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Multivalent antibody-recruiting macromolecules: linking increased binding affinities with enhanced innate immune killing

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Abstract

Antibody-recruiting molecules (ARMs) are a novel class of immunotherapeutics. They are capable of introducing antibodies on disease-relevant targets such as cancer cells, bacterial cells or viruses. This can induce antibody-mediated immune responses such as antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) and antibody dependent phagocytosis (ADCP) which can lead to killing of the pathogen. In contrast to the classic ARMs, multivalent antibody-recruiting macromolecules could offer an advantage in view of increasing the efficiency of antibody

recruitment and subsequent innate immune killing. Such compounds consist of multiple target binding termini (TBT) and/or antibody binding termini (ABT). Those multivalent interactions are able to convert low binding affinities into increased binding avidities. This review summarizes the current status on multivalent antibody-recruiting macromolecules and gives insight into possible benefits, still to overcome hurdles and future perspectives.

1. Introduction

The immune system is a defense mechanism comprising different cell types and molecules to protect the host from invading pathogens such as viruses and bacteria as well as providing protection against cancer. The system consists of 2 types of immune responses: innate immunity and adaptive immunity. Innate immunity has evolved over millions of years and provides immediate host defense. Although the innate immune response is rapid, it is antigen-independent meaning it lacks specificity leading sometimes to damaging of normal, healthy tissues.[1,2] Nevertheless, the innate immune system ensures protection against everyday invaders through recognition of conserved features of foreign pathogens called pathogen-associated molecular patterns (PAMPs), such as viral nucleic acids or lipopolysaccharide on cell walls, recognized by pattern-recognition receptors (PRRs).[2,3,4] The adaptive immune response on the other hand takes more time to develop, but is pathogen-specific. Moreover the adaptive immune system holds the capacity to elicit immunological memory, thereby inducing a more vigorous response to re-exposure of the same antigen.[5]

Pathogen-specific antigens can be recognized through antigen-specific receptors on T and B lymphocytes.[2,6] When B lymphocytes become activated by antigen-encounter, they will differentiate into plasma cells and this can result in, amongst other things, the secretion of large amounts of antibodies directed to the respective antigen.[7,8] These antibodies are able to bind to their antigens exposed on the surface of the pathogen. Through recognition of the Fc domain of the antibody by Fc receptors present on immune effector cells or proteins, the pathogen can be eliminated by several innate immune mechanisms such as complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC) and antibody dependent phagocytosis (ADCP) (**Figure 1**).[5] Hence, Fc receptor mediated antibody-dependent killing of pathogens and cells forms a bridge between the innate and the adaptive immune system by combining potent innate effector immune mechanisms with the diversity and specificity of the adaptive immune system.[9]

Importantly, besides playing a crucial role in pathogen clearance from the host, several studies indicated that CDC, ADCC and ADCP are a substantial component of tumor rejection in monoclonal antibody therapy, a form of targeted immunotherapy. Besides their potential direct anti-tumor effects such as blocking receptor signaling or sometimes delivery of a cytotoxic agent, they are also able to trigger the aforementioned immune mediated pathways that can operate in parallel to induce a cytolytic response.[10,11,12,13]

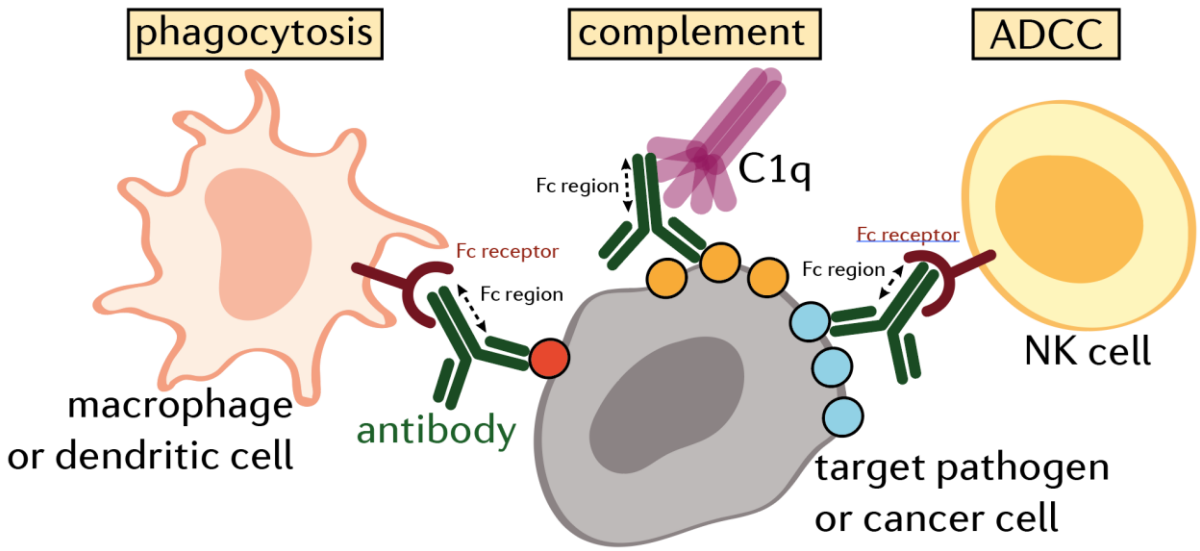

Figure 1. Innate immune killing mechanisms upon recognition of the Fc-domain of surface-bound antibodies on a target pathogen or cancer cell.

