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CD8+ T Cell-Independent Immune-Mediated Mechanisms of Anti-Tumor Activity

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Abstract

Despite the growing number of preclinical and clinical trials focused on immunotherapy for the treatment of malignant gliomas, the prognosis for this disease remains grim. Cancer immunotherapy seeks to recruit an effective immune response to eliminate tumor cells. To date, cancer vaccines have shown only limited effectiveness because of our incomplete understanding of the necessary effector cells and mechanisms that yield efficient tumor clearance. $CD8^+$ T cell cytotoxic activity has long been proposed as the primary effector function necessary for tumor regression. However, there is increasing evidence that indicates that components of the immune system other than $CD8^+$ T cells play important roles in tumor eradication and control. The following review should provide an understanding of the mechanisms involved in an effective antitumor response to guide future therapeutic designs. The information provided suggests an alternate means of effective tumor clearance in malignant glioma to the canonical $CD8^+$ cytotoxic T cell mechanism.

Keywords

Immunotherapy; cytotoxicity; glioblastoma

I. INTRODUCTION

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults, with an annual incidence of over 17,000 new cases in the United States.[1,2] The prognosis for this deadly disease is bleak, with a median survival of 18 to 21 months.[1,3,4] Complete tumor resection is difficult owing to the diffusely infiltrative nature of the tumor.[1] Combining postoperative radiation therapy with temozolomide chemotherapy has provided the greatest improvement in survival but only 2.5 additional months.[5] Recognition and understanding of

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the biology of GBM and its interactions with the immune system have led to novel immunotherapeutic approaches, including a variety of tumor vaccines.

However, there are challenges with immunotherapy for GBM because of specific features of the central nervous system (CNS) that limit an immune response. The blood-brain barrier limits entry of most immune cells to the brain parenchyma from peripheral blood, classical lymphatic vessels and nodes are absent in the CNS, and there are few T lymphocytes and limited antigen (Ag) processing and presentation, which all help prevent overwhelming CNS inflammation on a daily basis. There are resident immune cells that provide immune surveillance of the brain. Microglia are tissue-resident macrophages that screen and remove cellular debris and foreign material from the interneuronal spaces in the brain parenchyma.[6] Other macrophages and dendritic cells reside in the choroid plexus, perivascular spaces, and meninges. Cerebrospinal fluid that may contain specific Ags leaves the CNS through Virchow-Robin spaces to activate T lymphocytes in the cervical draining lymph nodes (DLNs).[7,8] These activated T cells can then return to and patrol the CNS.

In addition to the limitations of the immune system within the CNS, GBMs and their micro-environment are immune suppressive. Tumor-elaborated soluble factors, such as transforming growth factor beta (TGF-β) and prostaglandin E2 (PGE2)[9] act in the tumor or the DLNs to dampen T cell reactivity. Other mechanisms of immune suppression in the DLN include regulatory T cell–mediated killing of tumor Ag–presenting dendritic cells (DCs) T cell receptor nitration by myeloid-derived suppressor cells (MDSCs) and tolerogenic tumor-associated dendritic cells (TADCs).[10] Distinct types of suppressive immune cells, MDSCs, type 2 macrophages (M2), TADCs, and T regulatory cells (Tregs), are recruited to the tumor micro-environment by PGE2-dependent tumor expression of chemotactic molecules such as CXCL12.[11] Monocytes activated by cancer invasion signals migrate into the tumor and transform into tumor-associated macrophages (TAMs) with two distinct phenotypes.[12,13] Type 1 TAMs facilitate tumor killing, but type 2 macrophages (M2) promote tumor growth and vascularization by secreting epidermal growth factor, fibroblast growth factor, and vascular endothelial growth factor.[14–16] These immune suppressive cells in the tumor micro-environment induce tolerance and inactivate infiltrating T cells; TADCs, in particular, block an immune response by up-regulating the checkpoint inhibitor receptors PD-L1 and CTLA-4 and by expressing the inhibitory cytokines TGF-β and IL-10.[17]

Immunotherapy has the potential to greatly improve survival while providing good quality of life due to minimal toxicities. The majority of protocols rely primarily on the cytotoxic activity of $CD8^+$ T cells to affect tumor regression. However, many other components of the immune system that have been largely overlooked may play important roles in tumor clearance. This report is a review of CD8-independent means of immunotherapy that may provide potent tumor control in human GBM patients.

II. NATURAL KILLER CELLS

Natural killer (NK) cells are lymphoid cells that participate in both innate and adaptive immune responses to pathogens and cancer cells.[18–20] They use a variety of receptors to mediate different effects (Table 1). Examples of inhibitory receptors are major

histocompatibility (MHC) class I ligands, some killer cell immunoglobulin-like receptors (KIRs), and CD94/ NKG2A, which suppresses NK cell cytotoxicity. In contrast, activating receptors, including natural cytotoxicity receptors (NKp30, NKp44, and NKp46), initiate NK cell cytolytic activity.[20–22]

Healthy cells avoid attack by NK cells through the expression of MHC class I molecules and minimal expression of stress-induced self molecules.[20,23,24] In contrast, virally infected or malignant cells become susceptible to NK cell–mediated lysis by up-regulating stress-induced molecules and/or down-regulating MHC class I molecules.[25] To persist, tumors have evolved a variety of mechanisms to escape NK cell–mediated cytolysis. A few examples are secretion of immune regulatory molecules or immune suppressive modulators, which down-regulate NK cell effector functions, and modulation of NK cell receptor–ligand expression patterns.[26,27] The latter includes aberrant expression of nonclassical human leukocyte antigen (HLA) class I Ags.

