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

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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, ~ 4×10^6 / mouse) generated high-quality RNA suitable for gene expression analysis.

Keywords

Inflammation; Interleukin-5; Allergen

1.0 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 [1, 2]. Recent studies have underscored several unanticipated features of tissue eosinophils, notably, their heterogeneity and ability to respond to signals from distinct tissue microenvironments [3 - 6].

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Author Contributions

WEG designed and performed experiments, and reviewed the manuscript.

CMP provided critical assistance to WEG, analyzed data, and reviewed the manuscript.

HFR created the manuscript, and wrote the first and subsequent drafts.

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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, [7 - 10]). 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 [11, 12]). Siglec F is expressed universally on mouse eosinophils [11, 12] and provides reliable quantitative evaluation of eosinophils from multiple tissues [7]. 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* [13, 14]. 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 [11]. 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.

2.0 Methods

2.1 Mice

Wild-type BALB/c mice (8-10 weeks old) were from Charles River Laboratories, Frederick, MD. Interleukin-5 transgenic (*IL5*tg) mice [15] 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

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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 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 [3]. 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).

3.0 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 [16]). We have used this method to identify subpopulations of eosinophils in the lungs of allergen-challenged mice [4], as have Mesnil and colleagues [3] 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 [14], which precludes use of this reagent for eosinophil isolation for functional or gene expression studies.

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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 ~4×10^{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.

3.2 Evaluation of eosinophil purity

As shown in Fig. 2A, eosinophils isolated from *IL5*tg mice using this protocol also have normal staining properties, and have typical ring shaped or bow-tie nuclei [17]. The FACS method shown here results in purification of eosinophils from 43% in suspension to ~99% in isolate, as determined by visual inspection of stained cells by light microscopy [Fig. 2B].

We further evaluated eosinophil purification by flow cytometry, utilizing anti-Siglec F as a diagnostic marker. These results confirmed the light microscopic findings [Fig. 3A]; we find that the FACS isolates from lung tissue of *Af* sensitized and challenged mice and *IL5*tg mice included 96.5% and 98.2% Siglec F^{+} eosinophils, respectively.

3.3 Preparation of RNA from FACS-isolated eosinophils

Preparation of RNA from eosinophils can be challenging, as they are not highly synthetic cells, and cells undergoing activation or perturbation can release enzymatically-active secretory ribonucleases (RNases) from cytoplasmic granules [18].

As such, rapid preparation of intact, viable cells with minimal handling to avoid untoward activation is critical. Eosinophils isolated by the FACS method presented are not only of high purity, RNA samples isolated from these cells remain undegraded [Fig. 3B], and were suitable substrates for quantitative reverse transcriptase-PCR. As shown in Table 2, beta-glucuronidase (gusB) was amplified successfully from RNA isolated from live SSC^{hi}/FSC^{hi} $CD11c^{-}Gr1^{-/lo}MHCII^{-}$ eosinophils from the lungs of *IL5*tg mice and mice sensitized and challenged with *A. fumigatus*.

Thus far, we have utilized this FACS method to isolate viable eosinophils in substantial purity and yield from lungs of allergen-challenged and *IL5*tg mice, both conditions in which the non-leukocyte and non-eosinophil populations are relatively limited. This method may require adjustments in other tissues where less favorable conditions prevail. However, given the substantial focus on asthma and allergic inflammation in the the respiratory tract, utilization of this method under these (and related) conditions alone represent a substantial advance in our ability to examine eosinophils and their responses to specific provocation at the molecular level.

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4.0 Conclusions

We present a useful and generally applicable method for rapid FACS-mediated isolation of viable mouse eosinophils at high purity (97-99%) from lungs of allergen challenged mice. The method does not utilize anti-CD125, anti-CCR3 or anti-Siglec F, the latter antibody known to induce eosinophil apoptosis. Large numbers eosinophils (~4 × 10^6/mouse) isolated by this method yield high-quality RNA suitable for gene expression analysis.

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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*

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Highlights

- Mouse eosinophils are typically identified by flow cytometry by targeting Siglec F.
- Ligation with anti-Siglec F induces mouse eosinophil apoptosis.
- We present a method for FACS isolation which does not target Siglec F, CCR3 or IL5Rα.
- FACS-isolated eosinophils from *Af*-challenged mice are live $CD11c^{-}Gr1^{-/lo}MHCII^{-}$.
- Isolated eosinophils are high purity (98-99%) and generate high quality RNA.

