the influence of cytokines and transcription factors. Naive CD4 cells differentiate to Th17 cells in the presence of limiting TGF-β and the proinflammatory cytokine IL-6 [3–5]. During differentiation, the transcription factors BATF and IRF4 establish initial chromatin accessibility and in combination with STAT3 [6] and orphan nuclear receptor RORγt, the master Th17 regulator [7], and the negative regulator cMAF, function to establish a coherent transcriptional program to produce Th17 cells expressing a set of signature cytokines and cytokine receptors, including IL-23R [8] and IL-1R [9]. Overall, the dynamic regulatory program that coordinates Th17 differentiation involves sequential developmental stages coordinated by a network of highly interactive antagonistic interactions of Th17 promoting- and a smaller number of Th17 suppressing-modules [10].

Here we describe an additional layer of regulation whereby the differentiation of Th17 cells is controlled by a protease module that includes cathepsin L (catL), an endosomal/lysosomal cysteine protease, together with asparagine endopeptidase/legumain (AEP), which converts single chain catL (SC-catL) to the two-chain form, and serpinB1, a protease inhibitor previously characterized as a

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protective anti-inflammatory immune modulator. In prior studies, we correlated the increased inflammation and injury of influenza virus infected *serpinb1*$^{-/-}$ mice with increased numbers of IL-17-producing T cells, primarily γδ^{+} T cells [11]. Subsequently, we found that IL17^{+} γδ T cells, but not IFNγ^{+} γδ cells, are selectively expanded in naive *serpinb1*$^{-/-}$ mice [12], indicating a suppressive role for SerpinB1. Here we show, through the use of *in vivo* immunization and *in vitro* differentiation, that SerpinB1 deficient CD4 cells exhibit a cell-autonomous and selective deficit of suppression of Th17 cell differentiation. Further experimentation identified the SerpinB1-inhibitable protease that promotes Th17 differentiation as catL and identified AEP as an additional suppressive regulator.

2. Materials and methods

2.1. Animals

SerpinB1 deficient mice (*serpinb1a*$^{-/-}$, hereafter *serpinb1*$^{-/-}$) were generated in 129S6/SvEv/Tac (129S6) background [13]. CatH deficient mice (*ctsh*$^{-/-}$) in C57BL/6N background [14] were provided by Drs. Thomas Reinheckel (Albert-Ludwig University, Freiburg, Germany) and Johanna Joyce (Memorial Sloan-Kettering Cancer Center, New York). The mice were rederived at Boston Childrens Hospital by mating *ctsh*$^{-/-}$ males with WT C57BL/6J females and intercrossing the resulting heterozygotes. *Ctsh*$^{-/-}$ pups from the intercross were selected that carried the C57BL/6J-specific allele at the nicotinamide nucleotide transhydrogenase (*Nnt*) locus and were backcrossed to C57BL/6J for two additional generations. *Ctsh*$^{-/-}$ and *serpinb1*$^{-/-}$ mice were viable and fertile with no gross phenotypes. WT 129S6 mice (Taconic Labs) and WT C57BL/6J (Jackson Labs) were maintained together with *serpinb1*$^{-/-}$ and *ctsh*$^{-/-}$ mice in the animal facility of Boston Children's Hospital. Animal studies were approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital.

2.2. KLH immunization

WT and *serpinb1*$^{-/-}$ mice were immunized by injection *i.p.* of keyhole limpet hemocyanin (KLH) (200 μg, Sigma–Aldrich) in 200 μl of a 1:1 emulsion in Freund's adjuvant (Sigma–Aldrich). Seven days later, mice were sacrificed and splenocytes were cultured with or without KLH for 2 days with Brefeldin A present during the final 6 h. The cells were collected for flow cytometry and the supernatants for ELISA assay.

2.3. Isolation of naive CD4 cells

Single cell suspensions were prepared from spleens of 4–6wk old mice. After erythrocyte lysis, pooled splenocytes were depleted of CD11b^{+}, CD8α^{+} and CD19^{+} cells using biotinylated primary antibodies (BioLegend) and streptavidin-coated secondary magnetic particles (Stem Cell Technologies). The enriched cells were sorted on the FACS Aria for CD4^{+}CD25^{neg}CD44^{neg}CD62L^{+}. Purity was >98%.

