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An "Fc-Silenced" IgG1 Format With Extended Half-Life Designed for Improved Stability

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ABSTRACT

Multiple mutation combinations in the IgG Fc have been characterized to tailor immune effector function or IgG serum persistence to fit desired biological outcomes for monoclonal antibody therapeutics. An unintended consequence of introducing mutations in the Fc (particularly the C_H2 domain) can be a reduction in biophysical stability which can correlate with increased aggregation propensity, poor manufacturability, and lower solubility. Herein, we characterize the changes in IgG conformational and colloidal stability when 2 sets of C_H2 mutations "TM" (L234F/L235E/P331S) and "YTE" (M252Y/S254T/T256E) are combined to generate an antibody format lacking immune receptor binding and exhibiting extended half-life. In addition to significantly lowered thermostability, we observe greater conformational flexibility for TM-YTE in C_H2, increased self-association, and poorer solubility and aggregation profiles. To improve these properties, we dissected the contributions of individual mutations within TM-YTE on thermostability and substituted destabilizing mutations with new mutations that raise thermostability. One novel combination, FQQ-YTE (L234F/L235Q/K322Q/M252Y/S254T/T256E), had significantly improved conformational and colloidal stability, and was found to retain the same biological activities as TM-YTE (extended half-life and lack of antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity activity). Our engineering approach offers a way to improve the developability of antibodies containing Fc mutations while retaining tailored biological activity.

Introduction

The rapid increase in monoclonal antibody therapeutics approved for clinical application corresponds with an increase in engineering complexity used to generate them. In addition to humanization and optimization of binding affinities by mutagenesis of variable domains, the antibody Fc is increasingly being modified to customize effector function to best suit the intended physiological effect of the drug. For example, antibodies that rely on antibody-dependent cell-mediated cytotoxicity (ADCC) for *in vivo* efficacy can have this mechanism of action enhanced by Fc mutagenesis or glycoengineering.[1-3]

In many instances, it is desirable for a monoclonal antibody therapeutic or Fc-fusion protein to lack effector function. Such may be the case for antibodies with known Fc-mediated side effects (such as thrombocytopenia),[4] or those designed to block receptors without activating the immune system (so-called "benign blocker" antibodies) or with antibody drug conjugates where unanticipated interactions with nontumor cells may be detrimental. For these purposes, several types of "Fc-silenced" antibodies formats with varying degrees of reduced receptor binding have been developed.[5] These include aglycosylated IgG lacking the N297 glycan,[6-9] IgG4P,[10] IgG2/4,[11] IgG2m4,[12] "LALA" IgG1,[13] IgG2σ,[14] and the triple mutation L234F/L235E/P331S[15] (referred to as "TM") among others. A common design feature of all these formats is that undesirable Fc interactions with Fcγ receptors and the complement receptor C1q can now be decoupled from binding to the Neonatal Fc Receptor (FcRn) which conveys the sought after benefit of long serum persistence. As with effector binding, *in vivo* serum persistence conferred by FcRn is also a tunable property that can be modulated by mutations in the IgG Fc.[16] Mutations that increase FcRn affinity while retaining pH-dependent binding to FcRn can endow

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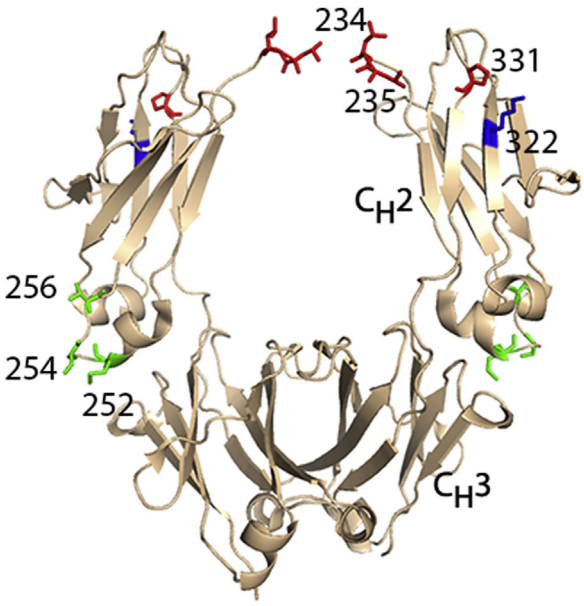

Figure 1. Location of mutations in the IgG Fc domain. The locations of TM mutations (red) and YTE mutations (green) as well as K322 (blue) were highlighted on the crystal structure of an IgG1 Fc (protein data base ID: 4X4M). The highlighted residue positions are all located in the C_H2 domain.

antibodies with extended serum persistence by optimizing FcRn recycling efficiency. Several sets of mutations have been identified that can extend IgG half-life in primates or human FcRn transgenic mice.[2,17-22] One set of mutations in the IgG1 C_H2 domain, M252Y/S254T/T256E (referred to as the YTE mutation hereafter),[15,23,24] has been shown to increase the half-life of a monoclonal antibody to greater than 90 days in humans.[25]

