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Project acronym: **DENDRITOPHAGES**

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Thematic Priority: **LSH-2002-1.2.4-6 "Development of vaccine technologies targeted to dendritic cells"**

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Project Objectives

The goal of cancer therapeutic cell vaccine is to prevent progression and tumor recurrence. Adoptive therapy in adjuvant settings will complement classical anti-cancer treatments. In this technology the patient's blood monocytes are transformed into effector monocyte-derived dendritic cells (dendritophages) which are activated to fight the patient's own disease. The therapeutic cell drug comprises dendritic cells loaded with cancer-specific antigens to activate the patient's immune system after re-injection.

This project aims to demonstrate at a multinational level, the immune and clinical efficacy, reproducibility and feasibility of anticancer cell vaccine by sequential steps: Choosing the best dendritic cell vaccination strategy by adequate pre-clinical studies (DC differentiation and maturation, tumor antigens selection and loading, dose delivered, site and vaccination schedule).

Monitoring the immune response in correlation after defining the most relevant immunomonitoring techniques.

Demonstrating the immunological efficacy of DC immunotherapy in prostate cancer after loading ex vivo dendritic cells with a tumor proteic antigen.

This required to set up the quality control criteria and data base design for the production of the cellular product, and to optimize a GMP process. It was planned to validate the most effective DC preparation under GMP and then to initiate a clinical trial to evaluate the cell drug on progressing prostate cancer patients.

Work performed

1 WP1 – COORDINATION, DATABASE ON CELL PREPARATION FOR CLINICAL TRIALS, REGULATORY REQUIREMENT

Eight scientific and project management meetings were organised, followed by reports

-Kick off meeting in Paris and Consortium agreement

-First scientific meeting in Roma

-Second scientific meeting in Regensburg

-Third scientific meeting in Catania

-The fourth scientific meeting was organised in Melbourne by the CBCT Peter Mac Callum Australian partner (M Prince) in Conjunction with a Dendritic Cell large meeting in October 05

-The fifth meeting was organised by CCRI in Vienna, in May 06

-The sixth meeting was organised by IDM in Saumur, in Nov 06

-A final scientific, administrative and steering committee meeting was held in Paris, in June 07.

Procedures for GMP production of DCs in appropriate facilities were delivered, allowing high quality standards and robustness.

Databases comparing the different DC populations obtained by 3 different methods, characterized by 5 independent partners on 4 preparations from healthy donors were obtained and exchanged. The logistics were organised for effective transfer of the preparations from the clinical setting and between the partners.

Status reports, intermediary and final reports as well as deliverables were finalised and distributed.

Regulatory requirements were discussed with national and EMEA authorities. Specifications for cell therapies and release criteria were discussed in several meetings. Clinical protocols were submitted to national ethical committees.

2. WP2 - PROCESS OPTIMISATION FOR DC MATURATION AND ACTIVATION

WP2 was designed to fulfil the following main objectives:

1 Validation of a procedure for production of clinical grade 3day-IFN-DCs by using IDM technology and comparison of the possible advantages of using these 3day-IFN-DCs versus standard Dendritophages.

2 Optimise techniques for DC manufacturing for clinical use with specific regard to: (i) IFN-α versus IL-4 versus IL-13 as inducers of DC differentiation; (ii) activation; (iii) maturation; (iv) functionality; (v) automated washing of contaminating cells and cryopreservation.

The contribution of ISS was: i) to validate a procedure for the preparation of clinical grade 3day-IFN-DCs, by applying the methodology developed by IDM for differentiation of Dendritophages; ii) to evaluate the phenotypic and functional properties (antigen up-take, cytokine and chemokine secretion) of 3day-IFN-DCs in comparison with Dendritophages or other standard DC preparations.

IDM has contributed to the validation of a procedure for the preparation of clinical grade 3day-IFN-DCs with material and training of the ISS personnel and to the comparative studies by generating Dentritophages (IL-13-DCs) and performing phenotypic and functional (allogeneic T cell proliferation) assays.

CCRI has contributed to the comparative studies generating IL-4-DCs and performing the phenotypic and functional (antigen-specific IFN-gamma secretion from autologous T lymphocytes) analyses of DCs obtained by different method.

Regensburg has contributed by performing the comparative evaluation of the phenotypic and functional (generation of antigen-specific CTL in vitro from autologous PBL) characteristics of the different kind of DCs.

2.1 RESULTS

2.1.1 Process optimisation

Validation of a procedure for the preparation of clinical grade 3day-IFN-DCs, by applying the methodology developed by IDM for differentiation of Dendritophage (ISS).

