Targeted Therapies: Immunologic Effects and Potential Applications Outside of Cancer

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The recent success of medications that enhance antitumor immune responses by disrupting “immune checkpoints” highlights the therapeutic dividends that can be paid by understanding the regulatory mechanisms of the immune system.\(^1\) Using this approach, commonly termed “immune checkpoint blockade” has yielded clinical advances across a spectrum of malignancies including melanoma, lung cancer, and renal cell carcinoma.\(^2\)–\(^4\)

The success of these agents illustrates the enormous translational potential of understanding and controlling the immune system and justifies additional research to identify other regulatory aspects of the immune system as candidates for new drug development.\(^5\) In addition, these clinical successes have underscored the need to define the immune effects of all forms of anticancer therapy for the following reasons: (1) a properly steered antitumor immune response can be curative even in patients with advanced cancer refractory to other therapies; (2) various anticancer agents have underappreciated immunomodulatory activity; (3) the optimal use of regimens that combine immune-based treatments with other forms of therapy will require a sophisticated and comprehensive understanding of all of the immune effects that are at play.\(^6\),\(^7\) In-depth reviews have highlighted these issues with memorable titles in which these effects have been described as “the secret ally” and in terms of what happens when “universes collide”.\(^8\),\(^9\) As the concept of cancer evolves from one that is cell-autonomous to one that incorporates the complex role of the tumor microenvironment on cancer growth and regression, the approach to therapy must also evolve.\(^7\),\(^10\)

By understanding the numerous interactions between tumor cells and nontumor cells in the microenvironment that underpin cancer pathogenesis, progression, and the response to therapy, we can continue to build on the exciting momentum that has been generated by newer anticancer therapies. Likewise, because most anticancer treatments are not immunologically null, characterizing the occult immune effects of therapies not originally developed as immunotherapies may facilitate the development of more precise and effective combinatorial treatment paradigms.

**The Immune Effects of Growth Factors**

Before considering how targeted therapies influence immune responses, it is worth providing some relevant background as it relates to the underlying biology of growth factors.

As described above, targeted therapies were developed to specifically block aberrantly activated signal transduction pathways within malignant cells, many of which arise from deregulated growth factor receptor tyrosine kinases (RTKs) or cellular protein kinases that can initiate oncogenic signaling.\(^11\),\(^12\) Many of these enzymes and signal transduction pathways were originally defined and conceptualized for their roles in growth factor (and growth factor receptor) biology rather than their roles in immunology.\(^13\) Thus, because many targeted therapies directly disrupt growth factor signaling (within tumor cells and potentially...
nontumor cells), it is important to revisit the immune effects of growth factors as a framework to understand the immune effects of targeted therapies. In the paragraphs below we use vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) ligand amphiregulin (AREG) as model growth factors and briefly review some of their reported immune effects. These growth factors were selected because pharmacologic inhibitors of VEGF and the EGFR are currently in clinical use.\textsuperscript{14,15}

VEGF is a proangiogenic growth factor that is crucial to maintaining tumor vascularity and nutrient supply.\textsuperscript{16} Its role in tumor angiogenesis fueled the development of bevacizumab, an anti-VEGF monoclonal antibody (mAb).\textsuperscript{17} In addition to its role in angiogenesis, VEGF has been shown to have multiple immunosuppressive effects including inhibition of T-cell development, inhibition of dendritic cell maturation, and an ability to promote the recruitment of immunosuppressive myeloid-derived suppressor cells (MDSCs).\textsuperscript{18–21} Moreover, VEGF has been shown to promote the induction of regulatory T cells (T regs) in the tumor microenvironment of mice as well as metastatic colorectal cancer patients.\textsuperscript{22} Recent studies have suggested VEGF expression by tumor cells promotes the expression of the inhibitory receptor programmed cell death (PD)-1 on the surface of CD8+ T cells, thereby promoting an “exhausted” phenotype of tumor-infiltrating cytotoxic T cells.\textsuperscript{23} Thus, despite being initially characterized for its effects on vasculature biology, VEGF expression can influence immune processes that may be central to the generation of an antitumor immune response.\textsuperscript{24} In support of this notion, higher pretreatment VEGF serum levels are associated with shorter overall survival times in melanoma patients treated with the immune checkpoint inhibitor ipilimumab, a mAb that blocks cytotoxic T lymphocyte antigen 4 (CTLA4).\textsuperscript{25} Moreover, regimens combining ipilimumab (anti-CTLA4) and bevacizumab (anti-VEGF) are being evaluated in patients with melanoma and glioblastoma.\textsuperscript{26–29} These studies have demonstrated that the combination of bevacizumab (anti-VEGF) and ipilimumab (anti-CTLA4) can be safely administered with predictable and manageable toxicity. Moreover, these studies illustrate that the combination of bevacizumab and ipilimumab can augment endothelial cell activation, lymphocyte infiltration into tumors, cytokine and chemokine expression, and antimelanoma humoral responses.

