Synthetic approaches to modulate and understand activation of the human complement system
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Chapter 6

Summary and general discussion

6.1 Summary

Immunotherapeutics induce clearance of malignant cells, this can be achieved by recruitment of complement of the human innate immune system via monoclonal antibodies (1-3). Such monoclonal antibodies have had clinical success and are under continuous development (4, 5). Similarly, bispecific complement agonists function to activate the complement system, however their molecular properties/structures differ from monoclonal antibodies (6, 7). In this thesis we have investigated and developed bispecific C1 agonists, which have been studied using minimal synthetic systems. This chapter summarizes the content and findings of all previous chapters and then discusses future directions for the projects that were presented, some of the project's limitations and then more broadly drug discovery via screening methods using both in vitro and in silico techniques.

Chapter 1 provides an overview of current strategies in complement modulation, with a strong focus on agonism via C1 targeting. In recent years, protein engineering of antibodies has gained traction in (pre-)clinical testing and underlines that complement is a viable target for such endeavours. In line with these observations is the development of synthetic C1 agonists, which comprise antibody fragments, both Fab domains and nanobodies.

Proteinaceous bispecific agonists of C1 inspired us to start developing fully synthetic C1 agonists. In **Chapter 2** a peptide was derived to bind and activate C1 using a high throughput screening method; random nonstandard peptides integrated discovery (RaPID). The 10^{12} peptide library was screened against the globular head domain of C1q and a single peptide sequence was found which could bind C1 in the presence of human serum. Next to the agonistic properties of the peptide, competition-based assays revealed antagonistic capabilities that inhibited the propagation of the complement pathway, but not C1 binding to native ligands.

In **Chapter 3** a bispecific nanobody agonist with proven efficacy in cellular models was tested in a synthetic system. This was done to explore some of the limits by which such a construct can effectively initiate complement activation. The C1 agonist comprised C1qNB75 to bind C1, and NBalfa to bind a synthetic peptide antigen. Simple DNA linkers enabled control over antigen positioning on increasingly long and flexible antigenic targets. It was found that a clear drop in complement activation was observed once the targets reach a threshold length of ~15 nm. These observations were also consistent for a protein system where the linker length between the nanobodies was changed.

Chapter 4 built on the findings of **Chapter 3** using a similar, though more intricate, DNA nanotechnology system. Here, the effect of antigen clustering was investigated. While a clear threshold such as in the previous chapter was not found, a marked inefficiency of the agonist was discovered in several conditions. It was speculated that the inefficiency was a consequence of strong binding of the bispecific. Indeed,

lowering binding affinity of the bispecifics by mutagenesis of specific residues in the C1qNB75-gC1q interface overcame the observed inefficiencies. Variable binding affinity of the bispecific nanobody yielded differential outcomes between differentially-positioned antigens. It would be interesting to keep pursuing antigen positioning and the effect this has on C1 agonism. However, such an investigation would benefit from an in-depth analysis of known antigen patterns of desirable membrane protein targets.

In **Chapter 5** production routes are presented by which (semi-)synthetic agonists can be produced. This was done to build a pipeline by which cell-targeting bispecifics could be generated. The methods that have been presented are not uniquely suitable for the production of these compounds, although these do provide a good starting-point. The production route of bispecific peptides, based on the cL3 peptide from **Chapter 2** yielded pure products, using a straightforward click-chemistry based method. Additionally, peptide-nanobody bispecifics were produced with a reaction efficiency that compares to similar methods in the literature. Currently non-proteinaceous molecules are typically not used to achieve complement agonism and may widen the scope of what is possible in membrane targeting.

6.2 RaPID

For the work in **chapter 2** RaPID technology was used. A selection was performed against gC1q, which was successful. From that selection, a single peptide sequence cL3 was derived. While for a proof-of-concept study this peptide works satisfactorily, the solubility and binding affinity of the peptide may be improved. Therefore, RaPID could be used to generate more gC1q targeting peptides. One of the major benefits of the RaPID selection is the incorporation of unnatural amino acids, which can be used to enhance target specificity or peptide stability (8, 9). Methylated amino acids for example could improve serum stability of the peptides. Additionally, an all-D amino acid library could be used to perform the selection and peptide size can be modified (10). From our selection, peptides were derived with hydrophobic patches within them and comprised mostly apolar residues. Tuning the amino acid mix toward more hydrophilic residues could improve the polarity of the hit sequences. The advantage of using the RaPID technique is that these selections can be performed in parallel, and that with the high throughput sequencing multiple selections can be analysed at once. Such an approach could potentially deliver >50 peptides to test, perhaps more, and therefore requires modalities to produce those peptides at small scale, preferably in parallel.

