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Charakterisierung intermediärer Monozyten als eigenständige proinflammatorische Zellpopulation

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Abkürzungsverzeichnis

ACE	*"Angiotensin I converting enzyme"*
ATP	Adenosintriphosphat
BHMT	Betain-Homocystein-Methyltransferase
CARE FOR HOMe	*"Cardiovascular and renal outcome in CKD 2-4 patients - The forth Homburg evaluation"*
CCR2	*"Chemokine (C-C motif) receptor 2"*
CCR5	*"Chemokine (C-C motif) receptor 5"*
CD	*"Cluster of differentiation"*
CKD	*"Chronic kidney disease"*
CKD-ND	„C*hronic kidney disease, no dialysis*"
CV	*"Cardiovascular"*
CX_3CR1	*"Chemokine (C-X3-C motif) receptor 1"*
DNA	*"Deoxyribonucleic acid"*
ENG	*"Endoglin"*
eNOS	*"Endothelial nitric oxide synthase"*
HOMe ALONE	*"Heterogenetiy of monocytes and echocardiography among allograft recipients in nephrology"*
HOM sweet HOMe	*"Heterogeneity of monocytes in subjects who undergo elective coronary angiography – The Homburg evaluation"*
IL	Interleukin
I Like HOMe	*"Inflammation, lipoprotein metabolism and kidney damage in early atherogenesis — The Homburg evaluation"*
IMT	*"Intima-media thickness"*
K/DOQI	*"Kidney disease outcomes quality initiative"*
LDL	*"Low density lipoprotein"*

LFA-1	*"Lymphocyte function-associated antigen"*
LPS	Lipopolysaccharid
MS	Methionin-Synthase
oxLDL	oxidiertes LDL
PBMC	*"Peripheral blood mononuclear cell"*
PSGL-1	*"P-selectin glycoprotein ligand-1"*
RefSeq	*"NCBI reference sequence"*
RNA	*"Ribonucleic acid"*
ROS	*"Reactive oxygen species"*
SAH	S-Adenosylhomocystein
SAM	S-Adenosylmethionin
SMSDK	*"SuperTAG methylation-specific digital karyotyping"*
SuperSAGE	*"Serial analysis of gene expression"*
TEK	*"Tyrosine kinase, endothelial"*
THF	Tetrahydrofolat
TLR2	Toll-like Rezeptor 2
TLR4	Toll-like Rezeptor 4
TNFα	Tumornekrosefaktor alpha
VEGFR	*"Vascular endothelial growth factor receptor"*
VLA-4	*"Very Late Antigen-4"*

Zusammenfassung

Chronisch nierenkranke Patienten weisen gegenüber nierengesunden Menschen eine deutlich erhöhte Mortalität auf, welche vor allem auf eine massiv beschleunigte Atherosklerose mit nachfolgend erhöhter kardiovaskulärer Morbidität zurückgeführt werden kann. Monozyten stellen als Mediatoren der Inflammation ein zentrales Bindeglied in der beschleunigten Atherogenese chronisch nierenkranker Menschen dar. Durchflusszytometrisch lassen sich drei Subpopulationen von Monozyten charakterisieren: klassische CD14++CD16-, intermediäre CD14++CD16+ und nicht-klassische CD14+CD16++ Monozyten. Vorarbeiten konnten eine zentrale Rolle der intermediären Monozyten in der Atherogenese nierenkranker Menschen aufzeigen und eine erhöhte Anzahl intermediärer Monozyten als Prädiktor kardiovaskulärer Ereignisse bei Dialysepatienten charakterisieren.

In der vorliegenden Arbeit konnte einerseits auf der Basis epidemiologischer Untersuchungen aufgezeigt werden, dass intermediäre Monozyten nicht nur in selektierten Kohorten von Dialysepatienten, sondern auch bei nicht-dialysepflichtigen chronisch nierenkranken Patienten und selbst bei nierengesunden Menschen Prädiktoren kardiovaskulärer Ereignisse sind. Andererseits erfolgte experimentell eine detaillierte Charakterisierung der drei Monozytensubpopulationen, welche selektiv proinflammatorische Eigenschaften intermediärer Monozyten aufzeigte. Schließlich wurden dysregulierte epigenetische Mechanismen bei chronisch nierenkranken Menschen identifiziert, die zu den monozytären Veränderungen und der beschleunigten Entstehung atherosklerotischer Läsionen bei diesen Patienten beitragen könnten.

Zusammenfassend erlauben die Ergebnisse dieser Arbeit eine bessere Charakterisierung der Monozytenheterogenität und schärfen das pathophysiologische Verständnis der hohen kardiovaskulären Morbidität chronisch nierenkranker Menschen. Auf dieser Basis werden intermediäre Monozyten als potentielles Ziel zukünftiger Behandlungsstrategien zur Prävention und Therapie kardiovaskulärer Erkrankungen bei chronisch nierenkranken Menschen diskutiert.

Abstract (Zusammenfassung in englischer Sprache)

Compared to individuals from the general population, patients with chronic kidney disease (CKD) suffer from a dramatically increased mortality, which is mainly due to accelerated atherosclerosis and subsequent elevated cardiovascular morbidity. As central cellular components of the immune system, monocytes may contribute to accelerated atherosclerosis in CKD patients. Three monocyte subsets are characterized *via* flow-cytometry: classical CD14++CD16-, intermediate CD14++CD16+ and nonclassical CD14+CD16++ monocytes. Recent studies revealed a central role of intermediate monocytes in CKD-associated atherosclerosis. Accordingly, high cell counts of intermediate monocytes predicted cardiovascular events in dialysis patients.

The present thesis first extends findings from epidemiological studies in dialysis patients, characterizing intermediate monocytes as predictors of cardiovascular events in cohorts of non-dialysis CKD patients and even in patients without overt CKD. Secondly, experimental analyses provided a detailed characterization of the three monocyte subsets, revealing subset-specific proinflammatory characteristics of intermediate monocytes. Finally, dysregulated epigenetic mechanisms were found in CKD patients, which may contribute to the shift in monocyte subsets and to accelerated atherosclerosis in these patients.

In summary, these results allow a better characterization of monocyte heterogeneity and broaden the pathophysiological understanding of the high cardiovascular morbidity of CKD patients. Based on these results, intermediate monocytes are currently discussed as potential targets for prevention and treatment of CKD-associated cardiovascular disease.

1. Einleitung

1.1 Die chronische Nierenerkrankung

Eine veränderte Altersstruktur und wachsende Komorbidität in westlichen Industrieländern konfrontiert unsere Gesellschaft mit einer progredienten Prävalenz der chronischen Nierenerkrankung (*chronic kidney disease* [CKD]), woraus für das Gesundheitswesen sowohl medizinisch als auch ökonomisch eine wachsende Belastung resultiert. So weisen in den Vereinigten Staaten von Amerika 26,3 Millionen Menschen eine chronische Nierenerkrankung auf [13]. Hingegen ist die Anzahl chronisch nierenkranker Menschen in Deutschland aufgrund fehlender epidemiologischer Untersuchungen in den letzten Jahren nicht detailliert bekannt; allerdings nahm allein die Anzahl der Dialysepatienten zwischen 1995 und 2007 von 41350 auf 66508 zu [7].

Die Lebenserwartung chronisch nierenkranker Menschen ist aufgrund einer ausgeprägten extrarenalen Komorbidität, die von der massiv beschleunigten Atherosklerose mit konsekutiv erhöhter kardiovaskulärer Morbidität und Mortalität dominiert wird, dramatisch verringert [16]. Dieser Zusammenhang zwischen chronischer Nierenerkrankung und dem Auftreten von Herz- und Gefäßerkrankungen wurde vor einigen Jahren als „Kardiorenales Syndrom“ definiert [67].

Das erhöhte Risiko für kardiovaskuläre Ereignisse bei chronisch nierenkranken Patienten kann dabei nicht allein durch die erhöhte Prävalenz klassischer kardiovaskulärer Risikofaktoren wie Bluthochdruck, Diabetes mellitus, Nikotinkonsum und Hypercholesterinämie [52,59,82] erklärt werden, sondern beruht additiv auf dem Einwirken sogenannter nicht-klassischer Risikofaktoren (**Tabelle 1**).

Tabelle 1. Klassische und Nicht-klassische Risikofaktoren bei chronischer Nierenerkrankung (modifiziert nach Sarnak et al [71])

Klassische Risikofaktoren	Nicht-klassische Risikofaktoren
Höheres Alter	Homocystein
Männliches Geschlecht	Anämie
Bluthochdruck	Dysregulierter Ca/P Metabolismus
Erhöhtes LDL Cholesterin	Oxidativer Stress
Diabetes mellitus	Chronische (Mikro)inflammation
Rauchen	Malnutrition
Physische Inaktivität	

LDL: *low-density-lipoprotein*; Ca: Calcium; P: Phosphat

Im Einklang hiermit vermag die isolierte Behandlung klassischer kardiovaskulärer Risikofaktoren wie die Senkung des LDL-Cholesterins mittels Statintherapie, oder die Gabe vaskuloprotekiver ACE- (*angiotensin I converting enzyme*) Hemmer, welche sich bei nierengesunden Menschen als kardioprotektiv erwiesen, keine substantielle Reduktion kardiovaskulärer Mortalität bei chronisch nierenkranken Patienten zu erreichen [19,88,91]. Daher erscheint ein besseres pathophysiologisches Verständnis der atherosklerotischen Gefäßveränderungen bei chronisch nierenkranken Menschen erforderlich, um effektivere Therapiestrategien definieren zu können. Ein zentraler Fokus zukünftiger Forschung zur kardiovaskulären Morbidität chronisch nierenkranker Patienten sollte hierbei auf nicht-klassische kardiovaskuläre Risikofaktoren gelegt werden.

1.2 Monozyten und Atherosklerose

Die chronische (Mikro)inflammation ist ein zentraler nicht-klassischer Risikofaktor des nierenkranken Patienten [68], welcher zentral durch eine Monozytendysfunktion charakterisiert ist. So konnte einerseits der lange vorbekannte urämische Immundefekt, welcher sich in einer verminderten Impfantwort und einer erhöhten Infektneigung manifestiert, auf eine Monozytenfunktionsstörung zurückgeführt werden, die durch gesteigerte Sekretion proinflammatorischer Zytokine und verminderte T-Zell Co-Stimulation gekennzeichnet ist [25]. Andererseits weisen Monozyten eine zentrale Bedeutung in der Entstehung und Progredienz atherosklerotischer Läsionen auf [33]. Bereits in der Frühphase der endothelialen Dysfunktion adhärieren Monozyten über distinkte Adhäsionsmoleküle (z.B. PSGL-1, VLA-4, LFA-1) an aktivierte Endothelzellen und migrieren in die Intima der Gefäßwand (siehe **Abbildung 1**). Hier differenzieren sie sich zu Dendritischen Zellen oder Makrophagen und induzieren die Entstehung atherosklerotischer Läsionen, indem sie proinflammatorische Zytokine und Wachstumsfaktoren sezernieren und dadurch weitere Immunzellen in die Gefäßwand locken [48]. Durch Aufnahme von oxLDL und weiteren Lipiden entstehen aus den Makrophagen Schaumzellen, die unter dem Endothel als „Fettstreifen“ („*fatty streaks“*) eine charakteristische Veränderung der Gefäßwand darstellen. Zusätzlich kommt es zum Einwandern glatter Muskelzellen aus der Media- in die Intima-Schicht der Gefäßwand und schließlich zur Ausbildung eines komplizierten atherosklerotischen Plaques, bestehend aus einem nekrotischen Kern, der von glatten Muskelzellen und einer Kollagenmatrix umgeben wird. Eine Ruptur der Plaques kann zur Freisetzung prothrombotischen Materials und zur Aktivierung der Koagulationskaskade mit nachfolgender Thrombusbildung führen, was sich klinisch bei Erkrankung von

Herzkranzarterien als akuter Myokardinfarkt, bei Erkrankungen von hinzuführenden Arterien als Apoplex manifestieren kann.

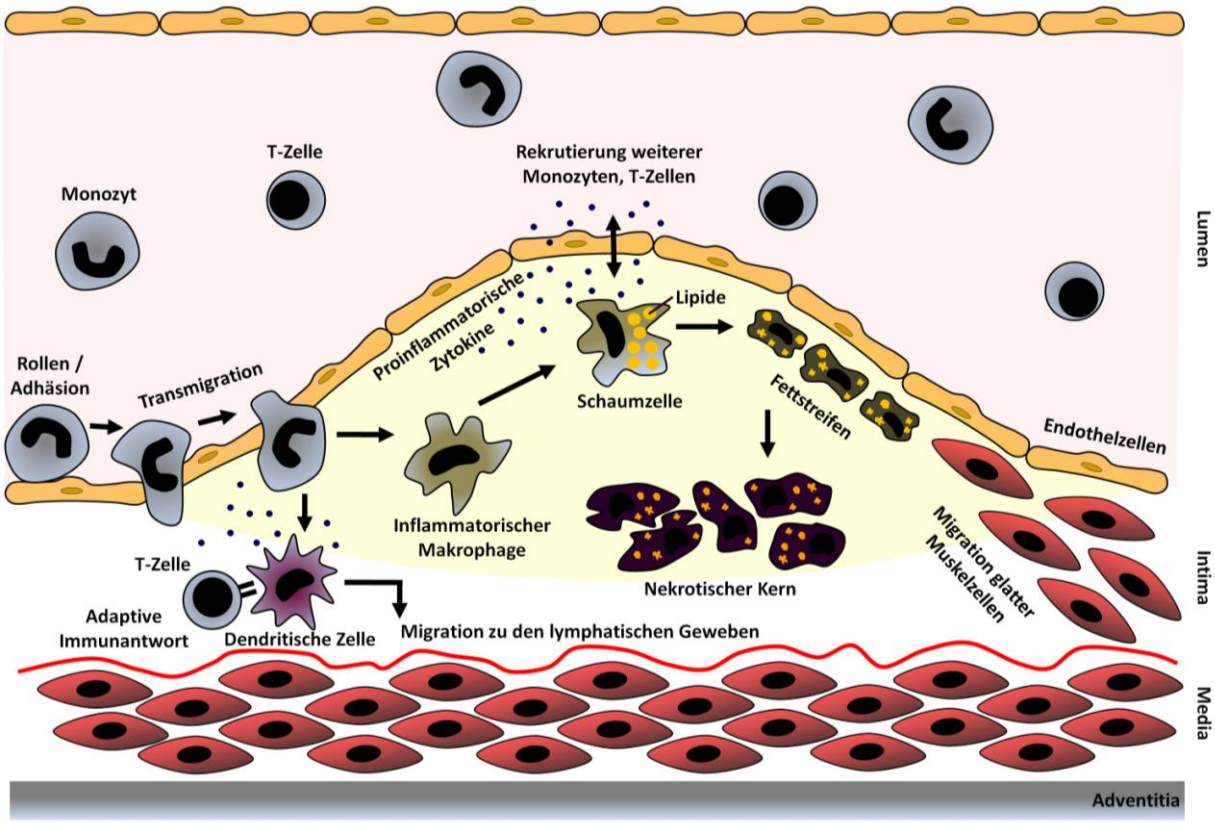

Abbildung 1. Rolle von Monozyten in der Entstehung atherosklerotischer Gefäßerkrankung. Monozyten adhärieren an aktivierte Endothelzellen und migrieren in den subendothelialen Raum, wo sie sich entweder zu Dendritischen Zellen oder Makrophagen differenzieren. Durch Sekretion proinflammatorischer Zytokine werden weitere Immunzellen sowie glatte Muskelzellen in die Intima angelockt und aktiviert. Außerdem nehmen Makrophagen Lipide auf und differenzieren sich zu Schaumzellen, die unter dem Endothel als „Fettstreifen“ zu erkennen sind. Dieser inflammatorische Zustand führt letztendlich zur Ausbildung eines komplizierten atherosklerotischen Plaques, der aus einem lipid- und makrophagenreichen nekrotischen Kern besteht. Eine Destabilisierung des Plaques kann zur Ruptur führen und sich nachfolgend als Myokardinfarkt oder Apoplex manifestieren.

Obgleich in experimentellen Studien eine zentrale Rolle von Monozyten in der atherosklerotischen Gefäßerkrankung zweifelsfrei nachgewiesen werden konnte, erwiesen sich jedoch in epidemiologischen Untersuchungen die zirkulierenden Zellzahlen der Monozyten vermeintlich überraschenderweise nicht als eigenständige Prädiktoren kardiovaskulärer Ereignisse [89]. Aus klinischer Sicht erscheint eine Aufklärung dieser diskrepanten Befunde wünschenswert, weil ein gleichermaßen diagnostisch zuverlässiger und pathogenetisch relevanter Marker der Mikroinflammation erlauben könnte, Patienten besser als bisher hinsichtlich ihres kardiovaskulären Risikos zu stratifizieren.

Zur Aufklärung dieses Widerspruchs zwischen experimentellen und klinisch-epidemiologischen Befunden trägt die Erkenntnis bei, dass humane Monozyten keine homogene Zellpopulation darstellen.

1.3 Monozytenheterogenität bei CKD und Atherosklerose

Die Existenz phänotypisch unterschiedlicher Monozytensubpopulationen wurde erstmals 1989 beschrieben [60]. Auf der Grundlage differentieller Expression des LPS-Rezeptors CD14 sowie des Immunglobulin Rezeptors FCγRIII CD16 wurden initial zwei unterschiedliche Monozytensubpopulationen charakterisiert und eine kleinere Subpopulation, die neben CD14 auch CD16 co-exprimiert (CD16-positive Monozyten, 7 – 15 % aller zirkulierender Monozyten), von der Mehrzahl der Monozyten, welche kein CD16 exprimieren (CD14++CD16- Monozyten), differenziert.

CD16-positive Monozyten wurden zunächst als proinflammatorische Monozyten bezeichnet, da sie einerseits in zahlreichen inflammatorischen Erkrankungen in erhöhter Zellzahl nachzuweisen sind [21,28,36,43,45,70,74,80] und andererseits im Vergleich zu CD14++CD16- Monozyten vermehrt proinflammatorische Zytokine wie TNFα und IL12p40/IL12p70 sowie vermindert das antiinflammatorische Zytokin IL10 bilden [4,22,79]. Zusätzlich verleiht die starke Expression distinkter Adhäsionsmoleküle (z.B. VLA-4, CX_3CR1) dieser Monozytensubpopulation eine hohe endotheliale Affinität sowie ein hohes Potential zur transendothelialen Migration [1].

Auch aus nephrologischer Sicht erscheint von besonderem Interesse, dass Dialysepatienten deutlich erhöhte Zellzahlen der CD16-positiven Monozyten aufweisen [6,8,44,58,64,72], eine jede Hämodialysebehandlung jedoch einen passageren Zellzahlabfall der CD16-positiven Monozyten induziert, der vermutlich auf eine vorübergehende Adhärenz an Endothelzellen zurückgeführt werden kann [65,72]. In Querschnittsanalysen bei chronisch nierenkranken Patienten zeigte sich außerdem eine Assoziation erhöhter Zellzahlen der CD16-positiven Monozyten mit subklinischen atherosklerotischen Gefäßerkrankungen [84].

Durch nähere Charakterisierung der Monozytensubpopulationen wurde im Jahre 2003 die Gesamtpopulation der CD16-positiven Monozyten erstmals in zwei funktionell und phänotypisch distinkte Monozytensubpopulationen unterteilt und diejenigen Monozyten, die den LPS-Rezeptor CD14 ebenso hoch exprimieren wie die Hauptpopulation der CD14++CD16- Monozyten, von Monozyten mit verringerter Expression von CD14 differenziert [1]. Eine Gruppe internationaler Experten bestätigte im Jahre 2010 die Existenz dieser drei distinkten Monozytensubpopulationen und führte die folgende offizielle Nomenklatur für Monozyten ein [96]:

klassische CD14++CD16- Monozyten,
intermediäre CD14++CD16+ Monozyten,
nicht-klassische CD14+CD16++ Monozyten.

Die Gesamtpopulation der intermediären und nicht-klassischen Monozyten wird dabei als „CD16-positive Monozyten“ bezeichnet.

Rezente klinische und experimentelle Studien deuten auf einen proinflammatorischen Charakter insbesondere der intermediären Monozyten hin. So konnte eine Hochregulation von CCR5 [1], ACE [83] und TLR2 [86] auf den intermediären Monozyten gezeigt werden. Gleichzeitig bilden diese intermediären Monozyten nach LPS Stimulation stärker als die klassischen und nicht-klassischen Monozyten TNFα und IL1β [14]. Umfassendere experimentelle Studien sind jedoch notwendig, um den proinflammatorischen Charakter intermediärer Monozyten detaillierter zu untersuchen [97].

Klinische Untersuchungen unserer Arbeitsgruppe zur prognostischen Bedeutung von Monozytensubpopulationen bestätigen den proinflammatorischen und präsumtiv proatherogenen Charakter der CD14++CD16+ Monozyten. So erwies sich in einer Kohorte von 94 Dialysepatienten die Zellzahl der CD14++CD16+ Monozyten, nicht jedoch der CD14+CD16++ Monozyten, als signifikanter und unabhängiger Prädiktor für das Auftreten kardiovaskulärer Ereignisse in einem Beobachtungszeitraum von 35 Monaten [32]. In zwei weiteren klinischen Studien bei Dialysepatienten konnte diese prognostische Bedeutung der CD14++CD16+ Monozyten bestätigt und sowohl die intradialytische Kinetik [65] als auch eine erhöhte ACE Expression [85] von intermediären Monozyten als weitere Prädiktoren erkannt werden.

In der Zusammenschau dieser Voruntersuchungen lässt sich eine pathophysiologische Bedeutung für die CD14++CD16+ Monozyten in der Atherogenese postulieren. Allerdings ist die Genese der Zellzahlzunahme der CD14++CD16+ Monozyten bei chronisch nierenkranken Patienten bislang ungeklärt. Zudem lassen sich diese Daten nicht ohne Weiteres auf nicht-dialysepflichtige chronisch nierenkranke Patienten oder gar die nierengesunde Allgemeinbevölkerung übertragen, da sich Dialysepatienten sowohl allgemein in der Pathogenese der Atherosklerose als auch spezifisch in der Verteilung der Monozytensubpopulationen von anderen Patientenkollektiven unterscheiden [49].

Deshalb sollte der prädiktive Charakter von CD14++CD16+ Monozyten auch bei anderen Patientenkollektiven untersucht werden.

1.4 Bedeutung epigenetischer Mechanismen für die beschleunigte Atherosklerose bei CKD

Die Ursachen für die urämische Monozytendysfunktion sowie für die Verschiebungen der Monozytensubpopulationen bei chronischer Nierenerkrankung sind bislang nicht hinreichend bekannt. In dem unphysiologischen urämischen Milieu können zahlreiche Faktoren wie die Hyperhomocysteinämie, chronische Inflammation, Dyslipidämie und oxidativer Stress eine Funktionsstörung der Monozyten induzieren. So konnten Zhang et al. [94,95] tierexperimentell zeigen, dass die Hyperhomocysteinämie die Bildung proinflammatorischer Monozyten induziert, welche nachfolgend in atherosklerotische Läsionen einwandern. Bisher wurde jedoch noch nicht untersucht, inwiefern die erhöhten Homocysteinspiegel bei chronisch nierenkranken Patienten, die aus einer verminderten renalen Exkretion und einem verminderten Katabolismus von Homocystein resultieren, die Entstehung der CD16-positiven Monozyten induziert.

Homocystein vermittelt pathophysiologisch zahlreiche proatherogene Prozesse, wie die Bildung reaktiver Sauerstoffspezies (*reactive oxygen species* [ROS]) [2], Migration von Leukozyten in atherosklerotische Läsionen [62], Proliferation von glatten Gefäßmuskelzellen [54] sowie Induktion eines prothrombotischen Zustands [5]. Im vermeintlichen Einklang zeigte sich in klinischen Studien eine Assoziation erhöhter Homocysteinspiegel mit kardiovaskulären Ereignissen bei chronisch nierenkranken Patienten [55].

Homocystein ist als zentraler Bestandteil des C1-Metabolismus direkt an der epigenetischen Genregulation beteiligt (**Abbildung 2**). Im C1-Metabolismus wird Homocystein zu S-Adenosylmethionin (SAM) umgewandelt; SAM ist der universelle Methylgruppendonor für zahlreiche zelluläre Methylierungsreaktionen, darunter auch für die DNA-Methylierung, die als eine zentrale epigenetische Komponente die Genexpression reguliert. Aus SAM entsteht nach erfolgter Methylierungsreaktion S-Adenosylhomocystein (SAH). Da SAH ein potenter kompetitiver Inhibitor von SAM-abhängigen Methyltransferasen ist [18], wird SAH physiologischerweise metabolisiert, um zelluläre Methylierungsreaktionen nicht zu behindern. Dies geschieht durch Hydrolyse von SAH zu Homocystein und Adenosin. Da diese Reaktion jedoch reversibel ist, führt jegliche Akkumulation von Homocystein auch zu einem Anstieg von SAH und somit zu einer potentiellen Inhibition der DNA-Methylierung. Physiologischerweise wird Homocystein deshalb entweder zu SAM (über die Folsäure/Vitamin B_{12}- oder Betain-abhängige Remethylierung) oder zu Cystathionin (über die Vitamin B_6-abhängige Transsulfurierung) metabolisiert; beide Prozesse sind jedoch bei chronisch nierenkranken Patienten gestört [61,93].

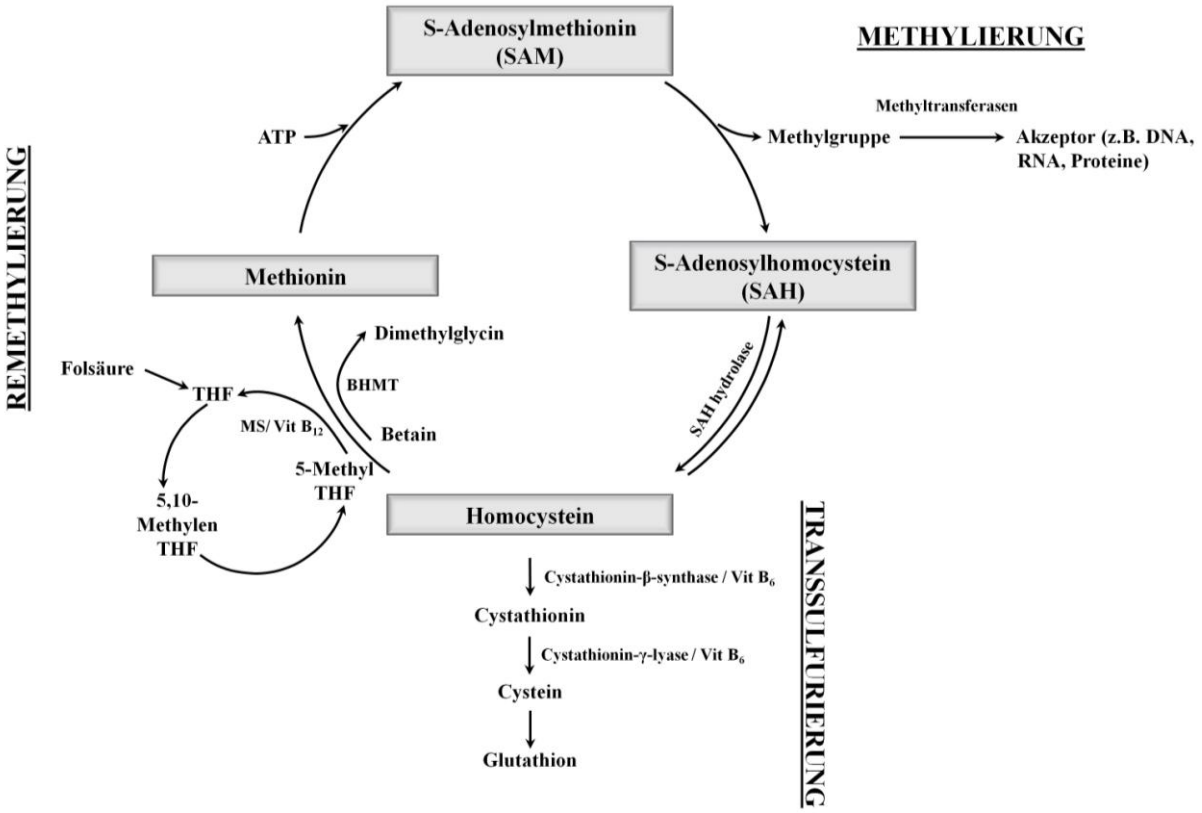

Abbildung 2. Rolle des C1-Metabolismus bei zellulären Methylierungsreaktionen. Homocystein kann entweder über den Transsulfurierungs- oder den Remethylierungspathway abgebaut werden. Über die Remethylierung entsteht S-Adenosylmethionin (SAM), der universelle Methylgruppendonor für zelluläre Methylierungsreaktionen, darunter auch für die DNA-Methylierung. Nach erfolgter Methylierung entsteht aus dem SAM das S-Adenosylhomocystein (SAH); SAH ist ein kompetitiver Inhibitor von Methyltransferasen und wird deshalb weiter zu Homocystein metabolisiert, um zelluläre Methylierungsreaktionen nicht zu behindern. Diese Reaktion ist jedoch reversibel, so dass eine Akkumulation von Homocystein auch zur Zunahme der SAH-Spiegel führt. Eine Eliminierung von Homocystein ist deshalb essentiell. THF: Tetrahydrofolat; MS: Methionin-Synthase; BHMT: Betain-Homocystein-Methyltransferase; Vit B_{12}: Vitamin B_{12}; Vit B_6: Vitamin B_6.

Außerhalb der Nephrologie weisen zahlreiche experimentelle und klinische Studien auf eine kausale Bedeutung einer veränderten DNA-Methylierung in der Atherogenese hin. So zeigen nierengesunde Patienten mit atherosklerotischen Gefäßerkrankungen einen gestörten globalen DNA-Methylierungsstatus in PBMCs (*peripheral blood mononuclear cells*) auf, der mit erhöhten Plasma-Homocystein- und SAH-Konzentrationen assoziiert ist [3,10,46,73]. In atherosklerotischen Läsionen treten DNA-Methylierungsveränderungen in Kontrollregionen verschiedener Gene auf, die an der Pathogenese von Atherosklerose beteiligt sind, wie etwa der Superoxid Dismutase (SOD), der endothelialen Stickstoffmonoxid-Synthase (eNOS) und dem Östrogen-Rezeptor-α (ESR1) [9,11,35,47,63].

Aus klinischer Sicht erscheint bedeutsam, den Einfluss des C1-Metabolismus auf die DNA-Methylierung bei chronisch nierenkranken Patienten zu untersuchen, obwohl in mehreren großen Interventionsstudien eine Senkung der Homocysteinspiegel mittels Substitution von

Folsäure, Vitamin B_6 und Vitamin B_{12} nicht zu einer Reduktion kardiovaskulärer Ereignisse führte [34,42,90,98]. Als mögliche Erklärung für diese vermeintlich widersprüchlichen Befunde kann im Einklang mit aktuellen experimentellen und klinischen Studien postuliert werden, dass SAH gegenüber Homocystein ein besserer Marker epigenetischer Fehlregulationen und daher ein geeigneterer Prädiktor kardiovaskulärer Erkrankungen ist [50,87]. Interessanterweise resultiert eine Substitution von Folsäure, Vitamin B_6 und Vitamin B_{12}, welche in den frustranen Interventionsstudien eingesetzt wurde, zwar in einer Homocystein-Senkung, nicht jedoch in einer Senkung von SAH [27,38]. Aus nephrologischer Sicht erscheint außerdem bedeutsam, dass SAH bei chronischer Nierenerkrankung deutlicher akkumuliert als Homocystein [41], da die Niere eine ausschlaggebende Rolle in der Eliminierung von SAH spielt [23]. Somit lässt sich ein direkter Zusammenhang zwischen chronischer Nierenerkrankung und der Kontrolle von Transmethylierungsreaktionen postulieren (zusammengefasst in [17,40,76-78]). Im Einklang damit konnten mehrere Studien eine Beeinträchtigung von Transmethylierungsreaktionen bei Hämodialysepatienten aufzeigen [24,39,51,75]. Darüber hinaus stellte sich eine gestörte DNA-Methylierung als unabhängiger Risikofaktor für das Auftreten kardiovaskulärer Todesfälle bei Hämodialysepatienten heraus [75].

Untersuchungen zur prognostischen Bedeutung erhöhter SAH-Spiegel und epigenetischer Fehlregulation sind notwendig, um die Bedeutung eines gestörten C1-Metabolismus in der beschleunigten Atherogenese chronisch nierenkranker Menschen weiter aufzuklären. Ebenso müssen weitere Studien untersuchen, inwiefern die Störung des C1-Metabolismus die Bildung präsumtiv proatherogener Monozyten bei chronisch nierenkranken Patienten induziert.

1.5 Weitergehende Zusammenfassung

Eine detaillierte Diskussion zum Thema Monozytenheterogenität und Epigenetik bei CKD-assoziierter Atherogenese erfolgte in folgenden zwei Übersichtsartikeln, die im Rahmen der Dissertationsarbeit erstellt wurden (siehe Anhang):

1) Zawada AM, Rogacev KS, Schirmer SH, Sester M, Böhm M, Fliser D, Heine GH. Monocyte heterogeneity in human cardiovascular disease. Immunobiology. 2012 Dec;217(12):1273-84.
2) Zawada AM, Rogacev KS, Heine GH. Clinical relevance of epigenetic dysregulation in chronic kidney disease. Nephrol Dial Transplant. Im Druck.

1.6 Zielsetzung der Arbeit

Die vorgelegte Dissertationsarbeit fokussiert vor dem ausgeführten wissenschaftlichen Hintergrund auf drei Fragestellungen:

Zunächst soll in epidemiologischen Untersuchungen die prognostische Bedeutung von CD14++CD16+ Monozyten bei chronisch nierenkranken, nicht-dialysepflichtigen Patienten sowie bei Nierengesunden erfasst werden. Hierbei wird postuliert, dass die Erfassung der Monozytenheterogenität den vermeintlichen Widerspruch aufzulösen vermag, dass trotz der zentralen Rolle von Monozyten in der Pathogenese der Atherosklerose bisherige epidemiologische Studien keine eindeutige Assoziation zwischen Monozytenzahlen und kardiovaskulären Erkrankungen aufzeigten. Nachfolgend soll über die epidemiologischen Studien hinaus eine genauere Charakterisierung der Monozytensubpopulationen erfolgen, um ein besseres Verständnis der Bedeutung von Monozytensubpopulationen bei Patienten mit chronischer Nierenerkrankung zu ermöglichen sowie um Faktoren zu charakterisieren, welche bei Urämie zur Veränderung der Verteilung von Monozytensubpopulationen beitragen.

Schließlich sollen epigenetische Regulationsmechanismen bei chronisch nierenkranken Patienten untersucht werden, da eine epigenetische Dysregulation bei nierenkranken Menschen zentral zur veränderten Monozytenbiologie sowie darüber hinaus zu proinflammatorischen Prozessen beitragen könnte.

2. Ergebnisse

2.1 CD14++CD16+ Monozyten als Prädiktoren kardiovaskulärer Ereignisse bei nicht-dialysepflichtigen chronisch nierenkranken Patienten

Diese Arbeit wurde publiziert als:
Rogacev KS, Seiler S, Zawada AM, Reichart B, Herath E, Roth D, Ulrich C, Fliser D, Heine GH. CD14++CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease. Eur Heart J. 2011 Jan;32(1):84-92.

Zusammenfassung

Die prognostische Bedeutung der CD14++CD16+ Monozyten ist bislang nur bei Dialysepatienten [32], nicht bei Patienten mit chronischer, nicht-dialysepflichtiger Nierenerkrankung („*chronic kidney disease, no dialysis*“ [CKD-ND]) untersucht. Letztere Patientengruppe überragt jedoch weltweit in ihrer Populationsgröße die Gruppe der Dialysepatienten deutlich und stellt ähnlich wie die Dialysepatienten eine kardiovaskuläre Hochrisikogruppe dar. Ziel dieser Studie war daher zu untersuchen, ob die Anzahl CD14++CD16+ Monozyten nicht nur bei Dialysepatienten, sondern auch bei CKD-ND ein Prädiktor kardiovaskulärer Ereignisse darstellt.

Bei 119 Patienten mit CKD-ND wurde die prognostische Bedeutung der präsumtiv proatherogenen CD14++CD16+ Monozyten erfasst. Über eine Nachbeobachtungszeit von durchschnittlich 4,9 Jahren wurde das Auftreten kardiovaskulärer Ereignisse bestimmt. Die Patientengruppe wurde 2004 rekrutiert; im Rahmen der Promotionsarbeit erfolgte einerseits die epidemiologische Auswertung der Patientendaten, andererseits die subpopulationsspezifische Charakterisierung der Expression der Chemokinrezeptoren CCR2, CX_3CR1 und CCR5 bei insgesamt 30 chronisch nierenkranken Patienten mittels Durchflusszytometrie.

Unabhängig vom Stadium der chronischen Nierenerkrankung zeigte sich eine differentielle Expression der Rezeptoren auf den Monozytensubpopulationen: während CD14++CD16- Monozyten eine starke Expression von CCR2 und CD14+CD16++ Monozyten eine starke Expression von CX_3CR1 aufwiesen, zeigten CD14++CD16+ Zellen neben der Co-Expression der beiden Rezeptoren CCR2 und CX_3CR1 eine subpopulationsspezifische Expression von CCR5. Einhergehend mit der hohen Expression an proatherogenen Markern stellte sich zudem heraus, dass chronisch nierenkranke Patienten mit erhöhter Anzahl der

CD14++CD16+ Monozyten ein erhöhtes kardiovaskuläres Risiko aufwiesen (Hazard Ratio für eine Zellzunahme von 10 Zellen/µL 1,26 [Konfidenzintervall: 1,04 – 1,52; p = 0,018]). Weiterhin konnte im Einklang mit der Hypothese, dass das urämische Milieu durch Beeinflussung des Epigenoms monozytärer Zellen eine Differenzierung zu CD16-positiven Monozyten induziert, eine Korrelation zwischen der Anzahl CD16-positiver Monozyten – insbesondere der CD14+CD16++ Monozyten (r = 0,201, p = 0,038) – und dem Serum-Homocystein festgestellt werden.

Diese Ergebnisse festigen die Bedeutung von CD14++CD16+ Monozyten in der Pathogenese der Atherosklerose und deuten auf eine Beteiligung epigenetischer Mechanismen an der Verschiebung der Monozytensubpopulationen bei chronisch nierenkranken Menschen hin.

Auf Basis dieser Ergebnisse soll innerhalb der CARE FOR HOMe Studie (Cardiovascular and Renal Outcome in CKD 2-4 Patients – The Forth Homburg evaluation) bei 444 chronisch nierenkranken, nicht-dialysepflichtigen Patienten die Bedeutung von SAM und SAH, als weitere zentrale epigenetische Regulationsfaktoren, im Kontext der Monozytenheterogenität bei CKD erfasst werden.

2.2 CD14++CD16+ Monozyten als Prädiktoren kardiovaskulärer Ereignisse bei Nierengesunden

Diese Arbeit wurde publiziert als:

Rogacev KS, Cremers B, Zawada AM, Seiler S, Binder N, Ege P, Große-Dunker G, Heisel I, Hornof F, Jeken J, Rebling NM, Ulrich C, Scheller B, Böhm M, Fliser D, Heine GH. CD14++CD16+ Monocytes Independently Predict Cardiovascular Events: A Cohort Study of 951 Patients Referred for Elective Coronary Angiography. J Am Coll Cardiol. 2012 Oct 16;60(16):1512-20.

Zusammenfassung

CD14++CD16+ Monozyten sind als unabhängige Prädiktoren kardiovaskulärer Ereignisse bei chronisch nierenkranken Patienten identifiziert worden [32,66]. Chronisch nierenkranke Patienten sind jedoch eine hoch selektive Patientenpopulation, die eine deutliche Verschiebung in den Monozytensubpopulationen aufweist. Zudem unterscheiden sich chronisch nierenkranke Patienten in der Pathogenese der Atherosklerose von Nierengesunden, weshalb sich epidemiologische Studienergebnisse nicht ohne Weiteres auf andere Patientengruppen übertragen lassen. Im Rahmen der HOM SWEET HOMEe Studie (Heterogeneity of Monocytes in Subjects Who Undergo Elective Coronary Angiography - The Homburg Evaluation) wurde nun die Hypothese überprüft, ob die Anzahl an CD14++CD16+ Monozyten auch bei Nierengesunden ein Prädiktor für kardiovaskuläre Ereignisse darstellt.

Bei 951 Patienten, die sich in der Klinik für Innere Medizin III (Kardiologie, Angiologie und Internistische Intensivmedizin) des Universitätsklinikums des Saarlandes einer elektiven Koronarangiographie unterzogen, wurden Monozytensubpopulationen durchflusszytometrisch analysiert und das Auftreten des primären Endpunktes (definiert als kardiovaskulärer Tod, akuter Myokardinfarkt, Nicht-hämorrhagischer Schlaganfall) jährlich nachverfolgt.

Die durchschnittliche Nachbeobachtungszeit betrug 2,6 ± 1,0 Jahre, in der 93 Patienten den primären Endpunkt erreichten. Während in der univariaten Kaplan-Meier-Analyse die Anzahl der Gesamtmonozyten ($p = 0,010$), der CD14++CD16- ($p = 0,024$) und der CD14++CD16+ Monozyten ($p < 0,001$) den primären Endpunkt prognostizierten, blieben nach Korrektur für weitere Risikofaktoren nur die CD14++CD16+ Monozyten unabhängige Prädiktoren für

kardiovaskuläre Ereignisse (Hazard Ratio viertes gegen erstes Quartil 3,019 [Konfidenzintervall: 1,315 – 6,928; p = 0,009]).

Da somit CD14++CD16+ Monozyten auch bei Nierengesunden als Prädiktoren kardiovaskulärer Ereignisse erkannt werden konnten, könnten diese Zellen ein spezifisches Ziel für zukünftige therapeutische Interventionen bei kardiovaskulären Risikopatienten darstellen. Hierzu sollten weitere Studien zunächst Monozytensubpopulationen detaillierter charakterisieren, um einerseits die Biologie der CD14++CD16+ Monozyten besser zu verstehen und um andererseits potentielle Mechanismen für eine selektive Modifikation oder Depletion dieser Zellen aufzuzeigen.

2.3 Charakterisierung humaner Monozytensubpopulationen

Diese Arbeit wurde publiziert als:

Zawada AM, Rogacev KS, Rotter B, Winter P, Marell RR, Fliser D, Heine GH. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. Blood. 2011 Sep 22;118(12):e50-61.

Zusammenfassung

Da auf der Basis epidemiologischer Vorarbeiten CD14++CD16+ Monozyten als ein potentielles Ziel für zukünftige therapeutische Interventionen postuliert wurden, war eine detaillierte Charakterisierung der drei Monozytensubpopulationen unabdingbar. Es sollte dabei geklärt werden, ob CD14++CD16+ Monozyten tatsächlich eine distinkte Zellpopulation darstellen, die sich nicht nur phänotypisch, sondern auch funktionell von den CD14++CD16- sowie den CD14+CD16++ Monozyten abgrenzen lässt. Dabei sollte weiterhin die Hypothese überprüft werden, dass die CD14++CD16+ Monozyten besonders proatherogene Komponenten des angeborenen Immunsystems sind.

Hierzu wurde erstmals erfolgreich eine Isolation der drei humanen Monozytensubpopulationen aus der Blutzirkulation etabliert. Bei nachfolgender genomweiter Expressionsanalyse mittels SuperSAGE (Serial Analysis of Gene Expression) wurden insgesamt 5 487 603 Sequenzabschnitte („tags“) sequenziert. Eine Annotation zur humanen RefSeq Datenbank erlaubte 97 selektive Marker der CD14++CD16+ Monozyten zu identifizieren ($p < 10^{-10}$). Mittels der Gene Ontology Analyse konnten distinkte immunologische Prozesse beschrieben werden, die in den CD14++CD16+ Monozyten präferentiell aktiviert sind, wie die Antigenprozessierung und –präsentation (z.B. *CD74*, *HLA-DR*, *IFI30*, *CTSB*), Inflammation (z.B. *TGFB1*, *AIF1*, *PTPN6*) und Angiogenese (z.B. *TIE-2*, *CD105*). Weiterhin erlaubte die Isolation von Monozytensubpopulation eine funktionelle Charakterisierung, wobei CD14++CD16+ Monozyten das größte Potential zur Aktivierung der CD4+ T-Zell-Proliferation, zur Angiogenese und zur ROS-Bildung aufwiesen. Schließlich konnte mithilfe von HLA-DR Microbeads eine selektive Depletion der CD14++CD16+ Monozyten erzielt werden. Eine Zusammenfassung der Funktionen der drei Monozytensubpopulationen ist in **Abbildung 3** dargestellt.

Zusammenfassend weisen diese Daten auf eine distinkte Funktion der CD14++CD16+ Monozyten im humanen Immunsystem hin und suggerieren eine entscheidende Rolle dieser

Zellen in der Entstehung und Progression atherosklerotischer Gefäßläsionen. Zudem wurden erstmals *in vitro* Möglichkeiten einer selektiven Depletion dieser Zellen aufgezeigt. Auf dieser Basis sollten zukünftige Studien Optionen einer Modifikation oder Depletion von CD14++CD16+ Monozyten *in vivo* zur Prävention kardiovaskulärer Erkrankungen ergründen.

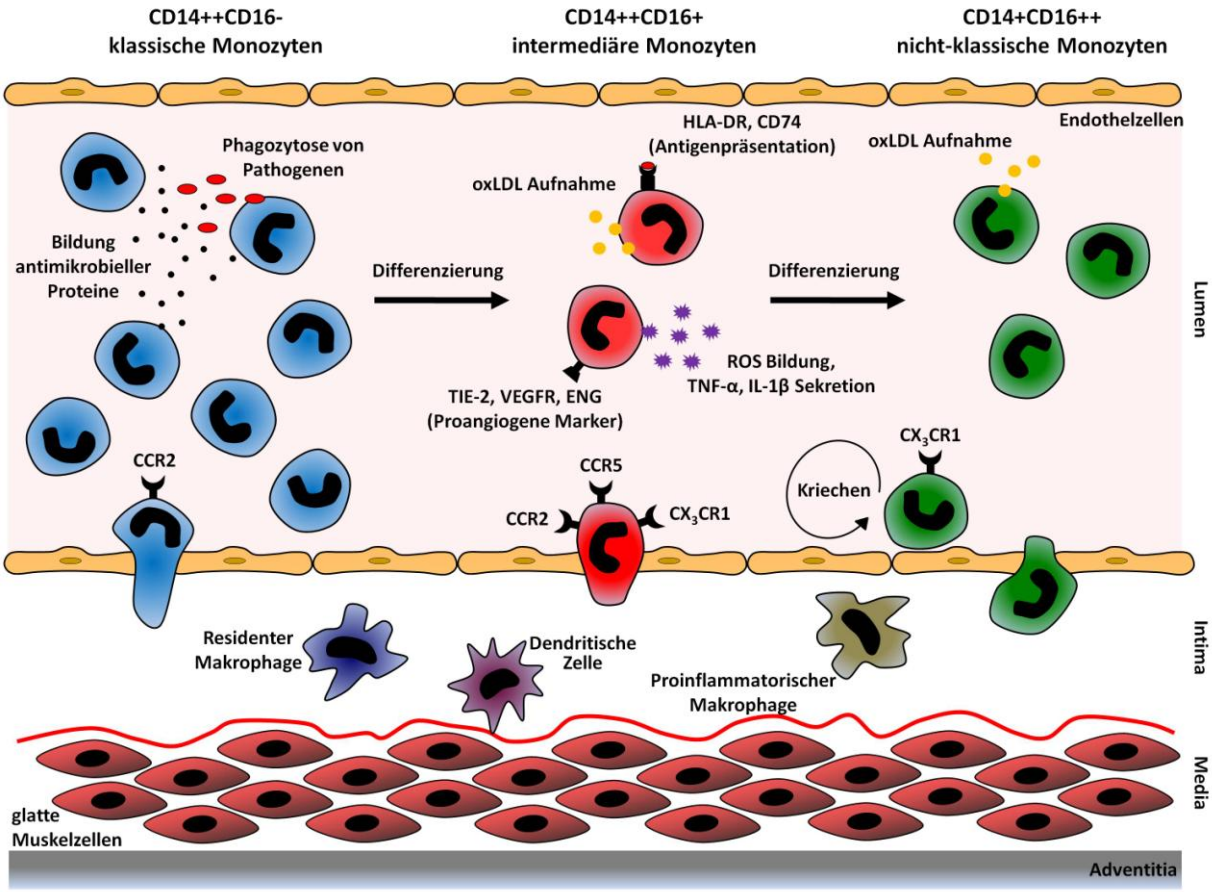

Abbildung 3. Funktionen der drei Monozytensubpopulationen (schematische Zusammenfassung rezenter eigener und externer Publikationen). CD14++CD16- Monozyten besitzen aufgrund der präferentiellen Bildung antimikrobieller Proteine und aufgrund ihrer hohen Phagozytoseleistung eine ausgeprägte antimikrobielle Kapazität [14,92]. Sie wandern nachfolgend entweder in Gefäße ein oder differenzieren sich weiter zu CD14++CD16+ Monozyten. CD14++CD16+ Monozyten sind prädisponiert für die Antigenprozessierung und –präsentation (HLA-DR, CD74) und besitzen das größte Potential zur Induktion der CD4+ T-Zell-Proliferation [69,92]. Sie bilden vermehrt proinflammatorische Zytokine (TNFα, IL1β) und reaktive Sauerstoffspezies (ROS) [14,92]. Die Fähigkeit zur Aufnahme von oxLDL sowie die Expression von proatherogenen Chemokinrezeptoren (CCR2, CCR5, CX_3CR1) verleiht den CD14++CD16+ Monozyten ein hohes proatherogenes Potential [1,57,66,69]. Außerdem exprimiert diese Monozytensubpopulation verstärkt proangiogene Marker (TIE-2, VEGFR, ENG) [92]. CD14++CD16+ Monozyten können ebenso in das Endothel einwandern und sich dort zu Makrophagen / Dendritischen Zellen bzw. in der Zirkulation zu CD14+CD16++ Monozyten differenzieren. CD14+CD16++ Monozyten patrouillieren das Endothel in kriechenden Bewegungen und sind ebenso in der Lage in das Endothel zu wandern [1,14]. Sie besitzen die kürzesten Telomere, so dass sie die am weitesten entwickelte Monozytensubpopulation darstellen [56].

2.4 Fehlregulation atherosklerose-assoziierter Gene als mögliche Ursache für die beschleunigte Atherosklerose chronisch nierenkranker Menschen

Diese Arbeit wurde publiziert als:

Zawada AM, Rogacev KS, Hummel B, Grün OS, Friedrich A, Rotter B, Winter P, Geisel J, Fliser D, Heine GH. SuperTAG Methylation-Specific Digital Karyotyping (SMSDK) Reveals Uremia Induced Epigenetic Dysregulation of Atherosclerosis-Related Genes. Circ Cardiovasc Genet. 2012 Dec 1;5(6):611-20.

