

# Regulation of complement and modulation of its activity in monoclonal antibody therapy of cancer

Saskia Meyer, Jeanette HW Leusen\*, and Peter Boross

Laboratory for Immunotherapy; Laboratory for Translational Immunology (LTI); University Medical Center Utrecht; Utrecht, The Netherlands

**Keywords:** antibody therapy, tumor, complement, complement-dependent cytotoxicity, complement-enhanced ADCC

**Abbreviations:** CRPs, complement-regulatory proteins; CDC, complement-dependent cytotoxicity; ADCC, antibody-dependent cell-mediated cytotoxicity; CDCC, complement-dependent cellular cytotoxicity.

The complement system is a powerful tool of the innate immune system to eradicate pathogens. Both *in vitro* and *in vivo* evidence indicates that therapeutic anti-tumor monoclonal antibodies (mAbs) can activate the complement system by the classical pathway. However, the contribution of complement to the efficacy of mAbs is still debated, mainly due to the lack of convincing data in patients. A beneficial role for complement during mAb therapy is supported by the fact that cancer cells often upregulate complement-regulatory proteins (CRPs). Polymorphisms in various CRPs were previously associated with complement-mediated disorders.

In this review the role of complement in anti-tumor mAb therapy will be discussed with special emphasis on strategies aiming at modifying complement activity. In the future, clinical efficacy of mAbs with enhanced effector functions together with comprehensive analysis of polymorphisms in CRPs in mAb-treated patients will further clarify the role of complement in mAb therapy.

## Introduction

Over the past three decades, an increasing number of monoclonal antibodies (mAbs) have been developed for the therapy of cancer. Nevertheless, the clinical application of mAbs remains a challenge. Antibodies represent a multibillion dollar market: currently, 15 mAb therapeutics for cancer are approved by the Food and Drug Administration, and the number is expected to increase in 2014. The majority of these products are IgG1, two are IgG2, and one IgG4. Most of these mAbs are used for the treatment of various malignancies, including both hematologic (e.g., CD20-targeting rituximab, CD52-targeting alemtuzumab), as well as

solid tumors (e.g., Her2-targeting trastuzumab, EGFR-targeting cetuximab). Although mAb treatment is generally effective, it does not provide a complete cure in most cases. Understanding the *in vivo* mechanism of action will likely help to improve the efficacy of mAbs.

Therapeutic mAbs can induce anti-tumor effects in direct and indirect manners. The direct mechanism (Fab-mediated) is based on either inducing anti-proliferative or pro-apoptotic signals in tumor cells or preventing the binding of ligands, such as growth factors or cytokines, to their natural receptors. The indirect effect is mediated by the Fc of the mAb, and leads to the activation of immune effector mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and complement-dependent cellular cytotoxicity (CDCC). ADCC is mediated by the recruitment of cytotoxic effector cells, such as natural killer (NK) cells, macrophages, and polymorphonuclear leukocytes (PMNs), that express Fc gamma receptors (FcγRs) on their surface. The activation of complement either leads to lysis of the antibody-opsonized malignant cell by CDC or CDCC.

The relative contribution of the different effector mechanisms to the clinical efficacy of individual mAbs remains unclear. Whereas ADCC and direct Fab-mediated effects are generally accepted as mechanisms of action, the contribution of complement to the anti-tumor effects of mAbs remains controversial.<sup>1,2</sup> A recent review by Taylor and Lindorfer discusses the role of complement in mAb treatment, focusing on rituximab, ofatumumab, and alemtuzumab, and provides a critical assessment of the *in vitro* methods used to study the complement system.<sup>3</sup> Here, the role of complement in mAb immunotherapy of solid and hematological cancers will be summarized based on *in vitro* and *in vivo* data and on clinical evidence. In addition, possible strategies for modifying complement-mediated anti-tumor activity of mAbs will be discussed.

© Saskia Meyer, Jeanette HW Leusen, and Peter Boross

\*Correspondence to: Jeanette HW Leusen; Email: jleusen@umcutrecht.nl

Submitted: 05/11/2014; Revised: 06/19/2014; Accepted: 06/20/2014

<http://dx.doi.org/10.4161/mabs.29670>

This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

## The Complement System in Immunotherapy

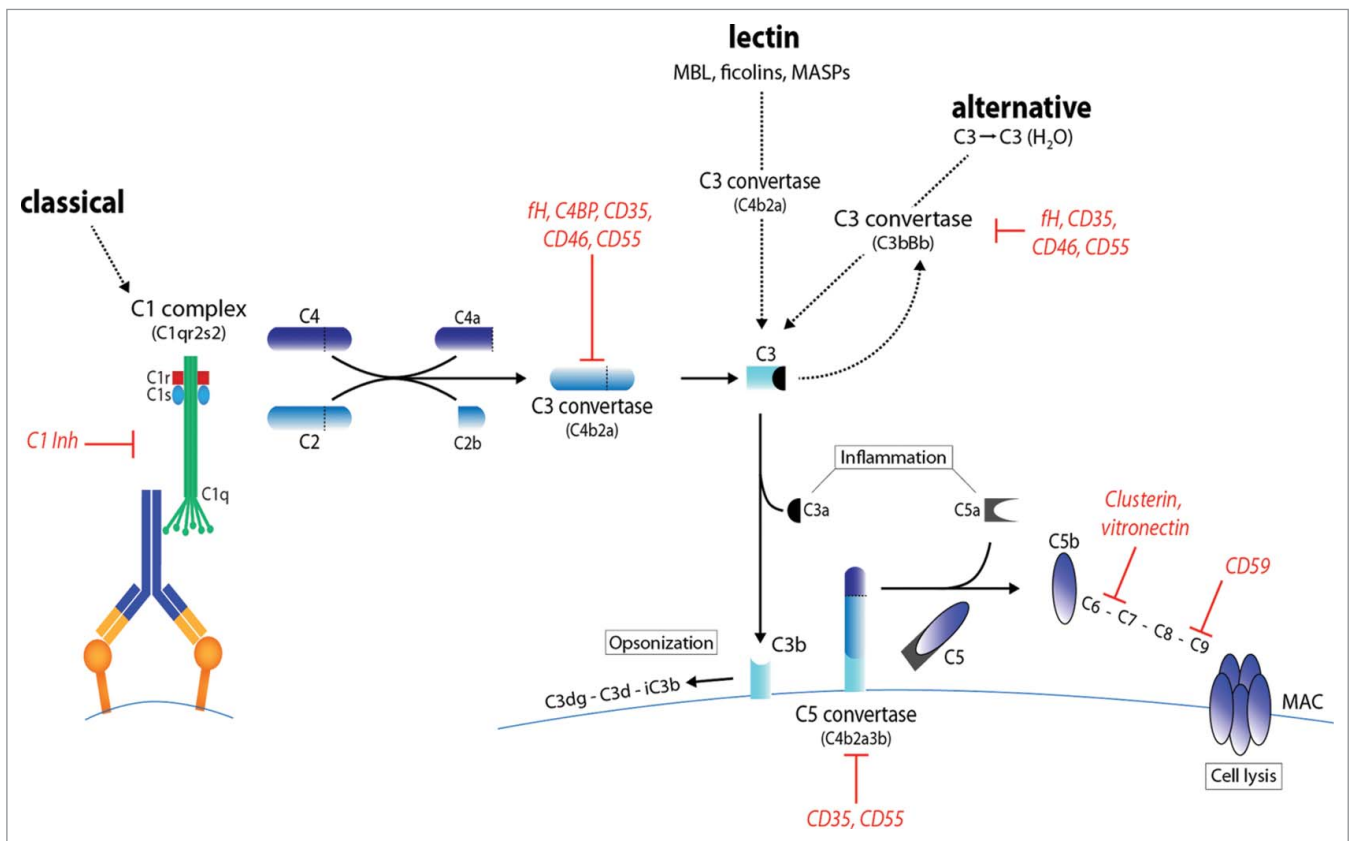
The complement system comprises more than 30 glycoproteins, from which 20 are present in plasma and 10 are cell-associated regulators or receptors. Complement proteins are mainly synthesized in the liver, but can also be produced locally

by various cell types, including macrophages, fibroblasts, and endothelial cells.<sup>4</sup>

Activation of the complement cascade induces diverse immune effector functions including cell lysis, phagocytosis, chemotaxis and immune cell activation. Complement can be activated by 3 different pathways: the classical, the alternative and the mannose-binding lectin pathway which all converge on the level of C3 (Fig. 1).<sup>5,6</sup> MAbs in immune complexes, such as opsonized tumor cells, activate the classical complement pathway. Upon C1q binding to the antibody Fc, the pro-enzymatic forms of the serine proteases C1r and C1s within the C1qr2s2 complex are activated. The exact mechanism of the auto-activation of the inactive serine proteases C1r remains unclear but data suggest that a possible conformational change in the C1 complex results in the auto-activation of each C1r molecule which in turn activates its counterpart in the tetramer. Subsequently, proteolytically active C1r cleaves C1s resulting in C1s activation. The serine protease C1s then cleaves C2 and C4 to

generate C2a, C2b, C4a, and C4b. C2a and C4b together form the C3 convertase. The cleavage of the central component C3 by the C3 convertase leads to formation of C3b, most of it reacts directly with water and the minority covalently binds to the plasma membrane of the target cell. Membrane-bound C3b interacts with the C3 convertase, yielding in the formation of the C5 convertase. Consequently, C5 is cleaved into C5a and C5b. The activation of the terminal pathway leads to the deposition of the components C5b-C9 into the opsonized target cell membrane forming the membrane-attack complex (MAC), eventually causing membrane disruption and cell lysis (CDC).

Next to cell lysis, complement activation can promote CDCC. C3b deposited on the plasma membrane of the target cell is quickly converted into inactive C3b (iC3b) and then further cleaved into C3d and C3dg. These fragments can engage complement receptors (CRs) on diverse cell types. Interaction of iC3b with CR3 on macrophages or NK cells resulted in enhanced ADCC.<sup>7,8</sup>



**Figure 1.** Schematic representation of the classical complement pathway initiated by mAbs and its inhibitors. All three complement pathways (classical, lectin, and alternative) converge on the level of C3 and lead to complement-dependent cytotoxicity (CDC) of the target cell by the formation of the membrane-attack complex (MAC). The classical pathway is induced upon binding of C1q to an antibody-opsonized target cell. Opsonin, ficolins and the carbohydrate binding protein mannose binding lectin (MBL) are expressed on foreign targets and their recognition leads to the activation of the lectin pathway. The alternative pathway is activated by water in the steady-state in a process known as tick-over. Furthermore, it functions as a positive amplification loop of the classical pathway as it initially does not require C1, C2, and C4 but processes C3b covalently bound to the target cell to generate more deposited C3b. Cleavage products in fluid phase and deposited on the target cell membrane recruit effector cells resulting in complement-dependent cellular cytotoxicity. The complement cascade is tightly controlled by soluble and membrane-bound complement-regulatory proteins (CRPs in red). CRPs function relies on their ability to adhere to or remove cleavage products required for the initiation of the next step in the complement cascade. Additionally, CRPs have cofactor functions for negative regulators of the complement cascade, hence enhancing their inhibitory effect on the convertases.

