

AUS DEM LEHRSTUHL FÜR CHIRURGIE

PROF. DR. MED. HANS J. SCHLITT,

DER FAKULTÄT FÜR MEDIZIN

DER UNIVERSITÄT REGENSBRURG

**HUMAN REGULATORY MACROPHAGES:
FROM BENCH TO BEDSIDE**

Inaugural – Dissertation
zur Erlangung des Doktorgrades der Medizin
der Fakultät für Medizin
der Universität Regensburg

vorgelegt von
Anne Christiane Broichhausen

2015

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Dekan:	Prof. Dr. Dr. Torsten E. Reichert
1. Berichterstatter:	Prof. Edward K. Geissler, PhD
2. Berichterstatter:	PD Dr. Norbert Ahrens
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Abstract

Administering immunoregulatory cells to patients as medicinal agents is a potentially revolutionary approach to the treatment of immunologically mediated diseases. By isolating and modifying cells *in vitro* before applying them to patients, specific cellular functions can be induced, opening astonishing new possibilities for antigen-specific treatments in autoimmunity, chronic inflammatory disorders and especially in transplantation medicine. During the last years our research group has developed a novel cell-based medicinal product containing human regulatory macrophages, called Mreg_UKR, for treatment of transplanted patients.

Developing these cells is a long and complicated process. It begins with a detailed understanding of macrophage biology and pathophysiology, including different states of polarisation and their function in allograft damage and repair. Based on this understanding, we have been able to identify an immunoregulatory macrophage phenotype that *in vitro* proved to be T-cell-suppressive. These so called Mregs reflect a unique state of macrophage differentiation, distinguished from macrophages in other activation states by their mode of derivation and robust phenotype. Owing to IFN- γ -induced indolamine 2,3-dioxygenase (IDO) activity and contact-dependant deletion of activated T-cells, Mregs are capable of suppressing mitogen-stimulated T-cell proliferation *in vitro* and drive the development of an activation induced regulatory T-cell (iTreg). No evidence of clinically significant adverse reactions was revealed when subjecting Mregs to conventional safety pharmacology and toxicology testing in mice. Two kidney transplanted patients have been treated with an Mreg containing suspension and show satisfactorily stable renal function with only low-dose Tacrolimus monotherapy more than five years after transplantation. These promising results and the need for a detailed clinical safety assessment warrant further clinical studies, which will be conducted within *The ONE Study*. My thesis depicts this process of developing an Mreg containing medicinal product from “bench to bedside”.

Übersicht

Die klinische Anwendung immunregulatorischer Zellen als medizinisches Produkt ist ein potentiell revolutionäres Therapiekonzept zur Behandlung immunologisch bedingter Erkrankungen. Durch *in vitro* Isolierung und Modifizierung der Zellen vor Applikation können spezifische Zellfunktionen induziert werden, die völlig neue Behandlungsmöglichkeiten bezüglich Autoimmun- und chronisch inflammatorischen Erkrankungen darstellen und insbesondere in der Transplantationsmedizin angewandt werden können. Unsere Forschungsgruppe hat in den letzten Jahren ein neuartiges Zell-basiertes medizinisches Produkt zur Therapie transplantierte Patienten entwickelt, das menschliche regulatorische Makrophagen enthält und als Mreg_UKR bezeichnet wird.

Die Entwicklung dieser Zellen ist ein langer und komplizierter Prozess. Er beginnt mit fundierten Kenntnissen von Physiologie und Pathophysiologie der Makrophagen, vor allem von verschiedenen Polarisierungszuständen der Zellen und ihrer Funktion in Abstoßungsreaktionen und Reparaturmechanismen im Transplantat. Auf dieser Basis konnten wir einen immunregulatorischen Makrophagentyp identifizieren, der sich *in vitro* als T-Zell-suppressiv bewiesen hat. Diese sogenannten Mregs sind charakterisiert durch einen einzigartigen Differenzierungsgrad und unterscheiden sich in ihrer Herkunft und dem robusten Phänotyp deutlich von anderen Makrophagen. Durch die IFN- γ -induzierte Indolamin-2,3-Dioxygenase-Aktivität und die Eliminierung aktivierter T-Zellen nach direktem Zellkontakt sind Mregs in der Lage, die Proliferation Mitogen-stimulierter T-Zellen zu supprimieren und darüber hinaus die Bildung eines regulatorischen T-Zelltyps anzuregen (iTreg). Pharmakologische und toxikologische Sicherheitsstudien in Mäusen gaben keine Hinweise auf klinisch signifikante Nebenwirkungen nach Mreg-Applikation. Zwei Nieren-transplantierte Patienten sind mit einer Mreg-haltigen Zellsuspension behandelt worden. Mehr als fünf Jahre nach der Transplantation weisen beide Patienten eine stabile Nierenfunktion auf, trotz einer nur niedrig dosierten Tacrolimus-Monotherapie. Aufgrund dieser vielversprechenden Ergebnisse sollen nun im Rahmen der *The ONE Study* detaillierte klinische Sicherheitsstudien durchgeführt werden. Meine Dissertation beschreibt den Entwicklungsprozess der Mregs als medizinisches Produkt vom „Labortisch bis hin zum Krankenbett“.

Zusammenfassung

Allgemeine Einleitung

In der experimentellen Forschung ist es bereits eine weit verbreitete Methode, immunregulatorische Zellen eines immunologisch toleranten Spenders einem nicht toleranten Empfänger zu übertragen, um dadurch eine immunologische Toleranz auf Seiten des Empfängers zu induzieren [1]. Dennoch hat die klinische Anwendung dieser Technik erst seit kurzem Aufmerksamkeit erlangt [2]. Zurzeit sind mehrere immunregulatorische Zelltypen im präklinischen Bereich so weit entwickelt, dass einige nun schon in frühen klinischen Studien als immunsuppressive Therapie geprüft werden. Zu diesen Zellen gehören unter anderem regulatorische T-Zellen [3], tolerogene dendritische Zellen [4] und die vielversprechenden regulatorischen Makrophagen, mit denen ich mich im Rahmen meiner Dissertation beschäftigt habe [5]. Ein breites Spektrum an Erkrankungen des Immunsystems könnte möglicherweise mit einer zellbasierten immunregulatorischen Therapie erfolgreich behandelt werden, wie beispielsweise T-Zell-vermittelte Autoimmunerkrankungen [6], chronische Entzündungsreaktionen [7], Graft-versus-Host-Reaktionen [8] und Abstoßungsreaktionen eines Transplantats [9]. Mit einer Zell-basierten immunregulatorischen Therapie könnte man die bis jetzt notwendigen immunsuppressiven Medikamente reduzieren oder gar umgehen und somit dem Patienten die bekannten gravierenden Nebenwirkungen der konventionellen immunsuppressiven Therapie ersparen. Da die immunologische Toleranz, die durch regulatorische Zellen induziert werden kann, von dominanter und selbsterhaltender Natur ist, besteht die fast unglaubliche Möglichkeit, Krankheiten zu heilen, die sonst eine lebenslange immunsuppressive Therapie erfordern würden.

Diese Dissertation gibt einen Überblick über die wichtigsten Beiträge zum Erfolg des „Mreg Projekts“ während meiner dreijährigen Mitarbeit in der Abteilung für experimentelle Chirurgie. Schwerpunkte dabei sind die genetische Charakterisierung von Zellen, die Entwicklung der technischen Herstellung des Zellprodukts sowie die Evaluierung der pharmakologischen Eigenschaften der Zellen in Tierversuchen. Während dieser Zeit konnte ich zwei wissenschaftliche Arbeiten als Erstautor veröffentlichen und brachte mich als Koautor in diverse weitere Veröffentlichungen ein (s.u.). Daten, die ich bereits erhoben habe und bis jetzt

noch nicht publiziert wurden, werden in weiteren Veröffentlichungen unserer Forschungsgruppe zu finden sein. Diese Dissertation stellt also eine zusammenfassende Abhandlung meiner Publikationen dar.

Liste der Publikationen - Erstautorschaft

Paper 1:

Broichhausen, Christiane; Riquelme, Paloma; Geissler, Edward K.; Hutchinson, James A. (2012): *Regulatory macrophages as therapeutic targets and therapeutic agents in solid organ transplantation*. In: *Curr Opin Organ Transplant* 17 (4), S. 332–342. DOI: 10.1097/MOT.0b013e328355a979

Impact factor: 2,818

Paper 2:

Broichhausen, Christiane; Riquelme, Paloma; Ahrens, Norbert; Wege, Anja K.; Koehl, Gudrun E.; Schlitt, Hans J. et al. (2014): *In question: the scientific value of preclinical safety pharmacology and toxicology studies with cell-based therapies*. In: *Mol Ther Methods Clin Dev* 1, S. 14026. DOI: 10.1038/mtm.2014.26

Impact factor: 6,425

Liste der Publikationen - Koautorschaft

Paper 3:

Hutchinson, James A.; Riquelme, Paloma; Sawitzki, Birgit; Tomiuk, Stefan; **Broichhausen, Christiane**; Miqueu, Patrick et al. (2011): *Cutting Edge: Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients*. In: *J. Immunol.* 187 (5), S. 2072–2078. DOI: 10.4049/jimmunol.1100762

Impact factor: 5,520

Paper 4:

Hutchinson, James A.; Ahrens, Norbert; Riquelme, Paloma; Walter, Lisa; Gruber, Michael; **Broichhausen, Christiane**; Böger, Carsten A. et al. (2014): *Clinical management of patients receiving cell-based immunoregulatory therapy*. In: *Transfusion*. DOI: 10.1111/trf.12641.

Impact factor: 3,568

Paper 5:

Lisa Walter, Paloma Riquelme, Stefan Tomiuk, Birgit Sawitzki, **Christiane Broichhausen**, Marcus N. Scherer, Michail Knauel, Norbert Ahrens, Bernhard Banas, Hans J. Schlitt, Edward K. Geissler and James A. Hutchinson. *Generation of BTNL8⁺ iTregs by Human Regulatory Macrophages is IDO- and B7-dependent*. Manuscript submitted.

Paper 6:

Managh, Amy J.; Hutchinson, Robert W.; Riquelme, Paloma; **Broichhausen, Christiane**; Wege, Anja K.; Ritter, Uwe et al. (2014). *Laser ablation-inductively coupled plasma mass spectrometry: an emerging technology for detecting rare cells in tissue sections*. In: *J. Immunol.* 193 (5), S. 2600–2608. DOI: 10.4049/jimmunol.1400869.

Impact factor: 5,520

Spezielle Einleitung

Makrophagen sind extrem vielseitige Zellen, die in der Lage sind, sowohl immunstimulatorische als auch immunsuppressive Effekte auszuüben. Diese Wandlungsfähigkeit kann nicht einfach durch eine strikte Einteilung in bestimmte Untergruppen erklärt werden, sondern spiegelt vielmehr die Fähigkeit von Makrophagen wider, zwischen verschiedenen Funktionszuständen zu wechseln. Ein solcher einzigartiger Differenzierungsgrad wird durch die regulatorischen Makrophagen (Mregs) beschrieben, die sich durch ihre Herkunft, den robusten Phänotyp und durch ein ausgeprägtes Potential zur Immunsuppression auszeichnen [5]. Durch eine INF- γ -induzierte Indolamin-2,3-Dioxygenase-Aktivität (IDO) sind Mregs *in vitro* in der Lage, den Proliferationsprozess Mitogen-stimulierter T-Zellen zu inhibieren und diese sogar nach direktem Kontakt aktiv zu eliminieren [10]. Dieses Potential macht Makrophagen zu einem durchaus interessanten und vielversprechenden Ziel in der Zell-basierten Therapie zur Toleranzinduktion in transplantierten Patienten.

Bereits in präklinischen Experimenten konnte anhand von Herz-transplantierten Mäusen nachgewiesen werden, dass eine Behandlung mit Spender-Mregs das Überleben eines Allografts in nicht immunsupprimierten Empfängern verlängerte [11]. Es stellte sich heraus, dass sich die allogenetischen Makrophagen nach intravenöser Infusion zunächst in den pulmonalen Gefäßen aufhielten, bevor sie dann nach kurzer Zeit in weitere Organe migrierten, wie Leber, Milz und Knochenmark, nicht aber in periphere Lymphknoten. In diesen Organen persistierten die Mregs für etwa vier Wochen. Nach Applikation von ¹¹¹Indium-Oxin-gelabelten, donorspezifischen Mregs wurden im menschlichen Organismus vergleichbare Ergebnisse erzielt [10]. Folglich scheinen Mregs nach Infusion in den Empfängerorganismus recht kurzlebig zu sein, sind aber dennoch befähigt, einen therapeutischen Nutzen zu erzielen, der weit über ihre Lebensdauer hinausgeht.

Auch im klinischen Stadium hat die Zell-basierte immunsuppressive Therapie mit Mregs bereits erste Erfolge erzielt. Einfache Mreg-haltige Zellpräparate wurden insgesamt 19 Nieren-transplantierten Patienten im Rahmen von Fallstudien und klinischen Studien infundiert [10, 12–15]. Zwei weitere Patienten erhielten vor Lebendspende einer Niere stark gereinigte, donorspezifische Mregs. Zum jetzigen Zeitpunkt, mehr als fünf Jahre nach der Transplantation,

erfreuen sich beide Patienten einer guten Nierenfunktion mit einer nur sehr niedrig dosierten Tacrolimus-Einnahme als einzige immunsuppressive Erhaltungstherapie [10]. Aufgrund dieser einzigartigen Ergebnisse sollen nun im Rahmen der *The ONE Study* weitere klinische Studien mit größeren Patientenzahlen durchgeführt werden.

Der genaue Wirkmechanismus von donorspezifischen Mregs nach Infusion in den Empfänger ist bislang ungeklärt. Jedoch gibt es einige Ansätze, die sowohl die Wirkung an T-Zellen als auch den anhaltenden Effekt nach Ableben der Mregs erklären können. Einerseits könnten Mregs direkt mit T-Zellen des Empfängers interagieren und so Effektorzellen anergisieren und sogar eliminieren, oder direkt die Bildung von regulatorische T-Zellen induzieren [9]. Andererseits ist bekannt, dass Alloantigene generell ein tolerogenes Potential entwickeln können [16]. Somit ist es sehr wahrscheinlich, dass Antigene von Mregs durch Antigen-präsentierende Zellen des Empfängers exprimiert werden, die so ihrerseits wiederum die T-Zell-Antwort modifizieren. Diese beiden generellen Mechanismen könnten selbstverständlich auch parallel zueinander ablaufen.

Bevor Mregs als Zell-basierte immunsuppressive Therapie zugelassen werden können, ist es noch ein weiter Weg. Dennoch hat sich unsere Forschungsgruppe diesem Ziel genähert, indem der exakte Phänotyp der Zellen und mögliche Wirkmechanismen genau analysiert wurden. Der Herstellungsprozess als medizinisches Produkt wurde präzise und reproduzierbar erläutert, mögliche Nebenwirkungen und Risiken für den Patienten identifiziert. In den folgenden Zusammenfassungen meiner wissenschaftlichen Arbeiten sollen genau diese Elemente abgehandelt werden.

Ergebnisse - Hauptprojekte

Meine zwei Veröffentlichungen mit Erstautorschaft repräsentieren den Hauptbestandteil meiner Doktorarbeit. Beide Projekte werden nachfolgend beschrieben. Anschließend sind die vier weiteren Arbeiten dargestellt, an denen ich mich mit meinen Ergebnissen beteiligt habe.

Paper 1: Regulatory macrophages as therapeutic targets and therapeutic agents in solid organ transplantation

Dieser Review gibt eine allgemeine Übersicht über die Physiologie der Makrophagen und ihre Rolle in Abstoßungsreaktionen bzw. Reparaturmechanismen nach Organtransplantation. Zudem ist es die Intention dieses Papers, die wichtigsten Forschungsergebnisse der letzten Zeit im Bereich der immunsuppressiven Makrophagen – also den „status quo“ – zu erfassen. Die Experimente in diesem Review, die ich mit konzipiert und im Labor durchgeführt habe, sind eine essentielle Grundlage für das Verständnis der Mregs und deren Weiterentwicklung zum Zell-basierten medizinischen Produkt: Via FACS- und qPCR-Validierungen konnte ich zunächst die verschiedenen Reifestadien von Monozyten des peripheren Blutes abgrenzen. Auf gleiche Weise konnte ich später den Genotyp der Mregs identifizieren. Basierend auf diesen handfesten Kriterien konnte ich den Herstellungsprozess von Mregs bezüglich Kulturdauer, Serum und Stimulation präzisieren.

Zuletzt werden in diesem Review verschiedene Möglichkeiten zur Manipulation von Makrophagen erklärt, um das Überleben eines Transplantats ohne hochdosierte immunsuppressive Medikamente zu ermöglichen.

Makrophagen sind extrem anpassungsfähige Effektorzellen, die mit völlig unterschiedlichen, oft sogar antagonistischen Aktivitäten im Immunsystem eine Rolle spielen: sie können eine Immunreaktion sowohl fördern als auch abmildern, Gewebe zerstören als auch regenerieren [17]. Neben der Fähigkeit zur Bildung von Abszessen und Granulomen reagieren Makrophagen äußerst sensibel auf pathologische Veränderungen in ihrer Umgebung. Dies ist auf ihre große Anzahl an Rezeptoren zurückzuführen, die sowohl einzelne Pathogene als auch Bestandteile abgestorbener Zellen erkennen können [18, 19]. Die so aktivierten Makrophagen sezernieren

daraufhin toxische Substanzen wie Perforin, Granzym und TNF- α . Außerdem dienen Makrophagen als professionelle Antigen-präsentierende Zellen und können hohe Konzentrationen an kostimulatorischen Molekülen exprimieren, wie beispielsweise verschiedene Zytokine [20]. Sie sind dadurch in der Lage, Immunantworten von naiven T-Zellen zu initiieren und so den Charakter von Reaktionen des erworbenen Immunsystems zu regulieren [21]. Umgekehrt sezernieren aktivierte T-Zellen wiederum Zytokine wie IL-12 und primen so weitere Makrophagen, wodurch die Immunantwort zusätzlich verstärkt wird. Nach dem gleichen Prinzip werden auch die Hemmung eines Immunprozesses, Geweberekonstruktion und Gefäßneubildungen durch anti-inflammatorische Eigenschaften von Makrophagen bewirkt. Hier spielt die Unterdrückung von T-Zellen durch IL-10 die tragende Rolle [22].

Abhängig von der Art des aktivierenden Stimulus und des Einflusses von anderen Effektorzellen ändern Makrophagen ihre Expression von Zytokinen, kostimulatorischen Molekülen und ihre zytotoxischen Mechanismen, um eine möglichst passende Immunantwort zu induzieren. Je nach Funktionsprinzip werden Makrophagen deswegen von einigen Autoren in M1- bzw. M2-polarisierte Zellen unterteilt, die entweder die Immunantwort von Th1-Zellen bzw. Th2-Zellen vorantreiben [23]. Diese Einteilung ist jedoch aus mehreren Gründen kritisch zu betrachten, nicht zuletzt weil sie keinen Raum lässt für die Differenzierung des regulatorischen/suppressiven Makrophagentyps [24–26]. Suppressivität in kultivierten Makrophagen wird durch verschiedenste Stimuli induziert, wie beispielsweise M-CSF, IL-10, 1,25-Dihydroxyvitamin D₃ und Glukokortikoide [27–31]. Besonders die Kombination aus 1,25(OH)₂D₃ und Glukokortikoiden erzeugt ein sehr stabiles immunsuppressives Stadium [32]. Paradoxe Weise können auch proinflammatorische Faktoren wie INF- γ , TLR-2, TLR-4, TLR-9 und PGE₂ über verschiedene Mechanismen suppressive Funktionen in Makrophagen induzieren und so die Proliferation von Effektor-T-Zellen verhindern, die Bildung von regulatorischen T-Zellen induzieren und immunologische Toleranz etablieren [7, 33–43].

Mit dem Ziel, ein Zell-basiertes medizinisches Produkt zur Induktion immunologischer Toleranz nach Transplantationen zu erhalten, hat sich unsere Forschungsgruppe auf die sogenannten regulatorischen Makrophagen (Mregs) konzentriert. Diese Zellen stellen durch ihren robusten

Phänotyp und ihre potente Suppression von T-Zellen *in vitro* entweder durch Indolamindioxygenase-Aktivität oder durch direkten Zell-zu-Zell-Kontakt einen einzigartigen Differenzierungsgrad dar. Mregs entstammen CD14⁺ peripheren Monozyten aus dem Blut und reifen in einer 7-tägigen Zellkultur. Während dieser werden die Zellen M-CSF, 10% humanem Serum und am Ende einem 24-stündigem Zusatz INF- γ ausgesetzt [44]. Die so erhaltenen Mregs zeichnen sich durch eine besondere Zellmorphologie aus und sind homogen CD14^{-/low} HLA-DR⁺ CD80^{-/low} CD86⁺ CD16⁻ CD64⁺ TLR2⁻ TLR4⁻ und CD163^{-/low} [10]. Außerdem sind sie befähigt, regulatorische T-Zellen zu induzieren (iTregs, siehe Paper 5).

Als eine vielseitige Zellart, die bei Transplantationen sowohl das angeborene als auch das erworbene Immunsystem aktivieren oder abschwächen kann, sind Makrophagen ein interessantes therapeutisches Ziel. Es können drei Mechanismen in Erwägung gezogen werden, um nutzbringende immunregulatorische Prozesse und Gewebsheilung in Gang zu setzen und gleichzeitig schädliche Immunprozesse zu unterbinden. Zunächst besteht die Möglichkeit – wie bereits an Tierversuchen gezeigt – Makrophagen zu eliminieren, die sonst das Transplantat schädigen. Klinisch einsetzbare Antikörper sind jedoch noch in der Entwicklungsphase und stehen derzeit nicht zur Verfügung [45]. Alternativ kann versucht werden, die Rekrutierung von Makrophagen zu verhindern [46–49]. Eine zweite Möglichkeit stellt die Transformation von Makrophagen in Richtung eines suppressiven Phänotyps *in vivo* dar. Einige Medikamente, die heute in der Transplantationsmedizin eingesetzt werden (mTOR-Inhibitoren, Mycophenolat Mofetil, IVIg), sind in der Lage, den Reifungsgrad und die Zytokinsekretion von Makrophagen zu verändern [50–60]. Eine dritte, sehr elegante Methode, stellt die Isolation von Monozyten dar, die in Kulturen zu suppressiven Makrophagen (Mregs) differenziert werden. Diese Zellen können dann als medizinisches Produkt in transplantierten Patienten angewandt werden. Unsere Forschungsgruppe kann als Vorreiter in der Anwendung dieser zellbasierten Therapie für Organ-transplantierte Patienten angesehen werden. Die generelle Durchführbarkeit und Verträglichkeit dieser Therapie wurde bereits an insgesamt 20 Nieren-transplantierten Patienten in zwei klinischen Studien der Phase I mit einfachen Zellpräparationen, die regulatorische Makrophagen enthielten, bestätigt (TAIC-I-/ TAIC-II-Studien) [12–15]. Zwei weitere Nieren-transplantierte Patienten wurden im Rahmen einer Fallstudie mit gereinigten Mreg-Präparaten des Spenders behandelt [10]. Genauere Details hierzu werden in der Zusammenfassung des Papers 3 beschrieben.

Paper 2: In question: the scientific value of preclinical safety pharmacology and toxicology studies with cell-based therapies

Im nachfolgenden Paper wurden die Versuche durch mich geplant und größtenteils durchgeführt. Lediglich die Transplantationen, das Infundieren der Zellpräparate sowie das Gewinnen von Gewebe post mortem wurden freundlicherweise von spezialisierten medizinisch technischen Assistenten übernommen.

Die Richtlinie der europäischen Arzneimittelagentur für Zell-basierte medizinische Produkte schreibt vor, dass neue Zellprodukte den allgemeinen pharmakologischen und toxikologischen Sicherheitsstudien unterzogen werden müssen, welche normalerweise an Tiermodellen erstellt werden. In dieser Arbeit werden deshalb die Pharmakokinetik und die Sicherheitspharmakologie eines Mreg-haltigen Zellprodukts an immundefizienten Mäusen überprüft.

Um Überlebensdauer von Mregs und deren Verteilung im Gewebe zu detektieren, wurden NSG Mäuse mit humanen Mregs behandelt. Die qualitative Anwesenheit in verschiedenen Organen wurde via Durchflusszytometrie überprüft. Mregs waren in der Lunge, im Blut und in der Leber bis sieben Tage nach der Infusion nachweisbar und behielten ihren Phänotyp während dieser Beobachtungsphase bei.

Athymische Nacktmäuse wurden in drei Testgruppen inkl. Kontrollgruppe mit unterschiedlich hohen Mreg Konzentrationen behandelt. Genaue Beobachtungen innerhalb drei Stunden nach Infusion ergaben keine Hinweise auf klinisch fassbare Reaktionen, v.a. bezüglich Herzfrequenz und -rhythmus. Weitere Untersuchungen in den nächsten sieben Tagen waren ebenfalls unauffällig: Todesfälle, Störungen im Herz-Kreislaufsystem, respiratorische, konstitutionelle und neurologische Probleme waren nicht zu vermerken. Auch post mortem zeigten sich keine Pathologien und kein Unterschied zwischen der Kontrollgruppe und den mit Mregs behandelten Mäusen.

Da Mregs scheinbar primär in die Leber wandern, wurden Empfängermäuse auf pathologische Leberwerte untersucht. Serumalbumin und alkalische Phosphatase (auch Parameter bei

systemischen Entzündungsreaktionen und erhöhtem Knochenstoffwechsel) waren in keiner Behandlungsgruppe signifikant erhöht. Jedoch zeigte sich eine erhöhte AST in der Gruppe mit der höchsten Mreg Exposition, wobei die Bedeutung dieses Phänomens bislang unklar ist. Wichtig ist dabei, dass in den mit Mregs behandelten menschlichen Patienten keine eingeschränkte Leberfunktion bemerkt wurde. Serumkreatininwerte als Parameter der Nierenfunktion und Blutzuckerspiegel, Indikator für Ischämie, Sepsis und endokrine Störungen, waren ebenso unauffällig.

Weiterhin ist es essenziell, das Risiko der Sensibilisierung zu erfassen. Dazu wurden in Herztransplantierten BALB/c Mäusen nach präoperativer Donor-Mreg-Therapie die donorspezifischen anti-MHC-Klasse-I Antikörper gemessen. Es war kein vorzeitiger Verlust der Transplantate zu vermerken, sodass davon ausgegangen werden kann, dass die präoperative Behandlung mit Mregs zu keiner Sensibilisierung geführt hat. Diese Schlussfolgerung wird dadurch gefestigt, dass sieben Tage nach Transplantation geringere Mengen an Alloantikörper in den mit Mregs behandelten Mäusen nachgewiesen wurden als in der Kontrollgruppe. Dieses Ergebnis kann jedoch nicht einfach auf den menschlichen Organismus übertragen werden, da Mäuse-Mregs nicht identisch mit denen von Menschen sind, also auch eine unterschiedliche Immunogenität aufweisen könnten.

Um die Toxizität und Karzinogenität von infundierten Mregs zu beurteilen, wurden in drei Gruppen C.B-17 SCID-Mäuse mit unterschiedlichen Dosierungen Mreg-haltiger Zellpräparationen behandelt. Innerhalb von 295 Tagen sowie post mortem konnten zu keinem Zeitpunkt Hinweise auf Tumore, biochemische oder hämatologische Störungen und histopathologische Veränderungen erfasst werden.

Es kann also davon ausgegangen werden, dass sich an Tiermodellen die Behandlung mit Mregs als sicher erwiesen hat und daher die Anwendbarkeit einer Mreg-basierten Therapie für Menschen zukünftig im Focus stehen sollte. Es bleibt fraglich, ob die Ergebnisse präklinischer Sicherheitsstudien mit einem immunologisch aktivem Zell-Produkt wie Mregs ohne Einschränkungen auf den Menschen übertragen werden können. Ein zentrales Problem stellt in diesem Fall die Inkompatibilität der unterschiedlichen Spezies dar, durch die entweder die biologische Relevanz ausbleibt oder keine direkte Information über das menschliche

Zellprodukt geliefert wird. Auch was das Risiko der Sensibilisierung betrifft, können aufgrund präklinischer Studien keine Rückschlüsse auf den menschlichen Organismus gemacht werden, da tierische und menschliche Mregs zwar ähnlich, nicht aber identisch sind und so ein unterschiedlich ausgeprägtes immunogenes Potential haben können.

Diese Schwachpunkte lassen auf den ersten Blick die Ergebnisse der Sicherheitsstudien weniger relevant erscheinen. Diese Problematik steht zunächst in Konflikt mit den Vorgaben des EMA Komitees (European Medicines Agency) für menschliche medizinische Produkte. Dennoch sollten aufgrund der medizinischen Relevanz und aufgrund der positiven Pilotstudien weitere klinische Studien gemäß der EMA Richtlinien durchgeführt werden, um eine vage Vorstellung über Sicherheit und Pharmakokinetik der Zellen zu erhalten.

Aus den o.g. Gründen sollte ein größerer Schwerpunkt auf klinische Studien mit Mregs gesetzt werden. Betrachtet man die präklinischen Ergebnisse zusammen mit der klinischen Erfahrung, die bereits mit Mregs gemacht wurde, so kann berechtigterweise gefordert werden, in Zukunft mehr explorative klinische Studien zuzulassen, ohne dass umfangreiche Tierexperimente durchgeführt werden müssen.

Ergebnisse - Nebenprojekte

Im Laufe meiner Mitarbeit in unserer Forschungsgruppe war ich in eine Reihe von Projekten involviert, die sich mit dem Thema Mregs beschäftigt haben. Die entsprechenden Veröffentlichungen werden im Folgenden beschrieben. Sie belegen mein Mitwirken am Projekt Mreg und die Tragweite meiner Experimente im weiteren Sinne zu belegen. Diese Veröffentlichungen stellen jedoch nicht den Hauptbestandteil meiner Doktorarbeit dar. Eingebbracht habe ich mich in diese Projekte hauptsächlich mit den Ergebnissen und Techniken meiner Versuche der beiden oben genannten Papers sowie Herstellung der diversen Zellkulturen.

Paper 3: Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients

In dieser Arbeit werden zwei Fälle Nieren-transplantierte Patienten beschrieben, die erfolgreich mit Mregs zur immunsuppressiven Therapie behandelt wurden. MM ist eine 23-jährige Patientin, die ein Spenderorgan von ihrer Mutter erhielt (HLA-B und HLA-DR) und sechs Tage vor der Transplantation einer zentralvenösen Infusion mit 8.0×10^6 Spender-Mregs unterzogen wurde unter Absicherung von Azathioprin sowie später Steroiden und Tacrolimus. Azathioprin und Steroide wurden einige Wochen nach Transplantation ohne gravierende Effekte abgesetzt, Tacrolimus bei konstant guter Nierenfunktion auf niedrigste Serumkonzentrationen reduziert. Vergleichbar wurde mit dem 47-jährigen Patienten CA verfahren, der die Niere eines nicht verwandten Spenders erhielt (kompletter HLA-Mismatch).

In Biopsien von Patientin MM konnten zu keinem Zeitpunkt Zeichen einer akuten Abstoßungsreaktion gefunden werden. In peripheren Blutproben war keine T-Zellexpansion zu erkennen, was als Anzeichen für eine fehlende Aktivität der T-Zellen gegenüber dem Transplantat gedeutet werden kann. Normale TOAG-1-Expression – bei akuten Abstoßungsreaktionen massiv vermindert – und das Fehlen von HLA-Antikörpern ist ebenfalls als Zeichen einer immunologischen Toleranz zu werten. Die Zunahme des Foxp3/ α -

Mannosidase-Verhältnis nach etwa vier Wochen kann darauf hindeuten, dass die Anzahl an regulatorische T-Zellen im Verhältnis zu aktivierten inflammatorischen T-Zellen relativ zunahm.

Neben den immunologischen Parametern wurde bei Patientin MM das Verhalten von Mregs im menschlichen Körper verfolgt. Hierzu wurde ein Teil der infundierten Mregs mit ¹¹¹Indiumoxin markiert und mittels Ganzkörperspektroskopie dargestellt. Die Mregs hielten sich zunächst im Lungenkreislauf auf, bevor sie sich nach etwa drei Stunden im Blutkreislauf verteilten und dann in Leber, Milz und Knochenmark anreicherten. Die Abwesenheit von Mregs im Harntrakt beweist, dass die meisten Mregs nach Infusion zunächst am Leben bleiben. Auch wenn uns diese Beobachtungen eine vorläufige Beschreibung über das pharmakodynamische Verhalten von infundierten Mregs geben, ist dennoch das weitere Schicksal dieser Zellen unklar.

Die immunologischen Parameter von Patient CA verhielten sich vergleichbar zu denen von Patientin MM. Die Genexpression beider Patienten wurde mehrfach genau analysiert und anhand des *Indices of Tolerance* (IOT) Forschungsnetzwerks evaluiert. Sowohl Patientin MM als (etwas später) auch Patient CA ähnelten in Bezug auf ihr genetisches Profil gesunden Testpersonen bzw. toleranten Patienten.

Auch wenn die Applikation von Zellpräparaten sicherlich nicht ohne Risiko ist, haben die Fälle beider Patienten die Realisierbarkeit dieser immunsuppressiven Therapie bewiesen. Weder MM noch CA zeigte unerwünschte Nebenwirkungen auf das Zellprodukt, und in beiden Fällen konnte schon nach wenigen Wochen die sonst übliche, hochdosierte immunsuppressive Therapie massiv reduziert werden, ohne dass es zu einer Abstoßungsreaktion des Transplantats kam. Sogar zum jetzigen Zeitpunkt, über fünf Jahre nach der Transplantation, sind die Nierenwerte beider Patienten stabil und zufriedenstellend. Aufgrund dieser überzeugenden Ergebnisse können an das Zellprodukt Mreg hohe Erwartungen gestellt werden. Im Rahmen der *The ONE Study* sollen deswegen weitere klinische Studien durchgeführt werden, wie beispielsweise die Studie *ONEMreg12*, in der 16 Patienten mit Spender-Mregs und begleitender MMF Therapie sieben Tage vor Transplantation behandelt werden sollen.

Paper 4: Clinical management of patients receiving cell-based immunoregulatory therapy

Auch wenn die Anwendung immunregulatorischer Zellen als medizinisches Produkt ein revolutionärer Ansatz zur Toleranzinduktion ist, muss dennoch Klarheit bestehen über die Anwendung, die pharmakologischen Eigenschaften und über potentielle klinische Risiken für den Patienten. Diese sollen im Folgenden anhand von Beispielen Nieren-transplantierte Patienten kritisch bewertet werden.

Wie Transfusionen mit Spendervollblut gezeigt haben [61], besteht nach präoperativem Kontakt mit Spender-Antigenen die Gefahr der Sensibilisierung. Spender-reaktive T- und B-Zellen könnten aktiviert werden und somit eine akute Abstoßungsreaktion in Gang setzen [62, 63]. Vollblut besteht jedoch aus Thrombozyten, Erythrozyten und Leukozyten mit einem großen Anteil potentiell aktivierender Antigen-präsentierender Zellen. Das Mreg-Zellprodukt hingegen enthält nur eine einzige homogene Zellpopulation und wird im Gegensatz zu donorspezifischen Bluttransfusionen nur einmalig appliziert, was das kumulative Risiko der Sensibilisierung abwendet. Außerdem haben präklinische und klinische Daten gezeigt, dass die Gefahr der Sensibilisierung gegen Spenderantigene nach Mreg-Applikation äußerst gering ist [10, 13–15].

Eine weitere Problematik stellen Hypersensitivitätsreaktionen nach Mreg-Infusion dar. Bis jetzt zeigte keiner der mit Mregs behandelten Patienten Zeichen einer IgE-vermittelten Hypersensitivität (Typ I-Reaktion). Da das Risiko dennoch bestehen bleibt, ist die Therapie mit Mregs bei Patienten mit Heparinallergie oder IgA-Mangel untersagt [64]. Zudem ist die Möglichkeit einer Antikörper- oder Komplement-vermittelten Typ II-Reaktion nicht ausgeschlossen, da fieberhafte und hämolytische Reaktionen auch nach Transfusion anderer Blutbestandteile beobachtet wurden [65, 66]. Auch wenn bei einer Antikörper-vermittelten Destruktion von infundierten Mregs nicht mit solch schwerwiegenden Folgen zu rechnen ist, müssen bei der Herstellung von Mreg-Infusionen sämtliche Antikörper gegen Blutbestandteile des Spenders herausgefiltert werden. Zur Vermeidung einer nicht-hämolytischen Typ II-Reaktion soll in weiteren klinischen Mreg-Studien prophylaktisch Paracetamol zur Antipyrese zum Einsatz kommen [67]. Im Falle einer fieberhaften Reaktion soll zunächst die Infusionsrate vermindert, bei Persistenz der Symptome muss die Infusion abgesetzt werden.

Auch ist das geringe Risiko einer Immunkomplex-vermittelten Reaktion (Typ III) nicht gänzlich auszuschließen, obwohl eine derartige Komplikation nach Bluttransfusionen und bei den mit Mregs behandelten Patienten nicht aufgefallen ist.

Eine seltene, aber gefürchtete Komplikation nach Bluttransfusionen ist die Graft-versus-Host-Krankheit (GvHD) [68]. Sie wird durch alloreaktive T-Zellen hervorgerufen, die vom Empfänger nicht abgewehrt werden können. Für die Herstellung von Mregs werden magnetische Polymerpartikel verwendet, um selektiv CD14⁺ Monozyten als Startmaterial zu erhalten und so die meisten anderen Leukozyten wie T-Zellen von der Zellpräparation auszumustern. Die Kontamination mit T-Zellen in einem Mreg-Produkt liegt typischerweise unter 1% und so ist die Wahrscheinlichkeit einer GvHD nach Infusion sehr gering.

Ein weiteres Problem nach Transfusionen im Allgemeinen ist das sogenannte Transfusionsbedingte Lungenödem (TRALI), hervorgerufen durch aktivierte neutrophile Granulozyten und Thrombozyten, die das Endothel in den pulmonalen Gefäßen schädigen [65]. Bis jetzt gibt es keinen Hinweis, dass Mregs neutrophile Granulozyten der Lunge aktivieren können. Dennoch sollen Mreg-Präparate nicht Patienten infundiert werden, die erhöhte Werte neutrophiler Granulozyten aufweisen.

Neben den immunologischen Risiken müssen auch physiologische Komplikationen reflektiert werden. Wird ein Zellprodukt zentralvenös appliziert, können durch Zellaggregate, Ablagerungen oder Embolisation pulmonale Gefäße verschlossen werden. Mregs in einer Suspension haben einen Durchmesser von etwa 15-30µm, Kapillaren bzw. Arteriolen der menschlichen Lunge ca. 7.5µm bzw. 15-150µm [69]. Demnach ist es denkbar, dass einzelne Mregs kleine Endarteriolen verschließen können. Experimente in Großtieren haben gezeigt, dass die Infusion von ca. 10⁸ Zellen/kg/KG oder ähnlich großer Partikel eine tödliche Dosis darstellt, während ca. 10⁷ Zellen/kg/KG nur einen signifikanten Anstieg des mittleren pulmonalarteriellen Drucks (PAP) bewirken [70–73]. Diese Dosen liegen jedoch weit über der empfohlenen Dosis einer Mreg-Infusion für Patienten (2.5 – 7.5 x 10⁶ Mregs/kg/KG). Somit kann nach langsamer Infusion der mittlere PAP ansteigen, jedoch ist ein Patient mit normaler Herzfunktion in der Lage, diese Veränderung zu kompensieren. Dennoch werden im Rahmen

der *ONEmreg12* Studie Patienten mit hochmolekularem Heparin voll antikoaguliert um das Risiko einer intrapulmonalen Koagulation zu verhindern.

Auch das Überangebot an Flüssigkeit durch die Infusion mit Gefahr eines Lungenödems muss kritisch evaluiert werden. Eine Mreg-Infusion beinhaltet 100ml, zusätzlich können bis zu 400ml Spülflüssigkeit benötigt werden. In kürzlich dialysierten Patienten sollte aber eine langsame Infusion dieser Menge keine klinisch signifikanten Ödeme hervorrufen.

Einige biochemische Veränderungen sollen noch erörtert werden: Nach Mreg-Infusion ist eine unmittelbare Zelllyse nicht auszuschließen, was theoretisch zur Hämolyse führen kann. Da jedoch die therapeutischen Dosen von Mregs sehr gering sind, ist eine derartige Komplikation nicht zu erwarten. Außerdem könnten Mregs aufgrund ihrer Expression von TNF- α die Produktion von Tissue-Faktor induzieren und so einen leichten pro-koagulatorischen Effekt im menschlichen Organismus bewirken. In den bis jetzt behandelten Patienten waren jedoch keine Anzeichen eines thrombotischen Geschehens zu erkennen. Ein höheres Risiko besteht in der Aktivierung der Bradykinin-Sekretion als Folge der aktivierten Gerinnungskaskade. Vor allem in Kombination mit ACE-Hemmern kann dies zu ausgeprägten hypotensiven Reaktionen führen.

Durch eine Mreg-Infusion könnten neoplastische Zellen übertragen oder das Wachstum autochtoner Tumore begünstigt werden [17]. Malignität wird jedoch meist durch primitive Formen der myelomonozytären Zellreihe und nicht durch post-mitotische Monozyten bzw. Makophagen hervorgerufen. Außerdem ergab die Behandlung immundefizienter Mäuse mit Mregs keine Hinweise auf maligne Entartungen (siehe Paper 2).

Die Übertragung von möglichen Keimen zählt ebenfalls zu den potentiellen Risiken der Mreg-Therapie. Deshalb wird im Spender und auch während des Herstellungsprozesses nach strengen Vorgaben regelmäßig nach bestimmten Erregern gefahndet. So kann das Infektionsrisiko minimiert werden.

Paper 5: Generation of BTNL8⁺ iTregs by Human Regulatory Macrophages is IDO- and B7-dependent

Neben der Fähigkeit zur T-Zell-Suppression bzw. Eliminierung sind Mregs in der Lage, regulatorische T-Zellen zu induzieren (iTregs). Eine signifikante Hochregulation von CD25 konnte in CD4⁺-Zellen nach Ko-Kultur mit Mregs detektiert werden. Um die Suppressionsleistung zu prüfen, wurden T-Zellen nach Mreg Ko-Kultur re-isoliert und mit Responder-T-Zellen kultiviert. Dabei wurde die Proliferation von stimulierten Responder-T-Zellen verhindert. Außerdem konnte gezeigt werden, dass CD4⁺ T-Zellen nach Mreg-Ko-Kultur die vollständige Reifung dendritischer Zellen unterbinden. Diese Erkenntnisse können Hinweis dafür sein, dass die therapeutischen Effekte von Mregs auch noch nach deren Ableben anhalten und so eine lebenslange Toleranz in transplantierten Patienten ausgebildet wird.

Die Genanalyse von T-Zellen nach Ko-Kultur mit Mregs zeigte Gene wie EB13 und FOXP3 hochreguliert, die klassische Treg-assoziierte Marker darstellen. Vergleichbare Ergebnisse waren in der Durchflusszytometrie zu erkennen. Die Stabilität der iTregs wurde anhand der FOXP3-Expression gemessen: nach 10 Tagen ohne Restimulation nahm die FOXP3-Expression bis auf ca. 64% des Ausgangswerts ab. Interessanterweise exprimieren Mregs während der Ko-Kultur mit T-Zellen relativ viel SOCS2 mRNA, die einen stabilisierenden Einfluss auf FoxP3⁺ Tregs hat [74].

Bei der genetischen Identifikation von iTregs fiel als immunologisch relevanter Marker vor allem das Butyrophilin-like Protein 8 (BTNL8) auf, das funktionell mit den B7-kostimulatorischen Molekülen verwandt ist [75]. Genaue Analysen haben gezeigt, dass in Vergleich zu CD4⁺ T-Zellen in iTregs die B7-like Splicevariante von BTNL8 exprimiert wird. Hingegen herkömmlicher Meinung ist BTNL8 auch in CD4⁺-Zellen des menschlichen Blutes nachweisbar, vor allem in nicht aktivierten CD4⁺-Zellen und zentralen T-Gedächtniszellen. Zudem ist bekannt, dass orthologe BTNL-Gene in Mäusen T-Zell-suppressive Eigenschaften aufweisen [76–78]. Mit dieser Information lässt sich spekulieren, dass BTNL8 die T-Zellaktivierung limitiert und somit Zellen wie iTregs einen immunsuppressiven Charakter verleiht.

Die genauen Mechanismen, mit denen Mregs eine T-Zell-Antwort beeinflussen, sind noch nicht geklärt. Klar ist, dass sich Mregs von allen bisher beschriebenen Polarisationszuständen in

Makrophagen unterscheiden, nicht zuletzt durch ihre ausgeprägte Expression von DHRS9, einer wenig bekannten Retinol-Dehydrogenase [79]. Retinolsäure spielt in der Induktion und Stabilisierung von Tregs eine tragende Rolle [80]. Es lässt sich also vermuten, dass die DHRS9 Expression von Mregs bei der Interaktion mit T-Zellen bedeutend ist.

Die Induktion von iTregs ist nicht Alloantigen-abhängig, denn sie wurde auch in autologen Ko-Kulturen beobachtet. Experimente haben bewiesen, dass zudem ein direkter physiologischer Kontakt zwischen Mregs und T-Zellen unabdinglich ist. Die Induktion von iTregs geschieht über den B7-kostimulatorischen Signalweg durch einen IDO-abhängigen Mechanismus, welcher durch Medikamente wie Tacrolimus oder Rapamycin nicht beeinflusst wird. Die IDO-Aktivität scheint aber nicht der einzige relevante Mechanismus zu sein: INF- γ -Makrophagen werden ähnlich wie Mregs kultiviert, jedoch nicht in Humanserum (HABS) sondern in fetalem Kälberserum (FCS). Sie sind aber nicht in der Lage, die Bildung von iTregs anzuregen. Folglich sind zusätzliche Wirkmechanismen zur Induktion von iTregs vonnöten, die nur Mregs zu eigen sind und entweder durch Faktoren in HABS induziert oder aber durch FCS inhibiert werden.

Treffen diese *in vitro* erhobenen Erkenntnisse über Mregs und iTregs auch auf ihre Eigenschaften *in vivo* zu, könnte dies eine Erklärung für den anhaltenden immunsuppressiven Effekt einer Mreg-Therapie sein. Es ist zu hoffen, dass die oben erwähnten Parameter im Rahmen der *The ONE Study* Mreg Studie auch in therapierten Patienten untersucht werden.

Paper 6: Laser ablation-inductively coupled plasma mass spectrometry: An emerging technology for detecting rare cells in tissue sections

Ausschlaggebend zur Bestimmung biologischer Effekte und Sicherheit eines Zell-basierten medizinischen Produkts ist die Pharmakokinetik, also die Verteilung und das Schicksal der Zellen im menschlichen Organismus. Im Falle von Mregs ist dies jedoch problematisch, da keine adäquaten Methoden zur Verfügung stehen, die Zellen nach Infusion im Patienten längerfristig zu verfolgen. Die *laser ablation inductively coupled* Massenspektrometrie (LA-ICP-MS) stellt ein Verfahren dar, das kleinste Proben, z.B. seltene Erdmetalle, mit einem fokussierten Laserpuls vaporisiert. Das vaporisierte Material wird dann mit Hilfe der Massenspektrometrie analysiert. Die in dieser Arbeit beschriebene Technik ermöglicht es, menschliche Mregs zu detektieren.

