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Is immunity in cancer the key to improving clinical outcome?

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The central importance of the immune system in the natural history of cancer control and progression is now clearly established. It can prevent growth and kill the cancer cells, but also facilitate tumour progression through selection of the most fit cells to survive in an immunocompetent host or through altering the local microenvironment that promotes tumour outgrowth.[1] Immunotherapy (IM) has now been clinically validated as an effective treatment for many cancers. The important breakthroughs were led by the impressive impact of blockade of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) or programmed cell death protein 1 (PD1) on survival of a proportion of patients with, for example, metastatic melanoma or non-small-cell lung cancer, which were previously relatively refractory to existing treatments.[2] However, objective tumour responses are only seen in a fraction of patients across different malignancies; many do not benefit at all and there can be significant toxicities. Numerous strategies are currently being evaluated both preclinically and clinically to better understand and combat the immune-suppressive factors significantly limiting patient response to therapy.[3] IM has usually been considered as an alternative to more traditional modalities. Previously the view has been that chemotherapy is inherently immunosuppressive and not suitable for combining with IM. These generalizations are being challenged by a new paradigm whereby immune surveillance is the agent that improves and even cures some patients with cancer, even those treated by conventional radio- or chemotherapy.[4]

This meeting of international clinical and scientific researchers addressed questions of importance to the optimization of different aspects of IM focused on improving patient benefits. The meeting addressed topics broadly in the following categories: (1) the influence of standard and novel chemo- and radiotherapies on immune responses; (2) the design and delivery of vaccines to stimulate antitumour immunity; (3) combinational immunotherapeutic regimes including with immune checkpoint inhibitors.

Influence of chemo- and radiotherapies on immune responses

Understanding how existing or experimental cancer treatment regimes may influence the functioning immune response in a positive way, for example by differentially impacting immunosuppressive cells or factors, can open opportunities for optimizing novel immunotherapy configurations.

To kick off the meeting, organizer *Angus Dalgleish (St George's, University of London, UK)* provided examples of drugs used in cancer treatment when their actions have been shown to include an influence on immune factors. For example, lower doses of the opioid antagonist naltrexone (NTX) are able to reduce tumour growth by interfering with cell signalling as well as by modifying the immune system.[5] The Immunomodulatory drugs(IMiDs), immunomodulatory compounds lenalidomide and pomalidomide, are agents with anti-inflammatory, immunomodulatory and anticancer activity. Indeed, lenalidomide is a very effective treatment for multiple myeloma.[6] Both pomalidomide and lenalidomide enhance tumour antigen uptake by dendritic cells (DCs) with an increased efficacy of antigen presentation, indicating a possible use of these drugs in DC vaccine therapies.[7] *Rachael Cant (St George's, University of*

London, UK) reported that peripheral blood mononuclear cell(PBMC) secretion of interleukin (IL)-6 induced by toll-like receptor (TLR)-2, 7, 8 and 9 but surprisingly not TLR4 ligands was inhibited by NTX in a dose-dependent manner. Further, NTX inhibited IL-6 and tumour necrosis factor (TNF)-α production in monocytes, B cells and plasmacytoid DCs after TLR7, 8 and 9 stimulation. This work offers some potential mechanisms for the positive experiences associated with the use of low-dose NTX in cancer as mediated through modulation of immunosuppressive cytokine production.

Michael Shurin (Boston University, MA, USA) noted that conventional radio- or chemo-therapeutic cancer treatment protocols in many cases provide benefit for only a proportion of patients, with failure associated with the development of tumour cell chemoresistance by multiple mechanisms. At maximum tolerated dose (MTD), the treatments can often be immunosuppressive, while reduced dosing may provide for immune stimulation by differential depletion of immunosuppressive populations, and even lower doses may have direct positive effects on tumour immunity. Levels of myeloid-derived suppressor cells (MDSCs) in the tumour and the circulation can predict response to chemotherapy.[8] Tumour MDSCs produce high levels of various immunosuppressive enzymes including myeloperoxidase (MPO). MPO contributes by increasing the degradation of drugs such as doxorubicine under oxidative conditions. One strategy to improve drug stability is the use of degradable carbon nanotube carriers that can be functionalized to transport and protect drugs. The nanotubes can be made into small nano-caps to allow loading of a drug and then closed with gold nanoparticles.[9] The tumour-associated MDSC MPO first targets the degradation of the nano-cap, releasing the drug (e.g. paclitaxel), which can then act locally to influence conversion of MDSCs into DCs. Proof-of-principle models in mice show a significant reduction of tumour MDSCs and inhibition of tumour growth.[10] The chemistry of the nanotubes can be varied to allow targeting of different drugs to modulate levels of various inhibitory cell types.[11] This type of approach emphasizes the challenges of balancing the pro- and antitumour effects of drugs in cancer treatment.