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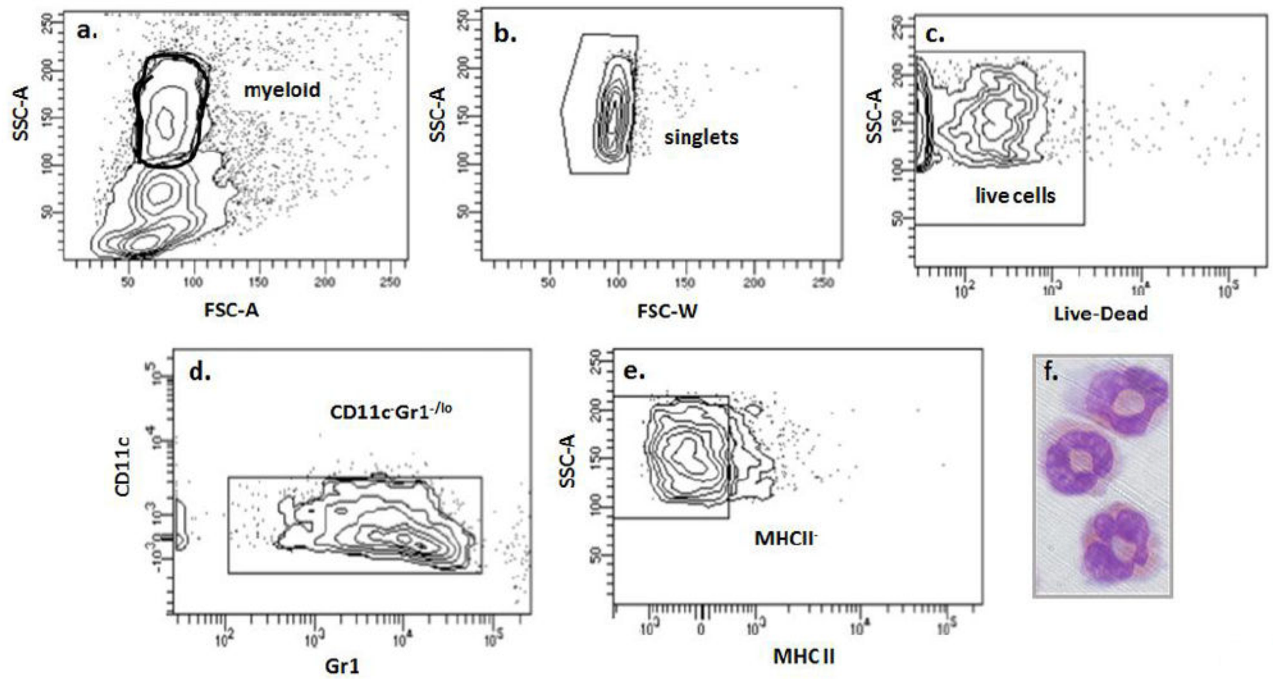

Fig. 1. Fluorescence-activated cell sorting (FACS) protocol without anti-Siglec F used to isolate eosinophils from mouse lung tissue

The protocol featured here does not require anti-Siglec F, which induces eosinophil apoptosis. Gating parameters (a. through e.) used to isolate eosinophils from single cell lung suspensions prepared from lungs of BALB/c mice sensitized and challenged with a filtrate from *Aspergillus fumigatus*. f. Cytospin preparation of cells identified by modified Giemsa-staining as live $CD11c^{-}Gr1^{-/lo}MHCII^{-}$ cells are morphologically eosinophils. Image photographed with a Leica DMI4000 microscope with camera; photographs taken at 100X magnification.

