The contributory effects of the VH families and CH regions of multimeric IgM on its interaction with FcμR, and antigen.

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Abstract

As the primary antibody and with increasing interest as a therapeutic antibody, IgM is also the largest antibody structure among the five major human isotypes. Spontaneously forming oligomers as pentamer and hexamers, IgM has avidity effects that could compensate for lower interactions as monomers. With recent findings of effects of the heavy chain constant region affecting antigen binding and the VH families of the V-regions affecting FcR engagement, we CDR grafted Trastuzumab and Pertuzumab CDRs to VH1-7 IgMs for biolayer interferometry investigations. From our panel of the 14 IgM variants, we found evidence of the V-regions holistically affecting FcμR binding and for the IgM C-region affecting Her2 engagements, mediated by the V-regions holistically and also by protein L binding at the light chain V-region. These findings suggest the oligomerization effect of IgMs that may affect both FcμR and antigen binding that are different from the other monomeric isotypes with relevance to guiding the development and protein engineering of IgM therapeutics.

Keywords: IgM, FcμR, Her2, Avidity, Hexamers, KD, Biolayer Interferometry, Protein L, Anti-IgM

Introduction

IgM is the primary response antibody isotype to be produced in the adaptive immunes response [1]. As the largest multimeric antibody among the major human isotypes (IgM, IgA, IgD, IgG and IgE), IgM can oligomerize via its Cμ4 domain at the heavy chain constant (C)-region and the tailpiece. IgM typically forms pentamers in the presence of J-chain and as

hexamers without [2, 3]. Due to the oligomerization, IgM has avidity effects that can compensate for weaker antigen binding present in monomeric antibody isotype to be stronger binders such as that observed between Pertuzumab and Trastuzumab IgG1s and IgMs [4]. While Trastuzumab had a stronger interaction with the Her2 antigen than Pertuzumab as IgG1s, the steric hindrances to binding multiple Her2 molecules due to epitope accessibility [5] resulted in Pertuzumab IgM interacting with Her2 stronger than Trastuzumab IgM [6].

As the primary response antibody, IgM contributes to mucosal immunity via the pIgR expressed on the epithelial cell surface that facilitates the transport for secretory IgM [7]. Yet, most importantly, the FcμR (also known as TOSO or FAIM3) is an IgM receptor expressed on lymphoid cells (B cells, T cells and NK cells) [8-10] that binds the CH4 domain of IgM [11] to activate the signalling cascade via the immunoglobulin tail tyrosine (ITT) phosphorylation motif [12].

With recent evidence showing that the antibody domains cross-influence each other where the variable (V-)regions, particularly the framework regions (FWR), can influence the FcR binding sites at the heavy chain constant regions (CH) for IgA [13-16], there is a need for a holistic [17] investigation for IgMs as well. With significant influence from non-antigen interacting regions such as the signal peptide [18] on antibody production and how the different antibody regions can come together form stretches and pockets that can bind non-antigens [19] [20], there remains much to investigate at the whole antibody level.

While the majority of current antibody therapeutics are currently IgGs, interest in other isotype such as IgM have risen in recent years due to their importance found with the recent SARS-CoV-2 pandemic [21-24] and with a listed company IGM Biosciences currently with therapeutic IgM candidates undergoing phase 1 and 2 clinical trials (ClinicalTrials.gov Identifier: NCT04553692 & CT04082936). As such, it is important to understand how the different regions in the IgM interact, and given its multimeric structure, how such distal effects can play out. To investigate such effects, the complementarity determining regions (CDRs) of Trastuzumab and Pertuzumab previously grafted onto the VH1-7 family FWRs [16, 18] were made into a panel of 14 IgMs for Biolayer Interferometry (BLI) measurements to investigate the VH family FWR effects on IgM hexamers binding to Her2 antigen and FcμR receptor respectively.

