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ABSTRACT BOOK

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IMPAIRED TOLL-LIKE RECEPTOR 8-MEDIATED IL-6 AND TNF-ALPHA PRODUCTION IN ANTIGEN-PRESENTING CELLS FROM PATIENTS WITH X-LINKED AGAMMAGLOBULINEMIA.

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The critical role of Bruton's tyrosine kinase (Btk) in B-cells has been documented by the block of B-cell development in X-linked agammaglobulinemia (XLA). Less is known about Btk function in myeloid cells. Several pieces of evidence indicate that Btk is a component of Toll-like receptors (TLRs) signaling. We analyzed whether Btk deficiency in XLA is associated with an impaired dendritic cells (DCs) compartment or defective TLR signaling. We analyzed the expression of TLRs 1-9 on myeloid DCs generated from XLA patients, and evaluated their response to activation by specific TLRs agonists. We show that XLA patients have normal numbers of circulating DCs. Btk deficient DCs have no defect in response to stimulation of TLRs 1/2, 2/6, 3, 4 and 5, but display a profound impairment of IL-6 and TNF-alfaproduction in response to stimulation by TLR-8 cognate agonist, ssRNA. These findings may provide an explanation for the susceptibility to enteroviral infections in XLA patients.

KINETICS OF DENDRITIC CELLS RECONSTITUTION AND COSTIMULATORY MOLECULES EXPRESSION AFTER MYELOABLATIVE ALLOGENEIC HEMATOPOETIC STEM CELL TRANSPLANTATION - IMPLICATIONS FOR THE DEVELOPMENT OF ACUTE GRAFT VERSUS HOST DISEASE.

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Allogeneic hematopoetic stem cell transplantation (allo-HSCT) with myeloablative conditioning represents a unique opportunity to monitor the kinetics of reconstitution of hematopoetic cells. Dendritic cells (DCs) are the most efficient antigen presenting cells. Two distinct subsets of DCs have been identified, myeloid (mDCs) and plasmacytoid (pDCs) that have different roles in the regulation of immunity. In immature state DCs express low levels of costimulatory molecules and have very low capacity to stimulate antigen specific T cells. After activation, DCs undergo a process termed maturation characterized by an increased expression of costimulatory molecules and cytokine production. Mature DCs migrate to lymphoid organs and activate antigen specific T cells. In this pilot study, we analyzed the kinetics of DCs subsets reconstitution in 5 patients (age range: 5-18 years) undergoing unmanipulated allo-HSCT from unrelated donor. Myeloid DCs were defined as lineage -, CD11c+, HLA-DR+ and pDCs as lineage -, CD123+ and HLA-DR+. We further evaluated activation status of circulating DCs by simultaneous monitoring of expression of costimulatory molecules CD80, CD83 and CD86. Peripheral blood DCs were monitored from the earliest phase of hematopoetic reconstitution. Both mDCs and pDCs appeared at earliest stages after engraftment and relative numbers within white blood cells compartment peaked between days 19-25 after HSCT. Their proportion then gradually declined and steady-state absolute levels found in the control group were reached between days 80-180 after BMT for mDCs and beyond day 180 for pDCs. Expression of costimulatory molecules, especially CD83 and CD86 transiently increased between days 15 and 35 and then went back to low steady state levels. Interestingly, patients who developed acute graft-versus-host disease (aGVHD) had lower absolute numbers of circulating DCs before appearance of clinical symptoms. Administration of glucocorticoids for the treatment of aGVHD abrogated circulating pDCs and led to a rapid decrease of mDCs within 24h similarly to the findings in systemic autoimmune diseases. In this pilot study the development of aGVHD was preceded by decreased numbers of circulating DCs though now we would like to confirm this hypothesis in larger series of patients.
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FUORESCENCE MICROSCOPY IMAGING TO MONITOR DENDRITIC CELLS' TUMOR LYSATE CAPTURING AND PROCESSING: PRELIMINARY DATA.

