Materials and Methods: We used the new automated two-dimensional chromatography system *PF2D* to fractionate the proteome of tumour tissues and tested protein fractions for recognition by pre-existing tumour-specific CD4+ T-helper cells and cytotoxic T-cells.
Results: Applying this method to the Ovalbumin (OVA)-specific, TCRtg OT-I mouse model demonstrate efficient separation, processing and cross-presentation to CD8+ T-cells by dendritic cells of OVA expressed by the OVA-transfected mouse lymphoma RMA-OVA. Applying this method to human tumour tissues we identified in patients with head and neck cancer MUC-1 and EGFR as tumour-associated antigens selectively recognized by patients' T-cells. Finally, we detected on an exemplary patient with a malignant brain tumour CD4 and CD8 T-cell responses against two novel antigens, transthyretin and calgranulin B/S100A9, which were expressed on tumour and endothelial cells. Immunogenicity of these antigens could be confirmed in 4 out of 10 other brain tumour patients.
Conclusions: This fast and cheap method appears suitable to identify candidate T-cell antigens in various disease situations, such as autoimmune and malignant diseases without restriction to their expression by a certain cell type or HLA allele.

293 Cyclooxygenase 2-driven inflammation in pancreatic cancer

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Prostanoids perform important tasks in normal and diseased tissues by acting as local signals which coordinate the concerted activities of different cell types. In our work, we substantially focus on the role related to inflammation and cancer. Chronically inflamed pancreas, urinary bladder, and colon represent high-risk environments for tumourigenesis.
Epidemiological, pharmacologic, clinical, and genetic studies show the cause/effect relationship between over-expression of pro-inflammatory cyclooxygenase (COX)-2, COX-2-mediated prostaglandin (PG) signaling and the development of epithelial cancers. Depending on the cellular context, PG stimulate growth, angiogenesis, and modulate immune functions in inflammation-driven cancers.
Keratin 5 promoter-driven COX-2 (K5 COX-2) transgenic mouse lines established in our group develop pre-invasive neoplasms in various epithelial tissues including pancreas. Pancreata of the K5 COX-2 mice develop ductal neoplasms which resemble, on molecular and morphological levels, human precursor lesions of pancreatic ductal adenocarcinoma (PDAC), i.e. cystic intra-ductal papillary mucinous neoplasms (IPMN) and pancreatic intra-epithelial neoplasias (PanIN) (Gastroenterology 130, 2006).
These phenotypic changes are associated with pronounced inflammatory infiltrates in pancreas, thus representing a putative high-risk environment for tumourigenesis. Cytokine gene expression analysis depicts the presence of TH-1 (elevated IFN-gamma, TBX21), TH-17 (elevated Il-6, Il-17A), and T-regulatory (elevated FOXP-3) cells. At the cellular level, diffuse inflammatory infiltrates are observed, besides prominent inflammatory clusters, in the diseased pancreata of K5 COX-2 mice. These are comprised of B-cells, T-cells, follicular dendritic cells, macrophages, and high endothelial venules decorated with lymphocyte adhesion molecules. In addition, a panel of TLO-relevant chemokines is expressed. Altogether are known to be hallmarks of ectopic tertiary lymphoid organs (TLO) which arise in chronic inflammatory diseases but with a yet un-known function. Such an inflammatory phenotype is suppressed by inhibition of COX-2 activity whereby celebrex-fed transgenics exhibit fewer and smaller clusters, indicating the involvement of COX-2/PG signaling in the establishment of TLO; a putative novel COX-2-effect on local immunity. Follicular clusters rich in B-cells and T-cells, with vessels that express peripheral lymph node addressin (PNAd) are also observed in COX-2-positive human PDAC. This hypothesizes that the induction of TLO might be relevant in humans as well.