The small soluble cleavage products C3a, C4a, and C5a act as pro-inflammatory anaphylatoxins and induce the recruitment and activation of effector cells, thereby potentiating the anti-tumor response. C5a binding to C5aR on macrophages induced the upregulation of the activating FcγRIII and downregulation of the inhibitory FcγRIIB resulting in enhanced mAb-mediated cellular effector functions.<sup>9</sup>

### Complement-regulatory proteins

Uncontrolled complement activation can result in severe pathology. Because of this putative danger its activity is regulated on multiple levels including both soluble and membrane-bound molecules (Fig. 1). As mAbs initiate the classical pathway, we will focus on the inhibitors of the classical and shared pathway. Complement-regulatory proteins (CRPs) act by binding to or displacing one of the interaction partners in the cascade. CRPs may also have cofactor function for Factor I, which further enhances their inhibitory effect on the C3 and/or C5 convertase.

#### *Soluble complement regulators*

***C1 inhibitor.*** The initiation of the classical pathway is controlled by the C1 inhibitor in two ways. First, in circulation, the C1 inhibitor is reversibly bound to the serine proteases C1s and C1r zymogens to prevent auto-activation of the C1qr2s2 complex. Upon C1q binding to the Fc part of an IgG, the C1 inhibitor is cleaved while C1s and C1r remain bound, leading to complement activation. Second, C1 inhibitor binds to the activated C1s and C1r proteases in the C1qr2s2 complex, resulting in C1r and C1s dissociation from the C1 complex, leaving C1q bound to the antibody. In addition, studies have shown that, in case of low avidity between an immobilized IgG and C1q, the entire C1qr2s2 complex is removed.<sup>10</sup> More than 275 mutations in the C1 inhibitor gene have been identified and associated with, for example, hereditary angioedema (HAE) or autoimmune diseases such as lupus erythematosus.<sup>11</sup> The majority of the mutations leads to low levels of circulating C1 inhibitor.

In addition to inhibiting activation of the classical and lectin pathways of complement, C1 inhibitor also inhibits Factor XIIa and kallikrein. Functional C1 inhibitor deficiency results in dysregulated bradykinin production, which leads to increased vascular permeability and angioedema.<sup>12</sup> Patients suffering from HAE are therefore treated with recombinant C1 inhibitor.

***Complement Factor H.*** Though Complement Factor H (fH) is mainly described as a regulator of the alternative pathway, it can also contribute to a decay-accelerating activity of the C3 convertase of the classical pathway. Furthermore, fH facilitates the cleavage of C3b and C4b into their inactive products iC3b and C4c/C4d, respectively, by acting as cofactor for Factor I. In human serum a second smaller splice variant (FHL-1 = factor-H-like protein) is present, exhibiting a reduced regulatory activity. Several mutations in the fH gene have been described leading to either impaired functional activity or more often to reduced interaction capacity with C3b. These mutations are located within a region of fH required to control complement activation

on cell surfaces but not in plasma.<sup>13</sup> Decreased protection of host cells from complement attack by these mutations typically leads to atypical hemolytic-uremic syndrome (aHUS). Anti-fH auto-antibodies have been shown to hamper the interaction with cell surface-bound C3b.<sup>14</sup>

Cancer cells were reported to either overexpress fH or bind fH on their surface to evade complement-mediated tumor killing.<sup>15</sup> A clinical study showed that a polymorphism in the fH gene is associated with event free survival after rituximab treatment in FL patients, although the study had relatively low power and this finding should be confirmed.<sup>16</sup> The same study also found associations in *CFHR1* and *CFHR5*, genes that are located near to *CFH* and encode fH-related proteins. Complement Factor H related (CFHR) proteins can also bind C3b but their biology is less known and therefore the significance of this is unclear.<sup>16</sup>

***C4b-binding protein.*** C4b-binding protein (C4BP) inhibits the classical pathway by accelerating the decay of C3 convertase (C4b2a) by irreversible displacement of C2a from C4b. It also has a cofactor function for the plasma protease Factor I. No full C4BP deficiency has been found yet. At present, only one functional non-synonymous polymorphism (R240H) has been identified that does not affect expression, but C3b binding and its cofactor functions both in fluid phase and on the cell surface are diminished.<sup>17</sup> This polymorphism has been associated with aHUS, a disease in which excessive complement activation was shown to play a pathogenic role. The anti-C5 mAb eculizumab is currently the only accepted treatment. To our knowledge this polymorphism was not studied in the context of mAb therapy.

***Clusterin / vitronectin.*** These two fluid phase proteins prevent the insertion of the MAC into the cell membrane by interacting with precursor complexes of the MAC. Data suggest that clusterin and vitronectin may form complexes in plasma with sC5b-7 and act in an additive manner by eventually resulting in a cytolytically inactive MAC.<sup>18</sup> The complement regulatory function of clusterin has been questioned.<sup>19</sup> Both proteins have additional functions beyond complement regulation, for example by acting as adhesion protein, potent inducer of cell aggregation, facilitating cell attachment and spreading. Several reports in the literature demonstrate vitronectin expression by tumor and surrounding cells as a connection to metastasis and high tumor grade.<sup>20</sup> These additional functions confound complement-associated functions of these proteins. Polymorphisms resulting in clusterin or vitronectin deficiencies have not yet been studied in the context of mAb therapy.

#### *Membrane-bound complement-regulatory proteins*

***CD35.*** CD35 (complement receptor type 1, CR1) is a membrane glycoprotein that binds C3b/C4b and is found on most circulating blood cells, but not on platelets. C3b or C4b binding by CD35 results in a decay-accelerating activity toward the C3 and C5 convertase, respectively. Besides its function as a receptor, CD35 has been identified as a major cofactor for the inactivation of C3b and C4b by Factor I. iC3b represents another interaction

partner for CD35, leading to its cleavage into the inactive forms C3c and C3dg and stopping further direct cell lysis. Reduced CD35 expression, but no complete deficiency has been described,<sup>21</sup> but no association with mAb therapy was established.

**CD46.** The membrane cofactor protein (MCP, CD46) is an integral membrane glycoprotein expressed on almost all circulating cells, as well as on epithelial and endothelial cells. CD46 blocks the formation of C3 convertase of the classical and alternative pathways by binding to C3b and exhibits cofactor properties for Factor I. CD46 in rodents is limited to the testis. CD46 KO mice, therefore, show no phenotype concerning complement. Several functional polymorphisms were identified and most were found to associate with aHUS,<sup>22</sup> but an association with mAb therapy in cancer has not been studied.

**CD55.** CD55 (decay-accelerating factor, DAF) is a glycosyl phosphatidylinositol (GPI)-linked glycoprotein expressed on peripheral blood cells, vascular endothelial cells, placenta, and many types of epithelial cells. A soluble form is found in many body fluids such as plasma, tears, and urine. CD55 accelerates the decay of C3 and C5 convertase of both the classical and alternative pathways. In contrast to CD35, DAF acts in a cis-fashion, i.e., CD55 is only able to inhibit complement activation on the same cell as it is expressed.

**CD59.** CD59 (protectin) is expressed on all circulating blood cells, endothelial cells, most epithelial cells and spermatozoa. Similar to CD55, CD59 is found as a GPI anchored protein, but it can also be detected in a soluble state. The receptor binds the C5b-8 complex thus preventing C9 input resulting in the inhibition of the formation of the polymeric C9 complex (MAC assembly). No polymorphisms have been described in the protein coding sequence of the CD59 gene.

Indirect loss of CD55 and CD59 on red blood cells is caused by a mutation in another gene, phosphatidylinositol glycan class A (PIGA), which is involved in the first step of GPI biosynthesis. This defect results in paroxysmal nocturnal hemoglobinuria (PNH) characterized by amongst others complement-induced hemolytic anemia. Red blood cells from PNH patients are more susceptible to autologous complement lysis.<sup>23</sup> Therefore, patients suffering from PNH are treated with eculizumab to reduce complement induced attack on erythrocytes.

### Complement-regulatory proteins in cancer

Tumor cells are known to develop immune evasion strategies either by the expression of membrane molecules or secretion of molecules into the tumor microenvironment. The secretion of sCRPs or the upregulation of membrane-bound CRPs (mCRPs) in cancer was shown for nearly all CRPs.

Secretion of the sCRP Factor H and its splice variant FHL-1 was shown for primary tumor cells and cancer cell lines, including ovarian cancer cells,<sup>24</sup> lung tumor cells,<sup>25</sup> and colon cancer cells.<sup>26</sup> High levels of the sCRP clusterin are found in several human cancers<sup>27-30</sup> compared with normal tissues with some exceptions.<sup>30,31</sup> Upregulation of clusterin was reported in cases

of *in vivo* breast cancer progression and tumor formation.<sup>32,33</sup> Secretion of vitronectin was detected in many human cancer cells, such as hepatic and non-hepatic cancer cell lines derived from the cervix, lung, pancreas,<sup>34</sup> and breast cancer tissue.<sup>35</sup> Since both clusterin and vitronectin have functions unrelated to complement, their specific role in cancer resistance with regard to complement activation is unclear.

Compared with sCRPs, the role of mCRPs was studied in more detail. Expression pattern analysis revealed that CD46, CD55, and CD59 are upregulated to a different extent on distinct tumor types compared with normal tissues,<sup>36-38</sup> with CD46 and CD59 being the most prominent ones. On human carcinoma cell lines, CD59 was identified as the main mCRP mediating protection from CDC induced by anti-carcinoma antiserum.<sup>38</sup> Ectopic expression of CD59 *in vitro* renders melanoma cells less susceptible to mAb-mediated CDC.<sup>39</sup> Contradictory to previous results one immunohistochemistry study showed that the loss of CD59 is associated with aggressiveness of breast carcinomas.<sup>40</sup> This study however did not assess expression of CD55, which—on colorectal cancer—is inversely correlated with the expression of CD59.<sup>41</sup>

CD55 expression is increased on epithelial cells derived from biopsies of patients with advanced prostate cancer and correlated with reduced efficacy of mAb-mediated complement lysis. Downregulation of CD55 by siRNA in prostate tumor epithelial cells resulted in a significant reduction of overall tumor burden *in vivo* in SCID mice.<sup>42</sup> Furthermore, high CD55 expression was correlated with worse survival of colorectal cancer patients.<sup>43</sup>

The expression level of CD46 was found to be relatively low on freshly isolated B cell leukemia samples compared with higher, but varying levels of CD55 and CD59. A low expression level of CD46 was associated with an increase in C3d deposition on renal cell carcinoma and a less advanced tumor growth.<sup>44</sup>

On fresh lymphoma cells (follicular lymphoma cells, mantle cell lymphoma, diffuse large cell lymphoma and small lymphocytic lymphoma) rituximab-induced CDC was correlated with the ratio of CD20 and CRPs (CD46, CD55, and CD59).<sup>45</sup> Blocking experiments clearly demonstrated the contribution of CRPs to protection from CDC, the CRP expression level however did not allow a prediction of the degree of rituximab-mediated CDC *ex vivo*.<sup>46</sup>

The role of CD35 as a complement-regulatory protein in cancer is less investigated. No CD35 expression could be detected on hematological malignancies<sup>36</sup> and gastric cancer cells,<sup>47</sup> whereas overexpression was observed in malignant endometrial tissues compared with controls.<sup>48</sup>

Taken together these studies convincingly show that CRP secretion and expression can be increased in cancer in the absence of mAb therapy suggesting that complement activation during mAb therapy is hampered by CRPs.

