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MEETING REPORT

Report on the sixth annual meeting of the Association for Immunotherapy of Cancer (CIMT), May 15 and 16, 2008 in Mainz, Germany

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Abbreviations

AML	Acute myeloid leukaemia
APCs	Antigen presenting cells
CFSE	Carboxyfluorescein succinimidyl ester
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DCs	Dendritic cells
DT	Diphtheria toxin
EP	Electroporation
ER	Endoplasmatic reticulum
Erb2	Estrogen receptor b2
FrC-DOM	Fragment C of tetanus toxin
GML	Genetically modified lymphocytes
GM-CSF	Granulocyte monocyte colony stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HEL	Hen egg lysosyme
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
ICAM-1	Intercellular adhesion molecule 1
IFNγ	Interferon gamma
IL	Interleukin
i.m.	Intramuscular
KO	Knockout
MHC	Major histocompatibility complex
NIH	National Institutes of Health
NK	Natural killer
ONTAK	Denileukin-diftitox
OVA	Ovalbumin
ORP1L	Oxysterol-binding protein homologue
pMHC	Peptide–MHC complexes
RHAMM	Receptor for hyaluronic acid mediated motility
$PMSA_{27}$	Prostate specific membrane antigen peptide 27
RILP	Rab-interacting lysosomal protein
TK	Thymidine kinase
Tregs	Regulatory T cells
TCR	T-cell receptor
TLR	Toll like receptor
TAP	Transporter associated with antigen processing
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

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Introduction

After one visit to Würzburg in 2007 the sixth annual meeting of the Association for Immunotherapy of Cancer (CIMT) was organized again in Mainz on May 15 and 16, 2008. More than 350 participants attended the scientific sessions with program topics ranging from fundamental research on antigen processing and mechanisms of breaking tolerance to more translational studies on adoptive T-cell transfer, vaccination and the use of adjuvants or immunomodulating agents. In addition, an extra session was organized which focused on the monitoring of immunotherapy-induced immune responses. Selected posters were discussed in short presentations during three parallel sessions. This year's meeting provided the attendees with a comprehensive overview of the most prominent work in the field.

It proved to be a very interesting and stimulating meeting, with plenty of opportunity to "meet the expert". In contrast to prior CIMT meetings, in which the great majority of participants came from Germany, this year's meeting attracted a much more international audience, reflecting the increasing interest in this meeting platform in- and out-side of Europe.

Antigen processing

The CIMT meeting kicked off with fundamental research on antigen processing. Three experts were invited to elaborate on this topic. The first speaker, *Emil Unanue (keynote lecture—St Louis, MO, USA),* presented his work on the biochemical rules of peptide selection for major histocompatibility complex (MHC) class II presentation. Working with the antigen hen egg lysosyme (HEL) in a mouse model, Unanue and co-workers dissected a number of key issues involved in antigen processing and presentation by antigen presenting cells (APCs) and the subsequent interactions of CD4+ T cells with peptide–MHC (pMHC) molecules. Two distinct sets of CD4+ T cells recognizing the same peptide fragment from the HEL protein have been identified. T cells of type A, reflecting "conventional" T cells, recognize both exogenously loaded and endogenously processed peptide. T cells of type B on the other hand only recognize MHC-complexes filled with exogenously loaded peptides. It was shown that differences in the conformation of the pMHC class II complex determined the recognition by either one of the two different T-cell types and that the class II-like accessory molecule H2-DM plays an important role in this, as it functions as a conformational "controller". The pMHC complexes edited by the abundantly present H2-DM in the lysosomes were more stable and held a conformation, which were only recognized by type A T cells. In contrast, pMHC complexes that have been filled with exogenous peptides or peptides from the early endosomes (so without being edited by H2-DM) were recognized by type B T cells. This was elegantly shown by the following experiment: if the peptide was delivered by liposomes into lysosomes of APC, there was extensive formation of type A pMHC, while the formation of the type B pMHC and their recognition by type B T cells was abolished. This effect did not take place when murine H2-DM-deficient APCs were examined. Thus it was concluded that type B T cells recognize pMHC complexes generated in recycling endosomes that can be eliminated by H2-DM in the late endosomal and lysosomal compartments. These type B T cells were shown to escape thymic negative selection in HEL transgenic mice, while type A T cells to the same epitope underwent thymic central deletion. It was stressed that type B T cells are rather abundant and can also be detected in high frequency in infections with intracellular bacteria like *Listeria monocytogenes*. The role of type B T cells in the development of autoimmunity in tissue sites prone to inflammation, such as tissues where extensive proteolysis and release of peptides occurs, was also discussed.

The immunological consequences of post-translational modification of peptides found in MHC class II in relation to autoimmune diseases was another issue which was briefly discussed. In this regard, three chemical modifications were described: (1) nitration of tyrosine residues, (2) citrullination of arginine residues and (3) oxidation of tryptophan residues. These modifications, which take place intracellularly in APCs that are activated by cytokines and pathogen-derived molecules, lead to stimulation of conventional type A T cells of which the recognition of pMHC complexes was highly specific for peptide modification. Moreover, CD4+ T cells highly specific for such modified peptides were detected in vivo, suggesting that these CD4+ T cells have escaped negative selection. Taken together, knowledge of unconventional type B T cells as well as T cells specific for post-translational modified antigens (type A) may be important for vaccine development.