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To dissect the biochemical and the cellular pathways of the antigen processing in dendritic cells (DCs) a fluorescence microscopy study was performed. The antigen capturing process by DCs was monitored within a phase I-II clinical trial of a therapeutic vaccination protocol on melanoma patients. The prime aim of this work was the transfer of the information obtained by in vitro studies to the clinic, and the second was the starting up of a proteomic characterization of the antigen effectors. Indeed, in our assay tumor tissue's lysates were used as the presented antigens. These extracts were labeled by FITC and used for cell imaging by conventional and confocal fluorescence microscopy. After loading with the fluorescent lysate, DCs were evaluated at different stages: after an 8-hours loading in immature DC (iDC), after an 18-h loading in iDC, after a 24-h maturation period in mature DC (mDC) and, finally, after a 48-h maturation period in mDC. In iDC, after an 8-h loading, an intensive and diffuse fluorescence was observed within the cytoplasmic compartment. After a 18-h loading, cell fluorescence intensity was significantly increased and lysate fluorescence appeared to be localized in a restricted, cloudy-shaded area with a typical polarized aspect. After 24 hours' maturation, the contour of the fluorescent cloud was more defined with an apparent decrease of the total emitted light. Moreover, the green fluorescence began to organize in small spots. After 48 hours' maturation, the fluorescence intensity of the cloudy-delimited area further decreased with a concomitant increase in the number and fluorescence intensity of the spot-like images. Colocalization with anti-MHCII IgG was tested. Our results confirm that overnight pulsing with tumor lysate is appropriate and represents a good practice in vaccination trials with DCs. Moreover, the morphological patterns obtained by fluorescence microscopy as a function of time from

pulsing and maturation of DCs loaded with a fluorescent lysate are in excellent agreement with the recent survey published by Műnz and coworkers, who showed the existence of constitutive autophagosome formation in MHC class II-positive cells. We propose that, in DCs, autologous tumor lysates could be processed and organized in autophagosomes.

Reference: Schmid D., Pypaert M. and Műnz C. *Immunity* 2007 26:79–92.

MODULATING B7-H MOLECULES ON RCC AND MELANOMA – A WAY OF SHAPING THE EFFECTOR T CELL RESPONSE.

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An adequate adaptive immune response against cancer involves two distinct signals: the first is mediated through the interaction of tumor-associated antigens (TAA) presented by MHC class I molecules and the specific T cell receptor (TCR), whereas the second signal involves the binding of costimulatory partners on both cells. Costimulatory molecules are known to efficiently shape the effector immune response towards silencing or activation of immune cells. Tumors evade the elimination by T lymphocytes due to diverse escape mechanisms including the differential expression of activating or inhibitory costimulatory molecules. The newly identified members of the B7-H family (B7-H1-4) represent important players in this process. We analyzed a large series of melanoma and RCC cell lines for the mRNA and protein expression of the B7-H molecules. The results show a heterogeneous constitutive expression pattern for B7-H1, -H2 and -H4 at the transcript and/or protein level. B7-H3 was homogenously expressed on all melanoma and RCC cell lines tested. Furthermore, 88% of primary RCC lesions exhibit B7-H3 expression, which significantly differed in intensity between the lesions. Strong B7-H3 expression correlated with a higher CD8 and inverse CD4 T cell infiltration when compared to lesions weakly expressing or lacking B7-H3 molecules. Moreover, high levels of B7-H3 expression are significantly associated with a low pT value in the RCC patients. In order to determine the role of B7-H3 expression on the immune response tumor specific CFSE based proliferation as well as degranulation (CD107a induction) assays with CD8 cells from either allogeneic PBMCs or autologous tumor infiltrating lymphocytes were performed using B7-H3^{+} or by specific siRNA downmodulated B7-H3^{-} RCC cell lines. So far no difference in the proliferative response of above mentioned T cells stimulated with B7-H3 modulated RCC cell lines was detected, whereas a small difference in CD107a induction was observed. The respective cytotoxicity assays as well as the cytokine production of the CD8's stimulated with B7-H3 modulated RCC cell lines are currently under investigation. B7-H1, -H2 and-H4 will be overexpressed in RCC cell lines using electroporation and subsequently the T cell assays will be performed.

