Rapid publication

Identification of SAF-2, a novel siglec expressed on eosinophils, mast cells, and basophils

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Background: Eosinophils, basophils, and mast cells are believed to be the central tenet cells in allergic conditions including allergic rhinitis, asthma, and eczema. The molecular mechanisms underlying the recruitment of these cells to sites of allergic inflammation are poorly understood.

Objectives: Our aim was to identify a common adhesion molecule that could potentially be responsible for mediating the recruitment of the allergic cell types to the lungs and other sites of allergy.

Methods: We have cloned a sialoadhesin molecule from a human eosinophil library with the use of expressed sequence tag technology and characterized its expression on allergic cells by the use of flow cytometry and specific mAbs.

Results: With the use of expressed sequence tag sequencing, we have identified a novel siglec molecule, SAF-2. SAF-2 has homology with other sialoadhesin family members (CD33 and siglec-5) and belongs to a subgroup of the Ig superfamily. SAF-2 is a 431–amino acid protein composed of 3 Ig domains with a 358–amino acid extracellular domain and a 47–amino acid tail. SAF-2 is highly restricted to eosinophils, basophils, and mast cells. Antibodies to SAF-2 do not modulate Ca^{++} mobilization or chemotaxis of human eosinophils induced by eotaxin.

Conclusion: SAF-2 is a highly restricted sialoadhesin molecule, which may be useful in the detection and/or modulation of allergic cells. (J Allergy Clin Immunol 2000;105:1093-1100.)

Key words: *Eosinophil, basophil, mast cell, allergy, cell surface molecule*

Abbreviations used

EST: Expressed sequence tag
ITAMs: Immunoreceptor tyrosine-based activation motifs
ITIMs: Immunoreceptor tyrosine-based inhibition motifs
RBC: Red blood cell

Eosinophils, basophils, and mast cells have been implicated as the major cell types producing inflammatory mediators in response to helminthic infections, as well as several diseases, particularly asthma, rhinitis, and atopic dermatitis.[1-3] In these situations the preferential accumulation and activation of these cells has been noted. Although considerable progress has been made in our understanding of eosinophil recruitment to the site of inflammation, several key points are still unclear, including the exact mediators used for localization to these sites during the migration process. For example, activation of vascular endothelial cell expression of adhesion molecules, notably vascular cell adhesion molecule-1, is believed to be a key event in this process during allergic inflammation.[4] In addition, a number of chemokines and other chemotactic factors, such as those acting via CCR3, have been implicated because of their involvement in eosinophil, basophil, and mast cell chemotaxis.[5-9] However, another possibility is that these cells are selectively recruited and activated in a specific way because of a unique cell surface phenotype. Although eosinophils, basophils, and mast cells are readily identifiable on the basis of their tinctorial properties, as yet, no cell surface marker that is unique to these cell subsets has been identified.[10,11] Here we describe the cloning and characterization of the first cell surface marker for eosinophils, basophils, and mast cells, *S*ialo*A*dhesin *F*amily-2 or SAF-2.

METHODS

Identification and characterization of SAF-2 complementary DNA

Three expressed sequence tags (ESTs) for SAF-2 were identified in a proprietary database (Human Genome Sciences) from an

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eosinophil complementary DNA library. The donor used to generate this library was diagnosed with asymptomatic hypereosinophilia. The assembly of ESTs had significant homology to CD33. The most 5′ clone, HEONN73, was obtained. Two independent clones were obtained by PCR using leukocyte Marathon cDNA as a tissue source (Clontech, Palo Alto, Calif).

TaqMan messenger RNA profiles

Poly A^+ RNA from multiple tissues of 4 different individuals (2 males and 2 females [except prostate]) was reverse-transcribed (with use of random primers), and specific gene messenger RNA levels were measured in each sample (with appropriate gene-specific primers) in an ABI7700 sequence detector with TaqMan real-time quantitative PCR (PE Applied Biosystems, Foster City, Calif) and genomic DNA standards. Primer/probe details are as follows: siglec-5, forward primer 5′-GGTGGGCTCAAATCTAGG-CC-3′, reverse primer 5′-CAGAATGGGAAACTGAGGCAC-3′, probe 5′-FAM-TGGCACTGTCATCAAGCAATTCACTGC-TAMRA-3′; SAF-2, forward primer 5′-AGTGACCTCTGGTCGTCCTCA-3′, reverse primer 5′-AACTTCCTGATGTTGCCATGG-3′, probe 6FAM-TGCTACACTCCTGACAGCACCATGACAGT-TAMRA; glyceraldehyde-3-phosphate dehydrogenase, forward primer 5′-CAAGGTCATCCATGACAACTTTG-3′, reverse primer 5′-CAAGGTCATCCATGACAACTTTG-3′, probe 5′-FAM-ACCACAGTCCATGCCATCACTGCCAT-TAMRA.

