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REVIEW

MITAP-compliant characterization of human regulatory macrophages

James A. Hutchinson[1], Nobert Ahrens[2] & Edward K. Geissler[1]

1 Department of Surgery, University Hospital Regensburg, Regensburg, Germany
2 Department of Transfusion Medicine, University Hospital Regensburg, Regensburg, Germany

Correspondence
Dr. James Hutchinson, Department of Surgery, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany.
Tel.: +49-941-944-6831;
fax: +49-941-944-6772;
e-mail: james.hutchinson@ukr.de

SUMMARY

This article provides a transparent description of Mreg_UKR cell products, including manufacture and quality-control processes, using the structure and vocabulary of the 'Minimum Information about Tolerogenic Antigen-presenting Cells' reporting guidelines. This information is intended as a resource for those in the field, as well as a stimulus to develop a new wave of immunoregulatory and tissue-reparative monocyte-derived cell therapies.

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Key words
cell-based therapy, minimum information about tolerogenic antigen-presenting cells, monocyte, Mreg, regulatory macrophage, solid organ transplantation, suppressor macrophage, tolerogenic antigen presenting cell (APC)

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Introduction

Administering cells with immunoregulatory function to patients to control unwanted immune reactions is not a new proposition[1]. From the earliest discovery that transferring regulatory cells from tolerant to nontolerant animals could establish tolerance in the recipient, it was suggested that the same principle could be applied therapeutically in man[2]. However, while adoptive transfer became a common experimental procedure, its translation to the clinic met many obstacles, not least the difficulties of identifying and isolating human regulatory cells. Despite the substantial challenges it presents, cell-based immunoregulatory therapies remain an attractive alternative to general immunosuppression for two main reasons. Firstly, cell therapy approaches offer the possibility of inducing antigen-specific immunological nonresponsiveness. Secondly, as peripheral regulation is a self-reinforcing state[3], it is possible that cell-based immunoregulatory treatments could have very long-lived effects, which would not necessarily be limited by the lifespan of the therapeutic cells. Critically, many of the technical challenges of manufacturing pharmaceutical-grade regulatory cell preparations have now been overcome, paving the way for early-phase clinical studies in solid organ transplantation[4].

When thinking about cell therapies, it is important to draw a distinction between *cell types* and particular cell-based *products* composed of those regulatory cell types [5–7]. Immunoregulatory cell products contain *populations* of cells that are inescapably heterogeneous in phenotype, function and degree of contamination with other cells. The exact processes used to manufacture a cell product affect these qualities to such an extent that alternative products containing ostensibly similar cell types may have quite different pharmacological properties[8]. Consequently, to compare between cell products, considerable information is needed about their production methods and biological characteristics. Until recently, however, there was no consensus as to what

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constituted a sufficient description of a cell product, particularly in the case of monocyte-derived regulatory cell-based products. To address this deficiency, Lord and Hilkens spearheaded an initiative through the A FACTT consortium (http://www.afactt.eu) to develop a minimum information model known as the *Minimum Information about Tolerogenic Antigen-presenting Cells* (MITAP) protocol[9]. MITAP reporting standards demand information in four parts, describing the manufacture of cell products [1] at the start of the production process, [2] during *in vitro* development, [3] at the end of the production process, and [4] in clinical application (http://w3id.org/ontolink/mitap). There is considerable support for MITAP in the field because it imposes greater transparency, a controlled vocabulary and makes it easier to interpret and integrate published information about regulatory myeloid cells.

Myeloid regulatory cells as therapeutic agents

Peripheral immunological nonreactivity mediated by regulatory T cells is sustained by professional and nonprofessional antigen-presenting cells with suppressor function, including myeloid regulatory cells, regulatory B cells and nonhaematopoietic lineages that express MHC Class II antigens. The contribution of myeloid regulatory cells to induction and maintenance of peripheral tolerance has been especially well studied[10–12]. Like regulatory T cells, different myeloid regulatory cell populations serve different physiological and pathophysiological purposes [13]. Under normal physiological, noninflammatory conditions, immature DCs and macrophages present self- or other innocuous antigens to T cells in a subimmunogenic context. Recognition of cognate antigen in the absence of costimulation causes effector T cells to die, become anergic or convert into regulatory T cells. Thereby, antigen presentation by unactivated myeloid APCs contributes to the steady-state maintenance of self-tolerance [14]. A second 'class' of myeloid regulatory cell arises as a consequence of persistent stimulation with pro-inflammatory mediators. Such activation-induced suppressor cells presumably serve as counter regulators that prevent auto-destructive inflammatory responses [15]. Activation-induced myeloid regulatory cells are phenotypically diverse and operate through a variety of mechanisms, including production of T-cell-suppressive soluble factors, receptor-mediated killing of effector T cells and the activation-dependent induction of pTregs.

