Considerations for Combined Immune Checkpoint Modulation and Radiation Treatment

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Recent advances indicate that new therapeutic strategies for the treatment of malignancies will be realized from combined radiation treatment and immune checkpoint modulation. Numerous biophysical properties must be considered for effective biologic development, including affinity, selectivity, oligomeric state and valency. High-resolution structural characterization contributes to our understanding of these properties and can lead to the realization of proteins with unique *in vitro* activities and novel *in vivo* therapeutic functions. In this article we focus on the importance of these factors for new potential biologics and consider these in the context of combination therapies with physical modalities, including radiation therapy. In particular, we examine the consequences of altered avidities and subset-specific ligand density on the rational modification of biological function in the immunoglobulin and tumor necrosis factor superfamilies and for new optimized combination therapies. © 2014 by Radiation Research Society

INTRODUCTION

This focus issue of *Radiation Research* presents several articles that highlight the potential opportunities for combining various immunotherapeutic regimens with radiation therapy. Here we review some of the key features relevant to the development of new combined therapeutic strategies, including biologic avidity, target molecule expression properties and the timing of biologic and radiation administration. Continued understanding of the mechanisms underlying these individual therapeutic modalities will be critical for the realization of new optimized combination strategies.

It is well appreciated that a wide range of secondary antigen-independent costimulatory signals involving cell

surface and secreted proteins from a number of families, such as the immunoglobulin (Ig), tumor necrosis factor (TNF), tumor necrosis factor receptor (TNFR) and lectin superfamilies, dictate the course, duration and strength of an immune response. The overall architecture and signaling potential of cognate receptor-ligand complexes are governed by numerous biochemical and biophysical factors including affinity, selectivity between multiple binding partners, oligomeric state and valency (Fig. 1). Equally important are the spatial and temporal expression patterns of these molecules, as well as the cell surface densities of the cognate receptors and ligands. These features control the organization and distribution of assemblies present at the immunological synapse formed between T cells and antigen presenting cells (APCs) (Fig. 1). While the organization of these assemblies is dictated by interactions involving the ectodomains of costimulatory receptors and ligands, these same spatial constraints are imposed on the noncovalently associated cytoplasmic signaling and scaffolding proteins responsible for propagating and amplifying extracellular signals. Thus, modulation of costimulatory pathways by alteration of extracellular interactions represents an enormous area of activity for the realization of therapeutic strategies directing the global modulation of immune function to treat malignancies, infectious diseases and autoimmune disorders. Classic examples include etanercept (Enbrel®), a soluble TNFR-Ig fusion protein, formed by the extracellular domain of TNFR and the Fc region of immunoglobulins that binds with high affinity to TNF (1). This interaction prevents TNF from binding to, and activating, cell surface-associated TNFR (2), resulting in reduced inflammatory responses in rheumatoid arthritis, juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis and ankylosing spondylitis (1, 3–5). Analogously, infliximab (Remicade®) is a monoclonal antibody against TNF that similarly results in blockade of the TNFR signaling pathway by sequestering TNF. Remicade is used to treat autoimmune disorders such as rheumatoid arthritis, ulcerative colitis and Crohn's disease (6, 7). Enbrel is the most widely used anti-TNF therapeutic in the field of rheumatology and Remicade is the most widely used anti-TNF drug when all U.S. Food and Drug Administration (FDA)-

