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Cysteine protease antigens cleave CD123, the α subunit of murine IL-3 receptor, on basophils and suppress IL-3-mediated basophil expansion

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ABSTRACT

Th2 type immune responses are essential for protective immunity against parasites and play crucial roles in allergic disorders. Helminth parasites secrete a variety of proteases for their infectious cycles including for host entry, tissue migration, and suppression of host immune effector cell function. Furthermore, a number of pathogen-derived antigens, as well as allergens such as papain, belong to the family of cysteine proteases. Although the link between protease activity and Th2 type immunity is well documented, the mechanisms by which proteases regulate host immune responses are largely unknown. Here, we demonstrate that the cysteine proteases papain and bromelain selectively cleave the α subunit of the IL-3 receptor (IL-3Rα/CD123) on the surface of murine basophils. The decrease in CD123 expression on the cell surface, and the degradation of the extracellular domain of recombinant CD123 were dependent on the protease activity of papain and bromelain. Pre-treatment of murine basophils with papain resulted in inhibition of IL-3-IL-3R signaling and suppressed IL-3- but not thymic stromal lymphopoietin-induced expansion of basophils in vitro. Our unexpected findings illuminate a novel mechanism for the regulation of basophil functions by protease antigens. Because IL-3 plays pivotal roles in the activation and proliferation of basophils and in protective immunity against helminth parasites, pathogen-derived proteases might contribute to the pathogenesis of infections by regulating IL-3-mediated functions in basophils.

1. Introduction

Th2 type immune responses play crucial roles in allergic disorders and in protective immunity against parasites. Helminth invasion induces Th2-related cytokines including IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), thereby mediating expanded populations of eosinophils, basophils, mast cells and other innate immune cell populations [1–3]. Basophils are basophilic granulocytes and represent <1% of peripheral blood leukocytes [4]. Basophils in humans and mice express FcεRI and readily generate large quantities of IL-4, in both an IgE-dependent and -independent manner, in response to various stimuli including parasite-derived antigens [5]. Recent studies have demonstrated the contribution of basophils to protection against both ecto- and endoparasites and to induction of Th2 type immune responses that are triggered after parasite infections [5]. Basophil numbers are increased in several animal models of helminth infections in an IL-3-dependent manner, and mice lacking IL-3 show delayed expulsion of certain helminths [6,7].

Helminth parasites secrete a variety of proteases, which play crucial roles in their virulence including in host entry, tissue migration and suppression of host immune effector cell functions [8]. Many allergens and allergen sources also have intrinsic protease activity [9]. A number of pathogen-derived antigens belong to the clan CA family (papain family) of cysteine proteases, as do allergens such as Der p 1 and Der f 1 from house dust mites and the papain from papaya (see MEROPS – [10]). Papain is a potent allergen, is associated with occupational allergy in humans [11],

Abbreviations: Ab, antibody; BMB, bone marrow-derived basophil; IL-3R, IL-3 receptor; LC-MS, liquid chromatography-mass spectrometry; MFI, mean fluorescence intensity; PAR, protease-activated receptor; rIL-3, recombinant IL-3; TSLP, thymic stromal lymphopoietin.

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and can cause strong Th2 type immune responses [12–15] and allergic airway inflammation in mice [13,14,16,17]. However, although the link between protease activity and Th2 type immunity is well documented, the mechanisms through which host immune responses are regulated are not fully understood.

Papain induces cytokine/chemokine production in various types of cells, including in basophils, which is partially mediated by the protease-activated receptor 2 (PAR2) [18,19]. Protease allergens also modulate host immune cell functions indirectly via cleavage of immunoreceptors such as CD25 and CD23 [9,20–22]. We therefore considered that there must be target molecules other than PAR-2 on basophils that can be cleaved by papain. In the present study, we found that the cysteine protease activity of papain and bromelain selectively cleaves the α subunit of the IL-3 receptor (IL-3Rα/CD123) on the surface of murine basophils, resulting in inhibition rather than activation of IL-3-IL-3R signaling. Our unexpected findings illuminate a novel mechanism for the regulation of basophil functions by pathogen-derived protease antigens.

2. Materials and methods

2.1. Antibodies (Abs) and other reagents

Biotinylated anti-mouse CD49b (DX5), and PE-conjugated anti-mouse CD11b (M1/70) and CD131 (JORO50) were purchased from BD Pharmingen (San Diego, CA). FITC-conjugated anti-mouse FcεRIα (MAR-1) was from eBioscience (San Diego, CA). PE-conjugated anti-mouse CD45 (30-F11), CD69 (H1.2F3), CD123 (5B11), CD200R (OX-110), and allophycocyanin-conjugated streptavidin were from Biolegend (San Diego, CA). These Abs were used for flow cytometric analysis. Purified polyclonal anti-STAT5 and phosphorylated-STAT5 were purchased from Cell Signaling Technology (Tokyo, Japan) and were used for immunoblot analysis. Papain and bromelain were purchased from Calbiochem (San Diego, CA) and, when required, were heat-inactivated by 10-min incubation at 100 °C [12]. Protein concentrations were measured using the BCA Protein Assay Kit (Pierce, Rockford, IL). Murine recombinant IL-3 (rIL-3) and rTSLP were purchased from Wako Pure Chemicals (Osaka, Japan) and R&D Systems (Minneapolis, MN), respectively.

