Report on the ISBTC Mini-symposium on Biologic Effects of Targeted Therapeutics

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Summary: The International Society for Biologic Therapy of Cancer held a mini-symposium on October 26, 2006 in Los Angeles to review current information regarding the biologic effects of both standard and targeted therapies. The purpose of the mini-symposium was to describe the existing knowledge regarding various biologic effects of current therapies, identify the most relevant issues and gaps in the knowledge base and discuss the optimal means of obtaining necessary missing information. Topics discussed included: (1) The impact of antitumor monoclonal antibody therapy on antigen presentation and adaptive immunity; (2) the effects of antiangiogenic/targeted therapy of the immune system; (3) the impact of chemotherapy on angiogenesis and immune function; (4) combination of antiangiogenic and immunotherapy at the clinical level; (5) the effects of tyrosine kinase inhibitors on T_H1/T_H2 response and T-regulatory cells; (6) the impact of farnesyltransferase inhibitors and other targeted agents on T-cell activation; (7) the impact of epigenetic modulators on biologic properties, and (8) the impact of the nature of cell death on the immune system. The ultimate goals of this mini-symposium were to use the above information to inform and influence basic science efforts and discussions, rationally design combination treatment regimens and optimally employ correlative studies in the context of ongoing and future clinical investigations.

Key Words: targeted therapy, biologic effects

(*J Immunother* 2007;30:577–590)

Received for publication February 17, 2007; accepted February 24, 2007.

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Financial Disclosure: David Carbone, MD, PhD, received a minimum speaking honoraria from Genetech and OSI. James Finke, MD was a previous Pfizer stockholder. All other authors have declared that they have no financial disclosures in regards to this manuscript.

Coordinated by the International Society for Biological Therapy of Cancer.

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Hanahan and Weinberg[1] described several alterations in cell physiology that are essential to malignancy. These included sensitivity to growth signals, insensitivity to antigrowth signals, limitless potential for replication, tissue invasion and metastasis, sustained angiogenesis, and evasion of apoptosis. Others have suggested the importance of chronic inflammation and acquired immune evasion. Recent investigations have identified molecular pathways that underlie these various properties and agents that potentially target those pathways. Taken together, this work has heralded the era of targeted therapy for malignancy with many agents available that target various pathways within the tumor cell or tumor microenvironment; several of which have already achieved Food and Drug Administration approval and are in widespread use.

Although these agents have, in many ways, begun to radically alter the way we view and treat cancer, they are mostly palliative with resistance typically developing over a period of months to a few years. The host-tumor interplay has generally been felt to be critical in achieving a durable tumor-free state. A number of such targeted agents inhibit multiple targets as well as similar or related pathways within host cells. Inhibition of such alternative targets within immune cells, endothelial cells, and cells or components of the tumor stroma might influence the tolerability, activity, duration of benefit, and ability to combine these various targeted agents. Understanding the resultant biologic effects of these targeted agents is critical to their optimal application.

The International Society for Biologic Therapy of Cancer held a mini-symposium on October 26, 2006 in Los Angeles to review current information regarding the biologic effects of both standard and targeted therapies (http://www.isbtc.org/meetings/am06/mini-symposium.php). The purpose of the mini-symposium was to describe the existing knowledge regarding various biologic effects of current therapies, identify the most relevant issues and gaps in the knowledge base, and discuss the optimal means of obtaining necessary missing information. The ultimate goals were to use the above information to inform and influence basic science efforts and discussions, rationally design combination treatment regimens and optimally employ correlative studies in the context of ongoing and future clinical investigations.

IMPACT OF ANTITUMOR MONOCLONAL ANTIBODY THERAPY ON ANTIGEN PRESENTATION AND ADAPTIVE IMMUNITY

Antitumor monoclonal Abs (mAbs) have emerged as effective antitumor therapies and can mediate antitumor effects by several mechanisms including a direct effect on tumor cells and recruitment of innate mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent lysis.[2] However, a growing body of evidence now supports the concept that mAbs may also enhance adaptive immunity.[3] Uptake of immune complexes or opsonized tumor cells by dendritic cells (DCs) leads to increased cross presentation of tumor antigens and the generation of antitumor T-cell responses.[4,5] This process depends on the engagement of Fc receptors (FcRs) on DCs. The FcR system consists of a balance of activating and inhibitory receptors. Selective blockade of inhibitory FcRs on DCs leads to DC maturation and is sufficient to drive cross presentation of antigens from phagocytosed opsonized tumor cells.[6] Harnessing the ability of mAbs to recruit adaptive immunity may enhance the durability of clinical responses, promoting cross-presentation and even targeting tumor cells lacking expression of the molecule targeted by the mAb. FcR polymorphisms that impact the balance of signaling via activating/inhibitory FcRs may have a major impact on the outcome of antibody therapy.[7]

Therapeutic applications of mAbs directed against human tumor-associated antigens have proven moderately successful as single modalities for the treatment of patients with a broad range of cancer types.[2] In addition to their abilities to down-modulate tumor cell membrane expression of their target antigens and focus immune responses or tumoricidal (chemotherapeutic or radionuclide) agents within the cancer microenvironment in vivo, additional, unexpected immunologic benefits may be linked to the administration of these drugs. Storkus et al have reported that agonist antibodies directed against receptor tyrosine kinases (RTKs) that are commonly overexpressed on the cell membranes of a broad range of tumor cell types results in specific RTK degradation mediated by the proteasome. This in turn enhances the level of RTK-derived peptides presented in tumor cell major histocompatibility complex (MHC) class I complexes, leading to the sensitization of antibody-treated tumor cells to lysis mediated by RTK-specific cytotoxic T cells in vitro and in vivo (in Hu-SCID models). Similar results were obtained using antibodies against the EphA2, epidermal growth factor receptor (EGFR) (cetuximab), and Her2/neu (trastuzumab) tumor antigens that are expressed coordinately on renal cell carcinoma and melanoma cell lines as well as other cancers. This suggests a general paradigm for enhancing the ability of moderate-to-low avidity, antitumor $CD8^+$ T cells to mediate clinically meaningful tumoricidal function as a result of antibody-based protocols. The success of this approach may be optimized in the context of combinational therapies that incorporate specific vaccination to accentuate the frequency of circulating RTK-specific CTL in cancer patients before, or concomitant with, the administration of clinical-grade antibodies.

EFFECTS OF ANTIANGIOGENIC/TARGETED THERAPY ON THE IMMUNE SYSTEM

Clinically evident cancers have acquired lesions in crucial growth regulatory pathways that enable unregulated growth, but to survive in an organism, they must also acquire a blood supply and avoid immune recognition and killing. It is becoming evident that these 2 characteristics that tumors acquire to survive in their host are often highly correlated and mediated by common factors and mechanisms. Two examples are the prostaglandins, particularly PGE_2, and the RTK signaling ligands vascular endothelial growth factor (VEGF) platelet derived growth factor (PDGF). Both of these factors have long been known to contribute to tumor growth and vascularization as well as tumor-associated immune suppression.