HLA-E and HLA-G are considered nonclassical HLA class I Ags because they are less polymorphic and bind more restricted peptide repertoires than the classical HLA-A, -B, and -C class I Ags.[28,29] HLA-E has a broad tissue distribution, similar to the classical HLA class I Ags, while HLA-G expression is much more restricted. However, both are expressed by fetal trophoblasts and, as such, were thought to function physiologically by protecting the fetus from allorecognition by maternal NK cells.[30] This belief holds true for HLA-E because it suppresses NK-mediated cytotoxicity via the inhibitory receptor CD94/NKG2A.[31]

Wischhuschen *et al.* first reported that HLA-E-mediated inhibition of NK function may contribute to the pathology of gliomas.[32] They found that HLA-E is expressed by glioma cell lines and primary glioma cells and that, relative to normal CNS tissue, HLA-E expression is enhanced in low-grade gliomas and is enhanced to even higher levels in GBMs. Subsequent studies confirmed and extended these findings by showing HLA-E expression to be associated with a subset of glioma cells with tumor-initiating properties.[33] HLA-E inhibited recognition and killing of these glioma-initiating cells by NK cells, interfering with HLA-E expression by siRNA-gene silencing restored NK killing.

The jury is still out on whether HLA-G suppresses NK responses. The long-held idea that HLA-G, like HLA-E, protects the fetus from maternal NK recognition is falling out of favor. Evidence shows (1) that HLA-G expression promotes HLA-E co-expression by stabilizing the HLA-E molecule upon binding of the HLA-G signal peptide, suggesting HLA-G may act indirectly through HLA-E to suppress NK responses; and (2) that, while HLA-G suppresses $CD8^+$ cytotoxic T cell function, it in fact activates NK cells via the KIR2DL4 receptor, leading to pro-inflammatory and pro-angiogenic responses.[29,30,34] The emerging paradigm for the role of HLA-G in pregnancy is that it indirectly inhibits NK allo-recognition through HLA-E and that it promotes uterine tissue remodeling, which is required for implantation. These findings suggest that we re-evaluate the reported immune inhibitory effects of HLA-G expressed on gliomas.

Wiendl *et al.* first reported that HLA-G expression by gliomas provided a means for immune escape.[35] They found HLA-G to be expressed in four of five glioma biopsies and in several

glioma cell lines, and that just a few HLA-G–positive tumor cells can inhibit anti-tumor responses. Subsequent studies confirmed and extended this work to show that HLA-G is frequently expressed by primary GBM biopsies (65 out of 108 in one study).[36,37] These and other studies suggest that HLA-G expression by tumors is a mechanism for immune evasion.

Emerging evidence muddies the waters, however. For example, neuroimaging analyses of patients with low grade gliomas show that high HLA-G expression correlates with large size and blurred boundaries, characteristics consistent with tumor invasiveness.[38] Together these studies confirm aberrant HLA-G expression by low- and high-grade gliomas, but do not clarify whether HLA-G expression contributes to tumor growth by immune suppression or remodeling of the tumor micro-environment.

Despite the ability of tumors to escape NK cell functions, many therapeutic trials for a variety of malignancies are exploiting NK cells for their functional responses.[39,40] Several approaches have been used for NK cell–based immunotherapy, including *in vivo* cytokine-mediated expansion of NK cells and adoptive transfer of autologous or allogeneic NK cells or of some NK cell lines such as NK-92.[41,42] Moreover, genetically modified NK cells expressing chimeric Ag receptors (CARs) are being investigated for clinical therapeutic use based on their cytotoxic function.[42,43]

III. NATURAL KILLER T CELLS (NKT)

There is another population of lymphocytes, natural killer T cells (NKTs), that are differentiated from NK cells. NKT cells are heterogeneous lymphoid cells that exhibit characteristics of both the innate and adaptive arms of the immune system. Similar to NK cells, these lymphocytes react quickly to stimuli that modulate the immune response.[44,45] NKT cells respond in an Ag-specific manner through an unconventional T cell receptor (TCR), which can react to multiple self and foreign Ags[46,47] through CD1b presentation.[45,48] Unlike traditional lymphocytes, NKT cells have the ability to simultaneously secrete helper T cell 1(Th1)/ pro-inflammatory (e.g., IFN-γ, TNF-α) and Th2/anti-inflammatory (e.g., IL-4, IL-10, IL-13) cytokines[49,50] that activate other NK cells as well as T and B cells.[45]

Because of the heterogeneity of TCR rearrangements, NKT cells are separated into two categories, type I and type II. Type I NKT cells are usually associated with the promotion of tumor immunity, whereas type II NKT cells appear to suppress tumor immunity.[51,52] A combination of activation variables dictates type I NKT cell function: the affinity of the Ag presented to the NKT TCR, the presence of co-stimulatory molecules, and the tissue environment in which the interaction takes place.[53] Type I NKT cells employ several mechanisms to promote cytolytic activity. For instance, both murine and human NKT cells can directly lyse tumor cells by a perforin-dependent mechanism,[54] and cell killing can be potentiated by intracellular granzyme B expression.[55] *In vitro* experiments have demonstrated that tumor cells expressing CD1d may be especially susceptible to direct NKT cell lysis.[56] This pattern has been observed *in vivo* in patients with B-cell lymphoma.[57] There is also evidence that high CD1d expression levels correlate with lower metastasis rates in a murine breast cancer model.[58]

Type I NKT cells are capable of mediating direct tumor lysis that is dependent on the activation of innate and adaptive immune cells.[59,60] The recruitment of anti-tumor cytolytic cell populations primarily involves the initiation of Th1 cytokine cascades. The first NKT cell ligand identified was α-GalCer, a potent activator of type I NKT cells. The clinical therapeutic potential of α-GalCer was demonstrated when application of a synthetic form of this ligand, KRN7000, increased survival in B16 melanoma–bearing mice.[56,61]

