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Review

Potential of Therapeutic Immune Responses against Malignancies with Monoclonal Antibodies

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Abstract

Immunotherapeutic monoclonal antibodies (mAbs) can be defined as those that exert their functions by tampering with immune system cell molecules, causing an enhancement of antitumor immune responses. Some of these antibodies are agonistic ligands for surface receptors involved in the activation of lymphocytes and/or antigen-presenting cells, whereas others are antagonists of mechanisms that normally limit the intensity of immune reactions. Several mAbs of this category have been described to display *in vivo* antitumor activity in mouse models. Only anti-CTLA-4 (CD152) mAb has entered clinical trials, but the preclinical effects described for anti-CD40, anti-CD137 (4-1BB), anti-CD102 (intercellular adhesion molecule-2), and regulatory T cell-depleting mAbs should lead to their prompt clinical development. Their use in combination with immunizations against tumor antigens has been reported to be endowed with synergistic properties. This new group of antitumor agents holds promise for at least additive effects with conventional therapies of cancer and deserves intensive translational research.

Introduction: Therapy of Malignant Diseases with mAbs¹

Some mAbs are therapeutic agents with progressive impact in the treatment of a wide array of conditions. Since their

discovery in the late 1970s (1), it became clear that these antibodies of defined specificity, and that could be produced in high amounts, had potential for the management of various diseases, including malignancies (2–4). In fact, cancer in its different forms stood out as the most suitable target (5–7), given that specific recognition of tumor-specific surface proteins was thought to yield “magic bullets” that would selectively guide effective mechanisms of cellular destruction to the malignant cells.

The key property of antibodies to be used as therapeutic tools is their behavior as high-avidity ligands to virtually any protein or glycoprotein of the organism. Classically, antibodies have been made immunizing rodents with the desired antigen and fusing their activated splenic B lymphoblasts to myeloma cell lines, resulting in clones of immortalized hybrid cells that would continuously produce the antibody (1). Caveats in this approach are that if the antigen is identical or very homologous between the rodent species and humans, such “anti-self” antibodies are difficult to obtain. This is important because active sites in functional proteins are typically highly conserved. In addition, rodent immunoglobulins have sequences that are recognized as strong antigens by the human immune system, thus preventing repeated administration. Genetic engineering has offered solutions to both problems, first by generating random libraries for the antigen-binding site of the antibodies that could be selected *in vitro* without self-tolerance bias (8). In addition, molecular engineering has permitted extensive replacement with human sequences, preserving only the antigen-binding site of the mouse immunoglobulin. Thus, modified antibodies are called humanized antibodies (9). Only recently, mouse strains have been generated that have silenced endogenous immunoglobulin genetic regions while having functional transgenic human immunoglobulin loci (10). Amazingly, those mice, when immunized, produce entirely human antibodies, and, therefore, completely human mAbs can be made from these mice by conventional techniques (11). In general, pharmacokinetics of antibodies are considered very favorable because they are protease resistant, stable in plasma, and, thus, generally endowed with long half-lives.

Anticancer antibodies can be classified according to the mechanism or mechanisms of action (Fig. 1) in the following groups: (a) Malignant cell-destroying mAbs: the meaning is that these antibodies bind tumor cells either specifically or selectively, causing their death (12). The antibody biomolecule has intrinsic functions that can mediate destruction of the tumor cell (13), such as complement fixation, antibody-dependent cellular cytotoxicity, and phagocytosis. In addition, artificial toxins or

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¹ The abbreviations used are: mAb, monoclonal antibody; APC, antigen-presenting cell; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; Treg, regulatory T cell(s); TNF, tumor necrosis factor; NK, natural killer; IL, interleukin; ICAM, intercellular adhesion molecule; TGF- β , transforming growth factor β ; VEGF,

vascular endothelial growth factor. CTLA, cytotoxic T lymphocyte antigen; PD-1, programmed cell receptor-1, BTLA, B- and T-cell attenuator; DC-SIGN, dendritic cell-specific ICAM-3–grabbing nonintegrin.

