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CIMT 2014: Next waves in cancer immunotherapy - Report on the 12th annual meeting of the Association for Cancer Immunotherapy

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Introduction

More than 900 scientists around the world visited the 12th Annual Meeting of the Association for Cancer Immunotherapy (CIMT) in Mainz, Germany from 6–8 May, 2014. Recent advancements in various specific fields of cancer immunotherapy were discussed in Europe`s largest meeting of this kind under the motto "Next Waves in Cancer Immunotherapy," the highlights of which are summarized in this meeting report.

Therapeutic Vaccination

Willem W. Overwijk (MD Anderson Cancer Center, Houston, U.S.A.) started the session by showing the pros (cheap, specific, safe and long-term protection) and cons (poor efficacy, requirement of functional immune system) of therapeutic cancer vaccines. Overwijk and colleagues focus on peptide vaccination against melanoma using gp100 peptide in Incomplete Freund's Adjuvant (IFA). He tried to answer the question why many vaccinated cancer patients do not experience tumor regression despite increased levels of cancer antigen-specific T cells. Overwijk stressed the point that one reason for the failure might be the use of IFA-formulated vaccines. His group showed that vaccination with peptides formulated in IFA led to accumulation of CD8 T cells at the injection site, resulting in dysfunction and apoptosis of these T cells.[1] In contrast, water-based vaccines permitted T cell accumulation at the tumor site and exhibited therapeutic anti-tumor effects while T cells induced upon IFA-formulated vaccination became exhausted at the injection site. He also shed light on why the IFA-formulated vaccine against gp100 did not synergize with anti-CTLA-4 therapy in the clinical setting, showing that the vaccination site also traps other tumor antigen-specific T cells which were activated by anti-CTLA-4 therapy. However, he highlighted that this is not always the case since virus-based vaccination synergizes with anti-CTLA-4 therapy. He concluded that not all cancer vaccines are created equal; persistent vaccine formulations can entrap tumor-specific T cells while short-lived formulations release the T cells to traffic to tumor sites.

Long-peptide (LP) vaccines (~20 amino acids) are a promising strategy as they require processing prior to MHC class-I presentation by dendritic cells (DCs), which may mimic tumor antigen presentation more accurately than short peptides that load directly on not only APCs but also all nucleated cells without prior processing.[2] **Yasuharu Nishimura** (Kumamoto University, Kumamoto, Japan) presented the development of LP-based cancer immunotherapy targeting both tumor antigen-specific CD4 and CD8 T cells. Using genome-wide cDNA microarray analyses, his group identified several new genes encoding for cancer testis antigens strongly expressed in oral and esophageal squamous cell carcinomas but not in many normal adult tissues. To select a candidate LP encompassing both Th cell and CTL epitopes, they combined prediction of tumor-associated antigen-derived HLA class II binding LPs with sequence data of known HLA-A2 or HLA-A24-restricted CTL epitopes, stimulated patient PBMCs to assess specific Th1 responses and confirmed cross-presentation of LPs in vitro and with HLA-I transgenic mice in vivo. They identified an LP from LY6K, HLA-A24-restricted $LY6K_{172–191}$, which naturally encodes the CTL epitope $LY6K_{177–186}$. $LY6K_{172–191}$ LP elicited Th1 responses in human PBMCs and cross-primed CTLs in HLA-A24 transgenic mice. In addition, the presence of $LY6K_{172–191}$ LP-specific Th cells in head and neck cancer patients vaccinated with the $LY6K_{177–186}$ short peptide (SP) suggests that tumor lysis induced by SP vaccination-activated CTLs accelerates uptake and processing of LY6K protein by APCs which in turn present

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LY6K-derived LPs to CD4 T cells. Nishimura observed a synergy between LY6K-specific Th and CTL responses in vitro and suggested that the same phenomenon may occur in vivo. Similar LPs were also identified in two other cancer testis antigens, CDCA1[3] and KIF-20A.[4] Taken together, TAA-derived LPs encoding both Th and CTL epitopes may be useful for immunotherapy of various types of cancer.[5]

Successful induction of T cell immunity by direct mRNA vaccination is associated with several challenges. **Ugur Sahin** (TRON, Translational Oncology at the University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany) outlined his approach of tackling these challenges and the clinical translation of personalized, mRNA-based cancer immunotherapy as performed in his institute. Pharmacological optimization of mRNA in-house[6-8] enabled intranodal delivery of naked mRNA, stimulating robust expansion of immunodominant CTLs, indicated by preclinical studies[9] as well as by preliminary immune evaluation of a phase I/II clinical trial against melanoma. Recently, Sahin and coworkers succeeded in translating this approach from local to systemic DC targeting using liposomal formulations which resulted in superior immunostimulation and rejection of established tumors in preclinical studies, providing the basis for several planned phase I/II clinical trials (melanoma, breast cancer, head and neck cancer) in the context of an RNA warehouse approach with pre-furnished RNA portfolios to be individually composed for each patient. According to Sahin, targeting tumor mutations is the key to successful personalized anti-cancer therapy. Using a complex mutation identification process[10] including NGS-sequencing of expressed mutations in tumor biopsies, prioritization and mutation immunogenicity testing with patient PBMCs and subsequent in-house product manufacture, immunization with an oligo-epitopic RNA vaccine is feasible within three months and is currently employed in a phase I/II clinical trial against melanoma (NCT01684241). Sahin concluded with stressing the importance of the regulatory development for actively personalized cancer immunotherapeutics and the active involvement of the CIMT regulatory research group, to facilitate clinical translation of individualized anti-tumor immunotherapies.

