Methods: Cytotoxic activity of control non transduced T cells (NT) and CAR^+ T cells was measured using a standard ^{51}Chromium release assay. Co-culture experiments of NT and CAR^+ T cells (1×10^6 cells/well) with viable LCLs (ratio 1:1) in the presence or in the absence of serial dilutions of B-CLL patients plasma enriched in soluble CD23 (sCD23) have been performed to assess sCD23 mediated inhibition of the CD23.CAR^+ T lymphocytes cytotoxic activity. The expansion of CAR^+ T lymphocytes in response to $CD23^+$ targets has been proved by weekly stimulation with allogeneic, g-irradiated (30 rads) LCLs (ratio 1:1), without addition of exogenous cytokines. IFN-g, TNF-a and TNF-b release was measured with a Flow Cytomix Assay, while IL-2 production was measured using a specific Enzyme-Linked Immunosorbent Assay. Soluble CD23 levels of B-CLL patients-derived plasma samples have been detected using a human CD23 ELISA kit.
Results: CD23.CAR^+ T cells showed specific cytotoxic activity against $CD23^+$ tumour cell lines (average lysis 54%, at Effector:Target (E:T) ratio 40:1) and primary $CD23^+$ B-CLL cells (average lysis 58%, at E:T ratio 20:1). This effect was obtained without any toxicity against normal B lymphocytes, differently from other CARs that target CD19 or CD20 antigens expressed by leukemic cells, but physiologically also by normal B lymphocytes. Moreover, CD23.CAR^+ T cells released inflammatory cytokines (4-fold more IFN-g, ??157-fold more TNF-a and 1445-fold more TNF-b) in response to $CD23^+$ target cells. IL-2 was also released (average release 2.681 pg/mL) and sustained the antigen-dependent proliferation of CD23.CAR^+ T cells.
Conclusions: Altogether these data suggest that gene modification of T cells to express the CD23.CAR represents a selective immunotherapy approach to eliminate CD23+ leukemic cells, while sparing normal B lymphocytes, in patients with B-chronic lymphocytic leukemia.

301 Combined immunogene therapy and Treg inactivation in treatment of weakly immunogenic solid tumours

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Background: The majority of cancers occur against a background of normal immune function and evidence suggests that host tumour antigen-specific effector and memory T cells should eliminate the neoplastic cells. Cancers avoid immune elimination by a number of interrelated mechanisms, which occur predominantly within the region of the tumour itself. Anti-tumour immune responses are subjugated by tumour-mediated immunosuppressive mechanisms that render the host tolerant to the cancer and thus create obstacles to immune therapies. This compartmentalised immune evasion does however provide an opportunity for the design of a regionally-based immunotherapy. Establishing an immune responsiveness at the level of the primary tumour would also inhibit progression of metastatic disease, as the antigen spectrum is similar on the primary and metastatic cancer cell.
We present an effective immune-based therapy of weakly immunogenic tumours using locally delivered immunogene therapy and systemic T regulatory (Treg) cells inactivation. The aims are obtained by promoting the development of immune effector responses in the tumour environment, and potentiate these responses by elimination of tolerogenic or immune suppressor influences.
Material and Methods: We investigated the tumour models murine fibrosarcoma (JBS) and colon carcinoma (CT26). Plasmids encoding GM-CSF and B7−1 were electrically delivered into tumours and Treg inactivation was accomplished by systemic administration of anti-CD25 antibody.
Results: Complete eradication of tumours was achieved at a level of 60% by immunogene therapy, 25% by Treg inactivation and 90% by the combined therapies. Cured animals by Treg inactivation, were resistant to re-challenge using the Winn assay. Cured mice displayed no signs of autoimmune disease after one year follow up and the antitumour responses were non cross reactive with normal tissues.
Conclusions: Combination of immunogene therapy and Treg inactivation constitutes an effective treatment which results in the eradication of weakly antigenic solid tumours. The combination augments the total effect of either treatment. The therapy was immune specific, tumour specific, transferable, safe and effective. This therapeutic model should be considered for clinical development as a primary or neoadjuvant therapy.

