suppressive cytokine milieu prevalent within the tumor environment as a major contributory factor (94). For instance, TGF-β can induce iTreg cells and it is well established that several tumor lines utilized in murine tumor studies secrete TGF-β (19, 95–97). Other tumor-derived soluble factors such as GM-CSF and VEGF may recruit or expand MDSCs which then secrete cytokines that could potentially induce Treg cells (98, 99). Additionally, tumor-associated macrophages or DCs may be instrumental in inducing Treg cells or recruiting discrete subsets of Treg cells with distinct phenotypes (83, 100).

Similar to the phenomenon of infectious tolerance (101), Treg cells may also directly enlist naïve T cells into the regulatory pool. In this regard, Treg cell production of IL-10 and TGF-β (102, 103) may also modulate some naïve CD4+ T cells, converting them to cells with inhibitory function. Another possibility is an indirect effect via modulation of DCs. Treg cells via CTLA-4 may keep DCs in an immature state by engaging CD80 and CD86 molecules on these antigen presenting cells (102). Such immature DCs may induce Foxp3 or Foxp+-like phenotype, in line with their demonstrated ability to efficiently induce iTreg cells *in vivo* (104). The modification of tumor-associated APCs is however not restricted to Treg effect alone. Other inhibitory agents produced by tumors such as IDO (105) may re-shape DCs to become tolerogenic and in turn promote induction of Foxp3+ Treg cells (106). Taken together, adaptive Treg cell generation may be promoted by tumor-related expression of key cytokines and soluble factors that have the potential to induce Foxp3+ cells from existing pool of tumor-infiltrating conventional CD4+ T cells or recruit discrete regulatory CD4+ T cells from distal sites.

In a nutshell, it is evident that the generation of adaptive Treg cells is likely a complex phenomenon and multiple pathways may be involved (**Figure 1**). Adding to this complexity is the tumor itself: its properties such as cytokine and chemokine milieu, angiogenic capabilities, etc. may determine or shape the generation of these peripherally induced adaptive Treg cells.

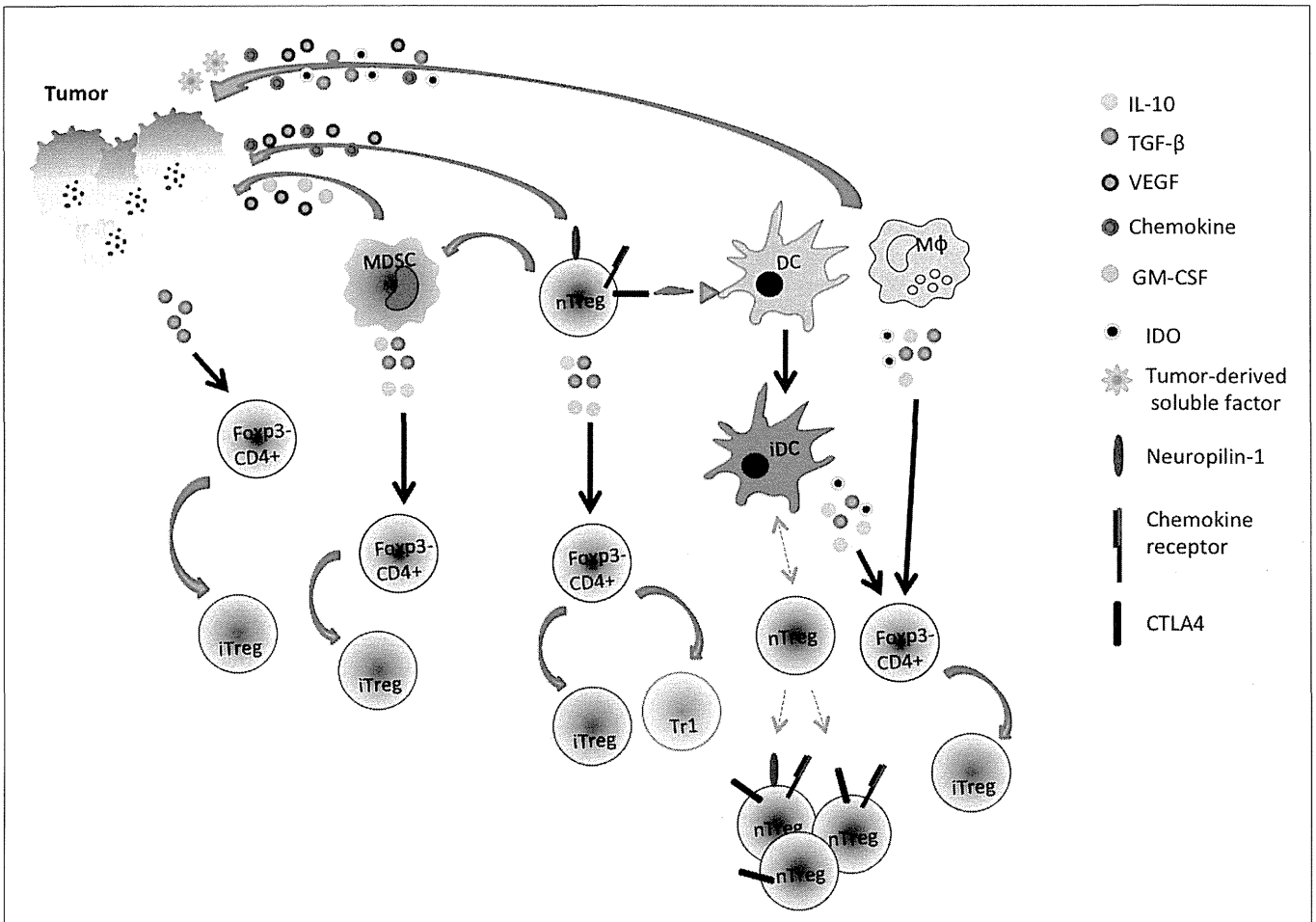

FIGURE 1 | Generation and recruitment of adaptive/induced Tregs in the tumor microenvironment. Tumor cells may secrete an array of cytokines and soluble factors that facilitate the induction of Foxp3 in Foxp3− cells or the recruitment of multiple cell types including natural Treg cells, myeloid-derived suppressor cells (MDSC), dendritic cells (DC), and macrophages. These cells in turn may secrete inhibitory and immune-suppressive factors such as TGF-β, IL-10, and indoleamine 2,3-dioxygenase (IDO) that could potentially convert some Foxp3− CD4+ cells into Foxp3+ cells. Additionally, tumor-derived factors or Treg interaction with DCs may promote generation of tolerogenic or immature DC (iDC) that recruit distinct populations of natural Tregs. nTreg is CD4+Foxp3+ cells while iTreg is CD4+Foxp3 variable.

Foxp3 STABILITY AS AN INDICATOR OF NATURAL VERSUS INDUCED TREG CELLS IN TUMORS?

Addressing the issue of Foxp3 stability within tumor-associated Treg cells, a recent report evaluated tumor-resident Treg cells. Using reporter mice that bear melanoma, authors were able to differentiate between "ex" and "current" Foxp3+ Treg cells (64). In this study, it was found that majority of the tumor-Treg cells retain Foxp3 expression and only a minor population lost its expression providing evidence that Foxp3 expression even in an inflammatory environment as the tumor remained stable. Since iTregs only show a partial DNA hypomethylation pattern unlike nTregs (68, 69), indicating a transient opening up of the Foxp3 locus, they do not to stably express Foxp3 and may even likely lose its expression in the absence of signals that elicited Foxp3 induction. Extrapolating from this, it is tempting to conclude that majority of tumor-Treg cells are likely nTregs based on their Foxp3 stability and not iTregs as Foxp3 unstable Treg cells would otherwise constitute a sizable fraction of tumor-Tregs if they were induced from conventional CD4+ T cells. Evaluations such as genetic profiling of Foxp3 locus thus may be useful in delineating what constituency Treg cells in different tumors belong to, i.e., the "i" or the "n" family.