Preparation of recombinant SAF-2

The full-length coding region of SAF-2 was subcloned into the mammalian expression vector pCDN with the use of PCR. The sequence of the insert was confirmed before being transfected into HEK293 cells with Ca^{++} phosphate. Clones were selected in 500 μg/mL G418 (Gibco BRL, Gaithersburg, Md) and evaluated for expression with Northern blot analysis, followed by fluorescence-activated cell sorter analysis. The extracellular domain of SAF-2 was subcloned by PCR and inserted in frame with a factor Xa cleavage site and the Fc portion of human IgG_1. The sequence was confirmed before electroporation of the vector into CHOEA1 cells. Stably expressing clones were selected, expanded, evaluated for Fc expression, and scaled up. SAF-2/Fc fusion was purified from supernatant with use of protein A Sepharose, and an aliquot was cleaved with factor Xa to generate the SAF-2 used for antibody generation.

Generation of antibodies to SAF-2, ELISA, and BIAcore analysis

Mice were immunized with SAF-2 (25 μg) in CFA and then received 2 booster injections (25 μg) at 2 and 4 weeks. On the basis of a good serum antibody titer to SAF-2, one mouse received a further immunization of 20 μg of SAF-2 in PBS administered intravenously. The spleen was harvested 4 days later and fused with myeloma cells according to the method described by Zola.[12] Positive hybridomas were tested for binding in 96-well microtiter plates coated with SAF-2/Fc at 0.5 μg/mL and detected with europium-conjugated anti-mouse IgG. Positive hybridomas were re-screened by immunoassay and BIAcore analysis and then cloned by the limiting dilution method. Several mAbs were purified by ProsepA chromatography (Bioprocessing, Consett, UK). Monoclonal antibody 2C4 used in this study was isotyped as IgG_1 κ. The antibodies were confirmed to be specific for SAF-2 by ELISA, BIAcore analysis, and flow cytometry with use of transfected cell lines.

Purification and culture of cells

Eosinophils were purified from peripheral blood after Percoll removal of PBMCs, lysis of red blood cells (RBCs), and immunomagnetic removal of neutrophils.[13] The resulting population was >95% eosinophils. In some experiments purified eosinophils were cultured for up to 2 days in complete RPMI medium containing 10% FCS and 1 or 10 ng/mL IL-5, or 10 or 50 ng/mL eotaxin (Peprotech, Rocky Hill, NJ), C3a, or C5a (Advanced Research Technologies).[14] Viability after 2 days or less of culture was >80%. Enrichment of peripheral blood for basophils was performed with a double-Percoll density gradient separation, increasing the number of basophils to 3% to 10% of the total leukocyte count,[15] or with further immunomagnetic negative selection to at least 50% (Miltenyi Biotec, Auburn, Calif). Human cord blood–derived mast cells were generated as previously reported.[16,17] The purified $CD34^+$ cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 ng/mL selenium, 5×10^{-5} mol/L 2-mercaptoethanol, 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 ng/mL stem cell factor (generously provided by Amgen, Thousand Oaks, Calif), and 50 ng/mL IL-6 (Biosource, Camarillo, Calif) for at least 10 weeks and 1 ng/mL IL-3 (Biosource) for the first 7 days. The purity of mast cells was determined by staining with May-Grünwald Giemsa reagents and routinely reached 99% to 100% by 14 to 16 weeks of culture. For these experiments, cells used were harvested at 16 to 17 weeks of culture. Bone marrow–derived eosinophils were cultured as follows: light density cells of human bone marrow in Ficoll were cultured in Iscove's modified Dulbecco's medium/20% FCS with 20 ng/mL recombinant human GM-CSF and 20 ng/mL recombinant human IL-5 (R&D Systems) at 1.5×10^6 cells/mL at 37°C in 5% carbon dioxide.[18] The cell lines HL-60 and EOL3 were treated with sodium butyrate to differentiate them to a more eosinophil-like phenotype.[19]

