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IL-33 enhances Siglec-8 mediated apoptosis of human eosinophils

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ABSTRACT

IL-33 activates eosinophils directly via the ST2 receptor. Like IL-5, IL-33 induces eosinophilia and eosinophilic airway inflammation in mouse models and primes human eosinophil responses. Previously, we reported that IL-5 priming enhances Siglec-8 mediated mitochondrial and reactive oxygen species (ROS)-dependent eosinophilic apoptosis and eliminates caspase dependence of this cell death process. Whether IL-33, like IL-5, augments pro-apoptotic pathways involving receptors such as Siglec-8 and in a similar manner has not been explored. Annexin-V labeling was performed to detect apoptosis in human eosinophils pre-incubated with or without a range of concentrations of IL-33 and/or IL-5 in the presence or absence of Siglec-8 monoclonal antibody (mAb) 2C4 and inhibitors of caspases. Tetramethyl-rhodamine staining was used as a marker of mitochondrial membrane potential loss and injury. ROS production was determined by measuring the superoxide dismutase-inhibitable reduction of cytochrome c. Cleavage of poly(ADP-ribose) polymerase (PARP) was assessed using Western blotting. Eosinophils cultured alone or with mAb 2C4 underwent low levels of apoptosis at 24 h. 2C4-induced eosinophil apoptosis was markedly and equally enhanced after culture for 24 h with either IL-33 or IL-5, although IL-5 was more potent. Effects on apoptosis with IL-33 and IL-5 were synergistic. In contrast, percentages of cells exhibiting reduced mitochondrial membrane potential were greater with IL-33 than IL-5 and effects of these cytokines were also synergistic. Antimycin, an inhibitor of mitochondrial electron transport, almost completely inhibited 2C4-induced apoptosis with either IL-33 or IL-5. Surprisingly, 2C4-induced eosinophil ROS production was significantly enhanced with IL-5 but not IL-33. Siglec-8-mediated apoptosis in the presence of IL-33 was more sensitive in magnitude than IL-5 to inhibition by the pan-caspase inhibitor Z-VAD-FMK, yet both cytokine conditions were associated with PARP cleavage. These data demonstrate that IL-33 is as effective but less potent than IL-5 in enhancing Siglec-8-mediated eosinophil apoptosis, and can synergize with IL-5. Eosinophils primed by IL-33 and/or IL-5 *in vivo* would be expected to display enhanced susceptibility to undergoing Siglec-8-induced apoptosis.

1. Introduction

Sialic acid binding immunoglobulin-like lectins (Siglecs) are single-pass transmembrane cell surface proteins found predominantly on leukocytes [1–3]. Among them, Siglec-8 was initially thought to be an eosinophil-specific cell surface protein, but it is also selectively expressed by mast cells and weakly by basophils [4,5]. Siglec-8 recognizes the glycan 6′-sulfo-sialyl Lewis X and its cytoplasmic domain contains an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) [6,7]. Engagement of Siglec-8 on eosinophils results in caspase- and mitochondrial-dependent apoptosis [8,9]. In addition, previous studies have shown that IL-5 priming potently enhances Siglec-8-mediated mitochondrial and ROS-dependent apoptosis with reduced caspase dependence [10]. Clinical use of IL-5 targeting therapies partially reduces eosinophilia in bronchial tissues and esophageal tissues, suggesting a role for other inflammatory molecules besides IL-5 in these disorders [11].

IL-33 is a newly identified cytokine that shares many biological properties as IL-5 including direct activating effects on eosinophils [12–15]. IL-33 has its own receptor, namely ST2, and stimulation through ST2 activates airway eosinophils and results in exacerbated eosinophilic airway inflammation. IL-33 drives the production of additional cytokines and IgE, and IL-33 administration

Abbreviations: ROS, reactive oxygen species; mAb, monoclonal antibody; TMRE, tetramethyl-rhodamine; PARP, poly(ADP-ribose) polymerase; Siglec, sialic acid binding immunoglobulin-like lectin; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; PMA, phorbol myristate acetate; $\Delta\psi m$, mitochondrial membrane potential loss; mCCP, carbonylcyanide *m*-chlorophenyl hydrazone.

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induces eosinophilia and hypertrophy of bronchial epithelial cells as well as mucus secretion in animal models *in vivo*. Such changes resemble pathologic findings in bronchial asthma. IL-33 also directly enhances eosinophil survival and primes a numbers of eosinophil responses via activation of NF-κB pathways [13].

Given the scope of IL-33 biology, much of which overlaps with IL-5, we hypothesize that IL-33 treatment of human eosinophils, like IL-5, will enhance Siglec-8-induced apoptosis, although their comparative efficacy, potency and mechanisms of enhancement may differ. In this paper, we demonstrate that IL-33 is as effective, but not as potent, as IL-5 in enhancing Siglec-8-mediated apoptosis. Effects of IL-33 and IL-5 on apoptosis are synergistic, and enhanced apoptosis mediated by both cytokines can be blocked with inhibitors of the mitochondrial respiratory chain or caspases. Surprisingly, IL-33 is more effective than IL-5 in enhancing Siglec-8-mediated mitochondrial injury yet less effective than IL-5 in enhancing Siglec-8-mediated ROS production.

