Identifizierung und Charakterisierung von myeloiden Suppressorzellen bei Patienten mit hepatozellulärem Karzinom

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Für meine Eltern,

mit all ihrer Liebe und Unterstützung

Μετὰ ταῦτα δή, εἶπον, ἀπείκασον τοιούτῳ πάθει τὴν ἡμετέραν φύσιν παιδείας τε πέρι καὶ ἀπαιδευσίας. Ἰδὲ γὰρ ἀνθρώπους οἷον ἐν καταγείῳ οἰκήσει σπηλαιώδει, ἀναπεπταμένην πρὸς τὸ φῶς τὴν εἴσοδον ἐχούσῃ μακρὰν παρ' ἅπαν τὸ σπήλαιον, ἐν ταύτῃ ἐκ παίδων ὄντας ἐν δεσμοῖς καὶ τὰ σκέλη καὶ τοὺς αὐχένας, ὥστε μένειν τε αὐτοῦ εἴς τε τὸ πρόσθεν μόνον ὁρᾶν, κύκλῳ δὲ τὰς κεφαλὰς ὑπὸ τοῦ δεσμοῦ ἀδυνάτους περιάγειν, φῶς δὲ αὐτοῖς πυρὸς ἄνωθεν καὶ πόρρωθεν καόμενον ὄπισθεν αὐτῶν, μεταξὺ δὲ τοῦ πυρὸς καὶ τῶν δεσμωτῶν ἐπάνω ὁδόν, παρ' ἣν ἰδὲ τειχίον παρῳκοδομημένον, ὥσπερ τοῖς θαυματοποιοῖς πρὸ τῶν ἀνθρώπων πρόκειται τὰ παραφράγματα, ὑπὲρ ὧν τὰ θαύματα δεικνύασιν.

Ὁρῶ, ἔφη.

Platon: Politeia 370 v. Chr

Erklärung zur Dissertation

Die Sortierung der Zellen wurde von Christina Reimer, Dr. Matthias Ballmaier und Dr. Mathias Rhein von der Zentralen Einrichtung für Zellsortierung durchgeführt.

Das Blut der Patienten wurde in der Gastroenterologisch- Onkologischen Tagesklinik entnommen.

Proliferationsversuche mit radioaktivem Thymidin wurden teilweise von Fr Buyny ausgelesenen.

Daten, die von Torsten Voigtländer erhoben wurden sind in die Abbildungen 1, 2, 3 und 4 der in der Zeitschrift Hepatology veröffentlichten Publikation „Myeloid Derived Suppressor Cells Inhibit Natural Killer Cells in Patients with Hepatocellular Carcinoma via the NKp30 Receptor“ eingeflossen.

Hierdurch erkläre ich, dass die Dissertation „Identifizierung und Charakterisierung von myeloiden Suppressorzellen bei Patienten mit hepatozellulärem Karzinom“ selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogenen Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, 22.03.2010

(Unterschrift)

Bastian Höchst

Zusammenfassung

Das Hepatozelluläre Karzinom (HCC) ist der fünft häufigste Tumor weltweit und steht an dritter Stelle der krebsbedingten Todesfälle. Dieser Tumor mit einer schlechten Heilungsaussicht zeigt eine steigende Inzidenz in Europa, den USA und Japan in den letzten 20 Jahren.

Derzeit bestehen die Behandlungsmethoden aus Resektion, Transplantation, lokal ablativen Verfahren, transarterieller Chemoembolisation und radiofrequenz- oder laserinduzierter Thermotherapie. Der Einsatz von Sorafinib zeigt erste Erfolge bei der systematischen Behandlung von HCC. Allerdings ist der Überlebensvorteil für die Patienten begrenzt.

Da das HCC eine Vielzahl von tumorspezifischen Antigenen exprimiert sind immuntherapeutische Behandlungen eine vielversprechende Option. tumorinduzierte Immunsuppression setzt Vakzinierungsansätzen und adoptiven T-Zelltransfer jedoch Grenzen. Während der Tumorprogression werden unter anderem myeloide Suppressorzellen (MDSC) induziert, die die Funktion und Proliferation von T-Zellen unterdrücken. Allerdings sind diese Zellen im Menschen nur sehr unzureichend charakterisiert. In dieser Arbeit wurden $CD14^{+}HLA\text{-}DR^{-/low}$-Zellen identifiziert, die sowohl im Blut als auch in Tumor von HCC Patienten signifikant vermehrt vorliegen. Wir konnten zeigen, dass MDSC die Funktion und Proliferation von $CD4^{+}$ T-Zellen hemmen. Der zugrunde liegende Mechanismus war hauptsächlich die Depletion von L-Arginin durch die Arginase I. Desweiteren zeigte sich, dass NK-Zellen ebenfalls durch MDSC hinsichtlich der Zytotoxizität als auch der Zytokinsekretion unterdrückt werden. Interessanter weise war die Suppression der NK- Zellen teilweise NKp30 vermittelt.

Neben der direkten Suppression durch MDSC zeigte sich ein weiterer Mechanismus, durch den eine Effektive Immunantwort gegen den Tumor unterbunden wird. Die hier beschriebenen MDSC waren *ex vivo* in der Lage $CD4^{+}CD25^{+}FoxP3^{+}$ T-Zellen zu induzieren, die ihrerseits wiederum suppressiv auf autologe T-Zellen wirkten. Dieser Mechanismus beruht auf der Expression von Membran-gebundenen TGF-β und der Fähigkeit der MDSC Retinsäure zu synthetisieren.

Somit stellen $CD14^{+}HLA\text{-}DR^{-/low}$-Zellen eine neue Population an suppressiven Leukozyten dar, die mehrere Schlüsselpositionen bei der Erkennung und Eliminierung von Tumoren hemmen. Für effektive immuntherapeutische Protokolle ist ein Verständnis dieser MDSC unerlässlich.

Schlagwörte: myeloide Suppressorzellen, Immunsuppression, hepatozelluläres Karzinom

Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and ranks third in cancer related death. This tumor with poor prospect of recovery shows a rising incidence in Europe, the USA and Japan in the last 20 years.

Currently, treating methods are resection, transplantation, local ablative procedure, transarterial chemoembolization and radiofrequency or laser-induced thermotherapy. The use of Sorafinib has shown limited success for the treatment of patients with HCC.

As the HCC expresses a variety of tumor-specific antigens, immunotherapeutic treatment options are a promising. Tumor-induced immune suppression, however, limits vaccination approaches and adoptive T cell transfer. Progressing tumors induces myeloid derived suppressor cells (MDSC), which suppress the function and proliferation of T cells. So far, these cells are characterized mainly in mice and only inadequately in humans. In this thesis, $CD14^{+}HLA\text{-}DR^{-/low}$-cells have been identified which are present both in blood and in tumors of HCC patients and are significantly increased compared to healthy donors. We have shown that MDSC inhibit proliferation of $CD4^{+}$ T cells. The underlying mechanism was mainly the depletion of l-arginine by arginase I. Furthermore, it became clear that NK cells are also suppressed by MDSC in terms of cytotoxicity and cytokine production. Interestingly, the suppression of NK cells was partially mediated by NKp30.

In addition to direct suppression of T cells, MDSC showed an additional mechanism by which an effective immune response against tumor is inhibited. The described MDSC were able to induce ex vivo $CD4^{+}CD25^{+}FoxP3^{+}$ T cells, which in turn their hand worked suppressive to autologous T cells. This mechanism was based on the expression of membrane-bound TGF-β and the synthesis to retinoic acid by MDSC.

Thus, $CD14^{+}HLA\text{-}DR^{-/low}$-cells represent a new population of suppressive leukocytes that inhibit several key positions in of anti-tumor immunity. For the generation of effective immunotherapeutic protocols is essential to understand the function of MDSC.

Catchwords: myeloid derived suppressor cells, immune suppression, hepatocellular carcinoma

Inhalt

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Abkürzungsverzeichnis

1-MT	1-Methyltryptophan
7AAD	7-Amino-Actinomycin D
Abb	Abbildung
ADCC	*antibody-dependent-cellular cytotoxicity,* Antikörper-abhängige zellvermittelte Zytotoxizität
AFP	α-Fetoprotein
AK	Antikörper
APC	*antigene-presenting-cell,* Antigen-präsentierende Zelle
APC	bei Ak: Allophyocyanin
atRA	*all-trans-retinoic* Acid trans- Retinsäure
$Aqua_{dest}$	destilliertes Wasser (aqua destillata)
Bq	Becquerel
BSA	*bovine serum albumin*, Rinderserumalbumin
bzw.	beziehungsweise
bspw.	beispielsweise
Ak	Antikörper
C	Celsius
D	Deutschland
CD	cluster of differentiation
CO_2	Kohlenstoffdioxid
CTLA-4	Zytotoxisches T-Lymphozyten Antigen 4
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonukleinsäure
EDTA	Ethylendiamintetraessigsäure
ELISA	enzyme linked immunosorbent assay
et al.	und andere
FACS	fluorescence activated cell sorting
FASL	FAS Ligand
Fc	*fragment crystalline,* c-terminales Fragment von Immunglobulinen
FCS	*fetal-calf-serum,* fötales bovines Serum
FITC	Fluoreszeinisothiocyanat
Foxp3	forkhead box P3
FSC	forward scatter channel, Vorwärtsstreulicht
g	Gramm

GITR	Glukokortikoid-induzierter Tumor Nekrose Faktor Rezeptor
GM-CSF	Granulozyten-Makrophagen Kolonie-stimulierender Faktor
h	Stunden
HBV	Hepatitis-B Virus
HCC	Hepatozelluläres Karzinom
HCV	Hepatitis-C Virus
HLA	humane Leukozytenantigene
H_2SO_4	Schwefelsäure
IDO	Indoleamine-2,3- Dioxygenase
IFN	Interferon
Ig	Immunglobulin
IL	Interleukin
IU	International unit
KIRs	Killer Immunglobulin-ähnliche Rezeptoren
l	Liter
LIRs	Leukozyten-immunglobulinähnliche-Rezeptoren
L-NMMA	L-NG-Monomethyl Arginin
L-NOHA	N (Omega)-hydroxy-L-Arginin
LPS	Lipopolysaccharid
µ	mikro (x 10^{-6})
m	milli (x 10^{-3})
MACS	magnetic activated cell sorting
MBq	Megabequerel
MDSC	*myeloid-derived suppressor cells*, Myeloide Suppressorzellen
MHC	*major histocompatibility complex,* Haupthistokompatibilitätskomplex
min	Minuten
mM	Millimolar
n =	Anzahl der Einzelbeobachtungen
N	Niederlande
NaCl	Natriumchlorid
NaN_3	Natriumazid
NCR	Natürliche Zytotoxizitätsrezeptoren
NKT-Zellen	Natürliche Killer T-Zellen
NK- Zellen	Natürliche Killerzellen
NO	Stickstoffmonoxid
RA	*retinoic acid* Retinsäure

PBMCs	peripheral blood mononuclear cells, mononukleäre Zellen
PBS	*phosphate buffered saline,* Phosphat-gepufferte physiologische Kochsalzlösung
PE	Phycoerythrin
PECy5	PhycoerythrinCy5
ROS	*reactive oxygen species*, reaktive Sauerstoffspezies
rpm	*rounds per minute*, Umdrehungen pro Minute
RPMI	Roswell Park Memorial Institute
RT	Raumtemperatur
S	Sekunden
SSC	side scatter channel
stim.	stimuliert
TACE	transarterielle Chemoembolisation
TCR	T-Zell Rezeptor
TGF	transforming growth factor
TIL	Tumor-infiltrierende Lymphozyten
TNF	Tumor Nekrose Faktor
TRAIL	TNF abhängiger Apoptose-induzierender Ligand
Treg	regulatorische T-Zelle
u.a.	unter anderem
unstim.	unstimuliert
v.a.	vor allem
v / v	Volumen pro Volumen

Einleitung

Das Hepatozelluläre Karzinom

Das hepatozelluläre Karzinom (HCC) ist weltweit die fünft häufigste Krebserkrankung und stellt die dritt häufigste krebsbedingte Todesursache dar (Parkin et al., 2005). Zwischen 85% und 90% aller primären Leberkrebse werden als HCC diagnostiziert. Die Entwicklung des HCC wird durch verschiedene epidemiologische Faktoren, wie Alter, Geschlecht, Zugehörigkeit zu ethnischen Gruppen oder die geographische Lage des Wohnortes, beeinflusst.

Globale Verteilung des HCC

Jährlich werden über eine Millionen HCC Neuerkrankungen diagnostiziert, auffällig ist eine ungleich verteilte Inzidenz. Über 80% aller weltweiten Fälle treten in Afrika und Fernost auf. Allein in China werden 50% aller HCC diagnostiziert.

In Nord- und Süd- Amerika sowie Europa tritt HCC mit einer wesentlich geringeren Häufigkeit auf. Für Europa konnten zudem signifikant unterschiedliche Fallzahlen im Norden und Süden beobachtet werden (Parkin et al., 2005).

Ursächlich für dieses Verteilungsmuster ist vor allem die hohe Prävalenz an chronischer Hepatitis-B-Virus-Infektion (HBV-Infektion) in den Gebieten hoher HCC Inzidenz (Amin et al., 2007). Durch die Impfung aller Neugeborenen in vielen asiatischen Hochrisikogebieten gegen das Hepatitis-B-Virus (HBV) zeigt sich hier bereits ein Rückgang der HCC Raten (Chang et al., 1997), allerdings nimmt die Anzahl der HCC-Fälle weltweit, besonders in den industrialisierten Ländern, weiter zu (Bosch et al., 2004). Diese Entwicklung wird hauptsächlich durch eine Zunahme chronischer Hepatitis-C-Virus-(HCV) Infektionen bedingt, wobei Männer hier etwa 3-4-fach häufiger betroffen sind als Frauen (Schott, 2008). Im Gegensatz zur HBV-Impfung liegt für die HCV-Infektion derzeit kein Impfstoff vor.

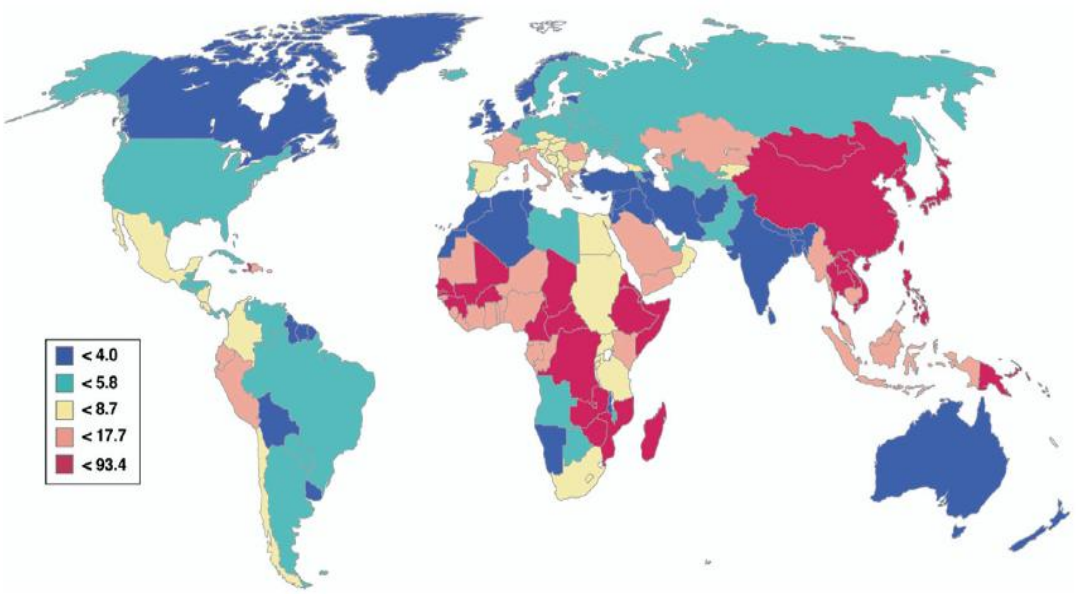

Abbildung 1: Altersangepasste Mortalitätsraten des HCC nach El-Serag et al. 2007. Die Raten sind pro 100 000 Personen angegeben.

Geschlecht

In nahezu allen Populationen zeigt sich bei Männern eine zwei und viermal höhere Erkrankungsrate von HCC gegenüber Frauen. Diese kann teilweise durch geschlechtsspezifische Verhaltensmuster in Hinsicht auf Risikofaktoren erklärt werden. Männer sind häufiger mit HBV und HCV infiziert, trinken mehr Alkohol, rauchen häufiger und haben einen erhöhten Eisenspiegel. Zudem zeigte sich im Mausmodel, dass männliche Tiere zwei bis achtmal häufiger HCC entwickeln als weibliche Kontrolltiere (Rudolph et al., 2000). Dies verweist auf einen zusätzlichen androgenen Einfluss bei Entstehung oder Entwicklung des HCC und relativiert die geschlechtsspezifischen Verhaltensmuster als Risikofaktoren. Der androgene Einfluss wurde auch im Humansystem durch Studien bekräftigt, in denen ein erhöhter Testosteronspiegel mit der Entstehung von HCC in Patienten mit HBV korreliert war (Yu and Chen, 1993; Yu et al., 2001).

HBV und HCV

HBV ist, wie bereits erwähnt, die häufigste Ursache für HCC mit ca. $3x10^8$ Personen weltweit, die an einer chronischen Infektion leiden. Studien ergaben, dass chronisch HBV- infizierte Personen ein 5-15 fach erhöhtes Risiko tragen ein HCC zu entwickeln. In 70% - 90% aller HBV assoziierten HCCs tritt parallel eine Leberzirrhose auf.

Während in den Regionen mit hoher Inzidenz des HCCs vor allem eine HBV Infektion vorliegt, ist in den industrialisierten Ländern eine HCV Infektion die vorherrschende Ursache (Bruno et al., 2001; Donato et al., 2001).

Alkohol

Ein weiterer Risikofaktor für HCC ist Alkoholabusus mit einer Aufnahme von 50-70 g Ethanol pro Tag (Donato et al., 2001). Bei Personen, die regelmäßig 60 g pro Tag konsumieren wurde eine lineare Zunahme des HCC Risikos nachgewiesen (Donato et al., 2001). Allerdings liegt die Hauptursache hierbei in der Entwicklung einer Leberzirrhose, während keine Hinweise für eine direkt karzinogene Wirkung des Alkohols sprechen.

Aflatoxin

Das Mycotoxin AFB_1 wird von *Aspergillus fungus* produziert. Dieser findet ideale Wachstumsbedingungen auf Getreide, wenn dieses warm und feucht gelagert wird. Im Gegensatz zu Alkohol ist AFB_1 direkt hepatokarzinogen.

Aflatoxin wird in der Leber metabolisiert, wodurch das aktive Intermediat AFB_1-exo-8,9-epoxid entsteht. Dieses kann in die DNA interkalieren und verursacht eine charakteristische Mutation des Tumorsuppressorgens p53 (p53 249^{ser}), die in 30-60% aller AFB_1 assoziierten HCC auftritt (Garner et al., 1972).

Die Entstehung des HCC ist im Allgemeinen das Endstadium einer typischerweise asymptomatisch verlaufenden Leberzirrhose. Diese beginnt meist mit einer unerkannten akuten bis subakuten Leberschädigung, die allmählich über eine Fibrotisierung entsteht (Seeff, 2004).

Therapie des HCC

Die Therapie des HCCs ist nicht nur von dem Tumorstadium abhängig, sondern wird erheblich von der in den meisten Fällen vorliegenden Zirrhose und der Leberrestfunktion beeinflusst. Dementsprechend wurden verschiedene Klassifikationssysteme entwickelt, die, neben dem Tumorstadium, die Leberfunktion berücksichtigen. Hierzu zählt die Okuda-Klassifikation des HCC, die die Tumorausdehnung, das Vorhandensein von Aszites sowie die Serumkonzentration an Albumin und Bilirubin einschließt. Der CLIP-Score (Cancer of the Liver Italian Group Programme) zur Stadienerfassung des HCC beinhaltet die Klassifikation der Leberzirrhose nach Child-Pugh, die Tumorausdehnung, den AFP-Spiegel sowie das etwaige Vorhandensein einer Portalvenenthrombose. Die BCLC-Kriterien (Barcelona-Clinic-Liver-Cancer) zur Definition des HCC-Tumorstadiums berücksichtigen den Allgemeinzustand der Patienten, die Tumorausdehnung, die Leberfunktion und die Einteilung der Patienten gemäß Okuda-Klassifikation. Bisher wird jedoch keines der vorhandenen Systeme universell angewandt (Talwalkar and Gores, 2004).

Zu den kurativen Therapiemethoden zählen die chirurgische Resektion der Tumor-Herde, lokal ablative Therapieverfahren und die Transplantation der Leber. Allerdings wird in westlichen Industriestaaten die Erkrankung durch den asymptomatischen Verlauf häufig in einem

fortgeschrittenen Stadium diagnostiziert, sodass für weniger als 5% der Patienten eine Resektion in Betracht kommt (Williams et al., 2007).

Bei eingeschränkter Leberfunktion wird immer die Möglichkeit einer Transplantation in Betracht gezogen, da hier nicht nur der Tumor entfernt wird, sondern die häufig vorhandene Zirrhose als Präkanzerose mit therapiert wird (Greten et al., 2006a). Die hohe Anzahl von Rezidiven kann bei dieser Therapieform durch die Milan-Kriterien minimiert werden, so dass hiermit eine 5-Jahres-Überlebensrate von 70% erreicht wird (Mazzaferro et al., 1996).

Eine weitere Option der HCC-Therapie stellen die perkutanen Therapieverfahren dar, die bei rechtzeitiger Diagnose des HCCs ebenfalls kurativ eingesetzt werden können. Es handelt sich um bildgestützte tumorabladierende Techniken unter Verwendung von Chemikalien, meistens Ethanol, oder Wärme. Sie stellen derzeit die beste Therapieoption für chirurgisch nicht behandelbare HCCs dar (Omata et al., 2004). Die Ethanolinjektion wird augenblicklich, aufgrund der relativ geringen Kosten und des günstigen Risikoprofils, am häufigsten genutzt. Daneben besteht die Möglichkeit Tumorgewebe mittels Radiofrequenzen, Mikrowellen, Lasern oder Kälteapplikation zu eliminieren. Einzelne noduläre Herde, die kleiner als 5 cm sind sowie bis zu 3 Herde, die jeweils kleiner als 3 cm sind, werden als Indikation zu dieser Therapieform akzeptiert. Die Ergebnisse zeigen, dass bis zu 90% der Tumoren, die kleiner als 2 cm sind, durch diese perkutane Verfahren abladiert werden können (Beaugrand et al., 2005).

Bei Therapie großer nicht resezierbarer Tumore kommt die transarterielle Chemoembolisation (TACE) zum Einsatz. Es handelt sich um ein Verfahren, bei dem ein Chemotherapeutikum direkt über eine tumorversorgende Arterie injiziert wird, sodass anschließend eine Embolisation des Gefäßes hervorgerufen wird. Die erzeugte Ischämie verstärkt die antitumorale Wirkung des Chemotherapeutikums. Insbesondere Patienten mit guter Leberfunktion (Child-Pugh A) und asymptomatischen multilokulären Tumoren ohne Gefäßinvasion profitieren von diesem Ansatz (Bruix et al., 2004). Weitere Anwendung findet die TACE als adjuvante Therapie nach erfolgter Resektion und zur „bridging"-Therapie vor einer Lebertransplantation, Für diese Indikation liegen jedoch nur wenige verlässliche Daten vor (Greten et al., 2006a). Die lokale Strahlentherapie des HCCs bleibt bisher Einzelfällen vorbehalten.

Der Einsatz konventioneller Chemotherapeutika zur systemischen Therapie des HCCs zeigt nur geringe Ansprechraten bei hohem Nebenwirkungsprofil. Gewöhnlich gilt das HCC als chemoresistent (Burroughs et al., 2004). Erstmalig konnte jedoch unter Therapie mit Sorafenib, einem neuen Multityrosinkinaseinhibitor, das Gesamtüberleben für Patienten mit fortgeschrittenem HCC von 7,9 auf 10,7 Monate verlängert werden (Llovet et al., 2008).

Immuntherapie des HCC

Eine retrospektive Analyse von 163 chirurgisch resezierten HCCs hat gezeigt, dass eine Lymphozyteninfiltration des Tumors mit einer besseren Prognose korreliert (Wada et al., 1998). Andere Studien zeigen ebenfalls, dass die Immuntherapie in Zukunft eine potentielle therapeutische Option für HCC Patienten sein kann (Butterfield, 2004).

Immuntherapeutische Behandlungsverfahren des HCC beinhalten einerseits die nicht-spezifische Aktivierung des Immunsystems durch die Gabe von Zytokinen im Rahmen der passiven Immuntherapie, jedoch ist auch eine antigenspezifische passive Immuntherapie durch Verabreichung monoklonaler Antikörper möglich. Unter Verwendung autologer und allogener Tumorzellen, Peptiden, Proteinen und DNA-Vakzinen wird bei einer aktiven Immuntherapie antigenspezifisch eine Immunreaktion gegen den Tumor initiiert (Greten et al., 2006b). Bei der adaptiven Tumorimmuntherapie werden NK-Zellen und T-Lymphozyten des Patienten oder eines allogenen Spenders *in vivo* unter GMP-Bedingungen stimuliert sowie amplifiziert und anschließend in den Patienten rücktransferriert. Bisher standen insbesondere die $CD8^+$ T-Zellen im Zentrum immuntherapeutischer Behandlungsversuche. Die zytotoxischen T-Zellen können mit ihrem T-Zellrezeptor das für sie spezifische Peptid, sofern dieses, zusammen mit ko- stimulatorischen Signalen präsentiert wird, erkennen. MHC-Klasse-I-Moleküle präsentieren intrazellulär synthetisierte Proteine in Form kleiner prozessierter Aminosäureketten. Im Rahmen von Tumorerkrankungen werden Proteine überexprimiert, liegen in mutierter Form vor oder sind viraler Genese und können somit eine Immunantwort generieren. Ein weiterer Aktivierungsweg zytotoxischer T-Zellen erfolgt über die zytokinvermittelte Stimulation von $CD4^+$ T-Zellen. $CD4^+$ T-Zellen interagieren mit professionellen Antigen-präsentierenden Zellen. Diese können exogene Peptide aufnehmen, prozessieren und gekoppelt an MHC-Klasse-II Moleküle präsentieren. Das Konzept der Vakzination wird dem des adoptiven T-Zelltransfers aus ökonomischen Gründen zumeist vorgezogen.

Effektorzellen des unspezifischen Immunsystems besitzen ebenfalls immuntherapeutisches Potential. Insbesondere für NK-Zellen ist seit längerer Zeit bekannt, dass sie *in vivo* spontan MHC-Klasse-I-defiziente Tumorzellen und ihre Metastasen eliminieren können (Whiteside and Herberman, 1995). Des Weiteren sind NK-Zellen eine der Hauptproduzenten von IFN-γ, welches pro-apoptotische und anti-proliferative Effekte auf Tumorzellen hat (Ikeda et al., 2002). Es wurde in Mausmodellen gezeigt, dass IFN-γ eine wichtige Rolle in der Abstoßung transplantierter Tumoren sowie in der Prävention der primären Tumorentwicklung spielt (Dighe et al., 1994; Kaplan et al., 1998).

Aktuell rücken suppressive Immunzellen vermehrt in den Fokus der Forschung.

Konzepte der Immuntherapie sind vorhanden, allerdings führen sie bisher nur zu sehr unzureichenden Ergebnissen. Um eine suffiziente Immuntherapie in der Klinik zu gewährleisten muss die immunsuppressive Aktivität von u.a. regulatorischen T-Zellen und myeloiden Suppressorzellen eliminiert und somit die Tumortherapie unterstützt werden (Kusmartsev and Gabrilovich, 2006b; Pure et al., 2005).

Tumorimmunologie

Viele spezifische Proteine, die zumindest potentiell immunogen sind wurden im HCC identifiziert. Dazu gehört das α-Fetoprotein (AFP), ein onkofetales Protein welches für die Diagnose und Verlaufskontrolle des HCC heran gezogen wird (Butterfield, 2004). Daneben finden sich typische Hodentumor-assoziierte wie MAGE (Chomez et al., 2001), SHP-1 (Chen et al., 2003), CTP11 (Dong et al., 2003) und NY-ESO1 (Zhang et al., 2005). Dennoch wird der Tumor nur unzureichend vom Immunsystem erkannt.

Immunosurveillance

Paul Ehrlich stellte 1909 als einer der Ersten, die Theorie auf, dass das Immunsystem in der Lage ist, das Wachstum von Karzinomen zu unterdrücken. In der Mitte des 20. Jahrhunderts wurde von F. M. Burnet und L. Thomas die Theorie der Immunosurveillance aufgestellt. Burnet war der Meinung, dass Tumor-spezifische Antigene eine effektive Immunantwort auslösen, die zur Elimination der entarteten Zellen führen (Burnet, 1957, 1971a, b, c; Burnet and Holmes, 1964). Thomas vertrat die Meinung, dass ein komplexer, langlebiger Organismus Mechanismen haben müsse, die, ähnlich wie bei der Abstoßung von Transplantaten wirkend, protektiv vor Neoplasien schützen (Thomas, 1959).

Später wurden dieser Theorie mit der Immunoediting-Hypothese noch zwei weitere Aspekte hinzugefügt (Dunn et al., 2002; Dunn et al., 2004a, b; Schreiber, 2005). Das Immunoediting beschreibt einen dynamischen Prozess der Antitumorimmunantwort bestehend aus drei Phasen: Elimination, Equilibrierung und Entkommen, einer Art Katz-und-Maus-Spiel, bei dem die Maus immer einen Schritt voraus ist (Melief, 2005).

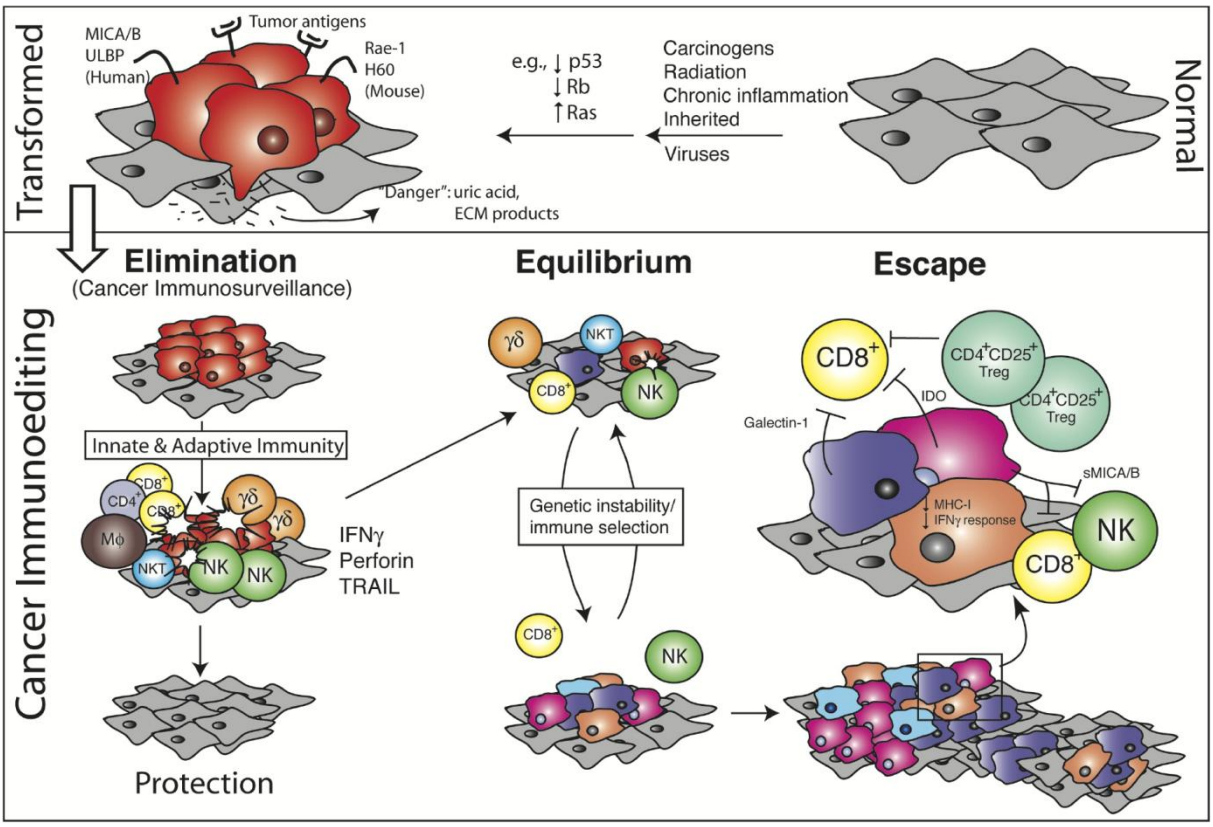

Abbildung 2: Die drei Phasen des Krebs-Immunoediting Prozess

Die erste Phase, die Elimination, entspricht der klassischen Immunosurveillance. Es kommt zu einer Immunantwort gegen den Tumor, an der sowohl das angeborene als auch das adaptive Immunsystem beteiligt sind. In dieser Phase ist das Immunsystem in der Lage, den Tumor vollständig zu beseitigen. In der zweiten Phase, der Equilibrierung, kommt es zu einem Gleichgewicht zwischen dem Immunsystem und den Tumorzellen, die der Elimination entgehen. Durch diese dynamische Interaktion und der genetischen Instabilität des Tumors kommt es zur Selektion von Tumorzellen, die in dem immunkompetenten Wirt wachsen können und nicht eliminiert werden. Die letzte Phase ist durch unkontrolliertes Wachstum nicht immunogener Tumorzellklone und eine zusätzliche tumorinduzierte Suppression des Immunsystems gekennzeichnet.

Tumor-Immune-Escape

Die Fähigkeit des Tumors trotz spezifischer Immunreaktionen unkontrolliert zu wachsen, wird durch eine Reihe vom Tumor induzierter Mechanismen ermöglicht.

