

“Siglec”ting the Allergic Response for Therapeutic Targeting

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Abstract

As a physician-scientist, I have pursued research related to translational immunology with the goal of improving our ability to diagnose and treat allergic, immunologic and other diseases involving eosinophils, basophils and mast cells. We have tried to delineate novel mechanisms of human disease, working whenever possible with primary human cells and tissues, attempting to identify targets that might be amenable to the development of new therapies. As a general strategy, we have compared eosinophils, basophils, mast cells and neutrophils to look for pathways in inflammation that were unique to distinct subsets of these cells. In doing so, the concepts of glycobiology did not enter my mind until we began noticing some intriguing functional differences involving selectins and their ligands among these cell types. One simple observation, that neutrophils were coated with a glycan that allowed them to interact with an endothelial adhesion molecule, while eosinophils lacked this structure, pried open the glycol-door for me. Fruitful collaborations with card-carrying glycobiologists soon followed that have forever positively influenced our science, and have irreparably altered our hypotheses, experimental design, research opportunities and discoveries. Within a few years we helped to discover Siglec-8, an I-type lectin expressed only on human eosinophils, basophils, mast cells. This receptor, together with its closest mouse counterpart Siglec-F, has been the primary focus of our work now for over a decade. If not for those in the fields of glycobiology and glycoimmunology, my lab would not have made much progress toward the goal of leveraging Siglec-8 for therapeutic purposes.

Introduction

My career in immunology, which led me to glycobiology, began as an undergraduate and expanded in medical school. My first exposure to research occurred in the Division of Allergy and Clinical Immunology at Johns Hopkins, where I worked as an undergraduate in the laboratory of Larry Thomas, a postdoctoral fellow at the time, and published my first paper on the biology of one of the histamine metabolizing enzymes, histaminase, and how a new class of drugs at the time, H₂ antihistamines, interfered with its function (Thomas et al. 1978). At the same time, I got my first exposure to glycobiology in my Biochemistry course at Johns Hopkins via lectures given by the late Saul Roseman (the “father” of sialic acid (Comb et al. 1960); also see Ron Schnaar connection below). A few years later, attending medical school at the University of Illinois College of Medicine in Chicago introduced me to Max Samter. I spent six glorious months working with this physician that I would soon learn was the one credited with describing aspirin-exacerbated respiratory disease, aka Samter’s Triad, consisting of asthma, nasal polyposis and aspirin hypersensitivity (Samter et al. 1968), a syndrome that is particularly eosinophilic in its inflammatory pattern. After these experiences, I knew I wanted to study allergy-immunology, but had little time or exposure during medicine internship and residency. And to be perfectly honest, glycobiology as a discipline or career focus was not on my radar yet.

Fellowship training and introduction to the concepts of inflammation and cell adhesion

In 1985, I was offered a fellowship position in Allergy and Clinical Immunology back at Johns Hopkins, in same Division where I had done my prior undergraduate lab work. I decided to work with Robert Schleimer, an immunopharmacologist and translational scientist interested in understanding the anti-inflammatory properties of glucocorticosteroids. I joined his lab at a time when cell adhesion molecules were just being discovered and characterized. Bob's lab had adapted methods to study leukocyte-endothelial interactions, and had just shown that various stimuli, including bacterial endotoxin and interleukin-1, caused umbilical vein endothelial cells to become more sticky for neutrophils (Schleimer et al. 1986). One adhesion molecule responsible for this property turned out to be E-selectin, initially called endothelial-leukocyte adhesion molecule-1 (ELAM-1) (Bevilacqua et al. 1987). My project was to explore ways by which mast cells might release substances that activated endothelial adhesiveness, attempting to forge a link between allergic cell activation in tissues and the subsequent inflammatory infiltrate characterized by the selective accumulation of basophils and eosinophils, the so-call late phase allergic response (Charlesworth et al. 1989). We identified such a factor, released from human lung tissue fragments, which induced endothelial adhesiveness for neutrophils. This turned out to be interleukin-1, and its release was effectively inhibited by steroids (Bochner et al. 1987a; Bochner et al. 1987b) suggesting that one way by which steroids reduce inflammation is by blocking release of cytokines that promote vascular adhesion molecule expression. This led to my first grant in 1988 on basophil adhesion, a so-called R29 "First" award, a predecessor of the modern

day K award. This was enough to get me onto the faculty, so I joined the Division of Allergy and Clinical Immunology as a junior faculty member.

