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Antigen profiles for the quantitative assessment of eosinophils in mouse tissues by flow cytometry

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Abstract

Much of our current understanding of eosinophil-associated pathologies has developed from the use of mouse models. While mouse eosinophils can be readily detected by flow cytometric methods, most studies do not document the efficiency of this process compared to direct counting of stained cells. Our intent was to address this knowledge gap by identifying one or more eosinophil-specific antigen profiles that yielded flow cytometric data that was statistically consistent with direct counts. We found that anti-CD193 (CCR3) and anti-CD125 (IL-5R α) antibodies were effective at detecting eosinophils in bone marrow of interleukin-5 transgenic mice, but these antibodies under-reported the percent positive cells. In contrast, anti-Siglec F alone or in combination with anti-CD45 can be used for the quantitative detection of eosinophils in mouse bone marrow and spleen. The antigen profile CD45⁺SiglecF⁺CD11c⁻ was effective at detecting eosinophils in the lung as well as bone marrow and spleen, and the results obtained correlated with direct morphometric counts under all conditions evaluated ($r^2 = 0.98 - 0.99$). To the best of our knowledge, this is the first systematic analysis presenting definitive correlations between percent eosinophils detected by cell surface markers and direct counting of stained cells in multiple tissues and at varying degrees of eosinophilia.

Keywords

Siglec F; CD11c; CD125; CD193; eosinophil; alveolar macrophage

1. Introduction

Eosinophils are tissue granulocytes that are recruited in response to Th2 cytokines and proinflammatory chemokines that contribute to the pathogenesis of helminth infections and allergic and hypersensitivity reactions (Rothenberg and Hogan, 2006; Hogan et al., 2008). Mouse models have played a crucial role in promoting the understanding of eosinophil recruitment, trafficking and function in relation to disease (Bochner and Gleich, 2010) and thus methods for identifying and quantifying eosinophils in blood and tissue have received prominent attention (Meyerholz et al., 2009; McGarry et al., 2010).

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Eosinophils are defined primarily by their morphology and staining characteristics in tissues, blood smears and cytopsin preparations (McGarry et al., 2010). Although nuclear morphology can vary, eosinophils are invariably identified by the pink to red-tinged cytoplasm and, at higher magnification, by their numerous cytoplasmic granules. Although human and mouse eosinophils are morphologically similar to one another, they have distinct granule proteins, respond differently to chemotactic and secretory stimuli, and express unique cell surface antigens (reviewed in (Rosenberg et al., 2007).

Antibodies directed against CD125 (IL-5R α subunit), CD193 (CCR3), and Siglec F have been used to detect mouse eosinophils by flow cytometry, however there have been few, if any, systematic studies documenting the efficiency and specificity of these reagents, nor any sense of how the results obtained correlate with direct inspection of stained cell preparations. For example, while CD125 is expressed primarily on eosinophils, it has also been detected on peritoneal B-1 cells (Hitoshi et al., 1990), a fact that might detract from specificity under certain experimental conditions. CCR3 is likewise expressed primarily on eosinophils in mice, but it has also been detected on mast cells and Th2 lymphocytes (Teixeira et al., 1997). Siglec F, a member of the sialic acid binding immunoglobulin-like lectins, is perhaps the most specific for murine eosinophils (Aizawa et al., 2003; Zhang et al., 2004), although Stevens and colleagues (Stevens et al., 2007) recently documented Siglec F expression on alveolar macrophages, and presented an antigen profile utilizing CD45, CD11c and Siglec F for quantitative identification of eosinophils in allergen and virus-challenged mouse lung tissue.

The goal of this study was to identify an antigen profile or profiles that would be useful for detection of mouse eosinophils in multiple tissues (lung, bone marrow and spleen) in naive mice and at varying degrees of eosinophilia. Likewise, our intent was to generate results that were statistically consistent with direct counts of stained cells from cytopsin preparations.

2. Methods

2.1 Mice

Six to eight-week old wild-type BALB/c mice were purchased from Division of Cancer Technologies (DCT, Rockville, MD). Interleukin-5 transgenic (IL-5Tg) mice on the BALB/c background (Dent et al., 1990) are maintained on site. This study was reviewed and all protocols were carried out in accordance with the Institute's Animal Care and Use Committee Guidelines (Animal Study Proposal LAD 7E).

