Cancer Immunotherapy: Whence and Whither

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Abstract

The current concepts and practice of cancer immunotherapy evolved from classical experiments that distinguished "self" from "non-self" and the finding that humoral immunity is complemented by cellular immunity. Elucidation of the biology underlying immune checkpoints and interactions between ligands and ligand receptors that govern the immune system's ability to recognize tumor cells as foreign has led to the emergence of new strategies that mobilize the immune system to reverse this apparent tolerance. Some of these approaches have led to new therapies such as the use of mAbs to interfere with the immune checkpoint. Others have exploited molecular technologies to reengineer a subset of T cells to directly engage and kill tumor cells, particularly those of B-cell malignancies. However, before immunotherapy can become a more effective method of cancer care, there are many challenges that remain to be addressed and hurdles to overcome. Included are manipulation of tumor microenvironment (TME) to enhance T effector cell infiltration and access to the tumor, augmentation of tumor MHC expression for adequate presentation of tumor associated antigens, regulation of cytokines and their potential adverse effects, and reduced risk of secondary malignancies as a consequence of mutations generated by the various forms of genetic engineering of immune cells. Despite these challenges, the future of immunotherapy as a standard anticancer therapy is encouraging.

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Background

Self versus non-self and immune tolerance

Much of our current understanding of the immunologic basis of disease, and now of immune-based therapies, derives from insights provided by classical experiments performed more than half a century ago. The concept that the body can differentiate between self and non-self and that foreign tissue when introduced into a recipient during early development can be "tolerated" originated with Sir Macfarlane Burnet (1). It was a series of skin transplantation experiments by Peter Medawar’s group that confirmed Burnet’s thesis, for which both scientists were jointly awarded the Nobel Prize for Medicine or Physiology in 1960. Using mice of different genetic backgrounds, they showed that skin grafts from a donor that was genetically unrelated to the recipient were rejected more rapidly if the recipient had previously rejected a graft from an animal genetically identical to the donor (2). They further described the acquisition of immunologic tolerance when a recipient was first exposed as an embryo or in early life to genetically disparate tissue that was genetically identical to the donor (2). They showed that T cells express a T-cell receptor (TCR) that is genetically unrelated to the recipient to the recipient and that foreign tissue when introduced into a recipient during early development can be "tolerated" originated with Sir Macfarlane Burnet (1). It was a series of skin transplantation experiments by Peter Medawar’s group that confirmed Burnet’s thesis, for which both scientists were jointly awarded the Nobel Prize for Medicine or Physiology in 1960. Using mice of different genetic backgrounds, they showed that skin grafts from a donor that was genetically unrelated to the recipient were rejected more rapidly if the recipient had previously rejected a graft from an animal genetically identical to the donor (2). They further described the acquisition of immunologic tolerance when a recipient was first exposed as an embryo or in early life to genetically disparate tissue that was genetically identical to the donor (2). They showed that T cells express a T-cell receptor (TCR) that is genetically unrelated to the recipient and that the tolerant state that enables tumors to evade immune detection and rejection.

Cellular immunity and the discovery of T cells, the T-cell receptor and the MHC

A student of Medawar’s, N.A. Mitchison, observed that tumor graft rejection was accelerated when he introduced lymph node cells from an immunized donor mouse that had previously rejected that tumor. This worked only with lymph node cells and not with serum from the immunized animal. This discovery implicated a cell-mediated immune response and presaged the era of cellular immunity (3). That thymus-derived cells (T cells) involved in such immune responses came from a series of experiments from Miller and Mitchell (4, 5). They showed the involvement of T cells by a sheep red blood cell hemolysis assay (6) using interstrain crosses and transplantation of T cells (antigen activated or not) into thymectomized mice. They demonstrated that T cells could be activated by antigen, separate from antibody-producing cells, and that the T cells likely collaborated with bone marrow-derived cells (B cells) in elaborating an immune response following an immunologic challenge (7).

It was not until the mid-1970s that Zinkernagel and Doherty first showed that T cells express a T-cell receptor (TCR) that recognizes antigen fragments in association with MHC-I or II (8, 9) and reviewed in ref. 10. The finding that combined stimulation of TCR by MHC-complexed antigen and of CD28 and other receptors by costimulators (11) can activate CTLs has ushered in an era of anticancer immunotherapy centered on T-cell activation.

MHC-dependent immunotherapy strategies

Several immunologic strategies for targeting tumors have recently emerged. Some are MHC dependent and some are MHC independent, but many involve manipulation of the tumor microenvironment (TME) to enhance T effector cell infiltration and access to the tumor, augmentation of tumor MHC expression for adequate presentation of tumor associated antigens, regulation of cytokines and their potential adverse effects, and reduced risk of secondary malignancies as a consequence of mutations generated by the various forms of genetic engineering of immune cells. Despite these challenges, the future of immunotherapy as a standard anticancer therapy is encouraging.

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independent. They all, however, involve mechanisms that either activate T cells, inhibit molecules that suppress T-cell activation, or modify T cells genetically to allow them to recognize and kill target cells (e.g., tumor cells) either in an MHC-dependent (TCR-modified T cells) or MHC-independent manner by genetically engineered Chimeric Antigen Receptor Modified T cells (CAR T cells).

Activation of T cells requires the participation of costimulatory molecules, of which CD28 is one of the most prominent. Binding of ligand to the TCR triggers a signaling cascade resulting in de novo T-cell activation and clonal expansion (11). Also key to CTL expansion is stimulation by cytokines, including IL2 to which CD8⁺ T cells respond in an autocrine and paracrine fashion (12). Clinically, high-dose administration of IL2 has produced prolonged survival in some patients with metastatic disease (13–15; reviewed in ref. 16). When CD28 on CD8⁺ T cells interacts with the surface glycoproteins CD80 (B7-1) and CD86 (B7-2), found predominantly on antigen-presenting cells (APC) such as macrophage and dendritic cells as well as B cells, the T cells are activated, increasing both in numbers and cytotoxic activity. To exploit this observation, CD80 was transfected directly into tumor cells and shown to be sufficient to stimulate T-cell–mediated cytolysis of tumor cells and tumor rejection (refs. 17–19; Fig. 1).

