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Review Article

Complement as effector system in cancer immunotherapy

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Abstract

The contribution of the complement system to the control of tumour growth has been neglected for a long time as the major emphasis has been put mainly on cell-mediated immune response against cancer. With the introduction of monoclonal antibodies in cancer immunotherapy complement has come into play with a great potential as effector system. Complement has a number of advantages over other effector systems in that it is made of molecules that can easily penetrate the tumour tissue and a large majority, if not all, of the components of this system can be supplied locally by many cells at tissue site.

Further advances are being made to increase the anti-tumour efficiency of the complements system using C-fixing antibodies that are modified in the Fc portion to be more active in complement activation. Another strategy currently investigated is essentially based on the use of a combination of two antibodies directed against different molecules or different epitopes of the same molecule expressed on the cell surface in order to increase the number of the binding sites for the antibodies on the tumor cells and the chance for them to activate complement more efficiently.

One of the problems to solve in exploiting complement as an effector system in cancer immunotherapy is to neutralize the inhibitory effect of complement regulatory proteins which are often over-expressed on tumour cells and represent a mechanism of evasion of these cells from complement attack. This situation can be overcome using neutralizing antibodies to target onto tumour cells together with the specific antibodies directed against tumor specific antigens. This is an area of active investigation and the initial data that start to be available from animal models seem to be promising.

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1. The complement system

The complement (C) system is an essential component of innate immunity and is actively involved in the host defense against infectious agents. It is also important in the removal of immune complexes and apoptotic cells [1]. Cancer cells may also be a potential target of C as suggested by the finding that activated C components and the terminal C complex are deposited on tumor masses such as breast and thyroid carcinoma [2,3].

The C system (Fig. 1) requires an activation process to release the biologically active products that are capable of recognizing and attacking neoplastic cells. The system can be directly activated by tumor cells through the alternative [4–6] or the lectin pathway [4,7]. However, antibody (Ab)-mediated activation of the classical pathway represents the most efficient way to tar-

get C activation products to tumor cells in sufficient amount to cause cell damage. Unfortunately, the humoral response in tumor-bearing patients is not very efficient and only low-titer and low-affinity Abs to tumor antigens are usually detected in cancer patients. In addition, these Abs are poor C activators and are unlikely to mediate C-dependent cytotoxicity (CDC) of neoplastic cells.

The C system has a definite advantage over cytotoxic cells as a defense system because it is made of soluble molecules that can easily reach the tumor site and diffuse inside the tumor mass. Moreover, C components are readily available as a first line of defense because they are synthesized locally by many cell types, including macrophages [8] fibroblasts [9] and endothelial cells [10,11]. Several neoplastic cells have also been shown to synthesize and secrete components of the C system [12,13].

Direct killing of tumor cells by the membrane attack complex (MAC) represents one of mechanisms used by the C system to control tumor growth. However, C may also exert its anti-tumor activity through additional non-cytotoxic effects. Thus,