2.2 Bone marrow cell suspension

The femur and tibia were flushed with RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA), the red blood cells were subjected to hypotonic lysis, and the remaining cells were suspended in Hanks' Buffered saline solution (HBSS, Invitrogen) prior to determination of total cell number and preparation of cytopsin. Approximately $10 - 20 \times 10^6$ cells were recovered from a wild-type naïve mouse.

2.3 Spleen cell suspension

Single cell suspensions were obtained by cutting the spleen into small pieces in Hanks' Buffered saline solution with 1% fetal bovine serum (Lonza BioWhittaker, Walkersville, MD) and 10 mM HEPES (Invitrogen). Fragments were passed through a 70 μ m strainer followed by a 40 μ m strainer. The red blood cells were subjected to hypotonic lysis, and the remaining cells were suspended in HBSS prior to determination of total cell number and preparation of slides. Approximately $2 - 10 \times 10^7$ cells were recovered from a wild-type naïve mouse.

2.4 Lung cell suspension

Prior to removal from the mouse, lungs were perfused by injecting the heart with 8 ml of perfusion media, PBS (KB Medical, Columbia, MD) with 10 mM EDTA (Quality Biologicals, Gaithersburg, MD) to remove peripheral blood cells. Lungs from 3 mice were pooled and then minced in a petri dish using a straight razor blade and transferred to a 50 ml beaker containing 15 ml of media (RPMI 1640, 0.05% DNase (Roche Diagnostics, Indianapolis, IN), 20 mM HEPES, 2mM L-glutamine (Invitrogen), 100 IU penicillin and 10 µg/ml streptomycin (Cellgro Mediatech, Inc., Manassas, VA). 60 µl of 50 mg/ml Liberase (Roche) were added and the contents stirred for 30 min at 37°C. After incubation, the contents of the beaker were removed and strained through a 70 µm cell strainer. The strainer was washed 2–3 times with 5 ml of media with 3% FBS and the cell/media mixture was strained a second time through a 40 µm strainer. Cells were spun for 10 min, the pellet was resuspended in 1–3 ml of ACK lysing buffer (Lonza) and incubated for 3–5 min after which 5–10 ml of media with 3% FBS was added and cells were spun again for 10 min. Pellet was resuspended in 8 ml of 40% Percoll (GE Healthcare, Piscataway, NJ) then overlayed on 3 ml of 70% Percoll and spun at 2500 rpm for 30 min at room temperature. Cells were isolated from the interface, washed with HBSS and counted using a hemacytometer prior to staining. Approximately $2-5 \times 10^6$ cells can be recovered from pooled lungs from three wild-type naive mice. Although ammonium chloride lysis (ACK) was reported to have an impact on eosinophil function (Ide et al., 1994), the different lysis methods have no apparent impact on marker expression, at least with respect to detection by the flow cytometric protocols addressed here.

2.5 Ovalbumin sensitization and challenge

Mice were immunized with ovalbumin (OVA, Sigma-Aldrich) according to the protocol of Gwinn *et al.* (Gwinn et al., 2006). Briefly, mice were injected intraperitoneally with OVA (50 µg) complexed with aluminum potassium sulfate (Imject Alum, Pierce, Rockford, IL) and then challenged intranasally (100 µg OVA in PBS) on days 7–10. The mice were sacrificed and bone marrow, spleen and lungs were collected on day 12.

2.6 Slide preparation and cell counts

Direct counting was accomplished by generating cytopsin slides from bone marrow, spleen and lung cell suspensions. Briefly, after hypotonic lysis of the red blood cells, the cells were counted and 50,000 cells in 100 µL of 0.1% BSA in PBS were placed in a cytofunnel (Thermo Scientific) and centrifuged at 500 g for 5 minutes. The slides were stained with Diff Quik (Siemens, Newark, DE) as follows: 5 minutes in fixative then 2 minutes air dry; 3 minutes in solution 1 (xanthene dye) followed by 3 washes in dH₂O then 2 minutes air dry; 10 seconds in solution 2 (thiazine) followed by 3 washes in dH₂O then air or blot dry and cover slip with cytoseal-60 (Richard-Allan Scientific, Kalamazoo, MI). Five hundred cells from each slide were counted under a 64× oil immersion objective and eosinophils are reported as percent of total cells. Eosinophils were detected based on the pink to red staining of their specific granules (Supplemental Figure 1)