AREG is a member of the EGF family of ligands that includes several growth factors (additional factors include EGF, transforming growth factor-α, epigen, and several others) that bind to and activate members of the human EGF receptor (HER) family that are worth briefly reviewing.\textsuperscript{30,31} The HER family includes 4 structurally related receptors that have distinct ligand-binding abilities, intrinsic tyrosine kinase activities, and abilities to form homo- and heterodimers with other members of this family. These RTKs have sequence and structural homology with the EGFR (also referred to as ErbB1/HER1) and include ErbB2/HER2/\textit{neu}, ErbB3/HER3 and ErbB4/HER4. The ErbB (or c-erb-B) designation stems from the fact that these RTKs share homology with the avian erythroblastic leukemia viral (v-erb-B) oncogene.\textsuperscript{32} The additional \textit{neu} designation for ErbB2/HER2 stems from the name given to this oncogene when it was originally cloned as an oncogene isolated from the neuro/glioblastomas that developed in the offspring of rats injected with the carcinogen ethylnitrosourea.\textsuperscript{33} Both the EGFR and ErbB2 have received enormous attention as therapeutic targets for cancers of the lung, colon, and head and neck (and others) for the EGFR, and breast cancer for ErbB2.\textsuperscript{34–37}
Like other ErbB ligands, AREG is known to bind to and activate the EGFR. AREG derives its name from its seemingly paradoxical ability to induce cell proliferation in some cell lines, whereas it induces growth arrest and differentiation in others. Although ErbB ligands such as AREG have been mainly studied in the context of epithelial development and epithelial cancers, their roles in the immune system have received less attention until very recently. For example, AREG is known to be expressed by epithelial cells, yet it is also expressed by cellular components of the immune system including dendritic cells, neutrophils, mast cells, and CD4+ T cells. Key immunologic roles for AREG have recently been uncovered by Zaiss and colleagues when they discovered that AREG modulates the activity of T regs in mouse models of colitis and melanoma. More recently, AREG has been implicated in the immune suppression mediated by ultraviolet radiation (UVR), which plays an important role in the development of UVR-induced skin cancers. Thus, canonical growth factor ligands have pleiotropic immune effects in part through their ability to modulate the function of immune cells such as T lymphocytes. As a result, targeted therapies that inhibit growth factor receptors and/or their downstream kinases influence processes within tumor cells and immune cells within the tumor microenvironment, and both are likely to be relevant to the generation of effective antitumor immunity.

**Effects of Targeted Therapies on Tumor Cells Relevant to Antitumor Immunity**

There is mounting evidence that inhibition of oncogenic signaling using targeted therapies can influence tumor:immune cell interactions. For example, the selective BRAFV600E kinase inhibitors vemurafenib and dabrafenib induce marked T lymphocyte infiltration into melanoma tumors. In addition, the EGFR blocking antibody cetuximab was shown to activate natural killer (NK) cells to promote dendritic cell maturation and CD8+ T-lymphocyte activation. Such effects likely depend on a variety of interrelated processes including those mediated by tumor-intrinsic factors, therapy-dependent factors, and host-dependent factors. Tumor-intrinsic factors will likely include the cellular origin of the tumor, its genomic and epigenetic landscape, and the activation status of signal transduction pathways within tumor cells. For example, recent studies have suggested that the load of neoantigens (altered peptides resulting from mutations, deletions, or translocations in the coding sequences of genes) expressed by a tumor can be relevant to antitumor immunity by increasing the likelihood that tumor cells are recognized as foreign (nonself) by the immune system.

There are several therapy-dependent factors that will likely influence how these medications modulate the generation of an anticancer immune response. For example, whether a medication is a therapeutic mAb or a small molecule kinase inhibitor will likely be relevant because (as discussed in more detail below) antibodies possess unique immunologic properties. Equally important will be the medication’s mechanism(s) of action. These may include the specific pathways/enzyme(s) that are inhibited as well as the cells (tumor cells and nontumor cells) that are impacted by the medication in question. It is also worth noting the possibility of off-target effects and differences in this regard between mAbs and small molecule kinase inhibitors.
molecule kinase inhibitors. The mAbs bind with high specificity to extracellular targets to disrupt growth factor receptor signaling. In contrast, small-molecule kinase inhibitors act on intracellular enzymes and may influence the activity of other targets than their intended one. This makes understanding and interpreting their immunologic impact on the tumor microenvironment more complex because both nontumor and off-target effects need to be considered.50

Host-dependent factors may include variables that influence immune responses in general such as age and comorbidities such as diabetes and obesity.51–53 It is also possible that a person’s genotype may play a role because genetic polymorphisms can influence the development of toxicity in response to targeted therapies.54 In the paragraphs below we discuss how the effects of targeted therapies on tumor cells may influence antitumor immune responses.

**Antibody-Mediated Effects From Therapeutic Monoclonal Antibodies**

As mentioned in the previous paragraphs, targeted therapies used in cancer can be broadly divided into small-molecule kinase inhibitors used to inhibit the activity of oncogenic enzymes and therapeutic mAbs that disrupt the activation of growth factor receptors or other receptors germane to cancer.55,56 Because antibodies are normal elements of the immune system, therapeutic mAbs not only disrupt oncogenic signaling but additionally provide a platform for interactions between tumor cells and immune cells. For example, therapeutic mAbs have been reported to enhance the ability of cells of the immune system to engulf tumor cells, a process known as opsonization.57 This occurs because many cells of the immune system express specialized antibody-binding receptors that bind to the Fc region of mAbs (Figure 1). These receptors, known as Fcγ receptors (FcγRs), are expressed by immune cells such as NK cells, monocytes, and granulocytes and can play a crucial role in the antibody-mediated recognition and killing of tumor cells.58 This antibody-mediated process is known as antibody-dependent cellular cytotoxicity (ADCC). The triggering of ADCC involves the release of cytotoxic granules containing perforin and granzyme, a theme common to other forms of antitumor immunity such as those mediated by cytotoxic T lymphocytes (CTLs). The ability to generate ADCC has been proposed as an important antitumor mechanism of action for mAbs that bind to the EGFR.59,60 Conceptually, this enables mAbs to both disrupt EGFR-mediated growth and survival signals within tumor cells while also initiating an immune-mediated attack at the surface of the tumor cell. Moreover, a better clinical response to the anti-EGFR mAb cetuximab has been linked to specific polymorphisms present within FcγRs.58,61,62 These genetic variations can influence the binding affinities of FcγRs for mAbs, which in turn can influence the potency of ADCC-mediated antitumor responses.53 This suggests that genetic variation within immunologic genes can influence the response of cancer patients to mAbs.61–64 Similar correlations of FcγR genotype and clinical outcome have been observed for trastuzumab, a HER2-specific therapeutic mAb used in treatment of breast cancer.65,66 Thus, through their ability to engage with immune cells, therapeutic mAbs are intrinsically able to mediate interactions between tumor cells and cells of the immune system to influence antitumor immunity.67
In line with the above concept, there is evidence that the specific isotype and/or glycosylation status of an individual therapeutic mAb is relevant in this regard because these differences influence the aforementioned interactions.  For example, the immunoglobulin G (IgG) isotype of EGFR-specific mAbs is thought to be important in determining which lineage of cells predominates in mediating ADCC. \[^{69}\] Fc \(\gamma R\) IIIA (CD16) is expressed on the surface of NK cells but not neutrophils and has a higher affinity for IgG1 over IgG2 suggesting that IgG2-based therapeutic mAbs would be less able to stimulate this form of cellular killing by NK cells. There is evidence to suggest that this is indeed the case. For example, panitumumab and zalutumumab are EGFR-specific antibodies of the human IgG2 and IgG1 isotypes respectively and NK cell-mediated ADCC occurs with zalutumumab and not with panitumumab. \[^{70}\] In contrast, both antibodies are equally effective at stimulating ADCC by neutrophils that express different Fc \(\gamma\) receptors than NK cells that have different IgG isotype affinities. \[^{69}\]

