IL-12. In untreated mice, the tumor growth started 120 days after study initiation. In contrast, in mice immunized with the multi-epitope vaccine the tumor free interval was extended up to 140–190 days; the tumor free interval in mice immunized with the peptides and IL-12 was prolonged even up to 235 days. Once tumors developed, those mice immunized with peptides+IL-12 showed a significantly slower tumor progression than mice not or sham immunized. Characterization of the immune responses revealed that mice immunized with peptides+IL-12 displayed higher IgG2a levels in serum and Th1 biased immune responses (IFN γ) *in vitro*.
From our data, we conclude that immunization with a multi-epitope vaccine in conjunction with IL-12 is very effective in preventing progression of Her-2/neu overexpressing tumors. Such a vaccine could be used in humans together with chemotherapy and/or for prevention of metastases.

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Immunization with genetic vectors expressing rhesus CEA efficiently breaks immune tolerance in mice and rhesus monkeys

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Background: CEA is a 180KDa glycoprotein over-expressed in a high percentage of adenocarcinomas, particularly those of the colon, pancreas, breast and lung. For this reason, it is currently under evaluation in clinical trials as target for immunotherapy in the treatment of colorectal cancer.
Materials and Methods: To demonstrate that genetic vaccination with vectors expressing this tumour antigen is capable of specifically breaking immunotolerance in non-human primates, it is necessary to use the equivalent of human CEA. Since the rhesus monkey (*macaca mulatta*) homologue of this human tumour associated antigen was not available, we have identified and cloned rhesus CEA (rhCEA) from colon tissue samples. rhCEA is an open reading frame of 2118 nucleotides encoding for a 705 aa polypeptide with 78.9% homology to human CEACAM-5 protein.
Results: Vaccination protocols using rhCEA expressing vectors were designed both for mice and rhesus monkeys.. To demonstrate the capability of xenogeneic vaccination to elicit an immune response against CEA as self-antigen in this model, we immunized CEA. Tg mice with vectors encoding either human (homogeneic) or rhesus CEA (xenogeneic).
After treatment of mice with DNA followed by EGT (Electro Gene Transfer) and adenovirus boosting, cross-reactive antibodies against human CEA protein were measured only in rhesus CEA immunized groups. Importantly, cellular immune-response against human CEA was observed upon immunization with rhesus CEA both in wild type and transgenic mice.
To further increase the level of antigen expression, we have constructed a synthetic codon usage optimized rhCEA cDNA (rhCEAopt). *In vitro* studies showed 10–50 fold greater protein levels than a similar vector carrying the native cDNA. Similarly, intramuscular injection of a DNA vector followed by EGT or Adenovirus expressing rhCEAopt in CEA. Tg mice resulted in greater protein levels than those detected upon injection of vectors encoding for rhCEA. Mice immunized with plasmid/ adenovirus vector mixed modality, both containing the cDNAopt showed strong cross reactive human CEA-specific antibody response, 300-fold higher than hCEA containing vectors. Cell mediated responses were two- and three-fold higher against rhesus or human protein, respectively, than using the vectors containing the native rhCEA.
To assess the efficiency of immunization of rhesus macaques with rhesus CEA, we injected vectors encoding for rhCEA or rhCEAopt in twelve monkeys. Both Ad vectors alone or in combination with DNA were efficient in breaking immune tolerance to CEA in immunized rhesus monkeys and maintain over time elicited immune response.
Conclusions: Our data show that use of rhesus CEA and development of modified expression cassettes that result in increased potency of Adenovirus, plasmid DNA and other gene delivery vaccine approaches may have significant impact on vaccine development against neoplastic malignancies expressing CEA.
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Synergistic antitumor activity of interleukin 23 and interleukin 2

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The recently discovered IL-23 shared with IL-12 similar heterodimeric structures and overlapping but distinct functions in the regulation of both innate and adaptive immunity. IL-12 has been shown to confer potent antitumor activity in a variety of murine tumor models. In our previous study, we showed that IL-23 also possessed potent antitumor activity. CT26 colon adenocarcinoma cells transduced with retroviruses carrying a single-chain IL-23 gene (CT26/IL-23) grew progressively until day 26 to an average size of 521 ± 333 mm^3 and then tumors started to regress in most animals, resulting in a final 70% rate of complete tumor rejection. In the present study, we seek a possible cooperative antitumor effect of IL-23 and IL-2. CT26 cells engineered to secrete both IL-23 and IL-2 (CT26/IL-23/IL-2) produced only a transient tumor growth, followed by complete rejection in all animals. Most significantly, transduction of both IL-23 and IL-2 resulted in significant reduction of lung tumor metastasis and led to 60% of mice survived the challenge, while all animals challenged i.v. with IL-23- or IL-2-transduced CT26 cells eventually died of lung metastasis. *In vivo* depletion experiment showed that rejection of CT26/IL-23/IL-2 tumor cells required both $CD4^+$ and $CD8^+$ T lymphocytes. Immunohistochemical analysis revealed tumor moderate infiltration of $CD4^+$ and $CD8^+$ T cells, and abundant infiltration of granulocytes ($Gr\text{-}1^+$) and macrophages ($Mac\text{-}1^+$) when tumors were in regression. We are currently investigating whether granulocytes and macrophages play a role in the IL-23/IL-2-mediating antitumor activity.