The cytotoxic T-lymphocyte antigen-4 (CTLA-4 or CD152) is another CD28-related protein on T cells that also interacts with CD80, but plays an opposing role to that of CD28 causing the suppression of previously activated T cells (11). This inhibition, known as an immune checkpoint, can be relieved by blocking the interaction between CD80 or CD86 with CTLA-4, primarily with inhibitory mAbs directed to CTLA-4. Alleviating the inhibitory immune checkpoint forms the basis for an anticancer immunotherapy approach that has produced some significant clinical efficacy, but also significant undesirable side effects (refs. 20, 21; Fig. 2).

A related immune checkpoint disruptive strategy that is now licensed for several clinical applications involves inhibition of the programmed cell death protein-1 (PD-1, or CD279), a cell surface
receptor found on activated T cells (22), or use of antibodies against the ligands for this receptor (PD-L1 and PD-L2; Fig. 2). The elevated expression of PD-1 contributes to the downregulation of immune responses (23). Shortly after its discovery by Tasuku Honjo, he and his collaborators, Clive Wood and colleagues, showed that PD-L1 (also designated B7-H1 or CD274) is the ligand for PD-1 and that it is a transmembrane surface antigen with immunoglobulin-like structure. It is widely distributed among tissues and organs, and its engagement with PD-1 leads to inhibition of TCR-mediated T-cell activation (24). The PD-L2 ligand (also designated B7-DC or CD273; refs. 25, 26) is structurally similar to PDL1 but differs somewhat in its physiologic role. Both are upregulated in response to different inflammatory cytokines such as IFNγ, IL4, and IL10 (27), but PD-L1 appears to be upregulated in diverse cell types, whereas PD-L2 is more commonly upregulated in dendritic cells (DC) and macrophage (27). Significantly, PD-L1 is also upregulated in some tumor cells and its interaction with PD-1 induces T-cell apoptosis (28). Furthermore, presentation of PD-L1 on tumor cells can be enhanced by IFNγ resulting in even greater inhibition of CTL activity. PD-L1 expression occurs in several solid tumor types including melanoma (29–31), bladder cancer (32), non-small cell lung cancer (33, 34), head and neck cancer (35, 36), and metastatic but not primary osteosarcoma (37), among others, and like PD-1 presents a therapeutic target.

As described earlier, expression of CD80 on APCs or tumor cells can engage CD28 to activate T effector cells and mediate tumor rejection (17–19, 38, 39). These types of intercellular interactions, however, do not operate in isolation. Cytokines, such as IL2, for example, play a complementary role by stimulating the expansion of T cells in vivo or ex vivo (Fig. 1; refs. 40–42). Historically, maintaining T-cell viability in culture had been challenging until the role of a lymphocyte-secreted factor that allowed longer term T-cell survival in culture was discovered. The fact that long-term culture of T cells was enabled by growth in medium conditioned by phytohemagglutinin (PHA)-stimulated lymphocytes ultimately proved to be a game changer for the deeper characterization of T-cell biology and
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clinchingly relevant adoptive T-cell therapy. By growing T cells in medium previously conditioned by culture of PHA-stimulated T cells, the cells remained viable for up to 9 months (41). The active factor, subsequently designated as IL2, has been used clinically now for several years to increase the antitumor efficacy of cytotoxic T cells by stimulating them to exit an anergic state and by promoting their expansion in vivo (40). Similarly, IL2 is also routinely used for promoting ex vivo T-cell expansion for adoptive cell transfer (42) although even serum-free media containing alternative cytokines can be equally effective (43).

The pioneering work of Mitchison (3) laid the foundation for adoptive T-cell transfer (ACT) as a significant anticancer therapy. One source of cytotoxic cells used for autologous ACT are tumor-infiltrating cells (TIL) whose anticancer efficacy was first described by Rosenberg and colleagues in a preclinical mouse model (44) and later in humans, most notably with metastatic melanoma (45, 46). Importantly, treatment of tumor-bearing mice with either TILs alone or with lymphodepleting agents such as cyclophosphamide alone had marginal benefit. A combination of both modalities, however, was far more effective than either alone, and efficacy was further enhanced by the administration of IL2 (44). These data suggested that presence of an intact immune system hindered therapeutic activity, and that lymphodepletion by cyclophosphamide (or radiation) allowed the TILs to exert their antitumor activity more effectively (47). Classically, TILs are recovered by culturing tumor fragments or disaggregated tumor activity more effectively (47). Furthermore, as discussed later, the development of efficient ex vivo T-cell expansion has served as a prelude to the emergence of genetically redirected T cells as an ACT-based anticancer therapy.

MHC regulation

Tumors utilize a variety of mechanisms to evade immune detection or mute immune response. A well-established immunoevasive mechanism is downregulation of MHC class I (MHC-1) complexes on tumor cells so that tumor antigen presentation is diminished and detection by CTLs is impaired (48). The mechanisms underlying diminished MHC-1 expression can occur at multiple levels including errors in proteasome-mediated antigen degradation, in chaperone accommodation of antigen fragments to MHC-1, in assembly of the complex with antigen fragments to be presented, and downregulation of MHC-1 component synthesis (48). It is noteworthy that transformation by some oncogenes is sometimes accompanied by diminished levels of MHC-1 at the cell surface (49). N-myc and C-myc, for example, have been reported to suppress MHC1 gene expression in solid tumors (50–54). In addition, other mechanisms, such as direct binding to and upregulation of the PD-L1 promoter, have been reported (55). Mutant BRAF (V600E) causes rapid internalization of MHC-1 which can be reversed by treatment with a MAPK inhibitor (56, 57). The HER2 oncogene, when overexpressed, can suppress expression of MHC-1 at the cell surface. Its reexpression can be achieved by silencing HER2 with an siRNA (58, 59) or by administration of a MAPK kinase (MEK) inhibitor (58). Treatment with metformin can alter MHC-1 expression on cancer cells. There is anecdotal evidence that diabetic patients with cancer who are treated with metformin for their diabetic condition respond better to cancer therapy than patients not treated with metformin. This relationship has recently received support from a large electronic records data mining effort showing that cancer risk is significantly reduced in patients receiving metformin compared with those who are not receiving the drug (60). Other retrospective studies have also found a reduced risk of cancer in patients treated with metformin (61–63). Several metabolic mechanisms for protection from cancer by metformin have been reviewed (64, 65), but most need further validation.