**Monoclonal Antibody-Based Anticancer Therapies Can Act via Complement Activation**

Another immune-related mechanism through which anticancer mAbs may be working is via the activation of complement and complement-dependent cytotoxicity (CDC). CDC can be initiated through interactions between the Fc regions of mAbs and complement proteins such as C1q. \[^{71}\] These interactions can promote the assembly of the membrane attack complex on the tumor cell surface, which can alter tumor cell membrane permeability and lead to tumor cell lysis. \[^{72}\] In vitro studies have demonstrated a complement-mediated cytotoxic effect on tumor cells for CD20-targeted mAbs rituximab and ofatumumab. \[^{73}\] Additional studies using EGFR-inhibiting mAbs have shown that when used alone these agents do not induce complement deposition or CDC. However, combination therapy of cetuximab and matuzimab, 2 EGFR-specific antibodies that bind to distinct parts of the receptor, elicits strong synergistic complement deposition and thus tumor cell lysis. \[^{74}\]

**Targeted Therapies Can Influence the Expression of Genes Involved in Antigen Processing, Presentation, and Tumor-Associated Antigens**

The generation of an antitumor immune response mediated by CD4+ and CD8+ T lymphocytes has been and continues to be a major goal of most forms of immunotherapy. Although the generation of a T-cell-mediated antitumor immune response is a complex process, a central theme involves the presentation of peptide antigens by tumor cells to CD4+ and/or CD8+ T lymphocytes; a pivotal step in the generation of antitumor immunity. A comprehensive review of antigen processing and presentation is beyond the scope of this review, but in the subsequent paragraphs we provide a brief overview and describe how targeted therapies may influence these processes.

Antigen processing involves the cleavage of proteins into peptides that are ultimately presented by major histocompatibility complex (MHC) class I (MHCI) and class II (MHCII) molecules. \[^{75}\] Peptides that are presented by MHCI molecules are derived from intracellular proteins, whereas those bound for presentation by MHCII molecules are derived from exogenous sources. An important caveat to this paradigm can occur within professional antigen-presenting cells, which involves a process called “cross-presentation” whereby exogenous antigens can be presented by MHCI molecules. \[^{76}\] Cross-presentation aside, the
typical generation of peptides for presentation by MHCI molecules that occurs within tumor cells and their normal counterparts involves 4 tasks that include peptide generation and trimming, peptide transport, assembly of the MHCI loading complex, and presentation of peptides at the cell surface.\textsuperscript{77,78} Cleavage of proteins into peptides occurs by proteolysis (via the ubiquitin-proteosome pathway), and peptide transport into the endoplasmic reticulum is mediated by the transporter associated with antigen processing (TAP) complex, which is composed of a complex of 2 proteins named TAP1 and TAP2. Importantly, targeted therapies can influence the expression of TAP proteins. For example, the anti-EGFR mAb cetuximab has been shown to increase the expression of TAP1, TAP2, and other proteins involved in antigen processing.\textsuperscript{79} In addition, the \textit{BRAF}^{V600E} kinase inhibitor vemurafenib was shown to enhance the induction (by interferon [IFN]-\textit{α}2b) of calnexin and calreticulin proteins involved in antigen processing.\textsuperscript{80} Thus, although tumor cells frequently downregulate proteins involved in antigen processing and presentation as a means of immune escape, there is evidence that targeted therapies can enhance the expression of these genes.\textsuperscript{81,82}

As noted above, once peptides are generated, their presentation at the cell surface ultimately involves them being “loaded” noncovalently onto MHCI or MHCII molecules.\textsuperscript{83,84} These peptide-MHC (pMHC) complexes at the cell surface are then able to engage the T-cell receptor on the surface of T lymphocytes, providing the antigen-specific signal (often referred to as “signal 1”) required for T-cell activation.\textsuperscript{85} In general, higher surface MHCI and/or MHCII expression is considered helpful for T-cell-mediated antitumor immunity and as a result is typically associated with better prognosis.\textsuperscript{86–90} This may reflect the fact that higher pMHC densities can promote T-cell activation.\textsuperscript{91,92} Not surprisingly, just as tumors downregulate proteins involved in antigen processing as a means of immune escape, they also downregulate the expression of MHC molecules via mechanisms that can be reversible or irreversible.\textsuperscript{78,93–96} As described below, in some contexts targeted therapies and the kinases they were developed to inhibit have been shown to alter the expression of MHC molecules (Figure 2).\textsuperscript{95,97–101}