Phenotypic and functional analysis

Expression of integrins or SAF-2 was evaluated in anticoagulated whole blood or in enriched cells with use of single-color indirect immunofluorescence and flow cytometry as previously described.[14,15] Dual-color detection of basophils was also performed.[15] Monoclonal antibodies used included the following: control IgG_1, CD18 (7E4), CD51 (AMF7, all from Coulter-Immunotech, Hialeah, Fla), CD9 (Immunotech), and an SAF-2 antibody (2C4, murine IgG_1). Also used was R-phycoerythrin–conjugated or FITC-conjugated F(ab′)2 goat-anti-mouse IgG (Biosource) and FITC-conjugated polyclonal goat anti-human IgE (Kierkegaard and Perry, Gaithersburg, Md). All samples were fixed in 0.1% paraformaldehyde (Sigma, St Louis, Mo) and analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, Calif). At least 1000 events were collected and displayed on a 4-log scale, yielding values for mean fluorescence intensity.

Functional responses (Ca^{++} and chemotaxis) were determined as previously described.[20]

RBC binding assay

COS-1 cells were transiently transfected by electroporation with full-length constructs of SAF-2 in pCDN, siglec-5 in pCDN, CD33 in pcDNA3,[21] and CD22 in pcDNA1.Amp[22] and then re-plated 24 hours later at 2×10^5 cells per well in 6-well tissue culture plates in Dulbecco's modified essential medium containing 0.5% FCS. Occasionally, cells were incubated with 2 mmol/L sodium butyrate overnight before the assays to enhance expression. Transfection efficiency was checked by flow cytometry before all assays. Binding assays with human RBCs were performed 48 to 72 hours after transfection, as previously described, with or without sialidase pretreatment of COS cells and human RBCs.[23] To quantify binding, the percentage of COS cell rosettes (defined as COS cell binding to more than 20 RBCs) was scored from counting at least 200 COS cells.

