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ORAL PRESENTATIONS

ANTIBODY THERAPY

S1
Bispecific costimulatory molecules for activation of tumor-killing lymphocytes

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The receptor tyrosine kinase ErbB2 (HER2) is overexpressed in multiple human tumors of epithelial origin. High ErbB2 expression is functionally involved in tumorigenesis and correlates with poor clinical prognosis. For immunotherapy of ErbB2 expressing tumors, we developed a strategy to supply the tumor cells with costimulatory activity. A bispecific fusion protein was constructed (BIg5), containing the IgV-like domain of huCD86, the CH2/CH3 domain of huIgG1 and the ErbB2-specific single chain antibody fragment scFv(FRP5). A similar fusion protein lacking the CD86 domain (Ig5) was used as a control. Upon binding of BIg5 to ErbB2 on tumor cells, these cells display CD86 on their surface and thus can deliver costimulatory signals for T-cell activation. In addition, NK cells could be activated by CD86 binding to CD28. BIg5 is secreted by eukaryotic cells as a homodimer with increased stability compared to monomers and possibly enhanced costimulatory activity due to crosslinking of CD28 on effector cells. By FACS analysis, specific binding of the scFv(FRP5) domain to ErbB2 as well as CD86 IgV binding to CTLA-4 could be demonstrated. Together with anti-CD3 antibody, BIg5 stimulates proliferation of human CD2-purified lymphocytes *in vitro*. After binding to ErbB2 on murine Renca-lacZ/ErbB2 tumor cells, about 50% of initially bound BIg5 is still present on the cell surface after 4 hours. For delivery of chimeric fusion proteins *in vivo*, we used syngeneic, stably transfected HC11 mammary epithelial cells continuously secreting the proteins. Inoculation of these bystander cells close to subcutaneously growing Renca-lacZ/ErbB2 tumors should provide a long-lasting source to achieve high local concentrations of BIg5 at the tumor site. *In vivo* HC11-BIg5 cells proved to be non-tumorigenic and secreted BIg5 for several weeks, causing a strong anti-BIg5 antibody response. Treatment of established Renca-lacZ/ErbB2 or ErbB2-negative Renca-lacZ tumors by peritumoral inoculation of either HC11-BIg5 or HC11-Ig5 cells led to rejection of all Renca-lacZ/ErbB2, but none of the Renca-lacZ tumors. HC11neo control cells had no effect on tumor growth. Rejection of $ErbB2^+$ tumors led to long-term protection also against subsequent challenge with intravenously injected $ErbB2^-$ tumor cells. Intraperitoneal injection of bystander cells secreting the fusion proteins did not lead to tumor regression suggesting that high local concentrations at the tumor site are necessary to target ErbB2 on tumor cells and to overcome elimination of BIg5 or Ig5 by neutralizing antibodies. The CD86 IgV domain of BIg5 did not play a major role in the observed antitumoral immune response suggesting NK-cell mediated ADCC as the initial effector mechanism followed by activation of tumor specific T cells. Targeting of ErbB2 on tumor cells with antibody fusion proteins that interact specifically with the host immune system could be an efficient and specific approach for therapy of solid $ErbB2^+$ tumors.

S2
Antibody-based immunoreceptors: the impact of signalling domain, binding affinity and costimulation.

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Cancer Cell International 2004, **4(Suppl 1):**S2

The immunoreceptor strategy is based on grafting of T cells with recombinant T cell receptor molecules that consist extracellularily of a scFv domain for MHC-independent antigen binding and intracellularily of the CD3 ζ- or FcεRI γ-signalling domain for cellular activation. Upon binding to antigen-positive cells, grafted T cells are activated, secrete IFN-γ, and lyse specifically antigen-positive, but not antigen-negative target cells. During the last years, we generated a panel of immunoreceptors with specficity for "tumor associated antigens" as targets for use in adoptive immunotherapy of malignant diseases: CEA, CA72-4, CA19-9 for gastrointestinal carcinomas, CD30 for Hodgkin's lymphoma and cutaneous T cell lymphoma, HMW-MAA and melanotransferrin for melanoma, and ErbB2 for a variety of carcinomas [1,2]. T cells taken from the peripheral blood of tumor patients and grafted with the appropriate immunoreceptor mediate a highly efficient immune response towards autologous, antigen expressing target cells *in vitro* [3].
One of the major advantages of the immunoreceptor strategy lies in the modular compositon of the receptor molecule. However, little is known about the impact of the individual receptor modules on cellular activation in a complex immunological context. We have identified several items that affect the efficacy of receptor mediated cellular activation including: the signalling domain that affects the stability of immunoreceptor expression and function in T cells [4]; the affinity of the scFv domain that

affects the efficacy of T cell activation; B7-1 and B7-2 costimulation that affects the quality of T cell activation [5–7].

The high complexity of the recognition and signalling process makes it unlikely that a universal configuration of the immunoreceptor exists. The design of the receptor molecule, however, has major impact on the stability and function in T cells and thereby on the efficacy of adoptive immunotherapy.

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S3

Tolerability and efficacy of the trifunctional antibody removab® (anti-EpCAM x anti-CD3) in patients with malignant ascites due to ovarian cancer: Results of a phase I/II study

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Introduction: Malignant ascites in patients with gynecological malignancies is associated with poor prognosis and poor quality of life. The bispecific trifunctional antibody removab® (anti-EpCAM x anti-CD3) belongs to a new class of intact antibodies that has been developed for targeted therapy of epithelial tumors. The two binding sites of removab® are directed against epithelial tumor cells (EpCAM$^+$) and T-cells (CD3$^+$) thus recruiting T-cells in the direct environment of tumor cells. Simultaneously, mediated by the intact Fc-fragment removab® binds to FcγI/IIIR$^+$ accessory cells (e.g. macrophages, natural killer cells, dendritic cells) that are mandatory for the induction of a tumor-specific immune response.

Patients and methods: In an open-label multicenter phase I/II-dose escalating study, a total of 23 patients with ovarian cancer and symptomatic ascites at FIGO stage III-IV were treated intraperitoneally with removab®. The patients had received a median of 3 (1–8) previous therapies. Their mean age was 62 (42–80) years. The treatment consisted of up to 5 intraperitoneal applications of the antibody within 13 days using increasing dosages.

Results: The intraperitoneal treatment with removab® was able to stop the production of ascites in 22 of 23 patients. These patients were ascites-free at the end of the study (day 37). Immunocytochemical quantification of tumor cells in the ascites fluid showed a dramatic reduction of EpCAM$^+$ cells (> log 5). In addition, clinically significant improvement of the quality of life was observed. The majority of adverse events was mild to moderate. The most common side effects observed in the study were fever (82.6%), nausea (60.9%), vomiting (56.5%), and abdominal pain (30.4%). The MDT (maximal tolerated dose) was reached at the increasing dosages of 10 μg–20 μg–50 μg–200 μg–200 μg.

Conclusion: In conclusion, intraperitoneal treatment with removab® was safe, well tolerated and showed encouraging efficacy in patients with malignant ascites due to ovarian cancer. Thus, the new concept of the anti-EpCAM x anti-CD3 antibody might offer a promising treatment option for patients with epithelial tumors.

S4

HLA DR-directed bispecific single-chain Fv antibodies for lymphoma therapy

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Cancer Cell International 2004, **4(Suppl 1):**S4

Fc receptors are important for the clinical efficacy of therapeutic antibodies. Bispecific antibodies (BsAb) are immunoglobulin-conjugates with two different binding specifities, targeting tumor antigens and effector cell trigger molecules. BsAb, produced by chemical coupling of one antibody against a tumor cell surface antigen with another against a Fc receptor, mediate effective interactions between effector and target cells.

Here, genetically coupled bispecific single chain Fv (bsscFv) were produced — as they easily enable further modifications of the molecule — directed against one of the effector cell antigens FcαRI(CD89)or FcγRIII(CD16) and against HLA class II or Lym-2. Lym-2 represents a variant form of the HLA-DR antigen and is highly expressed on the surface of malignant B cells, but only at low levels on normal cells. HLA class II and Lym-2 are both known as effective targets for effector cell-mediated lysis of malignant human B-lymphoid cells. CD89 is an interesting trigger molecule for BsAb therapy, as it recruits neutrophils as effector cells, which have tumor cytolytic potential against a broad spectrum of tumor cells and are the most abundant circulating blood leukocytes. Antibodies against CD16 have already shown biological activity *in vitro* and in tumor patients by recruting NK cells. The two component scFv were fused via a flexible 20aa linker. ScFv fragments were generated by producing phage display libraries from corresponding hybridomas, and screening the libraries with antigen-positive cells. Recombinant scFv against HLA class II, Lym-2, CD89 and CD16 were thus obtained from the hybridomas F3.3, Lym-2, A77 and 3G8 respectively. Functional bsscFv were expressed and secreted by insect cells and were purified via Nickel chelate chromatography. Purified BsAb reacted with HLA class II or Lym-2-positive target cells and one of the effector cell antigens, CD89 or CD16, respectivly. In ADCC experiments all constructs mediated specific lysis of HLA class II or Lym-2-

positive malignant human B-lymphoid cell lines with human MNC or PMN as effector cells. The [CD89 x HLA class II] and the [CD16 x HLA class II] bsscFv also mediated significant lysis of primary cells from patients with B-cell chronic lymphocytic leukaemia (B-CLL). In conclusion, these recombinant bsscFv may allow the specific recruitment of effector cells for an improved therapy in B-lymphoid malignancies.

S5

Increased frequency of CTLA4$^+$ TGFβ$^+$ CD4$^+$ CD25$^+$ T cells in peripheral blood of patients with chronic lymphatic leukemia and multiple myeloma

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A globally suppressed T cell function has been described for cancer patients including patients with chronic lymphatic leukemia (CLL) or multiple myeloma (MM). This has been mainly associated with inhibitory factors released by the tumor cells, while the role of recently characterized regulatory immune cells is not understood. Several different regulatory T cells such as CD4$^+$ CD25$^+$ T cells (T_{reg}), TGFβ producing TH3 or IL-10 producing TR1 cells have been described in murine models, and T_{reg} cells have also been implicated in the control of graft versus host disease after allogeneic transplantation in humans. In contrast, little is known about frequencies and function of regulatory cells in leukemias and lymphomas. To address this issue we have analyzed 82 peripheral blood samples from 24 CLL patients, 18 MM patients and 26 healthy individuals. By assessing CD4$^+$ CD25$^+$ T cells we established a strongly significant increase of this subpopulation in both CLL (13 ± 7% mean ± SD, $p < 0.01$) and MM (13 ± 9%, $p < 0.01$) when compared to healthy individuals (3.5 ± 1.5%). While CDC4$^+$ CD25$^+$ T cells could also comprise previously activated T cells, the expression of CTLA4 has been associated with T_{reg} cells. In fact, 80±14% respectively 67±26% of CD4$^+$ CD25$^+$ T cells in CLL resp. MM were also CTLA4$^+$, while only 30 ± 22% were found to be positive in healthy individuals strongly suggesting that these cells are mainly T_{reg} cells. In contrast, using the BDCA-4 specific antibody for Neuropilin-1, we were unable to detect this molecule recently described on murine T_{reg} cells on any of our samples. Interestingly, a significant proportion of CD4$^+$ CD25$^+$ T cells in CLL 28 ± 13% and MM 22 ± 12% also expressed intracellular TGFβ, which was only found in 6 ± 4% of these T cells in healthy individuals. Whether TGFβ production reflects a particular activation status of T_{reg} cells or whether these cells are a defined subpopulation requires further investigation. By analyzing co-expression of CCR7 and CD45RA we established that the fraction of CD4$^+$ CD25$^+$ T cells was particularly increased in the naïve and the central memory pool in peripheral blood of CLL and MM patients. Next we assessed T cell activation as a function of T_{reg} cells. As expected, there was already a significantly decreased proliferative response of CD4$^+$ T cells in many CLL patients even when CD25$^+$ cells were depleted. These findings are most likely explained by chronic exposure to inhibitory cytokines as well as T_{reg} cells. However, even under these conditions, coculture experiments of CD4$^+$ CD25$^-$ and CD4$^+$ CD25$^+$ T_{reg} cells supported the inhibitory role of T_{reg} cells in CLL. We therefore propose that immunotherapy in any malignancy including CLL and MM characterized by an increase of regulatory factors and cells will significantly benefit from strategies inhibiting immune repression.

Immune escape/NK cells

S6

MMP-7 inhibits CTL - tumor cell interaction

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MMP-7, the smallest member of the MMP-family, is a zinc-dependent metalloproteinase and is overexpressed in colon cancer and many other human cancers. Along with its prometastatic function, a fundamental role for MMP-7 has also been established in early tumor development, but the mechanism by which MMP-7 contributes to this, is still unknown.

Tumor specific cytotoxic T cells (CTLs) play a critical role in the control of tumor growth. They can induce apoptosis by CD95 as well as perforin/granzyme mediated pathways. Loss of CD95 may contribute to apoptosis resistance and immune escape of tumor cells leading to successful tumor outgrowth.

In our project we analyzed MMP-7 for its influence on CD95 mediated apoptosis and the cytolytic effector functions of CTLs. Furthermore we investigated the influence of MMP-7 cleavage activity on the adhesion and deadhesion of peptide specific CTL's to tumor targets.

In a cleavage assay with recombinant CD95 protein, we could show that MMP-7, cleaved approximately 2–3 kDa from the extracellular N-terminal end of CD95. In coculture experiments with ^{51}Cr-labeled HepG2-cells, we found a significant decrease of cytotoxic action of peptide specific CTLs in the presence of MMP-7. In addition MMP-7 leads to a higher adhesion of CTLs and inhibits their deadhesion from HepG2 cells. Considering that CTL's are serial killers, alteration in adhesion/deadhesion functions can be detrimental for tumor specific CTL killing.

Our results show, that MMP-7 can contribute to the apoptosis resistance of tumor cells by different mechanisms. These activities may explain the contribution of MMP-7 to early tumor growth.

S7

Genetically modified natural killer cells specifically recognizing the tumor-associated antigens ErbB2/HER2 and EpCAM

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The continuously growing natural killer (NK) cell line NK-92 is highly cytotoxic against malignant cells of various origin without affecting normal human cells. Based on this selectivity, the potential of NK-92 cells for adoptive therapy is currently being

investigated in phase I clinical studies. To further enhance the antitumoral activity of NK-92 cells and expand the range of tumor entities suitable for NK-92-based therapies, here by transduction with retroviral vectors we have generated genetically modified NK-92 cells expressing chimeric antigen receptors specific either for the tumor-associated ErbB2 (HER2/neu) antigen or the human Epithelial Cell Adhesion Molecule (Ep-CAM). Both antigens are overexpressed by many tumors of epithelial origin. The chimeric antigen receptors consist of either the ErbB2 specific scFv(FRP5) antibody fragment or the Ep-CAM specific scFv(MOC31), a flexible hinge region derived from CD8, and transmembrane and intracellular regions of the CD3 zeta chain.