Relevant to this review, metformin can restore expression of MHC-1 on the surface of breast cancer cells previously transfected with HER2, thereby rendering the cells more visible to CD8+ T cells (66). Conversely, pharmacologic inhibition of MEK appears to reduce HER2 expression and upregulate MHC-1 at the cell surface (58). One mechanism by which metformin may exert its immunomodulating effect might be by interfering with the MAPK pathway. The effects of metformin on the immune response, however, are pleiotropic. In addition to potentially targeting the diminished MHC expression on the tumor cell, the drug appears to also directly target cytotoxic CD8+ T cells, protecting them from anergy and restoring them to an activated state (67).

MHC-independent strategies

Recently, the complementary but vital roles of CD80 and IL2 in activating and mobilizing T cells for antitumor function have been exploited for generating cancer cell vaccines. In one example, a lentivirus vector has been designed to harbor a fusion gene encoding CD80 and IL2 as a single fusion peptide with a furin cleavage site separating the two proteins. When cells are transduced with this vector, the fusion protein is cleaved by endogenous furin to allow expression of CD80 at the cell surface and secretion of IL2 to promote T-cell expansion (68). This approach is being applied to patients with relapsed AML and requires ex vivo lentiviral transduction of patient-derived AML blasts and the subsequent autologous adoptive transfer. While the strategy may prove successful, there remain many unanswered questions. Transduction efficiency of AML blasts is only about 40 percent yet remission appears to be effectively induced. This may be due to the efficient expression of MHC-I and II molecules and a range of leukemia-associated antigens. Remission may also be aided by the machinery necessary for antigen processing and presentation, as well as enhanced expression of a range of adhesion and costimulatory molecules that are normally expressed by the professional APC. Notably, AML cells also express the costimulatory molecule CD86, but not CD80. Therefore, the genetic modification of AML cells to enable the expression of CD80 could allow them to directly stimulate T cells with appropriate TCR for engagement with MHC/leukemia-associated antigen complex (Fig. 1). Alternatively, the activation of natural killer (NK) cells by the CD80 and IL2-expressing AML cells, enables the NK-mediated lysis of AML cells (Fig. 1; ref. 45), resulting in the release of AML-associated antigens/neoantigens, their uptake by dendritic cells, and the subsequent activation of MHC/antigen-dependent T-cell responses against the endogenous AML cells expressing these antigens (69).
CAR as a path to MHC-independent anticancer therapy

The TCR is structurally similar to humoral immunoglobulin molecules in that it is a heterodimer comprised of a constant (C) region and a variable (V) antigen-binding domain. The majority of T cells have receptors comprised of \( \alpha \) and \( \beta \) chain heterodimers while a minority carry \( \gamma \) and \( \delta \) chains. The TCR differs from immunoglobulin molecules by having a transmembrane domain that anchors it to the cell surface, and a short intracellular domain. It is also associated with the CD3 multimeric protein complex which initiates a signaling cascade once the TCR is engaged with antigen/MHC complex (70). Initiation of the signaling cascade is mediated by immunoreceptor tyrosine-based activation motifs (ITAM) contributed by each of the various CD3 subunits (Fig. 1; ref. 71). The antigen-binding capacity of the TCR also differs from that of circulating antibody which can bind tertiary and quaternary antigen structures. Binding by TCR is restricted to short linear fragments of antigen presented in the context of the MHC class I or II.

The concept of facilitating direct T-cell–mediated but MHC-independent antitumor activity received a major boost when a group at the Weizmann Institute proposed replacing the TCR V regions with antibody V regions but retaining the extracellular C region, the transmembrane domain and the cytoplasmic domain of the receptor. The first such “chimeric” receptor was constructed by splicing the Vh and Vl chains of a mAb directed at 2, 4, 6-trinitrophenol (TNP) to the TCR \( \alpha \) and \( \beta \) constant domains leaving the remainder of the receptor intact. When introduced into allospecific T cells, the “chimeric” receptor was sufficient to promote T-cell proliferation, cytokine production, and target cell cytolysis (72).

While the first reported CAR used a double-chain antibody design (72), most current CAR designs utilize an extracellular single-chain antibody variable fragment (scFv) in which the variable heavy chain and the variable light chain are linked by a short flexible peptide. These in turn are linked to a flexible hinge region attached to a transmembrane domain and intracellular tail associated with a CD3 subunit (CD3\( \zeta \)) which provides ITAMs to affect intracellular signaling and T-cell activation. This architecture represents what is currently designated as a first-generation CAR design that has been supersedee by two later generations (Fig. 3). Following the description of the original prototype CAR (73), the first-generation CAR design contained the variable domains of the light and heavy chains of a mAb linked to a hinge region, a transmembrane, and the cytoplasmic domain of CD3\( \zeta \) (Fig. 3). Expression of this construct, however, was insufficient to sustain T-cell persistence as the T cells appeared to undergo rapid anergy (73, 74). To overcome this shortcoming, one (second generation) or two (third generation) costimulatory molecules were introduced in tandem with the CD3\( \zeta \) signaling domain. The costimulatory molecules most commonly used to date are CD28 and 4-1BB/CD137 (75, 76), which promote an increased production of cytokines, predominantly IL2 and IFN\( \gamma \), and which also promote proliferation and expansion of the genetically modified, redirected T cells (75, 77).

In addition to the intracellular signaling domains of the TCR and costimulatory molecules, CAR T cells express engineered...
antibody–variable chains, or alternatively ligands, that are able to bind molecules on the surface of target cells. Following engagement between CAR T-cell antibody/ligand and the target cell surface antigen, the genetically redirected T cell promotes target cell cytolysis by release of cytotoxic granules containing perforin and granzymes which can lyse a target cell, including drug-resistant tumor cells (78). A second cytolytic mechanism involves the interaction between Fas receptor (FasR/CD95) on the target cell and Fas ligand (CD95L) on the CD8+ T cell. When the Fas ligand and receptor are engaged, signaling pathways are activated in the target cell that trigger a caspase cascade resulting in target cell death (79). A series of recent reviews have given more complete descriptions of CAR T-cell biology, target antigens thus far selected, and a current list of CAR T-cell clinical trials underway and status to date (80–84).