The major rate-limiting step in PGE_2 production in tumors is cyclooxygenase-2 (COX2) enzyme expression, and both VEGF and COX2 can be induced by hypoxia. VEGF expression is controlled by the HIF1 transcription factor, but Carbone and colleagues[8] have recently demonstrated that the most important factor driving the overexpression of COX2 in lung cancer cells is also HIF1. In fact, TRX-1, another gene commonly overexpressed in lung cancer, can activate HIF1 even under normoxic conditions leading to constitutive overexpression of both VEGF and COX 2 in many tumors.

Many studies show that elevated levels of VEGF are associated with poor prognosis and increases in tumor vascularity.[9] Clinically, anti-VEGF antibodies have been shown to prolong patient survival in several different tumor types. In tumor-bearing animals, elevated VEGF is responsible for alterations in the differentiation and function of many hematopoietic lineages, including DCs[10] and T cells.[11] Carbone and colleagues[12] have also shown that these VEGF-induced immune cells can have both immune suppressive properties and directly promote angiogenesis. A recent study has confirmed these findings demonstrating that anti-VEGF strategies reduced the number of $CD4^+CD25^+$ T regulatory cells as well as decreasing FoxP3 expression, when administered in combination with a GM-CSF–secreting tumor vaccine. This resulted in increased CTL induction and improved vaccine efficacy.[13] Reversal of at least some of these effects is also observed in patients treated with antibodies against VEGF.[14] One single-arm clinical trial of a vaccine and anti-VEGF has shown that the combination is associated with a high rate of immune response induction.[15] A randomized trial testing this hypothesis is planned with a MUC-based vaccine in patients with locally advanced nonsmall cell lung cancer.

Cancer treatment strategies designed to inhibit PGE_2 production with COX inhibitors have met with limited success. However, COX inhibitors affect a

plethora of protumor and antitumor arachidonic acid metabolites, making this result not surprising. To target just those specific functions of PGE_2 that promote tumor growth, researchers at Vanderbilt Cancer Center have begun studying the immune and antitumor effects of selective inhibition of the 4 PGE_2 receptors. They have shown that immunity induction and DC differentiation[16] as well as homing of antigen-presenting cells to secondary lymphoid organs can be dramatically improved with selective EP2 receptor inhibition, and metastasis establishment and growth inhibited by selective EP4 antagonism.[17] Thus, with the blurring of the lines between antiangiogenic therapy and immunotherapy, new opportunities are presenting themselves for improving the outcomes of cancer patients. These are in the process of being tested in human clinical trials.

IMPACT OF CHEMOTHERAPY ON ANGIOGENESIS AND IMMUNE FUNCTION

Expansion of vasculature is critical for tumor growth. Tumors cannot grow beyond a few millimeters in the absence of angiogenic support, which is provided by VEGF-A or VEGF and other soluble factors.[18] Approaches to block tumor angiogenesis have therefore attracted significant attention and antibody therapy targeting VEGF has provided proof of principle in the clinic.[19–21] Although cytotoxic drugs have been used at maximum tolerated doses (MTDs) to target dividing tumor cells, endothelial cells are also damaged by chemotherapy. When chemotherapy is used at MTD, the intervals between chemotherapy cycles, necessary for normal tissue recovery, also allow for tumor endothelial cell repair and development of "resistance" to the antiangiogenic effect of the treatment.[22] Antiangiogenic or low-dose metronomic chemotherapy was designed to overcome this problem through the close, regular administration of low doses of chemotherapeutic drugs with no extended drug-free intervals, over prolonged periods.[23,24] Short-term antiangiogenic effects have been shown in vivo for vincristine, vinblastine, doxorubicin, mitoxantrone, etoposide, paclitaxel, 6-mercaptopurine, 9-amino-20(S)-camptothecin, topotecan, camptosar, and combrestatin A-4 in preclinical models. The clinical effectiveness of low-dose metronomic chemotherapy has been reported in several early phase clinical trials, including weekly low-dose taxanes in breast and ovarian cancer; low-dose, oral etoposide in nonsmall cell lung cancer, and germ-cell tumors refractory to intravenous etoposide; and daily antimetabolites in acute lymphoblastic leukemia.

Tumor neovessels are different from normal vasculature, both at a morphologic and molecular levels.[25] Tumor vascularization likely develops through 2 complementary mechanisms: angiogenesis, or sprouting of endothelial cells (ECs) from existing vessels, and vasculogenesis, or recruitment of endothelial progenitors that differentiate into endothelial cells.[18,26–28] The contribution of lineage negative myeloid progenitor cells or $CD45^+$ monocyte precursors to tumor vascularization has been established convincingly in the mouse.[29–31] The relative contributions of these mechanisms may vary considerably between mice and humans, and among tumor types. In ovarian cancer, $CD45^+$ $CD11c^+$ VE-cadherin$^+$ vascular leukocytes with vasculogenic potential have been identified at high frequency.[32,33] The antiangiogenic effect of metronomic chemotherapy is felt to be multifactorial. Potential mechanisms include reduction of VEGF-A levels and/or increase in endogenous antiangiogenic factors; killing of tumor endothelial cells and direct inhibition of angiogenic sprouting; suppression of circulating endothelial progenitor cells (CEPs) and/or circulating endothelial cells (CECs); suppression of recruitment and function of CEPs and/or CECs in tumor.[23,24,34] Remarkably, tumor endothelial cells seem to be more sensitive to very low doses of chemotherapy than bone marrow cells, thus metronomic chemotherapy is effective without causing substantial myelosuppression in vivo.