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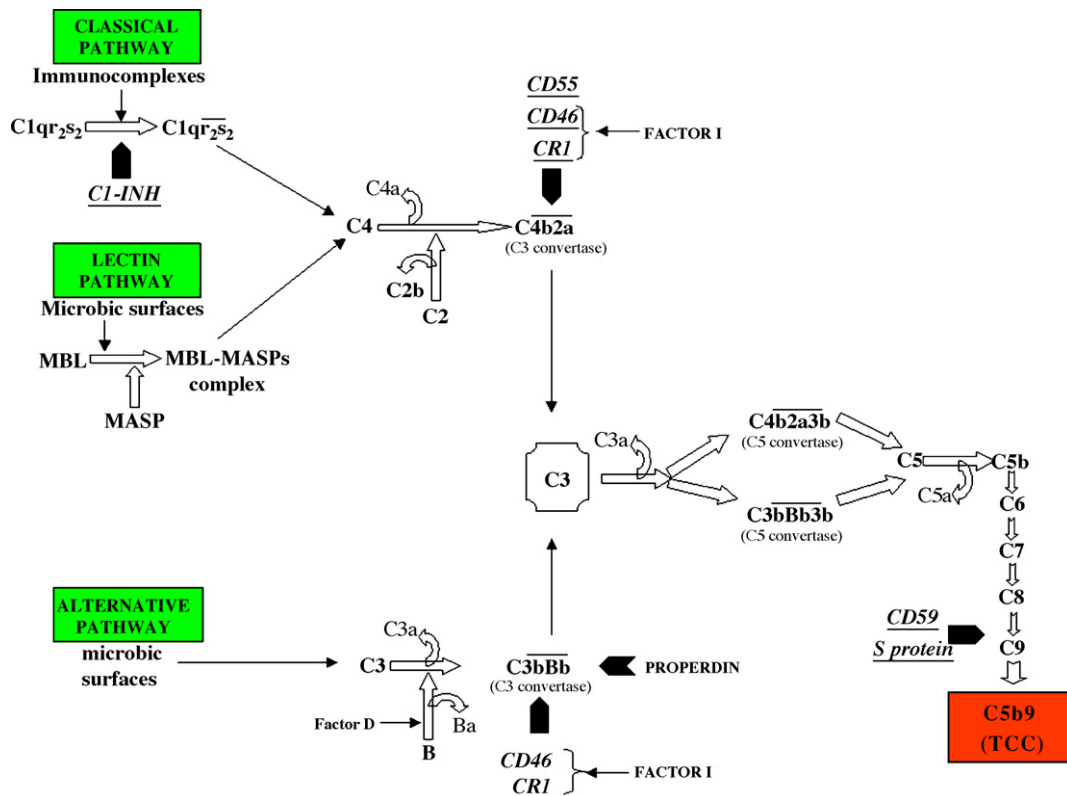


Fig. 1. The complement system.

C3b deposited on tumor cells and subsequently converted into iC3b promotes binding of these cells to the C receptors CR1 and CR3 expressed on human leukocytes. Although CR1 and CR3 fail to trigger the killing of tumor cells following their interaction with their respective ligands, C3b and iC3b, evidence collected both *in vitro* and *in vivo* indicate that the adhesion of iC3b-coated tumor cells to phagocytes and natural killer (NK) cells expressing CR3 (CD11b–CD18) results in C-dependent cell cytotoxicity (CDCC) provided that a second signal is delivered to tumor cells by anti-tumor Abs (Fc–FcγR) that mediate Ab-dependent cellular cytotoxicity (ADCC) [14,15].

These data suggest that the C system plays an important role in immunotherapy of cancer and acts as an additional weapon in support of the standard therapy provided by surgery, chemotherapy and radiation against tumor cells particularly in the control of the minimal residual disease. Optimal conditions are required for C to be effective, which include the level of expression of tumor antigens present on the surface of tumor cells, the class of Abs and the reduced expression of C inhibitors.

2. Tumor antigens and therapeutic antibodies

2.1. Tumor antigens

Identification of tumor antigens (TA) has been an essential step in the progress towards the development of successful cancer immunotherapy. The list of molecules to be considered potential good TAs (see Table 1) has grown over the last few decades and their properties has been investigated by several

groups [16–18]. It is now clear that the expression pattern as well as the temporal and tissue specificity of TA play a major role in determining its ultimate utility in immunotherapy. Ideally, TA should be expressed exclusively on the majority of cancer cells, and in any case the level of expression on tumor cells should be different from that on normal cells from which the tumor has originated. It is also important that a specific TA is expressed on metastatic cells because the primary tumor is most often removed surgically, and immunotherapy is currently used to prevent metastatic growth and recurrence of the tumor. To this end, it is highly desirable to select TAs that differ for mutations or expression pattern from the normal self protein. Needless to say that the Abs, to be effective, should be directed against extracellular TA, since intracellular antigens, though specific for tumor cells, can not serve as useful targets for immunotherapy.

Table 1
Major classification of tumor antigen

Viral-associated proteins	Derived from: EBV, HPV, HBV, HCV, HTLV-1, and others
Altered self TA	Expressed on normal tissue, and increased on tumors: HER-2/neu, tyrosinase, MART, and others
Tumor-specific antigens	Mutated version of self molecules: ras, p53, and others. Altered self epitopes: gangliosides and mucins
Abnormal levels of antigen expressed only in ontogeny and in restricted mature tissue such as testis	MAGE, PAGE, and so on. Oncofetal antigens (CEA and FP)

The rapid technological progress made over the last several years has provided effective means to identify a large number of potential TAs, and to test their ability to act as true tumor rejection antigen.

