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Untersuchungen zur Rolle von Chemokinen und IL-10-ähnlichen Zytokinen bei intestinaler Entzündung und Kanzerogenese

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Abkürzungen

µ	Mikro- (10^{-6})
α-	Anti-
$\gamma^{32}P$	radioaktiv markierter Phosphor
APC	Antigen-präsentierende Zelle
ATP	Adenosintriphosphat
BSA	Rinderserumalbumin (Bovine serum albumin)
CARD	Caspase activation recruiting domain
CED	Chronisch entzündliche Darmerkrankungen
cm	Zentimeter
CRC	Kolorektales Karzinom (Colorectal carcinoma)
CU	Colitis ulcerosa
d	Desoxy-
DAPI	4,6-diamidino-2-phenylindoldihydrochlorid
DC	Dendritische Zelle (Dendritic cell)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonukleinsäure
dNTP	Mix aus gleichen Teilen dATP, dGTP, dCTP, dTTP
ds	Doppelstrang
DTT	Dithiothreitol
ECM	Extrazelluläre Matrix
EDTA	Ethylendiamintetraeacetat
EGTA	Ethylenglykol-bis-(2-aminoethyl)-tetraacetat
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
EtBr	Ethidiumbromid
EtOH	Ethanol
FACS	Fluoreszenz-aktivierte Zellsortierung
FAE	Follikel-assoziiertes Epithel
FCS	Foetales Kälberserum
FITC	Fluorescein-Isothiocyanat
g	Gramm oder Beschleunigungskraft (Zentrifuge)

GAPDH	Glycerinaldehyddehydrogenase
GALT	subepitheliales Lymphgewebe (Gut-associated lymphoid tissue)
GI-	Gastrointestinal-
h	Stunde
HEPES	N-(2-Hydroxyethyl)-piperazin-N'-(2-ethansulfonsäure)
HRP	Merettichperoxidase (horse raddish peroxidase)
IEC	Intestinale Epithelzelle (Intestinal epithelial cell)
IFN	Interferon
IL	Interleukin
l	Liter
LPS	Lipopolysaccharid
M	Molar (mol/l)
m	Milli- (10^{-3})
MC	Morbus Crohn
MAP	Mitogen-activated protein
MDP	Muramyldipeptid
MetOH	Methanol
min	Minuten
MMP	Matrix-Metalloproteinase
MOPS	N-Morpholinopropansulfonsäure
mRNA	messenger RNA
MTS	3-(4,5-Dimethlythiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium
n	Nano (10^{-9})
nm	Nanometer
NOD	Nucleotide oligomerization domain
°C	Grad Celsius
OD	Optische Dichte
PAGE	Polyacrylamidgelelektrophorese
PAMP	Pathogen-assoziierte molekulare Muster (Pathogen-associated molecular pattern)
PBS	Phosphat-gepufferte Salzlösung (Phosphate buffered saline)
PCR	Polymerasekettenreaktion (Polymerase chain reaction)
PE	Phycoerythrin

PMS	Phenazinmethosulfat
PMSF	Phenylmethylsulfonylfluorid
PP	Peyer-Plaques
PRR	Pattern recognition receptor
PS	Penicillin-Streptomycin
PVDF	Polyvinylidenfluorid
RNA	Ribonukleinsäure
RT	Raumtemperatur
RT-PCR	Reverse Transkription-Polymerasekettenreaktion
SDF	Stromal cell derived factor
SDS	Natriumdodecylsulfat
sec	Sekunden
SNP	Einzelnukleotidpolymorphismus (single nucleotide polymorphism)
ss	Einzelstrang (single strand)
STAT	Signal transducer and activator of transcription
TAE	Tris-Acetat-EDTA
TBE	Tris-Borsäure-EDTA
TBS	Tris-gepufferte Salzlösung (Tris buffered saline)
TGF-β	Transforming growth factor beta
TLR	Toll-like-Rezeptor
TNBS	Trinitrobenzensulfonsäure
TNF-α	Tumornekrosefaktor alpha
Tris	Tris-(hydroxymethyl)-aminomethan
U	enzymatische Aktivitätseinheit (Units)
upm	Umdrehungen pro Minute
V	Volt
w/v	Gewicht pro Volumen (weight per volume)
WT	Wildtyp
x	-fach bzw. -mal

1. Einleitung

1.1 Das intestinale Immunsystem

1.1.1 Aufbau und physiologische Funktionen des intestinalen Immunsystems

Der Darm ist ein sehr komplex strukturiertes Organ und stellt mit mehr als 300 m^2 die größte Oberfläche im menschlichen Körper dar. Hierbei ist er nicht nur für die Verdauung und die Nährstoffaufnahme zuständig. Seine Oberfläche ist ständig der intestinalen Mikroflora ausgesetzt, die aus mehr als 400 verschiedenen Bakterienspezies und einer Gesamtzahl von 10^{14} Keimen besteht [Smith und Gorbach, 1995]. Daher spielt das intestinale Immunsystem eine wichtige Rolle, da es einerseits Toleranz gegenüber kommensalen Bakterien vermitteln, andererseits effizient Pathogene erkennen und eliminieren muss.

Das körpereigene Immunsystem besteht aus zwei Komponenten, dem angeborenen und dem erworbenen Immunsystem. Ersteres erkennt unspezifisch allgemeine, Pathogen-assoziierte molekulare Muster (pathogen-associated molecular patterns, PAMPs). Diese können Bestandteile mikrobieller Zellmembranen sein wie z. B. Muramyldipeptid (MDP), ein Peptidoglycan-Motiv in der Zellwand Gram-positiver und Gram-negativer Bakterien, oder Lipopolysaccharid (LPS) aus Gram-negativen Bakterien. Es werden aber auch bestimmte Nukleinsäure-Muster (CpG-Motive bakterieller DNA, ssRNA und dsRNA von Viren) und Proteine wie bakterielles Flagellin gebunden [Harris et al., 2006; Tlaskalova-Hogenova et al., 2004]. Die konstitutive Expression von antimikrobiellen Peptiden, den sogenannten Defensinen, trägt ebenfalls zur angeborenen Immunantwort gegen Bakterien bei [Fellermann und Stange, 2001].

Verantwortlich für das Erkennen von PAMPs sind sogenannte „Pattern recognition receptors“ (PRRs). Während MDP vom Rezeptorprotein „nucleotide-binding oligomerization domain-2" (NOD2) gebunden wird, werden LPS und CpG-Motive von sogenannten Toll-like-Rezeptoren (TLRs) auf der Zelloberfläche erkannt, wobei die LPS-Erkennung über TLR4 [Poltorak et al., 1998a; Poltorak et al., 1998b] und die CpG-Erkennung über TLR9 [Hemmi et al., 2000] vermittelt wird. Bakterielle Infektionen werden durch Entzündungszellen wie Neutrophile, die durch den Anstieg proinflammatorischer Zytokine wie IL-8 [Mitsuyama et al., 1994] oder Chemokine wie ENA-78/CXCL5 [MacDermott et al., 1998; Z'Graggen et al., 1997] rekrutiert werden, bekämpft. Außerdem kommt es zu einer gesteigerten Produktion von induzierbaren Defensinen, was ebenfalls der Eindämmung der Infektion dient [Bevins, 2006; Fellermann und Stange, 2001; Muller et al., 2005; Salzman et al., 2007]. Das erworbene Immunsystem leitet anschließend eine gezielte, Antigen-spezifische Immunabwehr ein. Diese beinhaltet die

Aktivität von Antigen-präsentierenden Zellen (APCs) wie Makrophagen und dendritischen Zellen (DCs), von T- und B-Lymphozyten und die klonale Selektion von reaktiven Zellen.
Das mukosale Immunsystem vereint angeborene und adaptive Immunantworten und besteht aus verschiedenen spezialisierten Zelltypen (siehe Abbildung 1).

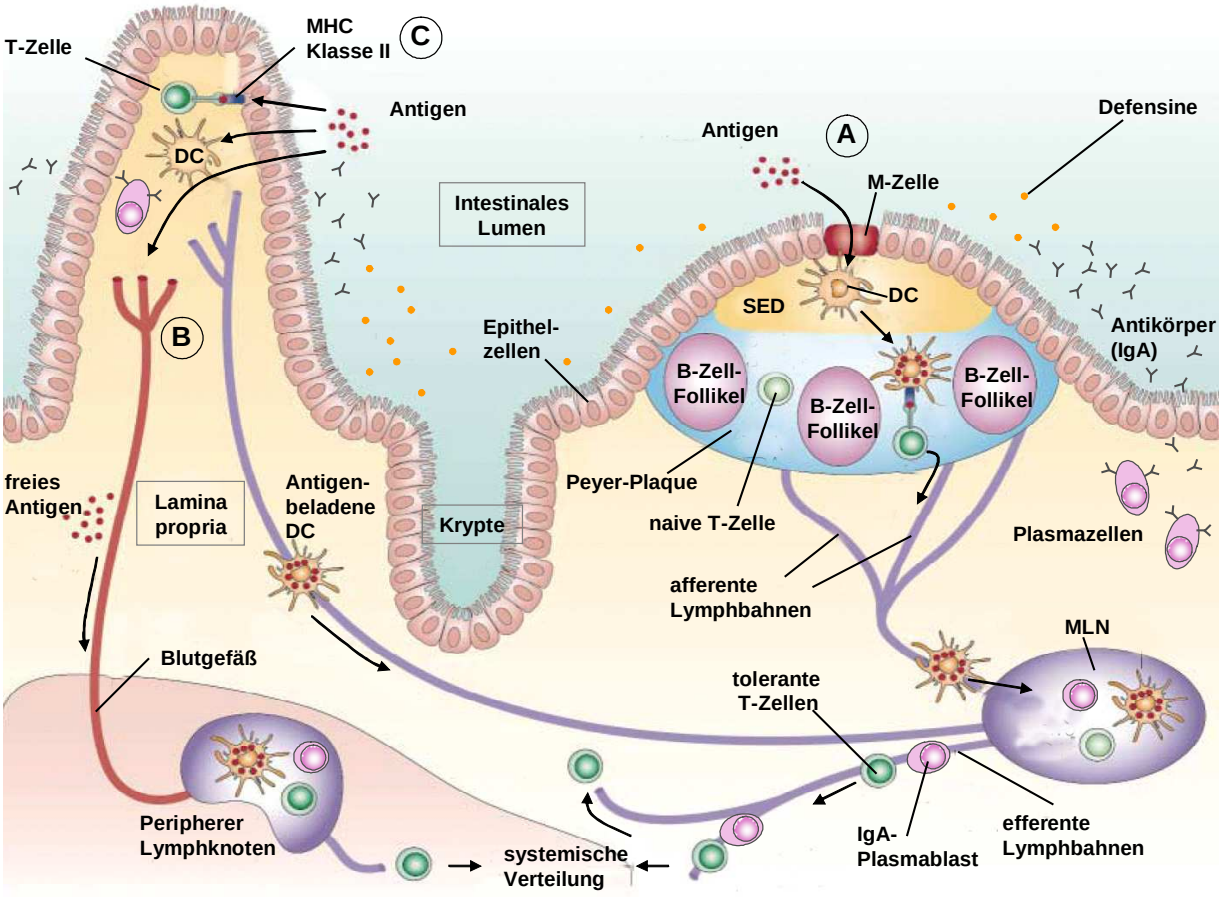

Abbildung 1. Übersicht über den Aufbau der intestinalen Barriere und des intestinalen Immunsystems. Die Erkennung von Antigenen kann über verschiedene Mechanismen erfolgen: (A) Die Antigene werden über die M-Zellen zu den DCs der Peyer-Plaques transferiert, welche die Antigene den dort präsenten B- und T-Zellen präsentieren. (B) Antigene oder Antigen-beladene DCs können über die Lymphe in die mesenterischen Lymphknoten (MLN) gelangen, wo sie B- oder T-Zellen präsentiert werden. (C) MHC Klasse II-positive IECs können als lokale APCs die Antigene T-Zellen präsentieren. In allen Fällen wandern T- und B-Zellen über die Lymphe in die Blutgefäße, wo sie zu Effektororganen wie der Lamina propria weitertransportiert werden (modifiziert nach [Miller et al., 2007]).

Die oberste Schicht der Darmwand stellen die intestinalen Epithelzellen (IEC) dar, die von einer Schleimschicht (Mucus) bedeckt sind. Unter diesen Zellen befindet sich das subepitheliale Lymphgewebe, auch GALT (gut-associated lymphoid tissue) genannt. Dieses enthält die Peyer-Plaques, spezialisierte Lymphknoten in der Darmwand, die von speziellen Epithelzellen (Follikel-assoziiertes Epithel, FAE) wie den M-Zellen bedeckt sind. Letztere transportieren luminale Antigene in das lymphoide Gewebe zu den dort präsenten

subepithelialen DCs, welche die Antigene wiederum naiven B- und T-Zellen präsentieren und diese dadurch aktivieren können [MacDonald, 2003; Muller et al., 2005].

Es ist von entscheidender Bedeutung, dass das Immunsystem zwischen kommensalen Mikroorganismen und pathogenen Bakterien unterscheiden kann, also eine Toleranz gegenüber Kommensalen zeigt, um eine chronische Entzündung zu vermeiden. Dies wird durch verschiedene Mechanismen gewährleistet. So exprimieren IEC z. B. eine geringere Anzahl von PRRs wie TLR4 und deren Co-Rezeptoren, aber auch die ständige Exposition gegenüber TLR-Liganden bewirkt eine Hyporesponsivität der Zellen [Michelsen und Arditi, 2007]. Ein weiterer Mechanismus ist die Polarität von IEC und die differentielle Expression von verschiedenen TLRs auf der apikalen bzw. der basolateralen Seite der Zellen. TLR5, der Rezeptor für bakterielles Flagellin, wird z. B. nur auf der basolateralen Seite exprimiert, so dass er nur dann in Kontakt mit seinem Liganden kommt, wenn pathogene Bakterien mit Flagellen bereits die Epithelschicht durchdrungen haben [Michelsen und Arditi, 2007]. Auf der anderen Seite ist die kontinuierliche Aktivierung von TLR-Signalwegen, verursacht durch kommensale Bakterien, essentiell für die intestinale Homöostase und den Schutz vor Gewebsverletzungen [Rakoff-Nahoum et al., 2004], weil dadurch gewebsprotektive Faktoren wie KC-1 oder Hitzeschock-Proteine produziert werden [Rakoff-Nahoum et al., 2004].

Durch den Kontakt mit nicht-pathogenen Bakterien werden in IEC außerdem immunregulatorische Zytokine wie transforming growth factor (TGF)-β gebildet [Haller et al., 2000]. Umgekehrt induzieren diese IECs dann die Produktion von IL-10 in mononukleären Zellen des peripheren Bluts [Haller et al., 2000]. Außerdem reagieren IEC auf den Kontakt mit pathogenen oder nicht-pathogenen Bakterien mit unterschiedlicher Zytokinproduktion [Haller et al., 2000]. Die Produktion von TNF-α beispielsweise wird durch pathogene Bakterien viel langandauernder induziert als durch nicht-pathogene Organismen [Haller et al., 2000].

Das Aufspüren von pathogenen Bakterien durch IEC ist also einer der integralen Mechanismen der fein abgestimmten Immunantwort auf krankheitserregende Mikroorganismen in der intestinalen Mukosa. Generell sind die mukosalen Epithelzellen die entscheidenden Zellen für die Koordination der Immunantwort. Denn durch ihren ständigen Kontakt mit dem Darmlumen „analysieren“ sie die Bedingungen und antworten auf den Kontakt mit pathogenen Mikroorganismen mit der Ausschüttung von proinflammatorischen Zytokinen und Chemokinen, darunter IL-8/CXCL8, IL-1β, GM-CSF, CXCL1, CXCL2, CXCL3 (GRO-α, -β, -γ) und CCL2 (MCP-1). Diese wiederum rekrutieren polymorphkernige Leukozyten, Makrophagen und Lymphozyten und initiieren eine Entzündungsreaktion [Jung

et al., 1995; Maaser und Kagnoff, 2002; Rumbo et al., 2004]. Bemerkenswerterweise ist die Produktion spezifischer proinflammatorischer Mediatoren wie TNF-α in IEC, die in Kontakt mit Bakterien stehen, von der Präsenz darunterliegender Leukozyten abhängig [Haller et al., 2000], was die Wichtigkeit der interzellulären Kommunikation zwischen IEC und Immunzellen unterstreicht.

Damit IEC diese vielfältigen Funktionen sowohl in der Koordination des intestinalen Immunsystems als auch bei der Aufrechterhaltung der intestinalen Barriere realisieren können, ist ein breites Spektrum von Rezeptoren auf den Zellen wichtig. Zellbotenstoffe wie Chemokine und Zytokine, die als biologische Mediatoren für die interzelluläre Kommunikation unentbehrlich sind, sowie deren Rezeptoren, spielen hierbei eine entscheidende Rolle. Daher sollte die Expression und die vermittelten Funktionen ausgewählter intestinal exprimierter Chemokin- und Zytokinrezeptoren (siehe auch Abschnitt 1.2 und 1.3) in der vorgelegten Arbeit genauer charakterisiert werden.

1.1.2 Dysregulation des intestinalen Immunsystems: Chronisch entzündliche Darmerkrankungen

1.1.2.1 Pathogenese und genetische Prädispositionen

Normalerweise zeigt das intestinale Immunsystem eine Toleranz gegenüber nicht-pathogenen, symbiotischen Bakterien und es herrscht ein Zustand kontrollierter Entzündung (siehe auch Abschnitt 1.1.1). Gerät dieses sensible Gleichgewicht außer Kontrolle („loss of tolerance“), so kann dies zu einer permanenten Entzündung des Gastrointestinal-(GI)-traktes und zum Entstehen von chronisch entzündlichen Darmerkrankungen (CED) führen [Duchmann et al., 1995; Duchmann et al., 1996; Sartor, 2006] (siehe Abbildung 2).

Unter dem Oberbegriff CED versteht man rezidivierende oder kontinuierliche, entzündliche Erkrankungen des Darms, die mit Verlust der Mukosa-Integrität, verminderter Resorptionsleistung und verstärkter Sekretion bzw. Exkretion einhergehen. Die beiden bekanntesten Vertreter sind der Morbus Crohn (MC) und die Colitis ulcerosa (CU).

A **B**

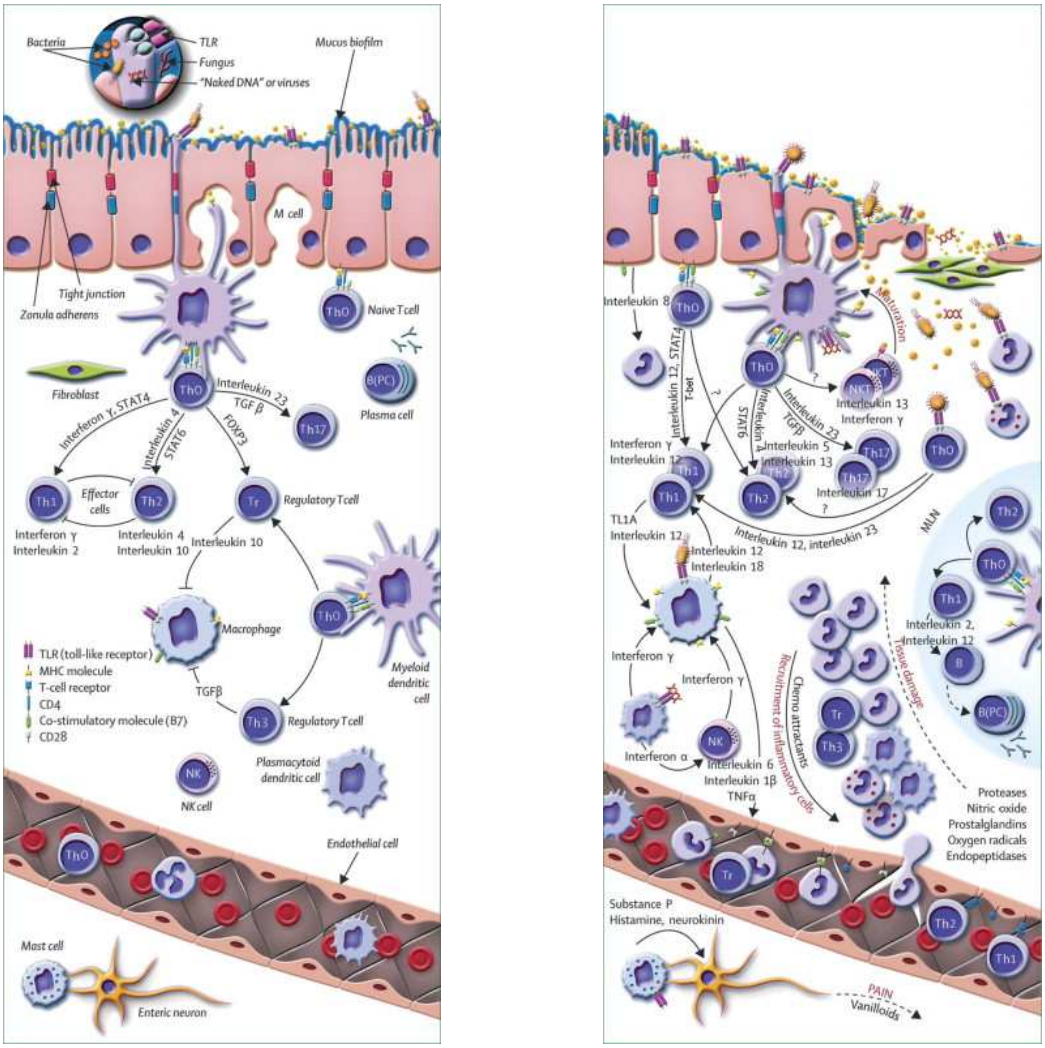

Abbildung 2: Übersicht über das intestinale Immunsystem unter physiologischen und pathologischen Bedingungen. (A) Unter physiologischen Bedingungen herrscht in der Anwesenheit von kommensalen Bakterien ein Gleichgewicht kontrollierter Entzündung zwischen Effektorzellen und regulatorischen Immunzellen, das durch ein streng reguliertes Chemokin- und Zytokinnetzwerk aufrechterhalten wird. (B) Unter Bedingungen chronischer Entzündung dringen luminale Antigene durch eine gestörte intestinale Barriere in darunterliegende Gewebeschichten ein, wo sie von DCs als Pathogene erkannt werden und in den DCs ein Reifungsprogramm auslösen, was wiederum zu einer Aktivierung von naiven T-Zellen (Th0) zu Effektor-T-Zellen (Th1, Th2, Th17) führt. IEC exprimieren nun vermehrt kostimulatorische Moleküle, wodurch sie als APCs fungieren und dadurch zur Effektor-T-Zell-Antwort beitragen (aus [Baumgart und Carding, 2007]).

Charakteristisch ist für den MC der diskontinuierliche, segmentale Befall (sogenannte "skip lesions") der Darmschleimhaut. Die Entzündung beschränkt sich hierbei nicht nur auf die oberen Schichten der Mukosa, sondern kann als transmurale Entzündung alle Ebenen der Darmwand betreffen. Jeder Abschnitt des Verdauungstraktes vom Mund bis zum Anus kann betroffen sein, meistens findet sich jedoch ein Befall im terminalen Ileum, Ileocolon und Colon ascendens. Im Gegensatz zum MC ist die CU durch einen kontinuierlichen Befall der Darmschleimhaut gekennzeichnet. Es handelt sich hierbei um eine auf die Oberfläche

begrenzte, mukosale Entzündung, die nur im Kolon auftritt, das Rektum ist hierbei fast immer betroffen. Die genauen Ursachen für beide Erkrankungen sind unklar, es wird jedoch angenommen, dass ein Zusammenspiel von äußeren Umwelteinflüssen, genetischer Prädisposition und gestörter Immunantwort verantwortlich ist.

Wie bereits in Abschnitt 1.1.1 erwähnt, ist das Immunsystem in einen angeborenen und einen adaptiven Teil untergliedert. Bei einer Aktivierung der adaptiven Immunantwort kommt es je nach Bedingungen entweder zu einer betont zellulären Antwort, die mit einer verstärkten Expression von sogenannten Th1-Zytokinen einhergeht, oder zu einer eher humoral betonten Reaktion, welche die Bekämpfung der Infektion über B-Zellen und von diesen sezernierte Antikörper einleitet und von einer verstärkten Th2-Zytokinexpression charakterisiert ist.

Die Differenzierung von naiven T-Zellen zu den verschiedenen Effektor-T-Zellen ist hierbei abhängig vom Zytokinmilieu, unter welchem sich die Zellen entwickeln. Je nach Bedingungen kommt es zur Differenzierung von Zellen zu Th1-, Th2- oder Th17-Effektorzellen oder zur Entwicklung von regulatorischen T-Zellen (T_{regs}) (siehe Abbildung 3). Während MC als eine Th1-vermittelte Erkrankung mit einer verstärkten Expression der Th1-Zytokine IL-1β, IL-12, TNF-α und IFN-γ angesehen wird, gilt die CU eher als eine atypische Th2-vermittelte Immunreaktion mit einer verstärkten Expression der Th2-Zytokine IL-5 und IL-13 [Bouma und Strober, 2003; Cobrin und Abreu, 2005; Monteleone et al., 2006; Sartor, 2006].

Neue Erkenntnisse legen eine Beteiligung von Th17-Zellen bei der Pathogenese von MC nahe [Bamias und Cominelli, 2007; McGovern und Powrie, 2007]. Diese Zellen sind durch eine Produktion von IL-17 charakterisiert und entstehen bei Mäusen unter dem Einfluss von TGF-β, IL-6, IL-21 und IL-23 [Bettelli et al., 2006; Mangan et al., 2006; Nurieva et al., 2007; Veldhoen et al., 2006; Zhou et al., 2007], während dieser Prozess in humanen Zellen unabhängig von TGF-β durch IL-23, IL-1β und IL-6 kontrolliert wird [Acosta-Rodriguez et al., 2007; Chen et al., 2007; Wilson et al., 2007] (siehe Abbildung 3).

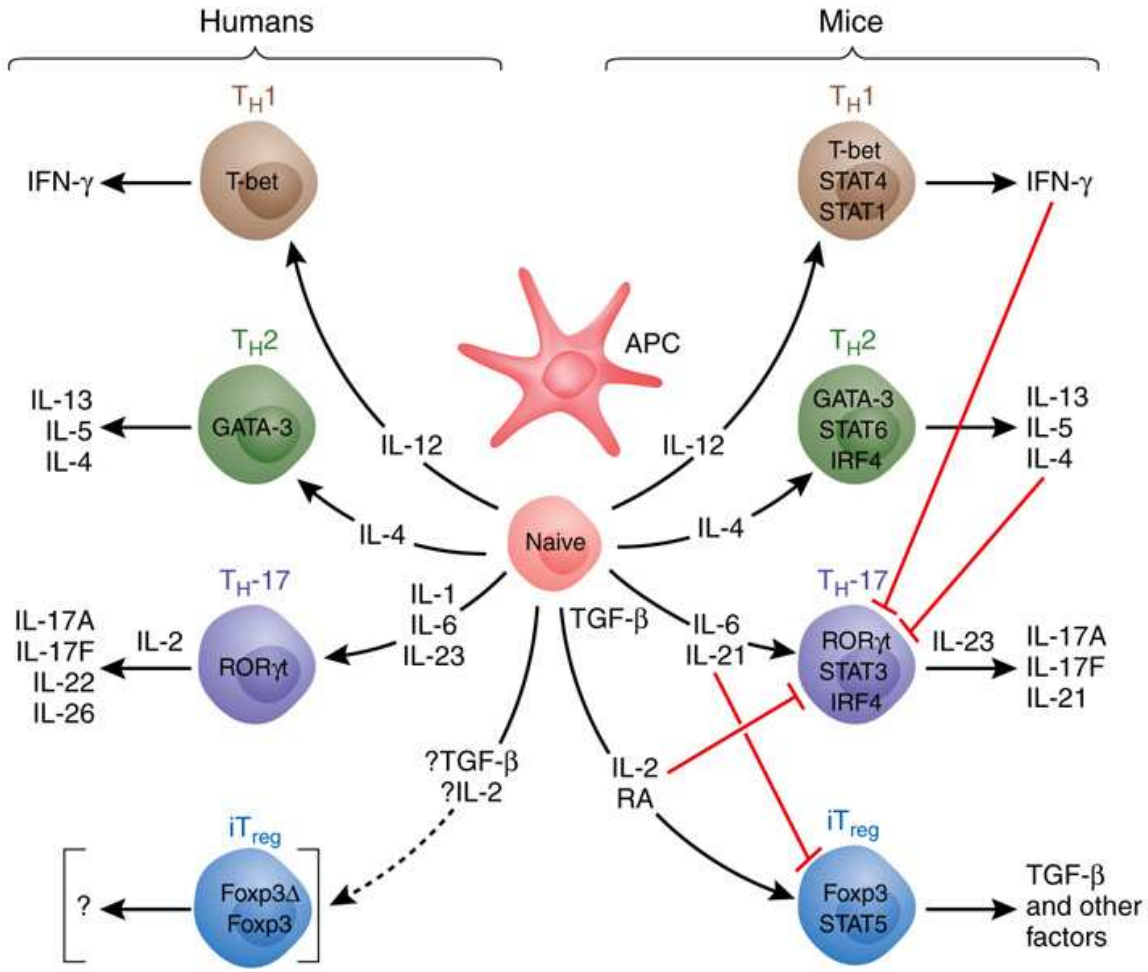

Abbildung 3: Übersicht über die verschiedenen T-Zell-Typen und ihre Differenzierung aus naiven humanen und murinen Vorläufer-T-Zellen. Während die Anwesenheit von IL-12 für die Differenzierung zum Th1-Typ verantwortlich ist, entstehen unter dem Einfluss von IL-4 Th2-Zellen. TGF-β ist wichtig für die Induktion regulatorischer T-Zellen. Bei der Entwicklung von Th17-Zellen zeigen sich Unterschiede zwischen den Spezies: Während murine Th17-Zellen unter dem Einfluss von TGF-β, IL-6, IL-21 und IL-23 differenzieren, wird dieser Prozess in humanen Zellen von IL-23, IL-1β und IL-6 kontrolliert (aus [Laurence und O'Shea, 2007]).

Th17-Zellen exprimieren unter anderem den Rezeptor für das Zytokin IL-23 [Mangan et al., 2006]. Bei IL-23 handelt es sich um ein Dimer aus IL-23p19 und IL-12p40 [Oppmann et al., 2000], wobei letztere Untereinheit auch ein Bestandteil von IL-12, einem Th1-Zytokin, ist [Manetti et al., 1993; Wolf et al., 1991]. IL-23, jedoch nicht IL-12, ist essentiell für eine T-Zell-vermittelte Kolitis im Mausmodell [Yen et al., 2006], und eine Neutralisierung von IL-23 mit anti-IL-23p19-Antikörpern verhindert das Entstehen bzw. mildert eine bereits vorhandene Kolitis [Elson et al., 2007]. Kürzlich wurde ein Polymorphismus im IL-23-Rezeptor-Gen (*IL23R*) beschrieben, der protektiv für das Entstehen eines MC ist [Duerr et al., 2006]. Eine Beteiligung von *IL23R*-Polymorphismen an der Prädisposition für MC konnte

von unserer Arbeitsgruppe bestätigt werden [Glas et al., 2007c]. All diese Daten unterstreichen die essentielle Rolle von IL-23 und Th17-Zellen bei der Pathogenese von MC.
Werden Th17-Zellen mit IL-23 stimuliert, so wird eine Reihe von spezifischen Genprodukten gebildet, darunter, neben dem namengebenden IL-17A sowie dem Th17-spezifischen Transkriptionsfaktor RORγt, auch die Zytokine IL-22, IL-26 und das Chemokin CCL20 (siehe Abbildung 4).

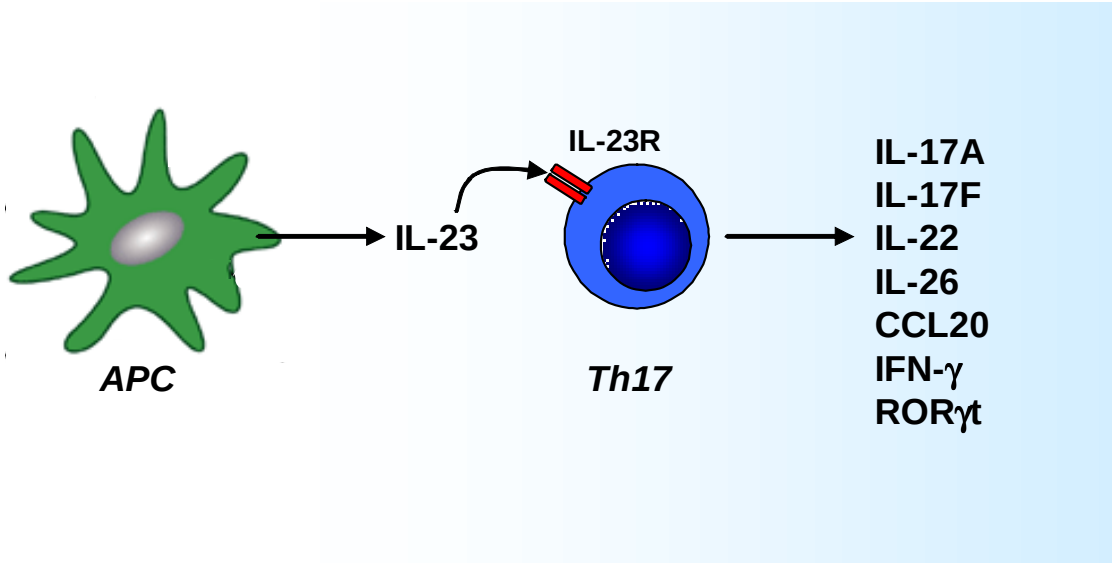

Abbildung 4. Übersicht über die von Th17-Zellen produzierten Zytokine nach Stimulation mit IL-23, das von APCs gebildet wird.

Die Funktionen von CCL20 sowie der IL-10-verwandten Zytokine IL-22 und IL-26 im GI-Trakt und ihre potentielle Rolle bei der Pathogenese von CED wurden im Rahmen dieser Arbeit näher untersucht.
Das Th17-stimulierende Zytokin IL-23 ist jedoch nicht nur bei der T-Zell-vermittelten Immunität von Bedeutung, sondern übernimmt auch wichtige Aufgaben bei der angeborenen Immunantwort im GI-Trakt [Hue et al., 2006; Uhlig et al., 2006]. Inzwischen gibt es viele Hinweise auf eine Beteiligung des angeborenen Immunsystems an der MC-Pathogenese [Eckmann, 2004; Muller et al., 2005; Sartor, 2006; Wehkamp et al., 2002]. Hierzu gibt es zwei Hypothesen [Comalada und Peppelenbosch, 2006]: (1) Das angeborene Immunsystem ist bei MC-Patienten aufgrund genetischer Defekte überaktiv, wodurch es zu einer ausgedehnten Entzündung kommt. (2) Die angeborene Immunantwort ist durch genetische Defekte geschwächt, was zu einer Akkumulation von Bakterien, zur Granulomformation und infolge dessen zu einer sekundären, adaptiven Immunantwort führt [Marks et al., 2006]. Bei beiden Hypothesen spielt jedoch die bakterielle Flora des Darmes eine wichtige Rolle. Im GI-Trakt befinden sich ca. 10^{14} Bakterien [Smith und Gorbach, 1995]. Die Beteiligung von

bestimmten luminalen Bakterienstämmen bei der Pathogenese von MC wird von Daten aus einer Reihe von Tiermodellen gestützt [Madsen et al., 1999; Rath et al., 1996; Sartor, 1997a; Sartor, 1997b; Sellon et al., 1998]. Es zeigt sich aber auch, dass bezüglich der Induktion intestinaler Entzündung nicht alle Bakterienstämme die gleichen Eigenschaften haben [Clavel und Haller, 2007; Haller, 2006].

Eine intakte intestinale Barriere ist ebenfalls von großer Bedeutung für die Aufrechterhaltung der intestinalen Homöostase und das Verhindern des Eindringens von Bakterien [Dignass, 2001; Podolsky, 1999]. Bei CED-Patienten ist diese epitheliale Barriere der Kolonmukosa gestört [Cobrin und Abreu, 2005; Sanders, 2005]. Dies äußert sich durch eine erhöhte Permeabilität, eine erhöhte Adhärenz von Bakterien sowie eine verminderte Defensin-Produktion [Cobrin und Abreu, 2005; Wehkamp et al., 2005]. Dadurch können Bakterien in die daruntergelegenen Gewebeschichten eindringen und dort eine Entzündung mit der Produktion von proinflammatorischen Zytokinen wie TNF-α oder IL-1β verursachen.

Obwohl die Ursachen, die zu der Entwicklung einer CED führen, bis heute nicht vollständig geklärt sind, gibt es doch Hinweise auf eine genetische Prädisposition, besonders bei MC-Patienten [Halfvarson et al., 2005; Halme et al., 2006; Russell und Satsangi, 2004]. Aufgrund der Heterogenität von CED ist davon auszugehen, dass eine Reihe von Genen in die Pathogenese bzw. die phänotypische Ausprägung der Krankheit verwickelt sind. In genetischen Kopplungsstudien wurden bisher 9 wahrscheinlich mit CED assoziierte genetische Risikomarker identifiziert (IBD1-IBD9, siehe Abbildung 5).

Der bis heute am besten untersuchte Locus ist IBD1 auf Chromosom 16q12. Das verantwortliche Suszeptibilitätsgen in dieser genomischen Region wurde 2001 durch mehrere unabhängige Arbeitsgruppen identifiziert. Hierbei handelt es sich um das Gen *CARD15*, das für den MDP-Rezeptor NOD2 codiert [Hampe et al., 2001; Hugot et al., 2001; Ogura et al., 2001].

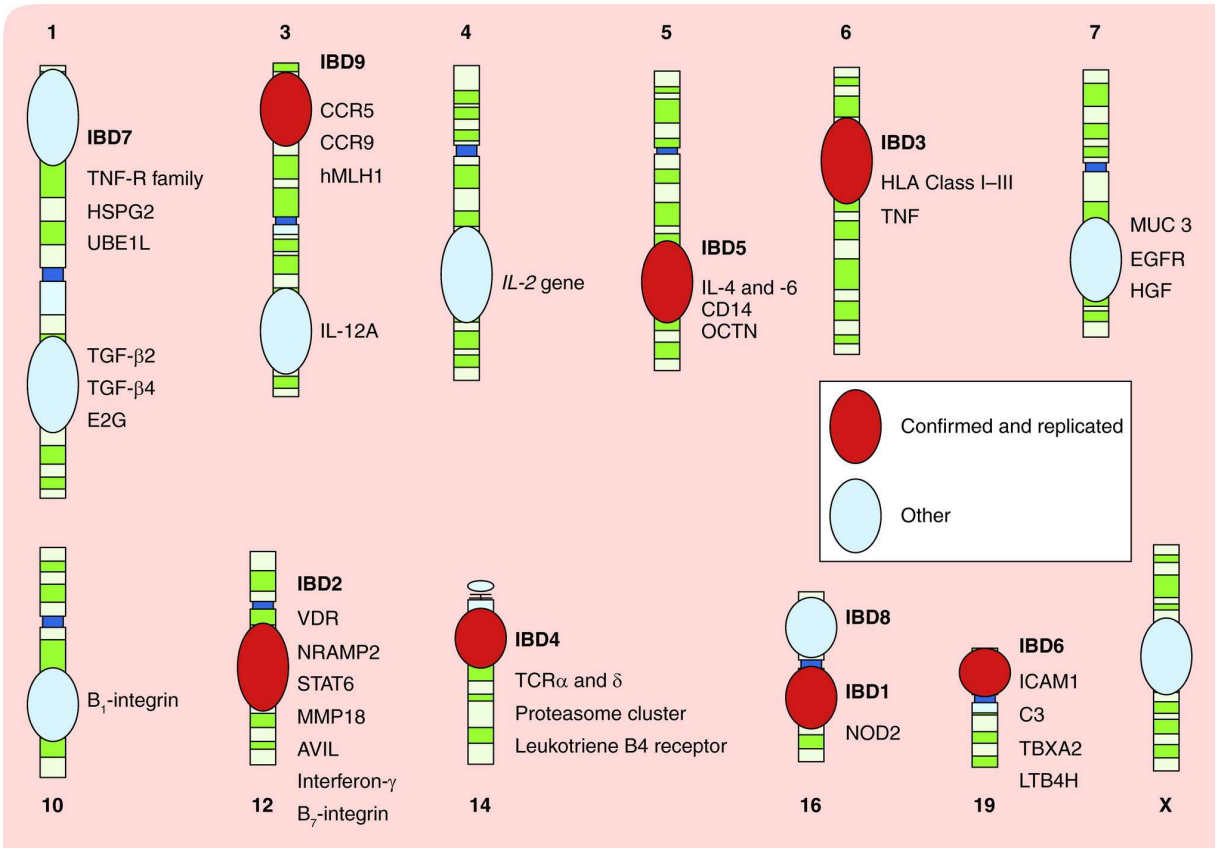

Abbildung 5. Übersicht über die mit CED assoziierten Genregionen (IBD1-9) (aus [Ahmed, 2006]).

Die drei häufigsten *CARD15*-Varianten (single nucleotide polymorphisms, SNPs) sind die Aminosäuresubstitutionen R702W in Exon 4, G908R in Exon 8 und eine Insertionsmutation 3020insC (1007fs) in Exon 11, bei der durch eine Leserasterverschiebung ein Stopcodon und dadurch bedingt ein verkürztes Protein entsteht. Diese Mutationen repräsentieren etwa 80 % aller mutierten Allele und sind bei MC-Patienten überdurchschnittlich oft zu finden [Hampe et al., 2001; Hugot et al., 2001; Lesage et al., 2002; Ogura et al., 2001]. Heterozygote Träger haben ein 2-4-fach erhöhtes Risiko, an MC zu erkranken, während Personen mit 2 Mutationen (homozygot für eine Mutation oder heterozygot für zwei verschiedene Mutationen =zusammengesetzt heterozygot) ein 20-40-fach erhöhtes Risiko tragen [Bonen und Cho, 2003]. Auch weisen Patienten mit *CARD15*-Mutationen allgemein ein früheres Erkrankungsalter, einen schwereren Verlauf mit Stenosen und Fisteln sowie eine präferentielle Entzündungslokalisation im terminalen Ileum auf [Abreu et al., 2002; Ahmad et al., 2002; Cuthbert et al., 2002; Economou et al., 2004; Lesage et al., 2002]. In einer Subgruppenanalyse zum genauen Beitrag der einzelnen *CARD15*-Mutationen zu spezifischen MC-Phänotypen konnten wir zeigen, dass homozygote Träger der 1007fs-Mutation einen signifikant früheren Erkrankungsbeginn und einen aggressiveren Phänotyp mit primär ilealem Befall sowie

häufigen Fisteln und Stenosen aufweisen [Seiderer et al., 2006]. Die anderen Mutationen R702W und G908R hingegen zeigten keine signifikanten Assoziationen mit bestimmten phänotypischen Krankheitsmerkmalen [Seiderer et al., 2006]. Interessanterweise konnte in einer kürzlich publizierten Studie ein Zusammenhang zwischen *CARD15*-Mutationen und der Entwicklung von Th17-Zellen gezeigt werden: DCs von MC-Patienten mit defizientem NOD2 waren im Gegensatz zu Wildtypzellen nicht in der Lage, über IL-23-Produktion die Entwicklung von Th17-Zellen zu stimulieren [van Beelen et al., 2007].

Neben den bereits erwähnten CED-Kopplungsregionen wurden in jüngster Zeit in genomweiten Untersuchungen neue, mit MC-assoziierte Gene beschrieben. Hierzu gehört der IL-23-Rezeptor (*IL23R*), der "organic cation transporter" (OCTN1/*SLC22A4*), die beiden in die Autophagozytose von Bakterien verwickelten Gene "autophagy-related 16-like“-Gen (*ATG16L1*) und "immunity-related p47 guanosine triphosphatases" (*IRGM*) sowie eine in der Nähe des Gens für den Prostaglandinrezeptor EP4 (*PTGER4*) gelegene Region, die dessen Expression reguliert. Die Assoziation von Polymorphismen in *IL23R*, *SLC22A4*, *ATG16L1* und *PTGER4* mit der Suszeptibilität für MC wurde von Untersuchungen unserer Arbeitsgruppe bestätigt [Glas et al., 2007a; Glas et al., 2007b; Glas et al., 2007c; Torok et al., 2005].

1.1.2.2 Intestinale Entzündung als Risikofaktor für die Entwicklung eines Kolonkarzinoms

CED-Patienten haben ein erhöhtes Risiko, ein kolorektales Karzinom (CRC) zu entwickeln. Dieses hängt jedoch eher mit der chronischen Entzündung der intestinalen Mukosa als mit einer klar definierten genetischen Prädisposition zusammen [Itzkowitz und Yio, 2004] (siehe Abbildung 6). Die Kolonmukosa von CED-Patienten zeigt einen verstärkten epithelialen „Umsatz“ und weist höhere Raten von Mitose sowie Apoptose auf, speziell in Arealen aktiver Entzündung im Vergleich zu Arealen ruhender Entzündung [Arai et al., 1999]. Obwohl ein erhöhter epithelialer Umsatz wahrscheinlich zur Kanzerogenese beiträgt, ist er als alleiniger krebsverursachender Faktor nicht ausreichend. Vielmehr trägt, im Kontext des erhöhten epithelialen Umsatzes, oxidativer Stress, der chronische Entzündungen begleitet, zur neoplastischen Transformation bei.

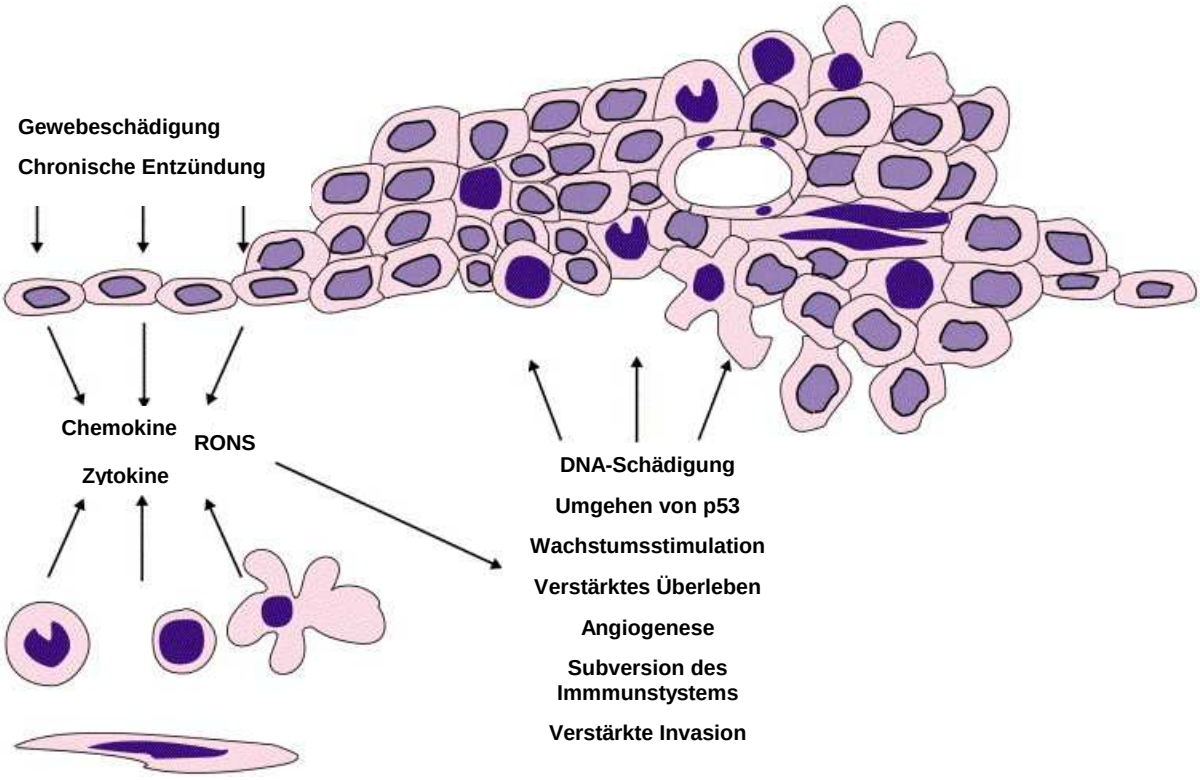

Abbildung 6. Entzündung als Risikofaktor für die Kanzerogenese. Chronische Entzündung sowie Gewebeschädigung führt zur Produktion von Zytokinen, Chemokinen sowie reaktiven Sauerstoff- und Stickstoffspezies (RONS), die zum Entstehen von malignen Erkrankungen beitragen (modifiziert nach [Balkwill und Mantovani, 2001]).

Entzündetes Gewebe von Patienten mit aktivem MC oder CU weist eine erhöhte Expression von reaktiven Sauerstoff- und Stickstoffspezies sowie ihres synthetisierenden Enzyms iNOS (inducible nitric oxide synthase) auf [Hussain et al., 2000; Hussain et al., 2003; Kimura et al., 1998]. Messungen von 8-hydroxy-2'-deoxyguanosin (8-OHdG), einem Marker für oxidative DNA-Schädigung, der durch den Angriff von Hydroxylradikalen aus der DNA-Base Desoxyguanosin entsteht, ergaben eine Erhöhung in der Mukosa sowohl von Patienten mit MC als auch mit CU [D'Inca et al., 2004; Lih-Brody et al., 1996]. Werden Schlüsselgene der Krebsentstehung, wie z. B. Tumorsuppressorgene, DNA-Mismatch-Repair-Gene oder DNA-Basenexzisionsreparatur-Gene, von reaktiven Sauerstoff- und Stickstoffspezies, die von Entzündungszellen produziert werden, angegriffen, kann dies zu Dysplasien und in Folge zur Entwicklung von Karzinomen führen. So weisen CU-Patienten eine erhöhte Mutationsrate im Tumorsuppressor-Gen p53 auf, was mit der Expression von NOS-2 korreliert [Hofseth et al., 2003; Hussain et al., 2000].

Freie Radikale können eine Vielzahl von metabolischen Prozessen beeinflussen, da sie neben DNA auch RNA, Proteine und Lipide angreifen [Hussain et al., 2003; Marnett, 2000].

Beispielsweise lässt sich in Kolonepithelzellen aus entzündeter Mukosa von MC- und CU-Patienten, jedoch nicht in nichtentzündeter Mukosa, eine Oxidation des Thiols im aktiven Zentrum des Enzyms Glycerinaldehyddehydrogenase (GAPDH) nachweisen, was in einer Hemmung der Enzymaktivität resultiert [McKenzie et al., 1996].

Außerdem fördern proinflammatorische Zytokine wie TNF-α, das bei CED-Patienten verstärkt exprimiert wird [Cobrin und Abreu, 2005], die Tumorentstehung und –progression. TNF-α kann direkte DNA-Schäden verursachen, antiapoptotische oder mitogene Signale übertragen, die Interaktion zwischen Tumor- und Stromazellen vermitteln sowie die Produktion von Matrix-Metalloproteinasen (MMPs), Zytokinen und Chemokinen stimulieren, die wiederum die Tumorentwicklung fördern. Außerdem vermag es synergistische Effekte mit Wachstumsfaktoren wie TGF-β oder EGF auszuüben [Balkwill, 2006; Szlosarek et al., 2006].

Der Transkriptionsfaktor NF-κB, der durch TNF-α aktiviert wird [Karin und Greten, 2005], stellt hierbei eine wichtige Verbindung zwischen Entzündung und damit assoziierter Kanzerogenese dar, indem er als Tumorpromotor wirkt [Pikarsky et al., 2004] und die Zellproliferation fördert bzw. Apoptose verhindert [Karin und Greten, 2005]. NF-κB ist in Tiermodellen intestinaler Entzündung sowie bei CED-Patienten verstärkt aktiv, und seine Hemmung verhindert das Entstehen einer Kolitis in Tiermodellen [Fiocchi, 1998; Neurath et al., 1998; Neurath et al., 1996; Schreiber et al., 1998].

In verschiedenen Mausmodellen, die entweder auf der Induktion von intestinaler Entzündung durch Chemikalien wie Dextransodiumsulfat (DSS) oder auf einer genetischen Prädisposition wie z. B. bei der IL-10-Knockout-Maus beruhen, lässt sich eine erhöhte Inzidenz von Kolonkarzinomen feststellen [Berg et al., 1996; Cooper et al., 2001; Cooper et al., 2000; Okayasu et al., 2002; Shattuck-Brandt et al., 2000], was die These unterstützt, dass eine chronische intestinale Entzündung ein wichtiger Risikofaktor für die Entwicklung eines CRC ist.

Es existiert eine Vielzahl von Studien am Menschen, die ein erhöhtes Risiko, an einem CRC zu erkranken, sowohl für MC- als auch für CU-Patienten nachweisen konnten. So wurde erst kürzlich in einer großen Meta-Analyse, die insgesamt 6 Studien einschloss, eine signifikant, zweifach erhöhte Inzidenz von CRC bei MC-Patienten belegt, wobei die Inzidenz für Dünndarmkarzinome mit 27.1-facher Erhöhung besonders hoch war [Jess et al., 2005].

Für CU-Patienten zeigte sich in einer großen Studie ebenfalls eine gesteigerte Inzidenz (5.7-fach) von CRC, die bei Patienten mit Pankolitis am höchsten war (14.8-fach) [Ekbom et al., 1990]. Allgemein sind bei CU-Patienten sowohl die Dauer als auch die Ausdehnung der Erkrankung wichtige Risikofaktoren für die Entwicklung eines CRC, genauso wie das

Vorhandensein einer primär sklerosierenden Cholangitis, familiäre Vorbelastung und ein frühes Diagnosealter [Croog et al., 2003; Eaden et al., 2000; Ekbom et al., 1990]. In einer anderen Studie war die Schwere der Entzündung, die durch koloskopische bzw. histologische Befunde bestimmt wurde, ein Risikofaktor für kolorektale Neoplasien [Rutter et al., 2004]. Allerdings zeigte sich in einer großen Metaanalyse von 116 Studien zwar eine Erhöhung des Karzinomrisikos bei Patienten mit CU (kumulatives Risiko 2 % nach 10 Jahren, 8 % nach 20 Jahren und 18 % nach 30 Jahren), die jedoch nicht signifikant war [Eaden et al., 2001]. Als protektive Faktoren gegen die Entwicklung eines CRC gelten die Einnahme antiinflammatorischer Medikation wie 5-Aminosalicylate (5-ASA) sowie eine Vorsorge durch Koloskopien und der Einsatz von Kolektomien [Croog et al., 2003; Eaden, 2003; Eaden et al., 2000; van Staa et al., 2005; Velayos et al., 2006].

1.1.3 Zytokine und Chemokine bei der kolorektalen Kanzerogenese und Metastasierung

Patienten mit fortgeschrittenem CRC weisen eine hohe Sterblichkeitsrate auf und überleben selten länger als fünf Jahre. Der Hauptgrund hierfür ist meist nicht der Primärtumor, sondern die entstandenen Metastasen, die früh in den abdominellen Lymphknoten und der Leber auftreten [Bird et al., 2006].

Die Metastasierung von Tumorzellen geschieht nicht zufällig, sondern ist ein hochorganisierter, Organ-selektiver Prozess. Hierzu müssen die Tumorzellen verschiedene Fähigkeiten akkumulieren. Man nimmt an, dass es im Primärtumor eine Subpopulation von Zellen mit metastatischem Potential gibt. Diese Zellen müssen sich vom Primärtumor loslösen, die Basalmembran durchbrechen und in die Lymph- bzw. Blutgefäße eindringen. Hier müssen sie dem Immunsystem entkommen und anschließend die Gefäße wieder verlassen, um sich im Zielorgan anzuheften, wo sie eine ausreichende Versorgung mit Blutgefäßen initiieren müssen, um weiter proliferieren zu können [Bird et al., 2006; Kopfstein und Christofori, 2006].

Neue Erkenntnisse deuten darauf hin, dass Chemokine und ihre Rezeptoren auf verschiedenen Stufen des Metastasierungsprozesses eine wichtige Rolle spielen. So sezernieren Tumorzellen MMPs und andere Proteasen, welche die Invasivität durch die extrazelluläre Matrix erleichtern [Vihinen und Kahari, 2002]. Chemokine sind hierbei die Schlüsselproteine, welche die Tumorzellen zur Produktion und Sekretion dieser Enzyme anregen. Zum Beispiel induziert IL-8 (CXCL8), das von Melanomzellen produziert wird, die transkriptionelle Aktivierung des Gens für MMP-2, was in einer erhöhten Invasivität durch die extrazelluläre

Matrix resultiert [Bar-Eli, 1999; Luca et al., 1997]. CXCL8-Überexpression in Androgen-unabhängigen Prostatakrebszellen aktiviert die Expression von MMP-9, was zu erhöhter lokaler Invasion von Tumoren in einem Nacktmaus-Modell führt [Inoue et al., 2000]. Das murine Chemokin MIP-2 (= humanes CXCL1) vermittelt im Mausmodell eine höhere Zellmigration und –adhäsion, außerdem fördert es die Angiogenese in von CRC stammenden hepatischen Metastasen [Kollmar et al., 2006]. In extrahepatischen Metastasen induziert es Proliferation und die Expression des Rezeptors CXCR2 [Kollmar et al., 2007]. In von Mammakarzinomen abstammenden, lungenspezifischen metastatischen Zellen findet sich eine erhöhte CXCL1-Produktion [Minn et al., 2005]. Für den Rezeptor CXCR3 konnte gezeigt werden, dass er die CRC-Metastasierung besonders in die Lymphknoten fördert [Kawada et al., 2007], d.h. Chemokinrezeptoren vermitteln auch eine Organspezifität der Metastasierung. Dies konnte in einer kürzlich veröffentlichten Studie über Mammakarzinomzellen bestätigt werden, in der die Expression verschiedener Chemokinrezeptoren mit dem Ort der Metastasierung assoziiert war: CX3CR1-Expression korrelierte mit Gehirn-Metastasen, CCR6 mit Pleura-Metastasen, CCR7 mit Haut-Metastasen, und eine CXCR4-Expression führte v. a. zu Metastasen in der Leber [Andre et al., 2006]. Hierbei korreliert der Ort der Metastasierung eindrucksvoll mit dem Expressionslevel der einzelnen Liganden in den Zielorganen.

Die wichtigste Rolle bei der Metastasierung verschiedenster Tumoren spielt CXCR4 (siehe auch Abschnitt 1.2.2). Für eine Vielzahl von verschiedenen Krebsarten wie z. B. Mamma-karzinom [Li et al., 2004b; Muller et al., 2001; Smith et al., 2004], Lungenkarzinom [Burger et al., 2003; Phillips et al., 2003], Melanom [Murakami et al., 2004; Murakami et al., 2002] Prostata- [Darash-Yahana et al., 2004] und Pankreaskarzinom [Saur et al., 2005] konnte ein Einfluss von CXCR4/CXCL12 auf das Tumorwachstum bzw. die Tumormetastasierung belegt werden (siehe Abbildung 7). Metastasen dieser Tumoren finden sich häufig in Organen mit einem hohen CXCL12-Expressionsniveau (Lunge, Leber, Knochenmark, Lymphknoten) [Zlotnik, 2006] (siehe Abbildung 7).

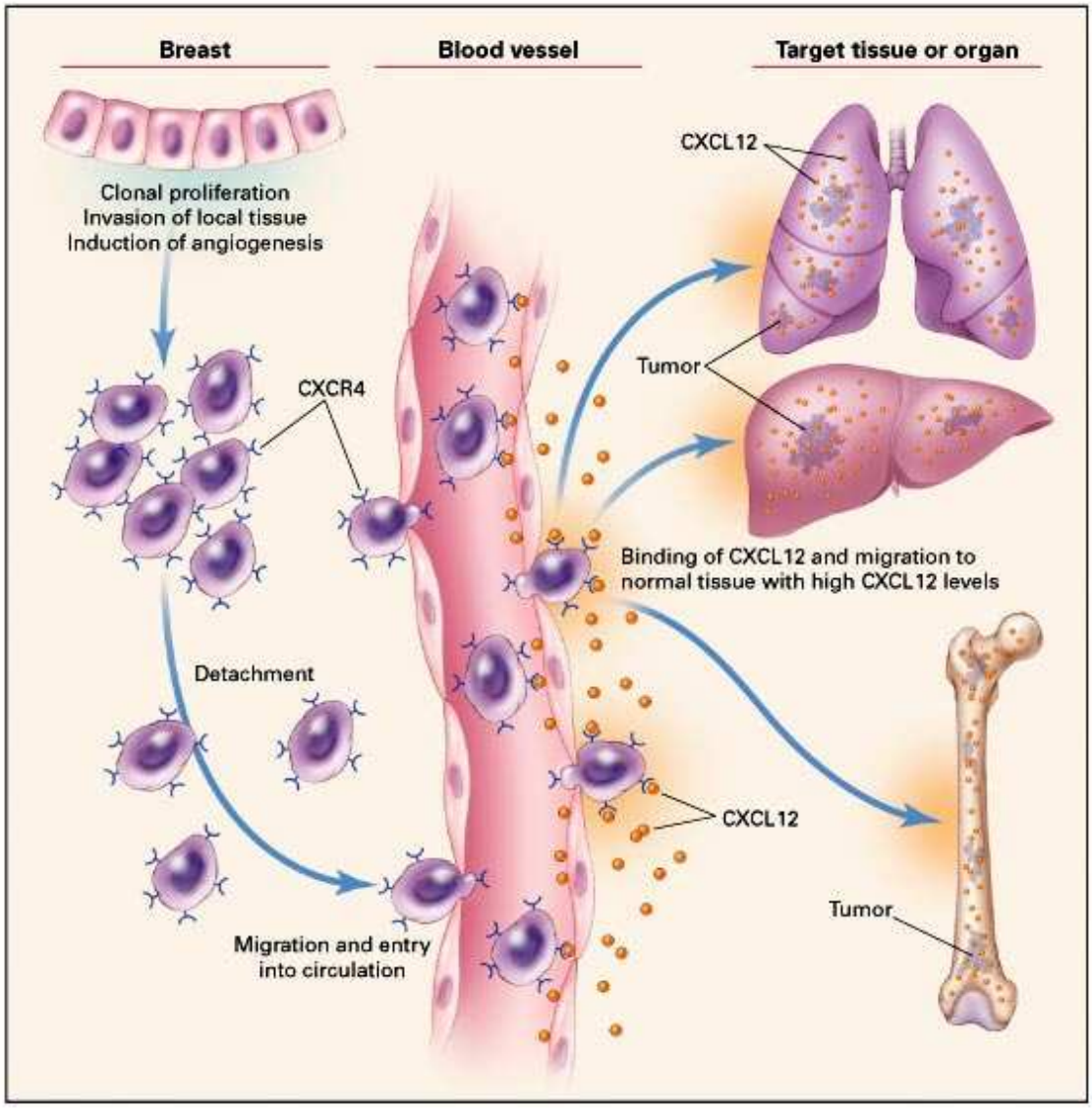

Abbildung 7. Übersicht über den Metastasierungsprozess von Mammakarzinomzellen unter dem Einfluss von CXCR4 und CXCL12 (aus [Murphy, 2001]).

Die Expressionshöhe von CXCR4 kann bei verschiedenen Tumorarten wie dem Melanom [Scala et al., 2005], Ovarkarzinom [Jiang et al., 2006], Osteosarkom [Laverdiere et al., 2005], Neuroblastom [Russell et al., 2004], Ösophaguskarzinom [Kaifi et al., 2005; Koishi et al., 2006], Mammakarzinom [Salvucci et al., 2006] oder der akuten myeloischen Leukämie [Rombouts et al., 2004] als ein prognostischer Marker angesehen werden; je höher die CXCR4-Expression, desto ungünstiger ist die Prognose bzw. desto schwerer die Erkrankung.

Als die vorliegende Arbeit begonnen wurde, gab es erste Hinweise auf eine CXCR4-Expression auch im Kolon bzw. in IEC [Dwinell et al., 1999; Heidemann et al., 2004]. Die genaue Rolle im GI-Trakt und speziell bei der kolorektalen Kanzerogenese war jedoch unklar und sollte daher im Rahmen dieser Arbeit näher charakterisiert werden.

1.2 Intestinal exprimierte Chemokinrezeptoren und ihre Liganden

1.2.1 Übersicht über das Chemokin-/Chemokinrezeptor-System

Chemokinrezeptoren sind Sieben-Helix-Transmembran-Rezeptoren, die ihre Signale über heterotrimere G-Proteine weiterleiten [Bacon et al., 2002; Murphy et al., 2000]. Bei ihren Liganden, den Chemokinen, handelt es sich um chemotaktische Zytokine, welche die gezielte transepitheliale Wanderung von Leukozyten zu Entzündungsherden oder zu Verletzungen dirigieren. Die verschiedenen Chemokine weisen eine Homologie von 20-70 % auf Aminosäureebene auf und lassen sich anhand konservierter Cystein-Strukturen in die 4 Unterfamilien C-, CXC-, CC- und CX3C-Chemokine einteilen [Fernandez und Lolis, 2002; Murphy et al., 2000; Nomenclature, 2003]. Hierbei steht "C" für Cystein und "X" für eine andere Aminosäure als Cystein. CXC- und CC-Chemokine stellen die beiden größten Untergruppen mit mehr als 15 bzw. 25 Mitgliedern dar, während bisher nur 2 verschiedene C- und nur ein einziges CX3C-Chemokin (Fractalkine) beschrieben ist [Nomenclature, 2003]. Die CXC-Chemokin-Gruppe wird in zwei weitere Subgruppen unterteilt, abhängig von der Existenz eines sogenannten ELR-Motivs (die Aminosäurefolge Glutamat [E]-Leucin [L]-Arginin [R]) in der Nähe des ersten aminoterminalen Cysteins [Belperio et al., 2000]. CXC-Chemokine mit dieser Sequenz wirken auf Neutrophile, während CXC-Chemokine ohne diese Sequenz auf Lymphozyten, z.T. auch auf dendritische Zellen wirken [Laing und Secombes, 2004; Luster, 1998]. Dagegen wirken CC-Chemokine vor allem auf Monozyten, Eosinophile und aktivierte T-Zellen und nicht auf Neutrophile und B-Zellen [Luster, 1998]. Entsprechend der Nomenklatur, die für die Chemokine eingeführt wurde, werden die Rezeptoren, abhängig von ihren Liganden, als XC-Rezeptoren (XCR), CXC-Rezeptoren (CXCR), CC-Rezeptoren (CCR) oder CX3C-Rezeptoren (CX3CR) bezeichnet [Murphy et al., 2000]. Bis zu diesem Zeitpunkt sind 10 CCRs, 7 CXCRs und jeweils ein XCR und CX3CR bekannt [Murphy, 2002] (siehe Abbildung 8).

Funktionell lassen sich Chemokine in inflammatorische und homöostatische Chemokine einteilen. Die Hauptfunktion inflammatorischer Chemokine ist die Rekrutierung von Leukozyten und anderen Immunzellen zu Entzündungsherden, während homöostatische Chemokine v. a. die Wanderung von Leukozyten in hämatopoietische Organe regulieren (sogenanntes „homing“) [Ben-Baruch, 2006]. Im Allgemeinen ist die Expression proinflammatorischer Zytokine induzierbar, während homöostatische Chemokine konstitutiv exprimiert werden [Ben-Baruch, 2006; Locati et al., 2002]. Diese funktionale Klassifizierung ist jedoch weniger strikt als die strukturelle, da es verschiedene Chemokine wie z. B. CCL20

gibt, die sowohl proinflammatorische als auch homöostatische Funktionen ausüben können [Charbonnier et al., 1999; Dieu-Nosjean et al., 2000; Izadpanah et al., 2001; Locati et al., 2002].

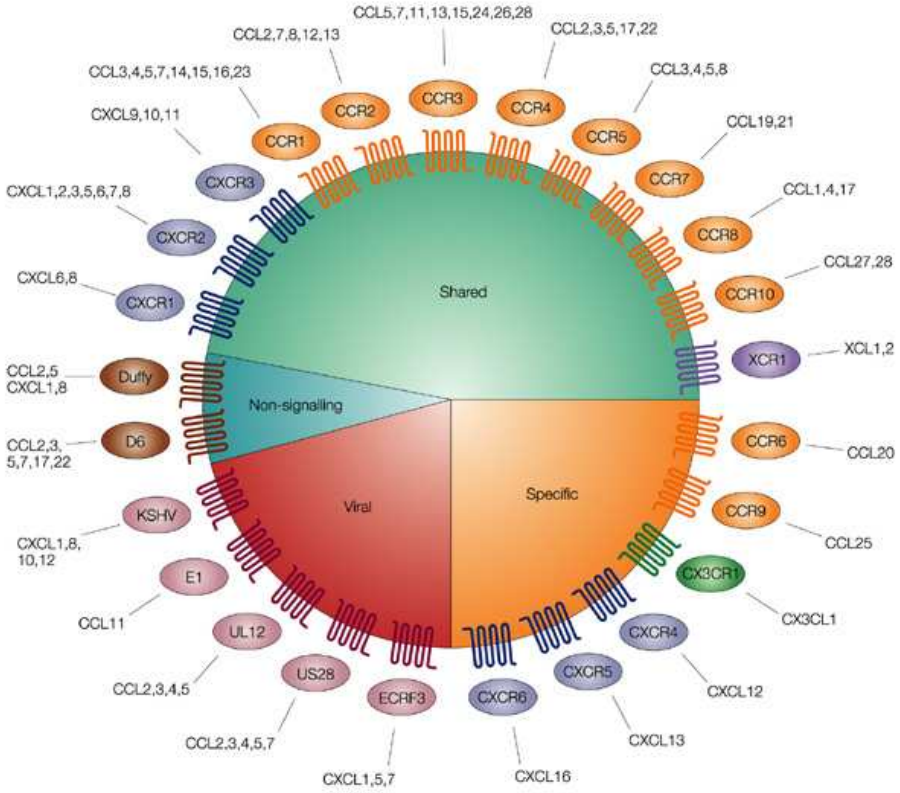

Abbildung 8: Übersicht über die Chemokinrezeptoren und ihre Liganden (aus [Balkwill, 2004]). CXCR7 (nicht dargestellt) wurde erst kürzlich als potentieller Rezeptor für CXCL12 und CXCL11 identifiziert [Balabanian et al., 2005; Burns et al., 2006].

Wurden Chemokine ursprünglich als chemotaktische Stoffe für Leukozyten entdeckt, so ist heute eine Vielzahl weiterer Funktionen bekannt. Chemokine und ihre Rezeptoren spielen eine Rolle bei der angeborenen und adaptiven Immunantwort, der Metastasierung und Vaskularisierung von Tumoren sowie bei verschiedensten entzündlichen Erkrankungen wie der rheumatoiden Arthritis, multipler Sklerose, chronisch obstruktiven Lungenerkrankungen, der Psoriasis oder bei allergischem Asthma [Ben-Baruch, 2006; Charo und Ransohoff, 2006; Luster, 1998; Rot und von Andrian, 2004]. Auch bei CED sind Chemokine wichtige Vermittler von Entzündungsreaktionen [Ajuebor und Swain, 2002; MacDermott, 1999; MacDermott et al., 1998]. Histologisch ist die intestinale Entzündung bei MC und CU durch die Anwesenheit von in die Mukosa infiltrierten Leukozyten gekennzeichnet, die durch Chemokine angelockt werden [Gerszten et al., 1999; MacDermott, 1999]. In logischer Konsequenz finden sich bei CED-Patienten auch erhöhte Expressionslevel verschiedener

Chemokine wie z. B. IL-8/CXCL8, Eotaxin/CCL11, ENA-78/CXCL5, MCP-1/CCL2, MCP-3/CCL7, RANTES/CCL5, MIP-1α/CCL3 oder IP-10/CXCL10 [Papadakis und Targan, 2000].

Das Chemokin-Ligand-Rezeptor-System ist generell von einer hohen Redundanz geprägt (siehe Abbildung 8). Viele Chemokine wie z. B. CXCL9 oder CCL16 sind in der Lage, mit hoher Affinität an verschiedene Rezeptoren zu binden, während umgekehrt verschiedene Rezeptoren wie z. B. CCR5 oder CCR3 eine Reihe von Chemokinliganden erkennen [Rot und von Andrian, 2004]. Insofern kommt es auch zu einer gewissen funktionellen Redundanz verschiedener Chemokine [Feng et al., 2006; Fu und Chen, 2004; Remick et al., 2001; Zhang et al., 2001]. Ausnahmen bilden die im Rahmen dieser Arbeit untersuchten Chemokin-/Chemokinrezeptor-Systeme CXCL12/CXCR4, CCL20/CCR6 und CXCL16/CXCR6. Bei diesen handelt es sich um eine exklusive Rezeptor-Liganden-Bindung. Die essentielle, überlebenswichtige Funktion solcher nicht-redundanter Chemokin-/Rezeptor-Paare wird am Beispiel CXCL12/CXCR4 deutlich. Knockout-Mäuse, die entweder in der Expression des Liganden CXCL12 oder des Rezeptors CXCR4 defizient sind, zeigen sehr ähnliche Symptome: Sie sterben perinatal mit schweren Defekten in der Vaskularisierung, besonders des GI-Trakts, außerdem weisen sie schwere Störungen in Hämatopoiese und Kardiogenese auf [Nagasawa et al., 1996a; Tachibana et al., 1998], was die essentiellen homöostatischen Funktionen dieses Chemokins und seines Rezeptors deutlich macht.

1.2.2 Das CXCL12/CXCR4-Ligand-Rezeptor-System

CXCL12 wurde zunächst als Pre-B-cell growth-stimulating factor (PBSF) oder stromal cell-derived factor-1 (SDF-1) bezeichnet, da es die Proliferation von Prä-B-Zellen stimuliert und konstitutiv von Stromazellen des Knochenmarks produziert wird [Nagasawa et al., 1994; Tashiro et al., 1993]. Es dient als Lockstoff u. a. für Lymphozyten und Monozyten [Bleul et al., 1996b] und wird fast ubiquitär exprimiert [Shirozu et al., 1995]. Diese Expression ist meist konstitutiv [Shirozu et al., 1995], d. h. es handelt sich bei CXCL12 um ein homöostatisches Chemokin.

