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Biomarkers of Eosinophil Involvement in Allergic and Eosinophilic Diseases: review of phenotypic and serum markers including a novel assay to quantify levels of soluble Siglec-8

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Abstract

There remains considerable controversy in the management of eosinophilic disorders, mainly due to a paucity of information regarding the clinical interpretation of total blood eosinophil counts versus surface activation markers versus eosinophil-derived or eosinophil-influencing mediator levels. Regrettably, few tests have been validated that define a unique clinical or prognostic phenotype that is more useful than simply monitoring total blood eosinophil counts. In this manuscript, phenotypic (cell surface) markers, along with serum and tissue-based markers that have been examined in the context of disease activity, are reviewed. We also report the development of a novel assay for detecting soluble Siglec-8 (sSiglec-8), a protein likely derived largely from eosinophils, as a potential serum biomarker. The assay consists of a competitive ELISA using a recombinant Siglec-8-Fc fusion protein. The goal of this preliminary study was to determine if sSiglec-8 is a useful biomarker that differentiates among patients with various eosinophil-associated diseases. In the final analysis, it is fair to say that further research is sorely needed to fully understand and validate the utility of various biomarkers, including sSiglec-8, before their use in clinical practice can be recommended with confidence.

Keywords

Eosinophil; granule proteins; ELISA; soluble Siglec-8

1. Overview of involvement of eosinophils in allergic diseases and asthma

Eosinophils are important effector cells in human allergic diseases. Eosinophil numbers generally increase or decrease in association with allergic and asthmatic disease exacerbations and resolution, respectively (Baigelman et al., 1983; Gibson et al., 1990; Zimmerman et al., 1993). Allergic subjects have a higher number and a more activated state

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of eosinophils compared to non-atopic control subjects. Increased eosinophils in the tissues, blood, and bone marrow are a hallmark of many asthma phenotypes and, in general, elevated numbers of eosinophils correlate with disease severity (Douwes et al., 2002).

The accumulation of and increase in eosinophils are by no means unique to allergies and asthma. Blood and tissue eosinophilia is a common feature of numerous disorders, such as drug reactions, helminth infections, atopic dermatitis, Churg Strauss syndrome, some malignancies, Addison's disease, hypereosinophilic syndrome (HES), eosinophilic gastrointestinal disorders (EGID) and others (Rothenberg and Hogan, 2006; Hogan et al., 2008). In some of these conditions, such as eosinophilic esophagitis, localized tissue eosinophilia can occur without peripheral blood eosinophilia (Simon et al., 2010), and eosinophil-related phenotypic and serum markers tend to be less helpful for diagnosis and in tracking disease activity.

Human studies with selective eosinophil-depleting biologics, such as anti-IL-5 (mepolizumab, reslizumab) and anti-IL-5 receptor (benralizumab) show rapid, profound and sustained reductions of blood eosinophils with a concomitant, but not necessarily as effective tissue depletion from, for example, the upper and lower airways, skin, gastrointestinal tract and bone marrow (Flood-Page et al., 2003a; Flood-Page et al., 2003b; Menzies-Gow et al., 2003; Phipps et al., 2004; Gevaert et al., 2006; Stein et al., 2006; Rothenberg et al., 2008; Stein et al., 2008; Haldar et al., 2009; Nair et al., 2009; Straumann et al., 2009; Busse et al., 2010; Kahn et al., 2010; Kim et al., 2010; Castro et al., 2011; Gevaert et al., 2011; Spergel et al., 2011). This represents a marked effect on their normal turnover, as eosinophils are felt to circulate in blood for about 12 hours before entering tissues, where they persist for about 8-12 days. These therapeutic tools are gradually helping to define the specific contribution of the eosinophil in a range of disorders (Bochner and Gleich, 2010).

1.1. Phenotypic markers of eosinophil activation

Eosinophils are known to display a number of characteristic alterations when activated *in vitro* or *in vivo*. Among the first to be identified were those involving microscopic morphology, such as those associated with the so-called “hypodense” phenotype. Such cells frequently appear in the circulation of patients with eosinophilia, including HES and have a characteristic cytoplasmic appearance in which the granules appear condensed and give the false impression that the cells are partially degranulated (Caulfield et al., 1990). They are called hypodense eosinophils because, on density gradient centrifugation, they are lighter than the 1.080 gm/L specific gravity normally used to separate granulocytes from mononuclear cells and therefore tend to float up into the mononuclear cell layers in such gradients. This microscopic phenotype can be reproduced *in vitro* under activation and priming conditions, especially those associated with exposure to eosinophil survival cytokines (Rothenberg et al., 1988).

