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Regulation of Siglec-8-induced intracellular reactive oxygen species production and eosinophil cell death by Src family kinases

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ABSTRACT

Rationale: Siglec-8 is a surface receptor predominantly expressed on human eosinophils where its ligation induces reactive oxygen species (ROS) formation and cell death. Since Siglec-8 has intracellular tyrosine-based motifs, we hypothesized that Src family kinases (SFKs) are involved in ROS formation and cell death induced by Siglec-8 engagement.
Methods: Human peripheral blood eosinophils were purified and incubated with anti-Siglec-8 monoclonal antibodies (mAb, agonist), IL-5, and SFK pharmacological inhibitors. We focused on Siglec-8-induced cell death in short-term IL-5-activated cells leading to a regulated necrosis-type cell death. ROS production was determined by dihydrorhodamine (DHR) 123 labeling and flow cytometry, or by chemiluminescence using Amplex red. Activation of SFK was determined using phospholuminex and Western blotting.
Results: In order to determine cellular localization of ROS production, we measured intra and extracellular ROS. While an ETosis stimulus (calcium ionophore A23187) led to extracellular ROS (ecROS) production, Siglec-8-engagement in short-term IL-5 activated cells led to intracellular ROS (icROS) accumulation. Consistently, inhibition of extracellular ROS by catalase inhibited ETosis, but not IL-5-primed Siglec-8-induced cell death. In order to determine signaling events for Siglec-8, we performed Western blotting and found SFK phosphorylation in lysates from eosinophils stimulated with anti-Siglec-8 mAb ± IL-5. In order to identify which SFKs were involved, we used the phospholuminex assay and found increased levels of phosphorylated Fgr in the cytoplasmic fraction of cells co-stimulated with anti-Siglec-8 and IL-5 for 3 hours compared with cells stimulated with IL-5 alone. To test the involvement of SFKs in ROS production and cell death, we used SFK inhibitors PP2 and dasatinib, both of which completely inhibited eosinophil ROS production and cell death induced by anti-Siglec-8 and IL-5 co-stimulation.
Conclusion: Siglec-8 engagement in short-term IL-5-activated eosinophils causes icROS production and SKF phosphorylation, and both are essential in mediating Siglec-8-induced cell death.

1. Introduction

Siglecs (sialic acid binding Ig-like lectin) are a family of I-type lectins that recognize sialic acid. Characterized by their preferential expression on hematopoietic and immune systems and their common structure in cytoplasmic domain bearing one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs), siglecs are assumed to have primarily inhibitory function in the immune system (Bochner and Zimmermann, 2015; Macauley et al., 2014). Except for a few instances such as Siglec-2 (CD22) mediated inhibition of BCR signaling in B cells, however, the roles and precise mechanisms of siglec function in immune responses are yet to be understood (Jellusova and Nitschke, 2012).

Siglec-8 is predominantly expressed on human eosinophils, although it is also found on the surface of basophils and mast cells (Floyd et al., 2000; Kikly et al., 2000). Interestingly, *in vitro* ligation of Siglec-8 induces apoptotic eosinophil cell death, and this function is paradoxically enhanced by co-stimulation or prior priming with IL-5, the latter being a prominent activation/survival factor for eosinophils (Nutku et al., 2003; Nutku-Bilir et al., 2008). Clinically, Siglec-8 has been implicated in asthma pathogenesis; a polymorphism of Siglec-8 was associated with asthma susceptibility in humans (Gao et al., 2010). Mice bearing a genetic deficiency for

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Siglec-F (the functional paralog of Siglec-8) (Cho et al., 2010; Zhang et al., 2007) or enzymes (*e.g.*, ST3Gal-III) responsible for generation of its putative ligand (glycans on Muc5b) were shown to have exacerbated eosinophilic inflammation in models of type 2 responses (Kiwamoto et al., 2014, 2015). Collectively, Siglec-8 and Siglec-F are suggested to have a role to prevent chronic lung inflammation of asthma by inducing cell death of activated eosinophils (Kiwamoto et al., 2012).

It has been demonstrated that Siglec-8 ligation induces apoptosis in resting cells, and a form of regulated necrosis, involving reactive oxygen species (ROS) accumulation and prolonged extracellular signal-regulated kinase (ERK) phosphorylation in short-term IL-5 pretreated cells (Nutku et al., 2005; Kano et al., 2013), consistent with the general notion that cells that receive a death signal but have apoptosis inhibited (*e.g.* by IL-5) activate backup cell death pathways such as regulated necrosis (Vanlangenakker et al., 2011). Recently, another form of ROS-dependent regulated necrosis was described in eosinophils, ETosis (Ueki et al., 2013). The authors demonstrated that extracellular ROS (ecROS) is produced in and required for the process of ETosis. On the other hand, previous results suggested intracellular ROS (icROS) is accumulated and promotes phosphorylation of nuclear ERK upon Siglec-8/IL-5 induced cell death. Whether the mechanism of regulated cell death induced by ETosis stimuli and Siglec-8/IL-5 are identical, *i.e.*, whether ecROS and icROS play similar roles in Siglec-8/IL-5 induced cell death as in ETosis, is the first focus of the present study. Furthermore, the mechanism of ROS induction by an ITIM-bearing receptor is not known. In other siglec pathways, such as those involving CD22 on B cells or CD33 on neutrophils, Lyn, a member of the non-receptor tyrosine kinase Src family kinases (SFKs), is implicated in signaling, suggesting an affinity of SFKs for the intracellular membrane-proximal ITIM found in most siglecs (Otipoby et al., 2001; Paul et al., 2000). In turn, SFKs are also implicated in chemoattractant-induced ROS production in neutrophils (Fumagalli et al., 2013). Therefore, to dissect the signaling pathways mediating eosinophil cell death following Siglec-8 engagement in short-term IL-5-primed eosinophils, we tested the hypothesis that SFKs are involved in ROS accumulation and cell death induced by Siglec-8.