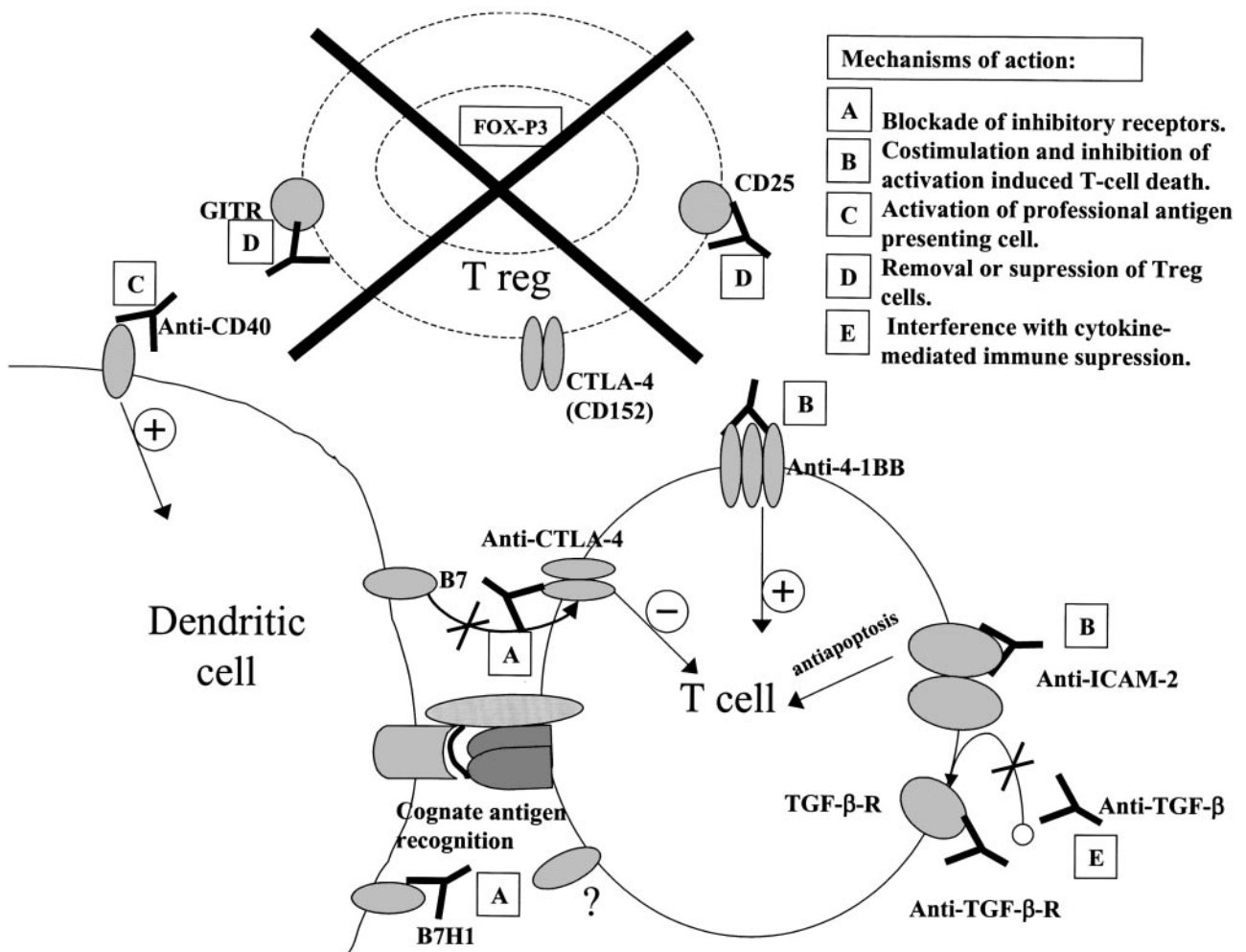


Fig. 1 Graphic representation of the different mechanisms of action postulated for immunotherapeutic mAbs. In several instances, a single mAb can operate through more than one general mechanism of action, as indicated in the legend on the right side of the graph.

radiative compounds can be coupled with the antibody to enhance cellular destruction (12, 14–16). (b) mAbs that interfere with signals transmitted from receptors for growth factors (17, 18): the rationale is that cancer cells sometimes need the constant signaling from mutated or nonmutated surface receptors both for survival and/or progression in the cell cycle (19). These antibodies block the signals required by tumor cells to survive and thrive (20). (c) Antibodies inhibiting angiogenesis: this subclass of mAbs targets vascular growth factors and their receptors (17, 21, 22) or surface proteins selectively expressed by proliferating endothelium (23, 24). Because malignancies rely on continuous angiogenesis and vasculogenesis to progress (19), these approaches have great potential. (d) mAbs that enhance the cellular immune response against cancer: these agents bind molecules on the surface of immune system cells. What they do is either provide activating signals to lymphocytes and APCs or block the action of receptors that normally down-regulate the immune response. Here, we focus on this last category (Fig. 1).