FORCEFUL ELECTROPORATED DCS UPREGULATE EXPRESSION OF COSTIMULATORY MOLECULES AND ARE MORE POTENT IN INDUCING INFLUENZA MATRIX PROTEIN M1-SPECIFIC T CELLS.

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Introduction

Dendritic cells (DC) are specialized in presenting foreign antigens to the immune system and thus are central to the initiation of immunity. The development of protocols to generate large numbers of DC in vitro has provided a rationale to design and develop DC-based vaccination studies for the treatment of infectious and malignant diseases. Previous studies have shown that mRNA electroporated DCs are able to process and present tumor associated antigens leading to the activation of tumor-specific T cells in vitro and in vivo. However, despite extensive experimental investigations and its widespread application in research and clinical environments, little is known as to what extent the immunological properties of DCs are influenced by electrical fields of critical strengths.

We have made series of experiments to determine whether the immunogenicity of DCs transfected under forceful electroporation conditions had consequences for the functional properties of DCs.

Results and Perspectives

We found that forceful electroporation of DCs resulted in loss of viability, but the level of transgene expression on DCs was four-fold upregulated. In addition, forceful electroporated DCs were more potent in inducing influenza matrix protein M1-specific T cells.

Interestingly, forceful electroporation induced further upregulation of costimulatory molecules on mature DCs, but did not promote phenotypic or functional maturation in immature DCs

Experiments are ongoing to determine the mechanism responsible for the enhanced immunological potency of DCs transfected under forceful electroporation conditions.

These findings indicate that the application of forceful electroporation should be considered in future development of protocols using mRNA transfected DCs.

LACTOFERRIN, A MAJOR DEFENSE PROTEIN OF INNATE IMMUNITY, IS A NOVEL MATURATION FACTOR FOR HUMAN DENDRITIC CELLS.

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Lactoferrin (LF) is an important protein component of the innate immune system that is broadly distributed within the body fluids. LF is endowed with multiple biological activities. Talactoferrin a recombinant human LF is in clinical development as an anti-cancer agent and is entering Phase III clinical trials. Here we show that TLF induces the maturation of human DCs derived from monocytes. Talactoferrin, at physiologically relevant concentrations (100 μg/ml) up-regulates the expression of HLA class II, CD83, CD80 and CD86 costimulatory molecule and CXCR4 and CCR7 chemokine receptors, acting primarily through the p38MAPK signaling

pathway. DCs matured by talactoferrin displayed an enhanced release of IL-8 and CXCL10, and a significantly reduced production of IL-6, IL-10, and CCL20. They also display a reduced ability to take up antigen, and increased capacity to trigger proliferation and release IFNγ in the presence of allogeneic human T cells. Talactoferrin-matured DC are able to prime naïve T cells to respond to KLH antigen and display a significantly increased capacity to present Flu-MA_{58-66} peptide to HLA-A2 matched T cells. These data suggest that a key immunomodulatory function that may be mediated by talactoferrin is to link the innate with adaptive immunity through DC maturation.

POST-APOPTOTIC TUMOR CELL DEATH ACTIVATES THE DENDRITIC CELL CROSS-PRESENTATION PATHWAY AND INCREASES THE GENERATION OF REGULATORY T LYMPHOCYTES.