**CAR T cells for B-cell malignancies**

Given the specificity of the redirected CAR T cell and its cytolytic capacity, the optimal target for a CAR T-cell strategy would be a tumor type that expresses an antigen unique to that tumor and that is absent from nontumor tissue. For this reason, B-cell malignancies were the initial cancer type to become the focus of a battery of clinical trials (85). The CD19 surface protein is a pan-B-cell marker that is expressed on essentially all B cells, from pro-B cells to memory B cells, but not on hematopoietic stem cells (82). Moreover, patients appear to be able to sustain persistent reduction in numbers and function of CD19+ B cells, providing that immunoglobulin replacement therapy is instituted (86). It is not surprising, therefore, that a large number of independent CAR T-cell clinical trials have emerged that target a range of CD19+ B-cell malignancies including non-Hodgkin lymphoma (NHL; refs. 81–83, 85), chronic lymphocytic leukemia (CLL; refs. 86–92), and acute lymphoblastic leukemia (ALL; refs. 93–97). Of all CAR T-cell therapeutic trials, the most successful to date has been that for recurrent or refractory B-cell ALL (95, 96). For a disease with historically poor prognosis and outcome, in the range of 7% 5-year survival, reports of 70% to 90% complete response rates are remarkable (95, 96).

**Immunotherapy for solid tumors and the immune microenvironment**

There have been fewer applications of CAR T-cell therapeutic approaches for solid tumors than for hematologic malignancies and progress has been less encouraging. There are multiple reasons to account for the less rapid advance. Unlike hematologic malignancies, solid tumors have more complicated microenvironments that can be highly immunosuppressive. In addition to the inhibitory effects of PD-1, PD-L1, and CTLA-4 on tumor rejection discussed above, the tumor microenvironment is replete with cells that interfere with antitumor activity. Despite recent advances, the complexity of the immune network in the context of tumor immunology is not fully understood.

**Cells and cytokines of the immune microenvironment**

The tumor milieu contains a variety of cell types that have activities that can be supportive of or antagonistic toward tumor maintenance or rejection. For example, a subpopulation of regulatory CD4+ T cells (Tregs) can effectively interfere with the function of APCs (98, 99). By blocking CTLA-4, one can render cytotoxic T cells (primarily CD8+) more responsive to antigen while suppressing the inhibitory effects of Tregs (100, 101). The complexity of the cancer-related immune network is further exemplified by studies showing that CD8+ T cells in a melanoma microenvironment can promote immunosuppression by mechanisms including recruitment of Tregs, the upregulation of PD-L1, and the upregulation of Indoleamine-pyrdrole 2, 3-dioxygenase (IDO), an enzyme that has tolerogenic capacity (102). Immunosuppressive mechanisms are mediated not only by direct intercellular contacts, but also by cytokines such as natural killer (NK) cell- and T-cell-derived IFNγ and tumor-associated macrophage (TAM)-derived VEGF and TGFβ (103). In addition to Tregs and TAMs in the tumor microenvironment, the immunosuppressive cell population often contains a heterogeneous population of myeloid-derived suppressor cells (MDSC) that also produce an immunosuppressive local environment (104–106). Among the generally immunosuppressive myeloid-derived cells in the tumor microenvironment are CD103+ (mouse)/CD141+ (human) dendritic cells (DC) that can cross-present tumor antigens to and activate cytotoxic CD8+ T cells. These cells, however, appear rare in the tumor microenvironment as their recruitment to the tumor site appears to be compromised (103, 106, 107). The intricacies of the tumor microenvironment, despite being complex, can offer possibilities for therapeutic intervention. Expansion of the CD141+ cell populations (107, 108) or the conversion of tolerogenic DCs to immune stimulatory DCs by over expression of IL12 (109), for example, represent two such potential antitumor therapeutic avenues.

The Treg cells, which have immunosuppressive activity in tumors, play a role that is directly counter to that of cytotoxic CD8+ T cells (103). When transiently ablated in a mouse model, the loss of suppressive Tregs impairs primary and metastatic tumor progression and sensitizes the tumors to radiotherapy (110). Similarly, interfering with Treg signaling by inhibiting the PI3(3)K isoform p110α activates CD8+ T cells and results in tumor regression in a wide range of cancers (111). A recent study suggests that for Tregs to render cytotoxic T cells dysfunctional, they first must reencounter antigen in the local environment and interact with APCs, which is accompanied by depression of CD80 and CD86 on the APC cell surface (112). The Tregs promote an anergic state in the cytotoxic T cells with accompanied impairment of cytokine secretion and granzyme release coincident with elevated expression of inhibitory PD-1 (112). Activation of cytotoxic T cells not only promotes the release of cytolytic enzymes (78), but also stimulates the release of cytokines and chemokines that impact NK cells that are resident in the tumor microenvironment and that also have cytotoxic function. The NK cells are also activated by interaction between cell surface receptors and cell surface ligands on the activating or target cell (113). Among the cytokines released by NK cells are IFNγ and TNFα, factors that can activate and recruit inflammatory cells to the local environment and regulate DCs, T cells, and B cells (113, 114). Upon target cell recognition, NK cells also release several members of the interleukin family and the chemokines MIP-1α, MIP-1β, and RANTES, which impact the immune circuitry (113–115). Thus, NK cells are central to maintaining the homeostatic balance between T-cell subsets. B cells, DCs, and myeloid populations by a plethora of cytokines and signaling molecules and their cognate receptors (113). Individually, and in combination, these cytokines exert their effects on cell function by both autocrine and paracrine mechanisms. As discussed earlier, IL2 is important for expansion of CD8+ T cells (12), and dendritic cells produce IL12 and promote CD8+ T-cell activation via...
chemokine production (116, 117). In addition, Tregs produce IL10 and TGFβ, both of which can act directly on CD8+ T cells to exert their inhibitory effects (118). Alternatively, TGFβ produced by a number of cell types can act on naïve T cells to induce Foxp3, a transcription factor vital to maturation into Tregs (119). In short, the immune network is extremely complex with much to be resolved.

Control of T-cell migration and tumor infiltration

To date, most immunotherapeutic approaches have involved autologous cell transfer with TILs, direct stimulation of CD8+ T cells by cytokines, by interference with inhibitory controls through antibody-mediated inhibition of PD-1, PD-L1, or CTLA-4, or by redirecting T cells with the aid of CARs that directly target tumor-associated antigens on the tumor cell surface. Less emphasis has been given to modulating the tumor microenvironment to enhance an antitumor immune response. One key to the efficacy of immunotherapeutic approaches is to ensure that effector T cells have access to and can infiltrate the tumor.