ADCC is triggered by recognition of the Fc domain of cell-bound antibodies by NK cells. NK cells are a subset of innate lymphocytes that are able to kill virus-infected and tumor cells. They express a series of transmembrane receptors including activating receptors, inhibitory receptors and co-receptors. These receptors control the activation, proliferation and effector functions of NK cells. One class of receptors present on the cell surface are the Fcγ receptors (FcγRs), more specifically FcγRIIc (CD32c) and/or FcγRIIIa (CD16a) that bind the Fc part of human immunoglobulins. Upon activation by antibodies bound to the target cell, NK cells are capable of forming immunological synapses with target cells allowing the release of cytotoxic granules containing perforin and granzymes to lyse the target cell. This mechanism, called ADCC, can be triggered within minutes without requiring transcription, translation or cell proliferation.[14,15,16,17]

The complement system, on the other hand, consists of a complex network of plasma and membrane-bound glycoproteins, cofactors, receptors and regulatory proteins. The system can be activated through three different pathways: the classical, the mannose-binding lectin and the alternative pathway. Antibodies bound to a cell surface are able to initiate the classical pathway. After binding of the C1 complex to the antibody Fc region, a cascade of proteolytic reactions is induced leading to the assembly of the membrane attack complex (MAC) which leads to lysis of the target pathogen or cancer cell. Furthermore, pro-inflammatory anaphylatoxins are generated and diverse complement opsonins can lead to target clearance.[10,11,18,19]

Phagocytosis is a complex process that is defined as the cellular uptake of large particles (> 0.5 µm) and includes the ingestion and elimination of pathogens but also of apoptotic cells. Professional phagocytes include neutrophils, monocytes, macrophages, dendritic cells, osteoclasts and eosinophils. Ingestion of the pathogens and/or apoptotic cells is initiated by the recognition through specific receptors present on the surface of such phagocyting cells. These receptors can be divided into two different groups: nonopsonic and opsonic receptors. Nonopsonic receptors will directly

recognize molecular motifs on the surface of the target whereas opsonic receptors will recognize host derived opsonins bound on the target. Two major examples of opsonins are antibodies and components of the complement. Fcγ receptors, present on leukocytes, are capable of recognizing Fc portions of IgG molecules whereas complement receptors will be able to recognize the components of the complement cascade. In this way, phagocytosis is induced. Initiation of a series of signaling cascades will lead to ingestion of the target. Upon the latter process, ingested materials will be located inside intracellular phagosomal vesicles that will subsequently mature through fusion with endosomes and lysosomes. This results in phagolysosomes, vesicles with a different membrane composition and a very acidic and degrative environment.[20,21,22]

2. Monoclonal antibodies in cancer therapy

Monoclonal antibodies are amongst the most important therapeutic agents for cancer treatment. They have been established as one of the most successful therapeutic strategies for both hematological malignancies and solid tumors. Antibodies that are specific for antigens that are expressed by the tumor itself can be used for targeted delivery of radioactive isotopes or chemotherapeutic agents when those are conjugated to each other. Unconjugated antibodies can have multiple mechanisms of action. They can exert their effect through direct tumor cell killing, for example through receptor blockade, aid in immune mediated killing such as immune checkpoint blockers, through specific effects on the tumor vasculature or stroma, or a combination of the above.[23,24,25] In the latter regard, the mechanism of action of immune checkpoint blocking anti-PD-L1 antibodies has been ascribed to a combination of both blocking of the PD-L1/PD-1 axis as well as by triggering innate effector killing against anti-PD-L1 targeted cancer cells.[26]

Although monoclonal antibodies can have inherent anti-tumor activity and thus improve the patient's outcome, they can be associated with severe and sometimes life-threatening side effects. These can range from organ-specific autoimmunity, such as gastro-intestinal toxicity (diarrhea, colitis), dermal toxicity (pruritus, exanthema), liver toxicity, endocrine toxicity or pneumonitis, which are transient and reversible on antibody clearance, to the life-threatening cytokine release syndrome which causes systemic inflammation through the generalized systemic induction of pro-inflammatory mediators.[24,27,28,29] Autoimmune toxicity is defined as on-target, off-tumor toxicity. It results from antigen-specific attack when tumor associated antigen is present on non-malignant tissue. The cytokine release syndrome, on the other hand, is not antigen-specific but occurs as a result of high-level immune activation.[30] Furthermore, therapy with mouse-derived monoclonal antibodies is restricted by immunogenicity issues. Even for chimeric, humanized and fully humanized monoclonal antibodies, generation of antibodies against the idiotype is still possible. This shows that alterations in particular amino acids at certain positions can influence immunogenicity and lead to adverse effects and loss in efficacy.[29] Another hurdle regarding monoclonal antibody therapy is the varying distribution in the tumor mass and limited penetration depth. A few typical characteristics of solid tumors such as heterogeneous antigen expression within the tumor mass, heterogeneous blood

supply, elevated interstitial pressure and mechanical barriers such as tight junctions can contribute to these hurdles. In addition, deep penetration in the tumor can be limited due to strong antibody binding to antigens shortly after leaving the capillaries, thereby preventing diffusion to more distant antigen sites in the tumor.[31,32] Together with a rather expensive and difficult production process, monoclonal antibodies undoubtedly have several disadvantages. Hence, more simple synthetic compounds that are able to recruit endogenous antibodies to a cancer cell surface could be a viable alternative.