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A critical issue in the preparation of effective dendritic cells (DC) vaccines is the source of tumor antigens. Recent literature data have shown that drug/radiation-induced tumor cell death increases the antitumor cytotoxic T cells in vivo. Using a renal carcinoma (RCC) cell line (K1), we have shown that the apoptotic (i.e. gamma-irradiated) tumor has a better access to the DC cross-presentation pathway than its necrotic (i.e. frozen/ thawed) counterpart (Int. J Immunopathol Pharmacol, in press). More recently, we have observed that post-apoptotic (secondary necrotic) K1 cells that have acquired a phospahtydilserine^{+}, calreticulin^{+}, propidium iodide^{+} phenotype after UVC radiation induce the highest CTL cross-priming by loaded, cytokine-matured DC. The mechanism underlying the stimulatory effect of UVC-K1 cells rested on increased tumor uptake and DC maturation, as judged by phenotypical analysis of membrane markers. Of interest, the death treatment reversed the inherited immunosuppression of the K1 cells. The K1 immune escape mechanism was not shared by another RCC line, whereas secondary necrosis induced by gamma-irradiation was the most immunogenic treatment in a gastric carcinoma cell line. Immunosuppression by the K1 cell line was independent of cell contact, was partially mediated by K1-derived TGF-beta and was not accompained by CD4+CD25+highFoxP3+ T regulatory (Treg) lymphocyte expansion. By contrast, efficient CTL cross-priming by DC loaded with UVC-treated K1 cells was characterized by marked Treg up-regulation, and was enhanced when the CD4+CD25+high cell population was depleted from cultures shortly after each cycle of stimulation Together, these data indicate that *i)* the death tumor treatment and the intrinsic characteristics of a given tumor may differentially affect the potency of ex-vivo generated DC and *ii)* optimal DC cross-presentation achieved in patients treated with DC vaccine or with genotoxic drugs should be combined with strategies aimed at controlling Treg cell expansion.

EXPRESSION AND REGULATION OF THE ISGYLATION IN TUMORS AND DENDRITIC CELLS.

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The Interferon-stimulated gene 15 (ISG15) is a member of the ubiquitin-like protein superfamily which is induced by type I interferons (IFN) and seems to exert dual functions as cytokine and as covalent modifier of a number of cellular and viral proteins. Secreted ISG15 is involved in the stimulation of innate immune cells as natural killer cells and neutrophils. In addition, ISG15 conjugation negatively interferes with ubiquitination of substrate proteins in tumor cells potentially contributing to a dysregulated proteasomal turnover and antigen presentation.

Our studies aim to clarify the role of ISG15 and the enzymes involved in ISG15 conjugation (Ube1L, UbcH8, and Herc5) in the modulation of innate immune responses and antigen-presentation in tumor cells and professional antigen-presenting cells (APCs). ISG15 but not the ISGylation enzyme machinery is upregulated in primary prostate and renal cell carcinomas when compared to the expression in corresponding non-malignant tissue samples. A potential mechanism of the elevated ISG15 expression in tumors is pro-apoptotic cell stress since exposure of melanoma cell lines to UV light and treatment with the chemotherapeutic agent paclitaxel resulted in significantly increased ISG15 mRNA and protein expression levels. In contrast, the ISGylation enzymes are not upregulated during stress response.

To evaluate the expression and regulation of the ISGylation system in APCs, the transcripts of ISG15 and ISGylation enzymes were analyzed in monocyte-derived dendritic cells (MoDCs) and myeloid blood DCs in the presence or absence of various maturation stimuli. All components of the ISGylation system were expressed in both DC populations. In MoDCs and myeloid DCs, a strong upregulation of all components was detected after maturation by poly(I:C). Moreover, treatment with IFN-γ or a combination of IFN-γ and TNF-α resulted in a moderate upregulation of the ISGylation system in MoDCs, whereas a downregulation of these components was observed in myeloid dendritic cells. LPS and a cocktail of TNF-α, IL-1, IL-6 and PGE_2 did not significantly alter the expression levels in MoDCs, but markedly reduced the expression of the components in myeloid dendritic cells.