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Strong Melan-A/MART-1 specific CD8+ T cell responses to peptide vaccination in young melanoma patients

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Tumor vaccines aim to induce strong specific T cell activation in vivo, which may well result in enhanced immune protection. It remains poorly understood why T cell responses are detectable in some but not all patients. We have previously shown that tumor-driven CD8+ T cell pre-activation is one of the factors associated with increased T cell responsiveness to peptide vaccination. In search for further parameters, we analyzed whether patient age may play a role. Eight stage III/IV melanoma patients (34–75 years old) were treated with four monthly low dose vaccinations with CpG oligodeoxynucleotide 7909, mixed with Melan-A analog peptide and Incomplete Freund's Adjuvant. We used fluorescent HLA-A2/Melan-A multimers (tetramers) to measure T cell frequency ex vivo in circulating blood by flow cytometry. High percentages (between 0.07 and 3.42%) of Melan-A peptide specific CD8+ T cells were found after vaccination, revealing strong T cell responses in all eight patients. Interestingly, we found a statistically significant (P <0.01) inverse correlation between T cell responses and patient age. Thus, besides tumor-driven T cell pre-activation, young patient age is an additional parameter predicting T cell responsiveness to vaccination with tumor peptides.

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First clinical evidences of antigen spreading in metastatic melanoma patients treated with a NGcGM3/VSSP/Montanide ISA 51 vaccine: A Phase I/IIb study

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Background: N-glycolyl GM3 ganglioside immunodominant epitope is expressed on human breast and melanoma tumours but absent from normal melanocytes. This unique feature renders this antigen very attractive for immunotherapy. NGcGM3 was non-covalently incorporated in the natural outer membrane vesicles of *Neisseria meningitidis* to form very small sized proteoliposomes (VSSP) and emulsified with Montanide ISA 51. In previous studies the immunogenicity and safety of this vaccine have been documented but in breast cancer patients. With this phase I/IIb study we intended to evaluate, in metastatic melanoma patients, the immunogenicity and safety of the preparation at two different dose levels. Patient's monitoring for clinical responses was also planned.
Methods: Twelve and nine metastatic melanoma patients received 0.2 or 0.4 mg of the vaccine in each of the 9 immunisations, respectively. The first 5 IM doses (induction phase) at two weeks intervals, while the remaining

of the treatment was given monthly. Serum Anti-NGcGM3 antibody titers were determined by ELISA. Patients were regularly submitted to CT scans and US during the study. WHO's toxicity criteria were applied.
Results: Vaccination induced specific antibody titers (IgM; range 640–2560 and IgG; range 80–2560) at both dose levels. Though anti-NGcGM3 serum antibodies increased with the higher dose of vaccine clinical outcome was more significant for the lower one. It was encouraging to observe objective responses in 3 out of 10 valuable patients of the 0, 2 mg dose level (regression of cutaneous metastases and stabilization of lung lesions for 22 months). Other patients showed mixed responses with elimination, stabilization or progression of cutaneous tumours. Main toxicities included erythema, mild local pain, and low grade fever. Most noteworthy vitiligo was developed by 4 patients after being injected at least 6 times with the lower dose of the vaccine.
Conclusion: For the first time evidences of the antigen spreading phenomena have been clinically observed (the appearance of vitiligo in patients only can be explained over the spreading basis because NGcGM3 epitopes are absent from normal melanocites but present in melanoma). The vaccine was safe and immunogenic also in advanced melanoma patients. These encouraging first clinical evidences of the NGcGM3/VSSP/Montanide ISA 51 vaccine effectiveness in stage IV (without surgical resection) melanoma patients guarantee further investigation.