In einer Vielzahl von Studien konnte gezeigt werden, dass Tumore nur noch unzureichend Antigene präsentieren. Solche „Antigen-loss-variants“ entstehen, wenn das zur Antigenpräsentation notwendige MHC-I-Molekül auf Tumorzellen nicht mehr exprimiert wird (Algarra et al., 2000). Außerdem können Defekte in anderen Komponenten der Antigenprozessierung

auftreten. Mutationen im „Transporters associated with antigen processing“ (TAP) und in den Immunoproteosom- Untereinheiten LMP2 und LMP7 wurden beschrieben (Selinger et al., 2000).

Eine zentrale Rolle bei der Unterdrückung der Tumor-spezifischen Immunantwort nehmen Zellen ein, die suppressiven Einfluss auf das Immunsystem haben. Ein durch die chronische Entzündungsreaktion hervorgerufenes spezifisches Zytokinprofil in der Tumormikroumgebung bewirkt eine Akkumulation von regulatorischen T-Zellen, tolerogenen dendritischen Zellen, Tumor-assoziierten Makrophagen und myeloiden Suppressorzellen.

Suppressive Immunzellen

Neben Veränderungen der Tumorzellen selbst wird die Effektivität der Immunantwort ebenfalls durch die Rekrutierung immunregulatorischer oder immunsuppressiver Zellen verändert. Diese Zellen, die in tumortragenden Organismen stark vermehrt vorliegen, sind modifizierte Immunzellen, die ihre ursprüngliche Funktion der Tumorabwehr nicht mehr erfüllen. Sie induzieren aufgrund unterschiedlichster Mechanismen Toleranz oder Anergie des Immunsystems gegenüber malignen Zellen.

Regulatorische T-Zellen (Tregs)

$CD4^+$ T-Zellen, die konstitutiv CD25, CD152, den glykokortikoid-induzierten TNF-Rezeptor (GITR) sowie den „forkhead box P3“- (FoxP3) Transkriptionsfaktor exprimieren, werden als regulatorische T-Zellen (Tregs) bezeichnet (Hori et al., 2003; Sakaguchi et al., 1995; Shimizu et al., 2002). Natürlich vorkommende Tregs reifen im Thymus und stellen 5-10% der peripheren $CD4^+$ T-Zellen dar (Wing et al., 2002). Die physiologische Rolle regulatorischer T-Zellen besteht in der Unterdrückung autoreaktiver Immunzellen, welche zu Autoimmunerkrankungen mit massiver Gewebeschädigung führen würden (Vieweg et al., 2007).

Neben natürlich vorkommenden Tregs sind bei Patienten auch von dem Tumor induzierte Tregs angereichert. Dies konnte für das Nierenzellkarzinom, das Melanom (Cesana et al., 2006; Dannull et al., 2005), das Lungenkarzinom (Woo et al., 2002) als auch für das hepatozelluläre Karzinom (Ormandy et al., 2005) nachgewiesen werden. Die Akkumulation von Tregs ist einer der meist Verbreitesten Mechanismen tumorinduzierter Immunsuppression, wobei der Einfluss in unterschiedlichen Neoplasien variiert (Quezada et al., 2008; Sutmuller et al., 2001).

Die Inhibition von gegen Tumorzellen gerichteten Lymphozyten durch Tregs erfolgt über mehrere Mechanismen. Dies können einerseits inhibitorische Zytokine sein, oder die Inhibition erfolgt über Zell-Zellkontakt mit der Zielzelle (Scheffold et al., 2007).

Es konnte gezeigt werden, dass TGF-β, Fas, Granzym-B, „lymphocyte activated gene“ (LAG)- 3 sowie „cytotoxic T lymphocyte antigen 4“ (CTLA-4) von regulatorischen T-Zellen exprimiert werden und Zielzellen nach direkten Kontakt in der Aktivität blockiert oder abgetötet werden (Bodor et al., 2007; Grossman et al., 2004; Nakamura et al., 2001; Paust et al., 2004). Die Sekretion von inhibitorischen Zytokinen wie TGF-β, IL-10 und IL-35 (Collison et al., 2007; Vignali et al., 2008) ist dagegen nicht von direktem Zell-Zellkontakt abhängig und kann auch zu einer „bystander“ Unterdrückung führen.

Durch CD39 und CD73 generieren Tregs Adenosin- Nukleotide, die Effektorfunktionen von T-Zellen blockieren (Deaglio et al., 2007; Kobie et al., 2006). Der Transfer des sekundären Botenstoffes zyklisches Adenosinmonophosphat (cAMP) über „gap junctions“ in T- Effektorzellen ist ein weiterer Mechanismus der Suppression (Bopp et al., 2007).

Über Wechselwirkungen mit dendritischen Zellen (DC) sind Tregs ebenfalls in der Lage, Immunantworten zu schwächen. In präklinischen Modellen konnte in einer Reihe von Studien gezeigt werden, dass Tregs und DCs interagieren (Vignali et al., 2008). In einem CTLA-4 abhängigen Prozess werden DCs in ihrer T-Zell Stimulation abgeschwächt (Oderup et al., 2006; Read et al., 2000). Des Weiteren konnte nachgewiesen werden, dass Tregs in DCs Indolamin-2,3-dioxygenase I- (IDO) Expression induzieren können, dessen Metabolite T-Lymphozyten hemmen (Fallarino et al., 2003; Mellor and Munn, 2004). Durch den von Tregs induzierten Verlust von CD80 und CD86 ist darüber hinaus keine effektive Ko-Stimulation von T-Zellen durch DCs möglich (Cederbom et al., 2000).

Tolerogene Dendritische Zellen

Dendritische Zellen sind nicht nur bei der Stimulation von T-Zellen wichtig, sie spielen auch eine Rolle bei der Erhaltung der peripheren Toleranz, indem autoreaktive T-Zellen unterdrücken (Steinman et al., 2003). Diese tolerogenen dendritischen Zellen präsentieren Autoantigene ohne zusätzliche Kostimulation. T-Zellen, die für die präsentierten Autoantigene spezifisch sind, werden von den DCs nicht stimuliert sondern in einen anergen Zustand versetzt. Dies wird durch die Ausschüttung immunsuppressiver Zytokine verstärkt (Fathman and Lineberry, 2007). Bei Tumorpatienten ist sowohl die Anzahl der DCs als auch ihre Fähigkeit T-Zellen zu stimulieren gegenüber gesunden Probanden deutlich verringert (Kim et al., 2006; Ormandy et al., 2006; Pinzon-Charry et al., 2007). Vom Tumor sezernierte Faktoren wie VEGF, TGF-β, IL-6 und IL-10 stören die normale Ausreifung von Monozyten zu funktionellen dendritischen Zellen und führen zur Entstehung eines tolerogenen Phänotypes (Enk et al., 1997; Gabrilovich, 2004; Geissmann et al., 1999; Steinbrink et al., 1997). Der in Tumorzellen überexprimierte Transkriptionsfaktor STAT3 induziert die Sekretion von VEGF und IL-10.

Dendritische Zellen exprimieren aufgrund der inhibitorischen Zytokine ebenfalls STAT3 und reifen nicht zu funktionellen antigenpräsentierenden Zellen heran (Gabrilovich, 2004).

Die Expression von MHC-II sowie kostimulatorischen Molekülen ist bei tolerogenen DCs herabgesetzt (Geissmann et al., 1999; Ormandy et al., 2006), sodass eine effektive T-Zellstimulation nicht mehr möglich ist. Erhöhte IDO-Expression in tolerogenen DC ist ein zusätzlicher Suppressionsmechanismus gegenüber T-Zellen (Lob and Konigsrainer, 2008). Durch den Kontakt von T- Lymphozyten mit tolerogenen DCs werden immunsuppressive Zytokine wie TGF-β und IL-10 sezerniert, welches bei $CD8^+$ T-Zellen eine Anergie auslöst (Gabrilovich, 2004) und $CD4^+$ T-Zellen zu Tregs differenzieren lässt (Enk, 2005; Mahnke et al., 2007). Es konnte ebenfalls gezeigt werden, dass tolerogene DCs, die durch VEGF beeinträchtigt wurden, zur Induktion von IL-10 produzierenden „T regulatory like cells“ (T_R1) beitragen (Jonuleit et al., 2000).

Tumor assoziierte Makrophagen

Makrophagen sind Teil des angeborenen Immunsystems und spielen eine wichtige Rolle in allen Bereichen des Immunsystems. Durch IFN-γ oder LPS „klassisch“ aktivierte Makrophagen zerstören Tumorzellen durch die Produktion von NO und Typ-I-Zytokinen sowie Chemokinen. Diese Makrophagen fungieren darüber hinaus ebenfalls als Antigen präsentierende Zellen und aktivieren zytotoxische $CD8^+$ T-Zellen (Sinha et al., 2007a). Im Gegensatz dazu begünstigen durch IL-4, IL-13 und TGF-β „alternativ“ aktivierte Makrophagen die Tumorprogression indem sie zu einer verstärkten Neovaskularisation führen und Typ II Zytokine und Chemokine sezernieren (Martinez et al., 2009). Die meisten progressiv wachsenden Tumore sind von vielen Makrophagen infiltriert. Diese Tumor-assoziierten-Makrophagen (TAMs) spielen eine wichtige Rolle im Tumorstroma und sind essentiell für die Angiogenese und die Remodellierung der Matrix. Hierfür sind vor allem VEGF und Metalloproteasen verantwortlich (Varin and Gordon, 2009). Durch letzeres erleichtern sie ebenfalls die Bildung von Metastasen, da sie die Intravasation begünstigen. TAMs exprimieren darüber hinaus das negative kostimulierende Molekül B7-H4 und damit zu einer Immunsuppression führt (Kryczek et al., 2006).

Myeloide Suppressorzellen

Myeloide Suppressorzellen (MDSC) wurden erstmals vor 25 Jahren als hematopoetische Suppressorzellen nichtlymphoiden Ursprungs beschrieben (Stober et al., 1984). Erste Hinweise, dass diese Zellen myeloiden Ursprung sind ergaben sich über 10 Jahre später, als man fest stellte, dass es bei Patienten mit Hals-Kopf-Karzinome (Pak et al., 1995b) sowie in Mäusen mit Lungenkarzinomen zu einer Anhäufung von $CD34^+$ Zellen im Blut kommt.

In späteren Arbeiten wurden MDSC dann in murinen Modellen als unreife myeloide Zellen charakterisiert, die Vorstufen von DC, Granulozyten oder Makrophagen beinhalten (Gabrilovich et al., 2007; Serafini et al., 2006a).

Phänotyp von MDSC und Morphologie

Identifiziert wurden MDSC in Patienten und Tumor-Maus-Modellen aufgrund ihrer Eigenschaft, Proliferation- und Zytokinsekretion von T-Zellen zu supprimieren.

In Mäusen werden MDSC durch die Expression von CD11b und Gr-1 charakterisiert.

Gr-1 beinhaltet die Makrophagen- und Neutrophilen- Marker LyG6C und LyG6G, CD11b wird dagegen von Makrophagen exprimiert. Diese Marker werden auf myeloiden Vorläuferzellen koexprimiert, weshalb man davon ausgeht, dass MDSC unreife Vorläuferzellen darstellen.

Darüber hinaus wurden noch IL-4Rα (Gallina et al., 2006; Umemura et al., 2008), der Makrophagenmarker F4/80 (Huang et al., 2006; Rossner et al., 2005; Umemura et al., 2008), der M-CSF-Rezeptor (CD115) und das kostimulatorische Molekül CD80 (Yang et al., 2006) auf MDSC beschrieben.

Hinsichtlich der Expression von Gr-1 kann man murine MDSC in zwei Subtypen unterteilen. Granulozytäre / neutrophile MDSC sind Gr-1^{high} (LyG6G^{+}, LyG6C^{low}) und weisen einen multilobulären / polymorphen Nukleus auf. Sie sind gering suppressiv gegenüber T-Zellen im Vergleich zu den „monozytären“ MDSC. Diese haben einen Mononukleus und sind durch die Expression von Gr-$1^{low/int}$ (LyG6$G^{+/-}$, LyG6C^{high}) gekennzeichnet (Movahedi et al., 2008; Sawanobori et al., 2008; Youn et al., 2008).

Im Gegensatz zu murinen MDSC sind die entsprechenden humanen Zellen nur sehr unzureichend charakterisiert. Im peripheren Blut von Patienten mit Kopf-Hals- sowie Lungen- und Brustkrebs wurden $CD34^{+}CD33^{+}CD15^{+}$ Zellen nachgewiesen, welche suppressiv auf T-Zellen wirken (Almand et al., 2001). Bei Patienten mit malignem Melanom wurden $CD11b^{+}CD14^{+}$ Suppressorzellen beschrieben (Filipazzi et al., 2007). Dieser Phänotyp wurde ebenfalls bei Patienten mit Kopf-Hals-Tumoren sowie mit multiplem Myelom charakterisiert (Serafini et al., 2006a). Neben diesen monozytären Zellen wurden ebenfalls MDSC beschrieben, die eher einen granulozytären Phänotyp aufweisen. Diese waren $CD11b^{+}CD14^{-}CD15^{+}$ (Schmielau and Finn, 2001; Zea et al., 1995). Die Expression von CD14 in humanen MDSC wird derzeit diskutiert (Filipazzi et al., 2007; Zea et al., 1995). Generell wird angenommen, dass humane MDSC Lin^{-}HLA-$DR^{-}CD33^{+}$ oder $CD11b^{+}CD14^{-}CD33^{+}$ sind (Gabrilovich and Nagaraj, 2009). Allerdings wurden in Melanompatienten Zellen identifiziert, welche einen $CD14^{+}$HLA-$DR^{-/low}$ Phänotyp aufweisen. Die Summe der Daten lässt darauf

schließen, dass unterschiendliche Tumore unterschiedliche Subtypen von MDSC induzieren (Filipazzi et al., 2007).

TABELLE 1: SUBPOPULATION VON HUMANEN UND MURINEN MDSC

	murine MDSC	humane MDSC
Häufige Oberflächenmarker	Gr-1, CD11b	CD33, CD11b $CD15^+$, $CD14^-$ HLA-DR II $^-$
Oberflächenmarker auf MDSC-Subtypen	CD80, F4/80, IL-4Rα, CD115, Ly6C, Ly6D	$CD14^+$, $HLA\text{-}DR^-$
Funktionelle Marker	ArgI, iNOS, ROS	ArgI, iNOS
Suppressiver Mechanismus	NO, ArgI, Nitrotyrosin ROS^- (monozytäre MDSC) NO^- (granulozytäre MDSC)	NO, ArgI, Nitrotyrosin

Immunsuppression

MDSC unterdrücken sowohl angeborene als auch adaptive Immunreaktionen. So konnte bei Patienten mit Kopf-Hals-Tumoren gezeigt werden, das intratumorale T-Zellen weniger IL-2 sezernieren, wenn sie mit anti-CD3 aktiviert werden (Almand et al., 2001; Diaz-Montero et al., 2008). Bei murinen MDSC wurde beobachtet, dass diese sowohl die Aktivität als auch die Proliferation von Antigen- stimulierten $CD8^+$- (Bronte et al., 2000; Bronte et al., 1998; Gabrilovich et al., 2001; Sinha et al., 2005b) und $CD4^+$- T-Zellen unterdrücken (Mazzoni et al., 2002; Sinha et al., 2005b). Des Weiteren wurde hier ebenfalls dargestellt, dass die Suppression Zell-Zell-Kontakt abhängig und Antigen spezifisch ist (Nagaraj et al., 2007a). Allerdings wurde in anderen Ansätzen gezeigt, dass Antigen aktivierte $CD4^+$ T-Zellen durch allogene MDSC supprimiert wurden, was für eine Antigen unabhängige Suppression spricht (Sinha et al., 2005a, b).

Im Hinblick auf NK-Zellen sind die vorhandenen Ergebnisse unterschiedlich. So konnte gezeigt werden, dass MDSC sowohl die Zytotoxizität als auch die IFN-γ Sekretion von NK-Zellen herab setzten und das dieser Effekt ebenfalls Zell-Zell-Kontakt abhängig ist (Li et al.,

2009; Liu et al., 2007a; Suzuki et al., 2005). MDSC bewirken eine geringere Expression von NKG2D, einem NK-Zell Rezeptor, der für die Aktivierung wichtig ist. (Li et al., 2009). Eine weitere Studie am murinen Lymphom ergab, dass MDSC den NKG2D-Liganden Rae-1 exprimieren. Die so aktivierten NK-Zellen waren in der Lage, MDSC zu lysieren (Nausch et al., 2008). Diese widersprüchlichen Ergebnisse machen deutlich, dass noch nicht alle Wechselwirkungen zwischen MDSC und NK-Zellen vollständig verstanden sind.

Mechanismen der Suppression

Die Suppression von T-Zellen durch MDSC erfolgt über mehrere Mechanismen. Die wichtigsten Effektorfunktionen werden dabei von der Arginase I (ArgI) und der induzierbaren NO-Synthase (iNOS / NOS2) vermittelt. Durch diese Enzyme wird die Konzentration der für T-Zellen essentiellen Aminosäure L-Arginin im Extrazellularraum erniedrigt (Bronte and Zanovello, 2005). Die Expression von ArgI wird durch TGF-β, MCP-1 und GM-CSF in einem STAT6-abhängigen Prozessstimuliert (Marigo et al., 2008). Die ArgI- Aktivität wurde sowohl im Serum als auch im Tumorstroma von Patienten mit verschiedenen Neoplasien detektiert (Porembska et al., 2003; Porembska et al., 2002).

Die Erniedrigung der Arginin- Konzentration führt über einen nicht vollständig geklärten Mechanismus zu einer verringerten Expression der ζ- Kette des T-Zell Rezeptors (TCR) und bewirkt Anergie der betroffenen T-Zellen (Bronte et al., 2005; Bronte and Zanovello, 2005).

Die Expression von iNOS wird vor allem durch IFN-γ induziert, jedoch üben auch TNF, IFN-α, IFN-β, IL-1, VEGF, GM-CSF und IL-6 einen Einfluss aus (Bronte and Zanovello, 2005; Serafini et al., 2006a). Das durch iNOS frei gesetzte NO nitrosyliert Cystein- Reste in Signalproteinen der T-Zell Aktivierungskaskade, wodurch die Phosphorylierung von essentiellen Signalmolekühlen verhindert wird. Des Weiteren werden durch NO die Guanylat- Zyklase und zyklische –GMP (cGMP-) abhängige Proteinkinasen aktiviert. Eine erhöhte cGMP- Konzentration verringert die Stabilität von IL-2- mRNA und führt somit zu einer verringerten Ausschüttung von IL-2 (Bronte and Zanovello, 2005).

Die Produktion reaktiver Sauerstoffspezies (ROS) durch das Zusammenspiel von ArgI und iNOS ist ein weiterer suppressiver Mechanismus gegenüber T-Zellen. Im Gegensatz zu NO, welches hauptsächlich in monozytären MDSC detektiert wird, wird ROS in granulozytären MDSC nachgewiesen (Movahedi et al., 2008; Youn et al., 2008). Beide Mechanismen werden durch IFN-γ induziert. Da MDSC von IFN-γ Rezeptor defizienten Mäusen ebenfalls suppressiv sind (Sinha et al., 2005b).ist zu vermuten, dass weitere Zytokine an der Rekrutierung oder Aktivierung von MDSC beteiligt sind

TGF-β wurde ebenfalls mit der immunsuppressiven Wirkung von MDSC in Zusammenhang gebracht. In Mausmodellen mit Fibrosarkom und Kolonkarzinom konnte dargestellt werden, dass monozytäre MDSC TGF-β sezernieren, wenn diese mit IL-13 aktiviert werden (Fichtner-Feigl et al., 2008; Terabe et al., 2003). In einem Leberkrebsmodel erfolgte die Suppression, von NK-Zellen, über membrangebundenes TGF-β (Li et al., 2009).

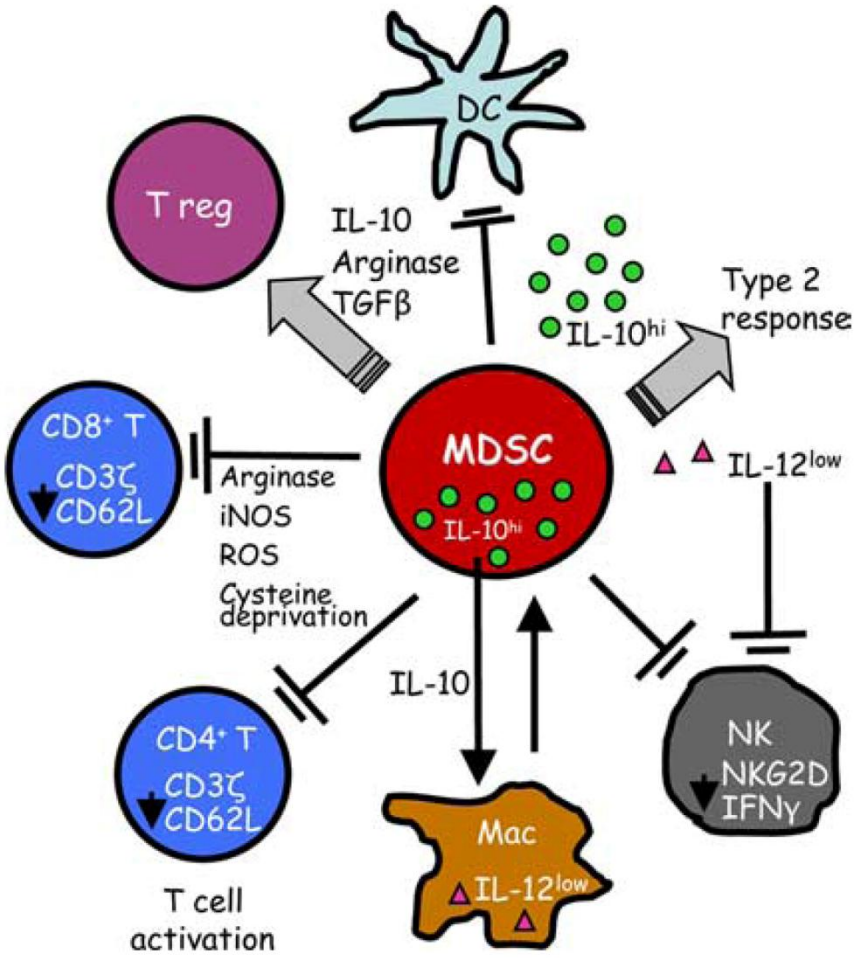

Abbildung 3: MDSC unterdrücken das Immunsystem durch verschiedene Mechansimen.

Interaktion mit regulatorischen T-Zellen

Es wurde berichtet, dass die Interaktion von CD80 auf MDSC und CD152 auf Tregs einen suppressiven Mechanismus bewirkt, mit dem die IFN-γ Sekretion von Lymphozyten verhindert wird (Marigo et al., 2008). Zahlreiche weitere Studien belegen eine Interaktion beider suppressiver / regulatorischer Zellpopulationen, allerdings ist der Mechanismus noch weitgehend unbekannt. In einem Lymphommodell wurde gezeigt, dass MDSC als antigenpräsentierende Zellen fungieren und die Proliferation von Treg induzieren (Serafini et al., 2008). In einem anderen Modell werden MDSC durch IFN-γ aktiviert, sezernierten TGF-β sowie IL-10 und induzieren somit suppressive Tregs (Huang et al., 2006).

Rekrutierung

Die Akkumulation und Aktivierung von MDSC wird durch verschiedene Faktoren verursacht, von denen viele mit einer chronischen Entzündung assoziiert sind. Chronische Antigenstimulation durch Neoplasien, Infektionen und andere Veränderungen im Immunsystem können ein verändertes Zytokinprofil verursachen, das auf myeloide Vorläuferzellen einwirkt und deren Ausreifung verhindert oder stört (Maecker et al., 2006; Serafini et al., 2006a). *In vitro* lösen tumorassoziierte Faktoren die Phosphorylierung der Januskinase- (Jak-) 2 und STAT3 aus. Dies verhindert die Ausreifung zu DC und führt zu einer Akkumulation von MDSC (Kusmartsev and Gabrilovich, 2006a). „Tumor-derived-soluble-factors“ (TDSF) bzw. Tumor-Exosome zeigen einen ähnlichen Effekt und wirken synergetisch auf die Migration von MDSC aus dem Knochenmark in die Milz und von dort in das Tumorstroma (Marigo et al., 2008; Serafini et al., 2006a).

In der folgenden Tabelle wird ein Überblick über Faktoren gegeben, die mit der Rekrutierung von MDSC assoziiert sind.

TABELLE 2: FAKTOREN, DIE MIT DER REKRUTIERUNG ASSOZIIERT SIND

„Epithelial-derived neutrophil-activated peptide 78"	(Yang et al., 2008)
"Granulocyte colony stimulating factor" (G-CSF)	(Parmiani et al., 2007; Sawanobori et al., 2008)
"Granulocyte macrophage colony stimulating factor" (GM-CSF)	(Filipazzi et al., 2007; Parmiani et al., 2007)
"Macrophage colony stimulating factor" (M-CSF)	(Bronte et al., 1999)
Interferon-γ (IFN-γ)	(Baniyash, 2004)
Interleukin 1β (IL-1β)	(Bunt et al., 2006)
Interleukin 6 (IL-6)	(Pan et al., 2008)
Interleukin 10 (IL-10)	(Chen et al., 2001)
Interleukin 12 (IL-12)	(Li et al., 2004)
Interleukin 13 (IL-13)	(Terabe et al., 2004)
"Monocyte chemotactic protein-1" (MCP-1)	(Huang et al., 2007)
"Postaglandine" (PG)	(Rodriguez et al., 2005; Sinha et al., 2007b)
"Stem cell factor"	(Pan et al., 2008)
"Stromal cell derived factor 1" (SCDF-1)	(Ozao-Choy et al., 2009; Xin et al., 2009)
"Vascular endothelial growth factor" (VEGF)	(Melani et al., 2003)

Blockierung der MDSC

In vielen experimentellen Modellen konnten die potentiellen Möglichkeiten der Beeinflussung der Immunantwort durch Blockade der MDSC gezeigt werden: Resektion des Tumors oder Depletion der MDSC verbessert die T-Zell-vermittelte Immunantwort (Bronte et al., 1999;

Bronte et al., 1998; Danna et al., 2004). Durch die Veränderung des Zytokinmilieus ließen sich MDSC zu Makrophagen, DCs und Granulozyten ausreifen (Bronte et al., 2000; Pak et al., 1995a). Durch Inhibierung der STAT3- Phosphorylierung konnte ebenfalls die Differenzierung der MDSC induziert werden und so die Suppression auf gehoben werden (Nefedova et al., 2004). Allerdings sind viele dieser Strategien zur Inhibition oder Ausreifung von MDSC aufgrund ihrer Nebenwirkungen nicht praktikabel (Serafini et al., 2006b). Vielversprechend sind dagegen Versuche mit „all-trans retinoic acid“ (atRA) die zu einem Ausreifen der MDSC führen (Kusmartsev et al., 2003) oder mit dem Vitamin D3- Metabolit „A25- dihydroxyvitamin D3“, dass zu einer Reduktion der MDSC führt (Young et al., 1996). Im Tumormodell des Lungenkarzinoms und Mesothelioms konnte mit Gemcitabin ebenfalls eine Neutralisierung der MDSC erreicht werden (Suzuki et al., 2005). Antiinflammatorische Wirkstoffe, wie Aspirin und dessen Derivate, waren ebenfalls vielversprechend (De Santo et al., 2005). Eine neue Studie konnte belegen, dass Phosphodiesterase-5 (PDE-5)- Inhibitoren die NO- Synthese bei MDSC hemmen. PDE-5- Blockade reichert intrazellulär cGMP an, welches über eine negative Rückkopplung auf RNA Ebene eine Reduktion der iNOS in MDSC bewirkt. Die verminderte Freisetzung von NO führt zu einer geringeren Suppression von T-Zellen (Serafini et al.,2006b)

TABELLE 3: THERAPEUTISCHE ANSÄTZE BEI MDSC

Therapieansatz	Mechanismus	Referenz
all-trans Retinsäure (atRA)	Induktion der Differenzierung der MDSC durch eine Akkumulation der Glutathion Synthase	(Kusmartsev and Gabrilovich, 2003; Nefedova et al., 2007)
Nitroaspirin	Rückkopplungsinhibition der induzierbaren NO-Synthase (iNOS)	(De Santo et al., 2005)
Phosphodieesterase-5-inhibitor (PDE-5-Hemmer) Sidenafil	Herunterregulation der ArgI und iNOS	(Serafini et al., 2006b)
Cytidin Analogon Gemcitabin	Elimination der MDSC	(Suzuki et al., 2005)
VEGF-Trap	Blockade des VEGF Signalweges	(Fricke et al., 2007)
COX-2 Inhibitoren	Herunterregulation der ArgI Expression	(Talmadge et al., 2007)
Tyrosine Kinase Inhibitor Sunitinib	Verhindern der MDSC Akkumulation durch Blockierung des SCF Rezeptor und des STAT3 Signalweges	(Ozao-Choy et al., 2009; Xin et al., 2009)
anti-Gr-1 Antikörper	Depletion der Gr-1$^+$ Zellen	(Seung et al., 1995)

Effektor Immunzellen

Natürliche Killerzellen

Natürliche Killerzellen (NK-Zellen) sind Effektorzellen des angeborenen Immunsystems. NK-Zellen interagieren mit dem adaptivem Immunsystem durch die Freisetzung von Zytokinen, die dendritische Zellen modulieren und zytotoxische Zellen aktivieren können. Man unterscheidet zwei verschiedene NK- Populationen, anhand ihrer Expressionsdichte von CD56.

Die Mehrheit der NK-Zellen (ca. 90 %) exprimieren geringe CD56 Level, sind CD16 positiv und werden als CD56dim bezeichnet. Im Gegensatz dazu weisen die CD56bright Zellen eine höhere CD56 Dichte auf und sind CD16 negativ. Sie verfügen über eine geringere natürliche Zytotoxizität, produzieren jedoch mehr pro inflammatorische Zytokine. Hierzu zählen IFN-γ, TNF-α, TNF-β, IL-10, IL-13 und GM-CSF (Moretta et al., 2000). NK-Zellen sind in der Lage, eine Vielzahl an Zielzellen spontan, ohne vorangegangene Sensibilisierung zu eliminieren. Sie verfügen nicht über rearrangierte, Antigen-spezifische Rezeptoren, wie beispielsweise B- und T-Lymphozyten, sondern erkennen ihre Zielzellen über inhibitorische und aktivierende Rezeptoren. Die Balance der entgegengesetzten Signale, hervorgerufen durch Bindung der Liganden an die verschiedenen Rezeptoren, bestimmt die resultierende biologische Antwort. Im Falle einer Herunterregulierung von MHC-Klasse-I-Molekülen, die häufig bei malignen oder virusinfizierten Zellen vorkommt, wird die Inhibition aufgehoben und das überwiegend aktivierende Potential führt zur Lyse der betroffenen Zelle („missing-self-theory“).

Andererseits können NK-Zellen auch durch induzierbare Liganden für ihre aktivierenden Rezeptoren stimuliert werden („induced-self-theory“). Hierzu gehören vor allem MIC-A, MIC-B und ULBPs, die von transformierten und Virus-Infizierten Zellen exprimiert werden (Moretta, 1995).

CD4+ und CD8+ T- Lymphozyten

$CD4^+$ und $CD8^+$ T-Zellen sind die Helfer- und Effektor- Zellen der adaptiven Immunität.

Die meisten Immuntherapien zielen darauf hin, diese Zellen zu aktivieren und so eine effektive Immunantwort gegen den Tumor zu induzieren und eine lang anhaltende Immunität zu gewährleisten.

Naive $CD4^+$ T-Zellen können durch unterschiedliche Einflüsse im Zytokinmilieu und der Art der Aktivierung durch DC oder Makrophagen zu unterschiedlichen Untergruppen von Helferzellen differenzieren. Th1 Zellen werden durch IFN-γ und IL-12 induziert. Dieser Subtyp von T-Helferzellen führt über eine Aktivierung der zytotoxischen $CD8^+$ T-Zellen zu einer Gewebeschädigung und Tumorregression. Auf der anderen Seite werden Th2 T- Helferzellen hauptsächlich durch IL-4 induziert und sezernieren ihrerseits IL-4, IL-5 und IL-13, welches zu einer B- Zell Antwort und Antikörperproduktion führt. Durch TGF-β werden neben Tregs auch Th17 T-Zellen induziert. Bei ihrer Differenzierung sind ebenfalls inflammatorische Zytokine wie IL-6, IL-17 und IL-23 beteiligt (O'Garra et al., 2008). Funktionell sind Th17 T-Zellen dadurch charakterisiert, dass sie zu einer akuten Gewebeentzündung und zu einer Infiltration von neutrophilen Granulozyten führen (Umemura et al., 2004). Ihre Rolle in Hinsicht der Tumorimmunologie wird derzeit noch kontrovers diskutiert. Allerdings scheint sich abzuzeichnen, dass sich Th17 T-Zellen positiv auf das Wachstum von Tumoren auswirken (Wu et al.,

2007). So konnte bei Patientinnen mit Ovariarkarzinomen einerseits eine Korrelation zwischen Th17 Zellen und der Angiogenese (Chen et al., 2007)andererseits eine Häufung von Th17 Zellen und Tregs in Blut und Tumor beobachtet werden (Meeran et al., 2007).

Im Gegensatz zu diesen deskriptiven Studien wurde in immunkompetenten Mausmodellen beobachtet, dass es durch die Expression von IL-17 zu einer gegen den Tumor gerichteten Immunität kommt, da hier der Effekt der CD8+ T-Zellen verstärkt wurde (Benchetrit et al., 2002; Hirahara et al., 2001).