### The role of complement in Ab immunotherapy

There are several lines of evidence showing that complement is involved in the anti-tumor effects of therapeutic mAbs. In the presence of human plasma, some anti-tumor mAbs readily activate complement *in vitro* leading to deposition of complement



fragments or CDC of tumor cells. The mAb ofatumumab is capable of lysing a range of rituximab-resistant targets, in particular CD20 low expressing cells.<sup>49,50</sup> In B-CLL cells, expressing both CD52 and low levels of CD20, alemtuzumab-mediated CDC can further be enhanced by the addition of ofatumumab.<sup>51</sup> The clinical response rate to rituximab has been shown to rely on the CD20 expression level, which is consistent with data on *in vitro* CDC induction. The CD20 mAb obinutuzumab (GA101) has a reduced ability to fix complement compared with rituximab. Nevertheless, at mAb concentrations, normally reached during mAb therapy, killing of CLL cells *in vitro* partially depended on complement activation.<sup>52</sup>

The anti-CD52 mAb alemtuzumab induced efficient CDC of freshly isolated leukemia/lymphoma cells *in vitro*<sup>53,54</sup> and complement consumption was demonstrated after infusion of alemtuzumab into patients with B cell lymphoma.<sup>55,56</sup> The anti-CD38 mAb daratumumab was selected for its efficient induction of CDC of multiple myeloma cells<sup>57</sup> and has broad anti-myeloma effects.

Despite convincing evidence that mAbs can activate complement *in vitro*, the contribution of complement activation to *in vivo* and clinical efficacy is less clear.

To study the involvement of complement mediated tumor cell lysis *in vivo*, researchers make use of complement deficient strains like C1q<sup>-/-</sup>, C3<sup>-/-</sup>, and C4<sup>-/-</sup> knockout mice. In particular B cell depletion studies using CD20 mAbs, capable of inducing complement mediated lysis *in vitro*, showed no contribution of complement to the therapeutic efficacy.<sup>58,59</sup> In a model for endogenous B cell depletion with human CD20 transgenic mice, CD20-targeting mAbs with reduced ability to induce complement activation (type II) outperformed complement-inducing anti-CD20 mAbs (type I). The *in vivo* efficacy of type I CD20 mAbs with abrogated complement binding capacity was still lower compared with type II CD20 mAbs.<sup>60,61</sup> Similarly, no role was found for complement in alemtuzumab treatment in a CD52 transgenic mouse model.<sup>62</sup> Treatment of liver metastasis in a metastatic melanoma model by the anti-gp75 mAb TA99 was not impaired in neither C1q nor CR3-deficient mice.<sup>63</sup>

Evidence for a beneficial role of complement was obtained in several independent animal models. The contribution of complement during rituximab monotherapy was shown in mouse models of lymphoma using C1q knockout animals<sup>64</sup> or complement depletion by cobra venom factor.<sup>65</sup> Additionally, at a limiting concentration of a mAb targeting the surface disialoganglioside GD2, reduced therapeutic effect was observed in a syngeneic model of mouse lymphoma in mice deficient in C3,<sup>66</sup> suggesting a supplemental role of complement as an antibody-mediated effector mechanism. In line with this, mAbs at limiting E:T ratios (in *in vivo* high tumor burden models) require all mAb effector mechanisms, including CR3 enhanced ADCC, for tumor killing.<sup>67</sup> That cytotoxic effector mechanisms can be reduced due to complement consumption further supports the role of complement in mAb therapy.<sup>68,69</sup> The use of fresh-frozen plasma as a source of complement in combination with rituximab has been evaluated in refractory CLL patients and resulted in a remarkable clinical response.<sup>70,71</sup>

Other data, however, suggest that complement can be detrimental in mAb therapy. A possible negative relation of complement fixation and ADCC was shown *in vitro*, as incubation of rituximab with complement-active serum blocked the adhesion of NK cells via the FcγRIII (CD16). Further functional assays confirmed that complement fixation upstream of C5 is responsible for the impaired ADCC activity mediated by NK cells in the presence of serum.<sup>72</sup> In addition, NK-mediated ADCC capacity of the glycoengineered mAb obinutuzumab (GA101), having a decreased ability to fix complement compared with rituximab, was not affected in the presence of complement.<sup>73</sup> To confirm those findings *in vivo*, a murine anti-idiotypic mAb directed against murine 38C13 B cell lymphomas was used to show that serum blocks murine NK cell activation. In the syngeneic mouse model, mice depleted of complement showed an improved anti-tumor effect.<sup>74</sup> It has also been observed that patients homozygous for a SNP in the C1qA gene that results in lower C1q levels (Gly70GGA) achieved a prolonged remission when treated with rituximab monotherapy compared with heterozygous or homozygous G genotyped patients (Gly70GGG).<sup>75</sup>

Taken together, *in vitro* complement activation by mAbs is well supported, yet the relative importance of complement to the anti-tumor effect is unclear. It is likely that a number of factors, such as the nature of tumor target, the location and stage of the tumor, characteristics of the mAb, microenvironment and the availability of other effector mechanisms together determine the contribution of complement.

## Enhancing Tumor Killing By Complement

Several strategies have been evaluated to enhance mAb-mediated complement-dependent tumor cell killing.

### Inhibition of complement regulators

Because tumor cells can overexpress CRPs, strategies aiming at neutralization or downregulation of mCRPs and sCRPs were studied.

On human melanoma cell lines, CD59 is expressed heterogeneously compared with CD46 and CD55. The application of a phosphatidylinositol-specific phospholipase C decreased CD59 expression and subsequently enhanced CDC.<sup>76</sup> However, *in vivo* enzymatic removal of mCRPs from the cell surface poses the risk of unspecific cleavage and side effects. Recently, the removal of CD46 by the application of a small recombinant protein causing its internalization enhanced efficacy of rituximab, alemtuzumab and trastuzumab, and was safe in a mouse and macaque model.<sup>77</sup>

Expression of mCRPs is modulated by cytokines.<sup>78</sup> For example, IL-1β downregulated the expression of CD46 and CD59, whereas IL-4 only affected CD46 expression. Cytokine-induced downregulation of mCRPs enhanced CDC of renal tumor cell lines *in vitro*.<sup>79</sup> Co-administration of mAbs and specific cytokines might therefore increase CDC *in vivo*, but as these cytokines have broad biological functions, it possibly also results in unwanted side-effects.

A more specific approach for targeting mCRPs is achieved by the use of siRNAs. Knock-down of single or multiple mCRPs resulted in a significant increase of CDC in vitro of tumor cell lines of different origin.<sup>80,81</sup> A combination of chemically-stabilized anti-mCRP siRNAs using cationic lipoplexes and treatment with a mixture of anti-Her2 mAbs (trastuzumab and pertuzumab) targeting different epitopes resulted in augmented CDC, complement-mediated cell death and caspase activity and CDCC by macrophages of breast, ovarian, and lung cancer cell lines.<sup>82</sup>

A potent CD59 inhibitor (rILYd4) sensitized normally rituximab-resistant lymphoma cells to CDC by rituximab<sup>83</sup> and ofatumumab.<sup>84</sup> Alternatively, mCRP function could be blocked by specific “neutralizing” antibodies. Blockage of CD55 and to a lesser extent CD59 with specific antibodies in vitro significantly increased CDC of B lymphoma cells by rituximab.<sup>85</sup> The use of intact anti-mCRP antibodies in vivo may lead to CDC of healthy host cells. Mini-antibodies, composed of single-chain variable fragments to CD55 and CD59 and the human hinge-CH2-CH3 domains of IgG1, did not induce CDC themselves, but increased rituximab-mediated CDC 2-fold in vitro.<sup>86</sup> Their application in an in vivo model of human CD20<sup>+</sup> B cell lymphoma in SCID mice markedly increased survival by rituximab treatment.<sup>87</sup> This study showed that B cell lymphomas are mainly protected from CDC by CD55 and CD59 and not by CD46 despite good expression, indicating that membrane expression of CRPs does not necessarily correlate with their functional activity.

Ideally, mCRPs should be blocked only at the tumor site to avoid unwanted damage of healthy tissues. On cervical cancer cell lines, CD55 and CD59 were identified as the most potent inhibitors of CDC. Therefore, bispecific antibodies targeting the tumor-associated antigen EpCAM expressed on cervical cancer cells and CD55 were generated. To reduce targeting of healthy cells, the antibody has a high affinity for the tumor-associated antigen and a medium to low affinity for CD55. In vitro data resulted in a 3-fold increase in complement activation.<sup>88,90</sup> The feasibility of this approach was tested in a rat model. In rodents, the expression of mCRPs is different compared with humans: CD46 and CD55 is mainly expressed in the testis, and an additional functional homolog Crry/p65, acting at the level of C3, was identified as the most important mCRP on tumor cells in rats. A bispecific antibody targeting a tumor-associated antigen and rat Crry markedly reduced lung tumor outgrowth in a syngeneic lung metastases model of colorectal cancer. This effect was attributed to increased complement activation due to the inhibition of Crry.<sup>90</sup>

Neutralization of sCRPs to enhance CDC was investigated to a lesser extent. fH is a crucial sCRP because it inhibits both the alternative and the classical pathways, and it is considered a tumor-associated antigen. Neutralization of fH with a specific antibody resulted in an increased C3 deposition<sup>25</sup> and increased CDC of colorectal cancer cells by the anti-CEA mAb up to 3-fold.<sup>26</sup> It was recently shown that the abrogation of fH function by using short-consensus repeat 18–20, representing the C-terminal ligand binding domain, in combination with ofatumumab or rituximab resulted in an increased susceptibility of primary CLL cells to CDC.<sup>91,92</sup> A further synergistic effect was seen

upon blockage of fH and CD55/CD59. The decay of C3b to iC3b is strongly mediated by Factor I, for which most of the described CRPs exhibit cofactor function. Inhibiting Factor I with a neutralizing mAb increased CDC induced by rituximab or ofatumumab of cell lines and primary CLL samples expressing CD20.<sup>93</sup>

These results demonstrate that targeting CRPs in vitro results in improved CDC; however, profound in vivo data are lacking in most cases. The role of complement and CRPs in rodent models is complicated by the fact that mCRPs are species-specific,<sup>94</sup> which brings into question the findings in a heterologous system involving complement and mCRPs from different species. Additionally, commonly used mouse strains C57BL/6 and Balb/C have been reported to exhibit a rather low complement activity.<sup>95</sup>

