Therapeutic potential of immunostimulatory monoclonal antibodies

Juliet C. GRAY*†, Peter W. M. JOHNSON* and Martin J. GLENNIE†
* Cancer Research UK Medical Oncology Unit, The Cancer Sciences Division, Southampton University School of Medicine, General Hospital, Southampton SO16 6YD, U.K., and † Tenovus Research Laboratory, The Cancer Sciences Division, Southampton University School of Medicine, General Hospital, Southampton, SO16 6YD, U.K.

ABSTRACT

The aim of cancer immunotherapy is to employ the specificity of the immune system to provide a more effective, less toxic, treatment compared with conventional therapies. Although many strategies have been used to try to generate effective anticancer immune responses, very few have reached mainstream clinical use. A new approach introduced over the last few years is to use immunostimulatory mAbs (monoclonal antibodies) to boost weak endogenous antitumour immune responses to levels which are therapeutic. Such agonistic or antagonistic mAbs bind to key receptors in the immune system acting to enhance antigen presentation, provide co-stimulation or to counteract immunoregulation. In animal models, this approach has been shown to promote powerful tumour-specific T-cell responses capable of clearing established tumour and leaving the animal with long-term immunity. In addition to this impressive therapy seen in tumour models, these same mAbs also have the potential to be therapeutically useful in autoimmune and infectious diseases. This review discusses the use of these mAbs as therapeutic agents, their advantages and disadvantages and the challenges that need to be overcome to use them clinically.

INTRODUCTION

Over a century ago, Paul Ehrlich predicted that antibodies could be used as ‘magic bullets’ to target and treat human disease. With the generation of the first mAbs (monoclonal antibodies) by Köhler and Milstein in 1975 [1], it was expected that their exquisite specificity and unlimited supply would ensure that therapeutic products would soon follow. Although clinical results were initially disappointing, in the last decade this expectation has begun to be realized with 18 mAbs now having FDA (Federal Drug Administration) approval for therapeutic use. Initial use of rodent mAbs in patients was limited by their immunogenicity and short plasma half-life. The engineering of almost human or fully human mAbs has largely overcome these obstacles and attention has now focused on selection of appropriate target antigens. In the field of cancer therapy, interest has concentrated on mAbs that target TAAs (tumour-associated antigens). It was initially envisaged that mAbs directed against TAAs...
would ‘opsonize’ cancer cells, recruiting host immune effector mechanisms and provoking eradication of the target cell. However, with a few notable exceptions, such as rituximab (anti-CD20) and trastuzumab (anti-HER/2neu), this has not proven to be the case and only a minority of such mAbs have reached clinical use [2].

The subject of this review is an exciting new group of mAbs which recognize key cell-surface receptors in the immune system in order to augment immune responses to cancer. Rather than targeting TAAs directly, such immunostimulatory mAbs potentiate T-cell responses by acting as surrogate ligands to co-stimulatory molecules, providing agonistic or counter-inhibitory signals [3]. The aim is to boost weak, ineffectual, endogenous antitumour immune responses to therapeutic levels. This potential has already been demonstrated in animal models with a number of these mAbs showing impressive activity [4–8]. Potent tumour-specific T-cell responses can be generated that are capable of eradicating established disease and providing long-term protection. Early clinical results suggest that this class of mAbs may also be used to generate antitumour immunity in patients [9–11]. As well as this clear promise as anticancer agents, immunostimulatory mAbs also show the potential to be therapeutic in infectious and autoimmune diseases. In this review we examine the benefits that this class of mAbs offer as therapeutic agents, and discuss the challenges and obstacles to using them clinically.

**REGULATION OF T-CELL RESPONSES**

T-cell activation is tightly controlled by a number of positive and negative signals which allow protective immunity against pathogens, but tolerance to self-antigens [12,13]. Antigenic signalling alone is generally insufficient to activate naïve T-cells. Co-stimulatory signals are also needed, functioning to enhance the proliferation, effector function and survival of T-cells in response to presented antigen. They are also important in ensuring generation of memory T-cell responses. Antigenic signalling in the absence of co-stimulation results in suboptimal activation and may lead to T-cell deletion or unresponsiveness (anergy). The first co-stimulatory molecule identified was the immunoglobulin family member CD28 [14]. Since then a number of accessory ligand-receptor pairs have been identified (Figure 1). T-cell activation can be regulated by changing the expression of either member of the receptor/ligand pair. Regulation can also be achieved by inhibitory molecules which block the normal interaction between the co-stimulatory molecule and its receptor. The need for co-stimulation is not an absolute requirement of the T-cell response but varies according to the strength of the TCR (T-cell receptor) signalling. Low-affinity TCR interaction in the context of limiting concentrations of antigen will need more co-stimulation to achieve effective T-cell responses. Different co-stimulatory molecules appear to be important at different time points in the T-cell response [15,16].
For example, CD28, which is expressed constitutively, seems to be important early in primary T-cell expansion, but becomes less significant later in the effector response and during secondary responses [17]. In contrast, evidence suggests that 4-1BB and OX40, which are not constitutively expressed, have an important role in maintaining the expanded T-cell population and in ensuring survival and responsiveness of the memory T-cell pool [16,18,19]. Most co-stimulatory molecules are either members of the Ig superfamily or members of the TNFR [TNF (tumour necrosis factor) receptor] family. Only a few of these co-stimulatory molecules [such as CD28, HVEM (herpes virus entry mediator) and CD27] are constitutively expressed on resting T-cells with the majority only appearing on the cell surface after TCR ligation.