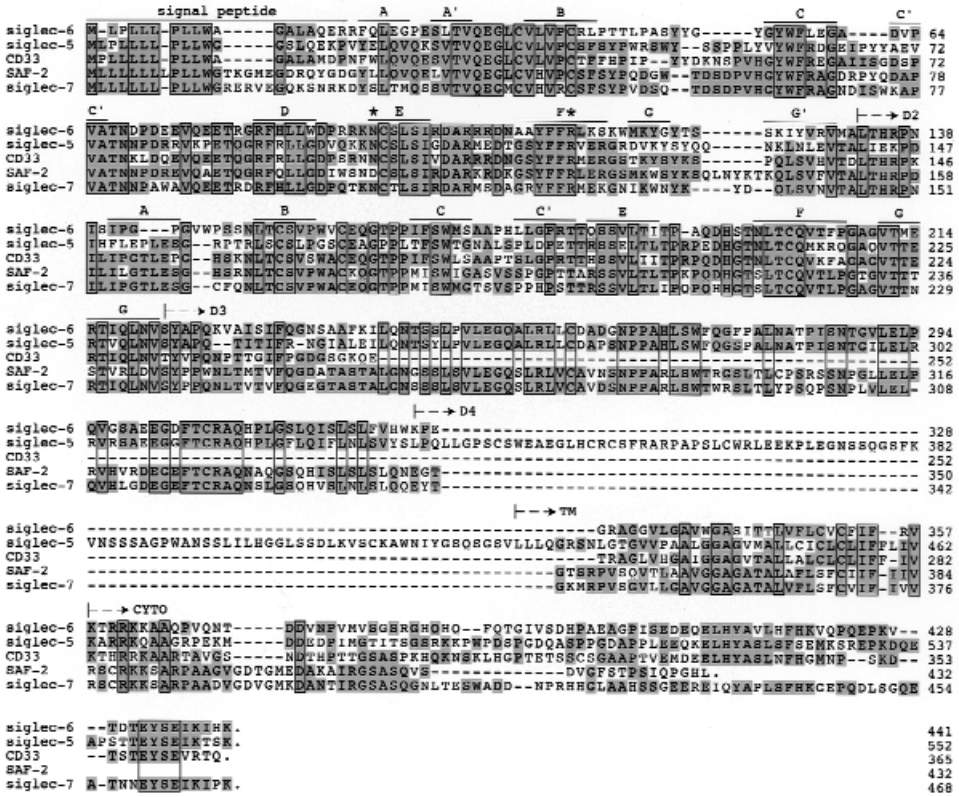

FIG 1. Predicted protein alignment of SAF-2 (GenBank No. AF223403), CD33 (GenBank No. M23197), siglec-5 (AF170484), siglec-6 (D86358), and siglec-7 (AF170485). Alignment was performed with Clustal analysis in MegAlign (Lasergene) and optimized by eye. Residues conserved in all sequences are boxed, and those conserved in 3 of the 5 are shaded. Strand predictions and domains were based on the work of Cornish et al.[24] The predicted signal peptide, strand predictions, Ig domains, TM, and cytoplasmic regions are marked above the alignment. The N-linked glycosylation site that has been shown to modulate sialic acid binding for CD22 and CD33 but is not conserved in SAF-2 is marked with the *first asterisk* (amino acid 106). The conserved R in the F strand of the N-terminal Ig domain is marked with the *second asterisk* (amino acid 125).

RESULTS

Identification of a cDNA for SAF-2 and homology to other siglecs

The cDNA for SAF-2 was identified from 3 ESTs that were assembled into a contiguous sequence with homology to CD33. The most 5′ clone, HEONN73, was obtained and sequenced. HEONN73 contained a 2875-bp insert coding for a 431–amino acid protein (Fig 1)[24] with homology to the sialoadhesin family. The extracellular domain is 358 amino acids folded into 3 Ig domains and contains 4 putative N-linked glycosylation sites. Following the transmembrane region, there is a 47–amino acid cytoplasmic domain with no known signaling motifs. SAF-2 has the highest homology to siglec-7 (68%), CD33 (49%), and siglec-5 (42%) (Fig 1).

The sialic acid binding site for CD22 and sialoadhesin requires an arginine in the N-terminal Ig domain.[25,26] All siglecs discovered thus far have an R at this position, including SAF-2 (Fig 1). Conversely, the conserved N-linked glycosylation site implicated in modulating sialic acid binding in other siglecs is not conserved in SAF-2 (Fig 1). When this site is mutated in CD22, sialic acid binding is completely lost; however, when mutated in CD33, it unmasks a ligand binding function.[27]

To determine the polymorphic nature of SAF-2, two independent clones were obtained from leukocyte cDNA by PCR. From these clones, 5 polymorphisms were identified. The polymorphisms were found at positions 31, 272, 374, 421, and 424 (Fig 1). At position 31, there is a conservative change from a V (as found in siglec-5 and CD33) to an M as found in siglec-7, whereas at position 272 there is an S (siglec-7) or a P (siglec-5 or -6). At position 374, one clone had an L, which is not found in any other sialoadhesin family member. The cytoplasmic region, which is difficult to align with other family members because of a lack of amino acid sequence conservation, has 2 polymorphisms, at position 421 (F→L) and 424 (P→L). Other siglecs also have polymorphisms; for example, comparison of siglec-5 with OB-BP-2 shows 2 amino acid differences,[24] and there are 3 known alleles for mouse CD22.[28]

