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Combination strategies to enhance antitumor ADCC

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Abstract

The clinical efficacy of monoclonal antibodies as cancer therapeutics is largely dependent upon their ability to target the tumor and induce a functional antitumor immune response. This two-step process of ADCC utilizes the response of innate immune cells to provide antitumor cytotoxicity triggered by the interaction of the Fc portion of the antibody with the Fc receptor on the immune cell. Immunotherapeutics that target NK cells, $\gamma\delta$ T cells, macrophages and dendritic cells can, by augmenting the function of the immune response, enhance the antitumor activity of the antibodies. Advantages of such combination strategies include: the application to multiple existing antibodies (even across multiple diseases), the feasibility (from a regulatory perspective) of combining with previously approved agents and the assurance (to physicians and trial participants) that one of the ingredients – the antitumor antibody – has proven efficacy on its own. Here we discuss current strategies, including biologic rationale and clinical results, which enhance ADCC in the following ways: strategies that increase total target–monoclonal antibody–effector binding, strategies that trigger effector cell ‘activating’ signals and strategies that block effector cell ‘inhibitory’ signals.

Keywords

$\gamma\delta$ T cells; ADCC; cancer; cytokines; IMiD; immunocytokines; immunomodulators; interleukins; monoclonal antibodies; NK cells; passive immunotherapy

ADCC

Monoclonal antibodies (mAbs) can target tumor antigens on the surface of cancer cells and have a favorable toxicity profile in comparison with cytotoxic chemotherapy. Expression of tumor antigens is dynamic and inducible through agents such as Toll-like receptor (TLR) agonists, immunomodulatory drugs (IMiDs) and hypomethylating agents [1]. Following binding of the mAb to the tumor antigen, the Fc portion of the mAb interacts with the Fc receptor (FcR) on the surface of effector cells (i.e., NK cells, $\gamma\delta$ T cells and macrophages),

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leading to antitumor cytotoxicity and/or phagocytosis of the tumor cell. FcR interactions can be stimulatory or inhibitory to the killer cell, depending on which FcR is triggered and on which cell. Stimulatory effects are mediated through Fc γ RI on macrophages, dendritic cells (DCs) and neutrophils, and Fc γ RIIIa on NK cells, DCs and macrophages. In murine models, the cytotoxicity resulting from FcR activation on a NK cell, $\gamma\delta$ T cell and macrophage is responsible for antitumor activity [2]. The role of DCs should be noted: although not considered to be primary ADCC effector cells, they can respond to mAb-bound tumor cells via their own FcR-mediated activation and probably play a significant role in activating effector cells. Preclinical models have shown that, although not the effector cell, DCs are critical to the efficacy of mAb-mediated tumor elimination [3]. Equally, mAb-activated ADCC effector cells can induce DC activation [4] and the importance of this crosstalk is an increasing focus of study [5].

The antitumor effects of mAbs are caused by multiple mechanisms of action, including cell signaling agonism/antagonism, complement activation and ligand sequestration, although ADCC probably plays a predominant role in the efficacy of some mAbs. In a clinical series, a correlation between the affinity of the receptor Fc γ RIIIa (determined by inherited FcR polymorphisms) and the clinical response to mAb therapy, supporting the significance of the innate immune response [6–10]. Several strategies could potentially improve the innate response following FcR activation by a mAb (Figure 1):

- Quantitatively increasing the density of the bound target, mAb or the effector cells;
- Stimulation of the effector cell by targeting the NK cell, $\gamma\delta$ T cell and/or macrophage with small molecules, cytokines or agonistic antibodies;
- Blocking an inhibitory interaction between the NK cell or macrophage and the tumor cell.

The ability of the combination approaches to enhance ADCC is largely determined by the capacity of the mAb to induce ADCC. Since the approval of the first mAb for the treatment of non-Hodgkin's lymphoma, rituximab (RTX), in 1997, several mAbs have become standard of care for the treatment of both solid tumors and hematologic malignancies, including trastuzumab (TRAST), alemtuzumab, cetuximab, panitumumab and ofatumumab [11]. As noted above, clinical series among lymphoma patients treated with an anti-CD20 mAb (RTX) [6,7], HER2-expressing breast cancer receiving anti-HER2 mAb therapy (TRAST) [8] or colorectal cancer patients treated with an anti-EGFR mAb (cetuximab) [9,10] observed a correlation between clinical benefit and Fc γ RIIIa genotype, with patients who have higher-affinity polymorphisms demonstrating superior clinical outcomes. By contrast, the anti-EGFR mAb panitumumab does not induce ADCC, owing to a different Fc isotype that does not bind to the Fc γ RIIIa. Therefore, when considering enhancement of ADCC, such approaches are limited to combinations with mAbs that activate the FcR. Nonetheless, an advantage of this dual therapy strategy is that mAbs yet to be discovered against currently unknown tumor antigens may be combined with the therapeutics discussed herein.

Increasing target–mAb–effector binding

As the central element in the target–mAb–effector cell unit, the mAb seems to be a probable candidate for improvements, either in its antigen-binding or its Fc-binding domains. This approach has been heavily pursued with some degree of success [12–15]. Antibody engineering to improve interaction between the target or FcR requires that each new antibody be individually developed and tested as a new entity.