CXCR4 wurde nach seiner Klonierung zunächst als HIV-Corezeptor beschrieben [Deng et al., 1996; Feng et al., 1996; Loetscher et al., 1994]. Entsprechend wirkt CXCL12 als ein Inhibitor der HIV-1-Infektion mit T-Zell-tropischen Virenstämmen [Bleul et al., 1996a; Oberlin et al., 1996]. CXCR4 wird besonders stark auf Leukozyten exprimiert [Loetscher et al., 1994], aber auch auf diversen anderen Zellen wie Endothel- und Epithelzellen [Murdoch, 2000].

Der hohe Homologie-Grad von über 90 % Aminosäure-Identität zwischen Maus und Mensch sowohl für CXCL12 [Shirozu et al., 1995] als auch für CXCR4 [Nagasawa et al., 1996b] weist auf ein hoch konserviertes Rezeptor-Liganden-System hin, das verschiedene, lebenswichtige Funktionen ausübt. Hierzu gehören die B-Lymphopoiese, die T-Zell-Rekrutierung zu Entzündungsorten sowie die T-Zell-Reifung, das „Homing“ von Stammzellen und Progenitorzellen von der Leber zum Knochenmark, die Hämatopoiese, Embryogenese und Organogenese sowie die Vaskularisierung [Kucia et al., 2005; Murdoch, 2000; Ratajczak et al., 2006]. Wie bereits unter Abschnitt 1.2.1 erwähnt, weisen CXCR4- oder CXCL12-Knockout-Mäuse Störungen in den eben erwähnten Funktionen auf und sind nicht lebensfähig [Nagasawa et al., 1996a; Tachibana et al., 1998]. Eine entscheidende Rolle spielt CXCR4 bei dem Wachstum und der Metastasierung verschiedener Tumoren (siehe Abschnitt 1.1.3). Die genaue Rolle von CXCR4 im GI-Trakt war zu Beginn dieser Arbeit unklar, weshalb deren Erforschung ein Hauptziel dieser Arbeit war.

1.2.3 Das CCL20/CCR6-Ligand-Rezeptor-System

Das Chemokin CCL20/MIP-3α wurde nach seiner Entdeckung zunächst als LARC oder Exodus bezeichnet [Hieshima et al., 1997; Hromas et al., 1997] und seine Expression wurde in Leber, Lunge sowie in Lymphozyten und Monozyten beschrieben [Hromas et al., 1997]. CCL20 wird im GI-Trakt konstitutiv von Follikel-assoziierten Epithelzellen (FAE) produziert, welche die Peyer-Plaques bedecken (siehe auch Abschnitt 1.1.1). Dadurch wird die Rekrutierung einer Untergruppe von DCs, die den Rezeptor CCR6 exprimieren, vermittelt [Cook et al., 2000; Iwasaki und Kelsall, 2000; Tanaka et al., 1999]. Außer im GI-Trakt findet sich CCL20 auch in anderen Geweben wie der Leber, Lunge oder Haut [Charbonnier et al., 1999; Hieshima et al., 1997]. Zusätzlich zur konstitutiven Produktion von CCL20 in FAE ist seine Expression auch durch proinflammatorische Zytokine oder bakterielle Infektionen induzierbar [Hromas et al., 1997; Izadpanah et al., 2001; Sierro et al., 2001; Sugita et al., 2002].

CCR6, der Rezeptor für CCL20 [Baba et al., 1997; Liao et al., 1997a], ist auf einer Vielzahl von Zellen wie Lymphozyten, Endothelzellen oder Fibroblasten zu finden. CCR6 wird auch auf einer Untergruppe von DCs in den Peyer-Plaques produziert. Folglich weisen CCR6-defiziente Mäuse eine defekte Akkumulation von DCs in der intestinalen Mukosa sowie eine defekte lokale humorale Immunantwort auf oral verabreichte Antigene auf [Cook et al., 2000]. Im Gegensatz dazu ist die systemische Immunantwort auf subkutane Antigengabe normal, was auf eine wichtige lokale Rolle von CCR6 im intestinalen Immunsystem hinweist

[Cook et al., 2000]. Kürzlich konnte gezeigt werden, dass CCR6+ DCs essentiell für die Aktivierung von Pathogen-spezifischen T-Zellen in den Peyer-Plaques sind [Salazar-Gonzalez et al., 2006]. Obwohl CCL20 der einzige bisher beschriebene Chemokinligand für CCR6 ist, können auch die antimikrobiellen Peptide β-Defensin-1 und -2, die strukturelle Ähnlichkeit zu CCL20 aufweisen [Hoover et al., 2002], an CCR6 binden [Yang et al., 1999]. Interessanterweise besitzt CCL20 umgekehrt auch antimikrobielle Aktivität [Yang et al., 2003].

Die aktuelle Datenlage deutet auch auf eine wichtige Rolle von CCL20/CCR6 bei intestinaler Entzündung hin. Während CCR6-defiziente Mäuse im DSS-Kolitis-Modell eine weniger schwere Pathologie aufweisen als Wildtyp (WT)-Mäuse, zeigt sich im Trinitro-benzensulfonsäure (TNBS)-Kolitis-Modell bei CCR6-defizienten Mäusen eine erhöhte Suszeptibilität in normalerweise resistenten Mausstämmen [Varona et al., 2003]. Außerdem findet sich im letzteren Modell eine erhöhte CCL20-Produktion im Kolon und eine entsprechend erhöhte Anzahl von CCR6+ T-Zellen in der Lamina propria [Katchar et al., 2007]. Eine Behandlung mit CCL20-neutralisierenden Antikörpern bewirkt hierbei eine Verbesserung der TNBS-induzierten Kolitis [Katchar et al., 2007]. Eine neue Studie beschreibt eine Expression von CCR6 auf Th17-Zellen [Acosta-Rodriguez et al., 2007], wichtigen proinflammatorischen T-Zellen, die mit der Pathogenese von MC in Verbindung gebracht werden (siehe auch Abschnitt 1.1.2.1). Weitere wichtige Funktion üben CCR6/CCL20 bei der Metastasierung verschiedener Tumoren wie Mammakarzinom oder dem CRC aus, wobei Tumorzellen bevorzugt in CCL20-exprimierende Gewebe wie die Leber metastasieren [Andre et al., 2006; Dellacasagrande et al., 2003; Ghadjar et al., 2006; Rubie et al., 2006b].

1.2.4 Das CXCL16/CXCR6-Ligand-Rezeptor-System

CXCL16 kommt sowohl als lösliches wie auch als zellgebundenes Chemokin mit einer Transmembran- und einer intrazellulären Domäne vor [Matloubian et al., 2000; Wilbanks et al., 2001]. Es ist auf der Oberfläche von APCs wie B-Zellen, Monozyten/Makrophagen und DCs zu finden, außerdem auf Endothelzellen und glatten Muskelzellen [Hofnagel et al., 2002; Matloubian et al., 2000; Wilbanks et al., 2001]. Eine Expression von CXCL16 im Kolon wurde ebenfalls beschrieben [Hase et al., 2006]. In membranständiger Form vermittelt es die Phagozytose von Gram-positiven und Gram-negativen Bakterien [Shimaoka et al., 2003] und trägt zur Bindung, Aufnahme und stimulatorischen Aktivität bestimmter bakterieller CpG-Motive bei [Gursel et al., 2006]. Außerdem dient es als Rezeptor für die Aufnahme von

oxidiertem low density lipoprotein (OxLDL) und wurde daher auch als "scavenger receptor that binds phosphatidylserine and oxidized lipoprotein" (SR-PSOX) bezeichnet [Shimaoka et al., 2000]. Membrangebundenes CXCL16 wird durch proteolytische Spaltung zum löslichen Chemokin [Abel et al., 2004; Gough et al., 2004], das chemotaktisch auf CXCR6-exprimierende Zellen, speziell Th1- sowie zytotoxische T-Zellen wirkt [Kim et al., 2001]. Die CXCL16-Expression wird durch proinflammatorische Stimuli wie IFN-γ induziert [Abel et al., 2004], und man findet eine erhöhte Expression in rheumatoiden Gelenken [Nanki et al., 2005; Ruth et al., 2006; van der Voort et al., 2005]. CXCL16-defiziente Mäuse zeigen keine Entwicklungs- oder morphologischen Abnormalitäten, jedoch wird wesentlich weniger OxLDL von den Zellen aufgenommen [Aslanian und Charo, 2006].

CXCR6, der Rezeptor für CXCL16, wurde 1997 von drei unabhängigen Arbeitsgruppen zunächst als HIV-Corezeptor identifiziert und als "seven transmembrane domain receptors from lymphocytes clone 33" (STRL33), Bonzo oder "T-lymphocyte-expressed seven-transmembrane domain receptor" (TYMSTR) bezeichnet [Deng et al., 1997; Liao et al., 1997b; Loetscher et al., 1997]. CXCR6 wird auf aktivierten T-Zellen [Kim et al., 2001; Wilbanks et al., 2001], speziell auf Th1-Zellen exprimiert [Kim et al., 2001; Unutmaz et al., 2000]. In chronisch entzündeten Geweben wie z. B. in rheumatischen Gelenken oder entzündeter Leber findet sich (aufgrund erhöhter CXCL16-Produktion) auch eine Anreicherung von CXCR6+ T-Zellen [Kim et al., 2001; Nanki et al., 2005; Ruth et al., 2006]. Eine CXCR6-Expression im Dünndarm und Kolon wurde ebenfalls beschrieben [Deng et al., 1997; Hase et al., 2006; Wagsater und Dimberg, 2004], die genauen Funktionen dieses Chemokinrezeptors und seines Liganden im GI-Trakt waren jedoch nicht bekannt.

1.3 Intestinal exprimierte Zytokinrezeptoren und ihre Liganden

1.3.1 Typ I-Zytokinrezeptoren: IL-31 und sein Rezeptorkomplex IL-31R/OSMR

Typ I-Zytokinrezeptoren werden auch als Hämopoietin-Rezeptoren bezeichnet. Sie besitzen ein konserviertes Aminosäuremotiv (WSXWS) in ihrem extrazellulären Anteil. Die Rezeptoren bestehen aus zwei unterschiedlichen Untereinheiten, und oft werden die Rezeptorketten, die für die Signalübertragung verantwortlich sind, von verschiedenen Zytokinen genutzt.

Auch der IL-6-Rezeptor gehört zur Gruppe der Typ I-Zytokinrezeptoren und besteht aus den zwei Untereinheiten IL-6R und gp130 [Taga et al., 1989; Yamasaki et al., 1988] (siehe Abbildung 9). Bei der Pathogenese von CED spielt IL-6 eine wichtige Rolle, indem es

antiapoptotische Signale an die Mukosa infiltrierende T-Zellen übermittelt und außerdem bei der Entwicklung von Th17-Zellen beteiligt ist [Mudter und Neurath, 2007], einer Untergruppe von inflammatorischen T-Zellen, die durch eine IL-17-Produktion gekennzeichnet sind und für ihre Entwicklung neben IL-6 auch TGF-β und IL-23 benötigen [Harrington et al., 2005; Mangan et al., 2006] (siehe auch Abschnitt 1.1.2.1).

Allgemein sind IL-6-ähnliche Zytokine in die Regulation der Akute-Phase-Antwort auf Gewebeverletzungen und Infektionen involviert [Heinrich et al., 2003]. Die Dysregulation des IL-6-Typ-Zytokin-Signalweges trägt zum Entstehen und Fortschreiten verschiedener Krankheiten wie rheumatoider Arthritis, Osteoporose, multipler Sklerose und verschiedener Krebsarten wie multiplen Myelomen oder Prostatakrebs bei [Heinrich et al., 2003].

Die anderen Zytokine der IL-6-Familie, deren funktionale Rezeptorkomplexe (mit einer Ausnahme) alle gp130 als eine Untereinheit enthalten, sind in Abbildung 9 dargestellt.

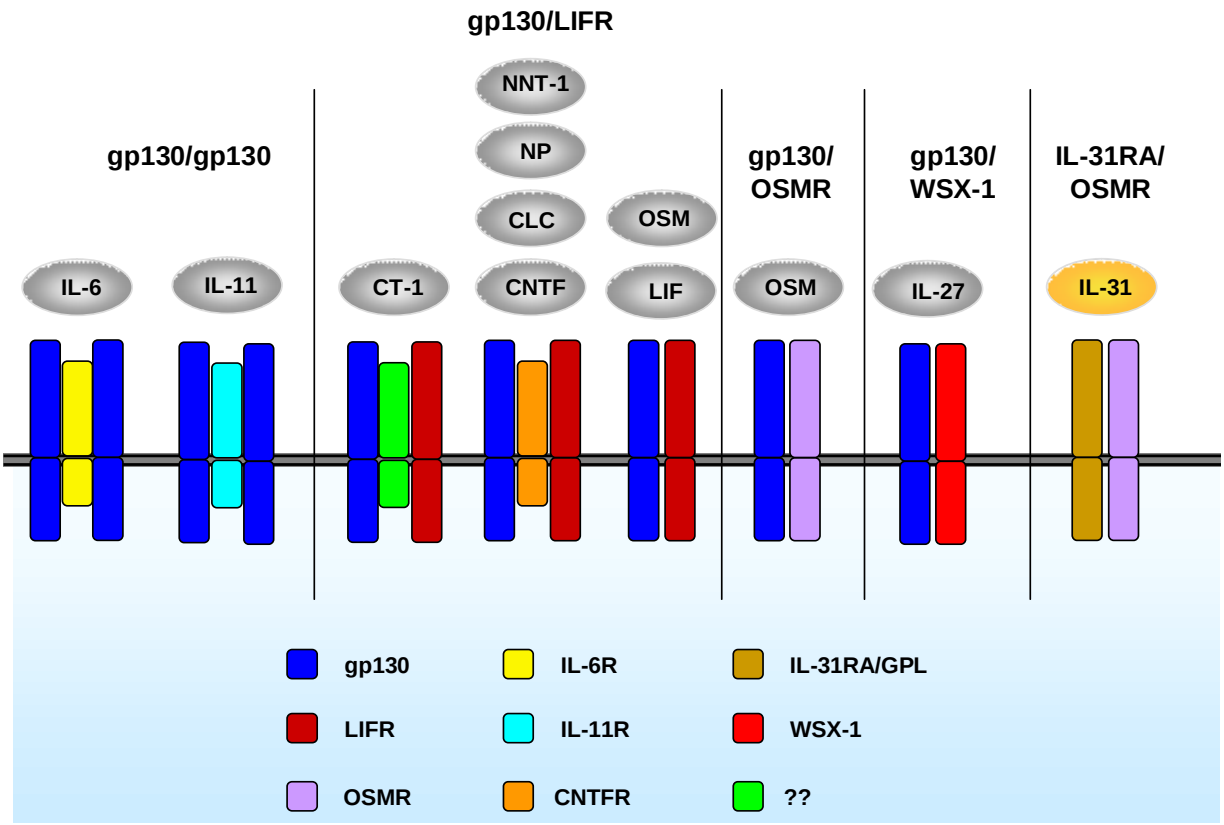

Abbildung 9: Schematische Übersicht über die IL-6-verwandten Zytokine und ihre Rezeptoren.

Vor kurzer Zeit wurde ein dem gp130 ähnlicher Rezeptor entdeckt, der als GPL (gp130-like) bezeichnet wird [Diveu et al., 2003]. Er ist identisch zu IL-31RA und stellt – neben OSMR – die zweite Rezeptor-Untereinheit für das neu entdeckte IL-6-verwandte Zytokin IL-31 dar [Dillon et al., 2004]. IL-31RA kommt in mindestens 4 verschiedenen Isoformen vor, wobei

die kurze Isoform als ein dominant negativer Repressor wirkt [Dillon et al., 2004; Diveu et al., 2004; Dreuw et al., 2004]. Die Signalübertragung der IL-6-ähnlichen Zytokine erfolgt über die Aktivierung von "Januskinasen / signal transducer and activator of transcription" (JAK/STAT)- sowie "mitogen activated protein kinase " (MAPK)-Kaskaden [Heinrich et al., 2003]. IL-31 ist, soweit bisher bekannt, der einzige Ligand für die IL-31RA-Untereinheit [Dillon et al., 2004; Dreuw et al., 2004], während OSMR auch Oncostatin M (OSM) bindet [Heinrich et al., 2003]. IL-31R-defiziente Mäuse weisen eine erhöhte T-Zell-Proliferation und Expression von Th2-Zytokinen auf, während die Th1-Antwort normal ist [Perrigoue et al., 2007]. Außerdem ist die absolute Anzahl von immaturen hämatopoietischen Progenitor-Zellen im Knochenmark und der Milz vermindert [Broxmeyer et al., 2007], was für eine wichtige Rolle von IL-31 bei der T-Zell-vermittelten Immunantwort spricht.

Die Funktionen von IL-31 wurden bisher primär im Zusammenhang mit atopischer Dermatitis untersucht. Transgene IL-31-Überexpression in Mäusen verursacht eine schwere Pruritis, Alopezie und Hautläsionen [Dillon et al., 2004]; ferner ist in Mausmodellen der atopischen Dermatitis die IL-31-Expression mit dem Kratzverhalten der Mäuse assoziiert [Takaoka et al., 2005]. Ebenso findet sich bei Patienten mit atopischer Dermatitis eine höhere IL-31-Expression bei pruritischer Dermatitis im Vergleich zu nicht-pruritischer Hautentzündung [Sonkoly et al., 2006]. Außerdem ist die IL-31-Expression mit der Expression der Th2-Zytokine IL-4 und IL-13 assoziiert [Neis et al., 2006]. In Lungenepithelzellen bewirkt IL-31 eine Hemmung der Zellproliferation durch eine veränderte Expression von Zellzyklusproteinen [Chattopadhyay et al., 2007].

Im Gegensatz zu obigen Ergebnissen existierten zur Rolle von IL-31 in IEC und bei intestinaler Entzündung bisher keine Daten, weshalb die Analyse dieser Funktionen, ausgehend von der wichtigen Rolle von IL-6 bei der Pathogenese von CED sowie der Verwandtschaft von IL-6 und IL-31, ein Teil der vorliegenden Arbeit war.

1.3.2 Typ II-Zytokinrezeptoren und ihre Liganden: IL-10-ähnliche Zytokine

Die Typ II-Zytokinrezeptoren ähneln in ihrer Struktur den Typ I-Zytokinrezeptoren mit der Ausnahme, dass ihnen das für letztere charakteristische Aminosäuremotiv WSXWS fehlt. Die Rezeptoren setzen sich (bis auf eine Ausnahme) aus zwei verschiedenen Untereinheiten zusammen [Langer et al., 2004] (siehe Abbildung 10).

Die Untereinheit mit der höheren Ligandenaffinität wird als R1 bezeichnet. Sie vermittelt auch die Spezifität der Signalübertragung. Sie hat eine größere intrazelluläre Domäne als die Untereinheit R2, die eine geringere Bindungsaffinität für den Liganden aufweist. Ausnahmen

hierzu bilden der IFN-α-Rezeptor, bei dem es sich genau umgekehrt verhält, und der Tissue Factor (TF)-Rezeptor, der aus nur einer Untereinheit besteht.

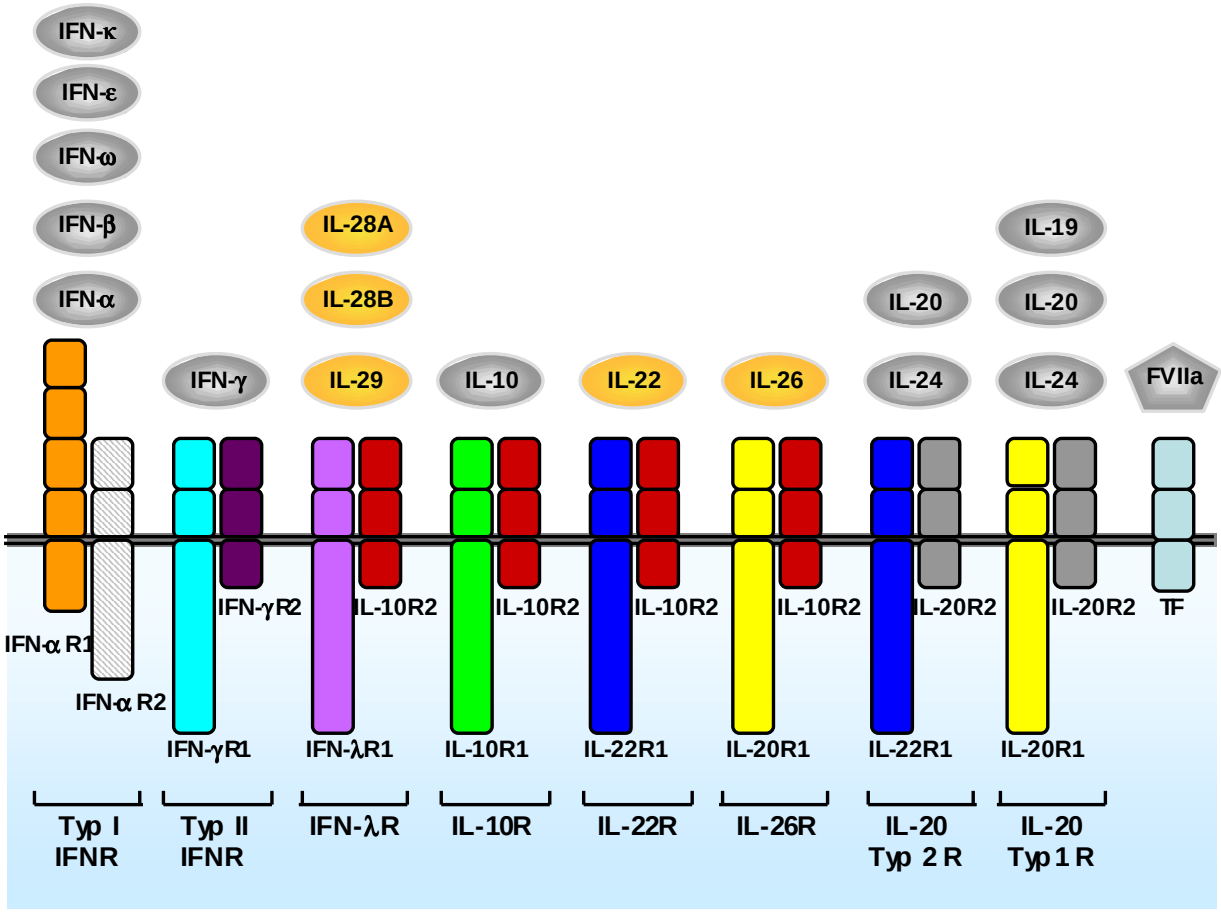

Abbildung 10: Schematische Übersicht über Klasse-II-Zytokinrezeptoren und ihre Liganden. Ein Charakteristikum dieser Familie ist, dass eine Rezeptorkette in verschiedene Rezeptorkomplexe involviert ist (z. B. IL-10R2), dass ein Ligand verschiedene Rezeptorkomplexe binden kann (z. B. IL-20), und dass verschiedene Liganden an den selben Rezeptorkomplex binden können (z. B. IL-19, IL-20, IL-24).

Zu den Liganden dieser Rezeptorfamilie gehören neben Typ I- und Typ II-Interferonen sowie dem Tissue Factor auch eine Gruppe von Zytokinen, die aufgrund ihrer Homologie zu IL-10 zu einer neuen Familie der IL-10-ähnlichen Zytokine zusammengefasst wurden. Diese besteht neben IL-10 aus den Zytokinen IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B und IL-29 [Conti et al., 2003; Fickenscher et al., 2002]. Letztere drei sind auch als λ-Interferone bekannt [Kotenko et al., 2003; Sheppard et al., 2003]. Während die Homologie der IL-10-Familie zu IL-10 auf Aminosäureebene nur mäßig hoch ist (zwischen 15 und 25 % Identität), so ist auf struktureller Ebene ein gemeinsames Muster basierend auf 6 α-Helices zu erkennen [Renauld, 2003].

Im Genom sind die Genorte dieser Zytokine in 3 verschiedenen Clustern angeordnet. Die Gene für IL-22 und IL-26 liegen zusammen mit IFN-γ auf Chromosom 12q15 [Kotenko,

2002]. IL-10, IL-19, IL-20 und IL-24 liegen auf Chromosom 1q32, während die λ-Interferone auf 19q13 lokalisiert sind [Kotenko, 2002]. Die analogen strukturellen Merkmale und der genomische Kontext lassen vermuten, dass die Gruppe der IL-10-ähnlichen Zytokine einen gemeinsamen phylogenetischen Ursprung hat [Renauld, 2003]. Die im Rahmen dieser Arbeit näher untersuchten Zytokine IL-22 und IL-26 gehören zusammen mit den λ-Interferonen zu einer Untergruppe der IL-10-ähnlichen Zytokine, die wie IL-10 die IL-10R2-Rezeptor-untereinheit als einen Bestandteil ihres Rezeptorkomplexes nutzt [Donnelly et al., 2004] (siehe Abbildung 10).

IL-10 selbst wurde erstmals als ein Faktor beschrieben, der die Aktivierung von Th1-Zellen und insbesondere deren Zytokinsynthese hemmt (cytokine sythesis inhibitory factor, CSIF) und von murinen CD4+ Th2-Zellen sezerniert wird [Fiorentino et al., 1989]. Die immunregulatorischen Effekte von IL-10 auf T-Zellen werden über verschiedene Mechanismen vermittelt. Zum einen kommt es auf APCs zu einer verringerten Expression von kostimulatorischen Oberflächenmolekülen wie MHC-Klasse II [de Waal Malefyt et al., 1991b] oder B7 [Ding et al., 1993]. Zusätzlich hemmt IL-10 in Makrophagen die Synthese einer Reihe von proinflammatorischen, T-Zell-aktivierenden Zytokinen wie IL-1β, IL-6, IL-12, IFN-α oder TNF-α [de Waal Malefyt et al., 1991a; de Waal Malefyt et al., 1991b; Fiorentino et al., 1991]. IL-10 beeinflusst auch die Produktion proinflammatorischer Zytokine in IEC nach Bakterienkontakt. Es hemmt in diesen Zellen die persistente Induktion von proinflammatorischen Zytokinen sowie die andauernde NF-κB-Aktivierung [Ruiz et al., 2005]. Außerdem induziert es über TGF-β-abhängige Signalübertragung die Degradation von TLR2 [Ruiz et al., 2005; Ruiz et al., 2006]. Obwohl die IL-10-vermittelten Effekte auf T-Zellen vor allem indirekter Natur sind, ist auch ein direkter Einfluss auf T-Zellen möglich, was z. B. zu einer verminderten T-Zell-Proliferation aufgrund einer Hemmung der IL-2-Produktion führt [de Waal Malefyt et al., 1993]. Die Hauptfunktion von IL-10 ist somit die Unterbindung einer überschießenden Immunreaktion. Diese wichtige Rolle wird in IL-10-defizienten Mäusen deutlich, die eine spontane Kolitis entwickeln und Merkmale aufweisen, die der MC-Erkrankung ähnlich sind wie z. B. transmurale Läsionen oder eine erhöhte Th1-Immunaktivität [Berg et al., 1996; Davidson et al., 1996; Kuhn et al., 1993]. Die Entwicklung der Kolitis ist hierbei abhängig von der Anwesenheit von Bakterien, da keimfrei gehaltene Mäuse nicht erkranken [Sellon et al., 1998], was die wichtige Rolle von Mikroorganismen bei der Pathogenese von CED unterstreicht. Aufgrund seiner immunregulatorischen Eigenschaften wurde IL-10 auch als potentieller Kandidat für die Behandlung von CED-

Patienten getestet, konnte sich aufgrund nur geringer Effekte aber nicht als Standardtherapie durchsetzen [Fedorak et al., 2000; Schreiber et al., 2000; van Deventer et al., 1997].

IL-10 wird von einer Vielzahl von Zellen hämatopoietischen und nicht-hämatopoietischen Ursprungs produziert, und seine Expression ist auf sowohl auf Transkriptionsebene als auch auf Ebene der mRNA-Stabilität reguliert [Moore et al., 2001]. Der funktionale IL-10 Rezeptorkomplex besteht aus der ligandenbindenden Untereinheit IL-10R1 und der akzessorischen Kette IL-10R2 [Kotenko et al., 1997; Liu et al., 1994] (siehe Abbildung 10). Während die IL-10R2 Untereinheit ubiquitär exprimiert ist [Kotenko et al., 1997], findet man die IL-10R1 Untereinheit vor allem auf hämatopoietischen Zellen [Ho et al., 1993; Liu et al., 1994]. Die Signaltransduktion der IL-10-Zytokinfamilie erfolgt über die Aktivierung von STAT-Proteinen, wobei IL-10, IL-22 und IL-26 vor allem STAT3 aktivieren, während λ-Interferone hauptsächlich STAT1 und STAT2 zur Signalweiterleitung nutzen [Donnelly et al., 2004].

Auch wenn IL-22 und IL-26 aufgrund struktureller Homologien mit IL-10 zu einer Gruppe zusammengefasst werden können, zeigen sich funktionell große Unterschiede.

1.3.2.1 IL-22 und sein Rezeptorkomplex IL-22R1/IL-10R2

IL-22 wurde erstmals als ein differentiell exprimiertes Gen in IL-9-stimulierten murinen T-Zellen beschrieben und als "IL-10-related T cell-derived inducible factor" (IL-TIF) bezeichnet [Dumoutier et al., 2000a]. Ein humanes Homolog, das auf Aminosäureebene zu 80,8 % identisch ist [Wolk und Sabat, 2006], wurde kurz darauf entdeckt und IL-22 genannt [Dumoutier et al., 2000b; Xie et al., 2000]. Die Aminosäureidentität von IL-22 zu IL-10 beträgt 22,8 % [Wolk und Sabat, 2006]. IL-22 ist ein T-Zell-spezifisches Zytokin, das von aktivierten T-Zellen, besonders von Th1-Zellen exprimiert wird [Gurney, 2004; Wolk et al., 2002]. Neue Daten weisen darauf hin, dass die Hauptquelle von IL-22 eine spezielle Gruppe von $CD4^+$-T-Zellen, die Th17-Zellen, sind [Liang et al., 2006] (siehe auch Abschnitt 1.1.2.1). IL-22 hat, im Gegensatz zu IL-10, das eine überschießende Immunantwort unterdrückt, proinflammatorische Funktionen. So wird z. B. in Keratinozyten die Transkription proinflammatorischer Gene (MMP-3, PDGFA, CXCL5) induziert [Boniface et al., 2005]. In subepithelialen Myofibroblasten zeigt sich ebenfalls eine Hochregulation proinflammatorischer Zytokine wie IL-6, IL-8 und leukemia inhibitory factor (LIF), außerdem eine Induktion von Chemokinen (CCL7, CXCL1, CXCL2, CXCL3, CXCL6) und anderen proinflammatorischen Mediatoren wie den MMPs [Andoh et al., 2005]. IL-22 spielt auch eine Rolle bei der Aktivierung des angeborenen, unspezifischen Immunsystems. Es stimuliert die

Transkription von β-Defensinen in der Haut und wird besonders stark in der Haut von Patienten mit T-Zell-vermittelten Dermatosen exprimiert [Wolk et al., 2004]. IL-22 leitet seine Signale v. a. über eine Aktivierung von STAT3 weiter [Lejeune et al., 2002; Xie et al., 2000].

Der IL-22-Rezeptor besteht aus den zwei Untereinheiten IL-22R1 und IL-10R2 [Kotenko et al., 2001a; Xie et al., 2000] (siehe Abbildung 10). Die IL-22R1-Untereinheit wird nicht auf Immunzellen exprimiert, dafür aber in Geweben wie der Haut oder dem Verdauungs- und Atemwegstrakt [Wolk et al., 2004]. Außerdem existiert ein lösliches, dem IL-22R1 ähnliches Protein (IL-22 binding protein, IL-22BP), dem jedoch die Transmembran- und die zytoplasmatische Domäne des Rezeptors fehlen. Es bindet IL-22 mit hoher Affinität und Spezifität und wirkt dadurch als endogener Zytokinantagonist, indem es z. B. die IL-22 vermittelte STAT-Aktivierung inhibiert [Dumoutier et al., 2001; Gruenberg et al., 2001; Kotenko et al., 2001b; Xu et al., 2001]. IL-22BP wird in einer Reihe von Geweben, u. a. auch im Kolon exprimiert [Dumoutier et al., 2001].

1.3.2.2 IL-26 und sein Rezeptorkomplex IL-20R1/IL-10R2

IL-26 wurde ursprünglich durch subtraktive Hybridisierung als eines der wenigen Gene entdeckt, das in Herpesvirus Saimiri (HVS)-infizierten T-Zellen überexprimiert wird [Knappe et al., 2000]. IL-26 weist 24,7 % Aminosäureidentität und 47 % Aminosäureähnlichkeit zu IL-10 auf [Fickenscher und Pirzer, 2004]. Trotz intensiver Suche konnte bisher kein murines Homolog zu IL-26 identifiziert werden, obwohl die benachbarten Chromosomenabschnitte, welche die Gene für IFN-γ und IL-22 beinhalten, stark konserviert sind [Fickenscher und Pirzer, 2004]. Es ist daher anzunehmen, dass ein IL-26-Gen in der Maus fehlt.

IL-26 wird auf basalem Level von aktivierten Monozyten und T-Zellen gebildet [Nagalakshmi et al., 2004a], speziell in aktivierten T-Zellen vom Typ Th1-Gedächtniszellen, zu einem gewissen Maße auch in NK-Zellen, jedoch nicht in Th2- oder B-Zellen [Wolk et al., 2002]. Neueste Daten zeigen eine IL-26-Expression auch in Th17-Zellen nach IL-23-Stimulation [Wilson et al., 2007]. Außerdem konnte es in frischen peripheren Blutzellen detektiert werden [Knappe et al., 2000].

Der IL-26 Rezeptorkomplex besteht aus den zwei Untereinheiten IL-20R1 und IL-10R2 [Hor et al., 2004; Sheikh et al., 2004] (siehe Abbildung 10). Im Gegensatz zum ubiquitär exprimierten IL-10R2 [Kotenko et al., 1997] wurde die spezifische Rezeptoruntereinheit IL-20R1 bisher nur auf nicht-hämatopoietischen Geweben wie Kolon, Leber, Pankreas und Haut gefunden [Hor et al., 2004; Nagalakshmi et al., 2004a; Wolk et al., 2002]. Die Bindung

von IL-26 an seinen Rezeptor resultiert in einer Aktivierung von STAT-Transkriptionsfaktoren [Hor et al., 2004]. Die Tatsachen, dass IL-26 bei Virusinfektion sowie nach anti-CD3-Stimulation von Th1-Zellen bzw. nach IL-23-Stimulation von Th17-Zellen exprimiert wird, weisen darauf hin, dass dieses Zytokin eine wichtige Rolle im adaptiven Immunsystem spielen könnte. Über die genauen Funktionen von IL-26 sind bisher keine umfassenden Untersuchungen durchgeführt worden, was somit ein Ziel der vorliegenden Arbeit war.

2. Zielsetzung

Ziel dieser Arbeit war eine umfassende Charakterisierung der Signaltransduktion und der biologischen Funktionen, die von intestinal exprimierten Rezeptor-Liganden-Systemen vermittelt werden. Außerdem sollte ihre Expression unter Entzündungsbedingungen *in vitro* und *in vivo* sowohl in Mausmodellen intestinaler Entzündung als auch bei Patienten mit CED untersucht werden. Es wurden Chemokinrezeptoren, welche die beiden größten Chemokinrezeptorfamilien (CXCR, CCR) repräsentieren sowie je ein Prototyp eines Typ I-und Typ II-Zytokinrezeptors untersucht. Hierbei waren folgende Gründe für deren Auswahl entscheidend:

- **Chemokinrezeptoren und ihre Liganden: CXCR4/CXCL12, CCR6/CCL20 und CXCR6/CXCL16**

  Die ausgewählten Rezeptoren stammen aus den 2 größten Chemokinrezeptorgruppen der CXC- sowie der CC-Rezeptorfamilie. Während CXCL12 ein typischer Repräsentant eines homöostatischen, konstitutiv exprimierten Chemokins ist, können CCL20 und CXCL16 sowohl homöostatisch wirken als auch durch proinflammatorische Stimuli induzierbar sein. Präliminäre Daten deuten auf wichtige Funktionen dieser Chemokine und ihrer Rezeptoren im GI-Trakt hin: Eine CXCR4- bzw. CXCL12-Defizienz führt u. a. zu einer gestörten Vaskularisierung des GI-Traktes. Eine CCR6-Defizienz hingegen resultiert in einer gestörten mukosalen Immunregulation im GI-Trakt. CCL20 wird u. a. von proinflammatorischen Th17-Zellen produziert, wichtigen Mediatoren intestinaler Entzündung. CXCL16 spielt eine wichtige Rolle bei der Phagozytose von Bakterien und der CpG-induzierten Immunantwort. Bei Patienten mit rheumatoider Arthritis, neben CED eine weitere entzündliche Autoimmunerkrankung, findet man erhöhte CXCL16-Expressionsspiegel.

- **Typ I-Zytokinrezeptor: IL-31R und sein Ligand IL-31**

  Ausschlaggebend für die Auswahl von IL-31 war dessen Verwandtschaft zu IL-6 und IL-23, die wichtige proinflammatorische Rollen bei der Pathogenese von CED und anderen entzündlichen Erkrankungen wie der rheumatoiden Arthritis spielen. Außerdem sind IL-6 und IL-23 Schlüsselzytokine bei der Entwicklung von Th17-Effektorzellen. Zu Funktionen von IL-31/IL-31R bei CED waren bisher keinerlei Daten bekannt.

- **Typ II-Zytokinrezeptoren und ihre Liganden: IL-22R/IL-22 und IL-26R/IL-26**
  IL-22 und IL-26 sind verwandt mit IL-10, das als bedeutendes anti-inflammatorisches Zytokin eine überschießende Entzündungsreaktion des Immunsystems, wie sie bei CED-Patienten auftritt, verhindert. Beide Zytokine werden von inflammatorischen T-Zellen, darunter Th17-Zellen, produziert. Die IL-10-Rezeptorunterheit IL-10R2 ist ein gemeinsamer Bestandteil der Rezeptorkomplexe dieser Zytokine.

Die Auswahl aller Rezeptoren beruhte außerdem auf der Tatsache, dass diese nur einen spezifischen Liganden besitzen, und umgekehrt der Ligand nur diesen einen einzigen Rezeptor bindet. In solchen Systemen lassen sich Ligand-Rezeptor-Wechselwirkungen einfacher und spezifischer charakterisieren als in komplexen Systemen mit mehreren Liganden/Rezeptoren.

3. Material

3.1 Geräte

Name	Bezugsquelle
ABI Prism 7700 Sequence Detection System	Applied Biosystems, Darmstadt
Autoklaven	Hirayama, Tokio, Japan
Brutschrank 37°C	Heraeus, München
DNA-Sequenziersystem ABI Prism 3100	Applied Biosystems, Darmstadt
FACS Calibur	BD Biosciences, Heidelberg
Feinwaage PL200	Mettler-Toledo, Giessen
Fluoreszenzmikroskop Zeiss Axiovert 135 TV	Carl Zeiss, Jena
Flüssigstickstofftank	Cryoson, Schöllkrippen
Gelelektrophoresekammer Sub Cell GT	Bio-Rad, München
Geltrockner	Bio-Rad, München
Mikroplattenleser	Tecan, GeniosPlus, Crailsheim
Mikroskop Olympus IX50	Olympus, Hamburg
Neubauer Zählkammer	Brand, Wertheim
PCR-Gerät PTC200	Bio-Rad, München
pH-Meter pH 530	WTW, Weilheim
Photometer UltrospecIII	Pharmacia, Freiburg
Pipetten	Eppendorf, Hamburg
Röntgen-Entwickler Agfa Curix 60	Agfa, Köln
Rotorgene RG-3000 Cycler	Corbett Research, Sydney, Australien
Schüttler	Stuart Scientific, Redhill, Großbritannien
Sterilbank	Heraeus, München
Stromgeber PowerPac Basic	Bio-Rad, München
Tischzentrifuge 5415R	Eppendorf, Hamburg
Tischzentrifuge Rotanta 63R	Hettich, Tuttlingen
UV-Dokumentationssystem Multi Doc-It	UVP, Upland, USA
Vortex Genie 2	Scientific Industries, Bohemia, USA
Western Blot Kammer Novex Mini Cell	Invitrogen, Karlsruhe

Tabelle 1: In dieser Arbeit verwendete Geräte

3.2 Chemikalien

Chemikalie	Bezugsquelle
β-Mercaptoethanol	Merck, Darmstadt
[γ^{32}P]-ATP	Perkin-Elmer, Rodgau-Jügesheim
Aceton	Merck, Darmstadt
Agarose	Invitrogen, Karlsruhe
Bradford-Reagenz	Bio-Rad, München
Bromphenolblau	Bio-Rad, München
Chloroform	Merck, Darmstadt
Coomassie Brilliant Blue R-250	Merck, Darmstadt
Dextrannatriumsulfat (DSS)	MP Biomedicals, Eschwege
Dimethylsulfoxid (DMSO)	Fluka, Taufkirchen
Dinatriumhydrogenphosphat	Merck, Darmstadt
Dithiothreitol (DTT)	Promega, Mannheim
Essigsäure	Merck, Darmstadt
Ethanol	Merck, Darmstadt
Ethidiumbromid (EtBr)	Sigma-Aldrich, Taufkirchen
Ethylendiamintetraacetat (EDTA)	Sigma-Aldrich, Taufkirchen
Ethylenglykol-bis-(2-aminoethyl)-tetraacetat (EGTA)	Sigma-Aldrich, Taufkirchen
Gelatine (bovine)	Sigma-Aldrich, Taufkirchen
Geneticin (G418-Sulfat)	Gibco, Karlsruhe
Glycerin	Merck, Darmstadt
Hoechst 33342	Sigma-Aldrich, Taufkirchen
Isopropanol	Merck, Darmstadt
Kaliumchlorid	Merck, Darmstadt
Magermilchpulver	Bio-Rad, München
Methanol	Merck, Darmstadt
Mowiol	Calbiochem, Darmstadt
N-(2-Hydroxyethyl)-piperazin-N'-(2-ethansulfonsäure) (HEPES)	Sigma-Aldrich, Taufkirchen
Natriumbutyrat	Sigma-Aldrich, Taufkirchen
Natriumchlorid	Fisher, Schwerte
Natriumdihydrogenphosphat	Merck, Darmstadt
Natriumdodecylsulfat (SDS)	Fisher, Schwerte
Natriumfluorid	Sigma-Aldrich, Taufkirchen

Natriumhydroxid	Merck, Darmstadt
Natriumorthovanadat ($NaVO_3$)	Sigma-Aldrich, Taufkirchen
Nonidet-P40 (NP-40)	Roche, Mannheim
Paraformaldehyd	Sigma-Aldrich, Taufkirchen
Phenylmethylsulfonylfluorid (PMSF)	Sigma-Aldrich, Taufkirchen
Salzsäure	Merck, Darmstadt
Tris-(hydroxymethyl)-aminomethan (Tris)	Fisher, Schwerte
Triton X-100	Sigma-Aldrich, Taufkirchen
Trizol	Invitrogen, Karlsruhe
Tween®20	Sigma-Aldrich, Taufkirchen

Tabelle 2: In dieser Arbeit verwendete Chemikalien

3.3 Verbrauchsmaterialien

Name	Bezugsquelle
Novex 10 % Gelatine-Zymogramm-Gele	Invitrogen, Karlsruhe
Novex 6 % DNA Retardation Gele	Invitrogen, Karlsruhe
NuPage Novex Bis-Tris Gele 4-12 %	Invitrogen, Karlsruhe
PVDF-Membran (Immobilon-P)	Millipore, Eschborn
Blotpapier	Bio-Rad, München
Röntgenfilme (X-OMAT AR)	Kodak, Stuttgart
Einmalkunststoffwaren	BD Biosciences, Heidelberg Eppendorf, Hamburg Saarstedt, Nümbrecht
Filterpipettenspitzen für PCR	Biozym, Hessisch Oldendorf
Kulturgefäße für die Zellkultur	BD Biosciences, Heidelberg
Einmalhandschuhe	Semperit Technische Produkte, Gevelsberg
Deckgläser (18x18 mm, Menzel)	Nunc, Wiesbaden
Objektträger	Marienfeld, Lauda-Königshofen
Chamber Slides Lab Tek II	Nunc, Wiesbaden

Tabelle 3: In dieser Arbeit verwendete Verbrauchsmaterialien

3.4 Reaktionssets (Kits)

Name	Bezugsquelle
BD OptEIA Human IL-8 ELISA Kit II	BD Biosciences, Heidelberg
BD OptEIA Human TNF ELISA Kit II	BD Biosciences, Heidelberg
Human CXCL12/SDF-1α Quantikine ELISA Kit	R&D Systems, Wiesbaden
Human CXCL16 Quantikine ELISA Kit	R&D Systems, Wiesbaden
Human VEGF Quantikine ELISA Kit	R&D Systems, Wiesbaden
Cell Titer 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay	Promega, Mannheim
First Strand cDNA Synthesis Kit for RT-PCR (AMV)	Roche, Mannheim
Omniscript RT Kit	Qiagen, Hilden
Quantitect SYBR Green PCR Kit	Qiagen, Hilden
Complete Proteaseinhibitormix	Roche, Mannheim
ECL (Enhanced chemoluminescence) Kit	Amersham Bioscience, Freiburg
Turbo DNA-free	Ambion, Huntingdon, Großbritannien

Tabelle 4: In dieser Arbeit verwendete Kits

3.5 Größenstandards

Name	Bezugsquelle
100 bp DNA ladder	Invitrogen, Karlsruhe
MagicMark Western Protein Standard	Invitrogen, Karlsruhe
MultiMark Multi-Colored Standard for SDS-PAGE	Invitrogen, Karlsruhe

Tabelle 5: In dieser Arbeit verwendete Größenstandards

3.6 Enzyme

Name	Bezugsquelle
Taq DNA Polymerase, rekombinant	Invitrogen, Karlsruhe
Omniscript Reverse Transcriptase	Qiagen, Hilden
DNase	Ambion, Huntingdon, Großbritannien
T4-Polynukleotidkinase (3 U/µl)	USB, Staufen
AluI	New England Biolabs, Frankfurt/Main

Tabelle 6: In dieser Arbeit verwendete Enzyme

3.7 Oligonukleotide und Primer

Alle Oligodesoxyribonukleotide für PCR-Reaktionen wurden bei der Firma MWG (Ebersberg) synthetisiert und direkt für PCR-Reaktionen eingesetzt.

Primer für die RT-PCR (h=human, m=murin):

Gen	Primersequenzen
hAktin	forward 5'-GCCAACCGCGAGAAGATGA-3' reverse 5'-CATCACGATGCCAGTGGTA-3'
hBD-2	forward 5'-CCAGCCATCAGCCATGAGGGT-3' reverse 5'-GGAGCCCTTTCTGAATCCGCA-3'
hCCL20	forward 5'-CTACTCCACCTCTGCGGCGAA-3' reverse 5'-TTTTACTGAGGAGACGCACAA-3'
hCCR6	forward 5'-ATTTCAGCGATGTTTTCGACTC-3' revers 5'-GGAGAAGCCTGAGGACTTGTA-3'
hCXCL12	forward 5'-AGAGCCAACGTCAAGCATCT-3' reverse 5'-CGTCTTTGCCCTTTCATCTC-3'
hCXCL16	forward 5'-GAGCTCACTCGTCCCAATGAA-3' reverse 5'-TCAGGCCCAACTGCCAGA-3'
hCXCR4	forward: 5'-GGTGGTCTATGTTGGCGTCT-3' reverse: 5'-TGGAGTGTGACAGCTTGGAG-3'
hCXCR6	forward 5'-CAGGCATCCATGAATGGGTGT-3' reverse 5'-CAAGGCCTATAACTGGAACATGCTG-3'
hGAPDH	forward 5'-CGGAGTCAACGGATTTGGTCGTAT-3' reverse 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'
hIFN-α	forward 5'-TTTCTCCTGCCTGAAGGACAGAC-3' reverse 5'-CTCTGACAACCTCCCAGGCACA-3'
hIFN-β	forward 5'-TGCTCTGGCACAACAGGTAG-3' reverse 5'-GCTGCAGCTGCTTAATCTCC-3'
hIL-10R2	forward 5'-GGCTGAATTTGCAGATGAGCA-3' reverse 5'-GAAGACCGAGGCCATGAGG-3'
hIL-20R1	forward 5'-TACACCCCTCAGCTCCAAGACT-3' reverse 5'-GAAGGAATACACAGCCTGCCAG-3'
hIL-22	forward 5'-GCAGGCTTGACAAGTCCAACT-3' reverse 5'-GCCTCCTTAGCCAGCATGAA-3'
hIL-22BP	forward 5'-AGGGTACAATTTCAGTCCCGA-3' reverse 5'-CGGCGTCATGCTCCATTCTGA-3'

hIL-22R1	forward:5’-CTCCACAGCGGCATAGCCT-3’ reverse: 5’-ACATGCAGCTTCCAGCTGG-3’
hIL-26	forward: 5’-GGCAGAAATTGAGCCACTGT-3’ reverse: 5’-TCCAGTTCACTGATGGCTTTG-3’
hIL-31	forward: 5’-GAACTCTGCCGTGATTCCTT-3’ reverse: 5’-AAGCCTGCAGAAGAAAAGCA-3’
hIL-31R	forward 5’-GGAGGGGAAAAGAATGGGTA-3’ reverse 5’-CGAGGGTCTCATGCTATGGT-3’
hIL-6	forward 5’-AAAGAGGCACTGGCAGAAAA-3’ reverse 5’-GAGGTGCCCATGCTACATTT-3’
hIL-8	forward 5’-CCAGGAAGAAACCACCGGA-3’ reverse 5’-GAAATCAGGAAGGCTGCCAAG-3’
hIRF-7	forward 5’-TGGTCCTGGTGAAGCTGGAA-3’ reverse 5’-GCTCCATAAGGAAGCACTCG-3’
hMxA	forward 5’-AGATCCAGGACCAGCTGAGCCTGT-3’ reverse 5’-GTGGAACTCGTGTCGGAGTCTGGTA-3’
h2’,5’-OAS	forward 5’-ATTGACAGTGCTGTTAACATCATC-3’ reverse 5’-AGATCAATGAGCCCTGCATAAACC-3’
hOSMR	forward: 5’-GGAATGTGCCACACACTTTG-3’ reverse: 5’-ACATTGGTGCCTTCTTCCAC-3’
hPKR	forward 5’-TTTGCTACTACGTGTGAGTCCCA-3’ reverse 5’-GTGAACAATATTTACATGATCAA-3’
hSOCS-1	forward 5’-CGCCAGCGCCGCTGTCGGCC-3’ reverse 5’-CTGCGGCCTCGTCTCCAGCC-3’
hSOCS-3	forward 5’-TTCTGATCCGCGACAGCTC-3’ reverse 5’-TGCAGAGAGAAGCTGCCCC-3’
hTNF-α	forward 5’-CCAGGCAGTCAGATCATCTTCTC-3’ reverse 5’-AGCTGGTTATCTCTCAGCTCCAC-3’
hVEGF	forward 5’-GCAGAATCATCACGAAGTGG-3’ reverse 5’-GCAACGCGAGTCTGTGTTTTTG-3’
mCXCL16	forward : 5’- TCTTCTGGCACCCAGATACC-3’ reverse : 5’-GGTTGGGTGTGCTCTTTGTT-3’
mCXCL16	forward 5’-AGCGCAAAGAGTGTGGAACT-3’ reverse 5’-GGTTGGGTGTGCTCTTTGTT-3’
mCXCL2	forward 5’-CCTGCCAAGGGTTGACTTCA-3’ reverse 5’-GTCAGTTAGCCTTGCCTTTG-3’
mCXCL5	forward 5’-CCTACGGTGGAAGTCATAGC-3’ reverse 5’-CTATTGAACACTGGCCGTTC-3’

mCXCR6	forward: 5’-TGTACGATGGGCACTACGA-3’ reverse: 5’-GTGAGAGAGGCAGCCGATA-3’
mCXCR6	forward 5’-TGTACGATGGGCACTACGA-3’ reverse 5’-GTGAGAGAGGCAGCCGATA-3’
mE-Selectin	forward 5’-ACCCGTCCCTTGGTAGTTG-3’ reverse 5’-CGTTGTAAGAAGGCACATGG-3’
mGAPDH	forward 5’-CGTCCCGTAGACAAAATGGT-3’ reverse 5’-TCTCCATGGTGGTGAAGACA-3’
mIL-17	forward 5’-CAAACACTGAGGCCAAGGAC-3’ reverse 5’-AGCTTCCCAGATCACAGAGG-3’
mIL-22	forward 5’-ACCTTTCCTGACCAAACTCA-3’ reverse 5’-AGCTTCTTCTCGCTCAGACG-3’
mIL-31	forward: 5’-CAGCTGTTTCAACCCACTGA-3’ reverse: 5’-CAGTTCTGCCATGCAGTTTG-3’
mIL-31R	forward 5’-GCGGTGGACACTTGGATAGT-3’ reverse 5’-ACCCTGGTCTCAGGACCTTT-3’
mIL-4	forward 5’-GAGTGAGCTCGTCTGTAGG-3’ reverse 5’-CAGCTTATCGATGAATCCAGG-3’
mIL-6	forward 5’-CAAAGCCAGAGTCCTTCAGAG-3’ reverse 5’-GGATGGTCTTGGTCCTTAGC-3’
mLactoferrin	forward 5’-GCCTGTTCCAGTCTAAAACCA-3’ reverse 5’-GTAAGAAAAGCGCAGGCTTC-3’
mOSMR	forward 5’-ACACCAAGTCCCTTCCACAG-3’ reverse 5’-ATGGTGACATTGGAGCCTTC-3’
mTNF-α	forward 5’-CCCCAAAGGGATGAGAAGTT-3’ reverse 5’-CACTTGGTGGTTTGCTACGA-3’
rat IL-22	forward 5’-GTTCTGCTCCCCCAGTCAG-3’ reverse 5’-TCTCTCCACTCTCTCCAAGC-3’
rat CXCL12	forward: 5’-AGAGCCAACGTCAAGCATCT-3’ reverse: 5’-TAATTTCGGGTCAATGCACA-3’

Tabelle 7: In dieser Arbeit verwendete Primer für semiquantitative und quantitative RT-PCR

Primer für die Genotypisierung:

Gen	Sequenz
CXCL16	forward 5'-ACTCGTCCCAATGAAACCAC-3' reverse 5'-CCACAGCTTCATCTCCCACT-3'

Tabelle 8: In dieser Arbeit verwendete Primer für die Genotypisierung

Oligonukleotide für den Electrophoretic mobility shift assay (EMSA)

Oligonukleotid	Bezugsquelle
NF-κB	Promega, Mannheim
AP-1	Santa Cruz, Heidelberg
poly(dI-dC)	Amersham, Freiburg

Tabelle 9: In dieser Arbeit verwendete Oligonukleotide für den EMSA

3.8 Chemokine/Zytokine

Name	Bezugsquelle
CXCL12, rekombinant	R&D Systems, Wiesbaden
CCL20, rekombinant	R&D Systems, Wiesbaden
CXCL16, rekombinant	R&D Systems, Wiesbaden
IL-22, rekombinant	R&D Systems, Wiesbaden
IL-26, rekombinant	R&D Systems, Wiesbaden
IL-31, rekombinant	R&D Systems, Wiesbaden
TNF-α, rekombinant	R&D Systems, Wiesbaden
IL-1β, rekombinant	R&D Systems, Wiesbaden
IFN-γ, rekombinant	R&D Systems, Wiesbaden
Lipopolysaccharid (LPS)	Sigma, Taufkirchen

Tabelle 10: In dieser Arbeit verwendete Chemokine und Zytokine

3.9 Hemmstoffe

Name	Bezugsquelle
PD98059 (ERK-Inhibitor)	Tocris Cookson, Bristol, Großbritannien
SP600125 (SAPK/JNK-Inhibitor)	Tocris Cookson, Bristol, Großbritannien
SB203580 (p38-Inhibitor)	Tocris Cookson, Bristol, Großbritannien
Wortmannin (Akt-Inhibitor)	Tocris Cookson, Bristol, Großbritannien

Tabelle 11: In dieser Arbeit verwendete Hemmstoffe

3.10 Antikörper

Antikörper	Bezugsquelle
α-CXCR4	R&D Systems, Wiesbaden
α-CXCL12	R&D Systems, Wiesbaden
α-CXCR6	R&D Systems, Wiesbaden
α-CXCL16	R&D Systems, Wiesbaden
α-IL-31RA	R&D Systems, Wiesbaden
α-phospho-ERK-1/2 (Thr183/Tyr185)	Cell Signaling, Frankfurt/Main
α-phospho-SAPK/JNK-1/2 (Thr183/Tyr185)	Cell Signaling, Frankfurt/Main
α-phospho-p38 (Thr180/Tyr182)	Cell Signaling, Frankfurt/Main
α-phospho-Akt (Ser473)	Cell Signaling, Frankfurt/Main
α-phospho-STAT1	BD Biosciences, Heidelberg
α-phospho-STAT3	Upstate Biotechnologies/Millipore, Schwalbach
α-phospho-STAT5	Cell Signaling, Frankfurt/Main
α-ERK-1/2	Cell Signaling, Frankfurt/Main
α-SAPK/JNK-1/2	Cell Signaling, Frankfurt/Main
α-p38	Cell Signaling, Frankfurt/Main
α-Akt	Cell Signaling, Frankfurt/Main
α-STAT1	Santa Cruz, Heidelberg
α-STAT3	Santa Cruz, Heidelberg
α-STAT5	Santa Cruz, Heidelberg
α-NFκB p65	Santa Cruz, Heidelberg
α-APO-1	Sigma, Taufkirchen
α-CXCR4, PE-konjugiert	BD Biosciences, Heidelberg
IgG Isotyp-Kontrollantikörper, PE-konjugiert	BD Biosciences, Heidelberg
α-Kaninchen, HRP-konjugiert	Amersham, Freiburg
α-Maus, HRP-konjugiert	Amersham, Freiburg
α-Maus, FITC konjugiert	Sigma, Taufkirchen
α-Hase, FITC konjugiert	Sigma, Taufkirchen
α-Ziege, FITC konjugiert	Sigma, Taufkirchen
α-Maus, Cy3 konjugiert	Sigma, Taufkirchen

Tabelle 12: In dieser Arbeit verwendete Antikörper

3.11 Zellkulturmedien und Zusätze

Medium/Zusatz	Bezugsquelle
DMEM (Dulbecco's Modified Eagle Medium) mit 4500 mg/l Glucose, L-Glutamin und Pyruvat	Gibco, Karlsruhe
RPMI-1640 mit L-Glutamin	Gibco, Karlsruhe
DMEM/F12 (1:1) (Dulbecco's Modified Eagle Medium/Ham's F12 Medium) mit L-Glutamin und 15 mM HEPES	Gibco, Karlsruhe
Dulbecco's PBS ohne $CaCl_2$ und $MgCl_2$	Gibco, Karlsruhe
Trypsin–EDTA	Gibco, Karlsruhe
100x PS (10000 U/ml Penicillin; 10000 µg/ml Streptomycin)	Gibco, Karlsruhe
Foetales Kälberserum (FCS) Gold	PAA, Pasching, Österreich
Trypanblau	Fluka, Taufkirchen
Gelatine (0,1 % in H_2O, autoklaviert)	Sigma-Aldrich, Taufkirchen
DMSO	Merck, Darmstadt
Insulin (10 mg/ml)	Sigma-Aldrich, Taufkirchen

Tabelle 13: In dieser Arbeit verwendete Zellkulturmedien und Zusätze

3.12 Zelllinien

Alle Zelllinien wurden über die American Type Culture Collection (ATCC) bezogen.

Zelllinie	Charakterisierung	Wachstumsmedium
HT-29	Humane kolorektale Adenokarzinomzellen adhärent	DMEM, 10 % FCS, 1 % PS
SW480	Humane kolorektale Adenokarzinomzellen adhärent	DMEM, 10 % FCS, 1 % PS
HCT116	Humane kolorektale Karzinomzellen adhärent	DMEM, 10 % FCS, 1 % PS
Caco-2	Humane kolorektale Adenokarzinomzellen adhärent	DMEM, 10 % FCS, 1 % PS
SW620	Metastatische Zellen von SW480 isoliert aus einer Lymphknotenmetastase adhärent	DMEM, 10 % FCS, 1 % PS
T84	Metastatische Zellen eines Kolonkarzinoms isoliert aus einer Lungenmetastase adhärent	DMEM/F12, 10 % FCS, 1 % PS

DLD-1	Humane kolorektale Adenokarzinomzellen adhärent	DMEM, 10 % FCS, 1 % PS
IEC-6	Normale Dünndarmepithelzellen aus der Ratte adhärent	DMEM, 10 % FCS, 1 % PS, 10 µg/ml Insulin
HepG2	Humane hepatozelluläre Karzinomzellen adhärent	RPMI, 10 % FCS, 1 % PS
Hep3B	Humane hepatozelluläre Karzinomzellen (enthalten ein integriertes HBV-Genom) adhärent	RPMI, 10 % FCS, 1 % PS
Huh-7	Humane differenzierte Hepatomzellen adhärent	RPMI, 10 % FCS, 1 % PS

Tabelle 14: In dieser Arbeit verwendete Zelllinien

3.13 Mausstämme

Mausstamm	Bezugsquelle
C57BL/6	Charles River Laboratories, Sulzfeld
C3H/HeJ	Charles River Laboratories, Sulzfeld

Tabelle 15: In dieser Arbeit verwendete Mausstämme

4. Methoden

4.1 Molekularbiologische Methoden

4.1.1 Isolation von RNA

Die Zellen (in 6 cm Kulturschalen) wurden mit PBS gewaschen und anschließend mit 1 ml Trizol von der Platte geschabt und in 1,5 ml Reaktionsgefäße überführt. Anschließend wurden sie 6 x durch eine 21G-Nadel gesaugt. Nach kurzer Inkubation auf Eis wurden 200 µl Chloroform zugegeben und die Reaktiongefäße für 20 sec geschüttelt. Nach einigen Minuten wurden die Lysate mit 12000 g für 15 min bei 4 °C zentrifugiert. Die entstandene klare obere Phase, welche die RNA enthielt, wurde vorsichtig in ein neues Reaktionsgefäß überführt. Es wurden 500 µl Isopropanol zugegeben, die Gefäße durch Schwenken gemischt und für 10 min bei Raumtemperatur (RT) inkubiert. Danach erfolgte eine erneute Zentrifugation mit 12000 g für 10 min bei 4 °C, um die gefällte RNA zu pelletieren. Das Pellet wurde 2 x mit je 1 ml 70 % EtOH gewaschen. Dazwischen erfolgte jeweils ein Zentrifugationsschritt (7500 g, 5 min, 4 °C). Am Ende wurde die RNA kurz getrocknet, in nukleasefreiem Wasser aufgenommen und die Konzentration photometrisch bestimmt. Die RNA wurde bei -80 °C gelagert.

4.1.2 Photometrische Bestimmung der RNA-Konzentration

Die Bestimmung der RNA-Konzentration in einer Lösung erfolgte mittels Messung der Absorption bei 260 nm Wellenlänge. Bei einer Schichtdicke von 1 cm entspricht ein Wert von 1 einer Konzentration von 40 µg/ml RNA. Als Maß für die Reinheit der RNA diente der Quotient aus den optischen Dichten bei den Wellenlängen 260 nm und 280 nm. Für reine RNA sollte er bei etwa 1,8 liegen. Niedrigere Werte bedeuten Verunreinigungen mit Proteinen, höhere Werte zeigen Verunreinigungen mit Salzen oder Zuckern an. Die Messungen erfolgten in einem UV-Spektralphotometer.

4.1.3 DNase-Verdau

Die RNA-Lösung wurde mit einem entsprechenden Volumen an 10 x DNase-Puffer und 2,5 U DNase versetzt. Nach 30-minütiger Inkubation bei 37 °C wurden weitere 2,5 U hinzugegeben und noch einmal wie oben inkubiert. Anschließend wurden 0,2 Volumen DNase-Inaktivierungsreagenz zugefügt und gut durchmischt. Nach kurzer Inkubation wurden

die Proben mit 10000 g für 1,5 min zentrifugiert und der Überstand in ein neues Reaktionsgefäß überführt.

4.1.4 Reverse Transkription

Die reverse Transkription wurde in einem 20 µl-Reaktionsansatz mit 2 µg RNA, 1 x Reaktionspuffer, 10 µM Oligo-p$(dT)_{15}$, 0,5 mM dNTP, 10 U RNAse-Inhibitor und 4 U reverser Transkriptase durchgeführt. Die Proben wurden nach dem Vermischen für 60 min bei 37 °C inkubiert.

4.1.5 PCR

Ein typischer PCR-Reaktionsansatz wurde in einem Gesamtvolumen von 20 µl unter folgenden Bedingungen durchgeführt: 1 x Reaktionspuffer, 2,5 mM $MgCl_2$, je 0,4 µM forward- und reverse-Primer, 0,4 mM dNTP und 2,5 U Taq-DNA-Polymerase. Ein typisches PCR-Programm sah folgendermaßen aus: 5 min initiale Denaturierung bei 95 °C, anschließend erfolgte die Amplifikation in 25-40 Zyklen von 30 sec Denaturierung bei 95 °C, 30 sec Anlagerung der Primer (Annealing) bei 55-61 °C und 30 sec Elongation bei 72 °C. Zur Analyse der PCR mittels Agarosegelelektrophorese wurden 6-10 µl des PCR-Ansatzes verwendet.

4.1.6 Quantitative PCR

Für die quantitative PCR wurde der QuantiTect SYBR Green PCR Kit eingesetzt, der neben dem PCR-Puffer auch bereits dNTPs, SYBR Green und eine Hotstart-DNA-Polymerase enthält. Ein typischer Reaktionsansatz enthielt 8 µl einer 1:20-Verdünnung der cDNA, 1 x Reaktionsmix und je 0,3 µM forward- und reverse-Primer in einem Gesamtvolumen von 20 µl. Für die PCR-Reaktion erfolgte zunächst eine 15-minütige Denaturierung bei 95 °C zur Aktivierung der Hotstart-Polymerase, anschließend wurde eine PCR mit 40-45 Zyklen durchgeführt: 30 sec Denaturierung bei 94 °C, 30 sec Annealing bei 60 °C und 30 sec Elongation bei 72 °C. Die PCR-Reaktionen wurden entweder in einem Rotorgene-RG3000-Gerät oder in einem ABI Prism 7700 Sequence Detection System durchgeführt und analysiert. Jede Messung wurde in Duplikaten durchgeführt. Eine vorher erstellte Standardkurve diente zur Kalkulation der relativen Genexpression anhand des Ct-Wertes. Die Expression der „Housekeeping-Gene“ Aktin oder GAPDH, deren Expression unter verschiedenen Bedingungen konstant ist, wurde zur Normalisierung der Genexpression verwendet.

4.1.7 Agarosegelelektrophorese

TAE-Puffer: 40 mM Tris-Acetat, 1 mM EDTA,

TE-Puffer: 10 mM Tris-HCl (pH 7,4), 1 mM EDTA

10 x DNA-Ladepuffer: 50 % Glycerin, 0,1 % Bromphenolblau in TE-Puffer

Je nach erwarteter Größe der aufzutrennenden Fragmente wurde ein 1-3 %iges (w/v) Agarosegel mit TAE-Puffer hergestellt. EtBr wurde in einer Endkonzentration von 0,5 µg/ml zugegeben. Die Proben wurden vor dem Auftragen mit einem entsprechenden Volumen an 10 x DNA-Ladepuffer versetzt. Die Auftrennung der Proben erfolgte bei konstanter Spannung von 10 V/cm Gellänge. Die Fragmente wurden unter einem UV-Transilluminator sichtbar gemacht und photographiert.

4.1.8 Restriktionsverdau

In einem typischen Restriktionsverdau wurden 5 µl eines PCR-Produkts mit 1-5 U des entsprechenden Restriktionsenzym in dem für das Enzym jeweils optimalen Puffer bei 37 °C über Nacht verdaut. Die daraus resultierenden Spaltprodukte wurden mit Hilfe der Agarosegelelektrophorese aufgetrennt und analysiert.

4.2 Proteinbiochemische Methoden

4.2.1 Proteinisolation aus Säugerzellen

Lysispuffer: 20 mM Tris-HCl (pH 7,4), 1 % Nonidet-P40, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM PMSF, 400 mM $NaVO_3$, 4 mM NaF, Proteaseinhibitormix

Zellen wurden in 6 cm Platten bis zu 70 %iger Konfluenz herangezogen. Sie wurden 3 x mit kaltem PBS gewaschen und in flüssigem Stickstoff schockgefroren. Anschließend wurden sie in 200-400 µl Lysispuffer aufgenommen und 6 x durch eine 21G-Nadel gesaugt. Nach einer 20-minütigen Inkubation auf Eis erfolgte eine Zentrifugation bei 10000 g für 20 min bei 4 °C. Der Überstand, der die Proteine enthielt, wurde in ein neues Reaktionsgefäß überführt und die Proteinkonzentration nach der Bradfordmethode bestimmt.

4.2.2 Isolation von zytosolischen Proteinen und Membranproteinen

Triton X-100-Puffer: 10 mM HEPES (pH 7,6), 1 % Triton X-100, 100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 4 mM $NaVO_3$, 40 mM NaF, Proteaseinhibitormix

Die Zellen wurden 3 x mit kaltem PBS gewaschen und anschließend in Triton X-100-Puffer lysiert. Nachdem die Zellen 6 x durch eine 21G-Nadel gesaugt wurden, wurden sie mit 15000 g für 30 min bei 4 °C zentrifugiert. Im Überstand, der in ein neues Reaktionsgefäß überführt wurde, befanden sich nun die Triton X-100 löslichen Proteine. Das Pellet wurde in Triton X-100-Puffer, der zusätzlich 1 % SDS enthielt, durch Ultraschall und mehrmaliges Saugen durch eine 21G-Nadel aufgelöst und mit 15000 g für 5 min bei 4 °C zentrifugiert. Der Überstand enthielt nun die Triton X-100 unlöslichen Proteine, die Membranproteinen entsprechen.

4.2.3 Konzentrationsmessung von Proteinen

Die Proteinkonzentration in einer Lösung wurde mit Hilfe des Bradford-Assays bestimmt. Das Prinzip beruht auf der Tatsache, dass der in der Lösung enthaltene Farbstoff (Coomassie Brilliant Blue G-250 [CBBG]) verschiedene Absorptionsmaxima zeigt, je nachdem ob er an Protein gebunden ist oder nicht. Die nicht gebundene kationische Form von CBBG zeigt ein Absorptionsmaximum bei 470 nm, während sich dieses bei der proteingebundenen anionischen Form bei 595 nm befindet. Mit Hilfe einer Standardkurve, die mit bekannten Proteinkonzentrationen erstellt wurde, konnte so die Proteinkonzentration einer unbekannten Probe spektrophotometrisch bestimmt werden. Für die Standardkurve wurden verschiedene Verdünnungen von Rinderserumalbumin (BSA) hergestellt (0; 0,1; 0,2; 0,5; 1; 2; 5; 10 mg/ml). Die Bradfordlösung wurde 1:5 mit Wasser verdünnt und 1 ml davon mit 2 µl Proteinlösung vermischt, kurz inkubiert und dann im Spektrophotometer bei 595 nm gemessen.

4.2.4 SDS-Polyacrylamidgelelektrophorese (PAGE)

Zur Auftrennung von Proteinen wurden Fertiggele verwendet. Die Proteinproben wurden mit 4 x Ladepuffer versetzt und bei 72°C für 10 min erhitzt. Danach wurden die Proben auf das Gel geladen und in MOPS-Laufpuffer aufgetrennt. Als Größenmarker dienten entweder der Magic Mark oder der Multi Mark Proteinstandard. An die Gele wurde eine konstante

Spannung von 110 Volt angelegt. Nachdem die Lauffront den unteren Rand des Gels erreicht hatte, wurde der Lauf gestoppt.

4.2.5 Coomassie-Färbung von PAGE-Gelen

Coomassie-Färbelösung: 40 % Methanol, 10 % Essigsäure, 0,5 % Coomassie Brilliant Blue R-250

Entfärbelösung: 40 % Methanol, 10 % Essigsäure

Das Gel mit den aufgetrennten Proteinen wurde für 45 min bei RT unter leichtem Schwenken mit Coomassie-Färbelösung inkubiert. Anschließend wurde die Lösung abgegossen und Entfärbelösung dazugegeben. Diese wurde während des Entfärbevorgangs (3-4 h) mehrfach gewechselt. Zuletzt wurde das Gel in einem Geltrockner getrocknet.

4.2.6 Gelatinezymographie

Renaturierungspuffer: 2,5 % Triton X-100

Coomassie-Färbelösung: 40 % Methanol, 10 % Essigsäure, 0,5% Coomassie Brilliant Blue R-250

Entfärbelösung: 40 % Methanol, 10 % Essigsäure

Zellen wurden in 24-Loch-Platten bis zu 70 % Konfluenz herangezogen. Nach Serumentzug über Nacht wurden sie mit entsprechenden Konzentrationen von CXCL12 stimuliert. Zu verschiedenen Zeitpunkten wurde das Medium entnommen, in vorher gewogene Reaktionsgefäße gegeben und bis zur Verwendung bei -20 °C eingefroren. Die Reaktionsgefäße mit dem enthaltenen Medium wurden gewogen und durch Abzug des Leergewichts das tatsächliche Gewicht des Inhalts bestimmt. Anschließend wurden die Proben in einer Vakuumzentrifuge auf die Hälfte bis ein Drittel ihres ursprünglichen Gewichts konzentriert. Nach erneutem Auswiegen konnte der Prozentsatz des Gewichtsverlustes bestimmt werden. Die Proben wurden auf einem 10 % Gelatine-Zymogramm-Gel (Invitrogen, Karlsruhe) in Tris-Glycin-Puffer (Invitrogen, Karlsruhe) aufgetrennt. Dann wurden die Gele für 60 min bei RT in Renaturierungspuffer geschwenkt. Anschließend erfolgte eine Inkubation für 24-48 h bei 37 °C in Entwicklungspuffer (Invitrogen, Karlsruhe). Die Gele wurden mit Coomassie-Blau gefärbt und anschließend entfärbt. Die Stellen, an denen die Gelatine durch entsprechende Enzyme in den aufgetragenen Proben verdaut wurde, erschienen weiß.