A variety of laboratories have described other phenotypic cell surface markers associated with eosinophil activation. Again, similar approaches have been used, such as comparing eosinophil surface markers on cells from normal donors versus those with allergic, parasitic or other disorders with peripheral blood eosinophilia. Table 1 lists a variety of surface markers that have been examined in activated eosinophils. Some of these surface proteins are decreased or shed, such as L-selectin (CD62L) (Matsumoto et al., 1998), CD23, CD31 and PSGL-1 (CD162) (Davenpeck et al., 2000), while the others, including CD35, CD11b, CD66, CD69 and CD81, are increased (Mawhorter et al., 1996; Pignatti et al., 2002; Wedi et al., 2002; Yoon et al., 2007; Suzukawa et al., 2008). In one study, Matsumoto *et al* used an extensive panel of monoclonal antibodies previously used to define leukocyte phenotypes including eosinophils (Ebisawa et al., 1995) and determined that CD44, which is normally

expressed on eosinophils, is increased about two-to-three-fold during activation, whereas CD69 goes from essentially undetectable to relatively high levels (Matsumoto et al., 1998). Many other molecules, such as CD9 (Fernvik et al., 1999) and CD49d/CD29 (VLA-4), which are expressed on eosinophils but not neutrophils, do not appear to change in level of surface expression very much, if at all, during *in vitro* activation. However, VLA-4 is reportedly lower on eosinophils of patients with eosinophilia (Kayaba et al., 2001), while activation of eosinophils enhances functional beta 1 integrin binding affinity that can be detected by epitope-specific activation antibodies (Werfel et al., 1996).

In vivo, cells that have undergone migration into tissues often display phenotypic evidence of activation. For example, following endobronchial allergen provocation in allergic individuals, eosinophils harvested from the lung by lavage have higher levels of CD11b and CD69 with reduced levels of CD62L (L-selectin) (Georas et al., 1992; Julius et al., 1999). The same pattern has been found on lung cells in eosinophilic pneumonia (Nishikawa et al., 1992). In peripheral blood from patients with parasitic diseases, most of the markers mentioned above are indeed altered, as well as in patients with HES and Churg-Strauss syndrome (CSS). Probably most consistent and most readily detectable and useful is surface expression of CD69, since it is not normally expressed on the surface of eosinophils except following activation. Otherwise, alteration of these phenotypic markers is more of a subtle change in the level of surface expression rather than presence versus absence. The same can be said *in vitro* in models of transendothelial and transepithelial migration, all of which tend to reproduce these phenotypic changes in cells undergoing the transmigration process (Ebisawa et al., 1997). Other antibodies can detect novel priming-related epitopes on eosinophils that can detect changes occurring on eosinophils in vivo (Luijk et al., 2005). Whether any of these changes are due exclusively to cytokine or chemokine activation *in vitro* or *in vivo* or other aspects of the migration process *per se* is difficult to determine. Also reported is the observation that markers of eosinophil activation, including reduced levels of IL-5 receptor and enhanced expression of the activated conformation of integrins such as CD49d/CD29 (so called VLA-4 or very late activation antigen-4) also accompanies diseases such as asthma and reverts to the non-activated forms in subjects treated with anti-eosinophil therapies such as mepolizumab (Johansson et al., 2006; Johansson et al., 2008; Stein et al., 2008; Johansson et al., 2012).

1.2. Serum markers related to eosinophil activation and disease activity

For decades, laboratories have utilized sensitive and specific ELISAs to measure levels of eosinophil-derived granule proteins, including eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), and major basic protein (MBP) (Kita et al., 2003). Because these measurements are performed on serum, it is possible that platelet activation and clotting per se could influence some of these measurements. Nevertheless, there is a fairly extensive literature in which these assays were used to measure levels in a variety of biological fluids. There are numerous examples of experimental and disease conditions correlating levels of various granule proteins with situations such as allergic disease, severity in asthma, and atopic dermatitis, as well as bronchial hyperreactivity. In an extensive literature review of ECP (Koh et al., 2007), the authors state that it can be quantified in many types of biological fluids, where it may, for example, correlate with airway inflammation but not airway hyper-responsiveness. However, in familial eosinophilia, EDN levels (and other measures of eosinophil activation such as CD69) did not correlate with total eosinophil count, but rather with clinical disease activity (Kay and Klion, 2004).