Combination Therapy

For decades combination therapy has been an important treatment modality in various diseases including infectious diseases, cardiovascular diseases and cancer to maximize therapy responses.[11] In cancer immunotherapy, combinatorial therapy approaches are still at the early stage of development, nevertheless already displaying great potential for future application as demonstrated during the "Combination Therapy" session.

The introduction of the checkpoint modulators Ipilimumab (anti-CTLA-4 antibody) and Nivolumab (anti-PD-1 antibody) revolutionized the field and had a tremendous impact for cancer immunotherapy,[12-14] but as pointed out by the first speaker of the session, **Michael Curran** (MD Anderson Cancer Center, Houston, U.S.A.), there are still opportunities to augment their therapeutic potential by joint action. The observation that blocking CTLA-4 leads to reciprocal upregulation of PD-1 on activated T cells suggested that a dual blockade of these non-redundant pathways might produce greater anti-tumoral activity than that observed for blockade of CTLA-4 alone. Curran was able to confirm that the joint action of CTLA-4 and PD-1 blockade in conjunction with a B16-Flt3-ligand vaccination in a preclinical B16-BL6 melanoma model augmented tumor infiltration of effector T cells, led to a favorable T effector to T regulatory (Treg) cell ratio and increased IFN-γ production in the tumor, resulting in rejection of 50% of tumors, compared with 10% achieved with CTLA-4 blockade alone.[15] Motivated by these promising preclinical results, a clinical phase I study was initiated combining Ipilimumab and Nivolumab in patients with advanced melanoma. In accordance with preclinical data, the trial revealed a superior outcome of the concurrent treatment with Ipilimumab and Nivolumab in comparison to Ipilimumab alone, but at an expense of a higher incidence of grade 3 and 4 adverse events.[16]

However, combining two checkpoint inhibitors as demonstrated above, is only one out of a plethora of possibilities. Whereas CTLA-4/PD-1 blockade focused on *"releasing the brakes"*, direct T cell stimulation is another interesting therapeutic approach. Targeting co-stimulatory receptors might additionally *"press the gas pedal"* for optimal T cell effector function and is currently under investigation at different stages.[17,18] In a B16-BL6 mouse model, concurrent administration of an agonistic 4–1BB antibody and CTLA-4 blockade, combined with a B16-Flt3-ligand vaccination, resulted in the rejection of 57% of the implanted tumors in comparison to 13% for 4–1BB activation and 20% for CTLA-4 blockade alone.[19] Despite the encouraging preclinical data for such a regimen, a planned phase I study combining Urelumab (anti-4–1BB antibody) and Ipilimumab has been withdrawn prior to enrollment due to multiple incidences of high grade hepatitis induced by Urelumab in a prior phase I study (NCT00803374), followed by the termination of another phase I/II trial (NCT00309023). In this regard, Curran stated that the metaphor "Effective tumor immunotherapy: start the engine, release the brakes, step on the gas pedal and get ready to face autoimmunity" nowadays still seems to hold true.[17] Nevertheless, it is worth mentioning that Kocak and colleagues provided first evidence that the combination of anti-4–1BB and anti-CTLA-4 therapy ameliorates their immune related side effects.[20] Curran demonstrated that the ability of 4–1BB activation to potently suppress Th17 responses explains its ability to attenuate anti-CTLA-4 induced autoimmunity.[21] Curran concluded that the clinical investigation should be initiated as therapeutic synergy was demonstrated in multiple preclinical tumor models. Given powerful combinations of immunotherapeutic antibodies like CTLA-4/PD-1 blockade or CTLA-4 blockade and 4–1BB agonist, Curran postulated that future combinations should involve vaccines to generate more tumor-specific T cells, and/or should utilize drugs which help break down the physical and suppressive barriers to T cell infiltration of tumors.

In addition to aforementioned targets which are partially approved or in late clinical trials, there are also potential new therapeutic targets on the horizon. **Mark Smyth** (QIMR Berghofer Medical Research Institute, Brisbane, Australia) and his colleagues identified a new checkpoint molecule to augment anti-tumoral natural killer (NK) cell function. CD96 is a member of the immunoglobulin-superfamily interacting with ligands of the nectin and nectin-like family and is expressed constitutively on resting NK cells.[22] So far little was known about its function, except that it shares the ligand CD155 with CD226 and TIGIT.[23] Using CD96-deficient mice, the group was able to demonstrate that CD96 competes with CD226 for CD155 binding and therefore limits CD226-driven secretion of pro-inflammatory mediators like IFN-γ by NK cells. Consequently, blocking of CD96 via a monoclonal antibody resulted in higher resistance against MCA-induced fibrosarcomas and fewer lung metastases after intravenous challenge with B16F10.[22]

The last speaker of this session, **Yutaka Kawakami** (Keio University, Tokyo, Japan) emphasized the importance of seeking for combinations outside the field of immunotherapy to intensify tumor immunity. Combining targeted therapies with immunotherapy can ideally lead to complementary response kinetics by rapidly inducing decrease of tumor burden and release of multiple endogenous tumor antigens. Moreover, he mentioned that the inhibition of the MAPK pathway in human melanoma cell lines by mutant BRAF inhibitors reduced the production of immunosuppressive cytokines (e.g., IL-6, IL-10 and VEGF), restoring the ability of DCs to produce high levels of IL-12 and TNF-α and subsequently to stimulate T cells.[24]