Result

<u>BLI measurements of FcμR to Pertuzumab and Trastuzumab IgM variants</u>

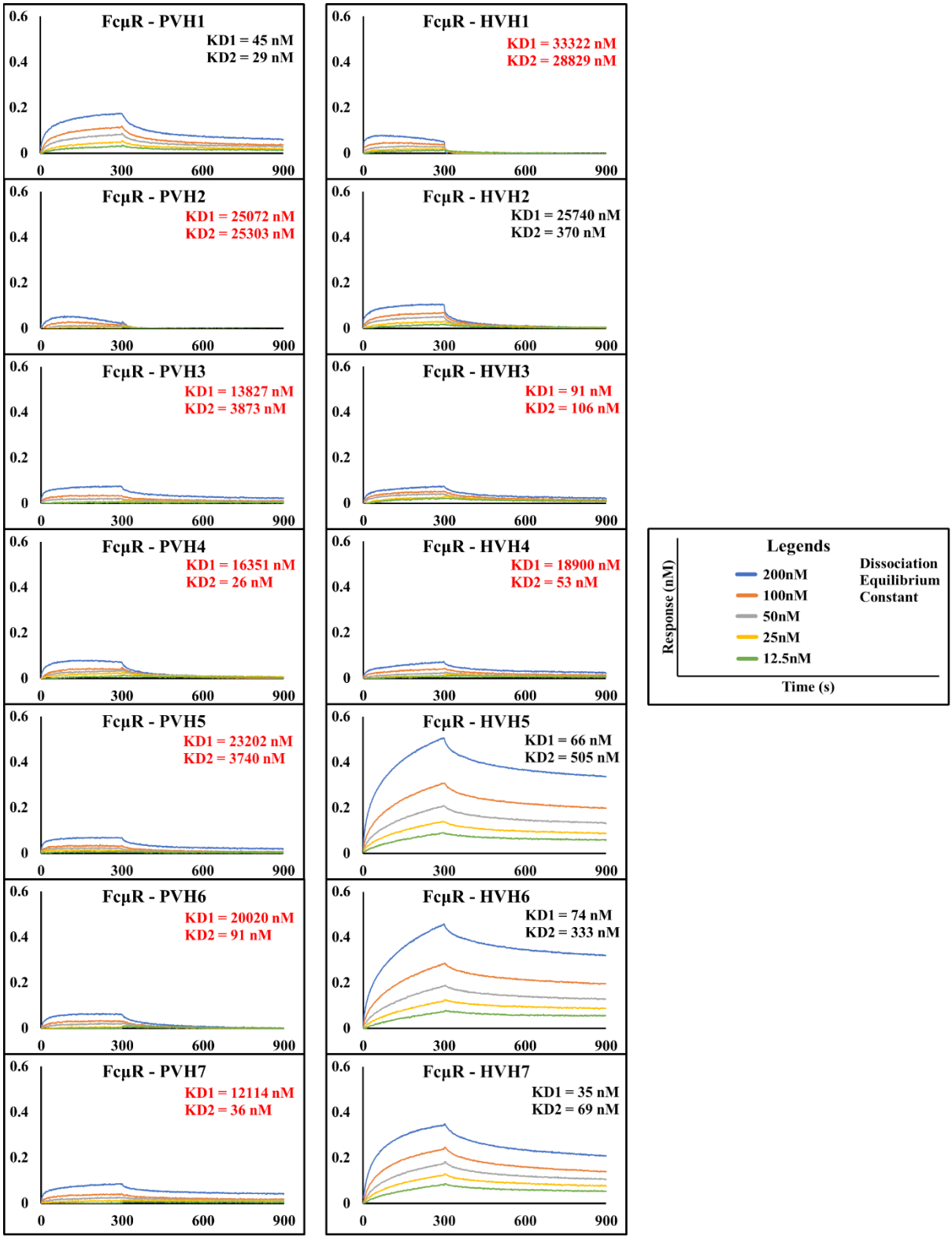

Figure 1. The BLI measurements of the different concentrations of Pertuzumab (PVH1-7) and Trastuzumab (HVH1-7) IgM variants to FcμR bound to the AHC biosensor. Two KD values were presented according to the 2:1 fitting model. The KD values highlighted in red were deemed unreliable due to low R^2 values (<0.9) and/or had poor response (<0.1). KD values are shown rounded off to the nearest whole number.

To investigate the distal allosteric effects of the V-region FWR VH families on the IgM CH region site to bind FcμR, we performed CDR grafting both Trastuzumab and Pertuzumab to the seven VH FWR recombinantly connected to the IgM C-region. All the VH1-7 variants were produced successfully, albeit at different concentrations, with them spontaneously forming hexamers as estimated using electron microscopy on selected samples from our panel shown in Supplementary Figure S1.

Based on fitting models of 2:1 antibody ligand interaction of the biolayer interferometry (BLI), we did not find evidence for the VH FWR to be the sole factor in influencing the engagement between the IgMs and FcμR given the lack of consistency between two antibody models of the same VH family. This suggests a contributory role for the combination of FWRs, CDRs and that of light chain.