Unlike conventional chemotherapy, where the MTD is viewed as the most effective dose, the optimal biologic dose of low-dose metronomic chemotherapy is harder to define and in the clinic doses have been arbitrarily chosen as fractions of the MTD. Optimization of low-dose metronomic chemotherapy may thus require painstaking clinical experimentation. Furthermore, the clinical end points to define therapeutic success for this form of chemotherapy may be quite different than for MTD-based chemotherapy. Unlike cytotoxic therapies, antiangiogenic therapies may not result in easily distinguishable decrease in tumor size. As a consequence, objective response rates may markedly underestimate the clinical benefit resulting from disease stabilization, increased progression-free or overall survival, palliation and increased quality of life. For example, a survival benefit from the addition of bevacizumab to cytotoxic chemotherapy has been apparent in metastatic colorectal cancer irrespective of whether patients achieve an objective response.[35]

Maraveyas et al[36] and Shaked et al[37] proposed that the optimal biologic dose of low-dose metronomic chemotherapy be the highest dose that can be delivered metronomically without causing bone marrow disruption or other major toxicity. Identification and validation of surrogate biomarkers would significantly accelerate the selection and clinical testing of optimal biologic dose. Circulating levels of endogenous proangiogenic factors such as VEGF, basic fibroblast growth factor (bFGF), or endogenous antiangiogenic factors such as thrombospondin-1 (TSP-1) and endostatin have been proposed as biomarkers of biologic efficacy of metronomic chemotherapy, as they partly mediate its effects.[38] Recently, CEPs and CECs have been proposed as reliable pharmacodynamic markers in preclinical studies. The optimal biologic dose of metronomic chemotherapy could be predicted by the maximum reduction in viable peripheral blood circulating VEGF receptor 2-positive (VEGFR-2^+) CEPs. Interestingly, MTD chemotherapy

was shown to induce robust CEP mobilization and tumors rapidly became drug resistant, whereas the administration of low-dose metronomic chemotherapy was associated with a consistent decrease in CEP numbers and viability and resulted in more durable suppression of tumor growth. Recently, a reduction in CECs below 11,000 cells/mL was associated with prolonged progression-free and overall survival in breast cancer patients receiving metronomic chemotherapy with methotrexate and cyclophosphamide.[39] Furthermore, achieving an objective response or stable disease with metronomic chemotherapy for breast cancer has been associated with a posttreatment increase in nonviable CECs.[40]

Owing to its low toxicity, metronomic chemotherapy is ideally suited for long-term combination with other drugs, including antiangiogenic drugs.[41] In preclinical models, metronomic chemotherapy enhances the effects of antibody or small-molecule antiangiogenic therapy.[34] Phase II trials of metronomic chemotherapy, sometimes used in combination with antiangiogenic drugs, have yielded encouraging results in patients with advanced cancer.[34]

Low-dose metronomic chemotherapy combinations with immunotherapy also seem promising. Combination of metronomic doses of cyclophosphamide with a DNA priming and vaccinia virus boost vaccine results in antitumor activity that is dramatically enhanced over either treatment alone. Remarkably, low-dose metronomic cyclophosphamide provides significantly greater synergism with vaccine than MTD cyclophosphamide. Both metronomic and MTD cyclophosphamide causes deletion of proliferating tumor-specific CTL in blood, but the metronomic dosing schedule causes deletion with slower kinetics and does not delete $CD43^{low}$ memory cells, which maintain substantial capacity to respond to repeat tumor challenge.[42] Recently, oral administration of metronomic cyclophosphamide was shown to induce a profound and selective reduction of circulating $CD4^+CD25^+$ regulatory T cells in advanced cancer patients. This was associated with suppression of their inhibitory functions on conventional T and NK cells, leading to a restoration of peripheral T-cell proliferation and innate killing activity.[43]

Thus, low-dose metronomic chemotherapy seems to be safe and convenient based on a large number of preclinical studies as well as an increasing number of early clinical trials. However, its clinical benefit remains to be verified. Despite lower cumulative doses of drugs, survival may be superior when compared with more dose intense regimens. Low-dose metronomic chemotherapy has been shown to produce antiangiogenic and immunostimulatory effects through a variety of effects. These biologic properties, together with its low toxicity profile, makes low-dose metronomic chemotherapy ideal for considering in combination with antiangiogenic and/or immunotherapies. Prospective randomized trials with translational pharmacodynamic end points are warranted to define the role of low-dose metronomic chemotherapy in combination with biologic therapy of cancer.

COMBINATION OF ANTIANGIOGENIC AND IMMUNOTHERAPY AT THE CLINICAL LEVEL

The interplay between cancer cells, stroma, blood vessels, and the cells of the immune system has profound implications on cancer behavior and therapeutic approaches. Identification of vascular and immune cell regulatory target molecules has allowed testing of specific agents that exploit these pathways for therapeutic benefit in cancer patients. For tumors to grow and expand, new blood vessels are induced to grow by a process of sprouting or splitting. The new lining of these vessels is recruited from endothelial stem cells, vascular leukocytes and even tumor cells mimicking endothelial cells.[32,44] Leukocytes traversing this vasculature have to attach to the endothelium, roll and adhere and eventually traverse the vessel to be able to attack the tumor. These processes are tissue and lymphocyte specific and are mediated by selectins, chemokine receptors and integrins on the leukocyte and adressins, chemokines, and immunoglobulin supper family molecules on the endothelial cells.

Clear cell renal cell carcinoma is, in part, controlled by the inactivation of the Von Hippel-Lindau gene with subsequent induction of hypoxic inducible factors including VEGF, a proangiogenic molecule, which has also been shown to have a regulatory influence on the immune system.[11,45,46] As noted previously, elevated levels of circulating VEGF have been shown to confer poor prognosis in RCC and other solid tumors as well as being associated with lower number of circulating immature DCs and immunosuppression.[47] Recently, a number of agents directed at VEGF or the VEGF receptor pathway have had clinical benefit in the treatment of metastatic RCC, including bevacizumab. It is conceivable that muting of VEGF effects in cancer patients with bevacizumab, will inhibit the immunosuppressive effects of VEGF, thereby potentiating immune therapies.

Bevacizumab is a recombinant, humanized mAb that was selected for its affinity to VEGF and inhibition of angiogenesis in murine xenografted human solid tumor models. In a randomized, double blinded placebo controlled phase II trial in previously Interleukin-2 (IL-2)–treated metastatic RCC patients, Yang and colleagues[48] reported improvement in time to progression for a 10 mg/kg dose bevacizumab administered every 2 weeks.

RCC-specific cytolytic T-lymphocytes (CTLs) are present in RCC patients, but in very low precursor frequencies.[49,50] CTL inactivation can be explained by multiple factors including a loss of T-cell receptor zeta chain (TCRζ) the signaling apparatus necessary for lysis,[51,52] defective signaling down stream from TCRζ and activation of inhibitory pathways (CTLA-4/CD28).[53–55] IL-2 is a central cytokine controlling lymphocyte function. IL-2 induces proliferation and the activation of effector T-cells.[56] Exposure of CTLs to IL-2 can correct the TCRζ signal defects and reverse the inhibitory effects of CTLA4, a rationale for the use of IL-2 as immunotherapy.[57]

IL-2 (Aldesleukin, Chiron/Novartis) has been approved by the US FDA for the treatment of patients with metastatic RCC since 1992 owing to the small number of patients having long lasting complete remissions.[58] However, single agent IL-2 treatment, with expansion and activation of T cells, is not sufficient to induce clinically relevant anti-RCC immune response in the majority of patients.