Transduced NK-92-scFv(FRP5)-zeta or NK-92-scFv(MOC31)-zeta cells express high levels of the fusion proteins on the cell surface as determined by FACS analysis. In europium release assays no difference in cytotoxic activity of NK-92 and transduced NK-92 cells towards ErbB2 or Ep-CAM negative targets was found. However, even at low effector to target ratios transduced NK-92 cells specifically and efficiently lysed established ErbB2 or Ep-CAM expressing tumor cells that were completely resistant to cytolytic activity of parental NK-92 cells. Similarly, ErbB2-positive primary breast cancer cells isolated from pleural effusions of patients with recurrent disease were selectively killed by NK-92-scFv(FRP5)-zeta. In an *in vivo* model in immunodeficient mice treatment with retargeted NK-92-scFv(FRP5)-zeta, but not parental NK-92 cells resulted in markedly delayed growth of ErbB2 transformed cancer cells.

These results demonstrate that efficient retargeting of NK-92 cytotoxicity can be achieved, and might allow the generation of potent cell-based therapeutics for the treatment of ErbB2 and Ep-CAM expressing malignancies. This therapeutic approach might be applicable for a large variety of different cancers where suitable cell surface antigens have been identified.

S8

Rapid functional exhaustion and deletion of cytotoxic T lymphocytes following immunization with recombinant adenovirus

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Replication-deficient adenoviruses (rec-AdV) expressing different transgenes are widely employed vectors for gene therapy and vaccination. We examined here the generation of β-galactosidase (βgal)-specific CTL following administration of βgal-recombinant adenovirus (Ad-LacZ). Using MHC class I tetramers to track βgal-specific CTL in different organs, we found that a significant expansion of βgal-specific CTL could only be achieved in a very narrow dose range ($2 \times 10^8 - 2 \times 10^9$ pfu). Functional analysis revealed that adenovirus-induced βgal-specific CTL produced only very low amounts of effector cytokines and were unable to lyse βgal peptide-pulsed target cells. Injection of optimal doses of Ad-LacZ into transgenic mice which express βgal exclusively in non-lymphoid organs, led to physical deletion of βgal-specific CTL. Our results indicate first that CTL deletion in the course of adenoviral vaccination is preceded by their functional impairment and second, that the outcome of rec-AdV vaccination depends critically on the antigen load in peripheral tissues. The presented findings thus impinge on the rationale to use adenoviral vectors in clinical vaccination.

S9

High frequency of functionally active Melan-A specific T cells in a patient with progressive immunoproteasome-deficient melanoma

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Tumor-reactive T cells play an important role in cancer immunosurveillance. Applying the multimer technology, we report here an unexpected high frequency of Melan-A-specific CTL in a melanoma patient with progressive lymph node (LN) metastases, consisting of 18% and 12.8% of total peripheral blood and tumor-infiltrating $CD8^+$ T cells, respectively. Melan-A-specific CTL revealed a high cytotoxic activity against allogeneic Melan-A-expressing target cells but failed to kill the autologous tumor cells. Loading of the tumor cells with Melan-A peptide reversed the resistance to killing, suggesting impaired function of the MHC class I Ag processing and presentation pathway. Mutations and/or down-regulation of the MHC class I heavy chain, the antigenic peptide TAP, and tapasin could be excluded. However, RT-PCR and immunohistochemical analysis revealed a deficiency of the immunoproteasomes low molecular weight protein (LMP)2 and LMP7 in the primary tumor cells, that affects the quantity and quality of generated T cell epitopes and might explain the resistance to killing. Overall, this is the first report of an extremely high frequency of tumor-specific CTL that exhibit competent T cell effector functions, but fail to lyse the autologous tumor cells. Immunotherapeutic approaches should not only focus on the induction of a robust anti-tumor immune response, but also have to target tumor immune escape mechanisms.

ADOPTIVE T CELL TRANSFER

S10

Adoptive T cell therapy using antigen-specific $CD8^+$ T cells for the treatment of patients with metastatic melanoma: a phase I clinical study

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The adoptive transfer of *in vitro* induced and expanded tumor antigen-specific cytotoxic T lymphocytes (CTL) provides a

promising approach to the immunotherpy of cancer. We have previously shown that Melan-A-specific CTL can be generated from HLA-A2.1$^+$ melanoma patients by 4 rounds of *in vitro* stimulation of purified CD8$^+$ T cells with autologous dendritic cells pulsed with a mutated HLA-A2 binding Melan-A (ELAGIGILTV) peptide. Based on these results we have initiated a pilot study of adoptive T cell therapy in advanced melanoma patients demonstrating that *in vitro* generated Melan-A specific CTL survive intact *in vivo* for several weeks and localize preferentially to tumor (Meidenbauer *et al*., *J Immunol* 2003, **170:**2161, 2003). Here we report on the clinical results of a phase I study of 12 HLA-A2$^+$ melanoma patients that received at least three i.v. infusions of Melan-A-specific CTL i.v. at 2-week intervals. Each T cell infusion was accompanied by a 6-day course of s.c. IL-2 (3 × 10^6 IU daily). A total of 51 T-cell infusions were administered, averaging 1.48 × 10^8 Melan-A multimer$^+$ T cells per infusion, with a range from 0.11 – 6.58 × 10^8 Melan-A-specific T cells per infusion. Clinical side effects were mild and consisted of chills and low-grade fever (WHO grade I-II) in 8 out of 12 patients that typically occurred within 6 to 8 h post infusion. Hematological effects, observed after T cell transfer, consisted of an increase in eosinophils up to 30% in 7 out of 12 patients, peaking 24h post transfer. Clinical and immunological responses consisted of antitumor responses in 3 out of 12 patients (2 PR, 1 mixed response), an elevated frequency of circulating Melan-A multimer$^+$ T cells up to 2% of total CD8$^+$ T cells up to 14 days post transfer, suggesting long-term survival and/or proliferation of transferred CTL, and a complete loss of Melan-A expression in lymph node metastases of 2 patients after T cell transfer. Our data indicate that the adoptive transfer of antigen-specific T cells in melanoma patients is capable of inducing clinical and systemic tumor-specific immune responses without provoking major side effects.

S11

Large scale *in vitro* expansion of polyclonal human CD4$^+$CD25^{high} regulatory T cells

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CD4$^+$CD25$^+$ regulatory T (T$_{reg}$) cells are pivotal for the maintenance of self-tolerance and their adoptive transfer protects from autoimmune diseases and pathogenic alloresponses after solid organ or bone marrow transplantation in murine model systems. *In vitro*, human CD4$^+$CD25$^+$ T$_{reg}$ cells display similar phenotypic and functional characteristics as murine CD4$^+$CD25$^+$ T$_{reg}$ cells, namely hyporesponsiveness to TCR stimulation and suppression of CD25$^-$ T cells. Thus far, the detailed characterization and potential clinical application of human CD4$^+$CD25$^+$ T$_{reg}$ cells was hampered by their paucity in peripheral blood and the lack of appropriate expansion protocols. Here we describe the up to 40,000-fold expansion of highly purified human CD4$^+$CD25^{high} T cells *in vitro* through the use of artificial APC for repeated stimulation via CD3 and CD28 in the presence of high dose IL-2. Expanded CD4$^+$CD25^{high} T cells were polyclonal, maintained their phenotype, exceeded the suppressive activity of freshly isolated CD4$^+$CD25^{high} T cells and showed characteristics of central memory T cells. The ability to rapidly expand human CD4$^+$CD25^{high} T$_{reg}$ cells large scale will not only facilitate their further exploration but also accelerate their potential clinical application in T cell-mediated diseases and transplantation medicine.

S12

Reversible HLA multimers (streptamers) for isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens

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Attempts to treat patients with tumor-reactive or viral-specific cytotoxic T lymphocytes (CTLs) have been limited due to the difficulty of isolating and expanding functionally active T cells present in low numbers in the peripheral blood. Recently developed MHC/peptide multimers mimic T cell receptor (TCR) ligands and, therefore, allow visualization and isolation of antigen-specific CTLs. However, the persistence of multimers leads to prolonged TCR signaling and subsequently to overstimulation and cell death. We have generated a new type of MHC/peptide multimers, termed streptamers, which can be dissociated from the TCR. In the mouse model, the dissociation of streptamers from the TCR, prevents T cells from multimer-induced cell death (Knabel *et al. Nature Med* 2002, **8:**631). In this study, we investigate the efficacy of reversible HLA/peptide multimers for isolation of human antigen-specific T cells. Melan-A and CMV have been chosen as representative tumor-associated and viral antigen (Ag), respectively. Specificity and reversibility of A2/CMV and A2/Melan-A streptamers was documented by staining of Ag-specific T cell clones and loss of staining after streptamer removal. Streptamer-stained Ag-specific T cells remained functionally active following dissociation, whereas lytic function of T cells was impaired in the presence of non-reversible multimers (tetramers). Furthermore, CMVpp65(495–503)-specific T cells were streptamer- or tetramer-sorted from HLA-A2-positive, CMV-seropositive donors either directly out of the blood or following repetitive peptide stimulations *in vitro*. Both attempts successfully led to the isolation of CMV-specific CTLs that were cloned by limiting dilution. Clonal proliferation was superior for CMV-specific streptamer-sorted T cells compared to tetramer-sorted T cells. CMV-specific T cell clones isolated with streptamers and tetramers displayed a similar TCR repertoire and avidity. Growing CTL clones were capable of lysing

CMVpp65(495–503)-pulsed as well as CMVpp65-transfected HLA-A2-positive target cells. For isolation of melanoma-reactive CTLs, the modified decapeptide Melan-A(26–35)A27L was chosen to construct streptamers respective tetramers. Again, streptamer-sorted Melan-A-specific CTL clones proliferated better than tetramer-sorted CTL clones. The isolated Melan-A-specific CTL clones displayed different TCR motifs, which can be

explained by the broad repertoire of Melan-A-specific T cells physiologically present *in vivo*. All Melan-A(26–35)A27L-specific CTL clones crossreacted with the naturally processed peptide Melan-A(27–35), but only some CTL clones lysed HLA-A2-matched, Melan-A-expressing melanoma cells. Of note, tumor recognition by some streptamer-sorted
CTL clones was superior to tumor lysis by tetramer-sorted CTL clones. Our current experiments focus on the isolation of T cells using reversible multimers coupled with microbeads allowing us to sort antigen-specific T cells under the guidelines of good manufacturing practice. Clinical goal is the adoptive transfer of antigen-specific T lymphocytes for treatment of patients with cancer or infectious diseases.

S13
Therapeutic efficacy of RAGE-1 and MAGE-9 peptide specific cytotoxic T cell clones in renal cell carcinoma

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Renal cell carcinoma (RCC) are supposed to be immunogenic and several clinical trials of immunotherapy using tumor-lysate pulsed dendritic cells have been performed. We here report on the generation and therapeutic efficacy of RAGE-1 and MAGE-9 peptide specific cytotoxic T cell clones.
RAGE-1 and MAGE-9 are expressed on 61% and 40% of RCC. Six MAGE-9 and 14 RAGE-1 derived peptides were found to be immunogenic in the context of the HLA-A2.1 MHC complex. CTL were generated by co-culture with peptide pulsed dendritic cells (DC) or peptide pulsed CD40-activated B cells. The latter are as efficient in antigen presentation as DC, but have the advantage of being easier to generate in great quantitities and to display functional activity for a prolonged period of time. Therefore, they are well suited for the generation of CTL clones to be used in adoptive transfer. Three MAGE-9 and RAGE-1 specific CTL clones were generated. The clones were strictly peptide-specific and displayed high cytotoxic activity not only against peptide-loaded T2 cells, but also against HLA-A2.1-positive RCC lines that naturally expressed MAGE-9, RAGE-1 or both. The *in vivo* efficacy of these MAGE-9 and RAGE-1 peptide specific CTL clones is currently being evaluated in human RCC-bearing SCID, which received after intraperitoneal RCC injection repeated applications of the CTL clones at weekly intervals. So far, the majority of control mice became moribund, but only one mouse receiving a MAGE-9 and a RAGE-1 specific CTL developed a tumor, that had retained MHC class I as well as MAGE-9 and RAGE-1 expression. CTL recovered from these mice at 2 and 3 weeks after the last T cell transfer revealed that CTL had retained their specificity and cytotoxic activity was only slightly reduced.
Thus, B cells appear well suited as antigen presenting cells for the generation of large quantities tumor peptide specific CTL as required for adoptive transfer and cancer testis antigens may well provide suitable target for immunotherapy of RCC.

S14
The role of glycosylation as a modulator for signalling strength in T cells

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Adoptive immunotherapy has proven efficiency in patients suffering from malignancies. However, the quality of *in vitro* generated tumor antigen-specific T cells which can be used for such therapies is limited. Therefore, methods have to be developed in order to increase potency of these tumor antigen-specific T lymphocytes. One possibility arises from the modification of glycoproteins such as the T cell receptor (TCR) or CD8. By their size and charge, sugar chains of these molecules play a role in the interaction of a T cell with the major histocompatibility complex (MHC). It is also reported that glycosylation decreases mobility of cell surface molecules by cross linking to carbohydrate binding proteins (so called lectins, e.g. Galectin-3). The resulting molecular lattice constrains TCR and CD8 clustering. Deglycosylation of the TCR and CD8 might be a possibility in order to increase mobility of the TCR or CD8 and to augment signalling strength. Unspecific global O- and N-deglycosylation were compared as well as a specific removal of selective glycosylation motives in defined proteins as the TCR.
For exogenous deglycosylation, T cells were treated with neuraminidase, which removes sialic acids at the end of sugar chains. This resulted in a better communication of the O-glycosylated CD8 with the MHC I molecule which was demonstrated by an increased specific tetramer-binding. Surprisingly, the ability to kill tumor cells was not increased. To inhibit the interaction of N-linked sugars of the TCR with Galectin-3 and so to increase mobility of the TCR, T cells were treated with lactose, which competes with the Galectin-binding sugars for Galectin-3. However, lactose-treatment did also not ameliorate target cell lysis. Thus, T cell deglycosylation could increase tetramer-binding, but this was not translated into a better T cell activity. We assumed, that other surface molecules which are important for signalling were negatively affected by these treatments. As consequence, we silenced N-glycosylation motives in the TCR molecule itself by point mutation. Wild-type and mutant TCRs were transduced into human T cells or the murine T cell hybridoma 58a-b- by retroviral gene transfer. Efficacy of TCR deglycosylation was ascertained by western blot analysis. Removing one or two defined sugar chains increased antigen-specific cytokine secretion. Deletion of three or more glycosylation sites impaired T cell function.
In summary, T cell deglycosylation is a sensitive mechanism. Deletion of defined glycosylation motives within a TCR is an efficient tool to increase signalling strength of a T cell.

respectively. Induction of high frequency T cell responses was detected in 3 of 4 patients with up to 0.92%, 0.43%, and 0.42% in peripheral blood and up to 0.8% in bone marrow by tetramer analysis. Detailed phenotypical analysis in patient 1 showed that WT1-specific peripheral blood T cells were almost exclusively CD45RA+CCR7-granzymeB+, and directly produced IFNγ in response to WT1 peptide, resembling cytotoxic effector T cells, while in the bone marrow both WT1-specific effector and CD45RA-CCR7- effector memory T cells were found. Patient 3 and 4 had progressive disease after 5 vaccinations. Patient 1 who had progressed during the first 4 weeks of vaccination with an increase of blasts to 30% in bone marrow was induced into complete remission after 6 vaccinations, which lasted for 12 months. Patient 2 is in continuous remission for currently 20 months. WT1 transcripts in bone marrow and peripheral blood were quantitated by PCR to monitor residual disease. In accordance with the clinical course in patients 1 and 2 we observed an approximately 100-fold, and 50-fold reduction, respectively, of WT1 transcripts following vaccination. No side effects as those typically seen with GM-CSF were noted. Taken together, these findings underline the efficacy of the vaccine, inducing high frequency WT1-specific effector and memory T cells in peripheral blood and bone marrow associated with complete remission of leukemia in the absence of hematological or renal toxicity.