Zusammenfassung

Daten unserer [66] und anderer Arbeitsgruppen [94,95] weisen auf eine zentrale Rolle eines gestörten C1-Metabolismus sowohl in der Monozytenbiologie als auch in der Pathogenese der Atherosklerose [2,5,54,62]. Erste Untersuchungen bei Patienten mit prävalenten atherosklerotischen Gefäßleiden aus der nierengesunden Allgemeinbevölkerung deuten zudem auf eine spezifische epigenetische Fehlregulation atherosklerose-assoziierter Gene sowohl in atherosklerotischen Läsionen als auch in mononukleären Zellen hin (zusammengefasst in [81]). Bislang fehlen jedoch jegliche Studien, die die Regulation atherosklerose-assoziierter Gene bei chronischer Nierenerkrankung untersuchten.

Deshalb erfolgte im Rahmen der vorliegenden Promotionsarbeit eine genomweite DNA-Methylierungsanalyse bei CKD Patienten mittels des SuperTAG methylation-specific digital karyotyping (SMSDK). Die DNA wurde aus mononuklären Zellen des peripheren Blutes von zehn männlichen Hämodialysepatienten im Alter zwischen 50-60 Jahren sowie von zehn alters- und geschlechtsgleichen Kontrollprobanden isoliert.

Die Analyse von 27 043 436 tags ergab insgesamt 4 288 Loci, die zwischen Dialysepatienten und Kontrollprobanden differentiell methyliert waren ($p < 10^{-10}$). Die Annotation der UniTags (sequenzgleiche tags) zu Promotordatenbanken und zur "Genetic Association Database" erlaubte eine differenzielle Methylierung von 52 Kandidatengenen, die mit kardiovaskulären Erkrankungen assoziiert sind, sowie von 72 Kandidatengenen, die mit Immun-/Infektionserkrankungen assoziiert sind, zu identifizieren. Mittels Gene Ontology Analyse konnten diese Kandidatengene zu distinkten proatherogenen Prozessen klassifiziert werden, wie dem Lipidmetabolismus und -transport (u.a. *HMGCR*, *SREBF1*, *LRP5*, *EPHX2*, *FDPS*), der Zellproliferation und Zellzyklusregulation (u.a. *MIK67*, *TP53*, *ALOX12*), der Angiogenese (u.a. *ANGPT2*, *ADAMTS10*, *FLT4*) sowie der Inflammation (u.a. *TNFSF10*,

LY96, *IFNGR1*, *HSPA1A*, *IL12RB1*). Diese Methylierungsveränderungen waren zudem mit einer differentiellen Expression distinkter Oberflächenmarker (CD43, CD86) sowie einer funktionellen Beeinträchtigung der Monozyten (ROS-Bildung, Phagozytosefähigkeit) bei Dialysepatienten assoziiert.

Somit erlaubte diese erstmalige genomweite DNA-Methylierungsanalyse bei Hämodialysepatienten eine Charakterisierung fehlregulierter atherosklerose-assoziierter Gene. Eine solche Charakterisierung, welche in den letzten Jahren von zahlreichen Experten wiederholt eingefordert wurde [17,40,76-78], bietet nun die Möglichkeit neue Biomarker für die atherosklerotische Gefäßerkrankung bei chronisch nierenkranken Menschen zu identifizieren. Da DNA-Methylierungsveränderungen bereits frühzeitig in der Atherogenese auftreten [53], besitzt ein solcher Biomarker das Potential, das kardiovaskuläre Risiko von chronisch nierenkranken Patienten frühzeitiger als bisher zu erfassen. Nachdem eine Assoziation zwischen fehlregulierter DNA-Methylierung und Monozytendysfunktion bei chronisch nierenkranken Menschen erkannt wurde, sollten zukünftige Studien untersuchen, inwiefern die DNA-Methylierungsveränderungen direkt zu Verschiebungen der Monozyten-subpopulationen bei chronisch nierenkranken Menschen beitragen.

3. Weiterführende Ergebnisse und Ausblick

3.1 Differenzierung humaner Monozytensubpopulationen

Diese Arbeit wurde zur Begutachtung eingereicht als:
Rogacev KS*, Zawada AM*, Achenbach M, Held G, Fliser D, Heine GH. Development of human monocyte subsets and its modulation by immunosuppressants.

* Co-Erstautorenschaft

Zusammenfassung

Die bisher veröffentlichen klinischen und experimentellen Untersuchungen weisen auf eine zentrale Bedeutung der CD14++CD16+ Monozyten in der Atherogenese hin. Vor diesem Hintergrund wird die Immunmodulation von Monozytensubpopulationen als innovative Therapie bei kardiovaskulären Erkrankungen diskutiert [26,37]. Um Mechanismen entwickeln zu können, die eine Modulation von Monozytensubpopulationen erlauben, ist jedoch zunächst ein besseres Verständnis der Entwicklung der Monozytensubpopulationen essentiell. So ist bisher nicht abschließend geklärt, wie der Differenzierungsprozess humaner Monozytensubpopulationen abläuft.

Daher wurden im Rahmen dieser Promotionsarbeit Monozytensubpopulationen bei Leukämiepatienten nach autologer und allogener hämatopoetischer Stammzelltransplantation im Zeitverlauf analysiert. Weiterhin wurde ein Zellkulturmodell zur Differenzierung von Monozytensubpopulationen aus CD34+ hämatopoetischen Stammzellen entwickelt. Hierdurch sollte einerseits die Differenzierung humaner Monozytensubpopulationen weiter untersucht werden, andererseits ein Modell generiert werden, welches die Wirkung verschiedener Substanzen auf die Differenzierung von Monozytensubpopulationen selektiv auszutesten vermag.

5-6 Tage nach Stammzelltransplantation waren CD14-CD16- Zellen die ersten Zellen, die in der Zirkulation erschienen. Anschließend war im zeitlichen Verlauf eine graduelle Zunahme zunächst der CD14++CD16-, gefolgt von den CD14++CD16+ und später den CD14+CD16++ Monozyten zu erkennen. Es gab keine Unterschiede in der Verteilung der Monozytensubpopulationen zwischen den autolog und den allogen transplantierten Patienten. Jedoch unterschieden sich die beiden Patientengruppen in der Expression distinkter monozytärer Oberflächenmarker (CCR2, HLA-DR, ENG, TEK und TLR4). Weiterhin konnte

in Übereinstimmung mit früheren Studien [15,20,31] eine selektive Depletion CD16-positiver Monozyten durch Kortikosteroide bei Patienten nach Stammzelltransplantation beobachtet werden. *In vitro* konnte eine Differenzierung der CD34+ hämatopoetischen Stammzellen zu CD14++CD16- und nachfolgend zu CD14++CD16+ Monozyten erreicht werden. Dies erfolgte durch einen zweistufigen Prozess, indem die CD34+ hämatopoetischen Stammzellen zunächst unter Einfluss von TPO, SCF, Flt-3 Ligand und IL-3 expandiert und diese anschließend in einem Differenzierungsmedium (Hematopoietic Progenitor Medium, PromoCell GmbH) kultiviert wurden. Die *in vitro* differenzierten CD14++CD16+ Monozyten zeigten vergleichbare Eigenschaften mit zirkulierenden Monozyten *in vivo* (Expression von Oberflächenmarkern, ROS-Bildung, Phagozytose, Induktion der CD4+ T-Zell-Proliferation). Konventionelle Immunmodulatoren (Rapamycin, Dexamethason) und der Aryl-Hydrocarbon-Rezeptor Aktivator Benzo(a)pyren erlauben, die Differenzierung von Monozyten zu inhibieren.

Zusammenfassend unterstützen diese Daten das Konzept eines gemeinsamen Vorläufers der Monozytensubpopulationen, aus dem sich zunächst CD14++CD16- Monozyten differenzieren, die sich anschließend in CD14++CD16+ und später in CD14+CD16++ Monozyten entwickeln. Anhand des aufgezeigten *in vitro* Modells zur Differenzierung humaner Monozytensubpopulationen können zukünftige Studien zur selektiven Beeinflussung von Monozytensubpopulationen auf diesen Forschungsergebnissen aufbauen.

3.2 Ausblick

Experimentell-klinische Untersuchungen:

Zukünftige Studien sollen untersuchen, inwiefern die Urämie *per se* die Differenzierung der einzelnen Monozytensubpopulationen aus CD34+ Vorläuferzellen beeinflusst, um Möglichkeiten einer selektiven Beeinflussung von CD14++CD16+ Monozyten aufzuzeigen.

Rezente Daten konnten den Transkriptionsfaktor NR4A1 (Nur77) als zentralen Regulator der Monozytendifferenzierung zu CD16-positiven Monozyten charakterisieren, so dass eine Modifikation dieses Transkriptionsfaktors ein potentielles Target für eine selektive Beeinflussung der CD14++CD16+ Monozyten darstellt [30]. Weiterhin sollten Optionen einer direkten zell-spezifischen Therapie mit monoklonalen Antikörpern gegen einzelne Monozytensubpopulationen erwogen werden, nachdem in anderen Bereichen der Inneren Medizin monoklonale Antikörper gegen spezifische Leukozytensubpopulationen bereits erfolgreich etabliert werden konnten [12]. Schließlich könnten Absorptionssäulen, die spezifisch gegen CD14++CD16+ Monozyten gerichtet sind und während einer Hämodialysebehandlung in den extrakorporalen Kreislauf integriert werden könnten, eine spezifische Depletion dieser Zellpopulation ermöglichen. Aktuell verfügbare Absorptionssäulen, die etwa bei der entzündlichen Darmerkrankung verwendet werden, erlauben bereits eine Reduktion der CD14++CD16+ Monozyten; jedoch werden durch diese Säulen neben den CD14++CD16+ Monozyten auch neurophile Granulozyten und Blutplättchen depletiert, so dass selektivere Säulen entwickelt werden müssen [29].

Weiterhin sollte überprüft werden, ob die Urämie DNA-Methylierungsveränderungen in den sich entwickelnden Monozyten induziert und somit eine veränderte Differenzierung von Monozyten bewirkt. Dazu wird eine genomweite DNA-Methylierungsanalyse in CD14++CD16+ Monozyten erfolgen, welche entweder unter Kontrollbedingungen oder unter Urämie differenziert werden.

Epidemiologische Untersuchungen:

Parallel sollte in epidemiologischen Untersuchungen die prognostische Bedeutung einerseits der Monozytensubpopulationen, andererseits von Parametern des C1-Metabolismus weiter untersucht werden.

Diese Fragestellung soll im Rahmen des HOMe Studienprojektes, welches die CARE FOR HOMe, die I Like HOMe, die HOM sweet HOMe und die HOMe ALONE Studie umfasst, bearbeitet werden (**Tabelle 2**).

So soll die prognostische Bedeutung erhöhter Zellzahlen der intermediären Monozyten über längere Beobachtungszeiträume als in bisherigen Studien untersucht werden; hierzu sollen alle Teilnehmer der CARE FOR HOMe und der HOM sweet HOMe Studie bis zum Jahr 2015 nachverfolgt werden. Die HOMe ALONE Studie wird erstmals die prognostische Bedeutung erhöhter Zellzahlen der intermediären Monozyten bei allogen nierentransplantierten Patienten aufzeigen. Schließlich erfolgte in der CARE FOR HOMe Studie eine differenzierte, subpopulationsspezifsche Analyse der Oberflächenexpression von Chemokinrezeptoren und weiteren Oberflächenmarkern, um zu untersuchen, ob eine qualitative Monozytenanalyse über eine reine Bestimmung der Zellzahlen einzelner Subpopulationen hinaus prognostisch relevant ist.

Weiterhin soll im HOMe Studienprojekt untersucht werden, ob SAH als renaler und kardiovaskulärer Prädiktor Homocystein überlegen ist. Zum Zeitpunkt der Einreichung der Dissertationsschrift suggerieren präliminäre Querschnittsanalysen der I Like HOMe Studie, dass SAH deutlicher als Homocystein mit der Nierenfunktion assoziiert ist; zusätzlich korreliert in diesen Zwischenanalysen SAH, nicht aber Homocystein, mit der IMT (Intima-Media-Dicke) als Marker der subklinischen Atherosklerose.

Die prognostische Bedeutung der beiden C1-Metabolite soll in der CARE FOR HOMe Studie überprüft werden. Das Studiendesign der prospektiven CARE FOR HOMe Studie erlaubt, erstmals in einer größeren epidemiologischen Untersuchung pathophysiologische Zusammenhänge zwischen zentralen Mediatoren der epigenetischen Fehlregulation (SAH / Homocystein) und kardiovaskulären Ereignissen an einem gut charakterisierten Kollektiv chronisch nierenkranker Menschen zu überprüfen.

Die Integration von experimentell-klinisch und epidemiologischen Untersuchungen soll ein besseres Verständnis der beschleunigten Atherosklerose nierenkranker Menschen erlauben, welches in neuen Möglichkeiten der Prävention und Therapie der inakzeptabel hohen kardiovaskulären Morbidität chronisch nierenkranker Patienten resultieren könnte.

Tabelle 2. Das HOMe Studienprojekt.

Studie				
Einschlusskriterien	Chronisch nierenkrane Patienten K/DOQI 2-4	Gesunde Probanden ohne manifeste CV Erkrankung	Patienten vor elektiver Koronarangiographie	Patienten nach allogener Nierentransplantation
Ausschlusskriterien	immunsuppressive Therapie klinisch manifeste Infekte bekanntes Malignom akutes Nierenversagen	Alter < 20 bzw. > 60 Jahre immunsuppressive Therapie CV Erkrankung	Alter < 18 Jahre	klinisch manifeste Infekte bekanntes Malignom akutes Nierenversagen
Erhobene Parameter (Einschluss)	klassische CV Risikofaktoren Calcium/Phosphat Parameter Echokardiographie Monozytensubpopulationen	klassische CV Risikofaktoren Intima-Media-Dicke Cholesterinhomöostase Monozytensubpopulationen	klassische CV Risikofaktoren Koronarangiographie / Lävographie Monozytensubpopulationen	klassische CV Risikofaktoren Calcium/Phosphat Parameter Echokardiographie Monozytensubpopulationen
Anzahl Studienteilnehmer	444	622	1368	222 (geplant)
Primäres kardiovaskuläres Outcome	jegliches CV Ereignis	-	akuter Myokardinfarkt ischämischer Schlaganfall kardiovaskulärer Tod	jegliches CV Ereignis
Primäres renales Outcome	Reduktion der eGFR um ≤ 50 % Nierenersatzverfahren ≥ 3 Mo Tod	-	-	Reduktion der eGFR um ≤ 50 % Nierenersatzverfahren ≥ 3 Mo Tod
Sekundäres kardiovaskuläres Outcome	akuter Myokardinfarkt ischämischer Schlaganfall kardiovaskulärer Tod	-	jegliches CV Ereignis	akuter Myokardinfarkt ischämischer Schlaganfall kardiovaskulärer Tod
Sekundäres renales Outcome	Reduktion der eGFR um ≤ 50 % Nierenersatzverfahren ≥ 3 Mo	-	-	Reduktion der eGFR um ≤ 50 % Nierenersatzverfahren ≥ 3 Mo

CV: kardiovaskulär; Mo: Monate; weitere Informationen unter www.uks.eu/home

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Anhang

Publikationen

Nachfolgend sind die Publikationen aufgeführt, die im Rahmen dieser Promotionsarbeit entstanden sind.

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$CD14^{++}CD16^{+}$ monocytes and cardiovascular outcome in patients with chronic kidney disease

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Aims Patients with chronic kidney disease (CKD) pose a worldwide growing burden to health care systems due to accelerated atherosclerosis and subsequent high cardiovascular (CV) morbidity. Atherogenesis is prominently driven by monocytes and monocyte-derived macrophages. The expression of CD14 and CD16 characterizes three monocyte subsets: $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{(+)}CD16^{+}$ cells; the latter two are often denoted as 'proinflammatory' $CD16^{+}$ monocytes. Despite an association between $CD16^{+}$ monocyte counts and higher CV risk in cross-sectional cohorts, the prognostic impact of elevated $CD16^{+}$ monocyte counts is poorly understood.

Methods and results We assessed monocyte heterogeneity using flow cytometry in 119 patients with non-dialysis CKD, who were prospectively followed for a median of 4.9 (inter-quartile range 4.8–5.0) years for the occurrence of CV events. In addition, we assessed expression of chemokine receptors on monocyte subsets. $CD14^{++}CD16^{+}$ monocyte were independently associated with CV events [hazard ratio (for an increase of 10 cells/μL) 1.26 (confidence interval: 1.04–1.52; $P = 0.018$)] after adjustment for variables that significantly affected $CD14^{++}CD16^{+}$ cell counts at baseline. Across the spectrum of CKD, $CD14^{++}CD16^{+}$ monocytes selectively expressed CCR5.

Conclusion We found that $CD14^{++}CD16^{+}$ monocytes were independently associated with CV events in non-dialysis CKD patients. Our results support the notion that $CD16^{+}$ monocytes rather than $CD16^{-}$ monocytes are involved in human atherosclerosis.

Keywords Monocyte heterogeneity • Cardiovascular outcome • Chronic kidney disease • Cardio-renal syndrome

Introduction

Monocytes and monocyte-derived macrophages are at the centre stage of the innate immune system, fulfilling important tasks in host-defence, immunoregulation, tissue repair, and regeneration.[1] Nonetheless, monocyte biology in health and disease is still poorly understood, and puzzling findings remain, such as the missing coherent association between monocyte counts and cardiovascular (CV) disease in large epidemiological studies.[2–4] This is somehow counterintuitive, as monocytes and macrophages are well-established key players in atherosclerosis.[5]

A possible explanation might be that human monocytes were considered to be a homogenous leucocyte subpopulation until 1989, when human monocyte heterogeneity was reported for the first time.[6] Presently, three human monocyte subsets are defined by the differential expression of the LPS receptor CD14 and the FcγIII receptor CD16, which are $CD14^{++}CD16^{-}$ cells, $CD14^{++}CD16^{+}$ cells, and $CD14^{(+)}CD16^{+}$ cells. In earlier studies, the latter two subsets are summarized as $CD16^{+}$ monocytes, which account for 10–20% of all circulating monocytes.[7] As opposed to classical $CD14^{++}CD16^{-}$ monocytes, $CD16^{+}$ monocyte counts are elevated in numerous inflammatory conditions,[8–15] including end-stage renal disease.[16] Therefore, $CD16^{+}$ monocytes have traditionally been termed 'proinflammatory' monocytes.[17]

In line, dialysis patients with high $CD14^{++}CD16^{+}$ monocyte counts are at increased risk for future CV events.[18] Obviously, dialysis patients are a highly selected population, so that findings from this population cannot be transferred to other patient groups. Therefore, we see a pressing need for further experimental and clinical research in human monocyte heterogeneity to clarify

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the significance of the respective monocyte subsets for human pathology and to test whether the predictive role of $CD14^{++}CD16^{+}$ monocytes in dialysis patients for CV events holds true in broader patient groups.

In the present study, we demonstrate that $CD14^{++}CD16^{+}$ monocytes are independently associated with CV events in patients with non-dialysis chronic kidney disease (CKD), even though $CD16^{+}$ monocyte counts in CKD patients are close to the range observed in healthy individuals and considerably lower than in patients on haemodialysis.

Methods

Study population

In a prospective cohort study on monocyte heterogeneity and CV outcome in CKD, 152 stable ambulatory patients with CKD K/DOQI 1–5 not receiving renal replacement therapy were screened. In all patients, comorbidity was determined by standardized interviews and by review of medical documentation. Thirty-one patients were excluded from the analysis as they were on immunosuppressive treatment, and in two patients, determination of monocyte subsets failed due to lost blood samples, leaving 119 patients in the study. In this cohort, CKD was due to diabetic nephropathy ($n = 26$), glomerulonephritis ($n = 20$), interstitial nephritis ($n = 14$), nephrosclerosis ($n = 13$), autosomal dominant polycystic kidney disease ($n = 8$), obstructive nephropathy ($n = 4$), other primary renal diseases ($n = 20$), and unknown conditions ($n = 14$).

Prevalent CV disease was defined as a history of myocardial infarction, coronary artery angioplasty/stenting/bypass surgery, stroke, carotid endarterectomy/stenting, non-traumatic lower extremity amputation, or lower limb artery bypass surgery/angioplasty/stenting. Diabetes mellitus was diagnosed if a patient had a history of diabetes mellitus, a spontaneous plasma glucose level of >200 mg/dL, self-reported diabetes mellitus, and/or received hypoglycaemic treatment. Patients were categorized as active smokers if they were current smokers or had stopped smoking <1 month before entry into the study. Systolic and diastolic blood pressures (BP sys and BP dia) were measured in a supine position. Pulse pressure was calculated as BP sys − BP dia.

Table 1 Baseline characteristics of the study participants

	Overall ($n = 119$)	No event ($n = 72$)	Event ($n = 47$)	*P*
Age (years)	66 (54–71)	63 (50–70)	70 (64–76)	<0.001
Women (%)	54 (45)	35 (49)	19 (40)	0.479
Smokers (%)	11 (9)	9 (13)	2 (4)	0.143
Diabetes mellitus (%)	42 (35)	17 (24)	25 (53)	0.001
History of CVD (%)	41 (34)	18 (25)	23 (49)	0.015
K/DOQI stage 1 (%)	2 (2)	2 (3)	0 (0)	0.008
K/DOQI stage 2 (%)	16 (13)	13 (18)	3 (6)	
K/DOQI stage 3 (%)	45 (38)	33 (46)	12 (26)	
K/DOQI stage 4 (%)	28 (23)	13 (18)	15 (32)	
K/DOQI stage 5 (%)	28 (23)	11 (15)	17 (36)	
Total cholesterol (mg/dL)	201 (163–231)	208 (163–233)	195 (161–228)	0.602
HDL cholesterol (mg/dL)	50 (40–59)	52 (40–61)	48 (41–53)	0.318
Body mass index (kg/m^2)	29 (26–33)	29 (26–33)	29 (25–33)	0.859
Plasma calcium (mmol/L)	2.4 (2.3–2.4)	2.4 (2.2–2.4)	2.4 (2.3–2.4)	0.327
Plasma phosphorus (mg/dL)	3.6 (3.1–4.4)	3.5 (3.0–4.2)	3.9 (3.3–5.0)	0.031
C-reactive protein (mg/L)	3.2 (2.9–7.6)	2.9 (2.9–6.4)	4.3 (2.9–13.2)	0.117
Plasma homocystein (μmol/L)	15 (12–21)	14 (11–20)	18 (13–27)	0.001
Proteinuria (g/g creatinine)	0.4 (0.0–2.2)	0.2 (0.0–1.4)	1.7 (0.0–3.3)	0.003
Systolic blood pressure (mmHg)	170 (150–185)	165 (146–184)	175 (155–195)	0.061
Diastolic blood pressure (mmHg)	95 (85–110)	95 (86–109)	95 (80–110)	0.752
Pulse pressure (mmHg)	70 (60–85)	68 (55–85)	75 (65–85)	0.011
Antiplatelet therapy (%)	41 (35)	20 (28)	21 (45)	0.049
Beta-blockers (%)	64 (55)	36 (51)	28 (61)	0.447
Angiotensin-receptor blockers (%)	66 (57)	38 (54)	28 (61)	0.442
ACE-inhibitors (%)	44 (38)	28 (40)	16 (35)	0.440
Statins (%)	41 (35)	23 (32)	18 (38)	0.695
Haemoglobin (g/dL)	13.0 (11.6–14.3)	13.3 (12.4–14.4)	12.0 (11.2–14.2)	0.014
Platelets ($\times 10^3$ cells/μL)	230 (188–268)	222 (183–261)	235 (189–268)	0.715
Leucocytes (cells/μL)	6800 (5600–7800)	6550 (5400–7550)	7000 (5800–8400)	0.257
Neutrophils (cells/μL)	4030 (3192–5037)	3838 (3188–4676)	4488 (3216–5548)	0.150
Lymphocytes (cells/μL)	1606 (1323–2072)	1628 (1358–2166)	1584 (1232–1974)	0.236

Variables are presented as percentage, or as median with inter-quartile range, as appropriate.

Informed consent was obtained from all patients, and the study design was approved by the local Ethics Committee.

All participants were followed from the baseline examination in 2004 until death or until 31 July 2009. One patient was lost to follow-up. The pre-specified combined clinical endpoint was the first occurrence of a CV event (defined as myocardial infarction, coronary artery angioplasty/stenting/bypass surgery, stroke with symptoms lasting >24 h, carotid endarterectomy/stenting, non-traumatic lower extremity amputation, lower limb artery bypass surgery/angioplasty/stenting, or death). All-cause mortality was assessed as a secondary endpoint.

To assess whether the reported shift in monocyte subset counts in CKD patients[16] occurs before or after the onset of dialysis treatment, we compared monocyte subset counts among 39 controls with intact renal function, 39 patients suffering from advanced CKD (stages 4/5, not yet undergoing renal replacement therapy), and 39 dialysis patients.

These groups were matched for age (±5 years), gender, prevalent CV disease, and diabetes mellitus, as defined above.

Laboratory methods

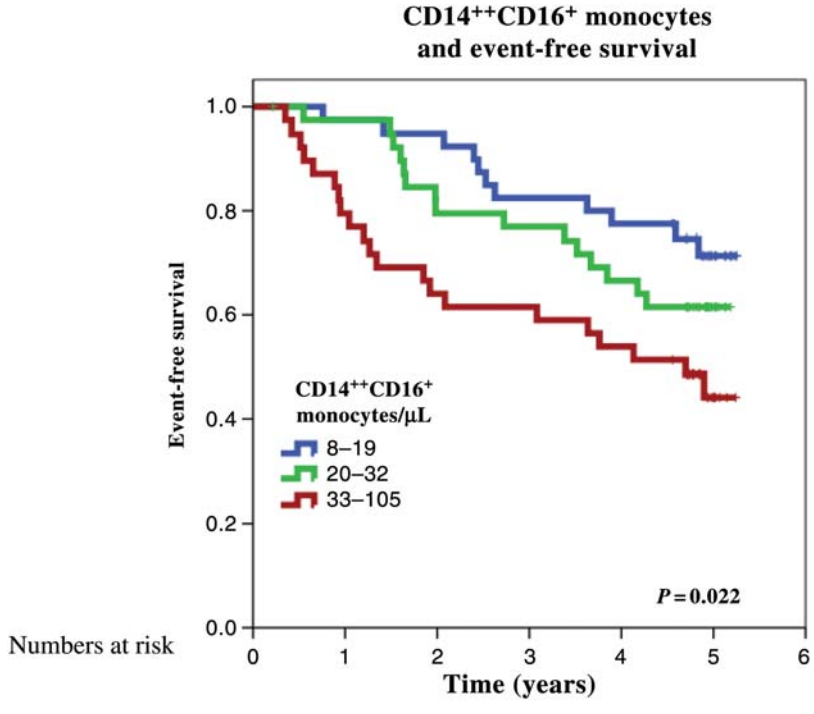
Total cholesterol, high-density lipoprotein cholesterol (HDL-C), calcium, phosphorus, albumin, and C-reactive protein were measured using standard techniques. Glomerular filtration rate was estimated (eGFR) using the MDRD study equation 4. Differential blood counts were determined with automated cell counters.

Via flow cytometry, monocyte subsets were identified according to our previously published standard staining and gating strategy[19] in a whole blood assay using 100 μL of heparin anticoagulated blood. Cells were stained with monoclonal antibodies—anti-CD86 (HA5.2B7, Beckman-Coulter, Krefeld, Germany), anti–CD16 (3G8, Invitrogen, Hamburg, Germany), anti-CD14 (Mϕ9, BD Biosciences, Heidelberg, Germany), and analysed by flow cytometry (FACSCalibur, BD Biosciences) using the Cell Quest software.

Monocytes were gated in a SSC/CD86^{+} dotplot, identifying monocytes as CD86^{+} cells with monocyte scatter properties. Subpopulations of CD14^{++}CD16^{-}, CD14^{++}CD16^{+}, and CD14$^{(+)}$CD16^{+} monocytes were distinguished by their surface expression pattern of the LPS receptor CD14 and the FcγIII receptor CD16. Using these basic panels, monocyte subpopulations were further examined for the expression of the chemokine receptors CCR2, CCR5, and CX_3CR1 in 30 patients, equally distributed across CKD stages 2–4. The following monoclonal antibodies were used: anti-CCR2 (48607, BD Biosciences), anti-CCR5 (2D7, BD Biosciences), and anti-CX_3CR1 (2A9-4, Biozol, Eching, Germany). Flow cytometrical data were measured as median fluorescence intensity and standardized against coated fluorescent particles (SPHERO^{TM}, BD Biosciences).

Statistical analysis

Categorical variables are presented as percentage of patients, and compared by Fisher's exact test. Continuous data are expressed as medians with inter-quartile range (IQR) and compared by



Numbers at risk

First tertile	40	39	38	33	31	12
Second tertile	40	38	31	30	26	7
Third tertile	39	31	25	24	21	7

Figure 1 Relationship between tertiles of CD14^{++}CD16^{+} monocyte counts and event-free survival in patients with chronic kidney disease (Kaplan–Meier analysis with log-rank test).

Mann–Whitney test (for two independent samples) or by Friedman test (for paired samples), as appropriate. Correlation coefficients were calculated by Spearman test. Kaplan–Meier survival curves were calculated, and event-free survival (i.e. time until first CV event as defined above) as well as overall survival (i.e. time until death of any cause) were compared by log-rank test. Cox proportional-hazards models were calculated to examine the relationship of monocyte subset cell counts with event-free survival after adjustment for variables that were associated with $CD14^{++}CD16^{+}$ monocyte counts at baseline, and for eGFR.

Data management and statistical analysis were performed with SPSS 17.0. The level of significance was set at $P \leq 0.05$.

Results

Baseline characteristic

The baseline characteristics of all 119 study participants are shown in *Table 1*. Forty-seven patients (39.5%) experienced a CV event before 31 July 2009. One patient was lost after a follow-up period of 1.8 years; the remaining 71 patients have been followed for a median of 4.9 (IQR 4.8–5.0) years. As expected, those patients who had a CV event were older, had a higher prevalence of diabetes mellitus at baseline, higher pulse pressure measurements, and more advanced CKD with lower eGFR, higher proteinuria and higher plasma phosphate levels (*Table 1*).

At baseline, eGFR correlated neither with total monocyte counts ($r = -0.048$, $P = 0.601$) nor with monocyte subpopulation counts ($CD14^{++}CD16^{-}$: $r = -0.012$, $P = 0.895$; $CD14^{++}CD16^{+}$: $r = -0.117$, $P = 0.205$; $CD14^{(+)}CD16^{+}$: $r = -0.094$, $P = 0.310$). Similarly, total monocytes and monocyte subset counts were not significantly associated with BP sys, pulse pressure, serum phosphate, and proteinuria (data not shown). Conversely, $CD14^{++}CD16^{+}$ and $CD14^{(+)}CD16^{+}$ monocytes were significantly correlated with age ($r = 0.187$, $P = 0.042$ and $r = 0.206$, $P = 0.025$, respectively), whereas total monocytes and $CD14^{++}CD16^{-}$ were not. Interestingly, $CD14^{++}CD16^{+}$ monocytes were the only monocyte subset to be significantly correlated with C-reactive protein ($r = 0.253$, $P = 0.006$), whereas $CD14^{(+)}CD16^{+}$ monocytes were significantly associated with serum homocystein levels ($r = 0.201$, $P = 0.038$).

At study enrolment, $CD14^{++}CD16^{+}$ monocyte counts were significantly elevated among patients with prevalent diabetes mellitus [diabetics: median 32 (IQR 21–39); non-diabetics: median 22 (IQR 17–34) cells/μL, $P = 0.009$], and prevalent CV disease [CV disease: median 27 (IQR 21–37); no CV disease median 23 (17–36) cells/μL, $P = 0.046$], whereas patients on statin treatment had higher counts of $CD14^{(+)}CD16^{+}$ monocytes [statin intake: median 75 (IQR 56–111); no statin intake: median 61 (IQR 47–86) cells/μL; $P = 0.028$], but not of $CD14^{++}CD16^{-}$,

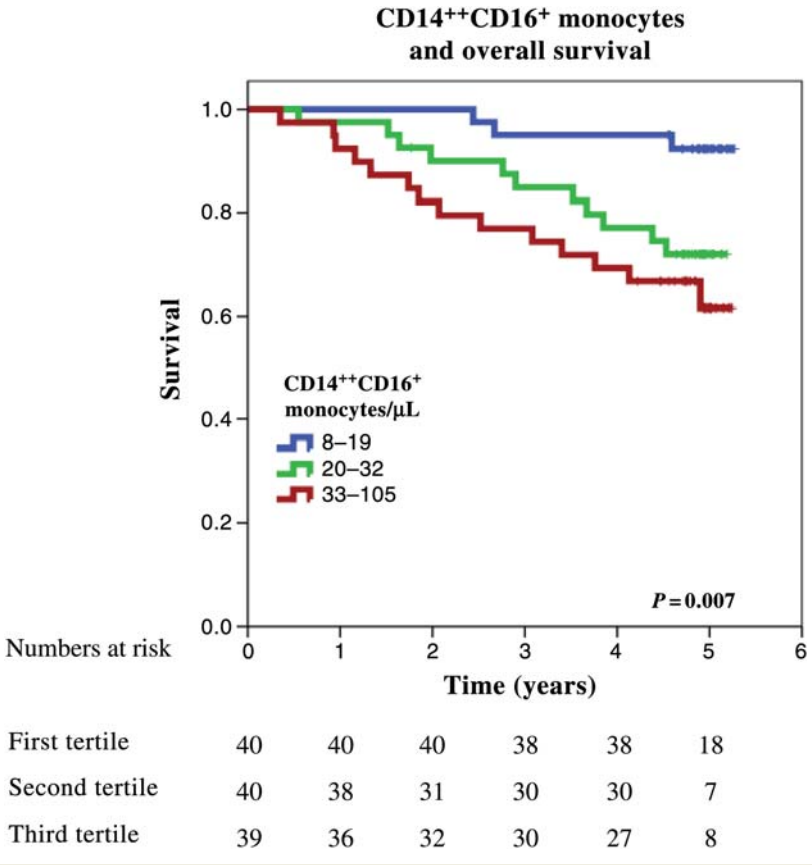


Figure 2 Tertiles of $CD14^{++}CD16^{+}$ monocyte counts and overall survival in patients with chronic kidney disease (Kaplan–Meier analysis with log-rank test).

$CD14^{++}CD16^{+}$ monocytes, or total monocytes, respectively. Intake of antiplatelet agents, angiotensin-converting enzyme (ACE)-inhibitors, angiotensin-receptor blockers, and beta-blockers was not associated with differences in monocyte (subset) counts.

$CD14^{++}CD16^{+}$ monocytes and their relation to cardiovascular outcome and mortality

Patients who experienced a CV event during follow-up had higher $CD14^{++}CD16^{+}$ monocyte counts compared with patients without an event, whereas counts of total monocytes, $CD14^{++}CD16^{-}$, and $CD14^{(+)}CD16^{+}$monocytes did not differ significantly. When stratifying patients by their $CD14^{++}CD16^{+}$ monocyte counts, patients in the highest tertile had the shortest event-free survival (*Figure 1*). Likewise, these patients had the shortest overall survival (*Figure 2*). The prognostic impact of this monocyte subset is even strengthened when stratifying patients by their percentage of $CD14^{++}CD16^{+}$ monocytes (defined as % of all circulating monocytes) instead of absolute counts of $CD14^{++}CD16^{+}$ monocytes (see Supplementary material online, *Figures S1* and *S2*).

In contrast, tertiles of $CD14^{++}CD16^{-}$ (*Figure 3*) and $CD14^{(+)}CD16^{+}$ monocytes (*Figure 4*) did not predict survival in our patient cohort. In Cox regression analysis, $CD14^{++}CD16^{+}$ monocyte counts remained significantly associated with event-free survival after adjustment for variables that were correlated with $CD14^{++}CD16^{+}$ monocyte counts at baseline (age, diabetes mellitus, prevalent CV disease, and C-reactive protein), and for eGFR (*Table 2*).

Chemokine receptor expression on monocyte subsets

The chemokine receptors CCR2, CCR5, and CX_3CR1 are relevant in subset-specific extravasation of monocytes in atherosclerotic plaques.[20] Therefore, we analysed monocyte surface expression pattern of these chemokines receptors in 30 CKD patients. Flow cytometry confirmed monocytic subset-specific expression of chemokine receptors across different stages of CKD. Although $CD14^{++}CD16^{-}$ monocytes expressed highest levels of CCR2, and $CD14^{(+)}CD16^{+}$ monocytes express highest levels of CX_3CR1, $CD14^{++}CD16^{+}$ monocytes are characterized by selective expression of CCR5 and coexpression of both CCR2 and CX_3CR1 irrespective of kidney function (*Figure 5*).

Comparison of chronic kidney disease patients to subjects with normal renal function and haemodialysis patients

As we observed no significant increase in monocyte subset counts with declining renal function—contrasting the known expansion of

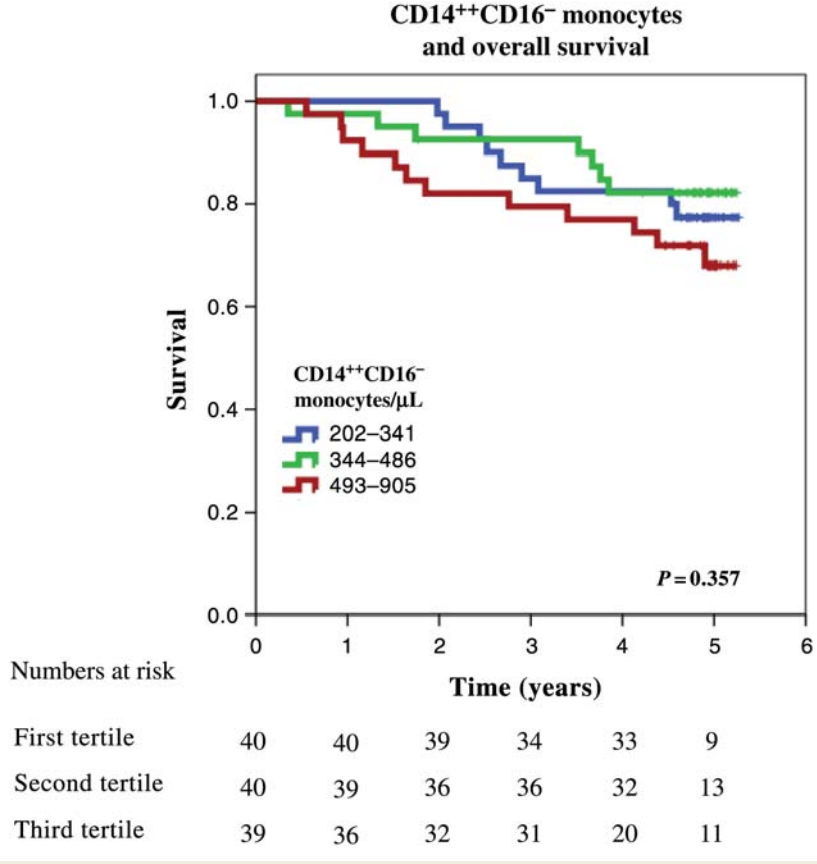


Figure 3 Tertiles of $CD14^{++}CD16^{-}$ monocyte counts and overall survival in patients with chronic kidney disease (Kaplan–Meier analysis with log-rank test).

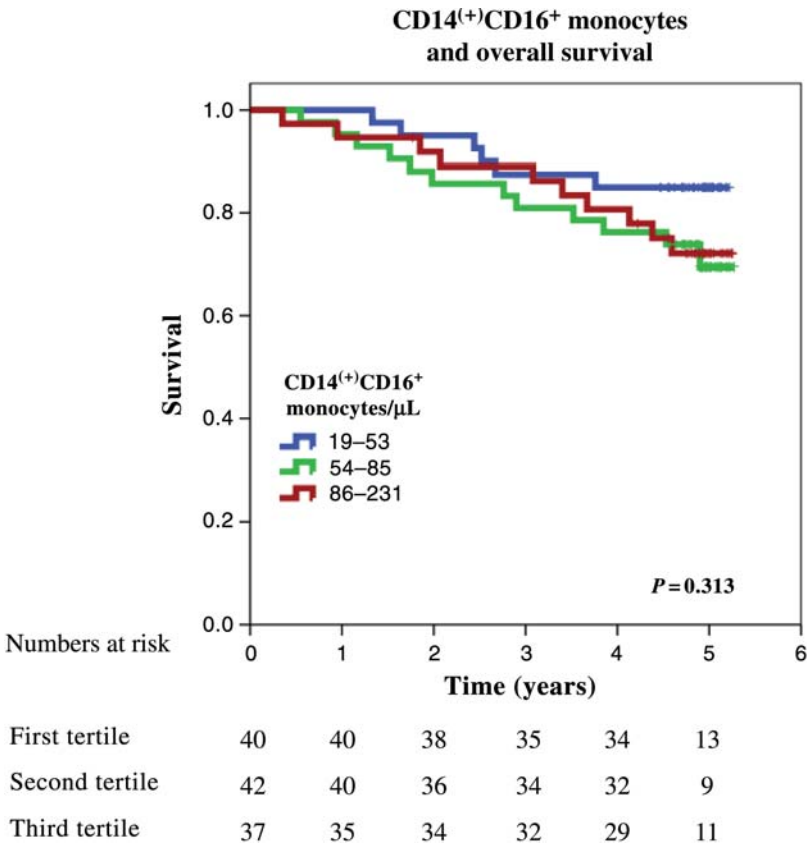


Figure 4 Tertiles of CD14(+) CD16+ monocyte counts and overall survival in patients with chronic kidney disease (Kaplan–Meier analysis with log-rank test).

Table 2 Cox proportional-hazards model (outcome variable: incident cardiovascular events)

Parameters	HR	CI	P
CD14++CD16+ monocytes (per 10 cells/μL)	1.26	1.04–1.52	0.018
Age (per 10 years)	1.52	1.14–2.03	0.004
eGFR (per mL/min/1.73 m^2)	0.97	0.95–0.99	<0.001
Diabetes mellitus (yes)	1.67	0.89–3.14	0.114
Prevalent cardiovascular disease (yes)	1.80	0.94–3.46	0.076
C-reactive protein (mg/L)	0.99	0.98–1.00	0.162

Indicated are hazard ratios (HR), their 95% confidence interval, and level of significance.

CD16+ monocytes in haemodialysis patients[16]—we compared 39 subjects with normal renal function, 39 patients with CKD (not receiving renal replacement therapy), and 39 haemodialysis patients matched for age, gender, prevalent CV disease, and diabetes mellitus.

In each group, 18 out of 39 (46%) patients were male, 22 patients (56%) were diabetics, and 14 patients (36%) had a history of CV disease. Counts of CD16+ monocytes significantly differed among the three groups, with lower counts of CD14++CD16+ and CD14(+)CD16+ monocytes in subjects with normal renal function and CKD patients compared with haemodialysis patients (*Figures 6* and *7*).

Discussion

Research interest in monocyte heterogeneity gained strong momentum in the last decade, as a subset-specific contribution of monocytes to atherogenesis has been postulated.[21] However, human monocyte heterogeneity and its relation to human CV disease are still poorly understood.

We have previously reported on the relationship of CD16+ monocytes and CV events in dialysis patients. In these studies, baseline CD14++CD16+ monocyte counts[18] and haemodialysis-induced CD16+ monocyte kinetics independently predicted CV outcome.[22] Of note, dialysis patients are a highly selected population, and epidemiological data from these patients cannot be extrapolated to other individuals. Specifically, dialysis patients experience a tremendous CV event rate. At the same time, they show a notable elevation of CD16+ monocyte counts compared with patients with intact renal function.[16]

It has been fully unknown whether this shift in monocyte subpopulations towards CD16+ monocytes occurs only in end-stage

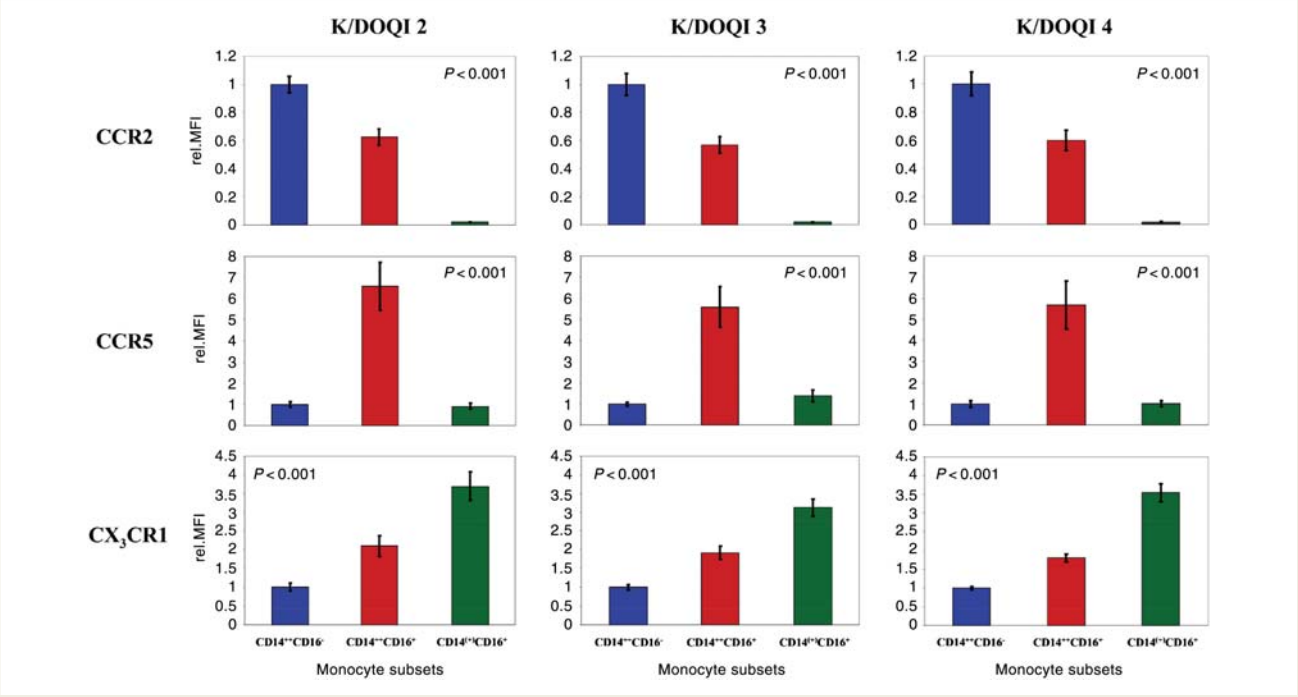


Figure 5 Expression of CCR2, CCR5, and CX3CR1 on $CD14^{++}CD16^{-}$ monocytes (blue columns), $CD14^{++}CD16^{+}$ monocytes (red columns) and $CD14^{(+)}CD16^{+}$ (green columns) across stages of chronic kidney disease. Flow cytometrical data were measured as median fluorescence intensity (MFI) and standardized on $CD14^{++}CD16^{-}$ monocytes. Results are presented as means ± SEM. Data were analysed using Kruskal–Wallis test.

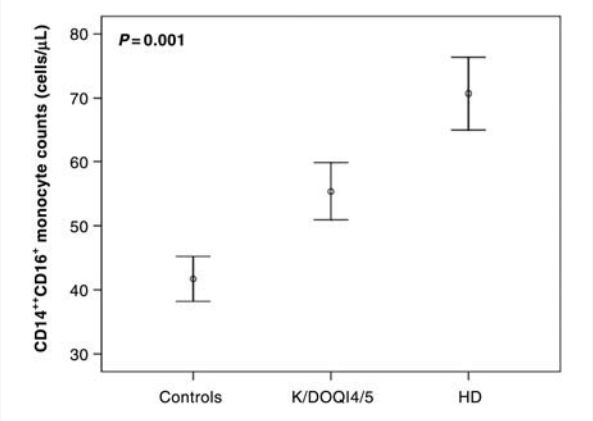


Figure 6 Counts of $CD14^{++}CD16^{+}$ monocytes in 39 matched triples of control subjects with normal renal function, patients with chronic kidney disease not on dialysis, and haemodialysis patients. Results are presented as means ± SEM.

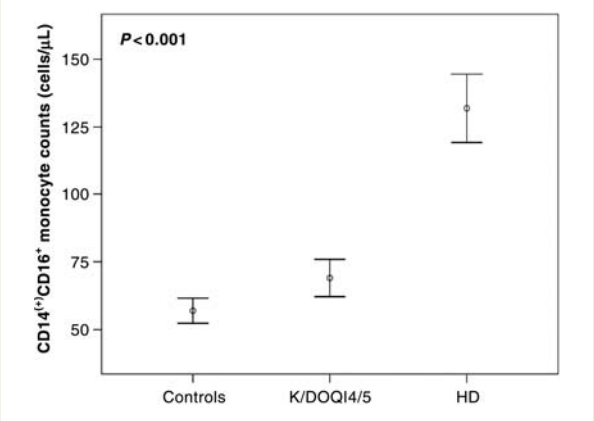


Figure 7 Counts of $CD14^{(+)}CD16^{+}$ monocytes in 39 matched triples of control subjects with normal renal function, patients with chronic kidney disease not on dialysis, and haemodialysis patients. Results are presented as means ± SEM.

renal disease, or at less severe renal impairment. Moreover, the prognostic impact of monocyte subset counts in non-dialysis CKD has been ignored before, despite the fact that non-dialysis CKD is an emerging global health issue, and that patients with less advanced CKD outnumber the dialysis population by far.

Albeit at lower CV risk compared with dialysis patients, individuals at earlier stages of CKD already suffer from an accelerated atherogenesis.[23] Recently, the strong bidirectional relationship between renal and CV morbidity has been underscored by the introduction of the classification of cardiorenal syndrome, in which the pathophysiological role of monocytes has been especially highlighted.[24]

We now report that non-dialysis CKD patients have $CD16^{+}$ monocyte counts close to the normal range observed in subjects with preserved renal function. Nonetheless, $CD14^{++}CD16^{+}$ monocyte counts are independently associated with CV events in patients with non-dialysis CKD in multivariate analysis.

In support of our present and earlier findings,[18,19,22] there are other cogent arguments for the prominence of $CD16^{+}$ monocytes but not $CD16^{-}$ monocytes in the inflammatory disease atherosclerosis: firstly, it is well established from epidemiological studies that $CD16^{+}$ monocyte counts are elevated in many other inflammatory conditions.[8–15] Secondly, $CD16^{+}$ monocytes are efficient producers of inflammatory cytokines, whereas they poorly secrete the anti-inflammatory interleukin (IL)-10.[25,26] Thirdly, several lines of evidence suggest a high endothelial affinity of $CD16^{+}$ monocytes conferred by their surface expression of chemokine receptors and adhesion molecules e.g. CX_3CR1, CCR5, VLA-4, and CD11c.[27,28] Interestingly, it has already been reported in 2000 that $CD16^{+}$ monocytes reside in the marginal pool where they can be rapidly mobilized in a catecholamine-dependent manner.[28] This observation has been later verified in an outstanding study demonstrating that the mouse counterparts of $CD16^{+}$ monocytes crawl along the endothelium and rapidly extravasate upon inflammatory stimuli.[29] The authors termed this the 'patrolling behaviour' of the murine counterparts of $CD16^{+}$ monocytes, and discussed these cells as a potential therapeutic target in inflammatory conditions such as atherosclerosis.[29] In line with this notion, are mechanistic data from studies by Ancuta *et al.*[30] indicating that $CD16^{+}$ monocytes home to sites of endothelial activation in a CX_3CR1-dependent manner, where they secrete MMP-9, CCL-2, and IL-6 with the ability to propagate further vascular injury through the recruitment of T-lymphocytes and additional monocytes. The relevance of CX_3CL1 and CX_3CR1 in atherosclerosis[31–33] additionally makes a good case for the role of $CD16^{+}$ in atherosclerosis, as CX_3CR1 is highly expressed on $CD16^{+}$ monocytes.