Among the Pertuzumab VH1-7 variants (Figure 1), PVH1-IgM is the only Pertuzumab variant with significant interactions with FcμR, yielding KD 1 and KD 2 values of 45 and 29 nM, respectively. For the Trastuzumab variants, four out of the seven VH families bind to FcμR with KD values ranging from 35 to 25740 nM with VH7, 6, 5 & 2 ranked from the strongest to weakest interacting IgMs. As all the VH variants within the respective antibody models had the same respective light chain, the light chain was also ruled out as to be a single contributing partner to FcμR engagement.

<u>BIL measurements of Pertuzumab and Trastuzumab IgM variants to Her2</u>

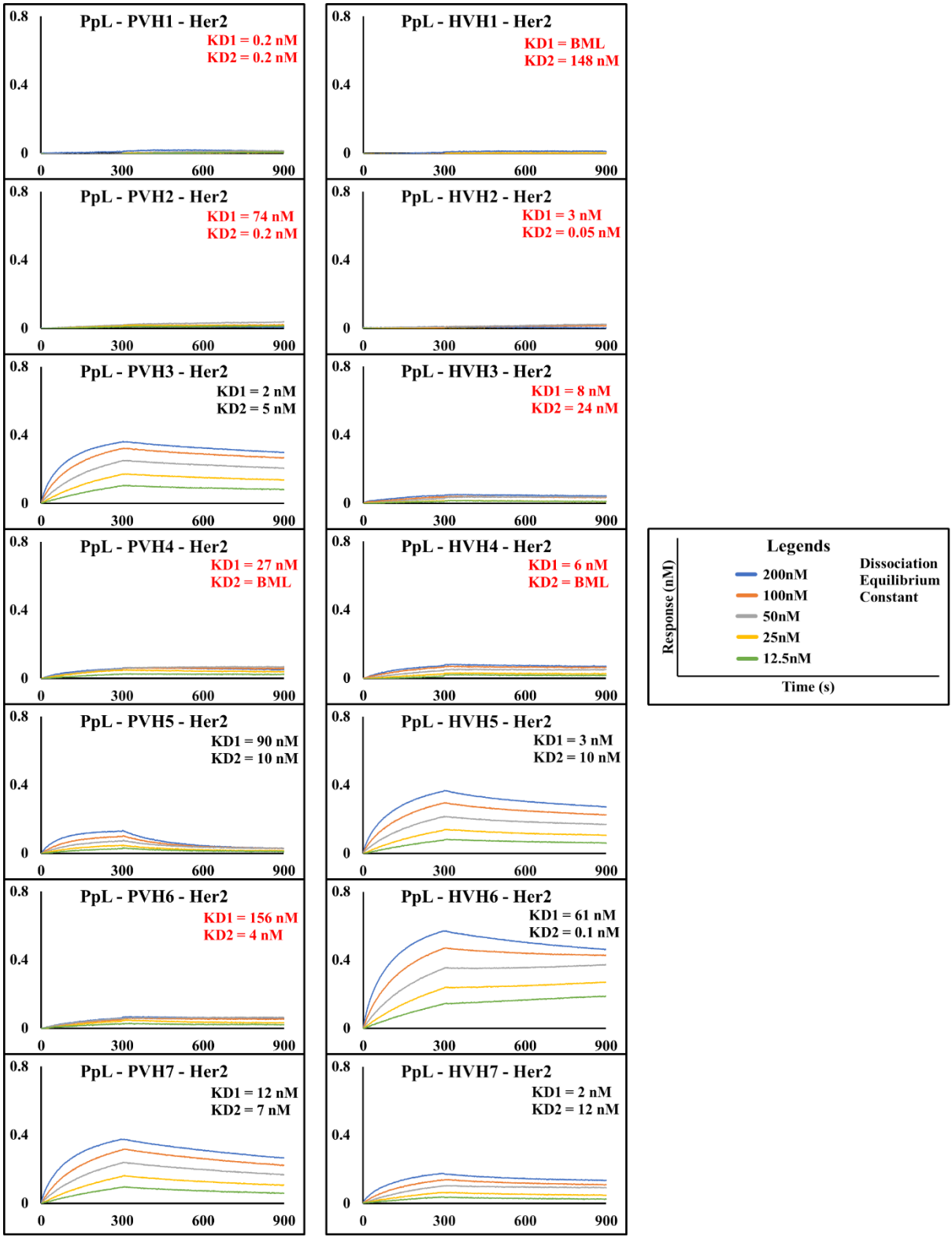

Figure 2. The BLI measurements of the different concentrations of Pertuzumab (PVH1-7) and Trastuzumab (HVH1-7) IgM variants to Her2 using the PpL biosensor. Two KD values were presented according to the 2:1 fitting model. The KD values highlighted in red were deemed unreliable due to low R^2 values (<0.9) and/or poor response (<0.1). KD values are shown