CD28, an Ig-like molecule, is the most important co-stimulatory molecule involved in the activation of naive T-cells. Its constitutive expression suggests a role early in T-cell activation and this is supported by studies in CD28-deficient mice. These mice show reduced T-cell responses with impaired immunity to some viruses and intracellular pathogens [20]. Ligation of CD28 by B7.1 or B7.2 greatly enhances TCR-induced proliferation, differentiation and survival particularly at low levels of antigen [15]. Increased secretion of IL (interleukin)-2 and up-regulation of anti-apoptotic proteins is also observed [21]. In addition, signalling through CD28 induces T-cell expression of other co-stimulatory molecules such as ICOS (inducible co-stimulator) and OX40, suggesting a degree of hierarchy within the co-stimulatory molecule system. Signalling through CD28 is antagonized by CTLA-4 [CTL (cytotoxic T-lymphocyte) antigen-4], a structural homologue of CD28 which binds to the B7 molecules with much higher avidity than CD28. A number of other molecules have now been identified which have structural similarity to CD28 [15]. These include ICOS, PD-1 (programme death-1) and BTLA (B- and T-lymphocyte activator). ICOS is only expressed at low levels on resting T-cells, but is up-regulated on T-cell activation [22]. Signalling through ICOS promotes effector function such as production of IL-10 and provision of B-cell help. PD-1 and BTLA are inhibitory molecules and may be important in maintaining self-tolerance [15].

4-1BB (CD137), OX40 (CD134), CD27, CD30 and HVEM are all members of the TNFR family that have been found to have co-stimulatory activity for T-cells [23]. These are all type I transmembrane glycoproteins expressed on APCs (antigen-presenting cells). CD27 is expressed constitutively on naive T-cells, whereas 4-1BB, CD30 and OX40 are only expressed upon activation. HVEM is expressed on resting T-cells and down-regulated upon T-cell activation. In general these co-stimulatory molecules appear to be important in sustaining, rather than initiating, immune responses [16]. 4-1BB and HVEM are reported to be most important for the activation and survival of CD8+ T-cells [23,24]. OX40 and CD30 act predominantly on CD4+ T-cells and promote Th2 responses [25,26]. CD27 ligation co-stimulates both CD4+ and CD8+ T-cells, promoting survival of proliferating T-cells early in the primary response and increasing the size of the memory T-cell pool [27].

Nearly all nucleated cells express MHC class I molecules and are able to present antigen to primed CD8+ T-cells and be potential CTL targets. However, only professional APCs, such as DCs (dendritic cells), express the co-stimulatory molecules necessary to activate naive T-cells. Immature DCs express only low levels of co-stimulatory molecules and activation, or 'licensing', is needed prior to efficient presentation of antigen [28]. Activation of DCs may occur in response to a number of different stimuli, including signals derived from CD4+ ‘helper’ T-cells, inflammatory cytokines, pathogens and from endogenous host-derived molecules released by tissue damage. The profile of co-stimulatory molecules expressed by DCs reflects the nature of these activating signals and directs the T-cell response accordingly. Effective responses, or T-cell immunity, will only be generated if signals for maturation have been received by the DC. Antigen presentation to naive T-cells by immature DCs is likely to result in tolerance, rather than immunity, to the antigen [29]. This default state of induction of tolerance unless DCs are activated (e.g. by pathogens, tissue damage or inflammatory cytokines) protects against inappropriate, damaging, immune responses being generated against self-antigens or non-noxious environmental antigens.

Inhibitory signals that T-cells receive through molecules such as CTLA-4, PD-1 and BTLA also help to ensure that tolerance to self-antigens is maintained and that T-cell responses are self-limiting. This is complemented by the effects of Treg-cells (regulatory T-cells), a subpopulation of CD4+ T-cells typically expressing CD25, FoxP3 (forkhead box P3) and CTLA-4. These cells normally constitute 5–10% of peripheral CD4 cells and have been shown to down-regulate the activation and expansion of self-reactive lymphocytes. They are sensitive to extremely low levels of antigen and, once stimulated, can suppress both CD4+ and CD8+ cells. The mechanism of suppression is not fully understood, but often involves cytokines such as IL-10 and TGF-β (transforming growth factor-β) [30,31]. Depletion of Treg-cells, for instance by thymectomy in neonatal mice,
Proposed mechanisms by which immunostimulatory mAbs enhance T-cell activation

Immunomodulating mAbs may augment T-cell responses by (i) enhancing antigen presentation (anti-CD40 mAbs), (ii) providing direct co-stimulation to T-cells [e.g. anti-(4-1BB) and anti-OX40 mAbs], or (iii) counteracting immunoregulation by deleting T_{reg}-cells (e.g. anti-CD25 mAbs) or blocking the regulatory influence of CTLA-4 [e.g. anti-(CTLA-4) mAbs].

removes this form of immunoregulation and provokes autoimmunity [32].