The rationale for combining high-dose bolus aldesleukin with bevacizumab includes potential positive interactions on the immune regulatory side, nonoverlapping toxicities, and potential for prolongation of tumor responses. The Cytokine Working Group has designed and is conducting a multicenter phase II study designed to estimate the efficacy of combination therapy of standard high-dose bolus IL-2 and bevacizumab therapy in metastatic RCC patients.

Patients with histologically confirmed metastatic renal cell carcinoma with predominantly clear cell histology with measurable or evaluable disease, a KPS of $\geq 80\%$, adequate end organ function, and no serious hemorrhage, bleeding diathesis, underlying coagulopathy, DVT, clinically significant peripheral vascular disease, or other thrombotic event are eligible for this study. One cycle consists of 84 days. Bevacizumab (10 mg/kg) IV is administered every 2 weeks, beginning at 2 weeks before the first dose of IL-2 and 1 to 2 hours before beginning IL-2 doses on subsequent weeks. High-dose bolus IL-2 (600,000 IU/kg) IV Q8 hours (maximum 28 doses) is given during two 5-day courses separated by 9 days (starting on day 15 and 29).

Fifteen patients have been enrolled with a median age is 54 (range 40 to 73) with 9 men and 6 women. The median number of bevacizumab doses during the first cycle was 7 out of a 7 (range 2 to 7) and the median number of IL-2 doses was 17 out of a maximum of 28 (range 6 to 26). There has been 1 treatment-related death. Typical IL-2 toxicities have been noted thus far.

Correlative biologic studies are embedded in this clinical trial. The nonessential amino acid L-arginine (L-arg) is converted to L-ornithine by arginase I activity. High levels of circulating ornithine, high levels of arginase I activity seen in myeloid suppressor cells, and low serum levels of arginine have been shown to induce loss of TCRζ, block T-cell proliferation, and reduce T-cell production of cytokines.[59] L-Ornithine levels are significantly elevated in RCC patients and thought to be related to VEGF's influence on arginase I and is another mechanism of immune escape. In this trial, L-ornithine levels were measured in the peripheral blood. In 4 patients tested to date, peripheral blood L-ornithine levels were dramatically decreased suggesting a treatment-related effect. Further analysis of the larger cohort will help establish the relationship this may have to response. This exploratory study is expected to reach its accrual goal of 60 patients within the next several months.

EFFECTS OF TKIs ON T_H1/T_H2 RESPONSE AND T-REGULATORY CELLS

Along with mAbs, small molecule tyrosine kinase inhibitors (TKIs) that antagonize the VEGF/PDGF receptors signaling have demonstrated significant antitumor activity in patients with metastatic renal cell carcinoma (mRCC) resulting in a paradigm shift in the treatment of this disease.[60,61] As clinical trials move forward with these multikinase inhibitors, it will be important to define their effect on the immune system as a prelude to combining them with various forms of immunotherapy.

The fact that clinical use of these TKIs can produce toxic effects, including lymphopenia, not directly attributable to VEGF/PDGF pathway blockade, raises the possibility that they will impair the ability of T lymphocytes to mount an antitumor immune response. Alternatively, these multikinase inhibitors may block the known immunosuppressive effects mediated by VEGF. VEGF effects in cancer patients include promoting the accumulation of myeloid suppressor cells, impairing DC antigen presentation and inhibiting the development of a type-1 cytokine response.[11,62,63] The accumulation of immature myeloid cells may in turn promote the development of T-regulatory cells in the tumor-bearing host.[64] Thus, inhibiting VEGF signaling may either enhance or inhibit antitumor immunity.

Finke et al have initiated studies to define the impact that treatment with the TKI, sunitinib, has on the type-1 cytokine response as well as Treg formation in patients with metastatic renal cancer. The rationale for targeting T_H1/T_H2 responses is based on the finding that a T_H2 bias exists in patients with renal cancer.[65–67] Tatsumi et al[65,66] had previously reported that antigen-specific $CD4^+$ T cells recognizing tumor-associated antigens, MAGE-6 and EphA2, in RCC patients produce primarily a type-2 cytokine (IL-5, IL-4) response with a significant reduction in a type-1 (IFNγ) cytokine response. Moreover, similar results are observed when RCC patient's peripheral blood mononuclear cells (PBMCs) were polyclonally activated.[67,68]

The analysis by Finke and colleagues[69] suggests that treatment with sunitinib can in fact promote a type-1 cytokine response (IFN-γ) and simultaneously decrease the type-2 response (IL-4) in patients with metastatic RCC. Peripheral blood from 23 metastatic RCC patients receiving sunitinib (50 mg daily dose) was obtained before treatment and on day 28 of a 42-day cycle. PBMCs were stimulated with anti-CD3 and anti-CD28 plate bound antibodies before assessing intracellular levels of IFNγ and IL-4 by a flow cytometry-based method (72 h). The percentage of T cells expressing intracellular levels of IFNγ increased significantly ($P = 0.001$) from day 1 to day 28 in 14 of 22 patients (64%). The corresponding number of T cells expressing intracellular IL-4 decreased in 14 of 22 patients, suggesting there was a shift from a type-2 bias to a type-1 response. Whether these findings reflect a conversion from a T_H2 bias to a T_H1 response in vivo and not the result of prolonged in vitro stimulation is being addressed by examining intracellular levels of

IFN-γ and T-bet (T_H1 transcription factor) in T cells of the patient after 4 hours of stimulation with anti-CD3/anti–CD28 antibodies. An analysis of the frequency of EphA2 and MAGE-6 specific $CD4^+$ T cells in the peripheral blood before and after sunitinib treatment using MHC class II tetramer/peptides complexes without any in vitro stimulation is currently in the works. It was also noted that the proportion of IFN-γ producing T cells at day 28 correlated with tumor shrinkage ($P = 0.02$). An important issue that remains to be resolved is whether the shift towards a T_H1 response in sunitinib-treated patients results from tumor shrinkage or is a direct effect of the treatment. Detection of changes in T_H1/T_H2 responses much earlier in treatment (eg, day 5), before any changes in tumor shrinkage, might help sort this out. It will also be important to test whether sunitinib or other TKIs have a direct effect on altering T_H1/T_H2 cytokine responses in vitro.