Mregs werden dual mit Gold und mit $^{154}\text{Samarium}$ -markierten Anti-HLA-DR oder $^{174}\text{Ytterbium}$ -markierten anti-CD54 Antikörpern gelabelt. Auf diese Weise können die Zellen mit Hilfe des LA-ICP-MS in Geweben äußerst präzise nachgewiesen werden. Mausmodelle zeigten auf diese Weise, dass sowohl eine als auch sieben Stunden nach Mreg-Infusion die Zellen in der Lunge, der Leber und der Milz nachgewiesen werden konnten.

Dieses Verfahren ist noch weit davon entfernt, als Standard in der immunologischen Forschung zu dienen. Nicht zuletzt die Auflösung der Bilder, die Markierungsverfahren sowie die zeitliche Verarbeitung sind verbesserungsbedürftig. Dennoch präsentieren die Daten ein außerordentliches Potential der LA-ICP-MS-Technologie, biologische Einzelproben zu detektieren, was als äußerst vielversprechend in Bezug auf klinische Mreg-Studien angesehen werden kann.

Ausblick

Mregs haben unserer Meinung nach ein ausgesprochenes Potential zur effektiven Zell-basierten immunsuppressiven Therapie. Wäre es möglich, mit einer Mreg-Therapie die jetzigen Abstoßungsraten nach Organtransplantation zu erhalten und gleichzeitig aber die schädlichen Wirkungen der herkömmlichen Immunsuppressiva zu vermeiden, so könnte die Morbidität und Mortalität transplantierte Patienten wesentlich reduziert werden. Genau dies ist das Ziel, das wir mit der Entwicklung der Mreg-basierten Therapie verfolgen. Die Pilotstudien mit den Patienten MM und CA haben die generelle Durchführbarkeit einer solchen Therapie in transplantierten Patienten bewiesen, auch wenn es noch weiterer größer angelegter Studien bedarf, um eine definitive Aussage zur Effektivität dieser Zellen zu treffen.

Die Wirkweise und Effektivität einer Mreg-Therapie, besonders in Bezug auf die T-Zell-Interaktion, konnte bereits an Tiermodellen gezeigt werden. Diese Ergebnisse sind jedoch nicht einfach auf den menschlichen Organismus zu übertragen: menschliche Mregs als Zellprodukt können im Menschen selber andere immunologische Reaktionen hervorrufen als in Tieren. Dennoch muss Klarheit geschaffen werden über die genaue Pharmakokinetik und die Wirkmechanismen im Menschen. Die einzige Möglichkeit, dieses Ziel zu erreichen, sind weitere klinische Untersuchungen. Doch wie können diese gerechtfertigt werden? Betrachtet man die wenigen bis jetzt durchgeführten klinischen Studien mit Mreg-haltigen Präparaten zusammen mit den Ergebnissen der präklinischen Sicherheitsstudien, so waren keinerlei schwerwiegende Komplikationen nach Mreg-Infusion erkennbar. Auch wenn dies nur eine vage Sicherheit ist, sollte in Zukunft der Fokus auf klinische Studien gesetzt werden.

Methoden wie das LA-ICP-MS sind bereits in Entwicklung, um genaue Details über das Verhalten von Mregs im menschlichen Körper nachzuvollziehen. Verschiedene Parameter in T-Zellen wurden identifiziert, um auch den Wirkmechanismus zwischen Mregs und Tregs im Menschen zu verstehen. Genau dieses Ziel verfolgt die *ONEmreg12*-Studie: unter genauer Überwachung sollen insgesamt 16 Nieren-transplantierte Patienten mit donorspezifischen Mregs unter MMF-Therapie sieben Tage vor Transplantation behandelt werden und genauere Auskunft über das Verhalten von Mregs und ihren anhaltenden immunsuppressiven Effekt

geben. Aufgrund der bis jetzt äußerst vielversprechenden Ergebnisse der Mreg-basierten Therapie kann dieser Studie – sowie auch weiteren klinischen Studien – durchaus positiv entgegengesehen werden.

Insgesamt kann also die Zell-basierte immunsuppressive Therapie mit Mregs als sehr aussichtsreich angesehen werden. Das erhebliche Potential dieser Zellen sollte genutzt werden und – wie in der *ONEmreg12*-Studie – weitere klinische Studien mit einer größeren Patientenzahl durchgeführt werden.

An Essay on the Biology of Macrophages

The role of macrophages in innate and adaptive immunity

Macrophages play an important role in innate immunity. They are located in areas which are easily reached by pathogens, such as the airway or gastrointestinal tract, and thus are often the first cells to initiate an immune response. Once macrophages encounter an antigen, they phagocytose it and the inflammatory cascade is launched, recruiting other immunological cells, which for their part are responsible for the further resolution of an infection. As part of this defence mechanism, macrophages are responsible for the formation of granulomata and abscesses, anticipating the diffusion of the pathogen from the infection site and thus reducing the risk of sepsis [81].

Recognition of a pathogen is the key process in generating an immune response. In order to recognize a pathogen, macrophages bear a diverse repertoire of pathogen-recognition receptors, so called pattern-recognition receptors, most notably, the Toll-like receptors (TLR) (figure 1). Different subtypes of TLR are responsible for the detection of different pathogen-associated molecules. TLR-2 recognizes many viral, bacterial and fungal antigens, including zymosan, bacterial peptidoglycan and lipoteichoic acid. TLR4 is an essential receptor for lipopolysaccharide (LPS) recognition and is also involved in the recognition of endogenous ligands such as heat shock proteins, fibrinogen, hyaluronic acid and heparan sulfate. TLR5 has been shown to recognize flagellin. TLR7, TLR8 and TLR9 recognize nucleic acid-like structures of viruses, especially uridine- or guanidine-rich single-stranded viral RNA. In contrast, TLR3 is capable of detecting double-stranded RNA, mostly produced during the replication phase of viruses [82]. Besides the TLRs, several other classes of pattern-recognition receptors have been identified. The macrophage mannose receptor (MMR, CD206) is a member of the C-type lectin receptor family, which recognizes carbohydrate patterns on the bacterial and viral surface. Dectin-1 is a β -glucan receptor responsible for the detection of fungi or fungal debris. Scavenger receptors, such as CD36, recognize modified low-density-lipoprotein and are responsible for the binding and phagocytosis of negatively charged macromolecules and senescent cells. Playing an important part in phagocytosis of pathogens, macrophages also bear

opsonic receptors such as Fc-receptors and complement-receptors which empower them to indirectly recognise and up-take opsonized pathogens.

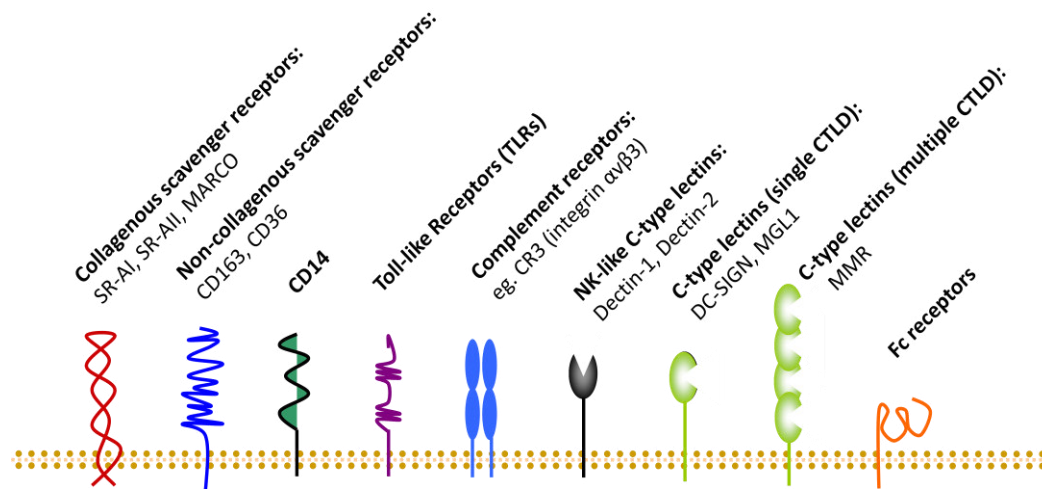


Figure 1: Macrophage receptor systems for detecting pathogen and tissue stress

A fundamental task of macrophages is phagocytosis of pathogens, dead cells and other foreign particular matter. Upon encountering a pathogen, it is ingested and directed into a phagosome, where various proteolytic enzymes and peroxides digest the particle. A small amount of the pathogen material is decomposed by proteases to small polypeptides and then transferred to the cell-surface in association with an MHC Class II molecule. Macrophages are also able to directly kill parasites, antibody-coated cells and neoplastic cells via release of various substances such as TNF- α , serine proteases and perforin.

Macrophages are part of the antigen-presenting cell (APC) system. Presenting the antigen particle via the MHC-II-complex, macrophages are able to stimulate responses to recall antigens by activating resting T-cells. However, it is the conventional immunological view that they are not able to initiate naïve T-cell responses, which is the sole preserve of dendritic cells (DC). The stimulating effect on T cells is dependent upon several co-stimulatory molecules, such as CD80 and CD86. Apart from providing TCR ligands and costimulation, macrophages are capable of polarizing T cell responses by secreting different cytokines and other inflammatory mediators, especially CCL2 and CCL5. Through IL-1 and IL-6 production, macrophages recruit other inflammatory cells to the site of inflammation, primarily neutrophils and T cells. Another cytokine produced by macrophages called TNF- α increases the vascular permeability to plasma

proteins and extravasating leucocytes. In turn, cytokines generated by activated lymphocytes, such as IFN- γ , feedback on macrophages and boost their antigen presenting capacity, accelerating the immune response [83].

Macrophages are not only responsible for the initiation of an immune response, but also participate in its resolution by activating anti-inflammatory features, clearing apoptotic or necrotic cells from the site of inflammation and promoting tissue repair. Regenerating activities include the promotion of new blood vessel formation and secretion of various tissue-trophic factors [84].

The origin of monocytes and macrophages

Hematopoietic stem cells (HSC) are the progenitors of the lympho-hematopoietic cell system including monocytes, characterized by an extremely high self renewal and proliferation potential. Focusing on the development of monocytes and macrophages, the common myeloid progenitor (CMP) gives rise to the granulocyte/monocytes progenitor (GMP) and the megakaryocyte/erythrocyte progenitor. After further development, the GMP differentiates into the monocytes colony forming unit (M-CFU), giving rise to monoblasts, promonocytes and finally monocytes. All the mentioned stages of development are induced by various colony stimulating factors (CSF), such as granulocyte-monocyte colony stimulating factor (GM-CSF) as well as a number of cytokines, most importantly IL-3 [5].

After maturation, monocytes exit the bone marrow and circulate in the peripheral blood for several days, until either being recruited to inflammatory sites or entering tissues to become tissue-resident macrophages, such as Kupffer cells or alveolar macrophages. Differentiation into macrophages entails loss or gain of various phenotypic markers and functions, depending on the particular tissue or inflammatory conditions (figure 2).

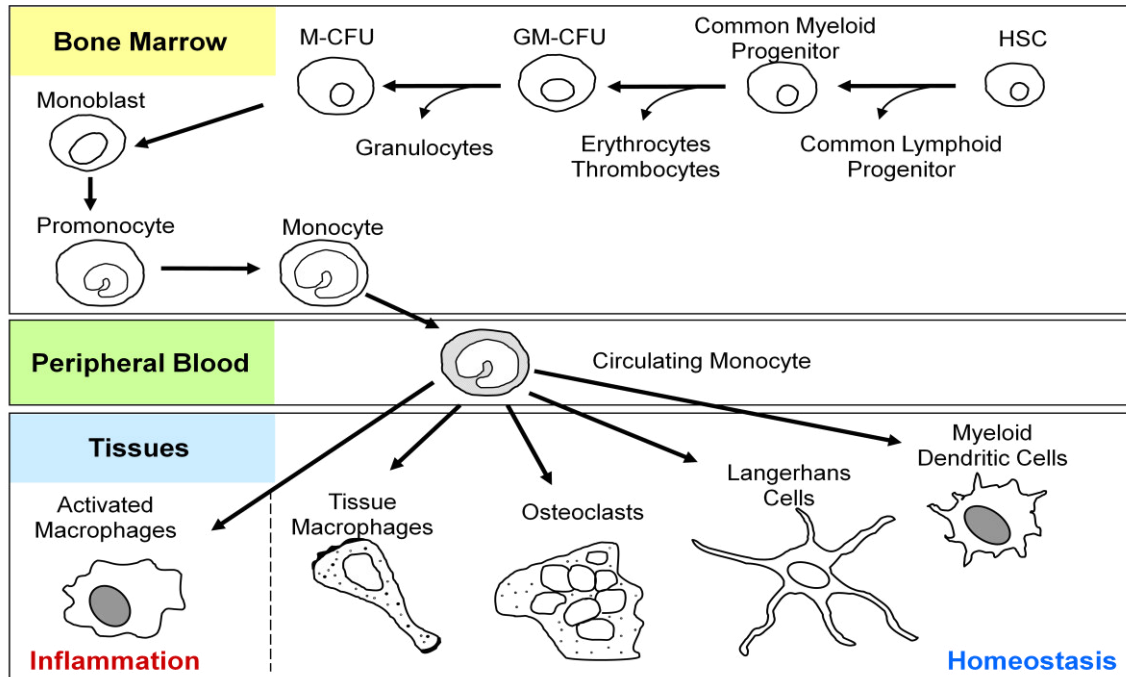


Figure 2: Monocytes are precursors to macrophages. Circulating monocytes ultimately derive from haematopoietic stem cells (HSC) and give rise to tissue macrophages.

Diversity of monocytes

Monocytes released from the bone marrow are morphologically heterogeneous with respect to size, granularity, nuclear morphology and antigenic markers. Based on the cell surface marker expression, monocytes are divided in two different subsets, the main markers being CD14 and CD16 (FcyRIII). “Inflammatory monocytes” are delineated by a CD14^{high} CD16⁻ phenotype, whereas CD14^{+/-} CD16⁺ cells define the group of “resident monocytes”. Both subsets express distinct chemokine, immunoglobulin, adhesion and scavenger receptors. For example, resident monocytes express the chemokine receptor 5 (CCR5), whereas inflammatory monocytes rather express CCR2 [85]. Based on the chemokine receptor CX3CR1 expression, the CD14^{+/-} CD16⁺ monocytes are believed to be the precursors to resident macrophages that are prone to differentiate into lymph node-resident DC. CD14^{high} CD16⁻ monocytes express lower levels of CX3CR1, but instead express a number of receptors that respond to inflammatory chemokines. Thus these cells are thought to be precursors to inflammatory macrophages [86, 87] (figure 3).

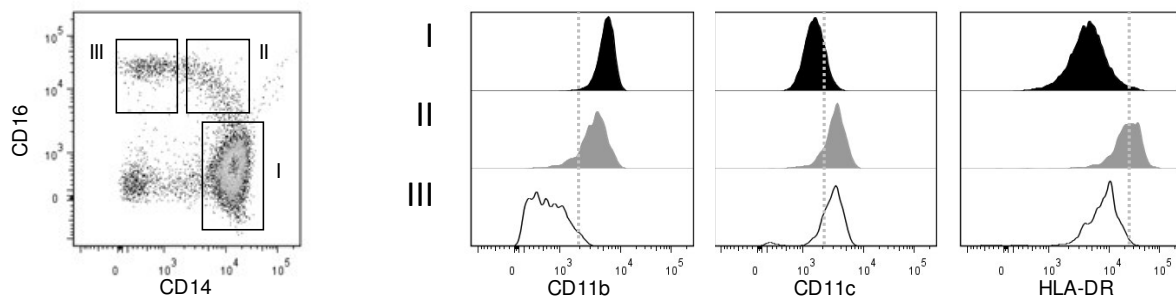


Figure 3: Distinct populations of human peripheral blood monocytes (Population I: inflammatory monocytes, population II/III: resident monocytes and dendritic cells).

Besides these two monocyte subsets, a third subset can be defined by a $CD14^{+/-} CD16^{+} CD64^{+}$ marker phenotype. This subset combines typical DC and monocyte characteristics, having a pronounced phagocytic activity and cytokine-producing capacity. This type of monocytes is suspected to have an immunoregulatory phenotype, which is based on an intermediate phenotype between monocytes and DCs [23, 88].

Diversity of macrophages

Macrophage populations in tissues may be renewed through the entry of new monocytes from the peripheral blood or by local proliferation. The physiological change of monocytes into macrophages features a series of individual phenotypic changes in order to fulfill the new functional activity of the particular tissue. Tissue-specific macrophages include osteoclasts, alveolar macrophages, Kupffer cells, mesangial cells, microglia as well as thymic, splenic and gut mucosal macrophages. It still remains uncertain, if monocytes are also versed to differentiate into DCs *in vivo*, as has been proven *in vitro*.

It is obvious that tissue specific macrophages fulfill different specialized tasks depending on their organ of residence. The most important macrophage groups and their functions are introduced in the paragraphs below: Thymus macrophages represent a significant component in the thymus physiology and play an essential role in negative selection of

developing T-cells. They are responsible for the clearance of apoptotic thymocytes, which are constantly deleted during the process of thymic selection.

The gut mucosal macrophage population constitutes the largest population of the human body. They reside within the lamina propria, fending for efficient phagocytosis and also antibacterial protection. Interestingly, gut mucosal macrophages do not respond to gut bacteria, which would cause perpetual intestinal inflammation; instead, these macrophages produce only non-inflammatory cytokines and are important in preventing gut inflammation.

Concerning the spleen, different subsets of macrophages have been identified, each of which occupies a discrete microanatomic location. Marginal zone macrophages express membrane receptors for bacterial polysaccharides which lead to efficient phagocytosis, characterizing them as an important part of the mobile surveillance system of the blood. In contrast to marginal zone macrophages, residing in the outer boarder of the marginal zone, metallophilic macrophages are found on the inner boarder adjacent to the white pulp. Metallophilic macrophages play a fundamental role in the response to viral infections [89]. The presence of Fc-receptors on so-called red pulp macrophages may facilitate recognition of senescent, antibody-sensitized cells [90]. Specialised phagocytic macrophages known as tingible body macrophages are located in the white pulp in the microenvironment of germinal centers. These presumably endocytose immune-complex-coated bodies and regulate the germinal center reaction by presenting this antigen [91].

Alveolar macrophages are found in the interstitium of the lung, where they clear inhaled microorganisms. Thus, alveolar macrophages are characterized by a very high expression of pattern-recognition receptors [92]. They are also responsible for tissue remodeling, as pulmonary fibrosis is thought to commence with a combination of pulmonary injury and alveolar macrophage recruitment.

Osteoclasts are a highly specialized population of macrophages, which are characterized by their multinucleated morphology and residence in bone. Unlike many other macrophages phagozytosing pathogens, osteoclasts resorb bone material. Hand-in-hand with this

remodeling process, they play a vital role in systemic electrolyte regulation, especially the Calcium concentration.

Kupffer cells are liver-resident macrophages that are exclusively located in the hepatic sinusoids. They play a role in homeostasis of the liver and participate in acute and chronic responses of the liver to noxious agents.

There are several different types of CNS macrophages, which are phenotypically different from most other macrophage populations. The largest population of CNS macrophages is known as microglia. When activated, these cells may have a detrimental effect in brain pathology through the promotion of inflammatory processes and neurotoxic substances, for instance in Alzheimer's disease and Parkinson's disease [93]. Bone marrow-derived perivascular cells are a small heterogeneous group of macrophages found in the CNS, located in the perivascular space of cerebral microvessels and thus situated at the intersection between brain parenchyma and blood [94]. Likewise, meningeal and choroid plexus macrophages are located at the blood-cerebrospinal interface, but these populations have not been well-studied yet.

Macrophage activation

As previously mentioned, circulating blood monocytes can be recruited to sites of inflammation to become activated macrophages, which are characterized by a high degree of functional heterogeneity. In response to different environmental signals, macrophages undergo various forms of polarised activation, depending on what kind of response foils the pathogen best. This polarisation forms the basis of two different macrophage groups, M1- and M2-polarised macrophages [95] (figure 4).

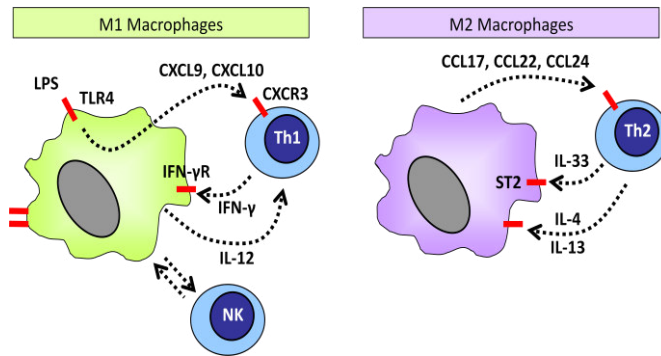


Figure 4: Macrophage polarisation. M1 macrophages promote Th1-type T cell responses, whereas M2 macrophages promote Th2-type T cell responses.

M1 macrophage activation (or classic activation) is defined by a high capacity to present antigens in response to IFN- γ or microbial products such as LPS. The secretion of high levels of IL-12 and IL-23 as well as the production of large amounts of proinflammatory cytokines (e.g. IL-1 β , TNF- α and IL-6) and chemokines (eg. CXCL9, CXCL10, CXCL11) particularly drive Th1-type T cell responses. M1 macrophages also produce nitric oxide and reactive oxygen intermediates, enabling them to be potential effector cells to kill intracellular micro-organisms and tumor cells.

M2-polarised macrophages are considered to be more heterogeneous than M1 macrophages. They can be divided into three different subtypes, namely M2a, M2b and M2c macrophages, and are often described as alternatively-activated macrophages [96]. M2a-polarised macrophages are generally induced by exposure to IL-4 or IL-13. On stimulation they produce CCL17 and develop a high endocytotic capacity as well as special receptors to scavenge tissue debris. They are also efficient in tissue remodeling and angiogenesis. M2b-polarised macrophages are activated by immune complexes or IL-1 β . Similar to M2a macrophages, they are responsible for phagocytosis and in addition play a role in microbicidal activities. M2b macrophages produce CCL1 and express sphingosine kinase 1. Both subtypes take over a major part in Th2-type T cell responses and strongly express MHC-II receptors. In contrast to the first two subtypes, M2c-polarised macrophages do not enhance immune responses, but instead seem to down-regulate other macrophage responses by secreting anti-inflammatory mediators such as IL-10, TGF- β , PGE2, CCL18, CCL16 or CXCL13. They are themselves activated by IL-10, TGF- β and glucocorticoids. Besides their capacity to down-regulate immune responses, M2c-

polarised macrophages are able to promote wound repair and angiogenesis. This subtype of macrophages is often found in chronically inflamed tissues [97] (figure 5).

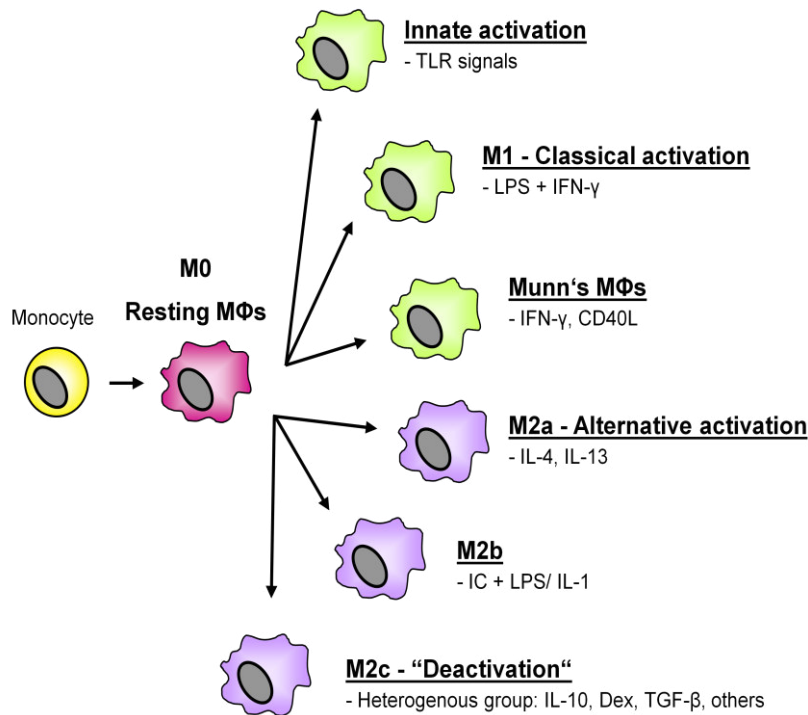


Figure 5: Macrophages as versatile effector cells. Depending on the stimulus, resting macrophages are either polarised to M1 macrophages or different subtypes of M2 macrophages and thus fulfill different functions upon stimulation.

All the above-mentioned differences in macrophage polarisation have been measured *in vitro*. It is unclear whether this paradigm applies so neatly to macrophage activation *in vivo*, where the cells are likely to be exposed to a mixture of polarising stimuli. However, it is known that the different kinds of macrophage polarisation are not just a definite state, but macrophages rather switch from one state to another depending on what stimuli they are activated by. Thus, macrophage polarisation can be considered a steady-state flow and not just a strict classification.

An essay on transplant immunology

Immunological tolerance

There are two principal mechanisms by which T cells are prevented from reacting against self-antigens: Firstly there is central tolerance, which is achieved by positive and negative selection of developing T cells in the thymus. This mechanism ensures that T cells are capable of recognizing self-MHC molecules. Secondly, in the periphery under non-inflammatory conditions, mature T cells with aberrant self-reactivity are continuously eliminated by deletional mechanisms or are actively suppressed by regulatory T cell populations.

Taking a closer look at the thymic selection of T cells, two basic steps can be identified. The first step comprises the positive selection of those T cells, which recognize self MHC-peptide complexes expressed by cortical epithelial cells. Approximately 95% of thymocytes (premature T cells) fail this selection and undergo “death-by-neglect” [98]. The thymocytes that survive this positive selection migrate into the thymic medulla, where they encounter a broad range of self-peptides presented by dendritic cells and macrophages. The broad expression of self-antigens of these cells is governed by a transcription factor called Auto-Immune Regulator Protein (AIRE). It has been shown in this context that mutations in AIRE cause a breakdown of central tolerance, generating a decrease of the expression of self antigens in the thymus [99] (figure 6).

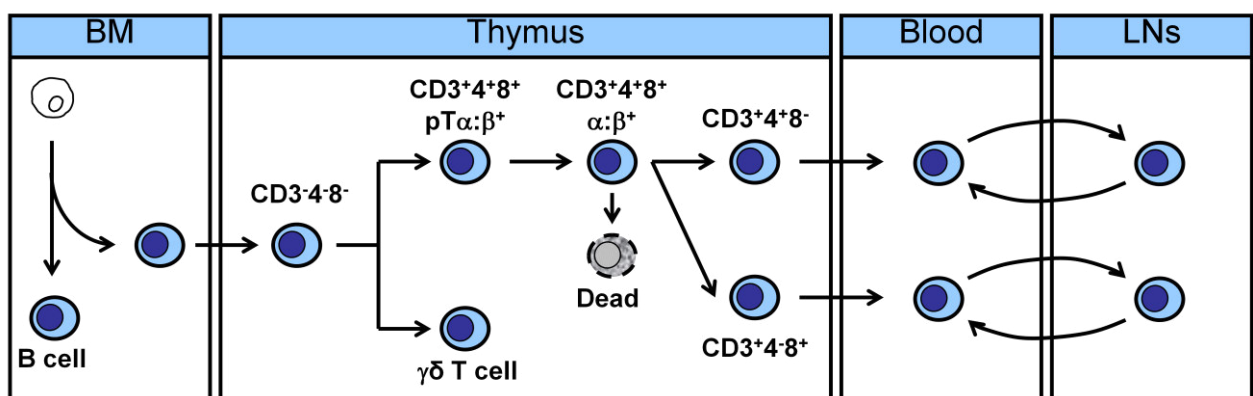


Figure 6: T cell development and restriction to self-MHC

Despite this precise system of T cell repertoire selection in the thymus, not all of the self-reactive T cells are eliminated before reaching the periphery. Consequently, there must be mechanisms to protect the body from autoimmune responses, which may be passive or active.

One passive mechanism is called immunological ignorance and describes the exclusion of T cells from certain tissues: This phenomenon operates in the testes and the central nerve system.

There are three active ways to achieve and conserve self tolerance in the periphery, namely clonal T cell deletion, T cell anergy and T cell-mediated regulation. It is important to understand that T cell activation comprises the recognition of an MHC-antigen-complex by the T cell-receptor and the subsequent costimulatory signal of the APC. In case of T cell anergy, there is a lack of costimulatory signals and the T cell fails to produce IL-2 and to differentiate into a T effector cell; consequently, no activation of further T cells takes place and they become refractory to further activating stimuli. Anergic T cells persist as inactive effector cells and hypothetically suppress T cell responses by occupying antigens presented by APCs [100].

The second mechanism, clonal T cell deletion, occurs through a process of activation-induced cell death (AICD) and is a crucial mechanism to maintain self tolerance. AICD of T cells results from the interaction of a Fas-bearing lymphocyte and Fas-ligands in tissues or other lymphocytes [101]. AICD can also be evoked by other death receptors, such as tumor necrosis factor receptor (TNFR) and death receptors 4 or 5 [102]. This kind of cell death is often described as clonal deletion, as self hazardous clones of T cells are eliminated on contact with a certain antigen.

The third mechanism, T cell mediated regulation, requires the existence of naturally occurring T regulatory cells (T regs). These cells seem to be essential for the downregulation of T cell responses. T reg deficiencies play a crucial role in a wide variety of autoimmune diseases and hypersensitivity responses [103]. An important factor decisive for T reg function is the Forkhead winged helix transcription factor (FOXP3). This transcription factor is responsible for the development and suppressive capacity of T regs. Notably, mutations or deficiencies in FOXP3 lead to the development of severe autoimmune syndromes [104]. On a molecular level, T regs are characterised by the CD4⁺ CD25⁺ FoxP3⁺ phenotype and arise during normal thymic T cell development [105]. T regs exert their suppressive function through a variety of mechanisms, including cytokine secretion and cognate interactions with APCs and other T cells. A crucial mechanism of T reg function is inhibition of B7-mediated T cell costimulation: CD28 interaction with one of its ligands (CD80 or CD86, also known as B7 molecules) is one of the best

characterized costimulatory pathways of T cells. Cytotoxic T-lymphocyte-associated protein (CTLA-4) is a competitive T cell inhibitor of CD28 binding to CD80 or CD86. Thus CTLA-4 expression by T regs down-regulates T cell activation by antagonizing CD28 signalling [106].

Transplantation rejection

In general, there are two principal mechanisms of graft rejection: cell-mediated (CMR) or antibody mediated rejection (AMR). Antibody-mediated rejection is based on antibodies against MHC molecules of the donor (which, in clinical transplantation are usually called Human Leucocyte Antigens or HLA). In case a kidney graft is transplanted into a patient with significant levels of antibodies directed against antigens of the kidney, the graft is likely to be rapidly killed in a process known as hyper-acute rejection [107]. Especially the foreign blood vessels in the graft are attacked by these antibodies. Consequently, the complement system is activated and phagocytic cells are recruited, resulting in vascular injury. The retraction of endothelial cells allows an interaction of platelets and underlying matrix [108]. Subsequent processes include coagulation, thrombosis, inflammation and ischemia (figure 7).

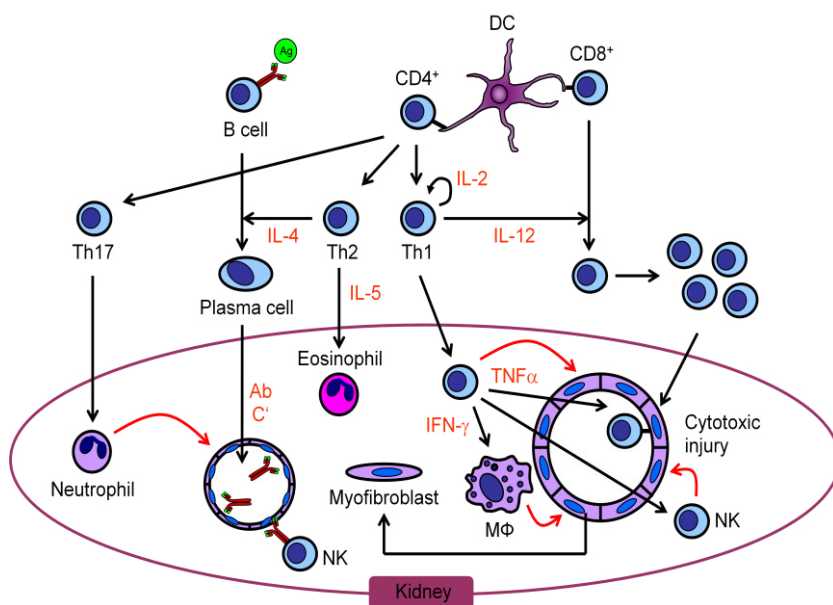


Figure 7: A summary of kidney transplant rejection. Multiple redundant effector mechanisms lead to allograft destruction. However, all are initiated by recognition of alloantigen by T cells.

Cell-mediated rejection is based on antigen-presenting cells (APC) from the donor activating alloantigen-reactive recipient T cells. Host T cells detect the foreign MHC from donor APCs in two ways (figure 8): Firstly, a proportion of recipient T cells own T cell-receptors that can directly bind to foreign MHC molecules, leading to T cell activation in the so-called *direct pathway*; secondly, MHC antigens can be processed like conventional antigens by recipient DCs, leading to recipient T cell activation via the *indirect pathway*. This process results in a typical immune reaction as described before.

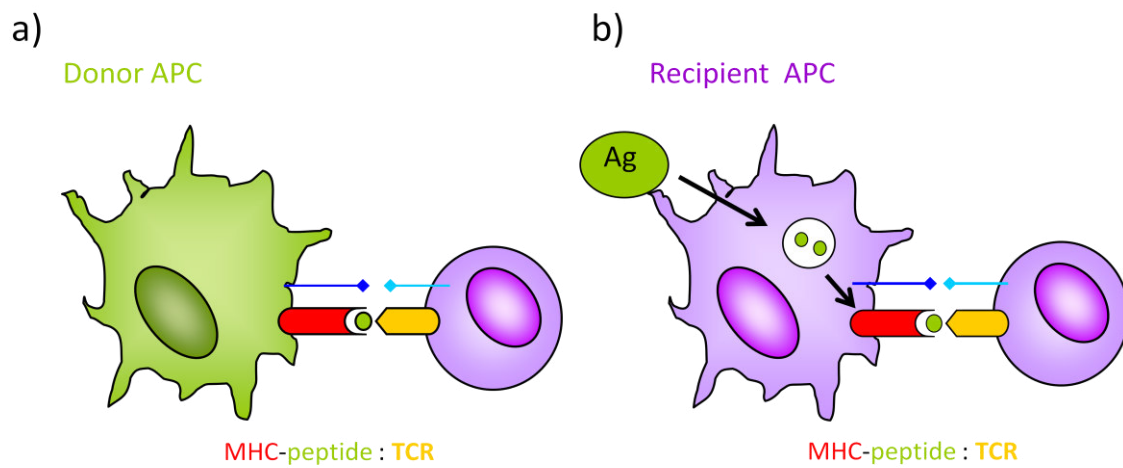


Figure 8: Direct (a) and indirect (b) pathways of antigen presentation

Immunosuppressive therapy in transplantation

Up to now, immunosuppressant drugs play the most important role in immunosuppression. In the next paragraphs, the historical development of four essential immunosuppressants – glucocorticoids, anti-proliferatives, calcineurin-inhibitors and mTOR-inhibitors – is depicted. A basic illustration of the function of these immunosuppressants is shown in figure 9.

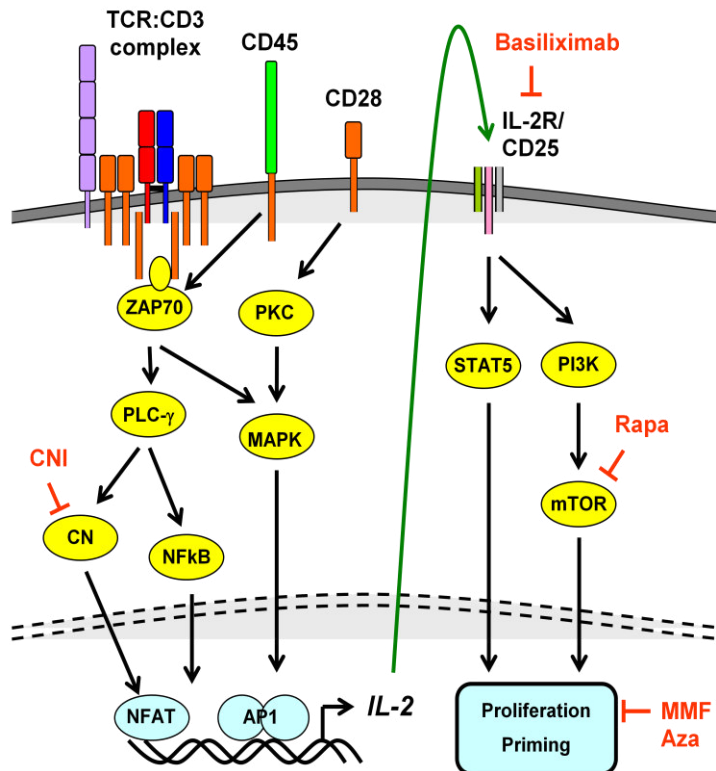


Figure 9: Points of action of classical immunosuppressants

In the early 1960s steroids like cortisone were the first drugs to be found successful in minimising acute rejection reaction in solid organ transplantation between genetically variant individuals [109]. At the same time, Azathioprine featured immunosuppressant effects in kidney transplanted patients by reducing de novo purine synthesis and dampening T- and B lymphocyte proliferation [110]. More than ten years later, Cyclosporine A, a fungal metabolite, proved to be an effective immunosuppressive drug increasing the one-year survival of transplanted patients enormously [111, 112]. Cyclosporine A is a calcineurin-inhibitor that inhibits T cell proliferation and is used as a basic immunosuppressant drug in transplanted patients until today.

In the next following years, several drugs have been developed for immunosuppressive therapy, none of which improving the outcome of transplanted patients significantly. It was not before 1982 that another promising drug was developed: the type 2 isoform inosine 5'-monophosphate dehydrogenase (IMPDH) inhibitor mycophenolate mofetil (MMF). Comparing

this drug to Azathioprine, being one of the standard immunosuppressants at that time, it could be shown that MMF is more lymphocyte-specific and more potent in preventing graft rejection [113, 114]. In addition to that, MMF proved to be even more effective in combination with other immunosuppressive drugs and thus has replaced Azathioprine in the daily clinical use [115].

Another promising drug, Tacrolimus, was developed in 1986. Tacrolimus is a calcineurin-inhibitor (CNI) like Cyclosporine A, but has been described to be up to 100-fold more potent in *in vitro* suppression assays [116]. Besides, Tacrolimus is capable of suppressing the activation, differentiation and proliferation of naïve and memory effector CD4⁺ and CD8⁺T cells by inhibiting the enzyme calcineurin [117, 118]. At the same time, successful T cell suppression does not affect myeloid cells on the same levels of concentration [119]. Comparing Tacrolimus with its CNI-antecessor Cyclosporine A, it turned out to be more potent in acute rejection episodes and graft loss [120, 121].

In 1989, a microbial product called Rapamycin (Sirolimus) was tested in transplantation models, featuring structural similarities to Tacrolimus. Though binding the same protein, Rapamycin does not inhibit calcineurin, but rather the mammalian target of Rapamycin (mTOR) [159]. This mechanism results in the arrest of the T cells' cell-cycle [122].

Besides glucocorticoids and small-molecule immunosuppressive drugs, a third group of immunosuppressants exist, namely biologicals. This group includes fusion proteins such as CTLA-4-Ig, depleting antibodies and non-depleting antibodies [115]. Antibody therapy as induction of immunosuppression reaches back to the early 1980's. Common antibody drugs are Anti-Thymocyte globulin (ATG) and Campath-1H (Alemtuzumab), both depleting T- and B cells, and also Basiliximab, which is a non-depleting anti-IL2R antibody. A great number of protein immunosuppressive drugs is now being developed (e.g. non-depleting CD40L antibodies ASKP1240 or 4D11) or in use (e.g. Belatacept) for blockade of the costimulatory CD40/CD40L or the CD28/CD80/CD86 pathways [123].

Keeping in mind the groups of immunosuppressants discussed in the previous paragraphs, it can be said that the common therapy protocol in transplantation consists of an induction phase

with an antibody such as Basiliximab and higher doses of CNI plus an anti-proliferative drug like MMF and tapered steroids. After this phase, the so called maintenance phase is mainly based on CNI, often combined with MMF or Rapamycin to reduce CNI doses and thus its toxicity [124, 125].

Immunosuppressive therapy and its toxicity

Even though short-term graft survival has been remarkably improved thanks to CNI, long-term outcomes of transplanted patients are still amendable [126]. Undesirable side effects like nephrotoxicity, neurotoxicity, disruption of glucose metabolism and even the risk of evoking malignancies have been associated with CNI treatment [127, 128]. Several studies aimed to reduce these adverse effects by combining or replacing CNIs with both MMF and Rapamycin. However, late conversion of a combined CNI - MMF treatment to MMF - Rapamycin therapy resulted in impaired kidney function, whereas early conversion was shown to initially improve it. Nevertheless, Rapamycin itself can cause severe side effects like proteinuria, bone marrow suppression and impaired wound healing [129]. The application of a Rapamycin - MMF combination without CNIs resulted in worse graft survival and acute rejection episodes compared to an addition of CNIs [124]. In contrast, treating stable renal transplant patients with low-dose Tacrolimus in combination with MMF, best results have been achieved in terms of graft survival, and impaired renal function could even be improved. Still, even though applying low dose immunosuppressants, general toxicity of Tacrolimus, Cyclosporin A or Rapamycin has obviously not been diminished and immunosuppressive therapy remains a tightrope walk between allograft tolerance and nephrotoxicity [130, 131].

Drug-free induction of tolerance

It has to be recognized that a major progress has been achieved in the management of acute rejection throughout the last decades. However, life expectancy and especially quality of life of transplanted patients is still low compared to the general population. Besides, long term graft survival has not been improved satisfactorily. The toxicity of immunosuppressive drugs, first of all CNIs, mainly account for these impairments [132, 133]. Thus, there is a strong need to find alternatives to the classic immunosuppressive therapy.

It was noted that some transplanted patients seem to accept their graft without any immunosuppressive therapy and with proper organ functions. In most cases, patients did not use immunosuppressant drugs due to non-compliance [134]. This state of drug-free acceptance is called operational tolerance, meaning stable graft function and immunocompetence at the same time [135]. In addition, operational tolerance describes the absence of a donor-specific response measured by donor-specific antibodies (DSA) [136]. Having substantiated examples of allograft tolerance at hand, it has become an essential aim to actively induce this condition in transplanted patients.

A well-known technique to achieve tolerance in transplanted individuals from rodent models to humans is to induce haematopoietic chimerism [137]. Up to now, this has been the only way to induce allograft tolerance in human renal transplant patients [138]. Nevertheless, haematopoietic stem cell transplantation prior to renal transplantation entails extremely radical preparative treatments and is associated with undesirable side effects like graft-versus-host-disease (GvHD). Thus, this kind of therapy must not be acquiesced in transplantation medicine without intensively searching for better alternatives.

Cell-based approaches to the induction of transplantation tolerance

Adoptive transfer of transplantation from tolerant to non-tolerant individuals with the transfer of populations of regulatory cells is a well-established method in experimental immunology, but only recently its clinical translation has received serious attention [2].

The cells of greatest interest in inducing cell-based tolerance in transplanted patients are T cells and APCs. Dendritic cells and macrophages, both APCs, influence the character of T cell responses and are potentially capable of inducing and maintaining peripheral tolerance. As described above, these cells receive both harmful and inoffensive signals from adjacent tissues and lymphocytes. After the integration of the signals, they are conveyed to T cells by a variation of co-stimulatory receptor and cytokine expression, while the T cells encounter their cognate antigen. In case of an apt stimulation, DCs and macrophages can acquire a tolerogenic phenotype, and on appropriately stimulating T cells they are capable of suppressing T cell activation or inducing the differentiation of peripheral T regulatory cells.

Besides DC, macrophages belong to the group of APCs. Since they operate similar to DCs, a large interest has been assigned to them in terms of inducing peripheral tolerance. Macrophages called transplant acceptance-inducing cells have been described, owning the capacity to dampen allograft rejection [139]. The original idea to apply macrophages for the induction of tolerance accrued from experiments, in which recipient DA rats were preoperatively treated with embryonic stem cells (ESC) derived from WKY strain animals, serving as donors. These experiments demonstrated that ESC treatment indefinitely prolonged DA-strain heart graft survival. This phenomenon was based on the development of the progeny of WKY-cells in the recipient rats. After infusion, the ESC gave rise to myelomonocytic cells, resulting in a limited but stable state of chimerism. This way, there was a continuous exposure of the recipient animals to alloantigenic stimuli of the donor without causing sensitization, which is necessary for the induction and maintenance of active peripheral tolerance after transplantation. Nevertheless, this method cannot be applied to human individuals, since the infusion of ESC is legitimately not contrivable. Thus, an alternative has been seen in the progeny of the infused ESC, namely a certain type of macrophage, which were called Transplant Acceptance-inducing Cells (TAIC). Treatment with TAICs has proven to be an effective method to ensure survival after various transplantations like heart, lung and kidney in mice, rats and miniature pigs. However, this model is only valid, if the TAIC derive from ESC of the donor animal, which also donates the graft. Applying this technique to third party allografts, transplantations result in acute rejection.

On the basis of these promising preclinical results, two early-phase clinical trials were performed, namely the TAIC-I and TAIC-II studies. In the TAIC-I study, 10 patients received in addition to conventional immunosuppression intravenous TAIC-infusion therapy five days after renal transplantation. The renal grafts were donated from deceased patients; TAICs were generated from donor splenocytes. This first TAIC study demonstrated that TAICs can be infused into patients without acute adverse reactions, verifying that TAIC treatment can be considered a safe clinical therapy. However, since the study cohort was very small, no conclusions about TAIC treatment efficiency after transplantations could be made [14].

In the second trial, the TAIC-II study, TAICs were generated from the donor's peripheral blood monocytes obtained by leukapheresis. The resultant TAICs were then administered to recipients five days prior to transplantation. As in the first trial, the TAIC-II study proved that it is feasible to administer TAICs as a pre-transplant therapy, but the study was not large enough to draw safety or efficacy conclusions. Nevertheless, the study gave hints that pre-transplantation exposure to donor specific antigen via TAICs renders the recipients hypo-responsive: Treated patients did not tolerate their kidney graft without immunosuppressants, but they only required very low doses of immunosuppression to maintain their transplants; moreover, immunological measurements of anti-donor reactivity (in MLR and biomarker studies) indicated attenuated responses [13].

Two living-donor renal transplant recipients have been treated with a suspension containing donor derived Mregs. Within 24 weeks post transplantation both patients were minimised to low-dose tacrolimus monotherapy and now, after more than five years, still maintain stable graft function. By one year post transplantation, both patients displayed patterns of peripheral blood gene expression converging upon the *Indices of Tolerance* (IOT-RASET) tolerance signature. These studies indicate that monocytes/macrophages are a potential target in inducing peripheral tolerance. However, before being able to apply macrophages as an approved therapy for transplant patients, more detailed and precise information about this type of cell and its characteristics has to be gained.