Viktor Umansky (DKFZ, Heidelberg, Germany) focused on overcoming immunosuppression induced by chronic inflammation and associated with poorer prognosis in melanoma.[12] Studies in a transgenic mouse melanoma model have shown increased levels of inflammatory factors [IL-1β, granulocyte macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)-γ] plus enrichment of MDSCs in melanoma lesions and lymphatic organs during tumour progression.[12] The MDSC infiltration was associated with a strong T-cell receptor ζ-chain downregulation in all T cells. A phosphodiesterase (PDE)-5 inhibitor, sildenafil, was able to reduce the levels of inflammatory mediators and correlated with decreasing MDSC density and immunosuppressive function. Pharmacological inhibition of MDSC-suppressive pathways is a potential strategy to overcome disease-induced immune defects, leading to enhanced effectiveness of immune-based therapies. Treatment with the PDE-5 inhibitor sildenafil inhibits the degradation of cyclic guanosine monophosphate(cGMP), which is a secondary messenger-activating protein kinase and a common regulator of ion channel conductance, glycogenolysis and apoptosis.[13] Importantly, it reduces nitric oxide and arginase-1(ARG1) production by MDSCs, which allows the partial recovery of ζ-chain expression, IL-2 production and proliferation of T cells, with increasing numbers detectable in the lymph nodes and tumour, and improved survival of tumour-bearing mice. CD8 T-cell depletion abrogated the sildenafil effect, suggesting the involvement of MDSCs and CD8 T cells in the observed therapeutic effects. These observations suggest that inhibition of chronic inflammation in the tumour microenvironment should be applied together with immunotherapies to increase their efficacy in melanoma treatment.[14] An open-label, dose de-escalation trial with the PDE-5 inhibitor tadalafil in pretreated patients with metastatic melanoma has now been performed and the treatment was well tolerated.[15] Stable disease was achieved in 3/12 patients (25%) with median progression-free survival of 4.6 months (range 0.7–7.1) and median overall survival of 8.5 months (range 2.7–23.7). Stable patients showed higher numbers of $CD8^+$ tumour infiltrating lymphocytes (TILs) in the middle of their metastases before treatment compared with patients with progressive disease. Following tadalafil treatment, $CD8^+$ and $CD4^+$ TILs and $CD8^+$ T cells in the peripheral blood showed increased CD3 ζ chain compared with baseline. These results support the use of tadalafil to improve clinical outcome of patients with advanced melanoma by enhancing antitumour immunity. Determining how best to deploy this drug in the sequencing of different components of combined melanoma IM will need to be addressed.[15]

Daniel Fowler (St George's, University of London, UK) presented work studying zoledronic acid (ZA) effects on macrophages. ZA is an aminobisphosphonate bone-specific agent that inhibits farnesyl diphosphate synthase. The strong affinity for bone, and not for other tissues, allows its use as a potent inhibitor of bone resorption and remodelling activity, with limited potential for side effects in non-skeletal tissues. It was reported that $V\delta2^{+}\gamma\delta$ T-cell perforin-dependent cytotoxicity of ZA-treated human M1 and M2 macrophages resulted from the upregulation of the specific target antigen, isopentenyl pyrophosphate. This may have implications for the use of osteoprotective therapies like ZA used in the management of patients with advanced prostate cancer as bone metastases have a major impact on morbidity and mortality.

Emily Webb (University of Southampton, UK) reported on the immune-modulating properties of chemotherapy in preclinical and *in vitro* murine models of neuroblastoma (NXS2; TH-MYCN; NB9464D). IM with a monoclonal antibody (mAb) against the tumour-associated GD2 (a disialoganglioside), GM-CSF and IL-2 was associated with a significantly improved outcome compared with standard therapy in patients with high-risk neuroblastoma.[16] However, short-term benefits have not translated to improvements in the longer-term survival rate and side effects of neuralgia are common. There are potential benefits of inducing immunogenic cell death in a tumour while also modulating the immunosuppressive tumour microenvironment. It was reported that mafosfamide and doxorubicin both alter immunogenic cell death markers in neuroblastomas (e.g. heat shock protein 70 and calreticulin detection at cell surface) while low-dose activated cyclophosphamide (CPA) significantly depleted regulatory T cells (Tregs) but not other CD4 and CD8 T cells in the infiltrate of the tumours. Further studies are exploring combination CPA treatment with anti-PD1 in the tumour models, with the results suggesting improved survival. A major challenge for the application of these approaches in the clinic is how such treatments will affect the many heavily pretreated patients.

Thomas Sayer (NCI, Frederick, MD, USA) discussed the targeting of the extrinsic apoptosis signalling pathway for cancer therapy. The TNF-related apoptosis-inducing ligand (TRAIL) appears to preferentially induce apoptosis of transformed cells and the use of TRAIL or agonist antibodies has been shown to be tolerable as a cancer therapy. However, cells can become resistant to TRAIL apoptosis, and identifying compounds to combine with TRAIL is a strategy to amplify the apoptotic effects.[17] A key rheostat of cell death signalling is the ripoptosome, a 2 MDa protein complex, with core components of caspase 8, Fas associated via death domain(FADD); Cellular Flice inhibitory protein(cFLIP); Receptor interacting protein(RIP1); it controls the activation of apoptotic and necroptotic cell death responses.[18] A high-throughput screen identified withanolide E, a steroidal lactone from *Physalis peruviana*, as highly active for sensitizing several human cancer cells to TRAIL-mediated apoptosis. This is mediated by depleting levels of cFLIP proteins through destabilization or aggregation, implicating an interference with associated chaperone proteins. Animal studies showed sensitization of human renal carcinoma cells to TRAIL-induced apoptosis by withanolide E with no toxicity.[19] Among diverse stimuli, TLR ligation is able to induce ripoptosome formation. This offers the prospect of combinational synergies with additional TLR immune stimulatory activities also contributing to cancer control.[20] More extensive tests of withanolide E on induction of apoptosis of human cancer cells and impacts on immune sensitization are in progress.

IL-2 treatment in melanoma treatment is widely practiced, with the mechanism of action believed to be through cellular immune effectors but with vascular leakage as an unwanted side effect. As discussed by *Brendon Coventry (University of Adelaide, Australia)* the clinical response rates from a meta-analysis of IL-2 therapy of metastatic melanoma are disappointing. The overall rates were complete response (CR) 4.0% [95% confidence interval (CI) 2.8%–5.3%], partial response 12.5% (95% CI 10.1%–15.0%) and overall response 19.7% (95% CI 15.9%–23.5%). The data demonstrated that CR rates were similar for intermediate *versus* high IL-2 dosing.[21] The main message here is that the natural history of any cancer involves a balance between activation and inhibition of tumour immunity, and this unstable system can deliver CRs in an unpredictable manner. This is evidenced by their occurrence even following repeated failures of some therapies. Such unexpected CRs might account for 1–10% of CRs irrespective of tumour type or particular therapy and CRs underpin 5-year survival statistics. The potential for applying mathematical modelling to account for this inherent variation might help measure the more precise impact of the different therapeutic interventions. Perhaps the lesson is that single

agents that influence only limited components of a patient's immune status that *de facto* allow tumour survival will mostly not be effective.