EGFR inhibitors (EGFRIs) including tyrosine kinase inhibitors (TKIs) as well as the anti-EGFR mAb cetuximab have been shown to increase surface expression of both MHC class I and class II in primary and malignant keratinocytes in vitro and in skin biopsies from cancer patients receiving anti-EGFR treatment.\textsuperscript{102} Conversely, EGFR ligands and other ErbB ligands can have the opposite effect and repress MHC induction.\textsuperscript{102–107} Several other studies have confirmed that inhibition of the EGFR or other members of the ErbB family enhances MHC expression.\textsuperscript{100,101,108,109} Likewise, vemurafenib, a selective inhibitor of the \textit{BRAF}^{V600E} mutant kinase, has been shown to augment the induction of MHC class I and II expression by interferons in some \textit{BRAF}^{V600E}-positive melanoma cells.\textsuperscript{80,110} In contrast, forced expression of \textit{BRAF}^{V600E} had the opposite effect and could repress MHC class I levels.\textsuperscript{110} This finding has been further supported by a study demonstrating that the \textit{BRAF}^ {V600E} mutant kinase can directly target MHC class I molecules for internalization and degradation, thereby promoting tumor growth and immune evasion.\textsuperscript{111} These effects are consistent with in vivo studies demonstrating that in response to \textit{BRAF}^ {V600E} inhibitors, tumor infiltration by CD8+ T cells is increased.\textsuperscript{112} Moreover, increases in tumor-infiltrating CD8+ T cells during treatment with the \textit{BRAF}^ {V600E} inhibitors vemurafenib and dabrafenib.

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were associated with a reduction in tumor size.\textsuperscript{47} It is worth noting that although an increase in MHCI expression on tumor cells could potentially enhance the recognition and killing of tumor cells by CD8\textsuperscript{+} T cells, it could have the opposite effect on antitumor NK cells, which express inhibitory receptors for MHCI molecules known as killer immunoglobulin-like receptors.\textsuperscript{113} Further complicating this picture is the fact that ligands for activating NK-cell receptors such as MHC class I–related chain A and B can be regulated by growth factor signaling.\textsuperscript{114} These findings illustrate the intimate yet complex relationship between canonical oncogenic signaling pathways and the cellular machinery that influences interactions between tumor cells and cells of the immune system. In addition to altering the expression of proteins involved in antigen processing and presentation, targeted therapies have also been shown to alter the expression of proteins and the peptides derived from them that can function as tumor-associated antigens (TAAs).

TAAs are usually defined as peptides that are expressed, processed, and presented by tumor cells.\textsuperscript{115} While they may not be entirely tumor specific and may be expressed by nontumor cells, TAAs have the potential to facilitate the recognition of tumor cells by T lymphocytes.\textsuperscript{116} Importantly, targeted therapies have been shown to influence antitumor immunity by altering the expression of TAAs. For example, melanocyte differentiation antigens (MDAs) are TAAs derived from gene products (proteins) that play a role in melanocyte differentiation and as such can be expressed by melanoma cells. In response to treatment with a selective BRAF\textsuperscript{V600E} kinase inhibitor (named PLX4720), the expression of MDAs was found to be augmented with resulting increases in antigen-specific immune responses against MDA-derived peptides.\textsuperscript{117} Thus, targeted therapies can influence antitumor immunity by changing the expression of proteins, and thus the pool of presented peptides, in a manner that can facilitate the generation of an antigen-specific antitumor immune response (Figure 3).

Recent studies have further highlighted the importance of the antigens presented by tumors by demonstrating that the presentation of neoantigens is pivotal for clinical responses to ipilimumab (anti-CTLA4 antibody).\textsuperscript{118} It is worth noting that neoantigen expression is influenced by the mutational burden of a tumor. Some tumors are associated with high mutational burdens (such as ultraviolet radiation–induced skin cancers) and therefore high neoantigen expression, whereas other tumors with lower mutational burdens may express few such neoantigens.\textsuperscript{49} Although the impact of targeted therapies on neoantigen expression is unknown, it is conceivable that through their ability to influence antigen processing, presentation, and gene expression in general, they may influence the expression of neoantigens.

**Targeted Therapies Can Influence PD-L1 Expression**

In settings of chronic antigen exposure, the normal development of activated CD8\textsuperscript{+} T cells into memory T cells can become disrupted. In such settings, CD8\textsuperscript{+} T cells can enter a state where they lose important functions such as the ability to secrete cytokines and kill (viroly infected or malignant) cells.\textsuperscript{119} In addition to these deficits, another important hallmark of this “exhausted” state is an increased expression of inhibitory receptors such as PD-1.\textsuperscript{120} When PD-1 binds its ligands programmed death ligand-1 and 2 (PD-L1 and PD-L2), T-cell...
activation is repressed. This “immune checkpoint” limits immune responses including those against tumors and chronic viral infections. Fortunately, the functional deficits exhibited by exhausted CD8+ T cells can be reversed by blocking the interactions between inhibitory receptors and their ligands. This is highlighted by the success and expanding use of anti-PD-1 therapies (such as nivolumab and pembrolizumab) to treat advanced-stage cancer. It also highlights the need to understand how oncogenic signaling and targeted therapies influence the expression of regulatory proteins such as PD-L1/L2 because such effects could potentially influence antitumor immune responses and/or the response to immunotherapies. This is particularly relevant when one considers that treatment regimens utilizing both targeted and anti-PD-1 therapies are currently undergoing clinical trials (phases 1 to 3).

There is evidence that oncogenic signaling and targeted therapies influence the expression of PD-L1 in some cellular contexts. For example, in non-small-cell lung cancer (NSCLC), treatment of cells with a preclinical mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor (U0126) was shown to decrease PD-L1 expression. In another study on NSCLC, increased expression of PD-L1 was noted in tumors with activating mutations in the EGFR and gefitinib, a small molecule inhibitor of the EGFR, attenuated the expression of PD-L1. Another study using NSCLC cell lines demonstrated that small-molecule kinase inhibitors of the EGFR, MEK, PI3 kinase, and anaplastic lymphoma kinase could repress PD-L1 expression. In the context of melanoma, the MAPK pathway has been implicated in regulating PD-L1 expression, although cellular context is important because inhibitors of BRAF, MEK, and phosphoinositide 3-kinase (PI3K) have shown variable effects with both reduction and induction of PD-L1 being reported. In triple-negative breast cancer, the phosphatase and tensin homologue (PTEN)/PI3K/protein kinase B (Akt) pathway has been linked to regulating PD-L1 expression as both an Akt inhibitor (MK-2206) and rapamycin decreased PD-L1 expression. Although the modulation of inhibitory receptor ligands such as PD-L1 on tumor cells by targeted therapies is incompletely understood, such effects have the potential to influence the response to immunotherapy and warrant further investigation.