2.2. Anti-tumor antibodies

The TAs that are currently used as targets of Abs in cancer immunotherapy of hematological malignancies include CD20 [19] and CD22 for B-cell non-Hodgkin's lymphoma [20], CD33 for acute myeloid leukemia [21], and CD52 for chronic lymphocytic leukemia [22]. Other TAs expressed on solid tumors represent good targets for Abs. Examples of these TAs include human epidermal growth factor receptor 2 (HER2, Her-2/neu or c-erbB-2) for breast cancer [23], epidermal growth factor receptor (EGFR) for colorectal or lung cancer [24], carcinoembryonic antigen (CEA) for gastrointestinal cancer [25,26], epithelial cell adhesion molecules (EpCAM or 17-1A) for colorectal cancer [27], CA72-4 (TAG-72) for gastrointestinal cancer [28], high-molecular weight melanoma-associated antigen (HMW-MAA) for malignant melanoma [29], and others [30] listed in Table 2.

The identification of new tumor-specific antigens and tumor-associated antigens and the control of tumor in preclinical models have raised a renewed interest in the use of TAs as target for both passive (Abs) and active (vaccine) immunotherapy.

Early attempts to use polyclonal Abs for immunotherapy have been limited due to the difficulty in achieving high titre and specificity of these Abs *in vivo*. The introduction of murine monoclonal Ab (mAb) (with the suffix “-momab” in the international

non-proprietary names) in immunotherapy represents a further advance that promised to overcome these difficulties [31], but did not solve the problem of immunogenicity encountered with the polyclonal Abs. A partial solution to this problem came with the production of mouse-human chimeric Abs (“-ximab”) by genetically fusing the mouse variable regions to the human constant domain. The anti-CD20 mAb Rituximab (RituxanTM) is an example of a chimeric Ab widely used in the treatment of non-Hodgkin's lymphoma [32]. Although chimeric Abs exhibit a reduced immunogenicity, they can still elicit a significant immune response. This issue was addressed with the production of humanized Abs (“-zumab”), in which the complementary determining regions responsible for the antigen binding within the variable regions are transferred to human frameworks [33]. Trastuzumab (HerceptinTM) and Alemtuzumab (CampathTM) are two examples of humanized Abs commonly used in the treatment of patients with metastatic breast cancer overexpressing HER2 [23] and with chronic lymphocytic leukaemia [22], respectively. Strategies have also been developed to generate fully human mAbs (“-umab”) to human TAs using transgenic mice or phage display library [34]. Recently, 46 fully human Abs have been isolated and characterized for their ability to react with CEA but not with other CEA gene family members [35].

2.3. Recombinant Abs and activation of the C system

The Abs are often used as a means to target radionuclides or chemical agents onto tumor cells, but they may also promote

Table 2
Examples of therapeutic mAbs

Target	Name	Mode of action	Company
Anti-idiotypic mAb, GD3 ganglioside mimetic	BEC2 (Mitumomab)	Vaccine mimicking GD3 glycopeptide	ImClone System, MERK KGaA
Anti-idiotypic mAb, CEA mimic	CeaVac	Stimulates immune response to CEA	Titan Pharmaceuticals
CA125	Ovarex	Induce an immune response against CA125	Altarex
CD20	Rituxan (Rituximab)	Lysis of B lymphocytes through activation of CDC and ADCC	IDEC Pharmaceuticals, Genentech
CD20	Zevalin (Ibritumomab tituxetan)	Radio-immunotherapy	IDEC Pharmaceuticals
CD20	Tositumomab (Bexxar)	Radio-immunotherapy and immune response	Corixa, Titan Pharmaceuticals, GlaxoSmith-Kline
CD22	Epratuzumab (LymphoCide)	Internalization and phosphorylation of the Ag	Immunomedics
CD33	Mylotarg (Gemtuzumab ozogamicin)	Chemo-immunotherapy	Wyeth Laboratories/AHP
CD33	Zamyl	Immune response	Protein Design Laboratories
CD52	Campath (Alemtuzumab)	Lysis of malignant lymphocytes through activation of CDC and ADCC	Millennium, BTG; ILEX Oncology; Hoffman-LaRoche
EpCam	Panorex (Edrecolomab)	Murine mAb targeting the epithelial cell adhesion molecule	GlaxoSmith-Kline, Centocor
Erb1/EGFR	Erbtix (Certuximab, IMC225)	Attach to and block EGFR	ImClone Systems, Merck KgaA
ErbB1/EGFR	EMD72000 (Matuzumab)	Attach to and block EGFR	Merck KgaA
ErbB1/EGFR	ABX-EGF (Panitumumab)	Attach to and block EGFR	Abenix
ErbB2/Her2/neu	Herceptin (Trastuzumab)	Blocks EGF by attaching to Her2	Genentech
ErbB2/Her2/neu X CD64 (FcγRI)	MDX-210	Bispecific Ab that induce immune response	Medarex, Immuno Designed Molecules
HMFG	TriAb	Immune response	Titan Pharmaceuticals
IL-2 receptor, CD25	Daclizumab (Zenapax)	Blocks the activation of IL-2 receptors	Protein Design Labs, Hoffman-LaRoche
PEM	Theragyn (Pemtumomab)	ADCC	Antisoma
VEGF	Avastin (Bevacizumab)	Angiogenesis inhibitor	Genentech BioOncology