Early translational research involving human granulocyte subsets, cell trafficking and the role of integrins, selectins and their ligands

One advantage of becoming a faculty member is additional flexibility as to which meetings one could attend each year. I had been regularly attending allergy-related meetings, but now I began attending FASEB meetings to expand my knowledge of cell biology and immunology. In the 1990's, I was fortunate to meet, and establish collaborations with, leading scientists in cell adhesion (including Thomas Tedder, Geoffrey Kansas, Michael Briskin and Rodger McEver), and through their generosity received valuable reagents that allowed us to explore the function of P-selectin (CD62P), L-selectin (CD62L) and PSGL-1 (CD162) on eosinophils and basophils (Bochner et al. 1994; Bochner et al. 1996; Davenpeck et al. 2000b; Davenpeck et al. 1997; Knol et al. 1994; Taylor et al. 2000; Wein et al. 1995).

What especially intrigued us around that time, however, was the simple question of how adhesion molecule expression and function might influence the selective recruitment of different granulocyte subsets. Two key observations occurred to us around the same time, one involving our own experiments and another involving some of the earliest descriptions of leukocyte adhesion molecule deficiencies. Regarding the former, while doing expanded kinetics experiments in which endothelial activation was allowed to occur for hours to days, we noted that endothelial cells acquired adhesiveness for human neutrophils at 4-6 hours, then lost much of that adhesiveness by 24 hours. In

marked contrast, endothelial cells maintained or even showed enhanced adhesiveness for human eosinophils and basophils at 24 hours, suggesting something was causing this attachment that did not occur with neutrophils (Lamas et al. 1988). The other observation we made was from simply reading a paper describing leukocyte adhesion molecule deficiency type 1 (LAD type 1), a newly defined, rare disorder of recurrent infections, impaired neutrophil trafficking and reduced pus formation due to a congenital deficiency of $\beta 2$ integrins. In Figure 1 of the paper, a part of the caption accompanying a photomicrograph read “Sections of infected umbilical cord surgically resected at 18 days of life [from a patient with LAD type 1 displaying an] inflammatory infiltrate of adjacent connective tissue...totally devoid of neutrophils but [containing] eosinophils in addition to macrophages, lymphocytes, and plasma cells” (Anderson et al. 1985). So clearly there were pathways for cell adhesion used by eosinophils and other cells that neutrophils, devoid of $\beta 2$ integrins, could not use. Within a short time, and the result of fruitful collaborations with William Luscinskas, Michael Gimbrone, Michael Briskin and Walter Newman, several papers followed that identified the integrins $\alpha 4\beta 1$ integrin (VLA-4) $\alpha 4\beta 7$ (LPAM-1), and their respective ligands VCAM-1 and MADCAM-1, in directing the trafficking of eosinophils and basophils, but not neutrophils (Bochner et al. 1995; Bochner et al. 1991; Bochner et al. 1994; Bochner et al. 1996).

Raising my awareness of glycobiology

My first serious foray into glycobiology began soon after one particular publication of mine in 1994 in the Journal of Immunology reporting that human eosinophils, unlike neutrophils, did not interact very well with E-selectin (Bochner et al.