Understanding the full range of factors that can influence tumour survival in its host is clearly important to developing the best ways to retrieve or stimulate effective immune control of a patient's cancer. *Jorg Wischhusen (University of Wurzburg, Germany)* highlighted that immunogenic mutations are selected against in some but not all cancers.[22] This emphasizes the role for additional escape strategies that can provide immune tolerance against neoantigens, a situation which is managed successfully during pregnancy when there is high expression of many immunosuppressive factors. Growth differentiation factor 15 ((macrophage inhibitory cytokine 1 (GDF-15(MIC-1)) is a divergent member of the transforming growth factor (TGF)-β family that is expressed at high levels in the placenta, seminal fluid and the prostate but otherwise only in stressed tissues. Low levels in the blood provide an indication of miscarriage in early pregnancy.[23] However, about 50% of human tumours show high levels of GDF15 expression. In patients with stage III/IV melanoma, high serum GDF-15 level is an independent predictor of poor survival.[24] There is evidence for a role of GDF-15 in immune escape from knockout glioma cells exhibiting improved tumour infiltration by T cells and macrophages and tumour control *in vivo*. GDF-15 is also highly expressed in the prostate and has been associated with inflammation and tumourigenesis.[25] Immune infiltration depends on lymphocyte function-associated antigen 1 (LFA-1)/intercellular adhesion molecule 1 interactions at the endothelial cells of blood vessels. GDF-15 prevents the activation of the LFA-1 integrin molecules on the T cells, thereby inhibiting adhesion to the endothelial cells. This action can explain the inverse association seen with intratumoural T cells in animal and human tumours, and correlates with the lack of response seen to checkpoint inhibitor anti-PD1 treatments in some patients with melanoma. Indeed GDF-15 is superior to lactate dehydrogenase level as a prognostic marker in patients with melanoma and independent of the mutational load. A recent melanoma cohort study was reported showing a statistically significant correlation with survival and lower GDF-15 levels that can also predict the survival of anti-PD1-treated patients. mAbs to mouse and human GDF-15 with high affinity and recognizing the mature dimers have been developed. In mouse tumour models, anti-GDF-15 cotreatment with anti-PD1 or anti-CD40/poly(I:C) significantly improves survival. A useful side effect of antibody treatment is the prevention of cancer cachexia. The wider safety of anti-GDF-15 and its potential impact in cancer treatment remain to be investigated. However, the modulation of this vascular checkpoint might have utility in improving the response rates to immune checkpoint blockade by allowing improved lymphocyte infiltration of tumours.

Key genetic mutations are known to drive the development of different cancers, and the influence of these changes on the immune response has largely been unexplored. *Samir Khleif (Augusta University, GA, USA)* focused on immune targeting of mutant genes like *KRAS* in human tumours in which, in spite of the immunogenicity of many experimental vaccines, there has been no evidence of altered clinical outcome. It appears that tumour cells with mutant *KRAS* induce the secretion of IL-10 and TGF-β1, which drive Treg induction.[26] Inhibition of KRAS reduces the infiltration of Tregs in KRAS-driven lung tumourigenesis even before tumour formation. So targeting KRAS and its downstream signalling pathways could be used as an immune modulatory strategy in cancer IM.[26] The challenge is to specifically target Tregs and not conventional T cells. Fortunately, there is a functional dichotomy in class IA phospoinositide-3 kinase(PI3K) isoforms in these two subsets that can be exploited. The PI3Kδ was shown as functionally critical in Tregs, acting to control T-cell-receptor signalling, cell proliferation and survival.[27] Coadministration of a PI3Kδ-specific inhibitor with a tumour-specific vaccine decreased numbers of suppressive Tregs and increased vaccine-induced CD8 T cells in the tumour microenvironment, promoting strong antitumour efficacy.[26] In other tumours the use of low-dose CPA is an alternative strategy to deplete Tregs while enhancing effector and memory cytotoxic T-lymphocytes (CTLs) within the tumour microenvironment.[28]

Vaccine design and delivery

Historically, immunotherapies have focused on the stimulation of the immune response to tumour-associated antigens (TAA) (cancer vaccines) or delivery by antibodies of immune activation or of drug payloads. The inherent genetic instability of cancers provides the capacity for

tumour evolution in the face of natural or induced antitumour immunity at the level of mutations of the TAA or its presentation in the context of the major histocompatibility complex (MHC).

Barbara Seliger (Martin Luther University of Halle-Wittenberg, Germany) reviewed the plethora of Major Histocompatibility Complex(MHC) and antigen-processing machinery (APM) downregulation immune evasion strategies that have been described in cancers.[29] In tumours of virtually all tissue origins, defects in the expression or function of these components have been found to facilitate immune escape. The underlying molecular mechanisms involve structural alterations of MHC class I antigens or APM components at the transcriptional, post-transcriptional or epigenetic level. In addition, signal transduction pathways, oncogenes, and putative tumour suppressor genes can influence the expression of MHC class I APM components in tumour cells. The local conditions of the tumour microenvironment of pH, oxygenation and metabolic activities can also affect MHC and related expression.[29] Knowledge of, or ability to modulate these downregulatory influences may be valuable in selection, or enhancement, of patients for specific immunotherapies.[30] For example, some MHC loss observed in tumours is hard wired by irreversible genetic changes while in other tumours the defect may be restored by the action of IFN.[29] However, there are also tumours that exhibit resistance to IFN treatment. Given the heterogeneity of tumours within an individual, determining a practical MHC phenotype for modulation might prove very difficult. The lesson could be that to stimulate effective antitumour immunity, the more targets recognized by the adaptive immune response the better.