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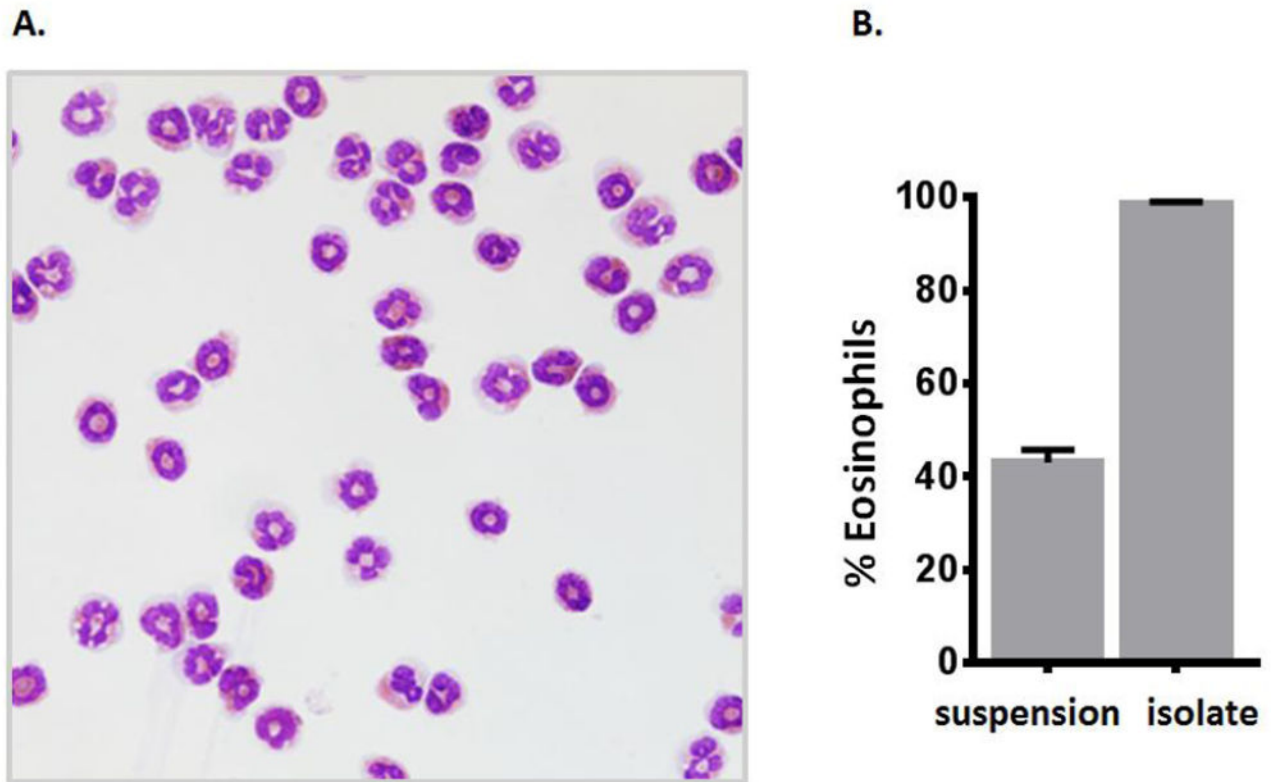

Figure 2. Evaluation of live CD11c$^-$Gr1$^{-/lo}$MHCII$^-$ eosinophils by light microscopy

A. Light microscopic image of a larger field of eosinophils isolated from lungs of *IL5*tg mice by the FACS protocol as shown in Fig. 1. Image photographed with a Leica DMI4000 microscope with camera; photographs taken at 40X magnification. B. Percent eosinophils in the initial single cell suspension and in the post-FACS isolate; > 200 cells counted per sample.

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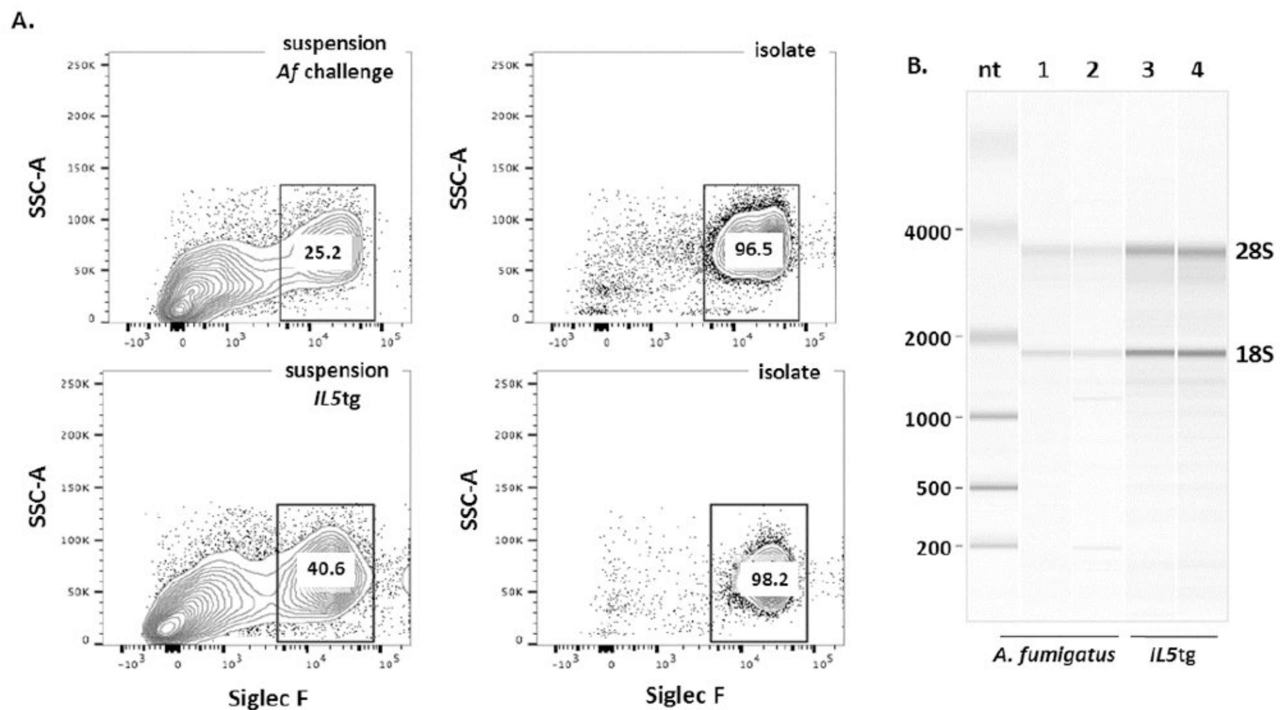