Increasing the antigen target

Tumor cells with a lower density of antigen targets are less responsive to mAbs than higher antigen-expressing diseases [16]. Therefore, it seems logical to try to increase the expression of the target on tumor cells. Antigen expression can be upregulated by cytokines [17], ionizing radiation [18], natural metabolites [19] and hypomethylating agents such as decitabine [20]. In addition, the family of TLR9 agonists known as CpG oligodeoxynucleotides (CpG ODN) can induce CD20 expression on malignant B cells [21–23]. Taken together with data showing the activating effect of CpG ODN on effector cells (discussed below), it seems reasonable that the combination of CpG ODN with mAb might have synergistic efficacy. Clinical series, however, have tested CpG ODN administered intravenously or subcutaneously and have observed little efficacy in Phase I and II studies [24–26] in low-grade lymphoma. One possible limitation of these studies has been their application to diseases (primarily follicular and mantle cell lymphoma) known to already have high expression of the relevant antigen (CD20). It is plausible that increasing antigen expression on low antigen-expressing diseases such as chronic lymphocytic leukemia could have a greater increase in relative efficacy. To this end, monotherapy studies have recently been undertaken [27,301] and should lead to combination trials.

Effector cells: NK cells

Given the importance of the effector cell in ADCC, it is conceivable that by increasing their number, in the context of adequate tumor antigens and mAbs, that the sum antitumor effect could be increased. One approach to test this hypothesis has been to generate high numbers of effector cells *ex vivo* using either IL-2 or a combination of IL-2, IFN- γ and anti-CD3 mAb, known as lymphokine-activated killer (LAK) cells and cytokine-induced killer cells, respectively, which both contain NK cells as the majority cell type present. Although LAK and cytokine-induced killer cell monotherapy have yet to show significant efficacy, their combination with mAbs has only recently been tested. Murine models have shown robust synergy between a variety of mAbs and transferred effector cells such as LAK and cytokine-induced killer cells [28–30]. The same approach was studied combining LAK cells with RTX in a cohort of patients with RTX-refractory lymphoma. In the study, adoptive transfer of LAK cells improved peripheral blood NK cell counts and ADCC, and even induced clinical responses in some patients [31]. A similar combination of LAK cells and RTX is being studied in an ongoing Phase II trial powered for clinical end points [302]. Perhaps the most ambitious – and potentially significant – ongoing test of this idea is a randomized trial comparing the 3-year event-free survival in patients with newly diagnosed diffuse large B cell lymphoma treated with RTX, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) versus R-CHOP plus autologous NK cell therapy [303]. To date, 273 out of the planned 276 have been enrolled [Cho SG, Pers. Comm.] and clinical results (as well as data describing the NK cell product) are pending.

Effector cells: $\gamma\delta$ T cells

The role of NK cells and macrophages in mediating ADCC has been well established; however, only recently have $\gamma\delta$ T cells been found to play a role as ADCC effectors. Typically, this population is considered as a minor subset (<5% of circulating T cells), although they may infiltrate tumors of epithelial origin preferentially and constitute a large portion of the tumor-infiltrating lymphocytes in cancers such as breast carcinoma. The combination of HLA-unrestricted cytotoxicity against multiple tumor cell lines of various histologies, secretion of cytolytic granules and proinflammatory cytokines such as TNF- α , IL-17 and IFN- γ make $\gamma\delta$ T cells potentially potent antitumor effectors [32,33]. Clinical evidence for $\gamma\delta$ T-cell function includes presence postchemotherapy predicting tumor response, and persistence following bone marrow transplantation correlating with survival [34,35]. The selectivity of $\gamma\delta$ T cells was seen following culture and expansion of tumor-

infiltrating lymphocytes that were unable to lyse normal tissues, but remained cytotoxic against autologous tumor [33].

Of significant relevance to ADCC, a subset of $\gamma\delta$ T cells upregulate CD16 (Fc γ RIII) upon activation [36,37]. CD16^{high} $\gamma\delta$ T cells constitutively express several NK cell receptors including NKG2A–CD94 and express high amounts of perforin, but low levels of IFN- γ [36]. They specifically respond to activation via CD16 and are capable of lysing tumors following exposure to mAbs including RTX [38–40], TRAST [39,40], alemtuzumab [39], ofatumumab [38] and GA101 [38], and may facilitate NK cell function [41]. The synergy between $\gamma\delta$ T cells and NK cells relies on an interaction between the CD137 ligand and CD137; activated $\gamma\delta$ T cells enhance antitumor cytotoxicity of NK cells through CD137 engagement [42].

Clinically, $\gamma\delta$ T cells are promising for translation as they are easily collected by leukapheresis and can be used as adoptive therapy in combination with mAbs [43,44]. The highly restricted T-cell receptor repertoire (over 90% limited to the $\gamma\delta$ subset) can be stimulated directly by synthetic compounds including bromohydrin pyrophosphate or indirectly by bisphosphonates [45]. Phase I and II clinical trials using $\gamma\delta$ T cells as monotherapy in solid tumors [46–51] and hematological malignancies [52] have been initiated with modest results to date [32]. Combination approaches with mAbs including RTX and TRAST are in development.