It is often the case that similar correlations are detected simply by assessing total eosinophil counts, although this is usually not the case in urinary analyses where soluble mediators are more readily detected. Other serum markers associated with eosinophilia include levels of

eosinophil survival-promoting, activity, likely representing IL-5 and/or GM-CSF (Ackerman and Bochner, 2007). Studies have begun to look at levels of IL-5 receptor and a soluble form of its receptor, but unfortunately, these molecules are not measurable in all conditions associated with eosinophilia (Wilson et al., 2011). Another soluble marker whose utility has been explored is the chemokine TARC (CCL17), which has been shown to correlate with disease activity in atopic dermatitis (Hijnen et al., 2004). One sizable retrospective study of hypereosinophilic syndrome suggested that higher levels of TARC were associated with a higher likelihood of steroid responsive disease, meaning that there disease activity was more likely to be well-controlled with oral corticosteroids if they had elevated serum TARC levels before treatment (Ogbogu et al., 2009). In another study, total blood eosinophil counts, EDN and eotaxin-3 levels correlated modestly with the density of esophageal eosinophils in children with eosinophilic esophagitis (Konikoff et al., 2006). Thus, despite some promising data, at this point, without prospective studies, one cannot confidently combine the use of peripheral blood eosinophil counts with any of these assays to make a definitive determination with regard to eosinophil activation and disease activity that would be clinically useful in diagnosis or disease management beyond that of simply following blood eosinophil counts.

1.3. Tissue markers related to eosinophil activation

For a time, it was in vogue to use a particular monoclonal antibody (EG2), which was purported to detect an activated or secreted form of ECP (Tai et al., 1984). This antibody was used to detect extracellular forms of these granule proteins that expressed neo-epitopes recognized by this particular monoclonal antibody. Despite initial enthusiasm, subsequent studies suggested that EG2 histochemical staining could occur under a variety of conditions and therefore this type of analysis and its interpretation are no longer considered reliable (Jahnsen et al., 1994; Nakajima et al., 1999). In performing tissue staining for eosinophils, it remains useful to use antibodies to eosinophil granule proteins, including ECP, as these are relatively specific for eosinophils.

Recent studies in mouse systems have embraced the use of antibodies to Siglec-F, the functional paralog of Siglec-8, as this is highly expressed on eosinophils (Tateno et al., 2005; Voehringer et al., 2007; Wu et al., 2011). In the mouse, unlike in humans, Siglec-F is not expressed on basophils or mast cells. Consequently, eosinophils are the only peripheral blood granulocyte that expresses Siglec-F. In lung tissues, however, the situation is not so simple, as alveolar macrophages in the mouse express Siglec-F (Stevens et al., 2007). Finally, it is important to point out that inducing eosinophil degranulation has been difficult to accomplish *in vitro*. Several laboratories have reported conditions for inducing eosinophil degranulation *in vitro*, including the use of IgA and cytokines, but a relatively modest percentage of total granule contents end up being released (Kita et al., 2003). Eosinophils also secrete sulfidopeptide leukotrienes and a variety of cytokines and chemokines , but again the latter two are generally produced in relatively small quantities compared to other cells. It is thus clear that more information is needed regarding mechanisms of eosinophil degranulation, before we can use levels of such mediators in tissues or other body compartments as useful biomarkers of disease activity.

1.4. Discovery of Siglec-8 and the development of a novel eosinophil-related biomarker assay to measure soluble Siglec-8 levels

In the late 1990's, our lab participated in a collaborative effort to identify novel eosinophil-specific markers. These efforts led to the discovery of Siglec-8, formerly known as sialoadhesin factor-2 or SAF-2 (Floyd et al., 2000; Kikly et al., 2000). Siglecs (sialic acid-binding, immunoglobulin-like lectins) are single-pass transmembrane cell surface proteins found predominantly on leukocytes (Varki and Angata, 2006; von Gunten and Bochner,

2008). Siglec-8 was initially thought to be an eosinophil-specific cell surface protein, but once antibodies were made to Siglec-8, it was quickly discovered that it is also expressed on mast cells and weakly on basophils (Kikly et al., 2000). Siglec-8 recognizes the glycan 6'-sulfo-sialyl Lewis X and contains an intracellular ITIM domain (Bochner et al., 2005; Bochner, 2009). Its engagement on eosinophils results in apoptosis; whereas, in mast cells it was shown to inhibit allergic degranulation responses activated via FcεRI (Nutku et al., 2003; Yokoi et al., 2008). While eosinophil surface levels of Siglec-8 do not appear to differ among donors with various allergic or eosinophilic conditions (Bolos-Sy et al., 2000), it was noted that Siglec-8 levels tend to decline as cells are handled *in vitro*. Indeed, a common paradigm for many siglecs is that their surface levels are reduced following external ligation (Tateno et al., 2007). This occurs as a result of internalization, with the receptors often getting recycled back to the cell surface. We therefore hypothesized that the reduction in surface Siglec-8 could be the result of the protein being internalized or shed from the cell surface. While Siglec-8 internalization remains actively under investigation, new tests were needed to look for shedding. This led to the development of a novel assay to detect and quantify soluble Siglec-8 (sSiglec-8) levels. Here we report the validation of this assay and its use to screen sera from normal donors, those with allergies, and subjects with a variety of hypereosinophilic disorders. The objectives of this study were two-fold: (1) to determine whether we could use sSiglec-8 as a biomarker to differentiate among patients with various disorders, especially those associated with eosinophilia; and (2) to use this information to aid in the development of a more extensive validation study. The ultimate goal of this work is the development of an assay with clinical and/or prognostic utility.