The Complex Set of Cellular and Molecular Mechanisms That Amplify, Inhibit, or Shape the Cellular Immune Response

The orchestration of an efficacious, cytotoxic immune response is the result of a complex interplay of many cell types that are controlled in their functions by signals received through surface receptors (25). Those receptors detect antigens, microbe-denoting patterns, or are involved in intercellular communication by cytokines or cell-to-cell contact (25).

Normally, an immunogenic antigen is picked up at peripheral inflamed tissues by DCs (26, 27). Those DCs are deployed and recruited to these tissues and are specialized in uptake, processing, and presenting foreign antigenic substances in MHC class I and II molecules, as well as in other antigen-presenting molecules such as CD1 (28). Besides, they sense the environment for cell damage (29), microbe patterns (30), and proinflammatory cytokines. If those stimuli are detected by the corresponding receptors, DCs migrate avidly to lymphoid tissue and up-regulate soluble and membrane-attached glycoproteins

that are necessary to productively activate antigen-recognizing T cells.

Once in lymphoid tissue, a bidirectional dialogue between APCs and lymphocytes takes place with the participation of many receptor-ligand pairs in opposed cell surfaces, cytokines, and chemokine gradients.

As a result of early activation of resting (naive or memory) lymphocytes, those cells express surface proteins that were undetectable before, called lymphocyte activation antigens. Those surface molecules are thereafter ready to receive information by their corresponding ligand or ligands.

The overall result of such many interactions is a bursting clonal expansion of responding T cells acquiring effector functions that are capable of destroying and clearing antigen-bearing cells.

DCs also receive activation signals by T-helper cells mainly, but not only, through the CD40L/CD40 pair. The consequence of this phenomenon is induction and/or maintenance of the active (proimmune or mature) state of DCs (31).

Most activated T cells will eventually die through programmed cell death pathways that regulate the number of antigen-specific lymphocytes (32). Some surface receptors are involved in triggering this apoptotic event, whereas other receptors signal to prevent or delay such an outcome (33, 34).

During the close contact of T cell/DC membranes, various types of molecules play a role in the so-called immunological synapse (35–38): (a) antigen receptor and antigen-presenting molecules; (b) adhesion molecules that hold the cells together in a regulated fashion; (c) chemokines that attract the leukocyte populations to the rendezvous tissue locations; (d) costimulatory receptors that promote division and acquisition of effector functions, supplying complementary signals to those arising from antigen receptors; (e) inhibitory receptors that down-regulate the size and potency of immune response; and (f) soluble activating or inhibiting cytokines.

Besides, there are T cells that are known to be specialized in regulating the extent of cytolytic responses, either by promoting it or by inhibiting it. T-helper cells are those that provide cytokines and activation of APCs for optimal CTL generation (39–41), whereas Treg are specialized in quenching early immune responses or in maintaining peripheral tolerance to autoantigens (42–45).

The principle of immunotherapeutic mAbs is that all those types of receptors can be potentially blocked or activated *in vivo* by mAbs and that specific subsets of Treg can be eliminated by specifically depleting antibodies (Fig. 1).

Anti-CTLA-4 (CD152) Antibodies

A humanized antibody against CTLA-4 was the first to reach clinical trials (46, 47). CTLA-4 is a surface protein that belongs to the CD28 family of receptors (48, 49). In fact, CTLA-4 shares its ligands with that molecule (50). However, in contrast to the costimulatory activity of CD28 (51), CTLA-4 ignites signals that inhibit cell cycle progression and cytokine secretion (52). CTLA-4 is expressed only by activated T cells and regulatory CD4⁺ CD25⁺ T cells (42, 53).

Knowledge of its key functional role as a brake system for T cells comes from observations in CTLA-4^{-/-} mice that

develop a lethal autoimmune syndrome with infiltration of multiple organs by activated lymphocytes (54, 55). The mechanism is the lack of control of CD28-mediated costimulation of T cells (56), and probably on dysregulation in the Treg cell activity, as suggested by the control of CTLA-4^{-/-} T cells on adoptive transfer into mice with CTLA-4^{+/+} T cells (57).