3. Antibody recruiting molecules

Antibody recruiting molecules (ARMs) are synthetic bifunctional molecules which are capable of introducing antibodies on a disease-relevant target. They consist of a target binding terminus (TBT) which is able to interact with the pathogen or cancer cell surface and an antibody binding terminus (ABT) being a hapten (a small molecule which is not able to provoke an immune response, unless it is attached to a larger entity such as e.g. protein) that is able to bind anti-hapten antibodies. By doing so, a ternary complex is formed between the pathogen or cancer cell of interest, the ARM and the antibody. The subsequent clustering of antibodies on a target surface is capable to induce antibody-mediated immune responses, including ADCC, CDC and phagocytosis, to kill the target.[33]

The first class of ABT are able to bind non-endogenous anti-hapten antibodies which means pre-immunization against the specific hapten is needed. For the latter, the hapten of interest is typically conjugated on a carrier protein (e.g. keyhole limpet hemocyanin (KLH)) which contains T-helper epitopes that aid to provoke a humoral immune response and the generation of anti-hapten antibodies. A significant benefit here is that the hapten can be chosen based on the desired physical and/or chemical properties.[33,34] An example of such a hapten is fluorescein, selected for its photophysical properties. Besides inducing antibody-dependent immune responses, fluorescein can be simultaneously used for fluorescence imaging of for example the tumor.[35,36]

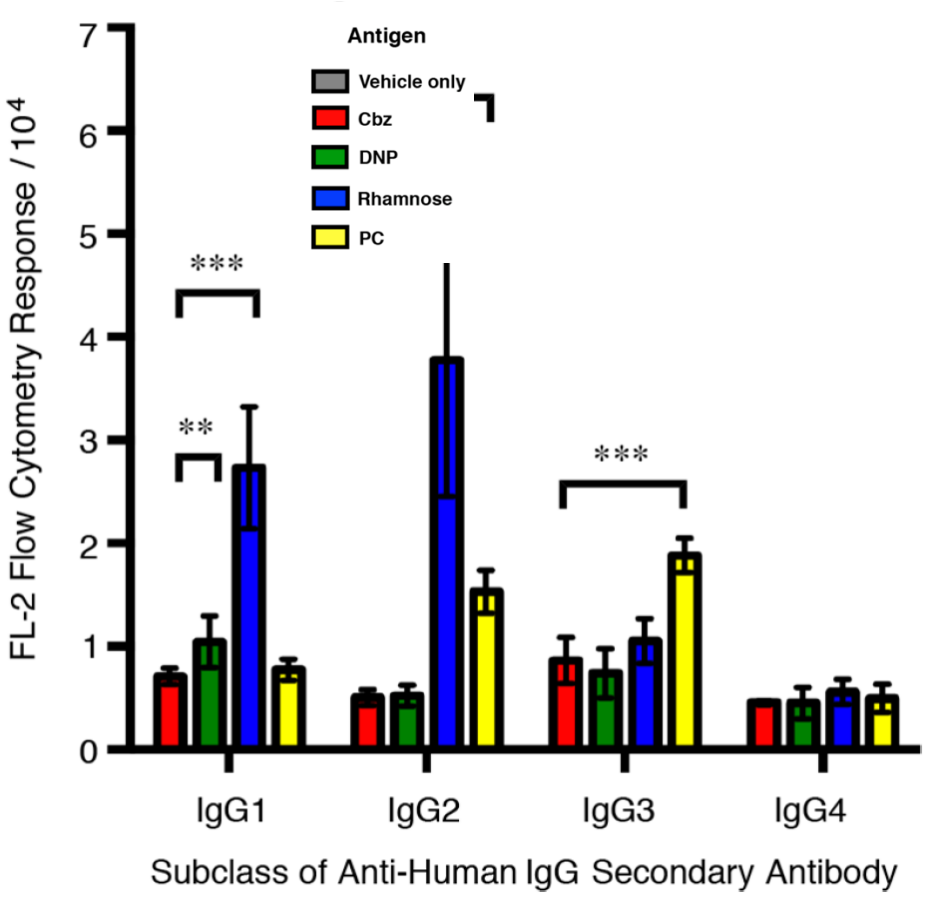

Figure 2. Flow cytometry analysis of binding of antibody isotypes from human serum to hapten-modified cells. Figure adapted with permission from ref [42].

Another option is to introduce haptens that can be recognized through endogenous antibodies, i.e. antibodies present in the serum of every human being. This approach seems particularly promising since healthy human serum can be turned into a cytotoxic agent against for example cancer cells. The most common used haptens in this context include 2,4-dinitrophenol (DNP), galactosyl-(1-3)-galactose (α-Gal) and L-rhamnose (rha).[33,34,37] The origin of anti-DNP antibodies is not fully known, but thought to be due to exposure to certain dyes, preservatives and pesticides.[38] The α-Gal epitope on the other hand is found in various tissues of non-primate mammals and bacteria. Hence human individuals, will recognize this epitope as non-self.[39] L-rhamnose is a deoxy sugar present in the cell wall of different microbes and plants. Both anti-α-Gal and anti-rhamnose antibodies are possibly generated through constant exposure to the epitopes present in the commensal gut flora.[40]

It is estimated that anti-α-Gal comprises up to 2 % of circulating IgM and 3-8 % of IgG. Naturally occurring anti-DNP antibodies on the other hand are present at rather low concentrations compared to other naturally occurring antibodies. Interestingly, it has been reported that L-rhamnose specific IgG antibody titers in human serum are significantly higher compared to DNP and α-Gal (**Figure 2**). Of all 3 haptens, endogenous anti-hapten antibodies span the different isotype classes, with according to Jakobsche et al. significant differences in isotype prevalence depending on the type of

hapten (**Figure 2**). With IgG1 in humans being the most potent isotype for inducing Fc-mediated innate immune killing[41], haptens such as DNP and rhamnose that correlate to high IgG1 anti-hapten titers might be of particular therapeutic relevance. Moreover, the affinity of the corresponding antigen-antibody complex has been reported to be higher for L-rhamnose, further advocating it as being an interesting candidate for the design of antibody recruiting molecules.[40,42] Despite this fact, of the 3 main haptens, DNP has received the most attention in the context of design antibody recruiting molecules, likely due to the commercial availability of affinity maturated anti-DNP antibodies, including mouse and human monoclonals that bind DNP with much higher affinity than endogenous anti-DNP in human serum[43]. However, it is also worth noting that DNP is also prone to non-specific binding to albumin[44] and to aromatic residues such as tryptophan in the antigen binding site of antibodies[45,46].