Tumor Microenvironment

The session was opened by **George Coukos** (Ludwig Center for Cancer Research, Lausanne, Switzerland) who presented an overview of his major discoveries implicated in novel anti-tumor therapies in cancer patients. He showed his important contributions in the characterization of tumor vasculature endothelial cells in cancer patients. His first study described the mechanism of paracrine dialog among tumor-located endothelial cells and T cells in ovarian cancer. Interestingly, he linked the short ovarian cancer patient survival time and absence of TIL to high expression of endothelin B receptor (ET_BR) on tumor endothelial cells. This discovery enabled the usage of new pharmacological compounds to enhance the efficacy of cell immunotherapies in cancer patients.[25] Moreover, Coukos showed a new molecular mechanism to disable tumor T cell infiltration due to a specific phenotype of tumor vasculatures.[26] In this recent discovery, the death mediator, Fas ligand (FasL) is described as a new factor on tumor endothelial cells involved in hijacking T cell effector functions. Selective expression of FasL on the vasculature of human and mouse solid tumors but not in normal vasculature might allow endothelial cells to kill tumor-infiltrating effector CD8 T cells but not Tregs. Interestingly, Tregs can survive despite this death mechanism thanks to constitutive expression of c-FLIP at high levels. He elegantly demonstrated that the overexpression of this transcription factor in CD8 T cells renders CD8 T cells resistant to FasL-mediated cell death. In addition, pharmacologic inhibition of VEGF (anti-VEGF antibody) and PGE2 (Aspirin), factors responsible for the induction of FasL expression on endothelial cells, produced a marked infiltration of CD8 T cells at the tumor site over Tregs. Combinations of pharmacological attenuation of FasL expression on tumor endothelial cells led to potent CD8-dependent tumor rejection.

Sergio Quezada (UCL Cancer Institute, London, U.K.) summarized his important findings linked to the success of Ipilimumab in melanoma patients. His preclinical work deeply dissected the mechanism of the mode of action and the potency of anti-CTLA-4 antibody therapy in the context of GVAX vaccination (irradiated B16F10 tumor cell–based vaccine that secretes GM-CSF) in melanoma mouse tumor models. His results clearly showed that the effectiveness of this therapy is linked to the antibody isotype, which drove intratumoral Treg cell depletion[27] by antibody-dependent cell-mediated cytotoxicity (ADCC). Quezada's team also demonstrated that intratumoral macrophages expressing Fcγ receptor IV are essential for the elimination of Treg cells targeted by anti-CTLA-4 antibody.[27] In addition, Quezada showed preliminary results from a study using patient samples to validate the potential relevance of his findings for the in vivo mode of action of Ipilimumab in melanoma patients and to characterize the expression pattern of Fc receptors in the tumor environment. In this regard, several immunomodulatory surface markers (such as PD-1, OX40, 4-1BB, ICOS and GITR) on human CD4 and CD8 T as well as Treg cells were characterized in TILs from melanoma patients. This study might be beneficial for screening potential responder patients for this type of immunotherapy[28] and allow intelligent combination with other immune modulatory antibodies.

Priti Hegde (Genentech, San Francisco, U.S.A.) showed a very interesting ongoing study to characterize the immune-cancer signature of the tumor environment across six different human tumors: colorectal cancer, melanoma, bladder cancer, non-small cell lung cancer (NSCLC), renal cell cancer and triple-negative breast cancer. The major aim is to clearly dissect the complexity of the tumor environment and to find key factors which can be targeted specifically for successful anti-tumor therapy. The work is based on immunohistochemical analysis run in parallel with highly sensitive immune gene expression assays (iCHIP) using the Fluidigm Biomark platform to interrogate the quality of the immune response across these six cancer types. The large amount of processed data showed how the complexity of the immune signature (based on factors such as effector, suppressor, Th1, Th2, Th17, immune check point markers) in relation to the vasculature and milieu (chemokines and cytokines components) is integrated specifically for each type and stage of tumor. Indeed, the analyzed tumors showed distinct immunoscores with respect to suppressor players, T effector/Treg ratio as well as chemoattractants. This large scale analysis will hopefully allow the planning of intelligent combination therapies in order to target specific components of the immune system dependent on the precise type and stage of human tumors.

Immunoguiding

The history of cancer immunotherapy is still short and so is the history of monitoring antigen-specific T cell responses. **Pedro Romero** (Ludwig Center for Cancer Research, Lausanne, Switzerland) reminded the audience of this fact by recalling that the IFN-γ ELISpot was invented in the late 80s and intracellular cytokine staining as well as MHC-tetramers for flow cytometry-based immune monitoring not until the 90s of the 20th century. He therefore stressed the need for further efforts in the direction of standardization and harmonization as it is, among others, also pursued by the CIMT Immunoguiding Program (CIP).[29-31] One important determinant of T cell function which is not yet regularly included in T cell immune monitoring is the micro RNA (miRNA) profiling of T cells. miRNAs are ~22 nucleotide, non-coding RNAs involved in the post-transcriptional regulation of gene expression. They can exert their effects through multiple mechanisms, including translational repression and mRNA degradation. Romero and coworkers already analyzed the miRNA profile of human CD8 T cells and found differentiation-associated patterns of miRNA expression.[32] Continuing this line of investigation, they set out to investigate the role of miRNA-155 for CD8 T cell differentiation and effector function utilizing the LCMV model system.[33] Having revealed that miRNA-155 is upregulated in effector CD8 T cells, they observed a massive reduction of T cell expansion in miRNA-155-negative animals while T cell differentiation was unaltered. In accordance with this finding, miRNA-155-negative T cells exhibited decreased proliferation accompanied by increased apoptosis. Importantly, Romero demonstrated the relevance of this regulator for efficient anti-tumoral T cell immunity in miRNA-155 knockout as well as overexpression studies. As target mRNA, the group was able to identify SOCS-1 that diminishes γ-chain cytokine signaling. The data warrant further studies to investigate the relevance of miRNA-155 in human cancer-reactive T cells as well as possibilities of active therapeutic intervention based on the presented findings.