CTLA-4 is retained in intracellular compartments (58) and directed to the cell surface area in which a productive interaction with a DC is taking place (59). The cytoplasmic tail of CTLA-4 recruits Tyr and Ser/Thr phosphatases (60, 61) that attenuate signals through activating receptors.

CTLA-4 has binding activity both for CD80 (B7-1) and CD86 (B7-2). The affinity of its interactions toward those receptors is much higher than that displayed by CD28 and, therefore, would compete with advantage for the ligands (38).

Anti-CTLA-4 antibodies can block the function of the molecule *in vitro*, even as Fab monovalent fragments (62, 63). *In vivo*, systemic treatment of mice with transplantable immunogenic colon carcinoma cells with anti-CTLA-4 mAb caused complete tumor regression of established tumors through an immune response found to be critically dependent on the activity of CTLs (64). The antitumor effect was also remarkable against spontaneous prostate carcinomas arising in mice transgenic for the SV40-T antigen under a tissue-restricted promoter (65). The effects of anti-CTLA-4 mAb were greatly potentiated by combining it with treatments based on vaccination with autologous tumor cells transfected to express GM-CSF (66, 67). GM-CSF transfection was previously known to favor immunization against tumor antigens by means of local recruitment and differentiation of bone marrow-derived APCs such as DCs (68–70). Other studies have confirmed the synergy between vaccination and anti-CTLA-4 mAb treatment (71).

A humanized mAb (MDX.010.Medarex) has entered clinical trials as a single agent in patients suffering from metastatic melanoma and ovarian cancer who are refractory to conventional treatments (46). Some of those patients had previously undergone vaccination with allogeneic melanoma cell lines secreting GM-CSF. CTLA-4 mAb induced tumor infiltration by mononuclear and polymorphonuclear leukocytes that, in some cases, led to a clinical response with a late onset after the single administration of the antibody. Indeed, doses in this study featured only a single dose of 3 mg/kg. In one case, the patient developed acute inflammation and swelling of a previously unnoticed central nervous system melanoma metastasis, which caused serious neurological complications and, ultimately, the death of the patient.

Other clinical trials are ongoing, and it has been recently reported that there is therapeutic synergy between vaccination with a peptidic antigen expressed by melanomas (gp100) and repeated, weekly doses of anti-CTLA-4 mAb (47). In these patients, events of severe autoimmunity beyond vitiligo (such as inflammatory bowel disease, hypophysitis, and eczema) have been observed, but they have been reversible in every case. Those trials stress the following points: (a) the effect of many among these novel, immunity-promoting mAbs can be potentiated by specific immunizations; and (b) autoimmunity is a real risk and possibly a price to pay. Interestingly, the two long-lasting complete responders from a total of 14 patients endured the most severe autoimmune adverse events: one case with

hypophysitis causing panhypopituitarism and the other with severe dermatitis (47).

Other molecules may exert a similar function. One potential target is the PD-1 molecule also expressed by activated T cells (72). PD-1^{-/-} mice also develop autoimmunity, but less intense than CTLA-4^{-/-} mice (72, 73). PD-1 also binds B7 family members B7-H1 (PDL1) and B7-DC (PDL2), although the exact function of these molecules is unclear. The suspected existence of yet unidentified alternative ligands defies the description of a more a clear-cut picture. To the best of our knowledge, PD-1-blocking mAbs have not been tried in mouse tumor models yet. B7-H1 is expressed by many mouse and human tumor cell types and is known to induce T-cell apoptosis (74). Indeed, the blocking of B7-H1 with mAbs has shown antitumor effects in those cases (74). In addition, B7-H1 expression is induced on intratumor DCs, as described in DCs found in malignant ascites, suggesting that this molecule greatly hampers DC T-cell-stimulating properties (75). Anti-B7-H1-blocking mAbs are definitive candidates for clinical impact in the future (76).

Only very recently, BTLA has been described as a relative of CTLA-4 that is expressed by activated T and B cells (77). It apparently binds the previously orphan B7 member B7x (B7-H4) and transmits inhibitory signals (78, 79) by Src homology domain-2 containing tyrosine phosphatase recruitment to its phosphorylated cytoplasmic tail (77). Interference of BTLA function in tumor immunotherapy is an obvious next step. BTLA^{-/-} mice do not develop spontaneous autoimmunity but are more prone to develop severe experimental allergic encephalitis than control littermates (77). Multiple, simultaneous, or sequential blockade of all those inhibitory pathways involving B7 family members is a direction worth taking to enhance therapeutic effects in the future.