The classification of myeloid regulatory cells as 'immature' or 'activation-induced' suppressor cells can be extended to *ex vivo*-generated, monocyte-derived cell types being used in cell-based medicinal products (CBMP). Because immature APCs play such a well-studied role in the maintenance of peripheral tolerance, most efforts to develop monocyte-derived regulatory cell products have naturally focused on myeloid regulatory cells in arrested states of immaturity[16]. Diverse anti-inflammatory treatments have been used to render immature monocyte-derived regulatory cells refractory to activation, including generation in the presence of IL-10 (DC10 cells) [17] or rapamycin (Rapa-DCs)[18], culture with low concentrations of GM-CSF (Tol-DCs) [19] or exposure to dexamethasone plus vitamin D [20].

This article concentrates on a type of activation-induced, monocyte-derived suppressor cells known as regulatory macrophages (Mreg)[21–23]. In preclinical studies, preoperative administration of donor-derived Mregs by intravenous injection prolonged allograft survival in nonimmunosuppressed, fully allogeneic recipients without lymphodepletion or other conditioning [21]. Early-stage clinical studies[24–27] demonstrated that pretransplant infusion of allogeneic Mregs was safe [28,29] and promoted regulatory responses in kidney transplant recipients maintained on very low-dose tacrolimus monotherapy[22]. Mreg cells are the active component of an immunosuppressive CBMP known as Mreg_UKR. A proprietory GMP-compliant process for manufacturing Mreg_UKR has been established at a commercial pharmaceutical manufacturing facility in Germany. Donor-derived Mreg_UKR is currently being investigated in a Phase-I/II trial as a means of promoting immune regulation in kidney transplant recipients to facilitate safe minimization of maintenance immunosuppression (clinicaltrials.gov: NCT02085629). Here, we provide a MITAP-compliant description of Mreg_UKR as a monocyte-derived immunoregulatory cell product.

PART 1: cells before

Part 1a: essential information about the donor

(i) *Species and strain*: Mreg_UKR is produced from adult human (*Homo sapiens*) peripheral blood $CD14^+$ monocytes. Both male and female donors have been used for full-scale, GMP-compliant production of Mreg_UKR. Experience with full-scale production of Mreg_UKR under clean room conditions is presently limited to donors of white, Northern European ethnic origin.

(ii) *Characteristics of the donor organism*: In the current *ONEmreg12* trial, Mregs are produced from

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monocytes isolated from healthy prospective living-donor kidney donors. In other clinical indications, Mreg_UKR may be derived from monocytes of healthy or diseased individuals and may be used as autologous or allogeneic therapies. Only individuals who comply with regulations concerning the donation, procurement and testing of human tissues imposed by the Commission Directive 2006/17/EC[30] are accepted as cell donors. Regulations pertaining to the German Transplantation Law, German Pharmaceutical Law and guidelines for the collection of blood and blood compounds and the use of blood products (2010) issued by the German Medical Association also apply. The following eligibility criteria for monocyte donation are based on those already applied to adult allogeneic stem cell donors at our centre and have been approved by the responsible authorities, namely *Regierung der Oberbayern* and the *Paul Ehrlich Institute*:

1 Donor bodyweight > 25 kg
2 Suitable venous access (peripheral or central)
3 Haematological and biochemical criteria

Results must not be greater than 48 hours old at the time of leucapheresis:

i ABO and RhD blood type determined
ii No irregular antibodies detectable
iii Differential blood count (including erythroblasts) determined.
iv Hb ≥ 8 g/dl.
v Leucocytes ≥ 2.5 × 10^3/μl.
vi Neutrophils ≥ 1.5 × 10^3/μl.
vii Thrombocytes ≥ 100 × 10^3/μl.
viii CRP ≤ 5 mg/l.
ix Normal coagulation (Quick/INR, aPTT, fibrinogen, antithrombin)

4 Microbiological and virological criteria

Results must not be more than 30 days old at the time of leucapheresis:

i All donors must be negative for anti-TP, HIV-1 + -2 Ab, HIV p24 Ag, HIV-PCR, HBsAg, HBcAb, HBV-PCR, HCV-Ab and HCV-PCR.
ii If indicated by history of possible exposure, donors must be shown to be uninfected with the following pathogens: HTLV-I/II, HHV-8, HSV, WNV, *Rickettsia spp., Borrelia spp., F. tularensis, B. pseudomallei, C. burnetii, M. tuberculosis, S. typhi, Brucella spp., Plasmodia spp., leishmania, trypansomes, schistosomes and babesia spp.* and strongyloides.
iii Potential donors are screened for prion disease by focused medical history-taking.

