1 **Review: Sagacious epitope selection for vaccines, and both antibody-based therapeutics**

2 **and diagnostics: Tips from virology and oncology.**

4 **Running Title:** Sagacity in Epitope Selection

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19 **Keywords:** Antigen selection, Epitope selection, Antibody targeting, Epitope Accessibility

21 **Abbreviations:** Ig, VH, CH, VL, CL, FWR, CDR, Fc, Fab, V-region, ADCC, AR.

22   **Statement of Significance:** The culmination and advances in protein engineering and antibody

23   development has allowed zooming in on the target antigen to epitope selection. Relevant to

24   vaccines, antibody-based therapeutics and diagnostics, the target epitope plays a major role in

25   the success of the interventions. This review discusses these epitope factors, and how with

26   sagacious epitope selection, better antibody-based therapeutics and diagnostics, and vaccines

27   can be potentially developed with higher success.

28   **ABSTRACT**

29   The target of an antibody plays a significant role in the success of antibody-based therapeutics

30   and diagnostics, and to an extent, that of vaccine development. This importance is focussed on

31   the target binding site – epitope, that sagacious epitope selection in a form of design thinking

32   beyond traditional antigen selection using whole cell or whole protein immunisation can

33   positively improve success. Intrinsic factors that can affect the functioning of resulting

34   antibodies can be more easily selected for with purified recombinant protein production and

35   peptide synthesis to display limited/selected epitopes. Many of these factors stem from the

36   location of the epitope that can affect the accessibility of the antibody to the epitope at a cellular

37   or molecular level, direct inhibition of target antigen activity, conservation of function despite

38   escape mutations, and even non-competitive inhibition sites. Through the incorporation of

39   novel computational methods for predicting antigen changes to model-informed drug design

40   development, superior vaccines and antibody-based therapeutics or diagnostics can now be

41   more easily designed, mitigating failures. With detailed examples, this review highlights the

42   new opportunities, factors and methods of predicting antigenic changes for consideration in

43   sagacious epitope selection.

Introduction

44

45   Antibodies and their fragments are increasingly important in diagnostics and

46   therapeutics development as evidenced in the ongoing COVID-19 pandemic [1,2]. An already

47   expensive process, diagnostics can fail due to escape mutations on the epitope that compromise

48   primer-based kits [2-4] or diagnostic antibody binding [5] even with sagacious rational antibody

49   design and engineering[6]. On therapeutics, antigenic epitope changes leading to escape

50   mutations contribute to the expensive drug failures. To improve success, Model Informed Drug

51   Discovery and Development (MID3) [7,8] was in pilot by the U.S. Food and Drug Administration

52   since 2018 [9] to support drug development [8,10].

53   The ability to selecting the right single antigen for diagnostics, therapeutics, and to an

54   extent that for vaccines targeting (e.g. choosing only the Spike over a whole virus), was

55   augmented through recombinant technology, where purified target antigens could be produced

56   and either injected into animals or used with *in vitro* antibody display methods e.g., phage

57   display for antibody selection. The same technology also supported the targeting of specific

58   epitopes on the antigen, where having antibodies specific to an epitope in a diagnostic kit can

59   improve selectivity and specificity. This specificity is useful when differentiating between

60   similar antigens e.g., between viruses such HIV and Hepatitis B (HBV) even when targeting

61   Reverse Transcriptase (RT) or distinguishing SARS-CoV-2 from MERS, and SARS when

62   targeting the RNA-dependent RNA polymerase (RdRp). Yet, being too specific in diagnostics,

63   as opposed for vaccinations, can also result in false negatives when the target epitope on the

64   antigen mutates beyond antibody recognition [11].

65   With advances in peptide technology, short/stapled peptides can also be used without

66   going to the DNA level for recombinant expression and over the commonly used whole cells

67   or antigens [12,13]. Recent developments in cyclic peptides and peptide vaccines allow for

68   immunization against specific conformational structures as epitopes instead of whole antigens

69   through mimotopes [14] as B cell peptides [15]. Such more selective methods can support the

70   development of therapeutics to reduce side effects due to off-target effects, although some level

71   of lower specificity could be of value for vaccines and to an extent, diagnostics to target

72   variants.

73   The selection of epitopes is thus a paradigm shift from chance-dependent antibody

74   development to a more rational purposeful approach, and it is dependent on the intended

75   application in guiding towards high specificity or to have inclusion of minor changes in which

76   this selection can occur as the two categories [16] of 1) linear/continuous, defined as a stretch of

77   amino acids sequences; 2) conformational/discontinuous, defined as sequence distal residues

78   in close proximity through protein folding, with the latter conformational type more prevalent

79   as B-cell epitopes.