302 The effect of differential in vitro regulation of NKG2D and CD161 NK cell receptors by IL-2 or IFN-a on activation of NK cells in metastatic melanoma patients

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Background: In metastatic melanoma (MM) immunomodulating agents such as IL-2 and IFN-α have shown therapeutic response. Considering that first line of antitumour immune defense is mediated by natural killer (NK) cells, and that NK cell activity is often impaired in MM it was of interest to study the effect of these cytokines on the less investigated CD16-defined NK cells and their dim and bright subsets.
Material and Methods: Peripheral blood lymphocytes (PBL) obtained from 45 MM patients in clinical stage IV prior to therapy treated for 18 h with 200 IU/ml IL-2 and 250 IU/ml IFN-α were evaluated for NK cell cytotoxicity. Expression of NKG2D, CD161, CD158a, CD158b receptors was analyzed on $CD3^-CD16^+$ NK cells by FACS flow, pSTAT1 and pSTAT5 protein expression by Western blotting, and mRNA for IFN regulatory factor-1 (IRF-1) after 4 h by rt-PCR.
Results: Both cytokines induced significant in vitro enhancement of NK cell cytotoxic activity. IL-2 induced NKG2D, while IFN-α induced NKG2D and CD161 receptor expression on NK cells and $CD16^{bright}$ subset, with no effect on the expression of investigated KIRs. Furthermore our results show that in MM patients only the induction of NKG2D with IL-2 on CD3-CD16+ NK cells and on $CD16^{bright}$ subset correlates with its enhancement of NK activity. Contrary to this, the induction of NKG2D by IL-2 on the regulatory $CD16^{dim}$ subset does not correlate with augmented NK activity. We found substantial specific inducibility of pSTAT1 and pSTAT5, as well as induction of IRF-1 transcription by IFN-α in PBL of investigated patients.
Conclusions: Although NK cell-killing of tumour cells depends on the balance of stimulatory and inhibitory signaling there is no data on IL-2 and IFN-α mediated NK cell activating receptor induction, especially on $CD3^-CD16^+$-defined NK cells or their cytotoxic $CD3^-CD16^{bright}$ subset. By showing differential induction of activating receptors with IL-2 and IFN-α, we found for the first time that IL-2 and IFN-α in vitro enhanced NK cell activity of MM patients would be in favor of IL-2, as it has more extensive correlation with NKG2D induction on $CD3^-CD16^+$ or cytotoxic $CD3^-CD16^{bright}$ NK cells. The obtained IL- enhanced NK cell cytotoxicity may result, as opposed to IFN-α, from its better up-regulation of numerous cytotoxic mediators. The results obtained in this study support the shown therapeutic effects in MM of these cytokines, applied in immunotherapy alone, or in combination with chemotherapeutic agents.

303 Cytotoxic treatment for rectal cancer reveals different innate immunity in responder and non-responder patients

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Background: Neo-adjuvant radio-chemo therapy for rectal cancer has dissimilar outcomes, with about 70% of partial/complete responses and 30% of no responses at surgery. Little is known on the role that innate immune responses play in the outcome of conventional therapies.
Material and Methods: We performed a prospective study in which we analyzed serial tumour and blood samples of 32 consecutive rectal cancer patients who underwent neo-adjuvant therapy. We characterized *in-situ* cell death, circulating monocytes, infiltrating macrophages and inflammatory molecules in the blood.
Results: 10 underwent complete pathological remissions, 10 had partial responses and 12 had no responses. In the responders we observed: after an initial expansion, a decrease in the number of circulating monocytes; an up-regulation of monocytes CD16 expression and a clear expansion of CD14+CD86+ monocytes at the earlier time points. The latter event was transient as it abated at the later time point. The therapy caused a decrease of hemoglobin concentration in all the pts. This was related to an expansion of CD14+CD163+ monocytes in responder pts only. In non-responder pts we observed: higher plasma concentration of C1q, C3 and C4; and a significant increase of CRP concentration at the end of the therapy and an expansion of Tie2 expressing monocytes.
Conclusions: The preliminary characterization of the macrophage infiltrate suggests a possible bias toward an alternative M2 polarization. These data suggest that neo-adjuvant therapy modulates the cellular and the humoral innate immune responses and that this response correlates with clinical outcomes. It could either reflect an heterogeneity in the response to primary inflammation signals, elicited as a consequence of cell death, or different patterns in the anti-inflammatory clearance of cell debris, that influence the long term outcome of the treatment.