Jacques Neefjes (Amsterdam, Netherlands) presented an innovative genome-wide approach to search for new targets involved in processing of MHC class II ligands. He used high throughput screening of a human melanoma cell line transfected with a siRNA library in combination with monitoring of changes in MHC class II expression on the cell surface by flow cytometry. Neefjes and colleagues found large series of candidate proteins involved in MHC class II presentation. The initial hits were normalized and matched with chemical libraries to make a selection for cell biological analysis. With this approach four genes have been validated and worked out in the last 10 years. One of them is dynein, a motor protein involved in movement of MHC class II containing vesicles via microtubules throughout the cell. Visualization of the movements showed that the vesicles move in a bi-directional way, first in the direction of the nucleus (by dynein), then back (by another motor protein: kinesin) and accelerate to the cell membrane. The specificity of the motor protein for MHC molecules is mediated by a protein called rab-interacting lysosomal protein (RILP). Another target in this molecular motor walking pathway is oxysterol-binding protein homologue (ORP1L), which interferes with the binding of the dynein motor to RILP. Depletion of cholesterol from the serum or blocking cholesterol synthesis modified ORP1L and its binding to RILP, showing that the formation and activity of the microtubule motor depends on the concentration of cholesterol. Because the validation of all gene target candidates identified by the screening would exceed a human life-span several fold, several hits were placed in a functional network,

which allows analyzing them in groups, reducing complexity and accelerating interpretation of the whole process.

Sven Burgdorf (Bonn, Germany) presented a mechanistic study on processing and presentation of protein antigen after receptor-mediated uptake. In contrast to MHC class I presentation of endogenous proteins, processing and MHC class I presentation of exogenous antigen is a special mechanism exclusively mediated by professional APCs such as dendritic cells (DCs). Cross-presentation is very important in the induction of immunity against tumors, because tumors lack the co-stimulatory molecules that are required to directly trigger a proper response of naïve T cells. Burgdorf and colleagues used a model antigen derived from chicken eggs, ovalbumin (OVA), to study the pathway of processing and presentation of exogenous protein. They had previously launched the concept that mannose receptor-mediated uptake of OVA leads to MHC class I presentation (cross-presentation), while uptake by micropinocytosis or scavenger receptor-mediated endocytosis leads to MHC class II presentation by non-overlapping pathways. At the meeting he elaborated on this. Early after mannose receptor-mediated uptake, the peptide antigen is loaded on MHC class I molecules in early endosomes. Inhibitor studies showed that the cross-presentation of OVA is dependent on the proteasome, indicating that processing takes place in the cytosol where the proteasome is located. Indeed, OVA could be purified from the cytosol when the proteasome was inhibited. Cross-presentation could also be blocked by US6, a cytomegalovirus-derived inhibitor of the transporter associated with antigen processing (TAP). This transporter is commonly believed to be located in the endoplasmic reticulum (ER) and to be involved in transport of processed endogenous-derived peptides from the cytoplasm into the ER, where the peptides then are loaded on MHC class I. To further elucidate the processing pathway of exogenous proteins, an endosome-specific inhibitor of TAP was created that could specifically block cross-presentation but not endogenous presentation of OVA. In addition, they showed that early endosomes contain TAP by fluorescence microscopy. Toll like receptor (TLR) triggering appeared to be crucial for recruitment of TAP to the early endosomes. This explains the finding that cross-presentation of endotoxin-free OVA was severely reduced. Strikingly, it was shown that cross-presentation and endogenous MHC class I presentation are spatially and mechanistically distinct. This may be important to limit competition for the proteasome between exogenous and endogenous derived MHC class I ligands. However, how to avoid this competition in vaccination studies using exogenous-derived antigens was not addressed in this study. Importantly, cross-presentation may be limited to situations where both antigen and a pathogen-derived danger signal are simultaneously present. On the other hand, this may also be true for induction of cross-tolerance.

Cellular therapy

Another main topic of the meeting was adoptive cellular therapy for the treatment of malignant diseases. T cells for adoptive transfer can be generated from autologous T cells by in vitro priming, T-cell receptor (TCR) transfer or T cells originating from an allogeneic donor in order to completely avoid immunological tolerance.

Stanley Riddell (Seattle, WA, USA) focused on adoptive T-cell therapy in the context of allogeneic stem cell transplantation. In his talk he pointed out the importance of allogeneic T cells for the induction of a high avidity T-cell response in vivo as they can account for a potent graft-versus-leukemia (GVL) effect. Riddell shed light on two major obstacles of allogeneic T-cell therapy. First, he illustrated methods to distinguish the GVL from graft-versus-host disease (GVHD). A suitable approach is the expansion of either minor-histocompatibility antigen or leukemia-specific clones in vitro. Secondly, he pointed out that durable T-cell response will have to be achieved in vivo for an effective therapy, which is not always the case in adoptive T-cell therapy. He presented data from a clinical trial where leukemia-specific T-cell clones were individually generated and applied to patients with acute leukemia. Although these T cells efficiently induced remissions, the responding patients were prone to a subsequent relapse. As shown by clonotypic PCR, the transferred T cells could not persist in vivo any longer than 3 weeks. To tackle this problem they explored the impact of the T-cell subpopulations from which the clones originate. Using CMV-specific CD8+ T cells in a primate model, they demonstrated that clones derived from the subset of effector memory T cells (CD62L-CD45RO+), did not infiltrate the tissues and rapidly underwent apoptosis after they had been transferred. In contrast, when CD8+ T cells were used that were derived from the CD62L+ fraction (central memory CD45RO+ and naïve CD45RA+ T cells) this resulted in long living T-cell clones that proliferate and last several weeks longer in vivo. Furthermore, it was observed that even in allogeneic adoptive T-cell therapy the induction of T-cell memory is prerequisite for sustained disease control. The CD62L+ subpopulation of CD8+ T cells is a promising compartment for the generation of such virus or leukemia-specific T cells.