Part 1b: source of cell material

Cell donors undergo mononuclear cell apheresis in accordance with a manufacturing authorization issued by *Regierung von Oberbayern*. Leucapheresis is performed with a Spectra Optia device using the continuous mononuclear cell collection programme according to the following specifications:

1.	Anticoagulant (citrate) dilution	1:10 (range from 1:9 to 1:14)
2.	Collection time	120–180 min
3.	Inflow rate	>55 ml/min
4.	Packing factor	4.5
5.	Collection pump	1 ml/min
6.	Collection preference	Haematocrit of 2–3%

Samples of the final apheresis product are taken for in-process quality-control testing. Total leucocyte and monocyte counts are determined by differential cell counting using a Sysmex device (Table 1). Sterility controls are performed using the BactAlert system for detection of aerobic and nonaerobic micro-organisms. Criteria for pharmaceutical release of mononuclear apheresates are as follows:

1.	Total monocyte content	≥0.6 × 10^9
2.	Microbiological control	Samples dispatched

After sampling for quality-control purposes, leucapheresis products are stored overnight at 22 ± 2 °C in the original collection bag with gentle horizontal agitation to prevent precipitation of cells from suspension. The next day, leucapheresis products are transported to a contract pharmaceutical manufacturing facility under temperature-controlled (22 ± 2 °C) conditions.

Part 1c: cell separation process

Human Mreg cells arise from isolated $CD14^+$ monocytes in the absence of other cell types. Mreg_UKR is manufactured from $CD14^+$ monocytes isolated directly from mononuclear cell apheresates using a CliniMACS™ device and CliniMACS™ CD14 reagent according to the manufacturer's standard operating procedure. Following the separation and washing steps, a sample is taken for assessment of $CD14^+$ monocyte purity and viability by flow cytometry.

CD14 microbeads are paramagnetic particles composed of a low-molecular dextran polymer with a Fe^{2+}/

Table 1. Results of ten consecutive mononuclear cell apheresis performed on a Spectra Optia device. Total leucocyte and monocyte recovery is presented in relation to donor- and process-associated parameters.

	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10
Donor-related										
Sex of cell donor	♂	♀	♀	♂	♂	♂	♂	♂	♀	♀
Age of cell donor	25	48	60	62	42	22	26	26	52	52
Leucocytes in blood (cells/nl)	4.47	6.05	6.47	7.70	7.06	5.63	6.40	6.55	5.83	5.99
Monocytes in blood (cells/nl)	0.37	0.57	0.50	0.62	0.59	0.45	0.57	0.66	0.60	0.33
Monocytes in blood (%)	8.3	9.4	7.7	8.1	8.4	8.0	8.9	10.1	10.3	5.5
Haematocrit (%)	40.7	36.1	39.0	48.1	43.8	44.8	44.7	44.4	39.7	38.2
Estimated blood volume (ml)	4727	3381	3627	5442	6944	6197	5959	5313	4144	3561
Process-related										
Duration of leucapheresis (min)	126	140	162	161	150	180	180	163	160	157
Processed blood volume (ml)	7452	6803	7061	7534	8239	9872	9845	9562	8744	7001
Mean inflow rate (ml/min)	65	53	48	51	60	60	60	64	60	49
Product-related										
End-product volume (ml)	119	115	126	136	133	146	155	133	141	133
Leucocytes in end-product ($x10^9$)	11.1	8.9	11.2	15.3	13.7	18.3	17.4	14.2	12.2	8.0
Monocytes in end-product ($x10^9$)	2.6	2.7	2.1	3.8	3.9	3.3	5.2	4.1	3.8	1.5
Monocytes in end-product (%)	23.4	30.3	18.8	24.8	28.5	18.0	29.9	28.5	31.1	19.1