Targeting effector cell activation: small molecules

TLR agonists

In addition to its aforementioned induction of CD20, CpG ODN also indirectly augments innate immune function. TLRs are specialized to recognize pathogen-associated molecular patterns; they stimulate plasmacytoid DCs and B cells [53], and one of many plasmacytoid DC responses to stimulation by CpG ODNs is activation of local NK cells, thus improving spontaneous cytotoxicity and ADCC [54]. CpG ODN effects on NK cells appeared to be indirect and IFN- γ production by T cells (possibly in response to plasmacytoid DC activation) has been hypothesized as the intermediary of NK cell activation. Preclinical data in lymphoma and HER2-expressing breast cancer show that CpG ODN augment antitumor efficacy of RTX [55,56] and TRAST [57–59]. Similar results have been seen with non-CpG ODN TLR9 agonists, referred to as immunomodulatory oligonucleotides, in augmenting both cetuximab [60] and TRAST [61]. Similar preclinical boosting of TRAST- and RTX-mediated ADCC and increases in NK cell cytotoxicity has been with agonists of TLR2 [62], TLR3 [63], TLR4 [64], TLR7 [65] and TLR8 [66]. While each of these TLRs have a distinct expression profile, many are broadly expressed on myeloid subsets including monocytes, macrophages and DCs. By contrast to other myeloid activators (such as GM-CSF, discussed below), there is evidence that TLR agonists may stimulate immune cell function without the concomitant activation of suppressive cell types such as myeloid-derived suppressor cells [67]. Even beyond direct TLR effects on ADCC effector cells, DC activation status is extremely sensitive to TLR agonist exposure and, as noted previously, these cells profoundly impact ADCC effector cell function.

Immunomodulatory drugs

IMiDs have shown clinical activity in multiple hematologic malignancies despite their primary mechanism of action being unclear. Among their biologic effects (particularly lenalidomide) there are demonstrable and pleiotropic effects on immune cells and signaling molecules. These include enhancement of *in vitro* NK cell- and monocyte-mediated ADCC on RTX-coated [68] as well as TRAST- and cetuximab-coated tumor cells [69]. *In vivo* studies in a human lymphoma severe combined immune deficiency mouse model

demonstrated significant increases in NK cell recruitment to tumors mediated via microenvironment cytokine changes and augmented RTX-associated ADCC [70]. Studies suggest that IMiD activation of NK cells occurs indirectly; partly via IL-2 induction by T cells [71]. Clinically, a recent study noted significant increases in peripheral blood NK cells, NK cell cytotoxicity and serum IL-2, IL-15 and GM-CSF [72], the potential ADCC-promoting effects of which are discussed below.

There have been several studies combining lenalidomide with RTX [73–75], as well as ongoing studies with cetuximab [304–306]. Among the most remarkable of these is a recent report of RTX plus lenalidomide for previously untreated indolent lymphoma, which yielded a complete response (CR) in 66% and partial response (PR) in 24% of the 70 patients evaluated [76]. Although interstudy comparisons are inherently difficult to interpret, lenalidomide monotherapy for relapsed disease has yielded CR in 7% and PR in 16% [77], while RTX for previously untreated disease has yielded CR in 37% and PR in 36% [78]. Taken together, these data suggest a degree of synergy between the agents, although whether this is by ADCC enhancement is unclear.

Inducers of NKG2D ligands

NKG2D is an activating immunoreceptor of primary importance on NK cells (also expressed by CD8 T cells, $\gamma\delta$ T cells and macrophages). Activating signals mediated through engagement of NKG2D by its ligands on target cells can bypass signals transmitted through inhibitory NK cell receptors, allowing NKG2D to function as a ‘master switch’ in determining the activation status of NK cells [79]. Expression of NKG2D ligands determines the sensitivity of lymphoma cell lines to RTX-mediated ADCC [80]. Forced expression of either murine [81] or human [82] NKG2D ligands on tumor cells sensitizes them to NK cell-mediated cytotoxicity. Various agents have been shown to upregulate expression of NKG2D ligands including IFN- α [83], all-*trans* retinoic acid [84], hydroxyurea [85], proteasome inhibitors [86] and various histone deacetylase (HDAC) inhibitors [87]. HDAC inhibitors augment cytotoxic activity of NK cells against tumor cells [88] and ADCC of RTX [89] and TRAST [90], although it is unclear whether this is due to upregulation of NKG2D ligands or target expression on tumor cells. Clinical studies combining HDAC inhibitors have been initiated, for example in chronic lymphocytic leukemia [91].

Targeting effector cell activation: cytokines

GM-CSF/G-CSF

GM-CSF is an important cytokine with profound effects on the differentiation and activation of myeloid cells and, as such, has been widely studied as an adjuvant with vaccines, although its utility in potentiating ADCC has also been seen in preclinical models. Both ADCC and phagocytosis are enhanced *in vitro* by GM-CSF among multiple leukocyte subsets, including monocytes and lymphocytes [92,93], as well as neutrophils and eosinophils [94], and these effects are significantly greater than that observed with the related cytokines G-CSF and M-CSF [93]. Similar results have been seen *in vivo* [95] and have been shown to be tumor-protective in the setting of mAb therapy. Potentially important for combination mAb therapy is the observation that GM-CSF induces CD20 upregulation on malignant B cells [96].