2.4. T-helper cell differentiation

Naive CD4 T cells (0.4×10^6) in 24 well plates (Costar) precoated with anti-CD3 (154-2C11, 5 μg/ml, BioXcell) and anti-CD28 (37.51, 2 μg/ml, BioXcell) were cultured in RPMI containing 10% FCS and polarizing cytokines. The cytokines were: Th1, mIL-12 (10 ng/ml, Biolegend) and anti-mIL-4 (11B11, 2 μg/ml, BioXcell); Treg, hTGF-β1 (3 ng/ml, Biolegend), mIL-2 (20 ng/ml, Biolegend), anti-mIFN-γ (XMG1.2, 2 μg/ml, BioXcell) and anti-mIL-4; Th17, mIL-6 (10 ng/ml, Biolegend), hTGF-β1 (2 ng/ml), anti-mIFN-γ, and anti-mIL-4. Cells stimulated in 'neutral' conditions (anti-mIL-4 plus anti-mIFN-γ without added cytokines) were considered Th0 cells.

Where studied, protease inhibitors, AEBSF (Pefabloc) and E64 (Sigma–Aldrich), E64D (Santa Cruz), z-Phe-Ala-fmk (Enzyme Systems), CA074-OMe (EMD Millipore), 1-naphthalenesulfonyl-IIe-Trp-aldehyde (IW–CHO, Enzo Life Sciences), CLIK195 (provided by Guo-Ping Shi), and the AEP inhibitor LI-1 [15], were added at the start of culture. Unless otherwise indicated, differentiated cells were harvested after 3 days for Western blot, peptidase assay or active site labeling or were restimulated for 4 h with PMA (50 ng/ml) and ionomycin (750 ng/ml) (Sigma–Aldrich) in the presence or absence of Brefeldin A for flow cytometry or ELISA, respectively.

2.5. Intracellular staining and flow cytometry

Harvested cells were stained with fluorochrome-conjugated antibodies to surface markers (Biolegend). The cells were fixed, permeabilized and stained intracellularly with fluorochrome-conjugated anti-mIL-17A (TC11-18H10) (hereafter IL-17), anti-mIFN-γ (XMG1.2) and anti-FoxP3 (FJK-16s) (all from Biolegend) using FoxP3 fixation/permeabilization reagents and protocols from eBiosciences. Data were acquired on a Canto II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

2.6. ELISA

IL-17A (hereafter IL-17) and IFN-γ were assayed using ELISA kits (eBioscience) according to the manufacturer's instructions.

2.7. Reverse transcription and qPCR analysis

RNA was isolated using RNeasy kits (Qiagen) and was digested with DNase I (Ambion) and reverse-transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad). The qPCR assays are detailed in Supplemental Materials and Methods.

2.8. Western blot

Differentiated cells were suspended at 12.5×10^6 per ml in PBS with 2 mM AEBSF and lysed with 5X SDS lysis buffer with mercaptoethanol and boiling for 10 min. Alternatively, cell homogenates prepared in NP40-containing buffer (described below) were similarly SDS-solubilized. Samples were resolved on 12% Tris-glycine gels and transferred onto PVDF. Membranes were blocked with 5% milk solids and stained with rabbit antiserum to human SerpinB1 [13], goat antiserum to mouse catL (AF1515, R&D Systems) or sheep antiserum to mouse AEP (AF2058, R&D Systems) followed by HRP-conjugated secondary antibodies (Cell Signaling). Bands were visualized by enhanced chemiluminescence (ECL-Plus, Amersham). Blots were stripped and restained with mouse anti-mouse β-actin antibody (Cell Signaling).

2.9. Enzymes, inhibitor, substrates and peptidase assays

Reagents and peptidase assays (Figs. 4 and 5C) are detailed in Supplemental Materials and Methods.

2.10. Active site labeling

Differentiated cells were washed and resuspended at 10×10^6/ml in RPMI without serum and incubated for 30 min with 10 μM DCG-04, a biotin moiety-containing activity-based probe for cysteine cathepsins [16]. The labeled cells were lysed, fractionated by SDS electrophoresis, transferred to PVDF, blocked with 1% BSA and stained with HRP-conjugated avidin. To evaluate the blocking ability of select cathepsin inhibitors, Th17 cells were preincubated