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Die Rolle von CD1a exprimierenden Monozyten und des Metabolismus in der Entzündung von Colitis ulcerosa

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München, 28.02.2021	Omar Al-Amodi
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Inhaltsverzeichnis

Abkürzungsverzeichnis

AA, AS	Aminosäuren
ATP	Adenosintriphosphat
CCL17	Chemokin Ligand 17, TARC
CD	Crohn’s disease, Morbus Crohn
CED	chronisch entzündliche Darmerkrankung, IBD
DC	dendritische Zelle
ECCO	European Crohn's and Colitis Organisation
FACS	fluorescence-activated cell scanning, Durchflusszytometrie
FoxP3	forkhead-box Protein 3, Transkriptionsfaktor
Glu	Glutaminsäure
GLUT4	Glukosetransporter Typ 4
HGF	hepatocyte growth factor
His	Histidin
HIV	humane Immundefizienz-Virus
IFNγ	Interferon γ
IL	Interleukin
Ma	Makrophage
MHC I	major histocompatibility complex I
MHC II	major histocompatibility complex II
Mo	Monozyt
moDCs	Monozyten-abgeleiteten dendritischen Zellen
NK	natürliche Killerzelle
NK-T	natürliche Killer T-Zelle
NOD Rezeptor	nucleotide-binding oligomerization domain Rezeptor
NSG-Maus	NOD-scid IL2Rγ-chainnull-Maus

PBMC	periphere mononukleäre Zellen des Blutes
PBS	phosphate buffered saline
PC	Phosphatidylcholin
PLA2	Phospholipase A2
PRRs	Pattern Recognition Receptors
SCCAI	simple clinical colitis activity index
TAG	Triglycerid
TARC	Thymusaktivitätsregulierte Zytokin
TGFß	transforming growth factor ß
TH0	naive T-Helferzelle
TH1	T-Helferzelle Typ 1
TH2	T-Helferzelle Typ 2
TH22	T-Helferzelle Typ 22
TH17	T-Helferzelle die IL-17 produziert
TNFα	Tumornekrosefaktor α
Treg	regulatorische T-Zellen
TSLP	thymic stromal lymphopoietin
TSLPR	Rezeptor für TSLP
UC	Ulcerative colitis, Colitis ulcerosa
qRT-PCR	quantitative real time Polymerasekettenreaktion

Publikationsliste für die kumulative Dissertation

Während meiner Doktorandentätigkeit unter Betreuung von Prof. Dr. med. Matthias Siebeck, und Dr. Roswitha Gropp sind folgende wissenschaftliche Artikel unter meiner Mitwirkung entstanden:

Originalarbeiten:

I. CD1a-Expressing Monocytes as Mediators of Inflammation in Ulcerative Colitis **Al-Amodi O,** Jodeleit H, Beigel F, Wolf E, Siebeck M, Gropp R. Inflammatory Bowel Diseases. 2018

   Fundstelle: https://academic.oup.com/ibdjournal/article/24/6/1225/4998980

II. Targeting ulcerative colitis by suppressing glucose uptake with ritonavir Jodeleit H, **Al-Amodi O,** Caesar J, Villarroel Aguilera C, Holdt L, Gropp R, et al. Disease Models & Mechanisms. 2018

   Fundstelle: https://dmm.biologists.org/content/11/11/dmm036210

Zusätzliche Publikationen und Kongressbeiträge

Autoantibodies as diagnostic markers and potential drivers of inflammation in ulcerative colitis.
PLOS ONE 2020, Henrika Jodeleit, Lisa Milchram, Regina Soldo, Gabriel Beikircher, Silvia Schönthaler, Omar Al-amodi, Eck hard Wolf, Florian Beigel, Andreas Weinhäusel, Matthias Siebeck, and Roswitha Gropp

CD1a expressing monocytes as potential therapeutic targets in ulcerative colitis
73. Jahrestagung der Deutschen Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten mit Sektion Endoskopie 2018. Al-amodi O., Jodeleit H., Beigel F., Wolf E., Gropp R., Siebeck M.

Targeting inflammation in ulcerative colitis by inhibition of glucose uptake
73. Jahrestagung der Deutschen Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten mit Sektion Endoskopie 2018. Jodeleit H., Al-amodi O., Caesar J., Villarroel Aguilera C., Holdt L., Gropp R., Beigel F., Siebeck M.

Failure to control inflammation as a driving force of symptoms and phenotype in the NSG mouse model of ulcerative colitis
European Cancer Congress (ECCO) 2018. H Jodeleit, P Palamides, O Al-amodi, G Beikircher, S Schönthaler, F Beigel, E Wolf, A Weinhäusel, M Siebeck, R Gropp

CD1a expressing monocytes as sensors and mediators of inflammation in ulcerative colitis,
European Cancer Congress (ECCO) 2018. O Al-amodi, H Jodeleit, F Beigel, E Wolf, M Siebeck, R Gropp

CD1a exprimierende Makrophagen als Biomarker und potentiell therapeutisches Target bei Colitis ulcerosa
134. Deutsche Gesellschaft für Chirurgie 2017, Omar alAmodi, H. Jodeleit, P. Palamides, R. Gropp, M. Siebeck

1. Einleitung

1.1 Allgemeines zu Colitis ulcerosa

Colitis ulcerosa (UC) gehört zu den chronisch entzündlichen Darmerkrankungen (CED). Die Inzidenz an Kolitis zu erkranken, ist weltweit steigend (1). Meist sind junge Patienten betroffen, die bei Diagnosestellung zwischen 16 und 25 Jahre alt sind (2). Die Krankheit ist durch Schübe aus wiederkehrenden Entzündungen des Dickdarms im Wechsel mit Remissionen gekennzeichnet (3). Die Patienten klagen oft über blutige Durchfälle, Bauchschmerzen, Gewichtsverlust und Ermüdung (4, 5). Morphologisch ist die UC durch multiple entzündliche Veränderungen, die sich in der Regel nur auf die Mukosa des Dickdarms beschränken gekennzeichnet (6). Die Krankheit verläuft chronisch und basiert in der Regel auf einer lebenslangen Therapie, was einen enormen Leidensdruck für die Patienten und eine Belastung für das gesamten Gesundheitssystems darstellt (7).

1.2 Ätiologie und Pathogenese

Sowohl die genaue Ursache als auch die genauen Abläufe der Entstehung von Colitis ulcerosa sind nicht eindeutig geklärt. Am Ehesten ist es eine Kombination aus genetischen, mikrobiotischen Faktoren und Umweltfaktoren, die zur dysregulierten Immunantwort führen (8). Der Darm ist ein wichtiger Bestandteil des menschlichen Immunsystems. Etwa 70 % aller Immunzellen befinden sich im Magen-Darmtrakt (9). Das gastrointestinale Immunsystem dient dazu, die Balance zwischen der Toleranz gegenüber Antigenen der physiologischen Darmflora einerseits und der Abwehr gegen pathogene Reize andererseits aufrechtzuhalten (10). Die Immunzellen des Darmepithels spielen dabei eine Schlüsselrolle. Das Epithel des Darms ist im ständigen Kontakt mit unterschiedlichen Antigenreizen und stellt eine physikalische Barriere zwischen dem intestinalen Mikrobiom und dem Lymphgewebe dar (11). Bei der UC kommt es zu einer Störung in der Barriere des intestinalen Epithels (12, 13). Es kommt zu einer erhöhten Permeabilität von Antigenen aus dem intestinalen Lumen, woraufhin vermehrt Immunzellen in der Lamina propria aktiviert werden (14). Durch weitere proinflammatorische Zytokine strömen neutrophile Granulozyten, Lymphozyten und Plasmazellen ein. Dieser ganze Prozess führt zu den typischen Gewebeschäden in Form von Kryptendestruktion, Abszessen und Ulzerationen (15, 16). Außerdem kommt es durch die Barrierestörung zu Ionenverlusten, sodass mehr Wasser osmotisch in das Lumen entzogen wird, was die Durchfälle verursacht (17). Insgesamt ist der Pathomechanismus dahinter sehr komplex. Vereinfacht lassen sich dadurch die gestörte Immunabwehr auf Epithelebene und die veränderte Reaktionen der angeborenen und erworbenen Immunantwort erklären (18).

1.3 Immunologische Grundlagen

1.3.1 Die angeborene Immunantwort

Die erste Immunantwort des Darms gegen eingedrungene Pathogene läuft über das angeborene Immunsystem. Diese Abwehrreaktion erfolgt sofort und unmittelbar, ist aber unspezifisch. Darmepithelzellen, Monozyten (Mo), Makrophagen, dendritische Zellen (DC) und natürliche Killerzellen (NKT und NK) sind Teil der angeborenen Immunreaktion (19). DC und Makrophagen erkennen via Toll-like receptor (TLR) oder NOD-like receptor (NLR) die Antigene und binden diese (20). Dadurch wird der Transkriptionsfaktor NFκB aktiviert (21, 22). Es kommt einerseits zur Ausschüttung der proinflammatorischen Zytokine (IL-6, IL-23, TNF) und anderseits zur Reifung der antigenpräsentierenden Zellen. Diese können die phagozytierten Antigene durch *major histocompatibility complex*-Moleküle (MHC Moleküle) an der T-Zelle präsentieren und aktivieren. Dann kann die intestinale Immunantwort weiter über das adaptive Immunsystem in Gang gesetzt werden (18, 23).

Monozyten und Makrophagen spielen eine entscheidende Rolle im Entzündungsprozess. Monozyten stammen aus hämatopoetischen Stammzellen ab. Mit Hilfe von IL-8 und TGFβ gelangen sie aus dem Blut in das Gewebe. Hier differenzieren sie weiter, und es entstehen Gewebemakrophagen (24). Durch Phagozytose bekämpfen sie unterschiedliche Pathogene. Außerdem können sie über ihre *Pattern Recognition Receptors* (PRRs) weitere immunologische Reaktionen einleiten. Die Colitis ulcerosa könnte demnach auch durch unpassende Aktivierung von PRRs und die daraus folgenden Entzündung entstehen (25). Im menschlichen Körper werden allgemein drei Subtypen von Monozyten mit jeweils verschiedenen Funktionen beschrieben (26). Die klassischen Monozyten können durch die Expression von CCR2 ins Zielgewebe auswandern und zu *tissue-resident* Makrophagen differenzieren (25).
Makrophagen üben ihre Aktivität auf verschiedene Arten aus. Sie können Zytokine freisetzten und dadurch ein entzündliches Milieu schaffen. Außerdem können sie bestimmte Signale vermitteln und lösen somit die Aktivierung von T-Zellen aus (27). Die Gewebe-Makrophagen der Lamina propria des Gastrointestinaltrakts können Pathogene phagozytieren, ohne eine weitere Immunreaktion auszulösen, was eine entscheidende Rolle bei der Homöostase im Darm darstellt (28). In diesem Gleichgewicht setzen sie TGFβ und IL-10 frei, um eine unregulierte Immunantwort zu verhindern (29). Inflammatorische Makrophagen werden nach ihrer Aktivierung in M1- und M2- Makrophagen unterschieden. Die Makrophagen vom M1-Typ wirken entzündlich, und in Kontrast dazu unterstützen Makrophagen vom M2-Typ die Wundheilungsprozesse (30).

Dendritische Zellen sind antigenpräsentierende Zellen in der Darmschleimhaut (31), wie auch in anderen Geweben des Körpers. Durch die Produktion von TGFβ tragen sie zur Differenzierung der TH0-Zellen zu regulatorischen T-Zellen bei (32). Werden die dendritischen Zellen zu stark stimuliert, kommt es zu einer Differenzierung der Effektorzellen und zu einer Vernachlässigung der regulatorischen T-Zellen. Es entsteht eine Immunantwort, die sich gegen die darmeigene Mikroflora oder gegen eigene Proteine richtet; man spricht dann von einem Toleranzverlust. Entsprechend dazu führt der Rückgang der T-regulatorischen Zellen in Colitis ulcerosa zu einem Verlust der peripheren Toleranz (18).

CD1a exprimierende Zellen haben die Fähigkeit, Lipidantigene an inaktive T-Zellen zu präsentieren. Dadurch werden die T-Zellen aktiviert und es kommt zur Freisetzung von IL-22, IL-13 und IFNγ. Diese T-Zellen werden als Th22-Zellen identifiziert.

CD1a ist ein Oberflächenglykoprotein und ähnelt in der Struktur MHC I. Als Marker für epidermale dendritische Zellen und Langerhans Zellen ist CD1a schon seit Längerem bekannt. CD1a erkennende T-Zellen gehören zum normalen aβ T-Zell-Repertoire und präsentieren einen Anteil von 0,02-0,4% im Körper eines gesunden Menschen (33). CD1a+ Zellen gehören also zum Abwehrmechanismus gegen Fremdpathogene, können aber gleichzeitig Eigenlipide wie Phosphatidylcholin (PC) oder Triglycerid (TAG) präsentieren, um autoreaktive T-Zellen zu aktivieren (33-36). Bei einer Entzündungsreaktion kann die Versorgung mit Fettsäureliganden durch Phospholipase A2 (PLA2) erfolgen. PLA2 setzt Fettsäuren aus Phospholipiden frei und kann somit die Entzündungsreaktion über CD1a verstärken. So können zum Beispiel Bienen- und Wespengifte die CD1a+ Zellen durch PLA2 aktivieren (37). Neuere Erkenntnisse legen nahe, dass das CD1a auf Langerhans-Zellen eine Entzündungsreaktion im Rahmen einer Kontaktdermatitis vermittelt, in dem die Freisetzung von IL-17 und IL-22 aus αß-CD4 + T-Zellen induziert wird (38). Darüber hinaus wurde eine CD1a-Expression auch in Monozyten-abgeleiteten dendritischen Zellen (moDCs) des Darms gefunden (39). Die moDCs sind durch die Expression von Interleukin 12 (IL-12) gekennzeichnet (40). Dies stützt die Idee eines proinflammatorischen Effekts von CD1a-exprimierenden Makrophagen (40-42).

1.3.2 Die adaptive Immunantwort

Die adaptive Immunreaktion ist zwar langsamer als die angeborene Immunantwort, hat aber im Gegensatz dazu einen hochspezifischen Ablauf. Als Hauptmitglieder haben sich die T- und B-Zellen etabliert. Die T-Zellen werden unterteilt in CD8+ zytotoxische T-Zellen und CD4+ T-Helferzellen. CD4+ Zellen vermitteln zwischen B- und T-Zellen. Antigenpräsentierende Zellen wie DC aktivieren über die spezifische Bindung des mit Antigen beladenen MHC II-Moleküls an den T-Zell-Rezeptor die naiven CD4+ TH0-Zellen. Sie differenzieren dann ja nach Zytokinmilieu zu TH1, TH2, TH17 oder regulatorischen T-Zellen (43). T-Zellen spielen eine wichtige Rolle bei der Produktion von proinflammatorischen Zytokinen und fungieren ebenfalls als Immunregulatoren (44).

TH1 Zellen sind für die Elimination von intrazellulären Pathogenen zuständig (45). Sie zeichnen sich durch die Produktion von IFNγ und IL-2 aus (46).

TH2 Zellen sind für die allergischen Reaktionen und den Schutz vor Parasiten verantwortlich (45). Durch die Produktion von IL-4, IL-5, IL-13 und IL-10 erfüllen TH2 Zellen ihre Aufgabe (47).

TH17-Zellen sind für die Immunantwort auf extrazelluläre Bakterien und Pilze verantwortlich. Sie produzieren IL-17, IL-17F und IL-22 (48).

Regulatorische T-Zellen sezernieren anti-inflammatorische Zytokine wie IL-10 und TGFβ, die Effektor T-Zellen inhibieren (49). Sie regulieren die Immunreaktion und wirken gegen das Entstehen von immunbedingten Entzündungen (50).

Colitis ulcerosa zeichnet sich durch eine TH2-Inflammation und die Freisetzung von IL-4, IL-5 und IL-13 aus (51, 52). Dadurch kommt es zur Barrieredysfunktion, Fibrose und epithelialer Hyperplasie (53). Außerdem konnte man vermehrt TH17 Zellen bei UC-Patienten feststellen. Diese haben einen proinflammatorischen Effekt und spielen eine wichtige Rolle bei Autoimmunerkrankungen (54, 55).

1.3.3 Zytokine und Chemokine

Als Regulatoren tragen die Zytokine und Chemokine zur Steuerung des Immunsystems bei. Sie sind spezifische Signalbotenstoffe und dienen der Kommunikation zwischen verschiedenen Immunzellen. Zytokine können sowohl pro-inflammatorische als auch anti-inflammatorische Wirkungen haben (56). Chemokine sind eine Gruppe von Zytokinen, deren Hauptaufgabe darin besteht, die Effektorzellen an den Entzündungsort anzulocken (57).

TGFβ (Transforming Growth Factor Beta) ist ein Wachstumsfaktor, der von dendritischen Zellen, B- und T-Lymphozyten sowie Makrophagen produziert wird. TGFβ reguliert die Proliferation und Differenzierung von diesen Zellen (58). Außerdem spielt der Faktor eine wichtige Rolle bei der Kontrolle der Autoimmunität. Über Foxp3 werden die regulatorische T-Zellen durch TGF induziert, um so die Homöostase aufrechtzuerhalten (59).

TARC (Thymusaktivitätsreguliertes Zytokin) oder auch CCL17 ist ein Chemokin, das von den dendritischen Zellen des Thymus, den Endothelzellen und den Fibroblasten sezerniert wird (60). Durch das TARC werden die T-Zellen in den Thymus angelockt, um ihre weitere Entwicklung zu regulieren (61). Die Expression von TARC im Darm ist dabei 20-fach erhöht bei Patienten mit aktiver UC im Vergleich zu gesunden Kontrollen (62). *In vivo* Versuchen kam es durch CCL17 zu einer Aktivierung von TH1 und TH17 Zellen sowie zu einer Bildung von inflammatorischen Zytokinen. Im Gegensatz dazu wurden die regulatorischen T-Zellen dadurch unterdrückt. Außerdem zeigten sich bei fehlender CCL17 Sekretion keine Kolitis-Symptome (63).

HGF (Hepatocyte Growth Factor) ist ein Wachstumsfaktor und wird hauptsächlich von mesenchymalen Stammzellen produziert (64). Er führt zur Proliferation der Epithelzellen und reguliert viele Wundheilungsprozesse (65, 66). In *in vivo* Versuchen wirkte die Therapie mit HGF anti-inflammatorisch gegen Kolitis, sodass es zu einer Besserung der Beschwerden kam (67).Vergleichend dazu konnte sich dieser positive Effekt auch histologisch bestätigen (68).