In patients, GM-CSF has been shown to increase cells expressing FcRI⁺ (CD64), FcRII⁺ (CD32) and FcRIII⁺ (CD16), and to cause transient increases in ADCC [97]. Despite this, other correlative clinical studies have suggested that high doses of GM-CSF may have a negative impact on ADCC [98]. However, a variety of studies combining GM-CSF with

different mAbs have not yielded clear evidence of a benefit in any disease, with the possible exception being neuroblastoma (see Table 1 and the 'IL-2' section below). Mechanistically, although GM-CSF augments important effectors including monocytes and macrophages, it may simultaneously activate suppressive immune elements such as myeloid-derived suppressor cells [99], which can inhibit NK cell function [100], and, thus, potentially negate the beneficial effects.

G-CSF induces the expression of FcRI on neutrophils, enhances neutrophil cytotoxic capacity and ADCC, both *in vitro* and *in vivo*, and greatly increases the number of peripheral blood neutrophils. Therefore, it is possible that G-CSF could enhance the antitumor effect of mAbs. A small study testing this approach with RTX for low-grade lymphoma yielded clinical responses apparently comparable to RTX monotherapy.

IFN- α

Type I interferons (IFN- α and IFN- β) are strong stimuli for NK cells, inducing proliferation [101], cytotoxicity via the STAT1 and STAT2 pathways and IFN- γ secretion via the STAT4 pathway [102]. Addition of IFN- α to mAb therapy augments antitumor ADCC *in vitro* [103–105], which could be attributed to effects on NK cells [106] as well as macrophages [107]. *In vivo* IFN- α has demonstrated significant enhancement of ADCC and antitumor mAb efficacy, particularly when combined with IL-2 [108]. Notably, NK cell cytotoxicity and ADCC from oral cancer patients could be augmented by IFN- α , although peritumoral NK cells were significantly impaired compared with peripheral blood NK cells [106].

Such preclinical data prompted an assessment of the effects of IFN- α on ADCC in early trials in cancer patients and, although these revealed minor augmentations of ADCC and NK cell cytolytic activity at lower doses, at higher doses, the predominant immunomodulatory effect was suppression of ADCC [109]. As another example of the potential inconsistencies between preclinical and clinical data (despite *in vitro* evidence that IFN- α induces CD20 upregulation on malignant B cells [110]) clinical studies showed no demonstrable CD20 upregulation in lymphoma tumor aspirates [111] and the combination of IFN- α with RTX has demonstrated poor clinical efficacy (Table 1).

IL-2

IL-2 was the first cytokine approved for the treatment of cancer (renal cell cancer and melanoma), based solely upon immunomodulatory effects (versus IFN- α , which has direct cytostatic effects as well). After its discovery as a T-cell growth factor [112], it was observed to induce cyto-toxicity in NK and T cells [113]. IL-2 acts via two distinct receptor complexes: the high-affinity receptor formed by IL-2R α -, β - and γ -chains, and an intermediate-affinity receptor formed by only the β - and γ -chains. Whereas the high-affinity receptor is expressed on activated T and NK cells, the intermediate receptor is constitutively expressed on CD3-CD56⁺CD16⁺ NK cells [114] and a minor subsets of T cells. Notably, a small subset of CD56^{high}CD16 NK cells constitutively expresses the high-affinity receptor and may respond to lower IL-2 concentrations [115]. These subtleties have prompted a variety of dosing schema in combination strategies with mAbs for a variety of tumor types (Table 1). One early-phase clinical study demonstrated significant proof of principle by showing the correlation between IL-2-mediated NK cell expansion and clinical response [116]. Ultimately, despite promising preclinical data [117,118], no study has shown a clear signal of greater efficacy than mAb monotherapy. Similar to the mechanistic concerns noted for GM-CSF, IL-2 also expands both immune stimulatory (e.g., effector T cell) and suppressive (Treg) components that may diminish the potential benefits.

However, one of the first mAbs to be tested in combination with IL-2, the antiganglioside chimeric mAb ch14.18, was recently shown to be part of a combination immunotherapy (mAb–GM-CSF–IL-2) that improved overall survival in patients with neuroblastoma [119]. Which of the three immunotherapeutic components (or which combination thereof) is needed for this effect is unclear, although the same regimen is currently being tested in a randomized trial with or without the mAb [307].

IL-12

IL-12 is a member of a small family of heterodimeric cytokines and an important immune regulatory cytokine that exerts potent antitumor activity in various preclinical contexts [14,15]. IL-12 (previously known as NK cell-stimulating factor) enhances the ability of NK cells to lyse target cells [120] and provides a link between innate and adaptive immunity [121], as seen in its augmentation of NK cell-mediated killing of HER2⁺ tumor cells combined with TRAST [122], shown in other studies to depend on NK cell IFN- γ production [123]. Signaling by IL-12p70 through the IL-12 signaling network leads to the polarization of naive CD4⁺ T cells into a Th1 phenotype [124], which indirectly enhances NK cell ADCC [125]. Several clinical studies have combined IL-12 with mAb (Table 1), demonstrating pharmacodynamic proof of principle, although no clear benefit over a mAb monotherapy. A recently initiated study may yield data on whether cetuximab and IL-12 can be combined to treat patients with advanced oropharyngeal cancer [308].