Adenosine levels in the tumor microenvironment modulate antitumor activity

About 20 years ago, it was noted that extracellular adenosine was elevated under tumor hypoxic conditions and was inhibitory to T-cell activation in a tumor environment (120–122). The effect of adenosine is mediated by adenosine receptors of which the A2a receptor is predominantly found on T and B lymphocytes (122, 123). Extracellular adenosine is accumulated by sequential phosphorylation of ATP to AMP with further hydrolysis to the adenosine nucleoside. This is accomplished by the membrane-associated ectonucleotidases CD73 and CD39 which are found on CD4+ Treg cells and other cells in the tumor microenvironment (124). With genetic ablation of A2a receptors, there is loss of the tumor-protective effect of adenosine accompanied by enhanced CD8+ cell activation and function (122–125). In a mouse tumor model with ablated A2a receptors, there was enhanced eradication of a lymphoma and improved efficacy of an anti-lymphoma tumor vaccine (126). As an alternative to blocking or deleting the adenosine receptor, others have reduced the production of adenosine by blocking the CD73 ectonucleotidase pharmacologically (127, 128) or by antibody-mediated blockade of the A2a receptor (129) with similar encouraging results.

Extracellular adenosine in the tumor environment and its A2a receptor are coupled to a K+ ion channel to regulate T-cell activity

The role of ion channels in immunity, the functional network that they form, and their history have been extensively reviewed (130–132), but the field remains relatively underexplored. Important to this review is that a subset of ion channels regulates T-cell function, including T-cell motility, and cytokine and granzyme production (130–133). When the TCR is engaged, there is a rapid release of Ca2+ from the endoplasmic reticulum, stimulating the formation and activation of Ca2+ release–activated Ca2+ (CRAC) channels in the plasma membrane allowing the further influx of extracellular Ca2+. Protection against depolarization of the membrane due to Ca2+ entry is provided in part by the Kv1.3 K+ channel, allowing outward movement of K+ ions. The Kv1.3 channel is activated by sensing membrane depolarization due to Ca2+ entry (134). A second type of K+ channel is the KCa3.1 channel that is activated directly by the increase in cytosolic Ca2+ rather than a change in membrane potential (135). Thus, the rise in cytosolic Ca2+ as a result of TCR engagement signals the KCa3.1 channel to open thereby allowing further K+ outward movement and membrane hyperpolarization. It is noteworthy that the Kv1.3 channel colocalizes with the TCR in human T cells (134, 135), and like the Kv1.3 channel, the KCa3.1 channel also localizes with the TCR at the immune synapse (136).

The requirement for Ca2+ involvement in CD8+ T-cell function and antitumor efficacy was established by knocking out the subunits that comprise the CRAC channel (137). The Ca2+ influx is important for CD8+–mediated inhibition of tumor growth. It supports its cytolytic activity and inhibits tumor engraftment in a mouse model (137). It does not appear to be required for CD8+ migration. A recent report has linked K+ channels to inhibition of adenosine A2a receptors by adenosine with consequent inhibition of mast cell migration (138). It appears that the A2a receptor is physically close to the KCa3.1 channel and in human mast cells effectively closes the channel pore in response to elevated adenosine and shuts down cell migration (138). In human activated T cells, adenosine selectively inhibits KCa3.1, but not Kv1.3 channels, mediated by the adenosine A2a receptor (139). Inhibiting KCa3.1, with either adenosine or a selective A2a agonist, interfered with T-cell motility and with IL2 secretion, both of which could be reversed by treatment with a selective A2a receptor antagonist (139).

As described earlier, elevated levels of adenosine can interfere with the infiltration of cytotoxic T cells into the tumor and ameliorate their antitumor effect (120–125). The intricate circuitry regulating tumor infiltration by cytotoxic T cells offers several potential therapeutic targets for increasing local CD8+ T-cell numbers to better enable tumor rejection. These include pharmacologic inhibition of the ectonucleotidases CD39 and CD73 to reduce the local concentration of inhibitory adenosine. Alternatively, pharmacologic inhibition of the A2a receptor or manipulation of the KCa3.1 K+ channel to prevent suppression of T-cell motility, are alternatives to enhance CD8+ T-cell tumor infiltration and promote IL2 secretion.

While Ca2+ involvement in ion channel function and T-cell activity is well accepted (130), its impact on T-cell membrane lipids has only recently been described. In an elegant set of experiments using live-cell fluorescence imaging and nuclear magnetic resonance spectroscopy, Xu and colleagues have shown an elevated local Ca2+ concentration proximal to the TCR following TCR engagement with consequent increase in cytosolic Ca2+. One outcome of this Ca2+ localization is to negate the regional phospholipid-associated negative charge, thereby exposing the CD3-associated ITAMs and facilitating the phosphorylation of their tyrosine residues (140). These data confirmed other work implicating membrane lipids in the regulation of T-cell activity (141–144), leading to the observation that availability of cholesterol can potentiate the antitumor activity of T cells (145). Cholesterol is stored as cholesterol esters to maintain homeostatic levels of membrane-associated cholesterol (146). The esterification of cholesterol is catalyzed by acyl-CoA acyltransferases 1 and 2 (ACAT1 and 2). Pharmacologic inhibition or deletion of ACAT1 differentially affects CD8+ T cells with little effect on CD4+ T cells. Consistent with the observation that activated CD8+ T cells are more robust in their synthesis of cholesterol than their nonactivated counterparts (147), pharmacologic inhibition of ACAT1 elevates the membrane cholesterol levels in CD8+ cells, increasing their cytotoxicity and enhancing the production of...
granzymes and cytokines. Notably, inhibition of ACAT1 has little effect on CD4+ T cells (145). Thus, enhancing cholesterol metabolism by inhibiting ACAT could be an additional means to potentiate the therapeutic benefit of current immunotherapy strategies such as the use of CAR T cells or immune checkpoint blockers.

Coming full circle: complementary humoral- and cellular-based roles in anticancer therapy

The majority of this review article has been based on cellular aspects of cancer immunity. Although the contribution of humoral immunity (i.e., antibodies) has been acknowledged for about 50 years (148, 149), it is only recently that the interaction of humoral- and cell-mediated anticancer mechanisms has been actively explored (150, 151). One example of this convergence is “antibody-dependent cell-mediated cytotoxicity” (ADCC), which refers to the ability of immune effector cells to engage and kill IgG antibody-coated target cells (reviewed in refs. 150, 151).