Further strong evidence derives from several studies showing that the CCR5 delta32 variant is associated with a more favourable CV outcome in the general population[34] and with better all-cause as well as CV survival in patients with end-stage renal disease.[35] CCR5 blockade in experimental atherosclerosis has been proved to be beneficial[36,37] and has been consecutively discussed as a potential therapeutic option in diabetics as a CV high-risk population.[38] Interestingly, CCR5 inhibition is feasible as it is an already applied therapeutic principle in HIV infection treatment.[39] As pointed out before, CCR5 is expressed by $CD16^{+}$ monocytes (especially $CD14^{++}CD16^{+}$ monocytes) but not by $CD14^{++}CD16^{-}$ monocytes as shown in the present study and previously.[27]

The cumulative evidence for a prominent role of CX_3CR1 and CCR5 in atherosclerosis—favouring $CD16^{+}$ monocytes as drivers of atherosclerosis—together with our clinical data on the association between $CD16^{+}$ monocytes, CV risk factors in low-risk subjects,[19] and CV events in high-risk individuals[18,22] and in the present trial, strongly suggest that $CD16^{+}$ monocytes are most likely the relevant monocyte subset in human atherosclerosis.

Furthermore, we feel that our data from non-dialysis CKD patients might be hypothesis generating for studies on monocyte heterogeneity and CV complications in the general population.

In summary, we report the role of $CD14^{++}CD16^{+}$ monocytes for future CV events in a cohort representative of a much larger patient group compared with earlier studies.[18,22] Extending previous knowledge on monocyte heterogeneity and CV outcome, we raise now the hypothesis that modulating $CD14^{++}CD16^{+}$ monocyte function—e.g. through interference with the CCR5–CCL5 axis—might be beneficial for preventing CV events.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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CD14++CD16+ Monocytes Independently Predict Cardiovascular Events

A Cohort Study of 951 Patients Referred for Elective Coronary Angiography

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Objectives The aim of this study was to analyze the yet ill-defined relationship of distinct human monocyte subsets with cardiovascular outcomes in a broad patient population at cardiovascular risk.

Background Monocytes, the most abundant immune cell type found in atherosclerotic plaques, are crucial promoters of atherogenesis. Three distinct human monocyte subsets exist: classical CD14++CD16−, intermediate CD14++CD16+, and nonclassical CD14+CD16++ monocytes. Immunomodulation of distinct monocyte subsets has recently been discussed as a new therapeutic avenue in atherosclerosis.

Methods Cardiovascular events in 951 subjects referred for elective coronary angiography were prospectively analyzed. Monocyte subset analysis was performed using flow cytometry, blinded to patients' clinical characteristics, and patients were categorized according to quartiles of total monocyte and monocyte subset counts. The primary endpoint was defined a priori as the first occurrence of cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke. Endpoint adjudication was done blinded to monocyte subset distribution.

Results During a mean follow-up period of 2.6 ± 1.0 years, 93 patients experienced the primary endpoint. In univariate Kaplan-Meier analysis, counts of total ($p = 0.010$), classical CD14++CD16− ($p = 0.024$), and intermediate CD14++CD16+ ($p < 0.001$) monocytes predicted the primary endpoint, whereas nonclassical monocytes did not ($p = 0.158$). After full adjustment for confounders, CD14++CD16+ monocytes remained the only monocyte subset independently related to cardiovascular events (fourth vs. first quartile: hazard ratio: 3.019; 95% confidence interval: 1.315 to 6.928; $p = 0.009$).

Conclusions CD14++CD16+ monocytes independently predicted cardiovascular events in subjects referred for elective coronary angiography. Future studies will be needed to elucidate whether CD14++CD16+ monocytes may become a target cell population for new therapeutic strategies in atherosclerosis. (J Am Coll Cardiol 2012;60: 1512–20) © 2012 by the American College of Cardiology Foundation

Monocytes are the central drivers of vascular inflammation in atherosclerosis. They contribute to atherogenesis from the formation of the earliest asymptomatic atherosclerotic lesions, namely fatty streaks, to final plaque rupture with potentially fatal outcomes (1,2). Although experimental studies have proven a causative role of monocytes in atherogenesis (3), epidemiological analyses have failed to unequivocally demonstrate an association between circulating monocyte counts and cardiovascular disease (4).

Importantly, monocytes display substantial heterogeneity, which is reflected by the differential surface expression of the lipopolysaccharide receptor (CD14) and the low-affinity Fcγ-III receptor (CD16). Although a subset-specific contribution of monocytes has been proposed in recent years on the basis of laboratory data (3), monocyte heterogeneity has not been analyzed thoroughly in the context of human atherosclerosis.

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The existence of 3 distinct monocyte subsets is acknowledged by recent consensus (5), namely, classical CD14++CD16− monocytes, intermediate CD14++CD16+ monocytes, and nonclassical CD14+CD16++ monocytes. However, most previous studies did not distinguish between intermediate CD14++CD16+ and nonclassical CD14+CD16++ monocytes but subsumed these 2 subsets as CD16-positive monocytes. Consequently, the intermediate monocyte subset was by far the most poorly characterized monocyte subset until recently (6). Although numerically the smallest monocyte subpopulation and seemingly displaying just an intermediate immunophenotype, CD14++CD16+ monocytes are a clearly distinguishable subset, as evidenced by the distinct gene expression profile recently been reported by Wong et al. (7) as well as our group (6), independently of each other.

We previously reported a predictive role of intermediate CD14++CD16+ monocyte counts in patients with chronic kidney disease (CKD) (8), a selected patient group at highest cardiovascular risk. Of note, CKD-associated immune dysfunction, which is characterized by profound shifts in monocyte subsets (9,10), and the CKD-specific pattern of accelerated atherosclerosis both preclude an uncritical generalization of our previous findings to the general population.

Against this background, we initiated the HOM SWEET HOMe (Heterogeneity of Monocytes in Subjects Who Undergo Elective Coronary Angiography—The Homburg Evaluation) study to test the hypothesis that counts of intermediate CD14++CD16+ monocytes predict cardiovascular events in subjects at cardiovascular risk referred for elective coronary angiography.

Methods

Between May 2007 and June 2010, subjects who were admitted to Saarland University Medical Center for elective coronary angiography were invited to participate in the HOM SWEET HOMe study. The study was approved by the local ethics committee, and all participants provided written informed consent.

At study inclusion, a history of smoking, diabetes, current drug intake, and cardiovascular comorbidities were recorded using a standardized questionnaire. Patient-reported comorbidities were confirmed by chart review. Prevalent cardiovascular disease was diagnosed in subjects with coronary artery disease (a history of myocardial infarction or coronary artery angioplasty, stent implantation, or bypass surgery), cerebrovascular disease (a history of major stroke [defined as acute onset of neurological symptoms persisting for >24 h] or carotid endarterectomy or stent implantation) or peripheral artery disease (a history of nontraumatic lower extremity amputation or lower limb artery bypass surgery, angioplasty, or stent implantation).

Subjects were categorized as active smokers if they were current smokers or had stopped smoking <1 month before study entry. Subjects with self-reported or physician-reported diabetes mellitus, with nonfasting blood glucose levels ≥200 mg/dl, with fasting blood glucose levels ≥126 mg/dl, or with current use of hypoglycemic medication were categorized as having diabetes. Body mass index was calculated as weight in kilograms divided by the square of height in meters.

Abbreviations and Acronyms

CKD = chronic kidney disease

CRP = C-reactive protein

Coronary angiography was performed on the day of hospital admission. Coronary artery disease was defined in subjects who had stenoses ≥ 50% in a major coronary artery on current coronary angiography and/or who had a history of coronary revascularization for coronary artery stenosis.

Because of their potential interference with monocyte subset counts, intake of systemic immunosuppressive agents was considered an exclusion criterion for the present analysis.

Laboratory measurements. Blood samples were obtained under standardized conditions. Blood levels of creatinine, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and C-reactive protein (CRP) were measured using standard methods. Estimated glomerular filtration rate was calculated using the 4-variable Modification of Diet in Renal Disease study equation.

Leukocyte and monocyte counts were measured with automated cell counters using standard techniques. Using flow cytometry, monocyte subpopulations were analyzed in a whole-blood assay using 100 μl of whole blood, as described and validated previously (6). Cells were stained by monoclonal antibodies (CD86 PE [HA5.2B7; Beckman-Coulter, Krefeld, Germany], CD16 PeCy7 [3G8; BD Biosciences, Heidelberg, Germany], CD14 PerCP [Mφ9; BD Biosciences]) and analyzed using flow cytometry (FACSCalibur and FACS Canto II; BD Biosciences) using Cell Quest and FACSDiva software (BD Biosciences).

In brief, monocytes were gated in an SSC/CD86+ dot plot, identifying monocytes as CD86+ cells with monocyte scatter properties. Subsets of CD14++CD16−, CD14++CD16+, and CD14+CD16++ monocytes were defined according to the surface expression pattern of the lipopolysaccharide receptor CD14 and the Fcγ-III receptor CD16 (compare Fig. 1 for a representative example). Nomenclature of monocyte subsets followed the recommendations of the Nomenclature Committee of the International Union of Immunological Societies (5).

Outcome analysis. After study inclusion, all study participants, or their next of kin, were contacted annually until death or until September 30, 2011, for outcome analysis.

We obtained medical records from the treating physicians for verification of all events reported by study participants or their next of kin. All events were adjudicated by the same investigators, who were blinded to monocyte data, according to the following definitions.

The composite primary endpoint was defined a priori as the first occurrence of cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke. Definition of

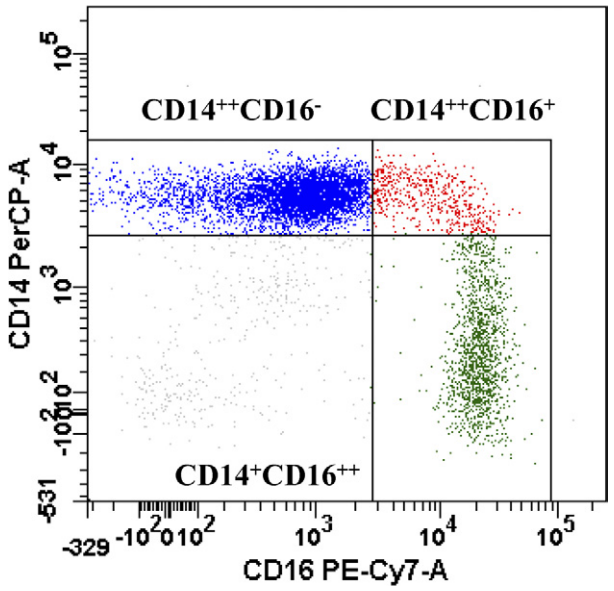

Figure 1 **Flow Cytometric Analysis of Monocyte Subpopulations**

Depicted are classical CD14++CD16− monocytes, intermediate CD14++CD16+ monocytes, and nonclassical CD14+CD16++ monocytes (representative example).

acute myocardial infarction followed the joint European Society of Cardiology, American College of Cardiology Foundation, American Heart Association, and World Heart Federation task force consensus (11). Stroke was defined as "rapidly developing clinical symptoms and/or signs of focal (or at times global) disturbance of cerebral function lasting > 24 hours (unless interrupted by surgery) or leading to death, with no apparent cause other than of vascular origin," in accordance with the World Health Organization (12). Those subjects without evidence of cerebral hemorrhage on cerebral imaging were defined to have nonhemorrhagic stroke. Deaths due to cardiovascular causes included sudden cardiac death, death from acute myocardial infarction, death from congestive heart failure, fatal stroke, fatal arrhythmia, and other fatal vascular events.

As a secondary endpoint, we defined the first occurrence of any cardiovascular event (defined as myocardial infarction; coronary artery angioplasty, stent implantation, or bypass surgery; stroke [with acute onset of neurological symptoms persisting for >24 h]; carotid endarterectomy or stent implantation; nontraumatic lower extremity amputation; or lower limb artery bypass surgery, angioplasty, or stent implantation) or death.

Statistical analysis. Data management and statistical analysis were performed using PASW Statistics 18 (SPSS, Inc., Chicago, Illinois) and the software environment for statistical computing R. Two-sided p values <0.05 were considered significant. Categorical variables are presented as percents of patients and were compared using chi-square tests. Continuous data are expressed as mean ± SD (or, in case of skewed distributions, as median [interquartile range]) and were compared using Mann-Whitney *U* tests. The associations between continuous variables were assessed using Spearman rank correlation testing.

Subjects were divided into 4 equally sized groups (quartiles) according to their total monocyte counts and monocyte subset counts. Kaplan-Meier survival curves were used to compare event-free survival (i.e., time until first occurrence of the primary or secondary endpoint) between groups. Participants with noncardiovascular death were censored at the time of death. The log-rank test was used to test the hypothesis that at least 1 of the survival curves differs from the others.

Cox proportional-hazards models were calculated to analyze the relationship of total monocyte and monocyte subset cell counts with event-free survival after adjustment for age and sex (model 1); further adjustment for systolic blood pressure, plasma cholesterol, diabetes, smoking, and body mass index (model 2); and finally further adjustment for estimated glomerular filtration rate, CRP, prevalent cardiovascular disease, and total leukocyte count (model 3).

To assess the predictive discrimination of each Cox model, we calculated the C-statistic (13), which provides the proportion of evaluable patient pairs that can be correctly classified by a model.

Results

Baseline characteristics. Monocyte subset analysis was intended in 999 HOM SWEET HOMe participants. Of these, 36 subjects were on systemic immunosuppressive medication, and 12 did not undergo planned coronary angiography after admission, leaving 951 subjects in the present analysis.

Baseline characteristics of these 951 patients are presented in Table 1. As expected, study participants had a high burden of risk factors and cardiovascular disease. Consequently, cardioprotective medication use was highly prevalent.

Study participants were followed for a mean of 2.6 ± 1.0 years; 3 patients were lost to follow-up. The predefined composite primary endpoint (cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke) occurred in 93 subjects, of whom 29 subjects had nonfatal acute myocardial infarctions, 24 had nonfatal nonhemorrhagic strokes, and 45 subjects died of cardiovascular causes. Study participants who experienced the primary endpoint were older, had more comorbidities, and had higher markers of systemic inflammation (Table 1).

Monocyte subset counts and cardiovascular risk factors. At study initiation, the mean monocyte count was 583 ± 211 cells/μl, of which 470 ± 179 cells were CD14++CD16− monocytes, 42 ± 24 cells were CD14++CD16+ monocytes, and 71 ± 37 cells were CD14+CD16++ mono-

Table 1 Baseline Characteristics of Study Participants

Variable	Total Cohort (n = 951)	No Primary EndPoint (n = 858)	Primary Endpoint (n = 93)	p Value
Age (yrs)	65.1 ± 10.3	64.7 ± 10.3	68.8 ± 9.1	<0.001
Body mass index (kg/m^2)	28.5 ± 4.6	28.4 ± 4.6	29.0 ± 5.0	0.396
Systolic BP (mm Hg)	147 ± 22	147 ± 22	150 ± 23	0.547
Diastolic BP (mm Hg)	82 ± 11	82 ± 10	81 ± 11	0.192
Hip circumference (cm)	103.5 ± 10.0	103.4 ± 9.7	104.2 ± 12.3	0.990
Waist circumference (cm)	101.5 ± 12.5	101.1 ± 12.1	105.6 ± 15.0	0.039
Creatinine (mg/dl)	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.5	<0.001
eGFR (ml/min/1.73 m^2)	75 ± 20	76 ± 19	69 ± 22	<0.001
CRP (mg/l)	1.9 (0.9–4.0)	1.9 (0.9–3.8)	3.7 (1.6–6.4)	<0.001
Total cholesterol (mg/dl)	187 ± 47	187 ± 47	181 ± 42	0.184
LDL cholesterol (mg/dl)	113 ± 41	114 ± 41	111 ± 37	0.561
HDL cholesterol (mg/dl)	49 ± 15	49 ± 15	48 ± 13	0.032
Triglycerides (mg/dl)	129 (93–182)	129 (92–182)	124 (96–182)	0.866
Leukocytes (1/μl)	6,709 ± 1,957	6,640 ± 1,931	7,344 ± 2,094	0.001
Total monocytes (1/μl)	583 ± 211	577 ± 211	633 ± 209	0.005
Women	305 (32.1%)	283 (33.0%)	22 (23.7%)	0.079
Smoking	122 (12.8%)	113 (13.2%)	9 (9.7%)	0.415
Diabetes mellitus	363 (38.2%)	312 (36.4%)	51 (54.8%)	0.001
Prevalent CVD	568 (59.7%)	496 (57.8%)	72 (77.4%)	<0.001
Prevalent CAD	513 (53.9%)	449 (52.3%)	64 (68.8%)	0.003
Cerebrovascular artery disease	94 (9.9%)	72 (8.4%)	22 (23.7%)	<0.001
Peripheral artery disease	67 (7.0%)	52 (6.1%)	15 (16.1%)	0.002
Beta-blockers	710 (74.7%)	639 (74.5%)	71 (76.3%)	0.906
ACE inhibitors	559 (58.8%)	494 (57.6%)	65 (69.9%)	0.067
Angiotensin receptor blockers	193 (20.3%)	169 (19.7%)	24 (25.8%)	0.372
Calcium-channel blockers	191 (20.1%)	169 (19.7%)	22 (23.7%)	0.653
Antiplatelet agents (%)	714 (75.1%)	645 (75.2%)	69 (74.2%)	0.912
Statins (%)	562 (59.1%)	501 (58.4%)	61 (65.6%)	0.399

Values are mean ± SD, median (interquartile range), or n (%).
ACE = angiotensin-converting enzyme; BP = blood pressure; CAD = coronary artery disease; CRP = C-reactive protein; CVD = cardiovascular disease; eGFR = estimated glomerular filtration rate; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

cytes. Associations of monocyte (subset) counts with traditional and nontraditional cardiovascular risk factors are specified in Table 2. Cell counts of all 3 monocyte subsets were correlated with traditional markers of inflammation (CRP and leukocyte counts). Additionally, CD14++CD16+ and CD14+CD16++ monocyte

Table 2 Correlation Coefficients of Monocyte (Subset) Counts With Traditional and Nontraditional Cardiovascular Risk Factors

	Total Monocytes		CD14++CD16− Monocytes		CD14++CD16+ Monocytes		CD14+CD16++ Monocytes	
Variable	r	p Value	r	p Value	r	p Value	r	p Value
Age	−0.019	0.564	−0.048	0.138	0.066	0.040	0.100	0.002
Body mass index	0.047	0.150	0.011	0.728	0.064	0.049	0.164	<0.001
Systolic BP	0.006	0.853	−0.019	0.563	0.044	0.174	0.137	<0.001
Diastolic BP	−0.042	0.196	−0.055	0.091	−0.013	0.682	0.057	0.078
Hip circumference	0.064	0.054	0.024	0.461	0.104	0.002	0.185	<0.001
Waist circumference	0.122	<0.001	0.086	0.010	0.128	<0.001	0.206	<0.001
Creatinine	0.084	0.010	0.058	0.075	0.152	<0.001	0.099	0.002
eGFR	−0.020	0.537	0.012	0.709	−0.145	<0.001	−0.089	0.006
CRP	0.213	<0.001	0.192	<0.001	0.233	<0.001	0.102	0.002
Total cholesterol	−0.048	0.136	−0.051	0.116	−0.045	0.163	−0.007	0.838
LDL cholesterol	−0.024	0.461	−0.025	0.451	−0.019	0.554	−0.007	0.819
HDL cholesterol	−0.150	<0.001	−0.148	<0.001	−0.131	<0.001	−0.052	0.112
Triglycerides	0.078	0.016	0.057	0.077	0.069	0.035	0.104	0.001
Leukocytes	0.629	<0.001	0.632	<0.001	0.443	<0.001	0.242	<0.001

Abbreviations as in Table 1.

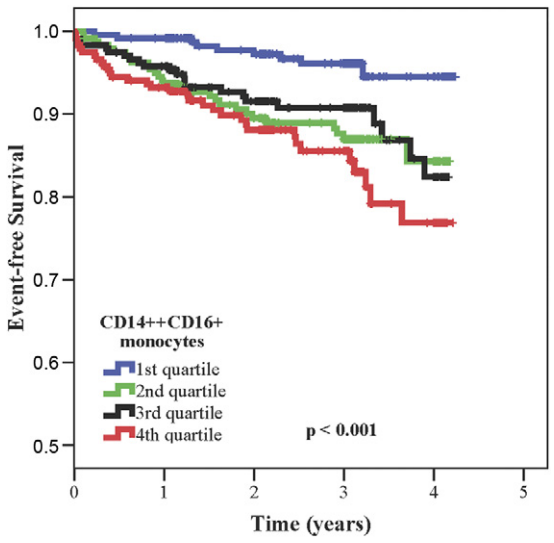

Figure 2 Relationship Between Quartiles of CD14++CD16+ Monocyte Counts and Event-Free Survival

Primary endpoint: cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke; Kaplan-Meier analysis with log-rank test.

counts showed weak, albeit significant, correlations with body mass index, renal dysfunction, age, and serum triglycerides.

Total monocyte and CD14++CD16− monocyte counts were higher in men than in women. In patients with diabetes, all monocyte subset counts were elevated (Online Table S1). Interestingly, prevalent cardiovascular disease was not associated with a shift in monocyte (subset) counts. Further associations between monocyte (subset) counts and intake of cardioprotective medication are listed in Online Table S1.

Total monocyte counts, monocyte subset counts, and cardiovascular outcomes. Patients who experienced events had a mean of 508 ± 178 CD14++CD16− monocytes/μl, 47 ± 22 CD14++CD16+ monocytes/μl, and 77 ± 43 CD14+CD16++ monocytes/μl. In contrast, patients who had uneventful follow-up had a mean of 466 ± 178 CD14++CD16− monocytes/μl ($p = 0.011$ compared with patients with events), 41 ± 24 CD14++CD16+ monocytes/μl ($p = 0.001$), and 70 ± 37 CD14+CD16++ monocytes/μl ($p = 0.105$).

After stratifying the study cohort by monocyte (subset) cell counts into quartiles, higher counts of total monocytes (log-rank test, $p = 0.010$), CD14++CD16− monocytes ($p = 0.024$), and CD14++CD16+ monocytes ($p < 0.001$), but not of CD14+CD16++ monocytes ($p = 0.158$), were univariately associated with the primary endpoint of cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke in Kaplan-Meier survival analysis (Fig. 2, Online Figs. S1A to S1C).

Similarly, higher CD14++CD16+ monocyte counts significantly predicted the occurrence of any cardiovascular event, pre-defined as a secondary endpoint, in Kaplan-Meier analysis ($p = 0.028$) (Fig. 3), whereas counts of total monocytes ($p = 0.098$), CD14++CD16− monocytes ($p = 0.066$), and CD14+CD16++ monocytes ($p = 0.062$) only tended to predict adverse cardiovascular outcomes.

In multivariate regression analysis, subjects with higher cell counts of CD14++CD16+ monocytes remained at higher risk for adverse outcomes after adjustment for age and sex (model 1); further adjustment for systolic blood pressure, plasma cholesterol, smoking, diabetes mellitus, and body mass index (model 2); and additional adjustment for renal function, CRP, prevalent cardiovascular disease, and total leukocyte counts (model 3), compared with individuals with the lowest counts of CD14++CD16+ monocytes (quartile 1) (Table 3).

In contrast to CD14++CD16+ cells, neither total monocyte counts nor counts of CD14++CD16− or CD14+CD16++ monocytes predicted adverse outcomes in fully adjusted models (Table 3).

In addition, Table 4 provides the C-statistics corresponding to the Cox analyses reported in Table 3. Discrimination overall improved for covariate adjustment. Regardless of adjustment, a specific benefit in terms of prediction performance was seen for CD14++CD16+ monocytes. Thus, the C-statistic analysis supports our conclusion that only CD14++CD16+ monocyte measurements improve risk prediction over the total monocyte count. To further illustrate that CD14++CD16+ monocytes might render prognostic information for cardiovascular outcome in addition to more conventional markers of inflammation, we cross-stratified the study cohort by the median of CD14++CD16+ monocyte counts and CRP higher than

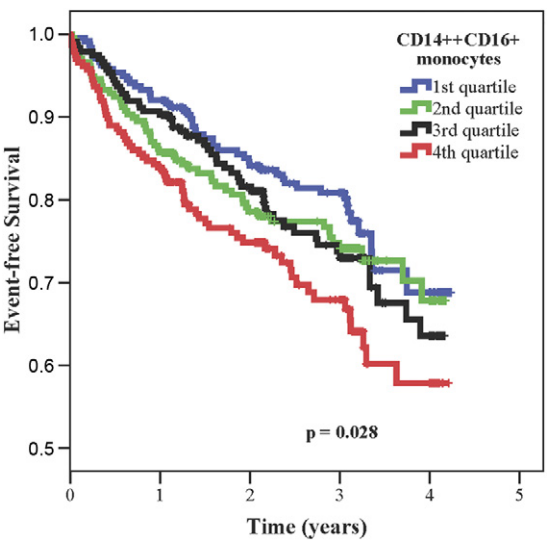

Figure 3 Relationship Between Quartiles of CD14++CD16+ Monocyte Counts and Event-Free Survival

Secondary endpoint: cardiovascular event; Kaplan-Meier analysis with log-rank test.

Table 3 Adjusted Cox Regression Analysis (Different Models) for Cardiovascular Events

Monocyte Subset	n	Events (n)	Model 1 HR	Model 1 95% CI	Model 1 p Value	Model 2 HR	Model 2 95% CI	Model 2 p Value	Model 3 HR	Model 3 95% CI	Model 3 p Value
Total monocytes											
Quartile 1	242	17	1.000			1.000			1.000		
Quartile 2	235	19	1.263	(0.656–2.432)	0.485	1.228	(0.636–2.368)	0.541	1.009	(0.515–1.976)	0.979
Quartile 3	236	24	1.528	(0.820–2.848)	0.182	1.487	(0.796–2.779)	0.213	1.229	(0.644–2.348)	0.532
Quartile 4	238	33	2.308	(1.280–4.162)	0.005	2.180	(1.195–3.977)	0.011	1.381	(0.681–2.801)	0.371
CD14++CD16− monocytes											
Quartile 1	238	18	1.000			1.000			1.000		
Quartile 2	238	21	1.186	(0.631–2.229)	0.597	1.182	(0.627–2.227)	0.605	1.051	(0.551–2.006)	0.880
Quartile 3	237	21	1.261	(0.671–2.369)	0.471	1.251	(0.665–2.352)	0.487	1.036	(0.540–1.988)	0.915
Quartile 4	238	33	2.144	(1.198–3.836)	0.010	2.039	(1.124–3.701)	0.019	1.259	(0.625–2.536)	0.520
CD14++CD16+ monocytes											
Quartile 1	238	9	1.000			1.000			1.000		
Quartile 2	238	28	3.191	(1.505–6.766)	0.002	3.121	(1.470–6.629)	0.003	3.218	(1.459–7.095)	0.004
Quartile 3	237	23	2.74	(1.267–5.926)	0.010	2.508	(1.154–5.454)	0.02	2.444	(1.074–5.563)	0.033
Quartile 4	238	33	3.899	(1.861–8.168)	<0.001	3.722	(1.770–7.829)	0.001	3.019	(1.315–6.928)	0.009
CD14+CD16++ monocytes											
Quartile 1	238	21	1.000			1.000			1.000		
Quartile 2	238	18	0.796	(0.424–1.495)	0.479	0.793	(0.421–1.494)	0.473	0.867	(0.456–1.649)	0.663
Quartile 3	237	25	1.225	(0.685–2.190)	0.494	1.193	(0.661–2.156)	0.558	1.138	(0.622–2.082)	0.675
Quartile 4	238	29	1.348	(0.767–2.372)	0.300	1.251	(0.705–2.220)	0.444	1.037	(0.571–1.883)	0.906

Model 1 includes monocyte (subset) counts, age, and sex; model 2 is further adjusted for systolic blood pressure, plasma cholesterol, diabetes, smoking, and body mass index; and model 3 is further adjusted for estimated glomerular filtration rate, C-reactive protein, prevalent cardiovascular disease, and total leukocyte count.
CI = confidence interval; HR = hazard ratio.

and lower than 2 mg/l, which corresponds to the CRP inclusion criterion of Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (14). Those subjects who had CD14++CD16+ monocyte counts higher than the median in conjunction with higher CRP levels had the worst outcomes (Online Fig. S1D) in Kaplan-Meier analysis ($p < 0.001$).

Discussion

The identification of a subset-specific involvement of monocytes in murine models of atherosclerosis was a breakthrough in cardiovascular research (15,16). Lately, specific immunomodulation of a distinct monocyte subset has been discussed as a promising therapeutic avenue in atherosclerosis (17). This notion is backed by the successful application of cell-targeted therapy in other fields of medicine, such as the introduction of rituximab in patients with lymphoma (18).

Unfortunately, advances in understanding of murine atherosclerosis are not paralleled by a better grasp of human pathology, given that the potential monocyte target subpopulation in atherosclerosis has not been convincingly identified yet. So far, most information derives from few cross-sectional studies: in a heterogenous cohort of 308 subjects, a shift toward CD16-positive monocytes was associated with the presence of coronary artery disease (19). Later, small cross-sectional studies linked high counts of CD16-positive monocytes with the presence of vulnerable atherosclerotic plaques in patients with stable angina pectoris (20) and with fibrous cap thickness in patients with unstable angina pectoris (21). Of note, none of these studies distinguished intermediate CD14++CD16+ monocytes from nonclassical CD14+CD16++ monocytes (as discussed in detail previously [22,23]). Consequently, so far, the respective roles of CD14++CD16+ and CD14+CD16++ monocytes in human atherosclerosis have not been fully discerned. Against this background, large prospective studies of monocyte subsets and cardiovascular outcomes have been demanded recently (17).

We previously set out to analyze the relationship between subset-specific monocyte counts and adverse cardiovascular outcomes in selected patient groups. Initially, we focused our analysis on patients with CKD receiving dialysis treatment (10,24,25), reasoning that this highest cardiovascular risk group would allow us to gather information in a limited sample size because of the high event rate. This allowed us to demonstrate CD14++CD16+ monocytes to be independent predictors of cardiovascular events in dialysis patients (10). However, a drawback of this approach was the observed strong shift in monocyte subsets toward CD16-

Table 4 C-Statistics Corresponding to the Cox Regression Analyses Reported in Table 3

Monocyte Subset	Model 1	Model 2	Model 3
Total monocytes	0.666	0.705	0.734
CD14++CD16− monocytes	0.661	0.701	0.732
CD14++CD16+ monocytes	0.699	0.723	0.748
CD14+CD16++ monocytes	0.666	0.698	0.731

positive cells compared with healthy controls (10), precluding a translation of these results to non-CKD populations.

Next we prospectively analyzed monocyte heterogeneity in subjects with earlier stages of CKD not requiring dialysis. At baseline, these patients had CD14++CD16+ cell counts between those of subjects with preserved renal function and those of patients with CKD receiving dialysis. Nonetheless, higher CD14++CD16+ monocyte counts again predicted cardiovascular outcomes in these patients (8).

To test the validity of these findings in a broad patient group not affected by the CKD-associated monocyte subset shift, we recruited subjects from the general population at cardiovascular risk, who were referred for elective coronary angiography. Our HOM SWEET HOMe study, the largest prospective cohort study on monocyte heterogeneity so far, thereby comprised a representative sample of subjects at cardiovascular risk with either prevalent coronary artery disease or a high burden of risk factors. At baseline, cell counts of all 3 monocyte subsets were correlated with traditional as well as nontraditional cardiovascular risk factors. Interestingly, CD14++CD16+ monocytes seem particularly to integrate cardiovascular risk burden, a notion that is supported by earlier data (26). Of most interest, CD14++CD16+ monocytes were the only independent predictors of adverse cardiovascular outcomes after full adjustment for traditional and nontraditional cardiovascular risk factors in the HOM SWEET HOMe study.

With regard to our fully adjusted Cox regression analysis, one might suspect a threshold effect of CD14++CD16+ monocyte counts, because in this model, quartiles 2 to 4 predicted adverse outcomes to a similar extent. A comparable conclusion could be also drawn from our previous study on CD14++CD16+ monocytes and survival in non-dialysis-dependent patients with CKD; here, the Kaplan-Meier curve of the second tertile clearly separated from the first tertile but ran close and parallel to the third tertile (8).

However, this concept is merely a hypothesis originating a posteriori from the present dataset. Future studies with predefined cell count cutoff values should therefore prospectively test this idea.

Circumstantial evidence suggesting a role of CD16-positive monocytes in atherosclerosis supports our present findings. Mechanistically, the significance of CD16-positive monocytes in atherosclerosis is emphasized by their proinflammatory capacity (6,27) along with their endothelial affinity (28). Their preferential adherence to activated endothelial cells (29) together with their potential to secrete interleukin-6, matrix metalloproteinase-9, and chemokine (C-C motif) ligand 2 and to attract T-lymphocytes and further monocytes (30) should be considered proatherosclerotic features.

Furthermore, the role of CD16-positive monocytes in atherosclerotic vascular disease is underscored by their association with subclinical atherosclerosis (31).

Of note, among CD16-positive monocytes, intermediate CD14++CD16+ monocytes can be viewed as particularly proatherogenic, as they selectively express C-C chemokine receptor type 5 (6,8,29), which has been associated with atherosclerosis in experimental (1) and large epidemiological (32–34) studies. Moreover, subset-specific high reactive oxygen species production and CD74 expression predispose CD14++CD16+ monocytes to propagate atherogenesis (6). These proatherogenic virtues are further extended by the proangiogenic capacity of intermediate CD14++CD16+ monocytes (6,35), linking them to potential plaque neovascularization as an important component in advanced stages of atherosclerosis (36).

Our study has several strengths that support the significance of CD14++CD16+ monocytes in cardiovascular disease.

First, the large cohort size provides firm ground for our results. Second, our monocyte analysis protocol, which has been validated (6) against a suggested reference method (37), reliably distinguishes between intermediate CD14++CD16+ and nonclassical CD14+CD16++ monocytes (6). Third, analysis of monocyte subsets was performed blinded to baseline characteristics, and endpoints were adjudicated blinded to the distribution of monocyte subpopulations.

As the major limitation, our study cannot prove causality, because of its nature as a cohort study.

However, our study provides an extensive dataset on human monocyte heterogeneity in atherosclerosis, a field in which a plethora of murine data on monocyte heterogeneity contrast with the paucity of human data. Considering the limitations of murine models (1,2,38), clinical studies have been demanded to fill this knowledge gap (17).

One might argue that our present findings are in contradiction to results from murine studies that favor a proatherosclerotic role of $Ly6C^{high}$ monocytes, conventionally regarded as counterpart of human CD14++CD16− monocytes, over $Ly6C^{low}$ monocytes, which are conventionally viewed as homologues of both human CD14++CD16+ and CD14+CD16++ monocytes (39).

However, this concept has recently been refined by Cros et al. (40), who reported that human CD14++CD16− and CD14++CD16+ monocytes both cluster together with murine $Ly6C^{high}$ monocytes, whereas CD14+CD16++ monocytes cluster together with $Ly6C^{low}$ monocytes. This finding is of great importance, because it could help reconcile murine studies with our present findings as well as with previous human studies that favored CD16-positive monocytes in cardiovascular disease.

In addition, 2 aspects deserve special consideration when interpreting results from murine studies. First, most murine studies on monocyte heterogeneity in atherosclerosis analyzed only 2 murine monocyte subsets: $Ly6C^{high}$ and $Ly6C^{low}$ cells (16). Second, many murine studies are performed in apolipoprotein $E^{-/-}$ mice on an atherogenic diet, which accordingly show massively elevated cholesterol levels

and a very profound rise of $Ly6C^{high}$ cells (up to 14-fold in individual studies [15]) without concomitant changes in $Ly6C^{low}$ monocyte counts (15). In humans, such extreme alterations are restricted to rare disease states such as familial hypercholesterolemia, in which a significant increase of CD14++CD16− monocytes has been reported (41). In contrast, wild-type mice on an atherogenic diet display significant increases neither of $Ly6C^{high}$ nor of $Ly6C^{low}$ cell counts and no atherosclerotic lesions despite persisting hypercholesterolemia (15). Accordingly, in a previous study in healthy volunteers, we did not observe associations between CD14++CD16− monocytes and cholesterol levels or subclinical atherosclerosis (31).

Taken together, we believe that our present results do not conflict with findings from earlier murine studies, if critical attention is paid to the respective experimental setting.

Ideally, studies on monocyte heterogeneity in other experimental animals, such as pigs, whose immune system is evolutionary closer to that of humans and which show a comparable monocyte subset distribution (42), will complement our existing knowledge in the future.

Conclusions

We report an association of CD14++CD16+ monocytes with cardiovascular events in subjects from the general population referred for elective coronary angiography. It remains to be proven whether the CD14++CD16+ monocyte subset represents a target cell population for new therapeutic strategies in atherosclerosis.

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Key Words: atherosclerosis ■ cardiovascular disease ■ CD14 ■ CD16 ■ monocytes.

APPENDIX

For a supplementary table and a figure and its legend, please see the online version of this article.

Beitrag der Mitautoren für die Arbeit:

Rogacev KS, Cremers B, Zawada AM, Seiler S, Binder N, Ege P, Große-Dunker G, Heisel I, Hornof F, Jeken J, Rebling NM, Ulrich C, Scheller B, Böhm M, Fliser D, Heine GH. CD14++CD16+ monocytes independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. J Am Coll Cardiol. 2012 Oct 16;60(16):1512-20.

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SuperSAGE evidence for $CD14^{++}CD16^{+}$ monocytes as a third monocyte subset

Adam M. Zawada, Kyrill S. Rogacev, Björn Rotter, Peter Winter, Rolf-R. Marell, Danilo Fliser and Gunnar H. Heine

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SuperSAGE evidence for CD14^{++}CD16^{+} monocytes as a third monocyte subset

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Monocytes are a heterogeneous cell population with subset-specific functions and phenotypes. The differential expression of CD14 and CD16 distinguishes classical CD14^{++}CD16^{-}, intermediate CD14^{++}CD16^{+}, and nonclassical CD14^{+}CD16^{++} monocytes. Current knowledge on human monocyte heterogeneity is still incomplete: while it is increasingly acknowledged that CD14^{++}CD16^{+} monocytes are of outstanding significance in 2 global health issues, namely HIV-1 infection and atherosclerosis, CD14^{++}CD16^{+} monocytes remain the most poorly characterized subset so far. We therefore developed a method to purify the 3 monocyte subsets from human blood and analyzed their transcriptomes using SuperSAGE in combination with high-throughput sequencing. Analysis of 5 487 603 tags revealed unique identifiers of CD14^{++}CD16^{+} monocytes, delineating these cells from the 2 other monocyte subsets. Gene Ontology (GO) enrichment analysis suggests diverse immunologic functions, linking CD14^{++}CD16^{+} monocytes to Ag processing and presentation (eg, *CD74*, *HLA-DR*, *IFI30*, *CTSB*), to inflammation and monocyte activation (eg, *TGFB1*, *AIF1*, *PTPN6*), and to angiogenesis (eg, *TIE2*, *CD105*). In conclusion, we provide genetic evidence for a distinct role of CD14^{++}CD16^{+} monocytes in human immunity. After CD14^{++}CD16^{+} monocytes have earlier been discussed as a potential therapeutic target in inflammatory diseases, we are hopeful that our data will spur further research in the field of monocyte heterogeneity. (*Blood*. 2011;118(12): e50-e61)

Introduction

Monocytes are cornerstones of the immune system linking innate and adaptive immunity and are critical drivers in many inflammatory diseases. They are known to originate from a common myeloid precursor in the BM and give rise to tissue macrophages and dendritic cells (DCs).[1,2]

As diverse as monocyte function is their immunophenotype. Based on the differential expression of the lipopolysaccharide (LPS) receptor CD14 and the FcγIIIR CD16, 2 subpopulations were initially defined (CD14^{++}CD16^{-} monocytes and CD16-positive monocytes).[3]

However, considerable heterogeneity within the minor CD16-positive monocyte population does exist, which had been neglected until 2003, when Ancuta et al reported that CD16-positive monocytes can be further subdivided into phenotypically distinct CD14^{++}CD16^{+} and CD14^{+}CD16^{++} cells.[4]

The recently updated classification of monocyte heterogeneity follows this differentiation of CD16-positive monocytes into CD14^{++}CD16^{+} and CD14^{+}CD16^{++} monocytes and acknowledges the existence of 3 monocyte subsets, that is, classical monocytes (CD14^{++}CD16^{-}), intermediate monocytes (CD14^{++}CD16^{+}), and nonclassical monocytes (CD14^{+}CD16^{++}).[5]

As reviewed recently, the intermediate monocyte subset remains poorly characterized because of the fact that most clinical and experimental studies either ignored these cells, or analyzed intermediate and nonclassical monocytes as a single subset.[6]

Although neglected in earlier studies, the intermediate monocytes are of major clinical importance: first, we found that elevated CD14^{++}CD16^{+} monocyte counts independently predict adverse outcome in patients at high cardiovascular risk.[7,8] Moreover, a host of data suggests that intermediate monocytes are of significance in HIV-1 infection,[9,10] given that—unlike classical and nonclassical monocytes—they selectively express CCR5, the coreceptor for HIV-1.[4,8]

Although CD14^{++}CD16^{+} monocytes show an intermediate phenotype in many chemokine receptors (eg, CCR2 and CX3CR1), they can be clearly distinguished by distinct identifiers from CD14^{++}CD16^{-} and CD14^{+}CD16^{++} monocytes, such as the subset-specific surface expression of CCR5[4,8] and of angiotensin-converting enzyme (ACE; CD143).[11] Admittedly, so far, proof for the existence of the intermediate monocyte subset derives mainly from flow-cytometrical surface expression analysis. Flow cytometry is limited in its analytical capacity, whereas gene expression profiling provides a much more profound characterization. In line, previous knowledge on the heterogeneity of 2 major subsets of CD14^{++}CD16^{-} and CD16-positive monocytes has recently been extended by gene expression profiling.[12-15]

However, no data on the gene expression profile of the intermediate CD14^{++}CD16^{+} monocyte subset exist so far because of the technically challenging procedure of separating CD16-positive monocytes into CD14^{++}CD16^{+} and CD14^{+}CD16^{++} cells.

To unravel heterogeneity of CD16-positive monocytes, we set out to develop a method to separate the 3 human monocyte subsets, and analyzed them in combination with an improved version of the SuperSAGE method[16] to characterize the monocyte subsets' transcriptomes. This method provides digital transcriptome data in a very high resolution, as rare transcripts which remain invisible on microarrays can also be exactly quantified.

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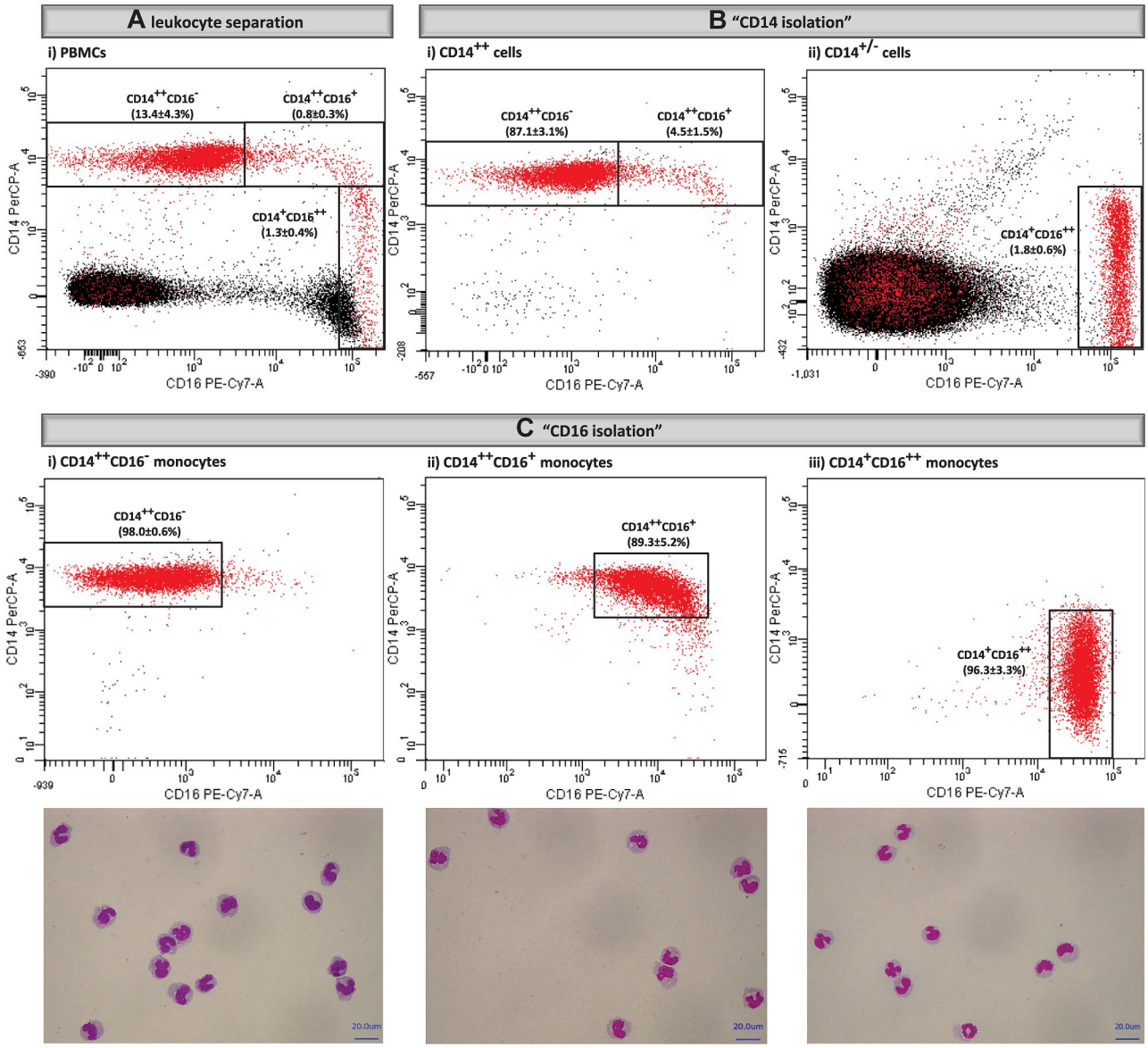

Figure 1. Purification of human monocyte subsets. (A) PBMCs were isolated by Ficoll-Paque and stained with anti-CD86, anti-CD14, and anti-CD16; CD86-positive cells (monocytes) are red, whereas CD86-negative (nonmonocytic) cells are black (i). NB: Percentages refer to CD86-positive monocyte subsets among all PBMCs, excluding CD86-negative cells (eg, CD16-positive NK cells and neutrophils) which protrude into the $CD14^{+}CD16^{++}$ monocyte gate in this dot plot. (B) After depletion of NK cells and neutrophils (CD16-positive nonmonocytic cells) using CD56 and CD15 MicroBeads (not shown), negatively isolated cells were separated into $CD14^{++}$ (i) and $CD14^{+/-}$ cells (ii) using FITC-conjugated anti-CD14 Ab and accordingly anti-FITC MultiSort MicroBeads. (C) Both fractions were incubated with CD16 MicroBeads to separate $CD14^{++}$ cells into $CD14^{++}CD16^{-}$ (i) and $CD14^{++}CD16^{+}$ monocytes (ii), and to purify $CD14^{+}CD16^{++}$ monocytes (iii) from $CD14^{+/-}$ cells. Top line: Flow cytometric analysis; bottom line: microscopic images (Keyence BZ-8000J [Keyence Deutschland] equipped with a Plan Apo 60×/1.40 oil objective lens [Nikon], magnification 30×, room temperature) after cytospin and May-Grünwald-Giemsa staining. Representative examples from 12 independent experiments are shown. In each dot plot, subset-specific percentages of monocytic cells among total cells are shown as means ± SD.

Methods

Isolation of human monocyte subsets

Twelve healthy volunteers were recruited for the study. All participants gave written informed consent in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Saarland University Hospital.

EDTA-anticoagulated blood was drawn by venopuncture and PBMCs were immediately isolated by Ficoll-Paque (Lymphocyte Separation Medium; PAA) gradient density centrifugation. Subsequently, NK cells and neutrophils were depleted using CD56 and CD15 MicroBeads (NonMonocyte Depletion Cocktail, $CD16^{+}$ Monocyte Isolation Kit; Miltenyi Biotec) according to the manufacturer's instructions. From these negatively isolated cells, human monocyte subsets were isolated according to their different CD14 and CD16 expression. First, the cells were incubated with FITC-conjugated anti-CD14 Ab (Miltenyi Biotec) and then with anti-FITC MultiSort MicroBeads (Anti-FITC MultiSort Kit; Miltenyi Biotec) to separate the pre-enriched monocytes in $CD14^{++}$ and $CD14^{+/-}$ cells. After release of anti-FITC MultiSort MicroBeads, both fractions were incubated with CD16 MicroBeads (Miltenyi Biotec) and separated into 3 monocyte subsets (compare Figure 1 for a representative example). All steps of monocyte subset isolation were performed at 4°C. After each single step, purity was analyzed flow-cytometrically.

RNA isolation and construction of SuperSAGE libraries

Isolated monocyte subsets were immediately lysed, frozen in liquid nitrogen, and stored at −80°C until RNA preparation. Total RNA from lysates was isolated

using the RNeasy Micro Kit (QIAGEN) including DNase treatment. From 12 donors, same aliquots of each sample were matched for SuperSAGE, leading to a total RNA amount of 12.0 μg ($CD14^{++}CD16^{-}$), 1.2 μg ($CD14^{++}CD16^{+}$), and 1.6 μg ($CD14^{+}CD16^{++}$), respectively.

SuperSAGE libraries were produced at GenXPro GmbH essentially as described by Matsumura et al,[17] but with the implementation of GenXPro PCR-bias-proof technology to distinguish PCR copies from original tags. Quality assessment of the tags was performed according to Qu et al[18] to reduce sequencing errors and artificial tag sequences. Tags were counted using the "GenXProgram." Likelihoods for differential expression of the tags were calculated according to Audic and Claverie.[19] The 26-bp tags were annotated to the human-refseq-database (National Center for Biotechnology Information [NCBI] from September 2010). Tags with no hits were annotated to all other mammalian refseq-mRNA databases (NCBI from September 2010). Tags matching to the same transcript were summed up to define a "transcript frequency"; *P* values for differential expression were also calculated based on the transcript frequencies. All SuperSAGE data are available in the Gene Express Omnibus (GEO) under accession number GSE30811.

Gene ontology information

Gene ontology (GO) information was obtained from www.GeneOntology.org for the refseq-mRNA entries. *P* values describing the likelihood for enrichment of GO terms were calculated by the Fisher exact test, based on transcripts that were differentially expressed with a $P < 10^{-10}$.[20]

Flow cytometric analysis

Via flow cytometry (FACS Canto II with CellQuest Software; BD Biosciences) monocyte subsets were identified according to our previously published gating strategy.[7] Briefly, monocytes were gated in a side scatter/CD86 dot plot, identifying monocytes as CD86-positive cells with monocyte scatter properties. Subpopulations of $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{+}CD16^{++}$ monocytes were distinguished by their surface expression pattern of the LPS receptor CD14 and the FcγIIIR CD16.