**ENHANCING T-CELL RESPONSES WITH mABs**

It has become clear that T-cell responses to both self and exogenous antigens can be enhanced using mAbs (Figure 2). mAbs targeting co-stimulatory molecules expressed on T-cells (e.g. CD28, 4-1BB and OX40) may act agonistically, functioning as surrogate ligands and augmenting T-cell proliferation and survival. Alternatively, rather than targeting T-cells directly, agonistic mAbs recognizing molecules expressed on DCs (e.g. CD40) may be used to mature and activate DCs, increasing the expression of both co-stimulatory ligands and MHC molecules. In addition, blocking mAbs have been employed to counteract the inhibitory effects of CTLA-4 and T_{reg}-cells. Each of these approaches has been shown to not only augment antigen-specific T-cell responses, but also to provide therapeutic benefit in murine syngeneic tumour models.

**GENERATING ANTITUMOUR IMMUNITY WITH IMMUNOSTIMULATORY mABs**

Evidence of immune response to a tumour is found in both animal models and patients. The cell-mediated arm of the immune system appears particularly important with T-cells, NK (natural killer) cells and NK T-cells all having significant roles [33,34]. Numerous human tumour antigens have now been identified [35], and T-cells recognizing these antigens can often be detected in patients. In melanoma patients, for instance, tumour-antigen-specific CD8^{+} CTLs can be detected in the tumour, peripheral blood and draining lymph nodes [33]. Furthermore, TILs (tumour infiltrating lymphocytes) isolated from such patients can be demonstrated to lyse autologous or HLA-matched tumour cells in vitro [36]. TILs have also been demonstrated in patients with a broad spectrum of other malignancies and their presence has been found to correlate positively with patient survival [33,37,38]. However, in patients who present with cancer, the immune system has failed to eradicate or control tumour growth. Tumour antigens are often weak, differing little, if at all, from self-antigens, but even when potentially immunogenic antigens are expressed the host may still fail to generate immunity. Many of the mechanisms which are beneficial to the host in maintaining tolerance to self-antigens become barriers to generating effective tumour immunity (Figure 3). Tumour cells themselves make poor antigen presenters as they usually lack co-stimulatory and MHC class II molecules. Cross presentation has been demonstrated to occur [39–41], but in the non-inflammatory tumour environment DCs are often immature and may not express a full complement
Figure 3 Failure to generate effective tumour immunity

Levels of tumour antigens and MHC molecules expressed on tumour cells may be low and cross-presentation of antigens by professional APCs is often inefficient. In addition, in the non-inflammatory environment of a tumour, antigens may be presented by immature, 'unlicensed', APCs lacking co-stimulatory molecules. Finally, any immune response that is generated may be counteracted by Treg-cells and immunosuppressive cytokines secreted by tumour cells.

Over the years numerous immunotherapeutic strategies have been used to try and enhance endogenous immune responses to cancer in order to generate long-term effective antitumour immunity. Because of the identified importance of cell-mediated immunity in protection against tumours, attempts have focused on augmenting this arm of the immune response. Vaccination with identified tumour antigens (either as peptides, proteins, naked DNA or whole-tumour cells) has been widely employed and has shown that it is possible to achieve tumour-specific T-cell responses. However, responses have usually been too weak or short-lived to attain clinical success. Non-specific immunostimulation (e.g. with cytokines or BCG) has also been utilized and, in some cases, reached mainstream clinical use. For instance, treatment with IL-2 has been found to induce long-term remission in a small proportion of patients with metastatic renal cell carcinoma [48]. Immunomodulating mAbs offer an alternative to these approaches and have a number of possible advantages. By enhancing antigen presentation, providing co-stimulation or counteracting immunoregulation, immunomodulating mAbs may be able to overcome the endogenous barriers to developing tumour immunity (Figure 4). Attractive aspects of this approach are that the immune response generated should be directed against multiple epitopes, reducing the chances of tumour-escape variants, and that the targeted antigens need not be identified. In animal tumour models, antibodies against a number of target molecules, including CD40 [5], 4-1BB [4], OX40 [8], CTLA-4 [7] and CD25 [6], have been shown to provoke powerful tumour-specific CTL responses capable of eradicating established tumour and, in some instances, leaving the animal immune to re-challenge.

ENHANCING ANTIGEN PRESENTATION

Anti-CD40 mAbs

CD40 is a 48 kDa transmembrane glycoprotein belonging to the TNFR superfamily. It is expressed on the surface of B-cells, DCs, macrophages, epithelial cells, haematopoietic progenitor cells and activated T-cells [49]. Its ligand, CD154, is a 34–39 kDa protein expressed mainly
By enhancing antigen presentation, providing direct co-stimulation to CD4\(^+\) or CD8\(^+\) T-cells or counteracting inhibitory signals, these mAbs may be used to expand a population of tumour-specific CTL effector cells. Tumour antigens released when tumour cells are lysed by such effector cells may be taken up by APCs and enhance the immune response further.