In ADCC, antibodies bound to tumor cells can associate with immune effector cells (e.g., NK cells, macrophage, DCs, other myeloid cells) by interaction of the Fc region of tumor-bound antibodies with Fcγ receptors (FcγRs) on the immune cell surface to promote tumor cell cytotoxicity (151–153). Several approved therapeutic mAbs that target various tumor types (150) have been used in this context, but with varying clinical success. One of the multiple reasons for indeterminate success or failure of antibody treatment includes polymorphic variants of the FcγRs that can affect the affinity between FcγRs on effector cells and the Fc moiety of antibody bound to the target tumor cells (154). This and other challenges are being addressed by reengineering the targeting antibody. For example, manipulating the Fc glycosylation state, specifically removing a fucose moiety, enhances binding to Fcγ receptors and enhances ADCC antitumor activity (155, 156).

While ADCC relies primarily on NK cells to mediate tumor rejection, a recently described antitumor mechanism in mouse also utilizes tumor-binding IgG antibodies but relies on tumor-associated dendritic cells (TADC) along with activated tumor-reactive T cells for its antitumor effect (157). While bone marrow–derived DCs efficiently internalized immune complexes of tumor cells and IgG and stimulated T-cell activation, TADCs did not unless stimulated with CD40 ligand and TNFα. Similarly, injection of allogeneic IgG directly into tumors was not sufficient to elicit an antitumor response unless combined with CD40 ligand and TNFα. When administered in combination, TADC activation and subsequent tumor-reactive T-cell activation were stimulated, resulting in the resolution of several tumor types and their metastases (157). Significantly, T cells from lung cancer patients were stimulated to proliferate in vitro in response to tumor antigens following coculture with allo-IgG–loaded TADCs CD40 ligand and TNFα, consistent with findings in mice. These data provide yet another promising alternative anticancer therapeutic strategy (157).

Challenges

Mutation as a double-edged sword

Each of the strategies to mobilize or redirect the immune system to eliminate liquid or solid tumors is not without its challenges. The role of mutation in tumorigenesis and progression and in cancer immunotherapy can be beneficial or detrimental. It is well accepted that activation of proto-oncogenes by mutation, amplification, or translocation can drive oncogenesis (158). As originally postulated, solid tumors in particular can assume a mutator phenotype (159) as a consequence of a mutation in genes responsible for maintenance of global genomic stability. These can, for example, include genes involved in high-fidelity replication or in repair of DNA damage. While thought to promote oncogenesis, a high tumor mutational load may actually enhance the effectiveness of some immunotherapy approaches, particularly those that involve disruption of the immune checkpoint (160–162). In addition to presentation of MHC-associated oncopeptides, presentation of mutation-derived neoantigens which likely arise from normal proteins that have incurred a missense mutation. These neoantigens can likely contribute to recognition by tumor-reactive T cells (160) when unleashed by inhibition of immune blockade (161, 162), suggesting that a heavier mutation load may predict a more successful outcome following inhibition of a PD-1 or CTLA-4 blockade.

A detrimental effect of mutagenesis can occur as a consequence of transducing immune cells with integrating viral vectors prior to ACT as such vectors can cause insertional mutations and cancer. In the earliest successful gene therapy trial (163), patients with X-linked severe combined immunodeficiency (SCID) were treated with a replication-deficient retrovirus expressing the missing gene. While the majority of patients experienced successful restoration of immune function (163), several patients developed a T-cell lymphoproliferative disease (164) caused by an activating insertional mutation event in the LAT02 proto-oncogene (165). Notably, HIV-infected patients who were treated with retroviral-modified CAR T cells experienced no hematologic malignancies for the following 10-year period (166). While insertional mutagenesis should remain a concern, the engineered self-inactivating lentiviral vectors currently in use appear to have more restricted integration sites that have minimal oncogenic bias and reduced risk (167). In addition, the risk of insertional mutagenesis is substantially lower for the terminally differentiated T cells compared with hematopoietic stem cells that were the target of common gamma chain modification for the treatment of SCID.

In a different context, there should be concern for mutations acquired during in vitro and ex vivo expansion of T cells for ACT. Mutation frequencies and rates in vivo have been difficult to establish. However, mouse models and human cells have yielded a frequency of between 10−7 and 10−8 per locus per cell (168–171) and the frequency of mutation appears to increase as a function of age (171–173). Thus, the older a patient, the greater will be the starting mutational burden in T cells harvested for expansion and autologous transfer. Most studies have measured frequency of acquisition of single nucleotide variants (SNV; refs. 168–170), whereas other studies have reported that loss of heterozygosity (LOH) due to mitotic recombination is the predominant form of mutation in vitro and in vivo (174–177). The concern of potential transformation arises from the very high rate of somatic mutation following ex vivo or induced in vivo expansion of immune cells for therapeutic purposes. Most mutations will be benign, and many individual mutations, or combinations of mutations, will be lethal, thereby removing these cells from the population and avoiding transformation. Similarly, but limited to in vivo expansion, many cells that express mutant proteins will be removed from the population in an MHC-dependent manner, thereby further reducing risk of transformation and consequent malignancy. However, patients with innately diminished DNA repair capacity have higher mutation rates and increased risk of...
Adverse effects of immunotherapies and their management

Most, but not all, immunotherapy-related adverse effects are of the short-term type such as cytokine storm. However, there are concerns of longer-term effects such as the potential for increased risk of autoimmune disease following treatment with PD-1 or CTLA-4 inhibitors (23, 182). In addition, there are possible risks for acquiring somatic mutations during ex vivo and induced in vivo T-cell expansion that is necessary for autologous or allogeneic adoptive T-cell strategies. Possible mutational risks associated with large-scale expansion of universal CAR T cells for "off-the-shelf" CAR T-cell therapy must also be considered. For autologous CAR T-cell therapy, a patient’s T cells are first recovered from peripheral blood, transduced with a retrovirus and ex vivo expanded in culture and the transduced cells.

CAR T cells and therapies

The intricacies of the immune signaling systems and their multiple regulatory feedback circuits are critical for maintaining a homeostatic balance in immune-mediated rejection and tolerance, which when perturbed can have serious consequences. The PD-1/PD-L1 inhibitory interaction, for example, serves not only the unwanted function of protecting the tumor, but is part of the mechanism that allows the body to discriminate between "self" as "non-self". As PD-1 is expressed in many tissues and cell types, alleviation of the PD-1/PD-L1 inhibitory mechanism runs the risk of inducing systemic adverse effects of varying degrees including autoimmune disease or "immune-related adverse events" (183). Similar concerns exist for suppressing immune inhibition mediated by CTLA-4 (184, 185).