1994), later confirmed under conditions of flow (Sriramarao et al. 1996). I received a phone call from Ronald Schnaar, a previously unknown (to me, that is) faculty member and glycobiologist in the Department of Pharmacology (and coincidentally, son-in-law to the late Saul Roseman mentioned above) located about 10 minutes away on the main Johns Hopkins Hospital campus. We soon arranged to meet at his office, and immediately began discussing something called “glycans” on various human leukocytes and ways to study their biochemistry, structure and function. Yes I had heard that something called sialyl Lewis X has been discovered as a ligand for E-selectin (Phillips et al. 1990), but I had not stopped to consider what that might mean. But our conversations turned on a light bulb in my brain, and I was hooked. Within months, Ron and I soon wrote our first grant together, receiving R01 funding to isolate, identify and characterize natural E-selectin ligands from human neutrophils. The work was challenging, but ultimately identified cell surface glycolipids carrying E-selectin ligands (Nimrichter et al. 2008). Around this time, Ron kindly introduced me to other glycobiologists, including Sen Hakomori, John Magnani and others at a Gordon Conference in the 1990’s in Ventura, California. I soon learned that companies were exploring ways to leverage glycobiology for pharmacologic purposes. Some even provided us with compounds for in vitro testing (Davenpeck et al. 2000a; Kim et al. 1998). Indeed, a pan-selectin antagonist is undergoing clinical trials for the treatment of acute sickle cell crisis (Wun et al. 2014).

Unexpected expedition into siglecs

Until 1999, I had never heard of siglecs (Crocker et al. 1998), or any of its prior nomenclature incarnations such as sialoadhesins. Instead, I had heard of I-type lectins (Powell et al. 1995), not because I studied them, but because I had studied C-type lectins in the form of selectins. By that time, my clinical interests extended beyond allergic diseases to include eosinophil-associated disorders, and I began to search for novel therapeutic targets on eosinophils. This opportunity arose in part because of an ongoing collaboration with our Allergy division and scientists at SmithKline Beecham. We had an IRB approved protocol to provide samples for further analysis. In fact, Siglec-8 was originally discovered using a human eosinophil cDNA library created from a patient of mine with hypereosinophilic syndrome (initially presenting with 97% peripheral blood eosinophils and splenomegaly) by employing high-throughput sequencing of expressed sequence tags. Using this approach, Kristy Kikly and colleagues at SmithKline Beecham (now GlaxoSmithKline) together with other scientists at Human Genome Sciences, identified a novel mRNA sequence encoding a putative protein whose predicted extracellular domain had high homology to Siglec-7 (68%), CD33 (49%), and Siglec-5 (42%). We called this protein SAF-2 (for sialoadhesin family member 2) and the work made the cover of the *Journal of Allergy and Clinical Immunology* (Kikly et al. 2000). However, the lab of Paul Crocker, using the same starting eosinophil material (ironically, in collaboration with others working at Human Genome Sciences, but fortunately not an impediment to many future collaborations between the Crocker and Bochner labs for which I am quite grateful) also published on this protein and correctly named it Siglec-8 (Floyd et al. 2000).

One thing that was odd about the original description of Siglec-8 was that it had an uncharacteristically short cytoplasmic domain devoid of any known intracellular signaling motifs. Subsequent investigations led to the discovery of an alternatively spliced form, initially called the “long form” but now known to be the predominant form, in which there are two putative tyrosine-based inhibitory motifs in the cytoplasmic domain. Indeed, both forms were present in eosinophils from our original hypereosinophilic patient and most other donors (Aizawa et al. 2002; Foussias et al. 2000). When antibodies to the extracellular region of Siglec-8 were generated, prominent and selective expression of Siglec-8 on human eosinophils was confirmed (Floyd et al. 2000; Kikly et al. 2000), but surprisingly, Siglec-8 was also found to be expressed on human mast cells and to a weak degree on human basophils (Figure 1) (Kikly et al. 2000). This work led to the development of intellectual property around Siglec-8 and antibodies to Siglec-8 that reside at Johns Hopkins.