TNFα (Der Tumornekrosefaktor-α) wird von den Makrophagen sezerniert (69). TNFα-Werte zeigen sich bei UC-Patienten erhöht, was eine wichtige Rolle bei der Entstehung von Colitis spielt. Dadurch wird die epitheliale Barriere geschädigt und es kommt zu einer erhöhten Durchlässigkeit des Epithels. Außerdem führt TNFα weiterhin zur Freisetzung von Metalloproteinasen und somit zu Gewebeveränderungen (70). Der monoklonale anti-TNFα-Antikörper Infliximab wurde als wirksame Therapie bei UC entwickelt (71).

TSLP (Thymic stromal lymphopoietin) wird von intestinalen Epithelzellen sezerniert (72) Durch seinen Rezeptor TSLPR wirkt TSLP auf unterschiedliche Immunzellen und unterstützt die Differenzierung naiver CD4+ T-Zellen zu TH2-Zellen (73, 74). Weiterhin werden Eosinophile, Mastzellen und B-Zellen aktiviert, sodass dem TSPL eine Rolle bei allergischen Reaktionen zugeschrieben wurde (75). Genauso ist TSPL wichtig für die Aufrechterhaltung der Homöostase, und es hat ebenfalls einen antiinflammatorischen Effekt (76). So induziert TSLP die Differenzierung von M2 Monozyten und Fibrozyten (77-79).

1.4 Die metabolischen Aspekte der Inflammation

Eine Entzündungsreaktion ist ein Prozess mit einem sehr hohen Energiebedarf. Aktivierung und Proliferation von Immunzellen benötigen reichlich Energie. Die Glykolyse und die Oxidation von Lipiden und Aminosäuren sind die Hauptquellen der Energieversorgung von Immunzellen. Im Zustand der Homöostase, wo die Hauptaufgabe von Immunzellen die Aufrechterhaltung der Toleranz ist, dient der Lipidstoffwechsel als bevorzugte Quelle der

Energiegewinnung. Die Lipidoxidation ist der effizienteste, aber auch der langsamste Weg zur Energiegewinnung der meisten Zellen (80). Im Gegensatz dazu ist die Glykose ein metabolischer Pfad, der als Endprodukt rasch zwei ATP Moleküle und zwei Pyruvat Moleküle entstehen lässt (81). Zudem liefert die Glykolyse Bausteine für die Aminosäure- und DNS Synthese. Deswegen basiert die Energieversorgung von aktivierten T-Zellen hauptsächlich auf der Glykolyse, die die sofortige Aktivierung, Proliferation und Differenzierung von Immunzellen sowie ihre Wanderung zu Entzündungsherden und die Expression von Zytokinen ermöglicht (82).

1.5 Das Tiermodell der vorliegenden Studien

In diesem Mausmodell wurden *non-obese diabetic-severe combined immunodeficiency interleukin-2Rnull* (NOD-scid IL2Rnull, NSG) Mäuse benutzt (83). Das Fehlen von IL-2 R *chain* hemmt die NK-Zellentwicklung und stört die angeborene Immunität. NSG Mäuse sind durch einen Mangel an B- und T-Lymphozyten, niedrige Zahl an NK-Zellen mit zytotoxischer Aktivität und unreife Makrophagen gekennzeichnet. Deswegen können sie keine eigene Immunität aufbauen und eignen sich gut dazu, menschliche Immunzellen anzunehmen und zu vermehren (84, 85). Diese Mäuse haben wir in unseren Experimenten mit mononukleären Zellen des humanen peripheren Bluts (PBMCs) von UC-Patienten rekonstituiert (NSG-UC).

Typische Symptome der Colitis ulcerosa werden durch eine rektale Applikation von Ethanol induziert und können mit Hilfe eines klinischen Scores der Mäuse gemessen werden. Die pathologischen Veränderungen des Kolons und der Kolonarchitektur wie Ödeme, Kryptabszesse oder Verlust der Krypten, können sowohl makroskopisch als auch histologisch bewertet werden. Ein wichtiger Aspekt dieses Mausmodells ist, dass die Entwicklung von pathologischen Veränderungen von der Krankheitsaktivität des Spenders abhängig ist. Je höher der klinische Aktivitätsscore der Spender (*simple clinical colitis activity index*; SCCAI) war, desto größer war die pathologische Ausprägung in den Mäusen (83). In unserem Mausmodell konnten CD1a exprimierende Makrophagen und Monozyten als biologische Marker für die Inflammation identifiziert werden. Außerdem zeigte sich im Rahmen der Inflammation ein Anstieg der Expression von TARC, HGF und TGF im distalen Abschnitt des Darms (83, 86)

2. Fragestellung und Zielsetzung der vorliegenden Forschungsarbeiten

Die Entwicklung biologischer Wirkstoffe wie anti-TNFα-Antikörper (Infliximab, Adalimumab und Certolizumab), und des anti-Integrin-α4β7-Antikörpers Vedolizumab hat zu einer wesentlichen Verbesserung der Therapie von Colitis ulcerosa beigetragen. Dennoch ist der medizinische Bedarf an neuen Therapeutika nach wie vor hoch, da viele UC-Patienten nur unzureichend auf diese Medikamente ansprechen, oder mit den Jahren eine sekundäre Resistenz entwickeln. Mögliche neue therapeutische Interventionspunkte können durch das bessere Verstehen der immunologischen Prozesse identifiziert werden.

Um die immunologischen Abläufe und die Funktion von beteiligten Zellen besser zu verstehen, haben wir in vorgegangenen Versuchen eine Immunprofilierung von UC-Patienten angestrebt. Mittels eines Panels bestehend aus Subtypen von Monozyten, Makrophagen, T-Zellen, B-Zellen, NK-Zellen, NKT-Zellen und unterschiedlichen Zytokinen wurden die PBMCs von Nicht-UC-Spendern mit PBMCs von UC-Patienten verglichen (87). Wir haben so zwei entzündlichen Zuständen identifiziert:

- Ein 'akuter' Zustand (aktive Form von Kolitis) ist durch CD11b+TSLPR+ -Makrophagen, CD14 + CD64+ und CCR2+ -Monozyten, HGF und TARC charakterisiert.
- Ein Umbau 'Remodeling' Zustand (Remissionsform von Kolitis), der durch CD14+TSLPR + -Monozyten, NK-T-Zellen, TGFß1 gekennzeichnet ist

TARC und TGFß1 können als biologische Marker genutzt werden, um zwischen diesen beiden Bedingungen zu differenzieren (87). Weiterhin wurden CD1a+CD11b+ Makrophagen und CD1a+CD14+ Monozyten als biologische Marker identifiziert, welche ein großes Potential haben, um zwischen UC-Patienten und nicht-UC-Personen zu unterscheiden. Auch waren diese beiden Zellpopulationen in Dickdarmproben von UC-Patienten im Vergleich zu Nicht-UC-Spendern erhöht. Auch im NSG-UC-Mausmodell wurden die beiden Zelltypen als Entzündungsmarker identifiziert (83, 87). Im NSG-UC-Modell korrelierten beide Zelltypen mit dem klinischen Aktivitäts-Score (88). Dies deutet darauf hin, dass CD1a+ Monozyten und CD1a+ Makrophagen eine proinflammatorische Immunantwort auslösen könnten.

Monozyten und Makrophagen erfüllen ihre Funktion unter anderem durch die Freisetzung von Zytokinen und durch T-Zellen, die sie aktivieren. Das gastrointestinale Immunsystem basiert auf den Zustrom von Monozyten aus dem Blut in die Schleimhaut. Hier entwickeln sich die meisten Monozyten zu Makrophagen. In Abhängigkeit vom Entzündungsmilieu können Monozyten aber auch als antigenpräsentierten Zellen fungieren und weitere immunologische Reaktionen einleiten oder sich zu Fibrozyten entwickeln (78, 89). Auch Makrophagen unterstützen die Schleimhautepithelzellen im Darm mit unterschiedlichen Funktionen, welche von der Beteiligung in Heilungs- und Modulationsprozessen bis zum Auslösen einer Entzündungsreaktion reichen (90). Die hohe Volatilität dieser Prozesse erlaubt es nicht durch das bloße Erkennen von CD1a auf Monozyten und Makrophagen und deren Korrelation mit der Krankheit auf eine pathologische Funktion zu schließen (88). In der vorliegenden Arbeit war es ein Ziel, die Rolle von CD1a exprimierenden Monozyten und Makrophagen *in vitro* und *in vivo* im NSG-UC Mausmodell zu untersuchen und herauszufinden, ob CD1a einen möglichen therapeutischer Interventionspunkt darstellt.

Ein weiterer möglicher therapeutischer Interventionspunkt bietet der Metabolismus. Die Entzündung ist ein Prozess, welcher umgehend ausreichende Energie zur Aktivierung und Proliferation von Immunzellen benötigt. Die Glukose ist die Hauptenergiequelle für die aktivierten Immunzellen.

So ist die Glykolyse bei Colitis ulcerosa (UC) und Morbus Crohn (CD) der bevorzugte Stoffwechselweg zur Energiegewinnung. Dies lässt sich im Stoffwechselprofil von Patienten im Vergleich zu nicht Erkrankten nachvollziehen. Hier wurden bei UC- und CD-Patienten erhöhte Laktatspiegel gefunden. (91, 92). In diesem Zusammenhang waren auch die Aminosäure (AS)- Spiegel bei den Patienten entweder erhöht (Glutaminsäure, Glu) oder erniedrigt (Histidin, His) (93). Daher könnte die gezielte Intervention in den Stoffwechsel einen neuen therapeutischen Ansatz für UC oder CD eröffnen und von Interesse für die Behandlung von chronisch entzündlichen Erkrankungen sein (94). Dieser Ansatz wurde bereits bei Tumorzellen erforscht, die ähnlich wie Immunzellen einen großen Energiebedarf haben (95). Da die Glukoseaufnahme der entscheidende Schritt zur Kontrolle der Glykolyse ist, könnte die Hemmung von Glukosetransportern (GLUTs) einen vielversprechenden therapeutischen Ansatz bieten.

Ritonavir wurde ursprünglich als Inhibitor des humanen Immundefizienz-Virus (HIV)-Protease entwickelt. Als Nebenwirkung hemmt Ritonavir zufälligerweise ebenfalls die Glukosetransporter Typ 1 und 4 (GLUT1, GLUT4), was folglich zu einem Abfall der Glykolyse führt (96-98). *In vitro* und *in vivo* Versuche haben bereits gezeigt, dass Ritonavir allein und in Kombination mit Metformin zu einer Hemmung der Proliferation multipler Myelom- und chronischer lymphatischer Leukämiezellen, welche beide einen abnormalen Glucosestoffwechsel aufweisen, führt (99, 100). Dieses positive Ergebnis führte zu der ersten klinischen Studie und damit zum Testen von Metforminhydrochlorid und Ritonavir bei Patienten mit rezidivierendem oder refraktärem multiplen Myelom oder chronischer lymphatischer Leukämie (101). In diesem Zusammenhang bietet sich eventuell eine Möglichkeit, durch Ritonavir in den Stoffwechsel der Inflammation einzugreifen.

3. Originalarbeiten der Kumulativen Dissertation

3.1 CD1a-exprimierende Monozyten als Entzündungsmediatoren bei Colitis Ulcerosa

In dieser Studie war es das Ziel, die Rolle von CD1a-exprimierenden Monozyten und Makrophagen *in vitro* und *in vivo* im NSG-UC Mausmodell zu untersuchen. Da Phosphatidylcholin (PC) als Ligand von CD1a identifiziert wurde, wurde der Effekt von PC auf den Monozyten und Makrophagen und ihre Funktion *in vitro* analysiert. Hierfür wurde besonders die Aktivierung von Monozyten, Makrophagen, T-Zellen, TH-Untergruppen sowie regulatorischen T-Zellen (Treg) genauer betrachtet. Die Inkubation von mononukleären Zellen des peripheren Blutes (PBMCs) mit PC führte zu einem Anstieg des Anteils von CD14+CD1a+ Monozyten auf Kosten von TSLPR-exprimierenden Monozyten. Dazu kam es zur Abnahme der Frequenz von den Tregs, was auf einen pathologischen Phänotyp von CD1a-exprimierenden Monozyten hindeutet. Zusätzlich induzierte PC die Aktivierung von CD4+ T-Zellen über CD1a und führte zu einer vermehrten Ausschüttung von IFNγ und IL-17. Dieses Expressionsmuster von CD1a-exprimierenden Monozyten wurde durch *ex vivo* Analyse bestätigt. Bei UC-Patienten war die Co-Expression von TSLPR im Vergleich zu nicht erkrankten Personen verringert. Da auch Triglycerid (TAG) und Cholesterin natürliche Liganden von CD1a sind, wurde die Korrelation zwischen TAG- und Cholesterinspiegeln mit CD14+ CD1a+ Monozyten im NSG-UC-Mausmodell analysiert. Beide Lipide wiesen eine positive Korrelation mit CD14+ CD1a+ Monozyten auf. Da diese Ergebnisse auf eine pro-inflammatorische Funktion von CD1a hinwiesen, wurde im NSG-UC Mausmodell die Wirkung von anti-CD1a Antikörpern getestet. Endpunkte zur Bestimmung der Wirksamkeit von anti-CD1a Antikörpern waren der klinische Aktivitätsscore und der histologische Score sowie Expressionsspeigel von TGFß, TARC, HGF und IFNγ im Darm. Um den Wirkmechanismus zu beschreiben, wurden zusätzlich die Frequenzen der CD1a exprimierenden Monozyten in der Milz bestimmt. Die Mäuse profitierten von der Behandlung, wie durch die Verminderung der Scores und des HGF Spiegels gezeigt werden konnte. Durch die Behandlung mit anti-CD1a-Antikörpern nahm die Frequenz von CD14+ CD1a+ Monozyten ab. Im Gegensatz dazu stieg der Anteil an TSLPR-exprimierenden Monozyten und Makrophagen sowie die mRNA-Expression-Level von TGFß1 und TARC in distalen Teilen des Dickdarms. Das Ganze deutet daraufhin, dass die Behandlung mit anti-CD1a-Antikörpern eine akute Entzündung unterdrückt und die Remodellierung beziehungsweise den Umbau des Dickdarms begünstigt.

Die vorgestellten Ergebnisse zeigen, wie stark die Stoffwechsel- und Entzündungswege miteinander verbunden sind und welchen Einfluss der Fettstoffwechsel auf die Differenzierung von Monozyten haben kann. Insgesamt werden die CD1a-exprimierende Monozyten als Vermittler der Entzündung bei Colitis ulcerosa und TSLPR als Gegenspieler dabei identifiziert.

Mein Beitrag zu dieser Publikation umfasst die ausführliche Literaturrecherche, Befragung der Patienten, Probenentnahmen, Durchführung aller *in vivo* und *in vitro* Versuche sowie die Aufbereitung und statistische Analyse der Ergebnisse. Außerdem habe ich zu der Erstellung von Abbildungen, kritische Revisionen und zum Schreiben des Manuskripts beigetragen.

Ziel I:
Untersuchung der Funktion von CD1a exprimierenden Monozyten sowie des metabolischen Effektes von Phosphatidylcholine (PC) auf die Immunzellen bei Colitis ulcerosa *in vitro*.

- **Hypothese 1:** PC fördert die Differenzierung und Proliferation von CD1a-exprimierenden Monozyten

  Methode: PBMC aus gesunden Spendern und UC-Patienten wurden isoliert und durchflusszytometrisch analysiert**.**

- **Hypothese 2:** PC aktiviert CD1a exprimierende Monozyten, die ihrerseits T Zellen aktivieren

  Methode: Dazu wurden isolierte PBMC von UC-Patienten und Nicht-UC-Spendern mit und ohne PC in einer Zellkultur inkubiert und eine vergleichende fluorescence-activated cell sorting (FACS)-Analyse durchgeführt. Zytokin Spiegel wurden durch intrazelluläre Durchflusszytometrie bestimmt.

- **Hypothese 3:** Die Aktivierung von T Zellen durch PC kann durch anti-CD1a Antikörper unterdrückt werden

  Methode: Dazu wurden isolierte PBMC, die mit PC stimuliert wurden, mit und ohne anti-CD1a Antikörpern in einer Zellkultur inkubiert und eine vergleichende FACS-Analyse durchgeführt.

Ziel II:
Identifizierung weitere Expressionsmuster der Immunzellen, die Rückschlüsse auf die Rolle von CD1+Monozyten bei UC erlauben.

- **Hypothese 4:** CD1a exprimierende Monozyten tragen zum Entzündungsprozess bei Colitis ulcerosa bei.

  Methode: Vergleich der FACS-Analyse der peripheren mononukleären Zellen des Blutes (PBMC) von UC-Patienten und einer gesunden Vergleichspopulation bezüglich der Expression von CCR2, CD86, TSLPR und CD1a.

Ziel III:
Analyse der CD1a exprimierenden Zellen und die Korrelation mit Serumspiegeln von TAG und Cholesterin im NSG-UC Mausmodell.

- **Hypothese 5:** TAG und Cholesterin sind als Liganden der CD1a exprimierenden Zellen beschrieben. Diese wurden ebenfalls bei chronischen Krankheiten als erhöht identifiziert (102). Nach rektaler Applikation von 50 % Ethanol sind die CD1a-exprimierenden Zellen im Vergleich zu der unbehandelten Kontrolle erhöht. Vergleichend damit sollten die TAG und das Cholesterin in der behandelten Gruppe erhöht sein.

  Methode: Vergleich der Spiegel von TAG und Cholesterin im Serum der unbehandelten Kontrollgruppe und der Ethanolgruppe. Bestimmung der Frequenz von CD1a- exprimierenden Monozyten, die aus der Milz isoliert wurden.

Ziel IV:
Validierung der *in vitro* generierten Ergebnisse im NSG-Mausmodell durch die i.p. Gabe von anti-CD1a Antikörper nach der rektalen Applikation von 50 % Ethanol.

- **Hypothese 6:** Die rektale Applikation von 50 % Ethanol und anti-CD1a Antikörper i.p. führten zu einer veränderten klinischen Aktivität im Vergleich zu der Kontrollgruppe.

  Methode: Vergleich der klinischen, makroskopischen und histologischen Scores der unterschiedlichen Gruppen im NSG-UC Mausmodell:
  I. 50 % Ethanol + anti-CD1a Antikörper
  II. 50 % Ethanol + Isotype
  III. Unbehandelte Kontrollgruppe

- **Hypothese 7:** Die Applikation von anti-CD1a Antikörper zieht einen antiinflammatorischen Effekt auf zellulärer Ebene in der NSG-Maus mit sich.

  Methode: Vergleichende FACS-Analyse im Hinblick auf die T-Zellen, der regulatorischen T-Zellen, der Monozyten und Makrophagen aus der Milz und des Darms der unterschiedlichen Gruppen.

