Turning Tumors into Vaccines: Co-opting the Innate Immune System

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Immunotherapy of cancer must be effective in the pre-established disease; i.e., in the therapeutic rather than prophylactic setting. Here, we review novel immunotherapeutic approaches for targeting established cancers. In addition to novel checkpoint-blocking antibodies, recent insight into innate immune sensors may further improve cancer immunotherapy protocols and help to overcome the limitations of conventional therapeutic immunization strategies. Specifically, the local induction of IL-12 and IFN-γ turns the immunosuppressive tumor microenvironment into an immunosupportive tissue, which is attained, for example, by local Toll-like receptor or RIG-I-like receptor triggering. Notably, the latter are endogenously expressed in all tumor cells and can have the advantage of turning tumors into tumor vaccines by inducing apoptosis and improving antigen presentation. Thus, immunostimulatory agents embody strong promise as a part of combinatorial cancer immunotherapies.

A major hurdle for therapeutic tumor immunotherapy is that the majority of tumor cells and their associated antigens are located in an immunosuppressive tumor environment. Despite the fact that large populations of tumor-reactive T cells can be raised in patients by active immunization or adoptive T cell transfer, T cells cannot fully realize their tumoricidal potential inside the tumor tissue. Therefore, an abundance of tumor antigen-specific T cells do not readily translate to tumor destruction (Mocellin et al., 2004).

Combinatorial immunoregimens are needed to augment the immune response at all levels. An important component will be the conversion of the tumor microenvironment into an immunosupportive tissue, thereby enabling the employment of tumor antigens in an immunostimulatory context. Immunogenic conversion of the tumor microenvironment can be achieved by intratumoral administration of appropriate immunostimulatory compounds. Such compounds include agents that induce IL-12 or type I IFN rather than simply inducing tumor inflammation. Unlike the classical type I IFN-inducing compounds targeting Toll-like receptors (TLRs) in immune cells, innate immune sensors of the RIG-I family are expressed in the cytoplasm of all cells, including tumor cells, and are not under the control of extracellular immunosuppressive cytokines such as IL-10 or TGF-β. The activation of RIG-I stimulates tumor cells to produce type I IFN and, ultimately, induces tumor cell apoptosis and antigen liberation. The resulting immunosupportive tumor microenvironment can be combined with therapeutic tumor vaccination strategies and checkpoint blockade inhibitors. In this review, we detail the rationale behind such combinatorial approaches.

Jus Ad Bellum

The infiltration of tumors with leukocytes, first observed by Rudolf Virchow in the 1800s, indicates that the immune system is engaged with the malignant tissue. Nevertheless, tumors progress while immunity fails. In an endeavor to induce active immune-mediated clearance of established tumors, many different strategies of immunotherapy have been developed over the past decades. Some of them have shown considerable activity in clinical trials, but many have turned out to be of limited therapeutic value. Strictly speaking, cancer immunotherapy has not met the expectations set forth since the pioneering work on Coley’s toxins in the 1880s (Coley, 1891; Fehleisen, 1883).

Now, the reasons are gradually being unveiled. Considerable progress has been made in the understanding of the immune system and tumor plasticity. This has provided an array of new targets and tools, and it is now very clear that the proper immune sensory input results in immune effector cells targeting and destroying autologous tumor cells. In addition, new antibody therapies were developed that release these immune effector cells from their natural restraints (anti-CTLA4 and anti-PD1). Now, multileveled immunotherapies are within reach, and it comes down to the right armament and tactics, given that the immune system appears to be quite capable of clearing tumors.

Tumor Plasticity and Immunity

A large body of evidence supports the concept that the immune system actively shapes tumor immunogenicity during tumorigenesis (Dunn et al., 2002). The immune system can protect from carcinogen-induced sarcoma and spontaneous epithelial carcinoma in the course of immunosurveillance (Shankaran et al., 2001). Immune pressure during tumorigenesis can mediate the selective outgrowth of tumor cell clones lacking immunogenic antigens (Matsushita et al., 2012) or exhibiting decreased sensitivity to immune attack (DuPage et al., 2012), a process called immunoediting. Immunoediting is facilitated by the high clonal heterogeneity present in established tumors and is multiplied by genetic instability and proliferative capacity. Thereby, immunoediting is the result of clonal tumor cell evolution in the face of selective immune- and therapy-induced pressures, a phenomenon that is discussed below.