In the previous selection a recombinant version of gC1q was used (11). This has a number of benefits, mainly a high yielding expression and the ability to imbue the protein with tags that are needed for the selection. Nevertheless, another selection round could be performed on native gC1q, which is purified from serum and cut from the collagen stalk domains by means of enzymatic digestion (12). Then the native

gC1q can be biotinylated and used as such. Depending on the digestion protocol, the glycosylation of the protein will be more native-like and native gC1q does not contain the proteinaceous loops that connect the A, B and C domain in single chain gC1q.

6.3 cL3-gC1q binding interface and specificity

Now that the initial characterization of cL3 has been performed, this should be expanded on. A structural characterization has not yet been achieved. Structural information on the binding interface between cL3-gC1q would open up many opportunities for rational optimization of the peptide. X-ray crystallography can be used to achieve this, as single-chain gC1q has been crystalized before (11, 13). We have indeed produced crystals of gC1q, however not a cL3-gC1q co-crystal, which was therefore not discussed in detail in this work. There are several other options for crystallography, mainly pre-formation of the protein crystal and subsequent soaking of the peptide into the crystals. It is important to keep in mind that the ~2500 Da size of cL3 may not be favourable for such an approach, depending on the crystal packing of gC1q. Furthermore, the solubility issues of cL3 may present an issue as well. Crystallography requires high protein and thus high peptide concentrations, at which cL3 is prone to precipitation.

CryoEM can be used as an alternative, however the 50 kDa size of gC1q is a limiting factor. This is a known issue and can be overcome by using other gC1q binders to make gC1q larger and more asymmetrical. The C1qNB75 may be used, although small (13, 14). There is an anti-nanobody Fab that could increase the size of the gC1q-NB complex further by binding the C1qNB75 as it is bound to gC1q. Of note, however, is that the binding interface of anti-NbFab on the generic Nb constant region differs from the C1qNB75 region, which could disturb the binding, which would require several point mutations in the C1qNb75 constant region for Fab binding (15).

Next to elucidation of the structural aspects of the cL3-gC1q interaction, the specificity of cL3 was not investigated. For that purpose, biotinylated cL3 can be used in pull-down assays and investigation by mass spectrometry. This would be helpful in decision making on whether to pursue optimization of cL3 or to perform a more elaborate peptide screen.

6.4 Generation of bispecifics

For the production of nanobody-peptide conjugates we utilized a click-chemistry based approach. Alternatively, a maleimide-based production would also have been viable (16-21). We achieved efficiencies that were approximately 50%, which is in line with the efficiency of similar strategies in the literature (16-21). Alternatively bispecifics can be generated that comprise only protein fragments such as antibodies, antibody fragments or nanobodies. A wide variety of designs are possible, ex-

emplified by the review in *mAbs 2017*, containing 19 classes of bispecifics and more than 100 examples of unique bispecific antibody designs (22). Therefore, bispecific design may be performed rationally, when the limitations towards a certain target are clear.

Even though, in our work, protein linkers had a limited effect on efficacy, in the design of bispecifics linkers can be an important consideration. Common GGS linkers for example are not efficient in maintaining distance, rather these form a "mushroom" conformation, similarly to PEG chains, and maintain relatively close end-to-end distancing (23, 24). Proline-rich or helical linkers maintain more defined distances (25-27). Therefore, linkers influence relative positioning of the bispecifics, which is of particular importance in multivalent systems (28-32). One other pragmatic consideration is that linkers matter in protein expression. Namely in **Chapter 3** the BC dual tandem alpha-helix (BCdta) construct expressed best, followed by BC60 and then BC16. These were produced in the same plasmid and expression systems and their only discernible difference was the linker connecting the nanobodies. The BCdta expression was about 2.5-fold higher than BC16. Interestingly, C1qNB75 was also produced with just the dta linker and that expressed with 4-fold the yield of the BC-dta construct. Such pragmatic considerations help designing the next generation of complement agonists. We believe that this dual helix motif functions as a solubility tag, but we can also speculate that the linker allows for spacing between the nanobodies during expression, which could be beneficial for correct folding.