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Lebenslauf

Anne Christiane Broichhausen, geb. 22.10.1987 in München

Aktuelle Tätigkeit

Seit April 2014: Assistenzärztin Klinikum Kempten, Abteilung für Allgemein-, Viszeral-, Gefäß-, Thorax- und Kinderchirurgie, Chefarzt Prof. Dr. Büchler

Ausbildung

Oktober 2013: Zweites Staatsexamen Humanmedizin

Seit Juni 2010: Promotion bei Prof. E. Geissler, PhD, Klinik und Poliklinik für experimentelle Chirurgie, Universitätsklinikum Regensburg
Thema: „ Human Regulatory Macrophages: From bench to bedside“

August 2009: Physikum (erstes Staatsexamen)

Seit Oktober 2007: Studium der Humanmedizin an der Universität Regensburg

Juli 2007: Abitur
Fächer: Biologie, Englisch, Mathematik

Sept. 2004 –Juli 2005: Auslandsjahr an der Western Reserve Academy, Ohio, USA („ASSIST“- Stipendium)

Sept. 1998 –Juli 2007: Gymnasium Gröbenzell (bei München)

Berufserfahrung

Aug. 2012 – Juni 2013: „Praktisches Jahr“:
– Chirurgie (Memorial University of Newfoundland und Universität Regensburg)
– Innere Medizin (Kantonspital St. Gallen, Schweiz)
– Pädiatrie (Universität Regensburg)

2010 – 2012: Diverse Famulaturen à 30 Tage:

- Anästhesie (Klinikum Bogenhausen, München)
- Notfallmedizin (Joondalup Health Campus, Perth, Australien)
- HNO (Marienkrankenhaus Hamburg)
- Chirurgie (Litembo Diocesan Hospital, Tansania)

Okt. 2008 –Jan 2009: Studentische Hilfskraft am Lehrstuhl für Humananatomie und Embryologie, Prof. Dr. Tamm (Universität Regensburg)

Sprachen

Deutsch (Muttersprache), Englisch, Latein, Französisch, Spanisch

Freizeit

Musik

2009 – 2013 : Mitglied des Universitätsorchesters Regensburg

2003 – 2007: Mitglied des Bayerischen Landesjugendorchesters,
Mehrfache Teilnahme an Wettbewerben im In- und Ausland,
Preisträger beim Musikwettbewerb „Jugend Musiziert“

1990 – 2007: Violinunterricht

Sport

- Leichtathletik: bis 2008 auf Wettkampfebene
- Bergsport, Langstreckenlauf, Segeln (amtlicher Schein)

Ehrenamt

1998 – 2006: Mitglied der Ministranten der Pfarrgemeinde Gröbenzell,
St. Johann Baptist

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Anhang

Im Anhang sind in folgender Reihenfolge die oben beschriebenen Veröffentlichungen zu finden:

- Regulatory macrophages as therapeutic targets and therapeutic agents in solid organ transplantation
- In question: the scientific value of preclinical safety pharmacology and toxicology studies with cell-based therapies
- Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients
- Clinical management of patients receiving cell-based immunoregulatory therapy
- Generation of BTNL8⁺ iTregs by Human Regulatory Macrophages is IDO- and B7-dependent
- Laser ablation-inductively coupled plasma mass spectrometry: An emerging technology for detecting rare cells in tissue sections



Regulatory macrophages as therapeutic targets and therapeutic agents in solid organ transplantation

Christiane Broichhausen, Paloma Riquelme, Edward K. Geissler, and James A. Hutchinson

Purpose of review

This review aims to provide a basic introduction to human macrophage biology and an appreciation of the diverse roles played by macrophage subsets in allograft damage and repair. Current and future strategies for therapeutically manipulating macrophage behaviour are discussed.

Recent findings

Macrophages are extremely versatile effector cells that exert both immunostimulatory and immunosuppressive effects. This adaptability cannot be explained by differentiation into committed sublineages, but instead reflects the ability of macrophages to rapidly transition between states of functional polarisation. Consequently, categorisation of macrophage subpopulations is not straightforward and this, in turn, creates difficulties in studying their pathophysiology. Nevertheless, particular macrophage subpopulations have been implicated in exacerbating or attenuating ischaemia-reperfusion injury, rejection reactions and allograft fibrosis. Three general strategies for therapeutically targeting macrophages can be envisaged, namely, depletion approaches, in-situ repolarisation towards a regulatory or tissue-reparative phenotype, and ex-vivo generation of regulatory macrophages (M reg) as a cell-based therapy.

Summary

As critical determinants of the local and systemic immune response to solid organ allografts, macrophage subpopulations represent attractive therapeutic targets. Rapid progress is being made in the implementation of novel macrophage-targeted therapies, particularly in the use of ex-vivo-generated M regs as a cell-based medicinal product.

Keywords

cell-based medicinal product, M reg, regulatory macrophage, the ONE study

INTRODUCTION

Before discussing their possible pharmacological manipulation, it is first necessary to familiarise the reader with the origin, development, phenotypic diversity and functions of the many diverse forms of macrophage that are known to exist [1]. This leads immediately into controversial territory, as there is no general agreement amongst experts as to what exactly constitutes a macrophage subset, how functionally-specialised macrophage populations might best be classified, or even whether macrophages and dendritic cells are distinct lineages or merely varieties [2,3]. Although categorising macrophages as either M1-polarised or M2-polarised may be unsatisfactory in a number of respects, it provides a workable description of macrophages present in solid organ allografts [4^{***}]. Macrophages contribute to the development of transplant pathology at many levels. Although the importance of

local proinflammatory macrophages in aggravating acute allograft injury is now well recognised, there remains much to be learnt about the role of intragraft macrophages in chronic injury and repair processes, as well as the often-overlooked participation of splenic and lymphoid tissue macrophages in promoting and regulating adaptive immune responses against alloantigens. As a therapeutic strategy in transplantation, biasing macrophage-mediated

Department of Surgery and Regensburg Center for Interventional Immunology, University Hospital Regensburg, University of Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg, Germany

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

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Tolerance induction

KEY POINTS

- Macrophages conduct both detrimental and beneficial processes during innate and adaptive responses against solid organ transplants.
- Macrophages represent an attractive therapeutic target in solid organ transplantation.
- Treatment with regulatory macrophages (M_{reg}) as a cell-based medicinal product is a promising clinical strategy for promoting immunological regulation against transplantation antigens.

responses towards regulation and the reestablishment of tissue homeostasis has much to commend it. Experimental methods of deleting activated macrophages, or preventing their recruitment into allografts, have shown potential therapeutic benefit, but no agents for specifically depleting macrophages are presently approved for use in patients. A number of drugs in current clinical use affect the activation state of macrophages, albeit not very selectively. A radically alternative approach to conventional pharmacological manipulation of macrophages is their use as a cell-based medicinal product after being driven to a regulatory state by ex-vivo culture [5¹¹]. The relative merits of these three macrophage-targeted therapeutic strategies – depletion, phenotypic modulation and ex-vivo manipulation – are discussed in this review.

A PRIMER IN MACROPHAGE BIOLOGY

Macrophages are highly adaptable effector cells that engage in diverse, often antagonistic activities: macrophages can both heighten and diminish immunological responses, and participate in both tissue-destructive and tissue-reparative processes [6]. Macrophages can be rapidly recruited from blood to sites of inflammation, but are also constitutive elements of normal tissue stroma, and specialised forms are essential cellular components of bone, kidney, liver and neuronal tissue [1,7]. This astonishing functional adaptability is reflected by the remarkable capacity of macrophages to inducibly express a very broad range of genes [8–10]. Macrophages play a critical role in innate immunity, constituting a first line of defence against many pathogens. Upon detecting a pathogen, macrophages are responsible for both eliminating that pathogen and initiating the inflammatory cascade which leads to recruitment of other immunological effector cells. In addition, macrophages are responsible for the formation of abscesses and granulomata, structures which limit the extent of acute

and chronic inflammatory reactions and prevent the wider dissemination of pathogens and other noxious agents.

Owing to their expression of an array of receptors recognising ligands normally associated with pathogens, but which also sense constituents of dying cells, macrophages are exquisitely sensitive to the pathological condition of their environment [11,12]. Binding of ligands to these receptors stimulates phagocytosis, whereupon macrophages destroy ingested material in phagosomes by several mechanisms, including acidification, fusion with protease-containing lysosomes, or production of reactive oxygen and nitrogen intermediates. Upon activation, macrophages also secrete toxic substances, such as perforin, granzyme and TNF- α . Macrophages serve as professional antigen-presenting cells and can inducibly express high levels of costimulatory molecules [13]. It was formerly held that macrophages, unlike dendritic cells, were incapable of priming naive T cell responses, but this old dogma has now been overturned [14,15]. Through the elaboration of cytokines and other soluble factors, macrophages are able to polarise T cell responses and thereby govern the nature of adaptive immune responses [16]. Reciprocally, activated effector T cells secrete cytokines that activate macrophages, enhancing their antigen-presenting capability and production of inflammatory mediators, thus amplifying adaptive immune responses. An example of this positive feedback is IL-12 production by activated macrophages, which drives Th1 cells to produce IFN- γ and this, in turn, further activates macrophages.

The resolution of inflammation also depends on anti-inflammatory properties of macrophages. Production of IL-10 by macrophages restrains T cell and macrophage responses, and promotes tissue-reparative processes through clearance of apoptotic or necrotic cells, stimulating new vessel formation, secretion of tissue-trophic factors and opposing fibrosis [17]. The conditions that dictate whether macrophages *in vivo* switch to a quiescent state and help to restore tissue homeostasis, or remain in an activated condition and promote chronic inflammation, are presently not well understood [18,19].

MONOCYTES ARE PRECURSORS TO MACROPHAGES

Macrophages ultimately derive from blood monocytes, which exit the bone marrow and circulate for several days before being recruited to inflamed tissues to become activated macrophages or entering noninflamed sites to become tissue-resident macrophages [1]. Human monocytes vary greatly

in morphology and marker phenotype, most notably in their expression of CD14 and CD16, but also with respect to their chemokine receptor expression profiles [20–22]. On the basis of these phenotypic differences, two principal subsets of human peripheral blood monocytes can be identified: inflammatory monocytes are CD14⁺ CD16⁻, whereas resident monocytes are CD16⁺ (Fig. 1). CCR2-expressing inflammatory monocytes traffic preferentially to sites of inflammation wherein they become activated macrophages. Resident monocytes, which likely develop from inflammatory monocytes under noninflammatory conditions, are believed to be precursors of tissue-resident macrophage populations and lymph node-resident dendritic cells [23].

MACROPHAGE ACTIVATION STATES

Inflammatory monocytes are recruited to sites of tissue injury where they become activated macrophages. Depending upon the nature of the activating stimulus and the influence of other immunological effector cells, activated macrophages alter their expression of cytokines, costimulatory molecules

and cytotoxic apparatus to promote the most appropriate response to the pathological insult. Thus, some authors draw a distinction between M1-polarised macrophages, which preferentially drive Th1 responses, and M2-polarised macrophages, which promote Th2 responses (Fig. 2) [1]. M1-polarisation, or classical activation, is induced by IFN- γ combined with microbial stimuli, such as lipopolysaccharide. M2-polarised macrophages have been subclassified into three groups: M2a, M2b and M2c macrophages. M2a macrophages, otherwise known as alternatively activated macrophages, are induced by IL-4 or IL-13 treatment [24]. M2b macrophages arise when resting macrophages are stimulated with immune complexes [25]. M2c macrophages, otherwise collectively known as deactivated macrophages, are a heterogeneous grouping of anti-inflammatory macrophages generated by exposure to such diverse stimuli as IL-10, glucocorticoids, activin A and IL-21 [26,27].

The convention of classifying activated macrophages according to their phenotypic similarity with classically or alternatively activated macrophages has held sway for many years. Yet this taxonomy fails in several important respects. Firstly,

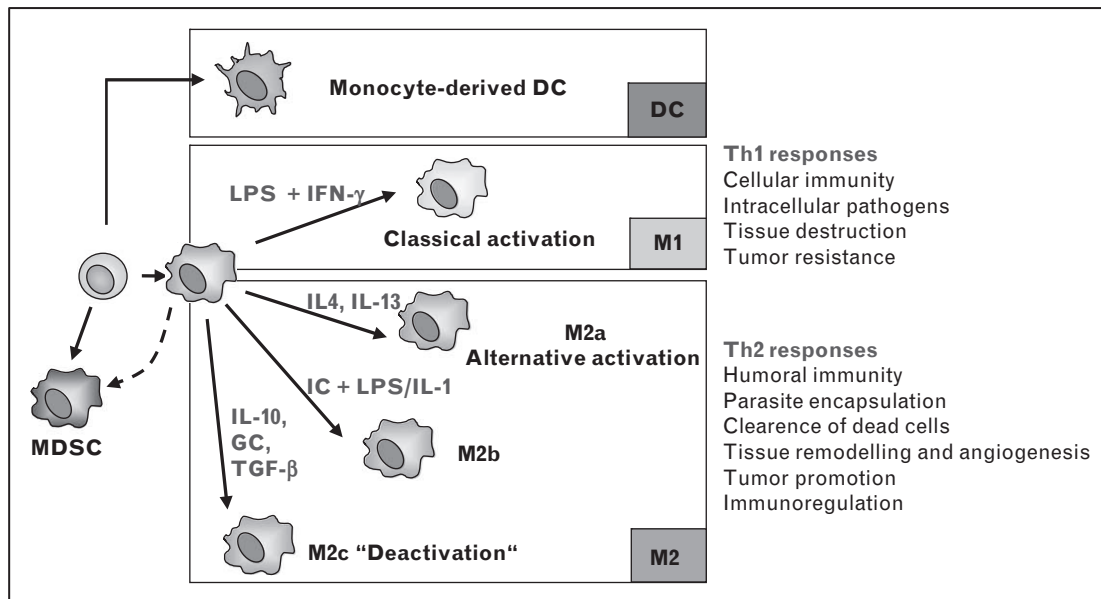


FIGURE 1. Classification of macrophage polarisation states. M1-polarised macrophages are induced by lipopolysaccharide and IFN- γ stimulation. M1 macrophages exhibit potent antimicrobicidal activities, including Reactive Nitrogen Species and Reactive Oxygen Species production, and secrete proinflammatory cytokines (IL-1 β , IL-6 and TNF α) and chemokines. M1 macrophages express high levels of MHC Class II, CD80 and CD86, and promote Th1 responses. M2a-polarised macrophages (also known as alternatively activated macrophages) are induced by IL-4 or IL-13 exposure. M2a macrophages have a high endocytic capacity, express high levels of antigen-capture receptors, such as Dectin-1 and MMR, and play crucial roles in resistance to parasitic infections and tissue repair. M2b-polarised macrophages arise after stimulation with immune complexes and are characterised by secretion of IL-10 and not IL-12. M2c-polarised macrophages comprise a heterogeneous group of 'deactivated' or suppressive macrophage phenotypes induced by a variety of stimuli, including IL-10, TGF- β , inhibitory receptor ligation (e. g. CD200R) or glucocorticoids. (For a detailed review of macrophage polarisation states see [1]).

Tolerance induction

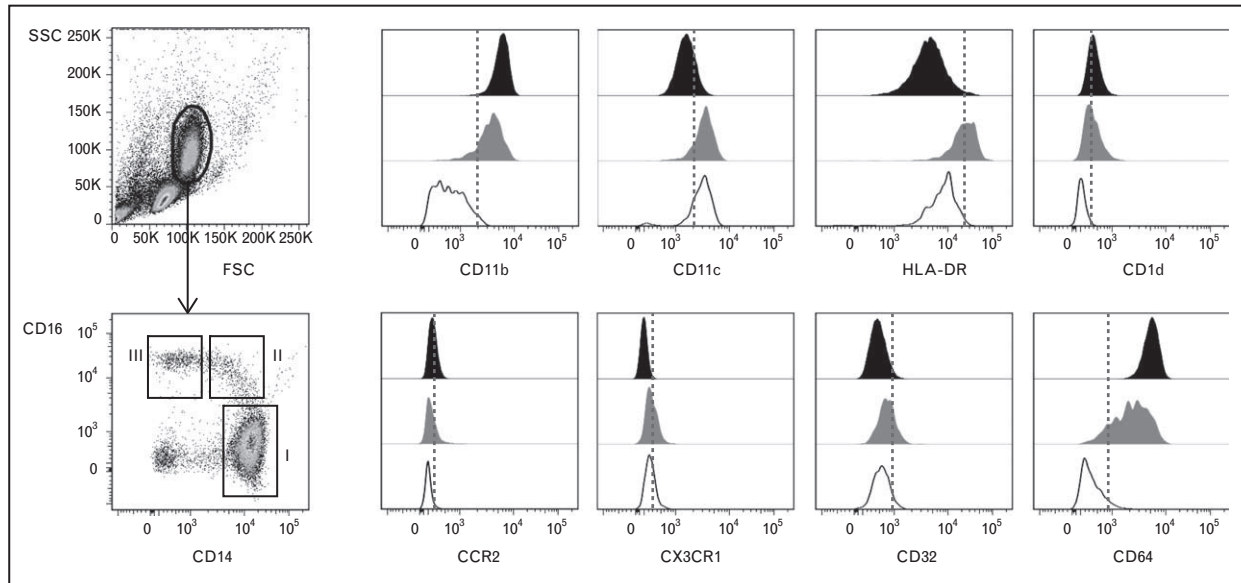


FIGURE 2. Subtyping of human peripheral blood monocytes. Human monocytes are conventionally divided into subsets according to their expression of CD14 and CD16. In fact, the recognised subsets of CD14⁺ CD16⁻ inflammatory monocytes (gate I and black traces), CD14⁺ CD16⁺ (gate II and grey traces) and CD14^{low} CD16⁺ resident monocytes (gate III and unfilled traces) probably represent stages in the maturation of inflammatory monocytes into resident monocytes. Inflammatory monocytes express higher levels of CD11b, CD1d, CCR2 and CD64 than resident monocytes. Conversely, CD16⁺ monocytes express higher levels of CD11c, HLA-DR and CX₃CR1 than inflammatory monocytes.

because macrophage phenotypes are so readily disturbed by extrinsic stimuli, in an inflamed-tissue environment, it is unreasonable to think that a phenotypically homogeneous macrophage population could arise that neatly corresponds to any in-vitro-induced state of activation [28]. Secondly, under normal physiological conditions, reactions by macrophages to inflammatory stimuli are checked by the constitutive action of anti-inflammatory tissue factors, so all ex vivo-generated macrophage types are necessarily artificial [29]. Thirdly, there is no clear place in the M1/M2 classification scheme for suppressor macrophages [30]. Nevertheless, much of our understanding of the roles of macrophages in transplant pathology is framed in terms of the preponderance of M1-polarised or M2-polarised macrophages within allografts because this scheme of classification is workable.

HUMAN SUPPRESSOR MACROPHAGES

The existence of anti-inflammatory, T cell-suppressive cells of the myeloid lineage has long been recognised. Broadly speaking, such myeloid suppressor cells are either characterised by an arrested state of immaturity, when they are known as myeloid-derived suppressor cells, or a more mature phenotype, reflecting the ability of myeloid

Antigen Presenting Cell (APCs) to switch into a suppressive mode under certain conditions [31]. Several excellent reviews concerning myeloid-derived suppressor cell biology have been published recently, so they need not be revisited here [32–34]. On the other hand, some of the diverse stimuli, which drive mature macrophages (or dendritic cells) to a suppressor phenotype, deserve mention. Suppressive activities in cultured macrophages are induced by a wide range of unrelated stimuli, including M-CSF, IL-10, immune complexes, vitamin D, glucocorticoids, IFN- γ , repetitive restimulation through TLR-2, TLR-4 or TLR-9, and prostaglandin E₂ (PGE₂). Accordingly, there is no unique phenotype or mechanism of action associated with suppressor macrophage populations.

Induction of suppressor macrophages

Treatment of cultured macrophages with M-CSF, in contrast to GM-CSF treatment, shifts them away from being efficient antigen-presenting cells and promotes an IL-10-producing suppressor phenotype [35^{***}]. Treatment of mice with M-CSF induces the expansion of immunosuppressive macrophage populations that hinder contact sensitivity reactions and impair protective immune responses against experimental brucellosis [36]. IL-10 is a broadly suppressive cytokine which acts

on macrophages to prevent their expression of IFN- γ and other proinflammatory cytokines, and to downmodulate expression of costimulatory molecules [37]. Importantly, autocrine stimulation by IL-10 reinforces its own expression in human macrophages in a STAT3-dependent manner, possibly through upregulation of TPL2 and consequent ERK activation, ensuring the phenotypic stability of IL-10-producing suppressor macrophages [27,38]. Classically, IL-10 produced by suppressor macrophages prevents the development of Th1-type T cell responses, but also inhibits Th2 cells and enhances the differentiation of IL-10-producing Treg cells [39]. 1,25-dihydroxyvitamin D₃ inhibits the expression of costimulatory molecules and MHC Class II by macrophages, and limits their production of IL-12 and IL-23 [40]. In addition, 1,25(OH)₂D₃ induces macrophages to express IL-10 and MIP3 α , thereby rendering them suppressive. Glucocorticoids have profound effects on many aspects of macrophage function, including inhibition of the NF κ B pathway [41]. The combination of 1,25(OH)₂D₃ and glucocorticoid treatment drives macrophages and dendritic cells to such a stably immunosuppressive state that some groups have proposed their use as a cell-based medicinal product [42].

Paradoxically, a number of classically proinflammatory factors can induce macrophage suppressor functions. Foremost amongst these is IFN- γ , which is secreted by activated T cells and normally enhances the antigen-presenting capability, IL-12 production and antimicrobicidal activity of macrophages. Surprisingly, in some circumstances, IFN- γ fails to induce upregulation of CD80 and MHC Class II in macrophages, but does induce expression of indoleamine 2,3-dioxygenase (IDO), programmed death ligand-1 (PD-L1) and FasL [43]. IDO is a tryptophan-catabolising enzyme which inhibits effector T cell proliferation through tryptophan deprivation and promotes T reg development by generating kynurenine [44,45]. In mice, IDO is indispensable for maintaining maternal tolerance of allogeneic conceptuses [46] and plays a critical role in the establishment of allograft tolerance by adoptive transfer of T regs [47]. When activated T cells expressing receptors for PD-L1 and FasL (PD-1 and Fas, respectively) encounter PD-L1- and FasL-expressing APCs, they are induced to undergo apoptosis. A vital role for both PD-L1 and FasL expression by macrophages in the induction and maintenance of transplant tolerance has been demonstrated, underscoring the importance of deletional mechanisms in the establishment of tolerance [48,49]. It is now well established that continuous physiological exposure of macrophages

and dendritic cells to TLR-2, TLR-4 or TLR-9 ligands drives them to a suppressive state [50–52]. In mice, repetitive sublethal challenge with lipopolysaccharide (LPS) up-regulates expression of heme oxygenase-1 (HO-1) in macrophages. HO-1 catalyses the degradation of heme, ultimately leading to the generation of carbon monoxide (CO), bilirubin and free iron. Amongst other effects, CO released by HO-1 renders macrophages suppressive through the inhibition of LPS-stimulated proinflammatory cytokine production [53]. PGE₂ is a potent inflammatory mediator, causing local vasodilation and recruitment of monocytes and neutrophils to sites of acute inflammation, but it also exerts immunosuppressive effects through macrophages [54^{***}]. Acting at the EP2 receptor, PGE₂ down-regulates the antimicrobial activities of macrophages, limits their phagocytic activity, induces their expression of IL-10 and antagonises TLR-dependent TNF- α expression.

Human regulatory macrophages

For some years now, efforts in our laboratory to develop a cell-based medicinal product for use in promoting transplant tolerance in renal transplant patients have focused on a type of suppressor macrophage, which we call regulatory macrophages (M reg). The human regulatory macrophage reflects a unique state of macrophage differentiation, distinguished from macrophages in other activation states by its particular mode of derivation, robust phenotype and potent T cell suppressor function. These cells arise from CD14⁺ peripheral blood monocytes during a 7-day culture period during which the cells are exposed to M-CSF, 10% human serum and a final 24-h pulse of IFN- γ [55]. M regs derived in this manner adopt a characteristic morphology and are homogeneously CD14^{-/low} HLA-DR⁺ CD80^{-/low} CD86⁺ CD16⁻ CD64⁺ TLR2⁻ TLR4⁻ and CD163^{-/low} [5^{***}]. Human M regs are potently suppressive of T-cell proliferation *in vitro*, both through IFN- γ -induced IDO activity and contact-dependent deletion of activated T cells [5^{***}]. After administration to a patient by central venous infusion, allogeneic M regs remain viable and rapidly partition into the liver, bone marrow and spleen, but do not accumulate in other lymphoid tissues.

MACROPHAGES AND TRANSPLANT PATHOLOGY

Acting within grafts, but also at distant sites, macrophages profoundly influence the development of various transplant pathologies. Early after

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transplantation, macrophages respond to ischaemia-reperfusion injury by mounting a rapid, stereotypical inflammatory response that leads to secondary tissue damage. Similarly, infiltration of allografts by macrophages during acute rejection episodes exacerbates tissue damage. In the longer term, macrophages are key mediators of chronic allograft inflammation and fibrosis. More than being simple effectors of the innate response, macrophages also serve as amplifiers of the adaptive immune response by supporting T cell reactions within allografts. On the contrary, not all macrophage-mediated processes are detrimental to allografts; in particular, re-establishment of tissue homeostasis by anti-inflammatory macrophages appears to be a determinant of long-term transplant survival.

The origin of macrophages within allografts

In the context of solid organ transplantation, macrophages found within allografts may stem from three immediate sources. Firstly, as a normal stromal component, tissue-resident macrophages may be of donor origin; however, these passenger macrophages usually disappear early after transplantation. Secondly, activation of graft-resident macrophages leads to their production of inflammatory mediators, resulting in a prompt migration of recipient macrophage precursors into the graft [56]. Infiltration of allografts by macrophages is a prominent feature of reperfusion injury and transplants undergoing acute rejection. The pool of circulating monocytes available for recruitment into allografts is reinforced by their release in large numbers from bone marrow as part of the generalised response to sterile tissue injury. Thirdly, activated macrophages can proliferate within inflamed grafts in response to M-CSF or IL-4 stimulation.

Ischaemia-reperfusion injury

Restoration of blood flow to an ischaemic organ graft causes cellular dysfunction, apoptosis and necrotic cell death within the transplanted tissues [57]. The consequent release of cellular constituents, such as nucleic acids and heat-shock proteins, triggers the activation of graft-resident dendritic cells and macrophages, initiating the inflammatory cascade [56]. Following reperfusion in experimental models of kidney ischaemia, infiltration by macrophages is first observed within 30 min (preceding lymphocyte infiltration) and peaks at 24–48 h [58]. This rapid influx, mediated by CCR2 and CX₃CR1, represents the earliest phase of a stereotyped reaction in which macrophages cause further tissue injury through the production of reactive oxygen

and nitrogen intermediates, release of extracellular matrix-degrading enzymes, secretion of cytokines and the activation of lymphocytes [59]. However, the destructive tendency of macrophages after reperfusion injury is short-lived, as both tissue-resident and infiltrating macrophages soon begin to exert anti-inflammatory and tissue-reparative effects [40]. This phasic contribution of macrophages to both reperfusion injury and its resolution is neatly illustrated by the effect of depleting macrophages using liposomal clodronate or the CD11b-DTR conditional ablation system. Such experiments demonstrate that depletion of macrophages prior to renal ischaemia-reperfusion injury in mice reduces the number of infiltrating macrophages in the organ at 48–96 h and helps to preserve short-term renal function [60]. However, the cost of preventing early macrophage-mediated injury is a poorer tissue repair response with impaired clearance of dead and apoptotic cells [61]. A similarly dichotomous role for macrophages in injury and repair after ischaemia-reperfusion has been demonstrated in lung [62,63], liver [64] and small intestine [65].

Acute allograft rejection

Macrophages are a prominent component of inflammatory infiltrates in acutely rejecting allografts. In the case of the rejecting renal allograft, infiltrating macrophages are to be found in the glomerular, vascular and tubulointerstitial compartments [66]. The total burden of infiltrating macrophages correlates with the severity of renal allograft rejection [67] and the density of glomerular infiltration by macrophages is predictive of a worse clinical outcome, independently of peritubular capillary C4d deposition [68]. Infiltration of renal allografts by macrophages within 7 days of transplantation is likewise associated with poorer clinical outcomes [69,70] and the presence of CX₃CR1-expressing macrophages in acutely rejecting kidney allografts is associated with resistance to steroid treatment and worse outcomes at 1 year [71]. Gene expression profiling studies of clinically indicated biopsies from renal allografts corroborate these histopathological findings, in that, high expression of macrophage-associated transcripts (especially M2-polarised macrophage-associated transcripts) correlates with T cell-mediated rejection [72]. Importantly, these studies show that macrophage infiltration is a feature of canonical T cell-mediated acute rejection, so is not merely a secondary consequence of antibody-mediated rejection. Macrophages enter acutely rejecting allografts in response to a wide range of chemotactic stimuli, including CCL2, CCL3, CCL7 and osteopontin [73–75]; in the case

of antibody-mediated rejection, C4d deposition also recruits macrophages [76]. In the acutely rejecting allograft, macrophages serve as direct effectors of tissue injury, but also aggravate the adaptive immune response through their presentation of alloantigen and secretion of T cell-stimulatory cytokines. In kidney transplantation models, macrophage depletion generally reduces the severity of acute rejection episodes, preserving function of the allograft and limiting lymphocytic infiltrates [77,78].

Chronic allograft injury

Precisely what governs the restoration of normal tissue homeostasis after an inflammatory episode is not well understood; in particular, the factors determining whether macrophages return to a quiescent, tissue-resident phenotype or persist in a chronically activated state are unknown [6]. Nevertheless, this decision is integral to long-term allograft outcomes because chronically activated macrophages play a profibrotic role and are central to the development of chronic transplant arteriopathy [79]. In general terms, the profibrotic response of macrophages can be characterised as a stereotyped response to chronic or repetitive injury [80]. In transplantation, the initiating insult is mediated by alloantigen-reactive T cells, but may later become a self-perpetuating condition. Studies of chronic allograft rejection have identified numerous mechanisms by which profibrotic macrophages may be induced; however, activation of myofibroblasts and vascular smooth muscle cells through secretion of cytokines, direct deposition of fibrotic matrix, and the promotion of Th2 and Th17 responses are common end pathways [79,81].

Evidence for the involvement of macrophages in chronic allograft injury comes from both experiments in animal models and correlative histopathological studies. Interstitial macrophage infiltrates are a feature of progressive glomerulosclerosis in the F344-to-LEW rat model of chronic rejection [82]. Using the same model, the presence of glomerular and vascular macrophage infiltrates was associated with glomerulosclerosis, tubular atrophy, interstitial fibrosis and arterial intimal proliferation [83,84]. In renal transplant biopsies from patients undergoing chronic rejection, infiltration of peritubular capillaries by macrophages is particularly associated with C4d deposition [76]. Profibrotic macrophages produce various mediators, including PDGF, IGF-1 and TGF- β_1 , which directly activate myofibroblasts and are; therefore, essential for normal wound healing [79]. In chronic allograft injury, these same factors play detrimental roles;

for instance, macrophages within the thickened intima of arteries with chronic transplant arteriopathy secrete high levels of PDGF, which drives the proliferation of intimal smooth muscle cells [85]. It has been suggested that macrophages within chronically rejecting allografts generally adopt an M2-polarised phenotype and promote Th2-type responses [4¹¹]. IL-13 produced by Th2 cells, in turn, drives macrophages to synthesise TGF- β_1 and thereby promote fibrosis [86]. Profibrotic macrophages can also generate IL-1 β , so inducing IL-17 production by Th17 cells, which promotes fibrosis, especially in the lung [87–90].

Although macrophages play a clear role in initiating and perpetuating fibrotic reactions, they are also implicated in the prevention and reversal of fibrosis [6]. Removal of dead cells and other debris from inflamed tissues by macrophages eliminates the stimulus for further inflammation. Remodelling of extracellular matrix by collagen-degrading enzymes produced by macrophages is vital to the restoration of normal tissue structure. Production of anti-inflammatory mediators, especially IL-10, by macrophages dampens adaptive immune responses and curtails fibrotic reactions [91]. Therefore, as is the case in reperfusion injury and acute rejection, whether allograft-infiltrating macrophages exert a beneficial or detrimental effect in chronic allograft injury depends on their particular state of activation and the pathological condition of the tissue in which they reside.

SUPPRESSOR MACROPHAGES AS A THERAPEUTIC TARGET

As an abundant, broadly distributed class of cells which serve indispensable roles in the amplification and attenuation of innate and adaptive immune responses to solid organ transplants, macrophages represent attractive therapeutic targets. It is perhaps unsurprising that many anti-inflammatory and immunosuppressive drugs in current clinical use exert pharmacologically important effects through their general action on macrophages or their products, including glucocorticoids, mTOR inhibitors and TNF α -blocking antibodies. The development of agents for selective targeting of macrophages in particular activation states could have important clinical implications. We can consider three general strategies for promoting beneficial immunoregulatory and tissue-repair processes while preventing detrimental macrophage-mediated processes. These general strategies are depletion of graft-damaging macrophages, in-situ induction of suppressor macrophage subsets, and ex vivo-generation of regulatory macrophages for re-administration to patients.

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Macrophage depletion

As discussed above, studies in mice have proven that depletion of monocytes and macrophages can limit the development of various allograft pathologies. Presently, no antibodies capable of depleting macrophages from tissues are clinically available, less so antibodies which specifically target inflammatory macrophage subpopulations while sparing suppressor macrophages, but efforts to develop such agents are underway [92]. In particular, the possibility of using antibodies against CSF-1R to either deprive macrophages of tonic M-CSF signalling or to bring about their direct killing has generated much recent interest [35¹¹,93]. An alternative strategy for depleting macrophages in tissues is to prevent their recruitment: one particularly promising approach is blockade of the CCR1, CCR2 and CCR5 chemokine receptors with monoclonal antibodies or small molecule inhibitors [94–97].

Manipulation of macrophage activation states

The strategy of driving macrophages within tissues towards a suppressor phenotype is not far-fetched. Indeed, many drugs in routine clinical use influence the polarisation state of macrophages, albeit unspecifically. Of the immunosuppressive drugs commonly used in solid organ transplantation, glucocorticoids and mTOR inhibitors have well documented effects on the maturation state of dendritic cells and macrophages [98–100]. Interestingly, mycophenolate mofetil (MMF) affects macrophages in several important respects, including limiting their ability to migrate into inflamed sites, restricting their maturation, inhibiting LPS-induced IL-12 production, and preventing expression of IL-1 β while inducing IL-1Ra expression [101].

Administration of intravenous immunoglobulin (IVIg) is a first-line treatment in certain acute immunologically mediated conditions and has a profound effect on the behaviour of macrophages [102]. IVIg stimulates the inhibitory Fc γ RIIb receptor, which leads to induction of the IL-10 expression and a regulatory phenotype in macrophages [103–105]. More experimentally, the immunosuppressive properties of mesenchymal stem cells (MSCs) appear to relate, at least in part, to their ability to induce IL-10 production by macrophages [106]. Several clinical trials of MSCs as an immunomodulatory therapy in solid organ transplantation are currently underway and are, in a sense, trials of a macrophage-targeting treatment [107].

Angiotensin II is generated locally by macrophages and fibroblasts, and plays an important role in fibrotic processes through stimulation of TGF- β ₁

production and induction of fibroblast differentiation into collagen-secreting myofibroblasts [79]. Furthermore, angiotensin II enhances TGF- β ₁ signalling by increasing SMAD2 expression and promoting the nuclear translocation of phosphorylated SMAD3. In the F344-to-LEW rat model of chronic renal allograft rejection, blockade of the angiotensin II type 1 receptor (AT₁R) with losartan decreases macrophage infiltration of allografts, prevents glomerulosclerosis and reduces tubulointerstitial injury [108].

REGULATORY MACROPHAGE PREPARATIONS AS THERAPEUTIC AGENTS

Pharmacological manipulation of macrophage activation states *in vivo* is a subtle art: an altogether more direct approach is to isolate monocytes and differentiate them to suppressor macrophages under controlled tissue culture conditions before administering them to patients as cell-based therapies. Cell-based immunomodulatory therapy is still very much in its infancy, although we are now witnessing a concerted international effort to bring such treatments into clinical practice (www.onestudy.org). Our group, at the cutting-edge of this effort, has pioneered the use of regulatory macrophages as a means of promoting allograft acceptance in solid organ transplant recipients. The feasibility of this approach was demonstrated in two clinical trials, the TAIC-I and TAIC-II studies, in which crude cell preparations containing regulatory macrophages were administered to a total of 20 patients [5¹¹,109–112].

TAIC-I and TAIC-II clinical studies

The TAIC-I study (www.clinicaltrials.gov: NCT00223093) conducted by Fändrich and colleagues was designed as a phase I trial to assess the clinical feasibility and tolerability of administering donor-derived M reg-containing cell preparations to deceased-donor renal transplant recipients [109]. Ten patients were recruited to the study and received between 0.5 and 7.5 $\times 10^6$ donor-derived cells/kg, administered by central venous infusion 5 days post-transplant. No acute complications of the infusion and no later adverse reactions relating to the cell infusion were observed. Thus, the TAIC-I trial demonstrated the clinical feasibility of producing and administering suppressor macrophages to kidney transplant patients.

The TAIC-II study (www.clinicaltrials.gov: NCT00223067) aimed to assess the feasibility, safety and immunological effects of administering

donor-derived M reg-containing cell preparations to living-donor renal transplant recipients [110]. Five patients were enrolled in the TAIC-II study and received between 1.7 and 10.4×10^6 cells/kg, 5 days prior to transplantation. Four patients were successfully minimised to low-dose tacrolimus monotherapy. No rejection occurred in two of five patients. One patient underwent a rejection episode at 36 weeks after reduction of tacrolimus treatment to less than 2 ng/ml for 6 weeks. The two remaining patients experienced acute rejection episodes only after complete cessation of immunosuppression for two and 34 weeks. In mixed lymphocyte reactions, all of the patients exhibited attenuated donor-specific responses, but not third-party responses.

Patients MM and CA

Since these early clinical trials, we have arrived at a detailed understanding of the derivation, phenotype and T cell-suppressive functions of in vitro-derived human regulatory macrophages. This knowledge has inspired methodological advances in regulatory macrophage manufacture, leading to a purer and more homogeneous cell product, which has now been applied to two further living-donor renal transplant recipients with encouraging results [5^{***}].

The first of these patients, MM, a 23 year-old female with renal failure owing to IgA nephropathy, was transplanted with a kidney donated by her 58 year-old mother. Donor and recipient had only single HLA-B and DR mismatches. Six days prior to transplantation, 8.0×10^6 donor-derived M regs/kg were administered to MM by slow central venous infusion under cover of 1 mg/kg per day azathioprine. Conventional treatment with steroids and tacrolimus was commenced at the time of transplantation. Azathioprine was discontinued from 8 weeks posttransplant and steroids were tapered to cessation by 14 weeks. Thereafter, MM was maintained on tacrolimus monotherapy with trough levels of less than 6 ng/ml. Protocol biopsies at 8 and 24 weeks showed no signs of rejection. At 3 years, MM was in a stable clinical condition and received tacrolimus 2 mg BD with trough levels of 4–5 ng/ml as her sole maintenance immunosuppression.

The second patient, CA, a 47 year-old man received a fully mismatched kidney from a 40 year-old living, unrelated male donor. CA was treated with 7.1×10^6 donor-derived M regs/kg, 7 days prior to transplantation under cover of 1 mg/kg per day azathioprine. At the time of transplantation, conventional treatment with tacrolimus and steroids was initiated. Protocol biopsies at 8, 24 and 52 weeks showed no signs of rejection. At 3 years, CA had

stable renal function and is now being maintained with sustained-release tacrolimus 5 mg OD with a trough tacrolimus level of 2.7 ng/ml.

A pattern of peripheral blood gene expression associated with a drug-free, tolerant state in renal transplant recipients has been defined by the IOT-RISSET consortium [113]. In order to assess the immunological impact of M reg therapy on patients MM and CA, expression of the ten most discriminatory gene markers of tolerance identified by the IOT-RISSET group was assessed in serial blood samples taken from both patients [5^{***}]. Over the first year posttransplant, the pattern of expression of these markers gradually converged upon the IOT-RISSET signature, suggesting that MM and CA might have achieved a degree of immunological regulation against their donors.

THE FUTURE OF REGULATORY MACROPHAGE THERAPY IS BRIGHT

Macrophages are phenotypically and functionally plastic effector cells with the potential to exacerbate or attenuate allograft pathology. A number of drugs in routine clinical use can profoundly influence macrophage activity, but their actions are not macrophage-specific. Agents in preclinical and clinical development for depleting or modulating the phenotype of particular macrophage populations hold great therapeutic promise; however, targeting drug treatments to the correct phase of allograft injury will not be without its challenges. In principle, ex-vivo production of regulatory macrophages for use as therapeutic agents solves many difficulties of pharmacologically altering macrophages *in vivo*. Early-stage clinical trials of M reg therapy in renal transplantation have proven the feasibility of treating patients with donor-derived M regs and the clinical outcomes have been encouraging. In our opinion, M reg therapy has significant therapeutic potential and warrants further clinical investigation.

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Conflicts of interest

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ARTICLE

In question: the scientific value of preclinical safety pharmacology and toxicology studies with cell-based therapies

Christiane Broichhausen¹, Paloma Riquelme¹, Norbert Ahrens², Anja K Wege³, Gudrun E Koehl¹, Hans J Schlitt¹, Bernhard Banas⁴, Fred Fändrich⁵, Edward K Geissler¹ and James A Hutchinson¹

A new cell-based medicinal product containing human regulatory macrophages, known as Mreg_UKR, has been developed and conforms to expectations of a therapeutic drug. Here, Mreg_UKR was subjected to pharmacokinetic, safety pharmacology, and toxicological testing, which identified no adverse reactions. These results would normally be interpreted as evidence of the probable clinical safety of Mreg_UKR; however, we contend that, owing to their uncertain biological relevance, our data do not fully support this conclusion. This leads us to question whether there is adequate scientific justification for preclinical safety testing of similar novel cell-based medicinal products using animal models. In earlier work, two patients were treated with regulatory macrophages prior to kidney transplantation. In our opinion, the absence of acute or chronic adverse effects in these cases is the most convincing available evidence of the likely safety of Mreg_UKR in future recipients. On this basis, we consider that safety information from previous clinical investigations of related cell products should carry greater weight than preclinical data when evaluating the safety profile of novel cell-based medicinal products. By extension, we argue that omitting extensive preclinical safety studies before conducting small-scale exploratory clinical investigations of novel cell-based medicinal products data may be justifiable in some instances.

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INTRODUCTION

Transferring immunoregulatory cells from a tolerant donor to nontolerant individual as a means of establishing tolerance in the recipient is a common technique in experimental immunology, but its clinical application is only now receiving serious attention.¹ Several classes of immunoregulatory cells are currently being developed as adjunct immunosuppressive agents for use in solid organ transplantation, including several types of regulatory T cells^{2–4} and suppressive myeloid cells.^{5–8} One particularly promising candidate cell type is the human regulatory macrophage.⁹ The regulatory macrophage (M reg) phenotype reflects a unique state of macrophage differentiation, distinguished from macrophages in other activation states by its mode of derivation, robust phenotype, and potent suppressor function.¹⁰ M regs prevent mitogen-stimulated T cell proliferation *in vitro* through IFN- γ -induced indoleamine 2,3-dioxygenase activity, as well as mediating a contact-dependent deletion of activated T cells.¹¹ In addition, M regs drive the development of activated induced regulatory T cells (iTreg) that, in turn, suppress the proliferation of effector T cells and inhibit the maturation of dendritic cells (Walter *et al.* unpublished data). Therefore, it is speculated that when M regs are administered to a transplant recipient, they initiate a feed-forward loop of allospecific regulation.

M reg-containing cell preparations have been administered to a total of 19 kidney transplant recipients in a series of case studies and two early-phase clinical trials.^{12–15} While these pilot studies do not provide conclusive evidence of the safety or efficacy of M reg treatment in renal transplantation, they do demonstrate the feasibility of delivering donor-derived M reg therapy to renal transplant recipients.¹⁶ A further two living-donor kidney transplant recipients have now been treated with $\sim 8.0 \times 10^6$ cells/kg bodyweight (BW) of highly purified donor-derived M regs.¹¹ These patients are now more than 5 years posttransplantation with stable renal function, receiving only low-dose tacrolimus monotherapy as maintenance immunosuppression. A newly developed therapeutic cell product containing M regs (known as Mreg_UKR) conforms to our expectations of a clinically applicable drug product. A further clinical trial of M reg therapy in living-donor renal transplantation is now authorized within the framework of the ONE Study (www.onestudy.org). This trial (ONEMreg12; EudraCT Nr.: 2013-000999-15; ClinicalTrials.gov: NCT02085629) aims to treat 16 patients with donor-derived Mreg_UKR at a dose of 2.5–7.5 $\times 10^6$ /kg BW under cover of 500 mg/day MMF on day 7 prior to surgery.¹⁷

Although cell-based medicines are quite different in nature from chemically synthesized drugs, many of the same general considerations apply to their clinical use. In order to use any therapeutic

¹Division of Experimental Surgery, Department of Surgery, University Hospital Regensburg, Regensburg, Germany; ²Department of Transfusion Medicine, Institute for Clinical Chemistry, University Hospital Regensburg, Regensburg, Germany; ³Department of Obstetrics and Gynaecology, University Hospital Regensburg, Regensburg, Germany; ⁴Nephrology Division, Department of Internal Medicine II, University Hospital Regensburg, Regensburg, Germany; ⁵Institute for Applied Cell Therapy, University Hospital of Schleswig-Holstein, Campus Kiel, Kiel, Germany. Correspondence: JA Hutchinson (james.hutchinson@klinik.uni-regensburg.de)

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agent safely and effectively, clinicians must know about its pharmacological properties and how these predict efficacy and safety in individual patients.¹⁸ Specifically, clinicians must know about the pharmacokinetics (*i.e.*, absorption, tissue distribution, metabolism, and elimination) and therapeutic dose-range of a drug, as well as having an understanding of its mechanism of action and potential adverse effects. These clinical considerations are now reflected in European Law^{19–21} and guidance issued by the European Medicines Agency.²²

Current European Medicines Agency guidelines on cell-based medicinal products (CBMPs) stipulate that novel cell products must be subjected to conventional toxicological and safety pharmacology studies.²³ Toxicology studies are principally concerned with defining the relationship between drug exposure and its adverse effects, usually taking structural changes to tissues upon postmortem examination as their major endpoint. Accordingly, a key objective of toxicological studies is defining the maximum tolerated dose of a drug in single and repeat doses. In contrast, safety pharmacology studies seek to predict whether a drug is likely to be found unsafe when administered to patients at therapeutic doses and thereby aim to prevent such occurrences. Within this remit, safety pharmacology studies try to predict the possible occurrence of rare adverse events.²⁴ In practical terms, this entails showing whether a drug is safe or unsafe using a core battery of pharmacological tests to assess adverse reactions affecting the central nervous, cardiovascular, respiratory, and other organ systems.²⁵ A general requirement for toxicological and safety pharmacology studies in the drug development process are now codified in International Conference on Harmonisation (ICH) guidelines (CPMP/ICH/539/00).²⁶

This article presents the results of preclinical studies into the pharmacokinetics, acute and chronic toxicity, carcinogenicity, and safety pharmacology of Mreg_UKR, which were presented to the German National Competent Authority, the Paul Ehrlich Institute (www.pei.de) as part of a successful application for authorization to conduct the *ONEmreg12* clinical trial. As an academic research group with no prior experience in drug development, we consulted an independent regulatory affairs advisor to devise a preclinical safety testing strategy that complied with all relevant regulatory obligations. This strategy was endorsed by the Paul Ehrlich Institute at a *scientific advice* meeting. On the basis of this advice and our own interpretation of European Medicines Agency guidelines,²³ a clinical trial application was lodged with the competent authority, which incorporated the pharmacokinetic, acute toxicity, chronic toxicity, and carcinogenicity studies presented in this article. This application was initially rejected owing to shortcomings in the clinical protocol and all three principal sections of the Investigational Medicinal Product Dossier. Notably, the Authority commented on the inadequacy of our biochemical and clinical investigations of mice treated with Mreg_UKR; hence, the safety pharmacology studies presented in this article were performed. In response to its critique, a revised clinical trial application was submitted to the Paul Ehrlich Institute and was granted approval.

