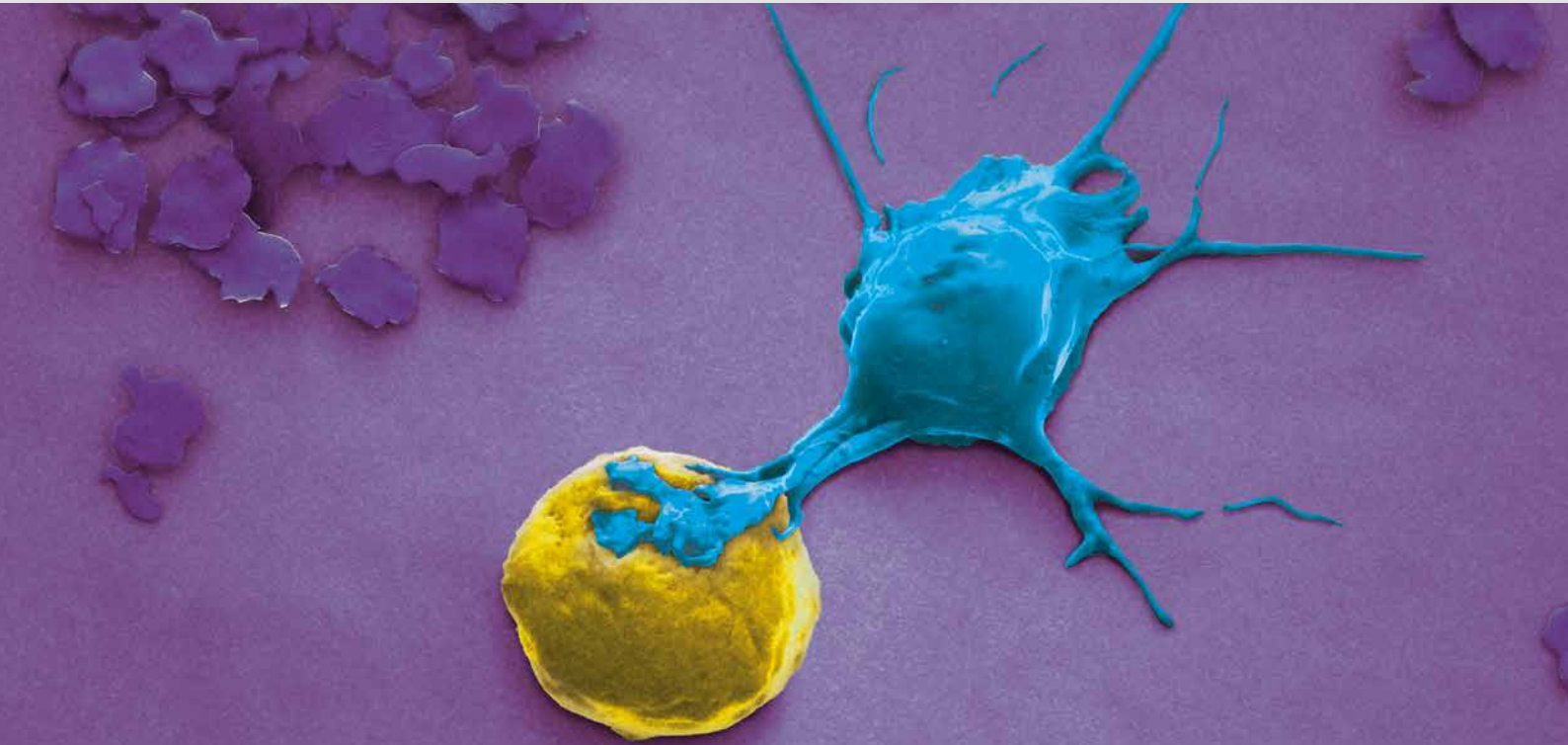# MACS & more

Designing a dendritic cell–based therapy for primary liver cancer

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Miltenyi Biotec

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Hepatocellular carcinoma – an introduction

Hepatocellular carcinoma (HCC) is the most common primary hepatic tumour and one of the most prevalent cancers worldwide. It is especially common in Asia and Sub-Saharan Africa. Risk factors for its development worldwide include hepatitis B, hepatitis C, alcohol abuse, aflatoxin exposure, and metabolic liver disease.

