Cytokine 111 (2018) 146–153

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Interleukin 33 regulates gene expression in intestinal epithelial cells independently of its nuclear localization

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ARTICLE INFO

Keywords:
IL33
Intestinal epithelial cells
Inflammation
Inflammatory bowel disease
RNA-Seq

ABSTRACT

Interleukin 33 (IL33) is a cytokine found in the extracellular space (mature IL33) or in the cell nucleus (full-length IL33). Nuclear accumulation of IL33 has been reported in intestinal epithelial cells (IEC) during intestinal inflammation and cancer, but a functional role for this nuclear form remains unclear. To study the role of nuclear IL33 in IEC, we generated transgenic mice expressing full-length IL33 in the intestinal epithelium (*Vfl33* mice). Expression of full-length IL33 in the epithelium resulted in accumulation of IL33 protein in the nucleus and secretion of IL33. Over-expression of full-length IL33 by IEC did not promote gut inflammation, but induced expression of genes in the IEC and lamina propria lymphocytes (LPL) that correlated negatively with genes expressed in inflammatory bowel diseases (IBD). Because the IL33 receptor ST2 is expressed by IEC, there was the potential that both the mature and full-length forms could mediate this effect. To specifically interrogate the transcriptional role of nuclear IL33, we intercrossed the *Vfl33* mice with ST2- deficient mice. ST2 deficiency completely abrogated the transcriptional effects elicited by IL33 expression, suggesting that the transcriptional effects of IL33 on IEC are mediated by its mature, not its nuclear form.

1. Introduction

Interleukin 33 (IL33), a member of the IL-1 family of cytokines [1], was originally described as a nuclear protein from human high endothelial venules [2]. Subsequent studies showed that IL33 acts as a cytokine, binding a heterodimeric receptor complex consisting of the ST2 receptor (ST2L) and the IL-1R accessory protein. The expression of this heterodimeric receptor has been detected on a variety of inflammatory cells [3,4], including eosinophils, basophils, macrophages, T helper 2 cells (Th2 cells), regulatory T cells, NK cells, B cells and group 2 innate lymphoid cells (ILC2) [5–7]. IL33 plays a role in the host defense against infection and has been reported to be involved in the pathogenesis of a wide range of diseases [8].

In the gastrointestinal tract, IL33 is normally expressed by stromal and immune cells, and IL33 protein has been detected in the nuclei of such cells [9,10]. IL33 is not normally expressed by epithelial cells, but recent evidence suggests that it can function as a novel epithelial "alarmin"[11], because it can be released as a danger signal by damaged, stressed, or necrotic cells to alert the immune system of a local threat. Epithelial expression of IL33 has been reported in samples from patients with ulcerative colitis [9,10,12–15] and cancer [16].

IL33 is believed to be a dual-function protein, functioning as conventional cytokine via its extracellular form (mature IL33) or as a transcriptional regulator via its nuclear form (full-length IL33). Although the molecular mechanism of release and processing of IL33 are not yet clear [17], it appears that the full-length IL33 released from injured or necrotic cells is biologically active [11,18–20], and this bioactivity can be transiently increased several-fold by limited proteolysis of the N-terminal domain (mature IL33) in inflamed tissue [21,22] before bioactivity is lost by destruction or oxidization of the C-terminal core tetrahedron structure [23]. The N-terminal domain of full-length IL33 is necessary for nuclear translocation, but it is unclear where it binds to the chromatin and whether it directly regulates gene expression in the intestinal epithelial cells (IEC). In this study, we investigate the biological properties of the full-length IL33, focusing on its transcriptional properties.

2. Materials and methods

2.1. Mouse strains

C57BL/6 mice were purchased from The Jackson laboratory (Bar Harbor, ME). ST2$^{-/-}$ mice were generated in our laboratory as described by He et al [16]. Mice were maintained under specific

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https://doi.org/10.1016/j.cyto.2018.08.009
Received 25 May 2018; Received in revised form 16 July 2018; Accepted 10 August 2018

pathogen-free conditions. All experiments involving animals were performed following guidelines of the Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai.

2.2. Ethics approval

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai, and were performed in accordance with the approved guidelines for animal experimentation at the Icahn School of Medicine at Mount Sinai (IACUC-2015-0004).

2.3. Generation of transgenic mice expressing IL33 in the intestinal epithelium

The cDNA of IL33 full-length form was cloned into a pBS-Villin vector that contained a 9 kb segment of the mouse villin promoter [24]. The pBS-Villin/IL33 plasmid was verified by sequencing, and the transgene was isolated from the plasmid by restriction enzyme digestion and gel purification. To generate transgenic mice, the transgene was microinjected into C57BL/6 mouse eggs. Identification of the transgenic *Vfl33* mice was done by PCR amplification using the following primers: 5′- ggctgtgatagcacacagga-3′ and 5′- ttcgcctgcggtgctgctgaac -3′.

2.4. Enzyme-linked immunosorbent assay

Small pieces of small intestine or colon (5 mm of mid-part) were isolated, rinsed in PBS, weighed, and cultured overnight in 12-well tissue culture plates (Costar) in 1000 µl complete DMEM at 37 °C in an atmosphere containing 5% CO_2. After centrifugation to pellet debris, culture supernatants were transferred to fresh tubes and stored at −80 °C. IL33 was quantified in the supernatant of intestinal explant cultures from *Vfl33* and WT mice by enzyme-linked immunosorbent assay (ELISA) according to standard manufacturer's recommendations (eBioscience) and the results were normalized to the weight of the intestinal explant.

