Identification of the antigen(s) being recognised (and any possible interacting proteins), by both antibodies, is being obtained through immunoprecipitation. Reactive bands will be identified using LCMS/LTQ. siRNA targeting, followed by proliferation and invasion assays, will be carried out in order to observe if any knockdown occurs.

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Tumour-derived high molecular weight M-CSF induces monocyte differentiation into M2- polarized macrophages

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Experimental and clinical evidence has highlighted that tumor-associated macrophages (TAM) represent the principal component of the leukocyte infiltrate and are usually associated with tumour growth, progression and metastasis. Macrophage population is generally divided into two distinct subsets: M1 and M2. M1 macrophages act as a first line of defence against pathogens whereas M2 cells participate in wound repair and maintenance of tissue integrity. In the tumour micro-environment TAM interactions with the extracellular matrix, neighboring cells, and soluble stimuli largely influence their gene expression and behavior.

To investigate the role of the tumor micro-environment on macrophage differentiation, we cultured freshly isolated human monocytes with pancreatic cancer cell line supernatants, in the absence of exogenous cytokine addition. In selected cultures, about 50% of the monocytes differentiated after 5 days into macrophages. The phenotype analysis of tumor-conditioned macrophages (TC-macro) demonstrated high expression of the mannose receptor, CD16, CD68 and low levels of MHC class II. TC-macro produced IL-10, IL-6, TNF but not IL-12, even after LPS stimulation. Moreover, TC-macro produced a panel of chemokines including CCL2, CXCL8, CCL17 and CXCL10. The transcriptional profile of TC-macro revealed that several genes in line with an M2 polarization are highly expressed. The nature of the tumor-derived factors inducing macrophage differentiation is currently under investigation; biochemical analysis indicated that the biological activity is excluded from exosomes and have a high molecular weight (>100.000 KDa). IL-3 and IL-6 were not detectable in tumor supernatants whereas M-CSF was present at low levels. By mass spectrometric techniques, we surprisingly found that the tumor-derived M-CSF had peculiar migration patterns which were different from those expected for the common human homodimeric glycosilated protein, suggesting an interesting structural differences for the tumor-secreted isoforms of this primary regulator of mononuclear phagocyte. The characterization of tumor-derived factors inducing macrophage differentiation could better clarify the intricate cross-talk between tumor cells and macrophages and thus might aid in the process of devising novel anti-tumor treatments.

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Neutralization of TGF-beta led to spontaneous elicitation of antitumor immune responses and elimination of tumors in mice administered of DNA encoding soluble TGF-beta receptor

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Tumor cells produce some cytokines to suppress the host immunity for the purpose of escaping from immunological attack by hosts. Among the immunosuppressive cytokines, TGF-beta is known as a key factor which weakens the host antitumor immunity by blocking activation and differentiation of immune cells or by accumulation of regulatory T cells. Although different kinds of cancer immunotherapy have been done, none of the treatments have reported successful clinical outcomes because of the difficulties in eliciting potent antitumor immune responses in cancer-bearing hosts with suppressed immunity. In this study, we tried to neutralize TGF-beta in tumor-challenged mice by administration of DNA encoding soluble TGF-beta type II receptor. B6 mice that were inoculated subcutaneously with EG.7 tumor cells were injected with plasmid DNA 10 to 12 days after tumor challenge. We monitored the tumor growth and examined for anti-tumor immune responses elicited after DNA administration in the mice. The treated mice acquired both humoral and cellular immune responses against the tumor. The frequency of tumor-specific cytotoxic T lymphocytes was significantly increased after treatment. Challenged tumors were eradicated in about 70% of the treated mice. In conclusion, potent antitumor immune responses can be elicited spontaneously by inhibiting TGF-beta function in cancer-bearing hosts. This strategy is applicable to clinical therapeutics against cancer.

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Implication of novel chemokine receptor CXCR7 in hepatocellular carcinoma

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The orphan chemokine receptor RDC1 was de-orphanized and re-baptized CXCR7 since the recent discovery in 2006 of its two ligands, CXCL11 and CXCL12. Membrane associated CXCR7 is expressed on many tumor cells types, promotes breast and lung tumor in-vivo, and increases invasiveness of prostate cancer cell lines. Hence, we investigated if and how CXCR7 is implicated in human hepatocellular carcinoma. To answer these questions we first studied the transcript expression of CXCR7, and of its two ligands in a cohort of 28 cases of human hepatocellular carcinoma. A significant 5 fold increase of CXCR7 was observed in HCC samples relative to normal liver (n=10), and of its two ligands only CXCL11 was over expressed in HCC. Thereafter, immunohistochemical staining performed for both CXCL11 and CXCR7 on HCC paraffin sections revealed that multiple cell types were positive for CXCR7 and CXCL11. Indeed, HCC cells, but as well hepatocytes in regeneration nodules, and proliferating biliary cells, were positive for CXCR7. CXCL11 showed a much broader tissue expression. Furthermore we investigated if in primary hepatic cells, notably hepatocytes and hepatic stellate cells, either CXCR7 or its ligands could respond to cytokines classically involved in the development of HCC. Our results showed that in isolated primary hepatocytes and hepatic stellate cells stimulated by IFN-g, TGF-b, IL-10 and IL-4, CXCL11 responds to IFN-g but no response was observed for either CXCL12 or CXCR7. Interestingly, quiescent human primary hepatocytes do not express membrane CXCR7. However HepaRG cell line, a human HCC cell line which can either differentiate into hepatocyte-like cells or remain in a proliferating phase, showed a strong up regulation of CXCR7 only during proliferation. When HepaRG cell line is cultivated in 0.1% serum conditions, CXCL12 induces proliferation. All together, our data shows that CXCR7 is over expressed in HCC and that activation of CXCR7 might induce pro-survival signals in malignant hepatic cells.