Figure 3. Evaluation of live $CD11c^{-}Gr1^{-/lo}MHCII^{-}$ eosinophils by flow cytometry and preparation of RNA

A. Fraction of Siglec F^{+} cells in the initial single cell suspensions (left-hand panels) and after isolation (FACS sorted cells as in Fig. 1, right-hand panels) from lung tissue from mice sensitized and challenged with a filtrate of *A. fumigatus* (upper panels) and *IL5*tg mice (lower panels). B. Independent samples from eosinophils isolated as in Fig. 1 from lungs of *A. fumigatus* sensitized and challenged mice (lanes 1 and 2) and from *IL5*tg mice (lanes 3 and 4) and collected into RNA-protect prior to RNA preparation (see Methods). RNA from samples (~3×10^6 cells) were evaluated on an Agilent RNA 6000 PicoChip on a Bioanalyzer 2100 Electrophoresis System and have RNA integrity (RIN) scores of greater than or equal to 7.

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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	77161
Single cell suspension	DNase I	Sigma-Aldrich	10104159001
	Collagenase D	Sigma-Aldrich	11088882001
Flow cytometry	anti-CD16/CD32	BD Biosciences	553142
	near infrared live-dead	ThermoFisher Scientific	L10119
	anti-CD11c alexaFluor 700	ThermoFisher Scientific	56-0114-82
	anti-Gr1 APC	BD Biosciences	553129
	anti-MHCII PE-cyanine 7	ThermoFisher Scientific	25-5321-82
	anti-Siglec F PE	BD Biosciences	552126
RNA preparation	RNA-protect	Qiagen	76526
	Trizol	Invitrogen	15596026
	Direct-zol RNA MiniPrep	Zymo Research	R2050
	RNeasy Mini Kit	Qiagen	74104
	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 (2X)	ThermoFisher Scientific	4391128
	TaqMan Gene expression assay (FAM) Gusb	ThermoFisher Scientific	ID: Mm01197698_m1 Cat. no. 4453320
	TaqMan Gene Expression Master Mix (2X)	ThermoFisher Scientific	4369016

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Table 2

Quantitative RT-PCR amplification of cDNA prepared from RNA isolated from live $CD11c^{-}Gr1^{-/lo}MHCII^{-}$ eosinophils

The housekeeping gene, beta-glucuronidase (gusB) was amplified from first-strand cDNA (+RT) generated from RNA samples shown in Fig. 3B prepared from FACS-isolated eosinophils from lungs of *IL5*tg mice and mice sensitized and challenged with *A. fumigatus* (*Af*) shown in Fig. 3B. No amplification product was detected from RNA samples in which reverse transcriptase was omitted (-RT) or in the absence of RNA template (ntc, no template control); undet, undetected, $C_t > 40$ cycles.

Well	Detector	cDNA sample	C_t (cycles)
B1	gusb	*IL5*tg +RT	27.00
B2	gusb	*IL5*tg +RT	26.98
B5	gusb	*IL5*tg - RT	*undet*
B6	gusb	*IL5*tg - RT	*undet*
B10	gusb	ntc	*undet*
D1	gusb	*Af* + RT	27.09
D2	gusb	*Af* + RT	27.10
D5	gusb	*Af* - RT	*undet*
D6	gusb	*Af* - RT	*undet*
D10	gusb	ntc	*undet*