80   About 96% of monoclonal antibody therapeutic candidates fail to make it to market [17],

81   costing close to tens of millions of dollars [18], augmenting the MID3 approach that includes

82   epitope prediction. Epitope prediction, traditionally based on amino acid physicochemical

83   properties such as hydrophobicity, flexibility, solvent accessibility and antigenicity [19-22] have

84   seen augmentation by machine learning methods to show promise for cancer [23] and even hybrid

85   experimental-computational approaches [24] involving deep neural network for MHC binding [25]

86   and attention-based LSTM networks [26].

87

88   **Epitopes factors**

89   **Accessible Epitopes (Cellular)**

90   For epitope selection particularly for therapeutics, accessibility of the epitope by the antibodies

91   is perhaps the first and foremost to be considered. For maximising success, extracellular targets

92   are typically picked for antibody therapeutic candidates with the exception for 'intrabodies'

93   where target cells produce the antibodies against intracellular antigens within itself [27]. Apart

94   from intrabodies, whole antibodies against intracellular targets often have limited penetration

95   of membranes (knowledge known to flow cytometry users) which will require intracellular

96   delivery methods [28,29] that are not always feasible for therapeutic use. Although unknown in

97   prevalence, there are instances where intracellular oncogenic markers can be targeted because

98   of externalization e.g. PRL3 [30]. Nonetheless, antibodies do have intrinsic advantages for

99   undruggable targets that are not suitable for small molecules [29] and the selection and

100   effectiveness of targeting intracellular proteins would require assessment on a case-by-case

101   basis.

102   With antibody intracellular delivery hurdles yet to be overcome, most current

103   therapeutics and diagnostic whole antibody targets are extracellular antigens. With

104   extracellular targets, there are many factors that can underlie the selection of epitopes. In one

105   example, glycosylation has been shown to impede antibody recognition in both the ongoing

106   COVID-19 pandemic [31] and HIV [32], with clear impact when occurring at epitope sites. Thus,

107   even with good epitope bioinformatics prediction scores or good experimental results from

108   bacterial produced proteins (where there are rare occasional exceptions), care must be taken

109   for the effects of post-translational modification on occluding or interfering with antibody

110   recognition.

111   Epitope occlusion in the form of cryptic epitope or cryptotopes [33] is reported in viruses

112   such as the Norovirus [34], Influenza [35], Ebola [36] and HIV **(Figure 1A)**, where apart from post-

113   translational modifications methods, HIV utilised the hypervariable regions in the Env to

114   occlude gp41 epitopes [37] from immune detection. On self-antigens, such occlusion of inducible

115   cryptic epitopes play a role in reducing autoimmunity [38], and in some cases, differentiating

116   disease states when exposed during pathogenesis e.g. prion disease [39]. In the unlikely situation

117   where targeting the viral receptor is not easily achieved, blocking the viral target (host cell

118   receptor) from viral spike binding can be performed, as in the case for SARS-CoV-2 [40,41] and

119   poliovirus [42], although care needs to be taken not to end up affecting host cell activity by the

120   antibody by over-activation (e.g. mimicking receptor stimulation and inducing of altered

121   signalling triggering uncontrolled microthrombosis, cell lysis and neutrophil activation [43]) or

122   by preventing activation (e.g. inhibiting hyaluronan clearance by liver cells [44]).

123   Antigens have the potential to bind antibodies in non-conventional CDR-dependent

124   manner by inducing binding pockets or the formation of stretches/patches on antibodies. This

125   phenomenon was observed for non-conventional immunogenic molecule such as nickel [45]

126   binding to Trastuzumab and Pertuzumab that can possibly underlie the disease pathogenesis of

127   nickel type-I allergy. The other example of induced binding is in the molecular dynamics (MD)

128   simulation of Trastuzumab binding to Her2, inducing a cryptotopes that facilitated Pertuzumab

129   binding [46]. While the synergism between the two clinical therapeutics were shown

130   experimentally to be due to the different epitopes on the same antigen without evidence of an

131   induced Pertuzumab epitope [47], the possibility of occluded epitopes ought to be sagaciously

132   considered depending on the desired application and utility.

