Specific Aims

Antibodies are crucial regulators of the immune response and particularly versatile therapeutic agents due to their ability to both bind with high affinity and direct the immune system. Indeed, antibodies comprise a broad range of approved therapies across disease indications, many of which are known to rely in large part on immune effector cell response. Those of the IgG isotype interact with FcγRs on effector cells. IgGs elicit effector response through multiple cell types (e.g., macrophages, monocytes) and through multiple processes, including removal of diseased cells through antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cell-mediated phagocytosis (ADCP). Many possible design parameters—constant region composition, FcγRs, cell populations, and antigen binding properties—make precisely predicting and manipulating effector function an elusive goal.

In a recently published study, we built a model of multivalent immune complex (IC—IgG-antigen complex) binding to FcγRs and showed that it can capture and predict experimentally measured binding and effector response of different IC compositions[1]. This model also predicted anti-tumor effector response to a single antibody of defined constant region *in vivo*. *Importantly, endogenous IgG responses are known to rely on antibodies of distinct constant region composition in combination. We hypothesize that IgGs of identical antigen binding, but different isotype or glycosylation status, can show synergistic effector-elicited cell killing and that a multi-IgG Fc binding model can effectively identify these combinations.* To identify potentially synergistic combinations of Fc domains, we propose to extend our model to incorporate binding and effector response of ICs comprised of IgG combinations.

Aim 1: Validate a multivalent binding model's ability to predict FcγR binding to mixed IgG composition immune complexes. *Hypothesis: An equilibrium multivalent binding model accurately represents FcγR interaction with ICs of mixed IgG composition.*

- Measure mixed composition synthetic IC binding to cells of defined FcγR expression.
- Generalize a multivalent binding model to account for ICs of mixed composition.
- Fit and verify that the model can predict effector response *in vitro*.

Aim 2: Map human and murine IgG isotypes to one another according to conserved effector response. *Hypothesis: Murine/human effector response regulation is conserved, even when single IgG isotypes are not.*

- Map IC composition to effector responses by tensor decomposition.
- Correlate murine and human effector responses according to similar cell population response.
- Verify this mapping predicts known, similar cross-species effector cell responses.

Aim 3: Link IgG effects and *in vivo* efficacy to identify and verify synergistic IgG-elicited cell killing. *Hypothesis: A binding model can identify synergistic effector interactions in vivo.*

- Regress single IgG treatments and *in vivo* cell clearance to identify synergistic combinations.
- Verify predicted cases of synergistic effector response *in vivo* within models of antibody-dependent, effector-mediated platelet and B cell depletion.
- Identify the relevant cell populations and mechanisms of synergistic effector response.

This investigation will considerably improve our ability to both engineer IgG with optimal effector cell killing response and inform how existing therapeutic and endogenous IgGs function. In particular, even existing monoclonal antibodies are mixtures of Fc compositions due to the cocktail of glycosylation forms present. A comprehensive view of how IgG Fc interact would therefore (1) improve our ability to match effector cell-mediated killing during antibody manufacturing, (2) provide insight into the role of the complex Fc cocktails created during an endogenous immune response, and (3) create the possibility of engineering logic into effector cell responses through antibody combinations.

Significance

The therapeutic potential of antibodies is demonstrated by their status as a broad class of effective agents across autoimmune diseases, infection, and cancer. Their versatility is enabled through an antibody's selectivity toward target antigen as determined by its variable region, along with the ability to elicit effector cell responses depending upon the composition of its constant Fc region. Antibodies of the IgG type direct effector response by binding to the FcγR family of receptors. FcγR activation is driven by multiple IgG clustering the receptors. Depending upon the configuration of receptors, this interaction may promote or prevent effector response. Thus, the mechanism of FcγR activation ensures that multiple IgG are present whenever eliciting effector response.

One capability elicited by effector cells is clearance of infected or otherwise pathogenic cells. Clearance can occur through two functionally distinct mechanisms: antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cell-mediated phagocytosis (ADCP). However, both mechanisms are (1) regulated by the family of FcγRs present on effector cells, (2) modulated by the identity of the Fc region present on an IgG[2], (3) performed by multiple cell types[3,4], and (4) influenced by properties of antigen engagement[5,6]. This multilayered complexity is a central challenge to engineering antibodies with desirable cell-killing functions, as well as understanding successful and dysregulated endogenous immunity. Our team recently demonstrated that a multivalent binding model of immune complex (IC—IgG-antigen complex) binding to FcγRs accurately captured and could predict *in vitro* binding across various IgG isotypes[1]. Further, it could accurately predict antibody-elicited tumor cell killing *in vivo* across antibodies of varied isotype, glycosylation status, and FcγR knockout animals[1]. Directly quantifying and predicting cell clearance made it possible to accurately predict and optimize for antibody-mediated cell clearance regardless of whether it occurred by ADCC or ADCP.

Endogenous antibody responses universally involve Fc of diverse isotype and glycosylation in combination. *The central hypothesis of our proposal is that antibodies of different Fc composition, but identical antigen binding, can have properties other than the additive combination of either alone.* A consequence of this is that, within a mixture, minor species (e.g., glycosylation variant) can have an outsized effect promoting or preventing cell killing. Even when recombinantly manufacturing a single monoclonal therapeutic agent, heterogeneity exists in the glycosylation forms derived[7,8]. Knowledge of how these different forms influence the behavior of one another would allow one to increase or reduce cell killing by adjusting the mixture of glycosylation forms. This would also help guide evaluation of biosimilars by determining whether glycosylation forms present at small fractions might influence overall therapeutic efficacy. On the side of the effector cells involved in mediating therapeutic antibody dependent effects, it has become clear that in addition to NK cells (expressing only one activating FcγR (FcγRIIIA), tissue resident macrophages and bone marrow derived monocytes participate in cytotoxic antibody dependent target cell clearance. In contrast to NK cells, these myeloid cell subsets express all activating (excepting inflammatory monocytes lacking human (h)FcγRIIIA or mouse (m)FcγRIV) and the inhibitory FcγRIIB. Thus, mixed IC may trigger all or specific subsets of activating/inhibitory FcRs, resulting in a further complexity. Despite the ability of multiple activating FcRs on myeloid effector cells our previous studies have demonstrated that individual IgG subclasses, such as mIgG2a/2c for example, may mediate their activity through select activating FcγRs despite their capacity to bind to other activating FcRs[2].

This work will only become more critical with recent advancements in our ability to experimentally characterize polyclonal IgG mixtures in ever finer resolution by making sense of this veritable data deluge[9,10]. Mapping interactions in effector response between pairs of antibodies will provide an essential first step toward more complex mixtures of Fc domains, and then integrating this information with variation in antigen binding. Thus, there is great potential to integrate this undertaking with other IgG engineering and antibody/effector cell characterization efforts.

Innovation

This research is a convergence of immunology, data analysis, biophysics, simulation, and experiment to develop a more predictive, mechanism-based, and quantitative picture of IgG-mediated cell killing. Each of these areas is absolutely critical in combination to ensure the success of this proposal. In response to an antigen, our body creates a cocktail of antibodies of diverse class, glycosylation, and

antigen binding[9,10]. The biophysical properties of these molecules have been extensively characterized but almost always on a component-by-component basis[11]. The subsequent combinatorial complexity that arises ensures that the whole is more than the sum of its parts.

Modeling innovation to calculate and visualize mixed IgG-FcγR binding Innovative modeling and analytical methods herein address key challenges in computationally predicting and visualizing IgG-FcγR binding. Branching processes provide an elegant analytical approach for overcoming high degrees of combinatorial complexity to calculate overall binding state. These have been applied successfully to study aggregation phenomena such as antibody-antigen binding and polymer networks where there are analogous calculation challenges[12,13]. A second key challenge will be interpreting the high-dimensional space of possible IgG treatment combinations and response across cell populations. Tensor factorization provides an efficient and parsimonious representation of high-dimensional space and indeed has accepted use within the machine learning community for other problems of capturing high-dimensional relationships such as topic modeling[14,15].

Builds upon earlier theory on Fc receptor activation Theoretical models have helped to understand Fc receptor activation, but critical gaps still exist in their application, especially when designing IgG therapies. Multivalent ligand/monovalent receptor binding models successfully represent activation of receptors such as FcεRI with similar binding configurations[16–21]. However, most cells express members of the FcγR family simultaneously in combination, meaning any manipulation of IC composition will necessarily have multivariate effects. Thus, while the underlying multivalent binding theory is long-standing, FcγR-IgG interactions are especially suited for developments in inference approaches to rigorously link these models to experimental observations and to visualize high-dimensional data[22]. Multivalent binding theory will be a critical companion to experiments mapping effector function; the baffling number of potential combinations preclude purely experimental searches or intuition from revealing precise answers[9]. For example, even considering 30 glycosylation variants, 4 IgG isotypes, pairs of two IgGs at 4 concentrations, and 5 antigen targets of varying valency, one is left with 9,600 design possibilities[9,10].