In some cases it might be possible to devise drugs that can potentiate immune responses to release polyclonal antitumour immunity. *Raj Chopra (Institute of Cancer Research, London, UK)* described examples of where small molecule inhibitors (SMIs) might have the potential to modulate immune responses. Thalidomide and its analogues, which are approved for treatment of multiple myeloma and myelodysplastic syndrome, have pleiotropic effects including inhibition of the release of TNFα and IFNγ from T cells.[6] The underlying molecular mechanisms centre around cereblon E3 ubiquitin ligase which is the primary target of thalidomide and its analogues. Drug binding increases the affinity of cereblon to the transcription factors IKAROS Family Zinc Finger(IKZF) and IKZF3 and casein kinase 1α (CK1α). Ubiquitination and degradation of these neosubstrates results in IL-2 release and growth arrest of multiple myeloma and myelodysplastic syndrome cells. These results offer opportunities to search for new SMIs to exploit ubiquitin ligases for specific degradation of disease-associated proteins.[31] A second example postulated that SMI inhibition of endoplasmic aminopeptidases of the APM (endoplasmic reticulum aminopeptidase(ERAP)1/2) could enhance DC presentation of tumour neoantigens by altering the antigen profile. ERAP1 is the principle target as ERAP2 cannot complement ERAP1 and 25% of the population have a null genotype. ERAP1 has polymorphisms that alter its trimming ability and some are associated with autoimmune ankylosing spondylitis. *Marina Natoli (Imperial College London, UK)* presented evidence of the effect of a hypomethylating agent guadecitabine (SGI-110) on the immunogenicity of ovarian cancer cells. The rationale of the approach is that epigenetic modifications might lead to improved immune activation in ovarian cancer.

One approach to maximizing the optimal antigen presentation is the use of DC-based vaccines. *Lisa Butterfield (University of Pittsburgh, USA)* reflected on aspects of the more than 200 DC vaccine clinical trials in patients with melanoma conducted since 1996. Past work has seen 5–10% clinical responses in some studies but overall no consistent efficacy and no correlation to vaccine antigen immune responses. This undoubtedly, in part, results from the variability between studies of the means of producing DCs, loading antigen, specific antigen selection, dosing, route of immunization, scheduling, assessment of potency and clinical impact.[32,33] Building on this experience, an ongoing trial of a vaccine of adenovirus encoding tyrosinase, Melanoma-associated antigen recognized by T cells -1(MART-1) and melanoma-associated antigen A6(MAGE-A6) antigens transduced into autologous DC, given three times followed or not by IFNα. Early results have seen evidence of a few clinical responses, CD4 and CD8 peptide responses (not as apparent against the full-length target) and determinant spreading to GP100 and NY-ESO in some patients. There was no evidence of an effect of IFN or the presence of adenovirus-neutralizing antibodies on either clinical or vaccine-related T-cell responses.[34] A transcriptional analysis of the DC vaccines is attempting to correlate the profiles with clinical outcomes with impacts on

MHC, costimulatory molecules and immune checkpoint-related expression, suggesting candidate combination treatment designs. There are currently five phase III studies of DC vaccine and five phase II studies of vaccine with anti-PD1 in progress that include patients with melanoma.

Kees Melief (Leiden University Medical Center, The Netherlands; and ISA Pharmaceuticals, Leiden, The Netherlands) stressed the central importance of the design and delivery of cancer vaccines with the ability to induce strong T-cell responses as the key components of success. Thus the choice of target antigen may influence the available T-cell repertoire (e.g. neoantigens rather than self-antigens); the vaccine platform needs to avoid antigen competition and provide for efficient processing by DCs to stimulate durable CD4 and CD8 T-cell responses [e.g. synthetic long peptides (SLPs), DNA, RNA] with an adjuvant to deliver a T-helper-1 polarized response.[35] Selection of vaccine target antigens that are obligatorily expressed in the cancer cells, such as viral oncogenes in human papillomavirus (HPV)-associated cancers, is clearly an advantage. These various factors were exemplified by overlapping SLPs of HPV16 E6 and E7 oncogenes with montanide adjuvant vaccine, which has shown efficacy in treating high-grade HPV-associated premalignant disease of the vulva but was unable to have significant impact on more advanced malignant disease.[36,37] A combination of HPV16-SLP vaccination with standard carboplatin and paclitaxel chemotherapy was investigated in mouse tumour models and in patients with advanced cervical cancer. Treatment of tumour-bearing mice with chemotherapy and vaccination significantly improved survival and was directly associated with the chemotherapy altering the myeloid cell population in the blood and tumour. Chemotherapy had no effect on tumour-specific T-cell responses. In patients with advanced cervical cancer, carboplatin paclitaxel also normalized the abnormal numbers of circulating myeloid cells, and this improved the T-cell responses of the patient. The lowest number of myeloid cells was seen at 2 weeks after the second cycle of chemotherapy and this point was chosen for vaccination and subsequently validated in patients who generated very strong and sustained HPV16-specific T-cell responses to a single dose of the vaccine.[38] A clinical trial [ClinicalTrials.gov identifier: NCT02128126] is now in progress that is assessing the safety, tolerability and the HPV-specific immune responses of different doses of the long peptide HPV16 vaccine with or without pegylated IFNα as combination therapy with carboplatin and paclitaxel. There is some evidence that treatment of larger tumours may benefit from the use of checkpoint inhibitors. The wider potential for using SLP vaccines, perhaps for neoantigens identified from tumour mutational analyses together with improved prediction programmes for MHC class I binding (ISABELLA) is now practically deliverable with the availability of a machine that can generate 100 SLPs to good manufacturing practice(GMP) in a single run. In this context, the winning poster presentation by *Derin Keskin (Dana-Farber Cancer Institute, MA, USA)* reported on a mass spectrometry based discovery of the human leucocyte antigen (HLA) peptidome approach with applications to neoepitope discovery and analysis of potential relevance to IM. While a bioinformatics and technical tour de force, the ultimate value of such approaches crucially depends on the power of the different algorithms used to comprehensively identify putative candidate targets. The individual tumour heterogeneity and potential for evolution will make it difficult to assess individual clinical utility. The major hurdles to widespread clinical application are likely to be logistics and most importantly the cost.