The preclinical studies described in this article found no evidence of acute or chronic adverse reactions to therapeutic doses of Mreg_UKR; accordingly, they present no impediment to the further development of Mreg_UKR as a pharmaceutical agent. However, this work brings into question the relevance of applying animal-into-animal (homologous) and human-into-animal (heterologous) safety testing strategies to CBMPs. In particular, this article illustrates how easily preclinical pharmacokinetic and safety pharmacology studies could lead to false conclusions about the probable pharmacological

properties of CBMPs in human recipients. Hence, a major conclusion of this work is that previous clinical experience from exploratory trials should be afforded far greater importance in assessing the potential clinical risk profile of Mreg_UKR therapy than preclinical animal experiments. By extension, we argue that there is a case for conducting small-scale exploratory clinical studies of novel CBMPs without extensive preclinical safety investigation, especially when similar CBMPs were already administered patients without adverse effects.

RESULTS

Tissue distribution and survival of Mreg_UKR in NSG mice

The eventual distribution of Mreg_UKR after intravenous infusion reflects their passive and active migration to different sites, their engraftment in those tissues, as well as their death and elimination. To track the survival and tissue distribution of M regs *in vivo*, human cells were injected into NSG mice and their presence in blood, spleen, bone marrow, liver, and lungs was assessed by flow cytometry on days 1–7 postinjection. Recipients were randomized to two treatment groups, which received either 5×10^6 viable M regs or vehicle-only. Prior to detection by flow cytometry, M regs were enriched from dissociated tissues by positive selection of CD11b⁺ cells with magnetic selection beads. Notably, this method of detection gives only qualitative information about the presence or absence of M regs in a tissue. M regs present in mouse tissues were identified by flow cytometry as living human CD45⁺ cells that coexpressed CD11b and HLA-DR. In previous work, we have shown that human M regs are homogeneously CD45⁺ CD11b⁺ HLA-DR⁺ CD14^{-/low} and CD16^{-/low} in phenotype.¹¹ To assess the stability of the M reg_UKR phenotype after administration to mice, expression of CD14 and CD16 by living M regs recovered from mouse tissues was also investigated.

Human M regs were detectable in lung, blood, and liver for up to 7 days postinfusion (Figure 1). It was not possible to reliably detect human M regs at any time point in spleen and bone marrow, either because human M regs were not present or because they were indiscriminable from the large populations of mouse macrophages present in those tissues. M regs retained their CD11b⁺ HLA-DR⁺ CD14^{-/low} phenotype throughout the 7-day observation period. In contrast, M regs upregulated CD16 expression within 1 day of infusion, which possibly reflects the absence of human immunoglobulins in NSG mice.²⁷

Clinical observation of NMRI-nude mice after Mreg_UKR injection

Fifteen age-matched, male NMRI-nude mice were randomized to three treatment groups of five animals. NMRI-nude mice are congenitally athymic, so are effectively T-cell deficient, but produce functionally normal B cells. Hence, NMRI-nude mice were chosen for safety pharmacology studies because they are incapable of T cell-mediated rejection of xenogeneic cells, while still being able to mount innate immune and IgM responses that might contribute to adverse reactions.

Recipient mice were anesthetized and fully anticoagulated with 60 IU heparin prior to slow (30–180 seconds) intravenous injection of M regs via the tail vein. For injection, M regs were suspended in Ringer's lactate solution plus 5% human albumin. Mice in treatment group 1 received 1 ml vehicle-only. Mice in groups 2 and 3 received 10^6 or 10^7 viable M regs suspended in a volume of 1 ml, respectively. These cell doses corresponded to $34.0 \pm 3.1 \times 10^6$ cells/kg BW and $356.8 \pm 31.9 \times 10^6$ cells/kg BW, respectively. Recipient mice

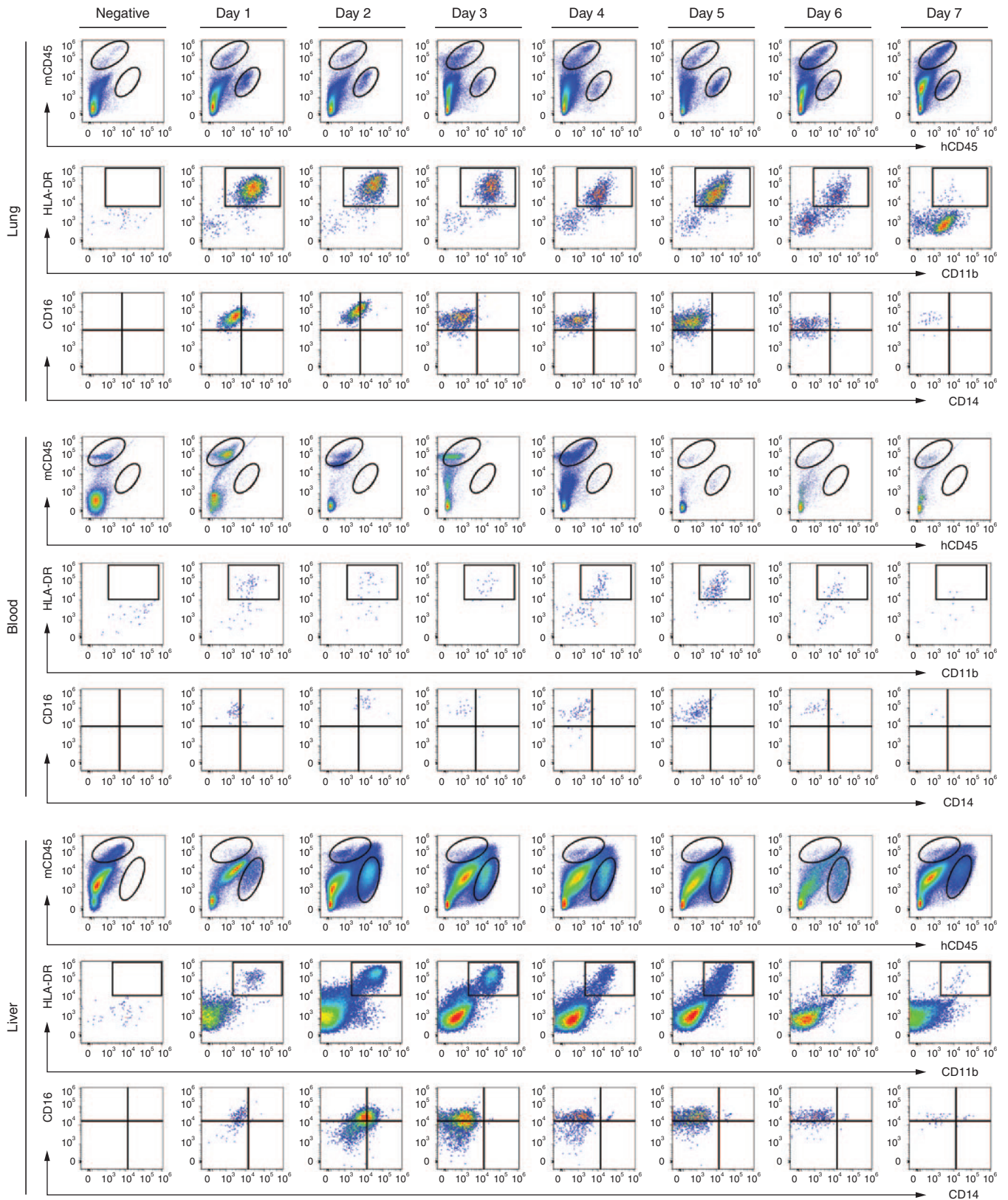


Figure 1 Distribution and fate of Mreg_UKR in NSG mice. Recipient mice were given 5×10^6 viable M regs or vehicle-only by slow intravenous injection. The tissue distribution of M regs was then assessed on days 1 to 7 post-injection by flow cytometry. Human M regs defined by expression of human CD45, CD11b and HLA-DR were detected in lung, blood and liver at all timepoints. Although the engrafted M regs remained CD14⁻, CD16 expression was regained by day 1. Data are representative of at least two animals per timepoint from at least two independent experiments.

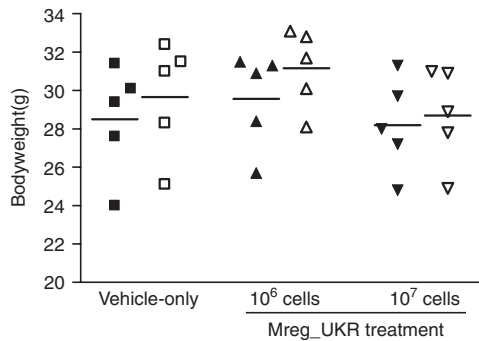


Figure 2 Weight gain in NMRI-nude mice was unaffected by human regulatory macrophages (Mreg_UKR) treatment. NMRI-nude mice were allocated to three groups of five animals. Mice received injections of either 10^6 or 10^7 viable human M regs resuspended in Ringer's lactate solution plus 5% human serum albumin and 60U heparin, or were given a vehicle-only injection. No significant difference in weight gain was observed over a 7-day observation period after Mreg_UKR administration. (Filled symbols indicate bodyweight on day 0; unfilled symbols indicate bodyweights on day 7.)

were closely observed for 3 hours following Mreg_UKR injection to assess their clinical responses, particularly with regard to respiratory rate and rhythm. Over the subsequent 7 days, recipient mice were checked daily for constitutional signs of adverse drug reactions and BW changes were recorded.

No acute adverse reaction to Mreg_UKR doses of 10^6 or 10^7 cells was detected. In particular, no deaths occurred following intravenous cell infusion, no change in respiratory rate or rhythm was detected, and no dyspnea, hemoptysis, or cyanosis was observed. This is perhaps a reassuring result because one theoretical concern with infusion of M regs, which have a diameter of 15–30 μm , is obstruction of pulmonary vessels by single cells or cell aggregates.¹⁸ Recipient mice in all groups recovered from general anesthesia within 30 minutes, and none showed signs of distress upon waking. At 3 hours postinfusion, mice from all treatment groups were normally active, and an abbreviated clinical examination revealed no respiratory or neurological abnormalities. No significant difference in weight gain between treatment groups was observed over the 7-day study (Figure 2). No delayed reactions, as assessed by changes in behavior or constitutional signs, were observed over the 7-day follow-up period, and no deaths occurred.

At 7 days postinfusion, mice in all treatment groups were normally active and showed no grossly unusual behavior. Specifically, recipient mice were examined using an adapted version of Irwin's comprehensive observational assessment, which assesses behavioral, neurological, and autonomic responses to drug treatments.²⁸ No clinically relevant differences in performance between treatment and control groups were detected (Table 1). No signs of dermatological disease were observed, although a few animals in each treatment group bore bite-marks. There was no sign of disturbed bowel function or rectal prolapse in any of the animals. Respiratory rate was not different between the treatment groups and respiratory rhythm was regular in all recipients (Table 1).

Postmortem examination of NMRI-nude mice on day 7 after Mreg_UKR injection

Upon thoracotomy under anesthesia, heart rate was not significantly different between the treatment groups and cardiac contraction was organized and regular in all recipients (data not shown). The lungs appeared pink and uniformly well perfused. No gross pathological

changes were evident in the Mreg_UKR-treated or control mice. Specifically, there was no sign of myocardial infarction or distension of the atria, ventricles or pulmonary arterial trunk in any animals. The abdomen contained no ascites, blood, tumors, or adhesions in any recipients or controls. The large and small intestines, spleen, urinary bladder, kidney, liver, pancreas, kidneys, and great vessels of all animals appeared grossly normal. No other gross abnormalities were noted. Organ weights were not significantly different between Mreg_UKR-treated and untreated recipients (Table 2).

Histological sections of brain, lung, heart, liver, spleen, duodenum, right colon, and kidney were prepared from paraformaldehyde-fixed, paraffin-embedded tissues and stained with hematoxylin and eosin. Tissue sections were evaluated blindly. No microscopic tissue pathology associated with Mreg_UKR administration was observed (Figure 3).

Biochemical investigation of NMRI-nude mice on day 7 after Mreg_UKR injection

Given that Mreg_UKR distributed primarily to liver, recipient mice were investigated for markers of liver injury: serum albumin (Figure 4a) and alkaline phosphatase (Figure 4b) levels were not significantly different between treatment groups; however, a marginal increase in serum aspartate transaminase (AST) was observed in group 3 (Figure 4c). The biological relevance of such a small difference serum AST levels is presently unknown. Notably, among the 21 patients treated with M reg-containing cell products, who are all more than 5 years posttreatment, no incidents of disturbed liver function tests were reported. Serum alkaline phosphatase is also a marker of increased bone resorption and serum albumin levels are typically reduced as part of the acute phase response; therefore, no biochemical evidence was found of increased bone turnover or systemic inflammation caused by Mreg_UKR. To investigate the possibility that Mreg_UKR affect renal function by embolising (in the form of individual cells, cell aggregates, dead cells, or immune complexes) to renal glomeruli, serum creatinine levels were measured as an indicator of filtrative capacity: no differences were observed between treatment groups (Figure 4d). Glucose levels are a sensitive, albeit very unspecific, parameter to screen for adverse drug reactions: Hypoglycemia might result from sepsis, disturbances of the hypothalamic–pituitary–adrenal axis resulting in reduced glucocorticoid production or disturbed insulin production (or IGF-2 production); hyperglycemia may result from pituitary, adrenal, or pancreatic dysfunction or could indicate ischemic disease or infections. No significant changes in glucose levels were observed (Figure 4e).

Immunogenicity of allogeneic mouse M regs in immunocompetent recipients

To formally assess the risk of humoral sensitization by M regs, donor-specific anti-major histocompatibility complex class I antibody responses were measured in BALB/c mice that received C3H cardiac allografts after preoperative treatment with donor strain-derived M regs. As previously published, no accelerated allograft lost was observed in the M reg-treated recipients, indicating that M reg administration on day 8 prior to transplantation did not sensitize recipients.¹⁰ Here, sera were harvested from mice 7 days after heart transplantation, and their alloantibody content was measured by flow cytometry cross-match. Consistent IgG responses were detected in transplanted mice without M reg treatment; in contrast, mice treated with 5×10^6 donor-derived M regs 8 days prior to transplantation had significantly lower levels of antidonor IgG

Table 1 Clinical examination of NSG mice treated with Mreg_UKR

	Vehicle-only	10 ⁶ M regs	10 ⁷ M regs
Behavioral			
Spontaneous activity			
Sleep	0.0±0.0	0.0±0.0	0.0±0.0
Body position	5.2 P0	5.6 P0	5.6 P0
Locomotor activity	2.0±0.0	2.0±0.0	2.8±1.1
Bizarre behavior	0.0±0.0	0.0±0.0	0.0±0.0
Motor-affective response			
Alley progression (cm)	35.5±14.7	43.9±20.5	45.0±22.3
Transfer arousal	4.0±0.0	4.0±0.0	3.2±1.8
Touch-escape	3.6±0.9	4.0±0.0	4.4±0.9
Positional struggle	2.0±0.0	2.0±0.0	2.0±0.0
Grasp irritability	0.8±0.4	0.8±0.4	1.0±0.7
Provoked biting	3.2±1.8	2.4±2.2	2.0±2.0
Provoked freezing	1.0±0.0	0.8±0.4	1.0±0.0
Finger approach	4.8±1.1	5.2±1.1	5.2±1.1
Positional passivity	3.2±1.1	3.6±1.7	2.4±0.9
Vocalization (events)	0.0±0.0	0.6±0.9	0.6±0.9
Urination (events)	0.2±0.4	0.2±0.4	0.2±0.4
Defecation (events)	2.4±2.1	1.0±1.7	4.0±1.9
Sensory-motor response			
Visual placing	6.0±0.0	6.0±0.0	5.6±0.9
Tail-pinch	2.6±2.3	1.0±0.0	2.4±1.5
Toe-pinch reflex	5.6±0.9	5.6±0.9	6.0±0.0
Corneal reflex	5.2±1.1	4.8±1.1	5.6±0.9
Pinna reflex	2.4±0.9	2.4±0.9	3.6±0.9
Startle	2.8±1.1	5.2±1.8	4.0±1.4
Neurological			
Posture			
Pelvic elevation	4.0±0.0	4.4±0.9	4.4±0.9
Tail elevation	2.0±0.0	2.0±0.0	2.0±0.0
Limb rotation	1.0±0.0	1.0±0.0	1.0±0.0
Muscle tone			
Body tone	6.0±0.0	6.0±0.0	6.0±0.0
Abdominal tone	4.0±0.0	4.4±0.9	5.2±1.1
Limb tone	2.0±0.0	2.0±0.0	2.0±0.0
Grip strength	6.0±0.0	5.6±0.9	4.8±0.8
Wire maneuver	0.4±0.9	0.8±1.8	0.4±0.9
Equilibrium and gait			
Righting reflex	0.0±0.0	0.0±0.0	0.0±0.0
Ataxic gait	0.0±0.0	0.0±0.0	0.0±0.0
Hypotonic gait	0.0±0.0	0.0±0.0	0.0±0.0
Other gait impairment	0.0±0.0	0.0±0.0	0.0±0.0
Total gait incapacity	0.0±0.0	0.0±0.0	0.0±0.0

CNS excitation			
Tremors	0.0±0.0	0.0±0.0	0.0±0.0
Twitches	0.0±0.0	0.0±0.0	0.0±0.0
Convulsions	0.0±0.0	0.0±0.0	0.0±0.0
Autonomic			
Eyes			
Palpebral closure	0.0±0.0	0.0±0.0	0.0±0.0
Exophthalmos	0.0±0.0	0.0±0.0	0.0±0.0
Secretion and excretion			
Lacrimation	1.2±2.7	0.0±0.0	0.2±0.4
Salivation	0.0±0.0	0.0±0.0	0.0±0.0
Diarrhea	0.0±0.0	0.0±0.0	0.0±0.0
General			
Hypothermia	0.0±0.0	0.0±0.0	0.0±0.0
Skin color	4.0±0.0	4.0±0.0	4.0±0.0
Resp. rate	6.0±0.0	6.0±0.0	6.0±0.0
Toxicity			
Acute death (events)	0.0±0.0	0.0±0.0	0.0±0.0

NMRI-nude mice were allocated to three groups of five animals. Mice received injections of either 10⁶ or 10⁷ viable human M regs resuspended in Ringer's lactate solution plus 5% human serum albumin and 60 U heparin, or were given a vehicle-only injection. Seven days after Mreg_UKR administration, recipient mice underwent clinical examination to identify possible signs of neurological impairment. No significant differences were found. Values represent mean ± SD.

(Figure 5). No antidonor IgM response was detected in either the control or M reg-treated group (data not shown). Therefore, there is no evidence that intravenous injection of allogeneic mouse M regs caused humoral sensitization.

Chronic toxicity studies in immunodeficient mice

Malignant disease after treatment with Mreg_UKR might, in principle, arise either as consequence of transferring neoplastic cells or as consequence of transferred cells promoting growth of autochthonous tumors.¹⁸ Neoplastic cells within Mreg_UKR products might originate from the donor, arise during *in vitro* culture or emerge after transfer into the recipient. Not only the therapeutically active cells within a cell product may lead to malignant disease but also cellular contaminants pose a risk of malignant transformation. In theory, immunosuppressive cell therapies might also promote recipient malignancies either by facilitating the growth of autochthonous tumors or by suppressing immune responses against cancerous cells.

To formally assess the risk of M regs causing malignancy or other chronic pathologies, conventional carcinogenicity and chronic toxicity studies were performed in immunodeficient mice. The purpose of this GLP-compliant study was to determine the chronic single-dose toxicity and tumorigenicity of M reg-containing cell preparations. Seventy-five male and 75 female C.B-17-scid mice were divided into three experimental groups (Table 3). Mice in group 1 served as vehicle-only controls. Mice in group 2 received M regs at a BW-adjusted dose (5 × 10⁶ cells/ kg BW) corresponding to the intended treatment dose in humans, whereas mice in group 3 received an eightfold excess cell dose (4 × 10⁷ cells/ kg BW). After treatment with M reg-containing

Table 2 Postmortem of NSG mice treated with Mreg_UKR

	Heart (mg)	Lungs (mg)	Liver (mg)	Left kidney (mg)	Spleen (mg)	Brain (mg)	Small intestine (mm)	Large intestine (mm)
Vehicle-only	176.2±39.0	180.2±21.4	1491.0±252.6	229.0±38.5	100.6±27.8	374.2±57.4	455.0±16.9	98.0±6.0
10 ⁶ M regs	186.0±17.1	202.6±15.4	1588.0±81.8	238.2±18.9	91.6±23.2	337.4±47.1	470.3±28.4	98.3±11.3
10 ⁷ M regs	189.0±33.3	173.4±18.9	1508.4±141.1	244.6±35.1	132.2±81.8	369.0±40.8	459.6±12.8	100.4±18.0

NMRI-nude mice were allocated to three groups of five animals. Mice received injections of either 10⁶ or 10⁷ viable human M regs resuspended in Ringer's lactate solution plus 5% human serum albumin and 60U heparin, or were given a vehicle-only injection. Seven days after Mreg_UKR administration, recipient mice were killed and organ weights or sizes were recorded. No significant differences were found. Values represent mean ± SD.

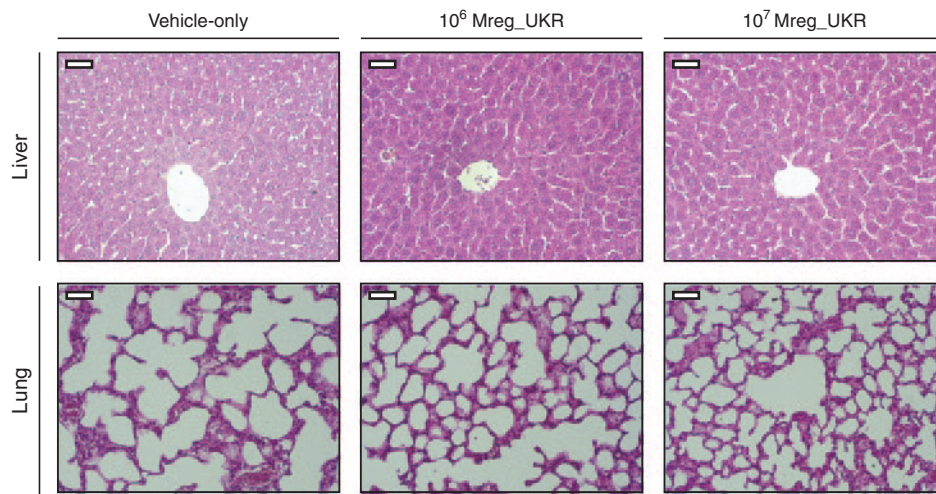


Figure 3 Histopathological survey of tissues from human regulatory macrophages (Mreg_UKR)-treated NMRI-nude mice. NMRI-nude mice were allocated to three groups of five animals. Mice received injections of either 10⁶ or 10⁷ viable human M regs resuspended in Ringer's lactate solution plus 5% human serum albumin and 60U heparin, or were given a vehicle-only injection. Seven days after Mreg_UKR administration, recipient mice were killed and tissues were harvested for histopathological examination. Three micrometer sections were cut from paraformaldehyde-fixed, paraffin-embedded tissues and then stained with hematoxylin and eosin. No tissue pathology associated with Mreg_UKR administration was observed. Representative images of liver and lung, the tissues in which M regs principally accumulate, are shown (Bar = 50 μm).

cell preparations, follow-up observations were made over 295 days. These studies showed no abnormal clinical or pathological findings that could be ascribed to M reg exposure. Specifically, clinical and postmortem examination on day 295 after M reg administration revealed no abnormalities of growth, tumor formation, biochemical or hematological disturbances, or any histopathological changes in any of the organs or tissues examined (data not shown).

DISCUSSION

The manufacture and application of medicinal products is strictly regulated to ensure an appropriate balance of risk and benefit to patients. Under European Union (EU) Law, CBMPs are governed by a legislative framework enacted through EU Regulation 1394/2007/EEC on Advanced Therapy Medicinal Products (ATMPs)¹⁹ and an amendment of Directive 2001/83/EEC on the Community code relating to medicinal products for human use.^{20,21} At once, this legislation both recognizes the inherent difficulties of studying cell-based therapies as pharmacological agents, but also imposes exacting standards for preclinical characterization of cell products, comparable to those applied to conventional pharmaceuticals.²³ Complying with these strict regulatory requirements is challenging, especially for academic centers with limited resources^{29,30}; moreover, the scientific value of the required safety studies is doubtful, as the Committee for Advanced Therapies (CAT) itself recognizes.³¹

Pharmacokinetic and safety pharmacology studies are performed during nonclinical drug development to assess drug exposure and to identify any possible unwanted drug effects, including rare adverse reactions. Information from such studies is then used to predict safe drug doses for early-phase trials in humans. However, as the results presented in this article illustrate, it is questionable whether preclinical safety testing in animals is a meaningful way of investigating immunologically active CBMPs. A core problem is one of interspecies incompatibility: either a cell product of human origin is tested in animals, which may lack biological relevance, or an analogous animal cell is tested, which does not give direct evidence about the safety of the human cell product. This article highlights the problems of applying animal-into-animal (homologous) and human-into-animal (heterologous) safety testing strategies to CBMPs. In our opinion, preclinical safety testing in animal models provides such poor-quality information that it is largely unhelpful in judging the probable safety profile of CBMPs in patients. Specifically, our confidence in the safety of administering Mreg_UKR to humans is not greatly increased by the safety studies presented here, or by previous studies in mice¹⁰ and miniature swine,³² despite no adverse effects having been identified. Accordingly, we argue that far greater emphasis should be placed on previous clinical experience with identical and closely related cell products when assessing probable clinical safety of novel CBMPs.

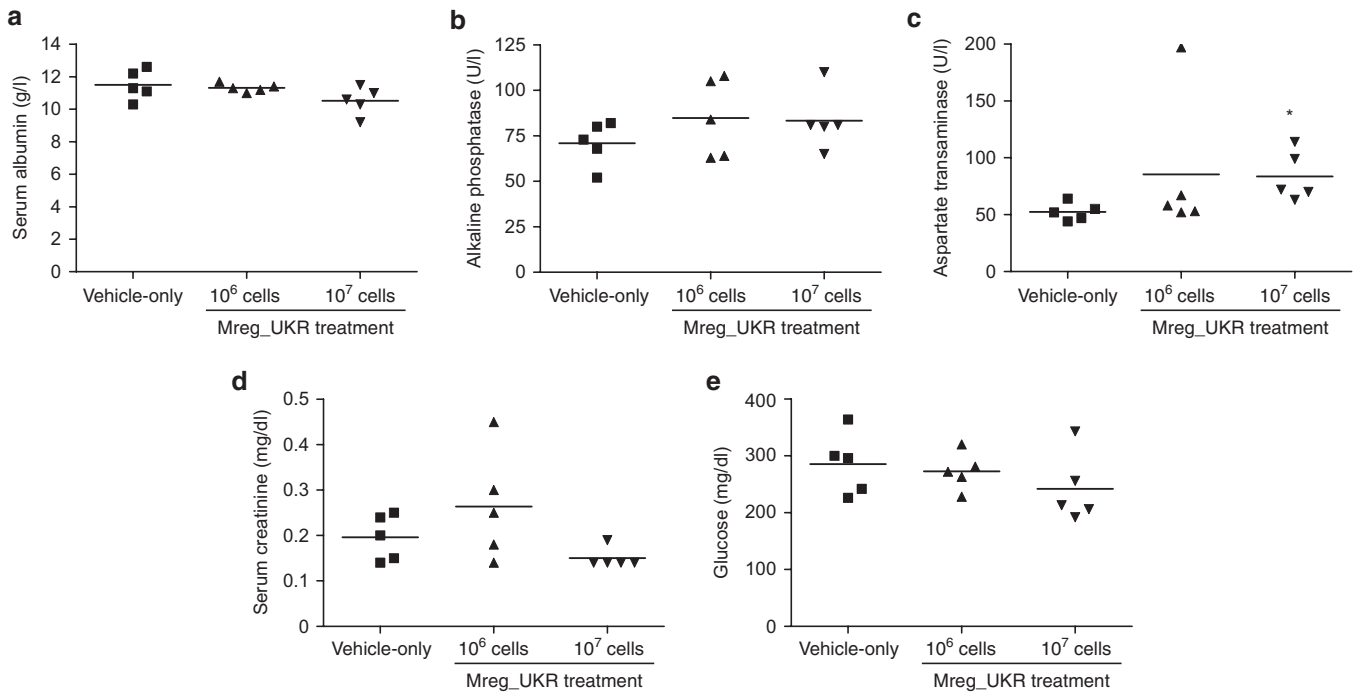


Figure 4 Biochemical investigation of NMRI-nude mice after human regulatory macrophage (Mreg_UKR) treatment. NMRI-nude mice were allocated to three groups of five animals. Mice received injections of either 10^6 or 10^7 viable human M regs resuspended in Ringer's lactate solution plus 5% human serum albumin and 60U heparin, or were given a vehicle-only injection. Seven days after Mreg_UKR administration, recipient mice were killed and serum levels of albumin, alkaline phosphatase, aspartate transaminase, creatinine and glucose were investigated. No significant differences were observed between treatment groups, except for a marginal increase in aspartate transaminase levels in mice treated with 10^7 M regs (Kruksall-Wallis test; 10^7 Mreg_UKR versus vehicle-only, $*P = 0.036$).

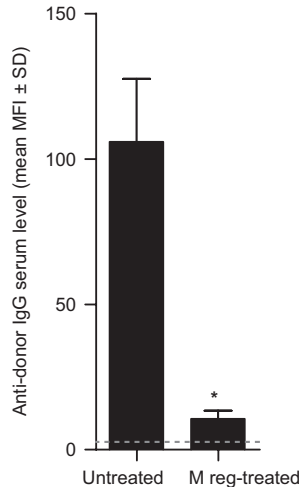


Figure 5 Assessing the immunogenicity of allogeneic mouse M regs in mice. BALB/c mice were either treated with 5×10^6 C3H-derived M regs ($n = 4$) or received no cells ($n = 3$). Eight days later, all mice were given a heterotopic heart transplant from a C3H donor. None of the transplants failed before day 7. On day 7, sera were harvested from all mice for measurement of antidonor IgG and IgM antibody titers by flow cytometry crossmatch. Mice treated with M regs 8 days before transplantation registered significantly lower levels of antidonor IgG than untreated controls (Mann-Whitney *U*-test; $*P < 0.001$). Gray line indicates limit of detection..

What can be concluded from the absence of adverse reactions in animals?

Few immunologists would contend that animal experiments are not valuable in proof-of-principle demonstrations of the efficacy of new

immunotherapies. Why then should we be critical about the value of safety pharmacology and toxicology studies, which use very similar models and techniques as those used for primary and secondary pharmacodynamic studies? One reason is that pharmacodynamic studies aim to detect *particular* biological effects that, in order to be regarded as therapeutically promising, should be relatively large and accrue to all recipients; by contrast, safety pharmacology studies aim to detect *any* adverse biological effects, which may be relatively small or restricted to only a subset of recipients. Self-evidently, proving the absence of detrimental effects requires more sensitive technical and statistical approaches than proving the presence of a beneficial effect.

Before examining the particular case of Mreg_UKR, it is useful to examine the logic of safety testing in animals. In general, it is argued that if a drug has an adverse effect in animal models then it is highly likely to elicit the same adverse reaction in patients; by extension, if a drug does not cause a given adverse reaction in animals, then it is correspondingly unlikely to elicit that reaction in humans. Clearly, this form of analogical reasoning hinges on the *biological relevance* of the animal model to the human system. In the case of chemically synthesized, small-molecule drugs acting at defined pharmacological targets, it may be uncontroversial to accept that its properties in animals are a *correct analogy* for its actions in humans; however, in the case of immunologically active CBMPs, this is often not obviously true. Human and mouse M regs are derived by analogous processes, express very similar phenotypes, and suppress effector T cell function; however, human and mouse M regs are not absolutely alike in phenotype and, whereas iNOS is indispensable for mouse M reg-mediated suppression of T-cell proliferation, it has no proven role in human M reg-mediated suppression.^{10,11} Thus, mouse and human M regs are equivalent cell types, but are not absolutely identical; it follows, for every

Table 3 Chronic toxicity studies of M reg-containing cell preparations in C.B-17-scid mice

Group	Cohort	Cell dose (cells/kg)	N	Cell density (cells/100 μ l)	Administration		Cotreatment	Mortality		
					(ml/kg)	Route		♂	♀	Total
1	1	0	13 ♂ 13 ♀	—	5	i.v.	None	4/13	9/13	13/26
	2	0	12 ♂ 12 ♀	—	5	i.v.	None	11/12	7/12	18/24
2	1	5×10^6	13 ♂ 13 ♀	1×10^5	5	i.v.	None	2/13	6/13	8/26
	2	5×10^6	12 ♂ 12 ♀	1×10^5	5	i.v.	None	11/12	3/12	14/24
3	1	4×10^7	13 ♂ 13 ♀	8×10^5	5	i.v.	None	4/13	8/13	12/26
	2	4×10^7	12 ♂ 12 ♀	8×10^5	5	i.v.	None	10/12	7/12	17/24

i.v. intravenously.

Seventy-five males and 75 females C.B-17-scid mice were divided into three experimental groups. Mice in group 1 served as vehicle-only controls. Mice in Group 2 received a bodyweight-adjusted dose of 5×10^6 cells/kg. Mice in group 3 received 4×10^7 cells/kg. After treatment with M reg-containing cell preparations, follow-up observations were made over 295 days.

pharmacological property studied using mouse M regs, it must be shown that human M regs possess a truly analogous property. Likewise, tolerogenic dendritic cell (DC), regulatory T cells and mesenchymal stem cells from mice and humans are divergent in phenotype and effector mechanisms, so the same argument could apply to safety testing of all these cell types.

Another way of interpreting preclinical safety studies is to regard them as a means for drug developers to screen-out potentially harmful cell products at a relatively early stage. This is a pragmatic approach, which concerns itself only with positive evidence of adverse reactions that lead to the conclusion that a product is *likely to be unsafe* in humans. If this is the purpose of preclinical safety studies, then it is crucial to recognize that finding a product is “not unsafe” is not the same as saying that it is “safe”; importantly, it follows that it is not valid to claim that screening for unsafe cell products increases the probable clinical safety of administering cell products found to be “not unsafe” to patients.

It is perhaps counterintuitive to think that extensive preclinical safety testing might not actually increase our confidence in the likely clinical safety of a cell product, but the conclusion can be proven by example. It is striking to note that mouse-into-mouse or human-into-mouse safety preclinical studies would not identify life-threatening acute hemolytic reactions as a consequence of ABO incompatible transfusion of erythrocytes.^{33,34} Similarly, in the field of adoptive transfer of antigen-specific T cells as a cancer therapy, there are many examples of “on-target, off-tumor” adverse effects, especially ocular and central nervous system (CNS) autoimmune reactions, that were not detectable in mice, but caused very serious complications in patients.³⁵ Also, in the field of embryonic stem cell transplantation, several groups have produced neural, neuronal or glial progenitors from human embryonic stem cells that were not tumor-forming in animals,³⁶ but gave rise to multifocal brain tumors in humans.³⁷ These three cases illustrate general reasons for unreliable safety conclusions from preclinical testing, which are: reactions

caused by antigens unique to human cell products; reactions caused by antigens unique to human recipients; and, reactions caused by the failure of human tumors to properly engraft in animals. To this general list, we might also add insidious adverse reactions (*e.g.*, immune complex deposition or fibrotic diseases) that may not present within a conventional 18-month toxicology study,³⁸ as well as infectious diseases that cannot be transmitted to rodents.

The EMA committee for human medicinal products’ guidelines (CHMP/410869/2006) on human cell-based medicinal products advocates a risk-based approach to safety pharmacology and toxicology studies.³⁹ The risk-based approach demands a focused investigation of possible adverse reactions predicted from the known pharmacodynamic and pharmacokinetic properties of that drug. For the most part, immunoregulatory cell types used as CBMPs are naturally occurring components of the immune system; therefore, possible adverse reactions elicited by such cells are predictable because they primarily relate to excessive immunological activity, triggering of unwanted immune responses, an abnormal distribution of cells, or dysregulated cell growth. A detailed risk assessment of Mreg_UKR administration to living-donor kidney transplant recipients has been published elsewhere.¹⁸ On the basis of this risk assessment, preclinical studies with Mreg_UKR concentrated upon the risk of pulmonary embolic disease and whether M regs cause nonspecific tissue injury at sites of accumulation. No clinically relevant detrimental effect was observed when Mreg_UKR were administered intravenously to NSG mice, either at therapeutic or supra-therapeutic doses. Specifically, the cell infusion had no apparent impact on respiratory, cardiac, renal, or neurological function. No gross or microscopic pathology was observed as a result of M reg administration, either at 7 or 295 days postinjection. On the basis of these negative results, there are no safety grounds for terminating development of Mreg_UKR as a CBMP; however, we regard this as a very weak conclusion with no definite implications for the management of patients receiving Mreg_UKR therapy.

So, what useful information can be drawn from safety pharmacology and toxicology studies of CBMPs? To answer this question, we have to establish which properties of our animal models represent correct analogies to the human condition: We are only entitled to draw safety conclusions regarding adverse species-nonspecific effects to which animals and humans are equally susceptible. As already mentioned, the major unwanted secondary pharmacodynamic effects and toxicities of immunologically active CBMPs are most likely to result from their influence over recipient immune responses; unfortunately, it is precisely these complicated and specific immunological interactions that are poorly modeled in animals.^{40–42} On the contrary, adverse reactions that affect systems that are highly conserved between animals and man can be usefully studied in animal models. Pulmonary embolism (PE) of Mreg_UKR is one such example of an analogous adverse reaction, since the diameter of pulmonary vessels and BW-adjusted pulmonary vessel numbers are very similar in all mammals. Accordingly, we can be somewhat reassured by the absence of PE in experimental mice, which implies that PE caused by Mreg_UKR in humans is unlikely at equivalent cell doses.¹⁸

How should the potential immunogenicity of CBMPs be assessed? In the context of solid organ transplantation, treatment with donor-derived M regs has a superior allograft-protective effect compared to recipient-derived M regs.¹⁰ However, sensitization is an inherent risk of administering allogeneic cells to a patient, which in solid organ transplant recipients could lead to accelerated transplant rejection.⁴³ ICH S6 recommendations on the preclinical safety evaluation of biotechnology-derived pharmaceuticals recognize the limitations of studying the immunogenicity of biopharmaceuticals intended for human use in animals.⁴⁴ Specifically, these guidelines acknowledge that induction of an antibody response in animals is not predictive of antibody formation in humans. In our experiments, it would clearly have been meaningless to assess the immunogenicity of human M regs in immunocompetent or immunodeficient mice; therefore, we investigated the potential of allogeneic mouse M regs to exacerbate or attenuate antibody responses in mice receiving an allogeneic heart transplant (*i.e.*, a homologous test system). These experiments showed that pretransplant M reg treatment significantly diminished humoral responses against allogeneic cardiac allografts, presumably by suppressing T-cell responses. Nonetheless, the conclusion that allogeneic M reg exposure does not normally elicit alloantibodies in mice cannot be neatly extrapolated to the human situation. Mouse and human M regs, although equivalent cell types, are not absolutely identical in phenotype, so may be differently immunogenic. Additionally, there may be preparation-related factors (*e.g.*, dead cell content or manufacturing process-related contaminants) or recipient-related factors (*e.g.*, concurrent inflammation or donor–recipient HLA-mismatches) that influence the immunogenicity of infused M regs in patients. Overall, these experiments provided no evidence that administering Mreg_UKR to patients is likely to cause humoral sensitization; however, they do not provide strong support for the conclusion that Mreg_UKR are unlikely to cause sensitization in patients.

ICH S6 recommendations on the preclinical safety evaluation of biotechnology-derived pharmaceuticals advise that toxicity studies in nonrelevant species (*i.e.*, species in which the test substance is not pharmacologically active) may be misleading and are discouraged.⁴⁴ Therefore, we are bound to ask whether our choice of animal models affected the strength of safety conclusions drawn about Mreg_UKR. Specifically, would testing Mreg_UKR in large

animal models have provided better evidence of safety? It is fair to assume that human M regs should be more similar to M regs from species with a closer evolutionary relationship to humans than M regs derived from more distantly related species. On this basis, one might expect safety testing of Mreg_UKR in large animals to be generally more informative than rodent experiments. However, experimental group sizes needed to detect clinically relevant adverse effects are often impractical in relevant large animal models. This is certainly true when considering the risk of sensitization: Historical rates of sensitization of patients receiving donor-specific blood transfusion prior to kidney transplantation under cover of azathioprine were 7–16%; therefore, it would necessary to observe 18–42 animals to have a 95% probability of detecting one or more sensitization event. Thus, while large animal experiments certainly provide the most convincing evidence of the efficacy of novel CBMPs, their actual value in toxicological and safety pharmacology studies is much less certain.

What can be learnt from pharmacokinetic studies in animals?

The EMA committee for human medicinal products' guidelines on human CBMPs recognize that conventional absorption, distribution, metabolism, and excretion studies are not usually relevant to CBMPs.²² However, these same guidelines mandate that pharmacokinetic studies of CBMPs should be carried out to demonstrate tissue distribution, viability, trafficking, growth, phenotype, and any alteration in phenotype due to factors in the tissue environment. After intravenous administration to NSG mice, human M regs partitioned to the lungs, blood, and liver, where a detectable fraction persisted up to 7 days. M regs were not found in spleen or bone marrow, either owing to technical limitations in their detection or because the cells were absent from those tissues.

Tellingly, the distribution of human M regs in NSG mice is not consistent with the distribution of human M regs in humans, since in previous work, we reported that ¹¹¹In-labeled M regs migrated via the blood from the lung to the liver, spleen, and bone marrow. Clearly, studying the distribution and survival of human M regs in NSG mice is unhelpful in predicting the pharmacokinetics of human M regs in patients. There may be many reasons for the discrepant behavior of human M regs in mouse and man, including species-specific differences in soluble mediators and adhesion molecules. Notably, when transferred into mice, human M regs are deprived of tonic M-CSF-stimulation, which is vital for macrophage survival *in vivo*; hence, the lifespan of human M regs in patients may be underestimated from this mouse model.⁴⁵ On the contrary, because NSG mice lack any effective means of rejecting M regs, the NSG mouse model may overestimate the lifespan of allogeneic M regs in an immunocompetent human recipient. The possible use of more sophisticated animal models, such as transgenic mice that better support engraftment of human M regs, does not address the fundamental problem that, without first characterizing the pharmacokinetics of human cells in human recipients, we cannot know whether their distribution and survival in a mouse is an accurate representation. The corollary of not knowing whether human M reg survival in immunodeficient mice is representative of their survival in humans is that only a weak interpretation of the absence of chronic adverse effects observed in our chronic toxicity and tumorigenicity study can be given.

Are there alternatives to safety pharmacology and toxicology studies in animals?

If, owing to interspecies differences, pharmacological and toxicological studies with mouse or human M regs are liable to produce

unreliable safety conclusions, how could the application of Mreg_UKR to patients ever be justified? Elsewhere we have argued that the likely clinical complications of administering cell products to patients are predictable and mitigable.¹⁸ There is now a substantial literature concerning the administration of therapeutic cell preparations (including regulatory T cells,⁴⁶ tolerogenic DCs,⁴⁷ M regs,⁸ and mesenchymal stem cells)⁴⁸ to patients for a variety of indications. In our opinion, these clinical experiences constitute a much more meaningful basis for assessing the safety profile of other novel cell-based therapies than safety pharmacology and toxicology studies in animals. Explicitly stated, any pharmacological differences between alternative preparations of the same immunoregulatory cell type are generally less important, at least from a safety perspective, than the pharmacological differences between the same immunoregulatory cell type from humans and animals. Therefore, greater confidence in the likely clinical safety of a novel immunoregulatory cell product can be taken from previous studies with similar preparations of the same class of immunoregulatory cell type in humans than from preclinical testing in animals. By extension, we argue that previous clinical experience with immunoregulatory CBMPs is a reliable basis for predicting safe doses of similar CBMPs; furthermore, patient safety information obtained about an immunoregulatory CBMP in one clinical indication is arguably a reliable basis for judging its likely safety profile in other indications. The obvious conclusion of this argument is that the growing body of clinical safety data relating to immunoregulatory CBMPs largely obviates the need for further safety testing of existing and new CBMPs in animal models.

In a recent article, members of the Paul Ehrlich Institute advocated two possible ways to circumvent the centralized European Marketing Authorization procedure pertaining to ATMPs.⁴⁹ In the first case, they suggest that some ATMPs could be reclassified as transplants or transfusion products, which are less stringently regulated under the Tissues and Cells Directive (Directive 2004/23/EC).⁵⁰ Specifically, cell products which have not undergone substantial manipulations and are intended for homologous use are contenders for classification as non-ATMPs. In the second case, they advocate the use of the Hospital Exemption Rule, a provision made under Article 28 of the EU Regulation on ATMPs for products prepared on a nonroutine basis for a specified patient to be excluded from the central authorization requirements for ATMPs.¹⁹ Notably, the Hospital Exemption Rule can be used by National Authorities as a regulatory tool for supporting the development and availability of eligible ATMPs, perhaps guiding a particular product into routine manufacturing and then later into central marketing authorization.⁴⁹

In summary, this article presents three lines of argument against the need for further preclinical safety studies of CBMPs in animal models. Firstly, safety pharmacology and toxicity testing of immunologically active CBMPs in heterologous or homologous animal models is questionably relevant to humans and may lead to misleading safety conclusions. Secondly, past clinical experiences with CBMPs are more informative about the probable safety of similar CBMPs in patients than preclinical safety testing in animal models. Thirdly, the burden of conducting preclinical pharmacokinetic, safety pharmacology, and toxicology studies may be great enough to deter development of novel CBMPs. Accordingly, we take a nuanced view of preclinical safety testing of CBMPs in animals: On the one hand, focused investigation of particular adverse reactions in genuinely analogous systems is valuable and, perhaps, there is a case for screening studies with truly novel cell products; on the other hand, it is evident that preclinical safety studies do not generally increase our confidence in the likely safety of CBMPs when

administered to patients. Therefore, we contend that there is an urgent need for a debate about the acceptability of trialing novel CBMPs in small-scale exploratory clinical studies with only minimal preclinical safety data, especially when similar CBMPs have been previously applied to patients without adverse effects.