CD40 has a central role in activating both humoral and cell-mediated immunity [51]. It is the main pathway through which CD4\(^+\) T-cells provide help to CTLs and B-cells. Engagement of CD40 on B-cells provokes clonal expansion, germinal centre formation, isotype switching and the generation of plasma cells [51]. It also enhances the ability of B-cells to present antigen. CD40 signalling on DCs brings about ‘licensing’ of the DC with enhanced antigen presentation, cytokine release and survival. Both B-cells and DCs up-regulate an array of co-stimulatory molecules in response to CD40 binding, which consequently enables effective activation of CD8\(^+\) T-cells. TLR agonists, such as LPS (lipopolysaccharide) and CpG DNA, dramatically enhance the effects of CD40 on DCs [52]. Agonistic anti-CD40 mAbs and soluble CD40L (CD40 ligand) have been shown to ‘license’ DCs in vitro and in vivo and bypass the need for CD4 help [53,54]. Administration of anti-CD40 mAb together with soluble non-immunogenic proteins such as ovalbumin greatly potentiates effective CTL priming and prevents the development of tolerance [55,56].

It has been postulated that one important reason why tumours fail to provoke effective CTL immunity is because of inadequate CD4 T-cell help [44]. By priming APCs directly, anti-CD40 mAbs may overcome this problem and avoid tolerogenic responses to tumour antigens. Impressive therapy has been demonstrated in a number of murine lymphoma and solid tumour models [5,57,58]. The immunity generated is mediated by CTLs and occurs even after depletion of CD4\(^+\) T-cells. A number of carcinomas and B-cell malignancies express CD40 and these seem to be most susceptible to therapy, possibly because anti-CD40 mAbs may have an additional direct antiproliferative or pro-apoptotic effect in these tumours [59,60].

The first clinical results of anti-CD40 mAb therapy were recently reported by Seattle Genetics [9,61]. Thirty-five patients, 12 with NHL (non-Hodgkin’s lymphoma) and 23 with multiple myeloma, were treated with a humanized anti-CD40 mAb in phase I dose-escalating studies [9,61]. Weekly doses of between 2 and 4 mg of antibody/kg of body weight were well tolerated with no serious toxicities reported. Encouragingly, two of the patients with NHL demonstrated partial responses, with one of these sustained for several months. In addition, a fall in the serum level of M-protein was seen in four myeloma patients, although criteria for objective responses were not met.

Phase I studies of rhuCD40L (recombinant human CD40L) also suggest that clinical antitumour responses may be generated by CD40 signalling [62]. Thirty-two patients with either NHL or solid tumours were treated with daily rhuCD40L in 5-day courses at doses of 0.05–0.15 mg of antibody·(kg of body weight)\(^{-1}\)·day\(^{-1}\). Two responses, one complete and one partial, were seen and 12 patients achieved some degree of stable disease. One patient with a laryngeal tumour, which had been resistant to multiple conventional and experimental therapies,
showed a partial response during 11 courses of treatment. After completing the rhuCD40L therapy, the patient was found to have a complete response and remained disease-free for a year following treatment. In general, mAbs have higher affinities and longer serum half-lives than soluble ligands and may thus prove more efficient as therapeutic agents.

**CO-STIMULATION TO T-CELLS**

**Anti-(4-1BB) mAbs**

4-1BB, or CD137, is another member of the TNFR superfamily. It is expressed on the surface of antigen-activated CD4⁺ and CD8⁺ T-cells and its most well-characterized function is as a co-stimulator of these cells. The natural ligand for 4-1BB, known as 4-1BB ligand, is constitutively expressed at low levels on resting B-cells, macrophages and DCs, and activation of these cell types increases its expression [63]. It has been clearly demonstrated that 4-1BB ligation augments the proliferation, survival, cytokine secretion and cytotoxic effector function of T-cells in response to TCR stimulation [64]. Although initial reports suggested that CD8⁺ T-cells responded preferentially to 4-1BB stimulation, more recently it has been shown that the proliferative effects are similar in CD4⁺ and CD8⁺ T-cells [65]. Agonistic anti-(4-1BB) mAbs lower by up to a 100-fold the amount of antigen needed to activate T-cells [24] and strongly promote the survival of T-cells, preventing activation-induced cell death [64]. Cytokine production by T-cells is also increased by 4-1BB co-stimulation, with CD4⁺ cells producing IL-2 and IL-4, and CD8⁺ cells producing primarily IFN-γ (interferon-γ) [66]. Agonistic anti-(4-1BB) mAbs markedly enhance CTL activity both in vitro and in vivo, primarily by increasing the number of T-cells with full effector function [67,68].

Important functions of 4-1BB in cells other than T-cells are emerging. Constitutive expression of 4-1BB has been found on DCs, NK cells, human monocytes, eosinophils, intestinal intraepithelial lymphocytes, mast cells and neutrophils [69]. Signalling through 4-1BB appears to have a stimulatory effect in most of these cells. Agonistic anti-(4-1BB) mAbs increase the proliferation and IFN-γ secretion of murine NK cells in response to IL-2. Importantly, this enhances the ability of NK cells to prime CD8⁺ CTL responses [70]. 4-1BB-stimulated DCs secrete increased amounts of IL-6 and IL-12, up-regulate B7-1 and B7-2 and show an enhanced ability to stimulate T-cell proliferation in vitro [71]. Cross-linking of 4-1BB on human monocytes with agonistic mAbs induces secretion of pro-inflammatory cytokines, including IL-8 and TNFα [69]. In contrast with these stimulatory functions, a number of inhibitory or regulatory effects are becoming evident. Agonistic anti-(4-1BB) mAbs inhibit T-cell-dependent humoral responses [72] and prevent the anti-apoptotic effects of GM-CSF (granulocyte/macrophage colony-stimulating factor) on neutrophils [69]. Additionally, 4-1BB signalling enhances the proliferation of CD25⁺ CD4⁺ regulatory T-cells both in vitro and in vivo [73], and 4-1BB-deficient CD4⁺ T-cells show exaggerated responses to some antigens [74]. Furthermore, agonistic anti-(4-1BB) mAbs are immunosuppressive and therapeutic in murine autoimmune disease models such as SLE (systemic lupus erythematosus), rheumatoid arthritis and Crohn’s disease [75–77].