Complementing the strong focus on adaptive immunity, **Eric Vivier** (Centre d' Immunologie de Marseille-Luminy, Marseille, France) introduced NK cells as a subset of cytotoxic innate lymphoid cells (ILC) that exhibits promising features for cancer immunotherapy.[34] He gave a comprehensive overview about the fundamentals of NK cell immunology and the current immunotherapeutic concepts linked to them. The important role of NK cells in defense against infections was clarified by a study showing that NK lymphopenia caused by MCM4 deficiency is connected to recurrent childhood viral infection as well as respiratory tract diseases.[35] But what are the fundamental mechanisms by which NK cell activity and target recognition is governed? The speaker rolled out the scenario of the "missing self" and the "stress-induced self" leading to NK cell activation. In the former case, the loss of MHC molecules on tumor cells resulting in a lack of inhibitory signaling causes NK cell activation. In the latter case, the upregulation of stress-induced ligands (e.g., by malignant transformation, infection, physical or chemical injury) is the functional trigger.[36] Based on the understanding of NK cell biology and interactions in the living organism, Vivier proposed different approaches allowing the harvest of the anti-tumoral potency of NK cells in immunotherapy. Currently being tested is the approach of allogeneic donor lymphocyte infusion (DLI). Preclinical data from the Vivier laboratory proved the feasibility of effective tumor therapy utilizing NK cells lacking inhibitory MHC class-I receptors. The concept awaits further development into the direction of clinical testing. Lirilumab, an antibody interfering at the same functional axis by blocking the binding of HLA molecules to killer inhibitory receptor KIR2DL3 is already in clinical testing.[37,38] Early clinical data have been promising and justify further clinical development.Vivier also pointed out that albeit in wild-type mice, NK cells reject an MHC-deficient bone marrow graft, there is no autoimmunity in MHC class-$I^{-/-}$ mice. Starting from the question how this observation could be explained, he diligently dissected the underlying mechanisms. It is known that in MHC class-I-deficient hosts, NK cells are hyporesponsive, which demonstrates their adaptive nature and highlights the relevance of inhibitory MHC class-I receptors for the tuning of NK cell reactivity.[39] As the phosphatase SHP-1 is responsible for downstream signaling of MHC class-I inhibitory signaling, Vivier and his colleagues created a Cre/lox-regulated transgenic mouse model allowing the deletion of the PTPN6 gene encoding SHP-1 in NK cells. In broad functional studies, the group proved that lack of functional SHP-1 induces a state of hyporesponsiveness in NK cells. SHP-1 is the decisive signal transduction modifier mediating the functional tuning of NK cells via MHC class-I receptors. Vivier resolved the apparently conflicting initial observation by the phrase "time matters, also for NK cells". He further proposed that NK cells detect sudden alterations of their environment, but if these alterations persist, they adapt by ceasing to react. The speaker put this notion into a broader context by stating that the concept of discontinuity can be applied to the immune system in general.[40]

Immunoinformatics and Genomics

Tumors exhibit reoccurring drivers and antigens as well as patient-specific mutations. Next Generation Sequencing (NGS) is able to identify both and thus represents a "game-changer" in cancer immunotherapy. **John Castle** (TRON - Translational Oncology at the University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany) presented the establishment of a genomics and bioinformatics platform for identifying and prioritizing somatic mutations for individualized cancer vaccines. An essential part was setting up a complete infrastructure including the establishment of a secure and fast way to transfer and process sequencing data, high performance computing, a labor information management system[41] and a clinical specimen biobank. The core platform developments comprise methods for RNA-Seq profiling of formalin-fixed, paraffin-embedded samples, statistical algorithms for NGS mutation detection, virus detection and HLA typing using standard RNA-Seq reads,[42] single cell genomics, T cell receptor profiling and the TRON Expression Atlas. With the genomics and

bioinformatics platform established, Castle and coworkers were able to identify the mutanome of B16F10 murine melanoma cells and prioritize mutations producing neo-epitopes for the elicitation of immune responses.[10] Furthermore, immunomic, genomic and transcriptomic characterization of CT26 colorectal carcinoma cells revealed that these tumor cells are primarily triploidy and tetraploidy.[43] In addition, they found that mutated tumor alleles are expressed according to their DNA frequency,[44] which has important implications in the design of individualized tumor vaccines. Clinical processes have been audited and defined in standard operating procedures. The established genomics and bioinformatics platform for individualized mutation-targeting cancer vaccines is being used in a first-in-human clinical trial for melanoma (NCT01684241).