FUNCTION OF NATURAL VERSUS INDUCED TREG CELLS

Several questions linger as we attempt to understand the role of iTreg cells versus nTreg cells in tumor immunobiology: is the role of iTregs largely redundant when nTreg cells are present? If not, do they possess similar specificity and or play similar roles as their natural counterparts? Two studies, one in a colitis model, the other in Foxp3-deficient mice, which succumb to lymphoproliferative disease, demonstrated that full protection from disease was only achieved when both nTreg cells and iTreg cells were present, suggesting that the function of each Treg cell group is complementary (49, 107). As Lafaille and colleagues surmised, a division of labor between nTreg cells and iTreg cells seems a plausible arrangement as far as their functional roles in regulating immune responses (13). One might speculate that given their sheer dominance and omnipresence, nTreg cells share the greater bulk of curtailing T cell responses while adaptive Treg cell contribution is solicited as needed and differs on a case-by-case à la cancer-by-cancer model. Relating to this principle, a study described the accumulation of nTreg cells and iTreg cells in the tumor microenvironment, with the latter possessing TCR specificity for a defined antigen expressed by the tumor. Suppression by cognate-antigen-specific iTreg cells was restricted to CD4+ T cells and occurred only within the local tumor environment while suppression of CD8+ T-cell response was independent of these tumor-antigen-specific iTreg cells (108). From this, one might deduce that iTreg cells evolve peripherally as in the tumor only to control some arms of the immune response while the nTreg cells control others.

In many colorectal cancer studies, the observation that increased Foxp3+ Treg cells correlate with good prognosis is particularly intriguing (109). An argument has been made that the Treg cells in this context may largely be involved in controlling potential inflammation that could ensue in response against the commensal bacteria present in the lower intestine if Tregs are absent (13). Given that GALT environment is permissive for induction of iTreg cells, it is tempting to speculate that the FOXP3+ Treg cells in colorectal cancer are mostly iTreg cells. To test this possibility, phenotypic characterization, TCR repertoire analysis, and FOXP3 methylation status of Treg cells in colorectal tumor tissues in parallel with solid tumors from sites not heavily associated with intestinal commensal bacteria could be a starting point.

Summarily, elucidating what environmental and molecular cues facilitate the generation of iTreg cells and the type of role they play particularly in various cancers would be eye-opening and may pave way for manipulating the immune system to prevent their generation in such context. At any rate, more studies are warranted to tease out who does what and to what degree is this division of labor shared.

TREG THERAPY: TARGETING NATURAL AND ADAPTIVE/INDUCED TREGS

To prime and/or boost anti-tumor immune response, selective removal or reduction of Treg cells have been carried out in a number of murine tumor studies (12). This depletion is generally achieved via the use of anti-CD25 mAb (PC61), anti-FR4 mAb, and diphtheria toxin, the latter to DEREG mice (which express diphtheria toxin receptor under the control of Foxp3 promoter (110–114). In humans, daclizumab (anti-CD25) and denileukin diftitox (ONTAK, a fusion protein of diphtheria toxin and recombinant human IL-2) treatment has also shown some efficacy in some cancers, consequent to their Treg cell depletion effect although with varying degrees of success (10, 115). Cyclophosphamide, a chemotherapy agent that is a part of treatment regimen in some cancers is also known to target Treg cells by reducing their frequencies or function (116–119). In combination with tumor vaccination, all three agents were tested in melanoma patients in one study. Interestingly, only modest reduction in Treg cells (as determined by methylation status of FOXP3 intron 1 within Treg cells) was noted in the peripheral blood of patients in the treatment groups (120). In a recent clinical trial utilizing multiple tumor-associated peptides as a therapeutic vaccine for renal cell cancer, T-cell responses of treated patients were associated with better disease control and correlated with lower numbers of FOXP3+ Treg cells prior to vaccination. This revelation prompted the incorporation of cyclophosphamide to the vaccine regimen in subsequent study which demonstrated that reduced Treg cell numbers achieved by this approach further improved patients' immune responses to the tumor antigens and importantly, their overall survival (121). The caveat to all these studies is that the effect of these Treg cell depletion/reduction protocols have not been evaluated on Treg cell subsets and essentially no information is available on whether iTreg cells are more susceptible to these regimen than nTreg cells or vice versa. Thus, critical evaluation of the residual Treg cell fractions not targeted by these agents is warranted as they may represent an induced population with phenotypic changes that make them evade depletion regimen.

On the other hand, there is some evidence that nTreg cells are more resistant to oxidative stress or apoptosis than conventional T cells (122). Based on this, nTreg cells, assuming they account for the majority of tumor-infiltrating Treg cells, may be the subset that is more resilient to therapeutic modalities aimed at eliminating tumor-Tregs. In this regard, multi-pronged approach combining

multiple agents targeting "i" and "n" Tregs may be necessary to achieve efficient elimination. While their differential expression is yet to be assigned to either iTreg or nTregs cells, CCR4, PD-1, and CTLA-4, which have been shown to be highly expressed on tumor-Treg cells (123) offer potential targets for treatment of cancers enriched in Treg cells with such phenotype. In alignment with this line of thinking, the combination of anti-CTLA-4 and anti-PD-1 antibody treatment in a mouse B16 melanoma study led to substantial reduction in Treg cells as well as myeloid cells with a concomitant increase in tumor-infiltrating effector T cells (124). Agonist antibody against Glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR), also expressed on Treg cells (125), is another treatment route that holds promise. In a murine model of melanoma, its administration promoted potent anti-tumor immune response (126). Similarly, in combination with anti-CTLA-4 antibody, anti-GITR administration evoked regression of established fibrosarcoma and colon carcinoma in other studies (127, 128). In either case, the positive outcomes were ascribed to anti-GITR antibody-mediated attenuation of Treg function or decreased intra-tumoral Treg cell accumulation, in addition to augmented CD+ T-cell effector response (126–128). For advanced melanoma, it is worth mentioning that administration of humanized anti-CTLA-4, ipilimumab improved survival of patients with metastatic melanoma in a clinical trial (129). In our recent investigations, we found that tumor-infiltrating T cells contained a higher frequency of effector Tregs with activated phenotypes compared with peripheral blood. Correspondingly, Tregs with a naive phenotype were barely detected in tumors while peripheral blood contained both naïve and effector Tregs. These tumor-infiltrating effector Tregs dominantly expressed CCR4, proposing CCR4 as a possible target for Treg control (manuscript in preparation).

The finding that human adaptive CD4+FOXP3+ Treg cells which express CD39, and CD73, and produce adenosine was described by Whiteside and co-workers (130). They demonstrated *in vitro*, the generation of iTreg cells with similar phenotype (except for FOXP3) in co-cultures simulating some of the features unique to the human cancer in which equivalent Treg cells were observed (131). They found that both adenosine and PGE2 produced by these iTreg cells co-operate in mounting strong suppressive function against autologous T effector cells. Thus, Whiteside proposed that targeting adaptive Treg cells by interfering with adenosinergic pathways and PGE2 production could be a viable therapeutic platform to disarm iTreg cells in human cancers (132).