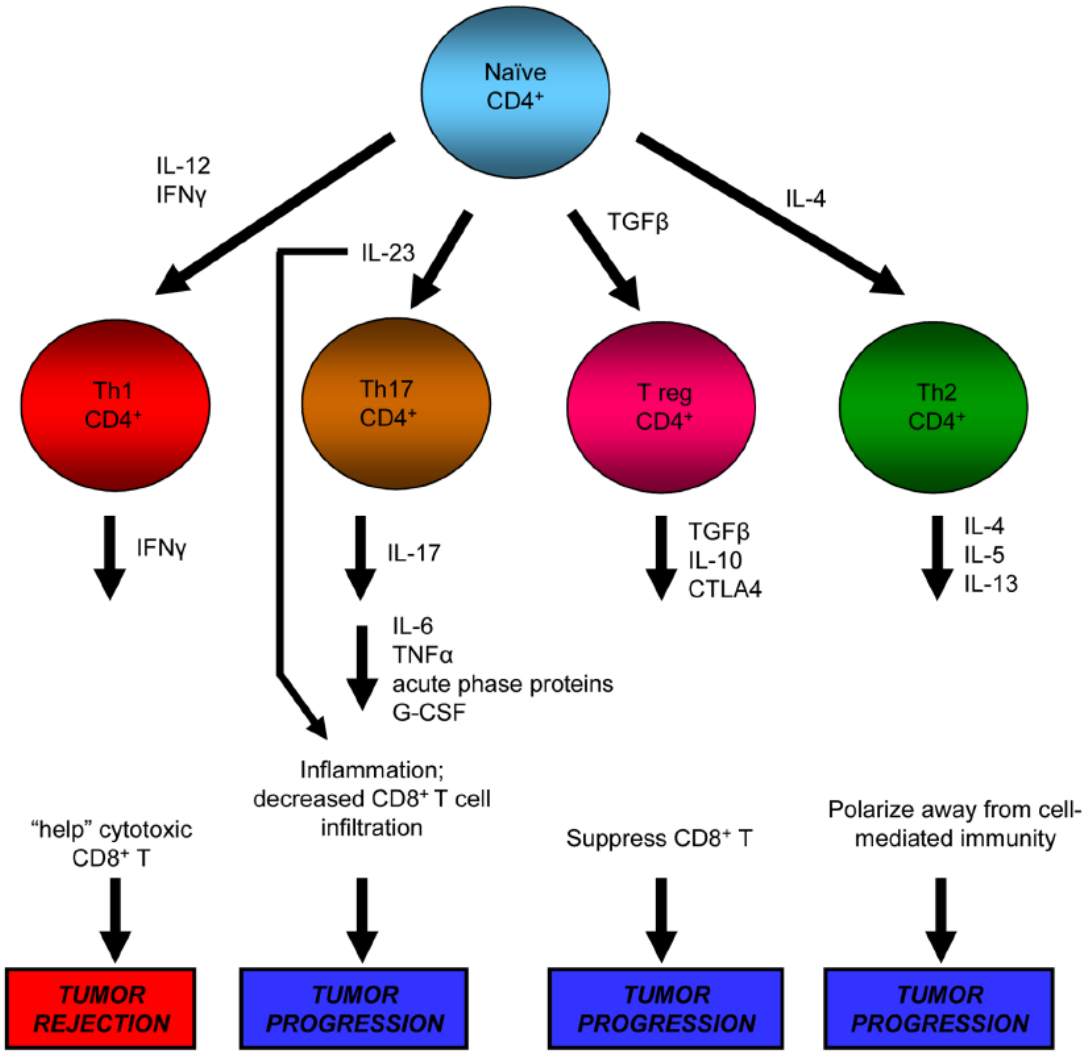

Abbildung 4: Ostrand-Rosenberg. 2009, Unterschiedliche Th-Subtypen und ihre Bedeutung in der Tumorimmunologie

Fragestellung

Das hepatozelluläre Karzinom (HCC) gehört weltweit zu den fünf häufigsten Krebserkrankungen und stellt die dritthäufigste krebsbedingte Todesursache dar (El-Serag and Rudolph, 2007). Die mittlere Überlebensrate nach fünf Jahren beträgt lediglich 5% bei einer Inzidenz von einer Millionen neuer Fälle weltweit. Die therapeutischen Optionen sind begrenzt und bestehen neben der Transplantation, hauptsächlich aus lokal-ablativen Therapien. Bedingt durch die Zirrhose und somit eingeschränkter Leberfunktion wird eine systemische Chemotherapie nur von weniger Patienten toleriert (Schott, 2008) und zeigt darüber hinaus nur ein geringes Ansprechen. Derzeit ist der Tyrosinkinase- Inhibitor Sorafenib das einzige Medikament, welches eine lebensverlängernden Effekt aufweist (Llovet et al., 2008).

Aufgrund der unbefriedigenden Therapieerfolge und der massiven Nebenwirkungen werden alternative Behandlungsmuster weiter entwickelt. Eine therapeutische Option in diesem Zusammenhang stellt die Immuntherapie des HCC dar. Allerdings sind hier die therapeutischen Effekte aufgrund immunsuppressiver Ausweichstrategien des Tumors immer noch gering. Bei den suppressiven Mechanismen handelt es sich neben Tumor assoziierten Makrophagen (Coffelt et al., 2009) hauptsächlich um regulatorischer T-Zellen (Zou, 2006). Eine weitere Gruppe immunsuppressiver Zellen stellen die myeloiden Suppressorzellen (MDSC) dar. Dieser Begriff umschreibt eine heterogene Gruppe suppressiver Zellen myeloiden Ursprungs. Dazu zählen Makrophagen, Granulozyten, dendritische Zellen und andere myeloide Zellen, die sich zumeist in einem unreifen Entwicklungsstadium befinden. In Mäusen sind diese Zellen durch die Koexpression der Marker CD11b und Gr-1 charakterisiert. In Patienten, die an Krebs erkrankt sind, wurden unterschiendliche Phänotypen von MDSC beschrieben. Auf der einen Seite wurden Zellen beschrieben, welche keinen Zelllinien typischen Marker expremieren, allerdings positiv für CD34, einem Stammzellmarker, sind. Auf der anderen Seite wurden Zellen beschrieben, welche Marker für Monozyten und oder Granulozyten aufweisen (Liu et al., 2007b; Serafini et al., 2006a; Viola and Bronte, 2007).

Die immunsuppressive Kapazität der MDSC beruht hauptsächlich auf dem Metabolismus des L-Arginin, welches essentiell für die T-Zell-Aktivität und Proliferation ist (Serafini et al., 2006a). Dies geschieht einerseits durch die Arginase I (Arg I) welche L-Arginin zu L-Ornithin und Harnstoff metabolisiert, zum anderen durch die induzierbare NO-Synthase (NOS2), welche neben L-Citrullin NO frei setzt (Viola and Bronte, 2007).

Zielsetzung

Bisher konnten MDSC beim Menschen im Gegensatz zur Maus nur unzureichend untersucht werden. Ziel dieser Arbeit war es daher zunächst die humanen Entsprechungen für murine $Cd11b^{+}Gr\text{-}1^{+}$ MDSC zu identifizieren. Dafür wurden Leukozyten aus dem Blut, Aszites und Tumorproben von Patienten mit HCC isoliert und mit denen von gesunden Spendern verglichen. Dabei wurden besonders mit Hilfe der Vielfarben- Durchflusszytometrie myeloide Zellen analysiert.

Im zweiten Teil wurden potentielle Kandidaten durch MACS-Sort und FACS-Sorten auf gereinigt und auf ihre suppressive Kapazität hin untersucht.

Hierfür wurden MDSC mit autologen $CD4^{+}$ T-Zellen inkubiert und die suppressive Wirkung auf T-Zellen anhand der Proliferation bzw. der Hemmung dieser gemessen. Eine mögliche Arginase-I-Aktivität wurde anhand geeigneter biochemischer Versuche bestimmt.

Basierend auf den so erhobenen Befunden sollten potentielle MDSC hinsichtlich des Phänotyps, als auch der Funktionen genauer untersucht werden. Dazu wurden neben molekularbiologischen Methoden, wie Microarray und quantitativer PCR, auch Vielfarben FACS-Analysen eingesetzt um mögliche weitere Marker zu identifizieren. Des Weiteren sollte mit diesen Methoden ebenfalls versucht werden genaue Wirkmechanismen der MDSC zu entschlüsseln.

Außerdem sollte untersucht werden, ob MDSC zu reifen bzw. aus ihnen Antigen präsentierende Zellen (APCs) generiert werden können. Dies geschah durch Zellkutur– Techniken, bei denen MDSC mit verschiedenen Inhibitoren oder Zytokinen kultiviert wurden. Dies sollte Hinweise darauf liefern, ob es möglich ist MDSC in Zellen zu konvertieren, die das Immunsystem aktivieren können und somit möglicher weise für eine Immuntherapie von Nutzen sind.

Im dritten Teil sollte die Interaktion der MDSC mit Effektor- Zellen des angeborenen als auch des adaptiven Immunsystems untersucht werden. Der besondere Schwerpunkt hier lag auf Faktoren, die an der Interaktion beteiligt sind als auch auf den Auswirkungen der beiden Zelltypen. Dies geschah mit Ko- Kulturen von MDSC und T- oder NK-Zellen, die zuvor durch FACS-Sort isolierten wurden.

A New Population of Myeloid-Derived Suppressor Cells in Hepatocellular Carcinoma Patients Induces $CD4^+$ $CD25^+$ $Foxp3^+$ T Cells

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Background & Aims: **Several studies have shown that development of hepatocellular carcinoma (HCC) generates a number of immune suppressive mechanisms in these patients. Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells that have been shown to inhibit T-cell responses in tumor-bearing mice, but little is known about these cells in humans owing to a lack of specific markers. In this study, we have investigated the frequency and function of a new population of MDSC denoted here as CD14 HLA-DR $^{/low}$ in HCC patients. We have also identified a novel, MDSC-mediated immune regulatory pathway in these patients.** ***Methods:*** **We have directly isolated and characterized MDSCs for phenotype and function from peripheral blood (n 111) and tumor (n = 12) of patients with HCC.** ***Results:*** **The frequency of $CD14^+HLA\text{-}DR^{-/low}$ cells in peripheral blood mononuclear cells (PBMC) from HCC patients was significantly increased in comparison with healthy controls. $CD14^+HLA\text{-}DR^{-/low}$ cells were unable to stimulate an allogeneic T-cell response, suppressed autologous T-cell proliferation, and had high arginase activity, a hallmark characteristic of MDSC. Most important, $CD14^+HLA\text{-}DR^{-/low}$ cells from HCC patients induced a $CD4^+CD25^+Foxp3^+$ regulatory T- cell population when cocultured with autologous T cells.** ***Conclusion:*** **$CD14^+HLA\text{-}DR^{-/low}$ cells are a new population of MDSC increased in blood and tumor of HCC patients. We propose a new mechanism by which MDSC exert their immunosuppressive function, through the induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells in cocultured CD4 T cells. Understanding the mechanism of action of MDSC in HCC patients is important in the design of effective immunotherapeutic protocols.**

Tumors have evolved different mechanisms to evade the host's immune response and generate a suppressive network.[1] Understanding the mechanisms behind this immune suppression is important to the success of immunotherapy protocols. Recently, myeloid-derived suppressor cells (MDSC) have been characterized as a population of cells that can negatively regulate T-cell function. MDSC are a heterogeneous population of myeloid cells including macrophages, granulocytes, and other cells that express both Gr-1 and CD11b in mice and suppress immune responses in vivo and in vitro.[2] In humans, MDSC have not been well characterized owing to the lack of specific markers. Only limited data are available on different myeloid cell populations with suppressor func- tion in patients with head and neck cancer, squamous cell carcinoma, non–small lung cancer, and colon and breast cancers.[3] The phenotype of these cells has been shown to be mainly $CD34^+$, $CD33^+$, $CD15^-$, and $CD13^+$, $CD14^-$/lin^-.[4–7] We have previously shown that although patients with hepatocellular carcinoma (HCC) mount tumor-specific humoral and cellular immune responses (detected in up to 50% of the patients), the disease progresses.[8] We have also shown that HCC, like many other types of cancer, has devised mechanisms to evade the hosts' immune response such as increase in regulatory T cells and defects in antigen-presenting cells.[9,10]

Herein, we have shown direct ex vivo isolation of a new subset of MDSC that are significantly increased in peripheral blood and tumor of HCC patients. These cells express CD14, have low or no expression of HLA-DR, and have high arginase activity. When cocultured with autologous T cells, MDSC from HCC patients induced $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells that were suppressive in vitro. Our study suggests that HCC patients develop $CD14^+$ $HLA\text{-}DR^{-/low}$ cells, which can modulate the immune response of these patients by inducing $CD4^+$ $CD25^+Foxp3^+$ regulatory T cells and inhibiting tumor-specific T-cell acti-

Abbreviations used in this paper: AFP, -fetoprotein; GM-CSF, granulocyte-macrophage colony–stimulating factor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN- , interferon- ; MDSC, myeloid derived suppressor cells; IL, interleukin; MHC, major histocompatibility complex; MoDCs, monocytes derived dendritic cells; PBMC, peripheral blood mononuclear cell; qPCR, quantitative polymerase chain reaction; TGF, transforming growth factor; TILs, tumor-infiltrating cells.

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vation. Understanding their mechanism of action is important for developing effective immunotherapy protocols.

Materials and Methods

Patients and Healthy Donors

Blood samples were collected from a total of 111 HCC patients seen at the Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School (Hannover, Germany). HCC was diagnosed according to the guidelines of the European Association for the Study of Liver. Written consent was obtained from all patients before blood and tumor sampling and the Ethics Committee of Hannover Medical School approved the study protocol. Table 1 shows the clinical characteristics of all HCC patients in this study.

Cell Isolation and Sorting

PBMC were isolated from freshly obtained blood by Ficoll density gradient centrifugation (Biochrom, Berlin, Germany) as described previously.[11] For isolation of $CD14^+$ $HLA\text{-}DR^{-/low}$ monocytes, PBMC were purified using CD14 Microbeads and AutoMACS separation unit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturers' instructions. Purified $CD14^+$ cells were sorted into $CD14^+$ $HLA\text{-}DR^{-/low}$ and $CD14^+$ $HLA\text{-}DR^+$ cells using the BD FACS Aria cell sorting system (Becton Dickinson, Heidelberg, Germany). The purity of the cells after sorting was >98%.

Flow Cytometry Analysis

To determine the frequency and phenotype of $CD14^+$ $HLA\text{-}DR^{-/low}$ cells in PBMC and tumor-infiltrating cells (TILs), multicolor fluorescence-activated cell sorting analysis was done using the following antibodies: anti-CD1c, anti-CD14 (Miltenyi Biotech); anti-HLA-DR, anti-CD11b, anti-CD16, anti-CD19, anti-CD33, anti-CD34, anti-CD80 (ImmunoTools, Friesoythe, Germany); anti–HLA-ABC, anti-CD206 (BD Pharmingen, Heidelberg, Germany); anti-CD83, anti-CD86, CD15 (Caltag, Hamburg, Germany); anti-CD11c (eBioscience, San Diego, CA); anti-CCR2 (R & D Systems, Minneapolis, MN); anti-CD1a (Abcam, Cambridge, UK). Flow cytometry was done on a Becton Dickinson FACS Calibur. Analysis of FACS-data was done using FlowJo software (TreeStar, Inc, Ashland, OR). Isotype-matched antibodies were used with all the samples as controls.

Table 1. Patient Characteristics

Diagnosis	n	Male/female	Age, y (mean)
HBV	24	19/5	64
HCV	26	20/6	61
HBV HCV Toxic	1	1/0	71
cirrhosis Hemach-	14	14/0	66
romatosis Un-	10	7/3	57
known Child–Pugh	36	30/6	67
score			
A	62		
B	38		
C	11		
CLIP 0 1	38		
CLIP 2 3	55		
CLIP 3	18		

CLIP, Cancer of the Liver Italian Program; HBV, hepatitis B virus; HCV, hepatitis C virus.

Mixed Lymphocyte Reaction

Allogeneic PBMC (5×10^5) from a healthy donor were incubated with indicated numbers of sorted $CD14^+$ $HLA\text{-}DR^{-/low}$ or $CD14^+$ $HLA\text{-}DR^+$ cells from an HCC patient in 96-well plates (Greiner, Frickenhausen, Germany) in complete RPMI media supplemented with 10% human AB serum. T-cell activation and expansion kit (Miltenyi Biotech) was used in separate wells as a positive control. After 72 hours, proliferation was measured by incorporation of ^{3}H-thymidine (Amersham, Freiburg, Germany) as described.[9]

Suppression Assay

$CD14^+$ $HLA\text{-}DR^{-/low}$ and $CD14^+$ $HLA\text{-}D^+$ cells were purified and sorted as described. PBMC (10^4) were stimulated with T-cell activation and expansion kit (Miltenyi Biotech) and incubated with $CD14^+$ $HLA\text{-}DR^{-/low}$ cells at different ratios. Proliferation was measured after 72 hours by ^{3}H-thymidine incorporation. For determination of interferon (IFN)-γ responses, culture supernatants from the suppression assay were removed after 48 hours and tested by ELISA (ImmunoTools) according to the manufacturer's instructions. L-Arginine was added where indicated in Figure 1. For functional analysis of in vitro– generated $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells, $CD14^+$ $HLA\text{-}DR^{-/low}$, $CD14^+$ $HLA\text{-}DR^+$, and $CD4^+$ T cells were sorted as described. The $CD4^+$ T cells were stimulated as described and were cocultured with either $CD14^+$ $HLA\text{-}DR^{-/low}$ or $CD14^+$ $HLA\text{-}DR^+$ cells for 3 days. T cells were sorted after 3 days gating on $CD4^+CD25^+$ population. Autologous CD4 T cells were stimulated and added to the sorted $CD4^+$ $CD25^+$ T cells at different ratios. Proliferation was measured after 72 hours by ^{3}H-thymidine incorporation as described previously.

Determination of Arginase Activity

The arginase activity of MDSC was determined as described before.[12] A standard curve consisting of serial dilutions of urea was run in parallel. The urea concentration was measured at 540 nm.

Proliferation Assay

PBMC were isolated from freshly obtained blood of HCC patients using Ficoll gradient purification. The PBMC were then stimulated with anti-CD2/CD3/CD28 beads (Miltenyi Biotech) for 5 days in 96-well plates; the

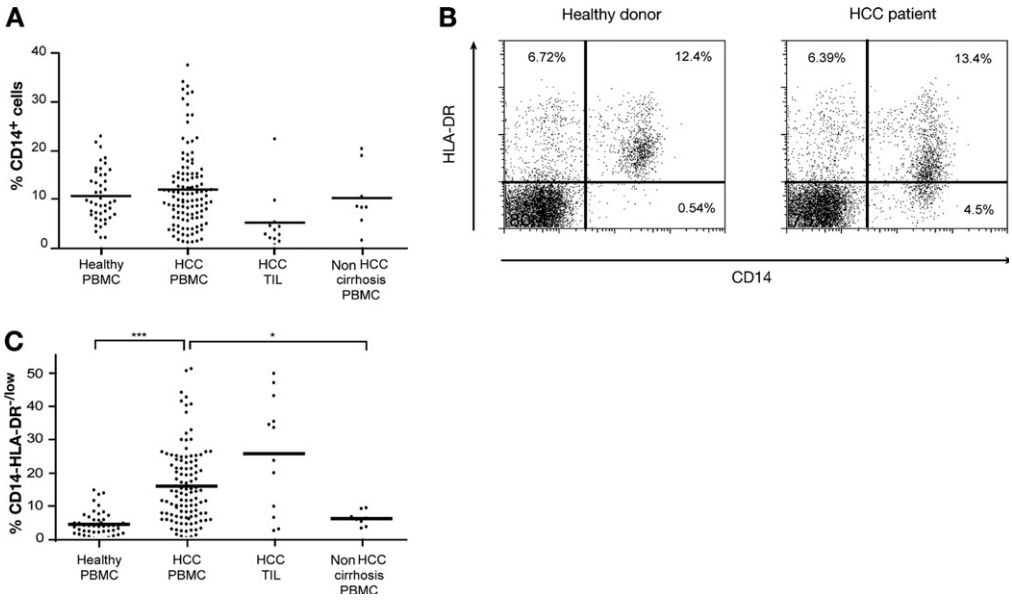

Figure 1. Frequency of MDSC is increased in HCC patients. (*A*) PBMC from 47 healthy do- nors, 8 HCV- or toxin-induced liver cirrhosis, 111 HCC pa- tients, and tumor-infiltrating lym- phocytes from 12 HCC tumors were analyzed for percentage of $CD14^+$ cells. (*B*) HLA-DR expres- sion on $CD14^+$ cells. PBMC were stained for CD14 and HLA-DR expression after CD19 exclusion. Representative *dot plots* of a healthy donor and an HCC patient are shown. (*C*) Frequency of $CD14^+HLA\text{-}DR^{-/low}$ population in PMBC of healthy donors, HCC patients, HCV- or toxic liver cirrhosis patients, and TILs from HCC patients were determined. **P* .05; ****P* .001.

IFN- γ concentration was determined in the supernatant by ELISA.

Restoration of -Fetoprotein–Specific T-Cell Responses

PBMC from HCC patients were depleted of $CD14^+$ $HLA\text{-}DR^{-/low}$ cells by sorting using the BD FACS Aria (Becton Dickinson) with a purity of 98%. The cells were stimulated with α -fetoprotein (AFP) peptides or an irrelevant peptide for 8 days in the presence of interleukin (IL)-2. Three AFP peptides (Biosyntan, Berlin, Germany) were used as described before[13]: $AFP_{137–145}$, $AFP_{249–258}$, and $AFP_{364–373}$. Peptide-specific T-cell responses were detected by IFN- γ secretion assay (Miltenyi Biotech) as described previously.[14]

Generation of Monocytes-Derived Dendritic Cells

Monocytes were isolated from PBMC using anti CD14-Beads and AutoMACS separation unit (Miltenyi Biotech) as described. $CD14^+$ cells were sorted into $HLA\text{-}DR^+$ and $HLA\text{-}DR^{-/low}$ fractions using FACS ARIA with a purity of >98%. Purified cells were cultured for 6 days in 96-well tissue culture plates (NUNC, Roskilde, Denmark) in AIM-V media (Invitrogen, Karlsruhe, Germany) supplemented with 1000 U/mL recombinant human granulocyte-macrophage colony–stimulating factor (GM-CSF) and 1000 U/mL rhIL-4 (ImmunoTools). Monocytes-derived dendritic cells (MoDCs) were also stimulated for 24 hours with 1 μg/mL lipopolysaccharide (LPS; Sigma-Aldrich, St Louis, MO) to induce maturation. Cells were analyzed for surface expression of CD1a (Abcam), CD14 (Miltenyi Biotech), CD80 (ImmunoTools), and CD83 and CD86 (Caltag) using flow cytometry.

Determination of FoxP3 and IL-10 Expression by Intracellular Staining and Quantitative Polymerase Chain Reaction

The $CD14^+$ $HLA\text{-}DR^{-/low}$ cells were sorted as described before from CD14 purified cells. We cultured 1×10^5 CD4 T cells in complete RPMI supplemented with 10% human AB-serum and activated with T-cell activation and expansion beads (Miltenyi Biotech). $CD14^+$ $HLA\text{-}DR^{-/low}$ or $CD14^+$ $HLA\text{-}DR^+$ cells were added. After 3 days of coculturing, the cells were stained for $CD4^+$ $CD25^+$ $Foxp3^+$ using anti Foxp3 (eBioscience) or an isotype control according to the manufacturers' instructions. For IL-10 staining, cells were prepared as described and stained for both FoxP3 and IL-10. For quantitative polymerase chain reaction (qPCR) analysis, RNA was isolated using RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA synthesis was done using SuperScript II (Invitrogen) and random hexamers. Real-time PCR was performed using the following primers (300 nmol each): Foxp3 forward 5=-CTACGCCACGCTCATCCGCTGG-3= and reverse 5=-GTAGGGTTGGAACACCTGCTGGG-3=, cyclophilin forward 5=-ATGCTCAACC CCACCGTGT-3=; cyclophilin reverse 5=-TCTGCTGTCTTTGGGACCTTGTC-3=. Reactions were done in triplicate using Sybr Green (Bio-Rad, München, Germany) and normalized to endogenous cyclophilin mRNA level for each reaction. Transwell inserts (Greiner) were used where indicated.

Statistical Analysis

Data are expressed as mean values ± standard error of the mean for percentages. Statistical analysis was done using Student *t* test to assess the differences between the study groups.

Results

Frequency of CD14$^+$ HLA-DR$^{-/low}$ Cells Is Increased in PBMC and Tumor of HCC Patients

The frequency of CD14$^+$ monocytes was analyzed in PBMC of 111 patients with HCC (Table 1) and healthy controls, patients with liver cirrhosis, and/or viral hepatitis but no HCC. As shown in Figure 1*A*, there was no difference in the frequency of CD14$^+$ monocytes in peripheral blood from patients with HCC, healthy controls, and liver cirrhosis. However, when the CD14$^+$ cells were further analyzed for HLA-DR expression (Figure 1*B*), we noticed a significant increase in the percentage of HLA-DR$^{/low}$ cells in both PBMC (16.2% 1.02%) as well as tumor-infiltrating lymphocytes (25.9% 4.9%) of HCC patients as compared to healthy donors (4.2% 0.52%) or nontumor cirrhosis patients (6.4% 0.78%; Figure 1*C*). No correlation between the frequency of CD14 HLA-DR$^{/low}$ cells and the stage of liver cirrhosis or hepatitis B/C virus (HBV/HCV) infection was seen (data not shown).

Characterization of CD14 HLA-DR$^{/low}$ Cells

To further characterize the CD14 HLA-DR$^{/low}$ cells in HCC patients, we compared their phenotype with CD14 HLA-DR cells. Representative histograms

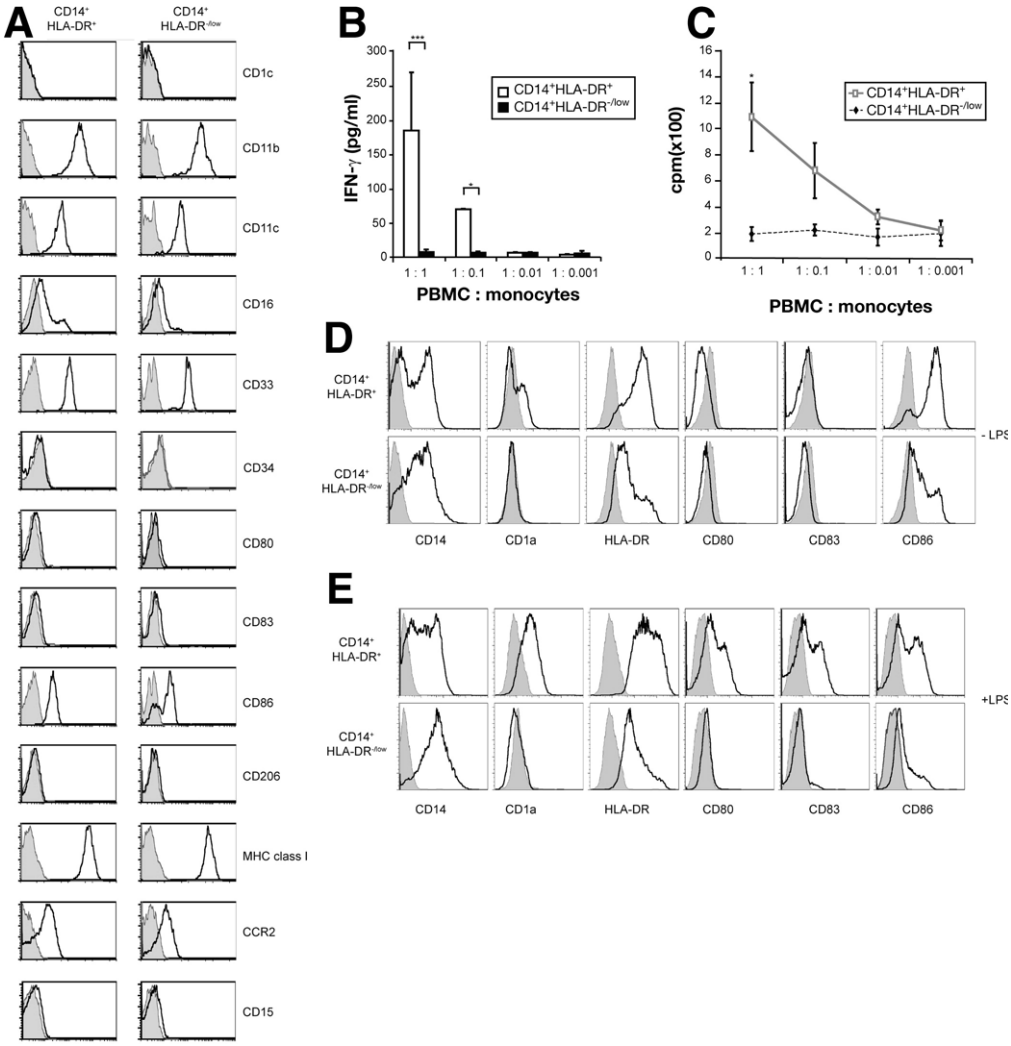

Figure 2. Analysis of CD14$^+$HLA-DR$^{-/low}$ cells in HCC patients. (*A*) FACS analysis of CD14$^+$HLA-DR$^+$ and CD14$^+$HLA-DR$^{-/low}$ cells from PBMC of HCC patients for different cell surface markers (*black line*) or isotype control (*filled histograms*) gated on CD19$^-$CD14$^+$HLA-DR$^+$ or CD14$^+$HLA-DR$^{-/low}$. Data shown are representative of >10 patients. (*B*) Stimulatory capacity of CD14$^+$HLA-DR$^{-/low}$ cells. CD14$^+$HLA-DR$^{-/low}$ cells (*black bars*) were cocultured with freshly isolated PBMC from a healthy donor for 3 days in different ratios. CD14$^+$HLA-DR$^+$ cells were used as a control (*white bars*). Supernatant was taken from the cultures before addition of ^{3}H-thymidine and measured for IFN-γ by ELISA. Results shown are representative of 5 independent experiments. *P < .05; ***P < .001. (*C*) T-cell proliferation was measured by ^{3}H-thymidine incorporation. Results shown are representative of 5 independent experiments. *P < .05. (*D* and *E*) Generation of dendritic cells from CD14$^+$HLA-DR$^{-/low}$ cells. CD14$^+$HLA-DR$^+$ or CD14$^+$HLA-DR$^{-/low}$ monocytes were FACS sorted from CD14$^+$ cells and incubated for 6 days with IL-4 and GM-CSF. Histograms of CD14, CD1a, CD80, CD83, CD86, and HLA-DR are shown before (*D*) and after (*E*) LPS stimulation (*black lines*) with isotype controls (*filled histograms*).

of the expression of different surface markers are shown in Figure 2*A*. Most of the cell surface activation markers such as major histocompatibility complex (MHC) class I, CD80, and CD83 were expressed at similar levels in both populations. Other monocytic markers such as CD11c, CD11b, CD33, and CD15 were also similar in both populations. CD16 and CD86 were expressed at lower levels on $CD14^{+}HLA\text{-}DR^{-/low}$ cells. For functional analysis, the stimulatory potential of $CD14^{+}HLA\text{-}DR^{-/low}$ monocytes was tested in a mixed lymphocyte reaction. $CD14^{+}HLA\text{-}DR^{-/low}$ and $CD14^{+}HLA\text{-}DR^{+}$ cells were sorted from PBMC of HCC patients and cultured with responder PBMC from a third healthy donor. As expected, $CD14^{+}HLA\text{-}DR^{+}$ monocytes induced strong IFN-γ secretion (Figure 2*B*) and T-cell proliferation (Figure 2*C*), whereas the $HLA\text{-}DR^{-/low}$ cells did not stimulate the allogeneic T-cell proliferation at any ratio.

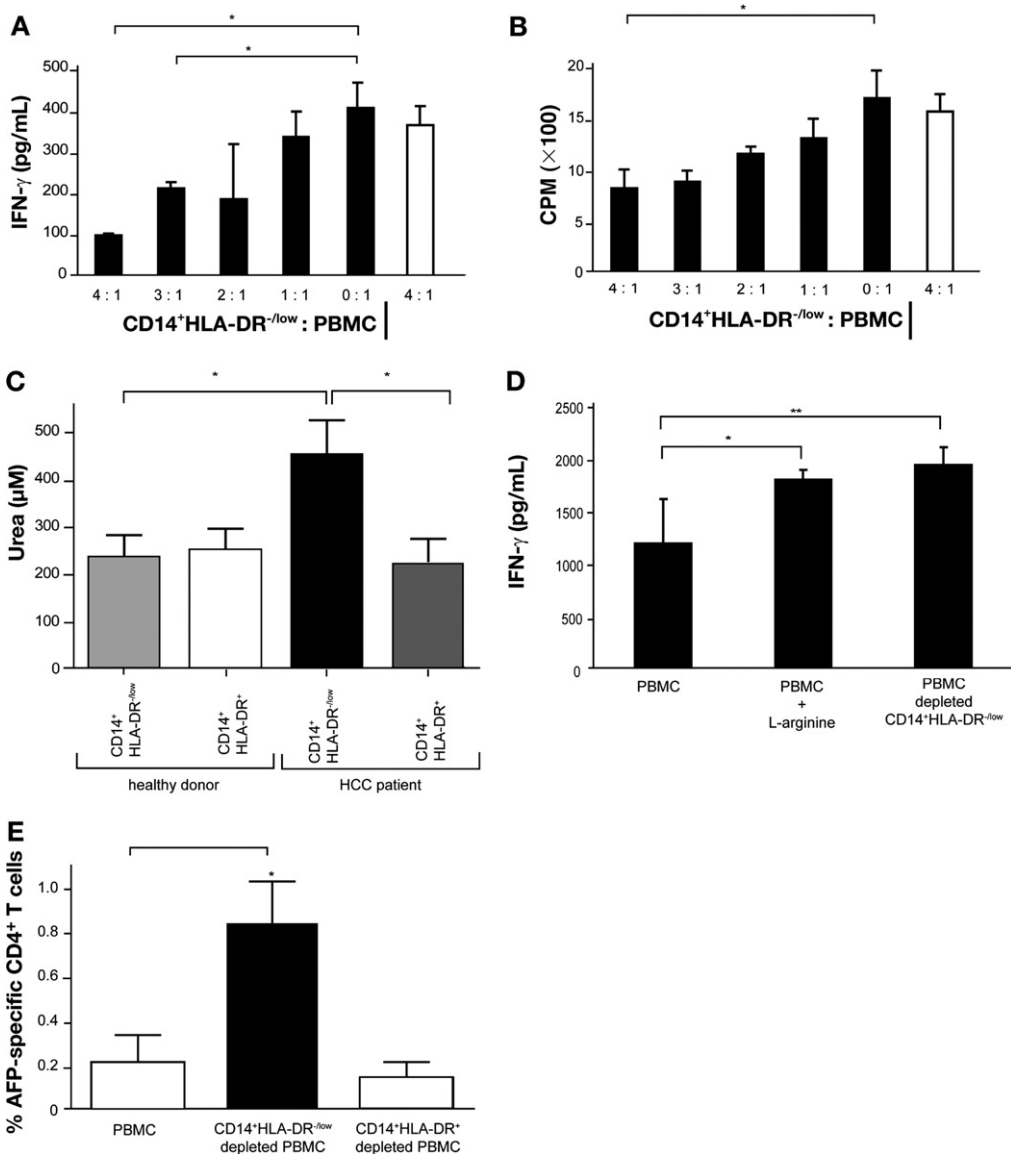

Figure 3. $CD14^{+}HLA\text{-}DR^{-/low}$ cells from HCC patients suppress autologous T-cell proliferation and have high arginase activity. (*A* and *B*) PBMC from healthy donors and HCC patients were stimulated and added to purified autologous $CD14^{+}HLA\text{-}DR^{-/low}$ cells at different ratios. IFN-γ (*A*) and ^{3}H-thymidine (*B*) incorporation was measured. No effect of $CD14^{+}HLA\text{-}DR^{+}$ cells on T-cell proliferation was seen even at the highest ratio (4:1; *white bar*). Data shown are representative of 6 independent experiments. $*P < .05$. (*C*) Arginase activity was determined in $CD14^{+}HLA\text{-}DR^{-/low}$ and $CD14^{+}HLA\text{-}DR^{+}$ cells from healthy donors and HCC patients. Results shown are combined from 5 independent experiments. $*P < .05$. (*D*) L-Arginine inhibits the suppressive function of $CD14^{+}HLA\text{-}DR^{-/low}$ cells. PBMC were either depleted of $CD14^{+}HLA\text{-}DR^{-/low}$ cells or L-arginine was added to the media. Cells were stimulated with CD2/CD3/CD28. $*P < .05$; $**P < .01$. Results from 5 independent experiments are shown. (*E*) Enhancement of AFP-specific $CD4^{+}$ T-cell responses. PBMCs or PBMC depleted of MDSC were stimulated with AFP peptides (AFP I, 137–145; AFP peptide II, 249–258; AFP peptide III, 364–373) and IFN-γ–secreting CD4 T cells were detected by FACS. Combined results from 8 different experiments are shown. $*P < .05$. PBMC depleted of $CD14^{+}$ $HLA\text{-}DR^{+}$ cells were used as controls.