6.5 C1 activation in synthetic systems

In **Chapters 3** and **4** synthetic systems were used to evaluate a bispecific agonist that recruits and activates C1 on synthetic bilayered membranes. These systems were compatible with serum and purified protein, allowing for detailed investigation of the activation process. Furthermore, the ability to adapt structural parameters via DNA nanotechnology on simple membranes provides a relatively high level of control compared to cellular membranes. One assay that gives insights into C1 activation was an enzymatic conversion assay determining the activity of C1s with a short LGR sequence. While satisfactory in its function, cheap and commercially available, some alternatives do exist that may be more suitable for other research questions. There are other, published peptide substrates that function similarly to the LGR substate that have higher affinity to C1s and may enhance sensitivity of the experiment (33). Furthermore, there exists different C1s activity assays that elucidate C1s activity in serum, rather than using purified C1 complex (34). While the latter is an indirect measurement, one could envision this being combined with ELISA based determination of C4 conversion, C3 conversion and C5b-9 conversion in parallel. Alternatively, although not shown in this work, we have tested conversion of purified C4 to be monitored with both western blot and SDS-PAGE and this approach is highly sensitive (35).

The DNA nanostructures that were used in those same chapters allowed for control over positioning of the bispecifics. The most significant benefit we found in our nanostructure designs was that these were folded with strands at stoichiometric equivalents, rather than designs that use excess staples which bind to a large scaffold strand (36-38). The latter process is a common method for DNA origami nanostructure production. When working with excess DNA staples in nanostructure production, these need to be removed using spin filters or PEG precipitation techniques, which are less than ideal for nanostructure integrity at high nanostructure concentrations. While the yield of a single reaction of such nanostructures may be as high as 90% (39), this technique does not scale well, and indeed larger scale purification is currently limiting DNA nanostructure use (40). Should one pursue plans of analysis via DNA nanostructures in biological systems the ease of production and long-term stability in storage should be a major consideration. Nevertheless, DNA nanostructure development and our understanding of them is expanding continuously, and their demonstrated biological efficacy provided by their structural control of molecular moieties is a motivation to keep pushing the limits of the technology (41, 42).

With our synthetic liposome-based assays, lysis of those liposomes is the main readout and measure of efficacy. However, the effector functions of complement are not limited to the lysis of cells. In particular, complement component C3 is a potent anaphylatoxin which induces phagocytosis, and C1 itself is a ligand that can bind cell surface receptors, with various effector functions. In fact, pushing for the activation of the terminal pathway and lysis of cells may have a detrimental effect. Active complement in the tumour-microenvironment is implicated in tumour growth (43). Indeed, native agonists of C1 such as C-reactive protein rarely induce lysis, and therefore using lysis as a readout is not so much wrong as it is incomplete. Induction of cellular phagocytosis by complement activation could have been investigated as well. E.g. streptavidin coated beads, or liposomes, could be used. Depleted sera (C1, C4, C3, C5b-9 depleted) could be used as checkpoint markers to investigate whether phagocytosis may take place with or without induction of lysis. Clearance of pathogenic cells via phagocytosis may lead to better patient outcomes since no severe, or less severe, inflammation is induced.

6.6 In vivo application of complement agonism

While the prospect of tuning activation is promising, one can question whether in vivo efficacy is dependent on fine-tuning. Rather, parameters such as target specificity, drug stability and drug-tissue dispersion after administration may be more important (44). Furthermore, complement regulator expression (e.g. CD59, CD55) is variable between cell-types (45). Knowledge of the limitations that complement inhibitors put on complement activation is highly relevant for CDC-inducing moieties. If the cell target requires it, perhaps a dual drug dosing strategy in which both complement agonists and inhibitors of regulators are administered will be most effective. Indeed, neutralizing complement regulators CD55 and CD59 have increased performance

of complement agonists in models of leukaemia (46), melanoma (47), breast cancer (48), renal cancer (49) and neuroblastoma (50). Drug bioavailability is another important factor, in particular because the distribution of complement in the human body varies, both spatially and temporally (51). The efficacy of immunotherapeutics will depend on a balance of all these factors, which dictate whether complement and the therapeutic can meet at the right location at the right time (phrasing adapted from inauguration speech prof. Leendert Trouw, Leiden 2024).