The transfer of the IDM technology/methodology to ISS was considered completed after the accomplishment of 4 preparations of Dendritophages at ISS. The results obtained from the first 3 preparations indicated that the ISS preparations are similar to the standard IDM preparations in terms of yield, viability, and phenotype. However, the purity of Dendritophages preparations obtained at ISS was lower as compared to the standard IDM preparations. An additional Dendritophage preparation was performed at ISS, with the assistance of IDM personnel. The purity of this preparation resulted to be in the range usually obtained in the standard IDM preparations. Thus, the validation of a clinically suitable procedure to generate 3day-IFN-DCs was initiated. The same apheresis product was used for the preparation of 3day-IFN-DCs by applying IDM technology in parallel with the standard ISS method. The results obtained indicated that both methods generated DC preparations similar in terms of yield, viability, phenotype. Concerning the purity, similar values were obtained with the two methods, when purity was calculated as the ratio between $CD11c^+$ cells and total $CD45^+$ cells. However, considering the ratio between $CD11c^+$ cells and total cells collected at the end of each procedure, the purity obtained with the ISS method was higher than that resulting from the IDM procedure. A second experiment was performed for the preparation of 3day-IFN-DCs by applying the IDM technology in parallel with the standard ISS method. In this experiment, IntronA (recombinant IFN-alfa2b, Schering Plough) was used for DC differentiation. A comparative evaluation of IntronA and Alfaferone (Wassermann) had indicated that IntronA at the dose of 10,000 IU/ml was as efficient as Alfaferone (the IFN-alfa routinely used at the dose of 10,000 IU/ml for IFN-DC preparation by the ISS method) in terms of ability to induce DC differentiation from purified $CD14^+$ monocytes. Two different doses of IntronA (10,000 and 1,000 IU/ml) were compared for the differentiation of 3day-IFN-DCs by the IDM technology. The results obtained have indicated that both ISS and IDM methods and both doses of IntronA generated DC preparations exhibiting similar yield, viability, and phenotype. As in the previous experiment, the purity of DCs differentiated with the ISS method was higher than that of the DC preparation obtained with the IDM procedure. The elutriation profiles clearly indicated that the 3day-IFN-DCs are of smaller size (10 µm) as compared to Dendritophages (12 µm). In a third experiment, a different protocol of elutriation suggested by the IDM team in order to obtain a higher purification of 3day-IFN-DC was followed. However, the purity of 3day-IFN-DC differentiated with the IDM technology was again very low. In order to define the best elutriation protocol for 3day-IFN-DC purification, the successive DC preparation was performed at IDM with the participa-

tion of IDM-trained ISS staff. The same apheresis product was used for the preparation of 3day-IFN-DCs and Dendritophages, with the aim of simultaneously completing the phase of comparison of the IDM vs. the ISS method for IFN-DC preparation and to start the phase of comparison of Dendritophages vs. 3day-IFN-DCs prepared according to IDM method. This experiment showed that 3day-IFN-DCs can be generated by using IDM technology and that pure 3day-IFN-DC (80%) can be obtained by elutriation even if they are smaller than Dendritophages. The 3day-IFN-DC phenotype was as expected. The subsequent DC preparation was performed at ISS. The same apheresis product was used for the preparation of 3day-IFN-DCs and Dendritophages. This experiment has confirmed that high purity (98%) 3day-IFN-DCs with the expected phenotype can be generated by using IDM technology.

Collectively, the results obtained have shown that 3day-IFN-DCs are:

i. similar to Dendritophages in terms of yield of differentiation, viability and allostimulatory capacity;
ii. smaller than Dendritophages;
iii. less efficient than Dendritophages in terms of phagocytosis.

Concerning cytokine and chemokine secretion, the results indicated that: i) IL12p70 is produced by both matured DC types, with Dendritophages secreting higher amounts than IFN-DCs; ii) IL10 and IL15 are produced at higher levels by matured Dendritophages as compared to IFN-DCs; iii) IP-10 and TARC are produced by both matured and not matured DC types, although at a significant higher extent by Dendritophages than by IFN-DCs.

Optimisation of monocyte enrichment for DC differentiation (CCRI).