the anti-tumour activity of biologically effector systems such as NK cells and the C system. Binding of multiple globular heads of C1q to closely spaced IgG on the cell surface is an absolute requirement for an effective activation of the classical pathways of the C system. This effect depends on the deposition of a high number of Ab molecules on tumor cells, which, in turn, is directly related to the expression of the antigenic epitopes on the cell surface.

We have recently investigated the ability of Abs directed against the folate receptor (FR) associated with epithelial ovarian carcinoma (EOC) to activate C [36]. FR is highly expressed on EOC cells and its level has been estimated to be around 1×10^6 molecules/cells on several cell lines [37]. We found that two chimeric mAbs directed against FR (cMOV18 or cMOV19) failed to induce C-dependent cytotoxicity (CDC) of tumor cells. These results were rather unexpected since B cells from patients with chronic lymphocytic and prolymphocytic leukemias express only 40,000–70,000 CD20 molecules/cells and still are highly susceptible to CDC mediated by Rituximab [38]. This clearly indicates that the density of the antigenic sites favours, but is insufficient to justify the mAb-mediated CDC. Additional factors may be required, besides the high number of antigenic targets, as suggested by the finding that CDC of B cells correlates with the segregation of CD20 into the lipid raft [39].

Several strategies have been devised to turn a non-C into a C-fixing Ab to be employed in immunotherapy including the selection of the Ig subclasses (IgG1 and IgG3), which are most efficient in activating C [40] and the production of IgG1 containing recombinant variants of Fc that exhibit increased capacity to induce CDC or ADCC [41,42]. The use of more than one Ab that recognize distinct epitopes of the same Ag is another way to favour deposition of closely spaced IgG on the surface of tumor cells. This was shown by Spiridon et al. [43], who examined the anti-tumor activity of several murine mAbs to Her-2 overexpressed on tumor cells and found that these mAbs were more effective in causing CDC as a mixture rather than as individual mAbs. Further evidence that interaction of Abs with multiple epitopes on target cells improves their biological activities was more recently provided by Meng and colleagues, who showed that a chimeric tetravalent mAb against human CD22 had an enhanced anti-tumor activity and an increased ability to bind C1q and to induce ADCC as compared to divalent mAb [44]. We reached a similar conclusion testing the C-fixing activity of the two chimeric Abs directed against different epitopes of FR, cMOV18 and cMOV19, and found that these Abs were able to induce C-dependent-CDC of EOC cell lines only if used as a mixture, but failed to do so when analysed individually [36].

3. Effect of the inhibitors of the complement system

Antibody-mediated C-dependent killing of tumor cells is not a very efficient effector mechanism, due to the overexpression of C regulatory proteins (CRPs) on tumor cells, which are in this way protected from C attack [45–47]. CRPs have been shown to be expressed on the surface of numerous cancer cells and cell lines [47]. They control C activation acting at different steps of the C cascade, and more specifically prevent deposition of

C3b, generation of C5a and MAC-mediated lysis [48–51]. Thus, complement receptor type 1 (CD35), membrane cofactor protein (CD46) and decay-accelerating factor (CD55) inhibit the generation and activity of C3 and C5 convertases [52]. CD59 acts at the level of C9, restricting the assembly of the membrane attack complex (MAC) [53]. CD46, CD55 and CD59 are thought to be the most important membrane C regulatory proteins (mCRPs) expressed both on normal and tumor cells, while the effect of CD35 seem to be mainly restricted to blood cells and glomerular podocytes.