2.5. Reverse-transcription polymerase chain reaction

Total RNA from tissues cells was extracted using the RNeasy mini Kit (Qiagen) according to the manufacturer's instructions. Complementary DNA (cDNA) was generated with Superscript III (Invitrogen). Quantitative PCR was performed using SYBR Green Dye (Roche) on the 7500 Real Time System (Applied Biosystems) machine. Thermal cycling conditions used were as follows: 50 °C for 2 min and 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, followed by dissociation stage. Results were normalized to the housekeeping gene Ubiquitin. Relative expression levels were calculated as $2^{(Ct(Ubiquitin)-Ct(gene))}$. Primers were designed using Primer3Plus software [26].

2.6. Histology and immunofluorescence staining

Tissues were dissected, fixed in 10% phosphate-buffered formalin, and then processed for paraffin sections. Five-micrometer sections were stained with hematoxylin and eosin (H&E) for histological analyses. For immunofluorescence staining, five-micrometer sections were dewaxed by immersion in xylene (twice for 5 min each time) and hydrated by serial immersion in 100%, 90%, 80%, and 70% ethanol and PBS. Antigen retrieval was performed by microwaving sections for 20 min in Target Retrieval Solution (DAKO). Sections were washed with PBS (twice for 10 min each time), and blocking buffer (10% BSA in TBS) was added for 1 h. Sections were incubated with primary antibody in blocking buffer overnight at 4 °C. After washing, conjugated secondary Abs were added and then incubated for 35 min. Cell nuclei were stained using 4′,6-Diamidino-2-Phenylindole (DAPI). The slides were next washed and mounted with Fluoromount-G (Southern Biotech). Images were captured using a Nikon fluorescence microscope. Colocalization was performed with ImageJ and the colocalization finder plug-in.

2.7. Western blot analysis

Intestine were opened longitudinally and thoroughly washed in PBS and then homogenized in ice-cold lysis buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1 µg/mL aprotinin). Lysates were then centrifuged at 12 000*g* for 15 min to remove insoluble cell debris. Protein content was quantified using the Bio-Rad protein assay (Bio-Rad) and 15 µg of protein was separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membrane was blocked for 1 h in buffer (TBS, 5% milk, 0.1% Tween 20) and then incubated with the primary antibody (Rat Anti-Mouse IL-33 Monoclonal Antibody) (Catalog # MAB3626, R&D Systems) in dilution buffer (TBS, 5% bovine serum albumin, 0.1% Tween 20) overnight at 4 °C. The membrane was then washed three times with wash buffer (TBS, 0.1% Tween 20), incubated with Rat IgG HRP-conjugated Antibody (Catalog # HAF005, R&D Systems) and visualized with the enhanced chemiluminescent detection system (Amersham Biosciences).

2.8. Isolation of IEC and LPL

Intestines were opened longitudinally and thoroughly washed in PBS. The intestine was then incubated in 30 ml PBS containing 1 mM dithiothreitol (DTT) on room temperature for 15 min. The intestine was then removed and briefly washed in PBS and incubated in 25 ml PBS containing 5.2 mM ethylenediaminetetraacetic acid (EDTA) at 4 °C at 200 RPM for 30 min. The cells were then subjected to 30 s vigorous shaking and the tissue removed. The cells were then centrifuged at 1000*g* for 5 min at 4 °C, washed in PBS containing 10% FBS and spun for a further 5 min at 4 °C at 1000*g*. These cells constituted the IEC population. To isolate lamina propria lymphocytes (LPL), the remaining tissues were performed as described before [16].

2.9. Cell sorting

Cell pellets were first pre-incubated with anti-mouse CD16/CD32 for blockade of Fc γ receptors, then were washed and incubated for 30 min with fluorescent conjugated antibodies against CD45 and EpCAM in a total volume of 500 µl PBS containing 2 mM EDTA and 2% (vol/vol) fetal bovine serum. DAPI (Invitrogen) was used to distinguish live cells from dead cells during cell sorting. Stained IECs ($DAPI^-CD45^-Epcam^+$) and LPL ($DAPI^-CD45^+$) were purified with a MoFlo Astrios cell sorter (DakoCytomation). Cells were > 95% pure after sorting.

2.10. Microarray analysis

Total RNA from the sorted intestinal $CD45^+$ cells from WT and *Vfl33* mice was extract using RNeasy Micro Kit (Qiagen). Microarrays were done and analyzed as described before [27,28]. In order to analyses the pathways that the differentially expressed genes are involved in, KEGG pathway enrichment analyses were performed using ClueGo [29,30]. A cut-off of 0.4 was set for kappa score and terms including at least 3 genes were retrieved.

2.11. RNA-seq

Following cell sorting into Trizol LS reagent, samples were shipped on dry ice to the Center for Functional Genomics and the Microarray & HT Sequencing Core Facility at the University at Albany (Rensselaer). Total RNA from sorted cells ($3-9 \times 10^5$ cells) was extracted using the RNeasy micro Kit (Qiagen) with an on-column DNAse digestion step