For validation, we compared this gating strategy to an alternative strategy which was recently suggested by Heimbeck et al.[21] As summarized in supplemental Figure 1 and supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the article), both gating strategies yielded virtually identical results.

For validation of SuperSAGE results, surface expression of different Ags was analyzed in 10 healthy subjects via a whole-blood assay using 100 μL of EDTA anticoagulated blood. Surface expression was quantified as median fluorescence intensity (MFI) and standardized against coated fluorescent particles (SPHEROTM; BD Biosciences). Histograms were created with FCS Express Version 3 Software (De Novo Software). Abs used in this study are summarized in supplemental Table 2.

Measurement of ROS

Isolated PBMCs were incubated with the cell-permanent carboxy-H_2DFFDA (Invitrogen) in a concentration of 10μM for 15 minutes at 37°C and 5% CO_2. Afterward, cells were stained with anti-CD14, anti-CD16, and anti-CD86. The intracellular reactive oxygen species (ROS) levels within the monocyte subsets were determined as MFI by flow cytometric analysis.

Phagocytosis assay

Fluoresbrite Yellow Green (YG) Carboxylate Microspheres (0.75 μm; Polysciences) were opsonized with heterologous serum (diluted to 50% with Krebs Ringers PBS) for 30 minutes at 37°C and adjusted to 10^8 particles/mL. One hundred microliters of citrate anticoagulated whole blood was mixed with 10 μL of opsonized particles and incubated with gentle shaking for 30 minutes at 37°C. Control samples were incubated at 4°C. Samples were stained as described in "Flow cytometric analysis," and counts of FITC-positive cells were determined flow-cytometrically in each monocyte subset.

In vitro angiogenesis assay

Angiogenic activity of monocyte subsets was assessed using a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma (Matrigel Basement Membrane Matrix; BD Biosciences). Matrigel was thawed at 4°C overnight and distributed on 24-well plates (200 μL/well). Afterward, Matrigel was allowed to solidify at 37°C for at least 1 hour. In 3 independent assays, freshly isolated monocyte subsets from healthy individuals were seeded on the polymerized matrix at a density of 1×10^5 cells/well and stimulated with human VEGF (10 ng/mL; Miltenyi Biotec). Cells were cultivated at 37°C in 5% CO_2. After 3 days, formation of tube-like structures was microscopically analyzed. HUVECs were used as positive control.

Proliferation assay

The monocyte subset-specific ability to induce $CD4^+$ T-cell proliferation was analyzed by measuring the cytoplasmic dilution of CFDA-SE (Vybrant CFDA-SE Cell Tracer Kit; Invitrogen). In detail, freshly isolated monocyte subsets from 5 healthy subjects were cultivated overnight in 96-well plates at a density of 5×10^4 cells/well in the presence of 2.5 μg/mL staphylococcal enterotoxin B (SEB; Sigma-Aldrich).

Within 24 hours, autogenic $CD4^+$ T cells were isolated using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). Purity of isolated $CD4^+$ T cells was > 97%. $CD4^+$ T cells were labeled with 5μM CFDA-SE at 37°C. After 10 minutes, RPMI (+5% FCS) was added in excess to stop labeling, followed by 2 washing steps. Afterward, 2×10^5 $CD4^+$ T cells were added to stimulated monocytes. After 3 days, counts of proliferating T cells were analyzed flow-cytometrically by measuring CFDA-SE dilution, identifying T cells by anti-CD3 APC. All experiments were performed in duplicate. For negative control, labeled T cells were cultivated without monocytes and without SEB, respectively.

Results

Generation of SuperSAGE libraries

Human monocyte subsets were purified with MACS technology based on the differential CD14 and CD16 expression, yielding a purity of 98.0% ± 0.6% for $CD14^{++}CD16^{-}$ monocytes, 89.3% ± 5.2% for $CD14^{++}CD16^{+}$ monocytes, and 96.3% ± 3.3% for $CD14^{+}CD16^{++}$ monocytes, with a mean viability of > 97% for all subsets (Figure 1).

Three independent SuperSAGE libraries were generated from isolated human monocyte subsets. After eliminating incomplete reads, twin ditags, ditags without complete library-identification DNA linkers, and tags which were detected only once (singletons), the total number of SuperSAGE tags was 5 487 603, comprising three 610 673 tags from $CD14^{++}CD16^{-}$ monocytes, 1 189 952 tags from $CD14^{++}CD16^{+}$ monocytes, and 686 978 tags from $CD14^{+}CD16^{++}$ monocytes. These tags accounted for 154 313 unique sequences (UniTags) in the combined libraries, of which 112 873 (73.1%) matched sequences corresponding to human refseq-RNA database entries and were considered for further analysis. The remaining UniTags hit either to a nonhuman database (4773 UniTags [3.1%]), or did not hit to the refseq databases (36 667 UniTags [23.8%]), and thus represented UniTags for mitochondrial transcripts, nonannotated transcripts, and sequencing artifacts.

Monocyte subset-specific genes

The 112 873 UniTags which matched to the human database represented 26 951 transcripts in the combined libraries (Figure 2). The complete list of identified transcripts in the monocyte subsets is shown in supplemental Table 5. Comparing gene expression profile in pairs of monocyte subsets, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes showed the highest similarity (Figure 2). Of 19 337 transcripts which were identified in the combined $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$

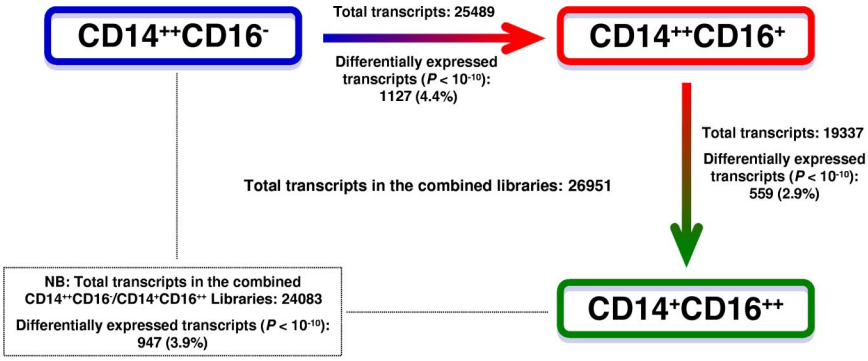

Figure 2. Schematic representation of differences in gene expression between the 3 monocyte subsets. For each pair of monocyte subsets, the number of total transcripts, and the number of differentially expressed transcripts that reached a level of significance of $P < 10^{-10}$ are depicted. Statistical analysis was performed according to Audic and Claverie.[19]

libraries, 559 (2.9%) were differentially expressed with a $P < 10^{-10}$, among which 258 genes were up-regulated in $CD14^{++}CD16^{+}$ monocytes, and 301 genes in $CD14^{+}CD16^{++}$ monocytes. $CD14^{++}CD16^{-}$ and $CD14^{++}CD16^{+}$ monocytes could be distinguished from each other by 1127 of 25 489 identified transcripts (4.4%), whereas $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{++}$ monocytes differed in the expression of 947 of 24 083 (3.9%) transcripts when applying the same cutoff value ($P < 10^{-10}$).

Comparison of SuperSAGE versus microarray

For validation of the SuperSAGE results, we compared our data with the previously published microarray results by Ancuta et al.[12] As the latter study did not distinguish between $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes, we recalculated the tag counts of CD16-positive monocytes in the present study by pooling data of $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes in a 1:1.6 ratio, based on the relative frequencies of these cells within the PBMCs.

Table 1 lists the 30 most differentially expressed genes between $CD14^{++}CD16^{-}$ and CD16-positive monocytes according to Ancuta et al.[12] Of note, microarray analysis and SuperSAGE found a strikingly similar expression pattern, even though some quantitative differences in the magnitude of fold-change values remain because of the different methods applied.

Table 1. Comparison of SuperSAGE results with microarray data by Ancuta et al[12]

	Ancuta et al[12]	SuperSAGE			
Gene symbol	$CD16^{+}/CD16^{-}$ ratio	$CD14^{++}CD16^{-}$ mo TPM	CD16-positive mo TPM	Ratio	*P*
S100A12	0.1	29.6	0.4	0.0	8.0×10^{-22}
VCAN	0.2	905.4	36.1	0.0	0
CD14	0.2	1701.9	240.0	0.1	0
CD36	0.2	106.1	9.4	0.1	9.6×10^{-56}
CD99	0.2	181.7	32.9	0.2	2.0×10^{-67}
METTL9	0.3	401.9	60.9	0.2	1.6×10^{-163}
CSF3R	0.3	410.7	77.0	0.2	8.7×10^{-147}
PLBD1	0.3	945.0	159.8	0.2	0
MS4A6A	0.3	852.5	105.3	0.1	0
ITGAM	0.3	111.3	27.4	0.2	2.0×10^{-33}
SELL	0.3	973.8	103.5	0.1	0
CRTAP	0.4	375.8	109.8	0.3	6.4×10^{-92}
S100A9	0.4	4796.3	627.8	0.1	0
GPX1	0.4	1038.6	428.5	0.4	1.7×10^{-156}
PLP2	0.4	142.1	36.4	0.3	1.2×10^{-40}
LST1	2.5	128.2	683.2	5.3	4.5×10^{-271}
IFITM3	2.5	173.9	923.9	5.3	0
SOD1	2.5	26.6	43.8	1.6	4.8×10^{-4}
IFITM2	2.5	578.0	2932.7	5.1	0
NAP1L1	2.8	37.1	82.7	2.2	7.2×10^{-12}
CSF1R	2.8	256.2	1227.6	4.8	0
MS4A7	2.8	387.5	1114.8	2.9	9.6×10^{-235}
TCF7L2	3.0	10.5	91.4	8.7	6.4×10^{-50}
TAGLN	3.2	6.1	54.1	8.9	1.8×10^{-30}
HMOX1	3.5	118.8	750.4	6.3	0
IFITM1	4.5	2.5	26.4	10.6	1.0×10^{-16}
SIGLEC10	4.8	7.8	55.4	7.1	4.3×10^{-28}
MTSS1	5.7	14.7	147.4	10.0	1.2×10^{-84}
CDKN1C	18.4	8.3	1712.4	206.3	0
FCGR3A	20.1	27.1	1911.6	70.5	0

$P < 10^{-310}$ is denoted as 0.
TPM indicates tags per million; and mo, monocytes.

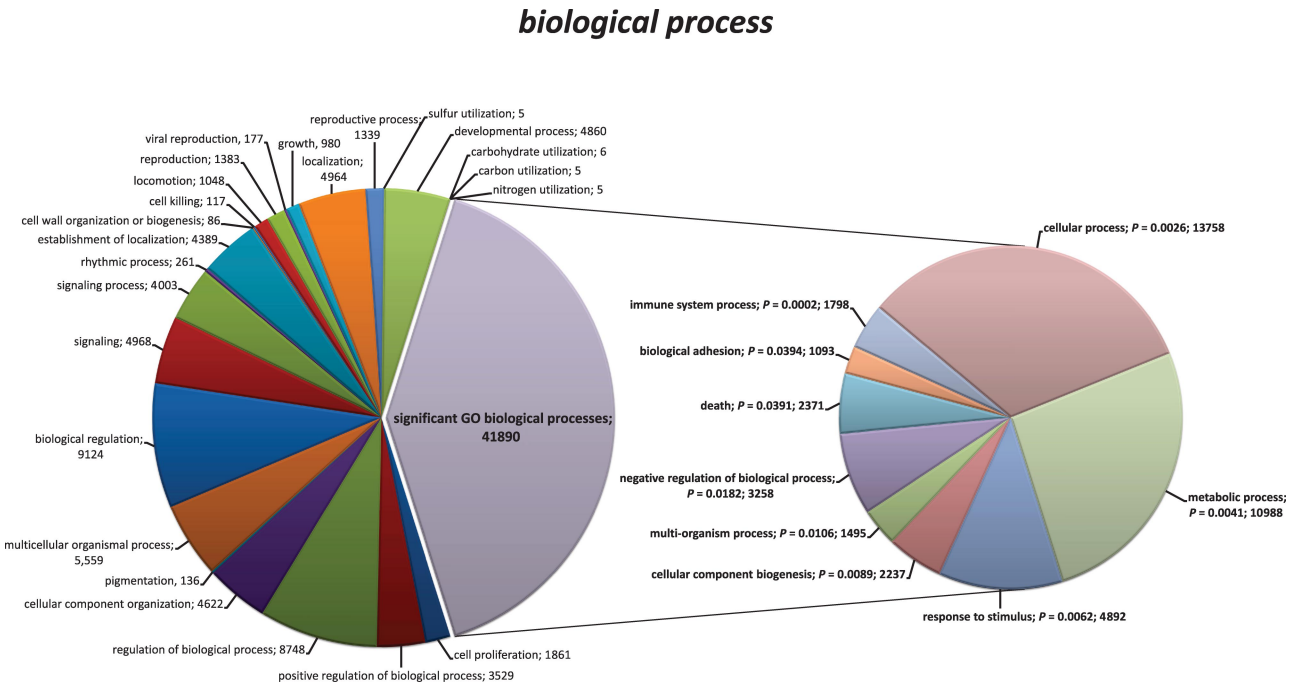

Figure 3. Pie charts of the functional annotation of identified transcripts from CD14^{++}CD16^{+} and CD14^{+}CD16^{++} monocytes based on GO categorization (biological process). Using GO categories, transcripts of CD14^{++}CD16^{+} and CD14^{+}CD16^{++} monocytes were categorized by the function of their encoded protein products. GO terms with statistical significant difference in gene expression are highlighted and projected into the right pie chart. Fisher exact test (2-tailed test) was used to compare groups for significant enrichment of particular GO classes. Numbers of transcripts for each GO term are given. All data are presented at level 2 GO categorization.

Genes differentially expressed between CD14^{++}CD16^{+} and CD14^{+}CD16^{++} monocytes

Because 4 earlier studies analyzed gene expression between CD14^{++}CD16^{-} and CD16-positive monocytes,[12-15] we deliberately chose to focus our data presentation on differences between the 2 subsets of CD16-positive monocytes (CD14^{++}CD16^{+} and CD14^{+}CD16^{++}), which had been neglected before. Comparisons between CD14^{++}CD16^{-} versus CD14^{++}CD16^{+} monocytes and CD14^{++}CD16^{-} versus CD14^{+}CD16^{++} monocytes—which are not the major topic of the present report—are summarized in supplemental Tables 3 and 4.

Those 30 genes defined by a 26-hit tag which differed most significantly between CD14^{++}CD16^{+} and CD14^{+}CD16^{++} monocytes are presented in Table 2. CD14^{++}CD16^{+} monocytes were distinguished from CD14^{+}CD16^{++} monocytes by significant higher expression of genes linked to defense against microbial pathogens (*LYZ*, *S100A8*, *CD14*, *S100A10* [for the ease of legibility, gene symbols are given in "Results"; gene titles are listed in supplemental Table 5]) and MHC II–restricted Ag processing and presentation (*HLA-DRA*, *CD74*, *IFI30*, *HLA-DPB1*, *CPVL*). In contrast, CD14^{+}CD16^{++} monocytes expressed higher levels of genes connected to MHC I–restricted processes (*HLA-B*, *B2M*) to migration and transendothelial motility (*LSP1*, *LYN*, *CFL1*, *MYL6*) and to cell-cycle progression (*CDKN1C*, *STK10*).

Biologic and functional differences between CD14^{++}CD16^{+} and CD14^{+}CD16^{++} monocytes

Gene ontology (GO) information was obtained from www.GeneOntology.org for the refseq-mRNA entries. *P* values describing the likelihood for enrichment of GO terms (enrichment *P* values) were calculated by the Fisher exact test, based on transcripts that were differentially expressed between the CD16-positive monocytes with a $P < 10^{-10}$. A total of 15 737 transcripts were annotated to biological process (Figure 3, with GO terms showing a significant difference [enrichment $P < .05$] highlighted), 16 260 transcripts to molecular function (supplemental Figure 2), and 16 615 to cellular component (supplemental Figure 3). In the following, those 4 GO terms within the biological process which showed the most pronounced differences in gene expression (according to *P* values) are further characterized.

Immune system process

Several genes involved in immune response were differently expressed between CD14^{++}CD16^{+} and CD14^{+}CD16^{++} monocytes, pointing to distinct functions of these monocyte subsets in immune defense.

In detail, transcripts up-regulated ($P < 10^{-10}$) in CD14^{++}CD16^{+} monocytes included those linked to the innate immune response (eg, *CD14*, *CFP*, *NCF2*) and to MHC II–restricted processing and presentation in adaptive immune response (eg, *IFI30*, *CD74*, and further *HLA-DR* paralogues).

In contrast, innate immune genes up-regulated in CD14^{+}CD16^{++} monocytes included those coding for complement factor D (*CFD*), signaling lymphocytic activation molecule family member 7 (*SLAMF7*), and GTP cyclohydrolase 1 (*GCH1*). Within adaptive immune response, CD14^{+}CD16^{++} monocytes predominantly expressed, for example, sialophorin (*SPN*), protein kinase C, δ (*PRKCD*), *STAT6*, and MHC I–associated mechanisms (eg, *B2M*, *HLA-B*, *HLA-E*, and *PSMB9*).

Moreover, CD14^{++}CD16^{+} and CD14^{+}CD16^{++} showed different expression of genes related to activation of monocytes, with higher expression of allograft inflammatory factor 1 (*AIF1*), *TGFB1*, *CD93*, and protein tyrosine phosphatase, nonreceptor type 6 (*PTPN6*) in CD14^{++}CD16^{+} monocytes, and higher expression of CD16, yes-1 Yamaguchi sarcoma viral-related oncogene homolog (*LYN*), heme oxygenase 1 (*HMOX1*), and Kruppel-like factor 6 (*KLF6*) in CD14^{+}CD16^{++} monocytes.

Table 2. Comparison of top genes differentially expressed between intermediate CD14^{++}CD16^{+} and nonclassical CD14^{+}CD16^{++} monocytes

Gene symbol	Gene title	Tag sequence	CD14^{++}CD16^{+} TPM	CD14^{+}CD16^{++} TPM	*P*	Fold change	Protein function
Top genes up-regulated in intermediate CD14^{++}CD16^{+} monocytes compared to nonclassical CD14^{+}CD16^{++} monocytes							
HLA-DRA	MHC complex, class II, DR alpha	CATGGGGCATCTCTTGTGTACTTATT	7564.9	2615.9	0	1.5	MHC II Ag presentation
LYZ	Lysozyme	CATGATGTAAAAAATACAAACATTCT	6770.6	1063.6	0	2.7	Antimicrobial agent
CD74	CD74 molecule, MHC class II invariant chain	CATGGTTCACATTAGAATAAAAGGTA	30 285.4	13 911.5	0	1.1	MHC II Ag processing; cell-surface receptor for MIF
IFI30	IFN γ-inducible protein 30	CATGATCAAGAATCCTGCTCCACTAA	5312.1	2128.7	2.1×10^{-253}	1.3	MHC II Ag processing
S100A8	S100 calcium-binding protein A8	CATGTACCTGCAGAATAATAAAGTCA	1557.0	283.7	8.6×10^{-171}	2.5	Calcium-binding protein with antimicrobial activity
HLA-DPB1	MHC complex, class II, DP beta 1	CATGTTCCCTTCTTCTTAGCACCACA	1497.9	340.2	6.3×10^{-140}	2.1	MHC II Ag presentation
TMSB10	Thymosin beta 10	CATGGGGGAAATCGCCAGCTTCGATA	3314.6	1650.4	1.8×10^{-104}	1.0	Organization of the cytoskeleton; cell motility, differentiation
SECTM1	Secreted and transmembrane 1	CATGACTCGAATATCTGAAATGAAGA	1756.7	803.6	3.5×10^{-67}	1.1	Hematopoietic and/or immune system processes
FAU	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	CATGGTTCCCTGGCCCGTGCTGGAAA	1384.8	566.0	2.7×10^{-65}	1.3	Fusion protein (ubiquitin-like protein fubi and ribosomal protein S30)
CPVL	Carboxypeptidase, vitellogenic-like	CATGATTAATCGATTCATTTATGGAA	470.5	57.9	5.1×10^{-65}	3.0	Processing of phagocytosed particles, inflammatory protease cascade
PPIA	Peptidylprolyl isomerase A (cyclophilin A)	CATGCCTAGCTGGATTGCAGAGTTAA	1817.5	900.2	8.7×10^{-59}	1.0	Accelerate the folding of proteins; formation of infectious HIV virions
CD14	CD14 molecule	CATGTGGTCCAGCGCCCTGAACTCCC	406.2	56.4	3.0×10^{-53}	2.8	Mediate the innate immune response to bacterial lipopolysaccharide
S100A10	S100 calcium-binding protein A10	CATGAGCAGATCAGGACACTTAGCAA	813.2	277.8	1.4×10^{-50}	1.5	Cell-cycle progression and differentiation; exo- and endocytosis
FOS	FBJ murine osteosarcoma viral oncogene homolog	CATGTGGAAAGTGAATTTGAATGAAA	287.1	17.8	2.3×10^{-50}	4.0	Forming the TF complex AP-1; proliferation, differentiation
TNFAIP2	TNF α-induced protein 2	CATGACTCAGCCCGGCTGATGCCTCT	757.5	268.9	6.5×10^{-45}	1.5	Mediator of inflammation and angiogenesis
Top genes up-regulated in nonclassical CD14^{+}CD16^{++} monocytes compared to intermediate CD14^{++}CD16^{+} monocytes							
CFL1	Cofilin 1	CATGGAAGCAGGACCAGTAAGGGACC	2871.6	6395.0	1.8×10^{-266}	−1.2	Actin dynamics; cell motility
IFITM2	IFN-induced transmembrane protein 2 (1-8D)	CATGACCATTCTGCTCATCATCATCC	1727.6	4362.8	3.7×10^{-230}	−1.3	Immune response
GNAI2	Guanine nucleotide-binding protein (G protein), α inhibiting activity polypeptide 2	CATGTTTTATGGAATTGTTCACCTGG	1270.8	3523.6	5.1×10^{-216}	−1.5	Regulation of adenylate cyclase
NPC2	Niemann-Pick disease, type C2	CATGTCTCTTTTTCTGTCTTAGGTGG	1286.2	3394.3	6.4×10^{-193}	−1.4	Transport regulation of cholesterol through endosomal/lysosomal system
MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle	CATGGTGCTGAATGGCTGAGGACCTT	2431.1	4885.7	6.2×10^{-163}	−1.0	Regulatory light chain of myosin
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CATGCCCATCTAGCTTGCAGTCTCTT	948.6	2611.5	6.2×10^{-159}	−1.5	Negative regulator of cell proliferation
LYN	v-yes-1 Yamaguchi sarcoma viral-related oncogene homolog	CATGATGTGTTTCACTTATGCTGTTG	1000.9	2602.6	6.2×10^{-145}	−1.4	Cell proliferation, migration
LAPTM5	Lysosomal protein transmembrane 5	CATGGCGGTTGTGGCAGCTGGGGAGG	1866.4	3905.3	2.4×10^{-143}	−1.1	Transmembrane receptor associated with lysosomes
OAZ1	Ornithine decarboxylase antizyme 1	CATGTTGTAATCGTGCAAATAAACGC	2649.6	4934.8	1.4×10^{-135}	−0.9	Regulation of polyamine biosynthesis
HLA-B	MHC, class I, B	CATGCTGACCTGTGTTTCCTCCCCAG	4623.1	7133.3	2.6×10^{-104}	−0.6	HLA class I heavy chain paralogue; Ag presentation
B2M	β2-microglobulin	CATGGTTGTGGTTAATCTGGTTTATT	6510.0	9267.9	2.0×10^{-93}	−0.5	Association with the MHC I heavy chain
STK10	Serine/threonine kinase 10	CATGGCAGAAGCACAGGTTCTGTACC	359.1	1137.9	3.4×10^{-85}	−1.7	Cell-cycle progression; involved in MAPKK1 pathway
PSAP	Prosaposin	CATGAAGTTGCTATTAAATGGACTTC	6670.3	9190.7	9.0×10^{-78}	−0.5	Catabolism of glycosphingolipids
CSTB	Cystatin B (stefin B)	CATGATGAGCTGACCTATTTCTGATC	424.2	1186.9	4.5×10^{-75}	−1.5	Thiol protease; intracellular degradation and turnover of proteins
LSP1	Lymphocyte-specific protein 1	CATGCAGGATGCTTGATGCTGCGTCC	1138.0	2237.1	8.7×10^{-72}	−1.0	Regulation of motility, adhesion, and transendothelial migration

$P < 10^{-310}$ is denoted as 0. Protein function: from Entrez Gene, UniProtKB/Swiss-Prot; most representative 26-hit tags are shown. Fold change: log2(CD14^{++}CD16^{+}/CD14^{+}CD16^{++} ratio).
TPM indicates tags per million.

Table 3. Top genes up-regulated in intermediate CD14^{++}CD16^{+} monocytes compared with classical CD14^{++}CD16^{-} and nonclassical CD14^{+}CD16^{++} monocytes

Gene symbol	Gene title	Tag sequence	CD14^{++}CD16^{-} TPM	CD14^{++}CD16^{+} TPM	CD14^{+}CD16^{++} TPM	Protein function
CD74	CD74 molecule, MHC class II invariant chain	CATGGTTCACATTAGAATAAAAGGTA	13 822.6	29 701.2	13 635.1	MHC II Ag processing; cell-surface receptor for MIF
IFI30	IFN γ–inducible protein 30	CATGATCAAGAATCCTGCTCCACTAA	2864.6	5212.8	2085.9	MHC II Ag processing
HLA-DPB1	MHC class II, DP β 1	CATGTTCCCTTCTTCTTAGCACCACA	404.4	1470.6	334.8	MHC II Ag presentation
HLA-DRA	MHC class II, DR α	CATGGGGCATCTCTTGTGTACTTATT	5090.7	7419.6	2566.3	MHC II Ag presentation
SECTM1	Secreted and transmembrane 1	CATGACTCGAATATCTGAAATGAAGA	614.0	1722.8	787.5	Hematopoietic and/or immune system processes
AIF1	Allograft inflammatory factor 1	CATGTCCCTGAAACGAATGCTGGAGA	733.4	2150.5	1362.5	RAC signaling; proliferation; migration; vascular inflammation
FAU	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	CATGGTTCCCTGGCCCGTGCTGGAAA	606.0	1358.0	554.6	Fusion protein (ubiquitin-like protein fubi and ribosomal protein S30)
TMSB10	Thymosin β 10	CATGGGGGAAATCGCCAGCTTCGATA	2257.8	3253.1	1618.7	Organization of the cytoskeleton; cell motility, differentiation
PPIA	Peptidylprolyl isomerase A (cyclophilin A)	CATGCCTAGCTGGATTGCAGAGTTAA	1058.3	1782.4	882.1	Accelerate the folding of proteins; formation of infectious HIV virions
PTPN6	Protein tyrosine phosphatase, nonreceptor type 6	CATGCCTCAGCCCTGACCCTGTGGAA	264.8	892.5	522.6	Regulation of cell growth, differentiation, mitotic cycle
TGFB1	TGF β 1	CATGGGGGCTGTATTTAAGGACACCC	170.9	569.8	263.5	Regulation of proliferation, differentiation, adhesion, migration
SAT1	Spermidine/spermine N1-acetyltransferase 1	CATGTTTGAAATGAGGTCTGTTTAAA	866.0	1979.1	1502.2	Catalyzes the acetylation of polyamines
CAPNS1	Calpain, small subunit 1	CATGCCCCAGTTGCTGATCTCTAAAA	261.4	657.2	331.9	Regulatory subunit of nonlysosomal thiol-protease
RHOB	ras homolog gene family, member B	CATGCACACAGTTTTGATAAAGGGCA	55.4	355.5	164.5	Intracellular protein trafficking
CTSB	Cathepsin B	CATGTGGGTGAGCCAGTGGAACAGCG	255.9	509.3	179.0	Degradation and turnover of proteins; maturation MHC II complex

Protein function: from Entrez Gene, UniProtKB/Swiss-Prot; most representative 26-hit tags are shown.
TPM indicates tags per million.

Cellular process

Within the GO term cellular process, CD14++CD16+ and CD14+CD16++ monocytes strongly differed in the expression of genes involved in cell adhesion, oxidative stress, and phagocytosis, pointing to a distinct transendothelial trafficking potential and a greater capacity of CD14++CD16+ monocytes for generation of ROS as well as for phagocytosis of pathogens.

In detail, while CD14+CD16++ monocytes expressed significantly higher levels of mRNA for numerous genes within the adhesion process (eg, *SLAMF7*, *RHOA*, *SPN*, *PECAM1*, *CYTH1*, *CYTIP*, *ITGAL*, *CD151*), CD14++CD16+ monocytes up-regulated expression of genes for distinct adhesion molecules such as *CD93*, *TGFBI*, parvin γ (*PARVG*), and *CSF3R*.

Moreover, CD14++CD16+ monocytes up-regulated genes linked to the generation of superoxide radicals (eg, *CYBA*, *TSPO*, *NCF2*) and down-regulated genes coding for enzymes in the detoxification of superoxide radicals (eg, *SOD2*, *PRDX1*, *GPX4*).

Finally, with regard to the process of phagocytosis, CD14++CD16+ monocytes expressed significantly higher levels of mRNA for *CD14*, ras-related C3 botulinum toxin substrate 1 (*RAC1*), and *CD93*.

Metabolic process

Although a large number of genes connected to the GO term metabolic process were up-regulated in CD14+CD16++ monocytes (eg, *STK10*, *GNAI2*, *CFL1*, *PSAP*), suggesting an increased potential of these cells for protein metabolism, CD14++CD16+ monocytes selectively up-regulated the expression of genes linked to Ag processing (eg, *CPVL*, *CTSB*, *IFI30*).

Response to stimulus

Immune cells respond to diverse stimuli, such as those evoked by bacterial infection or wounding. Again, numerous genes linked to the GO terms defense response and response to wounding were differentially expressed in monocyte subsets, with higher expression of, for example, lysozyme (*LYZ*), S100 calcium-binding protein A8 (*S100A8*) and complement component 1, q subcomponent, B chain (*C1QB*) in CD14++CD16+ monocytes, and higher expression of tumor necrosis factor receptor superfamily member 1B (*TNFRSF1B*), arachidonate 5-lipoxygenase (*ALOX5*), and carbohydrate sulfotransferase 2 (*CHST2*) in CD14+CD16++ monocytes, pointing to a differential role of monocyte subsets in dealing with biologic stress.

Unique identifiers of CD14++CD16+ monocytes

To further unravel CD14++CD16+ cells as a separate monocyte subpopulation, we aimed to identify unique markers which are selectively overexpressed in these monocytes.

Among those 258 genes which were up-regulated in CD14++CD16+ compared with CD14+CD16++ monocytes, 97 genes were likewise up-regulated in comparison to CD14++CD16− monocytes (defined as $P < 10^{-10}$). Of these 97 genes, 15 top genes defined by a 26-hit tag are presented in Table 3. The majority of these 15 top genes are linked to protein turnover and MHC II–restricted protein processing and presentation, such as *CD74* and other *HLA-DR* paralogues, IFNγ-inducible protein 30 (*IFI30*), calpain, small subunit 1 (*CAPNS1*), ras homolog gene family member B (*RHOB*), and cathepsin B (*CTSB*); others have a central role in monocyte activation, for example, protein tyrosine phosphatase nonreceptor type 6 (*PTPN6*), TGFβ1 (*TGFB1*), and allograft inflammatory factor 1 (*AIF1*).

Validation of monocyte subset specific markers identified by SuperSAGE

Finally, we aimed to test the biologic relevance of SuperSAGE data by flow cytometry and functional analyses.

Among the 97 genes which were found to be selectively overexpressed in CD14++CD16+ monocytes (reaching predefined level of statistical significance with a $P < 10^{-10}$), only few genes coding for surface proteins (eg, CD74 and HLA-DR) are accessible for flow cytometry. Therefore, we additionally analyzed further genes which again are up-regulated in CD14++CD16+ monocytes in SuperSAGE analysis, despite formally not reaching the strict statistical significance, such as the 2 proangiogenic markers endoglin (*ENG*) and the TEK tyrosine kinase (*CD202B*, angiopoietin receptor). As depicted in Figure 4A, flow cytometric analysis confirmed overexpression of these 4 markers on protein level.

To further underline the impact of these findings, we next demonstrated that those surface Ags which are selectively expressed in CD14++CD16+ cells might allow selective depletion of this monocyte subset in vitro, as shown exemplarily by using anti-HLA-DR MicroBeads (Figure 4B).

Because several genes involved in oxidative stress were up-regulated in CD14++CD16+ monocytes, we measured subset-specific spontaneous ROS levels using the ROS detection reagent H_2DFFDA, and confirmed CD14++CD16+ monocytes to be the main producers of ROS within the 3 monocyte subsets (Figure 4C).

After CD14++CD16+ monocytes showed selective up-regulation of genes linked to Ag processing and presentation, we next analyzed the subset specific ability of SEB stimulated monocytes to induce CD4+ T cell proliferation. Consistently with gene expression and flow cytometric analyses, CD14++CD16+ monocytes had the highest capacity to induce CD4+ T-cell proliferation (Figure 4D).

Remarkably, despite their low HLA-DR expression, CD14++CD16− monocytes likewise showed a substantial potential for CD4+ T-cell activation. To unravel this seeming contradiction, we analyzed the fate of isolated CD14++CD16− monocytes after SEB stimulation and found these cells to differentiate toward CD14++CD16+ monocytes. Concomitantly, after 72 hours of stimulation, CD14++CD16− monocytes up-regulated expression of HLA-DR (Figure 4E) and CD74 (data not shown) toward levels similar to unstimulated CD14++CD16+ monocytes. In contrast, CD14+CD16++ monocytes did not enhance HLA-DR expression on SEB stimulation (data not shown).

Next, after we found CD14++CD16+ monocytes to selectively up-regulate central proangiogenic markers as ENG and TEK, we analyzed surface expression of KDR (VEGFR2), which also significantly contributes to angiogenesis. In line with ENG and TEK, KDR was selectively up-regulated in CD14++CD16+ monocytes (Figure 4F). To confirm a proangiogenic character of CD14++CD16+ monocytes, we analyzed their capacity to form cordlike structures in Matrigel after VEGF stimulation. Unlike CD14++CD16− and CD14+CD16++ monocytes, CD14++CD16+ monocytes selectively collocated to clusters within 3 days (Figure 4F). However, no monocyte subset formed typical HUVEC-like structures.

Analogous to specific markers of CD14++CD16+ monocytes, we also validated our SuperSAGE results for markers of CD14++CD16− and CD14+CD16++ monocytes: for CD14+CD16++ monocytes, we confirmed subset-specific expression of the 4 adhesion molecules PECAM1 (CD31), SPN (CD43), ITGAL (CD11A), and CD47 by flow cytometry (Figure 4A). For CD14++CD16− monocytes, overexpression of CD93, FCGR1A (CD64), ITGAM (CD11B), and CD36 was flow-cytometrically

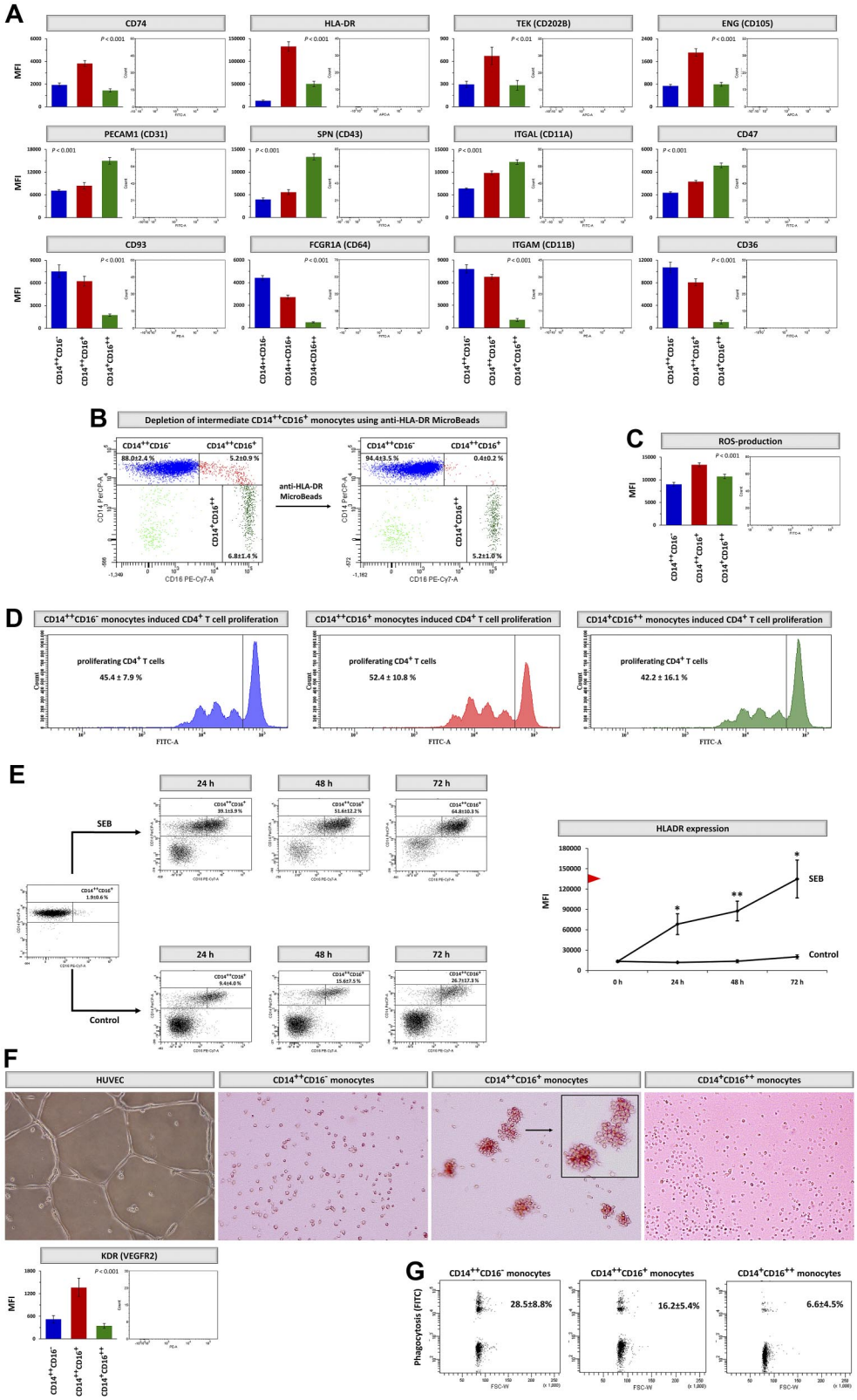

Figure 4. Monocyte subset-specific identifiers. (A) Surface expression of distinct markers on CD14^{++}CD16^{-} monocytes (blue columns), CD14^{++}CD16^{+} monocytes (red columns), and CD14^{+}CD16^{++} monocytes (green columns) performed by flow cytometry. Data were measured as median fluorescence intensity (MFI) and presented as means ± SEM. Background fluorescence (measured in negative controls) was subtracted. Statistical analysis was performed using the Kruskal-Wallis test. (B) NK cells and neutrophil-depleted PBMCs before (left dot plot) and after (right dot plot) incubation with anti–HLA-DR MicroBeads and subsequent negative isolation. (C) Flow cytometric analysis of spontaneous intracellular ROS levels within the 3 monocyte subsets using the ROS-detection reagent carboxy-H_2DFFDA. Data are presented and analyzed as described in panel A. (D) CD4^{+} T-cell proliferation, measured flow-cytometrically as cytoplasmic dilution of CFDA-SE. Monocyte subsets were isolated, stimulated with SEB (2.5 μg/mL), and cultivated with CFDA-SE–labeled CD4^{+} T cells for 3 days. After gating for CD3-positive cells, percentages of proliferating CD4^{+} T cells were determined and denoted as means ± SD. Representative examples of 5 independent experiments are shown. (E) Stimulation of isolated CD14^{++}CD16^{-} monocytes with 2.5 μg/mL SEB versus control. After 24, 48, and 72 hours, percentages of CD14^{++}CD16^{+} monocytes (left panels) and expression of HLA-DR (right panel) of total events was determined

confirmed (Figure 4A). As these 4 proteins are involved in the phagocytosis process, we tested the biologic relevance of this overexpression and confirmed that CD14++CD16− monocytes exhibit the highest phagocytosis potential (Figure 4G).

Discussion

During the past 2 decades a dichotomized view on human monocyte heterogeneity prevailed, distinguishing between classical (CD14++CD16−) and nonclassical (CD16-positive) monocytes.

However, the existence of an intermediate monocyte subset, which had been identified several years before,[4] has very recently been acknowledged in the International Consensus Statement on Monocyte Nomenclature.[5] From now on, we should accordingly differentiate 3 major monocyte subsets: classical CD14++CD16−, intermediate CD14++CD16+, and nonclassical CD14+CD16++ monocytes.

Of note, the intermediate CD14++CD16+ monocyte subset is only poorly characterized, as the 2 CD16-positive monocyte subsets (CD14++CD16+ and CD14+CD16++ cells) have been analyzed together in most studies so far, as reviewed recently.[6]

Moreover, CD14++CD16+ monocytes are of importance in the pathology of 2 global health issues, HIV-1 infection,[9,10] and cardiovascular disease.[7,8] Therefore, a better understanding of this subset is clearly needed. We aimed to characterize this subset more thoroughly with whole genome transcriptome analysis focusing on differences between the CD16-positive monocytes.

Using SuperSAGE, we analyzed the expression of approximately five 500 000 tags in the 3 human monocyte subsets, and found a high level of transcriptional similarity, mostly between intermediate CD14++CD16+ and nonclassical CD14+CD16++ monocytes (97.1%, $P < 10^{-10}$), arguing for a high developmental relationship. However, 559 genes showed strong differential expression between CD14++CD16+ and CD14+CD16++ monocytes; among those, 97 were strongly overexpressed in CD14++CD16+ monocytes compared with both CD14++CD16− and to CD14+CD16++ monocytes.

These 97 markers of CD14++CD16+ monocytes should be considered as only a fraction of all distinct identifiers, as we set a very strict level of significance to a $P < 10^{-10}$. Thus, many differentially expressed genes were excluded, for example, *CCR5* (*CD195*), *ENG* (*CD105*), and *TEK* (*CD202B*). Despite not formally reaching the strict predefined level of significance in SuperSAGE analysis, we could demonstrate selective expression of these molecules on protein level making them further identifiers of the intermediate monocyte subset.

Previously published whole genome expression analyses revealed biologic and functional differences between CD14++CD16− and CD16-positive monocytes.[12-15] These previous analyses linked CD14++CD16− monocytes to myeloid (eg, *CD14*, *MNDA*, *TREM1*, *CD1D*, *CD93*) and granulocyte lineage (eg, *FPR1*, *CSF3R*, *S100A8-9/12*),[12] and showed an increased antimicrobial potential of these cells (eg, *LYZ*, *MPO*, *RNASE3*, *PLBD1*).[15] In contrast, CD16-positive monocytes were shown by previous whole genome expression analyses to be at a more advanced stage of differentiation, and to have a more DC (eg, *SIGLEC10*, *CD43*, *RARA*) and macrophage (eg *CSF1R*, *MAFB*, *CD97*, *C3AR*) character, thereby possessing effector functions related to Ag processing and presentation (eg, *CTSL*, *CTSC*), and suggesting diverse patterns of transendothelial migration (eg, *CX3CR1*, *CD31*).[12]

As previous gene expression studies did not distinguish between the 2 CD16-positive subsets, our SuperSAGE data expand current knowledge about monocyte heterogeneity and help to unequivocally delineate CD14++CD16+ monocytes from CD14+CD16++ monocytes.

The herein presented SuperSAGE analysis revealed that CD14++CD16+ monocytes are likely to be predisposed for Ag presentation, as they express genes encoding MHC II molecules (eg, *CD74*, *HLA-DR*) and genes involved in Ag processing and turnover of proteins (eg, *IFI30*, *CAPNS1*, *RHOB*, *CTSB*). This assumption is strengthened by the selective expression of *CCR5* in CD14++CD16+ monocytes and the fact that DC precursors can be recruited directly from the blood to the lymphoid organs through signaling induced by CCR5-CCL3 interactions.[22] Consistently, we found SEB-stimulated CD14++CD16+ monocytes to have highest capacity to activate CD4+ T-cell proliferation in functional analysis.

GO enrichment analysis revealed further biologic and functional differences between the 2 CD16-positive subsets. Among biological processes, those genes which differed most significantly between intermediate and nonclassical monocytes were connected to the immune system process (eg, *CFP*, *NCF2*, *CFD*, *PRKCD*) arguing for specialized immunologic functions in vivo. In line, the 2 CD16-positive subsets harbor a contrasting capacity for modulating inflammatory responses; for example, the production of IL-1B and TNF-α on stimulation with LPS is restricted to CD14++CD16+ monocytes.[23]

Many clinical and experimental studies showed a proinflammatory role of CD16-positive monocytes, as their counts rise in numerous acute and chronic inflammatory conditions,[24-30] and as they represent the major producers of the inflammatory cytokines TNF-α[31] and IL-12.[32] In SuperSAGE analysis, CD14++CD16+ monocytes showed a high activation and inflammatory potential which is indicated by the selective up-regulation of genes linked to inflammatory processes (eg, *AIF1*, *TGFB1*). In line, we found this intermediate monocyte subset to be the main producer of ROS within the 3 monocyte subsets.

Within the transcriptome of CD14++CD16+ monocytes, the tag most frequently expressed was annotated to *CD74*. Interestingly, CD74 levels were recently suggested as a biomarker for atherosclerosis: in a clinical cohort study, CD74 levels were found to be associated with subclinical and overt atherosclerosis.[33] Animal data support this notion because CD74-deficient *Ldlr*−/− mice showed reduced atherosclerosis associated with an impaired adaptive immune response to disease-specific Ags.[34] These results are in line with our previous clinical data showing

Figure 4 (continued) flow-cytometrically. Percentages of CD14++CD16+ monocytes derived from CD14++CD16− monocytes are given as means ± SD. Representative examples of 5 independent experiments are shown. HLA-DR MFI was measured as described in panel A. Red arrowhead marks HLA-DR expression of unstimulated CD14++CD16+ monocytes (compare panel A). HLA-DR MFI of SEB-stimulated and control cells were compared by the paired Student *t* test; *$P < .05$, **$P < .01$. (F) Bottom panel: Surface expression of KDR (VEGFR2) on monocyte subsets measured by flow cytometry. Data are presented and analyzed as described in panel A. Top panel: Monocyte subsets cultivated for 3 days on Matrigel in the presence of 10 ng/mL VEGF (RPM1 medium/5% FCS). Representative examples of 3 independent experiments are shown. HUVECs were used as control cells (EGM-2 medium/5% FCS). Image acquistion was performed by the Keyence BZ-8000K microscope equipped with a Nikon Plan Apo 4×/0.2 objective and the BZ Viewer software, magnification 8-12×, room temperature. (G) Capacity to phagocyte opsonized carboxylate microspheres (0.75 μm, Yellow Green) by the 3 monocyte subsets within 30 minutes; counts of FITC-positive cells were determined flow-cytometrically and are denoted as means ± SD. Representative examples of 10 independent experiments are shown.

$CD14^{++}CD16^{+}$ monocyte counts to be independent predictors of cardiovascular outcome.[7,8]

As monocytes may further enhance atherogenesis via angiogenesis (eg, plaque neovascularization) and tissue remodeling, distinct angiogenic properties have been found in monocyte subsets.[35,36] Elsheikh et al identified a subset of human monocytes expressing the VEGFR-2 (KDR) to have endothelial-like functional capacity.[36] Furthermore, monocytes which express the angiopoetin-2 receptor TIE2 (TEK) have been characterized as highly proangiogenic cells specifically linked to tumor infiltration.[35] In the present study, we demonstrated that $CD14^{++}CD16^{+}$ selectively up-regulated the expression of TIE2, KDR, and ENG, arguing for an involvement of these cells in the process of angiogenesis. In functional analysis, $CD14^{++}CD16^{+}$ monocytes selectively formed clusters on Matrigel after VEGF stimulation, confirming data from Elsheikh et al who similarly reported clustering of presumably proangiogenic, VEGFR2-expressing monocytes.[36] As our results are also in accordance with the data of Murdoch et al,[37] who found TIE2 expression predominantly on $CD14^{++}CD16^{+}$ monocytes, it is tempting to speculate that the chemotactic effect of angiopoetin-2, which is released by vessels in inflamed or malignant tissues, could contribute to subset-specific recruitment of $CD14^{++}CD16^{+}$ monocytes.

Transendothelial trafficking is a prerequisite for the response of monocytes to inflammatory stimuli evoked, for example, by atherosclerosis or infection. It is well known that this process is mediated via different mechanisms between CD16-positive and $CD14^{++}CD16^{-}$ monocytes.[38] We here show that numerous genes coding for adhesion molecules and proteins involved in transendothelial migration were also differentially expressed between the 2 CD16-positive monocyte subsets, arguing for a diverse recruiting process and migratory behavior of $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes.

Indeed, Ancuta et al[4] demonstrated that fractalkine (CX3CL1)—the ligand for CX3CR1—mediates arrest and migration of CD16-positive monocytes. Notably, expression of CX3CR1 is highest in $CD14^{+}CD16^{++}$ monocytes[4,8]; additionally, SuperSAGE and FACS analysis demonstrated the highest expression of genes for further adhesion molecules in $CD14^{+}CD16^{++}$ monocytes, for example, integrin α L (ITGAL, CD11A), the integrin-associated protein CD47, sialophorin (SPN, CD43), which is a ligand for ICAM1 and the macrophage adhesion receptor sialoadhesin (SIGLEC1),[39,40] and PECAM1 (CD31), which triggers both leukoendothelial adhesion and integrin-mediated migration of leukocytes into surrounding tissues.[22]

After transendothelial migration, phagocytosis of pathogens is a hallmark of monocyte function. $CD14^{++}CD16^{-}$ were found to express a wide range of genes linked to the phagocytosis process (eg, *CD93*, *CD64*, *CD32*, *CD36*, *CD14*, *FCN1*, *SIRPA*). Accordingly, in functional analysis, we saw the highest phagocytic capacity in $CD14^{++}CD16^{-}$ monocytes, which is in line with previously published data.[23] Among the 3 monocyte subsets, genes coding for antimicrobial proteins (eg, *LYZ*, *S100A8/9*, *RNASE6*) were highest expressed in $CD14^{++}CD16^{-}$ monocytes; therefore, this subset is likely to be predisposed to exert the first line of innate immune defense against microbial pathogens.

In contrast to Cros and coworkers,[23] we found highest ROS levels in $CD14^{++}CD16^{+}$ monocytes rather than in $CD14^{++}CD16^{-}$ monocytes. This is most likely attributable to the fact that we measured basal ROS production from freshly isolated cells, whereas Cros et al analyzed ROS levels after stimulation with IgG-opsonized BSA.