The incidence of HCC is rising, both in the United States and the United Kingdom, and is likely to be a reflection of the increased prevalence of cirrhosis from three main causes: hepatitis C, fatty liver disease, and alcoholic liver disease (ALD). It is therefore considered to become a major health burden in the UK in the coming years.

Current treatment for HCC is limited and 5-year survival for all stages combined is less than 5%. At present, surgery (either tumour resection or liver transplantation) is the only potentially curative treatment for HCC. However, resection is only feasible in less than 10% of patients, as it requires small tumours, limited-stage disease, and good hepatic function. Hence, the majority of patients present with advanced disease that is deemed unresectable. The survival rate is relatively poor, even in those patients who undergo surgical resection, with high recurrence rates and a 5-year survival of about 30–60%.

For patients with unresectable but localised HCC, local ablative therapy can remain an option. However, for those patients who are unsuitable for surgery or local ablation but with liver-confined disease, palliative benefit may be gained from transarterial chemoembolisation (TACE). Two randomised, controlled trials have shown that TACE performed with doxorubicin[1] or cisplatin[2] improves survival in selected patients compared to best supportive care. Even so, TACE remains palliative and disease progression is inevitable, such that combination with novel therapies is attractive. Since TACE may liberate an abundance of tumour antigens it may lend itself to combination with immunotherapeutic strategies.

The immunotherapy of malignancy: antigens

As immune-mediated mechanisms play an important role in controlling the growth of some types of cancer, immunotherapy could in theory exploit such responses to generate therapeutic immune responses against tumour antigens. Many tumours have altered gene regulation resulting in expression of specific tumour antigens or overexpression of other proteins. One such category is the cancer/testis antigens, a category of tumour antigens that are normally expressed in male germ cells but not in adult somatic tissues. In malignancy, this gene regulation is disrupted, resulting in cancer/testis antigen expression in a proportion of tumours of various types. For instance alpha-fetoprotein (AFP) is a serum marker for HCC.

Proteins that are specifically or predominantly expressed by tumours are potential targets for immunotherapy. Standard vaccination strategies have had only limited success in stimulating anti-tumour immunity, leading to the use of adoptive therapy with T cells or dendritic cells (DCs) to stimulate more potent immune responses which selectively kill malignant cells expressing specific antigens. Tumour antigens expressed in varying degrees in HCC and thus potential targets for immunotherapy include AFP, MAGE-1 and 3, SSX-1 and 4, TSPY, NY-ESO-1, and Glypican-3.[3–5]

The immunotherapy of malignancy: DCs

DCs are potent antigen-presenting cells which exist in peripheral tissues where they take up and process antigens. Short peptide fragments of the antigens are then presented on the cell surface in association with the major histocompatibility complex (MHC). Signals released by infected or damaged tissues act through receptors on the DCs to promote their activation and maturation. Maturation is associated with expression of costimulatory molecules that allow the DCs to activate T cells and chemokine receptors that mediate DC migration from peripheral tissue into draining lymph nodes. In the lymph nodes DCs interact

with and activate T cells that recognise the presented epitopes, resulting in the generation of effector T cells that can mount antigen-specific anti-tumour immune responses.

Following the demonstration that activated DCs were potent inducers of immune responses when adoptively transferred into animals, the development of techniques to generate large numbers of monocyte-derived DCs (Mo-DCs) from peripheral blood enabled DC-based immunotherapy to be tested in clinical trials. We previously conducted a clinical trial[6] to assess the safety and efficacy of intravenous vaccination with mature Mo-DCs, pulsed *ex vivo* with a liver tumour cell line lysate (HepG2), in patients with advanced HCC. Furthermore, we have conducted tracking studies to assess the differential homing of Mo-DCs delivered intravenously or directly into the liver via the hepatic artery (manuscript in preparation). These studies demonstrated that autologous Mo-DC vaccination in patients with HCC is safe and well tolerated and that increased numbers of Mo-DCs can be retained in the liver when infused via the hepatic artery.