### Improve complement-mediated effector mechanisms

Better understanding of factors influencing complement activation, and the interaction of C1q with an antibody, led to the development of new approaches aiming at the enhancement of CDC. The epitope and the orientation of the mAb are believed to affect complement activation. Compared with rituximab, ofatumumab activates complement more efficiently, which is thought to be a consequence of the unique epitope it recognizes. The binding site is composed of residues on both the small and large extracellular loop of CD20, likely resulting in the localization of mAb Fc parts closer to the membrane, which helps deposition of complement in closer proximity of the target cell. Whether targeting a membrane proximal epitope always translates to good complement activation remains to be seen with a greater number of mAbs. A slow off-rate of the mAb is also thought to translate to better CDC activity.<sup>49</sup> Taking those mAb-specific properties during the screening process into account, possible good complement-activating candidates could be identified during the selection stage.<sup>96</sup> Human IgG3 activates complement most efficiently among the IgG subclasses. An IgG3 switch variant of rituximab induced better CDC of low CD20 expressing cells compared with its parental IgG1 counterpart.<sup>97</sup> The shorter serum half-life of IgG3 can be rescued by the introduction of the R435H mutation, resulting in a potent mAb for CDC.<sup>98</sup> IgG1/IgG3 chimera targeting CD20 showed stronger C1q binding, increased CDC capacity and more efficient B cell depletion in cynomolgus monkeys compared with the isotype-matched parental mAbs.<sup>99</sup>

Antibody amino-acid engineering influencing the binding of C1q to the Fc of an antibody can enhance complement activation. The binding site for the first complement component C1q is neither conserved among species nor among antibody classes.<sup>100</sup> For the human IgG1 subclass, key residues for C1q binding are centered in the CH2 domain. Mutations of amino acids located at the edges of the C1q binding region (K326W and E333S) had a profound influence on complement activation by increasing CDC (2-fold and 1.6-fold, respectively) and C1q binding (3-fold and 2-fold, respectively). The double mutant did not further improve CDC, but resulted in loss of ADCC.<sup>101</sup> The screen for superior complement activating antibodies led to the identification of three mutants with increased C1q binding;

the triple mutant (S267E-H268F-S324T) resulted in a significantly higher C1q affinity (47-fold) and a 6.9-fold enhanced CDC capacity. The decreased ADCC by the triple mutant could be restored by additional mutations known to enhance ADCC, yielding an antibody with 23-fold enhanced CDC activity compared with the wildtype.<sup>102</sup>

Though conventional single agent therapy with, for example, trastuzumab and cetuximab does not result in sufficient CDC, in vitro targeting of multiple epitopes of the same tumor-associated antigen led to an increased deposition of C3b on the cell surface and a higher mAb-mediated complement-dependent tumor cell lysis.<sup>103,104</sup> Interestingly, these strategies were tested for antibodies applied in the treatment of solid tumors that have been shown to upregulate mCRP expression levels. Currently available antibodies for the treatment of solid tumors largely are believed to act via direct Fab-mediated mechanisms (Her2, EGFR) and indirectly by the induction of ADCC. So far, complement has been considered to play a minor role in their anti-tumor activity. Nevertheless, complement may contribute to the mechanisms of action of currently clinically-applied mAbs targeting solid tumors, possibly by enhancing ADCC via the iC3b–CR interaction at suboptimal effector-to-target ratios.<sup>67</sup>

Recently, the initial steps of complement activation by IgG mAbs have been unraveled. Intermolecular interaction of the Fc parts between target-bound IgG molecules leads to the formation of hexamers. Those hexamers provide a platform for C1q binding.<sup>105</sup> The introduction of a mutation in the C terminus of the Fc (E345R) improved IgG hexamerization, thereby enhancing C1q binding and subsequently in vitro CDC capacity of mAbs targeting CD19, CD20, CD38, and CD52. CD20-targeting mAbs containing the E345R mutation mediated an improved therapeutic effect in vivo in a subcutaneous Burkitt lymphoma model using SCID mice.<sup>106</sup> This finding will likely help to explain why certain mAbs activate complement more effectively than others. The epitope an antibody recognizes could facilitate the natural formation of hexamers. At conditions of high target expression, the chance of formation of stable hexamers is higher, perhaps explaining why CDC is so dependent on the target expression level. Likewise the combination of two mAbs may lead to the formation of hexamers consisting of both types of mAbs, explaining the increased complement activating properties.

IgG antibodies have one conserved glycosylation site (D297) in the CH2 domain, which influences the antibody's ability to induce Fc-mediated effector functions. The attached carbohydrate chain starts with N-acetyl-glucosamine (GlcNAc) to which a fucose (Fuc) can be attached. Another GlcNAc residue is followed by a mannose (Man) residue, to which two mannose molecules bind to form two arms consisting of GlcNAc, followed by galactose and further a sialic acid residue. To study the effect of different glycoforms on the activation of complement, antibodies with defined carbohydrate structures were produced using, for example, specific expression systems or specific glycosidases. Complete deglycosylation of alemtuzumab abolished CDC.<sup>107</sup> The removal of any terminal galactose residues resulted in reduced CDC capacity of alemtuzumab, a consequence of loss of C1q binding.<sup>108</sup> In contrast, CDC activity remained unaffected

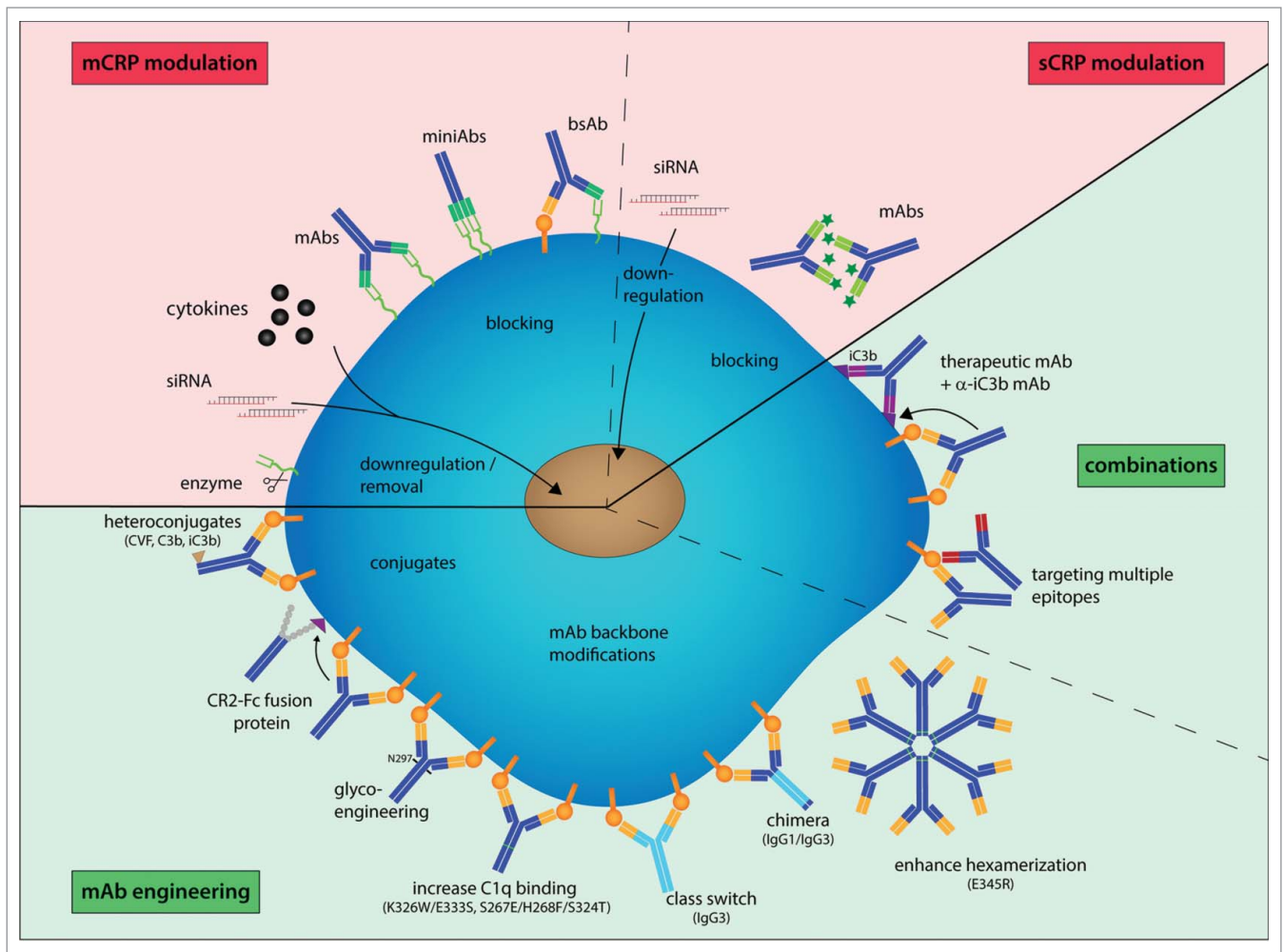
upon removal of terminal sialic acid.<sup>107</sup> Modifications of the carbohydrate structure leading to the exposure of mannose, GlcNAc and fucose, but not glucose, have resulted in the ability of MBL binding and the activation of the MBL pathway in vitro.<sup>109</sup> Antibodies carrying terminal mannose residues do not bind C1q.<sup>110</sup> The lack of fucose residues does not affect C1q binding.<sup>111</sup> Thus, different sugar residues can activate the MBL complement pathway, but at the same time abolish the activation of the alternative pathway. However, the contribution of MBL binding to the activity of a platelet depleting antibody could not be confirmed in vivo.<sup>112</sup>

It appears that all described changes in the glycan structure of IgG either do not affect or decrease complement activating capacity, whereas none actually enhanced it. The effect on CDC capacity should be tested for glycan-modifications aimed at altering FcR binding.

The conjugation of complement-activating agents to the therapeutic mAb might enhance mAb immunotherapy. Heteroconjugates were generated by coupling molecules such as cobra venom factor (CVF), C3b, or iC3b to an anti-tumor antibody. CVF permanently activates the alternative pathway via its attached oligosaccharide chains. It resembles C3 structurally and functionally, and, by binding to Factor B present in human serum, a fluid-phase C3/C5 convertase is formed. The enzyme is long-lived in serum because it is resistant to the regulatory proteins Factor H and Factor I. mAb-CVF conjugates thus direct a strong complement attack to the tumor site. Alternatively, C3b/iC3b conjugates were used to potentiate complement activity. mAb-C3b conjugates increased complement activation predominantly via the alternative pathway because mAb-conjugated C3b became resistant to inactivation by Factor H and I.<sup>113</sup> Covalently linking the F(ab')<sub>2</sub> of a normally non-cytotoxic anti-neuroblastoma mAb to CVF resulted in high cytotoxic activity against human neuroblastoma cells in vitro.<sup>114</sup> The combination of different CVF conjugated-anti-neuroblastoma mAb formats resulted in an additive effect.<sup>115</sup> CVF and C3b conjugated mAbs targeting the carcinoma antigen EpCAM-mediated higher C3 deposition and CDC of the colorectal carcinoma cell lines in vitro.<sup>88,89</sup> The same group demonstrated that β-glucan as adjuvant to mAbs activates CR3 on effector cells and results in CDCC (reviewed in ref. 116).