DNA-based vaccines encoding TAA have several advantages over protein- or peptide-based vaccines in that they can be produced more cheaply, reproducibly and, with electroporation delivery techniques, can be immunogenic in humans. *Vicky Brentville (Scancell)* described the ImmunoBody platform (Scancell, Nottingham, UK) for inducing high-avidity T-cell responses to TAAs. This DNA vaccine approach encodes CTLs and helper T-cell epitopes to replace complementary determining region(CDR) regions within the framework of a human immunoglobulin G1 (IgG1) antibody.[39] DNA vector immunization (gene gun or electroporation) allows for expression and direct processing by antigen-presenting cells (APCs) or following cross presentation of secreted immunoglobulin molecules and uptake *via* Fcγ receptors. Without the Fc component, there is a 10–100× reduction in vaccine potency. The first clinical lead product is SCIB1, an ImmunoBody with three epitopes from gp100 and one from TRP-2 engineered into its CDR regions. There are two HLA*0201 epitopes (TRP-2 and gp100) plus two CD4 epitopes (HLA-DR4 restricted gp100 and gp100 restricted by HLA-DR7, HLA-DR53 and HLA-DQ6). It was shown that the TRP-2 CD8 epitope breaks

tolerance and induces high-avidity T-cell memory responses to this self-epitope and the gp100 DR4 epitope stimulates strong CD4 T-cell responses. Recent studies have shown that there is a benefit in combining PD1 blockade with SCIB1 vaccination with the induction and proliferation of high-avidity T-cell responses at the tumour site leading to improved tumour control in animal models.[40] A clinical study in patients with stage III or IV melanoma with tumours found that the SCIB1-encoded epitope induced T-cell responses in 10/11 patients with no toxicity. Overall survival was 19 months, with patients showing clinical responses including two partial responses and stable disease. Results were even more dramatic in patients with fully resected disease as they all showed a T-cell response and are still alive with a current median observation time of 3 years.[41] SCIB2, encoding NY-ESO-1 epitopes, is a second clinical product for melanoma treatment with preclinical studies showing that the DNA vaccine induces potent antitumour immunity which is further enhanced by checkpoint blockade.[42] Some results from an early-phase vaccine dose escalation and safety clinical trial in patients with advanced melanoma were reported; there was no evidence of toxicity, confirmed immunogenicity and some objective clinical responses in three of nine patients.

Identifying suitable TAAs that are naturally recognized by the immune response and boosting this with vaccination is another approach to harnessing the full spectrum of potential antitumour immunity. Brentville also described such an opportunity through a platform called ModiTope. Both pathways of protein degradation, the proteasome for short-lived and autophagy/lysosomal for long-lived proteins and organelles, are also involved in antigen presentation or the effective delivery of peptides to MHC molecules for presentation to T cells. Stressful conditions of the tumour microenvironment promote autophagy in the cancer cells as a means of survival. This autophagy also causes post-translational modification of proteins (citrullination of arginine by Ca^{2+}-dependent peptidyl arginide deiminase(PAD) enzymes) and thus citrullinated peptides loaded onto MHC-II molecules can stimulate CD4+ T-cell responses.[43] It has been shown in mice that intermediate filament protein VIM (vimentin) citrullinated peptides induced CD4+ T cells in response to autophagic tumour targets. Indeed a single immunization with modified peptide induced long-term survival in mice bearing tumours for 2 weeks with no toxicity.[44] Therefore CD4+ cells can mediate potent antitumour responses against modified self epitopes presented on tumour cells providing the impetus for the use of citrullinated peptides produced during autophagy as vaccine targets for cancer therapy. The lead clinical target vaccine being developed contains three citrullinated peptides (two from vimentin and one from α-enolase restricted by DR4 or DP4) with target cancers of triple-negative breast cancer, ovarian carcinoma and osteosarcoma. Validation in appropriate mouse tumour models is in progress.

Another approach to maximizing the composition of the TAAs of vaccines exploited knowledge of the degradation processes in cells. *Bernard Fox (Earle A. Chiles Research Institute, OR, USA; and UbiVac, OR, USA)* described a novel multivalent vaccine that is created by disrupting degradation of intracellular proteins by the ubiquitin proteasome system. The DRibbles vaccine is composed of autophagosome vesicles containing defective ribosomal products (DRiPs) and short-lived proteins (SLiPs), known TAAs, mediators of innate immunity, and surface proteins that promote phagocytosis and cross presentation by APCs. The DRiPs and SLiPs are abundantly produced by tumour cells but are unstable, rapidly poly-ubiquitinated and degraded by the proteasomes. These DRiP/SLiP antigens, if delivered to APCs for cross presentation, could act to stimulate antitumour immune response. This can be achieved by simultaneously blocking proteosomal degradation and by stabilization of DRiP/SLiP proteins by modulating the cellular autophagy pathway which then produces autophagosome microvesicles containing DRiPs/SLiPs, plus other proteins that can facilitate cross presentation. These autophagosomes are then harvested by membrane disruption and fractionation to create the DRibbles vaccine.[45] This vaccine would be relevant in patients without pre-existing tumour immune recognition when immune checkpoint blockade would not be beneficial. Proof of principle was established using murine methylcholanthrene sarcomas with classical tumour-specific antigens where tumour immunized mice rejected only the homologous tumour, with no cross reactions. Mice were vaccinated with either the individual sarcomas or their derived autophagosomes and then challenged with the same or different sarcomas. In contrast to the whole-cell vaccines, the autophagosomes from the sarcomas treated with a proteasome inhibitor are able to prime T cells that cross react with the