Developing the next generation of Mo-DC vaccines in Birmingham

Following the successful completion of our previous clinical trials involving Mo-DCs, we looked to develop a new study to extend our understanding of these vaccines in patients with HCC. This led to the development of the ImmunoTACE clinical trial (long title: A randomised phase II clinical trial of conditioning cyclophosphamide and chemoembolisation with or without vaccination with dendritic cells pulsed with HepG2 lysate *ex vivo* in patients with Hepatocellular Carcinoma; EUDRACT # 2011-001690-62). This would combine the standard-of-care TACE with a Mo-DC vaccination and the addition of preconditioning in all patients with cyclophosphamide, which at low doses has been shown to selectively deplete regulatory T cells and increase antigen-specific immune responses in patients with HCC[7].

Changes to the regulatory landscape between the closure of our first HCC Mo-DC trial and the development of ImmunoTACE saw the introduction of new guidelines concerning the manufacture of Advanced Therapeutic Medicinal Products (ATMP). In order to be compliant with these current regulations it was necessary for us to develop our manufacturing process into a "closed" system.

Cell enrichment and culture process

The initial phase of this work included the use of CD14 MicroBeads from Miltenyi Biotec to isolate monocytes from peripheral blood samples. The CliniMACS® System provides a GMP-compliant platform for the isolation of CD14 monocytes, typically from leukocyte-rich apheresis. Based around a single-use, closed tubing set, the relative cost of CD14^{+} cell enrichment using this method is expensive and clearly would not be cost effective when beginning a new process development. With this in mind, we chose to begin the definition of our manufacturing process using CD14^{+} monocytes that were enriched from single units of whole blood with Miltenyi Biotec's autoMACS® Pro Separator and GMP-grade CliniMACS MicroBeads. The introduction of this automated step removed any operator bias in the preparation of the starting monocyte population before differentiation into Mo-DCs. As with many previous trials involving Mo-DCs, we chose to differentiate monocytes in the presence of GM-CSF and IL-4 (both 1,000 IU/mL). Here, we used premium-grade cytokines from Miltenyi Biotec which, whilst not certified as GMP grade, are prepared to the same standard and, importantly, are released with a lot-specific activity enabling us to ensure consistent dosing of cells in all experiments.

The next stage of our process development required transfer of our cell culture from traditional multi-well plates into a closed bag system. We compared culture bags and media from several manufacturers and found little variability in the quality of Mo-DCs prepared in each. Further development runs were performed in DendriMACS™ GMP Medium (Miltenyi Biotec). Usually a limitation of bag culture vessels is the need for a relatively high medium volume and consequently the requirement of high initial cell numbers. As an alternative to single-chamber bag systems we performed our initial culture experiments in Miltenyi Biotec's MACS GMP Cell Expansion

Bags. These have the advantage of multiple chambers that can be used flexibly, according to the required medium volume. This enabled us to begin process development with a bag material that is easily scalable to the next level. The initial monocyte number was set to 3×10^6 to account for medium additions on days 3 and 5.

On day 6 of culture the resulting immature DCs were loaded with antigen (see below) and matured in the presence of the TLR4 ligand monophosphoryl lipid A (MPLA). This maturation step was chosen as the material is available as a cGMP product and a single maturation agent lends itself to a simpler process with fewer interventions and is more cost effective. Furthermore, we have previously demonstrated that maturation via TLR4 ligation leads to potent up-regulation of costimulatory molecules as well as the lymph node homing receptor CCR7. This final maturation and antigen-loading phase continued for 48 hours until Mo-DCs are released on day 8.