IL-15

Despite numerous functional similarities to IL-2 and other members of the common γ -chain cytokines, IL-15 has been shown to be the primary signal responsible for NK cell differentiation and development [126–128]. Given the central role of NK cells in ADCC, there could be marked synergy between IL-15 and mAb therapy. This has been the case in a variety of preclinical models testing the combination of RTX [54,129] and cetuximab [130]. In addition, there are data demonstrating that some of the potential downsides of IL-2 such as amplification of Tregs might be avoided, or even counteracted, by IL-15 [131].

IL-21

IL-21 regulates the proliferation, maturation and function of multiple leukocyte subtypes including B cells, T cells, NK cells and DCs. IL-21 upregulates the expression of CD16, the Fc γ RIII subtype required for ADCC, costimulates the secretion of IFN- γ and upregulates the expression of granzyme and perforins, increasing the cytotoxic potential of NK cells [132,133]. Combinations of IL-21 with TRAST [134], cetuximab [135] and RTX [136] have demonstrated increases in *in vitro* NK cell-mediated ADCC, as well as a mAb-potentiating *in vivo* antitumor effect [137]. An early-phase study combining IL-21 with RTX demonstrated CRs and PRs (Table 1), even demonstrating efficacy in the setting of RTX-refractory disease [138].

Immunocytokines

A primary limitation of mAb therapy in combination with systemic cytokines is the dose-limiting toxicities of the latter; this may also be a cause of the disparity between lackluster clinical results and encouraging preclinical data. One potential solution is to target the cytokine to the tumor site by conjugating it to the mAb, the resulting fusion proteins frequently being referred to as immunocytokines (ICKs). Still, by localizing the cytokine to the tumor site, the aim has usually been to increase ADCC by activating effector (generally NK) cells. The majority of the constructs have thus utilized IL-2, given its NK cell-activating effects (discussed earlier).

Initial studies of an anti-GD2–IL-2 ICK have shown antitumor activity in patients with neuroblastoma [139] and have even gone on to show that this effect may be NK cell-mediated by virtue of its increased efficacy in patients with (autologous) killer immunoglobulin-like receptor (KIR)–KIR ligand mismatch [140]. The same molecule has also shown biological activity in the melanoma setting [141], although in this setting it appeared to induce tumor infiltration by T cells rather than NK cells. An additional clinical assessment is ongoing [309].

A similar approach targeting CD20 with an IL-2 ICK has shown efficacy in a severe combined immune deficiency mouse lymphoma model [142], and is being developed for combination with both RTX [310] as well as CD19-targeted chimeric antigen receptor-expressing cells [143]. Other IL-2 ICKs have been tested in the clinic in patients with prostate cancer. These have targeted the angiogenesis-associated alternatively-spliced extra domain B of fibronectin [144,145], and the tumor-associated A1 domain of tenascin-C [146] and EpCAM [147]. Meanwhile, ICKs utilizing other cytokines, such as IFN- α , are being developed preclinically [148], as well as conjugates of mAbs with other immune stimulants such as CpG ODN [149].

Targeting effector cell activation: agonistic antibodies

Innate immune effectors such as NK cells and macrophages increase expression of activation markers, such as CD69, following FcR triggering during ADCC. As the process is dependent on cell contact, the activation occurs preferentially at the site of the tumor, which is a potential therapeutic advantage of a combination strategy targeting an innate activation marker. Expression of the activation marker CD137, also known as 4-1BB, a member of the TNF receptor superfamily (TNFRSF), is increased following FcR triggering [150]. Unlike other activation markers, minimal expression of CD137 is observed on the surface of NK cells that have not been stimulated by antibody-coated tumor cells. Agonistic antibodies targeting CD137, in preclinical models, both *in vitro* and *in vivo*, augment NK cell function, including degranulation, secretion of IFN- γ and antitumor cytotoxicity [41,151,152]. The combination anti-CD137 antibody approach in addition to RTX and TRAST is supported by innate immune response-dependent murine models [42,151–153]. The selectivity of this approach to activated NK cells is clinically promising and being rapidly translated in a Phase I trial evaluating the combination of anti-CD137 mAbs with RTX in patients with lymphoma [311]. Taking a slightly different approach, Woo *et al.* have constructed a recombinant human 4-1BB ligand fusion protein which naturally dimerizes and activates human T cells *in vitro* [154]. As this molecule enters Phase I trials, it will be interesting to see whether it has a different activity and/or adverse effects profile compared with the mAb.