In summary, ISG15 is frequently upregulated in prostate and renal cell carcinomas possibly as a result of intratumoral stress response. The observed dysregulation may affect the ubiquitination and presentation of tumor antigens and consequently the antitumoral immune responses. In DCs, the ISGylation system is differentially expressed dependent on distinct maturation stimuli as well as the respective DC type. The observed modulations of the ISGylation system in DCs may have consequences for the ubiquitination, proteasomal processing and the pattern of presented peptides from individual antigens.

ADOPTIVE T CELL THERAPY FOR MALIGNANT MELANOMA PATIENTS.

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We have developed a protocol for expansion of tumor specific T cells using Dynabeads CD3/CD28 from patients receiving autologous dendritic cells (DC) vaccine. Based on the observation that high Treg numbers in cancer patients lead to poor immune responses in vaccinated patients, the patients in this clinical trail will receive Temodal treatment prior to the DC vaccine. Temodal, a drug used to treat melanoma patients, has been found to primarily affect the $CD4^+$ T-cell compartment, including the $CD4^+CD25^+$ subset (Treg).
Following vaccination with DCs the patients will receive ex vivo expanded T cells, containing tumour specific T cells generated during DC vaccination. Several different clinical trails have been conducted using adoptively transferred T cell or T cell subsets and therapeutic effects have been documented in these patients.
As a model system for expansion of antigen specific T cells we have used HLA-A2 positive donors (normal and melanoma patients) who had detectable numbers of CD8+ T cells against CMV, EBV or FLU. We have measured the number of antigen specific T cells generated during expansion using pentamers. To retain antigen specific T cells we have optimized the ratio of T cells to Dynabeads CD3/CD28. Based on these data we have developed a protocol for large scale T cell expansion that will be used in clinical trails.

COMPARISON OF DIFFERENT PROTOCOLS FOR PRODUCTION OF "VACCINE" DC WITH AN EYE ON THE ANTIGEN PROCESSING CAPABILITIES.

C. Massa

The gold standard of dendritic cell vaccine used in the clinic consists in DC differentiated from CD14+ blood monocytes by 5 days in GM-CSF and IL4 and then stimulated with the maturation cocktail made of IL1b, IL6, TNFa and PGE2. In light of the poor results obtained in the clinic, many different alternative protocols have been proposed in the last years but comparisons between the different "DC vaccine" have been mostly performed on the base of the costimulatory and Th polarising ability of the different DC. Since there is an increased consensus that providing DC with the full spectrum of tumor antigens, either in the form of protein lysates or mRNA, can be far superior to usage of defined peptides both on the level of applicability to different HLA patients and activation of T cells against a wider antigenic repertoire, including possibly patient specific epitopes, an important question is how the differently derived DC will process the tumor antigens and thus which epitope repertoire will be presented to the immune system by the vaccine. This is particularly important in light of the continuously increasing number of enzymes that are involved in the antigen processing pathway and of epitopes that have been demonstrated to be differently processed by tumor and professional APC.
To analyse this point, DC were differentiated from CD14+ blood monocytes using the different protocols and maturation cocktail proposed in the recent literature and characterised for expression of the subunits of the standard and immuno-proteasome, the cytosolic endopeptidases and aminopeptidases (namely TPPII, THOP, LAP, BH) and the ER-resident aminopeptidases ERAP1 and ERAP2. Analysis at the mRNA level indicates a differential modulation of the various APM component upon maturation by different stimuli, with microbial derived component that upregulate the expression of many enzymes with higher magnitude than the cytokine cocktail. With regard to the various differentiation protocols, the most strikingly difference are between the standard 7-days long protocol and the FastDC. Despite similar expression of costimulatory molecules on the surface and similar ability in inducing proliferation of allogeneic PBMC, FastDC do not display the strong modulation of APM component that is present in DC differentiated for 7 days. The functional consequences of these expression patterns on the processing of known antigen is under investigation.