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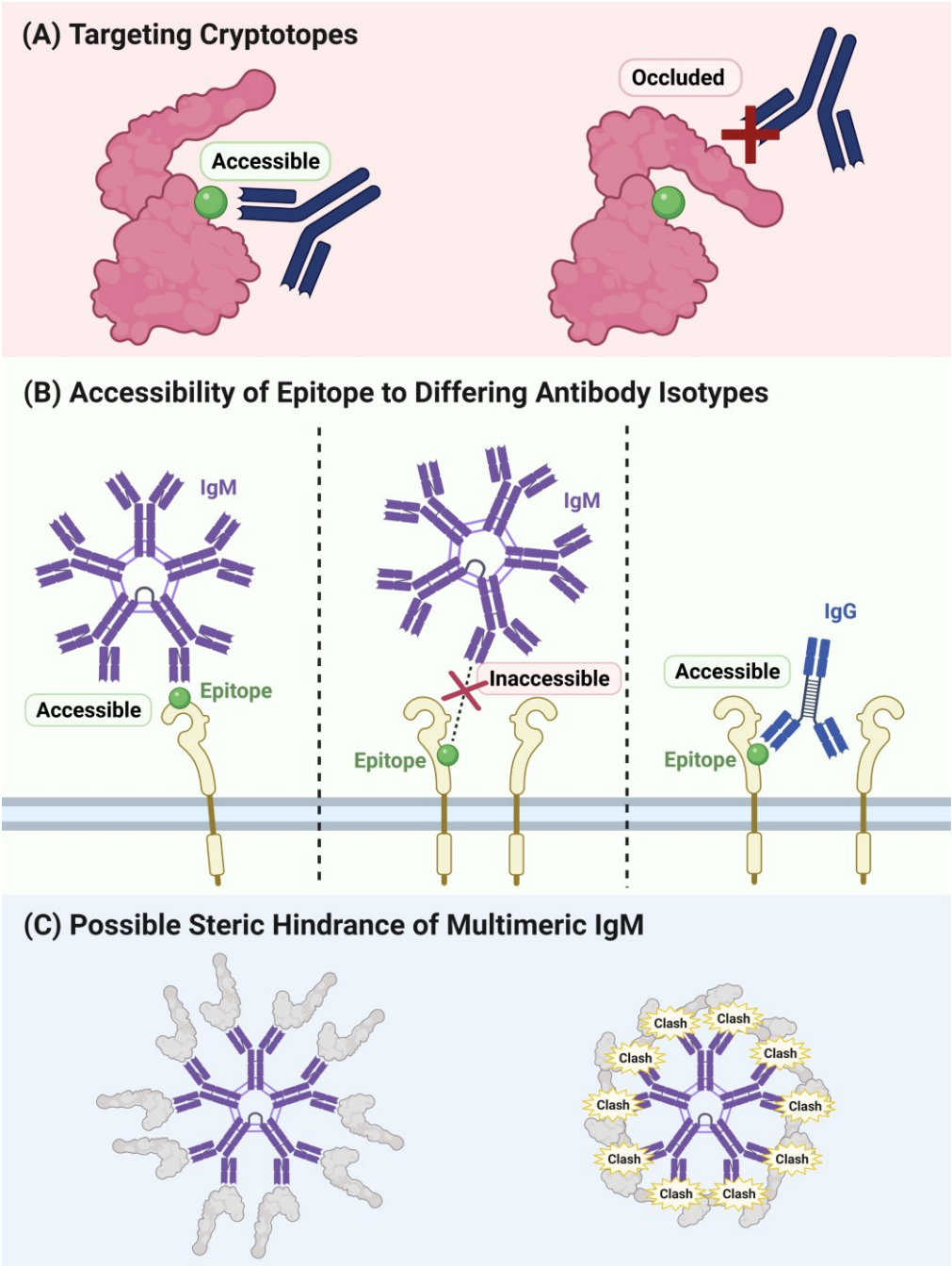
Figure 1. Accessibility of Epitopes. (**A**) Targeting cryptotopes. (**B**) Accessibility of epitope to differing antibody isotypes. The location of the epitope on the target antigen can affects its accessibility by antibodies that are conjugated or multimeric in nature due to steric hindrances. This is evident in internal epitopes that would not be accessible to multimeric IgMs but accessible to monomeric Igs or antibody fragments. (**C**) Steric hindrances for multi-valent binding by antibodies due to the location of the epitope on the target antigen. Created with BioRender.com.

