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Charakterisierung von immunregulatorischen NK-Zellen, non-T_{reg} T-Zellen und mesenchymalen Stammzellen

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Erklärung zur Dissertation

Die Zellsortierung muriner und humaner Lymphozyten wurde durch Christina Reimer, Dr. rer. nat. Mathias Rhein, Angelika Stucki-Koch und Dr. rer. nat. Matthias Ballmeier aus der zentralen Einrichtung für Zellsortierung durchgeführt.

Die Präparation von Nabelschnurgewebe sowie die Generierung mesenchymaler Stammzellen aus der Nabelschnur wurden von Tim Hatlapatka und PD Dr. rer. nat. Cornelia Kasper aus dem Institut für Technische Chemie (Universität Hannover) durchgeführt.

Zellzyklusanalysen wurden von Prof. Dr. rer. nat. Ralf Hass aus der Klinik für Frauenheilkunde und Geburtshilfe durchgeführt.

Die Quantifizierung der S100B-Konzentration im Überstand wurde von Johann Steiner aus der Universitätsklinik für Psychiatrie (Universität Magdeburg) durchgeführt.

Hiermit erkläre ich, die vorliegende Dissertation selbständig verfasst und alle Hilfsmittel, Quellen und zu Hilfsleistungen herangezogene Institutionen vollständig angegeben zu haben.

Die vorliegende Dissertation wurde noch nicht als Masterarbeit, Diplomarbeit oder ähnliche Prüfungsarbeit verwendet.

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(Unterschrift)
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ZUSAMMENFASSUNG

Zahlreiche immunregulatorische Zellpopulationen wurden bisher definiert. Die Interaktion zwischen verschiedenen Zellpopulationen führt hierbei entweder zur Aktivierung oder zur Inhibierung von Komponenten des Immunsystems, was wiederum weitreichende Auswirkungen etwa auf Verlauf und Therapie von Krankheiten oder der Rekonstitution nach Transplantationen haben kann. Dabei spielen nicht nur immunsystemassoziierte Zellen eine wichtige Rolle, sondern auch Zellen außerhalb des Immunsystems.

Im Menschen lassen sich zwei funktionell unterschiedliche natürliche Killer (NK)-Zellsubpopulationen unterscheiden: zytotoxische $CD56^{dim}$ NK-Zellen sowie $CD56^{bright}$ NK-Zellen, die aufgrund ihrer erhöhten Zytokinproduktion als „regulatorische“ NK-Zellen gelten. Murine NK-Zellen exprimieren hingegen kein CD56, weshalb *in vivo* Untersuchungen zur Entwicklung und zum Einfluss von regulatorischen NK-Zellen bisher nicht möglich waren. Wir konnten zeigen, dass entsprechende Korrelate in der Maus mittels CXCR3 und CD27 identifiziert werden können, was eine Basis für *in vivo* Experimente darstellt, deren Ergebnisse auf den Menschen extrapoliert werden können.

Des Weiteren haben wir immunregulatorische Eigenschaften von Nabelschnur-mesenchymalen Stammzellen (UC-MSC) auf NK-Zellen untersucht und, obwohl andere Studien den Einfluss von löslichen Mediatoren zeigten, dabei einen ausschließlich Zell-Zell-kontaktabhängigen Mechanismus der NK-Zellinhibition festgestellt. Hierbei spielt offensichtlich die Wechselwirkung zwischen LFA-1 und ICAM-1 keine Rolle für die Adhäsion von NK-Zellen an MSC, obwohl sie zentrale Zelladhäsionsmoleküle darstellen.

Zwei bisher nur wenig beschriebene T-Zellpopulationen, $CD20^+$ T-Zellen und $S100B^+$ T-Zellen, wurden im Rahmen dieser Arbeit bezüglich ihrer immunregulatorischen Funktionen näher charakterisiert.

Trotz der Expression des B-Zell-spezifischen CD20 Moleküls sind $CD20^+$ T-Zellen deutlich von B-Zellen sowohl auf RNA- als auch auf Proteinebene abgrenzbar und zeigen ein proinflammatorisches Potenzial. Dies könnte z.B. bei der Depletion von $CD20^+$ Zellen im Zuge einer Rituximabtherapie (anti-CD20-spezifische Antikörper) bisher unbekannte Auswirkungen auf den Erfolg einer Behandlung haben.

$S100B^+$ T-Zellen stellen eine kleine Population an $CD8^+$ T-Zellen im peripheren Blut gesunder Individuen dar. Diese Zellen exprimieren S100B intrazellulär und degranulieren schwächer als $S100B^-CD8^+$ T-Zellen. S100B wird von aktivierten $S100B^+$ T-Zellen sezerniert und kann an seinen Rezeptor „RAGE“ auf Zellen des angeborenen Immunsystems binden und sie so aktivieren. Möglicherweise repräsentieren $S100B^+$ T-Zellen daher eine Verbindung zwischen der angeborenen und der adaptiven Immunität.

ABSTRACT

Numerous immunoregulatory cell populations have been defined until today. The interaction between different cell populations results either in activation or in inhibition of several components of the immune system with considerable consequences on the outcome and therapy of diseases or reconstitution after transplantation.

In humans, two distinct NK cell subsets can be discriminated: cytotoxic $CD56^{dim}$ NK cells as well as $CD56^{bright}$ NK cells, which are regarded as “regulatory” NK cells due to their stronger ability to produce cytokines. In contrast, murine NK cells lack CD56 expression, making *in vivo* analyses of development and influence of regulatory NK cells impossible. We could show that corresponding correlates in mice can be identified by CXCR3 and CD27, constituting a basis for *in vivo* experiments whose results can be extrapolated to the human system.

Additionally, we determined the immunoregulatory characteristics of UC-MSC on NK cells. Although other studies reported a strong influence of soluble mediators such as IDO or PGE2, we observed an exclusively cell-cell contact-dependent mechanism of NK cell inhibition. In this context, interactions between LFA-1 and ICAM-1 are obviously not needed for NK-MSC adhesion although they represent important adhesion molecules.

Two so far only marginally described T cell subsets, $CD20^+$ T cells and $S100B^+$ T cells, were characterized in this study in detail for their immunoregulatory functions.

Although CD20 is believed to be a B cell-specific molecule, $CD20^+$ T cells are clearly distinguishable from B cells both on RNA and protein level, and they show proinflammatory potency. This could reveal a so far unknown influence of e.g. $CD20^+$ cell depletion in the course of treatment with Rituximab (anti-CD20-specific antibody).

$S100B^+$ T cells are a small population of $CD8^+$ T cells in peripheral blood of healthy individuals. These cells express S100B intracellularly and degranulate less than $S100B^-$ $CD8^+$ T cells. S100B is secreted by activated $S100B^+$ T cells and can bind to its receptor “RAGE” on cells of the innate immune system and thus activate them. Therefore, it is possible that $S100B^+$ T cells represent a bridge between innate and adaptive immunity.

SCHLAGWÖRTER/KEYWORDS

NK-Zellen/NK cells

Mesenchymale Stammzellen/mesenchymal stem cells

T-Zellen/T cells

INHALTSVERZEICHNIS

ABKÜRZUNGSVERZEICHNIS

ADCC	Antibody-dependent cellular cytotoxicity (antikörperabhängige zelluläre Zytotoxizität)
AIA	Activation-induced apoptosis (aktivierungsinduzierte Apoptose)
AICD	Activation-induced cell death (aktivierungsinduzierter Zelltod)
APC	antigen presenting cell (antigenpräsentierende Zelle)
APP	Akut-Phase-Protein
BCR	B cell receptor (B-Zellrezeptor)
BM-MSC	Bone marrow-MSC (Knochenmark-MSC)
cAMP	cyclic adenosine monophosphate (zyklisches Adenosin Monophosphat)
CCL	C-C-Motiv-Chemokinligand
CD	Cluster of differentiation (Differenzierungscluster)
CLL	Chronische lymphatische Leukämie
CRP	C-reaktives Protein
CRTAM	class-I-restricted T cell-associated molecule (Klasse-I-restringiertes T-Zell-assoziiertes Molekül)
CTL	cytotoxic T lymphocyte (zytotoxischer T-Lymphozyt)
CXCL	CXC-Motiv-Chemokinligand
CXCR	CXC-Motiv-Chemokinrezeptor
DC	dendritic cell (dendritische Zelle)
DNAM-1	DNAX accessory molecule (DNAX-akzessorisches Molekül)
EAE	Experimental autoimmune encephalomyelitis (Experimentelle autoimmune Enzephalomyelitis)
ESC	Embryonic stem cell (embryonische Stammzelle)
FasL	Fas Ligand
FcγR	Fcγ Rezeptor
FoxP3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony-stimulating factor (Granulozyten-Makrophagen koloniestimulierender Faktor)
GvHD	Graft versus host disease

	(Transplantat-gegen-Wirt-Reaktion)
GvL	Graft versus leukemia
	(Transplantat versus Leukämie)
HCV	Hepatitis C virus (Hepatitis C Virus)
HIV	Human immunodeficiency virus
	(Humanes Immundefizienz-Virus)
HLA	Human leukocyte antigen
	(Humanes Leukozytenantigen)
HSC	Hematopoietic stem cell
	(Hämatopoetische Stammzelle)
HSCT	Hematopoietic stem cell transplantation
	(Hämatopoetische Stammzelltransplantation)
HUVEC	Human umbilical vein endothelial cells
	(Humane Nabelschnurendothelzellen)
ICAM	Intercellular adhesion molecule
	(Interzelluläres Adhäsionsmolekül)
IDO	Indolamin-2,3-Dioxygenase
IFN	Interferon
Ig	Immunglobulin
IL	Interleukin
ILT	Immunoglobulin-like transcript
	(immunglobulinähnliches Transkript)
IP-10	IFN-γ-induced protein 10 kD
	(IFN-γ-induziertes Protein 10kD)
iPS	Induzierte pluripotente Stammzellen
ITAM	Immunoreceptor tyrosine-based activation motif
	(Immunrezeptor tyrosinbasierendes
	Aktivierungsmotiv)
ITIM	Immunoreceptor tyrosine-based inhibitory motif
	(Immunrezeptor tyrosinbasierendes
	Inhibierungsmotiv)
KIR	Killer-cell immunoglobulin-like receptor
	(Killer-Zell immunglobulinähnlicher Rezeptor)
KL	Kikuchi Lymphadenitis
KLR	Killer-cell lectin-like receptor
	(Killer-Zell lektinähnlicher Rezeptor)
LAIR	Leukocyte-associated immunoglobulin-like receptor

	(leukozytenassoziierter immunglobulinähnlicher Rezeptor)
LFA	Lymphocyte function-associated antigen (Lymphozyten funktionsassoziiertes Antigen)
mAb	Monoclonal antibody (monoklonaler Antikörper)
MAC	Membrane attac complex (Membran-Attacke-Komplex)
mDC	myeloid dendritic cell (myeloide dendritische Zelle)
MDSC	myeloid-derived suppressor cells (myeloide Suppressorzellen)
MHC	Major histocompatability complex (Hauptshistokompatibilitätskomplex)
MIP	Macrophage inflammatory protein (Makrophagen inflammatorisches Protein)
MLR	Mixed lymphocyte reaction (gemischte Lymphozytenreaktion)
MPA	Mycophenolate acid (Mycophenolsäure)
MSC	Mesenchymal stem cells (mesenchymale Stammzellen)
NCR	Natural cytotoxicity receptors (natürliche Zytotoxizitätsrezeptoren)
Necl2	Nectin-like protein 2 (nektinähnliches Protein 2)
NKT-Zellen	Natürliche-Killer-T-Zellen
PBMC	Peripheral blood mononuclear cells (mononukleäre Zellen des peripheren Blutes)
PD-1	Programmed cell death 1 (Programmierter Zelltod 1)
pDC	Plasmacytoid dendritic cell (plasmazytoide dendritische Zelle)
PD-L1	Programmed cell death 1 ligand (Programmierter Zelltod 1-Ligand)
PGE	Prostaglandin E
PHA	Phytohämagglutinin
PKC	Proteinkinase C
PMA	Phorbol-12-myristat-13-acetat
PVR	Poliovirusrezeptor
RAGE	Receptor for advanced glycation endproducts

	(Rezeptor für fortgeschrittene Glykierungsendprodukte)
RORγt	RAR-related orphan receptor γ (RAR-verwandter Waise-Rezeptor γ)
ROS	Reactive oxygen species (reaktive Sauerstoffspezies)
S100B	S100-beta
SLE	Systemischer Lupus erythematodes
SOC	Store-operated channels (speicherabhängige Kanäle)
TAM	Tumor-associated macrophages (tumorassoziierte Makrophagen)
TCR	T cell receptor (T-Zellrezeptor)
TGF	Transforming growth factor (Transformierender Wachstumsfaktor)
T_H-Zelle	T-Helfer-Zelle
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domains (T-Zellimmunrezeptor mit Immunglobulin- und ITIM-Domänen)
TLR	Toll-like receptor (Toll-ähnlicher Rezeptor)
TNF	Tumornekrosefaktor
T_{reg}-Zelle	regulatorische T-Zelle
UC-MSC	Umbilical cord-MSC (Nabelschnur-MSC)
VEGF	Vascular endotheloal growth factor (vaskulärer endothellialer Wachstumsfaktor)
ZNS	Zentrales Nervensystem

1 EINLEITUNG

1.1 IMMUNREGULATION

Eine Aktivierung einzelner Komponenten des Immunsystems führt zu einem Schutz vor Infektionen oder malignen Zellveränderungen. Nachfolgend ist allerdings auch eine entsprechende Suppression der Immunantwort notwendig, da es ansonsten in Folge von überschießenden Reaktionen zu Allergien und autoaggressiven Immunerkrankungen kommen kann. Entsprechend spielen sowohl aktivierende als auch inhibierende Signale bei der Immunregulation eine Rolle. Hierbei gibt es zahlreiche Mechanismen, mit deren Hilfe sowohl das angeborene als auch das adaptive Immunsystem gesteuert wird. Die Regulation des Immunsystems ist gekennzeichnet durch Wechselwirkungen zwischen verschiedenen Zellpopulationen via humoraler Faktoren oder direktem Zell-Zellkontakt.

Grundsätzlich sind alle Zellpopulationen des Immunsystems neben ihren spezifischen Aufgaben auch in der Lage, andere Zelltypen hinsichtlich ihres Aktivierungsstatus und ihrer Funktion zu beeinflussen. Das Netzwerk dieser Interaktionen erstreckt sich jedoch auch über die Komponenten des Immunsystems hinaus. Die Aktivierung oder Suppression von Lymphozyten und myeloiden Zellen ist auch durch Zellpopulationen möglich, die nicht direkt mit dem Immunsystem assoziiert sind wie z.B. Nervenzellen und Endothelzellen.

Die folgenden Unterkapitel bieten einen Einblick in die vielfältigen Mechanismen und Komponenten der Immunregulation mit Schwerpunkt auf die Übergruppen der in dieser Arbeit behandelten Zellpopulationen (NK-Zellen, T-Zellen, Mesenchymale Stammzellen) und ihrer funktionellen Charakteristika.

1.1.1 HUMORALE UND ZELLULÄRE IMMUNMODULATION

Diverse humorale oder lösliche Faktoren besitzen immunregulative Eigenschaften. Interferone, Interleukine, koloniestimulierende Faktoren und Tumornekrosefaktoren bilden die große Gruppe der pro- und antiinflammatorischen Zytokine. Sie werden auch zu therapeutischen Zwecken eingesetzt wie beispielsweise Interferone bei der Behandlung von Hepatitis B/C oder Multipler Sklerose (Pagliaccetti und Robek, 2010; Farrell und Giovannoni, 2010). Die immunregulatorische Funktion von Zytokinen wird u.a. bei der Reaktivität von NK-Zellen deutlich: werden diese mit den Interleukinen (IL)-12 und IL-18 stimuliert, führt dies zu einer sehr starken Produktion von Interferon-gamma (IFN-γ), das wiederum eine aktivierende Wirkung auf Makrophagen und andere

Zelltypen wie Mesenchymale Stammzellen (MSC) hat (Held et al., 1999; Polchert et al., 2008). Weiterhin setzen NK-Zellen Granulozyten-Makrophagen koloniestimulierenden Faktor (GM-CSF), Tumornekrosefaktor-alpha (TNF-α) und ebenso Chemokine wie Makrophagen inflammatorisches Protein- (MIP-)1α, MIP-1β oder CCL5 frei (Murphy et al., 1997; Wendt et al., 2006).

Eine weitere Gruppe immunregulierender löslicher Moleküle stellen Akut-Phase-Proteine (APP) dar. Sie werden hauptsächlich in der Leber gebildet und besitzen, ähnlich wie Zytokine, pro- oder antiinflammatorische Eigenschaften (Gabay und Kushner, 1999). Für den Entzündungsfaktor C-reaktives Protein (CRP) ist schon lange bekannt, dass er an T-Zellen binden und ihre Funktion inhibieren kann (Whisler et al., 1986). In einem Mausmodell für polymikrobielle Sepsis wurde erst kürzlich der Einfluss von APP auf die Immunregulation gezeigt – die Entzündungsreaktion in der Leber führt hier zur peripheren Akkumulation von myeloiden Suppressorzellen (MDSC), die wiederum ein Fortschreiten der Entzündung inhibieren (Sander et al., 2010).

Die dritte Gruppe löslicher immunregulativer Komponenten stellt das Komplementsystem dar. Eine Kaskade aus Zymogenen, also Proteasen, die durch Spaltung ihrerseits aktiviert werden, führt im Falle einer Aktivierung letztlich zur Opsonisierung von Pathogenen, zur Chemoattraktion von Phagozyten sowie zur Zerstörung der Zellmembran von Pathogenen mittels eines membranangreifenden Komplexes (MAC). Das Komplementsystem kann durch mannosebindendes Lektin (Lektin-Weg), C3-Konvertase (alternativer Weg) oder Antikörper (klassischer Weg) aktiviert werden. Der klassische Weg wird auch bei der Behandlung von Krankheiten genutzt. Der Einsatz der therapeutischen monoklonalen Antikörper (mAb) Rituximab, GA101 oder LFB-R603 (alle spezifisch für humanes CD20) induziert komplementvermittelte Zytotoxizität, antikörperabhängige zelluläre Zytotoxizität (ADCC) und Phagozytose (Pievani et al., 2010; Garff-Tavernier et al., 2010; Lim et al., 2010; Cartron et al., 2004).

Antikörper bzw. Immunglobuline werden von zu Plasmazellen differenzierten B-Zellen produziert und sezerniert. Die freigesetzten Antikörper entsprechen der membranständigen Form des B-Zellrezeptors (BCR). Die bereits oben beschriebenen anti-CD20 mAb werden beispielsweise bei der Behandlung von chronischer lymphatischer Leukämie (CLL) verwendet, bei der maligne monoklonale B-Zellen akkumulieren. Anti-CD20 mAb binden an diese Tumorzellen und induzieren damit u.a. über die Fc-Rezeptoren auf NK-Zellen ADCC (Garff-Tavernier et al., 2010). Im Rahmen der vorliegenden Arbeit wird eine T-Zellpopulation beschrieben, die CD20 auf der

Zelloberfläche exprimiert. Diese Zellen werden ebenso wie B-Zellen bei der Behandlung von vielen lymphoproliferativen und autoimmunen Erkrankungen, wie z.B. rheumatoider Arthritis, mit Rituximab depletiert und sind nach der Therapie nicht mehr im peripheren Blut detektierbar (Wilk et al., 2009).

Zusätzlich zu den oben erwähnten, in der Literatur viel beschriebenen Effektormolekülgruppen, gibt es eine lange Reihe weiterer löslicher Faktoren, die zwar nicht immer direkt mit dem Immunsystem assoziiert sind, es aber dennoch in unterschiedlicher Weise regulieren können. Darunter sind beispielsweise Mitglieder der calciumbindenden S100 Proteine wie S100-beta (S100B). Erhöhte S100B Konzentrationen im Serum von Patienten werden häufig bei Dysfunktionen des ZNS gemessen (Kanner et al., 2003; Marchi et al., 2003). Es wird u.a. in hohen Konzentrationen von Gliazellen, die Teil des zentralen und peripheren Nervensystems sind, gebildet, allerdings wurde es auch in anderen Zelltypen detektiert (Moore, 1965; Netto et al., 2006). In diesem Zusammenhang konnten wir im Rahmen dieser Arbeit $S100B^+$ T-Zellen in humanem peripheren Blut nachweisen, die auch in der Lage sind, S100B zu sezernieren (Steiner et. al., in Revision). Der Rezeptor für S100B, „receptor for advanced glycation end products“ (RAGE), wird auf einer Vielzahl von Zellen exprimiert, so auch auf Zellen des angeborenen Immunsystems (Granulozyten, Monozyten). Entsprechend könnte S100B immunregulatorische Effekte vermitteln, was aber bisher nicht untersucht worden ist.

Auch Gewebehormone wie Prostaglandine (PG), haben Einfluss auf das Immunsystem. Für PGE2 wird eine wichtige Rolle bei der Inhibierung von Lymphozyten durch Tumorzellen vermutet, und auch für MSC wurde ein PGE2-abhängiger Mechanismus beschrieben (Mulligan et al., 2010; Najar et al., 2010). PGE2 bindet an vier spezifische G-Protein-gekoppelte Prostaglandin E-Rezeptoren (EP1-4). EP1, EP2 und EP4 vermitteln zellaktivierende Signale (Mobilisierung intrazellulären Calciums und Aktivierung von cAMP). EP3 hingegen agiert als cAMP-inhibierender Rezeptor (Coleman et al., 1994).
Zusätzlich hat das tryptophandepletierende Enzym Indolamin 2,3-dioxygenase (IDO) einen verstärkenden Effekt bei der Inhibierung von Lymphozyten. Es wird wie PGE2 ebenfalls von MSC sezerniert (Meisel et al., 2004).

Immunmodulation kann nicht nur humoral vermittelt werden, sondern auch durch direkten Zell-Zellkontakt. Ein bekanntes Beispiel ist die Interaktion zwischen T- und B-Zellen, die zur Ausschüttung von Antikörpern führt. Ebenfalls ausführlich beschrieben ist

die Bildung der immunologischen Synapse auf NK-Zellen bei direktem Zellkontakt mit Targetzellen. Die Adhäsionsmoleküle „Lymphozyten funktionsassoziiertes Antigen-1“ (LFA-1) (auf NK-Zellen) und dem interzellulären Adhäsionsmolekül-1 (ICAM-1, auf Targetzellen) sind dabei von zentraler Bedeutung und erforderlich für eine erfolgreiche Lyse der Targetzelle (Barber et al., 2004). Weniger eindeutig sind Ergebnisse aus Untersuchungen zur Wechselwirkung zwischen MSC und Lymphozyten. Während wie oben erwähnt lösliche Faktoren wie PGE2 oder IDO in einigen Studien für die Inhibierung von Lymphozyten verantwortlich waren, konnten wir und andere dies in unseren Experimenten nicht bestätigen (Spaggiari et al., 2006; Krampera et al., 2006a; Aggarwal und Pittenger, 2005; Sotiropoulou et al., 2006).

1.1.2 Immunregulatorische Zellen innerhalb des Immunsystems

1.1.2.1 NK-Zellen

Wie alle anderen Lymphozyten werden NK-Zellen im Knochenmark gebildet. Sie sind Teil der angeborenen Immunität und sind anders als T-Zellen ohne vorherige Sensibilisierung in der Lage, entartete oder virusinfizierte Targetzellen zu lysieren. Ihnen fehlen die für T- oder B-Zellen spezifischen Marker CD3 bzw. CD19 oder CD20. Humane NK-Zellen werden durch einen $CD3^-CD56^+$ Phänotyp definiert, wobei jedoch auch $CD56^-$ NK-Zellen nachgewiesen wurden, die besonders bei HIV-1- oder HCV-Infektionen eine Rolle spielen (Björkström et al., 2010; Hong et al., 2010).

Wechselwirkungen diverser NK-Zellrezeptoren mit ihren Liganden bestimmen den Aktivierungsstatus der NK-Zellen. Neben den aktivierenden Rezeptoren CD16 (Fcγ-Rezeptor-III, FcγRIII) und den natürlichen Zytotoxizität-Rezeptoren (Natural cytotoxicity receptors, NCR) wie z.B. NKp46 gibt es eine Gruppe sowohl inhibierender als auch aktivierender Rezeptoren auf NK-Zellen. Im menschlichen Organismus werden die sogenannten Killer-Zell immunglobulinähnlichen Rezeptoren (KIR) exprimiert. Das murine funktionelle Homolog stellen die Killer-Zell lektinähnlichen Rezeptoren (KLR), zu denen z.B. Ly49-Moleküle gehören, dar. Diese Rezeptoren binden zum größten Teil spezifisch an Haupthistokompatibilitätskomplex- (MHC)-Klasse-I Moleküle auf Targetzellen und besitzen in ihrer zytoplasmatischen Domäne entweder „Immunoreceptor tyrosine-based inhibitory motifs“ (ITIMs) oder sind mit „Immunoreceptor tyrosine-based activation motif“ (ITAM) tragenden Adapterproteinen assoziiert (Abi-Rached und Parham, 2005). Bei nicht-aktivierten NK-Zellen ist das Gleichgewicht zugunsten inhibitorischer Signale verschoben. MHC-Klasse-I-defiziente Zellen werden von NK-Zellen lysiert, da kein inhibierendes Signal über KIR oder Ly49-

Moleküle vermittelt werden kann. Dies ist die Grundlage der „missing-self"-Theorie, nach der das Fehlen von MHC-Klasse-I auf Targetzellen zu einem Angriff durch NK-Zellen aufgrund fehlender inhibitorischer Signale führt (Ljunggren und Kärre, 1990; Kärre, 2002).
Eine weitere wichtige Gruppe von NK-Zellrezeptoren, die ausschließlich aktivierend sind, bilden die NCR. Einer dieser Rezeptoren, NKp46 (CD335), kann der Identifizierung von NK-Zellen in verschiedenen Spezies dienen, da er auf keiner anderen Zellpopulation exprimiert wird (Walzer et al., 2007). NKp46 bindet zwar an Hämagglutinine von virusinfizierten Zellen, aber es wird vermutet, dass noch andere Liganden existieren (Mandelboim et al., 2001). Auch die Liganden der beiden weiteren NCR, NKp30 (CD337) und NKp44 (CD336) sind bisher nicht bekannt, und alle drei Rezeptoren spielen außerdem eine Rolle bei der Lyse von Tumorzellen (Pende et al., 1999; Mandelboim et al., 2001; Arnon et al., 2004; Vitale et al., 1998).

Eine Reihe weiterer inhibierender oder aktivierender Rezeptoren auf NK-Zellen, darunter z.B. CD94/NKG2-Rezeptoren, CD226 (DNAM-1), CD305 (LAIR-1), CD355 (CRTAM), TIGIT und CD85j (immunglobulinähnliches Transkript 2, ILT2) spielen bei der Interaktion mit anderen Zellen ebenfalls eine Rolle. Während aktivierende Funktionen für DNAM-1 und CRTAM und supprimierende für LAIR-1, TIGIT und ILT2 beschrieben wurden, hängt die Funktion der CD94/NKG2-Rezeptoren von der Spezifität der jeweiligen NKG2-Isoform ab (Pende et al., 2001; Bottino et al., 2003; Meyaard, 2008; Arase et al., 2005; Stanietsky et al., 2009). Die entsprechenden Liganden wie HLA-G (u.a.), Poliovirus-Rezeptor (PVR), Kollagen und Necl2 werden auch auf MSC exprimiert (Selmani et al., 2009; Ku et al., 2006; Spaggiari et al., 2006; Sullivan et al., 2007).

NK-Zellen haben aufgrund der Eigenschaft, Tumorzellen lysieren zu können, u.a. klinische Relevanz bei der hämatopoetischen Stammzelltransplantation (HSCT). So vermitteln sie einen „Graft-versus-leukemia" (GvL)-Effekt, ohne eine „Graft-versus-Host-Disease" (GvHD) im Empfänger zu induzieren bzw. sind sogar in der Lage, GvHD zu reduzieren (Verheyden et al., 2005; Olson et al., 2010).

Anhand der Oberflächenexpressionsdichte von CD56 lassen sich im Menschen zwei unterschiedliche NK-Zellsubpopulationen definieren: $CD56^{dim}$ und $CD56^{bright}$ NK-Zellen. Sie unterscheiden sich sowohl in der Expression von CD16, KIR und diverser anderer Marker als auch in ihren Funktionen. So zeigen $CD56^{dim}$ NK-Zellen nicht zuletzt wegen ihrer höheren CD16 Expression eine stärkere Zytotoxizität, da die Quervernetzung dieses Fc-Rezeptors zur ADCC führt, wodurch die Targetzelle lysiert wird. Zusätzlich ist

die Fähigkeit, Zellkonjugate zu formen, bei $CD56^{dim}$ NK-Zellen stärker ausgeprägt, und sie besitzen ca. zehnmal mehr Perforin und Granzyme. Dabei handelt es sich um Moleküle, die im Rahmen der Zytotoxizität von NK-Zellen produziert und sezerniert werden und zur Lyse der Zielzelle führen. $CD56^{bright}$ NK-Zellen hingegen exprimieren nur wenig CD16 und sind im Vergleich mit $CD56^{dim}$ NK-Zellen zwar schwächere „Killer", haben aber ein größeres Potenzial zur Produktion proinflammatorischer Zytokine wie IFN-γ, TNF-α oder GM-CSF (Jacobs et al., 2001; Fehniger et al., 1999). Daher lassen sich $CD56^{bright}$ NK-Zellen generell als „regulatorische NK-Zellen" beschreiben.

Der prozentuale Anteil von $CD56^{dim}$ und $CD56^{bright}$ NK-Zellen verteilt sich sehr unterschiedlich auf die verschiedenen Kompartimente des Körpers. $CD56^{dim}$ NK-Zellen machen im peripheren Blut 90% aller NK-Zellen aus, während ihr Anteil in Lymphknoten nur 10% beträgt. In der frühen Rekonstitutionsphase nach allogener HSCT können jedoch überproportional viele funktionell ausgereifte $CD56^{bright}$ NK-Zellen im peripheren Blut der Patienten detektiert werden. (Jacobs et al., 1992; Vukicevic et al., 2010). Als möglicher Grund hierfür werden erhöhte Level an IL-15, ein starker Stimulus für NK-Zellen, diskutiert. Aus jüngsten Daten lässt sich außerdem vermuten, dass es sich bei $CD56^{bright}$ NK-Zellen nicht zwingend um unreife oder noch nicht vollständig ausdifferenzierte NK-Zellen handelt (Vukicevic et al., 2010). Zwar ist es die gegenwärtig vorherrschende Meinung, dass $CD56^{bright}$ Vorläuferzellen zu $CD56^{dim}$ NK-Zellen darstellen, aber weitere *in vivo* Untersuchungen sind notwendig, um die Reifungs- und Differenzierungsstadien von NK-Zellen besser verstehen zu können. Experimente in der Maus waren zu dieser Thematik bisher allerdings nicht möglich, denn murine NK-Zellen exprimieren kein CD56. Entsprechend sind keine NK-Zellsubpopulationen anhand dieses Markers in der Maus unterscheidbar. Auf Grundlage detaillierter Analysen von sortierten NK-Zellen wurde im Rahmen dieser Arbeit CXCR3 in Kombination mit CD27 als mögliches Unterscheidungsmerkmal funktionell differenter NK-Zellen in der Maus untersucht. Die Ergebnisse stellen die Basis für künftige *in vivo* Experimente dar, deren Ergebnisse auf den Menschen extrapolierbar sein sollen (Marquardt et al., 2010).

Des Weiteren spielen NK-Zellen auch für den Verlauf einer Schwangerschaft eine sehr wichtige Rolle. Während der frühen Schwangerschaft stellen deziduale NK-Zellen die anteilsmäßig stärkste Lymphozytenfraktion dar. Ihre Aufgabe ist vor allem die Regulation der Einwanderung von Trophoblasten in die uterine Dezidua und die Remodellierung uteriner Spiralarterien (Hanna et al., 2006; De Oliveira et al., 2010). Sind diese Mechanismen gestört, kann es zu beschränktem Wachstum des Fötus, Frühgeburt, Präeklampsie oder Fehlgeburt kommen (Khong et al., 1986; Kim et al., 2003; Pijnenborg

et al., 1991; Ball et al., 2006). Studien haben gezeigt, dass auch der lösliche Faktor IDO für die Aufrechterhaltung einer Schwangerschaft notwendig ist (Munn et al., 1998). In diesem Zusammenhang wird diskutiert, ob möglicherweise plazentale MSC IDO ausschütten und so zur Immunsuppression und zum Schutz des Fötus beitragen (Jones et al., 2007).

1.1.2.2 T-ZELLEN

T-Zellen werden durch die Expression von CD3 definiert und sind Teil der adaptiven Immunität. Ihre Aktivierung erfolgt über den T-Zellrezeptor (TCR), der im Allgemeinen über Haupthistokompatibilitätskomplex (MHC) -gebundene Antigene erkennt und als Heterodimer entweder aus je einer α- und β-Kette (TCR1, 95% der T-Zellen) oder γ-/δ-Kette (TCR2, 5% der T-Zellen) aufgebaut ist. Da der zytoplasmatische Teil des TCR sehr kurz ist, kann über diesen keine Signalgebung erfolgen. Stattdessen werden Signale über die TCR-akzessorischen Moleküle CD3 und die ζ-Kette weitergeleitet; zusammen mit dem TCR bilden sie den TCR-Komplex. Erst die TCR-Korezeptoren CD4 oder CD8 ermöglichen schließlich eine Aktivierung der T-Zellen. Ausgereifte T-Zellen exprimieren grundsätzlich nur einen der beiden Korezeptoren, wobei das CD4:CD8-Verhältnis im peripheren Blut normalerweise bei 2:1 liegt. Die beiden Moleküle unterscheiden sich neben ihrem strukturellen Aufbau vor allem in ihrer Spezifität. So binden CD4-Moleküle nur an MHC-Klasse-II-Moleküle, während $CD8^+$ T-Zellen MHC-Klasse-I-restringiert sind.

T-Zellen werden derzeit entsprechend der von ihnen gebildeten Zytokine in mindestens fünf unterschiedliche Effektorpopulationen unterteilt: zytotoxische T-Zellen (CTL), T-Helfer- (T_H1-) Zellen, T_H2-Zellen, T_H17-Zellen sowie regulatorische T-Zellen (T_{reg}). Bis auf zytotoxische T-Zellen, die durch die Expression von CD8 charakterisiert sind, exprimieren alle anderen Populationen fast ausschließlich CD4 auf ihrer Oberfläche. Ebenfalls zu dieser Gruppe gehörend, wurden unlängst sogenannte T_H9-Zellen und T_H22-Zellen beschrieben (Veldhoen et al., 2008; Eyerich et al., 2009).

T_H1-Zellen sind an der zellulären Immunantwort gegen intrazelluläre Pathogene beteiligt und produzieren überwiegend IFN-γ und Lymphotoxine, was bei Entzündungen zur Rekrutierung und Aktivierung von Makrophagen und CTL führt (Bennett et al., 1997). T_H2-Zellen hingegen besitzen gänzlich entgegengesetzte Effektorfunktionen. Zytokine wie IL-4, IL-5 und IL-13 werden durch diese Zellen sezerniert und führen u.a. zur

Aktivierung der humoralen Immunität durch B-Zellen. T_H1- und T_H2-Zytokine inhibieren zudem die Differenzierung von naiven T-Zellen in den jeweils anderen Zelltyp.

Pathogene wie *Klebsiella pneumoniae*, *Citrobacter* oder *Candida albicans*, die weder durch T_H1- noch durch T_H2-Antworten ausreichend bekämpft werden können, werden durch T_H17-Zellen neutralisiert (Bettelli et al., 2008). Es wird auch eine besondere Rolle dieser Zellen bei der Entwicklung von Autoimmunerkrankungen postuliert (Leipe et al., 2010; Kleinschek et al., 2007). T_H17-Zellen werden aus naiven T-Zellen durch „Transforming growth factor" (TGF)-β und IL-6 induziert und können durch IL-23 weiter expandiert werden (Veldhoen et al., 2006; Langrish et al., 2005). Sie sind in der Lage, neben IL-17 auch IL-22 zu sezernieren, welches wiederum eine Antwort der angeborenen Immunität infolge erhöhter Expression von Akut-Phase-Proteinen und β-Defensinen durch Epithel- und Parenchymzellen provoziert (Wolk et al., 2010). *In vivo* konnte zudem nachgewiesen werden, dass bei einer Infektion mit *Mycobacterium tuberculosis* zuerst T_H17-Zellen die Lunge infiltrieren und anschließend T_H1-Zellen durch Freisetzung von Chemokinen durch T_H17-Zellen rekrutiert werden (Khader et al., 2007).

T_{reg}-Zellen repräsentieren in Mensch und Maus 5-10% aller $CD4^+$ T-Zellen und exprimieren CD25 und den Transkriptionsfaktor FoxP3 (Slobodin et al., 2010). Im Gegensatz zu den meisten anderen T-Zellpopulationen wirken T_{reg}-Zellen supprimierend auf die übrigen T-Effektorzellen und sind wichtig für die Induktion und Erhaltung von Selbsttoleranz. Ein Defekt oder Fehlen dieser Zellen wurde bei vielen Autoimmunkrankheiten wie dem systemischen Lupus Erythematodus (SLE) im Menschen beobachtet (Alvarado-Sanchez et al., 2006). T_{reg}-Zellen werden aufgrund ihrer supprimierenden Eigenschaften derzeit allerdings auch intensiv auf ihr therapeutisches Potenzial hin untersucht. In einem allogenen Knochenmarkstransplantationsmodell verhinderten T_{reg}-Zellen eine GvHD (Hoffmann et al., 2002). Durch Interaktion mit natürlichen Killer-T-Zellen (NKT)-Zellen kann dieser nützliche Effekt durch *in vivo* Expansion der T_{reg}-Zellen noch verstärkt werden (Pillai et al., 2009).

Die Kombination von IL-4 und TGF-β führt zur Differenzierung von T_H9-Zellen, die 2008 zum ersten Mal definiert worden sind (Veldhoen et al., 2008). Die fehlende Expression der Transkriptionsfaktoren GATA-3, RORγt und FoxP3 weist auf eine unabhängige T-Zellsubpopulation hin (Dardalhon et al., 2008; Veldhoen et al., 2008). Der Rezeptor für IL-9 wird von vielen Immunzellen exprimiert, darunter T- und B-Zellen, dendritischen

Zellen (DC), Mastzellen und Makrophagen (Renauld et al., 1995). Somit kann es sich auch hierbei um eine immunregulatorische T-Zellpopulation handeln.

Erst 2009 wurden T_H22-Zellen von EYERICH et al. beschrieben (Eyerich et al., 2009). IL-22 wird auch von T_H17- und NK-Zellen sezerniert, allerdings sind T_H22-Zellen defizient für IL-17 und daher deutlich von der T_H-17-Population unterscheidbar (Kreymborg et al., 2007; Cupedo et al., 2009). Der Rezeptor für IL-22 wird ausschließlich auf nicht-immunsystemassoziierten Zellen wie Keratinozyten, Zellen des Verdauungs- und Respirationssystems exprimiert (Wolk et al., 2004). Im Zuge von inflammatorischen Prozessen der Haut wie z.B. Psoriasis infiltrieren T_H22-Zellen die Epidermis und setzen dort IL-22 und TNF-α frei, was zu einer Verstärkung der Entzündung führt (Eyerich et al., 2009).

Zwei unterschiedliche bisher nicht erwähnte T-Zelltypen mit potenziell immunmodulierenden Eigenschaften wurden in der vorliegende Arbeit näher untersucht: $CD20^+$ T-Zellen und $S100B^+$ T-Zellen (Wilk et al., 2009). Beide haben nur einen kleinen prozentualen Anteil in humanem peripherem Blut und sind bisher nur unzureichend beschrieben worden. Während $CD20^+$ T-Zellen besonders durch ihre Freisetzung an proinflammatorischen Zytokinen gekennzeichnet sind, kann S100B der letztgenannten T-Zellpopulation Zellen des angeborenen Immunsystems aktivieren und damit ebenfalls zu einer Entzündungsreaktion beitragen.

1.1.2.3 WEITERE IMMUNREGULATORISCHE LYMPHOZYTEN: NKT-ZELLEN, B-ZELLEN

NKT-Zellen bilden eine Zwischenstufe von NK- und T-Zellen bzw. angeborener und adaptiver Immunität (Taniguchi et al., 2003). Sie machen ca. 0.5% der T-Zellen in peripherem Blut aus und exprimieren sowohl NK-Zellrezeptoren wie CD56 oder CD16 als auch einen funktionellen TCR, der sich allerdings stark von dem anderer T-Zellen unterscheidet (Bendelac et al., 2007). Er setzt sich aus einer invarianten α-Kette ($V\alpha24J\alpha18$ im Menschen, „variable“ (V) und „joining“ (J)) und einer limitiert variablen β-Kette zusammen (meist $V\beta11$) (Godfrey et al., 2004; Bendelac et al., 2007). Diese Kombination macht sie, anders als die übrigen T-Zellen, nicht MHC- sondern CD1d-restringiert, folglich erkennen NKT-Zellen mit ihrem TCR Glycolipide anstelle von Peptiden (Borg et al., 2007). Trotz ihrer anteilsmäßig kleinen Zahl ist der Einfluss von NKT-Zellen auf die Immunmodulation sehr vielfältig: sie beeinflussen den Verlauf von Krebserkrankungen, Autoimmunreaktionen, Infektionen, Allergien, GvHD sowie Abstoßung von Allotransplantaten (Godfrey et al., 2010; Kohrt et al., 2010; Pillai et al.,

2009). Bei Aktivierung produzieren NKT-Zellen große Mengen sowohl prostimulatorische Zytokine wie IFN-γ und TNF-α als auch antiinflammatorisch wirkendes IL-4 und IL-10. Auch IL-17 kann von diesen Zellen produziert werden (Coquet et al., 2008). Aufgrund des beschriebenen gegensätzlichen Zytokinmusters lassen sich NKT-Zellen nicht eindeutig einer immunaktivierenden oder immunsupprimierenden Funktion zuweisen. Während eine Antitumoraktivität mehrfach nachgewiesen wurde, sind NKT-Zellen zugleich in der Lage, Autoimmunität zu supprimieren (Saito et al., 2010; Metelitsa et al., 2001; Beaudoin et al., 2002). Sie werden damit zu Recht häufig als „zweischneidiges Schwert“ bezeichnet, und weitere Untersuchungen sind nötig, um die Rolle dieser immunregulativen Zellpopulation besser zu verstehen.