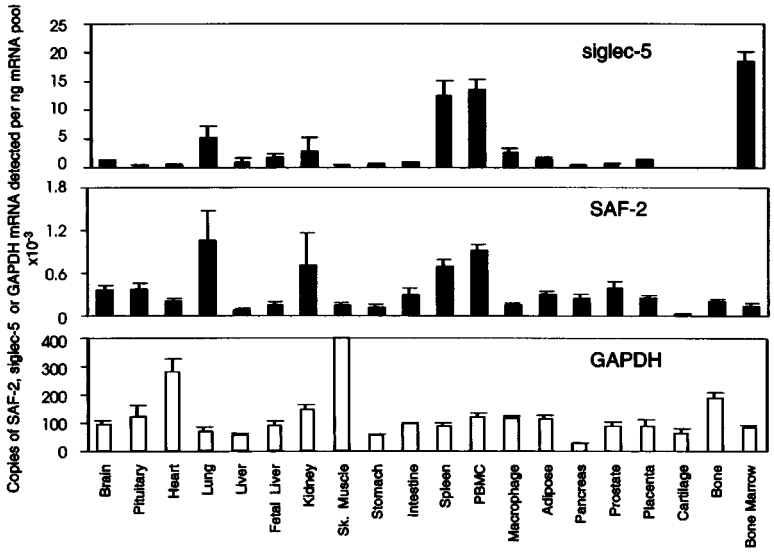

FIG 2. TaqMan analysis of SAF-2 and siglec-5. For each tissue, 4 different samples were measured for expression of SAF-2 and siglec-5 mRNA by using templates derived from 1 ng of polyA$^+$ RNA. Data are shown as mean ± SEM. Expression of the housekeeping gene *GAPDH* is shown for comparison.

Messenger RNA expression of SAF-2

To understand the potential role of SAF-2, TaqMan analysis (quantitative PCR evaluation of mRNA expression) of SAF-2 and siglec-5 was performed on mRNA obtained from tissues from 4 healthy donors. Analysis revealed that SAF-2 was expressed at very low copy numbers in several tissues with a slightly higher copy number in lung, kidney, spleen, and PBMCs (Fig 2). This contrasts in magnitude with siglec-5, which is expressed at a much higher copy number in the same tissues, as well as in bone marrow. This lower copy number in marrow may be due in part to SAF-2's expression pattern in "allergic" cells of the eosinophil, mast cell, and basophil lineages that are known to comprise a smaller percentage of the bone marrow milieu than other cell types. Northern blots were also performed and demonstrated a predominant band of ~3 kb in spleen, PBLs, and lung, in agreement with the TaqMan expression (data not shown).

Expression of SAF-2 on human eosinophils, basophils, and mast cells

Using whole blood, as well as purified leukocyte subpopulations, we analyzed the distribution of SAF-2 by FACS analysis with a specific antibody (2C4) as described in the Methods section. SAF-2 was localized to eosinophils (Fig 3) and was absent from other purified cell populations including neutrophils, monocytes, B cells, and T cells (data not shown).

Using dual-color flow cytometry and 6 separate blood donors, basophils in whole blood (n = 2, purity <1%), after density gradient centrifugation (n = 2, purity 5% in both) or further immunomagnetic enrichment (n = 2, 46% and 51% purity), all expressed low but consistently detectable levels of SAF-2. Using mean fluorescence intensity, the IgG controls averaged 6.6 ± 0.5, while SAF-2 staining was 9.5 ± 0.9 (mean ± SEM, n = 6, $P <$ .03 by paired *t* test). According to percent positive, this was equivalent to 19.3% ± 4.3% positive (n = 6, $P <$ .03 by paired *t* test). Mature human cord blood–derived mast cells also strongly expressed SAF-2, although the pattern of expression was somewhat more heterogeneous than for blood leukocytes in that the peaks were not perfectly unimodal (Fig 3).

Activation of purified eosinophils with optimal concentrations of eotaxin, C5a, C3a, or IL-5 for 1, 24, or 48 hours before analysis did not alter the levels of SAF-2 expression on the cell surface (data not shown). Two cells lines, HL-60 and EOL3, which have been reported to become more eosinophil-like after differentiation with sodium butyrate for 5 days, were examined for the expression of SAF-2.[29] Under these culture conditions, HL-60 and EOL3 failed to express SAF-2 (data not shown). Finally, when eosinophils were generated from bone marrow in vitro, no SAF-2 expression was noted. Eosinophils could be identified by day 14 by staining with CD9 (3%-12% of the cells) and Wright stain (data not shown). Thus it appears that SAF-2 expression may be a later marker for eosinophil differentiation.

