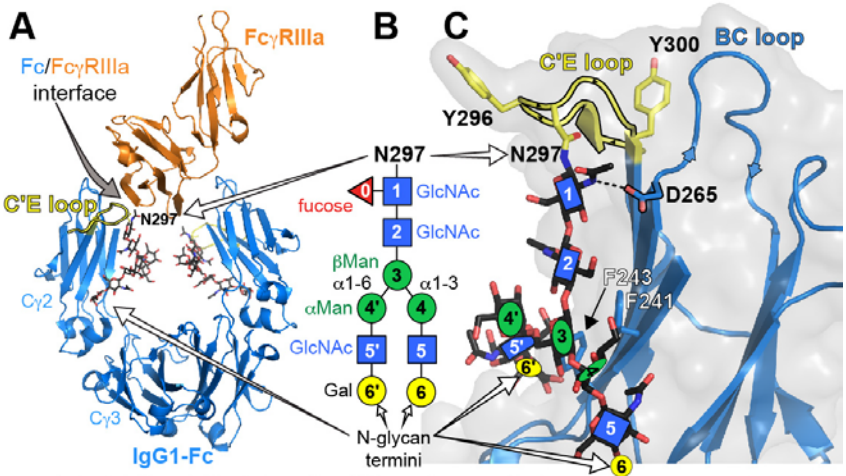


The IgG1:FcγRIIIa interaction (A) requires an N-glycan (shown as a cartoon in B). The N-glycan stabilizes the IgG Fc structure through intramolecular polypeptide/glycan interactions (C). Our laboratory has determined how this carbohydrate modification affects Fc structure.

I. Human antibody/receptor interactions

We made contributions in the area of immunology by describing critical structural features of immunoglobulins (antibodies) and identifying future areas for the improvement of disease treatments. *Therapeutic monoclonal antibodies* (mAbs) are the fastest growing class of new FDA-approved drugs that treat numerous cancers, autoimmune diseases, and transplant rejection; however, potentially grievous side effects that limit safety could be avoided by rational mAb design. Advances from our laboratory indicate mAb affinity for cell surface receptors can be increased; it is well known that increases in mAb affinity correlate with increased patient outcome. mAbs are expected to dominate the future of drug development due to their capabilities; however, mAbs are complex macromolecules and many details regarding how these drugs function remain undefined. Our primary objective is to answer basic questions about how mAbs, and thus a component of the human immune system, function to identify areas of the drug to target for improved efficacy and tolerance. The potential impact of this research is wide reaching.

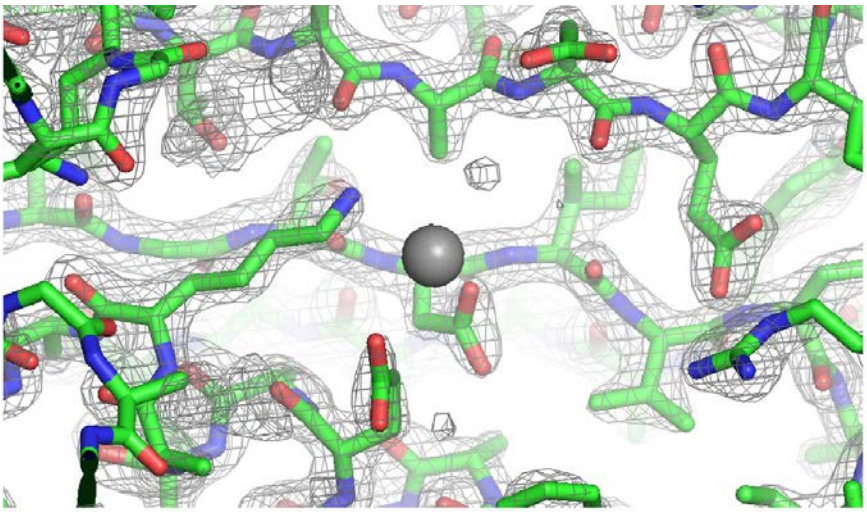
We strive to determine how a conserved and necessary post-translational modification of immunoglobulins modulates the immune response. The composition of a carbohydrate chain (an *N-glycan*) attached to the asparagine297 residue of the IgG Fragment crystallizable (Fc) influences the strength of the body's immune response. Shorter forms of this N-glycan are prevalent in patients with autoimmune disorders, notably rheumatoid arthritis, though it is unclear why. These same glycan forms also perturb the Fc – Fc γ receptor (FcγR) interaction, though when our work in this area began, a biophysical mechanism linking the Fc glycan and immune activation had not yet been described. Forty years ago, a structure of IgG1 Fc was solved by x-ray crystallography (Huber *et al*. Nature 1976). Over the intervening years many similar structures have been determined, but all of these fail to offer a testable hypothesis that addresses why the Fc N-glycan composition is important. In particular, a structure of Fc in complex with the Fc γ receptor IIIa (FcγRIIIa), a major inflammatory receptor, showed no direct contact between the Fc N-glycan and the FcγRIIIa polypeptide (Sondermann *et al.* Nature 2000).