6.7 In silico screening and the drug discovery pipeline

Screening technology is one of the most important methodologies in drug discovery. The capacity to generate a highly diverse and sufficiently large library of compounds (drug-leads) has proven to be an invaluable tool for many research fields (52). Library generation can be achieved with small molecules and peptides or biologics generated via immunization or phage/ribosome display (53). These screening tools use the power of numbers and, similarly, in silico methods can be utilized (54), including virtual compound screening, data mining and more recently the de novo generation of proteins e.g. nanobodies and enzymes using RFdiffusion (55, 56). The fact that de novo proteins can be designed is impressive and the success rate is increasing rapidly. RFdiffusion was only released last year, and already the hit-rate of RFdiffusion is challenged by the AlphaProteo software of Google's DeepMind. Their whitepaper reports a 9-88% success rate for seven clinically relevant targets (57). The stance of the software owners and governments regulations on ownership of in silico generated molecules will dictate their usefulness in drug discovery. In the next five years it will be interesting to monitor their success rate. Nevertheless it is already clear that protein structure prediction is receiving a tremendous amount of recognition by the scientific community. The Nobel prize for chemistry this year was shared between David Baker, Demis Hassabis and John M. Jumper, key figures in the development of computational protein design and structure prediction.

Currently in the drug discovery pipeline, only about 10% of candidate drugs that start in phase I trials make it through to regulatory approval (58). This number, 10%, seems almost a rule-of-thumb as it has virtually not changed in the last 20 years (59), with a high point of 24.5% and a low point of 5.9% in 2015 and 2022, respectively, according to the annual R&D report of the IQVIA Institute for Human Data Science. This indicates that drug testing and prediction of success is extremely complex, because an industry that invests ~70 billion dollars a year in R&D does not seem to be able to do better than a 90% failure rate.

In the initial phases of compound selection and characterization, the emphasis lies on molecular specificity, which concerns binding of the compound of interest to the molecular target exclusively (58). However, once molecular specificity is addressed, the complexity of the environment in which the drug has to operate only increases. In in-vivo trials the drug should be delivered to the right microenvironment. The rel-

ative abundance of the drug in organs and tissues is related to the drugs potency and off-target toxicity. An immense level of control is required to deliver the drug to the right organ, while simultaneously being able to distinguish between diseased and healthy tissue or cells. The balance of the properties such as aspecific binding, tissue dispersion and toxicity determine a successful outcome. A term which can be summarized as “off-target effects”.

In the drug development pipeline, prediction of off-target effects can be achieved using the microsomal stability test and cytotoxicity tests in relevant cell lines (60). Furthermore, developments of organ-on-a-chip and organoid technology provide the potential to administer these tests more reliably and even strive to replace animal testing (61, 62). It is interesting to speculate how early off-target effects can be addressed during compound selection and optimization. After initial hit-compounds have been generated, a rigorous negative selection regime could be implemented. An example of such an approach was performed in **Chapter 2**. A number of peptide sequences were obtained, then twelve sequences were synthesized and all of them bound to purified C1, however only one sequence bound C1 in the presence of serum. This showcases that serum is a complex medium and likely induced peptide instability or aspecific binding for most candidates. For future selections, C1 depleted sera could perhaps have been used to wash away any off-target peptides during the RaPID selection process. Doing such a selection during the screening process and before expensive methods such as high-throughput sequencing and solid-phase peptide synthesis are employed may make the process as a whole more efficient and cost-effective.

The question remains at which point stringent negative selection is most appropriate and what should be tested. One major caveat of stricter selection is that fewer leads are obtained. From a cost-benefit analysis in the broad perspective of the scientific field and society this is desirable; the earlier you remove false leads from the selection the more likely you are going to generate a successful compound during clinical trials. However, the researcher could end up empty handed quickly after a full selection cycle. In fact, if those models are effective, in some scenarios these *should* leave the researcher empty handed. In a real-world scenario that is a non-ideal outcome since only positive leads can be used to apply for grants and write papers. Thus, stringent negative selection in the earlier stages of compound generation are difficult to pursue, as these can “punish” the researcher. This does not even consider how challenging and trustworthy any negative selection may be as they are not in patient tests.

Even with extensive optimization we may not be able to increase the success of the drug discovery pipeline significantly. Clinical trials may also fail because of straightforward and practical considerations. During trials, up to 40% of patients withdraw because the trial location is inconvenient to reach (63). With trials costing 2.6 billion USD on average, this is a major limiting factor of pursuing them and leading success

factors seem to be time-management, patient availability and experience of the institute that performs the trial (63). Nevertheless, the scientific challenges remain, and some of the solutions to those challenges may be found in this work.

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