4.2.7 Electrophoretic mobility shift assay (EMSA)

5 x Bindepuffer:	50 mM Tris-HCl (pH 7,5), 5 mM $MgCl_2$, 20 % Glycerin, 2,5 mM EDTA, 250 mM NaCl
10 x TBE:	0,45 M Tris (pH 8,3), 0,01 M EDTA, 0,45 M Borsäure
Gelladepuffer:	250mM Tris-HCl (pH 7,5), 40 % Glycerin, 0,2 % Bromphenolblau
Puffer A:	10 mM HEPES (pH 7,9), 10 mM KCl, 0,1 mM EDTA, 0,1 mM EGTA, 1 mM DTT, 0,5 mM PMSF
Puffer B:	20 mM HEPES (pH 7,9), 0,4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 % Glycerin, 1 mM DTT, 1 mM PMSF

Für die radioaktive Markierung der NF-κB- oder AP-1-Oligonucleotide wurde auf Eis folgender 10 µl-Ansatz gemischt: 2 µl Oligonukleotidlösung, 1 µl 10 x Reaktionspuffer, 5 µl nukleasefreies Wasser, 1 µl [γ^{32}P]ATP, 1 µl T4-PNK (3 U/µl) und der Reaktionsansatz anschließend für 10 min bei 37 °C inkubiert. Durch Zugabe von 1 µl 0,5 M EDTA wurde die Reaktion gestoppt. Die Probe wurde mit Hilfe von mini Quick Spin Oligo-Säulen für 4 min bei 1000 g zentrifugiert. 2 µl der radioaktiven Probe wurden mit 10 ml Szintillationslösung vermischt und in einem Flüssig-Szintillationsmessgerät vermessen. Für die Isolation der nukleären Proteine wurden Zellen in 6-Loch-Platten entsprechend stimuliert, anschließend auf Eis gegeben und 3 x mit kaltem PBS gewaschen. Mit Hilfe eines Zellschabers wurden die Zellen mit 1 ml PBS in Reaktionsgefäße überführt. Sie wurden mit 1500 g für 5 min bei 4 °C zentrifugiert. Der Überstand wurde verworfen und das Pellet in 400 µl Puffer A resuspendiert. Nach 15-minütiger Inkubation auf Eis wurden 25 µl NP-40 (10 %) zugegeben und die Proben sofort für 10 sec auf einem Schüttler gemischt. Es erfolgte eine Zentrifugation mit 12000 upm für 1 min bei 4 °C. Der Überstand, der die zytosolischen Proteine enthielt, wurde verworfen und das Pellet in 50 µl Puffer B resuspendiert. Nach 15-minütigem Schütteln der Proben im Kühlraum wurden sie für 5 min mit 12000 upm bei 4 °C zentrifugiert. Der Überstand, der aus den extrahierten Zellkernproteinen bestand, wurde in ein neues Reaktionsgefäß überführt und die Proteinkonzentration mit Hilfe der Bradford-Methode (siehe Abschnitt 4.2.3) photometrisch bestimmt. Für die Bindereaktion wurden 450 µl 5 x Bindepuffer mit 50 µl Ladepuffer und 1,3 µl 1 M DTT vermischt (=fertiger Bindepuffer). Auf Eis wurden folgende Zutaten gemischt: 1 µl Poly(dI-dC), 3 µl 5 x fertiger Bindepuffer, x µl Zellkernextrakt (10-15 µg) und mit nukleasefreiem Wasser auf 14 µl aufgefüllt. Der Ansatz wurde für 5-10 min bei RT inkubiert. Anschließend wurde 1 µl der radioaktiv markierten DNA-Probe zugegeben und durch Auf- und Abpipettieren gemischt. Nach einer erneuten Inkubation von 30 min bei RT

wurden die Proben auf einem 6 % DNA Retardationsgel mit 0,5 M TBE-Puffer bei 100 Volt aufgetrennt. Nachdem die Lauffront im unteren Viertel des Gels angekommen war, wurde der Lauf gestoppt. Die Gele wurden aus der Apparatur herausgelöst und in einem Geltrockner für 90 min bei 80 °C unter Vakuum getrocknet. Die radioaktiven Signale wurden auf einem Kodak X-Omat AR Film durch Exponierung bei -80 °C sichtbar gemacht.

4.3 Immunologische und immunbiochemische Methoden

4.3.1 Western Blot

Waschpuffer (TBS-T):	20 mM TrisHCl (pH 7,6), 150 mM NaCl, 0,1 % Tween-20
Blockierlösung:	5% Magermilchpulver in TBS-T
Strippinglösung:	83 mM Tris-HCl (pH 7,5), 2 % SDS, 0,7 % β-Mercaptoethanol

Für den Western Blot wurden die Proteine aus dem Polyacrylamidgel auf eine PVDF-Membran, die zuvor für 1 min in Methanol eingeweicht wurde, übertragen. Dies geschah durch Transfer bei 25-30 V für 2 h. Um unspezifische Bindungsstellen abzublocken, wurde die Membran anschließend für 1 h bei RT (oder wahlweise über Nacht bei 4 °C) in Blockierpuffer geschwenkt. Nach 3 x 5-minütigem Waschen in Waschpuffer wurde die Membran mit dem primären Antikörper (verdünnt in 5 % BSA in TBS-T) über Nacht bei 4 °C inkubiert. Danach wurde erneut wie oben gewaschen und mit dem sekundären Antikörper (verdünnt 1:2000 bzw. 1:5000 in Blockierpuffer) für 1 h bei RT inkubiert. Es erfolgte ein weiterer Waschschritt. Die Detektion erfolgte mit dem ECL-System nach Angaben des Herstellers. Die Banden wurden durch Autoradiographie mit einem X-Omat AR Röntgenfilm visualisiert.

Falls die Membranen für eine weitere Antikörperdetektion verwendet wurden, wurden sie kurz in TBS-T gewaschen und dann für 30 min bei 50 °C unter Schwenken in Stripping-Puffer inkubiert, um gebundene Antikörper zu entfernen. Nach einem erneuten Blockierungsschritt in 5 % Milch wurden die Membranen wie oben beschrieben mit einem neuen Antikörper inkubiert und entsprechend weiterbehandelt.

4.3.2 Indirekte Immunfluoreszenzfärbung

Fixierlösung:	3,2 % Paraformaldehyd in PBS
Permeabilisierungslösung:	0,5 % Triton X-100 in PBS
Blockierlösung:	10 % Hasenserum in PBS
Einbettlösung:	6 g Glycerin, 2,4g Mowiol, 6 ml Wasser, 12 ml 0,2M Tris-HCl (pH 8,5)

Die Zellen wurden in 6-Loch-Platten auf Deckgläschen (18 x 18 mm, Menzel), die mit 0,1 %iger Gelatinelösung für 10 min bei RT beschichtet worden waren, herangezogen. Um Mediumrückstände zu entfernen, erfolgten 3 Waschschritte mit PBS. Anschließend wurden die Zellen für 20 min in Fixierlösung bei RT fixiert. Nach mehrmaligem Waschen mit PBS wurden die Zellen durch 5-minütige Inkubation bei RT mit 0,5 % Triton X-100 in PBS permeabilisiert. Nach erneutem Waschen wurden die Zellen für 60 min mit Blockierlösung inkubiert, um unspezifische Bindungsstellen abzusättigen. Die Antikörperinkubationen erfolgten für 60 min bei RT in einer feuchten Kammer. Die Antikörper wurden in Blockierlösung verdünnt. Anschließend wurden die Zellen zum Anfärben der Zellkerne für 10 min mit 4 µmol/l Hoechst 33342 inkubiert. Nach jedem Schritt wurden die Zellen mehrfach mit PBS gewaschen. Zum Schluss erfolgte noch ein Waschschritt mit destilliertem Wasser, um die Salze des PBS zu entfernen. Die Deckgläschen wurden mit einem Tropfen Einbettlösung auf einen Objektträger gegeben. Nachdem diese getrocknet waren, erfolgte eine Auswertung mit Hilfe der Fluoreszenzmikroskopie.

4.3.3 Detektion der Aktinpolymerisation

Fixierlösung:	3,7 % Paraformaldehyd in PBS
Permeabilisierungslösung:	0,5 % Triton X-100 in PBS
Einbettlösung:	6 g Glycerin, 2,4g Mowiol, 6 ml Wasser, 12 ml 0,2M Tris-HCl (pH 8,5)

HCT116-Zellen wurden auf “Chamber slides” ausgesät. Nach Serumentzug über Nacht wurden die Zellen mit 10 ng/ml CXCL12 für verschiedene Zeitintervalle stimuliert. Anschließend wurden die Zellen mit PBS gewaschen, in Fixierlösung für 20 min fixiert und für 5 min mit Permeabilisierungslösung inkubiert. Dann wurden sie für 30 min mit Rhodamin-markiertem Phalloidin und 4 µmol/l Hoechst 33342 inkubiert. Nach erneutem Waschen wurden die Zellen mit einem Tropfen Mowiol und einem Deckgläschen bedeckt und unter dem Fluoreszenzmikroskop ausgewertet.

4.3.4 Fluoreszenz-aktivierte Zellsortierung (FACS)

FACS-Puffer: 0,5 % BSA, 0,05 % NaN_3 in PBS

SW480- oder HT-29-Zellen wurden in 10 cm Platten bis zu einer Konfluenz von ca. 70 % herangezogen. Die Zellen wurden mit Hilfe von Trypsin losgelöst und anschließend 2 x mit PBS gewaschen (Zentrifugation 5 min, 300 g). Nach dem Bestimmen der Zellzahl wurden je 1-5 x 10^5 Zellen mit einem PE-markierten CXCR4-Antikörper oder einem PE-markierten Isotyp-Kontrollantikörper (20 µl Antikörper pro 1 x 10^6 Zellen) versetzt. Nach einstündiger Inkubation bei Raumtemperatur wurden die Zellen 3 x mit FACS-Puffer gewaschen, anschließend in 200 µl FACS-Puffer resuspendiert und sofort im FACS gemessen.

4.3.5 Enzyme-linked immunosorbent assay (ELISA)

Zellen wurden in 6 cm Zellkulturplatten bis zu einer Dichte von 70 % herangezüchtet. Nach Serumentzug über Nacht wurden 100 µl Medium entnommen (Nullwert). Anschließend erfolgte eine Stimulation der Zellen mit den entprechenden Chemokinen oder Zytokinen. Nach 1, 2, 4, 8, 12 und 24 h wurden jeweils 100 µl Medium entnommen und bei -20 °C bis zur Messung eingefroren. Humane Plasma- bzw. Serumproben wurden bis zur Verwendung ebenfalls bei -20 °C eingefroren. Für die Bestimmung der entsprechenden Zytokin-/ Chemokinkonzentrationen in den Proben wurde der jeweilige ELISA nach Angaben der entsprechenden Hersteller durchgeführt. Allgemein wurden 96-Loch-Platten, die mit einem immobilisierten Antikörper gegen das entsprechende Zytokin/Chemokin beschichtet waren, für 2 h mit den jeweiligen Proben inkubiert. Anschließend wurden die Platten gewaschen und mit einem Enzym-konjugierten Antikörper gegen das zu detektierende Zytokin/Chemokin inkubiert, wodurch ein Antikörper-Antigen-Antikörper-„Sandwich“ entstand. Nach einem weiteren Waschschritt wurde Enzym-Substratlösung dazugegeben, was in einer blauen Farbreaktion resultierte, deren Intensität direkt proportional zur gemessenen Zytokin-/Chemokinmenge war. Nach der Zugabe einer Stoplösung (2 M Schwefel- bzw. 1 M Phosphorsäure) und dem daraus resultierenden Farbüberschlag von blau zu gelb wurde die Absorption der Proben bei 450 nm gemessen. Anhand der Messwerte einer Standardkurve konnte die Zytokin-/Chemokinmenge in den Proben kalkuliert werden.

4.4 Zellkulturmethoden

4.4.1 Kulturbedingungen

Die verwendeten Zelllinien wurden bei 37 °C und 5 % CO_2 in wasserdampfgesättigter Atmosphäre in dem für sie optimalen Medium (siehe Tabelle 14) kultiviert. Ihr Wachstum wurde per Mikroskopie verfolgt. Waren die Zellen 70-80 % konfluent, wurde das Medium abgesaugt und die Platte einmal mit PBS gewaschen, um im Medium vorhandene Trypsininhibitoren zu entfernen. Anschließend wurden 1-2 ml Trypsin-EDTA zugegeben und die Zellen einige Minuten bei 37 °C inkubiert. Die von der Platte abgelösten Zellen wurden im entsprechenden Medium gesammelt und, je nach gewünschter Dichte, im Verhältnis 1:3 bis 1:10 auf neue Platten verteilt.

4.4.2 Auftauen und Einfrieren von Zellen

In flüssigem Stickstoff oder bei -80 °C eingefrorene Zellen wurden im Wasserbad bei 37 °C aufgetaut und sofort in 10 ml frischem Medium aufgenommen und auf 10 cm Zellkulturplatten ausgesät. Der Zustand der Zellen wurde mikroskopisch überprüft. Nach 24 h Inkubationszeit bei 37 °C wurde ein Mediumwechsel durchgeführt.

Zum Einfrieren wurden die Zellen nach dem Ablösen von der Platte gezählt, anschließend für 5 min bei 1200 upm pelletiert und in kaltem Gefriermedium (80 % FCS, 20 % DMSO) resuspendiert, so dass eine Dichte von 3-5 x 10^6 Zellen/ml erreicht wurde. Davon wurde je 1 ml in ein Gefrierröhrchen gegeben und dieses sofort auf Eis gestellt. Die Proben wurden bei -80 °C heruntergekühlt und anschließend bei -80 °C oder in flüssigem Stickstoff gelagert.

4.4.3 Bestimmung der Zellzahl

Die Zelldichte einer Lösung wurde mit Hilfe einer Neubauer-Zählkammer bestimmt. Hierfür wurden 10 µl der Zellsuspension im Verhältnis 1:1 mit Trypanblau gemischt, das von toten Zellen aufgenommen wird und sie dunkel färbt. Es wurde die Lebendzellzahl in einem Großquadrat bestimmt. Da das Volumen über einem Großquadrat 0,1 mm^3 beträgt, ergibt sich: Zellzahl pro ml = Zellen pro Großquadrat x 10^4 x Verdünnungsfaktor

4.4.4 Zellstimulation

Die Zellen wurden für 12-16 h in serumfreiem Medium bzw. Medium mit 0,1 oder 1 % FCS inkubiert. Je nach Versuch wurden die Zellen für 1 h mit entsprechenden Inhibitoren

präinkubiert, bevor die Stimulation mit den entsprechenden Chemokinen/Zytokinen, die dem Medium zugesetzt wurden, erfolgte.

4.4.5 Zellrestitutionsassay

Zellen wurden in 6-Loch-Platten bis zur vollständigen Konfluenz kultiviert. Mit Hilfe einer Rasierklinge wurden pro Loch 6-10 „Wunden“ gesetzt, indem die Zellen weggekratzt wurden. Die herumschwimmenden Zellen wurden durch mehrmaliges Waschen mit PBS entfernt. Die Wundränder wurden unter dem Mikroskop auf ihre Qualität hin begutachtet. Nur klar zu erkennende Ränder wurden für den Versuch ausgewertet. Zu den Zellen wurde nun Medium, das nur 0,1 % FCS enthielt, gegeben und sie wurden mit entsprechenden Konzentrationen an Zytokinen oder Chemokinen stimuliert. Nach 24 h wurden die Zellen erneut gewaschen und die über den Wundrand gewachsenen Zellen gezählt. Hierbei wurde darauf geachtet, dass die ausgezählten Stücke alle gleich lang waren.

4.4.6 Migrationsassay

Für die Bestimmung der Migration wurden Filtereinsätze für 24-Loch-Platten verwendet. Die Filtermembran hat eine Porengröße von 8 µm und besteht aus Polyethylenterephthalat (PET). Zellen wurden auf eine Dichte von 1,25 x 10^5 Zellen/ml in Medium mit 1 % FCS verdünnt. Davon wurden 300 µl oben in die Filtereinsätze gegeben. Unten in den Zellkulturplatten befanden sich 700 µl Medium (1 % FCS), entweder mit oder ohne Chemokin. Nach 24 h wurden die Zellen auf der Oberseite mit einem Wattestäbchen abgewischt und die Filter einmal mit PBS gewaschen. Anschließend wurden die Zellen in eiskaltem Aceton bei -20 °C für 10 min fixiert. Nach erneutem Waschen erfolgte eine Färbung der Zellen mit Hämatoxylin für 10 min. Nach Waschen mit Wasser wurden die Filter aus ihren Halterungen herausgeschnitten, auf Objektträger gegeben und mit einem Tropfen Einbettlösung und einem Deckgläschen bedeckt. Die Auszählung der gewanderten Zellen erfolgte unter dem Mikroskop.

4.4.7 Invasionsassay

Hierfür wurden Filtereinsätze verwendet, die mit Matrigel (Laminin 56 %, Collagen IV 31 %, Entactin 8 %) beschichtet waren. Die Zellen wurden auf eine Dichte von 5 x 10^4/ml verdünnt und davon 500 µl pro Filter eingesetzt. Die Fixierung geschah mit MetOH für 2 min bei RT. Die weiteren Schritte erfolgten wie unter Abschnitt 4.4.6 beschrieben.

4.4.8 Zellproliferationsassay

PMS: 0,92 mg/ml in PBS

MTS: 2 mg/ml in PBS

Die Zellen wurden in entsprechender Anzahl in 96-Loch-Platten ausgesät und mit entsprechenden Konzentrationen von Zytokinen/Chemokinen in 100 µl Medium (mit 0, 0,1 oder 1% FCS) stimuliert. Nach 48 h wurden 20 µl eines 1:20-Gemisches von PMS und MTS zugegeben und die Ansätze für 1-3 h bei 37 °C inkubiert. Anschließend wurde die Absorption bei 490 nm gemessen.

4.4.9 Apoptoseassay

Nicoletti-Puffer: 0,1 % Natriumcitrat, 0,1 % Triton X-100, 50 µg/ml Propidiumiodid

FACS-Puffer: 140 mM NaCl, 2 mM KH_2PO_4, 17 mM Na_2HPO_4, 4 mM KCl, 8 mM LiCl, 3 mM NaN_3, 1 mM Na_2EDTA, pH 7,37

SW480-Zellen wurden in 6-Loch-Platten mit einer Dichte von 0,2 x 10^6 Zellen/Loch ausgesät und mit verschiedenen Konzentrationen von α-APO-1 (+ 1/100 Protein A) und Zytokin/ Chemokin für 24 h stimuliert. Anschließend erfolgte die Ernte der Zellen in PBS. Nach einem Zentrifugationsschritt (2000 upm, 5 min) wurde das Zellpellet in 200 µl Nicoletti-Puffer resuspendiert und in diesem für mindestens 4 h und maximal 4 Tage bei 4 °C aufbewahrt. Die Bestimmung der Anzahl toter Zellen erfolgte per FACS-Analyse.

4.5 Tierexperimentelle Arbeiten

4.5.1 Tierversuchsethik und Haltung der Tiere

Alle Tierversuche wurden von der Tierversuchskommission der Regierung von Oberbayern genehmigt und gemäß den Richtlinien des „National Institutes of Health Guide for the Care and Use of Laboratory Animals“ durchgeführt. C57BL/6 und C3H/HeJ Mäuse wurden in Macrolon Typ III Käfigen unter einem 12 Stunden Licht-Dunkel-Zyklus gehalten. Wasser und Standardfutter (Sniff, Soest) standen ad libitum zur Verfügung. Die ausreichende Versorgung mit frischem Wasser und Futter wurde täglich kontrolliert. In gleichen Intervallen wurde auf die Sauberkeit der Käfige und den Zustand der Tiere geachtet. Die Käfige wurden mindestens zweimal pro Woche gereinigt und die Einstreu gewechselt. Die Mäuse waren in einem geeigneten Stall untergebracht, in dem die Temperatur von 20-22 °C und eine Luftfeuchtigkeit von 50-60 % konstant gehalten wurden. Bei einem Hell-Dunkel-Rhythmus

von 12 Stunden wurde darauf geachtet, Manipulationen an den Tieren immer zur gleichen Zeit durchzuführen.

4.5.2 Mausexperimente für das Dextransulfatsodium (DSS)-Kolitis-Modell

Die experimentelle Kolitis wurde durch Hinzufügen von 3 % und 4,5 % DSS zum Trinkwasser von C57BL/6- und C3H/HeJ-Mäusen über 5 Tage induziert. Am Tag 6 wurden die Mäuse durch CO_2-Narkose getötet und das Kolon für die weitere Analyse entnommen. RNA aus der Kolonschleimhaut wurde mit Hilfe des Qiagen RNeasy Kits nach Herstelleranweisung gewonnen.

4.5.3 Mausexperimente für die murine Zytomegalievirus (MCMV)-Infektion

C57BL/6-Mäuse wurden mit 1×10^6 pfu der MCMV-Linie Smith durch intravenöse Applikation infiziert. Kontrollmäusen wurde PBS injiziert. Nach 45 bzw. 67 h wurden die Mäuse durch eine CO_2-Narkose getötet und das Kolon sowie die Leber für die weitere Analyse entnommen. RNA aus der Kolonschleimhaut und der Leber wurde mit Hilfe von Trizol, analog zur RNA-Isolation aus Zellmaterial wie unter Punkt 4.1.1 beschrieben, gewonnen.

4.6 Biopsiegewinnung

Biopsien wurden von Patienten mit MC bzw. CU im Rahmen von Routinekoloskopien mit Hilfe einer Biopsiezange entnommen, sofort in 1 ml Trizol gegeben und bis zur Aufarbeitung bei -80 °C gelagert. Die Studien wurde von der Ethikkommission der Medizinischen Fakultät der LMU München genehmigt. Alle Patienten unterschrieben vor der Probenentnahme eine Einverständniserklärung. Von jedem Patienten wurden vier Biopsien entnommen, je zwei von makroskopisch entzündetem Gewebe und zwei von makroskopisch nicht entzündetem Gewebe. Humane Leberbiopsien wurden von Patienten gewonnen, denen aus medizinischen Gründen (z. B. Verlaufskontrolle bei chronischer Hepatitis C) eine Leberbiopsie entnommen wurde. Ein 3 mm langes Biopsiestück aus dem Stanzzylinder wurde sofort nach Entnahme in 1 ml Trizol gegeben und bei -80 °C bis zur weiteren Verarbeitung aufbewahrt. Die Studie wurde von der Ethikkommission der Medizinischen Fakultät der LMU München genehmigt. Alle Patienten gaben vor der Probenentnahme ihr schriftliches Einverständnis.

4.7 Kalkulation der Genexpression in Darmbiopsien

In jeder Biopsie wurde die Genexpression zur Aktin- bzw. GAPDH-Expression normalisiert. Zur genauen Quantifizierung wurde die durchschnittliche Expression in beiden Entzündungsbiopsien in Relation zu der in den nicht entzündeten gesetzt. Zur Berechnung des Korrelationskoeffizienten zweier Expressionsmuster wurde für jeden Patienten die mRNA-Expression in allen vier Biopsien korreliert.

4.8 Densitometrie

Die Quantifizierung von DNA-Banden in Agarosegelen erfolgte mit Hilfe der Software TINA, Version 2.09 (Raytest Isotopenmessgeräte GmbH, Straubenhardt) oder der Software ImageJ Version 1.36 des National Institute of Health (NIH).

4.9 Statistik

Statistische Analysen wurden mit Hilfe des zweiseitigen Student-t-Tests (für kontinuierliche Daten) oder des zweiseitigen Fisher's Exakt-Test (für kategorische Daten) durchgeführt. Die Berechnung von Konfidenzintervallen (CI) und des relativen Risikos (Odds Ratio OR) erfolgte mit der Software SPSS 14.0 (SPSS Inc., Chicago, USA). P-Werte $< 0,05$ wurden als signifikant gewertet.

5. In dieser Dissertation diskutierte Publikationen

5.1 Arbeiten zur Rolle von Chemokinen im Gastrointestinaltrakt

[1] Brand S*, **Dambacher J***, Beigel F, Olszak T, Diebold J, Otte JM, Göke B, Eichhorst ST. CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. *Exp Cell Res* 2005; Oct 15; 310(1): 117-30.

*** Geteilte Erstautorenschaft**

[2] Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Göke B, Eichhorst ST, **Dambacher J**. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK and Akt signaling resulting in proliferation and migration of colorectal cancer cells. *J Cell Biochem* 2006 Mar 1; 97(4): 709-23.

[3] **Dambacher J**, Seiderer J, Niess JH, Haller D, Diebold J, Göke B, Ochsenkühn T, Reinecker HC, Brand S. CXCL16 is a novel marker of intestinal inflammation in Crohn's disease. *Inflamm Bowel Dis* 2007; submitted.

[4] Seiderer J*, **Dambacher J***, Leistner D, Tillack C, Glas J, Niess JH, Pfennig S, Jürgens M, Müller-Myhsok B, Göke B, Ochsenkühn T, Lohse P, Reinecker HC, Brand S. The role of the CXCL16 p.Ala181Val polymorphism in inflammatory bowel disease. *Clin Immunol* 2007; submitted.

*** Geteilte Erstautorenschaft**

5.2 Arbeiten zur Rolle von Zytokinen im Gastrointestinaltrakt

[5] **Dambacher J**, Beigel F, Seiderer J, Haller D, Göke B, Auernhammer CJ, Brand S. Interleukin-31 mediated signals modulate intestinal epithelial cell proliferation and its expression is up-regulated in intestinal inflammation. *Gut* 2007 Sep; 56(9): 1257-65.

[6] Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkühn T, Göke B, Auernhammer CJ, **Dambacher J**. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* 2006 Apr; 290(4): G827 - 38.

[7] **Dambacher J**, Beigel F, Zitzmann K, de Toni E, Göke B, Diepolder H, Auernhammer CJ, Brand S. The role of the novel IL-10 like cytokine IL-26 in intestinal inflammation. *Gut* 2007; submitted.

6. Ergebnisse und Diskussion

Da die Ergebnisse der einzelnen Experimente in den jeweiligen Manuskripten bereits ausführlich beschrieben sind, soll im Folgenden vor allem auf übergreifende Probleme, Interpretationen, sowie auf die Einordnung der Arbeit in Bezug auf den derzeitigen Forschungsstand eingegangen werden.

6.1 Ausgewählte Chemokinrezeptoren und ihre Funktionen im Gastrointestinaltrakt

6.1.1 CXCR4 und CXCL12 werden in kolorektalen Tumorzellen invers exprimiert und vermitteln Zellmigration, Tumorinvasion und MMP-9-Aktivierung

Manuskript [1]: Brand S*, **Dambacher J***, Beigel F, Olszak T, Diebold J, Otte JM, Göke B, Eichhorst ST. CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. *Exp Cell Res* 2005; Oct 15; 310(1): 117-30. *** Geteilte Erstautorenschaft**

CXCR4 und CXCL12 haben großen Einfluss auf das Wachstum und die Metastasierung von Tumoren verschiedenen Ursprungs wie dem Mammakarzinom [Li et al., 2004b; Muller et al., 2001; Smith et al., 2004] oder dem Melanom [Murakami et al., 2004; Murakami et al., 2002]. Daher hypothetisierten wir, dass dieses Chemokinsystem ähnliche Funktionen in intestinalen Epithelzellen (IEC) und kolorektalen Karzinomzellen (CRC) haben könnte. In unseren Untersuchungen war der Rezeptor CXCR4 in allen untersuchten Tumor-Zelllinien auf basalem Niveau exprimiert, während wir CXCL12 nur in Caco-2-Zellen sowie in normalen Rattenepithelzellen (IEC-6) nachweisen konnten [Brand et al., 2005b], was auf eine differentielle Regulation der Expression dieses Chemokins hindeutete. In normalem epithelialem Gewebe war CXCL12 v. a. in Zellen im apikalen Teil der Darmzotten exprimiert, während die Zellen an der Basis der Krypten eine geringe Expression zeigten, was mit dem Differenzierungsgrad der Zellen (von der Basis der Krypten zur Spitze hin zunehmend) korrelierte [Brand et al., 2005b]. Entsprechend beobachteten wir einen Trend zu einer Herunterregulation der CXCL12-Expression in Tumorgewebe, das geringer differenziert ist [Brand et al., 2005b]. Übereinstimmend hierzu wurde von Wendt et al. demonstriert, dass die in Epithelzellen normalerweise konstitutive CXCL12-Genexpression in CRC-Zellen durch DNA-Hypermethylierung ausgeschaltet wird [Wendt et al., 2006]. Umgekehrt führt eine Inhibierung der Methylierung zu einem geringeren metastatischen Potential der Zellen [Wendt et al., 2006].

Bemerkenswerterweise wurden in einer kürzlich veröffentlichten Studie bei CRC-Patienten erniedrigte CXCL12-Plasma-Spiegel im Vergleich zu Kontrollen beobachtet; außerdem wiesen Patienten im fortgeschrittenem Tumorstadium (Dukes-Stadium B und C) niedrigere CXCL12-Level auf als Patienten im Dukes-Stadium A [Dimberg et al., 2007].

Bei der Untersuchung von Tumorgewebeschnitten fanden wir außerdem eine Tendenz zu einer inversen Expression von CXCL12 und CXCR4 [Brand et al., 2005b]. Dies ist übereinstimmend mit den Daten von Jordan et. al sowie Shibuta et al., die zeigen konnten, dass CXCR4 in HT-29-Zellen während einer durch Natriumbutyrat (NaBt) induzierten Zell-differenzierung [Chung et al., 1985], herunterreguliert wird [Jordan et al., 1999; Shibuta et al., 2002]. Umgekehrt konnten wir durch NaBt eine Hochregulation von CXCL12 beobachten [Brand et al., 2005b]. Außerdem zeigten Dwinell et al., dass die CXCR4-Expression von der Basis der Darmkrypten zur apikalen Seite hin abnimmt [Dwinell et al., 1999], während hier von uns eine entsprechende Zunahme der CXCL12-Expression gezeigt werden konnte [Brand et al., 2005b]. D. h. im Gegensatz zu CXCL12, das mit zunehmender Entdifferenzierung der Zellen abnimmt, wird CXCR4 verstärkt exprimiert. Diese erhöhte CXCR4-Expression konnte von verschiedenen Arbeitsgruppen in verschiedenen Tumorarten beobachtet werden, so z. B. auch in kolorektalen Lebermetastasen, die eine sehr hohe CXCR4-Expression aufwiesen [Rubie et al., 2006a]. Bemerkenswerterweise wird die CXCR4-Expression negativ durch den Transkriptionsfaktor p53 reguliert [Mehta et al., 2006], der eines der wichtigsten Tumorsuppressorgene darstellt und bei fast allen Tumorarten, u. a. auch dem CRC, im fort-geschrittenen Stadium mutiert ist [Vogelstein et al., 2000].

Sehr oft kann die CXCR4-Expression als ein prognostischer Marker für das Risiko einer Tumorinvasion bzw. die Prognose der Tumorentwicklung verwendet werden, so z. B. auch beim CRC [Kim et al., 2005; Ottaiano et al., 2006] oder dem Mammakarzinom [Salvucci et al., 2006], was auf eine entscheidende Rolle dieses Chemokinrezeptors bei der Tumor-metastasierung hindeutet. Für diesen Prozess der Metastasierung sind verschiedene Voraus-setzungen notwendig. Unsere Arbeitsgruppe konnte zeigen, dass CXCL12 neben einer gesteigerten Zellproliferation auch zu einer signifikant gesteigerten Migrationsrate von IEC über einen vorher definierten Wundrand führt [Brand et al., 2005b]. Dieser Effekt konnte durch eine gleichzeitige Behandlung der Zellen mit einem CXCR4-neutralisierenden Antikörper komplett inhibiert werden, was auf die CXCR4-Spezifität dieser CXCL12-vermittelten Funktion hinweist [Brand et al., 2005b]. Ferner konnten wir nachweisen, dass eine Stimulation von IEC mit CXCL12 zu einer Umorganisation des Aktin-Zytoskeletts mit Aktin-Polymerisation und der Ausbildung von „Spikes“ als eine wichtige Voraussetzung für

die Zellmigration führt [Brand et al., 2005b]. Außerdem beobachteten wir in Invasionsversuchen eine gesteigerte Invasionsfähigkeit von CRC-Zellen durch eine mit extrazellulären Matrix (ECM)-Proteinen beschichtete Membran [Brand et al., 2005b]. Bemerkenswerterweise war die von uns beschriebene, durch CXCL12-stimulierte IEC-Migration durch die Filtermembran absolut abhängig von der Präsenz von ECM-Proteinen [Brand et al., 2005b], was übereinstimmend mit beschriebenen Effekten in Caco-2-Zellen ist [Basson et al., 1992]. In Migrationsversuchen mit unbeschichteten Membranen beobachteten wir eine genau gegenteilige Wirkung von CXCL12 auf die Zellmigration [Brand et al., 2005b], indem es zu einem dosisabhängigen, die Zellmigration hemmenden Effekt von CXCL12 kam [Brand et al., 2005b]. Ähnliche negative Effekte von CXCL12 auf die Zellmigration wurden auch für Leukozyten beschrieben und als Fugetaxis oder Chemorepulsion bezeichnet [Papeta et al., 2007; Poznansky et al., 2002; Vianello et al., 2005a; Vianello et al., 2005b]. Durch eine gleichzeitige Inkubation mit CXCR4-neutralisierenden Antikörpern konnten wir zeigen, dass dieser fugetaktische Effekt von CXCL12 tatsächlich über CXCR4 vermittelt wird [Brand et al., 2005b]. Außerdem demonstrierten wir eine Abhängigkeit der migrationshemmenden Wirkung vom MEK-1/ERK-1/2-, jedoch nicht vom PI3-Kinase/Akt-Signalweg, wie Versuche mit gleichzeitiger Inkubation mit den entsprechenden Inhibitoren PD98059 bzw. Wortmannin ergaben [Brand et al., 2005b].

Eine wichtige Voraussetzung für das Durchdringen der Basalmembran stellt die Produktion von Matrix-verdauenden Enzymen in den Zellen dar. Zu diesen Enzymen gehören u. a. die sogenannten Matrix-Metalloproteinasen (MMPs). Wir konnten in Gelatinezymographieversuchen eine Aktivierung von MMP-9 nach CXCL12-Stimulation zeigen [Brand et al., 2005b]. Auch in anderen Tumorzellen wie Plattenepithelkarzinomen des Kopf-Hals-Bereiches [Samara et al., 2004], Prostatakarzinom [Chinni et al., 2006], chronisch-lymphatischer B-Zell-Leukämie [Redondo-Munoz et al., 2006] oder Melanomzellen [Parmo-Cabanas et al., 2006] induziert CXCL12 die MMP-9-Expression, was für die Invasionsfähigkeit von Zellen durch die Basalmembran erforderlich ist [Parmo-Cabanas et al., 2006].

Wenn Tumoren Metastasen ausbilden, sind diese in ihrem weiteren Wachstum von der Versorgung durch neue Blutgefäße abhängig [Rmali et al., 2007]. Hier spielen MMPs ebenfalls eine wichtige Rolle. An MMP-9-defizienten Mäusen wurde demonstriert, dass MMP-9 entscheidend für den sogenannten angiogenen „Switch“, d.h. eine Aktivierung von ruhenden Blutgefäßen, ist, indem es eine Ausschüttung des sehr wichtigen angiogenen Faktors „vascular endothelial growth factor“ (VEGF) bewirkt [Bergers et al., 2000]. Eine transgene MMP-9-Überexpression verursacht ebenfalls eine gesteigerte VEGF-Ausschüttung

und damit verbunden verstärktes Tumorwachstum und Tumorangiogenese [Mira et al., 2004]. Wir konnten in CRC-Zellen eine Induktion von VEGF durch CXCL12 sowohl auf RNA- als auch auf Proteinebene nachweisen [Brand et al., 2005b]. Diese Wirkung von CXCL12 bestätigt sich auch in anderen Zelltypen wie Prostatakarzinomzellen [Darash-Yahana et al., 2004], Endothelzellen [Neuhaus et al., 2003] oder lymphoiden Zellen [Kijowski et al., 2001]. Außerdem wirken CXCL12 und VEGF synergistisch in der Neoangiogenese [Kryczek et al., 2005]. Dies beruht vermutlich auf der durch VEGF induzierten CXCR4-Hochregulation [Bachelder et al., 2002; Salcedo et al., 1999], was auf einen positiven Feedback-Loop hindeutet. Ottaiano et al. konnten zeigen, dass eine gleichzeitige hohe Expression von CXCR4 und VEGF in CRC ein prognostisch besonders ungünstiger Marker für die Tumorentwicklung und die mittlere Überlebensrate der Patienten ist [Ottaiano et al., 2006]. Auch in Mammakarzinomzellen korreliert eine gleichzeitige Expression von VEGF, MMP-9 und CXCR4 in besonderem Maße mit dem Vorhandensein von Lymphknotenmetastasen [Hao et al., 2007]. Die synergistische Wirkung von VEGF, CXCL12 und CXCR4 ist sicherlich auch auf der Tatsache begründet, dass Hypoxie, wie sie häufig in schnell wachsendem Tumorgewebe zu finden ist, die Expression aller drei Gene, vermittelt über den Hypoxia-inducible factor-1 (HIF-1), induziert [Ceradini et al., 2004; Hitchon et al., 2002; Schioppa et al., 2003; Shweiki et al., 1992; Staller et al., 2003]. Bemerkenswerterweise aktiviert NaBt auch die Expression von HIF-1 in IEC [Pellizzaro et al., 2002], was vermuten lässt, dass die hier gezeigte Aktivierung der CXCL12-Expression nach NaBt-Gabe zumindest teilweise über HIF-1 vermittelt wird.

Aufgrund seiner Rolle als HIV-Corezeptor, aber auch aufgrund seiner entscheidenden Funktion bei der Tumorentwicklung und –metastasierung, stellt CXCR4 einen vielversprechenden therapeutischen Angriffspunkt dar. Bisher wurde eine Vielzahl von CXCR4-Antagonisten entwickelt. Der wohl bekannteste, AMD3100 [De Clercq, 2003], wird zur Zeit in klinischen Studien zur Mobilisation von hämatopoietischen Stammzellen getestet [Cashen et al., 2007]. Aber auch weitere Moleküle wie AMD070 [Stone et al., 2007], T140-Derivate [Tsutsumi et al., 2007] oder zyklische Pentapeptide wie FC131-Analoga [Tsutsumi et al., 2007], scheinen therapeutisch wirkungsvoll zu sein. Allerdings ist es bis zur routinemäßigen Anwendung beim Menschen noch ein langer Weg, und weitere Studien sind nötig, da durch diese CXCR4-Antagonisten nicht nur pathologische Zustände beeinflusst werden, sondern auch die normalen, lebenswichtigen Funktionen, die über CXCR4 vermittelt werden. Die letalen Auswirkungen einer CXCR4-Deletion bei Mäusen wurden bereits unter Abschnitt

1.2.1 beschrieben; die Nebenwirkungen dieser Therapeutika sind daher nur schwer kalkulierbar.

Zusammenfassend (siehe Abbildung 11) konnten wir zeigen, dass CXCL12 und sein Rezeptor CXCR4 in kolorektalen Tumorzellen und deren Metastasen funktional exprimiert sowie differenzierungsabhängig reguliert werden. CXCL12 induziert in CRC-Zellen eine gesteigerte Zellproliferation, Zellmigration sowie eine verstärkte Zellinvasionsfähigkeit mit erhöhter Produktion angiogener Faktoren wie MMPs und VEGF. Somit sind CXCR4 und sein Ligand in CRC von wichtiger Bedeutung sowohl für das lokale Tumorwachstum als auch die Tumorinvasion in andere Organe.

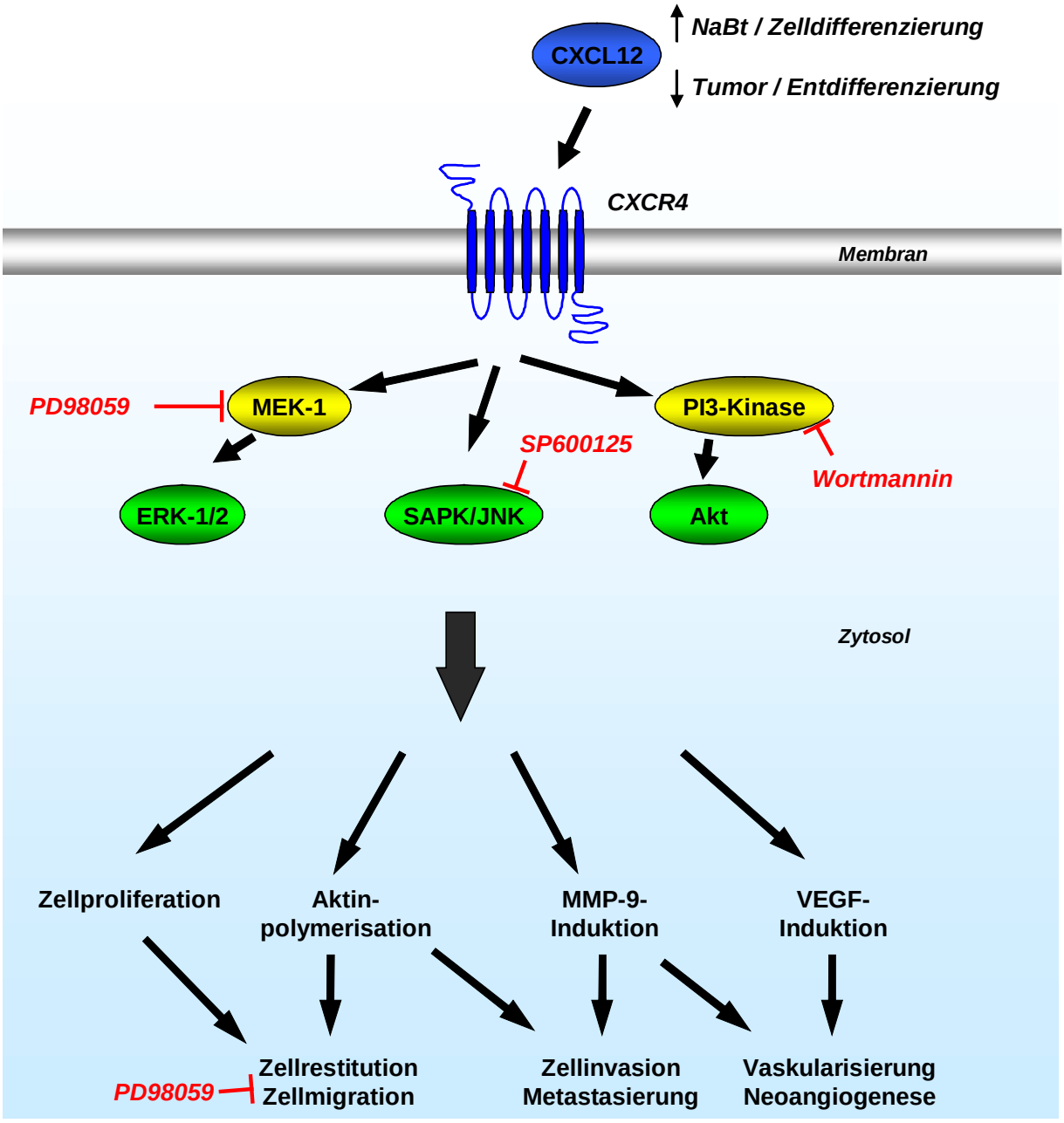

Abbildung 11. Übersicht über die von CXCR4/CXCL12 aktivierten Signalwege und vermittelten Funktionen in IEC.

6.1.2 Die zelldifferenzierungsabhängige Expression von CCR6 vermittelt ERK-1/2, SAPK/JNK und Akt-Aktivierung und führt zu gesteigerter Zellproliferation und Zellmigration von kolorektalen Karzinomzellen

Manuskript [2]: Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Göke B, Eichhorst ST, **Dambacher J**. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK and Akt signaling resulting in proliferation and migration of colorectal cancer cells. *J Cell Biochem* 2006 Mar 1; 97(4): 709-23.

CCR6-defiziente Mäuse zeigen Ausfälle in der Organogenese der Peyer-Plaques und weisen eine geschwächte mukosale Immunantwort auf orale Antigene und Pathogene auf [Cook et al., 2001; Varona et al., 2001], was auf einen wichtigen Beitrag von CCR6 zur Homöostase im GI-Trakt hinweist. Daher untersuchten wir die Funktionen dieses Chemokin-/Chemokinrezeptorsystems in IEC im Hinblick auf intestinale Entzündung sowie auf kolorektale Kanzerogenese. Der Chemokinrezeptor CCR6 wurde in allen von uns untersuchten IEC-Linien exprimiert [Brand et al., 2006d]. Seine Expression war differenzierungsabhängig, jedoch im Gegensatz zu CXCR4 mit einer verstärkten Expression in differenzierten Zellen [Brand et al., 2006d]. Übereinstimmend konnte von Dabelsteen et al. gezeigt werden, dass CCR6 ein später Marker für intestinale Zelldifferenzierung darstellt [Dabelsteen et al., 2003]. Konsistent hierzu fanden wir auch eine geringere Expression von CCR6 in weniger differenzierten Tumorzellen [Brand et al., 2006d]. Der CCR6-Ligand CCL20 wurde in allen untersuchten IEC-Linien nachgewiesen [Brand et al., 2006d], was konsistent mit einer bereits beschriebenen konstitutiven Expression in Kolonepithelzellen, speziell Follikel-assozierten Epithelzellen, ist [Izadpanah et al., 2001; Tanaka et al., 1999; Williams, 2006]. CCL20 übt hier wichtige homöostastische Funktionen aus, indem es für die Rekrutierung von unreifen DCs in die Peyer-Plaques sorgt [Cook et al., 2000; Iwasaki und Kelsall, 2000; Tanaka et al., 1999]. Ein Fehlen von CCR6 resultiert folglich in einer defekten Akkumulation von DCs in der intestinalen Mukosa [Cook et al., 2000] sowie einer gestörten CD4+ T_{reg}- und M-Zell-Entwicklung [Lugering et al., 2005].

Wir konnten zeigen, dass CCL20 in CRC-Zellen Proliferation, aber auch dosisabhängig Migration induziert [Brand et al., 2006d], was auf eine wichtige Rolle von CCR6 bei der zielgerichteten Migration, d.h. der Organ-spezifischen Metastasierung von kolorektalen Tumoren hindeutet. Andere Arbeitsgruppen demonstrierten ein Auftreten von CCR6-positiven Metastasen von kolorektalen Tumoren hauptsächlich in der Leber, die ein Hauptproduktionsort von CCL20 ist [Rubie et al., 2006b]. Darüber hinaus exprimieren CRC-

Patienten, die Lebermetastasen entwickelten, mehr CCL20 in der Leber als nicht betroffene Kontrollgruppen [Rubie et al., 2006b]. Außerdem ist eine verstärkte CCR6-Expression auf den Zellen des Primärtumors mit der Präsenz von Lebermetastasen assoziiert [Ghadjar et al., 2006]. Auch bei anderen Tumorarten wie dem Schilddrüsen- oder dem Ovarkarzinom findet sich eine CCR6-Überexpression in den Metastasen und damit assoziiert ein leberspezifisches Metastasierungsmuster [Dellacasagrande et al., 2003].

CCL20 hingegen war bei den von uns untersuchten Tumorgewebeschnitten in Lebermetastasen überhaupt nicht exprimiert [Brand et al., 2006d]. In kolorektalen Adenomen und CRC fanden wir eine Expression von CCL20 in fast allen Proben, wobei seine Expression invers mit der Invasivität der Tumoren, gegliedert nach der TNM-Klassifikation, korrelierte [Brand et al., 2006d]. Bemerkenswerterweise gibt es Ansätze, das Tumorwachstum durch die gezielte intratumorale Expression von CCL20 zu hemmen; denn CCL20 ist ein wichtiger chemotaktischer Faktor für unreife DCs, die den Tumor infiltrieren und dort eine tumor-spezifische Immunantwort (z. B. durch eine Aktivierung zytotoxischer T-Zellen) auslösen können [Crittenden et al., 2003; Furumoto et al., 2004; Fushimi et al., 2000]. Eine Herunterregulation der CCL20-Expression, wie von uns beobachtet [Brand et al., 2006d], könnte hier eine Reaktion des Tumors sein, der Erkennung und Eliminierung durch das Immunsystem zu entgehen. Ein weiterer, sehr interessanter therapeutischer Ansatz beruht auf der Applikation eines Fusionskonstrukts von CCL20 und einem normalerweise nicht immunogenen Tumorantigen [Biragyn et al., 2001]. Dieses rekrutiert unreife DCs und löst dadurch eine Reaktion des Immunsystems gegen die Tumorzellen aus [Biragyn et al., 2001], was die wichtige Rolle von CCL20 als Immunmediator unterstreicht.

Neben seiner konstitutiven Expression unter Normalbedingungen beobachteten wir unter Entzündungsbedingungen eine verstärkte Expression von CCL20, so nach der Inkubation mit proinflammatorischen Stimuli wie TNF-α, IL-1β oder LPS [Brand et al., 2006d]. Bemerkenswerterweise konnte CCL20 auch seine eigene Expression erhöhen [Brand et al., 2006d], außerdem induzierte es neben einer Aktivierung von MAP-Kinasen und Akt eine Expression von IL-8 [Brand et al., 2006d], was auf proinflammatorische Funktionen dieses Zytokins hinweist. Entsprechend fanden wir auch eine erhöhte Expression von CCL20 in entzündeten Kolonbiopsien von MC-Patienten im Vergleich zu nicht entzündeten Biopsien [Brand et al., 2006d], die stark mit der Expression von IL-8 korrelierte [Brand et al., 2006d]. Dies ist konform mit Daten anderer Studien, in denen ebenfalls eine verstärkte Expression von CCL20 in der Kolonmukosa von CED-Patienten sowie im peripheren Blut gezeigt wurde [Kaser et al., 2004; Kwon et al., 2002; Lee et al., 2005; Puleston et al., 2005]. Auch in einem

Mausmodell der Kolitis (TNBS-Kolitis) wurde eine verstärkte CCL20-Expression im Vergleich zu Kontrollmäusen beobachtet, wobei die Behandlung mit einem neutralisierenden CCL20-Antikörper eine Verbesserung der Kolitis bewirkte [Katchar et al., 2007], was auf eine wichtige Rolle von CCL20 bei der Pathogenese von CED hindeutet.

Interessanterweise hat CCL20 strukturelle Ähnlichkeiten mit Defensinen, kleinen antimikrobiellen Peptiden [Hoover et al., 2002]. Auf der einen Seite können diese ebenfalls CCR6 als Rezeptor nutzen [Yang et al., 1999]; auf der anderen Seite wurden auch für CCL20 antimikrobielle Eigenschaften beschrieben, die zum Teil stärker als die der Defensine waren [Hoover et al., 2002; Yang et al., 2003]. Die konstitutive Expression von CCL20 im intestinalen Epithel könnte hier einen Mechanismus darstellen, die intestinale Homöostase durch eine konstitutive antimikrobielle Aktivität aufrechtzuerhalten.

Weitere Implikationen für eine bedeutende Rolle von CCR6/CCL20 bei CED ergeben sich aus neueren Untersuchungen, in denen eine CCR6-Expression auf den erst kürzlich entdeckten Th17-Zellen beschrieben wurde [Acosta-Rodriguez et al., 2007]. Diese T-Zellen werden mit verschiedenen entzündlichen Erkrankungen, u. a. auch mit der Pathogenese von MC, in Verbindung gebracht [Fujino et al., 2003; Iwakura und Ishigame, 2006; Nielsen et al., 2003; Yen et al., 2006; Zhang et al., 2006]. Die Anwesenheit von CCR6 alleine jedoch definiert nicht den Th17-T-Zelltyp, da auch Th1-Zellen diesen Rezeptor exprimieren [Acosta-Rodriguez et al., 2007]. Eine gemeinsame Expression von CCR6 sowie CXCR3 ist spezifisch für Th1-Zellen, die neben IFN-γ auch geringe Mengen IL-17 produzieren [Acosta-Rodriguez et al., 2007]. Die Expression von CCR6 zusammen mit CCR4 hingegen charakterisiert Th17-Zellen, die IL-17, aber kein IFN-γ bilden [Acosta-Rodriguez et al., 2007]. In jedem Fall ist jedoch die Expression von CCR6 Voraussetzung für die Produktion der Th17-charakterisierenden Zytokine IL-17 sowie IL-22 [Acosta-Rodriguez et al., 2007]. Umgekehrt wird die Expression von CCL20 in Epithelzellen durch IL-17 hochreguliert [Kao et al., 2005], d. h. es könnte sich hier um einen positiven Feedback-Loop zwischen Th17-Zellen und Epithelzellen handeln. Bemerkenswerterweise produzieren Th17-Zellen neben IL-17, IL-22 und IL-26 auch CCL20 [Langrish et al., 2005; Wilson et al., 2007], was einen autokrinen Mechanismus nahelegt.

Zusammenfassend (siehe Abbildung 12) wurde von unserer Arbeitsgruppe demonstriert, dass CCR6 auf kolorektalen Karzinomzellen und auf von diesen abstammenden metastatischen Zellen exprimiert wird, während sein Ligand CCL20 nur im Primärtumor, jedoch nicht in Lebermetastasen vorkommt. Bindung von CCL20 an seinen Rezeptor resultiert in der Aktivierung von Akt über den PI3-Kinase-Weg sowie von ERK-1/2 und SAPK/JNK, was

teilweise über MEK-1 vermittelt wird. Daraus resultiert eine gesteigerte Zellproliferation und Zellmigration in IEC. Außerdem hat CCL20 proinflammatorische Funktionen und ist bei CED verstärkt exprimiert. Somit sind CCR6 und sein Ligand im Kolon von wichtiger Bedeutung sowohl bei intestinaler Entzündung als auch bei der zielgerichteten Migration von IEC unter physiologischen als auch pathologischen Bedingungen.

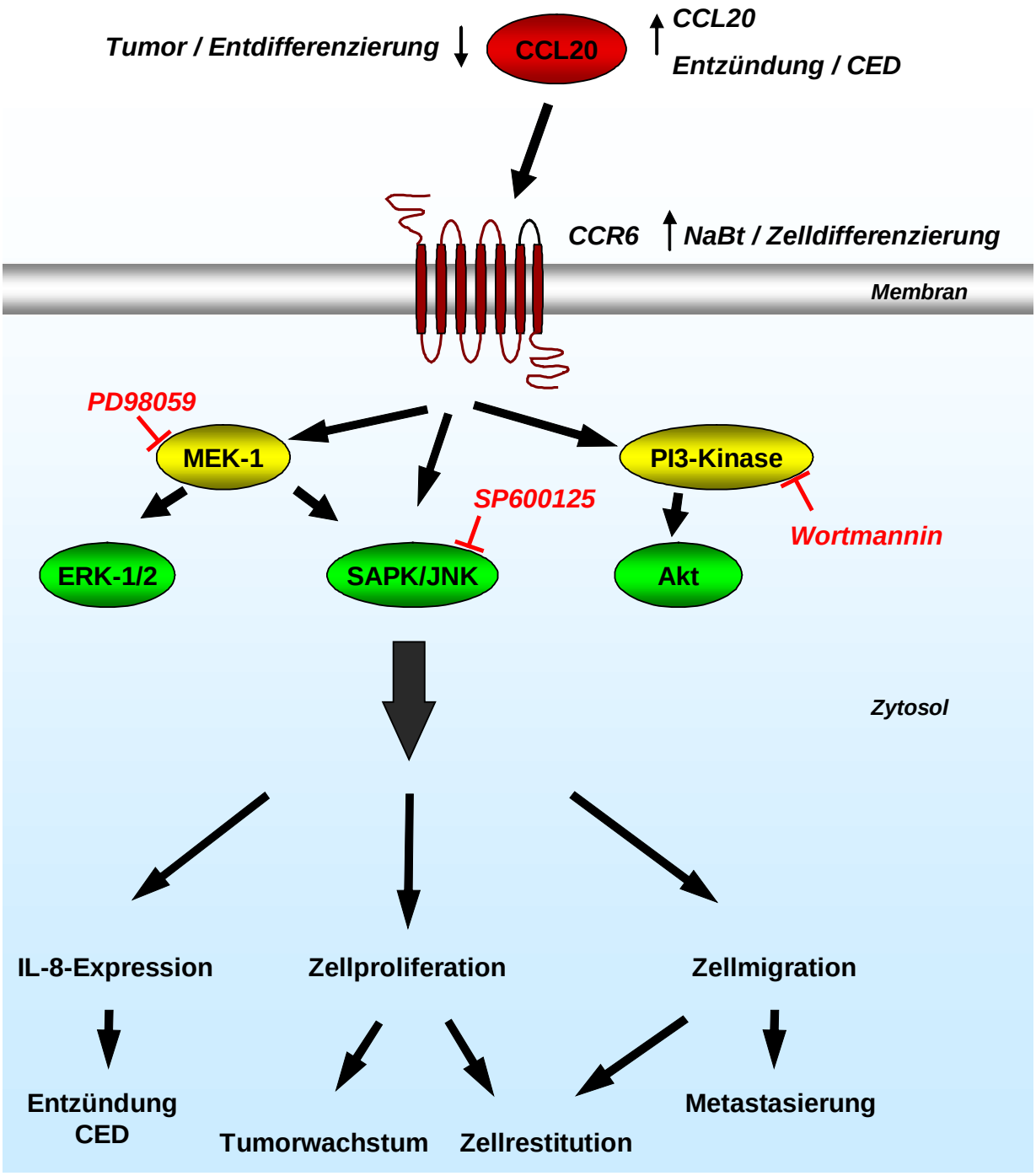

Abbildung 12. Übersicht über die von CCR6/CCL20 aktivierten Signalwege und vermittelten Funktionen in IEC.

6.1.3 CXCL16 ist ein neuer Marker intestinaler Entzündung mit einem prädisponierenden Polymorphismus für ileale Krankheitslokalisation bei Morbus Crohn-Patienten

Manuskript [3]: **Dambacher J**, Seiderer J, Niess JH, Haller D, Diebold J, Göke B, Ochsenkühn T, Reinecker HC, Brand S. CXCL16 is a novel marker of intestinal inflammation in Crohn's disease. *Inflamm Bowel Dis* 2007; submitted.

Manuskript [4]: Seiderer J*, **Dambacher J***, Leistner D, Tillack C, Glas J, Niess JH, Pfennig S, Jürgens M, Müller-Myhsok B, Göke B, Ochsenkühn T, Lohse P, Reinecker HC, Brand S. The role of the CXCL16 p.Ala181Val polymorphism in inflammatory bowel disease. *Clin Immunol* 2007; submitted. *** Geteilte Erstautorenschaft**

Das Chemokin CXCL16 kann sowohl membranständig als auch in löslicher Form auftreten. Präliminäre Daten wiesen auf eine proinflammatorische Rolle von CXCL16 bei entzündlichen Erkrankungen wie der rheumatoiden Arthritis hin, bei der sich erhöhte CXCL16-Spiegel in entzündeten Gelenken finden [Nanki et al., 2005; Ruth et al., 2006; van der Voort et al., 2005]. Daher wurde von uns hypothetisiert, dass dieses Chemokin und sein Rezeptor ebenfalls wichtige Funktionen bei intestinaler Entzündung ausüben. Eine Expression von CXCR6 und CXCL16 konnten wir in allen getesteten humanen und murinen Epithelzelllinien sowie in Geweben nachweisen [Dambacher et al., 2007c]. In letzteren ergab sich ein inverses Expressionsmuster: während CXCR6 am stärksten im Kolon exprimiert wurde, war CXCL16 v. a. im Duodenum, Jejunum und Ileum zu finden [Dambacher et al., 2007c]. Die Bindung von CXCL16 an seinen Rezeptor resultierte in der Aktivierung verschiedener Signaltransduktionswege wie den MAP-Kinasen ERK, SAPK/JNK und p38 sowie von Akt [Dambacher et al., 2007c].

Die Expression von CXCL16 (jedoch nicht von CXCR6) in IEC wurde durch proinflammatorische Signale wie TNF-α oder LPS reguliert [Dambacher et al., 2007c], was mit Daten aus anderen Zellsystemen übereinstimmt, in denen ebenfalls eine zunehmende CXCL16-Expression nach Stimulation mit IFN-γ, TNF-α oder IL-1β demonstriert wurde [Abel et al., 2004; Hosokawa et al., 2007; van der Voort et al., 2005; Wagsater et al., 2004; Wuttge et al., 2004]. Außerdem haben wir in einem Mausmodell der chronischen Ileitis (TNFΔARE-Mäuse, die durch eine erhöhte TNF-α-Produktion gekennzeichnet sind) [Kontoyiannis et al., 1999] in ilealen Epithelzellen eine signifikant höhere CXCL16-Expression im Vergleich zu Wildtyp (WT)-Mäusen gemessen [Dambacher et al., 2007c].

Bemerkenswerterweise bewirkt eine Behandlung mit anti-TNF-α-Antikörpern (Infliximab) bei Patienten mit rheumatoider Arthritis eine Reduktion der CXCL16-Serumkonzentration [Kageyama et al., 2006], wodurch der Einfluss von TNF-α auf die CXCL16-Produktion unterstrichen wird.

Auch in einem murinen Modell, das auf der Induktion einer intestinalen Entzündung durch murine Zytomegalievirus- (MCMV)-Infektion beruht, stellten wir signifikant höhere CXCL16-Spiegel im Vergleich zu nicht infizierten Kontrollmäusen fest [Dambacher et al., 2007c]. In Übereinstimmung zu den Tiermodellen intestinaler Entzündung fanden wir bei MC-Patienten ebenfalls eine gesteigerte CXCL16-Expression [Dambacher et al., 2007c]. Hierbei konnten wir bei diesen Patienten sowohl eine lokal erhöhte CXCL16-Expression in entzündeter Mukosa detektieren als auch eine systemische Erhöhung der CXCL16-Serumspiegel [Dambacher et al., 2007c]. Letztere waren bei Patienten mit aktivem MC signifikant höher als bei solchen mit nicht aktivem MC oder bei Kontrollpatienten [Dambacher et al., 2007c].

Diese Daten aus Mausmodellen und Patienten deuteten auf eine wichtige Rolle von CXCL16 bei der Pathogenese intestinaler Entzündung hin. Aus diesem Grund untersuchten wir zusätzlich den Einfluss eines kürzlich beschriebenen Polymorphismus' (p.A181V) im *CXCL16*-Gen [Lundberg et al., 2005] auf die Pathogenese und phänotypische Ausprägung von MC. Die verschiedenen Allele waren hierbei in unserer Kontrollgruppe und bei unseren MC-Patienten ähnlich verteilt, woraus sich folglich keine Assoziation dieses Polymorphismus' mit der Suszeptibilität für MC ableiten ließ [Seiderer et al., 2007]. Jedoch wiesen homozygote Patienten ein signifikant jüngeres Erkrankungsalter im Vergleich zu heterozygoten oder WT-Patienten auf [Seiderer et al., 2007]. Außerdem hatten diese Patienten ein erhöhtes Risiko für einen ilealen Befall, während WT-Patienten eine signifkant höhere Wahrscheinlichkeit für einen Befall des Kolons hatten [Seiderer et al., 2007].

Wurden die *CARD15*-Genotypen (p.Arg702Trp, p.Gly908Arg and p.Leu1007fsX1008) der Patienten in die Analyse mit einbezogen, so stellten wir bei Trägern einer der genannten *CARD15*-Varianten und des *CXCL16*-Valin-Allels eine signifikant höhere Inzidenz des stenosierenden oder penetrierenden Phänotyps fest im Vergleich zu Patienten, die nur eine *CARD15*-Mutation, aber das *CXCL16*-WT-Allel trugen [Seiderer et al., 2007]. Auch das Häufigkeit intestinaler Stenosen sowie ein Befall des Ileums war bei diesen Patienten signifikant höher [Seiderer et al., 2007].

Eine mögliche Erklärung für den ilealen Befall und den stenosierenden/penetrierenden Phänotyp bei Trägern des A181V-Polymorphismus' könnte in den physiologischen

Funktionen von CXCL16 bei der Erkennung und Eliminierung von Bakterien liegen. CXCL16 besitzt unter physiologischen Bedingungen *in vitro* eine ausgeprägte antimikrobielle Aktivität, die mit der von β-Defensin-2, einem antimikrobiellen Peptid, vergleichbar ist [Tohyama et al., 2007]. Die Pathogenese von MC wird allgemein mit einer veränderten Reaktion des Körpers auf intestinale Bakterien in Verbindung gebracht [Bamias und Cominelli, 2007; Comalada und Peppelenbosch, 2006; Sartor, 2006; Wehkamp und Stange, 2006]. Im terminalen Ileum, dem Übergang vom Dünndarm zum Dickdarm, kommt es zu einem plötzlichen starken Anstieg der luminalen Bakterienkonzentration. Eine gestörte Phagozytose von Bakterien bei MC-Patienten resultiert daher oft in einem Befall des terminalen Ileums. So konnte unsere Arbeitsgruppe zeigen, dass auch Mutationen in CX3CR1, dem Rezeptor für das Chemokin Fractalkine, in einem ilealen Krankheitsbefall resultieren [Brand et al., 2006c]. Die Expression von CX3CR1 auf DCs der Lamina propria ist entscheidende Voraussetzung für die Ausbildung von transepithelialen Dendriten zur Aufnahme von Bakterien aus dem Darmlumen [Brand et al., 2006c]. Zwar sind CX3CR1+ DCs in der gesamten Lamina propria zu finden, die intestinalen Dendriten bilden sich jedoch nur im terminalen Ileum aus, dem Darmabschnitt mit der höchsten Fractalkine-Expression [Niess et al., 2005]. Die von uns untersuchten Polymorphismen in CX3CR1 [Brand et al., 2006c] resultieren in einer schwächeren Affinität von CX3CR1 zu Fractalkine, außerdem in verminderter Rezeptorexpression [Faure et al., 2000] und beeinflussen somit möglicherweise die Aufnahme von Bakterien negativ. Auch die von CXCL16-vermittelte Phagozytose Gram-positiver und Gram–negativer Bakterien [Shimaoka et al., 2003] sowie sein Einfluss auf die CpG-induzierte Aktivierung der Immunantwort [Gursel et al., 2006] könnte durch den A181V-Polymorphismus beeinträchtigt sein und somit zu einem ähnlichen MC-Phänotyp führen. Die genaue funktionale Relevanz dieses Polymorphismus' muss allerdings noch geklärt werden.

Zusammenfassend (siehe Abbildung 13) zeigten wir eine Expression von CXCL16 und seinem Rezeptor CXCR6 in IEC, wobei CXCL16 verstärkt im proximalen Dünndarm, CXCR6 jedoch v. a. im distalen Kolon zu finden war. CXCL16 (jedoch nicht CXCR6) wird durch proinflammatorische Stimuli *in vitro* hochreguliert und ist auch in verschiedenen Mausmodellen intestinaler Entzündung sowie bei MC-Patienten lokal und systemisch verstärkt exprimiert. Der hier untersuchte Polymorphismus p. A181V im *CXCL16*-Gen ist bei MC-Patienten mit einem jüngeren Erkrankungsalter sowie einem ilealen Krankheitsbefall und dem Auftreten eines stenosierenden oder penetrierenden Phänotyps assoziiert, was die

wichtige Rolle von CXCL16 bei der spezifischen Krankheitsausprägung von MC unterstreicht.

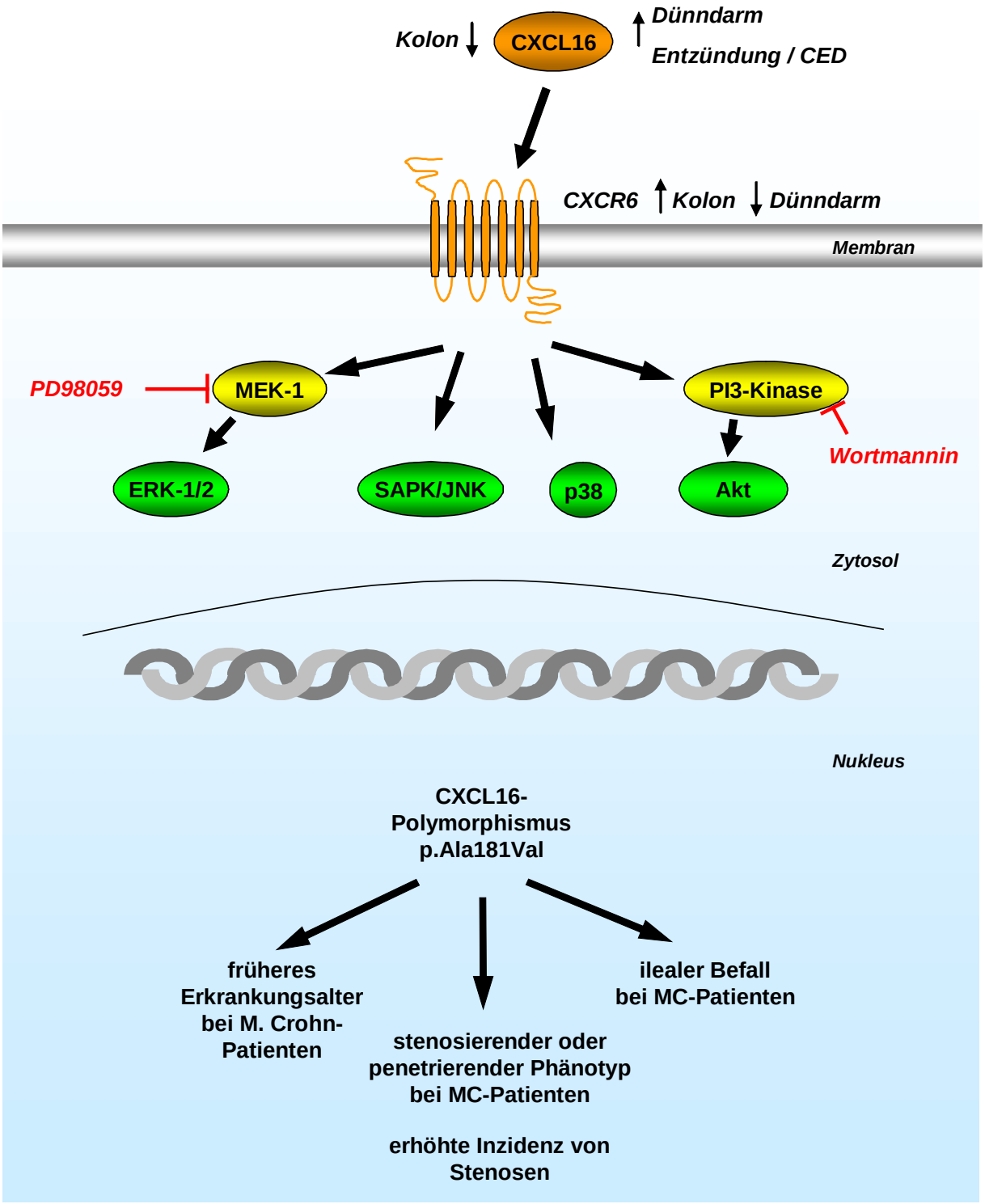

Abbildung 13. Übersicht über die von CXCR6/CXCL16 aktivierten Signalwege und vermittelten Funktionen in IEC.

6.2 Die Rolle von ausgewählten Zytokinen im Gastrointestinaltrakt

6.2.1 Typ I-Rezeptor-Zytokine: Das IL-6-verwandte Zytokin IL-31 ist ein zelldichteabhängiger Regulator der Proliferation von intestinalen Epithelzellen und Entzündungsmediator bei chronisch entzündlichen Darmerkrankungen

Manuskript [4]: **Dambacher J**, Beigel F, Seiderer J, Haller D, Göke B, Auernhammer CJ, Brand S. Interleukin-31 mediated signals modulate intestinal epithelial cell proliferation and its expression is up-regulated in intestinal inflammation. *Gut* 2007 Sep; 56(9): 1257-65.