In WP2, one of the CCRI goals was the comparative evaluation of DC manufacturing procedures using IL-4/GM-CSF, IL-13/GM-CSF, or IFN-α/GM-CSF for differentiation of monocytes into DCs (see below). In conducting these experiments it became apparent that CCRI method to manufacture DCs was partly inadequate for the challenge of WP2. The second goal was to implement cell enrichment by elutriation. The more conventional enrichment by adhesion is hampered by low yield; elutriation using the Elutra system has the advantage, in addition to the higher yield, of being conducted in a closed system in a single use plastic tubing system. CCRI team observed that monocytes enriched by elutriation were superior compared to DCs enriched by plastic adherence in terms of yield, purity, viability and showed higher IL-12 secretion, an optimal immune phenotype, and a sufficient induction of an allogeneic MLR. Altogether, these results indicate that elutriation using the Elutra system represents a superior enrichment procedure for monocytes and its use should be recommended for manufacturing DC-based cancer vaccines in the future.

2.2 Comparison of different methods for DC manufacturing

The experimental design for the DC comparison studies foresaw that the participating partner institutions used leukocytes donated by the same healthy individu-

als to allow the optimal comparison. In order to accomplish this goal, the leukocyte apheresis procedure was done in one centre, the leukocyte apheresis product was divided into three aliquots, and shipped to the participating laboratories.

Each partner laboratory enriched monocytes and differentiated them into DCs according to the laboratory standard procedures.

ISS differentiated IFN-DCs by using standard ISS method (IFN-DC preparation from purified $CD14^+$ monocytes cultured for three days in the presence of GM-CSF and IFN-α). CCRI used initially plastic adherence and later elutriation (see section 2.1.1) and cultivation in IL-4/GM-CSF supplemented medium. IDM generated Dendritophages (IL-13-DCs) by using IDM technology.

The DCs were charged with the test antigen OVA or the tumour antigen KSA or left untreated. Finally, the loaded and the unloaded DCs were either left untreated or matured for 6 hours with either a combination of LPS/IFN-γ (exclusively on IL-4-DCs) or FMKP/IFN-γ to enable them for IL-12 secretion. The matured or non-matured DCs, antigen-loaded or unloaded, were frozen and aliquots were sent to the other partners according to the work plan previously defined. Each partner institution comparatively evaluated different phenotypic and functional characteristics of the DCs according to the laboratory standard procedures, in order to provide complementary information.

IDM contribution. IDM was in charge of comparing IFN-DCs (ISS preparation), IL-4-DCs (CCRI preparation) and IL-13-DCs (IDM preparation) in terms of viability, recovery and phenotype.

Just after thawing, all DC types had a good viability (> 70%) according to the acceptance criteria used in clinical trial. In general, DC viability decreased after overnight culture. For IFN-DCs, the mortality was very important. Viability of DCs matured during 6h was also low. This can be explained by the higher fragility of matured DC compared to non matured DC. Nevertheless, 50% and 55% of viability for 6h matured frozen DC + overnight culture were acceptable values. In term of phenotype, no significant differences between the three non matured DC types were obtained.

ISS contribution. The ISS team was in charge of assessing the uptake of FITC-OVA and FITC-latex beads by the three types of non matured DCs as well as the chemokine (CXCL10/IP10, CCL17/TARC and IL-15) and cytokine (IL-12 and IL-10) production by IFN-DCs and by non-matured or 6h-matured DCs received from CCRI and IDM. Collectively, the results obtained have shown that: i) all DC types exhibit similar capability of latex beads uptake; ii) the 3 DC types differ in their ability of OVA uptake (IL-13-DCs > IL-4-DCs > IFN-DCs); iii) IL12p70, IL10 and IP-10 are secreted by all matured DCs, although at different extent (IL-13-DCs > IL-4-DCs ≥ IFN-DCs); iv) TARC is produced by all matured as well as not matured DC types, although at different extent (IL13-DCs > IFN-DCs and IL4-DCs); v) IL-15 is produced by matured IL-13-DCs.

CCRI contribution. The immune phenotype of DCs was measured 48 hours after maturation was initiated by exposure to FMKP/IFN-γ. All three types of DCs, IFN-DCs, IL-13-DCs, and IL-4-DCs, developed an immune phenotype typical for mature DCs. Expression of the monocyte marker CD14 was down-modulated; expression of MHC I and MHC II molecules, the B7 family co-stimulatory molecules CD80 and CD86, and the DC membrane molecule CD83 were up-regulated. No or only weak expression of CD1a molecules was found on all three types of DCs. A comparison of the maturation stimuli LPS and FMKP, both in combination with IFN-γ, for the effects on exclusively the IL-4-DCs, revealed an approximately equal capability to induce the secretion of IL-12 and to support T-lymphocyte activation in a mixed leukocyte culture.