MATERIALS AND METHODS

Manufacture of Mreg_UKR

Leucapheresis products used as starting material for Mreg_UKR generation were produced under a manufacturing license issued by the *Regierung von Oberbayern* (DE_BY_04_MIA_2013_0177/53.2 – ZAB – 2677.1 204). Mreg_UKR were produced under a separate manufacturing license (DE_BY_04_MIA_2013_0187/53.2–2677.1 A 220-O) by Apceth GmbH (Ottobrunn, Germany) according to a proprietary standard operating procedure, which was adapted from previously published protocols.⁵¹

Administration of Mreg_UKR to immunodeficient mice

Animal experiments were performed in accordance with permission Nr. 54-2532.1-10/12 granted by the *Regierung von Oberbayern*. NMRI-nude (NMRI-Foxn1^{nu}) mice were obtained from Charles River (Sulzfeld, Germany) and NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl/SzJ}) mice were bred in-house. Animals were kept in individually ventilated cages and fed a conventional diet. Recipient mice were anesthetized during Mreg_UKR infusion using 3.6 mg Xylazine plus 27.3 mg ketamine in 1000 μ l 0.9% NaCl, given by intraperitoneal injection at 40 μ l per 10 g BW. Immediately prior to injection, the concentration of M regs in Mreg_UKR products was adjusted to 10⁶ or 10⁷ viable cells/ml in Ringer's lactate solution plus 5% human serum albumin. Cell suspensions were injected through a 27-gauge needle into the tail vein of recipient mice over 30–180 seconds.

Clinical examination of Mreg_UKR recipients

Recipient mice were observed over 7 days for signs of adverse reactions to Mreg_UKR. Changes in BW were monitored. Behavioral, neurological, and autonomic responses to recipient mice were examined using an adaptive version of Irwin's comprehensive observational assessment.²⁸

Postmortem investigation of NMRI-mice after Mreg_UKR exposure

Recipient mice were killed on day 7 by thoracotomy and exsanguination under anesthesia. Organs were removed, weighed, and prepared for histology. Three micrometer histological sections were cut from paraffin-embedded tissues and stained with hematoxylin and eosin according to standard protocols. Sera were sent for analysis by the Institute of Clinical Chemistry at University Hospital Regensburg using routine diagnostic assays.

Flow cytometry to detect M regs in tissues from NSG mice

Recipient mice were killed on days 1 to 7 after M reg injection. Human leucocytes were recovered from tissues by physical (spleen and bone marrow) or enzymatic digestion (lung and liver) according to previously described methods.¹⁰ Single-cell suspensions were passed through a 40- μ m mesh (BD Biosciences, Heidelberg, Germany) before enrichment of CD11b⁺ leucocytes by positive human/mouse CD11b magnetic bead selection (Miltenyi, Bergisch-Gladbach, Germany) on an AutoMACS Pro device (Miltenyi).

Assessing the immunogenicity of mouse M regs in a cardiac transplant model

Abdominal heterotopic heart transplants from C3H donors into BALB/c recipients were performed as previously described in accordance with permission Nr. 54-2532.1-28/09.¹⁰ Graft rejection was defined as cessation of palpable cardiac contractions with verification by direct inspection of the allograft after laparotomy. Mouse M regs were generated as previously described. Recipient mice either received no additional treatment or received 5 \times 10⁶ donor-derived M regs on day 8 prior to transplantation. M regs were resuspended in 1 ml phosphate-buffered saline containing 62U heparin and administered by slow injection into the tail vein. Sera from all mice were harvested on day 7 posttransplant, and alloantibody levels were measured by flow cytometry cross-match. Briefly, C3H splenocytes were stimulated in overnight

culture with concanavalin A. Aliquots of 0.5×10^6 stimulated splenocytes were blocked with mouse FcR blocking reagent (Miltenyi) before incubation for 90 minutes on ice with 50 μ l of test serum diluted by 1:500 in Dulbecco's modified phosphate-buffered saline. Nonimmune sera from naive BALB/c mice were used as a negative control. After incubation, splenocytes were stained with antimouse IgG-FITC, antimouse IgM-APC, and anti-CD3-PE (antibodies from eBioscience, Frankfurt, Germany). For analysis, the CD3⁺ T cell population was gated, and geometric mean fluorescence intensity was determined (FlowJo v7.6.5, Miltenyi).

Chronic toxicity studies of M reg-containing cell preparations in C.B-17-scid mice

Responsibility for conducting good laboratory practice-compliant chronic toxicity studies was outsourced to a contract research organization (Aurigon Life Science, Gräfelting, Germany).

Statistics

Statistical analyses and curve fitting were performed with GraphPad software (La Jolla, CA). Values given in histograms and tables represent mean \pm SD. The Mann-Whitney *U*-test was used for nonparametric comparisons between two groups. The Kruskal-Wallis test was used for nonparametric comparisons among three groups.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Cutting Edge: Immunological Consequences and Trafficking of Human Regulatory Macrophages Administered to Renal Transplant Recipients

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Cutting Edge: Immunological Consequences and Trafficking of Human Regulatory Macrophages Administered to Renal Transplant Recipients

James A. Hutchinson,^{*,†} Paloma Riquelme,^{*,†} Birgit Sawitzki,^{†,‡} Stefan Tomiuk,^{†,§} Patrick Miqueu,^{†,¶} Maaz Zuhayra,^{||} Hans H. Oberg,[#] Andreas Pascher,^{**} Ulf Lützen,^{||} Uwe Janßen,^{†,§} Christiane Broichhausen,^{*} Lutz Renders,^{††} Friedrich Thaiss,^{‡‡} Ernst Scheuermann,^{§§} Eberhard Henze,^{||} Hans-Dieter Volk,^{†,‡} Lucienne Chatenoud,^{†,¶¶} Robert I. Lechler,^{†,|||} Kathryn J. Wood,^{†,##} Dieter Kabelitz,[#] Hans J. Schlitt,^{*} Edward K. Geissler,^{*,†} and Fred Fändrich^{†,***}

Regulatory macrophages (M regs) were administered to two living-donor renal transplant recipients. Both patients were minimized to low-dose tacrolimus monotherapy within 24 wk of transplantation and subsequently maintained excellent graft function. After central venous administration, most M regs remained viable and were seen to traffic from the pulmonary vasculature via the blood to liver, spleen, and bone marrow. By 1 y posttransplantation, both patients displayed patterns of peripheral blood gene expression converging upon the IOT-RASET signature. Furthermore, both patients maintained levels of peripheral blood *FOXP3* and *TOAG-1* mRNA expression within the range consistent with nonrejection. It is concluded that M regs warrant further study as a potential immune-conditioning therapy for use in solid-organ transplantation. The results of this work are being used to inform the design of The ONE Study, a multinational clinical trial of immunomodulatory cell therapy in renal transplantation. *The Journal of Immunology*, 2011, 187: 000–000.

Conditioning the response of organ transplant recipients to donor alloantigen using cell-based therapies is now becoming a clinically feasible strategy, and, as the potential risks are better understood and minimized, such

approaches are gaining credibility. Compared to longer established techniques, such as donor-specific blood transfusion and bone marrow transplantation, newer methods involving the ex vivo induction, expansion, and purification of tolerance-promoting cells offer the substantial advantages that the quality and dose of cell products can be tightly controlled. Moreover, by expansion or induction of cells in culture and by specifically purifying tolerogenic cell types, the number of viable suppressor cells administered to the patient can be maximized, and the dangers of sensitization and graft-versus-host reactions may be reduced.

Efforts in our laboratory to develop a cell product for promoting transplant tolerance in the clinical setting have focused on a type of suppressor macrophage, the human regulatory macrophage (M reg) (1–7). M regs exhibit a number of properties that might make them particularly suitable for clinical purposes, in particular, the cells are fully differentiated and potently T cell suppressive (8). M regs derive from CD14⁺ peripheral blood monocytes in the absence of other cell types when monocytes are cultured for 6 d in medium supplemented with human AB serum before stimulation with IFN- γ for a further 24 h. Cell populations generated in this manner are homogeneously CD14^{-/low}HLA-DR⁺CD80^{-/low}CD86⁺CD16⁻CD64⁺TLR2⁻TLR4⁻ and CD163^{-/low} (8).

*Laboratory for Transplantation Research, Department of Surgery, University Hospital Regensburg, Regensburg 93053, Germany; †The ONE Study Consortium of the European Union Seventh Framework Programme; ‡Institute for Medical Immunology, Berlin Charité University Hospital, Berlin 10117, Germany; §Miltenyi Biotec GmbH, Bergisch Gladbach 51429, Germany; ¶TeLand Expression, Nantes 44200, France; ||Department of Nuclear Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Kiel 24105, Germany; ††Institute for Immunology, University Hospital Schleswig-Holstein, Campus Kiel, Kiel 24105, Germany; **Department of Visceral and Transplantation Surgery, Charité University Medicine, Campus Virchow, Berlin 13353, Germany; †††Division of Nephrology, Department of Medicine, University Hospital Schleswig-Holstein, Campus Kiel, Kiel 24105, Germany; ††††Division of Nephrology, Department of Medicine, University Hospital Hamburg-Eppendorf, Hamburg 20246, Germany; †††††Division of Nephrology, Department of Medicine, University Hospital Frankfurt am Main, Frankfurt am Main 60590, Germany; ††††††Laboratory of Immunobiology, René Descartes University, Paris 75743, France; †††††††Medical Research Council Center for Transplantation, King's College London, London WC2R 2LS, United Kingdom; ††††††††Nuffield Department of Surgery, University of Oxford, Oxford OX3 9DU, United Kingdom; and †††††††††Clinic for Applied Cell Therapy, University Hospital Schleswig-Holstein, Campus Kiel, Kiel 24105, Germany

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The sequences presented in this article have been submitted to the National Center for Biotechnology Information/Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE24172.

Address correspondence and reprint requests to Dr. James A. Hutchinson, Department of Surgery, University Hospital Regensburg, Franz-Josef-Strauß-Allee-11, Regensburg 93053, Germany. E-mail address: james.hutchinson@klinik.uni-regensburg.de

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Abbreviations used in this article: 1-D/L-MT, 1-methyl-D/L-tryptophan; IOT, Indices of Tolerance; M reg, regulatory macrophage; RASET, Reprogramming the Immune System for Establishment of Tolerance; SPECT, single photon emission computed tomography.

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This report describes the treatment of two living-donor renal transplant recipients with preoperative infusions of donor-derived M regs. Both patients were successfully weaned to low-dose tacrolimus monotherapy within 24 wk of transplantation and remain in excellent clinical condition. Throughout their follow-up, the patients were intensively monitored using cutting-edge immunomonitoring assays provided by the European Union Framework Program 6 Reprogramming the Immune System for the Establishment of Tolerance (RISET) consortium, including flow cytometry, serial gene expression profiling, and analysis of TCR V β usage by peripheral blood T cells. The two patients are now 3 y posttransplantation with stable graft function and receive minimal maintenance immunosuppression.

Materials and Methods

Ethics

In a modification of the TAIC-II trial protocol (<http://www.clinicaltrials.gov>, NCT00223067), two patients were treated with M reg therapy at the discretion of two senior consultants as "individueller heilversuch" (individual healing attempts). The patients and their donors gave full, informed, written consent to the procedure and follow-up investigations. Human leukocytes for experimental use were obtained with approval of the local ethics committee (ethics vote 09/100) and informed consent of the donors.

In vitro characterization of human M regs

M regs were prepared according to published methods (1). For flow cytometry, harvested M regs were resuspended in ice-cold staining buffer (Dulbecco's modified PBS, 1% BSA, and 0.1% NaN₃) and blocked for 30 min with 10% FcR block (Miltenyi Biotec) before staining with fluorochrome-conjugated primary Abs for 1 h. 7-aminoactinomycin D exclusion was used for live/dead discrimination. To assess the T cell-suppressive capacity of M regs, CFSE-labeled CD3⁺ T cells and M regs were set in direct coculture for 5 d. Subsequently, T cell proliferation and absolute numbers were assessed by flow cytometry, as described elsewhere (1). The mechanism of M reg-mediated T cell suppression was investigated in direct 1:1 M reg/T cell cocultures. 1-methyl-D/L-tryptophan (1-D/L-MT; Sigma-Aldrich), an inhibitor of IDO, was completely dissolved in 5N HCl with gentle heating and agitation, before adjusting the solution to pH 7.2.

Production of M regs for infusion into patients

M regs for administration to patients were prepared under strict GMP conditions according to an adaptation of a previously published method (1). Briefly, donor PBMC were obtained by leucapheresis and Ficoll density gradient separation. Plastic-adherent PBMC were plated at a density of 35×10^6 monocytes/175 cm² culture flask (Cell+ T175 flask; Sarstedt) in 30 ml RPMI 1640-based medium without phenol red (Lonza) supplemented with 10% human AB serum (Lonza), 2 mM L-glutamine (Lonza), 100 U/ml penicillin, 10 μ g/ml streptomycin (Lonza), and recombinant human M-CSF (R&D Systems) at a final concentration of 5 ng/ml carried on 0.1% human serum albumin (Aventi). The cells were cultured for 6 d with complete medium exchanges on days 1, 2, and 4. On day 6, cultures were stimulated with 25 ng/ml recombinant human IFN- γ (Imukin; Boehringer Ingelheim). On day 7, the adherent cell fraction was recovered by trypsin-EDTA treatment (TrypLE Express without Phenol Red; Invitrogen) followed by gentle scraping. M regs from all flasks were pooled and resuspended in a physiological saline solution containing 5% human albumin for infusion.

Clinical management of patients CA and MM

M regs were administered to patients CA and MM in the context of an immunosuppressive protocol comprising tacrolimus, steroids, and azathioprine. To reduce the risk of preoperative infusion of M regs sensitizing recipients, infusions were given under cover of 2 mg/kg/d azathioprine, commencing 3 d prior to administration of M regs and continuing for 8 wk postoperatively (9). M regs were administered 6 (MM) or 7 (CA) d prior to transplantation by central venous infusion. From the day of transplantation onwards, the patients were immunosuppressed with tacrolimus and steroids; steroids were weaned over the first 10 wk postoperatively, leaving the patients with maintenance tacrolimus monotherapy, aiming for trough serum levels between 4 and 8 ng/ml.

Radiological investigations

Radiological investigations were performed by the Department of Nuclear Medicine at Universitätsklinikum Schleswig-Holstein according to standard clinical protocols. For short-term cell tracking studies, patient MM received a total of 5×10^7 M regs labeled with 45 MBq [¹¹¹In]-oxine (Covidien). Single photon emission computed tomography (SPECT) images were interpreted by an expert radiologist.

Gene expression profiling

Gene expression profiling data have been deposited in the National Center for Biotechnology Information/Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) with accession numbers GSE14655 and GSE24172.

Statistics

Reported values are mean \pm SD in all cases. All *n*-values signify the number of independent biological replicates using cells from separate donors. Error bars shown in Fig. 1 represent SEM. A paired *t* test was used for all significance testing. Statistical treatment of microarray data is described in the text and figure legends.

Results

Production and quality control of clinical M reg preparations

A basic scientific understanding of the development, function, and possible immunological role of naturally occurring counterparts of the human M reg has led to an optimized method of cell production for clinical purposes. The quality of M reg preparations produced by this method can be tightly specified in terms of cell surface phenotype, cell morphology, and potency in suppressing mitogen-driven T cell responses. Specifically, M regs are consistently CD14^{-/low}-HLA-DR⁺CD80^{-/low}CD86⁺CD16⁻TLR2⁻ and CD163^{-/low} (Fig. 1A).

M regs suppress T cell responses *in vitro*

To assess the T cell-suppressive capacity of M regs, a flow cytometric assay was used to quantify mitogen-driven CD4⁺ and CD8⁺ T cell proliferation and, in parallel, to make absolute counts of T cells in direct coculture with M regs. T cells cultured alone did not proliferate and, by the fifth day of culture, $17 \pm 6.5\%$ of CD4⁺ and $22.1 \pm 11.1\%$ of CD8⁺ T cells had died spontaneously (Fig. 1B, 1C). Direct coculture of M regs with allogeneic T cells did not stimulate an MLR reaction, nor did M regs rescue cocultured lymphocytes. The strong proliferation of both CD4⁺ and CD8⁺ T cells observed after PHA stimulation was profoundly suppressed by coculture with allogeneic M regs (Fig. 1B). Strikingly, fewer CD4⁺ and CD8⁺ T cells remained in coculture with M regs when stimulated with PHA ($p < 0.001$ and $p = 0.009$, respectively), which points to an M reg-mediated elimination of activated T cells (Fig. 1C). This disappearance of T cells during M reg cocultures is most likely due to phagocytosis of dying T cells by M regs because examination of M regs by transmission electron microscopy after coculture with T cells revealed the presence of numerous phagolysosomes (Supplemental Fig. 1). Suppression of T cell proliferation by M regs was observed at M reg/T cell ratios $>1:8$ (Fig. 1D). Inhibition of IDO activity with 1 mM 1-D/L-MT restored the ability of T cells cocultured in the presence of allogeneic M regs to proliferate in response to PHA (Fig. 1E).

Administration of M regs to patient MM

Patient MM, a 23-y-old female with renal failure owing to IgA nephropathy, received a kidney transplant from her 58-y-old mother, with whom she had single HLA-B and HLA-DR

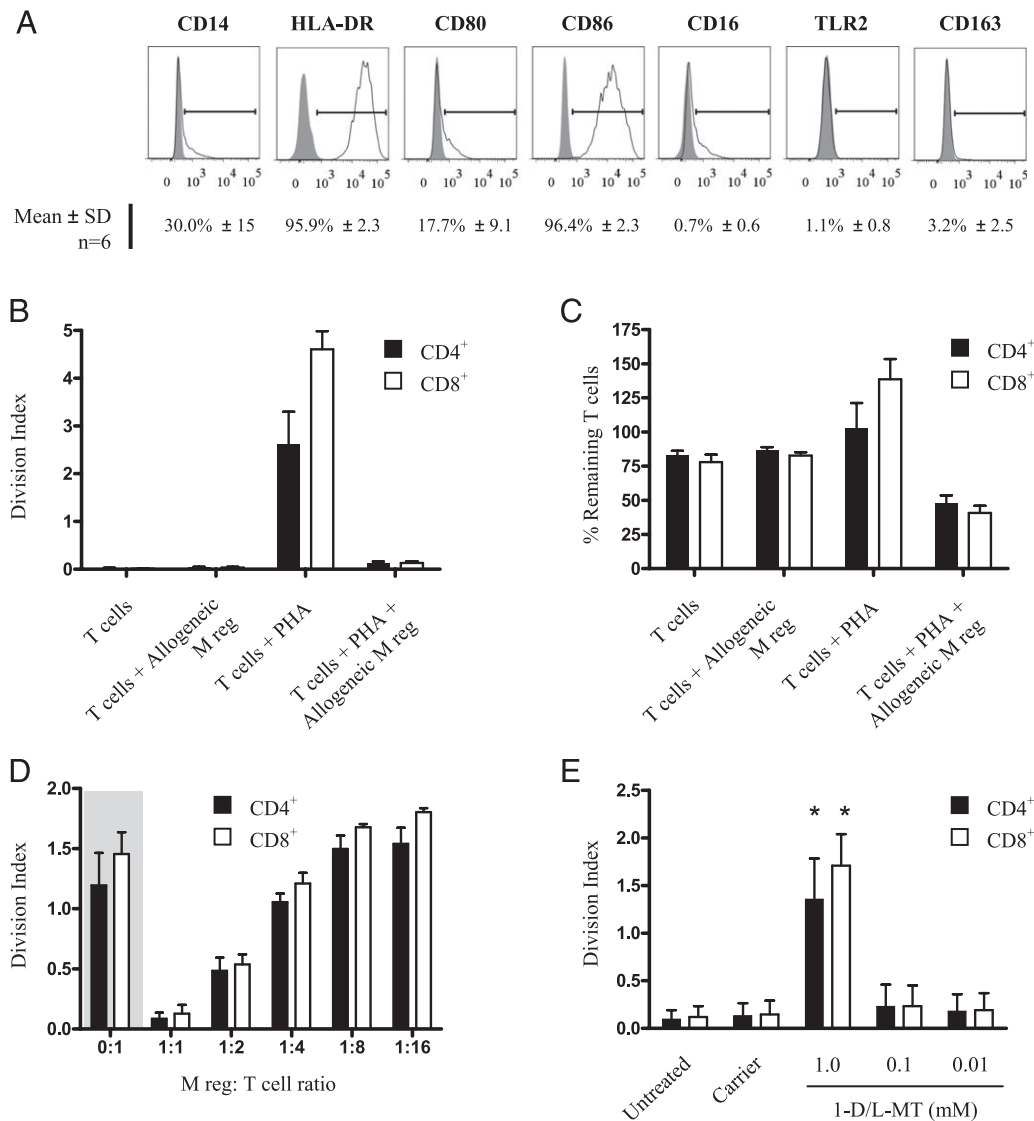


FIGURE 1. Phenotypic and functional attributes of M regs in vitro. *A*, M regs are uniquely identified by the CD14^{-low}HLA-DR⁺CD80^{-low}CD86⁺CD16⁻TLR2⁻ and CD163^{-low} phenotype. Expression ranges of these cell-surface Ags by M regs were determined in cell preparations from six normal healthy donors (mean \pm SD). Unfilled trace, specific staining; filled trace, isotype control. *B*, Over 5 d in direct 1:1 coculture, M regs did not stimulate allogeneic T cell proliferation and were suppressive of PHA-stimulated T cell responses ($n = 12$). *C*, Absolute quantification of CD4⁺ and CD8⁺ T cell numbers in 5-d 1:1 cocultures with allogeneic M regs suggest that M regs eliminated activated T cells. *D*, Suppression of T cell proliferation by M regs was observed at M reg/T cell greater ratios >1:8. *E*, 1-D/L-MT, an inhibitor of IDO, disinhibited the mitogen-stimulated proliferation of T cells in 1:1 cocultures with M regs ($n = 3$). * $p < 0.05$.

mismatches. CMV and EBV serology was negative in donor and recipient. Six days prior to transplantation, MM was given 4.3×10^8 viable donor-derived M regs (an equivalent of 8.0×10^6 cells/kg) by central venous infusion under cover of 1 mg/kg/d azathioprine (Fig. 2*A*, Supplemental Fig. 2). Transplantation was without complications and initial graft function was satisfactory, in that serum creatinine levels had fallen to 1.4 mg/dl within 1 wk. Azathioprine was discontinued from week 8 postoperatively without adverse effect, and steroid therapy was discontinued by week 14, after which MM was maintained on tacrolimus monotherapy. Protocol biopsies at 8 and 24 wk posttransplantation showed no signs of rejection. Currently, MM is 3 y posttransplantation and in a stable clinical condition on tacrolimus 2 mg twice daily with trough levels of 4 to 5 ng/ml.

Profiling of peripheral blood TCR V β usage showed no mono- or oligoclonal T cell expansion, which is indicative of

T cell nonreactivity to the transplanted kidney (Supplemental Fig. 3*A*) (10). Peripheral blood *TOAG-1* mRNA expression levels are usually reduced during acute rejection of kidney allografts, but MM consistently maintained levels of *TOAG-1* expression similar to healthy controls (Supplemental Fig. 3*B*) (11). No anti-HLA Abs, which were assayed on a monthly basis, were detected following the administration of M regs.

Trafficking of [¹¹¹In]-labeled M regs in patient MM

Knowing the fate of M regs after administration to a patient is central to any understanding of their potential immunomodulatory effects in vivo. Therefore, 5×10^7 (12%) of the 4.3×10^8 M regs given to patient MM were labeled with 45 MBq [¹¹¹In] oxine and administered at the same time as the unlabeled M regs. Immediately after the infusion, a whole-body SPECT study was performed and again at four later time points (Fig. 2*B*). Initially, the M regs were seen to be

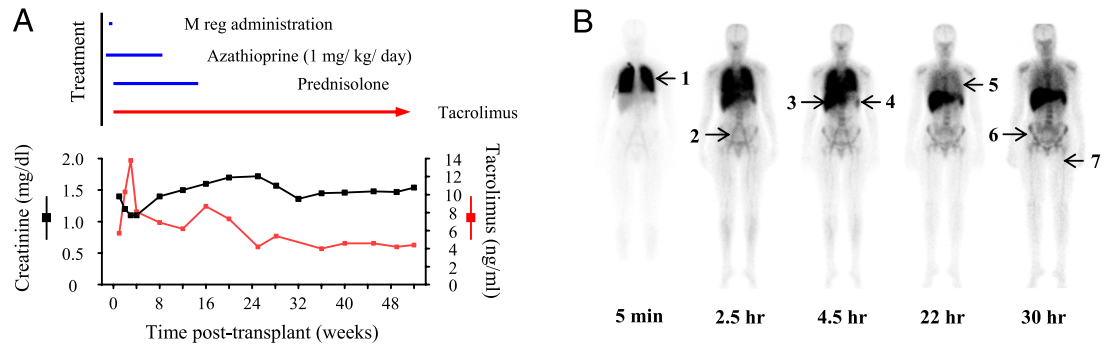


FIGURE 2. Administration of M regs to patient MM. *A*, The management and clinical course of patient MM. *B*, Anterior-posterior scintigrams reconstructed from SPECT imaging data show the distribution of ^{111}In -labeled M regs at various intervals after administration by central venous infusion: 1, M regs were initially trapped in the pulmonary vasculature; 2, by 2.5 h postinfusion, M regs were detected in circulation; 3 and 4, within 4.5 h, M regs had begun to accumulate in the liver and spleen; 5, on the day after M reg administration, the majority of M regs had exited the lungs and were no longer detectable in the blood; 6 and 7, M regs also accumulated in hematopoietically active bone marrow.

trapped in the lungs, but after 2.5 h, cells were detected in circulation and had begun to accumulate in the liver and spleen. Twenty-two hours after M reg administration, the signal from the lung fields had much diminished, the cells having accumulated in the liver, spleen, and hematopoietically active bone marrow. The absence of signal from the patient's urinary tract throughout the investigation indicates that the majority of infused M regs remained alive (12).

Administration of M regs to patient CA

Patient CA was an athletic 47-y-old male patient with severe chronic renal failure owing to nephrosclerosis as a consequence of long-standing arterial hypertension. CA was transplanted with a kidney from an unrelated 40-y-old living donor (Fig. 3*A*). Donor and recipient were fully HLA mismatched (donor: HLA-A3, -; HLA-B41, 55; HLA-DR11, 13; recipient: HLA-A2, -; HLA-B7, 35; HLA-DR4, 14), and both were CMV positive. One week prior to transplantation, a total of 7.5×10^8 viable donor-derived M regs (equivalent to 7.1×10^6 cells/kg) were administered to CA by slow central venous infusion (Supplemental Fig. 2). No evidence of impaired pulmonary perfusion caused by M reg administration was

found (Fig. 3*B*). Seven days later, the patient underwent transplantation without complications. Serum creatinine levels gradually declined over the subsequent 10 wk to <2 mg/dl. Azathioprine treatment was stopped after the 8th week postoperatively, and steroids were discontinued by the 10th week, leaving the patient with tacrolimus as his sole maintenance therapy. Protocol biopsies taken at weeks 8, 24, and 52 showed no signs of acute cellular rejection, although occasional clusters of $\text{CD}20^+$ B cells of unknown pathological significance were observed (data not shown). At 36 mo posttransplantation, CA had stable renal function with a creatinine of 1.43 mg/dl. Patient CA is being maintained on once-daily 5 mg sustained-release tacrolimus (Advagraf; Astellas Pharma) monotherapy; at 36 mo, his trough tacrolimus level was 2.7 ng/ml.

After administration of M regs, but prior to transplantation, the expression of *TOAG-1* mRNA in the peripheral blood of CA increased 5.5-fold (Supplemental Fig. 3*B*). A similar increase in *TOAG-1* was observed in patient MM after M reg treatment. As observed in MM, patient CA's expression of *FOXP3* mRNA began to rise from the fourth week postoperatively (Supplemental Fig. 3*B*), which corresponded

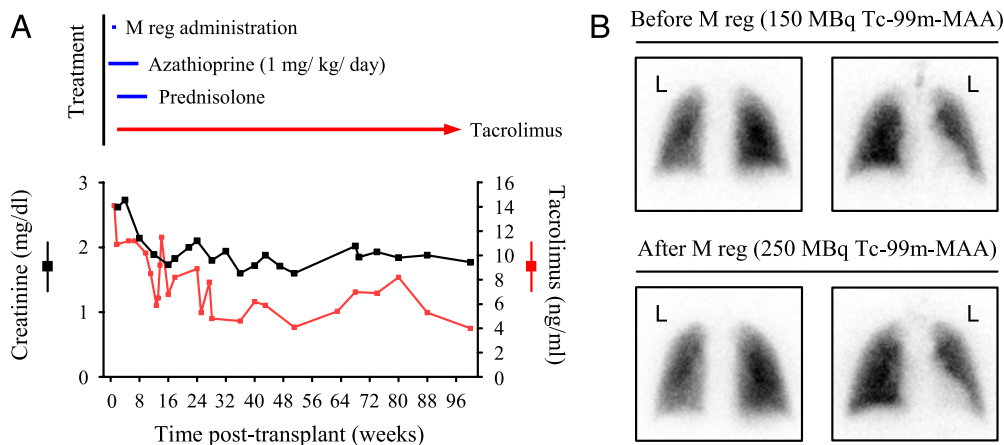


FIGURE 3. Administration of M regs to patient CA. *A*, The management and clinical course of patient CA. *B*, Total of 7.5×10^8 viable donor-derived M regs were administered to CA by slow central venous infusion using a giving set with a 200- μm cell filter. During and after infusion, the patient displayed no symptoms or signs of pulmonary embolism. To directly assess the degree to which M reg infusion might have impaired pulmonary perfusion, CA was investigated by ventilation and perfusion scintigraphy, both before and after M reg administration. Prior to cell administration, there was no evidence of a ventilation or perfusion defect: the perfusion fraction to the right lung was 59% and 41% to the left (L). After cell infusion, no localized or generalized perfusion defect was noted, and the perfusion fraction for each lung was unaltered.

to a small increase in the number of circulating T regs observed by flow cytometry (Supplemental Fig. 4).

Serial analysis of Indices of Tolerance marker gene expression in the peripheral blood of patients MM and CA

A pattern of gene expression in peripheral blood that correlates with a drug-free, tolerant state in kidney transplant recipients has been defined by the Indices of Tolerance (IOT) research network (13). Using the RASET 2.0 Agilent custom microarray platform (Agilent Technologies), serial analyses of gene expression in the peripheral blood of patients MM and CA were performed (Fig. 4). The dataset from MM and CA was then compared with the dataset obtained from the cohort of kidney transplant recipients studied by IOT. Expression profiles of the 10 most discriminatory biomarkers of tolerance identified by the IOT study were extracted from the complete quantile normalized, \log_2 -transformed dataset. From this data, pairwise correlation coefficients were calculated comparing the medians of the IOT-tolerant patient group with MM and CA at each separate time point, and heatmaps of reporter-wise median-centered \log_2 data were generated.

Throughout her postoperative course, the profile of gene expression displayed by patient MM was very similar to that of the tolerant patient group. Patient CA followed a different course, upregulating SH2D1B, HS3ST1, TCL1A, FCRL1, FCRL2, and CD79b and downregulating SLC8A1 and TLR5 only later in the follow-up period. In consequence, CA initially most resembled the group of chronically rejecting and stably immunosuppressed patients, but after week 32 closely resembled the clusters of patients classified as tolerant or healthy controls.

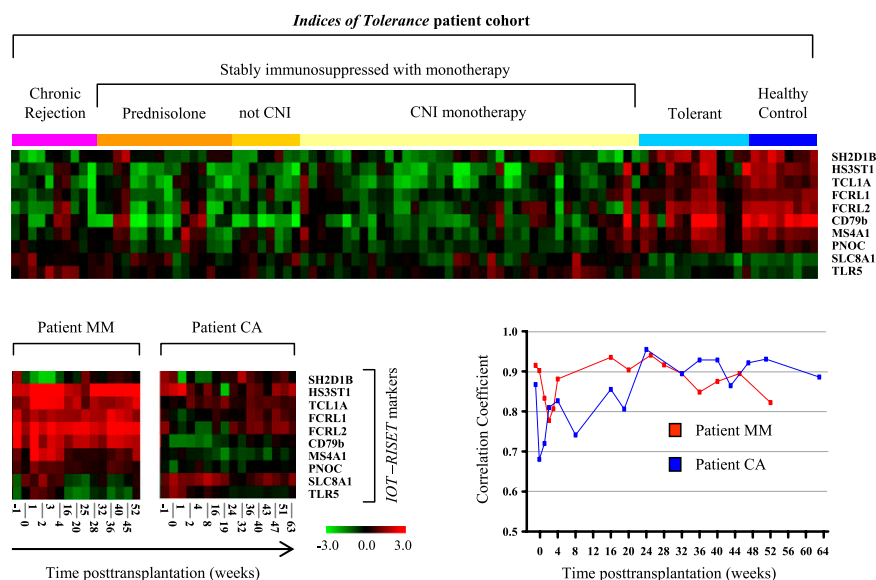
Discussion

Several alternative cell types are now approaching the point of preclinical development that might allow them to be properly investigated as adjunct immunosuppressive therapies in early-stage clinical trials, including certain tolerogenic DC subsets, regulatory T cells, mesenchymal stem cells, and M regs. Yet clinical studies using cellular therapies in transplantation remain controversial because many in the field doubt the clinical

practicality of such therapies and their safety. It is certainly true that administration of cell preparations to patients is not without potential complications, but these risks can be minimized by refining cell production and clinical monitoring of the recipient: in both these respects, we have learned much from the cases of MM and CA.

Should we be surprised by the clinical outcomes of MM and CA? Although a high proportion of renal transplant recipients with stable graft function several years after transplantation may be weaned to tacrolimus monotherapy, quite a different situation pertains to our patients. Both MM and CA underwent an early, rapid reduction in immunosuppression, such that both received tacrolimus monotherapy by 14 and 10 wk, respectively, and were further minimized to 4–6 ng/ml trough tacrolimus levels by 24 wk. At 3 y posttransplantation, patient CA is clinically stable with once-daily 5 mg sustained-release tacrolimus, and patient MM is being stably maintained with tacrolimus 2 mg twice daily. Despite their early minimization of immunosuppressive therapy, neither patient MM nor CA underwent an acute rejection episode during the 3-y follow-up period. Shapiro et al.'s study (14) remains a benchmark trial of minimized tacrolimus monotherapy in renal transplant recipients: In this study, 150 patients were treated with 5 mg/kg antithymocyte globulin with bolus prednisone as induction and were subsequently treated with tacrolimus monotherapy. Under this regimen, 37% of patients underwent acute rejection by 4 mo. After the fourth month, 113 patients underwent a stepwise minimization of tacrolimus dosing; these patients were followed up for a mean of 11 ± 5.4 mo, during which time 23% of patients underwent acute rejection. Other studies with the aim of establishing renal transplant patients on tacrolimus monotherapy after antithymocyte globulin induction (15) or alemtuzumab induction (16) achieved similar outcomes in terms of acute rejection rates and the proportion of patients tolerating monotherapy. Despite receiving no conventional induction therapy, neither MM nor CA underwent acute rejection or showed any signs of subclinical rejection at their last protocol biopsy. From clinical experience, for the patients to have tolerated such an abrupt weaning of

FIGURE 4. Using the RASET 2.0 Agilent custom microarray platform, serial analyses of gene expression in the peripheral blood of patients MM and CA were performed. The resulting dataset was then compared with that obtained from the IOT-RASET patients. Expression profiles of the 10 most discriminatory biomarkers of tolerance identified by the IOT-RASET study are presented. The color coding of the heatmaps represents reporter-wise median-centered \log_2 ratios of the combined IOT, CA, and MM datasets: red, relative upregulation; green, relative downregulation; black, no differential regulation. The convergence over time of patient MM and CA's marker gene expression upon the tolerance-associated IOT-RASET gene signature is best appreciated by considering pairwise Pearson correlation coefficients comparing the medians of the IOT-RASET drug-free, tolerant patient group with MM and CA at each sampling time point (*bottom right panel*).



immunosuppression is a surprising result and suggestive of attenuated antidonor reactivity.

Serial analyses of the immunological status of the two patients, especially by gene expression profiling, support the contention that the reactivity of MM and CA against their grafts was attenuated. As time progressed, the expression pattern of the IOT-RASET gene set in MM and CA tended toward that of a group of tolerant kidney transplant recipients or healthy untransplanted individuals (13). The frequency of individuals displaying the IOT-RASET gene signature among the cohort of stably immunosuppressed patients on calcineurin inhibitor monotherapy studied by IOT-RASET was only 14.7%. Considering that this group of patients were all 6 to 9 years after transplantation and had been purposefully selected for their stable clinical state, we can reasonably expect the frequency of patients displaying the IOT-RASET gene signature within the general pool of renal transplant recipients to be lower.

Could selection of MM and CA have biased our interpretation of their clinical outcomes? Patient CA was fully mismatched against his unrelated donor. Patient MM, who received a kidney from her 58-y-old mother, was at risk for being presensitized against noninherited maternal Ags (17). Therefore, neither donor-recipient pair could be said to be especially more likely to have a better outcome than the general pool of living-donor transplant recipients.

After systemic administration, human M regs could influence recipient responses against donor alloantigen by multiple mechanisms: M regs might have direct effects on alloreactive and regulatory T cells or may act indirectly through a systemic release of cytokines or alloantigen. At the cellular level, though, it has been shown that M regs profoundly suppress mitogen-stimulated T cell proliferation in vitro and that this activity is partly mediated by the tryptophan-catabolizing enzyme IDO. In mice, IDO is indispensable for maintaining maternal tolerance of allogeneic conceptuses (18) and participates in the establishment of allograft tolerance by adoptive transfer of T regs (19). Therefore, it is at least plausible that human M regs might influence responses to allografts through the action of IDO.

Central to any understanding of the possible mechanisms by which M regs might act is the question of whether they transiently or stably engraft in the recipient or die shortly after administration. Tracking studies with ¹¹¹In-labeled cells in patient MM revealed that donor-derived M regs migrated to specific sites and remained viable for at least the first 30 h posttransfusion, although their fate beyond this time is not known. However, given that M regs are believed to be fully differentiated, postmitotic cells, it must be assumed that they cannot establish a permanent state of chimerism.

In conclusion, these pilot case studies demonstrate the feasibility of treating renal transplant recipients with M regs. Reassuringly, no unexpected adverse reactions were observed in either patient. Tracking M regs has provided us with a preliminary description of their distribution after infusion, which is essential information in the assessment of any new therapeutic agent. Taken together, the clinical outcomes of the patients and their peripheral blood gene expression profiles must be seen as promising results, albeit of a preliminary nature. We are still years away from proving any clinical efficacy of M reg treatment as an adjunct immunosuppressive

therapy, which could only be properly demonstrated in a large-scale clinical trial. However, it is our opinion that further clinical trials of M reg treatment as an adjunct therapy aimed at establishing renal transplant recipients on minimal, calcineurin inhibitor-based maintenance immunosuppression are warranted. This concept is now being carried forward within The ONE Study, a European Union-funded multinational clinical trial.

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Disclosures

The authors have no financial conflicts of interest.

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Clinical management of patients receiving cell-based immunoregulatory therapy

James A. Hutchinson,¹ Norbert Ahrens,² Paloma Riquelme,¹ Lisa Walter,¹ Michael Gruber,³ Carsten A. Böger,⁴ Stefan Farkas,¹ Marcus N. Scherer,¹ Christiane Broichhausen,¹ Thomas Bein,¹ Hans-J. Schlitt,¹ Fred Fändrich,⁵ Bernhard Banas,⁴ and Edward K. Geissler¹

Administering immunoregulatory cells as medicinal agents is a revolutionary approach to the treatment of immunologically mediated diseases. Isolating, propagating, and modifying cells before applying them to patients allows complementation of specific cellular functions, which opens astonishing new possibilities for gain-of-function antigen-specific treatments in autoimmunity, chronic inflammatory disorders, and transplantation. This critical review presents a systematic assessment of the potential clinical risks posed by cell-based immunotherapy, focusing on treatment of renal transplant recipients with regulatory macrophages as a concrete example.

ABBREVIATIONS: APC(s) = antigen-presenting cell(s); BW = body weight; DCreg(s) = maturation-resistant, T-cell-suppressive dendritic cell(s); DST(s) = donor-specific blood transfusion(s); M reg(s) = regulatory macrophage(s); PAP = pulmonary arterial pressure; pDLI = preemptive donor lymphocyte infusion; PE = pulmonary embolism.

From the ¹Department of Surgery, Experimental Surgery Division, the ²Department of Transfusion Medicine, the ³Department of Anaesthesiology, and the ⁴Department of Internal Medicine II, Nephrology Division, University Hospital Regensburg, Regensburg, Germany; and the ⁵Institute for Applied Cell Therapy, University Hospital of Schleswig-Holstein, Kiel, Germany.

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Address reprint requests to: J.A. Hutchinson, Department of Surgery, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany; e-mail: james.hutchinson@ukr.de.

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TRANSFUSION **,**,**.*.

Transferring immunoregulatory cells from a tolerant donor to nontolerant recipient as a means of establishing tolerance in the recipient is a well-known technique in experimental immunology,¹ but its clinical application is only now receiving serious attention.² At present, several immunoregulatory cell types are reaching the point of preclinical development, which will allow them to be investigated as immunosuppressive agents in early-phase clinical trials, including regulatory T cells,³ tolerogenic dendritic cells,⁴ and regulatory macrophages (M regs).⁵ A broad spectrum of immunologic conditions may be amenable to treatment with cell-based immunoregulatory therapies, including T-cell-mediated autoimmune disease,⁶ chronic inflammatory conditions,⁷ graft-versus-host disease (GVHD),⁸ and transplant rejection.⁹ In these cases, cell-based immunoregulatory therapies might reduce or obviate the need for general immunosuppressive therapy, so sparing patients its attendant complications. Because the kind of immunologic tolerance supported by regulatory cells (as opposed to purely deletional or anergic mechanisms) is dominant and self-sustaining, there exists the staggering possibility that cell-based immunotherapy may offer a curative option in diseases that would otherwise require long-term general immunosuppressive therapy.

One particularly promising candidate cell type for use as an adjunct immunosuppressive agent in transplantation is the M reg. The M reg reflects a unique state of macrophage differentiation, which is distinguished from macrophages in other activation states by its robust phenotype and potent T-cell suppressor function.¹⁰ A newly developed therapeutic cell product containing M regs (known as Mreg_UKR) conforms to our expectations of a clinically applicable drug product in most respects.¹¹ Human M regs potently suppress mitogen-stimulated T-cell proliferation *in vitro*, which can be attributed to interferon (IFN)- γ -induced indoleamine 2,3-dioxygenase activity, as well as contact-dependent deletion of activated T cells.¹⁰ In addition, M regs drive the development of activated induced regulatory T cells that, in turn, suppress the proliferation of effector T cells and inhibit the maturation

of dendritic cells (L. Walter, submitted for publication). Therefore, when M regs are administered to an allogeneic recipient, it is hypothesized that a feed-forward loop of immunologic regulation is initiated leading to the long-term immunologic acceptance of a foreign transplant.

M reg-containing cell preparations have been administered to a total of 19 kidney transplant recipients in a series of case studies and two early-phase clinical trials (Table 1).^{10,12-15} While these pilot studies do not provide conclusive evidence of the safety or efficacy of M reg treatment in renal transplantation, they demonstrate the feasibility of this technique. An additional two living-donor kidney transplant recipients have now been treated with approximately 8.0×10^6 cells/kg of purer donor-derived M regs.¹⁰ These two patients are now more than 5 years posttransplantation with stable renal function on low-dose tacrolimus monotherapy as their sole maintenance immunosuppression. An additional clinical trial of M reg therapy in living-donor renal transplantation now has regulatory approval within the framework of the ONE Study (<http://www.onestudy.org>). This trial (*ONEMreg12*, EudraCT 2013-000999-15) aims to treat 16 patients with donor-derived Mreg_UKR at a dose of 2.5×10^6 to 7.5×10^6 /kg body weight (BW) under cover of 500 mg/day mycophenolate mofetil on Day 7 before surgery.

IDENTIFICATION OF CLINICAL RISKS ASSOCIATED WITH M REG THERAPY

Although cell-based medicines are quite different in nature from chemically synthesized drugs, many of the same general considerations apply to their clinical use.¹¹ An essential step in the safe implementation of any cell-based immunosuppressive treatment is an assessment of its associated risks and understanding how those risks relate to particular properties of the cell product.¹⁶ Naturally, evaluation of the risks associated with immunosuppressive cell therapy depends on the nature of the therapeutic cell type, the quality of the cell product, the route and timing of cell administration, and the condition and concomitant treatments of the recipient. This article examines the risk profile of Mreg_UKR treatment as a particular example of the risks of immunoregulatory cell therapy in living-donor renal transplantation. The following risk-benefit assessment has been adapted from the *ONEMreg12* clinical trial application, which was approved

by the Paul Ehrlich Institute (<http://www.pei.de>) on December 17, 2013. Although some risk factors and associated risks discussed here are unique to the administration of M regs to transplant recipients, others are of generic importance. The authors hope that this article might serve as template for other groups wishing to establish M reg therapy or other cell-based immunosuppressive therapies.

The type and rate of complications caused by Mreg_UKR have not yet been accurately assessed in clinical trials. Nevertheless, the limited available clinical evidence, as well as preclinical studies and theoretical considerations, serve as an adequate basis for a thorough risk assessment. In discussing the potential risks associated with M reg therapy, it is helpful to categorize them as immunologic, physiologic, malignant, or infectious (Table 2). Generally speaking, the potential complications of administering any immunoregulatory cell-based therapy overlap with those of Mreg_UKR therapy; nevertheless, it is valuable to consider possible differences between the safety profiles of alternative immunoregulatory cell products.

Potential immunologic complications of M reg therapy

The immunologic complications of M reg therapy are broadly similar to those associated with blood transfusions.¹⁷ When administering allogeneic cells to a transplant recipient, sensitization against donor antigens and the risk of accelerated graft rejection is a major concern; vice versa, transferring recipient-reactive immunologic effector cells might also cause disease. Additionally, non-specific reactions, such as massive cytokine release, may be responsible for adverse reactions.