The antitumour effects that could be achieved by 4-1BB signalling were first recognized by Melero et al. [4] in 1997. These workers showed that an agonistic mAb against 4-1BB provided effective therapy for mice with established Ag104A sarcoma and P815 mastocytoma. A tumour-specific CTL response was generated that provided effective therapy for even widely disseminated disease. Moreover, ‘cured’ mice were left immune to further challenge with the same tumour for up to 30 weeks. Depletion studies demonstrated that the antitumour effects of the antibody were CD4⁺⁺⁺, CD8⁺⁺ and NK-cell-dependent [78]. Splenocytes from treated mice showed enhanced in vitro proliferation (4-fold), IFN-γ secretion (30-fold) and tumour-specific CTL activity (65-fold) as compared with splenocytes from untreated mice bearing identical tumours. Since then anti-(4-1BB) mAbs have been used to generate therapeutic tumour immunity in a number of murine syngeneic tumour models, including some that are considered relatively poorly immunogenic [79–82]. The antigenic load and anatomical site of tumour also seem to be important in determining the immune response generated, and these become crucial when the tumour is of low immunogenicity. In one study of mice with B10.2 fibrosarcoma, 50% of animals were cured when treated with anti-(4-1BB) mAbs on days 3 and 6 after tumour inoculation. This figure was increased to 80% when treatment was delayed to days 13 and 16, presumably because the antigenic load is much greater [81,82]. The fact that effective CTL antitumour responses can be provoked from as early as day 3 after tumour inoculation to as late as day 24 suggests the continued functional expression of 4-1BB throughout this period, most probably as the result of continued antigenic stimulation by progressively growing tumours. Some tumours are resistant to anti-(4-1BB) mAb treatment and, in some instances, this may be due to immunological ignorance to the tumour antigens expressed. Wilcox et al. [83] showed that this may be overcome by simultaneous immunization with identified tumour peptides and anti-(4-1BB) mAb therapy.

The precise mechanism of the antitumour response generated by anti-(4-1BB) mAbs is unclear. Although it is clear that the CTLs provide the main effector response, there is also a variable dependence on CD4⁺ T-cells and NK cells [4,78,79,82]. CD4⁺ cells probably have a role in generating memory responses, and NK cells seem to promote the generation of antitumour CTLs, probably...
CTLA-4 is stored in intracellular vesicles until T-cell activation when it translocates to the cell surface. Expression counteracts co-stimulatory signals by competing with CD28 for binding to the B7-1/2 molecules and also by itself generating direct inhibitory signals.

There is little documented evidence of toxicity from anti-(4-1BB) mAb therapy in mice. Significantly, at least in animal models, it seems possible to induce tumour immunity without provoking autoimmunity. Several mAbs against human 4-1BB have been raised, but these have yet to enter clinical trials [84].

Anti-OX40 mAbs
OX40 is another member of the TNFR family of co-stimulatory molecules. It is expressed predominantly on CD4\(^+\) T-cells, but it has also been reported on CD8\(^+\) T-cells, B-cells, DCs and eosinophils [85]. OX40 is present on the surface of T-cells 12–24 h after antigenic stimulation, and persists for several days. Expression on memory T-cells is more rapid, occurring within 4 h of activation. OX40L (OX40 ligand) is expressed on activated APCs. Expression of OX40L on APCs can be induced by CD40 and CD28 signalling as well as by TLR ligands such as LPS. OX40- and OX-40L-deficient mice show impaired CD4 responses to viral infections. Reduced primary and memory CD4 responses to immunization with specific antigens are also seen. By contrast, agonistic anti-OX40 mAbs enhance antigen-specific primary and memory CD4 responses [86]. Transgenic CD4 cells lacking OX40 show normal initial proliferation and cytokine release in response to antigen, but reduced proliferation several days into the response. It seems that OX40 is particularly important for ensuring long-term survival of T-cells and this is achieved through up-regulation of the anti-apoptotic proteins Bcl-xL and Bcl-2 [87]. It seems therefore that CD28 signalling is important for the initial proliferation and cytokine secretion of naïve CD4\(^+\) T-cell and increases expression of OX40. OX40 signalling then ensures survival of the effector and memory population of T-cells.