Once somatic mutations are found and validated, which ones produce a neo-antigen which is (a) presented on a patient's HLA molecules and (b) likely to elicit an immune response? **Morten Nielsen** (Technical University of Denmark, Lyngby, Denmark) addressed this question by presenting his work on immunoinformatic methods for the prediction of peptide-MHC interactions and T cell epitopes. The primary principle of his algorithms is to learn underlying rules and patterns of experimentally validated MHC-binding peptide sequences by fitting mathematic models. In case of MHC class-I binding prediction, Nielsen showed that the field has reached a plateau where the accuracy does not improve with more data. The NetMHC algorithm covers more than 100 human and animal MHC class-I molecules.[45] Building models for MHC class-II predictions is more complicated, as the peptide binding groove is open at both ends, making it crucial to identify the core of an MHC class-II binding motif.[46] NetMHCII currently offers MHC class-II binding prediction models for more than 25 human and murine MHC-II alleles.[47] For the majority of MHC alleles, the binding specificity has not been characterized as there exists no or little peptide data for training. Pan-specific MHC prediction methods fill this gap as they are designed to deal with the immense MHC polymorphisms. NetMHCpan is trained on binding data covering more than 150 MHC class-I alleles, and allows prediction of peptide binding to any HLA molecule with known protein sequence.[48] NetMHCIIpan predicts binding for all HLA-DR, HLA-DP and HLA-DQ alleles.[49] To be loaded onto the MHC complex, proteins are first cleaved by the proteasome before entering the endoplasmatic reticulum via TAP molecules. Although both steps are modeled in prediction tools, the data suggest that adding those predictions has only a slight benefit[50] due to MHC class-I pathway coevolution.[51]

To be recognized by T cells, a peptide must be presented on MHC molecules. Thus, binding is a necessary but not sufficient step. What defines a T cell epitope? A predicted MHC binding affinity of smaller than 500 nM is routinely used as a threshold for likely immunogenic T cell epitopes. However, a recent study suggests that different alleles vary in epitope repertoire size and in binding affinity, both thresholds associated with immunogenicity.[52] Nielsen showed that peptide-MHC class-I stability is a better predictor of immunogenicity than binding affinity alone.[53,54] Integrating both predictors, combined with a recently described T cell propensity model[55], leads to a significant increase in T cell epitope prediction accuracy.[56] As more experimental data are generated, an improvement in prediction performance is anticipated. In summary, rational T cell epitope discovery, especially beyond MHC binding, is indeed feasible.

With a continuous decrease in costs of high-throughput techniques, a plethora of data, such as genomic and transcriptomic profiles, is emerging, often referred to as "big data". **Joel Dudley** (Icahn School of Medicine at Mount Sinai, New York, U.S.A.) started with the phrase "Let the data tell you about the biology" and showed examples of the chances and challenges of integrating and interpreting the digital universe of information for better models of disease. Given the rapid technological progress, it is possible to measure more than is known, which forms the basis of data-driven science. Taking advantage of existing data, combining bioinformatic tools and embracing complexity enables the full understanding of patient disease physiology and is leading to new paradigms redefining disease. Using existing disease-related clinical literature and disease-specific gene expression profiles, Dudley and coworkers recently integrated large public data sets to understand systematic patterns connecting regulatory variation with disease functional genomics.[57] A data-driven approach to connect drugs and disease using gene expression data profiles predicted new indications for established drugs (compound repositioning).[58] This approach identified tricyclic antidepressants as potential inhibitors of small-cell lung cancer and other neuroendocrine tumors.[59] One of the next steps will be to explore how approved drugs modulate networks within the complex immune system. Dudley concluded with emphasizing the large opportunity for systems biology, bioinformatics and systems medicine approaches[60] for the vast amount of big data.

Cellular Therapy

Adoptive Cell Transfer (ACT) therapies are among the most promising treatment strategies in the field of cancer immunotherapy. In general one can differentiate between adoptive transfer of autologous ex vivo expanded TILs and the reinfusion of genetically engineered leukocytes. In the latter case, T cells are either redirected by transfer of antigen-specific T cell receptors (TCRs) or by chimeric antigen receptors (CARs).[61] Most commonly, αβTCRs are used for this approach but there are also attempts to employ γδTCR-redirected T cells for ACT.[62] CARs, which recently came under the spotlight as a novel technology, recognize antigens via the antigen-binding site of a monoclonal antibody fused to intracellular signaling domains like the CD3-ζ chain.[63] CAR-expressing T cells are thereby able to recognize and kill tumor cells that express the cognate surface antigen without the need for its presentation on MHC molecules.

Carl June (Perelman School of Medicine at the University of Pennsylvania, Philadelphia, U.S.A.) presented his work on "CARs in the clinic for leukemia and beyond." He reported on different generations of CARs from CD4/8 CARs[64] and single-chain (sc) Fv CARs against CD19 depending on the signaling of

the CD3-ζ chain, to second and third generation CARs typically including signaling domains derived from costimulatory molecules like CD28, CD137 (4–1BB), CD134 (OX40) or ICOS. June pointed out that CAR-expressing T cells are long-lived and can be found after more than 11 years in treated patients.[65] The inclusion of costimulatory signaling domains permits the survival and proliferation in response to antigen in the absence of exogenous factors.[66] June mentioned that human T cells expressing CARs including 4–1BB costimulatory domains show a central memory phenotype, superior anti-tumoral efficacy and prolonged survival compared with CD28 CAR-expressing T cells.[67] CD19-targeting CARs that contain 4–1BB costimulatory domains also showed powerful effects in patients with CLL[68,69] and ALL.[70] Engineered T cells expanded more than 1000-fold and one CAR-expressing T cell killed ~1000 tumor cells. Treatment induced a complete remission in 2/3 patients with CLL and in 27/30 patients with ALL. Responders showed a strong proliferation of CAR-expressing T cells compared with non-responders. June also pointed out that engineered T cells were found in the central nervous system. This opens the opportunity to use CAR-transgenic T cells for the treatment of neuro-oncological diseases. CAR-engineered T cells recognizing targets like overexpressed mesothelin might also be used for the treatment of solid tumors[71] but this might raise potential on-target off-tumor immunotoxicity due to the expression of this antigen on healthy tissues.