2. Materials and methods

2.1. Eosinophil purification

Blood eosinophils were purified (to >95% purity) from healthy control subjects by using Percoll gradient separation and the CD16 magnetic bead negative selection system (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described. Written informed consent was obtained from all blood donors, and the study was approved by the Institutional Review Board of Cincinnati Children's Hospital.

2.2. Eosinophil culture and cell death measurement

Human eosinophils were cultured at 1×10^6/mL in RPMI 1640 containing 10% fetal bovine serum (FBS) (subsequently designated as "normal media"), or for some experiments, in RPMI 1640 containing 0.1% bovine serum albumin (BSA), in order to avoid the known ROS – scavenging effect of FBS [FBS(-) media]. To activate eosinophils, we added recombinant human IL-5 (PeproTech, Rocky Hills, NJ) (30 ng/mL), and anti-Siglec-8 or its isotype-matched control antibody (BioLegend, San Diego, CA) (2.5 μg/mL). Unless noted otherwise, IL-5 and anti-Siglec-8 were added simultaneously. Anti-Siglec-8 mAb 2C4 (mouse IgG1) was produced as previously described (Kikly et al., 2000). In some experiments the clone 7C9 anti-Siglec-8 antibody (BioLegend) was used; results were identical regardless of which anti-Siglec-8 antibody clone used. ETosis was induced by exposure to the calcium ionophore A23187 (Sigma-Aldrich, MO) (2 μM). The selective pharmacological inhibitor for SFKs (PP2) and its inactive control (PP3) were purchased from EMD-Millipore Chemicals (Merck, Darmstadt, Germany), and the pan-tyrosine kinase inhibitor dasatinib (DA) was obtained from Fisher Scientific (Pittsburgh, PA). Inhibitors or controls were added simultaneously with IL-5 and/or anti-Siglec-8, as specified in figure legends. When the ROS inhibitor diphenyleneiodonium (DPI; Sigma-Aldrich, St Louis, MO) was used, cells were preincubated with DPI for 5 min and then washed and resuspended with media prior to adding the indicated stimuli. Catalase (Sigma-Aldrich), which does not penetrate intact plasma membranes and thus works as an extracellular ROS scavenger, was added to the medium just prior to adding the indicated stimuli. Eosinophil apoptosis and death were assessed by means of flow cytometry with Annexin V and 7-aminoactinomycin D (7AAD) staining and a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For detecting ETosis, cells were cultured in an 8 well chamber slide (Thermo Scientific) for 3 h after stimulation, then stained with DAPI before determination of typical morphologic change of the nucleus by fluorescent microscopy.

2.3. Measurement of released eosinophil peroxidase (EPX) activity

EPX release was measured as described previously (Adamko et al., 2004). Briefly, following stimulation of eosinophils with indicated stimuli, the substrate *O*-phenylenediamine (OPD) was added directly to cell suspensions, reaction was stopped by H_2SO_4 and color intensity measured at 492-nm wavelength. Data are expressed as fold change, compared with level in supernatant of unstimulated cells.

2.4. ROS production measurements

To measure icROS, after stimulation for the indicated times, cells were incubated in normal media with 1 mM dihydrorhodamine (DHR) 123 (Invitrogen, Waltham, MA) at 37° C for 30 min, fixed with 1% PFA, washed with PBS, and subsequently analyzed by flow cytometry. In some experiments, cells were incubated with DHR 123 for 15 min before the addition of stimuli; at the end of stimulation period, cells were fixed with 1% PFA. For measuring ecROS, the Amplex-Red® Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used according to manufacturer's protocols. Briefly, isolated eosinophils were re-suspended in normal or FBS(-) media at 1×10^5/mL density, and following addition of stimuli, reagents and/or catalase, cells were transferred to a 96 well microplate. The microplate was kept at 37° C in a plate reader and the fluorescence was measured every 10 min.

2.5. Immunochemical detection of phosphorylated signaling proteins

Phosphorylated signaling proteins were measured using a multiplex bead assay and Western blotting. For the multiplex bead assay, an antibody panel kit for 8 phosphoproteins related to SFK (Lck, Lyn, Src, Yes, Fgr, Fyn, Blk and Hck) was used (Milliplex; Millipore, Temecula, CA). Briefly, eosinophils (1×10^6/mL) were cultured with or without stimuli for the indicated times, washed with cold PBS, and lysed in the presence of a cocktail of protease/phosphatase inhibitors (Thermo Fisher Scientific, Uppsala, Sweden) using manufacturer-provided lysis buffer to generate lysates equivalent to 1×10^7 cells/mL. Lysates were then filtered with centrifugation in provided filter plates. Total protein concentrations of the lysates were measured and adjusted by diluting with