2.7 Flow cytometric analysis

Bone marrow, spleen or lung cells were suspended in HBSS (Lonza, Walkersville, MD) at 10^6 /mL and stained with violet live-dead (Invitrogen) for 30 minutes at 4°C in the dark. After washing in 1% BSA (Sigma, St. Louis, MO) in PBS (BSA/PBS), cells were stained with antibody or isotype control. Each antibody was used in the presence of 0.5 µg blocking antibody, anti-CD16/CD32 (clone 2.4G2, BD Pharmingen) and incubated for 1 hour at 4°C in the dark. After washing in BSA/PBS, the cells were fixed overnight in 4% formaldehyde in PBS (Thermo Scientific). One-hundred thousand events were collected on a LSRII flow

cytometer (BD Biosciences) and the data was analyzed in FlowJo 7.5 (Tree Star, Ashland, OR). Compensation was performed in FlowJo post-collection. All analyses were performed on cells originally determined as live and the data are reported as percentage of live cells.

2.8 Statistical analysis

All analyses were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Each dataset was examined by ANOVA followed by either Bonferroni's or Tukey's post-test.

3. Results and Discussion

3.1 Detection and enumeration of mouse eosinophils by histological staining and visual inspection

Eosinophils are readily detected in cytospin preparations of cells isolated from bone marrow, lung and spleen by staining with modified Wright-Giemsa, (Diff-Quik; Figure 1A). Mouse eosinophil cytoplasmic granules stain pink to red in response to the xanthene dye while neutrophils, which can have similarly shaped nuclei, remain neutral and do not stain prominently under these conditions (Supplemental Figure 1; (McGarry et al., 2010)). As anticipated (Dent et al., 1990; Gwinn et al., 2006), we documented prominent eosinophilia in bone marrow, spleen and lungs of IL-5Tg mice, and in bone marrow and lungs of ovalbumin sensitized and challenged mice (Figure 1B – D). While cytological staining and visual inspection represent the standard for identifying eosinophils (Meyerholz et al., 2009; McGarry et al., 2010), and direct counting is a straightforward task, it is time-consuming and poorly-suited for large numbers of samples.

The eosinophils in our cytospin preparations were intact, and there was no evidence of degranulation under any of the conditions studied. Mouse eosinophils have a very limited propensity to undergo degranulation in response to physiologic stimuli (Lee and Lee, 2005). In the few *in vivo* mouse models in which degranulation is observed (Clark et al., 2004; Ochkur et al., 2007), eosinophils are still readily identified morphologically, as they retain most of their cationic granules.

3.2 Flow cytometric analysis of mouse bone marrow eosinophils

There are many examples in the literature of methods for flow cytometric detection of mouse eosinophils (Southam et al., 2005; Tateno et al., 2005; Stevens et al., 2007; Carlens et al., 2009); in most cases, these protocols have focused on a single tissue and/or one set of experimental conditions. Our goal was to develop a flow cytometric method for detection of eosinophils in multiple tissues with clear correlation to eosinophil counts obtained by direct visual inspection of stained cells. The list of antibodies used in our experiments is shown in Table 1. Our first experimental trial focused on flow cytometric detection of eosinophils in bone marrow from IL-5tg mice ($57 \pm 1\%$ eosinophils by visual inspection, Table 2). Gating on live cells (to avoid the high autofluorescence from dead cells), followed by either CD125 (the IL-5 receptor α chain) or CD193 (the eotaxin receptor, CCR3) resulted in substantial under-reporting, yielding $20 \pm 1\%$ and $16 \pm 2\%$ eosinophils, respectively. Furthermore, as neither anti-CD125 nor anti-CD193 detected eosinophils in the spleen from the IL-5Tg mice, these reagents were not considered further here, but they might be useful under other conditions or in other tissues. As noted in the methods, eosinophils were scored based on cytoplasmic staining; it is certainly possible that some of the eosinophils counted in the bone marrow of the IL-5Tg mice may be insufficiently matured, and may not yet have acquired CCR3- positivity. It is clear from the results reported by Voehringer et al. (Voehringer et al., 2007) that expression of this marker is delayed compared to Siglec F. Interestingly, Kudlacz et al. (Kudlacz et al., 2002) reported increased CCR3-dependent activation and augmented CCR3 expression on eosinophils isolated from IL-5 transgenic mice; IL-5 likewise regulates

CCR3 expression in human eosinophil promyelocyte cells (Tiffany et al., 1998). These complexities suggest that CCR3, while physiologically important, might not be the ideal target for this specific application.