Like overcoming the immune checkpoint for therapeutic purposes, the use of CAR T cells is not without its challenges and risks. Probably the most common adverse effect after CAR T-cell infusion is immune-mediated cytokine release syndrome (CRS) or "cytokine storm" which manifests as high fever, myalgia, anorexia, tachycardia, hypotension among other symptoms, and can sometimes be fatal (186–188). Although concerning, CRS can be fully reversed by corticosteroids, such as prednisone (95), which, however, runs the risk of compromising the therapeutic effect of the CAR T cells. An alternative approach based on the remarkable elevation of IL6 that has had considerable clinical success for managing and alleviating CRS symptoms is the administration of tocilizumab, a mAb directed to the IL6 receptor (189). Another challenge with CAR T-cell strategies is on-target (correct antigen target) off-target/off tumor (incorrect cell type target) concerns. Renal cell carcinomas (RCC), for example, express high levels of carboxy-anhydrase-IX (CAIX) at their cell surface (190). When patients with metastatic RCC were treated with CAIX CAR T cells, several developed liver toxicity due to CAR T-cell interaction with bile duct epithelial cells expressing CAIX (191). Similarly, a patient with a recurrent and metastatic ERBB2-expressing tumor, when treated with ERBB2 CAR T cells, displayed pulmonary toxicity due to ERBB2 expression in the lungs (192). Likewise, when patients with melanoma or myeloma expressing the MAGE-A3 antigen were infused with MAGE-A3 CAR T cells, they developed severe cardiotoxicity due to cross reactivity to a titin determinant that was expressed on cardiomyocytes (193).

Most attention in clinical CAR T-cell experience has been with B-cell malignancies where CD19 has been the redirected T-cell target. The approach has been relatively successful in eradicating that malignancy with the caveat that the strategy also elimates most of the normal cells of B-cell lineage as they also express the CD19 marker (94). The loss of normal B cells and consequent off-tumor target toxicity, however, can be managed by immunoglobulin transfer to compensate for lost B cells (94). A modification of the single chimeric antigen receptor that enhances specificity is the development of a dual CAR T-cell strategy. This modification, which involves inclusion of a second chimeric antigen receptor targeting CD123 (IL3 receptor α chain), has been instrumental in overcoming evasian of CD19 CAR T-cell cytokotoxicity or relapse by eliminating B-ALL cells that are CD19 negative (194). Evasion of CD19 CAR T cells by B-cell malignancies can arise due to a subset of cells that express an alternatively spliced CD19 variant that is not recognized by the CAR in the armed T cell. Inclusion of the CD123 chimeric antigen receptor eliminates the residual CD19-negative malignant B cells that also express the CD123 marker as well as those cells that have lost the CD 19 marker by acquired mutation and that contribute to CD19 CAR T-cell-resistant relapse (195). The CD123 marker is expressed on cells of the myeloid lineage and is a target for CD123 CAR T-cell therapy (196). One caveat with targeting CD123 for AML, for example, is that this marker is found on most myeloid cells and also on hematopoietic stem cells (197). Clinically, however, this may not be a serious problem (194), although this contention remains under debate (198, 199). In addition, in hematologic malignancies, where hematologic stem cell transplants (HSCT) are routinely employed to consolidate the often transitory remission following chemotherapy, CAR T-cell therapy could provide a so-called "bridge-to-transplant" allowing hematopoietic reconstitution by subsequent chemotherapy-mediated elimination of the CAR T cells and HSC transplantation.

The use of CAR T-cell approaches for solid tumors have been less successful than those for B-cell malignancies. This is in part
because useful surface markers that are unique to the tumor are not common. To overcome the issue of specificity, CAR T cells with dual specificity have been designed with the idea that requiring both antigens to be engaged would increase specificity of the CAR T cells to the intended target (199–201). In a mouse glioblastoma xenograft model, optimal epitopes for HER2 and IL13 receptor α2 were designed by in silico modeling, and CAR T cells with chimeric antigen receptors targeting both epitopes were generated. The dual specificity CAR T cells appeared to have greater antitumor efficacy than CAR T cells expressing either CAR alone, and the animals survived for a longer time (202). While this dual specificity CAR T cell was efficacious in a murine xenograft model, its utility in an immunocompetent environment is unclear.

A very elegant CAR T-cell model that overcomes this reservation utilizes a synthetic modular Notch receptor (203) designed to recognize one ligand on a tumor cell and a third-generation CAR that recognizes a second antigen on the same tumor cell (204). The extracellular domain of the synthetic Notch receptor, whose normal ligand is Delta, was replaced with an extra cellular domain that recognizes a tumor antigen (antigen A). The intracellular Notch domain that is cleaved after ligand binding to the extra cellular domain was replaced with a fragment that acts as a transcription factor that induces CAR expression to engage a second tumor antigen (antigen B) to initiate tumor cell cytolysis. To be killed selectively by the CAR T cell, therefore, the tumor must express both antigens. Any nontumor cell that expresses only one of the antigens will be spared. Of course, given tumor heterogeneity, it would not be surprising if some tumors contained a subpopulation of cells expressing only one or the other of the antigens, thereby escaping cytolysis by this approach and providing the seed for local tumor recurrence or metastasis. This concern has been addressed by the use of a bispecific CAR that enabled complete cytolysis of malignant B cells with no evidence of escape (205). These CAR T cells harbor reengineered single-chain bispecific antibodies that target both CD19 and CD20. Thus, malignant B cells that have lost CD 19, which normally would render them resistant to CAR T19 cell therapy, retain CD20 and are killed. It is also of course possible, though not yet directly demonstrated, that CAR-mediated lysis of a substantial population of tumor cells may in turn stimulate further immunologic responses against other tumor-associated antigens. This would produce an “antigen spread” and promote elimination of tumor cells that lack the CAR-targeted antigen(s). A related strategy involves direct administration of single molecule bispecific antibodies that target tumor cells (e.g., CD19) and T cells (e.g., a CD3 subunit TCR) to promote the engagement of the two cell types, activation of the coupled T cells and cytolysis of the target cells (206). This type of bispecific antibody, designated bispecific T-cell engager (BITE), is currently under assessment by several clinical trials (206, 207).