IL-31 ist ein erst vor kurzer Zeit neu beschriebenes Zytokin, das mit IL-6 verwandt ist. Präliminäre Daten weisen auf Funktionen dieses Zytokins und seines Rezeptors bei der Pathogenese von entzündlichen Hauterkankungen hin [Bilsborough et al., 2006; Dillon et al., 2004; Sonkoly et al., 2006; Takaoka et al., 2006; Takaoka et al., 2005]. Wir konnten in dieser Arbeit erstmals zeigen, dass IEC den Rezeptorkomplex für IL-31 und auch das Zytokin selbst exprimieren [Dambacher et al., 2007a]. Die Expression v. a. von IL-31RA, aber auch von IL-31 und OSMR, wurde durch NaBt, das eine Zelldifferenzierung bewirkt [Chung et al., 1985], stark aktiviert [Dambacher et al., 2007a]. Ähnliche Beobachtungen wurden auch für das verwandte Zytokin IL-6 und seine Rezeptoruntereinheit gp130 beschrieben, bei denen NaBt ebenfalls die Expression in Ösophagus- oder Nasopharyngialkarzinomzellen bzw. myeloiden Leukämiezellen induzierte [Chow et al., 2003; Wang et al., 1999; Xie et al., 1999].
Nach der Bindung von IL-31 an seinen Rezeptorkomplex beobachteten wir eine Aktivierung verschiedener Signaltransduktionswege wie der MAP-Kinase ERK, die Phosphorylierung von Akt sowie der Transkriptionsfaktoren STAT1 und STAT3 [Dambacher et al., 2007a], was auf eine funktionale Rolle von IL-31 in IEC hinwies. Eine ähnliche Signalübertragung durch IL-31 konnte auch in Glioblastom- und Melanomzellen [Chattopadhyay et al., 2007; Diveu et al., 2004] sowie in Lungenepithelzellen [Chattopadhyay et al., 2007] demonstriert werden.
Wir konnten differentielle Effekte von IL-31 auf die Zellproliferation nachweisen [Dambacher et al., 2007a]. Während sich IL-31 bei niedriger Zelldichte negativ auf die Zellproliferationsrate auswirkte, waren bei höherer Zelldichte eher proliferationsfördernde Effekte zu beobachten [Dambacher et al., 2007a]. Eine andere Studie wies für IL-31 antiproliferative Wirkungen durch eine veränderte Expression von Zellzyklusproteinen wie p27Kip1, Cyclin B1, cdc2, cdk6, mcm4 und Rb in Lungenepithelzellen nach [Chattopadhyay et al., 2007]. Diese Effekte wurden ebenfalls bei niedriger Zelldichte beobachtet [Chattopadhyay et al., 2007]. In IL-31R-defizienten Mäusen wurde eine gesteigerte T-Zell-Proliferation gemessen

[Perrigoue et al., 2007], was auch hier auf eine antiproliferative Rolle von IL-31 hinweist. Der von uns beobachtete antiproliferative Effekt von IL-31 ging bei höherer Zelldichte verloren und wurde sogar in einen, wenn auch schwachen, proliferationfördernden Effekt umgewandelt [Dambacher et al., 2007a]. Ähnliche bimodale Wirkungen auf die Zellproliferation sind auch für andere Zytokine wie TGF-β beschrieben [Boumediene et al., 2001]. Die Zelldichte spielt hierbei eine wichtige Rolle, da Zellen in dichten Kulturen der sogenannten Kontaktinhibition unterliegen. Hierbei kommt es zu einer veränderten Expression von Zellzyklusproteinen wie einer Herunterregulation von Zyklinen [Li et al., 2004a] und einer Hochregulation von p27Kip1 [Yanagisawa et al., 1999], wie sie auch von IL-31 vermittelt werden [Chattopadhyay et al., 2007]. Dies erklärt den relativen Verlust des antiproliferativen Effekts von IL-31 in dichten Zellkulturen, die per se schon eine niedrige Zellproliferation aufweisen.

Ähnlich wie in Keratinozyten wiesen wir für IL-31 in IEC proinflammatorische Funktionen nach, da es die Produktion des proinflammatorischen Chemokins IL-8 in den Zellen förderte [Dambacher et al., 2007a]. Dies ist übereinstimmend mit einer kürzlich publizierten Studie an subepithelialen Myofibroblasten, in denen IL-31 ebenfalls proinflammatorische Chemokine und Zytokine wie IL-8, IL-6, aber auch MMPs induzierte [Yagi et al., 2007]. Bemerkenswerterweise wurde in unseren Experimenten die Expression sowohl von IL-31 als auch von seinen beiden Rezeptoruntereinheiten, IL-31RA und OSMR, durch proinflammatorische Stimuli hochreguliert [Dambacher et al., 2007a], was auf einen positiven Feedback-Loop hinweist. Auf Keratinozyten, einer anderen Epithelzellart, wurde ebenfalls eine erhöhte IL-31R-Expression bei Patienten mit entzündlicher Hauterkrankung (atopischer Dermatitis) im Vergleich zu gesunden Kontrollen gemessen [Bilsborough et al., 2006].

Während wir in einem Mausmodell der Ileitis (TNFΔARE) keine signifikant erhöhte Expression von IL-31, IL-31RA oder OSMR im Vergleich zu WT-Mäusen feststellen konnten, zeigten wir in humanen Kolonbiopsien von CED-Patienten eine verstärkte Expression aller drei Gene im entzündeten Gewebe im Vergleich zu nicht entzündeten Abschnitten [Dambacher et al., 2007a].

MC gilt allgemein als eine Erkrankung mit einer verstärkten Th1- und Th17-Zytokinproduktion, während CU durch eine verstärkte Th2-Zytokinproduktion gekennzeichnet ist. Obwohl es sich bei IL-31 um ein Th2-Zytokin handelt [Dillon et al., 2004; Liang et al., 2006], war nicht nur bei CU-, sondern auch bei MC-Patienten eine verstärkte Expression von IL-31 und seinem Rezeptor zu beobachten. Dies kann dadurch erklärt werden, dass durch IL-31 auch eine Limitierung der Th2-Immunantwort vermittelt wird [Perrigoue et

al., 2007]. So findet sich z. B. in IL-31R-defizienten Mäusen eine erhöhte Th2-Zytokin-Produktion, die Th1-Immunantwort ist jedoch nicht beeinträchtigt [Perrigoue et al., 2007].

Im Gegensatz zu seinen Funktionen bei CED ist die proinflammatorische Rolle von IL-31 bei Hautentzündungen, speziell der atopischen Dermatitis, bereits umfangreicher von verschiedenen Arbeitsgruppen untersucht worden [Bilsborough et al., 2006; Dillon et al., 2004; Sonkoly et al., 2006; Takaoka et al., 2006; Takaoka et al., 2005]. Bei Patienten mit atopischer Dermatitis ist eine erhöhte IL-31- und IL-31R-Expression zu beobachten [Bilsborough et al., 2006; Sonkoly et al., 2006], ähnlich zu der hier beobachteten erhöhten Expression bei CED-Patienten [Dambacher et al., 2007a]. Bemerkenswerterweise gibt es eine Assoziation von intestinaler Entzündung und atopischer Dermatitis [Majamaa et al., 1996; Niwa et al., 2004]. Außerdem wurden bei Patienten mit atopischer Dermatitis Störungen in der intestinalen Permeabilität im Vergleich zu Kontrollgruppen berichtet [Dupont et al., 1989; Jackson et al., 1981]. IL-31-produzierende T-Zellen könnten daher die Hautreaktionen, die bei vielen CED-Patienten beobachtet werden, vermitteln. Obwohl wir präliminäre Daten für eine Rolle von IL-31 bei intestinaler Entzündung gezeigt haben [Dambacher et al., 2007a], sind weitere Studien nötig, um die exakten Funktionen und Wirkungen von IL-31 und seinem Rezeptor bei der Pathogenese von CED aufzuklären.

Zusammenfassend (siehe Abbildung 14) zeigte unsere Arbeitsgruppe eine Expression des IL-31-Rezeptorkomplexes, bestehend aus IL-31RA und OSMR, auf IEC sowie dessen zelldifferenzierungs- und entzündungsabhängige Regulation. Bindung von IL-31 an seinen Rezeptor resultiert in der Aktivierung von ERK, SAPK/JNK, Akt sowie von STAT1 und STAT3. IL-31 induziert IL-8-Expression und ist in entzündeten Arealen bei CED-Patienten hochreguliert. Außerdem beeinflusst es die Zellproliferationsrate in Abhängigkeit von der Zelldichte und induziert eine verstärkte Wundheilung in IEC.

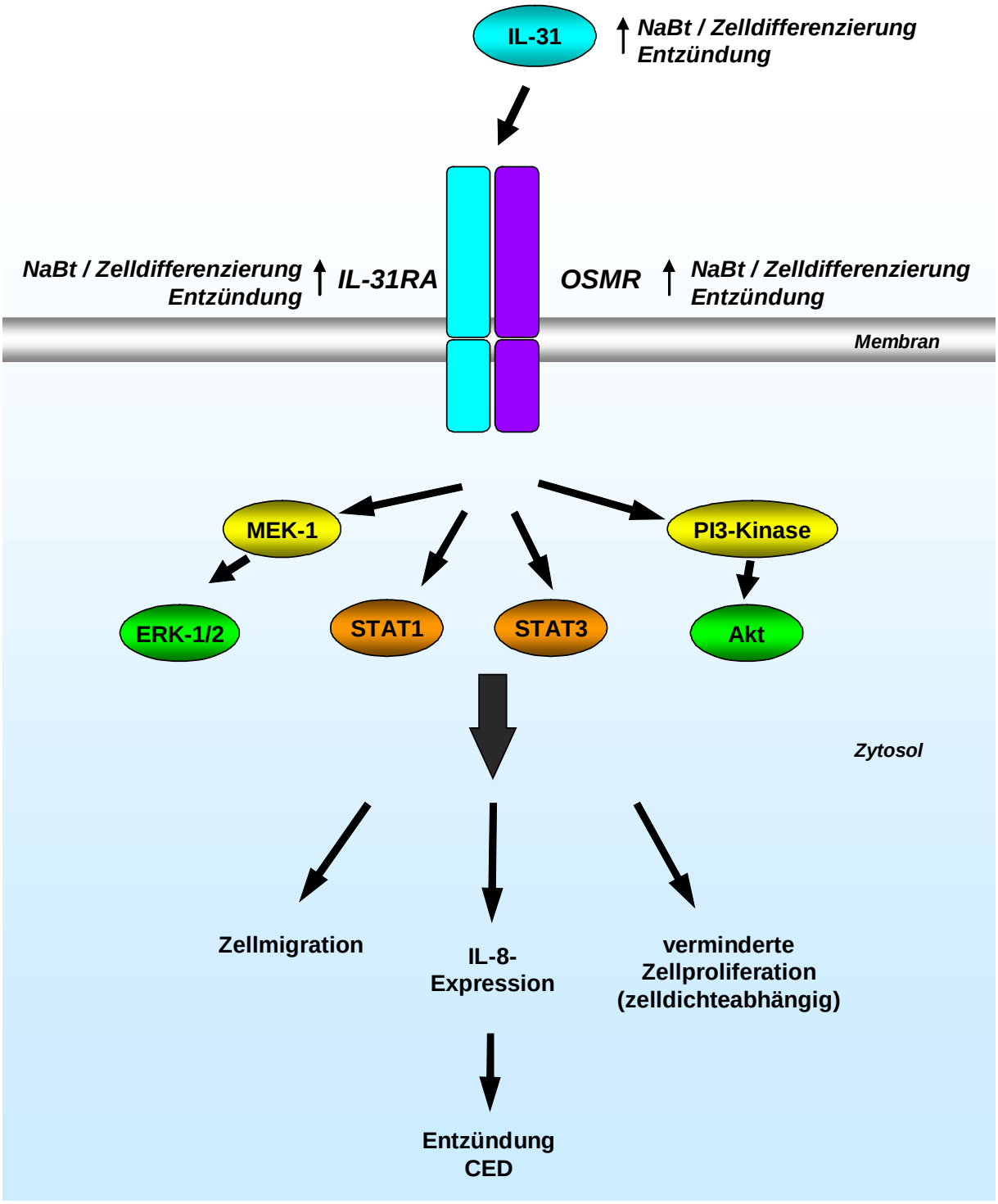

Abbildung 14. Übersicht über die von IL-31 vermittelten Funktionen in IEC.

6.2.2 Typ II-Rezeptor-Zytokine: IL-10-ähnliche Zytokine

6.2.2.1 IL-22, ein proinflammatorisches Zytokin, fördert die Integrität der intestinalen Barriere durch Stimulation von Zellproliferation, Zellmigration und β-Defensin-2-Expression in intestinalen Epithelzellen

Manuskript [5]: Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkühn T, Göke B, Auernhammer CJ**, Dambacher J**. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* 2006 Apr; 290(4): G827-38

Die proinflammatorische Rolle von IL-22 wurde bisher v. a. im Zusammenhang mit entzündlichen Erkrankungen der Haut demonstriert [Boniface et al., 2005; Wolk et al., 2004; Wolk et al., 2006]. Wir analysierten erstmals die Expression und die Funktionen von IL-22 im GI-Trakt, speziell unter Bedingungen intestinaler Entzündung [Brand et al., 2006a]. Wir konnten hier zeigen, dass IEC den funktionalen IL-22-Rezeptorkomplex exprimieren [Brand et al., 2006a], was auf den GI-Trakt als ein wichtiges Ziel- bzw. Wirkorgan von IL-22 hindeutet. Generell sind epitheliale Gewebe wie die Haut oder der Atemwegstrakt aufgrund ihrer IL-22R-Expression Zielorgane von IL-22 [Wolk et al., 2004; Wolk und Sabat, 2006].
Bemerkenswerterweise wurde in unseren Experimenten die spezifische Rezeptoruntereinheit IL-22R1 in IEC durch proinflammatorische Stimuli hochreguliert, während die Expression von IL-10R2 unverändert blieb [Brand et al., 2006a]. Demzufolge ist der IL-22-Rezeptor unter entzündlichen Bedingungen von wichtiger Bedeutung. Umgekehrt stellten wir eine aktivierende Wirkung von IL-22 auf die Expression proinflammatorischer Gene wie IL-8 und TNF-α fest. Aber auch die Expression von SOCS-3, einem negativer Regulator der STAT3-vermittelten Signaltransduktion [Kubo et al., 2003], wurde via STAT3 aktiviert [Brand et al., 2006a]. Bemerkenswerterweise zeigten Studien, dass STAT3 sowohl in einem Mausmodell der Kolitis (SAMP1/Yit-Mäuse) [Mitsuyama et al., 2006] als auch bei Patienten mit CED hochreguliert ist, nicht aber bei gesunden Probanden [Lovato et al., 2003; Musso et al., 2005; Suzuki et al., 2001]. Bei CED-Patienten ist es nur in Arealen aktiver Entzündung phosphoryliert [Musso et al., 2005]. In einem Mausmodell der Kolitis wiesen transgene Mäuse mit einem nicht funktionsfähigen SOCS-3-Protein eine stärkere STAT3-Aktivierung und eine stärkere Kolitis im Vergleich zu WT-Mäusen auf [Suzuki et al., 2001]. In hepatischen Zellen konnte unsere Arbeitsgruppe zeigen, dass IL-22 ebenfalls die Expression

von SOCS-3 aktiviert, und dass eine Überexpression von SOCS-3 die IL-22-vermittelte STAT-Aktivierung sowie die IL-22-vermittelte Zellmigration und Zellrestitution verhindert [Brand et al., 2007b]. Es ist zu vermuten, dass ein ähnlicher negativer Feedback-Loop auch in IEC die Funktionen von IL-22 reguliert.

Während der IL-22-Rezeptorkomplex auf IEC und hepatischen Zellen exprimiert war, konnten wir keine Expression von IL-22 selbst beobachten [Brand et al., 2006a; Brand et al., 2007b]. Dies ist übereinstimmend mit bereits bekannten Daten, die IL-22 als ein T-Zell-spezifisches Zytokin charakterisieren [Liang et al., 2006; Xie et al., 2000; Zheng et al., 2006], während der Rezeptor auf epithelialen Organen exprimiert wird [Wolk et al., 2004]. Zunächst wurden Th1-Zellen als Hauptquelle für IL-22 beschrieben [Gurney, 2004; Wolk et al., 2002], in neuesten Studien stellten sich jedoch Th17-Zellen als Hauptproduzenten für IL-22 heraus [Chung et al., 2006; Liang et al., 2006; Zheng et al., 2006]. Bemerkenswerterweise werden diese (relativ neu entdeckten) Th17-Zellen inzwischen als wichtige proinflammatorische Effektor-T-Zellen bei der Pathogenese diverser entzündlicher Autoimmunerkrankungen gesehen, wie an Mausmodellen der Kollagen-induzierten Arthritis oder der experimentellen Autoimmunenzephalitis gezeigt werden konnte [Iwakura und Ishigame, 2006]. Sie werden aber auch als kritischer Faktor bei der Pathogenese von MC betrachtet [Fujino et al., 2003; Nielsen et al., 2003; Yen et al., 2006; Zhang et al., 2006]. Die Entwicklung von Th17-Zellen ist abhängig vom Einfluss der Zytokine TGF-β, IL-6, IL-21 und IL-23 bei Mäusen [Bettelli et al., 2006; Mangan et al., 2006; Nurieva et al., 2007; Veldhoen et al., 2006; Zhou et al., 2007] bzw. IL-1β, IL-6 und IL-23 beim Menschen [Acosta-Rodriguez et al., 2007; Chen et al., 2007; Wilson et al., 2007]. Das Gen für den IL-23-Rezeptor (*IL23R*) wurde kürzlich als ein neues MC-Suszeptibilitätsgen beschrieben [Duerr et al., 2006], was auch von unserer Arbeitsgruppe in einer großen Kohorte von MC-Patienten bestätigt werden konnte [Glas et al., 2007c]. Interessanterweise konnten wir in weiteren Untersuchungen zeigen, dass die Proteinexpression von IL-22 im peripheren Blut mit dem Genotyp von *IL23R* korrelierte: Bei Trägern von MC Risiko-assoziierten Genotypen fanden wir signifikant höhere IL-22-Spiegel als bei Trägern risikovermindernder Allele, wodurch wir erstmal einen funktionalen Zusammenhang zwischen *IL23R*-Genotypen und Th17-Zytokinexpression zeigten [Schmechel et al., 2007].

Entsprechend der bei CED-Patienten verstärkt auftretenden Infiltration der Mukosa mit Th1- und Th17-Zellen fanden wir in den von uns untersuchten Kolonbiopsien von MC- und CU-Patienten eine signifikant erhöhte Expression von IL-22 in entzündetem im Vergleich zu nicht entzündetem Gewebe, die sehr stark mit der IL-8-Expression in diesen Proben korrelierte

[Brand et al., 2006a]. Hierbei war der Anstieg der IL-22-Expression bei MC-Patienten deutlich höher als bei CU-Patienten [Brand et al., 2006a], was durch die verstärkte Infiltration der Mukosa speziell mit Th17- und Th1-Zellen erklärt werden kann, ein Charakteristikum für MC. Zusätzlich konnten wir neben einer erhöhten IL-17-Produktion auch eine erhöhte IL-22-Expression im einem Maus-DSS-Kolitis-Modell nachweisen [Brand et al., 2006a]. In einer weiteren Studie wurde eine erhöhte Anzahl von IL-22-positiven Zellen in der Mukosa von CU- und MC-Patienten detektiert [Andoh et al., 2005]. Außerdem zeigte sich in Mausmodellen der Kolitis (DSS-Kolitis, CD45RB Transfer-Modell) eine erhöhte IL-22-Produktion [Te Velde et al., 2006]. Diese erhöhte IL-22-Expression und die Induktion proinflammatorischer Zytokine durch IL-22 in IEC deuten auf eine wichtige pro-inflammatorische Funktion dieses Zytokins bei intestinaler Entzündung hin. In einer zusätzlichen Studie zeigten wir, dass die Expression von IL-22-Protein im peripheren Blut bei MC-Patienten signifikant höher ist als bei einer Kontrollgruppe [Schmechel et al., 2007]. Zusätzlich korrelierten die IL-22-Serumspiegel signifikant mit dem Crohn's disease activity index (CDAI) der Patienten [Schmechel et al., 2007]. Zu ähnlichen Ergebnissen kam auch eine kürzlich veröffentlichte Studie mit einer kleineren Gruppe von Patienten [Wolk et al., 2007]. Interessanterweise korrelierte bei unseren Patienten nur die IL-22-Expression signifikant mit dem CDAI, nicht jedoch die Expression anderer Entzündungsmarker wie TNF-α oder IL-6 [Schmechel et al., 2007]. Somit könnte eine Messung von IL-22, nicht jedoch TNF-α oder IL-6, von klinischer Relevanz zur Bestimmung der Krankheitsaktivität bei MC-Patienten darstellen.

Im Gegensatz zu oben beschriebenen Daten, die auf entzündungsfördernde, die CED-Erkrankung verschlechternde Eigenschaften von IL-22 hinweisen, wurde auf der anderen Seite ein protektiver Effekt von IL-22 im DSS-Kolitis-Modell nachgewiesen [Sugimoto et al., 2006]. Dies könnte zum einen auf der Tatsache beruhen, dass IL-22 in IEC die Produktion von IL-10, einem antiinflammatorischen Zytokin, stimuliert [Nagalakshmi et al., 2004b]. Zum anderen konnten wir zeigen, dass IL-22 in IEC die Produktion von SOCS-3 induziert [Brand et al., 2006a], einem negativen Regulator des Jak/STAT-Signalweges. Eine IEC-spezifische SOCS-3-Defizienz bei Mäusen führt zu Krypten-Hyperproliferation und Hyperplasie nach DSS-Gabe [Rigby et al., 2007]. Somit könnte die Induktion von SOCS-3 in IEC eine weitere protektive Funktion von IL-22 vermitteln.

Aber auch auf systemischer Ebene besitzt IL-22 einen protektiven Effekt bei intestinaler Entzündung. Kürzlich wurde von Wolk et al. gezeigt, dass eine systemische Gabe von IL-22 die Produktion des LPS-bindenden Proteins (LBP) in der Leber anregt, und dass sowohl im

Mausmodell der DSS-Kolitis als auch bei MC-Patienten erhöhte Plasma-LBP-Spiegel zu finden waren [Wolk et al., 2007]. Obwohl LBP in niedrigen Konzentrationen für eine verstärkte Wirkung von LPS auf Zellen verantwortlich ist [Wright et al., 1990], wirkt es in hohen Konzentrationen neutralisierend [Kitchens und Thompson, 2005], indem es LPS auf Lipoproteine überträgt [Wurfel et al., 1994; Wurfel und Wright, 1995]. Daher könnte IL-22 über eine Induktion von hohen Plasma-LBP-Spiegeln wie von Wolk et al. gemessen [Wolk et al., 2007], zu einer Verhinderung einer LPS-induzierten systemischen Entzündung beitragen.

Wir konnten weitere protektive Funktionen von IL-22 bei intestinaler Entzündung demonstrieren, die auf der Wiederherstellung bzw. Aufrechterhaltung der intestinalen Barriereintegrität basierten [Brand et al., 2006a]. Eine intakte intestinale Barriere ist von entscheidender Bedeutung für die intestinale Homöostase [Cobrin und Abreu, 2005; Fasano und Shea-Donohue, 2005]. IL-22 förderte in unseren Experimenten in signifikanter Weise die IEC-Zellrestitution nach Verletzung, was über einen PI3-Kinase/Akt-abhängigen Mechanismus, ähnlich wie von uns auch in hepatischen Zellen demonstriert [Brand et al., 2007b], reguliert wurde [Brand et al., 2006a]. Zusätzlich bewirkte IL-22 eine gesteigerte Zellproliferation und eine verstärkte Expression von β-Defensin-2 [Brand et al., 2006a]. Letzteres gehört zur Gruppe der Defensine, kleine kationische Peptide mit antimikrobieller Wirkung, die ein wichtiger Bestandteil der angeborenen Immunantwort sind [Dann und Eckmann, 2007]. Neuere Daten deuten darauf hin, dass die Pathogenese von MC mit einer verminderten Defensin-Produktion und somit mit einer verminderten antimikrobiellen Aktivität zusammenhängen könnte [Nuding et al., 2007; Wehkamp und Stange, 2006]. Bemerkenswerterweise resultieren MC-prädisponierende *CARD15*-Mutationen in verringerter Defensin-Produktion [Voss et al., 2006; Wehkamp et al., 2004]. Außerdem ist eine niedrige Anzahl von β-Defensin-2-Genkopien mit MC assoziiert [Fellermann et al., 2006]. Somit könnte IL-22 durch die Induktion von β-Defensin-2 eine protektive Rolle in Bezug auf CED ausüben. Diese Hypothese wird von anderen Daten unterstützt, welche einen protektiven Effekt von IL-22 bei intestinaler Entzündung beschrieben: IL-22-Gabe im DSS-Kolitis-Modell reduzierte die Entzündung und führte zu einer verstärkten Produktion von Mucus-assoziierten Molekülen und einem Anstieg der Zahl von Goblet-Zellen, während durch IL-22-Neutralisation eine Verschlechterung der Kolitis vermittelt wurde [Sugimoto et al., 2007; Sugimoto et al., 2006; Sugimoto et al., 2005].

Ob aber die proinflammatorischen oder die protektiven Eigenschaften von IL-22 oder eine Kombination von beiden *in vivo* entscheidend sind, müssen weitere Studien zeigen. Interessant wäre in diesem Zusammenhang auch die Untersuchung von IL-22-defizienten

Mäusen, die in einer erst kürzlich publizierten Studie erstmals beschrieben wurden [Zenewicz et al., 2007]. Phänotypisch waren diese Tiere unauffällig und auch ihr Immunstatus war unverändert, jedoch wiesen sie eine erhöhte Anfälligkeit für Concanavalin-A-induzierte Hepatitis im Vergleich zu WT-Mäusen auf [Zenewicz et al., 2007].

Aber nicht nur im Kontext intestinaler Entzündung, sondern auch bei der Entwicklung von Tumoren könnte IL-22 von Bedeutung sein, wie neueste Daten vermuten lassen. Erstaunlicherweise wurde kürzlich demonstriert, dass IL-22 das Mammakarzinomwachstum im Mausmodell hemmt, indem es die ERK- und Akt-Phosphorylierung sowie die Zellproliferation inhibiert [Weber et al., 2006]. Dies steht in Kontrast zu den Daten dieser [Brand et al., 2006a] und auch anderer Arbeiten, die eine proliferationsfördernde Wirkung von IL-22 und/oder eine Aktivierung von ERK und Akt zeigen konnten [Andoh et al., 2005; Brand et al., 2006a; Brand et al., 2007b; Ikeuchi et al., 2005; Lejeune et al., 2002; Wolk et al., 2006; Xu et al., 2001]. Eine Expression von IL-22 in Colon-26-Zellen (murine Kolonkarzinomzellen), die Mäusen implantiert wurden, zeigte keinen Effekt auf das Tumorwachstum, obwohl sich die Überlebenszeit der Tiere deutlich verlängerte [Nagakawa et al., 2004]. Von Ziesche et al. wurde vor kurzem publiziert, dass IL-22, synergistisch mit IFN-γ, die Expression der „inducible nitric oxide synthase“ (iNOS) in den kolorektalen Zelllinien DLD-1 und Caco-2 induziert [Ziesche et al., 2007]. Wie bereits in Abschnitt 1.1.2.2 erwähnt, findet sich bei MC und CU-Patienten eine durch proinflammatorische Zytokine induzierte erhöhte iNOS-Expression und in Folge eine erhöhte Produktion von reaktiven Sauerstoff- und Stickstoff-Molekülen [Kolios et al., 2004], die als Risikofaktor für die Kanzerogenese gelten [Marnett, 2000]. Die Rolle von IL-22 bei der Kanzerogenese bleibt daher widersprüchlich und weitere Studien sind nötig, um diesen Aspekt aufzuklären.

Zusammenfassend (siehe Abbildung 15) konnte von uns gezeigt werden, dass der IL-22-Rezeptorkomplex, bestehend aus IL22R1 und IL-10R2, in IEC funktional exprimiert ist und die Bindung von IL-22 in der Aktivierung von MAP-Kinasen, Akt sowie STAT1 und STAT3 resultiert. Die Expression der spezifischen Rezeptoruntereinheit IL-22R1 auf IEC wird bei Entzündung hochreguliert und auch IL-22 selbst wird bei intestinaler Entzündung im Mausmodell sowie bei CED verstärkt exprimiert und induziert proinflammatorische Gen-expression. Außerdem ist IL-22 ein Barriere-protektives Zytokin, da es in IEC eine gesteigerte Zellproliferation sowie verstärkte Zellrestitution nach Verletzung bewirkt. Zusätzlich induziert es die Expression von β-Defensin-2, einem antibakteriellen Peptid mit wichtigen Funktionen im angeborenen Immunsystem.

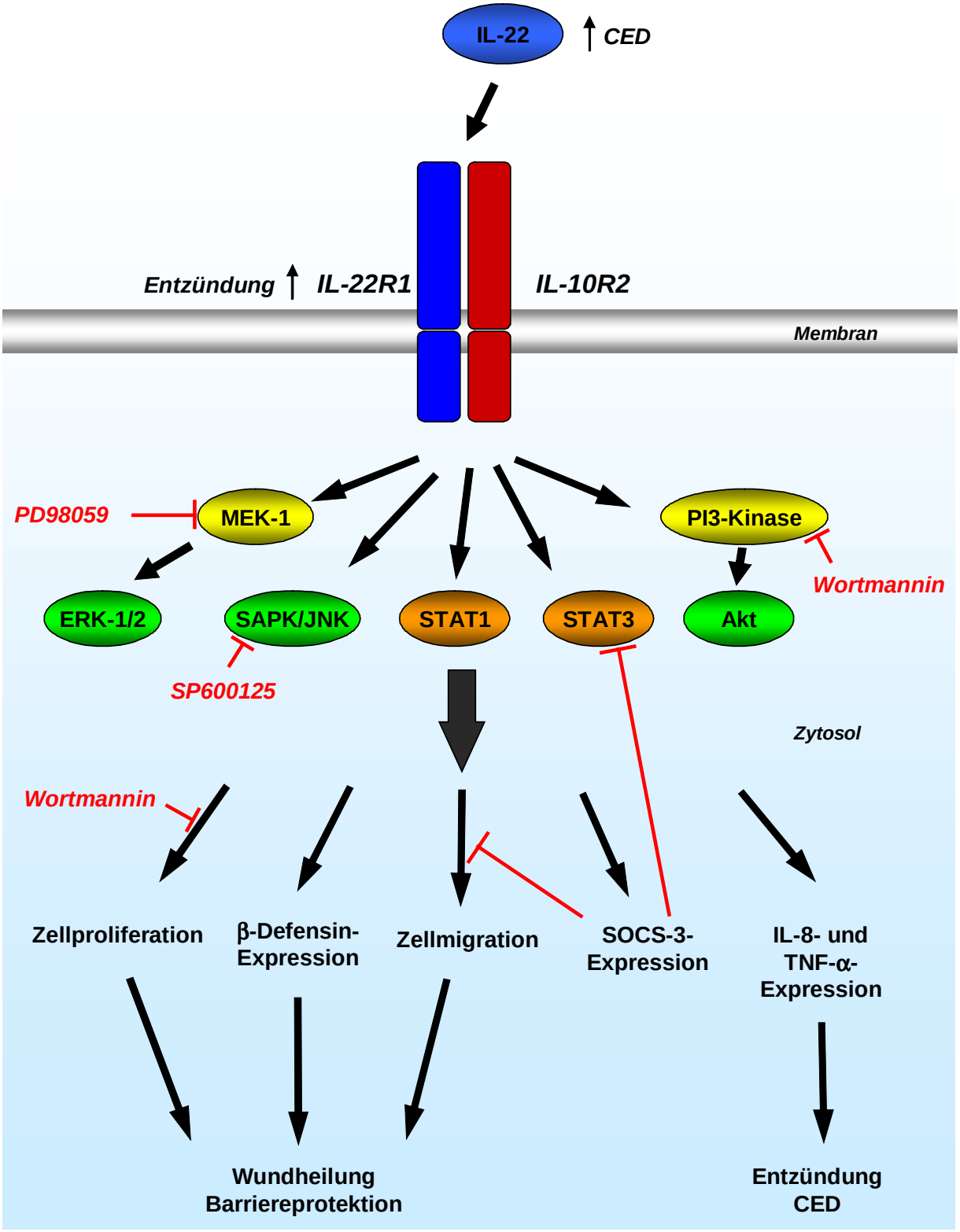

Abbildung 15. Übersicht über die von IL-22 vermittelten Funktionen in IEC.

6.2.2.2 IL-26: Ein Th1/Th17-Zytokin mit verstärkter Expression bei Patienten mit chronisch entzündlichen Darmerkrankungen und Vermittler pro-inflammatorischer, antiproliferativer Funktionen in intestinalen Epithelzellen

Manuskript [6]: **Dambacher J**, Beigel F, Zitzmann K, de Toni E, Göke B, Diepolder H, Auernhammer CJ, Brand S. The role of the novel IL-10 like cytokine IL-26 in intestinal inflammation. *Gut* 2007; submitted.

IL-26 ist ein Zytokin, das, ähnlich wie IL-22, von aktivierten T-Zellen, besonders von Th1- und Th17-Zellen, produziert wird [Nagalakshmi et al., 2004a; Wilson et al., 2007; Wolk et al., 2002]. Da bei der Pathogenese von CED Mukosa-infiltrierende T-Zellen eine wichtige Rolle spielen, könnte IL-26 unter Bedingungen intestinaler Entzündung wichtige Funktionen ausüben. Die Rolle dieses Zytokins in IEC und bei der Pathogenese von CED war bisher noch nicht erforscht und wurde von uns erstmals beschrieben. Wir konnten zeigen, dass der Rezeptorkomplex für IL-26, der aus den zwei Untereinheiten IL-20R1 und IL-10R2 besteht, in allen getesteten IEC-Linien exprimiert wurde [Dambacher et al., 2007b]. Außerdem war er auch in einigen hepatischen Zellen, jedoch nicht in HepG2-Zellen zu finden [Dambacher et al., 2007b]. Dies stimmt mit anderen Studien überein, die eine IL-20R1-Expression in epithelialen Geweben wie Lunge oder Haut demonstrierten, jedoch nicht auf hämato-poietischen Zellen [Hor et al., 2004; Nagalakshmi et al., 2004a; Sheikh et al., 2004]. Dieses Expressionsmuster ähnelt dem des verwandten Zytokin-Rezeptor-Systems IL-22/IL-22R, dessen Rezeptor ebenfalls auf epithelialem Gewebe zu finden ist [Wolk et al., 2004; Wolk und Sabat, 2006], während das Zytokin selbst von hämatopoietischen Zellen, insbesondere T-Zellen, gebildet wird [Gurney, 2004; Liang et al., 2006; Wolk et al., 2002]. In IEC induzierte IL-26 in unseren Experimenten die Genexpression der proinflammatorischen Zytokine IL-8, IL-6 und TNF-α [Dambacher et al., 2007b]. Insbesondere IL-6 und TNF-α spielen eine sehr wichtige Rolle bei der Pathogenese von MC [Clark, 2007; Mudter und Neurath, 2007] und eine Neutralisierung von TNF-α bzw. des IL-6-Rezeptors mit entsprechenden Antikörpern wird erfolgreich in der Therapie von CED und anderen entzündlichen Erkrankungen wie der rheumatoiden Arthritis eingesetzt [Siddiqui und Scott, 2005; Smolen und Maini, 2006].

Bei der im Rahmen dieser Arbeit untersuchten Expression von IL-26 in der Mukosa von CED-Patienten wurde eine verstärkte Expression in entzündetem Darmgewebe im Vergleich zu nicht entzündetem Gewebe beobachtet, die stark mit der IL-8-Genexpression korrelierte [Dambacher et al., 2007b]. Bemerkenswerterweise war der Anstieg in der Expression im

entzündeten Gewebe bei MC-Patienten höher als bei CU-Patienten [Dambacher et al., 2007b], was konsistent mit der Expression von IL-26 v. a. in Th1-Zellen ist, CU im Gegensatz zu MC jedoch als Th2-vermittelte Erkrankung gilt. Eine aktuelle Publikation berichtet eine IL-26-Expression auch in Th17-Zellen [Wilson et al., 2007], die ebenfalls vermehrt bei MC-Patienten zu finden sind.

Wir beobachteten in IEC eine Vermittlung der Signale von IL-26, ähnlich wie IL-22, über die Aktivierung der Transkriptionsfaktoren STAT1 und STAT3 [Dambacher et al., 2007b], die bei MC-Patienten verstärkt aktiviert sind [Lovato et al., 2003; Mudter et al., 2005; Schreiber et al., 2002]. IL-26 hemmte außerdem die Zellproliferationsrate, was kongruent zu den von uns beobachteten antiproliferativen Effekten von IL-28A und IL-29 ist [Brand et al., 2005a], zwei weiteren IL-10-ähnlichen Zytokinen [Brand et al., 2005a], jedoch im Gegensatz zur proliferationsfördernden Wirkung von IL-22 steht [Brand et al., 2006a].

Da die Expression von IL-26 in Herpesvirus saimiri-(HVS)-transformierten Zellen stark hochreguliert wird [Knappe et al., 2000], ließen sich antivirale Funktionen von IL-26, ähnlich wie von IL-28/IL-29 [Ank et al., 2006; Bartlett et al., 2005; Robek et al., 2005; Zhu et al., 2005], bei Virusinfektionen hypothetisieren. IL-26 induzierte in unseren Experimenten eine verstärkte Expression von IFN-α, IFN-β sowie von IRF-7 [Dambacher et al., 2007b], alles Gene, die antivirale Proteine hochregulieren. Auf der anderen Seite konnten wir jedoch keine Wirkung von IL-26 auf die Expression der Gene für die antiviralen Proteine MxA und 2’,5’-OAS beobachten [Dambacher et al., 2007b]. Entsprechend war in Experimenten mit HCV-Replikon-exprimierenden Zellen sowie in HCV-Infektionsversuchen keine direkte Hemmung der HCV-Replikation durch IL-26 festzustellen [Dambacher et al., 2007b]. Außerdem haben wir in mit HBV oder HCV infizierten Lebern keine verstärkte Expression von IL-26 im Vergleich zu nichtviralen Hepatopathien gemessen [Dambacher et al., 2007b]. Im Gegensatz hierzu konnten wir für die λ-Interferone IL-28A und IL-29, die wie IL-26 ebenfalls zur IL-10-Familie gehören, eine starke antivirale Aktivität in HCV-Infektions-und Replikationsexperimenten sowie bei CMV-Infektionen nachweisen, außerdem eine Hochregulation ihrer Expression bei HCV- oder MCMV-Infektionen *in vitro* und *in vivo* [Brand et al., 2007a; Brand et al., 2005a; Brand et al., 2006b]. Unsere Ergebnisse weisen daher für IL-26 auf eine Rolle bei intestinaler Entzündung, jedoch nicht bei Virusinfektionen hin. Die genauen Funktionen von IL-26 müssen jedoch noch geklärt werden. Die Tatsache, dass in der Maus bisher kein IL-26-Homolog nachgewiesen wurde [Fickenscher und Pirzer, 2004] und somit Studien an Mausmodellen nicht in Frage kommen, ist hierbei sicherlich limitierend.

Zusammenfassend (siehe Abbildung 16) demonstrierten wir, dass die beiden Untereinheiten IL-20R1 und IL-10R2, aus denen der IL-26-Rezeptorkomplex besteht, in IEC und hepatischen Zellen exprimiert werden. Durch IL-26 werden STAT-Proteine und MAP-Kinasen in IEC aktiviert, außerdem induziert es die Expression der proinflammatorischen Zytokine IL-8, IL-6 und TNF-α sowie von SOCS-3, einem negativen Regulator des STAT-Signalweges. In Darmbiopsien von CED-Patienten findet sich eine verstärkte Expression von IL-26 im entzündeten Gewebe. IL-26 aktiviert die antiviralen Gene IRF-7, IFN-α und IFN-β, jedoch nicht 2',5'-OAS oder MxA und zeigt keine direkte antivirale Wirkung in HCV-Replikations- und Infektionsexperimenten.

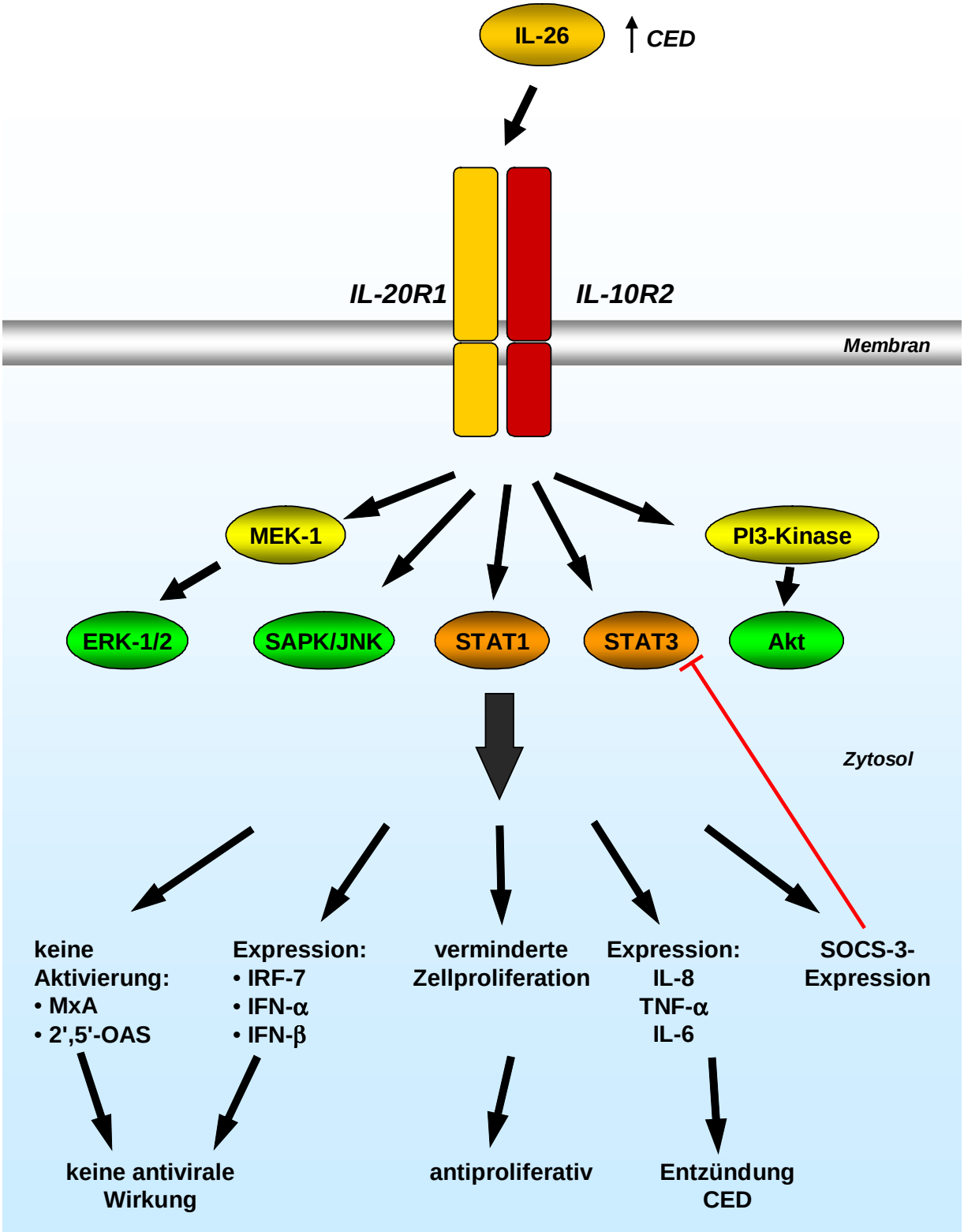

Abbildung 16. Übersicht über die von IL-26 vermittelten Funktionen in IEC.

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7. Zusammenfassung

Im Gastrointestinaltrakt bildet das intestinale Epithel die wichtigste Barriere zwischen Bakterien und Antigenen des Darmlumens einerseits und Immunzellen der Lamina propria andererseits. Intestinale Epithelzellen (IEC) sind ständig der Mikroflora des Darms ausgesetzt. Im Rahmen dieser Antigen-Epithelzell-Interaktion produzieren sie eine Vielzahl von Zytokinen und Chemokinen, unentbehrlichen biologischen Mediatoren der interzellulären Kommunikation zwischen infiltrierenden Leukozyten und Epithelzellen. Dysregulation der fein abgestimmten Immunantwort im Gastrointestinaltrakt kann zu Zuständen chronischer Entzündung und zur Entwicklung von chronisch entzündlichen Darmerkrankungen (CED) und in deren Folge auch zum Entstehen von kolorektalen Karzinomen führen.

Ziel der vorliegenden Arbeit war eine ausführliche Charakterisierung der Expression, der Signaltransduktion und der spezifischen biologischen Funktionen einer Auswahl der von IEC exprimierten **Chemokin-** sowie **Typ I- und Typ II-Zytokinrezeptoren**. Zusätzlich wurde die Expression der spezifischen Liganden unter Entzündungsbedingungen *in vitro* und *in vivo* analysiert. Hierfür wurden sowohl Versuche mit intestinalen Zelllinien durchgeführt als auch Mausmodelle intestinaler Entzündung sowie Patienten mit CED untersucht.

In den hier vorgelegten Arbeiten demonstrierten wir, dass intestinale Epithelzelllinien die Chemokinrezeptoren CXCR4, CXCR6 und CCR6 sowie die Rezeptoruntereinheiten für das Zytokin IL-31 und die IL-10-ähnlichen Zytokine IL-22 und IL-26 exprimieren. Alle untersuchten Chemokinliganden (CXCL12, CXCL16, CCL20) und das Zytokin IL-31 wurden in diesen Zellen ebenfalls gebildet, nicht jedoch die IL-10-ähnlichen Zytokine.

Die Analyse der Signaltransduktion ergab eine verstärkte Phosphorylierung der MAP-Kinasen SAPK/JNK und ERK-1/2 über MEK-1 sowie von Akt über den PI3-Kinase-Weg. Die Aktivierung der jeweiligen Signalwege konnte durch spezifische Inhibitoren (MEK-1-Inhibitor PD98059, SAPK/JNK-Inhibitor SP600125 sowie PI3-Kinase-Inhibitor Wortmannin) gehemmt werden. Außerdem vermittelten IL-31 und die IL-10-ähnlichen Zytokine eine Phosphorylierung der Transkriptionsfaktoren STAT1 und STAT3.

Die Aktivierung dieser Signaltransduktionswege führte zur verstärkten Transkription spezifischer Zielgene in IEC, wobei für **CXCL12** proangiogene Faktoren wie der "vascular endothelial growth factor" (VEGF) sowie Matrix-Metalloproteinasen (MMPs) als wesentliche Zielgene identifiziert wurden. CXCL12 vermittelte in IEC eine verstärkte Zellinvasion, Zellproliferation und beeinflusste MEK-1/ERK-1/2-abhängig die Zellmigration und trägt somit zur Metastasierung von kolorektalen Tumoren bei.

Das Chemokin **CCL20** förderte ebenfalls die gerichtete Zellmigration und die Zellproliferation. Ähnlich wie CXCL12 war es in Tumoren herunterreguliert und seine Expression korrelierte invers mit der Tumorinvasivität. CCL20 hatte zusätzlich, ähnlich wie **CXCL16**, entzündungsfördernde Eigenschaften, und die Expression beider Chemokine wurde durch proinflammatorische Stimuli in IEC *in vitro* hochreguliert. Bei Patienten mit CED, zu denen der Morbus Crohn und die Colitis ulcerosa gehören, fanden wir ebenfalls eine gesteigerte Produktion beider Chemokine. CXCL16 beeinflusst außerdem die spezifische Krankheitsausprägung bei Morbus Crohn-Patienten, da ein Einzelnukleotid-Polymorphismus (SNP) im *CXCL16*-Gen, der in einem Aminosäureaustausch (A181V) resultiert, mit einem jüngeren Erkrankungsalter sowie einem ilealen Krankheitsbefall und dem Auftreten eines stenosierenden oder penetrierenden Phänotyps bei diesen Patienten assoziiert war.

Aktuelle Studien zeigten, dass CCL20 außer in IEC auch in Th17-Zellen produziert wird, inflammatorischen T-Zellen, denen in jüngster Zeit eine wachsende Bedeutung bei der Pathogenese von CED zugesprochen wird und deren Entwicklung unter anderem von IL-23 abhängt. Auch die beiden IL-10-verwandten Zytokine **IL-22** und **IL-26** werden von Th17-Zellen gebildet. In unseren Versuchen induzierten beide Zytokine proinflammatorische Zielgene in IEC. Außerdem beeinflussten sie die IEC-Proliferation, wobei IL-26 proliferationshemmende Eigenschaften aufwies, während IL-22 die Zellproliferation in signifikanter Weise förderte. IL-22 stimulierte auch, vermittelt über den PI3-Kinase/Akt-Signalweg, die Migration von IEC und förderte somit die Wiederherstellung und die Integrität der intestinalen Barriere. Zusätzlich induzierte es die Expression von β-Defensinen, kleinen antimikrobiellen Peptiden, denen eine wichtige Bedeutung bei der angeborenen Immunantwort zukommt und deren Expression bei Morbus Crohn-Patienten vermindert ist. IL-22 war in von uns untersuchten Mausmodellen intestinaler Entzündung (DSS-Kolitis, CMV-Kolitis) verstärkt exprimiert und auch bei CED-Patienten fand sich eine signifikante Erhöhung der Expression von IL-22 und IL-26 im entzündeten Gewebe.

Ähnliche Beobachtungen machten wir auch für **IL-31**, ein kürzlich beschriebenes, mit IL-6 und IL-23 verwandtes Zytokin. Entzündete Mukosaareale von CED-Patienten exprimierten höhere Level von IL-31, aber auch von seinen beiden Rezeptoruntereinheiten IL-31RA und OSMR. Dies ist übereinstimmend mit unseren *in vitro*-Daten, die eine gesteigerte Expression dieser drei Gene nach Stimulation mit proinflammatorischen Stimuli wie TNF-α oder LPS zeigten. Im Gegensatz zur proliferationsfördernden Wirkung von IL-22 bzw. der hemmenden Wirkung von IL-26, stellten wir für IL-31 differentielle, zelldichteabhängige Effekte auf die

Zellproliferation fest. Vor allem bei niedriger Zelldichte wirkte dieses Zytokin proliferationshemmend, während dieser Einfluss bei höheren Zelldichten weitgehend verloren ging.

Zusammenfassend (siehe Abbildung 17) demonstrierten wir in den hier vorgelegten Arbeiten die Expression der Chemokinrezeptoren CXCR4, CCR6 und CXCR6 sowie der Rezeptoren für IL-22, IL-26 und IL-31 auf IEC. Durch sie werden vielfältige Funktionen vermittelt, wobei die Chemokin- und IL-22-induzierte Signaltransduktion zur verstärkten Proliferation und Migration von IEC führte, wie sie bei der Aufrechterhaltung bzw. Wiederherstellung der intestinalen Barriere bei Entzündung, aber auch bei der Migration kolorektaler Tumorzellen zu beobachten ist. Im Gegensatz hierzu wirkten die Zytokine IL-26 und IL-31 proliferationshemmend. Alle untersuchten Chemokine/Zytokine (mit der Ausnahme von CXCL12) fungierten als Entzündungsmediatoren im Gastrointestinaltrakt, indem sie proinflammatorische Genexpression in IEC induzierten. Außerdem war ihre eigene Expression in verschiedenen Mausmodellen intestinaler Entzündung sowie bei CED-Patienten stark hochreguliert, was die wichtige Rolle dieser Zellbotenstoffe bei Entzündungen im Gastrointestinaltrakt unterstreicht.

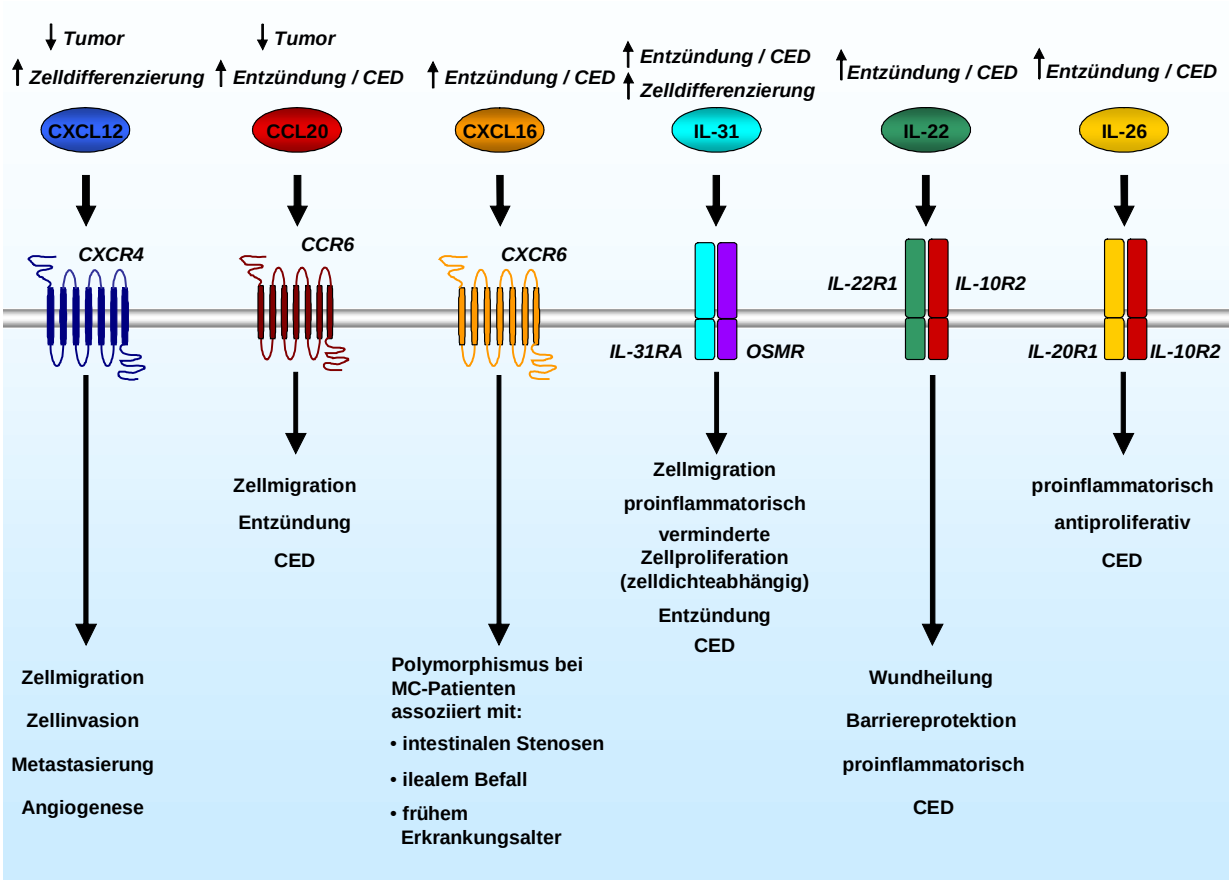

Abbildung 17. Überblick über die in dieser Arbeit untersuchten Chemokin- und Zytokinrezeptoren und die von ihnen vermittelten Funktionen im GI-Trakt.

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9. Erklärung zum Eigenanteil bei Koautorenschaft

Da es sich bei dieser Arbeit um eine kumulative Dissertation handelt, sind alle in dieser Zusammenstellung aufgeführten Originalarbeiten im Anhang beigefügt.

[1] Brand S*, **Dambacher J***, Beigel F, Olszak T, Diebold J, Otte JM, Göke B, Eichhorst ST. CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. *Exp Cell Res* 2005; Oct 15; 310(1): 117-30.

*** Geteilte Erstautorenschaft**

Eigenanteil 50 %: Entwicklung des Konzepts, Experimente (alle außer Immunhistochemie an Gewebeschnitten), Auswertung und Darstellung der Daten, Mitarbeit am Manuskript

[2] Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Göke B, Eichhorst ST, **Dambacher J**. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK and Akt signaling resulting in proliferation and migration of colorectal cancer cells. *J Cell Biochem* 2006 Mar 1; 97(4): 709-23.

Eigenanteil 30 %: Mitentwicklung des Konzepts, Anleitung eines Doktoranden, Experimente (komplett: qPCR, ELISA, Apoptoseassay; in Anteilen: RNA-Isolation, RT-PCR, Zellrestitutionsassay, Zellproliferationsassay), Auswertung und Darstellung der Daten, Mitarbeit am Manuskript

[3] **Dambacher J**, Seiderer J, Niess JH, Haller D, Diebold J, Göke B, Ochsenkühn T, Reinecker HC, Brand S. CXCL16 is a novel marker of intestinal inflammation in Crohn's disease. *Inflamm Bowel Dis* 2007; submitted.

Eigenanteil 40 %: Mitentwicklung des Konzepts, Experimente (komplett: qPCR, ELISA, Immunfluoreszenz; in Anteilen: RNA-Isolation, RT-PCR, Western Blot), Auswertung und Darstellung der Daten, Mitarbeit am Manuskript

[4] Seiderer J*, **Dambacher J***, Leistner D, Tillack C, Glas J, Niess JH, Pfennig S, Jürgens M, Müller-Myhsok B, Göke B, Ochsenkühn T, Lohse P, Reinecker HC, Brand S. The role of the CXCL16 p.Ala181Val polymorphism in inflammatory bowel disease. *Clin Immunol* 2007; submitted.

*** Geteilte Erstautorenschaft**

Eigenanteil 30 %: Mitentwicklung des Konzepts, Anleitung einer Doktorandin, statistische Analyse, Auswertung und Darstellung der Daten, Mitarbeit am Manuskript

[5] **Dambacher J**, Beigel F, Seiderer J, Haller D, Göke B, Auernhammer CJ, Brand S. Interleukin-31 mediated signals modulate intestinal epithelial cell proliferation and its expression is up-regulated in intestinal inflammation. *Gut* 2007 Sep; 56(9): 1257-65.

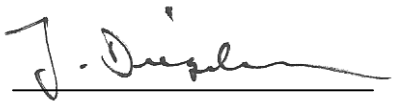
Eigenanteil 80 %: Entwicklung des Konzepts, Experimente (alle außer Epithelzellisolation aus TNFΔARE-Mäusen), Auswertung und Darstellung der Daten, Mitarbeit am Manuskript

[6] Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diepolder H, Marquardt A, Jagla W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkühn T, Göke B, Auernhammer CJ**, Dambacher J**. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* 2006 Apr; 290(4): G827-38.

Eigenanteil 30 %: Mitentwicklung des Konzepts, Koordination und Anleitung eines Doktoranden, Experimente (komplett: qPCR, ELISA, Zellmigrationsassay; in Anteilen: RNA-Isolation, RT-PCR, Proliferationsassay, Apoptoseassay), Auswertung und Darstellung der Daten, Mitarbeit am Manuskript

[7] **Dambacher J**, Beigel F, Zitzmann K, de Toni E, Göke B, Diepolder H, Auernhammer CJ, Brand S. The role of the novel IL-10 like cytokine IL-26 in intestinal inflammation. *Gut* 2007; submitted.

Eigenanteil 70 %: Mitentwicklung des Konzepts, Experimente (komplett: qPCR, ELISA; in Anteilen: RNA-Isolation, RT-PCR), Auswertung und Darstellung der Daten, Mitarbeit am Manuskript

München, 22.10.07 

Ort, Datum Unterschrift

Bestätigung durch den Betreuer:

München, 22.10.07 

Ort, Datum Unterschrift

10. Vollständiges Publikationsverzeichnis

Publizierte Originalarbeiten

1) Glas J, Seiderer J, Wetzke M, Konrad A, Török HP, Schmechel S, Tonenchi L, Grassl C, **Dambacher J**, Pfennig S, Maier K, Griga T, Klein W, Epplen J, Schiemann U, Folwaczny C, Lohse P, Göke B, Ochsenkühn T, Müller-Myhsok B, Folwaczny M, Mussack T, Brand S. rs1004819 is the main disease-associated *IL23R* variant in German Crohn's disease patients: Combined analysis of *IL23R*, *CARD15*, and *OCTN1/2* variants. *PLoS ONE* 2007 Sep 5; 2(9): e819

2) Seiderer J*, **Dambacher J***, Kühnlein B, Pfennig S, Konrad A, Haller D, Göke B, Ochsenkühn T, Lohse P, Brand S. The role of the selenoprotein S (SELS) gene -105G>A promoter polymorphism in inflammatory bowel disease and regulation of SELS gene expression in intestinal inflammation. *Tissue Antigens* 2007 Sep; 70(3): 238-46.

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3) **Dambacher J**, Beigel F, Seiderer J, Haller D, Göke B, Auernhammer CJ, Brand S. Interleukin-31 mediated signals modulate intestinal epithelial cell proliferation and its expression is up-regulated in intestinal inflammation. *Gut* 2007 Sep; 56(9): 1257-65.

4) Seiderer J, Brand S, **Dambacher J**, Pfennig S, Jürgens M, Göke B, Ochsenkühn T. Adalimumab in patients with Crohn's disease – safety and efficacy in an open-label single centre study. *Aliment Pharmacol Ther* 2007 Apr 1; 25(7): 787-96

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10) Brand S, Olszak T, Beigel F, Diebold J, Otte JM, Göke B, Eichhorst ST, **Dambacher J**. Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK and Akt signaling resulting in proliferation and migration of colorectal cancer cells. *J Cell Biochem* 2006 Mar 1; 97(4): 709-23.

11) Brand S, Hofbauer K, **Dambacher J**, Schnitzler F, Staudinger T, Pfennig S, Seiderer J, Tillack C, Konrad A, Göke B, Ochsenkühn T, Lohse P. Increased expression of the chemokine fractalkine in Crohn's disease and association of the fractalkine receptor T280M polymorphism with a fibrostenosing disease phenotype. *Am J Gastroenterol.* 2006 Jan; 101(1): 99-106.

12) Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Diebold J, Diepolder H, Adler B, Auernhammer CJ, Göke B, **Dambacher J**. IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression. *Am J Physiol Gastrointest Liver Physiol* 2005 Nov; 289(5): G960-8.

13) Brand S*, **Dambacher J***, Beigel F, Olszak T, Diebold J, Otte JM, Göke B, Eichhorst ST. CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. *Exp Cell Res* 2005; Oct 15; 310(1): 117-30.

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14) Brand S, Staudinger T, Schnitzler F, Pfennig S, Hofbauer K, **Dambacher J**, Seiderer J, Tillack C, Konrad A, Crispin A, Göke B, Lohse P, Ochsenkühn T. The role of Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms and CARD15/NOD2 mutations in the susceptibility and phenotype of Crohn's disease. *Inflamm Bowel Dis* 2005 Jul; 11(7): 645-52.

15) Brand S, Zitzmann K, **Dambacher J**, Beigel F, Olszak T, Vlotides G, Eichhorst ST, Göke B, Diepolder H, Auernhammer CJ. SOCS-1 inhibits expression of the antiviral proteins 2',5'-OAS and MxA induced by the novel interferon-lambdas IL-28A and IL-29. *Biochem Biophys Res Commun* 2005 Jun 3; 331(2): 543-8.

16) Dobreva G, **Dambacher J**, Grosschedl R. SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin mu gene expression. *Genes Dev* 2003 Dec 15; 17(24): 3048-61.

Zur Publikation angenommene Manuskripte

1) Schmechel S, Konrad A, **Diegelmann J**, Glas J, Wetzke M, Paschos E, Lohse P, Göke B, Brand S. Linking genetic susceptibility to Crohn’s disease with Th17 cell function: IL-22 serum levels are increased in Crohn’s disease and correlate with disease activity and *IL23R* genotype status. *Inflamm Bowel Dis* 2007; in press.

2) Glas J, Konrad A, Schmechel S, **Dambacher J,** Seiderer J, Schroff F, Wetzke M, Roeske D, Török HP, Tonenchi L, Pfennig S, Haller D, Griga T, Klein W, Epplen JT, Folwaczny C, Lohse P, Göke B, Ochsenkühn T, Mussack T, Folwaczny M, Müller-Myhsok B, Brand S. The ATG16L1 gene variants rs2241879 and rs2241880 (T300A) are strongly associated with susceptibility to Crohn’s disease in the German population. *Am J Gastroenterol* 2007; in press.

3) Seiderer J, Elben I, **Diegelmann J**, Glas J, Stallhofer J, Tillack C, Pfennig S, Jürgens M, Schmechel S, Konrad A, Göke B, Ochsenkühn T, Müller-Myhsok B, Lohse P, Brand S. The role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): Upregulated colonic IL-17F expression in active Crohn’s disease and analysis of the IL17F p.His161Arg polymorphism in IBD. *Inflamm Bowel Dis* 2007; in press.

Eingereichte Manuskripte

1) **Dambacher J**, Seiderer J, Niess JH, Haller D, Diebold J, Göke B, Ochsenkühn T, Reinecker HC, Brand S. CXCL16 is a novel marker of intestinal inflammation in Crohn's disease. *Inflamm Bowel Dis* 2007; submitted.

2) Seiderer J*, **Dambacher J***, Leistner D, Tillack C, Glas J, Niess JH, Pfennig S, Jürgens M, Müller-Myhsok B, Göke B, Ochsenkühn T, Lohse P, Reinecker HC, Brand S. The role of the CXCL16 p.Ala181Val polymorphism in inflammatory bowel disease. *Clin Immunol* 2007; submitted.

   *** Geteilte Erstautorenschaft**

3) **Dambacher J**, Beigel F, Zitzmann K, de Toni E, Göke B, Diepolder H, Auernhammer CJ, Brand S. The role of the novel IL-10 like cytokine IL-26 in intestinal inflammation. *Gut* 2007; submitted.

4) **Dambacher J**, Beigel F, Zitzmann K, Heeg M, Göke B, Diepolder H, Auernhammer CJ, Brand S. The role of Interleukin-22 in hepatitis C virus infection. *Cytokine* 2007; submitted.

5) Glas J, Seiderer J, Pasciuto G, Tillack C, **Diegelmann J**, Pfennig S, Roeske D, Jürgens M, Schmechel S, Konrad A, Wetzke M, Török HP, Griga T, Klein W, Epplen JT, Schiemann U, Mussack T, Lohse P, Göke B, Ochsenkühn T, Folwaczny M, Müller-Myhsok B, Brand S. The first two Crohn's disease susceptibility loci with a high degree of epistasis: PTGER4-expression-modulating polymorphisms in the 5p13.1 region enhance ATG16L1-associated susceptibility to Crohn's disease. *Nat Genet* 2007; submitted.

6) Glas J, Seiderer J, Pasciuto G, Tillack C, Konrad A, Schmechel S, Wetzke M, Török HP, **Diegelmann J**, Pfennig S, Jürgens M, Griga T, Klein W, Epplen JT, Schiemann U, Mussack T, Lohse P, Göke B, Ochsenkühn T, Folwaczny M, Müller-Myhsok B, Brand S. Significant differences in the genetic susceptibility to Crohn's disease between North American and European populations regarding gene variants in PHOX2B, NCF4, FAM92B, and in the intergenic region on chromosome 10q21.1. *Gastroenterology* 2007; submitted.

Publizierte Kurzfassungen (Abstracts)

1) Brand S, **Dambacher J**, Olszak T, Beigel F, Sisic Z, Eichhorst S, Göke B. CXCR4-induzierte Signaltransduktionswege beeinflussen Proliferation und Migration von intestinalen Epithelzellen.
59. Jahrestagung der Deutschen Gesellschaft für Verdauungs- und Stoffwechselkrankheiten, September 1-4, 2004, Leipzig, Germany.
Z Gastroenterol 2004; 42: 840.

2) Brand S, **Dambacher J**, Olszak T, Beigel F, Sisic Z, Eichhorst S, Göke B. CXCR4 mediated signals stimulate intestinal epithelial cell migration and cell proliferation.
12th United European Gastroenterology Week, Prague, Czech Republic, September 25-29, 2004
Gut 2004; 53 (Suppl. VI): A132.

3) Brand S, Staudinger T, Schnitzler F, Hofbauer K, **Dambacher J**, Seiderer J, Tillack C, Konrad A, Hölzel D, Göke B, Ochsenkühn T, Lohse P. The role of the Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in Crohn's disease.
12th United European Gastroenterology Week, Prague, Czech Republic, September 25-29, 2004
Gut 2004; 53 (Suppl. VI): A146.

4) Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Goeke B, Diepolder H, Adler B, Auernhammer C, **Dambacher J**. The Novel Lambda-Interferons IL-28A and IL-29 Mediate Proinflammatory, Antiproliferative, and Antiviral Signals in Intestinal Epithelial Cells.
Annual Meeting of the American Gastroenterological Association and Digestive Disease Week. May 14-19, 2005, Chicago, USA.
Oral Presentation at the Distinguished Late Breaking Abstract Session.
Gastroenterology. 2005 Jul; 129(1): 371.

5) Brand S, Beigel F, **Dambacher J**, Olszak T, Eichhorst ST, Zitzmann K, Diepolder H, Auernhammer C. SOCS-1 Inhibits Expression of the Antiviral Proteins 2,5-OAS and MxA Induced By the Novel Interferon-Lambdas IL-28A and IL-29.
Annual Meeting of the American Gastroenterological Association and Digestive Disease Week. May 14-19, 2005, Chicago, USA.
Gastroenterology 2005; 128 (4; Suppl. 2): A751.

6) Brand S, Hofbauer K, **Dambacher J**, Staudinger T, Schnitzler F, Pfennig S, Seiderer J, Tillack C, Konrad A, Crispin A, Göke B, Lohse P, Ochsenkühn T. Increased Expression of the Chemokine Fractalkine in Crohn's Disease and Association of the Fractalkine Receptor T280M Polymorphism With a Fibrostenosing Disease Phenotype.
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Anhang

Publizierte Originalarbeiten und eingereichte Manuskripte

Manuskript [1]

CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation

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Research Article

CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation

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Abstract

Colorectal cancer (CRC) is characterized by a distinct metastatic pattern resembling chemokine-induced leukocyte trafficking. This prompted us to investigate expression, signal transduction and specific functions of the chemokine receptor CXCR4 in CRC cells and metastases. Using RT-PCR analysis and Western blotting, we demonstrated CXCR4 and CXCL12 expression in CRC and CRC metastases. Cell differentiation increases CXCL12 mRNA levels. Moreover, CXCR4 and its ligand are inversely expressed in CRC cell lines with high CXCR4 and low or not detectable CXCL12 expression. CXCL12 activates ERK-1/2, SAPK/JNK kinases, Akt and matrix metalloproteinase-9. These CXCL12-induced signals mediate reorganization of the actin cytoskeleton resulting in increased cancer cell migration and invasion. Moreover, CXCL12 increases vascular endothelial growth factor (VEGF) expression and cell proliferation but has no effect on CRC apoptosis. Therefore, the CXCL12/CXCR4 system is an important mediator of invasion and metastasis of CXCR4 expressing CRC cells.

Introduction

Colorectal carcinoma (CRC) is the second leading cause of death due to cancer in the United States, accounting for more than 50,000 deaths annually [1]. The use of all currently available therapies has only modest impact on overall survival of patients with advanced-stage, metastatic disease. Although a number of molecules have been implicated in the metastasis of cancer cells, the precise mechanisms determining the directional migration and invasion of CRC cells into specific organs remain to be established. New evidence indicates that chemokines play a major role in this process of organ-selective metastasis [2].

Chemokine receptors are coupled to heterotrimeric G proteins and induce cell movement towards a concentration gradient of the cognate chemokine ligand [3]. Human stromal-cell derived factor-1 (SDF-1), also known as CXCL12 according to a new classification system [4] is thought to be the primordial chemokine. It binds and signals solely through the chemokine receptor CXCR4. Interestingly, it is the only chemokine that is essential for survival [5] as shown in mice genetically deficient in the chemokine receptor CXCR4 or its ligand CXCL12 which die perinatally with major defects in the vascular development (particularly in the gastrointestinal tract), hematopoi-

Abbreviations: CRC, colorectal cancer; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mAb, monoclonal antibody; MAP-kinase, mitogen-activated protein-kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; PI, phosphatidylinositol; RT-PCR, reverse transcriptase polymerase chain reaction; SAPK/JNK, stress-activated protein kinase/c-Jun-N-terminal kinase.

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esis and cardiogenesis [5,6]. Moreover, CXCR4 is essential in stem cell localization [6], serves as a chemoattractant for T cells and thymocytes in vitro and in vivo [7,8] and functions as a co-receptor for HIV-1 [9].

A recent study demonstrated that CXCR4 signaling plays a crucial role in metastasis of breast cancer by inducing chemotactic and invasive responses [2]. Metastasis was found predominantly in organs with abundant CXCL12 expression. Neutralization of CXCL12/CXCR4 interactions leads to a marked inhibition of lymph node and lung metastases [2]. The involvement of CXCR4 in metastasis is not limited to breast cancer, as CXCR4 is expressed in several other tumor cell lines [10–14] that also respond to CXCL12. Therefore, chemokine receptors expressed on tumor cells represent potential targets for therapeutic interventions.

The expression of CXCR4 in intestinal epithelial cells has been shown previously [15,16] and preliminary experiments in animal models indicate a role for CXCR4 in CRC metastasis particularly in triggering the outgrowth of micrometastases [17]. However, the mechanisms of CXCR4 signaling and its functional role in the migration and metastasis of human CRC cells are unsolved questions in the analysis of colorectal cancerogenesis. Here, we demonstrate that CXCR4 and CXCL12 are expressed in human CRC cells and sites of metastasis. Signal transduction experiments indicate that CXCR4 is functional in human CRC cells mediating specific functions such as CRC migration, invasion and proliferation.

Materials and methods

Reagents

Polyclonal antibodies to extracellular signal-regulated kinase (ERK)-1/2 (phosphorylated at Thr183/Tyr185 and total), stress-activated protein kinase (c-Jun N-terminal kinase) SAPK/JNK (phosphorylated at Thr183/Tyr185 and total), p38 (phosphorylated at Thr180/Tyr182 and total) and Akt (phosphorylated at Ser473 and total) were purchased from Cell Signaling (Beverly, MA). Horseradish peroxidase linked anti-rabbit secondary antibody was purchased from Amersham (Arlington Heights, IL). Recombinant human CXCL12 and monoclonal antibodies to human CXCR4 and CXCL12 were from R&D Systems (Minneapolis, MN). MEK-1 inhibitor PD98059, SAPK/JNK inhibitor SP600125, p38 inhibitor SB203580 and phosphatidylinositol3- (PI3) kinase inhibitor wortmannin were from Tocris Cookson (Bristol, U.K.).

Cell culture

The human CRC cell lines T84, SW480, Caco-2, HT-29, HCT116, the lymph node metastasis derived cell line SW620 and the transformed rat intestinal cell line IEC-6, were obtained from American Type Culture Collection (Rockville, MD). While T84 cells were grown in Dulbecco's modified Eagle medium/F-12 (GIBCO BRL/Life Technologies, Gaithersburg, MD), the other cell lines were grown in Dulbecco's modified Eagle medium (GIBCO) with 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated fetal calf serum (PAA, Pasching, Austria) in a humidified 5% CO_2 atmosphere at 37°C. For signal transduction experiments with CXCL12, cells were starved overnight in serum-free medium.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD) and treated with ribonuclease (RNase)-free deoxyribonuclease (DNA-free™-Kit, Ambion) to remove potential genomic DNA contaminants. Three micrograms of total RNA was reverse transcribed using Roche first strand cDNA synthesis kit. The following conditions were used for semiquantitative PCRs: 25 or 35 cycles (depending on the specific PCR) of denaturing at 95°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 45 s using previously published primers for CXCR4 [16], VEGF [18] and GAPDH [19]. The primers used for CXCL12 were forward 5′-AGAGCCAACGTCAAGCATCT-3′ and reverse 5′-CGTCTTTGCCCTTTCATCTC-3′. All PCR reactions included GAPDH primers to quantify PCR products. To control for genomic contamination, an identical parallel PCR reaction was performed containing starting material that had not been reverse transcribed.