2.2. Mice, and bone marrow cultures

C57BL/6 mice (7–10 weeks old) were purchased from CLEA Japan (Tokyo, Japan). All animals were used in accordance with the guidelines of the institutional committee of Juntendo University. To obtain a basophil-enriched population, bone marrow cells prepared from the tibias and femurs of mice were cultured for 8 days in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 2 mM L-glutamine, 0.05 mM 2-ME, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.3 ng/ml rIL-3 [23], yielding a cell population in which $CD49b^{+}Fc\varepsilon RI\alpha^{+}$ bone marrow-derived basophils (BMBs) represented 50–60% of the cells. For flow cytometric analysis, total IL-3-cultured cells or freshly-isolated bone marrow cells were adjusted to a final concentration of 1×10^6 cells/ml and were incubated at 37 °C with papain or bromelain in supplemented RPMI medium without FCS and rIL-3. After incubation, the cells were washed and used for subsequent analysis. For ELISA analyses of CD123, culture supernatants of papain-treated cells were collected and further incubated for 40 min at 37 °C with 10 μM E64 (Peptide Institute, Osaka, Japan) to inhibit the cysteine protease activity of papain. For immunoblot analysis, IL-3-cultured bone marrow cells were incubated for 16 h without rIL-3 prior to stimulation. After cell stimulation, cells were washed twice with PBS and lysed with lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40, protease inhibitor cocktail (Sigma–Aldrich), and phosphatase inhibitor cocktail (Sigma–Aldrich). Cell lysates were further treated with 10 μM E64 for 40 min at 37 °C before addition of sample buffer containing 2-mercaptoethanol for SDS-PAGE and heated for 3 min at 95 °C. For basophil counting, freshly isolated bone marrow cells were incubated for 1 h at 37 °C with 100 μg/ml papain or inactive papain in RPMI medium, washed, and cultured for 72 h in the presence of 0.3 ng/ml rIL-3 or 1 μg/ml rTSLP at a density of 250,000 cells/500 μl/well in 24-well plates. Non-adherent cells were collected and the number of live cells was counted by trypan blue exclusion. Basophils were identified as $CD49b^{+}Fc\varepsilon RI\alpha^{+}$ cells in flow cytometric analysis.

2.3. Flow cytometric analysis

Cells were pre-incubated with normal rat serum on ice for 15 min prior to incubation with the indicated combination of Abs, to prevent the nonspecific binding of irrelevant Abs. Stained cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed with FlowJo software (Tree Star, Ashland, OR). Dead cells stained with propidium iodide were excluded from the analyses.

2.4. Cleavage of rIL-3R

For ELISA analysis, a murine recombinant IL-3Rα-human IgG Fc protein (rIL-3Rα) (R&D Systems) was incubated for 1 h at 37 °C with papain or bromelain in PBS. For liquid chromatography-mass spectrometry (LC-MS), 2 mg/ml rIL-3Rα was incubated for 1 h at 37 °C with 20 μg/ml papain in PBS. All samples were further incubated for 40 min at 37 °C with 10 μM E64 prior to subsequent analyses.

2.5. ELISA

For detection of IL-3Rα, 96-well plates were coated with monoclonal anti-mouse CD123 Ab (5B11, Biolegend) overnight at 4 °C (1/200 dilution, 50 μl/well), and were blocked with 20% (v/v) ImmunoBlock (DS Pharma Biomedical, Osaka, Japan) (60 μl/well). Bound IL-3Rα was detected with polyclonal goat anti-mouse IL-3Rα (AF983, R&D Systems) (0.5 μg/ml, 50 μl/well) followed by horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG Ab (sc-2056, Santa Cruz Biotechnology, Santa Cruz, CA) (1/20000 dilution, 50 μl/well). HRP activity was detected by the color development for 20 min using tetramethyl benzidine (BD-OptEIA kit; BD-biosciences) and the reaction was stopped by adding sulfuric acid. The optical density at 450 nm, from which that at 570 nm was subtracted, was used as the signal.

2.6. Immunoblot analysis

Cell lysates prepared as described above were boiled in sample buffer, separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes, followed by immunoblot analysis with various primary and HRP-labeled secondary Abs. Blots were developed with Luminata Forte Western HRP Substrate (Millipore, Bedford, MA) and were analyzed with the LAS-4000 (FujiFilm, Tokyo, Japan).

2.7. Liquid chromatography/mass spectrometry (LC–MS) analysis and identification of several protease-cleaved fragments of IL-3Rα

Murine rIL-3Rα (2 mg/ml) was incubated for 1 h at 37 °C with 20 μg/ml papain in PBS. After incubation, the samples were