The following conclusions were drawn from the comparison experiments: (i) the manufacturing of DCs in using IL-4 or IL-13, both in combination with GM-CSF, gave similar results; (ii) maturation of IL-4-DCs with LPS or FMKP, both in combination with IFN-γ, was also comparable; (iii) it was possible to generate immune responses in vitro against the neo-antigen OVA to a similar extent as against the tumour antigen KSA.

Regensburg contribution. For the comparative DC study, 3 different DC preparations (FMKp/IFN-γ matured IL-4-DCs, immature IFN-DCs, FMKp/IFN-γ matured IL-13-DCs) were analyzed for their functional capability to induce antigen-specific CTL responses. A first series of experiments demonstrated that a defined antigen-specific CTL line responded to all the peptide-pulsed DC preparations in a similar pattern. To analyze the ability of the different DC preparations to generate antigen-specific CTL in vitro from autologous PBL, the 3 different DC preparations were pulsed with HLA-A2-restricted peptides and used as stimulator cells for autologous purified $CD8^+$ cells (selected by magnetic separation). After 2 weekly stimulations, T cells were harvested and analyzed for their phenotype (including MHC-peptide tetramer analysis) and functional activity (^{51}Cr release assay against peptide-pulsed T2 cells and allogeneic melanoma cells). FACS analysis revealed a similar frequency MHC-peptide-tetramer positive T cells. Cytotoxic activity against Melan-A-pulsed T2 cells could be demonstrated in all T-cell cultures. However, allogeneic melanoma cells, that endogenously express the Melan-A antigen, were only killed by T cell lines generated with IL-13 DCs but not by IL-4-DCs or IFN- DCs. Additional experiments with less immunogenic antigens are warranted to compare the functional capacities of the different DC preparations.

2 WP3 – ANTIGEN LOADING

1. Production and characterization of different formulations of virosome for DC loading

The virosome is derived from inactivated Influenza virus by detergent treatment, purification of the envelope components, and subsequent reconstitution of the virus envelope. Structurally, these "empty" virus particles are spherical proteoliposomes,

containing the Influenza transmembrane glycoprotein hemagglutinin (HA) and traces of neuraminidase (N), both in authentic and functional conformation anchored in the lipid bi-layer, which is predominantly composed of phospholipids. We developed procedures to introduce different antigens in the virosome structure.

Fluorescent labeling method has been established for HA, FITC-labeling resulting better than AEDANS-labeling for successive FACS analysis. The established labeling method can be applied to tumor antigen. Labeled virosomes have been produced with functional (fusion active) and pH-inactivated HA, characterized and tested for fusion activity. Labeled and non-labeled, fusion active/inactive virosomes have been sent to IDM and ISS for uptake, toxicity and maturations assay with dendritic cells.

Membrane proteins from LNCaP cells have been extracted and incorporated in virosomal formulation. The integration of membrane proteins into virosomes, in particular the KSA antigen, has been clearly demonstrated, indicating a spontaneous incorporation of the native protein during a standard formulation process.

Formulation of virosomes containing recombinant KSA antigen has been produced, as a simple co-formulation; however analysis of physical association indicated that the recombinant KSA, lacking the trans-membrane domain, was not incorporated into virosomal structure. In alternative to "spontaneous" incorporation, different chemical cross-linking strategies for loading have been investigated. Using the SMCC cross-linking, virosome formulations containing the recombinant KSA have been produced and purified. KSA is conjugated with PE using the heterobifunctional cross-linker SMCC, in a two-step procedure. In a first time PE is activated through its NH3 group and then conjugated through its acquired maleimide group and SH group from KSA. After optimization of the procedure a mid-scale formulation of KSA-virosome containing 0,3 mg/ml of associated KSA antigen, and a control formulation without KSA have been produced. Analytics performed on different formulations indicated that the presence of KSA does not induce changes in the physical-chemical characteristics (size and HA content) of virosome.

To select the most efficient technology to load DCs with antigen, it appeared necessary to switch to a more antigenic protein than the recombinant KSA. In a first attempt, we tried to prepare virosome containing recombinant CMVpp65 antigen as a simple co-formulation, and showed that no spontaneous incorporation occurs. Based on the protein properties a chemical cross-linking strategy has been designed. However due to solubility requirement of the recombinant protein that was not compatible with optimal reaction conditions it has not been possible to produce a formulation containing CMVpp65 antigen.Successively it has been decided to incorporate CMV IE 1 recombinant antigen. In absence of trans-membrane domain and on the basis of the protein characteristics a strategy for chemical cross-linking has been elaborated. Experiments, to determine the conditions for cross linking with pre-formed liposome containing SMCC-activated-PE and the formulation of resulting liposome in virosome, are actually running.