Alterations in Chemokine/Cytokine Expression by Targeted Therapies

In addition to altering the expression of molecules on the tumor cell surface (such as MHC molecules and PD-L1/2), oncogenic signaling and the targeted therapies developed to disrupt it can affect cytokine secretion within the tumor microenvironment. For example, the Ras oncoprotein has been shown to upregulate the expression of interleukin (IL-)1β, IL-6, and IL-8 in hematologic and epithelial cancers. The secretion of cytokines has been shown to be indispensable for tumorigenesis, and neutralization of both IL-6 and IL-8 has been shown to hinder angiogenesis and tumor growth and progression. Increased cytokine production is also elicited by the BRAFV600E mutation within melanoma cells, which has been shown to upregulate expression of IL-1α, IL-1β, IL-6, IL-8, IL-10, and VEGF. Treatment with the BRAFV600E kinase inhibitor vemurafenib is able to attenuate the production of these cytokines. It is worth noting that IL-10 and VEGF are immunosuppressive cytokines compared to the proinflammatory IL-1α, IL-1β, IL-6, and IL-8, which further obscures the impact of small-molecule inhibitors such as the
BRAFV600E inhibitor vemurafenib because the expression of both proinflammatory and immunosuppressive cytokines may be altered. The mechanism by which interleukins contribute to the immunosuppressive tumor microenvironment has been reported to depend heavily on cells known as tumor-associated fibroblasts (TAFs), which interact closely with tumor-infiltrating lymphocytes. TAFs isolated from melanoma patients were treated with IL-1 and were able to reduce the functional response of tumor-infiltrating lymphocytes in vitro by 4- to 5-fold compared to TAFs that did not receive IL-1 pretreatment. The mechanism of this response was shown to be dependent on the increased expression of COX-2, PD-L1, and PD-L2 by the TAF. Importantly, the BRAFV600E inhibitor was able to relieve this TAF-mediated immune suppression. The ability of targeted therapies to reduce the immunosuppressive ability of TAFs within the tumor microenvironment provides further evidence supporting the use of targeted therapies with immunotherapies that stimulate a tumor-specific T-cell response. These findings illustrate that targeted therapies influence the expression of soluble factors released by tumor cells that in turn can influence the recruitment and/or behavior of nontumor cells within the tumor microenvironment.

Effects of Targeted Therapies on Immune Cells Relevant to Antitumor Immunity

It has become clear that targeted therapies act on many cell types within the tumor microenvironment including those of the immune system. This is relevant because the tumor microenvironment contains immune cells (such as CD8+ and CD4+ T cells) that can recognize and eradicate tumor cells and other immune cells such as T regs and MDSCs that hinder antitumor immunity. Recent studies have demonstrated a role for some targeted therapies in combating the immunosuppressive tumor microenvironment by downregulating T regs and/or MDSC function, thus permitting the antitumor lymphocyte response to predominate (Figure 4). As previously mentioned, Zaiss et al showed in a murine model of melanoma that tumor-resident T regs expressed the EGFR on their surface, and EGFR ligands were able to stimulate the suppressive capacity of T regs. Further, treatment with the EGFR inhibitor gefitinib was able to abrogate the response seen by ligand addition. Elegant in vivo experiments using treatment with EGFRIs in mice bearing B16-F10 melanoma tumors (which lack EGFR expression) showed significant reduction in tumor size compared to peptide immunization alone, supporting an indirect antitumor activity of the EGFR mediated through its action on the tumor microenvironment. This may explain EGFRi efficacy in patients with EGFR-negative tumors. Similar effects have also been observed with sunitinib, an inhibitor of multiple tyrosine kinases including VEGF and platelet-derived growth factor receptors. In a murine model of colon carcinoma, sunitinib treatment was shown to decrease both the number and suppressive capacity of T regs from peripheral blood, and a 2-week interruption of therapy was accompanied by a rebound of suppressive activity mediated by MDSCs. A similar study in metastatic renal cell carcinoma patients showed a decrease in MDSCs and T regs in the peripheral blood in response to sunitinib therapy and a subsequent increase in effector cytokine production by circulating T lymphocytes. In mice, sunitinib administration prior to tumor-antigen vaccination also augmented tumor-specific T-lymphocyte responses, suggesting that targeted-therapy-mediated inhibition of existing T-reg responses is important for the priming...
and development of tumorlytic T-cell responses.\textsuperscript{141} Sunitinib has been shown to decrease frequencies of circulating Tregs and MDSCs in patients.\textsuperscript{142,143} This effect was perhaps potentiated by the increase in frequency of IFN-\(\gamma\)-producing type 1 helper T cells leading to an increased T-helper 1:T-helper 2 ratio and an overall augmented antitumor effect.\textsuperscript{143} Sunitinib has also been shown to lead to a decrease in expression of immunosuppressive cytokines including TGF-\(\beta\) and IL-10 and of negative regulatory immune checkpoint receptors, CTLA4 and PD-1 on tumor-infiltrating lymphocytes.\textsuperscript{144}

In addition to acting on Tregs, a role for the EGFR in the recruitment and response of innate immune cells was demonstrated in a study examining the role of the EGFR in hepatocellular carcinoma (HCC). In a series of conditional knockout experiments, it was shown that intact EGFR signaling leads to a substantial increase of serum CCL2, which directs the recruitment of liver macrophages or Kupffer cells.\textsuperscript{145} This study further demonstrated opposing roles for EGFR signaling in hepatocytes and Kupffer cells with EGFR inhibition in hepatocytes contributing to the deregulated growth observed in HCC while EGFR inhibition in Kupffer cells attenuated tumor growth by reducing their secretion of the hepatocyte growth factor IL-6. The polarizing effect EGFR inhibition has on these 2 cell types in the tumor microenvironment provides an explanation of why, despite the overexpression of the EGFR in 40\% to 70\% of human HCCs, EGFR therapies have yielded disappointing results in clinical trials.\textsuperscript{146,147}