S18

LUD00-014: Phase I Study of recombinant vaccinia-NY-ESO-1 (rV-NY-ESO-1) and recombinant fowlpox-NY-ESO-1 (rF-NY-ESO-1) in patients with NY-ESO-1 or LAGE positive cancers

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Objectives: To determine the toxicity and NY-ESO-1-specific immune responses induced by immunization with rV-NY-ESO-1 or rF-NY-ESO-1.

Study design: Eligible patients (pts) for the first part of the study were HLA-A2$^+$ with advanced NY-ESO-1/LAGE$^+$ tumors assessed by RT-PCR or immunohistochemistry. For the second part, pts were enrolled irrespective of the HLA type. Four injections of rV-NY-ESO-1, 3.1 × 10^7pfu i.d., or rF-NY-ESO-1, 7.41 × 10^7 pfu s.c., were administered four times at 4 week intervals in part 1. Pts enrolled to part 2 received 2 injections of rV-NY-ESO-1, 3.1 × 10^7pfu i.d., followed by multiple injections of rF-NY-ESO-1, 7.41 × 10^7 at monthly intervals in case there was no evidence for progressive disease during vaccination. Toxicity was evaluated every 2 weeks. Delayed-type hypersensitivity (DTH) against HLA-A2 binding NY-ESO-1 peptides was assessed at baseline and after the last vaccination in HLA-A2+ pts. Immunogenicity was assessed by ELISPOT and tetramer analysis against HLA-A2 binding NY-ESO-1 peptides.

Results: 29 pts with different types of cancer were enrolled, 9 pts are ongoing, 11 completed at least 4 cycles of treatment, 9 were withdrawn for disease progression or at their own discretion. No remarkable toxicities occured. Positive post-vaccination DTH reactions against 2 HLA-A2 binding NY-ESO-1 peptides occurred in 6/6 pts tested. At baseline all HLA-A2+ pts of part 1 were NY-ESO-1 serum antibody- and CD8$^+$ T-cell negative. In 2 pts a conversion of NY-ESO-1 serum antibody was observed after 3 vaccinations. In 9 pts for whom immune assay data are available, NY-ESO-1 specific CD8$^+$ T-cell responses were induced during vaccination. After 4 injections maximum ELISPOTS ranged between 160-800 per 50,000 CD8$^+$ T-cells in the 2 vaccinia pts and 100–500 in the 2 fowlpox pts as confirmed by tetramer assays. At 4 months 14 pts were found to have stable disease. 1 pt experienced a partial remission of subcutaneous and peritoneal melanoma metastases after 3 months of immunization. Assessments are underway for NY-ESO-1 specific T cell responses restricted by non-HLA-A2 and HLA class II alleles.

Conclusion: Immunization with recombinant vaccinia- and fowlpox-NY-ESO-1 is safe and induces NY-ESO-1 specific immune responses.

S19

Tumor vaccination after allogeneic bone marrow cell reconstitution of the non-myeloablatively conditioned tumor-bearing murine host

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Allogeneic bone marrow cell (BMC) reconstitution of the non-myeloablatively conditioned host is supposed to provide an optimized platform for tumor vaccination. We recently showed that an allogeneic T cell-depleted graft was well accepted if the tumor-bearing host was NK-depleted. Based on this finding a vaccination protocol in tumor-bearing, non-myeloablatively conditioned, allogeneically reconstituted mice was elaborated. Vaccination was most efficient when allogeneically reconstituted, tumor-bearing mice received tumor-primed, donor T cells, which had matured in the allogeneic host together with host-derived tumor lysate-pulsed dendritic cells. High frequencies of tumor-specific proliferating and cytotoxic T cells were recorded, the survival time of tumor-bearing mice was significantly prolonged and in over 50% of mice the tumor was completely rejected. Notably, GvH was not aggravated after vaccination with tumor-primed, donor-derived T cells that had matured in the host, i.e. T cells were tolerant towards the host, but not towards the tumor. The finding convincingly demonstrates the feasibility and efficacy of tumor vaccination of the allogeneically reconstituted, non-myeloablatively conditioned host after establishment of thymic tolerance and ceasing of initial GvH reactivities. Furthermore, they support the working hypothesis that reconstitution protocols favoring tolerance induction, which are time consuming, rather than a rapid establishment of full donor chimersim may allow more efficient tumor vaccination without potentially refreshing GvH reactivites.

POSTER PRESENTATIONS

S20
Limiting the risk of immunogenicity by identification and removal of T-cell epitopes (DeImmunisation™)

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Immunogenicity of non-human proteins such as plant derived toxin molecules and bacterial enzymes provide an almost complete barrier to their clinical implementation as novel therapeutic strategies for cancer. It is also recognised that the immunogenicity associated with therapeutic antibodies and certain human proteins can also limit their clinical effectiveness. For therapeutic antibodies a number of engineering options exist, and clearly have in part been developed to address the issue of immunogenicity. Examples include "classic" humanisation by CDR engraftment, use of human Ig libraries such as phage display systems and human Ig transgenic mice. However, in each case there should be an expectation that these can all result in antibodies still capable of eliciting an immune response in certain patients. This is connected to the process of affinity maturation during antibody generation that inevitably results in antibodies to which patients may not be naturally tolerant. Underlying this process is the presentation of T-helper peptide epitopes derived from the intracellular processing of the therapeutic antibody. Foreign T-cell epitopes are also present in plant toxins and bacterial enzymes and will cause a sustained immune response and the generation of immunological memory.

Our approach at Biovation has been the development of what we have termed DeImmunisation™ technology. This involves the identification and removal of helper T-cell epitopes from therapeutic antibody and protein candidates. We use *ex-vivo* human T-cell assays in a variety of formats to identify the T-cell epitopes, to direct the substitution of residues within epitopes and also to validate successful removal of epitopes from the protein.

Our start point is to use naïve T-cell assays to provide an outline epitope map of the protein of interest. T-cell assays are performed using healthy human PBMC preparations in MHC selected donor panels and synthetic peptide antigens. The epitope map can be further refined using the enzyme-linked immunospot (ELISPOT) assay to measure the frequencies of activated T-cells according to their particular cytokine profile. We use a software tool termed Peptide Threading to model the effect of amino acid substitutions on the ability of a T-cell epitope sequence to interact with multiple different MHC class II allotypes. This can guide the design of variant sequences and in some studies, the design phase is augmented by fine scale mapping of individual epitopes using alanine scanning mutational analysis. Antibodies and proteins engineered using this approach are tested for an improved immunogenicity profile using time course T-cell assays and selected donor panels.

DeImmunisation™ is widely applicable and we have engineered a number of therapeutic antibody and protein candidates some of which are now entering clinical trial. One such antibody in clinical development targets prostate specific membrane antigen (PSMA) and has shown no evidence of immunogenicity in >100 patients. Data will be presented on this example and other studies from our therapeutic antibody programmes and Fc-linked cytokine projects.

S21
Proteasome inhibition enhances HLA-class-II antibody-dependent polymorphonuclear cell mediated cytotoxicity against B-cell lymphoma

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Monoclonal antibodies (mAb) complemented the therapy of malignant lymphomas. Currently, mAb against CD20 or CD52 are approved and CD22 or HLA-class-II mAb are in clinical trials for the treatment for malignant B-cell lymphomas. Mechanism of action *in vivo* is not yet completely understood, but direct induction of apoptosis, activation of complement, and Fc-receptor dependent mechanisms including antibody-dependent-cellular-cytotoxicity (ADCC) seem to contribute to their clinical efficacy. At the same time proteasome inhibitors (PI) were developed as a novel class of anti-neoplastic agents. Their proposed mechanism of action include inhibition of the NFκB pathway by blocking the degradation of IκB in the proteasome, overriding of Bcl-2 mediated resistance in lymphoma cells and induction of apoptosis. MG262 (Z-Leu-Leu-Leu- $B(OH)_2$) is a PI belonging to the peptide boronate class of PIs including the clinically successfull PI Bortezomib (Velcade), which is approved as third line treatment of multiple myeloma.

We have previously demonstrated that polymorphonuclear granulocytes (PMN) are potent effector cells mediating ADCC against a wide range of malignant B-cell opsonized with HLA-class-II mAb. In the presence of suboptimal concentrations of HLA-class-II directed mAb F3.3 isolated PMN mediated modest amounts of cytotoxicity in Cr-release assays, which were significantly enhanced in the presence of the PI MG262 (31 ± 12%, 15 ± 3%, 68 ± 8% specific lysis for F3.3, MG262 and F3.3+MG262). In the absence of PMN as effector cells mAb F3.3 at the suboptimal concentration of 0.01μg/mL elicited no significant cytotoxicity, whereas MG262 mediated concentration dependent target cell death up to 15 ± 3% specific lysis at 2.5μM.

However, whereas PMN mediated significant lysis with HLA-class-II mAb, no significant lysis was observed with CD20, CD22 or CD52 directed antibodies. The promising results with the combination of HLA-class-II mAb and PI encourage us to extend our experiments to the combination of PIs, including bortezomib, with Rituximab (CD20), Epratzumab (CD22) or Alemtuzumab (CD52) mAb mediated cytotoxicity using PMN as effector cells.

S22
Increased effector functions of a monoclonal antibody by glycoform engineering

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Introduction: Igeneon is currently testing a humanized monoclonal antibody specific for Lewis Y (IGN311) in clinical

trials of passive cancer immunotherapy. The aim of the current study is to enhance the effector function of this antibody by genetically modifying the antibody producing cell line to express the glycosyl transferase GnTIII. The presence of this enzyme leads to glycosylation of the antibody with a bi-secting N-acetyl-glucosamine group and the absence of core fucosylation.

Methods: First, heavy and light chain genes of IGN311 were isolated, cloned into an expression vector and transfected transiently into EBNA cells: Genes for GnTIII tranferase expression were co-transfected resulting in a new expression product; an antibody, now called IGN312, with modified N-glycosylation pattern. A control wild-type antibody IGN311 wt. was expressed using exactly the same expression vectors and the same host but without co-transfection of genes for GnTIII expression. Both expression products were purified to homogeneity using an identical protein-A based down stream process. Expression products were characterized by SDS-PAGE, IEF and a target antigen specific sandwich ELISA.

Results: No degradation products could be detected and target affinity of the glyco-engineered antibody as well as assembling of heavy and light chains was not affected by GnTIII expression. *In vitro* experiments showed an up to 25 fold increased ADCC lysis activity of the glyco-engineered antibody IGN312 in comparison to the wild type expression product using six Lewis Y positive target cancer cell lines (SKBR5, SKBR3, LoVo, MCF7, OVCAR3 and Kato III). However, CDC activity measured on SKBR5 target cell line was 40% reduced. The reduction of CDC activity could be prevented by using a slightly different molecular-biological approach for increased levels of complex N-linked oligosaccharides of bisected, non-fucosylated type. With this approach, it could be shown to increase ADCC activity without reducing CDC activity. In fact, in this particular case the CDC activity was even 2- fold enhanced. Binding activity for this second-generation glyco-engineered antibody measured by specific sandwich ELISA was not affected.

Conclusions: Next steps will be the generation of a stable IGN312 expressing cell line that produces an antibody with enhanced effector functions. The long term medical and economical goal will be that, due to the enhanced potency of IGN312, the minimal effective dose needed for successful treatment of patients with epithelial Lewis Y expressing, cancers can be reduced significantly.

S23

Inhibition of signaling via erbB-receptors by antibodies that target the Lewis Y-antigen

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Introduction: IGN311 is a humanized monoclonal IgG1 antibody that binds to the Lewis Y (LeY) carbohydrate overexpressed on epithelial cancers. IGN311 potently mediates human effector functions (ADCC, CDC). The overexpression of fucosyltransferases in tumor cells also results in glycosylation of cell surface receptors by LeY. The anti-LeY antibodies IGN311 and its murine parent version ABL 364 therefore might influence the signaling in tumor cells via inhibition of growth factor receptor functions.

Methods: The present study was designed to provide a proof of concept *in vitro*: Two human tumor cell lines, A431 and SKBR3 (vulval carcinoma and breast cancer, respectively) were propagated under standard cell culture conditions, rendered quiescent by serum starvation and stimulated by EGF or heregulin. Cellular effector regulation, growth factor receptor expression and LeY expression were examined.

Results: Both ABL 364 and IGN311 blocked the stimulation of MAP kinase by EGF and heregulin in SKBR3 and A431 cells. The effect was comparable in magnitude with that of trastuzumab and was apparently non-competitive with respect to EGF. Finally, IGN311 and ABL 364 inhibited EGF-stimulated [3H]thymidine incorporation in A431 cells.

Conclusions: Taken together, the observations show that antibodies against carbohydrate determinants of erbB-familiy members are capable of blocking signaling. The clinical profiling of the humanized monoclonal anti Lewis Y antibody IGN311 has been initiated. Presently a Phase I dose-escalation trial in cancer patients is ongoing.

S24

Release of iC3b from apoptotic pancreatic tumor cells induces tolerance by binding to immature dendritic cells

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Background & Aims: Chemo- as well as immunotherapeutical approaches induce apoptosis in tumor cells. Apoptotic cells are known to activate homologous complement and to be opsonized with iC3b. Since maturation of dendritic cells (DC) can be inhibited by binding of iC3b to the complement receptor 3 (CR3, CD11b/CD18) and because immature DC induce tolerance, we investigated the induction of tolerance after pulsing DC with apoptotic cells in the presence or absence of native serum.

Methods: Apoptosis in pancreatic carcinoma cells was induced either by heat-stress, chemotherapy or anti-Her2 antibody. Monocyte-derived DC were pulsed with apoptotic cells with or without native serum. DC were analyzed for the maturation state by flow cytometry and the cytotoxic activity was determined. Tolerance was prevented by addition of substances such as anti CD11b or *N*-acetyl-D-glucosamine (NADG) which block iC3b binding to CR3.