Finke and colleagues[69] have also observed that sunitinib treatment decreased the expression of the transcription factor FoxP3 within the Treg subset ($CD3^+CD4^+CD25^{Hi}$). This was initially determined by defining the percentage of cells expressing FoxP3 within the $CD3^+CD4^+CD25^{Hi}$ population using 4-color flow cytometry. Similar results were obtained after the isolation of Treg cells from patient's peripheral blood before and during sunitinib treatment using high speed cell sorting and immunofluorescent staining with anti-FoxP3 antibody (Rayman et al, unpublished). Current studies are testing whether the decrease in FoxP3 levels corresponds to decreased suppressive activity of the Treg population. The reduction in FoxP3 expression was observed without any in vitro culturing suggesting that changes in FoxP3 levels were present in vivo in Treg cells. It will be important to determine to what extent the decrease in Treg FoxP3 expression is merely an indirect effect of sunitinib-induced tumor shrinkage and whether it is associated with comparable changes in type-1/type-2 cytokine responses. It will also be of interest to determine whether the reduction in FoxP3 expression is attributable to a direct effect of sunitinib on Treg cells or, alternatively, the result of sunitinib inhibiting the production of immature myeloid cells or other cell types that can promote Treg expansion.

Additional studies are needed to define the impact that sunitinib and other TKIs have on restoring T cell responsiveness in cancer patients and to explore the mechanism by which this occurs. These findings could have implications for furthering the clinical benefit of such therapies in RCC and other tumors and particularly for combining these agents with various forms of immunotherapy.

IMPACT OF FARNESYLTRANSFERASE INHIBITORS AND OTHER TARGETED AGENTS ON T-CELL ACTIVATION: IMPLICATIONS FOR FUTURE COMBINATION THERAPIES

The development of therapeutic agents that target-specific signaling molecules in oncogenic pathways has represented an important shift in oncology drug development. These molecular targets include Src kinases, the Ras/MAP kinase pathways, PI3K and Akt, NF-κB, the mTOR pathway, and Stat-family molecules. As agents that target these pathways enter clinical trial testing in melanoma, renal cell carcinoma, and other tumors in which immunotherapeutic strategies are also employed, it is important to take into consideration the effects of targeted agents on the activation of T lymphocytes. Many of the same pathways being targeted for cancer are also known to be important in signaling via the T cell receptor for antigen (TCR), cytokine receptors, or other immunologically relevant receptors.

Gajewski and colleagues[70] within the CALGB recently conducted a phase II clinical trial of the farnesyltransferase inhibitor (FTI) R115777 as first line therapy in patients with metastatic melanoma. The rationale for this study included the involvement in Ras-pathway signaling in melanomagenesis, even in tumors that lack activating Ras mutations,[71] and the notion that Ras family proteins require farnesylation for membrane targeting and biologic activity. This study included the requirement for excisional biopsies pretreatment and during week 7 of therapy, to measure FT activity at tumor sites and other biochemical effects of the drug. Fourteen patients were enrolled in the first stage of a standard Simon 2-stage phase II study design, and there were no clinical responses. Despite the lack of clinical activity, FT activity was found to be reduced by approximately 90% in all patients' tumors. Moreover, several tumors showed robust reduction in the phosphorylation status of the Ras effectors ERK and Akt. These surprising results indicate that potent inhibition of these pathways in melanoma metastases in patients might not be sufficient to obtain antitumor activity in vivo, at least with this drug as a single agent.

In parallel, they investigated whether R115777 administration might reduce the activation of T cells stimulated through the TCR. For this analysis, they employed an assay that uses whole blood stimulated with the superantigen staphalococcal enterotoxin A (SEA). This format allows the circulating drug to remain present during the analysis, as the blood elements are not separated. They found that IFNγ production by T cells in this assay was reduced in posttreatment samples compared with pretreatment samples, suggesting that R115777 does indeed exert an immunosuppressive activity in patients.[70]

The inhibitory effect on T-cell activation provided an opportunity to investigate the mechanism by which FTIs block cytokine production, using in vitro cell culture models. Gajewski et al[70] found that cytokine production by both T_H1 clones (that secrete IL-2 and IFN-γ) and T_H2 clones (that secrete IL-4 and IL-5) was inhibited by FTIs, with T_H1 cytokines being more sensitive. Although the expectation was that the mechanism of this inhibitory effect would be at the level of Ras/MAP kinase signaling, which would lead to a reduction in cytokine mRNA synthesis, surprisingly MAP kinase signaling was not

found to be inhibited, and that induction of cytokine mRNA also was not affected. Rather, it was determined that cytokine protein synthesis seemed to be blocked. These results argue that FTIs were inhibiting T-cell cytokine production at the posttranscriptional level, likely by inhibiting translation (Marks and Gajewski, submitted for publication). These results have implications for the putative farnesylated targets linked to the antineoplastic activity for these drugs in acute myelogenous leukemia[72] and other tumors.

In addition to FTIs, investigators have studied many signal transduction inhibitors for effects on TCR-mediated T-cell activation. Using $CD4^+$ and $CD8^+$ effector T cells as a model, Dr Gajewski's laboratory has seen that inhibitors of MEK/ERK, JNK, PI3K/Akt, and Src kinases inhibit IL-2 production in a dose-dependent fashion. In addition, proteasome inhibitors (which block NF-kB activation) also suppress cytokine production, and rapamycin (which blocks mTOR/p70S6K) inhibits T-cell proliferation.

Collectively, these results suggest that care must be taken in the future when planning to combine targeted inhibitors with immunotherapeutic interventions. Otherwise, the inhibitory effect of some of these agents on T-cell activation may be quite profound. It may not be necessary to avoid such combinations completely. Rather, proper timing of dose and schedule may be sufficient to allow optimal T-cell activation in response to a vaccine on the one hand, and antitumor activity of a targeted inhibitor on the other.

IMPACT OF EPIGENETIC MODULATORS ON BIOLOGIC PROPERTIES

The epigenetic silencing of gene expression has been implicated in the malignant phenotype of melanoma and renal cell cancer, including the resistance to apoptosis and the evasion of immune recognition. The treatment of melanoma cell lines and short-term melanoma cell cultures in vitro with the hypomethylating agent decitabine upregulates the expression of genes involved in apoptosis [including apaf-1, tumor necrosis factor receptor-1 (TNFR1), and caspases 1 and 8], immune recognition (HLA class I, *MAGE* genes, NY-ESO-1), and interferon response.[73–79] In RCC, decitabine upregulates *MAGE* genes, tumor suppressor genes such as *RASSF1A* and *KANK*,[80,81] and caspase-1. These effects of decitabine on melanoma and RCC may therefore have the potential to render cells more susceptible to the antitumor effects of immunomodulatory drugs. However, the impact of decitabine on cancer immunotherapy may also depend on its off-target effects.