The model used here is in essence a minimal pharmacologic model of IgG effector cell-elicited responses. By leaving out all but the most essential components, elegant pharmacologic models (e.g., competitive inhibition, additive interaction, etc) form the basis of analyzing compound effects from the most initial development stages through clinical evaluation[23,24]. Foundational models of effector-elicited responses will similarly allow for IgG therapies to be more rigorously engineered and evaluated.

IgG-mediated logic Identifying antibody constant regions with synergistic or antagonistic cell killing holds promise for more than just enhanced overall effector response. For example, a highly synergistic combination essentially provides AND logic between target antigens. Two constant regions that only lead to effector function in combination could help target cells for which a reliably specific antigen does not exist. In other words, if tumor cells are only unique in their expression of protein A and B, an anti-A/anti-B antibody combination would only signal for effector cell-elicited killing when both antigens are found in combination. This capability is similarly being pursued with chimeric antigen receptor cellular therapies in cancer, due to lack of completely specific tumor antigens, particularly in solid tumors[25,26]. By comparison, IgG-mediated logic would have significant benefits in cost, reliability, and likely toxicity as compared to cellular therapies. Other forms of logic may also be helpful and revealed by the approach here, such as A but not B to protect bystander antigen-expressing cells from an existing treatment. Therefore, synergistic constant region combinations hold promise both for enhancing the potency and avoiding side effects of therapeutic antibodies.

Methods development relevant to other receptor-ligand families Finally, the innovative methods here have immediate application in other areas of therapeutic engineering. Other immunotherapy and targeted therapy targets, such as the common γ-chain cytokines, FGF receptor tyrosine kinases, VEGF receptor tyrosine kinases, and bone morphogenic proteins, involve many ligands, large receptor families, and are expressed across many cell types[27–31]. The approach developed here—an activation model, parameterized through inference, and then mapped through tensor factorization—has immediate application in understanding the function of these other receptor families, learning how they respond to combinations of cues in the extracellular environment, and targeting their dysregulation.

Approach

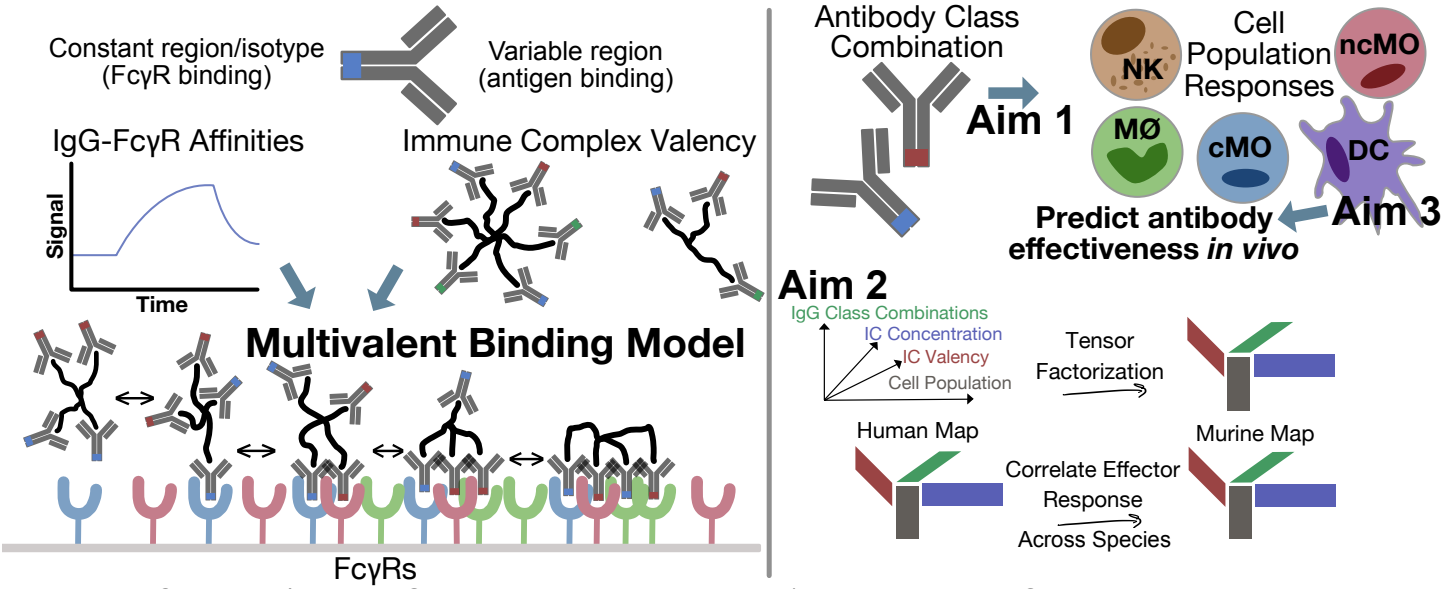

Figure 1: *Overview (also see Gantt chart at end of proposal).* Experimental IgG-FcγR binding measurements will be fit to a multivalent binding model extended to incorporate immune complexes with IgGs of mixed isotype and glycosylation. Binding and effector response predictions for these mixed isotype immune complexes will be tested *in vitro*. Using the computational model, we will then identify IgG combinations predicted to have synergistic effector-mediated killing and test on cell populations *in vitro* and in two *in vivo* models of platelet and B cell depletion. Using the model, we will also build a map of murine-human homology according to effector response.

Aim 1: Validate a multivalent binding model's ability to predict FcγR binding to mixed IgG composition immune complexes

Rationale FcγR activation and effector response occurs through multivalent immune complex (IC) binding and consequent receptor clustering. This aim will ensure that we are able to accurately model the binding and *in vitro* FcγR-dependent effector response of a cell population with defined FcγR expression, given we know the composition of an IC. At the same time, it will provide a helpful data compendium for examining the different factors that influence IC binding.

1.1. Measure mixed composition synthetic IC binding to cells of defined FcγR expression To start, we will utilize a panel of previously-generated CHO cell lines that express each human FcγRIA, FcγRIIA-131H, FcγRIIA-131R, FcγRIIIA-158V, FcγRIIIA-158F, or FcγRIIB individually (fig. 2)[1,32]. To ensure quantitative binding measurements, we will quantify receptor abundance in each cell line. These measurements are performed using by staining with FITC-coupled Abs directed against FcγRIA (CD64; clone 10.1; BD Pharmingen), FcgRIIA/IIB (CD32; clone 3D3; BD Pharmingen), and FcγRIIIA (CD16; clone 3G8; BD Pharmingen). Absolute quantitation is obtained by comparison to a panel of beads with defined numbers of antibody binding sites in each experiment. Cell lines with multi-modal distributions of receptor expression or variance greater than 50% of the expression level will be sorted again for more precise expression.

We will assemble ICs using TNP(2,4,6-trinitrophenyl)-conjugated BSA at valencies of 4 and 26. Anti-TNP antibodies of each IgG isotype will be bound to the TNP-BSA. Instead of creating IgG-TNP-BSA complexes of one isotype as we performed previously[1,32], we will use all pairs of 5:1 and 1:1 hIgG isotype mixtures, in duplicate. Binding will be quantified on cells using a PE-conjugated goat anti-human IgG F(ab')2. With four IgGs, six hFcγRs, and replicates, this corresponds to 528 independent binding measurements with which to ensure our model captures multi-IgG binding.

We anticipate that this data will show striking variation in the amount of cell binding, depending upon the valency of IC, affinity of each IgG used, ratio of IgGs present, and receptor expressed by the cell. In contrast to our earlier work, in this case there are two interaction affinities present, of an IgG present at higher and lower abundance. We expect this data will reveal that both affinities influence

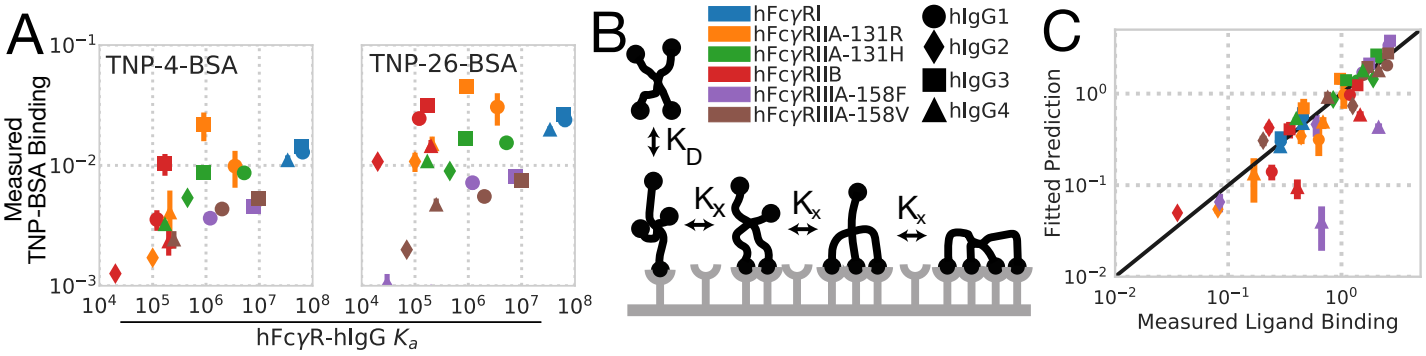

Figure 2: *A two-step model captures IgG immune complex binding.* A) Measurements of IC binding to cells expressing a single FcγR. Error bars indicate standard error of triplicate measurements. B) Diagram of binding model. An initial monovalent interaction then leads to multivalent interaction with partition coefficient K_x. C) Fit versus actual binding measurement across all IgG/FcγR pairs. Note that the most divergent pair—hFcγRIIIA-158F/hIgG4—was determined to be based on an unreliable affinity measurement (the equilibrium and kinetic binding assays in the source reference do not match for this case)[33].

binding, which we can inspect by plotting matched cases wherein one IgG identity is held constant and the other varies. Additionally, we expect that the relative abundance of each IgG will matter, which we can inspect by plotting matched pairs where the only difference is the ratio between each IgG.