- **Hypothese 8:** Durch die Gabe von anti-CD1a Antikörper wird auch das Profil der Zytokine zu Gunsten der Wundheilung beeinflusst.

  Methode: Untersuchung der Expression pro- und anti-inflammatorischer Zytokine mittels qRT-PCR aus dem Darm der behandelten NSG-Mäuse.

3.2 Targeting Colitis ulcerosa durch Unterdrückung der Glukoseaufnahme mit Ritonavir

Die Erhöhung des Cholesterinspiegels in Patienten mit chronisch entzündlichen Darmerkrankungen und in NSG-UC Mausmodell deuten auf einen metabolischen Effekt der Entzündung hin. Im Rahmen dieser Arbeit wurde untersucht, ob die Beeinflussung des Metabolismus einen therapeutischen Interventionspunkt darstellt, und ob Ritonavir durch die Hemmung der Glykolyse einen Effekt auf den Entzündungsprozess hat.

Im ersten Schritt wurde die Wirkung von Ritonavir auf CD4+ T-Zellen *in vitro* analysiert. Die mononukleären Zellen des peripheren Blutes (PBMCs) von UC-Pateinten wurden mit anti-CD3 stimuliert, was zu Aktivierung und Frequenzzunahme unterschiedlicher Subtypen von CD4+ T-Zellen führte. Nach anschließender Inkubation mit Ritonavir beobachtete man eine Abnahme der Frequenz von CD4 + T-Zellen auf das Ausgangslevel. Dies deutet daraufhin, dass Ritonavir eine Hemmung der Aktivierung von CD4+ T-Zellen bewirkt.

Im zweiten Schritt wurden NSG-UC-Mäuse, nach rektaler Applikation mit Ethanol, mit Ritonavir behandelt. Die Therapiewirksamkeit konnte anhand des klinischen und histologischen Scores und der Frequenz von humanen Immunzellen, die aus der Milz und dem Kolon der Mäuse isoliert wurden, gewertet werden.

Da die Aminosäuren (AS) als potentielle Marker für UC und CD vorbeschrieben sind, haben wir im Rahmen des Versuchs die AS-Spiegel im Serum von NSG-UC-Mäusen bestimmt Im Vergleich zur Kontrollgruppe waren die Glutaminsäure und die Asparaginsäure in der Ethanol-Gruppe signifikant erhöht. Außerdem zeigten beide Aminosäuren eine positive Korrelation mit dem klinischen- und histologischen Score, sowie mit den aktivierten Immunzellen in der Milz der Mäuse. Die Glutaminsäure und die Asparaginsäure wurden als potenzielle Biomarker für Entzündungen im NSG-UC-Mausmodell identifiziert.

Durch die Behandlung mit Ritonavir zeigten die Mäuse bzw. Därme einen verringerten klinischen, makroskopischen und histologischen Score. Außerdem führte die Behandlung mit Ritonavir zur Abnahme der in der Milz vorhandenen naiven CD4+T-Zellen, M2 Monozyten (CD14+CD163+) auf ihre Normallevel. Auf CD1a-exprimierende Monozyten zeigte Ritonavir keinen Effekt. Auch der erhöhte Spiegel von Glutaminsäure und Asparaginsäure durch die Behandlung mit Ethanol normalisierte sich nach der Behandlung mit Ritonavir. Die Ergebnisse deuteten darauf hin, dass Ritonavir ein ansprechendes Therapeutikum für UC sein kann und Glutaminsäure und Asparaginsäure als Biomarker für UC fungieren können. In weiteren Versuchen müsste jedoch der genaue Wirkmechanismus weiter geklärt werden.

Mein Beitrag zu dieser Publikation umfasst die Probenentnahmen, Durchführung von *in vitro* Versuchen und Assistenz bei den *in vivo* Versuchen. Außerdem nahm ich an der Korrektur und Literaturdurchsicht der Endfassung der Publikation teil.

Ziel I:
Verständnis der *in vitro* Wirkung von Ritonavir auf T-Zellen bei Colitis ulcerosa.

- **Hypothese 1:** Die Glykolyse ist die Hauptenergiequelle für die aktivierten Immunzellen bei Colitis ulcerosa. Ritonavir könnte zur Hemmung der Aktivierung und Proliferation von T-Zellen bei UC führen.

  Methode: Isolierte PBMC von UC-Patienten wurden mit anti-CD3 Antikörpern *in vitro* aktiviert. Die Aufnahme von Glukose wurde durch Ritonavir gehemmt. Die Aktivierung wurde durch FACS Analyse bestimmt.

Ziel II:
Der Einfluss der Inflammation auf den AS-Stoffwechsel im NSG-UC-Mausmodell.

- **Hypothese 2:** Die AS-Spiegel sollten nach rektaler Applikation von Ethanol im Serum von NSG-UC-Mäusen erhöht sein. Außerdem sollte sich eine positive Korrelation zwischen der AS-Spiegel und der Inflammationsstärke zeigen.

  Methode:
- Vergleich der unterschiedlichen Aminosäuren in Serum von der unbehandelten Kontrollgruppe und der mit 50 % Ethanol behandelten Gruppe.
- Vergleichende FACS-Analyse der Leukozyten aus der Milz von der unbehandelten Kontrollgruppe und der mit Ethanol behandelten Gruppe in Hinblick auf die Subtypen der T-Zellen.
- Korrelationsanalyse zwischen dem AS-Spiegel einerseits und dem klinischen, makroskopischen und histologischen Score sowie der aktivierten T-Zellen anderseits.

Ziel III:
Validierung der *in vitro* generierten Ergebnisse im NSG-Mausmodell durch die i.p. Gabe von Ritonavir nach der rektalen Applikation von 50 % Ethanol.

- **Hypothese 3:** NSG-UC Mäuse profitieren von der Behandlung mit Ritonavir.

  Methode: Vergleich des klinischen, makroskopischen und histologischen Scores der unterschiedlichen Gruppen:
  I. 50 % Ethanol + Ritonavir
  II. 50 % Ethanol + phosphate buffered saline (PBS)
  III. Unbehandelte Kontrollgruppe

- **Hypothese 4:** Die Applikation von Ritonavir zieht einen antiinflammatorischen Effekt auf zellulärer Ebene in der NSG-Maus mit sich.

  Methode: Vergleichende FACS-Analyse in Hinblick auf die T-Zellen, der regulatorischen T-Zellen, der Monozyten und der Makrophagen aus der Milz und dem Darm der unterschiedlichen Gruppen.

Zusammenfassung

Colitis ulcerosa (UC) ist eine der zwei wichtigsten entzündlichen Darmerkrankungen. Wiederkehrende Entzündungsschübe kennzeichnen den Verlauf der Erkrankung. Typische Symptome hierfür sind blutige Durchfälle und abdominelle Krämpfe. Bei Diagnosestellung sind die Patienten oft sehr jung und benötigen eine lebenslange Therapie. Viele Patienten leiden unter starken Nebenwirkungen der medikamentösen Therapie oder entwickeln Resistenzen dagegen. Als letzte therapeutische Option bleibt die chirurgische Resektion von Dickdarm und Enddarm. Die Entwicklung neuer Therapieoptionen verlangt nach einem besseren Verständnis der Krankheit. Die Pathogenese hinter dieser übersteigerten Immunantwort ist noch nicht vollständig verstanden.

Die Entzündung ist eine dynamische Antwort auf einen Reiz, bei der die Zellen des angeborenen sowie des erworbenen Immunsystems unterschiedliche Aufgaben erfüllen. Durch eine Immunprofilierung von Patienten mit Colitis wurde festgestellt, dass CD1a-exprimierende CD11b+ Makrophagen und CD14+ Monozyten im Blut und im Dickdarm von Patienten im Vergleich zu den gesunden Spendern erhöht waren (88). CD1a exprimierende Zellen präsentieren fremde und auch eigene Lipidantigene wie Phosphatidylcholin (PC) oder Triacylglycerin (TAG) an T-Zellen und spielen eine Rolle bei der Aktivierung autoreaktiver T-Zellen (33).

In der ersten Studie dieser Arbeit untersuchten wir den Zusammenhang zwischen Entzündungs- und Stoffwechselsignalen und CD1a-exprimierenden Monozyten *in vitro* und *in vivo*. Wir inkubierten Phosphatidylcholin (PC) mit mononukleären Zellen aus peripherem Blut (PBMCs) von UC-Patienten und Nicht-UC-Spendern für zwei oder sieben Tage. Die durchflusszytometrische Analyse zeigte, dass die Inkubation von PBMCs mit PC zu einer Erhöhung der Frequenz von CD1a+ CD14+ Monozyten auf Kosten von CCR2-, CD86- und TSLPR-exprimierenden CD14+ Monozyten führte. Dies deutet darauf hin, dass PC durch die Induktion von CD1a-Monozyten eine proinflammatorische Reaktion hervorruft. Außerdem induzierten CD1a-exprimierende Monozyten die Differenzierung von Th-Zellen und die Aktivierung von CD4 T-Zellen *in vitro* (88).

Wir verwendeten ein NSG-UC-Mausmodell, um die Funktion CD1a-exprimierender Zellen zu untersuchen. Die NOD / scid IL2Rγnull-Mäuse wurden mit PBMCs von UC-Patienten rekonstruiert (NSG-UC). In diesem Mausmodell haben wir die Frequenzen von CD14+ CD1a+ Monozyten und den Gehalt an Triglycerid (TAG), Cholesterinspiegel bestimmt. Nach rektaler Verabreichung von Ethanol zur Induktion einer Kolitis wurden die NSG-UC-Mäuse mit Anti-CD1a-Antikörpern behandelt. Wir konnten zeigen, dass der TAG- und Cholesterinspiegel im Rahmen einer Entzündung erhöht war und dieser korrelierte positiv mit CD14+ CD1a+ Monozyten. Die NSG-UC-Mäuse profitierten von der Behandlung mit Anti-CD1a-Antikörpern, was durch die verringerten klinischen und histologischen Scores dargestellt wird.

Darüber hinaus betrachteten wir eine Abnahme der Frequenz von CD1a-exprimierenden CD14-Monozyten, die aus der Milz und dem Dickdarm isoliert wurden, sowie eine Abnahme des HGF, was die positiven Auswirkungen der CD1a-Antikörper zeigte (88). HGF war zuvor mit einer akuten Entzündungsreaktion bei UC-Patienten und dem NSG-UC-Mausmodell assoziiert (87). Im Gegensatz dazu führte die Behandlung mit Anti-CD1-Antikörpern zu einem Anstieg der TGFß1-mRNA-Expressionslevels und der Frequenz von TSLPR-exprimierenden Makrophagen und Monozyten im Kolon, was darauf hindeutet, dass die Unterdrückung der CD1a-Aktivität das Gleichgewicht in Richtung einer umgestaltenden Entzündung verschieben

könnte, wie wir es zuvor beobachtet haben, als Patienten oder Mäuse mit Infliximab behandelt wurden (88). So schien die CD1a-Expression auf Monozyten einen proinflammatorischen Phänotyp zu verursachen.

Die Tatsache, dass die Frequenz von CD1a-exprimierenden Monozyten in NSG-nicht-UC-Mäusen ähnlich zunahm, ohne eine Entzündung auszulösen, lässt vermuten, dass das CD1a-exprimierender Monozyten allein nicht ausreicht. Diese Mäuse wiesen jedoch im Vergleich zu NSG-UC-Mäusen viel höhere Spiegel an TSLPR-exprimierenden Monozyten auf, was die Hypothese weiter stützt, dass die Störung des Gleichgewichts, die sich in einer unausgewogenen von Monozyten-Subtypen widerspiegelt, eine treibende Kraft bei der UC sein könnte (87, 88). Unsere Ergebnisse zeigten auch, dass die Stoffwechsel- und Entzündungswege eng miteinander verknüpft sind. Die CD1a-exprimierenden Monozyten könnten als Sensoren und Vermittler der UC-Entzündung fungieren. Die Mäuse profitierten von der Behandlung mit Anti-CD1a-Antikörpern, was CD1a zu einem potenziellen therapeutischen Ziel für die Behandlung der Colitis ulcerosa (UC) machte (88).

Entzündung ist ein Prozess, der eine prompte Energieversorgung erfordert. Dieser Bedarf wird durch eine metabolische Umstellung von oxidativer Phosphorylierung auf Glykolyse als Hauptenergiequelle gedeckt. Auf diese Weise wird schnell genug Energie erzeugt, die für die Differenzierung und Proliferation der Immunzellen notwendig ist. Die Glukoseaufnahme in die Zelle wird durch einen Glukosetransporter (GLUTs) sichergestellt (80, 81). Ein möglicher therapeutischer Ansatz könnte daher eine Intervention in den Stoffwechsel der Entzündung sein.

In der zweiten Studie dieser Arbeit untersuchten wir die Wirkung von Ritonavir auf die Colitis ulcerosa. Ritonavir ist ein spezifischer Proteaseinhibitor und wurde bereits zur Behandlung von AIDS- und HIV-Infektionen eingesetzt. Durch Hemmung des Glukosetransporters (GLUTs) reduziert Ritonavir als Nebenwirkung die Glykolyse. Wir inkubierten periphere mononukleäre Blutzellen (PBMCs) von UC-Patienten in Zellkultur nach Aktivierung durch Anti-CD3 drei Tage lang in Anwesenheit oder Abwesenheit von Ritonavir. Die anschließende FACS-Analyse zeigte, dass die Frequenz von CD4+-Zellen durch Ritonavir reduziert wurde (94).

Um den metabolischen Effekt der Entzündung *in vivo* zu verfolgen, wurde ebenfalls das NSG-UC-Mausmodell verwendet. Die Mäuse wurden mit PBMCs von Patienten mit Colitis ulcerosa (UC) rekonstituiert. Die UC-Symptome wurden durch rektale Applikation von Ethanol induziert. Die Entzündung führte im NSG-UC-Mausmodell zu einem Anstieg des Aminosäuregehalts. Damit bestätigten wir bekannte Ergebnisse bei UC-Patienten (91-93). Im Vergleich zur Kontrollgruppe in der Tierstudie waren Glutamin- und Asparaginsäure im Vergleich zur Ethanolgruppe signifikant erhöht.

Darüber hinaus wiesen beide Aminosäuren eine positive Korrelation mit dem klinischen und histologischen Score sowie mit den aktivierten Immunzellen in der Milz der Mäuse auf. Glutamin- und Asparaginsäuren wurden im NSG-UC-Mausmodell als potenzielle Entzündungsbiomarker identifiziert (94).

Schließlich wurde Ritonavir im selben Mausmodell getestet. Die Mäuse profitierten von der Behandlung mit Ritonavir, was sich in signifikant verringerten klinischen und histologischen Scores zeigte. Weiterhin fanden wir, wie in *in vitro* Experimente, eine Abnahme von aktivierten CD4+ T-Zellen sowie von M2 Monozyten. Außerdem normalisierte sich der Glutamin- und Asparaginsäure Spiegel unter der Therapie mit Ritonavir im Vergleich zur Kontrollgruppe (94)

Zusammenfassend lässt sich sagen, dass diese Studie weiterhin die metabolischen Auswirkungen von Entzündungen zeigt Aminosäure-Spiegel können potenziell als biologische Marker für Entzündungen benutzt werden. Dies könnte auch die Beurteilung der Wirksamkeit von Therapeutika verbessern. Die positiven Ergebnisse der präklinischen Studie im NSG-UC-Modell legen nahe, dass in Zeiten aktiver Erkrankung von UC-Patienten eine Therapie mit Ritonavir vorteilhaft sein könnte, was die Entwicklung neuer GLUT-Hemmer fördern könnte (94)

Summary

Ulcerative colitis (UC) is one of two major inflammatory bowel diseases. Relapsing inflammatory flares characterize the course of the disease. Typical symptoms include abdominal cramps and bloody diarrhea. When the diagnosis is made, patients are often very young and require lifelong therapy. Many patients suffer from strong side effects or develop resistance to medical treatment. Surgical resection of the colon and rectum remains the last therapeutic option. The development of new therapy options requires a better understanding of the disease. The pathogenesis behind this excessive immune response has not been fully understood.

Inflammation is a dynamic response to a stimulus, where the cells of the innate and acquired immune system perform different tasks. Through immunological profiling of patients with ulcerative colitis it was found that CD1a-expressing CD11b+ macrophages and CD14+ monocytes in the blood and colon of patients were increased compared to those of healthy donors (88). Cells expressing CD1a present foreign and self-lipid antigens such as phosphatidylcholine (PC) or triacylglycerol (TAG) on T-cells and have a role in the activation of autoreactive T-cells (33).

In the first study of this thesis, we examined the link between inflammatory and metabolic signals and CD1a-expressing monocytes *in vitro* and *in vivo*. We incubated phosphatidylcholine (PC) with peripheral blood mononuclear cells (PBMCs) from UC patients and non-UC donors for two or seven days. The flow cytometric analysis showed that the incubation of PBMCs with PC resulted in an increase of the frequency of CD1a+ CD14+ monocytes at the expense of CCR2-, CD86-, and TSLPR-expressing CD14+ monocytes. This indicates that PC triggers a proinflammatory reaction by inducing CD1a monocyte. In addition, CD1a-expressing monocytes induced the differentiation of Th cells and the activation of CD4 T cells *in vitro* (88).

We used an NSG-UC mouse model explore the function of CD1a-expressing cells. We reconstituted NOD/scid IL2Rγnull mice were with PBMCs from UC patients (NSG-UC). In this mouse model, we measured the frequencies of CD14+ CD1a+ monocytes and the level of triacylglycerol (TAG) and cholesterol. After rectal administration of ethanol to induce colitis, the NSG-UC mice were treated with anti-CD1a antibodies. We showed that TAG and cholesterol levels were elevated in inflammation and there was a positive correlation with CD14+ CD1a+ monocytes. The NSG-UC mice benefitted from treatment with anti-CD1a antibodies, which was shown by the reduced clinical and histological severity scores.

Furthermore, we registered a decrease in the frequency of CD1a-expressing CD14 monocytes isolated from the spleen and colon and the decrease in HGF which showed the positive effects of the CD1a antibodies (88). HGF was previously associated with an acute inflammatory response in UC patients and the NSG-UC mouse model (87). In contrast, the treatment with anti-CD1-antibodies lead to an increase of TGFß1 mRNA expression levels and frequencies of TSLPR-expressing macrophages and monocytes in the colon, suggesting that the suppression of CD1a activity might shift the balance toward a remodeling inflammation, as we have previously observed when patients or mice were treated with infliximab (88). Thus, CD1a expression on monocytes seemed to cause a proinflammatory phenotype.