Other activating molecules expressed by NK cells may become promising candidates for enhancing ADCC. OX40 (CD134) is another member of the TNFRSF, with some homology and chromosomal proximity to CD137. It is best known for its upregulation on activated T cells but has also been shown to be expressed (as has its ligand OX40L) on NK cells [155]. An early-phase trial of an anti-OX40 mAb was recently initiated for patients with prostate cancer [312]. Upon activation, NK cells upregulate ICOS (CD278), a member of the CD28 superfamily, which is then involved in induction of cytotoxicity and IFN- γ production, and ICOS-mediated stimulation allows activated NK cells to more efficiently lyse tumor cells [156]. Whether these observations will lead to an ADCC-enhancing role for ICOS stimulation remains to be seen.

Blocking effector cell inhibition: antagonistic antibodies

Expression of HLA molecules on normal tissue prevents NK cell-mediated cytotoxicity through interaction with the KIRs. In the setting of virus-infected or tumor-transformed cells

that have lost HLA expression, they become susceptible to NK cell spontaneous cytotoxicity [157]. However, by contrast to spontaneous cytotoxicity, ADCC results from the interaction of the activating FcR on the NK cell with the Fc portion of an antibody bound to a target cell. Following stimulation via the FcR, the NK cell becomes activated and lyses target cells through release of cytotoxic granules. Both preclinical and clinical evidence supports this role of NK cell function. A competent Fc receptor on the NK cell is required for the antitumor activity of mAbs such as RTX and TRAST [2,158]. The degree of NK cell infiltration into the tumor site as well as *ex vivo* function correlate with the clinical response to mAb therapy in patients with colorectal and breast cancer [159,160]. Despite the promising upfront efficacy of mAbs, patients heavily pretreated develop resistance to nearly all mAbs; for example, the response rate to TRAST in the metastatic HER2-expressing breast cancer setting is less than 10%. Multiple mechanisms may contribute to mAb resistance, including increased expression of HLA class I molecules. This results in a masked tumor cell appearing as 'self' and inhibition of NK cell cytotoxicity through KIR interactions [161]. KIR engagement also inhibits FcR signaling through inhibition of ZAP-70, Syk, phosphorylation of both the ζ - and γ -subunits and PLC- γ [162,163].

Killer immunoglobulin-like receptors

Given the inhibitory effects of HLA molecule interaction with KIRs, blocking antibodies may prevent resistance to mAb therapy and tumor escape. *In vitro*, anti-KIR mAbs that prevent HLA ligation to KIRs lead to increased degranulation, secretion of IFN- γ and spontaneous lysis of tumor cells [164]. When combined *in vivo*, treatment with RTX plus anti-KIR mAbs augmented NK cell degranulation and IFN- γ secretion [165]. This effect was specific to blockage of the KIR2DL receptor and has since been validated in combination with RTX plus a different anti-KIR2DL mAb, resulting in enhanced cytotoxicity against a RTX-coated lymphoma cell line [166]. The ability of KIR inhibition to enhance cytotoxicity in an autologous system was recently demonstrated using effector NK cells and EBV-transformed B cells as target cells from the same donor. Interestingly, although blockade of KIR2DL enhanced NK cell cytotoxicity, significantly less functional enhancement was observed by blocking KIR3DL. This may suggest that that KIR3DL provides a less potent inhibitory signal, and that patient-specific variance in the impact of KIR inhibition needs to be considered [167]. Early clinical studies have focused on anti-KIR mAb monotherapy in multiple myeloma, although preclinical data has already shown that this same approach can be enhanced by the myeloma-specific targeting anti-CD38 mAb daratumumab [168].

GITR

GITR is, like CD137 and ICOS, another member of the TNFRSF expressed in various cell types, but apparently with differential functions between different cell subsets (as well as between mice and humans). GITR is constitutively expressed on NK cells and is upregulated following activation. Its inhibitory role is evident in that high levels of expression of its ligand (GITRL), observed on multiple tumor types, impairs RTX-induced NK cell ADCC and IFN- γ production, both of which are restored by GITR-blocking antibodies [169,170]. An anti-GITR mAb in development (TRX518) was shown to block the interaction between GITR and its ligand, and to enhance the cytotoxicity of NK cells [171]. An early-phase monotherapy trial was initiated in 2010 [313].

CD47

Similar to KIR inhibition of NK cell function, SIRP α engagement on the surface of macrophages provides a negative signal preventing phagocytosis and macrophage-mediated cytotoxicity following mAb therapy [172]. Recent *in vitro* studies demonstrated the dependence of mAb efficacy on macrophage phagocytosis of human RTX-opsonized

lymphoma cells [173]. Murine studies have shown that macrophages play as important a role as NK cells in the therapeutic effect of anti-CD20 mAbs [174]. Clinical series have also shown that polymorphisms of Fc γ RIIa (CD32), expressed predominantly on macrophages, can predict the outcome after mAb therapy, including RTX [7], TRAST [175] and cetuximab therapy [10]. SIRP α on the macrophage binds to CD47, a transmembrane protein expressed on tumor cells, preventing their phagocytosis [176]. Although multiple tumor types express CD47, expression on tumor cells normally sensitive to ADCC and phagocytosis results in decreased efficacy of the mAbs such as RTX [177]. Blockade of CD47–SIRP α interaction *in vitro* results in enhanced phagocytosis and increased antitumor efficacy of RTX in mouse xenotransplant models. In a clinical correlative study, *ex vivo* RTX-mediated ADCC was impaired in CD47^{hi} versus CD47^{lo} patients. Anti-CD47 blocking mAb restored its function [178]. Anti-CD47 mAbs are in preclinical development with a Phase I trial anticipated.