In addition to the isotype itself, the sugar moiety attached to the N297 residue in each individual IgG heavy chain can alter FcγR binding. Although several hundred IgG glycovariants may exist, the most striking effect in altered binding of an IgG glycovariant to FcγRs has been observed for fucosylated/afucosylated IgG variants, which bind with altered affinity to hFcγRIIIA[2,32,34]. These IgG glycovariants can be generated via antibody production in cell lines deficient in the fucosyltransferase gene (LEC 13 CHO cells). To study how the abundance of certain IgG glycoforms affects FcγR binding, we will use pairs of 10:1, 3:1, and 1:1 hIgG1 glycovariant mixtures and study binding to hFcγRIIIA-expressing cell lines like above in triplicate. Depending on the outcome of these experiments we plan to extend these studies to other human IgG isotypes. Of special interest are hIgG2 and hIgG4, which are considered low FcγR binders, yet it is unclear how the presence of different amounts of afucosylated glycovariants affects their functional activity. This set of data will be instrumental in assessing how the abundance of individual glycoforms in a mixture affects FcγR binding and effector functions (e.g., how much this can be allowed to change during manufacturing).

1.2. Generalize a multivalent binding model to account for ICs of mixed composition To model these binding data, we will extend our published model of IgG-FcγR engagement to account for IgG isotype and glycovariant mixtures[1]. Briefly, we model FcγR engagement as a two-step process, wherein an immune complex first binds to a single receptor with kinetics equal to those of monovalent binding (fig. 2B)[16–21]. Subsequent binding events are governed by a partitioning parameter (K_x). This model of multivalent engagement successfully represents other receptors with a similar binding configuration such as FcεR and TCR[18,21]. A critical extension of this model that we made when applying it to the FcγR family is extending it for multiple receptors present. In doing so, K_x is proportional to the affinity of the receptor, which is necessary for the model to follow thermodynamic laws (detailed balance)[1].

The largest hurdle to applying this model for IgG mixtures is performing the binding configuration calculation. Though the number of microstates explodes in a combinatorial manner (we have to integrate over all possible mixtures of IC binding states—e.g., an IC bound at site 1, bound at site 1 and 2, etc—weighted by their individual likelihood), modeling the probabilities of these states as a branching process ensures we can efficiently calculate the macroscopic binding we expect to observe[12]. Importantly, despite many more possible binding configurations, there is no additional parametric uncertainty relative to our published model[1]. Indeed, because of this, we do not necessarily need to fit any new parameters and can directly make predictions based on our published parameterization (fig. 2). As previously, K_x must be proportional to the affinity of an interaction to satisfy detailed balance, and so there is only one K_x^* value we need to fit ($K_x = K_x^* K_a$)[1]. We will nevertheless perform fitting to this new

data, however, in case it can provide more exact parameterization for K_x^*.

1.3. Fit and verify that the model can predict effector response *in vitro* As validation of our model, predictions of binding and effector response will be evaluated in peripheral blood mononuclear cells (PBMC). PBMCs will be separated and differentiated into individual effector cell populations and stimulated with the same IC complexes[1,32,35–37]. As before, we will assume effector response is proportional to the amount of activating receptor minus the amount of inhibitory receptor found in bound complexes of multiple receptors[1]. This construction satisfies the criteria that increases in activating or inhibitory receptor multimerization have their expected effect, and that multimerization is essential for FcγR activation[1]. Though this is the most parsimonious construction that satisfies these rules, we will keep in mind this assumption and can easily explore alternative constructions.

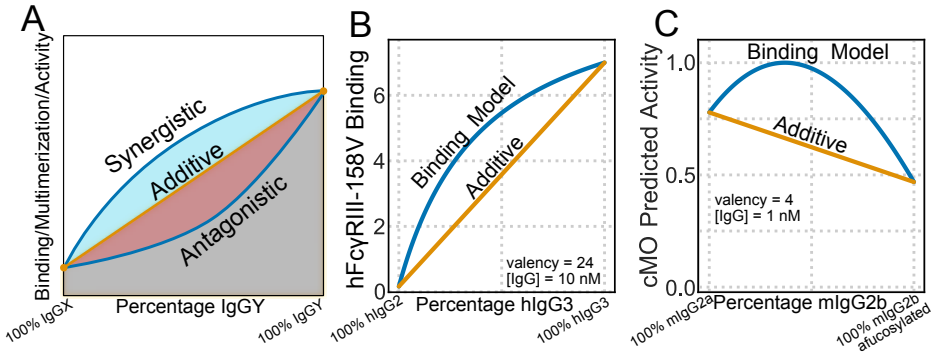

Figure 3: *Strategy for evaluating synergy, and preliminary evidence a multivalent binding model predicts non-additive interactions.* A) We expect that, when plotting a quantity from the binding model and varying the relative abundance of two IgG Fc compositions, the output quantity would have a linear relationship with mixture composition. We take deviations from this relationship to be either synergy or antagonism depending on whether they are above or below the line, respectively. This outcome will be summarized by quantifying the area under the curve for both the additive and actual case ($S = (A_{actual} - A_{additive})/A_{additive}$). B) Example of predicted synergy in IC binding. C) Example of predicted synergy in murine classical monocyte activity (receptor expression previously measured[1]).

Effector cell response will be quantified through induced cell type-specific cytokine secretion since we are stimulating with synthetic ICs (e.g., we cannot measure ADCC)[32]. Ten IC complex mixtures with large predicted variation in response, comprised of the isotypes and glycosylation forms from Aim 1.1, will be selected to test with each cell type. We will focus on monocytes, macrophages, and NK cells due to their ease of isolation and role of cytokine secretion during effector response[35]. The FcγR expression of these populations is well-characterized, but we can re-quantify this if needed using our well-established protocols[1,3,38]. We will measure a panel of >5 cytokines (including IL-6 and IFNγ) in parallel by bead-based ELISA to ensure our results are not cytokine specific and our ranking is similar across all IC-responsive cytokines within a cell type[32,35]. As different cell types might have very different sensitivities to activated FcγRs, we will test our model's predictive capacity by comparing predicted and actual *ranking* (Spearman correlation) of response strength to these 10 mixed ICs within a cell population. We expect close agreement between the IC compositions predicted to maximally induce a response in each cell population, and the responses measured. In addition to measuring cytokine secretion we will also study the phagocytosis of fluorescently labeled (FITC coupled TNP-BSA) IC. By using a combination of intracellular and extracellular FACS staining for FITC we will be able to distinguish between cell surface bound and intracellular antigen. As a second independent verification of IC phagocytosis of fluorescently labeled IC we will use cyto-spins of IC fed monocytes and macrophages and analyse intracellular IC via immunofluorescence microscopy. This work will demonstrate that a binding model can predict which effector cell populations will respond to ICs of mixed composition, helping to make sense of the vast number of possible IC compositions.

Finally, to validate use of this model for mIgG interventions in Aim 3, we will test a small subset of mixed ICs for our ability to predict relative effector response. From previous work we have both affinities and receptor abundance measurements with which to make model predictions[1]. We will select five mixtures of mIgG1/2c, with large variation in their predicted response, to test (see Aim 3.1). The same effector populations as above will be isolated from mouse spleens, and cytokine response measured by bead-based ELISA. We will test agreement of our measurements and model predictions with the same strategy as above.

Preliminary Data All of the methods used in Aim 1 are demonstrated in previous studies from our labs[1,32]. As exemplified in fig. 2, we have successfully measured binding in the panel of CHO cell lines used here and demonstrated that a multivalent binding model can account for binding of ICs comprised of single IgGs. This work successfully predicted *in vitro* and *in vivo* effector response both for human and murine ICs.