Accessible Epitopes (Molecular): Lessons from IgM for multi-specific antibodies

Apart from access at the intra/extracellular level and occluded/induced epitopes, obvious steric hindrances at the molecular level to access epitopes can impact the efficacy of the antibody **(Figure 1B)**. While the earlier example [46,47] showed synergistic binding of Trastuzumab and Pertuzumab simultaneously to their different epitopes on Her2, steric hindrances resulting from multiple whole or conjugated or multimeric antibodies are known to arise in flow cytometry[48].

When made into multimeric IgMs for multiple antigen binding, Trastuzumab IgM could not have full occupancy of its Fab regions due to steric hindrances binding to multiple Her2. This was however, not in the case for Pertuzumab IgM [49], which could then show higher avidity effects [50]. The sheer size of multimeric antibodies, while advantageous in agglutination for immune clearance and result in a better therapeutic or diagnostic than the traditional IgG, as evidenced in a nasal delivery for SARS-CoV-2 [51], need to be watched for possible steric hindrance **(Figure 1C)**. While IgM is used in hemagglutination assays [52-55], care needs to consider that the accuracy of such IgM based assays may be affected.

For this reason, the checklist to selecting any epitope for diagnostic or therapeutic application needs to ensure molecular accessibility, especially when intending towards large antibodies (whether conjugated or multimeric or whole). It should be noted that the textbook primary antibody response is typically IgM and steric hindrances may underlie (in part at least) why IgMs tend to have lower affinities for the antigen.

This concern can apply to the development of bi-specific antibodies requiring additional optimization [56]. Potentially limiting the effectiveness of multi-specific antibodies, especially those are intended to engage whole cells [57] despite the promises of doing so in bispecific T-cell engagers (BiTEs) for oncology [58], care must be taken of accessibility to maximize success. It is in this area that perhaps a more flexible antibody hinge at the CH1 of antibodies may alleviate some structural constraints as evidenced in IgA [59] and other isotypes [50].

166   **Conservation of Epitopes: Lessons from Viruses and Omalizumab-IgE**

167   Since escape mutations in the epitope results in antibody recognition failure, one key main

168   criterion of epitope selection is its conservation and this occurs in at least two levels of

169   conservation within the antigen even in the presence of mutations, or conservation within the

170   family to allow broad-spectrum targeting **(Figure 2)**. The earlier level of within the species or

171   viral type is exemplified in the recent COVID-19 pandemic where SARS-CoV2 spike

172   mutations led to decreased effectiveness of the early vaccines to novel variants [5]. On the level

173   for broad-spectrum protection, other viral vaccines such that against the human papillomavirus

174   (HPV) are found to induce cross-neutralizing antibodies [60].

175   Likely due to faster escape mutations in microbial pathogens, there are more

176   monoclonal antibody therapeutics against cancer than infectious diseases [61], where those

177   targeting the latter tend to be polyclonal [62] to cover more epitopes to mitigate risk of escape.

178   Nonetheless, in the search for conserved regions in pathogens, large sequences databases can

179   provide some insights (such as HBVdb [63], Los Alamos HCV [64], HFV [65], HIV [66,67], Stanford

180   HIV Drug Resistance [68], GISAID [69] and Nextstrain [70]) but bearing in mind the constraints of

181   extracellular targets for both neutralization of viruses for therapeutics and detection in

182   diagnostics that do not require pre-processing to release internal contents. Though it should be

183   noted that broad-spectrum targets of such nature are expectedly limited due to viral trophism.