In contrast to our findings with anti-CD125 and anti-CD193 flow cytometric detection with anti-Siglec F, an immunoglobulin-type lectin (Varki and Angata, 2006) yielded results that matched visual inspection of stained cytopsin preparations. Addition of antibody against the pan-leukocyte marker, CD45, alone, or in combination with negative selection with anti-CD11c had no adverse effect on detection of eosinophils in the bone marrow of IL-5tg mice.

3.3 Multiple antibody analysis

Although flow cytometric detection with anti-Siglec F (BD Pharmingen) correlated with direct counting of stained cells in bone marrow from IL5Tg mice, Stevens and colleagues (Stevens et al., 2007) demonstrated that mouse alveolar macrophages express the Siglec F antigen, and that eosinophils might be distinguished as a $CD45^{+}SiglecF^{+}CD11c^{low/-}$ cell population. We have examined this method as a means to identify eosinophils not only in lung, but also in bone marrow and spleen from un-manipulated (control, WT) BALB/c mice and from BALB/c mice that were sensitized and challenged with OVA (Figure 2A). As shown, a distinct $CD45^{+}CD11c^{-}SiglecF^{+}$ eosinophil population (quadrant 3) was detected in all tissues save for the spleen from control mice. As anticipated, $CD45^{+}CD11c^{+}SiglecF^{+}$ macrophages (~5% of total cells in each) were detected in mouse lung tissue only (quadrant 2).

The results of multiple trials using either one (Siglec F), two (Siglec F and CD45) or three (Siglec F, CD45 and CD11c) antigens to distinguish eosinophils in the bone marrow and in lung tissue, either in control or in OVA sensitized in challenged mice are shown in Figure 2B and 2C. Interestingly, when determining the percent eosinophils in the bone marrow, any one of these profiles is effective, as they all faithfully report percentages that match those revealed by visual inspection. When examining the lung, the use of anti-Siglec F alone, or anti-Siglec F together with anti-CD45 may result in over-reporting the percent eosinophils, a problem that can be eliminated by profiling eosinophils as $CD45^{+}SiglecF^{+}CD11c^{-}$. This over-reporting was only statistically significant among the control mice; the larger percentage of eosinophils observed with the OVA sensitized and challenged mice appeared to accommodate the smaller fraction of macrophages within the overall experimental variability.

3.4 Overall correlation between visual inspection and flow cytometric analysis

Overall, flow cytometric analysis performed as follows --gating first on live cells then CD45 followed by quadrant analysis of CD11c vs Siglec F leading to the identification of eosinophils as $CD45^{+}SiglecF^{+}CD11c^{-}$ -- correlates extremely well with staining and visual inspection in all tissues and over a full range of percent eosinophils (Figure 3). In the bone marrow, the correlation coefficient for this analysis is 0.98 and the slope of the linear regression is 1.2, suggesting a direct linear relationship between eosinophil number determined by visual inspection and that determined by flow cytometric analysis using the aforementioned profile; analogous results are shown for spleen and lung tissue.

4. Conclusion

Flow cytometric analysis using antibodies directed against CD45, CD11c and Siglec F permits unique identification of eosinophils in all tissues examined. Although Siglec F is clearly the best single marker of mouse eosinophils, and can be used alone or in combination with CD45 when evaluating eosinophils in bone marrow or spleen at all degrees of

eosinophilia, when examining cells in the lung it is necessary to differentiate between alveolar macrophages and eosinophils, hence the need to include CD11c. We demonstrated a strong correlation between the CD45⁺Siglec F⁺CD11c⁺ antigen profile and visual inspection in three major tissue types and over a substantial range of percent eosinophils. We cannot completely rule out the existence of additional cross-reacting cells (i.e. we cannot know for certain that all CD45⁺Siglec F⁺CD11c⁺ cells in these tissues are eosinophils). We can conclude from these data that if there are other cross-reacting cell types, using this methodology and these antigen profiles, these cells are unlikely to interfere with the analysis. While we did not validate our methodology in the gastrointestinal tract, Carlens et al. (Carlens et al., 2009) sorted SSC^{hi} CD45⁺CCR3⁺ cells from the intestinal lamina propria and identified them as eosinophils and reported that these cells also expressed Siglec F. While many researchers have utilized flow cytometric methods for eosinophil identification, this is the first presentation of a direct correlation between marker detection and visual inspection in multiple mouse tissues under a variety of conditions and varying degrees of eosinophilia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Percent eosinophils determined by staining and visual inspection