Most of my lab’s efforts since the discovery of Siglec-8 have focused on its biology, work made possible by grants from NIAID, NHLBI and the Dana Foundation and by access to a range of reagents including monoclonals and Ig fusion proteins involving Siglec-8 that Kristy Kikly provided. Using these precious reagents, a key initial finding was that Siglec-8 ligation, using an antibody since ligands were unknown, unexpectedly caused eosinophils to undergo cell death, especially under conditions when the eosinophils were activated and more likely to show enhanced, rather than reduced, survival (Na et al. 2012; Nutku et al. 2003; Nutku et al. 2005; Nutku-Bilir et al. 2008), findings that were confirmed in other studies (Kano et al. 2013; von Gunten et al. 2007). We also explored Siglec-8 function on human mast cells, where ligation inhibited high

affinity IgE receptor-mediated degranulation but did not affect survival (Yokoi et al. 2008). Again with Ron Schnaar's nudge, I began attending the annual meeting of the Society for Glycobiology, and learned about a new initiative, funded by NIGMS and lead by Jim Paulson at the Scripps Research Institute, called the Consortium for Functional Glycomics. Among the many amazing new efforts, reagents, mice and assays supported by the Consortium

(<http://www.functionalglycomics.org/glycomics/publicdata/primaryscreen.jsp>) was the availability of glycan arrays. Ron and I were fortunate to be among the first to get approval to submit Siglec-8 Ig fusion protein samples for ligand binding analysis, and soon reported a candidate ligand for Siglec-8, namely 6'-sulfated sialyl Lewis X (Figure 2) (Bochner et al. 2005), a structure reminiscent of the E-selectin ligand I had studied a decade ago. This finding led to additional studies with precious reagents provided by Nicolai Bovin and Paul Crocker in which we observed that a polyacrylamide polymer decorated with 6'-sulfated sialyl Lewis X not only selectively bound to eosinophils in human whole blood, but also induced apoptosis of cytokine-primed eosinophils in a Siglec-8 dependent manner (Hudson et al. 2009).

Once human Siglec-8 was discovered, the search was on for its counterpart in other species such as the mouse. While there was no true ortholog in mice, its closest functional paralog was found to be Siglec-F, in part because it was expressed on eosinophils but also because it too recognized 6'-sulfo-sialyl Lewis X (Aizawa et al. 2003; Tateno et al. 2005). This then led to a flurry of studies targeting Siglec-F in vivo by Ajit Varki, David Broide and others showing that mice deficient in Siglec-F had exaggerated eosinophilic inflammatory responses (Cho et al. 2010; Zhang et al. 2007),

while administration of anti-Siglec-F antibody reduced eosinophilic inflammation in models of lung or gastrointestinal inflammation (Rubinstein et al. 2011; Song et al. 2009a; Song et al. 2009b) or in models of hypereosinophilia, work done in collaboration with Nives Zimmermann at Cincinnati Children's Hospital (Zimmermann et al. 2008). Interestingly, the magnitude of apoptosis achieved via Siglec-F engagement was nowhere near the almost total cell death seen with Siglec-8 engagement, even with cytokine priming. Death via Siglec-F was purely caspase dependent with no role for SHP-1, NADPH oxidase or reactive oxygen (Mao et al. 2013), while for Siglec-8 cytokine-primed cells died via MAPK, ROS and mitochondrial pathways (Kano et al. 2013; Na et al. 2012; Nutku et al. 2003; Nutku et al. 2005; Nutku-Bilir et al. 2008; von Gunten et al. 2007).