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FIG. 1. Organization of the immunological synapse. Biochemical and biophysical properties of cell surface receptor and ligands are central to modulating immune responses. Engagement of the monomeric T-cell receptor (TCR) by the monomeric peptide-major histocompatibility complex (MHC) for example (1BD2) is necessary but not sufficient for T-cell activation. Additional stimulatory and inhibitory signals are required for a physiological response. Dimeric bivalent CTLA-4 can bind two copies of B7-1 (PDB: 1I8L) or B7-2 (PDB: 1I85) to deliver inhibitory signals. The related CD28 (~30% sequence identity to CTLA-4) is also dimeric, but due to small alterations at the dimer interface, it is thought to be monovalent and thus each CD28 dimer binds only a single molecule of B7-1 or B7-2 [modeled on the basis of 1I8L and 1YJD; (55)] to deliver its stimulatory signal. Additional complexity arises from the oligomeric properties of B7-1 and B7-2, and the monomeric PD-1 receptor, which delivers inhibitory signals upon engagement of a single molecule of PD-L1 or PD-L2 ligand (PDB: 3BIK and 3BP6), both of which are monomers. The various oligomeric properties of these receptors and ligands might lead to the formation of a range of assemblies, which impact the detailed organization of the associated cytoplasmic signaling complexes. These few examples highlight the involvement of factors such as oligomeric state, valency and selectivity in immune modulation.

approved uses of the drugs are considered, including Crohn's disease and ulcerative colitis. Notable, these strategies for disruption of the TNF:TNFR interaction have overlapping, but distinct clinical indications, highlighting the empirical nature of biologics development and underscoring the need for multiple approaches to target specific pathways.

CTLA-4 is an inhibitory receptor in the Ig superfamily that competes with CD28 for the B7 ligands, resulting in inhibition of T-cell activation. Ipilimumab (Yervoy®), a function-blocking mAb that targets CTLA-4 and directs global immune stimulation, received FDA approval in March 2011 for treatment of late stage melanoma (8, 9). In contrast, abatacept (Orencia®) is a human CTLA-4-Ig fusion protein that induces global immune suppression by competing with CD28 for engagement with the cell surface-presented B7 ligands and is currently a leading treatment for rheumatoid arthritis (10, 11). Belatacept (Nulojix®), a variant of Orencia containing two point mutations, received FDA approval in November 2011 for prevention of acute kidney transplant rejection, with equivalent efficacy compared to existing treatments, but with greatly reduced side effects and toxicity (12, 13). Notably, belatacept possesses only a twofold increase in apparent affinity for the B7 ligands, but exhibits a tenfold enhancement in its biological potency (14). Additional efforts involving library generation coupled with next generation sequencing have resulted in CTLA-4 variants with a wide range of affinities and selectivities towards B7-1 and B7-2, which exhibited distinct effects on T-cell activation (15). Taken together, such findings strongly support continued biochemical/structural analyses for the generation of molecules with

unique biochemical properties and the development of novel therapeutic approaches (Fig. 2). This article focuses on recent biochemical and high-resolution structural efforts that define the chemical and physical determinants responsible for affinity, specificity and oligomeric state and the exploitation of these properties for the generation of reagents with new biological properties.

AFFINITY OPTIMIZATION

The quest for ever-increasing affinity is a goal frequently shared by campaigns to discover both small molecules inhibitors and biologics. An outstanding recent example is the structure-guided generation of a signal regulatory protein α (SIRP α) variant selected by multiple rounds of yeast surface display that recognizes CD47 with ~50,000-fold higher affinity than the wild-type molecule [i.e., equilibrium dissociation constants (K_{d} s) of 11 pM vs. 5 μ M] (16). CD47 is an antiphagocytic “do not eat me” cell surface receptor that binds to the monomeric monovalent SIRP α inhibitory receptor on macrophages to transduce an antiphagocytic signal. This recognition process allows for the discrimination of living and dying cells, and many malignancies express high levels of CD47 to evade the phagocytic action of macrophages. This behavior also offers an important therapeutic opportunity, as antibodies that block the SIRP α :CD47 interaction result in significantly enhanced phagocytosis of cancer cells (9, 10, 12, 13).