Tumor cells can also evade C attack by binding soluble C inhibitors from serum such as factor H (fH) in much the same way as some microorganisms [54]. Sialic acid-rich proteins that bind fH are up-regulated by many tumors and overexpression of sialic acid has been associated with clinical severity [55]. It is also interesting to note that fH or a related protein is a marker for bladder cancer, suggesting a link between C resistance and escape from immune surveillance [56].

Overexpression of mCRPs on tumour cells has been shown to interfere with the C-mediated killing effect induced by therapeutic mAb [46,57]. The CDC of breast carcinoma cell lines induced by anti-HER2/neu mAb (Trastuzumab), increases from 10% to 80% following neutralization of mCRPs on tumor cells [58]. Similarly, the expression of CD55 and CD59 on colorectal carcinoma and lymphoma restricts C-mediated injury and determines the response rate *in vitro* for mAbs against Ep-CAM and CD20 [50,59].

Although *in vitro* studies indicate a role for mCRPs in determining the outcome of mAb immunotherapy, only a few studies have investigated the importance of mCRPs in appropriate experimental animal cancer models. Because mCRPs act in a species selective fashion [60,61], heterologous animal models involving C and mCRPs of different species might not be clinically relevant. For example, an anti-tumor mAb may be effective in a rodent model of human cancer simply because the human mCRPs expressed on the tumor cell do not protect from rodent C. Such protocols that mix human tumors and rodent C [e.g. human tumors in nude or severe combined immunodeficient (SCID) mice] explain why the same Ab that is active against tumors in mice is ineffective in a clinical (homologous) setting. As a consequence, a syngeneic model is more clinically relevant to investigate the efficacy of mAb and the effect of mCRPs expression [61–62].

Several clinical studies have provided evidence for mCRPs expressed on tumor cells providing protection from C attack. CD55 and CD46 on human tumor cells are believed to perform the same type of C inhibitory function as Crry on rodent cells. CD55 has been identified as a tumor-associated antigen and high expression levels of CD55 on colorectal cancer tissue is correlated with a significant decrease in survival [63]. Also, low CD46 has been found to be inversely related with high levels of C3 deposited on renal and cervical cancer tissue [64]. Furthermore, peripheral blood leukocytes isolated from patients with chronic lymphocytic leukemia (CLL) who had a poor response to anti-CD20 (Rituximab) treatment, were more sensitive to C lysis following *in vitro* neutralization of CD55 and CD59 than leukocytes isolated from patients who did respond to

Rituximab therapy [38]. In addition, significantly higher levels of CD59 have been found on CLL cells that were not cleared from the circulation following Rituximab therapy [65]. These data suggest that mCRPs have an important role in reducing the clinical effect of Rituximab treatment. This, however, remains a controversial issue. Thus, the expression level of CD55 and CD59 on tumor cells has been reported not to be correlated with the percentages of cell lysis and in another study the expression level of mCRPs was not predictive of the clinical outcome of Rituximab treatment [38]. Nevertheless, there is a general consensus on the important role played by C in the anti-tumour activity of Rituximab, and on the counteractive effect of mCRPs. The relative contribution of CDC and ADCC to the therapeutic effect of Rituximab and other therapeutic Abs remains to be established.

In summary, data from experimental models of cancer and clinical studies suggest that modulating C susceptibility of a tumor cell has the potential to increase therapeutic efficacy of a mAb by triggering C-dependent effector mechanisms, whether or not the primary mechanism of action is C-dependent.

4. Inhibition of mCRPs to enhance C-dependent killing of tumor cells

Based on *in vitro* and *in vivo* data, Ab-based immunotherapy of cancer appears to be greatly improved following neutralization of mCRPs. This goal can be achieved increasing the C-activating ability of mAbs, and also reducing the expression and/or the function of mCRPs.

The protective role of CD55 and CD59 from CDC is supported by the observation that cancer cells become more susceptible to C-dependent lysis after enzymatic removal of the GPI anchored CD55 and CD59 molecules from the cell surface by means of phosphatidylinositol-specific phospholipase C [66]. Down-regulation of expression of mCRPs resulting in enhancement of mAb-mediated C activation has also been obtained *in vitro* using various cytokines [67,68]. More recently, Zell and collaborators have succeeded in reducing the cell surface expression of these molecules and in enhancing the CDC of breast carcinoma and prostatic carcinoma cell lines using siRNA [69].