So far, data on monocyte heterogeneity are at times hard to interpret partly because of the lack of standards for monocyte gating. Therefore, it is unclear whether shifts in CD16-positive monocytes reported in many inflammatory diseases were caused by total rises of CD16-positive cells or rather selective increases of $CD14^{++}CD16^{+}$ or $CD14^{+}CD16^{++}$ monocytes. After the recently published consensus statement on monocyte heterogeneity nomenclature,[5] we would like to encourage other groups to analyze the selective contribution of $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes in inflammatory states, which might allow a more subtle understanding of the respective pathophysiologic role of both subsets. Moreover, we feel an imminent need to standardize the gating strategy for flow cytometric analysis of monocyte subsets. Of note, we were able to validate our CD86-based gating strategy against a proposed reference strategy recently published by Heimbeck et al.[21]

In summary, we provide first genetic evidence for the proposed discrimination of human monocytes in classical $CD14^{++}CD16^{-}$ monocytes, intermediate $CD14^{++}CD16^{+}$ monocytes, and nonclassical $CD14^{+}CD16^{++}$ monocytes. Although $CD14^{++}CD16^{+}$ monocytes show intermediate functional properties and expression of many genes, they can nevertheless be clearly distinguished by newly found unique identifiers from $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{++}$ monocytes, suggesting a distinct role in the immune system process. Of note, while considering $CD14^{++}CD16^{+}$ cells as a separate monocyte subpopulation, we do not want to negate strong developmental relationships between these subsets. In vivo, monocytes are assumed to leave the BM as $CD14^{++}CD16^{-}$ cells, to develop within few days to $CD14^{++}CD16^{+}$, and subsequently into $CD14^{+}CD16^{++}$, albeit formal evidence for this model is still lacking. Interestingly, we showed in vitro a differentiation of isolated $CD14^{++}CD16^{-}$ toward $CD14^{++}CD16^{+}$ monocytes in the present study.

Finally, after $CD14^{++}CD16^{+}$ monocytes have been discussed as potential therapeutic targets in inflammatory conditions such as atherosclerosis,[8,23,41] we are hopeful that our dataset will spur future research in this direction with the potential for new therapeutic avenues.

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Authorship

Contribution: The study was designed by A.M.Z., R.-R.M., and G.H.H.; A.M.Z., B.R., and P.W. performed research; data were analyzed and interpreted by A.M.Z., K.S.R., and G.H.H.; statistical analysis was performed by A.M.Z., B.R., and P.W.; D.F. supervised the project; the manuscript was written by A.M.Z., K.S.R., and G.H.H., and critically revised by D.F; and all authors read and approved the final version of the manuscript.

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SuperTAG Methylation-specific Digital Karyotyping Reveals Uremia-induced Epigenetic Dysregulation of Atherosclerosis-Related Genes

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Background—Accelerated atherosclerosis is a hallmark of chronic kidney disease (CKD). Although the role of epigenetic dysregulation in atherosclerosis is increasingly appreciated, only a few studies focused on epigenetics in CKD-associated cardiovascular disease, virtually all of which assessed epigenetic dysregulation globally. We hypothesized that gene-specific epigenetic dysregulation in CKD exists, affecting genes pertinent to inflammation and atherosclerosis.

Methods and Results—Ten clinically stable patients undergoing hemodialysis therapy and 10 healthy age- and sex-matched controls were recruited. Genome-wide analysis of DNA methylation was performed by SuperTAG methylation-specific digital karyotyping, in order to identify genes differentially methylated in CKD. Analysis of 27 043 436 tags revealed 4288 genomic loci with differential DNA methylation ($P<10^{-10}$) between hemodialysis patients and control subjects. Annotation of UniTags to promoter databases allowed us to identify 52 candidate genes associated with cardiovascular disease and 97 candidate genes associated with immune/infection diseases. These candidate genes could be classified to distinct proatherogenic processes, including lipid metabolism and transport (eg, *HMGCR*, *SREBF1*, *LRP5*, *EPHX2*, and *FDPS*), cell proliferation and cell-cycle regulation (eg, *MIK67*, *TP53*, and *ALOX12*), angiogenesis (eg, *ANGPT2*, *ADAMTS10*, and *FLT4*), and inflammation (eg, *TNFSF10*, *LY96*, *IFNGR1*, *HSPA1A*, and *IL12RB1*).

Conclusions—We provide a comprehensive analysis of genome-wide epigenetic alterations in CKD, identifying candidate genes associated with proatherogenic and inflammatory processes. These results may spur further research in the field of epigenetics in kidney disease and point to new therapeutic strategies in CKD-associated atherosclerotic disease. **(*Circ Cardiovasc Genet*. 2012;5:611-620.)**

Key Words: cardiovascular diseases ■ DNA methylation ■ epigenetics ■ genome-wide analysis ■ kidney

Patients with chronic kidney disease (CKD) suffer from accelerated atherosclerosis. Accordingly, cardiovascular events are the major cause of death in patients with CKD.[1]

It is widely accepted that nontraditional risk factors are major determinants of the exceedingly high burden of cardiovascular events in CKD.[2] Among such nontraditional risk factors, uremia-associated alterations in epigenetic regulation have recently been hypothesized to promote accelerated atherogenesis in patients with CKD.[3–8]

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Because epigenetic patterns are influenced by a host of environmental factors,[9] the unphysiological uremic milieu may trigger substantial alterations of the epigenome. More specifically, hyperhomocysteinemia,[10–12] inflammation,[13] dyslipidemia,[14,15] and oxidative stress[16] may promote an unbalanced DNA methylation, which is a major epigenetic modification of the genome. It contributes to transcriptional regulation, imprinting, X-chromosome inactivation, and genomic integrity.[3] Consequently, aberrations in genomic DNA methylation are associated with inappropriate gene expression and promotion of disease.[17]

Although the association among CKD, epigenetic dysregulation, and accelerated atherosclerosis has been proposed in several review articles, only a few pioneering studies analyzed global changes in the epigenome of patients with CKD[10–13,18] and yielded conflicting results: Ingrosso et al[11] reported global DNA hypomethylation in a small group of patients on hyperhomocysteinaemic hemodialysis (HD), whereas Stenvinkel et al[13] found global DNA hypermethylation in CKD, which was associated with both inflammation and poor outcome in patients on HD.

The association between CKD and global DNA methylation thus seems to be complex. Moreover, analyses of global DNA methylation will offer no information on regulation of

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specific genes, and only scarce data on site-specific epigenetic changes have been reported in patients with CKD so far.[10,11,18]

To further unravel the impact of epigenetic dysregulation in CKD, we set out to perform whole-genome analysis of DNA methylation in patients on HD. We, therefore, expanded the methylation-specific digital karyotyping (MSDK) method, which has first been described by Hu et al[19] and later been modified by Li et al,[20] into SuperTAG methylation-specific digital karyotyping (SMSDK). SMSDK uses those longer (26 bp) tags that are used in SuperSAGE,[21] and thus allows high-throughput and genome-wide DNA methylation mapping.

We now report substantial differences in the epigenome of patients on HD compared with healthy controls, comprising a fundamental dysregulation of atherosclerosis-related genes.

Methods

Study Participants

Clinically stable dialysis patients (n=10) undergoing standard HD therapy 3 times a week were recruited from the Department of Internal Medicine IV, Nephrology and Hypertension of the Saarland University Medical Center. In all patients, 20 mL ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood was drawn before a HD session after the long-interdialytic interval. Ten healthy age- and sex-matched hospital employees served as controls.

To circumvent age- and sex-specific impact on our epigenetic analyses, recruitment was confined to men subjects aged between 50 and 60 years. All participants gave written informed consent in accordance with the Declaration of Helsinki. The study protocol was approved by the local Ethics Committee.

Quantification of *S*-Adenosylmethionine/ *S*-Adenosylhomocysteine and Homocysteine

For the quantification of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH), EDTA samples were directly placed on ice and centrifuged immediately for 10 minutes at 2000*g*. After centrifugation, 1 mL of EDTA plasma was directly acidified with 100 µL of 1 N acetic acid to prevent SAM degradation, mixed thoroughly, and stored at −70°C until analysis. The high-performance liquid chromatography-mass spectrometry detection of SAH and SAM was carried out by using a Waters 2795 alliance HT, coupled to a Quattro Micro API tandem mass spectrometer (Waters Corporation, Milford, MA) as described by Kirsch et al.[22]

Homocysteine was measured in plasma with a fluorescence polarization immunoassay on the Abbott AxSYM system (Abbott Laboratories, North Chicago, IL).

DNA Isolation and Construction of SMSDK Libraries

Peripheral blood mononuclear cells (PBMCs) were immediately isolated from anticoagulated blood by Ficoll-Paque (Lymphocyte Separation Medium; PAA, Cölbe, Germany) gradient density centrifugation. DNA was isolated from peripheral blood mononuclear cells using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

Genome-wide analysis of DNA methylation was performed by SMSDK at GenXPro GmbH (Frankfurt, Germany). The libraries were generated essentially as described by Li et al[20] with modifications as described by Matsumura et al.[23] *Hinp1I* was used as the methylation-sensitive enzyme, recognizing non-CpG-methylated GCGC sites. After digestion by *Hinp1I*, a biotinylated adapter, containing the recognition site for the restriction enzyme *EcoP15I*, was ligated to the digested DNA. The resulting product was bound to streptavidin-coated magnetic beads (Dynal). After *NlaIII* digestion, unbound DNA was discarded, and a second adapter, containing another *EcoP15I* recognition site adjacent to a CATG overhang and a priming site for Illumina's p5 primer for high-throughput sequencing on the Illumina Genome Analyzer II, was ligated to the *NlaIII* site. The streptavidin-bound constructs of DNA fragments, flanked by the two *EcoP15I* recognition sites arranged in a head-to-head fashion, were digested with *EcoP15I* to cut off the *NlaIII* bound adapter and its adjacent 26 to 27 bp long tags from the streptavidin matrix. The resulting adapter-tags were then ligated to p7 adapters from Illumina's Genome Analyzer system. The constructs were sequenced by synthesis on Illumina's Genome Analyzer II system.

Quality Assessment and Statistics of SMSDK Data

Quality assessment of generated tags was performed according to Qu et al[24] with an in-house software to reduce sequencing errors and artificial tag sequences. Tags were counted using the GenXProgram. Statistical analysis was performed by using an R-script (http://search.cpan.org/~scottzed/Bio-SAGE-Comparison-1.00/lib/Bio/SAGE/Comparison.pm) based on statistics described by Audic and Claverie.[25]

Gene Ontology Information

Gene ontology (GO) information was obtained from www.GeneOntology.org for the annotated UniTags. *P* values describing the likelihood for enrichment of GO terms were calculated by the Fisher exact test based on UniTags that were differentially expressed with a $P<10^{-10}$.[26] GO analysis was performed by using an in-house software (http://genxpro.ath.cx).

Validation of DNA Methylation

Bisulfite sequencing was performed according to Geisel et al.[10] Briefly, 500 ng DNA was treated with sodium bisulfite (EpiTect Bisulfite Kit, Qiagen), and a section of the *METTL2B* promoter was amplified using the PyroMark polymerase chain reaction (PCR) Kit and the PyroMark CpG Assay (PM00031115, Qiagen, Hilden, Germany). Pyrosequencing was performed on the PSQ 96MA system (Qiagen); percentages of methylated (C) and unmethylated (T) CpGs were subsequently calculated. The mean of 2 CpG sites was used as a marker for *METTL2B* methylation.

Real-time Quantitative PCR

After isolating total RNA from PBMCs with the QIAamp DNA Blood Mini Kit (Qiagen), 500 ng RNA was reverse transcribed using the DyNAmo cDNA Synthesis Kit (Biozym, Hessisch Oldendorf, Germany). Real-time PCR was performed on the Mx3005P system (Stratagene, Waldbronn, Germany) using the DyNAmo ColorFlash SYBR Green qPCR Kit (Biozym) and predesigned primers (QuantiTect Primer Assay, Qiagen). Relative quantification was performed by the comparative ΔΔCt method. The housekeeping gene *GAPDH* was used as an internal standard (forward primer: 5′-ctcctccacctttgacgctg-3′; reverse primer: 5′-tcctcttgtgctcttgctgg-3′). Experiments were performed in duplicate.

Flow Cytometric Analyses

Flow cytometric analyses were performed according to our previous report[21] using the FACS Canto II with CellQuest Software (BD Biosciences, Heidelberg, Germany). Briefly, antigen expression on CD86-positive monocytic cells was analyzed *via* a whole blood assay using 100 µL of EDTA anticoagulated blood, quantifying surface expression as median fluorescence intensity standardized against coated fluorescent particles (SPHEROTM; BD Biosciences). Histograms were plotted with FCS Express Software (De Novo Software, Los Angeles, CA). For measurement of reactive oxygen species, 1×10^6 PBMCs were incubated (15 minutes, 37°C, 5% CO_2) with cell-permanent carboxy-H_2DFFDA (Invitrogen, Darmstadt, Germany). Afterward, cells were stained with anti-CD14, anti-CD16, and anti-CD86, and intracellular reactive oxygen species levels were determined as median fluorescence intensity in $CD14^{++}CD16^{+}$ monocytes (the major reactive oxygen species producing subset).

Phagocytosis assay was performed by using Fluoresbrite Yellow Green Carboxylate Microspheres (0.75 µm; Polysciences, Eppelheim,

Germany), which were opsonized with heterologous serum (diluted to 50% with Krebs Ringers PBS). About 100 µL of citrate anticoagulated whole blood was incubated with 10 µL of opsonized particles (10^8 particles/mL) with gentle shaking for 30 minutes at 37°C. Control samples were incubated at 4°C. Afterward, cells were stained with anti-CD86, anti-CD14, and anti-CD16, and counts of FITC-positive $CD14^{++}CD16^{-}$ monocytes (subset with highest phagocytosis potential) were determined flow cytometrically.

The following antibodies were used: CD86-PE (HA5.2B7, Beckman-Coulter, Krefeld, Germany), CD14-PerCP (MΦ9, BD Biosciences), CD16-PeCy7 (3G8, BD Biosciences), and CD43-FITC (eBio84-3C1, eBioscience, Frankfurt, Germany).

Statistical Analysis

We compared levels of SAM, SAH, and homocysteine, as well as data on pyrosequencing, real-time PCR, and flow cytometry between patients on HD and controls using the unpaired Student's *t* test. The Kolmogorov–Smirnov test was applied to test normality assumption. Both tests were performed with the IBM SPSS Statistics 18 software.

Results

Participants' Characteristics

Mean age was 56.1±3.9 years in patients on HD and 53.5±2.4 years in control subjects (P=0.100). As expected, patients on dialysis had significantly higher C-reactive protein (14.1±12.9 mg/L) compared with controls (2.0±1.3 mg/L; P=0.016). Consistent with the anticipated normal renal function, the average estimated glomerular filtration rate of controls was 86.0±14.3 mL/min/1.73 m^2.

Five patients on dialysis had prevalent atherosclerotic disease, defined as the presence of coronary artery disease (prior myocardial infarction or coronary revascularization), cerebrovascular disease (prior stroke with symptoms lasting longer than 24 hours or carotid revascularization), and/or peripheral artery disease (prior revascularization of lower-limb arteries). Controls were free from atherosclerotic disease.

Analysis of central modulators of DNA methylation—homocysteine, SAH, and SAM—demonstrated significantly higher levels of these metabolites in patients on HD (Table 1), which is in accordance with previous studies.[10,12] Among these central modulators, SAH levels differed most pronouncedly (30.9-fold increase in patients on HD; P<0.001) compared with homocysteine (2.4-fold increase; P<0.001) and SAM (4.8-fold increase; P<0.001). These exceedingly high SAH levels resulted in a dramatic decrease in the SAM/SAH ratio, an indicator of reduced cellular methylation capacity.

Table 1. Plasma Level of Homocysteine, SAM, and SAH in the Study Population

Parameter	Controls (n=10)	HD patients (n=10)	*P*
Homocysteine, µmol/L	12.6±3.0	30.4±9.3	<0.001
SAM, nmol/L	95.0±24.8	456.6±181.1	<0.001
SAH, nmol/L	13.7±3.7	424.2±202.2	<0.001
SAM/SAH ratio	7.1±1.4	1.2±0.4	<0.001

Statistical analysis was performed with the unpaired Student's *t* test. SAM indicates *S*-adenosylmethionine; SAH, *S*-denosylhomocysteine.

Generation of SMSDK Libraries

DNA from peripheral blood cells was used for the generation of 2 independent SMSDK libraries (control library and HD library). After eliminating low-quality reads (according to Qu et al[24] and further elimination of tags with a count <5) and trimming of adapter sequences, the total number of tags was 27 043 436, comprising 11 942 429 from control subjects and 15 101 007 from patients on HD (Table 2 and online-only Data Supplement Figure I). These 27 043 436 tags accounted for 575 744 unique sequences (UniTags), of which 551 002 UniTags were found in both SMSDK libraries, 7250 were uniquely found in the control library, and 17 492 in the HD library. As a result, the control library comprised 558 252 UniTags, and the HD library comprised 568 494 UniTags.

UniTags were classified in abundance groups according to their number of copies (Table 2). Most of the UniTags (>99.9%) were found in low frequency, corresponding to the low abundance group (<100 copies/million). Less than 0.1% UniTags were classified to the mid-abundance group (100–1000 copies/million) or to the high-abundance group (>1000 copies/million), respectively, which is in line with previous reports.[27]

Uremia-associated Dysregulation of DNA Methylation

Among all 575 744 UniTags, we calculated the likelihoods for different tag frequencies in the control library and HD library according to Audic and Claverie.[25] We a priori set a strict level of significance to $P<10^{-10}$ in order to selectively identify those loci in the genome with very pronounced differences in DNA methylation, avoiding false-positive or biologically

Table 2. Features of SMSDK Libraries From Control Subjects and Patients On HD

Library	Controls (%)	Patients on HD (%)	Total (%)
Sequenced tags	11 942 429 (44.2%)	15 101 007 (55.8%)	27 043 436 (100%)
Number of unique transcripts (UniTags)	558 252 (97.0%)	568 494 (98.7%)	575 744 (100%)
Abundance classes of UniTags*			
High-abundant (>1000 copies/million)	25 (<0.1%)	31 (<0.1%)	—
Mid-abundant (100–1000 copies/million)	235 (<0.1%)	215 (<0.1%)	—
Low-abundant (<100 copies/million)	557 992 (> 99.9%)	568 248 (>99.9%)	—

*Values normalized to 1 million tags. HD indicates hemodialysis.

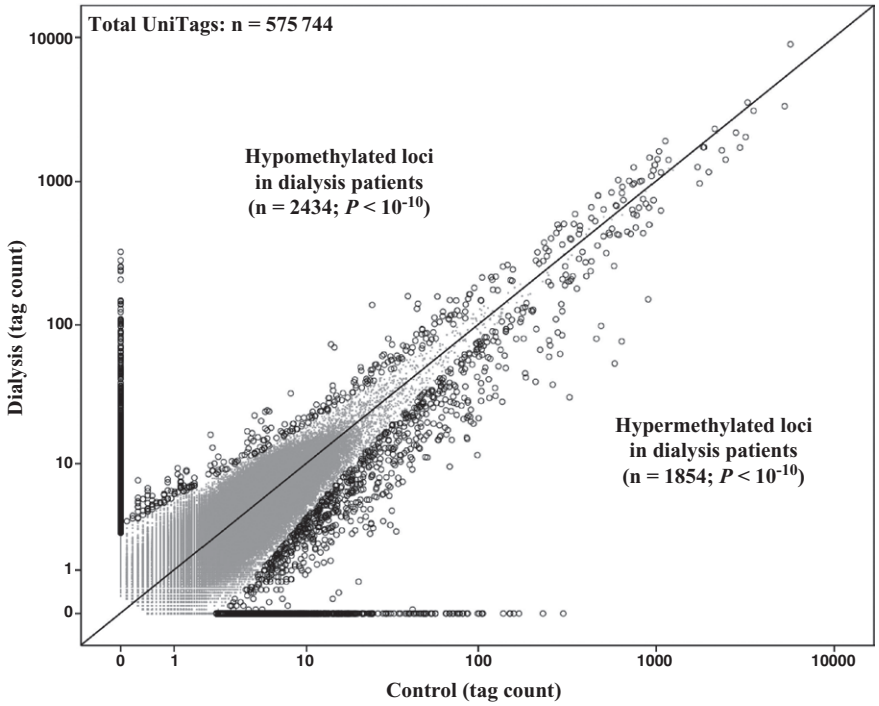

Figure 1. Scatter plot comparison (log scale) of UniTag frequencies in the control library (x-axis) and the HD library (y-axis). For all 575 744 UniTags, frequencies, which quantitatively illustrate the methylation level of the corresponding genomic loci, are displayed. Black circles represent those UniTags of which frequencies differ at a $P<10^{-10}$ between controls and patients on hemodialysis; all other UniTags are presented as grey dots.

irrelevant hits. We thus identified 4288 UniTags differing in their counts between patients on HD and control subjects (Figure 1). Among these 4288 UniTags, 1854 UniTags were found more frequently in control subjects, in line with hypermethylation in patients on dialysis, whereas 2434 UniTags were found more frequently in patients on dialysis, demonstrating hypomethylation in these patients.

Annotation of UniTags

Using an in-house version of the BLAST software (blastn version 2.2.21), we firstly annotated all 575 744 UniTags to different databases in hierarchical order as listed in Table 3 in order to match UniTags to their corresponding genomic loci. An e-value ≤0.001 was defined as a prerequisite for analysis.

Among all 575 744 UniTags, 79 574 (13.8%) UniTags could be annotated to upstream gene regions (databases 1–3) and were used for further analyses. The remaining UniTags matched to genomic loci not located in upstream gene regions (databases 4–6, totaling 265 459 UniTags, 46.1%) or could not be annotated at all (230 711 UniTags, 40.1%).

Table 3. Hierarchical Order of Databases for Annotation of UniTags

Database	UniTags (%)
1 upstream5000.fa_gcgc.fullDesC (UCSC)	51 607 (9.0%)
2 allHumanChromosome_AnnotatedPromoter (MPromDb)	12 848 (2.2%)
3 EPD_Human_DEZ10.fas (EPD)	15 119 (2.6%)
4 all_Human_chr.fa_gcgc (UCSC)	216 024 (37.5%)
5 human.rna.fna (NCBI)	16 376 (2.8%)
6 refseqgene.genomic.fna (NCBI)	33 059 (5.7%)
No hit	230 711 (40.1%)
Total	575 744 (100%)

For further stringency in our next analyses, we excluded those UniTags that did not match perfectly (number of matches <26/26) with sequences in databases 1 to 3. When applying these restrictions, 47 348 (59.5%) out of 79 574 UniTags could be used for further analyses (online-only Data Supplement Table I and Figure I).

Dysregulated Genes in Patients on Hemodialysis

When applying a strict *P* value ($P<10^{-10}$), 1089 out of these 47 348 annotated UniTags differed in their frequencies between patients on HD and control subjects. Thus, about a quarter of those 4288 UniTags that were found in significant different counts between patients on HD and healthy controls in SMSDK analysis could be annotated to a specific upstream gene region, of which the top 100 hits are summarized in online-only Data Supplement Table IIA (hypomethylated genes in patients on HD) and online-only Data Supplement Table IIB (hypermethylated genes in patients on HD). Additionally, all SMSDK data are presented in online-only Data Supplement Table III.

Several of these differentially methylated genes were linked to cell differentiation and cell-cycle regulation (eg, *DBF4B*, *TNFSF10*, and *PTPRN*) and especially to the p53 pathway (eg, *TP53*, *CDC14A*, *HIPK4*, and *BAG6*).

Moreover, other differentially methylated genes were connected to immune system processes including inflammation (eg, *CFB*, *LY96*, *SPN*, *NFKB2*, and *GPX4*), adhesion processes (eg, *ICAM2*, *CD300LG*, and *CTNNA3*), angiogenesis (eg, *ANGPT2*, *ADAMTS10*, and *FLT4*), cholesterol and lipid metabolism/transport (eg, *HMGCR*, *SLC27A1*, and *PCCA*), or other intracellular transport processes (eg, *KIF2C*, *SNX6*, and *TIMM8A*).

Finally, genes that are directly linked to epigenetic control (eg, *METTL2B*, *KDM6A*, *GADD45A*, and *JMJD5*) and transcriptional regulation (eg, *CSRNP2*, *ZNF382*, *ZNF251*, and *ZNF85*) were also differentially methylated in patients on HD.

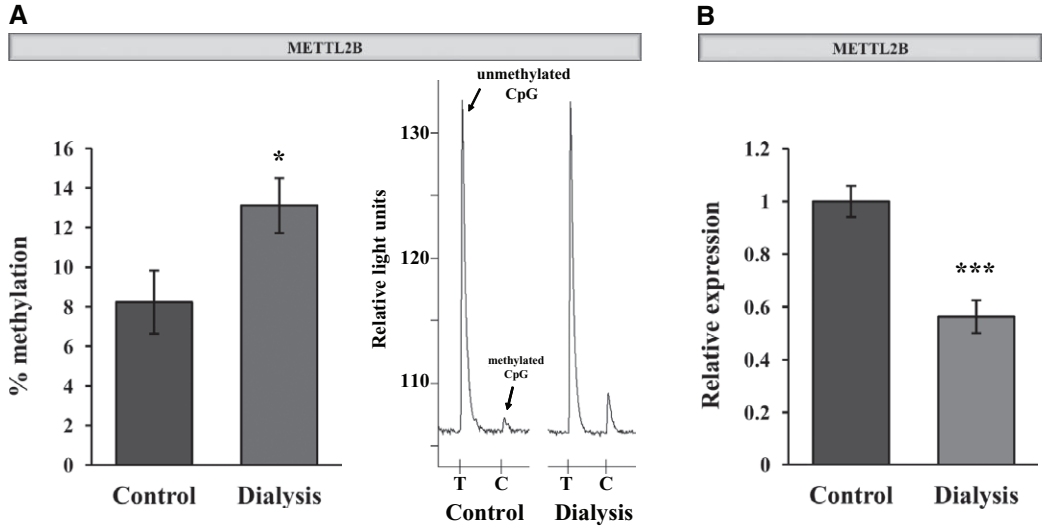

Figure 2. Validation of SuperTAG methylation-specific digital karyotyping data by bisulfite sequencing and real-time PCR. (**A**) Promoter methylation of *METTL2B* (methyltransferase-like 2B) was confirmed by bisulfite PCR and subsequent pyrosequencing. Representative pyrograms for 1 CpG are shown. (**B**) Gene expression of *METTL2B* was determined by real-time PCR using *GAPDH* as internal standard. Data are presented as mean±SEM and compared by Student *t* test. **P*<0.05; ****P*<0.001.

Validation of SMSDK Results

First, we compared our SMSDK results with the only prior genome-wide DNA methylation analysis in CKD. Focusing on renal markers, Sapienza et al[18] compared DNA methylation in saliva between patients with diabetes mellitus with and without CKD by the Illumina HumanMethylation 27 BeadChip array. Of 187 differentially methylated genes identified by Sapienza et al,[18] 70.4% were accordingly differentially methylated ($P<0.05$) in our analysis (online-only Data Supplement Table IV). Secondly, we aimed to validate our analysis by performing both bisulfite sequencing and real-time PCR of an arbitrary selected gene (*METTL2B*), which is linked to epigenetic regulation, and thereby confirmed SMSDK results (online-only Data Supplement Table IIB and Figure II). Thirdly, real-time PCR confirmed upregulation of *LY96* and *TNFSF10* (hypomethylated in SMSDK) and downregulation of *EPHX2* and *TRPV1* (hypermethylated in SMSDK) in patients on HD (online-only Data Supplement Figure II).

Finally, we aimed to assess the biological relevance of SMSDK results by flow cytometry and functional analyses. Firstly, in line with their CD43 promoter hypomethylation (online-only Data Supplement Table SIII), we demonstrated higher CD43 (SPN) protein expression on monocytic cells of

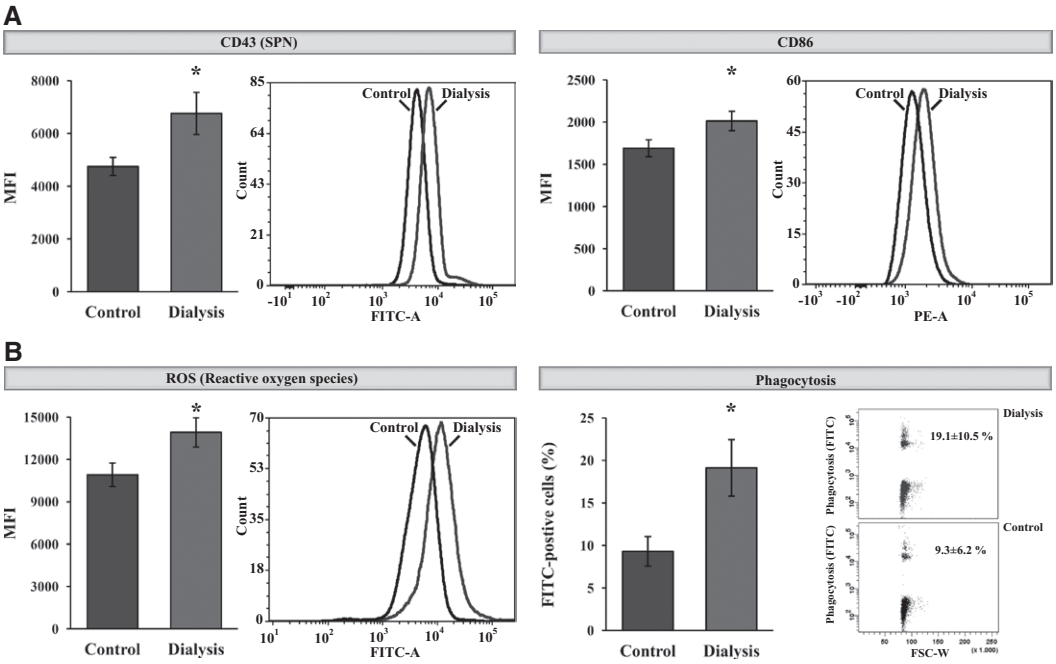

Figure 3. Validation of SuperTAG methylation-specific digital karyotyping data by flow cytometric analyses of monocytes from controls and patients on HD. (**A**) Surface expression of CD43 and CD86 (median fluorescence intensity after subtraction of background fluorescence). (**B**) Left: spontaneous intracellular ROS levels (ROS-detection reagent carboxy-H2DFFDA); right: phagocytosis of carboxylate microspheres (with subsequent counting of FITC-positive cells). Data are presented as means±SEM and compared by Student *t* test. **P*<0.05. ROS indicates reactive oxygen species.

patients on HD (Figure 3). Secondly, in line with their differential methylation of genes linked to inflammation and other immune system processes, patients on dialysis had higher protein expression of the monocytic activation marker CD86, higher cellular production of reactive oxygen species and higher monocytic phagocytosis rate (Figure 3).

Gene Ontology Analysis for Biological and Functional Differences

To investigate whether the above described epigenetic dysregulation may relate to biological and functional alterations of circulating immune cells of patients on dialysis, we next performed GO analysis. Among all 575 744 UniTags, 13 421 UniTags were annotated to the GO term biological process, 13 617 to molecular function, and 14 274 to cellular component. Figure 4 summarizes all GO terms that were assigned to the biological process at level 2 of GO categorization. GO terms showing significant differences (enrichment $P<0.05$) between control library and HD library are highlighted. These differing GO terms included several central biological processes like immune system processes ($P=0.001$), response to stimulus ($P=0.003$), cell proliferation ($P=0.006$), death ($P=0.016$), or metabolic processes ($P=1.2 \times 10^{-5}$), among others.

Dysregulation of Atherogenesis-related Genes in Patients on Hemodialysis

Following the postulate that uremia induces dysregulation of both atherosclerosis-protective genes and atherosclerosis-susceptible genes,[3–8] we finally analyzed whether the 1089 differentially methylated genes between patients on HD and controls can be directly linked to cardiovascular disease. Using the Genetic Association Database (accessible from the National Institutes of Health; http://geneticassociationdb.nih.gov/), we tested these genes for association with cardiovascular disease, as well as for immune/infection diseases, given that inflammation plays a central role in the pathogenesis of atherosclerosis.

Among all 1089 genes, 52 genes were associated with cardiovascular disease and 97 genes with immune/infection diseases. The most relevant genes are listed in Table 4 (cardiovascular disease) and Table 5 (immune/infection diseases). Of note, various genes connected to inflammation (eg, *TNFSF10*, *LY96*, *IFNGR1*, *HSPA1A*, and *IL12RB1*) were found to be hypomethylated in patients on dialysis. Further genes differentially methylated in patients on dialysis were connected to distinct cellular processes, including adhesion processes (eg, *PKD1*, *MADCAM1*, and *SPN*), cell proliferation and cell-cycle regulation (eg, *MIK67*, *TP53*, and *ALOX12*), apoptosis (eg, *CASP8*, *RAD51*, and *RAD51L1*), DNA repair (eg, *XRCC1* and *DDB2*), and lipid metabolism (eg, *HMGCR*, *SREBF1*, *LRP5*, *EPHX2*, and *FDPS*). Interestingly, hypermethylation—indicating downregulation of gene expression—affected genes that are supposed to be atherosclerosis-protective (eg, *TRPV1* and *GPX4*).

Discussion

Failure in epigenetic regulation substantially contributes to the onset and progression of vascular disease.[28] Among the 3 cornerstones of epigenetic regulation, namely histone modifications, RNA interference, and DNA methylation, the latter became the prime target for studies on interactions between disturbed gene regulation and promotion of vascular disease.

Accordingly, changes in global DNA methylation have been associated with future development of atherosclerosis in animal studies[14] and with prevalent vascular disease in cross-sectional clinical studies.[29]

Patients with CKD suffer from accelerated atherosclerotic vascular disease, which cannot completely be explained by traditional risk factors.[2] Of note, patients on dialysis display aberrations in global DNA methylation,[11,13] to which several features of the unphysiological uremic milieu, such as inflammation,[13] hyperhomocysteinemia,[10,11] oxidative stress,[16] and dyslipidemia,[14,15] may contribute.

Against this background, it has been speculated that disturbed DNA methylation in CKD may affect atherosclerosis-related genes with consequently higher susceptibility for vascular complications,[3–8] although information on site-specific regulation of these genes in CKD is virtually missing so far.

Beyond the field of nephrology, site-specific methylation analyses point to a dysregulation of several

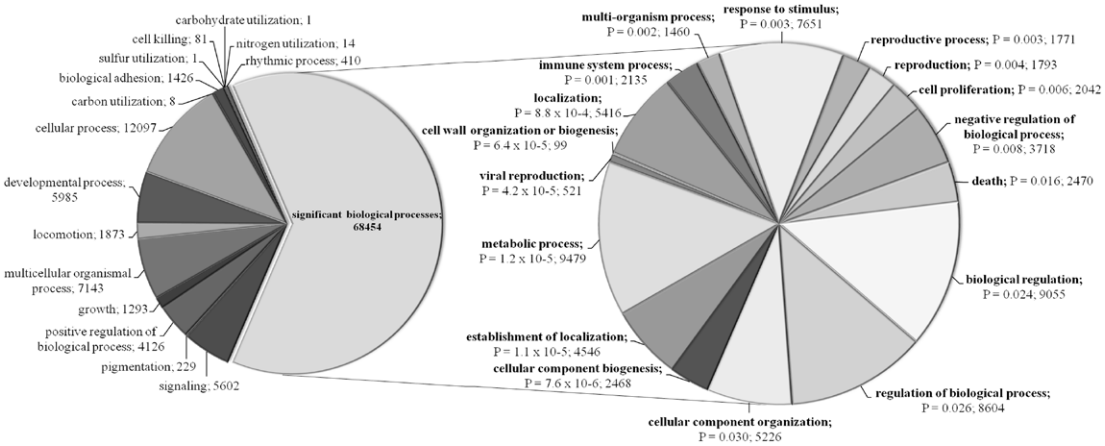

Figure 4. Pie charts of the functional characterization of annotated UniTags based on GO categorization (biological process). Using GO categories, annotated UniTags from the control library and the HD library were categorized by the function of the corresponding protein products. Among all UniTags categorized to level 1 GO term biological process, those level 2 GO terms with statistical significant difference ($P<0.05$) between control library and HD library are highlighted and projected into the right pie chart. Fisher exact test (2-tailed test) was used to compare groups for significant enrichment of particular GO classes. Numbers of UniTags for each GO term are given.

Table 4. Cardiovascular Disease

Gene symbol	Gene title	Tag sequence	Control TPM	Dialysis TPM	*P*	FC	Protein function
CFB	Complement factor B	CATGTTGCCCAGGCTGGTCTCAAACT	304.3	506.3	3.7E-148	0.7	Part of alternate pathway of complement activation; regulation of immune reaction
GCLM	Glutamate–cysteine ligase, modifier subunit	CATGTAGTTGTGCAGTTTTGAGTGAG	48.9	15.1	1.3E-57	–1.7	Glutathione synthesis
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase	CATGGTGGTGCACCCCTGTAATCCCA	0	14.9	1.3E-57	8.2	Control of cholesterol biosynthesis; rate-limiting enzyme of sterol biosynthesis
EPHX2	Epoxide hydrolase 2, cytoplasmic	CATGACCATTCACCCCTGGACCCCCT	37.8	12.0	1.2E-43	–1.7	Degrading toxic epoxides; associated with familial hypercholesterolemia
ECE2	Endothelin converting enzyme 2	CATGCCTGGTTAATTTTTGTATTTTT	0	10.7	1.1E-41	7.7	Type II metalloprotease
TP53	Tumor protein p53	CATGTTGGCCAGGCTGGTGTGGAACT	9.2	0	7.9E-40	–7.5	Responds to diverse cellular stresses to induce cell-cycle arrest, apoptosis, DNA repair
PKD1	Polycystic kidney disease 1 (autosomal dominant)	CATGCTGGCCAGGTTGGTCTCTAACT	4.6	22.7	1.2E-38	2.3	Regulator of calcium homoeostasis; cell–cell/matrix interactions; renal tubular development
SREBF1	Sterol regulatory element binding transcription factor 1	CATGCCGCTGCACTCCAGCCTGGGTG	0	7.4	5.1E-29	7.2	Regulates transcription of LDL receptor gene, fatty acid, and cholesterol synthesis pathway
ANGPT2	Angiopoietin 2	CATGGTGACTCACACCTGTAATCTCA	0	6.9	5.4E-27	7.1	Binds to TIE2 receptor and counteracts blood vessel maturation mediated by ANGPT1
IFNGR1	Interferon gamma receptor 1	CATGAGAGGCTGCCTGATAAACTGAT	0	6.2	3.3E-24	6.9	Receptor for interferon gamma
ALOX12	Arachidonate 12-lipoxygenase	CATGGTAAAACCCCATCTCTACCAAA	0	4.7	1.2E-18	6.6	Oxygenase and 14,15-leukotriene A4 synthase activity; has different physiological roles
HSPA1A	Heat shock 70 kDa protein 1A	CATGGAGACCAACACCCTTCCCACCG	0	4.4	2.2E-17	6.4	Stabilizes proteins against aggregation following stress-induced damage
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1	CATGTCAACGTCGTGGGCTTCGCCTG	8.0	1.7	5.2E-15	–2.3	Repair of DNA single-strand breaks
GPX4	Glutathione peroxidase 4 (phospholipid hydroperoxidase)	CATGTCTACAAAACAATAATTAGCCA	9.6	2.9	7.2E-13	–1.7	Protects cells against membrane lipid peroxidation and cell death
LRP5	Low-density lipoprotein receptor-related protein 5	CATGGTGAAACTCCATCTCTACTTAA	0	2.6	8.4E-11	5.7	Transmembrane low-density lipoprotein receptor

TPM indicates tags per million; FC: fold change (log2[dialysis/control ratio]).

atherosclerosis-related genes, such as the estrogen receptor α gene (*ESR1*), the inducible nitric oxide synthase gene (*iNOS*), and the extracellular superoxide dismutase gene (*SOD3*)—in the process of atherogenesis (reviewed in ref. 28).

We now aimed to identify atherosclerosis-related candidate genes in patients with CKD, extending our earlier studies that focused on methylation analysis of a single gene involved in oxidative stress-mediated atherosclerosis (*p66Shc* [*SHC1*]).[10]

Using SMSDK, we sequenced 27 043 436 tags. Despite choosing a very strict level of significance, we found >4000 UniTags differing between control subjects and patients on dialysis. This allowed us to identify disturbed methylation in 52 candidate genes associated with cardiovascular disease and in 97 genes associated with immune and infection diseases according to the NIH Genetic Association Database. These genes could be linked to diverse proatherogenic processes, including lipid metabolism and transport (eg, *HMGCR*, *SREBF1*, *LRP5*, *EPHX2*, and *FDPS*), cell proliferation and cell-cycle regulation (eg, *MIK67*, *TP53*, and *ALOX12*), angiogenesis (eg, *ANGPT2*, *ADAMTS10*, and *FLT4*), inflammation (eg, *TNFSF10*, *LY96*, *IFNGR1*, *HSPA1A*, and *IL12RB1*), and even epigenetic control (eg, *METTL2B*, *KDM6A*, *GADD45A*, and *JMJD5*).

Of note, epigenetic dysregulation not only affects genes associated with the promotion of atherosclerosis. Instead, we found hypermethylation of genes that have been characterized as atheroprotective in animal studies, namely *TRPV1* and *GPX4*.[30,31] Hypermethylation of these 2 genes points to a reduced transcription level of these protective factors in patients on dialysis.[32]

DNA methylation is centrally modulated by C1 metabolism, which itself is skewed in CKD. In C1 metabolism, the amino acid methionine is converted to SAM, which serves as

Table 5. Immune/Infection Disease

Gene symbol	Gene title	Tag sequence	Control TPM	Dialysis TPM	*P*	FC	Protein function
LY96	Lymphocyte antigen 96	CATGCCACTGCCCTCCAGCCTGGGTG	0	21.1	3.8E-81	8.7	Cooperates with TLR4 in the innate immune response to bacterial LPS
FCER1A	Fc fragment of IgE, high affinity I, receptor for; α polypeptide	CATGCCACTGCACTCCAGCCTGAGTG	0	16.5	1.1E-63	8.4	Responsible for initiating the inflammatory and immediate allergic response
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	CATGACTAAAACACCAAAAGCAATTG	0	15.6	2.1E-60	8.3	Induces apoptosis in transformed and tumor cells
COL2A1	Collagen, type II, α 1	CATGCTGGTCTCAAACTCCTGACCTC	0	11.0	1.1E-42	7.8	α-1 chain of type II collagen; specific for cartilaginous tissues
MADCAM1	Mucosal vascular addressin cell adhesion molecule 1	CATGTTGGGCAGGCTGGCCTCGAACT	0	7.8	1.5E-30	7.3	Endothelial cell adhesion molecule; interacts with LPAM-1, L-selectin, VLA-4
CSF3R	Colony stimulating factor 3 receptor (granulocyte)	CATGGTGAAACCCTTTCTCTACTAAA	0	5.4	2.0E-21	6.8	Proliferation, differentiation, and survival of cells along the neutrophilic lineage
TRPV1	Transient receptor potential cation channel, subfamily V, member 1	CATGGCGAAACCCTGACTCTACTAAA	4.4	0	1.4E-19	–6.5	Receptor-activated nonselective calcium permeant cation channel
IL12RB1	Interleukin 12 receptor, β1	CATGCCATCACGCCCAGCTAATTTTT	0	4.7	1.2E-18	6.6	Involved in IL12 and IL23 transduction
ARG2	Arginase, type II	CATGGGCCGGCCGCCTCCCGCGAAGG	0	4.4	2.2E-17	6.4	Catalyzes the hydrolysis of arginine to ornithine and down-regulates NO synthesis
MMP24	Matrix metallopeptidase 24 (membrane-inserted)	CATGGTGAAACCCTGTCTCTGCAAAA	0	3.6	1.4E-14	6.2	Breakdown of extracellular matrix
TNFRSF13C	Tumor necrosis factor receptor superfamily, member 13C	CATGGTGCCGACGCCGCCGCACAAGC	0	3.4	1.4E-13	6.1	B cell-activating factor; regulator of the peripheral B-cell population
CASP8	Caspase 8, apoptosis-related cysteine peptidase	CATGGAGAAAACCCGTCTCTACTAAA	0	3.2	8.0E-13	6.0	Responsible for TNFRSF6/FAS-mediated and TNFRSF1A induced cell death
RAD51	RAD51 homolog (*Saccharomyces cerevisiae*)	CATGTATATTACATTGCGCTTTAGAA	0	3.1	1.4E-12	6.0	Homologous recombination and repair of DNA
FDPS	Farnesyl diphosphate synthase	CATGTTGACCAGGCTGGTCTCAGATT	0	3.0	4.6E-12	5.9	Production of farnesyl pyrophosphate, a key intermediate in cholesterol/sterol biosynthesis
SPN	Sialophorin	CATGATCTCAGCTCATTGCAACCTCT	0	2.7	4.7E-11	5.8	Important for function of different immune cells; involved in T-cell activation

TPM indicates tags per million; FC: fold change (log2[dialysis/control ratio]).

a universal methyl group donor for methyltransferases. After transfer of its methyl group, SAM becomes SAH, which in turn binds to the active site of methyltransferases and thus strongly inhibits further methylation reactions. SAH may next be hydrolyzed to homocysteine. Of note, SAH and homocysteine are in equilibrium, so that hyperhomocysteinemia is inevitably associated with elevated SAH levels and with subsequent DNA hypomethylation.[11,33]

Although increased homocysteine levels are a common finding in CKD,[10,11,34] and although cohort studies associated hyperhomocysteinemia with subsequent cardiovascular events in CKD,[34] surprisingly none of the interventional studies targeting the high homocysteine levels in CKD proved a benefit,[35–39] which is in line with trials in the general population.[40,41]

Nonetheless, we reckon that those negative interventional trials neither rule out a causative role of a disturbed C1 metabolism in atherogenesis nor preclude epigenetic dysregulation from being a promising future therapeutic target in atherosclerosis.

Of note, epigenetic dysregulation has predominantly been addressed by measurement of the surrogate marker homocysteine in most clinical trials; this homocysteine-centered approach may result from the relatively convenient homocysteine analysis in contrast with the more cumbersome measurement of SAH. Based on our pathophysiological understanding of SAH as the direct inhibitor of methylation reactions, its measurement as a biomarker for epigenetic dysregulation in cardiovascular disease seems to be more meaningful than measuring its derivate homocysteine.[42,43]

Interestingly, all trials aiming at lowering homocysteine for primary or secondary prevention of cardiovascular disease used folate, vitamin B_6, and/or vitamin B_{12}, all of which efficiently

lower plasma homocysteine levels, but unfortunately do not affect SAH levels.[44] Furthermore, in patients with CKD, SAH accumulates more compared with homocysteine because the kidneys are the major site of SAH disposal in humans.[45] In accordance, in this study, we observed higher differences in SAH levels (30.9-fold increase in patients on dialysis) than in homocysteine levels (2.4-fold) and SAM levels (4.8-fold).

Importantly, chronic inflammation is commonly observed in patients on dialysis and associated with increased cardiovascular morbidity and mortality in this patient population (reviewed in ref. 46). As such, inflammation is a central uremic feature that—opposed to homocysteine—rather seems to trigger DNA hypermethylation[13] and thus may induce further aberrations in epigenetic regulation.

In line, Stenvinkel and coworkers[13] reported that global DNA hypermethylation was associated with both inflammation and poor outcome in CKD. Moreover, it was shown that the inflammatory cytokine IL-6 regulates a DNA methyltransferase gene[47] that may result in epigenetic dysregulation.

In this study, we demonstrated that epigenetic dysregulation in CKD comprises both DNA hypomethylation and DNA hypermethylation at different genomic loci. Of note, our data neither refute the finding of DNA hypomethylation nor challenge the reported DNA hypermethylation in CKD. Instead, our results underscore the importance of site-specific methylation analyses to deepen our knowledge of epigenetic regulation in CKD.

As a limitation, our present analysis neither allows to distinguish whether renal replacement therapy or uremia per se induces changes in DNA methylation in patients on HD nor to characterize the individual contribution of specific causative factors to epigenetic dysregulation.

Furthermore, no data on the prognostic impact of site-specific methylation in patients with CKD exist so far. We are, therefore, presently initiating a prospective study that shall confirm our hypothesis that dysregulation of predefined candidate genes may serve as early markers that predict future cardiovascular events in a large cohort of CKD.

Conclusions

A better understanding of the underlying causes of disease burden in CKD is desperately needed as conventional therapies failed to demonstrate a definite survival benefit in patients with CKD.[48–50] Against this background, we present genome-wide data on DNA methylation in patients on dialysis and characterize epigenetic dysregulation of candidate genes for accelerated atherosclerosis in CKD. We are hopeful that our findings may reveal relevant pathophysiological pathways that contribute to cardiovascular disease in patients with CKD, thus pointing toward potential new avenues for prevention and therapy.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Patients with chronic kidney disease (CKD) suffer from a tremendous burden of cardiovascular (CV) disease. Therapies focusing on classical CV risk factors, such as hypercholesterolemia, failed to substantially reduce this high CV morbidity. Therefore, a better pathophysiological understanding of CKD-associated CV disease is mandatory to define new therapeutic strategies. Against this background, we tested the hypothesis that epigenetic dysregulation of genes linked to CV disease occurs in CKD patients. Using SuperTAG methylation-specific digital karyotyping (SMSDK), we compared genome wide DNA methylation between 10 hemodialysis patients and 10 age- and gender-matched controls. We identified 52 genes linked to CV disease and 97 genes linked to immune/infection diseases to be differentially methylated in hemodialysis patients. These results point for the first time towards epigenetic dysregulation of atherosclerosis-related genes in hemodialysis patients, indicating a potential contribution of changes in DNA methylation to accelerated CV disease in CKD. Future studies should first analyse how far pre-defined candidate genes may serve as early markers for future CV events in CKD, and subsequently explore preventive and therapeutic strategies against CKD-associated epigenetic dysregulation.

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Das Manuskript wurde von Adam Zawada geschrieben und von Dr. Kyrill Rogacev, Prof. Dr. Gunnar Heine, Prof. Dr. Danilo Fliser und Prof. Dr. Jürgen Geisel überarbeitet. Alle Autoren haben die finale Version des Manuskripts gelesen und ihr zugestimmt.

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Monocyte heterogeneity in human cardiovascular disease

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ABSTRACT

Atherosclerosis has been characterized as an inflammatory process, in which monocytes and monocyte-derived macrophages are of paramount importance. Contrasting with their established role in atherosclerosis, monocytes have not unanimously been found to predict cardiovascular events in large epidemiological studies. However, in these studies human monocyte heterogeneity has been largely overlooked so far. Three human monocyte subsets can be distinguished: classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes. Of note, correct enumeration of subset counts requires appropriate staining and gating strategies that encompass a pan-monocytic marker (*e.g.* HLA-DR or CD86). In experimental studies on murine atherogenesis a monocyte subset-specific contribution to atherosclerosis has been established. However, major interspecies differences in atherogenesis itself, as well as in the immune system (including monocyte subset phenotype and distribution) preclude a direct extrapolation to human pathology. Experimental and pilot clinical studies point to a prominent involvement of intermediate $CD14^{++}CD16^{+}$ monocytes in human atherosclerosis. Future clinical studies should analyze monocyte heterogeneity in cardiovascular disease. If a specific contribution of intermediate monocytes should be confirmed, immunomodulation of this monocyte subset could represent a future therapeutic target in atherosclerosis.