In preliminary work we have implemented the most basic components of the multi-IgG binding model to demonstrate that calculating binding with this approach is indeed feasible (fig. 3). To verify correctness we have compared this new implementation to our published model for cases of a single IgG present and see agreement. The examples of non-additive interaction also fit intuitively with cases of synergy and antagonism we expected to observe. In fig. 3B, hIgG2 has almost no binding, while hIgG3 is a high affinity interaction. Sweeping between each IgG therefore is, in effect, changing the valency of the IC, and the first few hIgG3 added to the left of the plot have the greatest effect on the avidity of the interaction. One would expect adding a second binding site to have a larger relative avidity effect than adding a sixth site, like this suggests. In total, while additional work is necessary to make this binding model implementation usable for these studies, we do not expect challenges.

Challenges & Alternative Approaches We have previously used all of the methods in Aim 1 and so do not anticipate significant challenges in these experiments. If our binding measurements do not match our modeling predictions, we will first investigate whether the discrepancy is in a subset of the measurements (e.g., those with a certain IgG), specific outliers, or across all the data. The hFcγRIIIA-158F/hIgG4 case in fig. 2 provides an example, where we identified an outlier and traced it to the underlying affinity measurements[1]. If there are discrepancies across a subset of the data, we will investigate the underlying molecular mechanism. We can also use the single IgG measurements from our published work as a guide for whether discrepancies are modeling or experimental problems.

If our measured and predicted binding is consistent but our cell response measurements do not match our predictions, this will provide an opportunity to investigate additional mechanisms of effector regulation beyond binding. For example, recent reports have implicated clustering of hFcγRI as a mechanism of inside-out signaling[39]. If we observe divergent results from what we predict, we can investigate whether mechanisms such as inside-out signaling influence the relative response to different ICs. We can use a panel of blocking antibodies targeting each FcγR to isolate the influence of each FcγR expressed within a cell population[2,38]. Importantly, these other regulatory factors can contribute to effector cell response only after a cell has interacted with an IC, and so IC binding should still be a dominant factor in effector cell response, and certainly one for which we must first account.

Aim 2: Map human and murine IgG isotypes to one another according to conserved effector response

Rationale Unclear homology between the human and murine FcγR families stymies our ability to translate findings from murine models of disease[11]. Using the overarching hypothesis that there is conserved regulation at the level of cell type-specific effector response, we will use our interaction model to build a homology map between species.

2.1. Map IC composition to effector responses by tensor decomposition An inter-species map would greatly aid translation of findings in murine models of IgG-related diseases and IgG-elicited cell killing. Assembling such a map first requires a global view of how the family is regulated. Our model of FcγR engagement, besides fit parameters common to all FcγR-IgG pairs, requires affinities for each FcγR-IgG pair and the profile of FcγR expression within a cell. As these exist for effector cell populations within both murine and human cells[1,38,40], we will use these to assemble a data compendium of the predicted effector responses across IgG combinations in each species.

All combinations of IC concentration and composition will be varied to create a data tensor of model-predicted FcγR activity within each effector cell population. We will use the same constructed activity calculation as in Aim 1, as it successfully predicts effector response in our previously published work and will be further validated in Aim 1.3. We will simulate every possible combination of cell population (eosinophils, NK cells, dendritic cells, neutrophils, classical monocytes, and non-classical monocytes based on their FcγR expression), valency (1–26), concentration (log-scaled, 1 fM–1 μM), and IgG composition (combinations of 1:1, 1:2, 1:5, and 1:10 mixtures). We will start with the FcγRIIIA-158F

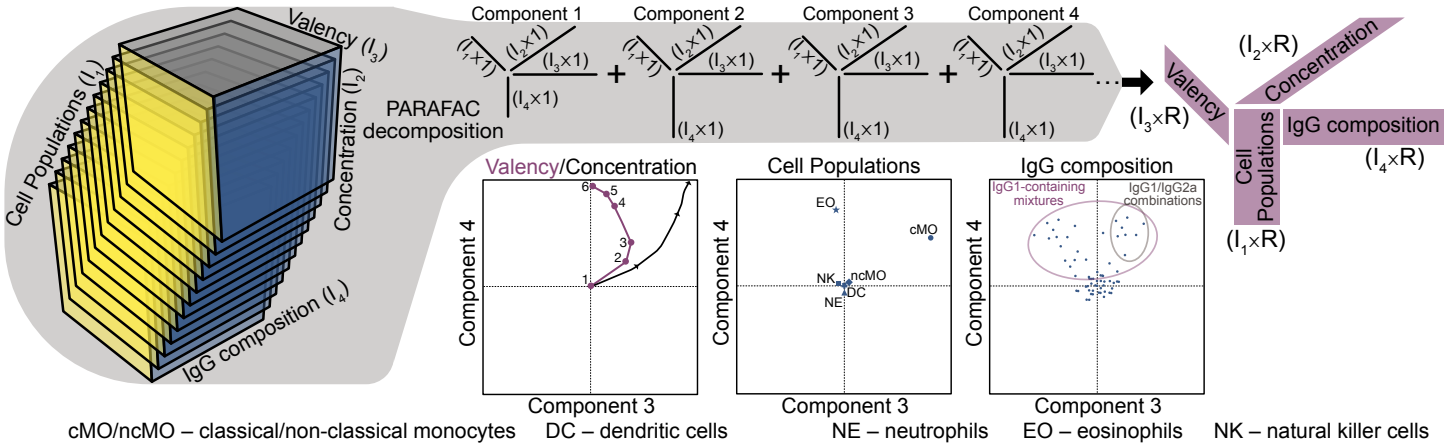

Figure 4: *Overview of tensor factorization to map effector response.* A central challenge in engineering IgG-elicited cell killing is that any intervention is pleiotropic on multiple levels. That is, an IC can have differing effects based on its IgG composition, concentration, and valency, with responses from distinct cell populations. With a binding model we can predict the outcome of any individual combination of factors but still have trouble mapping and visualizing the predominant axes of variation in these data. Through tensor factorization, this four-dimensional space can be decomposed into component factors that capture predominant axes of variation. For example, in the hypothetical factorization results shown, components three and four increase with concentration, and valency increases along component four. Component three, however, shows a bimodal valency relationship. Eosinophils and classical monocytes are activated along component four, while only the latter are activated along component three. Through the last components plot, we can see which mixtures drive movement along each component; IgG1-containing mixtures are positively associated with component four, while component three is exclusive to IgG1/IgG2a combinations. So, if we want to maximally activate eosinophils, these plots indicate we want a combination with IgG1 and higher valency. In this way, factorization provides a design schematic for variation in effector response.

and FcγRIIA-131R genotype; however, this method also provides an opportunity to look at predicted differences in regulation based on genotype in future studies. While we are starting with a selected set of effector cell populations for which we have existing FcγR abundance measurements, these results can quickly and easily be updated with new measurements (e.g., macrophages, dendritic cell subsets). These ~100,000 values for each species capture the variation in predicted effector function due to differences in binding propensity to each cell population but remain challenging to interpret due to the high-dimensional nature of the data.

We will then utilize canonical polyadic (CP) decomposition to visualize these data, a data reduction technique similar to principal component analysis in some aspects, representing the variation in effector response in a reduced dimensionality space[14]. Briefly, this method finds factors, or directions of variation in the data, and the relative contribution of each variable to that factor. The parallel plots of each factor and the variables involved are essentially a map of the variation present within the data. Importantly, with a sufficient number of factors, the data tensor can be perfectly reconstructed from the factors, and so information is preserved in the factorization process. We will determine the number of factors necessary to capture >95% of the variance in predicted response for each cell population upon reconstruction. This factorization will then be used as a "map" for regulation of the FcγR family.

2.2. Correlate murine and human effector responses according to similar cell population response We hypothesize that, while the individual FcγRs/IgGs are not directly conserved, there exists conserved regulation in the form of which cell populations are activated coordinately. That is, we can identify cross-species IC pairs targeted to have the same cell population responses. To do so, we will correlate each component of the cell population factorization between species. Identifying significant correlations here (Pearson correlation, with family-wise error rate correction through cell population randomization) will test our hypothesis that conserved regulation exists targeting the same cell populations.

As each resultant component of the factorization represents a separable subset of variation in FcγR/IgG regulation, we expect to observe one-to-one correspondence between components of the human and murine factorization. Therefore, we expect to see that each component of the cell population factorization has a single significant correlation pair (fig. 5).

2.3. Verify this mapping predicts known, similar cross-species effector cell responses Experimentally testing our mapping from Aim 2.2 would require isolation of many immune populations from both murine and human sources, along with cytokine measurements and functional characterization in both species. This scale of validation is outside the scope of this proposal but will be enabled in future studies by the present analysis. However, we will check for a few features we expect to observe: (1) hIgG1/hIgG3 and mIgG2a generally have greater effector responses and are more pro-inflammatory. We expect to identify linked components that involve mixtures of both these IgG[11]. (2) Both human and murine families have a single inhibitory receptor with similar expression patterns, hFcγRIIB/mFcγR2B, and so we expect that these will be aligned in the factorization (weighted similarly in linked components)[11]. (3) hFcγRIIIA/mFcγRIII are the sole Fc receptor on NK cells. Therefore, we expect to find linked components that represent activation of these receptors and includes NK cell response[11].