Results: All of the former strategies for apoptosis induction resulted in iC3b release. Pulsing DC with apoptotic cells in the presence of serum prevents maturation of DC and induces finally tolerance. This tolerance could be prevented almost completely by blocking the interaction of iC3b with the CR3 receptor.

S25

Bisphosphonic acid acts as Gamma/Delta T cell activating antigen and has direct cytotoxic activity against pancreatic carcinoma cells

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Background: T cells bearing the Gamma9/Delta2 T cell receptor (TCR) constitute two to ten percent of peripheral blood

T lymphocytes. They have recently raised much interest as non-MHC restricted effector cells against a variety of tumors. Gamma/Delta T cells are known to be stimulated by phosphoantigens without the need of professional antigen presenting cells. Furthermore, it is described that incubation with phosphoantigens increases their proliferation rate rapidly.

Materials & Methods: Apoptotic and anti-proliferative effects of two bisphosphonates (pamidronate and zoledronic acid) against eight different ductal pancreatic carcinoma cell lines were measured by Annexin-V/PI stain and MTT assay. Gamma/Delta T cells were enriched from peripheral blood of healthy donors and expanded by stimulation with anti-CD3 and IL-2. Cytotoxic activity of Gamma/Delta T cells of age of 14 days was tested against these cell lines. In the next step, we pulsed tumor cells prior to the ^{51}Cr release assay with bisphosphonates.

Results: Zoledronic acid induced even at lower concentrations inhibition of proliferation. Incubation with a 3 μM solution inhibits proliferation to 11–93%. Cell lines susceptible for this treatment had a higher apoptosis rate. Gamma/Delta T cells showed cytotoxic activity against pancreatic cell lines (cell lysis of 24–37% at an effector to target ratio of 80:1). Inhibition of proliferation correlated significantly with susceptibility against Gamma/Delta T cells ($P < 0.003$). Pulsing of target cells with bisphosphonates prior to the cytotoxicity assay increased the lysis rate (41–87%).

Discussion: Zoledronic acid has even at concentrations which could be achieved by normal dosage an anti-proliferative and apoptotic effect. Cell lines which are susceptible for bisphosphonates were also susceptible for treatment with Gamma/Delta T cells. The efficacy of Gamma/Delta T cells could be further enhanced by pulsing tumor cells with bisphosphonates.

Conclusion: At least for some pancreatic carcinoma cells a bisphosphonate-based therapy maybe useful, particular in combination with adoptive transfer of Gamma/Delta T cells

S26
Regulation of p53 tumor specific immune responses in colorectal cancer patients

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Anti-tumor specific immune responses are modulated during tumor development via different escape mechanisms abrogating the process of effective immunological tumor destruction. In this study we analyzed the p53 specific immune response in colorectal cancer patients (n = 24) depending on their UICC stage and characterized their regulatory T cell functions independent of the p53 mutational status.

Peripheral blood lymphocytes (PBMCs) from patients (UICC-stage I–IV) were stimulated with 10 pools of synthetic overlapping p53 peptides encompassing the full length wild-type (wt) p53 sequence and Il-10 and IFN-γ expression was assessed (ELISA and ELISPOT). PBMCs and tumor specific cells as well as tumor specimens of those patients were further characterized (cytospins, immunofluorescence/histology) and the expression of the following gene classes were analyzed: CD4, CD25, Foxp3, GITR, and GATA-3. After stimulation of the lymphocytes with the peptide pools distinct residues were found that induced a Th2 (IL-10, n = 24) or Th1 (IFN-γ, n = 10) type response. T cells from patients in UICC III (n = 7) and IV (n = 7) expressed higher IL-10 levels in response to p53 peptide (residues 291–330) than patients in UICC I and II (n = 6) (26 and 62 spots/10^5 cells versus 14 spots/10^5 cells, respectively), indicating that the UICC stage may play a crucial role in IL-10 production in response to p53 peptides. In contrast, other p53 peptides (residues 331–370) led to IFN-γ production but no correlation was observed between the UICC stage and the Th1 response. Markedly elevated amounts of CD4+ CD25+ cells in the PBMCs, as well as intensified staining for p53 (clone DO-7) within the tumor were observed in patients expressing higher levels of IL-10. Comparison of all tumor tissues using hierarchical clustering analysis showed a Th2 gene pattern. Dissimilarity between the tissues was due to differences in the tumor stage. Within the whole p53 protein sequence comparably more determinants inducing a Th2 type immune response were observed suggesting that the type of the tumor specific immune response to p53 depends on presentation and recognition of specific wt p53 residues. Furthermore, the level of IL-10 production seems to overweigh the IFN-γ production indicating that specific p53 epitopes may directly influence the outcome of immunological surveillance in colorectal cancer patients. This study offers new insights in a possible mechanism facilitating the tumor to escape immune surveillance by inducing rather a Th2 type (tolerance) than Th1 type response (destruction) through p53 overexpression.

S27
Assessment of genes associated with immune activation and regulation in pancreatic cancer patients

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Pancreatic adenocarcinoma is among the most fatal of gastrointestinal cancers. Recent *in vitro* studies showed that tumors are able to develop several escape mechanisms. We hypothesized that regulatory T cells (Treg) may impair anti-tumor immune responses. Therefore, we investigated the role of immune-mediated pathways in invasive pancreatic cancer.

Blood and tumor samples were analyzed in 14 patients undergoing surgery for pancreatic adenocarcinoma (UICC stages II to IV) by ELISA, immunohistology, and real-time RT-PCR techniques. The expression, distribution, and protein products of the following gene classes were analyzed: Treg (CD4, CD25, CD8, IDO, Foxp3), co-stimulatory molecules (CD28, CTLA-4), transcription factors (GITR, GATA-3), apoptotic markers (Bcl-2, Fas, FasL), cytokines (IFN-γ, IL-6/10), tumor suppressors (p53, APC), and tumor marker (CEA).

Immunohistogical/-fluorescence doublestaining corresponded with the PCR results for the various markers (CD4+CD25+,

CD8+CD28+, Bcl-2, Fas/FasL, p53/CD4+, CEA/CD4+, APC). Using hierarchical clustering methods the comparison of all tumor tissues revealed two samples with a highly different gene expression pattern. The dissimilarity was associated with the UICC stage of the patients. Compared to UICC III or IV, stage II showed a significantly lower expression of most genes (14 out of 19). The other characteristic difference in the gene expression pattern of the individual sample was due to the localization of the analyzed tissue within the tumor. Samples from the tumor center showed a markedly lower expression of the immune-response related genes than samples from the tumor border line. Gene expression related to immune activation and regulation was low (CD69<Foxp3 & CTLA-4<CD28 & IFN-γ & IL-10 & CD4<IDO & CD25 & GATA-3<Fas & IL-6) in all samples obtained from the tumor center. The anti-apoptotic genes Bcl-2 and GITR showed a higher expression than the pro-apoptotic genes Fas and FasL. The highest expression was measured for APC>CEA>Bcl-2>CD8. IFN-γ & CTLA4 & CD28; CD4 & IL-10 & Foxp3 & CD25; and Bcl-2 & APC were clustered together analyzing the patterns of gene expression among the different samples.

Invasive pancreatic carcinomas show relatively low levels in expression of genes associated with immune activation and regulation. This might indicate insufficient anti-tumor immune responses against the tumor. In addition, gene expression profiles differ significantly between samples obtained from the tumor center versus the tumor border line.

S28
Modulation of tumour directed B cell responses by NK cells

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Background and aim: Spontaneous natural killer cell (NK cell) activity against malignant cells is important for development of tumour directed Th1 biased and cytotoxic T cell (CTL) responses (Kelly *et al., Nature* 2002; Geldhof *et al, Blood* 2002) resulting in protection from tumour growth in mouse models. Aim of our investigations was to study the significance of NK cell activity on tumour directed B cell immunity.

Material and methods: Tumour directed B-cell responses were studied in C57Bl/6 mice, NK cells depleted in the experimental group by i.v. injection of anti-ASGM1 antibodies. Mice were immunized human IMR5-75 neuroblastoma cells and tumour specific antibody responses measured by flow cytometry and GD2 specific ELISA.

Results: Spontaneous murine NK activity against the IMR5-75 tumor model as a requested condition for analysing NK cells impact on adaptive responses were demonstrated in SCID mice lacking T- and B-cells, but having functional intact NK cells. Single subcutaneous injections of $3.45–4.0 \times 10^7$ IMR5-75 cells induced growth of tumor nodules in only 3/10 mice, but in 10/10 NK depleted mice ($P < 0.000$), demonstrating spontaneous murine NK activity against IMR5-75 cells. 4/4 C57Bl/6 mice immunized with irradiated IMR5-75 cells for two weeks developed significant global serum IgG responses (titer: 2 × 1:160, 2 × 1:320) against the immunizing cells (flow cytometry), while NK depleted control animals revealed only poor responsiveness (titer $< 1{:}40$; $P = 0.003$). Interestingly IgG responses in NK depleted mice after three weeks of immunisation were similar to the controls., This may reflect a delayed B cell answer in NK depleted mice. Dissection of IgG1 and IgG2a isotype specific responses demonstrated a strong impairment in Th1 biased IgG2a track and surprisingly in contrast a "compensatory" enforced Th2 oriented IgG1 response. ELISA based antibody measurement against the tumor specific GD2 ganglioside confirmed the flow cytometry data and demonstrated anti-GD2 responses only in NK depleted animals and only from the Th2 oriented IgG1 isotype. Dissection of GD2 specific IgG1 and IgG2a responses in 8 F004/C57 hybrid mice immunised with the GD2 expressing small cell lung cancer cell line H69 confirmed the Th2 biased GD2 specific B cell response in NK depleted mice as an independent control experiment.

Conclusions: Our data demonstrate an important NK cell regulatory function on the development of tumour directed B cell responses. Since Th1 biased IgG production results in antibodies with higher affinity (IgG1 and IgG3 in humans) for Fc-gamma-receptors than Th2 responses impairment of tumor directed ADCC will be a consequence. Tumour vaccination strategies will have to pay attention on the NK cell status of patients e.g. after chemo- or radiotherapy.

S29
NFκB activation and TGFβ loss signatures are prominent *ex vivo* responses of peripheral blood mononuclear cells

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Background: Homeostasis and activation of immune cells is tidily regulated. A well organized response of different players of the immune system is evoked by disturbance of the steady state homeostasis as seen in e.g. case of inflammation or cancer. One of the major methodologies to study the interactions in the immune system specially in humans is the *ex vivo* analysis of monocytoid cells. This *ex vivo* analysis of human immune cells however might be hampered by the fact that these cells are particularly flexible in reacting to their environment. E.g. specialized cells such as macrophages or DC constantly monitor their environment via so-called pattern recognition receptors. Furthermore several factors present *in vivo* are removed in the *ex vivo* system. For peripheral blood mononuclear cells, some aspects of the response to the *ex vivo* culture conditions have been established. Monocyte adherence was regarded as an activating event resulting in changes of gene expression and protein secretion and mainly increase in IL8 expression was shown to result from adherence of PBMCs to plastic. We and others have recently demonstrated that blood asservation and cell isolation techniques have to be closely controlled when monitoring cellular responses on a global level. Here we asked the question whether there is a global cellular

response to the *ex vivo* system itself. Gene expression profiling allows a large scale measurement of changes of gene expression and thereby an unbiased search of cellular changes.

Method: Whole genome expression profiling (HG-U133A, Affymetrix) of 79 samples from PBMCs and $CD4^+$ T-cells maintained for different time courses *in vitro* without further manipulation were performed. Differentially expressed genes where further evaluated using protein based assays.

Results: *In vitro* culturing did not change the quality of gene expression data. But time dependent changes of gene expression outweighted by far interindividual differences as demonstrated by unsupervised clustering methods. In total 827 genes showed significant changes of expression within the first 24h. During early time points a strong anti-apoptotic signature was observed. Later the transcription of numerous cytokines and chemokines was significantly upregulated while the expression of the respective receptors was diminished. Analysis of potential upstream elements responsible for the observed changes revealed that a third of the known NFκB target genes showed strong and time dependent regulation by simple *in vitro* culturing. Moreover, a prominent feature observed in *ex vivo* cultured $CD4^+$ T-cells was the early upregulation of several TGFβ antagonists and repression of TGFβ target genes. In summary a strong activation state of cells just maintained in culture was observed.

Conclusion: A rather strong and significant molecular response to the *in vitro* environment itself was detected in PBMCs and CD4+ T cells. Among others activation of NFκB target genes and withdrawal of TGFβ leads to an activation state of cultured cells. These data have significant impact on our interpretation of molecular and functional *in vitro* studies of human immune cells but might also be used as a molecular platform to optimize cellular culture systems for the *ex vivo* production of immunotherapeutically used cells.

S30

Enrichment of functional CD8 memory T cells specific for MUC1 in Bone Marrow of Multiple Myeloma Patients

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Objective: Recently, the bone marrow was shown to be a site were T cells are primed against blood borne antigens and tumor associated antigens. The common tumor-associated antigen (TAA) MUC1 has been shown to be expressed on about 90% of malignant plasma cells in multiple myeloma (MM). This study was performed to investigate the content and reactivity of MUC-1 specific memory T cells in BM compared to PB from MM patients with respect to possible use in immunotherapeutic strategies.

Methods: Paired BM and PB samples from 42 HLA-A2 pos. MM patients and 11 HLA-A2 pos. normal donors were tested for frequency of TAA-specific CD8 T cells by HLA-A2 tetramer-analysis using MUC1 derived peptide LLLTVLTV (12–20) as TAA or for frequency of tumor-reactive CD8 memory T cells in 40h short term IFNy ELISPOT assay. Antigen specific cytotoxic potential of 6 patients T cells was evaluated by Chr^{51}chromium release assay following single restimulation with MUC1 peptide pulsed DCs. Presence of MUC1 expressing cells and CD8 T cells in BM biopsies from MM patients was detected by immunohistochemistry.

Results: The frequencies of MUC1-specific CD8 T cells in PB and BM of 42 tested patients varied between 0–6.4% of CD8 T cells. In contrast, PB and BM of 11 normal donors contained only 0–0.25% tetramer binding CD8 T cells. Enrichment of MUC1 specific CD8 T cells (> 0.3% of CD8 T cells) was found in PB and BM from 16 out of 30 patients (53%).

Using short term IFNγ ELISPOT functional-assay we detected enrichment of MUC1-reacticve CD8 memory T cells in BM from 6 out of 12 patients (50%). In contrast, in corresponding PB, MUC1-reactive T cells were detected in only 1 out of 9 patients (11%). The frequencies of MUC1-reactive CD8 memory T cells varied between 1:390–1:3350 (BM) and 1:3340 (PB). BMTCs from MM patients were able to kill MUC1-peptide loaded Target cells in a dose dependent manner in contrast to corresponding PB T cells from the same patients. CD8 T cells were co-localized together with MUC1 expressing cells in the BM of MM patients.