The fact that frequencies of CD1a-expressing monocytes similarly increased in NSG-non-UC mice without inducing inflammation suggests that the presence of CD1a-expressing monocytes alone is not sufficient. These mice, however, exhibited much higher levels of TSLPR-expressing monocytes as compared with NSG-UC mice, further supporting the hypothesis that the disturbance of the equilibrium reflected by misbalanced expression of monocyte subtypes might be a driver in UC (87, 88). Our results also showed that the metabolic and inflammatory pathways were closely linked. The CD1a-expressing monocytes could act as sensors and mediators of UC inflammation. The mice benefitted from treatment with anti-CD1a antibodies, making CD1a a potential therapeutic target for the treatment of ulcerative colitis (UC) (88).

Inflammation is a process that demands prompt energy supply. This demand is met by a metabolic switch from oxidative phosphorylation to glycolysis as main energy source. In this way, enough energy is generated quickly that is required for the differentiation and proliferation of the immune cells. Glucose uptake into the cell is ensured by a glucose transporter (GLUTs) (80, 81). A possible therapeutic approach could therefore be an intervention in the metabolism of the inflammation.

In the second study of this thesis, we examined the effect of ritonavir on ulcerative colitis. Ritonavir is a specific protease inhibitor and has been in use for the treatment of AIDS and HIV infection. By inhibiting the glucose transporter (GLUTs), ritonavir reduces glycolysis as a side effect. We incubated peripheral blood mononuclear cells (PBMCs) from UC patients in cell culture after activation by anti-CD3 for three days in the presence or absence of ritonavir. Subsequent FACS analysis showed that the frequencies of CD4+ cells were reduced by ritonavir (94).

We used the NSG-UC mouse model to follow the metabolic effect of the inflammation *in vivo*. The mice were reconstituted with PBMCs from patients with ulcerative colitis (UC). UC-symptoms were induced through rectal challenge with ethanol. Inflammation led to an increase in the amino acid content (AA) in the NSG-UC mouse model. Thus, we confirmed known results in UC patients. Compared to the control group in the animal study, glutamic and aspartic acids were significantly increased compared to the ethanol group. In addition, both amino acids had a positive correlation with the clinical and histological score, as well as with the activated immune cells in the spleen of the mice. Glutamic and aspartic acids were identified as potential inflammatory biomarkers in the NSG-UC mouse model (94).

Finally, ritonavir was tested in the same mouse model. The mice benefited from ritonavir treatment as indicated by significantly decreased clinical and histological severity scores. Furthermore, like *in vitro* experiments, we found a decrease in activated CD4 + T cells and in M2 monocytes. In addition, the level of glutamic and aspartic acids normalized under the therapy with ritonavir compared to the control group (94).

In summary, this study continues to show the metabolic effects of inflammation. AA levels could potentially be used as biological markers for inflammation. This could also improve the assessment of the effectiveness of therapeutic agents. The positive results of the preclinical study in the NSG-UC model suggests that during times of active disease of UC patients a therapy with ritonavir might be beneficial and this might encourage the development of novel GLUT inhibitors (94).

Veröffentlichung I

ORIGINAL RESEARCH ARTICLE—BASIC SCIENCE

CD1a-Expressing Monocytes as Mediators of Inflammation in Ulcerative Colitis

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Background: CD1a-expressing CD14+ monocytes have been identified as inducers of autoreactive T cells. In this study, the link between inflammatory and metabolic signals and CD1a-expressing monocytes in vitro and in vivo was examined, and CD1a was evaluated as a potential therapeutic target for treatment of ulcerative colitis (UC).

Methods: Peripheral blood mononuclear cells (PBMCs) from UC patients and non-UC donors were incubated with phosphatidylcholine (PC) for 2 and 7 days and subjected to flow cytometric analysis. Triacylglycerol (TAG) and cholesterol levels and frequencies of CD14+ CD1a+ monocytes were determined in a mouse model of UC that is based on NOD/scid IL2Rγ^{null} mice reconstituted with PBMCs from UC patients (NSG-UC). NSG-UC mice were treated with anti-CD1a antibodies. Response to treatment was determined by clinical and histological scores, flow cytometric analysis of human leucocytes from the spleen and colon, and expression levels of TGFß1, HGF, IFNγ, and TARC.

Results: Incubation of PBMCs with PC resulted in an increase of the frequency of CD1a+ CD14+ monocytes at the expense of CCR2-, CD86-, and TSLPR-expressing CD14+ monocytes. CD1a+ CD14+ monocytes induced the activation of CD4+ T cells and differentiation of Th cells. In vivo, TAG and cholesterol levels increased upon inflammation and correlated positively with CD14+ CD1a+ monocytes. NSG-UC mice benefitted from treatment with anti-CD1a antibodies, as indicated by a reduced histological score and reduced frequencies of CD1a+ CD14+ monocytes in the colon and spleen of mice.

Conclusion: CD1a-expressing monocytes might act as sensors and mediators of inflammation in UC. Mice benefitted from treatment with anti-CD1a antibodies.

Key Words: ulcerative colitis, CD1a, macrophage, monocyte, metabolic, inflammation

INTRODUCTION

CD1a has long been known as a marker of Langerhans cells. Langerhans cells originate from yolk sac–derived erythro-myeloid progenitors, populate the epidermis during embryonic development, and maintain themselves during adulthood independent of hematopoiesis.[1,2] In contrast, embryonic macrophages of the intestine are replaced by monocyte-derived macrophages after birth and are constantly replenished by recruited monocytes during adulthood.[3] Recently, CD1a expression has also been found in monocyte-derived dendritic cells (moDCs) of the intestine.[4] One hallmark of these cells is the expression of IL-12.[5] CD1a belongs to the CD1 family of antigen-presenting proteins, which activate T cells by presenting lipids. It has long been considered an alternative system to complement the defense against pathogens that is mainly driven by peptide antigen-presenting major histocompatibility complex. In the meantime, however, it has been found that CD1a and b also present self-lipids such as phosphatidylcholine (PC) or triacylglycerol (TAG) to induce autoreactive T cells.[6–9] Unlike the CD1d restricted natural killer T cells, T cells recognizing CD1a belong to the normal αß T-cell repertoire and present a population of 0.02%–0.4% in healthy individuals.[8] T cells that reacted to presentation via CD1a with the release of IL-13, IFNγ, and IL-22 and were identified as Th22 cells. This analysis was performed with healthy donors, and as T cells expressed CLA, it has been suggested that auto-reactivity is a normal response in the skin provoked by injury to induce the wound healing processes. We have recently identified CD1a-expressing CD11b+ macrophages and CD14+ monocytes as biological markers to discriminate between patients with ulcerative colitis (UC) and non-UC donors with high sensitivity and specificity.[10] In addition, both cellular populations were found to be elevated in colon specimens of UC

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patients as compared with non-UC specimens. Furthermore, both cell types were identified as inflammatory markers in the NSG-UC mouse model,[11] suggesting that CD1a monocytes and macrophages elicit a proinflammatory immune response. In the inflammatory setting, the supply of fatty acid ligands could be provided by PLA2—an important component of inflammation and bee and wasp venom.[12] PLA2 releases fatty acids from phospholipids and thus could enhance inflammation via CD1a. This hypothesis is corroborated by the finding that PLA2 levels were increased in the NSG-UC mouse model (our own results). Alternatively, the metabolic switch observed in response to inflammation might provide lipids.

In this study, we examined the role of CD1a-expressing monocytes and macrophages in UC in vitro and in vivo. We first examined the effect of PC on monocytes and macrophages in vitro. Incubation of peripheral blood mononuclear cells (PBMCs) with PC resulted in increased frequencies of CD14+ CD1a+ monocytes at the expense of TSLPR-expressing monocytes and diminished frequencies of Tregs, suggesting a pathological phenotype of CD1a-expressing monocytes. In addition, PC induced the activation of CD4+ T cells via CD1a. This expression pattern of CD1a-expressing monocytes was corroborated by ex vivo analysis. In UC patients, co-expression of TSLPR was decreased as compared with nondiseased individuals. Furthermore, we analyzed the correlation of TAG- and cholesterol levels with frequencies of CD14+ CD1a+ monocytes in the NSG-UC mouse model. Both lipids correlated positively with CD14+ CD1a+ monocytes. Finally, NSG-UC mice were treated with an anti-CD1a antibody to corroborate the hypothesis that CD1a+ monocytes elicit a proinflammatory response. Mice benefited from treatment, as indicated by a diminished histological score. In addition, treatment with anti-CD1a antibodies specifically affected frequencies of CD14+ CD1a+ monocytes in the colon and the spleen and resulted in increased mRNA expression levels of TGFß1 and TARC in distal parts of the colon. In contrast, HGF mRNA levels decreased, suggesting that treatment with anti-CD1a antibodies suppresses acute inflammation and favors the remodeling of the colon.

METHODS

Isolation of PBMCs and Engraftment

Sixty milliliters of peripheral blood was collected from the arm vein of patients suffering from UC in trisodium citrate solution (S-Monovette, Sarstedt, Nürnberg, Germany). The blood was diluted with Hank's balanced salt solution (HBSS; Sigma Aldrich, Deisenhofen, Germany) in a 1:2 ratio, and 30 mL of the suspension was loaded onto LeucoSep tubes (Greiner Bio One, Frickenhausen, Germany). PBMCs were separated by centrifugation at 400*g* for 30 minutes with no deceleration. The interphase was extracted and diluted with phosphate buffered saline (PBS) to a final volume of 40 mL. Cells were counted and centrifuged at 1400 g for 5 minutes. The cell pellet was resuspended in PBS at a concentration of 4×10^6 cells in 100 µL.

Six- to 8-week-old NSG mice were engrafted with 100-µL cell suspension into the tail vein on day 1.

Cell Culture

PBMCs of UC patients and healthy individuals were isolated. The cell pellet was resuspended in RPMI (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 1×10^6 cells/mL. Additionally, 500-µL RPMI with 10% FCS and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) were added to each well and sample. Wells containing PBMCs and RPMI or PBMCs, RPMI and 10 µg of phytohemagglutinin (PHA)/mL served as negative and positive controls, respectively. For analysis of the effect of PC on PBMCs, 200 µg/mL PC (Sigma-Aldrich, St. Louis, MO, USA) was added. Cells were incubated for 48 hours and labeled for flow cytometry.

To analyze the effect of an anti-CD1a antibody on cells stimulated with PC, cells were incubated for 1 hour with 10 µg/mL of anti-human CD1a (Bio X Cell, West Lebanon, USA) and 10 µg/mL of a corresponding isotype antibody (Biolegend, San Diego, CA, USA) before 200 µg/mL of PC (Sigma-Aldrich, St. Louis, USA) was added. All wells additionally contained 15 ng/mL of IL-2 and IL-15 (PeproTech GmbH, Hamburg, Deutschland). Cells were incubated for 2 or 7 days and then labeled for flow cytometry.

To examine proliferation of CD14+ CD1a+ monocytes, cells were stained with the CellTrace CFSE Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Cells were incubated for 5 days before flow cytometry analysis was performed.

Animal Study Protocol

NOD/scid IL-2Rγ^{null} mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were kept under specific pathogen-free conditions in individually ventilated cages in a facility controlled according to the Federation of Laboratory Animal Science Association guidelines. Following engraftment (day 1), mice were presensitized by rectal application of 150 µL of 10% ethanol on day 8 using a 1-mm cat catheter (Henry Schein, Hamburg, Germany). The catheter was lubricated with Xylocain Gel 2% (AstraZeneca, Wedel). Rectal application was performed under general anaesthesia using 4% Isofluran. Postapplication, mice were kept at an angle of 30° to avoid ethanol dripping. On days 15 and 18, mice were challenged by rectal application of 50% ethanol following the protocol of day 8. Mice were killed on day 21.

Mice were treated by intraperitoneal application of 30 µg of anti-CD1a antibody (Clone OKT6, BioXcell, West Lebanon, USA) or 30 µg of isotype control (Biolegend, San Diego, CA, USA) on days 7, 14, and 17.

Clinical Activity Score

The assessment of colitis severity was performed daily according to the following scoring system: Loss of body weight: 0% (0), 0%–5% (1), 5%–10% (2), 10%–15% (3), 15%–20% (4). Stool consistency: formed pellet (0), loose stool or unformed pellet (2), liquid stools (4). Behavior: normal (0), reduced activity (1), apathy (4), and ruffled fur (1). Body posture: intermediately hunched posture (1), permanently hunched posture (4). The scores were added daily for a total score with a maximum of 18 points per day. Animals who suffered from weight loss >20%, rectal bleeding, rectal prolapse, self-isolation, or a severity score >7 were killed immediately and not taken into count.

Histological Analysis

Samples from distal parts of the colon were fixed in 4% formaldehyde for 24 hours, before storage in 70% ethanol, and were routinely embedded in paraffin. Samples were cut into 3-µm sections and stained with hematoxylin and eosin (H&E). Epithelial erosions were scored as follows: no lesions (0), focal lesions (1), multifocal lesions (2), major damage with involvement of basal membrane (3). Inflammation was scored as follows: infiltration of few inflammatory cells into the lamina propria (1), major infiltration of inflammatory cells into the lamina propria (2), confluent infiltration of inflammatory cells into the lamina propria (3), infiltration of inflammatory cells including tunica muscularis (4). Fibrosis was scored as follows: focal fibrosis (1), multifocal fibrosis and crypt atrophy (2). The presence of edema, hyperemia, and crypt abscess was scored with 1 additional point in each case. The scores for each criterion were added for a total score ranging from 0 to 12. Images were taken with a Zeiss AxioVert 40 CFL camera. Figures show representative longitudinal sections in original magnification. In Adobe Photoshop CS6, a tonal correction was applied in order to enhance contrasts within the pictures.

Isolation of Human Leucocytes

To isolate human leucocytes from murine spleen, spleens were minced and cells filtrated through a 70-µL cell strainer (Greiner Bio-One, Frickenhausen), followed by centrifugation at 1400 g for 5 minutes, and resuspended in FACS buffer (1× PBS, 2 mM EDTA, 2% FCS). For further purification, cell suspensions were filtrated using a 35-µm cell strainer (Greiner Bio-One, Frickenhausen) and then labeled for flow cytometry analysis.

Flow Cytometry Analysis

Labeling of human leucocytes was performed according to Supplementary Table 2. All antibodies were purchased from Biolegend (San Diego, CA, USA), with the exception of the intracellular staining of the Th subsets, which were purchased from R&D Systems (Heidelberg, Germany). All antibodies were used according to the manufacturer's instructions. Flow cytometry was performed using a BD FACS Canto II and analyzed with FlowJo 10.1-Software (FlowJo LLC, OR, USA). Cells were quantified according to the gating strategy depicted in Supplementary Figure 1.

RNA Analysis

RNA extraction and cDNA synthesis

Approximately 1 cm of distal parts of the colon was disrupted and homogenized with a TissueLyser LT (Qiagen, Hilden, Germany) followed by total RNA extraction according to the manufacturer's instruction using RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) and chloroform (Sigma-Aldrich, St. Lousi, MO, USA). No further treatment with DNase was needed as gDNA Eliminator Solution was included in the kit.

Reverse Transcription of RNA

Five µg of total RNA were used for cDNA synthesis. Reverse transcription was performed using a Mastercycler gradient (Eppendorf, Hamburg, Germany) and QuantiNova Reverse Transcription Kit (Qiagen, Hilden, Germany). To obtain a cDNA concentration between 10 pg and 100 ng, as required by the TaqMan Fast Advanced Master Mix protocol (Thermo Fisher Scientific, Waltham, MA, USA), samples were diluted with RNase-free water.

RNA and cDNA purity was assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative Real-time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (RT-PCR) was performed according to the TaqMan Fast Advanced Master Mix protocol (Thermo Fisher Scientific, Waltham, MA, USA) using the Applied Biosystems StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Single Tube TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA) included primers for the housekeeping genes GAPDH (Mm99999915_g1), GUSB (Mm00446953_m1), and TGFβ (Mm01178820_m1), HGF (Hs04329698_m1), CCL17 (Mm01244826_g1), and IFNγ (Hs00989291_m1). Analysis was performed using StepOnePlus Software v2.3.

The mean cycle threshold (CT) value was calculated for the housekeeping genes. Relative expression values for the analyzed genes were calculated as the difference between the mean cycle threshold (CT) of the housekeeping genes and the analyzed gene (delta CT) and depicted as the logarithmic value.

Detection of Cholesterol and Triglycerol Levels

Cholesterol and triglycerol levels were determined enzymatically by the Institute of Laboratory Medicine LMU,

Munich, Germany, using the Cobas analyzer (Roche Diagnostics, Mannheim, Germany).

Statistics

Statistical analysis was performed with R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/) and BRB Array Tools (https://brb.nci.nih.gov/BRB-ArrayTools/). Variables were represented with mean, standard deviation, median, and interquartile range values. A 2-sided Student *t* test and a confidence level of 0.95 were used to compare binary groups, and for more than 2 groups, analysis of variance (ANOVA), followed by TukeyHSD, was conducted. For correlation analysis, Spearman or Pearson correlation was used.

RESULTS

Phosphatidylcholine Affects Differentiation of CD14+ Monocytes and Regulatory T Cells

PC and derivatives of PC are important mediators of inflammation and resolution of inflammation. As PC has been described as a natural ligand of CD1a,[7] we examined the effect of PC on monocytes and macrophages; 1×10^6 / mL PBMCs derived from UC patients (n = 13) and non-UC donors (n = 28) was incubated in the presence (PC n = 41) or absence (control n = 39) of PC, as described in the Methods section. PHA served as a control antigen (n = 40). As shown in Figure 1A, frequencies of CD14+ CD1a+ monocytes increased significantly in the presence of PC, whereas PHA had no effect (for the complete data set, see Supplementary Table 3). In contrast, frequencies of CD14+ CD86+ declined upon incubation with PC, and CD14+ TSLPR+ declined in the presence of PC and PHA. PC had no effect on CD11b+ CD1a+ macrophages. In contrast to PHA, incubation with PC resulted in a significant decrease of regulatory T cells (Treg), indicating that PC elicits a proinflammatory response. As CD1a is thought to activate autoreactive T cells that are a considered a normal component of the human αß T cell repertoire, a correlation analysis was performed between frequencies of CD14+ CD1a+ monocytes and Th1 and Th2 cells (Fig. 1B). Frequencies of Th1 and Th2 cells correlated positively with frequencies of CD1a+ CD14+, and both correlations were significant, further corroborating the linkage between CD1a+ monocytes and T cells.

In order to examine whether PC induced the proliferation of CD1a-expressing monocytes, cell trace dilution was examined as described in the Methods section (Fig. 1C). Prior to incubation, PBMCs were stained with CellTrace CFSE. As shown in Figure 1C, PC induced the proliferation of CD14+ CD1a monocytes.