rounded off to the nearest whole number. BML stands for “beyond machine/measurement limit” where KD values generated are <1.0 x 10^{-12}.

As a control to determine if the IgM variants were folded correctly and functional, the IgM interactions with their known antigen Her2 were measured using two immobilization methods for the Octet Red96® system. The first method was to use the PpL biosensor to capture the IgM light chains (Figure 2) at the Vk1 FWR, whereas the second was to use the anti-IgM (recognizing IgM CH) immobilized with SA biosensors (Figure 3). The two immobilization methods considered the avidity effects of IgM, the need for correct folding of the IgM CH for recognition by anti-IgM, and the possible effects of PpL binding mitigating Her2 interactions.

While the majority of the Pertuzumab IgM VH variants did not interact with FcμR with the exception of Pertuzumab VH1 IgM (Figure 2), all of them were recognized by the anti-IgM and interacted with Her2 with the KD1 and KD2 values within a relatively narrow and expected nM range (Figure 3) and in readings were more consistent than PpL bound Pertuzumab IgMs to Her2 (Figure 2) suggesting that PpL binding modulated Her2 engagements for the IgMs.

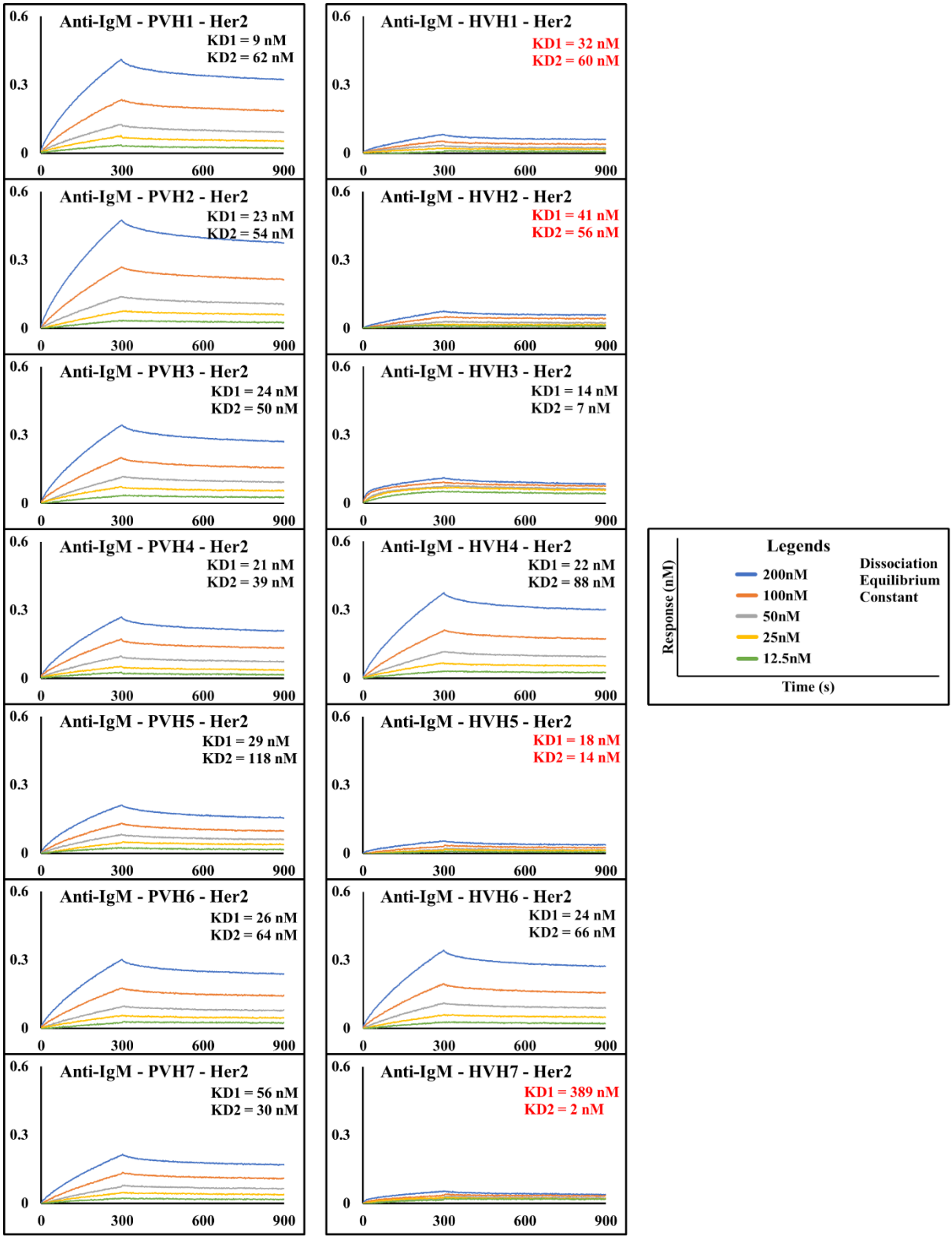

Figure 3. The BLI measurements of the different concentrations of Pertuzumab (PVH1-7) and Trastuzumab (HVH1-7) IgM variants to Her2 using the anti-IgM bound to SA biosensor. Two KD values were presented according to the 2:1 fitting model. The KD values highlighted in red were deemed unreliable due to low R^2 values (<0.9) and/or low response (<0.1). KD values are shown rounded off to the nearest whole number.

Using PpL immobilisation shown in Figure 2, we observed that three out of the seven VH Pertuzumab IgM variants interacted with Her2 with KD values ranging from 2 to 90 nM, where VH3, 7 & 5 were the strongest to weakest interacting IgMs.