Hiroshi Shiku (Mie University Graduate School of Medicine, Tsu Mie, Japan) reported on the ACT of TCR gene-transduced lymphocytes. He pointed out that the quality, specificity and quantity of transferred TCR-transgenic (TCRtg) T cells determines the efficacy of the treatment. In a TCRtg murine study, he and colleagues showed that ACT is more efficient in combination with vaccination or anti-GITR monoclonal antibodies. In a phase I clinical trial of TCR-engineered T cells that recognize MAGE-$A4_{143–151}$ on HLA-A24:02, the most common HLA haplotype in Japan, ten refractory esophageal cancer patients without previous lymphodepletion were treated with 2×10^8, 2×10^9 or 5×10^9 engineered T cells in combination with vaccination of the cognate peptides on day 14 and 28 after ACT. All patients showed transduced T cells in their peripheral blood 14 days after transfer and in four patients for more than 150 days. Three of the monitored patients showed stable disease or extended tumor-free status. Shiku and colleagues observed that the number of engineered T cells in the blood rather declined after administration of the short peptide vaccine. In murine studies they were able to show that this was not the case when a DNA or a long peptide vaccine was administered, and therefore plan to replace the intended peptide vaccine with a long peptide-containing alternative. Finally, Shiku presented a new generation of retroviral vectors for the transfer of TCRs that encode siRNA targeting the constant regions of endogenous TCR-α and TCR-β genes. This approach prevents the misspairing of transferred TCR chains with endogenous ones, increases the number of tumor antigen-specific TCRs and thereby the anti-tumoral effect, and reduces the risk of GvHD.

Jürgen Kuball (University Medical Center Utrecht, Utrecht, The Netherlands) presented his pioneering work on γδT cells. γδT cells only comprise a small subset of peripheral CD3 T cells but are abundant in epithelial tissue of the gastrointestinal and genital tract. Although γδT cells are able to rearrange TCR genes, the recognition largely resembles pattern recognition receptors of the innate immune system. For example, a subpopulation of $V_\gamma2^-$ T cells is able to recognize CMV infected cells but also cancer cells including primary leukemic blasts. This property might substantially contribute to the improved control of leukemia in patients with CMV infections.[72] It was shown that in leukemia patients that received T cell-depleted allogeneic stem cell transplantation, the risk for CMV infection is very high due to the lack of a T cell response needed for the control of CMV-infected cells. The transfer of CMV-reactive T cells on the other hand might lead to severe GvHD. Kuball and colleagues demonstrated that the transfer of αβT cell-depleted cells prevents from CMV reactivation without substantial GvHD due to the transfer of CMV-reactive $V_\gamma2^-$ T cells. Furthermore, he presented data on the transfer of γδTCRs into αβT cells. γ9δ2T cells recognize mevalonate metabolites like isopentenyl pyrophosphate (IPP) which are frequently overproduced in a broad range of tumor cells. This recognition depends on the expression of the surface antigen-presenting molecule BTN3A1 on target cells.[73] Kuball not only showed that it is possible to transfer γ9δ2TCRs into αβT cells but also explained that these T cells are able to specifically kill various leukemia cell lines in vitro and in vivo. He pointed out that there are differences in the anti-tumor reactivity among γ9δ2T cell clones explaining the so far limited tumor control of γ9δ2T cells in clinical studies.[74] Furthermore, he reported on a rho-GTPase that is involved in IPP presentation on BTN3A1 molecules. He and colleagues could show that this small GTPase co-localizes with BTN3A1 and is altered in leukemic but not in healthy stem cells. To evaluate the potency of the γδTCR approach, a clinical gene therapy trial with $\delta2^+$ TCRs is on its way.

Improving Immunity

Gunther Hartmann (University Hospital Bonn, Bonn, Germany) described the versatile use of blunt, short, double-stranded 5`triphosphate RNA (3pRNA), the ligand for retinoic-acid inducible gene 1 (RIG-I),[75] for the induction of immune responses against cancer. His group showed that RIG-I activation triggers cell death in human melanoma cells in vitro while endogenous Bcl-XL rescued non-melanoma cells from apoptosis induction.[76] 3pRNA also can exert its effects on NK cells. In human PBMCs, direct contact of RIG-I activated monocytes with NK cells is necessary for NK cell activation. Repetitive injections of 3pRNA were also effective in suppressing tumor growth in the B16 model of melanoma via secretion of IFN-γ by activated NK cells. Interestingly, RIG-I-activated melanoma cells secrete exosomes, small cell-derived vesicles, which are internalized by monocytes and can act as carriers of immunostimulatory molecules.[77] Hartmann and his group demonstrated that these

exosomes contain 3pRNA and RIG-I as well as costimulatory molecules like CD80 and CD86. Moreover, exosomes can also directly affect melanoma cells via direct apoptosis. In different murine tumor models, a nuclease-resistant 3pRNA which does not have TLR activity was delivered to tumor cells, epithelial cells and tissue resident DCs via formulation with PEI without signs of toxicity. Intratumoral injection of such formulated 3pRNA was effective in controlling melanoma growth and synergized with check-point inhibitors like anti-PD-1 antibody. Mice were also protected against lung metastases when the 3pRNA-PEI complexes were administered systemically. In addition, intraperitoneal injection of this formulation exhibited anti-tumoral activity in an ovarian cancer model where rechallenge of the surviving mice with ovarian cancer cells led again to rejection, indicating the existence of an effective T cell memory response. Such immunostimulatory agents activating RIG-I may be used as novel immunotherapeutic strategies against cancer.[78]