By varying the ABT and TBT, many different ARMs systems have been developed, acting against a broad variety of disease relevant targets, including viruses and virally infected cells[47,48,48], bacteria[36,49,50] and cancer cells[51,52,53].

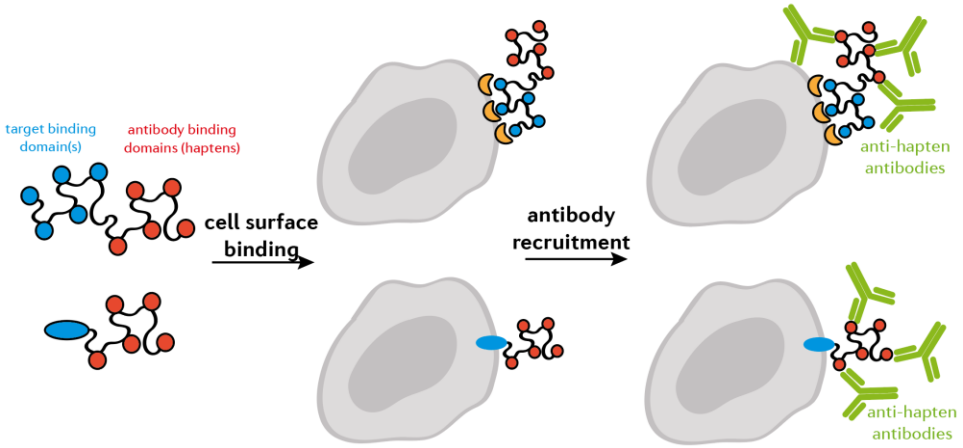

Figure 3. Antibody-recruiting macromolecules concept illustrated for systems with a single or with multiple target binding domains. Cell surface binding triggers recruitment of endogenous antibodies to the cell, thereby flagging the cell for destruction by innate immune effector mechanisms.

4. Multivalent antibody-recruiting macromolecules

In contrast to the classic ARMs consisting of one ABT and one TBT, multivalent macromolecules consisting of multiple ABTs and/or TBTs could be an added value in efficiently recruiting antibodies towards a cell surface and subsequent induction of innate cell killing. Multivalency is defined as the simultaneous binding of multiple ligands on one entity to multiple receptors on another. It is shown that multivalent interactions are able to convert low binding affinities into increased binding avidities due to this sequential and/or simultaneous binding of ligands and receptors. Furthermore, to achieve high avidities, high affinity of the monovalent binding is not necessarily required. This gives the opportunity to create high avidity ligands through multivalent constructs of low affinity ligands. As a consequence, these constructs will be effective at lower concentrations compared to the monovalent construct, often referred to as super-selectivity.[54,55,56] Interestingly, many biological systems utilize this concept. For example, the influenza virus uses this system for adhesion to the

glycocalyx to infect the cell. In doing so, the hemagglutinin, densely packed on the viral surface, will interact with multiple sialic acid motifs (a terminal sugar on many glycoproteins) on the surface of the target cell. The immune system has also implemented this concept, since all classes of antibodies have multiple binding sites.[57] Hence, exploring the possible benefits of multivalency in context of antibody recruitment (**Figure 3**) is an attractive strategy which has been explored by several research groups thus far.

The Wang group was the first to report on multivalent macromolecules for antibody recruitment. Their system consisted of multivalent glycopolymers bearing Galα1→3Gal as ABT that binds endogenous anti-α-Gal antibodies and α-mannosyl as TBT to target the bacterial cell wall. Numerous bacterial strains produce surface lectins in the form of fimbriae or pili, responsible for cell-cell interactions and infectious disease. One of those lectins are the type 1 fimbrial lectins which are specific for α-mannosides. As the binding affinity of this interaction falls in the millimolar range, multivalent polymers could strongly increase the binding avidity. Whereas the polymers, compared to a monovalent α-Gal-mannose conjugate, showed no significant benefit in binding to *Escheria coli* K-12 HB101 bacteria, they clearly showed a multivalent effect on the binding of anti-α-Gal antibodies.[58]

Interestingly, the same group also synthesized a series of polymers with varied densities of α-Gal for a different purpose. These synthetic carbohydrate polymers were designed to study the inhibition of the binding of endogenous anti-Gal antibodies to α-Gal antigens on the surface of mammalian cells for preventing rejection in the context of xenotransplantation. Evaluation of the binding affinity of the polymers against different isotypes of anti-α-Gal antibodies was found to be increased compared to monomeric α-Gal. Despite the expectation of increased affinity with increased density of α-Gal on the polymeric backbone, polymers with the highest density of α-Gal did not show the highest inhibition. This was probably due to formation of a non-cooperative spatial conformation of the polymer as well as to sterical hindrance. A flow cytometry assay, that provided a better mimic of the real situation, could only demonstrate high levels of inhibition towards the IgM isotype, thereby limiting the possible biomedical application of these polymers.[59]