Type I NKT cells recognize microbial glycolipids and self Ags.[62,63] As mentioned, α-GalCer is a potent activator of all type I NKT cells, causing them to produce copious amounts of IFN-γ, which facilitates the activation of $CD8^+$ T cells and Ag-presenting cells (APCs).[64] NKT cells specifically stimulate DCs through CD1d-TCR complexes and CD40-CD40L interactions, which induce DC maturation and IL-12 secretion.[65,66] IL-12 stimulates both NK and NKT cells, as well as other T cells, to produce more IFN-γ, and together these cytokines significantly impact the activation of downstream effector populations, such as NK cells, $CD8^+$ T cells, and γδ T cells.[67]

CD1d–restricted NKT cells that do not express the semi-invariant TCR are classified as type II. This NKT cell subset recognizes glycolipid Ags distinct from those recognized by type I NKT cells and is not as well characterized as its type I counterpart. In contrast to their role in enhancing an immune response to tumors, NKT cells, especially type II, have demonstrated suppressive activity in cancer immunology. Type II NKT cells were shown to be sufficient for down-regulating tumor immune surveillance in several studies using different tumor models.[57,68] $CD4^+$ type II NKT cells were shown to produce higher levels of IL-13 and IL-4 compared to type I NKT cells, and NKT cell–dependent IL-13 was found to be necessary for tumor recurrence in a growth-regression-recurrence-pattern 15-12RM fibrosarcoma tumor model.[69] The immunosuppressive effect appeared to be mediated by the sulfatide-reactive subset of type II NKT cells.[68]

Tumor immune surveillance is also blocked by increased production of TGF-β by a $CD11b^+Gr1^+$ population known as MDSCs.[70] The increase in TGF-β production is stimulated by IL-13–initiated signaling through the IL-4R-STAT6 pathway and TNF-α.[69,71]

IV. GAMMA DELTA T CELLS

B cells, alpha beta (αβ) T cells, and gamma delta (γδ) T cells are the three main lineages of lymphocytes in vertebrates that use genetically recombined receptors to survey their environment and mediate host defenses against disease.[72] Gamma delta (γδ) T cells have emerged as an evolutionarily conserved immune cell population,[73] with various percentages among species ranging 60–80% in cattle, pigs, and sheep, and 10–60% in humans depending on immune challenge.[74,75] Alpha beta (αβ) TCRs express either CD4 or CD8 co-receptors and interact with classical MHC molecules presenting peptides; they are used in adaptive immune responses. In contrast to αβ T cells, γδ T cells respond to Ags in a non-MHC manner through receptors including CD1, and they play an important role in innate immune surveillance as well as in adaptive immune responses.[76,77]

Because γδ T cells have multiple TCRs, they can recognize a wide range of Ags; however, in most human peripheral blood, they express a Vg9 Vd2 T cell TCR that recognizes cellular stress in a MHC-independent manner.[77] This population has three functionally distinct subsets: naïve ($CD45RA^+CD27^+$), central memory ($CD45RA^-$ $CD27^+$), and effector memory ($CD45RA^-$ $CD27^-$).[77] Vg9 Vd2 TCRs recognize nonpeptidic prenyl pyrophosphate metabolites, generically known as phosphoantigens.[78,79] Moreover, in response to antigenic stimulation, naive and central memory Vd2 cells will proliferate and secrete cytokines including high levels of IFN-γ and TNF-α.[80,81]

A. Gamma Delta T Cells and Cancer

Gamma delta T cells (γδ T cells) play an important role in immune surveillance and immune defense against tumors, including melanoma,[82] leukemia, lymphoma, neuroblastoma, and other types of carcinoma.[83,84] The antitumor activity of γδ T cells has been confirmed by *ex vivo* expansion followed by infusion to cancer patients.[85,86] Recently, *in vitro* activated γδ T cells have been shown to target a small number of colon cancer stem cells, which had been demonstrated to be responsible for the failure of conventional therapies. In addition, γδ T cells can kill chemotherapy (imatinib)–resistant chronic myelogenous leukemia lines. Due to the lack of appropriate animal models, there is no direct evidence to suggest that human Vd2 cells eradicate or reduce tumor burden *in vivo*. However, a number of studies imply that Vd2 cells may contribute to anti-tumor immunity, and are thus a promising target for cancer immunotherapy.

In vitro experiments, although limited in their extrapolation to physiological systems, have demonstrated that Vd2 cells are capable of recognizing and killing tumor cells through multiple pathways, including granule exocytosis, Fas/Fas-ligand (CD95/ CD178)-induced apoptosis, antibody-dependent cell-mediated cytotoxicity and TNF-related apoptosis inducing ligand.[87,88]

Moreover, γδ T cells rapidly produce large amounts of IFN-γ and TNF-α[80,81] while having strong cytotoxic activity as a result of perforin, granzymes, and death receptor ligands.[76] In response to tumors, NK cells and γδ T cells share similar properties of both innate and adaptive effector activity. NK and γδ T cells recognize similar ligands that are expressed by tumor cells.[89,90] Most hematopoietic cancer cells express various stress-induced molecules, acting as ligands for activation receptors such as NKG2D, CD94/NKG2A, CD94/NKG2C, DNAM-1, Ig-like transcript 2, CD161, KIR2DL1–3, NKp30, and NKp44, which all present on both NK and γδ T cells to regulate their activities.[91,92]