Utilizing another type of RNA, in this case long antigen-encoding mRNA, RNA-based vaccines hold promise as a new class of drug for the immunotherapy of cancer.[79] Several preclinical studies have shown that direct vaccination with naked antigen-encoding RNA can elicit efficient B and T cells responses as well as therapeutic immunity without causing toxicity, enabling clinical translation of this strategy.[9,80,81] **Ulrike Gnad-Vogt** (CureVac, Tuebingen, Germany) presented the clinical development of intradermally administered self-adjuvanted RNActive® vaccines which contain free and protamine-complexed mRNA for antigen expression and adjuvanticity, respectively.[82] In a phase I/IIa clinical study with castration-resistant prostate cancer patients, the CV9103 vaccine comprising PSA, PSMA, PSCA and STEAP1 encoding mRNAs was well-tolerated and induced antigen-specific immune responses in 79% of 33 immunologically evaluable patients (NCT00831467). Importantly, immune responses against multiple antigens were found to be associated with longer survival. Another phase IIb trial with CV9104 which includes PAP and MUC1 antigens in addition to the ones in CV9103 is ongoing to test the clinical efficacy for the treatment of prostate cancer (NCT02140138). After revealing that RNActive® vaccination induces chemokines like CXCL-10, CXCL-9 and CCL5 which play roles in the recruitment of immune cells to the tumor site,[83] analysis of tumor tissue for immune cell infiltration and cytokines/chemokines profiling is also planned within this study. The RNActive® platform was tested alone against NSCLC with a 65% immune response rate in a phase I/IIa CV9201 (NY-ESO1, MAGE C1, MAGE C2, Survivin and 5T4) trial with the addition of MUC1 (NCT00923312), and a phase Ib CV9202 trial is underway to test the combination of the RNActive® platform with irradiation (NCT01915524). Based on preclinical studies, Gnad-Vogt concluded that this platform can also be combined with other treatments including check-point blocking antibodies against CTLA-4 and PD-1 which opens new opportunities for clinical testing.

Looking at immune-mediators for improving immunity, **Qing Yi** (Lerner Research Institute, Cleveland, U.S.A.) investigated the role of IL-9 in the context of tumor immunotherapy. IL-9 is a pleiotropic cytokine that can act as a mediator of inflammation in autoimmune diseases as well as in allergic inflammation while it protomes a tolerant environment via enhancing the immunosuppressive functions of Tregs and mast cells. Yi presented data showing that endogenous IL-9 contributes to reduced tumor growth in a metastatic B16 model of mouse melanoma, as tumor growth was enhanced in the absence of IL-9.[84] Interestingly, adoptive transfer of tumor antigen-specific Th9 cells secreting IL-9 promoted a better tumor clearance than Th1 cells in vivo. Moreover, adoptively transferred Th9 cells persisted longer and differentiated into functional cytotoxic T cell-like effector cells with self-renewal capacity.[85] The transfer of Th9 cells also led to effector cell activation in tumor-draining lymph nodes and to their recruitment to the tumor where they exerted their cytotoxic effects. Yi and his group also explained the mechanism of action for this effect such that IL-9 induces tumor and lung epithelial cells to secrete CCL20 which attracts DCs to the tumor microenvironment through CCR6 in order to internalize tumor antigens.[86] These DCs then migrate to tumor-draining lymph nodes where they prime host effector cells and especially CD8 cytotoxic T cells which migrate to tumor sites via CCL20 chemoattraction to lyse tumor cells.

The microbial metabolome is comprised of the symbiotic microbes co-existing in close interaction with the immune system. **Laurence Zitvogel** (Institute Gustave Roussy, Villejuif, France) showed diligently how the gut microbiota help shape the immune response against cancer. Her group recently demonstrated that a commonly used chemotherapeutic agent, cyclophosphamide (CTX), disrupts mucosal integrity and induces translocation of $Gram^+$ gut-resident bacteria such as *L. johnsonii* and *E. hirae* to secondary lymphoid organs such as the spleen where they induce pathogenic Th17 (pTh17)-type T cell priming in a Myd88-dependent fashion.[87] Treatment of mice with the antibiotic Vancomycin led to reduced efficiency of CTX in the treatment of mastocytomas. In addition, the anti-tumoral effect of CTX in a sarcoma mouse model was found to be reduced in germ-free (GF) mice compared with specific pathogen-free (SPF) mice as a result of reduction in pTh17 responses. Interestingly, adoptive transfer of ex vivo differentiated polyclonal pTh17 cells reversed the Vancomycin-induced resistance against CTX. In line with these results, Zitvogel suggested that *L. johnsonii* and *E. hirae* can be used as probiotics to restore chemosensitivity in case of microbial dysbalance in the body.

Keynote Lecture

Josef Penninger (Institute of Molecular Biotechnology, IMBA, Vienna, Austria) presented two examples of how immune-related pathways can be explored to prevent and treat cancer.