294 Successful engraftment of glioblastoma biopsy spheroids in immunocompetent rats

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Standardized implantation of biopsy spheroids generated from GBM patients into athymic nude rats has now been well established and characterized, with tumour takes close to 100%. The resulting lesions resemble human glioblastomas in their presentation, containing invasive single cells, vascular proliferations, thrombotic vessels, and necroses. Given that the brain is a site of immune privilege, and human glioblastomas evade immune surveillance, we evaluated whether human GBM spheroids would survive in fully immunocompetent rats and kill their hosts. We compared the engraftment rates of spheroids generated directly from patients or prepared after several generations of passaging in nude rats. Spheroids originally derived from four GBM patients were implanted, followed up by weekly MRI, and engraftment rates and survival data were collected.
Xenografts that were generated directly from patient biopsy tissue appeared on MRI scans only in two cases and were thereafter rejected. In contrast, xenograft tumours based on glioblastoma tissue that has been previously passaged in nude rats displayed engraftment rates of over 50% in immunocompetent rats, and once appearing on MRI scans, the lesions invariably killed their hosts. The survival time lengths for immunocompetent animals were similar to those for nude rats when implanted with the same biopsy spheroids. We sought to further characterize the mechanisms that permitted the development of human tumours in immunocompetent rats, such as the nature of immune-inflammatory host cells present and the production of immunomodulatory cytokines by the tumour and the host. The current data suggests that the elevated numbers of both CD4+ and CD8+ lymphocytes together with higher serum levels of rodent IL-1a, IL-2 and IL18 were significantly correlated with tumour xenograft rejection in immunocompetent rats. Furthermore, survival of the xenografts was associated with the inability of activated lymphocytes to penetrate the tumour bed.
In conclusion, we established that passaging of human GBM biopsy spheroids in nude rats facilitates more efficient engraftment in immunocompetent rats.

295 Cyclooxygenase-2 (COX2) gene silencing with siRNA could enhance DNA vaccination to inhibit established ErbB-2 carcinomas

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Background: Cyclooxygenase-2 (COX2) is the key enzyme in the synthesis of prostaglandin E2 from its precursor, arachidonic acid. The level of COX2 is normally very low in cells but increased amounts of COX2 are commonly detected in both premalignant and malignant tissues. In order to investigate COX2 as a potential target for the prevention and treatment of cancer, we propose a novel immunotherapeutic approach for the prevention of ErbB-2 positive breast carcinomas, based on DNA vaccination against a specific antigen (ErbB-2) in combination with the silencing of COX2.
Material and Methods: The short hairpin RNA interference cassette targeting COX2 mRNA, containing the mouse RNA promoter U6, was amplified and cloned into the Eco72I site of pVAX-ratECTM (RRT), a vector carrying the extracellular and transmembrane domains of rat ErbB-2, and into pVAXI, to obtain respectively RRT-COX2 and pVAX-COX2. The gene silencing efficacy of both plasmids was checked by transfecting COX2 over-expressing A17 cells. The plasmids were used for electroporation-based vaccination of female BALB-neuT mice of different ages, corresponding to various stages of cancer progression, from atypical hyperplasia to invasive lobular carcinoma. We are evaluating mammary cancer progression in vivo, the titer of anti-ErbB-2 antibodies in the immune sera, and the ability of spleen cells to release IFNg in response to the $H2^d$ immune dominant peptide of ErbB-2.
Results: Vaccination of 10 week-old female BALB-neuT mice with RRT plasmid significantly triggers a protective immune response toward the development of autochthonous mammary cancer in BALB-neuT mice (Quaglino et al. 2004, Cans Res). 30% of treated mice were still fully free from palpable tumours one year after vaccination, when all control animals had already died because of mammary cancer. Till now (week 46 of age) we see a similar protection using RRT-COX2. The level of anti-ErbB-2 antibodies in the sera from RRT-COX2 vaccinated mice is slightly higher than that of RRT vaccinated mice, but not significantly different. We are now collecting spleen cells from vaccinated mice to evaluate the specific CD8 response against ErbB-2. Experiments with mice vaccinated when they already have lobular carcinomas are ongoing.
Conclusions: COX2 suppression induced by shRNA might help to overcome tumour-mediated immunosuppression and generate an effective anti-tumour immunity not only in prophylactic but also in therapeutic vaccination.