In order for decitabine to reverse CpG island methylation, it must be incorporated into DNA during S-phase of the cell cycle, with several rounds of replication required for demethylation of both strands of DNA.[82] Therefore, in addition to the expectation that decitabine would alter gene methylation in actively dividing tumors but not in dormant tumors, it would also likely alter methylation in other cell types actively dividing in cancer patients, including normal hematopoietic cells, activated lymphocytes, and vascular endothelial cells/pericytes involved in tumor neovascularization. From the standpoint of the biologic therapy of cancer, decitabine may be able to modulate the effect of immunomodulatory drugs on lymphocytes as well as the effects of antiangiogenic drugs and chemokines on tumor vasculature. However, it is important to note that the demethylation of CpG islands is not by itself sufficient to induce gene expression. The transcription factors required to activate a specific gene must also be expressed in order for a hypomethylated gene to be turned on. The off-target effects of decitabine will therefore depend on whether a given cell type is cycling as well as the make-up of the transcription factors expressed in that particular cell.

Some of the off-target effects of decitabine have been analyzed in a recently published phase I trial of low-dose subcutaneous decitabine combined sequentially with high-dose intravenous bolus IL-2 in patients with melanoma and renal cell cancer.[83] The low-dose decitabine regimen of 5 daily doses of decitabine administered for 2 weeks, used at a dose range of 0.1 to 0.3 mg/kg/dose, was the same schedule and dose used successfully to raise hemoglobin F (HbF) levels in sickle cell anemia patients.[84] In sickle cell patients, low-dose decitabine induced demethylation of the globin promoter in association with rises in HbF production.[85] In patients with melanoma and renal cancer, the addition of low-dose decitabine did not affect the ability to safely administer high-dose IL-2 and did not interfere with the antitumor activity of IL-2.[83] The only dose-limiting toxicity was prolonged grade 4 neutropenia attributed to the decitabine but not to the IL-2. Although grade 3-4 neutropenia was observed in sickle cell patients treated with low-dose decitabine, the frequency of grade 4 neutropenia was greater in melanoma and renal cancer patients, indicating that the off-target effect of decitabine on myelopoiesis was greater in solid tumor patients than in sickle cell patients. The mechanism underlying the neutropenia is currently undefined, as bone marrow biopsies have shown a decrease in myeloid progenitors but have not shown maturation arrest or provided any other clues as to why such low doses of decitabine selectively affect the myeloid lineage.[83,85] However, G-CSF did expedite neutrophil recovery in decitabine-treated patients.

Decitabine augmented HbF levels in melanoma and renal cancer patients, although this effect was very modest in comparison to the elevation observed in sickle cell patients.[83] This suggests that decitabine did alter globin gene methylation in erythroid cells, though the transcription factors required for HbF gene activation may have been relatively lacking in solid tumor patients compared with sickle cell patients. In the trial of decitabine plus high-dose IL-2, the effect of decitabine on DNA methylation was also more directly assessed by analyzing LINE methylation[86] in PBMCs. This assay showed a 13% reduction in DNA methylation after 2 weeks of

low-dose decitabine, and this demethylation overlapped with the period of high-dose IL-2 administration.[83] Importantly, the DNA hypomethylation was associated with the upregulation of > 1000 genes as well as the downregulation of > 1000 genes in PBMC. Among the 2 patients who had gene expression changes in PBMC analyzed using DNA spotted microarrays, there was a striking concordance between the genes affected in both patients, suggesting that decitabine elicits a specific pattern of gene expression changes in PBMC.

Approximately 5% of the genes upregulated by decitabine in PBMC had an immunomodulatory function, with the most notable including: interferon γ, interferon γ receptor-2, numerous interferon response genes, IL-8, IL-8 receptor α, multiple genes involved in tumor necrosis factor signaling, toll-like receptor 6, intercellular adhesion molecule-3 , IL-17 and IL-22 receptors, and FcGamma receptor 2A.[83] Downregulated genes included CTLA-4, IL-12 receptor β2, stat4, CCR7, CD2, and CD3-epsilon. It is not yet known whether these changes in gene expression are associated with changes in protein expression, or which cell subsets are affected. Likewise, it is not yet known whether these changes in immunomodulatory gene expression have functional significance. However, the findings show that decitabine does have off-target effects on PBMC that may either enhance or interfere with the generation of cellular immune responses. This may be pertinent not only to cytokine therapy with drugs like IL-2 and interferon α, but also to vaccine therapy and adoptive immunotherapy, and underscores the need to understand the off-target effects of hypomethylating agents like decitabine when using them to modulate the tumor response to other biologic agents.

IMPACT OF THE NATURE OF CELL DEATH ON THE IMMUNE SYSTEM

There are several factors which drive immunity, particularly in the setting of cancer.[87] Janeway proposed the existence of factors that initiated the adaptive immune response, which he termed "Signal 0's"[88] to distinguish them from Signal 1 (antigen/MHC), Signal 2 (costimulation, signaling intensity), and what we now recognize as Signal 3 (polarization to T_H1-T_H4 subsets dependent on the environment). Signals 4 (localization to individual epithelial sites mediated by integrins) and the largely unexplored Signal 5s, discriminating and integrating events during the effector phase are less well characterized. Furthermore, he described that the most critical property of immune system was its ability to discriminate self from nonself. Given that every cell division produces 30 to 100 mutations and the average colon cancer harbors approximately 12,000 mutations, this property requires exquisite precision. Janeway felt that infection and cell damage were the major signals involved in lymphocyte activation and hypothesized that the immune system functioned through the identification of pathogen-associated molecular pattern molecules (PAMPs) and Matzinger and others through "danger" or "damage"-associated molecular pattern molecules (DAMPs).[89,90] PAMPs include bacterial products such as lipopolysaccharides and CpG motifs derived from hypomethylated DNA.[91]

DAMPs are largely cytosolic intracellular or degraded matrix proteins that possess other distinct functions, but when released by dying cells or disturbed matrix can act extracellularly to induce inflammation.[92,93] The receptors[94–97] involved in recognizing DAMPs include, in addition to TLR4 and other TLRs, the receptor for advanced glycation end-products present on activated endothelium and inflammatory cells. In most cases, acute inflammation results in healing, initiated by either DAMPs or PAMPs. Higher numbers of microorganisms are associated with a longer period of healing and greater scarring. Molecules important for healing include high mobility group box 1 (HMGB1),[95,98] purine metabolites such as uric acid, and the degraded matrix components, hyaluronan and heparin sulfate found within the extracellular matrix.[90,97,99–102] Recruited macrophages, myofibroblasts, mast cells, and eosinophils promote healing depending on the site and nature of the insult. The polarized patterns of immune reactivity occur in response to environmental cues including both the DAMPs and PAMPs within the injured tissue.