Other groups have worked on avoiding GVDH in allogeneic T-cell therapy either by depleting GVH-mediating T cells or by the enrichment of favorable T-cell responses by activation induced markers. Presentations by *Marion Nonn (Mainz, Germany; P99)* and *Sonja Schmucker (Bergisch-Gladbach, Germany; P95)* both showed that CD137 can be

used for detection, enrichment or depletion of T cells after stimulation. Nonn shared experiences on the use of CD137 for depleting allo-reactivity. She also introduced an in vivo model for monitoring of residing allo-reactivity by the use of subcutaneously transplanted human fibroblast patches that substitute for human organs in NOD/SCID/IL2Rγc^{null} mice. The data underline that CD137 is a very interesting marker that might be used for depletion of unwanted T-cell reactivity as well as for the enrichment of antigen-specific CD4+ and CD8+ T cells. In addition, the use of chimeric mice reconstituted with human hematopoiesis such as NOD/SCID/IL2Rγc^{null} mouse might in the future be a helpful tool in preclinical studies of T-cell based therapies.

During the meeting, various stimulation protocols for the generation of autologous antigen-specific T cells were discussed. Some groups effectively replaced DC by other cell types to prime or expand T cells. Others optimized their stimulation protocol smartly by other means. *Silke Landmeier (Münster, Germany; P37)* showed that the application of $\gamma\delta$ T cells as substitute for DC could stimulate the expansion of peptide-specific CD8+ T cells. The fact that $\gamma\delta$ T cells are easy to obtain and expand, even up to high cell numbers, could make them a preferred option compared to large scale DC generation systems. In addition, this cell type is able to present an internally processed antigen after transduction by a viral vector. Thus these $\gamma\delta$ T cells might be good APC for expansion of low frequency pre-existing tumor-specific CD8+ T cells. *Agnieszka Wieczorek (Berlin, Germany; P72)* has identified a cytokine cocktail [including interleukin (IL)-2, IL-6, IL-7, IL-10, IL-12 and IL-15] that in vitro can induce CD8+ T cells that are specific for Human Immunodeficiency virus (HIV) and Hepatitis peptides. This was achieved by using mononuclear cells from peripheral blood of sero-negative donors. However, it remains to be demonstrated that these T cells can also recognize endogenously processed antigen.

Another approach of T cell therapy is to artificially introduce TCR with the desired specificity and avidity into autologous lymphocytes. *Beate Hauptrock (Mainz, Germany; P108)* introduced the use of a single chain TCR construct designed by fusing the Vα to the β chain of a TCR. This three-domain single chain receptor can be co-transfected with a truncated Cα domain to form a functional TCR. Although their first 3-domain TCR had a lower affinity, TCR function could be improved by inserting murine sequences to the human constant domains. However, many questions regarding the generation of the TCRs, the safety of the application of TCR-transgenic T cells, and their in vivo monitoring still have to be addressed.

With regard to the safety, *Ton Schumacher (Amsterdam, Netherlands)* demonstrated that TCR-transduced T cells can cause a GVHD-like disease in mice, even in the absence of the target antigen. The main reason for this is the pairing of a natural with a transgenic TCR α and β chain to form new chimeric TCR entities that indeed have new and unforeseeable specificity. It emerged that mis-pairing of endogenous and transgenic TCR chains can occur, which may lead to life-threatening side-effects.

Christina Pfirschke (Mainz, Germany; P40) suggested that switching interacting amino acids in the constant regions of α and β chain of a T cell might hinder the formation of these unwanted chimeric heterodimers. Another way of limiting toxicity by TCR transfer is to use in vitro transcribed RNA instead of lentiviral vectors as introduced by *Niels Schaft (Erlangen, Germany; P61)* for ErbB2/Her2neu and for CMV-pp65 by *Simone Thomas (Mainz, Germany; P93)*. Although both groups showed that mRNA transfection through electroporation (EP) is achievable for TCR transfer, the efficiency of the method requires further improvement.

In an interesting talk, *Wolfgang Uckert (Berlin, Germany)* illustrated how he transfected TCR supplemented with a 10-amino acid encoding sequence from human c-myc. An antibody targeting this "c-myc-tag" is able to deplete T cells that are expressing the tagged TCR and thereby limits the potential toxicity of transferred transgenic T cells. He also presented his advanced efforts to improve the effectiveness of TCR gene transfer. He analyzed different expression cassettes for the transduction of a WT-1 specific TCR and compared the transfection of TCR single chains as well α and β chains linked either by an IRES sequence or the picorna virus derived peptide P2A. The linkage of β chain-P2A-α chain resulted in the highest level of functionality of the transduced TCR. By transducing a gp100- or a Melan A-specific TCR into a CMV-specific T-cell clone, he further demonstrated that the probability of TCR-chain mis-pairing varies between different TCRs. He also showed that the TCR avidity and function can be increased by replacing parts of the human TCR by murine sequences. It was discussed that a pragmatic approach to reduce the risk of TCR-based gene-therapy would be to limit the time of transgenic TCR-expression by either mRNA-transfection or by tagging the transgenic TCR to allow depletion by an antibody. The use of mRNA beyond that is not associated with the risk of insertional mutagenesis and it is also not classified as gene-therapy by the authorities of some countries, such as Germany. However, efforts to improve the avidity of the TCR as well as avoiding peptide-chain mis-pairing for TCR integrated in the genome of the target cells are needed to allow for long lasting transgenic T cell responses. With regard to this, it might also be of interest to induce a T-helper response. *Stephanie Hoyer (Erlangen, Germany; P62)* presented data on the transfer of TCR encoding RNA, either class I or class II human leukocyte antigen (HLA)-restricted, to CD4+ T cells by EP. Both approaches eventually lead to a

lytic phenotype of the CD4+ T cells. In addition to the effector functions, the CD4+ T cells also induced maturation of DCs after antigen encounter indicating T-helper functionality. The in vivo efficiency of T-helper cells harboring both properties (antigen-specific helper as well as killing capacity) still needs to be studied.

In summary, the cellular therapy sessions reflected the substantial interest in T-cell based anti-tumor therapy with many new and promising approaches. While a few years ago, the generation of antigen-presenting DC vaccines dominated the field of immunotherapy, adoptive T-cell therapy has now become the major focus. With virus-specific T cells being already available for clinical application, protocols using malignancy-specific T cells will progressively find their way to the clinic.