Anti-4-1BB (CD137)

4-1BB (CD137) is a surface glycoprotein that belongs to the TNF receptor family. It is expressed by activated, but not resting, T and NK cells (80, 81). Recently, its expression on DCs has been reported also (82). There is only one known ligand for this molecule, named 4-1BBL, that belongs to the TNF family and is expressed by APCs such as macrophages, activated B cells, and activated DCs (83).

Ligation of 4-1BB *in vitro* by antibodies or natural ligands provides costimulation for lymphocyte proliferation and cytokine secretion (84–86). Costimulation by anti-4-1BB mAb is largely independent of that provided by CD28 (87), although CD28 ligation promotes 4-1BB surface expression (88). 4-1BB^{-/-} and 4-1BBL^{-/-} mice have mild immune defects in generation of CTLs against T-helper-dependent antigens (89, 90). These observations indicate the existence of other redundant pathways that possibly take over the function in the knock-out mice.

4-1BB activates several intracellular signaling pathways that include: TRAF-2, nuclear factor κ B, mitogen-activated protein kinases, BCL-xL, Bfl-1, and others (87, 91, 92). However, the exact signal or subset of signals responsible for its unique costimulatory properties remains elusive.

Treatment of mice bearing established transplanted tumors

with anti-4-1BB mAbs causes tumor regression even in tumor models considered to be poorly immunogenic (93). The effect depends on the activation of CTLs and is dependent on CD4⁺ T-helper cells in a tumor model-dependent fashion (93, 94). NK cells expressing 4-1BB are also important to trigger the therapeutic effects by means of their cytokine secretion activity rather than through direct cytotoxicity (81, 95). Normal function of IFN γ is absolutely required for the therapeutic effect, mainly to mediate the homing of effector T cells to tumor tissue (96). Antibodies against 4-1BB also greatly potentiate the effect of adoptive therapy with preactivated CD8⁺ T cells specific for tumor antigens (97–99). Some of the effects are clearly dependent on the ability of anti-4-1BB mAb to prevent programmed cell death in lymphocytes, thus extending their operative life (98, 100).

Transfection of tumors with the cDNA encoding for 4-1BBL also augments their immunogenicity (88, 101). However, such a treatment is less potent than the one mediated by artificial ligation of 4-1BB with mAbs (88, 102). This observation can be attributable to either different binding avidities or to the systemic, rather than local, distribution of the mAb. The issue of avidity is further supported by results showing that tumor cell transfection with a membrane-attached scFv form of an anti-4-1BB mAb results in a very high tumor immunogenicity (103). In these experiments, a crucial role for NK cells in the antitumor effect was also found.

Treatment with anti-4-1BB mAb synergizes with transfection of tumors to express IL-12. In particular, such a combined treatment eradicated advanced experimental colon cancer metastasis in mice (104, 105). Moreover, the combination of anti-4-1BB mAb with vaccination using chemically defined peptides encoding for tumor antigens was very efficacious in a synergistic fashion (106). It seems that anti-4-1BB mAbs can up-regulate a formerly weak, but present, immune response. However, they fail to initiate any immune response from zero.

In case there is a complete ignorance by the immune system of the relevant tumor antigens, vaccinations are probably required. Among other reasons, antigen stimulation is necessary to induce 4-1BB expression on specific T cells. Once antigen ignorance is broken, anti-4-1BB mAb can greatly promote antitumor immunity (106). Recently, we have found that potent synergistic effects are elicited by anti-4-1BB mAb combined with intratumoral injections of cultured DCs engineered to produce IL-12.² Such DCs cross-prime tumor antigens and induce an immune response that can be amplified by anti-4-1BB mAb.

Antibodies against another member of the TNF receptor family, named OX-40 (CD134), have antitumor effect, although much less intense. This fact has been attributed to the preferential costimulation of CD4⁺ lymphocytes by anti-OX-40, a situation that mirrors the preferential costimulation of CD8⁺ T cells by anti-4-1BB mAb (107).