Gel electrophoresis and immunoblotting

Cellular proteins, cytosolic and membrane fractions were extracted as described previously [20,21]. The protein concentration of each sample was quantified by the Bradford method. Immunoblotting was performed as previously described [22].

Enzyme-linked immunosorbent assay (ELISA)

For the quantification of IL-8 release, BD OptEIA Human IL-8 ELISA Kit II (BD Biosciences, Bedford, MA) and for quantification of VEGF and CXCL12, Quantikine ELISA Kits from R&D Systems (Minneapolis, MN) were used according to the manufacturer's instructions.

Evaluation of MMP-2 and MMP-9 activity by gelatin zymography

HT-29 and T84 cells were cultured in DMEM containing 0.1% FCS in the presence or absence of human CXCL12 for 24 and 48 h, respectively. Conditioned medium was

harvested and was stored at −20°C, and analyzed for MMP-2 and MMP-9 activity by gelatin zymography [23].

Immunohistochemistry

Surgical specimens from patients undergoing colectomy or lymph node resection were taken after obtaining patient's consent. Immunohistochemistry was performed as previously described [22]. The cells were incubated with anti-CXCR4 or anti-CXCL12. Fluorescein anti-mouse IgG from Sigma was used as secondary antibody. The cells were analyzed with an Axiovert 135 TV fluorescent microscope (Zeiss, Oberkochen, Germany).

Flow cytometry

HT-29 or SW480 cells were adjusted to a concentration of 2×10^7 cells/mL in FACS buffer (PBS containing 0.5% BSA and 0.05% sodium azide). Cells were washed three times and were incubated with a PE-conjugated anti-CXCR4 antibody or with a PE-conjugated isotype control antibody of irrelevant specificity. After washing three times with FACS buffer, cells were analyzed by flow cytometry.

Actin-polymerization assay

Actin polymerization assays were performed as described previously [24]. HCT116 cells were serum starved over night and then were stimulated with 100 ng/mL CXCL12 for different time intervals. Fluorescence microscopy was performed on a Zeiss axiovert 135 TV with the Axiovision software to obtain digital images.

Wounding assay

Wounding assays were performed as previously described [25]. The cells were stimulated with CXCL12 (10 and 100 ng/mL) or 0.1% FCS. The number of migrated cells (over the wounding edge) was counted under a microscope (Olympus IX50, Hamburg, Germany).

ECM invasion assay

Migration of CRC cells into micropore filters coated with ECM proteins (laminin 56%, collagen IV 31%, entactin 8%) was performed according to the manufacturer's instructions (BD Biosciences, Bedford, MA, USA). Briefly, HCT116 cells at a concentration of 50,000 cells/mL were placed in the top chamber of a two-chamber assay system and incubated for 24 h with and without CXCL12 placed in the lower chamber. After the incubation period, the cells on the upper side were removed using a cotton swab. Then, the coated filters were removed and stained. Intestinal epithelial cell invasion was quantified microscopically by counting the cells that had migrated into the filters.

Cell migration assay

Similar to the ECM invasion assays, cell migration assays were performed using BD Falcon cell culture inserts containing polyethylene terephthalate membranes (8 μm pore size) from BD Biosciences (Bedford, MA, USA). Similar to the invasion assays, the cells were placed in the top of the chamber while CXCL12 was given in the lower chamber.

Cell proliferation assay

HT-29 cells were seeded onto 96 well plates at a density of 10,000 cells/well and were allowed to attach overnight. The cells were stimulated with 10, 100 or 1000 ng/mL CXCL12 in serum-free medium, or with CXCL12-free serum-free medium (negative control). The cell proliferation rate was determined by MTS assay on day 2 using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions.

Apoptosis assays

SW480 cells, which are less resistant to Fas-induced apoptosis than HT-29 cells [26], were used in these assays. For induction of CD95-mediated cell death, ligand-specific anti-APO-1 mAb at concentrations of 100 and 500 ng/mL was used. Apoptosis assays were performed as described previously [27]. The nuclei were then analyzed for DNA content by flow cytometry.

Statistical analysis

Statistical analysis was performed using two-tailed Student's t test. P levels < 0.05 were considered as significant.

Results

CRC cells express CXCR4

To determine if CXCR4 is expressed in CRC cells and to utilize an intestinal epithelial cell model to study the CXCL12-CXCR4 ligand–receptor system, we analyzed CXCR4 expression in several human CRC derived cell lines (HT-29, SW480, Caco-2, HCT116, T84), the lymph node metastasis derived cell line SW620 and the rat intestinal epithelial cell line IEC-6 by RT-PCR, Western Blotting and immunohistochemistry. All cell lines expressed CXCR4 mRNA (Fig. 1A). CXCR4 protein was found predominantly in membrane fractions in HT-29 and T84 cells as shown by Western Blotting (Fig. 1B) and immunohistochemistry (Fig. 1C). Cell surface expression of CXCR4 in HT-29 cells was also

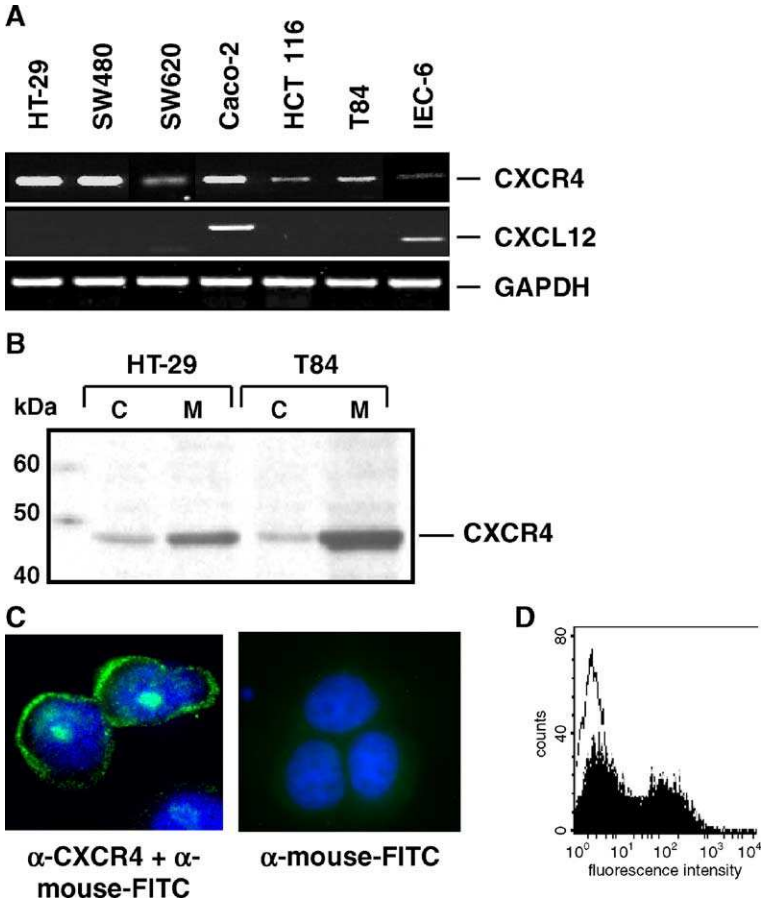

Fig. 1. CXCR4 is expressed in intestinal epithelial cells. (A) Expression of CXCR4 in various intestinal epithelial cell lines using RT-PCR analysis of mRNA derived from intestinal epithelial cells as indicated. CXCL12 was expressed only in the rat primary intestinal epithelial cell line IEC-6 and in differentiated Caco-2 cells but not in other colorectal cancer cell lines tested. (B) Expression of CXCR4 protein is found predominantly in the membrane fraction (M) and to a lesser degree in cytosolic fractions (C) of HT-29 and T84 cells, respectively. (C) Immunohistochemistry reveals a membrane associated staining of CXCR4 receptor (CXCR4: green staining, nucleus: blue staining with Hoechst 33342) in HT-29 cells. (D) FACS analysis showed expression of CXCR4 on the surface of 40% of HT-29 cells (shaded area: α-CXCR4, open area: isotype matched control antibody).

shown by flow cytometry with a PE-conjugated CXCR4 antibody (Fig. 1D, shaded area). An irrelevant isotype matched antibody was used as negative control (Fig. 1D, open area).

CXCR4 is expressed in primary CRC and CRC metastases

To analyze if CXCR4 and CXCL12 are expressed in primary human CRC tissue and CRC metastases, we performed a comprehensive immunohistochemical analysis which included tissue of a variety of colorectal cancers of different stages of invasion and differentiation (histological grading G1–G4) taken from 16 different patients (Table 1). In addition, we analyzed tissue from CRC lymph node metastases (n = 5), liver metastases (n = 4) and colorectal adenomas (n = 3). Ten out of the 16 CRC stained positive for CXCR4 (Table 1). In addition, four of five lymph node metastases, three of four liver metastases and all colorectal adenomas showed positive staining for CXCR4 expression demonstrating CXCR4 expression in the majority of CRC and CRC metastases (Table 1, Fig. 2).

CXCR4 and CXCL12 are inversely expressed in CRC

Although the CXCR4 ligand CXCL12 was expressed in all CRC and metastases analyzed, it was down-regulated or only partially expressed in 10 of 16 CRC (62.5%) compared to the surrounding normal colonic tissue (Fig. 2, Table 1). Similarly, the mRNA analysis of CRC cell lines (Fig. 1A) suggests that CXCL12 is rather down-regulated in CRC cell lines. This mRNA analysis demonstrated CXCL12 expression in only one of six CRC cell lines (Caco-2) and in the non-cancerous intestinal epithelial cell line IEC-6. Fig. 2A shows an example of a colorectal adenoma in which the CXCL12 protein expression is sharply down-regulated in the transition zone from normal to adenomous tissue (see arrows). While some CRC tissues showed overall high

Table 1
Clinical data and results of the immunohistochemical analysis of CXCL12 and CXCR4 protein expression in CRC, colorectal adenomas, lymph node and liver metastases

	TNM	Grading	CXCL12 expression	CXCR4 expression
CRC				
	T3N1M0	G2	+	++
	T2N0M0	G3	+	++
	T3N0M0	G2	+	++
	T3N2M0	G2	++	–
	T2N0M1	G2	++	++
	T3N0M0	G3	++	–
	T3N2M0	G2	+	–
	T3N0M0	G2	++	–
	T1N0M0	G1	+	–
	T3N0M0	G3	+	++
	T4N2M0	G3	+	++
	T4N1M0	G2	+	++
	T3N2M0	G4	+	–
	T3N1M1	G2	+	++
	T3N0M0	G2	++	(+)
	T3N0M1	G2	++	(+)
Colorectal adenomas				
	–	–	+	(+)
	–	–	+	+
	–	–	++	++
CRC liver metastases				
	T3N1M1	G2	+	++
	T3N1M1	G2	++	(+)
	T2N1M1	G3	++	++
	T3N1M1	G2	+	–
CRC lymph node metastases				
	T3N2M0	G2	++	(+)
	T3N2M0	G2	++	–
	T4N2M0	G3	+	(+)
	T4N1M0	G2	++	(+)
	T3N1M1	G2	+	++

–: no staining (no immunoreactivity for the specific antibody); (+): faint staining; +: only partial (focal) staining; ++: strong staining.

expression of CXCL12 and CXCR4 (Figs. 2C, D, G–J), others displayed a focal loss of CXCL12 and CXCR4 (Figs. 2A, B, E, F; Table 1). This loss of expression was not related to the grade of invasion, metastasis or differentiation as classified by TNM classification (Table 1). However, there was a trend towards an inverse expression pattern for CXCR4 and CXCL12. Three of the six CRC, which were overall highly positive for CXCL12 expression, were negative for CXCR4, two were faintly positive and only one was strongly positive for CXCR4 staining. Conversely, all of the six CRC negative for CXCR4 expression were positive for CXCL12, demonstrating a particular high CXCL12 expression in three cases. Similarly, all CRC cell lines expressed CXCR4 but only one CXCL12, while the semiquantitative RT-PCR analysis showed lower CXCR4 expression in non-cancerous IEC-6 cells compared to CRC cell lines but high CXCL12 expression.

CXCL12 expression increases with cell differentiation in intestinal epithelial cells

In contrast to CXCR4 expression, CXCL12 mRNA expression was found only in the primary intestinal epithelial cell line IEC-6 while Caco-2 was the only CRC cell line (out of the six CRC cell lines tested) with CXCL12 expression (Fig. 1A). CXCL12 protein expression in Caco-2 cells, which was measured by ELISA, revealed CXCL12 levels between 830 and 4900 pg/mL, depending on cell density (data not shown). Since it has been demonstrated that Caco-2 cells spontaneously differentiate in culture [28], we hypothesized that cell differentiation influences CXCL12 expression. Therefore, we analyzed the influence of cell differentiation on the CXCL12 mRNA expression levels using sodium butyrate stimulation as an established model of cell differentiation [15,29]. For these experiments, we used the human colonic epithelial cell line HCT116, which is a model for differentiation of intestinal mucosal epithelium [30]. As shown in Fig. 3A, increasing cell differentiation enhanced CXCL12 mRNA expression in HCT116 cells up to 90-fold after 72 h. Cell differentiation is a predominant feature of normal intestinal epithelial cells. The human colon is a constantly renewing tissue composed of intestinal epithelial cells of different differentiation stages. Therefore, we analyzed by immunohistochemistry if cell differentiation increases CXCL12 protein expression levels in normal intestinal epithelial cells. As shown in Fig. 3B, there was a clear distribution pattern of CXCL12 staining in normal intestinal epithelial cells beginning in the middle and increasing to the upper third of the crypt with highest expression in the most differentiated surface intestinal epithelial cells. This result is consistent with the RT-PCR analysis of HCT116 cells (Fig. 3A) and confirms that cell differentiation increases CXCL12 expression in intestinal epithelial cells.

Additionally, we examined a variety of other cancer cell lines including the hepatocellular cancer cell line HepG2, the melanoma cell line 624.38-MEL, the breast cancer cell line MCF-7, the pancreatic cancer cell line Panc-1 and the prostate cancer cell line LNCaP for up-regulation of CXCL12 mRNA expression after stimulation with sodium butyrate. After 24 h, there was a clear up-regulation of CXCL12 mRNA in 624.38-MEL, Panc-1 and LNCaP cells, which was less pronounced in HepG2 cells (Fig. 3C). MCF-7 was the only cell line, which expressed high basal levels of CXCL12 mRNA, similar to that observed in Caco-2 cells (Fig. 3C). This high CXCL12 mRNA expression did not further increase in MCF-7 cells after sodium butyrate stimulation (Fig. 3C).

CXCL12 induces MAP kinase and Akt activation

To test if CXCR4 expressed in CRC cells is functional, we analyzed major signaling pathways after stimulation of CXCR4 expressing HT-29 cells with CXCL12. Stimulation of chemokine receptors can result in transient activation of

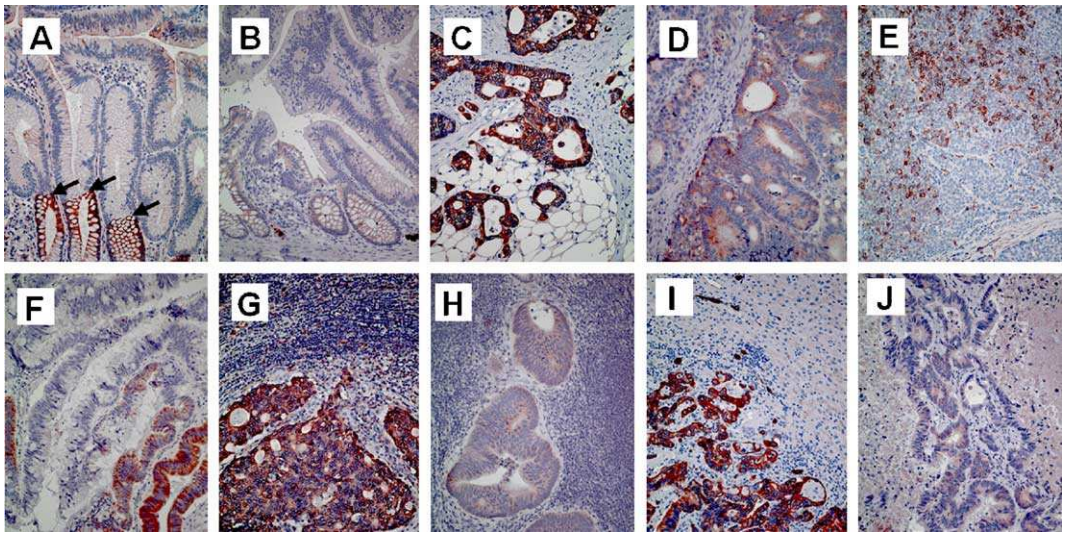

Fig. 2. CRC and CRC metastases express CXCL12 and its receptor CXCR4. However, individual cancer types varied in CXCL12 and CXCR4 expression levels. Examples of the immunohistochemical analysis of 3 colorectal adenomas, 16 CRC, 5 CRC lymph node metastases and 4 liver metastases are shown. (A) The image represents the border (marked by arrows) of normal colonic epithelial bordering a colorectal adenoma with decreased CXCL12 expression in a colorectal adenoma. (B) Low CXCR4 expression in a colorectal adenoma. CXCR4 is mainly expressed in the base of the crypts. (C) CXCL12 expression in CRC. (D) CXCR4 expression in CRC. (E) Focal loss of expression of CXCL12 in CRC. (F) Focal loss of expression of CXCR4 in CRC. (G) CXCL12 expression in CRC lymph node metastasis. (H) CXCR4 expression in CRC lymph node metastasis. (I) CXCL12 expression in CRC liver metastasis. (J) CXCR4 expression in CRC liver metastasis.

mitogen-activated protein (MAP) kinases [31]. 100 ng/mL CXCL12 induced a transient activation of ERK-1/2 in HT-29 cells (Fig. 4A). Similarly, SAPK/JNK kinases were phosphorylated following CXCL12 stimulation (Fig. 4B), while no p38 MAP kinase activation was observed (Fig. 4C). Similar results were obtained in SW480 cells (data not shown). To identify the respective upstream signaling events, we investigated the effect of the MEK-1 inhibitor PD98059, the SAPK/JNK inhibitor SP600125 and the PI3 kinase inhibitor wortmannin on MAP kinase phosphorylation. PD98059 down-regulated ERK-1/2 phosphorylation after CXCL12 stimulation (Fig. 5A), suggesting MEK-1 as an upstream signal transducer of the CXCL12-induced ERK activation. Crosstalk between the PI3 kinase and the MEK-ERK pathway has been proposed [32]. However, ERK activation after CXCL12 stimulation was not significantly affected by pretreatment with wortmannin. Similar results were obtained for SP600125 (Fig. 5B), suggesting a PI3- and SAPK/JNK kinase-independent activation of ERK-MAP kinases by CXCL12. On the other hand, while pretreatment with the JNK inhibitor SP600125 significantly suppressed phosphorylation of SAPK/JNK kinases (Fig. 5C), pretreatment with PD98059 or wortmannin did not influence phosphorylation levels suggesting a MEK-1 and PI3 kinase-independent pathway (Fig. 5D).

Activation of chemokine receptors may also result in activation of Akt [33,34]. Stimulation of HT-29 cells with CXCL12 caused an increased phosphorylation of Akt (Fig. 6A). Pretreatment with the PI3 kinase inhibitor wortmannin resulted in a complete dephosphorylation of Akt (Fig. 6B) while pretreatment with the SAPK/JNK inhibitor SP600125 partly decreased Akt activation (Fig. 6C) suggesting a PI3 kinase and partly SAPK/JNK-dependent pathway. Both MAP-kinase and PI3 kinase-Akt pathways have been linked to NF-κB activation [35,36]. CXCL12 did not significantly increase NF-κB and AP-1 DNA binding in the colorectal cancer cell line HT-29 (data not shown, Supplemental Fig. S1). Both transcription factors have been shown to increase IL-8 mRNA expression [37,38]. Consistent with the lacking NF-κB and AP-1 DNA binding, CXCL12 stimulation did not result in an increased IL-8 expression in this CRC cell line (data not shown, Supplemental Fig. S1).

CXCL12 does not influence Fas ligand-induced apoptosis but stimulates cell proliferation in CRC cells

We demonstrated that CXCL12 stimulation results in ERK-1/2 and Akt activation which has been shown to mediate anti-apoptotic pathways and increase cell proliferation [39,40]. Increased cell proliferation and decreased apoptosis are mechanisms found in cancerous tissue resulting in enhanced tumor growth and resistance to “apoptotic” and anti-proliferative therapy strategies [41]. Therefore, we investigated the chemokine-mediated effect on apoptosis using previously established experimental conditions [27]. In these experiments, SW480 cells were used which are less resistant to Fas-induced apoptosis than HT-29 cells [26]. However, no significant difference between the number of apoptotic cells in the chemokine stimulated group and the unstimulated group was found (Fig. 7A). In contrast, CXCL12 at concentrations of 10 ng/mL significantly increased cell proliferation ($P = 0.016$), while higher concentrations did not change the cell proliferation significantly (Fig. 7B).

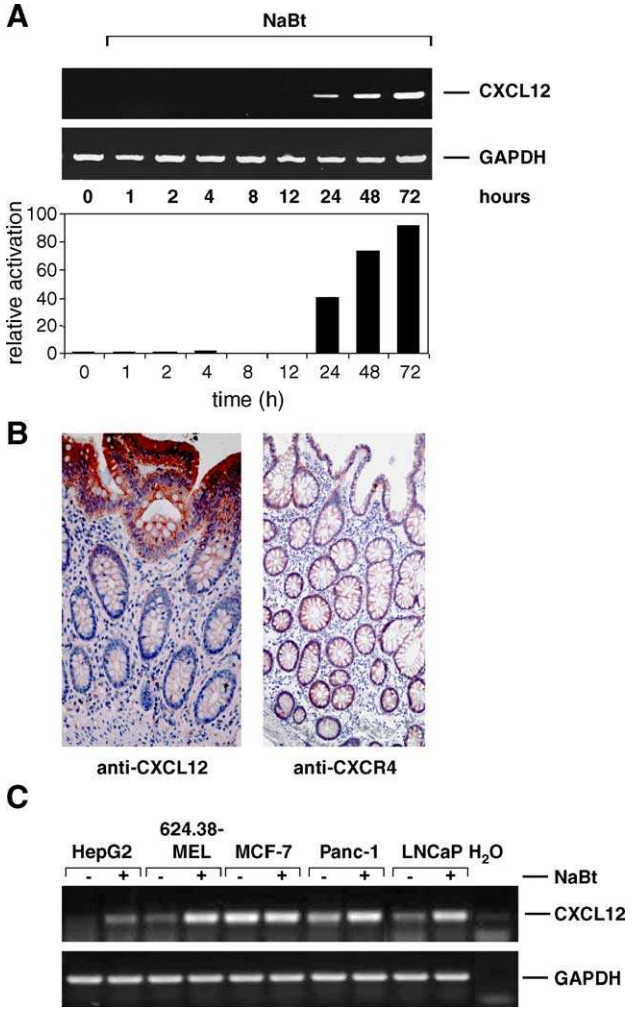

Fig. 3. CXCL12 mRNA expression increases with cell differentiation. (A) RT-PCR analysis of HCT116 stimulated with 5 mM sodium butyrate for time intervals as indicated. The RNA expression level of CXCL12 was normalized to GAPDH levels using TINA software. (B) Example of immunostaining with anti-CXCL12 antibody. A similar distribution pattern of CXCL12 staining in normal intestinal epithelial cells was observed in all patient samples demonstrating the highest CXCL12 expression in the apical intestinal epithelial cells and lower CXCL12 expression in the less differentiated epithelial cells at the base of the crypts. Normal intestinal epithelial cells also express the CXCL12 receptor CXCR4. (C) RT-PCR of cancer cell lines HepG2 (hepatocellular carcinoma), 624.38-MEL (melanoma), MCF-7 (breast carcinoma), Panc-1 (pancreatic carcinoma) and LNCaP (prostate carcinoma) stimulated with 5 mM sodium butyrate for 24 h. CXCL12 mRNA expression is up-regulated in all cell lines except MCF-7.

CXCR4-mediated activation of signaling pathways results in increased cell migration and invasion associated with actin polymerization and MMP-9 activation

The activation of MAP kinases such as ERK-1/2 and the activation of Akt has been linked to cell migration [42] which is a major characteristic of cancer growth. Therefore, we analyzed in cell migration assays if CXCL12 is chemotactic for intestinal epithelial cells. In these "wounding" assays, standardized, sterile wounds were created in IEC-6 cell monolayers which are a well established model of intestinal epithelial cell migration [25]. Twenty-four hours after wounding, the number of migrated cells over the wounding edge was counted under the microscope. To quantify the CXCL12-mediated cell migration, we analyzed a total of 24 fields in 12 separate dishes for each group containing more than 2000 migrated cells per group. This experiment demonstrated a statistically significant 80% increase of the cell migration rate in the CXCL12 stimulated cells ($P < 0.005$, Fig. 7C), which could be abrogated by the pretreatment with a CXCR4 neutralizing antibody.

Cytoskeletal reorganization is a requirement for cell motility and migration. Therefore, we analyzed the effect of CXCL12 on changes in the reorganization of filamentous actin (F-actin). Cells were starved overnight and displayed a disorganized cytoskeleton (Fig. 7D, left panel). CXCL12 caused a cytoskeletal rearrangement with stress fiber formation (Fig. 7D, right panel, see arrow) due to CXCR4 activation.

The ability of tumor cells to invasively infiltrate through the ECM characterizes their malignant growth potential. In addition, ECM is a vital component of the microenvironment in vivo, and its use in vitro has been shown to promote cell growth and enhance expression of cell-specific morphology and function [43]. To analyze the influence of CXCL12 on CRC cell migration into ECM proteins, we performed invasion assays using the

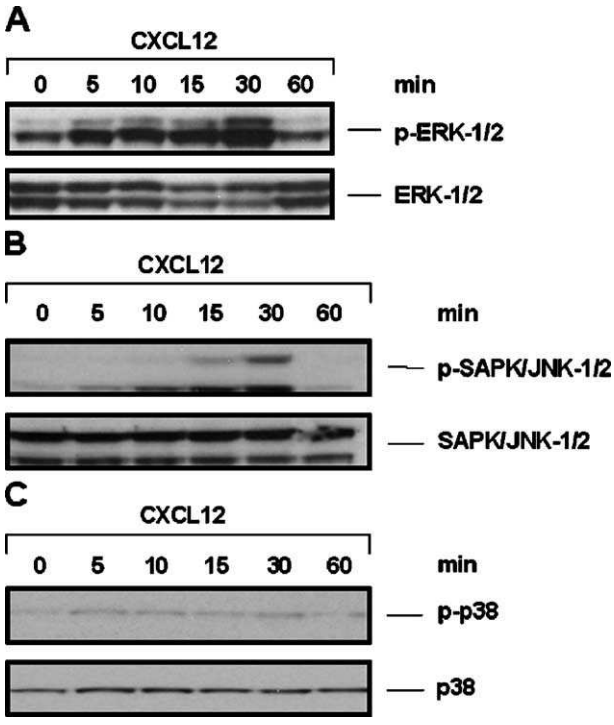

Fig. 4. CXCL12 activates MAP kinases in HT-29 cells. Activation and expression of phospho-ERK-1/2, phospho-SAPK/JNK and phospho-p38 were assessed by immunoblotting. (A) Phospho-ERK-1/2 activation after CXCL12 stimulation (100 ng/mL). (B) Stimulation of HT-29 cells with CXCL12 (100 ng/mL) resulted in increased phosphorylation of SAPK/JNK kinases. (C) No activation of p38 MAP kinase was observed after stimulation with CXCL12. One representative experiment ($n = 3$) is shown.

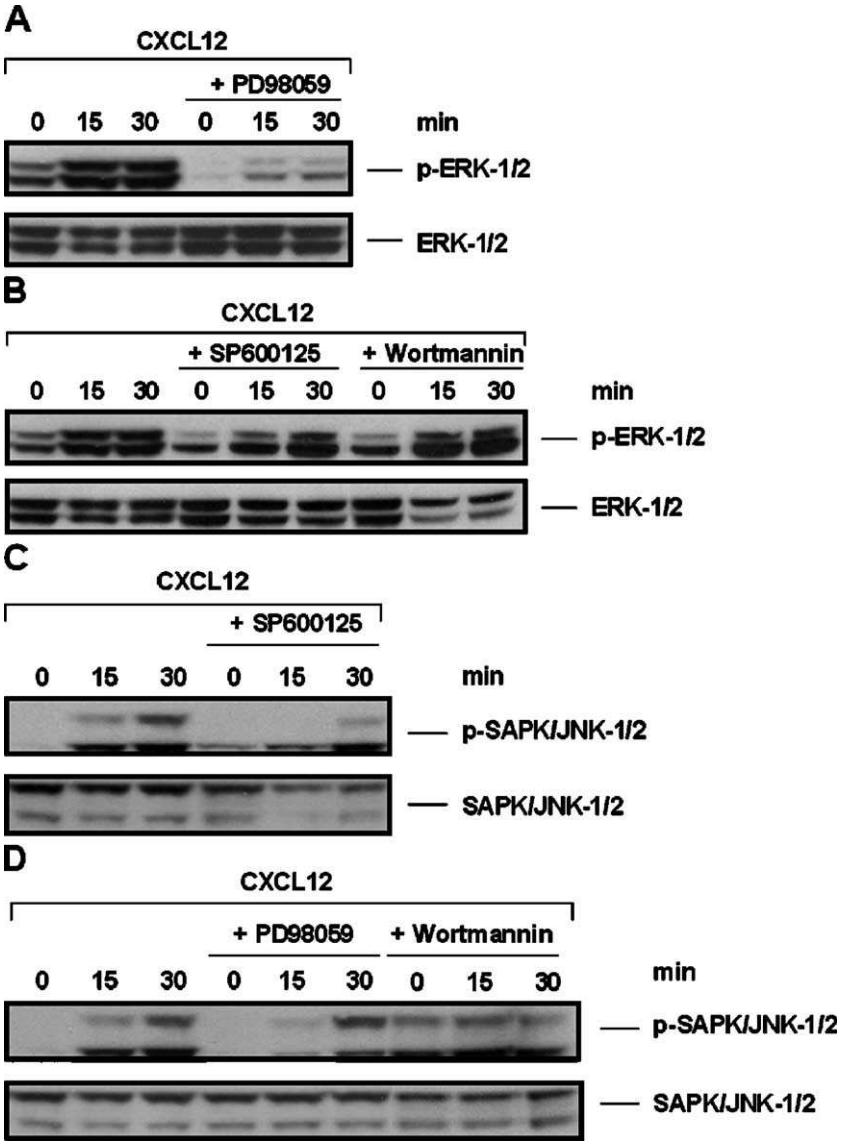

Fig. 5. Activation of MAP kinases can be blocked by specific inhibitors. (A) The CXCL12-induced ERK activation is MEK-1-dependent. Pretreatment with the MEK-1 inhibitor PD98059 (10 μmol/L one h before CXCL12 stimulation) resulted in a decreased ERK activation. (B) The CXCL12-induced ERK activation is SAPK/JNK and PI3 kinase-independent as shown by pretreatment with the SAPK/JNK inhibitor SP600125 (20 μmol/L) or the PI3 kinase inhibitor wortmannin (25 μmol/L) which did not influence ERK-1/2 activation. (C) Pretreatment with the SAPK/JNK inhibitor SP600125 (20 μmol/L) decreased SAPK/JNK activation. (D) SAPK/JNK activation is independent of MEK-1 or PI3 kinase. Pretreatment with the corresponding inhibitors PD98059 or wortmannin did not abrogate the CXCL12-induced SAPK/JNK activation. One representative experiment ($n = 3$) is shown.

CRC cell line HCT116 which was the CRC line with the highest invasion rate in a pilot experiment. Cells were grown in Matrigel Invasion Chambers which are coated with different ECM proteins such as collagen IV (31%), laminin (56%) and entactin (8%). CXCL12 induced a significant ($P < 0.05$), dose-dependent increase of cancer cell migration into the ECM filters (Fig. 7E).

Numerous studies have demonstrated the persistent localization of matrix metalloproteinase (MMP) expression to the interface between invading CRC cells and surrounding stroma supporting a role for MMPs in CRC invasion and metastasis [44]. Therefore, we analyzed the influence of CXCL12 on MMP-2 and MMP-9 secretion. Using gelatin zymography, we demonstrated that CXCL12 increases MMP-9 but not MMP-2 secretion in the colorectal cancer cell line HT-29 (Fig. 7F). Similar results were obtained in T84 cells (data not shown).

The direction of CXCL12-induced CRC cell migration is highly dependent on ECM proteins

Previous studies demonstrated that ECM proteins such as collagen IV are a requirement for CRC migration [45]. Thus, we repeated the cell migration assays in uncoated (ECM free) migration chambers. The two cell lines with the highest migration rate determined in pilot experiments, HCT116 and SW480, were chosen. Interestingly, CXCL12 blocked in these experiments the cell migration in a dose-

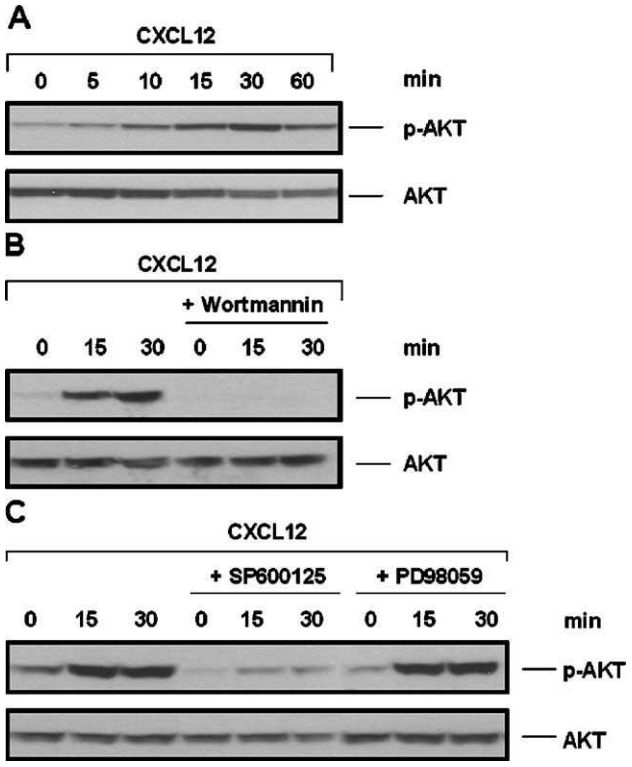

Fig. 6. CXCL12 induces Akt activation in intestinal epithelial cells. (A) CXCL12 induces PI3 kinase-dependent Akt phosphorylation. (B) Pretreatment with the PI3 kinase inhibitor wortmannin (25 μmol/L) resulted in a complete dephosphorylation of Akt. (C) Pretreatment with the JNK inhibitor SP600125 (20 μmol/L) partly decreased Akt activation whereas the MEK-1 inhibitor PD98059 had no effect on Akt phosphorylation. One representative experiment ($n = 3$) is shown.

dependent fashion in the two examined cell lines (Fig. 8A, $P < 0.0005$, total number of migrated cells counted: $n = 19543$). The results of the experiments in these two cell lines correlated highly ($r = 0.964$). This effect was, similar to the enhanced cancer cell invasion observed in the previous experiments (Fig. 7E), dependent on CXCR4 activation since anti-CXCR4 antibodies blocked the CXCL12-induced inhibition of migration (Fig. 8B). The migration was also highly dependent on MEK-1 kinase activation as demonstrated in preincubation experiments with the specific inhibitor PD98059 (Fig. 8B, $P < 0.0001$). The PI3 kinase inhibitor wortmannin had no specific effect on the CXCR4-mediated inhibition of migration but rather a general inhibitory effect on cell migration (Fig. 8B).

CXCR4 activation results in increased VEGF expression

Mice genetically deficient in the chemokine receptor CXCR4 or its ligand CXCL12 die perinatally with marked defects in vascularization of the gastrointestinal tract [5] suggesting a major role for CXCR4 in the process of angiogenesis. (Neo)angiogenesis also plays an essential role during carcinogenesis. Tumor tissue is characterized by increased vascularization. Vascular endothelial growth factor (VEGF) plays a key role in the process of (neo)angiogenesis. For several cancer cell lines, an increased VEGF expression has been shown after CXCR4 ligand stimulation [46,47]. Therefore, we analyzed the effect of CXCL12 stimulation on the VEGF gene transcription and protein expression in the CRC cell line HT-29. In these experiments, CXCL12 significantly up-regulated VEGF mRNA expression levels up to 8-fold (Fig. 8C). Accordingly, VEGF protein levels increased 5.8-fold after 48 h of CXCL12 stimulation (Fig. 8D). Similar results were obtained in HCT116 and SW480 cells (data not shown).

Discussion

A major function of chemokines is the recruitment of leukocytes to sites of infection or inflammation and homing of leukocytes [4,48]. New evidence indicates that chemokine receptors play a critical role in determining homing of metastatic tumor cells [2,49,50]. CRC is characterized by a specific pattern of organ metastasis with early metastasis into abdominal lymph nodes and liver. Tumor metastasis is of high clinical relevance since patients with advanced CRC rarely live beyond 5 years. In this study, we demonstrate that CRC cell lines express functional CXCR4 chemokine receptors for the chemokine SDF-1/CXCL12 which was also detected in normal intestinal epithelium, in primary CRC cells and metastatic tissue. Its ligand CXCL12 is expressed by normal intestinal epithelial cells possibly facilitating migration and invasion of cancer cells into the normal intestinal epithelium.

We demonstrate an inverse expression pattern of CXCR4 and CXCL12 in normal intestinal epithelium as well as in CRCs. Along the intestinal crypt axis, CXCL12 expression levels are low at the base of the crypts and increase in the more differentiated apical intestinal epithelial cells. An opposite expression pattern (high expression in undifferentiated cells at the base of the crypts and low expression in differentiated apical cells) has been described for CXCR4 in the colon [17] which was also found in some of our colonic tissue samples but less pronounced than the crypt axis distribution of CXCL12. Based on the intestinal epithelial cell migration induced by CXCL12 found in our experiments, this CXCL12 gradient within the intestinal crypt is likely a factor to be involved in the directed intestinal epithelial cell movement during cell differentiation from the base of the crypt to the apical part. In CRC tissue with high CXCR4 expression, we found only low or partially lost CXCL12 expression while CRCs with low CXCR4 expression had more often high CXCL12 expression. This is supported by previous studies describing a loss of CXCL12 in CRC and other malignant cell lines [15,51,52].

Similar to the increased CXCL12 mRNA expression in differentiated apical intestinal epithelial cells, stimulation with sodium butyrate, a histone deacetylase inhibitor, which increases cell differentiation, also increased CXCL12 mRNA expression. The differential expression of CXCL12

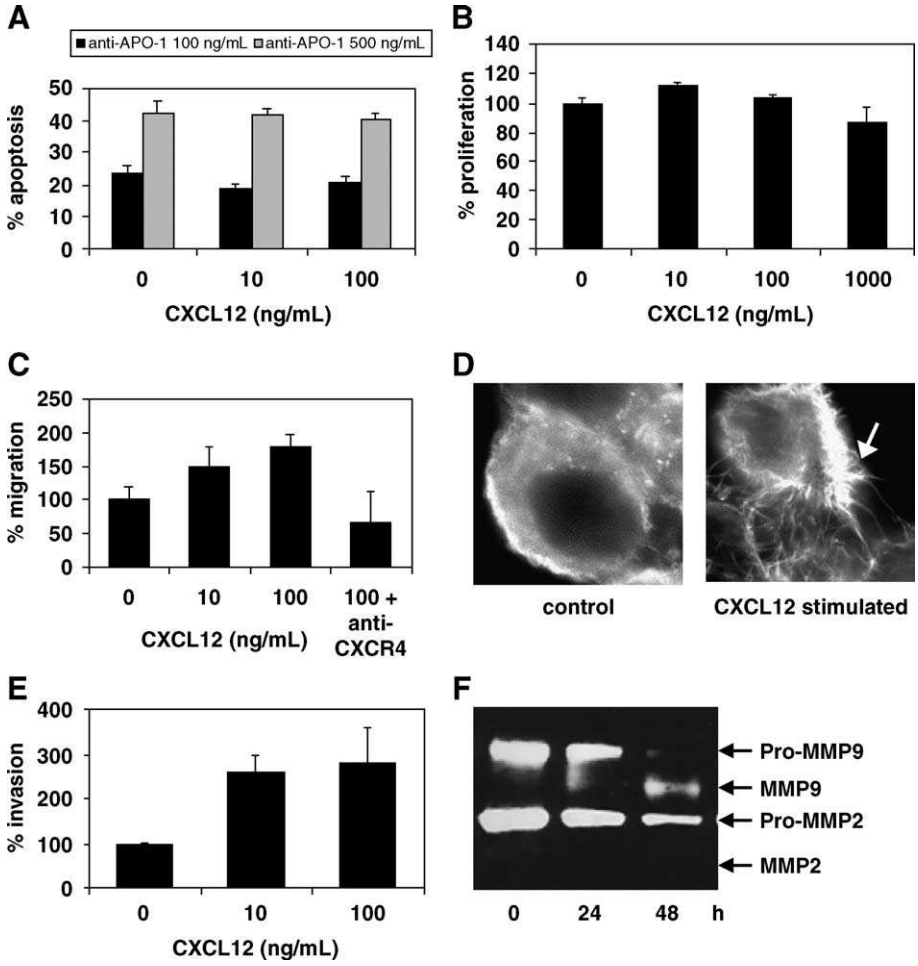

Fig. 7. CXCL12 does not regulate apoptosis but increases CRC cell proliferation, migration and invasion into extracellular matrix. (A) CXCL12 does not influence Fas ligand-mediated apoptosis in SW480 cells. Cells were treated with 100 and 500 ng/mL anti-APO-1 antibody and either CXCL12 stimulated or unstimulated for 24 h. Apoptosis was measured by FACS analysis. (B) CXCL12 increased cell proliferation in HT-29 cells. The cell proliferation rate was determined by MTS assay after 48 h. There was a statistical significant ($P < 0.05$) increase in cell proliferation when the cells were stimulated with lower doses of CXCL12 (10 ng/mL). Relative means ± SEM from 3 independent experiments are shown. (C) "Wounding" assays were used to analyze the influence of CXCL12 on intestinal epithelial cell migration. CXCL12 (100 ng/mL) induced a significant ($P < 0.005$) increase of the cell migration rate. Treatment with a neutralizing antibody against CXCR4 (10 μg/mL) resulted in decreased migration over the wounding edge. (D) CXCL12 stimulates actin polymerization in CRC cells. Immunohistochemistry of unstimulated (left panel) and CXCL12 stimulated (right panel) HCT-116 cells using phalloidin and Hoechst 33342 staining demonstrating actin cytoskeleton polymerization after 20 min of stimulation with CXCL12. (E) Directed cell migration was observed after CXCL12 stimulation in migration assays through 8 μm filters coated with ECM proteins. Stimulation with CXCL12 (10 or 100 ng/mL) resulted in an increased invasion of cells through the ECM protein coated membrane ($P < 0.05$). Relative means ± SEM from 3 independent experiments are shown. (F) Gelatin zymography demonstrated activation of MMP-9 but not MMP-2 in HT-29 cells.

mediated by sodium butyrate may also explain some of the anticancer activity of sodium butyrate which has been described for several malignancies such as melanomas although this might be also related to p53-dependent apoptosis mediated by sodium butyrate [53]. For example, a recent study demonstrated that sodium butyrate influences VEGF and hypoxia-inducible factor (HIF)-1α expression [54]. After 24 h of treatment, all the tested sodium butyrate concentrations reduced the HIF-1 protein level in the CRC line HT-29, whereas after a longer time of exposure, HIF-1 levels increased in the presence of a high sodium butyrate concentration with a concomitant increase in HIF-1 mRNA [54]. Interestingly, a recent study demonstrates that HIF-1 induces the expression of CXCL12 mRNA [55] suggesting that the effects of sodium butyrate are partly mediated by differential HIF-1 mRNA expression.

In addition, we demonstrated that stimulation with CXCL12 results in up-regulation of VEGF mRNA and protein expression in CRC cells. This is consistent with studies of CXCR4 in other cell lines [46,47] and might explain the marked defects in gastrointestinal vascularization and consecutive perinatal deaths observed in CXCR4 knockout mice [5]. The malignant potential of different cancers is defined by their ability to sustain growth even under hypoxic conditions. As demonstrated in this study, this ability is partly mediated by the angiogenic potential of CXCL12 which increases VEGF mRNA and protein expression which may directly influence the growth

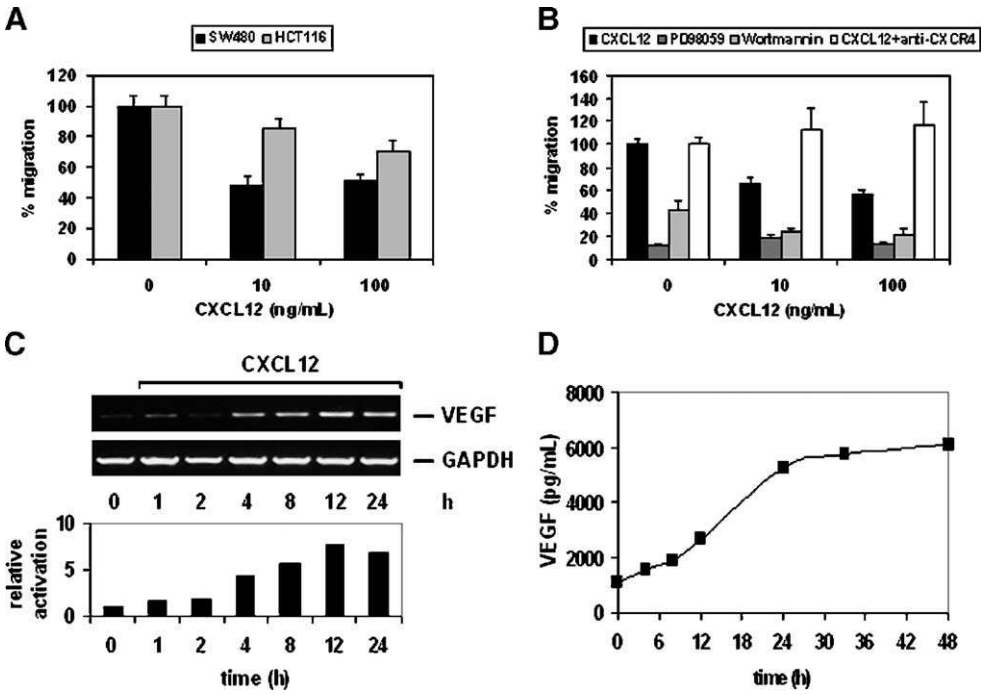

Fig. 8. CXCL12 mediates active CRC cell migration away from CXCL12 in the absence of ECM and increases VEGF expression in CRC cells. (A) CXCL12 reduced the migration of SW480 and HCT 116 cells through the filter in a dose-dependent manner. A total number of 10614 cells were counted for SW480 cells and 12718 cells for HCT116 cells. (B) CRC cell migration depends on CXCR4, MEK-1 and PI-3 kinases. The negative effect on migration in CXCL12 stimulated cells was abolished by the preincubation with an anti-CXCR4 antibody. Preincubation with PD98059 or wortmannin generally decreased the migration rate, whereas only PD98059 had an significant effect on CXCL12-mediated inhibition of migration; r(CXCL12/wortmannin) = 0.994; r(CXCL12/PD98059) = 0.438. (C) CXCL12 stimulation increases VEGF mRNA expression in CRC cells. RT-PCR analysis of HT-29 cells stimulated with CXCL12 (100 ng/mL) for time intervals as indicated. The CXCR4 mRNA levels were normalized to GAPDH. (D) VEGF protein expression is up-regulated after stimulation with 100 ng/mL CXCL12 as measured by ELISA according to the manufacturers instructions.

potential and prognosis of CXCR4 expressing tumors. Therefore, the level of CXCR4 expression may explain differences between different tumors regarding growth and tumor survival. In turn, previous studies demonstrated that VEGF increases CXCR4 mRNA expression [46,56]. For example, recently, it has been shown that VEGF up-regulates CXCR4 expression on vascular endothelial cells in human ovarian cancers synergizing CXCL12-mediated vascular endothelial cell migration [56]. Highly aggressive tumors rapidly outgrow their blood supply, leaving the cells in a hypoxic tissue environment. Tumor cells adapt to hypoxia by increasing their synthesis of HIF-1. However, as demonstrated by several recent studies, hypoxia itself increases CXCR4 and CXCL12 mRNA expression through HIF-1 [55,57,58]. Another recent study demonstrated that the von Hippel–Lindau tumor suppressor protein VHL negatively regulates CXCR4 expression owing to its capacity to target HIF-1 for degradation under normoxic conditions [59]. This process is suppressed under hypoxic conditions, resulting in HIF-dependent CXCR4 activation [59]. An analysis of clear cell renal carcinoma that manifests mutation of the VHL gene in most cases revealed an association of strong CXCR4 expression with poor tumor-specific survival [59]. These results suggest a mechanism for CXCR4 activation during tumor cell evolution and imply that VHL inactivation acquired by incipient tumor cells early in tumorigenesis confers not only a selective survival advantage but also the tendency to home to selected organs [59].

This CXCR4-mediated homing and CRC cell migration is according to our findings highly dependent on ERK and Akt signaling. CXCL12 predominantly activates MEK-ERK MAP kinase and Akt signaling. Interestingly, particularly the activation of ERK-MAP kinases and Akt has been implicated in cell migration [33]. For example, in T lymphocytes, CXCL12-induced chemotaxis is dependent on ERK-1/2 and PI3 kinase activation [33] which is consistent with our results. Our experiments demonstrated that CXCR4 activation results in increased intestinal epithelial cell migration which could be blocked using PI3 kinase and MEK-1 inhibitors. Increased cell migration is a major feature of malignant tumor growth.

Interestingly, we observed a bidirectional migration of CRC cells depending on the presence of ECM proteins. While CXCL12 was chemotactic for CRC cells in the presence of ECM proteins such as collagen IV and laminin, CXCL12 induced movement away from the chemokine without these proteins. A similar bidirectional migratory response of T cells dependent on the CXCL12 concentration has been described previously [60]. The active movement away from CXCL12 has been named “chemofugetaxis” [60]. Both the chemotactic and “chemofugetactic” effects

were CXCR4-dependent in our experiments since anti-CXCR4 antibodies inhibited these effects. Moreover, we demonstrated that CXCL12 is an activator of MMP-9 secretion which has been shown to be an important mediator of CRC invasion and metastasis [44]. CXCR4 activation also resulted in increased actin polymerization which is an important prerequisite for cell motility.

In summary, we demonstrate that the chemokine receptor CXCR4 is expressed by CRC and CRC metastases. Upon stimulation with its ligand CXCL12, several distinct signaling pathways including ERK-MAP kinases and Akt are activated. This results in increased cell migration, actin polymerization and increased expression of angiogenic mediators such as VEGF. CXCL12 secreted by intestinal epithelial cells therefore triggers local tumor invasion and may enhance CRC metastasis into other organs. Future studies will have to evaluate whether antagonists of CXCR4 activation on CRC cells, such as AMD3100 [61], can affect CRC cell survival and chemosensitivity potentially leading to new therapeutic avenues for patients with CRC.

Acknowledgments

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Appendix A. Supplementary data

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Manuskript [2]

Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK and Akt signaling resulting in proliferation and migration of colorectal cancer cells

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Cell Differentiation Dependent Expressed CCR6 Mediates ERK-1/2, SAPK/JNK, and Akt Signaling Resulting in Proliferation and Migration of Colorectal Cancer Cells

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Abstract The expression of CCL20 (MIP-3α), which chemoattracts leukocytes to sites of inflammation, has been shown in intestinal epithelial cells (IEC). Aim of this study was to analyze the role of the CCL20 receptor CCR6 in IEC and colorectal cancer (CRC) cells. Expression of CCR6 and CCL20 was analyzed by RT-PCR and immunohistochemistry. Signaling was investigated by Western blotting, proliferation by MTS assays and chemotactic cell migration by wounding assays. The effect of CCL20 on Fas-induced apoptosis was determined by flow cytometry. CCR6 and its ligand CCL20 are expressed in IEC. Moreover, CRC and CRC metastases express CCR6, which is upregulated during IEC differentiation. Stimulation of IEC with CCL20 and proinflammatory stimuli (TNF-α, IL-1β, LPS) significantly upregulates CCL20 mRNA expression. CCL20 expression was significantly increased in inflamed colonic lesions in Crohn's disease and correlated significantly with the IL-8 mRNA expression in these lesions ($r = 0.71$) but was downregulated in CRC metastases. CCL20 activated Akt, ERK-1/2, and SAPK/JNK MAP kinases and increased IL-8 protein expression. The CCL20 mediated activation of these pathways resulted in a 2.6-fold increase of cell migration ($P = 0.001$) and in a significant increase of cell proliferation ($P < 0.05$) but did not influence Fas-induced apoptosis. In conclusion, IEC and CRC express CCL20 and its receptor CCR6. CCL20 expression is increased in intestinal inflammation, while CCR6 is upregulated during cell differentiation. CCR6 mediated signals result in increased IEC migration and proliferation suggesting an important role in intestinal homeostasis and intestinal inflammation by mediating chemotaxis of IEC but also in mediating migration of CRC cells. J. Cell. Biochem. 97: 709–723, 2006.

Key words: chemokine; chemokine receptor; CCL20; CCR6; cell migration; colorectal cancer; Crohn's disease

The interaction between intestinal epithelial cells (IECs) and immune cells is an important component of the intestinal immune response coordinating the recruitment and activation of leukocytes at sites of intestinal injury, inflammation, and wound repair through the expression of chemokines and adhesion molecules. Moreover, in addition to their function as chemoattractants for leukocytes, chemokines were recently identified as potential regulators

Abbreviations used: CRC, colorectal cancer; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEC, intestinal epithelial cell; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MAP kinase, mitogen-activated protein-kinase; MEK, mitogen-activated protein kinase kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PI, phosphatidylinositol; RT-PCR, reverse transcriptase polymerase chain reaction; SAPK/JNK, stress-activated protein kinase/c-Jun-N-terminal kinase; TNF-α, tumor necrosis factor alpha.
Stephan Brand and Torsten Olszak contributed equally to this work.

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of IECs [Yang et al., 1997; Dwinell et al., 1999; Brand et al., 2002, 2005a; Niess et al., 2005].

Chemokines are chemotactic cytokines that induce mammalian cell movement through binding to seven transmembrane receptors [Luster, 1998]. These chemokine receptors are coupled to heterotrimeric G proteins and induce cell movement towards a concentration gradient of the cognate ligand [Luster, 1998]. Depending on their chemokine motif, chemokines can be classified into four different subfamilies: CC, CXC, XC, and CX3C chemokines.

The CC chemokine ligand (CCL)20 (MIP-3α, LARC, Exodus) is primarily produced by the gut epithelium, particularly in the dome epithelium of Peyer's patches where it induces local migration of dendritic cell (DC) subsets expressing its receptor CCR6 [Tanaka et al., 1999; Iwasaki and Kelsall, 2000] but also in liver, lung, appendix, and tonsillar crypts [Hieshima et al., 1997; Hromas et al., 1997; Dieu et al., 1998; Dieu-Nosjean et al., 1999; Tanaka et al., 1999]. The chemokine receptor CCR6 is expressed on immature DCs, B-cells, and memory T-cells expressing gut-homing (α4β7) and skin-homing (CLA) integrins [Liao et al., 1999; Nakayama et al., 2001]. In contrast, we recently demonstrated that the chemokine receptor CX3CR1 is expressed on mature DCs and has essential functions in the luminal sampling of intestinal bacteria [Niess et al., 2005]. Therefore, these two chemokine receptors are important mediators of trafficking of intestinal DCs. CCL20 is the only known chemokine ligand for CCR6, but recent studies have shown that this protein can also act as a functional receptor for the antimicrobial peptides β-defensin 1 and 2 [Yang et al., 1999]. CCR6 knockout mice show deficiencies in Peyer's patch organogenesis and impairment of mucosal responses to both oral antigens and enteric pathogens, and disturbed cutaneous hypersensitivity reactions but maintain normal systemic responses [Cook et al., 2000; Varona et al., 2001]. Interestingly, experiments in CCR6 knockout mice demonstrated that absence of CCR6 resulted in less severe intestinal pathology in animals treated with dextran sodium sulfate [Varona et al., 2003] suggesting that the CCR6/CCL20 axis has a critical, non-redundant role in the in vivo control of immune responses in the intestine.

In addition to their function as chemoattractant for inflammatory cells, chemokines mediate migration of epithelial cells. This function is likely to be important in cancer cell migration and metastasis. For example, signaling through the chemokine receptor CXCR4 mediates actin polymerization and pseudopodia formation in breast cancer cells and induces chemotactic and invasive responses [Muller et al., 2001]. In addition, organs that represent important sites of breast cancer metastasis are the most abundant sources of chemokine ligands for these tumor-associated receptors [Muller et al., 2001]. Recently, we demonstrated similar functions for CXCR4 in colorectal cancer (CRC) cells [Brand et al., 2005a,b].

The expression of CCR6 in IECs has been shown previously [Yang et al., 2005] but the detailed signaling mechanisms and specific biological functions of CCR6 in IEC are unsolved questions in determining its role in IEC biology including its role in intestinal inflammation and colorectal cancerogenesis which was the purpose of this study.

MATERIALS AND METHODS

Reagents

Polyclonal antibodies to phosphorylated extracellular signal-regulated kinase (ERK)-1/2 (Thr183/Tyr185), phosphorylated stress-activated protein kinase (c-Jun N-terminal kinase) SAPK/JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), and phospho-Akt (Ser473) were purchased from Cell Signaling (Beverly, MA). Anti-ERK-1/2, anti-SAPK/JNK, anti-p38, and anti-Akt antibodies were also from Cell Signaling. Horseradish peroxidase linked anti-rabbit secondary antibody was purchased from Amersham (Arlington Heights, IL). Previously characterized [Dellacasagrande et al., 2003] monoclonal antibodies against human CCR6 (Clone 53103) and CCL20 (Clone 67310) were from R&D Systems (Minneapolis, MN). Recombinant human CCL20, TNF-α, and IL-1β were obtained from R&D Systems (Minneapolis, MN). MEK-1 inhibitor PD98059, SAPK/JNK inhibitor SP600125, p38 inhibitor SB203580, and phosphatidylinositol3-(PI3) kinase inhibitor wortmannin were from Tocris Cookson (Bristol, UK). Lipopolysaccharide (LPS) from *Escherichia coli* (O26:B6) prepared by phenol extraction was purchased from Sigma (St. Louis, MO) and prepared as dispersed sonicate in endotoxin-free water (Life Technologies, Rockville, MD) before diluting to final concentration in supplemented media.

Cell Culture

The human CRC cell lines T84, SW480, Caco-2, HT-29, HCT116, and DLD-1 were obtained from American Type Culture Collection (Rockville, MD). While T84 cells were grown in Dulbecco's modified Eagle medium/F-12 (GIBCO BRL/Life Technologies, Gaithersburg, MD), the other cell lines were grown in Dulbecco's modified Eagle medium (GIBCO) with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (PAA, Pasching, Austria) in a humidified 5% CO_2 atmosphere at 37°C. For signal transduction experiments with CCL20, cells were starved overnight in serum-free medium.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD). For RT-PCR, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNA-free™-Kit, Ambion) to remove potential genomic DNA contaminants. Three micrograms of total RNA were reverse transcribed using Roche first strand cDNA synthesis kit. To control for genomic contamination, an identical parallel PCR reaction was performed containing starting material that had not been reverse transcribed. The following conditions were used for semi-quantitative PCRs: 25 or 35 cycles (depending on the specific PCR) of denaturing at 95°C for 45 s, annealing temperature at 61°C for 45 s, extension at 72°C for 45 s. All PCR reactions included GAPDH primers to quantify PCR products. The primers for the PCR reactions were as follows: CCR6 forward and reverse 5′-atttcagcgatgttttcgactc-3′ and 5′-ggagaagcctgaggacttgta-3′, CCL20 forward and reverse 5′-ttgctcctggctgctttgatg-3′ and 5′-tctttctgttcttggcgtatg-3′, GAPDH forward and reverse 5′-catgtgggccatgaggtccac-3′ and 5′-tgaaggtcggaagtcaactgat-3′. The PCR products were subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

Quantitative PCR

Real-time PCR was performed with a Rotorgene RG-3000 cycler (Corbett Research, Sydney, Australia) using the Quantitect SYBR Green PCR Kit from Qiagen (Hilden, Germany) following the manufacturer's guidelines. Oligonucleotide primers were designed according to the published sequence, and the following primer pairs were used: CCL20: forward 5′-ctactccacctctgcggcgaa-3′, reverse 5′-ttttactgaggagacgcacaa-3′; beta-actin: forward 5′-gccaaccgcgagaagatga-3′, reverse 5′-catcacgatgccagtggta-3′; interleukin-8 (IL-8): forward 5′-ccaggaagaaaccaccgga-3′, reverse 5′-gaaatcaggaaggctgccaag-3′ (MWG-Biotech, Ebersberg, Germany). CCL20 mRNA expression was normalized to beta-actin expression in the respective cDNA preparation. To compare CCL20 expression levels between inflamed and non-inflamed colonic lesions, CCL20 expression in non-inflammatory tissue was arbitrarily set to 1.0 (i.e., 100%).

Gel Electrophoresis and Immunoblotting

Cells were solubilized in lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and phosphatase inhibitors (400 mM sodium orthovanadate and 4 mM NaF) and were passed six times through a 21G needle. After 30 min on ice, lysates were cleared by centrifugation at 10,000*g* for 20 min. The protein concentration of each sample was quantified by the Bradford method. Immunoblotting was performed as previously described [Muehlhoefer et al., 2000].

Enzyme-Linked Immunosorbent Assay (ELISA)

For the quantification of IL-8 release, BD OptEIA Human IL-8 Elisa Kit II (BD Biosciences, Bedford, MA) was used according to the manufacturers instructions.

Immunohistochemistry

Surgical specimens from patients undergoing colectomy or lymph node resection were taken after obtaining patient's consent. Immunohistochemistry was performed as previously described [Muehlhoefer et al., 2000] using a standard streptavidin-peroxidase technique. Previously characterized [Dellacasagrande et al., 2003] monoclonal antibodies against human CCR6 and CCL20 were from R&D Systems (Minneapolis, MN) and were used according to the manufacturer's guidelines.

Cell Proliferation Assay

HT-29 cells were seeded onto 96-well plates at a density of 10,000 cells/well and were allowed to attach overnight. The cells were stimulated

with 10, 100, or 1,000 ng/ml CCL20, or with CCL20-free medium (negative control). The cell proliferation rate was determined by MTS assay on day 2 using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions.

Apoptosis Assays

Apoptosis assays were performed as we described previously [Eichhorst et al., 2004]. SW480 cells, which are less resistant to Fas induced apoptosis than HT-29 cells [Abreu et al., 2000], were used in these assays. For induction of CD95-mediated cell death ligand specific anti-APO-1 mAb at concentrations of 100 and 500 ng/ml was used.

Wounding Assay

Wounding assays were performed as previously described [Dignass and Podolsky, 1993]. Briefly, SW480 cells, which were the most suitable CRC cell line in pilots experiments, were grown in 6-well plates to complete confluence. Using a sterile razor blade, six standardized wounds were created in each plate. Detached cells were removed by three washes with PBS, and the cell medium was changed from 10% fetal calf serum (FCS) containing medium to 1% FCS containing medium. The cells were stimulated with CCL20 (10 and 100 ng/ml) or 1% FCS. The cells were washed with PBS after 24 h and fixed with ethanol. The number of migrated cells (over the wounding edge) was counted under a microscope (Olympus IX50, 10× magnification). For each group (CCL20 stimulated and medium stimulated) three dishes were analyzed, whereas for each dish six separate fields were counted.

Statistical Analysis

Statistical analysis was performed using two-tailed Student's *t*-test. *P*-levels <0.05 were considered as significant.

RESULTS

Expression of CCR6 and its Ligand CCL20 in IEC and CRC Cells

To determine if CCR6 is expressed in IEC and CRC cells and to utilize an IEC model to study the CCL20-CCR6 ligand-receptor system, we analyzed CCR6 and CCL20 mRNA expression in several human CRC derived IEC cell lines (HCT116, Caco-2, DLD-1, HT-29, SW480, T84). RT-PCR analysis demonstrated CCR6 and CCL20 mRNA expression in all cell lines tested (Fig. 1A). Using colonic biopsy samples, we demonstrated by immunohistochemical analysis that CCR6 is expressed in primary IEC (Fig. 1B).

Next, we analyzed the influence of cell differentiation on the CCR6 expression levels

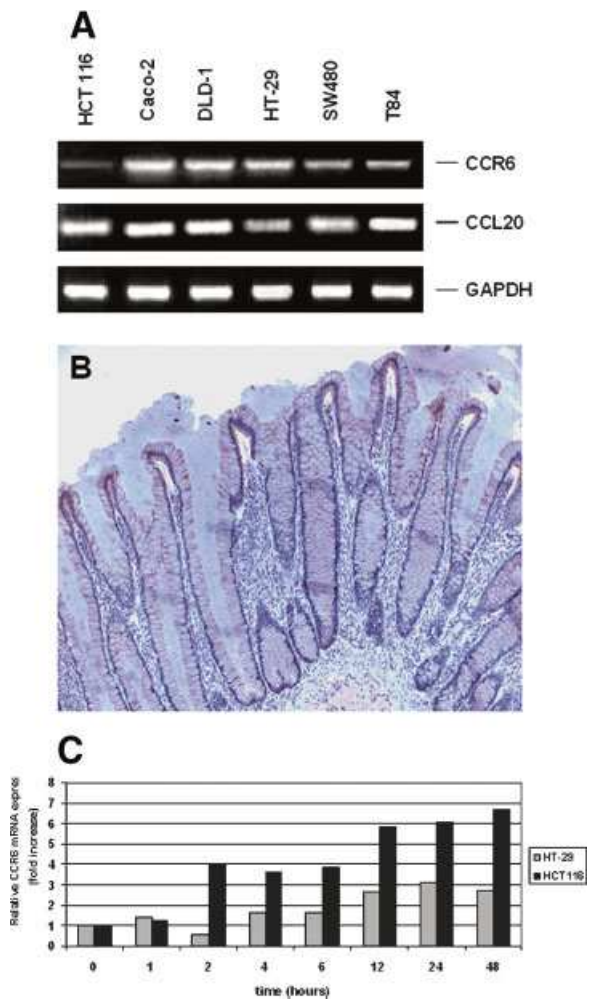

Fig. 1. CCR6 is expressed in IECs and its expression increases with cell differentiation. **A**: Expression of CCR6 and CCL20 in various IEC lines using RT-PCR analysis of mRNA derived from IECs as indicated. **B**: Immunohistochemistry of normal colonic tissue using a previously characterized [Dellacasagrande et al., 2003] human monoclonal anti-CCR6 antibody. CCR6 is predominantly expressed in differentiated, apical intestinal epithelial cells. **C**: Quantitative PCR analysis of HT-29 cells and HCT116 cells stimulated with 5 mM sodium butyrate for time intervals as indicated. The mRNA expression level of CCR6 was normalized to actin levels and is presented as -fold increase compared to CCR6 mRNA levels at time point 0 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in IEC using sodium butyrate stimulation as an established model of IEC differentiation [Chung et al., 1985; Jordan et al., 1999]. Increasing cell differentiation enhanced CCR6 mRNA expression in HT-29 cells up to 3.1-fold after 24 h and in HCT116 cells up to 6.7-fold after 48 h (Fig. 1C). Similarly, there was a clear distribution pattern of CCR6 staining in normal IECs beginning in the middle and increasing to the upper third of the crypt with highest expression in the most differentiated apical IECs (Figs. 1B and 2A).

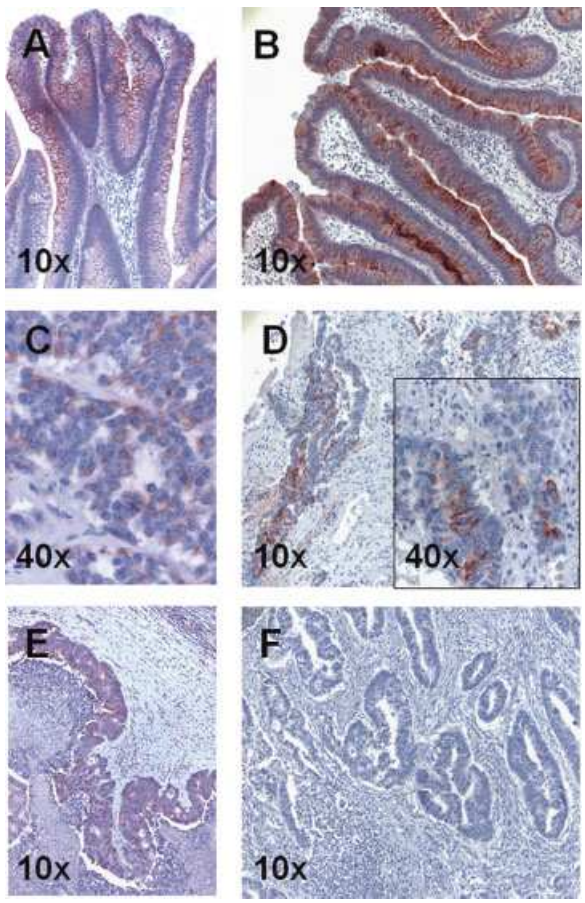

Fig. 2. Intestinal epithelial cells in colorectal adenomas, CRC, and CRC metastases express CCR6. Examples of the immunohistochemical analysis of 4 colorectal adenomas, 15 CRC, 4 CRC lymph node metastases, and 6 CRC liver metastases are shown. **A**: Immunostaining with a human monoclonal anti-CCR6 antibody (R&D Systems, Minneapolis, MN) of a histological section of a colorectal adenoma demonstrating that CCR6 expression is increased in the more differentiated, apical IEC. **B**: Colorectal adenoma stained with a human monoclonal anti-CCL20 antibody (R&D Systems, Minneapolis, MN) demonstrating high CCL20 expression. **C**: CRC stained with anti-CCR6. **D**: CRC stained with anti-CCL20. **E**: Lymph node metastasis stained with anti-CCR6. **F**: CRC section stained with anti-CCL20 demonstrating loss of CCL20 expression. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Having demonstrated that CCR6 and its ligand CCL20 are expressed in CRC derived cell lines (Fig. 1A), we next performed a comprehensive immunohistochemical analysis to study CCR6 and CCL20 expression in primary human CRC tissue and CRC metastases. This included tissue of a variety of CRCs of different stages of invasion and differentiation (histological grading G1-G4) taken from 15 different patients. In addition, we analyzed tissue from CRC lymph node metastases ($n = 4$), liver metastases ($n = 6$), and colorectal adenomas ($n = 4$). While normal colonic mucosa and all four colorectal adenomas demonstrated staining positive for CCL20 protein expression (Fig. 2B), none of the liver metastases and only half of the lymph node metastases expressed CCL20 according to the immunohistochemical analysis. Similarly, in 9 of 15 CRCs CCL20 expression was lost or decreased in comparison to normal colonic tissue suggesting that CCL20 is downregulated in cancerous and metastatic tissue (Table I, Fig. 2D,F). Moreover, the more invasive the specific CRC or CRC metastases were according to the TNM classification, the less likely was CCL20 expression (Table I). For example, none of the three T4 tumors/metastases expressed CCL20 (Table I). All CRC and CRC metastases stained, at least focally, positive for CCR6 (Table I, Fig. 2C,E). However, the number of high or moderate CCR6 expressing tissue samples decrease from 100% in colorectal adenomas to 66.6% CRC to 50% in lymph node metastases and only 33.3% in liver metastases suggesting, similar to the results of Figure 1B,C, that CCR6 expression is higher in differentiated tissues.

Proinflammatory Cytokines Increase CCL20 mRNA Expression

Several chemokines have been shown to be upregulated by proinflammatory cytokines. Therefore, we analyzed if LPS and the proinflammatory cytokines TNF-α and IL-1β regulate CCL20 mRNA expression. In these experiments, the human IEC cell line HT-29 was stimulated with LPS (1 μg/ml), TNF-α (50 ng/ml), or IL-1β (10 ng/ml). LPS increased CCL20 mRNA expression nearly twofold with a peak after 4 h (Fig. 3A). Similar to LPS, the proinflammatory cytokines TNF-α and IL-1β upregulated CCR6 mRNA expression up to 2.4- and 2.5-fold, respectively (Fig. 3B,C). In addition to proinflammatory stimuli (Fig. 3A–C), CCL20 increased its own mRNA expression in

TABLE I. Results of Semiquantitative Immunohistochemical Analysis of CCL20 and CCR6 Protein Expression in Colorectal Adenomas, CRC, CRC Lymph Node Metastases, and Liver Metastases

	TNM	Grading	CCL20 expression	CCR6 expression
Colorectal adenomas (CRA)				
CRA1	N/A	N/A	++	+
CRA2	N/A	N/A	++	+
CRA3	N/A	N/A	+	+
CRA4	N/A	N/A	+	+
Colorectal cancers (CRC)				
CRC1	T1N0M0	G1	−	(+)
CRC2	T2N0M1	G2	+	++
CRC3	T3N0M0	G2	(+)	+
CRC4	T3N0M0	G2	(+)	+
CRC5	T3N0M0	G2	+	(+)
CRC6	T3N0M0	G3	−	+
CRC7	T3N0M0	G3	+	+
CRC8	T3N0M1	G2	+	(+)
CRC9	T3N1M0	G2	(+)	+
CRC10	T3N1M1	G2	(+)	+
CRC11	T3N2M0	G2	+	+
CRC12	T3N2M0	G4	−	++
CRC13	T3N2M1	G3	+	(+)
CRC14	T4N2M0	G3	−	(+)
CRC15	T4N1M1	G2	−	++
Lymph node metastases (LNM)				
LNM1	T3N2M0	G2	+	++
LNM2	T3N1M1	G2	+	+
LNM3	T3N2M1	G3	−	(+)
LNM4	T4N2M0	G3	−	(+)
Liver metastases (LM)				
LM1	T2N0M1	G2	−	(+)
LM2	T3N0M1	G2	−	+
LM3	T3N1M1	G2	−	(+)
LM4	T3N1M1	G2	−	(+)
LM5	T3N1M1	G3	−	(+)
LM6	T3N1M1	G3	−	+

CRCs and CRC metastases are ranked according to the results of their TNM classification. All samples were scaled regarding their CCL20 and CCR6 immunostaining intensity by two senior investigators blinded to clinical, TNM, and treatment information. Scale: −, no expression; (+), partial/low expression; +, moderate expression; ++, strong expression.