Therapeutic vaccinations

The pre-clinical and clinical use of vaccination combined with immunoregulatory antibodies in melanoma was presented by *Jeffrey Weber (Tampa, FL, USA).* Although melanoma is considered to be an immunogenic tumor, clinical studies of vaccines against this tumor have had very little impact on this disease. This can be explained in part by altered tumor environmental factors in general, and by the expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4) on effector and regulatory T cells (Treg) in particular. It is now clear that expression of CTLA-4 at the tumor site can block antigen-specific T-cell responses. It has already been demonstrated that anti-CTLA-4 antibody treatment in combination with vaccination cures melanoma in mice. For the experimental treatment of patients, two anti-CTLA-4 antibodies are under investigation: Tremelimumab, which is an IgG2 from Pfizer and Ipilimumab, an IgG1 antibody from Bristol-Myers, Squibb and Medarex. In phase I/II clinical trials, the administration of the antibody, either alone or in combination with peptide vaccination, has shown promising results. The use of Ipilimumab resulted in various immune tolerance-breaking events. According to Weber the side effects recorded so far were considered an acceptable risk in patients with cancer given the severity of the disease. Importantly, the patients with immune-related adverse events were those who benefit most from this adjuvant treatment with Ipilimumab in combination with peptide vaccination. The response kinetics of anti-CTLA-4 antibodies are unique, in that it may take weeks for anti-tumor effects to develop. In addition, increase in tumor volume after treatment due to infiltration of lymphocytes might be misinterpreted as progression and precede clinical responses. A phase III trial using Tremelimumab in advanced melanoma patients has been discontinued following an interim analysis showing that this treatment did not result in better overall survival than conventional treatment. The full consequences of this recent failure for clinical development of ongoing clinical trials combining vaccination or chemotherapy with Ipilimumab are still disputed in the field. Nevertheless, current Ipilimumab trials are still continued. So far, overall anti-CTLA-4 treatment showed clinical responses with a duration of more than 1 year in 5–17% of cancer patients. However, the adverse events are serious and present in about 40% of the patients, and this is strongly related to the dose and administration schedule. Biomarkers of anti-tumor activity induced by this treatment are still unclear, except for data supporting an association between adverse events or autoimmunity and better clinical outcome.

Other strategies to improve the efficacy of anti-tumor vaccines were presented by *Craig Slingluff Jr (Charlottesville, VA, USA),* who gave a comprehensive overview of different interventions to improve cancer immunotherapy. The main approach was to vaccinate against melanoma with multiple MHC class I ($n = 12$) and class II ($n = 6$) binding peptides combined with Montanide ISA-51 and granulocyte monocyte colony stimulating factor (GM-CSF). In the first part of his presentation, he introduced the concept of lymphoid neogenesis: establishment of a transient tertiary lymphoid organ at the vaccination site. A privileged site for T-cell priming is created dependent on the combination of the antigen and adjuvant. In the second part, he addressed the trafficking of T cells to the tumor. Peptide vaccination in Montanide ISA-51 was associated with upregulation of chemokine (C-X-C motif) receptor CXCR3 expression on CD8+ T cells. This is important for migration into the tumor because tumor cells have been shown to express its ligands CXCL9, 10 and 11. Upregulation of CXCR3 on T cells can also be induced by low dose of IL-2 or intra-tumoral application of interferon gamma (IFNγ), of which the latter is now tested in melanoma patients. Another challenge is to interfere with the tumor microenvironment. Vascular endothelial growth factor (VEGF) produced by melanoma cells has a negative influence on the immune response (paralyzes antigen presentation, blocks DC maturation and induces myeloid suppressor cells), and forms an autocrine growth loop together with VEGF receptor 2 resulting in proliferation of melanoma cells. VEGF blockade by Bevacizumab may be a potential targeted therapy in combination with immunotherapy. In accordance with increasing numbers of colleagues in the field Slingluff concluded with the advice to combine different intervention strategies (such as the recently published combination of anti-VEGF and Rapamycin in melanoma patients) to improve immunotherapy of cancer.

Lindsey Low (Southampton, UK; P45) gave an update of the results obtained with the DNA fusion gene vaccine, now using the plasmid domain of fragment C of tetanus toxin (FrC-DOM) linked to prostate specific membrane

antigen peptide 27 ($PSMA_{27}$). In a phase I/II trial, HLA-A2 patients with recurrent prostate cancer were injected intramuscularly (i.m.; dose 800, 1,600, and 3,200 μg) or by i.m. EP (dose 400, 800, and 1,600 μg) with this vaccine. So far, humoral and T-cell responses have been assessed for the first dose level of each group. In 14/20 patients at least a twofold increase in anti-FrC-DOM antibody level were found, of which 9 patients received the vaccine by EP and the other 5 switched from three times DNA alone to boost at weeks 24 and 48 with EP, suggesting that EP results in an increased humoral response. In nine out of ten evaluated patients FrC-DOM specific CD4+ T-cell responses were detectable in peripheral blood mononuclear cell samples, which sustained longer in the EP patient group. CD8+ T-cell responses against $PSMA_{27}$ as determined by IFNγ-ELISPOT analysis after 8 days of pre-stimulation were found in 6/10 patients, demonstrating that the fusion vaccine approach is capable of inducing a strong immunity against the inserted tumor antigen.