Lastly, methods aimed at disrupting iTreg cell induction such as interfering with TFG-β signaling in relevant tumors could be complementary approaches to vaccination. Using siRNA-mediated downregulation of TGF-β production by B16 melanoma cells, this idea was explored by Mills and colleagues and they reported that tumor growth was hampered (133). This coincided with reduced tumor-Treg cell numbers although it was not clear as to whether this reduction affected iTreg cells as we might postulate based on experimental design.

Worth mentioning is the issue of Treg function at the interface of autoimmunity and cancer. The pivotal and positive role of Treg cells is exemplified in mice as well as IPEX patients in which impaired Foxp3+ Treg cell development culminates in wholesale breakdown of immune tolerance (1, 134, 135). When placed in the context of tumors however, Treg suppressive function appears for the most part, to result in unfavorable prognosis. In fact, studies that portray Treg presence within the tumor in a bad light, i.e., inhibiting anti-tumor response outweigh those demonstrating they may have favorable contributions in cancer (10–12). In a recent report, melanoma patients who had better response following treatment with high dose IL-2 plus vaccine had higher Treg frequencies portraying a correlation between Tregs and better response against tumor (136). Thus, therapeutic strategies that are focused on Treg reduction in order to promote tumor clearance need to take this apparent duality in Treg function into account. More importantly is the effect such depletion may have on elevating a patient's risk for developing autoimmune conditions especially if systemic Treg depleting routes are utilized. In this regard, localized Treg reduction by intratumoral administration of Treg depleting agents which has shown efficacy at reducing tumor burden in mice (127) may offer a more favorable treatment platform without the inherent risk of the global Treg elimination assuming the tumor is accessible. Furthermore, since Treg cells in tumor environment appear to be of the effector Treg phenotype and may exhibit augmented suppressive activity when compared to those in circulation (64, 137–139), localized Treg modulation approach could be a viable option to target only a subset of highly suppressive, effector Treg cells based on specific molecules which they uniquely upregulate in response to tumor antigens. By so doing, the bulk of nTreg cells are left intact while only those "in action" are removed. This should be a feasible approach as we have recently tested the effect of anti-CCR4 antibody on subsets of human Treg cells in melanoma patients and found it to efficiently eliminate a population of CCR4-expressing effector Tregs while sparing naïve Treg populations (manuscript in preparation). Until we have some evidence of the nature and extent of the contributions of nTregs and iTregs in various tumors, treading carefully on indiscriminate Treg depletion for cancer therapy however seems a reasonable proposition.

PERSPECTIVES

Different subsets of Treg cells may be committed to regulate specific arms of immune responses (140). Understanding the functional capabilities of both iTreg cells and nTreg cells will no doubt help in guiding future treatment platforms. A number of possibilities exist: their elimination from the tumor microenvironment, blocking their ability to produce a number of immune-suppressive/immune-altering molecules such as adenosine, PGE2, perforin, and granzyme B, targeting anti-apoptotic pathways, disrupting their ability to proliferate and or persist in tumors, etc. The list is not conclusive as our understanding continues to expand about the nature of Treg cells that prevail in different cancer types. Thus, additional investigations are necessary to first determine whether the variabilities seen among different cancer studies with respect to phenotype associated with the tumor-Treg cells relate to their origin, i.e., are they natural or peripherally iTreg cells. From such information, we may be able to optimize Treg cell-targeted approaches to reduce or eliminate not just a major subset

that is prevalent within the tumor, but a minor subset that could contribute to hindering optimal therapeutic success in the settings where their presence is related to poor survival. To this end, designing antibodies against some of the molecules that appear to preferentially mark Treg cells infiltrating tumors may be a good investigational direction worth pursuing in our quest to treat cancers. It will be interesting to see whether such studies reveal information about the effect of treatment on subsets of Treg cells that are affected, and those that are resistant to modulation. At any rate, treatment modalities focused on elimination of Tregs or disruption of their function to bolster anti-tumor immunity should take into account the differences between cancer types, the subset of the Tregs that predominate within the tumor, and their recruitment dynamics.

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Antibody-based therapy in colorectal cancer

Treatment in patients with nonresectable and resectable colorectal cancer at the advanced stage is challenging, therefore intensive strategies such as chemotherapy, signaling inhibitors and monoclonal antibodies (mAbs) to control the disease are required. mAbs are particularly promising tools owing to their target specificities and strong antitumor activities through multiple mechanisms, as shown by rituximab in B-cell non-Hodgkin's lymphoma and trastuzumab in breast cancer. Three mAbs (cetuximab, bevacizumab and panitumumab) have been approved for the treatment of colorectal cancer in the USA and many other mAbs are being tested in clinical trials. The potential of antibody therapy is associated with several mechanisms including interference of vital signaling pathways targeted by the antibody and immune cytotoxicity selectively directed against tumor cells by tumor-bound antibody through the Fc portion of the antibody, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Moreover, recent experimental findings have shown that immune complexes formed by therapeutic mAbs with tumor-released antigens could augment the induction of tumor-specific cytotoxic $CD8^+$ T cells through activation of APCs. In addition, antibodies targeting immune checkpoints on hematopoietic cells have recently opened a new avenue for the treatment of cancer. In this review, we focus on mAb treatment in colorectal cancer and its immunological aspects.

KEYWORDS: antibody antibody-dependent cellular cytotoxicity antibody-facilitated T-cell immunity colorectal cancer complement-dependent cellular cytotoxicity Fcγ receptor immune checkpoint

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Colorectal cancer (CRC) can now be diagnosed at earlier stages owing to the advance of screening methods, such as the fecal occult blood test, colonoscopy and colonography, and some patients with resectable CRC are operated on less invasively with the development of new surgical techniques such as endoscopic, laparoscopic and robotic procedures. However, for patients with advanced-stage CRC, it is still difficult to control their disease and intensive therapies including irinotecan or oxaliplatin-based chemotherapies, signal inhibitors, and antibodies are required [1]. In addition to the conventional drugs, immunotherapy has recently emerged in the clinic and could be another tool for CRC therapy. While preclinical studies have provided promising evidence, most immunotherapies aimed at harnessing antitumor immune responses selectively through immune mechanisms have not yet achieved the ultimate acceptance. Indeed, only two drugs have been approved by the US FDA – although very recently – sipuleucel-T for prostate cancer and ipilimumab for malignant melanoma. At the moment, there is no approved immunotherapeutic antibody available for CRC in the clinic. However, accumulating data indicate that conventional therapeutic antibodies may provoke antitumor immunity through the Fc fragment of the antibody. Therefore, it may be necessary to reconsider the immune system as a potent strategy for the treatment of CRC.