Anti-OX40 mAbs and OX40L–Ig fusion proteins have both shown therapeutic benefit in murine tumour models [8]. Modest therapy has been seen for a number of syngeneic tumour models, including the poorly immunogenic B16-F10 melanoma. Anti-OX40 mAb therapy as a single agent seems less able to generate therapeutic tumour immunity than anti-(4-1BB) mAb alone [80]. In adoptive transfer models, anti-OX40 mAbs were synergistic with systemic IL-2 in expanding transferred T-cells and promoting tumour rejection [88]. Although there is as yet no evidence of a therapeutic effect of anti-OX40 mAbs in humans, expression of OX40 is found on tumour-infiltrating lymphocytes in patients with breast cancer, melanoma and head and neck cancer [89].

COUNTERACTING IMMUNOREGULATION

Anti-(CTLA-4) mAbs
CTLA-4 is an inhibitory receptor with structural homology with CD28. CTLA-4 is stored in intracellular vesicles and, unlike CD28, is only expressed on the surface of activated T-cells (Figure 5) [90]. CTLA-4 binds B7.1 and B7.2 with significantly higher affinity than CD28. Whereas CD28 can only interact with one dimer of B7 at a time, CTLA-4 can form a stable lattice. CTLA-4 inhibits T-cell proliferation and IL-2 secretion in response to antigenic stimulation. This is partly because CTLA-4 out-competes CD28 for binding to the B7 ligands. In addition, binding of ligand to CTLA-4 generates inhibitory signals which down-regulate T-cell function. The importance of CTLA-4 in maintaining self-tolerance and T-cell homeostasis is demonstrated in CTLA-4-deficient mice [91]. After a few weeks, these
mice develop massive polyclonal T-cell proliferation with multi-organ lymphocyte infiltration and autoimmunity. CTLA-4 is also expressed on CD25+ Treg- cells, but does not seem to be essential for their regulatory function [92].

CTLA-4 function can be inhibited by antagonistic anti-(CTLA-4) mAbs. The antitumour effect of these mAbs was first demonstrated in murine colon carcinoma and fibrosarcoma models [93]. CTL-dependent rejection of established tumours was demonstrated and long-term protection was achieved. Therapy has also been shown in other syngeneic tumour models, including prostatic carcinoma, lymphoma, renal carcinoma and colon carcinoma [94]. Non-immunogenic tumours, such as B16 melanoma, do not seem to be susceptible to therapy with anti-(CTLA-4) mAbs alone. However, therapy has been achieved in B16 melanoma when the anti-(CTLA-4) mAb is administered with GM-CSF-transfected tumour vaccine [95].

A human anti-(human CTLA-4) mAb (MDX.010; Medarex) has become the first immunomodulating mAb to enter clinical trials, with several phase I/II studies in patients with cancer now reported. In the largest series, 56 patients with metastatic melanoma received anti-(CTLA-4) mAb together with vaccination with two peptides from the melanoma antigen gp100 [10]. All patients received an initial infusion of 3 mg of anti-(CTLA-4) mAb/kg of body weight and subsequent doses of either 1 or 3 mg of anti-(CTLA-4) mAb/kg of body weight every 3 weeks. An overall response rate of 13% was reported, with two complete and five partial responses. Both the complete responses and three of the partial responses were sustained beyond 2 years. Significant toxicity was observed, with 25% of patients developing grade III/IV autoimmune-like manifestations, including colitis, dermatitis, uveitis, hepatitis and hypophysitis. However, in all cases, these side effects resolved, or were controlled, with corticosteroids and supportive medical care. Interestingly, although there was no correlation between the dose of anti-(CTLA-4) mAb given and either clinical response or toxicity, there was a significantly higher incidence of clinical response in those patients developing autoimmune (36% in patients with autoimmune toxicity compared with 5% in those with no signs of toxicity). This, and other similar early clinical trials of anti-(CTLA-4) mAbs [11,96], supports the idea that immunomodulating mAbs may be sufficiently potent to generate therapeutic antitumour immunity in patients, but that the price for this is likely to be a degree of autoimmunity.

**Anti-CD25 mAbs**

CD25 is a 55 kDa transmembrane protein which constitutes the high-affinity α subunit of the IL-2R (IL-2 receptor) [97]. The IL-2R itself is composed of three subunits (α, β and γ), all of which are required for high-affinity binding of IL-2. In the absence of the α subunit, the β and γ subunits form an intermediate-affinity receptor which retains signalling function and is expressed by resting T-cells and NK cells. The high-affinity trimeric receptor is expressed by activated T-cells and Treg-cells. It appears to confer opposing functions in these two different populations of cells. In activated T-cells, the high-affinity IL-2R allows the T-cells to proliferate and develop effector function in response to IL-2. In Treg-cells, the high-affinity IL-2R seems to be important in ensuring both thymic development of Treg-cells and also their peripheral expansion and survival [31]. The importance of this regulatory role is demonstrated in CD25-deficient mice which are phenotypically indistinguishable from IL-2-deficient mice [98]. In the first few weeks of life these mice behave immunologically relatively normally and can mount anti-viral T-cell responses. However, by a few weeks of age they develop deregulated polyclonal B- and T-cell expansion and die from multisystem autoimmune disease. This disease is prevented by adoptively transferring CD25+ CD4+ T-cells into the mice. This suggests that, although there might be some redundancy in the role of IL-2 in activation of T-cells, its role in immunoregulation is fundamental.