2. Materials and methods

2.1. Reagents

Monoclonal Siglec-8 antibody 2C4 (IgG1 mouse anti-human Siglec-8) was generated using a Siglec-8-human IgG1 Fc fusion protein (Siglec-8-Fc) as previously described (Kikly et al., 2000). This dimeric Siglec-8-Fc fusion protein is the same reagent previously used to identify putative Siglec-8 glycan ligands (Bochner et al., 2005; Guo et al., 2011).

2.2. Biotinylation of Siglec-8-Fc

Siglec-8-Fc was dialyzed against PBS and biotinylated using 1 mg of biotin X-NHydroxysuccinimide ester (biotin-X-HSE, EMD Chemicals, Gibbstown, NJ), dissolved in N, N Dimethylformamide (100 μl), per mg of Siglec-8-Fc. The reaction mixture was stirred at room temperature for 1 h and then overnight at 4°C. After dialyzing again to remove free biotin, bovine serum albumin was added (10 mg per mg of Siglec-8-Fc-biotin), filtration sterilized and stored frozen at -20°C until used.

2.3. Eosinophil purification and generation of serum samples from various donors

Eosinophils from normal and allergic donors were purified from peripheral blood after density-gradient centrifugation using Percoll (Pharmacia, Uppsala, Sweden) for separation of mononuclear cells, followed by erythrocyte hypotonic lysis and immunomagnetic negative selection with CD16 negative selection employing Dynabeads™ from Invitrogen (Carlsbad, CA) (Matsumoto et al., 1997) or negative selection with Miltenyi microbeads (Auburn, CA). Eosinophil purity and viability were consistently higher than 98%, with neutrophils being the only contaminating cells.

Serum samples were obtained from normal donors (n=5) and those with mild allergies (n=5). Subjects from both groups had normal eosinophil counts < 300/mm^3. Serum also was collected at the time of diagnosis, prior to initiation of therapy, and stored frozen at -80°C, from patients with benign eosinophilia (defined as subjects with eosinophil counts > 1,500/

mm^3 for ≥ 5 years without any symptoms or evidence of end organ involvement in the absence of theraphy) (n=3) eosinophilic gastrointestinal disease (EGID, n=2), the lymphocytic variant of HES (LHES, n=3), the myeloproliferative variant of HES associated with a deletion mutation on chromosome 4 involving FIP1L1-PDGFR (Cools et al., 2003), now best referred to according to the World Health Organization classification of PDGFRA-associated myeloproliferative neoplasm (Vardiman and Hyjek, 2011) (PDGFRA-associated MPN, n=2), other patients meeting criteria for HES but not classifiable as either LHES or PDGFRA-associated MPN (HES, n=7), and those diagnosed with helminthic parasitic diseases (PARA, n=3). The two parasitized subjects with high eosinophil counts (3272 and 1929/mm^3) had Loa Loa infections, while the third subject (eosinophil count 710/mm^3) had strongyloides. All sera were obtained from pre-treatment on the day of the eosinophil count provided.

2.4. Quantification of sSiglec-8 using a competitive binding ELISA

Due to the lack of two antibodies that bind different determinants on Siglec-8 to set up a two-site immunoenzymetric assay, a competitive binding ELISA was developed to quantify sSiglec-8 levels in human serum. In this assay, 2C4 mAb was bound to plastic microtiter plates (optimal at 2 μg/ml in PBS, overnight at room temperature). The plate was then blocked with PBS-1% bovine serum albumin (1 h, room temperature, 0.3 ml/well). Following a buffer wash, 50 μl of the test sera or reference serum dilutions containing known amounts of Siglec-8-Fc were pipetted into their respective wells. Thirty minutes later, 50 μl of biotinylated Siglec-8-Fc (optimal at 30 ng/ml) was added. The plate was gently tapped to mix the contents in each well. If unlabeled Siglec-8-Fc was present, it would bind to the 2C4 mAb attached to the plate and thus proportionally block the binding of biotinylated Siglec-8-Fc. After 1 h incubation at room temperature, the primary binding reaction was stopped with a buffer wash (PBS-0.05% Tween20) and streptavidin-horseradish peroxidase (HRP, Sigma-Aldrich, St. Louis, MO) (1 μg/ml) was added to all wells to detect bound biotinylated Siglec-8-Fc. Following a final 1 h incubation at room temperature, the plate was washed with PBS-0.05% Tween20 and the quantity of bound biotinylated Siglec-8-Fc-avidin-HRP complexes was detected with the addition of ABTS substrate containing H_2O_2 (Sigma-Aldrich, St. Louis, MO). Optical density (OD_{405nm}) was measured using a microtiter plate reader (Dynatech MR4000) and plotted as a function of the optical density obtained with the standards containing known quantities of unlabeled Siglec-8-Fc. All analyses were done in triplicate.