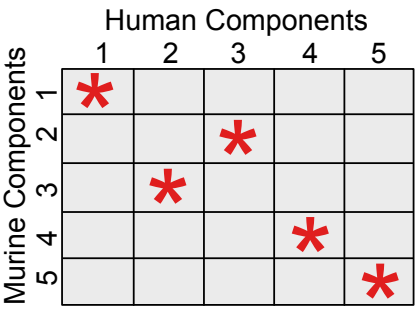

Figure 5: *Schematic for expected results of human–murine cell population correlations.* We expect to observe one-to-one links between each component of the factorization across species. However, this need not be the same component number in each species. For example, here, murine component three and human component two might be correlated due to shared weighting of classical monocytes and NK cells.

In addition to the individual component-specific relationships we expect to find above, we will test our ability to apply our homology model for "translating" between murine and human IgG compositions in Aim 3.3. This will evaluate the most translationally-valuable aspect of our results here.

Challenges & Alternative Approaches Importantly, while Aim 2 aids translation of Aim 3 and addresses a fundamental question about conservation of this receptor-ligand family, execution of both can proceed independently. As CP decomposition efficiently and parsimoniously captures variation in the original data tensor, we expect the factors identified to clearly display any correspondence between species. However, if we do not identify components with corresponding variation between species, we can take a more targeted approach. With an IC composition that leads to a certain set of predicted effector cell response in humans (e.g., NK cells, but not other cell types), we can then vary murine IC composition to look for compositions that have a matching profile in mice (or vice versa). This will still identify homology across species.

We expect CP decomposition to be the most useful and easily interpretable method for tensor factorization, given that it provides parallel components along each dimension. However, many other factorization methods exist which may have benefits depending upon the variation found in the data. For example, Tucker decomposition is a more flexible generalization of CP decomposition that allows for linking between components through a core tensor[41]. This creates a tradeoff of fewer components being necessary to explain the data, but additional challenge in visualizing the core tensor. Additionally, both CP and Tucker decomposition can be forced to have only non-negative components. By constraining the factorization in this way, this often makes the resulting components much easier to interpret (since it separates out balanced negative and positive effects)[42]. In total, there is a rich toolbox we can apply to further explore the data here to create an effective map of IgG-FcγR regulation.

Aim 3: Link IgG effects and *in vivo* efficacy to identify & verify synergistic IgG-elicited cell killing

Rationale Successfully identifying examples of synergistic effector-elicited killing will demonstrate that IgG isotypes have unique properties in combination. Moreover, it will show that a binding model can successfully identify these cases to engineer response and that this synergy can be employed successfully *in vivo*.

3.1. Regress single IgG treatments and *in vivo* cell clearance to identify synergistic combinations We will use a passive mouse model of immunothrombocytopenia (ITP) and a model of cytotoxic antibody-mediated B cell depletion (CD20 specific) as model systems for IgG-elicited cell killing[43]. These model systems have beneficial properties, including that effector cell-elicited killing (platelet or B cell depletion) can be assessed and quantified rapidly, and do not involve long-term inflammation with unknown compensatory changes. Moreover, the responsible effector cells are liver resident Kupffer cells and/or resident monocytes, which have a well-defined FcγR expression pattern[3,4].

To predict cell killing, we will use a similar regression approach to the one we recently performed for anti-tumor IgGs in a B16F10 melanoma model[1,2]. Briefly, for each intervention, the binding model predicts a level of FcγR activity for each cell population, and then those "activities" are regressed against fractional reduction in cell number (fig. 8)[1]. This regression will be performed using a $y = 1 - exp(-X \cdot p)$ relationship, where X is the matrix of activities and y is the fractional reduction in cell number. This construction corresponds to an exponential survival distribution and thus an underlying random process wherein every cell is at uniform risk of clearance. The structure of the regression portion of the model is unchanged by considering mixed composition ICs; therefore, with our updated binding model we will be able to immediately make predictions about *in vivo* response after fitting. Like with the B16F10 model, we will use a panel of pre-existing experimental results in which different IgG isotypes, glycosylation variants, and FcγR knockouts have been evaluated. A wider panel of these experiments exists for both the ITP and CD20-depletion models than the B16F10 case, in fact, which will aid exact parameterization of the model and therefore accurate predictions (e.g., fig. 6)[2,43,44]. Model prediction will be quantified through leave-one-out (LOO) and leave-one-isotype-out crossvalidation. The significance and distribution of derived quantities will be estimated by bootstrap[45].

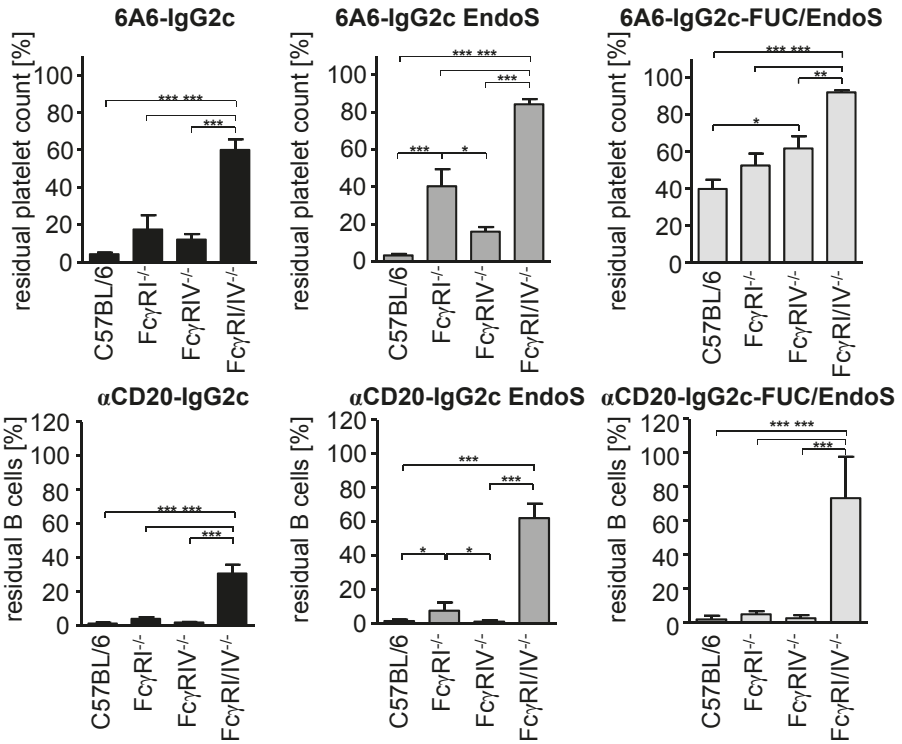

Figure 6: *Both platelet and B cell IgG-mediated depletion are FcγR-, Fc isotype-, and Fc glycosylation-dependent[43].* Quantification of platelet and B cell depletion using either 6A6 or anti-CD20 IgG, respectively.

We will identify a predicted case of synergistic interaction between mIgG isotypes and/or glycosylation variants (fig. 7) for each target cell. Synergy will be calculated according to the Bliss independence rule[23]. That is, in the absence of synergy, we will assume each antibody has an independent, proportional decrease in the number of platelets or CD20-positive cells observed. IgG combinations with the greatest predicted reduction over that from an additive effect will be selected. If these cases are widespread we will also consider the disease relevance of the glycosylation and/or isotype combination (e.g., prioritize fucose/non-fucose combinations due to their therapeutic manufacturing relevance). We also expect the combinations identified will be comprised of mIgG1 and/or mIgG2c, given the efficacy of these as single agents[2,43,44].

Note that this approach is not limited to synergy in cell killing arising through synergy in FcγR binding (e.g., fig. 3C). For example, high affinity IgGs tend to also have a higher affinity for the inhibitory hFcγRIIB/mFcγR2B. However, these higher affinity IgGs could play an outsized role on initial monovalent binding of ICs. ICs with a small number of high-affinity IgGs along with many lower affinity (but mFcγR2B non-binding) IgGs therefore might lead to greater activation than ICs with either IgG alone.

3.2. Verify predicted cases of synergistic effector response *in vivo* within models of antibody-dependent, effector-mediated platelet and B cell depletion All four human and mouse IgG isotype variants are available for both *in vivo* model systems[2,43,44,46,47]. Afucosylated IgG isotype glycovariants can be generated by recombinant antibody production in LEC13 cells as we have done before[48]. The selected combinations will be evaluated in C57BL/6 mice in parallel to matched treatments with either IgG alone. Platelet and B cell depletion in the blood will be assessed before, 4 hours after, and 24 hours after the corresponding IgG isotype mixture injection by FACS analysis. We will use 8 mice per treatment, or 32 total (control, each IgG alone, and the combination), to provide sufficient power (0.8) for each test below.