The first mAbs recognizing CD25 were developed almost 25 years ago [99]. These antibodies block binding of IL-2 to the receptor and may also cause depletion of CD25+ cells. In vitro activation, proliferation and cytotoxic effector function of activated human T-cells is inhibited. The initial in vivo effects demonstrated with these antibodies were immunosuppressive with therapeutic benefits shown in a number of murine models of transplant rejection and autoimmunity [100,101]. These benefits have since been demonstrated in patients [102]. There are now two anti-(human IL-2) mAbs in clinical use, basiliximab and daclizumab [103]. These mAbs have been shown to reduce the risk of acute rejection in renal transplant patients without major toxicity. Clinical benefit has also been seen in psoriasis, rheumatoid arthritis and uveitis [104].

In contrast with these immunosuppressive effects seen in humans, others have shown that anti-CD25 mAbs can actually be used to provoke autoimmune disease in mice [105]. In addition, anti-CD25 mAbs have also been shown to enhance murine CD8+ T-cell responses to influenza A and vaccinia viruses [106]. These effects suggest that in some instances the dominant effect of anti-CD25 mAbs is removal of immunosuppression. Antagonistic anti-CD25 mAbs do not seem to directly inhibit suppressor function of Treg-cells [107] and it may be that this effect is only seen if the antibody results in significant depletion of CD25+ cells. The effect of anti-CD25 mAbs may also depend on the level of immune activation of the host when the antibody is given. If there are significant numbers of activated T-cells, as in organ rejection, then inhibition or removal of this cell population may be the dominant...
The fact that depletion of CD25+ cells with a specific mAb could be used to remove self-tolerance and generate autoimmunity prompted investigation as to whether tolerance to tumour antigens could be similarly overcome. Shimizu et al. [108] demonstrated that mice treated with the depleting anti-CD25 mAb PC61 developed protective immunity when subsequently inoculated with RL61 melanoma or B16 melanoma. Depletion of 70–80% of CD25+ T-cells seemed to be sufficient to see a therapeutic effect. It was also shown that CD8- and NK-cell-mediated cytotoxicity could be generated in vitro by culturing tumour with splenocytes depleted of CD25+ T-cells. Therapy has been observed in a number of other syngeneic tumour models [6,109]. In most cases the anti-CD25 mAb is only effective if given prophylactically and does not induce regression of established tumours. In some cases inoculation with one tumour has generated cross-protection against histologically distinct tumour lines [109]. This is presumably because of shared tumour antigens on a number of murine transplanted tumours.

Interestingly, as a general rule, the mice successfully treated with anti-CD25 mAbs do not appear to develop autoimmunity. It would seem that, for syngeneic mouse tumours at least, the level of immunoregulation that needs to be removed to generate tumour immunity is less than needed to provoke autoimmunity.

OTHER POTENTIAL USES

Viral infection

Although research has focused on the anticancer potential of immunostimulatory mAbs, they may also prove clinically useful as agents that can enhance antiviral immune responses, either in a prophylactic or therapeutic setting. Potentially, co-stimulatory mAbs could be a valuable adjunct to vaccination strategies against viruses such as hepatitis C and HIV, against which it is difficult to generate protective immunity. In mice, agonistic anti-(4-1BB) mAbs enhance the cellular immune response to an adenovirus vaccine encoding hepatitis C antigens, affording protective immunity in mice receiving both vaccine and mAb [110]. Similarly, anti-CD40 mAbs improve response to immunization against viral infections in a number of animal models [111,112]. In a therapeutic setting, treatment of mice with agonistic anti-(4-1BB) mAbs not only increases the CD8+ T-cell response to the immunodominant influenza epitopes, but also broadens the anti-viral T-cell response, expanding T-cell populations recognizing subdominant epitopes [113]. In addition, an anti-CD40 mAb has been demonstrated to activate intrahepatic APCs and enhance viral clearance in a murine model of chronic hepatitis B infection [114]. Although these antiviral effects of immunostimulatory mAbs have as yet only been demonstrated in animal models, there is no reason to think that their effects in humans will be any less potent.

Autoimmune disease

Paradoxically, the same immunostimulatory mAbs that enhance immune responses to cancer and viruses may also prove to be therapeutic in some autoimmune diseases. In the last few years it has become clear that agonistic co-stimulatory mAbs, particularly anti-(4-1BB), can be immunosuppressive in several different autoimmune disease models, including SLE, Crohn’s disease, uveitis and rheumatoid arthritis. In many of these models agonistic anti-(4-1BB) mAbs not only prevents the onset of disease, but also reverses established pathology [76,77,115–117]. In contrast with most immunosuppressive therapies used to treat autoimmune diseases, the effects of the anti-(4-1BB) mAb appear to be long-term and do not seem to require continuous administration of mAb for the therapy to persist. The precise mechanism by which anti-(4-1BB) mAbs suppresses these autoimmune diseases is not clear and seems to vary in the different models studied. In SLE models, autoantibodies have an important pathogenic role and these may be suppressed by an anti-(4-1BB) mAb [76]. In a cell-mediated inflammatory bowel disease model, representative of Crohn’s disease, an anti-(4-1BB) mAb promotes activation-induced cell death in CD4+ T-cells and also activates CD25+ Treg- cells [77]. Interestingly, in this model, there is also an increase in the number of IFN-γ-secreting CD8+ T-cells. Treatment of mice with CIA (collagen-induced arthritis) with an anti-(4-1BB) mAb results in expansion of a population of CD11c+ CD8+ T-cells which inhibit antigen-specific CD4+ cells through an IFN-γ and indoleamine 2,3-dioxygenase-dependent mechanism [75]. It is not entirely understood why these immunosuppressive effects of anti-(4-1BB) mAbs are dominant in these autoimmune diseases, but not in syngeneic tumour models.