A breakthrough came in 2011 when my mentor and I published a stunning observation: though the Fc N-glycans appeared immobilized by contacts with the Fc polypeptide in images determined using x-ray crystallography, under physiologically-relevant conditions the N-glycan termini exhibit significant motion (Barb and Prestegard, Nature Chemical Biology).

As a new laboratory we tested whether Fc:FcγRIIIa affinity is modulated through motion of the Fc N-glycan, and how the composition of the Fc N-glycan directly affects motion. We determined that Fc with specific amino acid changes (mutations) at the interface between the N-glycan and polypeptide residues, distinct from the Fc/FcγRIIIa interface, showed greater N-glycan mobility (Subedi, Hanson and Barb, Structure 2014). This mobility directly correlated with FcγRIIIa affinity. Thus, as the Fc N-glycan experienced greater motion, the Fc affinity for FcγRIIIa decreased with a linear relationship. This provided direct evidence that the three-dimensional conformation of the N-glycan contributes to Fc function through an allosteric mechanism. Besides proving a novel role for N-glycans in biology, we published the first description linking Fc N-glycan conformation to function.

Our result linking N-glycan motion with receptor binding was fundamental, however, a question remained unanswered of *how* the conserved N-glycan affected the three dimensional structure of IgG Fc and contributed to receptor binding. One predominant hypothesis permeated the literature: The Fc N-glycan physically separates two Fc domains creating an optimal binding site for the FcγR. We found evidence to the contrary that suggested an alternative mechanism: the N-glycan affects ***localized*** Fc protein structure around the point of N-glycan attachment (asparagine297) and does not alter domain orientation. This work was published in a (Subedi and Barb, Structure 2015). As a result, we were able to directly relate the structure of the Fc to the composition of the N-glycan, and provide a powerful structure-activity link that for the first time that unambiguously links immunoglobulin structure with function.

We are continuing our work by focusing on the role of receptor N-glycosylation and an atomic-level description of the role of N-glycosylation in stabilizing the Fc polypeptide structure. Our work is pioneering and lends a high-resolution description of how glycans influence antibody structure/function and uncovers a new, potentially widespread, mechanism to control immune activation. Non-nutritive carbohydrates are found on every living cell, and the technical capability to describe structure/activity relationships is nascent.



Electron density of the holo ACP synthase from *Escherichia coli* calculated from diffraction to 1.8 Å. Our laboratory is investigating interactions between the acyl carrier protein and enzymes of the fatty acid biosynthesis pathway.