By using tetramers, previous studies have identified a natural subset of CD4+ as target cells of CD1a.[8] To corroborate these results and the correlation analysis, PBMCs from 4 different donors were incubated with PC, and CD4+ T cells were analyzed for intracellular expression of IFNγ, IL-4, and IL-17. We observed an increase of all 3 populations, and this increase was significant for CD4+ IFNγ+. This effect could partially be reversed by anti-CD1a antibody.

To further examine whether CD1a mediates responses to CD4+ T cells, PBMCs from 2 different donors were incubated for 7 days with PC in the presence of absence of anti-CD1a antibodies adapted to the protocol described in de Jong et al. [8]. PHA served as a positive control (for the complete data set, see Supplementary Table 3). As observed after incubation for 2 days, frequencies of CD1a-expressing monocytes were significantly increased in the presence of PC (Fig. 2). Anti-CD1a antibodies had no effect. This also applies to regulatory T cells (CD4+ CD25+ CD127 low), indicating that this effect was not mediated by CD1a. The effect of PC on activated CD4+ T cells (CD4+ CD69+ and CD4+ CD134+) and mucosal CD4+ T cells (CD4+ CD103+), however, seemed to be mediated by CD1a as frequencies of these cell types increased and the effect was abolished in the presence of anti-CD1a antibodies. In contrast to previous results, which described an induction of Th22 cells by CD1a,[7] a decline of Th22 was observed.

CD14+ CD1a+ Monocytes Display a Proinflammatory Phenotype Ex Vivo

As in vitro data might not reflect the in vivo situation, the CD14+ CD1a+ monocyte population was further analyzed for expression of CCR2, CD86, TSLPR, and CD1a in PBMCs from UC (n = 21) and non-UC donors (n = 9). As shown in Figure 3, there was a significant difference in the CD14+ CD1a+ coexpression pattern between non-UC and UC patients. The CD14+ CD1a+ population from UC patients displayed a higher activation status, higher CCR2 levels to direct them to sites of inflammation, and lower levels of TSLPR. The latter observation is in agreement with the results obtained in vitro and supports the assumption that CD1a+ monocytes shift the balance toward a proinflammatory phenotype in UC. Assuming that the coexpression pattern of the non-UC patient reflects the normal situation, CD14+ CD1a+ monocytes are furnished to convey signals toward a proinflammatory or immune-tolerating and remodeling response depending on the level of expression of receptors on CD14+ CD1a monocytes and factors released from the inflammatory milieu signals.

TAG and Cholesterol Levels Correlate With CD14+ CD1a+ Monocytes In Vivo

The strong response to exposure with PC suggested that CD1a+ CD14+ monocytes might respond to inflammation by proliferation and differentiation. As TAG and/or cholesterol have been found elevated in chronic diseases[13] and have

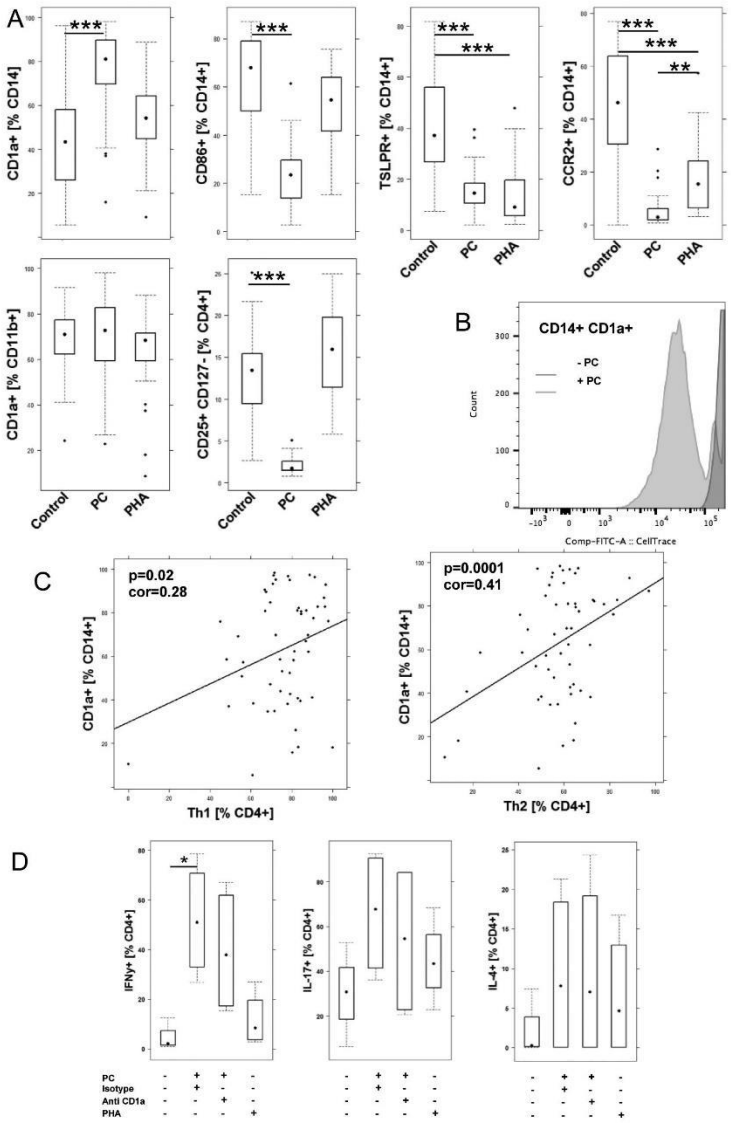

FIGURE 1. Incubation of PBMCs with PC affects frequencies of monocytes, macrophages, and CD4 T cells. A, Flow cytometric analysis of PBMCs from UC patients (n = 13) and non-UC donors (n = 28) that were incubated in the absence (control n = 39) or presence of PC (n = 41) and PHA (n = 40) for 48 hours depicted as boxplot diagrams. B, Cell trace CFSE dilution of CD14+ CD1a+ monocytes. C, Correlation analysis of frequencies of CD14+ CD1a+ monocytes and Th1 of Th2 cells depicted as scatter plots. The method was Pearson; numbers display correlation coefficients (cor-values) and *P* values. D, Flow cytometric analysis of CD4+ T cells for intracellular expression of IFNγ, IL-4, or IL-17 depicted as boxplots. PBMCs (n = 4) were incubated in the absence or presence of PC and anti-CD1a antibody. For comparison of groups, ANOVA followed by Tukey's HSD was conducted. Boxes represent upper and lower quartiles, whiskers represent variability, and outliers are plotted as individual points (***0.001; **0.01; *0.05).

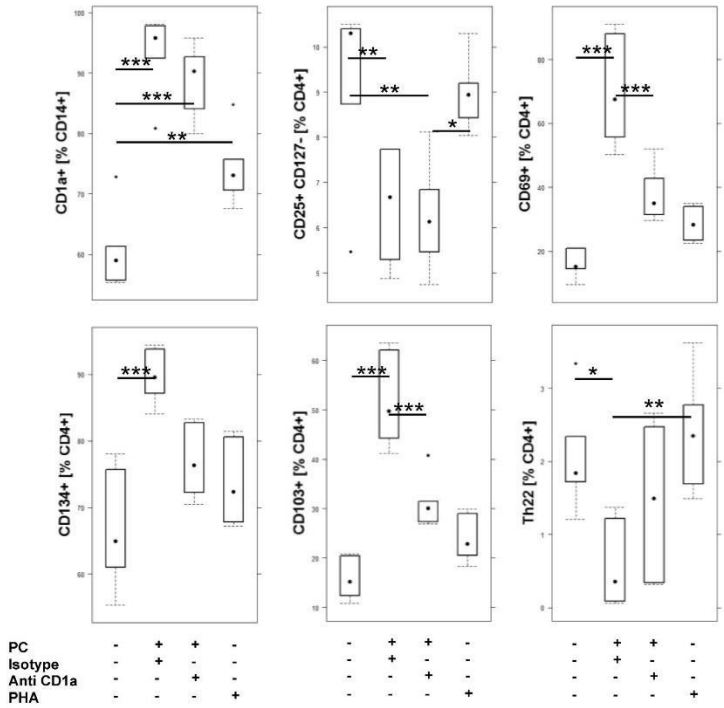

FIGURE 2. Anti-CD1a antibodies affect frequencies of CD4+ T cells. Flow cytometric analysis of PBMCs from 2 different donors that were incubated in triplicate for 5 days with PC in the presence (n = 6) or absence of anti-CD1a antibodies (n = 6) or PHA (n = 6) depicted as boxplot diagrams. For comparison of groups, ANOVA followed by Tukey's HSD was conducted. Boxes represent upper and lower quartiles, whiskers represent variability, and outliers are plotted as individual points (***0.001; **0.01; *0.05).

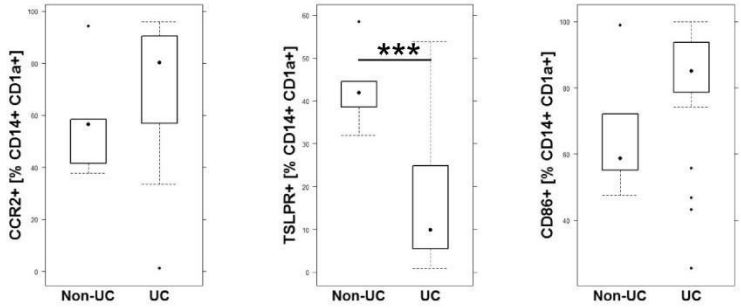

FIGURE 3. Ex vivo analysis of CD14+ CD1a+ monocytes for co-expression of CCR2, CD86, and TSLPR. Flow cytometric analysis of PBMCs from UC patients (n = 21) and non-UC (n = 9) donors depicted as boxplot diagrams. Boxes represent upper and lower quartiles, whiskers represent variability, and outliers are plotted as individual points. For comparison of groups, a Student *t* test was performed (***0.001; **0.01; *0.05).

furthermore been identified as natural ligands of CD1a, we wanted to examine whether we could detect a correlation between TAG and CD1a+ CD14+ monocytes in the NSG-UC mouse model. We first examined whether TAG or cholesterol become elevated in response to challenge. Mice were reconstituted with PBMCs derived from 4 different UC patients.

Following reconstitution with 4×10^6 PBMCs, mice were separated into 2 groups: unchallenged control (n = 11) and challenged control (n = 14). As shown in Figure 4A, challenge with ethanol results in significantly increased TAG (control: mean ± SD, 75.36 ± 31.46; ethanol: mean ± SD, 131.0 ± 48.98; $P = 0.003$) and cholesterol (control: mean ± SD, 74.0 ± 31.46; ethanol: mean ± SD, 87.14 ± 13.82; $P = 0.003$) levels. In these mice, TAG and cholesterol levels positively correlated with frequencies of CD14+ CD1a+ monocytes (TAG: *rho* = 0.5, $P = 0.008$, n = 24; Cholesterol: *rho* = 0.49, $P = 0.007$, n = 25), indicating that these cells might sense inflammation on a metabolic level to mediate the inflammatory signal to T cells (Fig. 4B).

Anti-CD1a Antibodies Ameliorate Symptoms and Phenotype in NSG-UC Mice

So far, all data indicated a strong proinflammatory response mediated by CD14+ CD1a+ monocytes. In addition, previous studies have shown that CD1a-expressing CD11b+ macrophages and CD14+ monocytes are highly correlated with the clinical activity score in UC patients and that both cellular populations were found to be elevated in the colon of UC patients.[10] Moreover, both populations had been identified as inflammatory markers in the NSG-UC model (our own results). In order to examine whether inhibition of CD1a affects symptoms and phenotype, anti-CD1a antibodies were tested in the NSG-UC mouse model. Mice were reconstituted with PBMCs derived from 3 different UC patients, 2 of whom were treated with adalimumab and exhibited moderate Simple Clinical Colitis Activity Index (SCCAI) scores of 3 and 5, respectively,[14] and 1 patient was treated with vedolizumab and exhibited a high SCCAI score of 9. In a control experiment, mice were reconstituted with PBMCs derived from a non-UC donor. Following reconstitution with 4×10^6 PBMCs, mice were separated into 3 groups: unchallenged control (non-UC: n = 4; UC: n = 8), challenged control (non-UC: n = 4; UC: n = 16), and the study group (non-UC: n = 4; UC: n = 16), containing challenged mice additionally treated by intraperitoneal injection of anti-CD1a antibodies (30 µg) on days 7,

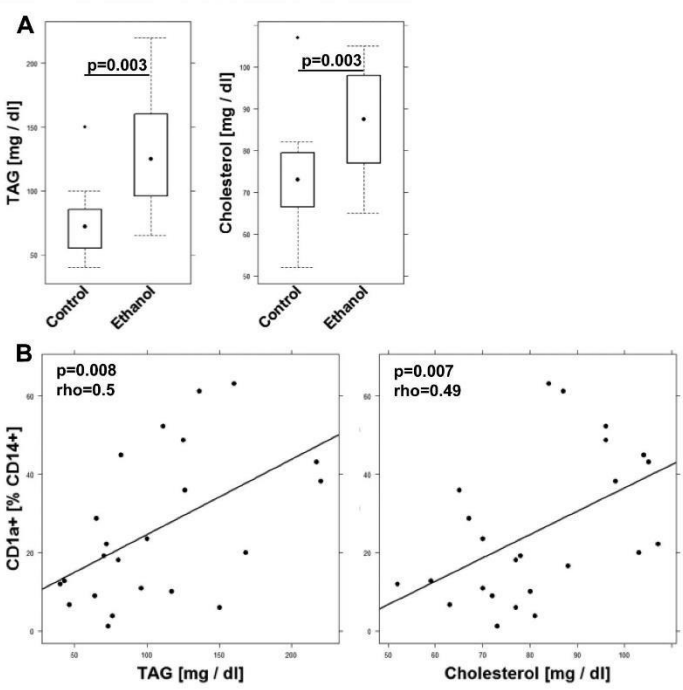

FIGURE 4. TAG and cholesterol levels increase in the NSG-UC mouse model and correlate with CD1a-expressing monocytes. NSG-UC mice were engrafted with PBMCs derived from UC patients (n = 4), challenged with 10% ethanol at day 8 and 50% ethanol at days 15 and 18. A, TAG and cholesterol levels depicted as boxplot diagrams; nchallenged group (control, n = 12), ethanol challenged group (ethanol, n = 21). Whiskers represent variability, and outliers are plotted as individual points. B, Correlation analysis of CD14+ CD1a+ monocytes with TAG and cholesterol levels depicted as scatter plots. (TAG n = 24, cholesterol n = 25). The method was Spearman; numbers display correlation coefficients (*rho* values) and *P* values.

14, and 17, as described in the Methods section. The challenged control group was treated with 30 µg of isotype control antibodies. Symptoms of colitis were induced by intrarectal challenge of 10% ethanol on day 8, followed by 50% ethanol on days 15 and 18. Mice were monitored throughout the experiment, and symptoms were classified according to a clinical score, as described in the Methods section. As shown in Figure 5A, mice developed a clinical activity score upon challenge with ethanol, which was slightly decreased in the study group. As previously described,[11] NSG-non-UC mice hardly responded to challenge with ethanol. Anti-CD1a antibodies further diminished the effect of ethanol. Mice were killed on day 21, and the colon was removed and subjected to visual inspection. As shown in Figure 5B, the colons of mice in the challenged group revealed lack of pellets, soft pellets, and dilatation, whereas the appearance of the colon of the study group was similar to the unchallenged control group. Colons of the NSG-non-UC mice appeared normal. To further analyze the effect of anti-CD1a antibodies, distal parts of the colon were subjected to histological analysis. As shown in Figure 5C, the challenged control groups revealed edema and influx of inflammatory cells, whereas the unchallenged control and treated study group exhibited reduced influx of inflammatory cells and less edema. Some of the mice, however, appeared to have augmented fibrosis (data not shown). This was also reflected in the histological score (Fig. 5A).

To analyze the effect of anti-CD1a antibodies on a cellular level, human leucocytes isolated from distal parts or the colon of NSG-UC mice were isolated and subjected to flow cytometric analysis. Due to the low number of cells, samples were pooled from each group. As shown in Figure 5D, treatment of mice with anti-CD1a antibodies resulted in a specific decline of CD14+ CD1a+ monocytes whereas frequencies of TSLPR-expressing CD11b+ macrophages and CD14+ monocytes increased, suggesting a favoring of the anti-inflammatory response by anti-CD1a antibodies. This hypothesis was also corroborated by RT-PCR analysis performed on distal parts of the colon (for the complete analysis, see Supplementary Table 4). As previous studies had identified HGF, IFNγ, TGFß1, and TARC as markers associated with the acute or remodeling condition of inflammation in NSG-UC mice or UC patients, mRNA expression levels were determined. As shown in Figure 5E, levels of mTARC and mTGFß1 increased in response to treatment, suggesting that inflammation was not completely suppressed but that anti-CD1a antibodies favored a remodeling condition. In contrast, treatment resulted in decreased HGF mRNA, indicating that the acute inflammatory condition was suppressed. Treatment had no effect on IFNγ mRNA levels.

To further examine the effect of anti-CD1a antibodies on the cellular level, human leucocytes were isolated from mouse spleens and subjected to flow cytometric analysis. Previous studies had identified CD14+ CD1a+ monocytes and CD11b+ CD1a+ macrophages as cellular markers associated with inflammation in the NSG-UC model. Thus, the increase of frequencies of both cell types in response to challenge was in agreement with previous results (Fig. 6A). Unexpectedly, the same effect was observed in the NSG-non-UC mice. These mice, however, exhibited higher frequencies of TSLPR-expressing monocytes. No difference between healthy and UC donor was observed with respect to monocytes expressing CD64+. Treatment of mice with anti-CD1a antibodies resulted in a decrease of CD14+ CD1a+ monocytes whereas CD11b+ CD1a+ macrophages remained unaffected. In order to find some evidence on which cellular population CD1a+ CD14+ monocytes and CD14+ macrophages evoked an activity, a correlation analysis was performed (Fig. 6B). A positive correlation was observed between CD14+ CD1a+ and central memory CD8+ T cells and unswitched B cells (CD19+ CD27+ IgD+) whereas CD1a+ monocytes correlated negatively with switched B cells (CD19+ CD27+ IgD-) and Treg (CD4+ CD25+ CD127-), indicating that CD14+ monocytes elicit a CD8 response while suppressing the maturation of B cells. This result is in agreement with previous studies.[5] In contrast, CD11b+ macrophages correlated positively with activated CD4+ T cells (CD69+), suggesting an entirely different mode of action of these 2 cell types.