Introduction

The global burden of cardiovascular morbidity is steadily growing, and cardiovascular disease (CVD) is nowadays the leading cause of death worldwide (Murray and Lopez 1997). Atherosclerotic vascular disease, representing the largest proportion of the cardiovascular disease spectrum, has been acknowledged as a chronic inflammatory condition in recent decades (Ross 1999), in which monocytes and monocyte-derived macrophages are the protagonists of vascular inflammation (Hansson and Hermansson 2011).

Monocytes have been considered as a homogenous cell population until the late 1980s, when Bernward Passlick, Dimitri Flieger and Löms Ziegler-Heitbrock first demonstrated the existence of distinct subsets of monocytes (Passlick et al. 1989). Since then, much effort was undertaken to understand the implications of monocyte heterogeneity in the pathogenesis of cardiovascular disease (Woollard and Geissmann 2010).

In the present review, we will first summarize how monocytes contribute to the initiation and progression of atherosclerotic lesions. Next, we will discuss how far the identification of monocyte heterogeneity may broaden our understanding on the involvement of monocytic cells in atherogenesis. We will focus on the impact of monocyte subsets in human atherosclerosis, summarizing evidence from clinical studies. Congruent results from murine studies will be briefly discussed when examining fundamental aspects of atherosclerosis. For more detailed discussion on murine monocyte heterogeneity in atherosclerosis, the interested reader is referred to excellent reviews published in the last years (Auffray et al. 2009; Gautier et al. 2009; Swirski et al. 2009; Weber et al. 2008; Woollard and Geissmann 2010).

Monocytes in atherosclerosis

Perpetual (micro)inflammation and its cellular hallmark – monocyte activation – are the major underlying pathological processes in atherogenesis (Ross 1999). Activation of endothelial cells, which is the initiating event in atherogenesis, is driven by traditional risk factors such as smoking, hyperglycaemia and hypertension, and mediated by proinflammatory stimuli such as TNFα (tumor necrosis factor alpha), IL1β (interleukin 1, beta), oxLDL

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(oxidized low density lipoprotein) and angiotensin II (Alamanda et al. 2012; Alvarez et al. 2004; Eriksson et al. 2000; Hansson and Hermansson 2011; Riou et al. 2007; Wang et al. 2008). These stimuli induce expression of adhesion molecules – namely selectins and integrin ligands – on the endothelial surface, which allow recruitment of circulating monocytes (Hansson and Hermansson 2011; Zernecke et al. 2008). Expression of endothelial selectin (E-selectin), which interacts with monocytic P-selectin glycoprotein ligand-1 (PSGL-1), mediates rolling of monocytes on activated endothelial cells (Mestas and Ley 2008). Subsequently, endothelial integrin ligands – such as VCAM1 (vascular cell adhesion molecule 1) and ICAM1 (intercellular adhesion molecule 1) – bind to monocytic VLA-4 (very late antigen-4, $\alpha4\beta1$ integrin; dimer of CD49D and CD29), or to LFA-1 (lymphocyte function-associated antigen 1; composed of CD18 and CD11A) and MAC-1 (macrophage-1 antigen; composed of CD18 and CD11B), respectively, thus allowing firm attachment (Huo et al. 2000; Ley et al. 2011).

Following adhesion, monocytes are recruited into the intimal layer of the vascular wall (intima) by diapedesis, which is primarily dependent on PECAM-1 (platelet–endothelial-cell adhesion molecule-1, CD31) and CD99 (Muller 2003). In the subendothelial space, monocytes differentiate into macrophages under the influence of endothelium-derived MCSF (macrophage colony-stimulating factor) (Hansson and Hermansson 2011). Macrophages further amplify the inflammatory process by proliferation and production of proinflammatory cytokines and growth factors (Libby 2002).

Furthermore, a broad range of pattern recognition receptors, such as scavenger receptors, enables macrophages to ingest oxLDL and other lipids, resulting in the formation of lipid-laden foam cells, a defining feature of atherosclerotic lesions (Greaves and Gordon 2009; Kunjathoor et al. 2002). Central scavenger receptors involved in lipid uptake process are scavenger receptors class A (SR-AI, AII, and AIII) and scavenger receptors class B (SR-BI and CD36) (Kunjathoor et al. 2002). Notably, alternative pathways for lipid uptake may exist (Manning-Tobin et al. 2009; Moore et al. 2005).

Other pattern recognition receptors mediate oxLDL-induced inflammatory responses, among which toll-like receptors 2 and 4 (TLR2 and TLR4) are of particular importance (Miller et al. 2003; Seimon et al. 2010). Beside these membrane-bound receptors, the intracellular pattern recognition receptor NALP3 inflammasome (NLR family, pyrin domain containing 3) exists. Activated by cholesterol crystals present in macrophages, this multi-protein complex induces the secretion of inflammatory cytokines such as IL1β and IL18 (Duewell et al. 2010; Schroder and Tschopp 2010).

In aggregate, these inflammatory processes attract further immune cells from the vascular lumen and smooth muscle cells from the media layer into the intima. This continuous cell recruitment promotes the formation of advanced atherosclerotic plaques, consisting of a necrotic core region surrounded by a cap of smooth muscle cells and collagen-rich matrix. Subsequently, this fibrous cap may gradually be thinned by macrophage-derived matrix metalloproteinases (MMPs), rendering the plaques prone to rupture (Newby 2005). As a final process, such disintegration of the fibrous cap exposes subendothelial prothrombotic material that activates the intravascular coagulation cascade and induces thrombus formation with subsequent vascular occlusion (Fuster et al. 2005).

Although pathophysiological models unequivocally establish monocytes as major players in atherogenesis, disparate findings were reported in epidemiological studies, which did not consistently find an association between cell counts of blood monocytes and cardiovascular disease (Grau et al. 2004; Johnsen et al. 2005; Wheeler et al. 2004). We hypothesized before that acknowledgement of monocyte heterogeneity may allow reconciling pathophysiological models with epidemiological studies (Heine et al. 2008).

Human monocyte heterogeneity

Heterogeneity of human monocytes was first described in 1989 based on the differential cell-surface expression of the LPS receptor CD14 and the FcγIII receptor CD16 (Passlick et al. 1989). Initially, two different monocyte subsets were characterized, namely classical $CD14^{++}CD16^{-}$ monocytes and monocytes co-expressing CD14 and CD16 (CD16-positive monocytes). According to the Nomenclature Committee of the International Union of Immunological Societies, these CD16-positive monocytes should nowadays be subdivided into intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes by their different expression of CD14 (Ziegler-Heitbrock et al. 2010). Throughout this review article, this official nomenclature is applied.

While flow cytometric *differentiation* of monocyte subsets is based on quantification of surface CD14 and CD16 expression, correct *identification* of monocytes inevitably requires staining of a third pan-monocytic marker. Two different approaches either using CD86 (compare Fig. 1) or HLA-DR (compare (Heimbeck et al. 2010)) as pan-monocytic markers have been suggested, which yield identical results (Zawada et al. 2011). Other staining protocols which only analyze CD14 and CD16 expression will inevitably fail to correctly distinguish monocytes from other leukocyte subsets: these protocols usually rely on cellular physical characteristics (flow cytometrically measured as forward and side scatter) for defining monocytes. As depicted in Fig. 2, following such an approach, nonclassical $CD14^{+}CD16^{++}$ monocytes cannot properly be separated from other CD16-expressing leukocytes, namely neutrophils and natural killer cells, leading to an inaccurate assessment of monocyte subset cell counts.

Importantly, for CD16 staining the use of the 3G8 clone is recommended. Other clones – such as B73.1/Leu11c – may lead to incorrect enumeration of CD16-postive monocytes, after the CD16 B73.1/Leu11c epitope is lost in some primary immunodeficiency diseases (Lenart et al. 2010).

Since the early years of research on monocyte heterogeneity, CD16-positive monocytes were considered proinflammatory cells due to their pronounced expansion in many inflammatory conditions (Fingerle et al. 1993; Grip et al. 2007; Horelt et al. 2002; Katayama et al. 2000; Kawanaka et al. 2002b; Saleh et al. 1995; Soares et al. 2006; Thieblemont et al. 1995) and their selective expression of proinflammatory cytokines such as TNFα and IL12p40/IL12p70 (Belge et al. 2002; Szaflarska et al. 2004). A high expression of distinct adhesion molecules such as VLA-4 endows CD16-positive monocytes with a high endothelial affinity, which allows their homing to the marginal vascular pool, from where they can be mobilized by exercise in a catecholamine dependent manner (Heimbeck et al. 2010; Steppich et al. 2000). This high endothelial affinity is further amplified by interactions of fractalkine (CX_3CL1) with its receptor CX_3CR1, which is highly expressed on CD16-positive monocytes and which subsequently mediates transendothelial migration (Ancuta et al. 2003).

More than one decade after the seminal work by Bernward Passlick, Dimitri Flieger and Löms Ziegler-Heitbrock (Passlick et al. 1989), a more refined understanding of monocyte heterogeneity emerged: by analyzing chemokine receptor expression on CD16-positive monocytes, Ancuta et al. (2003) were able to distinguish a minor subset of $CD14^{++}CD16^{+}$ monocytes from $CD14^{+}CD16^{++}$ monocytes. $CD14^{++}CD16^{+}$ were subsequently characterized by their up-regulation of CCR5 (chemokine (C–C motif) receptor 5) (Ancuta et al. 2003), ACE (angiotensin converting enzyme) (Ulrich et al. 2006) and TLR2 (Urra et al. 2009). Nonetheless, a dichotomized view on human monocyte heterogeneity – distinguishing classical $CD14^{++}CD16^{-}$ monocytes from CD16-positive monocytes – prevailed until 2010, when the existence of three monocyte subsets was finally acknowledged (Ziegler-Heitbrock et al. 2010).

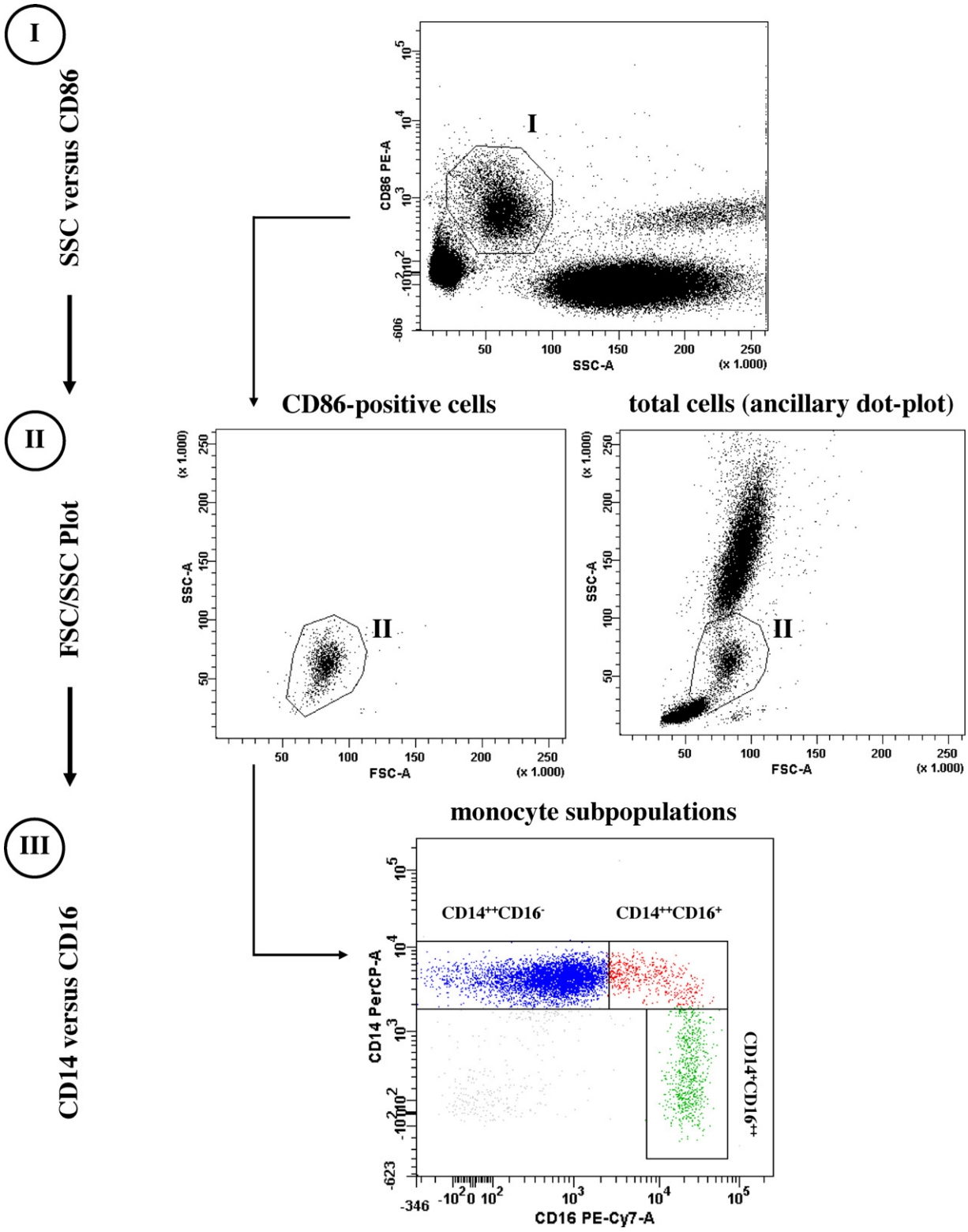


Fig. 1. CD86-based gating strategy for human monocyte subpopulations. After whole blood staining with anti-CD86, anti-CD14 and anti-CD16, monocytes are first identified as CD86-positive cells with monocyte-specific side scatter properties in a SSC/CD86 dot-plot (gate I). Next, CD86-positive leukocytes are depicted in a forward/sideward scatter plot. Here, monocytes are gated according to their characteristic scatter properties (gate II); all leukocytes are presented in an ancillary dot-plot. Cells identified by CD86 expression in gate I and by monocyte-specific scatter properties in gate II are defined as monocytes, and depicted in a separate dot-plot. Here, monocytes are finally subdivided into classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes.

We (Zawada et al. 2011) and other groups (Cros et al. 2010; Shantsila et al. 2011; Wong et al. 2011) set out to further characterize the three human monocyte subsets and demonstrated subset specific functions for all three subsets. Fig. 3 summarizes major markers and functional characteristics of the three monocytes subsets.

By SuperSAGE analysis, we found 97 genes which are selectively expressed in intermediate $CD14^{++}CD16^{+}$ monocytes (Zawada

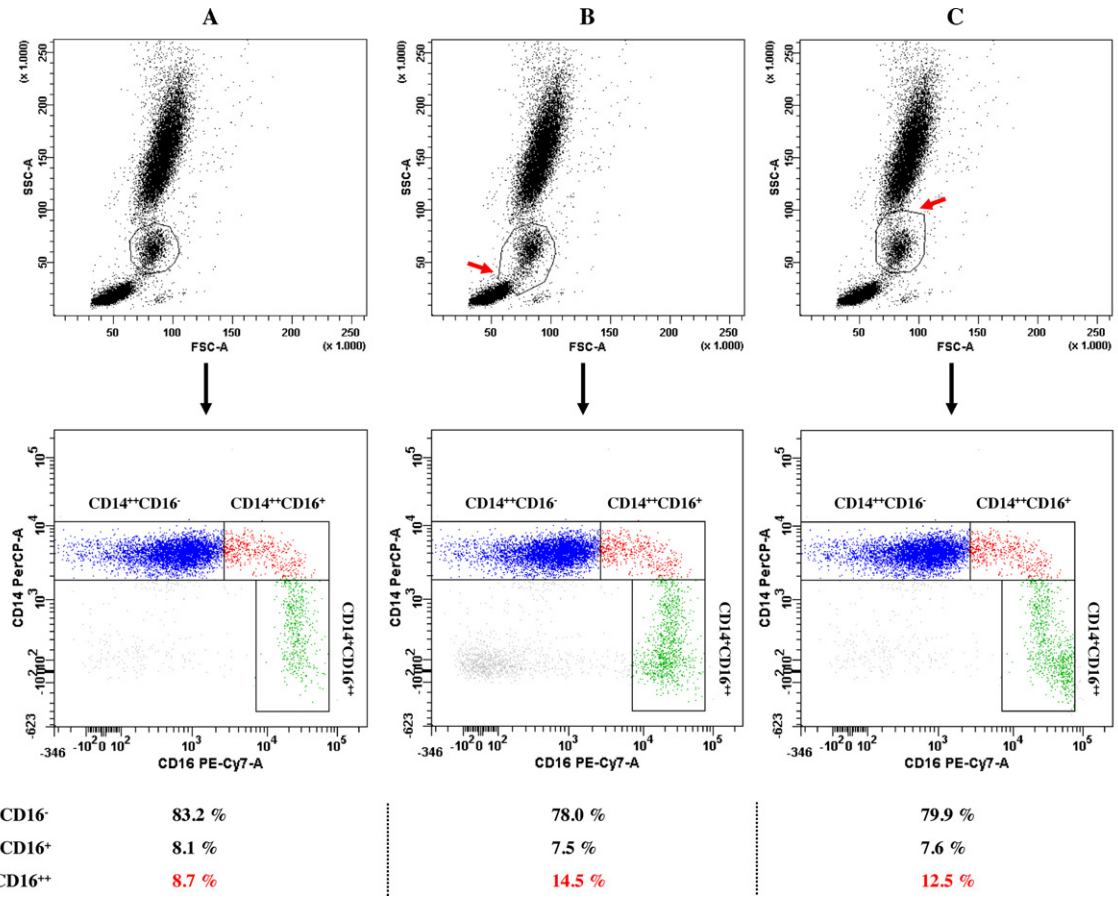


Fig. 2. Evidence for the need of a pan-monocytic marker in flow cytometric analysis of monocytic subpopulations. This representative example illustrates that a gating strategy which merely relies on monocytic scatter properties results in incorrect cell count measurements. This particularly affects nonclassical monocyte counts, as these cells overlap with non-monocytic cells in the FSC/SSC dot-plot. Compared to the CD86-based gating strategy, which specifically identifies monocytes, an exclusively scatter-based monocytic gating strategy will therefore result either in falsely low counts of nonclassical monocytes – when a narrow gate is drawn around monocytes (A), or to falsely high counts of nonclassical monocytes – when a wider gate is drawn, which will inevitably include CD16-positive natural killer cells (B) and/or neutrophils (C). Numbers in parentheses gives percentages of monocyte subsets from the same individual analyzed by a CD86-based gating strategy.

et al. 2011). Additionally, we identified further surface markers for $CD14^{++}CD16^{+}$ monocytes, namely CD74, HLA-DR, TEK (CD202B, TIE2), ENG (CD105) and KDR (VEGFR2), which allow their discrimination *via* flow cytometry. Concurring results were independently presented by Wong et al. (2011) who performed microarray analysis of monocyte subsets and found a selective upregulation of distinct markers in intermediate monocytes (*e.g.* HLA-ABC, CLEC10A (CD301), GFRA2).

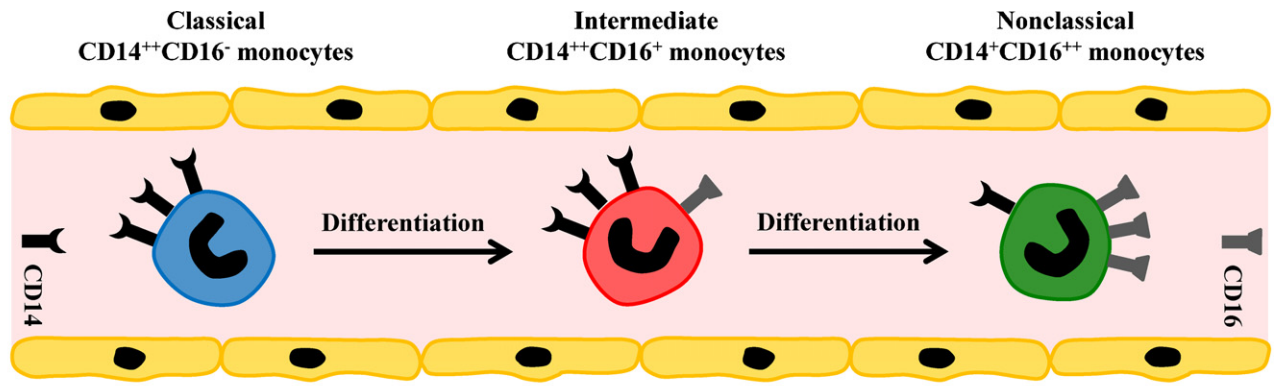
These descriptive analyses are supplemented by functional studies that confirm unique characteristics of $CD14^{++}CD16^{+}$ monocytes, demonstrating their high efficiency to stimulate CD4 T-cell proliferation and their high proangiogenic capacity (Rossol et al. 2011; Zawada et al. 2011). Moreover, they possess a high inflammatory potential, as they are the main producers of ROS in unstimulated conditions (Zawada et al. 2011) and selectively produce IL1β and TNFα upon LPS stimulation (Cros et al. 2010).

In contrast, secretion of IL1β and TNFα by nonclassical $CD14^{+}CD16^{++}$ monocytes is triggered by viruses and nucleic acids (*via* the TLR7-TLR8-MyD88-MEK pathway) rather than by LPS stimulation (Cros et al. 2010). Furthermore, nonclassical monocytes, which express a broad range of adhesion molecules, are predisposed to innate local surveillance of tissues, as they patrol the endothelium in a LFA-1 dependent, crawling behavior (Cros et al. 2010).

Gene expression analyses also suggest developmental relationships between the three monocyte subsets. Transcription of genes that are associated with maturation progressively increases from classical over intermediate to nonclassical monocytes (Wong et al. 2011). These data are in line with clinical studies that reported a consecutive increase, first of intermediate monocytes followed by nonclassical monocytes after treatment with MCSF (Weiner et al. 1994). Finally, nonclassical monocytes have shorter telomere lengths (Merino et al. 2011) arguing for a more mature stage of these cells. Thus, monocyte differentiation is currently considered as a gradual process in which classical monocytes differentiate into intermediate and subsequently into nonclassical monocytes as the most mature cells. In this developmental process, all monocyte subsets have their specialized immunologic functions allowing a fast adaption to altered microenvironments. Of note, formal proof of this concept remains to be provided.

Monocyte heterogeneity in atherogenesis: implications from murine models

At present, the concept of a subset-specific contribution of monocytes to atherosclerotic vascular disease is largely derived from rodent models. The Nomenclature Committee of the International Union of Immunological Societies acknowledged the same number of monocyte subsets in mice as in men – namely three (Ziegler-Heitbrock et al. 2010). However, as CD14 and CD16 are not applicable for the discrimination of murine monocyte subsets, alternative markers – namely Ly6C and CD43 – are used for



Major markers of monocyte subsets		
CCR2[a,b,c]	CCR5[a,b,c,d]	CX_3CR1[a,c]
CD93[e]	CD74[e]	PECAM1 (CD31)[e]
FCGR1A (CD64)[b,e]	HLA-DR[b,e]	SPN (CD43)[e]
ITGAM (CD11B)[e]	TEK (CD202B, TIE2)[e]	ITGAL (CD11A)[e]
CD36[b,e]	ENG (CD105)[e]	CD47[e]
CXCR2[b,c]	KDR (VEGFR2)[e]	CSF1R (CD115)[b]
SELL (CD62L)[b,c]	TLR2[f]	PTGDR2 (CD294)[b]
CCR1[b]	HLA-ABC[b]	SIGLEC10[b]
CXCR1[b]	CLEC10A (CD301)[b]	SELPLG (CD162)[b,e]
CD99[b]	GFRA2[b]	CD86[f]
CLEC4D[b]	ACE (CD143)[g]	ITGA4 (CD49D)[f]
CLEC5A[b]		
IL13RA1[b]		

Functional characteristics of monocyte subsets		
High phagocytosis potential[e]	MHCII processing and presentation; Stimulation of CD4 T cell proliferation[d,e]	Transendothelial migration in response to fractalkine (CX_3CL1)[c]
Production of antimicrobial agents[e]	High basal ROS production[e]	Patrolling endothelium in LFA-1 dependent manner[h]
Strong interactions with platelets[f]	Strong interactions with platelets[i]	Sensing of viruses and nucleic acids *via* TLR7 and TLR8[h]
CCL2 and IL10 production after LPS stimulation[h]	TNFα and IL1β production after LPS stimulation[h]	Short telomere lenghts[m]
	High oxLDL uptake[j]	High oxLDL uptake[j]
	Mobilisation from the marginal pool by exercise[k,l]	Mobilisation from the marginal pool by exercise[k,l]
	Depletion by glucocorticoids[l]	Depletion by glucocorticoids[l]
	Proangiogenic capacity[e]	

[a]Rogacev et al., 2011; [b]Wong et al., 2011; [c]Ancuta et al., 2003; [d]Rossol et al., 2011; [e]Zawada et al., 2011; [f]Urra et al., 2009; [g]Ulrich et al., 2006; [h]Cros et al., 2010; [i]Tapp et al., 2011; [j]Mosig et al., 2009; [k]Steppich et al., 2000; [l]Heimbeck et al., 2010; [m]Merino et al., 2011

Fig. 3. Major markers and functional characteristics of human monocyte subsets summarized from several publications. It is generally assumed that the three human monocyte subpopulations are developmentally related; nonetheless, they are characterized by distinct markers and functional properties.

distinction of monocytes into classical (defined as $Ly6C^{++}CD43^{+}$), intermediate ($Ly6C^{++}CD43^{++}$) and nonclassical ($Ly6C^{+}CD43^{++}$) cells. While the intermediate monocyte subset has been ignored in most rodent experiments, few reports point to a distinct role of intermediate monocytes in murine immunity and suggest that these cells are predisposed to become lymphatic-migrating dendritic cells (Qu et al. 2004).

Importantly, differences in distribution in cell counts of monocyte subsets and in subset-specific cellular functions preclude uncritical transfer of findings from murine experiments to human research (Heine et al. 2012; Rogacev and Heine 2010; Strauss-Ayali et al. 2007). Such differences may partly explain why the term "proinflammatory monocytes" has widely been applied to classical monocytes in murine studies, contrasting with the literature

on human monocyte heterogeneity, where intermediate and/or nonclassical monocytes have been denoted as "proinflammatory monocytes". In line, while gene expression analyses suggest certain similarities between human and murine monocyte subsets, several important markers were found to be conversely expressed between the two species' monocyte subsets, such as CD9, CD36 and TREM1 (triggering receptor expressed on myeloid cells 1). Moreover, subset-specific PPARγ (peroxisome proliferator activated receptor γ) signature and expression of receptors for uptake of apoptotic cells differed between species (Ingersoll et al. 2010); as a shortcoming, CD16-positive monocytes were not differentiated into intermediate and nonclassical monocytes.

Nonetheless, research on murine monocyte biology may add to our understanding of human monocyte heterogeneity. For obvious reasons, studies on the developmental relationship of monocyte subsets are far more advanced in mice than in men, and depletion studies identified classical monocytes as the first monocytes which appear in the circulation, followed by intermediate and nonclassical monocytes (Sunderkotter et al. 2004).

Both humans and mice express the chemokine receptor CCR2 on classical monocytes. In rodent experiments, this receptor induces the egress of classical monocytes from the bone marrow, and it maintains normal blood monocyte counts in the circulation (Serbina and Pamer 2006; Tsou et al. 2007). In contrast, homeostasis of nonclassical monocytes requires CX_3CR1 rather CCR2, given that the CX_3C-axis provides an essential survival signal for this monocyte subset (Landsman et al. 2009).

Murine classical monocytes can give rise into inflammatory macrophages and dendritic cells during inflammation as well as under homeostatic conditions (Geissmann et al. 2003; Woollard and Geissmann 2010). In contrast, nonclassical monocytes were termed "patrolling monocytes" as they crawl on the endothelium in a LFA-1 dependent manner, allowing rapid tissue invasion in case of damage or infection, and subsequent differentiation into macrophages (Auffray et al. 2007).

In line with their distinct roles in homeostasis and inflammation, murine monocyte subsets may have different implications in the process of atherogenesis. In hypercholesterolemia, cell counts of classical monocytes rise dramatically (Swirski et al. 2007; Tacke et al. 2007). At the same time, classical monocytes display an enhanced adhesion capacity to the activated endothelium, which is followed by their infiltration into atherosclerotic lesions and by their differentiation into lesional macrophages (Swirski et al. 2007; Tacke et al. 2007). Nonclassical monocytes enter the atherosclerotic plaques in a CCR5-dependent manner, albeit to a lesser extent than classical monocytes (Tacke et al. 2007). Moreover, due to their patrolling behavior and their upregulation of central adhesion molecules – such as CX_3CR1 and LFA-1 –, nonclassical monocytes may be further predisposed to contribute to early atherogenesis (Woollard and Geissmann 2010).

Importantly, most rodent studies which analyzed monocyte heterogeneity in atherogenesis focused on the distinction between classical and nonclassical monocytes, ignoring the impact of intermediate monocytes. We are convinced that the recent appraisal of monocytic trichotomy may allow an even more thorough understanding of murine atherogenesis.

Monocyte heterogeneity in human atherosclerosis

Both the limited homology in monocyte heterogeneity in different species, as well as distinct pathophysiological patterns of atherogenesis between men and mice (Libby et al. 2011) preclude an uncritical transfer of results from murine studies to human pathology. This may explain why most experts consider classical monocytes as central drivers of murine atherogenesis, while CD16-positive monocytes – *i.e.* intermediate and/or nonclassical monocytes – have been clinically and mechanistically implicated in the pathology of human atherosclerosis. Unfortunately, most reports on monocyte heterogeneity in human atherogenesis did not consider monocytic trichotomy; instead, in analogy to murine studies, human intermediate and nonclassical monocytes were often analyzed as a single population.

This is a matter of concern because the contribution of intermediate and nonclassical monocytes to atherogenesis may differ substantially. Compared to nonclassical monocytes, intermediate monocytes may possess a higher proinflammatory armamentarium, as *in vitro* studies found an enhanced production of ROS, TNFα and IL1β by intermediate monocytes (Cros et al. 2010; Zawada et al. 2011). Moreover, these cells selectively express the chemokine receptor CCR5 (Ancuta et al. 2003; Rogacev et al. 2011), a marker which has been associated with atherosclerosis in large epidemiological studies (Gonzalez et al. 2001; Muntinghe et al. 2009; Pai et al. 2006).

Beyond such *in vitro* experiments, several clinical studies aimed to assess the implication of monocyte heterogeneity in human cardiovascular disease. In the following, we first summarize cohort studies which assessed relationships between cell counts of monocyte subsets and specific cardiovascular risk factors, namely obesity, hypercholesterolemia and chronic kidney disease. Secondly, we review cross-sectional studies that analyzed associations of cell counts of monocyte subsets with prevalent (sub)clinical cardiovascular disease. Finally, prospective studies that assessed cell counts of monocyte subsets as predictors of cardiovascular outcome are discussed.

Epidemiological studies either report absolute cell counts of monocytes subsets (as cell/μl blood), or relative cell counts (percentages among all monocytes). Throughout the following article, we will use the term "cell counts" when absolute cell numbers are reported, and "frequency" when relative cell numbers are given.

Cardiovascular risk factors

Obesity and monocyte heterogeneity

Obesity – which is increasingly acknowledged as a strong cardiovascular risk factor – is a chronic inflammatory condition, in which adipose tissue generates diverse proinflammatory mediators (Rocha and Libby 2009). We (Rogacev et al. 2010) and others (Poitou et al. 2011) have suggested that monocytes may link obesity and cardiovascular disease.

Cottam et al. (2002) first demonstrated a shift towards CD16-postive monocytes in a small group of 26 morbidly obese (WHO obesity class III; BMI > 40 kg/m^2) patients. Only recently, Poitou et al. (2011) extended these findings in a second, larger cohort which comprised 105 patients with WHO obesity class II and III (BMI > 35 kg/m^2) involved in a gastric surgery program, 39 preobese and WHO obesity class I subjects who underwent a weight reduction program (BMI 25–35 kg/m^2), and 32 lean healthy controls. Frequencies and cell counts of intermediate and nonclassical monocyte subsets were substantially higher in WHO obesity classes II and III, but not in less severe obesity, compared to controls. Similar associations were found between CD16-positive monocyte subsets and fat mass, assessed by DXA (dual-energy X-ray absorptiometry). Finally, homeostasis model assessment (HOMA) insulin resistance (HOMA-IR), as an indicator of insulin resistance, was associated with intermediate and nonclassical monocytes in univariate, but not in multivariate analysis.

Notably, serial monocyte measurements in 36 obese subjects who underwent RYGB-(Roux-en-Y gastric bypass) for weight loss detected a strong decrease of intermediate and nonclassical

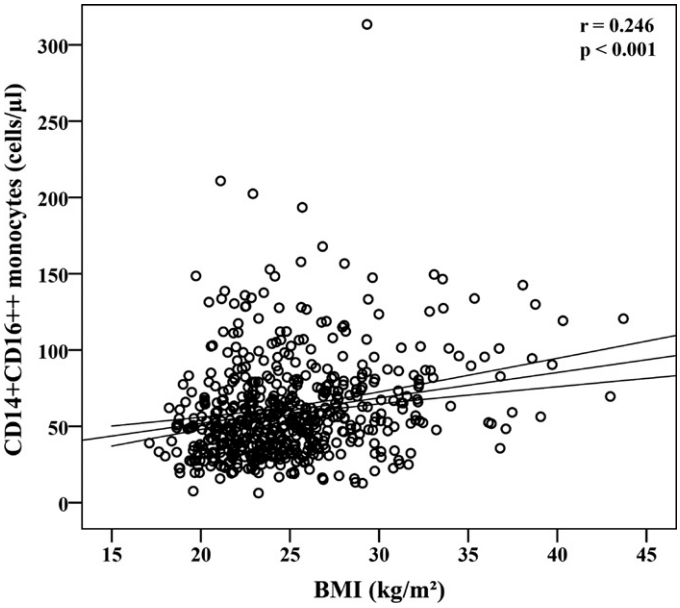


Fig. 4. Correlation of body-mass-index (BMI) with counts of nonclassical monocytes in 569 I LIKE HOMe study participants. To allow better visualization, data from a single super-obese participant (BMI = 58.5) are not depicted in the figure. Correlation coefficients were calculated by Spearman test.

monocyte frequencies, which was more pronounced in the latter subset. Congruent findings were reported by Cottam et al. (2002).

By far the largest database on the associations of monocyte heterogeneity with body weight and other cardiovascular risk factors in apparently healthy subjects comes from our I LIKE HOMe (Inflammation, Lipoprotein Metabolism and Kidney Damage in early atherogenesis – The Homburg Evaluation) study (Rogacev et al. 2010). Monocyte subset counts were analyzed in 569 healthcare workers aged between 25 and 60 years without prevalent cardiovascular disease, systemic immunosuppressive treatment, diabetes mellitus, or advanced chronic kidney disease.

Among I LIKE HOMe participants, BMI correlated with cell counts of nonclassical monocytes ($r = 0.246$; $p < 0.001$) (Fig. 4), whereas no correlation was found with cell counts of classical and intermediate monocytes. Interestingly, the correlation between BMI and nonclassical monocytes remained significant after exclusion of participants with BMI > 35 kg/m^2.

Presently, it remains enigmatic which factors drive the shift in monocyte subsets in obese individuals.

Hypercholesterolemia and monocyte heterogeneity

Interactions between lipid metabolism and human monocyte subset biology have been suggested by several *in vitro* studies, which found a different pattern of scavenger receptors across monocyte subsets, along with a subset-specific uptake of native, oxidized and enzymatically degraded LDL-cholesterol (Draude et al. 1999; Kapinsky et al. 2001; Mosig et al. 2009; Stohr et al. 1998).

In a clinical study, Rothe et al. (1996) demonstrated that frequencies of nonclassical monocytes negatively correlate with the concentration of HDL-C in a small group of hypercholesterolemic patients, but not among healthy controls. Of note, correlations of CD16-positive monocyte frequencies with total and LDL-cholesterol were not reported. Polymorphism analysis of the ApoE genotype suggested that dyslipidemia was the inducer rather than the consequence of low counts of nonclassical monocytes. Interestingly, in a separate cohort of 79 hypercholesterolemic patients with coronary artery disease, high total cholesterol and high serum triglycerides rather than low HDL-cholesterol was correlated with high frequencies of nonclassical monocytes (Rothe et al. 1999). In their analysis on the association between obesity and monocyte heterogeneity, Poitou et al. (2011) very recently confirmed this association of high frequencies of nonclassical monocytes with high triglycerides and low HDL-cholesterol in univariate analysis. Interestingly, adjustment for BMI virtually eliminated the impact of lipid parameters on monocyte frequencies in this highly selected population.

To further investigate associations between lipid metabolism and monocyte heterogeneity, we re-analyzed data from 565 I LIKE HOMe participants who did not receive lipid-lowering drugs at study inclusion. We confirmed a weak – albeit significant – negative correlation between plasma HDL-cholesterol and nonclassical monocyte counts ($r = -0.095$; $p = 0.025$), as well as a positive correlation between plasma triglycerides and these cells ($r = 0.156$; $p < 0.001$). In line with Poitou et al., adjustment for body mass index eliminated these significant associations (partial correlation coefficients: HDL-cholesterol: $r = 0.016$; $p = 0.698$; triglycerides $r = 0.054$; $p = 0.199$). Fig. 5 illustrates the interaction between HDL-cholesterol (Fig. 5A), triglycerides (Fig. 5B), nonclassical monocyte counts, and BMI.

Results from studies that analyzed the fate of monocyte subsets after therapeutic interventions yielded very conflicting results. Again, Rothe et al. (1999) were the first to report a statin-induced shift in monocyte subset frequencies when patients were randomized to receive a cholesterol lowering therapy using fluvastatin, or placebo, both combined with cholesterol lowering diet counseling. Surprisingly, an increase in nonclassical monocytes was observed in both study groups, which was even more pronounced under statin therapy; patients on statin treatment additionally experienced an increase in intermediate monocytes (Rothe et al. 1999). In striking contrast, Coen et al. (2010) found no change in CD16-positive monocyte frequencies in patients after therapy with 10 mg rosuvastatin. Finally, cell counts of classical and CD16-positive monocytes remained stable in patients who were admitted to hospital for unstable angina pectoris, and who initiated statin treatment, while a shift towards CD16-positive monocytes was observed in a control group (Imanishi et al. 2010).

In summary, conflicting data on the associations between lipid metabolism, intake of lipid lowering agents and monocyte heterogeneity were reported. Furthermore, associations between nonclassical monocytes and lipid parameters vanished after adjustment for BMI in epidemiological studies. Therefore we believe that other obesity-related factors than hypo-HDL-cholesterinemia or hypertriglyceridemia causally induce shifts in monocyte subset distribution in metabolic disease.

Chronic kidney disease and monocyte heterogeneity

Chronic kidney disease (CKD) has been acknowledged as a cardiovascular risk factor in the last decade and may soon be classified as a cardiovascular disease equivalent (Sarnak et al. 2003). CKD patients suffer accelerated atherosclerosis, leading to a high incidence of cardiovascular events (de Jager et al. 2009; Go et al. 2004).

Ongoing microinflammation has been identified as a hallmark of CKD. Similar to patients with other chronic inflammatory diseases (Fingerle et al. 1993; Grip et al. 2007; Horelt et al. 2002; Katayama et al. 2000; Kawanaka et al. 2002b; Saleh et al. 1995; Soares et al. 2006; Thieblemont et al. 1995), dialysis patients face a shift towards CD16-positive monocytes, which has first been described by Nockher and Scherberich (1998) and subsequently confirmed by several other studies (Brauner et al. 1998; Carracedo et al. 2006; Kawanaka et al. 2002a; Ramirez et al. 2005; Sester et al. 2001). This expansion comprises both CD16-positive monocyte subsets, intermediate and nonclassical monocytes.

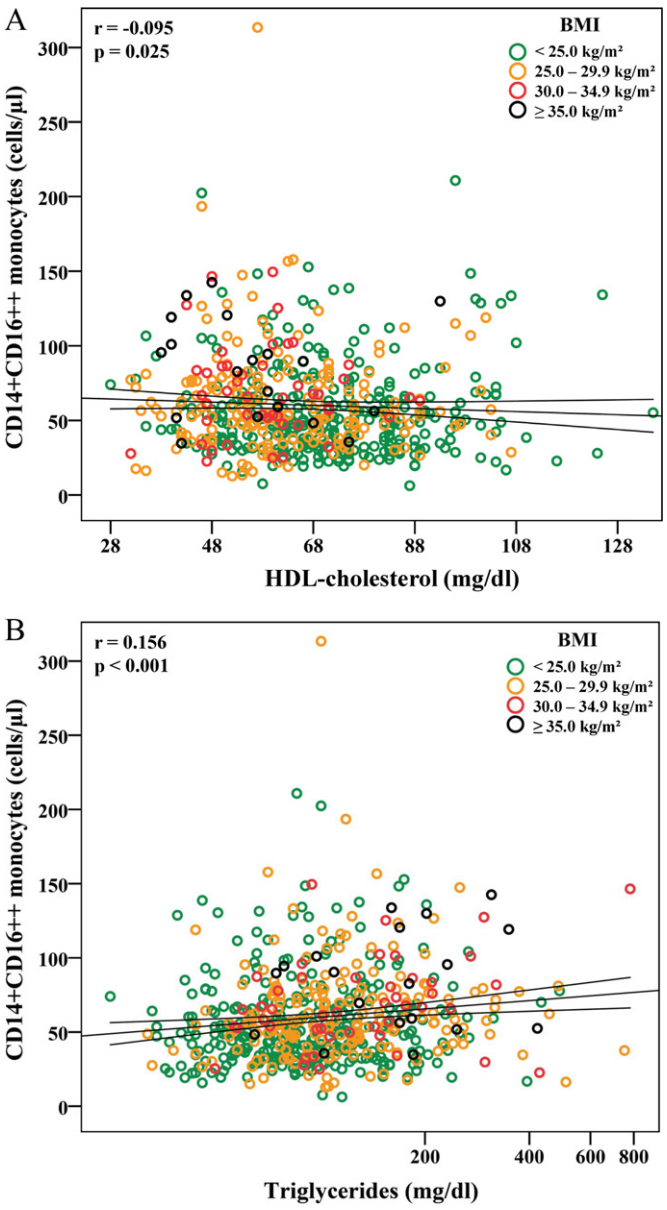


Fig. 5. Correlations of plasma triglycerides (Fig. 5A) and plasma HDL-cholesterol (Fig. 5B) with counts of nonclassical monocytes in 565 I LIKE HOMe study participants (after exclusion of four individuals on lipid lowering therapy). To illustrate interactions between nonclassical monocyte counts, parameters of lipid metabolism and obesity, BMI categories of all participants are marked. Because of skewed distribution, plasma triglycerides are depicted on a logarithmic scale. Correlation coefficients were calculated by Spearman test.

For a detailed review on monocyte heterogeneity and other components of immune dysregulation in CKD patients, the interested reader is referred to recent review articles (Heine et al. 2012; Rogacev and Heine 2010). In brief, the underlying pathological processes for this shift in monocyte subpopulations are not fully understood. It is speculated that the dialysis procedure – which comprises immunostimulation by dialysis membranes and fluids – *per se* significantly contributes to this shift. In line, patients at earlier CKD stages not requiring dialysis have markedly lower CD16-positive monocyte counts than dialysis patients (Rogacev et al. 2011). Moreover, each dialysis session induces a strong transient drop of CD16-positive monocytes (Nockher et al. 2001; Rogacev et al. 2009; Sester et al. 2001), which is thought to result from monocyte activation and their subsequent attachment to endothelial cells. At the end of each hemodialysis session, counts of CD16-positive monocytes return to baseline values.

Monocyte heterogeneity in prevalent cardiovascular disease

In line with the associations between cardiovascular risk factors and monocyte heterogeneity, a growing body of cross-sectional epidemiological studies indicates a central role of CD16-postive monocytes in early subclinical atherosclerosis as well as in advanced cardiovascular disease.

Subclinical atherosclerosis

The largest cohort study among apparently healthy individuals is the I LIKE HOMe study, which sonographically assessed subclinical atherosclerotic vascular disease by measuring common carotid intima media thickness (IMT) (Rogacev et al. 2010). Among its 569 participants, the I LIKE HOMe study found a significant correlation of cells counts of CD16-positive monocytes and IMT. When subdividing CD16-positive monocytes into intermediate and nonclassical monocytes, only the latter remained significantly associated with IMT. Notably, the association between CD16-positive monocytes (or nonclassical monocytes) and IMT lost statistical significance after adjusting for BMI, again pointing towards a substantial interaction between obesity, inflammation and cardiovascular disease.

Conflicting results on the association between common carotid atherosclerosis and monocyte subsets were reported in smaller and highly selected patient cohorts: in line with the I LIKE HOMe study results, frequencies of nonclassical monocytes correlated with carotid IMT among stable renal allograft recipients, while frequencies of intermediate monocytes did not (Ulrich et al. 2008). In contrast, hemodialysis patients with advanced carotid plaques tended to have higher counts of classical and intermediate monocytes compared to dialysis patients without advanced plaques (Ulrich et al. 2011). Finally, in WHO obesity classes II and III patients, no association was found between frequencies of CD16-positive monocytes and subclinical atherosclerosis. Three months after bariatric surgery, a decreasing intermediate monocyte population size was univariately correlated with a reduction in IMT; this finding again depended on BMI variation (Poitou et al. 2011).

Clinically manifest cardiovascular disease

Few cross-sectional studies analyzed monocyte heterogeneity in patients with manifest cardiovascular disease. The first study – and up to now the largest – reported higher frequencies of CD16-positive monocytes in a heterogenous cohort of 247 patients at various stages of coronary artery disease (CAD), including both stable CAD patients and patients with acute coronary syndrome (Schlitt et al. 2004). As a methodological shortcoming, a less sophisticated gating strategy than nowadays recommended was applied, which did not use a pan-monocytic marker such as CD86 or HLA-DR for identifying monocytes. Moreover, intermediate and nonclassical monocytes were not separately analyzed. Subsequently, two small cross-sectional studies confirmed a shift towards CD16-positive monocytes (Tallone et al. 2011; Wildgruber et al. 2009). However, subsuming both studies, a total of only 25 healthy controls and 32 CAD patients were analyzed.

When subdividing CAD patients with stable angina pectoris by plaque characteristics (assessed by multidetector computed tomography), elevated frequencies of CD16-positive monocytes were only found in patients who had at least one vulnerable plaque (Kashiwagi et al. 2010). Finally, a decrease in the CD16-positive monocyte population size was associated with long-term

plaque stabilization in CAD patients with instable angina pectoris (Imanishi et al. 2010).

Intermediate monocytes as predictors of cardiovascular outcome

Following these cross-sectional studies, the question arises whether a specific monocyte subset might serve as a biomarker for prediction of cardiovascular events. First prospective studies on this issue were performed in CKD patients, which unanimously found that high counts of intermediate monocytes independently predicted adverse outcome among 94 dialysis patients as well as among 119 patients at earlier stages of CKD (Heine et al. 2008; Rogacev et al. 2011). Two subsequent cohort studies suggested that analysis of intradialytic monocyte kinetics or monocytic expression of ACE may yield additional prognostic information (Rogacev et al. 2009; Ulrich et al. 2010).

Because of altered monocyte subset distribution and the distinct pattern of accelerated atherosclerosis in CKD, these findings should not uncritically be extrapolated to the general population. Currently, data on the predictive role of monocyte subsets in non-CKD populations are scarce.

Therefore we initiated the HOM Sweet HOMe (Heterogeneity of Monocytes in subjects who undergo elective coronary angiography – The Homburg Evaluation) study which analyzed monocyte heterogeneity and cardiovascular events in 954 patients referred for elective coronary angiography. First results have been presented at the 2011 EMDS meeting in Brussels; in this preliminary analysis intermediate monocyte counts were predictors of cardiovascular events in univariate analysis.

These findings are seemingly in conflict with a second cohort study from Sweden, where Berg et al. (2012) randomly selected 700 subjects from the Malmö Diet and Cancer study, in whom monocyte subsets were measured from isolated, frozen stored mononuclear leukocytes. At univariate analysis, counts of all three monocyte subsets were higher in cases *versus* controls. After adjustment for confounders, high classical monocyte counts – but not frequencies – predicted cardiovascular events. Notably, the accompanying editorial advocates a cautious interpretation of these data (Mehta and Reilly 2012). It is critical to note that determination of monocyte subsets was not performed from fresh blood, which contrasts with current recommendations (Ziegler-Heitbrock et al. 2010). Isolation and long-term storage of monocytes could have caused alterations in monocyte subset distribution and phenotype. For example, contrasting with its signature character (Ancuta et al. 2003; Rogacev et al. 2011; Rossol et al. 2011; Wong et al. 2011), CCR5 was not selectively expressed on intermediate monocytes in this study. Furthermore, a yet not characterized CD14dimCD16dim subpopulation was found (as illustrated in a representative dot-plot from a long-term frozen sample) which may have arisen from the preparation procedure or from storage. Finally, gating of monocytes was not performed *via* a validated gating strategy (Heimbeck et al. 2010; Zawada et al. 2011), possibly resulting in inaccurate assessment of CD16-positive monocyte counts (see Fig. 2).

Key features of the Malmö Diet and Cancer study, and of the HOM SWEET HOMe study, are summarized in Table 1.

Table 1
Features of the Malmö Diet and Cancer study and the HOM SWEET HOMe study.

	Malmö Diet and Cancer study	HOM SWEET HOMe study
Study design	Retrospective analysis (recruitment 1991–1994)	Prospective analysis (recruitment 2007–2010)
Sample preparation	Frozen peripheral blood mononuclear cells	Fresh whole blood
Number of Patients	700	954
Follow-up	Until 12/2008	Ongoing
Definition of endpoints	*Endpoints*: Myocardial infarction; ischemic stroke; death attributable to underlying coronary heart disease	*Combined primary endpoint*: Cardiovascular death, acute myocardial infarction or non-hemorrhagic stroke *Secondary endpoints*: Death of any cause, any cardiovascular event
Endpoint adjudication	Registry analysis by ICD-Code	Diagnosis confirmed by chart review
Outcome predictor	$CD14^{++}CD16^{-}$ monocytes	$CD14^{++}CD16^{+}$ monocytes

ICD-Code: International Classification of Diseases Code.

Monocyte heterogeneity in human myocardial infarction

Following reports on a subset-specific contribution of monocytes to murine myocardial infarction (Nahrendorf et al. 2010), monocyte heterogeneity in human myocardial infarction (MI) has attracted substantial interest in recent years. Animal data point to a sequential contribution of monocyte subsets to injury and repair (Nahrendorf et al. 2010).

The first human study that serially assessed monocyte subsets in human myocardial infarction was reported by Tsujioka et al. (2009) from Wakayama (Japan), who analyzed 36 patients suffering from acute myocardial infarction (AMI). In line with animal studies, a sequential mobilization of monocyte subsets was found. An increase of classical monocytes counts occurred very early after hospital admission. Cell counts peaked 2.6 days after AMI, and decreased afterwards. In contrast, CD16-positive counts were low at hospital admission (compared to patients with stable angina pectoris) and gradually increased until day 4.8. Afterwards, their cell counts remained elevated until the end of the observation period on day 12 compared to baseline values. Interestingly, peak cell counts of classical monocytes were negatively associated with myocardial salvage (assessed *via* by cardiovascular magnetic resonance imaging [CMRI] at day 7) and negatively correlated with recovery of left ventricular (LV) function (assessed *via* CMRI at day 7 and month 6), while peak counts of CD16-positive monocytes were not.