different sarcomas and protect a significant proportion of vaccinated hosts from a nonhomologous tumour challenge.[46] A combination of intranodal delivery of autologous or allogeneic DRibbles together with anti-OX40 antibody has been shown to induce strong memory and effector T-cell responses. This vaccine-induced cross priming of $CD8^+$ T cells that recognized shared tumour antigens in the context of host MHC class I molecules. This supports the potential to combine allogeneic 'off-the shelf' DRibble vaccines together with antibodies against costimulatory molecules in the clinic.[47] The development of a standard product for clinical use is under way with the type of clinical trial envisaged for patients with stage III or IV non-small cell lung carcinoma given pretreatment with CPA, then vaccinated. The use of protein arrays and patient antibody responses may provide a useful surrogate for immune monitoring.[48] The affinity of T cells may not be an overriding feature of useful tumour-specific immunity as it is the biology of the effectors in the tumour environment that is critical, and this may allow a wider range of T-cell recognized neo- and unaltered antigens to be viable targets.[49]

Combinatorial approaches

To be effective, cancer vaccines will most likely need to stimulate polyclonal antitumour-specific immune responses. They will also need to avoid stimulating immune suppressive components. Combinatorial approaches that aim to remove or reduce existing immune suppressive factors can potentially maximize the recovery of existing or novel stimulated antitumour activity, changing the balance in favour of cancer control and elimination. Several interesting investigations on the timely use of chemotherapy, radiation treatment and immune modulators such as checkpoint inhibitors in combinatorial approaches were presented.

Ignacio Melero (Clinica Universidad de Navarra, Madrid, Spain) discussed the potential for synergistic combinations of IM agents and for combining IM agents with conventional cancer treatments. Successful licensing of treatment following clinical trials evaluating combining blockade of CTLA4 and PD1 might be able to instruct future approaches to immuno-oncology combination therapy.[50,51] The range of potential combinations is very large, since they include costimulatory antibodies (CD137, OX40, CD40, GITR), conventional agents inducing immunogenic cell death (chemotherapy, radiotherapy, antiangiogenics, targeted therapies), checkpoint inhibitors (CTLA4, lymphocyte activation gene-3(LAG3); T-cell immunoglobulin and mucin-domain containing-3(TIM3); B & T lymphocyte attenuator-4(BTLA4); T cell immunoreceptor with Ig and ITIM domains(TIGIT)), cancer vaccines (neoantigens), functional modification of immunosuppressive enzymes [Indoleamine 2,3 dioxygenase-1 (IDO1); inducible nitric oxide synthase (iNOS)], Treg targeting or inhibition, adoptive cell therapy and myeloid cell modulation. Additional issues that will be important in optimizing benefits and need to be investigated include the route of delivery, dosing levels, duration, cycles, coadministration or sequential administration, and planned or reactive protocol designs, with most of these often quite arbitrary. However, while there are over 900 clinical trials of combination therapies in progress, unfortunately these have not been rationally or coordinately designed. Nevertheless, individual trials are reporting encouraging results, for example, the use of checkpoint inhibitors with anti-CD30 linked to the antimitotic agent monomethyl auristatin E (MMAE) in Hodgkin lymphoma.[52] CD137 (4-1BB) is a surface costimulatory glycoprotein on activated T lymphocytes; stimulation by mAbs or other agonist moieties therapeutically augments the cellular immune response against tumours, irrespective of CD137 expression on tumour cells.[53] Another study has provided preclinical and clinical evidence that the proimmune effects of radiotherapy can be synergistically augmented with immunostimulatory anti-PD1 and anti-CD137 mAbs.[54] Animal models have shown that CD8 T cells, induced through cross presentation by particular DCs and requiring type I IFNs, mediated the therapeutic activity. Further, there is evidence of synergy through radiotherapy with impact even for tumour sites outside the field of irradiation. These mechanisms support the clinical development of combination therapies using anti-PD1 and anti-CD137 mAbs and radiotherapy.[54] This type of approach exemplifies the concept of acting locally but with global benefits.

David Waxman (Boston University, MA, USA) discussed metronomic drug-delivery schedules. CPA is a bifunctional alkylating agent prodrug frequently used in cancer treatment. CPA can lead to immunogenic tumour cell death by inducing innate immune-alerting signals like calreticulin and High mobility group box 1(HMGB1) by tumour cells, thereby stimulating cross presentation of tumour antigens to T cells. In addition,

CPA depletes immune-suppressive Treg cells and boosts cytokine responses, including production of type I interferons that boost the differentiation and mobilization of mature DCs and expand T cells with a memory phenotype. For CPA, immunogenic responses can be achieved when using metronomic drug-delivery schedules, whereby lower doses of drug are given at regular but more frequent intervals than conventional MTD chemotherapy.[55] Studies of CPA administration on an intermittent, every-6-day metronomic schedule were shown to deliver a strong, innate antitumour immune response in glioma models in immunodeficient and immune-competent mice.[56,57] Tumour regression involves activation of innate immunity and this required the 6-day drug break, whereas more frequent CPA delivery reduced antitumour activity. Further studies using two cycles of CPA metronomic treatment documented sustained upregulation of tumour-associated CD8+ CTL cells, natural killer (NK) cells, macrophages and other immune cells. Detection of the CTL and NK effectors peaked on day 6, and then declined by day 9 following the second CPA injection, and there was an inverse correlation with levels of FoxP3-marked Tregs. CPA also mediates cytotoxic tumour damage and this leads to dsRNA formation, including activation of endogenous retrovirus sequences that can elicit IFNαβ production. Within hours, tumour-derived IFN stimulates the production of CXCL11 and other cytokines that by day 3 have impacted the recruitment of CXCR3-expressing immune cells. The six-day cycle balances the continued immune activation/recruitment and tumour damage. Tumour-specific ablation was achieved after several treatment cycles and required CD8+ T cells detectable in the tumour and blood and consistent with the induction of long-term, tumour-specific CD8+ T-cell memory. Codepletion of CD8+ T cells and NK cells did not inhibit tumour regression beyond CD8+ T-cell depletion alone, suggesting that the metronomic CPA activated NK-cell function *via* CD8α T cells. These data support the use of single-agent chemotherapy delivered by a metronomic schedule to treat established tumours and induce long-term immune memory.[56,57] There are immune responsive and unresponsive gliomas to metronomic CPA treatment and gene expression comparisons identified lack of production of IFNαβ or downstream pathways as key factors. The cancer cells are sensitive to the latter, suggesting the possibility of poly(I:C) treatment with such tumours. The challenge now is to translate these findings into the clinic.