Although the cytokine-induced anti-tumor activities of γδ T cells appear more prominent against hematological cancers than other types of malignancies,[93,94] γδ T cells have demonstrated efficacy against solid tumors through the recruitment of immune cells including small peritoneal macrophages that respond directly against cancer immunosurveillance.[95] In clinical studies, γδ T cells have been reported to infiltrate into various solid of tumors including lung carcinoma,[96] renal cell carcinoma,[97] and breast carcinoma.[98] In patients, both positive and negative correlations have been made between clinical responses and tumor-infiltrating Vd2 cells. γδ T cells, consisting of both $Vd1^+$ and

Vd2$^+$ cells, were predominant tumor-infiltrating lymphocytes in melanoma lesions, and that low numbers of tumor infiltrating γδ T cells correlated with advanced disease.[82]

V . TUMOR-REACTIVE B CELLS AND ANTIBODIES

B cells are lymphocytes that express a unique membrane-anchored antibody B cell receptor (BCR).[99] Unlike CD8 T cells, B cells do not require interaction with APCs to engage Ag to their BCR. However similar to CD8 T cells, B cells require costimulatory and cytokine signals to achieve full activation. Costimulation occurs when a CD4 T helper cell recognizes its cognate peptide-MHC II complex on the surface of the B cell.[100] Costimulation is followed by CD40L-CD40 interactions with T cells that activate an antibody response.[101,102] Activated B cells then undergo an immunoglobulin class switch by DNA recombination to produce specific isotypes of membrane-bound antibody/BCR (IgM to IgG, IgA, or IgE) and differentiation into short-lived plasma cells for antibody production,[103] long-lived plasma cells or memory B cells,[104] or long-lived memory B cells.[105]

Vaccine-based immunotherapy involves tumor cell vaccinations that stimulate production of tumor-specific antibodies that circulate and bind to tumor Ags in the blood or at the tumor site. Antibody binding to tumor-specific Ag induces opsonization of the Ag, which in turn facilitates its uptake by APCs. Antibody binding to Ag on the surface of live tumor cells can trigger a multitude of responses including neutralization of the target protein function,[106] tumor clearance by phagocytosis and/or adaptive immunity,[107] complement-dependent cytotoxicity,[108,109] chemoattraction of other leukocytes, or antibody-dependent cell-mediated cytotoxicity.[109,110]

VI. CD4$^+$ T CELL ANTI-TUMOR ACTIVITY

A. CD4$^+$ T Cell Subsets

Naïve CD4$^+$ T cells can differentiate into one of several functionally distinct subsets that directly mediate, or indirectly stimulate or suppress, tumor-specific immunity.[111,112] Here we focus on conventional TCR α/β CD4$^+$ T cell subsets that contribute to anti-tumor immunity via Ag recognition of peptides presented by MHC class II molecules, and not on subsets that recognize other types of Ags (e.g., glycolipids presented by CD1d to NKT cells) or suppress immunity (e.g., inducible and natural CD4$^+$ T regulatory cells).

The choice of which immune stimulatory pathway a naïve CD4$^+$ T cell takes occurs in the periphery when its TCR first engages its cognate peptide/ MHC class II Ag. The pathway choice is dictated during activation by Ag concentration, the type of APC engaged by the CD4$^+$ T cell, the costimulatory molecules the APC presents, and most importantly, on the cytokine milieu of the microenvironment.[113,114] Together these factors lead to epigenetic changes and the corresponding expression of key transcription factors, the balance of which determine the gene expression and cytokine secretion profiles that define subsets of activated CD4$^+$ Th cells.[115–121] The cytokines secreted by CD4$^+$ Th cells then shape ensuing immune responses by signaling through cytokine receptors on other immune effector cells. Table 2 summarizes the CD4$^+$ Th subsets known in mice and humans, their cytokine and effector molecule secretion profiles, and their dominant, defining transcription factors. Note that, in

the T follicular helper subset family, specialized T follicular helper cells secrete the various cytokines.

Activated $CD4^+$ Th cells also indirectly stimulate Ag-specific $CD8^+$ T cells by "licensing" APCs such as DCs. Licensing results from engagement of CD40 on the DC with its ligand (CD154) on the activated $CD4^+$ Th cell.[122,123] CD40-mediated signaling stimulates the DC to provide all three signals necessary for the differentiation of Ag-specific naïve $CD8^+$ T cells to effector cytotoxic T lymphocytes (CTLs): Ag, co-stimulation, and pro-inflammatory cytokines.[124–126]

CD40-mediated signaling promotes Ag cross-priming of $CD8^+$ T cells to exogenous proteins taken up, processed, and cross-presented as selected peptides with MHC class I molecules by DCs.[127–129] CD40-mediated signaling also enhances DC expression of the costimulatory molecules CD80 and CD86 while stimulating the secretion of pro-inflammatory cytokines, most notably IL-12.[125] The effects on DCs mediated by Th cells are critically important in stimulating, amplifying, and directing effector and memory Ag–specific $CD8^+$ T responses.[130]

In addition to helping $CD8^+$ CTLs, Ag-specific $CD4^+$ T cells may themselves directly lyse MHC class II positive cells. Mouse and human $CD4^+$ Th cytotoxic (ThCTL) cell lines were first reported over three decades ago, but were discounted as artifacts because they were derived from long-term Ag stimulation *in vitro*.[131–134] Now ThCTLs are considered a naturally occurring $CD4^+$ T cell subset; they exist in many species and in healthy individuals (about 2% of peripheral blood $CD4^+$ T cells).[135,136] This subset is expanded in people seropositive for chronic viral infections such as human immunodeficiency virus 1, human cytomegalovirus, and hepatitis viruses, and in mice chronically infected with gamma-herpes and lymphocytic choriomeningitis viruses.[136–140] In addition to chronic Ag exposure, acute Ag exposure expands ThCTLs as their frequencies are increased in mice within a week after infection with influenza virus A or the intracellular bacterial pathogen *Brucella abortus*.[141,142] Increased numbers of ThCTLs are also detectable in mice and humans with various malignancies, as discussed next.