The high-affinity SIRP α mutant possesses nine mutations, many of which are located on the CD47 recognition surface and are predicted to directly influence binding. Direct crystallographic analysis of the mutant SIRP α :CD47

FIG. 2. Expanded CD28 and B7 families of costimulatory receptors and ligands. Well characterized receptor:ligand interactions are highlighted (e.g., CD28:B7-1/2, CTLA-4:B7-1/2, ICOS:ICOS-L, PD-1:PD-L1/2), as well as more recently discovered interactions (e.g., PD-L1:B7-1, CD28:ICOS-L). Also illustrated are members of the B7 family [B7-H3, B7-H4 (56), HHLA2 (31)] for which cognate receptors have not been reported. The outcomes of the various receptor:ligand interactions are depicted as stimulatory (+) or inhibitory (-).

complex demonstrates no significant structural alterations (16). Although the high-affinity SIRP α monomers exhibited greater potency in binding and blocking CD47 on cancer cells, they failed to promote phagocytosis of cancer cells upon coculturing with macrophages. In fact, no significant effect on the phagocytosis of tumor cells was observed upon treatment with the high affinity SIRP α monomers, SIRP α dimers lacking Fc chains or with a F_{ab} fragment of a high affinity CD47 blocking antibody, B6H12. Phagocytosis was only observed when CD47 was engaged and blocked by the high-affinity SIRP α monomers in presence of human IgG4 Fc domains either as a fusion protein or in combination with cancer targeted therapeutic monoclonal antibodies, such as trastuzumab, cetuximab and rituximab. The SIRP α engineered reagents with affinities greatly exceeding normal physiological values allows for sequestration of the targeted cell surface molecule (e.g., CD47), prevents engagement of the cell surface resident binding partner (wild-type SIRP α) and results in blockade of the associated signaling pathways. Coupling of these activities with immunoglobulin Fc-dependent macrophage-mediated engagement and phagocytosis of tumor cells represents a new potential therapeutic strategy.

A critical consideration in normal physiology and therapeutic design is the fact that optimal binding required for the desired biological effect need not always be the highest achievable binding (i.e., very high affinity). This concept is illustrated by numerous physiological examples. For instance, various members of the signaling lymphocyte activation molecule (SLAM) family exhibit homophilic interactions involving immunoglobulin variable domains,

which are characterized by K_d s spanning nearly three orders of magnitude (e.g., CD84, sub- μ M; NTB-A, 2 μ M; and CD150, 200 μ M) (17, 18). Thus, each SLAM family member has evolved an affinity that supports signaling properties that are biologically optimal. Equally important factors are tissue expression patterns and cell surface expression levels, which must be considered within the context of biochemical affinities. These considerations are reminiscent of the optimal, but not maximal cell surface densities, affinities and dwell times of T-cell receptor-major histocompatibility complex (TCR-MHC) interactions that contribute to efficient T-cell activation (19–21). These same principles are directly relevant to the development of therapeutic strategies.

A particularly important example is provided by the recent report of the mechanism of action of AMP224, a PD-L2 Ig-fusion protein composed of the extracellular domain of the programmed cell death ligand 2 (PD-L2) and the Fc region of human IgG that binds to the programmed death receptor 1 (PD-1) on T cell surfaces (22). When PD-1 binds to its ligands, B7-H1 (PD-L1) and B7-DC (PD-L2), it inhibits T-cell activation, proliferation and cytotoxicity and further induces apoptosis of T cells, thus contributing to the immunosuppressive character of the tumor microenvironment. Unlike recently described monoclonal antibodies directed against PD-1, AMP-224 does not act as a simple blocking agent. Preclinical studies using an orthologous surrogate for AMP-224 (murine AMP-224), in which the extracellular domain of murine B7-DC is fused to the Fc portion of murine IgG2a, demonstrated that a combination of low-dose cyclophosphamide and the murine AMP224 significantly induced E7-specific, CD8⁺ cytotoxic T lymphocytes (CTLs) and enhanced the therapeutic efficacy of a HPV16 E7 peptide vaccine in a murine model of a syngeneic E7-expressing, TC-1 lung carcinoma. This dimeric, bivalent reagent appears to possess relatively modest apparent affinity and has been reported to engage only those T cells presenting a high cell surface density of PD-1 receptors (i.e., >80,000 PD-1 molecules per cell) (22). Following murine AMP-224 administration, the proliferation of tumor infiltrating PD-1^{hi} CD4⁺ regulatory T cells (Tregs) and PD-1^{hi} CD8⁺ “exhausted” T cells was inhibited, thus reducing the population of dysfunctional, PD-1^{hi} T cells, while increasing the population of the “nonexhausted” PD-1^{lo} CD8⁺ effector T cells. Thus, AMP-224 tipped the balance of infiltrating lymphocytes from the immunosuppressive Tregs to immune effector cells leading to strong antitumoral immunity and tumor regression, which mechanistically distinguishes AMP-224 from typical neutralizing mAbs⁴. This example underscores the need to achieve optimal, not maximal, affinities/avidities to realize the desired biological/therapeutic activity of immune checkpoint inhibitors. It also highlights the significance of the levels and timing of the expression of cell surface target molecules on various T-cell subtypes for optimal design of immunotherapies.