DISCUSSION

The role of CD1a in homeostasis or inflammation is far from being understood. The vagueness concerns both the CD1a-expressing macrophages/monocytes and T cells to which these cells convey their signals. In the intestine, where homeostasis, repair, and defense against pathogens relies on the influx of immune cells from the blood into the mucosa, plasticity of immune cells and especially of monocytes might be imperative. Most monocytes develop into macrophages; however, depending on the inflammatory milieu, monocytes can adopt a DC phenotype or develop into fibrocytes and cells with hepatocyte characteristics.[15, 16] And even macrophages have different functions ranging from inflammatory to healing and modulatory. For that reason, macrophages are considered accessory cell types that support their client cells—notably mucosal epithelial cells in the gut—with various functions.[17] Hence, the identification of CD1a on monocytes and macrophages cannot per se be associated with a certain pathological function. In UC patients, CD1a-expressing CD11b+ macrophages and CD14+ monocytes have been found elevated in the blood and in the colon.[10] While TSLPR-expressing monocytes and macrophage could be associated with the acute condition or the remodeling inflammatory condition, respectively, no such correlation was found for the CD1a-expressing macrophages and monocytes. This was different in the NSG-UC model, where both cell types were correlated with clinical activity score. Macrophages exert their activities in 2 ways: They release cytokines and thus shape the inflammatory milieu, and they convey their signals by inducing T cell activation. Various studies based on in vitro

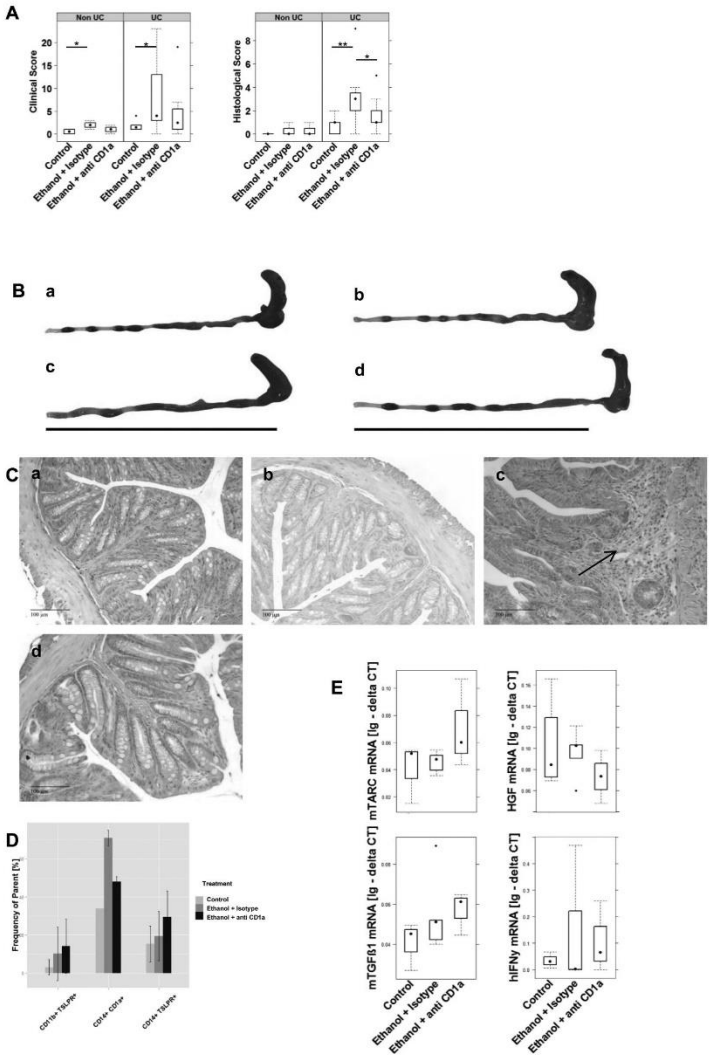

FIGURE 5. NSG-UC mice benefit from treatment with anti-CD1a antibodies. NSG mice were engrafted with PBMCs derived from UC patients (NSG-UC, n = 3) and 1 non-UC donor (NSG-non-UC). Mice were challenged with 10% ethanol at day 8 and 50% ethanol at days 15 and 18 and treated with 30 µg of anti-CD1a antibody or isotype control at days 7, 14, and 17. Control: NSG-non-UC, n = 4; NSG-UC, n = 8. Challenged control (ethanol + isotype): NSG-non-UC, n = 4; NSG-UC, n = 16. Challenged and treated with anti-CD1a antibody (ethanol + anti-CD1a): NSG-non-UC, n = 4; NSG-UC, n = 16. A, Clinical colon and histological scores of NSG-UC mice depicted as boxplot diagrams. B, Macrophotographs of colons at autopsy. a, NSG-UC unchallenged control. b, NSG-non-UC challenged control. c, NSG-UC challenged control, NSG-UC. d, Challenged and treated with anti-CD1a antibody. C, Photomicrographs of H&E-stained sections of distal parts of the colon from mice. Arrows indicate edema and influx of inflammatory cells. D, Frequencies of human leucocytes isolated from colons of mice. Samples from each group were pooled. Mean values are given; error bars are SD. Quantification was performed using flow cytometric analysis. Sample sizes: control n = 3, ethanol + isotype n = 3, ethanol + anti-CD1a n = 3. E, mRNA expression levels of mTARC, mTGFß1, HGF, and hIFNγ depicted as boxplots. Lg: delta CT, logarithmic delta cycle threshold. Boxes represent upper and lower quartiles. Whiskers represent variability, and outliers are plotted as individual points. Labels given on x-axes on the bottom row apply to all charts. For comparison of groups, ANOVA followed by Tukey's HSD was conducted.

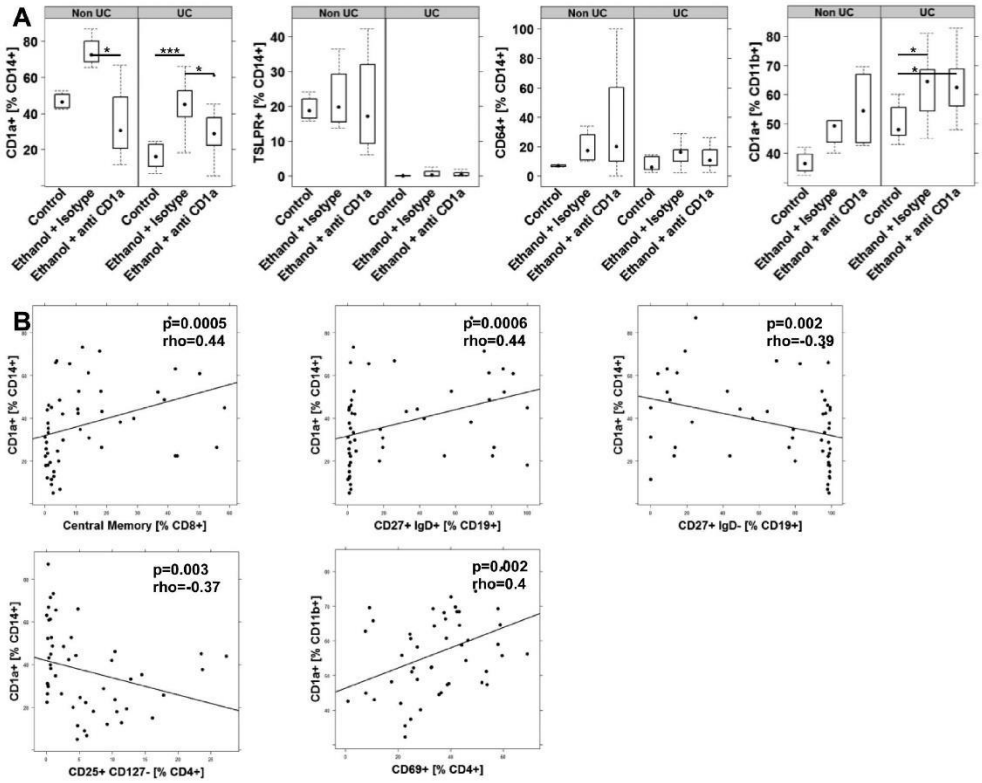

FIGURE 6. Treatment of NSG-UC mice with anti-CD1a antibodies affects CD14+ CD1a+ monocytes. NSG mice were engrafted and treated as in Figure 5. Human leucocytes were isolated from mouse spleen and subjected to flow cytometric analysis. A, Frequencies of CD14+ CD1a+ and CD11b+ CD1a+ cells depicted as boxplot diagrams. Boxes represent upper and lower quartiles. Whiskers represent variability, and outliers are plotted as individual points. B, Correlation analysis of frequencies of CD14+ CD1a+ monocytes with subsets of B and T cells cells depicted as scatter plots. The method was Spearman; numbers display correlation coefficients (*rho* values) and *P* values.

differentiation of DCs from CD34+ hematopoietic stem cells or CD14+ blood monocytes have shown that CD1a expression defines IL-12-producing DCs,[5, 18, 19] supporting the idea of a proinflammatory phenotype of CD1a-expressing macrophages. In our in vitro study, we have shown that frequencies of CD1a-expressing monocytes were enhanced in the presence of PC, indicating that PC might additionally fuel inflammation by inducing the development of CD1a+ monocytes. The effector function of monocytes and macrophages not only relies on the released cytokines but also on T-cells, which are activated by these cells. It has been shown by in vitro analysis that CD1a expressed on K562 cells to present self-lipids activate Th22 cells to express IL-22, which is thought to play a role in the wound healing processes.[8] This effect was donor dependent, as only 3 of 6 donors reacted to exposure to CD1a-expressing K526 cells with elevated levels of IL-22. Some of these autoreactive T cells, however, also expressed IFNγ and IL-13, suggesting that the response might not be restricted to Th22 cells. Other in vitro experiments have shown that DC-expressing CD1a directed a Th1 response whereas CD1a- dendritic cells favored a Th2 response.[18] In addition, recent findings suggest that CD1a expressed on Langerhans cells mediate inflammation in poison ivy dermatitis by inducing the release of IL-17 and IL-22 from αß CD4+ T cells.[20] Furthermore, the fact that memory CD4+ cells isolated from psoriasis patients responded to exposure with autologous CD1a-expressing DCs by increased expression of IL-17 and IL-22 suggests that CD1a-mediated inflammation might also play a role in psoriasis. In the in vitro experiment presented here, CD1a-expressing monocytes induced the activation of CD4+ cells and the frequencies of Th17, Th1, and

Th2 cells. As CD1a is not expressed in mice, in vivo experiments to elucidate the function of CD1a cannot be performed in conventional mouse models. Therefore, the NSG-UC mouse model was used in this study. Mice benefited from the treatment with anti-CD1a antibodies, as indicated by the decreased clinical and histological scores. The beneficial effect was also reflected in the decrease of frequencies of CD1a-expressing CD14+ monocytes isolated from the spleen and colon and the decrease of HGF, which had previously been found to be associated as a marker of acute inflammation in UC patients and the NSG-UC mouse model.[10] In contrast, frequencies of TSLPR-expressing monocytes and macrophages and TGFß1 mRNA expression levels increased in the colon in the presence of anti-CD1a antibodies, suggesting that the suppression of CD1a activity might shift the balance toward a remodeling inflammation, as we have previously observed when patients or mice were treated with infliximab. Thus, CD1a expression on monocytes seemed to provoke a proinflammatory phenotype. The fact that frequencies of CD1a-expressing monocytes similarly increased in NSG-non-UC mice without inducing inflammation suggests that the presence of CD1a-expressing monocytes alone is not sufficient. These mice, however, exhibited much higher levels of TSLPR-expressing monocytes as compared with NSG-UC mice, further supporting the hypothesis that the disturbance of the equilibrium reflected by misbalanced expression of monocyte subtypes might be a driver in UC. The proinflammatory phenotype of CD1a+ monocytes was also corroborated by correlation analysis. CD1a+ CD14+ monocytes correlated positively with central memory CD8+ T cells and negatively with Tregs. Both CD1a+ and CD1a- macrophages have previously been shown to have the capacity to elicit a cytotoxic T-cell response, although this response was weaker in CD1a+ macrophages.[19] Of note, CD1a-expressing monocytes correlated positively with unswitched B cells and negatively with switched B cells, supporting data obtained from in vitro studies that had shown that CD1a DCs mediate T-cell priming whereas CD1a- DCs promote B-cell activation.[21]

This observation, together with the finding that CD14+ CD1a+ monocytes responded to PC by proliferation or differentiation and correlated positively with TAG levels, suggests that these monocytes not only relay signals but also might sense inflammation and in turn evoke a proinflammatory response. This observation prompted us to further characterize the phenotype of CD1a-expressing monocytes. And indeed, flow cytometric analysis revealed that these populations also expressed TSLPR, CCR2, and the activation marker CD86 to various degrees. Assuming that the expression pattern of non-UC donors reflects the homeostatic situation and that TSLP and, in consequence, TSLPR are essential for maintaining homeostasis in the intestinal mucosa,[22] the equilibrium could be maintained by almost equal expression levels of TSLPR and CD1a simultaneously relaying signals toward opposing pathways required by the constant regeneration of the mucosa. In case of inflammation as in UC, the balance would be shifted toward a proinflammatory phenotype by increased levels of PC or TAG at the expense of a tolerating phenotype mediated by TSLPR. This hypothesis is corroborated by our findings that auto-antibody levels are significantly increased in UC patients (our own results). Metabolic and inflammatory pathways are known to be highly integrated to provide swift energy supply in times of high energy expenditure by immune cells.[23] In addition, the link between monocyte differentiation and lipid metabolism has previously been found and assigned to the activity of PPAR-γ, whose inhibition induces the differentiation of M2 macrophages.[24] Furthermore, inducing PPAR-γ activation leads to suppression of TSLP expression, reinforcing the link between TSLP and lipid metabolism.[25] The results presented here further elucidate the link by defining CD1a-expressing monocytes as sensors and mediators of inflammation and TSLPR and CD1a as opposing actors in the inflammatory "mobilée."

SUPPLEMENTARY DATA

Supplementary data is available at *Inflammatory Bowel Diseases* online.

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Ethical considerations: All donors gave informed written consent, and the study was approved by the Institutional Review Board of the Medical Faculty at the University of Munich (2015–22). Animal studies were approved by the ethics committee of the government of Upper Bavaria, Germany (55.2-1-54-2532-65-11 and 55.2-1-54-2532-76-15), and performed in compliance with German Animal Welfare Laws.

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RESEARCH ARTICLE

Targeting ulcerative colitis by suppressing glucose uptake with ritonavir

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ABSTRACT

Glucose is the preferred source of energy in activated inflammatory cells. Glucose uptake into the cell is ensured by a family of glucose uptake transporters (GLUTs), which have been identified as off-target molecules of the HIV protease inhibitor ritonavir. In this study, we examined the effect of ritonavir on inflammation *in vitro* and *in vivo*. Peripheral blood mononuclear cells (PBMCs) were activated with anti-CD3 in the presence or absence of ritonavir and analyzed by flow cytometric analysis. Frequencies of CD4+ cells were significantly affected by ritonavir (CD69+ *P*=3E-05; CD134 *P*=4E-06; CD25+ *P*=E-07; central memory *P*=0.02; effector *P*=6E-03; effector memory *P*=6E-05). To corroborate that inflammation has a metabolic effect *in vivo*, a mouse model was used that is based on immunocompromised NOD-scid *IL-2Rγ*^{null} mice reconstituted with PBMCs from patients with ulcerative colitis (UC). Inflammation had a significant effect on amino acid (AA) levels (Glu *P*=1E-07, Asp *P*=1E-04). Principal component analysis (PCA) discriminated between unchallenged and challenged groups. Finally, the efficacy of ritonavir was tested in the same mouse model. Dependent variables were clinical and histological scores, frequencies of human leukocytes isolated from spleen and colon, and levels of AA in sera of mice. Mice benefited from treatment with ritonavir as indicated by significantly decreased colon (*P*=7E-04) and histological (*P*=1E-04) scores, frequencies of M2 monocytes (CD14+ CD163; *P*=0.02), and Glu levels (*P*=2E-05). PCA discriminated between control and challenged groups (*P*=0.026). Thus, inhibition of glucose uptake might be a promising therapeutic intervention point for active UC.

KEY WORDS: Ulcerative colitis, Metabolic switch, Glucose transporter, Ritonavir, NOD-scid *IL-2Rγ*^{null}, Mouse model, Inflammation

INTRODUCTION

The energy supply of inflammatory cells relies on three sources: glycolysis, oxidation of lipids, and amino acid (AA) metabolism. In homeostasis, when the major task of inflammatory cells is the maintenance of tolerance, lipids are the preferred source as lipid oxidation is the most efficient albeit slowest pathway to generate ATP (for a review, see O'Neill et al., 2016). The response to an assault, however, requires the immediate activation, proliferation and differentiation of inflammatory cells, their migration to sites of inflammation, and expression of cytokines, growth factors and chemokines. These processes demand prompt energy supply, which is met by a metabolic switch from lipid oxidation to glycolysis to ensure swift ATP generation and the synthesis of biosynthetic intermediates, albeit at the expense of efficiency. Therefore, the dependence on glycolysis might offer an Achilles' heel of inflammatory cells.

This aspect is of great interest for treatment of chronic inflammatory diseases. Although great therapeutic improvement has been made with the approval of highly effective biologicals like anti-TNFα antibodies (infliximab, adalimumab and certolizumab), the anti-integrin-α4β7 antibody vedolizumab and the anti-IL12/23 antibody ustekinumab, many patients respond insufficiently to these drugs or develop a secondary loss of response and require additional or different treatments. Metabolic profiling of patients with ulcerative colitis (UC) and Crohn's disease (CD) in comparison to non-diseased individuals corroborated the assumption that, in these diseases, glycolysis is the preferred metabolic pathway (Dawiskiba et al., 2014; Bjerrum et al., 2017). Hence, targeting glycolysis might open up entirely novel treatment options for UC or CD. In addition to metabolites related to glycolysis, these studies have shown that, in sera of UC and CD patients, levels of AAs were either up [glutamic acid (Glu)] or down [histidine (His)] (Hisamatsu et al., 2015).

Targeting glucose uptake has already been followed for the treatment of tumors whose energy demands and resources are similar to those of inflammatory cells. The dependence on glycolysis of tumor cells was observed by Otto Warburg approximately 100 years ago and is known as the Warburg effect (Warburg, 1956). As glucose uptake is the key rate-limiting step of glycolysis, inhibiting glucose transporters (GLUTs) offers a promising therapeutic approach. GLUTs encompass a wide family of integral membrane proteins that are differentially expressed in various tissues. The development of GLUT inhibitors for the treatment of cancer has further gained momentum by the observation that ritonavir, which has originally been developed as an inhibitor of HIV protease, coincidentally inhibits the members 1 and 4 (Murata et al., 2000; Hertel et al., 2004; Vyas et al., 2010) of the GLUT family.