For the Trastuzumab IgM variants, three out of the seven VH families interacted with the Her2 with lower KD values (stronger interactions) than the Pertuzumab variants with KD values ranging from 0.1 to 61 nM. Compared to the Pertuzumab IgMs with the order of 3,7 and 5 for strongest to weakest binders, the Trastuzumab IgMs were in the order of VH5/7 & VH6 being the strongest and weakest binders (based on the average KD1 and KD2 values). VH5 and VH7 FWRs from both Pertuzumab and Trastuzumab IgM variants consistently interacted with Her2 but VH5 exhibited greater variability (KD1 and KD2 average = PVH5: 50 nM to HVH5: 6.5 nM) than VH7 (KD1 and KD2 average = PVH5: 9.5 nM to HVH5: 7 nM).

Anti-IgM captured IgM showed that all Pertuzumab VH IgM variants bound to Her2 with KD values ranging from 9 to 118 nM with VH4, VH1/2/3, VH6/7 & VH5 as the strongest to the weakest binders (Figure 3). Among the seven Trastuzumab IgM variants, only the VH3, 6, and 4 (in order of lowest KD to highest) yielded reliable KD1 and 2 averages ranging from 7 to 88 nM. It is noteworthy that the VH6 FWR on both Trastuzumab (HVH6) and Pertuzumab (PVH6) showed relatively consistent KD1 and KD2 values.

Discussion

To investigate the effects of seven VH families on the distal binding of IgM CH to its receptor FcμR, and the proximal effects of PpL binding of the light chain on antigen Her2 binding, we used the CDRs of two well-studied cancer therapeutic antibodies, Pertuzumab and Trastuzumab. The CDR-grafted V-regions, previously characterized on other heavy chain isotypes [4, 13-16, 18, 20], were stitched onto the human IgM CH [4] and made into whole IgMs. From previous reports [3, 25, 26] and from our electron microscope analyses of selected samples (Supplementary Material Figure S1), the IgMs expectedly formed multimers.