Fe^{3+} iron oxide/hydroxide core coated with a mouse monoclonal antibody specific for human CD14. CliniMACS CD14 Reagent is a dark amber, nonviscous solution composed of CD14 microbeads buffered with phosphate-buffered saline (PBS) containing ethylene diamine tetraacetic acid (EDTA) and Poloxamer 188, which are added as stabilizers. CliniMACS CD14 reagent is supplied sterile and endotoxin-free in glass vials. CliniMACS CD14 reagent is a medical device class III (Rule 17, Annex IX) and is CE-certified by TÜV SÜD Product Service GmbH, Munich. It is assessed according to Annex II of the Medical Device Directive 93/42/EEC and fulfils the essential requirements of this Directive. CliniMACS CD14 reagent is certified under EC Design Examination (Certificate No. 07 09 08 31072 025). The conformity of the CliniMACS CD14 reagent according to the Council Directive is ensured and declared by Miltenyi Biotec GmbH.

During Mreg culture, CD14 microbeads are initially bound to the surface of monocytes, but are completely internalized within 150 min[31]. It is well-established that CD14 delivers its ligands into phagosomes of macrophages[32], so CD14 microbeads are fated to undergo lysosomal degradation through exposure to an acidic environment (~pH 4.8) and proteases. Under these conditions, antibody and dextran are rapidly degraded, whereas iron simply dissolves. Our group has performed experiments to measure the amount of iron retained by Mregs generated from human peripheral blood monocytes after CD14 microbead selection. Iron content was measured by dissolving Mregs in nitric acid and hydrogen peroxide before quantification of iron in solution by inductively coupled plasma mass spectrometry (unpublished results; collaboration with Dr. Amy Managh). Mregs contained up to 1.42×10^9 atoms of iron (equivalent to 1.32×10^{-13} g/cell iron) per cell. This is ~100-fold greater than the normal iron content of a monocyte-derived macrophage, so it appears CD14-selected monocytes sequester iron from microbeads[5]. Here, we summarize our risk assessment for CD14 microbeads and their possible implications for the safety of Mreg_UKR therapy:

1 *Iron-dextran colloids:* Intravenous administration of iron-dextran colloids is an established treatment for iron deficiency anaemia. A number of licensed iron-dextran colloid solutions for *iv* infusion are licensed for clinical use in Europe, including CosmoFer® (Pharmacosmos, Holbæk Denmark). The normal dosing schedule for CosmoFer® is 100–200 mg iron given two to three times per week; in comparison, the total iron content of a maximum dose of Mreg_UKR is

~13.2 mg. In general, adverse reactions to colloidal iron are rare or very rare, but mild anaphylactoid reactions resulting in urticaria, rashes, pruritus, nausea or shivering may occur in 1/100 to 1/1000 patients. Severe anaphylactoid reactions are very rare. It is important to note that Mreg_UKR patients do not receive direct infusions of iron-dextran colloids because the iron-dextran is fully taken up and degraded by the cells during culture. Overall, the clinical risks associated with infusion of such a small quantity of iron-dextran are considered to be very small. Moreover, the patient management plan anticipates possible adverse reactions and allows for their prevention or treatment.

2 *Particulates:* Miltenyi microbeads measure 50 to 100 nm in diameter, so are too small to obstruct blood vessels and do not pose an embolic risk.

3 *Mouse antibodies:* Mouse monoclonal antibodies are potentially immunogenic. Therefore, previous immunization against mouse antibodies (perhaps by previous treatment with cells isolated using CliniMACS reagents) is a relative contraindication to Mreg_UKR therapy. Theoretically, patients might generate anti-mouse Ig antibody responses after Mreg_UKR administration, but this is considered extremely unlikely because any anti-CD14 microbeads attached to developing Mregs are likely to be completely degraded within lysosomes. Nonetheless, during the process of informed consent, patients receiving Mreg_UKR should be advised that they may be at future risk of reactions against drug products that contain mouse Ig.

4 *Virological safety:* Transmission of zoonoses with CD14 microbeads is essentially excluded by measurements of viral contamination performed by Miltenyi GmbH.

Part 1d: phenotype

(i) *Morphology*: Upon inspection of Trypan blue-stained samples in a haemocytometer, $CD14^+$ monocytes isolated by CliniMACS exhibit typical morphology of human blood monocytes.

(ii) *Cell-surface and intracellular markers*: After CliniMACS isolation, $CD14^+$ monocyte purity is typically >97% and contamination with $CD3^+$ T cells is consistently <1%. The majority of $CD14^+$ monocytes belong to the 'classical' $CD14^+$ $CD16^-$ subset; however, a variable proportion of 'resident' $CD14^{+/int}$ $CD16^+$ monocytes are also present.