The same study group later associated peak classical monocyte counts with microvascular obstruction in AMI, again assessed *via* CMRI (Tsujioka et al. 2010), whereas CD16-positive monocyte counts rather than classical monocytes were reported to predict in-stent restenosis after coronary stenting in AMI (Liu et al. 2010). Associations between classical monocyte counts in AMI, impaired left ventricular function and microvascular obstruction were independently confirmed by van der Laan et al. (2012). For logistic reasons, an unconventional gating strategy for monocyte subsets was applied; moreover, despite the sequential mobilization of monocyte subsets after AMI (Tsujioka et al. 2009), the time-point of flow cytometric analysis was not standardized, and no serial measurements were performed.

Finally, the Wakayama group reported that intake of the direct renin inhibitor aliskiren may dampen the peak of classical monocyte counts after AMI, and may associate with higher myocardial salvage. The impact of aliskiren on CD16-positive monocytes was not explicitly reported (Ozaki et al. 2012).

Unfortunately, neither the Wakayama group (Liu et al. 2010; Ozaki et al. 2012; Tsujioka et al. 2009; Tsujioka et al. 2010), nor van der Laan et al. (2012) used a pan-monocytic marker – CD86 or HLA-DR – for monocyte identification. Moreover, both did not provide separate data on intermediate and nonclassical monocytes counts.

The impact of this limitation is illustrated by a very recent work of Tapp et al. (2012), who differentiated intermediate and nonclassical monocytes in patients suffering from myocardial infarction (Tapp et al. 2012). Indeed, they found unique dynamics and functional characteristics of the intermediate monocyte subset in patients after STEMI (ST-elevation myocardial infarction): cell counts of intermediate monocytes increased dramatically in these patients compared to control groups, whereas no differences were seen in the counts of nonclassical monocytes.

Conclusions

23 years after the initial description of human monocyte heterogeneity, a subset-specific contribution of monocytes to health and disease is unequivocally acknowledged. Unfortunately, uncritical transfer of animal studies to human (patho)physiology, and lack of uniform denomination of monocyte subsets as well as discrepant laboratory techniques between different scientific groups impede a clear understanding on how monocyte subsets contribute to cardiovascular disease in men. We therefore advocate general application of the monocyte subset nomenclature that has been suggested by the Nomenclature Committee of the International Union of Immunological Societies, and general utilization of validated flow cytometry protocols.

Despite these limitations, several experts started to propose subset-specific immunomodulation of monocytes as future treatment strategies in human cardiovascular disease. For the time being, evidence from *in vitro* experiments and from clinical cohort studies identified intermediate monocytes as most promising targets for therapeutic interventions in stable patients with atherosclerotic vascular disease. In contrast, the impact of monocyte subsets in acute myocardial infarction needs further preclinical studies before subset-specific therapeutic interventions should be proposed.

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Full Review

Clinical relevance of epigenetic dysregulation in chronic kidney disease-associated cardiovascular disease

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ABSTRACT

Across the spectrum of clinical medicine, the field of epigenetics has gained substantial scientific interest in recent years. Epigenetics refers to modifications in gene expression which are not explained by changes in DNA sequence. Classical components of epigenetic regulation comprise DNA methylation, histone modifications and RNA interference. In chronic kidney disease (CKD), several features of uraemia, such as hyperhomocysteinemia and inflammation, may contribute to changes in epigenetic gene regulation. It has been suggested that these changes may affect genes related to cardiovascular disease. Thereby, a uraemia-associated disturbance in epigenetic regulation may contribute to the substantial increase in cardiovascular morbidity in CKD patients. The present review aims to summarize current knowledge of epigenetic dysregulation in cardiovascular disease from a nephrological perspective, with a special focus on DNA methylation. We first describe the impact of altered epigenetic regulation in non-CKD-associated arteriosclerosis, and next characterize uraemic features which may affect epigenetic gene regulation in the context of cardiovascular disease. Finally, we conclude that substantial additional work is needed before epigenetic regulatory mechanisms may become therapeutic targets in CKD-associated cardiovascular disease.

INTRODUCTION

Patients with chronic kidney disease (CKD) have an unacceptably high risk for cardiovascular events, which is mainly attributable to dramatically accelerated vascular disease. Traditional cardiovascular risk factors, such as hypercholesterolaemia, hyperglycaemia, arterial hypertension, smoking, obesity and physical inactivity, can only partly explain this high cardiovascular risk [1]. In line, conventional cardiovascular treatment strategies failed to improve cardiovascular survival in CKD patients substantially [1, 2].

Thus, future therapies will have to focus on non-classical cardiovascular risk factors, among which uraemia-associated alterations in epigenetic gene regulation attracted some interest in recent years. Epigenetic mechanisms are crucial regulators of cellular homeostasis, which control gene expression and maintain cell identity during subsequent cell divisions. Consequently, dysfunctional epigenetic gene regulation may substantially contribute to the onset and progression of diverse pathologies such as cancer or arteriosclerosis.

It is increasingly recognized that environmental factors may influence epigenetic regulation. In line, it is common opinion that in CKD patients, long-term exposure to the unphysiological uraemic milieu may affect epigenetic mechanisms, which may eventually comprise the regulation of arteriosclerosis-related genes.

The present review summarizes recent findings in the field of epigenetics regarding arteriosclerosis and CKD. Notably, epigenetic dysregulation may affect many other aspects of renal medicine not covered in this article. Starting in early life, when maternal–foetal epigenetic interactions are of paramount importance for human development [3], epigenetic mechanisms are of essential importance for renal physiology, and their dysregulation may induce and perpetuate renal disease [4].

Admittedly, research on epigenetic regulation in CKD is still an evolving field in its very beginnings, whereas epigenetics has been extensively studied in other disciplines of

internal medicine. In oncology, epigenetic research has entered the scene as early as 1983, when Feinberg and Vogelstein first demonstrated epigenetic dysregulation in cancer cells [5]. Since then, a steady increase in the understanding of epigenetic mechanisms in the pathogenesis of human cancer allowed to define new therapeutic strategies in oncology (summarized in [6–8]). In 2004, the Food and Drug Administration approved the DNA methyltransferase inhibitor azacitidine for treatment of subtypes of myelodysplastic syndrome (MDS), where it proved to prolong survival compared with standard care [9]. Thus, adequate understanding of epigenetic dysregulation in human disease may allow to improve outcome in affected patients.

DNA METHYLATION AS A CENTRAL EPIGENETIC REGULATOR

The term 'epigenetics' refers to changes in gene expression which are caused by altered DNA accessibility without affecting the nucleotide sequence. In contrast to mutations in the DNA sequence, epigenetic marks are dynamic; they can be altered by exogenous factors including nutrition and environmental influences. Nonetheless, epigenetic mechanisms are sufficiently stable to transmit information on gene expression from one cell generation to the next; they control diverse biological phenomena such as X-chromosome inactivation, silencing of transposable elements or genomic imprinting [10].

Epigenetic mechanisms comprise several levels of gene regulation, which include DNA methylation, histone modifications and RNA interference. The present review will focus on DNA methylation, which is the best understood epigenetic modification in the context of CKD. For space constraints, the role of histone modifications and RNA interference in epigenetic regulation are not explained in detail, as excellent reviews have been published recently, to which the interested reader may refer to [11–13].

In mammals, DNA methylation occurs at the C5 position of cytosines predominantly in the context of CpG dinucleotides (cytosines followed by guanines). The 5′ regulatory regions of many genes are enriched in CpG dinucleotides which form so-called CpG islands, and their methylation generally prevents gene transcription. In contrast, transcriptionally active DNA regions are typically unmethylated (Figure 1) [14]. This mechanism of activation and repression of gene transcription by differential DNA methylation is mediated via alterations in chromatin configuration and accessibility of the transcription machinery to the promoter region.

DNA is associated with histones and other chromosomal proteins, which themselves can also be modified. Different modifications of histones exist such as acetylation or methylation, which regulate the degree of chromatin condensation and consequently the level of transcription. Of note, a cross-talk between DNA methylation and histone modifications exists, so that silencing of gene expression by DNA methylation is often associated with, e.g., deacetylation of histones in the same genomic region (Figure 1).

DNA METHYLATION AND ARTERIOSCLEROSIS IN NON-CKD

Within the spectrum of arteriosclerotic vascular disease, atherosclerosis defines a chronic inflammatory process characterized by endothelial cell activation, by infiltration of circulating monocytes and other leukocytes into the subendothelial space, by subsequent differentiation of monocytes towards macrophages and dendritic cells and by migration and proliferation of smooth muscle cells (SMCs) (Figure 2). Thus, transformation of several distinct cell types essentially contributes to the development of atherosclerosis (atherogenesis), and these cellular transformations necessarily require reprogramming of gene expression. Therefore, it is highly probable that epigenetic mechanisms may be centrally involved in atherosclerosis.

In recent years, several clinical and experimental studies analysed the implications of disturbed DNA methylation in arteriosclerosis. Until now, only few studies focussed on CKD patients. All the more, a critical appraisal even of non-CKD studies appears fruitful to the nephrological community, augmenting our understanding of potential implications of epigenetic regulation in accelerated arteriosclerosis. In the following, we first discuss human studies which analysed global DNA methylation rather than methylation of specific genes. Secondly, we review human studies on gene-specific DNA methylation. Finally, we summarize human studies that assessed global or gene-specific DNA methylation in the context of CKD.

GLOBAL ASSESSMENT OF DNA METHYLATION IN ARTERIOSCLEROSIS

Global DNA methylation in cardiovascular disease has been assessed by analysing either peripheral blood cells, or vascular tissue. A small cohort study reported lower DNA methylation in peripheral blood cells (measured by cytosine extension assay) among 17 male patients with prevalent cardiovascular disease compared with 15 male healthy controls [15]. Such an association between global DNA hypomethylation and prevalent cardiovascular disease was confirmed in the larger Normative Aging Study, when analysing DNA methylation of long interspersed nucleotide element-1 (LINE-1) repetitive elements in peripheral blood mononuclear cells as a marker for global DNA methylation among 712 elderly men [16]. Moreover, lower LINE-1 methylation at study initiation predicted cardiovascular mortality in this cohort.

Contrarily, global DNA hypermethylation rather than hypomethylation in peripheral blood cells was reported among 137 Indian coronary artery disease (CAD) patients compared with 150 controls (assessing global DNA methylation by cytosine extension assay) [17], and among 101 Singapore Chinese Health Study participants with prevalent myocardial infarction and/or stroke compared with 185 controls (assessing leukocyte DNA methylation of repetitive elements as global DNA methylation marker) [18]. In the latter study,

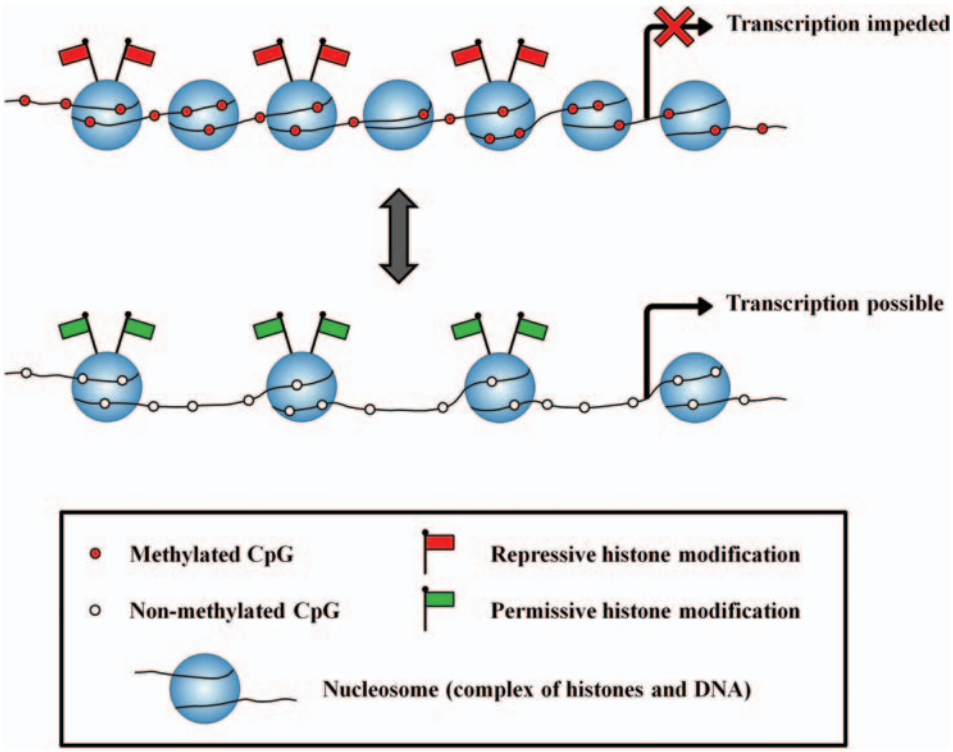

FIGURE 1: Mechanism of epigenetic gene regulation. Two major components of epigenetic regulation are DNA methylation and histone modifications. (1) DNA methylation occurs at the C5 position of cytosines in the context of CpG dinucleotides (indicated as filled circles). An open chromatin structure (feature of transcriptionally active genes) is characterized by unmethylated CpGs (indicated by open circles). (2) Further regulatory mechanisms are provided by histone modifications, which may either allow ('permissive modifications') or silence ('repressive modifications') gene transcription. Of note, interactions between both regulatory pathways exist.

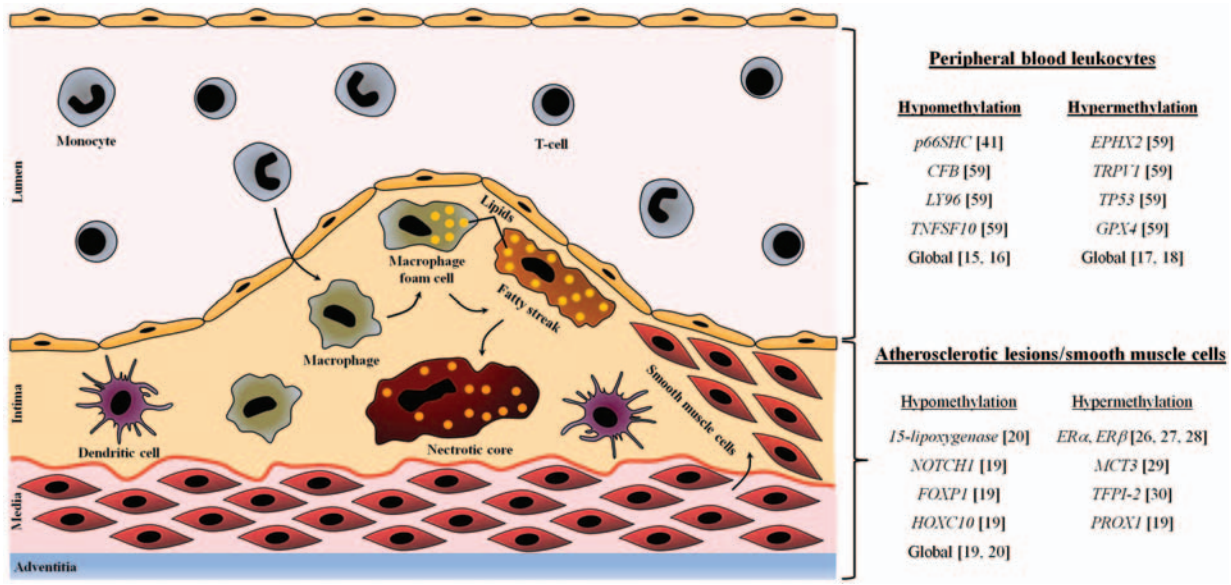

FIGURE 2: DNA methylation in atherosclerosis. Following initial endothelial activation, circulating monocytes and other leukocyte subsets are recruited into the subendothelial space. There, monocytes differentiate into macrophages, which take up lipids to form foam cells and thus give rise to fatty streaks, which are the earliest ultrastructural alterations in atherosclerosis. These early atherogenic lesions may subsequently gradually develop into advanced atherosclerotic plaques, which are characterized by a lipid- and macrophage-rich necrotic core; migration of SMCs from the tunica media into the tunica intima may further contribute to this atherogenic process. Current epigenetic knowledge from analyses of global and/or gene-specific DNA methylation suggests alterations (comprising both hypo- and hypermethylation) to occur in early as well as in advanced atherosclerotic lesions. Moreover, epigenetic changes in peripheral blood leukocytes were characterized in the context of atherosclerosis, which may become a clinical marker for epigenetic dysregulation in subjects at cardiovascular risk.

male patients with incident cardiovascular events during follow-up had higher global DNA methylation than subjects with event-free survival, again contrasting to findings from the Normative Aging Study.

When globally analysing DNA methylation in vascular tissue samples rather than in peripheral leukocytes, current data point towards an association between atherosclerotic lesions and hypo- rather than hypermethylation. With a microarray-based approach, Castillo-Díaz *et al.* [19] compared 45 human atherosclerotic coronary artery samples from patients undergoing revascularization surgery and 16 control aortic fragments from patients undergoing aortic valve replacement; a near-complete demethylation of normally hypermethylated CpG islands was found in advanced human atherosclerotic lesions. In line, Hiltunen *et al.* [20], who analysed DNA methylation via high performance liquid chromatography in 55 human arterial samples obtained from autopsy or amputation, found reduced global DNA methylation in advanced atherosclerotic arteries compared with normal arteries.

These human studies on epigenetic dysregulation in cardiovascular disease are complemented with data from animal models. DNA hypomethylation was detected in atherosclerotic lesions from New Zealand White rabbits and from ApoE knock-out mice on a Western-type diet [20, 21]. Interestingly, similar DNA hypomethylation occurred during rabbit aortic SMC transdifferentiation [20]. Further animal data point to very early dysregulation of DNA methylation in atherogenesis, as differential DNA methylation—both hypomethylation and hypermethylation—is present in aortas and PBMCs of ApoE−/− mice before any signs of atherosclerotic lesions [22]; thus, changes in DNA methylation may serve as very early markers of atherosclerotic vascular disease.

Presently, it remains enigmatic why some reports suggested DNA hypomethylation in human arteriosclerosis, while other studies yielded contradictory findings. Differences in study size, definition of arteriosclerotic, respectively, atherosclerotic disease and different technical approaches for assessment of global DNA methylation may partly contribute to these discrepancies. It is beyond the scope of the present review to critically discuss limitations of different methods for methylation analysis in detail. Taken together, only application of sophisticated methods for analysis of DNA methylation, which may be standardized across different laboratories, and recruitment of subjects from well-characterized cohorts will provide robust data that may confirm the importance of epigenetic dysregulation in the pathogenesis of cardiovascular disease.

However, the most notable limitation of current studies is the fact that any measurement of global DNA methylation will inevitably provide an oversimplified assessment of epigenetic dysregulation, as it neither quantitatively nor qualitatively acknowledges the co-existence of hypo- and hypermethylation of distinct genes within the same cell. In line, it has been suggested earlier that hypermethylation of arteriosclerosis-protective and hypomethylation of arteriosclerosis-susceptible genes may exist in arteriosclerotic disease [12, 23–25].

Thus, although changes in global DNA methylation status may point towards a pathological condition, a better understanding of the interplay between epigenetic dysregulation and accelerated arteriosclerosis mandates DNA methylation analyses of specific genes.

SITE-SPECIFIC ASSESSMENT OF DNA METHYLATION IN ARTERIOSCLEROSIS

Most studies on site-specific epigenetic regulation in arteriosclerosis somewhat arbitrarily selected single genes and reported their methylation status.

In this context, methylation of oestrogen receptor α and β genes (*ERα* and *ERβ*) was investigated repeatedly. Oestrogen receptors are present in SMCs and endothelial cells, where they may mediate vasculoprotective effects of oestrogens. Hypermethylation of the promoter regions of the *ERα* and *ERβ* genes was detected in coronary plaques and—more specifically—in SMCs during their transformation from a normal to a proliferative state [26–28]. Similarly, hypermethylation of monocarboxylate transporter 3 (MCT3) was found in transforming SMCs [29].

Moreover, in human atherosclerotic lesions, hypomethylation of the promoter region of the 15-lipoxygenase gene [20], and hypermethylation of the tissue factor pathway inhibitor-2 (TFPI-2) [30] were described.

Beyond such single-gene analyses, Castillo-Díaz *et al.* [19] performed a broader, microarray-based approach that revealed a total number of 142 hypomethylated and 17 hypermethylated CpG islands in human atherosclerotic arteries. Many of these CpG islands could be linked to genes coding for signalling and transcription factors such as *PROX1*, *NOTCH1* or *FOXP1*, while others were annotated to genes connected to angiogenesis, SMC modulation and inflammation.

Further research is clearly needed to confirm and expand results of these pioneering studies, before specific epigenetic biomarkers for arteriosclerosis may be defined on a more solid basis. Next, it will have to be tested in how far experimental data from tissue analysis are mirrored by similar changes in samples which can be obtained in convenient, less invasive manner—such as circulating leukocytes—before these experimental findings may become clinically relevant.

DNA METHYLATION IN CKD

General consensus exists by most experts that the toxic uraemic milieu may exert a crucial impact on epigenetic gene regulation and may thus perpetuate CKD-associated accelerated arteriosclerosis [12, 23–25, 31, 32]. Nevertheless, surprisingly few experimental and clinical studies on this topic have been reported, most of which analysed altered DNA methylation in the context of CKD-associated hyperhomocysteinemia [33] and inflammation [34].

HYPERHOMOCYSTEINEMIA, CARDIOVASCULAR DISEASE AND DNA METHYLATION IN CKD

Homocysteine is a central component of the one-carbon metabolism, which regulates DNA methylation. Its derivative *S*-adenosylmethionine (SAM) is the universal methyl group donor for >100 different cellular methylation reactions, including DNA methylation (Figure 3). After transfer of a methyl group to its target, SAM is converted to *S*-adenosylhomocysteine (SAH), which is a powerful competitive inhibitor of SAM-dependent methyltransferases. Therefore, efficient removal of SAH is essential for cellular methylation reactions, which require hydrolysis of SAH into homocysteine and adenosine via SAH hydrolases. Importantly, this reaction is reversible, with the equilibrium favouring SAH formation rather than its hydrolysis, and only rapid removal of homocysteine and adenosine allows this reaction to proceed in the hydrolytic direction. In contrast, any accumulation of homocysteine would directly increase SAH levels and thereby subsequently inhibit transmethylation reactions. Homocysteine may either be removed through the remethylation pathway, in which methionine synthase (in a folate/vitamin B_{12}-dependent reaction) or betaine-homocysteine methyltransferase (using betaine as methyl group donor) will convert homocysteine into methionine. Alternatively, homocysteine can undergo transsulfuration to cystathionine in a vitamin B_6-dependent pathway (cystathionine-β-synthase).

In CKD, homocysteine levels are elevated because of both decreased renal excretion and impaired capacity to metabolize homocysteine. In clinical cohort studies, CKD patients with highest homocysteine levels suffered most cardiovascular events [35]. This inspired numerous groups to explore possible pathophysiological pathways which underlie such detrimental effects of hyperhomocysteinemia. Earlier studies first focussed on functional pathways, such as production of reactive oxygen species [36], promotion of leukocyte recruitment [37] and SMC proliferation [38], as well as induction of a pro-thrombotic state [39]. Only in the last years, the implications of homocysteine in epigenetic mechanisms came into scientific focus.

Ingrosso *et al.* [33] were the first to analyse DNA methylation in the context of CKD-associated hyperhomocysteinemia. Assessing global DNA methylation by cytosine extension assay and southern blotting, 32 male hyperhomocysteinemic haemodialysis patients were found to have significantly lower DNA methylation compared with 11 healthy controls. Moreover, in haemodialysis patients, DNA hypomethylation correlated with plasma homocysteine concentrations, and folate therapy partly restored DNA methylation.

In contrast, Nanayakkara *et al.* [40] failed to reproduce the association between homocysteine and DNA methylation in 93 patients with less advanced CKD (CKD stage 2–4), when analysing global leukocyte DNA methylation by tandem mass spectrometry. Nor did they find an association of global DNA methylation with renal function, subclinical arteriosclerosis

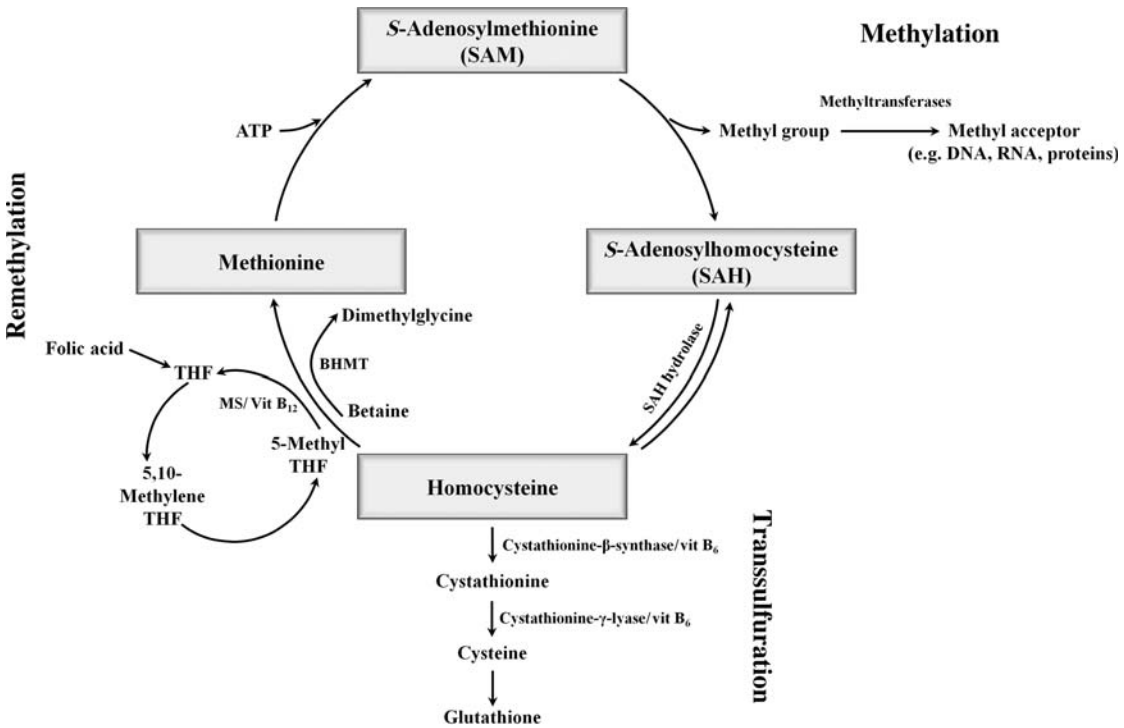

FIGURE 3: Role of homocysteine metabolism in methylation reactions. The homocysteine derivative SAM is the universal methyl group donor for a multitude of different methylation reactions, such as DNA methylation. After transfer of its methyl group, *S*-adenosylhomocysteine (SAH) is formed, which is a competitive inhibitor of methyltransferases. Removal of SAH requires its hydrolysis into homocysteine and adenosine. Since this reaction is reversible, rapid removal of homocysteine is crucial, as accumulation of SAH may inhibit cellular methylation reactions. This removal is achieved either via the remethylation or via the transsulfuration pathway.

(measured as common carotid intima media thickness) and endothelial function. Additionally, homocysteine-lowering vitamin treatment had no effect on global DNA methylation. These discrepant findings may partly be explained by different study designs, as a folate wash-out period was mandatory in the study by Ingrosso *et al.* [33], but not in the latter trial [40].

Finally, our group measured parameters of the one-carbon metabolism and leukocyte LINE-1 methylation as a surrogate marker of global DNA methylation in 22 haemodialysis patients and 26 healthy, age- and sex-matched controls. Surprisingly, haemodialysis patients had higher rather than lower LINE-1 methylation compared with controls [41]; a correlation between SAH and LINE-1 methylation was neither found within the cohort of haemodialysis patients, nor within the group of healthy controls. Admittedly, this study is limited by the small study cohort size, and by the use of a rather crude surrogate marker for estimation of global DNA methylation.

In summary, the use of differing laboratory techniques, and diverse study designs, yielded controversial data on the impact of a disturbed one-carbon metabolism on DNA methylation in CKD. In our opinion, a broader understanding will require more refined methological approaches, which should comprise analysis of gene specific rather than global DNA methylation. In this regard, a focus on arteriosclerosis-related genes appears worthwhile.

From a clinical point of view, it may be argued that further analyses of the implications of one-carbon metabolism on DNA methylation in CKD seems futile, since several interventional trials which aimed to attenuate hyperhomocysteinemia in CKD via supplementation of folate, vitamin B_6 and/or B_{12} failed to affect cardiovascular morbidity in CKD patients [42–46].

We nevertheless reckon that these disappointing results do not preclude a pathophysiological role of one-carbon metabolism in cardiovascular disease: while supplementation with folate, vitamin B_6 and/or B_{12} may reduce homocysteine levels in CKD, it fails to affect plasma SAH [47]. This is of particular interest for two reasons: first SAH rather than homocysteine is increasingly considered the real culprit in cardiovascular disease [48, 49], as SAH, but not homocysteine, directly inhibits methylation reactions. Secondly, SAH accumulates in excess to homocysteine with declining renal function, given that the kidney is the major site of SAH disposal in humans [50, 51].

INFLAMMATION, CARDIOVASCULAR DISEASE AND DNA METHYLATION IN CKD

A second focus of epigenetic research in CKD centres on inflammation-induced disturbances in DNA methylation. Chronic (micro)inflammation is a common feature in CKD, which drives the development and progression of atherosclerotic lesions and thus contributes to elevated cardiovascular morbidity and mortality in CKD patients [52].

Beyond the field of nephrology, several studies suggested chronic inflammation to trigger DNA hypermethylation a decade ago [53, 54]; in line, proinflammatory cytokines were shown to regulate a DNA methyltransferase gene [55].

In CKD, Stenvinkel *et al.* [34] assessed global DNA methylation in peripheral blood leukocytes from 37 patients in CKD stages 3 and 4, 98 incident dialysis patients, 20 prevalent haemodialysis patients and 36 controls by the Luminometric Methylation Assay (LUMA) method. When patients were subdivided into inflamed (CRP $\geq$ 10 mg/L) and noninflamed (CRP < 10 mg/L) groups, inflamed patients ($n = 62$) had significantly higher global DNA methylation than noninflamed patients ($n = 93$) or controls. When incident dialysis patients were followed for 36 ± 2 months, DNA hypermethylation was significantly associated with all-cause and cardiovascular mortality.

A first study on gene-specific DNA methylation analysis in the context of inflammation and CKD focussed on DNA methylation of the *p66Shc* (*SHC1*) gene [41]. p66Shc is a stress response protein involved in reactive oxygen species metabolism. In murine studies, *p66Shc* deletion renders resistance to oxidative stress, thus prolonging life span and protecting against age-related endothelial dysfunction [56, 57]. Recruiting 22 haemodialysis patients and 26 controls, we found that the *p66Shc* gene is hypomethylated in human CKD, which may lead to enhanced expression of this gene and subsequently contribute to oxidative stress-mediated arteriosclerosis in CKD.

Admittedly, more comprehensive data on epigenetic dysregulation in the context of CKD-associated inflammation are needed, which should provide both more specific information than global methylation analysis, and a broader data set than analysis of (arbitrarily selected) single-gene methylation.

DNA METHYLATION PROFILING IN CKD

Against this background, two recent studies aimed to identify epigenetic biomarkers in CKD in a whole genome approach. Using the Illumina HumanMethylation27 Bead Chip array, Sapienza *et al.* [58] performed DNA methylation profiling in 24 diabetic haemodialysis patients and 24 diabetic patients without diabetic nephropathy. After extracting DNA from saliva, methylation was measured at 27578 CpG sites, which allowed to analyse methylation of >14 000 genes. One hundred and eighty-seven of these genes were found to be differentially methylated at least at two CpG sites, many of which were implicated in diabetic nephropathy and/or kidney disease; in pathway analysis, these differentially methylated genes could be linked to inflammation, oxidative stress, ubiquitination, fibrosis and drug metabolism. Of note, this study deliberately aimed to focus on the identification of epigenetic biomarkers for kidney disease rather than on the characterization of dysregulated genes in the context of CKD-associated arteriosclerosis.

Therefore, we set out to resolve this question by performing genome-wide DNA methylation analysis using SuperTAG methylation-specific digital karyotyping (SMSDK) in 10 male haemodialysis patients and 10 matched controls without kidney disease [59]. With this method we analysed 575744

loci, 4288 of which displayed differential methylation with a P-value of 10^{-10}. Differentially methylated genes were linked to distinct proatherogenic processes such as inflammation (e.g. *TNFSF10*, *LY96*, *IFNGR1*, *HSPA1A* and *IL12RB1*), lipid metabolism and transport (e.g. *HMGCR*, *SREBF1*, *LRP5*, *EPHX2* and *FDPS*), proliferation and cell-cycle regulation (e.g. *MIK67*, *TP53* and *ALOX12*) as well as angiogenesis (e.g. *ANGPT2*, *ADAMTS10* and *FLT4*). Importantly, by using the 'Genetic Association Database' we identified 52 genes which are associated with cardiovascular disease (e.g. *HMGCR*, *TP53*, *ANGPT2*, *IFNGR1* and *HSPA1A*) and 97 genes with immune/infection diseases (e.g. *TNFSF10*, *IL12RB1*, *MMP24*, *CASP8* and *SPN*). These results point for the first time towards a dysregulation of arteriosclerosis-related genes in CKD, and may thus indicate an implication of epigenetic dysregulation in accelerated arteriosclerosis in CKD.

CONCLUSIONS AND FUTURE PERSPECTIVES

After a significant contribution of epigenetic dysregulation to cardiovascular disease has been suggested in preliminary studies, the issue of potential therapeutic interventions arises.

Interestingly, unspecific epigenetic modifications have already entered contemporary cardiovascular medicine, as some of the pleiotropic effects of routinely used drugs have been attributed to epigenetic modifications [60]. However, a more tailored approach in cardiovascular medicine is still not in sight. Of note, in other fields of internal medicine, direct targeting of epigenetic regulatory mechanisms—e.g. by DNA methyltransferase inhibitors and histone deacetylase inhibitors—are already integrated in clinical medicine albeit at the price of unspecific intervention [60].

Against the background of the dramatically high cardiovascular disease burden, which cannot be satisfactorily lowered by conventional treatment strategies, transfer of these novel therapeutic avenues to the field of nephrology should become a research priority in the future. Given the present paucity of data in this field, we suggest the following next steps.

First, after gene-specific changes in DNA methylation have been characterized very recently, epidemiological studies should aim to characterize those site-specific epigenetic modifications that will predict cardiovascular events in a large cohort of CKD patients. Such approach would allow clinicians to identify high-risk individuals who may specifically benefit from preventive and therapeutic interventions. Even though such interventions are presently limited to conventional therapeutic strategies, comprising stringent blood pressure control and proteinuria lowering, more specific interventions into epigenetic regulatory pathways might become available in future years. Therefore, we aim to analyse prognostic implications of specific changes in DNA methylation of pre-defined genes among 444 CKD patients in our ongoing CARE FOR HOMe study.

Secondly, to characterize potential therapeutic approaches in epigenetic medicine, a better understanding of a disturbed C1-metabolism in CKD-associated cardiovascular disease is needed. After folate, vitamin B_6 and/or B_{12} failed to improve the high cardiovascular risk in CKD patients despite lowering homocysteine levels, the role of other C1-metabolites such as SAH will gain substantial interest in forthcoming years. Against this background, we are studying the association between kidney function, C1-metabolites and cardiovascular disease in our epiGEN HOMe project, comprising our I LIKE HOMe and CARE FOR HOMe trials. We postulate that elevated SAH may surpass homocysteine as cardiovascular outcome marker in CKD. If this hypothesis holds true, strategies to efficiently lower SAH levels in CKD patients should be explored.

Finally, future experimental and clinical studies should aim to explore further areas of epigenetic regulatory mechanisms beyond DNA methylation, including histone modifications and RNA interference, as both mechanisms still remain very poorly characterized in the context of CKD-associated cardiovascular disease.

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CONFLICT OF INTEREST STATEMENT

None declared.

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Development of human monocyte subsets and its modulation by immunosuppressants

Running Title:

Monocyte subset differentiation

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Abstract

Three human monocyte subsets exist: $CD14^{++}CD16^{-}$ and CD16-positive monocytes, comprising $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes. Immunomodulation of distinct monocyte subsets has been proposed as an innovative treatment for atherosclerosis. Therefore, we characterized monocyte development and analyzed potential immunomodulators of $CD14^{++}CD16^{+}$ monocytes, which are established predictors of cardiovascular outcome.

We analyzed monocyte subsets in patients following autologous and allogenic stem cell transplantation. *In vivo* $CD14^{++}CD16^{-}$ monocytes were the first to arise, followed by $CD14^{++}CD16^{+}$ and later by $CD14^{+}CD16^{++}$ monocytes. Monocyte subset distribution did not differ significantly in patients after allogenic compared to autologous transplantation ($P > 0.05$). Corticosteroids considerably depleted $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ cells *in vivo*, but left $CD14^{++}CD16^{-}$ monocytes unaffected. Calcineurin inhibitors, mycophenolic mofetil and methotrexate did not influence monocyte subset development, but modified surface receptor expression (CCR2, HLA-DR, ENG, TEK and TLR4). Furthermore, human monocytes were generated *in vitro* from $CD34^{+}$ progenitor cells. The impact of conventional immunomodulators – steroids, rapamycin, calcineurin inhibtors – and of the aryl hydrocarbon receptor (AHR) activator benzo(a)pyrene upon monocyte subsets was studied. Only steroids, rapamycin and benzo(a)pyrene significantly affected CD16-positive monocyte counts.

We report for the first time *in vivo* the developmental relationship of all three monocyte subsets and the effects of established and experimental immunomodulators *in vivo* and *in vitro*.

Keywords: monocyte differentiation, CD14, CD16, aryl hydrocarbon receptor, immunosuppression, vascular biology

Introduction

The role of monocyte heterogeneity in cardiovascular medicine is increasingly acknowledged (1-3). In murine models of atherosclerosis inhibition of chemokine receptors reduces circulating monocyte counts and almost completely inhibits atherogenesis (4). Consequently, immunomodulation of monocyte subsets has been proposed as an innovative treatment in cardiovascular disease (5, 6). Three human monocyte subsets exist: classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes, the latter have been previously summarized as CD16-positive cells (7). Pioneering experimental (8) and clinical data suggested a prominent role of intermediate $CD14^{++}CD16^{+}$ monocytes in cardiovascular disease in selected patient groups at highest cardiovascular risk (9, 10). Of note, the predictive role of $CD14^{++}CD16^{+}$ monocytes for cardiovascular events has been recently confirmed in a large cohort from the general population (11).

In contrast to the extensively studied murine monocyte subsets, human monocyte heterogeneity still remains poorly understood (1, 3). Specifically, human monocyte subset differentiation and the impact of immunomodulating drugs on human monocyte heterogeneity have not been thoroughly studied so far.

We reasoned that analyzing the developmental relationship and the influence of immunosuppressants on human monocyte subsets would advance the notion of therapeutic interference with monocyte subpopulations. As a model of *de novo* human monocyte differentiation, we chose to study hematologic reconstitution after allogenic and autologous hematopoietic stem cell transplantation (HSCT). In addition, the developmental course and the impact of immunosuppressants was analyzed in an *in vitro* model of human monocyte subset development.

Material and Methods

In vivo differentiation of monocyte subsets

We prospectively analyzed monocyte subpopulation differentiation in 19 patients after HSCT, of whom nine patients received allogenic HSCT after myeloablative conditioning regimens, and another ten patients underwent high dose chemotherapy with subsequent autologous HSCT. Indications for HSCT and conditioning regimes are presented in **Table 1A and 1B**.

The local ethics committee approved the study and all patients gave their written consent.

In order to analyze the effect of immunosuppressive drugs on monocyte subsets, we compared patients after autologous transplantation (AutoTx, n = 10) with patients undergoing allogenic stem cell transplantation (AlloTx, n = 9). All patients received peripheral blood mononuclear cells (PBMCs). AlloTx patients received anti-thymocyte globulin (ATG Genzyme; 4.5 mg/kg with related and 7.5 mg/kg with unrelated donors, respectively) on days -4 through -2 for prophylaxis of graft versus host disease. During the study period all AlloTx patients were on immunosuppression comprising a calcineurin inhibitor (either cyclosporine A [target trough level 150-200 ng/ml] or tacrolimus [target trough level: 8-12 ng/ml]) either as monotherapy, or combined with either mycophenolate mofetil (2 x 1 g/d from day +1 to day +28) or methotrexate (15 mg/m² (d +1), 10 mg/m² (d +3; +6). All patients received 50 mg prednisolone prior to stem cell transfusion. Afterwards corticosteroids were selectively administered for treatment of nausea, for prevention of allergic reaction to blood transfusions, or (in AlloTx) for treatment of graft versus host disease. Monocyte subset analysis was performed blinded to clinical characteristics of the respective patient.

Flow cytometric analysis

Monocyte subsets were identified *via* flow cytometry (FACS Canto II with FACSDiva Software; BD Biosciences, Heidelberg, Germany) according to our standardized and validated gating strategy (12) in cell culture or in a whole-blood assay using 100 µl of EDTA anticoagulated blood. Briefly, monocytes were gated in a side scatter/CD86 dot plot, identifying monocytes as CD86-positive cells with monocyte scatter properties. $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocyte subpopulations were then distinguished by their surface expression pattern of CD14 (LPS receptor) and CD16 (FcγIII receptor).

Surface expression of different antigens was quantified as median fluorescence intensity (MFI) and standardized against coated fluorescent particles (SPHEROTM; BD Biosciences). The following antibodies were used: anti-CD14 PerCP (Mφ9), anti-CD16 PeCy7 (3G8), anti-CD195 APC (2D7/CCR5), anti-CD282 Alexa Fluor 647 (11G7) and anti-CD192 Alexa Fluor 647 (48607) from BD Biosciences, Heidelberg, Germany; anti-CD74 FITC (5-329) and anti-CD105 APC (SN6) from eBioscience Frankfurt, Germany; anti-HLA-DR FITC (L243) and anti-CD202b Alexa Fluor 647 (Ab33) from BioLegend, Fell, Germany; anti-CD143 FITC (9B9) and anti-CX_3CR1 FITC (2A9-1) from Biozol, Eching, Germany; anti-CD86 PE (HA5.2B7) from Beckman-Coulter, Krefeld, Germany and anti-CD284 FITC (HTA125) from AMS Biotechnology, Abingdon, United Kingdom.

In vitro generation of monocytes from hematopoietic $CD34^+$ stem cells

For isolation of $CD34^+$ hematopoietic stem cells, EDTA-anticoagulated blood was drawn from healthy volunteers by venopuncture and PBMCs were immediately isolated by Ficoll-Paque (Lymphocyte Separation Medium; PAA, Cölbe, Germany) gradient density centrifugation. $CD34^+$ cells were isolated using the CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The mean purity of isolated $CD34^+$ cells was 80.4 ± 6.4 % as determined flow cytometrically after anti-CD34 (CD34 APC; 581; BD Biosciences) and anti-CD45 (CD45 PE; HI30; BD Biosciences) staining.

Monocytes were generated from isolated $CD34^+$ cells in a two step culture: first, $CD34^+$ hematopoietic stem cells were expanded in 6-well plates (1×10^4 cells/ml) for 13 days in the Hematopoietic Progenitor Cell Expansion Medium DXF (PromoCell GmbH, Heidelberg, Germany) supplemented with the Cytokine Mix E (PromoCell GmbH) which contains the recombinant human growth factors TPO, SCF, flt3-ligand and IL-3. A 25 fold expansion rate of hematopoietic stem cells was observed within the 13 days. In the second step, expanded cells (2×10^4 cells/ml) were seeded in 6-well plates in the Hematopoietic Progenitor Medium (PromoCell GmbH). Differentiation of hematopoietic stem cells into monocytes was flow cytometrically monitored after anti-CD86, anti-CD14 and anti-CD16 staining, subdividing *in vitro* differentiated monocytes into $CD14^-CD16^-$, $CD14^{++}CD16^-$ and $CD14^{++}CD16^+$ cells.

For experiments with immune modulators, rapamycin, cyclosporine A and dexamethasone (all purchased from Biomol, Hamburg, Germany) as well as benzo(a)pyrene and

α-naphthoflavone (both purchased from Sigma Aldrich, Munich, Germany) were added to the Hematopoietic Progenitor Medium.

Phagocytosis assay

For phagocytosis assays, Fluoresbrite Yellow Green (YG) Carboxylate Microspheres (0.75 µm; Polysciences, Eppelheim, Germany) were first opsonized for 30 minutes at 37°C with serum from healthy donors (diluted to 50% with Krebs Ringers PBS) and adjusted to 10^8 particles/ml.

At day 7 of differentiation, 1 x 10^4 cells in 100 µl of culture medium were mixed with 10 µl of opsonized particles and incubated with gentle shaking for 30 minutes at 37°C. Control samples were incubated at 4°C. Cells were stained with anti-CD86, anti-CD14 and anti-CD16 and counts of FITC positive cells were determined flow cytometrically in each cell type ($CD14^-CD16^-$, $CD14^{++}CD16^-$ and $CD14^{++}CD16^+$ cells).

Measurement of reactive oxygen species (ROS)

1 x 10^4 cells from day 7 of differentiation were incubated with the cell-permanent carboxy-H_2DFFDA (Life Technologies, Darmstadt, Germany) in a concentration of 10 µM for 15 minutes at 37°C and 5% CO_2. Afterwards, cells were stained with anti-CD86, anti-CD14 and anti-CD16 and ROS levels were determined flow cytometrically as MFI within the three cell types ($CD14^-CD16^-$, $CD14^{++}CD16^-$ and $CD14^{++}CD16^+$ cells).

Proliferation assay

The ability of distinct cell types ($CD14^-CD16^-$, $CD14^{++}CD16^+$) to induce $CD4^+$ T-cell proliferation was analyzed by measuring the cytoplasmic dilution of CFDA-SE (Vybrant CFDA-SE Cell Tracer Kit; Life Technologies). Therefore, cells at day 7 of differentiation were separated into $CD14^-CD16^-$ and $CD14^{++}CD16^+$ using CD14 Microbeads (Miltenyi Biotec) and cultivated overnight in 96-well plates at a density of 5 x 10^4 cells/well in the presence of 2.5 µg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich, Munich, Germany). Within 24 hours, CD4+ T-cells were isolated from healthy donors using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec) and labeled with 5 µM CFDA-SE for 10 minutes at 37°C.

2 x 10^5 labeled CD4+ T-cells were added to SEB stimulated cells and counts of proliferating T-cells were measured after 4 days as CFDA-SE dilution, identifying T-cells after anti-CD3 (CD3 APC; SK7; BioLegend) staining.

Statistics

Categorical data were presented as counts (percentages), and continuous data as mean (standard deviation), unless indicated otherwise. We compared continuous variables with Mann-Whitney test or with Kruskal-Wallis test (followed by Dunn's test as post-hoc test), as appropriate.

Results

Differentiation of human monocyte subsets in patients after HSCT

In all patients, counts of monocyte subsets were analyzed from day +1 to hospital discharge. **Figure 1A** depicts representative examples from a patient after autologous HSCT and a patient after allogenic HSCT. $CD14^{-}CD16^{-}$ cells were the first cells appearing in the peripheral blood (day 5-6). Over the course of time we observed a gradual increase of $CD14^{++}CD16^{-}$ monocytes, seamlessly followed by the appearance of $CD14^{++}CD16^{+}$ and later of $CD14^{+}CD16^{++}$ monocytes. Monocyte subpopulation frequencies after HSCT plateaued at day 8-10 for $CD14^{++}CD16^{-}$ monocytes, at day 12-14 for $CD14^{++}CD16^{+}$ monocytes and at day 14-16 for $CD14^{+}CD16^{++}$ monocytes.

Interestingly, the developmental course of monocyte subsets in patients after autologous transplantation was similar to the pattern observed in patients after allogenic transplantation (**Figure 1A**), suggesting that the immunosuppressants given to patients after allogenic transplantation had no impact on monocyte subset development. Percentages of monocyte subsets determined at the end of the study period differed not between patients after allogenic HSCT *versus* patients after autologous HSCT (classical: 67.5 % *vs* 60.3 %; intermediate: 15.7 % *vs* 16.3 %; nonclassical: 11.5 % *vs* 15.4 %; $P > 0.05$, respectively).

Of note, previous studies reported that glucocorticoids induce depletion of CD16-positive but not of classical $CD14^{++}CD16^{-}$ monocytes (13-15); however, these reports subsumed intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ monocytes as a single population. Instead, following recent consensus recommendations (7), we now subdivided CD16-positive monocytes into $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ cells, and found both subpopulations to be depleted following high dose corticosteroid treatment in HSCT patients (**Figure 1B**).

Expression of surface markers on differentiating monocytes in patients after HSCT

In a recent report, we described distinctive surface markers of human monocyte subsets characterizing their respective role in immunity (12). To investigate whether immunosuppressants have an impact on functional monocyte markers, we analyzed surface expression of these markers (CCR2, CD74, HLA-DR, ENG, TEK, CCR5, ACE, TLR2, TLR4, CX_3CR1) on three monocyte subsets in 9 patients at day 14-16 after autologous HSCT and in 4 patients at the same timeframe after allogenic HSCT. These markers are centrally involved

in distinct physiological and pathological processes such as inflammation and host defense (CD74, HLA-DR, TLR2, TLR4), atherosclerosis (CCR2, CCR5, CX3CR1, ACE) and angiogenesis (ENG, TEK). We previously described these markers as signature markers of the distinct monocyte subsets; CCR2 was a signature for $CD14^{++}CD16^{-}$ monocytes, whereas CD74, HLA-DR, ENG, TEK, CCR5, ACE, TLR2, TLR4 were signatures for $CD14^{++}CD16^{+}$ monocytes and CX_3CR1 was a signature for $CD14^{+}CD16^{++}$ monocytes; the subset-specific expression pattern of the respective markers in patients after HSCT matched our previous description of the surface expression in healthy donors (12).

When comparing patients after allogenic with patients after autologous HSCT, subset-specific expression of all surface markers – except for CD74 – was lower (**Figure 2**); specifically CCR5, ACE, TLR2 and CX_3CR1 tended to be downregulated, whereas expression of CCR2, HLA-DR, ENG, TEK and TLR4 was significantly lower ($P < 0.05$).

In vitro differentiation of monocytes from $CD34^{+}$ hematopoietic stem cells

To further analyze monocyte subset differentiation and its modulation by routinely applied and experimental immunosuppressants, we established an *in vitro* model of monocyte differentiation.