The ability of an antibody to bind its receptor affects its activation of downstream signalling on the immune cells. With IgM being the primary antibody and FcμR found on lymphocytes such as natural killer cells, B cells and T cells [8-10], impaired receptor engagement can lead to lower efficacy in IgM therapeutics. While the FcμR binding site is located on the CH region of the IgM and is distal from the antigen-binding V-regions, recent investigations using whole antibodies have demonstrated that the V-regions (Both VH and VL

in combination) can influence FcR binding sites on the CH region. Such distal allosteric effects were found for IgG1 [16], IgA [13] [14], IgE [15], and was expectedly found here in IgM. However, IgM differed from the mentioned antibody isotypes in its oligomerization. Apart from being a B-cell receptor as a monomer, it commonly exists as an multimer that can influence its structural rigidity and thereby, the allosteric communications as was found in the difference between IgA1 and 2 [13] at the hinge. At the same time, the close proximity of the V-regions due to the IgM oligomerization can also affect its binding to the known antigens due to epitope accessibility and steric hindrances [5, 6] of binding multiple antgens that could be further influenced by the binding of superantigens [13, 27], particularly at the V-regions. Such factors can influence the efficacy of therapeutic antibodies and the sensitivity and accuracy of diagnostic antibodies. With the growing importance of IgM as a therapeutic antibody and its use already in diagnostics (such as in haemagglutination kits), it was timely to investigate IgM and how its FcμR binding can be influenced by the choice of FWR in CDR-grafting in humanization [28].

From our IgM panel (Figure 1), the BLI results showed that the V-regions of the IgMs can abolish FcμR engagement at the CH region from the expected ~10 nM avidity interaction [29]. Although this work used a different measurement method from the Scatchard plot analysis with the assumption of 1:1 stoichiometry [29], our 2:1 fitting model for the BLI system given that 1:1 could not yield reasonable KD values, reported similar range of values for a number of our IgMs. With the lack of patterns specific to the VH families of the two antibody models, the results reveal that the FWRs alone were not responsible for the distal allosteric effect, but also having contributions from the CDRs. With the same light chain within each antibody model exhibiting the differing effects, the light chain by itself was also ruled out, although it is expected that it contributes to the holistic effects from the VH-VL pairing.

Such combinatorial V-region effects were shown where the VH1 FWR, producing a FcμR binding IgM with Pertuzumab CDRs (PVH1) and light chain, did not show significant FcμR engagement with the Trastuzumab CDRs (HVH1) and light chain. The reversal of such patterns was also observed with VH7, 5 and 6 FWRs interacting well for Trastuzumab but not Pertuzumab. It is interesting that apart from PVH1, all the other Pertuzumab variants did not have significant interactions with FcμR.

As a control for the produced recombinant IgMs being correctly folded and functional, the binding of the V-regions was tested using PpL capture while the correct folding of the CH regions was tested using anti-IgM captures. With loading of the IgMs by the two capture

methods (Supplementary Figure S2-6) demonstrating recognizable IgM binding sites, we were confident that non-bindings were not due to mis-folded or non-functional recombinant IgMs.

Using the PpL capture method, only PVH3, 5 & 7 of the Pertuzumab IgM variants were able to bind to Her2 (Figure 2) whereas with anti-IgM capture of the IgMs, all the Pertuzumab variants (PVH1-7) were able to bind to Her2. This finding demonstrates the immunomodulation effects of microbial superantigens of normal flora (discussed in [27] and [30]) on IgM where similar superantigens can inactivate otherwise functional IgMs that is also expected to extend to other isotypes given the binding at the light chain Vk-FWR1 [19].

Within the anti-IgM captured IgM panel, it should be noted that the log-fold difference of Pertuzumab VH5 IgM and Trastuzumab VH5 IgM were reflective of the difference between Pertuzumab and Trastuzumab IgGs previously determined [31]. On the other hand, the compensatory effect of Pertuzumab VH7 IgM to have its KD match with that of Trastuzumab VH7 IgM, reflects the avidity effects [4] and even epitope accessibility issues to bind Her2 as discussed previously [6] [5].

Analysing the Trastuzumab dataset, we noticed trends where not all the Trastuzumab VH IgMs bound to Her2 regardless of whether they were captured by PpL or anti-IgM. There were also clear interferences from PpL in preventing the HVH1-4 from binding Her2. Curiously, HVH1 and 2 were consistently unable to bind Her2 despite of the capture method, but the same grafted method on Pertuzumab IgM captured by anti-IgM could. Given that the two HVH V-regions could bind to Her2 when made into IgG1s [16], IgAs [13] and IgEs [15] using the same V-regions and light chains, there was a clear structural interference from the IgM CH in the Her2 binding. With the pattern for the rest of the Trastuzumab VH families binding Her2 in some form either captured by PpL or anti-IgM, the recombinant IgMs were certainly functional, and that the oligomerization of IgM somehow abolished the antigen binding.