2. Development of In vitro assays

As a read-out system for the optimal antigen carrier system for loading of DC, the tumor-associated antigenic protein KSA/EpCAM was introduced in virosomes or lipo-

somes or used as native protein. After loading on immature DCs cells were analyzed for apoptosis induction (annexin-V/PI staining) and maturation via modulation of the expression of different surface molecules expressed on dendritic cells (CD1a, CD80, CD83, CD86, CD209, HLA-DR). Furthermore, we have developed a functional read-out system for induction of KSA-specific T cell responses after stimulation of peripheral blood lymphocytes with immature DCs loaded either with the native KSA protein or liposome or virosome-encapsulated KSA. Data clearly indicate that loading of immature DC with the native protein or liposomal or virosomal formulations is not toxic for DC and does not induce maturation upon 24h after protein loading. Comparative functional T-cell studies suggest that loading of DCs with the KSA liposomes is superior to the KSA protein and KSA virosomes for induction of antigen-specific immune responses.

3. Cellular biology of the stimulatory CD40/CD40L interaction

The main goal of all delivery strategies is to introduce tumour antigens into the MHC I processing and presentation pathway. Only this allows the presentation of tumour antigens to CTLs and their priming resulting in expansion and enabling them to search and destroy tumour cells. A critical issue in that context is cross-presentation of exogenous antigens. A DC that is enabled for cross-presentation is capable of shuttling essentially every antigen into the MHC I pathway. The interaction of CD40 with its ligand CD154 (CD40L) is known to be the key stimulus to enable DCs for cross-presentation and thus for CTL activation. Instead of optimising the source and nature of the tumour antigen, we elected to focus on mechanisms that allow a DC to present antigen from any source to CTLs by investigating the conditions under which the CD40/CD40L stimulatory interaction might be exploited in the design of novel cancer vaccines.

Thus we re-interpreted the aims of WP3 and focused our efforts on understanding the cellular biology of the stimulatory CD40/CD40L interaction. The classical understanding of the biology of DCs is to a certain extent based on a rather static picture. Certain functional capabilities are ascribed to distinct developmental phases, conventionally referred to as immature (iDC) or mature DCs (mDC). Accumulating evidence now indicates the existence of additional functional developmental stages of DCs. Most importantly, cross-presentation of exogenous antigens to CTLs depends on a "semi-mature" status of DCs (smDC). These smDCs are characterised by active IL-12 secretion, which is down modulated in mDCs. Another only lately appreciated developmental stage is the tolerance inducing capacity of mDC. Particularly, the up-regulation of indolamine dioxygenase (IDO) is associated with immune suppressive feedback loops. Thus, the same DC that in its immature status maintains tolerance and during its first day after exposure to an appropriate maturation stimulus releases IL-12 and supports cross-presentation will from the third day on act immune suppressive priming the differentiation of regulatory T-cells.

Here we re-evaluated the kinetics of IL-12 secretion from DCs. Immature DCs were initially exposed to maturation stimuli mediated by TLR engagement, an inflammatory cytokine cocktail, or a CD40/CD40L mediated signal. After 48 hours, fully mature DCs that had exhausted their capacity to secrete IL-12 were re-exposed to the same panel of maturation stimuli. CD40/CD40L interaction was the only stimulus that had

the potential to trigger a secondary burst of IL-12 secretion from DCs, which at a molecular level correlates with a down modulation of the expression of molecules involved in TLR signalling in fully matured DCs. Such DCs were also re-enabled to support T-lymphocyte proliferation in an allogeneic mixed leukocyte reaction (MLR). This led us to conclude that DCs after maturation preserve some developmental flexibility and are not simply terminally differentiated cells that only fulfil one final purpose. These observations may impact on the design of DC-based cancer vaccines that are an area of intense investigation in tumour immunology.