1. $CD4^+$ Th1 Cells and Anti-Tumor Immunity—$CD4^+$ Th1 cells are traditionally thought to be secondary to cytolytic effector cells in anti-tumor immunity. Ascribing "second class citizen" status to $CD4^+$ Th1 cells is misleading, though, because they are usually necessary to eradicate tumors by licensing DCs and providing cytokine support to $CD8^+$ CTLs and NK cells. Evidence for this comes from reports of limited anti-tumor effects of cytolytic effectors alone.[143–145] Consequently, the paradigms of immunizing cancer patients with vaccines containing peptides only recognized by $CD8^+$ T cells, or adoptively transferring *ex vivo* expanded autologous $CD8^+$ lymphocytes alone, have shifted to strategies that include inducing or adoptively transferring tumor-specific $CD4^+$ Th1 cells.

One such strategy is to immunize with long peptides containing both $CD4^+$ and $CD8^+$ T cell epitopes. Harao *et al.* used genome-wide microarray analysis to identify a novel cancer-testis Ag, cell division cycle–associated 1 (CDCA1), overexpressed in lung, head-and-neck, and other cancers.[146,147] They reported CDCA1 contained immunogenic HLA-A2 (A*02:01) –

restricted peptides that induce Ag-specific $CD8^+$ CTLs from the peripheral blood mononuclear cells (PBMCs) of lung cancer patients.[147] The investigators then employed a recently developed computer algorithm to find long CDCA1-derived peptides (24–26 amino acids) that include one of these defined HLA class I epitopes plus novel peptides predicted to bind HLA class II molecules.[148] They identified two long peptides, $CDCA_{139–64}$-LP and $CDCA_{155–78}$-LP, that induce both $CD4^+$ Th1 and $CD8^+$ CTL responses from PBMCs isolated from head-and-neck cancer patients but not healthy controls. This approach also successfully defined long peptides derived from the kinesin family member 20A, a protein frequently overexpressed in many solid tumors, including bladder, breast, gastric, lung, and pancreatic cancers.[149] Again, these long peptides induced both $CD4^+$ Th1 and $CD8^+$ CTL responses from PBMCs isolated from cancer patients but not healthy individuals. Together these findings should inform future vaccine-based or adoptive cell transfer clinical trials for patients with solid tumors.

The approach arguably having the most profound success in cell-based cancer immune therapy is the adoptive cell transfer (ACT) of autologous, *ex vivo* expanded, tumor-recognizing T cells. Recognition is conferred either by the endogenously expressed TCR on tumor-infiltrating lymphocytes (TILs) or by genetically engineered CARs expressed by transduced PBMCs.[150,151] TIL-based ACT typically employs $CD8^+$ T cells with some stunning positive results: ~50% objective clinical responses in patients with advanced melanoma refractory to standard therapies.[152] However, many patients do not achieve objective tumor responses despite the persistence of adoptively transferred $CD8^+$ TIL clones that retain *in vivo* Ag responsiveness.[153] These data suggest that the specificity of the selected TILs is not optimal, that $CD4^+$ Th cells are required, or both.[154]

CAR-based ACT uses transduced PBMCs that contain a mixture of $CD4^+$ and $CD8^+$ T cells; no direct clinical comparisons between pure $CD4^+$ and $CD8^+$ CAR-transduced cells are yet available. However, recent preclinical data suggest that early activation of $CD4^+$ CAR cells is critical for potent and durable anti-tumor immunity in an orthotopic model of mesothelioma.[155] These and other data are redirecting attention to the inclusion of $CD4^+$ T cells in ACT trials for cancer.

Hunder *et al.* adoptively transferred *ex vivo* expanded autologous, PBMC-derived, $CD4^+$ Th1 cell clones into a patient with melanoma refractory to conventional chemotherapy.[156] The clones were specific for a peptide derived from the melanoma-associated Ag NY-ESO-1 presented by the HLA class II molecule HLA-DOB1*0401. The patient did not require exogenous cytokine therapy because the clones produced autocrine IL-2. The entire tumor regressed even though only 50–75% of the cells expressed NY-ESO-1, which suggests that responses against other epitopes were also elicited. Epitope spreading was confirmed because the patient had PBMCs specific for melanoma Ags MART-1 and MAGE-3 after therapy. Responses to these Ags were undetectable prior to ACT.

What this case report has in common with most clinical trials describing objective tumor responses following ACT of TILs or CAR-transduced T cells is that it targeted the “low-hanging fruits” on the cancer Ag tree. These include differentiation Ags overexpressed on malignant versus healthy tissues (e.g., MART-1), Ags shared by malignancies and

nonessential healthy tissues (e.g., CD19), and cancer-testis Ags, which are shared tumor Ags with expression on healthy cells limited to male germ cells in the testis (e.g. NY-ESO-1 and MAGE-3). For ACT therapies to be maximally effective, they should spare healthy tissues by targeting truly tumor-specific Ags that arise by mutations, ideally by mutations that drive the malignant phenotype.[154]

Tran *et al.* used whole exomic sequencing to show that TILs isolated from a patient with meta-static cholangiocarcinoma contained $CD4^+$ Th1 cells specific for a mutated peptide derived from ERBB2IP ($ERBB2IP^{E850G}$), an ERBB2IP-interacting protein mutated in that patient's cancer.[157] About 25% of the polyclonal, *ex vivo* expanded $CD4^+$ Th1 cell TILs transferred back into the patient recognized $ERBB2IP^{E850G}$ presented by HLA-DQB1*0601. Tumor regression lasted for approximately one year and after recurrence the patient was infused with a highly purified (>95%) population of $ERBB2IP^{E850G}$/ HLA-DQB1*0601–specific Th1 cells. Tumor regression occurred more quickly than before (within one month) and remained stable for at least six months (the time of publication). These and other clinical data support expanding autologous $CD4^+$ T cells specific for tumor-associated and, more important, tumor-specific Ags for ACT therapy.