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Targeting CD4+ CD25+ FOXP3+ Treg cells abrogates established mechanisms of immune tolerance, reshuffles the T cell repertoire and results in effective anti-tumor immunity

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The presence of regulatory mechanisms that down-regulate the immune response to ErbB2 oncogene in the periphery has been recognized in human patients and transgenic mice. BALB-neuT mice genetically predestined to develop multiple, fast-growing, invasive, and metastasizing carcinomas are one of the most aggressive models of autochthonous mammary carcinogenesis. These mice are transgenic for the transforming rat-ErbB2 oncogene under the transcriptional control of the mouse mammary tumor virus. Due to Erbb2 transgene expression in the thymus and its over-expression in the mammary gland, CD8+ T cell clones reacting at high affinity with dominant Erbb2 epitopes are deleted. Despite the lack of such a crucial component of immune reactivity, DNA electroporation of a plasmid coding the extracellular and transmembrane (EC-TM) domains of Erbb2 markedly delays the onset of mammary carcinomas when microscopically detectable diffuse in situ carcinomas are present ("early vaccination") but fails to block the progression of invasive carcinomas ("late vaccination"). The protection afforded rests on the activation of CD4+ T cells releasing IFN-gamma and the induction of anti-neu antibodies. Nevertheless, when "early vaccination" is coupled with temporary Treg depletion trough the administration of anti-CD25 mAb, long lasting tumor immunity is induced and the antibody response is enhanced. BALB-neuT mice treated with anti-CD25 mAb and elelctroporated with EC-TM plasmids display a CTL response against the neu immunodominant peptide due to reshuffling of their CD8 T cell repertoire. This new CD8 T cell repertoire is different from that of vaccinated wild type BALB/c mice. Temporary interference with Treg is also instrumental for the induction of an effective immune response in BALB-neuT mice already bearing invasive carcinomas

("late vaccination"). In conclusion, our data suggest that the combination of Treg temporary interference with ErbB2 specific DNA tumor vaccine reshuffles the T cell repertoire, and lead to both preventive and therapeutic anti-tumor immunity.

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Methods for the isolation and identification of MHC-presented peptides from leukaemic cells

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Aberrant kinase activity is implicated in the majority of malignancies. As specific immune responses may be generated to phosphopeptides presented by MHC molecules, the isolation of these peptides from the surface of cancer cells may form a basis for immunotherapy. In chronic myeloid leukaemia (CML) the majority of cases (95%) are caused by the fusion of the bcr and abl genes which results in the production of a deregulated tyrosine kinase, hypothetically resulting in aberrant MHC phosphopeptide expression.

Here we describe the development of a mild acid elution technique to selectively release peptides from MHC class I or II complexes, with minimal contamination by intracellular material or serum proteins. Unlike previous approaches, this method is compatible with immobilised metal ion affinity chromatography (IMAC), a powerful tool for fractionation of peptides and the subsequent simplification of mass spectrophotometric profiles. Fractionation of cell surface eluates from the CML cell line K562-A3 by a range of methods including IMAC and characterization by tandem mass spectrometry lead to the identification of numerous peptides and phosphopeptides, many of which bear strong links to malignancy. These include peptides from: ephrin-A4 precursor, elongation factor 1-alpha 1, MYEOV, and Myc binding protein 2; and phosphorylated peptides from nuclear receptor coactivator 2 and membrane-associated protein HEM-1.

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Analyses of novel tumour antigens as targets for cancer immunotherapy

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The efficiency of current immunotherapy approaches is still far from that expected. In order to identify immunogenic proteins of melanoma and prostate cancer that could be used as target genes for cancer therapy we screened phage-displayed cDNA libraries derived from melanoma prostate cancer, and testis with sera from 76 cancer patients. This resulted in the identification of >1000 different clones, however, only about 10% of them represented cDNAs fused in-frame to the T7 phage coat protein thus ensuring the exposition of natural products of genes on the phage surface. The rest likely represent mimotopes, albeit, there might be true tumour antigens among them that resulted from frame-shifting mutations or defective regulation of alternative splicing or translation.