Generation of MoDC From CD14⁺HLA-DR⁺ and HLA-DR⁻/low Cells

We also looked to see whether it is possible to generate dendritic cells in vitro from CD14⁺ HLA-DR⁻/low cells. CD14⁺ HLA-DR⁻/low and HLA-DR⁺ cells were sorted and incubated with IL-4 and GM-CSF for 6 days before LPS was added to mature dendritic cells. Expression of CD14, CD1a, CD80, CD83, CD86, and HLA-DR was evaluated. As shown in Figure 2*D*, CD14⁺ HLA-DR⁻/low cells remained mainly CD14⁺ upon incubation with GM-CSF/IL-4 for 6 days and did not express CD1a, CD80, or CD83. The HLA-DR expression was not changed. LPS stimulation of these cells also failed to up-regulate any of the costimulatory markers tested (Figure 2*E*). In contrast and as expected, the CD14⁺HLA-DR⁺ cells down-regulated their CD14 expression and underwent maturation upon LPS treatment, similar to dendritic cells generated from CD14⁺ cells treated with IL-4/GM-CSF.

CD14⁺HLA-DR⁻/low Cells Inhibit Autologous T-Cell Proliferation

Next, we tested whether CD14⁺ HLA-DR⁻/low monocytes from HCC patients can suppress autologous T-cell responses. Sorted CD14⁺ HLA-DR⁻/low cells were added at different ratios to autologous anti-CD3/CD28 – stimulated PBMC and IFN-γ release and proliferation was analyzed. CD14⁺ HLA-DR⁻/low cells suppressed the proliferation and IFN-γ production of autologous PBMC in a dose-dependent manner (Figure 3*A* and *B*). When CD14⁺ HLA-DR⁺ monocytes were used as controls, they failed to suppress proliferation or IFN-γ secretion of the responding PBMC as expected.

Suppression by CD14⁺ HLA-DR⁻/low Cells Is Mediated Through Arginase Activity

In murine models, it has been shown that mainly macrophages and immature myeloid cells metabolize arginine to urea and ornithine,[15,16] We therefore tested arginase activity in CD14⁺ HLA-DR⁻/low and CD14⁺ HLA-DR⁺ cells from HCC patients and healthy controls. As shown in Figure 3*C*, CD14⁺ HLA-DR⁻/low monocytes from HCC patients have >2-fold higher arginase activity (451 ± 75 µmol urea/10^5 monocytes) than CD14⁺ HLA-DR⁺ (222 ± 55 µmol urea/10^5 monocytes). The arginase activity of CD14⁺ HLA-DR⁻/low and CD14⁺ HLA-DR⁺ cells from healthy donors was similar (250 ± 47.8 and 234.5 ± 51.32 µmol urea/10^5monocytes, respectively). To further confirm the relevance of arginase expression for the suppressive function of CD14⁺ HLA-DR⁻/low cells, L-arginine was included in the suppression assay. Addition of L-arginine or depletion of MDSC resulted in enhanced IFN-γ secretion (Figure 3*D*). These results show that CD14⁺ HLA-DR⁻/low cells from HCC patients have arginase activity and inhibitory features similar to murine MDSC and therefore represent a human MDSC population.

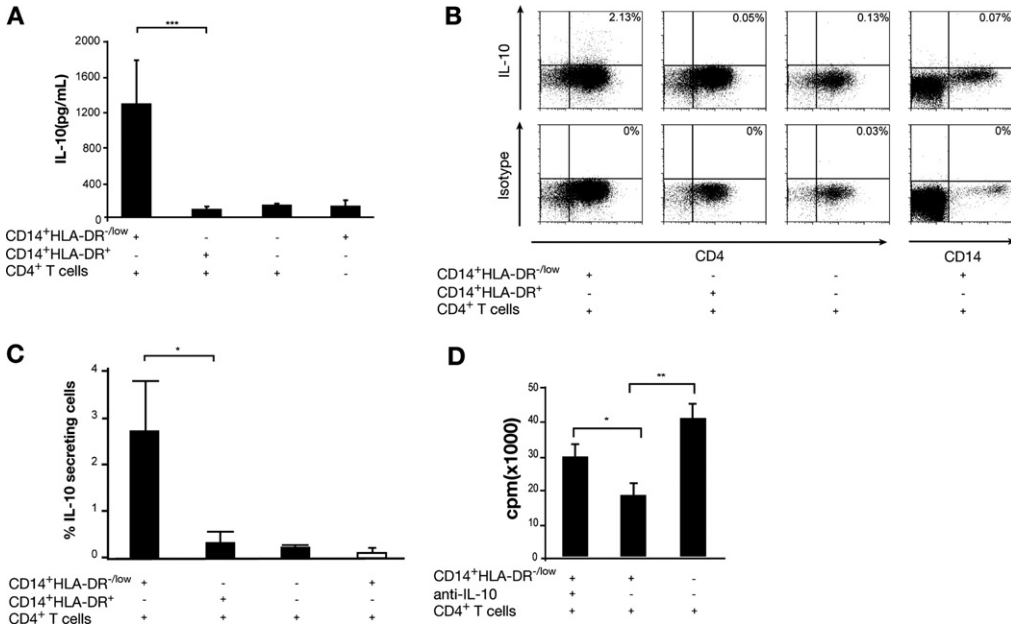

Figure 4. CD14⁺HLA-DR⁻/low cells induce IL-10 secretion in cocultured T cells. (*A*) CD4⁺ T cells were stimulated and incubated with sorted CD14⁺HLA-DR⁻/low or CD14⁺HLA-DR⁺ cells. IL-10 concentration was measured in the supernatant by ELISA after 24 hours. ***$P < .001$. (*B*) CD4⁺ T cells were stimulated and incubated with sorted CD14⁺HLA-DR⁻/low or CD14⁺HLA-DR⁺ cells. IL-10 secretion was analyzed by intracellular FACS analysis as shown in the representative *dot plots*. (*C*) Cumulative results of 4 independent experiments (*$P < .05$) are shown. IL-10 secretion was analyzed by gating on CD4⁺ T cells (*black bars*) and CD14⁺ cells (*white bar*). (*D*) CD14⁺HLA-DR⁻/low cells were cultured with CD2/CD3/CD28-stimulated CD4⁺ T cells in the absence or presence of anti–IL-10 antibody. The proliferation was analyzed by ^{3}H-thymidine incorporation. Shown are cumulative results from 3 independent experiments. *$P < .05$; **$P < .01$.

CD14⁺ HLA-DR⁻/low Depletion Enhances AFP-Specific T-Cell Responses

We sought to determine whether the CD14⁺ HLA-DR⁻/low cells could also inhibit tumor-specific immune responses in HCC patients. AFP is considered to be a potential tumor antigen for HCC and AFP-specific T-cell responses have previously been shown in HCC patients.[17] PBMCs were isolated from blood of 8 HCC patients and depleted of CD14⁺ HLA- DR⁻/low cells. The remaining PBMC (purity 95%) were stimulated with a pool of 3 AFP-derived peptides ($AFP_{137–145}$, $AFP_{249–258}$, and $AFP_{364–373}$) as previously described.[13] As shown in Figure 3*E*, AFP-specific CD4 T-cell responses were increased up to 4-fold in 5 out of 8 patients after depletion of CD14⁺ HLA- DR⁻/low cells. In 3 cases, AFP-specific CD4⁺ T-cell responses were only detected after depletion of CD14⁺ HLA- DR⁻/low cells. In 2 cases, the frequency of AFP-specific CD4⁺ T-cell responses increased after depletion of CD14⁺ HLA- DR⁻/low cells and in 3 cases no AFP-specific CD4⁺ T-cell responses were detected before and after depletion of CD14⁺ HLA- DR⁻/low cells (data not shown). No enhancement of AFP-specific responses was seen in

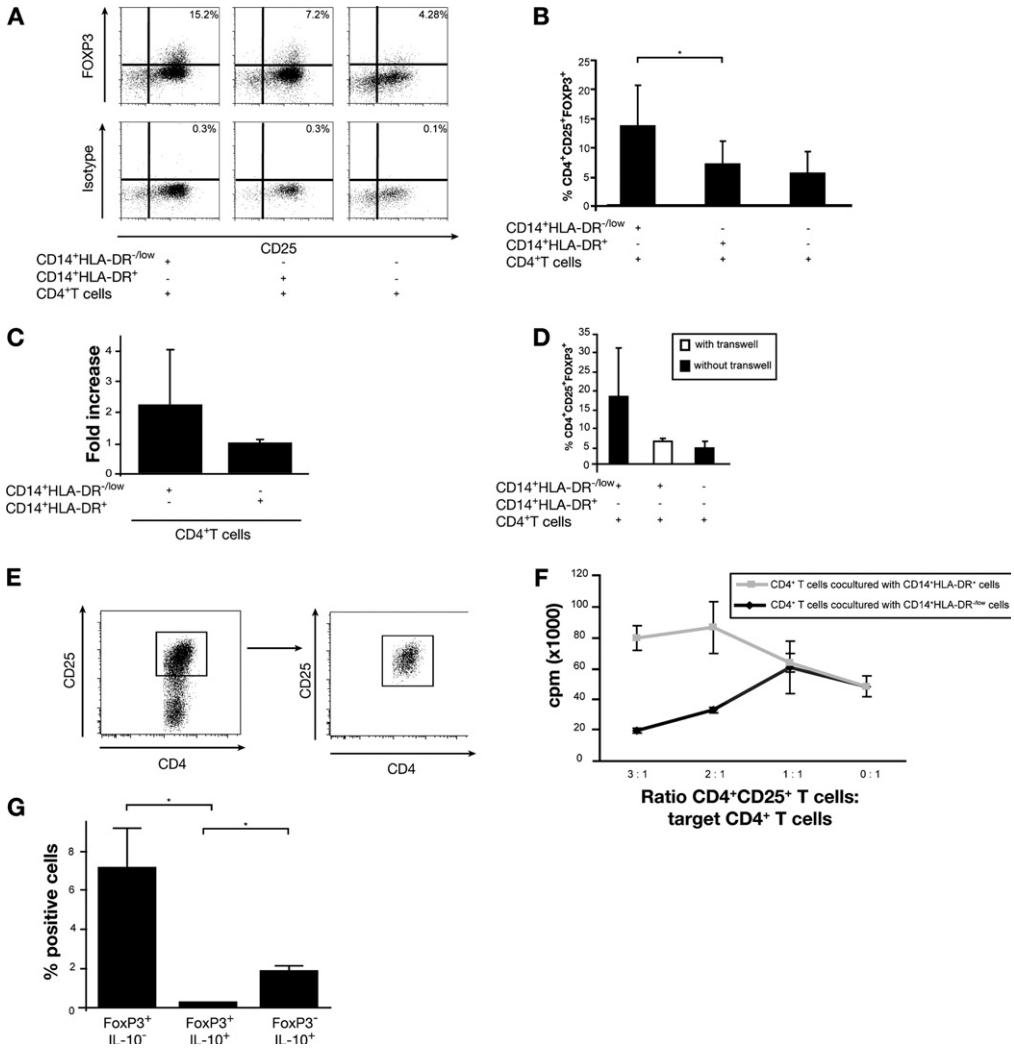

Figure 5. CD14⁺ HLA-DR⁻/low cells induce Foxp3 expression. (*A*) CD14⁺ HLA-DR⁻/low or DR⁺ cells were cocultured with CD4⁺ T cells for 3 days. Staining for Foxp3 was performed on CD4⁺ CD25⁺ T cells. Representative *dot plot* (*A*) or cumulative results from 5 independent experiments (*B*) are shown. **P* < .05. (*C*) qPCR analysis of Foxp3 mRNA upon culturing CD14⁺HLA-DR⁻/low or DR⁺ cells with CD4⁺ T cells for 3 days. Cyclophillin mRNA was used as control. Shown are results of 2 independent experiments. (*D*) Induction of Foxp3 expression is cell-contact dependent. CD14⁺ HLA-DR⁻/low cells were cultured with CD4⁺ T cells as described and transwell inserts were used as indicated. Shown are cumulative results from 2 independent experiments. (*E* and *F*) Suppressive function of CD14⁺ HLA-DR⁻/low -induced T cells. CD2/CD3/CD28-activated CD4⁺ T cells were cultured with CD14⁺ HLA-DR⁻/low or CD14⁺HLA-DR⁺ cells for 3 days. CD4⁺CD25⁺ T cells were resorted from these cocultures. Shown is the gating and the purity of the cells sorted after the coculture (*E*). (*F*) Suppression of autologous CD4⁺ T cells by in vitro induced CD4⁺ CD25⁺ T cells. The resorted CD4⁺CD25⁺ T cells from (*E*) were cocultured with autologous stimulated CD4⁺ T cells for 3 days at different ratios. Proliferation was measured by ^{3}H-thymidine incorporation. As a control, CD4⁺ T cells were cocultured with CD14⁺HLA-DR⁺ cells at the same ratios. Shown are representative results of 2 independent experiments. (*G*) Foxp3 and IL-10 expression by MDSC-induced CD4⁺ T cells. CD4⁺ T cells and CD14⁺HLA- DR⁻/low cells were cocultured as described. CD4⁺ T cells were stained for IL-10 and Foxp3. Shown are results from 3 independent experiments. **P* < .05.

control experiments when PBMC were depleted of $CD14^+HLA\text{-}DR^+$ cells (Figure 3*E*).

$CD14^+HLA\text{-}DR^{-/low}$ Cells Induce IL-10 Secretion in Cocultured T Cells

Next, the cytokine profile of the $CD14^+HLA\text{-}DR^{-/low}$ cells was analyzed. $CD14^+HLA\text{-}DR^{-/low}$ cells did not secrete transforming growth factor (TGF)-β, IL-4, IL-12, or IL-10 as measured by ELISA (data not shown). However, IL-10 was detected in cell supernatants of $CD14^+HLA\text{-}DR^{-/low}$ cells cocultured with autologous CD3/CD28-stimulated PBMC (Figure 4*A*). Intracellular IL-10 staining was done to investigate which cells secreted IL-10 in the cocultures. Figure 4*B* is a representative FACS analysis of the IL-10 staining. As shown, neither the MDSC nor the T cells alone secreted IL-10; however, upon coculturing the 2 populations, there was significant IL-10 secretion by the $CD4^+$ T cells. No IL-10 was secreted when $CD14^+HLA\text{-}DR^+$ cells were cocultured with T cells. Cumulative results from 4 experiments are shown in Figure 4*C*. To test if IL-10 secreted by $CD4^+$ T cells impaired the proliferation of CD3/CD28-stimulated PBMC in the coculture experiments, we incubated CD3/CD28-stimulated $CD4^+$ T cells with $CD14^+HLA\text{-}DR^{-/low}$ cells in the presence of an IL-10 –neutralizing antibody, which resulted in a 60% increase in proliferation (Figure 4*D*) and IFN-γ secretion (data not shown). This recovery was similar to when L-arginine was included in the reaction (Figure 3*D*). However, when IL-10 and L-arginine were both included in the assay, no synergistic effect was seen (Supplementary Figure 1; see supplementary material online at www.gastrojournal.org).

MDSC Induce $CD4^+CD25^+Foxp3^+$ Regulatory T Cells

Because we saw an increase in IL-10 production by T cells upon coincubation with $CD14^+HLA\text{-}DR^{-/low}$ cells, we further examined the T cells from the cocultures for regulatory phenotype. $CD14^+HLA\text{-}DR^{-/low}$ and $CD14^+HLA\text{-}DR^+$ cells were incubated with autologous CD3/CD28-stimulated $CD4^+$ T cells for 3 days and Foxp3 expression was evaluated in $CD4^+$ T cells by intracellular staining and qPCR. A 2-fold higher frequency of $Foxp3^+CD25^+$-positive cells was detected when $CD14^+HLA\text{-}DR^{-/low}$ cells were coincubated with CD3/CD28-stimulated cells in comparison with $CD14^+HLA\text{-}DR^+$ cells (Figure 5*A* and *B*). Similar results were obtained when Foxp3 expression was analyzed by qPCR analysis (Figure 5*C*). The increase in $Foxp3^+CD4^+$ T cells in the cocultures was seen as early as 36 hours after coculture (data not shown). Finally, induction of Foxp3 expression was cell contact dependent and was abrogated $CD14^+HLA\text{-}DR^{-/low}$ cells and CD3/CD28- stimulated cells were separated in a transwell experiment (Figure 5*D*).

To see whether the induced regulatory T cells were also functional, the $CD4^+CD25^+$ T cells were resorted after coculturing with MDSC (Figure 5*E*) and tested in an autologous suppression assay. As shown in Figure 5*F*, the $CD4^+$ T cells cocultured with $CD14^+HLA\text{-}DR^{-/low}$ cells could suppress autologous T-cell proliferation in a ratio-dependent manner. No suppression was seen with $CD4^+CD25^+$ T cells resorted from cocultures of $CD4^+$ with $CD14^+HLA\text{-}DR^+$ cells. Finally, we stained $CD4^+$ T cells after coculturing with $CD14^+HLA\text{-}DR^{-/low}$ cells for Foxp3 and IL-10 in parallel. As shown in Figure 5*G*, the induced $CD4^+FoxP3^+$ cells did not secrete IL-10. However, MDSC also induced generation of another type of regulatory T cell, $CD4^+FoxP3^-$ cells that secreted IL-10. $CD14^+HLA\text{-}DR^+$ cells did not induce either of the 2 subtypes of regulatory T cells (data not shown).

To test the role of L-arginine in regulatory T-cell induction, we included L–arginine in cocultures of MDSC with $CD4^+$ T cells. There was no effect of L-arginine on induction of $CD4^+CD25^+Foxp3^+$ cells cocultured with MDSC (Supplementary Figure 2).

Discussion

MDSC have been identified as a subset of inflammatory monocytes with immunosuppressive activity on $CD8^+$ T cells.[18] These cells are well characterized in mice, but only limited information is available on MDSC in humans. In this study, we describe a new subset of MDSCs in patients with primary liver cancer. MDSC in HCC patients have a $CD14^+HLA\text{-}DR^{-/low}$ phenotype and their frequency is increased significantly in peripheral blood and tumor of these patients. $CD14^+HLA\text{-}DR^{-/low}$ cells from HCC patients have increased arginase activity and directly suppress the response of tumor-specific $CD4^+$ T cells ex vivo. In addition, our study shows that $CD14^+HLA\text{-}DR^{-/low}$ cells suppress T-cell function not only directly through arginase, but possibly also indirectly through the induction of $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells.

So far, human MDSC have been described mainly as $CD34^+$, $CD33^+$, and $CD15^-$ cells detected in patients with head and neck cancer, non–small cell lung cancer, and breast cancer.[4,2,7] In renal cancer patients, a population of cells that were $CD11b^-CD15^+$, but $CD14^-$ were shown to have characteristics of MDSCs.[5] Others have reported $CD14^+$ monocytes as MDSC in patients with different types of cancer.[19,20]

We have identified an increase in a population of CD14 cells that have low or no expression of HLA-DR. However, our cells did not express any CD15, in agreement with others who also have found expression of CD15 on the CD14, but not on the CD14 cells[5] or CD14 HLA-DR $^{/low}$ cells.[19]

Supporting our data, $CD14^+HLA\text{-}DR^{-/low}$ cells have recently been identified in patients with melanoma.[19] However, in contrast with our study, they could not find any arginase activity in $CD14^+HLA\text{-}DR^{-/low}$ cells, a hallmark characteristic of MDSC. In addition, their study showed that TGF- played a key role in immunosuppres

sive activity of $CD14^+$ HLA-$DR^{-/low}$ cells as opposed to MDSC from HCC patients that did not secrete TGF-β . We have performed a number of functional assays to demonstrate similar immunosuppressive functions as previously described for murine MDSC. $CD14^+$HLA-$DR^{-/low}$ MDSC suppressed proliferation and IFN-γ secretion of CD3/CD28-stimulated autologous PBMC as well as tumor-specific $CD4^+$ T cells.

A number of studies have shown that $CD4^+CD25^+$ regulatory T cells are increased in patients with cancer and that their presence correlates with disease progression.[21–23] Different in vitro studies have reported that dendritic cells can induce Foxp3-expressing regulatory T cells.[24–28] It has been shown that immature $Gr-1^+CD115^+$ myeloid cells in mice induce regulatory T cells[29] in vitro, but a direct link between the MDSC and regulatory T cells has not been demonstrated so far. Our data demonstrate that MDSC are capable of inducing not only $CD4^+$ $FoxP3^+$IL-10^- cells, but $CD4^+FoxP3^-$IL-10- secreting cells (Tr1) as well. Tr1 cells are another subset of regulatory T cells that also play an important role in the control of immune system. The role of each of these populations and their exact mechanism of action in HCC patients remains to be defined.

Different mechanisms have been described as to how MDSCs exert their immunosuppressive function. In a recent study, it was shown that T-cell tolerance can be induced by MDSCs through production of reactive oxygen species and peroxynitrite causing a nitration of the TCR–CD8 complex, which prevents CD8 T cells from binding to synthetic MHC complexes.[30] Our study suggests that MDSCs in HCC patients inhibit T-cell responses both through arginase activity as well as induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells. The MDSC-mediated regulatory T-cell induction is a complex process, and one mechanism might be arginase activity. Our data suggest that arginase plays a role in suppressive function of MDSC, but does not affect induction of regulatory T cells in vitro. In addition, transwell experiments showed that additional factors possibly acting through direct cell– cell contact also play a crucial role in generation of regulatory T cells. It will be important to determine which factors are responsible for induction of regulatory T cells by MDSC in HCC patients. Identification of more specific markers for regulatory T cells as well as MDSC will help in the future to understand the biology of these 2 cell types with immune suppressor functions. In addition, targeting these cells in vivo might help in boosting antitumor responses in HCC patients.

In summary, we describe an increase in the frequency of immunosuppressive MDSC in peripheral blood and tumor of patients with HCC. MDSC, characterized by their $CD14^+$HLA-$DR^{-/low}$ phenotype in these patients, can suppress tumor-specific $CD4^+$ T-cell responses and induce $CD4^+CD25^+Foxp3^+$ regulatory T cells, suggesting a new immunosuppressive pathway of MDSC developed in cancer patients.

Dissecting the mechanisms behind these suppressive mechanisms will be helpful for the design of effective immunotherapeutic protocols, probably not only for patients with HCC but also for patients with other types of cancer.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.03.020.

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Supplementary Figures

Figure S1

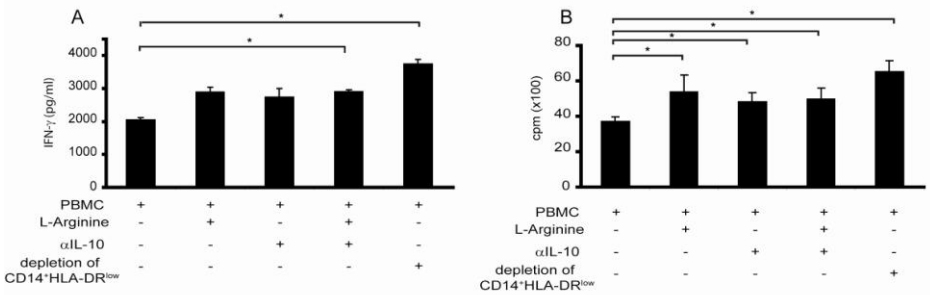

Figure S2

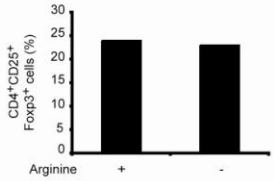

ABBILDUNG 10: SUPPLEMENTARY FIGURE 1 & 2: EFFECT OF L-ARGININE AND BLOCKING IL-10 ANTIBODY ON THE SUPPRESSIVE EFFECT OF CD14$^+$HLA-DR$^{-/LOW}$ CELLS

S1- Effect of L-arginine and blocking IL-10 antibody on the suppressive effect of CD14$^+$HLA-DR$^{-/low}$ cells. A and B- PBMC were stimulated with CD2/CD3/CD28. Where indicated, L-arginine, anti-IL-10 blocking antibody or both were added to the media. IFN-γ was measured using ELISA (A) and proliferation (B) was measured by ^{3}H-thymidine incorporation (*$p < 0.05$).

Figure S2: L-arginine does not affect the induction of CD4$^+$CD25$^+$Foxp3$^+$ cells *in vitro*. CD4$^+$ T cells were cultured with CD14$^+$HLA-DR$^{-/low}$ cells in the presence or absence of L-arginine for 3 days. Staining for Foxp3 was done on CD4$^+$CD25$^+$ cells as described.

Myeloid Derived Suppressor Cells Inhibit Natural Killer Cells in Patients with Hepatocellular Carcinoma via the NKp30 Receptor

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Several immune suppressive mechanisms that evade the host immune response have been described in patients with hepatocellular carcinoma (HCC); one of these mechanisms is expansion of myeloid-derived suppressor cells (MDSCs). MDSCs have been shown to inhibit T cell responses in tumor-bearing mice, but little is known about these cells in humans. Here, we have analyzed and characterized the effect of MDSCs on the innate immune system, in particular, their interaction with natural killer (NK) cells in patients with HCC. MDSCs from patients with HCC inhibited autologous NK cell cytotoxicity and cytokine secretion when cultured together in vitro. This suppression was dependent on cell contact, but did not rely on the arginase activity of MDSCs, which is a hallmark function of these cells. However, MDSC-mediated inhibition of NK cell function was dependent mainly on the NKp30 on NK cells. *Conclusion:* Our study suggests a new role for MDSCs in patients with HCC in disarming the innate immune system and further contributing to the immune suppressor network in these patients. These findings have important implications when designing immunotherapy protocols. (HEPATOLOGY 2009;50:000-000.)

Tumors have evolved different mechanisms to generate a suppressive network and evade the host's immune response. One such mechanism is an increase in myeloid-derived suppressor cells (MDSCs). MDSCs are a heterogeneous population of myeloid cells including macrophages, granulocytes, and other cells that express both Gr-1 and CD11b in mice and suppress immune responses in vivo and in vitro.[1] In humans, MDSCs have not been well characterized owing to the lack of specific markers. Only limited data are available on dif- ferent myeloid cell populations with suppressor function in patients with head and neck cancer, squamous cell carcinoma, non–small cell lung cancer, and colon and breast cancers. The phenotype of these cells has been shown to be mainly CD34, CD33, CD15, and CD13, $CD14^{-}/lin^{-}$.[2-5]

Our earlier studies have demonstrated that hepatocellular carcinoma (HCC), similar to other types of cancer, induces a suppressive network to evade the host immune response. We have shown an increase in regulatory T cells[6] as well as defective antigen-presenting cells[7] as some of the suppressive mechanisms in patients with HCC. Recently, we have characterized a new population of MDSCs that are $CD14^{+}$ human leukocyte antigen (HLA)-$DR^{-/low}$, significantly increased in peripheral blood and tumors of patients with HCC, have arginase activity, and suppress autologous T cell proliferation.[8] Interestingly, our study showed that MDSCs in patients with HCC induce $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells, which also suppress antitumor immune responses.[8]

Natural killer (NK) cells are effector cells of the innate immune system, where they have the ability to kill tumors

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; IFN, interferon; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; NK, natural killer cell; PBMC, peripheral blood mononuclear cells; STAT, signal transducer and activator of transcription.

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or virus-infected cells, secrete cytokines, and regulate both innate and adaptive immune responses.[9] It has previously been shown that NK cells in patients with cancer are reduced in their cytotoxic function.[10,11] In particular, several studies in patients with HCC have shown that NK cells from these patients are defective in their lytic function and cytokine secretion.[12,13] However, the cause for the impaired function of NK cells is not known.

We hypothesized that MDSCs can also exert their inhibitory function on the innate arm of the immune system and its effector cells, the NK cells. In order to investigate if MDSCs can regulate NK cell function, we analyzed their interaction with NK cells when isolated directly from peripheral blood of patients with HCC and healthy donors. In this study, we show that NK cells in patients with HCC are defective in their lytic function and cytokine secretion as compared to healthy donors. We also provide evidence for the potential linkage between the suppression of NK cell function and increase in MDSCs seen in patients with HCC. We show that MDSCs inhibit autologous NK cell cytotoxicity and cytokine release in patients with HCC when cocultured in vitro. This inhibition is cell contact– dependent and is mediated through the NKp30. We suggest that MDSCs are able to inhibit and regulate NK cells as effectors of the innate immune system contributing further to immune suppressor mechanisms in patients with HCC. These finding have important implications when designing immunotherapy protocols in patients with HCC.

Patients and Methods

Patients and Healthy Donors. Blood samples were collected from patients with HCC seen at the Department of Gastroenterology, Hepatology and Endocrinology, Han- nover Medical School (Hannover, Germany). HCC was di- agnosed according to the guidelines of the European Association for the Study of the Liver. Written consent was obtained from all patients before blood and tumor sampling, and the Ethics Committee of Hannover Medical School ap- proved the study protocol. Table 1 shows the clinical char- acteristics of all patients with HCC in this study.

Cell Isolation and Sorting. Peripheral blood mononucleocytes (PBMCs) were isolated from freshly obtained blood by Ficoll density gradient centrifugation (Biochrom, Berlin, Germany) as described.[8] $CD14^{-}$ cells were purified using CD14 Microbeads and AutoMACS (magnetic cell sorting) separation unit (Miltenyi Biotech, Bergisch Gladbach, Germany). $CD14^{-}$ $HLA\text{-}DR^{-/low}$ and $CD14^{-}$ $HLA\text{-}DR^{-}$ cells were isolated from $CD14^{-}$ cells using BD FACS (fluorescence-activated cell sorting) Aria cell sorting system (Becton Dickinson, Heidelberg, Germany) as described.[8] $CD56^{+}$ $CD3^{-}$ NK cells were purified using the CD14-depleted fraction or PBMCs. The purity of the cells after sorting was 98%.

Table 1. Patient Characteristics

Characteristic	Value
Male/Female	25/5
Average age	69 years
Cause of cirrhosis	
HBV HCV	7
Ethanol	6
Other	9
Unknown	3
Liver cirrhosis	5
No cirrhosis	
Child-Pugh A	3
Child-Pugh B	17
Child-Pugh C	9
Tumor stage	1
BCLC A	
BCLC B	6
BCLC C	7
BCLC D	16
	1

HBV, hepatitis B virus; HCV, hepatitis C virus; BCLC, Barcelona Clinic Liver

Isolation of Tumor-Infiltrating Lymphocytes. Tumor specimens were collected at the time of surgery and processed by cutting into small pieces and digested with 3000 U/mL collagenase (Sigma-Aldrich, St. Louis, MO) and 130 U/mL dispase I (Roche, Mannheim, Germany) for 30 minutes. Resulting cells were washed with phos- phate-buffered saline, and lymphocytes were isolated by Ficoll density gradient as described.[8]

Cell-Mediated Cytotoxicity Assay. Lytic function of NK cells was determined by a standard 4-hour chromium-release assay. Briefly, FACS-sorted NK cells, NK cells cocultured with $CD14^{+}HLA\text{-}DR^{-/low}$ cells, or PBMCs depleted of $CD14^{+}$ cells were added to ^{51}Cr-labeled

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K562 target cells in triplicate at different ratios of effector to target cell.

Flow Cytometry Analysis and Blocking Antibodies. To determine the phenotype of MDSCs and NK cells ex vivo and upon coculturing, FACS analysis was done using the following antibodies: anti-CD16, anti–HLA-DR (ImmunoTools, Friesoythe, Germany); anti-CD14, anti-CD56, anti-69, anti-CD314 (anti-NKG2D), anti-CD336 (anti-NKp44) (Miltenyi Biotech, Bergisch Gladbach, Germany); anti-CD94, anti–HLA-ABC (Becton Dickinson, Heidelberg, Germany); anti-CD86 (Caltag, Hamburg, Germany); anti-CD337 (NKp30) (Beckman Coulter, Fullerton, CA); and anti-NKp80 (R&D Systems, Minneapolis, MN). For intracellular staining, cells were lysed using BD Cytofix/Cytoperm Fixation/Permeabilization Kit and stained with anti–in- terferon- (IFN-), (Becton Dickinson, Heidelberg, Germany). For blocking experiments, the following anti- bodies were used: anti-CD337 (2-20 g/mL, Clone P3015), anti-CD336 (5 g/mL, Clone P44-8) (Bioleg- end, San Diego, CA); anti-CD94 (20 g/mL, Clone 131412), and anti-CD314 (1 g/mL, Clone 149810) (R&D Systems, Minneapolis, MN). Flow cytometry was done using Becton Dickinson FACSCalibur. Analysis of FACS data was done with FlowJo software (TreeStar Inc., Ashland, OR). Isotype-matched antibodies were used as indicated.