3. Results and discussion

Figure 1 shows that purification of eosinophils using the DynaBeads system often results in a loss of Siglec-8 surface expression compared to baseline on cells in whole blood. This does not appear to occur using the Miltenyi microbeads negative selection system. These data suggest that under certain circumstances, levels of Siglec-8 decline with cell handling. While many siglecs are internalized after ligation (Tateno et al., 2007), no ligands were likely present under our experimental conditions. Thus, we hypothesized that Siglec-8 could be actively shed from the eosinophils *in vivo* and to test this hypothesis, an assay to measure sSiglec-8 was developed.

In Figure 2, two modes of reagent addition and selection of reagent concentrations are compared: either simultaneous addition of unlabeled (1-500 ng/ml) and biotin labeled (20 versus 40 ng/ml) Siglec-8-Fc for 1.5 h into a plate coated with mAb 2C4 at 1.25, 2.5, 5 and 10 μg/ml (panel A), or sequential addition of unlabeled (1-500 ng/ml, 30 min) Siglec-8-Fc followed by a 1 h incubation with biotin-labeled (20 versus 40 ng/ml) Siglec-8-Fc (without washing) into a plate coated with mAb 2C4 at 1.25, 2.5, 5 and 10 μg/ml. In comparison to simultaneous addition (Fig. 2A), the sequential addition of these reagents (Fig. 2B) resulted

in an assay with the greatest analytical sensitivity. Therefore, in subsequent experiments, sequential addition of reagents was employed using 2.5 μg/ml for mAb 2C4 coating and 40 ng/ml for biotin-labeled Siglec-8-Fc. This resulted in an analytical sensitivity below 10 ng/ml (Figure 2B).

Figure 3 shows further efforts towards optimization of the calibration curve. In these experiments, the concentrations of unlabeled Siglec-8-Fc were varied, while comparing either 2 or 2.5 μg/ml of immobilized 2C4 mAb and either 30 or 40 ng/ml of biotinylated Siglec-8-Fc. These experiments showed that the addition of 30 ng/ml of biotin-Siglec-8-Fc into a plate coated with 2C4 at 2 μg/ml produced the most analytically sensitive ELISA (Figure 3A). These conditions were then replicated in three separate experiments, yielding the sSiglec-8 calibration curve shown in Figure 3B, with a potential range of detection from 2-500 ng/ml.

Levels of sSiglec-8 were then determined in ten serum samples from subjects with normal eosinophil counts (<300/mm^3, five from atopics, five from non-atopics), and in all cases sSiglec-8 levels were undetectable (data not shown). Next, 17 serum samples from patients with various forms of eosinophilia were masked and assayed for sSiglec-8 levels. As shown in Figure 4A, sSiglec-8 was detectable in some but not all subjects with eosinophilia and weakly correlated with total blood eosinophil counts across all diagnoses. Although the numbers were small, more impressive correlations (those with R^2 values displayed in the Figure 4A legend) were seen between total eosinophil counts and sSiglec-8 levels among subjects with HES, benign eosinophilia (albeit with low sSiglec-8 levels) and LHES. When these same sSiglec-8 levels were re-plotted by diagnosis (Figure 4B), it became more readily observable that subjects with PDGFRA-associated MPN and PARA (2 subjects were infected by Loa loa, 1 subject by strongyloides) had undetectable sSiglec-8 levels, while LHES sera contained the highest sSiglec-8 levels measured and values for HES and EGID varied.

There are several shortcomings of the data in its current form. First, the exact source of the measured sSiglec-8 in these serum samples cannot be determined (e.g., they could come from mast cells or basophils). Second, the mechanism of sSiglec-8 generation is unknown and will require additional studies, including those that explore whether cell surface shedding is occurring. Certainly the present findings require further verification in larger cohorts of patients both prospectively and before and after medical intervention. Studies would also need to be done in which disease activity and severity are monitored along with total blood eosinophil counts and sSiglec-8 levels in order to ascertain whether the latter measurements would be useful either as a disease-specific biomarker or as a clinical test that influences medical management, perhaps by reflecting disease control. Nevertheless, these results suggest that sSiglec-8 levels correlate with simultaneously obtained peripheral blood total eosinophil counts in some disorders, such as HES and LHES. It is especially tempting in future studies to explore the diagnostic utility of sSiglec-8 levels >60 ng/ml in the diagnosis of LHES.