304 Myeloid response and macrophage polarization in mouse melanoma lung metastasis

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Myeloid derived cells contribute to tumour growth by suppressing immune responses and providing the tumour cells with inflammatory cytokines. Monocytes acquire different functional traits during polarization to the classical

pro-inflammatory M1 or the alternative anti-inflammatory/pro-tumoural M2 macrophage. The macrophage mannose receptor (MR) has been found up-regulated in the M2 phenotype and has been shown to be essential for cytokine production. MR might also interact with other canonical pattern recognition receptors in order to mediate intracellular signalling.
In an experimental model of mouse melanoma lung metastasis, we aim to describe the recruitment of myeloid cells into the lung after tumour cell challenge in a time dependent manner. Twenty-four hours after injection of B16F10 cells into the tail vein of C57BL/6 mice we observed a marked infiltration of $CD68^+CD11b^+CD11c^-$ monocytes into the lung. A fraction of these monocytes was $Gr\text{-}1^+$. The infiltration ceased within 48 h. In C57BL/6 mice lacking MR ($MR^{-/-}$), recruitment of these monocytes was abrogated. Three weeks after tumour cell injection, fewer lung colonies were scored in the $MR^{-/-}$ than in the wild type mice, suggesting a possible role for MR both in early and late stages of metastasis formation.
We aim to further characterize the monocyte and macrophage populations involved in lung colony formation with particular interest in the expression of macrophage polarization-related genes. We are using PCR arrays on monocytes and macrophages sorted from the mouse lungs following tumour cell challenge. The candidate polarization genes as potential targets in melanoma lung metastasis will be discussed.

305 Detection of circulating galectin-1 in the microvesicle fraction of serum from breast cancer patients

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Background: Galectin-1 is a β-galactose binding lectin implicated in tumour progression through its ability to regulate tumour cell migration, metastasis, and tumour-immune escape. Galectin-1 can be produced either by tumour cells or tumour stroma. High expression of galectin-1 by stromal cells in the tumour milieu has been associated with breast cancer invasiveness and progression.
Objective: To further evaluate the role of galectin-1 in breast cancer, we analyzed its expression in the microvesicle fraction of patients' serum.
Material and Methods: This study included serum samples from 12 breast cancer patients from I, II and III stages; also, 6 healthy women serum samples were analyzed as controls. Six milliliters of serum were centrifuged at $15{,}000 \times g$ for 30 minutes at 4ºC; supernatants were subjected to CL2B agarose column gel chromatography; void volume was ultracentrifuged at $105{,}000 \times g$ for 2 h at 4ºC and the pellet (microvesicles fraction, MV) was washed and resuspended in PBS. Further analysis by means of Western blot, electron microscopy and EpCAM+ magnetic isolation was performed. MV fractions were also applied to sucrose gradient centrifugation.
Results: 6 out of 12 patients showed galectin-1 expression in MV by Western blot; in these patients, galectin 1 was also detected in their EpCAM+ enriched fraction. MV electron microscopy showed the presence of a heterogeneous collection of membranous vesicles and nonmembranous particles ranging from 40 nm to 1 micron in diameter. Control samples did not show presence of microparticles in the pellets. Sucrose gradient centrifugation confirmed the presence of galectin-1 in both low and high density fractions although CD63 and Hsp70 exosome markers were not detected. Electron microscopic of low density fractions included typical MV and lipoprotein images.
Conclusions: Circulating galectin-1 in sera from breast cancer patients may be associated with tumour derived microvesicles which could control antitumour responses.