(iii) *Secreted molecules*: Secreted products of $CD14^+$ monocytes are not routinely assessed during manufacture of Mreg_UKR.

Part 1e: cell numbers

(i) *Absolute cell number*: The number of monocytes obtained by CliniMACS sorting depends upon the number of monocytes present in the leucapheresis product and may be limited by the capacity of CliniMACS columns. Typically, $0.5–1.5 \times 10^9$ viable $CD14^+$ monocytes are recovered.

(ii) *Viability*: $CD14^+$ monocyte viability is assessed by 7-AAD exclusion in flow cytometry and typically exceeds 95%.

PART 2: differentiation and induction of tolerogenicity

Manufacture of Mreg_UKR is carried out in accordance with current GMP principles for manufacturing sterile medicinal products. The essential culture conditions for Mreg_UKR were established at University Hospital Regensburg (UKR). The GMP-compliant handling steps necessary for producing a sterile cell product under clean room conditions were developed by a contract research organization in Germany. Assays for Mreg_UKR identity, purity and potency were first established at UKR and then transferred to the contract manufacturer. Here, we describe the principles of Mreg_UKR production and quality control; however, some details of the process are proprietory 'know-how'.

Part 2a: preculture conditions

The manufacture of Mreg_UKR products is a continuous process; hence, no formal distinction is made between drug substance and drug product. $CD14^+$ monocytes isolated by CliniMACS are not stored prior to further manipulation. Isolated monocytes are washed with culture medium to remove CliniMACS separation buffer before monocyte density is adjusted to 10^6 cells/ml in culture medium. Samples are then taken for analysis of CD14 expression and cell viability by flow cytometry.

Part 2b: culture conditions

(i) *Cell numbers*: The number of monocytes used for each Mreg_UKR production run depends upon the number of culture vessels to be seeded. A typical production run uses three to eight vessels that are seeded at 180×10^6 viable $CD14^+$ monocytes per vessel. Accordingly, total starting cell numbers may vary between 0.54×10^9 and 1.44×10^9 viable $CD14^+$ monocytes after CliniMACS isolation.

(ii) *Cell concentration*: $CD14^+$ monocytes are suspended at 10^6 viable cells/ml in culture medium. This suspension is distributed into culture vessels at a density of 2×10^6 cells/cm^2 of vessel surface area.

(iii) *Culture medium*: Mreg cells are generated in an RPMI-based medium supplemented with human AB serum, monocyte colony-stimulating factor (M-CSF) and stabilized L-glutamine. On day 6, Mreg cultures are stimulated with IFN-γ. No antibiotics are used at any step in Mreg_UKR production. The composition of Mreg_UKR culture medium is given in Table 2. The particular reagents used for Mreg_UKR manufacture are specified in Table 3.

Serum for Mreg_UKR manufacture is sourced from a commercial provider in Germany that supplies recalcified plasma-derived sera pooled from at least 20 male donors of blood group AB. The supplier operates according to Directive 2006/17/EC and guidelines for collection of blood and blood compounds and use of blood products (2010) issued by the German Medical Association. Donors are screened for anti-HIV-1/2 antibodies, anti-HCV antibody, HBs antigen and anti-HBc antibody, as well as screening for syphilis using the *Treponema pallidum* particle agglutination (TPHA) assay. Additionally, collected sera are tested for HIV-1 and HCV by PCR-based methods. Prior to use in Mreg_UKR culture, human serum is heat-inactivated to destroy complement and then stored frozen to prevent degradation. No virus-inactivation steps are performed. Male-only serum is preferred to female serum because it is theoretically less likely to contain anti-HLA antibodies, which may be elicited by pregnancy. Surprisingly, in a recent study, three commercially sourced, male-only human AB serum batches contained measurable amounts of HLA Class I- and Class II-reactive antibodies[33]. Considering these antibodies were diluted by pooling of sera, it seems that one or more of the plasma donors were truly sensitized. Hypothetically, anti-HLA antibodies could affect Mreg development by opsonizing the cells, leading to activation of other FcR-bearing monocytes, or by inappropriately activating monocytes by retrograde signal transduction through MHC molecules themselves[34]. However, in five separate production runs under research or clean room conditions, Mregs were found to develop normally in the presence of donor-specific antibodies. Moreover, these antibodies did not mediate complement-dependent cytotoxicity in conventional cross-match assays. Manufacturers should be aware of the unexpected presence of anti-HLA antibodies in certain commercially sourced human sera, although these antibodies do not appear to affect the quality of Mreg_UKR products.