4. Conclusions

Over the years, a variety of serum, cell surface, tissue-based and other markers of eosinophil activation and involvement in disease have been studied. So far, none have been clearly found to be superior to simply measuring blood eosinophil counts as a way to diagnose allergic or eosinophilic diseases, or to follow disease activity and response to treatment. Here we report the development of a sensitive competitive ELISA to detect sSiglec-8 in human serum samples obtained from patients with various forms of eosinophilia. This analysis has allowed us to identify sSiglec-8 as a potential biomarker for certain

hypereosinophilic diseases such as HES and LHES. Whether these measurements would be useful in distinguishing LHES from other forms of eosinophilia, or for predicting disease exacerbations, remains to be determined. Further research is required to validate the utility of all eosinophil-related biomarkers before their use in clinical practice can be recommended with confidence.

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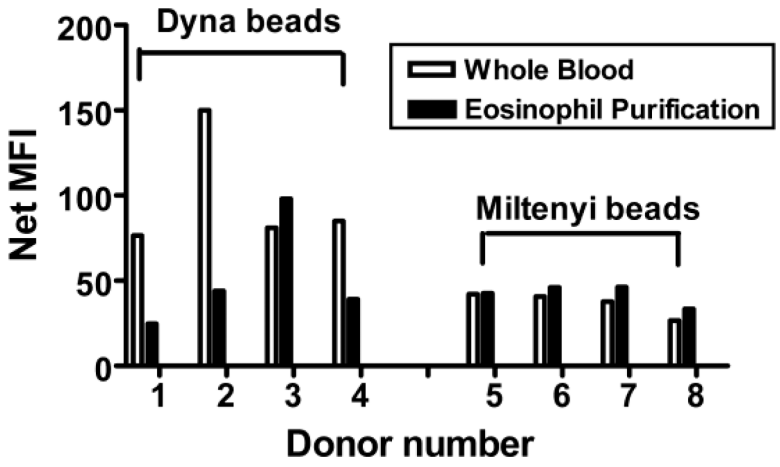

Figure 1.
Effect of eosinophil purification on cell surface levels of Siglec-8. Open bars represent data in whole blood (n=8) while black bars represent data after eosinophil purification (n=8). MFI, mean fluorescence intensity.

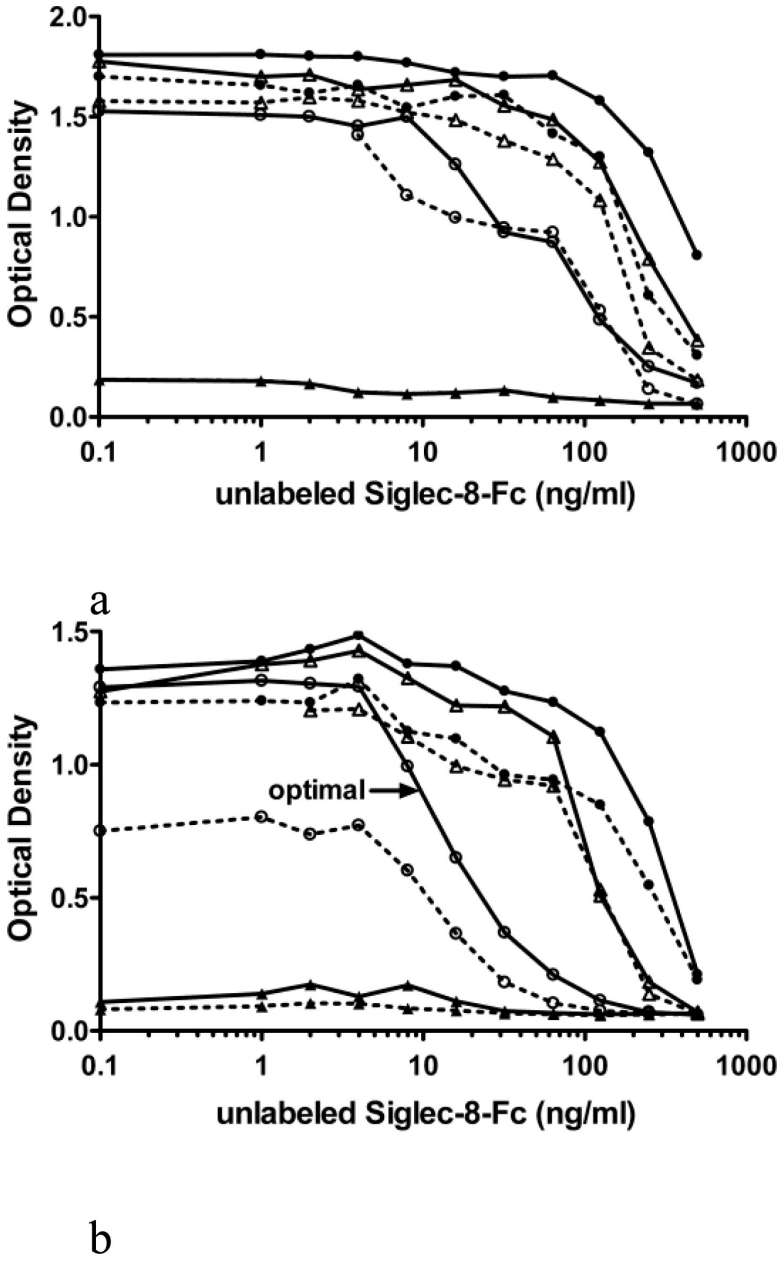

Figure 2.