OLIGOMERIC STATE AND AVIDITY

Another important factor in the development of biologics is the oligomeric state (and its modulation) because of its direct impact on avidity, which refers to the apparent affinity observed in multivalent molecules due to the contributions of multiple binding interactions. Avidities are difficult to predict, as in addition to the intrinsic binding energies associated with each individual interaction, there can be considerable entropic and enthalpic contributions from the effective local concentration and relative organization of multiple binding sites, as well as binding-induced strain and structural reorganization. Thus the avidity of multivalent molecules cannot be assumed to represent the simple summation of binding free energies for the individual sites. These factors are particularly relevant to the members of the TNF and TNFR superfamilies. TNF ligands are type-II membrane proteins that typically form tight noncovalent trimers on the cell surface. The protomers exhibit a classical jelly-roll topology, with loops at each of the interprotomer interfaces forming the binding sites for three receptor molecules composed of 1–4 cysteine-rich domains (CRD), resulting in a 3:3 receptor:ligand assembly (Fig. 3). These specific geometric constraints organize the receptor cytoplasmic tails for the recruitment of the signaling/scaffolding proteins, including TNF receptor-associated factors (TRAFs) and death domains, resulting in the activation of downstream signaling pathways. Of particular note are the relatively stable compact trimers formed by most TNF ligands and affinities for receptors in the low nM range, suggesting that soluble versions of these ligands might represent important high avidity, multivalent therapeutic reagents to modulate the functions of TNFR superfamily members. For example considerable effort has been focused on the development of CD40L and its variants for targeting CD40-related signaling pathways (23–26).

Notable exceptions to these generalities (e.g., compact, stable, high affinity for receptor) are the members of a small atypical subfamily that includes OX40L, GITRL and CD30. These proteins possess shorter TNF homology domains than other members of the TNF superfamily (~120–130 vs. ~150 residues) and direct structural characterization shows that GITRL and OX40L adopt less compact quaternary structures with far fewer interprotomer contacts than observed in other members of the superfamily (27–28). In the case of human GITRL, this modest interface results in a “weak trimer” with a readily detectable trimer $\rightleftharpoons$ monomer equilibrium (K_d of $\sim 10 \mu M^2$), in contrast to TNF, which shows no detectable dissociation. GITRL is further distinguished by a relatively weak K_d of $\sim 0.5 \mu M$ for its cognate receptor, GITR, which is approximately two orders of magnitude weaker than typical TNF:TNFR interactions. Based on these observations, it was suspected that the weak receptor binding activity was a direct consequence of the dissociation process, as receptor recognition requires an intact interprotomer interface. In this scheme, some of the

intrinsic binding energy for the receptor:ligand association would be “wasted” on driving the dynamic oligomerization behavior of GITRL towards productive trimer. Indeed, the N-terminal fusion of a GCN4-derived trimerization module resulted in a nondissociating species that exhibited substantially enhanced receptor binding ($K_d \sim 4$ nM; i.e., $\sim$ two orders of magnitude) and significantly greater *in vitro* T-cell activation (27).