Eindeutig der adaptiven Immunität zuordbar sind hingegen B-Zellen. Neben ihrer Funktion als antigenpräsentierende Zellen (APC), ist die Produktion von Antikörpern einer der wichtigsten immunregulatorischen Funktionen von B-Zellen. Zusätzlich wurde aber nun eine immunregulative B-Zellpopulation nachgewiesen, die IL-10 produziert und im Mausmodell die Symptome experimenteller autoimmune Enzephalitis (EAE) mildern können (Yanaba et al., 2008; Matsushita et al., 2008).

1.1.2.4 Myeloide Zellen: Monozyten und Makrophagen, Dendritische Zellen, Mastzellen, Granulozyten

Neben den non-myeloiden Zellen beinhaltet die Gruppe der Leukozyten auch mehrere myeloide Linien, die ebenfalls immunregulatorisches Potenzial aufweisen.

Monozyten zirkulieren im Blut und sind außerdem im Knochenmark und vor allem in der Milz detektierbar. Nach 1-3 Tagen Zirkulation wandern sie in die Gewebe ein und differenzieren dabei zu Makrophagen aus. Je nach Lokalisierung unterscheidet sich die Bezeichnung dieser Makrophagen, wie z.B. Kupffer-Zellen in der Leber und Alveolarmakrophagen in der Lunge. Neben ihrer phagozytären Funktion induzieren Makrophagen entweder pro- oder antiinflammatorische Immunantworten (Crofton et al., 1978; Knolle et al., 1995; Sibille und Reynolds, 1990; Valstar et al., 2006; Haslett, 1999). Eine weitere Makrophagenpopulation bilden tumorassoziierte Makrophagen (TAM). Sie werden über Chemoattraktoren wie dem C-C-Motiv-Chemokinligand 2 (CCL2), CXC-Motiv-Chemokinligand 12 (CXCL12) und vaskulärem endothelialen Wachstumsfaktor (VEGF) zu Tumorzellen rekrutiert, was zu einer Verstärkung des Tumorwachstums führen kann (Pollard, 2004; Mantovani et al., 1992).

Klassische dendritische Zellen (DC) sind spezialisierte antigenprozessierende und -präsentierende Zellen. Sie sind hochmotil und wandern zu den T- und B-Zellzonen lymphatischer Gewebe, wo sie als ausgereifte DC z.B. T-Zellantworten regulieren können (Banchereau und Steinman, 1998). Wie bei anderen Zelltypen auch wurden hier sowohl aktivierende als auch supprimierende Mechanismen beobachtet (McLellan et al., 1996; Quaratino et al., 2000). DC werden aufgrund ihrer unterschiedlichen Herkunft und Funktionen grundsätzlich in zwei Subpopulationen unterteilt: myeloide DC (mDC) und plasmazytoide DC (pDC) (Ardavin et al., 2001). Während mDC wie z.B. Langerhans-Zellen der Haut besonders als APC agieren und T-Zellen in der T-Zellzone von Lymphknoten aktivieren, exprimieren pDC vor allem die „Toll-like"-Rezeptoren (TLR) 7 und 9, mit denen sie einzelsträngige RNA oder bakterielle bzw. virale nichtmethylierte CpG DNA erkennen (Colonna et al., 2004; Akira und Hemmi, 2003; Heil et al., 2004; Hemmi et al., 2000). Infolge dieser Stimulation können pDC große Mengen an Typ 1-IFN freisetzen, das beispielsweise NK-Zellen aktivieren kann (Yoneyama et al., 2004; Szabo und Dolganiuc, 2008). DC können außerdem NK-Zellen zu drainierenden Lymphknoten rekrutieren, durch Zell-Zellkontakt und die Produktion von IL-12, IL-15, IL-18 und IFN-α/β NK-Zellen aktivieren und eine IFN-γ-Antwort induzieren, die wiederum eine T_H1-Polarisation auslösen kann (Fernandez et al., 1999; Yu et al., 2001; Martin-Fontecha et al., 2004).

Eine, obwohl bereits 1984 erstmals beschrieben, noch wenig untersuchte Zelllinie stellen regulatorische myeloide Suppressorzellen (MDSC) dar (Strober, 1984). Diese Zellen können aus Knochenmarkzellen in Gegenwart von G-CSF und GM-CSF generiert werden und spielen eine Rolle bei der Unterdrückung von Tumorzellwachstum, was in einem Lymphom-Mausmodell gezeigt worden ist. Zugleich zeigten NK-Zellen in diesem Modell eine gesteigerte IFN-γ Produktion (Highfill et al., 2010; Nausch et al., 2008). In anderen Studien wiederum wurde eine Induktion von T_{reg}-Zellen, aber gleichzeitig eine NK-Zell-inhibierende Wirkung nachgewiesen. Der letztgenannte Effekt war unabhängig von immunsupprimierenden Faktoren wie Arginase I oder IDO, vielmehr war direkter Zell-Zellkontakt vonnöten (Hoechst et al., 2009; Hoechst et al., 2008).

1.1.3 Immunregulatorische Zellen außerhalb des Immunsystems

Immunregulatorische Interaktionen sind nicht nur auf Zellen des Immunsystems beschränkt. Infolge vielseitiger Einsatzmöglichkeiten bei Transplantationen rücken vor allem Stammzellen in den Fokus der Forschung. Ein Aspekt der vorliegenden Studie liegt dabei auf den Wechselwirkungen von NK-Zellen mit MSC. Ebenfalls erwähnt

werden sollen an dieser Stelle aber auch andere immunmodulierende Zellen wie z.B. Endothelzellen.

1.1.3.1 STAMMZELLEN

Stammzellen sind eine Klasse undifferenzierter Zellen, die die Fähigkeit besitzen, in mehrere unterschiedliche spezialisierte Zelltypen zu differenzieren. Man kann zwei unterschiedliche Arten an Stammzellen unterscheiden: embryonale Stammzellen (ESC), die sich in jede mögliche Körperzelle ausdifferenzieren lassen, und adulte Stammzellen bzw. Vorläuferzellen, die nur ein bestimmtes Repertoire an Gewebetypen bilden können. Seit 2006 ist man zudem in der Lage, aus somatischen Zellen induzierte pluripotente Stammzellen (iPS) mittels Transfektion zu reprogrammieren.

Bisher sind die immunregulativen Fähigkeiten von ESC und iPS nicht ausreichend untersucht worden. Zwar sind ESC immunprivilegiert und rufen daher keine Immunantwort hervor, so sind sie doch in der Lage, Zellen des Immunsystems zu beeinflussen. Sie können z.B. T-Zellantworten unterdrücken und ESC-konditioniertes Medium kann die Ausreifung von APC aus dem Knochenmark verhindern (Li et al., 2004; Koch et al., 2008). Uneindeutig ist, ob ESC von NK-Zellen lysiert werden (Drukker et al., 2002; Dressel et al., 2010). Aus ESC gewonnene Extrakte führten bei DC zu einem unreifen Phänotyp und einer verringerten Kapazität, allogene T-Zellen zu aktivieren. Zusätzlich war die Proliferation in allogenen gemischten Lymphozytenreaktionen (MLR) reduziert (Mohib et al., 2010).

Adulte oder somatische Stammzellen wurden bereits in den 1950er Jahren definiert, sie teilten sich in zwei Gruppen: hämatopoetische Stammzellen (HSC), die die Vorläuferzellen für Zellen des Immunsystems enthalten, sowie MSC. Erst 40 Jahre später wurden außerdem sogenannte neuronale Stammzellen im menschlichen Gehirn nachgewiesen (Reynolds und Weiss, 1992).

MSC existieren in beinahe allen Geweben wie Knochenmark, Nabelschnurblut, Wharton’s jelly (Nabelschnur), Fettgewebe, Haarfollikeln, Zahnwurzeln, Haut, peripherem Blut, Lunge, Leber und Milz (Pittenger et al., 1999; Kogler et al., 2004; Majore et al., 2010; Zuk et al., 2001; Shih et al., 2005; Trubiani et al., 2005; Zvaifler et al., 2000; in 't Anker et al., 2003). Da sie nicht mittels eines spezifischen Markers identifiziert werden können, wurden bestimmte Kriterien aufgestellt, um eine uniformere Klassifizierung von MSC zu ermöglichen: 1) MSC müssen plastikadhärent sein, 2) MSC müssen CD73, CD90 und CD105 exprimieren bei gleichzeitigem Fehlen von z.B. CD14,

CD11b, CD45, CD133 und HLA-DR und 3) MSC müssen die Fähigkeit besitzen, in chondrogene, osteogene und adipogene Zelllinien differenzieren zu können (Dominici et al., 2006). Letztgenannte Eigenschaft macht sie wiederum zu einer wichtigen Ressource für Tissue Engineering und regenerative Medizin. In zahlreichen klinischen Studien wurden MSC bereits bei Knochen-, Knorpel- oder Rückenmarksschäden, kardiovaskulären oder hämatologischen Erkrankungen oder Osteogenesis imperfecta eingesetzt (Gangji und Hauzeur, 2010; Wakitani et al., 2002; Sykova et al., 2006; Stamm et al., 2004; Fouillard et al., 2007; Horwitz et al., 2001). Dabei ist es möglich, auch über allogene Grenzen hinaus MSC zu transplantieren, ohne Abstoßungsreaktionen zu provozieren (Le Blanc et al., 2003). Entsprechend müssen Mechanismen durch MSC vermittelt werden, die einen immunsupprimierenden Effekt haben. Auch dieser Sachverhalt lässt sich für die Klinik nutzen – MSC wurden bereits zur Vermeidung akuter GvHD oder zur Unterstützung des Anwachsens hämatopetischer Stammzellen bei HSCT erfolgreich eingesetzt (Ringden et al., 2006). *In vitro* konnte die MSC-vermittelte Inhibierung von Lymphozyten nachgewiesen werden, allerdings sind die Ursachen hierfür nicht eindeutig geklärt. Während in einigen Fällen PGE2 und IDO die entscheidenden Faktoren zu sein scheinen, wurde in anderen Untersuchungen Zell-Zellkontakt als Auslöser der Supprimierung vermutet (Krampera et al., 2006a; Sotiropoulou et al., 2006; Aggarwal und Pittenger, 2005; Augello et al., 2005).

Die meisten Studien wurden mit aus dem Knochenmark stammenden MSC (BM-MSC) durchgeführt. Andere Quellen für die Isolierung von MSC erfordern jedoch weniger invasive Eingriffe bzw. werden die entsprechenden Gewebe sonst meist wegen fehlender nachfolgender Verwendung entsorgt. So lassen sich z.B. aus der Nabelschnur, sofern innerhalb kurzer Zeit nach der Geburt verarbeitet, MSC ohne großen Aufwand isolieren (Majore et al., 2009). Ob ähnlich immunsupprimierende Funktionen durch Nabelschnur-MSC (UC-MSC) vermittelt werden, wurde in der vorliegenden Arbeit mit dem Schwerpunkt auf NK-Zell-vermittelte Funktionen wie Zytokinproduktion und Zytotoxizität näher untersucht.

1.1.3.2 Sonstige Zellen

Im Phänotyp den MSC sehr ähnlich sind Fibroblasten (Haniffa et al., 2007). Allerdings werden letztgenannte Zellen nicht den Stammzellen zugeordnet, da es sich um final ausgereifte Stromazellen handelt und sie sich *in vitro* nicht in Osteoblasten und Adipozyten ausdifferenzieren lassen (Cappellesso-Fleury et al., 2010). Bei längerer Expansion werden Fibroblasten tumorigen, was ein großes Risiko für Transplantationen

mit MSC darstellt: kontaminierende Fibroblasten könnten im Empfänger die Bildung von Tumoren hervorrufen (Prockop und Olson, 2007). Gleichzeitig sind Fibroblasten, wie auch MSC, immunprivilegiert, induzieren also keine Abstoßungsreaktion, und besitzen immunmodulatorische Fähigkeiten, obwohl auch hier die genauen Mechanismen noch weiter untersucht werden müssen (Cappellesso-Fleury et al., 2010).

Endothelzellen sind ebenfalls nicht mit dem Immunsystem assoziiert und dennoch für dessen Funktionalität mitentscheidend. Bei Entzündungen wird das Endothel lokal aktiviert, was zur Sekretion zahlreicher löslicher Mediatoren und zur Expression u.a. von ICAM führt. Leukozyten binden mit spezifischen Oberflächenrezeptoren wie LFA-1 an die Endothelzellen und können schließlich durch die Gefäßwand in Richtung des entzündeten Gewebes transmigrieren (Extravasation). Als Modell für *in vitro*-Experimente werden auch hier häufig Endothelzellen aus der Nabelschnur gewonnen, die sogenannten Human Umbilical Vein Endothelial Cells (HUVEC) (Tissari et al., 2005; Coupel et al., 2007).

2 FRAGESTELLUNG UND ZIELSETZUNG

Besonders hinsichtlich zellulärer Therapien beim Menschen ist eine genaue Kenntnis der zu erwartenden Effekte oder Komplikationen unerlässlich. Viele Mechanismen und Wechselwirkungen sind dennoch bisher völlig unbekannt. Für die vorliegende Arbeit wurden definierte Zellpopulationen auf ihre immunregulatorischen Eigenschaften hin untersucht. Der Fokus lag hierbei auf NK-Zellen, MSC und T-Zellen.

Derzeit werden insbesondere regulatorische T-Zellen intensiv auf ihre Anwendung als Alternative zu Immunsuppressiva bei Transplantationen hin untersucht. In dieser Hinsicht könnten aber auch NK-Zellen und MSC für den Erfolg von Transplantationen eine nicht unerhebliche Rolle spielen. Dies bezieht sich sowohl auf HSCT als auch auf die Transplantation rekonstruierter Gewebe wie beispielsweise Knochen- oder Knorpelimplantate (Tissue Engineering) (Olson et al., 2010; Krampera et al., 2006b).
Humane NK-Zellen können in zwei funktionell unterschiedliche Subpopulationen unterschieden werden (Jacobs et al., 2001). Äquivalente Populationen in der Maus lassen sich aufgrund fehlender CD56 Expression nicht detektieren, entsprechend waren *in vivo*-Experimente zur Untersuchung der NK-Zellentwicklung und -differenzierung bisher nicht möglich. Während andere Studien CD27 als möglichen Marker vorschlagen, zeigten eigene Vorarbeiten eine Rolle für CXCR3 für die Definition von Korrelaten in der Maus (Hayakawa und Smyth, 2006; Wendt et al., 2006). Auf dieser Basis sollte ein Mausmodell etabliert werden, mit dem es möglich ist, Untersuchungen zu NK-Zellsubpopulationen *in vivo* durchzuführen und dabei besonders die Rolle regulatorischer $CD56^{bright}$ NK-Zellen, die nach HSCT überproportional im peripheren Blut detektierbar sind, im Menschen besser zu verstehen (Marquardt et al., 2010).

Da sowohl für NK-Zellen als auch für MSC ein therapeutischer Effekt nach HSCT durch das Entgegenwirken einer GvHD gezeigt wurde, ist die Anwendung im Menschen eine mögliche Alternative zum Einsatz von Immunsuppressiva (Ringden et al., 2006). Obwohl MSC aus vielen unterschiedlichen Geweben isoliert werden können, wurden für die meisten Studien MSC aus Knochenmark generiert und immunsupprimierende Wirkungen durch diese Stammzellen demonstriert. Für das vorliegende Manuskript wurden hingegen UC-MSC eingesetzt. Die Isolierung dieser Zellen erfordert verständlicherweise keinen invasiven Eingriff und ist daher ethisch eher vertretbar als die Verwendung von BM-MSC. Ob infolge von Koinkubation mit UC-MSC ebenfalls eine Inhibierung des Immunsystems, speziell nicht-voraktivierter NK-Zellen, stattfindet und welcher Mechanismus hier entscheidend ist, wurde im Rahmen dieser Arbeit untersucht.

Des Weiteren wurden zwei unterschiedliche T-Zellsubpopulationen, die nicht den regulatorischen T-Zellen angehören, hinsichtlich ihrer immunregulatorischen Funktionen analysiert. Es handelt sich dabei um $CD20^+$ T-Zellen sowie $S100B^+$ T-Zellen. Beide Zelltypen sind im Menschen nur zu kleinen prozentualen Anteilen detektierbar und sind bisher nur sehr unzureichend beschrieben worden. Sie spielen aber möglicherweise bei verschiedenen Erkrankungen bzw. Therapien eine Rolle.

CD20 ist ein unglykolisiertes Transmembranprotein und ist das erste Differenzierungsantigen, das spezifisch für B-Zellen identifiziert wurde (Tedder et al., 1988; Stashenko et al., 1980). Es wird auf Pro- und ausgereiften B-Zellen mit Ausnahme von Plasmazellen exprimiert (Tedder und Engel, 1994). CD20 spielt in der Regulation von B-Zell-Proliferation, -Aktivierung und Apoptose ein Rolle, wobei aber kein natürlicher Ligand bekannt ist (Tedder et al., 1986). Des Weiteren fungiert CD20 als transportregulierender Calciumkanal, was ursprünglich mittels Patch Clamp Analysen transfizierter Zelllinien gemessen wurde (Kanzaki et al., 1997; Bubien et al., 1993).

Der therapeutische anti-CD20 Antikörper Rituximab wird sehr vielseitig eingesetzt, ohne dass tatsächlich alle möglichen Neben- oder Wechselwirkungen bekannt sind. Mittels dieses Antikörpers werden B-Zellen, die typischerweise CD20 exprimieren, *in vivo* depletiert (Golay et al., 2000). Wir konnten im humanen peripheren Blut sowie in diversen Kompartimenten (Leber, Lymphknoten, Thymus) $CD20^+$ T-Zellen nachweisen, die nach Behandlung mit Rituximab ebenso wie B-Zellen nicht mehr detektierbar waren. Sollten die $CD20^+$ T-Zellen pro- oder antiinflammatorische Funktionen besitzen, kann dies Auswirkungen auf den Therapieerfolg haben bzw. einen alternativen Wirkmechanismus darstellen. Es sollte daher eine detaillierte Analyse des Phänotyps sowie funktioneller Charakteristika von $CD20^+$ T-Zellen erstellt werden.
T-Zellen produzieren außerdem, je nach Subpopulation, eine Vielzahl bestimmter löslicher Mediatoren. Das Protein S100B, von dem sonst nur eine Sekretion durch Gliazellen und andere nicht-immunsystemassoziierte Zellen bekannt war, konnten wir intrazellulär in zirkulierenden T-Zellen nachweisen (Moore, 1965). Der Rezeptor für S100B, RAGE, wird auf einer Vielzahl von Zellen auch innerhalb des Immunsystems exprimiert (Leclerc et al., 2009). Um Aufschluss über die biologische Funktion dieser $S100B^+$ T-Zellen zu erhalten, sollten wiederum phänotypische und funktionelle Eigenschaften und zudem die Wirkung von S100B auf andere Zellen des Immunsystems analysiert werden.

In dieser Arbeit soll durch verschiedene Beispiele gezeigt werden, dass nicht nur regulatorische T-Zellen, die derzeit Gegenstand intensiver Forschung sind, die Immunantwort beeinflussen können, sondern dass sowohl im Menschen als auch in der Maus weitere Zellpopulationen existieren, die eine große Bedeutung bezüglich Immunregulation besitzen. Folglich kann das Verständnis über das Netzwerk der Interaktionen zwischen Zellen innerhalb und außerhalb des Immunsystems auf Basis der vorliegenden Arbeit erweitert werden.

3 MURINE CXCR3^{+}CD27^{BRIGHT} NK CELLS RESEMBLE THE HUMAN CD56^{BRIGHT} NK-CELL POPULATION

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Murine CXCR3^{+}CD27^{bright} NK Cells Resemble the Human CD56^{bright} NK Cell Population

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Abbreviations:

neCXCR3$^{-/+}$ NK cells: newly emerged CXCR3$^{-/+}$ NK cells

sCXCR3$^{-/+}$ NK cells: stable CXCR3$^{-/+}$ NK cells

LN: lymph nodes

BM: bone marrow

Summary

Human Natural Killer (NK) cells can be subdivided into $CD56^{dim}$ and $CD56^{bright}$ NK cells, which exhibit different phenotypical and functional characteristics. As murine NK cells lack CD56 or a distinct correlate, direct comparative studies of NK cells in mice and humans are limited. Although CD27 is currently proposed as a feasible subset marker in mice, we assume that the usage of this marker alone is insufficient. We rather investigated the expression of the chemokine receptor CXCR3 for its suitability for distinguishing murine NK cell subsets with simultaneous consideration of CD27. Compared to $CXCR3^-$ NK cells, exerting stronger cytotoxic capability, $CXCR3^+$ NK cells displayed an activated phenotype with a lower expression of Ly49 receptors, corresponding to human $CD56^{bright}$ NK cells. Also in common with human $CD56^{bright}$ NK cells, murine $CXCR3^+$ NK cells exhibit prolific expansion as well as robust IFN-γ, TNFα and MIP-1α production. We additionally demonstrated changes in both CXCR3 and CD27 expression upon NK cell activation.

In summary, CXCR3 serves as an additional applicable marker for improved discrimination of functionally distinct murine NK cell subsets that comply with those in humans.

Introduction

Natural Killer (NK) cells are bone marrow-derived granular lymphocytes that are distinct from T and B cells. As a part of the innate immune system, NK cells play a role in early defence of infection and tumor rejection without prior sensitization. The most prominent function of NK cells is the ability to kill target cells by releasing lytic substances from intracellular granules. However, NK cells also produce several cytokines and their role as mediators in regulating innate and adaptive immune response is a main topic of current research [1-6].

Human NK cells are defined as $CD3^-CD56^+$, whereas murine NK cells, which lack CD56, are discriminated as $CD3^-NK1.1^+$. Recently, NKp46 (CD335) has been identified as a common marker for NK cells in both species, simplifying the future definition of NK cells [7].

In contrast to other lymphocytes, it is mainly the balance of activating and inhibitory signals, mediated by respective receptors, that regulates NK cell function [8]. Human NK cells express two structurally unrelated MHC class I-specific receptor families, the killer cell immunoglobulin-like receptors (KIR) and the killer cell lectin-like receptors (KLR). Mouse NK cells lack KIR, but they possess functional homologues with a lectin-like structure (Ly49 receptors). Inhibitory receptors bind classical and non-classical MHC class I and prevent the killing of MHC class I-bearing target cells.

Research over the last two decades revealed that NK cells do not represent a homogeneous lymphocyte fraction but can be subdivided into functionally distinct populations [9-13]. In humans, the two common NK cell subsets are defined according to the density of the surface marker CD56. As reviewed in WILK et al. (2008), $CD56^{dim}$ NK cells represent the classical cytotoxic NK cell subset, whereas $CD56^{bright}$ NK cells exert only marginal cytotoxic capacity and produce higher amounts of cytokines such as IFN-γ and TNFα [14;15]. The predominant function of $CD56^{bright}$ NK cells as cytokine producers indicates a primary role of these cells in immune regulation. Recently, a new approach to categorize NK cells by differentiating between "target cell responsive" and "cytokine responsive" was proposed [16].

The proportions of the NK cell subsets vary between the different compartments of the body. For instance, the ratio of $CD56^{dim}$ and $CD56^{bright}$ NK cells in peripheral blood is inverted in lymph nodes (ca. 10:1 in blood vs. ca. 1:10 in lymph nodes) [12;17;18]. The particular phenotype of decidual NK cells ($CD56^{superbright}KIR^+$) also hints to a specialized ‘equipment’ of NK cells in certain locations [18-20].

The lack of identical or comparable surface molecules is a major obstacle when transferring information from mouse models to human biology. Several attempts have been made to find markers defining mouse NK cell subsets equivalent to those in humans. Murine IFN-γ-producing killer DCs (IKDCs) with a $B220^{+}CD11c^{+}NK1.1^{+}$ phenotype are suggested to belong to the NK cell lineage and overlap with human $CD56^{bright}$ NK cells in cytokine production and lymphoid tissue distribution. However, lysis of YAC-1 target cells did not differ from $CD11c^{-}$ NK cells [21]. Recent data indicate CD127 as a potential marker for murine thymic NK cells that correspond to the human $CD56^{bright}$ NK cell subset [22]. Currently, CD27 is discussed as a potential NK cell subset marker for murine as well as for human NK cell subsets since CD27 is almost exclusively expressed on $CD56^{bright}$ NK cells. [23-26]. A similar expression pattern was detected for CXCR3. The chemokine receptor for CXCL9-11 has a crucial role for recruitment of NK cells to sites of inflammation and accumulation in tumors [27;28]. Microarray data revealed that CXCR3 might also be suitable for distinguishing mouse NK cell populations [29].

In this study we evaluated phenotype and function of $CXCR3^{-}$ and $CXCR3^{+}$ NK cells for their suitability for comparisons with human NK cell subsets with particular emphasis on the compartment-specific distribution and coexpression of CXCR3 with CD27.

Murine $CXCR3^{-}$ NK cells displayed higher CD16 and Ly49 receptor expression and stronger cytotoxicity than $CXCR3^{+}$ NK cells, which proliferated stronger and produced higher amounts of cytokines such as IFN-γ. Additionally, we found that $CD27^{+}$ NK cells can be subdivided into $CD27^{dim}CXCR3^{-}$, $CD27^{bright}CXCR3^{-}$ and $CD27^{bright}CXCR3^{+}$ and that both CD27 and CXCR3 expression changes upon stimulation of mouse NK cells.

In conclusion, our data suggest that murine NK cell subsets, complying in phenotype and function with those of humans, could be identified best by differential expression of CXCR3 and CD27. The definition of functionally distinct NK cell subsets in mice is useful for further *in vivo* analyses of NK cell development, activation and migration with respect to their human counterparts.

Results

Murine NK cells display compartment-specific CXCR3 and CD27 expression plus coexpression of CXCR3 on CD27^{bright} but not CD27$^{dim/-}$ cells.

Murine NK cells lack CD56 expression, the major marker for discrimination of functionally different NK cell subsets in humans. CD56^{dim} and CD56^{bright} NK cell ratios vary between the compartments. If equivalent NK cell subsets also exist in mice, one or more corresponding surface markers should be expressed at different levels when comparing the compartments. The surface receptor CD27 is discussed as a feasible marker for distinguishing murine NK cell subsets and is also a current focus in human NK cell research [25;26]. Microarray analyses of sorted human CD56^{dim} and CD56^{bright} NK cells also revealed a role for CXCR3, which is exclusively expressed on CD56^{bright} NK cells [29]. Therefore, we determined expression levels in different compartments in mice (Fig. 1). The expression patterns of CD27 and CXCR3 were relatively similar (Fig. 1A). The two markers were expressed in lower percentages on blood-derived and splenic NK cells as compared to NK cells from lymph nodes, bone marrow and liver. Notably, exclusively lung-derived NK cells were not consistent in the ratio of CD27 and CXCR3 expression. The majority of NK cells from the lung expressed CD27 (65%), whereas only 10% of lung NK cells were CXCR3^{+}. Further phenotypic analyses revealed that CXCR3 is predominantly expressed on CD16$^{-/dim}$ but not CD16^{bright} NK cells (Fig. 1B). Remarkably, CXCR3 was almost exclusively expressed on CD27^{bright} NK cells. This was consistent throughout all compartments (Fig. 1C). CD27^{-} NK cells never expressed CXCR3 (Fig. 2). All these and the following experiments were performed with B6 mice, however, similar results in phenotype and function were obtained with lymphocytes derived from Balb/c mice (data not shown). Therefore, murine NK cell subsets could be defined as CXCR3^{-}CD16^{bright}CD27$^{-/dim}$ and CXCR3^{+}CD16$^{-/dim}$CD27^{bright}.

The four NK cell subsets CXCR3^{-}CD27^{-}, CXCR3^{-}CD27^{dim}, CXCR3^{-}CD27^{bright} and CXCR3^{+}CD27^{bright} differ in their phenotype

Murine NK cell subsets are currently discriminated by the presence or absence of CD27 and CD11b [23]. Since CD27^{+} NK cells can be further subdivided into CD27^{dim}, CD27^{bright}CXCR3^{-}, and CD27^{bright}CXCR3^{+}, we next determined the expression of several activation markers, the maturation marker CD11b, and KLRs on these subsets. The percentages of receptor positive NK cells are depicted in Fig. 2. FACS analyses

confirmed similar tendencies in marker expression in spleen, bone marrow and peripheral blood (Fig. 2 and data not shown).

Compared to CXCR3$^-$ NK cells, CD27^{bright}CXCR3$^+$ NK cells displayed a higher percentage of CD69$^+$ and lower percentage of CD62L$^+$ NK cells. However, 2B4 expression did not differ within the CD27$^+$ NK cell subset. Furthermore, we detected increased expression of CD94 on CD27^{bright}CXCR3$^+$ NK cells. Percentages of CD11b and Ly49 receptor expression were slightly reduced compared to the other subsets. These results clearly show that NK cell subset phenotypes differ not only between CD27$^-$ and CD27$^+$ NK cells. Combinatory analyses of CD27 and CXCR3 revealed different phenotypical characteristics of CD27^{dim} and CD27^{bright} and CXCR3$^-$ and CXCR3$^+$ NK cells, respectively. In addition, CD62L, CD16, and 2B4 were coexpressed with CD11b, whereas CD69 and CD94 expression negatively correlated with CD11b expression (data not shown). Ly49 receptors were generally stronger expressed on CD11b$^+$ and CD16$^{-/dim}$ NK cells.

Surface expression of CXCR3 and CD27 changes upon activation

Before performing *in vitro* and *in vivo* activation assays with subsequent analyses of NK cell subsets, the expression stability of the defining subset marker was determined. Thus, the phenotypes of CXCR3$^-$ and CXCR3$^+$ NK cells after activation with IL-15 (used in the proliferation assay), IL-12 and IL-18 (used for the IFN-γ assay) or YAC-1 target cells (cytotoxicity assay) were analyzed.

When NK cells were stimulated with cytokines or target cells, downregulation of CXCR3 was observed in the sorted CXCR3$^+$ NK cell subset (Fig. 3A). Up to 50% of all CXCR3$^+$ NK cells exhibited decreased CXCR3 expression, representing a newly emerged CXCR3$^-$ (neCXCR3$^-$) NK cell population. Notably, a newly emerged CXCR3$^+$ (neCXCR3$^+$) NK cell subset appeared in IL-15-cultured CXCR3$^-$ NK cells after three days. However, neCXCR3$^+$ NK cells did not completely correspond to fresh CXCR3$^+$ NK cells because of their low CD27 expression (Fig. 3B). In contrast, sorted CXCR3$^+$ NK cells maintained high CD27 expression even after CXCR3 downregulation. When NK cells were stimulated with IL-12 and IL-18, CXCR3$^-$ NK cells upregulated CD27, whereas the CD27 expression decreased on CXCR3$^+$ NK cells (Fig. 3C). The activation potential and maturation level of murine NK cells has been shown to be associated with CD11b expression [30]. All fresh splenic CXCR3$^-$ NK cells expressed CD11b, whereas only 66% of CXCR3$^+$CD27^{bright} expressed this maturation marker (Fig. 3D, E). After three days in

culture in presence of IL-15, the percentage of $CD11b^+$ NK cells was significantly reduced in both subsets, particularly in the $CXCR3^+$ NK cell subset. These data demonstrate that NK cell subsets are able to modify their phenotype under certain conditions. Consequently, before performing functional assays of $CXCR3^-$ and $CXCR3^+$ NK cells, sorting of the two subsets was necessary.

IL-21-dependent proliferation of murine NK cell subsets

We previously reported that sorted human $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets differ in IL-21-dependent proliferation [31]. In order to investigate if this also holds true for murine NK cell subsets, we determined the proliferation of sorted $CXCR3^-$ and $CXCR3^+$ splenic NK cell subsets in response to activation with IL-21 and/or IL-15 in [^{3}H]thymidine and CFSE assays (Fig. 4). Upon stimulation, $CXCR3^+$ NK cells displayed a stronger proliferative response than $CXCR3^-$ NK cells, regardless of the combination of stimulating cytokines. Both IL-15 and IL-21 alone had comparable effects on $CXCR3^+$ NK cells, whereas $CXCR3^-$ NK cells proliferated poorly when stimulated with IL-21. In contrast, $CXCR3^-$ NK cells proliferated well in response to IL-15. As measured with [^{3}H]thymidine, the combination of IL-15 and IL-21 resulted in drastically increased proliferation of both subsets, especially in $CXCR3^+$ NK cells (Fig. 4B). This additive effect was not clearly detectable in CFSE assays where $7\text{-}AAD^-$ NK cells were analyzed to exclude apoptotic cells. In contrast to $CXCR3^-$ NK cells, however, almost all $CXCR3^+$ NK cells responded to stimulation with IL-15 and IL-21 alone or in combination.

Elevated cytotoxicity in $CXCR3^-$ mouse NK cells

In order to investigate if murine $CXCR3^-$ and $CXCR3^+$ NK cells display differential cytotoxic ability like human $CD56^{dim}$ and $CD56^{bright}$ NK cells, standard 4h ^{51}Cr-release assays and CD107a assays were performed (Fig. 5). Cytotoxic activity of $CXCR3^-$ NK cells against YAC-1 target cells was twice as high as $CXCR3^+$ NK cell-mediated cytotoxicity (Fig. 5A). Although $CXCR3^-$ NK cells also degranulated stronger than $CXCR3^+$ NK cells, a relatively high proportion of the latter subset was also $CD107a^+$ (Fig. 5B). We further analyzed degranulation of sorted $CXCR3^+$ NK cells and discriminated $neCXCR3^-$ NK cells from NK cells that maintained CXCR3 on their surface (stable; sCXCR3), revealing that NK cells which downregulated CXCR3 expression displayed stronger degranulation than $sCXCR3^+$ NK cells (Fig. 5C). Strongly

reduced percentages of degranulating NK cells were measured when using negatively sorted NK cells which had no contact to anti-NKp46 antibody (data not shown).

Production of IFN-γ, TNFα, and MIP-1α is increased in stimulated CXCR3$^+$ NK cells

As human CD56^{bright} NK cells are known to produce higher amounts of cytokines such as IFN-γ than CD56^{dim} NK cells, cytokine production of sorted murine CXCR3$^-$ and CXCR3$^+$ NK cells was determined both on mRNA and protein levels (Fig. 6) [14;15].

Upon stimulation with PMA/ionomycin or IL-12 and IL-18 (15h), mRNA levels of MIP-1α, TNFα, and IFN-γ were higher in CXCR3$^+$ as compared to CXCR3$^-$ NK cells (Fig. 6A). Also on the protein level, CXCR3$^+$ NK cells were the main IFN-γ producing subset when stimulated with IL-12 and IL-18 (Fig. 6B). In CXCR3$^-$ NK cells, CD27$^+$ NK cells displayed slightly stronger IFN-γ production than CD27$^-$ NK cells, whereas in CXCR3$^+$ NK cells no difference was detected between CD27$^-$ and CD27$^+$ NK cells. CD27$^{-/dim/bright}$ NK cells appeared in the CXCR3$^+$ subset after stimulation of the NK cells which downregulated CD27 expression (see also Fig. 3C).

Induction of IFN-γ was also detected upon contact with YAC-1 cells as assessed by the CD107a assay (data not shown). In general, CXCR3 expression correlated positively with IFN-γ, TNFα, and MIP-1α production. We did not detect any cytokine production in unstimulated NK cells (data not shown).

Discussion

In humans, $CD56^{dim}$ and $CD56^{bright}$ NK cells represent functionally distinct subsets [9;12;13]. In contrast, mouse NK cells express neither CD56 nor a correlate. This limits investigations of equivalent murine NK cell subsets and therefore extrapolations of murine data to the human system. Thus, the definition and characterization of NK cell subsets in mice is a major topic of current NK cell research. Recently, markers such as CD94 or CD27 were proposed as potential markers for murine NK cell subsets corresponding to the human $CD56^{dim}$ and $CD56^{bright}$ paradigm [23;32]. Based on microarray gene analyses, we previously demonstrated the almost exclusive coexpression of CXCR3 on human $CD56^{bright}$ NK cells [29]. Therefore, CXCR3 (CD183) could be described as a molecule that allows comparisons between human and mouse NK cell subsets [14;29]. In this study, CXCR3 expression, and particularly coexpression of CD27 on murine NK cells, was analyzed in order to determine the optimal marker constellation to define a murine NK cell subset.

The percentages of NK cell subsets in humans and mice vary considerably among the compartments. For instance, in humans 90% of circulating and 85% of splenic NK cells are $CD56^{dim}CD16^{bright}$, whereas in lymph nodes up to 90% of NK cells display a $CD56^{bright}CD16^{-/dim}$ phenotype [18;33]. In mice, we also detected higher percentages of $CXCR3^{+}$ NK cells in lymph nodes and other compartments such as bone marrow, uterus, and liver. We observed a similar distribution pattern for CD27, except for lung-derived NK cells, which presented a very low CXCR3 but high CD27 expression. In healthy humans, the majority of lung NK cells displays a $CD56^{dim}$ phenotype [34]. However, the similar expression patterns of CXCR3 and CD27 suggest a coexpression of both markers. In fact, CXCR3 was exclusively expressed on $CD27^{bright}$ NK cells, although this could not be shown for human NK cells [26]. In recent publications, mouse NK cell subsets were defined as $CD27^{+(high)}$ and $CD27^{-(low)}$ [23]. According to our data regarding CXCR3 and CD27 expression, murine NK cell subsets can be more precisely differentiated into $CD27^{-}CXCR3^{-}$, $CD27^{dim}CXCR3^{-}$, $CD27^{bright}CXCR3^{-}$, and $CD27^{bright}CXCR3^{+}$ NK cells. These populations are constitutively present in several organs with varying abundances. Regarding the phenotype, the $CXCR3^{+}CD27^{bright}$ NK cell subset contained a greater proportion of $CD69^{+}$, $CD94^{+}$, $CD62L^{-}$, $CD16^{-/dim}$, $CD11b^{-}$, and $Ly49s^{-}$ NK cells as compared to $CXCR3^{-}CD27^{bright}$ NK cells. The putative corresponding human $CD56^{bright}$ NK cell subset is similarly defined as $CXCR3^{+}$, $CD27^{++}$, $CD69^{++}$, $CD16^{-/dim}$, $CD11b^{-/+}$, and KIR^{-} [14;25;26;35;36]. However, CD62L is reported to be more strongly expressed

on $CD56^{bright}$ NK cells [29;37]. CD62L, like CXCR3, is suggested to play a role in NK cell migration and recruitment. A lower expression of the adhesion molecule CD62L could be substituted by the expression of other receptors. This can also be suggested for CCR7, which is expressed on human $CD56^{bright}$ NK cells but not $CD56^{dim}$ NK cells. CCR7 was not detected on any murine NK cell population, illustrating the limits in comparability of murine and human NK cells ([23;38] and data not shown).

Utilization of certain markers for *in vivo* and *in vitro* studies is limited by expression stability. For instance, activation of human NK cells results in upregulation of CD56, which impairs the distinction of activated $CD56^{dim}$ NK cells from resting $CD56^{bright}$ NK cells [14;39]. We analyzed CD27 and CXCR3 expression upon activation of NK cells and demonstrated downregulation of CXCR3 on $CXCR3^{+}$ NK cells. Rapid ligand-induced internalization and degradation of CXCR3 as well as its *de novo* synthesis has been reported for both NK and T cells [40;41]. A physiological role of changes in CXCR3 expression during maturation and trafficking of NK cells was suggested based on *in vitro* and *in vivo* data [41;42]. Notably, 72h activation of sorted $CXCR3^{-}$ NK cells with IL-15 induced expression of this marker on about 50% of the cells. This ne$CXCR3^{+}$ NK cell population expressed CD27 at lower density than fresh $CXCR3^{+}$ NK cells and therefore did not completely correspond to resting $CXCR3^{+}$ NK cells. Comparable results were reported for sorted human $CD27^{+}$ NK cells, which lost CD27 expression upon stimulation with IL-15. This new $CD27^{-}$ subset was highly cytotoxic [25]. Importantly, $CXCR3^{+}$ NK cells that downregulated CXCR3 expression in our experiments, displayed stronger degranulation than stable $CXCR3^{+}$ NK cells. Thus, the phenotype still correlated with the capacity to kill target cells. However, if and to which degree expression changes also occur *in vivo*, has to be determined. CXCR3 downregulation can be assumed at least for tumor-infiltrating NK cells [28]. Regarding the maturation level of the NK cell subsets, analyses of CD11b expression revealed an immature phenotype of a fraction of $CXCR3^{+}$ NK cells. Recent studies showed that KLRG1 is acquired by developing NK cells, which are entirely $CD27^{-}$ [43]. $CD27^{-}$ NK cells never expressed CXCR3, supporting the suggestion, that $CXCR3^{-}$ display a more mature NK cell subset. However, as already discussed by DI SANTO, "immature" NK cells may mediate effector functions different from those of their "mature" counterparts [44]. The decrease of CD11b expression in culture after three days could result from differential proliferative capacities. Because CXCR3 is essential for recruitment of NK cells in response to infection, it is very likely that $CXCR3^{-}$ and $CXCR3^{+}$ NK cell subsets fulfil different

functional roles in the immune system. To clarify whether or not murine CXCR3$^-$ and CXCR3$^+$ NK cells differ in their functional characteristics like human CD56^{dim} and CD56^{bright} NK cells, we determined proliferative capacity, cytolytic activity, degranulation and cytokine production of the NK cell populations in response to physiological stimulation. In general, CXCR3$^+$ NK cells displayed stronger proliferative capacity and cytokine production (IFN-γ, TNFα, MIP-1α), whereas CXCR3$^-$ NK cells more efficiently lysed YAC-1 target cells. HAYAKAWA et al. reported a stronger cytotoxicity in CD27$^-$ NK cells compared to CD27$^+$ NK cells, which are in part CXCR3$^+$ [23]. However, only purified CD11b$^+$ NK cells were used in the cytotoxicity assays they performed. CD11b has been associated with elevated levels of cytolytic function of mature NK cells. Whereas all CXCR3$^-$ NK cells were CD11b$^+$ and highly cytolytic, a fraction of CXCR3$^+$ NK cells lacked CD11b expression. CXCR3$^+$ NK cells displayed lower cytotoxicity, and this could be due to their different developmental stage. Interestingly, the proliferative response of CXCR3$^+$ NK cells to IL-21 was far greater than that of CXCR3$^-$ NK cells. Although inhibiton of proliferation of mouse NK cells by IL-21 has been reported, the effect was not analyzed for different NK cell subsets [45]. For human NK cells we showed that CD56^{bright} NK cells exhibited a strong proliferative response towards IL-21 when combined with IL-2, although IL-21R is equally expressed on CD56^{dim} and CD56^{bright} NK cells [31]. These results also correspond to our murine data. Compared to CXCR3$^-$ NK cells, slightly higher percentages of CXCR3$^+$ NK cells displayed IL-21R expression (data not shown). As shown for the human system, a specific role for STAT proteins can be suggested for the induction of proliferation of murine NK cells by IL-21 and IL-2. The two cytokines may induce the formation of particular STAT protein dimers, which could differentially affect the proliferation of CXCR3$^-$ and CXCR3$^+$ NK cells. The combination and properties of STAT complexes still have to be determined in detail. In addition, signalling via IL-21R requires receptor heterodimerization with the γ chain (CD132), which is also shared by IL-2R and IL-15R [46;47]. In humans, the high affinity IL-2R, comprising CD25, CD122 and CD132, is only expressed on CD56^{bright} but not CD56^{dim} NK cells. The stronger proliferation of CXCR3$^+$ NK cells could be due to the higher expression of CD122 on CXCR3$^+$ NK cells (data not shown). In addition, CD11b$^-$ NK cells are reported to proliferate faster than CD11b$^+$ cells *in vivo* [30]. Since a fraction of CXCR3$^+$ NK cells was negative for CD11b, it is plausible that these cells proliferate more strongly. A major role of NK cells is to kill malignant tumor cells. Accumulation of NK cells in certain tumor tissue is dependent on

CXCR3 expression and the presence of IFN-γ [28]. In this context, $CXCR3^+$ NK cells are probably important for immunosurveillance, since these NK cells are also more potent IFN-γ producers than $CXCR3^-$ NK cells when stimulated with IL-12 and IL-18.