Michael Schmitt (Ulm, Germany; P10) presented the results from their phase I/II vaccination trial in patients with hematological malignancies, in which the tumor cells expressed the receptor for hyaluronic acid mediated motility (RHAMM). Patients with minimal residual disease were vaccinated with a vaccine consisting of the CD8+ T-cell epitope peptide of RHAMM emulsified in incomplete Freund's adjuvant combined with GM-CSF injections. Immunomonitoring revealed specific T-cell responses in 70% of the patients in terms of IFNγ and granzyme B production and by R3 peptide-tetramer positive CD8+ T cells. In this dose-escalating clinical trial (300 and 1,000 μg R3 peptide) Schmitt demonstrated that a higher dose of the R3 peptide vaccine resulted in a weaker T-cell immune response, suggesting that more peptide does not necessarily lead to better response. Moreover, the elevated numbers of IFNγ-producing T cells observed after three vaccinations in patients with acute myeloid leukemia (AML) decreased again after four vaccinations. A similar observation was made with respect to granzyme B-releasing CD8+ T cells in vaccinated patients with multiple myeloma. Objective clinical responses were observed in 50% of the vaccinated patients. The next question is whether there is a correlation between clinical efficacy and vaccine-induced immunity. In four out of ten patients with clinical responses elevated levels of IL-2 were found in serum at 3 weeks after the fourth vaccination. However, the elevated numbers of functional CD8+ T cells were not long-lasting and AML patients relapsed suggesting that booster vaccination might be warranted.

A novel vaccination approach was presented by *Raffaella Fontana (Milan, Italy; P49)*, who made use of autologous lymphocytes and genetically modified them to express the tumor antigen MAGE-3. These genetically modified lymphocytes (GML) also expressed the Herpes Simplex Virus (HSV)-thymidine kinase (TK) antigen which can be used as a reporter antigen as well as a suicide gene that can be activated by the anti-viral drug ganciclovir in case of severe side effects. In three out of the ten treated stage III/IV melanoma patients a strong and durable (up to 1 year) functional MAGE-3 specific CD8+ T-cell response was measurable both in the circulation and in the tumor infiltrated lymphocytes. Moreover, these patients showed skin reactivity against the MAGE-3 antigen and displayed a favorable clinical outcome. In responding patients a new MAGE-3 epitope restricted by two HLA class I molecules was identified. This was recognized by the CD8+ T cells that heavily infiltrated the tumor but was absent in the pre-treatment tumor sample lymphocyte culture. CD4+ T-cell responses against the HSV-TK antigen preceded the CD8+ T-cell response against MAGE-3. Although in one patient the TK-specific T-cell frequency was 400 times higher than that against MAGE-3, it did not divert the immune system from recognizing the MAGE-3 antigen. The results of this clinical trial showed that the infusion of GML expressing tumor associated antigens leads to anti-tumor effector T cells that are able to traffic from the lymphoid organ where they are primed to inflamed tissue (the delayed-type hypersensitivity site) and more importantly to the tumor. Although some patients appeared to show clinical benefit from the increase in effector T-cell response, the relation between immune response and clinical efficacy needs to be established in a larger group of patients.

Enhancing immunity and adjuvants

The short talk presentation section on enhancing immunity and adjuvants, started with a presentation by *Kris Thielemans (Brussels, Belgium; P83)* showing preliminary data based on a phase I clinical trial using TriMix-DC vaccine in advanced melanoma patients. The TriMix vaccine being composed of monocyte derived DCs co-electroporated with mRNA encoding CD70, CD40L and a constitutive active TLR4 ligand and antigen encoding mRNA was superior in the ability to secrete IL-12 after cryopreservation compared to DCs prepared by conventional cytokine cocktail. Moreover, the TriMix-DCs had the potency to induce naïve CD4+ T cells to produce the T-helper type 1 (Th1) cytokine IFNγ, and the strength of the TriMix-DC vaccine was further shown by the ability of peptide loaded TriMix-DCs (where the peptides were derived from MAGE-A3, MAGE-C2, or gp100) to induce CD8+ T-cell expansion compared to immature DCs. In this regard, a strong immunological response, in terms of IFNγ and tumor necrosis factor alpha (TNFα) production by infiltrated T cells and upregulation of CD137 and CD107 expression after re-exposure to

antigen-expressing autologous EBV lines, was observed in the biopsy taken at the vaccination site of the patients. Taken together, the results presented suggest TriMix-DCs could be an attractive candidate in vaccine development.

The use of bispecific recombinant single chained antibodies as a device to induce a potent immune response was discussed by two speakers. *Ute Burkhardt (Frankfurt, Germany; P22)* presented results on a bispecific conjugate, consisting of a fusion of bacterial produced recombinant single-chain monoclonal antibody Fv fragments, which bind to CD40 on the DC surface and a fragment of the tumor antigen derived from the estrogen receptor b2 (Erb2). Although the construct was proved to efficiently induce DC maturation and deliver the Erb2 peptide to DCs, one potential danger of using bacterial expressed recombinant single chained antibodies as a vaccine modality is that of endotoxin contamination, which was also reported to be present in the single chain antibody preparation used in this study. *Claudia Franke (Dresden, Germany; P28)* discussed the use of mammalian expressed bispecific antibodies (thereby overcoming the potential endotoxin contamination) to enhance the lysis of tumor cells by natural killer (NK)-T cells. They generated a bispecific antibody, directed against the tumor specific La protein and the human cytomegalovirus glycoprotein UL16 binding protein that binds and activate NK cells. This antibody very efficiently increased the specific lysis of the La-expressing tumor cells by NK-T cells in vitro. It would be interesting to investigate the anti-tumor effect of this bispecific antibody in vivo. Taken together, bispecific antibodies show promising effects in pre-clinical studies, although particular caution should be taken to minimize endotoxin contamination during the production of such molecules.