Enhanced oligomerization has been a widely exploited strategy to achieve enhanced avidity and increased stability, as in the cases of numerous dimeric bivalent Ig-fusion proteins (e.g., Enbrel, Orencia). Many additional scaffolds have been examined to enhance avidity, including a variety of trimerization (24), pentamerization (29), hexamerization 7 and dodecamerization (25) domains. Even greater valency can be achieved by the use of modified nanoparticles or engineered cells expressing high levels of the desired proteins target (30). Although the highest avidity constructs are likely to represent excellent affinity tools for imaging and quantitation, these reagents must be carefully evaluated for the optimal therapeutic outcome, including the strength of the interaction and the immunogenicity of the oligomerization scaffold.

FUTURE CONSIDERATION: DISCOVERY/ EVALUATION OF NEW COSTIMULATORY MOLECULES

While current efforts in industry and academia appear to focus on a small number of costimulatory molecules, new and important activities continue to be identified [e.g., see ref. (31)]. There is a pressing need for the sustained and systematic evaluation of the many cell surface and secreted proteins that provide novel opportunities for immune modulation and potential therapeutic application (32). The New York Structural Genomics Research Consortium (NYSGRG; <http://www.nysgrg.org/psi3-cgi/index.cgi>) offers one such paradigm for achieving this goal. The NYSGRG is one of four large-scale structure discovery centers supported by the National Institute of General Medical Sciences’ (NIGMS) Protein Structure Initiative (PSI; <http://www.nigms.nih.gov/research/specificareas/PSI/pages/default.aspx>). These centers are charged with developing and applying technologies for the production and structural characterization of biomedically relevant and challenging classes of proteins. The NYSGRG, in collaboration with the Immune Function Network (<http://sbkb.org/center/atoms-to-animals-the-immune-function-network>), has emphasized infrastructure for high-throughput expression and purification of secreted proteins and the ectodomains of integral membrane proteins that control adaptive and innate immunity (33). These efforts rely on automated tissue culture capabilities that support the capacity to generate and evaluate the small-scale expression (i.e., 4 mL scale) properties of hundreds of baculoviral and lentiviral constructs per week (Fig. 4). This strategy is

FIG. 3. Dynamic self-assembly of human GITRL effects receptor affinity. Panel A: Ribbon representation of human tumor necrosis factor (TNF; PDB Code 1TNR) exhibiting the compact bell-shaped trimeric organization characteristic of the TNF superfamily. Panel B: Structure of human GITRL (PDB Code 2Q1M) exhibiting an atypical expanded trimeric architecture. Panel C: Representation of the reversible monomer-trimer equilibrium of the soluble human GITRL ectodomain. As only the trimeric GITRL is competent to bind receptor, this self-association behavior imposes an energetic penalty that reduces the apparent receptor-binding affinity. Panel D: Ribbon diagram of the constitutively trimeric (i.e., nondissociating) human GITRL construct (PDB Code 2R32), formed by the addition of a GCN4-derived coiled-coil trimerization module, which does not exhibit measurable dissociation and binds GITR with an affinity that is approximately two orders of magnitude greater than wild-type. The linker region connecting the coiled-coil trimerization domain and the TNF domain are not represented in the electron density; these covalent connections are depicted by dashed line segments.

critical as it allows for the rapid, efficient and cost effective identification of those proteins, constructs and complexes that are likely to yield actionable quantities of material in large-scale expression (e.g., liters), which represents the most costly step in the entire production pipeline in terms of both time, effort and resources (33). These capabilities offer considerable promise for large-scale evaluation of new

mechanistically informative tool reagents and potential therapeutics.