Although Fc mutation combinations have allowed researchers to tailor the biology of potential therapeutic antibodies or Fc fusions beyond that provided by existing IgG isotypes, mutations in the Fc region (particularly in the C_H2 domain) can inadvertently decrease the biophysical stability of the antibody. Several Fc mutations have been shown to lower antibody thermostability[26-30] and this introduced instability can correspond with an increased propensity for aggregation.[27,31,32] Limiting the propensity for aggregation for biologics is important for a number of reasons. Protein aggregation can hamper the production, formulation, and storage of drug products. Beyond manufacturing, aggregation and other post-translational modifications (such as oxidation and deamination) can result in reduced drug efficacy, altered pharmacokinetics, and increased immunogenicity.[33,34] Managing potential aggregation is particularly important in high-concentration liquid formulation to facilitate convenient subcutaneous dosing.[35]

In this study, we describe the biology and consequences on antibody biochemical and biophysical stability when 2 sets of mutations, both residing in the C_H2 domain of the Fc (TM and YTE), are combined in one antibody (Fig. 1). It has previously been reported that the TM-YTE IgG1 backbone causes an ~12°C reduction in T_m1[36] and an increased protein decay coefficient.[30] In this study, we confirm the destabilizing effects of TM-YTE on thermostability by differential scanning calorimetry (DSC) and differential scanning fluorimetry (DSF). We further show that TM-YTE has increased conformational flexibility by hydrogen/deuterium exchange (HDX) as well as reduced colloidal stability, and an increased aggregation rate when compared the parental IgG. We then go on to engineer an improved set of mutations with the same biological properties of TM-YTE, but with improved stability profiles. To accomplish this, we dissected the effects on thermostability of individual mutations within TM. We identified the most destabilizing mutations and replaced them with more stable alternatives. The result is an antibody format with extended *in vivo* half-life and reduced effector binding, with enhanced stability compared to the original mutation combination.

Materials and Methods

Reagents, Antibody Expression, and Purification

All chemicals were of analytical grade. Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Soluble extracellular domains of FcγRIIIa (158V and 158F), FcγRIIa, FcγRIIb, FcγRIa, and FcRn were all generated at MedImmune. Human C1q was purchased from Quidel (San Diego, CA). Antibody positions are listed according to the Kabat EU numbering convention.[37]

All antibody constructs were transiently expressed in HEK 293FT cells using 293fectin™ and Freestyle™ medium from Life Technologies (Carlsbad, CA). The culture medium was collected 10 days after transfection. Antibodies were purified by standard protein A affinity chromatography. Monomer content for all antibodies was determined by analytical size-exclusion chromatography (SEC). Preparative SEC was used (when necessary) to isolate >97% monomeric antibody used in all experiments.

DSC and DSF

DSC measurements were carried out using a MicroCal VP-DSC differential scanning microcalorimeter (MicroCal, Northampton, MA). All solutions and samples used for DSC were filtered using a 0.22-μm filter. Antibodies used for the DSC studies were >97% monomeric as judged by analytical gel filtration chromatography. Prior to DSC analysis all samples were exhaustively dialyzed (at least 3 buffer exchanges) in 25 mM histidine-HCl, pH 6. Buffer from this dialysis was then used as reference buffer for subsequent DSC experiments. Prior to sample measurement, baseline measurements (buffer-vs.-buffer) were obtained to be subtracted from the sample measurement. Dialyzed samples (at a concentration of 1 mg/mL) were added to the sample well and DSC measurements were performed at a 1°C/min scan rate. Data analysis and deconvolution were carried out using the Origin™ DSC software provided by MicroCal. Selected deconvoluted thermograms are shown in Supplementary Figure 1 with full results summarized in Supplementary Table 1.

For DSF measurements, SYPRO® Orange dye (5000× stock; Invitrogen, Inc., Carlsbad, CA) was added at a 5× final dye concentration to antibodies at 0.5 mg/mL concentration in 25 mM histidine-HCl (pH 6).[38] Twenty-five microliters of prepared samples were transferred in triplicate to polypropylene 96-well PCR plates (Bio-Rad, Hercules, CA). A CFX real-time PCR system with C1000 module was used as a thermal cycler. The fluorescence emission was monitored using the FRET scan mode in the CFX Manager software (Bio-Rad; software version 3.01224.1015). The PCR plate containing the test samples was subjected to an initial 20°C equilibration for 10 min, followed by a temperature ramp from 20°C to 90°C in 0.2°C increments with a 10 s equilibration step prior to taking fluorescence measurements. The temperature of hydrophobic exposure (T_h) was calculated by the software using a mathematical second-derivative method (−d(RFU)/dT) to calculate the inflection point of the curve. The reported T_h is an average of 3 measurements.