Suppression Assay. NK cells, CD14 HLA-DR $^{/low}$ and CD14 HLA-DR cells were purified as described. NK cells were stimulated with 250 IU/mL interleukin-2 (IL-2; Chiron, Amsterdam, Netherlands) and CD14 cells were added as indicated. IFN- secretion was measured in super- natants after 48 hours by enzyme-linked immunosorbent assay (ELISA; ImmunoTools, Friesoythe, Germany). Trans- well inserts, blocking antibodies, or inhibitors were added as indicated.

Determination of STAT Expression by Quantitative Polymerase Chain Reaction. NK cells and CD14 cells were purified as described. Cells were cocultured for 12 hours, and RNA was isolated using RNeasy Micro Kit (Qiagen, Hilden, Germany). Complementary DNA synthesis was done with iScript Kit (Bio-Rad, München, Germany). Quantitative polymerase chain reaction was performed using the following primers (300 nmol each): signal transducer and activator of transcription 1 (STAT1) forward, 5 -tgc aaa acc ttg cag aac ag-3 ; STAT1 reverse, 5 -ggg cat tct ggg taa gtt ca-3 ; STAT3 forward, 5 -ctg gcc ttt ggt gtt gaa at-3 ; STAT3 reverse, 5 -ctc tgc cca gcc tta ctc ac-3 ; STAT4 forward, 5 -agc ctt gcg aag ttt caa ga-3 ; STAT4 reverse, 5 -aca ccg cat aca cac ttg ga-3 ; STAT5 forward, 5 -aca aag att gtt ggg gca ag-3 ; STAT5 forward, 5 -atg ctc aac ccc acc gtg t-3 ; cyclophilin A reverse, 5 -tct gct gtc ttt ggg acc ttg tc-3 . Reactions were done in triplicate using Sybr Green (Bio-Rad, München, Germany) and normalized to endogenous cyclophilin A messenger RNA level.

Statistical Analysis. Data are expressed as mean standard error of the mean (SEM) for percentages. Statistical analysis was done using Student *t* test to assess the differences between the study groups. *P* values 0.05 were considered statistically significant.

Results

NK Cells from Patients with HCC Are Reduced in their Cytotoxicity. To characterize NK cells from patients with HCC, CD56 CD3 cells were sorted from PBMCs and tumor-infiltrating lymphocytes of patients with HCC. Lytic activity was compared to NK cells from healthy donors in a ^{51}Cr-release assay against K562 target cells at different ratios. Cytotoxicity of NK cells from peripheral blood and tumor of patients with HCC was significantly reduced as compared to healthy donors at all ratios tested (Fig. 1). At the 4:1 ratio, there was more than 50% reduction in lysis by NK cells isolated from PBMCs and tumor of patients with HCC as compared to that from healthy donors.

MDSCs Inhibit NK Cell Cytotoxicity and IFN- Release. We have previously shown a significant increase in frequency of CD14 HLA-DR $^{/low}$ MDSCs in peripheral blood and tumor of patients with HCC. In order to

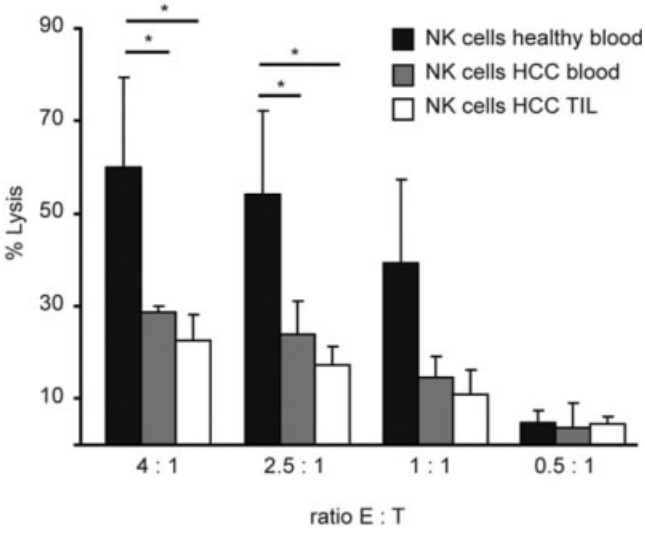

Fig. 1. NK cell cytotoxicity is reduced in patients with HCC. NK cells were FACS-sorted from PBMCs of either healthy donors (n 10) or patients with HCC (n 10), or from tumor-infiltrating lymphocytes (n 5) by gating on CD56 CD3 population and used in a ^{51}Cr-release assay against K562 target cells. Figure shown is average of four inde- pendent experiments (*P 0.05).

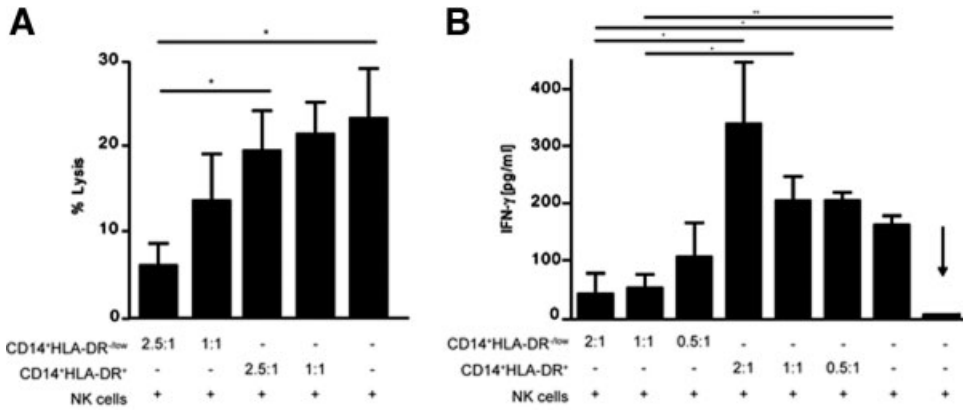

Fig. 2. CD14 HLA-DR$^{-/low}$ cells suppress NK cell function. (A) Purified NK cells were cultured in the absence or presence of different ratios of CD14 HLA-DR or CD14 HLA-DR$^{-/low}$ cells as indicated. After 12 hours, K562 cells were added at a ratio of 2.5:1 (E:T) and lysis was determined by standard ^{51}Cr-release assay (P 0.05). Shown are cumulative results from four independent experiments. (B) NK cells were stimulated with IL-2 and cultured in the presence or absence of CD14 HLA-DR$^{-/low}$ or CD14 HLA-DR cells as indicated. IFN- release was determined after 48 hours by ELISA. Shown are cumulative results from five independent experiments ($*P$ 0.05, $**P$ 0.001). NK cells cultured without IL-2 were used as background (arrow).

see whether MDSCs can impair NK cell cytotoxicity, MDSCs and NK cells were sorted by FACS from peripheral blood of patients with HCC and cultured together. After 12 hours, the NK cells were tested in a standard ^{51}Cr-release assay. CD14 HLA-DR monocytes were used as a control. NK cell cytotoxicity was significantly impaired after coculture of NK cells with MDSCs, which was still observed at a 1:1 ratio (Fig. 2A). However, no suppression was observed when NK cells were cocultured with CD14 HLA-DR cells. In addition, MDSCs did not lyse K562 cells at any ratio tested, and there was no lysis of MDSCs by the NK cells detected (data not shown). Next, MDSCs or CD14 HLA-DR cells from peripheral blood of patients with HCC were cocultured with NK cells, and the supernatant was tested for IFN- secretion. As shown in Fig. 2B, IFN- release by NK cells was also more than 60% at 0.5:1 ratio) when cocultured with MDSCs but not with CD14 HLA-DR cells. Intracellular FACS analysis was performed in order to investigate the source of IFN- . As shown in Fig. 3A, NK cells released IFN- (53.3% IFN- CD56 cells) when cocul- tured with CD14 HLA-DR cells, but not with MDSCs (0.7% IFN- CD56 cells). Analysis of MDSCs revealed that CD14 HLA-DR cells released more IFN- after coincubation with NK cells (13.8% IFN- CD14 cells) than with MDSCs (5.4%) (Fig. 3A,B).

Phenotypic Characterization of MDSCs and NK Cells. Here, the phenotype of NK cells and MDSCs was analyzed before and after coculture. FACS-sorted MDSC and NK cells were incubated together for 24 hours, and the number and phenotype of both NK cells and MDSCs were analyzed by FACS. The numbers of MDSCs or NK cells did not change significantly upon cocul-

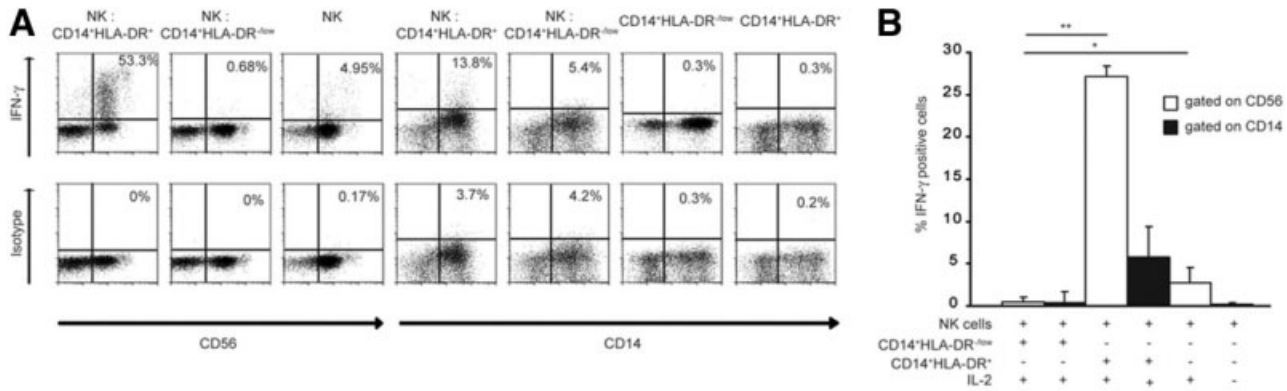

Fig. 3. Intracellular cytokine analysis of CD14 HLA-DR$^{-/low}$ in the presence or absence of NK cells. (A) NK cells were stimulated with IL-2 and cultured either alone, with CD14 HLA-DR$^{-/low}$, or with CD14 HLA-DR cells. IFN- was analyzed after 48 hours by intracellular FACS as shown in representative dot plots. (B) Cumulative results of three independent experiments are shown ($*P$ 0.05; $**P$ 0.001). Cytokine secretion was analyzed gating on CD14 cells (black bars) and CD56 cells (white bars).

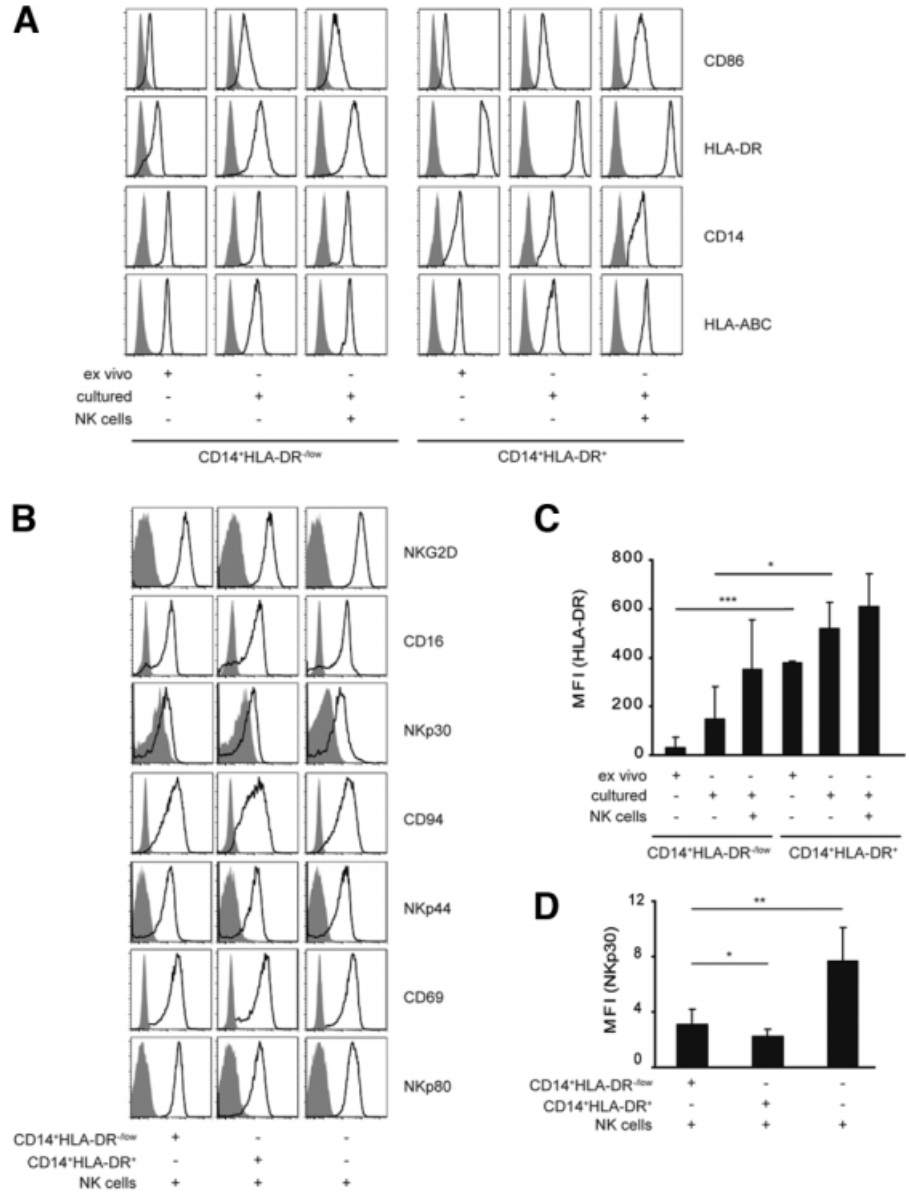

Fig. 4. Phenotypic analysis of NK cells and CD14 HLA-DR /low cells upon coculture. Purified CD14 HLA-DR /low or CD14 HLA-DR cells were cultured alone or with FACS-sorted autologous NK cells as indicated, and the expression of different surface markers (black lines) or isotype control (filled histograms) was analyzed by FACS. Representative histograms for (A) CD14 HLA-DR , CD14 HLA-DR /low or (B) NK cells are shown. Cumulative results of seven independent experiments for (C) HLA-DR and (D) NKp30 are shown (*P 0.05; **P 0.001, ***P

(data not shown). MDSCs retained the expression of all their surface markers; these markers were similar when the MDSCs were cultured alone or with NK cells (Fig. 4A). There was an up-regulation of HLA-DR expression on MDSCs upon coculture with NK cells (Fig. 4A,C). Similarly, NKG2D, CD16, CD94, NKp44, CD69, and NKp80 expression did not change on NK cells upon coculture with MDSCs (Fig. 4B). In contrast, there was a significant reduction in expression of NKp30 on NK cells upon coculture with MDSCs (Fig. 4B,D). It should be noted that NK cells also had a reduction in expression of NKp30 when cocultured with monocytes.

Suppression of NK Cells by MDSCs Is Dependent on Cell Contact. In order to investigate the possible mechanisms as to how MDSCs inhibit NK cell function, MDSCs and NK cells were incubated either together or in separate wells of a transwell, and IFN- concentration in cell super- natants was measured after 48 hours. Figure 5A shows that the inhibition of NK cells by MDSCs is mainly dependent on cell contact, because up to 80% of the inhibition was reversed when the cells were separated by use of a transwell.

Suppression of NK Cells by MDSCs Is Independent of Arginase or Inducible Nitric Oxide Synthase Func-

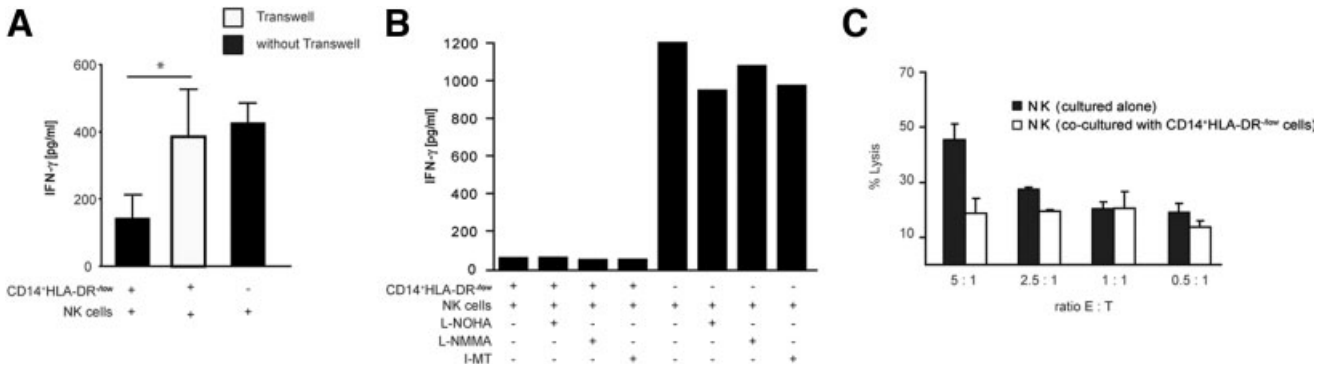

Fig. 5. Inhibition of IFN- production by MDSC is mainly cell contact– dependent, but independent of arginase and NO. (A) CD14 HLA-DR /low cells were cocultured with NK cells as described and transwell inserts were used as indicated (white bar). IFN- secretion was determined by ELISA. Shown are cumulative results from three independent experiments (*P 0.05). (B) Purified NK cells and CD14 HLA-DR /low cells were cocultured in the absence or presence of *N*-omega-hydroxy-L-arginine (L-NOHA), *N*(G)-monomethyl-L-arginine (L-NMMA), 1-MT (10 mol/L each), or media alone as indicated. IFN- production was measured by ELISA after 48 hours. Figure shown is a representative of three independent experiments. (C) Purified NK cells and CD14 HLA-DR /low cells were cocultured for 36 hours. NK cells were reisolated, and a ^{51}Cr-release assay against K562 target cells was performed. Shown is representative data from three independent experiments.

ible NO synthase on MDSC-mediated suppression of NK cells. We have previously shown that MDSCs express high levels of arginase.[8] MDSCs were treated with *N*-omega-hydroxy-L-arginine, *N*(G)-monomethyl-L-arginine, and 1-Methyl-Tryptophane (I-MT) (specific inhibitor for indoleamine 2,3-deoxygenase [IDO]) and incubated with NK cells. None of these inhibitors affected MDSC mediated inhibition of cytokine secretion by NK cells (Fig. 5B). These results show that MDSCs inhibit NK cells through another pathway independent of arginase I, inducible NO synthase, and IDO.

The Suppressive Effect of MDSCs on NK Cells Is Long-Lasting. We also incubated NK cells with MDSCs for 24 hours, then re-sorted NK cells from these cocultures and used them in a chromium-release assay against K562 cells. NK cells re-isolated from MDSC cocultures had a reduced lytic function as compared to uncultured NK cells and NK cells cocultured with CD14 HLA-DR cells (Fig. 5C). This suggests that the suppressive effect of MDSCs on NK cells is long-lasting and not a transient outcome.

MDSCs Inhibit NK Cell Function Using the NKp30 Receptor. To analyze the molecular basis of NK cell inhibition by MDSCs, we tested to see if blocking of any of the receptors on NK cells could affect the interaction between MDSCs and NK cells and subsequent inhibition. MDSCs and NK cells were coincubated in the presence of anti-NKG2D, anti-CD94, anti-NKp44, anti–major histocompatibility complex I (MHC I), anti-MHC-II, and anti-NKp30. Addition of anti-NKp44, anti-NKG2D, anti-CD94 monoclonal antibody or anti-MHC I or anti-MHC II had no effect on MDSC-mediated inhibition of IFN- release by NK cells (Fig. 6). In addition, there was no effect on NK cell lysis (data not shown). However,
blocking NKp30 substantially reduced the inhibitory function of MDSCs on NK cells. Indeed, in the presence of anti-NKp30, both cytolytic activity and IFN- release by NK cells were reversed. Similar amounts of IFN- were measured in supernatants from NK cells alone and NK cells cocultured with MDSCs when NKp30 blocking antibody was used (Fig. 7A,B). There was no effect of anti-NKp30 antibody on NK cells alone (data not shown). These results suggest that NKp30 engagement is involved in MDSC-mediated inhibition of NK cell function.

Role of STATs in MDSC-Mediated Inhibition of NK Cells. Because the STAT signaling pathways are known to be important in NK cell function and recently have been shown to be involved in MDSC-mediated inhibition of NK cells in mice,[14] we investigated the expression of messenger RNA level of different STATs in our cocultures. Quantitative polymerase chain reaction was performed on NK cells and CD14 HLA-DR /low cells under different conditions, as indicated. A significant up- regulation of STAT1 expression was observed when NK cells and CD14 HLA-DR /low cells were cultured to- gether (Supporting Fig. 1A; 0.56 0.03 to 0.08 0.02), but no differences in expression level could be detected for STAT3, STAT4, and STAT5 (Supporting Fig. 1B-D). However, adding the nucleoside analogue fludarabine as an inhibitor for STAT1 phosphorylation[15] showed no effect on the IFN- secretion of NK cells (Supporting Fig.
1E). Intracellular staining for p-STAT1 showed an activation of STAT1 in CD14 HLA-DR /low cells (Supporting Fig. 1F, panel I) cocultured with NK cells but also

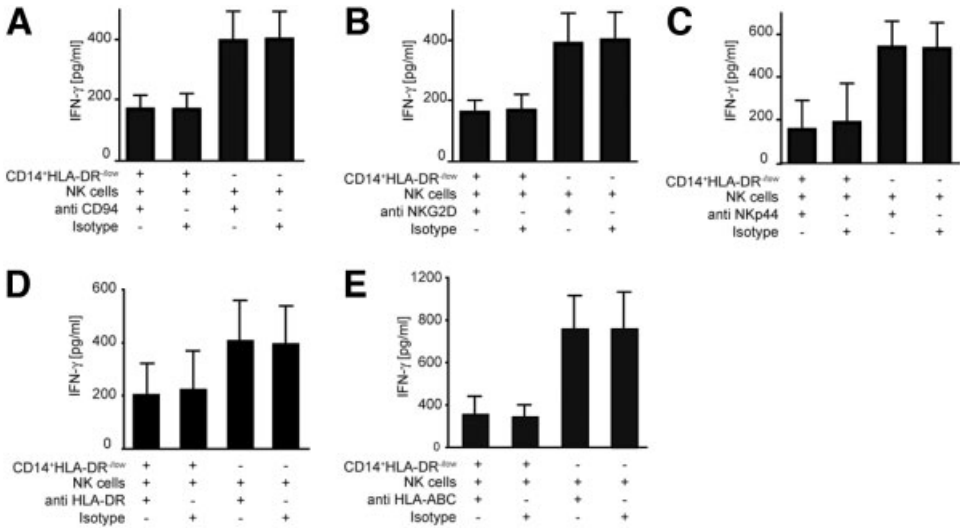

Fig. 6. MDSC-mediated suppressive mechanism is independent of anti-CD94, anti-NKG2D, anti-NKp44, anti-HLA-DR, and anti-MHC class I. Purified NK cells were cultured with IL-2 in the absence or presence of different blocking antibodies as indicated. (A-E) IFN- secretion was measured in supernatants after coincubation in the presence of the indicated antibodies by ELISA. Shown are cumulative results from two independent experiments.

(Supporting Fig. 1F, panels II,IV). These findings suggest that STAT1 is not involved in the suppression of NK cells by CD14 HLA-DR /low cells in humans.

Discussion

It has become increasingly clear that host-tumor interactions are quite complex, leading to tumor escape mechanisms in patients with cancer.[16,17] MDSC expansion is one of the mechanisms that HCC tumors develop to evade the host immune response seen in both mice and humans.[8] This study demonstrates a novel role for MDSCs in patients with HCC.

We have previously shown that patients with HCC mount spontaneous tumor-specific immune responses, but still progress with their disease,[6] suggesting that immune-suppressor mechanisms might counterbalance antitumor immune responses. Indeed, we have been able to detect different types of immune escape mechanisms such as an increase in CD4 CD25 reg- ulatory T cells and defective dendritic cell function in patients with HCC.[7,18] Recently, we have identified a new population of MDSCs in patients with HCC; these cells are CD14 HLA-DR /low, are significantly increased in peripheral blood and tumors of HCC pa- tients, and can inhibit autologous T cell proliferation.[8]

More importantly, we could also demonstrate that these cells induce CD4 CD25 FoxP3 regulatory T

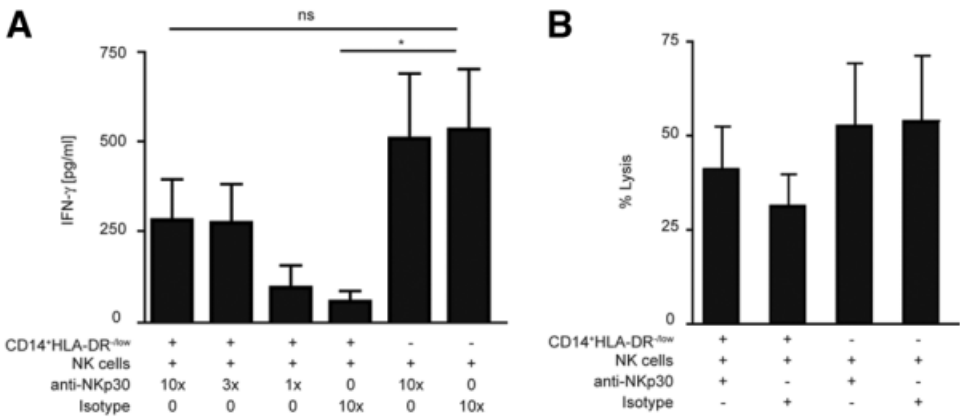

Fig. 7. MDSC-mediated suppressive mechanism is dependent on NKp30. (A) NK cells were cultured with IL-2, CD14 HLA-DR /low in the absence or presence of anti-NKp30 antibody or isotype control. IFN- secretion was measured by ELISA. Cumulative results SEM of three independent experiments are shown (*P 0.05). (B) NK cells were cultured with CD14 HLA-DR /low in the presence or absence of anti-NKp30 antibody or isotype control. K562 cells were added at a ratio of 2.5:1 (E:T) and lysis was measured. Cumulative results SEM of three independent experiments are

Here, we show a novel role for MDSCs in patients with HCC, where MDSCs contribute to the immune suppression in these patients by inhibiting NK cells, the effector cells of the innate immune system. NK cells as the effectors of the innate arm of the immune system play an important role in eliminating virus-infected cells as well as in controlling tumor cell growth.[19] It has also been shown that human NK cells kill immature autologous dendritic cells.[20] Therefore, defective NK cell function in patients with HCC not only impairs clearance of tumor cells with MHC class I down-regulation but also can lead to accu- mulation of these immature cells, which can be tolero- genic to T cells. Therefore, MDSCs play a pivotal role in influencing anti-tumor immune responses by regulating both adaptive and innate immunity.

Impairment in the function of NK cells in HCC patients has recently been described supporting our observations.[12,13] However, until now the mechanism by which NK cells in patients with HCC are impaired has not been clear. We have previously shown that MDSCs induce regulatory T cells in patients with HCC. Interestingly, it has also been shown that regulatory T cells are inhibitors of NK cell function in patients with cancer,[21] suggesting a possible additional pathway of MDSC-me- diated inhibition of NK cell function in patients with HCC.

It is important to mention that our study does not address how the cross-talk between NK cells and MDSCs contributes to HCC pathogenesis in vivo. It has been shown that NK cells are also impaired in their function in patients with liver cirrhosis.[22] We have tested MDSC-NK interactions in patients with liver cirrhosis as well as patients with hepatitis C virus (HCV). In either case, MDSCs were able to suppress NK cell function (Supporting Fig. 2). In addition, there were no differences between HCC patients with or without HCV. We suggest that MDSC-mediated NK cell impairment is a general mechanism regardless of the patient population, whether healthy donors, patients with liver cirrhosis, or patients with hepatitis. However, in our previous study, we have shown that the frequency of MDSCs is only increased in peripheral blood of patients with HCC but not in patients with liver cirrhosis or HCV infection.[8] Therefore, we suggest that the increase in frequency of MDSCs in patients with HCC is one mechanism responsible for suppression of NK cell function in these patients.

Several previous murine studies have demonstrated a tumor-dependent increase of MDSCs.[1,23] In mice, conflicting results have been described on the MDSC-NK crosstalk in mouse tumor models. In one study, CD11b Gr-1 cells activated NK cells through a STAT1-medi-

inhibitory role of these cells in mice with tumors.[24,25] However, nothing is known about the NK-MDSC crosstalk in humans and in particular in patients with HCC.

The mechanism by which MDSCs inhibit NK cell function in patients with HCC is not clear. Our previous study showed that MDSCs in these patients have high arginase activity, which is a hallmark function of MDSCs.[8] However, in this study, the inhibition of NO production as well as the blocking of arginase function did not affect the inhibitory effect of MDSCs. Blocking direct cell contact between MDSCs and NK cells, however, re- versed the inhibitory effect of MDSCs on NK cells. Inhi- bition of NK cell function by MDSCs was mainly mediated through the NK-activating receptor NKp30, one of the three natural cytotoxicity receptors on NK cells. NKp30 has been shown to be primarily responsible for the NK cell–mediated lysis of autologous immature dendritic cells.[26] Because inhibition of NK cells by MDSCs was dependent on cell contact, it suggests that MDSCs express one or more ligands for NKp30. How- ever, at this point, the ligand(s) for NKp30 on MDSCs is not known. Nonetheless, it cannot be ruled out that there are additional factors possibly acting through direct cell contact that play a role in MDSC-mediated NK cell sup- pression. It is also important to note that NKp30 is an activating receptor which here, in interaction with MDSCs, results in inhibition of NK cell function. We believe that the level of stimulation or inhibition has to do with the density of NKp30 ligand expressed on the surface of MDSCs, which remains to be identified.

Further work is required to elucidate the exact mechanism used by MDSCs in this suppression. Additional studies are also needed to see whether the NK cells can also affect the MDSCs in humans and in patients with HCC. The multifaceted role of MDSCs illustrates further the complexity of the immune-suppressive mechanisms in patients with HCC, and as such, the need to design new immune-based therapies targeting these pathways.

In summary, our study has uncovered another new mechanism of immune evasion for HCC tumors whereby MDSCs regulate the innate immunity and its effector cells, the NK cells. Impaired NK cells can affect antitumor immune responses, which contributes further to tumor escape from both innate and adaptive immune responses

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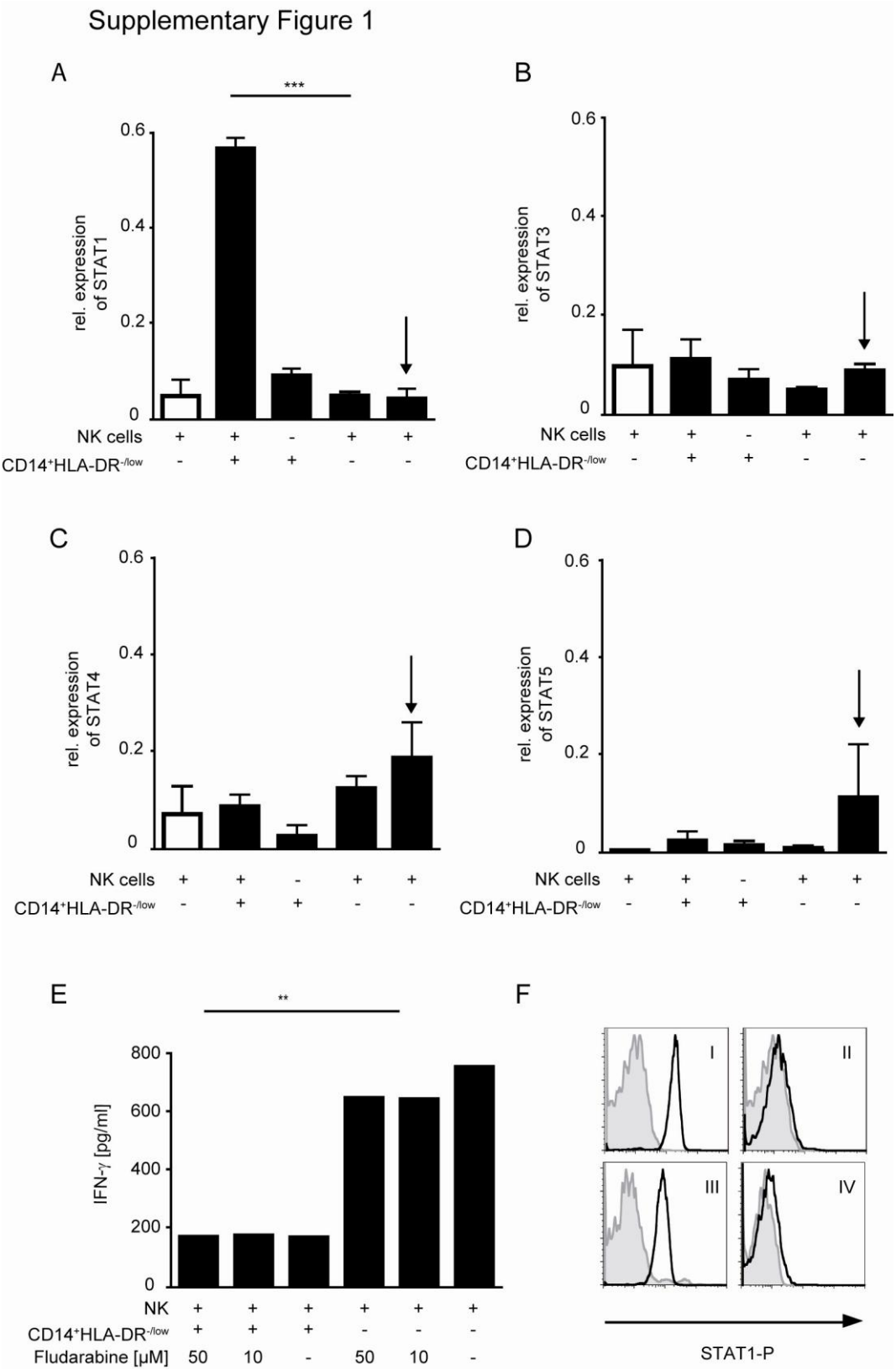

ABBILDUNG 18: FIGURE S1: STAT´S SIGNALLING PATHWAY IS NOT INVOLVED IN MDSC MEDIATED SUPPRESSION OF NK CELLS

Figure S1

STAT´s signalling pathway is not involved in MDSC mediated suppression of NK cells. (A-D) Co-culture of NK cells with $CD14^{+}HLA\text{-}DR^{-/low}$ cells results in increased STAT1 expression. Purified NK cells were stimulated with IL-2 in the presence or absence of $CD14^{+}HLA\text{-}DR^{-/low}$ cells for 6 hours. The relative expression of STAT1, STAT3, STAT4 and STAT5 was tested on either NK cells *ex vivo (*white bars), on different co-cultures as indicated and on NK cells without IL-2 (arrows) (**$p < 0.001$). Shown are cumulative results from 3 independent experiments. (E) Blocking STAT1 phosphorylation does not leads to enhanced IFN-γ secretion. Fludarabine was titrated into $CD14^{+}HLA\text{-}DR^{-/low}$: NK cells co-culture as indicated and IFN-γ secretion was measured by ELISA after 48 hours. Shown is one of two representative experiments (*$p < 0.05$). Data shown are representative of 2. (F) Phosphorylation of STAT1 occurs in $CD14^{+}HLA\text{-}DR^{-/low}$ cells. NK cells and $CD14^{+}HLA\text{-}DR^{-/low}$ cells were co-cultured (I+II) or $CD14^{+}HLA\text{-}DR^{-/low}$ cells (III) or NK cells (IV) were cultured alone for 6 hours. The STAT1 phosphorylation was analysed by intracellular FACS ex vivo (filled histograms) or after culture (black lines). Shown is one of 2 representative histograms.