IEC (Fig. 3D). This induction of CCL20 mRNA expression was biphasic with peak expression levels at 2 and 24 h, an expression pattern previously described for other proinflammatory cytokines including the chemokine ENA-78/CXCL5 after cell stimulation with proinflammatory cytokines [Schnyder-Candrian and Walz, 1997] suggesting that CCL20 increases the expression of proinflammatory cytokines resulting in the second CCL20 mRNA peak. This hypothesis was analyzed in the following experiments (Fig. 4).

CCL20 mRNA is Increased in Inflammatory Colonic Lesions in Crohn's Disease and Correlates Highly With IL-8 mRNA Expression Levels

Next, we analyzed if CCL20 mRNA expression correlates with the level of inflammation in vivo using real-time PCR. In these experiments, we compared CCL20 mRNA expression levels in biopsy samples taken from 14 different sites (of 7 patients with Crohn's disease) with endoscopically (macroscopic) inflamed colonic mucosa with those of endoscopically non-inflamed colonic mucosa taken from 14 different sites of the same 7 patients. The IL-8 expression, which was used as a control marker for inflammation, was significantly increased ($P < 0.05$) in the inflamed biopsy samples (Table II). The increase in IL-8 mRNA expression ranged from 1.4 up to 31.1-fold compared to the non-inflamed tissues. Similarly, CCL20 mRNA expression levels were significantly higher ($P < 0.05$) in biopsy samples with inflamed mucosa when compared with non-inflamed lesions (increase between 1.9- and 16.7-fold; Table II). Moreover, the CCL20 mRNA levels correlated highly with the IL-8 mRNA expression levels ($r = 0.71 \pm 0.09$), demonstrating its association with intestinal inflammation.

Having demonstrated that CCL20 mRNA expression correlates highly with IL-8 levels in Crohn's disease and that CCL20 upregulates its own mRNA expression in IEC, we analyzed next

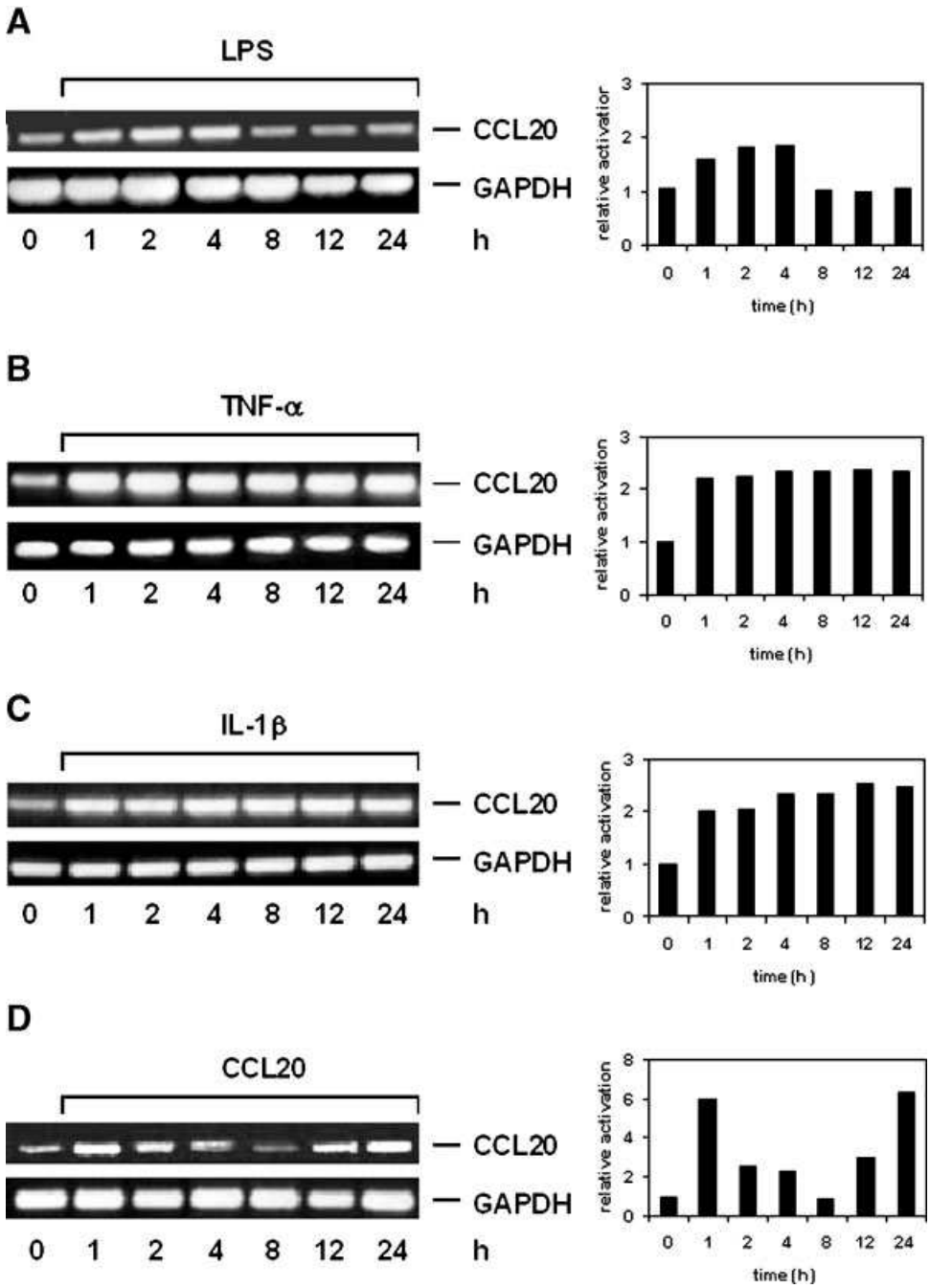

Fig. 3. CCL20 mRNA expression is upregulated after stimulation with various proinflammatory stimuli. Regulation of CCL20 mRNA expression after stimulation with LPS (1 μg/ml; **Panel A**) with TNF-α (50 ng/ml; **Panel B**), IL-1β (10 ng/ml; **Panel C**) and CCL20 (100 ng/ml; **Panel D**). The expression levels of CCL20 mRNA were normalized to GAPDH mRNA levels using TINA software.

if CCL20 increases IL-8 levels in IEC. In these experiments, HT-29 cells were stimulated with 100 ng/ml CCL20 and IL-8 protein expression was measured by ELISA. CCL20 stimulation increased IL-8 protein levels 4.6-fold after 24 h (Fig. 4).

CCL20 Induces ERK-1 and ERK-2 Activation by MEK-1 Dependent Mechanism

Having demonstrated that CCR6 is functional in IEC resulting in increased expression of the proinflammatory chemokines CCL20 and

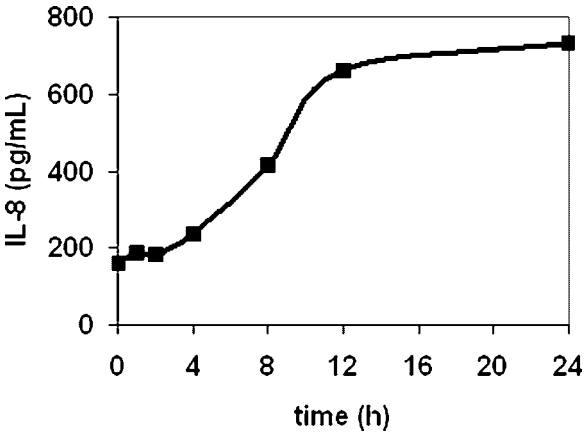

Fig. 4. CCL20 increases IL-8 protein expression in HT-29 cells. IL-8 protein expression measured in ELISA assays increased 4.6-fold after stimulation with recombinant human CCL20 (100 ng/ml).

IL-8, we next analyzed major signaling pathways of CCR6 in IEC. In these experiments, CCR6 expressing SW480 and HT-29 cells were stimulated with the CCR6 ligand CCL20. Stimulation of chemokine receptors can result in transient activation of mitogen-activated protein (MAP) kinases [Ganju et al., 1998a; Tilton et al., 2000]. Similarly, 100 ng/ml CCL20 induced transient activation of ERK-1/2 (Fig. 5A). To identify the upstream signaling events, we investigated the effect of the MEK-1 inhibitor PD98059 on the CCL20 mediated ERK regulation. PD98059 downregulated ERK-1/2 phosphorylation after CCL20 stimulation (Fig. 5B), suggesting MEK-1 as an upstream signal transducer of the CCL20 induced ERK activation. Crosstalk between the PI3-kinase and the MEK-ERK pathway has been proposed [Rommel et al., 1999]. However, ERK activation after CCL20 stimulation was not significantly affected by pre-treatment with wortmannin (Fig. 5C), suggesting a PI3-kinase-independent activation of ERK-MAP kinases by CCL20. Similarly, the JNK kinase inhibitor SP600125 did not influence the CCL20 induced ERK activation (Fig. 5D).

CCR6 Activation Results in SAPK/JNK- and Akt Phosphorylation

Activation of chemokine receptors may also result in activation of p38 and SAPK/JNK kinases and Akt [Ganju et al., 1998b; Sotsios et al., 1999; Robledo et al., 2001]. Stimulation of SW480 and HT-29 cells with CCL20 resulted in phosphorylation of SAPK/JNK kinases

TABLE II. Comparison of CCL20 and IL-8 mRNA Expression in Inflamed and Non-Inflamed Colonic Lesions of Patients With Crohn's Disease

Patient no.	Current medication	Anatomic site of biopsy sampling		CCL20 expression level inflamed versus non-inflamed	IL-8 expression level inflamed versus non-inflamed	Correlation CCL20/IL-8
		Non-inflamed	Inflamed			
1*	AZA, IFX	Cecum*	Terminal ileum*	1.93	4.50	0.985
2	Mesalazine, AZA	Descending colon	Descending colon	3.69	11.67	0.636
3	AZA	Cecum	Cecum	16.73	11.08	0.372
4*	Mesalazine, cortico-steroids	Cecum*	Terminal ileum*	3.41	1.52	0.497
5	No medication	Descending colon	Descending colon	2.15	1.41	0.999
6	MTX	Transverse colon	Transverse colon	12.27	31.12	0.955
7	AZA	Transverse colon	Transverse colon	2.00	2.24	0.542
Average ± SEM				6.03 ± 2.11	9.08 ± 3.76	0.71 ± 0.09

CCL20 expression was measured by quantitative PCR and normalized with respect to beta-actin expression levels in the respective cDNA preparation. To compare expression levels between inflamed and non-inflamed colonic lesions, mRNA expression in non-inflamed tissue was arbitrarily set to 1.0 (100%). The current medical therapy, under which biopsy samples were taken, and the anatomic site from which the samples were taken, are given for all patients. Biopsy sampling for inflamed and non-inflamed lesions was intended to be performed in the same colonic or ileal segment. This was not possible in patients marked with an asterisk (*) due to severe inflammation in the whole anatomic segment. Therefore, biopsies from a bordering (non-inflamed) segment were included for comparison. Abbreviations: AZA, azathioprine; IFX, infliximab; MTX, methotrexate.

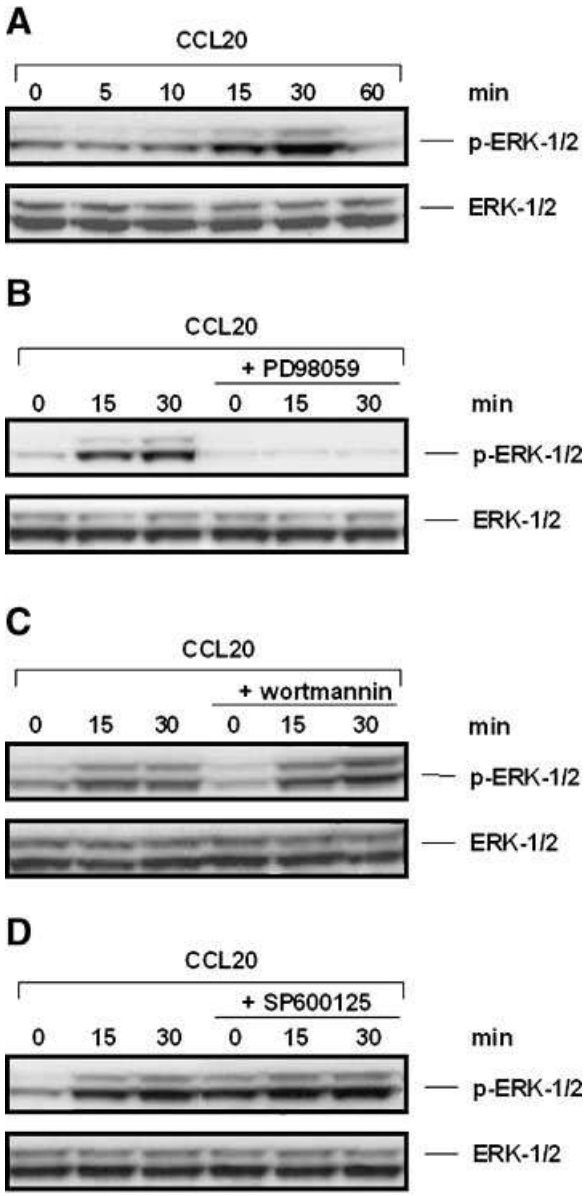

Fig. 5. CCL20 activates ERK-MAP kinases in SW480 cells. Activation and expression of phospho-ERK-1/2 was assessed by immunoblotting. **A**: Phospho-ERK-1/2 activation after CCL20 stimulation (100 ng/ml). **B**: The CCL20-induced ERK activation is MEK-1 dependent. Pretreatment with the MEK-1 inhibitor PD98059 (10 μmol/L 1 h before CCL20 stimulation) resulted in decreased ERK activation. **C**: The CCL20 induced ERK activation is PI3 kinase independent. Pretreatment with the PI3 kinase inhibitor wortmannin (25 μmol/L) did not influence ERK-1/2 activation. **D**: Similarly, ERK activation is independent from SAPK/JNK signaling. Pretreatment with the SAPK/JNK inhibitor SP600125 (20 μmol/L) did not influence ERK-1/2 activation. Similar results were found in HT-29 cells (data not shown). One representative experiment (n = 3) is shown.

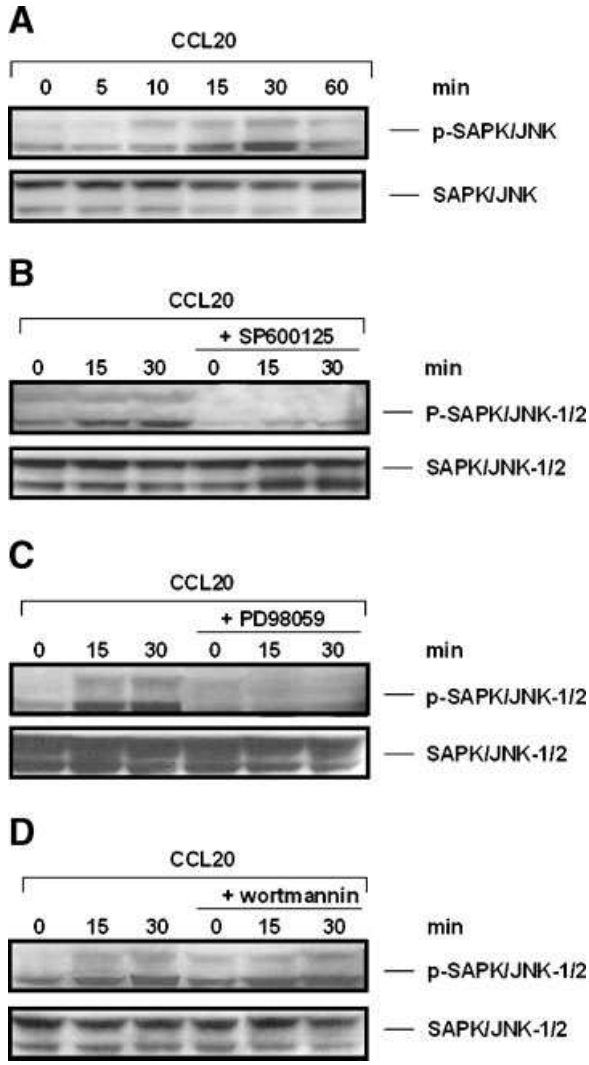

Fig. 6. CCL20 stimulation activates SAPK/JNK. **A**: Stimulation of SW480 cells with CCL20 (100 ng/ml) resulted in increased phosphorylation of SAPK/JNK kinases. **B**: Pretreatment with the SAPK/JNK inhibitor SP600125 (20μmol/L) decreased SAPK/JNK activation. **C**: SAPK/JNK activation is MEK-1 dependent. Pretreatment with the MEK-1 inhibitor (PD98059, 10 μmol/L) decreased JNK activation. **D**: The PI3 kinase inhibitor wortmannin (25 μmol/L) did not abrogate the CCL20 induced SAPK/JNK activation. Similar results were found in HT-29 cells (data not shown). One representative experiment (n = 3) is shown.

(Fig. 6A). Pretreatment with the JNK inhibitor SP600125 significantly suppressed phosphorylation of SAPK/JNK kinases (Fig. 6B), as did pretreatment with the MEK-1 inhibitor PD98059 suggesting an MEK-1-dependent pathway (Fig. 6C). In contrast, the PI3 kinase inhibitor wortmannin did not influence phosphorylation levels (Fig. 6D). In addition, CCL20 stimulation resulted only in a weak increase of phosphorylation levels of p38 (data not shown)

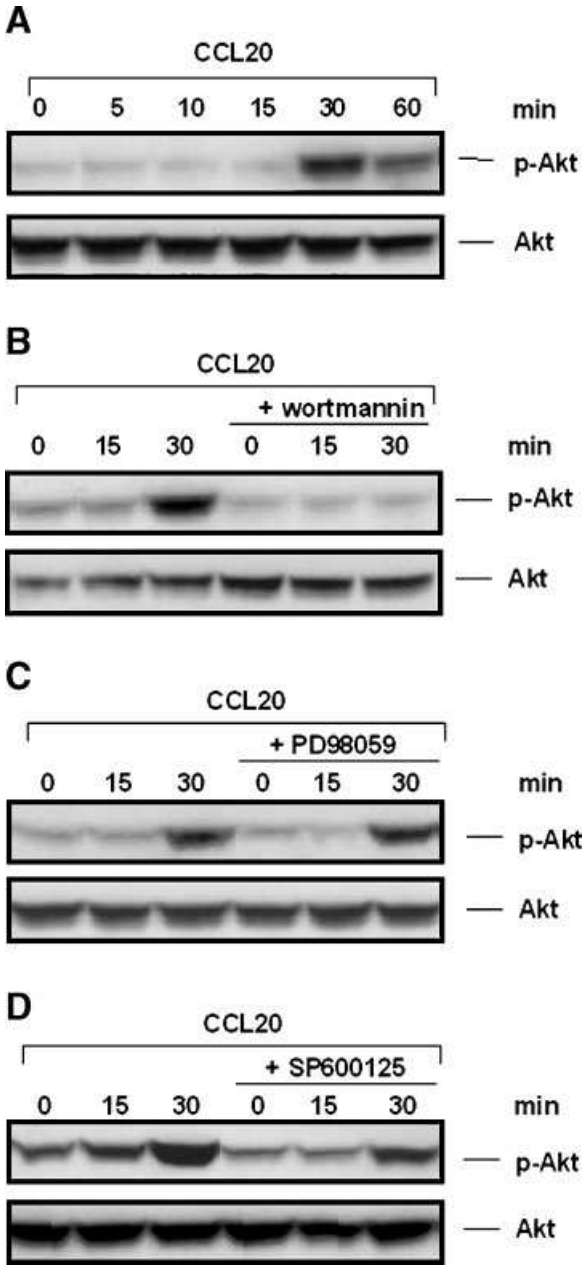

Fig. 7. CCL20 induces Akt activation in SW480 cells. **A**: CCL20 (100 ng/ml) induces Akt phosphorylation. **B**: Pretreatment with the PI3 kinase inhibitor wortmannin (25 μmol/L) inhibited Akt phosphorylation. **C**: Pretreatment with the MEK-1 inhibitor PD98059 had no effect on Akt phosphorylation levels. **D**: Similarly, the JNK inhibitor SP600125 (20 μmol/L) did not influence Akt phosphorylation levels. Similar results were found in HT-29 cells (data not shown). One representative experiment (n = 3) is shown.

suggesting that CCL20 activate primarily ERK and JNK MAP kinase signaling in IEC. Furthermore, CCL20 binding to its receptor CCR6 also resulted in increased phosphorylation of Akt (Fig. 7A). Pretreatment with the PI3 kinase inhibitor wortmannin completely inhibited CCL20-induced Akt phosphorylation (Fig. 7B), while pretreatment with the MEK-1 inhibitor PD98059 (Fig. 7C) and the SAPK/JNK inhibitor SP600125 (Fig. 7D) did not influence Akt activation suggesting a PI3 kinase and SAPK/JNK-independent pathway.

CCL20 Does not Influence Fas Ligand Induced Apoptosis but Stimulates IEC Proliferation

We demonstrated that CCL20 stimulation results in ERK-1/2 and Akt activation which have been shown to mediate anti-apoptotic pathways and increase cell proliferation [Fridell et al., 1996; Dudek et al., 1997]. Both, cell proliferation and apoptosis are important mechanisms by which the intestinal barrier and its restitution (e.g., in intestinal inflammation) are controlled. Moreover, increased cell proliferation and decreased apoptosis have been demonstrated in cancerous tissue resulting in enhanced tumor growth and resistance to "apoptotic" and antiproliferative therapy strategies [Eichhorst and Krammer, 2001]. Therefore, we investigated the chemokine-mediated effect on apoptosis using experimental conditions which we previously established [Eichhorst et al., 2004]. In these experiments, SW480 cells were used which are less resistant to Fas induced apoptosis than HT-29 cells [Abreu et al., 2000]. However, no significant difference between the number of apoptotic cells in the chemokine stimulated group and the unstimulated group was found (Fig. 8A). In contrast, CCL20 at concentrations of 10 ng/ml and 100 ng/ml increased significantly cell proliferation ($P < 0.05$ and $P = 0.006$, respectively), while higher concentrations did not change the cell proliferation rate significantly (Fig. 8B).

CCR6 Activation Results in Increased Cell Migration

The activation of MAP kinases such as ERK-1/2 and the activation of Akt have been linked to cell migration [Bonacchi et al., 2001] which is a major mechanism of wound healing after injury of the IEC barrier, for example, in severe inflammation but also a main characteristic of CRC cancer growth. Therefore, we analyzed in cell migration assays if CCL20 is chemotactic for IECs. In these "wounding" assays, standardized sterile wounds were created in SW480 cell monolayers, which were the most suitable CCR6-expressing IEC line for wounding assays

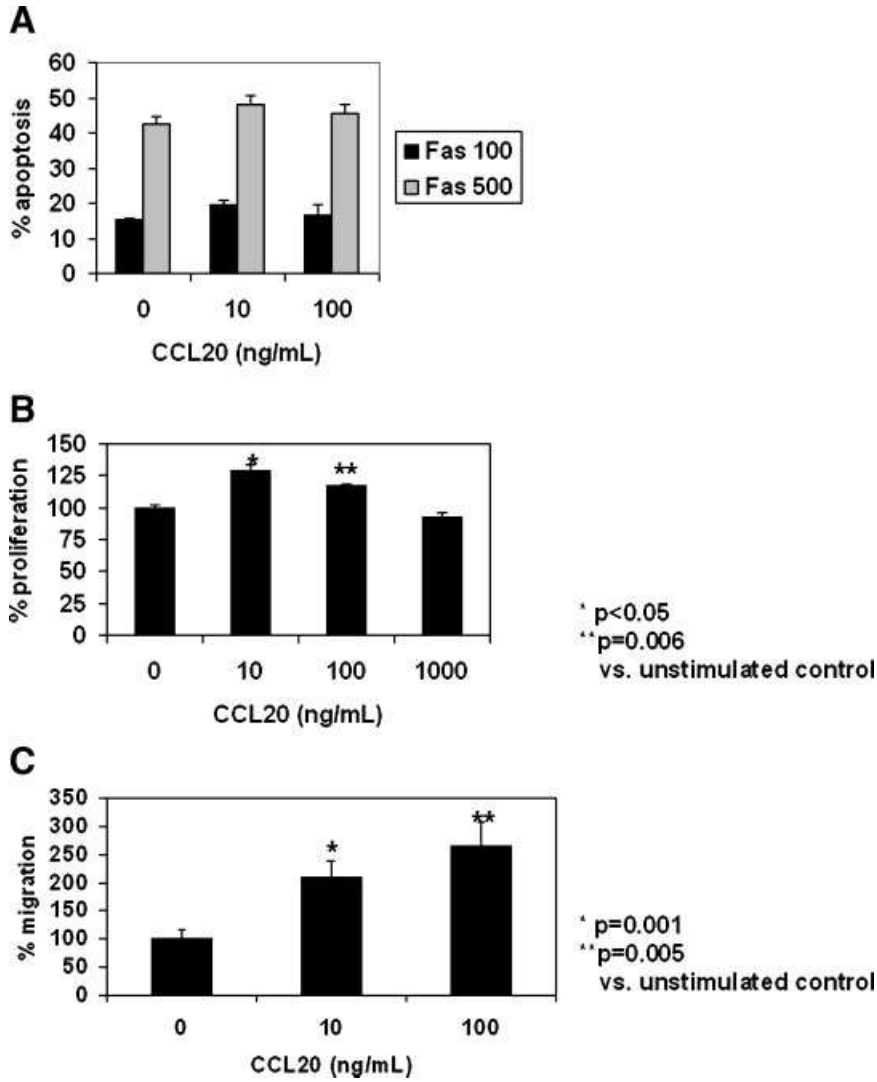

Fig. 8. CCL20 does not regulate apoptosis but increases cell proliferation and cell migration in IEC cells. **A**: CCL20 does not influence Fas ligand mediated apoptosis in SW480 cells. Cells were treated with 100 and 500 ng/ml anti-APO-1 antibody and either CCL20 stimulated or remained unstimulated for 24 h. Apoptosis was measured by FACS analysis. **B**: CCL20 increases cell proliferation in HT-29 cells. HT-29 cells were seeded onto 96 well plates at a density of 10,000 cells/well and were grown for 1 day. After starvation in serum-free medium overnight, the cells were stimulated with CCL20 as indicated, or with CCL20-free medium (negative control). The cell proliferation rate was determined by MTS assay after 48 h. There was a statistical significant ($P < 0.05$ and $P = 0.006$) increase in cell proliferation when the cells were stimulated with CCL20 (10 ng/ml and 100 ng/ml, respectively). Relative means ± SEM from three independent experiments are shown. **C**: ''Wounding'' assays were used to analyze the influence of CCL20 on IEC migration. Standardized, sterile wounds were created in SW480 cell monolayers as described in the Method section. Twenty-four hours after wounding the number of migrated cells (over the wounding edge) was counted under the microscope. CCL20 induced a significant increase of the cell migration rate (10 ng/ml CCL20: $P = 0.001$; 100 ng/ml CCL20: $P = 0.005$ vs. medium stimulated controls).

in pilot experiments. Twenty-four hours after wounding, the number of migrated cells over the wounding edge was counted under the microscope. To quantify the CCL20 mediated cell migration, we analyzed a total of 18 fields in 6 separate dishes for each group containing more than 300 migrated cells per group. This experiment demonstrated a statistically significant increase of the cell migration rate in the CCL20 stimulated cells in comparison to medium stimulated controls ($P = 0.001$ for 10 ng/ml CCL20; $P = 0.005$ for 100 ng/ml CCL20; Fig. 8C).

DISCUSSION

Chemokines are chemotactic cytokines, which recruit leukocytes to sites of infection

or inflammation [Butcher and Picker, 1996; Baggiolini, 1998; Zlotnik and Yoshie, 2000; Niess et al., 2005]. However, recent studies indicate that certain chemokines and chemokine receptors also play an important role in IEC biology [Dwinell et al., 1999; Jordan et al., 1999; Brand et al., 2002, 2005a,b]. Moreover, several studies demonstrated that chemokine receptors are critical in determining homing of metastatic tumor cells [Geminder et al., 2001; Muller et al., 2001; Robledo et al., 2001; Taichman et al., 2002; Brand et al., 2005a].

Previous studies demonstrated that IEC express the CCR6 ligand CCL20 [Tanaka et al., 1999; Iwasaki and Kelsall, 2000; Kwon et al., 2002; Puleston et al., 2005]. Here, we show that IEC also express its receptor CCR6. Furthermore, we demonstrated that CCR6 is functional in IEC. Stimulation of IEC with CCL20 resulted in activation of distinct signaling pathways. We also demonstrated that this chemokine ligand receptor pair is an important mediator of intestinal inflammation. Stimulation of IEC with proinflammatory cytokines and LPS significantly upregulated CCL20 mRNA expression. Moreover, CCL20 increased its own mRNA expression, a mechanism previously shown for other proflammatory cytokines and chemokines such as fractalkine [Brand et al., 2002]. In addition, we demonstrated that CCR6 activation in IEC resulted in increased protein expression of the proinflammatory chemokine IL-8. Quantitative PCR analysis demonstrated that CCL20 is upregulated in inflamed colonic biopsy samples taken from patients with Crohn's disease. The CCL20 mRNA levels correlated highly with the IL-8 mRNA levels in these biopsy samples suggesting an important role in intestinal inflammation. These results are consistent with previous studies demonstrating increased CCL20 expression levels in colonic biopsy samples of patients with inflammatory bowel disease [Kwon et al., 2002; Kaser et al., 2004; Puleston et al., 2005].

A multitude of studies indicates that chronic intestinal inflammation as seen in ulcerative colitis and Crohn's disease is a risk factor for developing CRC [Katzka et al., 1983; Ekbom et al., 1990; Choi and Zelig, 1994; Gillen et al., 1994]. CRC is characterized by metastasis into abdominal lymph nodes and liver. Tumor metastasis is of high clinical relevance since patients with advanced CRC rarely live beyond 5 years. In this study, we demonstrate that CRC cells express functional CCR6 chemokine receptors, which was also detected in all primary CRC cells and in all metastases analyzed. The liver is a main producer for CCL20 [Hieshima et al., 1997], therefore liver metastasis of CCR6-expressing CRC cells is likely to be facilitated by this chemokine system. This is supported by a recent study, which demonstrated that CCR6 is over-expressed in liver metastases of colon, thyroid, and ovarian carcinomas compared with normal liver [Dellacasagrande et al., 2003]. In contrast to CCR6, the expression of CCL20 was downregulated in several CRCs and CRC metastases (Table I). Stimulation of CRC cells by the CCR6 ligand CCL20 triggers responses that are similar to leukocyte responses to chemoattractants, such as CRC migration. Our results demonstrating a role for CCR6 in CRC cell migration are supported by a very recent study [Yang et al., 2005], which showed that apical stimulation of polarized CCR6 expressing CRC cells with CCL20 resulted in tyrosine phosphorylation of the p130 Crk-associated substrate (Cas), an adaptor/scaffolding protein that localizes in focal adhesions and has a role in regulating cytoskeletal elements important for cell attachment and migration.

Moreover, we demonstrate that CCL20 is expressed by normal IECs possibly facilitating migration and invasion of cancer cells into the normal intestinal epithelium. In addition to CRC, functional CCR6 receptors have been detected in several other malignancies such as pancreatic cancer [Kleeff et al., 1999; Kimsey et al., 2004; Campbell et al., 2005], hepatic cancer cells [Fujii et al., 2004], multiple myeloma [Moller et al., 2003], and certain B-cell non-Hodgkin's lymphomas [Rodig et al., 2002] suggesting similar mechanisms of tumor cell migration and metastasis in a variety of malignancies.

Interestingly, we demonstrate an increasing CCR6 expression along the intestinal crypt axis with low expression levels at the base of the crypts to high expression levels in the more differentiated apical cells. Although CCR6 expression increased in normal IEC with cell differentiation, this association was less clear in CRC tissue. However, high CCR6 expression was less common in CRC metastases than in normal colonic tissue and colorectal adenomas supporting the observation that CCR6 expression is higher in differentiated tissues. Our observation that CCR6 is a marker of normal

IEC differentiation, is also supported by a recent complex gene analysis of markers of differentiation in keratinocytes and IECs which found CCR6 primarily in IEC at later stages of differentiation [Dabelsteen et al., 2003]. Based on the IEC migration induced by CCL20 found in our experiments, this CCR6 gradient within the intestinal crypt is likely to be a factor involved in the directed IEC movement during cell differentiation from the base of the crypt to the apical part.

CCL20 predominantly activates the MEK-ERK MAP kinase signaling pathway in IEC which has also been shown to be the major signaling pathway of other chemokine receptors such as CX3CR1 [Brand et al., 2002] and CXCR4 [Tilton et al., 2000; Brand et al., 2005a,b]. However, in contrast to our analysis of CX3CR1 signaling [Brand et al., 2002], CCR6 activation also resulted in increased phosphorylation of SAPK/JNK kinases as well as increased Akt phosphorylation levels. Interestingly, two recent studies demonstrated that SAPK/JNK is activated in Crohn's disease [Hommes et al., 2002; Waetzig et al., 2002], and that inhibition of SAPK/JNK resulted in significant clinical benefit and rapid endoscopic ulcer healing [Hommes et al., 2002].

Particularly, the activation of ERK-MAP kinases and Akt has been implicated in cell migration [Sotsios et al., 1999; Bonacchi et al., 2001; Yap, 2001; Graness et al., 2002]. Our experiments demonstrated that CCR6 activation results in increased IEC migration. Increased cell migration is a major feature of malignant tumor growth. In addition, CCR6-induced IEC migration is also important during normal IEC development and under inflammatory conditions. For example, an impairment of the integrity of the mucosal epithelial barrier is observed in the course of various intestinal disorders including inflammatory bowel diseases. As demonstrated in wounding assays in this study, CCL20 stimulation facilitates the epithelial restitution.

In summary, we demonstrate that the chemokine receptor CCR6 is expressed by normal IEC and CRC cells. Upon stimulation with its ligand CCL20 several distinct signaling pathways including ERK-MAP kinases and Akt are activated. CCL20 mRNA expression is upregulated in intestinal inflammation but downregulated in CRC metastases. CCR6 expression in CRC and CRC metastases indicate a role for this chemokine system in CRC migration and metastasis. This is supported by our results of in vitro models demonstrating increased CRC proliferation and migration after CCR6 activation. Therefore, CCR6 and its ligand, which are essential in controlling immune cell trafficking in response to inflammatory stimuli, may have also an important role in determining the metastasis of CRC cells in vivo.

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CXCL16 is a novel marker of intestinal inflammation in Crohn's disease

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CXCL16 is a novel marker of intestinal inflammation in Crohn's disease

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Key words:

Crohn's disease, ulcerative colitis, inflammatory bowel disease, chemokine, CXCL16, CXCR6, mouse model, intestinal inflammation, intestinal epithelial cells, cytokine, signaling

Abbreviations

APC, antigen-presenting cell; CD, Crohn's disease; CpG, cytosine-phospho-guanine; DC, dendritic cell; ERK, extracellular signal–regulated kinase; FCS, fetal calf serum; GALT, gut-associated lymphoid tissue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCMV, human cytomegalovirus; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MAP kinase, mitogen-activated protein-kinase; MCMV, murine cytomegalovirus; MEK, mitogen-activated protein kinase kinase; NK, natural killer cell; PI, phosphatidylinositol; SAPK/JNK, stress-activated protein kinase/c-Jun-N-terminal kinase; STAT, signal transducer and activator of transcription; UC, ulcerative colitis; vs., versus; wt, wild-type

Abstract

Background/Aims: In this study, we determined the so far unknown expression and signal transduction of the novel CXCL16-CXCR6 chemokine-ligand receptor system in intestinal inflammation.

Methods: In intestinal epithelial cells (IEC) and human and murine intestinal tissues, expression analyses were performed by Northern blotting, PCR, Western blotting, and immunohistochemistry. CXCL16 mRNA was measured by quantitative PCR in human colonic biopsies of patients with Crohn's disease (CD) as well as in the TNFΔARE mouse model of ileitis and in murine cytomegalovirus (MCMV)-induced colitis. CXCL16 serum levels were analyzed by ELISA. CXCL16-induced signal transduction was analyzed in IEC with phospho-specific antibodies for MAP kinases and Akt.

Results: CXCL16 mediated intestinal immune responses are region specifically regulated, given the inverse expression pattern of CXCL16 and CXCR6 with highest CXCL16 mRNA levels in the proximal small intestine and highest CXCR6 mRNA expression in the distal colon. CXCL16 and CXCR6 mRNA was expressed in colorectal cancer-derived IEC lines (T84, HT-29, Caco-2, SW480, CMT93). CXCR6 was also expressed by apical primary human IEC. IEC-expressed CXCR6 was functional as demonstrated by CXCL16-induced MAP kinase and Akt activation. Intestinal CXCL16 expression was elevated in the TNFΔARE mouse model of ileitis and in MCMV-induced colitis ($p<0.05$) as well as in the sera and colons of patients with CD ($p<0.05$), where its expression correlated with IL-8 levels ($r=0.672$).

Conclusion: IEC express the CXCL16 receptor CXCR6. CXCL16 expression is up-regulated in CD suggesting that this chemokine acts as chemoattractant for CXCR6+ T cells and as modulator of barrier function in intestinal inflammation.

Introduction

Intestinal epithelial cells are not a passive barrier as previously assumed, they also act as sensitive indicators of infection that initiate defense responses. Noninvasive as well as invasive organisms have been demonstrated to elicit production of chemoattractants. Epithelial cells have been found to express both chemokines and chemokine receptors [1-6]. Some of these chemokines appear to be important in the uninflamed intestine but become increased during disease. The combined cellular functions of intestinal epithelial cells (IEC) and classical immune cells co-ordinate, through the expression of adhesion molecules and chemokines, the recruitment and activation of leukocytes at sites of intestinal injury, inflammation and wound repair. In addition to their function as chemoattractant for leukocytes, chemokines were recently identified as potential regulators of intestinal epithelial cells [1-3, 5, 6]. However, the mechanisms of chemokine receptor signaling and discovery of the functional role of chemokines and their receptors in the control of the intestinal epithelial immune function are outstanding questions for the full understanding of the role of chemokines in the intestine.

Recently, a new chemokine ligand receptor pair (CXCL16-CXCR6) was identified and added to the growing family of chemokines. CXCL16 is a unique chemokine with characteristics of CC chemokines and a structure similar to that of fractalkine in having a transmembrane region and a chemokine domain suspended by a mucin-like stalk [7]. CXCL16 is expressed on the surface of antigen presenting cells (APCs), including subsets of CD19+ B cells and CD14+ monocytes/macrophages [7]. Membrane-bound CXCL16 mediates adhesion and phagocytosis of both Gram-negative and Gram-positive bacteria [8]. Importantly, anti-CXCL16 antibodies, which suppress chemotactic activity of CXCL16, significantly inhibit bacterial phagocytosis by human APCs [8]. Therefore, CXCL16 may play an important role in facilitating uptake of various pathogens.

CXCL16 binds to the chemokine receptor CXCR6 [7, 9]. CXCR6 was first recognized as receptor for simian and human immunodeficiency viruses [10]. This chemokine receptor was originally cloned as an orphan receptor by three independent groups who assigned three different names to it: STRL33 (seven transmembrane receptor-like from clone 33), Bonzo, and TYMSTR (T lymphocyte-expressed seven-transmembrane domain receptor) [10-12]. In mice, CXCR6 is expressed by subsets of CD4+, CD8+ and natural killer T cells, whereas in humans CXCR6 is expressed by small subsets of Th1 or T-cytotoxic 1 (Tc1) cells, establishing CXCR6 as a differential marker of polarized type 1 T cells [13]. CXCR6+ T cells are dramatically enriched among T cells at sites of tissue inflammation, such as rheumatoid joints and inflamed livers [13]. CXCR6 mRNA expression has been detected primarily in lymphoid tissue such as spleen and thymus [10] but also in the small intestine and the colon [10, 14, 15]. However, the signal transduction and biological functions of this chemokine receptor system in IEC and its role in intestinal inflammation are unknown. We therefore analyzed the expression of the CXCL16-CXCR6 chemokine-ligand receptor system in *in vitro* and *in vivo* models of intestinal inflammation including its expression in sera and colonic biopsies of IBD patients. Furthermore, we determined if CXCR6 is similar to other chemokine receptors such as CXCR4 [1, 2, 5], CCR6 [6] and CX3CR1 [3] expressed in IEC suggesting functions for this chemokine system in the maintenance of the intestinal epithelial barrier. In addition, we analyzed the CXCR6 mediated signal transduction in IEC.

Patients and Methods

Reagents

Specific polyclonal antibodies to phosphorylated ERK-1/2 (Thr183/Tyr185) were obtained from Promega (Madison, WI); antibodies against phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), phospho-Akt (Ser473), SAPK/JNK, p38 and Akt and the MEK-1 inhibitor PD98059 were purchased from New England Biolabs (Beverly, MA). ERK-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase linked anti-rabbit secondary antibody was purchased from Amersham (Arlington Heights, IL). Human CXCR6 antibodies and recombinant mouse CXCL16 were from R&D Systems (Minneapolis, MN), and PI3 kinase inhibitor wortmannin was from Sigma (St. Louis, MO).

Cell culture

The human colorectal cancer cell lines T84, SW480, Caco-2, HT-29 and the murine colorectal cancer cell line CMT93 were obtained from American Type Culture Collection (Rockville, MD). While T84 cells were grown in Dulbecco's modified Eagle medium/F-12 (Cellgro, Mediatech Inc., Herndon, VA), the other cell lines were grown in Dulbecco's modified Eagle medium (Cellgro) with 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated FCS (Sigma, St. Louis, MO) in a humidified 5% CO_2 atmosphere at 37°C. For stimulation experiments with CXCL16, cells were starved overnight in serum-free medium.

Gel electrophoresis and immunoblotting

Total protein was isolated by solubilizing cells in lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 μg/mL aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and phosphatase inhibitors (400 mM sodium orthovanadate and 4 mM NaF) and passing the lysates six times through a 21G

needle. After 30 minutes on ice, lysates were cleared by centrifugation at 10,000 x *g* for 20 minutes. Cytosolic and membrane protein fractions were isolated as previously described [3, 16]. The protein concentration of each sample was quantified by the Bradford method. Immunoblotting was performed as previously described [17].

Immunohistochemistry

Intestinal biopsy specimens were taken during diagnostic endoscopy after informed consent. Immunohistochemistry was performed on 3 µm sections. After pretreatment with Pro Taqs I Antigen Enhancer (Quartett, Berlin, Germany) in the microwave, the slides were rinsed in Tris-buffered saline (TBS) for 2 x 5 min and incubated with 7.5% H_2O_2 for 10 min, followed by 10 min in water. Sections were immersed in TBS for 2 x 5 min. The primary antibody (human-anti-CXCR6, R&D Systems) was visualized using the Vectastain ABC Elite system (Vector Laboratories, Burlingame, USA). In negative controls, sections were stained with an isotype-matched mouse IgG2b antibody. After brief rinsing in 0.05% Tris buffered in 35-Brij Solution (Merck), sections were immersed in 3-amino-9-ethyl-carbazole substrate (Zytomed, Berlin, Germany) for 15 min, and counterstained with haematoxylin and cover-slipped with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany). Immunohistochemical analysis of CXCR6 and CXCL16 expression in the IEC cell line HT-29 was performed adopting a previously established staining protocol using FITC-conjugated anti-mouse and anti-goat secondary antibodies (Sigma, Taufkirchen, Germany) and Hoechst 33342 (Sigma) staining [5, 17]. In negative controls, cells were stained omitting the primary antibody.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD). For RT-PCR, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNA-free™-Kit, Ambion, Austin, TX) to remove potential genomic DNA contaminants. The

following conditions were used for all PCRs: 35 cycles of denaturing at 95˚C for 1 min, annealing temperature for 30 sec, extension at 72˚C for 1 min. The primers for the PCR reactions are shown in Table 1. The PCR products were subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

Quantitative PCR

Real-time PCR was performed with a Rotorgene RG-3000 cycler (Corbett Research, Sydney, Australia) using the Quantitect SYBR Green PCR Kit from Qiagen (Hilden, Germany) following the manufacturer's guidelines. Oligonucleotide primers were designed according to published sequences, and the following primer pairs were used: human CXCL16: forward 5'-GAG CTC ACT CGT CCC AAT GAA-3', reverse 5'-TCA GGC CCA ACT GCC AGA-3'; human beta-actin: forward 5'-GCC AAC CGC GAG AAG ATG A-3', reverse 5'-CAT CAC GAT GCC AGT GGT A-3'; human interleukin-8 (IL-8): forward 5'-CCA GGA AGA AAC CAC CGG-A-3', reverse 5'-GAA-ATC-AGG-GCT-GCC-AAG-3' (MWG-Biotech, Ebersberg, Germany). CXCL16 expression was normalized to beta-actin expression in the respective cDNA preparations.

Northern blotting

Northern Blot analysis was performed as previously described [3]. Mouse CXCR6 and CXCL16 cDNA were generated by RT-PCR using primers as listed in Table 1. For generation of GAPDH cDNA, previously published primers were used [18].

Enzyme-linked immunosorbent assay (ELISA)

For the quantification of CXCL16 in serum samples of IBD patients and healthy controls, human CXCL16 Quantikine Elisa Kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions.

Quantitative measurements of serum levels of proinflammatory cytokines in patients treated with infliximab

Quantitative serum levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, and TNF-α were measured with a FlowCytomix kit © (Bender Medsystems, Vienna, Austria) according to the manufacturer's instructions using a FACSCalibur System (Becton Dickinson, Heidelberg, Germany). Serum levels were measured 24 hours before and 72 hours after anti-TNF-α therapy using 5 mg/kg infliximab (Remicade, Centocor Inc., Malvern, PA) given as i.v. infusion over a period of 2 hours. In addition, serum CRP concentrations were measured using a latex based high-sensitivity immunoassay (N Latex CRP mono / Behring Laser Nephelometer, Dade Behring GmBH, Marburg, Germany) before and after infliximab therapy.

Isolation of primary ileal epithelial cells from heterozygous TNFΔARE mice

Heterozygous TNFΔARE/+ mice (a generous gift from Dr. G. Kollias; Hellenic Pasteur Institute, Athens, Greece), which gradually develop chronic inflammation in the ileum from moderate to severe levels at 8 and 18 weeks of age [19], and wild-type TNF+/+ mice (WT) were killed at the age of 8 and 18 weeks. Primary IEC from the ileal epithelium of WT and TNFΔARE/WT mice were purified as previously described [20].

Murine cytomegalovirus (MCMV) infection in vivo

C57BL/6 mice were infected i.v. with $1x10^6$ pfu murine cytomegaly virus (MCMV) of the Smith strain [21] in PBS as previously described [22]. Control mice received an injection of PBS only. After 45 h, mice were euthanized by CO_2 asphyxiation. Total RNA of the colon was isolated using Trizol reagent.

Statistics

Fisher's exact test or χ^2 test was used, where appropriate, for comparison between categorical variables. Student's t-test was applied for quantitative variables. All tests were two-tailed and p-values < 0.05 were considered as significant. Statistical analyses were performed using the SAS 8.2 software for Windows.

Results

CXCR6 and CXCL16 mRNA are expressed in IEC of the murine and human intestine

First, we determined the organ-specific mRNA expression of CXCR6 and CXCL16 analyzing murine tissues derived from BALB/c mice by Northern blot. CXCL16 mRNA expression was detected as a major 1.8 kb and a 2.5 kb transcript in small and large intestine, kidney, spleen and liver (Fig. 1A). While CXCR6 mRNA expression was highest in the ileum, cecum and colon, CXCL16 mRNA expression was highest in the duodenum and jejunum but still detectable in the colon demonstrating a segment specific CXCL16 expression pattern inverse to that of CXCR6 (Fig. 1A). CXCL16 and CXCR6 mRNA were expressed in the human colorectal cancer derived IEC lines (T84, HT-29, Caco-2, SW480), as well as in the murine colorectal cancer cell line CMT93 (Fig. 1B). Anti-CXCR6 antibodies detected CXCR6 in the membrane fraction of the IEC line HT-29 (Fig. 1C) which was consistent with the high CXCR6 expression in the membrane of these cells in the immunohistochemical analysis (Fig. 1D; negative control Fig. 1E). In addition, we demonstrated by Western blot analysis (Fig. 1F) and immunohistochemistry (Fig. 1G) CXCL16 protein expression predominantly in the membrane fraction and to a lesser degree in the cytosol of the IEC line HT-29 (negative control: Fig. 1H). Similar results for CXCR6 and CXCL16 protein expression were obtained in SW480 cells (data not shown). We also detected CXCR6 expression in primary IEC of the normal human colon (Fig. 1I, 1K; negative controls Fig. 1J, 1L). CXCR6 was predominantly expressed in apical IEC (Fig. 1K).

CXCL16 induces MAP kinase and Akt phosphorylation in IEC

We next investigated whether CXCR6 is functionally active in IEC by analyzing signaling pathways in the murine IEC line CMT93 after stimulation with murine CXCL16. As shown in Fig. 2A/B, CXCL16 increased MEK-1 dependent ERK-1/2 phosphorylation in CMT93 cells,

independent of PI3 kinase activity. Since phosphorylation of p38, SAPK/JNK kinases and Akt has been shown in response of chemokine receptor dependent signaling [5, 6, 23-25], we assessed the phosphorylation of these kinases following CXCL16 stimulation. CXCL16 induced weak phosphorylation of SAPK/JNK kinases (Fig. 2C) and only very weakly p38 phosphorylation (Fig. 2D). Moreover, CXCR6 activation resulted in PI3-kinase dependent and partly MEK-1 dependent phosphorylation of Akt (Fig. 2E, 2F).

CXCL16 mRNA expression in human IEC is under the control of inflammatory signals

Given the important role of chemokines in intestinal inflammation, we analyzed whether proinflammatory cytokines up-regulate CXCR6 and CXCL16 mRNA expression in human IEC. The human colorectal cancer cell line HT-29 was stimulated with either TNF-α (50 ng/mL) or LPS (10 μg/mL). As shown in Fig. 3A, TNF-α up-regulated CXCL16 mRNA expression in HT-29 cells, while CXCR6 expression remained unchanged (data not shown). Similar to TNF-α, LPS enhanced CXCL16 mRNA expression (Fig. 3B) without altering CXCR6 expression levels (data not shown).

CXCL16 mRNA expression is up-regulated in murine models of ileitis and colitis in vivo

We next examined CXCL16 expression in two murine models of intestinal inflammation *in vivo*. Using heterozygous TNFΔARE mice, which develop chronic ileitis [19], we demonstrated that CXCL16 mRNA expression in ileal epithelial cells was significantly increased 3.5-fold in 18 week old mice compared to wildtype mice of the same age ($p<0.05$, Fig. 3C), while no significant change was observed in 8 week old mice (data not shown). Similar to our results in BALB/c mice (Fig. 1A), in the combined group of TNFΔARE and WT mice (n=18), CXCL16 expression was 3.1-fold higher in epithelial cells derived from the small intestine (ileum and jejunum) than in colonic epithelial cells ($p=0.025$; data not shown).

Next, we analyzed colonic CXCL16 mRNA expression in intestinal inflammation following viral infection *in vivo* using the murine model of MCMV infection. C57BL/6 mice were infected with 10^6 pfu MCMV of the Smith strain [21]. Forty-five hours after infection, CXCL16 mRNA levels were upregulated 1.8-fold in the colon of infected mice compared to non-infected mice (p=0.03; Fig. 3D).

CXCL16 mRNA expression is increased in the inflamed colonic mucosa of CD patients

Following the analysis in murine models of intestinal inflammation, we compared CXCL16 mRNA expression levels in biopsy samples taken from 20 different sites of 10 CD patients with endoscopically (macroscopic) inflamed colonic mucosa with those of endoscopically non-inflamed colonic mucosa taken from 20 different sites of the same 10 patients (total number of biopsies: n=40). IL-8 expression, which was used as a control marker for inflammation, was significantly increased (6.9-fold, $p< 0.05$) in the inflamed biopsy samples compared to the biopsies taken from non-inflamed areas. The increase in IL-8 mRNA expression ranged from 1.4 up to 31.1-fold compared to the non-inflamed tissues. Similarly, CXCL16 mRNA expression levels were higher in biopsy samples from inflamed mucosa when compared with non-inflamed lesions (increase between 1.3 and 16.3-fold; Table 2). Moreover, the CXCL16 mRNA levels correlated with the IL-8 mRNA expression levels (r=0.672), demonstrating its association with intestinal inflammation in CD patients.

CXCL16 serum levels are increased in active CD

We next analyzed CXCL16 protein expression in sera of IBD patients. Compared to a healthy control population (n=30), the CXCL16 serum levels were significantly higher ($p<0.005$) in the 47 IBD patients but without a significant difference between 30 CD patients and 17 patients with UC (4.07 vs. 3.95 ng/mL, Fig. 3E). High CXCL16 serum levels were detected

particularly in 13 patients with active IBD compared to patients in remission and healthy controls (4.35 vs. 3.50 ng/mL CXCL16; p=0.04). In patients with active IBD, CXCL16 serum levels correlated with CRP serum levels (r=0.485). However, in a subgroup analysis of this patient group, increased CXCL16 expression was detected only among patients with active CD, for whom CXCL16 levels were significantly higher compared to the control population (p=0.02), while CXCL16 serum levels in CD patients in remission (Crohn's disease activity index, CDAI < 150) were not significantly different from the control population (p=0.29, Fig. 3F), whereas for patients with severe active UC no difference compared to the control group was found (data not shown).

Anti-TNF therapy has no immediate effect on CXCL16 serum levels

In a subcohort of n=12 IBD patients we further analyzed serum CXCL16 levels before and 72 hours after infliximab therapy to characterize the immediate effect of anti-TNF therapy on CXCL16 protein expression. However, in these patients, the mean CXCL16 serum levels before infliximab therapy (3.89 ng/mL, range: 3.11 – 5.51) did not differ significantly from the mean CXCL16 level 72 hours after infliximab therapy (3.83 ng/mL, range: 2.95 – 4.87; p=0.84). Since only four of these 12 patients had elevated CRP levels of > 1.5 mg/dl before therapy, there was also no correlation between CRP levels and CXCL16 levels (data not shown). Similarly, an additional analysis of serum levels of the cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, and IFN-γ before and after infliximab therapy showed for most patients only low or undetectable cytokine levels and no significant correlation to the CXCL16 serum levels (data not shown). However, before infliximab therapy, elevated TNF-α and IL-8 serum levels were found in the majority of patients which correlated highly with each other (r=0.907) but not with the CXCL16 levels (r=-0.288 and r=-0.264, respectively). Similar to the CXCL16 levels, there was no significant difference between the TNF-α and IL-8 serum

levels measured before and 72 hours after infliximab therapy (TNF-α: p= -0.26; IL-8: p= 0.68).

Discussion

IEC form an active barrier and are sensitive indicators of infection that initiate defense responses based on their capacity to express both chemokines and chemokine receptors. We [3-6, 17, 26] and others [1, 27] have identified chemokines and their receptors expressed on IEC as key regulators involved in the inflammatory response of the gastrointestinal mucosa which therefore potentially play a role in the pathogenesis of IBD. However, the exact mechanisms of chemokine receptor signaling and their specific functions in the intestinal epithelium are still largely unknown.

In this study, we provide evidence that the CXCL16-CXCR6 chemokine ligand receptor system is expressed in the intestinal mucosa and plays a role in intestinal inflammation and in human IBD. Our results demonstrate that the CXCL16-CXCR6 chemokine ligand receptor system is an autocrine regulator of IEC immune functions and demonstrate that CXCR6 is expressed by IEC. This is consistent with two studies analyzing surgical specimens of colorectal cancer tissue and normal controls [15, 28]. CXCR6 is predominantly expressed in surface apical IEC while only weak expression was found in crypt IEC, which is consistent with the expression pattern described for other proinflammatory cytokine receptors [6, 29]. The high expression of CXCR6 in intraepithelial lymphocytes [9], and the presence of CXCL16 mRNA in the intestine indicate that CXCL16 and CXCR6 might have roles in retaining T cells in the intestinal epithelium. In addition, for the first time this study provides evidence that IEC-expressed CXCR6 is functional. CXCL16 predominantly activated the MEK-ERK MAP kinase signaling pathway which has also been shown to be the major signaling pathway of other chemokine receptors such as CCR6, CXCR4 and CX3CR1 [3, 5, 6, 30]. However, in contrast to CX3CR1 signaling [3], CXCR6 activation also resulted in increased Akt phosphorylation levels and – less strongly – increased phosphorylation of SAPK/JNK and p38 MAP kinases. Recent studies focusing on these signaling pathways demonstrated that SAPK/JNK and p38 MAP kinases are activated in

CD [31, 32], and that inhibition of SAPK/JNK and p38 activation resulted in significant clinical benefit and rapid endoscopic ulcer healing [32].

A recent detailed immunohistochemical analysis revealed that CXCL16 is not expressed in all IECs, but primarily on the follicle-associated epithelium [14], which, together with APCs such as CD14+ monocytes and macrophages, form the primary source of CXCL16 production in the intestine [7]. Here, we showed that stimulation of IEC with LPS and TNF-α increased CXCL16 mRNA expression. This is in agreement with a previous study in which stimulation of CXCL16 secreting human macrophages with LPS and TNF-α increased the chemotaxis of CXCR6/L1-2 transfectants [7]. Moreover, all IEC analyzed in our study were colorectal cancer derived. Consistent with our results, a very recent study demonstrated CXCL16 in human colorectal cancer cells and suggested that high-level expression of CXCL16 in tumor cells correlates with a good prognosis and increased tumor-infiltrating lymphocytes in colorectal cancer [33]. In addition, we demonstrated increased colonic CXCL16 expression in murine *in vivo* models of intestinal inflammation including TNFΔARE mice and MCMV infected mice. TNFΔARE mice are characterized by an overexpression of TNF-α and develop a chronic transmural ileitis with characteristics of human CD [19]. Interestingly, CXCL16 expression is increased in the ileum of these mice compared to wildtype control mice. Similarly, colonic CXCL16 expression is also increased in MCMV infected mice compared to uninfected mice. Moreover, recent studies demonstrated up-regulation of CXCL16 in herpes simplex virus type 1 (HSV-1) [34] infection and an influence of CXCL16 on the nature and specificity of CpG-induced immune activation [35], suggesting a role for this chemokine in the immune response following viral infection.

Similar to murine intestinal inflammation, we observed increased CXCL16 expression in the colon and serum of patients with active CD. We therefore analyzed the immediate effects of anti-TNF therapy on CXCL16 serum levels in a subgroup of IBD patients but could not detect a significant effect of infliximab on serum CXCL16 levels measured before and 72

hours after anti-TNF-α therapy. This does not exclude long-term effects of infliximab on the expression levels of this chemokine since a very recent study demonstrated in patients with rheumatoid arthritis that CXCL16 serum levels decreased 14 and 30 weeks after infliximab treatment [36]. However, this may be related rather to disease remission than being a direct effect of infliximab on the expression levels of this chemokine given the lack of an immediate effect of anti-TNF therapy on CXCL16 expression within 72 hours as seen in our study. Similarly, we did not observe significant changes in serum TNF-α levels before and after infliximab therapy which is in agreement with a previous study [37]. Another study analyzing the effect of infliximab on TNF-α serum levels in patients with psoriasis demonstrated an effect on TNF-α expression only at later time points [38] and in one study even increased TNF-α levels were recorded following 72 hours after infliximab therapy in patients with rheumatoid arthritis [39]. However, in both CD [37] and rheumatoid arthritis [40], high circulating TNF-α levels correlated with active disease and lack of response to infliximab.

In summary, this is the first comprehensive analysis of the CXCL16-CXCR6 chemokine ligand receptor system in intestinal inflammation. We have shown that the chemokine receptor CXCR6 is expressed by IEC. Upon stimulation with CXCL16, several distinct signaling pathways including ERK-MAP kinases and Akt are activated. Proinflammatory stimuli such as LPS and TNF-α increase CXCL16 mRNA expression. Similarly, CXCL16 mRNA expression is increased in murine intestinal inflammation and human IBD. However, anti-TNF therapy has no immediate effect on CXCL16 serum levels in IBD patients. We hypothesize that CXCL16 plays an important role in regulating mucosal innate and adaptive immune responses. Further functional studies are necessary to define exact roles of this novel chemokine system in the intestine; however, the chemoattractation of CXCR6+ T cells during intestinal inflammation through highly expressed CXCL16 protein seems to be an important function in human IBD.

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Tables

Table 1

PCR Product	**Size (bp)**	**Forward and Backward Primer**	**Annealing temperature**
hCXCL16	566	5’-GCA GCG TCA CTG GAA GTT GTT AT-3’ 5’-TGC GGT GAG GAT GAA GAT GAT GA-3’	61˚C
mCXCL16	217	5’-AAA CAT TTG CCT CAA GCC AGT-3’ 5’-GTT TCT CAT TTG CCT CAG CCT-3’	60˚C
hCXCR6	767	5’-CAG GCA TCC ATG AAT GGG TGT-3’ 5’-CAA GGC CTA TAA CTG GAA CAT GCT G-3’	62˚C
mCXCR6	932	5’-TGT ACG ATG GGC ACT ACG A-3’ 5’-GTG AGA GAG GCA GCC GAT A-3’	58°C

Table 1. PCR primers and conditions used.

Table 2

Pat. #	CXCL16 mRNA expression (fold increase) inflamed vs. non-inflamed	Correlation CXCL16/IL-8	Anatomic site of biopsy sampling		Current Medication
			non-inflamed	inflamed	
1 *	1.3	0.258	cecum *	terminal ileum *	mesalazine, cortico-steroids
2 *	16.3	1.000	cecum*	ileocecal valve*	MTX
3 *	1.7	0.967	cecum *	terminal ileum *	AZA, IFX
4	2.5	0.447	descending colon	descending colon	mesalazine, AZA
5	2.1	0.705	cecum	cecum	AZA
6 *	2.0	0.807	ascending colon*	terminal ileum*	AZA
7	2.3	0.651	descending colon	descending colon	no medication
8	1.7	0.986	transverse colon	transverse colon	AZA
9 *	4.2	0.554	cecum*	terminal ileum*	AZA, cortico-steroids
10	1.9	0.341	ascending colon	ascending colon	AZA
Average ±SEM	3.60 ± 1.31	0.672 ± 0.078			

Table 2. CXCL16 and IL-8 mRNA expression in inflamed and non-inflamed colonic lesions from patients with CD determined by quantitative RT-PCR and normalized to beta-actin expression levels. The current medical therapy during biopsy sampling and the anatomic site, from which the samples were taken, are given for all patients. While it was intended to take biopsies from inflamed and non-inflamed lesions in the same intestinal segment, in patients with severe inflammation biopsies from neighbouring sites were taken that are indicated by asterisk (*). Abbreviations: AZA, azathioprine; IFX, infliximab; MTX, methotrexate

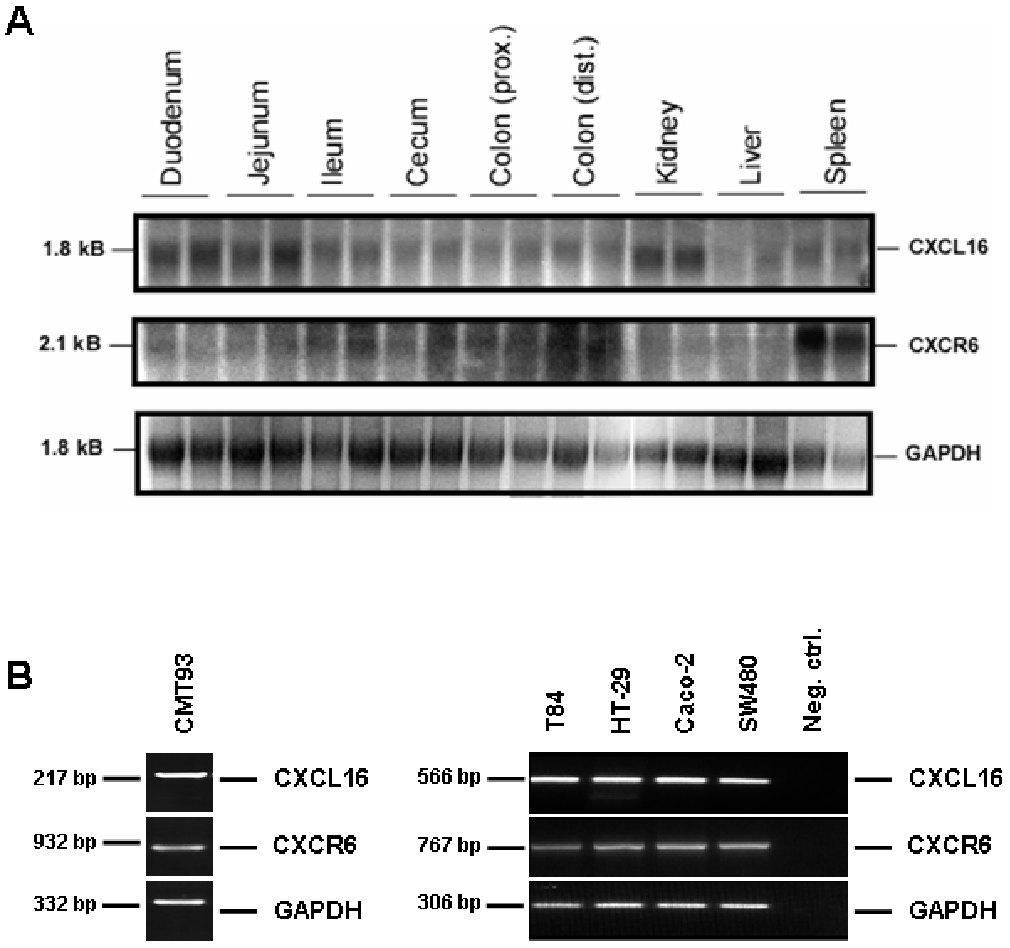
Figure legends

Figure 1. IEC express CXCR6 and CXCL16. (A) Northern Blot analysis of mRNA derived from murine tissue of two wildtype BALB/c mice as indicated. Note the inverse segment-specific expression of CXCL16 and CXCR6 mRNA in the murine gastrointestinal tract with high CXCL16 expression in the small intestine and high CXCR6 expression in the colon. **(B)** CXCL16 and CXCR6 mRNA expression in human IEC lines and the murine IEC line CMT93 were analyzed by RT-PCR. **(C)** Western blot analysis of CXCR6 protein expression in cytosolic *(C)* and membrane *(M)* protein fractions of HT-29 cells. **(D)** Immunohistochemical staining reveals a membrane associated expression of CXCR6 in HT-29 cells (anti-CXCR6 antibody detected with a FITC conjugated secondary antibody, nucleus: stained with Hoechst 33342). **(E)** No CXCR6 specific staining was obtained using the secondary antibody (FITC conjugated) only. **(F)** Western blot analysis of CXCL16 protein expression in cytosolic *(C)* and membrane *(M)* protein fractions of HT-29 cells. **(G)** Immunohistochemical staining with a CXCL16 specific antibody. **(H)** No CXCL16 specific staining was detected using the secondary antibody only. **(I), (K)** CXCR6 staining was detected predominantly in (apical) surface colonic epithelial cells. **(J), (L)** Staining with corresponding isotype-matched mouse IgG2b. (I), (J) original magnification 20x. (K), (L) original magnification 40x.

Figure 2. CXCL16 activates MAP kinases and Akt in IEC. (A) Detection of phosphorylated ERK-1/2 in CMT93 IEC lines after CXCL16 stimulation (100 ng/mL). **(B)** Phospho-ERK1/2 activation by CXCL16 in CMT93 cells pretreated with the MEK-1 inhibitor PD98059 (5 μmol/L) and the PI3 kinase inhibitor wortmannin (200 nmol/L). **(C)** Phosphorylated SAPK/JNK kinases in CMT93 cells after CXCL16 stimulation (100 ng/mL). **(D)** CXCL16 stimulation of CMT93 cells resulted also in very weak phosphorylation of p38. **(E)** Phosphorylated Akt in CXCL16 stimulated CMT93 cells (including wortmannin

pretreated cells) and in **(F)** PD98059 pretreated cells. For all signaling experiments (Fig. 2A-F), one representative experiment of three performed is shown.