Preparation of antigen

The choice of antigen to be loaded onto Mo-DC vaccines has long been a source of much discussion because tumour antigens are often shared self-antigens and thus elicit a tolerogenic response. As our understanding of tolerance within the immune system increases, the combination of inhibitors of checkpoint molecules such as CTLA-4 and PD-1/PD-L1[8,9] and perturbation of regulatory cells has led to a resurrection of host tumour as an antigen source. The ImmunoTACE clinical trial was developed ahead of these exciting new changes. For this reason, and for pragmatic reasons associated with access to host tumour, we decided to use a cancer cell line lysate as a source of antigen. In our case, the hepatoblastoma cell line HepG2 was chosen, partly due to our previous vaccine trial experience and because this cell line shares some antigens associated with HCC.

Typically, preparation of lysates for clinical application makes use of repeated freeze/thaw cycles to induce cell lysis. This method, whilst simple, often leads to low total protein yields and can result in residual whole-cell contamination. We again chose to make

use of a frequently used tool in our research laboratory from the Miltenyi Biotec stable. We have used the gentleMACS™ Dissociator for many years to generate single-cell suspensions from whole tissue and to prepare material for molecular biology protocols. Here, we used gentleMACS M Tubes with the RNA_01.01 protocol to generate a lysate for clinical use. Briefly, HepG2 cells were harvested from Corning® CellStack® – 5 Chambers using a closed bag system (Macopharma) and cGMP trypsin substitute (TrypLE™ Select, Invitrogen™) before being washed in PBS and resuspended in normal saline supplemented with calcium and magnesium. Individually wrapped sterile gentleMACS M Tubes were taken into a grade A processing area and 7 mL of the HepG2 cell suspension (approx. 35×10^6 cells) were added via injection through the septa to keep the process closed. The lids were then firmly closed and each tube processed on a gentleMACS Dissociator using protocol RNA_01.01, repeated 5 times. Following lysis, 500 IU DNAse I (Pulmozyme®, Roche) was added and the tubes incubated at 37 °C for 10 minutes. The lysate was then recovered using a long needle and syringe via the septa and transferred to a sterile bag and washed in normal saline. Following final resuspension in saline the lysate was aliquoted into 2 mL crimp-sealed vials and stored at –80 °C. A representative sample of the batch produced was removed and tested for sterility, endotoxin contamination, and presence of whole-cell

contamination before being released for use in the clinical manufacturing process.

Final release and cryopreservation

Choosing a suitable assay to define the release of cell therapy products can be a complex process. Parameters such as viability, sterility, and presence of endotoxin can be measured using easily validated tests, whilst surface phenotype is a more complex proposition. In Birmingham, we have integrated a MACSQuant® Flow Cytometer into our ATMP manufacturing facility QC lab as a single-platform cytometer for the enumeration and phenotypic analysis of monocytes and mature DCs.

Following apheresis, total leukocyte count and percentage of monocytes are calculated using the MC CD14 Monocyte Cocktail and "Express mode" on the MACSQuant Analyzer 10 (fig. 1). This requires minimal operator input and gives a rapid determination of key parameters required for enrichment of CD14+ monocytes using the CliniMACS Platform. This same cocktail and protocol are used after enrichment to determine absolute monocyte count and purity before beginning differentiation in culture.

Following differentiation of Mo-DC as described previously, an analysis of phenotype is made on day 6, prior to addition of antigen and MPLA. Specifically, we measure the

expression of HLA-DR, CD11c, CD86 and CD14 (fig. 2). Final release of the cells is performed on day 8 with samples taken for sterility testing, endotoxin contamination and repeat phenotypic analysis. Our release criteria stipulate an increase in expression of HLA-DR and CD86 on dual-positive cells between day 6 and day 8, consistently high expression of CD11c, and absence of CD14. Cells that meet these criteria are released in four batches. An initial batch will be delivered directly to the patient for administration via the hepatic artery whilst the remaining three batches are resuspended in an appropriate volume of cryoprotectant (CryoStor® CS10, BioLife Solutions®) before being transferred to individual CryoMACS® Freezing Bags (Miltenyi Biotec). Controlled-rate freezing is carried out according to a previously validated protocol and cells are stored in a –152 °C mechanical freezer until required. Repeat peripheral infusions will be thawed at the patient bedside and delivered via a peripheral vein monthly for 3 months.