It is generally assumed that DCs upon encountering danger signalling follow a predetermined differentiation pathway. We extend this notion by providing evidence that DCs may retain some developmental plasticity permitting them to respond to secondary immune modulatory stimuli. We specifically investigated the expression kinetics of IL-12 that is produced by DCs for about one day after maturation and ceases afterwards, which is referred to as DC exhaustion. DCs were exposed to a panel of primary maturation stimuli, including TLR-mediated stimuli (LPS) or CD40/CD40L-mediated signalling, both of which induce secretion of IL-12, or a cocktail of inflammatory cytokines comprised of tumour necrosis factor TNF-α, prostaglandin (PG) -E2, IL-1β, and IL-6 that does not trigger biologically relevant IL-12 release. Importantly, exposure to secondary CD40/CD40L signalling, but not to any other maturation agent, after 24, 48, or 72 hours resulted in a second phase of IL-12 secretion. Down modulated expression of molecules involved in TLR mediated signalling in DNA micro array analysis of LPS/IFN-γ activated DCs were found as a molecular correlate for this observation. We also investigated the potential of mature DCs subjected to secondary CD40/CD40L signalling after 48 hours to activate T-lymphocytes in an allogeneic mixed leukocyte reaction (MLR). Such secondary CD40/CD40L signalling enabled DCs to act as efficiently as primary matured DCs to induce T-lymphocyte proliferation. In contrast, not re-stimulated control DCs did not efficiently prime T-lymphocyte proliferation. We conclude that the concept of DC exhaustion may need amending, as our data indicate that mature DCs maintain some potential to respond to secondary T-cell mediated stimuli. This observation may impact on the design of DC-based cancer vaccines.

3 WP4 PHARMACOKINETICS AND INJECTION ROUTE STUDIES

The principal objective was to examine the tracking and localisation of DC once administered to the patient using both qualitative and quantitative (dosimetric) approaches to determine the strategy which was most effective in delivering DC to lymph nodes. A further objective was agreed upon with the partners to develop novel labels for tracking human cells in animal models, allowing a comparison of different manufacturing approaches for human DCs based upon their subsequent tracking in-vivo in mouse using small animal positron emission tomography (SAPET).

Using ^{111}In-oxine DC-labeling and utilizing planar whole body SPECT scans we observed reliable tracking via SC and ID routes, and observed a rate of mDC traffic

to regional lymph nodes similar to that previously demonstrated by others, who demonstrated rates between 0.00% to 3.14% A key finding in this study was that although mDC appeared to have superior migration capacity, *both* mDC and nmDC were able to track to regional lymph nodes when injected via both the ID and SC routes. We were unable to observe any trafficking to adjacent nodes following IN injection. These results indicate that local injection and intranodal visualization should not neccesarily be interpreted as effective DC localization sufficient for effective antigen presentation.

We discovered that mDC did not exit the lungs as rapidly as nmDC. These types of tracking studies will be able to evaluate if DC modifications alter the dynamics of trafficking. Although we did observe superior retention of ^{64}Cu-PTSM compared to ^{18}F-FDG, there was still substantial efflux observed *in vitro*. We found this label *qualitatively* useful for tracking DC administered via the SC/ID/IN routes. Following studies to quantitate the extent of ^{64}Cu-PTSM efflux, compensation studies for this efflux were possible and we were able to obtain approximations of cell migration. Indeed, the spatial resolution of the PET images allowed detection of only a few thousand cells. The quantitation indeed indicated substatially lower levels of migrated cells to those calculated by Indium labeling. With Indium we calculated 0.48% – 1.3% of injected cells migrated to the regional node whereas ^{64}Cu-PTSM labeling showed this to be only 0.14% - 0.3% when equivalent time points were considered.

We conclude that it is possible to label both mature and non-matured DC with ^{111}In-oxine and ^{64}Cu-PTSM and track their fate *in vivo*. Mature DC demonstrated superior ability to migrate to regional lymph nodes and both SC and ID injection routes were similar. As modifications to DC production are developed, such strategies of tracking will become increasingly important. ^{111}In-oxine remains the most reliable tracer although the improved resolution with ^{64}Cu PTSM which allows quantitation of very low numbers of DC, may be useful for very short-term tracking. Although approximately 10-25 million mDC can be generated and injected, consistently less than 2% of cells successfully migrate to the regional LN following administration at a single site with substantial inter- and intra-patient variability.This study comprehensively demonstrated that we can deliver DC to the regional node, and thus if we aim to achieve therapeutically effective DC vaccination in the future we must focus on modulating the inherent patient-related factors that are inhibiting those DC.

4 WP5: INNUNOMONITORING OF THE CLINICAL TRIALS

The main goals achieved in work package 5 was the optimization of existing and novel methods for the detection of tumor-associated antigen-specific $CD8^+$ and $CD4^+$ T cells, the preparation of antigen-specific T cell lines stimulated either with defined KSA/Ep-CAM-derived peptide or protein-loaded dendritic cells and the implementation of these techniques for a quality assessment system for immunomonitoring.