Figure 3. CXCL16 expression is upregulated in intestinal inflammation. (A) TNF-α (50 ng/mL) increases CXCL16 mRNA expression as shown by densitometric analysis of semiquantitative PCR but has no significant effect on the CXCR6 mRNA expression (data not shown). One representative experiment of three performed is shown. **(B)** LPS (10 µg/mL) up-regulates CXCL16 mRNA expression. No effect on CXCR6 mRNA expression was found (data not shown). One representative experiment of three performed is shown. **(C)** CXCL16 mRNA expression is increased 3.5-fold in ileal epithelial cells from TNFΔARE heterozygous mice (n=9) compared to wildtype mice (n=9) as determined by quantitative PCR (*$p<0.05$ vs. wt). Additionally, there was a higher CXCL16 expression in ileal and jejunal epithelial cells compared to colonic epithelial cells (data not shown). **(D)** Colonic CXCL16 mRNA expression is increased in $1x10^6$ pfu MCMV i.v. infected C57BL/6 mice (n=11) in comparison to PBS injected C57BL/6 control mice (n=4, * $p=0.03$ vs. controls). **(E)** CXCL16 serum levels are increased in CD and correlate with CRP serum levels. In the sera of patients with IBD (n=47), significantly higher CXCL16 protein levels were detected in patients with CD (n=30) compared to healthy controls (n=30; * $p<0.01$ vs. controls; UC: $p=0.06$ vs. controls). **(F)** CXCL16 expression was particularly high in CD patients with active disease (* $p=0.02$ vs. controls).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Duodenum
Jejunum
Ileum
Cecum
Colon (prox.)
Colon (dist.)
Kidney
Liver
Spleen
1.8 kB
CXCL16
2.1 kB
CXCR6
1.8 kB
GAPDH
B
CMT93
T84
HT-29
Caco-2
SW480
Neg. ctrl.
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CXCL16
932 bp
CXCR6
332 bp
GAPDH
566 bp
CXCL16
767 bp
CXCR6
306 bp
GAPDH

Figure 1

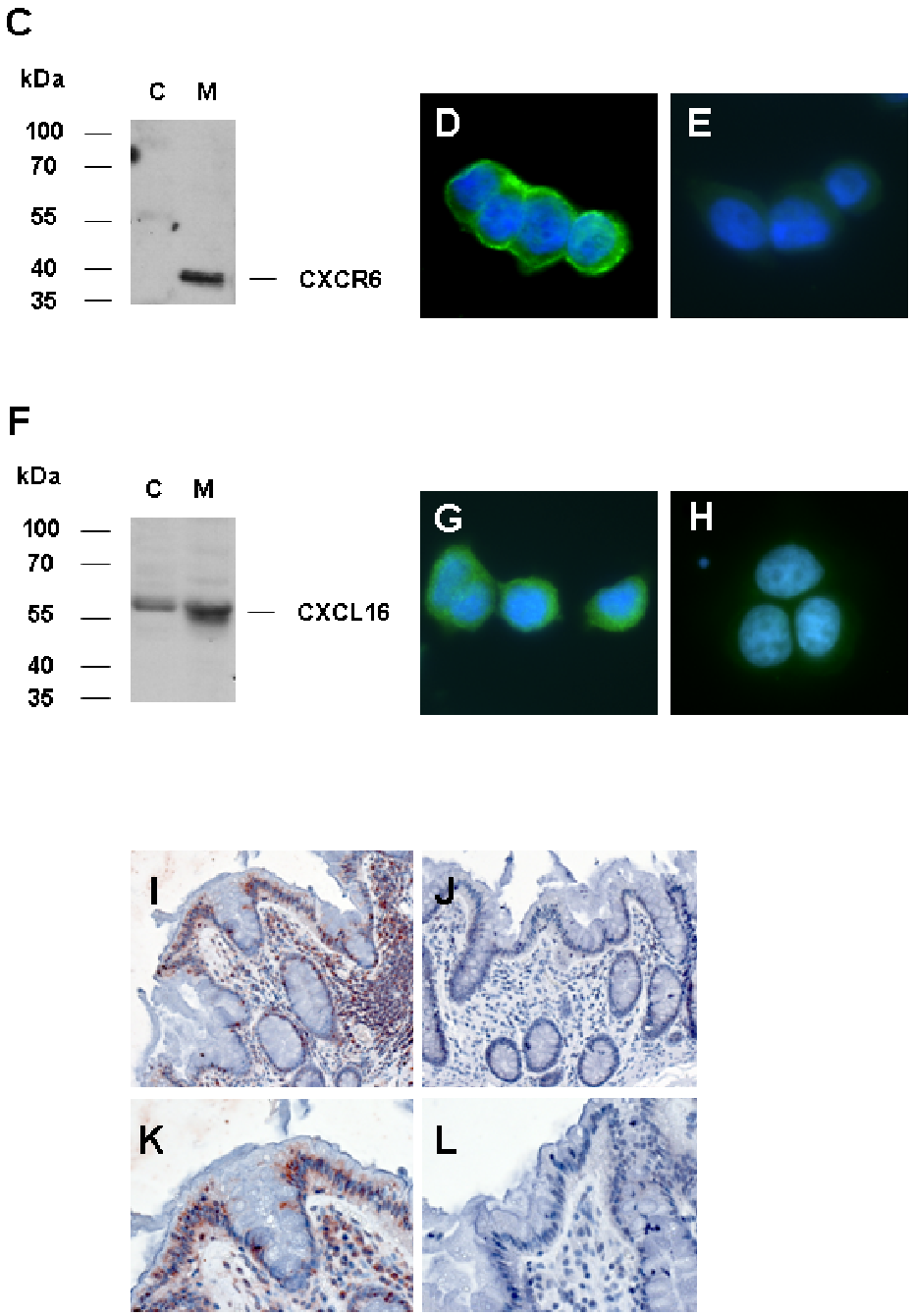
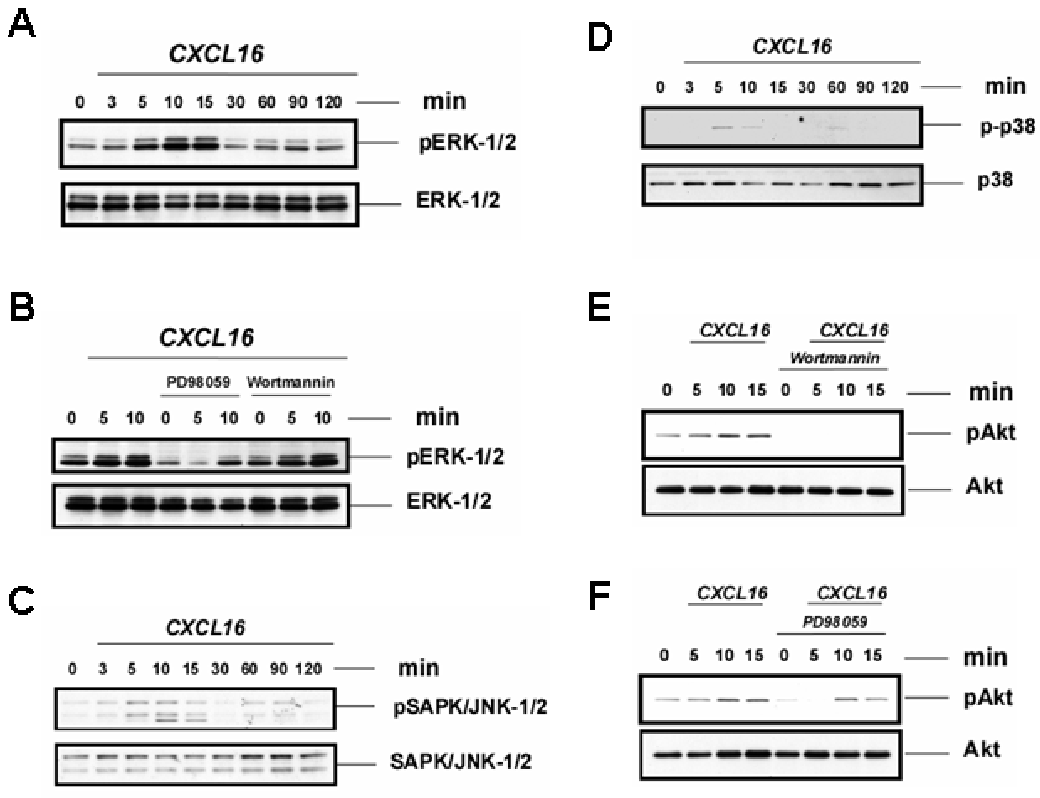

C
kDa
C M
100
70
55
40
35
CXCR6
D
E
F
kDa
C M
100
70
55
40
35
CXCL16
G
H
I
J
K
L

Figure 1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CXCL16
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min
pERK-1/2
ERK-1/2
B
CXCL16
PD98059
Wortmannin
0 5 10 0 5 10 0 5 10
min
pERK-1/2
ERK-1/2
C
CXCL16
0 3 5 10 15 30 60 90 120
min
pSAPK/JNK-1/2
SAPK/JNK-1/2
D
CXCL16
0 3 5 10 15 30 60 90 120
min
p-p38
p38
E
CXCL16
CXCL16
Wortmannin
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min
pAkt
Akt
F
CXCL16
CXCL16
PD98059
0 5 10 15 0 5 10 15
min
pAkt
Akt

Figure 2

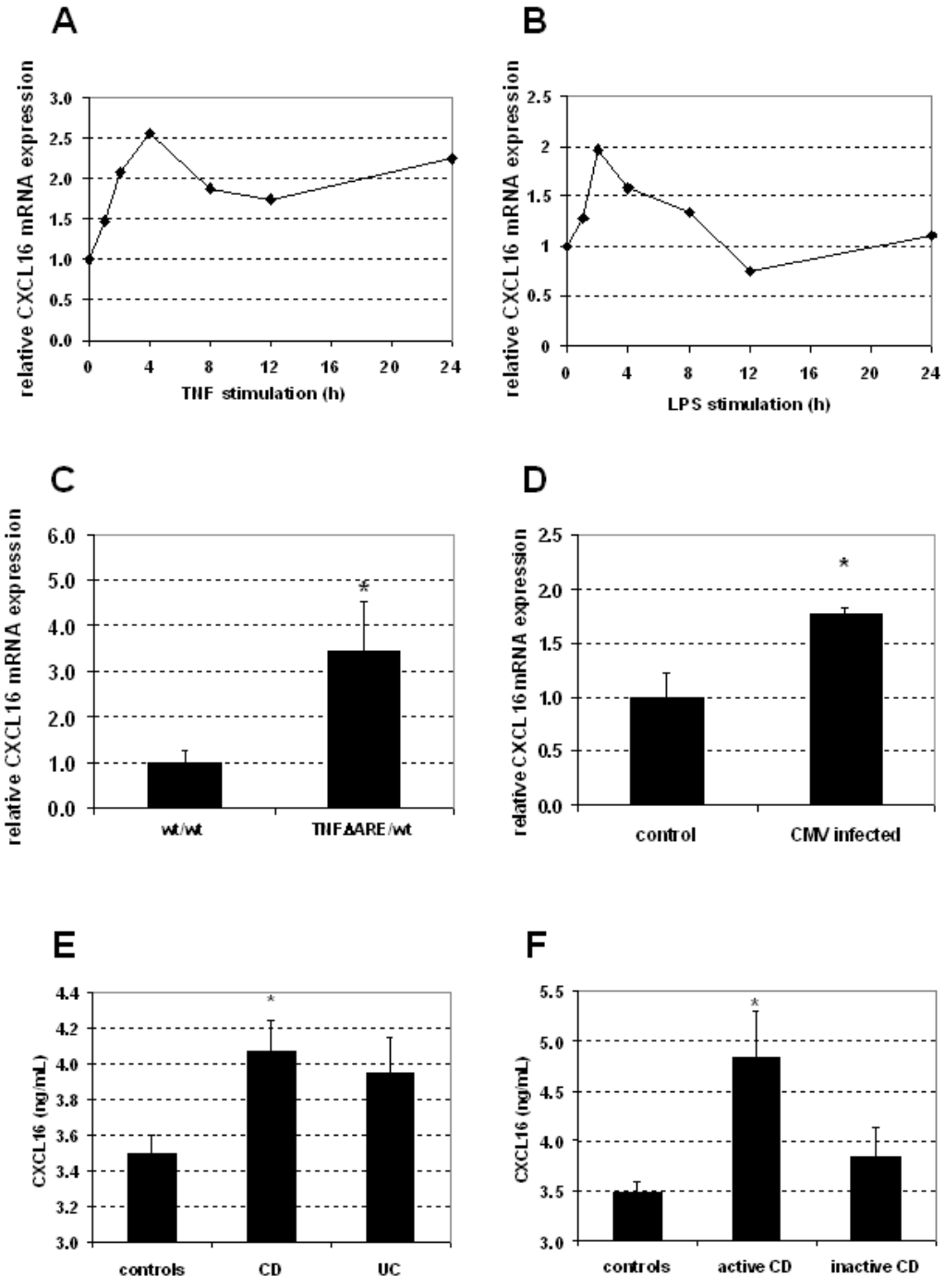
A
relative CXCL16 mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
TNF stimulation (h)
B
relative CXCL16 mRNA expression
2.5
2
1.5
1
0.5
0
0
4
8
12
16
20
24
LPS stimulation (h)
C
relative CXCL16 mRNA expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
wt/wt
TNFΔARE/wt
D
relative CXCL16 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
control
CMV infected
E
CXCL16 (ng/mL)
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
*
controls
CD
UC
F
CXCL16 (ng/mL)
5.5
5.0
4.5
4.0
3.5
3.0
*
controls
active CD
inactive CD

Figure 3

Manuskript [4]

The role of the CXCL16 p.Ala181Val polymorphism in inflammatory bowel disease

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*** Geteilte Erstautorenschaft**

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The role of the CXCL16 p.Ala181Val polymorphism in inflammatory bowel disease

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Short title: CXCL16 polymorphism in Crohn's disease

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Key words:

Crohn's disease, ulcerative colitis, inflammatory bowel disease, chemokine, CXCL16, CXCR6, polymorphism, genetics, intestinal inflammation, CARD15, NOD2, intestinal stenosis

Abbreviations

APC, antigen-presenting cell; CARD, caspase-activation recruitment domain; CD, Crohn's disease; DC, dendritic cell; fs, frameshift; GALT, gut-associated lymphoid tissue; IBD, inflammatory bowel disease; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; NK, natural killer cell; NOD, nucleotide-binding oligomerization domain; OR, odds ratio; TACE, tumour necrosis factor-alpha-converting enzyme; TLR, Toll-like receptor; TNF, tumor necrosis factor; UC, ulcerative colitis; vs., versus; wt, wild-type

Abstract

Background/Aim: CXCL16 is a chemokine with increased expression in inflammatory bowel diseases (IBD). The aim of this study was to identify genetic determinants of CXCL16 modulating the susceptibility and phenotype of IBD.

Methods: Genomic DNA from 574 individuals (365 IBD patients, 209 healthy controls) was analyzed for the CXCL16 p.Ala181Val polymorphism and a detailed genotype-phenotype analysis was performed. Genotyping included also *CARD15*/NOD2 variants p.Arg702Trp, p.Gly908Arg, and p.Leu1007fsX1008.

Results: In Crohn's disease (CD), the CXCL16 p.Ala181Val polymorphism is not a disease susceptibility gene but associated with younger age at disease onset (p=0.016) and higher frequency of ileal involvement (p=0.024; OR 2.17; 95% CI 1.12-4.21) in ValVal carriers compared to a higher frequency of colonic involvement in AlaAla carriers (p=0.009; OR 2.60; CI 1.29-5.25). Carriers of at least one Val allele and one CARD15/NOD2 variant had a higher incidence of a stricturing and penetrating phenotype (p=0.030, OR 4.04, CI 1.27-12.84) and of stenoses (p=0.014; OR 3.97; CI 1.38-11.40) than patients carrying NOD2 variants only. In contrast, in ulcerative colitis, there were no associations of the CXCL16 p.Ala181Val SNP with disease susceptibility and disease phenotype.

Conclusion: The CXCL16 p.Ala181Val polymorphism is not an IBD susceptibility gene but can modify the CD phenotype alone or in conjunction with CARD15/NOD2 variants.

Introduction

The pathogenesis of inflammatory bowel diseases (IBD) is still not completely understood, but it is widely accepted that the disease results from an exaggerated mucosal immune response to luminal antigens in a genetically susceptible host [1, 2]. Since the first report of *CARD15* as a major susceptibility gene in Crohn's disease (CD) [3, 4], polymorphisms in several proteins involved in inflammation and bacterial host defense such as toll-like receptor (TLR) 4 [5], the fractalkine receptor CX3CR1 [6], C-reactive protein (CRP) [7] or macrophage migration inhibitory factor (MIF) [8] have been shown to contribute to certain CD phenotypes.

Recently, it has been shown that chemokines and chemokine receptors are also important in the intestinal immune response [6, 9-13]. Another chemokine contributing to the defense against bacteria is CXCL16, the most novel member of the CXC chemokine family. Interestingly, CXCL16 shares structural similarities with CC chemokines and the CX3C chemokine fractalkine. CXCL16 and fractalkine are the only chemokines with a transmembrane region and a chemokine domain suspended by a mucin-like stalk [14]. Therefore, CXCL16 may exist as membrane bound or cleaved, soluble form. CXCL16 expression has been shown in antigen presenting cells (APCs), including subsets of CD19+ B cells and CD14+ monocytes/macrophages [14]. A recent study demonstrated that membrane-bound CXCL16 mediates adhesion and phagocytosis of both Gram-negative and Gram-positive bacteria [15]. Moreover, suppression of chemotactic activity of CXCL16 with anti-CXCL16 antibodies significantly inhibited bacterial phagocytosis by human APCs [15]. These findings suggest that CXCL16 may play an important role in facilitating the uptake of bacterial pathogens.

CXCL16 binds a distinct receptor recently identified and designated as CXCR6 [14, 16] which was first recognized as a receptor for simian and human immunodeficiency viruses [17]. CXCR6 was originally described under three different names: STRL33 (seven transmembrane

receptor-like from clone 33), Bonzo, and TYMSTR (T lymphocyte-expressed seven-transmembrane domain receptor) [17-19]. CXCR6 is expressed by CD4+, CD8+ and natural killer T cells in mice, whereas in humans CXCR6 is expressed by small subsets of Th1 or T-cytotoxic 1 (Tc1) cells [20]. Most interestingly, CXCR6+ T cells are particularly found among T cells at sites of tissue inflammation, such as rheumatoid joints and inflamed livers [20]. CXCR6 mRNA has been detected particularly in lymphoid tissue such as thymus and spleen [17] but also in the small and large intestine [17, 21, 22]. Interestingly, we also demonstrated functional CXCR6 expression on intestinal epithelial cells [23] suggesting a role for this chemokine system in modulating the intestinal barrier function.

Recently, we demonstrated increased CXCL16 expression in the colon and sera of patients with active CD [23] pointing to a role for this chemokine system in the pathogenesis of IBD. This is supported by a very recent study showing that anti-CXCL16 antibody treatment significantly suppressed IL-1β production in a murine model of dextran sulfate sodium (DSS) induced colitis [24]. Most recently, a sequence alteration has been described in exon 4 of the *CXCL16* gene [25] resulting in a C→T missense mutation in codon 181 replacing alanine (GCT) by valine (GTT; CXCL16 p.Ala181Val) [25]. This SNP has been shown to be associated with the severity of coronary artery stenosis [25] but its effect in IBD patients has not been investigated so far. Given our preliminary results suggesting a role for this chemokine in CD [23], we therefore analyzed the effect of the CXCL16 p.Ala181Val polymorphism on IBD susceptibility and disease phenotype in a large German cohort (n=574) including 365 IBD patients.

Patients and Methods

Study population of the genotype-phenotype analysis

The study population (n=574) was comprised of 201 patients with CD, 164 patients with ulcerative colitis (UC), and 209 healthy unrelated controls of Caucasian origin and sex-matched to the IBD group (Table 1). Diagnosis of CD or UC was assessed according to established criteria [26]. Demographic and routine clinical data were recorded by patients' chart analysis and an interview at the time of enrolment. CD phenotypes were determined according to the Vienna classification [27]. In UC patients, anatomic location was determined based on the criteria pancolitis, left-sided colitis or colitis limited to the rectum (proctitis). The study was approved by the local Ethics committee. All participating patients gave written, informed consent prior to genetic analysis.

Genotyping of the CXCL16 p.Ala181Val polymorphism

Genotyping of the C→T substitution underlying the CXCL16 p.Ala181Val polymorphism was performed by restriction fragment length polymorphism (RFLP) analysis as previously described [25]. The presence of the three common CARD15/NOD2 mutants p.Arg702Trp, p.Gly908Arg, and p.Leu1007fsX1008 was determined by direct DNA sequence analysis as described previously [28, 29].

Statistics

For comparison between categorical variables, Fisher's exact test or χ^2 test was used where appropriate. Student's t test was used for quantitative variables. All tests were two-tailed, considering p-values < 0.05 as significant.

Results

The CXCL16 p.Ala181Val polymorphism is not associated with IBD susceptibility but with disease onset at young age and ileal involvement in CD patients

First, we analyzed if the recently described CXCL16 p.Ala181Val polymorphism contributes to disease susceptibility and certain phenotypic characteristics of IBD patients. The distribution of the different *CXCL16* genotypes in IBD patients did not differ significantly from that observed in healthy controls and showed no differences between CD and UC patients (Table 2). However, genotype-phenotype analysis revealed a significantly younger age at diagnosis in ValVal homozygous carriers (mean 24.3 ± 9.1 yrs) compared to wild-type AlaAla (29.1 ± 12 yrs) or heterozygous AlaVal carriers (29.2 ± 12.4 yrs; p=0.028 homozygous vs. wild-type and p=0.016 homozygous vs. heterozygous; Table 3). AlaAla patients had a higher frequency of colonic involvement compared to patients carrying at least one Val substitution (p=0.009; OR 2.60; CI 1.29-5.25; Table 3). In addition, there was a significantly increased risk for disease localization in the ileum for patients carrying at least one Val allele compared to patients with wild-type AlaAla status (p=0.024; OR 2.17; 1.12-4.21; Table 3). In contrast, the genotype-phenotype analysis in patients with UC revealed no differences in male-to-female-ratio, BMI, age, age at diagnosis, disease duration, location of disease or family history between carriers and non-carriers of the p.Ala181Val substitution (data not shown).

CD patients carrying both the CXCL16 p.Ala181Val polymorphism and CARD15 variants are at higher risk of ileal involvement and stricturing or penetrating phenotype

Based on previous studies demonstrating severe phenotypic consequences in CD patients carrying CARD15 variants [29, 30], we next determined in all CD patients whether the three common CD-associated CARD15/NOD2 variants p.Arg702Trp, p.Gly908Arg and

p.Leu1007fsX1008 were present and analyzed the phenotypic consequences. The combined prevalence of these three variants in the CD cohort was 36.8%. There was no significant difference in their prevalence between the AlaAla wild-type group and patients carrying the Val allele. Genotype-phenotype analysis in the four subgroups (CXCL16-/NOD2-, CXCL16-/NOD2+, CXCL16+/NOD2-, CXCL16+/NOD2+) revealed no differences in age, disease duration, family history or BMI (Table 4). However, when comparing CD patients carrying both the Val allele and at least one of the three main NOD2 mutants (CXCL16+/NOD2+) with patients carrying only a NOD2 variant (CXCL16-/NOD2+), we found a significantly higher incidence of a stricturing or penetrating disease phenotype (Vienna B2/B3 versus B1; p= 0.030, OR 4.04, CI 1.27-12.84) and of intestinal stenoses (78.4%; p=0.014; OR 3.97; CI 1.38-11.4) in the CXCL16+/NOD2+ group compared to the CXCL16-/NOD2+ group (Table 4). In addition, the CXCL16+/NOD2+ group demonstrated the highest rate of ileal involvement (L1+L3: 76.4%) and the lowest frequency of colonic manifestations (11.8%, p=0.027 compared to the CXCL16-/NOD2+ group).

Discussion

This is the first analysis investigating the role of the CXCL16 p.Ala181Val polymorphism on IBD susceptibility and phenotype. Although this study demonstrated no significant difference in the allele frequency between IBD patients and controls, we observed a significantly younger age at onset of CD and higher rate of ileal involvement in carriers of the Val allele including an association with stricturing and penetrating disease behaviour for carriers of both the Val allele and NOD2 mutants. In contrast to CD, no significant association of the CXCL16 p.Ala181Val polymorphism and an UC phenotype was found, supporting previous studies demonstrating the CXCL16-CXCR6 chemokine system as a differential marker of Th1-mediated inflammation as observed in CD [20]. The current pathogenic concepts of CD [1, 2] and the higher frequency of ileal involvement and stricturing/penetrating disease behaviour found in carriers of the CXCL16 p.Ala181Val polymorphism suggests that this amino acid substitution may lead to an impaired recognition of intestinal bacteria, particularly since membrane-bound CXCL16 mediates adhesion and phagocytosis of both Gram-negative and Gram-positive bacteria by human APCs [15]. In addition, CXCL16 mediates chemotaxis of T and NKT cells and is involved in lymphocyte compartmentalization in the GALT [21]. A detailed immunohistochemical analysis revealed that CXCL16 is not expressed in all IECs, but primarily on the follicle-associated epithelium [21], which, together with APCs such as CD14+ monocytes and macrophages, form the primary source of CXCL16 production in the intestine [14]. One may therefore hypothesize that the CXCL16 p.Ala181Val polymorphism leads to an impaired recognition of bacteria which would explain phenotypic similarities between carriers of this polymorphism and those of the p.Thr280Met CX3CR1 substitution and the 1007fs *CARD15* variant [6, 29].

Defects in the recognition of bacterial antigens seem to be the underlying defect for many CD-associated genes. For example, a recent study revealed for the p.Thr300Ala variant in the *ATG16L1* gene, which is involved in the autophagocytosis of bacteria [31], an association

with an ileal disease phenotype [32]. Similarly, we demonstrated for the SNP p.Leu1007fsX1008 in NOD2, which is involved in the recognition of bacterial muramyl dipeptide, a strong association with ileal CD [29, 30]. We recently demonstrated that recognition and phagocytosis of luminal bacteria by dendritic cells (DCs) is highly dependent on the chemokine receptor CX3CR1 [33]. Interestingly, we also demonstrated for the p.Thr280Met SNP in the CX3CR1 gene a strong association with an ileal CD phenotype [6]. However, ileal DCs are a main source of IL-23 production [34] which may link *IL23R*, another recently discovered CD susceptibility gene [35], to the events involved in the recognition of intestinal bacteria. We recently demonstrated that 93.2% of TT homozygous carriers of the *IL23R* rs1004819 variant, the main CD-associated *IL23R* variant in the German population, as compared to 78% of CC wildtype carriers had ileal involvement [P = 0.004; OR 4.24; CI (1.46-12.34)] [36].

It is of note, that the functional consequences of the CXCL16 p.Ala181Val polymorphism have not been determined thus far. Membrane-bound CXCL16 is proteolytically cleaved for chemotactic activity, a process which is mediated by the disintegrin-like metalloproteinase ADAM10 and TNF-α converting enzyme (TACE) [14, 37]. Given the location of the CXCL16 p.Ala181Val polymorphism within the spacer region between the chemokine and transmembrane region involved in the chemokine release by TACE [25], further studies on the influence of this polymorphism on CXCL16 cleavage and function *in vivo* will be of great interest.

Similar to our results, the CXCL16 p.Ala181Val polymorphism has been shown to be associated with the severity of coronary artery stenoses [25], and CXCL16 serum levels are increased in inflammation, atherosclerosis, and acute coronary syndromes [38], while we demonstrated that CXCL16 serum levels are increased in active CD [23]. This suggests that the effects of the CXCL16 chemokine system are not limited to intestinal inflammation but rather involved in various types of inflammatory responses.

In summary, we demonstrated that CXCL16 does not influence the susceptibility to IBD although the CXCL16 p.Ala181Val polymorphism is associated with a severe disease phenotype in CD patients characterized by disease onset at an early age with ileal involvement and an increased risk of stenoses in the presence of additional *CARD15* variants. However, further replication studies and functional analyses of the CXCL16 p.Ala181Val variant are necessary to confirm our results.

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	CD (n=201)	**UC** (n=164)	**Controls** (n=209)
Gender			
Male (%)	93 (46.3%)	83 (50.6%)	99 (47.4%)
Female (%)	108 (53.7%)	81 (49.4%)	110 (52.6%)
Age (yr)			
Mean ± SD	40.3 ± 11.9	42.8 ± 14.7	48.6 ± 15.2
Range	17 - 75	18 - 84	23 - 86
Body mass index			
Mean ± SD	23.2 ± 4.0	24.1 ± 4.3	
Range	16 - 40	16 - 41	
Age at diagnosis (yr)			
Mean ± SD	28.3 ± 11.9	31.9 ± 13.7	
Range	7 - 71	9 - 81	
Disease duration (yr)			
Mean ± SD	11.9 ± 8.2	10.7 ± 7.8	
Range	1 - 43	0.5 - 39	
Positive family history of IBD (%)	25 (12.4%)	21 (12.8%)	

Table 1. Demographic characteristics of the study population.

CXCL16 p.Ala181Val genotype status	AlaAla	AlaVal	ValVal
Controls	76 (36.4%)	102 (48.8%)	31 (14.8%)
CD	67 (33.3%)	99 (49.3%)	35 (17.4%)
UC	46 (28.1%)	84 (51.2%)	34 (20.7%)

Table 2. Frequencies of the CXCL16 p.Ala181Val polymorphism in patients with CD or UC and in controls. None of the differences was statistically significant ($p>0.05$). AlaAla = wild-type, AlaVal = heterozygous carriers, ValVal = homozygous carriers.

	(1) AlaAla (n=67)	(2) AlaVal (n=99)	(3) ValVal (n=35)	(1) vs. (2) p-value	(1) vs. (3) p-value	(2) vs. (3) p-value	(1) vs (2)+(3) p-value
Male sex n=(%)	31 (46.3%)	45 (45.4%)	17 (48.6%)	p=1.000	p=0.838	p=0.844	p=1.000
Body mass index (kg/m^2)							
Mean ± SD	23.2 ± 4.1	23.0 ± 3.9	23.9 ± 4.1	p=0.675	p=0.443	p=0.249	p=0.969
Range	17-40	16-37	16-31				
Age at diagnosis (yr)							
Mean ± SD	29.1 ± 12.0	29.2 ± 12.4	24.3 ± 9.1	p=0.962	**p=0.028**	**p=0.016**	p=0.512
Range	12-66	13-71	7-49				
Disease duration (yr)							
Mean ± SD	12.3 ± 8.4	11.3 ± 7.9	13.1 ± 8.8	p=0.401	p=0.653	p=0.260	p=0.633
Range	1-34	2-33	3-43				
Age (yr)							
Mean ± SD	41.4 ± 12.1	40.4 ± 12.2	37.5 ± 10.6	p=0.602	p=0.091	p=0.178	p=0.324
Range	19-69	20-75	17-61				

Location							
Terminal ileum (L1)	6 (9.0%)	7 (7.1%)	3 (8.6%)	p=0.771	p=1.000	p=0.721	p=0.784
Colon (L2)	21(31.3%)	14 (14.1%)	6 (17.1%)	**p=0.011**	p=0.158	p=0.783	**p=0.009**
Ileocolon (L3)	29 (43.3%)	56 (56.6%)	20 (57.2%)	p=0.114	p=0.214	p=1.000	p=0.099
Upper GI (L4)	11 (16.4%)	22 (22.2%)	6 (17.1%)	p=0.430	p=1.000	p=0.633	p=0.571
Any ileal involvement*	44 (65.7%)	80 (80.8%)	28 (80%)	**p=0.031**	p=0.171	p=1.000	**p=0.024**
Behaviour							
Non-stricturing, Non-penetrating (B1)	14 (20.9%)	20 (20.2%)	6 (17.1%)	p=1.000	p=0.795	p=0.807	p=0.852
Stricturing (B2)	15 (22.4%)	27 (27.3%)	9 (25.7%)	p=0.586	p=0.807	p=1.000	p=0.606
Penetrating (B3)	38 (56.7%)	52 (52.5%)	20 (57.2%)	p=0.636	p=1.000	p=0.696	p=0.764
Use of immunosuppressive agents	58 (86.6%)	75 (75.8%)	29 (82.9%)	p=0.113	p=0.769	p=0.483	p=0.185

Table 3. Associations between the CXCL16 p.Ala181Val polymorphism and certain CD characteristics.

** = (L1)+(L3)+(L4-patients with ileal involvement)*

	(1) **CXCL16- / NOD2-** (n=44)	**(2)** **CXCL16- / NOD2+** (n=23)	**(3)** **CXCL16+ / NOD2-** (n=83)	**(4)** **CXCL16+ / NOD2 +** (n=51)
Male sex n=(%)	17 (38.6%)	14 (60.9%)	40 (48.2%)	22 (43.1%)
Body mass index (kg/m^2)				
Mean ± SD	23.2 ± 4.4	23.3 ± 3.6	23.6 ± 4.3	22.6 ± 3.3
Range	17-40	18-32	16-37	17-31
Age at diagnosis (yr)				
Mean ± SD	28.1 ± 10.7	30.9 ± 14.3	29.0 ± 12.2	26.1 ± 11.1
Range	12-58	15-66	13-71	7-61
Disease duration (yr)				
Mean ± SD	13.2 ± 8.6	10.8 ± 8.1	10.9 ± 7.0	13.3 ± 9.6
Range	1-32	2-34	2-32	3-43
Age (yr)				
Mean ± SD	41.3 ± 11.5	41.8 ± 13.3	39.9 ± 12.1	39.4 ± 11.5
Range	19-69	20-68	18-75	19-71

Location				
Terminal ileum (L1)	2 (4.5%)	4 (17.4%)	3 (3.6%)	7 (13.7%)
Colon (L2)	13 (29.5%)	8 (34.8%)	14 (16.9%)	6 (11.8%); ***p=0.027 vs (2)***
Ileocolon (L3)	19 (43.2%)	10 (43.5%)	44 (53.0%)	32 (62.7%)
Upper GI (L4)	10 (22.7%)	1 (4.3%)	22 (26.5%)	6 (11.8%)
Behaviour				
Non-stricturing, Non-penetrating (B1)	5 (11.4%)	9 (39.1%)	19 (22.9%)	7 (13.7%); ***p=0.030 vs (2)***
Stricturing (B2)	12 (27.3%)	3 (13.0%)	23 (27.7%)	13 (25.5%)
Penetrating (B3)	27 (61.4%)	11 (47.8%)	41 (49.4%)	31 (60.8%)
Use of immunosuppressive agents	41 (93.2%)	17 (73.9%)	67 (80.7%)	37 (72.5%)
Surgery because of CD	30 (68.2%)	11 (47.8%)	41 (49.4%)	37 (72.5%)
Fistulas	27 (61.4%)	11 (47.8%)	41 (49.4%)	31 (60.8%)
Stenoses	34 (77.3%)	11 (47.8%)	54 (65.1%)	40 (78.4%); ***p=0.014 vs. (2)***
Abscesses	21 (47.7%)	7 (30.4%)	29 (34.9%)	19 (37.2%)
Extraintestinal manifestations	38 (86.4%); ***p=0.011 vs (4)***	16 (69.6%)	61 (73.5%)	32 (62.7%)
Positive family history of IBD	4 (9.1%)	4 (17.4%)	7 (8.4%)	10 (19.6%)

Table 4. Associations between the CXCL16 p.Ala181Val polymorphism, NOD2 mutants, and CD disease characteristics.

Manuskript [5]

Interleukin-31 mediated signals modulate intestinal epithelial cell proliferation and its expression is up-regulated in intestinal inflammation

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Interleukin 31 mediates MAP kinase and STAT1/3 activation in intestinal epithelial cells and its expression is upregulated in inflammatory bowel disease

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INFLAMMATORY BOWEL DISEASE

Interleukin 31 mediates MAP kinase and STAT1/3 activation in intestinal epithelial cells and its expression is upregulated in inflammatory bowel disease

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Background/aim: Interleukin 31 (IL31), primarily expressed in activated lymphocytes, signals through a heterodimeric receptor complex consisting of the IL31 receptor alpha (IL31Rα) and the oncostatin M receptor (OSMR). The aim of this study was to analyse IL31 receptor expression, signal transduction, and specific biological functions of this cytokine system in intestinal inflammation.
Methods: Expression studies were performed by RT-PCR, quantitative PCR, western blotting, and immunohistochemistry. Signal transduction was analysed by western blotting. Cell proliferation was measured by MTS assays, cell migration by restitution assays.
Results: Colorectal cancer derived intestinal epithelial cell (IEC) lines express both IL31 receptor subunits, while their expression in unstimulated primary murine IEC was low. LPS and the proinflammatory cytokines TNF-α, IL1β, IFN-γ, and sodium butyrate stimulation increased IL31, IL31Rα, and OSMR mRNA expression, while IL31 itself enhanced IL8 expression in IEC. IL31 mediates ERK-1/2, Akt, STAT1, and STAT3 activation in IEC resulting in enhanced IEC migration. However, at low cell density, IL31 had significant antiproliferative capacities ($p<0.005$). IL31 mRNA expression was not increased in the TNFΔARE mouse model of ileitis but in inflamed colonic lesions compared to non-inflamed tissue in patients with Crohn's disease (CD; average 2.4-fold increase) and in patients with ulcerative colitis (UC; average 2.6-fold increase) and correlated with the IL-8 expression in these lesions ($r=0.564$ for CD; $r=0.650$ for UC; total number of biopsies analysed: $n=88$).
Conclusion: IEC express the functional IL31 receptor complex. IL31 modulates cell proliferation and migration suggesting a role in the regulation of intestinal barrier function particularly in intestinal inflammation.

Recently, interleukin 31 (IL31) has been identified as a four helix bundle cytokine that is produced by activated T cells, particularly by T helper type 2 cells.[1] IL31 is closely related to the IL6-type cytokines oncostatin M (OSM), leukaemia inhibitory factor (LIF), and cardiotrophin-1 and signals through a heterodimeric receptor complex composed of the IL31 receptor alpha (IL31Rα) and the oncostatin M receptor (OSMR).[1] IL31Rα is identical to the gp130-like receptor (GPL).[1] At least four splice variants of IL31Rα have been described.[1] The short isoform of IL31Rα exerts a strong inhibitory effect on the signalling of IL31 and behaves as a dominant negative receptor.[2] The tyrosine residues 652 and 721 in the cytoplasmic region of the long isoform of IL31Rα/GPL(745) have been identified as the major STAT5 and STAT3 activating sites, respectively.[3] IL31Rα has sequence and domain homologies with gp130, LIFR, and granulocyte colony stimulating factor receptor.[1] Expression of IL31Rα and OSMR mRNA has been shown to be induced in activated monocytes while epithelial cells express mRNA for both receptors constitutively.[1] Activation of the receptor complex resulted in recruitment of Jak1, Jak2, STAT1, STAT3, STAT5, and PI3 kinase signalling pathways in glioblastoma and melanoma tumour cells, and lung epithelial cells.[2 4] While IL31 is the only ligand for the IL31Rα receptor subunit identified so far, the OSMR subunit is also used by OSM, which signals through a receptor complex consisting of OSMR and gp130.[5]

So far, biological functions of this novel cytokine were mainly analysed in certain skin diseases such as atopic dermatitis.[6] Transgenic mice overexpressing IL31 develop a skin disorder characterised by severe pruritus, alopecia, and skin lesions suggesting a role for IL31 in skin inflammation.[1] A similar phenotype was induced after subcutaneous delivery of recombinant IL31 in mice.[1] Peripheral lymph nodes from IL31 transgenic mice and IL31 protein treated mice were enlarged compared to non-treated and non-transgenic mice.[1] In humans, IL31 was significantly overexpressed in pruritic atopic skin lesions.[7] In vivo, staphylococcal superantigen rapidly induced IL31 expression in atopic individuals.[7] Comparisons between skin from patients with atopic dermatitis and healthy skin showed high IL31Rα expression on epidermal keratinocytes and increased IL31 expression in infiltrating cells in skin samples taken from atopic patients.[8] Moreover, an increased IL31Rα expression in diseased tissues derived from an animal model of airway hypersensitivity has been described.[1]

While the role of IL31 in skin inflammation is well characterised,[1 7–10] its biological properties in other tissues are largely unknown. Although IL31 expression has been described in the small intestine and colon,[1] the expression of the IL31

Abbreviations: CD, Crohn's disease; ERK, extracellular signal regulated kinase; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPL, gp130-like receptor; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFN, interferon; IL, interleukin; IL31Rα, interleukin 31 receptor alpha; LIF, leukaemia inhibitory factor; LIFR, leukaemia inhibitory factor receptor; LPS, lipopolysaccharide; MAP kinase, mitogen activated protein kinase; MCMV, murine cytomegalovirus; MEK, mitogen activated protein kinase kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; OSM, oncostatin M; OSMR, oncostatin M receptor; PCR, polymerase chain reaction; PI, phosphatidyl-inositol; RT-PCR, reverse transcriptase polymerase chain reaction; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription; TNF-α, tumour necrosis factor alpha; UC, ulcerative colitis

receptor complex has not been demonstrated in intestinal epithelial cells (IEC) so far. Based on the expression of IL31Rα in epithelial tissues such as skin and lung epithelium and similarities between skin and gut demonstrated by us for other STAT inducing cytokines such as IL22,[11] we analysed the IL31 receptor expression in the intestinal epithelium and characterised its signal transduction and its specific biological functions in IEC, particularly in intestinal inflammation.

MATERIALS AND METHODS

Reagents

Polyclonal antibodies to phosphorylated extracellular signal regulated kinase (ERK) 1/2 (Thr183/Tyr185) and phospho-Akt (Ser473) were purchased from Cell Signaling (Beverly, MA, USA). Anti-ERK-1/2 and anti-Akt antibodies were also from Cell Signaling. Phospho-STAT1 antibody was from BD Transduction Laboratories (Franklin Lakes, NY, USA), phospho-STAT3 antibody from Upstate Biotechnology (Lake Placid, NY, USA), and antibodies against STAT1 and STAT3 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase conjugated secondary antibodies to mouse or rabbit IgG and chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate) were from Pierce (Rockford, IL, USA). Human IL31Rα antibody was from R&D Systems (Minneapolis, MN, USA). Recombinant human IL31, tumour necrosis factor (TNF)-α, IL1β, and interferon (IFN)-γ were obtained from R&D Systems (Minneapolis, MN, USA). Lipopolysaccharide (LPS) from *Escherichia coli* (O26:B6) prepared by phenol extraction was purchased from Sigma (St Louis, MO, USA) and prepared as dispersed sonicate in endotoxin-free water (Life Technologies, Rockville, MD, USA) before diluting to final concentration in supplemented media.

Cell culture

The human colorectal cancer derived IEC lines HT-29, SW480, HCT116, T84, CaCo-2, and DLD-1 were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle medium (Gibco BRL/Life Technologies, Gaithersburg, MD, USA) with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat inactivated fetal calf serum (PAA, Pasching, Austria) in a humidified 5% CO_2 atmosphere at 37℃. For signal transduction experiments with IL31, cells were starved overnight in serum-free medium.

Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed as previously described.[12] Briefly, total RNA was isolated using Trizol reagent (Gibco BRL/Life Technologies, Gaithersburg, MD, USA). For RT-PCR, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNA-free-Kit, Ambion) to remove potential genomic DNA contaminants. A volume of 2 µg of total RNA was reverse transcribed using Qiagen Omniscript RT Kit. To control for genomic contamination, an identical parallel PCR reaction was performed containing starting material that had not been reverse transcribed. The following conditions were used for semi-quantitative PCRs: 40 cycles denaturing at 95℃ for 30 seconds, annealing at 60℃ for 30 seconds, extension at 72℃ for 30 seconds. The primers (MWG Biotech, Ebersberg, Germany) for the PCR reactions are shown in table 1. The PCR products were subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and sequenced.

Table 1 Primers used for PCR analysis

hIL31Rα forward:	5′-ggaggggaaaagaatgggta-3′
hIL31Rα reverse:	5′-cgagggtctcatgctatggt-3′
hIL-OSMR forward:	5′-ggaatgtgccacacactttg-3′
hIL-OSMR reverse:	5′-acattggtgccttcttccac-3′
hIL31 forward:	5′-gaactctgccgtgattcctt-3′
hIL31 reverse:	5′-aagcctgcagaagaaaagca-3′
hIL-8 forward:	5′-ccaggaagaaaccaccgga-3′
hIL-8 reverse:	5′-gaaatcaggaaggctgccaag-3′
hβ-actin forward:	5′-gccaaccgcgagaagatga-3′
hβ-actin reverse:	5′-catcacgatgccagtggta-3′
mGAPDH forward:	5′-CGTCCCGTAGACAAAATGGT-3′
mGAPDH reverse:	5′-TCTCCATGGTGGTGAAGACA-3′
mIL31R forward:	5′-GCGGTGGACACTTGGATAGT-3′
mIL31R reverse:	5′-ACCCTGGTCTCAGGACCTTT-3′
mOSMR forward:	5′-ACACCAAGTCCCTTCCACAG-3′
mOSMR reverse:	5′-ATGGTGACATTGGAGCCTTC-3′
mIL31 forward:	5′-CAGCTGTTTCAACCCACTG-3′
mIL31 reverse:	5′-CAGTTCTGCCATGCAGTTTG-3′

h: human; m: mouse.

Quantitative PCR

Real time PCR was performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) using the Quantitect SYBR Green PCR Kit from Qiagen (Hilden, Germany) following the manufacturer's guidelines. Oligonucleotide primers (table 1) were designed according to the published sequences. All mRNA expression levels were normalised to β-actin or GAPDH expression in the respective cDNA preparation.

Signal transduction experiments, gel electrophoresis, and immunoblotting

The signal transduction experiments were performed in overnight serum starved IEC lines as indicated. Cells were stimulated with 100 ng/ml IL31, unless indicated otherwise. This concentration was based on pilot experiments demonstrating a significantly higher effect of 100 ng/ml for the activation of certain kinases and cell migration than lower concentrations. Cells were solubilised in lysis buffer containing 1% Nonidet P-40, 20 mM TRIS-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 µg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin and phosphatase inhibitors (400 mM sodium orthovanadate, and 4 mM NaF) and were passed six times through a 21 gauge needle. After 30 minutes on ice, lysates were cleared by centrifugation at 10 000 *g* for 20 minutes. The protein concentration of each sample was quantified by the Bradford method. Immunoblotting was performed as previously described.[13]

Immunofluorescence staining

Immunofluorescence analysis of IL31Rα expression in the IEC cell line HCT116 was performed as follows. Cells were fixed with 3.2% paraformaldehyde/PBS for 20 minutes and were then permeabilised with 0.5% Triton X-100/PBS for 5 minutes. Cells were blocked with 10% rabbit serum/PBS for 1 hour and then incubated with the primary antibody (anti-IL31Rα, R&D Systems) for 1 hour. After washing with PBS, cells were incubated with a FITC conjugated anti-goat secondary antibody (Sigma, Taufkirchen, Germany) and Hoechst 33342 (Sigma) for 1 hour. Cells were washed with PBS, mounted with Mowiol and were analysed under a fluorescence microscope. In negative controls, cells were stained omitting the primary antibody.

Enzyme linked immunosorbent assay

For the quantification of IL-8 release, BD OptEIA Human IL-8 ELISA Kit II (BD Biosciences, Bedford, MA, USA) was used according to the manufacturer's instructions.

Cell proliferation and cell restitution assays

HCT116 cells were seeded onto 96 well plates at densities of cells per well as indicated and were allowed to attach overnight.

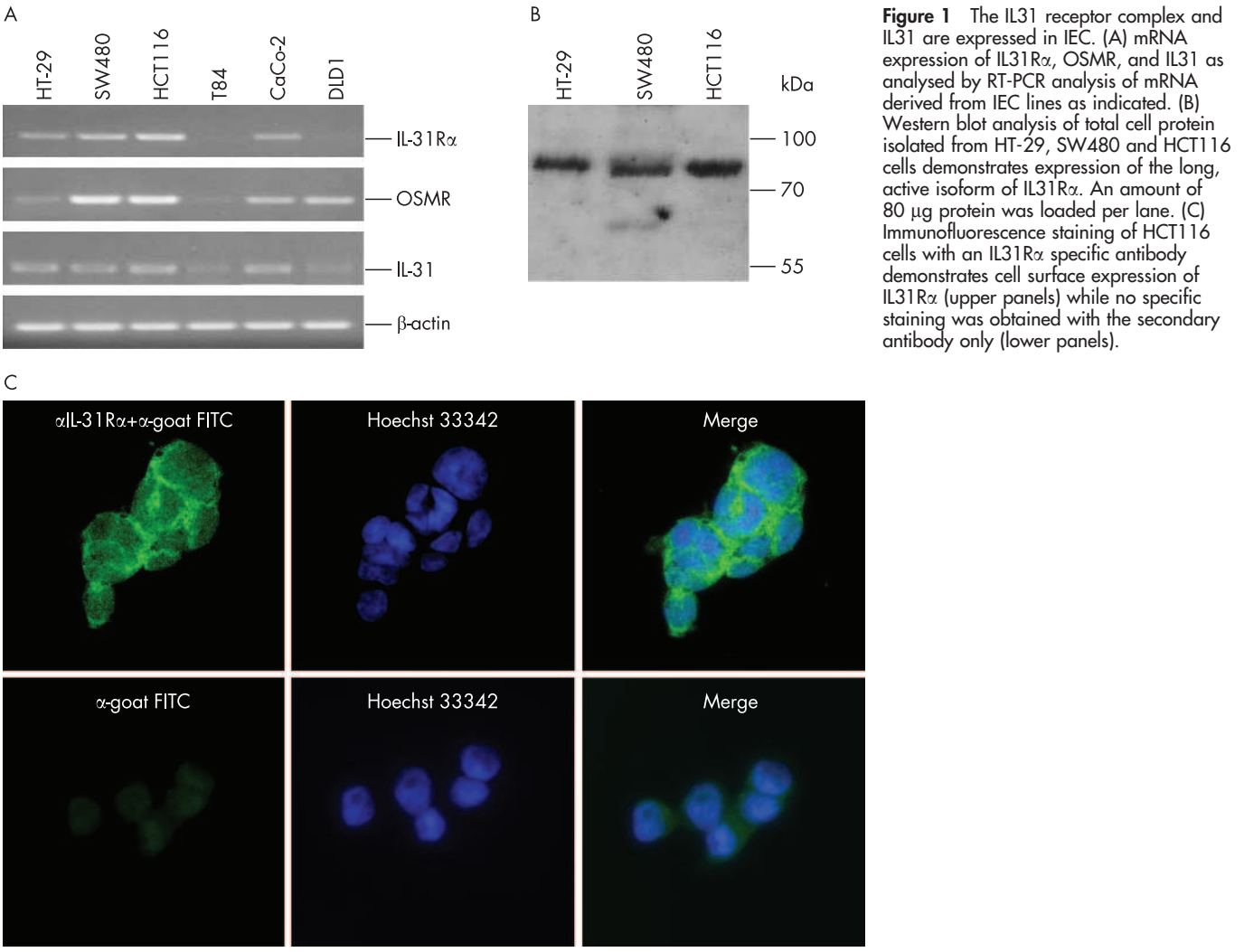

Figure 1 The IL31 receptor complex and IL31 are expressed in IEC. (A) mRNA expression of IL31Rα, OSMR, and IL31 as analysed by RT-PCR analysis of mRNA derived from IEC lines as indicated. (B) Western blot analysis of total cell protein isolated from HT-29, SW480 and HCT116 cells demonstrates expression of the long, active isoform of IL31Rα. An amount of 80 μg protein was loaded per lane. (C) Immunofluorescence staining of HCT116 cells with an IL31Rα specific antibody demonstrates cell surface expression of IL31Rα (upper panels) while no specific staining was obtained with the secondary antibody only (lower panels).

Cells were then stimulated with 10, 50, and 100 ng/ml IL31, or with cytokine-free medium (negative control) for 48 hours. The cell proliferation rate was determined by MTS assay on day 2 using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Cell restitution assays were performed as wounding assays as previously described.[14] Briefly, SW480 cells, which were the most suitable IEC line in pilot experiments, were grown in six well plates to complete confluence. Using a sterile razor blade, eight standardised wounds were created in each plate. Detached cells were removed by three washes with PBS, and the cell medium was changed from 10% FCS containing medium to 0.1% FCS containing medium. The cells were stimulated with IL31 (100 ng/ml) or PBS. The cells were washed with PBS after 24 hours and the number of migrated cells (over the wounding edge) was counted under a microscope. Five dishes were analysed for each group (IL31 stimulated and PBS stimulated), whereas for each dish eight separate fields were counted.

Isolation of primary ileal epithelial cells from heterozygous TNFΔARE mice

Heterozygous TNFΔARE mice (a generous gift from Dr G Kollias; Hellenic Pasteur Institute, Athens, Greece), which gradually develop chronic inflammation in the ileum from moderate to severe levels at 8 weeks and 18 weeks of age,[15] and C57BL/6 wild type (WT) mice were killed at the age of 18 weeks. Primary IEC from the ileal and colonic epithelium of WT and TNFΔARE/WT mice were purified as previously described.[16] Briefly, the intestinal tissue was cut into pieces and incubated at 37°C in DMEM containing 5% FCS and 1 mM dithiothreitol (DTT) for 30 minutes. The remaining tissue was incubated in 30 ml PBS containing 1.5 mM EDTA for additional 10 minutes. The supernatants were filtered, centrifuged for 5 minutes at 400 *g*, and the cell pellet was resuspended in DMEM containing 5% FCS. Finally, the primary IEC suspension was purified by centrifugation through a 25%/40% discontinuous Percoll gradient at 600 *g* for 30 minutes. Cell purity was assessed by determining the absence of CD3+ T cell contamination. Trypan blue exclusion confirmed the presence of at least 80% viable cells after the 2 hour isolation procedure. Primary IEC from the ileum were collected in sample buffer for subsequent RNA isolation.

Murine cytomegalovirus infection in vivo

C57BL/6 mice were infected intravenously with 1×10^6 pfu murine cytomegalovirus (MCMV) of the Smith strain[17] in PBS as previously described.[18] Control mice received an injection of PBS only. After 45 hours, mice were killed by CO_2 asphyxiation. Total RNA of the colon was isolated using Trizol reagent. The mice in vivo studies were approved by the animal care and use committee of the State of Bavaria (Regierung von Oberbayern) following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

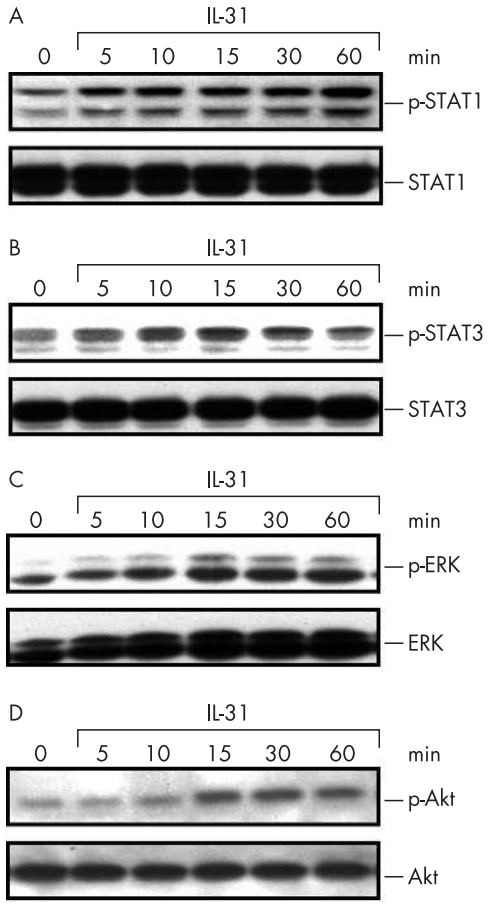

Figure 2 IL31 induces STAT1, STAT3, ERK-1/2, and Akt phosphorylation in IEC. Upon stimulation of HCT116 cells with IL31 (100 ng/ml), (A) STAT1 and (B) STAT3 proteins are phosphorylated. (C) Phospho-ERK-1/2 (p-ERK) activation after IL31 stimulation (100 ng/ml). (D) IL31 induces Akt phosphorylation. Similar results were obtained for SW480 cells (data not shown). One representative experiment (n = 3) is shown.

Colonic biopsies

Biopsies were taken from patients with CD and UC undergoing diagnostic colonoscopy. The study was approved by the ethics committee of the Medical Faculty of the University of Munich. All participating subjects gave written, informed consent before biopsy sampling. Four biopsies were collected from each patient: two from macroscopically non-inflamed sites and two from macroscopically inflamed mucosa. IL31, IL31Rα, OSMR, and IL8 mRNA levels were measured in each individual biopsy. For quantification, the average IL31, IL31Rα, or OSMR and IL8 mRNA expression of the two non-inflamed biopsies was compared to the average expression in the two inflamed biopsies. For calculation of the correlation coefficient, IL31 mRNA expression was correlated with the IL8 mRNA expression in the four individual biopsies for each patient.

Statistical analysis

Statistical analysis was performed using two tailed Student's *t* test. p Values <0.05 were considered as significant.

RESULTS

The IL31 receptor complex is expressed in IEC lines

Firstly, we determined if the IL31 receptor complex consisting of IL31Rα and OSMR is expressed in IEC using RT-PCR and cDNA derived from the human IEC lines HT-29, SW480, HCT116, T84, CaCo-2, and DLD-1. In addition, we examined IL31 expression in these cell lines. RT-PCR analysis demonstrated weak IL31 mRNA expression in all cell lines tested (fig 1A). Using primers specific for the long isoform of this receptor, IL31Rα expression was found in all IEC lines analysed with the exception of T84 and DLD-1 cells (fig 1A). OSMR was expressed in all cell lines although only at low levels in HT-29 and T84 cells (fig 1A). The long isoform of IL31Rα was also expressed on protein level in IEC as demonstrated by western blot (fig 1B) and immunofluorescence staining (fig 1C) with an IL31Rα specific antibody. The strongest expression of both receptor subunits was demonstrated in SW480 and HCT116 cells (fig 1A and 1B), which we therefore used in the following signal transduction experiments.

IL31 induces STAT1/3, ERK-1/2, and Akt phosphorylation

After confirming IL31Rα and OSMR expression in IEC, we next analysed if this complex is functional in IEC investigating various signalling pathways after stimulation of HCT116 and SW480 cells with recombinant IL31. Recent studies in other cell lines reported activation of STAT and MAP kinase signalling by IL31,[1 4] which we therefore analysed in IEC. These experiments demonstrated moderate activation of both STAT1 (fig 2A) and STAT3 (fig 2B) in IEC after stimulation with IL31 (100 ng/ml). Moreover, IL31 (100 ng/ml) increased phosphorylation levels of ERK-1/2 (fig 2C) and Akt proteins (fig 2D).

IL31 modulates IEC proliferation and migration

The activation of ERK MAP kinases and Akt has been linked to increased cell proliferation,[11 12 19 20] while a recent study demonstrated antiproliferative effects for IL31 in lung epithelial cells mediated via STAT3 activation.[21] We therefore analysed the effect of IL31 on IEC proliferation using MTS assays and the IEC line HCT116. At cell densities of <5000 cells/well, IL31 strongly decreased cell proliferation ($p = 0.001$ for 500 cells/well stimulated with 50 ng/ml or 100 ng/ml IL31, respectively, vs medium stimulated cells), while this effect was lost at higher cell densities and even an opposite, but much less pronounced proliferation-stimulating effect of IL31 (100 ng/ml) was observed with 10 000 cells/well and 15 000 cells/well compared to medium stimulated controls ($p = 0.046$ and $p = 0.013$, respectively; fig 3A).

Recently, we demonstrated that activation of Akt kinases and STAT proteins is also involved in IEC migration.[11] Therefore, we next analysed the influence of IL31 on IEC migration. As demonstrated in figure 3B, 100 ng/ml IL31 increased the migration of HCT116 cells significantly ($p = 0.005$).

IL31, IL31Rα, and OSMR mRNA expression is upregulated in an in vitro model of cell differentiation

Given the influence of cell density on the IL31 mediated effects on cell proliferation, we next analysed potential effects of IEC differentiation on IL31 and IL31 receptor expression in sodium butyrate treated IEC, which is an established in vitro model of cell differentiation.[22 23] We measured IL31, IL31Rα, and OSMR mRNA expression by quantitative PCR in HCT116 cells after sodium butyrate treatment. There was an increased expression of all three genes that was most pronounced for IL31Rα mRNA (270-fold upregulation after 48 hours, fig 3C), while IL31 and OSMR were upregulated up to 50-fold and 12-fold, respectively (fig 3C).

IL31, IL31Rα, and OSMR mRNA expression is upregulated by proinflammatory cytokines and LPS

Given the high expression of IL31 in T cells, which are consistently found in various forms of intestinal inflammation, we next characterised expression and potential functions of IL31 in inflammation. Firstly, we analysed IL31, IL31Rα, and OSMR mRNA expression in HCT116 cells after stimulation with

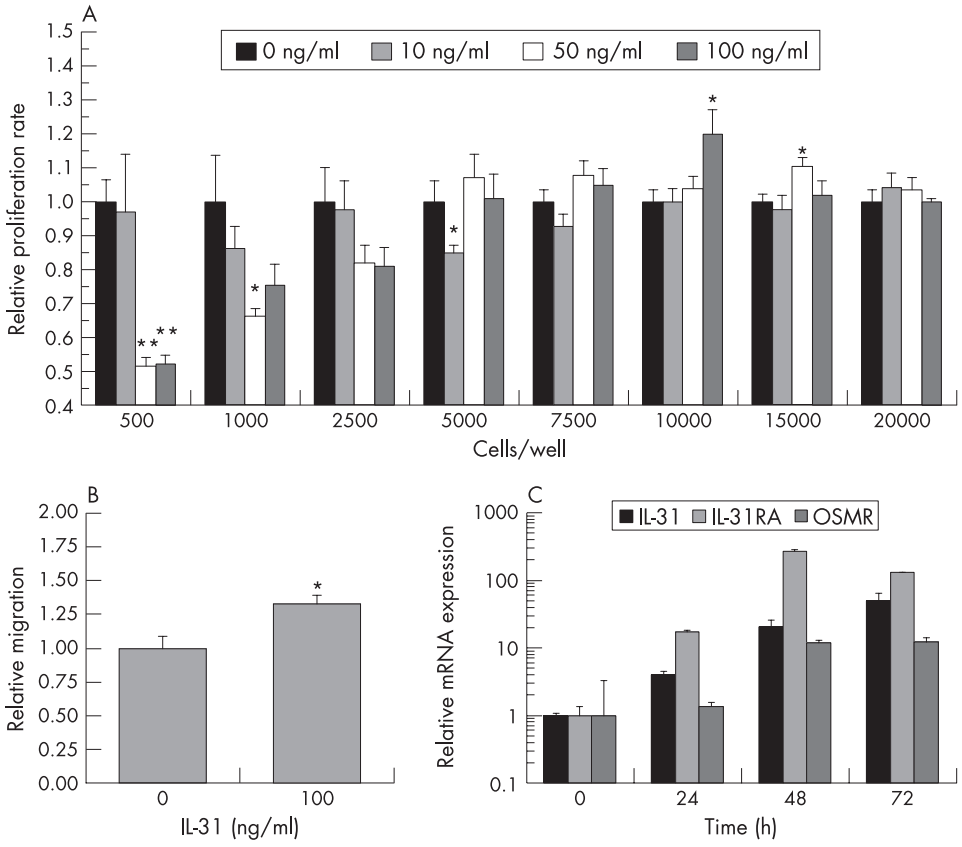

Figure 3 IL31 receptor expression is modulated by cell differentiation and influences IEC proliferation and migration. (A) IL31 influences cell proliferation depending on cell density as determined by MTS assay (n = 6 for each cytokine concentration and cell density; *p<0.05, **p<0.005). Proliferation in unstimulated cells was arbitrarily set as 1.0 for each cell density. Data are presented as mean (SEM). (B) IL31 stimulation results in significantly increased cell migration in cell restitution assays (*p = 0.005). Migration of unstimulated cells was defined as 1.0. (C) IL31, IL31Rα, and OSMR mRNA expression is strongly upregulated during cell differentiation induced by sodium butyrate.

LPS and the proinflammatory cytokines TNF-α, IL1β, and IFN-γ. The mRNA expression of all three genes was upregulated 24 hours after stimulation with these proinflammatory cytokines (fig 4). The strongest effect on the transcriptional regulation of all three IL31 related genes was seen after stimulation with IFN-γ, which increased OSMR expression 5.4-fold, IL31R mRNA 3.5-fold, and IL31 mRNA 12.1-fold after 24 hours of stimulation.

IL31 increases the expression of proinflammatory cytokines in IEC

Based on the transcriptional upregulation of IL31 and its receptor complex by proinflammatory cytokines, we next analysed if IL31 itself regulates the expression of proinflammatory proteins. In this experiment, we studied in the IEC line HCT116 the effect of IL31 on protein expression of IL8, a prototypic inflammatory cytokine produced by IEC.[24] As shown in figure 5, IL31 increased IL8 expression 3.1-fold after 24 hours of stimulation.

IL31 mRNA expression does not correlate with TNF-α mRNA expression in a Th1 mediated model of murine ileitis

Having shown that IL31, IL31Rα, and OSMR mRNAs are upregulated under inflammatory conditions in vitro, we next examined their expression in murine models of intestinal inflammation in vivo. Using heterozygous TNFΔARE mice as a model of chronic ileitis,[15] we demonstrated no significant differences in IL31 mRNA expression in these mice compared to wild type mice of the same age and genetic background (fig 6A); however, there was a trend towards higher IL31 expression in colonic epithelial cells compared to ileal epithelial cells (p = 0.088). Similarly, OSMR expression was not significantly higher in TNFΔARE mice in comparison with wild type mice (fig 6B). In contrast, TNFΔARE mice had a significantly higher TNF-α expression than wild type mice (p<0.01; fig 6C).

IL31 mRNA expression is not upregulated in MCMV infection in vivo

In another set of experiments, we investigated colonic IL31, IL31Rα, and OSMR mRNA expression in viral infection using the murine in vivo model of MCMV infection. C57BL/6 mice were infected with 10^6 pfu MCMV of the Smith strain[17] as previously described.[18] However, 45 hours after infection, IL31 and IL31Rα mRNA levels were not significantly different compared to non-infected mice (p = 0.73; fig 6D) while there was a trend towards a higher OSMR expression in infected mice (p = 0.06; fig 6D). In contrast, in TNFΔARE mice and corresponding wild type mice, IL31Rα mRNA levels were lower and just above the detection threshold in both mice strains without significant differences between the two strains (data not shown). However, in comparison with the cDNA isolation of the MCMV infected mice (fig 6D), the isolation procedure of ileal and colonic epithelial cells of TNFΔARE mice yielded much lower RNA and cDNA amounts, respectively. Using similar reaction volumes, there was GAPDH detection already after 18 PCR cycles in the MCMV infected mice, while GAPDH was detected in TNFΔARE mice only after 25 PCR cycles, explaining the different levels of IL31Rα expression in two mouse experiments.

IL31 mRNA expression is increased in the inflamed colonic mucosa of patients with inflammatory bowel disease

We next analysed IL31 and its receptor expression in human intestinal inflammation in vivo using real time PCR. In this analysis, we compared IL31, IL31Rα, and OSMR mRNA expression levels in a total of 88 biopsy samples taken from 22 different patients with inflammatory bowel disease (IBD)

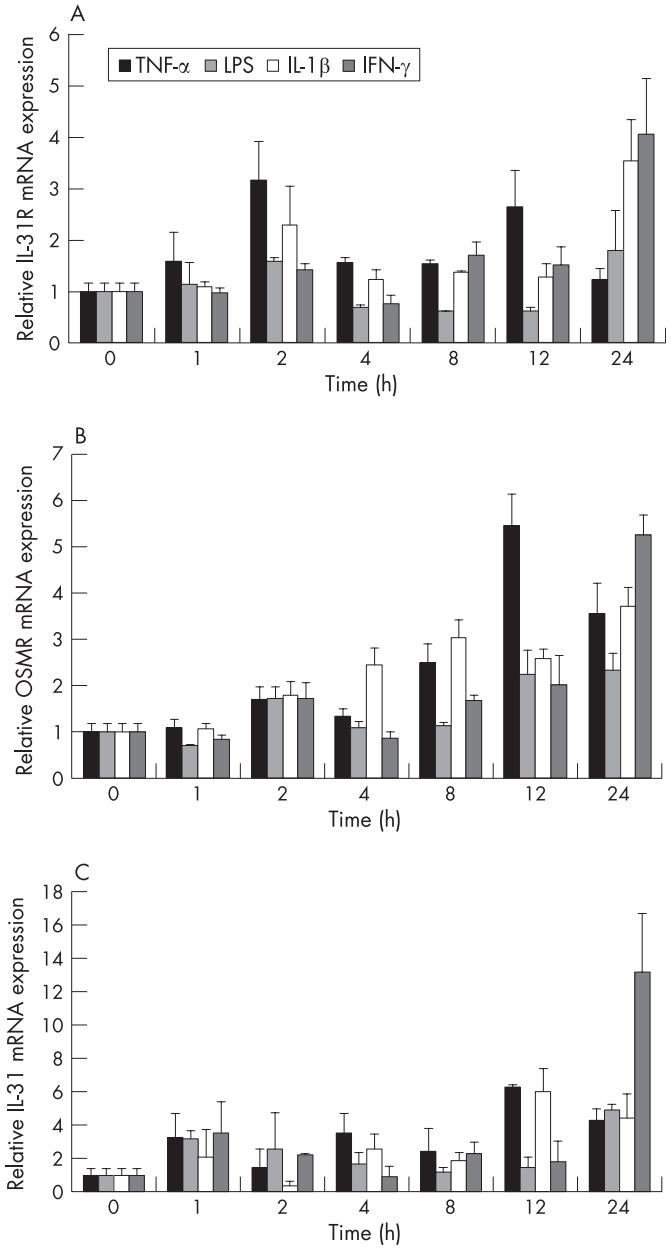

Figure 4 IL31Rα, OSMR, and IL31 mRNA expression are upregulated by proinflammatory stimuli. (A) Regulation of IL31Rα, (B) OSMR, and (C) IL31 by LPS (1 μg/ml) and the proinflammatory cytokines TNF-α (50 ng/ml), IL1β (10 ng/ml), and IFN-γ (1000 U/ml). HCT116 cells were stimulated for time intervals as indicated and mRNA expression was measured by quantitative PCR and normalised to β-actin expression.

including 12 patients with CD and 10 patients with UC. The biopsies were sampled from sites with endoscopically (macroscopic) inflamed colonic mucosa and compared to those of endoscopically non-inflamed colonic mucosa taken from the same 22 patients. We used the IL8 mRNA expression in these biopsies as a control marker of inflammation. IL8 mRNA expression was significantly increased ($p<0.05$) in the inflamed biopsy samples in CD (fig 7A). Moreover, in CD IL31 mRNA expression levels were higher in inflamed colonic biopsy samples than in non-inflamed colonic lesions (mean increase 2.4-fold; fig 7A) and correlated with IL8 mRNA expression in these patients ($r = 0.564$; fig 7C). There was also a twofold higher IL31Rα and OSMR mRNA expression in inflamed biopsies compared to uninflamed tissue (fig 7A). Similarly, in eight of the 10 patients with UC the IL31 mRNA

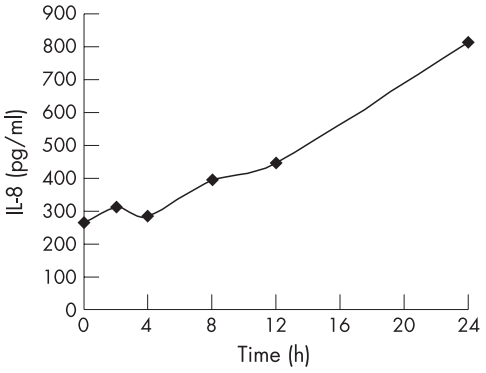

Figure 5 IL31 increases IL8 expression in IEC. IL8 protein levels increase and reach a maximum after 24 hours of IL31 stimulation. IL8 protein concentration was determined by ELISA.

expression was increased in the inflamed lesions (mean increase 2.6-fold) and correlated with the IL8 mRNA expression in these lesions ($r = 0.650$; fig 7B, D). In addition, IL31Rα and OSMR mRNA expression was slightly increased in the inflamed tissue of the UC patients (1.5-fold and 1.7-fold, fig 7B).

DISCUSSION

Crohn's disease and ulcerative colitis, the two main forms of inflammatory bowel disease, are defined as idiopathic, chronic relapsing, inflammatory conditions that are immunologically mediated.[25] Despite intensive research their aetiology is only incompletely understood.[25 26] However, several cytokines such as TNF-α, IL1β, and IL23 play a key part in the aggravation of intestinal inflammation.[25–30] Given its important role in inflammatory disorders of the skin,[1 6–10] we therefore analysed in this study the novel cytokine IL31 focusing on expression, signalling, and its role in intestinal inflammation.

While activated T cells are the main source of IL31,[1] we here demonstrate that IEC lines express the functional IL31 receptor complex consisting of IL31Rα and OSMR, which is consistent with the expression pattern shown for other epithelial cell types such as keratinocytes[1] and lung epithelial cells.[4] After stimulation with IL31, ERK, Akt, and STAT proteins are activated in IEC. Similar to our findings, activation of STAT1/3 and PI3 kinase signalling has been observed in glioblastoma and melanoma tumour cells,[2] and in lung epithelial cells[4] following IL31 stimulation. However, in agreement with a recent study in lung epithelial cells, in which strong activation was only obtained by IL31 receptor overexpression,[4] the level of STAT activation in non-transfected IEC was only moderate.