Apart from immunoediting acting on a clonal basis, individual tumor cells can also respond with evasive plasticity. For example, T-cell-induced inflammation is not necessarily beneficial...
to tumor immunity. Using the HGF-CDK4(R24C) mouse model of autochthonous murine melanoma, Landsberg et al. (2012) closely recapitulated the clinical adoptive cell transfer (ACT) protocol used as melanoma immunotherapy regimen. Although this treatment was highly effective in initially eradicating the melanomas, after approximately two months, tumors recurred aggressively in a subgroup of mice (Kohlmeyer et al., 2009; Landsberg et al., 2012; 2010). Relapsed tumors showed a dedifferentiated mesenchymal-like phenotype with loss of gp100 and melan-A antigens and 23 other pigmentation genes along with an upregulated nerve growth factor expression (CD271). Strikingly, isolation and retransplantation of these dedifferentiated melanomas into naïve animals resulted in a tumor phenotype that was again susceptible to the same ACT protocol. However, as in the first round, the tumors gained resistance to ACT treatment. This reversible dedifferentiation was mediated by TNF-α produced by tumor-infiltrating immune cells. Selective proliferation and selection of dedifferentiated subclones was excluded in the study design. These results highlight the plasticity of individual melanoma cells in functionally evading immunotherapy-induced T cell attack with no need for genetic adaptation. Interestingly, the TNF-α-induced resistance to T cell recognition was confined to the melanocyte differentiation antigens and did not affect T cell recognition of the mutated CDK4 protein. Besides broadening the immunoeediting principle, and thereby adding flexibility to the clonal evolution theory (Greaves and Maley, 2012; Nowell, 1976), these findings call for a broader therapeutic antigen scope and emphasize the careful coordination of inflammatory responses within the tumor ecosystem.

**Tumor Therapy Resistance and Clonal Revolution**

Enduring tumor inflammation facilitates progressive tumor growth, a highly complex polyclonal process. Nearly four decades ago, Nowell (1976) put forward the theory of tumorigenesis as an evolutionary process. More recent reviews by Greaves and Maley (2012) and Merlo et al. (2006) address the current understanding of the subject and emphasize the fact that each therapy introduces a new selective pressure on the clonal evolution within the tumor. Thereby, basically all therapies will induce resistance by mediating the natural selection of resistant tumor cells. The clonal evolution theory is supported by the observation that intratumoral mutational heterogeneity, the prerequisite for such evolution, is extensive (Gerlinger et al., 2012). Gene expression profiles of good and bad prognosis were found dispersed over a single tumor mass, and multiple distinct mutations of typical tumor suppressor genes were found spatially separated over the tumor, illustrating convergent phenotypic evolution. The study demonstrates the presence of branched evolutionary tumor growth and Darwinian selection during tumorigenesis. In addition to clonal evolution, reversible dedifferentiation illustrates that individual cell clones can vividly adapt to their surroundings in a struggle for survival. This adds a “clonal revolution” perspective to the clonal evolution theory, in which individual cancer cells can revolt to escape immune attack and death.

A clear example of therapy-induced clonal evolution is the acquired resistance of melanomas to Vemurafenib, a B-Raf enzyme inhibitor. Vemurafenib is highly successful in eradicating melanomas carrying the BRAF (V600E) mutation (Flaherty et al., 2010), which is found in roughly 60% of melanomas and 7% of other cancers. Vemurafenib can achieve an impressive 80% response rate in melanoma treatment. Nonetheless, recurrent tumor cells employ either PDGFRα upregulation as an alternate survival pathway or utilize mutated N-RAS as an alternate MAPK activation route. Interestingly, the primary BRAF (V600E) kinase target is not mutated in Vemurafenib-resistant clones (Nazarian et al., 2010). Similar mechanisms account for the clonal escape of other targeted therapies, such as Imatinib (Gorre et al., 2001), or of conventional therapies, such as chemotherapy (Wang et al., 2004) or irradiation (Luzhna et al., 2013). Tumor adaptation to microenvironmental changes such as oxygen or nutrition deprivation occurs in a similar fashion by selecting for the clones best fit to adapt to the new survival conditions. As indicated by Gerlinger et al. (2012), these tumor escape mechanisms are driven by the extreme genetic instability and heterogeneity within tumors, which can be enhanced by genotoxic treatments such as irradiation and chemotherapy.

Besides clonal evolution and clonal revolution, tumors actively produce a microenvironment that counteracts antitumor immune responses. To accomplish immune suppression, tumor cells employ various suppressive mechanisms, including the expression of programmed cell death ligand 1 (PD-L1), the production of indolamine 2,3-dioxygenase, or the induction of T cell anergy (Gajewski, 2012). Alternatively, tumor cells can attract other cell types that suppress invading immune cells and support tumor growth and survival, such as tumor-infiltrating macrophages, cancer-associated fibroblasts (Madar et al., 2013), and, in particular, myeloid-derived suppressor cells (MDSCs).