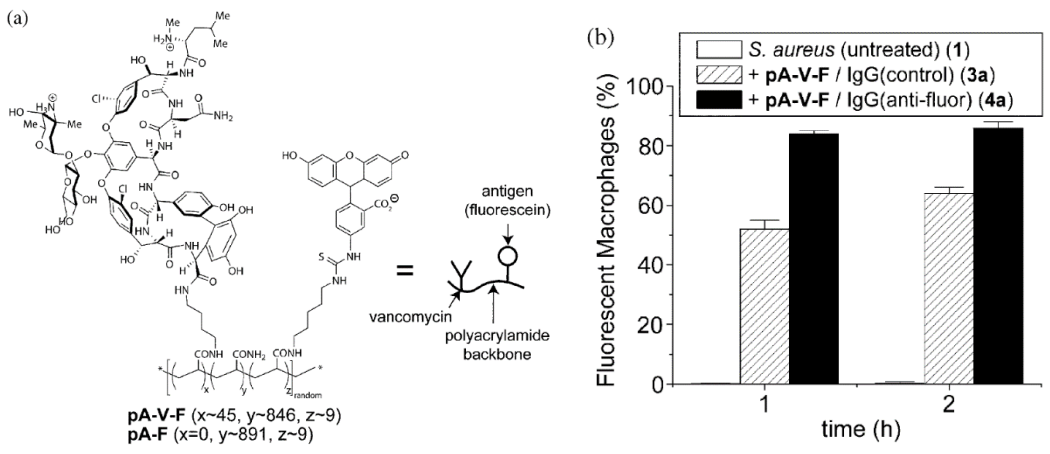

Figure 4. (A) Chemical structure of pA-V-F, a polyacrylamide presenting both vancomycin and fluorescein groups and the control polymer pA-F lacking vancomycin groups. **(B)** Phagocytosis of *S. aureus*. *S. aureus* labeled with polyacrylamide presenting both vancomycin and fluorescein groups (pA-V-F) were treated with anti-fluorescein IgG or isotype-control IgG. The percentage of macrophages that are fluorescent demonstrates greater phagocytosis for pA-V-F labeled *S. aureus* treated with anti-fluorescein IgG. Figure adapted with permission from ref [36].

The Whitesides group reported on bifunctional polyacrylamides containing multiple vancomycin and fluorescein motifs. Vancomycin is able to recognize D-Ala-D-Ala on the bacterial cell wall of Gram-positive bacteria whereas fluorescein is a low molecular weight hapten, able to recognize anti-fluorescein antibodies. Furthermore, it can be used as an agent to visualize the binding of the polymer to bacterial cells. In a first paper, the ability of these polymers to bind and attract anti-fluorescein antibodies towards surfaces presenting D-Ala-D-Ala groups on self-assembled monolayers was demonstrated. Additionally, polymers were also able to bind to *Escheria faecalis*, a Gram-positive bacteria.[60] In a second study, opsonization of polymer-labeled *Staphylococcus aureus* and *Staphylococcus pneumoniae* by anti-fluorescein was established. Moreover, these antibodies were able to interact with macrophages and promote phagocytosis of polymer-labeled *S. aureus*, as shown in Error! Reference source not found. **4**.[36] As no monovalent control was included in both studies, the potential effect of multivalency remains elusive.

The first report of multivalent antibody-recruiting structures for targeted destruction of tumor cells was based on rhamnose-functionalized liposomes. Although the focus is not on the contribution of multivalency, it can possibly have a beneficial effect on recruitment of anti-rhamnose antibodies and subsequent cytotoxicity. Liposomes consisted of different ratios of folic acid (FA) as TBT and L-rhamnose (rha) as ABT. After showing specific recruitment of anti-rhamnose antibodies to folate receptor expressing cells incubated with FA-rha-functionalized liposomes, complement-dependent lysis of tumor cells was examined. Interestingly, the degree of cytotoxicity was dependent on the FA/rha ratio incorporated into the liposomes. A higher content of rhamnose gave rise to a higher percentage of cytotoxicity. Even though not indicated here, these findings can possibly be related to an increase in binding avidity towards anti-rhamnose antibodies. On the other hand, liposomes with a FA/rha ratio of 1:1000 did not perform any better compared to a monovalent construct, hence making it difficult to confirm the aforementioned hypothesis. Nevertheless, as shown in **Figure 5,** liposomes (FA/rha 1:2500) were able to significantly delay tumor growth with minimal toxicity in rhamnose immunized mice, thereby demonstrating the therapeutic benefits of this approach.[61]

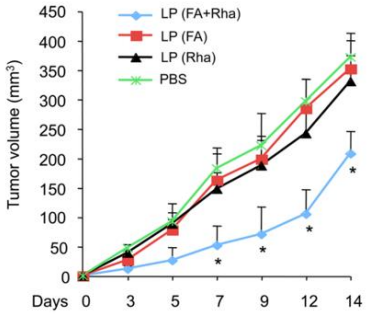

Figure 5. Tumor growth rate of 4T1 breast cancer in mice. Treatment with targeted liposomes significantly decreased tumor growth compared to control liposomes and PBS. Figure adapted with permission from [61].

Recently, the De Geest group published several papers on so-called antibody recruiting polymers (ARPs) in the context of cancer immunotherapy. The first question that was addressed is to what extent polymeric scaffolds that contain multiple antibody-recruiting hapten motifs allow for a more efficient antibody recruitment to the cell surface with prolonged cell surface persistence and efficient induction of innate effector killing. For proof-of-concept, efforts were focused on ARPs bearing a lipid motif at one of the polymer chain-ends that is able to insert into the phospholipid cell membrane through hydrophobic interaction and DNP as antibody recruiting motif (**Figure 6**). Biolayer interferometry (BLI) revealed a dramatic increase in binding avidity with increasing amount of DNP on the polymer backbone (**Figure 7A**). Notably, a more than 4-log increase in the K_D value of a polymer bearing 10 DNP units compared to a monovalent construct was observed. Based on these findings several polymers were synthesized with different types of lipid motifs and 10 DNP hapten motifs substituted onto the polymer backbone. It was found that only polymers that contained both a lipid tail and DNP motifs were able to recruit anti-DNP antibodies to a cell surface. In addition, ARPs bearing a dimyristyl lipid motif outperformed monoalkyl lipid as well as cholesterol motifs in terms of cellular association and consequently antibody binding (**Figure 7B**). Taking this into consideration, dialkyl-polyDNP (**Figure 6**) was utilized to demonstrate the ability to trigger phagocytosis of ARP-treated cancer cells by human macrophages. Interestingly, phagocytosis was induced to a similar extent as the clinical-grade monoclonal antibody therapeutic Cetuximab, known to exert its mechanism of action through Fc-mediated innate killing (**Figure 7C**). This highlights the potential of the ARP approach in an immune-oncological context. Despite the fact that the lipid DNP polymers operate in a receptor-independent way, requiring direct intratumoral injection, this can also be a disadvantage. Lipid motifs are known to be mobile on cell surfaces and shuttle between cells. Moreover they can bind hydrophobic pockets of serum proteins and be carried away by the bloodstream, which will lower the concentration of the ARP at the tumor site, thereby potentially decreasing the innate immune killing efficacy.[62]