SAF-2 mediates sialic acid—dependent binding to human RBCs

One of the defining properties of previously characterized members of the siglec family is their ability to mediate sialic acid–dependent binding of human RBCs. SAF-2, expressed as a full-length protein on COS cells, can bind human RBCs (Fig 4); as with other siglecs, this binding is abolished by sialidase pretreatment of the

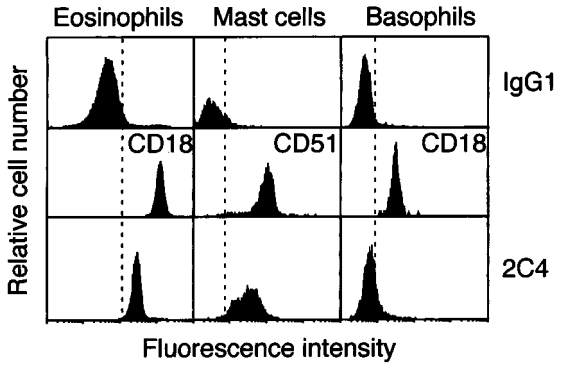

FIG 3. Expression of SAF-2 on human peripheral blood eosinophils, basophils, and 16-week-old cord blood–derived cultured mast cells. Histograms shown are representative of 3 to 4 experiments with virtually identical results for each cell type. Monoclonal reagents used as positive and negative controls are also shown.

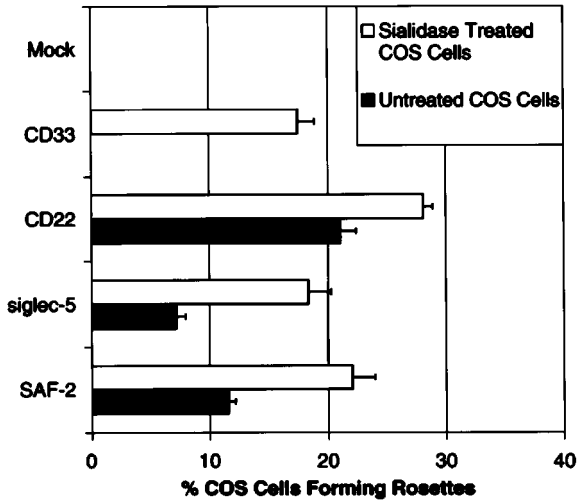

FIG 4. Binding of human RBCs to SAF-2 expressed on COS cells. Binding assays were performed with COS cells mock-transfected or transiently expressing SAF-2, siglec-5, CD22, or CD33. Human RBCs were added to untreated *(black bars)* or sialidase-pretreated *(white bars)* COS cells and allowed to bind for 30 minutes at 4°C. After washing, cells were fixed and percentage of COS cells with RBC rosettes (defined as COS cell binding more than 20 RBCs) was scored by counting at least 200 COS cells per well. All constructs were expressed at comparable levels as determined by flow cytometric analysis before assays (data not shown). Results shown are mean ± SD of triplicate wells from a representative experiment.

RBCs (data not shown). There is growing evidence that the lectin activity of the siglecs can be masked and hence regulated by sialylation on the same cell surface.[23, 24,30,31] We therefore tested whether this was also true for SAF-2 (Fig 4). As previously reported, CD33, the smallest member of the sialoadhesin family with only 2 Ig-like domains, could only bind RBCs when COS-CD33 cells were pretreated with sialidase. Siglec-5, which has 4 Ig-like domains, showed some binding on untreated COS cells, although less so than CD22, which has 7 Ig-like domains and showed almost equivalent binding with or without sialidase pretreatment. Binding activity of COS-SAF-2 cells was also inhibited by endogenous sialic acids, because sialidase pretreatment of the transfected COS cells augmented red cell adhesion by about 50% (Fig 4). However, perhaps surprisingly because SAF-2 has only 3 Ig-like domains, untreated COS-SAF-2 cells showed significant binding of RBCs. This may be explained by SAF-2 having a higher binding avidity than other siglecs or a different sialic acid–binding specificity. However, another interesting potential contributing factor is that SAF-2 lacks the conserved N-linked glycosylation site (aa 106), which has been shown to unmask sialic acid–binding activity when mutated in CD33.[26] Although this is only a model system, these results suggest that SAF-2 on eosinophils might participate in interactions with other cells, although this is likely to be regulated at least in part by endogenous eosinophil sialylation.