The exact mechanism of this IgM CH effect remained enigmatic, requiring future structural and modelling studies that can guide antibody engineering mitigation methods. Nonetheless, our panel show the VH7 FWR to be a suitable FWR for CDR grafting for retaining the bulk of the IgM antigen binding abilities. This consensus is absent for FcμR engagement, suggesting the need for future studies to investigate IgM candidates individually yet holistically due to the combinatorial factors involved in mechanisms that are yet to be fully elucidated.

To fully investigate these, there will also be a need to examine the proximal effects elicited by PpL on Her2 binding as found for IgEs and IgAs of the same V-regions [13, 15]

suggesting unique characteristics of IgM CH oligomerization that may explain its status as a primary antibody requiring class-switching for affinity maturation.

In conclusion, we found that not all IgMs would engage FcμR efficiently to elicit the necessary downstream effects and that unlike the grafting of the other Ig isotypes, the CH region of IgM somehow affected the ability of V-regions to bind to Her2 effectively as well. And with further contributory effects from PpL to affect antigen binding, there is a need for detailed antibody engineering at a holistic whole antibody manner to detect and mitigate for such effects. In addition to providing insights to engineering IgM therapeutics, the results here also provide a potential clue to the functioning of the antibody generation process in the immune system to possible underlying rationale for class switching and how isotype switching can play a role in affinity maturation in humans.

Materials and Methods

The synthesis of the recombinant IgM VH1-7 variants

The recombinant IgM variants were recombinantly generated by joining the CDR grafted variants of Trastuzumab (PDB 1N8Z) and Pertuzumab (PDB 1S78) sequences from the previously IgG1 VH1-7 variants [16, 18] to the IgM CH [4] with signal peptides as previously described [18, 19, 32, 33]. The light chains were created by grafting the respective Pertuzumab and Trastuzumab Vκ1 genes with the κ-constant region as were performed for the heavy chains. Briefly, the dual plasmids were prepped [34] and transformed into DH5α competent cells for plasmid production as previously described [35]. The plasmids were then transfected into HEK293 cells for production and purified as previously optimized [36] and [37]. The purified recombinant IgMs were then subjected to size exclusion for collection of the spontaneously formed hexamer fractions as previously described [4].

BLI Measurements

KD measurements of Fc-tagged Human FcμR (Cat: 13556-H02H, SinoBiological) immobilised on Anti-Human IgG Fc (AHC) (Cat: 18-5060, Sartorius) biosensors were performed on the Octet Red96® system with the loading threshold set at 1.0 nm and utilizing the recombinant Pertuzumab and Trastuzumab IgM VH whole antibodies variants in five serial diluted concentrations (12.5 to 200nM) solutions.

KD measurements of the Pertuzumab and Trastuzumab IgMs to their Her2 antigen (Cat: 10004-HCCH, SinoBiological) were performed by first immobilizing them on Protein L (Cat: 18-5085, Sartorius) and CaptureSelect™ Biotin Anti-IgM Conjugate (Cat: 7102892500,

Thermofisher Scientific) bound Streptavidin (SA) biosensors (Cat: 18-5019, Sartorius) (loading threshold set at 1.0 and 0.5 nm, for Protein L and Anti-IgM SA biosensor, respectively) and subject to the five serially diluted Her2 solutions (12.5 to 200nM).

The program and steps used were described as followed. The Octet Acquisition v10.0 program was used to set the following steps for KD measurements: Pre-conditioning (0.2 M glycine, pH 1.52 & 10x kinetic buffer (KB) (Cat 18-1105, Sartorius), 30 s); Initial Baseline (10x KB, 60 s); Loading (ligands, 600s with threshold) Baseline (10x KB, 120 s); Association (analyte, 300 s); Dissociation (10x KB, 600 s); Regeneration (0.2 M glycine, pH 1.52 & 10x KB), 30 s). The equilibrium dissociation constant (KD) values were automatically calculated from the ka and kd using the Data Analysis v10.0 program using the 2:1 fitting model. KD measurements were deemed reliable if the R^2 value is above 0.9 and the response value is above 0.1. All measurements were performed in triplicates as previously performed [4, 13-20, 31, 38]. The 2:1 fitting models were used to analyse the IgM interactions and shown in Results as well as Supplementary Materials.

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Contributions

Conceptualization, Methodology, Investigation, Methodology, Writing were by W.L.L. and S.K-E.G. Funding Acquisition, S.K-E.G. Supervision, S.K-E.G.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The datasets GENERATED/ANALYZED for this study are available upon request.

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