Conclusions: MUC1 specific T cells are highly enriched in PB and BM of about 50% of MM patients indicating induction and maintainance of tumor cell directed immune responses during the course of disease. We detected high amounts of MUC1-derived peptide specific CD8 memory T cells capable of IFNγ secretion and cytotoxicity upon appropriate restimulation in BM but not PB of MM patients. Thus, autologous BM-derived memory T cells reactivated *in vitro* with MUC1 pulsed dendritic cells might be useful for future immunotherapy of MM.

S31

Isolated γδ T cells express natural cytotoxicity receptors

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Introduction: γ/δ T cells account for up to ten percent of T lymphocytes in the peripheral blood of healthy donors. They can be activated by cytokines like interleukin(IL)-2 and IL-15, express natural killer (NK) cell markers such as NKG2D and show cytotoxic activity against several tumor cells. Natural cytotoxicity receptors (NKp30, NKp44 and NKp46) have so far been regarded as specific NK receptors. Resting NK cells express NKp30 and NKp46 whereas NKp44 is expressed by activated NK cells only. To date, only two γ/δ T cell clones expressing NKp44 have been described. There is no description of polyclonal γ/δ T cells expressing natural cytotoxicity receptors.

The aim of the presented study was to evaluate the expression of activating NK cell markers including NKp30, NKp44 and NKp46 on isolated γ/δ T cells, since these may play a role in the cytotoxic activity of γ/δ T cells.

Methods: γ/δ T cells were isolated from healthy donors to a purity of >90% by magnetic activated cell sorting and cultured in the presence of anti-CD3, IL-15 and high dose IL-2. On days 0, one and seven the expression of activation markers including NKG2D

and natural cytotoxicity receptors on γ/δ T cells were evaluated immunophenotypically. For further confirmation, RT-PCR was performed on a light cycler for mRNA of β-actin, NKp44 and DAP 12, the signal transduction molecule of NKp44.
Results: On day 0 there was little expression of NK cell marker NKG2D on isolated polyclonal γ/δ T cells. Expression of NKG2D was up to 85 % after one day of stimulation with cytokines IL-2 and IL-15 and did not change significantly during further culture.
On day one of culture there was a slight expression of NKp30 and NKp46 (14 ± 9 % and 18 ± 11 %, n = 4), which decreased rapidly after 7 days of culture (3 ± 2 % for NKp30 and 2 ± 1 % for NKp46). Although there was no expression of NKp44 on day one of activation with cytokines, on day 7 a surface expression of up to 26 % could be determined (n = 22, mean 8 ± 7 %).
In contrast isolated CD4+ and CD8+ α/β T cells were consistently negative for surface expression of NKp44 after culture with high dose IL-2 and IL-15. These results could be confirmed by detection of NKp44 and DAP12 mRNA by RT-PCR from RNA from γ/δ T cells but not from α/β T cells.
Summary and conclusion: To our knowledge this is the first description of natural cytotoxicity receptors on polyclonal γ/δ T cells. NK cells markers are known to play an important role in the cytotoxic activity of γ/δ T cells. NKp44 has so far only been described on two γ/δ T cell clones, however, in these, an activating role could also be determined (Cantoni *et al.*, *J Exp Med*, 1999). From our results we conclude, that natural cytotoxicity receptors are not only expressed by NK cells but also by isolated γ/δ T cells. In further studies the role of these receptors in the cytotoxic activity of γ/δ T cells should be evaluated.

S32
The kinetics of accumulation of adoptively transferred ovalbumin-specific T cells in a transgenic, ovalbumin-expressing murine tumor

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We have studied the recruitment of adoptively transferred tumor-specific CD8-positive T cells in a mouse model system. Subcutaneous tumors of the transgenic, ovalbumin-expressing murine melanoma cell line B16-OVA were established in mice, which subsequently received intravenous transfer of ovalbumin-specific T cells, derived from the mouse strain OT-I, transgenic for a T cell receptor recognizing an ovalbumin-derived peptide, SIINFEKL.
Specific T cells from OT-I spleen were expanded in culture in the presence of SIINFEKL peptide prior to transfer. The homing of adoptively transferred cells to B16-OVA tumors was demonstrated using several methods, and the kinetics of the accumulation of the cells in the tumor tissue was delineated by flow cytometry. Quantitative measurements of the content of SIINFEKL/H-2 Kb tetramer and CD8 double positive cells in single cell suspensions from extirpated, collagenase treated tumors were performed each day for 8 consecutive days following adoptive transfer. The presence of double positive cells increased gradually until day 5, when an average number of 3.3×10^6 (n = 9) double positive cells per gram tumor tissue was found. The number of double positive cells per gram tumor remained fairly constant until day 8, which was the latest day examined.
Our results establish a baseline for the tumor accumulation of transferred T cells in this model, and form a foundation for studies of different experimental protocols for adoptive transfer of T cells. Monitoring the effect on the specific accumulation of transferred T cells in this model we are presently studying various strategies for injecting and supporting the transferred cells. Results from these investigations could hopefully contribute towards optimizing adoptive immunotherapy of cancer in human subjects.

S33
Tumor-specific murine T cell receptors displace endogenous TCRs in human T cells

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A novel approach in immunotherapy of human malignancies is based on universal tumor associated antigen (TAA)-specific T cell receptor (TCR) gene delivery into human T cells. The MDM2 oncoprotein is overexpressed in a multitude of human (Hu) malignancies, counter-balancing p53 as major tumor suppressor protein. We took advantage of HuCD8xA2K^b transgenic (Tg) mice to bypass human MDM2-specific self-tolerance: the chimeric MHC-molecule of the xenogenic host enables the selection of HLA-A2.1-restricted lymphocytes thereby allowing the contribution of murine CD8 for TCR affinity maturation in the thymus and the subsequent deployment of non-tolerogenic lymphocytes into the periphery.
The TCR-encoding genes were retrovirally transduced in human T cells *in vitro*. Quantitative expression frequency of the heterodimeric TCR on a per cell basis was accomplished by introducing independent IRES selection cassettes into either TCRα/β construct so as to verify subtle phenotype/structure/function characteristics among different TCR constructs.
A major concern was to determine as to whether or not murine TCRs are able to form hybrid TCRs with the endogenous ones potentially raising the issue of autoimmunity. Based on empirical data one may hypothesize that surface expression of a certain heterodimeric TCR construct reflects it's intrinsic stability guided by it's propensity to heterodimerize and to be incorporated into the multi-step assembly of the functional TCR/CD3 complex so as to be sheltered from down-modulation and proteolytic degradation. Amino acid substitutions that affect interchain affinity will have an impact on surface expression, the formation of hybrid TCRs and functionality in terms of cytokine secretion and cytolysis in chromium release assays. For this a multitude of TCR constructs, designed as either murine and partially humanized double chain TCRs or as single chain TCRs, have been assayed on their functional outcomes and consequences for the endogenous TCRs. The expression of single murine TCR chains documents their capability to form hybrid TCRs a tendency that can be further increased by the humanization of the constant domains. Point mutations that impair pairing as deduced from protein structure reduce their surface expression and inversly accumulate in the cytoplasma.

Murine double chain TCRs as opposed to humanized TCRs are capable to compete with and to replace a significant fraction of the endogenous TCRs in an exogenous TCR (exoTCR) dose dependent fashion irrespective of the clonal TCR subfamily diversity of the particular donor. Subsets of endogenous TCR high (endoTCR^{hi} correlates with exoTCR^{lo}) and TCR low (endoTCR^{lo} correlates with exoTCR^{hi}) expressing T cells have been FACS-sorted and proceed to be verified for phenotype maintainance and functional potency. One major intriguing topic is to pinpoint to the murine TCR structure determinants that are responsible for this competing stringency in order to introduce them into tumor-specific human TCRs for their favourable interchain pairing and subsequently their exclusively heterologous expression in human T cells.

S34

Induction of leukemia reactive, allogeneic donor lymphocytes

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The therapeutic value of donor lymphocyte infusions (DLI) in patients who relapse with AML is limited by a low efficacy and a high risk of GVHD. Therefore, we aim at generating leukemia reactive donor T cells for patients with AML. So far, 4 patient-donor pairs were evaluated (Table 1). Donor PBMC were stimulated with mature donor dendritic cells (DC), pulsed with irradiated and anti-CD34 MAb coated patient leukemic blasts (LB), or directly with cytokine (GM-CSF, IL-4, TNFalpha) treated LB. A 2–3-fold expansion of donor T cells occured in all experiments. Immunophenotyping showed a predominant outgrowth of CD4+ cells (80–95% of CD3+ cells) in all but one patient (75% CD8), with 0–1% CD56+/CD3– cells. After three weekly stimulations, donor cells were tested for IFNgamma secretion by ELISPOT assay after stimulation with irradiated leukemic blasts cells (LB) and non-leukemic controls, depending on the availability of patient cells. In 4/5 donors, cells with reactivity against patient LB with low or no reactivity against patient non-leukemic cells could be generated. In the remaining patient, the resulting donor T cell line showed a higher IFNgamma secretion after stimulation with patient PHA blasts than LB, but none after stimulation with third party LB. Direct stimulation with cytokine treated LB was performed in one donor (04), resulting in reactivity against LB and not PHA blasts, but high reactivity against 3rd party EBV-transformed B cells (LCL). In one patient so far (02), leukemia reactive donor T cells were expanded with a recently developed system using gene-modified K562 cells loaded with anti-CD3 and anti-CD28 antibodies [1,2]. One week of expansion resulted in a 10-fold increase of reactivity with sustained specificity of the resulting T cell line (not shown). Characterisation of tumor reactive donor T cells by MHC blocking experiments in one donor (02) and separate analysis of T cell subsets in a second donor (04) indicated that specific anti-leukemic reactivity was mainly mediated by CD4+ T cells in those donors.

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Table 1

Donors	Targets: Leukemic blasts	PHA– T cell blasts	CD34 neg. cells	EBV– B cells	3rd party leukemic blasts
1	252			77	21
2	11 ± 0		2 ± 1		
4	33 ± 1	4 ± 2			
7	70	113			16
10	36 ± 4	7 ± 0			

Numbers shown are spots/1e5 effector cells (IFNgamma ELISPOT) ± std err

S35

Identification of a melanoma-associated chondroitin sulfate proteoglycan (MCSP) peptide recognized by CD4+ T lymphocytes on human melanoma cells

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The identification of tumor antigens recognized by cytolytic CD8+ T cells (CTLs) on human tumor cells has opened new avenues in cancer immunotherapy. There is consensus, that the induction of both, tumor-specific CTLs and CD4+ T helper cells is necessary for an optimal antitumor immunity. Unfortunately, only a few tumor-specific helper T cell epitopes have been described so far. We therefore have focused our research on the identification of melanoma antigens recognized by CD4+ T cells. One interesting candidate antigen is the human melanoma-associated chondroitin sulfate proteoglycan (MCSP), which is expressed on > 90% of human melanoma tissues and induces strong humoral responses in mice.

In the present study, we describe the induction of MCSP-specific CD4+ T cell clones from the peripheral blood of a healthy human donor and the subsequent identification of the T cell epitope which is located in the core protein. The identified peptide was presented to the T helper cells by HLA-DR11 molecules, which are expressed by approximately 13% of Caucasians. The T cells directly recognized HLA-matched MCSP-expressing melanoma cells and produced high amounts of IFN-gamma, a cytokine with important antitumoral effects. To the best of our knowledge, this is the first MCSP-derived T cell epitope described and it should be useful for melanoma immunotherapy.

S36

Chimeric carrier proteins for targeted delivery of tumor antigens to professional antigen presenting cells

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Tumor-specific T lymphocytes can be regarded as a highly effective mechanism for tumor rejection. A substantial number of T-cell defined tumor antigens including mutated oncoproteins and differentiation antigens have been identified. However, while most spontaneous tumors appear to be antigenic, few are immunogenic. Activation of tumor-specific cytotoxic T cells (CTL) requires presentation of tumor antigens by professional antigen presenting cells (APCs) via MHC I molecules. Due to their crucial role in T-cell activation, APCs are being exploited for active cancer immunotherapy. Present experimental strategies include the incubation of dendritic cells with synthetic, tumor specific peptides to achieve uptake of tumor antigens and presentation in the context of MHC molecules. Alternatively, gene therapeutic approaches are aimed at the endogenous expression of tumor antigens in APCs upon transfer of suitable vector constructs.

Our strategy for the presentation of tumor antigens by APCs is based on the intracellular delivery of tumor antigens as part of a fusion protein specifically targeted to APC cell surface receptors. We have constructed prototype molecules that contain a soluble fragment of CTLA-4 for cell binding via interaction with B7 molecules, genetically fused to a protein fragment derived from the tumor-associated antigen ErbB2. To improve uptake and direct the antigenic determinant preferentially to the MHC class I pathway, in one of these protein vaccines also the translocation domain of the bacterial *Pseudomonas* exotoxin A has been included. In the parental toxin this protein domain facilitates escape from the endosomal compartment to the cytosol upon receptor mediated endocytosis.

Here we have investigated the *in vitro* cell binding activity of such reagents and their antitumoral activity in immunocompetent murine model systems. Specific binding to B7 molecules and uptake of bacterially expressed protein vaccines could be demonstrated. *Ex vivo* restimulation with an ErbB2-derived peptide of splenocytes from Balb/c mice injected with the fusion proteins resulted in enhanced IFN-γ production by T cells. Protective and therapeutic effects of ErbB2 protein vaccines were also investigated. Vaccinated animals were protected against subsequent challenge with syngeneic ErbB2 expressing tumor cells. Likewise, s.c. injection of ErbB2 protein vaccines in the vicinity of established tumors resulted in tumor rejection and long lasting protection indicating that immunological memory was induced.

Our results suggest that chimeric proteins combining a tumor antigen and specific recognition of APCs in a single molecule are suitable for targeted delivery of antigens to professional APCs and might become valuable tools for cancer immunotherapy.

S37

Particulate antigenic structures: highly immunogenic carriers for T cell epitopes derived from tumour antigens

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Polyomavirus-like-particles (PLPs) are empty, non-replicative, non-infectious particles that represent a potent antigen-delivery system [1]. Due to the high immunogenicity of heterologous PLPs consisting of the major polyomavirus coat protein VP1 and a foreign CD8 T cell epitope at its C-terminus it is possible to protect mice against B16-OVA melanoma [2]. Here we show that in mice protective anti-tumour immunity can be already induced by means of subcutaneous vaccination with particulate antigens, heterologous VP1-pentamers (8–9 nm in size). These VP1-pentamers carrying an immunodominant H-2K^b ovalbumin $(OVA)_{257-264}$ epitope evoked full protection in C57BL/6 mice against lethal B16-OVA melanoma challenge upon twice subcutaneous immunisations in a weekly interval. Furthermore, 60 % of mice vaccinated with VP1-pentamers carrying an immunodominant H-2K^b-restricted self-epitope of tyrosinase-related protein 2 $(TRP2)_{180-188}$ survived to lethal B16-OVA challenge. This experiment additionally underlines the capacity of PLPs to break T cell tolerance against a differentially expressed self-antigen. More importantly, heterologous capsoids of VP1-$OVA_{252-270}$ (~ 45 nm in size) cured mice from B16-OVA melanoma cells that had been administered 5 days prior to the first therapeutic treatment. As correlate for protection the number of $OVA_{257-264}$-specific CD8 T cells were significantly increased within the splenocyte population of treated mice even in the absence of an adjuvant (QuilA) as measured by H-2K^b-$OVA_{257-264}$-PE tetramers. The weekly treatment intervals appeared to be crucial for vaccine efficacy due to VP1-specific antibody interference. These results reveal that heterologous PLPs and even chimerical polyomavirus-specific pentamers represent highly efficient antigen carriers for inducing cell-mediated immunity against malignant diseases underlining their potency in the fight against cancer.