In addition, treatment with anti-4-1BB mAb has inhibitory activity on some pathogenic functions mediated by T-helper

² I. Tirapu, Improving efficacy of interleukin-12-transfected dendritic cells injected into murine colon cancer with anti-CD137 monoclonal antibodies and alloantigens, submitted for publication.

cells by yet undefined mechanisms (108). These T-helper-inhibitory effects of anti-4-1BB mAb clearly ameliorate experimental autoimmunity conditions, such as mouse models of lupus (109, 110) and experimental allergic encephalitis models (111). The precise role of the *in vivo* effects of anti-4-1BB mAb on CD4⁺ T cells in tumor immunotherapy remains to be elucidated and might involve Treg cells. Costimulation via 4-1BB has also been implicated in shaping the T-helper response toward a Th1 pattern of cytokine secretion (112).

A humanized anti-4-1BB mAb has been tested in nonhuman primates without toxicity and with interesting observations of inhibition of T cell-dependent antibody responses in these animals (113). Clinical development and eventual trials with mAbs of this specificity are planned in various institutions, including ours.

Anti-CD40

CD40 is a TNF receptor family member that plays a crucial role in shaping both the cellular and the humoral immune response (114). It is chiefly expressed on B cells, DCs, and macrophages. Its specific ligand (CD40L) is expressed by activated T-helper cells in a highly restricted fashion (114). CD40L genetic deficiency in mice and humans causes both humoral and cellular immunodeficiency attributable to both failure of immunoglobulin class switch in B cells and lack of DC activation to prime T-cell responses (115). Therefore, CD40 is a key molecule in the instructive activity of T-helper cells (116). Recently, it has been found that CD40 is also expressed by CD8⁺ T cells and plays a key role in the activation of memory but not naive CTL precursors (117).

Treatment of mice bearing B-cell malignancies with an antibody against CD40 leads to complete cure in many cases (118). The mechanism of action seems to be double. On one hand, the antibody stimulates an antitumor CTL-mediated immune response. In contrast, direct binding of CD40 on the surface of lymphoma and myeloma cells seems to kill tumor cells and might also enhance the antigen-presenting functions of these neoplastic B cells (118, 119). It should be also mentioned that tumor cell transfection with CD40L also increases immunogenicity and exerts therapeutic activity (120–122).

The property of expressing surface CD40 is not limited to hematological malignancies but extends to many other types of tumors such as carcinomas (123). This explains the reason for strategies based on targeting CD40 with immunotoxins (mAb-toxin conjugates) that are currently being explored with some encouraging results (124).

Nonetheless, such an antitumor activity can be found even in tumors in which CD40 expression is completely undetectable (125). Even more, the best therapeutic effects are achieved if anti-CD40 antibodies are combined with vaccinations with antigenic peptides shared by tumor cells (125). The mechanism involves the general maturation of endogenous DCs that evokes productive proliferation and effector differentiation of T cells. In this study, an important warning call has been made, because mice treated *i.v.* with anti-CD40 antibodies may experience a shock syndrome that resembles endotoxin-caused shock (125). Importantly, though, the same antibody doses given *s.c.*

malignant nodules achieves identical therapeutic activity without the lethal adverse events (125).

Clinical grade, antihuman CD40 is about entering Phase I clinical trials for different types of lymphoma in Southampton, United Kingdom. The synergistic effects of anti-CD40 mAb with radiotherapy observed in preclinical lymphoma models are to be developed in the clinic as well (126). These combined effects might have something to do with the reported opsonizing activity of antitumor antibodies that favors cross-presentation of tumor antigens by DCs (127).

Anti-ICAM-2 (CD102)

ICAM-2, a member of the immunoglobulin superfamily encompassing two immunoglobulin domains, was originally described as a counterreceptor for the leukocyte integrin leukocyte function antigen-1 (128, 129). Its expression is restricted to endothelial cells and lymphocytes (130). The surface expression on lymphocytes is up-regulated on activation from low basal levels and, importantly, many malignancies derived from both T and B lymphocytes are ICAM-2 positive. Recent studies have established that mannose-rich carbohydrate structures attached to ICAM-2 interact with the DC-specific lectin DC-SIGN (CD209) (131, 132), playing, therefore, a potentially important role both in DC-T-cell interactions and DC trafficking through endothelial barriers.