Regarding cytotoxicity, specific lysis of YAC-1 target cells by $CXCR3^-$ NK cells was twice as high as by $CXCR3^+$ NK cells. Furthermore, degranulation corresponded well to this result in several compartments, corroborating the specific role of $CXCR3^-$ NK cells in terms of cytolytic ability. Interestingly, NK cells exerted killing in our experiments even without prestimulation or presence of activating cytokines. This could be due to the binding of NKp46 mAbs used for sorting and which increased the degranulation of NK cells compared to negatively sorted NK cell subsets (data not shown).

However, we did not detect "all-or-none" responses in the two murine NK cell subsets. NK cells from all subsets have overlapping functional characteristics, and it was reported in humans and mice that e.g. IFN-γ production can change over a short period of time [29;30]. This demonstrates the variability of NK cell functions.

In conclusion, our data suggest the applicability of the surface marker CXCR3 for a better discrimination of murine NK cell subsets resembling those in humans. Characteristics of the discussed NK cell subsets are summarized in Fig. 7.

The introduction of CXCR3 as a marker for the discrimination of NK cell populations is useful for comparing murine NK cells with the equivalent human cells. This will form the basis for *in vivo* analyses of defined NK cell subsets in animal models. The differential coexpression patterns of markers such as CXCR3 and CD27 on NK cells enables a more detailed characterization of NK cell populations and indicates that the entire NK cell compartment is composed of more than just the two subsets, which have been the focus of recent NK cell research.

Materials and Methods

Mice

For all experiments, 8 to 16 week old female C57BL/6 mice (Charles River Laboratories, Wilmongton, MA, USA and animal facility Hannover Medical School, Hannover, Germany) were used. Mice were bred under specific pathogen free conditions and maintained in filter-topped cages under conventional conditions. Experiments involving animals were performed in compliance with federal and institutional guidelines (according to FELASA).

Preparation of cell suspensions

Peripheral blood was taken from the retro orbital plexus and collected into heparinized tubes. White blood cells were prepared by hypotonic lysis of red blood cells (RBC lysis buffer, containing NH_4Cl) and washed in PBS containing 3% fetal calf serum (FCS, PAA Lab., Cölbe, Germany).

Mice were euthanized by CO_2 asphyxiation or cervical dislocation. Organs (lymph nodes, spleen, uterus, thymus, liver, and lung) were extracted, sliced and homogenized with a 40µm nylon (BD Pharmingen, Heidelberg) or steel mesh. For isolation of bone marrow cells, femurs and tibiae were flushed with PBS using a 27G syringe. When necessary, cell suspensions were enriched for lymphocytes via density gradient (Lympholyte M, Cedarlane, Ontario, CN) or treated with red blood cell lysis buffer (0.146 M NH_4Cl, 0.1mM EDTA-Na_2, 1g $NaHCO_3$, pH 7.3).

Antibodies and cytokines

The mouse-specific mAbs Ly49D (4E5, FITC), Ly49G2 (4D11, FITC), Ly49C/I (5E6, FITC), NK1.1 (PK136, FITC, PE, APC), CD3 (145-2C11, FITC, PE, PerCP), CD16 (2.4G2, PE), CD27 (LG.3A10, PE), CD45 (30-F11, FITC, PerCP), CD107a (1D4B, FITC), CD122 (TM-β1, PE) and IFN-γ (XMG1.2, PE) were purchased from BD Biosciences. In addition, the following mAbs were used: CD3 (145-2C11, AlexaFluor® 647), CD27 (LG.3A10, PerCP/Cy5.5, Biolegend, San Diego, CA, USA), CD11b (M1/70.15, FITC, Eurobiosciences, Friesoythe, Germany), CD127 (A7R34, PE-Cy7), NKp46 (29A1.4, FITC, PE, eBioscience, San Diego, CA, USA) and CXCR3 [anti-CD183] (220803, PE, APC, R&D Systems, Minneapolis, USA). Isotype-matched mAbs were used as negative controls. To block FcγRII/III receptor-mediated unspecific

binding, CD16/32 mAb (2.4G2) from purified hybridoma supernatants was used for FcR blocking. The antibody-producing hybridomas were obtained from American Type Cell Collection (ATCC, Rockville, MD, USA).

The following recombinant cytokines were reconstituted and stored according to the manufacturers' recommendations and used as indicated in the text: human IL-2 (Eurocetus, Amsterdam, NL), murine IL-12, murine IL-15 (both ImmunoTools), murine IL-18 (MBL, Woburn, MA, USA) and murine IL-21 (R&D Systems).

Flow cytometric analysis of cell surface markers

After pre-incubation with 2.4G2 mAb or mouse serum, cells were incubated for 20 min at 4°C in the dark with the respective mAbs.

After washing, cells were analyzed on a multicolor flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany) using Cell Quest Pro software. Controls of medium and isotypes were performed simultaneously. Forward and side scatter properties of the cells were used to gate on the lymphocyte population. FACS data were analyzed using SUMMIT 5.1 software (Dako, Hamburg, Germany).

Cell sorting

In order to obtain pure NK cell populations or subpopulations ($CXCR3^+$ and $CXCR3^-$ NK cells), cell suspensions were sorted after staining with anti-NKp46 or anti-CD3, anti-NK1.1 and anti-CD45 (+ anti-CXCR3) mAbs using a FACSAria Cell Sorting System (BD Biosciences, Heidelberg, Germany) at the Hannover Medical School FACS facility (purity of the populations at least 95%). Data acquisition was performed using DIVA software (BD Biosciences).

Cell culture conditions

For stimulation assays, sorted NK cells or NK cell subpopulations were cultivated at 37°C and 5% CO_2 in complete R10 medium consisting of RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 50U/ml penicillin, 50µg/ml streptomycin, 1mM L-glutamine, 0.5mM sodium pyruvate (Biochrom) and 0.001% β-mercaptoethanol (ME; Merck, Darmstadt, Germany). To ensure the survival of NK cells, rIL-2 was added in a final suboptimal concentration of 100U/ml as indicated.

CFSE assay

Sorted splenic CXCR3$^-$ and CXCR3$^+$ NK cells were labelled with 1.5 µM (final concentration) CFSE (Molecular Probes, Invitrogen, Eugene, OR, USA) according to the manufacturer's recommendation. In detail, following CFSE labelling for 10 min at 37°C in PBS containing 0.1% BSA (Sigma-Aldrich), five volumes of ice cold medium were added and cells were incubated on ice for additional five min. After two washes, cells were resuspended in R10+ME supplemented with IL-2 (100U/ml), split into round-bottom 5ml-tubes (BD Biosciences) and stimulated with IL-15 (50ng/ml) and/or IL-21 (40ng/ml) for five days. 7-AAD$^-$ (Immunotech, Beckman Coulter, Marseille, France) cells were gated for analysis.

[^{3}H]thymidine assay

Sorted CXCR3$^-$ and CXCR3$^+$ NK cells (1 x 10^5/ml) were incubated in triplicates in R10+ME medium supplemented with 100U/ml IL-2. For stimulation, 50ng/ml IL-15 and/or 40ng/ml IL-21 were used. These optimized cytokine concentrations were determined by earlier dose titrations. After 48h, cultures were pulsed with 0.4 µCi [^{3}H]thymidine (Amersham Biosciences, Braunschweig, Germany), and incubated for another 24h. After harvesting, incorporated DNA was measured in a beta-counter (Perkin Elmer, Rodgau, Germany).

Cytotoxicity assay

Cytotoxicity of freshly sorted splenic CXCR3$^-$ and CXCR3$^+$ NK cells (5 x 10^5/ml) against YAC-1 target cells was assessed by standard 4-hr chromium release assay. Target cells were labelled with 3 MBq $Na^{51}CrO_4$ (Hartmann Analytic, Braunschweig, Germany), incubated for 1h at 37°C, washed two times and used for the assay within 1h. Cells were plated in V-bottom 96-well plates. Background values were determined by incubating target cells without effector cells. Maximal values were obtained by lysing target cells with 1% Triton X-100 (Sigma-Aldrich). After 4h, cells were pelleted and 100µl supernatant of each well was used for measurement of ^{51}Cr release in a gamma counter (MicroBeta/PerkinElmer, Waltham, MA, USA) in triplicates with effector:target (E:T) ratios of 10:1, 5:1, 2.5:1, and 1.25:1. Specific lysis was calculated by: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] x 100.

CD107a degranulation assay

CD107a (lysosome-associated membrane protein [LAMP-]1) is a marker for lysosomal granule exocytosis. Therefore, NK cell degranulation in response to stimulation with target cells was studied using an anti-CD107a mAb. For this experiment, lymphocytes (E:T ratio 10:1) or sorted $CXCR3^-$ and $CXCR3^+$ NK cells (E:T ratio 2:1) were incubated at 37°C in 5% CO_2 together with YAC-1 cells for 4h. Anti-CD107a mAb was added directly to the cell suspensions at a final concentration of 0.01mg/ml. After 1h of incubation, Monensin (BD Biosciences) was added as a golgi block at a final concentration of 5µg/ml and incubation was continued for additional 3h. In case of subsequent intracellular cytokine staining, brefeldin A (Sigma-Aldrich, München, Germany) was added at a final concentration of 2µg/ml for the last 3h of incubation time. Samples were finally surface-stained and analyzed via multicolor flow cytometry.

Intracellular staining

In order to determine the IFN-γ production, sorted $CXCR3^-$ and $CXCR3^+$ NK cells were cultured in 96-well round-bottom culture plates (Greiner, Frickenhausen, Germany) in the presence of rIL-2 (100U/ml), rIL-12 (10ng/ml) and rIL-18 (5ng/ml) for 15-17h. Optimal cytokine concentrations were determined by earlier dose titrations. Brefeldin A (Sigma-Aldrich, München, Germany) was added at a final concentration of 2µg/ml for the last 2h of incubation time.

Analysis of intracellular IFN-γ was preceded by surface staining at 4°C. After 30min, cells were washed twice and resuspended in PBS containing 3% FCS. After fixation with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 10min, cells were perforated with 0.1% saponin buffer (PBS supplemented with 0.1% saponin (Riedel-de Haën, Seelze, Germany) and 0.01 M HEPES (Roth, Karlsruhe, Germany)) and anti-IFN-γ mAb was added. After 30min of incubation and three washes, cells were analyzed as described above.

RNA isolation, reverse transcription and qPCR

Total RNA was isolated from overnight-stimulated (for IFN-γ: rIL-2 (100U/ml), rIL-12 (10ng/ml) plus rIL-18 (5ng/ml), for TNFα and MIP-1α: PMA (50ng/ml) plus ionomycin (500ng/ml), both obtained from Sigma Aldrich) sorted $CXCR3^-$ and $CXCR3^+$ NK cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany) with on-column DNase treatment. Reverse transcription (RT) was performed with Sensiscript or Omniscript RT Kits (Qiagen) in a thermocycler (Biometra, Göttingen, Germany), according to the

manufacturer's instructions. To quantify PCR results, 12.5µl SYBRGreen Supermix (Bio-Rad, Hercules, CA, USA) were added to primers (final concentration 250nM) and equal amounts of cDNA in a total volume of 25µl. QuantiTect® Primer Assays for β-actin, IFN-γ, TNFα, and MIP-1α were purchased from Qiagen. PCR was performed using an iCycler (Bio-Rad). Control cDNA from stimulated splenic lymphocytes was used to generate standard curves.

Statistics

Statistical analyses were performed as unpaired two-tailed *t*-tests, unless otherwise stated, using GRAPHPAD PRISM V5.00 software. Levels of significance are given as *P*-values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Plotted data represent mean plus/minus standard error of mean (SEM).

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Conflict of interest

The authors have declared that no competing interests exist.

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Figure legends

Fig. 1: CXCR3 and CD27: Compartment-specific expression and co-expression patterns on murine NK cells. Resting NK cells ($NK1.1^+CD3^-$) from different compartments were analyzed by flow cytometry. **A:** NK cells were analyzed for CXCR3 and CD27 expression (n=4-12). **B:** Peripheral blood-derived NK cells were gated. For analysis of CXCR3 expression on the subsets, gates were placed as indicated ($CD16^{dim}$ NK cells were analyzed together with $CD16^-$ NK cells). Each symbol indicates one subject, small horizontal bars represent the mean. **C:** $CD27^{dim}$ and $CD27^{bright}$ NK cells ($NK1.1^+CD3^-$) were gated and analyzed for CXCR3 expression. Mean values of percentages ± SEM of six experiments are depicted. ***P<0.001, **P<0.01 (Student's unpaired *t*-test). LN= lymph node, BM= bone marrow.

Fig. 2: The receptor expression differs between the four NK cell subsets $CD27^-CXCR3^-$, $CD27^{dim}CXCR3^-$, $CD27^{bright}CXCR3^-$, and $CD27^{bright}CXCR3^+$. Expression of several activation markers and Ly49 receptors on splenic $CXCR3^-$ (□) and $CXCR3^+$ (■) NK cells ($NKp46^+$) was determined by flow cytometry. Mean values of the percentages ± SEM of three experiments with different mice are shown. ***P<0.001, **P<0.01, *P<0.05 (one-way ANOVA, Bonferroni post-hoc test).

Fig. 3: CXCR3 and CD27 expression changes upon NK cell activation. Murine splenic NK cells ($NKp46^+$) were sorted into $CXCR3^-$ and $CXCR3^+$ subsets and activated with different stimuli in the presence of IL-2 (100U/ml). **A:** Sorted subsets were activated with IL-12 and IL-18 (15h), YAC-1 target cells (4h) or IL-15 (3d and 7d), respectively. CXCR3 expression before (shaded) and after (open) stimulation are overlaid. The corresponding percentages are depicted in the histograms. **B:** Sorted $CXCR3^-$ and $CXCR3^+$ NK cells were activated with IL-15 for 3d *in vitro* and were compared with resting NK cells with respect to their CD27 and CXCR3 expression. **C:** $CXCR3^-$ (left) and $CXCR3^+$ (right) NK cells were analyzed for their CD27 expression when cultured overnight with or without IL-12 and IL-18. **D**: Resting and IL-15-activated $CXCR3^-$ (shaded) and $CXCR3^+$ (black histogram, open) NK cells ($NKp46^+$) were compared for their CD11b expression levels (isotype control: grey histogram, open). **E**: Comparison of the percentages of $CD11b^+$ cells in resting and IL-15-activated $CXCR3^-$ (□) and $CXCR3^+$ (■) NK cell ($NKp46^+$) subsets. Mean values of three experiments using different mice ±

SEM are shown. ***P<0.001, **P<0.01, *P<0.05 (Student's unpaired *t*-test). Histograms and dot plots are representative of at least three independent experiments.

Fig. 4: Murine CXCR3$^-$ and CXCR3$^+$ NK cells proliferate differentially in response to IL-15 and /or IL-21. Murine splenic NK cells were sorted into CXCR3$^-$ and CXCR3$^+$ subsets and activated with IL-15 and/or IL-21. IL-2 (100U/ml) was supplemented in every experiment. **A:** Sorted NK cell (NK1.1$^+$CD3$^-$) subsets were activated in triplicates with IL-15 and/or IL-21, and proliferation was quantified by [^{3}H]thymidine assay. Mean values of three to five assays and mice ± SEM are shown. ***P<0.001, **P<0.01, (Student's unpaired *t*-test). **B:** Proliferation of 7-AAD$^-$ NK cells (NKp46$^+$) was determined by CFSE staining for flow cytometry after five days of culture. Histograms are representative of three independent experiments.

Fig. 5: Murine CXCR3$^-$ and CXCR3$^+$ NK cells display different cytotoxic ability. A: Sorted splenic CXCR3$^-$ and CXCR3$^+$ NK cells (NKp46$^+$) were incubated without target cells or in the presence of YAC-1 target cells at different effector:target (E:T) ratios in absence of stimulatory cytokines. Cytotoxicity was determined by standard ^{51}Cr release assay and specific lysis was calculated. Results were obtained from three independent experiments, each done in triplicate, with five mice in each experiment. Mean values ± SEM are shown. Significant differences between the mean values were determined by two-parameter ANOVA. ***P<0.001, **P<0.01. **B:** Lymphocytes from several compartments were incubated for 4h with or without YAC-1 target cells at an E:T ratio of 10:1. CXCR3$^-$ (□) and CXCR3$^+$ (■) NK cells were gated and analyzed for CD107a expression. **C:** Sorted CXCR3$^+$ NK cells (NKp46$^+$) were incubated for 4h with or without YAC-1 target cells and were afterwards discriminated into newly emerged CXCR3$^-$ (hatched bar) and stable CXCR3$^+$ (■) NK cells. Mean values ± SEM of at least three experiments and mice are shown. ***P<0.001, **P<0.01, *P<0.05, (Student's unpaired *t*-test). All cytotoxicity and degranulation assays were performed in the absence of stimulatory cytokines.

Fig. 6: Cytokine production differs between murine CXCR3$^-$ and CXCR3$^+$ NK cells. A: Sorted CXCR3$^-$ (□) and CXCR3$^+$ (■) NK cells (NKp46$^+$) were stimulated with PMA/ionomycin (or IL-12+IL-18 for IFN-γ) for 15h. mRNA transcription levels of MIP-1α, TNF, and IFN-γ were determined via RT-qPCR, each done in triplicate. β-actin was

used for normalization. Mean values ± SEM of at least three experiments with different mice are shown. *P<0.05 (Wilcoxon signed rank test). **B:** Sorted splenic $CXCR3^-$ (□) and $CXCR3^+$ (■) NK cells were stimulated with IL-12 (5ng/ml) and IL-18 (10ng/ml) in the presence of IL-2 (100U/ml). Intracellular IFN-γ was measured by flow cytometry. Mean values ± SEM of at least three experiments are shown.

Fig. 7: Comparison of human and murine NK cell subsets with regard to phenotype and function. While human NK cell subsets can be subdivided by CD56 expression density, equivalent murine NK cell subsets can be described by CXCR3 and CD27 expression.

Fig. 1

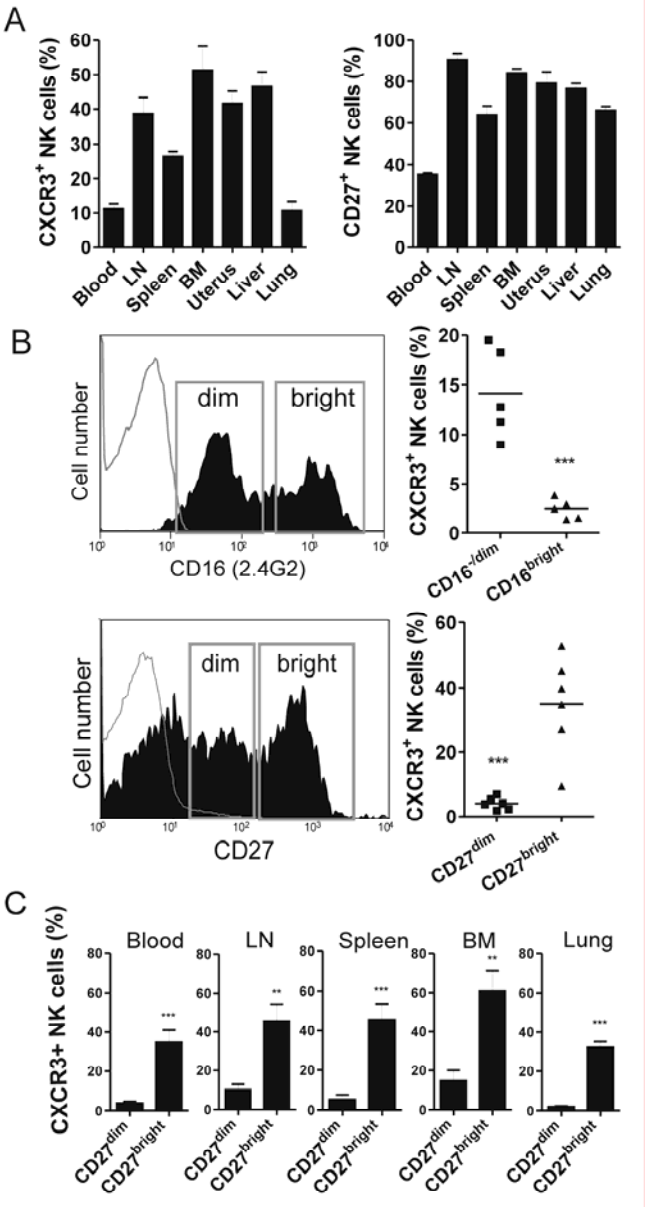

A
CXCR3+ NK cells (%)
CD27+ NK cells (%)
Blood
LN
Spleen
BM
Uterus
Liver
Lung
B
Cell number
dim
bright
CD16 (2.4G2)
CXCR3+ NK cells (%)
CD16-/dim
CD16bright
CD27
CD27dim
CD27bright
C
CXCR3+ NK cells (%)
Blood
LN
Spleen
BM
Lung

Fig. 2

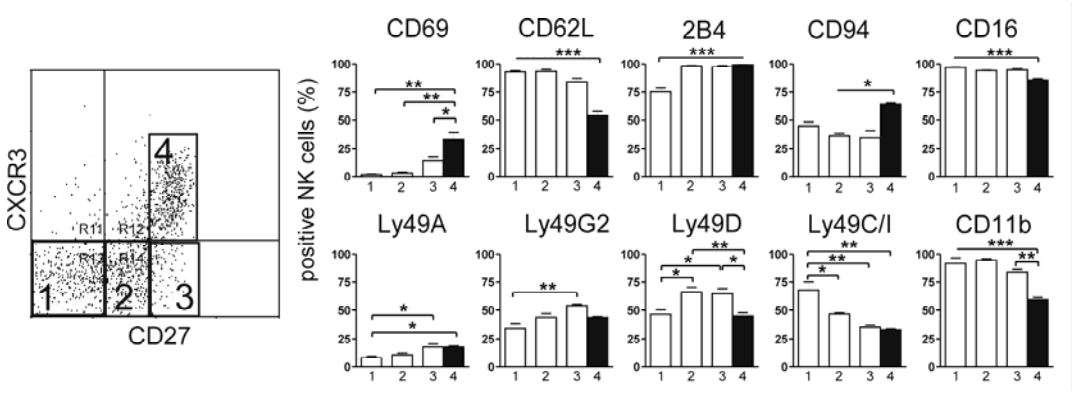

CXCR3
CD27
R11
R12
R13
R14
1
2
3
4
positive NK cells (%)
CD69
CD62L
2B4
CD94
CD16
Ly49A
Ly49G2
Ly49D
Ly49C/I
CD11b

Fig. 3

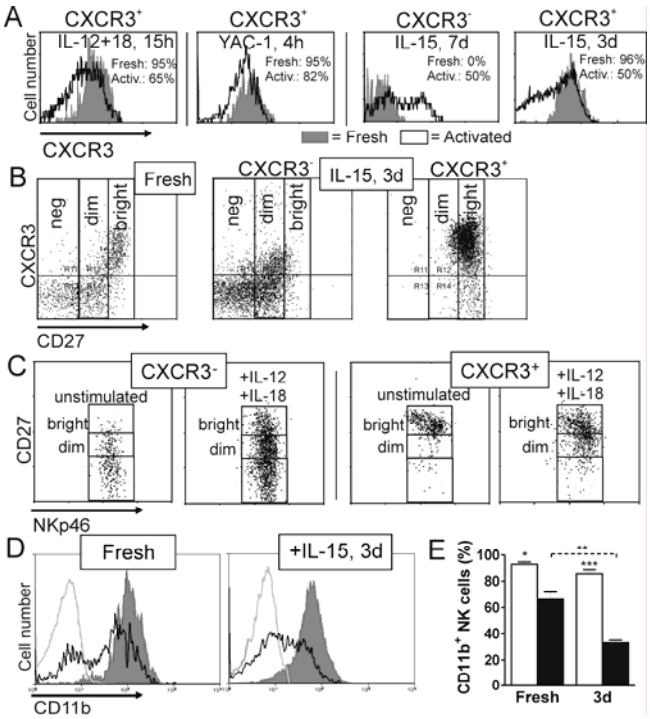

A
CXCR3+
IL-12+18, 15h
Fresh: 95%
Activ.: 65%
CXCR3+
YAC-1, 4h
Fresh: 95%
Activ.: 82%
CXCR3-
IL-15, 7d
Fresh: 0%
Activ.: 50%
CXCR3+
IL-15, 3d
Fresh: 96%
Activ.: 50%
Cell number
CXCR3
= Fresh
= Activated
B
Fresh
CXCR3-
IL-15, 3d
CXCR3+
neg
dim
bright
CXCR3
CD27
C
CXCR3-
+IL-12
+IL-18
unstimulated
bright
dim
CXCR3+
CD27
NKp46
D
Fresh
+IL-15, 3d
Cell number
CD11b
E
CD11b+ NK cells (%)
Fresh
3d

Fig. 4

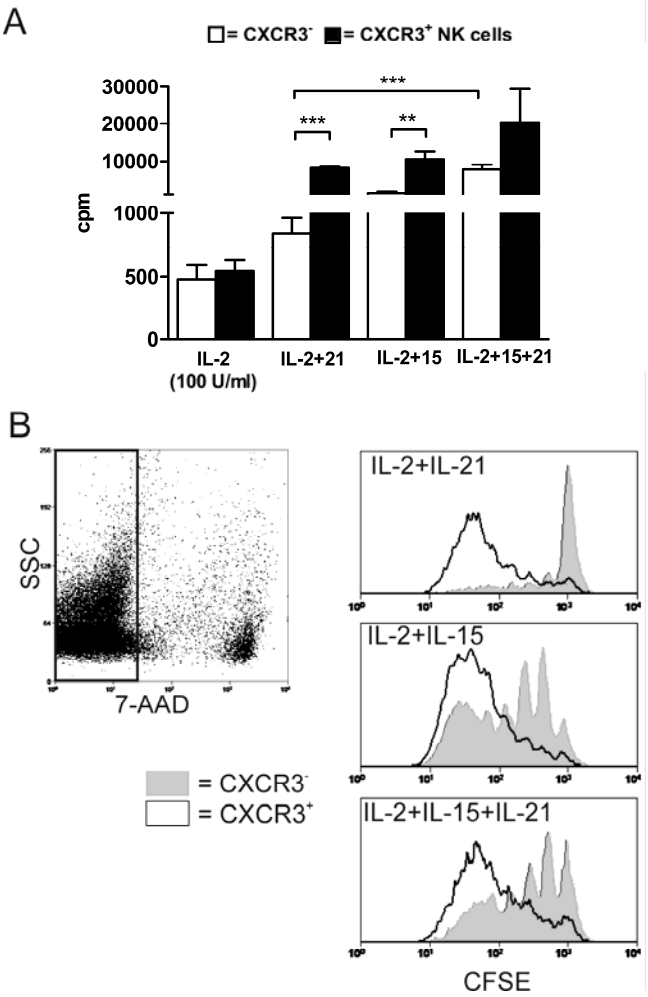

A
□= CXCR3⁻ ■= CXCR3⁺ NK cells
30000
20000
10000
1000
500
0
cpm
***
***
**
IL-2
(100 U/ml)
IL-2+21
IL-2+15
IL-2+15+21
B
SSC
7-AAD
= CXCR3⁻
= CXCR3⁺
IL-2+IL-21
IL-2+IL-15
IL-2+IL-15+IL-21
CFSE

Fig. 5

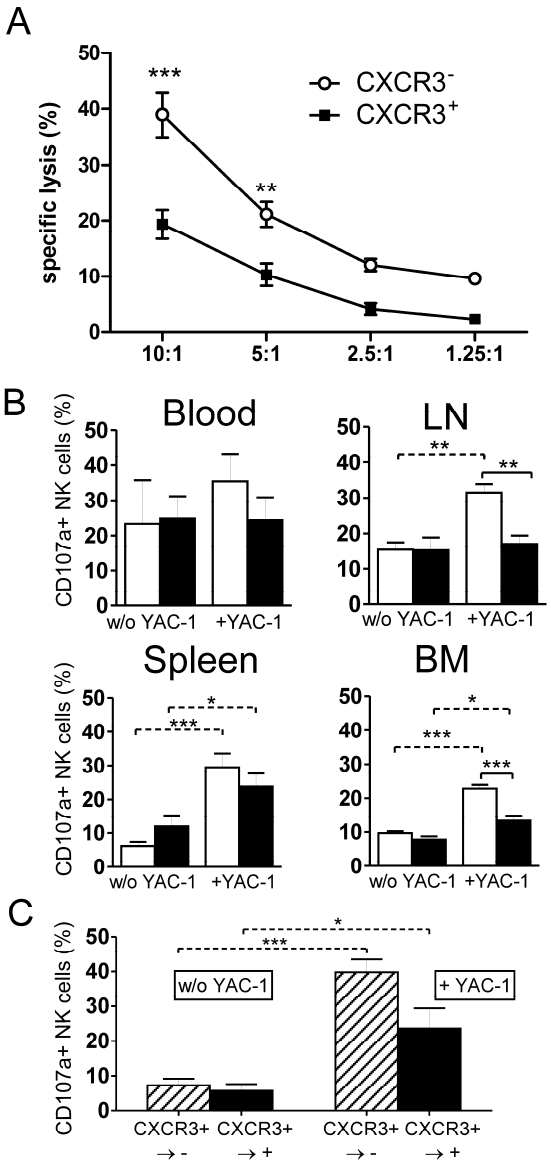

A
specific lysis (%)
CXCR3-
CXCR3+
10:1
5:1
2.5:1
1.25:1
B
Blood
LN
Spleen
BM
CD107a+ NK cells (%)
w/o YAC-1
+YAC-1
C
w/o YAC-1
+ YAC-1
CXCR3+ → -
CXCR3+ → +

Fig. 6

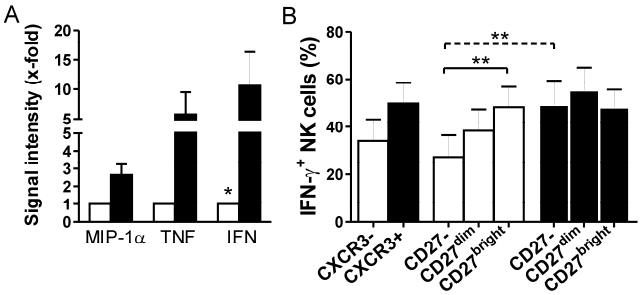
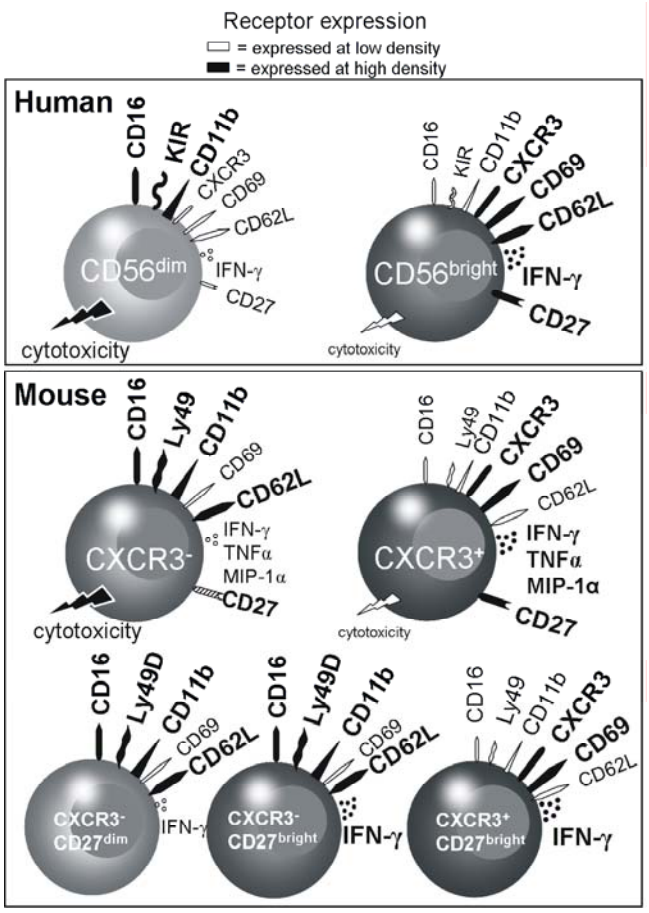

A
Signal intensity (x-fold)
20
15
10
5
5
4
3
2
1
0
*
MIP-1α
TNF
IFN
B
IFN-γ+ NK cells (%)
80
60
40
20
0
**
**
CXCR3-
CXCR3+
CD27-
CD27dim
CD27bright
CD27-
CD27dim
CD27bright

Fig. 7


Receptor expression
= expressed at low density
= expressed at high density
Human
CD16
KIR
CD11b
CXCR3
CD69
CD62L
IFN-γ
CD27
CD56dim
cytotoxicity
CD56bright
Mouse
Ly49
TNFα
MIP-1α
CXCR3-
CXCR3+
Ly49D
CXCR3- CD27dim
CXCR3- CD27bright
CXCR3+ CD27bright

4 UMBILICAL CORD MESENCHYMAL STEM CELLS INHIBIT NK CELL EFFECTOR FUNCTIONS BY DIRECT CELL-CELL-CONTACT INDEPENDENTLY FROM LFA-1- OR LAIR-1-LIGATION

Nicole Marquardt, Cornelia Kasper, Ralf Hass, Tim Hatlapatka, Reinhold E. Schmidt, Roland Jacobs

Vorbereitet zur Einreichung bei *Blood*

Wissenschaftlicher Beitrag von Nicole Marquardt an der Publikation:

Expansion isolierter MSC, Präparation von PBMC-Suspensionen und angereicherter NK-Zellsuspensionen, Zellkultur, intrazelluläre Analysen, CFSE und CD107a Assays, Apoptose Assay, Zytotoxizitätsassay (auch teilweise von Frau Sabine Buyny durchgeführt), Auswertung der Daten, Verfassen des Manuskriptes.

Umbilical-Cord Mesenchymal Stem Cells inhibit NK cell effector functions by direct cell-cell-contact independently from LFA-1- or LAIR-1-ligation

Short title: UC-MSCs mediate suppression of NK cell functions

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Reference count (*max. 50 references*): 42 references

Abstract

Mesenchymal stem cells (MSCs) are currently discussed for their potential as immunosuppressant in hematopoietic stem cell transplantation (HSCT). Natural killer (NK) cells on the other hand comprise the main lymphocyte population in the early reconstitution phase after HSCT. Therefore, both cell types may come into direct contact after transplantation.

To date, most data about MSC-NK cell interactions are based on data obtained with bone marrow-derived MSCs (BM-MSCs) and report an inhibition of NK cell functions mainly due to soluble factors. Here, we investigated the effects of umbilical cord-derived MSCs (UC-MSCs) on NK cell phenotype and function.

In spite of no NK cell activation or alteration in their phenotype, direct cell-cell contact with UC-MSCs resulted in decreased proliferative capacity, reduced intracellular IFN-γ production, and suppressed cytotoxicity of NK cells. In contrast, UC-MSC supernatant or NK-MSC-conditioned supernatants were non-effective, indicating a requirement of distinct membrane interaction for UC-MSC-mediated NK cell suppression. However, the involved signaling molecules still have to be identified.

We conclude that NK cell function is negatively regulated by direct cell-cell contact with UC-MSCs and that this potential of UC-MSCs might be used for therapeutic approaches in patients that underwent HSCT, where conventional treatment is ineffective.

Introduction

MSCs are capable to differentiate into various cell phenotypes such as osteoblasts, adipocytes, and chondrocytes and can be isolated from many different tissues including fat tissue, dental pulp tissue, fetal liver, lung, umbilical cord and umbilical cord blood [1-6]. They have high potency for the use in tissue repair and regeneration [7,8]. In addition, immunosuppressive effects triggered by MSCs may prevent graft-versus-host disease (GvHD) in patients that underwent hematopoietic stem cell transplantation (HSCT) and support the engraftment of hematopoietic stem cells [9,10]. MSCs can be transplanted across allogeneic barriers what makes them applicable to adoptive immunotherapy [11]. Therefore, MSCs are currently of special emphasis within several research fields. However, the mechanisms of their immunoregulatory activity are still under investigation.

NK cells belong to the innate immunity and play a fundamental role in early defence of viral infection and tumor rejection. Similar to MSCs, NK cells are suggested to represent a cell population that improves the outcome of haploidentical HSCT due to NK cell-mediated graft-versus-leukemia (GvL) effect [12].

Interactions via cell-cell contact and soluble factors between MSCs and NK cells have been suggested [13-16]. Whereas proliferation and cytokine production of NK cells has been shown to be decreased in NK:MSC co-cultures, the influence of MSCs on NK cell cytotoxicity is more controversial. Some groups demonstrated a MSC-dependent decrease in NK cell-mediated cytolysis, however, other studies could not prove this effect on NK cells [13,15,17,18]. However, most of the experiments of other groups were performed with bone marrow-derived MSCs (BM-MSCs) although other sources for MSC isolation require less invasive interventions.

In this study, we used umbilical cord-derived MSCs (UC-MSC) to investigate their immunosuppressive effects on NK cells. The mechanisms of UC-MSC-associated inhibition of NK cells is so far undescribed. Since most studies report that both soluble factors as well as cell-cell contact in combination lead to decrease of effector cell functions, we analyzed whether or not MSCs, MSC supernatant or MSC-NK-conditioned supernatant affect phenotype and function of NK cells. We placed special emphasis on the influence of LFA-1- or LAIR-1 ligation on NK cells, since the ligands for both receptors (ICAM-1 and collagens, respectively) are expressed on MSCs [19,20]. Whereas LFA-1 plays a crucial role for NK cells for cellular adherence and for composing an immunological synapse, LAIR-1 is reported to represent one of the major inhibitory receptors being expressed on leukocytes [21,22].

However, the role of LFA-1 and LAIR-1 for the contact between lymphocytes and MSCs has not been described so far.

The results of this study indicate a major role for direct cell-cell contact but not for soluble factors regarding inhibition of NK cell proliferation, cytokine production and cytotoxicity. However, NK cell suppression by UC-MSCs seems to be independent from LFA-1 or LAIR-1 binding.

Results

Phenotypic analysis and differentiation potential of UC-MSCs

Analysis of specific surface antigen expression patterns on plastic adherent MSCs were performed by flow cytometry (Supplementary Fig. 1a). Cells were strongly positive for CD29, CD44, CD73, CD90, CD105, and CD166 and lacked expression of CD3, CD11b, CD14, CD45, CD133, and HLA-DR on their cell surface. In addition, the potential of the MSCs to differentiate into chondrogenic, adipogenic and osteogenic lineages was determined as described before (data not shown) [23]. Therefore, the cells meet the criteria for defining multipotent mesenchymal stromal cells recommended by the International Society for Cellular Therapy (ISCT) [24].

Phenotype of NK cells does not change upon co-culture with MSCs

In order to determine whether or not NK cells shape their phenotype upon co-culture with MSCs for 15-18 hours, we analyzed NK cells cultured with or without MSCs by flow cytometry (Fig. 1A). Neither activating receptors such as natural cytotoxicity receptors (NCR: CD335 [NKp46], CD336 [NKp44], CD337 [NKp30]), ζ chain, CD16, CD27, CD69, and CD244 (2B4), nor killer cell immunoglobuline-like receptors (KIRs: CD158) or CD94/NKG2A were affected in their expression level. In addition, the content of perforin and the granzymes A, B and K in NK cells was not affected by MSCs as determined by intracellular staining.