COMBINATION THERAPY OF COSTIMULATORY BLOCKADE AND RADIATION THERAPY

Radiation therapy has traditionally been used as a locoregional therapy where local tumor control has been

FIG. 4. High-throughput expression efforts for costimulatory molecule discovery. Panel A: The New York Structural Genomics Research Consortium (NYSGR) Cell::Explorer System, a custom-built solution for the automation of cell culture processes, is comprised of a pair of incubators, allowing parallel incubation of mammalian and insect cells, a JANUS® workstation and Multidrop™ Combi for liquid handling and analytical instruments (an EnVision® plate reader and a Guava® flow cytometer). Cells are grown in plates (6-, 24- or 96-well) that are moved between the different stations of the system by means of a six-axis articulated robot arm. Panel B: Representative small-scale screening outcomes for expression of ectodomains in insect cells. High Five™ cells, 4 ml volume in 24 deep-well blocks, were infected with baculovirus encoding eighteen (murine) members of the TNF receptor superfamily and fourteen CTLA-4 orthologs. Four days post-infection, the culture supernatant was harvested, incubated with Ni-IDA resin (His60, Clontech Laboratories Inc.) and the eluant analyzed by Coomassie stained SDS-PAGE. These small-scale expression evaluations readily identify those constructs appropriate for scale-up.

the primary goal. However, recent reports from several investigators have highlighted the systemic effects of radiation therapy in preclinical (34–40) and clinical settings (41, 42), primarily due to radiation-enhanced antitumoral immune responses. Cancer cells are more efficiently recognized and cleared by the immune system after irradiation, in part because radiation produces a spatially confined, oxidative environment by generating reactive oxygen and nitrogen species inside the tumor. The ionizing radiation-induced oxidative stress contributes to the increased antigenicity of irradiated tumor cells and augments the release of necessary activating signals for dendritic cells (DCs), also known as endogenous danger associated molecular pattern (DAMP) molecules from irradiated tumor cells. Cross-presentation of tumor antigens released from irradiated, dying tumor cells and presentation of radiation-induced neoantigens by DCs can potentially induce

antitumoral immunity that contributes to control of the primary tumor, as well as metastatic disease. This systemic effect of radiation therapy, also referred to as the abscopal (ab-scopus, away from the target) effect, has been occasionally described in cancer patients undergoing radiation therapy (43). With the recent advent of the combination of immunotherapy and radiation therapy, abscopal effects of radiation exposure have been invoked when clinical investigators have noted regression of nonirradiated metastatic disease after irradiation of a distant metastasis, usually in patients treated previously with an immune checkpoint inhibitor, such as anti-CTLA-4 antibody (e.g., ipilimumab) (41, 42). Although the term “abscopal effect” has a certain mystique, it fails to highlight the mechanisms responsible for the systemic effects of radiation therapy. As we embark on designing combination clinical trials of radiation therapy and immunotherapy, it will be

important to define the systemic immunomodulatory properties of radiation therapy and their contributions to radiation-enhanced autologous *in situ* vaccines (39, 44). A recent clinical trial of stereotactic body radiation therapy in combination with ipilimumab, named RADVAX, [ClinicalTrials.gov Identifier: NCT01497808 (<http://clinicaltrials.gov/show/NCT01497808>)], highlights the importance of radiation-enhanced vaccination effects in combination therapies. That irradiated tumor cells can be used, as an autologous tumor antigen depot, was first suggested in 1999 (34). In a spontaneous metastatic model of Lewis lung adenocarcinoma, these investigators demonstrated that a combination of ablative high-dose radiation therapy and Flt3L, a cytokine that stimulates DCs proliferation and circulation in the blood, resulted in enhanced long-term cures relative to radiation therapy controls. Subsequently, elegant studies by the Kroemer laboratory demonstrated that radiation therapy induces immunogenic cell death (ICD) (45). Irradiation of the CT26 colon carcinoma cell line leads to the translocation of cytosolic calreticulin to the cell membrane and provides a phagocytosis signal to DCs (46). Radiation also induces tumor cells to secrete HMGB1, a ligand for Toll-like receptor (TLR) 4, which subsequently leads to DC recruitment and activation (45). Further, the level of interferon (IFN)- γ in the tumor microenvironment and IFN- γ inducible genes increased after radiation therapy, resulting in the inhibition of cellular proliferation, enhanced angiogenesis, an increase in caspase-mediated cell killing and an increase in MHC class I expression on nonirradiated tumor cells (47). Radiation therapy can also affect the tumor vasculature by inducing an increase in the expression of endothelial adhesion molecules: VCAM-1, ICAM-1, P-selectin and E-selectin (48), thereby promoting increased infiltration of immune effector cells into the irradiated tumor microenvironment.