MDSCs are a heterogeneous population of immature myeloid cells that originate from the bone marrow and normally differentiate into macrophages, neutrophils, or dendritic cells (DCs). During certain pathologies, including cancer, their natural differentiation is halted, and they expand and infiltrate the tumor site as MDSCs, attracted and activated by the local cytokine milieu (Gabrilovich and Nagaraj, 2009; Heusinkveld and van der Burg, 2011). MDSCs induce a state of chronic tissue inflammation and immune suppression (Bunt et al., 2006; Meyer et al., 2011) that is characterized by the production of reactive oxygen species (ROS), nitric oxide (NO), Arginase 1 (ARG-1), and cytokines such as TNF-α, IL-1, and IL-6 (Gabrilovich and Nagaraj, 2009). They are forceful modulators of T and NK cell immunity, can induce regulatory T (Treg) cells de novo in the periphery or attract thymic-derived Treg cells (Fortin et al., 2012; Lindau et al., 2013), and can give rise to suppressive tumor-associated macrophages (Gabrilovich and Nagaraj, 2009). For these reasons, they will be in the focus of the following sections.

MDSCs rely strongly on the local NF-κB-driven inflammatory milieu for their suppressive capabilities (Karín and Greten, 2005). At the early stages of tumor growth, NF-κB has a direct impact on chronic inflammation-induced carcinogenesis (Greten et al., 2004; Karín and Greten, 2005; Pikarsky et al., 2004), and a vicious inflammatory circle exists between NF-κB activation in MDSCs and subsequent NF-κB activation in malignant and premalignant cells through the cytokines released from MDSCs, as depicted in Figure 1. This ensures enduring NF-κB activation in the malignant cells and continuous upregulation of antiapoptotic and proliferative genes within the tumor, both of which
New insights into the mechanisms of MDSC-mediated immunosuppression in mice (Sumida et al., 2012) and ovarian cancer patients (Coward et al., 2011). Several recent studies provide evidence that breaking the stronghold of MDSCs in the tumor microenvironment is a key step toward effective antitumor immunity. For example, the use of a monoclonal antibody against the IL-6 receptor eliminates MDSC-mediated immunosuppression in mice (Sumida et al., 2012) and ovarian cancer patients (Coward et al., 2011). Furthermore, cimetidine suppresses lung tumor growth in mice through apoptosis induction in MDSCs (Zheng et al., 2013). Notably IFNγ and IL-12 have the capacity to convert MDSCs into functional, nonsuppressive antigen-presenting cells. IL-12 reprograms MDSCs, forcing them to support CD8+ T cell attack of solid tumors (Kerkar et al., 2011). Additionally, this IL-12-induced reprogramming of MDSCs resulted in the upregulation of costimulatory markers such as CD80, CD86, the differentiation marker F4/80, and MHC-II as well as the inhibition of ARG-1 and NO synthase 2 (Steding et al., 2011). IL-12 treatment decreased in vivo MDSC infiltration of tumors and increased CD8+ T cell infiltration and survival. The beneficial in vivo effects of IL-12 on MDSC and tumor immunity were also seen in combination with chemotherapy, such as oxaliplatin (Hernandez-Alcoceba and Berraondo, 2012).

Like IL-12, the cytokine IFNγ can potently induce MDSC differentiation, promoting tumor immunity. IFNγ used in vitro or systemically in vivo stimulated the differentiation and maturation of MDSCs within tumor tissue in the C26 colon carcinoma model. Differentiated MDSCs displayed phenotypical changes characteristic of maturation and exhibited a significantly reduced T-cell-suppressive phenotype. The systemic use of cytokines comes with drawbacks, including toxicity and cost. In mice, IL-12- or IFNγ-dependent MDSC differentiation is also achieved with the TLR9 ligand CpG (Zogmeier et al., 2011). In the C26 colon carcinoma model and in spontaneous gastric tumors in CEA424-Tag mice, CpG administration induced MDSC maturation and differentiation via the stimulation of TLR9 in plasmacytoid DC, resulting in the reduced T-cell-suppressive activity of MDSCs and tumor regression. In addition to intratumoral CpG administration,

Figure 1. Tumor Immune Suppression by MDSC Recruitment, Reciprocal NF-kB Signaling, and Reversible Dedifferentiation