MSCs inhibit NK cell proliferation without induction of apoptosis

Proliferation of CFSE-labelled NK cells was induced by stimulation with IL-15 and was analyzed by flow cytometry after three and six days of culture, respectively (Fig. 2). Direct cell-cell contact with MSCs resulted in strong inhibition of proliferation already after three days of co-culture. After six days, more than 90% of control NK cells cultured without MSCs proliferated, whereas only 52% of co-cultured NK cells displayed cell division capacity. Interestingly, when NK cells were cultured in MSC supernatant or MSC-NK-conditioned medium, no decrease in NK cell proliferation could be observed after six days (Fig. 2A). T and B cells have been shown to be arrested in the G0/G1 phase of the cell cycle when they were coincubated with MSCs [25,26]. However, this has not been investigated for NK cells so far. The results were further confirmed after cocultivation of IL-15-activated NK cells with MSCs for six days and we determined by cell cycle analysis more than 90% NK cells being arrested in the G0/G1 phase, whereas NK cells in single culture displayed

also a normal G_2/M phase (Fig. 2B). Although a slightly increased subG_1 phase indicated an elevated level of cell death, we could not detect increased apoptosis, neither after 24h in unstimulated NK cells nor after six days in IL-15-activated NK cells (Fig. 2C).
Together, these results indicate that the suppression of NK cell proliferation is not mediated by soluble factors and that the lack of proliferation does not result from NK cell apoptosis.

MSCs do not induce cytokine production in resting NK cells but rather inhibit cytokine production in stimulated NK cells

To determine, whether soluble factors are released by resting NK cells upon co-culture with MSCs, the supernatants were analyzed by cytokine array (Fig. 3A). No significant NK cell-associated induction of cytokine or chemokine release could be observed after co-culture with MSCs.
In contrast to resting NK cells, activated NK cells normally produce cytokines such as IFN-γ and TNFα. Since IFN-γ has been demonstrated to activate MSCs, we analyzed intracellular cytokine production by flow cytometry [27]. Co-culture with MSCs did not induce IFN-γ or TNFα production in NK cells (data not shown). When NK cells were stimulated with PMA/ionomycin after co-culture, cytokine production was not abrogated as compared to NK cells cultured without MSCs (Fig. 3B). However, in a more physiological stimulation setting using IL-12+IL-18, NK cells clearly displayed a decrease in IFN-γ production when previously co-cultured with MSCs.

MSCs inhibit NK cell degranulation and cytotoxicity

The major function of NK cells is the lysis of target cells. In addition to cytotoxicity, we also determined NK cell degranulation, since this function may not directly correlate to killing of target cells. An efficient degranulation but decreased cytotoxicity could e.g. indicate a defect in cell polarization or synapse formation. Upon MSC-NK co-culture with direct cell-cell contact, both NK cell degranulation and cytotoxicity were strongly reduced (Fig. 4A,B). In contrast, neither MSC supernatant, MSC-NK-conditioned medium, nor MSCs co-cultured in transwell systems affected subsequent NK cell cytotoxicity (Fig. 4B,C). Thus, NK cell-mediated lysis is already suppressed on degranulation level mediated by direct cell-cell contact but not soluble factors. Importantly, allogeneic MSCs were lysed only by preactivated but not resting NK cells as assessed by chromium release assay (Fig. 4D).

NK cell inhibition by MSCs is not LFA-1- or LAIR-1-dependent

Since NK cell inhibition by MSCs seems to be dependent on cell-cell contact, we determined the role of the integrin LFA-1. This receptor is expressed on almost all leukocytes and is the most important component for forming the immunological synapse upon cell-cell adhesion. One of the LFA-1 ligands, ICAM-1, is expressed on MSCs [19]. Blocking of LFA-1 should provide an indication of the role of the immunological synapse in NK-MSC interactions. We determined proliferative capacity and cytokine production by NK cells after co-culture with MSCs in presence or absence of anti-LFA-1 mAb (Fig. 5A, B). However, no effect in LFA-1-blocking on NK cells was observed in proliferation or cytokine production. Therefore, the LFA-mediated adhesion is not the critical factor in the interplay of NK cells with MSCs.

Another receptor with inhibitory function is LAIR-1. It is expressed on most leukocytes and functionally binds to collagens [22,28]. Since collagens are expressed by MSCs and could therefore represent ligands for LAIR-1 on NK cells, we first determined LAIR-1 expression on NK cells (Fig. 5C) [20]. In every donor, all fresh NK cells were LAIR-1^{+}. The interaction of LAIR-1 with collagens can be efficiently blocked by soluble LAIR-1 or LAIR-2 proteins [29,30]. For activation assays, we therefore blocked the binding sites of the collagens by preincubating the MSCs with soluble LAIR-1 or LAIR-2 protein at different concentrations. However, no NK cell-rescuing effects were observed regarding cytokine release (Fig. 5D). Thus, the results reveal that ligation of LAIR-1 is not responsible for the suppression of NK cell functions.

Discussion

In the present study, we determined the effects of human UC-MSCs on NK cell functions after co-culture. Both cell types are currently under investigation regarding their use for cellular therapy, for example for the treatment of GvHD after HSCT or support of hematopoietic stem cell engraftment [9,10]. In addition, MSCs represent an important tool for tissue engineering and transplantation of regenerated tissues [7,8]. MSCs can be isolated from different adult or post-natal birth-associated tissues. Whereas most studies or clinical trials were performed using adult BM-MSCs, these cells suppress lymphocyte activation, although the immunoregulatory mechanisms are not clearly defined yet. Post natal/embryonic UC-MSCs represent a promising alternative for adult BM-MSCs, and comparative studies of the two cell types have already been performed by using PBMC [31]. However, research on mechanisms of UC-MSC-mediated inhibition of NK cells is limited. Since interactions between NK cells and MSCs upon transplantation may occur, detailed analyses would be necessary to predict the outcome of cell-based therapies.

The phenotype of NK cells can give information on their activation level. Therefore, we analyzed the expression pattern of a panel of surface receptors and content of perforin and granzymes in NK cells after co-culture with UC-MSCs and could not detect any significant changes. However, after 6 days in culture in presence of MSCs, a marginal downregulation of CD56 expression was observed (data not shown), confirming recent data [16]. Evidence for activation or inhibition of NK cells could not be determined at the protein level. These results are contradictory to those of other groups, reporting on a decrease in expression of natural cytotoxicitiy receptors, NKG2D or Granzyme B [13,16,32]. This discrepancy could be explained by different culture and stimulation conditions. We co-cultured resting NK cells and MSCs for phenotypic analysis over night, whereas in other studies NK cells were activated and incubated with MSCs for at least four days.

CFSE assays revealed a strong inhibitory influence of UC-MSCs on the proliferative capacity of NK cells. This effect was observed upon direct cell-cell contact and only marginally when supernatants from MSC cultures or MSC-NK-conditioned medium were used, clearly indicating membrane interaction-dependent mechanisms which involve distinct surface receptors. The proliferative defect was also described for antigen-stimulated T cells by other groups, and this result was strengthened by cell cycle analyses [25]. We proved in the present study for the first time that also NK cells are arrested in the G0/G1 phase when co-cultured with MSCs, maybe due to cyclin D2 inhibition. Furthermore, we could confirm that MSCs do not induce apoptosis in NK cells [16,18].

The secretion of cytokines is one major function of NK cells, in particular of the regulatory CD56^{bright} NK cell subset. One of the most prominent cytokines produced and secreted in relatively high amounts by stimulated NK cells is IFN-γ. This cytokine has been shown to activate MSCs, which in turn secrete the inhibitory factors IDO and PGE2 [27]. To test whether the co-culture of NK cells and MSCs induces secretion of IFN-γ or any other cytokine by NK cells, we analyzed cell-culture supernatants revealing no IFN-γ secretion (data not shown). Only one potentially NK cell-inhibiting factor, namely IL-10, was measured in detectable amounts [33]. Although most molecules produced by MSCs such as IL-6, MCP-1 (CCL2), MCP-2 (CCL8), IP-10 (CXCL10) and sTNFR1 have rather activating effects on NK cells [34-37]. Other co-culture results from our and other groups suggest an inhibitory effect mediated by the MSCs. Following stimulation with IL-12 and IL-18 after co-culture with MSCs, NK cell production of IFN-γ reduced to 50%. Hence, a strong inhibitory effect is probably overruling the stimulation mediated by the released factors since NK cell functions are diminished after direct cell-cell contact with UC-MSCs.

The capability of NK cells to lyse target cells is a very important issue in HSCT since NK cells can mediate GvL effects [12]. Parallel infusion of MSCs for therapeutic reasons could not only have beneficial effects but could also abrogate the NK cell-mediated GvL effect. We proved that direct cell-cell contact with UC-MSCs but not MSC-supernatant or MSC-NK-conditioned medium caused a significant decrease in degranulation and cytolysis in NK cells. Furthermore, IL-15 preactivated NK cells were able to kill UC-MSC target cells, confirming data of BM-MSCs obtained by other groups [15,16]. Recently, increased levels of IL-15 were suggested to induce the expansion of CD56^{bright} NK cells after HSCT[38]. Whether MSCs are lysed by activated NK cells in these settings or would rather suppress NK cell function and thus negatively affect the NK cell-mediated GvL effect has still to be clarified.

Our results reveal that only direct cell-cell contact strongly inhibits NK cell functions. Although some interaction molecules have been suggested so far to be important in MSC-mediated lymphocyte suppression, no distinct receptor has been identified so far.

Since the functional inhibition is not only restricted to NK cells but also to a multitude of leukocyte subsets, we suggest that a distinct surface molecule might contribute to the observed effects. This may apply for LFA-1 and also for LAIR-1 on NK cells. LFA-1 is necessary for cellular adhesion and forming an immunological synapse with ICAM-1 on target cells, and LAIR-1 is one of the most distributed inhibitory receptors and binds to collagens [21,22]. However, blocking antibodies against a subunit of LFA-1, CD11a, or soluble LAIR-1/LAIR-2 proteins used to block collagen binding sites on MSCs did not rescue NK

cell functions. Therefore, another inhibitory receptor or a combination of receptors on NK cells must play a role for the MSC-mediated inhibition. One conceivable molecule is PD-1. A PD-1/PD-L1-dependent pathway was shown for the interactions between MSCs and T and B cells, however, NK cell activity was not analyzed [39]. Both PD-1 on NK cells and its ligand PD-L1 on MSCs are only expressed upon activation of the respective cell population [27,40,41]. In our experiments NK cells were not (pre)activated, hence, an influence of PD-1 is unlikely. Another possible receptor is the T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) on NK cells, which interacts on one hand with the activating receptors DNAM-1 and CD96, but on the other hand after binding via the poliovirus receptor (PVR, CD155) inhibition of NK cells has been reported recently [42]. Further experiments are needed to determine the role of TIGIT on NK-MSC-interactions. An additional specific pathway for PBMC-MSC-interaction was shown recently. The knockdown of galectin-1 in MSCs restored proliferation and IFN-γ production in PBMC, but not NK cells [43]. Thus, different lymphocyte populations or at least their functions may be affected by MSCs in different ways.

In summary, we show that NK cell proliferation, cytokine production and cytotoxicity are suppressed by direct cell-cell contact with UC-MSCs but not soluble factors. The UC-MSC-mediated effects are very similar to those obtained with BM-MSC and should be addressed when the combination of NK cells and MSCs is considered for cell-based therapeutic applications.

Material and methods

Isolation and culture of human umbilical cord MSCs

The research was approved by the Institutional Review Board, project #3037 in an extended permission #443 on 26th February, 2009. With consent of the parents, human umbilical cord (UC) was obtained from full-term (38-40 weeks) infants by Caesarean section at Hannover Medical School. Red blood cells were removed with PBS enriched with 5g/l glucose (Sigma Aldrich, Deisenhofen, Germany), 50µg/ml gentamicin (PAA Laboratories, Pasching, Austria), 2.5µg/ml amphotericin (Sigma), 100U/ml penicillin and 100µg/ml streptomycin (PAA Laboratories). The UC tissue was minced into approx. 0.5cm^3 pieces and incubated in αMEM (Invitrogen, Karlsruhe, Germany) supplemented with 15% of allogeneic human serum (kindly provided by the Division of Transfusion Medicine, Hannover Medical School) and 50µg gentamicin under humidified conditions at 37°C and 5% CO_2. Medium was replaced every second day. An adherent cell layer was observed after 10 days. The UC tissue was removed after 2 weeks, and the adherent cells were detached by accutase (Gibco/Invitrogen) treatment for 5min at 37°C. UC-MSCs were washed, resuspended and subcultured in αMEM culture medium supplemented with 10% human serum, 50µg/ml streptomycin, 50U/ml penicillin, 1mM glutamine and 0.5mM sodium pyruvate (Biochrom, Berlin, Germany) at a density of 4x10^3 cells/mm^2. After passage 3 or 4, UC-MSCs were phenotypically characterized or cryconserved in human serum containing 10% (v/v) DMSO in liquid nitrogen.

Isolation of peripheral blood mononuclear cells (PBMC) and NK cells

Blood samples were collected from healthy consenting donors and 1:2 diluted with phosphate buffered saline (PBS). PBMC were obtained by Ficoll Hypaque density gradient centrifugation (1000 x *g*, 20min). NK cells were isolated from PBMC by magnetic depletion (iMag, BD Biosciences, Heidelberg, Germany) or by cell sorting of CD56$^+$CD3$^-$ lymphocytes using a FACSAria Cell Sorting System (BD Biosciences, Heidelberg, Germany) at the Hannover Medical School FACS facility (purity of the NK cell populations at least 95%). Data acquisition was performed using DIVA software (BD Biosciences).

Monoclonal antibodies

The following anti-human mAbs were used for multicolour flow cytometry: CD3 PE (clone UCHT1), CD158a FITC (EB6), CD158b FITC (GL183), CD159a (Z199), CD166 PE (3A6), CD244 PE (C1.7), CD335 PE (NKp46, BAB281), CD336 PE (NKp44, Z231),

CD337 PE (NKp30, Z25) (all purchased from Beckman Coulter, Hamburg, Germany), CD3 PerCP (SK7), CD11b PE (ICRF44), CD14 APC (M5E2), (1C6/CXR3), CD27 FITC (M-T271), CD45 PerCP (2D1), CD56 PE (MY31), CD56 APC (B159), CD73 PE (AD2), CD107a FITC (H4A3), CD183 PE (1C6/CXR3), IFN-γ PE (4S.B3), TNFα FITC (MAb11), Granzyme A FITC (CB9), Perforin FITC (δG9) (all from BD Biosciences), CD16 FITC (DJ130C), CD94 PE (HP-3D9), HLA-DR FITC (CR3/43) (Dako, Glostrup, Denmark), CD29 APC (MEM-10A), CD44 FITC (MEM-85), CD62L FITC (LT-TD180), CD105 FITC (MEM-226), Granzyme B PE (GB11), Granzyme K Alexa647 (24C3) (Immunotools, Friesoythe, Germany), CD69 PE (CH/4, Invitrogen, Darmstadt, Germany), CD90 APC eBio5E10, eBioscience, Frankfurt, Germany), CD133 APC (293C3, Miltenyi Biotec, Bergisch Gladbach, Germany), CD355 PE (Cr24.1, Biolegend, San Diego, CA, USA). For determination of apoptosis by flow cytometry, a FITC-conjugated annexin V/7-aminoactinomycin D (7-AAD) kit (Beckman Coulter) was used. Blocking of LFA-1 was performed with anti-CD11a (HI111, Biolegend).
Isotype-matched mAbs were used as controls for every flow cytometric experiment.

Flow cytometric analysis of NK cells and MSCs

Fresh or thawed NK cells were used. After pre-incubation with 1:5-diluted commercial human IgG (Octagam, Octapharma, Langenfeld, Germany), cells were incubated for 20min at 4°C with the respective mAbs. After washing, the cells were analyzed on a multicolour flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany) using Cell Quest Pro software. Medium and isotype controls were performed simultaneously. Forward and side scatter properties of the cells were used to gate on the lymphocyte population. FACS data were analyzed using Summit 5.1 software (Dako, Hamburg, Germany).

MSC-NK co-culture

MSCs were thawed and plated onto 24- or 48-well plates at $2x10^4$ cells/cm^2 in 400µl complete medium. Adhesion of cells was allowed for 24h. MSC-conditioned medium was removed immediately before adding the NK cells from unrelated donors in complete medium at a MSC:NK-ratio of 1:10. NK cells cultured without MSCs were used as controls. In case of LFA-1-blocking, NK cells were preincubated with anti-LFA-1 mAb for 30min at 37°C and then directly added to the MSCs. In LAIR-1-blocking experiments, soluble LAIR-1 or LAIR-2 proteins (Abnova, Heidelberg, Germany) preincubated in the final concentrations with MSCs for 1 hour before adding the NK cells without removing the

proteins. For transwell experiments, MSCs adhered in the lower well before NK cells were added in transwell inserts (0.4µm pore diameter, Nunc, Langenselbold, Germany). After 16-18h of co-culture, the NK cells were separated from the MSCs by gentle pipetting. The purity of NK cells was determined by flow cytometric analysis and supernatants of the cultured cells were frozen for further analyses or immediately used as MSC-NK-conditioned medium. For determination of the influence of MSC-NK-conditioned medium, NK cells from the same isolation were cultured in the absence of any cytokines for 16-18h and then resuspended in the supernatant of the parallel co-culture. These NK cells were cultured for additional six days or 16-18h, respectively, in the MSC-NK-conditioned medium and subsequently analyzed for their proliferative and cytotoxic abilities. When necessary, MSCs were detached by incubation with accutase at 37°C for 5min.

Intracellular detection of cytokine production in NK cells

After co-culture with MSCs, NK cells were removed from the MSCs and activated with phorbole-myristate-acetate (PMA)/ionomycin or IL-12 (10ng/ml) and IL-18 (10ng/ml) for a total time of 4h. After 1h of incubation time, Brefeldin A (Sigma Aldrich) was added at a final concentration of 2µg/ml. After surface staining, intracellular staining of cytokines was performed. After fixation with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 10min, cells were perforated in 0.1% Saponin buffer (PBS supplemented with 0.1% Saponin (Riedel-de-Haën, Seelze, Germany) and 0.01M HEPES (Roth, Karlsruhe, Germany)) and anti-IFN-γ or anti-TNFα mAb were added. After 30 minutes of incubation and three washes, cells were analyzed as described above.

Detection of cytokines in culture supernatant

Collected supernatants from single NK cell or MSC cultures or NK-MSC-cocultures were thawed and pooled. For detection of released cytokines, RayBio® Human Inflammation Antibody Array 3 (Raybiotech, Norcross, GA) was used following the manufacturer's recommendations and as described previously [44].

CFSE assay

PBMC or NK cells were labelled with 2.5µM (final concentration) carboxyfluorescein diacetate succinimidyl esther (CFSE, Molecular Probes, Invitrogen, Eugene, OR, USA) according to the manufacturer's recommendations. In detail, following CFSE labelling for 10min at 37°C in PBS containing 0.1% BSA (Sigma Aldrich), five volumes of ice-cold

medium were added and cells were incubated on ice for additional 5min. After two washing steps, cells were resuspended in αMEM culture medium and added to the adherent MSCs. For proliferative stimulation of NK cells, recombinant IL-15 (Peprotech, Hamburg, Germany) was added at a final concentration of 10ng/ml. After 3 and 6 days, 7-AAD$^-$ NK cells were gated and analyzed by flow cytometry.

Cell cycle analysis

NK cells were cultured with or without MSCs for 6 days in presence of IL-15 (10ng/ml). The NK cells were then harvested, washed, and about 1x10^5 cells were fixed in 70% (v/v) ice-cold ethanol at 4°C for 24h. Thereafter, the fixed cells were stained with CyStain DNA 2 step kit (Partec, Münster, Germany) and filtered through a 50µm filter. The samples were then analyzed in a Galaxy flow cytometer (Dako, Hamburg, Germany) using FloMax analysis software (Partec) and the MultiCycle cell cycle software (Phoenix Flow Systems, San Diego, CA). Accordingly, all cell cultures were quantified for the cell number in the flow cytometer to obtain the proliferation data.

Detection of apoptosis

After culture with or without MSCs for 15-18h, the level of apoptosis in NK cells was determined by flow cytometry using an annexin V/7-aminoactinomycin D (7-AAD) kit (Beckman Coulter). Positive controls were performed with paraformaldehyde-treated cells.

CD107a degranulation assay

Lysosomal granule exocytosis was determined by CD107a expression. NK cells precultured with or without MSC were incubated at 37°C in 5%CO_2 together with K562 target cells for 4h. Anti-CD107a mAb was added directly to the cell suspensions for the total time of incubation. After 1h of incubation, Monensin (BD Biosciences) was added at a final concentration of 6µg/ml. Finally, samples were surface-stained and analyzed via flow cytometry.

Cytotoxicity assay against K562 target cells

Cytotoxicity of NK cells cultured with or without MSC, against K562 target cells was assessed by standard 4hr chromium release assay. Target cells were labelled with 3 MBq $Na^{51}CrO_4$ (Hartmann Analytic, Braunschweig, Germany), incubated for 1h at 37°C, washed two times and used for the assay within 1h. Cells were plated in V-bottom 96-well plates.

When MSCs were used as target cells, cells were plated in flat-bottom 96 well plates and were allowed to adhere over night, before NK cells were added.

Background values were determined by incubating target cells without effector cells. Maximal values were obtained by lysing target cells with 1% Triton X-100 (Sigma-Aldrich). After 4h, cells were pelleted and 25µl supernatant of each well was used for measurement of ^{51}Cr release in a gamma counter (MicroBeta/PerkinElmer, Waltham, MA, USA) in triplicates at effector:target (E:T) ratios of 10:1, 5:1 (and 2.5:1). Specific lysis was calculated by: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] x 100.

Statistical analyses

Statistical analyses were performed as unpaired two-tailed *t*-tests, unless otherwise stated, using GRAPHPAD PRISM V5.00 Software. Levels of significance are given as *P*-values (*** $P<0.05$, ** $P<0.01$, * $P<0.001$). Plotted data represent mean plus/minus standard error of mean (SEM).

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Competing Interest

The authors declare no financial, personal, or professional conflict of interest.

Abbreviations

BM-MSCs – bone marrow mesenchymal stem cells

LAIR – Leukocyte-associated immunoglobulin-like receptor

LFA – Lymphocyte function-associated antigen

NK cells – Natural killer cells

UC-MSCs – Umbilical cord mesenchymal stem cells

Figure Legends

Supplementary Fig. 1: The phenotype of the MSCs conforms to criteria of the ISCT. MSCs after passage 3 or 4 were detached by accutase treatment and analyzed for their phenotype by flow cytometry. One representative example is shown.

Fig. 1: The NK cell phenotype is not altered by co-culture with MSCs. Purified NK cells were cultured in the absence (□) or presence (■) of MSCs. After 15-18h of culture, NK cell phenotype was analyzed by flow cytometry. Mean values of the percentages ± SEM of at least 3 different experiments are shown. Gr. = Granzyme.

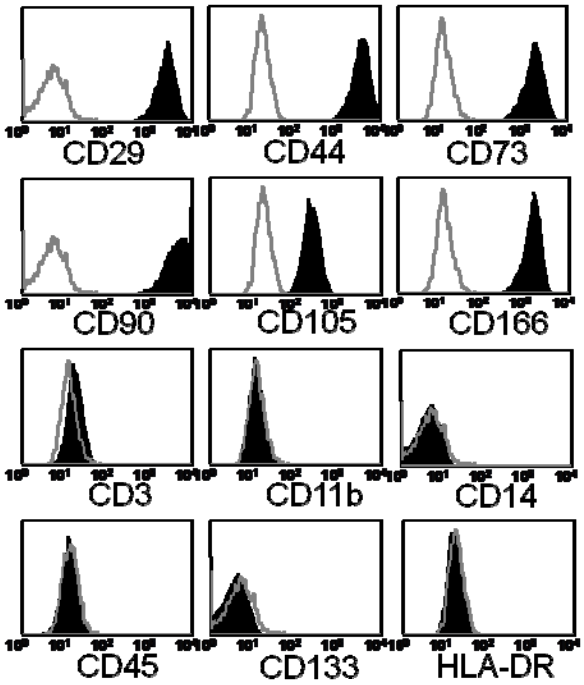
Fig. 2: MSCs inhibit NK cell proliferation without induction of apoptosis. A: NK cells were stained with CFSE and cultured for 3 or 6 days in presence of IL-15 (upper row: representative example after 6 days). NK cells were directly added onto MSCs, or cultured in MSC-supernatant or MSC-NK-conditioned supernatant (MSC-NK-S). Mean values of the percentages ± SEM of 3 experiments are shown. ***p<0.001, *p<0.05 (student's unpaired *t*-test) **B:** NK cells were co-cultured with or without MSCs in presence of IL-15. After 6 days, NK cells were harvested and cell cycle analysis was performed by flow cytometry. **C:** Apoptosis of NK cells was analyzed by flow cytometry after co-culture in absence (□) or presence (■) of MSCs for 24h or 6d. Mean ± SEM of 3 different experiments is depicted.

Fig. 3: Interaction of UC-MSCs and NK cells affects the production of soluble mediators. A: Supernatants of 3 different cell culture experiments of NK cells in absence of MSCs (□), MSCs in absence of NK cells (▨), or co-culture of NK cells and MSCs (■) were pooled and analyzed for the presence of soluble effector molecules by inflammation cytokine array. **B:** Sorted NK cells were cultured in absence (□) or presence (■) of MSCs for 15-18h and subsequently removed from MSCs. The following 4h, NK cells were stimulated with PMA/ionomycin or IL-12 and IL-18, respectively, and finally analyzed for intracellular IFN-γ by flow cytometry. Mean values ± SEM of 5 experiments are shown. *p<0.05 (student's *t*-test).

Fig. 4: NK cell-mediated degranulation and cytotoxicity are decreased after direct cell-cell contact with MSCs. A: Sorted NK cells were cultured in absence (□) or presence (■) of MSCs. After 15-18h, NK cells were removed from MSCs and CD107a assay with K562

target cells was performed. Mean values ± SEM of 3 experiments are shown. **B-D:** After culture without MSCs (○), coculture in presence of MSCs (■), in MSC supernatant (●) or MSC-NK-conditioned supernatant (◇), NK cells were tested for cytotoxicity in chromium release assay. Mean values of at least 3 experiments are shown. **C:** NK cells and MSCs were separated by tanswell inserts. **D:** Specific lysis of K562 target cells (white symbols) or MSCs (black symbols) was determined. Resting NK cells in fresh medium (○, ▼) or MSC-NK-conditioned supernatant (◇) and IL-15-preactivated NK cells (▲, △) were used as effector cells. ***$p<0.001$, *$p<0.05$ (student's *t*-test).

Fig. 5: Inhibition of NK cell functions is not affected by blocking of LFA-1 or LAIR-1. NK cell functions after culture in absence (□) or presence (■) of MSCs were determined. **A:** Proliferation of IL-15-stimulated CFSE-stained NK cells was determined by flow cytometry after 3 days and 6 days. Mean values ± SEM of 3 experiments are shown. **B:** Before starting the co-cultures, NK cells were preincubated with a blocking mAb specific for CD11a. After 15-18h of co-culture, NK cells were removed from MSCs and stimulated with IL-12 and IL-18 for 4h. Intracellular IFN-γ was determined by flow cytometry. Mean ± SEM of 3 experiments are shown. **C:** Expression of LAIR-1 on fresh peripheral blood-derived $CD3^-$ cells was determined by flow cytometry. Percentages are displayed in the respective quadrants. One representative of 3 samples is shown. **D:** MSCs were preincubated with soluble LAIR-1 and/or LAIR-2 protein in different concentrations. NK cells were added without removing the medium supplemented with LAIR-proteins and incubated 15-18h on MSCs. NK cells were then removed from MSCs and stimulated with IL-12 and IL-18 for 4h. Intracellular IFN-γ was determined by flow cytometry.



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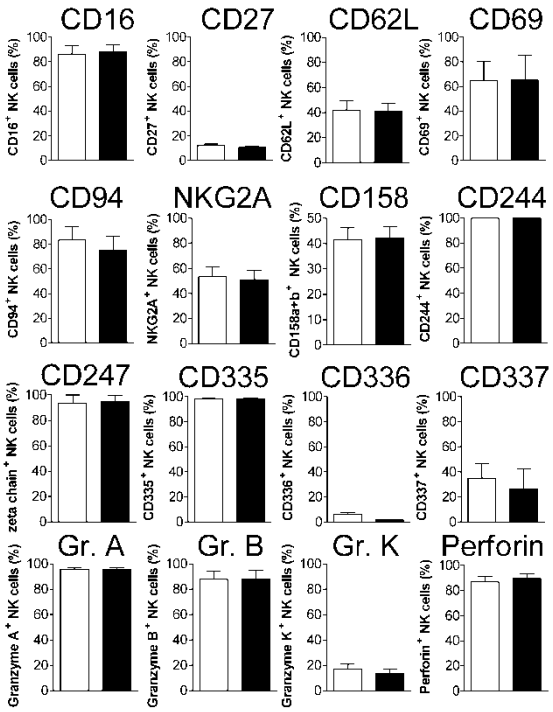

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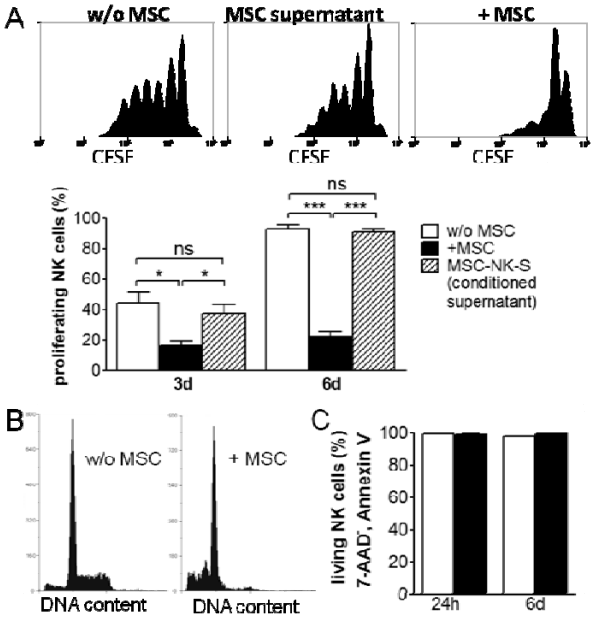

Fig. 2: MSCs inhibit NK cell proliferation without induction of apoptosis. A: NK cells were stained with CFSE and cultured for 3 or 6 days in presence of IL-15 (upper row: representative example after 6 days). NK cells were directly added onto MSCs, or cultured in MSC-supernatant or MSC-NK-conditioned supernatant (MSC-NK-S). Mean values of the percentages ± SEM of 3 experiments are shown. ***p<0.001, *p<0.05 (student's unpaired t-test) **B:** NK cells were co-cultured with or without MSCs in presence of IL-15. After 6 days, NK cells were harvested and cell cycle analysis was performed by flow cytometry. **C:** Apoptosis of NK cells was analyzed by flow cytometry after co-culture in absence (□) or presence (■) of MSCs for 24h or 6d. Mean ± SEM of 3 different experiments is depicted.

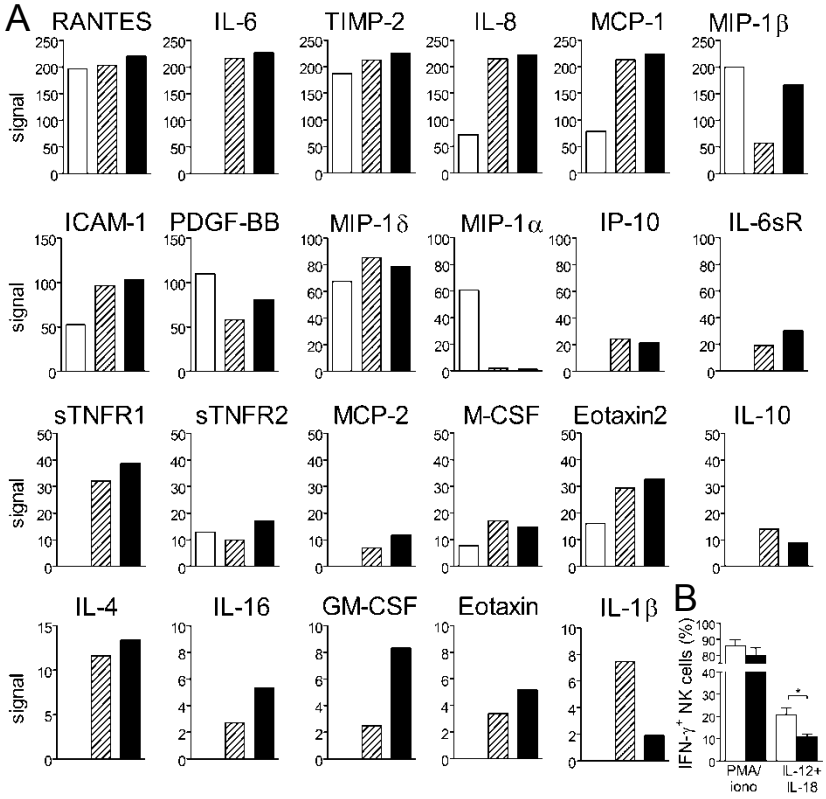

Fig. 3: The presence of MSCs does not affect cytokine production by stimulated NK cells. A: Supernatants of 3 different cell culture experiments of NK cells in absence of MSCs (□), MSCs in absence of NK cells (▨), or co-culture of NK cells and MSCs (■) were pooled and analyzed for the presence of soluble effector molecules by inflammation cytokine array. **B:** Sorted NK cells were cultured in absence (□) or presence (■) of MSCs for 15-18h and subsequently removed from MSCs. The following 4h, NK cells were stimulated with PMA/ionomycin or IL-12 and IL-18, respectively, and finally analyzed for intracellular IFN-γ by flow cytometry. Mean values ± SEM of 5 experiments are shown. *p<0.05 (student's t-test).

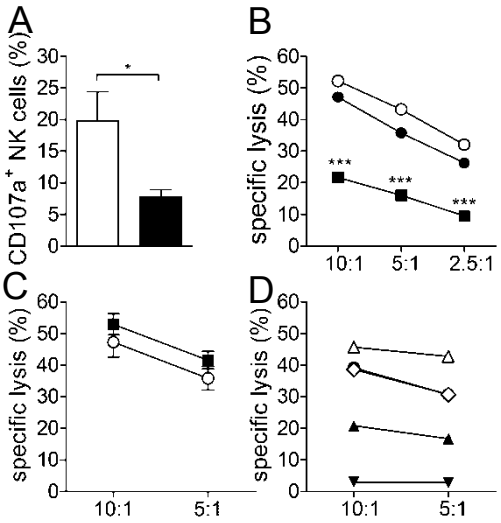

Fig. 4: NK cell-mediated degranulation and cytotoxicity is decreased after direct cell-cell contact with MSCs. A: Sorted NK cells were cultured in absence (□) or presence (■) of MSCs. After 15-18h, NK cells were removed from MSCs and CD107a assay with K562 target cells was performed. Mean values ± SEM of 3 experiments are shown. **B-D:** NK cell-mediated cytotoxicity under different conditions was determined by chromium release assay. Mean values of at least 3 experiments are shown. **B:** NK cells were precultured in absence (○) or presence of MSCs (■) or in MSC supernatant (●). **C:** NK cells were precultured in absence (○) or presence of MSCs (■). NK cells and MSCs were separated by tanswell inserts. **D:** Specific lysis of K562 (white symbols) or MSC (black symbols) target cells was determined. Resting NK cells in fresh medium (○, ▼) or MSC-NK-conditioned supernatant (◇) and IL-15-preactivated NK cells (△, ▲) were used as effector cells. ***p<0.001, *p<0.05 (student's t-test).

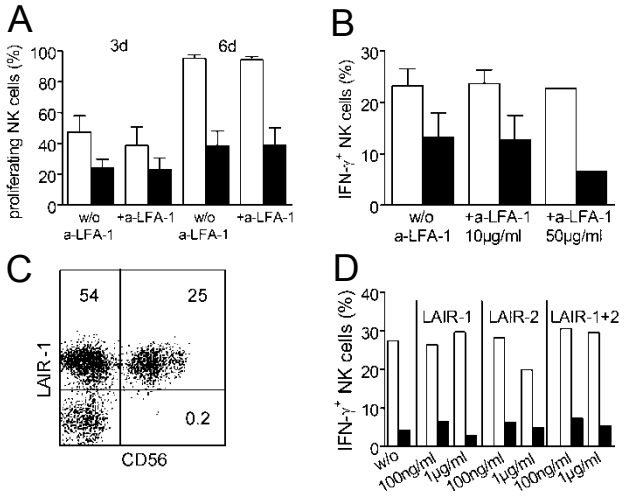

Fig. 5: Inhibition of NK cell functions is not affected by blocking of LFA-1 or LAIR-1. NK cell functions after culture in absence (□) or presence (■) of MSCs were determined. **A:** Proliferation of IL-15-stimulated CFSE-stained NK cells was determined by flow cytometry after 3 days and 6 days. Mean values ± SEM of 3 experiments are shown. **B:** Before starting the co-cultures, NK cells were preincubated with a blocking mAb specific for CD11a. After 15-18h of co-culture, NK cells were removed from MSCs and stimulated with IL-12 and IL-18 for 4h. Intracellular IFN-γ was determined by flow cytometry. Mean ± SEM of 3 experiments are shown. **C:** Expression of LAIR-1 on fresh peripheral blood-derived $CD3^-$ cells was determined by flow cytometry. Percentages are displayed in the respective quadrants. One representative of 3 samples is shown. **D:** MSCs were preincubated with soluble LAIR-1 and/or LAIR-2 protein in different concentrations. NK cells were added without removing the medium supplemented with LAIR-proteins and incubated 15-18h on MSCs. NK cells were then removed from MSCs and stimulated with IL-12 and IL-18 for 4h. Intracellular IFN-γ was determined by flow cytometry.

5 DEPLETION OF FUNCTIONALLY ACTIVE CD20+ T CELLS BY RITUXIMAB TREATMENT

Esther Wilk, Torsten Witte, Nicole Marquardt, Tibor Horvath, Katy Kalippke, Kirsten Scholz, Nadine Wilke, Reinhold E. Schmidt, Roland Jacobs

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Wissenschaftlicher Beitrag von Nicole Marquardt an der Publikation:

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Depletion of Functionally Active CD20+ T Cells by Rituximab Treatment

Esther Wilk, Torsten Witte, Nicole Marquardt, Tibor Horvath, Katy Kalippke, Kirsten Scholz, Nadine Wilke, Reinhold E. Schmidt, and Roland Jacobs

Objective. **Rituximab is a therapeutic anti-CD20 antibody used for in vivo depletion of B cells in proliferative and autoimmune diseases. However, the mechanisms of action are not fully understood, since not all of the therapy-mediated effects can be explained by the depletion of antibody-secreting cells. In addition to B cells, there is also a small population of T cells coexpressing CD20 in all individuals. This study was conducted to examine the phenotype and function of CD3+CD20+ T cells in patients with rheumatoid arthritis (RA) and healthy controls.**

Methods. **The phenotype and apoptosis of peripheral blood mononuclear cells from healthy donors and RA patients were examined by 4-color fluorescence-activated cell sorting analyses. Cytokine production was determined by intracellular staining and measurement of cytokines in the supernatants. Proliferation of sorted T cell populations was analyzed using ^{3}H-thymidine uptake assays.**

Results. **In healthy individuals, 0.1–6.8% of peripheral blood T cells (mean 1.6%; n = 142) coexpressed CD20, which was not significantly different from that in the peripheral blood of RA patients, in whom 0.4–2.6% of T cells (mean 1.2%; n = 27) were CD20+. During rituximab therapy, the CD20+ T cells along with the B cells were eliminated from the RA peripheral blood. Among the CD20+ T cells, 45% coexpressed CD8 and 55% coexpressed CD4. Polyclonal CD3+CD20+ cells were functionally characterized by constitutive cytokine production (i.e., interleukin-1β and tumor necrosis factor α), a low proliferative capacity, a high activation state, and enhanced susceptibility to apoptosis.**

Conclusion. **These findings suggest that CD20+ T cells represent a terminally differentiated cell type with immune-regulatory and proinflammatory capacities. Depletion of CD20+ T cells may be an additional mechanism by which anti-CD20 therapy functions in patients with RA.**

In vivo depletion of CD20+ B cells using rituximab therapy is a very effective treatment for many lymphoproliferative and autoimmune diseases, such as lymphoma, idiopathic thrombocytopenic purpura (ITP), multiple sclerosis (MS), and rheumatoid arthritis (RA), as well as for immunosuppression in renal transplantation (1–4). However, aside from the elimination of B cells, the mechanisms of action of this biologic agent are still not fully understood. Rituximab is a humanized monoclonal antibody (mAb) specific for the CD20 antigen and induces Fc-mediated lysis of the target cells (5,6). CD20, a 35–37-kd tetra-spanning integral membrane protein on pre, naive, and mature B cells, regulates the early steps of cell cycle progression in B cells, including B cell proliferation and apoptosis (7). CD20 has been shown to be integrated into lipid rafts in response to antibody binding (8). The molecule can also act as an ion channel (9).

However, expression of CD20 is not restricted to B lymphocytes. A small population of T cells in all individuals, accounting for ~1.6% of all T cells, coexpress CD20 (CD20+ T cells). A slightly higher frequency (2.4%) of this T cell population has been reported by another group (10). Although the density of CD20 surface expression on T cells is lower than that on B cells, CD20+CD3+ T cells are completely eliminated

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along with the B cells in patients during rituximab therapy. We therefore analyzed the phenotype and functional capabilities of CD3+CD20+ T cells, in order to evaluate whether the loss of this T cell fraction could contribute to the success of rituximab therapies in diseases in which B cell depletion alone is unlikely to account for the therapeutic effect.

PATIENTS AND METHODS

Cell separation. Blood samples were collected from RA patients attending the outpatient clinic of the Clinic for Immunology and Rheumatology at Hannover Medical School, and from healthy donors whose samples were provided by the Institute for Transfusion Medicine at Hannover Medical School (details on the patients' characteristics are available at http://www.mh-hannover.de/11358.html). The study was approved by the Ethics Review Board of Hannover Medical School. Cadaveric tissue samples were obtained from the Institute of Legal Medicine at Hannover Medical School and were limited to fresh samples from healthy tissue that had been harvested within 24 hours of death.