Antibody therapy targeting tumor-associated antigens in cancer has been widely accepted in the clinic. Rituximab, the classic example, was approved for the treatment of B-cell non-Hodgkin's lymphoma in 1997 in the USA and has provided great success for lymphoma treatment, thus opening a new era for antigen-targeted therapy with monoclonal antibodies (mAbs). Currently, three mAbs, cetuximab, bevacizumab, and panitumumab, are approved for the treatment of CRC by the FDA, and approximately 40 mAbs have been or are being tested in clinical trials in CRC patients (Table 1) [2,3]. Two of the approved mAbs in CRC recognize the EGF receptor (EGFR); cetuximab is a chimeric human IgG1 mAb, whereas panitumumab is a fully human IgG2 mAb. Efficacy of cetuximab was observed in patients with irinotecan-refractory metastatic CRC when combined with irinotecan [4] and in patients with best supportive care [5]. Compared with bolus fluorouracil/leucovorin/irinotecan (FOLFIRI) in CRC patients, the first-line therapy with FOLFIRI plus cetuximab

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Table 1. Antibodies approved by the US FDA or under clinical trials for colorectal cancer.

Name of mAb	Target	Completed phase
US FDA approved		
Bevacizumab	VEGF	–
Cetuximab	EGFR	–
Panitumumab	EGFR	–
In clinical trials		
Adecatumumab	EpCAM	II
Cixutumumab	IGF-1R	II
Conatumumab	DR5	II
Dalotuzumab	IGF-1R	I
Drozitumab	DR5	I
Edrecolomab	EpCAM	III
Ensituximab	MUC5AC	I and II
Etaracizumab	αVβ3 integrin	I and II
Figitumumab	IGF-1R	II
Ganitumab	DR5	II
Necitumumab	EGFR	II
Nimotuzumab	EGFR	II
Ramucirumab	VEGFR-2	I
Tigatuzimab	DR5	I
Trastuzumab	HER2/neu	II
Tremelimumab	CTLA-4	II
Zalutumumab	EGFR	I and II
AMG386	Angiopoetin 1/2	II
CDX-1127	CD27	I
CEP37250/KHK2804	Glycolipids	I
CNTO328	IL-6	II
CT-011	PD-1	II
GS-6624	Lyayl oxidase-like2	II
hA33	A33	I
Hu3S193	Lewis-Y	I
IMC 18F1	VEGFR-1	II
ING-1	EpCAM	I
IVIG	NA	II
KRN330	A33	I
L19	Fibronectin	I and II
MDX-1105	PD-L1	I
MDX-1106	PD-1	I
MEHD7945A	EGFR/HER3	II
MGA271	B7-H3	I

Data were summarized with the search of 'colorectal' and 'antibody' as of August 2012 at ClinicalTrials.gov [202].
EGFR: EGF receptor; mAb: Monoclonal antibody; VEGFR: VEGF receptor.

Table 1. Antibodies approved by the US FDA or under clinical trials for colorectal cancer (cont.).

Name of mAb	Target	Completed phase
In clinical trials (cont.)		
OMP-21M18	Cancer stem cell	I
RO5083945	EGFR	I

Data were summarized with the search of 'colorectal' and 'antibody' as of August 2012 at ClinicalTrials.gov [202].
EGFR: EGF receptor; mAb: Monoclonal antibody; VEGFR: VEGF receptor.

improved progression-free survival (PFS) in the cetuximab-treated group and prolonged overall survival (OS) only in patients with *KRAS* wild-type tumors [6]. Clinical evidence for the efficacy of panitumumab in CRC is more limited than that for cetuximab due to the developmental delay of this drug. However, some clinical trials of panitumumab provided similar evidence to cetuximab, with its clinical efficacy confined to patients with wild-type *KRAS* [7]. One of the major mechanisms of anti-EGFR mAbs is inhibiting cell proliferative signals through EGFR, reflecting a correlation between the treatment efficacy and the *KRAS* mutation status. Interestingly, some clinical studies demonstrated the impact of Fcγ receptor (FcγR) polymorphisms on the clinical outcomes in CRC patients treated with cetuximab, regardless of the *KRAS* status [8,9], suggesting a possible involvement of immune mechanisms in cancer patients. In addition, these polymorphisms could be regarded as potential biomarkers in CRC patients treated with cetuximab or panitumumab. Additional studies with large cohorts are warranted.

Another approved mAb for treatment of CRC is bevacizumab, a humanized IgG1 mAb against VEGF-A [10]. Bevacizumab plus irinotecan/florouracil/leucovorin (IFL) as first-line treatment for patients with metastatic CRC provided longer median OS and improved response rate and PFS compared with an IFL plus placebo control group [11]. Subsequently, FOLFIRI plus bevacizumab improved OS better than modified IFL plus bevacizumab in patients with untreated metastatic CRC, although the comparison was in sequential cohorts [12]. Similarly, another clinical trial demonstrated that FOLFIRI plus bevacizumab was superior to IFL plus bevacizumab in median OS and PFS. In the second-line salvage setting, adding bevacizumab to oxaliplatin/fluorouracil/leucovorin showed a modest improvement in OS, response rate and PFS [13]. Such clinical benefits of bevacizumab are mainly derived from neutralizing VEGF-A and inhibiting its binding to VEGF receptor 2, which then results in angiogenesis inhibition [14]. It is less likely that bevacizumab acts directly on the host immune system by complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) considering its target localization. Nevertheless, given that Th1 cytokines, such as IFN-γ and TNF-α, inhibit tumor angiogenesis [15], bevacizumab may cooperate in its antiangiogenesis activity with certain immune responses.

mAb therapy targeting immunostimulatory/inhibitory molecules on immune cells has recently emerged as a novel strategy in cancer treatment [16,17]. The efficacy of mAbs that block immune checkpoints, such as CTLA-4, PD-1 and PD-L1, has been under investigation in clinical trials including CRC. Indeed, the humanized anti-CTLA-4 mAb ipilimumab was approved by the FDA for the treatment of unresectable or metastatic melanoma in 2011, and ipilimumab plus dacarbazine showed significantly prolonged OS as a fist-line treatment for previously untreated melanoma patients compared with dacarbazine plus placebo in a Phase III clinical trial (11.2 vs 9.1 months) [18]. Ipilimumab has clearly shed new light on the cancer immunotherapy field and such immune checkpoint blockade antibodies are now being tested extensively in a variety of different cancer types.

In this review, we will discuss recent clinical and experimental results, and underlying mechanisms of antibody therapies in CRC such as:

- Antibodies targeting cell-surface tumor-associated antigens;
- Antibodies targeting intracellular tumor-associated antigens;
- Antibodies targeting immune checkpoints.

Antibody therapies targeting cell-surface antigens in CRC

Antibody therapies targeting cell-surface antigens in CRC have been explored for more than 30 years. Murine-derived mAbs were originally administered in humans a few decades ago;

however, it soon became clear that patients frequently developed human anti-mouse antibody (HAMA)-eliciting hypersensitive reactions due to the allogeneic immune responses. To prevent this problem, chimeric, humanized and fully human antibodies exhibiting less immunogenicity and enhanced efficacy in immune effector functions including ADCC have been developed, and are currently used in the clinic.