In vitro combination of rituximab and an anti-iC3b antibody led to higher iC3b deposition and lysis of Raji cells than by rituximab alone due to additional complement activation by the iC3b mAb.<sup>68</sup> Intravenous infusion of rituximab followed by anti-iC3b antibody into a cynomolgus monkey model confirmed rapid rituximab binding to B cells expressing CD20 on the cell surface, and deposition of iC3b, finally resulting in the co-localization of both antibodies. Nevertheless, final evidence for a better therapeutic outcome in a tumor animal model remains missing.

Another strategy to overcome complement inhibitory activity and amplify complement activation on tumor cells involves the co-administration of a CR2-Fc fusion protein with anti-tumor mAbs. CR2 is a member of the C3-binding protein family. The receptor, which is expressed on B cells and dendritic cells, binds inactivated C3 fragments (iC3b, C3d, and C3dg). Those relatively long-lived CR2 ligands might be present at a high



**Figure 2.** Strategies employed to boost complement activation in mAb cancer therapy. Many cancer cells upregulate the expression of membrane-bound and soluble complement-regulatory proteins (mCRPs/sCRPs) to circumvent complement-mediated killing. Thus several approaches blocking target cell derived inhibitory complement components (red) have been successfully developed to overcome complement resistance. On the one hand CRP function can be “neutralized” by targeting them directly using antibodies of different formats (e.g., full size, miniAbs, bsAbs). On the other hand their expression can be modulated by interfering with their expression level (siRNA, enzymatic removal, and cytokines). Alternatively, therapeutic antibody-mediated enhanced complement activation (green) can be achieved by mAb backbone modifications. Here mutations in the Fc part were identified to enhance C1q binding or increase IgG hexamerization which in turn facilitates efficient complement activation. Additionally, IgG3 antibodies or IgG1/IgG3 chimeras have a higher complement activating capacity. Furthermore, the use of CR2-Fc fusion proteins together with a therapeutic antibody and heteroconjugates (mAbs coupled to, for example, C3b and iC3b) boost the complement cascade. Moreover, combinations of antibodies effectively trigger complement. Targeting multiple epitopes with different mAbs increases the opsonization of the target cell and the combination of a therapeutic mAb with a mAb recognizing the inactive complement component iC3b further enhanced complement-mediated cell lysis.

concentration at the site of complement activation. Moreover, the Fc of the fusion protein is expected to result not only in extra complement deposition, but also additional recruitment of effector cells, resulting in enhanced CDC and ADCC. Complement deposition, CDC and ADCC were significantly increased in the presence of a human CR2-Fc fusion protein.<sup>117</sup> In vivo biodistribution studies with the radioactively-labeled fusion protein confirmed its tumor specificity. In the final therapeutic study, mice bearing EL4 T cell lymphoma were treated with a tumor-specific mAb, the fusion protein or the combination. Mice receiving the combination therapy showed prolonged survival (80%) compared with mice receiving just the mAb (20%). The same approach was further evaluated in a syngeneic model using the

murine CR2 sequence coupled to the murine IgG2a Fc.<sup>118,119</sup> In vitro data were comparable with results obtained with the human fusion protein. Co-administration with anti-tumor mAbs led to an improved anti-tumor response in two syngeneic mouse models (metastatic EL4 T cell lymphoma and melanoma B16), with CDC playing a key role as effector mechanism.

## Conclusions

The role of complement in mAb therapy of tumors is complex, which results from the multifaceted nature of complement activation. Several factors influence complement activation by



mAbs and most mAbs act via multiple effector mechanisms, and their relative importance may change during the course of treatment. Nonetheless, despite these variables, the overwhelming evidence suggests that induction of complement activation is beneficial. Several mAbs readily activate complement in vitro and there is strong evidence that CRPs are used by tumor cells to evade complement-mediated attack. This could potentially hamper complement-mediated anti-tumor effects.

In many cases, polymorphisms in CRPs causing excessive complement activation were linked to inflammatory conditions. Only a few studies have examined these variations in the context of mAb therapy. Based on in vitro results showing the inhibitory effect of CRPs on mAb-mediated complement activation, it is expected that similar associations could be found if complement does play a role in the clinical effect.

As we gain better understanding of the factors that influence complement activation by IgG mAbs, it will be possible to select

and engineer mAbs with improved complement activating properties. Stronger complement activation by ofatumumab compared with rituximab does not seem to translate into clinical benefit, although data on direct comparison with rituximab is not yet available.

In the future, clinical efficacy by mAbs rationally optimized for certain effector functions will enable better insight into their mechanisms of action and the role of complement. Several strategies are being developed to enhance complement activation, and these may also advance to the clinic (Fig. 2). Genetic screenings of large mAb-treated populations for polymorphisms in complement proteins and CRPs is expected to provide the much lacking human data.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### References

1. Beers SA, Cragg MS, Glennie MJ. Complement: help or hindrance? *Blood* 2009; 114:5567-8, author reply 5568; PMID:20035044; <http://dx.doi.org/10.1182/blood-2009-10-249466>
2. Introna M, Golay J. Complement in antibody therapy: friend or foe? *Blood* 2009; 114:5247-8; PMID:20018922; <http://dx.doi.org/10.1182/blood-2009-10-249532>
3. Taylor RP, Lindorfer MA. The role of complement in mAb-based therapies of cancer. *Methods* 2014; 65:18-27; PMID:23886909; <http://dx.doi.org/10.1016/j.ymeth.2013.07.027>
4. Macor P, Tedesco F. Complement as effector system in cancer immunotherapy. *Immunol Lett* 2007; 111:6-13; PMID:17572509; <http://dx.doi.org/10.1016/j.imlet.2007.04.014>
5. Holers VM. Complement and its receptors: new insights into human disease. *Annu Rev Immunol* 2014; 32:433-59; PMID:24499275; <http://dx.doi.org/10.1146/annurev-immunol-032713-120154>
6. Wagner E, Frank MM. Therapeutic potential of complement modulation. *Nat Rev Drug Discov* 2010; 9:43-56; PMID:19960015; <http://dx.doi.org/10.1038/nrd3011>
7. Gelderman KA, Lam S, Sier CF, Gorter A. Cross-linking tumor cells with effector cells via CD55 with a bispecific mAb induces beta-glucan-dependent CR3-dependent cellular cytotoxicity. *Eur J Immunol* 2006; 36:977-84; PMID:16525993; <http://dx.doi.org/10.1002/eji.200535653>
8. Boross P, Jansen JH, de Haij S, Beurskens FJ, van der Poel CE, Bevaart L, Nederend M, Golay J, van de Winkel JG, Parren PW, et al. The in vivo mechanism of action of CD20 monoclonal antibodies depends on local tumor burden. *Haematologica* 2011; 96:1822-30; PMID:21880632; <http://dx.doi.org/10.3324/haematol.2011.047159>
9. Shushakova N, Skokowa J, Schulman J, Baumann U, Zwirner J, Schmidt RE, Gessner JE. C5a anaphylatoxin is a major regulator of activating versus inhibitory FcγR3s in immune complex-induced lung disease. *J Clin Invest* 2002; 110:1823-30; PMID:12488432; <http://dx.doi.org/10.1172/JCI16577>
10. Chen CH, Boackle RJ. A newly discovered function for C1 inhibitor, removal of the entire C1q2s2 complex from immobilized human IgG subclasses. *Clin Immunol Immunopathol* 1998; 87:68-74; PMID:9576012; <http://dx.doi.org/10.1006/clin.1997.4515>
11. Bowen B, Hawk JJ, Sibunka S, Hovick S, Weiler JM. A review of the reported defects in the human C1 esterase inhibitor gene producing hereditary angioedema including four new mutations. *Clin Immunol* 2001; 98:157-63; PMID:11161971; <http://dx.doi.org/10.1006/clin.2000.4947>
12. Cugno M, Zanichelli A, Foienni F, Caccia S, Cicardi M. C1-inhibitor deficiency and angioedema: molecular mechanisms and clinical progress. *Trends Mol Med* 2009; 15:69-78; PMID:19162547; <http://dx.doi.org/10.1016/j.molmed.2008.12.001>
13. Rodríguez de Córdoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sánchez-Corral P. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol* 2004; 41:355-67; PMID:15163532; <http://dx.doi.org/10.1016/j.molimm.2004.02.005>
14. Kopp A, Hebecker M, Svobodová E, Józsi M, Factor H. A Complement Regulator in Health and Disease, and a Mediator of Cellular Interactions. *Biomolecules* 2012; 2:46-75; <http://dx.doi.org/10.3390/biom2010046>
15. Junnikkala S, Jokiranta TS, Friese MA, Jarva H, Zipfel PF, Meri S. Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1. *J Immunol* 2000; 164:6075-81; PMID:10820293; <http://dx.doi.org/10.4049/jimmunol.164.11.6075>
16. Charbonneau B, Maurer MJ, Fredericksen ZS, Zent CS, Link BK, Novak AJ, Ansell SM, Weiner GJ, Wang AH, Witzig TE, et al. Germline variation in complement genes and event-free survival in follicular and diffuse large B-cell lymphoma. *Am J Hematol* 2012; 87:880-5; PMID:22718493; <http://dx.doi.org/10.1002/ajh.23273>
17. Blom AM, Bergström F, Edey M, Diaz-Torres M, Kavanagh D, Lampe A, Goodship JA, Strain L, Moghal N, McHugh M, et al. A novel non-synonymous polymorphism (p.Arg240His) in C4b-binding protein is associated with atypical hemolytic uremic syndrome and leads to impaired alternative pathway cofactor activity. *J Immunol* 2008; 180:6385-91; PMID:18424762; <http://dx.doi.org/10.4049/jimmunol.180.9.6385>
18. Murphy BF, Saunders JR, O'Bryan MK, Kirszbaum L, Walker ID, d'Apice AJ. SP-40,40 is an inhibitor of C5b-6-initiated haemolysis. *Int Immunol* 1989; 1:551-4; PMID:2489042; <http://dx.doi.org/10.1093/intimm/1.5.551>
19. Hochgrebe TT, Humphreys D, Wilson MR, Easterbrook-Smith SB. A reexamination of the role of clusterin as a complement regulator. *Exp Cell Res* 1999; 249:13-21; PMID:10328949; <http://dx.doi.org/10.1006/excr.1999.4459>
20. Gladson CL, Cheresch DA. Glioblastoma expression of vitronectin and the alpha v beta 3 integrin. Adhesion mechanism for transformed glial cells. *J Clin Invest* 1991; 88:1924-32; PMID:1721625; <http://dx.doi.org/10.1172/JCI115516>
21. Liu D, Niu ZX. The structure, genetic polymorphisms, expression and biological functions of complement receptor type 1 (CR1/CD35). *Immunopharmacol Immunotoxicol* 2009; 31:524-35; PMID:19874218; <http://dx.doi.org/10.3109/08923970902845768>
22. Richards A, Kemp EJ, Liszewski MK, Goodship JA, Lampe AK, Decorte R, Müslümanoğlu MH, Kavukcu S, Filler G, Pirson Y, et al. Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc Natl Acad Sci U S A* 2003; 100:12966-71; PMID:14566051; <http://dx.doi.org/10.1073/pnas.2135497100>
23. Botto M, Kirschfink M, Macor P, Pickering MC, Würzner R, Tedesco F. Complement in human diseases: Lessons from complement deficiencies. *Mol Immunol* 2009; 46:2774-83; PMID:19481265; <http://dx.doi.org/10.1016/j.molimm.2009.04.029>
24. Junnikkala S, Hakulinen J, Jarva H, Manuelian T, Bjørge L, Bützow R, Zipfel PF, Meri S. Secretion of soluble complement inhibitors factor H and factor H-like protein (FHL-1) by ovarian tumour cells. *Br J Cancer* 2002; 87:1119-27; PMID:12402151; <http://dx.doi.org/10.1038/sj.bjc.6600614>
25. Ajona D, Castaño Z, Garayoa M, Zudaire E, Pajares MJ, Martínez A, Cuttitta F, Montuenga LM, Pio R. Expression of complement factor H by lung cancer cells: effects on the activation of the alternative pathway of complement. *Cancer Res* 2004; 64:6310-8; PMID:15342420; <http://dx.doi.org/10.1158/0008-5472.CAN-03-2328>
26. Wilczek E, Rzepko R, Nowis D, Legat M, Golab J, Glab M, Gorlewicz A, Konopacki F, Mazurkiewicz M, Sładowski D, et al. The possible role of factor H in colon cancer resistance to complement attack. *Int J Cancer* 2008; 122:2030-7; PMID:18183578; <http://dx.doi.org/10.1002/ijc.23238>
27. Steinberg J, Oyasu R, Lang S, Sintich S, Rademaker A, Lee C, Kozłowski JM, Sensibar JA. Intracellular levels of SGP-2 (Clusterin) correlate with tumor grade in prostate cancer. *Clin Cancer Res* 1997; 3:1707-11; PMID:9815554
28. Parczyk K, Pilarsky C, Rachel U, Koch-Brandt C. Gp80 (clusterin; TRPM-2) mRNA level is enhanced in human renal clear cell carcinomas. *J Cancer Res*