Murine mAbs are commonly used to neutralize mCRPs expressed on tumour cells. Golay and colleagues [59] analysed several B lymphoma cell lines and a few samples of fresh follicular non-Hodgkin's lymphoma cells for their sensitivity to CDC induced by Rituximab. They observed that the C resistance of these cells was dependent on the expression level of CD55 and CD59, since neutralization of the two mCRPs by BRIC216 and YTH53.1 mAbs respectively rendered the cells more susceptible to CDC. Similar results were obtained studying *in vitro* the immunotherapy of several tumors [45,58].

To overcome problems of immunogenicity related to the *in vivo* administration of mouse Ab against mCRPs, we have isolated neutralizing single chain fragment variables (scFvs) to CD55 and CD59 from a human phage display library [70] to be used in combination with C-fixing Rituximab and other C-

activating mAb [71]. The human phage Ab libraries offer the advantage over conventional mAb to provide a large Ab repertoire not shaped by the constraints of the immune system with a dramatic increase in the chances of isolating Ab to self-antigens [72]. Furthermore, these molecules have a better chance to penetrate the tumor mass and are characterized by a faster clearance than conventional Abs due to their smaller size [73]. Addition of the Fc domain to scFv in designing therapeutic Abs helps to prolong their antigen binding activity and serum half-life [74]. In our case, the anti-CD55 and anti-CD59 scFvs were fused to the Hinge-CH2-CH3 sequence of human IgG1, forming two full human miniantibodies with the specificity for CD55 and CD59 and the functional activity of the Ig. The results of *in vitro* studies have clearly shown that the killing effects of Rituximab on lymphoma cell lines doubled in the presence of the miniantibodies opening the way to their use in combination with other C-fixing anti-tumor Abs.

5. Targeting of blocking antibodies Abs on tumors cells

The wide distribution of mCRPs on circulating and tissue cells is a real drawback for the therapeutic use of mAbs to these molecules. Under these conditions, binding of mAbs to tumour cells will be insufficient to be clinically effective and may also cause undesired side effects. An important issue that needs to be addressed for the therapeutic use of Abs against mCRPs in cancer patients is to devise a strategy to target the blocking Abs to tumour cells.

The three-step biotin–avidin system, employed by Paganelli and co-workers in the clinic to target radionuclides to breast cancer [75–77], is a possible approach to address this issue. One drawback of this system is that the procedure of biotin-labelling may impair the functional activity of anti-tumour mAbs, as shown for biotin-labelled anti-GD3-ganglioside mAb by Jokiranta and Meri [78]. In addition, repeated injections of avidin may cause an Ab response in the recipient and avidin purified from different sources or produced as a recombinant protein are now being screened in several laboratories to find the least immunogenic for human use [79–81].

Bispecific Abs that recognize both TA and a mCRP represent another tool to address this issue providing the anti-tumor arm that directs the bispecific mAb to the tumor cells and the other arm that neutralizes the most important mCRPs at the tumor cells surface. Preferential homing of the bispecific mAb can be obtained by using a high affinity anti-tumor variable regions and medium/low affinity blocking mCRP variable regions, thus minimizing binding of the anti-mCRP arm to normal cells. Gelderman and colleagues developed a bispecific mAbs directed against human CD55 and EpCAM, a colorectal cancer TA, and another bispecific mAbs directed against human CD55 and G250, a renal cell carcinoma Ag. Both these molecules induce an increased deposition of C3b, and enhance CDC and CDCC as compared to the original Abs [50,82]. The bispecific Ab recognizing rat colorectal cells and Crry, the most important rat mCRP, was shown in *in vivo* experiment to prevent growth of tumor cells in a rat model of colorectal metastasis [83].

In conclusion, C has raised a novel interest in the control of tumour growth following the introduction of chimeric or humanized Ab in cancer immunotherapy. Work is in progress in several laboratory to explore new ways to improve the functional efficiency of this system selecting the most appropriate subclass of Ab, increasing the density of Ig deposition on the target cells and removing the inhibitory block provided by mCRPs over-expressed on the surface of tumour cells.

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