Supplementary figure 2

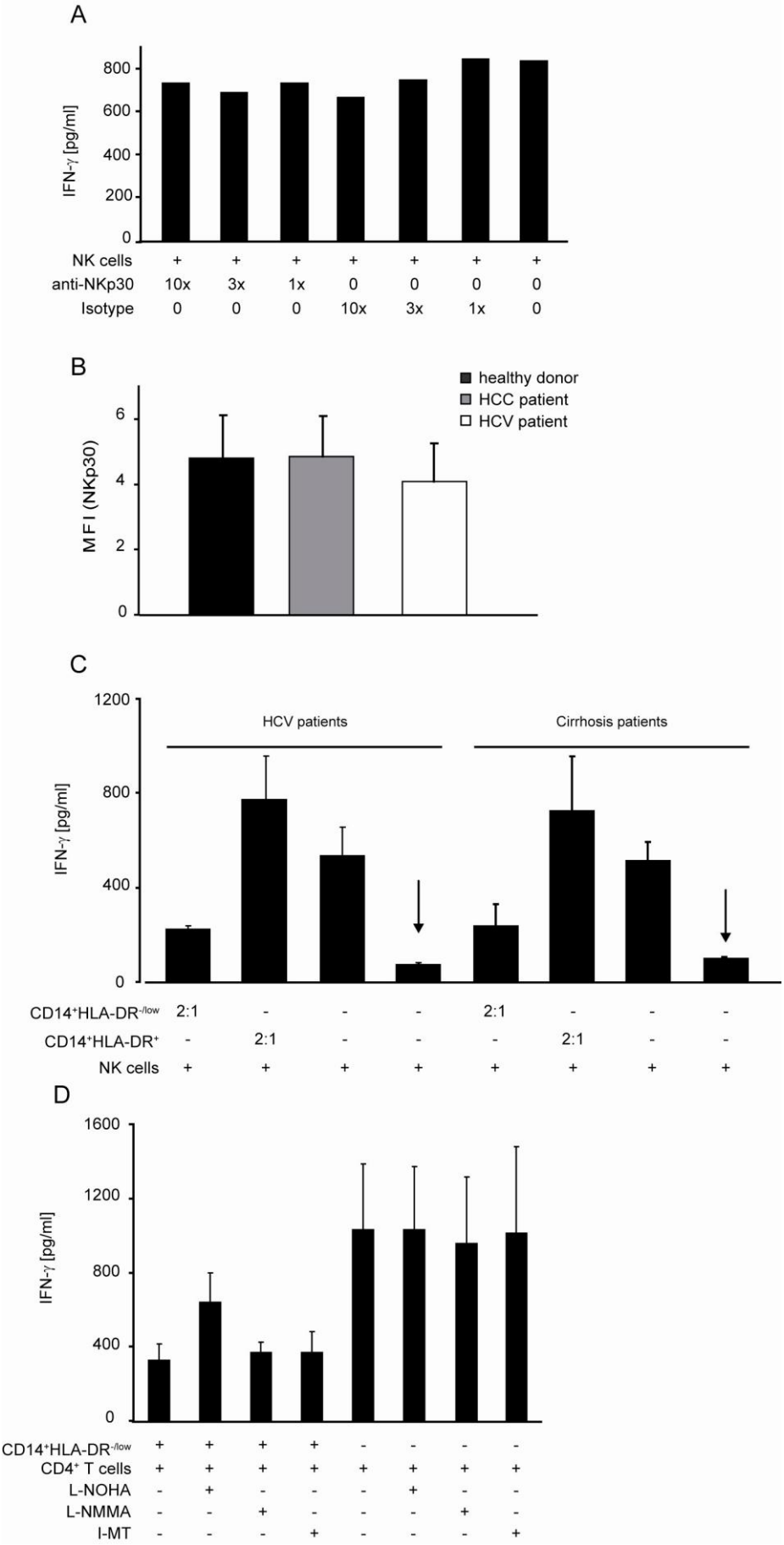

ABBILDUNG 19: FIGURE S2: CD14^{+}HLA-DR$^{-/LOW}$ CELLS SUPPRESS NK CELL FUNCTION

Figure S2

CD14^{+}HLA-DR$^{-/low}$ cells suppress NK cell function

NK cells were stimulated with IL-2 and cultured in the presence or absence of CD14^{+}HLA-DR$^{-/low}$ or DR^{+} cells as indicated. The amount of IFN-γ was determined after 48 hours by ELISA. Shown are cumulative results from 2 independent experiments. NK cells cultures without IL-2 were used as background (black arrows).

Plasticity of regulatory T cells and Th17 cells can be triggered by subsets of monocytic cells

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$CD4^+$ T cells develop, upon activation, into different T helper subtypes, depending on the stimuli in the environment. These subsets are characterized by different cytokine profiles and distinct functions. Until recently, T cells were mostly divided into Th1 and Th2 cells, depending on the cytokines they produce (Hung et al., 1998). A third subset of IL17 secreting effector T helper cells has recently discovered (Korn et al., 2009; Reiner, 2007). It has been described, that TGF-β and IL-6 are essential for the differentiation of Th17 cells (O'Garra et al., 2008). The exact function in case of tumour-immunity is controversial discussed (Bronte, 2008) but in most cases, Th17 cells seem to be important in clearing pathogens during host defense reactions and induce tissue inflammation in autoimmune disease (Horlock et al., 2009).

The participation of TGF-β in the differentiation of Th17 cells places the Th17 cells in close relationship to induced regulatory T cells (iTregs) as TGF-β can also induce the differentiation of naive T cells into $FoxP3^+$ Tregs in the peripheral compartments (Dumitriu et al., 2009; Mucida and Cheroutre, 2007). Regulatory T cells (Tregs) play an important role in the control of destructive inflammatory response (Zheng et al., 2009) as well as in the suppression of anti-tumour immune response (Audia et al., 2007; Ormandy et al., 2005; Wang and Wang, 2007). Most frequently studied Tregs are the naturally occurring population of $CD4^+CD25^+FoxP3^+$ Tregs that develops in the thymus (Hori et al., 2003). Nevertheless, T cells with regulatory function can also be generated in the periphery from the naïve T cell

pool after administration of the antigen (Coombes et al., 2005; Kretschmer et al., 2005; Thorstenson and Khoruts, 2001).

Myeloid derived suppressor cells (MDSC) were originally described as a heterogeneous population of immature cells derived from myeloid progenitors with suppressive capacity in tumour-bearing mice (Bronte et al., 2000; Bronte et al., 2001; Serafini et al., 2006a). Here these cells are mainly characterized by the co-expression of CD11b, a monocytic marker, and Gr-1, a granulocytic marker. In contrast, human MDSC are much less characterized because of a lack of specific markers. Recently MDSC has been described, which express the monocytic marker CD14 but lack the antigen-presenting molecule MCH class II (Haile et al., 2008; Hoechst et al., 2008; Rodriguez et al., 2009). These cells are not only directly suppressive on T cells and NK cells, they have also the capacity to induce $CD4^+CD25^+FoxP3^+$ regulatory T cells ex vivo (Hoechst et al., 2008; Hoechst et al., 2009).

Although subsets of DCs and macrophages has been described in intestinal microenvironment to induce immune tolerance (Bogunovic et al., 2009; Coombes et al., 2007; Denning et al., 2007; Rescigno, 2009; Varol et al., 2009). Their functions in the induction of protective immunity versus immune tolerance remain poorly understood. It was shown that $CD103^+$ DC have the capacity to induce regulatory T cells whereas $CD103^-$ DC induces inflammatory Th17 cells (Coombes et al., 2007).

Much attention has been paid to the DC subsets in mice but far less is known about cell with these properties in human.

Material and Methods

All experiments using peripheral blood were performed with freshly drawn samples. Peripheral blood was obtained from healthy volunteer donors.

Cell isolation and sorting

PBMC were isolated from freshly obtained blood by Ficoll density gradient centrifugation (Biochrom, Berlin, Germany) as described previously. $CD14^+HLA\text{-}DR^{-/low}$ or $CD14^+HLA\text{-}DR^+$ cells were isolated from pre-isolated $CD14^+$ cells using BD FACS Aria II cell sorting system (Becton Dickinson, Heidelberg, Germany). $CD4^+$ cells were isolated from CD14 depleted fraction of PBMC using Microbeads and AutoMACS separation unit (Miltenyi, Biotech, Bergisch Gladbach, Germany) according to the manufacturers´ instruction. The purity of the cells after separation was >98%.

Flow cytometry analysis

Cells were surface-labeled with antibodies for 15 min at 4°C. Samples for intracellular staining were additionally fixed and permeabilizated using Human FoxP3 Buffer Set (Becton Dickinson, Heidelberg, Germany) according to manufactures protocol. FACS acquisition was performed on LSR-II and analyzed with FlowJo software. FACS-analysis was done using following antibodies: CD4-Vioblue (Miltenyi, Clone VIT4)) CD14-PasificOrange (Clone TüK4, Invitrogen), FoxP3-AlexaFlour648 (Clone 259D/C7, Becton Dickinson), IL-17-PE (Clone SCPL1362, Becton Dickinson), CD103-FITC (Clone Ber-ACT8, Becton Dickinson D), TGF-β-PE (Clone 9016, R&D). Isotype-matched antibodies were used as indicated.

Cytokine Bead Array

Cytokines were tested on cell culture supernatant at different time points as indicated. This was done using Cytokine Bead Array (CBA) (Bender MedSystems, Vienna, Austria).

Induction of T helper cells subtypes

$CD4^+$ T cells were isolated as described and stimulated with anti-CD3/CD28 in the presence of MDSC for 3 days. For Th17 cells, stimulated $CD4^+$ T cells were co-cultured with monocytes for 6 days.

Isolation of Th17 cells

$CD4^+$ T cells were co-cultured with autologous monocytes for 6 days. Towards it, cells were stimulated with Cytostim and Th17 cells were isolated using IL-17-Secretion Assay-Detection Kit (Miltenyi) according to manufactures protocol. The process was repeated once to enhance purity.

Determination of mRNA expression by Quantitative Polymerase Chain Reaction

T cells and $CD14^+$ cells were purified as described. RNA was isolated using RNeasy Micro Kit (Qiagen, Hilden, Germany). Complementary DNA synthesis was done with iScript Kit (Bio-Rad, München, Germany) Quantitative polymerase chain reaction was performed using the following primers (300 nmol/l each): IL-17RA forward, 5´-GAG CAC ATG CAC CAC ATA CC-3´; IL-17RA reverse,5´-CGG AAT TGG TTC TGG AGT GT-3´; GATA-3 forward, 5´-GAA CCG GCC CCT CAT TAA G-3´; GATA-4, reverse, 5´-ATT TTT CGG TTT CTG GTC TGG A-3´; T-Bet forward, 5´-GAT CAT CAC CAA GCA GGG ACG-3´;T-Bet reverse,5´-TCC ACA CTG CAC CCA CTT GC-3´; RORc forward, 5´-CAT TTT CTG CCT CTG CCT TC-3´;RORc reverse, 5´-TCT TGG CCT TCA TTG TAC CC-3´; IL-17A forward, 5´-AAT TCT GAG GAC AAG AAC TTC CC-3´; IL-17A reverse, 5´-ATA GTC TAA CTG CTT TGG GGA GTG-3´; TGF-β1 forward, 5´-GGG ACT ATC CAC CTG CAA GA-3´; TGF-β1 reverse, 5´-CCT CCT TTG

GCG TAG TAG TCG-3´; IL-10 forward; 5´-CAA AAC CAA ACC ACA AGA CAG ACT T-3´; IL-10 reverse, 5´-GAG GAC CAG GCA ACA GAG CA-3´; Cyclophilin A forward, 5´-ATG CTC AAC CCC ACC GTG T-3´; Cyclophilin A reverse, 5´-TCT GCT GTC TTT GGG ACC TTG TC-3´; RDH12 forward, 5´TCT TGA CCC TTC TGG GGA ATG-3´; RHD12 reverse, 5´-CAA TTT CTC GGG CTC TCT G-3´; RHD16 forward, 5´-AGC TGA GAA ACA GGG ACC AA-3´; RHD16 reverse, 5´-TGG CCC AGA ATT AAC ACA CA-3´; RHD5 forward, 5´-GGG GCT ACT GTG GTC TCC AAA-3´; RHD5 reverse, 5´-CTG CAG GGT TTT CTC CAG AC-3´; RHD8 forward, 5´-CTG TCT CTG GGA AAG CAA GG-3´; RHD8 reverse, 5´-CTG GTG TAT GCA TGG AGG TG-3´; RDH11 forward, 5´- GAG ATG GAT GTG GTG GCT TT-3´; RDH11 reverse, 5´- ATT ACG AGC TTG GGC AGA GAA TT-3´; ALDH8A1 forward, 5´-AGG CTC CTC CCA GGT TAT GT-3´; ALDH8A1 reverse, 5´-CTC AAG TGA TCC CAA CAC CT-3´;

Statistical Methods

Statistical analysis was performed with Graphpad Prism 5 (Graphpad Software). Data are shown as the mean ± SEM, unless otherwise indicated. Two-tailed paired t test was used for comparison between matched paired groups.

RESULTS:

Subsets of CD14$^+$ monocytes induces regulatory T cells or IL17 secreting T cells

We have previously shown that CD14$^+$HLA-DR$^{-/low}$ cells from human peripheral blood induce CD4$^+$CD25$^+$Foxp3 regulatory T cells when cultured with autologous CD3/CD28 stimulated CD4$^+$ T cells. In contrast, CD14$^+$HLA-DR$^+$ monocytes co-cultured with autologous CD4$^+$ T cells, did not generate significant levels of Foxp3$^+$ regulatory T cells, but induced a high number of IL-17 secreting CD4$^+$ T cells (Figure 1A). We further characterized the induced Foxp3$^+$regulatory and the IL-17 secreting CD4$^+$ T cells for expression of specific markers by real time PCR. The induced regulatory T cells by MDSC expressed Foxp3 and secrete TGF-β, whereas CD4$^+$ T cells cultured with monocytes expressed the Th17 specific transcription factor, ROR-c and IL-17 (Figure 1B).

MDSC express genes involved in RA metabolism and express membrane bound TGF-β

A microarray analysis of CD14$^+$HLA-DR$^{-/low}$ MDSC showed an up regulation of several genes involved in the metabolic pathway of retinoic acid (Data not shown). To confirm these results,

freshly sorted $CD14^+HLA\text{-}DR^{-/low}$ and $CD14^+HLA\text{-}DR^+$ cells were tested for the expression of multiple genes identified through microarray by real time PCR. Human $CD14^+HLA\text{-}DR^{-/low}$ MDSC had a higher expression level of all the genes tested as compared to $CD14^+HLA\text{-}DR^+$ monocytes, in particular *aldh8* and *dhrs9* genes (Figure 2a). Retinoic acid (RA) has been shown to enhance TGF-β dependent induction of regulatory T cells but suppress the development of IL17 secreting T cells (Mucida and Cheroutre, 2007).

Since we did not detect any TGF-β in co-cultures of MDSC or monocytes with T cells, we tested the two cell populations for membrane bound TGF-β. Both $CD14^+HLA\text{-}DR^{-/low}$ MDSC and monocytes expressed membrane bound TGF-β, with MDSC up-regulating it over time when co-cultured with autologous T cells (Figure 2B).

Since cytokines may be involved in the induction of regulatory as well as Th17 T cells, the secretion of different cytokines was analyzed by cytokine bead array (CBA). MDSC did not secrete any of the inflammatory cytokines tested; IL-6, IL-1β, IL-17 and TNF-α whether alone or when co-cultured with autologous $CD4^+$ T cells. This is in contrast to monocytes that secreted significant amounts of all of these cytokines either alone or when cultured with autologous $CD4^+$ T cells (Figure 2 C-F).

Induction of regulatory T cells and Th17 T cells by $HLA\text{-}DR^+$ monocytes or $HLA\text{-}DR^-$ MDSC is TGF-β dependent and effected by RA

To understand the mechanisms behind the induction of $Foxp3^+$ Treg cells by MDSC and Th17 cells by monocytes, we investigated the role of retinoic acid (a vitamin A metabolite) in this process. To this end, we used LE540, a synthetic retinoic acid receptor antagonist, AM580, a retinoic acid agonist, as well as all trans-RA (atRA) and added them to co-cultures of $CD14^+HLA\text{-}DR^{-/low}$ MDSC and $CD14^+HLA\text{-}DR^+$ monocytes with $CD4^+$ T cells. Addition of increasing concentrations of atRA, enhanced induction of $Foxp3^+$regulatory T cells by MDSC more than 30% consistent with the role of retinoic acid in Treg induction (Figures 3A & E). atRA agonist, AM 580 also enhanced the induction of $Foxp3^+$ regulatory T cells by more than 30% (Figures B & F). Consistent with these results, addition of the RAR inhibitor, LE540 reduced the frequency of $Foxp3^+$Treg cells induced by MDSC more than 40% (Figures 3 C & G).

A7980 did not lead to an enhanced expression of FoxP3 indicating that RAR gamma is not involved in the induction of regulatory T cells (Figures D & H).

To assess the role of TGF-β in induction of regulatory T cells, we added TGF-β blocking antibody to MDSC- T cell co-cultures. Blocking of TGF-β led to a reduction in frequency of $Foxp3^+$ regulatory T cells by almost 40% in a dose dependent manner (Figures 4A & B).

Combination of anti-TGF-β and LE540 has a synergistic effect as well as addition of IL1-β, IL-6 and TNF-α did not affect the induction of Tregs by MDSC (data not shown).

Induction of Th17 cells is cytokine dependent and can be blocked by RA

All the experiments done so far have looked at induction of regulatory T cells after three days of co-culture. However, we observed that a longer culture time is required to get the optimal Th17 induction by monocytes. We therefore cultured monocytes for 6 days with autologous $CD4^+$ T cells to analyze the induction of Th17 cells in the presence and absence of atRA, AM580 and LE540.

Addition of atRA had also a significant effect on induction of TH17 cells by monocytes, with a more than 60% reduction in the IL-17 secreting T cells consistent with effect of RA on suppression of IL-17 expression (Figures 5A & E). As expected, LE540 had no effect on Th17 induction by monocytes (Figures 5B & F). AM580 also resulted in reduction in frequency of IL-17 secreting cells comparable to atRA (Figures 5C & G). Similar to the induction of FoxP3, A7980 also has no effect on induction of IL-17 secretion by monocytes (Figures 5D & H)

The ability of $CD14^+HLA\text{-}DR^+$ monocytes to induce IL-17 producing $CD4^+$ T cells was also dependent on TGF-β since blocking of TGF-β reduced the percentage of IL-17 secreting $CD4^+$ T cells by 50% in a dose dependent manner (Figure 6A &E).

Since we found significant concentrations of IL1-b, IL-6 and TNF-α in the cultures of monocytes alone or with T cells, blocking antibodies against these cytokines were used to analyze their role in Th17 induction.

Blocking the IL-6 receptor alpha in the co-culture of monocytes and T cells led to a reduction of IL-17 secreting cells by 40% (Figures 6B & F).

Blocking of IL-1β and TNF-α had no effect on Th17 induction by monocytes. (Figures 6C, D, G, H)

$CD14^+HLA\text{-}DR^{-/low}$ MDSC convert Th17 into regulatory T cells

Our data so far indicates that MDSC and monocytes according to their stage of development and phenotype can differently regulate naïve T cell responses. Next we asked whether these cells affect already differentiated Th17 or iTreg cells as well thereby orchestrating their plasticity.

We performed a series of experiments where $CD14^+HLA\text{-}DR^+$ monocytes were co-cultured with autologous $CD4^+$ T cells for 7 days. After 7 days, Th17 cells were purified twice to get a

high purity of at least 80%. These cells were then cultured with autologous $CD14^{+}HLA\text{-}DR^{-/low}$ cells and tested for Foxp3 and IL-17 expression.

As shown in Figure 7, the frequency of IL-17 secreting $CD4^{+}$ T cells declines as early as 12 hours after co-culture and continue to decrease by almost 40% after 72 hours (Figure 7A &D,C). At this time point (12 hours), double positive $IL\text{-}17^{+}Foxp3^{+}$ cells are detected which increase by almost 3 fold after 72 hours. In addition, there is a gradual increase in frequency of $Foxp3^{+}$ $CD4^{+}$ T cells after 48 hours which doubled after 72 hours. After 48 hours show a decrease of $FoxP3^{+}IL17^{+}$ cells, while the frequency of the iTregs continues to increase to 8% (Figure 7 D & E).

To see if the Foxp3 cells can also be induced from the double negative cells, these cells were sorted and cultured with MDSC for 72 hours. The double negative cells do not induce $FoxP3^{+}$ regulatory T cells and their frequency remains relatively constant as shown in Figure 7 B. It was also tested if Th17 cells become $FoxP3^{+}$ if they were cultured with $CD14^{+}HLA\text{-}DR^{+}$ cells or alone. In none of these experimental approaches showed an increase of iTregs. Therefore, although it is possible that some Th17 negative cells can also become regulatory T cells, the majority of Th17 cells induced by monocytes go through a double positive stage and become $FoxP3^{+}$ cells upon culturing with $CD14^{+}HLA\text{-}DR^{-/low}$ MDSC.

To investigate the different development stages of Th17 cells to iTregs, purified Th17 cells were incubated with $CD14^{+}HLA\text{-}DR^{-/low}$ cells and FACS-sorted for IL17 and FoxP3 on day 2 and analyzed by real time PCR (Figure 7F).

As shown in Figure 7G, subsequent qPCR indicate that the up-regulation of FoxP3 (R2) is the first step in the conversion of Th17 cells to iTregs, followed by the down regulation of RORc (R3). Afterwards IL17AR (R3-4) and IL17A (R4) decreases while IL10 (R3) and TGF-β (R5) is up-regulated.

Blocking RA or TGF-β reduces conversion of Th17 cells

To ensure, that atRA and TGF-β are also important in the conversion of Th17 cells into iTregs, we blocked both pathways using the atRA antagonist LE540 or an blocking antibody. Adding LE540 leads to a decrease of $FoxP3^{+}$ as well as $FoxP3^{+}IL17^{+}$ cells. On the other hand, adding atRA affects a slide increase of the double positive cells and a decrease of the Th17 cells. However, the effect was not statistically significant. Blocking TGF-β leads to a significant reduction of $FoxP3^{+}IL17^{+}$ cells. Also, the frequency of double negative cells is increased, suggesting that TGF-β is also important for the stability of Th17 cells.

Discussion

Here we have shown that different subtypes of monocytic cells are able induce regulatory T cells or Th17 cells. Whereas MDSC can induce Treg differentiation with anti-inflammatory properties, “classical” monocytes induce Th17 cells with an pro-inflammatory immune response (Kuang et al., 2010).

In mice two different populations of gut lamina propria DCs are described (Bogunovic et al., 2009; Varol et al., 2009), which differ in their origin, as well as in their phenotype and function. It is found that $CD103^{+}CX_3CR1^{-}$ lpDCs are developed from Macrophage-DC precursors, whereas $Ly6C^{hi}$ monocytes give rise to $CD103^{-}$ CX_3CR1^{+} DCs (Bogunovic et al., 2009; Varol et al., 2009). The function of these cells seems to be different as well. Namely, It has been reported (Coombes et al., 2007) that mucosal $CD103^{+}$ DCs induce $FoxP3^{+}$ Treg cells, whereas (Atarashi et al., 2008) $CD103^{-}$ CX_3CR1^{+} DCs were able to drive Th-17 cell differentiation in the presence of bacteria-derived ATP. At the same time Varol et al (Varol et al., 2009) found severe intestinal inflammation in mice reconstituted with $CD103^{-}$ CX_3CR1^{+} DCs indicating on the pivotal role of these 2 subpopulations in the regulation of Treg as well as Th17 cell mediated immune responses. Another study described lamina propria macrophages and DCs to be responsible for different regulatory and IL-17 producing T cell generation (Denning et al., 2007). In this study Treg development was RA IL-10 and TGF-β dependent.

In our study we found that cells with different myeloid origin isolated from human PBMCs posses the ability to modulate $CD4^{+}$ T cell developmental program. $HLA\text{-}DR^{-/low}$ MDSCs are able to drive $FoxP3^{+}$ T cell induction, whereas $HLA\text{-}DR^{+}$ monocytes enhance Th-17 cell generation. These two populations of myeloid cells are showing similarities as well as differences on genetic level. Exactly these characteristics are realized in their potential to trigger different developmental programs. Both are expressing membrane bound TGF-β, known to be important for Th-17 as well as $FoxP3^{+}$ iTreg differentiation. At the same time, already *ex vivo*, MDSCs have unregulated genes of RA metabolism, but they are missing inflammatory cytokines (IL-6, IL-1β), which in combination with mTGF-β creates environment beneficial for $FoxP3^{+}$ iTreg induction. Monocytes, in opposite to MDSCs lack RA metabolism genes but secrete IL-6, IL-1β and define Th-17 pool differentiation.

These findings suggest the complexity of the induced immune response according to the subset or the stage of differentiation of monocytes.

Several studies have described intestinal DCs and Macrophages and have detailed the function of these cells in the induction of tolerance and immunity in mice (Denning et al., 2007; Kelsall and Rescigno, 2004). However, so far they are no reports of human monocytes with similar function. We have demonstrated here that a subset of human monocytes have an immunosuppressive phenotype by retinoic acid. A different subset of monocytes induced pro-

inflammatory Th17 cells by secreting inflammatory cytokines as IL6. For both effects TGF-β was essential, that was expressed on the surface of the CD14 positive cells (Wang et al., 2009).

MDSCs and monocytes with T cell modulatory characteristics resemble to the subpopulations of lpDCs described in mice. The observation that they are present in human peripheral blood can indicate that such influence on the fate of T cells might be rather general mechanism and not characteristic solely for the intestinal mucosa. In line with this observation we found MDSCs infiltration into the tumor sites in HCC patients, whereas ascitic fluid is enriched in monocytes. Exact mechanism and biologic relevance of this phenomenon still need to be elucidated although we think that depending on the prevalence of different myeloid cells in various environments one can have different type of immune response at the same time.

Thus, the balance between retinoic acid and IL-6 instructs the lineage specification of anti- or pro-inflammatory T cells activated in the presence of TGF-β.

Opposed to HLA-DR positive monocytes, the MDSC failed to secrete any inflammatory cytokines cultured alone or in combination with $CD4^+$ T cells. This is in agreement with human intestinal (Smythies et al., 2005) and murine lamina propia macrophages (Coombes et al., 2007; Denning et al., 2007) which do not secrete pro-inflammatory cytokines when stimulated with TLR ligands. Differently to these studies, MDSC do not secrete any IL-10.

It has been shown, that mucosal DC mediates the induction of CD103 via TGF-b (Annacker et al., 2005; Hadley et al., 1997) in murine models. It has also demonstrated that the expression of CD103 on DC is required for the induction of CD4+CD25+ regulatory T cells (Coombes et al., 2007; Jaensson et al., 2008). However, neither iTregs nor MDSC showed any expression of CD103 in our model.

The fact that the differentiation of both induced Tregs and Th17 cells requires TGF-β provided the first evidence, that the development of these subsets might be linked (Bettelli et al., 2006; Veldhoen et al., 2006). More recently, direct interactions between the specific transcription factors of these lineages have been described (Ziegler and Buckner, 2009), showing a plasticity that allow iTregs to acquire functions of Th17 cells cultured under Th17 conditions (Locksley, 2009; Zhou et al., 2009). However, we were not able to induce IL-17 production in iTregs since these cells were sensitive for culture condition and we were not able to isolate iTregs satisfactory because of missing specific markers.

Another important finding we think is that MDSCs can change initial, Th17 developmental program triggered in $CD4^+$ T cells and lead to differentiation of Th17 cells into $FoxP3^+$ iTregs.

This might be of particular importance to respond ‘quick and correctly’ to the environments where immune response is triggered.

Which cell from a pool of $IL17^+$ T cells converts from Th17 into $FoxP3^+$ regulatory T cells in our experimental system is not known. It might be that $IL\text{-}17^+$ T cells still contain ‘intermediate’ cells with double differentiation potential which under the influence of changes in ‘differentiation microenvironment’ (in this case Monocytes vs MDSCs) will up regulate FoxP3 and switch towards iTreg development. Another possibility is that even terminally differentiated Th17 cells might have potential to re-differentiate into other subpopulation of the cells in this case into $FoxP3^+$ or $FoxP3^+IL17^+$ cells.

However, adding IL6, IL1β and TNF-α to the co-culture of MDSC and T cells do not repress the induction of FoxP3 expression which could occur by an inhibition of the signal-transduction by RA (Hill et al., 2008).

In addition, our data suggest that MDSC can counteract the ability of Monocytes to induce the differentiation of IL17 producing T cells by induction of FoxP3 in the TH17 cells.

Through the incubation of Th17 cells with MDSC, FoxP3 is detectable within the first 12 hours. Interestingly, this happened primarily in the $IL17^+$ cells. Only after 24 hours, single $FoxP3^+$ cells are detectable. Since it comes firstly to an expression of FoxP3, and then adjusted to each other the Th17 specific properties are down regulated, it seems reasonable to conjecture that the development of iTregs from Th17 cells pass through a double positive phase.

However, not all th17 cells are converted into iTregs. One possibility is, that not enough MDSC were added to the culture or that the MDSC mature during culturing (data not shown). Another option is that only a subtype of Th17 cells can be converted into regulatory T cells. This is a fact that still needs to be investigated.

Another open question is the route through which the switch of Th-17 towards $FoxP3^+$ is occurring. Are IL-17 single positive cells going through double positive stage and later turn into $FoxP3^+$ cells or do they first become double negative and only after this start to transform into Treg cells.

Several reports have shown the potential of IL17 producing cells to develop into $IFN\text{-}\gamma^+$ Th1 cells and to drive pathological processes, although the possibility to raise $FoxP3^+$ Tregs was not reported. It seems that stability of Th17 differentiation program as well as of $IL17^+$cells widely depends on the microenvironment and timing of response. Depending on cell interactions occurring or other unknown factors influencing naïve or committed Th17 lymphocytes

they might show either Th1 inflammatory character or can be accompanied by more regulatory, anti-inflammatory components of the immune response. This might be of particular importance in the rapid and adequate switch of type of reaction to the specified immune trigger as well as to resolved immune response.

The conflicting effect of RA and IL6 in TGF-β- dependent generation of iTregs and TH17 cells might be a self-controlling / correcting mechanism to regulate the anti- and pro-inflammatory immunity.

In our study we characterized 2 cell populations with different myeloid origin derived from human PBMCs. Their genetic/phenotypic characteristics as well as functional difference resembles to the populations already described in mice, although in our case we found these two cells in PBMCs and they were showing different genotype/phenotype already ex vivo. We think that control of immune response shown at the mucosal surfaces might be characteristic not for intestine only but can be rather general phenomenon and act in different compartments.

Alternatively cells found in our study might further differentiate into different DC subtypes. Although directly *ex vivo* these cells are already present in blood, there might be cells with both origins having balancing effect on the immune response generation.

Vitamin A deficiency causes immune dysfunction and increased mortality in children (Sommer, 1993). In murine models it has been shown, that vitamin A metabolite in combination with TGF-β is important to induce tolerance and that a deficiency of RA are associated with an imbalance of TGF-β induced Tregs and Th17 cells in vivo (Mucida et al., 2007).

These findings suggest new approaches for the treatment of immune disorders in which the imbalance mediated by TGF-β is involved.