During manufacturing-process development, M-CSF concentrations in the range 5–100 ng/ml were tested. No consistent effect of M-CSF concentration on cell viability, yield, phenotype or suppressive function was observed. Time-course experiments revealed that M-CSF was consumed or degraded over time in culture, such that cultures with an initial dose of 5 ng/ml M-CSF contained subphysiological concentrations by day 2 of culture, whereas cultures with an initial dose of 25 ng/ml M-CSF maintained concentrations >10 ng/ml throughout the 7-day culture period.

(iv) *Culture container*: In the current process, proper development of the Mreg phenotype requires that monocytes adhere at least transiently to the surface of a tissue culture vessel. The exact nature of the closed-system culture vessels used for Mreg_UKR is proprietory information.

(v) *Culture environment*: Cultures are maintained at 37 ± 1 °C and buffered against 5 ± 1% CO_2. The exact nature of the incubation system is proprietory information.

Part 2c: differentiation or tolerization process protocol

Human Mregs derive from $CD14^+$ peripheral blood monocytes when cultured in the presence of M-CSF and high concentrations of heat-inactivated human serum for more than 4 days prior to stimulation with IFN-γ. In the current manufacturing process, monocytes are cultivated in Mreg culture medium for 6 days prior to stimulation with 25 ng/ml recombinant human IFN-

Table 2. Composition of 500 ml Mreg_UKR basal culture medium.

Component	Stock concentration	Volume (ml)	End concentration
RPMI-1640 without phenol red or L-glutamine	–	444.75	–
Heat-inactivated, male-only human AB serum	–	50	10%
GlutaMAX, stabilized L-glutamine	200 mM	5	2 mM
rhM-CSF carried on 0.1% human albumin in RPMI-1640 without phenol red or L-glutamine	50 μg/ml	0.25	25 ng/ml

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Table 3. GMP-grade reagents specified for preparation of Mreg_UKR culture medium.

Material	Application	Specification
RPMI-1640 without phenol red	Basal culture medium	Sterile Endotoxin levels ≤ 1 EU/ml Animal free Manufacturer: Lonza RPMI 1640 w/o L-Gln and PR Cat.no.: BE 12-918F
Pooled male-only, human AB serum, heat-inactivated	Basal culture medium	Sterile Endotoxin levels ≤ 10 EU/ml Mycoplasma-negative Virus testing negative for HIV, HBV, HCV and TPHA Manufacturer: ZKT Tübingen Cat.no.: P-HS/Tü
Recombinant human M-CSF	Basal culture medium	Sterile Endotoxin levels ≤ 1 EU/μg Animal free Purity ≥ 97% Manufacturer: R&D Systems Recombinant human M-CSF Cat.no.: AFL216
GlutaMAX, stabilized L-glutamine	Basal culture medium	Sterile Endotoxin levels ≤ 1 EU/ml Animal free Manufacturer: Gibco GlutaMAX-I CTS, 100x Cat.no: A12860
Human IFN-γ	Stimulation	Sterile Endotoxin levels ≤ 1 EU/ml Imukin 0.1 mg IFN-γ/0.5 ml Approved medicinal product: B.Braun Melsungen AG Registration number: 26289.00.00

γ for a further 18–24 h. On day 7, Mregs are harvested, washed extensively, resuspended in infusion solution and packaged in a transfusion bag. The duration of culture was optimized to achieve a phenotypically homogeneous population of Mregs. IFN-γ results in stable upregulation of indoleamine 2,3-dioxygenase (IDO) that confers suppressive activity to Mreg_UKR over a period of >18 h[22]. At harvest, Mregs are detached from vessel surfaces by purely mechanical means according to a proprietory protocol; notably, trypsinization can reduce overall cell yield and viability, whereas other enzyme preparations (*e.g.* accutase) can alter the cell-surface phenotype of Mreg cells.

Part 2d: antigen

Mreg_UKR is not pulsed with exogenous antigen. For application in the *ONEmreg12* trial in kidney transplantation, Mreg_UKR is generated from peripheral blood monocytes collected from a prospective kidney transplant donor and then administered to the intended kidney transplant recipient. In this setting, Mreg_UKR bears allogeneic tissue antigens, which may include MHC Class I and –II antigens, as well as multiple minor antigens.