306 Identification of novel cancer-testis antigens by studying humoral response against cancer

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Exploration of humoral responses to cancer may reveal diagnostic and prognostic biomarkers and may help to discover potential immunotherapeutic targets. In a previous study we identified a set of 1321 antigens eliciting humoral responses in patients with melanoma, prostate or gastric cancer by phage display-based SEREX approach and studied the frequency of autoantibody responses to these antigens by exploiting phage-displayed antigen microarrays. The goal of the current study is to identify novel cancer antigens that could be used for development of polyantigenic immunotherapy approaches.
An initial set of 49 potential therapeutic targets was selected by including antigens with cancer-associated autoantibody responses, low or absent mRNA expression in normal tissues or the presence of cancer-associated splice variants determined by *in silico* analysis and no previous knowledge of their immunogenicity. These genes were subjected to sequential analysis of their mRNA expression in 14 different normal human tissues by real-time RT-PCR, followed by cancerous and adjacent normal tissue pairs from 50 patients with melanoma, breast, colon or gastric cancer and then by immunohistochemistry.
mRNA expression analysis in normal tissues revealed that 15 out of 49 antigens were expressed preferentially in immunoprivilleged tissues such as testis. Nine of them, including SPAG8, SPAG16, CFL1 etc, were expressed at various levels and frequencies in cancerous tissues. Restricted expression of SPAG8 in normal tissues was confirmed by IHC on tissue arrays. Interestingly, 4 of them are encoded by testis-restricted splice variants of ubiquitously expressed genes. We propose that deregulation of splicing controls in cancer cells may result in the production of splice variants that are normally produced only in germ cells and if these protein isoforms are expressed in cancers they may elicit immune response in cancer patients, hence representing novel category of tumour antigens − "cancer-testis spliced" antigens.
Thus, the systematic analysis of humoral responses to cancer revealed 5 novel cancer-testis antigens and a novel category of tumour antigens – cancer-testis spliced antigens and all of them are a subject to further analysis of their immunogenicity and relevance as immunotherapeutic targets.

307 Anti-cancer immune reaction induced by cryo-ablation therapy

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Background: Cryogenic treatment sometimes stimulates the immune system by releasing intracellular antigens. We evaluated anti-tumour immune response after cryotherapy by analyzing alterations in serum cytokine levels.
Methods: Percutaneous cryosurgery was performed in 13 patients with unresectable advanced tumours. The size of the ice ball was confirmed by intraoperative ultrasound 15 minutes freezing to make a 3 cm ice ball. The therapy was performed for three freeze/thaw cycles per a tumour per a treatment and was continued eight times for once a week. Evaluation of serum factors was done before and after ablation therapy, and serum tumour markers were measured after every four treatments. Tumours were evaluated by abdominal computed tomography after eight treatments. Serum levels of interleukin (IL) -4, -6, and -10, tumour necrosis factor (TNF)-alpha, and interferon (IFN)-gamma were measured by ELISA. The Th1/Th2 ratio was estimated from the IFN-gamma/IL-4 ratio.
Results: In five cases, tumour necrosis was identified not only in the treated area but also away from the treated area, and then these cases belonged to immune reaction (IR) group. In other cases, just local effect was noted (LE), and then levels of serum factors were compared with those in IR. Serum levels of AA and CRP were increased in both the IR and LE groups after the third treatment, and that of IL-6 paralleled CRP increases. No differences in the level of serum IL-2 was observed after treatment in any of the patients. The serum level of IL-10 was low in three patients in the IR group and in one patient in the LE, but it group tended to increase with the number of treatments. In contrast, the level of TNF-alpha was increased in the IR group but showed no remarkable changes in the LE group. The Th1/Th2 ratio was increased in the IR group, compared to that in the LE group. To evaluate the clinical significance of these alterations in serum cytokines, pretreatment levels, maximum levels in response to therapy, and the number of treatments necessary to induce maximum levels were compared between the two groups. Pretreatment levels of IL-10 in the LE group were significantly greater than those in the IR group ($p = 0.0071$), and the maximum value (67.9 ± 6.3 pg/mL) was greater than that for the IR group (58.4 ± 8.1 pg/mL), but no significant difference was found between the two groups. In contrast, both pretreatment levels and maximum levels in response to treatment of TNF-alpha were significantly greater in the IR group than in the LE group. The maximum Th1/Th2 ratio was significantly greater in the IR group than in the LE group, despite the factor that pretreatment levels and treatment times to induce maximum levels were similar between the two groups.
Conclusion: It might be possible to evaluate the appearance of immune responses to cryosurgery by monitoring serum cytokine levels.

308 Characteristics of NK cells isolated from regional lymph nodes of melanoma patients

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Background: Melanoma is an aggressive but also immunogenic malignant tumour. The first line of antitumour immune defense is mediated by natural killer (NK) cells that able to lyse malignantly transformed cells and also may play an important role in lymphoid organs in the control of spreading of malignant tumours. As NK cell activity against malignantly transformed cells is regulated by the balance between activating and inhibitory signals mediated by NK cell receptors, the aim of this study was to investigate the expression