Using the preferential targeting of lytic viruses for tumour cells can also have indirect influences on antitumour immunity and be further enhanced by combinatorial approaches. *Alan Melcher (The Institute of Cancer Research, London, UK)* described the development of immunovirotherapy. Reovirus, an oncolytic herpes simplex virus type, is a genetically unmodified, nonpathogenic double-stranded RNA virus with anticancer activity mediated by both direct targeting of malignant cells with activation of the ras pathway and stimulation of antitumour immunity. The reovirus is protected from neutralizing antibodies after systemic administration by immune cell carriage (probably mostly by the monocytes), which delivers reovirus to tumour, where there is preferential expression in malignant cells compared with the surrounding normal tissue.[58] These findings suggest new preclinical and clinical scheduling and treatment combination strategies to enhance *in vivo* immune evasion and effective intravenous delivery of oncolytic viruses to patients *in vivo*. T-VEC (talimogene laherparepvec), a second-generation oncolytic herpes simplex virus type 1 armed with GM-CSF, is now approved as the first oncolytic virus drug in the USA and Europe.[59] The phase III trial showed that local intralesional injections with T-VEC in patients with advanced malignant melanoma suppresses both the growth of the injected lesions but also impacts on systemic disease, prolonging overall survival.[60] T-VEC with ipilimumab showed a tolerable safety profile, and the combination appeared to have greater efficacy than either T-VEC or ipilimumab monotherapy in the treatment of patients with advanced melanoma.[61] In ongoing work, patients with glioma or other brain metastasis were injected with 1×10^{10} virions 3–17 days before surgery and virus was detectable in the tumour with levels appearing to correlate with Ki67 expression. A comparison of the gene expression patterns in the virus-treated patient biopsies and those from untreated tumour biopsies is being performed. Within the approximately 100 gene changes, altered activities related to apoptosis, viral transcription, cytokines and more than 30 IFN-responsive entities were seen. Further, immunohistochemistry of tumour specimens and *in vitro* analysis of tumour and TILs is investigating any correlation of the upregulation of programmed death ligand 1 (PD-L1) and the level of type 1 and IFNγ. Reovirus treatment with PD1 blockade in a mouse glioma therapy model suggested that the combined treatment can provide improvement in survival. If the patient

tumour is 'cold' (low $CD8^+$ T-cell infiltration and high PD-L1 expression), it may not respond to checkpoint blockade: the virotherapy can act to convert the tumour to a 'hot' state, when the inflamed T-cell phenotype provides for improved tumour regression in more patients. Given that many tumour patients receive concomitant steroid treatment that nominally blocks aspects of cellular immunity, virotherapy may circumvent the latter by focusing the immune activity to the local microenvironment. The progression of these types of protocols will be dependent on the outcome of appropriate clinical trials.

Sandra Tuyaerts (KU, Leuven, Belgium) described the PRIMMO clinical study design, which aims to combine PD1 blockade, radiation and several different agents with known immunomodulatory properties to tackle cervical and endometrial carcinoma or uterine sarcoma. The immunomodulators include vitamin D3, aspirin, lansoprazole and low-dose CPA, all with potential influences on tumour-induced immunosuppression. The rationale is to reduce immunosuppression, induce immunogenic death, facilitate T-cell priming and block immune-controlling checkpoint limiters. Treatment will start with the cocktail of immunomodulatory drugs given orally every 24 h for 2 weeks, followed by intravenous anti-PD1 (200 mg) every 3 weeks for six cycles, with three fractionated doses of radiation to a primary lesion during the first course. Monitoring will include tumour and blood sampling through the protocol, with clinical responses assessed by Response Evaluation Criteria in Solid Tumors(RECIST) criteria at a primary endpoint of 26 weeks. A major issue will be how to correlate any clinical responses with the different elements of the complex treatment.