Preclinical studies *in vitro* and *in vivo* have shown that ritonavir alone and in combination with metformin inhibits proliferation of multiple myeloma and chronic lymphocytic leukemia cells, both of which exhibit abnormal glucose metabolism (Adekola et al., 2015; Dalva-Aydemir et al., 2015). The positive outcome of these experiments resulted in a first clinical trial to test metformin hydrochloride and ritonavir in patients with relapsing or refractory

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multiple myeloma or chronic lymphocytic leukemia (http://clinicaltrials.gov/ct2/show/NCT02948283).

Recently, we have developed a mouse model of UC, which is based on NOD-scid *IL-2Rγ*^{null} (NSG) mice reconstituted with peripheral blood mononuclear cells (PBMCs) from patients with UC (NSG-UC mice) (Palamides et al., 2016; Jodeleit et al., 2017; Al-amodi et al., 2017). In this model, the development of symptoms is induced by rectal challenge with ethanol and requires immune reconstitution with PBMCs from patients with active disease.

In light of the metabolic switch observed in UC patients, we evaluated ritonavir as a potential therapeutic for treatment of UC. First, the effect of ritonavir on CD4+ T cells was tested *in vitro*. Results indicate that ritonavir inhibited the activation of CD4+ T cells. Secondly, glutamic and aspartic acid were identified as potential biomarkers of inflammation in the NSG-UC mouse model. Finally, ritonavir was tested in the NSG-UC mouse model. Results indicate that ritonavir might be an attractive therapeutic for UC and that AAs are powerful biomarkers in this model.

RESULTS

Ritonavir affects subsets of CD4+ T cells

Previous studies have shown that ritonavir inhibits proliferation and survival in multiple-myeloma and chronic-lymphocytic-leukemia cell lines *in vitro* and *in vivo* by shutting down the glycolytic pathway (Adekola et al., 2015; Dalva-Aydemir et al., 2015). However, these cell lines *per se* exhibit an abnormal glucose metabolism – a condition that we would not expect in unchallenged inflammatory cells. We therefore examined the response to ritonavir in subtypes of CD4+ T cells challenged with anti-CD3. PBMCs were isolated from five donors and experiments performed in triplicates. A total of 1×10^6 cells were incubated with or without anti-CD3 antibodies for 72 h in the presence and absence of ritonavir and subjected to flow cytometric analysis as described in the Materials and Methods. (For a definition of cells and gating strategy, see Table S2 and Fig. S1). As shown in Fig. 1A and Table S3, frequencies of CD4+ cells increased in response to anti-CD3; however, this effect was not significant. Upon the addition of ritonavir, frequencies of CD4+ cells returned to levels of the control group, and the difference between the ritonavir-treated group and the anti-CD3-treated group was significant, whereas that between the ritonavir-treated group and the control group was not. Exposure to anti-CD3 antibodies resulted in significantly increased frequencies of CD25-, CD134- and CD69-expressing CD4+ cells, indicating a general activation of CD4+ T cells. Ritonavir reversed these effects with the exception of CD4+ CD69+ cells, whose frequencies even further increased. Challenge with anti-CD3 antibodies had no effect on naïve or effector CD4+ T cells, and only a minor effect on effector memory CD4+ T cells, whereas the frequencies of central memory CD4+ T cells increased significantly. Ritonavir had opposing effects on effector, central memory and effector memory CD4+ T cells. Ritonavir decreased frequencies of effector and central memory CD4+ T cells, whereas frequencies of effector memory cells increased. To ensure that ritonavir did not affect the viability of PBMCs, PBMCs from two different donors were analyzed for LIVE CD4+ and LIVE CD14+ cells using Zombie Green™ Fixable Viability Kit. As shown in Fig. 1B, the viability of CD4+ T cells was not impaired by ritonavir.

Glutamic and aspartic acid are metabolic markers for inflammation in the NSG-UC mouse model

As previous studies have identified AAs as potential markers of inflammation in UC and Crohn's disease (Dawiskiba et al., 2014; Bjerrum et al., 2017), we measured AAs in serum of NSG-UC mice in response to challenge with ethanol. The study was performed as described previously (Palamides et al., 2016; Jodeleit et al., 2017). NSG mice were reconstituted with PBMCs and challenged according to a standard protocol as described in the Materials and Methods. At 8 days post-reconstitution, the mice were divided into two groups: one was left unchallenged (control), the other was challenged by rectal application of ethanol. Each group contained four to six animals and the experiment was repeated with four donors. Donors exhibited a simple clinical colitis activity index (SCCAI) (Walmsley et al., 1998) from one to seven. One donor was therapeutically naive, one was treated with mesalazine and vedolizumab, one with mesalazine, vedolizumab and glucocorticoids and one with glucticorticoids. Upon challenge with ethanol, mice stools became soft or liquid, the animals lost weight and the clinical activity score was elevated. Control animals displayed hardly any symptoms. Symptoms were classified according to a clinical score described in the Materials and Methods. Upon challenge, the clinical score increased significantly (Fig. 2A; for complete data set see Table S4). This observation was also corroborated by the colon score, which also increased significantly. As shown in previous studies (Palamides et al., 2016; Jodeleit et al., 2017; Al-amodi et al., 2017), histological analysis of the colon revealed the influx of a mixed infiltrate of leukocytes, edema, crypt loss and changes in the colon architecture (not shown). The histopathological manifestations were classified according to a histological score (Fig. 2A) and confirmed a significant response to ethanol. Prior to autopsy, serum samples were collected and subjected to AA analysis. Inflammation resulted in significantly elevated levels of glutamic and aspartic acid (Fig. 2A). Receiver operating characteristic (ROC) analysis identified both AAs as biomarkers with high potential to discriminate between challenged and unchallenged mice (glutamic acid: AUC=0.91; aspartic acid: AUC=0.87) (Fig. 2B). For further analysis, leukocytes were isolated from spleen and subjected to flow cytometric analysis as described in the Materials and Methods. Correlation analysis revealed a high correlation coefficient between both AAs and the clinical score, colon score and histological score, and with activated CD4+ CD69+ and CD4+ CD103+ T cells (Table 1), and thus corroborated the *in vitro* experiments. As observed in the human studies, levels of histidine declined upon challenge (Hisamatsu et al., 2015).

To further evaluate the combined power of the parameters to discriminate between the two groups, a principal component analysis (PCA) was performed to include the clinical, colon and histological scores, and levels of glutamic and aspartic acid. Scatter plots of PCA scores of single mice, with ellipses indicating confidence regions, depict mice of the control group as a closely clustered group, whereas ethanol-challenged mice are more distributed (Fig. 3). Multivariate regression analysis of the five parameters revealed an R^2 predicted value of 0.62 and discriminating analysis based on partial least square that 95% of all predictions were correct.

Treatment with ritonavir ameliorates disease phenotype in NSG-UC mouse model

To examine the effect of ritonavir *in vivo*, NSG-UC mice were treated with ritonavir. The study was performed as in the previous experiment; this time, however, the challenged group was treated with vehicle (ethanol+vehicle) and a third group was added consisting of mice challenged with ethanol and treated with ritonavir (ethanol+ritonavir). The study was repeated with PBMCs from four different UC patients (Table 2). (For the

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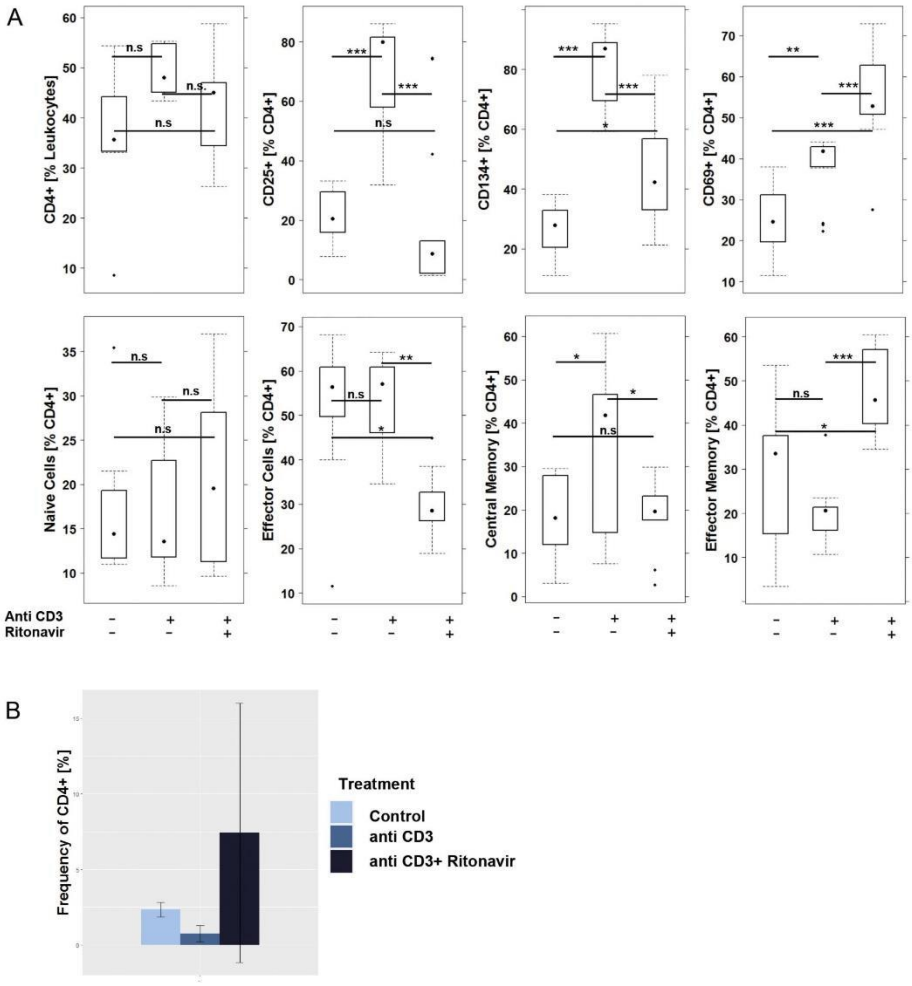

Fig. 1. Ritonavir affects frequencies of CD4+ T-cell subtypes. Flow cytometric analysis of PBMCs that were incubated for 3 days in the presence or absence of anti-CD3 antibodies and ritonavir. Experiments were performed in triplicate. (A) Frequencies of CD4+ T cells and subtypes thereof depicted as boxplot diagrams (donor n=5). For comparison of groups, ANOVA followed by Tukey's HSD was conducted. Boxes represent upper and lower quartiles, whiskers represent variability and outliers are plotted as individual points (***P≤0.001, **P≤0.01, *P≤0.05, n.s., non-significant). Labels given on x-axes on the bottom row apply to all charts. (B) Frequencies of LIVE CD4+ cells depicted as barplots. Mean values are given; error bars are s.d. Results from two different donors are given.

complete data set, please see Table S5.) The clinical score increased upon challenge and almost normalized with treatment of ritonavir (Fig. 4C). This observation was corroborated by macroscopic inspection of the colons. As shown in Fig. 4Ac, colons of ritonavir-treated mice appeared normal as compared to colons of ethanol-challenged mice (Fig. 4Ab), which exhibited the typical dilation and soft stools. Histological analysis further confirmed these results. Challenge with ethanol resulted in severe colon pathology to include crypt alterations, edema and fibrosis (Fig. 4Bb), whereas histological sections of ritonavir-treated mice appeared almost normal (Fig. 4Bc) (for scoring data, see Table S6). This observation was corroborated by the histological score, which was significantly reduced in the ritonavir-treated group (Fig. 4C).

As subtypes of monocytes had been previously identified as inflammatory markers in the NSG-UC mouse model (H.J., O.A.-A. and G.B. et al., unpublished), human leukocytes were isolated from the spleen of mice and subjected to flow cytometric analysis. The most profound effect of ritonavir was found on naïve CD4+ T cells and on M2 monocytes (CD14+ CD163+), whose frequencies were significantly elevated upon challenge with ethanol and reduced to

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RESEARCH ARTICLE Disease Models & Mechanisms (2018) 11, dmm036210. doi:10.1242/dmm.036210

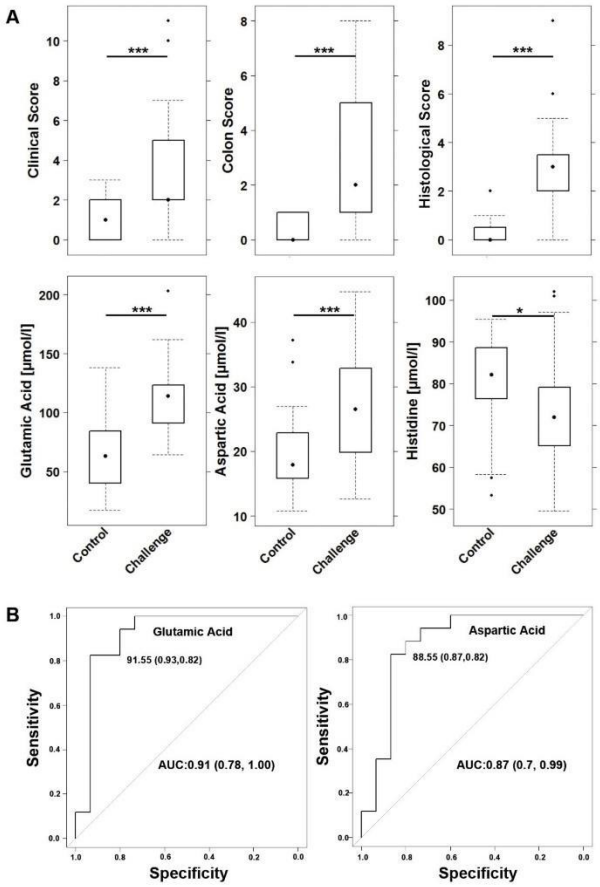

Fig. 2. Glutamic and aspartic acid are inflammatory markers in the NSG-UC mouse model. NSG-UC mice were engrafted with PBMCs derived from UC patients, challenged with 10% ethanol at day 8, and 50% ethanol at days 15 and 18. (A) Amino acid (AA) levels and clinical and histological scores depicted as boxplot diagrams [donors n=3; unchallenged control group (control n=10); ethanol-challenged group (Challenge n=11)]. For comparison of groups, ANOVA followed by Tukey's HSD was conducted. Boxes represent upper and lower quartiles, whiskers represent variability and outliers are plotted as individual points (***P≤0.001, *P≤0.05). Labels given on x-axes on the bottom row apply to all charts. (B) ROC curve for glutamic and aspartic acid in discriminating control and challenged groups.

normal levels in the presence of ritonavir (Fig. 4D). In contrast, frequencies of CD1a-expressing CD14+ monocytes were not significantly affected by treatment with ritonavir (Table S5). The anti-inflammatory effect of ritonavir was corroborated by analysis of AA levels. Glutamic and aspartic acids levels normalized upon treatment with ritonavir (Fig. 4E). Histidine levels increased upon treatment; however, this effect was not significant.

Table 1. Correlation analysis of aspartic and glutamic acid serum levels with clinical, colon and histological scores and activated CD4+ T cells.

	Correlation coefficients	
Cells	Aspartic acid (P;CI)	Glutamic acid (P;CI)
Clinical score	0.3 (0.007; 0.1-1)	0.44 (0.001; 0.18-1)
Histological score	0.42 (0.0007; 0.21-1)	0.58 (2E-6; 0.4-1)
Colon score	0.39 (0.0005; 0.2-1)	0.46 (5E-5; 0.28-1)
CD4+ CD69+		0.36 (0.008; 0.12-1)
CD4+ CD103+	0.61 (7E-6; 0.42-1)	0.41 (0.03; 0.17-1)

Method=Pearson; numbers display correlation coefficients, followed by P-values and confidence intervals (CIs) in parentheses. Sample size: n=71.

To examine the effect of ritonavir on inflammation in the compartment of the colon, leukocytes were isolated and subjected to flow cytometric analysis (Fig. 5). Frequencies of human CD11b + macrophages, CD14+ monocytes, CD19+ B cells, and neutrophils were reduced upon treatment with ritonavir, whereas CD4+ T cells were unaffected (for gating strategy see Fig. S2). The strongest effect was observed on CD103- and CD134-expressing CD4+ T cells, mature CD11b+ macrophages and CD14+ monocytes, and M1 (CD14+ CD64+) and M2 (CD14+ CD163) monocytes; however, differences did not reach significance.

As observed previously, PCA clearly segregated the challenged group from the control, and the treated group clustered with the control group when clinical score, histological score, and aspartic and glutamic acid levels were used as parameters (Fig. 6). Ordinal regression analysis revealed the significance of this model [Pr (Chi)=0.004], and these parameters resulted in a significant discrimination of the control group and the ethanol-challenged group (P=0.02), and the ethanol-challenged group and the ritonavir-treated group (P=0.05).

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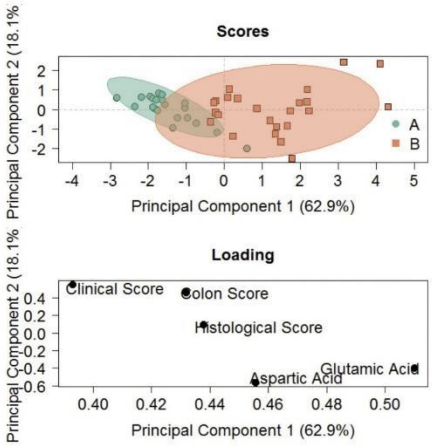

Fig. 3. PCA discriminates between control and ethanol-challenged group. PCA score and loading plots (donors *n*=4, each group *n*=17). Sample size: donor *n*=4, ('A', green) control *n*=17, ('B', orange) challenge *n*=17. Ellipses display confidence areas of 0.95.

DISCUSSION

The metabolic requirements of cancer cells differ from immune cells with regard to their capacity to modulate and adapt energy supply. Whereas cancer cells are continuously dependent on glycolysis due to their proliferative and migratory nature, immune cells acquire these traits upon challenge and modulate metabolic pathways accordingly. Therefore, the observation that activation of CD4+ T cells and the increase of frequencies of central memory CD4+ T cells in response to anti-CD3 were reversed by ritonavir did not come as a surprise. We did not expect, however, for ritonavir to increase the frequencies of effector memory cells. Central memory and effector memory cells differ with regard to expression of CD62L and CCR7, and, hence, ritonavir would impair the ability of memory cells to migrate to lymph nodes to be exposed to dendritic cells. We also did not expect that ritonavir increases the frequencies of early activated CD4+ CD69 + T cells. Further studies have to show which subtypes of cells are activated to understand the specific responses to ritonavir.