Sensitization against donor antigens

With a mouse heterotopic heart transplant model, donor-derived M regs were found to afford a greater graft-protective effect than recipient- or third party-derived M regs.¹⁸ Administration of M regs before transplantation was more effective than posttransplant treatment, which is consistent with our current understanding of the function of M regs in promoting T-cell regulation. Accordingly, our clinical studies of M reg therapy have focused on the use of donor-derived cells given preoperatively.¹⁰

TABLE 1. Overview of clinical studies with human M regs

Study	Number	Total cell number	Cells/kg BW	Reference
Patient KW	1		1.1×10^6	Hutchinson et al. ¹⁵
TAIC-I Study	12	$0.9-5.0 \times 10^8$	$1.0-5.0 \times 10^6$	Hutchinson et al. ¹²
Patient FR	1	4.8×10^9	6.9×10^7	Hutchinson et al. ¹⁴
TAIC-II Study	5	$1.4-5.9 \times 10^9$	$1.7-10.4 \times 10^7$	Hutchinson et al. ¹³
Patients MM and CA	2	$4.3-7.5 \times 10^8$	$7.0-8.0 \times 10^6$	Hutchinson et al. ¹⁰

TABLE 2. Overview of possible adverse reactions to Mreg_UKR

Class of adverse effect	Description of adverse effect	Evidence
Immunologic	Sensitization against donor alloantigen	Theoretical
	Type I hypersensitivity reactions	Theoretical
	Type II hypersensitivity reactions	Theoretical
	Type III hypersensitivity reactions	Theoretical
	Mild febrile reactions	Theoretical
	GVHD	Theoretical
	TRALI	Theoretical
Physiologic	PE (overt or subclinical)	Preclinical toxicology
	Infusion-related circulatory overload	Theoretical
	Hypotension due to bradykinin activation	Theoretical
	Paradoxical embolism	Theoretical
Biochemical changes	Elevated LDH	Theoretical
	RBC lysis causing elevated free Hb and Hp	Theoretical
	Elevated TAT complexes or D-dimer	Theoretical
	Elevated K ⁺ (consequence of K ⁺ infusion)	Theoretical
	Elevated BNP, D-dimer, troponin due to PE	Theoretical
Infectious diseases	Local or systemic infection by opportunistic bacteria, viruses, fungi, or parasites owing to compromised immunity through generalized immunosuppression	Theoretical
Malignant disease iatrogenic disease	Promotion of autochthonous tumor growth	Theoretical
	Allograft dysfunction or rejection owing to reduction in immunosuppressive therapy	Theoretical
	Allograft dysfunction owing to biopsy	Theoretical
	Allograft dysfunction owing to surgical complications	Theoretical
Other	Higher rate of noncompliance with immunosuppressive therapy	Previous experience

Deliberate preoperative challenge of transplant recipients with donor alloantigen carries a risk of priming donor-reactive T cells¹⁹ and B cells,²⁰ potentially leading to accelerated graft rejection. Induction of donor-specific antibody production is especially undesirable because preformed donor antibody could lead to hyperacute, acute, or chronic rejection. There is an important historical precedent for patients being sensitized by pretransplant exposure to donor antigen in the form of HLA-disparate blood transfusions.²¹ Pretransplant challenge of prospective transplant recipients with donor-specific blood transfusions (DSTs) used to be common practice in renal transplantation and was associated with fewer acute rejection episodes and improved allograft survival when donor and recipient shared at least one HLA-DR antigen.²² Unfortunately, DST was also associated with a relatively high rate of sensitization, so the practice was largely abandoned after the introduction of cyclosporin. Sensitization rates in prospective kidney transplant recipients receiving DST without concomitant immunosuppression ranged from 8% to 29%, but this could be reduced by cotreatment with azathioprine to 7% to 16%.²³

Recent studies performed by Ezzelarab and colleagues²⁴ in rhesus macaque monkeys are important in assessing the potential risk of monocyte-derived antigen-presenting cells (APCs) of donor origin in causing alloantigenic sensitization. This group generated maturation-resistant, T-cell-suppressive dendritic cells (so-called DCregs) by differentiating monocytes in the presence of vitamin D3 and interleukin (IL)-10. Allogeneic kidney transplant recipients were treated with 3.5×10^6 to 10×10^6 donor-derived DCregs/kg on Day -7 before transplantation under cover of belatacept and rapamycin. After

transplantation, belatacept was continued for 10 or 56 days, and rapamycin treatment was continued for 180 days. DCreg treatment prolonged allograft survival compared to untreated control animals. Importantly, neither anti-donor immunoglobulin (Ig)G nor IgM antibodies were observed in the sera of the six DCreg-treated recipient monkeys. These data indicate that transfusing relatively high numbers of donor-derived myeloid APCs does not necessarily lead to sensitization, although the experiment was not large enough to detect the rates of sensitization observed in clinical studies of DST.

Sensitization rates observed in DST-treated patients are likely to overestimate the risk of sensitization in patients treated with Mreg_UKR. The composition of whole blood products and Mreg_UKR preparations are very different: A 200-mL DST comprises 0.8×10^9 to 2.2×10^9 white blood cells (WBCs; of which 2%-10% are monocytes), 0.8×10^{12} to 1.3×10^{12} red blood cells (RBCs), and 30×10^9 to 80×10^9 platelets; by contrast, M reg preparations are homogeneous and a single treatment dose of 2.5×10^6 to 7.5×10^6 M regs/kg represents a total dose of 1.25×10^8 to 7.5×10^8 cells. Importantly, the WBC fraction of a DST contains many potentially activatory APCs, whereas the Mreg_UKR product is an essentially pure preparation of suppressive cells.¹⁰ Additionally, classical DST protocols often involved multiple rounds of treatment and the risk of sensitization increased with each rechallenge: In a series of 61 patients, the rate of sensitization after a first DST was 5%, but this increased to 10% after a second DST was given 2 months later. To avoid any cumulative risk of sensitization with multiple treatments, Mreg_UKR will only be given as one-time therapy at 7 days before transplantation.

In previous clinical studies, none of the 12 patients treated postoperatively and the nine patients treated preoperatively with M regs (or M reg-containing cell preparations) developed measurable donor antibody responses and none showed signs of antibody-mediated rejection.^{10,12-15} Taken together, these preclinical and clinical data suggest that the risk of sensitization against alloantigen as consequence of Mreg_UKR administration is low. Nevertheless, as a precautionary measure, patients receiving Mreg_UKR should be treated with mycophenolate mofetil 250 mg BD from the day of cell infusion until transplantation. Additionally, patients will be screened for donor-specific antibody on the day before transplantation and surgery will be cancelled if there is evidence of de novo reactivity.

In a broader context, several groups working with immature dendritic cells have shown that recipient-derived cells (with or without donor antigen pulsing) have tolerogenic properties.⁴ In addition, most groups working with therapeutic nTreg and Tr1 preparations use cells of recipient origin. Using recipient-derived cells circumvents any risk of anti-donor sensitization; however, at least in the case of M regs and DCregs, the far greater efficacy of donor-derived cells in promoting allograft survival appears to outweigh the possible risk of sensitization.

Antibody-mediated hypersensitivity and nonspecific reactions

Discussion of Type I, Type II, and Type III hypersensitivity reactions and nonspecific adverse immunologic reactions are presented as Appendix S1 (available as supporting information in the online version of this paper).

Transfusion-associated GVHD

GVHD is an extremely rare complication of blood transfusions, characterized by fever, hepatic dysfunction, gastrointestinal disturbances, pancytopenia, and rash.²⁵ Despite its rarity, because transfusion-related GVHD is fatal in approximately 90% of cases, it deserves special consideration. GVHD is caused by alloreactive donor T cells that cannot be rejected by the recipient. Immunocompromised recipients and recipients of transfusions from donors with closely matched HLA types are especially susceptible to transfusion-related GVHD because they are less capable of eliminating donor T cells. We note that an HLA-homozygotic recipient given T-cell-containing cell preparations from a haploidentical, HLA-heterozygotic donor represents a particularly high-risk constellation. To mitigate the risk of GVHD, the Mreg_UKR manufacturing process uses CD14+ monocytes enriched by magnetic bead sorting as a starting material, thereby largely excluding T cells and other WBCs from the preparation.²⁶ Nevertheless, as it is impossible to remove all donor T cells from

M reg preparations, there may be a very small residual risk of GVHD. As a basis for assessing the risk of GVHD after infusing T cells into an allogeneic recipient, we must turn to the extensive literature on preemptive donor lymphocyte infusion (pDLI) as a treatment for hematologic malignancy.²⁷ In a meta-analysis of pDLI, CD3+ T-cell doses greater than 100×10^6 cells/kg were found to be associated with an increased risk of GVHD.²⁸ In the case of unrelated donors, CD3+ T cell doses of $\geq 10 \times 10^6$ cells/kg were associated with a higher risk of GVHD.²⁸ In a typical M reg cell product, contamination with CD3+ T cells is consistently less than 1%, which implies a maximum exposure of 0.075×10^6 CD3+ T cells/kg in M reg recipients; this equates to approximately 100-fold fewer T cells than typically used for pDLI. Therefore, the risk of GVHD as a result of Mreg_UKR is considered to be extremely low.

In a broader context, reaction of autologous immunoregulatory cell products against a recipient is extremely unlikely, especially in the case of self-derived tolerogenic DCs. However, there are concerns that transfused T regs could lose their suppressive capacity and differentiate into graft-reactive effector T cells if restimulated in an inflammatory environment.²⁹ It is well documented that nTregs can redifferentiate into Th17 cells,³⁰ but conversion into Th1,³¹ Th2,³² and Tfh³³ cells has also been reported. Various strategies to avoid nTreg products becoming potentially pathogenic effector cells have been proposed, including selection of the CD45RA+ subset that is completely demethylated at the Treg-specific demethylated region (TSDR) of the FOXP3 gene, limiting the number of divisions undergone by T regs in vitro, and performing T reg expansion in the presence of rapamycin and/or vitamin D3.³⁴ Little is known about the behavior of nTregs (or other regulatory T-cell products) after infusion into patients and, of course, it is not possible to control the fate of nTregs in vivo. Of significant concern are experiments in mice that show Foxp3 expression in T regs is unstable under inflammatory conditions and that T regs can spontaneously redifferentiate into a IFN- γ - and IL-17-producing T cells that exacerbate autoimmune disease.

Transfusion-related acute lung injury

Discussion of transfusion-related acute lung injury (TRALI)-like reactions is presented as Appendix S1.

Potential physiologic complications of M reg therapy

Potential physiologic complications of preoperative M reg administration to renal transplant recipients include embolization of cellular aggregates or cellular debris, fluid overload, and biochemical disturbances owing to release of cellular constituents.

Pulmonary embolism

When a cell product is administered systemically by intravenous (IV) infusion, the potential for embolism of cells, aggregates of cells, or debris to the pulmonary vasculature is a major concern. Pulmonary vascular obstructions caused by cell infusion may be widespread throughout the pulmonary tree and are more likely to affect small end-arteriolar branches or capillaries than larger vessels; therefore, pulmonary embolism (PE) after cell infusion is unlikely to present with classical clinical signs of thromboembolic PE. Depending on its extent, PE may be a life-threatening condition and all possible measures must be taken to avoid its occurrence.

Human M regs in suspension are approximately spherical and have a diameter of 15 to 30 μm . In humans, an estimated 2.8×10^{11} individual capillary segments are distributed between 10^8 alveoli.³⁵ Human pulmonary capillaries have a mean diameter of $7.5 \pm 2.3 \mu\text{m}$, whereas partially muscular and nonmuscular arterioles associated with respiratory bronchioles have internal diameters ranging from 15 to 150 μm .³⁶ Rigid polystyrene particles with a diameter of greater than 10 μm cannot pass through the lung vasculature, whereas the majority of particles less than 6 μm in diameter pass within 48 hours.³⁷ Polymorphonuclear leukocytes (PMNs), which have a mean diameter of $6.8 \pm 0.8 \mu\text{m}$, take 60 to 100 times longer than RBCs to transit through the lung capillary bed owing to their size.³⁸ Because of hydrostatic pressure, capillaries toward the lung bases are dilated and offer less resistance to the passage of PMNs; importantly, this effect is sufficient to cause a relative retention of PMNs in the upper lung vasculature.³⁵ On this evidence, it seems probable that passage of single M regs traveling through the pulmonary vasculature may be resisted by pulmonary capillaries and it is conceivable that aggregates of M regs might obstruct small end-arterioles.

What might be the clinical consequence of administering a treatment dose of 2.5×10^6 to 7.5×10^6 viable cells/kg on pulmonary perfusion? Studies performed in adult dogs after lung embolization with 22- μm aggregated albumin particles revealed no significant increase in mean pulmonary arterial pressure (PAP) when up to approximately 9×10^6 particles were administered.³⁹ In contrast, administration of approximately 3.5×10^6 polystyrene beads with a mean diameter of $58.9 \pm 5.2 \mu\text{m}$ to dogs caused an increase in mean PAP, increased shunt fraction, and reduced cardiac output.⁴⁰ Particles with a mean diameter of $116.6 \pm 13.1 \mu\text{m}$ likewise caused an increase in mean PAP, shunting, and reduced cardiac output and was ultimately fatal in some cases.⁴⁰ The mean lethal dose of splenocytes in heparinized dogs is 4.5×10^8 cells/kg BW and the mean lethal dose of adult hepatocytes in dogs is 1.7×10^8 cells/kg BW.⁴¹ Recently, Kobayashi and colleagues⁴² found that slow IV infusion of 10^7 mesenchymal stem cells/kg BW into heparinized miniature swine

caused a 30 mmHg increase in PAP. Therefore, infusion of approximately 10^8 cells or cell-sized particles per kilogram can be lethal in large animals and infusion of approximately 10^7 cells or cell-sized particles per kilogram can cause a significant increase in PAP.

In conclusion, it is possible that slow infusion of 2.5×10^6 to 7.5×10^6 M regs/kg BW could increase pulmonary vascular resistance, thereby increasing PAP; however, recipients with normal right-heart function are unlikely to be symptomatically compromised by M reg infusion. As a precautionary measure, patients receiving Mreg_UKR should be fully anticoagulated with high-molecular-weight heparin to reduce the risk of intrapulmonary coagulation resulting from vascular stasis. In a general sense, the risk of any given therapeutic cell type administered by IV injection causing PE depends on the size of the cells, their adherence to pulmonary vascular endothelium, their propensity to aggregate into clusters, and perhaps the degree of contamination with activated thrombocytes. Without experimental evidence, one might imagine that patients would tolerate higher doses of nTregs, which have a diameter of approximately 8.5 μm when quiescent, better than myeloid cells, such as M regs and tolerogenic DCs, which are larger.

Circulatory overload and biochemical disturbances

Discussion about infusion-related circulatory overload and biochemical disturbances is presented as Appendix S1.

Potential for transmission of malignant disease

Malignant disease after treatment with an immunosuppressive cell product might, in principle, arise either as consequence of transferring neoplastic cells or as consequence of transferred cells promoting growth of autochthonous tumors.⁴³ Neoplastic cells within the cell product might originate from the donor or arise during in vitro culture or after transfer into the recipient. Importantly, not only the therapeutically active cells within a cell product might cause malignant disease, but the cellular contaminants may also pose a risk. Immunosuppressive cell therapies might promote recipient malignancies either by directly influencing the growth of malignant cells or by suppressing immune responses against cancerous cells.

Human M regs are prepared from isolated CD14+ blood monocytes. Blood monocytes stem from a myelomonocytic precursor in marrow, known as the promyelocyte. As the promyelocyte differentiates into a blood monocyte, it passes through a series of intermediate stages, gradually losing promyelocyte characteristics and gaining features of mature monocytes. Blood monocytes are the immediate precursors of interstitial histiocytes, connective tissue macrophages, and various specialized,

tissue-specific macrophage populations. Malignancies can arise from cells at any stage of myelomonocytic development, although malignancies of primitive forms are very much more common than those of blood monocytes and macrophages.⁴⁴ In our view, it is extremely unlikely that a myelogenous leukemia could be transmitted from a donor with an undiagnosed malignancy to a recipient through Mreg_UKR treatment. Likewise, we consider the risk of inadvertently isolating malignant cells from prospective donors with any other undetected malignancy as negligible. Division of human M regs during in vitro generation cannot be reliably detected, so it appears that M regs are fully differentiated postmitotic cells. Although the possibility of there being a small fraction of M regs with some proliferative potential cannot be absolutely excluded, it is our opinion that the general non-proliferative condition of M regs means that the likelihood of them undergoing neoplastic transformation during culture is very low.

The arguments presented above for the low risk of malignancy after Mreg_UKR equally apply to tolerogenic DC preparations. Proliferating T cells accumulate genetic aberrations, both in vivo and in vitro, but the pathologic relevance of such mutations are unclear. nTreg-derived malignancies do occur naturally, notably in approximately 50% of patients with cutaneous T-cell lymphoma;⁴⁵ nevertheless, this is a very rare condition. To the best of the authors' knowledge, there is no evidence that nTregs expanded using any common clinical protocol are immortalized by the procedure; moreover, we know of no toxicologic studies showing that ex vivo expanded nTregs give rise to T-cell malignancies in immunodeficient animal models.

Potential for transmission of infectious diseases

Transmission of viral, bacterial, or fungal disease through the application of cell products is a potential risk of any cell therapy.⁴⁶ Hypothetically, there are three potential sources of infective contaminants that must be controlled, namely, pathogens from the cell donor, microbial contaminants of culture reagents or materials, and adventitious agents introduced during the manufacturing process. Therefore, we are concerned not only with excluding primary pathogens and opportunistic pathogens of transplant recipients, but also adventitious agents which may be favored by the cell product manufacturing process. Guidelines issued by the Amsterdam Forum prescribe that all potential live kidney donors are screened for cytomegalovirus and Epstein-Barr virus, hepatitis B virus (HBV) and hepatitis C virus (HCV), human immunodeficiency virus (HIV) and human T-lymphotropic virus 1 and 2, *Treponema pallidum*, *Toxoplasma gondii*, and urinary tract infections. When indicated by the patient's history or other investigations, screening for human herpes virus 8

and herpes simplex virus, *Mycobacterium tuberculosis*, *Salmonella typhi*, *Brucella* spp., *Trypanosoma cruzi*, malaria, schistosome, and strongyloides infections should also be undertaken. To exclude the presence of pathogens in the leukapheresis product used as starting material for the generation of Mreg_UKR, testing will be performed for HBV, HCV, HIV, and *T. pallidum*. Microbiologic control of Mreg_UKR preparation is performed on a manufacturing intermediate using the BacTAlert system, which detects aerobic and nonaerobic bacteria and fungi. Sterility of the final product is ascertained by testing a membrane filtrate from the Mreg_UKR culture medium and washing solutions. In addition, testing for endotoxin and mycoplasma contamination are performed to pharmacopoeial standards. Together, the measures taken to avoid transmission of infectious diseases with the Mreg_UKR product represent a stringent approach to ensuring microbiologic safety. With appropriate microbiologic screening of intermediate and final cell products, there is no reason to think that any particular immunoregulatory cell product is more or less likely to transmit infectious diseases than Mreg_UKR.

RISK-BENEFIT BALANCE OF CELL THERAPY IN TRANSPLANTATION

Were it possible to maintain the acceptable rates of rejection achieved with conventional calcineurin inhibitor-based immunosuppression, but avoid the toxicity of such treatment by dose minimization, we would expect to substantially reduce the morbidity and mortality of renal transplant recipients. One possible route to achieving this goal is to strengthen regulatory immune responses against donor alloantigens, which might lead to a lower overall requirement for maintenance immunosuppression. This is precisely what we hope may be achieved with Mreg_UKR or other cell-based immunosuppressive therapies.

Compared to standard therapy, patients in the forthcoming *ONEmreg12* trial will receive lower doses of tacrolimus and will be weaned from mycophenolate mofetil completely, if no clinical or histologic signs of rejection are evident. In consequence, M reg-treated patients established on low-dose maintenance tacrolimus monotherapy should benefit from better glomerular filtration rates, less neurotoxicity, fewer opportunistic infections, and a reduced incidence of adverse cardiovascular events. Aside from probable reductions in patient morbidity and mortality, reducing immunosuppressive therapy should reduce the cost of drugs given to transplant recipients and the costs incurred by treating complications of generalized immunosuppression. Similar therapeutic benefits may be anticipated with other immunoregulatory cell products with a very similar safety profile to Mreg_UKR. The *ONEmreg12* trial takes a relatively conser-

vative approach to minimization of tacrolimus dosing; however, if patients in this study were to show a promising immunologic profile, more radical reductions in maintenance immunosuppression might be considered in future clinical trials, with even greater potential benefits.

AUTHORS' CONTRIBUTIONS

All authors made a substantial intellectual contribution to the preparation of this article.

CONFLICT OF INTEREST

The authors report no conflicts of interest or funding sources.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's Web site:

Appendix S1. Clinical management of patients receiving cell-based immunoregulatory therapy

Generation of BTNL8⁺ iTregs by DHR9⁺ Human Regulatory Macrophages is IDO- and B7-dependent

Lisa Walter¹, Paloma Riquelme¹, Stefan Tomiuk², Birgit Sawitzki³,
Christiane Broichhausen¹, Marcus N. Scherer¹, Michail Knauel², Norbert Ahrens⁴,
Bernhard Banas⁵, Hans J. Schlitt¹, Fred Fändrich⁶, Edward K. Geissler¹
and James A. Hutchinson^{1*}

1. Department of Surgery, Experimental Surgery Division, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany
2. Miltenyi Biotec GmbH, 51429 Bergisch Gladbach, Germany
3. Institute for Medical Immunology, Berlin Charite University Hospital, 10117 Berlin, Germany
4. Department of Transfusion Medicine, Institute for Clinical Chemistry, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany
5. Department of Internal Medicine II, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, Regensburg 93053, Germany
6. Institute for Applied Cell Therapy, University Hospital Schleswig-Holstein, Campus Kiel, Arnold-Heller-Str. 3, Kiel 24105, Germany

* *Corresponding author:*

Dr. J.A. 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
email: james.hutchinson@klinik.uni-regensburg.de

Running title: Human M regs generate BTNL8⁺ iTregs

ABSTRACT

Human regulatory macrophages (M regs) represent a unique and stable state of macrophage activation, characterized by a potent T cell-suppressive capacity and DHRS9 expression. These cells have already been investigated as an immunosuppressive cell-based therapy in renal transplantation with promising preliminary results. This article examines the phenotypic and functional changes imposed by M regs on allogeneic T cells, which were characterized by flow cytometry, microarray and secondary suppression assays. M reg-exposed CD4⁺ T cells were induced to express FoxP3, CD25, CTLA4, OX40, GITR, ICOS, EB13 and BTNL8. These M reg-induced iTregs suppressed T cell proliferation and inhibited dendritic cell maturation. Generation of iTregs by M regs depended upon indoleamine 2,3-dioxygenase and B7-signalling, but was unaffected by tacrolimus or rapamycin. These data support the concept that pre-transplant treatment of recipients could engender a pro-tolerogenic immunological milieu and hint at a feed-forward mechanism by which this favorable state could be perpetuated.

Keywords: Cell therapy; M reg; T reg; EB13; BTNL8; DHRS9.

Word count: **3983**

Reviewer link: The gene expression data presented in this article have been submitted to the National Center for Biotechnology Information/Gene Expression Omnibus:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=bhyjdugmkkecwre&acc=GSE49369>

HIGHLIGHTS

- The human regulatory macrophage represents a unique and stable state of macrophage activation.
- DHRS9 is a novel marker that discriminates M regs from other monocyte-derived macrophage types.
- M regs drive CD4⁺ T cells to become Foxp3⁺ iTregs through IDO- and B7.
- BTNL8 is a novel, specific marker of T cells that have directly interacted with M regs.

INTRODUCTION

Transferring immunoregulatory cells from an immunologically tolerant donor to non-tolerant individual as a means of establishing tolerance in the recipient is a common technique in experimental Immunology, but its clinical application is only now receiving serious attention (1). Several classes of immunoregulatory cells are currently being developed as adjunct immunosuppressive agents for use in solid organ transplantation, including different types of regulatory T cell and suppressive myeloid cells (2). One particularly promising candidate cell type is the human regulatory macrophage (3-5). Preclinical experiments in a heterotopic mouse heart transplant model have demonstrated the capacity of mouse M regs to prolong allograft survival (6). A single iv dose of 5×10^6 donor-strain M regs at 8 days prior to transplantation significantly prolonged allograft survival in unconditioned, non-immunosuppressed recipients. Importantly, this graft-protective effect was not simply due to alloantigen exposure, but was mediated by inducible nitric oxide synthase-expressing cells. Following iv injection into mice, allogeneic M regs were initially trapped in the pulmonary vasculature, but rapidly redistributed to other peripheral organs, especially the liver and spleen, but not lymph nodes, where they persisted for up to 4 weeks. Similar pharmacokinetics were observed in one patient after administration of ^{111}In indium oxine-labelled cells (5). Thus, M regs appear to be relatively short-lived after transfer, but are nevertheless capable of exerting a therapeutic benefit that endures beyond their lifespan.

In principle, donor-derived M regs might influence the response of recipient T cells against alloantigen in two ways: Infused M regs might interact directly with recipient T cells to anergise or delete antigen-specific effector T cells, or induce regulatory T cells; alternatively, M regs might give-up donor antigen to recipient antigen presenting cells that then modify the recipient T cell response (1). Of course, these two general mechanisms are not mutually exclusive and M regs might contribute actively or passively to both. The tolerogenic properties of alloantigen administered by intravenous injection without adjuvant are well-documented and it is highly probable that presentation of M reg-derived antigens by recipient APCs (via the indirect or semi-direct pathway) contributes to the therapeutic benefit of M reg treatment (7). Previous observation that prolongation of allograft survival through M reg treatment depends upon active properties of M regs is not inconsistent with the idea that M regs exert suppressive effects through recipient APCs, as one function of M regs may be to die and release donor antigen into a suitably self-conditioned environment (6). On the other hand, the high likelihood that M regs act, at least in part, through recipient APCs does not negate the possibility that M regs might interact directly with recipient T cells to suppress or regulate their reactivity to donor alloantigen. Clearly, determining whether direct interaction between M regs and recipient T cells occurs, and

what therapeutic consequences this interaction may have, is critical to our further understanding of the suppressive activity of M regs.

This article examines the phenotypic and functional changes wrought upon T cells exposed to M regs *in vitro* with a view to identifying properties that might serve as specific markers of T cells that have interacted with M regs. A comparative characterisation of T cells cultured with M regs or under control conditions revealed that M regs drove T cells to become FoxP3⁺ iTregs, which were uniquely identified by butyrophilin-like protein 8 (BTLN8) expression. Patients receiving M reg therapy in the forthcoming *ONEmreg12* trial (EudracCT-Nr.: 2013-000999-15) will be monitored for T cells expressing BTNL8 as a putative measure of pharmacodynamic effect.

MATERIALS AND METHODS

Generation of M regs and IFN- γ -M ϕ

CD14⁺ monocytes were isolated from Ficoll-prepared PBMC and positive-selection using anti-CD14 microbeads (Miltenyi, Bergisch-Gladbach). Monocytes were plated in 6-well Cell+ plates (Sarstedt, Nümbrecht) at 10⁶ cells/well in RPMI-1640 (Lonza, Cologne) containing 10% heat-inactivated human AB serum (Lonza), 2 mM Glutamax (Invitrogen, Karlsruhe), 100 U/mL penicillin (Lonza), 100 ug/mL streptomycin (Lonza), and rhM-CSF (R&D Systems, Wiesbaden-Nordenstadt) at 25 ng/ml carried on 0.1% human albumin (CSL-Behring, Hattersheim-am-Main). On day 6 of culture, cells were stimulated for 18 hours with 25 ng/mL rhIFN γ (Chemicon, Billerica, MA). IFN- γ -M ϕ were generated under identical conditions, except 10% human serum was substituted with 10% FCS.

Coculture of M regs and T cells

On day 7 of culture, M reg and IFN- γ -M ϕ medium was exchanged with serum-free X-vivo-10 medium (Lonza) supplemented with 2 mM Glutamax and 25 ng/mL rhM-CSF. MACS-sorted CD3⁺ T cells were added to macrophages at a 1:1 ratio for 5 days. For transwell experiments, inserts with 0.4 μ m pore size were used (Greiner-BioOne, Frickenhausen). Where indicated, cocultures were performed in the presence of tacrolimus (Astellas, Munich), 1-methyl-L-tryptophan (Sigma), recombinant CTLA-4-Ig fusion protein (Bio X Cell, West Lebanon, NH) or control human immunoglobulin (CSL-Behring).

Flow cytometry

Surface staining (Table S1) was performed at 4°C in DPBS/1% BSA/0.02% NaN₃/10% FcR-block (Miltenyi). Foxp3 Fixation-and-Permeabilization buffers (eBioscience) were used for intracellular staining. Dead cells were excluded with 7-AAD (BD-Biosciences) or Aqua-LIVE/DEAD[®] (Invitrogen).

Western blotting, immunoprecipitation and protein sequencing

SDS-PAGE and immunoblotting were performed using conventional methods. Protein A/G-sepharose (Sigma-Aldrich) was used to immunoprecipitate the ASOT1 antigen before sequencing by MALDI-MS (Proteome-Factory, Berlin).

Rabbit polyclonal anti-serum against DHRS9

Rabbit polyclonal antisera were raised against a peptide corresponding to residues 85-105 of human DHRS9 (CTDPENVKRTAQWVKNQVGEKG).

Microarrays

Highly-purified populations of CD4⁺ or CD8⁺ T cells were isolated using a FACS-Aria flow-sorter (BD-Biosciences). Total RNA was extracted using an RNeasy-Plus Mini-Kit (Qiagen, Hilden). Assessment of RNA quality, labeling and hybridization to 8x60K Agilent Whole-Human-Genome-Oligo-Microarrays was performed according to published methods¹⁰. Fluorescence signals from hybridized microarrays were detected using Agilent's Microarray Scanner System (G2505C, Agilent-Technologies, Palo Alto). The Agilent Feature Extraction Software 10.7.3.1 was used to extract the microarray image files. Background-corrected intensity-values were quantile-normalized and log₂-transformed. Only reporters with at least three valid signal intensity values in at least one sample group were considered. For visualisation in heatmap format, the log₂ intensity values were median-centered for each reporter. One-way ANOVA was performed using GeneSpring-GX v.11.5.1 (Agilent).

qPCR

SuperScript-III (Invitrogen) was used for reverse-transcription. qPCR was performed with a LightCycler™ real-time PCR system using the FastStart DNA Master SYBR Green I kit (Roche-Diagnostics, Penzberg). DHRS9 qPCR primers: 5'-TGACCGACCCAGAGAATGTCAA-3' and 5'-GCCGGGAACACCAGCATTATT-3'. Specificity was confirmed by amplicon sequencing (MWG-Biotech, Ebersberg).

TSDR methylation analysis

CD4⁺ T cells were positively isolated by MACS. Genomic DNA was extracted with the QIAamp DNA blood mini-kit and bisulfite-treated (EpiTect, Qiagen) before real-time PCR quantification of Foxp3 TSDR (29).

Statistics

Statistical analyses and curve-fitting were performed with GraphPad software. As indicated, values given in histograms either represent mean±SD, mean±SEM or median±MADM. A Mann-Whitney test was used for all significance tests.

RESULTS

The steady-state phenotype of human M regs

Human M regs derive from purified CD14⁺ blood monocytes when cultured at a density of 10⁵ cells/cm² in RPMI-1640 supplemented with 10% human AB serum (HABS) and 25 ng/ml M-CSF for six days prior to stimulation with IFN- γ for a further 18 hours. M regs generated in this manner are consistently CD14^{-/low} HLA-DR⁺ CD80^{-/low} CD86⁺ CD16⁻ TLR2⁻ and CD163^{-/low} (Fig.1a). In culture, M regs exhibit a very distinctive morphology with the cells adopting a tessellating, epithelioid morphology to form almost confluent monolayers (Fig.1b). Individual M regs 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 μ m. Ultrastructural examination of M regs by transmission electron microscopy confirmed the impression of a large, flattened cell adhering very closely to the underlying surface (Fig.1c-f).

Human M regs only arise in human serum

IFN- γ -stimulated macrophages (IFN- γ -M ϕ) are generated by growing monocytes under identical conditions to M regs, but with substitution of FCS for HABS. Notably, IFN- γ -M ϕ did not acquire the marker phenotype (Fig.1g) or typical morphology (Fig.1h) of M regs. As previously reported, M regs efficiently suppress mitogen-stimulated allogeneic CD4⁺ and CD8⁺ T cell proliferation in 1:1 cocultures (5); however, IFN- γ -M ϕ were found to be significantly less suppressive under the same conditions (Fig.1i). IFN- γ -M ϕ exhibited general characteristics of M1-polarised macrophages, including promotion of Th1-cytokine production by cocultured T cells (Fig.S1). Therefore, it appears the phenotype and suppressive function of human M regs is a feature of human monocytes cultivated in human serum. Because IFN- γ -M ϕ are generated under very similar conditions from M regs, but have a different phenotype and function, IFN- γ -M ϕ represent an appropriate and useful experimental control cell type for M regs.

The M reg phenotype is distinct from previously described M ϕ polarisation states

To establish the phenotypic proximity of M regs to macrophages in previously described states of activation, a panel of macrophage types was generated for comparison with M regs in terms of their morphology, cell-surface markers, cytokine production, and global gene expression profiles. M regs were readily distinguished from all these other macrophages by their characteristic morphology (Fig.2a) and distinct cell-surface phenotype (Fig.2b). M regs and the panel of comparator macrophages were also distinguished by their cytokine and chemokine production profiles (Fig.2c).

DHRS9 expression uniquely identifies M regs amongst comparator M ϕ types

ASOT1, a monoclonal antibody raised in mice against human M regs, reacted strongly against M regs, but not other macrophages (Fig.3a). By immunoprecipitating and sequencing its antigen, the ASOT1 mAb was shown to recognise DHRS9, a little-studied retinol dehydrogenase of the SDR family (8-10) (Fig.3b). Quantitative PCR confirmed that *DHRS9* mRNA expression was restricted to M regs within the panel of comparator macrophages (Fig.3c). Importantly, *DHRS9* mRNA expression was not extinguished by lipopolysaccharide treatment of M regs. A rabbit polyclonal antibody generated against an N-terminal epitope of DHRS9 reacted against a protein of ~35-kD immunoprecipitated by ASOT1 (Fig.3d). As a commercially-available monoclonal antibody (clone 3C6) which recognises DHRS9 also reacted with the same protein detected by the rabbit antibody, it is concluded that both ASOT1 and the rabbit polyclonal antibody recognise DHRS9. Using this rabbit pAb, DHRS9 protein expression was shown to be unique to M regs (Fig.3e). PCR analysis showed that M regs express all the necessary enzymes to convert β -carotene and retinol to retinoic acid (Fig.3f). Naturally-occurring DHRS9⁺ macrophages were detected in human spleen (Fig.3g).

M regs induce T cell-suppressive T cells

That administration of M regs affords allograft-protective effects that persist beyond the lifespan of the M regs implies the establishment of recipient regulatory T cell responses. As a first step in testing this hypothesis, the impact of exposure to M regs on allogeneic T cell phenotype and function was investigated in coculture experiments (Fig.4a). When unstimulated, polyclonal human T cells were cocultured with equal numbers of allogeneic M regs for 5 days, no bias was seen in the CD4/CD8 ratio compared to IFN- γ -M ϕ -cocultured T cells (Fig.4b). CD25 was significantly up-regulated by CD4⁺ T cells after M reg-coculture compared to IFN- γ -M ϕ -coculture (Fig.4c). To gauge their suppressive capacity, M reg-cocultured T cells were re-isolated and set in culture with autologous CFSE-labelled responder T cells. In such assays, proliferation of responder T cells stimulated with plate-bound anti-CD3 was relatively inhibited by M reg-cocultured T cells (Fig.4d).

M reg-cocultured T cells inhibit monocyte-derived DC maturation

To test whether M reg-cocultured T cells inhibit DC maturation, allogeneic M reg-cocultured CD4⁺ T cells were flow-sorted and added at a 2:1 ratio to immature monocyte-derived DCs (mo-DC) from the same donor as the M regs. These T cell-cocultured mo-DCs were matured with TNF α over 2 days and then expression of maturation markers was assessed. CD4⁺ T cells from M reg cocultures inhibited the upregulation of CD80 and CD83 by maturing mo-DCs (Fig.4e).

M reg coculture drives CD4⁺ T cells to become activated iTregs

To better characterise the influence of M reg co-culture on T cells, whole-genome gene expression profiling by microarray was performed. M regs and IFN- γ -M ϕ were generated from monocytes of 5 healthy donors. Polyclonal CD3⁺ T cells were isolated from a further 5 unrelated, healthy, HLA-discrepant donors and were added into direct co-culture with M regs or IFN- γ -M ϕ , or were cultured alone for 5 days without stimulation. Following co-culture, CD4⁺ and CD8⁺ T cells were FACS-sorted and total RNA was extracted. In addition, freshly isolated CD3⁺ T cells were also sorted into CD4⁺ and CD8⁺ fractions for RNA isolation. These RNA samples gave rise to a microarray dataset comprising triplicate samples of eight cell types produced by single-colour hybridisations. From this dataset, genes that were highly (>20-fold) and significantly ($p < 0.01$ after Benjamini-Hochberg correction for multiple testing) differentially expressed in any two of the four sample sets were identified by one-way ANOVA (Fig.5a). By presenting these differentially expressed genes as a hierarchically clustered heatmap, it was evident that T cells cocultured with either M regs or IFN- γ -M ϕ are more similar to one another than to either fresh T cells or T cells cultured alone; however, many notable differences between M reg-cocultured CD4⁺ T cells and IFN- γ -M ϕ -cocultured CD4⁺ T cells were discovered.

Discriminatory gene analyses identified a large number of reporters up- and down-regulated in M reg-cocultured T cells compared to IFN- γ -M ϕ -cocultured T cells (Table S2). Enriched among those genes up-regulated in M reg-exposed CD4⁺ T cells were T reg-associated markers, such as CD25, CTLA4, TNFRSF4/OX40, TNFRSF18/GITR, ICOS and LAG3. Notably, EB13 was 18.6-fold up-regulated in M reg-cocultured CD4⁺ T cells compared to IFN- γ -M ϕ -cocultured T cells. EB13 encodes one subunit of heterodimeric cytokines IL-27 and IL-35, which are both principally regarded as immunosuppressive cytokines (11). FOXP3 was also significantly up-regulated in M reg-cocultured CD4⁺ T cells compared to all control conditions (Fig.5b), but key transcriptional regulators of Th1, Th2, and Th17 differentiation, namely TBX21/TBET, GATA3, and RORC, were not differentially expressed between M reg-cocultured CD4⁺ T cells and controls (data not shown).

Flow cytometry demonstrated a relative up-regulation of FoxP3, CD25, CTLA4, OX40, GITR, CD28, PD1 and ICOS by M reg-cocultured CD4⁺ T cells compared to IFN- γ -M ϕ -cocultured CD4⁺ T cells or T cells cultured alone (Fig.5c). Some M reg-cocultured CD4⁺ T cells expressed LAG3, but LAG3 expression was not entirely coextensive with OX40 expression. FoxP3⁺ T cells represented 50 \pm 8 % of M reg-cocultured CD4⁺ T cells and these were predominantly CD45RA⁻ (Fig.5c). M reg-cocultured T cells secreted ~4-fold more IL-10 than IFN- γ -M ϕ -cocultured T cells during a 24-hour restimulation with anti-CD3, although the absolute amounts of IL-10 produced were

small (Fig.5d). Only marginally more cell-surface TGF- β -expressing T cells were detected after M reg-coculture than after IFN- γ -M ϕ -coculture (Fig.5e). Analysis of the methylation status of Treg-specific demethylated region (TSDR) of the FOXP3 gene in M reg-cocultured CD4⁺ T cells revealed only 5.3% demethylation, which was not different to that found in CD4⁺ T cells cultured alone (Fig.5f). This low degree of TSDR methylation proves that M reg-cocultured CD4⁺ T cells are not nTregs. Therefore, we conclude that M regs are capable of driving the development of allogeneic T cells into activated iTregs, which are characterised by a FoxP3⁺ CD25⁺ CTLA4⁺ OX40⁺ GITR⁺ CD28⁺ PD1⁺ ICOS⁺ CD45RA⁻ CD62L^{-/low} CCR7^{-/low} and CD127^{-/low} phenotype. Because the expression of Foxp3 in M reg-generated iTregs may only be transient, its stability was investigated in iTregs cultured for 10 days without further restimulation (Fig.5g). FoxP3 expression decayed slowly over this period, such that 64% \pm 26 of the initial pool of FoxP3⁺ T cells was present at day 10. In this context, it is interesting to note that M reg cocultured CD4⁺ T cells expressed relatively high levels of *SOCS2* mRNA, which has been shown to stabilise the phenotype of FoxP3⁺ T regs (12) (Table S1).

Generation of activated iTregs by M regs is B7- and IDO-dependent

Induction of iTregs by M regs was not alloantigen-dependent, as no difference in iTreg generation was observed between allogeneic and autologous cocultures (Fig.6a). Nevertheless, transwell separation of M regs and T cells in coculture demonstrated the need for direct physical interaction for M reg-driven iTreg development (Fig.6b). By blocking B7-mediated signaling using 10 μ g/ml CTLA4-Ig, it was shown that iTreg generation by M regs is substantially dependent upon the B7 costimulatory pathway (Fig.6c). Because T cell suppression by M regs is principally IDO-mediated, the effect of blocking IDO activity with 1-methyltryptophan was also examined. Generation of T regs in cocultures treated with 1mM 1-L-MT was significantly reduced, but not completely abolished (Fig.6d). R041-5253, an antagonist of the retinoic acid receptor-alpha, did not affect iTreg generation (Fig.6e). Importantly, from a translational perspective, neither 10 ng/ml tacrolimus nor 10 ng/ml rapamycin prevented iTreg development in the presence of M regs (Fig.6f&g). Taken together, these results show that M regs drive an antigen non-specific, but B7- and contact-dependent induction of iTregs through an IDO-dependent mechanism that is unaffected by therapeutically-relevant concentrations of tacrolimus or rapamycin.

BTNL8 is a marker of iTregs arising in M reg cocultures

With the intention of identifying novel markers of iTregs generated by coculture with M regs, we re-examined the list of genes highly and significantly up-regulated in M reg-cocultured T cells (Fig.7a). The 10 most discriminatory reporters included 5 genes encoding proteins with known function (CYS1, OXCT2, RET, GNG4 and EBI3), one cell-surface receptor of uncharacterized function (BTNL8), two predicted proteins of unknown function (SEC14L6 and C11orf96) and one lincRNA (represented by reporters A_19_P00316371 and A_19_P00810403). Of these discriminatory genes, Butyrophilin-like protein 8 (BTNL8) stood out as a potential marker of M reg-generated iTregs of possible immunological importance (13). BTNL8 belongs to the 4-member butyrophilin-like gene family, which is closely related to the butyrophilin family, both of which families are structurally and functionally related to the B7-costimulatory molecules (Fig.7b).

The specific up-regulation of *BTNL8* mRNA expression in M reg-cocultured T cells observed by microarray studies was corroborated by qPCR using primers specific for both the BTN-like and B7-like variants (Fig.7c). Using isoform-specific primer sets and sequencing of amplicons, it was shown that M reg-cocultured T cells express only the B7-like variant of BTNL8 (data not shown). A selection of polyclonal antibodies raised against the extracellular portion of full-length BTNL8 is commercially available; however, none of these reagents are well-characterised. Therefore, the specificity of two pAbs were assessed by IP-Western (Fig.7d). BTNL8 was precipitated from lysates of M reg cocultured T cells using a goat pAb (sc-245053). As a negative control, β -actin was precipitated using a goat pAb (sc-1616). BTNL8 protein was then detected by Western blotting using either the goat α -BTNL8 pAb or a rabbit α -BTNL8 pAb (PA5-24933). In agreement with the PCR data, both blotting antibodies detected a precipitated protein at ~37-kD, but no protein at 57-kD. Therefore, we can be confident that M reg-cocultured T cells express the B7-like isoform of BTNL8, but not the BTN-like variant, and that both the goat and rabbit pAbs are reactive with this isoform. Western blotting using the goat α -BTNL8 pAb revealed strongest expression of a 37-kD isoform of BTNL8 in M reg-cocultured T cells, compared to IFN- γ M ϕ -cocultured T cells or T cells cultured alone (Fig.7e). Likewise, Western blotting using the rabbit α -BTNL8 pAb revealed much stronger BTNL8 expression in M reg-cocultured T cells than in IFN- γ M ϕ -cocultured T cells (Fig.7f). To demonstrate cell-surface expression of BTNL8, CD4⁺ T cells were stained with goat α -BTNL8 pAb and analysed by flow cytometry (Fig. 7g). By this approach, M reg-cocultured CD4⁺ T cells were shown to uniformly express cell-surface BTNL8, in contrast to control CD4⁺ T cells.

DISCUSSION

This work seeks to identify particular characteristics of human CD4⁺ T cells acquired through direct contact with M regs. Through IDO-dependent mechanisms, M regs potently suppress mitogen-stimulated T cell proliferation and drive conventional T cells to a regulatory phenotype. Regulatory T cells arising from M reg-cocultures exhibit many typical properties of *in vitro*-induced T reg (iTreg), including a FoxP3⁺ CD25⁺ CTLA4⁺ OX40⁺ GITR⁺ CD28⁺ PD1⁺ and ICOS⁺ phenotype and the capacity to inhibit mitogen-stimulated proliferation of responder T cells (15). Suppressive FoxP3⁺ T cells have been generated *in vitro* by various means, most commonly by treatment with exogenous TGF- β or coculture of T cells with DCs or macrophages that produce TGF- β (16;17). Such TGF- β -induced iTreg typically express TGF- β themselves, which accounts for their suppressive function (18). Similarly, activation of T cells in the presence of IL-10, or in coculture with IL-10-producing DCs, generates regulatory T cells that act through IL-10 production (19). In contrast to these TGF- β - and IL-10-dependent regulatory T cells, M reg-induced T regs produce little IL-10 or TGF- β and no clear evidence was found that either cytokine contributes to their suppressive activity. Thus, although it cannot be claimed that M reg-induced T reg are a novel type of T reg, these cells are sufficiently distinctive that, were an increase in phenotypically similar T regs observed in M reg-treated patients, it would point to a direct effect of M regs on recipient T cells *in vivo*.

In previous work, M regs were found to suppress mitogen-driven T cell proliferation through the action of IDO (5). Here, it was shown that IDO activity also contributes to the generation of iTregs in M reg cocultures. The principal candidate mechanism for IDO-dependent induction of T regs is ligation of the aryl hydrocarbon receptor (AHR) by tryptophan metabolites generated through the action of IDO (20;21). A second possibility is that tryptophan degradation products might exert effects through G protein-coupled receptor 35 (GPR35), which microarray analyses found was 9.3-fold up-regulated in M reg-cocultured T cells compared to IFN- γ M ϕ -cocultured T cells (22). Notably, the demonstration that iTreg generation was inhibited by CTLA4-Ig and required M reg-T cell contact argues that IDO activity alone is not sufficient to account for iTreg induction by M regs.

The overarching objective of this work was to identify unique markers of CD4⁺ T cells that have encountered M regs. In this context, expression of the B7-like isoform of BTNL8 is potentially useful, but it is also an intriguing receptor from a basic scientific perspective (13). The butyrophilin and butyrophilin-like proteins are B7-related costimulatory molecules (as are CTLA-4, ICOS, etc.) and probably serve as negative regulators of immune responses. The human genome encodes four BTNL genes (designated BTNL2, -3, -8 and -9) of which only BTNL2 and

BTNL9 have clear orthologues in mice (23). Ig-fusion proteins of mouse Btl1, Btl2 and Btl9 have T cell-suppressive properties *in vitro* through unknown counter-receptors (24-26); however, there is sparse information about the functions of human BTNL family members. In particular, human BTNL8 has received very little attention. BTNL8 is predicted to be type-I membrane protein with an extracellular region comprising an IgV and IgC domain (13). Various isoforms of BTNL8 have been reported, including a 57-kD isoform, which incorporates an intracellular B30.2 domain that is lacking from the 37-kD isoform. Expression of both *BTNL8* mRNA splice variants was previously reported in neutrophils and eosinophils, but was not detectable in freshly-isolated T cells, B cells, NK cells or monocytes (23;27). Here, BTNL8 was found to be a highly discriminatory marker of M reg-cocultured CD4⁺ T cells, which leads us to speculate that BTNL8 could limit T cell activation during inflammatory responses through a negative feedback loop (28).