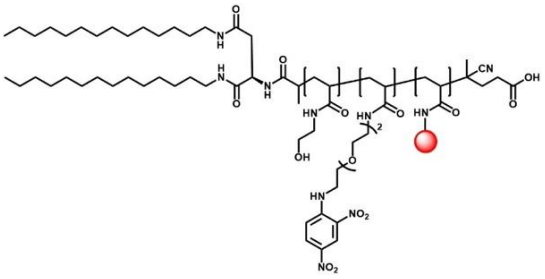

Figure 6. Chemical structure of the antibody-recruiting polymer (ARP) containing a dialkyl lipid and multiple DNP functionalized groups. The red sphere represents a fluorescent label.

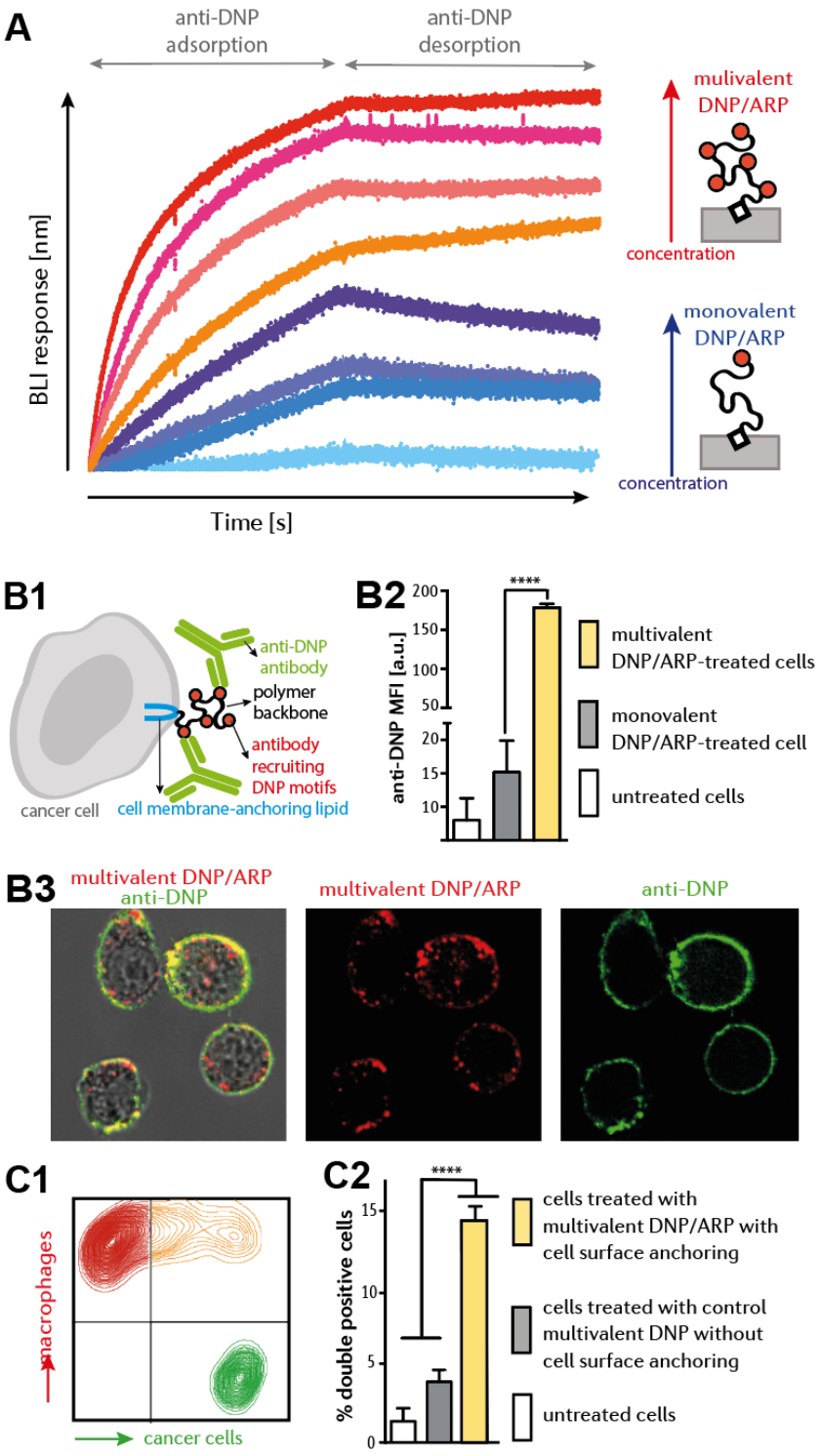

Figure 7 (A) Multivalency effect on antibody binding to ARPs. Biolayer interferometry sensorgrams of (polyclonal rabbit) anti-DNP binding to streptavidin-coated sensors functionalized with biotinylated polyDNP. **(B)** Antibody recruitment to ARP-treated cells (B1) ARPs anchor to the cell surface by hydrophobic insertion of a lipid tail at the ARP chain end into the phospholipid membrane. Cell surface anchoring of ARPs triggers recruitment of endogenous antibodies to the cell, thereby flagging the cell for destruction by innate immune mechanisms. (B2) Flow cytometry histogram of anti-DNP recruitment to dialkyl polyDNP treated CT26 cells. (B3) Confocal microscopy images of cells treated with dialkyl-polyDNP (red) and anti-DNP (green). **(C)** Innate effector killing of ARP-treated cells. (C1) Phagocytosis of cancer cells by macrophages produces a population of double

positive cells in the upper right quadrant of the flow cytometry scatter plot. (C2) Quantification of ADCP efficiency by calculating the percentage of double positive cells.