296 Macrophage migration in tolerance

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Tumour Associated Macrophages (TAM) and lipopolysaccharide- (LPS)-tolerant macrophages share several characteristics, such as a massive accumulation of the p50NF-kB homodimer in the nucleus and the incapacity to express strong inflammatory programs (eg. impaired TNFa production) in response to inflammatory signals such as LPS (tolerance). Our recent study has described that both TAM and LPS-tolerant macrophages express

an M2 polarized phenotype, associated with strong anti-inflammatory and immunosuppressive functions.
Due to the high capability of TAM to infiltrate solid tumours, here we investigated the effect of tolerance on macrophage migration, *in vitro* and *in vivo*.
For the *in vitro* study we assessed the capabilty of tolernat cells to responde to chemotactic stimuli both by western blot, measuring the phosphorylation of ERK protein, and by Boyden chambre. The air pouch mouse model help us to investigate *in vivo* the behaviour of macrophages from tolerant or non-tolerant animals during an inflammatory response.
Our study shows that LPS-tolerant macrophages maintain their capacity to respond to the chemotactic C5a complement factor, in terms of both cell migration and ERK1/2 phosphorylation. In contrast, LPS-tolerant macrophages did not respond to the chemokines CCL2 and CCL5. By using the air pouch model in mice treated systemically with LPS (in vivo tolerance) we further demonstrated a differential regulation of different leukocyte populations recruitment. In particular, a $F4/80^{+}C5aR\ (CD88)^{+}$ macrophage population was still recruited in response to C5a, in the air pouch of LPS-tolerant mice, supporting the functional activity of this pathway in *in vivo* tolerant conditions.
Future studies in our group will address the role of tolerance in driving selective accumulation of distinct polarized macrophage populations in pathological sites (eg. cancer, chronic inflammatory diseases). We speculate that selective recruitment of tolerant M2 populations may contribute to the extinction of the inflammatory response, thus contributing to restoring tissue homeostasis.

297 Therapy of murine HPV 16-associated TC-1 tumours: suppression of T regulatory and myeloid derived suppressor cells

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Background: Myeloid-derived suppressor cells (MDSC) interfere with tumour immunity, promote tumour growth by inhibiting tumour cell cytotoxicity and facilitate immune suppression and tumour progression. Immunosuppressive CD4+/CD25+ T regulatory (Treg) cells, which play a role in suppressing the function and proliferation of tumour-specific CD4+ and CD8+ T effector cells, represent another major mechanism by which tumours can escape immune recognition. To overcome these effects and improve the effect of immunotherapy, we used gemcitabine or all-trans-retinoic acid (ATRA) for induction of myeloid cell maturation in vivo, or administration of antiCD25 antibody (PC61) for depletion of Treg cells.
Material and Methods: Mice bearing established TC-1 tumour transplants (ca 0.02 cm2) were treated with gemcitabine (120 mg/kg, i.p.) or ifosphamide derivative CBM-4A (150 mg/kg, i.p.) in combination with ATRA (10 mg/kg, s.c., 2 cycles for 5 days) and cytokine (IL-2 or IL-12)-producing irradiated cellular vaccines (ca 40 mg cytokine/105 cells/ml/48 h). Treg cells were removed with PC61 Ab (antiCD25), day 4 after chemotherapy (i.p., 0.3 mg/mouse). For in vitro monitoring of immune response, ELISA and Elispot assays were used according to the manufacturer's instructions.
Results: The treatment with gemcitabine led to a significant tumour-inhibiting effect, to a decrease of the number of MDSC in the spleens of treated animals and to an improved effect of subsequent immunotherapy. The cytoreductive chemotherapy with CBM-4A, which resulted in strong upregulation and accumulation of immunosuppressive immature myeloid Gr-1+/CD11b+ cells in the spleens of the treated animals, was significantly decreased after subsequent therapy with ATRA. Moreover, this drug combination was able to improve subsequent immunotherapy with irradiated TC-1-IL-12 tumour vaccine. Further, it has been found that the removal of Treg cells with P61 Ab exhibited an additive effect to the subsequent immunotherapy with the IL-12-producing vaccine.
Conclusions: Taken together, removal of MDSC and Treg cells in vivo contribute to the boosted efficacy of cytokine-producing cellular vaccines for the therapy of early established tumour transplants minimized after chemotherapy and provided useful information for elaborating the optimal immunotherapeutic strategies for the treatment of HPV 16-associated tumours.
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298 Cartilage oligomeric matrix protein DNA vaccine in transgenic mice developing autochthonous mammary carcinomas