Administration of anti-ICAM-2-specific mAbs to mice bearing ICAM-2-negative established tumors induces complete regressions through a mechanism dependent on CD8⁺ T cells, which correlates with induction of tumor-specific CTLs (133). It has been described recently that, on ligation, human ICAM-2 ignites a signaling pathway that inhibits programmed cell death (134). Those signals involve intracytoplasmic transient interactions of ICAM-2 with ezrin as well as a series of downstream events that interfere with the mitochondria apoptosis pathway through inositol-3-phosphate kinase and Akt (protein kinase B) activation (134). In addition, binding of immunotherapeutic mAbs to ICAM-2 can enhance its adhesiveness to its cognate ligand DC-SIGN (133), although the real impact of this adhesion phenomenon on the antitumor effects remains elusive. Additional research in the mouse tumor models has demonstrated that immunotherapy with anti-ICAM-2 mAbs promoted the survival of activated T lymphocytes that recognize tumor antigens both *in vivo* and *in vitro* (135). As a result of this mechanism of action, these therapeutic antibodies enhance the antitumor activity of immunization procedures against tumor antigens, such as the *in vivo* gene transfer of IL-12 to malignant cells (135). As a consequence, two important experimental directions are being taken in this field to: (a) elucidate the physiological functions of ICAM-2 as a player in immunological synapses as well as the control on T-cell survival; and (b) further exploit ICAM-2 artificial ligation with antibodies as a therapeutic tool to enhance T-cell immunity against tumors and infectious agents.

Importantly, potential toxicity problems have been identified with regard to a cytokine storm syndrome on combination of anti-ICAM-2 mAb with both *IL-12* gene therapy and with adoptive transfer of T cells (135). Nonetheless, two interesting pieces of information came out in those studies: (a) a nontoxic

therapeutic window of doses can be found; and (b) *in vivo* blockade of IFN γ activity, once the tumors had regressed, was capable of preventing the mentioned lethal toxicity.

Anti-ICAM-2 and anti-4-1BB mAbs are agents that act, at least partially, on the contraction phase of the immune response that physiologically follows clonal expansion (34). Interfering with activation-induced cell death renders a more numerous army of tumor-specific T lymphocytes that can deal with greater tumor burdens but can also lead to a dangerously higher output of cytokines.

Possible antiangiogenic mechanisms were searched for, because ICAM-2 is expressed on vascular cells with some degree of selectivity in mouse tumor vessels. Although experiments have failed to show a decrease in vascularization of implanted matrigel plugs. Some delay in nude mice was found in the growth of transplanted angiomas. These results indicate that an antiangiogenic component in the overall antitumor effect cannot be ruled out (133).

Depletion or Inactivation of Regulatory T Cells

Silence to endogenous antigens is enforced by thymic mechanisms that delete autoreactive clones (136). In addition, there exist peripheral mechanisms that maintain unresponsiveness to self-antigens. In particular, a population of CD4⁺ CD25⁺ T cells has been identified as a key mediator of these functions (42, 45, 137). Either their depletion, or their genetic absence caused by lack of Fox-P3 (138–140), shows their importance at preventing and ameliorating autoimmune conditions (141). In fact, Treg-adoptive transfer prevents or treats experimental autoimmunity in various models (142). These Treg cells constitutively express CTLA-4 (42) and the orphan TNF receptor family member *GITR* (glucocorticoid-induced TNF receptor family-related gene; Refs. 143 and 144). They also selectively express the Fox-P3 transcription factor (138–140). Transfection of this gene is sufficient to induce thymocytes to differentiate into Treg cells (140). Treg cells inhibit proliferation and activation of effector T lymphocytes through actions performed in close membrane contact-based functions (42, 45, 137).

Depletion of Treg cells with anti-CD25 mAbs exerts antitumor effects (145) by permitting the activation of antitumor CD8⁺ and CD4⁺ effector T cells (145, 146). At least part of the antitumor effect is mediated by CD4⁺ effector T lymphocytes that promiscuously recognize various unrelated tumor cell lines and secrete IFN γ .⁴ The antitumor activity of those CD4⁺ cells was found to be largely dependent on the ability of IFN γ to inhibit tumor vascularization. The combination of CD25 depleting mAb, anti-CTLA-4 mAb, and vaccination with a GM-CSF-transfected variant of a poorly immunogenic melanoma causes synergistic antitumor effects (71).

Anti-*GITR* antibodies have been reported to functionally interfere with Treg functions (143), thus defining a potential target for this kind of immunotherapeutic agent in cancer treatment. Translational research on this idea has already started.