As none of the identified clones contained a mutation we further looked for novel cancer-germ cell or overexpressed antigens. The criteria for selecting an antigen for expression analyses were: it (1) contains an uncharacterised splice variant, (2) represents a novel gene, (3) shows a cancer associated EST profile or (4) plays an important role in oncogenesis. The expression was tested in a panel of 15 normal tissues and in paired cancerous and adjacent normal tissues of 46 melanoma, breast, prostate and gastric cancer patients using qPCR. We have so far tested the expression of 18 antigens. We saw a testis specific expression for 2 of 3 genes with cancer associated EST profile, but no overexpression in cancerous tissues comparing to the normal counterparts was observed. 2 of 3 novel genes showed a predominant expression in testis and one so far tested also showed a cancer associated overexpression (clone 284). None of the 5 tested functionally relevant genes showed an elevated expression in cancer. 4 out of 7 novel splice variants of known genes were testis specific, and one of these tested so far (clone 29) showed also a cancer-associated overexpression.

The alternative splicing of immuno-privileged tissues like testis is very extensive, hence it is possible that due to splicing defects often observed in cancerous tissues such testis-associated isoforms could be formed and recognised by the immune system, leading us to hypothesise that analogously to cancer-germ cell expressed antigens a category of cancer-germ cell spliced antigens might exist. Clones 29 and 284 will further be subjected to T cell activation assays to test their potential to be used in cancer immunotherapy applications.

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CD8+ cytotoxic T lymphocytes generated against a WT1 peptide analog enhance the lytic activity of leukemic cells

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Background: The WT1 antigen performs an oncogenic function in various types of cancer. It is overexpressed in human leukemias and therefore it has been considered as an attractive target for immunotherapy. Most WT1-specific CTLs described displayed a low avidity and exerted minimal lytic activity against cancer cells.

Materials & Methods: We used an approach to improve the immunogenicity of CTL epitopes consisting of substituting the first-amino-acid, of 2 known HLA-A0201-restricted WT1-derived peptides (Db126 and WH187), with tyrosine (Y).

Results: This modification resulted in the enhancement of the binding ability of the 126Y analog and CTL generated against this peptide exerted a significantly lytic activity against the 126Y peptide-loaded target cells and importantly cross-reacted with the 126N native peptide. Another interesting finding is the significant high lytic activity recorded for the 126Y CTL against freshly isolated HLA-A0201-matched leukemic cells expressing the WT1 antigen. This data confirms that T cells generated against the 126Y analog peptide cross-react also with the naturally processed 126N native peptide. Moreover, it seems that stimulation with the peptide analog induced CTLs with a high TCR avidity. Finally, the high lytic activity provoked by the 126Y CTL may be also attributed to the significant high number of anti-126 T cell frequencies in this T cell line as demonstrated by IFN-gamma production in the ELISPOT assay.

Conclusions: This study provide evidence that peptide modification results in a better immune response against cancer and further support the use of this strategy as a potential approach for the development of a leukemia-vaccine.

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Study of Lewis y expression and anti Lewis y immune response through Lewis y-circulating immune complexes detection in breast cancer patients

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The aims of this study were: 1-to detect Lewis y (Ley) antigen in breast tumor samples; 2- to determine the role of MUC1 as a carrier of Ley and 3- to investigate the induction of humoral immune response through the detection of circulating immune complexes (CIC) carrying Ley in breast sera. Materials and methods: 137 breast tissue and serum samples: 72 malignant tumours, 30 benign diseases and 35 normals. The expression of Ley was determined by standard immunohistochemistry (IHC); percentage of stained cells, intensity and pattern of the reaction were analyzed. An immunoprecipitation was performed in order to determine if MUC1 may behave as a possible carrier for Ley. HMFG1, an anti MUC1 monoclonal antibody (MAb) was used to precipitate MUC1 from breast cancer sera. Immunoprecipitates (IP) obtained were run in SDS-PAGE and Western blot (WB) assays. Sheets were incubated with C14 (anti Ley) and HMFG1. An ELISA was developed to study the presence of Ley/CIC. Briefly, C14 was adsorbed in multiwell microplates and incubated overnight at 4ºC. After washing, 1% bovine serum albumin/PBS was added for 3 hours at 37ºC. Serum samples were incubated overnight at 4ºC and 1:2000 anti human IgM or 1:3000 IgG reacted with the complexes and revealed with ABTS and 30% H2O2 in sodium citrate buffer, pH 5.0; OD was measured at 405 nm. Results: By IHC with C14, positive results were found in 34% malignant tumors; 33% benign diseases and 35% normal samples; no statistical difference was found. The pattern of expression differed between malignant and non malignant samples: cancer specimens showed more frequently a cytoplasmic and membrane non apical reaction while non malignant samples showed an apical membrane reaction. By WB, IP displayed a band at >200KDa with both C14 and HMFG1 MAbs. By ELISA, mean OD for IgM/LeyCIC in breast cancer sera, benign and normal samples were: 0.538, 0.949 and 0.942, respectively. By ANOVA, significant statistical differences between breast cancer and normal and benign samples were found. Mean OD for IgG/LeyCIC were: 0.414, 0.438 and 0.492,