DAMPs including HMGB1 and heat shock proteins can function as nominal "superadaptor" molecules. HMGB1 is mainly a nuclear protein, but it is also present in the cytosol, vesicles, and cell membrane, as well as found within the extracellular space. HMGB1 has both nuclear and extracellular functions.[95,98,100,103] Its ubiquitous presence in cellular compartments and adaptive basic/basic/acidic domain substructures that enables it to bind many different partners, indicate that HMGB1 might act as a superadaptor molecule, promoting various cellular functions and signaling depending on its location. Similarly, heat-shock proteins chaperone damaged and unfolded proteins within the cell and at the cell membrane, and shuttle these proteins into antigen-presenting cells. DAMPs share features such as basic domains, sugar-binding domains and a molten globule-like state that favor their interaction with many different molecules. This promiscuous binding might explain their pleiotrophic effects in biologic systems including as the ability to bind individual partners, correlating with an extracellular proinflammatory activity.[94,96] Several DAMP receptors function as uniquely configured sensors and adaptor molecules initiating stereotyped and integrated responses.[95] Stress sensors are typically sensitive to genomic, metabolic, ER-unfolded protein or membrane stress. Sensors of cellular stress communicate to cells of the innate and adaptive immune response.[93,96,98,104–107]

HMGB1 is released by dying tumor cells or secreted by activated NK cells or macrophages.[94,108] Recent experiments suggest that cytolytic cells release HMGB1 when lysing tumor targets.[109] Furthermore the mechanism of cell death, either apoptotic or necrotic, might influence the degree of HMGB1 release. Lotze et al have

studied HMGB1 release in association with melanoma cell (451Lu, WM9, FEM X, MEL397) death induced by LAK cells, tumor-specific cytolytic T lymphocytes, TRAIL, or granzyme B delivery. HMGB1 release from melanoma cells (451Lu, WM9) was observed within 4 and 24 hours after incubation with IL-2–activated PBMCs (LAK cells). Tumor-specific cytolytic T lymphocytes also induced HMGB1 release in FEM X, T2 cells pulsed with gp100 peptide. This HMGB1 release was only partially blocked by the pancaspase inhibitor zVAD-FMK indicating that this cytolytic death was at least in part necrotic in nature. TRAIL treatment induced HMGB1 release within 24 hours and this extrinsic pathway-mediated cell death was completely blocked with zVAD-FMK. Conversely, granzyme B delivery induced apoptosis, but did not induce HMGB1 release. Thus HMGB1 release may elicit local inflammatory responses and be important for the emergence of some tumors arising as the consequence of chronic inflammation.[87,93,101,110–117]

Modern imaging cytometry allows for automated spatial and temporal detection of multiple flourophores simultaneously in fixed cells in high-throughput microplate formats. Image analysis algorithms can be used to quantify subcellular localization of specific fluorescent labels throughout the nucleus, cytoplasm, and cell membrane at the single cell level. Modes of cell death can be distinguished using this approach by using markers that correlate with apoptosis, autophagy, and necrosis. Lotze and colleagues have developed imaging cytometric assays to distinguish these modes of cell death in human fibroblasts and tumor cells induced by commercially available reagents, using the Cellomics ArrayScan V^{TI} imaging cytometer. Levels of cytoplasmic SR-VAD-FMK and mitotracker are used to identify apoptotic cells, cytoplasmic monodansylcadaverine (MDC), MAP-LC3, or Lysotracker to label autophagic cells, and nuclear Sytotx Orange, 7-AAD, HMGB1, or Toto-3 to measure necrotic cells.

Using imaging cytometry it is possible to categorize type I (apoptosis), type II (autophagy), and type III (necrosis) cell death and to distinguish them from cellular senescence. These high content screening assays can be used to examine the nature of cell death mediated by chemotherapy, LAK/T-cell, vaccinia oncolysis,[102,118,119] and irradiation as well as for novel targeted agents. Their robustness, ease of use, and validity when compared with other standard assay methodologies promotes their serious consideration in developing and assessing novel therapies.

DISCUSSION

This mini-symposium highlighted an emerging appreciation and understanding of the mechanistic complexity associated with the use of targeted therapies. This complexity takes many forms. For example, some targeted agents, such as imatinib mesylate, engage multiple intracellular kinases, and thus possess the capacity to disrupt multiple signaling networks simultaneously. In some cases this can produce multiple indications for a single agent. In the case of imatinib mesylate, a single drug targets the kinases that drive each cancer (ie, bcr-abl and c-kit, respectively) to effectively treat patients with vastly different malignancies (eg, chronic myelogenous leukemia and gastrointestinal stromal tumors). Alternatively, multitargeted agents might engage a primary target as well as a downstream, parallel, or upstream modifier of the target or targeted pathway's function; or, such a therapy might influence a particular pathway as well as a completely different mechanism of tumor sustenance. One such example comes from the actions of EGF receptor inhibitors, which can modify proangiogenic signaling by VEGF. Needless to say, these off-target effects can contribute to toxicity as well as efficacy[120] so care must be exercised when these agents and combinations are developed. Furthermore, how cells die as a consequence of this therapy, can no longer be discounted as unimportant and this may critically affect the quality and nature of the host response.

It is important to recognize that this discussion of "off-target effects" encompasses 2 distinct settings. In the first instance, the off-target effect is the direct consequence of the multiple specificities of the targeted agent, such as the multiple targets of agents such as sunitinib. The second setting can be described as secondary consequences of an initial targeted effect, such as the effects of EGFR signal inhibition on tumor cell-initiated secretion of VEGF.

The goal of cancer therapy should be durable benefit for the patient, and it has been long presumed and experimentally demonstrated that immune control contributes to this benefit. It is widely appreciated that immune therapies such as cytokines, vaccines, adoptive transfer of immune cells, and allogeneic cell infusion all mediate their effects via immune mechanisms. Several investigators (eg, L.W. and M.D. in this mini-symposium) have hypothesized and demonstrated that some cancer-directed antibodies indeed initiate adaptive immune responses directed against the targeted cancer antigen and, in some cases, to other cancer-related targets.[2] However, it is certainly plausible that so-called standard therapies may induce host-protective immunity that contributes to long-term control of cancers. The induction of such immunity may be uncommon for conventional cytotoxic agents that cause significant immunosuppression. However, the increasing use of nonimmunosuppressive targeted therapies with pleiotropic effects on tumor proliferation and apoptosis (see below) may warrant reexamination of this premise. Frequently, such efforts are confounded by a lack of a priori knowledge of the tumor regression antigen that might be relevant for a given individual. However, proof of concept can be obtained in animal models, where tumor rechallenge experiments can be supplemented by increasingly feasible searches for regression antigens and effector cell specificities. The work of Coukos, Lotze, and others, some of which was discussed in this mini-symposium, suggests

that efforts to induce tumor infiltration by activated T-lymphocytes and DCs may constitute an appropriate surrogate of immune control of cancer.

The method of tumor cell death also may prove to be a pivotal determinant of whether effective cancer therapy induces host-protective adaptive immune responses. As discussed by Lotze in this mini-symposium, probing the relationships among types of treatments, modes of cell death and immune responses is likely to identify mechanisms that can be amplified or selectively suppressed to promote the induction of effective antitumor immunity in preclinical models. The relevance of these findings can then be determined in appropriately designed clinical studies.