- Clin Oncol 1994; 120:186-8; PMID:8263017; <http://dx.doi.org/10.1007/BF01202200>
29. Hough CD, Cho KR, Zonderman AB, Schwartz DR, Morin PJ. Coordinately up-regulated genes in ovarian cancer. *Cancer Res* 2001; 61:3869-76; PMID: 11358798
  30. Kumar S, Vinci JM, Pytel BA, Baglioni C. Expression of messenger RNAs for complement inhibitors in human tissues and tumors. *Cancer Res* 1993; 53:348-53; PMID:7678074
  31. Behrens P, Jeske W, Wernert N, Wellmann A. Down-regulation of clusterin expression in testicular germ cell tumours. *Pathobiology* 2001; 69:19-23; PMID: 11641613; <http://dx.doi.org/10.1159/00048753>
  32. Redondo M, Villar E, Torres-Muñoz J, Tellez T, Morell M, Petito CK. Overexpression of clusterin in human breast carcinoma. *Am J Pathol* 2000; 157:393-9; PMID:10934144; [http://dx.doi.org/10.1016/S0002-9440\(10\)64552-X](http://dx.doi.org/10.1016/S0002-9440(10)64552-X)
  33. Trougakos IP, Gonos ES. Clusterin/apolipoprotein J in human aging and cancer. *Int J Biochem Cell Biol* 2002; 34:1430-48; PMID:12200037; [http://dx.doi.org/10.1016/S1357-2725\(02\)00041-9](http://dx.doi.org/10.1016/S1357-2725(02)00041-9)
  34. Yasumitsu H, Seo N, Misugi E, Morita H, Miyazaki K, Umeda M. Vitronectin secretion by hepatic and non-hepatic human cancer cells. *In Vitro Cell Dev Biol Anim* 1993; 29A:403-7; PMID:7686142; <http://dx.doi.org/10.1007/BF02633989>
  35. Kadawaki M, Sangai T, Nagashima T, Sakakibara M, Yoshitomi H, Takano S, Sogawa K, Umemura H, Fushimi K, Nakatani Y, et al. Identification of vitronectin as a novel serum marker for early breast cancer detection using a new proteomic approach. *J Cancer Res Clin Oncol* 2011; 137:1105-15; PMID:21253761; <http://dx.doi.org/10.1007/s00432-010-0974-9>
  36. Seya T, Matsumoto M, Hara T, Hatanaka M, Masaoka T, Akedo H. Distribution of C3-step regulatory proteins of the complement system, CD35 (CR1), CD46 (MCP), and CD55 (DAF), in hematological malignancies. *Leuk Lymphoma* 1994; 12:395-400; PMID:7514063; <http://dx.doi.org/10.3109/10428199409073780>
  37. Niehans GA, Chervitz DL, Staley NA, Knapp DJ, Dalmasso AP. Human carcinomas variably express the complement inhibitory proteins CD46 (membrane cofactor protein), CD55 (decay-accelerating factor), and CD59 (protectin). *Am J Pathol* 1996; 149:129-42; PMID:8686736
  38. Donin N, Jurianz K, Ziporen L, Schultz S, Kirschfink M, Fishelson Z. Complement resistance of human carcinoma cells depends on membrane regulatory proteins, protein kinases and sialic acid. *Clin Exp Immunol* 2003; 131:254-63; PMID:12562385; <http://dx.doi.org/10.1046/j.1365-2249.2003.02066.x>
  39. Coral S, Fonsatti E, Sigalotti L, De Nardo C, Visintin A, Nardi G, Colizzi F, Colombo MP, Romano G, Altomonte M, et al. Overexpression of protectin (CD59) down-modulates the susceptibility of human melanoma cells to homologous complement. *J Cell Physiol* 2000; 185:317-23; PMID:11056001; [http://dx.doi.org/10.1002/1097-4652\(200012\)185:3<317::AID-JCP1>3.0.CO;2-L](http://dx.doi.org/10.1002/1097-4652(200012)185:3<317::AID-JCP1>3.0.CO;2-L)
  40. Madjd Z, Pinder SE, Paish C, Ellis IO, Carmichael J, Durrant LG. Loss of CD59 expression in breast tumours correlates with poor survival. *J Pathol* 2003; 200:633-9; PMID:12898600; <http://dx.doi.org/10.1002/path.1357>
  41. Koretz K, Brüderlein S, Henne C, Möller P. Expression of CD59, a complement regulator protein and a second ligand of the CD2 molecule, and CD46 in normal and neoplastic colorectal epithelium. *Br J Cancer* 1993; 68:926-31; PMID:7692919; <http://dx.doi.org/10.1038/bjc.1993.456>
  42. Loberg RD, Day LL, Dunn R, Kalikin LM, Pienta KJ. Inhibition of decay-accelerating factor (CD55) attenuates prostate cancer growth and survival in vivo. *Neoplasia* 2006; 8:69-78; PMID:16533428; <http://dx.doi.org/10.1593/neo.05679>
  43. Durrant LG, Chapman MA, Buckley DJ, Spendlove I, Robins RA, Armitage NC. Enhanced expression of the complement regulatory protein CD55 predicts a poor prognosis in colorectal cancer patients. *Cancer Immunol Immunother* 2003; 52:638-42; PMID: 12811528; <http://dx.doi.org/10.1007/s00262-003-0402-y>
  44. Blok VT, Daha MR, Tijmsma OM, Weissglas MG, van den Broek LJ, Gorter A. A possible role of CD46 for the protection in vivo of human renal tumor cells from complement-mediated damage. *Lab Invest* 2000; 80:335-44; PMID:10744069; <http://dx.doi.org/10.1038/labinvest.3780038>
  45. Manches O, Lui G, Chaperot L, Gressin R, Molens JP, Jacob MC, Sotto JJ, Leroux D, Bens JC, Plumas J. In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* 2003; 101:949-54; PMID:12393572; <http://dx.doi.org/10.1182/blood-2002-02-0469>
  46. Golay J, Lazzari M, Facchinetti V, Bernasconi S, Borleri G, Barbui T, Rambaldi A, Introna M. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* 2001; 98:3383-9; PMID:11719378; <http://dx.doi.org/10.1182/blood.V98.12.3383>
  47. Inoue T, Yamakawa M, Takahashi T. Expression of complement regulating factors in gastric cancer cells. *Mol Pathol* 2002; 55:193-9; PMID:12032231; <http://dx.doi.org/10.1136/mp.55.3.193>
  48. Murray KP, Mathure S, Kaul R, Khan S, Carson LF, Twigg LB, Martens MG, Kaul A. Expression of complement regulatory proteins-CD 35, CD 46, CD 55, and CD 59-in benign and malignant endometrial tissue. *Gynecol Oncol* 2000; 76:176-82; PMID: 10637067; <http://dx.doi.org/10.1006/gyno.1999.5614>
  49. Teeling JL, French RR, Cragg MS, van den Brakel J, Ployter M, Huang H, Chan C, Parren PW, Hack CE, Dechant M, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood* 2004; 104:1793-800; PMID:15172969; <http://dx.doi.org/10.1182/blood-2004-01-0039>
  50. Bologna L, Gotti E, Da Roit F, Interemesoli T, Rambaldi A, Introna M, Golay J. Ofatumumab is more efficient than rituximab in lysing B chronic lymphocytic leukemia cells in whole blood and in combination with chemotherapy. *J Immunol* 2013; 190:231-9; PMID:23225880; <http://dx.doi.org/10.4049/jimmunol.1202645>
  51. Baig NA, Taylor RP, Lindorfer MA, Church AK, Laplant BR, Pavey ES, Nowakowski GS, Zent CS. Complement dependent cytotoxicity in chronic lymphocytic leukemia: ofatumumab enhances alemtuzumab complement dependent cytotoxicity and reveals cells resistant to activated complement. *Leuk Lymphoma* 2012; 53:2218-27; PMID:22475085; <http://dx.doi.org/10.3109/10428194.2012.681657>
  52. Bologna L, Gotti E, Manganini M, Rambaldi A, Interemesoli T, Introna M, Golay J. Mechanism of action of type II, glycoengineered, anti-CD20 monoclonal antibody GA101 in B-chronic lymphocytic leukemia whole blood assays in comparison with rituximab and alemtuzumab. *J Immunol* 2011; 186:3762-9; PMID:21296976; <http://dx.doi.org/10.4049/jimmunol.1000303>
  53. Golay J, Manganini M, Rambaldi A, Introna M. Effect of alemtuzumab on neoplastic B cells. *Haematologica* 2004; 89:1476-83; PMID:15590398
  54. Zent CS, Chen JB, Kurten RC, Kaushal GP, Lacy HM, Schichman SA. Alemtuzumab (CAMPATH 1H) does not kill chronic lymphocytic leukemia cells in serum free medium. *Leuk Res* 2004; 28:495-507; PMID:15068903; <http://dx.doi.org/10.1016/j.leukres.2003.09.011>
  55. Hale G, Swirsky DM, Hayhoe FG, Waldmann H. Effects of monoclonal anti-lymphocyte antibodies in vivo in monkeys and humans. *Mol Biol Med* 1983; 1:321-34; PMID:6438435
  56. Dyer MJ, Hale G, Hayhoe FG, Waldmann H. Effects of CAMPATH-1 antibodies in vivo in patients with lymphoid malignancies: influence of antibody isotype. *Blood* 1989; 73:1431-9; PMID:2713487
  57. de Weers M, Tai YT, van der Veer MS, Bakker JM, Vink T, Jacobs DC, Oomen LA, Peipp M, Valerius T, Slootstra JW, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol* 2011; 186:1840-8; PMID: 21187443; <http://dx.doi.org/10.4049/jimmunol.1003032>
  58. Minard-Colin V, Xiu Y, Poe JC, Horikawa M, Magro CM, Hamaguchi Y, Haas KM, Tedder TF. Lymphoma depletion during CD20 immunotherapy in mice is mediated by macrophage Fc gammaRI, Fc gammaRIII, and Fc gammaRIV. *Blood* 2008; 112:1205-13; PMID:18495955; <http://dx.doi.org/10.1182/blood-2008-01-135160>
  59. Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, Tedder TF. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* 2004; 199:1659-69; PMID:15210744; <http://dx.doi.org/10.1084/jem.20040119>
  60. Beers SA, Chan CH, James S, French RR, Attfield KE, Brennan CM, Ahuja A, Shlomchik MJ, Cragg MS, Glennie MJ. Type II (totitumomab) anti-CD20 monoclonal antibody outperforms type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. *Blood* 2008; 112:4170-7; PMID:18583569; <http://dx.doi.org/10.1182/blood-2008-04-149161>
  61. Beers SA, French RR, Chan HT, Lim SH, Jarrett TC, Vidal RM, Wijayawera SS, Dixon SV, Kim H, Cox KL, et al. Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. *Blood* 2010; 115:5191-201; PMID:20223920; <http://dx.doi.org/10.1182/blood-2010-01-263533>
  62. Hu Y, Turner MJ, Shields J, Gale MS, Hutto E, Roberts BL, Siders WM, Kaplan JM. Investigation of the mechanism of action of alemtuzumab in a human CD52 transgenic mouse model. *Immunology* 2009; 128:260-70; PMID:19740383; <http://dx.doi.org/10.1111/j.1365-2567.2009.03115.x>
  63. Otten MA, van der Bij GJ, Verbeek SJ, Nimmerjahn F, Ravetch JV, Beelen RH, van de Winkel JG, van Edmond M. Experimental antibody therapy of liver metastases reveals functional redundancy between Fc gammaRI and Fc gammaRIV. *J Immunol* 2008; 181:6829-36; PMID:18981101; <http://dx.doi.org/10.4049/jimmunol.181.10.6829>
  64. Di Gaetano N, Cittera E, Nota R, Vecchi A, Grieco V, Scanziani E, Botto M, Introna M, Golay J. Complement activation determines the therapeutic activity of rituximab in vivo. *J Immunol* 2003; 171:1581-7; PMID:12874252; <http://dx.doi.org/10.4049/jimmunol.171.3.1581>
  65. Cragg MS, Glennie MJ. Antibody specificity controls in vivo effector mechanisms of anti-CD20 reagents. *Blood* 2004; 103:2738-43; PMID:14551143; <http://dx.doi.org/10.1182/blood-2003-06-2031>
  66. Imai M, Landen C, Ohta R, Cheung NK, Tomlinson S. Complement-mediated mechanisms in anti-GD2 monoclonal antibody therapy of murine metastatic cancer. *Cancer Res* 2005; 65:10562-8; PMID: 16288049; <http://dx.doi.org/10.1158/0008-5472.CAN-05-1894>
  67. Boross P, Jansen JH, de Haij S, Beurskens FJ, van der Poel CE, Bevaart L, Nederend M, Golay J, van de Winkel JG, Parren PW, et al. The in vivo mechanism of action of CD20 monoclonal antibodies depends on local tumor burden. *Haematologica* 2011; 96:1822-