In the NSG-UC mouse model, the induction of inflammation had a significant effect on glutamic and aspartic acid, tryptophan and histidine levels, and corroborated results obtained in UC patients (Dawiskiba et al., 2014; Hisamatsu et al., 2015; Bjerrum et al., 2017). ROC analysis indicated that glutamic and aspartic acid were biological markers to discriminate with high sensitivity and specificity between unchallenged control and ethanol-challenged mice. PCA of the colon and histological scores, and glutamic and aspartic acid levels revealed a clustering of these two groups. This result is a major advantage as it most probably allows for evaluating more subtle differences in efficacy.

Table 2. Basic patient demographics

Donor	Age (years)	Gender	Medication	SCCAI
UC1	26	M	Adalimumab	9
UC2	80	F	Mesalazine	5
UC3	54	M	None	3
UC4	30	M	Vedolizumab	11

M, male; F, female; SCCAI, simple clinical colitis activity index.

Mice benefitted significantly from treatment with ritonavir as indicated by the four parameters: clinical and histological scores and glutamic and aspartic acid levels in serum. PCA distinguished between control and challenged groups, and treatment with ritonavir resulted in clustering closer to the control group. Analysis of the human leukocytes isolated from spleen revealed that the most pronounced effect of ritonavir was observed in monocytes. Unlike in the *in vitro* experiments, ritonavir had no effect on activation of CD4+ T cells (data not shown).

An effect of ritonavir was, however, observed when human leukocytes isolated from colon were analyzed. As observed in the *in vitro* experiments, frequencies of activated CD4+ T cells declined with the exception of CD4+ CD69+ T cells. Likewise, frequencies of mature CD11b+ macrophages and CD14+ monocytes decreased, as well as M2 monocytes.

In summary, this study shows that inflammation in the NSG-UC mice has a significant metabolic effect in the NSG-UC model, which partially reflects the metabolic effects observed in UC patients. AA levels are potential biological markers of inflammation so might improve the evaluation of efficacy of therapeutics. The positive outcome of the preclinical study in the NSG-UC model suggests that UC patients might benefit from adjuvant treatment with ritonavir during times of active disease, and might encourage the development of novel GLUT inhibitors.

MATERIALS AND METHODS

Ethical considerations

All donors gave informed written consent and the study was approved by the Institutional Review Board (IRB) of the Medical Faculty at the University of Munich (2015-22).

Animal studies were approved by the ethics committee of the government of Upper Bavaria, Germany (55.2-1-54-2532-76-15) and performed in compliance with German Animal Welfare Laws.

Isolation of PBMCs and engraftment

Isolation of PBMCs and engraftment was performed as described previously (Jodeleit et al., 2017). Sixty milliliters of peripheral blood were collected in trisodium citrate solution (S-Monovette, Sarstedt, Nürnberg, Germany) from the arm vein of patients suffering from UC or healthy control subjects. The blood was diluted with Hank's balanced salt solution (HBSS, Sigma-Aldrich, Deisenhofen, Germany) in a 1:2 ratio and 30 ml of the suspension were loaded onto LeucoSep tubes (Greiner Bio One, Frickenhausen, Germany). PBMCs were separated by centrifugation at 400 ***g*** for 30 min and no acceleration. The interphase was extracted and diluted with phosphate buffered saline (PBS) to a final volume of 40 ml. Cells were counted and centrifuged at 1400 ***g*** for 5 min. The cell pellet was resuspended in PBS at a concentration of 4×10^6 cells in 100 µl.

Six- to eight-week-old NSG mice were engrafted with 100 µl cell suspension into the tail vein on day 1.

Cell culture

PBMCs of healthy individuals and UC patients were isolated. The cell pellet was resuspended in RPMI (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 1×10^6 cells/ml. Additionally, 500 µl RPMI with 10% FCS and 1% penicillin-streptomycin (Sigma-Aldrich, St Louis, MO, USA) were added to each well and sample. Wells containing PBMCs and RPMI or PBMCs, RPMI and anti-CD3 (1 µg/ml, BioLegend, San Diego, CA, USA) served as negative and positive controls, respectively. Ritonavir was dissolved in 100% ethanol (10 mg/ml). For analysis of the effect of ritonavir, 100 µg/ml ritonavir (Sigma-Aldrich, Deisenhofen, Germany) was added. Cells were incubated for 72 h. The content of each well was centrifuged at 1400 ***g*** for 5 min. The pellet was resuspended in FACS buffer and labeled for flow cytometry.

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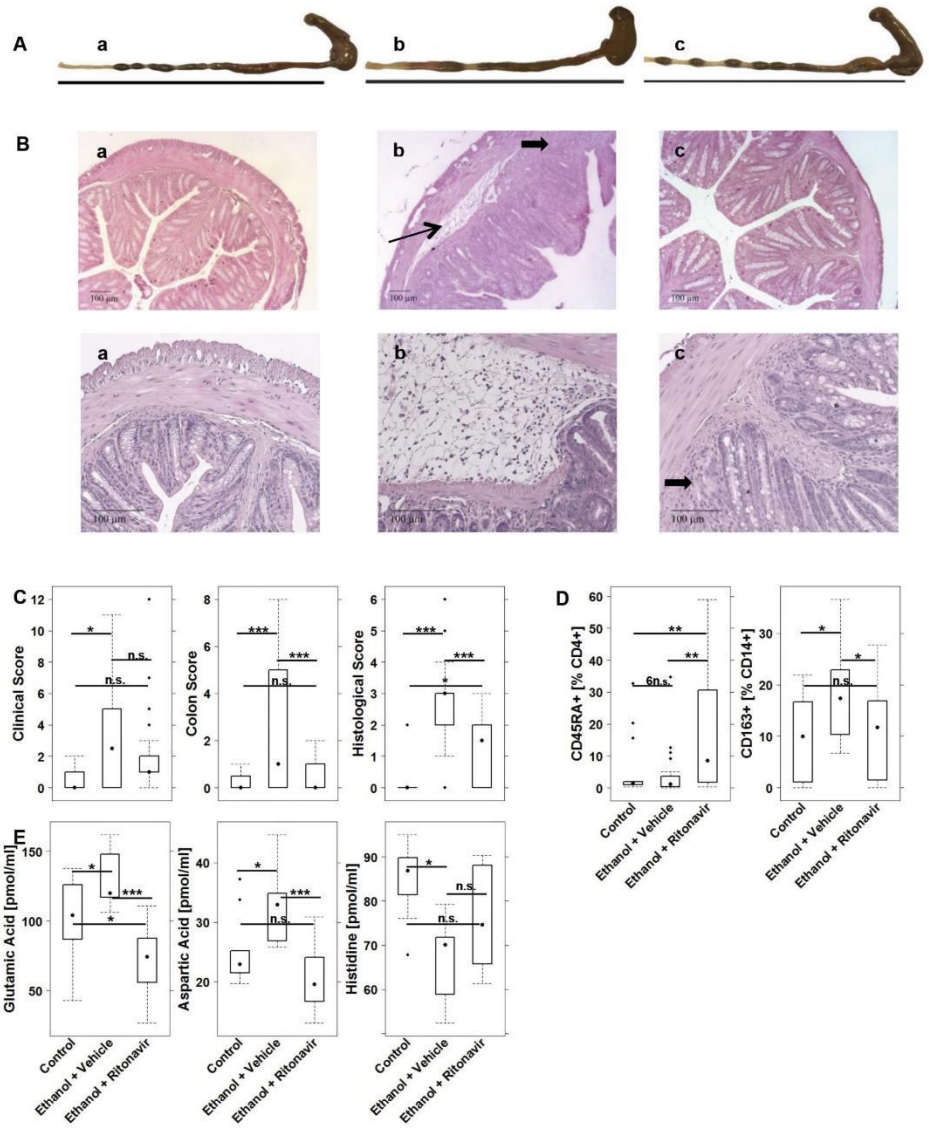

Fig. 4. NSG-UC mice benefit from treatment with ritonavir. NSG-UC mice were engrafted with PBMCs derived from UC patients (*n*=4), challenged with 10% ethanol at day 8, and 50% ethanol at days 15 and 18, and treated with 20 mg/kg body weight ritonavir or carrier at days 7, 8 and 14-20. (a) Unchallenged control (Control *n*=15); (b) challenged control (Ethanol+Vehicle, *n*=21); (c) challenged and treated with ritonavir (Ethanol+Ritonavir, *n*=27). (A) Macrophotographs of colons at autopsy. (B) Photomicrographs of H&E-stained sections of distal parts of the colon from mice reconstituted with the same donor. Thin arrow indicates edema and influx of inflammatory cells; bold arrows indicate fibrosis. (C) Clinical, colon and histological scores of NSG-UC mice. (D) Frequencies of CD14+ subtypes isolated from spleens of mice. (E) Aspartic and glutamic acid serum levels depicted as boxplot diagrams (donor *n*=2, Control *n*=10, Ethanol +Vehicle *n*=10, Ethanol+Ritonavir *n*=11). For comparison of groups, ANOVA followed by Tukey's HSD was conducted. Boxes represent upper and lower quartiles, whiskers represent variability and outliers are plotted as individual points (***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, n.s., non-significant). Labels given on *x*-axes on the bottom row apply to all charts.

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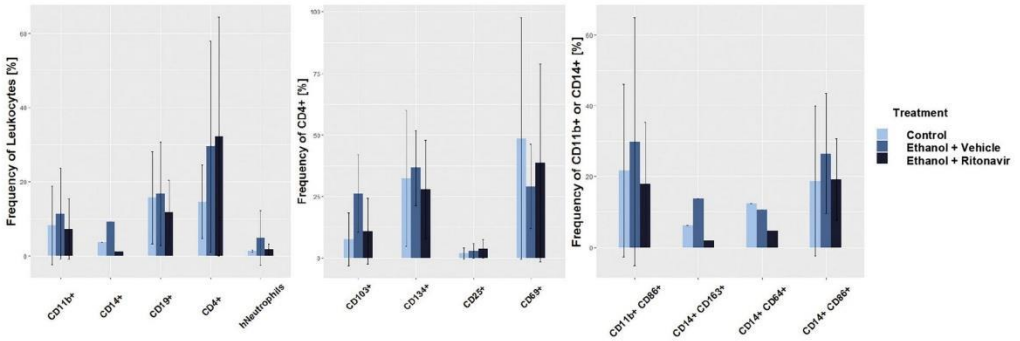

Fig. 5. Treatment with ritonavir affected frequencies of human leukocytes isolated from colon of NSG-UC mice. Leukocytes were isolated from mice described in Fig. 4. Data were combined from four different experiments and samples from each group were pooled from six mice (Control *n*=4, Ethanol+Vehicle *n*=4, Ethanol+Ritonavir *n*=4). Mean values are given; error bars are s.d. Quantification was performed using flow cytometric analysis.

To differentiate between live and dead cells, staining with Zombie Green™ Fixable Viability Kit (BioLegend) was performed according to the manufacturer's instructions.

Animal study protocol

NOD-scid *IL-2Rγ*^{null} (NSG) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were kept under specific pathogen-free conditions in individually ventilated cages in a facility controlled according to the Federation of Laboratory Animal Science Association (FELASA) guidelines. Following engraftment (day 1), mice were pre-sensitized by rectal application of 150 µl of 10% ethanol on day 8 using a 1 mm cat catheter (Henry Schein, Hamburg, Germany), which is normally used for urinary catheterization in cats. In our case, we used it for rectal application of ethanol because of a similar diameter of a mouse rectum and a cat's urethra. The catheter was lubricated with Xylocain©Gel 2% (AstraZeneca, Wedel, Germany). Rectal application was performed under general anesthesia using 4% isofluran. Following application, mice were kept at an angle of 30° to avoid ethanol dripping. On days 15 and 18, mice were challenged by rectal application of 50% ethanol following the protocol of day 8. Mice were sacrificed by cervical dislocation on day 21.

Ritonavir was dissolved in 100% ethanol (100 mg/ml) and diluted with PBS to a final concentration of 5 mg/ml of 5% ethanol. Mice were treated by intraperitoneal application of 500 µg or 5% ethanol in PBS (vehicle) on days 7, 8 and 14-20. The dosage corresponds to 20 mg/kg body weight, which is recommended for maintenance therapy of HIV patients and calculated for a 25 g mouse.

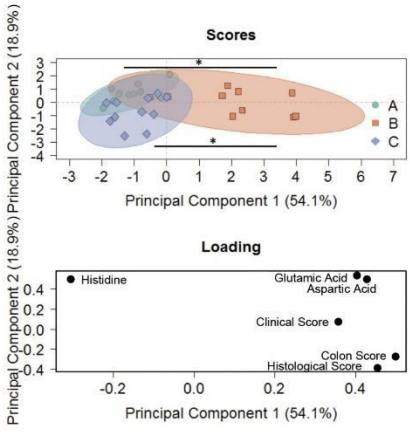

Fig. 6. PCA discriminates between the control group, ethanol-challenged group and ritonavir-treated group. PCA scores and loading plots ('A', green=Control; 'B', orange=Ethanol+Vehicle; 'C', blue=Ethanol+Ritonavir). For comparison of groups, ordinal regression analysis was performed (*$P \leq 0.05$). Control group versus Ethanol+Vehicle P=0.02; Ethanol+PBS P=0.05.

Clinical activity score

The assessment of severity of colitis was performed daily according to the following scoring system: loss of body weight: 0% (0), 0-5% (1), 5-10% (2), 10-15% (3), 15-20% (4). Stool consistency: formed pellet (0), loose stool or unformed pellet (2), liquid stools (4). Behavior: normal (0), reduced activity (1), apathy (4) and ruffled fur (1). Body posture: intermediately hunched posture (1), permanently hunched posture (4). The scores were added daily into a total score with a maximum of 18 points per day. Animals who suffered from weight loss >20%, rectal bleeding, rectal prolapse, self-isolation or a severity score >7 were euthanized immediately and not taken into count.

Histological analysis

Samples from distal parts of the colon were fixed in 4% formaldehyde for 24 h before storage in 70% ethanol and were routinely embedded in paraffin. Samples were cut into 3-µm sections and stained with hematoxylin and eosin (H&E). Epithelial erosions were scored as follows: no lesions (0), focal lesions (1), multifocal lesions (2), major damage with involvement of basal membrane (3). Inflammation was scored as follows: infiltration of few inflammatory cells into the lamina propria (1), major infiltration of inflammatory cells into the lamina propria (2), confluent infiltration of inflammatory cells into the lamina propria (3), infiltration of inflammatory cells including tunica muscularis (4). Fibrosis was scored as follows: focal fibrosis (1), multifocal fibrosis and crypt atrophy (2). The presence of edema – minimal (1), moderate (2), severe (3), hyperemia (1) and crypt abscess (1) – was scored with additional points in each case. The scores for each criterion were added to give a total score ranging from 0 to 12. Images were taken with a Zeiss AxioVert 40 CFL camera. Figures show representative longitudinal sections in original magnification. In Adobe Photoshop CS6, a tonal correction was applied in order to enhance contrasts within the pictures.

Isolation of human leukocytes

To isolate human leukocytes from murine spleen, spleens were minced and cells filtrated through a 70 µl cell strainer (Greiner Bio-One, Frickenhausen, Germany) followed by centrifugation at 1400 ***g*** for 5 min and resuspended

in FACS buffer (1× PBS, 2 mM EDTA, 2% FCS) (Jodeleit et al., 2017). For further purification, cell suspensions were filtrated using a 35-µm cell strainer (Greiner Bio-One) and then labeled for flow cytometry analysis.

Isolation of LPMCs

For isolation of lamina propria mononuclear cells (LPMCs), a modified protocol of Weigmann et al. (2007) was used. The washed and minced colon was pre-digested for 20 min in pre-digestion solution containing 1× HBSS (Thermo Scientific, Darmstadt, Germany), 5 mM EDTA, 5% FCS, 100 U/ml penicillin-streptomycin (Sigma-Aldrich) in an orbital shaker with slow rotation (40 ***g***) at 37°C. Epithelial cells were removed by filtering through a nylon filter. Following washing with PBS, the remaining colon pieces were digested twice for 20 min in digestion solution containing 1× RPMI (Thermo Scientific), 10% FCS, 1 mg/ml collagenase A (Sigma-Aldrich), 10 KU/ml DNase I (Sigma-Aldrich) and 100 U/ml penicillin-streptomycin (Sigma-Aldrich) in an orbital shaker with slow rotation (40 ***g***) at 37°C (Weigmann et al., 2007). Isolated LPMCs were collected by centrifugation at 1400 ***g*** for 5 min and resuspended for FACS analysis. Cell suspensions were filtrated one more time using a 35-µm cell strainer for further purification before labeling the cells for flow cytometry analysis.

Flow cytometry analysis

Labeling of human leukocytes was performed according to Table S1. All antibodies were purchased from BioLegend and used according to the manufacturer's instructions. Antibodies were diluted 1:200. Flow cytometry was performed using a BD FACS Canto II™ and analyzed with FlowJo 10.1 software (FlowJo LLC, OR, USA). Cells were quantified according to the gating strategy depicted in Fig. S1.

Detection of amino acids

Samples were prepared according to the manufacturer's instructions. Following incubation of 100 µl of serum with internal standards for 5 min, 25 µl of 15% 5-sulfosalicylic acid was added and samples were centrifuged at 9000 ***g*** for 15 min at 4°C. Supernatants were filtered through a 0.2-µm membrane and 75 µl of lithium loading buffer was added. Samples were analyzed by the AA analyzer Biochrom 30+ (Biochrom Ltd, Cambridge, UK).

Statistics

All statistical analysis was performed with R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/) and BRB Array Tools (https://brb.nci.nih.gov/BRB-ArrayTools/). Variables were represented with mean, standard deviation and median values. A two-sided Student's *t*-test and a confidence level=0.95 was used to compare binary groups and, for more than two groups, ANOVA followed by Tukey's HSD was conducted. For correlation analysis, Pearson correlation was used. To minimize the risk of overfitting, multivariate regression models were performed applying the kernelpls fit method and performing a leave one out cross validation procedure. For PCA, a confidence interval of 0.95% was used.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.S.; Methodology: H.J., O.A.-A.; Formal analysis: C.V.A., R.G.; Investigation: H.J., O.A.-A., J.C., L.H.; Resources: F.B.; Data curation: R.G.; Writing - original draft: R.G.; Writing - review & editing: M.S.; Supervision: R.G.; Project administration: R.G., F.B., M.S.; Funding acquisition: F.B., M.S.

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Supplementary information

Supplementary information available online at
http://dmm.biologists.org/lookup/doi/10.1242/dmm.036210.supplemental

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