Targeted therapies have also been noted for their unintended deleterious effects on immune cells and immune responses. In vitro studies have shown that imatinib inhibits T-cell proliferation and results in reduced expression of activation markers CD25 and CD69, leading some to posit its immunosuppressive activity.\textsuperscript{148–150} Likewise, sorafenib, a small-molecule tyrosine kinase inhibitor targeting RAF as well as the VEGF receptor, the platelet-derived growth factor receptor, Flt-3, and c-KIT, has been shown to inhibit the maturation and costimulatory activity of dendritic cells, which results in decreased ability to stimulate T cells.\textsuperscript{151} This effect was reversible on discontinuation of the drug. Thus, having a detailed understanding of the effects of targeted therapies on immune cells will be crucial to allow their optimal use alone and in combination with immune-based therapies.\textsuperscript{152} Indeed, the hepatotoxicity seen in patients receiving the BRAF\textsuperscript{V600E} inhibitor vemurafenib with the anti-CTLA4 ipilimumab underscores the challenges that can arise when targeted therapies are combined with immunotherapy.\textsuperscript{153}

Effects of Targeted Therapies on Normal Tissue Homeostasis: The Skin as a Model

In addition to acting within the tumor microenvironment, the immune effects of targeted therapies are also at play within normal cells and tissues remote from the area of a tumor. This is evidenced by the common immune-mediated side effects of these medications such as those affecting the skin and gastrointestinal tract.\textsuperscript{154–158} These effects are not trivial because they can interfere with anticancer therapy and quality of life. Changes induced within the skin by treatment with EGFRIs have received the most attention and, as outlined
below, have shed light on how EGFR signaling influences immune homeostasis within the context of a normal tissue.

The effects of EGFRIs in patients were predicted by murine knockout studies that illustrated the role of the EGFR in the maintenance of healthy follicular structures and epidermal barrier function and have recapitulated the effects of EGFRIs in patients. Additional studies have highlighted the profound impact of EGFR inhibition on immune cell trafficking into the skin (Figure 5). For example, dense immune infiltrates can be found in the affected skin of erlotinib (EGFR TKI)-treated patients consisting of macrophages, Langerhans cells, and both CD4+ and CD8+ lymphocytes. These changes in immune cell recruitment are likely mediated by alterations in the expression of chemokines such as CCL2, CCL5, CCL27, and CXCL14 within normal keratinocytes, which are crucial to the recruitment of monocytes, T cells, and other innate and adaptive effector immune cells to the skin. In addition, treatment of primary epidermal keratinocytes with EGFR inhibitor erlotinib decreased the expression of antimicrobial peptides such as human β-defensin 3, cathelicidin, and ribonuclease 7, suggesting that EGFR TKIs also alter host defenses against bacteria. In studies using mice with a conditional knockout of the EGFR, it was shown that, despite their recruitment to the skin, neither T cells nor Langerhans cells were solely responsible for the cutaneous inflammatory phenotype, but rather, it was a combination of innate immune cells (macrophages, granulocytes, and mast cells) that when trafficked to the skin could elicit a phenotype comparable to the EGFR-related rash observed in patients. The immune alterations described above further illustrate the immune impact of targeted therapies and shed light on observations from clinical trials using EGFRIs that indicated a relationship between rash severity and efficacy of therapy. This relationship is in line with the notion that EGFRIs and other targeted therapies influence immune events within normal tissues as well as within the tumor microenvironment (Figure 6). Understanding the impact of targeted therapies on the immune system will help to (1) facilitate their optimal use in the treatment of cancer and (2) promote the development of approaches to mitigate their immune-mediated side effects.

The Use of Targeted Therapies Outside of Advanced Cancer

At present, the medications discussed above are used exclusively for the treatment of advanced cancer. However, it is important to consider that the ability to pharmacologically disrupt specific signaling pathways may provide additional therapeutic opportunities. The repurposing of medications is a well-recognized approach to develop new treatments for diseases with limited therapeutic options. In the subsequent paragraphs we seek to provide a broad overview of how targeted therapies (such as EGFR inhibitors) have been applied in settings outside the treatment of advanced cancer (Figure 7).

Use of EGFR Inhibitors as Antiviral Agents

As described in earlier sections of this article, the EGFR plays diverse roles in physiologic processes and in the pathogenesis of several forms of cancer. In addition, the EGFR has also been shown to play important roles in the pathogenesis of viral infections. The EGFR has
been implicated as a host factor that is relevant to infections caused by poxviruses, human cytomegalovirus (CMV), influenza A virus (IAV), and hepatitis C virus (HCV).\textsuperscript{170–173}

Many viruses exploit the EGFR as a means to internalize by using it as a coreceptor for entry into host cells. For example, the principal envelope glycoprotein of human CMV preferentially binds to an EGFR monomer and oligomerizes with ErbB3, which then allows the virus to infect human fibroblast HEL cells.\textsuperscript{172} Recently, Kim et al demonstrated that human CMV binds to the EGFR and induces signaling via PI3K in CD34+ human progenitor cells, which mediate HCMV entry and viral trafficking as well as the establishment of viral latency.\textsuperscript{174} IAV also uses the EGFR as a coreceptor for binding to host cells. IAV utilizes the EGFR in a sialic acid–dependent manner for internalization.\textsuperscript{170} In regard to HCV, a screen using siRNAs identified the EGFR as a key mediator of its entry.\textsuperscript{171} Last, the EGFR has been shown to act as a coreceptor for adeno-associated virus serotype 6 entry, an adeno-associated virus vector used for gene therapy.\textsuperscript{175} These findings illustrate that the EGFR is an important growth factor receptor that facilitates viral infection.