In both target cell models we will evaluate the outcomes of the experiment in a few different ways. First, to evaluate the predictive capacity of our model in this independent cohort, we will test that there is significant correlation between the predicted and actual target cell depletions (Pearson correlation). Second, we will test that (1) the deviation observed with the combination is toward synergy as predicted, and (2) the synergy is statistically significant (mixed effects model, significance tested by bootstrap). Within Aim 3.3 we will additionally test the mechanism by which synergy arises. In total, this work will demonstrate that mixtures of IgG have unique properties of effector-elicited cell clearance in combination.

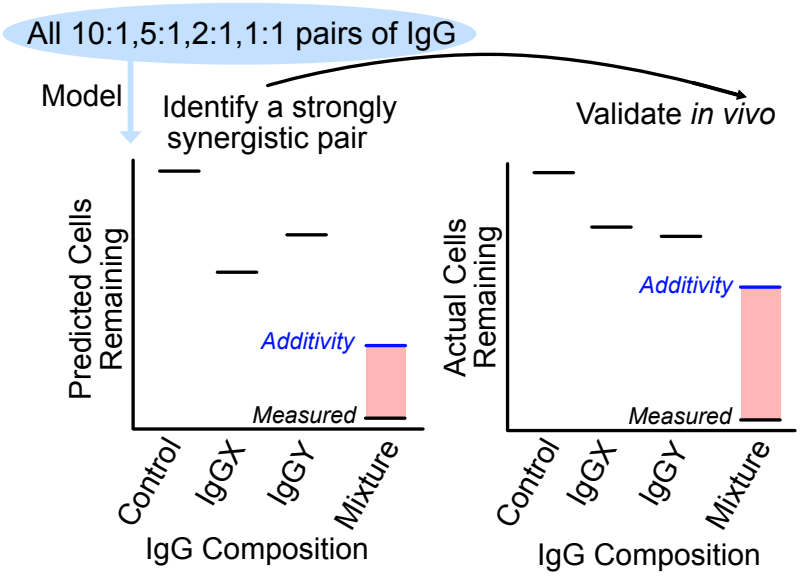

Figure 7: *Synergy strategy.* We will verify our cases of predicted synergy through: (1) correlation between prediction & response, (2) significant synergy in the direction predicted, and (3) mechanism of synergy (Aim 3.3).

Lastly, to test our predictions of homology between the human and murine FcγR families, we will test these combinations in humanized mouse models as we have done before[46,47]. All single and mixture conditions used above will be "translated" from murine to human IgG compositions using the results of Aim 2. These 6 conditions (3 interventions for either B cell or platelet targeting) will be quantified for target cell depletion at the same times as above. We will test for significant correlation between the results from the C57BL/6 and humanized models (Pearson correlation). This will serve as partial validation of the modeling in Aim 2 and demonstrate the value of the human-mouse homology model.

3.3. Identify the relevant cell populations and mechanisms of synergistic effector response Broadly, there are four possible underlying mechanistic sources of synergy/antagonism between IgGs: (1) at the level of binding to an individual effector cell population, (2) not in binding, but in the resultant response of an individual cell population, (3) additivity on previous levels, but with two IgGs targeting a differing complement of cell populations, or (4) additivity across cell populations, but some other emergent interaction through cell communication.

To resolve the underlying mechanisms of interaction, we will use two initial experiments. First, we will use TNP-BSA binding studies as outlined in Aim 1, with primary effector cell populations, to identify whether mechanism (1) might explain the interaction we observe. Individual effector cell populations will be separated and then incubated with each IgG separately, or with the indicated mixture of both IgGs. Significant interactions between the IgGs in binding will be quantified through deviation from additivity. Second, we will quantify the isobologram of ADCC/ADCP effector response in each cell population to address mechanism (2). For B cells, we will incubate each population with B cells and anti-CD20 IgGs of the same mixtures as those tested *in vivo*. The number of remaining B cells will be quantified through B220 staining after 72 hours incubation[44]. For platelets, we will incubate them with each cell population and 6A6 antibodies of the same Fc compositions. The number of remaining platelets will be quantified at 24, 36, and 72 hours of incubation using calcein staining then flow cytometry. Finally,

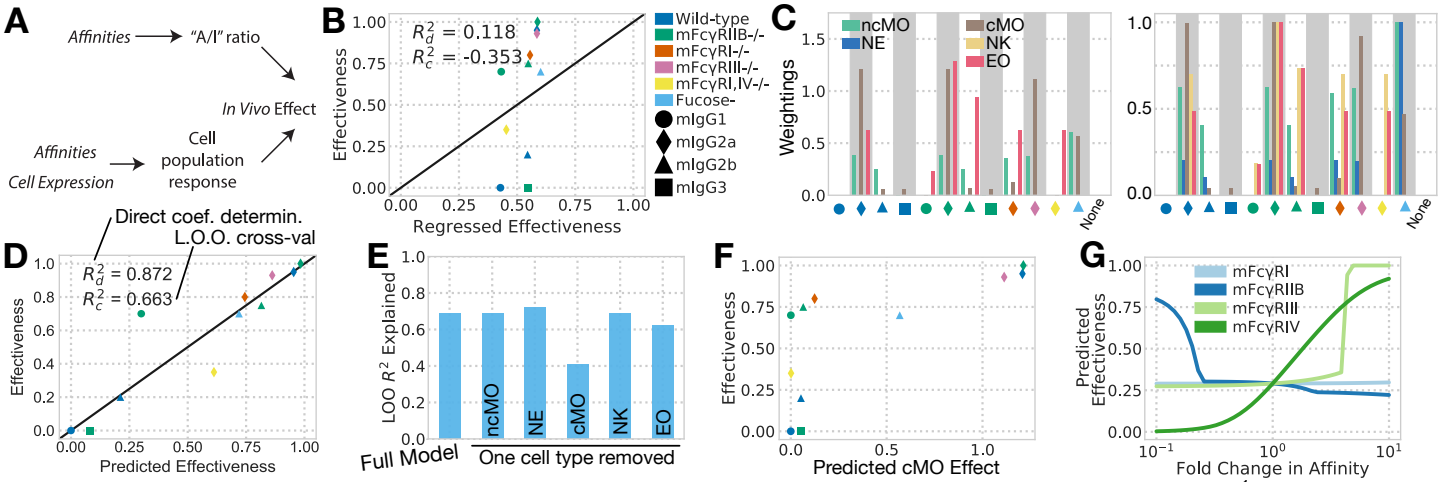

Figure 8: *An FcγR-IgG binding model accurately predicts in vivo IgG-mediated tumor cell killing*[1]. A) Schematic of earlier IgG isotype experiments (top) and our approach (bottom). In earlier work, the ratio of the highest affinity activating receptor to that of the inhibitory receptor (A/I ratio) was proposed to predict response[2]. B) Effectiveness (proportional reduction in lung metastases, i.e. no reduction is 0.0, complete is 1.0) of individual mIgG interventions versus the A/I ratio for each mIgG constant region. C) Individual cell activities calculated for each intervention using receptor multimerization predicted by multivalent binding model. Each quantity is scaled according to the weighting applied by the fitted regression model (left) or by maximum cell type response observed (right). D) Predicted versus observed effectiveness. E) R_c^2 with individual input components removed. F) Calculated activity index for cMO versus overall effectiveness of each intervention. G) Predicted effect of modulating each individual mFcγR affinity of mIgG2b. Regression performed with exponential survival relationship in contrast to published work, due to benefits explained in Aim 3.1[1]. EO: eosinophil, cMO: classical monocyte, ncMO: non-classical monocyte, NK: natural killer, NE: neutrophil.

we will investigate mechanism (3) by determining whether additive combinations of the individual cell population effector response measurements can explain the overall responses we observe *in vivo*.

We strongly expect these first three mechanisms will explain the synergy we observe, as these are the three mechanisms captured by our modeling prediction. Verifying the relevant cell populations involved will further validate the accuracy of our model. Based on our previous *in vivo* studies in both the ITP and B cell depletion model, we would expect that liver resident Kupffer cells and/or resident monocytes are the relevant effector cell populations. Both cell subsets express all activating and the inhibitory FcγRIIB, making it difficult to distinguish the contribution of both cell subsets purely based on using individual FcγR knockout mice. However, by selectively depleting bone marrow derived resident monocytes through small doses of clodronate liposomes[3] or by using a titrated irradiation approach to generate mice with a selective lack of FcγRs on bone marrow derived monocytes or liver resident Kupffer cells[40] we will be able to delineate if B cell or platelet depletion through mixed IgG subclass antibodies behaves differently compared to the use of one IgG subclass *in vivo*. Briefly, animals will be injected with select ratios of IgG subclass mixtures of platelet (6A6) and B cell (CD20) specific antibodies. B cell and platelet counts will be assessed in the blood 4 and 16 hours after antibody injection. To assess if tissue resident Kupffer cells are involved in B cell and platelet depletion, we will inject mice with 10 µL of clodronate liposomes, which shows a rather selective depletion of bone marrow derived resident monocytes[3]. Moreover, we will generate bone marrow chimeric animals selectively expressing activating FcγRs either on tissue resident Kupffer cells or bone marrow derived monocytes by irradiating FcγR deficient or sufficient animals with 6Gy followed by a reconstitution with bone marrow of FcγR sufficient or deficient mice[40]. Should mixed IgG subclass dependent target cell depletion involve other cell populations, we can also study the involvement of NK cells or neutrophils by using either NK- or neutrophil-depleting antibodies or NK cell- or neutrophil-deficient mouse strains. We expect to observe a reduction in the degree to which either platelets or B cells are depleted that is consistent with our model's weighting for that population with the given mixture. Our model treats cell populations as having separable contributions to platelet or B cell depletion. Therefore, if the effects

of depleting effector cell populations is other than we expect, and depleting either effector population individually has a greater effect than expected, we will take this as evidence of cell communication or other emergent behavior. In total, from these studies we will have a mechanistic view of how synergy between IgGs arises.