PD-1

PD-1 is a negative regulatory member of the CD28 superfamily expressed on the surface of activated T cells, B cells, NK cells and macrophages, similar to but more broadly regulatory than CTLA-4. Its two known ligands, PD-L1 and PD-L2, are both expressed on a variety of tumor cell lines. The PD-1–PD-L1 axis modulates the NK cell versus multiple myeloma effect, as seen by its blockade enhancing NK cell function against autologous primary myeloma cells, seemingly through effects on NK cell trafficking, immune complex formation with myeloma cells and cytotoxicity specifically toward PD-L1⁽⁺⁾ tumor cells [179]. Two anti-PD-1 mAbs (BMS-936558 and CT-011) are currently in clinical trials, the latter in a combination study with RTX for patients with low-grade follicular lymphoma [314].

Conclusion

The recent approval of an anti-CTLA4 mAb has demonstrated that modulating the immune response can improve patient survival [180,181]. As the immune response is a major determinant of mAb efficacy, the opportunity now exists to combine mAb therapy with IMiDs to enhance their antitumor efficacy. Remarkable advances in the basic science of cellular immunology have increased our understanding of the effector mechanisms of mAb antitumor efficacy. Whereas the earliest iterations of such combinations, for example IL-2 and GM-CSF, may have augmented both effector and suppressive cells, newer approaches such as IL-15 and TLR agonists may more efficiently activate effector cells while minimizing the influence of suppressive cells. Despite these encouraging rationale and preliminary data, clinical evidence is still required to demonstrate whether combination therapies will increase the antitumor effects of mAb.

Still, this approach is unique in combining a tumor-targeting therapy, the mAb, with an immune-enhancing therapy. If successful, these therapies may be combined with multiple mAbs in routine practice, as well as novel mAbs yet to be developed. Various approaches including augmenting antigen expression, stimulating the innate response and blocking inhibitory signals are being explored to determine the optimal synergy with mAb therapies. Therapies targeting NK cells, $\gamma\delta$ T cells, macrophages and DCs may ultimately be used in combination to further augment ADCC. Encouraging preclinical studies have led to a number of promising therapeutics, and the results of proof-of-concept clinical trials are eagerly awaited.

Future perspective

Over the next decade it is certain that some combinations of mAbs with IMiDs will become an important weapon in the arsenal of anticancer therapies, with RTX and lenalidomide being one clear example. Ongoing preclinical investigations of the potential synergy of such combinations, and the underlying mechanisms thereof, will allow for rational design of early-phase clinical trials with rigorous measurement of meaningful biomarkers (e.g., increase in NK cell subsets, phenotypic activation state or cytolytic function). Those combinations that demonstrate proof of principle will be brought forward to later-phase trials assessing clinical end points that can confirm true synergy; examples of this could include patients who are refractory to either mAb or the IMiD monotherapy. The greater the effort towards higher-quality biomarker assessments and studying such ‘high-bar’ clinical settings, the more easily combination therapies will be proven when they are brought, ultimately, to randomized, controlled trials.

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■ of interest

■ ■ of considerable interest

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310. Fusion Protein Cytokine Therapy After Rituximab in Treating Patients With B-Cell Non-Hodgkin Lymphoma. <http://clinicaltrials.gov/ct2/show/NCT00720135>
311. A Study Of PF-05082566 As A Single Agent And In Combination With Rituximab. <http://clinicaltrials.gov/ct2/show/NCT01307267>
312. Monoclonal Antibody to OX40, Cyclophosphamide (CTX) and Radiation in Patients With Progressive Metastatic Prostate Cancer. <http://clinicaltrials.gov/ct2/show/NCT01303705>
313. Trial of TRX518 (Anti-GITR mAb) in Unresectable Stage III or Stage IV Malignant Melanoma (TRXMel-1). <http://clinicaltrials.gov/ct2/show/NCT01239134>
314. Monoclonal Antibody CT-011 in Combination With Rituximab in Patients With Relapsed Follicular Lymphoma. <http://clinicaltrials.gov/ct2/show/NCT00904722>

Executive summary

ADCC

- There are several potentially important mechanisms of antibody-mediated tumor killing, including ADCC, cell signaling agonism/antagonism, complement activation and ligand sequestration.
- ADCC appears to be a primary antitumor mechanism for the majority of clinically approved monoclonal antibodies (mAbs), including rituximab, trastuzumab and cetuximab.
- ADCC depends upon the binding of mAbs to Fc receptors on effector cells, which include NK cells, $\gamma\delta$ T cells, macrophages and dendritic cells.

Increasing the number of mAbs bound to effector cell receptors

- Antigen target density can be increased by tumoral exposure to cytokines, cytotoxins, hypomethylating agents and Toll-like receptor agonists, such as CpG oligodeoxynucleotides.
- Effector cells (e.g., NK or $\gamma\delta$ T cells) can be increased either by *ex vivo* expansion with cytokines and activating mAbs or by *in vivo* expansion with cytokines or other pharmacologies including bisphosphonates.