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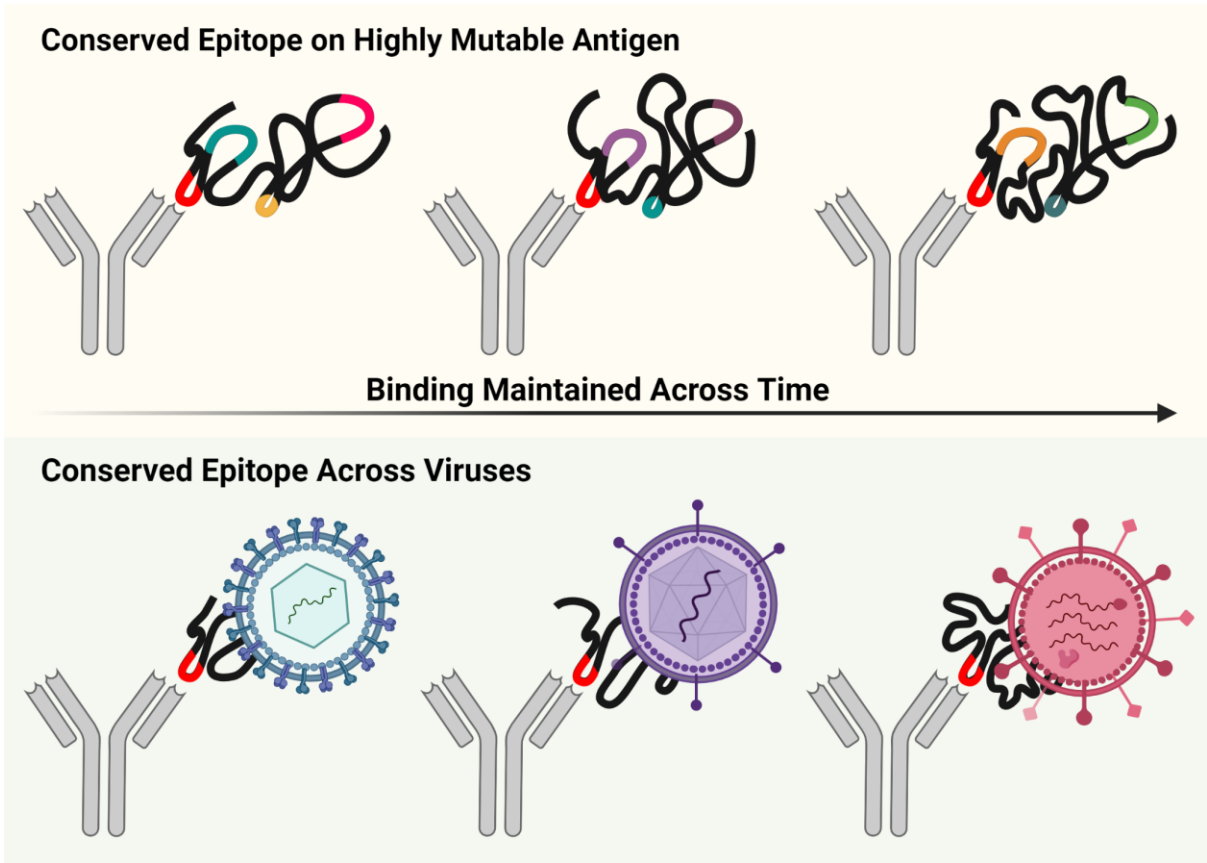
Figure 2. Conservation of epitopes across highly mutable target antigens (top) would reduce the chance of escape mutations even as the pathogen accumulates mutations in the target antigen to prolong the effectiveness of the antibody in either detecting or neutralizing the target. Similarly, targeting conserved epitopes that are conserved in similar proteins (e.g., Reverse Transcriptase) across viruses or pathogens can allow broad-spectrum detection or therapeutic intervention (bottom), improving its market use while also useful against emerging viruses utilizing enzymes of the same protein family. Created with BioRender.com.

192   Foresightedly, the overcoming of cellular level accessibility issues e.g. intrabodies or

193   novel delivery methods to target intracellular targets, conserved viral epitopes can expand to

194   viral enzymes [71] for better search of potential broad-spectrum antivirals. This can be performed

195   through a search of substrate analogues sites that indicate the presence of a functional domain

196   [72,73] that would be more conserved due to the preservation of enzyme functions.

197   In antibodies, the broad conserved target of Omalizumab (Xolair®) to IgE Fc allows it

198   to be effective against various IgE type I allergies [74]. Since the C-region of antibodies is

199   generally more conserved, Omalizumab was sagaciously raised against the C-region,

200   overlapping with the FcεRIα binding site. While the existence of allotypes in the Cε-regions

201   [75] can be an issue, another possible effects recently reported that can affect binding is the

202   allosteric communication from the V-region that could influence FcεRIαa interaction [76].

203   Despite overlapping with that of FcεRI, the V-region distal allosteric effects had negligible

204   effects on Omalizumab binding. This make the Omalizumab binding side a sagacious epitope

205   that displayed the following benefits: 1) at a conserved region across a variety of pathogenic

206   factors; 2) at the allosterically insulated regions that is robust to changes at hypervariable

207   regions; and 3) relatively immutable.

208   **Predicting New Mutated Epitopes: Lessons from Viruses**

209   The need for conserved epitopes in the face of mutations is amplified and shown to be crucial

210   by the challenge to existing COVID-19 vaccines by new variants [77]. In the lack of conserved

211   epitopes, the next best is to pre-emptively predict possible escape mutations in the epitopes and

212   design interventions against them. This is not only easily performed through recombinant or

213   peptide methods discussed above, but even easier to implement as vaccines with the rise of

214   mRNA vaccines [78].