Premalignant lesions can secrete cytokines such as IL-1 and IL-6 in order to attract myeloid-derived suppressor cells (MDSCs) from the bone marrow. Upon infiltration of the tumor mass, MDSCs install a highly immunosuppressive environment, characterized by the production of reactive oxygen species (ROS), nitric oxide (NO), and Arginase 1 (ARG-1), which are all potent repressors of T cell immunity. In turn, MDSCs also produce cytokines such as IL-1, IL-6, and TNF, which activate NF-κB in local tumor cells. This stimulates the tumor cells to upregulate antiapoptotic and proliferative genes and incite them to produce more cytokines such as TNF, IL-1, and IL-6. In turn, this again activates NF-κB in MDSCs, upregulating their cytokine production and augmenting their secretion of ROS, NO, and ARG-1. Thereby, a vicious circle exists between tumor cells and MDSCs, which is based on reciprocal NF-κB activation through a cytokine secretion loop, firmly maintaining a strong immunosuppressive tumor microenvironment. Importantly, by these cytokines, tumor cells can undergo reversible dedifferentiation, a potent mechanism for escaping antigen-specific T cell recognition.
systemic poly(I:C) injections showed similar results. The use of IFNα-neutralizing antibodies and type I IFN receptor-deficient mice demonstrated that the strong MDSC-modifying effects of CpG and poly(I:C) are dependent on type I IFN (Zoglmeier et al., 2011). Similar results were obtained in large established CT26 colon carcinomas (Shirota et al., 2012). Although Zoglmeier et al. (2011) did not find significant direct effects of CpG on tumor-associated MDSCs, De Santo et al. (2008) reported a screen of TLR agonists that did directly affect influenza-associated MDSC differentiation. In their work, the TLR7-8 ligand R848, the TLR3 ligand poly(I:C), and the TLR9 agonist CpG A-type 2216 were all individually able to relieve the suppression of T cells by isolated MDSCs. Importantly, the suppressive capacity of MDSCs was further reduced when they were coincubated with invariant NKT (iNKT) cells, thus maximizing IL-12 production. This interaction between MDSCs and iNKT cells was CD1d and CD40 dependent. Direct exposure of MDSCs to different IL-12- and/or IFNα-inducing TLR ligands or influenza virus infection, particularly in the presence of iNKT cells, was capable of inducing MDSC maturation and differentiation. In line with these findings, the combinatorial use of CD40 activation and poly(I:C) potently reverts the immune suppressive activity of MDSCs and converts tumor-resident tolerogenic DCs into active antigen-presenting cells. Upon anti-CD40 and poly(I:C) tumor treatment without additional antigenic immunization, these cells migrated from the tumor stroma to local lymph nodes, activated significant T cell immunity, and induced the rejection of otherwise lethal intraperitoneal ID8 ovarian carcinomas. Again, IL-12 and IFNα were the predominant cytokines mediating these effects (Scarlett et al., 2009). Besides individual cytokines and TLR ligands, an oncolytic adenovirus engineered for increased TLR9 stimulation has recently achieved similar TLR9-dependent, MDSC-modulating, and T-cell-immunity-stimulating results in a xenograft model of lung cancer and a syngeneic melanoma model (Cerullo et al., 2012). Furthermore, anti-CD40 treatment combined with gemcitabine, a chemical nucleoside analog that is incorporated in proliferating tumor cells alternative to cytidine, thereby arresting tumor growth, was reported to induce tumor regression upon MDSC reprogramming in a small cohort of pancreatic cancer patients (Beatty et al., 2011).

The studies above clearly demonstrate that immune modulators such as the TLR ligands CpG, R848, and poly(I:C) as well as the cytokines IL-12 and IFNα are capable of converting the IL-1α-, IL-6-, and TNFα-dominated immunosuppressive tumor microenvironment into a T-cell-immunity-promoting tumor. Notably, such tumor microenvironmental conversions attract T cells to the tumor tissue. Inevitably, activated T cells release TNFα in the tumor microenvironment, and, in principle, this may tip the balance back toward MDSC formation and tumor cell dedifferentiation. However, the studies above and their practical outcomes demonstrate that microenvironmental changes are maintained in favor of antitumor immunity. Nonetheless, more scrutiny into the exact kinetics and temporal stability of MDSC conversion is warranted.

Importantly, the use of TLR ligands, MDSC conversion still predominantly depends on the recruitment and ample presence of (plasmacytoid) DCs at the tumor site. TLR ligands usually have no direct effects on tumor cells, and, consequently, the efficacy of such interventions heavily depends on the ability of the immune system to localize functional antigen-presenting cells to the tumor tissue (Palucka and Banchereau, 2013). Moreover, most solid tumors are poorly vascularized and contain areas of hypoxia. Besides attracting MDSCs (Murdoch et al., 2004), poor tumor vascularization limits the access of systemically administered cytokines (such as IL-12 or IFNα) or antibodies (such as CD40-agonistic mAb or anti-IL-6 mAb). Therefore, the efficacy of such interventions is limited by the local access to and availability of the target cell population.

**Novel Compounds Targeting Immune Receptors Expressed in Tumor Cells**

The innate cytosolic immune sensor RIG-I is structurally related to MDAs, and both belong to the RIG-I-like receptor (RLR) family of helicases that recognize double-stranded RNA (dsRNA) upon the viral infection of their host cell. Importantly, RLRs reside in all nucleated cells in the body, including tumor cells. Although MDAs recognize long dsRNA structures, such as those found in poly(I:C), the hallmark activating structure for RIG-I has been defined as short, blunt-ended 5′-triphosphate dsRNA (3pRNA) (Hornung et al., 2006; Pichlmair et al., 2006; Schlee et al., 2009).