2. Materials and methods

2.1. Antibodies, reagents, and recombinant proteins

Murine monoclonal IgG1 mAb recognizing Siglec-8 (2C4) was generated as previously described [5]. Recombinant human IL-33 and IL-5 were from R&D Systems (Minneapolis, MN). Tetramethylrhodamine ethyl ester perchlorate (TMRE) was purchased from Molecular Probes (Eugene, OR). Mouse anti-human CD44 mAb (clone J-173, IgG1) and mouse anti-human Fas mAb (clone 7C11, IgM) were purchased from Beckman–Coulter (Hialeah, FL). Cytochrome c, antimycin, phorbol myristate acetate (PMA) and erythrosin-B were from Sigma–Aldrich (St. Louis, MO). A pan-caspase inhibitor was also tested (Z-VAD-FMK from EMD Chemicals, San Diego, CA).

2.2. Eosinophil purification and culture

Written informed consent for blood donation using an IRB-approved protocol was obtained before enrollment. Eosinophils were purified from peripheral blood after density-gradient centrifugation using Percoll (Pharmacia, Uppsala, Sweden) for separation of mononuclear cells from granulocytes, followed by erythrocyte hypotonic lysis and immunomagnetic negative selection with CD16 antibody Miltenyi microbeads (Auburn, CA). Eosinophil purity and viability were consistently higher than 98%, with neutrophils being the only contaminating cells. Purified peripheral blood eosinophils were cultured in RPMI 1640 medium (Life Technologies, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (High Clone Laboratories, Logan, UT), 100 U/ml penicillin G and 0.1 mg/ml streptomycin sulfate (Life Technologies, Invitrogen). After pre-incubation for 30 min with or without various cytokines or inhibitors, eosinophils were harvested at different time points over 3–24 h of co-culture with saturating concentrations of 2C4 mAb to Siglec-8 (10 μg/ml) or other mAbs as indicated in the text.

2.3. Assessment of apoptosis and mitochondrial membrane potential

Eosinophil apoptosis and changes in mitochondrial membrane potential were quantified as previously described [9,10]. Briefly, FITC-Annexin-V labeling was performed to detect apoptosis in eosinophils. To measure changes in mitochondrial membrane potential, cells were loaded for 30 min at 37 °C with 100 nM TMRE, a lipid-soluble calcein ester that accumulates in mitochondria and provides an index of the inner membrane potential [16]. Reduction in TMRE staining was used as a marker of mitochondrial membrane potential loss ($\Delta\psi m$) [9]. Separate aliquots of eosinophils in each culture were incubated with the mitochondrial membrane uncoupler carbonylcyanide *m*-chlorophenyl hydrazone (mCCP, Sigma–Aldrich, at 10 μM, 15 min, 37 °C) as a positive control to induce maximal $\Delta\psi m$, and then stained cells were analyzed by flow cytometry as described [9].

2.4. Assay for ROS production

Using a modification of previously reported methods [17], 0.5 mg/ml fibronectin (Sigma–Aldrich) was used to pre-coat the wells (to minimize background eosinophil activation and ROS production) and 200 μl eosinophil suspension (0.5×10^6 cells/ml) in HBSS (pH 7.4) containing 10 mM HEPES, and 10 mg/ml cytochrome c were seeded into the wells of 96-well tissue culture plates. After stimulation with IL-33 or IL-5 and 2C4 mAb (or PMA at 30 ng/ml as a positive control), the absorbance of reaction wells was repeatedly determined at 550 nm in a microplate reader for up to 3 h. Between absorbance measurements, plates were kept at 37 °C. Superoxide anion production was calculated using an extinction coefficient of 21.1×10^{-3} M/cm for reduced cytochrome c, and was expressed as nanomoles of cytochrome c reduced per 10^5 cells.

2.5. Western blot assays

Antibody to poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technologies, Beverly, MA) was used to detect endogenous levels of the full-length PARP-1 (116 kDa), as well as the large fragment of PARP-1 resulting from caspase cleavage (89 kDa). After electrophoresis of eosinophil lysates, proteins were transferred to PVDF membranes and then incubated for 1 h at RT with 1× TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk. The membranes were incubated overnight at 4 °C with PARP Ab (1:1000). After washing, the membranes were incubated with horseradish peroxidase-linked anti-rabbit IgG (1:2500) for 45 min at RT. Bands were visualized using the ECL western blotting detection system (GE Healthcare, Piscataway, NJ).

2.6. Statistical analysis

Data are presented as the means ± standard error of the mean (SEM) from three independent experiments. Statistical significance between treatment and control group was assessed either by using Student's t test or analysis of variance (ANOVA), as appropriate. *P* values <0.05 were considered significant.

3. Results

3.1. IL-33 enhances Siglec-8-induced eosinophil apoptosis and alters mitochondrial membrane potential

We have previously shown that IL-5 and GM-CSF enhance Siglec-8-medicated eosinophil apoptosis [8]. Therefore the ability of IL-33 to alter Siglec-8-mediated apoptosis was compared to IL-5. As shown in Fig. 1, both IL-33 and IL-5 enhanced Siglec-8-induced apoptosis in a concentration-dependent manner. While IL-33 and IL-5 had comparable efficacy, IL-5 was approximately tenfold more potent in inducing enhanced apoptosis (left portion of Fig. 1). To determine whether there was any additivity or synergy between IL-33 and IL-5, coincubation with a range of concentrations of each cytokine was compared, as shown in the right portion of Fig. 1. Under some experimental conditions, synergy was seen. For example, synergy was observed with a range of IL-5 concentrations plus 30–300 pM concentrations of IL-33, since 3–30 pM IL-33 by itself did not significantly enhance apoptosis.