Transfer to GMP and validation

We are currently completing the final phase of validations to begin delivery of this Mo-DC cancer vaccine in the near future. Engineering runs under full GMP conditions are underway using both the CliniMACS Plus Instrument and the CliniMACS Prodigy®, though intention is to use the CliniMACS Prodigy for all cell enrichments. This automation of the enrichment process is a key forward step in the manufacturing of Mo-DC therapies, saving considerable hands-on time and being relatively simple to scale out with the provision of additional machines. Furthermore, as the development of the CliniMACS Prodigy continues, the introduction of a compliant culture process to take patient apheresis samples through to immature Mo-DCs without the need for additional manual manipulations will simplify the delivery of novel cellular therapies.

Conclusion

In common with many similar facilities throughout the UK, our experience in Birmingham of developing an ATMP manufacturing facility has been challenging. The commitment of a strong team has enabled
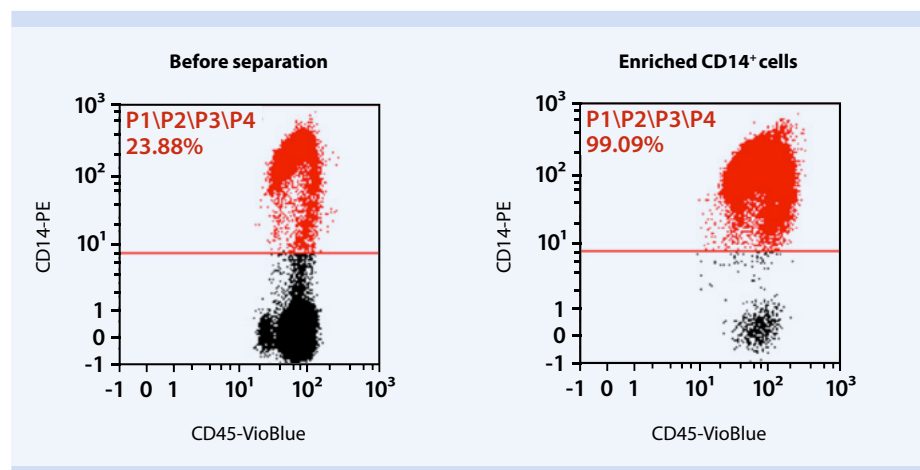