To overcome the challenges listed above, such as the need for intratumoral injection and the removal of the ARPs at the tumor site by binding to hydrophobic pockets of serum proteins, covalent ligation to the cell surface could be a viable alternative. Therefore, the same group of authors looked into metabolic labeling of cancer cells. Azido sugars are able to metabolically label cells[63] and, in this way, introduce azide motifs into the glycocalyx. These azides can selectively react with cyclooctynes through strain-promoted azide-alkyne cycloaddition (SPAAC) and form a stable triazole bond. To establish a proof-of-concept, showing that ARPs can bind to metabolically azide-labeled cells, Ac_4ManN_3 was chosen as a simple readily available azido sugar. Whereas this sugar has little or no selectivity towards cancer cells, this approach could find clinical translation when using a newer generation of azido sugars, such as those developed by the Cheng group.[64] In these sugars, the anomeric acetyl group is converted into a caged ether protecting group, which allows for a more selective metabolization by cancer cells that overexpress the enzymes histone deacetylase and cathepsin L, required to release the protecting group. Taking this into account, the ARP design was optimized, including presence of both DNP and azide-reactive dibenzocyclooctyne (DBCO) motifs. *In vitro* analysis showed that only polymers containing both DBCO and DNP were capable to recruit antibodies to an azide-labeled cancer cell surface in 2D and 3D cell cultures (**Figure 8**).[65] It is also interesting to note that very recently the Rullo group has reported on antibody recruiting molecules that were able to covalently conjugate to endogenous antibodies through proximity driven ligation[44] which could also open up interesting avenues when translated to macromolecular antibody recruiting entities.

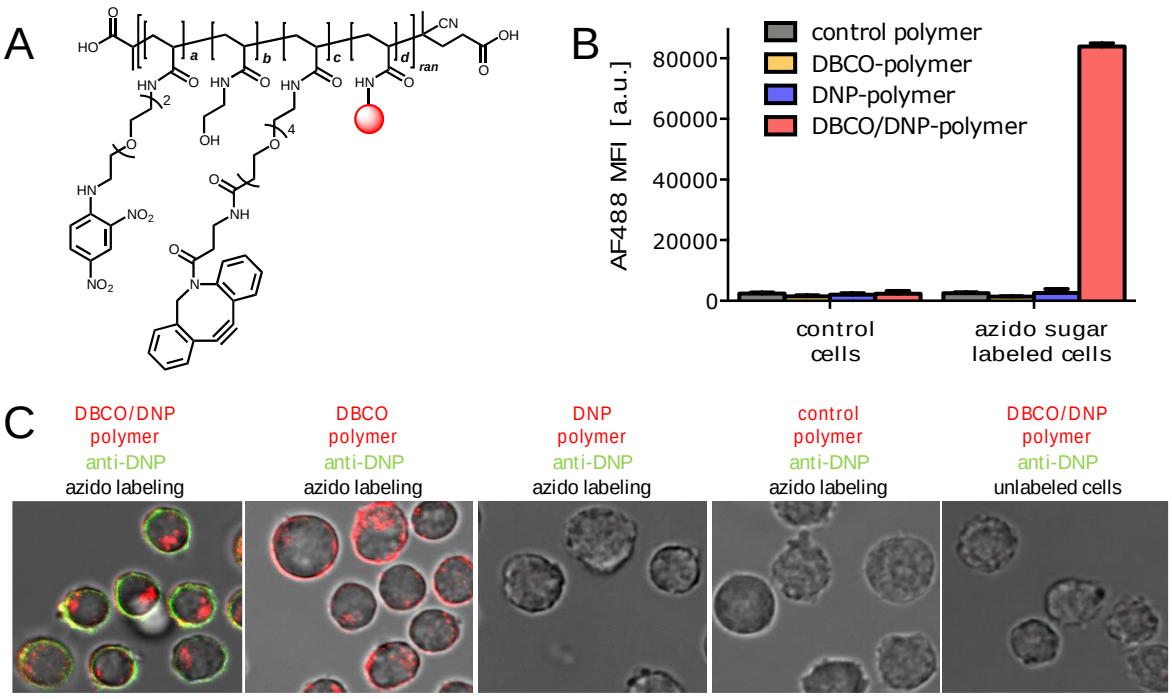

Figure 8. (A) Chemical structure of the antibody-recruiting polymer (ARP) containing multiple DBCO and DNP functionalized groups. **(B)** Flow cytometry analysis of anti-DNP antibody recruitment to azido sugar labeled cells pulsed with polymers. Non-labeled cells were used as control. **(C)** Corresponding confocal microscopy images. Red fluorescence (polymer) and green fluorescence (AF488 anti-DNP) are shown separately together with the overlay of both fluorescence channels and the transmitted light (TL) channel.