Antibodies targeting cell-surface molecules expressed by tumor cells, such as rituximab and trastuzumab, have made an enormous impact in the oncology field and they are now widely accepted in the clinic [19,20]. An initial idea of antibody cancer therapy was the selective interruption of vital signaling pathways in which the targeted antigens are critically involved. However, a series of studies soon showed that CDC and ADCC are also critically associated with the efficacy of the treatment (FIGURE 1A–C) [21,22]. In patients with follicular lymphoma, polymorphisms in the *C1qA* gene influenced the response to rituximab therapy [23,24], suggesting the involvement of CDC in rituximab treatment (FIGURE 1B). When therapeutic IgG mAbs recognize and dock to cell-surface antigens on the tumor cell, the binding affinity of the antibody Fc portion to immunostimulatory/inhibitory FcγRs determines the magnitude of ADCC (FIGURE 1C) [25]. This mechanism has been investigated in mice that have three activating FcγRs: FcγRI (CD64), FcγRIIIA (CD16A) and FcγRIV, and one inhibitory receptor, FcγRIIB (CD32B). Clynes *et al.* reported that FcγRIIB-deficient mice significantly augmented antitumor efficacy of TA99 (mouse IgG2a anti-gp76 mAb) compared with wild-type mice in a murine B16 melanoma lung metastasis model, although mice deficient in activating FcγRs completely lost the efficacy of

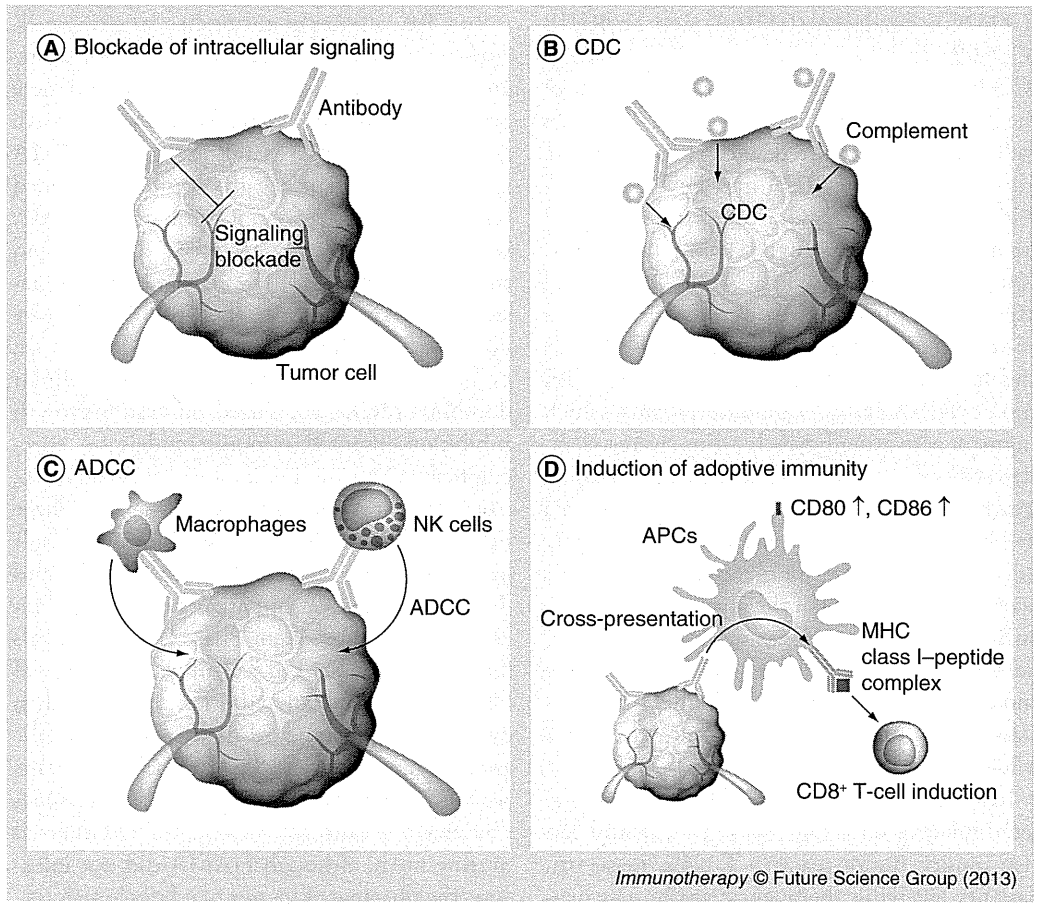

Figure 1. Antitumor mechanisms of antibodies targeting cell-surface antigens.
(A) Antibodies block intracellular signaling resulting in tumor growth inhibition. **(B)** Complement complexed with antibodies attack tumor cells, called CDC. **(C)** NK cells and macrophages attack tumor cells in an Fc–Fcγ receptor-dependent manner called ADCC. **(D)** APCs such as dendritic cells take up immune complexes through Fcγ receptors and cross-present the antigen with upregulation of costimulatory molecules, such as CD80 and CD86, to MHC class I pathway resulting in antitumor $CD8^+$ T-cell induction.
↑: Increase; ADCC: Antibody-dependent cellular toxicity; CDC: Complement-dependent cytotoxicity.

TA99 [26]. Antitumor efficacy of trastuzumab and rituximab observed in BALB/c nude mice lacking T cells was abrogated in mice deficient in activating FcγRs and the engineered antibody unable to bind any FcγRs lost the antitumor activity, indicating the crucial role of ADCC associated with activating and inhibiting FcγRs [26]. Further studies from the same group showed that, in the B16 melanoma model, IgG2a TA99 was the most efficacious at inducing an antitumor response than the other three subclasses (IgG2a ≥ IgG2b > IgG1 >> IgG3) due to its much higher affinity to activating FcγRs (mainly associated with FcγRIV) and less affinity to an inhibitory FcγRIIB [27]. In another animal model using anti-mouse CD20 mAb subclasses, similar subclass-dependent interactions with distinct FcγRs were also observed (IgG2a/c > IgG1/IgG2b > IgG3) [28]. In humans, there are five activating FcγRs: FcγRI (CD64), FcγRIIA (CD32A), FcγRIIC (CD32C), FcγRIIIA (CD16A) and FcγRIIIB (CD16B), and one inhibitory receptor, FcγRIIB (CD32B). The biological activities of each of the subclasses of human IgG (hIgG) are not well explored compared with those in mice. hIgG1 binds to FcγRIIA and FcγIIIA 4–40-fold better than hIgG2 and hIgG4. hIgG3 strongly binds to FcγRIIIA (3 × hIgG1, 100 × hIgG2 and 24 × hIgG4) [29]. hIgG1 and hIgG3 are considered to be more potent for Fc-mediated lysis of target cells than hIgG2a and hIgG4. Accordingly, FcγRIIA 131 H/H and FcγRIIIA 158 V/V polymorphisms, which augment activating FcγRs binding, were associated with better clinical outcomes in rituximab treatment for B-cell non-Hodgkin's lymphoma [30], in trastuzumab for metastatic breast cancer [31], and in cetuximab for metastatic CRC [8]. As several modifications of the glycosylation status on the Fc portion, such as defucosylation and sialylation, alter the affinity between antibody and FcγRs [32,33], modified antibodies may provide better antitumor responses compared with wild-type antibodies.

In addition to CDC and ADCC, there are accumulating data that antibody therapy can also induce tumor-reactive T cells (FIGURE 1D). Patients receiving therapeutic antibodies usually maintain their clinical benefit for much longer than the injected antibody can be detected after the last antibody administration, suggesting an involvement of memory T-cell responses. In accordance with this, dendritic cells (DCs) pulsed with antigen–antibody immune complexes induced far stronger antitumor immune responses compared with DCs pulsed with protein antigen [34]. An inhibitory FcγRIIB in DCs also negatively regulates antigen presentation to effector cells [35]. In addition, a HER2/neu-expressing, GM-CSF-secreting whole-cell vaccine combined with systemic injection of anti-HER2/neu mAb protected tolerized HER2/neu transgenic mice from subsequent challenge of HER2/neu-expressing tumor cells [36]. The augmented $CD8^+$ T-cell responses and maturation of DCs were associated with this strong antitumor effect in mice treated with the vaccine and the mAb in an Fc portion-dependent manner [37]. Saenger *et al.* reported that a combination of gp100 DNA vaccine and TA99 mAb augmented tumor growth inhibition compared with either a single DNA vaccine or TA99 treatment by activating FcγRs [38]. Importantly, higher numbers of F4/80 macrophages and $CD8^+$ T cells infiltrated tumors in mice treated with gp100 DNA vaccine and TA99 [38].