Part 2e: storage

The Mreg_UKR cell product consists of Mreg cells suspended in 95 ml Ringer's lactate solution plus 5% human albumin at 1.25–7.5 × 10^6 viable Mregs/ml packaged in a gas permeable transfusion bag. In this condition, Mregs remain viable and phenotypically stable for at least 24 h. In the *ONEmreg12* trial, the final cell product is normally administered within 6 hours.

PART 3: cells after

Part 3a: phenotype

(i) *Morphology*: In culture, Mregs exhibit a distinctive morphology with the cells adopting a tessellating, epithelioid morphology to form almost confluent monolayers. Individual Mregs are large, densely granular cells with a prominent central body and a thin cytoplasmic skirt, which spreads symmetrically over the surface of the culture vessel, reaching diameters of up to 50 μm. Mregs exhibit a single, roughly spheroid nucleus located centrally within the cytoplasm (Fig. 1a).

(ii) *Cell-surface and intracellular markers*: To be released as a pharmaceutical product for administration to patients, each batch of Mreg_UKR must conform to 'release specifications' that describe the identity, purity and potency of its constituent cells. In the case of Mreg_UKR, these properties are measured by flow cytometry-based assays according to GMP principles. Markers used for Mreg_UKR release include CD14, CD16, CD80, CD85h, CD86 and CD258 (Fig. 1b). The release assay for Mreg_UKR potency is based on IDO expression as a surrogate marker of suppressor activity[22]. The exact specifications used for product release are proprietory information.

Riquelme *et al.* [35]recently reported that dehydrogenase/reductase 9 (DHRS9), a little-studied retinol

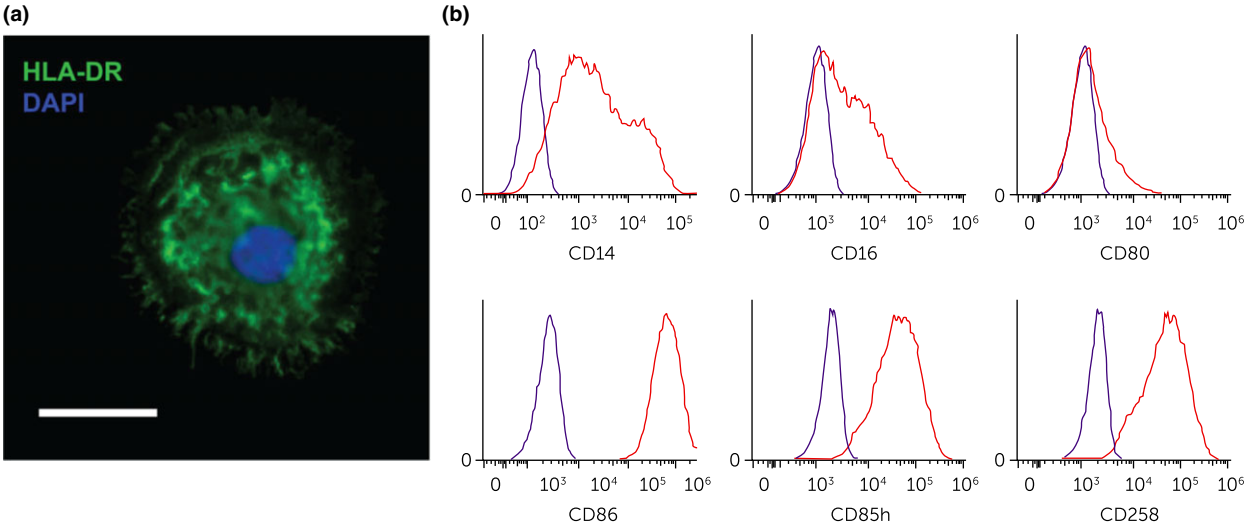

Figure 1 *Morphology and cell-surface markers of human Mreg_UKR.* (a) Fluorescence micrograph of an HLA-DR-expressing human Mreg cell adhering to a glass slide. Bar = 50 μm. (b) Markers of Mreg identity used for product release include CD14, CD16, CD80, CD86, CD85 h and CD258.