215   Prediction attempts are seen in the polio virus RdRp in vitro platform leading to the

216   development of its vaccine [79] with similar attempts for HFMD causative Enterovirus (EV) A71

217   [80]. These methods of predicting new mutations exhibit great promise despite being *in vitro* and

218   can benefit from *in silico* augmentation involving network analysis [81], robust statistical model

219   building [82], time series [83], stacking models [84] and random signature analysis [85,86]. Nonetheless,

220   it should be noted that EVs and lentiviruses like HIV, mutate through recombination whereas

221   Influenza use the Orthomyxovirus family method of assortment, requiring further research to

222   get to a unified computation method capable of dealing with RNA viruses of different mutation

223   methodologies.

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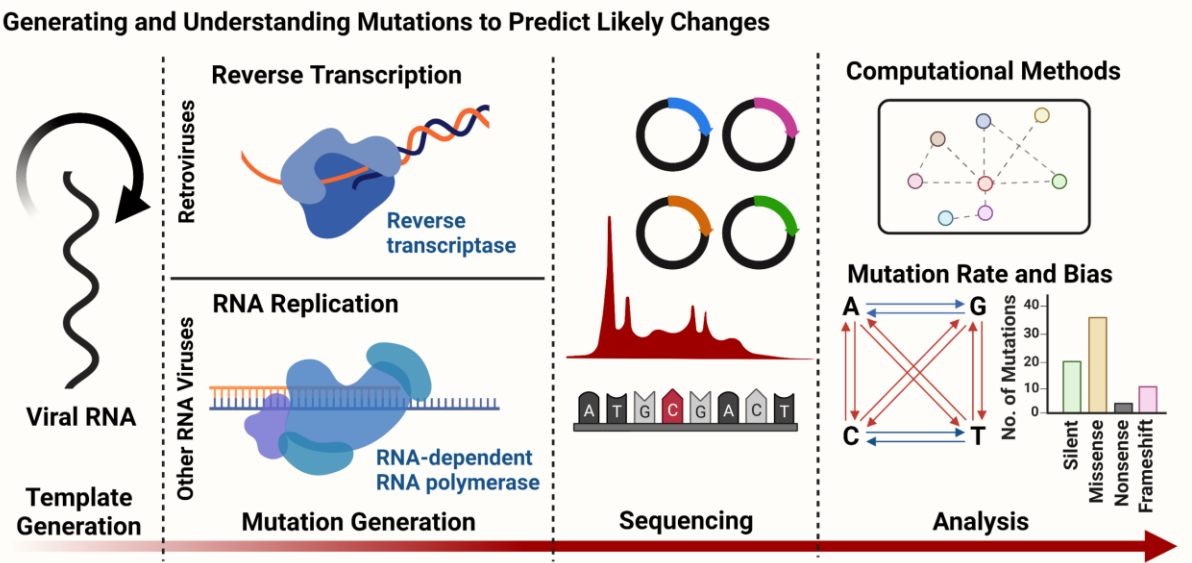
225   **Figure 3**. Schematic of mutation prediction of RNA viruses that can be generated *in vitro*

226   (adapted from [87]) to determine innate viral polymerase mutation rates, hotspots and biases for

227   the incorporation into computational methods for better prediction. Such a platform would not

228   only provide insights to the viral polymerases for augmentation towards lethal mutagenesis,

229   but also improve preparedness to identify conserved regions and identify potential mutations

230   of concern that can lead to escape to guide drug and diagnostic kit development. Created with

231   BioRender.com.

232   Experimentally, there is already notable progress in predicting mutations through the

233   understanding of molecular biology and the innate biases of pathogen enzymes involved in

234   replication (**Figure 3**). While such predictions are often deemed stochastic and unpredictable

235   [88], recent evidence has shown certain propensities in the genetic code [89] that can confer a certain

236   degree of predictability. It is certain that with more knowledge about the target biology, the

237   less stochastic and more predictable mutations in the given target can be. This is demonstrated

238   recently where even without immune or drug selection pressures, HIV reverse transcriptase

239   displayed clear mutational biases to induce certain types of mutations in specific locations in

240   HIV genes within a mimic of a single replication cycle [87]. Given that a high percentage of the

241   generated mutations were found in patients, the rise of drug-resistant mutations in the HIV

242   genes were not entirely stochastic nor required intense immune or drug selection pressures to

243   emerge. As the 'Godzilla' of fast mutating viruses, studies showed that the cross resistance for

244   protease inhibitors [90] and reverse transcriptase inhibitors [91] could be linked by network analysis.