In CRC, a series of antibodies targeting cell-surface antigens have been tested in clinical trials (summarized in TABLE 1) and additional new antigenic targets are currently being explored. Among these, EpCAM (also known as 17–1A antigen) is a cell-surface glycoprotein expressed on a wide range of tumors including CRC, which was originally isolated from a colon tumor cell line [39,40]. Besides cell–cell adhesion, EpCAM promotes cell proliferation by upregulating c-myc and cyclin A and E [41,42], or by proteolytically releasing its intracellular domain EpICD [43]. Based on tumor-growth inhibition observed in mouse models [44], edrecolomab, a murine IgG2a anti-EpCAM mAb, has been investigated in clinical trials in a large number of CRC patients. While initial trials have shown a significantly improved OS and disease-free survival in patients receiving edrecolomab [45], subsequent Phase II and III trials failed to show any clinical impact in Stage II CRC patients and any additional benefit when compared with standard fluorouracil and folic acid chemotherapy in Stage III CRC patients [46–48]. A plausible explanation is that HAMA hindered the antitumor effects of the injected murine mAb, although HAMA did not influence the pharmacokinetics of edrecolomab [49]. Alternatively, ADCC by human immune cells mediated through the murine antibody may not have been strong enough to elicit tumor-growth inhibition in patients. In fact, transpired out later that two other anti-EpCAM mAbs, adecatumumab (a fully human IgG1) and ING-1 (a humanized IgG1) exerted much

higher ADCC activities than edrecolomab [50]. Of those, only adecatumumab showed inhibition of MCF-7 (a breast carcinoma cell line) and/or cell proliferation *in vitro* in the absence of complement and immune cells [51]. In patients with prostate cancers after radical prostectomy in a Phase II study, adecatumumab delayed disease progression in a subgroup of patients with baseline prostate-specific antigen levels ≤1 ng/ml and EpCAM-expressing tumors [51]. Efficacy of adecatumumab after complete resection of CRC metastases is now under investigation [201]. By contrast, a Phase I trial of ING-1 with 14 patients, including ten CRC patients, exhibited a higher risk of pancreatitis and a marginal antitumor efficacy, thus further clinical trials with ING-1 were abandoned [52].

Lewis-Y is a type 2 blood group-related difucosylated oligosaccharide antigen expressed by more than 70% of epithelial cancers including CRC [53]. Recent reports suggest that Lewis-Y promotes the proliferation of ovarian carcinoma-derived RMG-I cells through the PI3K–AKT pathway and by upregulating TGF-β1, VEGF and β-FGF [54,55]. BR96, a chimeric human IgG1 mAb, showed CDC and ADCC against a Lewis-Y-positive colon tumor cell line. Additionally, the antibody was internalized after cell-surface binding [56]. This peculiarity determined a direction of subsequent development of the antibody and doxorubicine-conjugated BR96 elicited tumor-growth inhibition in various types of tumor-bearing mice, including colon tumor [57]. Nevertheless, further clinical development of doxorubicine-conjugated BR96 was terminated since two Phase II clinical trials had demonstrated a limited antitumor efficacy of the antibody in patients with breast cancer and gastric cancer [58,59]. A Phase I study focusing on a humanized IgG1 anti-Lewis-Y mAb hu3S193 [60], in which 15 patients (including eight CRC patients) were enrolled, verified the safety and selective distribution of the mAb to Lewis-Y expressing tumors [61]. In a Phase II study in patients with advanced platinum-resistant/refractory ovarian cancer, primary peritoneal cancer or fallopian tube cancer, a favorable result by single hu3S193 injection, namely 42% of disease stabilization in heavily pretreated patients was reported [62]. Kircheis *et al.* have recently developed a new humanized anti-Lewis-Y mAb MB314 using a plant-based expression system, whose Fc portion contains reduced core fucose content compared with the wild-type mAb MB311 [63]. MB314 gained higher ADCC function and augmented TNF-α secretion from mononuclear cells when incubated with Lewis-Y-positive tumor cells [63]. Clinical trials testing the efficacy of Fc-engineered mAbs are warranted.

A33 is a cell-surface glycoprotein of the immunoglobulin superfamily. Its physiological function is not fully understood [64,65]. A33 is expressed by normal intestinal epithelium and 95% of primary and metastatic CRCs [66]. The normal bowel mucosa eliminates cell-bound A33 mAb within a few days, whereas significant amounts of the mAb are retained in tumor sites up to 6 weeks after mAb A33 injection in humans [67,68]. Therefore, this antigen has been considered to be a suitable target of mAb therapy in CRC. Two Phase I clinical trials with a humanized IgG1 anti-A33 mAb huA33 have been conducted in CRC patients, and safety and specific distribution of the mAb at tumor sites were confirmed [69,70]. In addition to this treatment aspect of huA33, it may be available as a high-resolution diagnostic tool of micrometastatic tumors in the body. ^{124}I-huA33, a positron-emitting mAb, was well tolerated in patients and showed favorable imaging selectivity, especially for metastatic tumors in the liver where a conventional PET with ^{18}F-fluorodeoxyglucose is not available [71]. A Phase I/II study with the fully human mAb KRN330 in patients with metastatic CRC has been recently completed [72]. Following the evidence of its safety in Phase I, the mAb demonstrated antitumor efficacy in the interim report in 2011 (two partial response, eight stable disease and six progressive disease of 16 patients) [72]. A final report is awaited.

Taken together, antibodies that target cell-surface antigens exhibit antitumor effects through both the Fab and the Fc regions. Therefore, in addition to an antibody's binding specificity with tumor cells, an antibody's capacity to trigger antitumor immunity through the Fc portion needs to be considered for further augmenting antitumor activity.

Antibody immunotherapy targeting intracellular molecules of CRC

Antibody therapies targeting intracellular molecules, such as mutated antigens and cancer–testis antigens in tumor cells, have not been extensively explored due to the potential difficulty of mAbs to access intracellular antigens. With cancer–testis antigens, such as NY-ESO-1 that is frequently expressed by various types of cancer cells but not in normal somatic cells except germ cells in the testis, there is a close correlation between spontaneous anti-NY-ESO-1 antibody