First we established a new approach to the analysis of T cell-mediated functional activity, the MHC multimer technology. Multimers allow for direct visualization of anti-

gen-specific T cells by flow cytometry. Second, antigen-specific T cell lines were generated from CD 8 T cells stimulated with mature dendritic cells (DC) pulsed with the HLA-A2-restricted Ep-CAM/KSA peptides and human β_2-microglobulin (β_2m) (10µg/ml) at a responder to stimulator ratio of 10:1 in 96-well plates. CTL lines were cultured in complete medium (RPMI 1640 medium). Alternatively, purified CD4+ or CD8 + T cells were stimulated with immature dendritic cells loaded with different recombinant KSA/Ep-CAM protein preparartions (native protein, liposomes, virosomes).

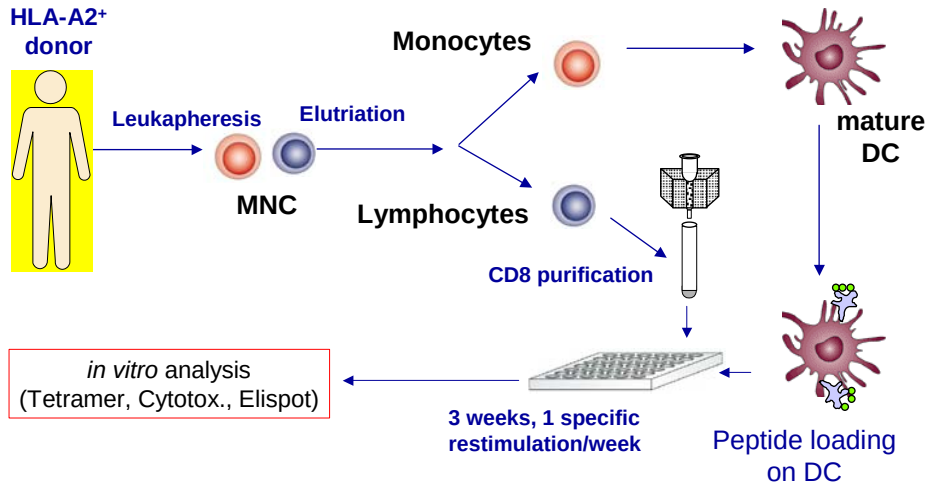

Figure 1: Protocol for in vitro generation of antigen-specific CTL

Results achieved: We developed also full GMP procedures for the immunomonitoring of T cell responses by Elispots against groups of specific antigenic peptides.

Ep-CAM specific CD8+ T cells were generated in vitro based on the in vitro stimulation of CD8+ T cells from HLA-A2+ healthy donors with autologous monocyte-derived fully matured DCs pulsed with different HLA-A2-binding Ep-CAM peptides. We were able to induce Ep-CAM-specific CTL that were able to kill HLA-A2+ Ep-CAM expressing target cells

To ascertain the capacity of DC loaded with different KSA/Ep-CAM protein formulations for generation of antigen-specific T cells, PBL from healthy donors were stimulated twice (1 stimulation per week) with monocyte-derived DC loaded with KSA protein, KSA liposomes, KSA virosomes, or empty virosomes as control T-cell lines stimulated with DC-KSA-liposomes showed a specific response against the KSA protein while the T cells stimulated with DC loaded with the native KSA protein or virosomes revealed no specific activity.

5 WP6 - PHASE I CLINICAL STUDY IN PROSTATE CANCER

The aim of WP6 was to set up a clinical pilot trial for the treatment of prostate cancer in order to evaluate the aspects of this project in a clinical setting. The specific aims were:

- Initiation of a clinical trial for the treatment of prostate cancer using a DC-based cancer vaccine. This included preparation of the study protocol, investigator's brochure, patient's information, and case report forms.
- Treatment of patients with DCs loaded with PSA, KSA, or KSA/liposomes,if possible compared to KSA liposomes injected subcutaneously.
- Documentation of the feasibility and safety of such a cancer vaccine design.
- Optimisation of in vitro monitoring procedures to study the anti-tumour immune response induced in prostate cancer patients.

In order to obtain permission to conduct this trial, a formal application was submitted first to the Ethics Committee of the City of Vienna, which approved the study. The cancer vaccines were manufactured at the GMP facilities of the St. Anna Children's Hospital in Vienna, A. This facility has a manufacturing authorisation from the Austrian government. All reagents used to manufacture the DC cancer vaccine were GMP certified; no bovine proteins are used in the DC cultures. A second larger study with in vivo KSA control vaccine was planned in Regensburg and submitted to the Paul Erlich Institute and the local Ethical Committee.