245   Such analysis showed how mutations can confer not just resistance to one inhibitor, but to

246   others in cross-resistance. Thus, in the vein of MID3, structural modelling studies can study

247   the possible effects of likely emerging mutations with respect to drug resistance for this is not

248   an inexhaustive range of possibilities given that the mutations would be constrained by the

249   function of the target. And it is also in this area that therapeutics that augment lethal

250   mutagenesis for error catastrophe [92,93] could be an alternative strategy for undruggable targets.

251   **Enabling better direct inhibition: Lessons from Her2 and IgM**

252   While alternative strategies exist as in non-competitive non-nucleoside RT inhibitors or

253   NNRTIs [94], the related above-mentioned lethal-mutagenesis-based methods [95], direct inhibition

254   of targets is the most common approach of therapeutics. While it is expected that epitopes at

255   activation sites are key to this, consideration of the antibody can augment the success of this

256   where larger molecules may inhibit binding better.

257   In the above mentioned example of steric hindrances of Trastuzumab and Pertuzumab

258   IgM to Her2, Pertuzumab IgM inhibited cell proliferation better than Pertuzumab IgG1 [49]. This

259   was interesting because Pertuzumab IgM being larger/multimeric may have prevented Her2

260   homodimerization/activation, necessary for its oncogenic effects [96] better than its IgG1

261   counterpart based on the stoichiometry of binding sites. This is especially given that the

262   experiments were performed *in vitro* without effector immune cell effects and that unlike the

263   Trastuzumab binding site [97], the Pertuzumab binding site directly inhibits homodimerization

264   [98].

265   Thus, the selection of epitopes directly at the dimerization sites or near them while

266   allowing direct therapeutic inhibition as opposed to simply binding the antigen for just immune

267   tagging for effector cells, can be coupled by the use of multimeric or conjugated antibodies for

268   better direct effects (**Figure 4A**). And this is relevant not only in oncology where over-

269   expressed biomarkers lead to proliferation, but also in infectious diseases where most

270   therapeutics aim to neutralize virus attachment and entry [99] and now more recently, narrowing

271   to possible superantigenic areas in the spike. With further understanding of viral spike proteins,

272   epitopes that facilitate direct inhibition of potentially deadly viral pathological effects ought to

273   be considered. With recent evidence showing the SARS-CoV2 spike to also possess

274   superantigenic characteristics that can cause hyperinflammation leading to multisystem

275   inflammatory syndrome in children (MIS-C) and cytokine storms in adult COVID-19 [100], such

276　　regions can be blocked through epitope selection of the region or mitigating the clinical

277　　morbidity. Such an approach may provide the needed paradigm shift in treating viral infections

278　　[101] that have superantigenic root pathogenesis. Although antibodies may already bind to such

279　　superantigenic motifs sites, further in-depth consideration of such recognition sites [102] will

280　　facilitate epitope selection for direct viral pathogenicity blocking in addition to that of

281　　attachment and entry.

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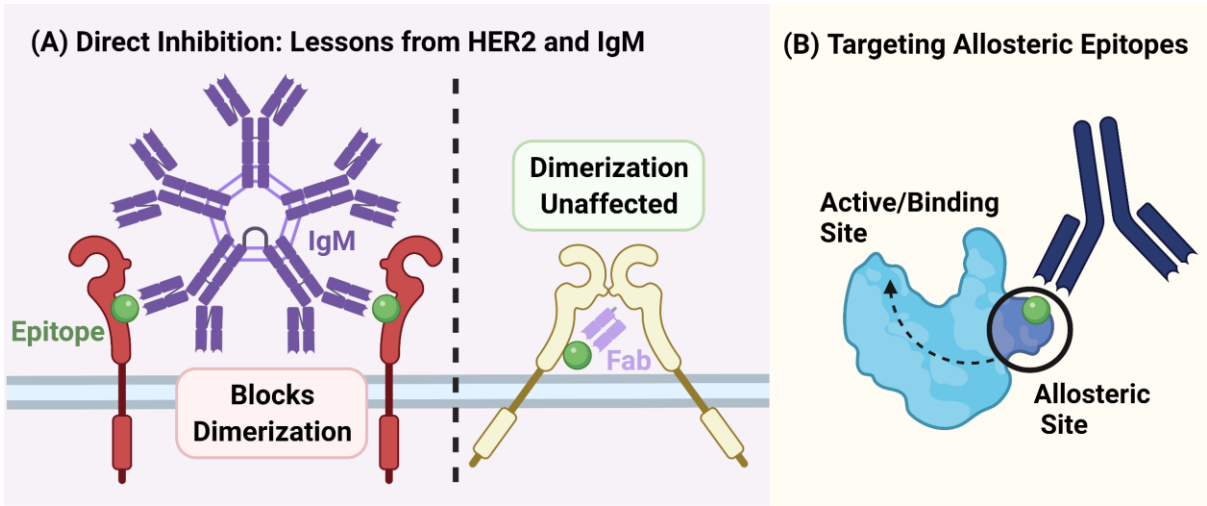
283   **Figure 4**. Choosing the epitopes for their potential functionality to (**A**) directly inhibit