Other possible functions of SAF-2 on eosinophils

In an attempt to determine the role of SAF-2 in eosinophil biology, specific SAF-2 mAbs were analyzed for their ability to affect eosinophil function. First, the antibodies were tested for their ability to cause a Ca^{++} flux in purified eosinophils, either on their own or after cross-linking with a second antibody. Compared with eotaxin, which produced a robust Ca^{++} response, none of the mAbs to SAF-2 caused a Ca^{++} flux in eosinophils over a 15-minute time course (data not shown). The mAbs were then tested for the ability to modulate the Ca^{++} response to eotaxin in purified eosinophils. The eosinophils were pre-incubated with anti-SAF-2 with or without a cross-linking antibody and then simulated with eotaxin. Again, the mAbs did not influence the Ca^{++} flux in response to eotaxin (data not shown). In addition, we also evaluated the mAbs in an eosinophil chemotaxis assay, using eotaxin as the chemotactic agent; again, the mAbs failed to modulate eosinophil function (data not shown). Finally, SAF-2 mAbs had no effect on spontaneous or IL-5–induced eosinophil survival (data not shown).

DISCUSSION

SAF-2 was one of several clones identified when an EST database was searched for novel sialoadhesin family members. Its expression pattern is distinct from that of other family members because it is found on eosinophils, mast cells, and basophils. Although CD33 has been found to be expressed on basophils and mast cells as well, it is not exclusive to "allergic" cells.[32,33] Basophils express very low levels of SAF-2 compared with