2. $CD4^+$ Th17 Cells and Anti-Tumor Immunity—$CD4^+$ Th17 cells can infiltrate solid tumors, but their contribution to anti-tumor immunity is unclear.[158] This is because the cytokines that Th17 cells secrete can be both proinflammatory and immune suppressive, and Th17 differentiation and function are affected by the tumor micro-environment. Melanoma is one tumor in which Th17 TILs appear to stimulate anti-tumor immune responses. Martin-Orozco *et al.* reported that poorly immunogenic B16/F10 melanoma cells colonized the lungs of IL-17–deficient mice in significantly higher numbers than the lungs of wild-type controls, and that Th17 ACT triggered a strong tumor-specific CTL response in wild-type mice.[159] The transferred Th17 cells promoted DC infiltration of the tumors and subsequent Ag cross-presentation and cross-priming of naïve $CD8^+$ T cells. The Th17 TILs also stimulated stromal cells to produce chemokines (e.g., CCL20) that recruited CTLs to the tumor. These findings are consistent with others showing that Th17 cells are more effective than Th1 cells in eradicating melanomas in ACT mouse models, most likely because Th17 cells are superior to Th1 cells in promoting $CD8^+$ T cell cross-priming.[160,161]

3. ThCTLs and Anti-Tumor Immunity—Ag-specific $CD4^+$ Th cells can differentiate into MHC class II–restricted cytotoxic lymphocytes (ThCTLs) that are comparable to $CD8^+$ CTLs in potency.[112,162,163] Like $CD8^+$ CTLs, ThCTLs kill Ag-expressing target cells via the perforin/granzyme B pathway or CD95/ CD95L (Fas/FasL) engagement. $CD4^+$ ThCTLs comprise TILs in solid tumors such as melanoma and hepatocellular carcinoma (HCC).[164,165] In a study of 547 patients with hepatitis B virus (HBV) –related HCC, Fu *et al.* found significantly higher frequencies of circulating and liver-infiltrating ThCTLs in early stage HCC patients compared to healthy controls and patients chronically infected with HBV.[165] Granzyme A and B and perforin expression in ThCTLs and CD107a mobilization (a lysosomal-associated membrane glycoprotein associated with the release of cytolytic granules) were also higher in HCC patients than in patients chronically infected with HBV. ThCTLs from HCC patients secreted IFN-γ and TNF-α, cytokines associated with the Th1

subset. Despite the overall elevated numbers of ThCTLs in HCC patients, the frequency of ThCTLs in the tumor itself was significantly lower than in normal liver, suggesting that the tumor micro-environment was immune suppressive. Finally, both ThCTL frequency and lytic function decreased as HCC progressed. The loss in ThCTL frequency correlated significantly with disease progression and high mortality. Because the levels and functions of circulating ThCTLs mirrored those of liver-infiltrating ThCTLs, the frequency of the former may prove to be a useful biomarker for HCC progression.

4. CD4$^+$ Th Plasticity and Anti-Tumor Immunity—A hallmark of CD4$^+$ T cell biology is subset plasticity.[111] The expression of subset-defining activating and inhibitory transcription factors that drive subset differentiation is not terminal but dynamic, and it is the balance that dictates CD4$^+$ Th phenotype and function. The result of this dynamic is plasticity: a Th1 cell may become a Th17 or a ThCTL as the balance of transcription factors shifts as a result of changes in the *in vivo* micro environment or in the *ex vivo* culture conditions. In a mouse model of late-stage melanoma, Quezada *et al.* transferred low numbers of naïve CD4$^+$ T cells bearing a transgenic TCR specific for the melanoma-associated Ag Trp1 into radiation-induced lymphopenic mice bearing established B16 melanoma.[166] The naïve CD4$^+$ T cells proliferated and differentiated into effector ThCTLs *in vivo* and eradicated the tumors in a MHC class II–restricted manner, which was enhanced by CTLA-4 blockade.

These preclinical results were replicated clinically when Kitano *et al.* generated MHC class II-restricted, NY-ESO-1-specific CD4$^+$ T cell clones from four advanced melanoma patients treated with Ipilumimab.[164] The clones secreted Th1-associated cytokines (IL-2, IFN-γ, and TNF-α) but not Th2-associated cytokines. They also expressed the Th1-defining transcription factor T-bet as well as the transcription factor Eomesodermin (Eomes). T-bet and Eomes are members of the T-box family of transcription factors, which is closely associated with CD8$^+$ T cell differentiation into cytolytic effectors. Correspondingly, the NY-ESO-1–specific clones expressed the degranulation markers perforin, granzyme B, and CD107a, and lysed NY-ESO-1–positive target cells in an MHC class II–restricted manner. For all intents and purposes, these clones are poly-functional CD4$^+$ Th1/ThCTLs.