Emphasized by knockout studies in mice, the TNF-family molecule RANKL (Receptor Activator of Nuclear Factor κ B Ligand, also known as osteoprotegrin ligand (OPGL)) and its receptor RANK are well-known as essential regulators for the development and activation of osteoclasts and thus play a crucial role in bone

remodeling.[88] Furthermore, RANK/RANKL signaling has been initially associated, among others, with DC-T cell interaction,[89] lymph node formation,[90] body temperature regulation[91] and formation of lactating mammary glands.[92] Dysfunction of this pathway explained bone-related pathologies like osteoporosis and arthritis[93] but also bone loss in leukemia or chronic obstructive pulmonary disease (COPD).[94] The importance of re-establishment of the physiological balance of the RANK/RANKL pathway is underlined by the fact that Denosumab, an IgG2-anti-RANKL-antibody mimicking the interceptive role of the molecular decoy OPG, has already been approved for osteoporosis, skeletal-related events in cancer and giant cell tumors.[95]

The diagnosis of osteoporosis is most commonly made in postmenopausal women. The observed bone loss in females was attributed to the finding that sex hormones control the RANK/RANKL pathway. While estrogen levels decreasing postmenopausally led to less OPG expression and therefore augmented osteoclastogenesis and osteoporosis,[96] progesterone was found to induce RANKL expression in mammary epithelial cells eliciting their proliferation.[97,98] Hence, the potential involvement of the RANK/RANKL pathway in breast cancer and its associated bone metastasis was investigated. Penninger and his colleagues showed that the synthetic progesterone MPA, by activation of the RANK/ RANKL pathway, has a tumor promoting effect as it leads to increased mammary epithelial cell proliferation and survival, and also affects mammary cell stems. Moreover, his group provided the first genetic evidence that inactivation of RANK on mammary epithelial cells markedly delays the incidence and onset of MPA/DMBA-driven breast cancer.[99] These findings are of high interest as especially women receiving hormone replacement therapy (HRT) or taking hormonal contraceptives manifest an increased risk for the development of breast cancer[100], and anti-RANKL therapy might be used in these patients to prevent or treat disease.[101] Further benefit of this therapeutic approach may be expected for bone metastasis, as data indicate that the inhibition of RANKL results in reduced tumor burden in bone and abolishment of paralysis in a mouse model of melanoma metastasis.[102]

Penninger also talked about the role of RING finger E3 ubiquitin-protein ligase Cbl-b (Casitas B cell lymphoma (CBL)-proto-oncogene-b); in immune system activation and tolerance. Cbl-b is a key regulator of activation thresholds in mature T lymphocytes, underlined by numerous studies in Cbl-b$^{-/-}$ mice, which develop spontaneous, albeit mild, autoimmunity[103] or arthritis even in the absence of microbacterial adjuvant stimulation.[104] Cbl-b is involved in signaling pathways that determine T cell activation vs. T cell tolerance. Since immune suppression and insufficient activation of T cells are central limitations of tumor immunotherapy, the question was raised whether Cbl-b displayed any role in anti-tumor immunity. Indeed, it was found that Cbl-b$^{-/-}$ mice spontaneously reject TC-1 tumors and UVB-induced skin tumors. This tumor rejection is controlled by CD8 T cells which kill tumor cells in a perforin-dependent way. Strikingly, proven by therapeutic transfer, naïve Cbl-b$^{-/-}$ CD8 T cells are sufficient to mediate rejection of established TC-1 tumors. Furthermore, these CD8 T cells display impaired proliferative suppression by Tregs, and long-term anti-tumoral immunity is established in mice lacking functional Cbl-b.[105] However, Cbl-b is not exclusively controlling T cell anti-cancer immune responses but also possesses functions in innate immune cells, as in a control experiment, TC-1 tumor growth was significantly delayed compared with Cbl-b$^{-/-}$ Rag$^{-/-}$ control mice. It appears that in these mice, the absence of Cbl-b in NK cells licenses these cells to spontaneously reject tumor mass.

In the B16F10 melanoma model, Cbl-b$^{-/-}$ mice showed significantly reduced lung metastases, while depletion of NK cells led to a large increase in metastases count. The therapeutic transfer of Cbl-b$^{-/-}$ NK cells into B16F10 tumor-bearing mice conveyed fewer metastases in the lungs of wild-type mice. Remarkably, within the NeuT^{+} mouse model where female mice develop spontaneous metastatic breast tumors, the cross-bred Cbl-b$^{-/-}$ NeuT^{+} mice were able to control tumor growth with reduced metastatic tumor/lung ratios. This effect was absent when NK cells were depleted, implying that NK cells defective in Cbl-b are sufficient to inhibit the progression of tumor and distant metastases.[106] It appears that Cbl-b affects multiple regulatory circuits in anti-tumor immunity, making it an interesting target for tumor immunotherapy. Indeed, Penninger's group further explored molecular Cbl-b-mediated ubiquitylation targets resulting in the identification of the TAM (Tyro3, Axl, Mer) family of tyrosine kinase receptors. Utilizing a novel, selective TAM inhibitor LDC1267 (blocking Cbl-b ubiquitylation and thus its activity) to treat mice challenged with B16F10 melanoma or 4T1 mammary tumor cells markedly reduced the number of metastases. Furthermore, adoptive transfer of wild-type NK-cells treated with LDC1267 ex vivo significantly improved the anti-metastatic responses to levels observed in mice transplanted with Cbl-b$^{-/-}$ NK cells. Interestingly, in a last set of experiments, it was shown that the long-known anti-metastatic activity of the anti-coagulation drug warfarin, a known inhibitor of the TAM receptor ligand Gas6, depends on NK cells and their functional Cbl-b.[106] Penninger concluded that various signaling pathways are regulated by Cbl-b in innate and adaptive immune cells and therefore an efficient manipulation of Cbl-b might give hope for emerging cancer immunotherapies.

Conclusion

The promise of cancer immunotherapy has recently been recognized as "breakthrough of the year" at the end of 2013.[107] Topics covered at CIMT2014 and summarized in this report represent various novel anti-cancer approaches and their clinical translation as next waves of cancer immunotherapy. We anticipate that the advancements in the field will come more close to clinical application and lead to approval of novel immunotherapeutic drugs in the coming year, from which we will hear at the 13th Annual CIMT Meeting (May 11–13 2015, Mainz, Germany).

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