Heparinized human peripheral blood was diluted 1:2 with phosphate buffered saline (PBS) and separated by Ficoll-Hypaque centrifugation (for 20 minutes at 1,000*g*). Cells from the interphase (peripheral blood mononuclear cells [PBMCs]) were isolated, washed twice (10 minutes at 1,000*g* and 10 minutes at 200*g*), and resuspended in PBS. For some experiments, cells were sorted into CD20+ and CD20− T cells using a FACSAria cell sorter (Becton Dickinson, Heidelberg, Germany).

Isolation of leukocytes from lymphatic tissues. Tissue samples from mesenterial lymph nodes as well as from the spleen, thymus, and liver were cut into small pieces with scissors. The pieces were homogenized by pressing them through a stainless steel mesh with a plunger of a 10-ml syringe, and then suspending the extract in PBS. Mononuclear cells were isolated via density-gradient centrifugation, as described above.

Monoclonal antibodies. For analyses of human leukocytes, various fluorochrome-labeled mAb directed against different antigens were used, as follows: fluorescein isothiocyanate (FITC)–conjugated CD3, phycoerythrin (PE)–conjugated CD8, FITC-conjugated CD25, FITC- and PE-conjugated CD95, FITC-conjugated CD154, and PE-conjugated CD166 (all from Dako, Hamburg, Germany); FITC-conjugated CD3, FITC-conjugated CD4, and FITC-conjugated CD11a (all from Immunotech, Hamburg, Germany); peridinin chlorophyll A protein (PerCP)– and allophycocyanin (APC)–conjugated CD3, APC- and FITC-conjugated CD4, APC-conjugated CD19, FITC- and PE-conjugated CD20, CD21, FITC-conjugated CD26, CD28, FITC-conjugated CD29, APC-conjugated CD38, FITC-conjugated CD40, PE-conjugated CD45RA, PE- and FITC-conjugated CD45RO, R-PE–conjugated CD49a, PE-conjugated CD56, IgD, IgM, FITC-conjugated T cell receptor 1 (TCR-1) (γ/δ), and PE-conjugated TCR-2 (α/β) (all from Becton Dickinson); FITC-conjugated CD2, FITC-conjugated CD3, APC-conjugated CD20, FITC-conjugated CD27, APC-conjugated CD56, PE-conjugated CD69, FITC-conjugated CD161, FITC-conjugated CD57, CyChrome-conjugated CD69, FITC-conjugated CD71, and FITC-conjugated CD83 (all from PharMingen, Hamburg, Germany); and FITC-conjugated CD24, FITC-conjugated CD62L, and FITC-conjugated CD98 (all from ImmunoTools, Friesoythe, Germany).

For TCR-2 typing, a Beta Mark $TCRV_\beta$ repertoire kit (Beckman Coulter, Hamburg, Germany) was used. Stainings of intracellular molecules were performed using Alexa for detection of interferon-γ (IFNγ; Caltag, Hamburg, Germany), FITC and PE for tumor necrosis factor α (TNFα), PE for interleukin-2 (IL-2), IL-4, IL-10, and IL-17, FITC for monocyte chemotactic protein 1 (MCP-1) (all from BD PharMingen, Heidelberg, Germany), FITC and PE for IL-1β (Becton Dickinson), FITC and PE for IL-8 (R&D Systems, Wiesbaden, Germany), and FITC for transforming growth factor β (TGFβ) (IQ Products, Groningen, The Netherlands).

For determination of apoptosis by fluorescence-activated cell sorting (FACS), a FITC-conjugated annexin V/7-aminoactinomycin D (7-AAD) kit (Beckman Coulter) was used. For all cytofluorometric experiments, appropriate isotype control antibodies (conjugated with Alexa, APC, CyChrome, FITC, PE, or PerCP) were utilized.

Unlabeled CD20 (Becton Dickinson) was used for blocking experiments. For cell activation, CD3 was purified from cell culture supernatants of antibody-secreting OKT3 hybridomas (American Type Culture Collection, Wesel, Germany) and adjusted to a concentration of 10 μg/ml. For calcium flux experiments, cells were activated by adding CD3 (clone MEM-92; a generous gift from Vaclav Horejsi, Prague, Czech Republic) at a concentration of 8 μg/ml.

Phenotype analyses. Analyses of cell phenotype were performed using multicolor immunofluorescence of Ficoll-Hypaque–separated cell samples utilizing directly labeled mAb, as described previously (11). Briefly, 3×10^5 cells/well of a 96-well U-bottomed plate were incubated with murine mAb against the appropriate antigens at an optimal dilution for 20 minutes at 4°C. Nonspecific binding was eliminated by mixing the samples with a 1:5 solution of a commercial human IgG (Octagam; Octapharma, Langenfeld, Germany). Samples were washed 3 times in PBS/bovine serum albumin (BSA), and at least 10^4 cells per appropriate gate were analyzed using a dual-laser cytometer (FACSCalibur; Becton Dickinson) with CellQuest software or Summit 4.3 (Dako). For blocking of CD20 fluorescence, PBMCs were incubated for 30 minutes with unlabeled CD20 antibodies in varying concentrations, prior to phenotyping. After 3 washes, the cells were stained as described above.

Cell stimulation. Activation of cells was performed using various stimuli, as previously described (12). Briefly, PBMCs or sorted CD20+ and CD20− T cells were resuspended in R10 medium (RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 m*M* L-glutamine, and 1 m*M* sodium pyruvate) at a concentration of 2×10^6 cells/ml. Cells were then stimulated by the addition of phorbol 12-myristate 13-acetate (PMA) (10 ng), ionomycin (1 m*M*), phytohemagglutinin (25 μg/ml), pokeweed mitogen (0.5 μg/ml), and concanavalin A (10 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2.

For CD3 stimulation, 96-well MaxiSorp plates were coated by incubation with 100 μl CD3 (OKT3), or PBS as

negative control, for 30 minutes. The wells were washed 3 times with PBS and replenished with the cell suspension.

In some experiments, the transport inhibitor brefeldin A (2.5 m*M*) was added 1 hour after the cells were added, in order to prevent the secretion of induced cytokines into the supernatant, as described elsewhere (12). After 4 hours of culture at 37°C in 5% CO_2, the cells were harvested for further analyses.

Quantitative determination of cytokines. Four different populations of sorted CD3+ cells (CD4+CD20+, CD4+CD20−, CD8+CD20+, and CD8+CD20−) were activated with 10 ng PMA/1 m*M* ionomycin per ml in R10 medium for 18 hours. Supernatants were harvested and stored at −80°C until analyzed. The supernatants were subjected to a cytometric bead array analysis (Becton Dickinson) in order to concomitantly determine the concentrations of secreted IL-2, IL-4, IL-6, IL-10, IFNγ, and TNFα. The assays were performed according to the manufacturer's protocol.

Staining of intracellular proteins. After stimulation, the cells were washed with PBS/BSA and stained for surface markers (CD3 and CD20) for 20 minutes. After 2 washes with PBS/BSA, the cells were fixed for 10 minutes at room temperature in PBS containing 4% paraformaldehyde. Cells were then washed once and resuspended in saponin buffer (PBS supplemented with 5 m*M* HEPES and 0.1% saponin) to perforate the cell membranes. Subsequently, aliquots were stained with mAb against intracellular cytokines. Nonspecific binding of the mAb via Fc receptors was discerned by adding human IgG solution. After 30 minutes of incubation at 4°C, the cells were washed once with saponin buffer and twice with PBS/BSA. After resuspension, the cells were ready for FACS analysis. Analyses of resting PBMCs were performed in the same manner, but without prior stimulation.

Determination of apoptotic cells. Freshly isolated or overnight CD3–stimulated PBMCs were stained for CD3 and CD20 surface markers, as described above. After 3 washes in PBS/BSA, the stained cells were resuspended in annexin binding buffer (10 m*M* HEPES/NaOH, 140 m*M* NaCl, 2.5 m*M* $CaCl_2$, pH 7.4) and incubated with 10 μl FITC-conjugated annexin V and 10 μl 7-AAD for 10 minutes at 4°C. The cells were washed in annexin binding buffer and analyzed by flow cytometry.

RNA isolation and reverse transcription (RT). Total RNA of purified human CD20+ and CD20− T cell populations, B cells, or control cells was isolated using RNeasy Micro and RNeasy Mini kits (Qiagen, Hilden, Germany), after homogenizing the preparations with QIAshredder columns (Qiagen) according to the manufacturer's instructions. Additional on-column DNase treatment was conducted (RNase-Free DNase; Qiagen). RT was performed with an Omniscript or Sensiscript RT kit (Qiagen).

Real-time quantitative RT–polymerase chain reaction (PCR). RT-PCR (iCycler; Bio-Rad, Hercules, CA) was performed with iQ SYBR Green Supermix (Bio-Rad, Munich, Germany) according to the manufacturer's protocol. A total volume of 25 μl comprising 12.5 μl of iQ SYBR Green Supermix, 2.5 μl QuantiTect primers (human CD20; Qiagen) or human β-actin primers (forward 5′-AGTACTCCGTGTGGATCGGC-3′, reverse 5′-GCTGATCCACATCTGGTGGA-3′), or human CD19 primers (forward 5′-AGGAATACAAAGGGGACTGG-3′, reverse 5′-GTCTTATGAGAACGAGGATG-3′) was prepared in a final concentration of 250 n*M*. Equal amounts of complementary DNA (cDNA) were added and the volume was adjusted with PCR Grade Water (Ambion, Huntingdon, UK). Control cDNA was used to generate a standard curve.

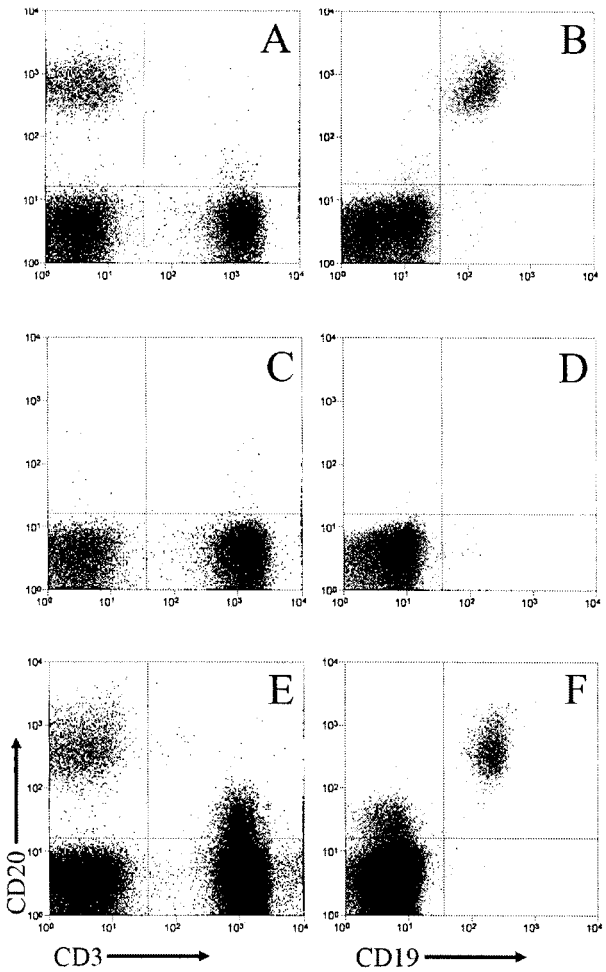

Figure 1. In vivo depletion of CD20+ cells by rituximab treatment. Peripheral blood samples were analyzed by fluorescence-activated cell sorting. Representative dot plots from a patient before (**A** and **B**) and 1 day after (**C** and **D**) rituximab depletion are depicted. Cells were stained with CD3 and CD20 antibodies (**A** and **C,** respectively) or CD19 and CD20 antibodies (**B** and **D,** respectively), and gated on lymphocytes according to the forward scatter versus side scatter properties of the cells. For comparison, cells from a healthy donor were analyzed (**E** and **F**).

RESULTS

Depletion of CD3+CD20+ T cells by rituximab treatment. In patients receiving the rituximab antibody, not only B cells but also T cells coexpressing CD20 were efficiently depleted. Analyses of 10 patients treated with

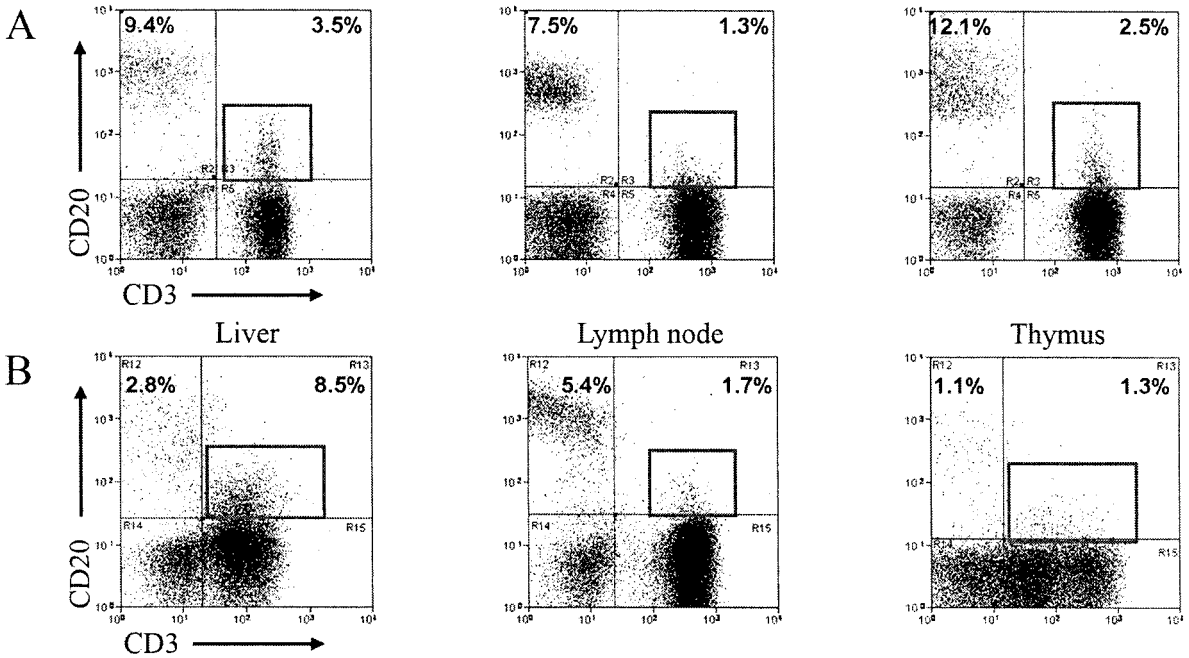

Figure 2. Coexpression of CD20 on T lymphocytes. A fraction of lymphocytes from different compartments coexpressed CD20 at low density. Cells from the peripheral blood of 3 healthy donors (**A**) and from various tissues (**B**) were isolated and stained with CD3 and CD20 antibodies. The cells were gated on lymphocytes according to the forward scatter versus side scatter properties. The boxed areas indicate CD20+ T cells, while the percentages of CD20+ cells are given in the corresponding quadrants.

rituximab for different reasons (e.g., lymphoproliferative syndrome or Epstein-Barr virus–induced B cell expansion) demonstrated complete depletion of CD20+ cells. In addition to B cells, CD20+ T cells were also completely eliminated in the blood samples from all subjects after application of the antibody. CD20+ T cells in patients (Figures 1A–D) as well as healthy controls (Figures 1E and F) did not express other B cell markers such as CD19.

Frequency of CD3+CD20+ T cells. FACS analyses of freshly isolated PBMCs from healthy donors revealed that a mean frequency of 1.6% of all CD3+ T cells (range 0.1–6.8%; n = 142) coexpressed the CD20 antigen, which is normally referred to as a B cell marker (results from 3 healthy donors shown in Figure 2A). In absolute numbers, the CD20+ T cells accounted for ~28 cells/μl, on average, in healthy individuals. In RA patients, the frequency of CD20+ T cells (mean 1.2%, range 0.4–2.6%; n = 27) was not significantly different from that in healthy controls.

In comparison with B cells, CD20 was coexpressed on the surface of T cells at a considerably lower density. In addition to the findings in the peripheral blood, we detected comparable percentages of CD20+ T cells in the lymphocytes isolated from various tissues or organs, including the lymph nodes, thymus, and liver, indicating that there was ubiquitous distribution throughout the body (Figure 2B). In addition, we analyzed T cells in synovial fluid obtained from 10 RA patients, and we found that a mean ± SEM 5.3 ± 1.6% of the T cells were CD20+, of which 6% and 6.6% constitutively produced IL-1β and TNFα, respectively. Thus, these cells from the synovial fluid were quite similar to blood lymphocytes, although the percentages of these cells were higher in synovial fluid.

Blocking experiments using unlabeled CD20 antibodies prior to CD20 fluorescence analyses effectively impeded binding of labeled CD20 antibodies to B cells and CD20+ T cells, indicating a specific binding of CD20 antibodies to the respective T cells (for more details, see http://www.mh-hannover.de/11358.html). The expression of CD20 protein by peripheral blood T cells was additionally confirmed on the RNA level. Real-time RT-PCR using messenger RNA (mRNA) isolated from sorted CD3−CD20+ B cells, CD3+CD20+ T cells, and CD3+CD20− T cells revealed adequate amounts of CD20-encoding mRNA in the 2 CD20+ cell populations but none in the CD20− T cells in all samples analyzed. PCR was also performed using CD19-specific primers to further confirm the T cell nature of

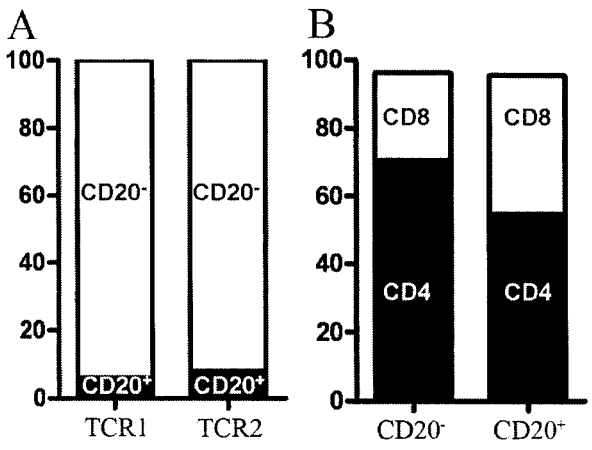

Figure 3. Coexpression patterns of CD20+ and CD20− T lymphocytes, as assessed by flow cytometry. **A,** Peripheral blood mononuclear cells (PBMCs) were gated on γ/δ (T cell receptor 1 [TCR-1]) and α/β (TCR-2) T cells. The percentages of CD20+ and CD20− T cells within the 2 T cell subsets are depicted. **B,** PBMCs were gated on CD3+CD20+ and CD3+CD20− cells. The expression patterns of CD4 and CD8 in the 2 T cell populations are shown.

CD3+CD20+ cells. In contrast to the findings in B cells, the 2 T cell subpopulations (CD20− and CD20+) lacked any CD19 message (for more details, see http://www.mh-hannover.de/11358.html).

Phenotype of CD3+CD20+ T cells. We next questioned whether coexpression of CD20 is restricted to a specific subset of T cells. We therefore performed 4-color FACS analyses using PBMCs from healthy donors. With the exception of CD20, T cells did not express any other B cell markers (e.g., CD19, CD21, CD24, or soluble IgM) (results not shown). Analysis of CD28 expression on the 2 T cell subsets revealed that 83.9% of CD20+ T cells and 81.1% of CD20− T cells coexpressed CD28. There were no significant differences between CD20− and CD20+ T cells with respect to either the presence or the expression density of CD28 (results not shown).

Comparable ratios of CD20+ T cells were determined in the TCR-1 (γ/δ)– and TCR-2 (α/β)–expressing T cell populations (Figure 3A). Among the CD20+ T cell population, 45% coexpressed CD8 and 55% coexpressed CD4 (Figure 3B); this indicates a preferential coexpression of CD20 by CD8+ T cells. In contrast, CD20− T cells were composed of 70% CD4+ T cells and 30% CD8+ T cells, approximately complying with the common CD4:CD8 ratio of 2:1 in peripheral blood T cells.

Expression patterns of other surface markers were analyzed by gating on the appropriate T cell populations. We observed clear differences in the expression patterns of the differentiation/activation-associated markers CD29, CD38, CD45RO, CD49a, CD56, CD69, CD154, CD161, and CD166, which were preferentially expressed on CD20+ T cells. A high percentage of CD20+ T cells (22%) were also CD95+, indicating susceptibility to apoptosis via the Fas–FasL pathway (Table 1).

To exclude the possibility that the development of CD20+ T cells is a result of clonal expansion, we analyzed the α/β TCR repertoire of CD20+ and CD20− T cells in 5 healthy individuals and 8 RA patients. By gating on the respective populations, we determined differential usage of at least 5 V_β rearrangements by CD20+ and CD20− T cells in each individual. Comparing the different donors, there was no preferential TCR V_β-chain expression in CD20+ T cells when compared with CD20− T cells (detailed results provided at http://www.mh-hannover.de/11358.html). However, CD20+ and CD20− T cells both favored the expression of the $V_\beta 2$ chain in most patients.

Calcium flux. CD20 is a tetraspan protein that assembles into oligomeric complexes and forms or regulates a calcium entry channel. To investigate whether the calcium flux of CD20+ T cells differs from that of CD20− T cells, we measured calcium mobilization in the 2 T cell populations by flow cytometry. Cells were labeled with fluo-4, a dye that increases fluorescence intensity after Ca^{++} ion binding. Calcium flux was induced by crosslinking CD3 with the mAb MEM-92. Due to its IgM isotype, no secondary antibodies were required for the crosslinking experiments. The mean fluorescence intensity (MFI) of fluo-4 was determined in the different T cell populations before and after Ca^{++} flux induction. We measured a higher average increased

Table 1. Coexpression patterns of CD20+ and CD20− T cells*

	CD3+CD20+ T cells	CD3+CD20− T cells
CD29	83	57
CD38	7	19
CD45RO	72	42
CD49a	40	3
CD56	9	8
CD69	21	16
CD95	22	2
CD154	8	2
CD161	18	11
CD166	23	13

* Values are the percent of coexpressing cells in each subset.

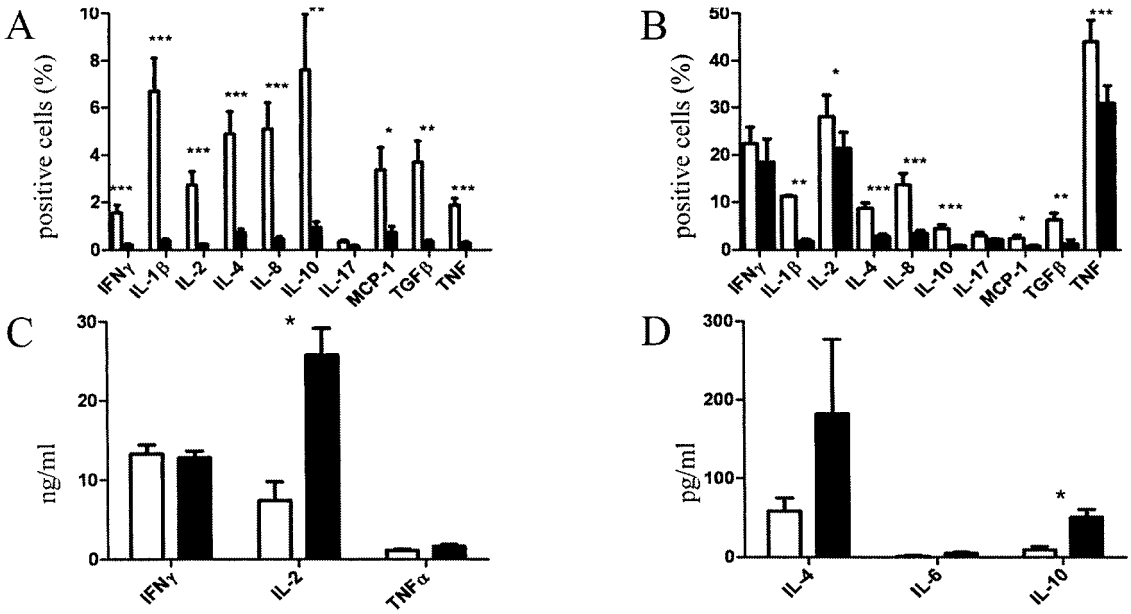

Figure 4. Cytokine production in the T cell subpopulations. **A,** Freshly isolated peripheral blood mononuclear cells were stained for surface markers and intracellular cytokines. Cells were gated on CD20+ T cells (open bars) and CD20− T cells (solid bars). The percentages of cytokine-positive cells in the 2 T cell populations are shown. **B,** Cells were activated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 4 hours in the presence of a transmembrane transport blocker (brefeldin A), and then analyzed for cytokine production as described above. **C** and **D,** Equal numbers of sorted CD20+ T cells (open bars) and CD20− T cells (solid bars) were stimulated with PMA/ionomycin for 24 hours, and the supernatants were analyzed for the concentration of the indicated cytokines. Bars show the mean and SEM. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$, versus CD20+ T cells. IFNγ = interferon-γ; IL-1β = interleukin-1β; MCP-1 = monocyte chemotactic protein 1; TGFβ = transforming growth factor β; TNF = tumor necrosis factor α.

MFI in CD20+ T cells (MFI 44.2) compared with that in CD20− T cells (MFI 20.0; $P < 0.01$).

Functional capabilities of CD3+CD20+ T cells. In order to compare the functional capacities of the T cells, we determined the proliferative response and cytokine production of CD20− and CD20+ T cells. Intracellular staining of freshly isolated lymphocytes revealed cytokine-producing cells among the CD20+ T cell fraction, which expressed IFNγ, IL-1β, IL-2, IL-4, IL-8, IL-10, IL-17, MCP-1, TGFβ, and TNFα in varying percentages (2–8%) (Figure 4A). In contrast, freshly isolated CD20− T cells hardly expressed any of these cytokines.

We next tested the 2 T cell populations for their ability to produce the cytokines after polyclonal stimulation with PMA/ionomycin (Figure 4B). The 2 T cell subsets were able to respond to the stimulus, but different patterns of cytokine expression were observed. Higher numbers of cytokine-producing cells were determined in the CD20+ T cell subset for all of the above-mentioned mediators. With the exception of IL-17 and IFNγ, the differences in expression of all other cytokines between the 2 T cell subsets reached statistically significant levels. In contrast to substantial IFNγ production in both T cell subsets, IL-17 was detected in only 3% and 2% of CD20+ and CD20− T cells, respectively.

We also tested supernatants of PMA/ionomycin-activated CD20+ and CD20− T cells that had been previously sorted and found to have a purity >95%. Supernatants were harvested after 24 hours and subjected to cytometric bead array analysis for quantification of the cytokines produced.

With regard to the levels of inflammatory cytokines in the supernatants, we determined similar concentrations of IFNγ and TNFα in the 2 T cell subsets, whereas supernatants harvested from CD20+ T cells contained significantly less IL-2 ($P < 0.05$) compared with the levels in supernatants from CD20− T cells (Figure 4C). Among the other cytokines analyzed by cytometric bead array (IL-4, IL-6, and IL-10), IL-10 was detected at higher concentrations in supernatants from CD20− T cells ($P < 0.05$), whereas no significant differences were determined for IL-4 and IL-6 (Figure 4D).

To determine the proliferative response of the

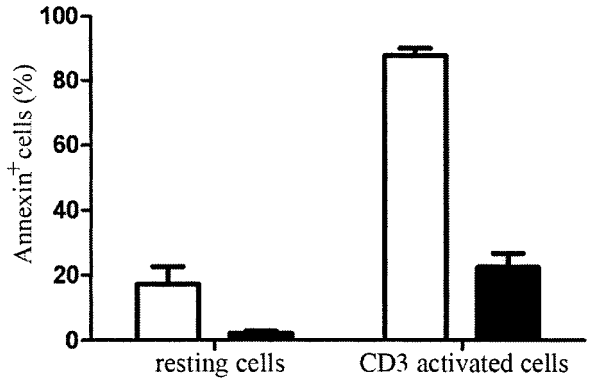

Figure 5. Spontaneous and induced apoptosis of CD20+ and CD20− T cells. Peripheral blood mononuclear cells were stained with a cocktail of CD3 and CD20 monoclonal antibodies, annexin V, and 7-aminoactinomycin D. Fluorescence-activated cell sorting analysis was performed by gating on CD3+CD20+ cells (open bars) and CD3+ CD20− cells (solid bars). The percentages of apoptotic (annexin V–positive) cells were determined. The cells were analyzed directly after isolation (resting cells) and after 24 hours of activation via CD3 crosslinking. Bars show the mean and SEM.

2 T cell subsets, cells were sorted into CD3+CD20+ and CD3+CD20− cells and 2×10^4 cells/well of each fraction were stimulated with immobilized CD3 antibodies for 72 hours, in triplicate. Tritiated thymidine was added 24 hours before the cells were harvested. The ^{3}H-thymidine incorporation assay revealed a significantly stronger proliferative response in the CD20− T cell population as compared with the CD20+ T cell population (1,074 counts per minute versus 554 cpm; $P < 0.05$) (for more details, see http://www.mh-hannover.de/11358.html).

Levels of apoptosis among CD20+ and CD20− T cells. In order to assess the numbers of apoptotic cells, we analyzed the surface expression of phosphatidyl serine (PS) on CD20− and CD20+ T cells. Based on this assay, CD20+ T cells were found to be more prone to apoptosis than CD20− T cells (Figure 5). About 17% of freshly isolated CD20+ T cells expressed PS on the surface, which was strikingly higher than the percentage of freshly isolated CD20− T cells expressing PS (<3%).

Apoptosis is a physiologic mechanism to limit immune responses in vivo by inducing cell death in activated immune cells (known as activation-induced cell death). We were therefore interested in determining whether apoptosis of CD20+ T cells could further increase upon activation. Stimulation with CD3 crosslinking, which mimics the physiologic activation pathway via the TCR, induced a strong enhancement of apoptosis of CD20+ T cells (88% apoptotic cells) and also induced apoptosis of CD20− T cells, but to a lower extent (22.5% apoptotic cells).

DISCUSSION

Rituximab therapy is standard in the treatment of malignant lymphomas and is currently being utilized for several systemic autoimmune diseases, including ITP, MS, and RA. An immunosuppressive effect of rituximab treatment has recently been demonstrated in renal transplantation (4). The antibody is specific for CD20 and its application efficiently depletes B cells in vivo. The initial rationale for treating patients with rituximab was to reduce the number of autoantibody-producing B cells. However, it has become evident that the observed curative effects of this treatment cannot be explained simply by the depletion of antibody-secreting cells (13,14).

Several B cells not only serve as antibody-producing cells but also can be effective in antigen presentation, and they communicate, via CD40, with T cells expressing the ligand CD154. Therefore, the removal of B cells reduces the costimulatory effects on T cells (15,16). Furthermore, B cells can produce several cytokines, including IL-10, and depletion of B cells has been shown to restore the numbers of regulatory T cells (17,18). Thus, an indirect effect of rituximab on T cell function has been postulated.

Our study focuses on a different mechanism by which rituximab can influence the functional properties of T cells. Our approach was to analyze the small population of T cells that express CD20 at low density. The CD20+ T cell population is present in all individuals and comprises ~1.6% of peripheral blood lymphocytes. These T cells are not restricted to the peripheral blood, as was shown by the detection of CD20+ T cells in varying percentages in several tissues such as the thymus, liver, and lymph nodes, indicating a ubiquitous distribution. In addition, in the synovial fluid, we observed increased numbers of CD20+ T cells, suggesting a pivotal role of these cells in the inflamed joints. In addition to the proof on the protein level, CD20 expression in the respective T cell subset was additionally confirmed by quantitative PCR, revealing CD20 mRNA transcription only in sorted CD20+ T cells, but not in CD20− T cells, whereas CD19-specific mRNA was absent in both T cell subsets.

Periodic analyses of patients' blood revealed that rituximab treatment depleted the CD20+ T cell population as well as the B cells. Although the number of CD20+ T cells represents a small fraction of all T cells,

they constitute a substantial pool of cells, the depletion of which might significantly contribute to the therapeutic effect of rituximab in certain diseases. We therefore analyzed the phenotype and function of CD20+ T cells in more detail. Patients with autoimmune diseases such as RA often display increased frequencies of CD28− T cells (19,20). Therefore, we analyzed the CD28 coexpression patterns on CD20− and CD20+ T cells. In the blood samples from the RA patients in this study, ~17% of the T cells were CD28−, but there was no significant difference between the CD28 coexpression on the CD20− and CD20+ T cell subsets.

Since the CD20+ T cell pool comprises both CD4+ and CD8+ cells and exhibits a considerable variability in the coexpression patterns of several surface molecules, mono- or oligoclonality of this T cell subpopulation is unlikely. To rule out this possibility, we determined the V_β clonotypes of 5 healthy donors and 8 RA patients. Each of these subjects expressed a variety of V_β chains in both CD20− and CD20+ T cells, thus confirming the polyclonality of the CD3+CD20+ T cell population. Comparing the 5 healthy donors with respect to V_β patterns of CD20+ T cells revealed that no specific chain was preferred, also indicating a polyclonal antigen specificity of the cells. Thus, we can conclude that CD20+ T cells represent a heterogeneous polyclonal T cell population with either a class I or a class II major histocompatibility complex restriction. However, in most RA patients, we observed a preferred $V_\beta 2$ expression in both T cell subsets. $V_\beta 2$ has been reported to be preferably expressed in T cell lines generated from the PBMCs of RA patients, suggesting that a disease-related increase in $V_\beta 2$-chain expression occurs (21).

We further compared the expression of several differentiation and activation markers on the 2 T cell subsets, revealing that activation markers, in particular, such as CD25, CD45RO, CD49a, CD69, and CD166, were more strongly expressed on CD20+ T cells. In contrast, the expression of CD38 (also referred to as an activation marker) was significantly lower on CD20+ T cells. CD38 is a multifunctional ectoenzyme, which also acts on cell adhesion, signal transduction, calcium signaling, and organization of lipid rafts (22). Because most of these functions can also be mediated by CD20, this molecule might thus serve on T cells as an alternative to CD38.

Functional analyses uncovered considerable differences between the 2 T cell subsets. Intracellular staining for cytokines revealed that a notable number of resting CD20+ T cells were constitutively producing several cytokines, whereas resting CD20− T cells were mostly negative. Additional stimulation induced cytokine production in both T cell subsets, although the number of positive cells remained significantly higher in the CD20+ T cell fraction.

Since we detected constitutive cytokine production and also higher percentages of cytokine producers among CD20+ T cells, we were interested to know to what degree CD20+ T cells could contribute to the total cytokine production. We therefore sorted CD3+ cells into CD20+ and CD20− T cells and stimulated the cells separately. The supernatants harvested from the cultures were then subjected to quantitative analysis by cytometric bead array. To our surprise, the concentrations of 2 of the 6 cytokines produced by CD20+ T cells, IL-2 and IL-10, were only equal to or lower than those measured in CD20− T cells.

We hypothesized that CD20+ T cells could be in an already highly activated state, as indicated by the coexpression of activation markers and constitutive production of several cytokines, and therefore could be undergoing activation-induced apoptosis. Therefore, we analyzed the proliferative response and degree of apoptosis in the cells. Comparison of the proliferation of sorted CD20+ and CD20− cells revealed a much stronger response of CD20− T cells. Concomitantly, our results clearly showed that freshly isolated CD20+ T cells were already apoptotic, which could be accelerated by activation to a larger extent than that of CD20− T cells.

Our findings show that CD20+ T cells represent a highly activated cell population. They coexpress several activation markers, strongly mobilize Ca^{++} ions, and produce cytokines without additional stimulation. However, the low proliferative capacity, exhaustion of cytokine production, and the advanced degree of apoptosis suggest that CD20+ T cells are undergoing downregulation by a mechanism known as activation-induced apoptosis (23). Nevertheless, before undergoing apoptosis, CD20+ T cells comprise an effector cell population capable of producing several cytokines. Among these mediators are IL-1β and TNFα, both of which are cytokines involved in the pathogenesis of RA. One could argue that the CD20+ T cell population is small and, to some extent, already apoptotic, and therefore insignificant. However, CD20+ T cells are constantly present in each individual, indicating a steady state in which apoptotic CD20+ T cells are constantly replaced with preapoptotic CD20+ T cells; thus, this cell population constitutes a pool of activated T cells releasing proinflammatory factors. We can therefore assume that the

CD20+ T cell population substantially contributes to the severity of disease, and the depletion of this cell subset might represent a mechanism, in addition to the elimination of B cells, by which rituximab treatment is effective in diseases such as RA.

ACKNOWLEDGMENT

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Jacobs had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Witte, Schmidt, Jacobs.

Acquisition of data. Wilk, Witte, Marquardt, Horvath, Kalippke, Scholz, Wilke, Jacobs.

Analysis and interpretation of data. Wilk, Witte, Marquardt, Horvath, Kalippke, Scholz, Wilke, Schmidt, Jacobs.

