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Research paper

FACS isolation of live mouse eosinophils at high purity via a protocol that does not target Siglec F

Wendy E. Geslewitz, Caroline M. Percopo, Helene F. Rosenberg*

Inflammation Immunobiology Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

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ABSTRACT

Flow cytometry protocols designed to identify mouse eosinophils typically target Siglec F, an α-2,3-sialic acid binding transmembrane protein expressed universally on cells of this lineage. While a convenient target, antibody-mediated ligation of Siglec F induces eosinophil apoptosis, which limits its usefulness for isolations that are to be followed by functional and/or gene expression studies. We present here a method for FACS isolation which does not target Siglec F and likewise utilizes no antibodies targeting IL5Rα (CD125) or CCR3. Single cell suspensions are prepared from lungs of mice that were sensitized and challenged with *Aspergillus fumigatus* antigens; eosinophils were identified and isolated by FACS as live SSC^{hi}/FSC^{hi} $CD11c^{-}$ $Gr1^{-/lo}MHCII^{-}$ cells. This strategy was also effective for eosinophil isolation from the lungs of *IL5*tg mice. Purity by visual inspection of stained cytospin preparations and by Siglec F-diagnostic flow cytometry was 98–99% and 97–99%, respectively. Eosinophils isolated by this method (yield, $\sim 4 \times 10^6$/mouse) generated high-quality RNA suitable for gene expression analysis.

1. Introduction

While long-perceived as end-stage cells with limited function, eosinophils are now appreciated as immunomodulatory leukocytes with complex roles in health and disease (Rosenberg et al., 2013; Lee et al., 2010). Recent studies have underscored several unanticipated features of tissue eosinophils, notably, their heterogeneity and ability to respond to signals from distinct tissue microenvironments (Mesnil et al., 2016; Percopo et al., 2017; Geslewitz et al., 2017; Wicher et al., 2017).

In order to explore these issues further, it will be critical to have a means to isolate live eosinophils for comparative gene expression and functional studies. There are several protocols available for flow cytometry and FACS isolation of eosinophils generated by our lab and by others (for example, (Dyer et al., 2011; Stevens et al., 2007; Berek et al., 2016; Kastenschmidt et al., 2018)). These protocols typically target eosinophil-specific or eosinophil-enriched cell surface receptors. Among the most prominent of these receptors is Sialic acid-binding immunoglobulin-type lectin F (Siglec F), a type I transmembrane protein with an immunoreceptor tyrosine-based inhibitory motif (ITIM) that binds α-2,3-linked sialic acids and is the functional paralog of human Siglec 8 (reviewed in (O'Sullivan et al., 2017; Kiwamoto et al., 2013)). Siglec F is expressed universally on mouse eosinophils (O'Sullivan et al., 2017; Kiwamoto et al., 2013) and provides reliable quantitative evaluation of eosinophils from multiple tissues (Dyer et al., 2011). While its biological function has not been fully clarified, antibody-mediated ligation via anti-Siglec F induces eosinophil apoptosis both *ex vivo* and *in vivo* (Zimmermann et al., 2008; Zhang et al., 2007). As such, anti-Siglec F may be useful as a means to control eosinophil overabundance in allergic responses and may ultimately be an important clinical tool (O'Sullivan et al., 2017). Nonetheless, this is of course a negative attribute of this antibody, or any antibody that one would choose to use for FACS-mediated isolation of live, healthy eosinophils for functional studies.

Here, we present a FACS isolation protocol that does not target Siglec F. Eosinophils are isolated at high yield, at 97–99% purity and full viability.

2. Methods

2.1. Mice

Wild-type BALB/c mice (8–10 weeks old) were from Charles River

Abbreviations: FACS, fluorescence-activated cell sorter; *IL5*tg, interleukin-5 transgenic; Siglec F, sialic acid-binding immunoglobulin-type lectin F; ITIM, immunoreceptor tyrosine-based inhibitory motif; gusB, beta-glucuronidase; *Af*, *Aspergillus fumigatus*

* Corresponding author at: Building 50, Room 6241, 50 South Drive, NIAID, NIH, Bethesda, MD 20892, USA.
E-mail address: hrosenberg@niaid.nih.gov (H.F. Rosenberg).

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Laboratories, Frederick, MD. Interleukin-5 transgenic (*IL5*tg) mice (Dent et al., 1990) on the BALB/c background are maintained by the NIAID/Taconic consortium and the 14BS vivarium at NIAID. The National Institute of Allergy and Infectious Diseases Division of Intramural Research Animal Care and Use Program, as part of the National Institutes of Health Intramural Research Program, approved the experimental procedures herein as per protocol LAD 8E.

2.2. Allergen sensitization and challenge

Mice under isoflurane anesthesia were sensitized on days 0 and 7 with intraperitoneal injections of *Aspergillus fumigatus* extract (*Af*, 20 μg/mouse, HollisterStier) emulsified with aluminum/magnesium hydroxide (ImjectAlum, ThermoFisher) followed by intranasal inoculation with *Af* (25 μg/mouse in PBS) on days 12, 13, and 14; mice were euthanized and lungs were removed for preparation of single cell suspensions as described below on day 17.

2.3. Preparation of single-cell lung suspensions

Single cell suspensions were prepared from lungs of *IL5*tg mice and mice sensitized and challenged with *A. fumigatus* as described above. After perfusion *in situ* via the right ventricle with phosphate-buffered saline (PBS) with 500 mM EDTA, the lungs were removed from the body cavity, minced and incubated for 90 min at 37°C with RPMI 1640 and 5% fetal calf serum with DNase I (20 mg/mL, Sigma-Aldrich) and Collagenase D (40 mg/mL, Sigma-Aldrich). After incubation, red blood cells were lysed with sterile dH_2O, counted on a hemocytometer with trypan blue exclusion to evaluate viability, and placed in PBS with 0.1% bovine serum albumin (BSA, Sigma-Aldrich) prior to fluorescence-activated cells sorting (FACS). The materials used for preparation of single cell suspensions and for all the methods to follow are listed in Table 1

Table 1
Reagents used in experimental work in this manuscript. Shown here are reagents as listed in the Methods Section, together with commercial source and current catalog number.