Preliminary Data In a recently published study, we employed a model of multivalent IC binding to FcγRs and showed that it can capture and predict experimentally measured binding and effector response with differing antigen valency, isotypes, and glycosylation variants[1]. With this model, we could quantitatively predict anti-tumor cell killing in response to a single TA99 antibody of defined Fc region *in vivo*. While predicting outcome, our approach also accurately identified the cell population driving response in this model[1,49]. We have verified an identically-constructed model can similarly predict platelet depletion (crossvalidation $R^2 > 0.8$).

Challenges & Alternative Approaches If IgG combinations selected in Aim 3 do not show synergistic responses, the mechanism-focused measurements in Aim 3.3 will be extremely valuable to diagnose any inconsistencies from the model predictions.

Many alternative constructions exist for defining synergy, each based on underlying definitions for how two agents additively interact[23]. Bliss synergy is a useful definition of synergy for our purposes due to its simplicity and derivation from a statistical definition of additivity that fits well with our model of predicting *in vivo* effect. Further, because it is defined based on a probabilistic interaction of individual agent's effects, Bliss synergy is likely to be interpretable alongside the factorization results of Aim 2. However, we can explore other definitions of synergy to identify which are most informative of therapeutically meaningful interactions. In particular, Lowe synergy defines additive interactions through the expectation that no drug should be synergistic with itself. To test whether this definition would be more helpful, we can test how frequently Bliss synergy arises with IgGs mixed with themselves. Bliss synergy most often indicates that an agent has synergy with itself when a dose response curve is especially sensitive with respect to concentration[50], which we have not seen in our binding measurements. Finally, Lowe synergy yields no close formed solution, and so has some added difficulty when calculating. Thus, in total, Bliss synergy is a well-justified starting definition, but alternative definitions such as Lowe synergy may be informative and provide an alternative strategy.

To relate the activities of each effector cell population to depletion of B cells and platelets, we have to define a survival function, or a function for the relative risk of each additional target cell to be cleared. We propose using an exponential distribution, because this corresponds to the outcome observed when each target cell is at identical "risk" of being cleared. However, alternative survival distributions exist, corresponding to underlying definitions of relative risk. In particular, the Gompertz and Weibull functions correspond to multiplicative and additive differences in "risk" among target cells. Therefore, using these distributions could capture, for example, if platelets and B cells lie on a continuum from easy to hard to clear for reasons unrelated to their antigen availability.

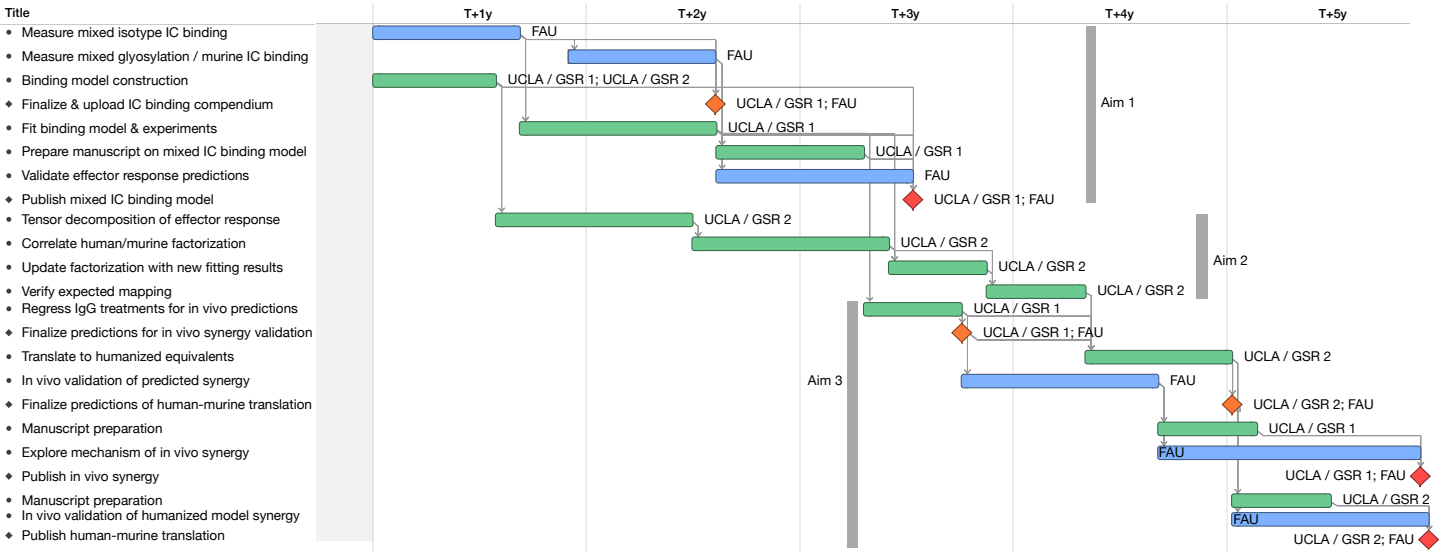

Figure 9: *Gantt chart of proposal timeline.*

References

1. Robinett RA, Guan N, Lux A, Biburger M, Nimmerjahn F, Meyer AS. Dissecting FcγR regulation through a multivalent binding model. Cell Systems. 2018;6(7):1–8. PMCID: PMC6062446

2. Nimmerjahn F, Ravetch JV. Divergent IgG subclass activity through selective Fc receptor binding. Science. 2005 Dec;310(5753):1510–1512. PMID: 16322460

3. Biburger M, Aschermann S, Schwab I, Lux A, Albert H, Danzer H, Woigk M, Dudziak D, Nimmerjahn F. Monocyte subsets responsible for immunoglobulin G-dependent effector functions in vivo. Immunity. 2011;35(6):932–944. PMID: 22169040

4. Montalvao F, Garcia Z, Celli S, Breart B, Deguine J, Rooijen NV, Bousso P. The mechanism of anti-CD20–mediated B cell depletion revealed by intravital imaging. The Journal of Clinical Investigation. 2013 Dec;123(12):5098–5103. PMCID: PMC3859399

5. Bakalar MH, Joffe AM, Schmid EM, Son S, Podolski M, Fletcher DA. Size-Dependent Segregation Controls Macrophage Phagocytosis of Antibody-Opsonized Targets. Cell. 2018;174(1):131–142.e13. PMID: 29958103

6. Tang Y, Lou J, Alpaugh RK, Robinson MK, Marks JD, Weiner LM. Regulation of antibody-dependent cellular cytotoxicity by IgG intrinsic and apparent affinity for target antigen. The Journal of Immunology. 2007;179(5):2815–2823. PMID: 17709495

7. Higel F, Seidl A, Sörgel F, Friess W. N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. European Journal of Pharmaceutics and Biopharmaceutics. 2016 Mar;100:94–100. PMID: 26775146

8. Olivova P, Chen W, Chakraborty AB, Gebler JC. Determination of N-glycosylation sites and site heterogeneity in a monoclonal antibody by electrospray quadrupole ion-mobility time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry. 2008 Jan;22(1):29–40. PMID: 18050193

9. Chung AW, Kumar MP, Arnold KB, Yu WH, Schoen MK, Dunphy LJ, Suscovich TJ, Frahm N, Linde C, Mahan AE, Hoffner M, Streeck H, Ackerman ME, McElrath MJ, Schuitemaker H, Pau MG, Baden LR, Kim JH, Michael NL, Barouch DH, Lauffenburger DA, Alter G. Dissecting Polyclonal Vaccine-Induced Humoral Immunity against HIV Using Systems Serology. Cell. 2015 Nov;163(4):988–998. PMCID: PMC5490491

10. Mahan AE, Jennewein MF, Suscovich T, Dionne K, Tedesco J, Chung AW, Streeck H, Pau M, Schuitemaker H, Francis D, Fast P, Laufer D, Walker BD, Baden L, Barouch DH, Alter G. Antigen-specific antibody glycosylation is regulated via vaccination. PLOS Pathogens. 2016;12(3):1–18. PMCID: PMC4794126

11. Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease models. Blood. 2012 Jun;119(24):5640–5649. PMID: 22535666

12. Macken CA, Perelson AS. Branching processes applied to cell surface aggregation phenomena. Berlin: Springer-Verlag; 1985.