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Figure 1

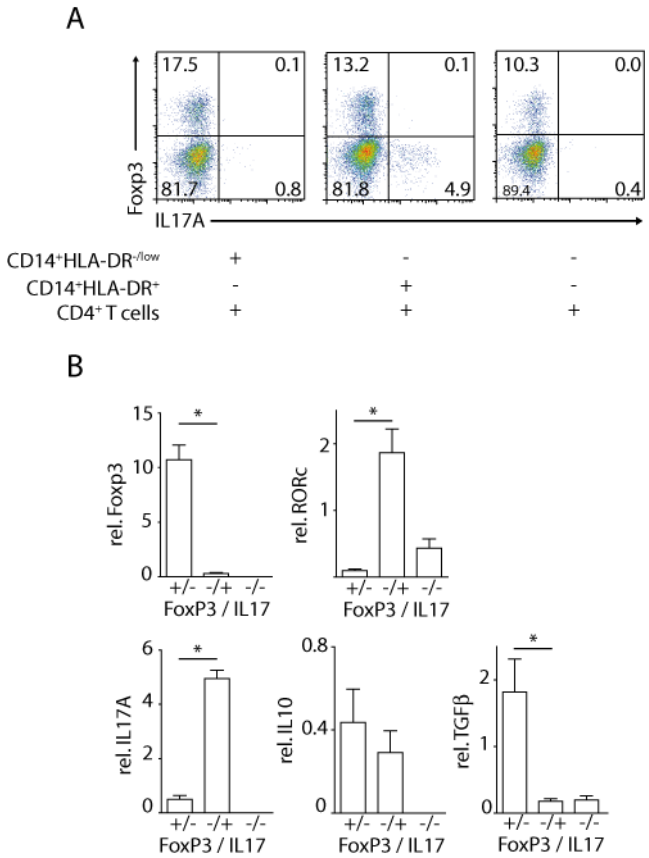

ABBILDUNG 20: FIGURE 1: INDUCTION OF TREG / TH17 T CELLS BY MDSC / MONOCYTES

Figure 1: Induction of Treg / TH17 T cells by MDSC / Monocytes

(A) $CD4^+$ T cells were stimulated with anti CD3/CD28 beads cultured in the presence or absence of $CD14^+HLA\text{-}DR^{-/low}$ or $CD14^+HLA\text{-}DR^+$ cells. ICS for IL17 and FoxP3 was performed after 3 days gating $CD4^+$ cells. Shown dotplot is representative for more than 5 independent experiments. (B) Different T helper subtypes from the co-culture were isolated using FACS-Sort. qPCR analysis of FoxP3, RORc and IL17A. Expression was set relative to Cyclophilin A mRNA. Shown are cumulative results of 2 independent experiments. *P < 0.05.

Figure 2

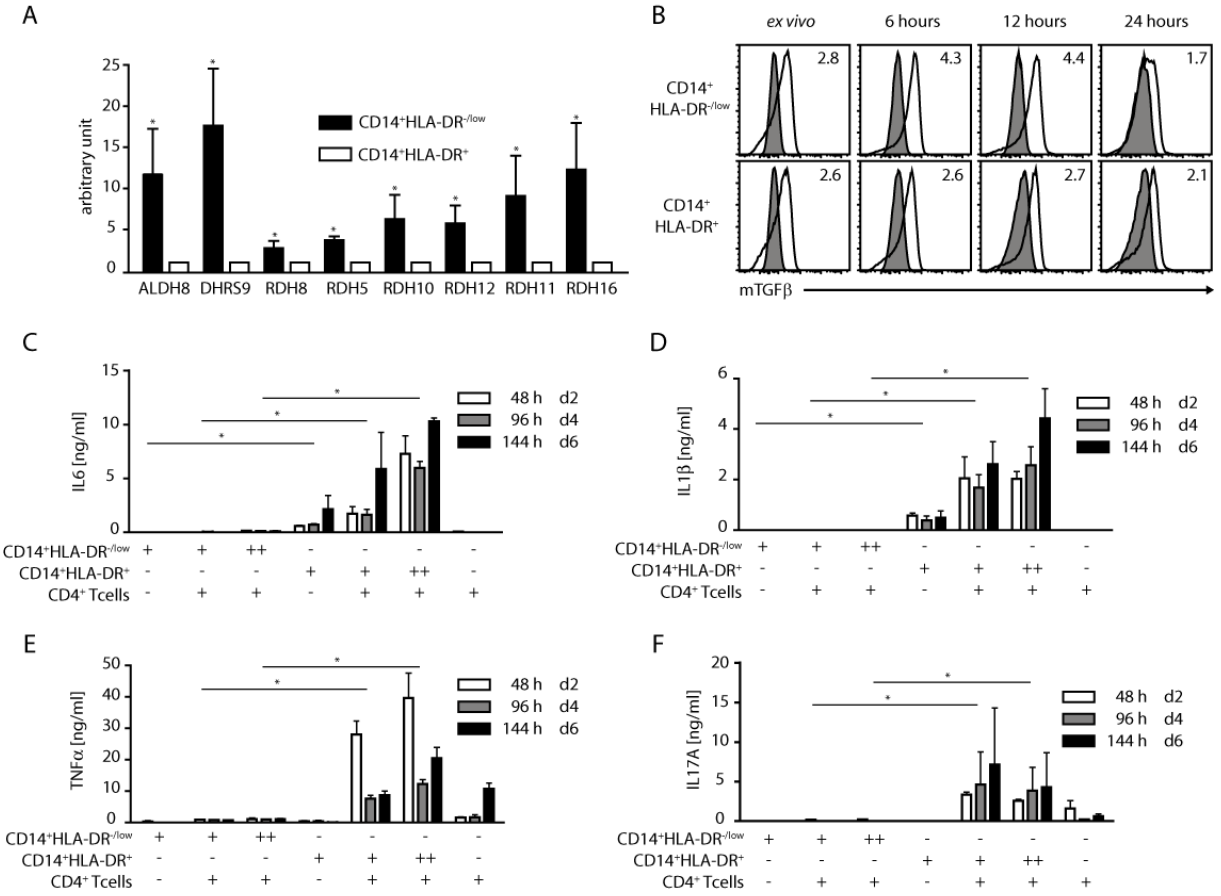

ABBILDUNG 21: FIGURE 2: MDSCS EXPRESS RA GENES, MEMBRANE BOUND TGF-B BUT

Figure 2:

MDSCs express RA genes, membrane bound TGF-β but do not secrete inflammatory cytokines

(A) MDSC expresses genes involved in retinoic acid synthesis. $CD14^+HLA\text{-}DR^{-/low}$ and $CD14^+HLA\text{-}DR^+$ cells were purified from freshly obtained blood. Gene expression was assayed by qPCR and normalized relative to expression of Cyclophilin. Data shown are cumulative results from at least 4 independent experiments. (B) MDSC express membrane bound TGF-β. $CD14^+HLA\text{-}DR^{-/low}$ and $CD14^+HLA\text{-}DR^+$ cells were purified and co-cultured with autologous $CD4^+$ T cells. Cells were stained for membrane bound Transforming growth factor β (mTGF-β) (black line) or Isotype control (filled histogram) gated on $CD14^+$ cells. Data shown are representative for two independent experiments. (C) MDSC does not secrete inflammatory cytokines. $CD14^+HLA\text{-}DR^{-/low}$ $CD14^+HLA\text{-}DR^+$ cells were cultured in the presence or absence of autologous $CD4^+$ T cells at different ratios. Cytokines were tested at different time points using cytokine-bead-array. $CD4^+$ T cells alone were used as controls. Shown are cumulative results from two independent experiments. *P < 0.05.

Figure 3

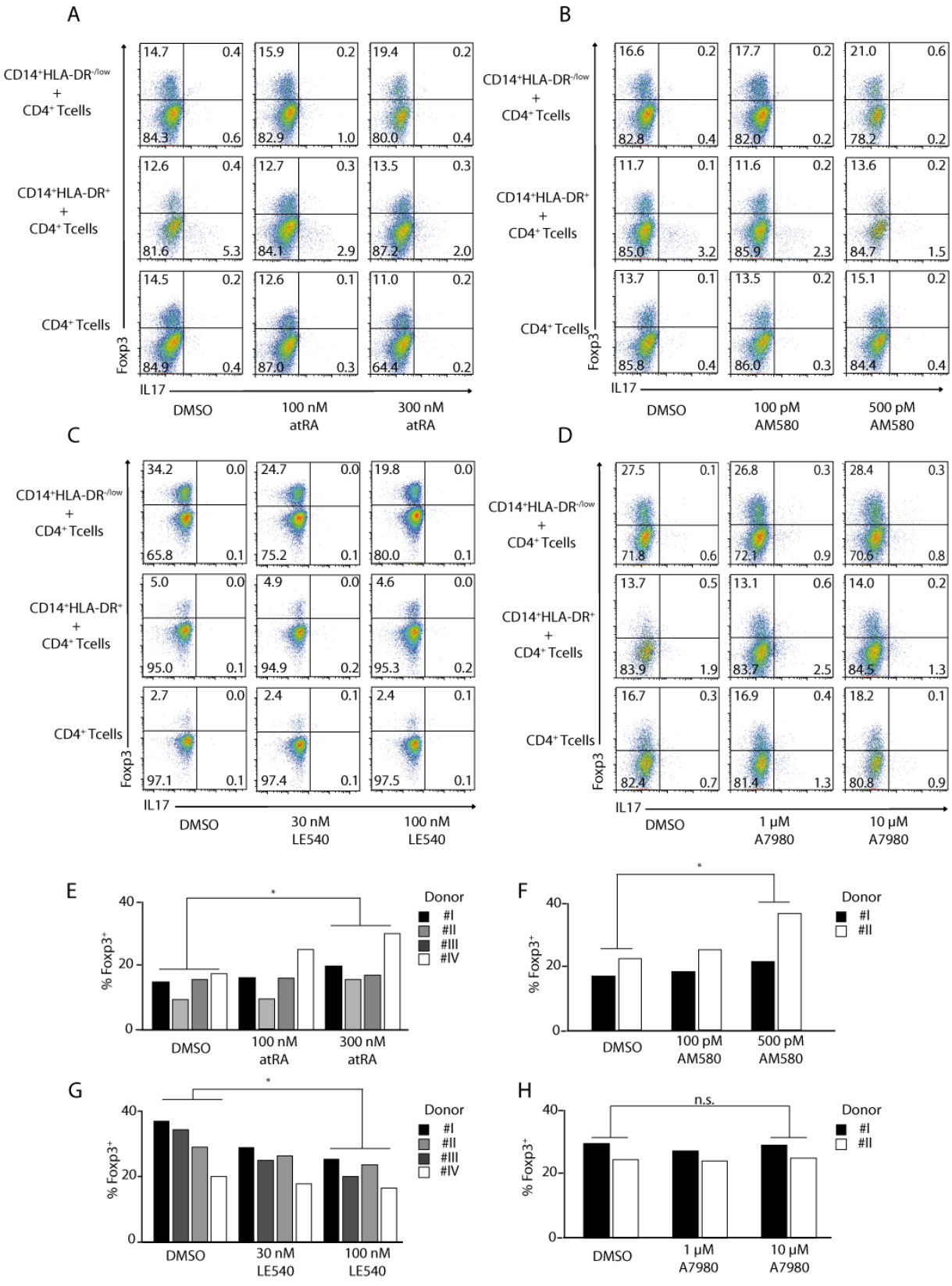

ABBILDUNG 22: FIGURE 3: INDUCTION OF REGULATORY T CELLS BY MDSC IS RA DEPENDENT.

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Figure 3:

Induction of regulatory T cells by MDSC is RA dependent.

(A-H) $CD4^+$ T cells were stimulated with anti-CD3/CD28 coated beads and co-cultured in the absence or presence of $CD14^+HLA\text{-}DR^-$ or $CD14^+HLA\text{-}DR^+$ cells. As indicated atRA (A & E), AM580 (B & F), LE540 (C & G) or A7980 (D & H) were titrated in. ICS for IL-17 and FoxP3 was performed after 3 days gating on $CD4^+$ T cells. Shown are representative dotplots from four (A & C) or two (B & D) independent experiments. (E-H) Results from single experiments were plotted as bars gating on $CD4^+$ T cells. *P < 0.05, n.s. : not significant.

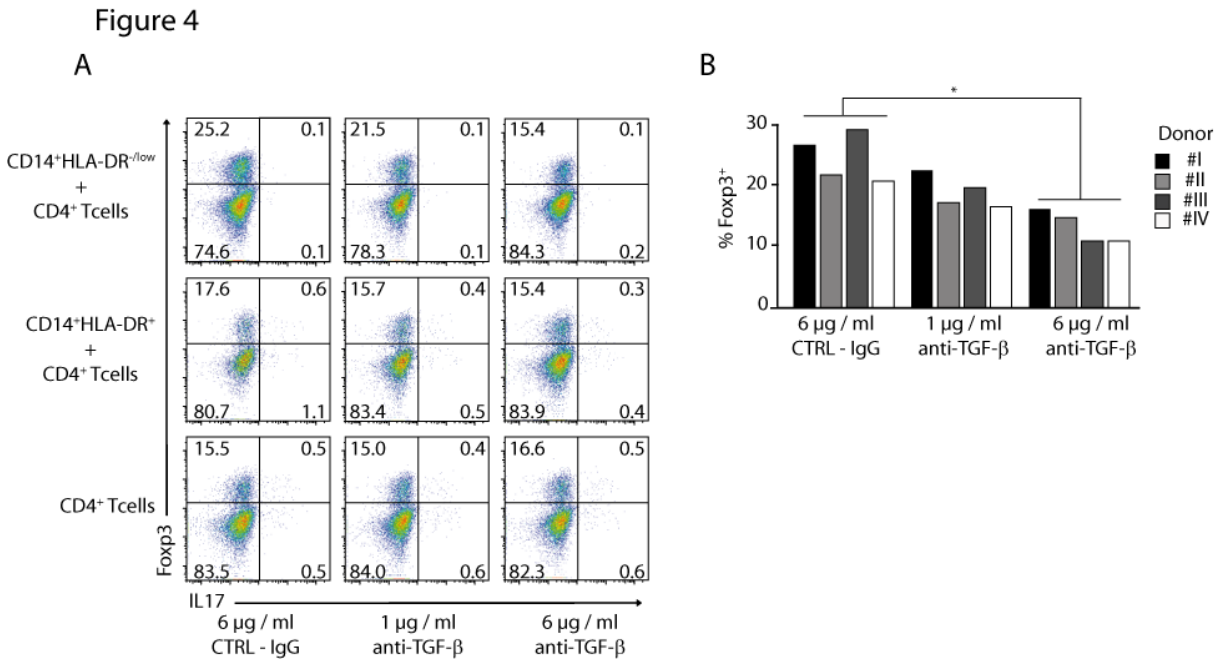

ABBILDUNG 23: FIGURE 4: INDUCTION OF FOXP3 EXPRESSION IS TGF-B DEPENDENT.

Figure 4

Induction of FoxP3 expression is TGF-β dependent.

(A & B) $CD4^+$ T cells were stimulated with anti-CD3/CD28 beads and co-cultured in the absence or presence of MDSC or Mono. Anti-TGF-β was added in different concentration as indicated. The expression of FoxP3 and IL-17 was performed by ICS on day 3 gating on $CD4^+$ T cells. Shown are representative dotplots for 4 independent experiments (A) or results for single donors were plotted as bars gating on $CD4^+$ T cells (B). *P < 0.05.

Figure 5

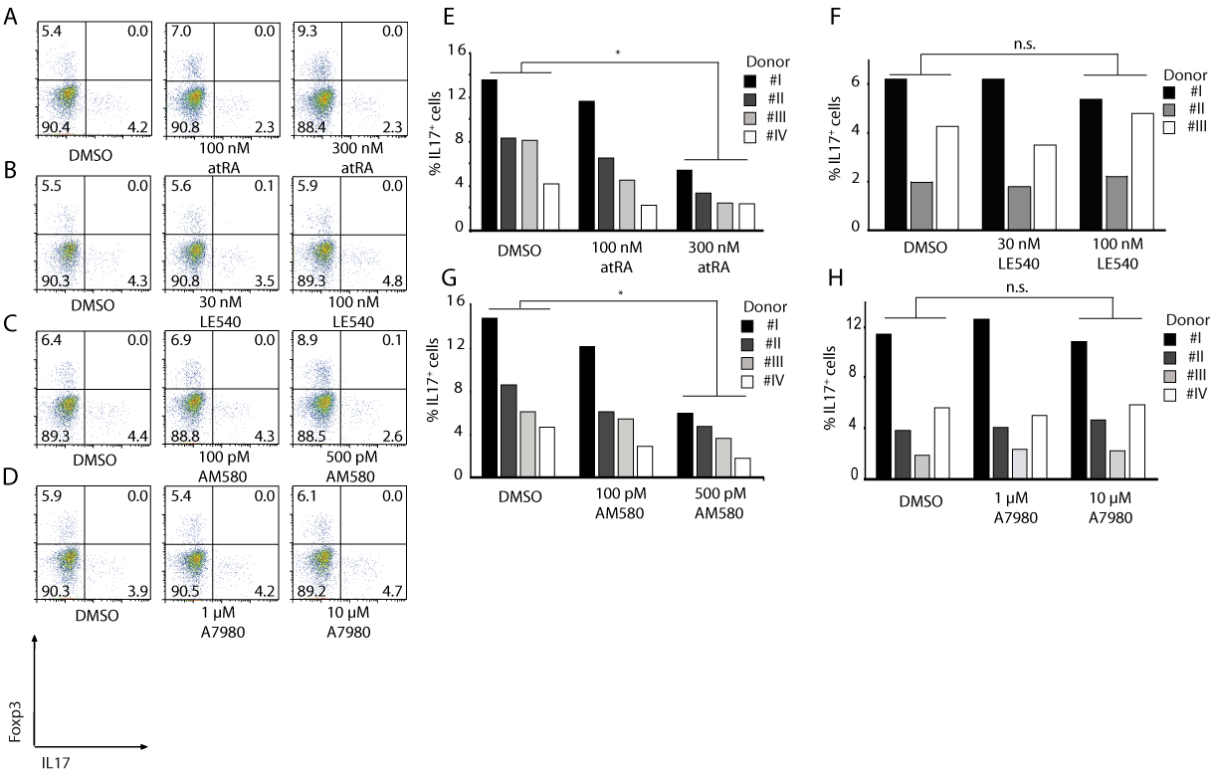

ABBILDUNG 24: FIGURE 5: INDUCTION OF TH17 T CELLS CAN BE BLOCKED BY RA.

Figure 5

Induction of Th17 T cells can be blocked by RA.

CD3/CD28 stimulated $CD4^+$ T cells were co-cultured with $CD14^+HLA\text{-}DR^+$ monocytes for 6 days. As indicated atRA (A & E), LE540 (B & F), AM580 (C & G) or A7980 (D & H) was added to the culture. Shown are representative dotplos for FoxP3 and IL17 gating on $CD4^+$ T cells (A-D) or single results are shown in bars (E-H). *P < 0.05, n.s. : not significant.

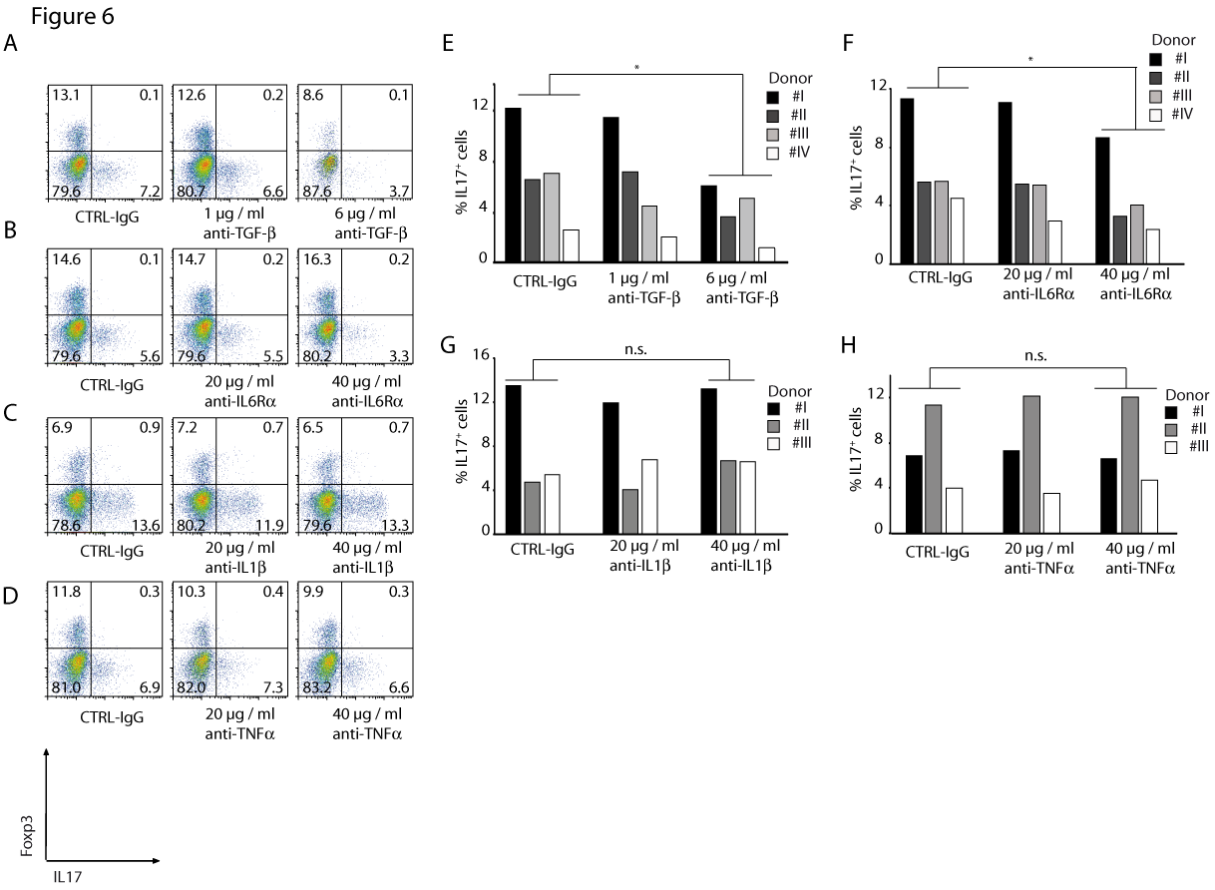

ABBILDUNG 25: FIGURE 6: INDUCTION OF TH17 T CELLS IS TGF-B AND IL6 DEPENDENT.

Figure 6

Induction of Th17 T cells is TGF-β and IL6 dependent.

$CD4^+$ T cells were CD3/CD28 stimulated and co-cultured with $CD14^+HLA\text{-}DR^+$ monocytes for 6 days. As indicated blocking, blocking antibodies were added to the co-culture. Induction of Th17 T cells were measured by ICS FACS analysis gating on $CD4^+$ T cells. Shown are representative dotplots (A-D) or single results for every donor as bars (E-H). *P < 0.05, n.s. : not significant.

Figure 7

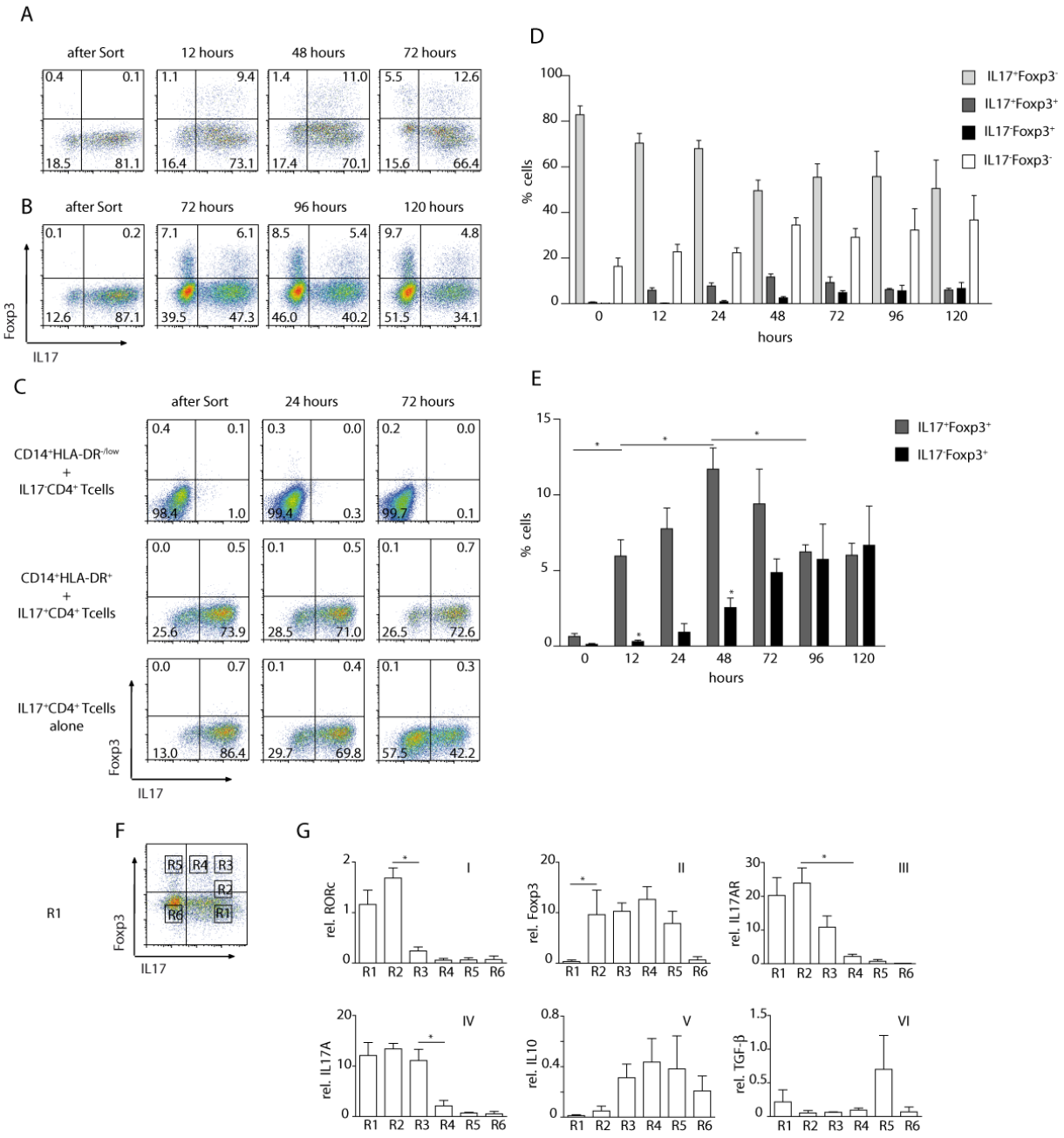

ABBILDUNG 26: FIGURE 7: MDSC CONVERT TH17 T CELLS TO REGULATORY T CELLS.

Figure 7

MDSC convert Th17 T cells to regulatory T cells.

(A) Th17 T cells were isolated as described in material & methods and incubated with $CD14^+HLA\text{-}DR^-$ cells for indicated time points. Cells were analysed for the expression of FoxP3 and IL-17 by intracellular FACS staining. As a control, $IL\text{-}17^-$ T cells were either treated under similar conditions or $IL\text{-}17^+$ cells were cultured alone or with $CD14^+HLA\text{-}DR^+$ (B). Shown are representative dotplots (A & B) or cumulative results from 4 independent ex-

periments (C & D). *P < 0.05. (F & G) Th17 cells were isolated as described before and cultured with $CD14^{+}HLA\text{-}DR^{-}$ cells for 24 hours. Quantitative PCR was performed on different gates shown in figure 7F. Expression level for different genes was shown relative the Cyclophilin A. Shown are cumulative results of two independent experiments.

Figure 8

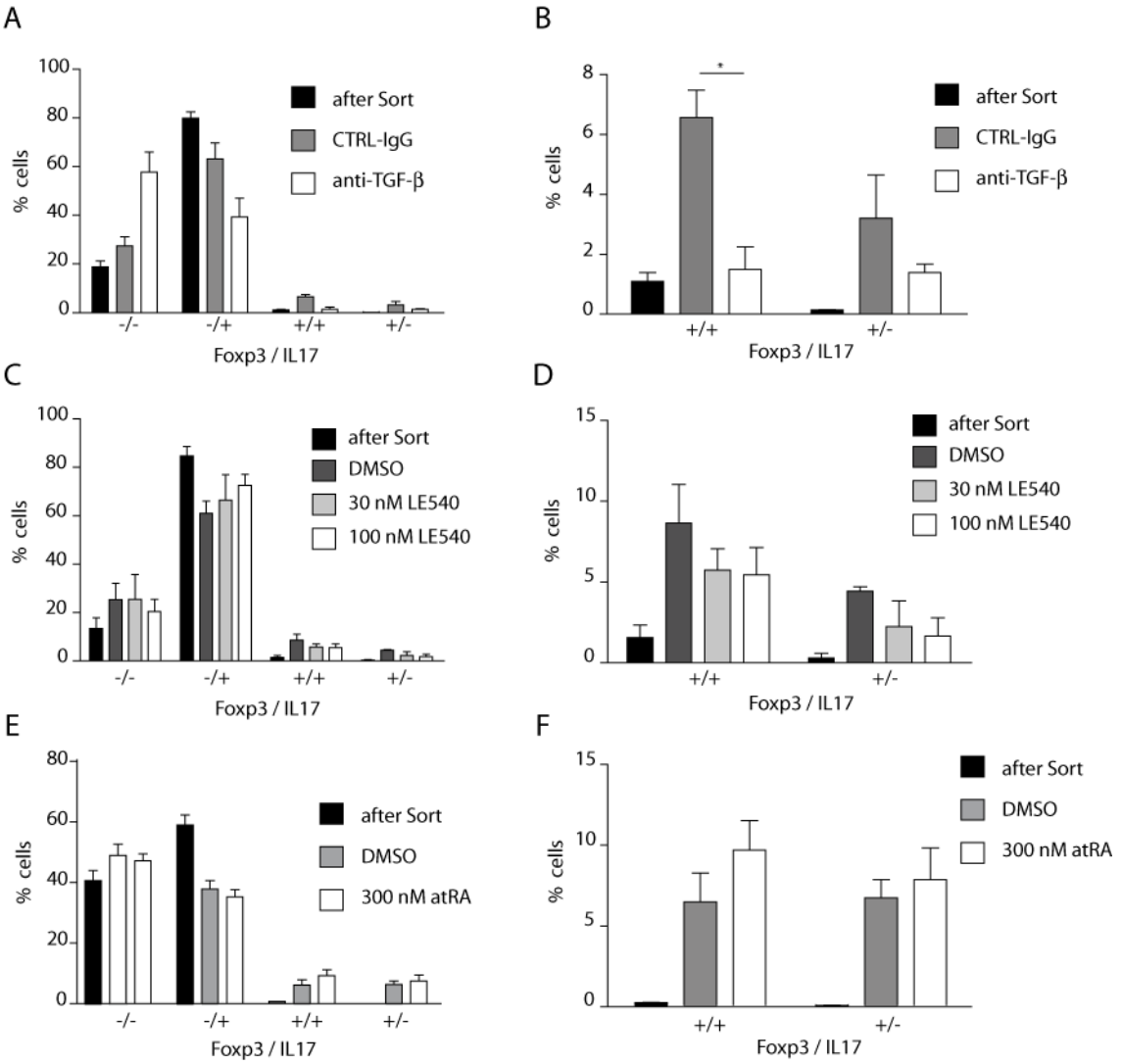

ABBILDUNG 27: FIGURE 8: BLOCKING TGF-B OR ATRA PREVENT TH17 CELLS TO BE CONVERTED BY MDSC.

Figure 8

Blocking TGF-β or atRA prevent Th17 cells to be converted by MDSC.

Th17 T cells were incubated with $CD14^{+}HLA\text{-}DR^{-}$ MDSC for 72 hours. As indicated, blocking antibody for TGF-β or LE540 or atRA were added to the co-culture. The expression of IL-17 and FoxP3 was performed by intracellular FACS staining. Shown are cumulative results from 2 independent experiments.

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Supplementary Figure 1

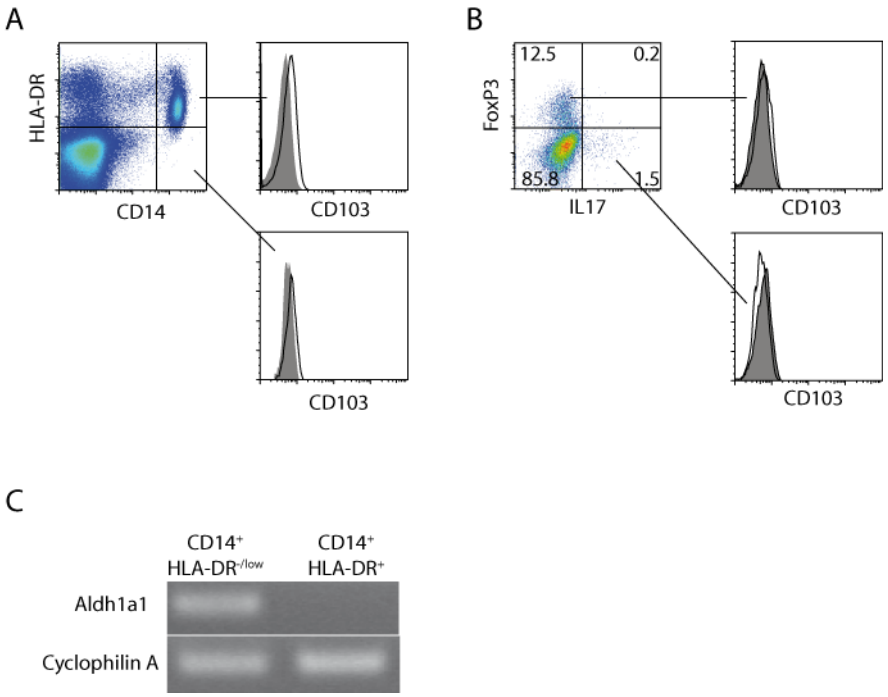

ABBILDUNG 28: SUPPLEMENTARY FIHURE 1: NEITHER CD14+ CELLS NOR INDUCED T CELLS EXPRESS CD103.

Supplementary Figure 1

Neither $CD14^+$ cells nor induced T cells express CD103.

(A) PBMC were stained for CD103 gated on $CD14^+$ HLA-DR $^-$ or HLA-DR^+ cells (black line) or isotype control (filled histogram). (B) Induced Th17 cells and Tregs were stained for CD103 (black line) or isotype control (filled histogram) gated on IL-17^+ cells or $FoxP3^+$ cells. (C) Expression level of ALDH1a1 (RALD1) was analysed by qPCR. Representative gel picture from 3 independent experiments is shown.