- 30; PMID:21880632; <http://dx.doi.org/10.3324/haematol.2011.047159>
68. Kennedy AD, Solga MD, Schuman TA, Chi AW, Lindorfer MA, Sutherland WM, Foley PL, Taylor RP. An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of CD20+ cells by rituximab. *Blood* 2003; 101:1071-9; PMID:12393727; <http://dx.doi.org/10.1182/blood-2002-03-0876>
  69. Beurskens FJ, Lindorfer MA, Farooqui M, Beum PV, Engelberts P, Mackus WJ, Parren PW, Wiestner A, Taylor RP. Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. *J Immunol* 2012; 188:3532-41; PMID:22368276; <http://dx.doi.org/10.4049/jimmunol.1103693>
  70. Klepfish A, Gilles L, Ioannis K, Rachmilewitz EA, Schattner A. Enhancing the action of rituximab in chronic lymphocytic leukemia by adding fresh frozen plasma: complement/rituximab interactions & clinical results in refractory CLL. *Ann N Y Acad Sci* 2009; 1173:865-73; PMID:19758239; <http://dx.doi.org/10.1111/j.1749-6632.2009.04803.x>
  71. Xu W, Miao KR, Zhu DX, Fang C, Zhu HY, Dong HJ, Wang DM, Wu YJ, Qiao C, Li JY. Enhancing the action of rituximab by adding fresh frozen plasma for the treatment of fludarabine refractory chronic lymphocytic leukemia. *Int J Cancer* 2011; 128:2192-201; PMID:20635386; <http://dx.doi.org/10.1002/ijc.25560>
  72. Wang SY, Racila E, Taylor RP, Weiner GJ. NK-cell activation and antibody-dependent cellular cytotoxicity induced by rituximab-coated target cells is inhibited by the C3b component of complement. *Blood* 2008; 111:1456-63; PMID:18024795; <http://dx.doi.org/10.1182/blood-2007-02-074716>
  73. Kern DJ, James BR, Blackwell S, Gassner C, Klein C, Weiner GJ. GA101 induces NK-cell activation and antibody-dependent cellular cytotoxicity more effectively than rituximab when complement is present. *Leuk Lymphoma* 2013; 54:2500-5; PMID:23452151; <http://dx.doi.org/10.3109/10428194.2013.781169>
  74. Wang SY, Veeramani S, Racila E, Cagley J, Fritzinger DC, Vogel CW, St John W, Weiner GJ. Depletion of the C3 component of complement enhances the ability of rituximab-coated target cells to activate human NK cells and improves the efficacy of monoclonal antibody therapy in an in vivo model. *Blood* 2009; 114:5322-30; PMID:19805620; <http://dx.doi.org/10.1182/blood-2009-01-200469>
  75. Racila E, Link BK, Weng WK, Witzig TE, Ansell S, Maurer MJ, Huang J, Dahle C, Halwani A, Levy R, et al. A polymorphism in the complement component C1qA correlates with prolonged response following rituximab therapy of follicular lymphoma. *Clin Cancer Res* 2008; 14:6697-703; PMID:18927313; <http://dx.doi.org/10.1158/1078-0432.CCR-08-0745>
  76. Brasoveanu LI, Altomonte M, Fonsatti E, Colizzi F, Coral S, Nicotra MR, Cattarossi I, Cattelani A, Natali PG, Maio M. Levels of cell membrane CD59 regulate the extent of complement-mediated lysis of human melanoma cells. *Lab Invest* 1996; 74:33-42; PMID:8569195
  77. Beyer I, Cao H, Persson J, Wang H, Liu Y, Yumul R, Li Z, Woodle D, Manger R, Gough M, et al. Transient removal of CD46 is safe and increases B-cell depletion by rituximab in CD46 transgenic mice and macaques. *Mol Ther* 2013; 21:291-9; PMID:23089733; <http://dx.doi.org/10.1038/mt.2012.212>
  78. Varsano S, Rashkovsky L, Shapiro H, Radnay J. Cytokines modulate expression of cell-membrane complement inhibitory proteins in human lung cancer cell lines. *Am J Respir Cell Mol Biol* 1998; 19:522-9; PMID:9730881; <http://dx.doi.org/10.1165/ajrcmb.19.3.3181>
  79. Blok VT, Gelderman KA, Tijmsma OH, Daha MR, Gorter A. Cytokines affect resistance of human renal tumour cells to complement-mediated injury. *Scand J Immunol* 2003; 57:591-9; PMID:12791098; <http://dx.doi.org/10.1046/j.1365-3083.2003.01265.x>
  80. Geis N, Zell S, Rutz R, Li W, Giese T, Mamidi S, Schultz S, Kirschfink M. Inhibition of membrane complement inhibitor expression (CD46, CD55, CD59) by siRNA sensitizes tumor cells to complement attack in vitro. *Curr Cancer Drug Targets* 2010; 10:922-31; PMID:20879979; <http://dx.doi.org/10.2174/156800910793357952>
  81. Terui Y, Sakurai T, Mishima Y, Mishima Y, Sugimura N, Sasaoka C, Kojima K, Yokoyama M, Mizunuma N, Takahashi S, et al. Blockade of bulky lymphoma-associated CD55 expression by RNA interference overcomes resistance to complement-dependent cytotoxicity with rituximab. *Cancer Sci* 2006; 97:72-9; PMID:16367924; <http://dx.doi.org/10.1111/j.1349-7006.2006.00139.x>
  82. Mamidi S, Cinci M, Hasmann M, Fehring V, Kirschfink M. Lipoplex mediated silencing of membrane regulators (CD46, CD55 and CD59) enhances complement-dependent anti-tumor activity of trastuzumab and pertuzumab. *Mol Oncol* 2013; 7:580-94; PMID:23474221; <http://dx.doi.org/10.1016/j.molonc.2013.02.011>
  83. Hu W, Ge X, You T, Xu T, Zhang J, Wu G, Peng Z, Chorev M, Aktas BH, Halperin JA, et al. Human CD59 inhibitor sensitizes rituximab-resistant lymphoma cells to complement-mediated cytotoxicity. *Cancer Res* 2011; 71:2298-307; PMID:21252115; <http://dx.doi.org/10.1158/0008-5472.CAN-10-3016>
  84. Ge X, Wu L, Hu W, Fernandes S, Wang C, Li X, Brown JR, Qin X. rILYd4, a human CD59 inhibitor, enhances complement-dependent cytotoxicity of ofatumumab against rituximab-resistant B-cell lymphoma cells and chronic lymphocytic leukemia. *Clin Cancer Res* 2011; 17:6702-11; PMID:21918174; <http://dx.doi.org/10.1158/1078-0432.CCR-11-0647>
  85. Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, Tedesco F, Rambaldi A, Introna M. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* 2000; 95:3900-8; PMID:10845926
  86. Ziller F, Macor P, Bulla R, Sblattero D, Marzari R, Tedesco F. Controlling complement resistance in cancer by using human monoclonal antibodies that neutralize complement-regulatory proteins CD55 and CD59. *Eur J Immunol* 2005; 35:2175-83; PMID:15971270; <http://dx.doi.org/10.1002/eji.200425920>
  87. Macor P, Tripodo C, Zorzet S, Piovani E, Bossi F, Marzari R, Amadori A, Tedesco F. In vivo targeting of human neutralizing antibodies against CD55 and CD59 to lymphoma cells increases the antitumor activity of rituximab. *Cancer Res* 2007; 67:10556-63; PMID:17975000; <http://dx.doi.org/10.1158/0008-5472.CAN-07-1811>
  88. Gelderman KA, Blok VT, Fleuren GJ, Gorter A. The inhibitory effect of CD46, CD55, and CD59 on complement activation after immunotherapeutic treatment of cervical carcinoma cells with monoclonal antibodies or bispecific monoclonal antibodies. *Lab Invest* 2002; 82:483-93; PMID:11950905; <http://dx.doi.org/10.1038/labinvest.3780441>
  89. Gelderman KA, Kuppen PJ, Bruin W, Fleuren GJ, Gorter A. Enhancement of the complement activating capacity of 17-1A mAb to overcome the effect of membrane-bound complement regulatory proteins on colorectal carcinoma. *Eur J Immunol* 2002; 32:128-35; PMID:11754353; [http://dx.doi.org/10.1002/1521-4141\(200201\)32:1<128::AID-IMMU128>3.0.CO;2-P](http://dx.doi.org/10.1002/1521-4141(200201)32:1<128::AID-IMMU128>3.0.CO;2-P)
  90. Gelderman KA, Kuppen PJ, Okada N, Fleuren GJ, Gorter A. Tumor-specific inhibition of membrane-bound complement regulatory protein Cry with bispecific monoclonal antibodies prevents tumor outgrowth in a rat colorectal cancer lung metastases model. *Cancer Res* 2004; 64:4366-72; PMID:15205353; <http://dx.doi.org/10.1158/0008-5472.CAN-03-2131>
  91. Hörl S, Banki Z, Huber G, Ejaz A, Müllauer B, Willenbacher E, Steurer M, Stoiber H. Complement factor H-derived short consensus repeat 18-20 enhanced complement-dependent cytotoxicity of ofatumumab on chronic lymphocytic leukemia cells. *Haematologica* 2013; 98:1939-47; PMID:23850806; <http://dx.doi.org/10.3324/haematol.2013.089615>
  92. Hörl S, Bánki Z, Huber G, Ejaz A, Windisch D, Müllauer B, Willenbacher E, Steurer M, Stoiber H. Reduction of complement factor H binding to CLL cells improves the induction of rituximab-mediated complement-dependent cytotoxicity. *Leukemia* 2013; 27:2200-8; PMID:23760402; <http://dx.doi.org/10.1038/leu.2013.169>
  93. Lindorfer MA, Beum PV, Taylor RP. CD20 mAb-Mediated Complement Dependent Cytotoxicity of Tumor Cells is Enhanced by Blocking the Action of Factor I. *Antibodies* 2013; 2:598-616; <http://dx.doi.org/10.3390/antib2040598>
  94. Miwa T, Song WC. Membrane complement regulatory proteins: insight from animal studies and relevance to human diseases. *Int Immunopharmacol* 2001; 1:445-59; PMID:11367529; [http://dx.doi.org/10.1016/S1567-5769\(00\)00043-6](http://dx.doi.org/10.1016/S1567-5769(00)00043-6)
  95. Ong GL, Mattes MJ. Mouse strains with typical mammalian levels of complement activity. *J Immunol Methods* 1989; 125:147-58; PMID:2607149; [http://dx.doi.org/10.1016/0022-1759\(89\)90088-4](http://dx.doi.org/10.1016/0022-1759(89)90088-4)
  96. Gerritsen AF, Bosch M, de Weers M, de Winkel JG, Parren PW. High throughput screening for antibody induced complement-dependent cytotoxicity in early antibody discovery using homogeneous macroconfocal fluorescence imaging. *J Immunol Methods* 2010; 352:140-6; PMID:19891971; <http://dx.doi.org/10.1016/j.jim.2009.10.009>
  97. Rösner T, Derer S, Kellner C, Dechant M, Lohse S, Vidarsson G, Peipp M, Valerius T, An IgG3 switch variant of rituximab mediates enhanced complement-dependent cytotoxicity against tumour cells with low CD20 expression levels. *Br J Haematol* 2013; 161:282-6; PMID:23294176; <http://dx.doi.org/10.1111/bjh.12209>
  98. Stapleton NM, Andersen JT, Stemerding AM, Bjarneron SP, Verheul RC, Gerritsen J, Zhao Y, Kleijer M, Sandlie I, de Haas M, et al. Competition for FcRn-mediated transport gives rise to short half-life of human IgG3 and offers therapeutic potential. *Nat Commun* 2011; 2:599; PMID:22186895; <http://dx.doi.org/10.1038/ncomms1608>
  99. Natsume A, In M, Takamura H, Nakagawa T, Shimizu Y, Kitajima K, Wakitani M, Ohta S, Satoh M, Shitara K, et al. Engineered antibodies of IgG1/IgG3 mixed isotype with enhanced cytotoxic activities. *Cancer Res* 2008; 68:3863-72; PMID:18483271; <http://dx.doi.org/10.1158/0008-5472.CAN-07-6297>
  100. Thommesen JE, Michaelsen TE, Løset GA, Sandlie I, Brekke OH. Lysine 322 in the human IgG3 C(H)2 domain is crucial for antibody dependent complement activation. *Mol Immunol* 2000; 37:995-1004; PMID:11395138; [http://dx.doi.org/10.1016/S0161-5890\(01\)00010-4](http://dx.doi.org/10.1016/S0161-5890(01)00010-4)
  101. Idusogie EE, Wong PY, Presta LG, Gazzano-Santoro H, Totpal K, Ultsch M, Mullkerrin MG. Engineered antibodies with increased activity to recruit complement. *J Immunol* 2001; 166:2571-5; PMID:11160318; <http://dx.doi.org/10.4049/jimmunol.166.4.2571>
  102. Moore GL, Chen H, Karki S, Lazar GA. Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. *MAbs* 2010; 2:181-9; PMID:20150767; <http://dx.doi.org/10.4161/mabs.2.2.11158>
  103. Spiridon CI, Ghetie MA, Uhr J, Marches R, Li JL, Shen GL, Vitetta ES. Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antitumor activity of a human breast cancer cell line