Stimulation of the effector cell

- Small molecules including Toll-like receptor agonists, immunomodulatory drugs and inducers of NKG2D ligands can induce activation and proliferation of effector cells, including NK cells.
- Cytokines such as GM-CSF, G-CSF, IFN- α , IL-2, IL-12, IL-15 or IL-21 can induce activation of effector cells including macrophages, NK cells and dendritic cells.
- Immunocytokines are a novel class of therapeutics that target cytokines such as IL-2 or IFN- α to the tumor-infiltrating leukocytes by conjugating them to an antitumor mAb, limiting cytokine-mediated effector cell stimulation to the tumor site.
- Agonistic antibodies to surface molecules such as the costimulators CD137, OX40 and ICOS can be used to activate effector cells such as NK cells. Some of these costimulators are upregulated upon FcR ligation such that the agonistic mAb specifically activate those effector cells exposed to the tumor-mAb target.

Blocking effector cell inhibition

- Antagonistic antibodies can be used to block effector cell interactions that would otherwise inhibit ADCC or phagocytosis, such as: ligation of killer immunoglobulin-like receptor, GITR or PD-1 on NK cells to their respective ligands, which can be expressed on tumor cells or tumor-infiltrating leukocytes, and ligation of SIRP α on macrophages to the 'don't eat me signal' CD47 molecule on tumor cells.

Figure 1. Enhancing ADCC

Figure 1: Enhancing ADCC
FcR: Fc receptor; HDACi: Histone deacetylase inhibitor; IMiD: Immunomodulator; KIR: Killer immunoglobulin-like receptor;
MΦ: Macrophage; mAb: Monoclonal antibody; pDC: Plasmacytoid dendritic cell; TLR: Toll-like receptor.

Table 1

Clinical studies combining cytokine and monoclonal antibody therapy.

Cytokine	mAb	Patients (n)	Responses (%)	Ref.
IFN- α 2b	RTX	64	CR: 33; PR: 37	[182]
		38	CR: 11; PR: 34	[183]
IFN- α 2b	RTX	36 (–) IFN- α vs 33 (+) IFN- α	CR $\uparrow$: 20 vs 21 [†]	[184]
PEG-IFN- α 2b	RTX	9	CR: 11; PR: 22	[111]
GM-CSF	RTX	39	CR: 45; PR: 25	[185]
GM-CSF	RTX (+) chemotherapy	39	CR: 64; PR: 23	[186]
		38	CR: 53	[187]
		20	CR: 60; PR: 15	[188]
		15	CR: 80; PR: 20	[189]
GM-CSF	TRAST	17	CR: 0; PR: 0	[190]
GM-CSF	17-1A (anti-EpCAM)	20	CR: 10; PR: 0	[191]
GM-CSF	ch17-1A (chimeric anti-EpCAM)	24	CR: 0; PR: 0	[192]
GM-CSF	3F8 (anti-GD2)	15	CR in BM: 80	[193]
GM-CSF + IFN- α 2b	17-1A (anti-EpCAM)	27	CR: 0; PR: 4	[194]
G-CSF	RTX	19	CR: 16; PR: 26	[195]
IL-2	RTX	54	CR: 2; PR: 7 [‡]	[196]
		34	CR: 18; PR: 21	[116]
		12	CR: 8; PR: 0	[197]
IL-2	TRAST	10	PR: 10	[198]
		45	CR: 4; PR: 4	[199]
		13	CR: 0; PR: 0	[200]
IL-2	14.2a (anti-GD2)	33	CR: 3; PR: 3	[201]
IL-2	ch14.18 (anti-GD2)	15	CR: 7; PR: 7	[202]
IL-2 + GM-CSF	ch14.18 (anti-GD2) (+) BMT/ISO	113 (–) mAb/cytokine vs 113 (+) mAb/cytokine	2-year EFS: 46 vs 66	[119]
IL-12	RTX	45	CR: 4; PR: 4	[199]
		13	CR: 0; PR: 0	[200]
		25 (–) IL-12 vs 30 (+) IL-12	CR: 20; PR: 32 vs CR: 13; PR: 23	[203]
IL-12	TRAST	13	CR: 0; PR: 0	[200]
		15	CR: 8	[204]
		25 (–) IL-12 vs 30 (+) IL-12	CR: 20; PR: 32 vs CR: 13; PR: 23	[203]
IL-12	TRAST (+) chemotherapy	21	CR: 5; PR: 19	[205]
IL-21	RTX	21	CR: 10; PR: 29	[138]

[†] Denotes increase in CR rate after induction RTX with continued RTX (–) or (+) IFN- α .

[‡] Study limited to previously RTX-refractory patients.

(+): Received the experimental agent; (–): Did not receive the experimental agent; BM: Bone marrow; BMT: Autologous bone marrow transplant; CR: Complete response; EFS: Event-free survival; ISO: Isotretinoin; mAb: Monoclonal antibody; PEG: Polyethylene glycol; PR: Partial response; RTX: Rituximab; TRAST: Trastuzumab.