In addition to type I IFN induction in immune, nonimmune, and tumor cells, tumor cells are highly susceptible to RIG-I-induced apoptosis via the activation of the BH3-only proteins Puma and Noxa. Importantly, nonmalignant cells are rescued from RIG-I-induced apoptosis by their ability to upregulate Bcl-xL (Besch et al., 2009). Thus, unlike TLR ligands, 3pRNA is a direct tumoricidal agent. When used in vivo against melanoma, 3pRNA induces high amounts of IFNα, IFNβ, and IL-12 and mediates effective antitumor immunity in melanoma and other cancer types (Poeck et al., 2008). RIG-I activation can be combined with gene silencing, and such so-called bifunctional small interfering RNAs (siRNAs) (for example, RIG-I activation combined with Bcl2 silencing) have shown efficacy in a melanoma lung metastasis model (Poeck et al., 2008). Another successful bifunctional siRNA is RIG-I triggering combined with the silencing of TGF-β in pancreatic cancer in mice, which leads to the recruitment of CD8+ T cells to the tumor site along with a reduction of MDSC tumor infiltration (Ellermeier et al., 2013). 3pRNA is either generated by in vitro transcription or chemically synthesized. Such RNAs usually trigger TLR7 in DCs, a process which has a deteriorating effect on the RIG-I-induced IFN response (Forbach et al., 2012). With the discovery of the exact minimal RIG-I ligand (Schlee et al., 2009) and the specific interaction between 3pRNA and RIG-I (Wang et al., 2010), RIG-I-selective RNA agonists can be designed with optimized characteristics for cancer immunotherapy. Collectively, RIG-I triggering in tumor cells has the unique property of exhibiting selective tumoricidal activity in addition to strong type I IFN induction in both 3pRNA-stimulated tumor cells and immune cells.

In addition to its strong IFN signature, RIG-I in myeloid cells activates the inflammasome via the direct association of RIG-I and the ASC adaptor protein (Poeck et al., 2010). For RIG-I, inflammasome signaling is independent of NLRP3 (Kanneganti et al., 2006; Poeck et al., 2010; Rajan et al., 2010). Although NLRP3-dependent inflammasome activation in MDSCs leads to the release of IL-1β, promoting immune suppression by MDSCs (Bruchard et al., 2013), in the case of RIG-I, type I IFN blunts the caspase-1-mediated production of IL-1β. IFNs induce...
Optimizing Therapeutic Tumor Vaccination

Although the conversion of the tumor microenvironment into an immune-fostering state is a pivotal step toward the induction of protective tumor immunity, the simultaneous administration of a more conventional cancer vaccine distant from the tumor site can broaden and peak the specific activation of antitumor immune responses (Bijker et al., 2008). Optimal vaccine combinations often rely on the presence of an antigenic fraction that is capable of being presented by professional antigen-presenting cells. Several tactics exist for orchestrating combined CD4+ and CD8+ T cell immunity. Although live vaccines are considered to be the most potent forms of immunization because of their limited replicative potential and seamless colocalization of antigens and multiple PAMPs, TLR ligand-protein conjugates exert a stimulating synergy that comes close to that of a live vaccine (Heit et al., 2005). Although containing the whole array of suitable epitopes for (cross-)presentation, protein antigens are difficult to generate, especially on a good manufacturing practice (GMP) level, given that protein production often involves bacterial synthesis, which is inevitably prone to endotoxin contamination. Therefore, synthetic peptides such as synthetic long peptides (SLPs) can be used for vaccination purposes because they mimic the antigenic properties of a whole protein antigen; i.e., they contain an array of antigens for both helper CD4+ and cytotoxic CD8+ T cell responses irrespective of the patient’s human leukocyte antigen (HLA) type, allow direct antigen presentation by professional antigen-presenting cells (Bijker et al., 2008), and were shown to induce strong, effective CD8+ T cell responses (Quakkelaar and Melief, 2012). In clinical trials, SLP vaccines in montanide ISA-51 adjuvant have been exceptionally effective (for an overview of vaccine adjuvants, see Table 1). An HPV16-SLP vaccine for patients with high-grade vulvar intraepithelial neoplasia has shown strong T cell immunity and impressive clinical responses (Kenter et al., 2009). Additionally,
which enable DCs to engage in prolonged CD8+ T cell cross-prime-ning and processing, DCs provide an array of receptors specialized in the uptake of specific antigenic matter. For example, CD8α+ DCs can potently cross-present antigens from necrotic cells, such as virally infected cells or dying tumor cells, using the C-type lectin domain family member 9a (CLEC9a or DNGR1) (Sancho et al., 2009). Although CLEC9a favors the cross-presentation of antigens in viral infection (Ibora et al., 2012; Zelenay et al., 2012), the uptake of antigens via CLEC9a allows for the tuning of the resulting antigen presentation, depending on the modulation of the induced CD4+ T cell response. The coupling of antigens to CLEC9a-specific antibodies without the addition of adjuvants mainly induces Foxp3+ CD4+ Treg, whereas, in the same immunization, the addition of polyI:C resulted in strong cross-presentation and IL-12-dependent induction of CD8+ T cell immunity. In contrast, curdian adjuvants induced a Th17-cell-focused response (Joffre et al., 2010). Additional antigen uptake receptors exist that can be effectively exploited for enhancing antigen cross-presentation, including CLEC7a (Dec-tin-1), DC-SIGN (CD209), DEC205 (CD205), and the mannose-1 receptor (CD206). Moreover, other cell types, such as NK and NKT cells, can influence DC cross-presenting capabilities. NK cells can inhibit cross-priming and IL-12-dependent induction of CD8+ T cells to become effective memory cells (Xiao et al., 2009). They upregulate a set of approximately 350 genes through epigenetic changes that program CD8+ T cell effector function and memory development (Agarwal et al., 2009). To prevent bystander activation of naive CD8+ T cells in an IL-12 or IFNα cytokine milieu, the process is under the control of CD4+ T cell help and is dependent on a paracrine IL-2 loop (Raüé et al., 2013).