responses in the serum and NY-ESO-1-specific CD8+ T-cell responses in patients with NY-ESO-1-expressing tumors [73]. In fact, NY-ESO-1 protein/IgG antibody immune complexes are efficiently cross-presented to the MHC class I pathway in a FcγR-dependent manner [74]. However, until very recently, it had not been addressed whether exogenous therapeutic antibodies targeting intracellular antigens could facilitate CD8+ T-cell antitumor immune responses by augmenting antigen presentation of the MHC class I pathway and inhibit tumor growth. We have explored this concept and found that mAb treatment against intracellular antigens resulted in efficient induction of CD8+ T cells recognizing this antigen. We termed this promising novel approach for cancer immunotherapy antibody-facilitated T-cell immunity (FIGURE 2) [75]. To better study this concept, we established syngeneic tumor models in BALB/c mice using CT26 colon carcinoma cells and CMS5a sarcoma cells that were stably transfected with cancer–testis antigens such as NY-ESO-1 or MAGE-A4 [76]. Tumor cells treated with a chemotherapeutic drug, such as 5-fluorouracil, accelerated release of intracellular antigens and injected antigen-specific mAbs distinctively accumulated at tumor sites. Combination treatment of chemotherapeutic drugs and anti-NY-ESO-1 mAb in mice bearing NY-ESO-1-expressing tumors exhibited an augmented antitumor effect. This antitumor effect was associated with an Fc receptor-dependent DC maturation and enhanced NY-ESO-1-specific CD8+ T-cell induction [75]. Our data clearly proposes the feasibility for the combination of mAb-targeting intracellular tumor-associated antigens and chemotherapy. To allow this strategy to be explored in clinical settings, a human anti-NY-ESO-1 mAb 12D7 has been developed. This human mAb showed a similar antitumor efficacy against NY-ESO-1-positive tumor cells in a preclinical model [77] and a clinical trial with 12D7 mAb is now being planned.

Similar to cancer–testis antigens, mutated antigens highly specific for CRC could also be good candidates for mAb therapy since gene mutations particularly associated with tumor development are confined to tumor cells and are absent in normal tissues. Indeed, a recent study by the Cancer Genome Atlas Network found that 16% of CRC were hypermutated: three-quarters of these had high microsatellite instability with hypermethylation and MLH1 silencing, and one-quarter had somatic mismatch-repair gene and polymerase E mutations. By contrast, other nonhypermutated cancers had considerably similar patterns of genomic alterations. The authors identified 24 genes that were significantly mutated, including *ARID1A*,

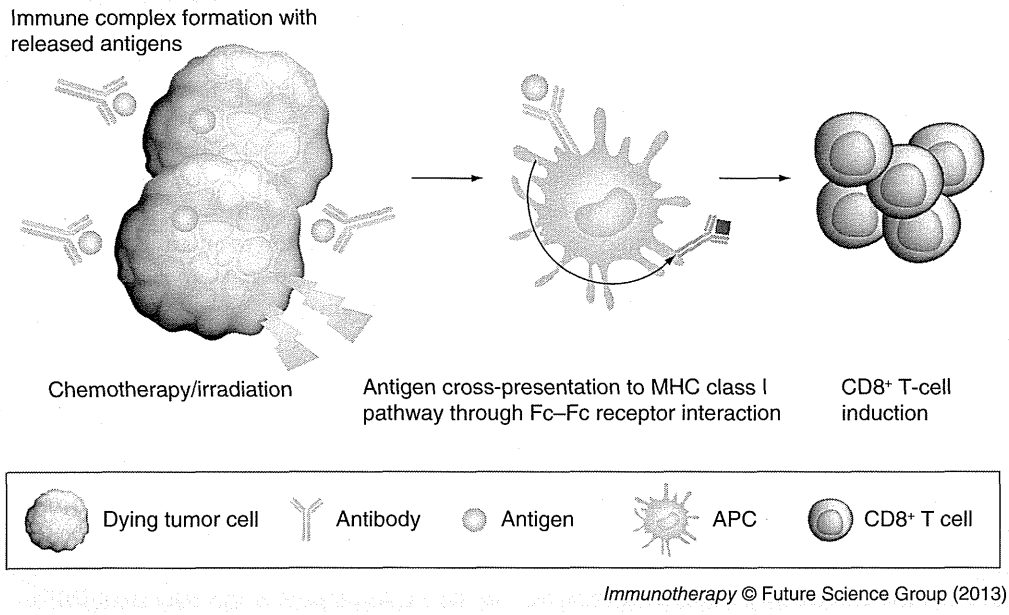

Figure 2. Antitumor mechanisms of antibodies targeting intracellular antigens. Intracellular tumor antigens released from dying tumor cells following chemotherapy or radiotherapy are captured by an antibody, and antigen–antibody immune complexes are generated. These immune complexes are taken up by APCs such as dendritic cells and augment antigen presentation by the MHC class I pathway. These APCs efficiently induce CD8+ T-cell responses that can efficiently kill tumor cells. We propose this concept as antibody-facilitated T-cell immunity.

SOX9 and *FAM123B*, as well as the expected *APC*, *TP53*, *SMAD4*, *PIK3CA* and *KRAS* [78]. However, it is still controversial whether those *de novo* mutated antigens are immunogenic (a critical feature for antibody-facilitated T-cell immunity) and thus could be tumor rejection antigens recognized by the host immune system [79,80].

Antibody immunotherapy targeting T-cell checkpoints for CRC

For optimal T-cell activation, T-cell receptor ligation to peptide–MHC complexes combined with costimulatory (i.e., CD28) signals is required. However, inhibitory (i.e., CTLA-4 and PD-1) molecules (so-called immune checkpoints) on T cells play a crucial role in regulating T-cell activation to maintain the immune homeostasis in our bodies. A disturbed balance of costimulatory and inhibitory signals may result either in the failure of eliminating exogenous pathogens or in autoimmune diseases. The tumor microenvironment establishes complex networks to escape immune attacks where cytotoxic T-cell activity against tumors are perturbed by downregulated stimulatory signals, by upregulated inhibitory signals or by both [81]. The development of mAbs with either activating costimulatory signals (agonistic mAbs) or suppressing inhibitory signals (antagonistic mAbs) has induced the recovery of their cytotoxicity against exogenous pathogens and tumor cells [16,17]. Such mAbs have been studied extensively in preclinical animal models, with several of them now also being explored in clinical trials. The pioneer of these antibodies, ipilimumab, a fully humanized anti-CTLA-4 mAb, was approved by the FDA for the treatment of unresectable or metastatic melanoma in 2011 based on the survival benefit in a Phase III clinical trial [18]. Clinical trials using T-cell checkpoint mAbs have also been performed in other types of cancer including CRC. Ongoing clinical trials of T-cell checkpoint mAbs in CRC include mAbs targeting CTLA-4, PD-1 and PD-L1. Antagonistic T-cell checkpoint mAbs, compared with mAbs with agonistic T-cell stimulating properties, are currently the primary focus of clinical trials, partly because an agonistic anti-CD28 mAb (TGN-1241) showed severe toxicity, such as multiorgan failures due to cytokine storm in a Phase I dose-escalation trial in 2006 [32].