Polyfunctionality is a point on a continuum between one Th subset and another. Such subset plasticity results from transcriptional reprogramming of mature CD4$^+$ Th in the periphery. Mucida *et al.* identified a population of CD4$^+$CD8a$^+$ ThCTLs in the gut that lack expression of Th-inducing POZ-Kruppel Factor (ThPOK); this is a transcription factor turned on during thymic development that helps flip the differentiation switch to the CD4$^+$ lineage in double positive thymocytes.[167] These ThCTLs arose from CD4$^+$ Th cells that extinguished ThPOK expression during their migration from the thymus to the gut. Reis *et al.* showed ThPOK gene silencing begins with upregulation of Runx3, a transcription factor that orchestrates expression of specific genes by CD8$^+$ CTLs.[168] The nascent CD4$^+$CD8a$^+$ ThCTLs remain functionally dormant in the gut until activated by the proinflammatory cytokine IL-15. ThPOK and Runx3 negatively regulate each other, as ThPOK is required by mature CD4$^+$ Th cells to repress Runx3.[169]

As our knowledge of $CD4^+$ T cell biology grows, so does our ability to manipulate these cells for therapy. Further insights into the signals and transcription factors that drive differentiation of $CD4^+$ Th into anti-tumor effector cells will no doubt lead to more effective *in vitro* culture conditions to drive functionally heterogeneous TILs into effector subsets, or will permit differentiation of PBMCs into long-lived T cells for CAR-based therapies.

VII. CONCLUSIONS

Despite increasing interest in using immunotherapeutic approaches in cancer treatment, the prognosis for patients diagnosed with malignant glioma remains poor. Advances in cancer immune therapy have been limited, and an inherent hurdle to progress is the lack of a basic understanding of the mechanisms needed for an effective immune-based anti-tumor response. Understanding the many facets of the immune system and examining the immune components involved in an effective anti-tumor response can provide insight into these mechanisms (Figure 1).

Many cancer vaccines have been designed to induce a robust $CD8^+$ T cell response. Although $CD8^+$ T cell cytotoxicity may play a role in effecting an antitumor response, at least one study in a murine model of glioma demonstrated effective tumor control that was $CD8^+$ cell–independent and showed that $CD8^+$ cell depletion actually improved overall survival.[110] Even in $CD8^+$ T cell–mediated tumor clearance, recruitment of additional immune cells such as $CD4^+$ Th1 cells that maintain the $CD8^+$ T cell response is necessary. It is likely that a concerted effort of a variety of immune cells may be necessary to elicit and maintain an effective anti-tumor response.

In this article, we reviewed several potential mechanisms to stimulate an anti-tumor immune response other than $CD8^+$ T cell cytotoxicity. CD4+ T cells are capable of cytolytic function through their direct and indirect killing in tumor models. $CD4^+$ T cells activated through OX40 ligation or direct activation by OX40L may mediate cytokine secretion for the recruitment and activation of NK cells, NKT cells, and neutrophils. Other subsets of $CD4^+$ T cells, such as $CD4^+$ Th1 cells, may play an important and necessary role in tumor eradication by licensing APCs and providing cytokines that support $CD8^+$ CTLs and NK cells.

The importance of B cells in a successful response to therapy has been largely overlooked. B cells can act as APCs for $CD4^+$ T cell activity and can differentiate into plasma cells and secrete tumor-reactive antibodies or antibodies required for ADCC. Glioma-bearing mice generated tumor-reactive antibodies that were shown to bind to tumors after immunotherapy with tumor lysate vaccine and OX40L.[110] The infiltration of innate immune cells in vaccinated animals points the possibility that NK cells, neutrophils, and other immune cells actively kill antibody-coated tumor cells. For this reason, it is likely that many different mechanisms are at play for successful tumor clearance.

A deeper understanding of immune-mediated mechanisms of tumor clearance should drive the design of new immunotherapy regimens. The interplay of many components is likely

necessary to fully harness the power of the immune system to mediate lasting tumor clearance with memory.

ABBREVIATIONS

α-GalCer	α-galactosylceramide
Ag	antigen
APC	antigen-presenting cell
CAR	chimeric Ag receptor
CD	cluster of differentiation
CNS	central nervous system
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DLN	draining lymph node
GBM	glioblastoma multiforme
HBV	Hepatitis B virus
HLA	human leukocyte antigen
IFN-γ	interferon gamma
IL	interleukin
KIR	killer cell immunoglobulin-like receptor
M2	type 2 macrophage
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
NK cell	natural killer cell
NKT cell	natural killer T cell
PBMC	peripheral blood mononuclear cell
PGE2	prostaglandin E2
si-RNA	small interfering ribosomal nucleic acid
TADC	tolerogenic tumor-associated dendritic cell
TAM	tumor-associated macrophage
TCR	T cell receptor
TGF-β	transforming growth factor beta
Th	helper T cell
ThPOK	Th-inducing