Besides linking an antigen to a TLR ligand to enhance uptake and processing, DCs provide an array of receptors specialized in the uptake of specific antigenic matter. For example, CD8α+ DCs can potently cross-present antigens from necrotic cells, such as virally infected cells or dying tumor cells, using the C-type lectin domain family member 9a (CLEC9a or DNGR1) (Sancho et al., 2009). Although CLEC9a favors the cross-presentation of antigens in viral infection (Ibora et al., 2012; Zelenay et al., 2012), the uptake of antigens via CLEC9a allows for the tuning of the resulting antigen presentation, depending on the modulation of the induced CD4+ T cell response. The coupling of antigens to CLEC9a-specific antibodies without the addition of adjuvants mainly induces Foxp3+ CD4+ Treg, whereas, in the same immunization, the addition of polyI:C resulted in strong cross-presentation and IL-12-dependent induction of CD8+ T cell immunity. In contrast, curdian adjuvants induced a Th17-cell-focused response (Joffre et al., 2010). Additional antigen uptake receptors exist that can be effectively exploited for enhancing antigen cross-presentation, including CLEC7a (Dec-tin-1), DC-SIGN (CD209), DEC205 (CD205), and the mannose-1 receptor (CD206). Moreover, other cell types, such as NK and NKT cells, can influence DC cross-presenting capabilities. NK cells can inhibit cross-priming and IL-12-dependent induction of CD8+ T cells to become effective memory cells (Xiao et al., 2009). They upregulate a set of approximately 350 genes through epigenetic changes that program CD8+ T cell effector function and memory development (Agarwal et al., 2009). To prevent bystander activation of naive CD8+ T cells in an IL-12 or IFNα cytokine milieu, the process is under the control of CD4+ T cell help and is dependent on a paracrine IL-2 loop (Raüé et al., 2013).

### Table 1. Characteristics of Several Different Immune Adjuvants

<table>
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<th>Receptor</th>
<th>Immune Skewing</th>
<th>Main Target</th>
<th>Cell</th>
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<tr>
<td>Non-Tumor-Targeting Adjuvants in Clinical Trials*</td>
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<tr>
<td>Alum</td>
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<td>Th2</td>
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<td>Ampligen</td>
<td>TLR3</td>
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<tr>
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<tr>
<td>CpG 7909</td>
<td>TLR9</td>
<td>Th1, Th2</td>
<td>APC</td>
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<tr>
<td>Imiquimod</td>
<td>TLR7,8</td>
<td>Th1</td>
<td>APC</td>
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<td>Iscomatrix</td>
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<td>Th1, Th2</td>
<td>APC</td>
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<tr>
<td>MDP</td>
<td>NOD2, NLRP3</td>
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<td>Experimental Tumor-Targeting Adjuvants</td>
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<tr>
<td>3pRNA (in vivo transfected)</td>
<td>RIG-I, TLR7</td>
<td>Th1</td>
<td>tumor, APC</td>
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<td>MDA5, TLR3,7</td>
<td>Th1</td>
<td>tumor, APC</td>
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*Source: National Cancer Institute.

a p53-SLP vaccine has proven effective in inducing strong T cell responses in different trials of ovarian cancer patients (Leffers et al., 2009; 2012) as well as colorectal cancer patients (Zeestraten et al., 2013). Interestingly, these SLP vaccines perform well in combination with classical therapies such as chemother-apy (Vermeij et al., 2012), and, in particular, the addition of IFNα results in the augmentation of the induced T cell response (Zeestraten et al., 2013). Notably, IFNα also improves cross-presentation by DCs (Lattanzi et al., 2011).