13. Stockmayer WH. Theory of Molecular Size Distribution and Gel Formation in Branched-chain Polymers. The Journal of Chemical Physics. 1943;11(2):45–55.

14. Kolda TG, Bader BW. Tensor decompositions and applications. SIAM Review. 2009;51(3):455–500.

15. Anandkumar A, Foster DP, Hsu DJ, Kakade SM, Liu Y-K. Two SVDs Suffice: Spectral decompositions for probabilistic topic modeling and latent Dirichlet allocation. arXiv. 2012;abs/1204.6703.

16. Perelson AS. Receptor clustering on a cell surface. III. Theory of receptor cross-linking by multivalent ligands: Description by ligand states. Mathematical Biosciences. 1981;53(1-2):1–39.

17. Hlavacek WS, Posner RG, Perelson AS. Steric Effects on Multivalent Ligand-Receptor Binding: Exclusion of Ligand Sites by Bound Cell Surface Receptors. Biophysical Journal. 1999 Jun;76(6):3031–3043. PMCID: PMC1300273

18. Hlavacek WS, Perelson AS, Sulzer B, Bold J, Paar J. Quantifying aggregation of IgE-FcεRI by multivalent antigen. Biophysical Journal. 1999;76(5):2421–2431. PMCID: PMC1300214

19. Perelson AS, DeLisi C. Receptor clustering on a cell surface. I. Theory of receptor cross-linking

by ligands bearing two chemically identical functional groups. Mathematical Biosciences. 1980;48(1-2):71–110.

20. Perelson AS. Receptor clustering on a cell surface. II. Theory of receptor cross-linking by ligands bearing two chemically distinct functional groups. Mathematical Biosciences. 1980 May;49(1):87–110.

21. Stone JD, Cochran JR, Stern LJ. T-cell activation by soluble MHC oligomers can be described by a two-parameter binding model. Biophysical Journal. 2001 Nov;81(5):2547–2557. PMCID: PMC1301723

22. Salvatier J, Wiecki TV, Fonnesbeck C. Probabilistic programming in Python using PyMC3. PeerJ Computer Science. 2016 Apr;2:e55.

23. Greco WR, Bravo G, Parsons JC. The search for synergy: A critical review from a response surface perspective. Pharmacol Rev. Department of Biomathematics, Roswell Park Cancer Institute, Buffalo, New York, USA. 47(2):331–385. PMID: 7568331

24. Palmer AC, Sorger PK. Combination cancer therapy can confer benefit via patient-to-patient variability without drug additivity or synergy. Cell. 171(7):1678–1691. PMCID: PMC5741091

25. Roybal KT, Rupp LJ, Morsut L, Walker WJ, McNally KA, Park JS, Lim WA. Precision Tumor Recognition by T Cells With Combinatorial Antigen-Sensing Circuits. Cell. 2016;164(4):770–779. PMCID: PMC4752902

26. Cho JH, Collins JJ, Wong WW. Universal chimeric antigen receptors for multiplexed and logical control of T cell responses. Cell. 2018;173(6):1426–1438.e11. PMCID: PMC5984158

27. Gonnord P, Angermann BR, Sadtler K, Gombos E, Chappert P, Meier-Schellersheim M, Varma R. A hierarchy of affinities between cytokine receptors and the common gamma chain leads to pathway cross-talk. Science Signaling. 2018;11(524). PMID: 29615515

28. Antebi YE, Linton JM, Klumpe H, Bintu B, Gong M, Su C, McCardell R, Elowitz MB. Combinatorial Signal Perception in the BMP Pathway. Cell. 2017 Sep;170(6):1184–1196.e24. PMCID: PMC5612783

29. Lee S, Choi J, Mohanty J, Sousa LP, Tome F, Pardon E, Steyaert J, Lemmon MA, Lax I, Schlessinger J. Structures of β-klotho reveal a 'zip code'-like mechanism for endocrine FGF signalling. Nature. 2018;553(7689):501–505. PMID: 29342135

30. Simons M, Gordon E, Claesson-Welsh L. Mechanisms and regulation of endothelial VEGF receptor signalling. Nature Reviews Molecular Cell Biology. 2016;17:611–625. PMID: 27461391

31. Belov AA, Mohammadi M. Molecular mechanisms of fibroblast growth factor signaling in physiology and pathology. Cold Spring Harbor perspectives in biology. 2013;5(6):10.1101/cshperspect.a015958 a015958. PMCID: PMC3660835

32. Lux A, Yu X, Scanlan CN, Nimmerjahn F. Impact of immune complex size and glycosylation on IgG binding to human FcγRs. The Journal of Immunology. 2013 Apr;190(8):4315–4323. PMID: 23509345

33. Bruhns P, Iannascoli B, England P, Mancardi DA. Specificity and affinity of human Fcγ receptors and their polymorphic variants for human IgG subclasses. Blood. 2009; PMID: 19018092

34. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Gloria Meng Y, Weikert SH, Presta LG. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. Journal of Biological Chemistry. 2002;277(30):26733–26740. PMID: 11986321

35. Schrier SB, Hill AS, Plana D, Lauffenburger DA. Synergistic Communication between CD4+ T Cells and Monocytes Impacts the Cytokine Environment. Scientific Reports. 2016;6:34942. PMCID: PMC5056362

36. Li C, Levin M, Kaplan DL. Bioelectric modulation of macrophage polarization. Scientific Reports. 2016;6:21044. PMCID: PMC4751571

37. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. BMC Cancer. 2015;15(1):577. PMCID: PMC4545815

38. Biburger M, Trenkwald I, Nimmerjahn F. Three blocks are not enough—Blocking of the murine

IgG receptor FcγRIV is crucial for proper characterization of cells by FACS analysis. European Journal of Immunology. 2015;45(9):2694–2697. PMID: 26138319

39. Brandsma AM, Schwartz SL, Wester MJ, Valley CC, Blezer GLA, Vidarsson G, Lidke KA, Leusen JHW. Mechanisms of inside-out signaling of the high-affinity IgG receptor FcγRI. Science Signaling. 2018;12. PMID: 30042128

40. Lehmann B, Biburger M, Brückner C, Ipsen-escobedo A, Gordan S, Lehmann C, Voehringer D, Winkler T, Schaft N, Dudziak D, Sirbu H, Weber GF, Nimmerjahn F. Tumor location determines tissue-specific recruitment of tumor-associated macrophages and antibody-dependent immunotherapy response. Science Immunology. 2017;2(January):eaah6413. PMID: 28783667

41. Tucker LR. Some mathematical notes on three-mode factor analysis. Psychometrika. 1966 Sep 1;31(3):279–311. PMID: 5221127

42. Yang Z, Michailidis G. A non-negative matrix factorization method for detecting modules in heterogeneous omics multi-modal data. Bioinformatics. 2016;32(1):1–8. PMCID: PMC5006236

43. Kao D, Danzer H, Collin M, Groß A, Eichler J, Stambuk J, Lauc G, Lux A, Nimmerjahn F. A monosaccharide residue is sufficient to maintain mouse and human IgG subclass activity and directs IgG effector functions to cellular Fc receptors. Cell Reports. 2015;13(11):2376–2385. PMID: 26670049

44. Hamaguchi Y, Xiu Y, Komura K, Nimmerjahn F, Tedder TF. Antibody isotype-specific engagement of Fcγ receptors regulates B lymphocyte depletion during CD20 immunotherapy. The Journal of Experimental Medicine. 2006 Mar;203(3):743–753. PMCID: PMC2118227

45. Efron B. The Jacknife, the Bootstrap and Other Resampling Plans. SIAM; 1982.

46. Schwab I, Lux A, Nimmerjahn F. Pathways Responsible for Human Autoantibody and Therapeutic Intravenous IgG Activity in Humanized Mice. Cell Reports. 2015 Oct;13(3):610–620. PMID: 26456831

47. Lux A, Seeling M, Baerenwaldt A, Lehmann B, Schwab I, Repp R, Meidenbauer N, Mackensen A, Hartmann A, Heidkamp G, Dudziak D, Nimmerjahn F. A Humanized Mouse Identifies the Bone Marrow as a Niche with Low Therapeutic IgG Activity. Cell Reports. 2014 Apr;7(1):236–248. PMID: 24685130

48. Patnaik SK, Stanley P. Lectin-resistant cho glycosylation mutants. Methods Enzymol. 2006;416:159–82. PMID: 17113866

49. Zhu EF, Gai SA, Opel CF, Kwan BH, Surana R, Mihm MC, Kauke MJ, Moynihan KD, Angelini A, Williams RT, Stephan MT, Kim JS, Yaffe MB, Irvine DJ, Weiner LM, Dranoff G, Wittrup KD. Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended serum half-life IL-2. Cancer Cell. 2015 Apr;27(4):489–501. PMCID: PMC4398916

50. Berenbaum MC. What is synergy? Pharmacological Reviews. American Society for Pharmacology; Experimental Therapeutics; 1989;41(2):93–141. PMID: 2692037