If *ex vivo*-generated allogeneic human M regs were capable of modifying recipient T cells *in vivo* in a similar fashion as they do *in vitro*, this would help to explain persistence of the therapeutic effects of M regs beyond their lifespan. In this regard, it is interesting that M reg-induced T regs were capable of preventing the full maturation of monocyte-derived DCs in response to TNF α . Thus, although M regs and M reg-induced T regs may only be transient populations, it is conceivable these cells might initiate a feed-forward regulatory loop that is perpetuated by recipient DCs and T regs. Flow cytometry and qPCR assays to measure the frequency of OX40⁺ LAG3⁺ T cells and BTNL8-expression by T cells in peripheral blood have now been established. It is hoped that monitoring these parameters in patients treated with M regs during the *ONEmreg12* trial will corroborate our *in vitro* findings, thereby demonstrating a pharmacodynamic effect of M reg therapy in a clinical setting.

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DATA DEPOSITION

The gene expression data presented in this article have been submitted to the National Center for Biotechnology Information/Gene Expression Omnibus as dataset GSE49369.

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FIGURE LEGENDS

Figure 1: *The steady-state phenotype of human M regs. (a)* Human M regs exhibit a CD14^{-/low} HLA-DR⁺ CD80^{-/low} CD86⁺ CD16^{-/low} TLR^{-/low} CD163^{-/low} cell-surface phenotype (data representative of n=3 donors). **(b)** M regs in culture acquire a distinctive morphology (bar = 50 μ m). **(c-f)** Transmission electron micrographs of M regs that show a close adherence to the culture surface, active nuclei with abundant fine chromatin, numerous cell processes, lipid inclusions [L], and bilaminar annular structures [\rightarrow] within the cytoplasm that resemble confronting cisternae. **(g)** IFN- γ -stimulated macrophages (IFN- γ M ϕ) are generated by growing monocytes under identical conditions to M regs, but with substitution of FCS for HABS. In contrast to M regs, IFN- γ M ϕ are CD14⁺ HLA-DR⁺⁺ CD80⁺ CD86⁺ CD16⁺ TLR⁺ CD163⁺ (data representative of n=3 donors). **(h)** IFN- γ M ϕ are readily distinguished from M regs by their irregular, elongated form (bar = 50 μ m). **(i)** In direct 1:1 co-cultures, M regs strongly suppress PHA-stimulated CD4⁺ and CD8⁺ T cell proliferation, whereas equal numbers of IFN- γ M ϕ do not (n=3; CD4⁺: p=0.036; CD8⁺: p=0.036).

Figure 2: *Within the spectrum of previously described states of macrophage polarisation, the human M reg phenotype is unique. (a)* M regs are reliably distinguished from macrophages in other polarisation states by their characteristic morphology in culture. **(b)** Human M regs can be distinguished from all other macrophage types by a constellation of four cell-surface markers: CD14^{-/low} CD16⁻ TLR2^{-/low} and CD163⁻ (n=6; values are mean \pm SD). **(c)** The 24-hour constitutive cytokine secretion profile of M regs was typical of neither M1- or M2-polarised M ϕ (n=6). M regs constitutively produced relatively small amounts of TNF- α and IL-6, and did not secrete detectable amounts of IL-12p40. M regs expressed detectable levels of TGF- β and relatively greater amounts of IL-1Ra than most other macrophage types, but notably less IL-10.

Figure 3: *DHRS9 expression uniquely identifies the M reg phenotype. (a)* The ASOT1 mAb recognised an antigen expressed by M regs, but not other macrophage types. **(b)** An antigen of \sim 35 kD was specifically precipitated by ASOT1. This precipitated antigen was identified by mass spectrometry as DHRS9. **(c)** Strong *DHRS9* mRNA expression was detected in M regs, but not other macrophage types (n=6). **(d)** ASOT1 precipitated an antigen which was also recognised by an anti-DHRS9

rabbit pAb and an anti-DHRS9 mouse mAb, confirming that ASOT1 recognises DHRS9. **(e)** Immunoblotting with a rabbit anti-DHRS9 pAb demonstrated that DHRS9 expression at the protein level distinguishes M regs from other macrophage types. **(f)** The SDR family of retinol dehydrogenases, which can be classified as NAD⁺ or NADP⁺ dependent enzymes, are responsible for conversion of retinol to retinal (10). Retinal is further metabolised to retinoic acid by retinal dehydrogenases: Expression of *ALDH1A1* and *ALDH1A2* mRNA was detectable in M regs. Retinal is liberated from β -carotene through the action of beta-carotene monooxygenases: Expression of *BCO2*, but not *BCMO1*, was detected in M regs. Therefore, M regs appear to express the necessary machinery to convert β -carotene and retinol to retinoic acid. (Expression of enzymes responsible for retinoid metabolism was detected by PCR in M regs from 3 donors: D1-3.) **(g)** To ascertain whether DHRS9-expressing macrophages occur naturally, an immunohistochemical staining procedure for fixed tissues was established using the rabbit anti-DHRS9 pAb. Immunohistochemical staining of spleen identified a minor population of DHRS9-expressing macrophages. Although it is not possible to conclude that these splenic macrophages are a physiological counterpart of the human M reg, the existence of naturally-occurring DHRS9⁺ macrophages argues that the DHRS9⁺ M reg phenotype is not purely an *in vitro* artefact. (Arrow; original magnification 400x).

Figure 4: *M reg-cocultured T cells inhibit T cell proliferation and mo-DC maturation.* **(a)** Human M regs were cocultured for 5 days with either allogeneic or autologous T cells. These T cells were then analysed by flow cytometry, qPCR and microarray, or were used to suppress T cell proliferation or inhibit TNF α -stimulated DC-maturation. **(b)** 5-day coculture of positively-isolated CD3⁺ T cells with allogeneic M regs at a 1:1 ratio did not affect the proportion of CD4⁺ and CD8⁺ T cells compared to coculture with IFN- γ M ϕ (n=11). **(c)** 5-day coculture of positively-isolated CD3⁺ T cells with allogeneic M regs at a 1:1 ratio led to a relative increase in the proportion of CD25⁺ T cells compared to coculture with IFN- γ M ϕ (n=11). **(d)** Division of CFSE-labelled responder CD4⁺ T cells stimulated with plate-bound anti-CD3 was relatively inhibited by M reg-cocultured T cells, but not by control T cells (n=6). **(e)** CD4⁺ T cells re-isolated from M reg cocultures by FACS-sorting inhibited the upregulation of CD80 and CD83 by maturing mo-DCs (data representative of n=6).

Figure 5: *M reg coculture drives CD4⁺ T cells to become activated iTregs.* **(a)** Hierarchical clustering of the reporter sets returned by one-way ANOVA which were significantly ($p < 0.01$) and highly differentially (> 20 -fold) regulated in any two of the comparator T cell populations. This analysis shows that T cells cocultured with either M regs or IFN- γ M ϕ are more similar to one another than to either fresh T cells or T cells cultured alone; however, many notable differences between M reg-cocultured CD4⁺ T cells and IFN- γ M ϕ -cocultured CD4⁺ T cells are evident. Red shading indicates a higher expression of a certain reporter in the respective sample compared to the median of all samples, whereas green shading indicates down-regulation. **(b)** Relative expression of a biased selection of T reg-related genes in CD4⁺ T cells cocultured with allogeneic M regs or IFN- γ M ϕ , or cultured alone ($n=5$; median \pm MADM). **(c)** Confirmation by flow cytometry of upregulated expression of T reg-related markers by M reg-cocultured CD4⁺ T cells compared to IFN- γ M ϕ -cocultured CD4⁺ T cells and T cells cultured alone (data representative of $n=5$ donors). **(d)** 24-hour secretion of IL-10 by α -CD3-stimulated CD4⁺ T cells reisolated from allogeneic M reg cocultures ($n=4$). **(e)** Cell-surface expression of TGF- β by CD4⁺ T cells cocultured with allogeneic M regs or IFN- γ M ϕ (representative of $n=4$ donors). **(f)** Quantification of demethylation status of the *T reg-specific Demethylated Region* (TSDR) in CD4⁺ T cells cocultured with allogeneic M regs or IFN- γ M ϕ , or cultured alone ($n=3$; mean \pm SEM). **(g)** Assessment of the stability of FoxP3 expression in M reg-induced iTregs over 7 days in culture without restimulation ($n=3$; mean \pm SEM).

Figure 6: *Generation of activated iTregs by M regs is B7- and IDO-dependent.* Induction of FoxP3⁺ iTregs in 5-day allogeneic M reg cocultures was assessed by flow cytometry. Plots are gated on live CD3⁺ CD4⁺ T cells. Quadrants were set strictly according to FMO-isotype controls. **(a)** Generation of iTregs in M reg cocultures was not alloantigen dependent. **(b)** Nevertheless, direct contact between M regs and T cells was necessary for the generation of FoxP3⁺ iTregs. **(c)** The requirement for direct interaction between M regs and T cells is partly explained by the dependence of FoxP3⁺ iTreg generation on B7-mediated signals, which could be inhibited with CTLA-4-Ig. **(d)** Generation of FoxP3⁺ iTregs was inhibited when IDO activity was blocked with 1 mM 1-L-methyltryptophan. **(e)** An antagonist of RAR α did not affect FoxP3⁺ iTreg generation. **(f)** 10 ng/mL tacrolimus or **(g)** 10 ng/mL rapamycin did not affect FoxP3⁺ iTreg induction. (Data are representative of $n \geq 3$ donors in all cases.)

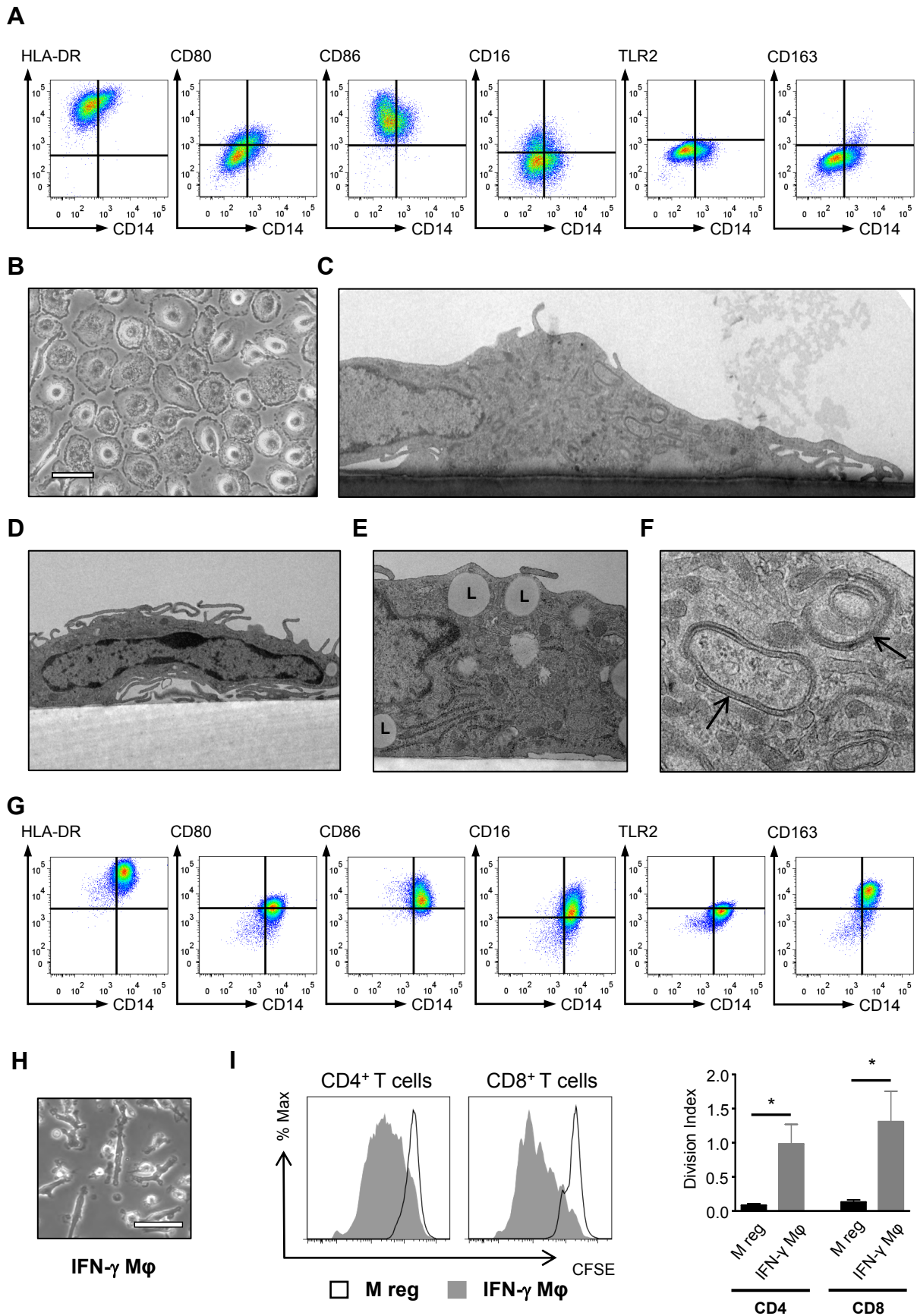
Figure 7: *BTNL8 is a marker of iTregs arising in M reg cocultures. (a)* Top ten most highly upregulated reporters in allogeneic M reg-cocultured CD4⁺ T cells compared to IFN- γ M ϕ -cocultured CD4⁺ T cells (n=5; median \pm MADM). **(b)** Six putative alternatively-spliced isoforms of BTNL8 have been reported, ranging in predicted molecular weight from 37 kD to 57 kD. These isoforms can be categorized as B7-like or BTN-like according to their structure. The BTN-like variant comprises an extracellular IgV and IgC domain, a transmembrane domain and an intracellular B30.2 domain (14). The B7-like variant share the same extracellular IgV and IgC domains, but owing to alternative splicing resulting in a frameshift, it possesses a different transmembrane region and only a short intracellular C-terminal domain. **(c)** qPCR for BTNL8 mRNA in CD4⁺ T cells cocultured with M regs or IFN- γ M ϕ , CD4⁺ T cells cultured alone and freshly-isolated CD4⁺ T cells (n=9; mean \pm SEM). **(d)** BTNL8 was precipitated from a lysate of M reg-cocultured T cells using a goat α -BTNL8 pAb and detected by Western blotting using either the α -BTNL8 pAb or a rabbit α -BTNL8 pAb. This experiment shows that the goat and rabbit pAbs both recognise the same 37 kD variant of BTNL8. **(e)** Western blot for BTNL8 using a goat α -BTNL8 pAb from T cells cocultured with allogeneic M regs or IFN- γ M ϕ and T cells cultured alone. A band corresponding to BTNL8 was detected at 37 kD. **(f)** Western blot for BTNL8 using a rabbit α -BTNL8 pAb from T cells cocultured with allogeneic M regs or IFN- γ M ϕ . A band corresponding to BTNL8 was detected at 37 kD. **(g)** M reg-cocultured T cells and control T cells were stained for analysis by flow cytometry with either a goat α -BTNL8 pAb or a rabbit α -BTNL8 pAb.

LIST OF SUPPLEMENTARY MATERIALS

Figure S1: IFN- γ M ϕ -cocultured T cells expressed Th1-type cytokines.

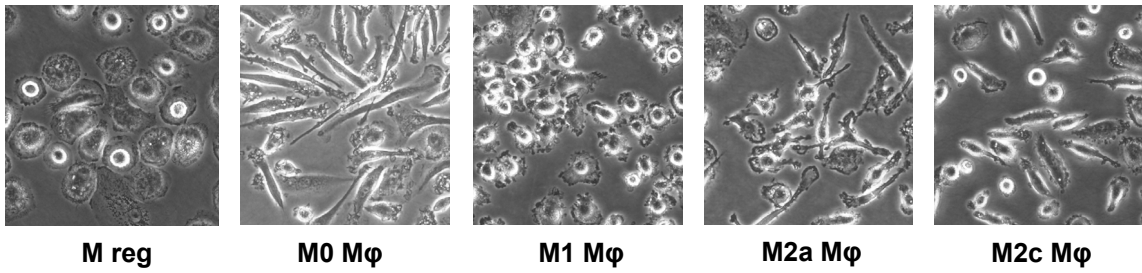
Table S1: A subset of 30 T reg-associated markers selected from the 572 reporters significantly up-regulated in M reg-cocultured T cells compared to IFN- γ M ϕ -cocultured T cells that were returned by SAM.

Table S2: Antibodies used for flow cytometry studies.

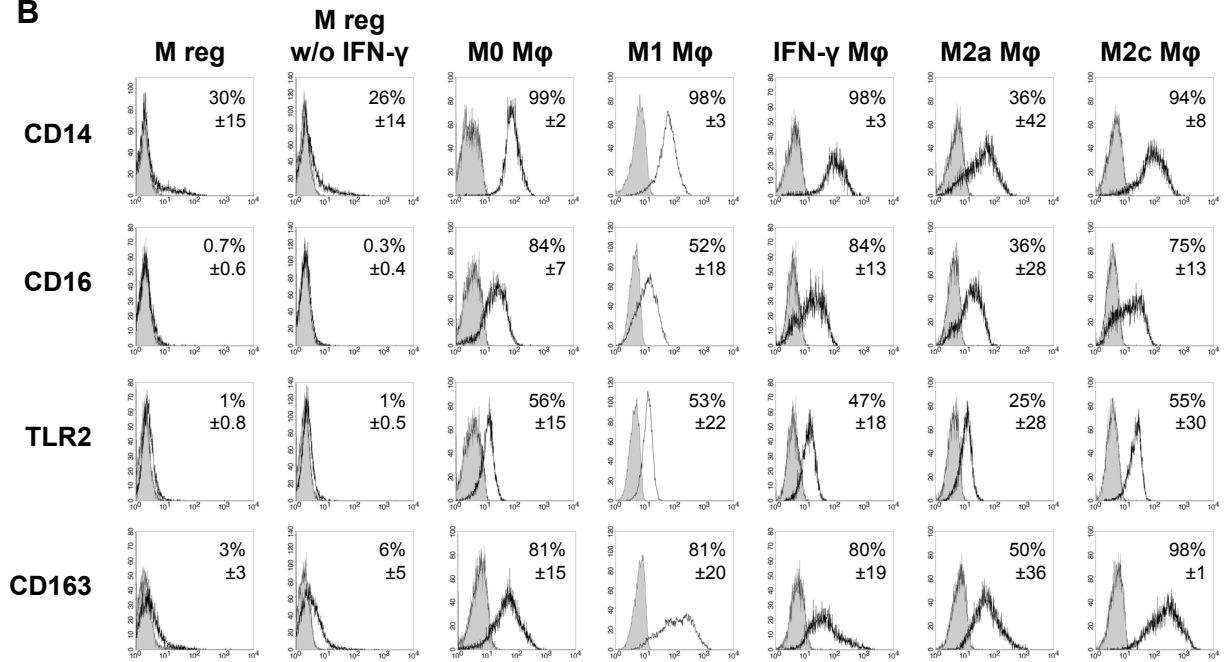


Walter, L. *et al.* (2013) Figure 1

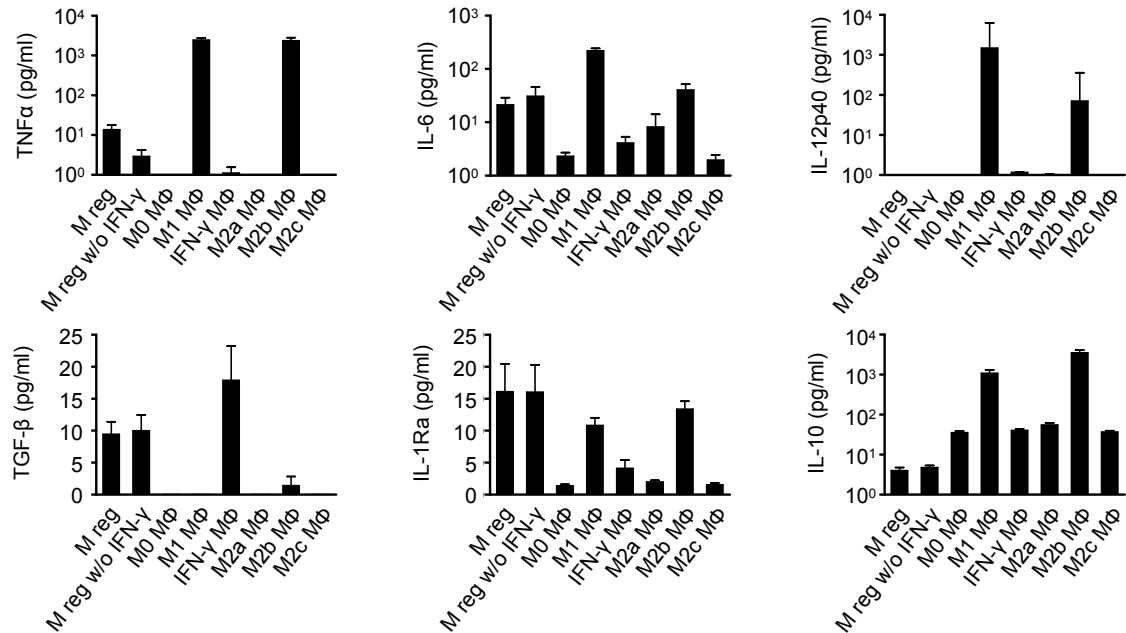
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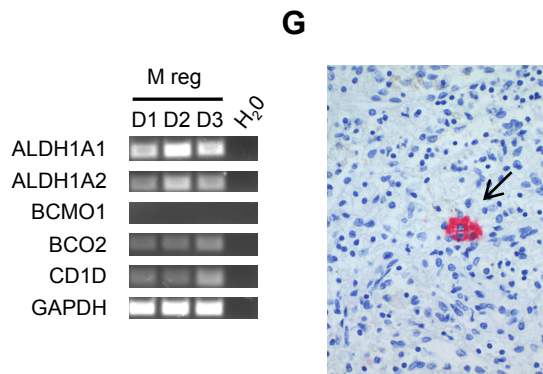
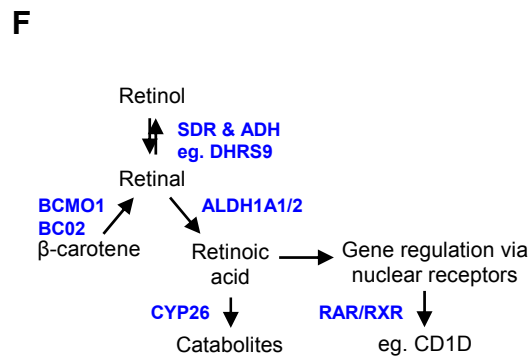
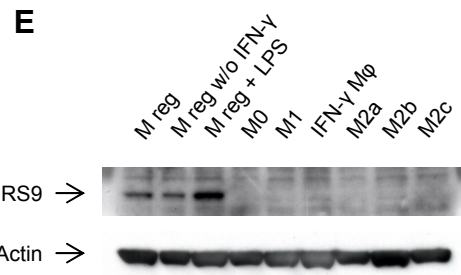
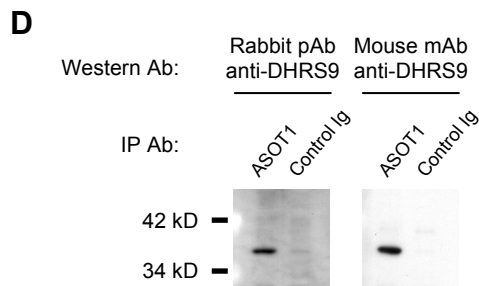
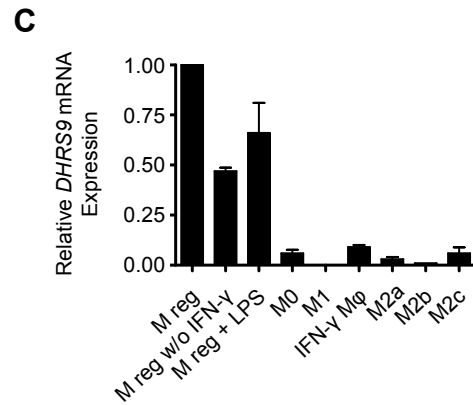
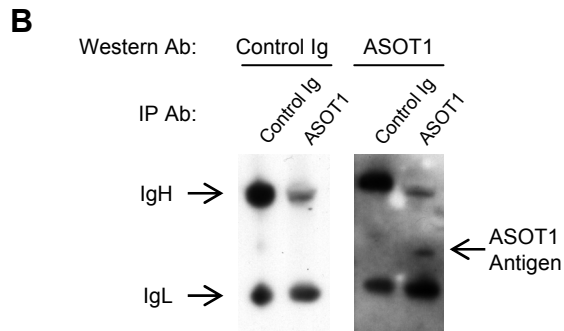
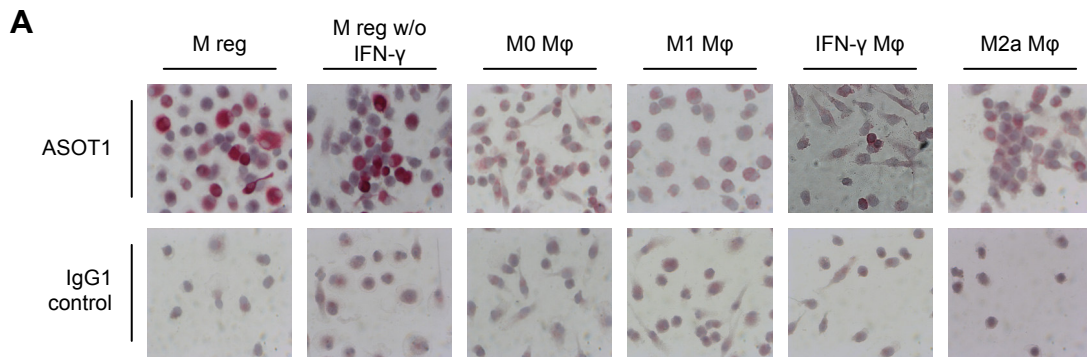
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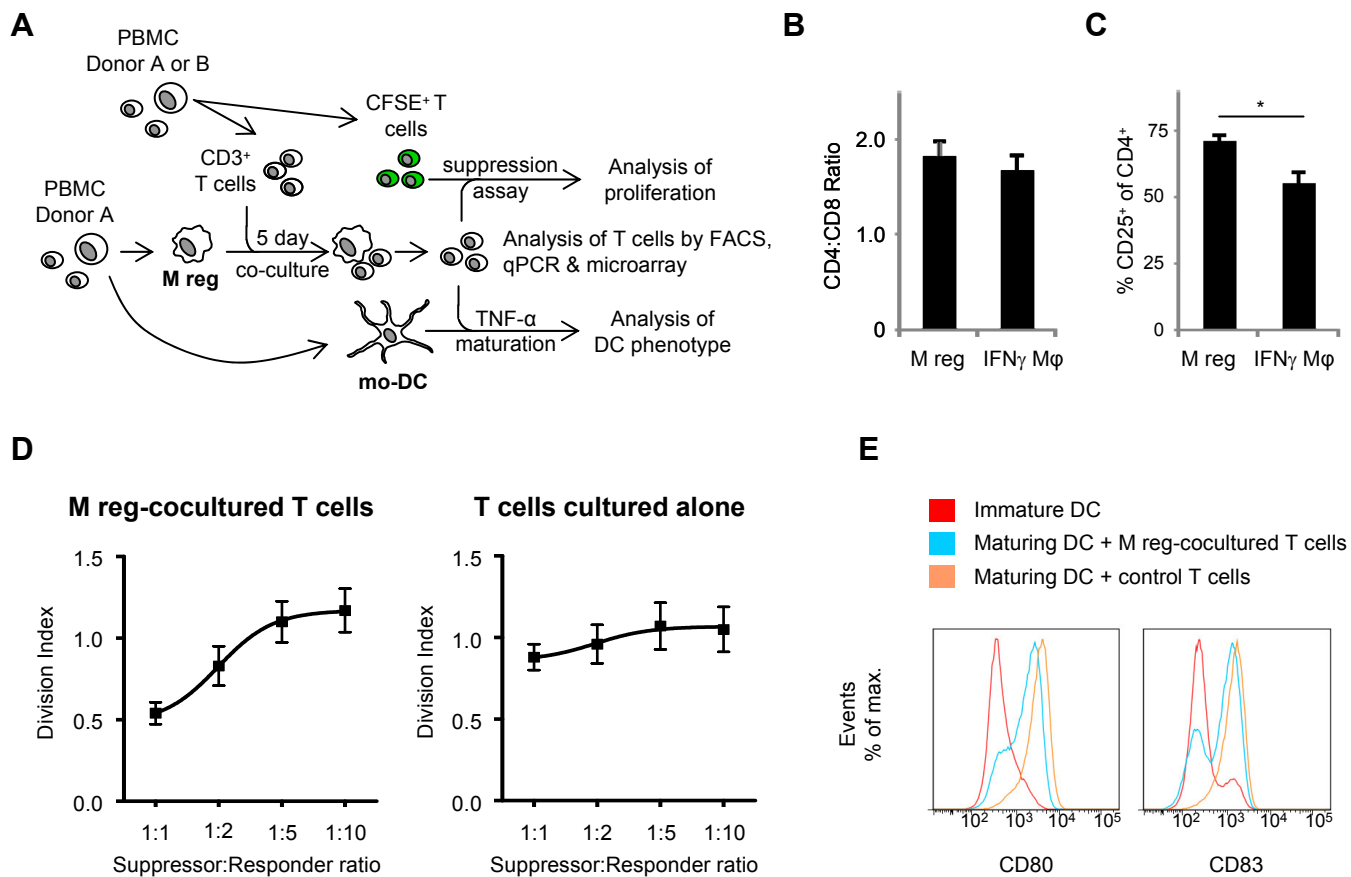
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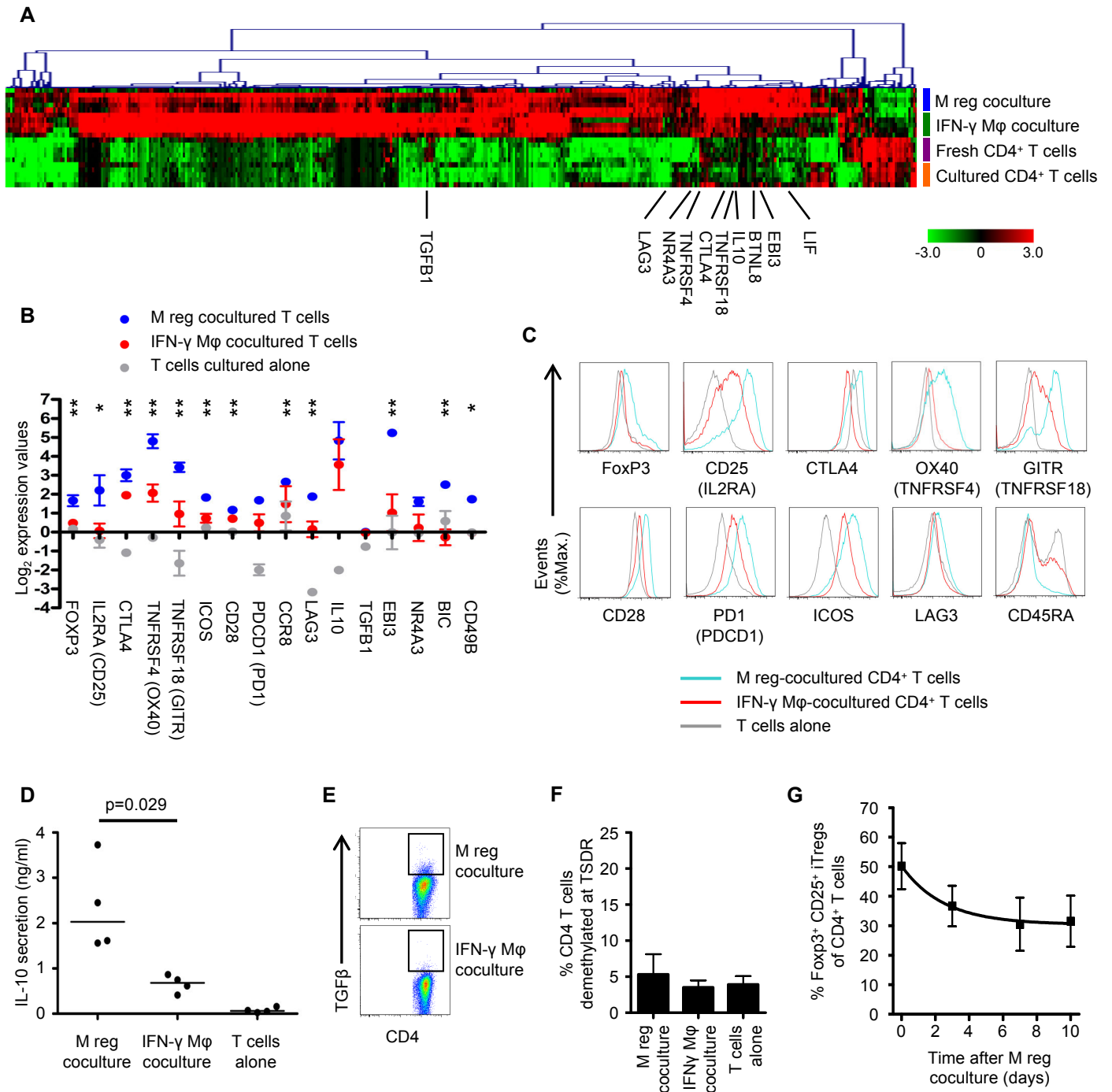
Walter, L. *et al.* (2013) Figure 2



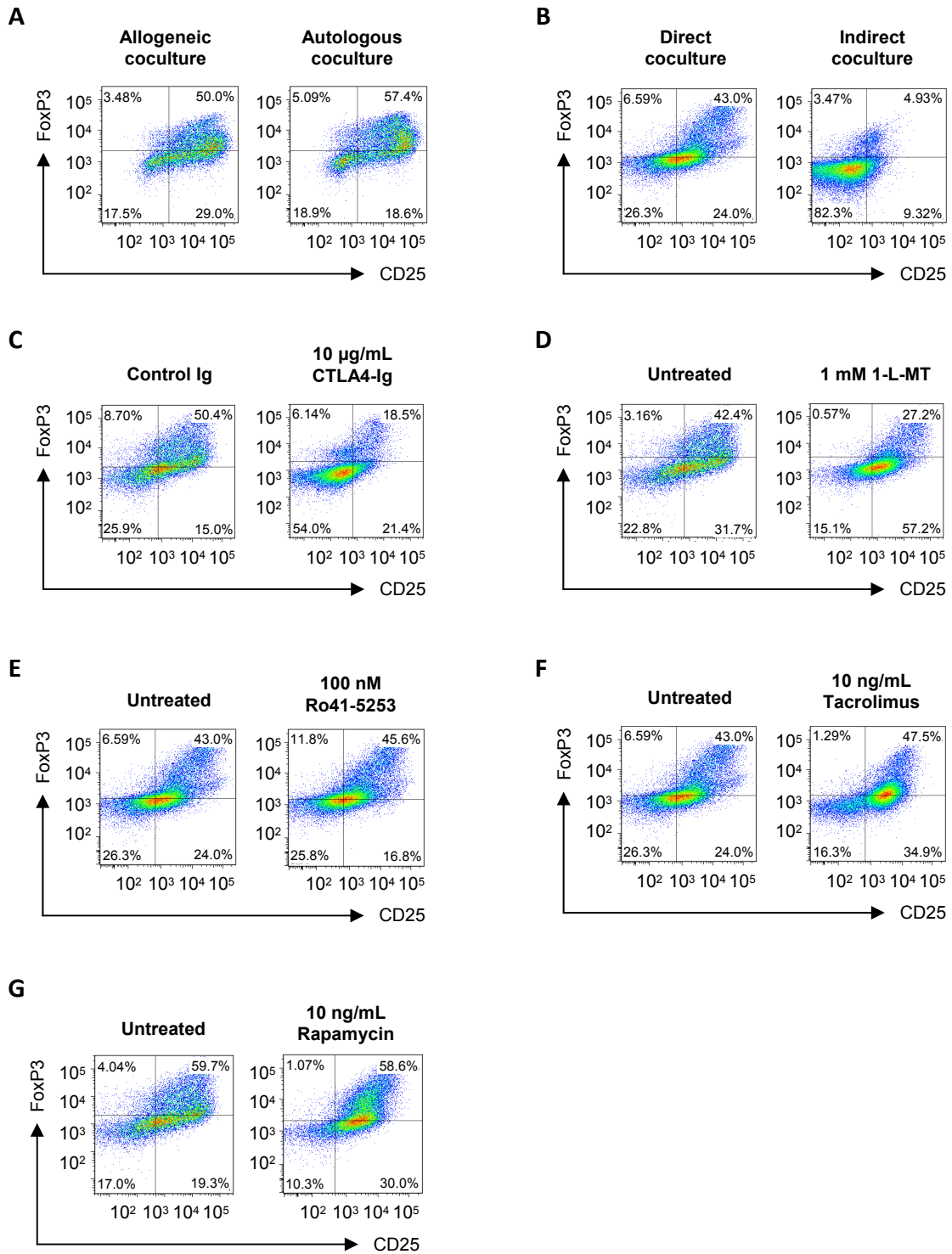
Walter, L. *et al.* (2013) Figure 3



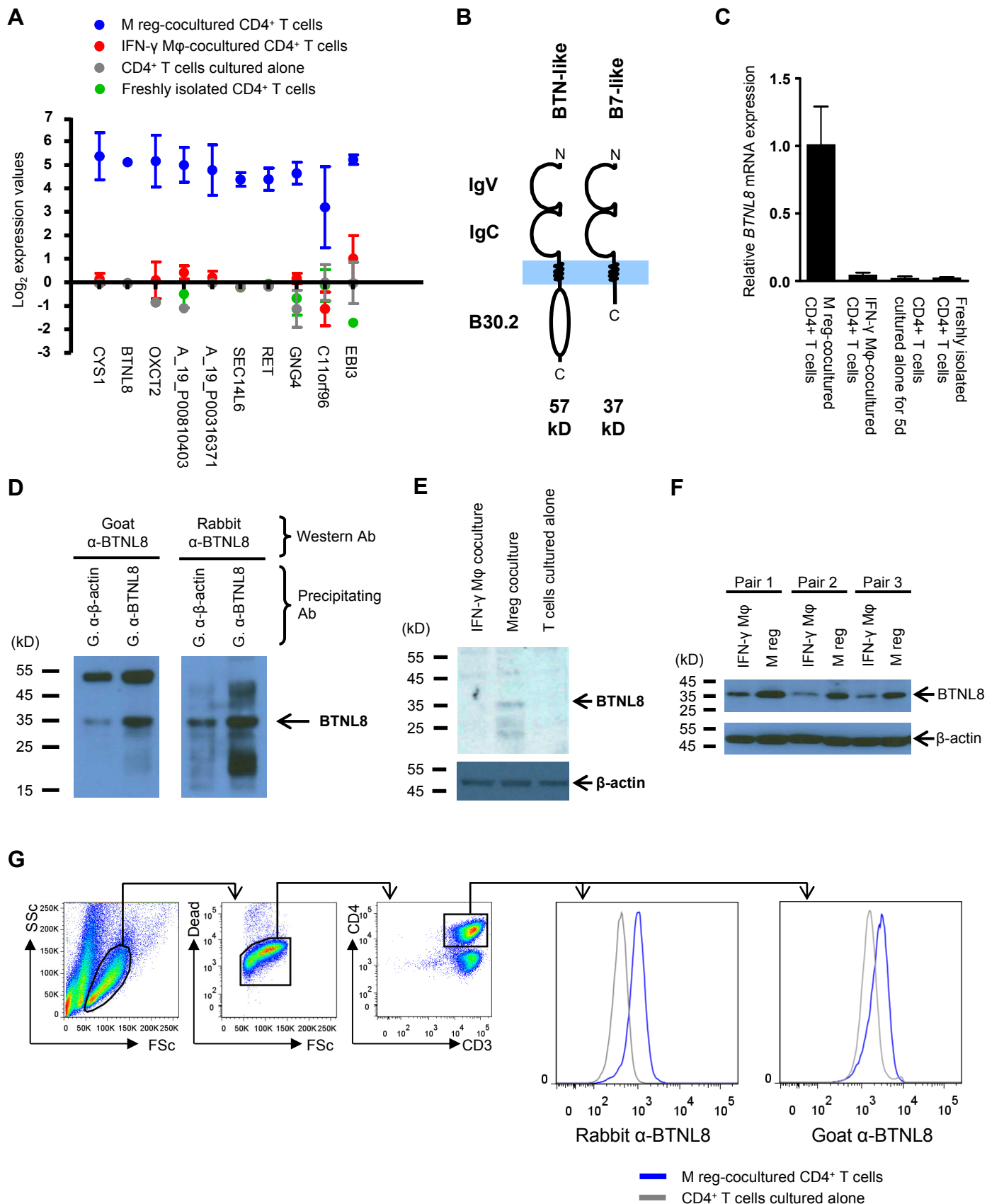
Walter, L. *et al.* (2013) Figure 4



Walter, L. *et al.* (2013) Figure 5



Walter, L. *et al.* (2013) Figure 6



Walter, L. *et al.* (2013) Figure 7

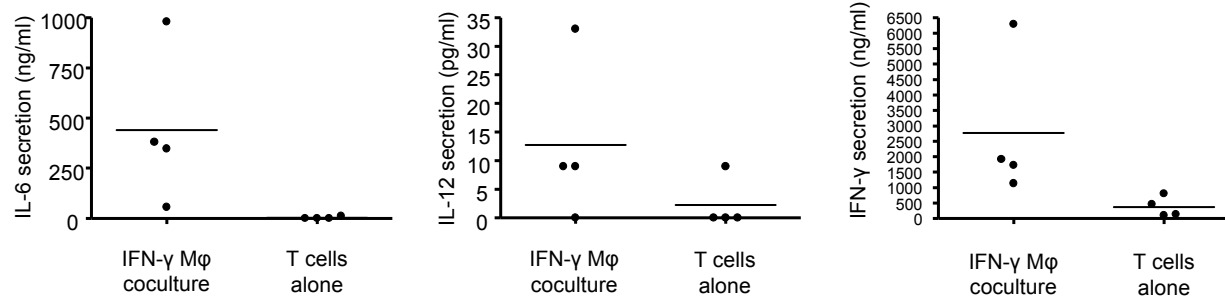


Figure S1: IFN- γ M ϕ -cocultured T cells expressed Th1-type cytokines.

Table S1. Antibodies used for flow cytometry studies.

Specificity	Supplier	Clone
CD3	BD Pharmingen	SK7
CD4	BD Horizon	RPA-T4
CD8	BD Pharmingen	SK1
CD11b	Beckman Coulter	Bear1
CD11c	BD Horizon	B-ly6
CD14	BD Horizon	M ϕ P9
CD16	BD Pharmingen	3G8
CD25	BD Pharmingen	M-A251
CD28	BD Biosciences	L293
CD45	Beckman Coulter	J33
CD45RA	BD Pharmingen	HI100
CD45RO	BD Pharmingen	UCHL1
CD57	BD Pharmingen	NK-1
CD62L	BD Pharmingen	DREG-56
CD80	BD Pharmingen	L307.4
CD83	BD Pharmingen	HB15e
CD86	BD Pharmingen	2331/FUN-1
CD127	BD Pharmingen	HIL-7R-M21
CD134/OX40	Becton Dickinson	L106
CD152/CTLA4	BD Pharmingen	BNI3
CD163	BD Pharmingen	GHI/61
CD197/CCR7	BD Pharmingen	3D12
CD223/LAG-3	R&D Systems	Goat polyclonal
CD278/ICOS	eBioscience	ISA-3
CD279/PD1	BD Pharmingen	EH12.1
CD282/TLR2	eBioscience	TL2.1
CD357/GITR	eBioscience	eBioAITR
Foxp3	eBioscience	PCH101
HLA-DR	BD Bioscience	G46-6
LAP/TGF β 1	R&D Systems	27232

GeneSymbol	Alternative name	Fresh CD4+ T cells	CD4+ T cells cultured alone for 5d	IFN- γ M ϕ cocultured CD4+ T cells	M reg cocultured CD4+ T cells	fold-change (M reg > IFN- γ M ϕ)	p-value (t-test of M reg vs. IFN- γ M ϕ)
BTNL8		-0,027	-0,033	-0,050	5,119	35,970	0,00001
EBI3		-1,709	-0,021	1,019	5,233	18,552	0,00008
TNFRSF8	CD30	0,773	-0,213	0,542	3,856	9,948	0,00034
GPR35		-0,857	-0,640	0,120	3,342	9,332	0,00033
BIC		-1,101	0,575	-0,276	2,499	6,847	0,00095
TNFRSF4	CD134; OX40	1,630	-0,297	2,059	4,796	6,667	0,00004
VDR		-0,691	-0,756	2,855	5,515	6,321	0,00019
TNFRSF18	GITR	-0,212	-1,647	0,953	3,419	5,527	0,00315
CD38		-3,003	-4,069	1,538	3,786	4,748	0,00168
IL1R2		-0,395	0,770	1,786	4,002	4,644	0,00257
CXCR6		-2,701	-2,817	1,338	3,486	4,433	0,01533
IL2RA	CD25	0,702	-0,724	0,248	1,926	4,400	0,02805
CCR8		0,575	0,404	0,812	2,750	3,832	0,00552
TNFRSF9	CD137	-2,366	-1,396	-1,025	0,748	3,418	0,04786
FCRL3		-0,517	-1,492	-1,363	0,372	3,329	0,00217
LAG3		-2,821	-3,173	0,150	1,871	3,298	0,00142
PRDM1	BLIMP1	0,404	-1,018	-0,289	1,144	2,700	0,00116
NR4A3		-2,959	-0,055	0,225	1,604	2,600	0,02970
TNFRSF1B	CD120b; TNFR2	0,101	-0,422	0,248	1,362	2,442	0,00953
DUSP16		0,609	-0,479	0,369	1,591	2,333	0,00079
FOXP3		0,485	0,169	0,477	1,658	2,268	0,00095
CCR8		0,575	0,404	0,812	2,750	2,254	0,01503
ICOS		0,013	0,234	0,727	1,824	2,138	0,00053
SOCS2		1,581	-1,331	-0,609	0,487	2,137	0,03470
CTLA4		-0,037	-1,089	1,947	2,999	2,073	0,00445
CD274	PDL1	-0,909	0,232	0,410	1,449	2,055	0,04800
DUSP10		-0,463	-0,861	0,157	1,176	2,027	0,00216
IL1R1		0,401	-0,139	1,278	2,296	2,025	0,01317
CCR4		1,170	0,095	0,245	1,256	2,015	0,00309

Table S2: A subset of 30 T reg-associated markers selected from the 572 reporters significantly up-regulated in M reg-cocultured T cells compared to IFN- γ M ϕ -cocultured T cells. Values are median normalised log₂-transformed intensities.