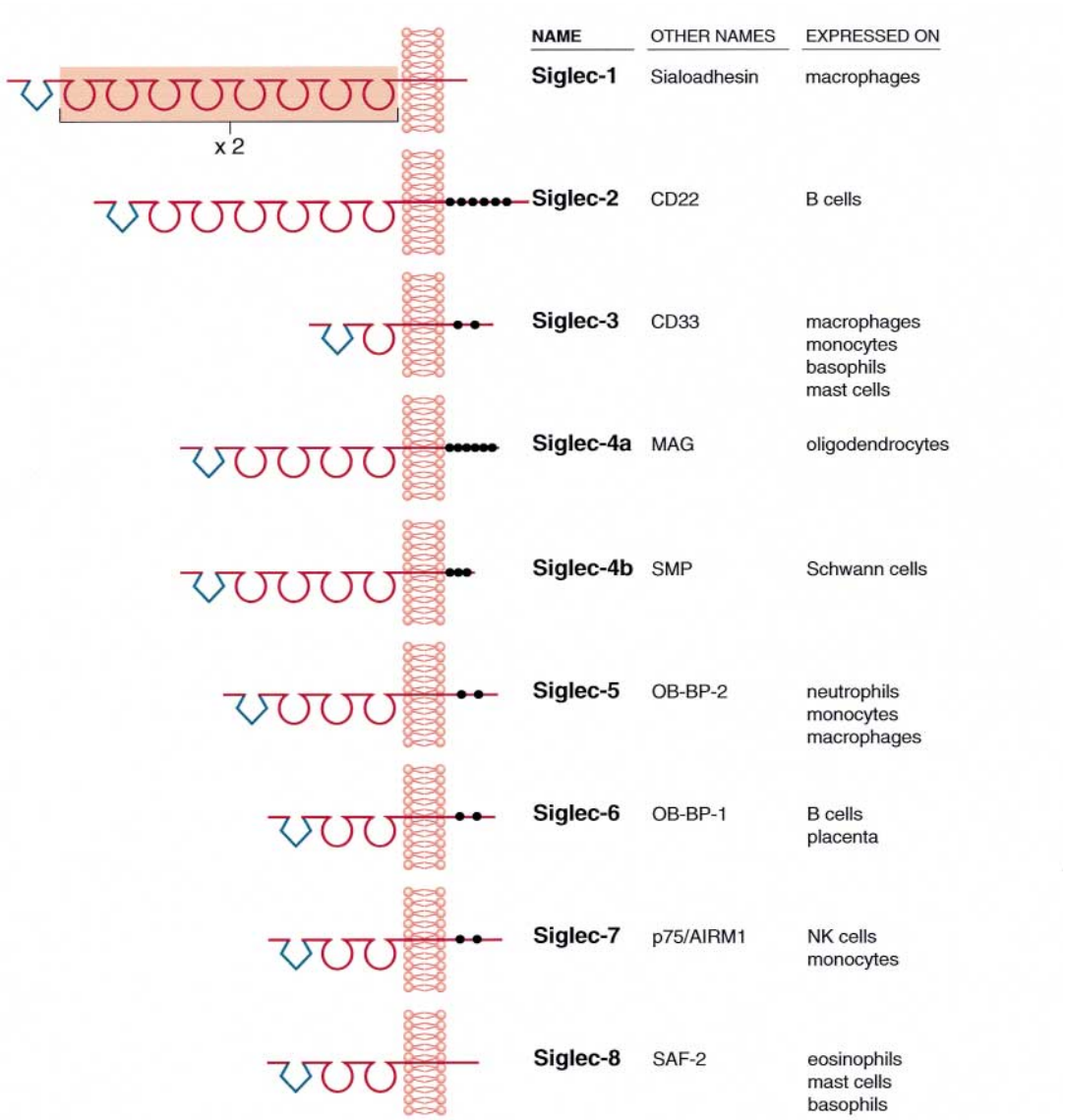

FIG 5. Schematic representation of siglec family. The most N-terminal Ig domain belongs to the V-set subtype and is represented by a *dark blue structure*. This is followed by varying numbers of C2-set Ig domains represented by *red circles*. Cytoplasmic domains are drawn to scale relative to each other. Tyrosines in the cytoplasmic domain are represented as *black dots*; most tyrosines fall into ITIMs or ITAMs and are thought to participate in signaling.

eosinophils and mast cells; perhaps this reflects differences in maturation state or activation of the basophils. Eosinophils differentiated from bone marrow fail to express SAF-2, indicating that it may be a later marker for eosinophil differentiation. An examination with specific mAb has failed to detect any change in SAF-2 surface expression on eosinophils after activation in vitro. SAF-2 is a member of the sialoadhesin family of proteins, also known as the I-type lectins and recently renamed *the siglec family* (sialic acid–binding Ig-like lectins).[34] The siglecs are an ever-expanding family of cell surface molecules expressed almost exclusively on hematopoietic cells, but their function remains poorly understood. Other siglec family members include sialoadhesin (siglec-1), CD22 (siglec-2), CD33 (siglec-3), myelin-associated glycoprotein (MAG or siglec-4), siglec-5,[24] OB-BP-1/siglec-6,[35] and AIRM1 or siglec-7[36,37] (Fig 5). With the exception of siglec-4, all are expressed on various subsets of hematopoietic cells. Siglecs belong to the Ig supergene family and have an N-terminal V-set Ig domain, followed by 1 to 16 C2-set Ig domains. Siglecs mediate sialic acid–dependent adhesion with other cells generally preferring either α2,3 linkages (siglec-1, -3, and -4) or α2,6 linkages (siglec-2).[34]

Most family members have either immunoreceptor tyrosine-based inhibition motifs (ITIMs) or immunoreceptor tyrosine-based activation motifs (ITAMs), which participate in signaling through Src homology 2 (SH2) domain binding to the phosphotyrosine of the ITIM or ITAM (Fig 5). This has been demonstrated for CD22, CD33, and AIRM1.[23,36,38] However, SAF-2 lacks any cytoplasmic ITIMs or ITAMs. Whether SAF-2 can act as a signaling molecule is not known. So far, these mAbs do not induce Ca^{++} flux in purified eosinophils, affect Ca^{++} flux or migration responses to eotaxin, or alter eosinophil survival (data not shown). Given the sialic acid–binding capability of this molecule and its unique distribution on eosinophils, basophils, and mast cells, it is tempting to speculate that it may serve as a ligand for foreign organisms, especially helminths. Clearly, the function of SAF-2 on these cells will require further study. However, in keeping with the siglec nomenclature, we propose that SAF-2 be renamed *siglec-8*. Although it represents a specific marker for the "allergic" cell types, more work is needed to understand the biology of this molecule and its role in various disease processes.

Note added in proof. An article describing the same siglec was recently published (Floyd et al. Siglec-8: a novel eosinophil-specific member of the immunoglobulin superfamily. J Biol Chem 2000;275:861-6).

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