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Background: Through transcriptional profiling of mammary cancer appearing in mice transgenic for the Erbb-2 oncogene (BALB-neuT mice) (Cavallo et al. 2006) and bioinformatics meta-analyses on human genome-wide tumour transcription profile studies (Cavallo et al. 2007) new putative targets for anti-tumour vaccination were identified (Calogero et al. 2008). Among these, cartilage oligomeric matrix protein (COMP), that potently suppresses apoptosis in transformed cells by inducing the transcriptional up-regulation of survivin, one of the Inhibitor of Apoptosis Protein family members overexpressed in virtually all human cancer (Altieri 2003). COMP immunogenicity was tested in vaccination-protection assays in transgenic mice developing autochthonous mammary carcinomas: The Erbb-2 transgenic BALB-neuT mice and PyMT mice, transgenic for the polyoma middle T oncogene (Guy et al. 1992).
Material and Methods: COMP mouse transcript was cloned in pVAX1 vector (Invitrogen®, Milano, Italy). Mice were vaccinated with 50 mg COMP plasmid diluted in 20 μl sterile water with 0.9% NaCl injected twice with a two weeks interval into the quadriceps muscle, followed by electroporation using the CLINIPORATOR™ (Igea, Carpi, Italy). Mammary glands were palpated at weekly intervals to note tumour appearance. The induction of specific anti-COMP antibodies was evaluated in the sera using an ELISA kit (Kaminia®, Seattle, USA).
Results: BALB-neuT mice develop palpable mammary carcinomas starting by week 22 of age, while PyMT mice develop palpable carcinomas starting by week 11 of age. Mice were vaccinated when their mammary glands display atypical hyperplasia and in situ carcinomas (weeks 10 and 12 for BALB-neuT mice; weeks 6 and 8 for PyMT mice). In both BALB-neuT and PyMT mice vaccinated with COMP plasmid, a significant increase in tumour free survival and a significant decrease in tumour multiplicity were observed. Moreover, vaccinated mice developed specific anti-COMP antibodies in the sera.
Conclusions: This study show that COMP is a good target for antitumour vaccination, and that DNA vaccination targeting COMP is a suitable way for breaking immune tolerance.

299 Hypoxia inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing sustained hypoxia

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Background: Ischemia exists in many diseased tissues including arthritic joints, atherosclerotic plaques and malignant tumours. Macrophages accumulate in these sites and upregulate hypoxia-inducible transcription factors (HIFs) 1 and 2 in response to the hypoxia present.
Material and Methods: We used microarrays, qRT-PCR and cytokine release assay to study gene expression in hypoxic primary human macrophages, and then siRNA to block the hypoxic upregulation of HIFs 1α and 2α in order to study their role in regulating gene expression in these cells. We also investigated the role of NF-kB signaling in this phenomenon using both a synthetic inhibitor of p65 nuclear translocation and an adenoviral dominant negative inhibitor of IKKβ.
Result: We show that the expression of a number of genes, including the cell surface receptors, CXCR4 and GLUT1, and potent, tumour-promoting cytokines, VEGFA, interleukins 1β and 8 and adrenomedullin was upregulated in these cells in response to sustained (18h) hypoxia – and that this was regulated by both HIFs 1α and 2α. While hypoxia also stimulated the expression and/or phosphorylation of various proteins in the NF-κB signaling pathway, blockade of NF-kB signaling had little or no effect on the upregulation of these genes in sustained hypoxia.
Conclusions: These studies showed that both HIFs 1 and 2, but not NF-kB, are important transcriptional effectors regulating the responses of macrophages to sustained hypoxia. Further studies using experimental mouse models are now warranted to investigate the role of such macrophage responses in the progression of various diseased tissues like malignant tumours.

300 A novel immunotherapy approach for B-CLL by use of Chimeric TCR

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Background: B-cell chronic lymphocytic leukemia (B-CLL), the most common form of leukemia in adults in Western countries, is characterized by a progressive accumulation of mature $CD19^{+}CD5^{+}CD20^{dim}$ B lymphocytes that over-express the B-cell activation marker CD23. Here we cloned and expressed in T lymphocytes a novel chimeric antigen receptor (CAR) that targets the CD23 antigen (CD23.CAR).