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S38

Development, monitoring and first immunological results of a phase I/II vaccination trial using genetically modified allogeneic breast cancer cells

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The rational behind the use of allogeneic tumor cell lines as therapeutical vaccines is that multiple antigens common to both, the immunizing cell line and the patient's tumor are presented by shared human leukocyte antigen (HLA) molecules. The cell line "KS" was established from a malignant effusion of a breast cancer patient and found to express an array of ubiquitous tumor associated antigens (TAA) such as MUC-1, CEA, SSX-2, and members of the MAGE-family. As a result of interferon(IFN)-γ stimulation KS cells express high levels of HLA molecules, which probably present multiple peptide ligands for polyclonal T cell activation. This KS cell line was genetically modified to express CD80 providing costimulatory signals to T lymphocytes, and further transfected to overexpress Her-2/neu in order to avail a well characterized TAA as a marker for immunodiagnostic. *In vitro* studies demonstrated: a) KS24.22 transfectants can induce allospecific responses through direct priming; b) activate cytotoxic T cells (CTL) and T_H cells; c) stimulate antigen-specific HLA-A*02-restricted CTL after transfection with viral antigens; and d) present TAA epitopes restricted by HLA-A*02. These results supported the realisation of a phase-I/II clinical trial, where KS24.22 cells are used to vaccinate patients with breast cancer.

KS24.22 were expanded under GMP conditions and safety testing was done following FDA criteria. The primary objectives of the study are toxicity and feasibility. To correlate vaccine-induced immune responses with clinical responses we evaluated KS24.22-associated and TAA-specific T cells with a combination of molecular and cellular immunodiagnostic tests (qRT-PCR, ELISpot). Eligibility criteria included the following: (1) measurable metastatic breast cancer; (2) patient already received either anthracyclin- or taxan-based chemotherapy; (3) HLA-A*0201-positivity; (4) *in vitro* activation of patient's T cells by mitogen antibodies and KS24.22 vaccine cells; (5) written informed consent. Patients were excluded for following reasons: (1) immunosuppressive or autoimmune diseases; (2) acute or systemic infections; (3) chemotherapy or radiotherapy within 4 weeks; (4) antibodies, cytokines, or other immune therapies within 6 weeks. After irradiation, 10^7 cells were injected i.d. four times at 2-week intervals and four times monthly. The protocol was approved by the the local ethic-committees, the Paul-Ehrlich-Institut and the Committee for Somatic Genetherapy of the Deutsche Ärztekammer.

So far, 10 patients were included in this study receiving together more then 70 vaccinations. Vaccinations showed only minor side effects like flulike symptoms and injection site reactions. Here, the immunohistochemical analysis of biopsies showed infammatory cell infiltration consisting of macrophages, dendritic cells and predominantly $CD4^+$ T cells. Three patients had to discontinue therapy because of progression. Disease stabilisation was observed in seven patients. Of these, postvaccination PBMCs showed increasing KS24.22-reactive T cell responses detected by quantifying antigen-induced IFN-γ-mRNA. Two patients clearly developed HLA-A*02-restricted, Her-2/neu-specific $CD8^+$ T cells alongside with KS24.22-related alloresponses. CEA- and MAGE-1-specific $CD8^+$ T cells could be detected as well.

In summary, this immunization strategy proved to be safe and feasible, and induced TAA-specific immune responses. However, no objective tumor regressions were observed so far. The qRT-PCR method proved to be highly sensitive and can be used to perform an "immunologic staging" under vaccination.

S39

Dendritic cell-based immunotherapy of hormone refractory prostate carcinoma (HRPCa) – a pilot trial

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Rationale: Prostate cancer is one of the most common cancer found in man. For patients with hormone-refractory, metastatic tumour no effective therapy is available and prognosis is very poor. Therefore we designed a clinical study using a novel approach for the treatment of patients with advanced stages of prostate cancer. Preliminary data concerning dendritic cell (DC) based vaccination revealed promising results but the clinical relevance is still questioned. Thus, we decided to develop a clinical protocol for the treatment of 12 patients with hormone-refractory prostate carcinoma using prostate-specific antigen (PSA)-derived peptides loaded on DC.

Methods: PBMC from HLA-A2 positive patients are isolated from the peripheral blood by Leucapheresis. Monocytes are seperated by adherence and differentiated into immature DC by incubation with GM-CSF and IL-4 under serumfree conditions over six days. Then DC are matured with TNF-α and PGE_2 and pulsed with three different PSA-Peptides (PSA-1 [FLTPKKLQCV]; PSA-2 [KLQCVDLHV]; PSA-3 [VISNDVCAQV]). For vaccination 6×10^6 Peptide-pulsed DC are applied intracutaneously after previous subcutaneous application of INF-γ (50 μg/m²). Vaccination is performed 4 times in 3 week intervals. Patients are included after informed consent and sufficient hematological liver and renal function. Primary end point of the trial is the evaluation of PSA, measurable tumour parameter after 4 vaccinations and clinical benefit. Secondary end points are safety, quality of life (EORTC-QLQ-C30) and immunological parameters.

Results: Until now 8 patients are enrolled. 4 patients completed all 4 scheduled vaccination. 3 patients are still under vaccination. One patient dropped out due to early progress. The vaccination is well tolerated without any therapy related adverse event. Response rates are under evaluation and will be presented.

Conclusion: Vaccination with PSA-peptide-pulsed autologous DC combined with INF-γ is feasible and well tolerated. Further evaluation is in progress.

S40

The European Searchable Tumour Cell and Databank, ESTDAB, as a tool for research in cancer immunology

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Collections of tumour cell lines continue to represent valuable resources in cancer research. However, cell banks rarely provide detailed immunological characterisation of the lines that they offer. To redress this omission, ESTDAB was founded as an EU infrastructures project, (see www.medizin.uni-tuebingen.de/estdab). It now provides the world's largest collection of melanoma cell lines, and is the only cell bank to offer the researcher the ability to seek cells with combinations of immunologically-relevant characteristics (go to http://www.ebi.ac.uk/ipd/estdab/index.html). These include HLA class I and II high resolution typing, HLA expression patterns, tumour antigen expression, cytokine secretion, adhesion molecule expression, apoptosis resistance, surface glycosylation patterns and other parameters. These lines are available to bona fide investigators for research purposes. Examples of studies being performed using this resource are elution of HLA class I- and II-bound peptides to identify novel melanoma-associated antigens, assessment of mechanisms of MHC antigen loss, defects in antigen processing pathways, secretion of suppressive factors, influence of the culture environment and presence of other cell types on cancer cell characteristics, and activity of glycosyltransferases creating tumour-type glycan structures. Together with the EU project OISTER (Outcome and Impact of Specific Treatment in European Research on Melanoma, coordinated by D. Schadendorf), we are continuously collecting new cell lines generated from patients entered into immunotherapy trials. The appropriateness of these lines for monitoring purposes is being established. Here, we report studies on four melanoma cell lines which were chosen for propagation and elution of HLA-bound peptides by "acid-stripping" from the surface of viable cells. FACS analysis of HLA expression before and after this procedure confirmed that HLA class I expression decreased after peptide elution. The peptide content of the eluted samples was established by mass spectrometric analysis, and the eluates screened for immunogenicity *in vitro* on T cells of normal donors selected for sharing at least one HLA-I allele with the melanoma cell line. T cells were cultured in the presence of different cytokines and titrated amounts of eluate, using either autologous antigen-presenting cells, APC (eluate-pulsed irradiated PBMC, dendritic cells generated from the same donor, or HLA-matched B-cells). After several rounds of restimulation, T cell lines recognising eluate-pulsed autologous APC were obtained. Investigation of their exact antigenic specificity is now in progress. For studies on MHC class II-bound peptides, monoclonal antibodies were used to isolate HLA-DR, DQ and DP molecules from lysates of at least 10^{10} tumor cells. From these, HLA class II-bound peptides were eluted at low pH and fractionated by HPLC. After screening T cell reactivity, positive fractions were sequenced by MALDI-TOF in order to determine potential tumor-specific antigens and identify related T cell epitopes. Potentially immunogenic candidates thus far identified include an HLA-DR-restricted sequence derived from a possibly mutated stress protein (grp78) NVMRIINEPTAAAIA. Several non-mutated peptides from overexpressed molecules did not show evidence of immunogenicity *in vitro*. The availability of the ESTDAB collection will facilitate immunological investigation of *in vitro* human melanoma models.

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S41

Efficient *in vitro* expression of human reverse transcriptase (hTERT) in dendritic cells of lung cancer patients using RNA electrotransfection

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Patients with advanced lung cancer have a poor prognosis after standard radiochemotherapy. An innovative treatment strategy is the targeting of tumor associated antigens with cellular effector cells. The telomerase catalytic subunit (hTERT) is an attractive target of cytotoxic T cells as it is highly expressed in both NSCLC and SCLC cells.

To activate tumor antigen specific CD4+ helper and CD8+ cytotoxic T lymphocyte responses genetically modified dendritic cells (DC) are increasingly used. In this study, we used electro-transfection of DCs with hTERT mRNA that enables an HLA independent whole antigen approach potentially targeting a wide range of hTERT epitopes. Immature, i.e. non-proliferating human DCs were prepared from peripheral blood monocytes in serum-free growth medium, GM-CSF and IL-4. Subsequently the DCs were electroporated in transfection buffer with mRNA and matured in a cytokine cocktail consisting of IL-1beta, IL-6, TNF-alpha and PGE_2.

To establish and optimize mRNA electroporation conditions the DCs of a healthy individual were transfected with green fluorescent protein (GFP) mRNA. The percentage of electro-transfected DCs was 35% determined by selecting GFP expressing cells using flow cytometry. Importantly the RNA electrotransfected DCs retained their typical morphological and immunophenotypical characteristics, expressing high levels of HLA-DR and no lineage markers. CD83 as an indicator of maturation was expressed in 44%. The costimulatory molecules CD80 and CD86 were expressed in 73% and 97%. Having established electroporation parameters we transfected monocyte derived DCs with hTERT mRNA in 2 lung cancer patients. Verification of transfection efficiency was performed by analyzing the induction of telomerase activity with the TRAP assay. Twenty-four hours after electrotransfection the measured activity in the DCs was equivalent to HL60 cells that biologically express high levels of hTERT. Non-transfected DCs did not show any

telomerase activity. These data show that strong hTERT expression can be achieved in DCs of lung cancer patients using mRNA electrotransfection. They provide the basis for further preclinical and clinical studies.

S42

Parvovirus H1-induced tumor cell death enhances human immune response via crosspresentation of dendritic cells

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Introduction: Certain autonomous parvoviruses and their derivates are currently under evaluation as antitumor vectors since they preferentially replicate and kill *in vitro*-transformed cells and may reduce the incidence of spontaneous and implanted tumors in animals. However, their oncolytic properties are not yet fully understood and seem to involve more than tumor cell killing. H1-mediated tumor cell lysates may trigger antigen presenting cells to augment host immune responses. Therefore, we analysed maturation, activation and crosspresentation of dendritic cells (DC) after phagocytosis of H1-induced tumor cell lysates using the known human SK29-Mel melanoma cell model.

Methods: For detection of cell death Annexin V/Propidiumjodide assay in FACscan analysis was used. To analyse phagocytosis by DC PKH-2 stained immature DC were cocultured with PKH-26 stained H1-, UV- induced or freeze thaw cycled (and combinations of these) melanoma cells and quantified via FACscan and fluorescence microscopy. Comparably, FACscan was used for the analysis of DC maturation and activation . DC were labeled with CD45-FITC, CD14-, CD80-, CD83-, CD86-PE and 7-AAD. Crosspresentation of tumor cell antigens to CTL via DC were detected by IFNγ-release with a HLA-A2 negative subclone of SK29Mel-1.

Results: We first established that the HLA-A2 positive (SK29-Mel-1) and an HLA-A2 negative (SK29Mel-1.22) melanoma cell lines were equally susceptible to H1-induced cell killing. Secondly, we found that monocyt-derived, immature DC pagocytosed H1-mediated lysates better than mechanically destroyed cells. These DC were more perceptive to late than to early H1 infected cell lysates. DC cocultured with late apoptotic H1-induced SK29Melcells presented typical markers of maturation and activation. Furthermore SK29Mel-1.22 activated DC confirmed crosspresentation to autologoues CTL.

Conclusion: We revealed to the first time that parvovirus-induced tumor cell killing stimulates DC and CTL. The immune system may rapidly detect and respond to H1-infected tumors, thus making the clinical perspectives of parvoviral vectors even more promising

S43

Optimizing the antigen loading of dendritic cells with exogenous peptides

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The central role of dendritic cells (DC) in the immune system and their unique potency to induce tumor-specific killer and helper T cells has been demonstrated in numerous studies and is today unequivocal. Therefore, DC-based immunotherapy represents one of the most promising approaches to fight cancer and since the first vaccination study in 1996 numerous trials have been performed with more than 30 DC-based vaccination trials published only in the past 3 years. In principle, antigen can be delivered to DC by various strategies, but most commonly HLA class I or II restricted peptides derived from defined tumor antigens have been used. Because peptides can be readily obtained in clinical grade quality, are easily standardized and facilitate the immuno-monitoring during clinical trials, they can still be considered as gold standard of DC antigen loading.

Nevertheless, several issues concerning the use of peptide-loaded DC still have to be addressed. In the present study we carefully analyzed different parameters such as peptide concentration, stability of HLA/ peptide complexes on immature (i-DC) versus mature-DC (m-DC) or antigen competition in order to optimize the loading of DC with HLA class I and II peptides.