284   dimerization in the case of Pertuzumab IgM inhibiting Her2 dimerization better than its IgG

285   counterpart due to its larger size[49]. In such cases, an antibody fragment such as a Fab alone

286   may not necessarily provide sufficient steric hindrance to block dimerization. In the event

287   where direct blocking may not be suitable, the targeting of (**B**) allosteric epitopes to influence

288   active or functional sites can also be explored to increase target areas in the same target antigen.

289   Created with BioRender.com.

290   Evident from oncology and HIV virology requiring multi-pronged interventions, where

291   in the former combinatorial synergistic use of Trastuzumab and Pertuzumab showed promise

292   [47,103], and that combinatorial antiretroviral therapy (ART) remains the dominant intervention

293   for HIV [104] in the latter, there are additional factors in epitope selection.

294   To add to additional targetable sites even within the same antigen and in the absence of

295   ideal epitopes for direct intervention, allosteric epitopes can be both an additional and

296   alternative solution. Evident in the use of NNRTI and recent evidence of non-enzymatic

297   subunit being potentially druggable in HIV [105], distal epitopes can be used to influence the

298   activate of the enzyme/target's active sites by non-competitive antibodies (**Figure 4B**). While

299   allosteric sites may not necessary elicit obvious shape changes [106] thus often being modulators

300   rather than activators/inhibitors (see examples: IgE [76], FcεRI [107], IgA [59], microbial targets [108]),

301   dampening of oncology or virology effects may sometimes provide the necessary gain needed

302   for the immune system. Furthermore, targeting allosteric sites in microbial and viral proteins

303   could drive function-crippling mutations through drug resistance mechanisms at the drug

304   binding site. Evidence of allosteric sites are present in the HIV Gag protein where non-cleavage

305   site mutations contributed to drug resistance associated protein fitness compensation [109,110].

306   These sites could be epitopes for intrabodies that in this example, either prevent drug-resistance

307   or reduce protein fitness Such epitopes can be leveraged upon to dissociate already bound

308   complexes or simply to increase dissociation for a modulation of the effects (e.g. in the case of

309   IgE-FcERI [107]).

310   With additional areas to target apart from active/functional sites, it may be easier to find

311   common allosteric sites to allow development of broad-spectrum targeting antibodies, and such

312   widening of the search field may overcome constraints present in extracellular localization.

313   While the allosteric analysis is currently most effectively performed computationally, it is

314   beneficial to consider the entire protein structure/model as holistically as possible to study the

315   distal effects in a counterintuitive manner when raising antibodies based on the reductionist

316   approach in experiments of using purified recombinant proteins, regions or peptides. While

317   many new databases now include annotated posttranslational modification such as PTM-SD

318   [111,112], the majority of crystal/NMR structures in structural databases are without post-

319   translational modifications which can have mismatches with *in vitro* expressed cell lines with

320   varying glycosylation patterns [113].

321   **Applications, Solutions, and Conclusion - Holistic Epitope selection**

322   Sagacity in target and epitope can maximize the success for vaccine, therapeutics, and

323   diagnostic development. Apart from deep understanding of the target biology, there are

324   numerous factors from accessibility at the cellular and molecular level, the inducibility,

325   occlusion, conservation at family and species levels, influence by distal allosteric sites, that can

326   now be considered not only individually but together. Coupling such efforts with sagacious

327   antibody development [6] that can involve swapping of elements [114], the very expensive

328   developmental processes can be made more cost and time efficient.

329

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331

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