(A) Representative photos of eosinophils from indicated sources; OVA, ovalbumin sensitization and challenge; IL5Tg, interleukin-5 transgenic mouse. Representative eosinophils are marked with red asterisks and neutrophils with black asterisks. Original magnification, 40 $\times$. (B – D) Percent eosinophils were determined by visual inspection of Diff –Quik stained cells (500 counted per slide) from (B) bone marrow, (C) spleen and (D) lung from BALB/c-IL-5Tg, BALB/c (WT) and BALB/c mice sensitized and challenged with ovalbumin (OVA). The percent eosinophils detected in the spleen of the OVA sensitized and challenged mice is not significantly different than the WT. Each point represents percent eosinophils counted from an individual mouse; bar, mean $\pm$ SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ANOVA followed by Bonferroni's multiple comparison test.

B. Bone marrow

C. Lung

Figure 2. Flow cytometric detection of eosinophils in bone marrow, spleen and lung
 (A) Samples are from naive mice (control) or mice sensitized and challenged with ovalbumin. Live cells were selected as CD45⁺, followed by CD11c and Siglec F as shown. The cells in Q3 (CD45⁺CD11c⁻Siglec F⁺) are eosinophils. Cells from (B) bone marrow or (C) lung were evaluated by visual inspection (Diff-Quik) or flow cytometric analysis with antibodies directed against indicated antigen. Mean $\pm$ SEM; n= 4–10 mice per group; * p < 0.05.

Figure 3. Correlation between visual inspection and flow cytometric analysis (CD45⁺ CD11c⁺ Siglec F⁺)

Percent eosinophils was determined in (A) bone marrow, (B) spleen and (C) lung by flow cytometry (y-axis, CD45⁺ CD11c⁺ Siglec F⁺) and visual inspection of matched Diff-Quik stained slides (x-axis). Each point represents eosinophils enumerated from an individual mouse. Slopes and correlation coefficients (r^2) were determined by best-fit line linear regression as per algorithms in GraphPad Prism 5.

Antibodies and isotypes

Table 1

Each antibody or isotype control was used at the indicated concentration. The titer of each antibody and isotype control was determined in experiments with 10^6 cells in 100 μ l. Antibody reagents that are most effective at detecting eosinophils in mouse tissues in our protocols are demarcated in bold.

Antigen	Dilution	Fluorochrome	Vendor	Clone	Isotype	Comments
LIVE/DEAD	1:1000	Violet	Invitrogen	None	Unstained	
CD16/CD32	5 μ g/ml	None	BD Pharmingen	2.4G2	None	Blocks Fc γ receptors
Siglec F	1 μ g/ml	PE	BD Pharmingen	E50-2440	Rat IgG2a	
CD193	10 μ g/ml	AF-647	BD Pharmingen	83103	Rat IgG2a	Useful on live cells only
CD125	1 μ g/ml	AF488	BD Pharmingen	T21	Rat IgG1	
CD11b	1 μ g/ml	APC-Cy7	BD Pharmingen	M1/70	Rat IgG2b	Useful on live cells only
CD45	2 μ g/ml	Pe-Cy5	BD Pharmingen	30-F11	Rat IgG2b	Pe-Cy5 is difficult to compensate
	2 μ g/ml	AF700	BD Pharmingen	30-F11	Rat IgG2b	
CD11c	1 μ g/ml	AF700	BD Pharmingen	HL3	Hamster IgG1	
	5 μ g/ml	AF488	eBioscience	N418	Hamster IgG1	

Surface marker analysis in bone marrow

Table 2

Data presented are percent of live cells (after live/dead staining, see Methods).

Percent Positive (IL5Tg Bone Marrow)									
Sample	Diff Quik	SF ⁺	CD125 ⁺	CD193 ⁺	CD45 ⁺ SF ⁺	CD45 ⁺ CD11c ⁻ SF ⁺			
1	54	58	20	14	57	57			
2	57	63	20	13	61	63			
3	55	59	20	20	57	58			
4	60	73	18	18	72	71			
Mean ±SEM	57 ±1	63±3	20 ±1	16 ±2	62±4	62 ±3			
Significance vs. Diff Quik	---	ns	***	***	ns	ns			

Parallel samples were stained with Diff-Quik and counted directly to determine the percent eosinophils; SF, Siglec F; ns, not significant; *** p < 0.001.