POZ	Kruppel Factor
Treg	T regulatory cell

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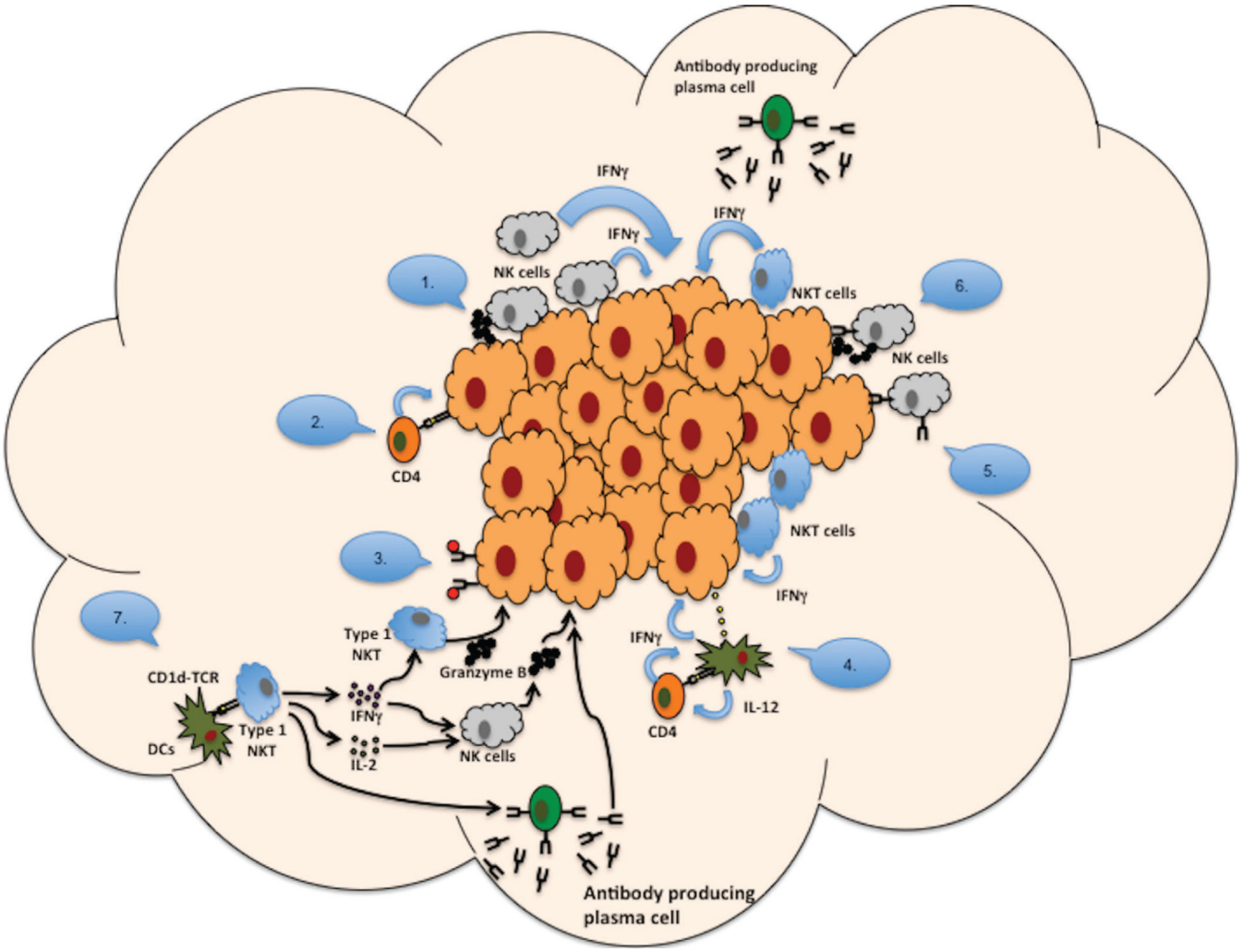

FIG. 1.

Mechanisms used by immune cells to attack tumors: (1) NK cells can release cytotoxic granules at sites of direct contact with Ag-bearing tumor cells. (2) Cytotoxic $CD4^+$ T cells can kill tumor cells that express Ag on MHC II. (3) Antibodies bind to target Ags (e.g., CD20) and complement binds to antibodies, resulting in a complement cascade that lyses tumor cells. (4) Following Ag presentation, IFN-γ–activated macrophages can acquire a tumoricidal phenotype and up-regulate CTL markers, including granzyme A/B and NKG2D. (5) Fc receptors on NK cells can bind to tumor cells opsonized by plasma cell–secreted antibodies. (6) FcγRIII receptors on NK cells can bind to tumor cells by plasma cell–secreted antibodies, inducing ADCC; tumor cells are lysed by perforins and granzymes secreted by NK cells. (7) NKT cells can respond through CD1b presentation to DC and secrete a variety of cytokines (e.g., IFN-γ, IL-2) that activate other NKT cells, NK cells, and antibody-producing plasma cells.

TABLE 1

Receptors used by NK cells to mediate different effector functions

Activation receptors	Inhibitory receptors	Dual function receptors
NKG2D	KIR2DL1	CD94-NKG2
NKp30	KIR2DL2	2B4
NKp44	KIR2DL3	NTB-A
NKp46	KIR3DL1	KIR2DL4
NKp80	KIR3DL2	
DNAM1	LILRB1 [LIR-1]	
CD96	KIRG1	
CD16 [Fcg RIIIA]	NKR-P1A	
KIR2DS1	Siglec7 and/or Siglec9	
KIR2DS2		
KIR3DS1		
BY55 [CD160]		
CD2		

Note: Alternative receptor nomenclature is given in brackets. DNAM1 = DNAX accessory molecule 1; KIR2DL2 = killer cell immunoglobulin-like receptor (KIR), two domains, long cytoplasmic tail, 2; KIR2DS1 = KIR, two domains, short cytoplasmic tail, 1; KLRG1 = killer cell lectin-like receptor G1; LILRB1 = leukocyte immuno-globulin-like receptor, subfamily B, member 1; NKG2 = NK group 2; NKp = NK cell protein; NKR-P1A = NK cell receptor protein 1A; Siglec = sialic-acid-binding immunoglobulin-like lectin.

TABLE 2

Subsets of $CD4^+$ Th cells and their known effector molecules and defning transcription factors

Th Subset	Secreted effector molecules	Transcription factors
Th1	IL-2, IL-10, IFN-γ, TNF-α, TNF-β/LT-α	T-bet
Th2	IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IL-25, IL-31	GATA3
Th9	IL-9, TNF-α, and IL-10 in mice)	PU.1/Spi-1 and IRF4
Th17	IL-17A, IL-17F, IL-17A/F, IL-21, IL-22 (and IL-26 in humans)	RORγt
Th22	IL-13, IL-22, TNF-α	AHR
Thf	IL-4, IL-10, IL-12, IL-21, IFN-γ	Bcl-6
ThCTL	IL-2, IFN-γ, TNF-α, granzyme/perforin	Runx3