Rolf Kiessling (Karolinska Institute, Stockholm, Sweden). Adoptive transfer of *in vitro* expanded TILs has shown clear clinical benefit in some patients with malignant melanoma. However, the conditioning chemo- or radiotherapy and postinfusion IL-2 injections can cause toxicities. Using a combination of TIL infusion with DC vaccination could obviate the requirement for conditioning and IL-2, and reduce side effects. In a small study of eight patients (MAT01), vaccination with autologous tumour-lysate-loaded DCs was followed by TIL infusion, which yielded a single grade 3 adverse event.[62] Mature DCs were effectively generated from monocytes and the TILs infused were predominantly effector memory $CD8^+$ CTLs, with particular clones still detectable in the blood for weeks after injection. However, after evaluating both clinical and immunological parameters, it seems unlikely that this approach will be a substitute for conditioning chemotherapy and IL-2 in adoptive transfer of TILs.[62] In addition, the transferred T cells will need to overcome the influences of the tumour milieu with its abundant reactive oxygen species that substantially impair antitumour activity. A means to making antitumour T cells more resilient towards reactive oxygen species by coexpressing catalase along with a tumour-specific chimeric antigen receptor (CAR) to increase their antioxidative capacity by metabolizing H_2O_2 was described. Such CAR-CAT T cells also supported protection of nontransfected immune effector cells as measured by CD3ζ chain expression in bystander T cells or NK cytotoxicity even in the presence of high H_2O_2 concentrations. This approach represents a novel means for protecting TILs from tumour-associated oxidative stress-mediated repression.[63] Other work has shown a central role of tumour-derived prostaglandin E2(PGE2) in inducing MDSCs and suggested improved efficacy for combining cyclooxygenase 2 targeted therapy with adoptive NK cell transfer in patients with cancer.[64] Anti-CTLA4 treatment has been shown to decrease levels of MDSCs, Tregs, ARG-1 and iNos but with measurably different kinetics. It would be of great interest to analyse the influence of checkpoint inhibition on T-cell responses to neoantigens. In principle, the methodology to analyse this is available. For example, exome sequencing of two tumours identified approximately 2500 mutations and 300 insertions that with neoepitope and MHC binding predictions yielded up to 4000 HLA-A2 putative targets was reported. Successive analyses of pools of peptides reduced this to two mutation-related epitopes of ETV6 and NUP210 recognized by 4% and 10% of TILs, respectively. However, these specific T cells could not recognize the naturally processed target on autologous tumour cells, although in one case (EVT6) this was rescued by IFNγ treatment of the target cells. This is an immense amount of work to identify putative targets of TIL and illustrates that some candidate antigens may not be able to deliver a useful antitumour activity through additional escape strategies acquired by the tumour.

Paul M. Sondel (University of Wisconsin, USA) focused on the concept of an *in situ* vaccine approach in addressing the challenge of IM for 'cold' tumours. In this scenario, in spite of adequate tumour immunogenicity, the absence of T cells *in situ* is not necessarily reversible by many of

the current immunotherapeutic approaches. The action of agents that utilize antibody-dependent cell-mediated cytotoxicity by innate immune cells, including NK cells, might not be so restricted. NK cells express killer immunoglobulin-like receptors (KIRs) that bind KIR ligands (KIR-Ls), MHC class I molecules and the combinations influence the activation or inhibition of NK activity. An analysis of 174 patients with high-risk neuroblastoma who received either isotretinoin (RA) alone or RA + IM: anti-GD2 mAb, IL-2 and GM-CSF investigated whether KIR/KIR-L genotype was associated with outcome. For the inhibitory KIRs, the impact of all KIR-Ls present *versus* missing at least one KIR-L on outcome was analysed. The results showed that IM therapy benefited those patients with KIR-L present as opposed to those with KIR-L missing, suggesting that KIR/KIR-L genotyping might be used prospectively to identify patients most likely to benefit from this therapy. The best setting for use of this IM is minimal residual disease, but that can take 5 months to achieve and only 70% of patients make it, and then about 35% of patients show any benefit of this IM and most will still relapse. Improvements in the IM may result from the use of an anti-GD2-IL-2 fusion (immunocytokine) where the IL-2 component allows IL2Rs to function not only as receptors but also as facilitators of NK cell binding to immunocytokine-coated tumour cells.[65] Animal model studies support the use of combining anti-CD40/CpG and immunocyokine/anti-CTLA-4.[66] In addition, radiation and intratumoural immunocytokine potentiated further by T-cell checkpoint blockade is a very effective novel IM strategy.[67] Interestingly the cured animals do have antitumour T-cell memory and the immunocytokine, Fc receptor expression, GD2 tumour expression and upregulation of FasL by the radiation all contribute while NK cells are not required. The working hypothesis is that the mAb/FcR interaction provides for tumour antigen uptake and presentation rather than mediating antibody-dependent cell-mediated cytotoxicity. Another complication is that in some tumour types a small unirradiated tumour mass can prevent useful therapy of a larger treated and irradiated tumour. This reciprocal tolerance is mediated by Tregs that can be depleted by using diphtheria toxin-IL-2 or anti-CTLA4 treatment. Clinical trials designed to test these observations in the clinic are in development.

Thomas Neβelhut (Praxisgemeinschaft fuer Zelltherapie, Duderstadt, Germany) discussed combining DC and checkpoint blockade in solid tumour therapy. There are many anecdotal examples of DC vaccine-associated clinical responses but overall response rates are low. Immunosuppressive pathways, including Tregs and CTLA-4 expression on T cells, can be overcome with combinations of vaccine, low-dose CPA and PD1 blockade.[68] Similar treatment regimes are showing a 13% response rate in patients with a variety of different tumours.

Concluding remarks

The consensus of the meeting was that considerable progress in the successful application of IM to cancer is being achieved. A key challenge ahead is how to rationally prioritize and then adequately investigate the large number of potential combinatorial therapeutic regimes. Some clear principles can be achieved in preclinical models but this needs to translate into focused clinical evaluation that can be speedily delivered. Greater coordination of clinical trial design based on all the data, both negative and positive, would aid this process. The current protocols using different immune checkpoint blockers are not necessarily optimal and a more rational approach to their use is required, not the least because with current protocols demanding sustained use, all healthcare systems will be bankrupt. It is apparent that many standard-of-care cancer radio- or chemotherapy components can have important influences on promoting more effective and perhaps pivotal antitumour immunity. This is clearly an area for wider investigation as, for example, an optimization of CPA treatment in cancer (dose, timing, combination scheduling) would be a very cost-effective addition to the treatment regimens. Most importantly it will be necessary to address the future evaluation of treatment regimes in cancer with an open mind. This could allow the repositioning and dosing of old drugs with immune potential in treatment sequencing regimes aimed at optimizing the immune response as a major factor in all cancer outcomes. Preaching the importance of the immune response to clinical outcome in cancer to the wider oncological community should be the continuing mission, as there is still much oncology practice from pathology to treatment that does not understand the importance and implications of this paradigm shift.

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