Comparison of simultaneous (Panel A) versus sequential addition (Panel B) of unlabeled and biotinylated Siglec-8-Fc for optimization of the sSiglec-8 ELISA. Compared were four concentrations of 2C4 mAb (1.25 μg/ml, close triangle; 2.5 μg/ml, open circle; 5 μg/ml, open triangle; and 10 μg/ml, close circle) and two different concentrations of Siglec-8-Fc-biotin (20 ng/ml, dashed lines; 40 ng/ml, solid lines) with addition of non-biotinylated Siglec-8-Fc (1-500 ng/ml). The designation of “optimal” in Panel B refers to the best assay conditions, namely sequential addition of unlabeled Siglec-8-Fc, followed by addition of 40 ng/ml Siglec-8-Fc biotin, using 2.5 μg/ml of mAb 2C4 as the capture reagent.

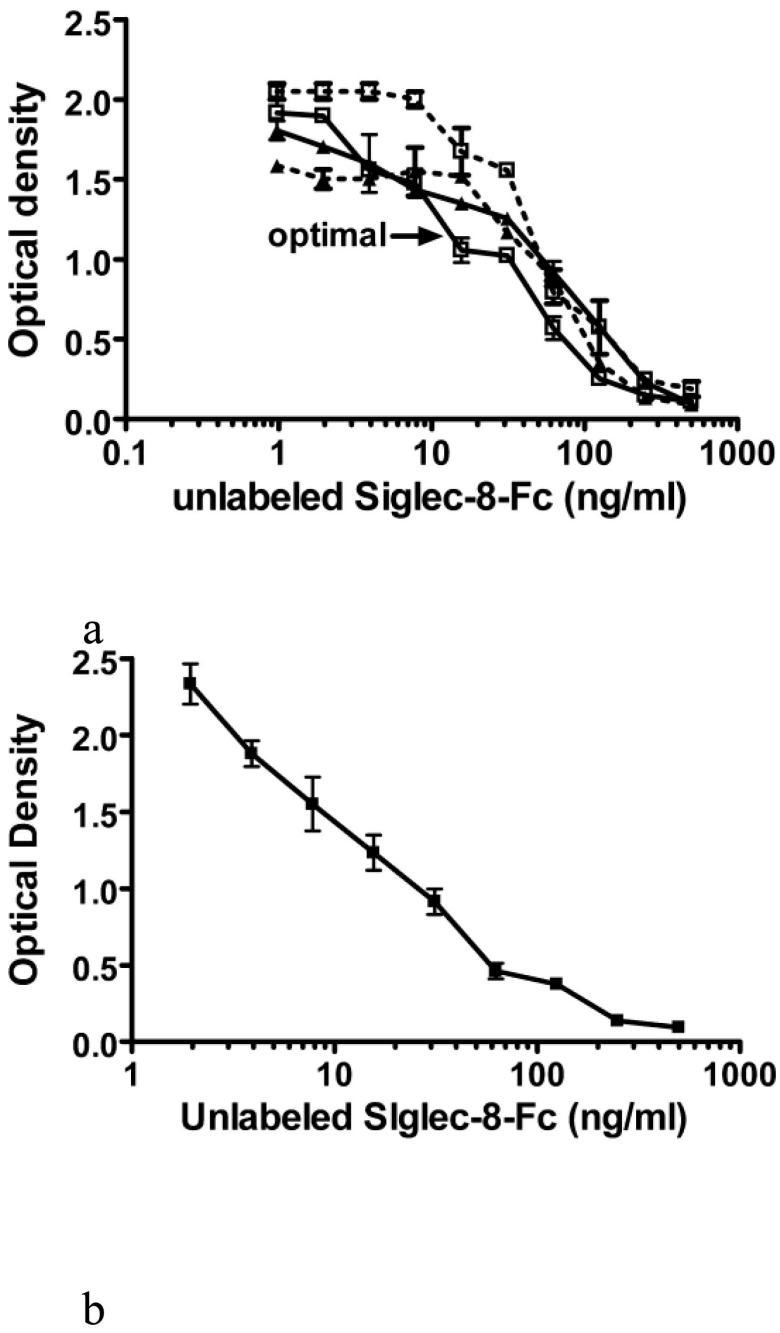

Figure 3.