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Biological characterisation of CD40-activated B cells 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 challenging, e.g. pediatric patients or 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 and efficient presentation of antigen to $CD8^+$ T-cells.
However, the induction of naïve and the amplification of memory $CD4^+$ T cell responses is most likely a prerequisite of potent cellular adjuvants. Antigen uptake and presentation by B-cells is highly epitope-specific. It therefore remains unclear whether CD40-B, have the capacity to take up, process and present a broad range of antigens in the context of MHC class II.
Here we address, whether CD40-B process antigens in the context of MHC class II and induce secondary and 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 and KLH, as model protein antigens, 2) the artificial promiscuous MHC class II binding peptides PADRE-AKF and PADRE-AKX as model peptide-neoantigens. While specific cells were successfully expanded for all antigens studied, INF-γ and IL-4 ELISPOT profiles did not indicate a dominant TH_1 or TH_2 bias.
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. 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 T-cells.
Taken together, CD40-B cells efficiently induce primary MHC class I and II restricted T-cell responses, have potential to home to secondary lymphoid organs and induce T-cell chemotaxis. These data further support the potential of these autologous APC as adjuvants in cancer immunotherapy.

Cellular therapies and cytokines

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Pharmacokinetics and pharmacodynamics of recombinant human IL-18 (rhIL-18) in patients with solid tumors

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Background: rhIL-18, a Th-1 inducing cytokine, has demonstrated anti-tumor activity in a variety of preclinical models. Exposure and biomarker response data from the first human trial are described below.
Materials and Methods: Patients with solid tumors received doses of rhIL-18 (SB485232) ranging from 3–1000 mcg/kg administered as 2h infusions daily for 5 consecutive days. Assessments included tolerability, immunogenicity, anti-tumor activity, pharmacokinetics, and biomarkers of immunomodulatory activity to define a potentially biologically effective dose range. Plasma drug concentrations were measured by ELISA. Cytokine/protein biomarkers were measured by ELISA and cytokine biochip. Cell activation biomarkers were measured by flow cytometry.
Results: Twenty-six patients were evaluable for these analyses, including 21 with metastatic renal cell carcinoma, 4 with melanoma, and 1 with Hodgkin's lymphoma. Plasma concentrations of rhIL-18 exhibited 2.5-fold accumulation with daily dosing, a dose-independent accumulation half-life of 35h, and a tri-phasic nonlinear dose-concentration relationship. Clearance and volume of distribution were dose-dependent, largely due to saturable binding to IL-18 binding protein, a high-affinity circulating modulator of rhIL-18 activity induced through INF-gamma. Dose-related induction of INF-gamma and GM-CSF were observed, as well as increased levels of downstream chemokines. Lymphopenia was maximal by 8h after the first infusion at all dose levels. Drug related increases were observed in expression of FasL on CD4, CD8, and NK cells, expression of CD69 on $CD8^+$ cells, and expression of CD11b on NK cells and monocytes. Evidence of clinical activity was observed in two patients at the 100 mcg/kg dose, and associations with biomarkers were explored.
Conclusions: These data demonstrate the complex pharmacokinetic behavior, immunomodulatory activity, and therapeutic potential of rhIL-18.

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P43/Endothelial monocyte-activating polypeptide-II: protein exaptation provides a novel counterattack mechanism in colorectal tumours

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P43 is an auxiliary component of the mammalian multisynthase complex, playing a central role in protein synthesis. This 34kDa protein is normally retained intracellularly; however, in what appears to be an example of protein exaptation (appropriation of a molecule with one function for a different purpose), tumour cells may express this protein in a soluble extracellular form, known as Endothelial Monocyte-Activating Polypeptide II. EMAP-II has multiple cytokine-like activities, inducing procoagulant activity on the surface of endothelial cells, increasing expression of E-and P-selectins and tumour necrosis factor (TNF) receptor-1, and directing migration of monocytes and neutrophils. Since it is not biologically advantageous to a tumour to attract lymphocytes, we hypothesized that EMAP-II might simultaneously attract phagocytic cells while suppressing the activity of lymphocytes. We therefore investigated the effects of p43/EMAP-II on lymphocytes. Recombinant protein induced apoptosis in mitogen-activated peripheral blood lymphocytes, and in Jurkat T-cells. We then examined EMAP-II expression in the HT29 and DLD-1 colorectal cancer cell lines. Both express extracellular p43/EMAP-II; however HT29 retain it on the external cell surface, while DLD-1 cells release a soluble, biologically active 20kDa fragment of the molecule. Co-culture of Jurkat cells with HT29 cells induced activation of caspase 8 and apoptosis in the Jurkat cells, which was partially blocked by addition of neutralizing antibodies against p43/EMAP-II. Conditioned medium from DLD-1, but not HT29 cells, had similar effects, suggesting that both membrane-bound and soluble forms of p43/EMAP-II can induce lymphocyte apoptosis. We conclude that p43/EMAP-II in its extracellular form plays multiple roles in the tumour micro-environment, one of which is to assist in immune evasion by providing a counterattack against cytotoxic T-cells. Tumour-associated exaptation may provide a new source of therapeutic targets.