Another way to improve the immunotherapy is by optimizing the presence of DCs in DC compartments (lymph node, spleen) as exemplified by basic research shown by *Natalio Garbi (Heidelberg, Germany)*. He showed the results of a very elegant study on the homeostasis of DC numbers in mice. He dissected if the stable DC numbers in the lymphoid organs are maintained by active or passive mechanisms. He used a mouse model where conventional CD11c+ DCs can be depleted with diphtheria toxin (DT). By making a bone marrow chimera of this mouse with a GFP+ mouse, he could study re-population of DCs after depleting with DT. He stressed that in absence of DCs new precursor DCs are recruited and activated. The presence of and properly activated DCs (expressing MHC class II) is essential for the awareness of naïve T cells to recognize foreign antigens as he observed a poor capacity to stimulate naïve T cells that had been in an environment with reduced numbers of DCs or DCs lacking MHC class II expression. Low number and bad quality of DC may be overcome by targeting antigens more efficiently to DC or deliver antigens loaded on ex vivo generated DC.

New strategies

Sebastian Amigorena (Paris, France) visualized the interaction between T cells and DC in intact lymph nodes in movies made by optical 3-dimensional imaging using 2-photon confocal microscopy. The expression of the intercellular adhesion molecule 1 (ICAM-1) was shown to be essential for long lasting interaction between these two cell types and subsequent induction of an immune response. When cells isolated from ICAM-1 knockout (KO) mice were used the contacts between mature DC and naïve T cells were short and unstable resulting in a failure to prime an effective T-cell response. This was demonstrated by the adoptive transfer of carboxyfluorescein succinimidyl ester (CFSE)-labeled OT-1 cells. Although similar proliferative responses of transferred OT-1 cells were obtained in OVA expressing tumor-bearing wild type mice or ICAM-1 KO mice, the OT-1 cells injected in the ICAM-1 KO mice failed to produce IFNγ and insufficiently survived. Using the same technology, the effect of anti-CD25 antibodies to suppress natural Tregs was investigated. Mice were injected with MCA_{101}-OVA (fibrosarcoma) tumor cells and treated with anti-CD25 at the same time. Ten days later OT-1 cells were injected and tumor growth was controlled only in the group of mice treated with anti-CD25 antibody. Interestingly, the frequency of Foxp3+ cells was not different between the two groups of mice, but the phenotype and function of the Tregs was altered, in that CD25 was down-regulated, CD44 was upregulated and they produced more IFNγ. It appeared that the motility of T cells was lower and the arrest time was higher in the tumor draining lymph node of anti-CD25 antibody treated mice. This suggests that Tregs inhibit stable contacts of T cells with DC during priming. Notably, no effect of Treg depletion was found on the motility of OT-1 cells inside the tumor itself.

The observation that Tregs may play an adverse role in therapeutic anti-cancer treatment, suggests that the manipulation of these cells might improve the efficacy of therapy. *Bernard Fox (Portland, OR, USA)* showed two ways to intervene with Tregs. In mice, vaccination in lymphopenic environment enhances the efficacy of the treatment. In addition, adoptive transfer of T cells isolated from tumor-bearing mice followed by CD25+ T-cell depletion resulted in eradication of established tumors in the recipient mice. Furthermore, in multiple murine vaccination models anti-CD4 treatment prior to immunotherapy reduced the absolute numbers of CD3+, CD4+, Foxp3+ cells in the spleen but the remaining effector T cells were found to be more effective. GVAX is a vaccine that consists of autologous tumor cells genetically modified to secrete GM-CSF. In prostate cancer patients, treated with GVAX, in combination with cyclophosphamide and fludarabine to deplete Tregs, it was shown that the majority of Tregs were from the adoptive

transfer product when the lymphopenic patient was reconstituted. In conclusion, Fox demonstrated the importance of interfering with Tregs either by depleting CD25+ T cells which are present in the adoptive transfer product or by anti-CD4 treatment.

Another new strategy to deplete Tregs before vaccinating with tumor-specific peptides or peptide-loaded DC was presented by *Michael Erdmann (Erlangen, Germany; P98).* He showed results of administration of denileukin-diftitox (ONTAK) in melanoma stage III/IV patients in order to deplete Tregs cells prior to peptide-loaded DC treatment. So far 7 of the intended 17 patients were evaluable for immunomonitoring. Using ELISPOT and mixed lymphocyte-peptide culture method (as reported by Pierre Coulie, Brussels, Belgium) it was revealed that DC vaccination alone was more effective in eliciting an immune response than the ONTAK treatment followed by DC therapy. No significant expansion of tumor antigen-specific T cells was observed after ONTAK pre-treatment neither were the numbers of CD4+, $CD25^{high}$ or Foxp3 expressing T cells decreased. Currently they are trying to determine the cause of this unexpected lower immunity when ONTAK was used. The dose and schedule of ONTAK still has to be established. Importantly, the T-cell responses against common recall antigens such as influenza and tetanus need to be determined to be sure that patients in the study were not immunosuppressed.

The fine specificity of CD4+, Foxp3 + Tregs mediating suppression of anti-tumor immunity was explored by *Hyam Levitsky (Baltimore, MD, USA)* and his colleagues. Naïve T-cells can be committed to Tregs by DC educated in the anti-inflammatory tumor environment and it was shown that Tregs can even be induced by vaccination. At the tumor site Tregs can prevent the function of infiltrating effector T cells. Where does the specificity of Tregs to exert their specific suppression come from? When A20-HA and A20-OVA tumors both were established on the same flank of the mice and HA-specific Th1 cells were adoptively transferred, the HA tumor was prevented from growing but not the OVA tumor. Furthermore, in this model HA-specific Tregs were able to block HA-specific effector T cells from exerting their eradicative function. Thus, by using this mixed tumor model it could be demonstrated that Tregs need to be activated by the same antigen as the effector T cells. When the Tregs outnumber the effector T cells (two Tregs to one effector T cell) the suppression became less antigen-specific as additional antigen-specific Th responses were blocked. Importantly, a slight change in the balance of the Treg/effector T-cell ratio could have a major impact on clinical outcome as discussed by Levitsky.