Because resting Treg cells express CD25, which is the α chain of the high-affinity receptor of IL-2, an IL-2-toxin chimeric protein that had been developed for cutaneous lymphoma treatment is being used as a Treg-depleting agent (147, 148). To achieve depletion, antihuman CD25 mAbs are also available for development (149).

Other T cells with regulatory functions exist, such as CD4⁺ cells (called Tr1 cells), that secrete IL-10 and TGF- β (44, 150). Interference with their functions has not been reported to enhance tumor immunity but it might be important in some instances.

Interference with Cytokines That Down-Regulate the Immune Response

Certain soluble factors are known to depress the cellular immune response. TGF- β and its receptors are the best known (151, 152). TGF- β inhibits T-cell activation at many levels, including lymphocyte proliferation and the function of APCs (153). Tumors frequently produce high amounts of this factor, and there is mouse genetic evidence that TGF- β receptors on lymphocytes are involved in the failure of immune surveillance against tumors (154). Other soluble factors such as VEGFs (155–157) and IL-10 (158–160) are endowed with similar properties. Interference of the function of these mediators can be achieved with blocking antibodies, decoy receptors, peptides, and other means.

The T-cell activation antigen CD69 has been implicated recently in the control of TGF- β secretion (161). CD69 exerts a “brake” function on the immune response. CD69^{-/-} mice mount stronger responses against certain experimental tumors. Moreover, *in vivo* down-modulation of CD69 from the surface of lymphocytes with specific mAbs increase antitumor immunity in some models (161). It remains to be seen whether interference with TGF- β through CD69 would be effective under conditions in which tumor cells were producing high amounts of this soluble mediator.

In the case of anti-VEGF (162) and anti-VEGF receptor (22) humanized antibodies that are being clinically tested, future experimentation should evaluate the real effect of interfering VEGF on the antitumor immune response.

Conclusions: The Road Map to Clinical Development

The road map to clinical development includes: (a) Immunotherapeutic mAbs emerge as a new family of agents with potential in cancer treatment. Among their virtues, the ability to synergize with other more conventional therapies stands out. (b) Augmentation of immune responses is also a goal in other conditions such as chronic viral infections (*i.e.*, chronic hepatitis, herpes virus chronic, or latent infections, and so on). Therefore, other indications for these antibodies might lie ahead. (c) Several specificities of mAbs with these properties have been examined in murine models of cancer, but many other molecules of the immune system might still hold exploitable secrets for

³ N. Casares, L. Arribillaga, P. Sarobe, J. Dotor, A. Lopez-Diaz De Cerio, I. Melero, J. Prieto, F. Borrás-Cuesta, and J. J. Lasarte. CD4⁺/CD25⁺ regulatory cells inhibit activation of tumor-primed CD4⁺ T cells with IFN γ -dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J. Immunol.*, in press.

mAbs. Currently, the clinical experience involves only early trials with anti-CTLA-4 mAbs, indicating that this field is at its infancy. (d) Autoimmunity is a clear danger of immunotherapy, and it is becoming more evident when relevant efficacy results are being achieved (162). Learning how to manage such complications and assessing the balance of adverse *versus* beneficial immunity will be an important and difficult clinical topic in the near future of immunotherapy. (e) If immunotherapy of cancer were a car, a formula to its racing success might be (163, 164) to start the engine (immunization/vaccination), jump on the accelerators (exaggerate costimulation), and release the brakes (tamper with the mechanisms that enforce immune tolerance and keep ongoing immune responses under control). mAbs can be very useful tools, particularly for the last two steps. Mutual potentiation by combinations of agents acting at each step is gaining experimental support both in preclinical and clinical settings. The more regulatory checkpoints are tampered with, the more the risk for unwanted inflammation will be increased. Skillful and wary clinical steering will be much needed. (f) Clinical development requires resources and expertise that involve bioprocessing, GMP manufacturing, toxicology, regulatory affairs, both scientists and clinicians directly involved in translational research, experts in complex intellectual property issues, and so on. Those resources are normally only available to industry. Those companies already in business with humanized mAbs have the advantage and only need to recruit or outsource expertise in monitoring antitumor immune responses. Cellular immunology and immunohistochemistry tests are to be used as surrogate end points for clinical trials. Industrial liaisons are a must, and the current state of the field should attract investment for development of at least some of these novel therapeutic options. The prediction of which of these will finally pay off is clearly a difficult forecast, but there exists profound reasons for optimism in many of them.

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