II. A biochemistry approach towards generating biorenewable chemicals

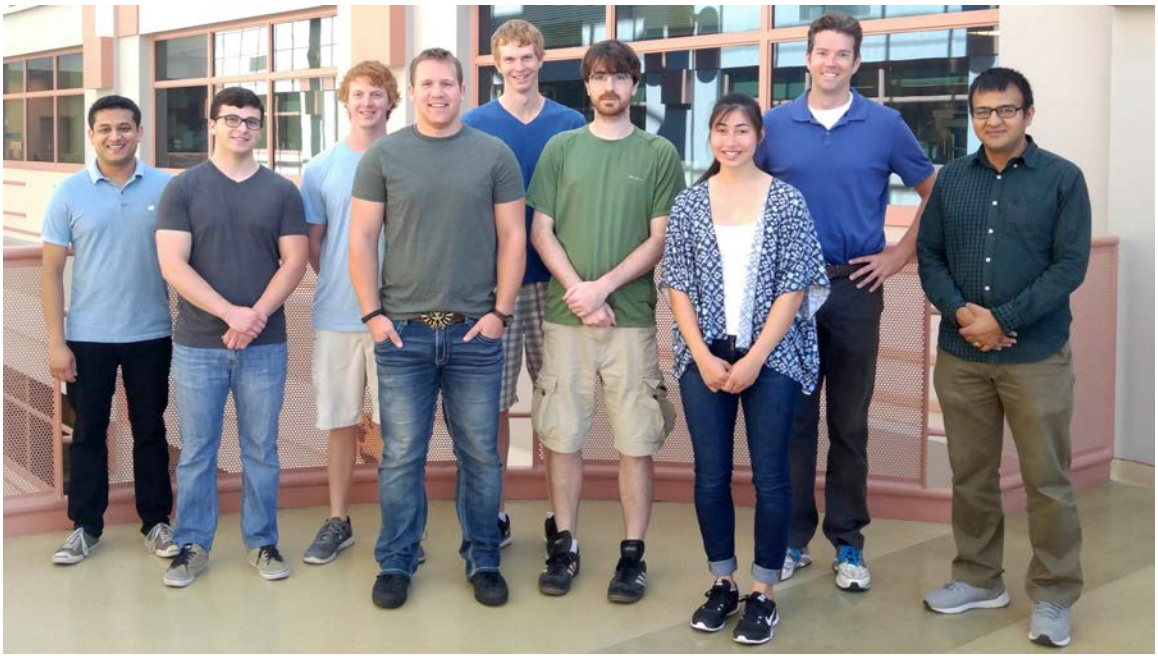
The goal of this project is to generate engineered biocatalysts and develop synthetic metabolic pathways that are based on bacterial fatty acid biosynthesis but operate in parallel. This approach will permit the production of novel biorenewable chemicals in industrial fermentation applications. The parallel pathways will be used to produce novel compounds from endogenous pools of acyl-CoAs (a carbon-donating metabolite). The unique aspect of this project is that like many polyketide metabolic pathways, compounds will be built upon an acyl-carrier-protein (ACP) scaffold and enzymatically hydrolyzed to generate a free carboxylic acid. We are currently exploring the engineering potential of three *E. coli* fatty acid biosynthesis enzymes (holo-ACP synthase, FabD, and FabH). In so doing, we will develop a defined set of rules for modifying these enzymes that will be predictive and permit custom chemical production based upon this theoretical platform.

The primary achievements towards our goal of developing an engineered and parallel synthetic pathway include advances in protein preparation, structure determination and assay development. We established methods to prepare pure holo-, acetyl- and malonyl-ACP that are published in the journal Protein Expression and Purification (Marcella, Jing and Barb, 2015). We were able to efficiently prepare high yields of pure forms of these proteins and use them as reagents for assays with the FabD and FabH enzymes mentioned above. This material also allowed us to explore designs of the FabD enzyme that exhibited dramatically broader substrate recognition than the wild-type enzyme and represents a crucial step to designing our novel parallel pathways.

An additional barrier to designing a parallel metabolic pathway has been a lack of high-resolution information about our enyzymes targets. We are addressing this by x-ray crystallography studies of the holo-ACP synthase (in our lab) and FabH enzymes (primarily in the Honzatko lab). We have had remarkable results with the holo-ACP synthase and have a high-resolution model of the enzyme at 1.8Å resolution and a diffraction map, collected in the end of February to 3.0Å, that contains reflections from the holo-ACP synthase in complex with its ACP substrate.

Our efforts to perform a precise functional characterization were limited by assays reported in the literature. Many of these relied upon radioactive nuclei and were not high-throughput. We developed a fluorescence-based assay to detect CoA formation in a FabD reaction with malonyl-CoA and holo-ACP as substrates. Currently, we are measuring the K_M/k_{cat} values for the wild type and designed enzymes with a range of synthetic CoAs produced in the lab. These values will represent a quantitative analysis of the effect of the FabD mutations on substrate specificity. The high throughput assay as proven to be robust, sensitive, flexible and adaptable to other enzymes. We characterized the activity of the *E. coli* FabH enzyme with the new assay and developed a platform to screen engineered FabH variants.

Lab members (6/2016):



Ganesh P. Subedi, MS PhD (post doc)
Aaron M. Marcella (PhD candidate)
Daniel Falconer (PhD student)
Jacob Roberts (PhD student)
Kashyap Patel (PhD student)
Mark Larson (lab technician)
Damien Cano (undergrad)
Holly Senebandith (summer REU undergrad)