S44

Phase I/II study of vaccination with immature and mature dendritic cells in patients with melanoma and renal cell carcinoma

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Dendritic Cells (DC) may influence the development of a natural immune response and could be potentially effective in specific treatments for cancer patients. A phase I-II vaccination trial for patients with advanced melanoma and renal cell carcinoma (RCC) is ongoing in our Department of Oncology. In the present study we compared the therapeutic use of intradermally administered immature DC (iDC) and mature DC (mDC) pulsed with autologous tumor lysate (ATL) and keyhole limpet hemocyanin (KLH). iDC were differentiated from adherent PBMC obtained by leukapheresis and cultured with IL-4 (1000 IU/ml) and GM-CSF (1000 IU/ml) for 6 days. ATL and KLH were added on the 6th day. On the 7th day iDC were used for therapeutic infusion in 9 patients, while mDC, obtained after a further 48-hour stimulation with IL-1β, IL-6, TNFα and PGE2, were administered in 10 successive patients. Treatment schedule was as follows: vaccination on days 0 and 16, and once a month thereafter for at least 5 cycles or until progression occurred. Patients received IL-2 subcutaneously at a dose of 3,000,000 IU/die from days 2 to 6 of each cycle. Of the 9 patients (8 melanoma and 1 RCC) treated with iDC, 4 showed stable disease (SD) of 19, 6, 6, and 6 months' duration, and 5 progressed. Four of these patients also had weak

delayed-type hypersensitivity (DTH) skin reactions for KLH or ATL. The 10 patients (9 melanoma and 1 RCC) treated with mDC obtained the following results: 1 complete response (abdominal lymph nodes) of 10+ months' duration, with positive DTH for both ATL and KLH and onset of vitiligo, 1 partial response (lung and lymph nodes) of 3 months' duration, with subsequent development of brain metastases, 1 mixed response (skin and lymph nodes) lasting 6 months with positive DTH for both ATL and KLH and subsequent development of brain metastases, 3 SD of 9 (RCC patient), 9 and 7+ months' duration with positive DTH only for KLH (one patient progressed with brain metastases). Four patients with negative DTH for ATL progressed (1 brain metastases). mDC vaccination appears to be more effective in inducing clinical and immunological responses than iDC. In particular, 2 responder patients showed evidence of an immunological response that was specific for ATL. The disease would seem to have a high propensity to spread to the Central Nervous System, and a form of protection with liposoluble chemotherapy could therefore be hypothesized in future.

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S45
Survival and homing of *ex vivo* expanded donor derived dendritic cells after allogeneic BMT

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Introduction: Little is known about survival and trafficking of *ex vivo* expanded dendritic cells (eDCs) after adoptive transfer in animals. We investigated the trafficking patterns of eDCs in mice that had received allogeneic BMT.

Methods: C57BL/6 ($H2^b$) BM was depleted of $B220^+$, $CD3^+$ and $GR1^+$ cells and was expanded for 7 days with GM-CSF, IL-4 and Flt3L. A retroviral vector encoding for luciferase (luc) and gfp was used for transduction. EDCs were analyzed by FACS and functionality was tested with MLR. EDCs from C57BL/6 donor mice were injected i.v. at 4×10^6per animal into BALB/c recipients that had received either allogeneic myeloablative BMT (BALB/c-mbl) or non-myeloablative BMT (BALB/c-n/mbl) conditioning. Survival and *in vivo* trafficking of gfp/luc^+ eDCs were monitored by bioluminescent imaging (BLI). Tissues were examined for gfp^+eDCs using immunofluorescence microscopy. Additional experiments were performed with eDCs generated from gfp-transgeneic animals (C57BL/6-TgN(ActbEGFP)1OSB, $H2^b$).

Results: Expansion of depleted BM with GM-CSF, IL4 and FLT3L induced a polyclonal population of $CD11c^+CD11b^+$ and $CD11c^-CD11b^+$ eDCs. Both populations expressed CD40, CD80, CD86 and MHC II. No $CD3^+$ or $NK1.1^+$ cells were found, the number of $CD19^+$ cells ranged from 0–2.5%. After transduction, gfp^+ cells represented up to 36% of viable cells. EDCs were functional in a MLR assay. After injection, transduced cells were monitored *in vivo* with BLI for up to 100 days. In BALB/c-mbl and BALB/c-n/mbl eDCs were initially detected in the area of the lungs and later in the area of the gut, spleen and thymus. Using immunofluorescence microscopy, gfp/luc^+ eDCs and gfp-transgenic eDCs were detected at different time points in the spleen, Peyers patches, thymus and lymph nodes. During the observation period no animal exhibited signs of GvHD.

Conclusion: Here, we show that donor-derived, *ex vivo* expanded DCs survive in hosts after allogeneic, MHC mismatched BMT for at least 6 weeks. EDCs migrate to lymphoid organs like spleen, Peyers patches, lymph nodes and thymus. Myeloablative or non-myeloablative conditioning does not significantly affect trafficking patterns. Mice that had received eDCs developed no clinical signs of GVHD. The ability to visualize DC survival and trafficking gives new insight into the biology of adoptively transferred DCs and will help to optimize DC based treatment strategies.

S46
PBMCs transfected with RNActive stimulate specific T-cells

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Vaccination with messenger RNA (mRNA)-transfected dendritic cells (DCs) is a very potent and versatile strategy for immune intervention. It has recently been reported to trigger specific T-cell responses and clinical benefit when used as anti-tumor vaccine in cancer patients. Towards the improvement of this promising technology, most researchers studied the optimal transfection methods, maturation signals and application sites of DCs. On the contrary, we focused our research on the improvement of the other active component of this vaccine that is mRNA. We studied the stability and efficiency of translation of different mRNA designed to have improved futures. Through this work, we could develop an optimal mRNA vector called RNActive™ which enhances the efficiency of mRNA-based immune stimulations. Besides, we tested the possibility of replacing DCs by some other immune cells as a recipient of the mRNA. We found that under certain conditions, mRNA can be transfected in PBMCs preparations. The expression of relevant antigens through this mRNA transfection technology results in the presentation of MHC-associated epitopes as demonstrated by the stimulation of antigen-specific T-cells. This method is a blood-saving replacement of mRNA-transfected DCs. It is useful especially in the context of immunomonitoring: a small amount of PBMCs is enough to generate mRNA-transfected autologous cells. These cells can be used as targets in *in vitro* assays where the T-cell response that developed in vaccinated patients is being studied. Thus, our work on mRNA-technologies offers new tools to improve and study the triggering of immune responses using mRNA-based vaccines.

S47

Addition of histamine to IL-2 treatment augments T1 cell-function in melanoma patients *in vivo*: results from a randomized clinical trial of IL-2 with or without histamine (MP 104)

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Histamine is used as an adjunct to interleukin-2 (IL-2) in tumor immunotherapy due to its protective effect on NK and T cell inhibition by monocyte-derived reactive oxygen metabolites *in vitro*. Results from a first randomized phase III trial showed an increase in survival in stage IV melanoma patients with liver involvement (Agarwala SS, *J Clin Oncol* 2002, **20**). Here we have analyzed the effect of histamine on T cell cytokine production in patients treated with IL-2 without or with histamine within a second randomized multicenter phase III trial. A significant increase (mean 2.2-fold) in frequencies of CD3+ T cells producing IFN-γ (T1 cells) in response to mitogen stimulation was detected in patients treated with histamine plus IL-2 (n = 7 patients) while IL-2 alone (n = 10 patients) had no effect on the frequency of IFNγ-producing CD3+ T cells. In contrast, frequencies of CD3+ T cells producing IL-13 (T2 cells) significantly increased in patients receiving IL-2 (mean 2.7-fold) and this effect was not modulated by histamine (mean increase 2.9-fold). These effects were observed for both CD3+CD8+ as well as CD3+CD4+ T cells. *In vitro* experiments using separated T cells and monocytes from healthy subjects show that while histamine does not induce IFN-γ production in T cells it protects T cells from monocyte-induced down-regulation of IFN-γ. Melanoma-specific T cell responses were analyzed in the 9 HLA-A2+ patients (IL2 + histamine, n = 4; IL-2, n = 5) against HLA-A2+ melanoma cell lines using intracellular cytokine staining. Induction or augmentation of melanoma-reactive IFN-γ and IL-13-producing T cells could be shown in 2 HLA-A2-positive melanoma patients treated with histamine and IL-2, but in none of the patients in the IL-2 arm. Both patients had received previous vaccination with HLA-A*0201 binding tyrosinase peptide and also tyrosinase-specific T cell responses were detected after IL-2 plus histamine treatment. In summary, treatment with histamine in combination with IL-2 increases T1 responses and stimulates melanoma-specific T cells.

S48

Peptide vaccination induces specific effector and memory T cells but fails to enhance preexisting T cell immunity

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Peptide vaccines were shown to induce specific T cell reponses in the majority of patients with resected primary melanoma. We evaluated the immunogenicity of tyrosinase peptides in the combination with the adjuvants GM-CSF and KLH in patients with relapsed resected stage III/IV melanoma (median 4.5 (± 4.2) previous relapses, range 1–20 relapses). Twentythree patients received tyrosinase peptides mixed with KLH administered 4 times 2 weekly and then 2 times 4 weekly with GM-CSF daily for 4 days. Using intracellular cytokine and tetramer staining Tyrosinase specific IFNγ-producing T cells ranging from 0.05 to 1.4% of CD3+CD8+ T cells were already detected in 13 of the 23 patients before vaccination was initiated. In 12 of these patients we were unable to boost the frequency of tyrosinase-specific T cells by 6 vaccinations. In contrast, induction of tyrosinase-specific T cells was achieved in 7 out of 10 patients without preexisting immunity with 0.08–0.3% specific T cells of CD3+CD8+ T cells after 6 vaccinations. Prolonged immunization for a total of 12 cycles resulted in induction of further increase of the frequency of peptide-specific T cells up to 1.9% in 5 of 6 patients. The phenotypic characterization of preexisting as well as of vaccine-induced T cells showed the presence of both tyrosinase-specific memory and effector T cells and vaccination did not result in a major phenotypic shift in the prevaccination T cells. There were also no major differences in the proliferation capacity of Tyrosinase specific T after short-term in-vitro stimulation with IL-2 between the group with inducible T cell responses and patients with a preexisting T cell response. In summary our results indicate that a preexisting T cell response to a peptide significantly impairs the ability of the vaccine to expand specific T cells.

S49

Biological characterization of CD40-activated B cells as a prerequisite for their use as cellular adjuvant in cancer vaccines

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Cellular immunotherapy is a promising approach to specific treatment of cancer. Dendritic cells (DC) are the best-studied antigen presenting cells (APC) and have been tested in multiple clinical trials over the last years. To extend this strategy to clinical situations in which DC therapy might be more challenging, e.g. pediatric patients or to approaches with frequent vaccinations, we have established CD40-activated B cells (CD40-B cells) as a complementary autologous APC, due to the simple generation of large amounts of highly efficient CD40-B cells from small amounts of peripheral blood. Similar to the pharmaceutical development of DC to become a standardizable adjuvant, several important biological aspects need to be addressed for CD40-B cells prior to "product development" according to GMP conditions.

We have identified efficient presentation of antigen in the context of MHC class I and MHC class II as well as homing capabilities to secondary lymphoid organs as essential biological criteria for this cellular adjuvant to merit further development. Recently, we showed that antigen-loaded CD40-B cells expand memory and induce primary $CD8^+$ T cell responses in healthy donors and cancer patients alike.

Here we address, whether CD40-B do process antigens in the context of MHC class II and induce secondary as well as primary

CD4+ T-cell responses: We developed a T-cell expansion system that uses CD40-B cells as sole APC to induce antigen-specific responses of purified CD4+ T-cells: 1) tetanus toxoid (TT) and keyhole limpet hemocyanin (KLH), were used as a model for whole protein antigens, 2) the artificial promiscuous MHC class II binding peptides PADRE-AKF and PADRE-AKX served as model peptide-neoantigens. After 2 to 5 rounds of stimulation with antigen-loaded CD40-B cells ELISPOT technology was used to determine the presence of antigen specific CD4+ T-cells. While specific cells were successfully expanded for all antigens studied, INF-γ and IL-4 cytokine secretion profiles did not indicate a dominant polarization towards TH_1 or TH_2 lineage.

Similarly important, we addressed, if CD40-B cells have the potential to home to lymph nodes and induce T-cell chemotaxis: CD40-B lack receptors important for relocating to peripheral tissue but do express CD62L, LFA-1, CCR7 and CXCR4, receptors implied in homing to secondary lymphoid organs. Migration experiments using their cognate ligands CXCL12, CCL19 and CCL21 demonstrated that these receptors are fully functional and mediate migration of CD40-B cells. Furthermore, CD40-B cells express several important T-cell attractants including IP-10, Rantes, MCP-1 and ENA-78. Correspondingly supernatant from CD40-B cultures induces strong chemotaxis of CD4+ and CD8+ T-cells.

Taken together, CD40-B cells efficiently induce primary MHC class I and II restricted T-cell responses and have potential to home to secondary lymphoid organs. Based on these findings pre-clinical models have been developed to assess the *in vivo* capacity of these cells to efficiently migrate to T cell rich areas of secondary lymphoid organs and to induce efficient T cell activation.

S50

Identification of a novel epitope derived from the cancer-germline antigen, HAGE, displaying both in-vitro and in-vivo immunogenicity

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It is now well established that both CD4+ and CD8+ tumour-specific T-lymphocytes play critical roles in anti-tumour immunity; thus there is a desirability to identify both MHC class I and II-restricted tumour antigens that induce immunogenic responses in both in-vitro models and more significantly in an in-vivo environment. Many current tumour antigens that are potential targets fall into the category of cancer-germline (CG) antigens, and are considered to represent good candidate antigens for tumour immunotherapy based on their lack of expression on normal somatic tissues. HAGE is a novel CG gene expressed in a wide range of solid tumour tissue (eg. around 20% of melanomas, one-third of lung cancers) but also in haematological malignancies (in >50% and >20% of chronic and acute myeloid leukaemias, respectively). Here we describe the use of a combination of computer algorithms to identify potentially immunogenic peptides from the HAGE protein based on both predicted HLA affinity and proteosomal cleavage sites. An HLA-A2-binding motif contained within a longer HLA-DR-binding sequence was identified. Two peptides representing either the class I motif alone, or a longer peptide containing the class I motif within the class II motif, were then screened in in-vitro T cell sensitisation experiments using PBMC or monocyte-derived dendritic cells from healthy donors or CML patients; the class I peptide was also used in-vivo to vaccinate HLA-A2-transfected mice. We demonstrate that both these peptides are immunogenic in-vitro not only for T cells from healthy donors, but also from CML patients, as assessed by functional assays such as cytokine secretion and cytotoxicity. Moreover, immunogenicity was confirmed by using MHC/peptide tetramers to show specific expansion of sensitised T-cells. Furthermore, the class I peptide also demonstrates immuno-genicity in-vivo following vaccination of HLA-A2-transfected mice. Spleen cells isolated from these mice showed specific cytotoxicity ex-vivo. We conclude that the HLA-DR-binding peptide and the HLA-A2 motif contained therein may represent potential vaccines for the immunotherapeutic treatment of cancer, particularly CML, targeting the HAGE expression of a high proportion of tumours.