Overcoming a hostile tumor environment

Overcoming a hostile immunosuppressive tumor microenvironment represents a major hurdle for CAR T-cell therapy. The tumor microenvironment is a highly complex network of tumor cells, stromal cells, activating and inhibitory cells of the immune system, vasculature, cytokines, and intercellular milieu that is generally immunosuppressive and an environment that favors tumor growth. As previously discussed, elevated local adenosine levels are inhibitory to CTLs and their ability to infiltrate the tumor. Reducing the local adenosine concentration by inhibiting ectonucleotidases (126–128) or suppressing adenosine uptake by blocking the adenosine receptor (122–124) represent alternative approaches to restoring CD8$^+$ T-cell, or CART-cell infiltration and activity. An additional approach for modifying the local tumor environment utilizes CART cells armed with the ability to secrete a cytokine (208). A major focus of this approach has centered on IL12 which exerts its antitumor activity by acting on NK cells and CD8$^+$ cells, and inducing the local production of IFNγ (209). CAR T cells that were modified to secrete IL12 have enhanced antitumor activity in a preclinical murine model (210, 211). However, a clinical trial in which melanoma patients were treated with TILs genetically modified to secrete IL12, encountered significant toxicities (212). Another recent report describes a phase I clinical trial in which hMIC16 CAR T cells were further modified to secrete IL12 for treatment of patients with advanced, recurrent ovarian cancer (213), but no adverse effects have been reported to date. Tumor-associated stromal cells also contribute to the immunosuppressive microenvironment, mediated in part by elevated expression of fibroblast activation protein-α (FAP; ref. 214). When FAP expressed in stromal cells was targeted with CAR T cells in a preclinical cancer model, tumor cell growth was inhibited (215). Similarly, when stromal cells that express FAP in a murine model were eliminated, tumor-infiltrating CD8$^+$ T cell activity and longer survival of mice harboring tumors were enhanced (216). Thus, adapting the tumor microenvironment to favor tumor eradication is fraught with obstacles but presents an encouraging approach.

Patient safety is paramount as evidenced by the recent temporary halting of a phase II clinical trial by the FDA. Patients with refractory ALL had received fludarabine plus cyclophosphamide prior to CD19 CAR T-cell infusion. Three patients less than 25 years of age developed cerebral edema and died while treated under this protocol and the FDA halted the trial. The fludarabine had been added to the preconditioning protocol and appeared to be the cause of the deaths. Shortly thereafter, the FDA allowed the trial to continue, but only after fludarabine was removed from the preconditioning protocol.

Patient safety and alleviation of therapeutic cost

While patient safety remains a constant concern, probably the biggest obstacle for autologous CAR T-cell therapy is the cost, which has been estimated to be as high as $400,000 to $500,000. Even if this price is exaggerated, the high cost should not be surprising given the degree of personalized clinical care required for current autologous CART-cell therapy protocols. As a first step, patient-derived T cells are recovered, often by leukapheresis, and the recovered T cells are stimulated to proliferate to facilitate their genetic modification by infection with a γ-retrovirus or lentivirus encoding the CAR construct. The ex vivo–transduced T cells are then expanded about 10-fold at which point the cell preparation is ready for patient infusion. This multistep process must be repeated for each patient, accounting in part for the very high cost.

To alleviate the high price of individualized CAR T-cell therapy, “universal” engineered T cells have been generated. These have the advantage that they can be expanded into large batches and used as “off the shelf” therapeutic allogeneic CAR T cells with broad applicability (216). The TCR has been disrupted by TALEN-mediated site-specific mutagenesis to eliminate the risk of graft versus host disease. Similarly, CD52, which is broadly expressed on multiple hematologic cell lineages, is also eliminated, thus rendering these cells resistant to the anti-CD52 mAb.
alumtuzumab, which is frequently employed in the treatment of hematologic malignancies. Therefore, these cells can be used in the context of alumtuzumab conditioning of the patient, which reduces the leukemic burden while concomitantly conferring a selective advantage to the allogeneic CAR T cells, including the reduced risk of rejection of these HLA-mismatched cells by the host (217). In brief, donor T cells are collected by leukapheresis, activated in culture, TCR is inactivated by site-directed mutagenesis, and transduced with a lentivirus vector harboring the CAR of interest. The cells are expanded about 10 fold to yield about 10^{10} genetically engineered “universal” T cells. From each batch prepared, aliquots are frozen, and thawed for use as needed. Treatment of an 11-month girl with relapsed CD19+ B-ALL provided an opportunity for clinically relevant proof of principle. The patient received a single dose of the “universal” CAR T cells, designated UCART19 cells, and has undergone complete molecular and clinical remission, within the limits of detection (218).

The advances in our understanding of the biology of how our bodies distinguish “self” from “non-self” during the last 60 years have been remarkable, and the degree to which this understanding is being applied to anticancer therapies is encouraging. As Helen Keller once remarked, “Optimism is the faith that leads to achievement. Nothing can be done without hope and confidence” (219). Given the many distant and recent accomplishments, optimism is clearly applicable to the future of cancer immunotherapy. As described above, the hurdles that remain for achieving a uniformly curative anticancer immunotherapy strategy are significant but it is likely that all can be overcome or circumvented. Particularly encouraging is the idea that immunotherapy approaches can be complemented by or combined with surgery or other developing strategies. These include targeting of biochemical pathways that are key to tumor survival or manipulating the tumor microenvironment rendering it hostile to tumor viability. Decisions by oncologists regarding optimal treatment strategies in the future will require a personalized or “precision” approach taking into account variations in genomic, transcriptomic, proteomic, and metabolic profiles for a given tumor and patient. While currently in its infancy, the promise of such a comprehensive approach is enormous, and the expectation that cancer will be managed as a chronic disease, if not commonly cured, is becoming reality.

Disclosure of Potential Conflicts of Interest

J. Maher is a chief scientific officer at Leucid Bio. F. Farzaneh reports receiving a commercial research grant from Autolus, Cellectis, speakers bureau honoraria from Autolus and Cellectis, has ownership interest (including patents) in Autolus, and is a consultant/advisory board member for Autolus. No potential conflicts of interest were disclosed by the other authors.

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