Application	Reagent	Source	Catalog no.
Animal study	*A. fumigatus* extract	HollisterStier	5021JF
	ImjectAlum	ThermoFisher scientific	77,161
Single cell suspension	DNase I	Sigma-Aldrich	10,104,159,001
	Collagenase D	Sigma-Aldrich	11,088,882,001
Flow cytometry	Anti-CD16/CD32	BD Biosciences	553,142
	Near infrared live-dead	ThermoFisher Scientific	L10119
	Anti-CD11c alexaFluor 700	ThermoFisher Scientific	56-0114-82
	Anti-Gr1 APC	BD Biosciences	553,129
	Anti-MHCII PE-cyanine 7	ThermoFisher Scientific	25-5321-82
	Anti-Siglec F PE	BD Biosciences	552,126
RNA preparation	RNA-protect	Qiagen	76,526
	Trizol	Invitrogen	15,596,026
	Direct-zol RNA MiniPrep	Zymo Research	R2050
	RNeasy mini kit	Qiagen	74,104
	Agilent RNA 6000 pico kit	Agilent	5067-1513
cDNA synthesis	First-strand cDNA synthesis kit for RT-PCR (AMV)	Roche	11,483,188,001
qPCR	RT^2-preAMP master mix (2×)	ThermoFisher Scientific	4,391,128
	TaqMan Gene expression assay (FAM) Gusb	ThermoFisher Scientific	ID: Mm01197698_m1 Cat. no. 4453320
	TaqMan Gene Expression Master Mix (2×)	ThermoFisher Scientific	4,369,016

together with the commercial source and catalog numbers.

2.4. FACS, flow cytometry, and cellular visualization

Immediately following preparation of single cell suspensions, cells were incubated with Near-Infrared Live-Dead (ThermoFisher) followed by anti-CD16/CD32 (BD Biosciences), anti-CD11c (ThermoFisher), anti-Gr1 (BD Biosciences), and anti-MHCII (ThermoFisher) at 1 μL per 10^6 cells. Cells categorized as $CD11c^-Gr1^{-/lo}MHCII^-$ were isolated via fluorescence-activated cell sorting (FACS) on a FACSAria II (BD Biosciences) and sorted into PBS with 0.1% bovine serum albumin (BSA). Purity was assessed by visualization via cytospin preparation stained with modified Giemsa (Diff-Quik; ThermoFisher). Other samples were incubated with anti-Siglec F and evaluated by flow cytometry.

2.5. RNA isolation from FACS-derived eosinophils

Eosinophils isolated via FACS were sorted directly into RNAprotect (Qiagen) at 750 μL per 1.5×10^5 cells eluted. Cells were then lysed with cold Trizol (Invitrogen) and 100% ethanol. RNA was then isolated with the Direct-zol RNA Miniprep Kit (Zymo Research) and purified with the RNeasy Mini Kit (Qiagen) as previously described (Mesnil et al., 2016). RNA quality was determined by using the Agilent RNA 6000 Pico Kit (Agilent) on the Bioanalyzer 2100 Electrophoresis System (Agilent).

2.6. Quantitative PCR amplification of beta-glucuronidase (GusB) from mouse eosinophil RNA

Two ng of RNA per sample was converted into cDNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche) and then pre-amplified with the TaqMan PreAMP Master Mix (ThermoFisher) and beta-glucuronidase (GusB-FAM) TaqMan Gene Expression Assay (ThermoFisher) for 10 cycles as per manufacturers' instructions. Amplified cDNA products were then amplified further with TaqMan Gene Expression Master Mix (ThermoFisher) on an Applied Biosystems 7500 Real-Time PCR Cycler (Applied Biosystems).

3. Results and discussion

3.1. Fluorescence-activated cell sorting (FACS) without targeting Siglec F for isolation of eosinophils from mouse lung tissue

FACS is a useful, rapid method for isolating pure populations of cells (reviewed in (Herzenberg et al., 2002)). We have used this method to identify subpopulations of eosinophils in the lungs of allergen-challenged mice (Percopo et al., 2017), as have Mesnil and colleagues (Mesnil et al., 2016) who recently defined resident populations of regulatory eosinophils in mouse lung tissue. While useful and effective for identification purposes, antibody-mediated ligation of the cell surface antigen, Siglec F, initiates eosinophil apoptosis (Zhang et al., 2007), which precludes use of this reagent for eosinophil isolation for functional or gene expression studies.

A FACS strategy and appropriate gating for isolating eosinophils from single cell suspensions prepared from lung tissue of mice sensitized and challenged with *A. fumigatus* is shown in Fig. 1. With this strategy, eosinophils are isolated largely by negative selection, as live $CD11c^-Gr1^{-/lo}MHCII^-$ cells, with a yield of $\sim 4 \times 10^6$ eosinophils per mouse. This strategy was also used to isolate eosinophils from of lungs *IL5*tg mice.

As shown, we found that it was not necessary to include anti-CD45 (typically utilized as a pan-leukocyte positive selection marker) as this had no impact on eosinophil purity or yield (see below). Similarly, while the Gr1 gate has been set to be fully inclusive, it can be limited to include only $Gr1^-$ eosinophils, but this will reduce yield.