- in vitro and in vivo. *Clin Cancer Res* 2002; 8:1720-30; PMID:12060609
104. Dechant M, Weisner W, Berger S, Peipp M, Beyer T, Schneider-Merck T, Lammerts van Bueren JJ, Bleeker WK, Parren PW, van de Winkel JG, et al. Complement-dependent tumor cell lysis triggered by combinations of epidermal growth factor receptor antibodies. *Cancer Res* 2008; 68:4998-5003; PMID:18593896; <http://dx.doi.org/10.1158/0008-5472.CAN-07-6226>
  105. Diebold CA, Beurskens FJ, de Jong RN, Koning RI, Strumane K, Lindorfer MA, Voorhorst M, Ugurlar D, Rosati S, Heck AJ, et al. Complement is activated by IgG hexamers assembled at the cell surface. *Science* 2014; 343:1260-3; PMID:24626930; <http://dx.doi.org/10.1126/science.1248943>
  106. Beurskens FJ, de Jong RN, Verploegen S, Voorhorst M, Strumane K, Lindorfer MA, Taylor RP, Saphire EO, Burton D, Schuurman J, et al. Enhanced IgG Hexamerization Mediates Efficient C1q Docking and Complement-Dependent Cytotoxicity; Preclinical Proof Of Concept On Primary CLL and Burkitt Lymphoma. *Blood* 2013; 122:375.
  107. Boyd PN, Lines AC, Patel AK. The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H. *Mol Immunol* 1995; 32:1311-8; PMID:8643100; [http://dx.doi.org/10.1016/0161-5890\(95\)00118-2](http://dx.doi.org/10.1016/0161-5890(95)00118-2)
  108. Tsuchiya N, Endo T, Matsuta K, Yoshinoya S, Aikawa T, Kosuge E, Takeuchi F, Miyamoto T, Kobata A. Effects of galactose depletion from oligosaccharide chains on immunological activities of human IgG. *J Rheumatol* 1989; 16:285-90; PMID:2498512
  109. Malhotra R, Wormald MR, Rudd PM, Fischer PB, Dwek RA, Sim RB. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. *Nat Med* 1995; 1:237-43; PMID:7585040; <http://dx.doi.org/10.1038/nm0395-237>
  110. Wright A, Morrison SL. Effect of C2-associated carbohydrate structure on Ig effector function: studies with chimeric mouse-human IgG1 antibodies in glycosylation mutants of Chinese hamster ovary cells. *J Immunol* 1998; 160:3393-402; PMID:9531299
  111. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fc gamma RIII and antibody-dependent cellular toxicity. *J Biol Chem* 2002; 277:26733-40; PMID:11986321; <http://dx.doi.org/10.1074/jbc.M202069200>
  112. Nimmerjahn F, Anthony RM, Ravetch JV. Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity. *Proc Natl Acad Sci U S A* 2007; 104:8433-7; PMID:17485663; <http://dx.doi.org/10.1073/pnas.0702936104>
  113. Reiter Y, Fishelson Z. Targeting of complement to tumor cells by heteroconjugates composed of antibodies and of the complement component C3b. *J Immunol* 1989; 142:2771-7; PMID:2522969
  114. Juhl H, Petrella EC, Cheung NK, Bredehorst R, Vogel CW. Complement killing of human neuroblastoma cells: a cytotoxic monoclonal antibody and its F(ab')<sub>2</sub>-cobra venom factor conjugate are equally cytotoxic. *Mol Immunol* 1990; 27:957-64; PMID:2233757; [http://dx.doi.org/10.1016/0161-5890\(90\)90118-J](http://dx.doi.org/10.1016/0161-5890(90)90118-J)
  115. Juhl H, Petrella EC, Cheung NK, Bredehorst R, Vogel CW. Additive cytotoxicity of different monoclonal antibody-cobra venom factor conjugates for human neuroblastoma cells. *Immunobiology* 1997; 197:444-59; PMID:9413745; [http://dx.doi.org/10.1016/S0171-2985\(97\)80078-2](http://dx.doi.org/10.1016/S0171-2985(97)80078-2)
  116. Xiang D, Sharma VR, Freter CE, Yan J. Anti-tumor monoclonal antibodies in conjunction with  $\beta$ -glucans: a novel anti-cancer immunotherapy. *Curr Med Chem* 2012; 19:4298-305; PMID:22834812; <http://dx.doi.org/10.2174/092986712802884303>
  117. Imai M, Ohta R, Varela JC, Song H, Tomlinson S. Enhancement of antibody-dependent mechanisms of tumor cell lysis by a targeted activator of complement. *Cancer Res* 2007; 67:9535-41; PMID:17909064; <http://dx.doi.org/10.1158/0008-5472.CAN-07-1690>
  118. Elvington M, Huang Y, Morgan BP, Qiao F, van Rooijen N, Atkinson C, Tomlinson S. A targeted complement-dependent strategy to improve the outcome of mAb therapy, and characterization in a murine model of metastatic cancer. *Blood* 2012; 119:6043-51; PMID:22442351; <http://dx.doi.org/10.1182/blood-2011-10-383232>
  119. Boross P, Leusen JH. Boosting antibody therapy with complement. *Blood* 2012; 119:5945-7; PMID:22730521; <http://dx.doi.org/10.1182/blood-2012-04-420760>