Further fine optimization of the sSiglec-8 ELISA (Panel A) and validation of the selected assay conditions in replicate experiments (Panel B). Panel A shows sequential addition (30 min) of unlabeled Siglec-8-Fc (1-500 ng/ml) followed by a 1 hour incubation of Siglec-8-Fc biotin (30 ng/ml, solid lines or 40 ng/ml, dashed lines) without washing into a plate coated with 2C4 mAb at either 2 μg/ml (squares) or 2.5 μg/ml (triangles). The designation of "optimal" in Panel A refers to the best assay conditions, namely sequential addition of unlabeled Siglec-8-Fc, followed by addition of 30 ng/ml Siglec-8-Fc biotin, using 2 μg/ml of mAb 2C4 as the capture reagent, the conditions used to generate the averaged data (n=3, mean ± SD) shown in Panel B.

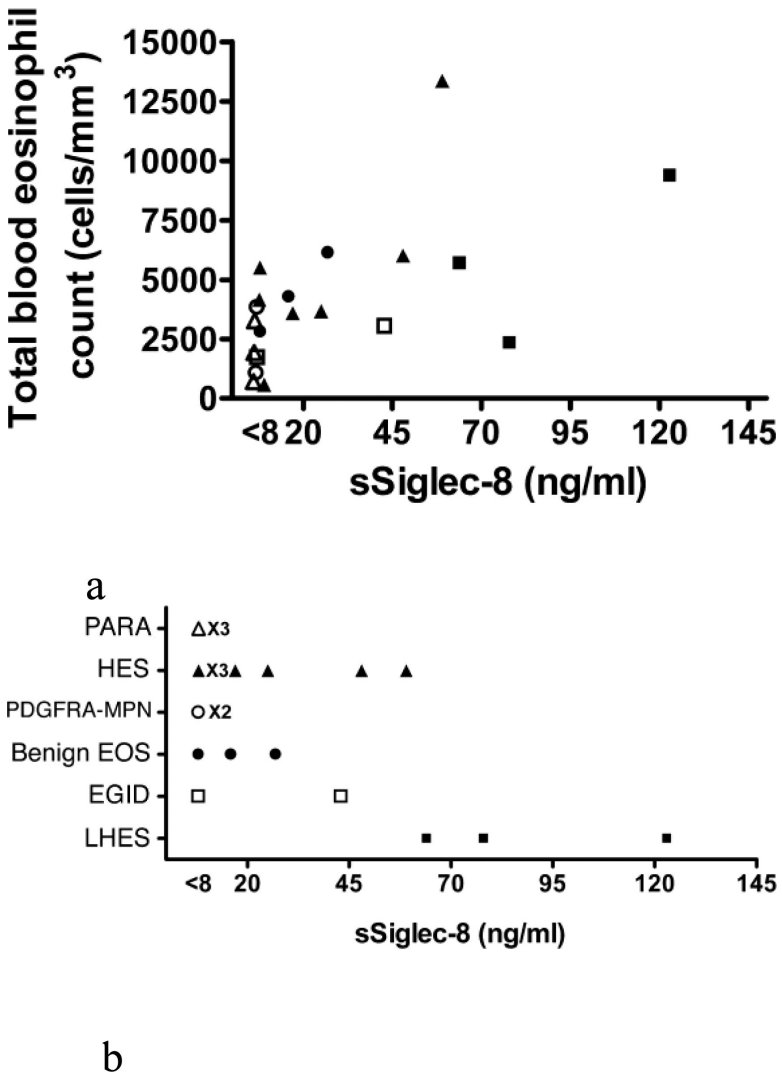

Figure 4.

Analysis of serum samples for levels of sSiglec-8 using the conditions in Figure 3B. Sera from 17 newly diagnosed and untreated patients with various disorders including parasitic diseases (PARA, △), hypereosinophilic syndrome (HES, ▲), PDGFRA-associated MPN (PDGFRA-MPN, ○), benign eosinophilia (benign EOS, ●), eosinophilic gastrointestinal disorders (EGID, □) and lymphocytic HES (LHES, ■). Panel A. Correlations between total eosinophil counts and levels of sSiglec-8. Although the numbers are small, statistically significant correlations (those with R^2 values) are seen for some disorders. Panel B: Replotting of data in Panel A by diagnosis to better show the disease-related levels of sSiglec-8.

Table 1

Examples of changes in eosinophil surface markers after activation

Decreased	Increased	No change
CD31	CD11b	CD9
CD62L	CD35	CD11a/CD18
CD125	CD44	CD49d/CD18
CD162	CD66	Siglec-8
	CD69	
	CD81	