Figure 1 Enrichment of CD14+ monocytes. CD14+ monocytes were enriched from PBMCs using the CliniMACS System. The percentage of monocytes was calculated using the MC CD14 Monocyte Cocktail and "Express mode" on the MACSQuant Analyzer 10.
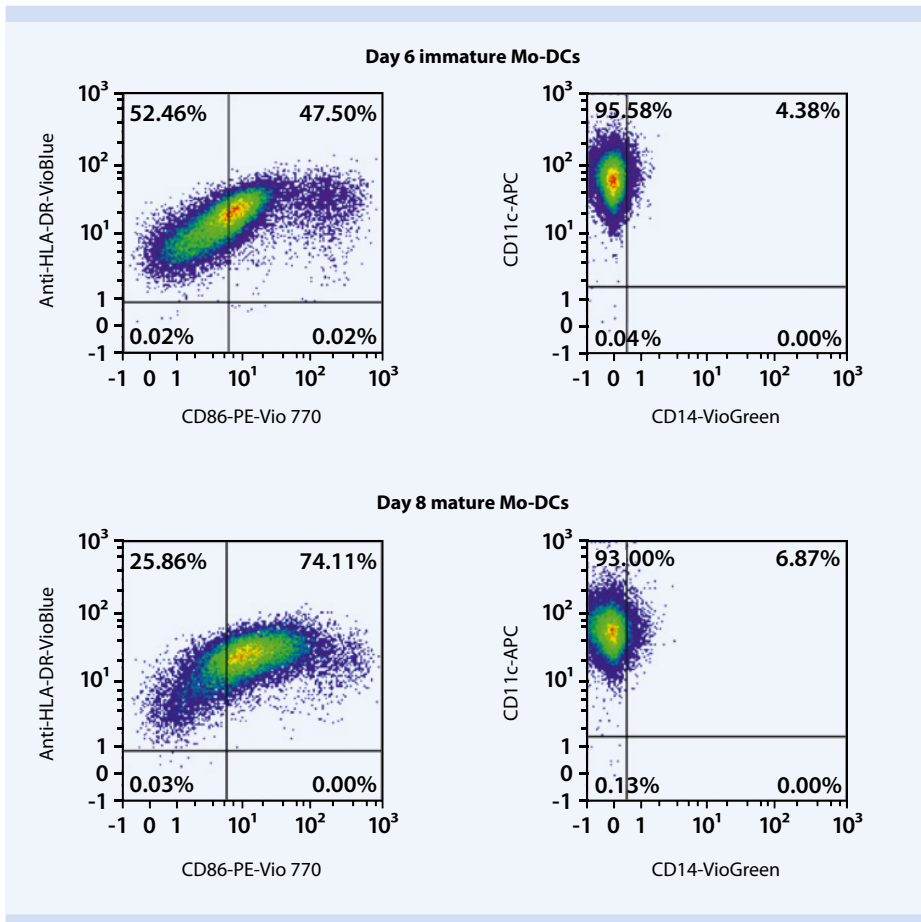
Figure 2 Phenotypic characterization of mature Mo-DCs. Enriched CD14⁺ cells were differentiated for 6 days and the resulting immature Mo-DCs were matured for two days. Cells were stained with Anti-HLA-DR-VioBlue®, CD86-PE-Vio® 770, CD11c-APC, and CD14-VioGreen™ on day 6 and day 8, and analyzed by flow cytometry on the MACSQuant Analyzer 10.

MACS Product	Order no.
CliniMACS Plus Instrument	151-01
CliniMACS Prodigy	200-075-301
CliniMACS CD14 MicroBeads	130-019-101
CliniMACS CD14 Reagent	272-01
autoMACS Pro Separator	130-092-545
Human GM-CSF – premium grade (100 µg)	130-093-866
Human IL-4 – premium grade (100 µg)	130-093-922
DendriMACS GMP Medium	170-076-302
MACS GMP Cell Expansion Bags	170-076-403
gentleMACS Dissociator	130-093-235
gentleMACS M Tubes	130-096-335
MACSQuant Analyzer 10	130-096-343
MC CD14 Monocyte Cocktail, human	130-092-859
Anti-HLA-DR-VioBlue, CD11c-APC, CD86-PE-Vio 770, CD14-VioGreen	*
CryoMACS Freezing Bags	**

* Visit www.miltenyibiotec.com/antibodies

** Visit www.miltenyibiotec.com/cryomacs

us to bring the ImmunoTACE clinical trial into reality and has demonstrated how research laboratory tools can, with careful planning, be integrated into validated manufacturing processes for the preparation of ATMPs.

With a rapidly increasing incidence of liver disease in the UK, the development of novel therapies has never been more pressing. Moreover, as we gather a greater understanding of the interplay between the human immune system and tumour microenvironment we have at our disposal a greater armoury to manipulate the immune response to cancer. For many years cell therapy has promised much, but failed to deliver comprehensively. Combination of improved manufacturing techniques with immunomodulation is moving the field into an exciting era that may finally see us translate some of the early potential into meaningful treatments for the many patients living with the burden of cancer.

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