Although SLP vaccines are effective in inducing immunity and are capable of mediating therapeutic immunotherapy against virus-associated cancer (Kenter et al., 2009), responses to self-antigens such as p53 have shown strong immune activation but have done so without significant beneficial clinical outcome. As recently reviewed by Zom et al. (2012), a means for further improving immune activation and processing of antigens to CLEC9a-specific antibodies without the addition of adjuvants mainly induces Foxp3+ CD4+ Treg, whereas, in the same immunization, the addition of polyI:C resulted in strong cross-presentation and IL-12-dependent induction of CD8+ T cell immunity. In contrast, curdian adjuvants induced a Th17-cell-focused response (Joffre et al., 2010). Additional antigen uptake receptors exist that can be effectively exploited for enhancing antigen cross-presentation, including CLEC7a (Dec-tin-1), DC-SIGN (CD209), DEC205 (CD205), and the mannose-1 receptor (CD206). Moreover, other cell types, such as NK and NKT cells, can influence DC cross-presenting capabilities. NK cells can inhibit cross-priming and IL-12-dependent induction of CD8+ T cells to become effective memory cells (Xiao et al., 2009). They upregulate a set of approximately 350 genes through epigenetic changes that program CD8+ T cell effector function and memory development (Agarwal et al., 2009). To prevent bystander activation of naive CD8+ T cells in an IL-12 or IFNα cytokine milieu, the process is under the control of CD4+ T cell help and is dependent on a paracrine IL-2 loop (Raüé et al., 2013).

Importantly, an effector environment dominated by IFNα and IL-12 induces antigen-independent activation and proliferation of CD8+ memory T cells prior to the recognition of their cognate antigen (Raüé et al., 2013; 2004). According to the model proposed by Raüé et al. (2013), this phenomenon allows memory CD8+ T cells to initiate differentiation and effector functions upon entering the periphery of an infection site and its inflammatory microenvironment, enhancing the host’s response to the microbial invasion by “preheating” the T cell for action. In the case of tumors, not only do compounds such as the RIG-I agonist 3pRNA revert MDSC immune suppression, induce tumor cell apoptosis, and liberate tumor-associated antigens for immunity, but memory CD8+ T cells residing in or near the tumor site are simultaneously enticed in order to activate their IL-12-IFN-programmed effector...
functions. Moreover, the CD8+ T cells that are newly raised against the liberated tumor antigens will encounter a tumor cytokine environment that is fully supportive for effector functions and memory development.

Thus, peptide vaccines can set the stage for strong T cell activation simultaneous with 3pRNA tumor treatment. T cells induced by such vaccines are enabled to exert and sustain their cytotoxic antitumor functions within a tumor microenvironment that is liberated from MDSC suppression through intratumoral 3pRNA treatment. However, the induction and effector functions of these T cells are still restrained by natural regulatory mechanisms.

Releasing Natural Inhibition by Checkpoint Blockade

T cell numbers that engage in tumor attack can be maximized in two different phases: during T cell priming and during recognition of the target cell. The priming phase of a T cell response involves three stimulatory signals: MHC:TCR interaction, CD80 and CD86:CD28 cosimulation, and the cytokine milieu. As one of the natural limitations to T cell proliferation, primed T cells up-regulate CTLA4 upon cognate antigen recognition, which competes with CD28 for interaction with the costimulatory molecules and effectively shuts down the priming phase. In addition to this brake on T cell activation, activated DCs and tumor cells express ligands such as PD-L1 (also known as CD274 or B7-H1). PD-L1 interacts with PD1 expressed on activated T cells. When PD1 is engaged by PD-L1, it inhibits TCR-driven proliferation and cytokine production (Freeman et al., 2000) and also severely shortens TCR:MHC interactions (Fife et al., 2009). Thereby, interactions such as PD1-PD-L1 have a deteriorating effect on T cell priming and target cell recognition and killing. Indeed, the active inhibition of CTLA4 (Leach et al., 1996) or PD1 interactions (Nishimura et al., 1999) produces exaggerated immune responses. Besides CTLA4 and PD1, a collection of additional checkpoints and receptors exist, and a comprehensive review on the subject is published in this issue of *Immunity* (Chen and Mellman, 2013).

In the clinical setting, CTLA4 blockade by Ipilimumab was approved by the FDA for the treatment of metastatic melanoma in 2010. Ipilimumab has achieved significant immunological as well as clinical responses, nearly doubling the median overall survival time irrespective of an additional gp100 peptide vaccination, in patients with unresectable late-stage melanoma (Hodi et al., 2010). As expected, grade 3 or 4 adverse autoimmune side effects accompany Ipilimumab treatment, and they appear to coincide with its antitumor effect. Adverse effects could be reversed by appropriate treatment. Also, in patients with previously untreated metastatic melanoma, Ipilimumab acts in combination with the standard-of-care chemotherapeutic agent dacarbazine (Robert et al., 2011). Likewise, Ipilimumab significantly increased progression-free survival in combination with paclitaxel and carboplatin used in non-small-cell lung cancer (NSCLC) (Lynch et al., 2012) and, as a single agent, shows efficacy in renal cancer treatment (Yang et al., 2007). Clearly, the inhibition of the CTLA4 immune checkpoint is a fruitful intervention for boosting antitumor immunity.