During initial expansion of $CD34^{+}$ hematopoietic stem cells over 13 days, no upregulation of CD14 and CD16 surface expression was observed. Following this expansion period, $CD14^{-}CD16^{-}$ cells were cultivated in differentiation medium for 18 days, where they differentiated first into $CD14^{++}CD16^{-}$ monocytes (day 2-3; with maximal cell counts at day 7-9), and subsequently into $CD14^{++}CD16^{+}$ monocytes (**Figure 3A**), thus reflecting the developmental process observed *in vivo* after HSCT. However, no further differentiation of $CD14^{++}CD16^{+}$ monocytes into $CD14^{+}CD16^{++}$ monocytes was observed *in vitro.*

Characterization of *in vitro* generated monocyte subsets

$CD14^{++}CD16^{+}$ monocytes are characterized as highly proinflammatory cells predicting cardiovascular mortality in patients at high cardiovascular risk (9, 10, 12). We now analyzed whether *in vitro* differentiated $CD14^{++}CD16^{+}$ monocytes show functional properties

comparable to circulating $CD14^{++}CD16^{+}$ monocytes, which would allow their use for further *in vitro* research in the field of monocyte subset biology.

We first measured expression of surface antigens (CD74, HLA-DR, ENG, TEK, CCR5, ACE, TLR2 and TLR4), which are *in vivo* signature markers of circulating $CD14^{++}CD16^{+}$ monocytes (12), on monocyte subsets on day 4 to 18 of *in vitro* differentiation. Throughout the *in vitro* differentiation period, $CD14^{++}CD16^{+}$ monocytes showed highest expression of all those signature surface markers (**Figure 3B**).

Furthermore, we verified functional characteristics of *in vitro* differentiated monocytes subsets: $CD14^{++}CD16^{+}$ monocytes had a high capacity to phagocyte (**Figure 3C**) and to induce $CD4^{+}$ T cell proliferation after SEB stimulation (**Figure 3D**); moreover, compared to $CD14^{-}CD16^{-}$ and $CD14^{++}CD16^{-}$ cells, $CD14^{++}CD16^{+}$ monocytes produced highest levels of reactive oxygen species (ROS) (**Figure 3E**), resembling functional characteristics of their circulating $CD14^{++}CD16^{+}$ monocytic counterparts *in vivo* after HSCT.

Impact of conventional immunosuppressants on *in vitro* differentiation of human monocyte subsets

We assessed the impact of different immunosuppressants on the differentiation process of monocyte subsets. Rapamycin (100 nM), cyclosporine A (250 ng/ml) or dexamethasone (250 nM) were added to the differentiation medium after initial expansion of $CD34^{+}$ hematopoietic stem cells, and their differentiation towards $CD14^{++}CD16^{+}$ monocytes was monitored for 8 days (**Figure 4A**). The proliferation inhibitor rapamycin reduced the development of $CD14^{++}CD16^{+}$ monocytes more potently (3.7 fold reduction in $CD14^{++}CD16^{+}$ monocyte percentage at day 8) than dexamethasone (2.2 fold reduction, respectively), while development of CD14++CD16+ monocytes was not significantly inhibited by cyclosporine A.

Impact of aryl hydrocarbon receptor activation on *in vitro* differentiation of human monocyte subsets

Since activation of the aryl hydrocarbon receptor (AHR) was shown to influence hematopoietic stem cells (16) and to modulate T cell subsets (17), we hypothesized that AHR activation might influence monocyte subset differentiation.

Benzo(a)pyrene (10 µM) was added as an AHR activator to the differentiation medium and differentiation of monocytes was monitored for 8 days (**Figure 4B**). Interestingly, of all cells $CD14^{++}CD16^{+}$ monocytes were preferentially depleted by benzo(a)pyrene (e.g. 12.8 ± 1.9 % control vs 4.4 ± 2.7 % benzo(a)pyrene on day 8). This effect could be partially antagonized by addition of α-naphthoflavone (5.8 ± 3.1 % benzo(a)pyrene and α-naphthoflavone on day 8). (**Figure 4B**).

Discussion

Monocyte heterogeneity has attracted much scientific interest especially in cardiovascular research within the last couple of years (3).

Of special interest may be the intermediate $CD14^{++}CD16^{+}$ monocyte subset, since circumstantial evidence suggests pro-atherogenic virtues of $CD14^{++}CD16^{+}$ monocytes; among those are the combined expression of the chemokine receptor triad, CCR5, CCR2 and CX_3CR1 (10), that has been shown to be crucial in experimental atherogenesis (4), the potential to home to activated endothelial cells and to attract further monocyte and T-lymphocytes (8) and their highest inflammatory capacity of all monocyte subsets (12, 18). This is underscored by cross-sectional studies associating CD16-positive monocytes with cardiovascular risk (19, 20) and by studies reporting that specifically $CD14^{++}CD16^{+}$ monocytes were independent predictors of cardiovascular outcome (9-11, 21).

However, human monocyte heterogeneity still remains poorly characterized, while a plethora of experimental data on murine monocyte subpopulations emerged in recent years (3). In view of general immunological interspecies differences in mice and men (22), which are to some extent also seen in monocyte heterogeneity (23), experimental data from murine models should not be uncritically transferred to humans. Therefore, a validated *in vitro* model for researching human monocyte subsets is needed. Previously monocytes and macrophages have been generated from $CD34^{+}$ progenitor cells (24). However, no study so far compared the *in vitro* generated monocytes regarding phenotypic and functional properties with circulating human monocytes. In this regard, hematopoietic stem cell transplantation (HSCT) provides a unique validation tool for the *in vivo* evaluation of the temporal sequences of human monocyte subset differentiation and for further phenotypic and functional analyses. Interestingly, only one report looked at monocyte reconstitution of two subsets ($CD14^{++}CD16^{-}$ and CD16-positive cells) early after autologous stem cell transplantation (25); however the developmental course of the *three* subsets has not been rigorously followed. Of note, as discussed by Wrigly et al. (26) and our group (3) distinction of the two CD16-positive monocyte subsets into intermediate $CD14^{++}CD16^{+}$ and nonclassical $CD14^{+}CD16^{++}$ is essential due to the different functional characteristics of these subsets (12, 27). Moreover, the effect of immunomodulating drugs on monocyte heterogeneity has been largely overlooked so far; therefore we analyzed monocyte reconstitution following allogenic HSCT in comparison to autologous HSCT.

We report here that classical $CD14^{++}CD16^{-}$ monocytes are the first monocyte subset to emerge after HSCT, followed by intermediate $CD14^{++}CD16^{+}$ monocytes and finally nonclassical $CD14^{+}CD16^{++}$ monocytes. The observed developmental course and characteristics of *in vitro* generated monocyte subsets are in accordance with the analysis following HSCT.

These findings support the so far unproven concept (1) that classical $CD14^{++}CD16^{-}$ monocytes are precursors of CD16-positive monocytes.

In vitro only rapamycin and steroids – representing established immunomodulators – significantly inhibit development of $CD14^{++}CD16^{+}$ monocytes. The observations following HSCT support these findings. *In vivo* glucocorticosteroids deplete CD16-positive monocytes (13-15), whereas immunosuppressive drugs such as the calcineurin-inhibitors cyclosporine A and tacrolimus, mycophenolate mofetil and methotrexate do not substantially alter monocyte subset distribution but merely modulate cell surface receptor expression of CCR2, HLA-DR, ENG, TEK, CCR5, ACE, TLR2, TLR4 and CX_3CR1. Functional characteristics of monocyte subsets could thus be altered since these receptors are implicated in various physiologic and pathophysiologic processes, ranging from inflammation and host defense (CD74, HLA-DR, TLR2, TLR4) to atherosclerosis (CCR2, CCR5, CX3CR1, ACE) and angiogenesis (ENG, TEK). Of note, upregulation of ACE on the surface of intermediate monocytes has been associated with atherosclerosis (28) and was reported to be a predictor of outcome in dialysis patients (29).

In addition, we report for the first time that immunomodulation *via* the AHR activator benzo(a)pyrene rather selectively inhibited the differentiation of $CD34^{+}$ progenitor cells to $CD14^{++}CD16^{+}$ monocytes. This result could point to new strategies regarding monocyte subset modulation, since a variety of AHR agonists exist (30).

Limitations of our study are explained by its design as an observational study in humans. Thus, we could neither test the effect of rapamycin *in vivo* since this drug was not part of the established immunosuppressive regimen after HSCT, nor could we test the effect of AHR agonists *in vivo* since none of these compounds obtained a license for use in humans.

In conclusion, we report the developmental course of human monocyte subsets *in vivo* and *in vitro*; the validated *in vitro* model of monocyte subset differentiation may allow to assess the effects of novel pharmacological agents on monocyte heterogeneity.

Acknowledgements

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Conflict of interest

The authors have nothing to disclose.

What is known about this topic?

- Three monocyte subsets exist: classical $CD14^{++}CD16$, intermediate $CD14^{++}CD16^{+}$ and non-classical $CD14^{+}CD16^{++}$ monocytes
- Monocyte heterogeneity plays a central role in cardiovascular medicine
- $CD14^{++}CD16^{+}$ monocytes are independent predictors of cardiovascular outcome
- In contrast to the well researched biology of murine monocyte subsets, human monocyte subset biology is still poorly understood

What does this paper add?

- Developmental relationship of the three human monocyte subsets has been better characterized
- Validation of an *in vitro* model for differentiation of monocyte subsets
- Impact of routinely applied immunosuppressants on monocyte subset function and on their differentiation was characterized

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Table 1A Characteristics of patients undergoing allogenic stem cell transplantation

No.	Age	Gender	Indication	Conditioning	Immunosuppression	Steroid
1	22.8	m	AML	FLAMSA	CSA/MMF	yes
2	37.1	m	CML	BU/CY	CSA	no
3	54.6	m	AML	FLAMSA	CSA/MMF	yes
4	58.8	f	AML	CLAMSA	CSA/MMF	no
5	63.9	m	DLBCL	FLU/BU/CY	TAC/MMF	no
6	39.1	f	cALL	TBI/CY	CSA/MTX	yes
7	63.8	f	AML	FLAMSA	CSA/MMF	yes
8	52.7	f	DLBCL	FLU/BU/CY	TAC/MMF	no
9	45.3	m	T-ALL	TBI/CLAMSA	CSA/MMF	no

m: male, *f:* female; *AML:* acute myeloid leukemia, *CML:* chronic myeloid leukemia in blast crisis, *DLBCL:* diffuse large B-cell lymphoma, *c-ALL:* common acute lymphatic leukemia, *T-ALL:* T-acute lymphatic leukemia

TBI: total body irradiation; *FLAMSA:* fludarabine 30 mg/m^2 (d -12 to -9), cytarabine 2000 mg/m^2 (d -12 to -9), amsacrine 100 mg/m^2 (d -12 to -9), TBI 2 x 2 Gy (d -5), cyclophosphamide 60 mg/kg (d -4 to -3); *BU/CY:* busulfane 3,2 mg/kg (d -7 to -4), cyclophosphamide 60 mg/kg (d -3 to -2); *CLAMSA:* clofarabine 30 mg/m^2 (d -12 to -9), cytarabine 2000 mg/m^2 (d -12 to -9), amsacrine 100 mg/m^2 (d -12 to -9), TBI 2 x 2 Gy (d -5), cyclophosphamide 60 mg/kg (d -4 to -3); *FLU/BU/CY:* fludarabine 25 mg/m^2 (d -8 to -4), cyclophosphamide 60 mg/kg (d -3 to -2), busulfane 3,2 mg/kg (d -6 to -4); *TBI/CY* TBI 2x2 Gy (d -3 to -1), cyclophosphamide 60 mg/kg (d -5 to -4); *TBI/CLAMSA:* Total body irradiation 2x2 Gy (d -12 to -11), cranial irradiation 24 Gy (d -10) / clofarabine 30 mg/m^2 (d -9 to -6), cytarabine 2000 mg/m^2 (d -9 to -6), amsacrine 100 mg/m^2 (d -9 to -6), cyclophosphamide 60 mg/kg (d -4 to -3).

CSA: cyclosporine A 3 mg/kg (beginning d -1; subsequently blood target level 150 to 200 ng/ml), *MMF:* mycophenolate mofetil 2g/d (d +1 to +28), *TAC:* tacrolimus 0,05 mg/kg (beginning d -1;subsequenly blood target level 8-12 ng/ml), *MTX:* methotrexate 15 mg/m^2 (d +1), 10 mg/m^2 (d +3; +6)

Table 1B Characteristics of patients undergoing autologous stem cell transplantation

No.	Age	Gender	Indication	Conditioning	Steroid
1	54.9	f	DLBCL	HD-TTBE	no
2	39.1	m	MM	MEL	yes
3	51.3	m	MM	MEL	yes
4	43.7	m	FL	BEAM	no
5	56.3	f	T-NHL	BEAM	no
6	53.0	m	MM	MEL	yes
7	57.9	m	FL	TBI/CY	yes
8	64.9	m	DLBCL	MEL	yes
9	66.4	m	MM	MEL	yes
10	60.4	m	T-NHL	BEAM	no

m: male, *f:* female, *DLBCL:* diffuse large B-cell lymphoma, *MM:* multiple myeloma, *T-NHL:* T-Non Hodgkin´s lymphoma

HD-TTBE: thiotepa 2 x 5 mg/kg (d -4 to -3), carmustine 400 mg/m^2, etoposide 150 mg/m^2 (d -5 to -3); MEL melphalane 100 mg/m^2 (d-3 to -2); *BEAM:* Carmustine 2 x 150 mg/m^2 (d -6), cytarabine 2 x 200 mg/m^2 (d -6 to -3), etoposide 2 x 100 mg/m^2 (d -6 to -3), melphalane 140 mg/m^2 (d -2); TBI/CY: Total body irradiation 2 x 2 Gy (d -6 to -4), cyclophosphamide (60 mg/kg d -3 to -2).

Figure Legends

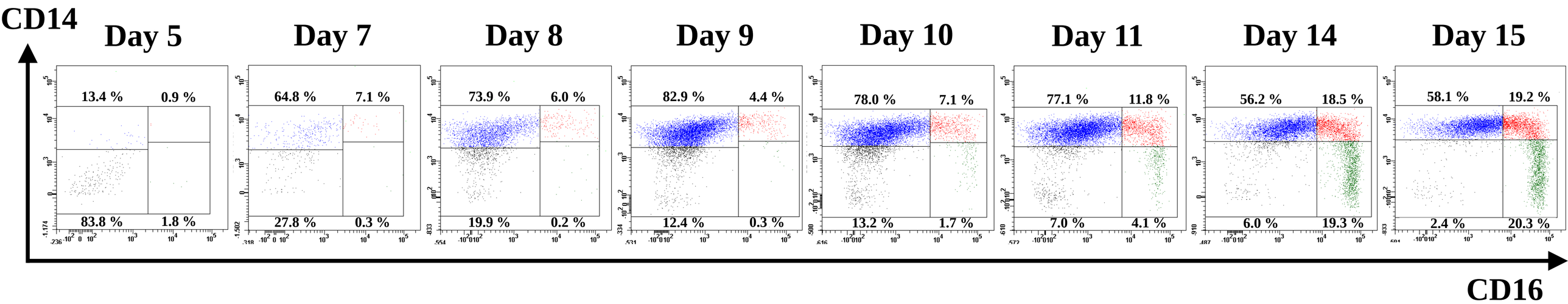
Figure 1. Monocyte subpopulations in patients after autologous and allogenic HSCT. (A) *Via* flow cytometry, monocyte subsets were analyzed in patients from day one after autologous and allogenic HSCT until hospital discharge. According to our validated gating strategy (12), monocyte subsets were defined and divided into classical $CD14^{++}CD16^{-}$ monocytes (blue), intermediate $CD14^{++}CD16^{+}$ monocytes (red) and nonclassical $CD14^{+}CD16^{++}$ monocytes (green). $CD14^{-}CD16^{-}$ cells are displayed in black. Representative examples are shown. **(B)** Impact of steroids on monocyte subsets is shown in one representative patient after allogenic HSCT.

Figure 2. Expression of signature marker proteins on monocyte subsets in patients after HSCT. Expression of surface proteins was determined as median fluorescence intensity (MFI) in 9 patients after autologous HSCT and in 4 patients after allogenic HSCT. Statistical analysis was performed using the Mann-Whitney-U-Test. *P < 0.05; **P<0.01.

Figure 3. Differentiation of monocyte subsets *in vitro*. (A) After initial expansion of CD34+ cells, differentiation was initiated and the process was monitored flow cytometrically for 18 days. **(B)** Expression of surface proteins on $CD14^{-}CD16^{-}$ cells (black), $CD14^{++}CD16^{-}$ monocytes (blue) and $CD14^{++}CD16^{+}$ monocytes (red) on day 4 to 18 of *in vitro* differentiation, measured as MFI. **(C)** Capacity to phagocyte opsonized carboxylate microspheres by $CD14^{-}CD16^{-}$ cells, $CD14^{++}CD16^{-}$ monocytes and $CD14^{++}CD16^{+}$ monocytes at day 7 of differentiation; counts of FITC-positive cells were determined flow cytometrically and are denoted as means ± SD. **(D)** Ability to induce $CD4^{+}$ T cell proliferation by $CD14^{-}CD16^{-}$ cells and $CD14^{++}CD16^{+}$ monocytes at day 7 of differentiation measured flow cytometrically as cytoplasmic dilution of CFDA-SE. **(E)** Measurement of spontaneous intracellular ROS levels in $CD14^{-}CD16^{-}$ cells, $CD14^{++}CD16^{-}$ monocytes and $CD14^{++}CD16^{+}$ monocytes by flow cytometry using the ROS-detection reagent carboxy-H_2DFFDA. Data were measured as MFI and presented as means ± SEM. Statistical analysis was performed using the Kruskal-Wallis test. Blood was taken from five healthy donors.

Figure 4. Impact of immunosuppressants and of aryl hydrocarbon receptor activation on *in vitro* monocyte differentiation. (A) Differentiation of monocytes was flow cytometrically monitored for 8 days after the addition of rapamycin (100 nM), cyclosporine A (250 ng/ml) or dexamethasone (250 nM) to the differentiation medium after initial expansion of $CD34^+$ cells. Data are presented as means ± SEM. Representative dot plots for day 8 are shown. Statistical analysis was performed using Kruskal-Wallis followed by Dunn's test as post-hoc test to compare treated samples *vs* control. * $P < 0.05$; ** $P < 0.01$. **(B)** Differentiation of monocytes after supplementation of the differentiation medium with the AHR activator benzo(a)pyrene (10 µM) and / or α-naphthoflavone (20 µM). Representative examples for day 1, 5 and 8 are shown including mean values ± SD of 3 independent experiments with blood of three healthy donors.

Patient after autologous stem cell transplantation



Patient after allogenic stem cell transplantation

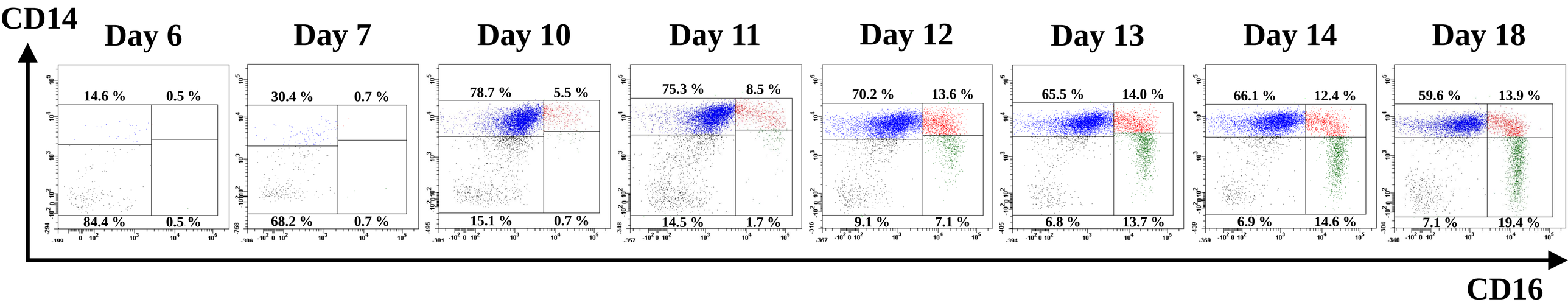


Figure 1A

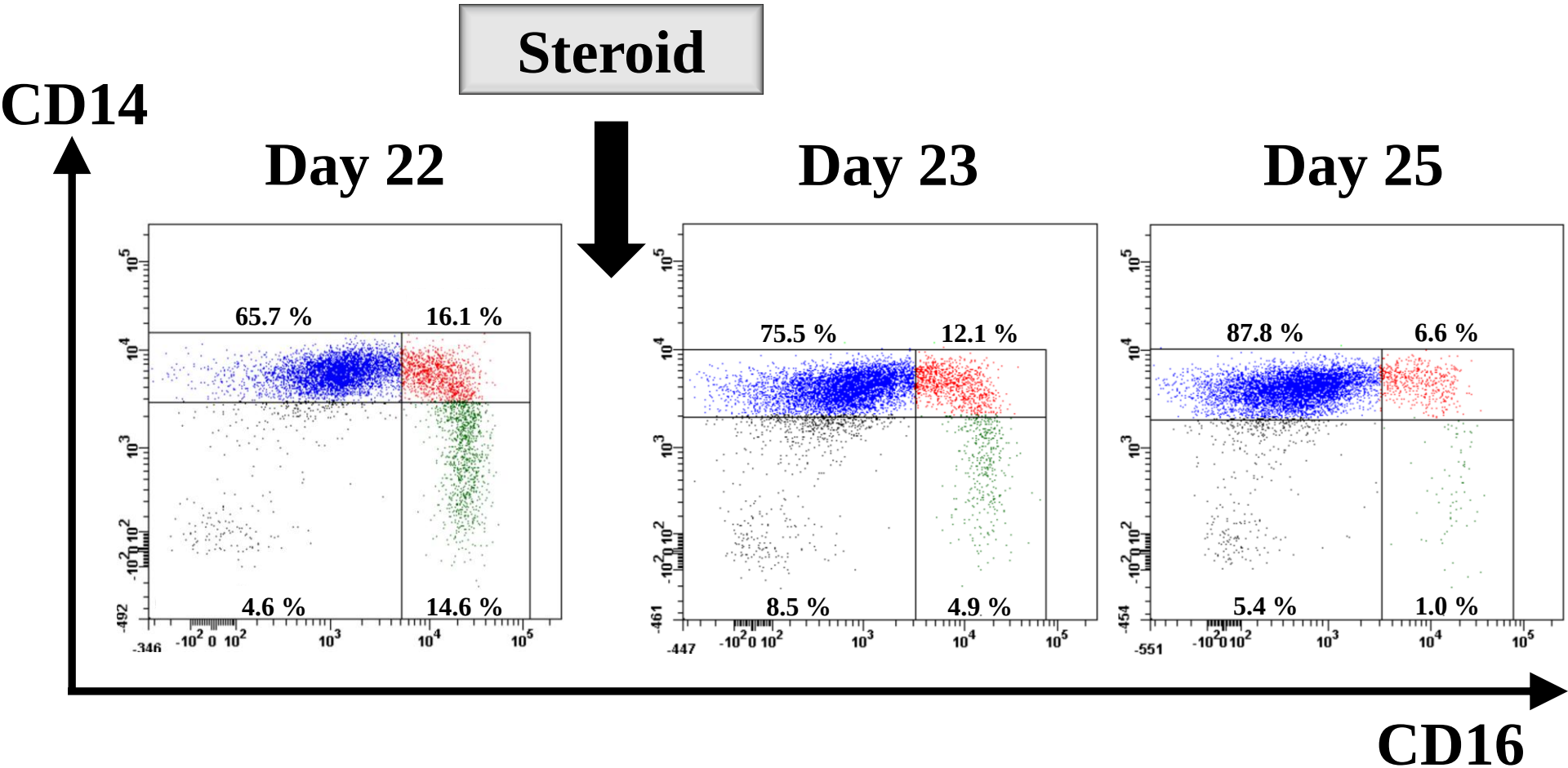

Steroid
CD14
Day 22
Day 23
Day 25
65.7 %
16.1 %
4.6 %
14.6 %
75.5 %
12.1 %
8.5 %
4.9 %
87.8 %
6.6 %
5.4 %
1.0 %
CD16

Figure 1B

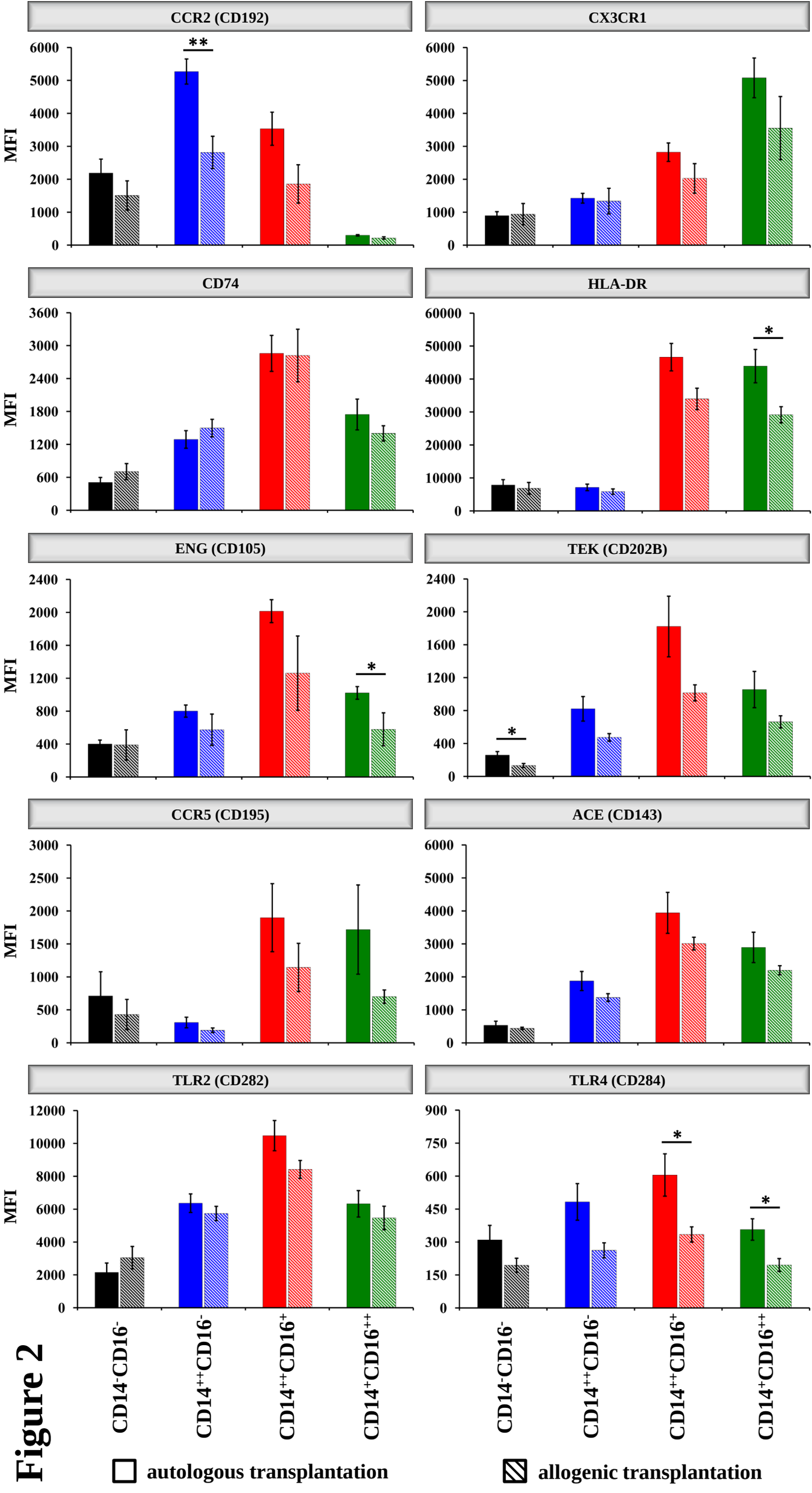

CCR2 (CD192)
CX3CR1
CD74
HLA-DR
ENG (CD105)
TEK (CD202B)
CCR5 (CD195)
ACE (CD143)
TLR2 (CD282)
TLR4 (CD284)
MFI
**
*
CD14-CD16-
CD14++CD16-
CD14++CD16+
CD14+CD16++
autologous transplantation
allogenic transplantation

Figure 2

In vitro differentiation of monocytes from CD34⁺ hematopoetic stem cells

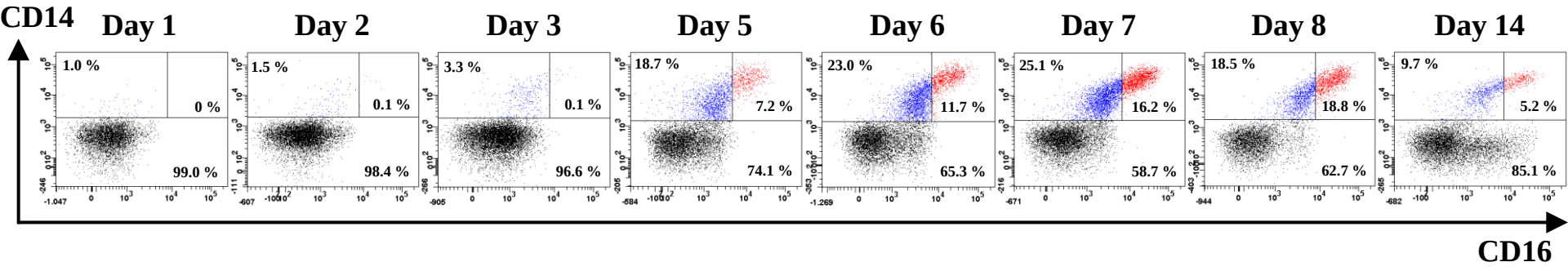


Figure 3A

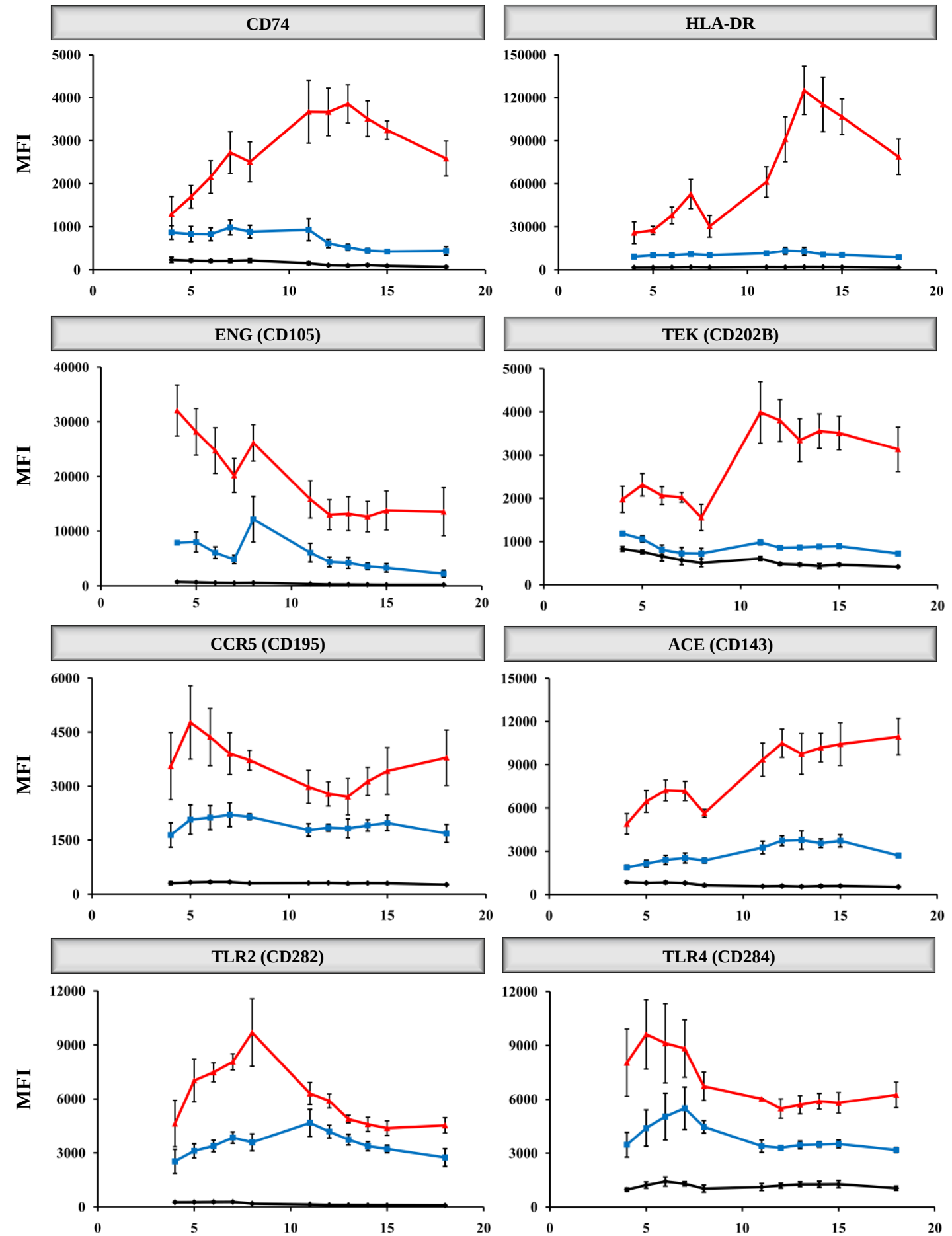

CD74
HLA-DR
ENG (CD105)
TEK (CD202B)
CCR5 (CD195)
ACE (CD143)
TLR2 (CD282)
TLR4 (CD284)
MFI
Day of differentiation

Figure 3B

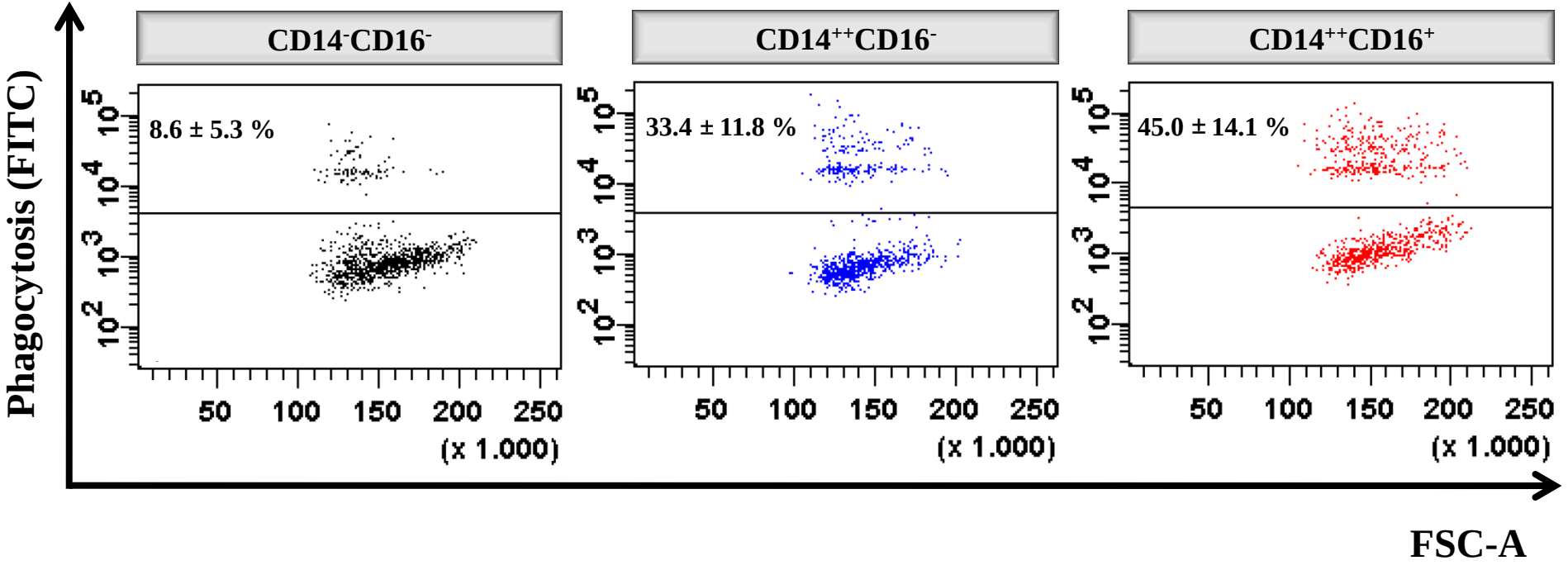

CD14-CD16-
CD14++CD16-
CD14++CD16+
8.6 ± 5.3 %
33.4 ± 11.8 %
45.0 ± 14.1 %
Phagocytosis (FITC)
FSC-A
50
100
150
200
250
(x 1.000)

Figure 3C

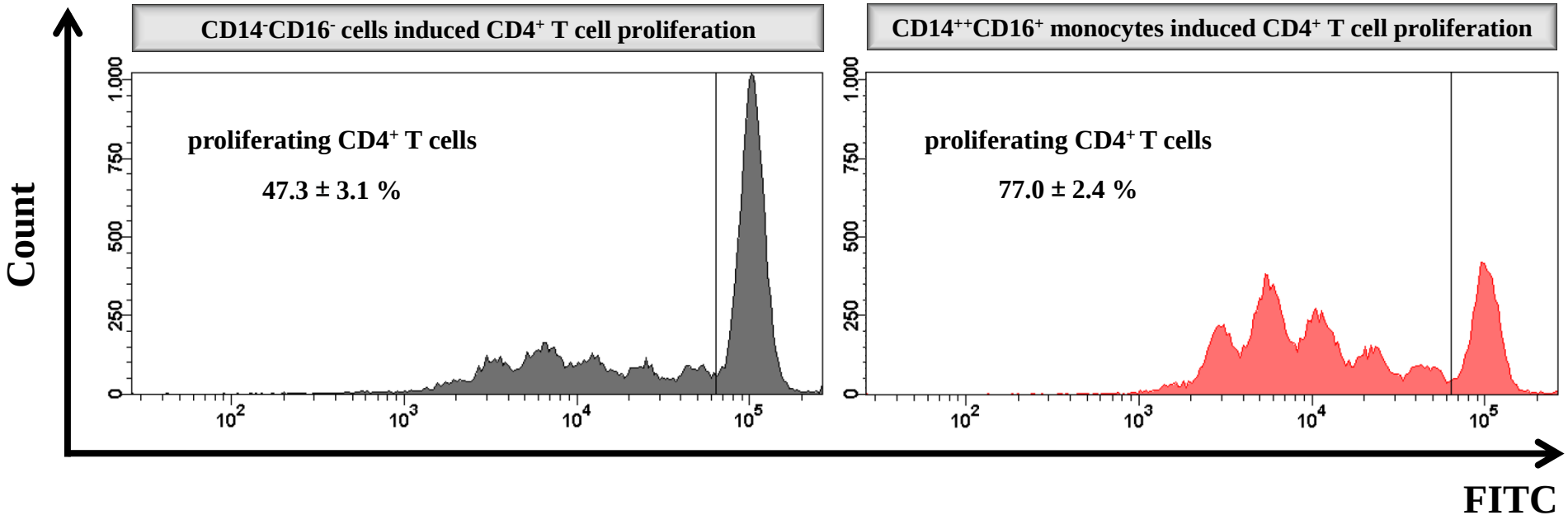
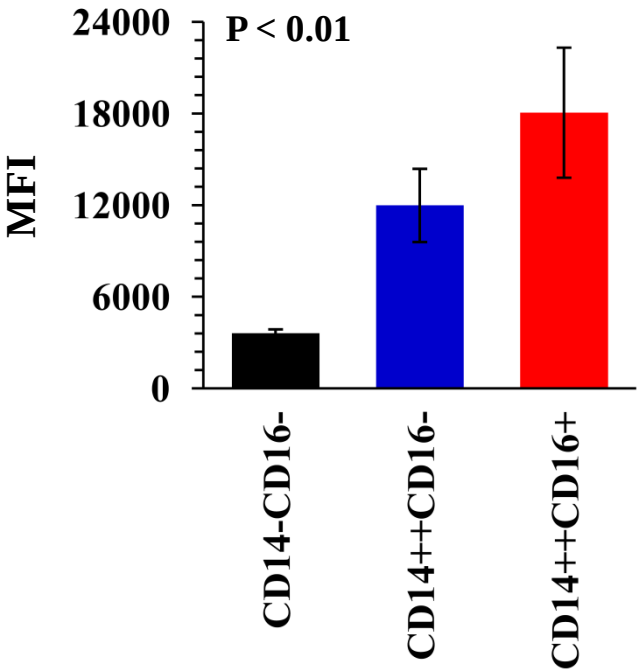

CD14-CD16- cells induced CD4+ T cell proliferation
proliferating CD4+ T cells
47.3 ± 3.1 %
CD14++CD16+ monocytes induced CD4+ T cell proliferation
proliferating CD4+ T cells
77.0 ± 2.4 %
Count
FITC
0
250
500
750
1.000
10^2
10^3
10^4
10^5

Figure 3D


P < 0.01
MFI
24000
18000
12000
6000
0
CD14-CD16-
CD14++CD16-
CD14++CD16+

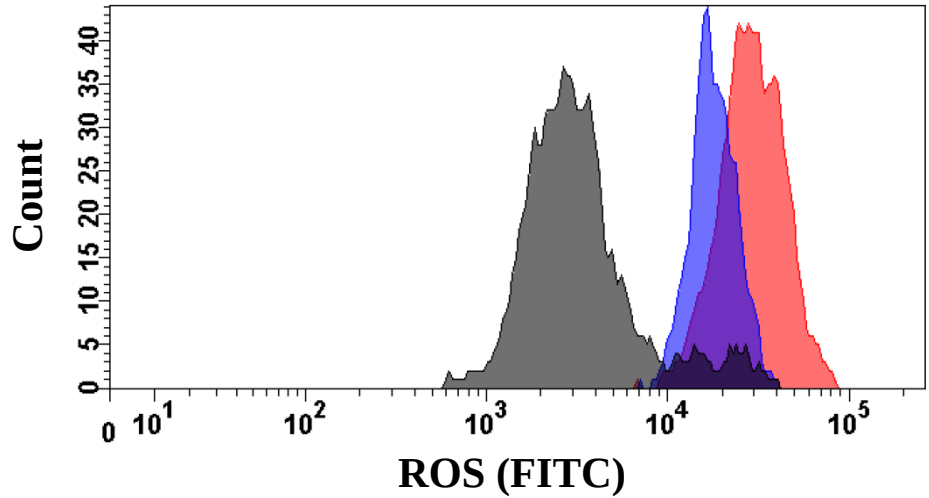

Count
0
5
10
15
20
25
30
35
40
0
10^1
10^2
10^3
10^4
10^5
ROS (FITC)

Figure 3E

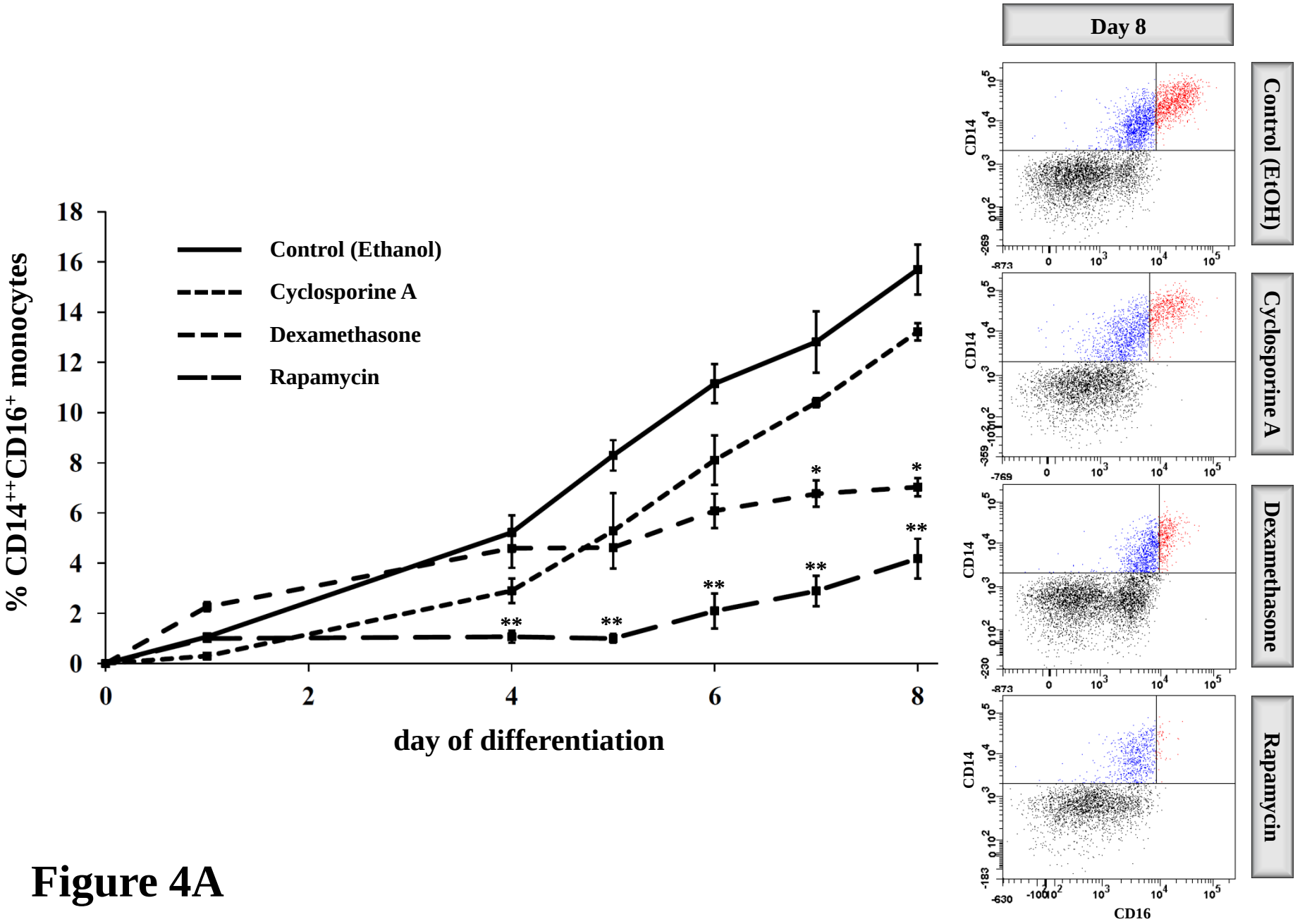

Day 8
Control (EtOH)
Cyclosporine A
Dexamethasone
Rapamycin
CD14
CD16
% CD14++CD16+ monocytes
day of differentiation
Control (Ethanol)
Cyclosporine A
Dexamethasone
Rapamycin
0
2
4
6
8
10
12
14
16
18
*
**

Figure 4A

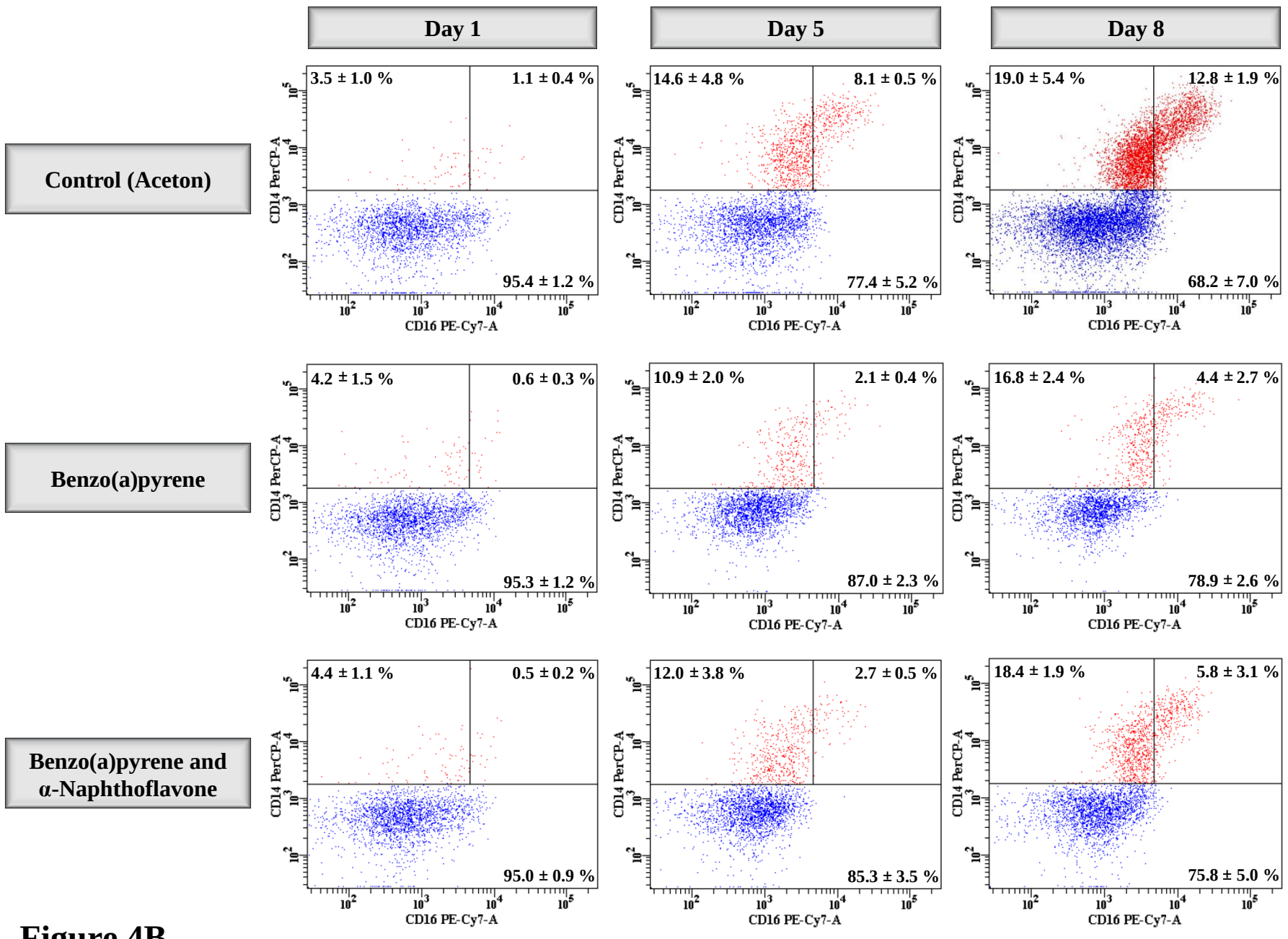

Day 1
Day 5
Day 8
Control (Aceton)
Benzo(a)pyrene
Benzo(a)pyrene and α-Naphthoflavone
3.5 ± 1.0 %
1.1 ± 0.4 %
95.4 ± 1.2 %
14.6 ± 4.8 %
8.1 ± 0.5 %
77.4 ± 5.2 %
19.0 ± 5.4 %
12.8 ± 1.9 %
68.2 ± 7.0 %
4.2 ± 1.5 %
0.6 ± 0.3 %
95.3 ± 1.2 %
10.9 ± 2.0 %
2.1 ± 0.4 %
87.0 ± 2.3 %
16.8 ± 2.4 %
4.4 ± 2.7 %
78.9 ± 2.6 %
4.4 ± 1.1 %
0.5 ± 0.2 %
95.0 ± 0.9 %
12.0 ± 3.8 %
2.7 ± 0.5 %
85.3 ± 3.5 %
18.4 ± 1.9 %
5.8 ± 3.1 %
75.8 ± 5.0 %
CD14 PerCP-A
CD16 PE-Cy7-A

Figure 4B