In addition to mediating viral entry into cells, EGFR activation by respiratory viruses inhibits antiviral defense mechanisms. IFN-\(\lambda\), a type III interferon, provides an antiviral mucosal response to viral infection via IRF1 signaling.\textsuperscript{176,177} IAV and rhinovirus were able to activate the EGFR, and its activation suppressed IRF1 induction of IFN-\(\lambda\).\textsuperscript{178} In a related way, EGFR activation by respiratory viruses including IAV, rhinovirus, and respiratory syncytial virus decreased the production of CXCL10, a lymphocyte-recruiting chemokine, in an IRF1-dependent manner in airway epithelial cells.\textsuperscript{179} Given that the EGFR plays an important role in viral entry and host antiviral suppression, it is not surprising that EGFR inhibitors have been shown to have antiviral activity as described in more detail below.

Blockade of EGFR kinase activity utilizing the EGFR TKI erlotinib has been investigated in the context of HCV infection. Lupberger et al showed that erlotinib could effectively block viral transmission in vitro utilizing cocultures involving HCV-producing and uninfected Huh-7.5 human hepatocytes.\textsuperscript{171} They also demonstrated that treatment with erlotinib could delay the kinetics of HCV infection and decrease viral load in a human-liver chimeric mouse model.\textsuperscript{171} Erlotinib has since been found to synergistically enhance the anti-HCV effects of IFN-\(\alpha\) as well as the viral protease inhibitor telaprevir and the cyclophilin A inhibitor alisporivir.\textsuperscript{180,181} Utilizing erlotinib with sofosbuvir, the recently FDA-approved HCV NS5B polymerase inhibitor, reduced the IC\(_{50}\) of sofosbuvir by 210-fold in vitro HCV-infection assays.\textsuperscript{181} The use of EGFR inhibitors in HCV-infected patients showed that treatment with erlotinib could reduce HCV viral load in sporadic case reports.\textsuperscript{182} These data prompted the initiation of phase 1/2a clinical trials investigating the safety and efficacy of erlotinib in HCV infections (NCT01835938).\textsuperscript{183} In addition to HCV, treatment with the preclinical EGFR tyrosine kinase inhibitor AG1478 prevented CMV-induced PI3K activation and subsequent expression of viral proteins in host target cells.\textsuperscript{172} In IAV models, blockade of EGFR signaling by anti-EGFR antibodies or treatment with EGFR tyrosine kinase inhibitors diminished virion uptake into human lung epithelial cells in vitro.\textsuperscript{170} The EGFR TKI gefitinib was found to decrease respiratory viral burden in C57Bl/6 mice infected with IAV in both prophylactic and therapeutic models, a mechanism dependent on higher levels of IFN-\(\lambda\) produced by respiratory epithelial cells in gefitinib-treated animals.\textsuperscript{178,179}
EGFR blockade has also been found to inhibit the pathogenesis of poxvirus, dengue virus, and viral hemorrhagic fevers in in vitro studies. Due to its importance in maintaining cellular homeostasis, it is unsurprising that many viruses have evolved mechanisms to co-opt the EGFR signaling pathway for their gain. The use of EGFR inhibitors as antiviral agents will likely grow as additional interactions between clinically relevant viruses and this critical receptor complex are uncovered. It is worth noting that EGFR inhibition may not always be beneficial in the setting of viral infection because treatment with the EGFR kinase inhibitor AG1478 or gefitinib was shown to disrupt EGFR signaling in CMV-infected primary bone-marrow–derived CD34+ hematopoietic progenitor cells resulting in viral reactivation.

EGF and EGFR Inhibitors in Models of Sepsis

The effects of EGF and inhibition of the EGFR have been studied in different animal models of sepsis. Early studies looking at the effect of EGF on the cecal ligation and puncture (CLP) polymicrobial model of sepsis in mice showed increased expression levels of EGFR and EGF within the gut tissue after CLP. In septic mice, exogenous EGF treatment was able to attenuate expression of proapoptotic proteins Bid, Fas-associated death domain, and p21 as well as decrease sepsis-mediated mortality by 30%. More recently, EGF was shown to improve intestinal integrity and mortality following sepsis in a CLP model incorporating chronic alcohol ingestion. Alternatively, studies using LPS treatment to model endotoxemia demonstrated that the EGFR is crucial to the production of TNF-α within the heart. Treatment with an irreversible EGFR inhibitor (PD168393) or the EGFR inhibitor erlotinib was able to decrease TNF-α production, improve cardiac ejection fraction, and improve survival rates in endotoxemic mice. Likewise, treatment with the EGFR inhibitor gefitinib was also shown to protect mice from LPS-mediated septic shock by blocking aspects of toll-like receptor 4 signaling. The results of these studies, using different models, indicate a role for the EGFR in regulating the response to sepsis. The nature of the influence of the EGFR in sepsis, whether regenerative or pathologic, may depend on the presence and location of injury among other factors. Taken together, the aforementioned studies clearly imply that EGFR signaling can influence the response to sepsis and underscore the importance of further elucidating the role of EGFR signaling in this context.

Neurobiology and Targeted Therapies

Growth factors play vital roles within the nervous system, and the signaling pathways at play within tumor cells (and thus inhibited by targeted therapies) are also important to the biology of many cell types within the central and peripheral nervous systems. It is therefore not surprising that targeted therapies have shown activity in several models relevant to neurobiology. For example, early studies seeking to identify pathways involved in inhibiting nerve regrowth following injury revealed that EGFR kinase inhibitors can promote nerve regrowth after injury. Similarly, EGFR inhibitors enhanced recovery in models of spinal cord injury. In line with these neurologic effects, there is intriguing evidence that EGFRIs can alleviate neuropathic pain in humans. More recently, ErbB2 blockade with trastuzumab has been reported to enhance peripheral nerve regeneration after nerve injury. With regard to the central nervous system, a MEK inhibitor (PD325901) has been recently reported to reduce cocaine-mediated behaviors in a murine model of cocaine addiction.
addiction. These studies further illustrate how the development of targeted therapies for cancer can potentially be used to address unmet needs in other areas of medicine.