Anita Gomes (Lisbon, Portugal; P21) found two interesting new targets as potential activators or inhibitors of $\gamma\delta$ T cells, the non-classical MHC molecule ULBP1 and the costimulatory molecule B7H1 (PD-L1). These membrane proteins popped up in a screening of a high number of lymphoma and leukemia cell lines versus not target cells for killing by $\gamma\delta$ T cells. Microarray analysis of targets versus non-targets revealed 51 common down-regulated and 37 upregulated membrane proteins as potential inhibitors or activators of $\gamma\delta$ T cells. Although the candidate genes have not been validated yet, this study deserves the attention because of its original approach.

Clinical trial endpoints

New insights have been gained for clinical trial endpoints for cancer immunotherapy trials through community-wide efforts. *Axel Hoos (BMS, Wallingford, CT, USA)* showed that adjusted endpoints for development of cancer immunotherapy should be based on community consensus and need to be data-driven. Three topics were covered. First, T-cell immune responses measured by sophisticated assays have always been used to describe biological activity of immunotherapies. However the large variability of results has so far complicated our ability to draw reliable conclusions from such data. To address this, an international consensus working group, initiated by the Cancer Vaccine Consortium (CVC), conducted several meetings and workshops, proposed that immunoassays in clinical trials should be performed at least at three different time points during the study, one baseline and two follow-up time points. Assays used should be established, reproducible and technically (not clinically) validated. In addition, at least two assays should be used in parallel to demonstrate the same findings. This is particularly important if developmental decisions would be based on immune response data. Moreover, the cut-off values for a positive immune response should be defined prospectively in terms of what defines an immune response and in how many patients in the study population does it need to be seen to constitute a positive outcome. Second, anti-tumor response patterns are insufficiently described by the standard criteria such as WHO/RECIST. Similar to immune response, the Cancer Vaccine Clinical Trial Working Group addressed this topic and suggested that a possible delayed onset of a clinically measurable therapeutic effect is not captured with standard criteria. Furthermore, durable stable disease might also represent benefit, which is not adequately captured. It was announced that suggestions for new anti-tumor response criteria based on clinical data will be first presented to the scientific community during ASCO 2008. Third, the detection of survival differences in randomized trials may also be impacted by possible delayed effects of immunotherapies and may lead to a delayed separation of Kaplan–Meier survival curves with

implications for the statistical power of the study. Again, based on community discussions during a workshop of the CVC in 2006, all accessible data from randomized phase 3 trials with immunotherapies were analyzed suggesting the real possibility of a delayed separation of curves. This phenomenon would need to be accounted for in the statistical design of future studies for ensuring the adequate powering of the study. This may require a different approach to calculating hazard ratios for these trials and may require larger clinical effects after the separation of curves to achieve overall statistical significance. Overall, the community-based work as conducted by CIMT and CVC offers new insights to improve clinical endpoints for immunotherapy studies and may translate into more successful developmental strategies.

Immunomonitoring

The last session of the meeting was organized by the CIMT Immunomonitoring working group. This provided an opportunity to update and discuss the three major issues in the immunotherapy field that had been identified at the 4th annual meeting. (1) So far, a correlation between data from immunomonitoring and clinical responses has only been found in a minority of patients and published trials. (2) There are so many different assays available that they cannot all be applied as both, patient material and financial resources are limited. It is still a highly disputed question which tests should be used. (3) The third challenge is the existence of a broad variety of different local protocols for each of the available assays and the lack of standardization that prevents comparability of results obtained by different labs world-wide.

More practical aspects of immunomonitoring had already been discussed in a workshop on HLA-peptide multimer staining. It was demonstrated that dump channel and dead cell markers can be efficiently used to gate out cells from final analysis that can contribute to non-specific binding of HLA-peptide multimers. Secondly, protocols are now available that allow functional analysis of tetramer positive cells. The fact that over 100 participants attended this first workshop organized by the CIMT working group shows that the there is a great interest in the technical details and more practical aspects which should also be respected in future meetings.

Correlation of immunomonitoring and clinical events

Mario Roederer (Bethesda, MD, USA) used multicolor flow cytometry assays to follow immune responses after immunization with different Leishmania vaccines in mice and in HIV-infected patients with different courses of disease. In the Leishmania major model the quantification of antigen-specific CD4+ T cells simultaneously producing three cytokines (IFNγ, IL-2 and TNFα) allowed to predict protection against infection in immunized mice.

The National Institutes of Health (NIH) group could also detect more polyfunctional HIV-specific CD4+ and CD8+ T cells in patients with delayed disease progression. In this study the quality of the HIV-specific T-cell response was determined by simultaneously monitoring for five functional T-cell markers (IFNγ, IL-2, TNFα, MIP-1β [CCL4], and CD107a). The combined use of 12–14 colors also allowed determining the phenotype of fractions of antigen-specific T cells simultaneously producing one to five of the investigated markers in detail but so far clear correlations between function and phenotype could not be systematically established. Both, in vaccinated mice and in HIV-infected individuals, multifunctional effector cells produced up to tenfold increased amounts of cytokines on a per cell basis. The presented data suggest that recent advances in the use of multi-color flow-cytometry make the assay a very powerful tool to monitor immune responses in detail and to identify patterns of immunity that will soon allow predicting success or failure of immunizations applied.

With more and more functional and phenotypic subgroups of cells to be investigated at the same time, there is a requirement for higher initial cell numbers so that sufficient events are obtained in each of these subgroups. Investigators working in tumor immunology might be confronted with relatively low frequencies of antigen-specific T cells and restricted amount of patient material for immunomonitoring. In addition to these biological considerations it has to be kept in mind that an assay with 12 or even more colors is very complex and extremely difficult to control, standardize and validate, which might so far only be possible for a few expert labs worldwide with sufficient budget, manpower and qualification. Tests that use up to nine colors can probably be established with acceptable investments and might be sufficient to generate results that correlate with clinical events. The development of a validated kit for nine colors that can be used by a larger number of labs has already begun.