The choice to use DNP as a hapten was mainly based on the commercial availability of anti-DNP antibodies, which facilitates experimental readout. On the other hand, DNP is a hydrophobic molecule which complicates reaching high hapten densities onto polymer backbones. For this reason, it could be beneficial to exploit other possible haptens for endogenous antibodies to utilize as ABT. Rhamnose in particular is an attractive candidate since it is highly water soluble and has successfully been explored in the context of antibody-recruiting small molecules.[37,40,66] Hence, a polymer backbone solely composed out of L-rhamnose repeating units will not be inflicted by solubility issues in aqueous medium as would be the case for polymers composed of a large number of DNP motifs. Another interesting benefit is that rhamnose-specific antibody titers show a population-wide prevalence in human individuals and are significantly higher in titer and/or affinity compared to endogenous antibodies against other common haptens.[37,40] Therefore, glycopolymers were designed that contained rhamnose as a repeating unit with a lipophilic motif, cholesterolamine, at the chain end to drive spontaneous cell-surface insertion. Those polymers were able to specifically attract endogenous anti-rhamnose IgG antibodies from human serum to a target cell surface, thereby outperforming a monomeric construct and thus showing the superior ability of polymeric constructs (**Figure 9**).[67]

More recently, researchers in the Berthet and Renaudet group reported on the synthesis of tetra- and hexadecavalent rhamnose-based antibody recruiting molecules by using peptide carriers. As TBT, a well-known cyclopeptide Arg-Gly-Asp (cRGD) was selected. ELISA analysis confirmed the beneficial effect of the multimeric presentation of L-rhamnose compared to a monovalent control. Similar to multivalent presentation of the ABT, multivalent presentation of the TBT could increase the binding avidity of the antibody recruiting macromolecule. Making use of fluorescent mono- and tetravalent cRGD-based conjugates that could recognize $\alpha_v\beta_3$ integrins on the cell surface of M21 melanoma cells, it was confirmed that the tetravalent cRGD had a higher potency of binding. Taking this into consideration, tetra- and hexadecavalent L-rhamnose motifs were conjugated to a tetravalent cRGD motif (**Figure 109**) and used for *in vitro* assays. No specific binding of the anti-rhamnose IgG to the tetravalent rhamnose construct on M21 cells was demonstrated in contrast to the hexavalent construct, thereby indicating the importance of multivalent presentation.[68]

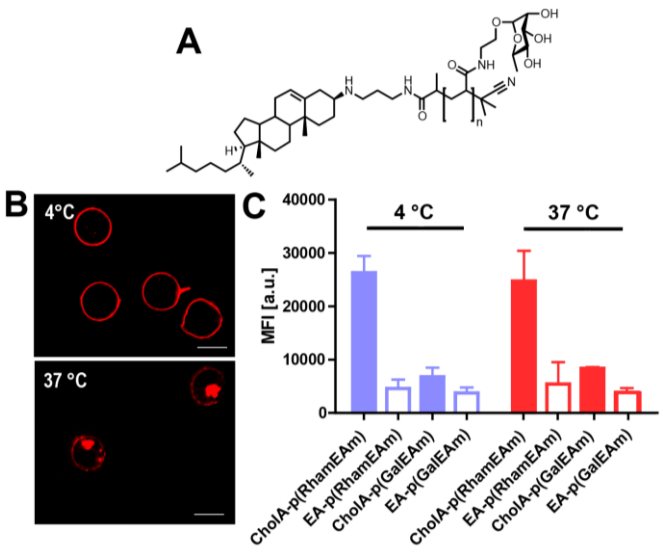

Figure 9. (A) Chemical structure of the antibody-recruiting polymer (ARP) containing a cholesterolamine lipid and multiple rhamnose functionalized groups. **(B)** Confocal microscopy images of the ARP for 1h at 4 and 37 °C. **(C)** Antibody recruiting activity of Jurkat T cells pulsed with the ARP CholA-p(RhamEAm) or control polymers.

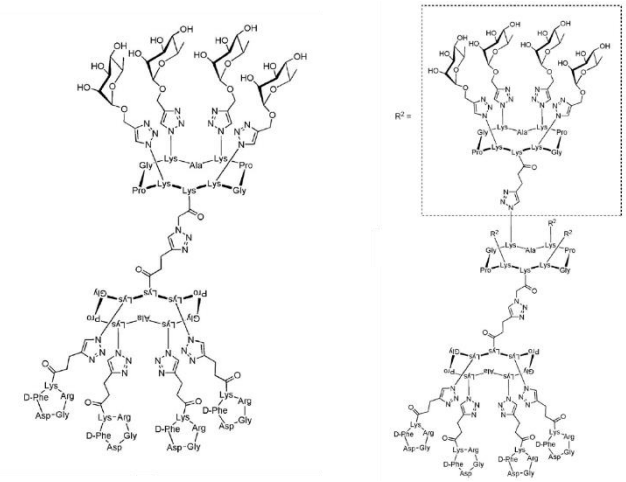

Figure 10. Chemical structures of antibody-recruiting molecules with tetra- or hexadecavalent rhamnose motifs. Figure adapted with permission from ref [68].

5. Conclusion and outlook

This review highlighted that multivalent structures may offer an advantage compared to monovalent structures in terms of efficient antibody recruitment. This could potentially be of benefit to patients with lower concentrations of circulating endogenous antibodies when considering treatment with an antibody-recruiting therapeutic. Where the efficacy of antibody recruiting small molecules could potentially be hampered by low binding affinity, multivalent structures have the potential to still attract antibodies as a result of an increased binding avidity. There is, however, still a need for more research into suitable TBT that exhibit a highly selective expression on the target cell surface to decrease the likeliness of off-target binding. In this regard, super-selectivity induced by multivalent

binding of low affinity ligands could be of great relevance. In parallel, strategies that, upon direct administration to the diseased tissue (e.g. by intratumoral administration) could allow for highly efficient anchoring to tumor cells, with minimal leakage into systemic circulation. With regard to the latter it is also important to avoid immune-complex formation in the bloodstream through binding of multiple endogenous antibodies to multiple multivalent antibody-recruiting macromolecules. Hence, it is clear that despite the fact high appeal of such multivalent structures, more detailed studies are needed. At present, the benefit has mainly been demonstrated *in vitro*, and *in vivo* testing is definitely needed to embrace their full potential and elucidate potential challenges that require further molecular engineering.

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