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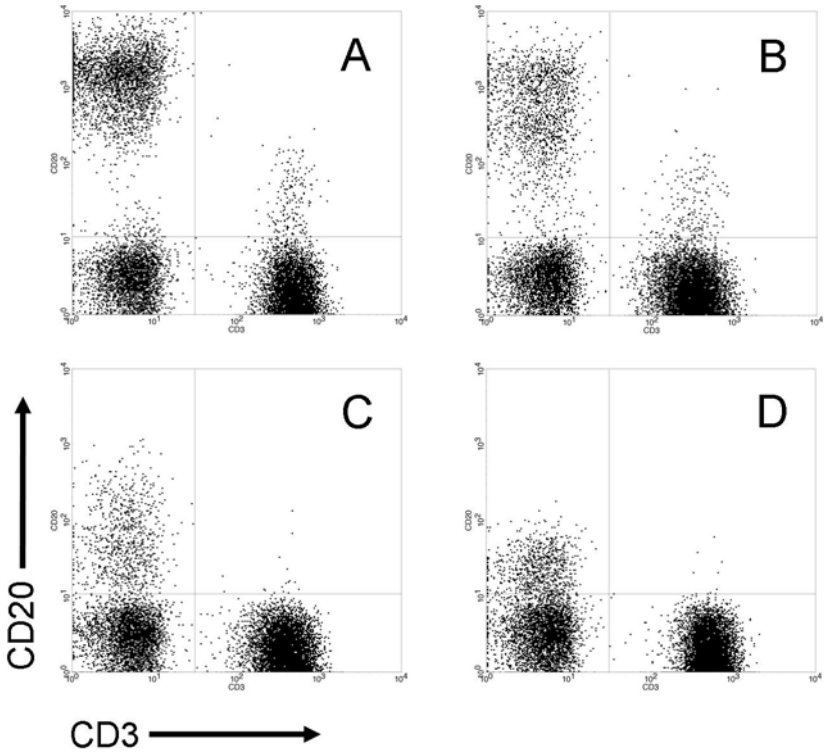
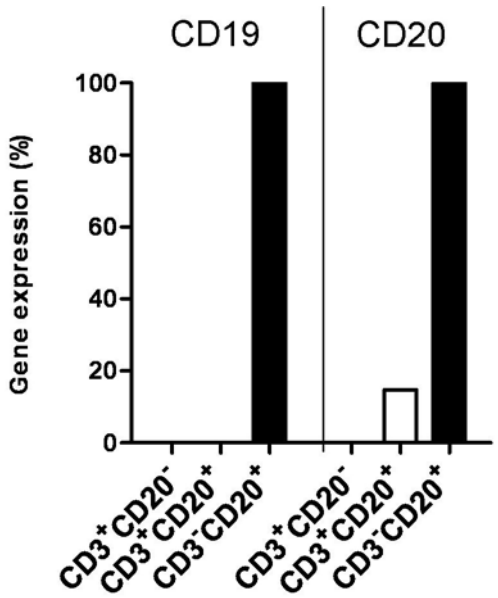
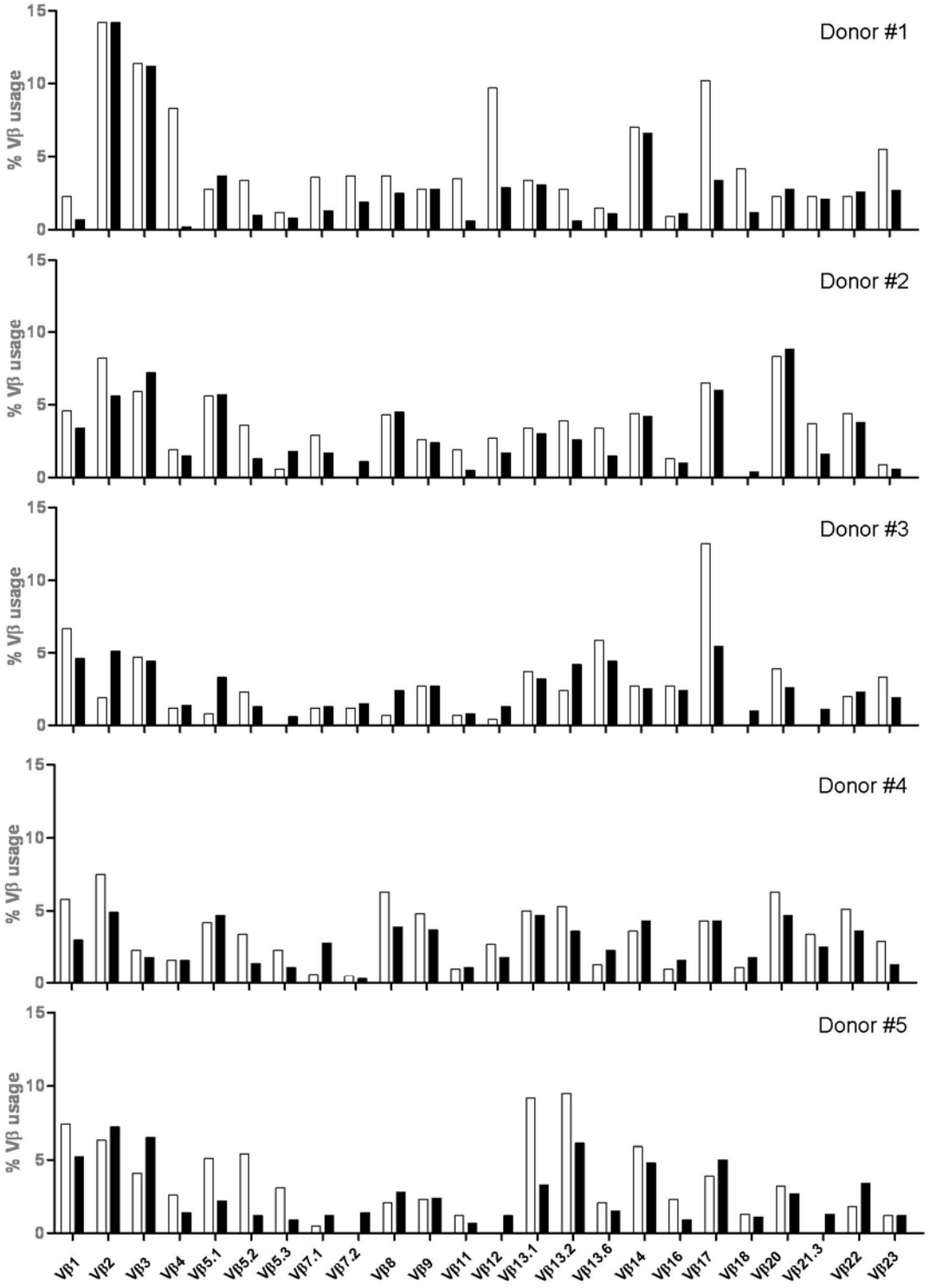
Supplementary Information



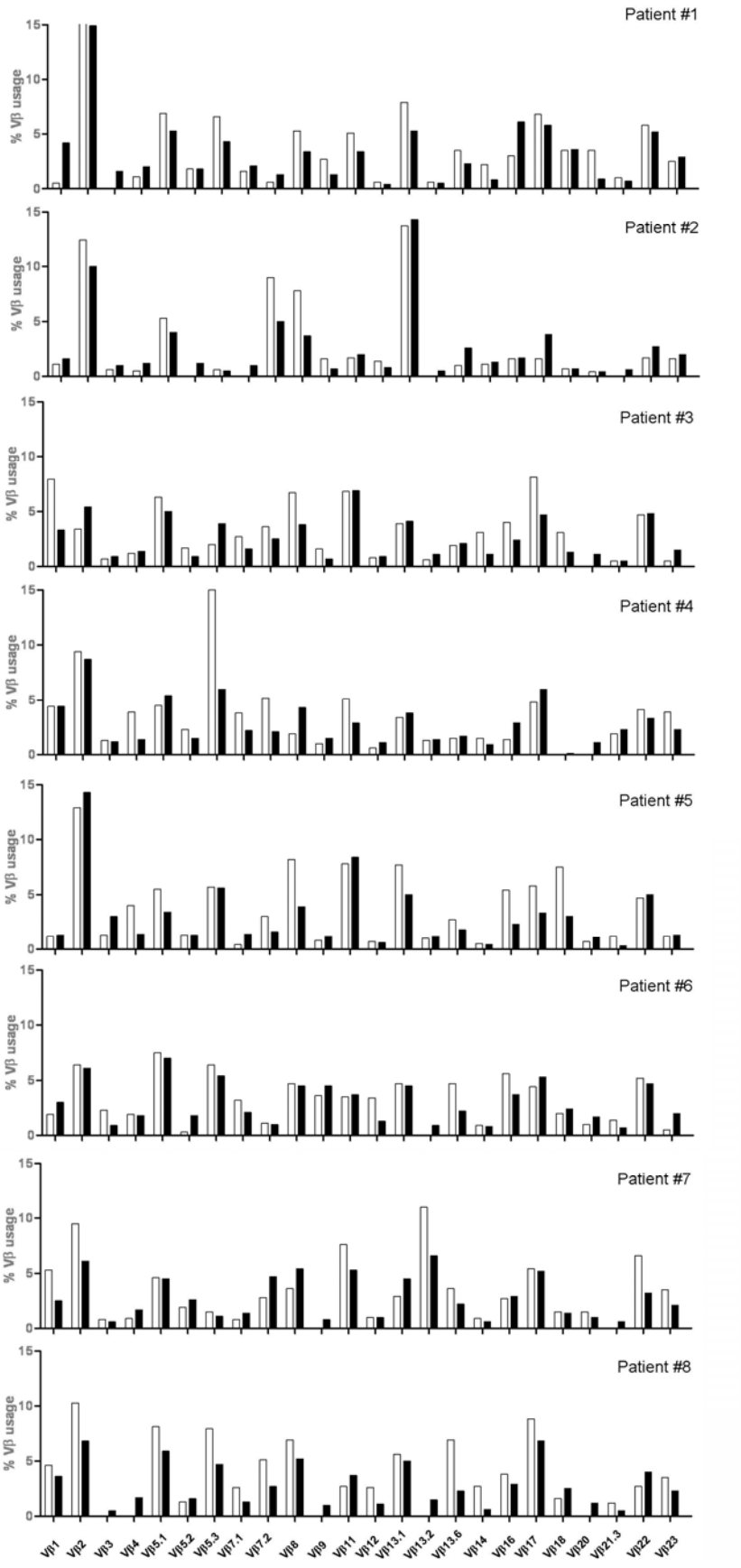
Suppl. Fig. 1: Blocking of CD20 expression in human B and T cells.
Human PBMC were preincubated with increasing amounts of unlabeled CD20 antibodies or IgG (as control) for 30 min. After washing, cells were stained with CD20PE and CD3PerCP antibodies. A: 20µl IgG control; amount of blocking CD20 antibodies: B= 1µl, C= 10µl, D=20µl



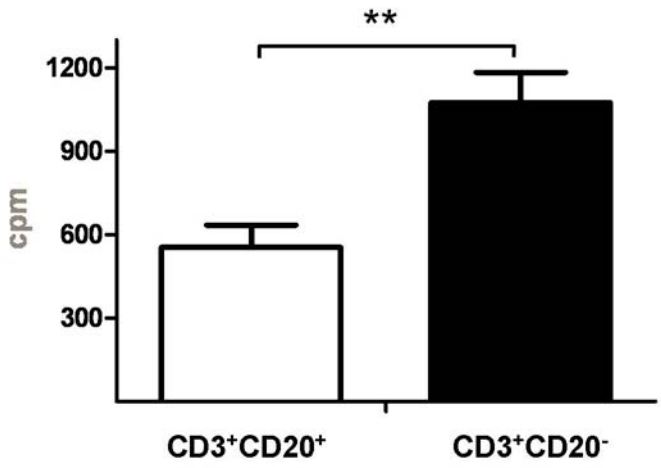
CD19 and CD20 mRNA expression in human T and B cells. Human PBMC were sorted into 3 populations (CD3+CD20+, CD3+CD20- and CD3-CD20+) with a FACSAria cell sorter. CD19 and CD20 specific mRNA expression was analyzed by quantitative real time PCR using an iCycler (Biorad, Munich, Germany). Results of three independent experiments are shown.



Suppl. Fig. 3a: Receptor repertoires of α/β T cell populations. PBMC were gated on $CD3^+CD20^+$ and $CD3^+CD20^-$ cells and the usage of Vβ chains were determined by multicolour flow cytometry. percentages of positive T cells for the different Vb chains (as indicated on the x-axis) are depicted for $CD20^+$ (white bars) and $CD20^-$ T cells (black bars), which were analyzed from peripheral blood of five healthy individuals.



Suppl. Fig. 3b: Receptor repertoires of α/β T cell populations. PBMC were gated on $CD3^+CD20^+$ and $CD3^+CD20^-$ cells and the usage of Vβ chains were determined by multicolour flow cytometry. Percentages of positive T cells for the different Vβ chains (as indicated on the x-axis) are depicted for CD20+ (white bars) and $CD20^-$ T cells (black bars), which were analyzed from peripheral blood of eight RA patients.



Suppl. Fig 4: Proliferative capacity of $CD20^+$ and $CD20^-$ T lymphocytes. PBMC were sorted into $CD3^+CD20^+$ (white bar) and $CD3^+CD20^-$ (black bar) T cells. Cells were activated by CD3 cross-linking for 72h and H3-thymidine was added 24h before harvesting. Incorporation of the H3-nuclides is given in counts per minute (cpm).

Table 1 Characteristics of RA patients

Patient #	Activity (DAS28)	RF	CCP	CRP	$CD3^+CD20^+$ T cells (%)
1	3.9	-	-	1.2	0.7
2	3.5	+	+	0.8	1
3	3.7	+	+	1.2	1
4	4.9	+	+	15	1.4
5	5.6	+	+	14.5	0.7
6	6.8	+	+	35.6	0.9
7	6.1	+	-	2	2.4
8	4.4	+	+	1.2	0.5
9	6.1	+	-	5.6	1.3
10	7.2	+	+	9.3	1.2
11	6.2	+	+	11	1.8
12	6.52	-	+	40.7	2.2
13	3.8	-	-	6.8	1.3
14	4.06	-	-	1.4	0.4
15	3.6	+	+	2.7	0.6
16	4.7	+	+	7	2.6
17	3.5	+	+	5.2	0.8
18	6.5	+	-	33.2	0.7
19	3.5	+	+	6.9	1
20	3.9	+	+	118	1.4
21	3.34	+	+	7.4	1.9
22	3.57	+	+	5.4	0.5
23	3.29	+	+	3.8	1.9
24	3.47	+	+	5.1	1.9
25	4.6	+	+	30.6	1.7
26	3.73	+	+	2.2	0.9
27	1.2	+	+	0.8	0.8

Abbr.: DAS28: Disease activity score 28 joints count
RF: Rheumatoid factor
CCP: anti-cyclic citrullinated peptide
CRP: c-reactive protein

6 S100B SECRETION BY HUMAN CD8+ T CELLS ACTIVATES MONOCYTES AND GRANULOCYTES: NOVEL CROSSTALK BETWEEN THE ADAPTIVE AND INNATE IMMUNE SYSTEMS

Johann Steiner, Nicole Marquardt, Inga Pauls, Kolja Schiltz, Hassan Rahmoune, Sabine Bahn, Bernahrd Bogerts, Reinhold E. Schmidt, Roland Jacobs

Eingereicht bei Brain, Behaviour, and Immunity. In Revision.

Wissenschaftlicher Beitrag von Nicole Marquardt an der Publikation:
Aktivierung von Monozyten und Granulozyten durch S100B + durchflusszytometrische Analysen, Auswertung der entsprechenden Daten und teilweise Verfassen des entsprechenden Ergebnisteils.

S100B secretion by human CD8+ T cells activates monocytes and granulocytes: novel crosstalk between the adaptive and innate immune systems

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Running title: Lymphocytic S100B expression and secretion

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Word count - Abstract: 242

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Abstract

The calcium-binding protein S100B has originally been detected in the nervous system and melanocytes. Therefore, S100B serum levels have been employed as a surrogate marker for brain-related pathologies and melanoma progression. Inflammatory processes play an important role in these disorders. This study addresses the potential relevance of S100B+ lymphocytes in mediating such inflammatory responses.

S100B expression was determined in human peripheral blood leukocytes isolated from healthy volunteers using automated flow cytometry. S100B+ lymphocytes were characterised for cytotoxic activity, cytokine production and S100B secretion. In addition, we investigated whether secretion of S100B modulates the immune activity of monocytes and neutrophils expressing the S100B receptor RAGE (receptor of advanced glycation end products).

S100B expression was observed in CD8+ lymphocytes, which comprise about 2-4% of all lymphocytes. Comparison of S100B+ and S100B- CD3+CD8+ cells revealed no differences in production of interferon gamma (IFNγ) and interleukin-2 (IL-2). However, S100B+ lymphocytes lacked cytotoxic activity, as assessed by a CD107a degranulation assay. Stimulation of these cells with anti-CD3 or phytohaemagglutinin resulted in release of S100B and triggered increased expression of the adhesion molecule CD11b and membrane shedding of CD62L from granulocytes and monocytes.

These findings set the stage for a new field of research addressing a S100B-mediated crosstalk between the innate and the adaptive immune system, encompassing various physiological and pathological conditions which have been associated with increased S100B serum levels. S100B might function as an interface to immunological processes, distinct from the known cytokine- and chemokine-mediated pathways.

Keywords: S100, S100B, T cells, lymphocytes, granulocytes, monocytes, RAGE.

Abbreviations

APC, allophycocyanin
ANOVA, analysis of variance
BSA, bovine serum albumin
Ca^{2+}, calcium ion
CD, cluster of differentiation
Cox-2, cyclooxygease-2
CRP, C-reactive protein
DAMP, damage-associated molecular pattern
E-rosetting, erythrocyte rosetting using sheep red blood cells
FITC, fluorescein isothiocyanate
FSC, forward scatter
HLA, human leukocyte antigen
IFNγ, interferon gamma
IL, interleukin
LPS, lipopolysaccharide
MCP-1, monocyte chemoattractant protein-1
MFI, mean fluorescence intensity
Ndr, nuclear Dbf2-related
PBMC, peripheral blood mononuclear cells
P53, tumour suppressor protein with a molecular weight of 53 kDa
PBS, phosphate buffered saline
PE, phycoerythrin
PerCP, peridinin chlorophyll protein
PHA, phytohaemagglutinin
PK, protein kinase
PMA, phorbol myristate acetate
RAGE, receptor of advanced glycation end products
RPMI, Roswell Park Memorial Institute medium
R10, RPMI medium supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate.
S100, family of calcium binding proteins that are soluble in 100% ammonium sulphate at neutral pH
SD, standard deviation
SEM, standard error of mean
SSC, side scatter
TNFα, tumour necrosis factor alpha
VCAM-1, vascular cell adhesion molecule-1

1. Introduction

S100B is a calcium ion (Ca^{2+})-binding protein of the S100-calmodulin-troponin superfamily which was originally found in high concentration within the nervous system, particularly in astrocytes.[1] Therefore, S100B serum levels have been considered as part of a damage-associated molecular pattern (DAMP) and used as a biomarker of brain- or astrocyte-related pathologies in clinical neuroscience.[2-6] This protein is also widely employed as a surrogate marker for melanoma progression by dermatologists, due to its abundant expression in melanoma cells.[7] However, S100B expression has also been observed in other cell types including oligodendrocytes, ependymal and plexus epithelial cells, adipocytes, chondrocytes, dendritic cells, Langerhans cells, injured myocardium, satellite cells of dorsal root ganglia, and Schwann cells of the peripheral nervous system.[8-10]

Recently, we draw scientific attention to the expression of S100B in lymphocytes again,[9] which had previously been explored more than 20 years ago. In 1982, Kanamori and colleagues found that S100 protein was present in white blood cells and particularly in T lymphocytes.[11] Takahashi and colleagues demonstrated more precisely that these cells contained the β fraction of the S100 protein (S100B), which was localised to the cytoplasm and partially the nucleus by immunocytochemistry.[12,13] The lymphocyte subset was identified as $CD8^+$ T cells, which are also positive for CD2 and CD3 and negative for CD4, CD57, and HLA-DR. $S100B^+$ T cells comprise 1-4% (mean 3.4%) of peripheral blood mononuclear cells (PBMCs) and 5-22.5% of $CD8^+$ cells under normal conditions. Interestingly, the proportion of $S100B^+$ T cells is lower in patients with different types of carcinoma, comprising only 0.5% of PMBCs.[13] In 1987, Sansoni et al. further defined $S100B^+$ lymphocytes as lacking the CD4 antigen.[14] Based on immunofluorescence and immunoelectron microscopy, Ferrari and colleagues showed that S100B+ lymphocytes are always $CD11b^+$ and suggested that these cells may play a role in modulating the immune system.[15] In accordance with this, De Panfilis et al. observed S100B expression in $CD8^+CD11b^+$ cells, and these exerted immunosuppressive effects.[16] Notably, $S100B^+$ T cells have not only been found in the peripheral blood, but also in the thymus, lymph nodes, and spleen, indicating a wide distribution in lymphatic organs and thus suggesting a specific immunological role.[17]

S100B is involved in a variety of *intracellular processes*, including regulation of cytoskeletal dynamics and protein kinase activity, and interacts with growth-associated target proteins such as the tumour suppressor P53 and nuclear Dbf2-related (Ndr) kinase, thereby indirectly controlling growth, proliferation, and morphology of the cells.[8] In the context of such growth-regulating mechanisms, it is of particular importance that

lymphoproliferative disorders characterised by S100B overexpression have been associated with aggressive behaviour and unfavourable clinical outcomes,[18-20] a finding supported by a histopathological case control study.[21]

S100B is also a *secretory protein* with cytokine-like features. Its effects are mainly facilitated by binding to the receptor for advanced glycation end products (RAGE), a multi-ligand receptor that amplifies inflammatory responses and mediates the effects of some neurotrophic and neurotoxic factors.[22] RAGE is expressed in a wide variety of cell types in the nervous, immune and other systems such as hepatocytes, cardiac myocytes and vascular endothelial and smooth muscle cells (see Table 1).[22-25] Astrocytes and oligodendrocytes have been shown to release S100B, which can affect neighbouring cells in a dose-dependent manner.[25,26] Nanomolar levels of S100B protein stimulate neurite growth and neuron survival, whereas micromolar levels have the opposite effect and can even induce apoptosis.[27] Similar dose-dependent trophic or suppressive effects have also been observed in melanoma cell lines.[28] However, according to Klein et al., cultures of normal peripheral blood lymphocytes proliferate even at high doses of externally administered S100 protein (maximum stimulation at 31 µg/ml, proliferation even at 125 µg/ml), suggesting that the growth-regulating effects of S100B may be cell type dependent.[28]

Most previous publications have concentrated on the role of S100B in the brain and melanocytes and S100B expression in peripheral lymphocytes has not been addressed adequately. However, this could be important with regard to the study of inflammation, cardiovascular and neuropsychiatric diseases. Early studies which first demonstrated the occurrence of $S100B^+$ lymphocytes used Ficoll-Hypaque centrifugation combined with erythrocyte rosetting to enrich T cells from peripheral blood. Subsequently, conventional immunocytochemistry using light, fluorescence and electron microscopy, was used to study putative $S100B^+$ lymphocytes. These approaches are limited by the possibility of technical artefacts and subjective bias. Therefore, we have assessed the lymphocyte subset-specific S100B distribution by flow cytometry using blood cells isolated from healthy human volunteers. It was also of importance to characterize $S100B^+$ lymphocytes within the immune system by studying S100B secretion and determining the associated cytokine response and cytotoxic capability. Moreover, we determined whether secreted S100B modulates the proinflammatory activity of monocytes and neutrophils to identify a hitherto unknown crosstalk between cells of the adaptive and innate immune systems.

2. Materials and methods

2.1 Blood samples

Heparinised blood samples from 20 healthy donors were obtained from the Institute for Transfusion Medicine at Hannover Medical School. Experiments were performed on either whole blood or mononuclear cells, which were prepared as follows. Blood was diluted 1:2 in phosphate buffered saline (PBS) and separated by Ficoll-Hypaque centrifugation for 20 min at 1000 g. PBMCs were collected from the interphase, washed by two rounds of suspension in PBS and centrifugation for 10 min at 1000 g and 200 g. The final pellets were resuspended in RPMI 1640 medium supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (all reagents: Biochrom, Berlin, Germany), also known as R10.

2.2 Antibodies

The antibodies used in this study were CD3 PerCP, CD4 PE, CD4 APC, CD8 PE, CD8 APC, CD19 PE, CD45 PerCP, CD56 PE, IL-2 APC (Becton Dickinson, Heidelberg, Germany), CD3 APC, CD11b PE, CD56 APC, CD107a PE, IFNγ PE (BD Pharmingen, Heidelberg, Germany), CD62L APC (Immunotools, Friesoythe, Germany), polyclonal rabbit anti-human S100B, and pig anti-rabbit serum FITC (Dako, Hamburg, Germany).

2.3 Phenotyping

Phenotypic analyses were performed using the CD antibodies indicated above in $3x10^5$ cells. After 20 min incubation with the antibodies of interest, cells were washed three times with PBS/BSA and at least 10000 events per sample were acquired using a FACSCalibur cytometer and CellQuest software (Becton Dickinson, Heidelberg, Germany). Cytometric data were analysed offline using Summit Version 4.1 software (Beckman Coulter, Hamburg, Germany).

2.4 Intracellular S100B staining

For detection of intracellular S100B, surface-stained cells were fixed for 10 min at room temperature by resuspension in 100 µl of 4% paraformaldehyde solution (Merck, Darmstadt, Germany). Cells were then washed and resuspended in PBS containing 0.1% saponin (Riedel-de Haën, Seelze, Germany), 0.01M HEPES (Applichem, Darmstadt, Germany) and 10 µl rabbit anti human S100B serum (1:1000). After 20 min of incubation, the cells were washed three times in PBS and then incubated with FITC-labelled pig anti-rabbit serum for 20 min. After three additional washes, the cells were subjected to cytometric analysis.

2.5 Degranulation assay

$1x10^6$ PBMCs in 1 ml R10 medium were stimulated with 5 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich, Munich, Germany) at 37°C as described previously in the presence of 20 µl anti-CD107a.[29] After 1 h, 6 µg/ml monensin (Sigma-Aldrich, Munich, Germany) was added to inhibit secretion and allow for intracytoplasmic accumulation. Three hours later, cells were stained for surface markers and S100B as described above, and the samples were analysed by flow cytometry.

2.6 Intracellular cytokine staining

Prior to intracellular staining for cytokine production, cells were stimulated with 5 ng/ml PMA and 500 ng/ml ionomycin. Briefly, PBMCs ($2x10^6$) were suspended in 1 ml R10 and brefeldin A (BFA; Sigma-Aldrich: Munich, Germany) was added after 1 h of culture in a humidified incubator at 37°C to inhibit further cytokine secretion. After an additional 3 h of incubation, the cells were immunostained with plasma membrane markers as described above. Cells were fixed and permeabilised as described above and antibodies directed against S100B, IL-2 and IFNγ were added. After incubation for 20 min and three washes, cells were analysed by flow cytometry.

2.7 Polyclonal T cell activation

PBMCs ($1x10^6$ cells/ml) were incubated in R10 supplemented with 25 µg/ml phytohaemagglutinin (PHA; HA16; Remel, Dartford, UK) or suspended in a 96-well flat-bottom plate coated with anti-CD3. The coating was produced by addition of 100 µl of 10 µg/ml anti-CD3 in PBS to each well, followed by incubation for 30 min at 37°C and washing with PBS. Supernatants and cells were harvested and analysed at different time-points.

2.8 Quantification of S100B in cell culture supernatants

Cells were harvested and centrifuged for 5 min at 1000g. S100B concentrations in cell-free supernatants were measured by an immunoluminometric assay using directly coated magnetic microparticles (LIAISON S100™; DiaSorin, Dietzenbach, Germany), as described previously.[30] The lowest measurable concentration of S100B was 0.02 µg/l. Intra- and inter-assay coefficients of variation were <5%. Measurements were performed in duplicate.

2.9 Activation of monocytes and granulocytes by recombinant human S100B protein

The recombinant human S100B protein (Abcam, Cambridge, UK) used in this study was produced in genetically modified, Gram-negative *Escherichia coli*. One disadvantage of this production method is a possible contamination with endotoxins, such as

lipopolysaccharide (LPS). To exclude an LPS-mediated activating effect, whole blood samples were preincubated with the antibiotic peptide polymyxin B (P-4932; Sigma-Aldrich, Munich, Germany), which neutralises LPS by binding to the major lipid A component. After 20 min of incubation at 37°C with or without 10 µg/ml polymyxin B, S100B protein was added at 10 µg/ml to whole blood.[28] Addition of LPS (100 ng/ml; Sigma-Aldrich, Munich, Germany) was used as a positive control. The possibility of an activating effect by other endotoxins was controlled by testing the effect of immunodepleting S100B from the protein buffer. After incubation of S100B with rabbit anti-human S100B antibodies, anti-rabbit MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and the immunocomplexes were removed magnetically. Cells were then incubated for 1 h at 37°C and surface-stained with the appropriate antibodies. Finally, red blood cells were lysed with BD FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) and $CD45^+$ granulocytes or monocytes were gated via side scatter (SSC) versus forward scatter (FSC) characteristics in flow cytometric analyses.

2.10 Statistical analysis

Statistical analyses were performed using the GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, California, USA). The data showed normal distributions, as indicated by Kolmogorov-Smirnov tests. Therefore, parametric tests such as unpaired two-tailed Student's *t*-tests and analysis of variance (ANOVA) were employed. Bonferroni correction was applied to control for Type I error. A probability level of $P < 0.05$ was considered statistically significant.

3. Results

3.1 S100B shows a predominantly intracellular localisation

Freshly isolated PBMCs were stained for lymphocyte surface markers and for S100B immunoreactivity. Flow cytometry using non-permeabilized cells showed little or no S100B surface expression in all lymphocyte populations examined (i.e., T, B, and NK cells). However, combined surface phenotyping and intracellular S100B staining after permeabilisation revealed a discrete population of cells which expressed S100B. $S100B^+$ cells comprised 2-4% of the lymphocyte population and the majority displayed a $CD3^+CD8^+$ phenotype, whereas a relatively small percentage of B and NK cells coexpressed S100B (Figure 1). Meanwhile, CD11b was coexpressed in 42 ± 22% of all $S100B^+$ lymphocytes.

3.2 $S100B^{\pm}$ cells represent a subpopulation of mainly non-cytotoxic T cells

As most of the S100B lymphocytes presented a $CD8^+$ T-cell phenotype, we initially restricted the functional characterisation studies to the $CD8^+$ T cell subset. To determine the cytotoxic potency of $S100B^+$ T cells, we used a CD107a degranulation assay. CD107a is expressed in intracellular granules and becomes detectable as soon as activated T cells release their cytotoxic contents. Addition of the polyclonal activating compound PMA showed that $S100B^+$ lymphocytes show little or no degranulation and thus represent a non-cytotoxic subpopulation of $CD8^+$ cells. Consistent with this finding, $CD107a^+$ cells were restricted to the $CD3^+CD8^+$ $S100B^-$ population (Figure 2).

3.3 S100B$^{\pm}$ and S100B$^-$ T cells express similar cytokine profiles

Another functional capability of $CD8^+$ T cells is the production of cytokines such as IFNγ and IL-2. To determine whether $S100B^+$ and $S100B^-$ T cells differ in cytokine profile, cells were stimulated with PMA/ionomycin for four hours in the presence of the transport blocker BFA to cause intracellular cytokine accumulation. Cytokine production was measured by intracellular flow cytometry and this revealed no significant difference between the two cellular subsets (Figure 3).

3.4 S100B is secreted by activated CD8$^{\pm}$ T cells

S100B expression over time was investigated after stimulating cells with anti-CD3 and PHA. Control cells were not stimulated.$S100B^+$ T-cell numbers decreased with time (PHA versus control, P <0.01; anti-CD3 versus control, P <0.05), while the $S100B^-$ T-cell fraction showed a concomitant increase (PHA versus control, P <0.01; anti-CD3 versus control, P <0.05) after stimulation. Non-stimulated controls did not show altered S100B expression (Figures 4A and 4B). No significant necrosis or apoptosis was detected in the total cell population (data not shown), suggesting that the decrease of $S100B^+$ T-cell frequency was due to secretion and/or intracellular degradation. Accordingly, we determined whether the cells converted to a $S100B^-$ state due to loss of the protein. Supernatants of total $CD8^+$ T cells were harvested at different time-points and S100B concentration was measured by immunoluminescence. The percentage of $S100B^+$ cells in the culture was determined in parallel. An inverse pattern between decreased $S100B^+$ cell number (PHA versus control, P <0.01; anti-CD3 versus control, P <0.05) and S100B concentration in the supernatant (PHA versus control, P <0.05; anti-CD3 versus control, P <0.05) was observed (Figure 4C), suggesting that $CD8^+$ T cells secrete S100B.

3.5 S100B activates granulocytes and monocytes

We next investigated the potential regulatory role of S100B on immune system function. First, we treated monocytes and granulocytes with recombinant S100B since these cells are known to express RAGE, which can be stimulated by S100B.[22-24,31] Whole blood

samples were activated with recombinant S100B or LPS as a control in the absence or presence of polymyxin B, which inhibits the biological activity of LPS, a possible contaminating factor in recombinant protein preparations. Activation of granulocytes and monocytes was determined based on the expression of the adhesion molecules CD11b and CD62L (Figure 5). Granulocytes and monocytes showed increased expression of CD11b and shed CD62L after stimulation with LPS or S100B. In contrast, LPS inactivation by polymyxin B prevented changes in CD11b and CD62L expression. However, increased CD11b and decreased CD62L levels were still detected after S100B stimulation in the presence of polymyxin B. Therefore, the observed effect was not due to contamination with LPS. Furthermore, we also depleted the media of S100B and this was no longer able to stimulate the cells. This demonstrated that S100B, and not endotoxins or other components, activated the cells in these experiments.

4. Discussion

In the present study, we established a new link in which non-cytotoxic CD8+ lymphocytes interact with cells of the mononuclear phagocytosis system. This effectively demonstrates a new mechanism linking the adaptive and innate immune systems, which is mediated by the S100B protein. Interestingly, this protein appears to play a hybrid role of a neurotrophic factor in one case and that of a cytokine on the other.[22,27]

$S100B^+$ lymphocytes were first detected more than 20 years ago. While early publications attempted to phenotype the $S100B^+$ subpopulation by conventional immunocytochemistry at the light, fluorescence, or electron microscopic level, virtually no functional characterisation of these cells' cytotoxic capability, cytokine production, and S100B secretion had been achieved.

Using automated flow cytometric S100B intra and extra-cellular immunolabelling, the present study confirms the predominant intracellular localisation of S100B. However, a few cells that secrete S100B showed surface expression of the protein. In accordance with previous studies, $S100B^+$ lymphocytes comprised about 2-4% of all lymphocytes and the majority of these cells were of a $CD3^+CD8^+$ phenotype.[12,13,32] We were not able to confirm the finding of Ferrari et al., which suggested that all $S100B^+$ T lymphocytes are $CD11b^+$.[15] Instead, we identified a variable coexpression of CD11b in only 42 ± 22% of $S100B^+$ lymphocytes.

Based on S100B expression in $CD8^+CD11b^+$ cells, De Panfilis et al.[16] concluded that $S100B^+$ T lymphocytes may exhibit immunosuppressive activities since $CD8^+CD11b^+$ cells act to suppress both B-cell differentiation and T-cell proliferation.[33,34] Accordingly, using a CD107a degranulation assay, the present study showed that $S100B^+$ lymphocytes

did not degranulate, and are therefore non-cytotoxic cells, and we demonstrated that $CD107a^+$ cells are restricted to the $CD3^+CD8^+S100B^-$ subpopulation.

Further functional characterisation of $S100B^+$ and $S100B^-$ T-lymphocyte subsets showed that these cells are similar in their IFNγ and IL-2 production upon stimulation with PMA/ionomycin. An important novel finding of the present study was that $S100B^+$ lymphocytes released S100B upon stimulation with anti-CD3 and PHA. Release of S100B secretion has been observed in other cell types, such as astrocytes, oligodendrocytes and adipocytes.[10,25,26] Notably, RAGE, the predominant receptor for S100B, has been detected on immune cells such as endothelial cells, microglia, monocytes, macrophages, granulocytes and lymphocytes.[22] Previously, glial and necrotic melanoma cells were considered a major source of S100B and secretion of this molecule leads to induction of (neuro)inflammatory processes through activating expression of cyclooxygease-2 (Cox-2), IL-1β, and nitric oxide synthase and TNFα in microglia and macrophages.[8,35,36] S100B also engages RAGE on endothelial cells, thereby increasing expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1).[8] Moreover, S100B enhances the interaction of RAGE with the leukocyte integrin Mac-1, thus increasing leukocyte adhesion to endothelial cells.[37]

Beyond the many known immune-activating functions of S100B, the present study detected novel crosstalk between the adaptive and innate immune systems mediated by S100B. More specifically, S100B expressing T cells may amplify pro-inflammatory responses via RAGE on innate immune cells. Our experiments showed that S100B activates granulocytes and monocytes, as indicated by increased expression of the adhesion molecule CD11b and membrane shedding of CD62L. It is possible that serum S100B may not attain concentrations which are high enough for induction of inflammatory gene expression in circulating white blood cells. However, locally concentrated S100B may be sufficient to modulate the immune system. Of note, S100B has been shown to induce chemoattraction of RAGE-expressing encephalitogenic $CD4^+$ T cells in a model of experimental autoimmune encephalomyelitis, which mimics multiple sclerosis, promoting T-cell infiltration of the central nervous system.[38] S100B may serve as an alarmin not only in pathologic, but also in various physiologic conditions, signalling tissue and cell damage, and thereby inducing activated immune surveillance in order to prevent harm amplification.

Increased S100B levels have been found in the cerebrospinal fluid and blood of patients suffering from Alzheimer's disease, Down's syndrome, and other neurodegenerative processes, as well as in cerebral autoimmune diseases, infection, and

trauma.[2-6,39] Interestingly, inflammation has been discussed as a pathophysiological factor in these disorders, but can also be conceptualized as a restorative response. Similarly, major psychiatric disorders such as schizophrenia and affective disorders are accompanied by elevated S100B levels in some body fluids.[6] Increased S100B levels may mediate such inflammatory mechanisms via microglial activation, i.e. activation of the mononuclear phagocytosis system cells of the brain, particularly in acutely ill and suicidal cases.[40-45] Thus, a paper entitled "S100B in neuropathologic states: the CRP [C-reactive protein] of the brain?" could gain new meaning in view of the above-described and formerly unknown close interactions between S100B and the immune system.[46] However, abnormal systemic conditions like melanoma progression, metabolic syndrome and diabetes are also linked to S100B levels and immune processes.[7,47,48] S100B release into the serum may also be the result of normal physiological conditions, such as physical exercise,[49] stress,[50] or fasting.[10] When designing clinical studies to assess the functional role of S100B it will be important to take these considerations into account.

In conclusion, we provide robust evidence showing that stimulated CD8+ T cells release S100B which leads to activation of granulocytes and monocytes. This indicates a novel key regulatory mechanism of innate immune functions by S100B+ T cells distinct from cytokine- and chemokine-mediated pathways. It may depend on the local inflammatory micromilieu, if S100B is acting rather pro- or anti-inflammatory *in vivo*. Due to the emerging role of S100B as an interface with the immune system, the results provide the ground for a wide array of future studies in physiological and pathological conditions that have been associated with increased S100B levels, including potential novel therapeutic avenues.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1: Examples of cell types that are capable of expressing RAGE [22-25,31].

Nervous system	Neurons (constitutively) Glia (astrocytes, oligodendrocytes, microglia)
Immune system	B and T lymphocytes Dendritic cells Monocytes Granulocytes
Other cell types	Vascular endothelial and smooth muscle cells Mesangial cells Hepatocytes Cardiac myocytes Lung epithelial cells

Figure 1: **Intracellular coexpression of S100B in lymphocytes**.

PBMCs were stained for CD8 and S100B. Flow cytometric analyses of extracellular (A) and intracellular (B) protein expression. C) S100B expression in $CD8^+$ (white bar) and $CD8^-$ (black bar) T cells from 20 donors. Data are presented as mean ± SEM. D) Typical dot plots for three healthy controls (HC) are shown. Cells were gated on lymphocytes according to SSC versus FSC properties.

Figure 2: Degranulation of cytotoxic granules by $CD8^+$ T cells.

Cells were stimulated for four hours with PMA/ionomycin in the presence of anti-CD107a, and were gated on $CD3^+CD8^+$ T cells. Coexpression of S100B and CD107a was analysed by flow cytometry. Typical dot plots for two healthy controls (HC) are shown.

Figure 3: Intracellular cytokine expression in $CD8^+$ T cells.

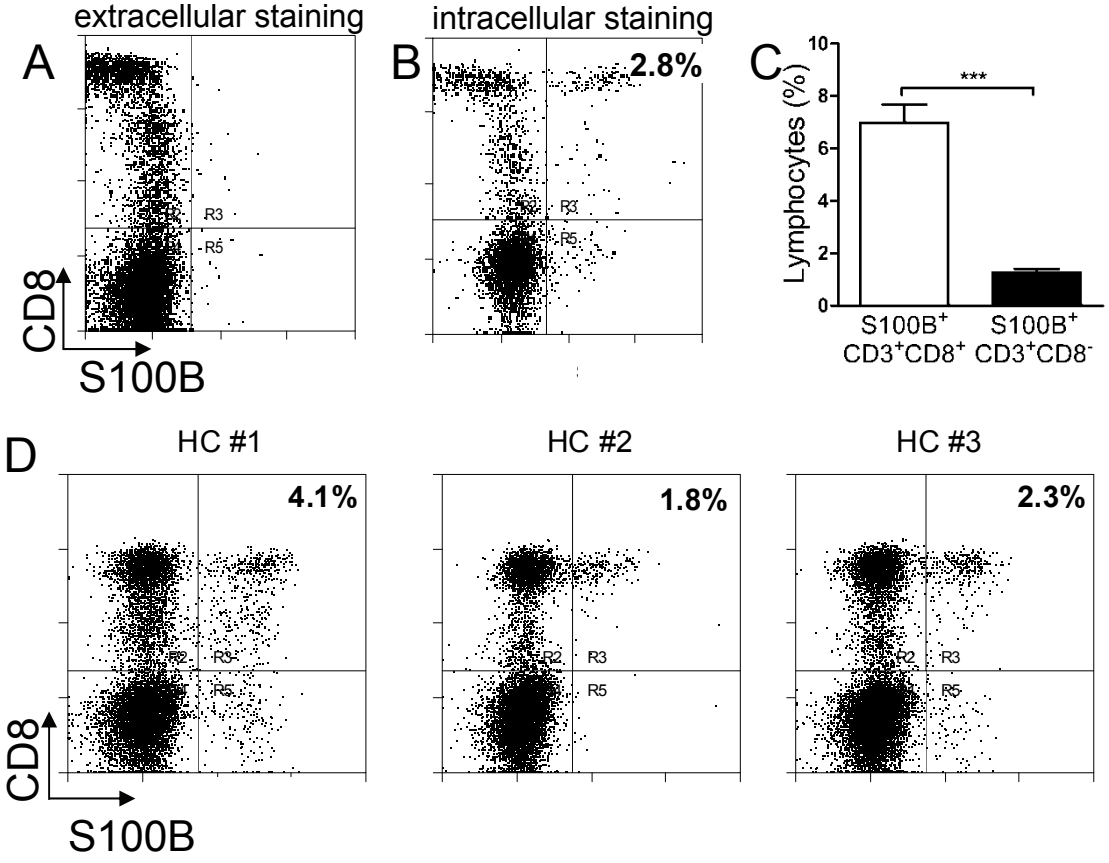
PBMCs were stimulated with PMA/ionomycin for four hours in the presence of brefeldin A, which prevents intracellular transport and secretion of induced cytokines. Cells were stained for CD3, CD8, S100B, and IL-2 or IFNγ, gated on $CD3^+CD8^+S100B^+$ (left panel) and $CD3^+CD8^+S100B^-$ (right panel). Typical expression of IL-2 (top panel) and IFNγ (bottom panel) for one representative individual is shown.

Figure 4: Secretion of S100B by activated $CD8^+$ T cells.

PBMCs were either not stimulated or stimulated with PHA and antibodies against CD3. A) Expression of S100B was determined after 24 and 96 h, gating on CD3+CD8+ cells. Percentages of $S100B^+$ cells are shown in the upper right box of each plot. B) Changes in $S100B^-$ (upper panel) and $S100B^+$ (lower panel) $CD8^+$ T-cell numbers are shown for different donors over time. C) S100B protein concentration (upper panel) and $S100B^+CD8^+$ T-cell number (lower panel) for the same cultures. *Annotation:* Data are presented as mean ± SEM.

Figure 5: Activation of granulocytes and monocytes by S100B.

A) Whole blood samples were either preincubated alone (□) or with polymyxin B (■). Blood samples were stimulated with LPS and S100B (control not stimulated) and expression of CD11b (right panel) and CD62L (left panel) was determined by flow cytometry. B) To confirm that the activating effect was S100B-specific, cells were stimulated with either S100B, anti-S100B (a-S100B) or S100B solution depleted of S100B (Depletion). *Annotation:* The mean fluorescence intensities (MFI) ± SEM of four experiments are shown. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.