**Local Application of Targeted Therapies**

Although targeted therapies are currently used systemically, there is preclinical evidence that small-molecule TKIs can be locally applied to manipulate immune responses. The recent development of topical formulations of Janus kinase inhibitors illustrates how locally applied kinase inhibitors can be used to treat autoimmune skin diseases and inflammation of the eye. In addition, inhaled kinase inhibitors are also being explored for the treatment of lung diseases. Several studies have reported the immunologic impact of topically applied EGFR inhibitors. For example, topical EGFR inhibitors have been shown to enhance the effector phase of contact hypersensitivity. Moreover, topical application of an EGFR inhibitor has been shown to prevent UVR-induced skin tumors and block the immunosuppressive effects of UVR. Our group has shown that the topical application of an EGFR inhibitor can enhance the immune response to cutaneous vaccination. More recently, the topical application of the BRAFV600E-selective inhibitor vemurafenib has shown promise as a treatment to accelerate wound healing through its paradoxical ability to activate the MAPK pathway. Thus, in addition to their use as systemic agents, locally applied targeted therapies may harbor potential for the treatment of diseases affecting accessible epithelial tissues.

**Summary and Conclusions**

There is ample evidence that targeted therapies influence immunologic processes through varied mechanisms. These effects are at play within malignant cells, nontumor cells within the tumor microenvironment, and normal tissues remote from the site of a tumor (Figure 6). Although many of these effects likely contribute to the adverse effects of targeted therapies, there is a growing appreciation that targeted therapies modulate antitumor immunity and that the immune system plays a role in the response to targeted therapies. Understanding these immunologic effects will likely inform how to optimally use these medications (alone and in combination) for the treatment of cancer. Some of these effects seen with small-molecule TKIs may represent off-target effects, but they may still be clinically relevant. In addition, there is growing evidence that because of their ability to influence immune responses and/or to disrupt key pathogenic signaling events, targeted therapies have the potential to be repurposed for use in the treatment of other human diseases.

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Monoclonal antibody (mAb)-based targeted therapies can facilitate interactions between tumor cells and immune cells. Therapeutic mAbs can bind to growth factor receptors (such as members of the ErbB family of receptor tyrosine kinases). In addition to disrupting oncogenic signals emanating from these receptors, mAbs mediate interactions between tumor cells and immune cells (such as natural killer [NK] cells shown above). Cells such as NK cells can recognize the Fc regions of therapeutic mAbs via their Fcγ receptors. This can lead to antibody-dependent cellular cytotoxicity and tumor cell lysis.

Figure 1.
Figure 2.
Targeted therapies can increase the expression of MHC molecules. Before treatment (left panel), tumor cells can evade detection by CD8+ T lymphocytes due to low expression of MHC class I molecules. During treatment with a targeted therapy (right panel) the expression of MHC class I molecules may be increased either directly or by enhancing the induction of MHC molecules by cytokines present within the tumor microenvironment. This leads to enhanced recognition of tumor cells by CD8+ T lymphocytes, the activation of antitumor T lymphocytes, and enhanced antitumor immunity. Increases in MHC class II may also occur, leading to enhanced CD4+-mediated T-cell responses (not shown).
Figure 3.
Targeted therapies can increase expression of tumor-associated antigens. Targeted therapies may influence antitumor immunity by increasing the expression of tumor-associated antigens that can be recognized by host T lymphocytes. Before treatment (left panel) the tumor cell is not recognized by host T lymphocytes. In response to treatment with a BRAFV600E inhibitor (right panel) there is a change in the expression of tumor-associated antigens derived from melanocyte differentiation antigens that can promote the recognition of tumor cells by T lymphocytes. The recognition of tumor-associated antigens by T lymphocytes facilitates their activation and the generation of an antitumor immune response.
Targeted therapies can enhance antitumor immunity by disrupting the function of suppressive immune cells. Cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) block antitumor immune responses by inhibiting the function of antitumor T lymphocytes. By disrupting the function of MDSCs and/or Tregs, targeted therapies can enhance the function of antitumor T lymphocytes, thereby augmenting antitumor immunity.
Figure 5.
EGFR inhibitors alter immune homeostasis within the skin via numerous mechanisms. Studies in humans and mice have shown that disruption of the EGFR genetically or pharmacologically can influence numerous immune-related processes. These include changes in the expression of chemoattractant chemokines within epidermal keratinocytes such as CCL2, CCL5, CXCL14, and CCL27. These changes likely influence the alterations in immune-cell recruitment seen within the skin in patients treated with EGFR inhibitors. Other reported effects induced by EGFR inhibition on epidermal keratinocytes include alterations in MHC expression and the expression of antimicrobial peptides. EGFR indicates epidermal growth factor receptor.
Figure 6.
Targeted therapies influence immune-related events by acting on diverse cell types within the tumor microenvironment and remotely. Targeted therapies can influence immune-related processes within numerous cell types. In doing so, they can influence immune responses against tumor cells and immune homeostasis within normal tissues. These changes may potentially enhance (green arrows) and/or hinder (red arrows) antitumor immunity and the response to immunotherapy. In addition, these effects may contribute to the immune-related side effects of targeted therapies.
Figure 7.
The use of targeted therapies in settings other than the treatment of cancer. The EGFR and its downstream signaling pathways play complex roles in numerous processes in addition to their roles in cancer. As a result, inhibitors of the EGFR and other targeted therapies are being tested systemically and locally for diverse applications. For example, targeted therapies are being evaluated as antiviral agents, vaccine adjuvants, to enhance wound healing, to promote nerve regrowth following nerve injury, and in other settings. EGFR indicates epidermal growth factor receptor.