S51

New TRP-2-derived T helper epitopes identified in HLA-DRB1*0301 transgenic mice elicit spontaneous T cell responses in HLA-DRB1*03 and HLA-DRB1*04 melanoma patients

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Antigen-specific cytotoxic CD8+ T lymphocytes (CTLs) are effective mediators of destructive anti-melanoma immunity but primary CTL-sensitisation and establishment of CTL-memory are dependent on the helper activity of antigen-specific CD4+ T lymphocytes. Therefore, active immunotherapy of melanoma patients should ideally target antigen-specific CD8+ and CD4+ T cells in order to achieve effective anti-tumor CTL immunity. Identification of tumor antigen epitopes is a prerequisite for specific T-cell targeting in the course of vaccination and for vaccine evaluation. But in contrast to HLA-class I restricted peptide ligands only a few HLA-class II-presented epitopes are characterized. We used computer algorithms and HLA-DRB1*0301 (HLA-DR3) transgenic mice to identify epitopes derived from the differentiation antigen tyrosinase-related protein-2 (TRP-2). Three potential HLA-DR3-restricted epitopes were predicted from the TRP-2 protein sequence, two of the corresponding synthetic peptides exhibited HLA-DR3 binding capacity, but only one sequence (Pep1) specifically activated CD4+ T cells in HLA-DR3 transgenic mice after peptide immunization. Processing of an epitope located in the Pep1 sequence from the TRP-2 antigen was subsequently demonstrated by TRP-2 protein immunization. Pep1-specific CD4+ T cells could also be induced *in vitro* in human T cell cultures obtained from the peripheral blood of normal HLA-DRB1*0301 donors. Interestingly, *in vivo* sensitized Pep1-specific T cells were also detectable in the peripheral blood

of HLA-DRB1*03 and HLA-DRB1*04 melanoma patients verifying Pep1 as a target of spontaneous T cell responses.

S52

Use of immunopotentiating reconstituted influenza virosomes (IRIV) as vector to deliver antigens into dendritic cells

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Dendritic cells (DC) are the most potent antigen presenting cells and therefore represent a promising tool for cancer immunotherapy. We developed single use cell processors, allowing generation of large numbers of clinical grade monocyte-derived DC in serum free medium containing GM-CSF and IL-13. We have previously demonstrated that these DC cross present antigens to specific CD8 T cells on MHC class I molecules *in vitro*. In order to improve specific CD8 T cell responses, we tested Immunopotentiating Reconstituted Influenza Virosomes (IRIV) as vehicles to deliver antigens into DC. IRIVs are spherical, unilamellar vesicles with a mean diameter of 150 nm derived from the envelope of the Influenza virus. IRIVs could enter into DC via binding of hemagglutinin flu protein present at the surface of IRIV to sialic acid. It has been shown that antigens encapsulated into IRIVs are delivered to the cytosol and enter the MHC class I pathway inducing a CD8 T cell immune response. In this study, we demonstrated efficient uptake of PKH-26 labeled IRIVs by DC. IRIVs did not affect DC viability and did not induce DC maturation. In addition, IRIVs did not prevent maturation induced by bacterial extract (FMKp) and IFNγ. In order to study the capacity of IRIV associated with antigen to present epitopes at the DC cell surface, we developed a model antigen containing the highly immunogenic mutated Melan-$A_{26-35(27L)}$ peptide encapsulated into IRIVs

S53

Preclinical investigation of DNA immunization with a rearranged human papillomavirus type 16 (HPV16) E7 oncogene

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Infection with human papillomaviruses (HPV) is the major risk factor for the development of cervical cancer. The HPV E7 oncogene is constitutively expressed in infected cells hence it represents a promising target for immune therapy of HPV-related disease. For safety reasons the use of a transforming gene for DNA vaccination is not feasible. Therefore, we have generated a rearranged ('shuffled') HPV16 E7 artificial gene (HPV16 E7SH) specifically dissected at the sites associated with cell transformation. Sequence duplications were added in order to supply all original T cell epitopes. The potential risk of back to wild type recombination was reduced by the use of different codons in the duplicated sequences.

Our objective was to investigate whether the HPV16 E7SH gene is lacking detectable transforming properties and shows E7-specific tumor rejection in mice and immunogenicity in humans.

The E7SH genes were generated by fusion-PCR using overlapping primers. For DNA immunization, the genes were cloned into vector pTHamp (kindly provided by T. Hanke, Oxford, UK) and injected intramuscularly (i.m.) into female C57BL/6 mice. CD8+ T cell responses were measured by ELISPOT and ^{51}Cr-release assays. Tumor regression experiments in immunized mice were performed after inoculation of HPV16 E7 wild type-expressing syngeneic C3 cells. Soft-agar transformation assays were performed to compare transforming capacity of wild type and artificial E7SH genes in murine cells. E7SH recombinant retroviral vectors were generated for infection of NIH3T3. Human monocyte-derived dendritic cells were transfected by nucleoporation and HPV16 E7SH expression was investigated by RT-PCR, Western blot, and specific T cell stimulation *in vitro*.

Intramuscular immunization with the pTHamp-E7SH expression plasmid induced E7-specific cytotoxic T cell responses as detected *ex vivo* by IFN-γ ELISPOT and after *in vitro* restimulation by ^{51}Cr-release assays. Two i.m. immunizations with pTHamp-E7SH were sufficient to mediate regression of established C3 tumors in C57BL/6 mice. The E7SH gene has lost its transforming properties as analyzed by *in vitro* soft-agar-transformation assays. After transfection of immature and mature human monocyte-derived dendritic cells recombinant HPV16 E7SH expression was detected by RT-PCR, Western blot analysis, and *in vitro* stimulation of autologous E7-specific T cells suggesting potential immunogenicity in humans. Our data indicate that the HPV16 E7SH gene efficiently induces cytotoxic T cells directed against the wild type E7 antigen as measured *in vitro* and *in vivo*. The construct will be tested in clinical phase I trials to proof safety and immunogenicity in patients.

S54

Immunomediated growth and regression of pancreatic tumors *in vivo*

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We are using a novel spontaneous pancreatic adenocarcinoma tumor model to investigate immunotherapeutic approaches against pancreatic cancer.

Crossbreeding of p53 knockout mice with TGF-α transgenic mice which overexpress TGF-α in the pancreas and thus develop fibrosis and ductual pancreatic cancer at the age of one year dramatically accelerates tumor development and represents the first model of pancreatic adenocarcinoma with genetic alterations as well as characteristics similar to the human disease.

We have established a total of 28 murine adenopancreatic cell lines (mPACs) derived from 6 different TGF-α p53–/– mice. *In vivo* growth kinetics were analysed in normal syngenic mice and showed that some cell lines progress after *in vivo* injection to form lethal tumors while others grow during the first 10 days and then regress. Next, these tumors were injected into scid beige and in nude mice. In these mice progressors and regressors grow

progressively indicating that the regression in normal euthymic mice is an immunemediated response.
Cytotoxic T cell responses against MHC I positive mPACs are induced after immunization with irradiated mPACs and can be found in mice with spontaneous pancreatic carcinomas. Injection of mPAC leads to the induction of IFN-γ secrting CD8 T cells *in vivo*, which can also be found in tumor bearing mice.
This new model opens the possibility to investigate sponatneous immune responses against pancreatic cancers in a gentically well defined tumor model, which mimicks human adenocarcinoma.

S55

The receptor for hyaluronic acid mediated motility (RHAMM/CD168) is a leukemia associated antigen eliciting both humoral and cellular immune responses in patients with acute myeloid leukemia (AML)

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To improve the clinical outcome of AML patients, immune therapies targeting leukemia associated antigens (LAAs) might be an approach additionally to chemotherapy and transplantation of hematopoietic stem cells. Ideal LAAs should be preferentially expressed in leukemic blasts, but neither on stem cells of normal hematopoiesis.
RHAMM/CD168 was defined as a LAA using the approach of serological screening of expression libraries (SEREX), eliciting IgG responses in 42% of patients with AML, but not in healthy volunteers (HV) or patients with autoimmune diseases. mRNA for RHAMM was demonstrated by qRT-PCR to be expressed in leukemic blasts of more than 80% of the AML patients, but not in PBMN or CD34+ stem cells of healthy volunteers. Among normal tissues, only testis, placenta and thymus showed significant mRNA expression for the antigen, therefore sharing the expression profile of some other SEREX antigens.
Immunostaining of cytospins and western blots of naive AML blasts and AML cell lines (e.g. K562) confirmed the RHAMM expression on the protein level in 70% of the patients.
To define T cell epitopes of RHAMM, 10 peptides were synthesized following the SYFPEITHY and PAProC algorithms and subjected to ELISPOT assays for interferon gamma and granzyme B. CD8+ T cells taken from the peripheral blood of AML patients and presensitized with peptide R3 or R5 were specifically reactive in the assays against T2 cells pulsed with R3/R5 or COS7 cells co-transfected with HLA-A*0201 and RHAMM. The successful co-transfection was confirmed by flow cytometry and immunocytology. Cross-reactivity was excluded. These results were confirmed using a Cr-51 release assay.
In an AML patient having received blast-derived dendritic cells, a higher frequency of RHAMM-directed T cells was observed after four vaccinations when compared to the status before vaccination. We are going to initiate a phase-I vaccination trial using peptide R3 on a carrier molecule as specific AML vaccine. There is evidence that RHAMM is also expressed in other leukemia types (CML, CLL) which might encourage futher clinical trials.

S56

Sphingosine-kinase and sphingosine-1-phosphate regulate migration of immature dendritic cells

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Introduction: Currently, dendritic cells (DC) are tested as vectors for cancer immunotherapy. However, little is known about the mechanisms regulating DC migration. Sphingosine-kinase (SphK) and its catalytic product Sphingosine-1-phosphate (S1P) play a central role in processes such as cellular differentiation, survival and migration, which are often disregulated in cancer. Here, we examined the role of SphK and S1P in migration of DC.
Methods: DC were generated in the presence of GM-CSF and IL-4 for 5 days, then matured with PGE_2, TNF-α and IL1-β for 2 days. Expression of SphK and S1P-receptors was examined by RT-PCR. In transwell assays migration of immature (i) and mature (m) DC towards SDF-1, MiP-1a, MCP, 6Ckine, MiP-3β [100 ng/ml] and S1P [10^{-5}M] was tested for being dependent on SphK using the SphK inhibitor Dihydrosphingosine [DHS, 10^{-6}M]. The role of $S1P_3$ receptor in S1P-induced migration was tested using the $S1P_3$-inhibitor Suramin [10^{-6}M]. In parallel, Ca^{2+}-flux was assessed by FACS with Fura Red.
Results: SphK expression was declining from iDC to mDC to antigen-loaded mDC. Expression levels of S1P receptors were $S1P_1 > S1P_2 = S1P_3$, unrelated to maturation stage or antigen uptake. IDC migrated on SDF-1, MIP-1α, MCP and S1P, whereby S1P combined with a chemokine acted synergistic. MDC migrated on 6Ckine and MIP-3β, but not on S1P. Pre-treatment with DHS inhibited migration of iDC but not mDC, showing that SphK is required for iDC migration. Pre-treatment with Suramin inhibited iDC migration in response to S1P, demonstrating a mediation via $S1P_3$. Chemokine induced Ca^{2+}-flux was inhibited by DHS, indicating that SphK-mediated migration might be Ca^{2+}-dependent.
Conclusion: Our results suggest a role for SphK/S1P in accumulation of peripheral iDC at sites of antigen invasion. These findings could provide a new approach to optimise DC-based cancer immunotherapy by therapeutic modulation of SphK/S1P and have to be verified in an animal model in the next step.
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S57

Immunization of Rhesus monkeys with a conjugate vaccine IGN402 induces immune responses against carbohydrate and protein antigens, and cancer cells

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Introduction: Tumor-associated antigens resulting from aberrant glycosylation, such as the Sialyl-Tn carbohydrate antigen, are over-

expressed or exposed on cancer cells and provide potential targets for cancer vaccination. However, as T-cell independent antigens carbohydrates are poorly immunogenic, and fail to induce memory.

Methods: In order to increase the immunogenicity we have coupled the Sialyl-Tn carbohydrate antigen to a highly immunogenic carrier molecule. The carrier molecule, the mAb17-1A antibody, provides additional antigens for vaccination. An immunogenic formulation of mAb17-1A-Sialyl-Tn conjugate on alhydrogel, IGN402, with or without additional adjuvants was tested in Rhesus monkeys for tolerability and immunogenicity.

Results: A significant antibody response against the mAb17-1A antibody was found by ELISA. Furthermore, also a specific immune response against the Sialyl-Tn carbohydrate was induced, and immune sera showed binding reactivity to a variety of cancer cells.

Conclusion: Immunization in the presence of additional adjuvants, such as QS-21, strongly enhanced the immune response against the carbohydrate antigen, and importantly also resulted in the production of carbohydrate-specific IgG antibodies. The data indicate that carrier-induced T-cell help was sufficient for carbohydrate specific class switch induction.

S58

The Tübingen approach: identification, selection and validation of tumor-associated HLA peptides for cancer therapy

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There is a substantial need for molecularly defined tumor antigens to prime cytotoxic T cells *in vivo* for cancer immunotherapy, especially in the case of tumor entities for which only few tumor antigens have been defined so far. Here, we present the "Tübingen approach" to identify, select and validate large numbers of HLA class I-associated peptides derived from tumor-associated antigens. Step 1 is the identification of naturally presented HLA-associated peptides directly from primary tumor cells. Step 2 is selection of tumor-associated peptides from step 1 by differential gene expression analysis and data mining. Step 3 is validation of selected candidates by monitoring *in vivo* T-cell responses. Our approach combines methods from genomics, proteomics, bioinformatics and T-cell immunology. The aim is to develop effective immunotherapeutics consisting of multiple tumor-associated epitopes in order to induce a broad and specific immune response against cancer cells.

immatics biotechnologies, a privately owned spin-off from the University of Tübingen, is dedicated to the development of immunotherapeutics based on several approaches, of which one is presented here. In February 2004, the company received substantial private funding in its first financing round to start operations. (http://www.immatics.com)

S59

Molecular characterization of virus-induced autoantibody responses

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We present here a comprehensive molecular mapping of virus-induced autoimmune B cell responses obtained by SEREX analysis (serological identification of recombinantly expressed antigens). Immunoscreening of cDNA expression libraries of various organs (lung, liver, and spleen) using sera from mice infected with cytopathic (vaccinia virus, VV) or non-cytopathic (lymphocytic choriomeningitis virus, LCMV) viruses revealed a broad specificity of the elicited autoantibody response. Interestingly, the majority of the identified autoantigens have been previously described as autoantigens in humans. We found that induction of virus-induced autoantibodies of the IgG class largely depends on the CD40-CD40L-mediated interaction between T and B cells. Furthermore, antibody titers against a number of autoantigens were comparable to the concomitantly induced antiviral antibody response. Comparison of serum reactivity against a selected panel of autoantigens after infection with VV, LCMV or vesicular stomatitis virus showed that the different virus infections triggered distinct autoantibody responses suggesting that virus infections may leave specific "autoantibody fingerprints" in the infected host.