Other co-stimulatory mAbs have also been shown to be therapeutic in autoimmune disease models. The same anti-CD40 mAbs which enhance antitumour immune responses in lymphoma models have been demonstrated to ameliorate disease in CIA [118]. CIA is a Th1 cell-mediated model of arthritis and it seems that anti-CD40 mAbs induce secretion of IL-10 by B-cells which generates a less pathogenic Th2-type immune response [119]. Finally, agonistic anti-CD28 mAbs have been shown to prevent the onset of autoimmune insulitis in NOD (non-obese diabetic) mice [120]. This also seems to be mediated by swaying the pathogenic immune response away from a Th1-type towards a Th2-type response.

CLINICAL USE OF IMMUNOSTIMULATORY mABs

With the exception of anti-(CTLA-4) mAbs, clinical experience of immunostimulatory mAbs is very limited.
A number of obstacles must be overcome in order to translate the impressive therapies seen in animal models into effective clinical treatments. The first issue, the immunogenicity of rodent antibodies administered to humans, has been largely overcome. Chimaeric humanized or fully human mAbs can now be routinely generated which can be generally administered to patients without HAMA (human anti-mouse antibody) responses. The second problem is that the target molecules of most immunostimulatory mAbs are species-specific. Although it is relatively straightforward raising mAbs recognizing human targets, it is more problematic finding good pre-clinical models to evaluate their in vivo functional effects. The latter is important as binding affinity and in vitro effects do not necessarily correlate with agonistic in vivo effects. In the case of the anti-(CTLA-4) mAbs, hu-PBL SCID (human peripheral blood lymphocyte-engrafted severe combined immunodeficient) mice, transgenic human CTLA-4 knockin mice and primates have all been used as pre-clinical models [121–123]. All of these models have limitations and none predicted the degree of autoimmunity that was seen when anti-(CTLA-4) mAbs were used clinically. A third issue is whether this class of mAbs will be potent enough to generate therapeutic antitumour immune responses in patients. Spontaneous human tumours differ from syngeneic murine tumours in that they may have been present for months or years prior to diagnosis. This, and perhaps their lower level of intrinsic immunogenicity, may mean that the levels of tolerance that need to be overcome in order to generate effective antitumour immunity are substantially greater in patients. Potentially this could be overcome by using these mAbs in combination with other therapies such as peptide immunization. Indeed, using immunostimulatory mAbs to enhance such antigen-specific therapies may prove to be particularly useful and efficacious. A final concern is that the immunosuppressive effects that have now been recognized for a number of these mAbs have the potential to be detrimental and to counteract the development of tumour immunity. Since it is not fully understood why these effects predominate in some models but not others, it is impossible to be confident that they will not be functionally important in patients with cancer.

Since the preparation of the review, the drug trial of TGN 1412 at Northwick Park Hospital in London, U.K. has highlighted the use of immunostimulatory mAbs, and anti-CD28 mAbs are discussed briefly in the Supplementary text available at http://www.clinsci.org/cs/111/cs1110093add.htm.

CONCLUSIONS

Immunostimulatory mAbs have emerged over the last few years as a new group of agents which show the potential to be useful in the treatment of cancer and autoimmune and infectious diseases. They enhance immune responses by binding to key receptors in the immune system providing either agonistic or antagonistic signals. In animal models, their antitumour effects look particularly promising, with mAbs recognizing a number of different target molecules demonstrating impressive therapeutic effects. They can be used to generate powerful, tumour-specific, T-cell responses in many cases eradicating established disease and generating long-term protective tumour immunity. Importantly, these mAbs offer a means of establishing effective antitumour immune responses without identifying tumour antigens or tailoring therapies to individual HLA types, thus potentially providing convenient and ‘off the shelf’ therapies.

Clinical experience of this group of mAbs is, as yet, limited and there are undoubted obstacles to translating the promising effects seen in animal models into useful therapies for patients. However, several immunostimulatory mAbs have now entered clinical trials and early results suggest that they can be used to enhance antitumour responses in patients without promoting unacceptable autoimmunity. The therapeutic results seen so far in patients are extremely encouraging and, whether used alone or in combination with other immunotherapies, this novel group of agents offers a very real potential to provide an effective means of generating therapeutic immunity to cancer.

ACKNOWLEDGMENTS

We thank Cancer Research UK and Tenovus (Cardiff) for their generous financial support.

REFERENCES

104 J. C. Gray, P. W. M. Johnson and M. J. Glennie


64 Cannons, J. L., Lau, P., Ghumman, B. et al. (2001) 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy. J. Immunol. 167, 1313–1324
86 Rogers, P. R., Song, J., Gramaglia, I., Killeen, N. and Croft, M. (2001) OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity 15, 445–455