Numerous investigators have demonstrated important interactions between tumor-derived angiogenesis and immunosuppression, as discussed by Carbone and Coukos at this mini-symposium. Coukos described the rationale for metronomic chemotherapy, with particular reference to the antiangiogenic and immunomodulatory properties of this approach to cancer therapy. Carbone amplified on the immunomodulatory effects of such therapies, and described the rationale and promise of selectively manipulating prostaglandin activity to promote antiangiogenic effects. Thus, it is important to determine the "off-target" effects of diverse therapies, with a goal of optimizing those effects that can promote tumor control. Thus far, some of these approaches have been explicitly tailored to achieve a potentially off-target effect, such as combining an anti-VEGF antibody with a tumor vaccine to promote tumor immunity, or as described by Ernstoff at this mini-symposium, combining IL-2 and bevacizumab. In another variation on this theme, Gollob has described the use of a combination of the demethylating agent decitabene and high-dose IL-2. However, it may be at least as fruitful to identify clinically effective approaches to treat cancer with chemotherapy, vaccines or angiogenesis inhibitors and determine the diverse mechanisms that might underlie clinical benefit. For example, if combining a metronomic chemotherapy approach with an antiangiogenesis agent improves clinical outcomes, it would be interesting and potentially important to determine whether patients develop antitumor immune responses that could be beneficially shaped, amplified, or sustained by the addition of other treatment modalities. Equally important, it will be useful to determine whether compensatory mechanisms that promote tumor growth (eg, the induction of Treg by long-term antiangiogenesis therapy, leading to immune suppression and escape from immune control), contribute to clinical benefit, or primary or acquired drug resistance. For example, Finke discussed at this mini-symposium that sunitinib therapy decreases T-regs. However, it remains to be demonstrated that this is a primary mechanism as opposed to a reacquisition of immune competence resulting from decreased tumor volume.

The perspective of biologic therapists differs from some other cancer treatment disciplines in that tumor: host interactions are clearly viewed as being central to the establishment, maintenance, and treatment of cancer. However, this broad viewpoint has extended only minimally into the realms of chemotherapy, surgery, and radiation therapy. Small-molecule signaling inhibitors are viewed almost exclusively based on their known cellular targets, and are rarely considered beyond that limited context. Their influences on tumor-host interactions require careful study. In another example, medical oncologists routinely combine antibodies with small molecule chemotherapy agents, but rarely if ever consider how those antibodies might interact with the host immune system. For example, Dhodapkar pointed out that antibodies can induce adaptive responses, and it is now widely accepted that polymorphisms in CD16 influence treatment outcomes in B-cell lymphoma patients treated with rituximab.[7] However, surprisingly little effort has been expended to understand the mechanisms that underlie such effects. Do the findings establish ADCC as the relevant mechanism of action for this antibody, or do other factors, such as FcR-facilitated antibody-directed crosslinking of CD20 play a role in the observed variations in clinical response? How does the intrinsic sensitivity of B-lymphoma cells to apoptosis regulate their capacity to undergo immune-mediated apoptosis through complement fixation or ADCC? Can a systems biology approach resolve some of the conceptual ambiguity regarding the interactions of tumors, host genotype, and the host immune system? And indeed can clinical investigations be designed to address these questions efficiently and productively?

Implicit in all these studies are the caveats provided by Finke at this mini-symposium regarding the lack of correlation of measurable immune responses with clinical outcomes. Finke's presentation highlights the challenges faced by researchers who wish to use biomarkers to measure biologic effects of any form of cancer therapy. Most of the tests used to measure biologic effects are unvalidated, have large confidence intervals, and suffer from variability in sample acquisition and processing. For example, the effects of small molecule TKIs may be lost in the time it takes to process cells and conduct the relevant assays. It has long been known that the examination of events in the peripheral blood or skin may not provide an appropriate surrogate of events that take place in tumors or tumor-draining lymph nodes. Even if such tests can be designed and executed with a measure of precision, relating the results to clinical outcomes is a precarious endeavor at best. Moreover, attempts to correlate biologic measures and clinical benefit suffer from the proverbial "chicken and egg" question. Does the inhibition of a tumor angiogenesis marker cause or result from a treatment-related antiangiogenesis effect? Despite these obvious but important limitations, such tests can provide a proof of concept regarding the biologic effects of a treatment, as in the sunitinib therapy-induced decrease in Treg cell numbers reported by Finke, or the loss of immune function reported by Gajewski for treatment with a farnesyltransferase inhibitor. Even with fairly objective measures such as cell numbers, such data still

must be interpreted with caution as cell numbers may in many cases prove to be unrelated to cellular phenotype or clinical outcomes.

It is recognized that many of the animal models used to create proofs of concept or preclinical justifications of dose and schedule for biologic therapies are simply inadequate. To replace xenograft models in immunodeficient mice, chemically induced cancer models and occasional syngeneic models derived from cell lines, elegant transgenic and knock-in mouse models have been designed to at least partly model the development of human malignancies. However, virtually all of these models incompletely replicate the timing, interactions of genes and environment, inflammatory anlage, and even the complement of genetic abnormalities that culminate in the vast majority of human cancers. Orthotopic transplants of human tumors in relevant rodent organs are feasible but highly cumbersome. Despite these limitations, the development of improved animal models should prove pivotal in translating fundamental observations into the clinic. Continuing efforts to generate improved versions of models that are relevant to human cancers remain high priorities for development. The Mouse Model Consortium provides an important resource for researchers in this field, and the design and implementation of new models should be a high priority, particularly to examine multifaceted tumor: host interactions.

While we describe many conceptual and practical challenges to understanding and leveraging the off-target effects of biologic therapy, there is no doubt that these effects are frequent, can be crucial in contributing to clinical outcomes, and thus can be manipulated to improve cancer treatment. Surprisingly little work has been done so far to elucidate these off-target effects, and all participants in the mini-symposium agreed that this dearth of information creates huge opportunities to identify new targets and improve cancer treatment. The challenge before researchers in the field is to seek out these effects, define them, and determine how they contribute to or detract from either efficacy or toxicity. This will require a commitment to develop innovative concepts, to test the concepts in appropriate animal models, and to design clinical studies with unambiguous end points that can validate biomarkers and demonstrate their relationships to clinical outcomes. It should not be forgotten that it may be easier to dissect the reasons for success than it is to try and develop a new treatment approach from the ashes of prior failures. Accordingly, an examination of how clinically effective targeted therapies affect the tumor stroma, modify angiogenesis, and influence the host immune response may yield important clues that guide the development of this field.

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