All patients that were recruited to participate in the pilot trial had hormone refractory metastatic prostate cancer. PBMCs were collected from the patients by leukocyte apheresis. The iDCs were charged with PSA, KSA, or KSA/liposomes.

Exposure to LPS/IFN-γ was used to trigger DC maturation. After 6 hours the DCs reached a semi-mature status characterised by active IL-12 secretion. At this stage the DCs were frozen in suitable aliquots. One of these aliquots was used for quality control. The treatment schedule comprised six applications of the cancer vaccine at weekly intervals. DCs were injected directly into lymph nodes for the pilot trial.

The conclusions drawn from this pilot trial were: (i) The feasibility and safety of treating metastatic prostate cancer using tumour antigen charged DCs enabled for IL-12 release was confirmed. (ii) A comparison between the utility of different tumour antigen preparations is not yet possible for efficacy but GMP feasibility and safety were demonstrated.

A larger trial to assess efficacy compared to non cellular vaccine was prepared . All quality controls for KSA formulation were done and regulatory files prepared as well as investigator brochures and clinical protocol. This second trial could however not be effectively conducted during the timeframe of the project due to extensive regulatory issues to be complied. Therefore, more research and development activities and less demonstration activities than initially planned were achieved.

Progress beyond the state of the art

Although still at an early stage of clinical development, the concept of applying rationally designed cancer vaccines may have the potential to change cancer treatment. DCs as the principal regulators of immunity received special attention as a novel form of adjuvant for cancer immune therapy. An important issue in the design of a DC-based cancer vaccine concerns the decision to use immature or mature DCs.. We have therefore developed a DC culture system designed to maximize IL-12 secretion by maturation with a bacterial membrane fraction (FMKP, P Fabre), or lipopolysaccharide (LPS), a potent bacterial endotoxin, and IFN-γ. Because IL-12 secretion from DC1 is limited to the first 24 hours after maturation, we applied DCs after maximal 6 hours of cultivation in the presence of FMKP or LPS and IFN-γ as semi-mature actively IL-12 secreting type 1 DCs (smDC1). Our strategy is supposed to polarise a type 1 immune response that supports cytolytic anti-tumour immunity, which has never before been attempted in a formalised clinical setting. Here we successfully delivered first feasibility and safety data for this novel approach.

In vivo tracking of the DCs in humans according to different injection routes showed that accumulation of DCs in lymph nodes could be achieved after subcutaneous, intradermous or intranodal injections, but not after intravenous injection.

Furthermore, we have developed efficient methods for generating large numbers of antigen-specific T cells by repetitive in vitro stimulation with defined peptide or protein loaded dendritic cells that may be used for functional analysis of tumor-associated antigen-specific $CD8^+$ and $CD4^+$ T lymphocytes. Immunomonitoring technologies, and mostly specific Elispots could be standardised.

Standardised GMP procedures developed during the project were implemented in the new cell therapy production centers developed by the 5 partners (IDM, CCRI, ISS, Regen, Peter Mac) which have been successfully approved for 4 of them for the moment for clinical preparations by their respective regulatory authorities.

Conclusion

This complex project required many steps to proof the validity and safety of a dendritic cell vaccine optimised by the different partners.

The very close collaboration in full confidence and friendly relations between the partners allowed timely exchanges of materials and information. We could determine the best approach for this vaccine, differentiation, maturation, tumor antigen loading. Large scale manufacturing procedures in the right environment were produced in all centers. We could show that these DCs effectively migrate to lymph nodes after injection. Almost all project objectives were successfully achieved.

Our main project deviation is that we could not effectively complete the multicentric clinical trial comparing the efficacy and safety of the DC vaccine (loaded with KSA) on prostate cancer patients, due to length of interaction with regulatory authorities and complexity of the drug substance and manufacturing files and all the quality and validity controls required. However a mono-centric trial comparing DCs loaded with free or liposome-KSA could be conducted successfully in Vienna by CCRI.

In parallel, using the same optimised mature dendritic cells, with the GPM procedures, IDM conducted a multi-centric phase 2 clinical trial in melanoma patients. In this case, DCs were loaded with tumor lystes. 80 patients received DC vaccine injections (25 million cells each) and for most of them immune responses against tumor peptides could be documented. No patient experienced severe (grade 3 or 4) side effects.

We can therefore conclude that the DC vaccine that we have optimised and developed during the "Dendritophage" project can effectively and safely be administered to cancer patients.