Besides tumor ablation, even radiation therapy at sublethal doses increases the cell surface expression of MHC class I molecules and amplifies the diversity of peptide antigen presentation by tumor cells (49). It also increases the expression of death receptors, such as Fas/CD95, which leads to an increased susceptibility of tumor cells to CTL-mediated cell killing (35). The ability of sublethal doses of radiation to induce or suppress the expression of cell surface immunogenic costimulatory and inhibitor molecules has been termed immunomodulation. Although the immunomodulatory doses of radiation may not eradicate the tumor completely, when combined with adoptive cell therapy or immune checkpoint inhibitors sublethal doses of radiation have been successful in eradicating both primary and metastatic tumors (35, 37). A search of the ClinicalTrials.gov website (<https://clinicaltrials.gov/>) shows a total of fifteen clinical trials using radiation therapy and ipilimumab for metastatic cancer patients. While empirical design of clinical trials would answer several questions about the RADVAX approach, preclinical studies and clinical trials should

attempt to address a number of critical parameters to optimize immunomodulation of radiation therapy, including total dose, fractionation, sequencing and type of immunotherapy. Preclinical studies highlight the importance of defining the time course and radiation dose response of the immunomodulation of cell surface costimulatory molecules that could result in a more rational design of combination therapy trials with immunostimulatory antibodies and radiation.

A number of biomarkers have been developed for predicting responses to immune checkpoint therapies (50). Several studies point towards an immune contexture of the tumor microenvironment that is associated with the type, density and distribution of tumor infiltrating immune cells [(51), reviewed in ref. (52)]. Based on these defining features, patients could be stratified according to immunoscore of the tumor microenvironment (53) for prognostic and predictive markers for immunotherapy (54, 55). For example, patients with high basal expression of FoxP3 and indoleamine 2,3-dioxygenase in tumors and high numbers of tumor infiltrating lymphocytes respond to ipilimumab therapy. Sublethal immunomodulatory doses of radiation could amplify the response to immunotherapy when the immunoscore indicates the presence of tumor infiltrating lymphocytes. In contrast, the absence of these cells in the tumor microenvironment suggests that a tumor vaccination approach has to be invoked to generate effector immune cells against nonimmunogenic tumors. In this situation, an ablative fractionation of radiation may be necessary to generate an autologous *in situ* vaccination. The partnership of immunotherapy with radiation therapy can be maximized if radiation exposure can augment antigen release and enhance the cell surface targets for immune attack without increasing immune suppressive factors, such as induction of CD4⁺CD25⁺ Treg and infiltration of tumor-associated macrophages and bone marrow derived suppressor cells.

In summary, we have described some of the key biochemical and biophysical features that are currently impacting the design of effective biologics for immunotherapy. At the same time, considerable opportunities continue to emerge in the area of radiation therapy. Given the complementarity and synergy of these approaches, combined therapies involving both checkpoint modulation and radiation therapy are now attracting considerable attention. To fully exploit these opportunities, future efforts must focus on fundamental studies that further elaborate the mechanistic underpinnings including: aspects of affinity/avidity; the expression characteristics of cell surface target molecules; and the coordinated timing of biologic and radiation administration. Given the largely empirical nature of these treatments and the challenges associated with the prediction of specific mechanisms, we anticipate that careful systematic evaluation of a range of biologics with distinct properties and their (spatial and temporal) combination with radiation will be required for the realization of new optimized treatments.

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