Diskussion

In der vorliegenden Arbeit wurden myeloide Suppressorzellen in Patienten mit hepatozellulärem Karzinom identifiziert und charakterisiert. Weiterhin wurde die Interaktion mit $CD4^+$ T-Zellen als Vertreter der adaptiven Immunität, und NK-Zellen, als Effektorzellen des angeborenen Immunsystems untersucht.

MDSC in HCC Patienten werden in dieser Arbeit als $CD14^+$ $HLA\text{-}DR^{-/low}$ definiert, ihre Frequenz ist im Blut als auch im Tumor erhöht. Dieser Subtyp von MDSC wirkt *ex vivo* suppressiv über eine erhöhte Arginase-Aktivität und induziert funktionelle regulatorische T-Zellen. Des Weiteren inhibieren $CD14^+HLA\text{-}DR^{-/low}$ Zellen NK-Zellen über eine NKp30 Interaktion.

Frequenz und phänotypische Charakterisierung

In murinen Modellen werden MDSC durch die Expression der Marker CD11b und Gr-1 charakterisiert.

Im Menschen sind diese Zellen dagegen nur sehr unzureichend beschrieben. In den meisten Studien werden MDSC als $CD11b^+$, $HLA\text{-}DR^-$, $CD34^+$ und $CD33^+$ Zellen definiert, die keine weiteren Differenzierungsmarker aufweisen (Almand et al., 2001; Garrity et al., 1997; Pak et al., 1995b; Serafini et al., 2006a). Daneben werden auch MDSC beschrieben, die den Granulozytenmarker CD15 tragen, allerdings ebenfalls $CD14^-$ sind (Filipazzi et al., 2007; Mirza et al., 2006; Zea et al., 2005).

Die hier beschriebene Population von MDSC wird durch die Expression von CD14 und einer geringen oder fehlenden Expression von HLA-DR charakterisiert. Ähnliche phänotypische Merkmale sind auch von anderen Arbeitsgruppen identifiziert worden (Filipazzi et al., 2007; Serafini et al., 2006b). In der Arbeit von Filipazzi wurden die MDSC in Melanompatienten ebenfalls als $CD14^+HLA\text{-}DR^{-/low}$ beschrieben, diese wiesen jedoch keine Arginaseaktivität auf. Hinsichtlich weiterer Marker konnten keine Unterschiede zwischen HLA-DR positiven oder negativen Monozyten identifiziert werden. Weder CD80, welches im Mausmodel für die Suppression verantwortlich ist (Yang et al., 2006), noch der negative kostimulatorische Ligand B7-H4 (Kryczek et al., 2006) konnten nach gewiesen werden. Da die MDSC negativ für den Makrophagenmarker CD206 sind, kann aus geschlossen werden, dass es sich um TAMs handelt (Umemura et al., 2008).

Generierung dendritischer Zellen aus Monozyten

In verschiedenen Studien wurde gezeigt, dass dendritische Zellen in Patienten mit Tumorerkrankungen einen unreifen Phänotyp aufweisen (Enk, 2005; Enk et al., 1997; Kim et al., 2006; Ormandy et al., 2006). Dendritische Zellen sind die potentesten Antigen präsentieren-

den Zellen und nehmen eine Schlüsselposition in der Tumorantigenerkennung ein. Sie spielen eine wichtige Rolle sowohl bei anti-Tumor-Vakzinierung als auch bei der Immuntherapie bereits etablierter Tumoren (Figdor et al., 2004; Fricke and Gabrilovich, 2006; Kusmartsev and Gabrilovich, 2002; Maecker et al., 2006; Nagaraj et al., 2007b). Da die Frequenz dendritischer Zellen im Blut gering ist, wurde versucht, Monozyten-generierte dendritische Zellen (MoDC) aus $CD14^{+}HLA\text{-}DR^{-/low}$ und $CD14^{+}HLA\text{-}DR^{+}$ Zellen generieren. Während dieses mit den Klasse II positiven Monozyten gelang wiesen die Zellkulturen mit MDSC kaum Merkmale dendritischer Zellen auf. Die Expression von CD14 blieb unverändert, MHC-II und das kostimulatorische Molekül CD86 wurden nur gering exprimiert. CD80, CD83 und der MoDC Marker CD1a fehlten völlig. Dies kann ein Hinweis darauf sein, dass die Entwicklung von DCs aus $CD14^{+}$ Vorläuferzellen in Patienten mit erhöhtem Anteil an MDSC in der Monozytenpopulation beeinträchtigt ist (Enk et al., 1997; Kim et al., 2006; Ormandy et al., 2006).

MDSC zeigen Suppression von autologen $CD4^{+}$ T-Zellen und Arginaseaktivität

Untersuchung der $CD14^{+}HLA\text{-}DR^{-/low}$ Zellen auf funktioneller Ebene zeigte eine Suppression der Proliferation und IFN-γ Sekretion von $CD4^{+}$ T-Zellen ähnlich der bei murinen Modellen (Bronte et al., 1998; Delano et al., 2007; Kusmartsev et al., 2005; Kusmartsev et al., 2004). Als Kontrollgruppe wurden $CD14^{+}HLA\text{-}DR^{+}$ Monozyten eingesetzt. Diese zeigten keinerlei suppressive Aktivität.

Die Aktivität der Arginase I (ArgI) wird in Mausmodell als einer der wichtigsten Marker für MDSC angesehen (Kusmartsev et al., 2004; Ochoa et al., 2007; Popovic et al., 2007; Rodriguez et al., 2005; Rodriguez and Ochoa, 2008; Zhao et al., 2009). In der vorliegenden Arbeit wurde die Aktivität der ArgI in $CD14^{+}HLA\text{-}DR^{-/low}$ Zellen aus dem Blut von HCC-Patienten gemessen und es zeigte sich eine deutlich erhöhte Produktion von Harnstoff. Ähnliches konnte auch von anderen Arbeitsgruppen beobachtet werden, hingegen wurde in dieser Studie nur die Aktivität im Serum von Patienten bestimmt oder es handelte sich bei den betrachteten MDSC um $CD15^{+}CD14^{-}$ Zellen, die nur bedingt mit den in unsere Studie charakterisierten Zellen verglichen werden können (Porembska et al., 2003; Zea et al., 2005). Um die Relevanz der ArgI und somit der damit verbundenen Depletion von L-Arginin weiter zu überprüfen wurde L-Arginin in den Suppressionsversuchen im Überschuss zugegeben. Hier zeigte sich ebenfalls eine gesteigerte IFN-γ Sekretion der kokultivierten T-Zellen und somit geringere suppressive Aktivität der MDSC.

Depletion von MDSC führt zu einer gesteigerten Immunantwort auf α-Fetoprotein (AFP)

Die Depletion von immunsuppressiven Zellen stellt bei Patienten mit Karzinomen eine mögliche Therapie dar, um eine verstärkte Immunantwort gegen tumorspezifische Antigene zu erreichen. Bei dem in der Studie verwendeten α-Fetoprotein (AFP) handelt es sich um ein onkofetales Antigen welches für den klinischen Verlauf bei HCC Patienten heran gezogen wird. Des weiteren wurde ebenfalls eine T-Zell Immunreaktion gegen AFP beschrieben (Greten et al., 2006b). Durch die Depletion der MDSC mittels FACS-Sort konnte die Frequenz der AFP spezifischen $CD4^+$ T-Zellen im Rahmen dieser Arbeit um den Faktor 4 gesteigert werden. Vergleichbare Ergebnisse lieferten *in vitro* Studien an Patienten mit Nierenkarzinom (Dannull et al., 2005) und in Maus-Modellen des Melanomen (Sutmuller et al., 2001).

Ausgehend von den hier beschriebenen *in vitro* Versuchen und korrelierenden Daten aus murinen Modellen ist es vielversprechend MDSC *in vivo* zu depletieren um eine Verbesserung der anti-Tumor-Immunantwort zu erreichen. Allerdings ist dies im Patienten zurzeit aufgrund mangelnder oder nicht ausreichender Marker nicht möglich.

MDSC induzieren $CD4^+CD25^+FoxP3^+$ und $CD4^+IL10^+FoxP3^-$ regulatorische T-Zellen

In Patienten mit HCC wurde eine erhöhte Frequenz von regulatorischen T-Zellen beschrieben (Ormandy et al., 2005). Ähnliches konnte ebenfalls für andere Tumore gezeigt werden (Cesana et al., 2006; Dannull et al., 2005; Pan et al., 2008; Wang and Wang, 2007). Eine mögliche Ursache für diese erhöhte Frequenz von Tregs stellt die Induktion dieser Zellen durch tolerogene DCs dar (Enk, 2005; Mahnke et al., 2007). Bei der Bestimmung des Zytokinprofils der MDSC in der Kokultur mit autologen T-Zellen fiel auf, das erhöhte Konzentration von IL-10 vorlagen. Um zu bestimmen welche Zellen für die erhöhte IL-10-Sekretion verantwortlich sind, wurden die kokultivierten Zellen durchflusszytometrisch untersucht. Dabei zeigte sich, dass das IL-10 nicht von den MDSC (Steinbrink et al., 1997) sondern von den T-Zellen sezerniert wird. Um die Auswirkungen der IL-10-Produktion *in vitro* abschätzen zu können wurde das Zytokin durch blockierende Antikörper neutralisiert. Eine gesteigerte T-Zell Proliferation in den entsprechenden Kulturen spricht für suppressive Eigenschaften der IL-10-sezernierenden T-Zellen, wie sie bereits in der Vergangenheit beschrieben wurden (Roncarolo et al., 2006; Wang and Wang, 2007). Eine weitere Untersuchung der T-Zellen ergab, dass diese FoxP3 exprimieren. Die $CD4^+$ T-Zellen, die mit $CD14^+HLA\text{-}DR^{-/low}$ Zellen inkubiert wurden hatten eine zweifach erhöhte Frequenz an $FoxP3^+$ T-Zellen im Vergleich mit den beiden Kontrollgruppen, in der T-Zellen mit $CD14^+HLA\text{-}DR^+$ Zellen oder alleine kultiviert

wurden. Auch in murinen Modellen konnte gezeigt werden, dass MDSC für die Induktion von FoxP3$^+$ Tregs verantwortlich sind (Huang et al., 2006; Serafini et al., 2008). Allerdings wurde hier kein genauer Mechanismus beschrieben. Neben der Induktion von FoxP3$^+$ Tregs durch CD14$^+$HLA-DR$^{-/low}$ Zellen kann ebenfalls eine Induktion von FoxP3$^-$ IL-10$^+$ T-Zellen beobachtet werden (Roncarolo et al., 2006). Durch die Zugabe von L-Arginin im Überschuss und die Verwendung eines neutralisierendem Antikörper für IL-10 wurde die Induktion der FoxP3$^+$ T-Zellen nicht unterbunden, weshalb man darauf schließen kann, dass der Mechanismus unabhängig von diesen Faktoren ist. Allerdings benötigt die Induktion einen direkten Zell-Zell Kontakt. Ist dieser durch einen permeablen Einsatz unterbunden ist ebenfalls die Induktion von FoxP3$^+$ T-Zellen unterbunden.

Der genaue Mechanismus soll an späterer Stelle im Rahmen dieser Arbeit noch eingehend diskutiert werden.

NK-Zellen von HCC Patienten weisen eine geringere Zytotoxizität auf

Im Vorfeld wurde die Zytotoxizität von NK-Zellen von HCC Patienten und gesunden Spendern verglichen. Dabei fiel eine signifikant geringere Lyse bei NK-Zellen von HCC Patienten auf. Da bekannt ist, das Monozyten und NK-Zellen interagieren (Welte et al., 2006) und in murinen Modellen eine Suppression von NK-Zellen durch MDSC gezeigt werden konnte (Li et al., 2009) wurde die mögliche Interaktion von humanen NK-Zellen und MDSC genauer untersucht.

MDSC inhibieren die Zytotoxizität und IFN-γ Sekretion von NK-Zellen

Um herauszufinden, ob MDSC eine suppressive Wirkung auf NK-Zellen haben, wurden beide Zelltypen für 12 Stunden gemeinsam inkubiert. Bei den anschließenden Chrom-Freisetzungsversuchen zur Ermittlung der zytotoxischen Aktivität zeigte sich eine signifikante Reduktion der Aktivität der NK-Zellen, welche mit MDSC inkubiert wurden. Im Gegensatz dazu zeigten CD14$^+$HLA-DR$^+$ Zellen keinen inhibitorischen Effekt auf die lytische Aktivität der NK-Zellen. In Hinsicht auf die IFN-γ Sekretion zeigte sich ein ähnliches Bild. Sowohl die Menge von IFN-γ als auch die Frequenz der NK-Zellen, die das Zytokin sezernieren, werden durch MDSC deutlich reduziert. Die MHC-II positiven Monozyten bewirken einen deutlich abweichenden Effekt. In dieser Kokultur sezernierten die NK-Zellen deutlich mehr IFN-γ verglichen mit der Probe, in der NK-Zellen alleine gemessen wurden. Dies spricht für eine zusätzliche Induktion der IFN-γ- Sekretion bei NK-Zellen durch Präinkubation mit CD14$^+$HLA-DR$^+$ Monozyten.

Phänotypische Charakterisierung von MDSC und NK-Zellen

Das Expressionsniveau verschiedener Oberflächenmarker auf NK-Zellen und $CD14^+$ Zellen wurde vor und nach Inkubation der Zellen gemessen. Dies sollte Aufschluss über mögliche Interaktionspartner liefern. Auf Seiten der MDSC zeigte sich eine gesteigerte Expression von HLA-DR. Dies kann einerseits durch eine direkte Zell-Zell Interaktion über NKp80 und ACIL erklärt werden, bei der beide Interaktionspartner aktiviert werden (Welte et al., 2006), andererseits ebenfalls durch Zytokine, die von den NK-Zellen ausgeschüttet werden (Gerlini et al., 2008; Hildenbrand et al., 2008). In Bezug auf die NK-Zellen zeigte sich eine erniedrigte Expression von NKp30. Dieser aktivierende Marker auf NK-Zellen, der zu den drei natürlichen Zytotoxizitätsrezeptoren gehört, wird in den meisten Studien mit der Lyse von unreifen DCs in Verbindung gebracht (Ferlazzo et al., 2002).

Die Suppression von NK-Zellen ist Zell-Zell-Kontakt abhängig aber unabhängig von ArgI und iNOS

Um den Mechanismus der Suppression von MDSC auf NK-Zellen zu eruieren wurden spezifische Inhibitoren eingesetzt, die die Funktion der ArgI, iNOS oder IDO herab setzen. Diese Enzyme sind bereits als suppressorische Mediatoren in MDSC oder tolerogenen Dendritischen Zellen bekannt (Bronte and Zanovello, 2005; Capuano et al., 2009; Johansson et al., 2009; Kai et al., 2003; Lob and Konigsrainer, 2008; Robinson et al., 2008). Um die suppressorische Aktivität der MDSC auf NK-Zellen nach Inhibitorgabe abzuschätzen wurde die IFN-γ-Sekretion als Indikator für NK-Zell Aktivität bestimmt. Allerdings zeigte weder L-NOHA (ArgI- Inhibitor), L-NMMA (NO-Synthase- Inhibitor) noch I-MT (IDO- Inhibitor) einen Effekt hinsichtlich der Zytokinsekretion. Eine völlige Aufhebung der Suppression von MDSC gegen NK-Zellen konnte erreicht werden, wenn der direkte Zell-Zell-Kontakt durch eine permeable Membran im Transwell-System aufgehoben war. Diese Ergebnisse wurden in der Vergangenheit bereits an murinen Modellen bestätigt (Li et al., 2009).

Während die Suppression von T-Zellen durch MDSC in murinen Modellen hauptsächlich ArgI und iNOS abhängig ist (Kusmartsev et al., 2000; Makarenkova et al., 2006; Popovic et al., 2007) und ein gleicher Mechanismus ebenfalls bei Patienten vorliegt (Porembska et al., 2003; Zea et al., 2006; Zea et al., 2005) scheint die Suppression der NK-Zellen durch MDSC auf andere Weise reguliert zu sein.

STAT Signalkaskaden sind nicht an der Suppression beteiligt

Da die Signalübertragung durch STAT- (signal transducer and activator of transcription) bei NK-Zellen eine wichtige Rolle spielt und gezeigt wurde, dass die dieser Weg bei der Suppression durch MDSC beeinträchtig ist (Liu et al., 2007a), wurde ein möglicher Einfluss der STAT-Signalkaskadenauf die inhibitorische Aktivität von humanen MDSC untersucht. Hierfür wurde die Expression der mRNA von STAT1, 3, 4 und 5 in der Kokultur von NK-Zellen und

MDSC untersucht. Dabei zeigte sich eine signifikant vermehrte Expression von STAT1 mRNA. Quantifizierung der mRNA für die anderen STAT-Moleküle ergab keinen Unterschied. Um zu überprüfen, ob das auf mRNA-Ebene überexprimierte STAT1 bei der Inhibition von NK-Zellen durch MDSC beteiligt ist wurde das Nukleosidanalog Fludarabin eingesetzt und anschleißend die IFN-γ-Sekretion der NK-Zellen gemessen. Fludarabin verhindert die Phosphorylierung von STAT1 und unterbindet so die STAT1 vermittelte Signaltransduktion (Frank et al., 1999). Allerdings zeigte sich kein Effekt hinsichtlich der IFN-γ Sekretion der NK-Zellen durch die Inhibition von STAT1.

Zusätzlich zur Analyse der Auswirkung der STAT1 Inhibition wurde STAT1, das in phosphorylierter und somit aktiver Form vorliegt, quantifiziert.

Intrazelluläre FACS-Analyse des phosphorylierten-STAT1 zeigte eine Aktvierung von MDSC in der Kokultur mit NK-Zellen. Dieser Effekt trat jedoch auch auf bei MDSC, die ohne NK-Zellen kultiviert wurden. Dies legt die Vermutung nahe, dass die Signalübertragung durch STAT1 nicht an der Suppression von NK-Zellen beteiligt ist.

Die Inhibition durch MDSC ist NKp30 abhängig

Um den genauen Mechanismus der Inhibition von MDSC gegenüber NK-Zellen zu verstehen, wurden blockierende Antikörper gegen unterschiedliche aktivierende als auch inhibitorische Rezeptoren auf NK-Zellen und gegen die MHC-Moleküle der Klassen I und II auf MDSC eingesetzt. Dies sollte Aufschluss darüber geben, welche Moleküle auf NK-Zellen Angriffspunkte suppressorischer Aktivität der MDSC darstellen. Dabei zeigte sich keinerlei Effekt durch die Zugabe der blockierenden Antikörper gegen NKp44, NKG2D, CD94 oder der MHC-Moleküle. Erstaunlicherweise erbrachte die Blockierung des aktivierenden Rezeptors NKp30 auf NK-Zellen in der Kokulture mit MDSC eine signifikante Erhöhung der IFN-γ Sekretion. Die lytische Kapazität der NK-Zellen wurde ebenfalls teilweise wieder hergestellt. Da die Suppression der Zytokinsekretion als auch der Lyse nicht vollständig wiederhergestellt wurde, liegt die Vermutung nahe, dass ein oder mehrere weiterer Faktoren an dem Mechanismus beteiligt ist, wobei membrangebundenes TGF-β einen potentiellen Kandidaten darstellt. NKp30 gilt als aktivierender Marker auf NK-Zellen und wird eher mit der Lyse von unreifen myeloiden Zellen in Zusammenhang gebracht (Balsamo et al., 2009). Da TGF-β auf der Oberfläche von MDSC also auch von Monozyten detektierbar war und somit dies nicht der Unterschied der beiden Subpopulationen sein kann, der über Suppression oder Aktivierung entscheidet, bedarf dieser Interaktion noch weitere Aufmerksamkeit.

In murinen Modellen sind die Ergebnisse hinsichtlich der Interaktion von MDSC und NK-Zellen zum Teil widersprüchlich. Einerseits wurde gezeigt, dass MDSC suppressiv auf NK-Zellen wirken, andererseits wurden auch MDSC beschrieben, die NK-Zellen aktivierten. Die

suppressive Wirkung wird durch membranständiges TGF-β verursacht, die Aktivierung erfolgt dagegen über RAE-1 (Li et al., 2009; Nausch et al., 2008).

Subtypen von CD14+ monozytären Zellen induzieren regulatorische T-Zellen oder Th17 Zellen über einen TGF-β und Retinsäure abhängigen Mechanismus

In murinen Modellen wurden unterschiedliche DC- Subtypen identifiziert, welche wiederum unterschiedliche $CD4^+$ T Helfer Typen induzieren. Coombes et all. konnten zeigen, dass Darm assoziierte $CD103^+$ DCs in der Lage sind, regulatorische T-Zellen in einem TGF-β abhängigen Mechanismus zu induzieren (Coombes et al., 2007). Andere Gruppen zeigten ebenfalls, dass $CD103^+CX_3CR1^-$ ipDCs in der Lage sind, regulatorische T-Zellen zu induzieren. Desweiteren konnte hier ebenfalls bezeigt werden, dass $CD103^-CX_3CR1^+$ DCs die Differenzierung von Th17 Zellen fördern (Atarashi et al., 2008; Varol et al., 2009). Im Gegensatz zu diesen DC-Subtypen konnte eine Expression von CD103 nicht auf MDSC nicht nach gewiesen werden. Eine weitere Studie beschrieb Makrophagen und DC, die regulatorische und IL-17 sezernierende T-Zellen induzieren (Denning et al., 2007). Die Induktion der regulatorischen T-Zellen war abhängig von Retinsäure, TGF-β und IL-10.

In dieser Arbeit wurden Zellen myeloiden Ursprungs aus humanen PBMCs isoliert, die die Fähigkeit aufweisen, $CD4^+$ T-Zellen in unterschiedliche T-Helfer- Subtypen zu induzieren. Während $HLA\text{-}DR^-$ MDSC die Entwicklung von regulatorischen T-Zellen fördern, induzieren $HLA\text{-}DR^+$ Monozyten die Ausbildung von Th17 T-Zellen.

Die beiden monozytären Populationen expremieren membran- gebundenes TGF-β, welches für die Differenzierung der iTregs als auch der Th17 Zellen entscheidend ist (O'Garra et al., 2008). MDSC expremieren *ex vivo* Gene des RA Metabolismus (Marill et al., 2003; Yang et al., 2002), allerdings sezernieren sie keine inflammatorischen Zytokine (IL-1β und IL-6). Diese Mikroumgebung aus TGF-β und RA und das Fehlen von IL-6 fördert die Entwicklung von Tregs. Auf der anderen Seite sind die Gene für die Synthese von RA in Monozyten nicht exprimiert, dafür sezernieren sie IL-6 und IL-1β und bewirken somit eine TH17 Differenzierung (Fujishima et al., 2010; Mus et al., 2010).

Somit stellt das Gleichgewicht aus Retinsäure und IL-6 eine wichtige Weiche für die Entwicklung von anti- oder pro- inflammatorischen T-Zellen in der Anwesenheit von TGF-β dar.

MDSC konvertieren Th17 Zellen in regulatorische T-Zellen

Die Tatsache, dass die Differenzierung von sowohl Th17 als auch iTregs TGF-β benötigt, lieferte den Hinweis, dass die Entwicklung dieser Subtypen mit einander verknüpft ist (Korn et al., 2009; Veldhoen et al., 2006). Vor kurzen veröffentlichte Studien konnten eine direkte

Interaktion der für die jeweilige T-Zell-Population spezifischen Transkriptionsfaktoren nach weisen (Ziegler and Buckner, 2009). Dadurch konnte eine Plastizität gezeigt werden, bei der iTregs, wenn sie unter Th17 Konditionen kultiviert werden Charakteristika von TH17 Zellen annahmen (Locksley, 2009; Zhou et al., 2009). Bei den hier gezeigten Systemen war es immer erforderlich, die benötigten Bedingungen für die Konvertierung von iTregs in Th17 Zellen in Form von Zytokinen zuzugeben. Demnach spiegeln diese Bedingungen die *in vivo* Situation nur sehr unzureichend. Der Mechanismus, der in dieser Arbeit vorgestellt wird verzichtet völlig auf exogene Faktoren.

Allerdings bleibt zu klären, warum nicht alle Th17 Zellen in iTregs konvertieren. Eine Möglichkeit besteht darin, dass nicht genug MDSC vorhanden waren oder das diese in der Kultur zu schnell reifen und somit die für MDSC typischen Charakteristika verlieren. Eine weitere Möglichkeit ist die, dass nur ein Subtyp der Th17 Zellen in der Lage ist den Phänotyp zu ändern. Ein weiterer kritischer Punkt ist die Tatsache, dass $IL17^{+}FoxP3^{+}$ T-Zellen nicht direkt im Blut von Patienten oder gesunden Spendern nach gewiesen werden konnten. Dies lässt sich möglicherweise damit erklären, dass sich die Konvertierung der Th17 Zellen im Gewebe abspielt und die doppelt positiven Zellen somit nicht in der Peripherie nachweisbar sind.

Biologische / Medizinische Relevanz

Die Immuntherapie stellt eine zusätzliche Option bei der Behandlung des HCC dar. Einerseits sind die bisherigen therapeutischen Optionen gering, andererseits stellt das HCC durch die Expression von spezifischen Antigenen ein potentielles Ziel dar. Derzeit laufen verschiedene Ansätze hinsichtlich einer immuntherapeutischen Behandlung. Diese beruhen hauptsächlich auf den Versuchen, generell das Immunsystem zu aktivieren, den Tumor stärker immunogen zu machen, eine antigenspezifische Immunreaktion zu induzieren als auch das immunsuppressive Milieu zu beeinflussen (Butterfield, 2007; Butterfield et al., 2008; Butterfield et al., 2006; Butterfield et al., 2007). Einige klinische Studien konnten eine erste Erfolge verzeichnen (Kuang et al., 2004; Lee et al., 2005; Peng et al., 2005), allerdings sind die Ergebnisse bei weitem nicht ausreichend. Neben der Induktion einer effektiven tumorspezifischen Immunantwort wird in Zukunft die Unterbindung immunsuppressiver Mechanismen von großer Bedeutung sein. Erste Ansätze dies bezüglich befinden sich bereits in der ersten klinischen Phase (Beyer et al., 2005; Frank et al., 1999; Kusmartsev et al., 2008; Takaku et al., 2009; Xin et al., 2009).

Das immunsuppressive Netzwerk, das von Tumoren induziert wird, ist lange noch nicht vollständig identifiziert und verstanden. Zudem sind die Interaktionen der einzelnen Komponenten ebenfalls nur unzureichend bekannt.

Die in dieser Arbeit beschriebenen MDSC stellen einen weiteren, wichtigen Aspekt hinsichtlich der Immunsuppression dar. Es wurde gezeigt, dass sie direkt suppressiv auf $CD4^+$ T-Zellen und NK-Zellen einwirken und somit eine effektive Immunreaktion unterbinden. Unsere Ergebnisse zeigen zum ersten Mal, dass eine wichtige Wechselwirkung der MDSC mit $CD4^+$ T-Zellen vorliegt, die zur Differenzierung von regulatorischen T-Zellen führt. So konnte experimentell die erhöhte Frequenz von regulatorischen T-Zellen in HCC Patienten erklärt werden, was in anstehenden Therapieprotokollen berücksichtigt werden muss.

Für eine Effektive Immuntherapie wird es in Zukunft unerlässlich sein ebenfalls die in dieser Arbeit beschriebenen MDSC zu berücksichtigen und Methoden zu entwickeln, ihre immunsuppressiven Effekte zu unterbinden.

Desweiteren ermöglicht ein genaues Verständnis dieser Mechanismen ebenfalls einen möglichen Therapieansatz bei Autoimmunreaktionen.

Zusammenfassung

Mehrere Studien haben gezeigt, dass die Entwicklung des HCCs mit dem Auftreten von verschiedenen immunsupprimierenden Mechanismen bei den Patienten assoziiert ist. In murinen Modellen konnte gezeigt werden, dass diese T-Zellen inhibieren. Die Ergebnisse, in Bezug auf die Suppression von NK-Zellen sind im Mausmodell widersprüchlich. Beim Menschen liegen, aufgrund des Mangels an charakteristischen Oberflächenmarkern, weitaus weniger Daten vor. Ziel dieser Arbeit war es, MDSC in Patienten mit HCC zu identifizieren und zu charakterisieren. Desweiteren sollte die Interaktion dieser Zellen mit $CD4^+$ T-Zellen und NK-Zellen untersucht.

Es wurde eine Population von HLA-$DR^{-/low}$ $CD14^+$ Monozyten identifiziert, die bei HCC Patienten sowohl im peripheren Blut als auch im Tumor signifikant erhöht ist. Eine weitergehende phänotypische Charakterisierung dieser Zellen ergab keinerlei Unterscheide hinsichtlich der Expression der untersuchten Oberflächenmarkern verglichen mit $CD14^+$HLA-DR^+ Monozyten.

Bei dem Versuch aus $CD14^+$HLA-$DR^{-/low}$ Zellen MoDC zu generieren zeigte sich, dass den resultierenden Zellen typische Marker von DC´s fehlten. CD1a wurde nicht, die kostimulatorischen Marker CD80, CD83 und CD86 nicht oder nur sehr schwach exprimiert. CD14 hingegen wird nicht herunter reguliert.

Funktionelle Untersuchungen haben gezeigt, dass $CD14^+$HLA-$DR^{-/low}$ Zellen in der Lage sind $CD4^+$ T-Zellen und NK-Zellen zu inhibieren.

Die T-Zellen wurden hinsichtlich ihrer Proliferation und IFN-γ Sekretion gehemmt. Der hier zugrunde liegende Mechanismus beruht hauptsächlich auf der Depletion des L-Arginins durch die Arginase I. Durch Zugabe von L-Arginin, Inhibition der Arg I oder durch Depletion der $CD14^+$HLA-$DR^{-/low}$ Zellen konnte sowohl die Proliferation als auch die IFN-γ Sekretion von $CD4^+$ T-Zellen wieder hergestellt werden. Darüber zeigte sich, dass die Depletion der MDSC ebenfalls zu einer signifikant höheren Frequenz von AFP-spezifischen $CD4^+$ T-Zellen führte.

Die Suppression der NK-Zellen dagegen ist dagegen Zell-Zell-Kontakt abhängig und wird, zumindest teilweise, durch den NK-Rezeptor NKp30 vermittelt. Die Hemmung von ArgI, iNOS oder IDO durch spezifische Inhibitoren zeigte keinerlei Effekt hinsichtlich der lytischen oder sekretorischen Aktivität der NK-Zellen. Generell zeigte sich, dass NK-Zellen von gesunden Probanden eine signifikant höhere Zytotoxizität aufweisen.

Ein weiterer suppressiver Mechanismus der $CD14^{+}HLA\text{-}DR^{-/low}$ Zellen zeigte sich bei dem Versuch, das Zytokinprofil dieser Zellen zu charakterisieren. MDSC sind *in vitro* in der Lage, regulatorische T-Zellen zu induzieren. Dies wird durch membrangebundenes TGF-β verursacht und durch Retinsäure verstärkt.

Schlussfolgernd lässt sich festhalten, dass die hier identifizierte Population von MDSC einen weiteren immunsuppressiven Mechanismus bei Patienten mit HCC darstellt.

Durch die direkte Inhibition von $CD4^{+}$ T-Zellen als auch von NK-Zellen werden zwei wichtige Bereiche sowohl der adaptive, als auch der angeborenen Immunität unterdrückt und somit eine effektive Reaktion gegen den Tumor massiv abgeschwächt. Die erhöhte Frequenz an regulatorischen T-Zellen, die bei HCC aber auch bei anderen Tumorerkrankungen beschrieben wurde, kann durch $CD14^{+}HLA\text{-}DR^{-/low}$ Zellen erklärt werden. Da $CD14^{+}HLA\text{-}DR^{-/low}$ Zellen eine Schlüsselposition bei der Suppression des Immunsystems einnehmen ist das Verständnis der MDSC bei der Entwicklung neuer immuntherapeutischer Protokolle bei der Behandlung von Tumorerkrankungen von großer Bedeutung. Darüber Hinaus ist zu prüfen, ob MDSC auch bei Autoimmunerkrankungen eine Rolle spielen und hier genutzt werden können.

Abbildungsverzeichnis

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Publikationen

A new population of myeloid derived suppressor cells in hepatocellular carcinoma patients induces $CD4^+CD25^+FoxP3^+$ Tcells

Bastian Hoechst, Lars A. Ormandy, Matthias Ballmaier, Frank Lehner, Christine Krüger, Michael P. Manns, Tim F. Greten, Firouzeh Korangy

Gastroenterology 2008

Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor

Bastian Hoechst, Torsten Voigtlaender, Lars Ormandy, Jaba Gamrekalashvili, Fei Zhao, Heiner Wedemeyer, Frank Lehner, Michael P. Manns, Tim F. Greten, Firouzeh Korangy

Hepatology 2009

Low dose cyclophosphamide treatment impairs regulatory T cells and unmasks AFP-specific $CD4^+$ T cell responses in patients with advanced HCC

Tim Greten, Lars Ormandy, Annika Fikuart, Bastian Höchst, Sylvana Henschen, Monique Hoerning, Michael Manns, Firuozeh Korangy

J Immunother. 2009

In vitro polarized human $CCR4^+CCR6^+$ TH17 cells suppress autologous $CD8^+$ T cell responses

Fei Zhao, Bastian Hoechst, Jaba Gamrekelashvili, Lars Ormandy, Michael Manns, Tim Greten, Firouzeh Korangy

Submitted

Plasticity of regulatory T cells and Th17 cells can be triggered by subsets of monocytic cells

Bastian Hoechst, Fei Zhao, Jaba Gamrekelashvili, Michael Manns, Tim Greten, Firouzeh Korangy

In preparation

Kongressbeiträge

Characterisation of myeloid suppressor cells in patients with Hepatocellular carcinoma

Bastian Höchst, L. Ormandy, MP Manns, T. Greten, F. Korangy

Cancer Immunotherapy (CIMT), 12. – 14. April 2007, Würzburg.

Myeloid derived suppressor cells in patients with hepatocellular carcinoma induces $CD4^+CD25^+FoxP3^+$ cells.

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Cancer Immunotherapy (CIMT), 15. – 17. Mai 2008, Mainz.

Vorträge

Myeloid derived suppressor cells induces regulatory T cells in patients with HCC

Colloquium of Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, 24 Juni 2008, Hannover

Immune suppressor mechanism in hepatocellular carcinoma.

Helmholtz Alliance on Immunotherapy of Cancer. 2nd Retreat. 19. Mai 2009, Grünstadt