For the PD1 pathway, first reports indicated that anti-PD1 treatment induces durable objective responses as a single agent in one out of four to five patients with melanoma, renal cancer, or NSCLC (Topalian et al., 2012). In patients with advanced-stage cancer, anti-PD1 treatment induced durable regressions (Brahmer et al., 2012), and Lambolizumab (the antibody against PD1 previously known as MK-3475) treatment of advanced melanoma recently achieved response rates up to 52% with low-grade adverse effects (Hamid et al., 2013). Treatment outcome appears to correlate with tumor-associated PD1 expression (Topalian et al., 2012), and adverse immune side effects seen with anti-PD1 treatment appear to be less common and less severe than those seen following Ipilimumab treatments (Brahmer et al., 2012).

With effective checkpoint blockade, augmented therapeutic vaccination, and tumor microenvironmental conversion, we now have a broad range of tools at hand that spans the immune response from its priming to its effector phase. New combinatorial immunoregimens can be shaped, such as deploying RLR and TLR immunostimulatory agents to augment different phases of the antitumor immune response. Hereby, checkpoint inhibition permits the T cell response to be released from its natural brakes and be propelled in order to exert its full force.

Advanced Clinical Protocols for Tumor Immunotherapy

The majority of the interventions discussed above are independent of patient HLA haplotype, can be made in large quantities on a GMP level, and can be combined with other therapies. Also, conventional anticancer regimens such as chemotherapy and irradiation can be incorporated in such therapeutic combinations. It is well known that certain types of chemotherapy, for example anthracyclines, can induce immunogenic cancer cell death. This occurs predominantly via the exposure of calreticulin, acting as a phagocyte “eat me” signal, and the release of HMGB1 (Orsini et al., 1977; Zitvogel et al., 2008). Moreover, local irradiation of a tumor mass sometimes leads to the regression of metastases distant from the radiation field, a phenomenon referred to as the abscopal effect (Demaria and Formenti, 2012). Rarely sufficient to induce systemic cancer regression, the abscopal effect probably represents a systemic immune response triggered by the irradiation of tumor cells. Indeed, the abscopal effect appears to coincide with systemic immune activation (Postow et al., 2012), which could be initiated by the irradiation-induced modulation of the tumor peptide repertoire and MHC class-I expression (Reits et al., 2006).

Besides these conventional approaches, targeted therapies, such as vemurafenib (Flaherty et al., 2010) in melanoma and tasquinimod (Pili et al., 2011) in prostate cancer, can lead to partial tumor regression, providing a time window for immunological intervention such as adoptive transfer therapies like sipuleucel-T (Kantoff et al., 2010), adoptive T cell transfer therapy (Restifo et al., 2012), or therapeutic vaccination (Kenter et al., 2009). Depicted in Figure 3, an immunotherapeutic intervention should influence all levels of the immune response simultaneously, attending its entirety. As an essential step accompanying any tumor immunotherapy regimen, the tumor microenvironment has to be converted from an immunosuppressive stroma into a tissue milieu in ruthless support of CD8+ T cell infiltration and effector function. Key cytokines to this reversal are type I IFN and IL-12. Targeting innate nucleic acid sensors expressed in tumor cells, such as RIG-I, allows such a switch in the tumor microenvironment, given that tumor cells themselves start to make type I IFNs, release tumor-specific antigens, and undergo cell death.
Simultaneous therapeutic vaccination protocols should contain a broad spectrum of tumor antigens, which is needed for the synchronized induction of ample CD4+ helper T cell and CD8+ cytotoxic T cell immunity and preferably contains antigens that span different antigenic subclasses in order to counteract reversible tumor cell dedifferentiation. Such broad antigen panels can be incorporated into overlapping SLP vaccines. Then, to enhance the vaccine-induced generation of T cell immunity, the SLPs can be conjugated to TLR ligands that strongly induce IFNα and IL-12 secretion by the responsive DCs, such as CpG. This augments CD8+ T cell activation and effector function and allows for programmed proliferation of these cells upon reaching the RIG-I-converted tumor environment dominated by IFNs. Furthermore, including additional adjuvants such as α-galactosylceramide in the vaccination can exploit NKT-cell-mediated stimulation of such antigen cross-presentation. Finally, the T cell response should be released from its natural inhibitions by blocking CTLA4 or PD-1, thus allowing maximized T cell effector function.

The bottleneck of such combinatorial immune interventions in cancer is its immunosuppressive shield. The great advance we are now witnessing is that, by targeting the heart of the tumor cell’s own antiviral defense system (i.e., RIG-I), this shield can not only be broken, but the entire tumor can be turned into a vaccine. Once this is achieved, all other immunotherapeutic regimens synergize to further strengthen the resulting antitumor response. In combination with classical therapies such as irradiation or chemotherapy or as a stand-alone regimen, we are likely to see such combinatorial immunotherapeutic strategies enter the clinical routine in the future.

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