CTLA-4 (CD152), a homolog of the costimulatory molecule CD28, is a receptor for CD80 and CD86, and is essential for T-cell homeostasis and tolerance. CTLA-4 is translocated onto the cell surface from the intracellular compartment upon T-cell activation and competes with CD28 for CD80 or CD86, or delivers an inhibitory signal directly through its cytoplasmic tail [82]. In CTLA-4 knockout (KO) mice, massive lymphocyte proliferation followed by autoimmune disease development was observed [83]. In addition to effector T cells, CTLA-4 on Tregs also play an important role for the maintenance of T-cell homeostasis. Mice conditionally knocked out for CTLA-4 expression in Tregs exhibited lethal myocarditis and other focal lymphocyte infiltrations, although their disease progression was much slower than CTLA-4 KO mice. By contrast, mice conditionally knocked out for CTLA-4 expression in Tregs showed enhanced antitumor immunity and upregulation of CD80 and CD86 on APCs [84]. Antitumor efficacy of antagonistic anti-CTLA-4 mAb was demonstrated in a murine colon tumor model [85] and CTLA-4 signaling blockade improved T-cell proliferation, Th1 cytokine secretion and decreased the threshold of tumor-associated antigen recognition [86]. Based on these promising preclinical data, anti-CTLA-4 mAb treatment has been tested in clinical trials in patients with CRC [87]. A Phase II study evaluated the safety and efficacy of tremelimumab, a fully human IgG2 anti-CTLA-4 mAb, in 45 patients with treatment-refractory CRC; however, the study did not provide any clinical benefit in this patient population. While the finding that 43% of the patients survived longer than 6 months is of interest, it is not clear whether this reflects clinical benefit with tremelimumab or with other factors such as patient selection [87]. IgG subclasses, such as IgG2 for tremelimumab and IgG1 for ipilimimab, that have different affinities to activating FcγRIIA and FcγRIIIA may influence the clinical benefits. Clinical trials with a large number of patients are warranted.

PD-1 (CD279), also a homolog of CD28, is a receptor induced on the cell surface of activated T cells, B cells and myeloid cells. It is also known as an 'exhaustion' marker of effector T cells under tumor-bearing conditions [17] and PD-1 expression is inversely associated with impaired cytokine secretion such as IFN-γ, TNF-α and IL-2 of tumor antigen-specific $CD8^+$ T cells [88]. The PD-1 receptor has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). The interaction between PD-1 and PD-L1 or PD-L2 dampens effector function of T cells, and PD-1 KO mice develop autoimmune glomerulonephritis and a

dilated cardiomyopathy due to an autoantibody against cardiac troponin [89–91]. These ligands are expressed by APCs, as well as CD80 and CD86, which are the ligands for CTLA-4, and PD-L1 expression is also found in many types of cancer tissues including CRC [92,93]. In preclinical animal models, blockade of the interaction between PD-1 and PD-L1 with antagonistic anti-PD-1 mAb or anti-PD-L1 mAb showed profound antitumor activity [94,95]. In a Phase I study of a fully human IgG4 anti-PD-1 mAb treatment in refractory solid tumors, safety was confirmed and one out of 14 patients with CRC achieved a complete response [96]. Subsequently, in a Phase Ib study with the same mAb, objective responses were observed in a substantial proportion of patients with melanoma (26 out of 94 patients), non-small-cell lung cancer (14 out of 76) and renal cell cancer (nine out of 33), but not in patients with prostate cancer or CRC [97]. In addition, a clinical trial with anti-PD-L1 mAb is ongoing and an interim report showed that objective clinical responses were observed in patients with melanoma (nine out of 52), non-small-cell lung cancer (five out of 49), renal cell cancer (two out of 17) and ovarian cancer (one out of 17), but not in patients with CRC or pancreatic cancer [98].

These initial clinical results give us a notion that single T-cell checkpoint blockade may not be sufficient for the treatment of patients with CRC, partly due to the poor immunogenicity of CRC. Indeed, a single anti-CTLA-4 treatment was not efficacious for poorly immunogenic tumors, but when combined with a GM-CSF-transduced cellular vaccine, it exhibited strong antitumor activity in an animal model [86]. Combination of several T-cell checkpoint mAbs may be a more efficacious strategy. We reported that large established tumors in mice were controlled successfully by a combination of antagonistic anti-CTLA-4 mAb and agonistic anti-GITR mAb, which was not achieved by single mAb treatment. While anti-CTLA-4 mAb augmented proliferation of $CD8^+$ T cells, anti-GITR mAb enhanced IFN-γ production from $CD8^+$ T cells [99]. T-cell activation through the multiple different pathways may effectively contribute improved antitumor immunity, although it remains to be investigated what combination is best for the patients with CRC.

Conclusion

Antibody therapy in CRC was initially applied to interfere with the vital signaling pathways targeted by the antibody, such as EGFR or VEGF-A pathways. Additional mechanisms of antibody-based therapies in CRC engage the patient's immune system. In the case of mAb-targeting tumor-associated antigens, immune-mediated antitumor effects are largely determined by the interaction between the Fc portion and FcγRs. With respect to subclasses and modification of the Fc portion, therapeutic antibodies should be designed to have a high affinity to activating FcγRs, but limited affinity to an inhibitory FcγRIIB. It is also essential to understand which types of immune cells dominantly access the immune complexes in the tumor microenvironment, since each population has different profiles of FcγRs expressions. Another aspect of mAb-targeting tumor-associated antigens is the use of mAbs conjugated to toxins and radioisotopes for treatment and diagnosis. Data from a Phase III trial have recently shown that an antibody–drug conjugate, trastuzumab ematansine, significantly prolonged PFS and OS with less toxicity than lapatinib plus capecitabine in patients with HER2-positive advanced breast cancer, previously treated with trastuzumab and a taxane (the EMILIA trial) [100]. In the case of mAbs directly manipulating immune responses, immune checkpoints are of particular interest and may be especially suitable for cancer treatment as they may overcome a severe immune suppressive microenvironment shaped by the tumor. Indeed, some mAbs have already shown encouraging results in clinical trials. Yet, several issues will have to be addressed to optimize mAb treatment: which immune checkpoints (single or combinations) should be targeted for each tumor? How are these mAbs accumulated in tumor sites? And how can one isolate patients sensitive for the mAbs? Interestingly, immune checkpoint mAbs targeting TNF superfamily receptors, such as agonistic anti-CD40 antibody, may require a particular structure to be able to bind to an inhibitory FcγRIIB, otherwise the antibodies lose immune stimulatory function, such as the induction of antigen-specific $CD8^+$ T cells and tumor growth inhibition [101,102]. A similar FcγRIIB requirement has been observed in a mAb targeting death receptor 5 on tumor cells, as well as for CD40 [103].

Improved quality and quantity of antitumor immune responses are essential for effective immunotherapy in patients with CRC [104,105]. It is, therefore, necessary to better understand the effect of CRC on the immune system, as well as the cancer cell biology of CRC.

Future perspective

Increasing new data reveal that antibody therapy induces antitumor activity, not only through direct blocking of vital signals provided by the target molecules, but also through stimulating ADCC and CDC activity. Thus, there will be a focus on antibody-engineering methods of how to maximize these responses in patients with CRC. In addition, mAb therapy targeting immune checkpoints will become standard in the clinic. Finding combinations of such mAbs with other drugs to achieve long-lasting clinical responses will be another critical issue and will be explored in future clinical trials. Identifying biomarkers for predicting the effects of antibody therapy will be critical for developing better treatment strategies.

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Executive summary

- Three monoclonal antibodies (cetuximab, bevacizumab and panitumumab) have been approved for the treatment of colorectal cancer in the USA and a large number of other monoclonal antibodies are being tested in clinical trials.
- Accumulating data implicate the potential contribution of the host immune system to antitumor activity induced by cetuximab and panitumumab, although no specifically designed 'immunotherapeutic' antibody is currently available in the clinic for colorectal cancer.
- Antibodies induce antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity and efficient cross-presentation by APCs through the Fc portion. Novel technologies aiming at maximizing these reactions are applied and studied in the clinic.
- Immune checkpoint blockade antibodies, such as ipilimumab, are considered another therapeutic strategy against cancer due to their different mode of the antitumor mechanism and they have recently emerged in clinical settings.

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