Tailor the adaptive immune response with

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Laser Ablation–Inductively Coupled Plasma Mass Spectrometry: An Emerging Technology for Detecting Rare Cells in Tissue Sections

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Amy J. Managh, Robert W. Hutchinson, Paloma Riquelme, Christiane Broichhausen, Anja K. Wege, Uwe Ritter, Norbert Ahrens, Gudrun E. Koehl, Lisa Walter, Christian Florian, Hans J. Schlitt, Helen J. Reid, Edward K. Geissler, Barry L. Sharp and James A. Hutchinson

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Laser Ablation–Inductively Coupled Plasma Mass Spectrometry: An Emerging Technology for Detecting Rare Cells in Tissue Sections

Amy J. Managh,* Robert W. Hutchinson,[†] Paloma Riquelme,[‡] Christiane Broichhausen,[‡] Anja K. Wege,[§] Uwe Ritter,[¶] Norbert Ahrens,^{||} Gudrun E. Koehl,[‡] Lisa Walter,[‡] Christian Florian,[‡] Hans J. Schlitt,[‡] Helen J. Reid,* Edward K. Geissler,[‡] Barry L. Sharp,* and James A. Hutchinson[‡]

Administering immunoregulatory cells to patients as medicinal agents is a potentially revolutionary approach to the treatment of immunologically mediated diseases. Presently, there are no satisfactory, clinically applicable methods of tracking human cells in patients with adequate spatial resolution and target cell specificity over a sufficient period of time. Laser ablation–inductively coupled plasma mass spectrometry (LA-ICP-MS) represents a potential solution to the problem of detecting very rare cells in tissues. In this article, this exquisitely sensitive technique is applied to the tracking of gold-labeled human regulatory macrophages (Mregs) in immunodeficient mice. Optimal conditions for labeling Mregs with 50-nm gold particles were investigated by exposing Mregs in culture to variable concentrations of label: Mregs incubated with 3.5×10^9 particles/ml for 1 h incorporated an average of 3.39×10^8 Au atoms/cell without loss of cell viability. Analysis of single, gold-labeled Mregs by LA-ICP-MS registered an average of 1.9×10^5 counts/cell. Under these conditions, 100% labeling efficiency was achieved, and label was retained by Mregs for ≥ 36 h. Gold-labeled Mregs adhered to glass surfaces; after 24 h of culture, it was possible to colabel these cells with human-specific ^{154}Sm -tagged anti–HLA-DR or ^{174}Yb -tagged anti-CD45 mAbs. Following injection into immunodeficient mice, signals from gold-labeled human Mregs could be detected in mouse lung, liver, and spleen for at least 7 d by solution-based inductively coupled plasma mass spectrometry and LA-ICP-MS. These promising results indicate that LA-ICP-MS tissue imaging has great potential as an analytical technique in immunology. *The Journal of Immunology*, 2014, 193: 000–000.

Administration of regulatory or suppressor cell populations to nontolerant recipients as a means of establishing tolerance is a well-known technique in experimental immunology, but its clinical application is only now being seriously investigated (1). In principle, a broad spectrum of immunological conditions might be amenable to treatment with cell-based immunoregulatory therapies, including T cell–mediated autoimmune diseases (2, 3), chronic inflammatory conditions (4), graft-versus-host disease (5), and solid organ transplant rejection (6). In such conditions, cell-based immunoregulatory therapies might reduce or obviate the need for conventional immunosuppressive therapy,

sparing patients its attendant complications. Moreover, because tolerance achieved through peripheral regulation (as opposed to purely deletional mechanisms or immunological ignorance) is dominant and self-sustaining, there exists the staggering possibility that cell-based immunotherapy might offer curative treatments for diseases that would otherwise require long-term general immunosuppressive therapy (7).

Several immunoregulatory cell types are now reaching the point of preclinical development that would allow them to be investigated as immunosuppressive agents in early-phase clinical trials (8–11). One particularly promising candidate cell type for use as an adjunct immunosuppressive agent in transplantation is the regulatory macrophage (Mreg) (12). The Mreg reflects a unique state of macrophage differentiation, distinguished from macrophages in other activation states by its mode of derivation, robust phenotype, and potent T cell suppressor function (13). Mregs potently suppress mitogen-stimulated T cell proliferation in vitro, which can be attributed to IFN- γ -induced IDO activity, as well as contact-dependent deletion of activated T cells. In addition, Mregs drive the development of activated induced regulatory T cells that, in turn, suppress the proliferation of effector T cells and inhibit the maturation of monocyte-derived dendritic cells in response to TNF- α (14). Therefore, it is thought that when Mregs are administered to an allogeneic recipient they can initiate a feed-forward loop of immunological regulation, potentially leading to the long-term acceptance of a foreign transplant (15).

To use any therapeutic agent safely and effectively, clinicians must be able to predict its effectiveness and safety in individual patients (16). An important determinant of the biological effect and safety of any therapeutic cell product is its pharmacokinetics

*Centre for Analytical Science, Department of Chemistry, Loughborough University, Loughborough, Leicestershire LE11 3TU, United Kingdom; [†]Electro Scientific Industries, Huntingdon, Cambridgeshire PE29 6XS, United Kingdom; [‡]Division of Experimental Surgery, Department of Surgery, University Hospital Regensburg, Regensburg 93053, Germany; [§]Department of Obstetrics and Gynaecology, University Hospital Regensburg, Regensburg 93053, Germany; [¶]Institute for Immunology, University of Regensburg, Regensburg 93053, Germany; and ^{||}Department of Transfusion Medicine, University Hospital Regensburg, Regensburg 93053, Germany

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Address correspondence and reprint requests to Dr. James A. Hutchinson, Department of Surgery, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany. E-mail address: james.hutchinson@ukr.de

The online version of this article contains supplemental material.

Abbreviations used in this article: DPBS, Dulbecco's PBS; LA-ICP-MS, laser ablation–inductively coupled plasma mass spectrometry; Mreg, regulatory macrophage.

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(i.e., its distribution, survival, and fate in the recipient). Typically, pharmacokinetic studies in humans are performed as part of the early clinical pharmaceutical development process; however, in the case of Mregs, and cell-based medicinal products in general, adequate methods for reliably detecting infused cells in tissues over days or weeks after transfer are not available (17). In a previous study, we used single-photon emission computed tomography to track ^{111}In oxine-labeled allogeneic human Mregs in one patient: Mregs given by central venous infusion were first concentrated in the pulmonary vasculature but by 3 h postinfusion had migrated to the liver, spleen, and hematopoietically active bone marrow via the blood (13). Although this radiological tracing study provided valuable information, it tells us very little about the precise distribution of Mregs within tissues, and, owing to the short half-life of ^{111}In , the survival and fate of Mregs after 30 h postinfusion could not be characterized.

Cell tracking in patients presents a number of substantial obstacles (18). To identify cells after administration to a recipient, it is necessary that they are specifically and durably labeled. A variety of cell labeling reagents and corresponding detection technologies exist, notably radioisotopic labeling (19), stable isotope labeling (e.g., deuterium), nuclear magnetic resonance labels (20), nanoparticles (21), and luminescent dyes (22); however, to some degree, each of these approaches is limited by detection sensitivity, signal nonspecificity, and the impermanence or toxicity of labels. Thus, there is an unfulfilled need for a cell labeling and cell detection technology capable of reliably quantifying very low-frequency cells (perhaps as few as 1 in 10^7 cells) in tissues with a very low false-positive detection rate. Most importantly, none of the existing cell-tracking techniques are capable of reliable single-cell detection at a suitable level of resolution to identify single cells and their microanatomical context. Judged by these performance requirements, existing technologies are not suitably sensitive or specific, so alternative approaches must be considered.

Laser ablation–inductively coupled plasma mass spectrometry (LA-ICP-MS) is an analytical technique that is predominantly used in earth and material sciences, but it is also beginning to be applied to analysis of biological samples (23). In principle, LA-ICP-MS entails vaporizing small parts of a sample using a focused pulse of laser energy. The elemental composition of this vaporized material is analyzed by mass spectrometry, which provides exquisitely sensitive detection. By taking contiguous samples across a specimen, a map of the distribution of elements within a complex sample can be constructed. This strategy has been used to visualize endogenous elements in biological tissues (24), and elemental tagging prior to LA-ICP-MS enables specific tissue features, such as amyloid deposits in brain tissue (25), to be distinguished. Applications of this remarkable technique to the problem of tracking rare cells would overcome many of the limitations associated with other methodologies: A “microscope” that used LA-ICP-MS to map tissue components labeled with rare earth metals would be incredibly sensitive and specific, because most rare earth metals are completely absent from tissues. Hence, the objective of the work presented in this article was to develop an LA-ICP-MS method for identifying labeled human Mregs in tissues specimens.

Materials and Methods

Experiments in animals

Animal experiments were performed in accordance with permission Nr. 54-2532.1-10/12 granted by the Regierung von Oberbayern. NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice were bred in-house. Animals were kept in individually ventilated cages and fed a conventional diet.

Generation and gold labeling of human Mregs

Human Mregs were generated as previously described (26). Briefly, CD14⁺ monocytes were isolated from Ficoll-prepared PBMCs by positive selection using anti-CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were plated in six-well Cell+ plates (Sarstedt, Nümbrecht, Germany) at 10^6 cells/well in RPMI 1640 (Lonza, Cologne, Germany) containing 10% heat-inactivated human AB serum (Lonza), 2 mM GlutaMAX (Invitrogen, Karlsruhe, Germany), 100 U/ml penicillin (Lonza), 100 $\mu\text{g}/\text{ml}$ streptomycin (Lonza), and recombinant human M-CSF (R&D Systems, Wiesbaden-Nordenstadt, Germany) at 25 ng/ml carried on 0.1% human albumin (CSL-Behring, Hattersheim-am-Main, Germany). On day 6 of culture, cells were stimulated for 18 h with 25 ng/ml recombinant human IFN- γ (Chemicon, Billerica, MA). To label Mregs with 50-nm gold nanoparticles (Sigma, Munich, Germany), human Mregs were harvested, washed twice in Dulbecco's PBS (DPBS), and resuspended at 10^6 cells/ml in prewarmed X-Vivo 10 (Lonza) containing 3.5×10^9 gold nanoparticles/ml. The cell suspension was incubated at 37°C for 1 h in a closed 15-ml tube with end-over-end rotation. Thereafter, the cells were washed three times in DPBS before further processing.

Flow cytometry

Cell surface staining was performed at 4°C in DPBS/1% BSA/0.02% NaN₃/10% FcR-block (Miltenyi Biotec), as described elsewhere (13). Dead cells were excluded with 7-aminoactinomycin D (BD Biosciences, Heidelberg, Germany). Data were collected using a FACSCanto II cytometer (BD Biosciences), and the proportion of live Mregs, gated by physical characteristics, was analyzed using FlowJo v7.6.5 software. To assess the T cell-suppressive capacity of Mregs, allogeneic PHA-stimulated CFSE-labeled CD3⁺ T cells and Mregs were set in direct 1:1 coculture for 5 d. Subsequently, T cell proliferation was quantified by flow cytometry, as previously described (26).

Culture of gold-labeled Mregs in chamber slides

Mregs were resuspended in Mreg-generation medium (as above) before replating into chamber slides (BD Falcon, Heidelberg, Germany) at a density of 10^6 viable Mregs/ml. Cells replated in chamber slides were allowed to adhere over 24 h. For counterstaining, adherent Mregs were washed three times in ice-cold DPBS, air dried, and stained with Diff-Quik Giemsa reagents (Medion Diagnostics, Munich, Germany).

Administration of gold-labeled human Mregs to NSG mice and tissue preparation

Immediately prior to injection, the concentration of gold-labeled Mregs was adjusted to 5×10^6 viable cells/ml in DPBS, without Ca²⁺ or Mg²⁺, supplemented with 60 IU/ml heparin. Cell suspensions were injected through a 27-gauge needle into the tail vein of recipient NSG mice over 30–180 s. For solution-based ICP-MS and LA-ICP-MS analyses, organs were harvested at 24 h or 7 d postinfusion by postmortem dissection. Tissues for analysis by LA-ICP-MS were fixed in paraformaldehyde and embedded in paraffin wax, and 6- μm sections were cut. Sections were mounted on SuperFrost glass microscope slides (Thermo Fisher Scientific, Schwerte, Germany) and stored in a dry environment at 37°C for several days prior to further processing. Sections were dewaxed with Roti-Histol (Carl Roth, Karlsruhe, Germany) and progressively rehydrated in increasingly dilute ethanol. Following conventional immunohistochemical techniques, tissues were blocked with 5% normal mouse serum in TBS for 1 h. Tissues were stained overnight at 4°C using primary metal-tagged MaxPar Abs (Fluidigm Sciences, Sunnyvale, CA) diluted in TBS + 5% normal mouse serum, according to the manufacturer's recommendations. Specifically, tissues were stained with ^{154}Sm -tagged anti-CD45 (clone H130) or ^{174}Yb -tagged anti-HLA-DR (clone L243). Sections were counterstained with H&E before progressive dehydration in alcohol and air drying.

Solution-based ICP-MS

Analyses were performed on a sector-field ICP-MS instrument (Element 2XR; Thermo Scientific, Bremen, Germany). The instrument was fitted with a cyclonic spray chamber, a conical glass concentric nebulizer (both from Glass Expansion, West Melbourne, VIC, Australia), and a 0.25-mm inner diameter probe (Elemental Scientific, Omaha, NE). Prior to analysis, the samples were digested in small volumes of concentrated acid using the procedure reported previously by our group (23). Following evaporation of the acid, a 5% aqua regia solution was used to reconstitute the samples. Gold is known to have a high affinity for the surfaces of the sample introduction system (27). Therefore, to minimize memory effects, solutions of 5% aqua regia and 10 mM cysteine were aspirated between samples.

LA-ICP-MS

Analyses were performed on a laser ablation system (UP-213, Nd:YAG, λ 213 nm; ElectroScientific Industries) coupled to a sector-field ICP-MS instrument (Element 2XR; Thermo Scientific) in external trigger mode. The laser ablation system was fitted with a low-volume, teardrop-shaped ablation cell, which was described elsewhere (28). Helium was used as the ablation gas, at a typical flow rate of 0.6 l/min, with an argon make-up flow introduced, at a flow rate of 0.8 l/min, after the ablation cell. Single-cell identification was demonstrated by ablating 55- μ m-diameter areas at locations corresponding to individual cells. Only single cells were targeted; cells present in clusters or $<55 \mu\text{m}$ apart were discounted. The presence of label was determined from the time-resolved ^{197}Au signal-intensity profile. Imaging of the cells and tissue sections was accomplished by performing adjacent line scans over sections of the slide, while measuring ^{197}Au , ^{154}Sm , or ^{174}Yb in time-resolved mode. Images were constructed using Iolite version 2.15 (Melbourne Isotope Group, Melbourne, VIC, Australia), a freeware data deconvolution package that runs on the IGOR-Pro 6.22A platform (WaveMetrics, Lake Oswego, OR). The software converts each raw data point into a color-coded pixel; thus, the color profile of the resulting image depicts the distribution of the respective elements across the sampled region.

Results

Labeling of human Mregs for detection by LA-ICP-MS

From an analytical chemistry perspective, most d-block and f-block elements can be equally easily quantified by LA-ICP-MS. Metal labels can be incorporated into living cells by various biological processes, including phagocytosis, pinocytosis, receptor-mediated endocytosis, or cell surface labeling. Retention of a metal label may be passive or active, and it may or may not require reaction of the metal with cellular constituents. The choice of metal label, its chemical form, and the mechanisms by which it is taken up and retained by living cells determine its properties as a tracer label. From a biological perspective, the qualities that we expect of a useful living cell label for *in vivo* tracking studies are durability, biological inertness, and specificity; hence, absence of label from normal tissues. Omniscan, a gadolinium-based nuclear magnetic resonance contrast agent, was shown to be a suitable reagent for labeling human regulatory T cells, which presumably acquired label by fluid-phase pinocytosis (23).

In the current study, 50-nm gold particles were selected as a label because Mregs are known to be highly phagocytic (15). Optimal conditions for labeling were determined by incubating Mregs with gold particles at a range of concentrations for various periods prior to measuring cellular gold content by solution-based ICP-MS (Table I). Label uptake was time and concentration dependent and did not reach saturation within the range of tested conditions; nevertheless, gold incorporation after 1 h of incubation with 3.5×10^9 particles/ml was >500 -fold the lower limit of detection. To ascertain whether gold labeling had any effect on Mreg viability, cell surface phenotype, or *in vitro* function, gold-labeled and unlabeled Mregs were analyzed by flow cytometry and suppression assays (Fig. 1). After labeling, no differences in dead cell

frequency, marker phenotype, or suppressor activity were observed. Accordingly, gold nanoparticles at concentrations of 3.5×10^9 particles/ml are suitable for labeling human Mregs without obvious toxic effects.

Laser ablation of single Mregs

To assess the uptake of gold label by individual cells, gold-labeled Mregs were washed extensively after labeling and replated in chamber slides. The majority of replated Mregs readhered to the vessel surface within 1 h and acquired typical Mreg morphology (Fig. 2A). After 1 h, the adherent cells were washed extensively and Giemsa stained prior to analysis by LA-ICP-MS. Single Mregs were ablated with single shots of a 55- μ m-diameter laser. This beam size ensured complete ablation of an individual cell, while reducing the chance of inadvertently ablating neighboring cells. LA-ICP-MS of the labeled cells yielded an average signal intensity of 1.9×10^5 counts/cell (77% relative SD). Each signal was clearly distinguishable from the low background signal ~ 0 –10 counts (Fig. 2B). No signals above background were observed for the ablation of nonlabeled cells or from control areas of the slide containing no cells (Fig. 2C). This demonstrated that labeled cells can be rapidly and reliably distinguished from their non-labeled counterparts, a point that was subsequently confirmed during the analysis of mixed populations of labeled/nonlabeled cells (Table II). To prove that gold nanoparticles are retained by living Mregs, labeled cells were cultured for 36 h in chamber slides before LA-ICP-MS analysis. Clear signals, with an average signal intensity of 1.6×10^5 counts/cell (69% relative SD), were obtained for this analysis (Fig. 2D). Therefore, it is concluded that living, adherent Mregs can be labeled with 100% labeling efficiency and retain gold nanoparticles for ≥ 36 h after labeling.

The above experiments sampled entire cells to demonstrate 100% cell labeling and detection capability. For tissue imaging, it is clearly beneficial to decrease the diameter of the laser spot to less than the size of the cell and, hence, increase the resolution of the image. To demonstrate that the cells internalize sufficient label for high-resolution imaging, 8- μ m-wide line scans were performed across a cluster of labeled cells (Fig. 3). The count rates remained sufficient to enable detection of the cells, whereas the 8- μ m spot size was sufficient to discriminate between the signals from individual cells.

Gold-labeled Mregs can be dual labeled with ^{154}Sm -tagged or ^{174}Yb -tagged Abs

When gold-labeled Mregs are administered to a recipient, release of gold nanoparticles and subsequent reuptake by recipient phagocytes could lead to misidentification of those recipient cells as Mregs. One solution to this problem is to establish that cells bearing the tracer label express the anticipated marker phenotype by immunohistochemical “postlabeling” using metal-conjugated Abs.

Table I. Optimizing conditions for labeling human Mregs with 50-nm gold particles

Gold Concentration (particles/ml)	Incubation Time		
	10 s	1 h	2 h
0	Not detected	Not detected	Not detected
3.50×10^7	Not detected	1.79E+06	1.75E+06
1.75×10^8	Not detected	1.06E+07	9.52E+06
3.50×10^8	1.54E+06	3.09E+07	3.76E+07
1.75×10^9	1.05E+07	1.71E+08	3.64E+08
3.50×10^9	1.51E+07	3.39E+08	4.36E+08

Human Mregs were incubated with various concentrations of gold nanoparticles, and their gold uptake was determined by ICP-MS. Data are the average number of gold atoms internalized/cell for each labeling condition.

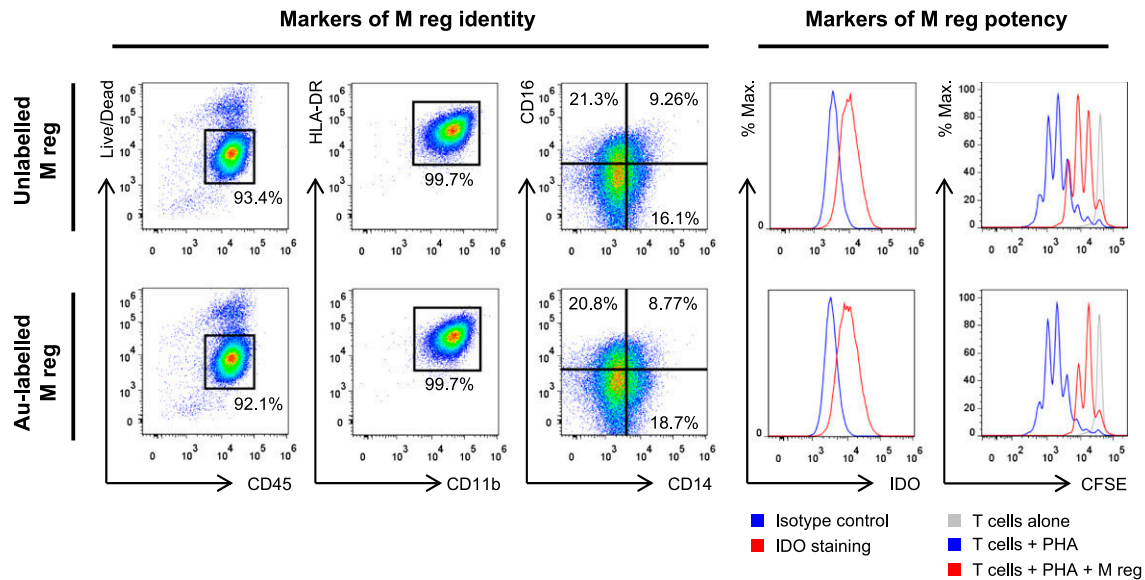


FIGURE 1. Labeling human Mregs with 50-nm gold nanoparticles did not affect their viability, phenotype, or suppressor function. Human Mregs were incubated for 1 h in the presence or absence of 3.5×10^9 gold nanoparticles/ml. Subsequently, the phenotype and viability of unlabeled and gold-labeled Mregs were assessed by flow cytometry. Data are representative of $n = 3$ independent allogeneic pairs. No difference was observed in the viability of gold-labeled Mregs compared with unlabeled Mregs. Likewise, no differences were observed between gold-labeled and unlabeled Mregs in the expression of CD11b, HLA-DR, CD14, or CD16. Both gold-labeled and unlabeled Mregs expressed similar levels of IDO, which is indispensable for the T cell-suppressive function of human Mregs. Accordingly, gold labeling of Mregs did not affect their capacity to suppress polyclonal proliferation of CFSE-labeled, PHA-stimulated allogeneic CD4⁺ T cells in direct 1:1 cocultures.

Therefore, we examined the technical feasibility of codetecting Mregs labeled with gold and ¹⁷⁴Yb-tagged anti-HLA-DR (Fig. 4A) or ¹⁵⁴Sm-tagged anti-CD45 (Fig. 4B) Abs. Mregs were labeled with gold nanoparticles prior to overnight culture in chamber slides. Unfixed, adherent Mregs were counterlabeled by incubation with

metal-conjugated Abs. After extensive washing, the dual-labeled Mregs were analyzed by LA-ICP-MS. Coextensive signals were obtained for gold labeling and the postlabels, proving that this method could, in principle, be used to detect dual labeling of cells in tissues.

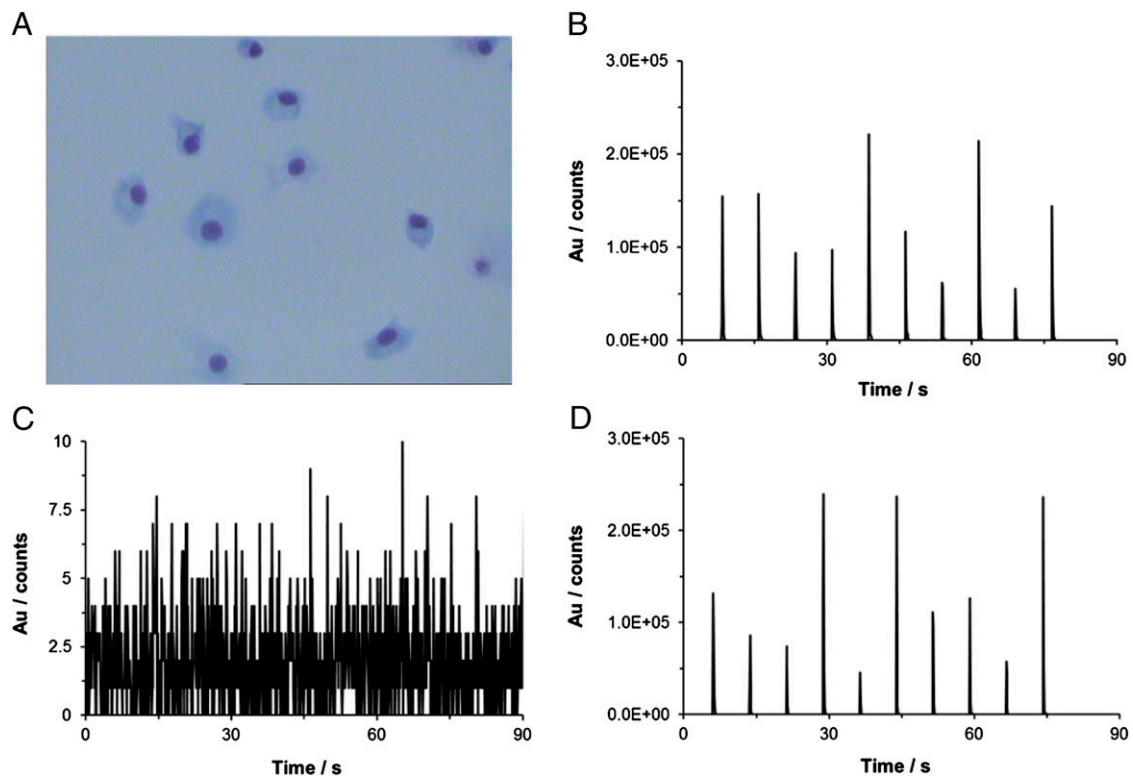


FIGURE 2. One hundred percent labeling efficiency was achieved when Mregs were incubated with 3.5×10^9 gold nanoparticles/ml for 1 h. **(A)** After labeling with gold nanoparticles, Mregs were able to readhere to a plastic tissue culture surface. Cells were visualized using a Giemsa counterstain. **(B)** Single gold-labeled Mregs were ablated with single shots of a 55- μ m-diameter laser, yielding an average signal intensity of 1.9×10^5 counts/cell (77% relative SD). **(C)** No signals above background were observed when unlabeled Mregs were ablated. **(D)** Labeled Mregs retained gold for ≥ 36 h after labeling.

Table II. Specificity of ^{197}Au labeling of human Mregs

Ratio of Labeled/Nonlabeled Cells	Culture Time after Labeling (h)	No. of Cells Ablated	No. of Labeled Cells Detected
1:0	1	100	100
0:1	1	100	0
1:1	1	100	45
1:0	36	100	100
0:1	36	100	0
1:1	36	100	43

Various proportions of gold-labeled Mregs were plated in chamber slides and allowed to adhere for 1 or 36 h. The relative numbers of labeled and unlabeled Mregs were assessed by LA-ICP-MS. Labeled and unlabeled Mregs were clearly discriminable. There was no significant transfer of gold label from labeled to unlabeled Mregs over the 36-h incubation period.

Tracking of human Mregs in NSG mice

The eventual anatomical distribution of Mregs after i.v. infusion reflects their passive and active migration and engraftment into different tissues, as well as their death and elimination. To track the survival and tissue distribution of Mregs in vivo, gold-labeled human Mregs were injected into NSG mice. The presence of label in brain, heart, kidney, liver, spleen, small intestine, large intestine, skeletal muscle, and tail skin was assessed on days 1 and 7 postinjection by solution-based ICP-MS (Table III). At both time points, gold label was principally detected in lung, liver, and spleen (although above-background signal was also detected in kidney, heart, brain, and intestine), which is consistent with previous results from flow cytometry experiments that showed transient engraftment of Mregs in these tissues (28).

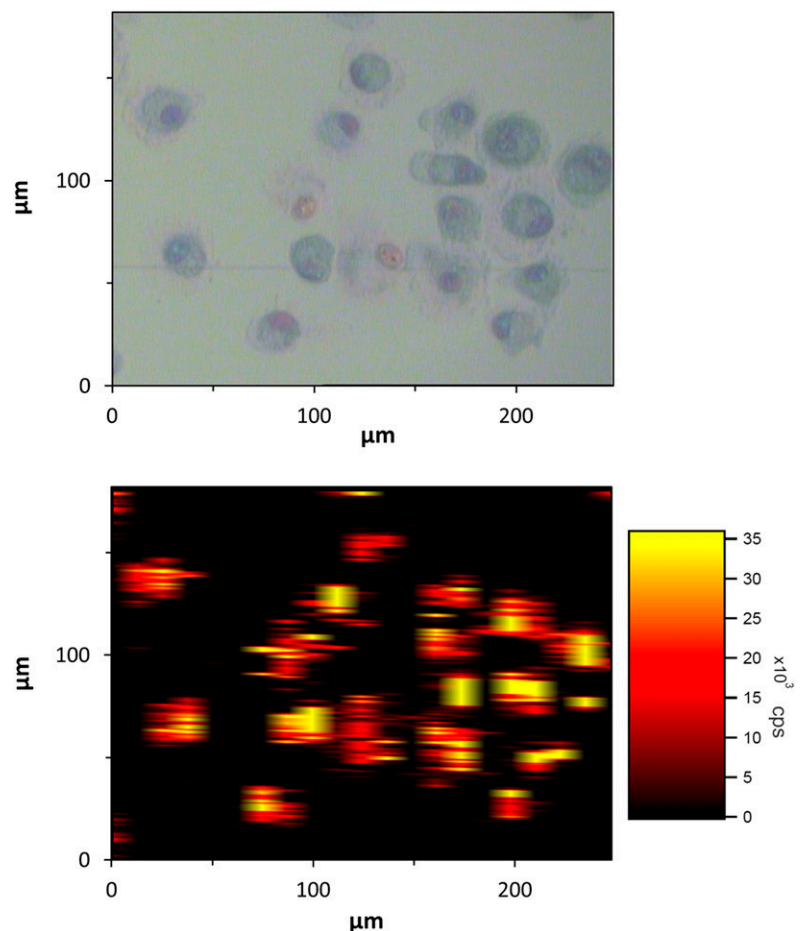
To investigate whether LA-ICP-MS permits detection of individual gold-labeled cells in tissues, recipient tissues were

harvested at day 1 or 7 postinjection of gold-labeled Mregs. These tissues were fixed with paraformaldehyde and embedded in paraffin. Six-micron sections of lung, liver, and spleen were conventionally counterstained with H&E prior to analysis by LA-ICP-MS. Sampled areas were $320 \times 245 \mu\text{m}$ in size, with two sampled areas selected per tissue section. As predicted by solution-based ICP-MS, label was detected in all three tissues at 1 and 7 d postinjection (Fig. 5). Within the relatively small areas of tissue examined, no particular pattern of signal distribution was perceived.

Discussion

Faced with the problem of detecting very rare cells in tissues, the aim of this work was to demonstrate that LA-ICP-MS can be used to identify metal-labeled cells in tissue samples with high sensitivity and specificity. In principle, this objective has been realized;

FIGURE 3. An 8- μm laser spot-size is sufficient to allow detection of gold-labeled Mregs. Eight-micrometer-line scans were made across a group of gold-labeled, Giemsa-counterstained Mregs. Count rates were adequate to detect labeled cells, whereas the spot size was small enough to allow spatial resolution of adjacent cells. The upper panel shows optical images and the lower panel shows the distribution of gold label.



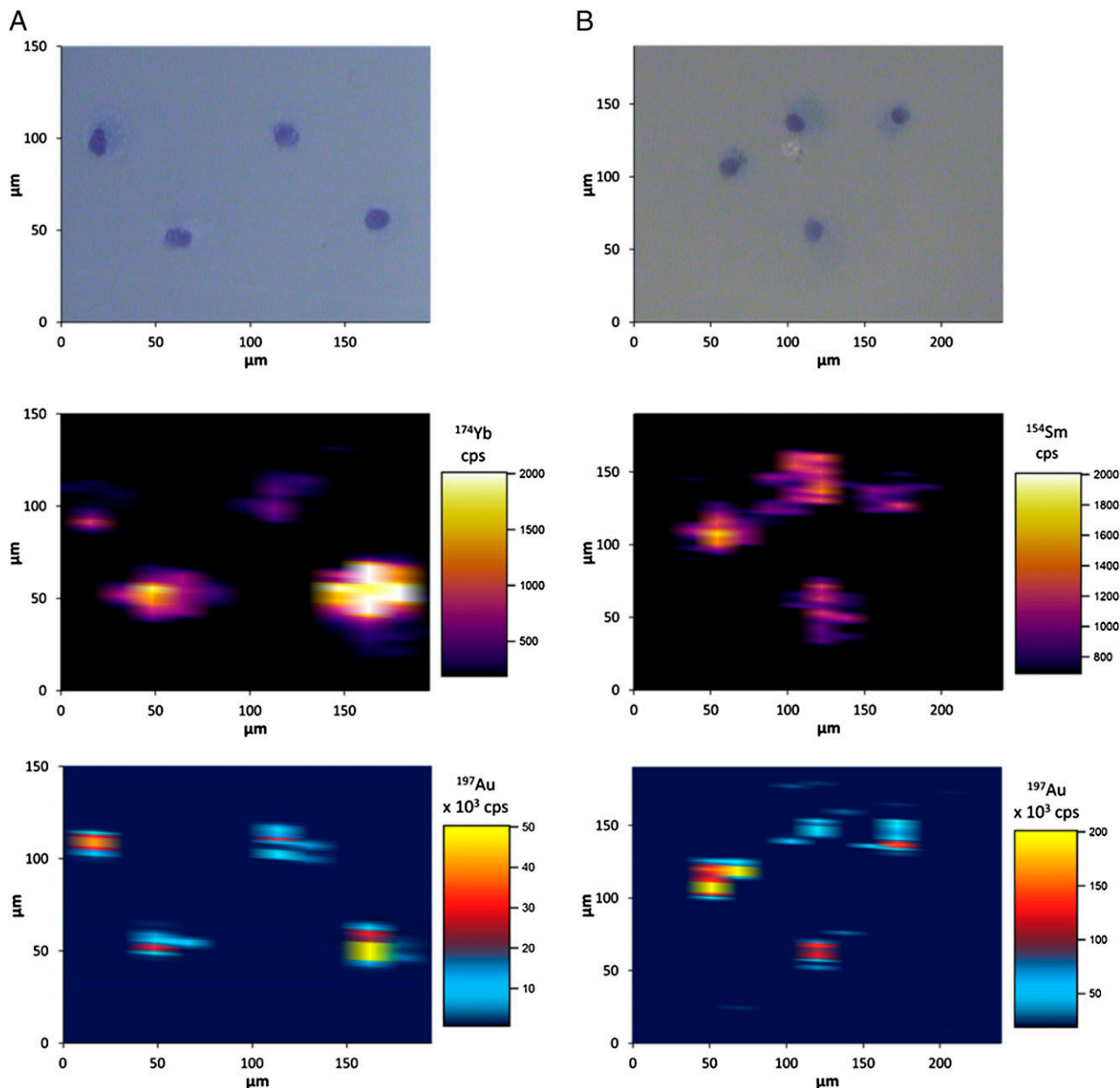


FIGURE 4. Gold-labeled Mregs can be dual labeled with ^{174}Yb -tagged or ^{154}Sm -tagged Abs. Gold-labeled Mregs were cultured overnight in chamber slides prior to postlabeling with either ^{174}Yb -conjugated anti-HLA-DR (**A**) or ^{154}Sm -conjugated anti-CD45 (**B**) mAbs. After extensive washing, the dual-labeled Mregs were counterstained with Giemsa and subjected to LA-ICP-MS analysis.

in practice, the technology is not ready to be commonly used in immunological research. Nevertheless, the results presented in this article indicate the enormous future potential of LA-ICP-MS in imaging biological specimens at a level of resolution suitable for identifying individual cells. Although this study focused on the narrow application of this approach to detecting gold-labeled Mregs, clearly LA-ICP-MS tissue imaging could be useful for a much broader range of applications. Therefore, we must ask ourselves how the current system might be improved to better serve the needs of biologists.

The resolution of images generated by LA-ICP-MS is dictated primarily by laser parameters (e.g., laser spot diameter, line spacing, repetition frequency, and scanning speed). Finer resolution is necessarily better when imaging histological specimens. In the current study, a beam width of 8 μm and scan speed of 8 $\mu\text{m/s}$ were used for the analysis of tissue sections, which provided sufficient resolution to identify single cells. In principle,

the resolution of images could be increased to ≤ 1 μm . However, the choice of laser parameters must take into account additional factors, such as the instrument sensitivity and the required analysis speed.

Presently, the speed of analyzing specimens is slow: to image an area of 1 mm^2 using an 8- μm spot diameter and 8- $\mu\text{m/s}$ scan speed takes ~ 4.5 h. The speed of analysis is largely limited by the washout time of the laser ablation chamber, which determines the duration of the signal from each laser pulse. Commercially available systems typically provide washout times in the order of a few seconds; however, with custom modifications, many users have reduced this time, in some cases to < 100 ms. With improved instrumentation, washout times might be reduced to ~ 10 ms, which would drastically improve both the sensitivity and the speed of analysis.

Using our current LA-ICP-MS system, it was possible to detect two labels on cultured cells; however, for many applications it

Table III. Presence of ^{197}Au -labeled human Mregs in tissues of recipient mice on days 1 and 7 postinjection by solution-based ICP-MS

Tissue	Day 1		Day 7	
	Au-Labeled Mregs	Unlabeled Mregs	Au-Labeled Mregs	Unlabeled Mregs
Lung	251.40 ± 13	0.15 ^a	122.47 ± 9	0.09 ^a
Spleen	147.85 ± 12	0.04 ^a	181.25 ± 11	0.07 ^a
Liver	120.46 ± 26	0.02 ^a	132.15 ± 29	0.02 ^a
Kidney	8.23 ± 12	0.23 ^a	2.56 ± 3	0.12 ^a
Heart	3.27 ± 56	0.23 ^a	1.09 ± 124	0.12 ^a
Skeletal muscle	0.48 ^a	0.12 ^a	0.49 ± 97	0.21 ^a
Brain	1.46 ± 22	0.04 ^a	0.06 ^a	0.07 ^a
Tail skin	35.70 ± 134	2.10 ^a	0.47 ± 24	nt
Large intestine	0.67 ^a	0.06 ^a	0.63 ± 44	0.45 ^a
Small intestine	1.90 ± 58	0.43 ^a	0.86 ± 47	0.50 ^a

Data are pg Au/mg tissue ± % RSD; $n = 3/\text{group}$.

^aBelow the limit of detection.

nt, not tested; RSD, relative SD.

would be useful to measure many more parameters. In principle, this could be achieved by combining the existing LA-ICP-MS with conventional fluorescence microscopy or by developing the system to detect multiple metal labels. The principal restriction on analyzing a greater number of metal labels is the rate at which sector field mass spectrometers detect each element, which they must do sequentially. Therefore, the capability of the current system to detect multiple metal labels could be improved by using a simultaneously detecting mass spectrometer, such as a time-of-flight mass spectrometer.

In this study, we applied LA-ICP-MS to the real-world biological problem of identifying rare cells in tissues. In doing so, we demonstrated the potential usefulness and power of this method in biological research. The technology presented in this article allowed detection of gold-labeled cells in tissues with very high specificity and sensitivity, as well as revealing their microanatomical relationship to recipient tissue structures. The specificity of LA-ICP-MS tissue imaging reflects the large signal-to-background ratio afforded by using rare earth metals or biologically inert nanoparticles as labels. The sensitivity of LA-ICP-MS tissue imaging (i.e., the ability to reliably detect very rare cells) is a reflection of the incredible sensitivity of mass spectrometry as an analytical technique. Unsurprisingly, there is now great interest in exploiting mass spectrometry as a specific and sensitive detection method in various biological applications, most notably in flow cytometry (30). A commercially available device known as the CyTOF is being adopted by many groups interested in clinical immune monitoring, with astonishing results (31, 32). The coupling of laser ablation with ICP-MS-based detection presented in this article takes this technology to a new level by combining detection of specific Ags with spatial resolution, allowing accurate, sensitive, and specific mapping of Ag distribution within tissues.

Concurrent detection of signals from Mregs prelabeled with gold and postlabeled with metal-tagged Abs would imply the presence of intact human Mregs in mouse tissues. Dual labeling and LA-ICP-MS detection of gold-labeled Mregs grown on chamber slides with either ^{174}Yb -tagged anti-HLA-DR or ^{154}Sm -tagged anti-CD45 was successful. Unfortunately, when sections of spleen from mice that had received gold-labeled Mregs were counterstained with the ^{174}Yb -conjugated anti-HLA-DR Ab before being analyzed at low resolution by LA-ICP-MS, very high and spatially diverse background signals for ^{174}Yb were detected (Supplemental Fig. 1). Therefore, no correlation in the distribution of ^{174}Yb and ^{197}Au signals could be established. We are unable to explain this nonspecific staining in mouse tissues. One possibility is that our metal-tagged mAbs are unsuitable for use on tissue sections,

possibly owing to the destruction of Ags during tissue fixation. Also, we cannot discount the possibility that MaxPar reagents have a greater propensity for nonspecific binding to tissues than do Abs with alternative labels. Because of the very high sensitivity of LA-ICP-MS, postlabeling with rare earth metal-labeled Abs might require development of more suitable metal-conjugated Abs, possibly labeled using different chemistries, as well as better histological techniques to block nonspecific binding of Abs. Importantly, our inability to identify colabeled Mregs does not detract from the potential usefulness of LA-ICP-MS in detecting colabeled cells in tissues; rather, it speaks to the current limitations of our metal-labeled Abs and histological techniques, which are ultimately soluble issues.

Albeit a niche application of LA-ICP-MS tissue imaging, characterizing the distribution, survival, and fate of therapeutic cells in patients will be critical to their future development as pharmaceutical agents (29, 33). LA-ICP-MS offers an unrivaled combination of sensitivity, specificity, and spatial resolution, which makes it an especially suitable method for cell tracking. Moreover, using LA-ICP-MS as a detection method makes available a variety of nonradioisotopic tracers, which may be safer and more convenient than currently available labels in the manufacture and application of cell-based therapies. Another exciting possibility of LA-ICP-MS cell tracking is that colabeling cells with two or more metal-containing compounds that are metabolized, degraded, or excreted by different mechanisms might make discrimination of live and dead cells possible, because only live cells would carry both labels. This article is proof-of-concept that gold-labeled Mreg can be detected in tissues for at least 7 d after infusion. Serious consideration is being given to the feasibility and clinical safety of labeling Mreg with gold nanoparticles for infusion into patients; indeed, clinical translation of cell tracking by LA-ICP-MS is a key objective of The ONE Study initiative (www.onestudy.org).

We conclude that, although LA-ICP-MS tissue imaging is a nascent technology, it has the potential to be a very powerful analytical technique in biology. If the present limitations of resolution and number of labels can be overcome, then LA-ICP-MS-based tissue imaging has a bright future.

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Disclosures

The authors have no financial conflicts of interest.

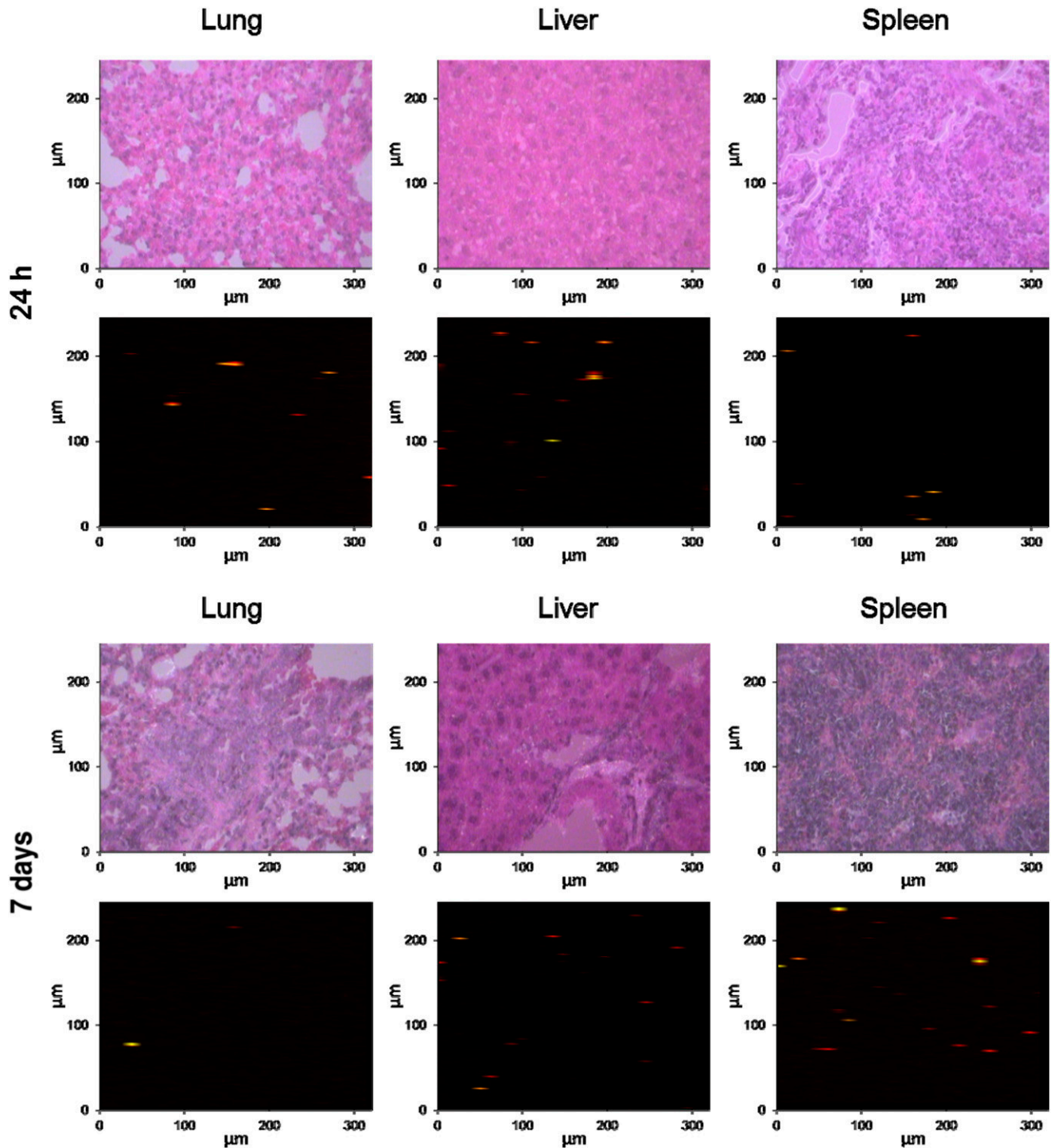


FIGURE 5. High-resolution imaging of small sections of mouse liver, lung, and spleen tissue by LA-ICP-MS. Optical and corresponding LA-ICP-MS images of mouse liver, lung, and spleen show the distribution of gold within each tissue. Regions of high gold intensity appear as colored spots in the LA-ICP-MS images. Tissues were harvested at 24 h (*upper panels*) and 7 d (*lower panels*) after administration of gold-labeled Mregs and were subsequently counterstained with H&E.

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