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dehydrogenase of the short-chain dehydrogenase/reductase (SDR) family of NAD(P)(H)-dependent oxidoreductases, is a stable and specific marker of human Mregs. DHRS9 mRNA and protein expression discriminated human Mregs from a panel of *in vitro*-derived macrophages in other polarization states. Likewise, DHRS9 expression distinguished Mregs from a variety of human monocyte-derived tolerogenic antigen-presenting cells in current development as cell-based immunotherapies, including Tol-DC, Rapa-DC, DC-10 and PGE_2-induced MDSC. Importantly, stimulating Mregs with 100 ng/ml lipopolysaccharide for 24 h did not extinguish DHRS9 expression. Expression of DHRS9 is not currently specified as a criterion for Mreg_UKR product release.

(iii) *Secreted molecules*: Secreted factors are not routinely measured as part of Mreg_UKR manufacture.

Part 3b: cell behaviour

Human Mregs suppress mitogen-stimulated T-cell proliferation *in vitro* through interferon-gamma (IFN-γ)-induced indoleamine 2,3-dioxygenase (IDO) activity, as well as contact-dependent deletion of activated T cells [22]. In addition, Mregs drive the development of activated-induced regulatory T cells that, in turn, suppress the proliferation and activity of effector T cells (Riquelme-P *et al.*, unpublished). After intravenous administration, human Mregs traffic via the blood to lung, liver, spleen and haematopoeitically active bone marrow[22,29,36].

Part 3c: cell numbers

(i) *Absolute cell numbers*: The resulting number of cells in each Mreg_UKR batch reflects starting cell numbers and technical variations. By specification, Mreg_UKR products must contain a total of 118.75×10^6–712.50×10^6 viable Mregs, as determined by the Trypan Blue counting method.

(ii) *Viability*: By specification, Mreg_UKR products must contain >75% viable Mregs, as determined by 7-AAD exclusion in flow cytometry; however, cell viability is typically >90%.

PART 4: about the protocol

Part 4a: regulatory authority

Production of mononuclear cell apheresates by the Department of Transfusion Medicine at University Hospital Regensburg was authorized by the *Regierung der Oberbayern* according to licence DE_BY_04_MIA_2013_0177/53.2-ZAB-2677.1_204. Production of Mreg_UKR by a contract manufacturing organization was authorized by the *Regierung der Oberbayern* under licence DE_BY_04_MIA_2013_0187/53.2-2677.1_A_220-0. The *ONEmreg12* clinical study was authorized by the *Paul Ehrlich Institute* under Vorlage-Nr. 1887/05 and the Local Ethics Committee under Votum 14-111-0016.

Part 4b: purpose

The *ONEmreg12* clinical trial (Eudra-CT Nr. 2013-000999-15) is a noncommercial, investigator-initiated, monocentre, single-arm, phase-I/II trial. The *ONEmreg12* trial was designed with the dual objectives of [1] assessing the safety of administering allogeneic Mreg_UKR to prospective kidney transplant recipients and [2] assessing the efficacy of Mreg_UKR treatment as a means of minimizing conventional, tacrolimus-based maintenance immunosuppression after living-donor kidney transplantation[28,29]. Under the *ONEmreg12* protocol, on day 7 prior to transplantation, patients receive a single dose of donor-derived Mreg_UKR ($2.5–7.5 \times 10^6$ viable Mregs/kg bodyweight) by slow central venous infusion under cover of 500 mg/day mycophenolate mofetil.

Part 4c: relationship between the source organism of the cells and the target organism

For the *ONEmreg12* trial, Mreg_UKR is produced from monocytes collected from a prospective living-donor kidney transplant donor and administered to the intended kidney transplant recipient. The *ONEmreg12* trial protocol stipulates that donor and recipient must have at least one mismatched allele for HLA-A, -B or -DR; therefore, all participants in the *ONEmreg12* study are treated with allogeneic cell products. In other clinical indications, Mreg_UKR may be autologous or 3rd-party origin.

Conclusion

This article provides a transparent, technical description of Mreg_UKR products, including the processes used for manufacture and quality control. This article is not a laboratory protocol or standard operating procedure; however, it contains enough information for any skilled person to appreciate the similarities and dissimilarities between Mreg_UKR and other monocyte-derived

immunosuppressive cell products[16,37–40]. Accordingly, this article should be a useful resource for those in the field[41,42], as well as stimulating development of the next generation of immunoregulatory and tissue-reparative monocyte-derived cell therapies.

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Conflict of interest

J.A.H. and E.K.G. are the named inventors on European Patent Office (EPO) filing 16159985.6-1402 dated 11.03.2016, 'Immunoregulatory cells and methods for their production'. All other authors have no conflict of interest to declare. Trizell GmbH holds all commercial rights to Mreg_UKR cell products.

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