With expanding interests in siglecs and glycobiology, I increased the participation of my lab's trainees at the annual Glycobiology Society meetings, where they presented our latest work. At these meetings, new collaborations were established, including contributing human cells to Stuart Haslam's lab for comparative glycomic analyses of human eosinophils, basophils, and mast cells (North et al. 2012). We also pursued collaborations with Richard Cummings, David Smith and others to further exploit the glycan arrays for its ability to define carbohydrate epitopes recognized by human immunoglobulins in sera and in the form of commercial products used for immunoglobulin replacement, showing that there was more to anti-carbohydrate responses than just the IgG2 subclass (von Gunten et al. 2009); detecting an unexpected pattern of antibodies to sulfated glycan in the sera of patients with scleroderma (Grader-Beck et al. 2011); and identifying unique patterns of glycan recognition that defined

human immune antibody responses to microbes (Schneider et al. 2015). As a result of the recognition of our growing expertise in glycobiology and eosinophil cell biology, we and our collaborators were kindly asked to contribute multiple reviews on Siglec-8 function, Siglec-8 targeting as part of future strategies for treatment of eosinophil-associated diseases, and on broader aspects of glycobiology and glycoimmunology (Bochner 2009; Bochner 2015; Bochner et al. 2012; Bochner et al. 2015; Kiwamoto et al. 2013; Schnaar 2015; Shin et al. 2012; von Gunten et al. 2008; Wechsler et al. 2012).

Ongoing efforts on Siglec-8 and Siglec-F biology, ligands and function

Current experiments are exploiting and optimizing antibody and glycan targeting of Siglec-8 and Siglec-F on eosinophils and Siglec-8 on mast cells (note that mouse mast cells do not express Siglec-F), while exploring sialylated natural tissue ligands for these molecules. It was these efforts that fully integrated and indoctrinated my lab into the field of glycobiology, expanding ongoing collaborations with the Schnaar lab to include the lab of Jim Paulson, Corwin Nycholat and colleagues at the Scripps Research Institute and the lab of Mike Tiemeyer and colleagues at the University of Georgia Complex Carbohydrate Research Center as a result of being fortunate to receive the Lung Inflammatory Diseases Program of Excellence in Glycobiology (LID-PEG) program project grant funded by NHLBI (<http://lidpeg.jhmi.edu>) headed by Ron Schnaar. Studies supported by the LID-PEG and other NIH grants have shown that the α 2,3 sialyltransferase ST3Gal-III (*St3gal3* gene product) is required for constitutive Siglec-F lung ligand synthesis (Guo et al. 2011) thanks to access to deficient mice from Jamey Marth's lab at UC Santa Barbara. We and others subsequently found that after allergen

sensitization and challenge, *St3gal3*^{+/-} and *St3gal3*^{-/-} mice have more intense allergic eosinophilic airway inflammation and less sialylated Siglec-F ligands in their airways, and that the most likely explanation for these findings is that levels of sialylated airway ligands for Siglec-F were diminished in these mice, resulting in a reduction in a natural pro-apoptotic pathway for controlling airway eosinophilia (Kiwamoto et al. 2014; Suzukawa et al. 2013).

Siglec-F recognizes the same sialylated, sulfated glycans as Siglec-8 in vitro, but the identities of endogenous sialoside ligands and their glycoprotein carriers in vivo were unknown. Lungs from normal and mucin-deficient mice, as well as mouse tracheal epithelial cells, were investigated for the expression of Siglec-F ligands. We detected mouse lung glycoproteins that bound to Siglec-F, and binding was sialic acid dependent. Proteomic analysis of Siglec-F binding material identified Muc5b and Muc4, and analysis of lungs from mucin-deficient mice confirmed the identity of Muc5b as one glycoprotein ligand for Siglec-F. Purified mucin preparations carried sialylated and sulfated glycans bound to eosinophils and induced their death in vitro. Mice conditionally deficient in Muc5b display exaggerated eosinophilic inflammation in response to intratracheal installation of IL-13. These data identified a previously unrecognized endogenous anti-inflammatory property of airway mucins by which their glycans controlled lung eosinophilia through engagement of Siglec-F (Kiwamoto et al. 2015) (Figure 3).