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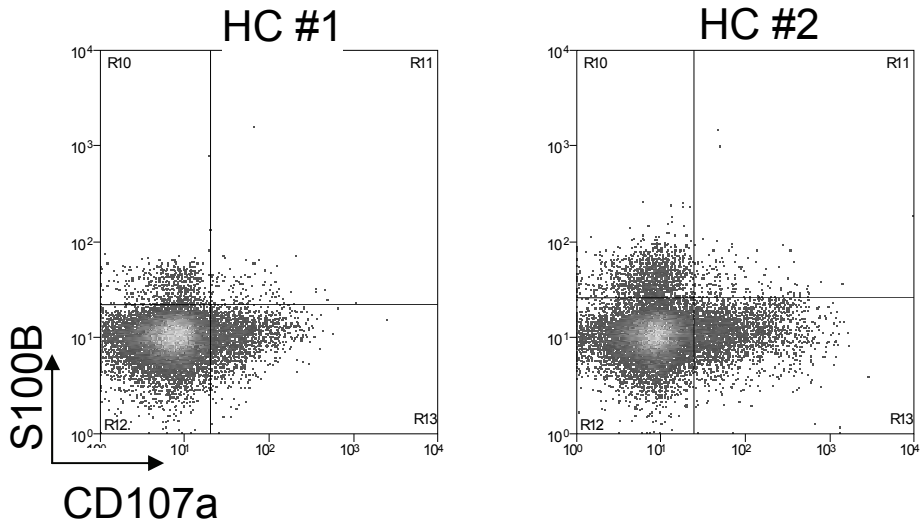

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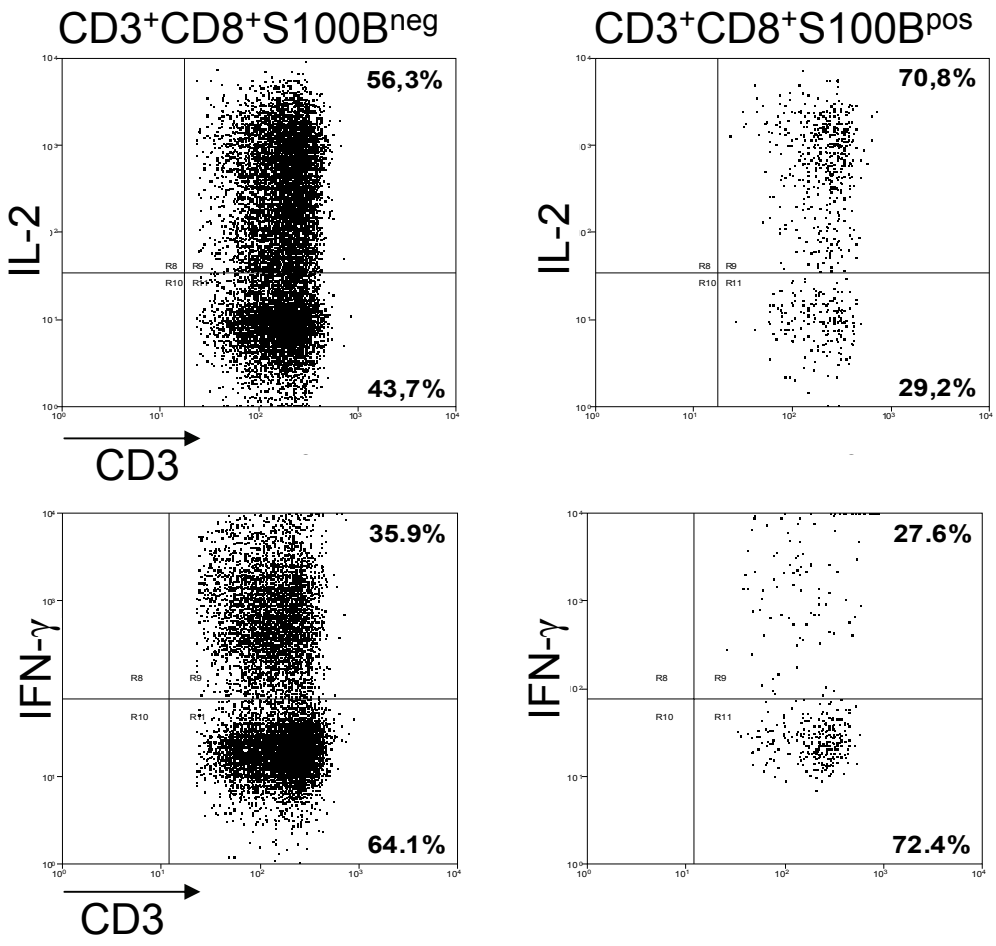

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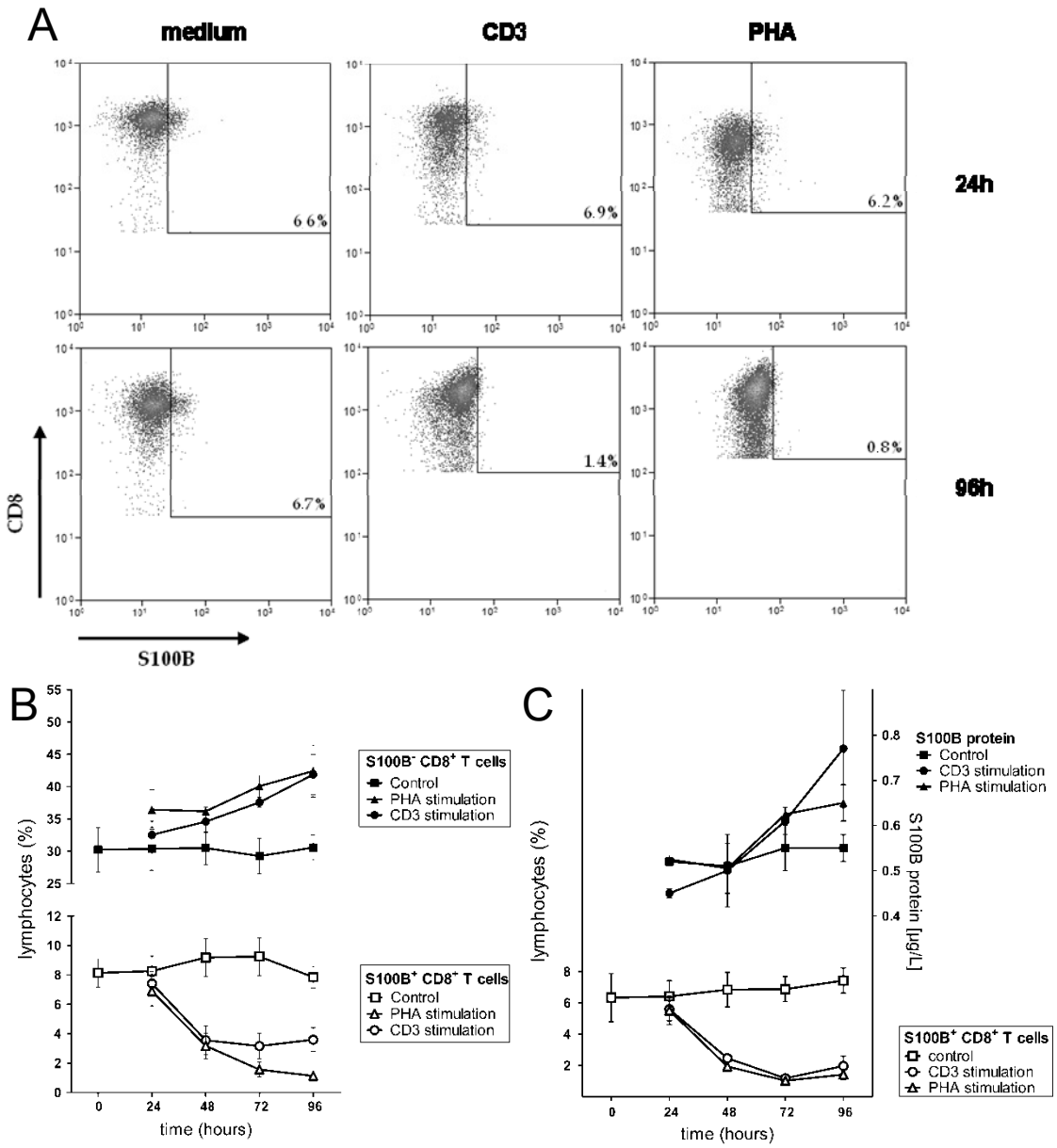

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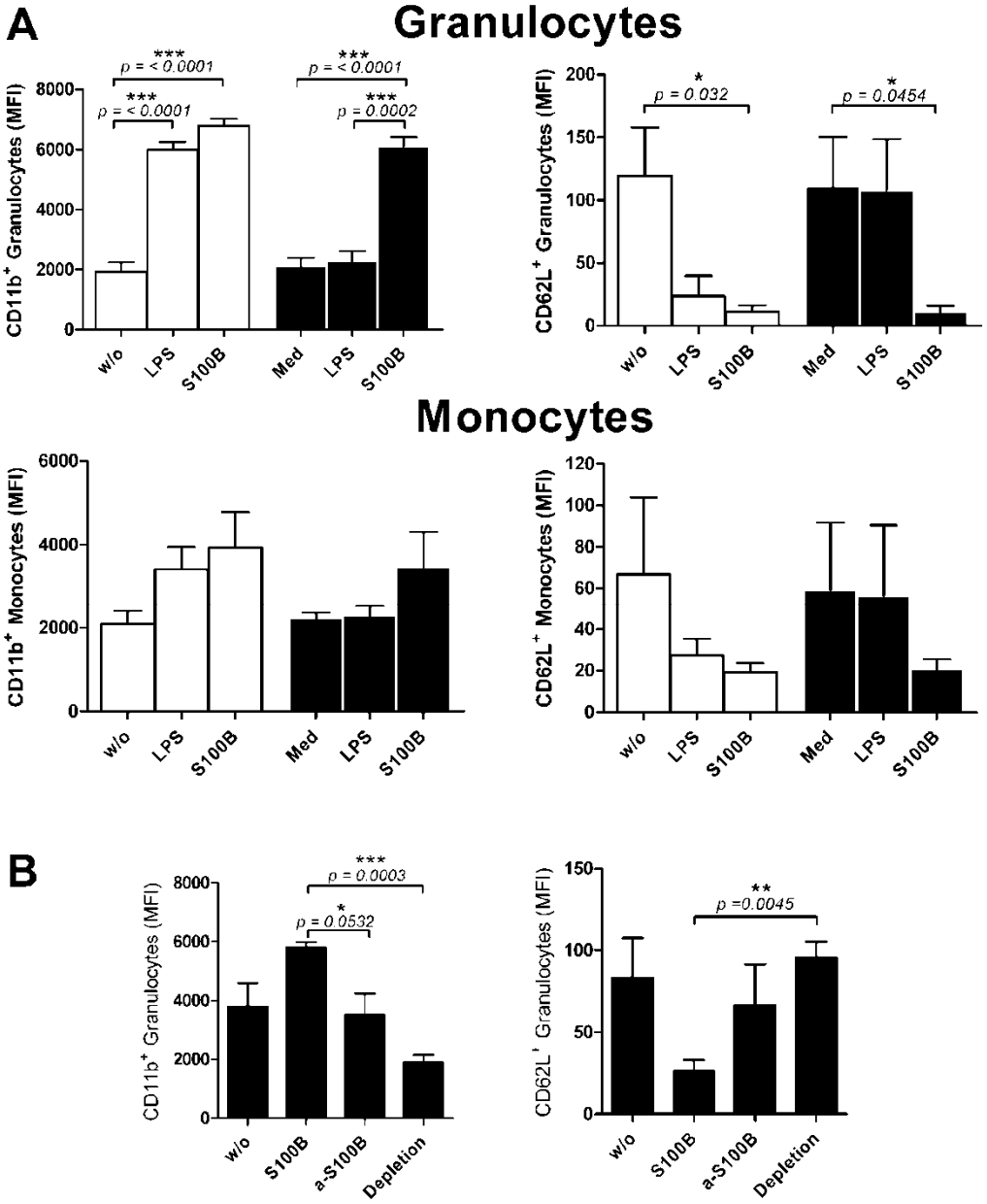

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7 DISKUSSION

Im Rahmen der vorliegenden Arbeit wurden verschiedene Zellpopulationen auf ihr immunregulatorisches Potenzial hin untersucht. Die Immunregulation kann in diesem Zusammenhang entweder inhibierend oder aktivierend auf andere Zelltypen wirken. Während T_{reg}-Zellen derzeit intensiv für einen möglichen klinischen Einsatz im Bereich der Toleranzinduktion nach Transplantationen diskutiert werden, existieren jedoch weitere Zelltypen, die nicht unbedingt eine immunsupprimierende Funktion im eigentlichen Sinne haben, deren Interaktionen aber ebenso Auswirkung auf den Erfolg einer Therapie haben können.

7.1 REGULATORISCHE NK-ZELLSUBPOPULATIONEN IN MENSCH UND MAUS

NK-Zellen stellen einen natürlichen Schutz gegen virusinfizierte und entartete Zellen dar, indem sie diese ohne vorherige Sensibilisierung direkt lysieren können. Dieser Mechanismus wird vor allem über KIR gesteuert, kann aber auch durch weitere Rezeptoren auf NK-Zellen wie NKG2D vermittelt werden (Guerra et al., 2008). Immunregulatorische Eigenschaften werden den NK-Zellen besonders durch ihre Fähigkeit, Zytokine zu produzieren, zugeschrieben. Allerdings sind auch zytotoxische NK-Zellen in der Lage, das adaptive Immunsystem zu regulieren. So wurde in einem Mausmodell nach HSCT die Expansion von alloreaktiven T-Zellen aus dem Transplantat durch NK-Zellen verhindert, indem mittels Fas-Ligand (FasL) und Perforin die Lyse von Spender-T-Zellen eingeleitet wurde, was einer GvHD entgegengewirkte (Olson et al., 2010). Gleichzeitig vermitteln Spender-NK-Zellen einen für den Empfänger vorteilhaften Transplantat versus Leukämie (GvL)-Effekt. Während also maligne Empfängerzellen oder -gewebe zerstört werden, vermitteln zytotoxische NK-Zellen gleichzeitig keine GvHD, sondern sind in der Lage, diesen schädigenden Prozess sogar einzudämmen (Ruggeri et al., 1999).

Der Begriff „regulatorische NK-Zellen“ wurde bisher allerdings wie erwähnt auf Grundlage der Zytokinexpression für unterschiedliche NK-Zellpopulationen verwendet. So wurden IL-10-produzierende NK-Zellen in der Maus-Leber nachgewiesen oder tolerogene NK-Zellen im Blut und in der Dezidua gefunden (Yoshida et al., 2010; Saito et al., 2008; Deniz et al., 2008). Es wurde zudem demonstriert, dass aktivierte NK-Zellen je nach sezerniertem Zytokin- und Chemokinmuster entweder unterstützend oder inhibierend auf T-Zellantworten wirken können (Santoni et al., 2007). Als typische regulatorische NK-Zellen werden derzeit aber meistens die $CD56^{bright}$ NK-Zellen betrachtet, da sie eine stärkere Zytokinproduktion als $CD56^{dim}$ NK-Zellen aufweisen (Jacobs et al., 2001; Cooper et al., 2001). Zusätzlich zeigen $CD56^{bright}$ NK-Zellen ein

stärkeres *Homing*, also die Migration in die Lymphknoten und andere Gewebe (Fehniger et al., 2003). Entsprechend sind in sekundären lymphatischen Geweben wie Lymphknoten oder Tonsillen auch ein höherer Anteil von $CD56^{bright}$ NK-Zellen nachweisbar (Ferlazzo et al., 2004).

In vivo Experimente könnten Aufschluss über Entwicklung und Differenzierung regulatorischer NK-Zellen geben. Murine NK-Zellen sind beispielsweise schon ausführlich untersucht worden, insbesondere hinsichtlich ihrer Expression von Ly49-Molekülen, die in ihrer Funktion den KIR im Menschen entsprechen. Eine fehlende CD56 Expression auf Maus-NK-Zellen machte es jedoch bis jetzt unmöglich, regulatorische NK-Zellen, die denen im Menschen entsprechen, zu identifizieren. Als entsprechendes Korrelat zu CD56 in der Maus wurde von HAYAKAWA et al. CD27 vorgeschlagen (Hayakawa und Smyth, 2006). Tatsächlich zeigen murine $CD27^{+}$ NK-Zellen funktionelle Ähnlichkeiten mit $CD56^{bright}$ NK-Zellen. Allerdings konnten wir bei *in vitro*-Analysen zeigen, dass CXCR3 ebenso als möglicher Marker für funktionell unterschiedliche NK-Zellpopulationen, die den humanen $CD56^{dim}$ bzw. $CD56^{bright}$ NK-Zellen entsprechen, fungieren könnte (Marquardt et al., 2010). $CXCR3^{+}$ NK-Zellen waren beispielsweise vermehrt in Lymphknoten und anderen Kompartimenten nachweisbar, während im Blut nur ein geringer Prozentsatz an NK-Zellen diesen Marker exprimierte. Gleichzeitig konnten wir zeigen, dass CXCR3 ausschließlich auf $CD27^{bright}$ NK-Zellen exprimiert wird, was wiederum deutlich macht, dass auch $CD27^{+}$ NK-Zellen in der Maus weiter in funktionelle Subpopulationen unterscheidbar sind. CXCR3 ist ein Rezeptor für die Chemokine CXCL9, CXCL10 und CXCL11 und ist wichtig für die Migration von NK-Zellen (Martin-Fontecha et al., 2004). Eine verringerte Migrationsfähigkeit wurde für $CD27^{-}$ NK-Zellen demonstriert (Hayakawa und Smyth, 2006). In weiteren Experimenten konnten wir zeigen, dass $CXCR3^{-}$ NK-Zellen bessere „Killer“ von Lymphomzellen als $CXCR3^{+}$ NK-Zellen waren, während letztere stärker proliferierten und ein deutlich höheres Potenzial zur Produktion von Zytokinen wie IFN-γ, TNF-α oder MIP-1α aufwiesen. Die $CXCR3^{+}$ NK-Zellen der Maus stimmen in ihrer Funktion also mit humanen regulatorischen $CD56^{bright}$ NK-Zellen überein. Bei Aktivierung von murinen NK-Zellen wird CXCR3 jedoch von der Zelle internalisiert, was für die *in vivo* Identifizierung von NK-Zellsubpopulationen in Mausmodellen mit NK-Zellaktivierung problematisch wäre. Tatsächlich wurde eine Herunterregulation von CXCR3 für tumorinfiltrierende NK-Zellen gezeigt (Wendel et al., 2008). Weiter kommt erschwerend hinzu, dass auch CD27 in seiner Expression bei Aktivierung der NK-Zelle nicht stabil bleibt. Allerdings wurde durch unsere Analysen deutlich, dass die CXCR3-Expressionsdichte aktivierter NK-Zellen mit der Funktion korreliert. Das bedeutet, dass Zellen, die ehemals $CXCR3^{+}$

waren und nach Aktivierung CXCR3$^-$ sind, anschließend beispielsweise eine höhere Degranulierung aufweisen bzw. CXCR3$^+$ NK-Zellen trotz Herunterregulation der CD27 Expression eine immer noch erhöhte Zytokinproduktion zeigen. Gleichzeitig sind aber immer CD27^{bright} NK-Zellen die stärksten Zytokinproduzenten.

Entsprechend lassen sich in der Maus folgende NK-Zellsubpopulationen unterscheiden, die funktionelle Homologien zu zytotoxischen CD56^{dim} bzw. immunregulatorischen CD56^{bright} NK-Zellen im Menschen aufweisen: zytotoxische CXCR3$^-$CD27$^-$ NK-Zellen, CXCR3$^-$CD27^{dim} (als intermediäre Stufe) und zytokinproduzierende CXCR3$^+$CD27^{bright} NK-Zellen.

Einen weiteren Ansatz, funktionell verschiedene NK-Zellsubpopulationen in der Maus zu definieren, machten 2009 Yu et al., indem sie CD94 als Marker einsetzten (Yu et al., 2009). CD94$^+$ NK-Zellen zeigten hier eine erhöhte Kapazität zu proliferieren, IFN-γ zu produzieren sowie Targetzellen zu lysieren, was am deutlichsten bei CD94$^+$CD27$^+$ NK-Zellen der Fall war. In unseren Analysen wurde CD94 am stärksten auf CXCR3$^+$CD27^{bright} NK-Zellen exprimiert, die ebenfalls dominant in der Proliferation und Zytokinproduktion waren, allerdings schwächer in der Zytotoxizität als CXCR3$^-$ NK-Zellen. In der Studie von Yu et al. war allerdings ein Teil der CD94$^-$ NK-Zellen CD27$^{+/bright}$, und diese Zellen zeigten wiederum eine verringerte Lysekapazität gegenüber CD94$^+$CD27$^+$ NK-Zellen. Daraus wird deutlich, dass CD27 nicht alleine als Marker für funktionelle NK-Zellsubpopulationen dienen kann. Die Daten der Studie lassen außerdem vermuten, dass die Expression von CD94 im Laufe der Zellreifung zunimmt und dementsprechend Schlüsse auf die NK-Zellentwicklung gemacht werden könnten, was aber weiter untersucht werden müsste (Yu et al., 2009).

Die Entwicklungsfolge von NK-Zellen ist bisher nicht eindeutig definiert, obwohl zahlreiche Ansätze zur phänotypischen Identifizierung der unterschiedlichen Reifungsstadien sowohl in der Maus als auch im Menschen gemacht worden sind (Huntington et al., 2007; Freud und Caligiuri, 2006; Freud et al., 2006; Poli et al., 2009; Cooper et al., 2001a). Humane CD56^{bright} NK-Zellen werden derzeit als Vorläufer zu vollständig ausgereiften CD56^{dim} NK-Zellen betrachtet (Nagler et al., 1989; Chan et al., 2007; Romagnani et al., 2007). In der Maus wird die Expression von CD27 sowie des Oberflächenrezeptors CD11b (Mac-1) zur Identifizierung verschiedener Entwicklungsstadien von NK-Zellen herangezogen (Hayakawa und Smyth, 2006). Hiernach wären CD11b$^-$ NK-Zellen weniger weit entwickelt als CD11b$^+$ NK-Zellen. Unsere Ergebnisse zeigen, dass murine CXCR3$^+$ NK-Zellen zum Teil CD11b$^-$ sind und

sich daher theoretisch in einem früheren Entwicklungsstadium befinden als $CXCR3^-$ NK-Zellen. In einem *in vivo*-Mausmodell ließe sich demnach überprüfen, ob der Anteil von $CD11b^+$ NK-Zellen tatsächlich mit der Rekonstitution nach HSCT einhergeht.

Gleichzeitig ist denkbar und überprüfbar, ob ein überproportionaler Anteil $CXCR3^+$ NK-Zellen nach allogener HSCT in der Maus nachweisbar ist, da im Menschen nach HSCT in der Rekonstitutionsphase ungewöhnlich viele $CD56^{bright}$ NK-Zellen im Blut detektierbar sind (Jacobs et al., 1992; Dulphy et al., 2008; Vukicevic et al., 2010). Dies könnte Folge einer erhöhten IL-15-Konzentration sein, die im Serum von Transplantationspatienten nachgewiesen worden ist und zur Expansion besonders der $CD56^{bright}$ NK-Zellen führen würde (Boyiadzis et al., 2008; Dulphy et al., 2008). Die entsprechenden $CD56^{bright}$ NK-Zellen zeigten regulatorische Funktionen wie die Sekretion von IFN-γ, aber nur wenige phänotypische Charakteristika noch nicht vollständig entwickelter NK-Zellen (Vukicevic et al., 2010). Es kann aber dennoch nicht ausgeschlossen werden, dass sich diese Zellen in einem weniger reifen Zustand befinden.

Folglich ergibt sich daraus ein theoretischer Ansatz, in einem Mausmodell die mögliche Expansion von $CXCR3^+$ NK-Zellen sowie deren Expression von CD11b und CD27 nach HSCT zu überprüfen. Tatsächlich zeigten erste Vorversuche eine Zunahme der ausgereiften $CD11b^+CD27^-$ bzw. einen Abfall der intermediär entwickelten $CD11b^+CD27^+$ NK-Zellen im peripheren Blut innerhalb von 4 Wochen nach der Transplantation. Erst 5-6 Wochen nach HSCT wurde zudem eine steigende CXCR3-Expression gemessen (Daten nicht gezeigt), obwohl in anderen Studien gezeigt wurde, dass im Menschen bereits 10 Tage nach der HSCT ein sehr hoher Anteil $CD56^{bright}$ NK-Zellen auftritt. Da aber auch im humanen System das Auftreten der $CD56^{bright}$ mit einiger Verzögerung bis zu mehreren Monaten zu beobachten ist, kann hier die Versuchsdauer noch verlängert werden (Jacobs, persönliche Kommunikation).

Schlussfolgernd existieren ebenso wie im Menschen auch in der Maus regulatorische NK-Zellen, die allerdings durch unterschiedliche Oberflächenmarker identifiziert werden. Im Menschen handelt es sich um $CD56^{bright}$, in der Maus um $CXCR3^+$ NK-Zellen. Ihre phänotpyischen und funktionellen Charakteristika sind Tabelle 1 zusammengefasst. Durch Sekretion proinflammatorischer Zytokine wie IFN-γ oder TNF-α sind sie in der Lage, sowohl Zellen des angeborenen und adaptiven Immunsystems als theoretisch auch z.B. MSC zu aktivieren. Mittels der vorliegenden Arbeit wird es möglich sein, die Rolle von NK-Zellen und ihren Subpopulationen auch *in vivo* zu untersuchen.

Tab. 1: Charakteristika funktionell äquivalenter muriner und humaner NK-Zellsubpopulationen

Eigenschaft	Maus		Mensch	
NK-Zellsubpopulation	***CXCR3$^-$***	***CXCR3$^+$***	***CD56^{dim}***	***CD56^{bright}***
CD16 Expression	CD16^{bright}	CD16$^{-/dim}$	CD16^{bright}	CD16^{dim}
CD27 Expression	CD27$^{-/dim}$	CD27^{bright}	CD27^{dim}	CD27^{bright}
Prozentanteil im peripheren Blut	90%	10%	90%	10%
Prozentanteil im Lymphknoten	60%	40%	10%	90%
Frequenz in der Lunge	+++	+	+++	+
Proliferation	-	+++	+	+++
Zytokinproduktion	+	+++	+	+++
Degranulierung/Zytotoxizität	+++	++	+++	+

7.2 NK-Zellen und mesenchymale Stammzellen

Den MSC werden ähnlich den NK-Zellen immunregulatorische Fähigkeiten zugeschrieben. Mononukleäre Zellen des peripheren Blutes (PBMC), isolierte T-Zellen, B-Zellen, DC sowie NK-Zellen wurden bei Kokultur mit MSC in ihren Funktionen inhibiert, und MSC verhinderten im *in vivo*-Modell die Entstehung einer GvHD und unterstützten das Anwachsen des Transplantats (Joo et al., 2010; Maitra et al., 2004). Welchen Mechanismen die Immunsuppression zugrunde liegt, ist derzeit aber noch nicht eindeutig geklärt bzw. gibt es derzeit viele unterschiedliche Lösungsansätze. In mehreren Studien wurden die löslichen Mediatoren PGE2 und IDO als auslösende Faktoren vermutet, andere Gruppen zeigten wiederum eine rezeptorabhängige Inhibierung z.B. durch HLA-G oder durch die Inhibierung der Formation einer immunologischen Synapse (Selmani et al., 2009; Aldinucci et al., 2010). Dieses Forschungsfeld ist noch immer nicht vollständig abgedeckt, so wurde 2010 beispielsweise nachgewiesen, dass das Lektin Galectin-1 von MSC sezerniert werden kann und die Proliferation von T-Zellen, nicht aber die von NK-Zellen einschränken kann (Gieseke et al., 2010).

Die meisten dieser Studien wurden mit MSC durchgeführt, die aus dem Knochenmark isoliert wurden. MSC repräsentieren allerdings eine nur sehr kleine Fraktion von 0.001-0.01% aller nukleären Zellen im Knochenmark. Es stehen jedoch auch andere Quellen zur Isolation von MSC zur Verfügung, bei denen weniger stark invasive Eingriffe notwendig sind und zusätzlich eine größere Ausbeute an MSC bieten (Pittenger et al.,

1999). Ein Beispiel hierfür sind MSC aus dem Nabelschnurblut oder dem Nabelschnurgewebe (Wharton's jelly) (Majore et al., 2010). Für die vorliegende Arbeit wurden UC-MSC eingesetzt, und die Analysen zeigten, dass die Proliferation, Zytokinproduktion, Degranulierung sowie Zytotoxizität von NK-Zellen durch UC-MSC inhibiert wurden, auch wenn im Allgemeinen phänotypisch weder eine Aktivierung noch eine Inhibierung von NK-Zellen angezeigt wurde. In anderen Studien, die allerdings mit BM-MSC durchgeführt wurden, wurde z.B. eine Reduzierung der Granzym B-, NKp30-, NKp44- und NKG2D-Expression demonstriert (Cappellesso-Fleury et al., 2010; Spaggiari et al., 2008). Diese Ergebnisse konnten wir in unseren Analysen nicht bestätigen. In unseren Experimenten war die Expression von CD56 nach 6 Tagen Kokultur mit MSC allerdings leicht herunter reguliert, was wiederum mit Daten aus anderen Gruppen übereinstimmt (Daten nicht gezeigt) (Sotiropoulou et al., 2006).

Eine Inhibierung der NK-Zellen nach Kokultur mit MSC konnten wir aber indirekt auf mRNA-Ebene nachweisen. Die Genexpression von *CRTAM* war deutlich reduziert (Daten nicht gezeigt). CRTAM wird als Protein nur auf aktivierten CTL und NK-Zellen exprimiert und bindet an „nectin-like protein 2" (Necl2), was wiederum zur Aktivierung dieser Zellen führt (Boles et al., 2005). Im Vergleich zu unstimulierten NK-Zellen ist die *CRTAM* Genexpression in NK-Zellen aus Kokultur mit MSC noch niedriger, was für eine allgemeine Inhibierung der NK-Zellen spricht.

Einige NK-Zellen, die nur einen einzelnen spezifischen KIR-Typ exprimieren und daher nur durch die entsprechenden spezifischen MHC-Klasse-I-Moleküle blockiert werden, können durch allogene Zellen dazu aktiviert werden, diese anzugreifen. Diese auf der „missing-self"-Theorie basierende Tatsache könnte dazu führen, das allogene MSC von NK-Zellen lysiert werden. In unseren Experimenten wurden MSC allerdings nur von präaktivierten NK-Zellen lysiert, nicht jedoch von unstimulierten NK-Zellen, was Daten anderer Studien bestätigt bzw. widerspricht (Rasmusson et al., 2003; Sotiropoulou et al., 2006; Jewett et al., 2010). Die Zytotoxizität scheint unabhängig von autologen oder allogenen Settings zu sein und kann über Maskierung der aktivierenden Rezeptoren NKp30, NKG2D und DNAM-1 via Antikörper aufgehoben werden (Spaggiari et al., 2008; Spaggiari et al., 2006). In einer anderen Studie wurde die Lyse der MSC durch Zugabe von anti-CD16 (FcγRIII) mAb blockiert (Jewett et al., 2010). Der Antikörper induzierte hierbei Apoptose in bis zu 40% der NK-Zellen und führte demnach zur Depletion eines großen Teils der zytotoxischen NK-Zellen, was die verminderte Lyse der MSC erklären kann.

Wie bereits erwähnt, produzieren regulatorische NK-Zellen auf Stimulation hin Zytokine wie z.B. IFN-γ. Besonders für dieses Zytokin ist eine aktivierende Funktion auf MSC bekannt, infolgedessen eine erhöhte Sekretion von PGE2 und IDO durch MSC wiederum in einem rückgekoppelten Mechanismus zu einer Inhibierung der NK-Zellen führen könnte (English et al., 2007). Andererseits sind, wie wir gezeigt haben, aktivierte NK-Zellen in der Lage, MSC zu lysieren. Ob generell nun eher eine Lyse der MSC durch aktivierte NK-Zellen oder aber die Aktivierung der MSC durch IFN-γ zur Inhibierung der NK-Zellen führt, konnte bisher nicht geklärt werden. Der bloße Kontakt mit allogenen MSC induzierte in unseren Untersuchungen jedoch keine Produktion von IFN-γ in ruhenden NK-Zellen, somit kann eine inhibierungsverstärkende Wirkung durch IDO und PGE2 aus der Kokultur mit MSC ausgeschlossen werden.

Untersuchungen von Zellkulturüberständen ergaben keine Induktion der Freisetzung von Zytokinen oder Chemokinen. Bei der Untersuchung von Zellkulturüberständen wurden hauptsächlich Faktoren detektiert, für die eine NK-Zell-aktivierende Funktion nachgewiesen wurde. Einzig IL-10 wirkt inhibierend, obwohl auch für dieses Zytokin ein aktivierender Effekt auf NK-Zellen nachgewiesen worden ist, sofern es mit IL-18 kombiniert wurde (Cai et al., 1999). Insgesamt wäre aufgrund der detektierten löslichen Effektormoleküle im Überstand eher eine NK-Zellaktivierung zu erwarten, die jedoch ausbleibt. Dementsprechend muss ein stärkerer inhibitorischer Faktor vorliegen.

Unsere Experimente im Transwellsystem oder mit MSC- bzw. MSC-NK-konditioniertem Überstand lassen vermuten, dass keine löslichen Faktoren maßgeblich an der Inhibierung beteiligt sein können, da hier keine Suppression der NK-Zellaktivität stattfand. Entsprechend weisen die Ergebnisse auf einen Zell-Zell-kontaktabhängigen Mechanismus hin, wobei allerdings nicht ein einzelner Rezeptor, sondern höchstwahrscheinlich eine Kombination aus mehreren Rezeptor-Ligand-Interaktionen entscheidend ist. Da verschiedenste NK-Zellfunktionen betroffen sind, bei denen unterschiedliche aktivierende Rezeptoren eine Rolle spielen, ist es unwahrscheinlich, dass die Herunterregulation der Expression eines einzelnen aktivierenden Rezeptors für die Inhibierung verantwortlich ist. Vielmehr spricht vieles für die Existenz eines inhibierenden Rezeptors auf NK-Zellen und anderen Lymphozyten, dessen inhibierende Signale die der aktivierenden Rezeptoren überwiegen. Da die MSC-vermittelte Suppression unspezifisch für alle Lymphozyten gezeigt worden ist, handelt es sich möglicherweise außerdem um einen oder mehrere Rezeptoren, die ubiquitär exprimiert werden.

Ein wichtiger Lymphozytenrezeptor nicht nur für die Migration zu Entzündungsherden, sondern auch zur Etablierung und Aufrechterhaltung eines Zell-Zellkontakts beispielsweise mit einer Targetzelle ist das Adhäsionsmolekül LFA-1, das auf der Mehrheit der Lymphozyten nachweisbar ist (Luo et al., 2007; Ley et al., 2007). Einer seiner Liganden, ICAM-1, wird auf der Oberfläche von MSC exprimiert (Chamberlain et al., 2007). LFA-1/ICAM-1-Wechselwirkungen sind ausschlaggebend für die Formation einer immunologischen Synapse, durch die weitere Zell-Zell-Interaktionen ermöglicht werden (Orange, 2008). ICAM-1 und VCAM-1 hatten in einem *in vitro*-Modell einen entscheidenden Einfluss auf die MSC-vermittelte Inhibierung von T-Zellen (Ren et al., 2010). Desweiteren wurde die LFA-1-abhängige Lyse von MSC durch IL-2-aktivierte NK-Zellen gezeigt (Poggi et al., 2007). Ob aber der Zell-Zell-Kontakt zwischen unstimulierten NK-Zellen und MSC ebenfalls durch LFA-1/ICAM-1-Interaktion vermittelt wird, haben wir mittels blockierender Antikörper gegen eine Untereinheit von LFA-1, nämlich CD11a untersucht. Sowohl der therapeutische Antikörper Efalizumab (Daten nicht gezeigt) als auch ein kommerziell erhältlicher Antikörper konnten allerdings weder die durch MSC supprimierte Proliferation noch die Synthese von IFN-γ in NK-Zellen wiederherstellen. Daraus wird deutlich, dass zumindest LFA-1 als Hauptbestandteil der immunologischen Synapse bei NK-Zellen keinen Einfluss auf die MSC-vermittelte NK-Zellinhibierung hat. Entsprechend müssen andere Rezeptoren für die Suppression verantwortlich sein.

Ein weiteres Protein, dessen Einfluss auf die NK-MSC-Interaktionen von uns untersucht worden ist, ist LAIR-1. Es wird auf den meisten Leukozyten wie T-Zellen, B-Zellen, NK-Zellen, Monozyten, basophilen und eosinophilen Granulozyten, Mastzellen sowie $CD34^+$ hämatopoetischen Vorläuferzellen exprimiert und ist einer der am stärksten vertretenen inhibitorischen Rezeptoren (Meyaard, 2008; Meyaard et al., 1997; Ouyang et al., 2003; Poggi et al., 1998; Verbrugge et al., 2006; Florian et al., 2006). Strukturell ähnelt es den KIR, bindet sowohl an transmembrane als auch extrazelluläre Matrixkollagene (Kollagentypen II und III) und spielt eine Rolle bei der Inhibierung von Immunzellen bei der Extravasation durch kollagenreiches Subendothelium (Farndale et al., 2008). Auch MSC exprimieren Matrixkollagene, genauer die Kollagentypen I, II, III, IV, VI und VII, weshalb eine Interaktion von NK-Zellen mit MSC über LAIR-1/Kollagen theoretisch möglich ist (Juneja et al., 1992). Die Bindung von LAIR-1 an Kollagen kann durch das lösliche Protein LAIR-2 blockiert werden, was wir für unsere Experimente ausgenutzt haben (Lebbink et al., 2008). Kommerziell erhältliche lösliche LAIR-1- und LAIR-2-Proteine wurden mit MSC vor der Zugabe von NK-Zellen inkubiert. Obwohl die meisten oder alle Kollagenbindungsstellen für LAIR-1 auf NK-Zellen so blockiert wurden, wurde dennoch die NK-Zellsuppression nicht verhindert, sowohl die Produktion von IFN-γ als

auch die Zytotoxizität waren nach MSC-Kokultur weiterhin vermindert. Folglich ist die Rolle von LAIR-1 bei NK-MSC-Interaktionen zu vernachlässigen.

Auch Interaktionen zwischen CD155 (Poliovirusrezeptor, PVR) auf MSC und den entsprechenden Liganden auf NK-Zellen könnten für eine MSC-vermittelte Suppression verantwortlich sein. Bei diesen Liganden handelt es sich einerseits um den aktivierenden Rezeptor DNAM-1 und um CD96, der die Adhäsion mit Targetzellen unterstützt (Fuchs et al., 2004). Andererseits bindet auch TIGIT an CD155 und induziert eine Inhibierung in NK-Zellen (Stanietsky et al., 2009). TIGIT wird nicht nur auf NK-Zellen, sondern auch auf $CD8^+$ T-Zellen, T_{reg}-Zellen, $CD4^+$ Gedächtniszellen und NKT-Zellen exprimiert. Weiterführende Untersuchungen sind aber noch notwendig, um den Einfluss von TIGIT für die NK-MSC-Wechselwirkung definieren zu können.

Ein weiterer infragekommender Rezeptor ist „Programmed Death 1“ (PD-1). PD-1 ist ein inhibierender Rezeptor, der auf T- und B-Zellen exprimiert wird. Neueste Publikationen zeigen, dass PD-1 auch auf humanen NK-Zellen exprimiert wird, die mit IL-2 aktiviert worden sind (Plege et al., 2010). Auf frischen NK-Zellen ist PD-1 allerdings nicht exprimiert (Benson, Jr. et al., 2010). Mit murinen BM-MSC wurde ein PD-1/PD-L1-abhängiger Mechanismus für T- und B-Zellen nachgewiesen, bei dem die Aktivierung von Lymphozyten inhibiert wurde, aber die Aktivität von NK-Zellen wurde hier nicht näher betrachtet (Augello et al., 2005). Zusätzlich exprimieren MSC den Liganden für PD-1, PD-L1, erst nach Kokultur mit aktivierten Splenozyten oder unter direkter Zugabe von IFN-γ (English et al., 2007). Da die von uns eingesetzten NK-Zellen nicht prästimuliert waren und entsprechend kein IFN-γ sezerniert wurde, ist also eine Inhibierung über PD-1/PD-L1-Ligation eher unwahrscheinlich.

Im Gegensatz dazu stellt ist die Interaktion zwischen HLA-G auf MSC und seinen Liganden auf NK-Zellen einen durchaus möglichen Weg der NK-Zell-Inhibierung dar. HLA-G gehört zu den nicht-klassischen MHC-Klasse-I-Molekülen und seine Expression wurde vor allem auf Trophoblasten und Tumorzellen gezeigt (Favier et al., 2007; Rouas-Freiss et al., 2007). HLA-G wurde aber auch auf MSC nachgewiesen (Nasef et al., 2007 und Daten nicht gezeigt). Bisher wurden drei Liganden für HLA-G identifiziert, genauer ILT2 (CD85j/Leukozyten-immunglobulinähnlicher Rezeptor B1, [LILRB1]), ILT4 (CD85d/LILRB2) und KIR2DL4 (CD158d) (Shiroishi et al., 2003; Rajagopalan und Long, 1999). Sowohl ILT2 als auch KIR2DL4 werden von NK-Zellen exprimiert und besitzen eine inhibitorische Funktion. Dementsprechend ist denkbar, dass HLA-G auf MSC an ILT2 und/oder KIR2DL4 auf NK-Zellen binden und sie somit in ihren Funktionen

supprimieren kann. Die Ligation von ILT2 durch HLA-G führt bei NK-Zellen zu verminderter Zyototoxizität, IFN-γ-Produktion sowie verringerter intrazellulärer Calciummobilisierung (Favier et al., 2010). Hämatopoetische Stammzellen werden durch HLA-G-Expression vor NK-Zell-vermittelter Zytolyse geschützt (Wang et al., 2010). Die Blockade von HLA-G führte in MLR/MSC-Kokulturen zu einer Wiederherstellung der Proliferation der Lymphozyten (Nasef et al., 2007). Entsprechende Versuche mit NK/MSC-Kokulturen bieten einen vielversprechenden Ansatz zur Untersuchung der MSC-abhängigen NK-Zellsuppression.

Nicht nur direkter Kontakt von MSC führt zur Suppression von Lymphozyten. Es konnte kürzlich gezeigt werden, dass auch Monozyten als Mediatoren für die MSC-vermittelte Inhibierung von T-Zellen oder auch NK-Zellen verantwortlich sein können (Cutler et al., 2010; Jewett et al., 2010). Zudem wird vermutet, dass MSC zu einer Induktion und Expansion von T_{reg}-Zellen führen (Patel et al., 2010). Ohne die Präsenz von MSC wurde hier bei Kokultur von PBMC mit Brustkrebszellen eine T_H1-Zytokinantwort provoziert, die zu einer Aktivierung von CTL und NK-Zellen und damit zur Beseitigung der Tumorzellen führte. MSC in diesem Ansatz veränderten hingegen das Zytokinprofil in Richtung T_H2, was wiederum die Effektorfunktionen von CTL und NK-Zellen einschränkte und gleichzeitig die Expansion von T_{reg}-Zellen unterstützte. Somit könnten MSC die Immunantwort derart herunter regulieren, dass eine normale Immunosurveillance nicht mehr möglich ist und es zum uneingeschränkten Wachstum maligner Zellen kommt.
Dies sind denkbare Wege der gegenseitigen, indirekten Regulation des Immunsystems durch MSC. Da aber selbst direkte Wechselwirkungen noch nicht vollständig verstanden sind, wurden in unseren Experimenten ausnahmslos sortierte bzw. angereicherte NK-Zellen verwendet, weshalb ein regulierender Effekt durch andere Zellpopulationen ausscheidet.