The results generated at the NIH, together with additional data that is now emerging from infectious diseases and tumor immunology, strongly suggests that vaccine induced immune responses will be complex and patterns of effective immunity will probably only be reflected by monitoring several functions of the immune system simultaneously. Although results from the basic assays that monitor one single function of the immune system will probably not be sufficient to reach a correlation between immunomonitoring, a well-planned combination of different mono-parametric tests that measure complementary functions might lead to as conclusive results as the use of

far advanced multi-functional assays that monitoring several markers at once.

Selection of assays

Sjoerd van der Burg (Leiden, Netherlands) has been using a whole arsenal of different techniques to monitor samples from patients vaccinated against human papillomavirus (HPV)-induced pre-malignant vulvar lesions and cervical cancer to guide the development of long peptide vaccines against HPV E6 and E7 at the Leiden University Medical Center. Based on his many years experience he proposed recommendations for assay selection in three steps—starting simple, going up to highest reachable complexity and ending with the minimum number of tests required to represent a surrogate for clinical events. In the very early phases of clinical development the main purpose of immunomonitoring should be to show that a newly designed vaccine is immunogenic. This can be obtained by use of one or two simple mono-parametric assays ("*start simple*"). During these early phases more assays will already have to be established and validated in order to be ready for use when development of the new vaccine proceeds. The selection of each additional assay should strongly depend on the type of vaccine approach chosen and should allow to monitor as many functional markers as feasible with budget and samples ("*intensive use*"). The more knowledge about the mechanism of action and the specificities of expected immune responses the investigator has, the more rational the choice of assays that should come to use can be.

All results from such intensive immunomonitoring can be used to guide further development of the product and to support decisions to proceed with or stop the current development plan. Once a vaccine is developed to stages where is has been shown to be effective, patterns of immunity that correlate with effective immune responses should be identified and defined as surrogate markers. Consequently, only those assays necessary to make predictions will have to be applied in later stages.

Standardization of immunoassays

There must be a clear distinction between using immunoassays as established surrogate markers to predict clinical endpoints and the more basic application of using immunoassays as biomarkers. One pre-requisite to turn results from immunoassays into biomarker of value will be the careful standardization and validation of the tests before use in clinical trials. The CIMT monitoring panel is aiming to guide the standardization of commonly used assays and to provide a tool for external test validation by organizing inter-laboratory testing projects. During the immunomonitoring sessions members of the organizing team presented first preliminary results from panel phase for HLA-peptide tetramer staining (*Cécile Gouttefangeas, Tübingen, Germany*), ELISPOT assay (*Ann Mander, Southampton, UK*) and intracellular cytokine staining (*Marij Welters, Leiden, Netherlands*). Each panel has a specific and unique design that allows for the systematic investigation of distinct variables expected to influence assay sensitivity in addition to providing external validation of locally applied protocols to all participating labs. Once data collection is accomplished the group will prepare final reports for participants and will make all findings available to the scientific community.

Sylvia Janetzki (New York, NY, USA) introduced the immunoassay proficiency panel program of the Cancer Vaccine Consortium (CVC), which is the largest program of this kind world-wide and covers ELISPOT, HLA-peptide multimer staining, intracellular cytokine staining and following T-cell proliferation by CFSE-dilution. First initial harmonization guidelines for ELISPOT assays were presented that are based on results form the first two ELISPOT panels with 36 and 29 participating labs. Data from a third panel that was recently conducted revealed that the consequent application of the guidelines reduced variation and increased the performance of the group. Especially, all labs with top panel performance complied with the harmonization guidelines, whereas 33% of labs who did not, missed to detect at least one response correctly. Since January 2008 the CVC is part of the Cancer Research Institute, enabling the newly organized group to continue with the current panels and to extend its program. The aim of the group is to offer an external quality assurance program and to enhance assay harmonization by introducing specific guidelines for investigated assays. As both CIMT and CVC use different panel designs and partly generate independent data sets on same questions the comparison of results can be used to confirm or question findings and help to distil the mechanisms and rules underlying test performance. For instance, their data clearly shows the importance of low background in ELISPOT assays which is dependent on the choices for medium and serum additives used for freezing, thawing and testing the cells. The correct choice of medium and additions is still an open question. The CVC plans to investigate the serum/medium issue in its upcoming panel in fall 2008. It was proposed to co-ordinate these efforts as collaboration between CIMT and CVC.

Jeffrey Smith (Merck and Co., West Point, NY, USA) presented his approach to reach stringent acceptance criteria for cell material after thawing chosen by a big pharmaceutical company with a huge vaccination program. First, different tests to determine the quality of cells based on viability, rate of apoptosis and phytohemaglutinin-induced cytokine production were established, validated and compared. These assays were then used to systematically monitor the influence of (1) time to processing of

blood samples, (2) transport storage temperature conditions to reach optimal and stable cooling and (3) temperature changes during long-term storage of frozen cell material on the results of the quality tests applied. In the last part of the talk it was shown that all conditions that lead to a reduced quality of cells based on the proposed readout tests clearly impaired the detection of antigen-specific T cell responses within the samples. The data from quality testing and functional assays for antigen-specific T cell responses were combined and clear cut acceptance criteria for cells after thawing were defined. It became clear that validated quality control steps for cells should be implemented by all labs that monitor clinical material but it is still an open question which techniques to use and which thresholds to set for acceptance criteria. Although the study conducted by Merck will be of great help to guide the process to reach study specific acceptance criteria each center will have to establish appropriate tests and define the acceptance criteria to be applied for its center or study specific setting.

Final conclusions

It is clear that the field is defined by progress in small steps rather than unexpected breakthroughs. Nevertheless, it was also clear that step by step developments emerge from all areas of immunotherapy, ranging from fundamental research to large clinical trials with combination therapies. The participant was able to hear about new and innovative tools to solve some of the biological and regulatory problems that they encounter somewhere early or late in an approval process.

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