Finally, as a result of a new collaboration between Bob Schleimer and his colleagues here at Northwestern, and the Schnaar lab at Johns Hopkins, one additional paper was published (Jia et al. 2015) showing that in normal human turbinates from the upper airway, Siglec-8 ligands were prominently expressed in submucosal gland

epithelial cells, probably by serous cells. No expression of Siglec-8 ligand was detected in mucous cells. All of the staining was eliminated when the tissue was treated with sialidase, suggesting that it was due to sialic acid moieties in the tissue sites. Evaluation of turbinate tissue sections from patients with chronic rhinosinusitis revealed a marked increase in the level of staining for Siglec-8 ligand, suggesting that inflammatory cells or mediators involved in the disease have activated the submucosal gland cells to increase their expression of the ligands. What carries Siglec-8 ligand is as yet unknown, and is the focus of ongoing studies. While Muc5b is a ligand for Siglec-F, based on studies of upper airway human samples, MUC5B is a ligand for Siglec-9, not Siglec-8 (Jia et al. 2015), so it will be interesting to explore these differences. Indeed, we now realize that Siglec-F can recognize sialylated tri- and tetra-antennary bisected glycans while Siglec-8 does not (Kiwamoto et al. 2015).

Future prospects and concluding remarks

Where is my lab headed in this field? Our latest studies presented at the 2015 Society for Glycobiology meeting involve analysis of Siglec-8 signaling (Janevska et al. 2015) and Siglec-8 internalization (O'Sullivan et al. 2015), the latter providing the framework for therapeutic targeting of Siglec-8-expressing cells. With the skills of Zhou Zhu and colleagues at Yale, we now have access to Siglec-8 transgenic mice and will soon complete their initial characterization before employing them in models of eosinophil and mast cell associated diseases. Since these diseases include hypereosinophilic syndromes, eosinophilic gastrointestinal disorders, asthma, urticaria, atopic dermatitis, food allergy, eosinophilic leukemia, systemic mastocytosis and others, our goal is to facilitate the

development of Siglec-8 directed therapies for such disorders. Efforts include the development of forms of anti-Siglec-8 antibodies that will induce inhibitory Siglec-8 signals in these cells, resulting in their apoptosis or a reduction in mediator release. Other approaches could involve depletion of such cells, delivery of toxic or inhibitory payloads via nanoparticles decorated with Siglec-8 ligands, or enhancement of in vivo and in situ endogenous ligands for Siglec-8, as is the focus of our ongoing work supported by grants AI72265 and HL107151 from the National Institutes of Health.

I hope this review has clearly emphasized that the intellect, openness and power of collaboration among those in the field of glycobiology is tremendous, and has hugely benefitted my clinical practice and my own basic and translational research. I also want to acknowledge the incredible impact provided by the 20 or so trainees, fellows, postdocs and visiting scientists whose work I have cited, several of whom were directly supported by the LID-PEG, and to whom I owe a enormous debt of gratitude for their insight, skills, and dedication, and I hope and anticipate that they will continue the trend of incorporating glycobiology and glycoimmunology into their scientific endeavors.

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Figure legends

Figure 1. Expression of SAF-2 [Siglec-8, as detected by monoclonal antibody 2C4] 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. Reproduced from (Kikly et al. 2000) with permission.

Figure 2. Results from the Siglec-8-Ig glycan binding array analysis. A total of 172 glycans were screened for binding along with positive and negative controls, as described under “Experimental Procedures.” Error bars represent mean $\pm$ S.D. replicate determinations from a single experiment. Note that ligand 181 (6'-sulfo-sLex) stands out among all the others for binding activity. RFU, relative fluorescence units. Reproduced from (Bochner et al. 2005) with permission.

Figure 3. Too many eosinophils in the airway can worsen asthma (A). Murine studies by Kiwamoto et al (Kiwamoto et al. 2015) found that specific sialoglycans on the mucus protein Muc5b, normally found in the airway, engage Siglec-F and cause eosinophil death (B). This represents a potential pathway but which endogenous airway mucins, via specific glycosylation patterns, can influence innate immune responses. Illustration by Jacqueline Schaffer.