Einen denkbareren Ansatz für weitere Analysen bietet die Frage, ob MSC, die aus unterschiedlichen Geweben isoliert wurden, unterschiedliche immunregulatorische Funktionen oder Signalwege besitzen. Einige Untersuchungen hinsichtlich der Vergleichbarkeit zwischen MSC aus z.B. Knochenmark und Nabelschnur(blut) wurden bereits durchgeführt (Malgieri et al., 2010; Prasanna et al., 2010; Potian et al., 2003). Tatsächlich inhibierten beispielsweise UC-MSC Lymphozyten stärker als BM-MSC. In immunkompetenten Mäusen wurden entsprechend BM-MSC schneller abgestoßen als UC-MSC (Deuse et al., 2010). Wie sich dies in immunsupprimierten Organismen verhält, ist allerdings unklar. Darüber hinaus waren die verwendeten BM-MSC dieser Studie aus Patienten, die älter als 65 Jahre waren. Inwiefern MSC altersabhängige Änderungen

durchlaufen, ist bisher nicht eindeutig geklärt, da es widersprüchliche Ergebnisse zu dieser Thematik gibt. Tendenziell lassen aber die publizierten Ergebnisse darauf schließen, dass Proliferations- und Differenzierungsfähigkeit von MSC mit steigendem Alter des Knochenmarkspenders abnehmen und die Zellseneszenz der MSC zunimmt (Zhou et al., 2008; Stolzing et al., 2008; Coipeau et al., 2009; Choumerianou et al., 2010).

Aus oben erwähnter Studie wird deutlich, dass trotz immunregulativer Eigenschaften der MSC vermutlich dennoch Immunsuppressiva eingesetzt werden müssen. Besonders alloreaktive T-Zellen sowohl aus dem Spender aber auch aus dem Empfänger müssen unter Kontrolle gehalten werden, da die MSC abgestoßen werden könnten oder deren immuninhibierenden Funktionen nicht ausreichen. Dasselbe gilt für die Transplantation von rekonstruierten, ausdifferenzierten Geweben, die sich von MSC ableiten. Die derzeit am häufigsten verwendeten immunsupprimierenden Medikamente, darunter Tacrolimus, Rapamycin, Mycophenolsöure (MPA), haben dabei unterschiedliche Wirkungsweisen. Tatsächlich scheinen sich die oben genannten Therapeutika und MSC gegenseitig zu beeinflussen, obwohl die bisherigen Ergebnisse teilweise differieren.
Tacrolimus etwa wirkt in hohen Konzentrationen toxisch auf MSC, Rapamycin und MPA inhibieren in therapeutischen Dosen die Proliferation von MSC. Andererseits erhöht die Präinkubation mit Tacrolimus oder Rapamycin das immunsupprimierende Potenzial der MSC, während die supprimierende Effektivität von beiden Immunsuppressiva abnimmt (Hoogduijn et al., 2008). In einer anderen Studie hatten Calcineurin-Inhibitoren, also auch Cyclosporin und Tacrolimus, einen antagonistischen Effekt auf die inhibitorische Wirkung von MSC. Das heißt letztendlich, dass die supprimierende Wirkung der Medikamente durch MSC zumindest teilweise aufgehoben werden kann (Buron et al., 2009). Welche Auswirkungen die entsprechenden Immunsuppressiva auf die Interaktion von NK-Zellen und MSC haben, ist bisher nicht untersucht worden, müsste aber unbedingt berücksichtigt werden, sofern NK-Zellen und MSC parallel therapeutisch eingesetzt werden sollen.

Zusammenfassend können wir ein vorläufiges Modell der NK-Zellinhibierung erstellen, bei dem direkter Zell-Zellkontakt zwischen ruhenden NK-Zellen und MSC entscheidend ist. Der entsprechende Rezeptor auf NK-Zellen muss allerdings noch definiert werden (Abb. 1).

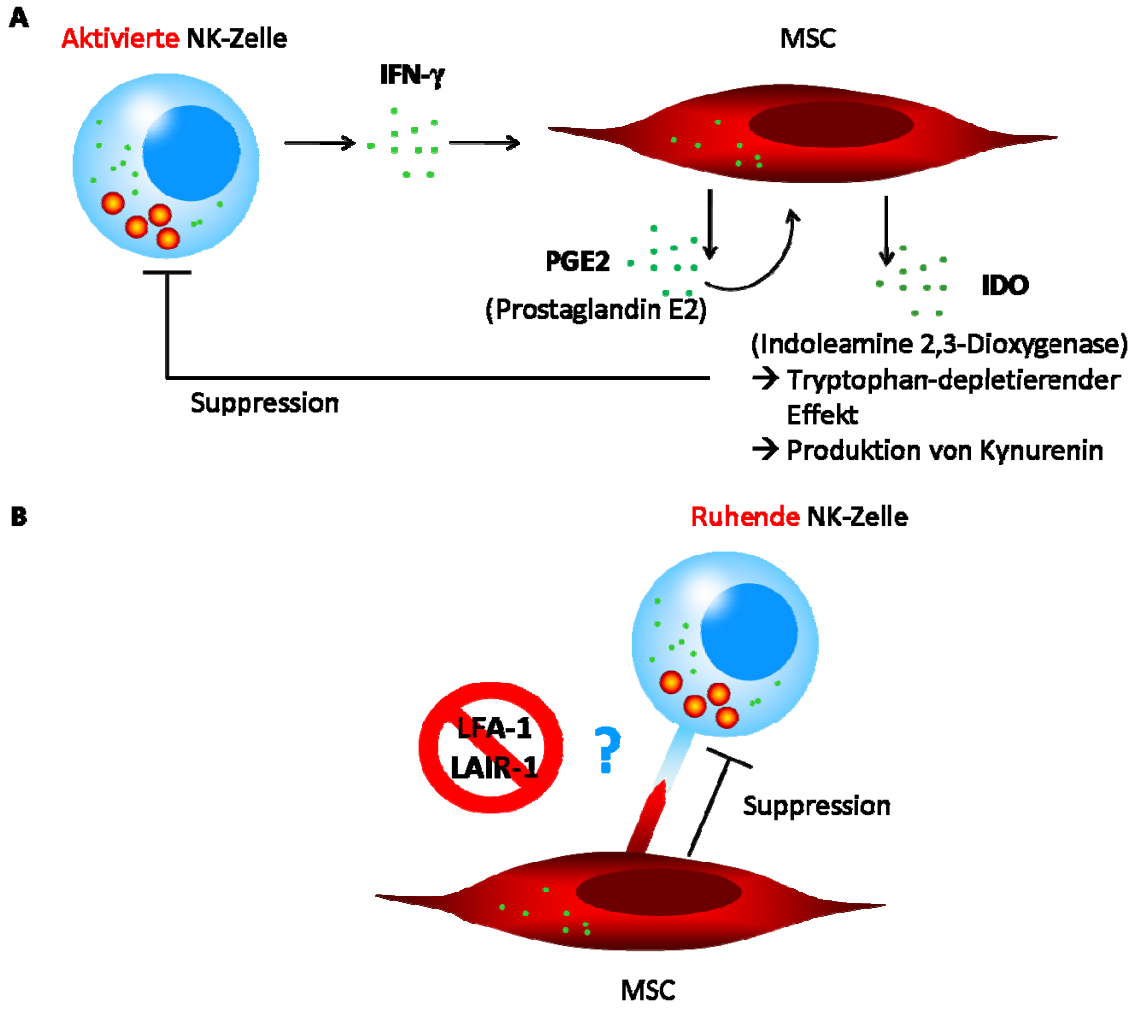

Abb. 1: Modelle der NK-Zellsuppression durch MSC.
Sowohl aktivierte als auch ruhende NK-Zellen werden durch MSC in ihren Funktionen supprimiert. **A:** Aktivierte NK-Zellen sezernieren IFN-γ, das wiederum aktivierend auf MSC wirkt. Aktivierte MSC produzieren erhöhte Level der inhibitorischen Mediatoren PGE2 und IDO, die supprimierend auf Lymphozyten bzw. NK-Zellen wirken (English et al., 2007; Meisel et al., 2004). **B:** In unseren Experimenten wurden ruhende NK-Zellen ausschließlich durch direkten Zell-Zellkontakt inhibiert. Ein Einfluss von LFA-1 und LAIR-1 auf NK-Zellen konnte durch Blockierexperimente hierbei ausgeschlossen werden. Der bzw. die entscheidenden Rezeptoren konnten noch nicht eindeutig geklärt werden.

7.3 $CD3^+CD20^+$ T-Zellen

Obwohl CD20 ursprünglich als B-Zellspezifisches Differenzierungsantigen definiert wurde, wird dieser Marker auch auf T-Zellen exprimiert. In Patienten mit T-Zelllymphomen konnte mehrfach eine $CD3^+CD20^+$ Zellpopulation nachgewiesen werden, aber auch in gesunden Individuen lassen sich $CD20^+$ T-Zellen detektieren (Miyazaki et al., 2009; Hultin et al., 1993; Wilk et al., 2009). In unseren Analysen hatten $CD20^+$ T-Zellen mit ca. 1.6% aller T-Zellen im peripheren Blut einen relativ geringen Anteil, und vergleichbare Werte fanden sich in Geweben wie Lymphknoten, Thymus und Leber. Die fehlende Expression von B-Zellmarkern wie CD19, CD21, CD24 oder IgM wurde auf

Proteinebene mittels Durchfluss- und Chipzytometrie und für CD19 zusätzlich auf RNA-Ebene demonstriert, womit ausgeschlossen werden konnte, dass es sich um Dubletten aus T- und B-Zellkonjugaten handelt, die bei konventionellen durchflusszytometrischen Analysen als Artefakte gemessen werden könnten. Der natürliche Ligand zu CD20 ist unbekannt, allerdings wurde der Einfluss von CD20 auf B-Zell-Proliferation und -Apoptose beschrieben (Shan et al., 1998). Neben der Aufgabe als Calciumkanal könnte CD20 auch Teil der Tyrosin-Protein-Kaskade sein, da die Bindung monoklonaler Antikörper gegen CD20 die Tyrosinphosphorylierung verschiedener Proteine auslöst (Deans et al., 1993).

Differenzierungs- und Aktivierungsmarker wurden generell stärker auf $CD20^+$ T-Zellen exprimiert, was auf einen erhöhten Aktivierungsstatus dieser Zellen schließen lässt. Da wie erwähnt $CD20^+$ T-Zellen auch besonders in T-Zelllymphomen nachgewiesen wurden, könnte klonale Expansion dieser Zellen auch eine Rolle im gesunden Individuum spielen. Neben einer gleichmäßigen Verteilung in TCR1- und TCR2-exprimierenden T-Zellen, ergab die Analyse des α/β TCR Repertoires keine Akkumulation einer bestimmten Kombination, wenngleich in Patienten mit rheumatoider Arthritis im Allgemeinen die $V_\beta 2$ Kette bevorzugt exprimiert wurde. Eine klonale Expansion dieser Zellen kann außerdem durch ihre im Vergleich zu $CD20^-$ T-Zellen verringerte Proliferationskapazität ausgeschlossen werden.

Der Calciumdurchfluss war in $CD20^+$ T-Zellen höher als in $CD20^-$ T-Zellen und lässt eine funktionelle Rolle des Calciumkanals für $CD20^+$ T-Zellen vermuten (Kanzaki et al., 1997). Ein dem CD20-Molekül in seinen Funktionen ähnelnd ist CD38 (Malavasi et al., 2006). Dieser Rezeptor wird auf $CD20^+$ T-Zellen in geringerer Dichte exprimiert, möglicherweise stellt CD20 in diesem Zusammenhang eine Alternative zu den Funktionen von CD38 dar.
Die Aktivierung von Zellen erfordert eine Erhöhung der zytoplasmatischen Calciumkonzentration, was sowohl durch die Freisetzung von Calcium aus intrazellulären Speicherorten wie dem endoplasmatischem Retikulum als auch durch Zufluss extrazellulären Calciums ermöglicht wird. Sind die intrazellulären Speicher erschöpft, kann CD20 in B-Zellen als „Store-operated cation-“ (SOC-) Kanal dienen (Li et al., 2003).

Auf B-Zellen sind CD20 und der BCR kolokalisiert (Petrie und Deans, 2002). Bei der Kreuzvernetzung mit Rituximab assoziiert CD20 mit dem BCR, und es kommt zur Erhöhung des Calciumeinstromes (Walshe et al., 2008). Ob dementsprechend CD20 auf

T-Zellen mit dem TCR assoziiert ist, wurde bisher nicht untersucht. Im Falle einer Aktivierung dieser Zellen bei der Rituximab-Therapie käme es möglicherweise noch vor der Depletion der $CD20^+$ T-Zellen zu deren Aktivierung und folglich zu einer erhöhten Sekretion proinflammatorischer Zytokine. Tatsächlich sind die nach einer Rituximab-Behandlung auftretenden Nebenwirkungen wie Fieber, Schüttelfrost und Hautausschlägen auf eine vermehrte Zytokinproduktion zurückzuführen. Es kommen zwar zahlreiche Quellen für diese proinflammatorisch wirkenden Zytokine in Frage, jedoch könnten auch $CD20^+$ T-Zellen einen Beitrag hierfür leisten.

$CD20^+$ T-Zellen zeigten konstitutiv, also ohne vorherige Stimulation, stärkere intrazelluläre Expression für eine Reihe von Zytokinen, darunter proinflammatorische Zytokine wie IFN-γ, IL-1β, IL-2, IL-8, MCP-1 und TNF, allerdings auch antiinflammatorisch wirkendes IL-4, IL-10 und TGF-β. Das immunregulatorische Zytokin IL-17 hingegen wurde nicht in stärkerem Maße von $CD20^+$ T-Zellen exprimiert. In gesammelten Überständen von $CD20^+$ T-Zellen wurde im Gegensatz zu den intrazellulären Messungen aber weniger IL-2, IL-4 und IL-10 sowie äquivalente Konzentrationen von IFN-γ bzw. TNF-α, verglichen mit Überständen von $CD20^-$ T-Zellen, gefunden. Ein möglicher Grund hierfür ist die sehr geringe Anzahl an $CD20^+$ T-Zellen, die folglich auch nur sehr geringe Mengen an Zytokinen sezernieren können. Zusätzlich ist ein großer Teil der $CD20^+$ T-Zellen nicht mehr sehr vital bzw. ist präapoptotisch. Möglicherweise sezernieren diese Zellen insgesamt nur noch einen kleinen Teil Zytokine, während $CD20^-$ nicht-apoptotische Zellen über einen längeren Zeitraum stimulierbar sind und ihre Zytokine freisetzen können. Nicht auszuschließen ist auch eine unterschiedliche Sekretionskinetik, die z.B. auch für $CD56^{dim}$ und $CD56^{bright}$ NK-Zellen gezeigt worden ist (Wendt et al., 2006). Demnach könnte es sein, dass $CD20^+$ T-Zellen die intrazellulär vorhandenen Zytokine erst nach längerer Inkubationszeit sezernieren. Für die Überstände wurden die Zellen mit PMA/ionomycin, also direkt über das Signaltransduktionsenzym „Proteinkinase C“ (PKC), stimuliert, was nicht einer physiologischen Stimulation entspricht. Unstimulierte oder mittels TCR stimulierte T-Zellen könnten die oben genannten Zytokine in anderen als den gemessenen Konzentrationen sezernieren. Dies müsste in weiteren Experimenten getestet werden.
Generell lassen sich aber anhand des Zytokinmusters die $CD20^+$ T-Zellen keiner der einleitend erläuterten spezifischen T-Zellpopulation wie T_H1-, T_H2-, T_H9- oder T_H17-Zellen zuordnen. Auch wird es sich nicht um T_{reg}-Zellen handeln, da weder CD25 noch FoxP3 exprimiert wurden (Daten nicht gezeigt). Die Produktion von IL-22 durch $CD20^+$ T-Zellen wurde zwar nicht überprüft, aber es ist unwahrscheinlich, dass es sich um T_H22-Zellen handelt, da diese Zellen im Gegensatz zu $CD20^+$ T-Zellen keine Synthese

von IL-4 oder IFN-γ aufweisen (Eyerich et al., 2009).

Da der natürliche Ligand von CD20 bis heute nicht bekannt ist, basiert das meiste Wissen auf dem Gebrauch von anti-CD20 mAb wie z.B. Rituximab. In diesem Zusammenhang wurde herausgefunden, dass CD20 mit Lipid rafts assoziiert ist und Kreuzvernetzung von CD20 auf B-Zellen zur Apoptose dieser Zellen führt (Li et al., 2004a; Shan et al., 1998; Hofmeister et al., 2000). Bei Lipid rafts handelt es sich um sphingolipid- und cholesterolreiche Membranmikrodomänen, in denen selektiv Rezeptoren und Signaleffektoren akkumulieren (Brown und London, 2000). Lipid raft-assoziierte Kinasen der src-Familie sind verantwortlich für die Induktion von Apoptose durch CD20 Ligation (Hofmeister et al., 2000). Ob es sich ähnlich in $CD20^+$ T-Zellen verhält, ist bisher nicht geklärt. Es erklärt außerdem nicht direkt die erhöhte Apoptoserate in $CD20^+$ T-Zellen, da die T-Zellen in unseren Experimenten nicht mit CD20, sondern mittels CD3-Kreuzvernetzung aktiviert worden sind. Entsprechende Zusammenhänge wären aber denkbar und müssten weiter untersucht werden.

Eine Form des apoptotischen Zelltodes stellt die aktivierungsinduzierte Apoptose (AIA) bzw. Zelltod (AICD) dar, der für T-Zellen bereits beschrieben wurde (Zhang et al., 1996; Varadhachary et al., 1997). AICD ist die Folge wiederholter Stimulation über CD3/TCR und wird über Fas/FasL gesteuert. Als eine Art Feedbacksystem wird so eine laufende Immunantwort beendet und periphere Toleranz induziert (Fisher et al., 1995). Ein relativ großer Anteil an $CD20^+$ T-Zellen exprimierte in unseren Experimenten CD95, was für einen Fas-abhängigen Mechanismus der Apoptoseinduktion in $CD20^+$ T-Zellen spricht.

Insgesamt ist zu schlussfolgern, dass es sich um eine hochaktivierte T-Zellpopulation handelt, die in allen gesunden Individuen detektierbar ist. Ihre funktionellen Eigenschaften sind in Abb. 2 zusammengefasst. Eigene Voruntersuchungen zeigen, dass auch ein Teil muriner T-Zellen CD20 auf der Oberfläche exprimiert (Daten nicht gezeigt). Durch die Eigenschaft, proinflammatorische Zytokine konstitutiv ohne vorherige Stimulation zu exprimieren, besitzen $CD20^+$ T-Zellen einen potenziell großen Einfluss auf die Regulation des Immunsystems. Mehr Untersuchungen sind aber notwendig, um die Rolle dieser $CD20^+$ T-Zellen und vor allem die Folgen ihrer Depletion bei Rituximabtherapie besser verstehen zu können.

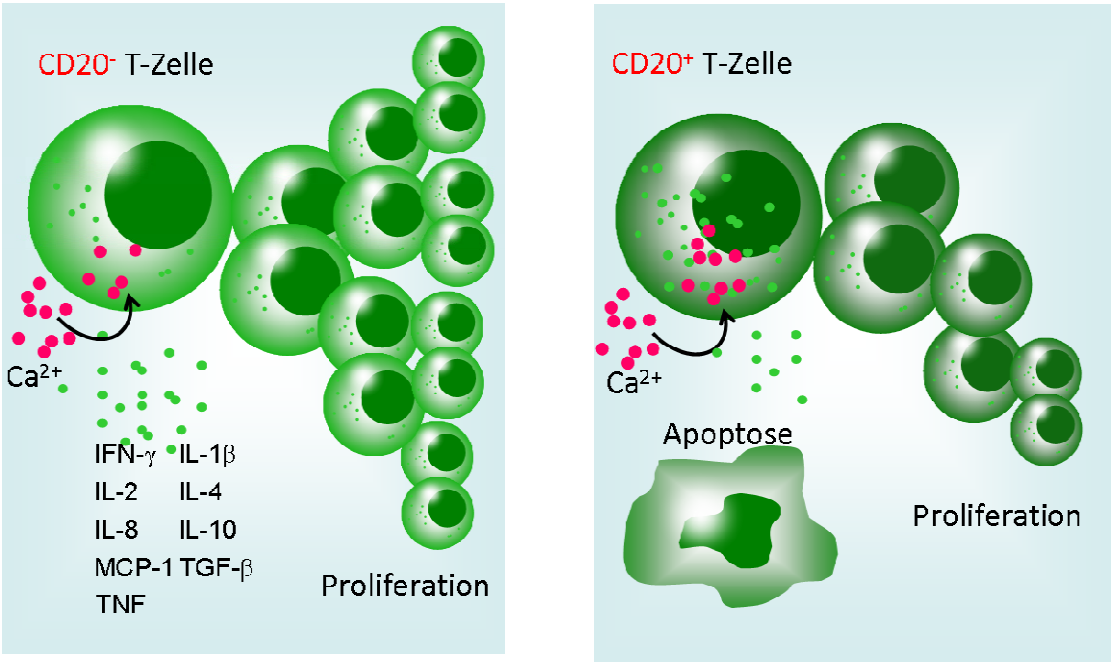

Abb. 2: CD20$^-$ und CD20$^+$ T-Zellen unterscheiden sich in Calciumeinstrom, Zytokinsekretion, Proliferation und Zellzyklus.

7.4 S100B$^+$ T-Zellen

Das Ca^{2+}-bindende Protein S100B wird nicht ubiquitär exprimiert, sondern gewöhnlich von Zellen des zentralen und peripheren Nervensystems wie z.B. Astrozyten oder Schwann-Zellen. In Leukozyten konnten wir S100B in einer kleinen Population von CD8$^+$ T-Zellen detektieren. Diese T-Zellpopulation haben wir eingehend auf ihren Phänotyp und ihre T-Zell-spezifischen Funktionen hin untersucht.

S100B wurde von Lymphozyten ausschließlich intrazellulär exprimiert, und zwar bevorzugt von CD8$^+$ T-Zellen. Bereits 1988 wurde die Expression von einigen Oberflächenrezeptoren auf S100B$^+$CD8$^+$ T-Zellen untersucht und berichtet, dass CD11b immer mit S100B koexprimiert wird (De Panfilis et al., 1988). Diese Daten konnten wir nicht bestätigen, da durchschnittlich mit hoher Variabilität nur knapp die Hälfte aller S100B$^+$ T-Zellen CD11b$^+$ war. Möglicherweise ist dies die Folge unterschiedlicher Aufreinigungs- und Färbeprotokolle oder Messtechniken.

Es wurde gezeigt, dass der kostimulatorische Rezeptor CD28 (früher als „9.3“ bezeichnet) nicht auf S100B$^+$ T-Zellen exprimiert wird (De Panfilis et al., 1988). CD28 wird von der Mehrheit der T-Lymphozyten exprimiert, bindet an B7 und stellt ein Schlüsselmolekül für die T-Zellaktivierung dar (Linsley et al., 1991). Für CD28$^-$CD8$^+$ T-Zellen wurden zwar einerseits supprimierende Funktionen, andererseits eine erhöhte Zytotoxizität und ein aktivierter Phänotyp gezeigt sowie die Expression von aktivierenden

NK-Zellrezeptoren wie z.B. KIR, CD16, CD56, NKG2D und eine erhöhte Expression von Perforin und Granzym B (Ciubotariu et al., 1998; Azuma et al., 1993; Speiser et al., 1999). Desweiteren könnten $CD28^-CD8^+$ Effektor-T-Zellen während einer HCV-Infektion hepatozelluläre Schäden verursachen, was ebenfalls gegen eine supprimierende Funktion spricht (Kurokohchi et al., 2003). Die Expression von CD28 auf $S100B^+$ T-Zellen ebenso wie deren Gehalt an Perforin und Granzymen müsste von uns weitergehend analysiert werden.

$CD8^+$ T-Zellen sind wie einleitend erklärt in der Lage, Targetzellen zu lysieren. Hierfür ist es notwendig, dass es zur Degranulation intrazellulärer Granula in der immunologischen Synapse kommt. Diese Degranulation lässt sich mit Hilfe des CD107a-Assays messen. Auffälligerweise zeigten $S100B^+$ T-Zellen keine Degranulation, weshalb sie eine Sonderstellung innerhalb der zytotoxischen $CD8^+$ T-Zellpopulation einnehmen. Das spricht auch dagegen, dass $CD28^-$ T-Zellen generell wie oben erwähnt stärker zytotoxisch sind als $CD8^+CD28^+$ T-Zellen (Ciubotariu et al., 1998; Azuma et al., 1993). Andererseits unterscheiden sich $S100B^+$ T-Zellen in ihrer Zytokinproduktion zumindest in Bezug auf IL-2 und IFN-γ scheinbar nicht von $S100B^+$ T-Zellen. Dennoch zeigen sie immunregulatorisches Potenzial, indem sie S100B auf Aktivierung hin sezernieren. Der Rezeptor von S100B, RAGE, wird u.a. von Zellen des angeborenen Immunsystems (Monozyten/Makrophagen) exprimiert (Brett et al., 1993; Leclerc et al., 2009; Collison et al., 2002; Moser et al., 2007b). Die Ligation von RAGE auf Monozyten induziert die Expression inflammatorischer Gene und erhöht die mRNA-Stabilität des IFN-γ-induzierbaren Proteins (IP-10), das in vielen entzündlichen und autoimmunen Krankheiten verstärkt sezerniert wird (Shanmugam et al., 2006). In unseren Experimenten konnten wir ebenfalls zeigen, dass S100B Granulozyten und Monozyten aktiviert, was wiederum schlussfolgern lässt, dass $S100B^+$ T-Zellen einen proinflammatorischen Effekt ausüben können und theoretisch in der Lage sind, die angeborene Immunität zu beeinflussen.

Mithilfe von Kokulturexperimenten sortierter $S100B^+$ T-Zellen ließe sich diese Hypothese genau untersuchen. Ebenso müsste beispielsweise per Chrom-Freisetzungsassay ($[^{51}Cr]$ Assay) überprüft werden, ob das verringerte CD107a Signal tatsächlich mit einer geringeren Zytotoxizität korreliert. Neue Ergebnisse aus Analysen mit NK-Zellen lassen vermuten, dass dies nicht immer der Fall ist (Jacobi et al., 2009). Eine verminderte Zytotoxizität mit parallelem Anstieg der CD107a Expression wurde hier durch Zugabe von intravenösem Immunglobulin provoziert, es kommt hierbei vermutlich zu einer „Erschöpfung“ des NK-Zellzytotoxizitätsmechanismus’ (Bhat und Watzl, 2007).

Leider ist eine Zellsortierung von S100B$^+$ T-Zellen derzeit nicht möglich, da S100B nur intrazellulär exprimiert wird und die Zelle zur Detektion des S100Bs fixiert und permeabilisiert werden muss und folglich nicht mehr zur Zellkultur zur Verfügung steht. Ausführliche durchflusszytometrische Analysen der Koexpression von Oberflächenmolekülen mit S100B haben gezeigt, dass kein bekannter Surrogatmarker existiert, mit dessen Hilfe S100B$^+$ T-Zellen identifiziert werden könnten, ohne sie funktionell zu beeinträchtigen (Daten nicht gezeigt).

In anderen Zellen des Immunsystems als T-Zellen wird S100B kaum exprimiert. Eine Ausnahme bilden DC, für die S100B zusammen mit dem Protein Fascin ein gebräuchlicher immunhistochemischer Marker ist (Ross et al., 2000). Derzeit wird diskutiert, ob mittels S100B möglicherweise experimentell induzierte plasmazytoide DC von myeloiden DC, die sowohl in Herkunft als auch Funktionen stark differerieren, unterschieden werden könnten (Nishikawa et al., 2009). Hiernach wären mDC S100B$^+$, während S100B in pDC fehlen würde. In derselben Studie wurde ein Zusammenhang von S100B mit der seltenen Krankheit „Kikuchi-Fujimoto-Lymphadenitis“ (KL) hergestellt. Ätiologie und Inzidenz dieser Krankheit sind bisher unbekannt, das Leitsymptom ist Fieber, begleitet von grippeähnlichen Symptomen. KL kann über Lymphknotenbiopsien diagnostiziert werden (Hutchinson und Wang, 2010). Dabei sind zahlreiche S100B$^-$ DC detektiert worden, und anstelle von S100B$^+$ mDC fanden sich bemerkenswerterweise große Mengen an S100B$^+$CD8$^+$ T-Zellen, deren Expansion in KL bereits früher nachgewiesen worden ist (Bosch et al., 2004; Nishikawa et al., 2009). Diese S100B$^+$CD8$^+$ T-Zellen in Lymphknoten bildeten Komplexe mit S100B$^-$Fascin$^+$ DC, gleichzeitig wurden aber auch T_{reg}-Zell-/DC-Komplexe gefunden. DC haben eine Aufgabe als APC, und während exogene Proteine über MHC-Klasse-II-Moleküle den CD4$^+$ T-Zellen präsentiert werden, werden über MHC-Klasse-I-Moleküle intrazelluläre Proteine prozessiert und CD8-positiven T-Zellen präsentiert. Mittels eines als „cross presentation“ bezeichneten Mechanismus können exogene Proteine allerdings auch über den endogenen Weg präsentiert werden (Ridge et al., 1998). Es wurde gezeigt, dass die Präsentation von Gewebeantigenen mittels DC Toleranz in peripheren CD8$^+$ T-Zellen induziert (sog. „crosstolerance“). War die Aufnahme apoptotischen Materials durch DC gestört, kam es zu einer Akkumulation funktioneller, autoreaktiver CTL (Luckashenak et al., 2008). Möglicherweise ist eine Induktion der Toleranz bzw. Anergie eine Erklärung, warum S100B$^+$ T-Zellen in unseren Experimenten eine verminderte Degranulation aufwiesen. Allerdings würde man dann auch eine verminderte Zytokinproduktion vermuten, was wir allerdings nicht nachweisen konnten.
Welche Rolle also S100B$^+$ T-Zellen in diesem Zusammenhang genau spielen, muss

weiter untersucht werden, aber es ist eine immunregulatorische Funktion zu vermuten. pDC exprimieren den Rezeptor RAGE zwar nur in geringem Maße, aber konstitutiv, wodurch eine Interaktion zwischen S100B-sezernierenden T-Zellen und pDC möglich wäre (Popovic et al., 2006).

Die eigentliche Funktion von $S100B^+$ T-Zellen ist bis dato nicht geklärt. Besser bekannt ist hingegen die Kapazität der intrazellulären S100 Proteine, Ca^{2+} zu binden. Diese ist generell eher schwach, steigt aber mit Bindung der entsprechenden Zielproteine wie z.B. dem Transkriptionsfaktor p53 um 200-400fach an (Prosser et al., 2008; Wright et al., 2008). Die drastische Änderung der intrazellulären Ca^{2+}-Konzentration wird einen biologischen Effekt auf die $S100B^+$ Zelle haben. Es ist bekannt, dass intrazelluläres S100B einen Einfluss auf verschiedenste Funktionen wie Proteindegradation, Zellproliferation und -differenzierung, Enzymaktivitäten, Rezeptorfunktionen und Proteinphoshorylierung hat (Tsoporis et al., 2010). Darüber hinaus wurde gezeigt, dass bei Bindung von S100B in mikromolaren Konzentrationen mit RAGE Proliferation und die Bildung reaktiver Sauerstoffspezies (ROS) in Neuroblastomzellen ausgelöst wurde (Leclerc et al., 2009). S100B induziert außerdem die Chemoattraktion von RAGE-exprimierenden enzephalitogenen $CD4^+$ T-Zellen in einem Modell für Multiple Sklerose und begünstigt so die T-Zellinfiltration in das ZNS (Yan et al., 2003). RAGE wird nicht nur auf Zellen des Nervensystems exprimiert, sondern auch auf aktivierten T-Zellen (Moser et al., 2007). Auf $S100B^+$ T-Zellen konnten wir jedoch kein RAGE nachweisen, weshalb eine autokrine aktivierende Wirkung des S100Bs auf diese Zellen auszuschließen ist. Andere Zellen, die RAGE exprimieren wie z.B. Astrozyten, sind in der Lage, S100B selbst zu sezernieren, folglich kann hier S100B auch autokrin wirken (Ponath et al., 2007). Je nach Konzentration des S100B-Proteins werden jedoch unterschiedliche Mechanismen in Gang gesetzt: bei nanomolaren Konzentrationen kommt es zur Proliferation der Astrozyten, während im mikromolaren Bereich die Produktion von IL-6 und TNF-α unterstützt wird (Huttunen et al., 2000). Die Konzentrationen, die von PHA- oder CD3-stimulierten $S100B^+$ T-Zellen sezerniert werden, lagen bei 0,5-1µg/L, also sehr gering, wobei die höchsten Konzentrationen bei Stimulation mit CD3 gemessen wurden.

Schlussfolgernd stellen $S100B^+$ T-Zellen eine kleine Population CD8-positiver T-Zellen dar, die eine erniedrigte Degranulierung zeigen und in der Lage sind, ihr intrazelluläres S100B zu sezernieren. S100B aktiviert Granulozyten und Monozyten, dementsprechend ist eine Regulation des angeborenen Immunsystems durch $S100B^+$ T-Zellen denkbar. Diese Eigenschaften sind als Modell in Abb. 3 dargestellt.

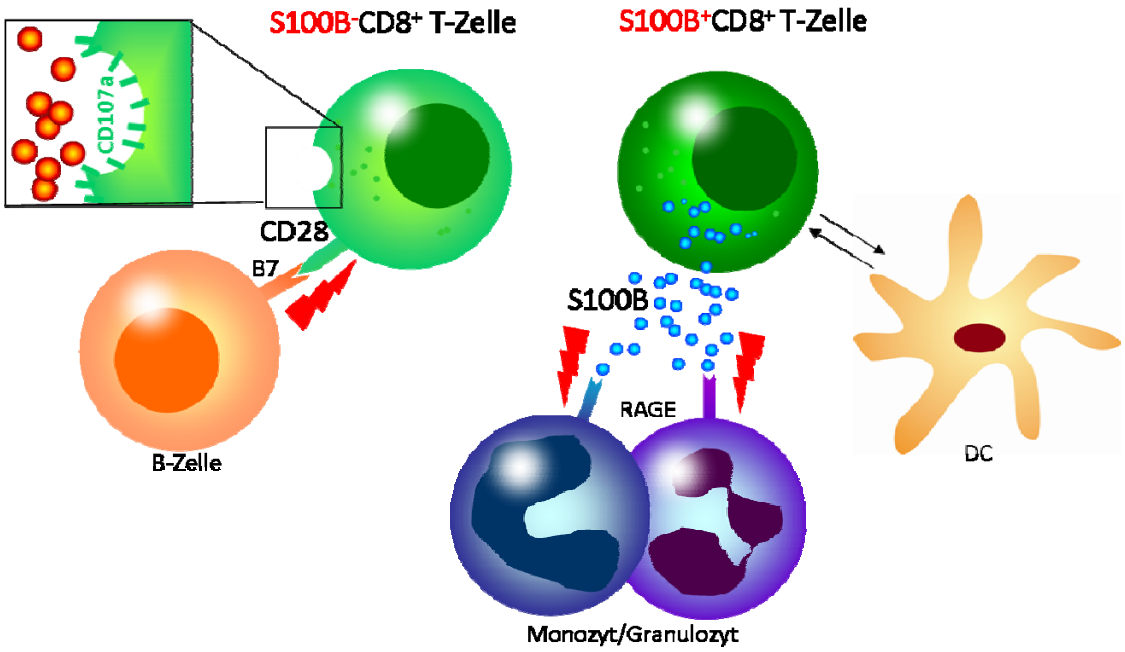

Abb. 3: S100B$^-$ und S100B$^+$ T-Zellen unterscheiden sich in Rezeptorexpression und Effektorfunktionen. Während für S100B$^-$CD8$^+$ T-Zellen eine normale Degranulierung und entsprechend die Freisetzung von Perforin und Granzymen über ein positives CD107a-Signal detektierbar war, konnte dies für S100B$^+$CD8$^+$ T-Zellen nicht bestätigt werden. S100B$^+$CD8$^+$ T-Zellen wurden als CD28$^-$ beschrieben (De Panfilis et al., 1988). CD28 ist ein kostimulierender aktivierender Rezeptor, der an B7 auf B-Zellen bindet. S100B wird sowohl intrazellulär in T-Zellen exprimiert als auch von ihnen sezerniert. Lösliches S100B ist in der Lage, an seinen Rezeptor RAGE z.B. auf zirkulierenden Monozyten und Granulozyten zu binden und die Zellen somit zu aktivieren. Zusätzlich wurde eine Aggregatbildung von S100B$^+$ T-Zellen und DC in Lymphknoten bei der Krankheit KL nachgewiesen (Nishikawa et al., 2009).

8 FAZIT

Abschließend kann zusammengefasst werden, dass die unterschiedlichen in dieser Arbeit präsentierten Zellpopulationen verschiedenste sowohl inhibierende als auch aktivierende immunregulatorische Funktionen aufweisen.

Auf Grundlage unserer Daten ist es möglich, Entwicklung und Differenzierung der regulatorischen NK-Zellsubpopulation *in vivo* zu untersuchen. Ferner gibt die vorliegende Arbeit Hinweise auf die Art der Interaktion von NK-Zellen mit UC-MSC. Sollte sich herausstellen, dass auch NK-Zellsubpopulationen unterschiedlich auf die Wechselwirkung mit MSC reagieren, könnte das von uns erstellte *in vivo*-Modell außerdem für weiterführende Experimente von Nutzen sein. Zusätzlich kann der eventuelle therapeutische Nutzen von NK-Zellen und MSC bei der HSCT nun detaillierter untersucht werden. Die UC-MSC-vermittelte Immunsuppression ist eindeutig nachgewiesen, allerdings müssen die entsprechenden rezeptorabhängigen Mechanismen noch identifiziert werden, bevor UC-MSC in der Klinik Verwendung finden können.

In weiteren Ansätzen wurden zwei unterschiedliche T-Zellsubpopulationen im Menschen näher charakterisiert. $S100B^+$ T-Zellen und $CD20^+$ T-Zellen kommen im peripheren Blut gesunder Individuen vor und können aktivierende Signale vermitteln, stehen aber in keinem direkten Verhältnis zueinander. Ihr immunregulatorisches Potenzial wurde bisher nicht berücksichtigt. Die Depletion von $CD20^+$ T-Zellen im Rahmen einer Behandlung mit anti-CD20 mAb eliminiert neben den erwarteten B-Zellen auch unbeabsichtigterweise $CD20^+$ T-Zellen. Die Konsequenzen daraus sind nicht absehbar, da die biologische Funktion dieser T-Zellpopulation bisher nicht bekannt ist. Dies trifft auch für $S100B^+$ T-Zellen zu. Im Rahmen dieser Arbeit konnten aber ihre funktionellen Fähigkeiten eingegrenzt werden, was eine Basis für zukünftige Untersuchungen darstellt.

ABBILDUNGSVERZEICHNIS

Murine CXCR3$^{\pm}$CD27^{bright} NK cells resemble the human CD56^{bright} NK-cell population

Umbilical cord mesenchymal stem cells inhibit NK cell effector functions by direct cell-cell-contact independently from LFA-1 or LAIR-1

Depletion of Functionally Active CD20+ T Cells by Rituximab Treatment

S100B secretion by human CD8+ T cells activates monocytes and granulocytes: novel crosstalk between the adaptive and innate immune systems

Diskussion

TABELLENVERZEICHNIS

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TEILNAHME AN WISSENSCHAFTLICHEN VERANSTALTUNGEN

- Spring School on Immunology 2008 (Ettal, Deutschland)
- Posterpräsentation: "Murine NK Cell Subpopulations"
- NK Cell Symposium 2008 (Bad Herrenalb, Deutschland)
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- Annual Meeting of the DGfI 2008 (Wien, Österreich)
- Posterpräsentation: "CXCR3 expression patterns on murine NK cells characterize phenotypic and functional homologues to human NK cell subsets"
- 2nd European Congress of Immunology 2009 (Berlin, Deutschland)
- Poster presentation: "Direct comparisons of human and murine NK cell subsets based on CD27 and CXCR3 coexpression patterns"
- Koautor (Poster): "CD20^{+} T cells: functional and phenotypical characteristics of a highly differentiated effector lymphocyte subset"
- Koautor (Poster): "Association of a NKp44 allele with primary Sjögren's syndrome"
- Koautor (Poster): "Rituximab depletes functionally active CD20^{+} T cells in rheumatoid arthritis: an additional mechanism of action?"
- Combined Meeting of the ESGCT, GSZ, DG-GT and ISCT 2009 (Hannover, Deutschland)
- Besuch der Konferenz
- 12th Meeting of the Society for Natural Immunity 2010 (Cavtat, Kroatien)
- Posterpräsentation: "CD56^{intermediate} NK cells: A link between CD56^{dim} and CD56^{bright} NK cells?"
- Posterpräsentation: "Murine regulatory CXCR3^{+} CD27^{bright} NK cells resemble human CD56^{bright} NK cells in phenotype and function"
- 40th Annual Meeting of the German Society for Immunology (DGfI), 2010 (Leipzig, Deutschland)
- Posterpräsentation: "Differential expression of S100B by human leukocytes"

PUBLIKATIONEN

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- **Marquardt, N.**, Wilk, E., Schmidt, RE., Jacobs, R., CXCR3 expression patterns on murine NK cells characterize phenotypic and functional homologs to human NK cell subsets. Wien Klin Wochenschr (2008) 120/15-16 [Suppl 1] 1-200.
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