Recently, we demonstrated that activation of Akt and STAT proteins mediates IEC proliferation and migration.[11 12 19 20] Similarly, our experiments demonstrated that IL31 receptor activation increases IEC migration. Interestingly, IL31 is located on chromosome 12q24 in close proximity to chemokine-like factor 1, which has been very recently shown to be a functional ligand for CCR4.[31] Given the results of our experiments, IL31 also has chemotactic properties influencing the migration of IEC. Moreover, an influence on cell migration has been demonstrated for the IL31 related cytokine IL-6[32 33] and several other IL6-like cytokines such as LIF.[34] In addition, it has been shown that LIF may upregulate the gene expression of chemokines such as MCP-1.[34] Similarly, we here show that IL31 increases the expression of the proinflammatory chemokine IL8. Particularly the activation of STAT3 is a common signalling pathway mediated by these migration stimulating cytokines.[35] For example, we recently demonstrated that cell migration mediated by the IL-10-like cytokine IL22 is dependent on STAT3 activation.[11] A very recent study

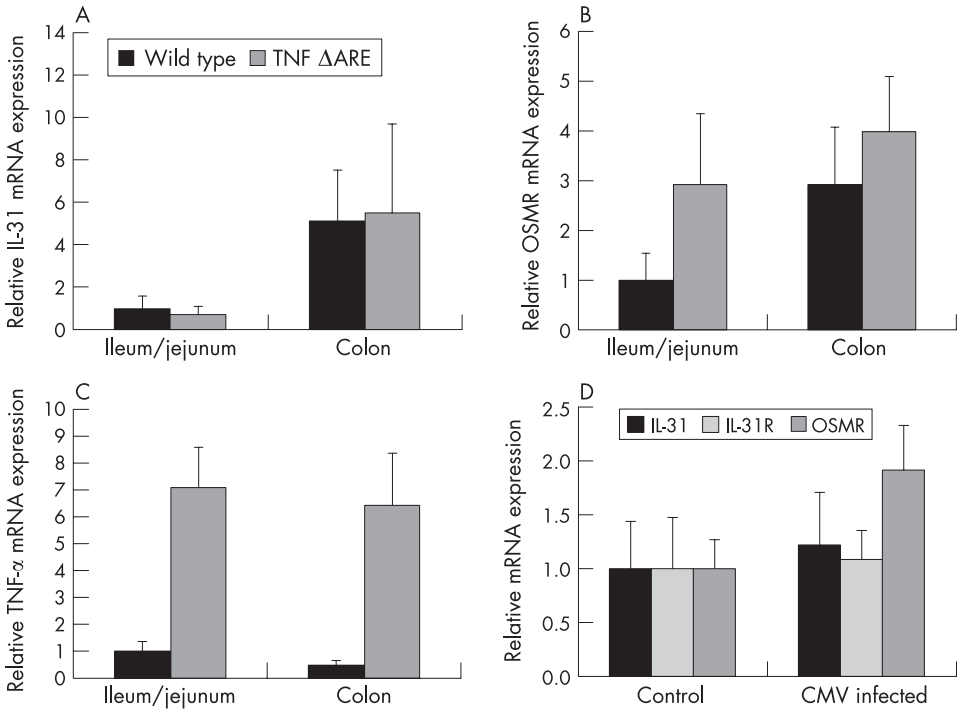

Figure 6 IL31 mRNA expression is not increased in murine models of Th1 mediated intestinal inflammation. (A) IL31 expression was similar in ileal and colonic epithelial cells of TNFΔARE mice (n = 9) compared to C57BL/6 wild type mice (n = 9). Ileal mRNA expression in wild type mice was arbitrarily set to 1.0 and all other expression levels were calculated proportionally as fold increase. Data are presented as mean (SEM). (B) OSMR mRNA expression was not significantly different in TNFΔARE mice compared to wild type mice ($p = 0.26$). (C) TNF-α mRNA expression was significantly higher in TNFΔARE mice compared to wild type mice ($p < 0.01$). (D) IL31, IL31Rα, and OSMR mRNA expression were not significantly increased in C57BL/6 mice infected with murine cytomegalovirus (n = 10) compared to uninfected C57BL/6 mice (n = 4).

established that OSMR mediated signal transduction is negatively regulated by SOCS3 through a receptor tyrosine independent mechanism.[36] Consistently, we demonstrated that SOCS3 can also inhibit signalling of other STAT activating cytokines.[37–42]

IL31Rα belongs to the IL6 cytokine receptor (IL6R) family. IL6Rs have divergent effects on cell proliferation in different epithelial cell types. While suppression of proliferation has been described for IL6R mediated signalling in some epithelial cells,[43] it enhances cell growth in other epithelial cell lines.[44 45] Here, we demonstrate that IL31 at high doses and low cell density significantly decreases cell proliferation in the colorectal cancer cell line HCT116. Similarly, a very recent study demonstrated that IL31 was highly effective in suppressing proliferation by altering expression of cell cycle proteins including upregulation of p27Kip1 and downregulation of cyclinB1, cdc2, cdk6, mcm4, and Rb in lung epithelial cells.[4] Here, we demonstrate that the antiproliferative effect of IL31 in HCT116 cells was lost in dense cell cultures and under certain conditions even reversed into a proliferation stimulating effect. A similar bimodal effect on cell

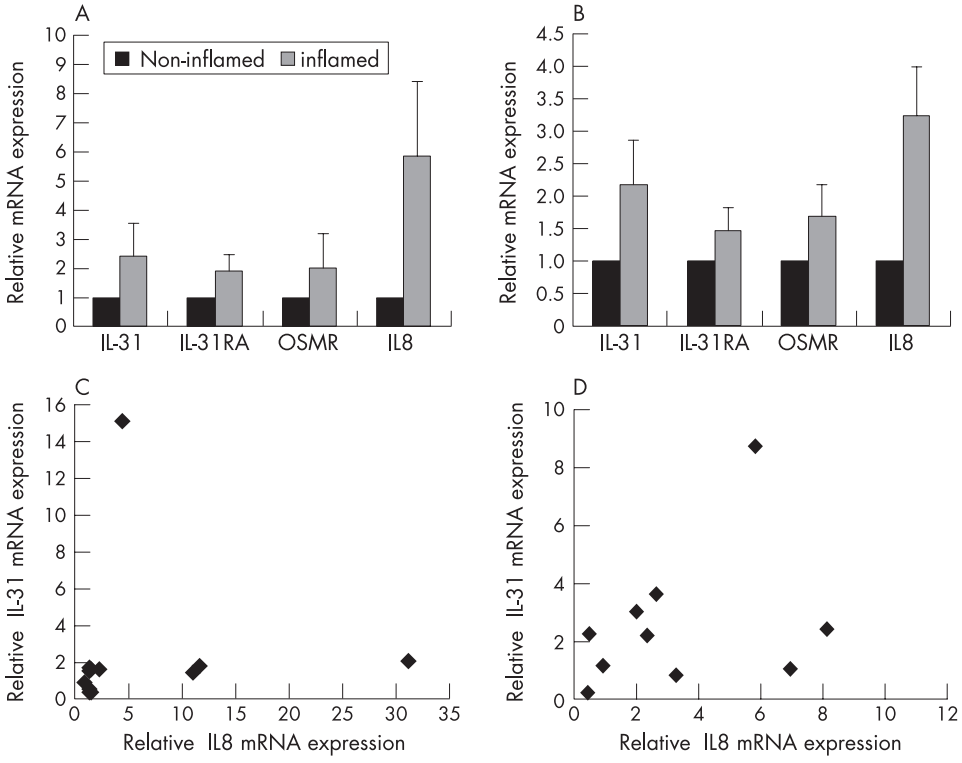

Figure 7 Colonic IL31 mRNA expression is increased in human inflammatory bowel disease. (A, C) IL31 expression is increased 2.4-fold in inflamed colonic biopsies from patients with Crohn's disease compared to uninflamed biopsies while IL31Rα and OSMR expression were increased twofold. IL31 mRNA expression correlated with the IL8 expression ($r = 0.56$), which was measured as a marker for inflammation. (B, D) In patients with ulcerative colitis, a 2.6-fold increased IL31 mRNA expression was observed in inflamed biopsies in comparison with uninflamed tissue with a high correlation to IL8 mRNA levels ($r = 0.65$). IL31Rα und OSMR were increased 1.5-fold and 1.7-fold, respectively.

proliferation dependent on cell density has also been described for other cytokines such as TGF-β.[46] However, a number of studies demonstrated that unstimulated cells in dense cultures undergoing contact inhibition are characterised by similar alterations of the expression of cell cycle proteins as caused by IL31 stimulation[4] including downregulation of cyclins[47] and upregulation of p27Kip1,[48] which explains the relative loss of the antiproliferative effect of IL31 (in comparison with unstimulated cells) in dense cultures considering the overall low cell proliferation in (unstimulated) dense cultures. Similar to IL31, other proliferation inhibiting agents such as bone morphogenetic protein (BMP-7)[49] and the MEK inhibitors PD98059 and U0126[47] have much higher effects on cell proliferation inhibition at low cell density compared to dense cultures. In contrast, cell proliferation stimulating cytokines such as hepatocyte growth factor (HGF) lose their proliferation stimulating properties in cell cultures with low cell density.[47] It has been demonstrated that a single STAT3 recruitment site (Tyr-721) in the cytoplasmic domain of IL31Rα exerts a dominant function in the entire receptor complex and is critical for gene induction and growth inhibition.[4] Similarly, we demonstrated upregulation of STAT3 following IL31 stimulation in our study. However, in the initial study,[4] an IL31Rα overexpressing cell line was used explaining the stronger antiproliferative effects of IL31 observed in that study compared to our experiments. In addition, it has been demonstrated that high cell density itself is associated with ligand independent STAT3 activation mediating growth inhibition,[50] which further explains the loss of the antiproliferative properties of IL31 in dense cell cultures. Moreover, we demonstrate that IL31 and IL31 receptor mRNA expression are cell differentiation dependent, which further supports the strong influence of cell density on the biological functions of IL31.

In this study, we demonstrate that proinflammatory cytokines increase the expression of IL31 and its receptor complex. The strongest upregulation of IL31, IL31Rα, and OSMR gene expression was seen after stimulation with IFN-γ. Similarly, in monocytes, gene upregulation of IL31Rα was stronger following IFN-γ compared to LPS stimulation.[1] Given our quantitative PCR results of primary murine IEC, which demonstrated in comparison to colorectal cancer derived IEC only weak IL31Rα mRNA expression, it is therefore likely that proinflammatory cytokine activated monocytes are a major IL31Rα expressing cell type and target of IL31 in IBD.

Depending on the T cell source of cytokine production, cytokines have been differentiated in Th1 and Th2 cytokines. It has been proposed that CD represents a Th1 and Th17 mediated intestinal inflammation, while ulcerative colitis resembles more a Th2 mediated colitis.[30 42 51] IL31 is mainly produced by activated Th2 cells[1] and a very recent study implicates IL31R signalling as a novel negative regulatory pathway that specifically limits Th2 mediated inflammation[52] while development of intestinal pathology in the TNFΔARE mouse model depends on Th1-like cytokines such as IL12 and IFN-γ.[53] In contrast, the generation of CD4(+) T cell mediated Th1 responses were normal in IL31Rα(−/−) mice.[52] Consistently, we demonstrated no increased IL31 mRNA expression in TNFΔARE mice, a Th1 mediated murine model of ileitis.[15 53] In these mice, baseline colonic IL31 expression was higher than ileal IL31 expression. This is in agreement with the first report on IL31 expression in normal tissues demonstrating a higher IL31 expression in the colon than in the small intestine.[1] However, in comparison with uninflamed colonic tissue, the IL31 mRNA expression was upregulated in inflamed colonic tissue in both forms of IBD suggesting that in humans IL31 mediated effects are not completely restricted to Th2 mediated types of inflammation. Moreover, the IL31 mRNA expression correlated with IL8 mRNA expression in both forms of intestinal inflammation.

While this is the first report on IL31 in intestinal inflammation, the role of IL31 in skin inflammation, particularly in atopic dermatitis is well established.[1 7–10] Interestingly, there is an association between intestinal inflammation and atopic dermatitis.[54 55] Moreover, in patients with atopic dermatitis, disturbances in intestinal permeability compared to normal controls have been reported.[56 57] Therefore, IL31 producing T cells may mediate skin reactions as observed in many IBD patients but this and the role of IL31 on intestinal permeability need further investigation. Given its effect demonstrated here on IEC proliferation and migration, it is likely that this novel cytokine system modulates IEC barrier function. However, while most colorectal cancer derived IEC expressed considerable levels of IL31Rα, its mRNA expression levels in unstimulated murine primary IEC were low suggesting that cancerogenesis might be another process in which this novel cytokine is involved. This is supported by IL31Rα expression in other malignancies such as glioblastoma and melanoma,[2] which also secrete a soluble form of OSMR which may bind IL31 when combined with soluble IL31Rα,[58] but further studies are required to elucidate potential functions of IL31 in cancerogenesis.

In summary, we demonstrated that IEC express the functional IL31 receptor complex. Binding of IL31 to its surface receptor complex in IEC leads to phosphorylation of STAT1/3, Akt, and ERK MAP kinases. IL31 inhibited cell proliferation in low density cell cultures and increased IEC migration. Moreover, the mRNA expression of IL31 was upregulated in inflamed colonic lesions of IBD patients but not in a Th1 mediated model of murine ileitis. Taken together, our data indicate a role for this cytokine in promoting proinflammatory gene expression and modulating IEC barrier function suggesting a role in the pathogenesis of IBD.

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Competing interests: None.

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Manuskript [6]

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IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration

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Brand, Stephan, Florian Beigel, Torsten Olszak, Kathrin Zitzmann, Sören T. Eichhorst, Jan-Michel Otte, Helmut Diepolder, Andreas Marquardt, Wolfgang Jagla, Andreas Popp, Stéphane Leclair, Karin Herrmann, Julia Seiderer, Thomas Ochsenkühn, Burkhard Göke, Christoph J. Auernhammer, and Julia Dambacher. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol* 290: G827–G838, 2006; doi:10.1152/ajpgi.00513.2005.—IL-22 is produced by activated T cells and signals through a receptor complex consisting of IL-22R1 and IL-10R2. The aim of this study was to analyze IL-22 receptor expression, signal transduction, and specific biological functions of this cytokine system in intestinal epithelial cells (IEC). Expression studies were performed by RT-PCR. Signal transduction was analyzed by Western blot experiments, cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay and Fas-induced apoptosis by flow cytometry. IEC migration was studied in wounding assays. The IEC lines Caco-2, DLD-1, SW480, HCT116, and HT-29 express both IL-22 receptor subunits IL-22R1 and IL-10R2. Stimulation with TNF-α, IL-1β, and LPS significantly upregulated IL-22R1 without affecting IL-10R2 mRNA expression. IL-22 binding to its receptor complex activates STAT1/3, Akt, ERK1/2, and SAPK/JNK MAP kinases. IL-22 significantly increased cell proliferation ($P = 0.002$) and phosphatidylinsitol 3-kinase-dependent IEC cell migration ($P < 0.00001$) as well as mRNA expression of TNF-α, IL-8, and human β-defensin-2. IL-22 had no effect on Fas-induced apoptosis. IL-22 mRNA expression was increased in inflamed colonic lesions of patients with Crohn's disease and correlated highly with the IL-8 expression in these lesions ($r = 0.840$). Moreover, IL-22 expression was increased in murine dextran sulfate sodium-induced colitis. IEC express functional receptors for IL-22, which increases the expression of proinflammatory cytokines and promotes the innate immune response by increased defensin expression. Moreover, our data indicate intestinal barrier functions for this cytokine-promoting IEC migration, which suggests an important function in intestinal inflammation and wound healing. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and IEC migration.

interleukin-10-like cytokines; interleukin-22; defensin

IL-22 WAS ORIGINALLY DESCRIBED as an IL-9-induced gene and was named for IL-10-related T cell-derived inducible factor (IL-TIF) (22). This cytokine shows 22% amino acid identity with IL-10 and belongs to a family of cytokines with limited homology to IL-10, namely IL-10, IL-19, IL-20, IL-24, IL-26, IL-28A, IL-28B, and IL-29 (the latter 3 also known as IFN-λs). IL-22 binds at the cell surface to a receptor complex composed of two chains belonging to the class II cytokine receptor family (CRF2): IL-22R1 and IL-10R2 (23, 32, 56). The ligand-binding chains for IL-22, IL-26, IL-28A/B, and IL-29 are distinct from that used by IL-10. However, all of these cytokines use a common second chain, IL-10 receptor-2 (IL-10R2; CRF2–4) to assemble their active receptor complexes. The binding of IL-22 to its respective R1 chain induces a conformational change that enables IL-10R2 to interact with the newly formed ligand-receptor complexes. This, in turn, activates a signal-transduction cascade that results in rapid activation of several transcription factors, including STAT proteins in several cell lines such as mesangial cells, lung epithelial cells, melanomas, hepatomas, and keratinocytes (6, 22, 23, 35, 53, 56).

Major sources of IL-22 are activated T and natural killer cells (54). As discovered thus far, IL-22 activities include upregulation of acute-phase reactants in the liver and hepatoma cells (22, 23) as well as induction of pancreatitis-associated protein in pancreatic acinar cells (2), suggesting a role for this cytokine in inflammatory processes. To date, no comprehensive analysis of IL-22-inducible genes has been published; however, several IL-22-inducible genes have been identified including chemokine genes in hepatocytes such as IFN-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), and IL-8 (19).

IL-22Rs are expressed on a number of tissues, including kidney, pancreas, and liver (32). The biological activities induced by IL-22 are only beginning to be defined. For example, a recent study (42) supports a potential therapeutic role for IL-22 as a protective factor in hepatocellular injury.

Although expression of the IL-22 receptor complex has been demonstrated in colonic epithelial cells (38), the regulation of the receptor expression, its detailed signal transduction including its specific biological functions in intestinal epithelial cells (IEC) and role in human gastrointestinal disease still have to be established, which was therefore the aim of this study.

Here, we demonstrate that the expression of IL-22 is upregulated in intestinal inflammation as seen in patients with Crohn's disease. IL-22 signaling in IEC resulted in increased

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proinflammatory gene transcription. Importantly, IL-22 promotes the intestinal barrier integrity in vitro through stimulation of IEC migration and defensin expression. Overall, our data indicate a role for this cytokine system in protecting the intestinal barrier by enhancing IEC migration, suggesting an important function in intestinal inflammation and wound healing.

MATERIALS AND METHODS

Reagents. Polyclonal antibodies to phosphorylated extracellular signal-regulated kinase (ERK)1/2 (Thr183/Tyr185), phosphorylated stress-activated protein kinase (c-Jun NH_2-terminal kinase), SAPK/JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), and phospho-Akt (Ser473) were purchased from Cell Signaling (Beverly, MA). Anti-ERK1/2, anti-SAPK/JNK, anti-p38, and anti-Akt antibodies were also from Cell Signaling. Horseradish peroxidase-linked anti-rabbit secondary antibody was purchased from Amersham (Arlington Heights, IL). Recombinant human IL-22, TNF-α, and IL-1β were obtained from R&D Systems (Minneapolis, MN). LPS from *Escherichia coli* (O26:B6) prepared by phenol extraction was purchased from Sigma (St. Louis, MO) and prepared as dispersed sonicate in endotoxin-free water (Life Technologies, Rockville, MD) before diluting to final concentration in supplemented media. MEK-1 inhibitor PD98059, SAPK/JNK inhibitor SP600125, and phosphatidylinositol 3 (PI3)-kinase inhibitor wortmannin were from Tocris Cookson (Bristol, UK).

Cell culture. The human colorectal cancer-derived IEC lines SW480, Caco-2, HT-29, HCT116, and DLD-1 were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (GIBCO-BRL/Life Technologies, Gaithersburg, MD) with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (PAA, Pasching, Austria) in a humidified 5% CO_2 atmosphere at 37°C. For signal-transduction experiments with IL-22, cells were starved overnight in serum-free medium.

RT-PCR. RT-PCR was performed as previously described (13). Briefly, total RNA was isolated using TRIzol reagent (GIBCO-BRL/Life Technologies). For RT-PCR, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNA-free Kit, Ambion) to remove potential genomic DNA contaminants. Three micrograms of total RNA were reverse transcribed using Roche first-strand cDNA synthesis kit. To control for genomic contamination, an identical parallel PCR reaction was performed containing starting material that had not been reverse transcribed. The following conditions were used for semiquantitative PCRs: 25–36 cycles (depending on the specific PCR) of denaturing at 95°C for 45 s, annealing at 61°C for 45 s, extension at 72°C for 45 s. The primers for the PCR reactions are shown in Table 1. The PCR products were subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. Densitometric analysis was performed using software TINA (version 2.10g, Raytest Isotopenmessgeräte, Straubenhardt, Germany).

Quantitative PCR. Real-time PCR was performed with a Rotorgene RG-3000 cycler (Corbett Research, Sydney, Australia) using the Quantitect SYBR Green PCR kit from Qiagen (Hilden, Germany) following the manufacturer's guidelines. Oligonucleotide primers were designed not to amplify genomic DNA, according to the published sequences, and the following primer pairs were used: human IL-22 forward 5′-gcaggcttgacaagtccaact-3′, reverse 5′-gcctccttagccagcatgaa-3′; β-actin: forward 5′-gccaaccgcgagaagatga-3′, reverse 5′-catcacgatgccagtggta-3′; IL-8 forward 5′-ccaggaagaaaccaccgga-3′, reverse 5′-gaaatcaggaaggctgccaag-3′; TNF-α forward 5′-ccaggcagtcagatcatcttctc-3′, reverse 5′-agctggttatctctcagctccac-3′ (MWG-Biotech, Ebersberg, Germany). IL-22 mRNA expression was normalized to β-actin expression in the respective cDNA preparation. To compare IL-8 and IL-22 expression levels between inflamed and noninflamed colonic lesions in patients with inflammatory bowel disease (IBD), expression in noninflammatory tissue was arbitrarily set to 1.0.

Table 1. *PCR primers used for RT-PCR analysis*

hIL-22R forward:	5′-CTCCACAGCGGCATAGCCT-3′
hIL-22R reverse:	5′-ACATGCAGCTTCCAGCTGG-3′
hIL-10R2 forward:	5′-GGCTGAATTTGCAGATGAGCA-3′
hIL-10R2 reverse:	5′-GAAGACCGAGGCCATGAGG-3′
hIL-22BP forward:	5′-AGGGTACAATTTCAGTCCCGA-3′
hIL-22BP reverse:	5′-CGGCGTCATGCTCCATTCTGA-3′
hIL-8 forward:	5′-ATGACTTCCAAGCTGGCCGTGGCT-3′
hIL-8 reverse:	5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′
hTNF-α forward:	5′-ATGAGCACTGAAAGCATG-3′
hTNF-α reverse:	5′-TCACAGGGCAATGATCCC-3′
hBD-2 forward:	5′-CCAGCCATCAGCCATGAGGGT-3′
hBD-2 reverse:	5′-GGAGCCCTTTCTGAATCCGCA-3′
hSOCS-3 forward:	5′-TTCTGATCCGCGACAGCTC-3′
hSOCS-3 reverse:	5′-TGCAGAGAGAAGCTGCCCC-3′
hGAPDH forward:	5′-CGGAGTCAACGGATTTGGTCGTAT-3′
hGAPDH reverse:	5′-AGCCTTCTCCATGGTGGTGAAGAC-3′
mLactoferrin forward:	5′-GCCTGTTCCAGTCTAAAACCA-3′
mLactoferrin reverse:	5′-GTAAGAAAAGCGCAGGCTTC-3′
mE-Selectin forward:	5′-ACCCGTCCCTTGGTAGTTG-3′
mE-Selectin reverse:	5′-CGTTGTAAGAAGGCACATGG-3′
mCXCL2 forward:	5′-CCTGCCAAGGGTTGACTTCA-3′
mCXCL2 reverse:	5′-GTCAGTTAGCCTTGCCTTTG-3′
mCXCL5 forward:	5′-CCTACGGTGGAAGTCATAGC-3′
mCXCL5 reverse:	5′-CTATTGAACACTGGCCGTTC-3′
mIL-4 forward:	5′-GAGTGAGCTCGTCTGTAGG-3′
mIL-4 reverse:	5′-CAGCTTATCGATGAATCCAGG-3′
mIL-6 forward:	5′-CAAAGCCAGAGTCCTTCAGAG-3′
mIL-6 reverse:	5′-GGATGGTCTTGGTCCTTAGC-3′
mIL-17 forward:	5′-CAAACACTGAGGCCAAGGAC-3′
mIL-17 reverse:	5′-AGCTTCCCAGATCACAGAGG-3′
mIL-22 forward:	5′-ACCTTTCCTGACCAAACTCA-3′
mIL-22 reverse:	5′-AGCTTCTTCTCGCTCAGACG-3′

h, Human; m, murine.

Signal-transduction experiments, gel electrophoresis and immunoblotting. The signal-transduction experiments were performed in overnight serum-starved intestinal epithelial cell lines as indicated. Cells were stimulated with 100 ng/ml IL-22, unless indicated differently. This concentration was used based on pilot experiments demonstrating a significantly higher effect of 100 ng/ml for the activation of certain kinases and cell migration than lower concentrations. Cells were solubilized in lysis buffer containing 1% Nonidet P-40, 20 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and phosphatase inhibitors (400 mM sodium orthovanadate and 4 mM NaF) and were passed six times through a 21-gauge needle. After 30 min on ice, lysates were cleared by centrifugation at 10,000 *g* for 20 min. The protein concentration of each sample was quantified by the Bradford method. Immunoblotting was performed as previously described (37).

ELISA. For the quantification of IL-8 release, BD OptEIA Human IL-8 Elisa kit II (BD Biosciences, Bedford, MA) was used according to the manufacturer's instructions.

Colonic biopsies. Biopsies were taken from patients with Crohn's disease and ulcerative colitis undergoing diagnostic colonoscopy. The study was approved by the Ethics Committee of the Medical Faculty of the University of Munich. All participating subjects gave written, informed consent before biopsy sampling. From each patient four biopsies were collected: two from macroscopically noninflamed sites and two from macroscopically inflamed mucosa. IL-22 and IL-8 mRNA levels were measured in each individual biopsy. For quantification, the average IL-22 and IL-8 mRNA expression of the two noninflamed biopsies was compared with the average expression in the two inflamed biopsies. For calculation of the correlation coefficient, for each patient, mRNA expression of IL-22 was correlated to expression of IL-8 mRNA in the four individual biopsies.

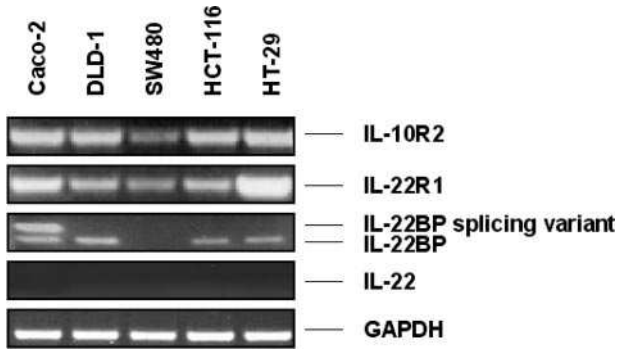

Fig. 1. IL-10 receptor 2 (R2), IL-22R1, and IL-22 binding protein (IL-22BP) but not IL-22 are expressed in intestinal epithelial cells (IEC). mRNA expression of IL-10R2, IL-22R1, IL-22BP, and IL-22 as analyzed by RT-PCR analysis of mRNA derived from IEC lines as indicated.

Wounding assay. Wounding assays were performed as previously described (18). Briefly, SW480 cells, which were the most suitable human IEC line in pilot experiments, were grown in six-well plates to complete confluence. With the use of a sterile razorblade, eight standardized wounds were created in each plate. Detached cells were removed by three washes with PBS, and the cell medium was changed from 10% FCS-containing medium to 1% FCS-containing medium. The cells were stimulated with IL-22 (10 and 100 ng/ml) or 1% FCS. The cells were washed with PBS after 24 h, and the number of migrated cells (over the wounding edge) was counted under a microscope (Olympus IX50, ×10 magnification). For each group (IL-22 stimulated and medium stimulated), three dishes were analyzed, whereas for each dish, eight separate fields were counted containing more than 300 migrated cells per group.

Cell proliferation assay. HT-29 cells were seeded onto 96-well plates at a density of 10,000 cells per well and were allowed to attach overnight. Cells were then stimulated with 10, 100, or 1,000 ng/ml IL-22 or with cytokine-free medium (negative control) for 48 h. The cell proliferation rate was determined by MTS assay on *day 2* using the CellTiter 96 aqueous non-radioactive cell-proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions.

Apoptosis assays. Apoptosis assays were performed as described previously (24). For induction of CD95-mediated cell death, ligand-specific anti-APO-1 mAb at concentrations of 100 and 500 ng/ml was used. Cells were trypsinized and lysed in a hypotonic lysis buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 50 μg/ml of propidium iodide. After incubation at 4°C overnight, the nuclei were then analyzed for DNA content by flow cytometry.

Dextran sulfate sodium colitis model. C57BL/6 and C3H/HeJ mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Experimental colitis was induced by adding 3% and 4.5% dextran sulfate sodium (DSS; molecular weight 36,100–45,500; TdB Consultancy, Uppsala, Sweden) to the drinking water of C57BL/6 and C3H/HeJ mice, respectively, for 5 days. At *day 6*, mice were euthanized by CO_2 asphyxiation and the large intestine was removed for further analysis. Total RNA of the colon was isolated using Qiagen RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. This study was approved by the Animal Care and Use Committee of the State of Bavaria (Regierung von Oberbayern) following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Statistical analysis. Statistical analysis was performed using two-tailed Student's *t*-test. *P* values <0.05 were considered as significant.

RESULTS

IEC express the IL-22 receptor complex and IL-22 binding protein. To determine whether the IL-22 receptor complex consisting of IL-10R2 and IL-22R1 is expressed in IEC and to use an IEC model to study this ligand-receptor system, we analyzed IL-10R2 and IL-22R1 mRNA expression in several human IEC lines (Caco-2, DLD-1, SW480, HCT116, and HT-29). RT-PCR analysis demonstrated IL-10R2 and IL-22R1 mRNA expression in all cell lines tested (Fig. 1). Moreover, all cell lines with the exception of SW480 cells expressed mRNA for IL-22 binding protein (IL-22BP). Interestingly, a previously described alternative splicing variant of IL-22BP (21) was found in Caco-2 cells (Fig. 1). However, none of the cell lines analyzed expressed IL-22 (Fig. 1).

IL-22 induces STAT1/3-, ERK1/2-, SAPK/JNK-1/2, and Akt phosphorylation. Having demonstrated that IEC lines express the IL-22 receptor complex, we next analyzed whether this complex is functional in IEC investigating various signaling pathways activated by IL-22. Previous studies (6, 22, 23, 35, 53, 56) in other cell lines reported activation of STAT signaling by IL-22. Therefore, we investigated the influence of IL-22 on phosphorylation levels of STAT1 and STAT3 in IEC. Compared with basal levels of tyrosine phosphorylation of STAT1 in unstimulated controls, tyrosine phosphorylation of STAT1 was clearly induced by 100 ng/ml IL-22 (Fig. 2*A*). Similarly, IL-22 strongly induced tyrosine phosphorylation of STAT3 with a maximal phosphorylation level after 15–30 min (Fig. 2*B*).

Moreover, IL-22 (100 ng/ml) induced a transient activation of ERK1/2 (Fig. 3*A*). During the observed time interval, total ERK1 and ERK2 levels remained unchanged (Fig. 3*A*). To identify upstream signaling events, we investigated the effect of the MEK-1 inhibitor PD98059 on the IL-22-mediated ERK regulation. PD98059 downregulated ERK1/2 phosphorylation after IL-22 stimulation significantly (Fig. 3*B*), suggesting MEK-1 as an upstream signal transducer of the IL-22-induced ERK activation. Cross talk between the PI3-kinase and the MEK-ERK pathway has been proposed (43). However, ERK activation after IL-22 stimulation was not significantly affected by pretreatment with wortmannin (Fig. 3*C*), suggesting a PI3-kinase-independent activation of ERK-MAP kinases by IL-22. Similarly, the JNK kinase inhibitor SP600125 did not influence the IL-22-induced ERK activation (Fig. 3*C*).

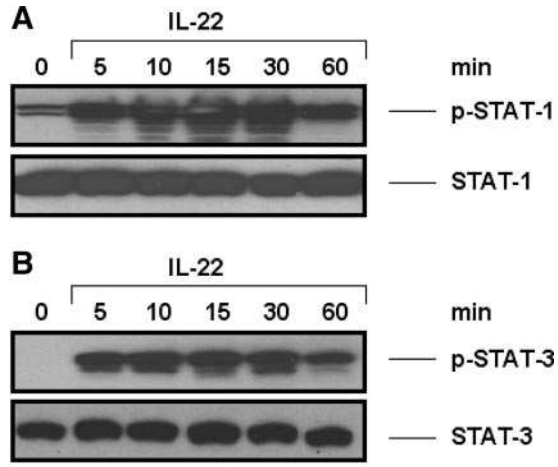

Fig. 2. IL-22 induces STAT-1 and STAT-3 phosphorylation in IEC. On stimulation of HT-29 cells with IL-22 (100 ng/ml), STAT1 (*A*) and STAT3 (*B*) proteins are strongly phosphorylated. One representative experiment ($n = 3$) is shown.

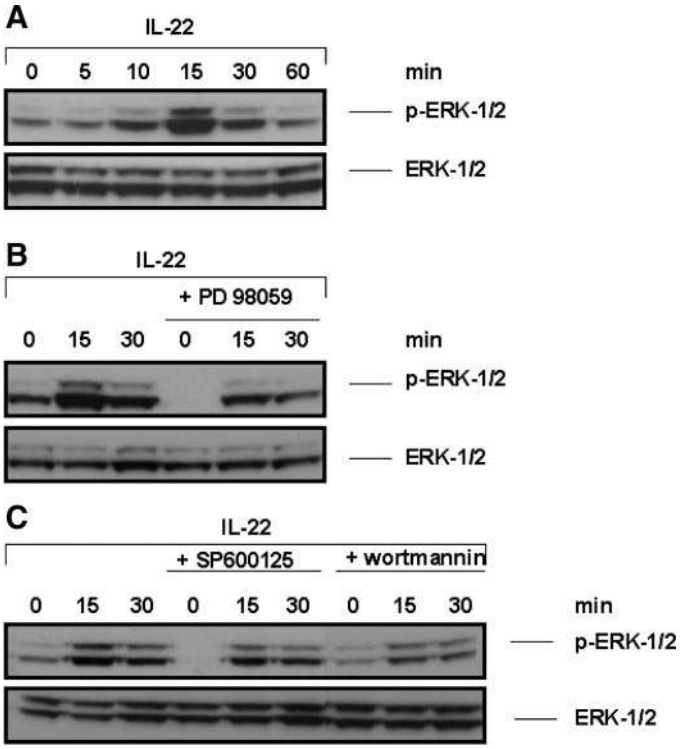

Fig. 3. IL-22 activates ERK-MAP kinases in IEC. Activation and expression of phospho-ERK1/2 in HT-29 cells was assessed by immunoblotting. *A*: phospho-ERK1/2 activation after IL-22 stimulation (100 ng/ml). *B*: the IL-22-induced ERK activation is MEK-1 dependent. Pretreatment with the MEK-1 inhibitor PD98059 (10 μM 1 h before IL-22 stimulation) resulted in a decreased ERK activation. *C*: The IL-22-induced ERK activation is SAPK/JNK and phosphatidylinositol 3 (PI3)-kinase independent. Pretreatment with the SAPK/JNK inhibitor SP600125 (20 μM) or the PI3-kinase inhibitor wortmannin (25 μM) did not influence ERK1/2 activation. One representative experiment ($n = 3$) is shown.

Activation of the IL-22 receptor in hepatocytes results in activation of p38 and SAPK/JNK kinases (35). Similarly, stimulation of IEC with IL-22 resulted in the phosphorylation of SAPK/JNK kinases (Fig. 4*A*), which was significantly suppressed by pretreatment with the JNK inhibitor SP600125 (Fig. 4*B*). However, stimulation of IEC with IL-22 did not result in a significant phosphorylation of p38 (data not shown). Furthermore, IL-22 binding to its receptor complex also resulted in increased phosphorylation of Akt (Fig. 4*C*). Pretreatment with the PI3-kinase inhibitor wortmannin caused a complete dephosphorylation of Akt (Fig. 4*D*).

IL-22 increases expression of proinflammatory cytokines in IEC. After having established that the IL-22 receptor complex is functional in IEC, we next examined transcriptional targets of this cytokine. First, we analyzed mRNA levels for SOCS-3, which we previously identified as an immediate-early STAT1/3-dependent gene (4). As shown in Fig. 5*A*, IL-22 treatment induced SOCS-3 mRNA expression with maximal levels detected after 12 h of stimulation.

Furthermore, previous studies in other cell lines demonstrated that IL-22 increases gene expression of acute phase proteins (19, 38). Therefore, we focused on the proinflammatory cytokines TNF-α and IL-8, two major mediators of inflammatory responses in IECs, as a downstream readout of IL-22-mediated gene expression. The mRNA expression of both cytokines was significantly increased (TNF-α: 5.2-fold; IL-8: 27.7-fold) as measured by quantitative PCR (Fig. 5*B*). Accordingly, IL-8 protein expression measured in ELISA assays increased 4.2-fold after IL-22 stimulation (Fig. 5*C*).

IL-22R1 mRNA expression is upregulated after stimulation with proinflammatory cytokines. Having established that IL-22 upregulates the expression of proinflammatory cytokines, we next analyzed whether mRNA expression of the IL-22 receptor subunits IL-22R1 and IL-10R2 is regulated by proinflammatory cytokines. Although IL-10R2 is a promiscuous receptor subunit, which is not only used by IL-22 but also by IL-26, IFN-λs, and the anti-inflammatory cytokine IL-10, signaling through IL-22R1 is restricted to IL-22. As shown in Fig. 6, LPS, IL-1β, and TNF-α upregulated IL-22R1 mRNA expression but had no effect on IL-10R2 mRNA expression levels. Maximal induction of IL22R1 was detected after 2–4 h of stimulation.

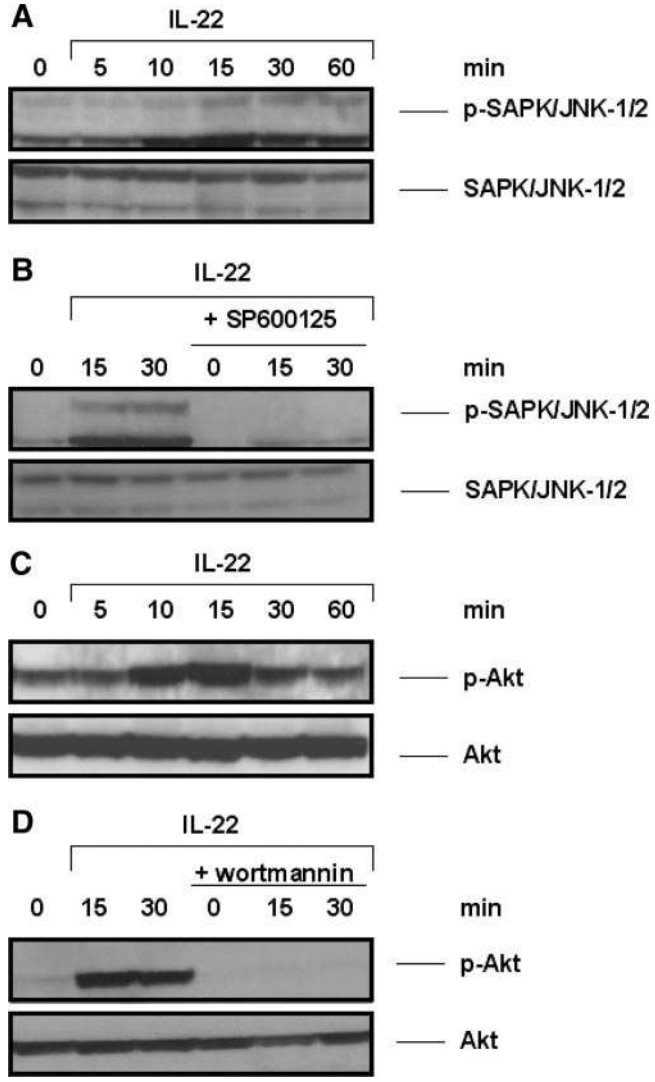

Fig. 4. IL-22 activates SAPK/JNK MAP kinases and Akt in IEC. *A*: stimulation of HT-29 cells with IL-22 (100 ng/ml) resulted in increased phosphorylation of SAPK/JNK kinases. *B*: pretreatment with the SAPK/JNK inhibitor SP600125 (20 μM) decreased SAPK/JNK activation. *C*: IL-22 induces PI3-kinase-dependent Akt phosphorylation. *D*: pretreatment with the PI3-kinase inhibitor wortmannin (25 μM) resulted in a complete dephosphorylation of Akt. One representative experiment ($n = 3$) is shown.

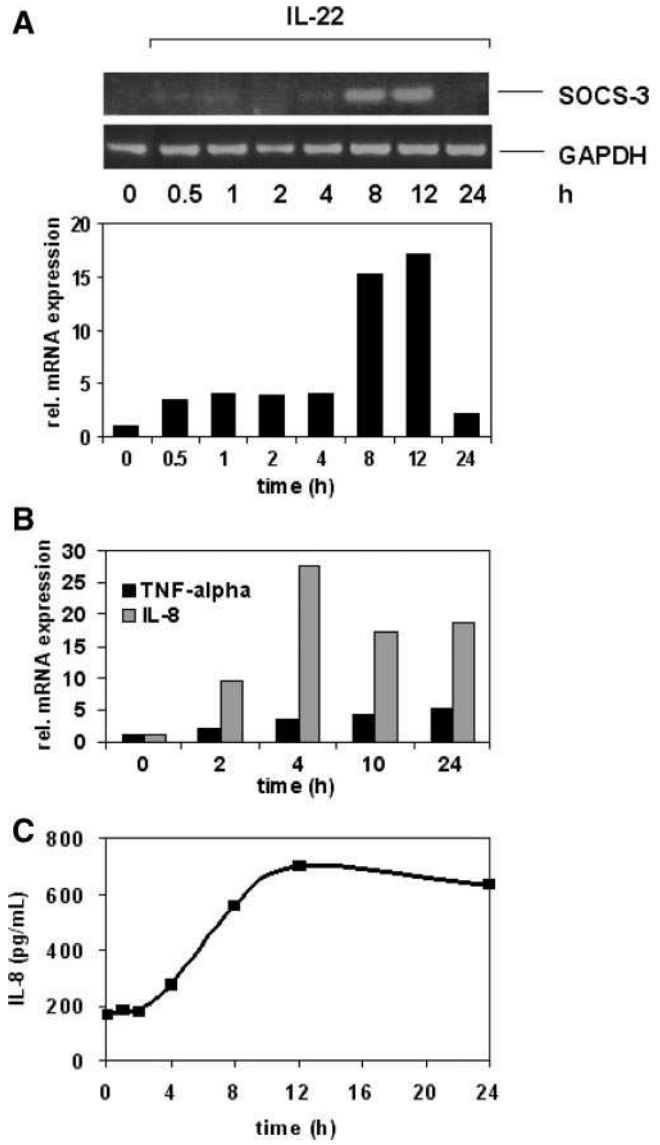

Fig. 5. IL-22 increases expression of proinflammatory cytokines and SOCS-3 in IEC. *A*: SOCS-3 mRNA expression after stimulation of HT-29 cells with IL-22 (100 ng/ml). The mRNA expression levels were normalized to GAPDH mRNA levels using TINA software. *B*: upregulation of IL-8 and TNF-α mRNA expression after stimulation of HT-29 cells with 100 ng/ml IL-22. TNF-α and IL-8 mRNA expression was measured by quantitative PCR and normalized with respect to β-actin expression levels in the respective cDNA preparation. mRNA expression at the start of the experiment (0 h) was arbitrarily set to 1.0. *C*: IL-8 protein levels increase and reach a maximum after 12 h of IL-22 stimulation. IL-8 protein concentration was determined by ELISA. Rel., relative.

IL-22 mRNA expression is increased in inflamed colonic mucosa of patients with IBD. Because we have shown that IL-22 upregulates the expression of proinflammatory cytokines and that the expression of the specific receptor subunit IL-22R1 is increased by proinflammatory stimuli, we analyzed its expression in intestinal inflammation in vivo using real-time PCR. In these experiments, we compared IL-22 mRNA expression levels in 80 biopsy samples from 20 patients with IBD (Crohn's disease: $n = 9$; ulcerative colitis: $n = 11$) taken from sites with endoscopically (macroscopic) inflamed colonic mucosa with those of endoscopically noninflamed colonic mucosa taken from the same 20 patients. The IL-8 mRNA expression, which was used as a control marker for inflammation, was significantly increased ($P < 0.05$) in the inflamed biopsy samples in Crohn's disease (Table 2, Fig. 7*A*). The increase in IL-8 mRNA expression ranged from 1.4- to 31-fold compared with the noninflamed tissues. Similarly, IL-22 mRNA expression levels were significantly higher in inflamed colonic biopsy samples than in noninflamed colonic lesions ($P < 0.05$; increase between 1.4- and 13.6-fold; Table 2, Fig. 7*A*). Interestingly, the highest IL-22 mRNA levels were found in a patient with severe Crohn's disease (Fig. 7*A*, red mark) affecting the ileocecal valve and terminal ileum (Fig. 7*C*), which also had the highest IL-8 mRNA expression levels (Fig. 7*A*). His-

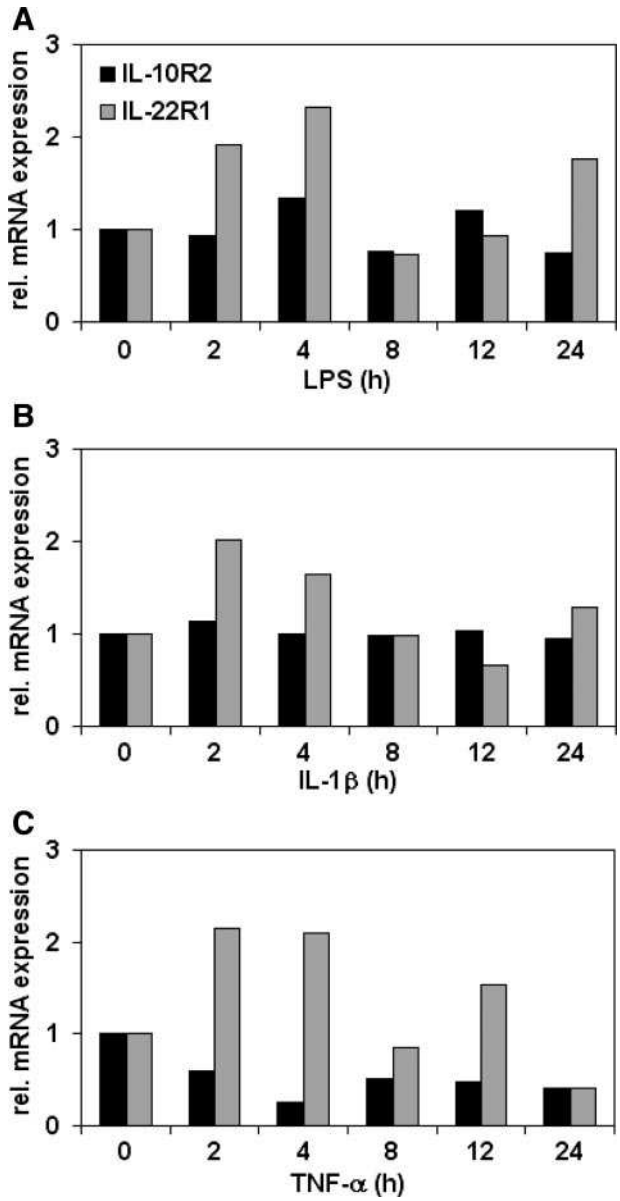

Fig. 6. IL-22R1 mRNA expression is upregulated after stimulation with various proinflammatory stimuli. Regulation of IL-10R2 and IL-22R1 mRNA expression in HT-29 cells after stimulation with LPS (1 μg/ml; *A*) with IL-1β (10 ng/ml; *B*) and TNF-α (50 ng/ml; *C*). The expression levels of IL-10R2 and IL-22R1 mRNA were normalized to GAPDH mRNA levels using TINA software.

Table 2. *Comparison of IL-22 and IL-8 expression in inflamed and noninflamed colonic lesions of patients with CD and UC*

Patient Number	Diagnosis	Current Medication	Anatomic Site of Biopsy Sampling		IL-22 Expression Level Inflamed vs. Noninflamed	IL-8 Expression Level Inflamed vs. Noninflamed	Correlation IL-22/IL-8
			Noninflamed	Inflamed			
*1**	CD	AZA, IFX	cecum*	terminal ileum*	1.59	4.50	−0.207
2	CD	mesalazine, AZA	descending colon	descending colon	3.67	11.67	0.926
3	CD	AZA	cecum	cecum	1.92	11.08	0.976
*4**	CD	mesalazine, corticosteroids	cecum*	terminal ileum*	1.50	1.52	0.956
5	CD	no medication	descending colon	descending colon	2.81	1.41	0.977
6	CD	MTX	cecum*	terminal ileum/ ileocecal valve*	13.58	31.12	0.941
7	CD	AZA	transverse colon	transverse colon	3.07	2.24	0.994
8	CD	corticosteroids	cecum*	terminal ileum*	1.37	1.45	1.000
9	CD	AZA	cecum	cecum	3.35	4.26	1.000
Average ± SE			CD		3.65 ± 1.41	7.69 ± 3.62	0.840 ± 0.010
10	UC	corticosteroids	sigmoid*	rectum*	0.52	6.97	−0.675
11	UC	mesalazine, budenosid	descending colon	descending colon	0.97	2.37	−0.199
12	UC	AZA	rectum	rectum	0.72	0.94	0.849
13	UC	none	tranverse colon	tranverse colon	3.25	2.01	1.000
14	UC	none	cecum	rectum	5.74	8.12	0.999
15	UC	AZA, corticosteroids, mesalazine	cecum	cecum	1.82	2.64	0.959
16	UC	6-MP	sigmoid	sigmoid	2.42	5.82	0.912
17	UC	none	sigmoid	sigmoid	1.76	3.27	0.527
18	UC	MTX	transverse colon*	descending colon*	0.98	0.71	0.529
19	UC	6-MP, mesalazine, corticosteroids	cecum*	ascending colon*	0.84	0.49	0.860
20	UC	MTX	sigmoid*	rectum*	1.25	2.44	0.818
Average ± SE			UC		1.84 ± 0.46	3.25 ± 0.78	0.598 ± 0.165

IL-22 and IL-8 mRNA expression in 80 inflammatory bowel disease biopsy samples were measured by quantitative PCR and normalized with respect to β-actin expression levels in the respective cDNA preparation. To compare expression levels between inflamed and noninflamed colonic lesions, mRNA expression in noninflamed tissue was arbitrarily set to 1.0. The current medical therapy and the anatomic site from which the samples were taken are given for all patients. Biopsy sampling for inflamed and noninflamed lesions was intended to be performed in the same colonic or ileal segment. This was not possible in patients marked with an asterisk (*) due to severe inflammation in the whole anatomic segment. Therefore, biopsies from a bordering (noninflamed) segment were included for comparison. AZA, azathioprine; IFX, infliximab; MTX, methotrexate; 6-MP, 6-mercaptopurine; CD, Crohn's Disease; UC, ulcerative colitis.

topathological analysis of the biopsies demonstrated severe mucosal ulceration with massive infiltration of neutrophils and other inflammatory cells (Fig. 7, *D* and *E*). Magnetic resonance imaging enteroclysis demonstrated wall thickening of the terminal ileum resulting in ileal stenosis (Fig. 7, *F* and *G*).

In patients with ulcerative colitis, the average IL-8 and IL-22 expression levels in inflamed tissues were lower compared with expression levels in patients with Crohn's disease (Table 2, Fig. 7, *A* and *B*). Although IL-8 expression was significantly upregulated to eightfold in the inflamed lesions of patients with ulcerative colitis ($P = 0.02$), the difference of IL-22 mRNA expression in inflamed and noninflamed biopsies did not reach statistical significance ($P = 0.09$). Interestingly, IL-22 was downregulated in 5 of 11 patients with ulcerative colitis, with 3 of these patients also having low IL-8 expression levels (Table 2, Fig. 7*B*).

Overall, the upregulation of IL-22 expression was more pronounced in inflamed lesions of patients with Crohn's disease compared with patients with ulcerative colitis (3.65- vs. 1.84-fold), confirming its role in T_H1-mediated inflammation (54). Moreover, the IL-22 mRNA levels correlated highly with the IL-8 mRNA expression levels in patients with Crohn's disease ($r = 0.840$, Table 2). In eight of nine samples, the correlation coefficient was even higher than 0.90. In patients with ulcerative colitis, the observed correlation between IL-22 and IL-8 mRNA expression was lower (0.598; Table 2), and only 4 of 11 patients showed correlation coefficients >0.90.

IL-22 mRNA expression is increased in murine DSS colitis. Next, we studied the IL-22 mRNA expression levels in intestinal inflammation in vivo in the acute phase of colitis in the murine DSS colitis model. As shown in Fig. 8, 6 days after DSS treatment, IL-22 was among the genes most strongly upregulated, whereas it was almost undetectable in untreated mice. This effect was more pronounced in C3H/HeJ mice than in C57BL/6 mice. The IL-22 mRNA expression correlated with the expression of other proinflammatory cytokines and chemokines such as IL-6 and MIP-2α (Fig. 8).

IL-22 induces intestinal epithelial restitution and promotes intestinal barrier integrity in vitro. Having established that IL-22 plays a role in mucosal injury in vivo, we next analyzed whether IL-22 itself promotes IEC injury or is a counterregulatory cytokine released to promote wound healing. This was analyzed in previously established IEC restitution assays (11). In these "wounding assays," standardized, sterile wounds were created in SW480 cells, which were shown to be the most suitable human IEC line for migration experiments, forming evenly distributed monolayers in pilot experiments (11). Twenty-four hours after wounding, the number of migrated cells was counted under the microscope. This experiment demonstrated a highly significant, dose-dependent 290% increase of the cell migration rate in the IL-22-stimulated cells ($P = 0.00000002$ for 10 ng/ml and $P = 0.00000006$ for 100 ng/ml IL-22 vs. unstimulated controls, Fig. 9*A*).

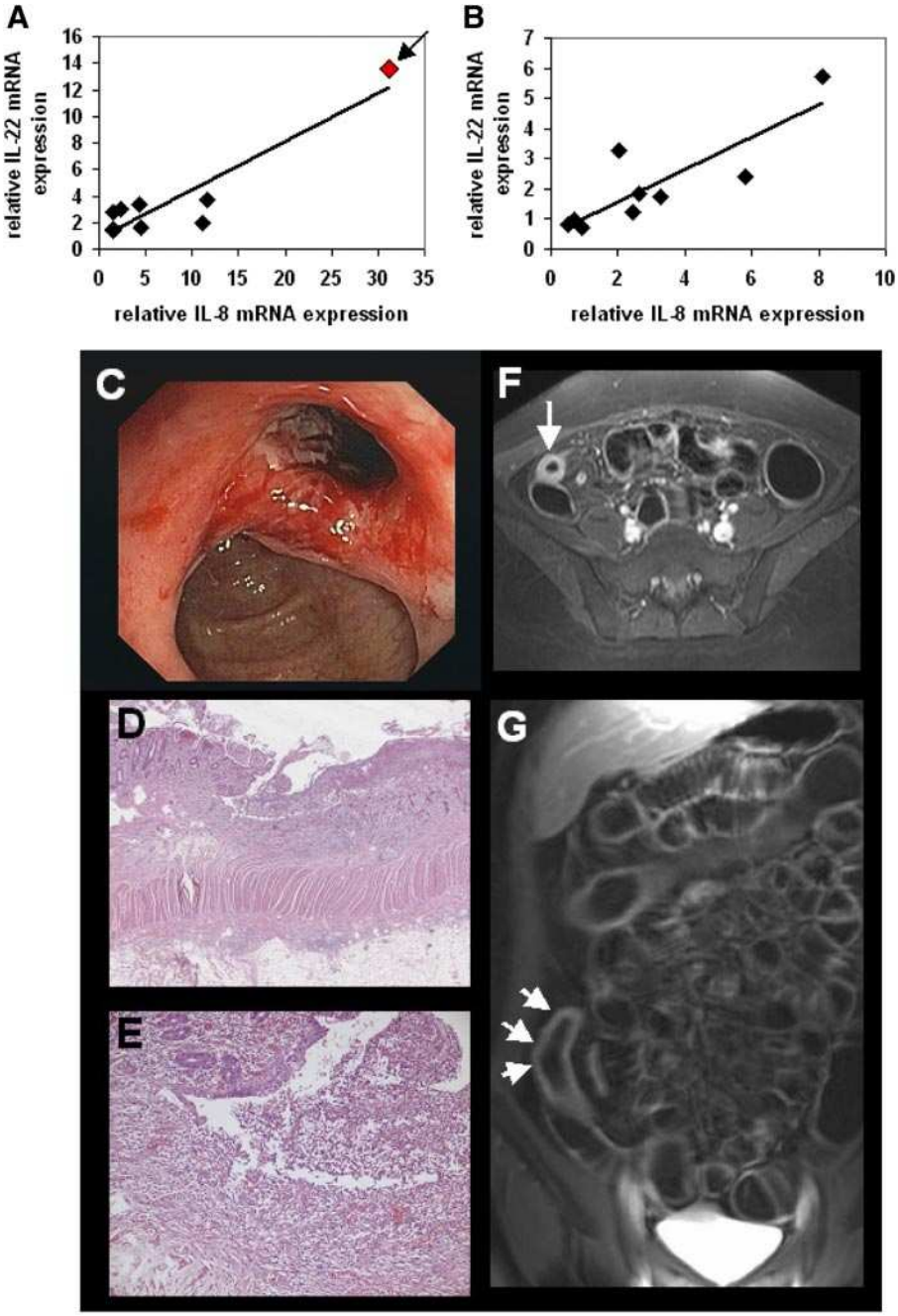

Fig. 7. IL-22 is upregulated in inflammatory bowel disease. IL-22 mRNA expression in colonic and ileal biopsies correlated with IL-8 mRNA expression in Crohn's disease (*A*) and ulcerative colitis (*B*). The highest IL-22 mRNA levels of all patients examined in this study (*A*; Table 2) were found in a patient with severe Crohn's disease (*A*; see arrow) particularly affecting the ileocecal valve and terminal ileum (*C*), which had also the highest IL-8 mRNA expression levels (*A*). Histopathological analysis of the biopsies demonstrated severe inflammation with extensive mucosal ulcerations and massive infiltration of neutrophils (*D* and *E*). Magnetic resonance imaging enteroclysis demonstrated wall thickening of the terminal ileum and ileal stenosis (*F* and *G*).

IL-22 promotes intestinal barrier integrity by PI3-kinase-dependent mechanisms and through increased IEC proliferation and β-defensin-2 production. Next, we analyzed the mechanisms by which IL-22 promotes IEC restitution and intestinal barrier integrity. The activation of MAP kinases such as ERK1/2 and the activation of Akt have been linked to cell migration (5, 48). Therefore, we repeated the restitution assays using a specific MEK-1 inhibitor (PD98059) and the PI3-kinase inhibitor wortmannin. The inhibition of IL-22-induced Akt activation through pretreatment with wortmannin significantly decreased IL-22-mediated cell migration ($P < 0.00001$ compared with cells stimulated with IL-22 only; Fig. 9*B*). In contrast, the inhibition of ERK activation using the MEK-1 inhibitor PD98059 did not block IL-22-mediated cell migration (Fig. 9*B*; $P < 0.00001$ compared with unstimulated cells, $P = 0.19$ compared with cells stimulated with IL-22 only), suggesting that IL-22-induced cell migration is PI3-kinase but not MEK-1 dependent. Furthermore, we also analyzed whether the IL-22-dependent IEC restitution is caused by increased cell proliferation or decreased apoptosis, particularly because ERK1/2 and Akt activation have also been shown to mediate antiapoptotic pathways and to increase cell proliferation (20, 25). Therefore, we investigated the IL-22-mediated effect on apoptosis using previously established experimental conditions (24). In these experiments, SW480 cells were used, which are less resistant to Fas-induced apoptosis than HT-29 cells (1). However, no significant difference between the number of apoptotic cells in the IL-22-stimulated group and the unstimu-

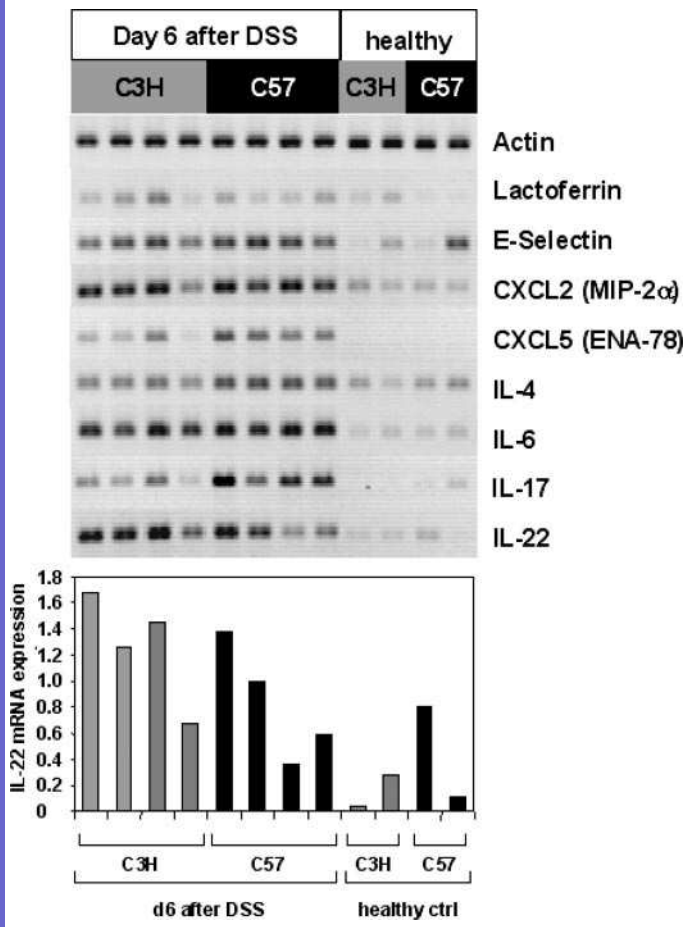

Fig. 8. IL-22 mRNA expression is upregulated in murine dextran sulfate sodium (DSS) colitis. C57BL/6 and C3H/FeJ mice were stimulated with 3% and 4.5% DSS, respectively, added to the drinking water for 5 days. Six days (d6) after DSS treatment, mice were euthanized and colonic gene expression was determined by semiquantitative RT-PCR as described in MATERIALS AND METHODS using the primers listed in Table 1. The PCR for actin was performed with 28 PCR cycles, whereas for all other genes, 36 cycles were used. IL-22 mRNA expression was calculated relative to actin mRNA expression in the respective cDNA preparation using TINA software. Control mice received no treatment ("healthy").

lated group was found (Fig. 9*C*). In contrast, IL-22 at concentrations of 10 and 100 ng/ml significantly increased cell proliferation ($P = 0.002$ and $P = 0.001$, respectively), whereas there was a trend for an antiproliferative effect using higher IL-22 concentrations ($P = 0.07$, Fig. 9*D*).

Finally, intestinal barrier integrity is also mediated by the expression of "barrier protective" proteins such as defensins. Therefore, we analyzed whether IL-22 regulates human β-defensin-2 (hBD-2) expression in IEC. As demonstrated in Fig. 9*E*, IL-22 upregulated hBD-2 mRNA expression in the IEC line HT-29 up to sixfold.

DISCUSSION

IBD, such as Crohn's disease and ulcerative colitis, are chronic inflammatory disorders of the gastrointestinal tract. Although the etiology is incompletely understood, initiation and aggravation of the inflammatory process seem to be due to a massive local mucosal immune response. IL-10 is a regulatory cytokine that inhibits both antigen presentation and subsequent proinflammatory cytokine release. IL-10-deficient mice spontaneously develop severe intestinal inflammation (34). Therefore, the use of IL-10 has been proposed as anti-inflammatory biological therapy in chronic IBD (36).

In contrast, we showed in this study that the novel IL-10-related cytokine IL-22, which shares with IL-10 the IL-10R2 subunit for signaling, has proinflammatory functions in IEC and is upregulated in Crohn's disease. Moreover, we demonstrated that the IL-22 receptor complex consisting of IL-22R1 and IL-10R2 is expressed in IEC and is functional in these cells. After stimulation with IL-22, MAP kinases, Akt, and STAT proteins are activated resulting in an increased expression of proinflammatory cytokines and hBD-2. An increased hBD-2 expression after IL-22 stimulation has also been demonstrated in keratinocytes (55), suggesting that IL-22 increases the innate immunity of epithelial tissues such as skin and intestine. Interestingly, an increased mRNA expression of hBD-2 has been recently described in inflamed colonic lesions of patients with Crohn's disease (51). This is consistent with the increased IL-22 mRNA expression in inflamed colonic lesions in Crohn's disease found in this study. The IL-22 mRNA expression correlated highly with the IL-8 mRNA expression ($r = 0.840$). It has been shown that activated T cells are major sources of IL-22 (54). Polarization of T cells toward the type 1 (T_H1) phenotype further increases the activation-induced IL-22 expression, whereas polarization toward type 2 (T_H2) reduces it (54). Interestingly, it has been proposed that Crohn's disease represents a T_H1-mediated intestinal inflammation, whereas ulcerative colitis resembles more a T_H2-mediated colitis (7). Consistent with this observation, IL-22 expression was significantly increased in inflamed lesions in Crohn's disease but overall only slightly elevated in active ulcerative colitis, further illustrating the potential importance of IL-22 in the pathogenesis of Crohn's disease. These results are supported by a very recent study that also demonstrated increased IL-22 expression in IBDs, particularly in Crohn's disease (3). Furthermore, this study characterized IL-22R1-expressing subepithelial myofibroblasts as additional targets of IL-22 in the intestine (3).

PCR analysis revealed that the mRNA expression of the IL-22 specific subunit of the receptor complex (IL-22R1), but not the promiscuous IL-10R2 subunit, which is also part of the anti-inflammatory IL-10 receptor complex, is under transcriptional control of proinflammatory cytokines. Moreover, IL-22 increased IL-8 mRNA and protein expression in IEC. Similarly, IL-22 upregulated expression of several chemokine genes in hepatocytes, including IL-8, IP-10, and MCP-1 (19). In addition to liver (19) and intestine, proinflammatory properties of IL-22 were also reported in the skin (6, 55), lung (53), and pancreas (2, 28). Hence, consistent with the IL-22R1 receptor expression, epithelial tissues seem to be a preferred target of IL-22. For example, IL-22 overexpression in mice causes neonatal lethality with skin abnormalities reminiscent of psoriatic lesions in humans (17).

Furthermore, we demonstrated that IEC also express mRNA for IL-22BP, which specifically binds IL-22 and does not bind other IL-10-related cytokines (21, 33). This suggests that IEC may regulate the intensity of IL-22 signaling by differential expression of IL-22BP. This is further supported by their ability to secrete anti-inflammatory IL-10 on IL-22 stimulation as demonstrated in a recent study (38).

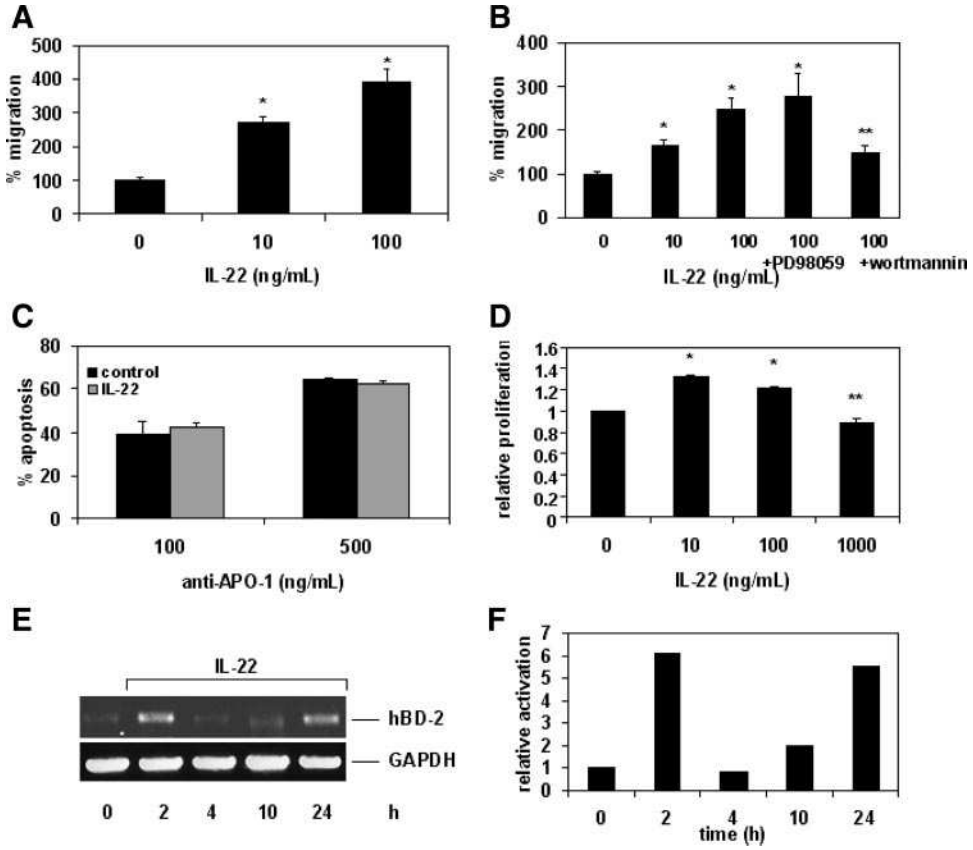

Fig. 9. IL-22 promotes intestinal barrier integrity in vitro by enhancing IEC restitution, cell proliferation, and human β-defensin-2 expression. *A*: "wounding" assays were used to analyze the influence of IL-22 on IEC migration. IL-22 (10 and 100 ng/ml) induced a significant increase of the cell migration rate (*$P <$ 0.00001 vs. unstimulated control). *B*: preincubation with the PI3-kinase inhibitor wortmannin (25 μM) inhibited IL-22-mediated IEC migration, whereas the MEK-1 inhibitor PD98059 (10 μM) did not affect the IL-22-induced cell migration (*$P < 0.00001$; **$P = 0.002$ vs. unstimulated control). *C*: IL-22 does not influence Fas ligand-mediated apoptosis in SW480 cells. Cells were treated with 100 and 500 ng/ml anti-APO-1 antibody and either IL-22 stimulated or unstimulated for 24 h. Apoptosis was measured by FACS analysis. *D*: low doses of IL-22 increased cell proliferation in HT-29 cells. HT-29 cells were seeded onto 96-well plates at a density of 10,000 cells per well and were grown for 1 day. After starvation in serum-free medium overnight, the cells were stimulated with IL-22 as indicated or with cytokine-free serum-free medium (negative control). The cell proliferation rate was determined by MTS assay after 48 h. With lower doses of IL-22 (10 and 100 ng/ml), there was a statistical significant increase in cell proliferation whereas on stimulation with high doses of IL-22 (1,000 ng/ml) a decrease of cell proliferation was observed (*$P < 0.005$; **$P = 0.07$ vs. unstimulated control). *E*: after stimulation with IL-22 (100 ng/ml), human β-defensin-2 (hBD-2) mRNA is upregulated 6-fold in HT-29 cells as determined by semiquantitative PCR. *F*: densitometric analysis of the results shown in *E*.

In this study, IL-22 activated STAT1 and STAT3, which resulted in increased SOCS-3 mRNA expression in IEC, confirming our previous results that SOCS-3 is a transcriptional target of STAT1/3 (4). This is also in agreement with increased SOCS-3 mRNA in a hepatoma cell line following IL-22 stimulation (33) and a very recent study demonstrating STAT1/3 activation after stimulation with IL-22 in the colonic epithelial cell line Colo205 (38). In this study, IL-22 signaling could be inhibited by IL-22BP and a neutralizing antibody against IL-10R2 (38). Interestingly, colonic tissue samples of patients with Crohn's disease demonstrated increased STAT1 phosphorylation levels and, compared with samples taken from ulcerative colitis patients, increased SOCS-3 levels (44) further underlining the proinflammatory properties of IL-22 in Crohn's disease. Interestingly, in a murine colitis model SOCS-3 has been demonstrated to play a negative regulatory role in STAT3 activation and intestinal inflammation (46). Moreover, we recently demonstrated that signaling of other IL-10-like cytokines such as IL-28A and IL-29 is abrogated by increased expression of SOCS proteins (15), suggesting that signaling of IL-10-like cytokines is regulated by SOCS proteins. Interestingly, we found a similar mechanism for the signaling mediated by IFN-α (49).

In addition, IL-22 activates ERK and SAPK/JNK MAP kinases in IEC, which is similar to the signaling described for IL-22 in hepatic cells (35). However, in contrast to IL-22 signaling in hepatocytes (35), IL-22 did not significantly alter the phosphorylation levels of p38 MAP kinases in IEC. Particularly, signaling via SAPK/JNK in IEC has gained interest because two recent studies demonstrated that SAPK/JNK is activated in Crohn's disease (30, 50) and that inhibition of SAPK/JNK resulted in significant clinical benefit and rapid mucosal healing (30). In this study, the IL-22-mediated ERK activation was MEK-1 dependent, whereas the activation of Akt was entirely dependent on PI3-kinase.

Importantly, the activation of ERK-MAP kinases and Akt has been implicated in cell migration (5, 11, 27, 45, 57). Similarly, our experiments demonstrated that IL-22-receptor activation results in increased IEC migration and epithelial wound healing, which could be blocked using a PI3-kinase inhibitor. Generally, the integrity of the intestinal mucosal surface barrier is rapidly reestablished even after extensive

destruction because of an enormous regenerative capability of the mucosal surface epithelium. As demonstrated in wounding assays in this study, IL-22 stimulation may facilitate this epithelial restitution.

The IL-22-mediated barrier integrity in the IEC wounding assays was partly due to an increased cell proliferation rate. Interestingly, only low IL-22 concentrations (10 and 100 ng/ml) increased the IEC proliferation rate, whereas high doses (1,000 ng/ml) decreased cell proliferation. This inverted U-shaped dose-response curve is similar to the biological response observed for other cell migration-mediating cytokines such as the chemokine CXCL12 (41). We recently demonstrated similar antiproliferative properties for high doses of IFN-λs in IEC and hepatic cells, which also belong to the IL-10-like cytokine family (8–10). However, similar to IFN-λs (9, 10), no effect on cell apoptosis could be demonstrated for IL-22 in IEC. This is in contrast to studies in the hepatic cell line HepG2, where stable overexpression of IL-22 induced the expression of several antiapoptotic genes including Bcl-2, Bcl-x, and Mcl-1 (42).

There is also increasing evidence that Crohn's disease is a polygenic disease with several genes being involved in its pathogenesis. Interestingly, CARD15/NOD2, the first susceptibility gene of Crohn's disease (29, 31, 40), has been linked to increased intestinal permeability (16) and diminished defensin production (52). Although our in vitro data demonstrated that IL-22 increases defensin expression and promotes the integrity of the intestinal barrier, additional in vivo experiments are necessary to clarify the role of IL-22 in intestinal inflammation.

Recently, we demonstrated that in addition to CARD15/NOD2, there are several other genes [e.g., Toll-like receptor 4 (14), organic cation transporter cluster (47), and fractalkine receptor (CX3CR1) polymorphisms (12)] involved in the pathogenesis of Crohn's disease. The high expression of some of these receptor proteins in monocytes and dendritic cells (DC) suggests a central role of these cell populations in the pathogenesis of Crohn's disease. As recently demonstrated by us and others, DCs are able to sample bacteria from the intestinal lumen particularly in the ileum (39). Although IL-22 is not expressed by DCs, a very recent study demonstrated that a particular DC subset, which expresses Nectinlike protein-2, regulates IL-22 expression in activated CD8(+) T cells (26).

In summary, we demonstrated that IEC express the IL-22-receptor complex. Binding of IL-22 to its surface receptor in IEC leads to phosphorylation of STAT1/3, Akt, ERK-, and SAPK/JNK MAP kinases. In addition, IL-22 upregulated the mRNA expression of proinflammatory cytokines and of hBD-2. IL-22 also increased IEC migration but had no effect on apoptosis. Moreover, the mRNA expression of IL-22 is upregulated in inflamed colonic lesions in patients with Crohn's disease. Taken together, our data indicate a role for this cytokine in promoting proinflammatory gene transcription and IEC migration, suggesting an important function in intestinal inflammation and wound healing.

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Manuskript [7]

The role of the novel IL-10 like cytokine IL-26 in intestinal and hepatic inflammation

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Abbreviations

aP, alkaline phosphatase, CRP, C-reactive protein; ERK, extracellular signal–regulated kinase; EBV, Epstein-Barr virus; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGT, Gamma-glutamyl transferase; GOT, Glutamate-Oxalate Transaminase; GPT, Glutamate-Pyruvate Transaminase; HCV, hepatitis C; HVS, herpesvirus saimiri; IEC, intestinal epithelial cell; IFN, interferon; IL, interleukin; MAP kinase, mitogen-activated protein-kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MxA, myxovirus resistance protein A; 2’,5’-OAS, 2',5'-oligoadenylate synthetase; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; RT-PCR, reverse transcriptase polymerase chain reaction; SAPK/JNK, stress-activated protein kinase/c-Jun-N-terminal kinase; SEM, standard error of the mean; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; Th cells, T helper cells; TNF-α, tumor necrosis factor alpha

Keywords

IL-26, inflammatory bowel disease, Crohn’s disease, ulcerative colitis, hepatitis C

Abstract

Background/Aims: IL-26 is a novel IL-10 like cytokine without murine homologue expressed in activated Th1 cells. Currently, its role in human disease is completely unknown. Therefore, the aim of this study was to analyze its role in intestinal and hepatic inflammation and viral infection.

Methods: Expression studies were performed by RT-PCR. Signal transduction was analyzed by Western blot experiments and ELISA. Cell proliferation was measured by MTS assay.

Results: All examined IEC lines and the hepatic cell lines Huh-7, Hep3B and primary hepatocytes express both IL-26 receptor subunits IL-20R1 and IL-10R2. IL-26 activates ERK-1/2 and SAPK/JNK MAP kinases, Akt and STAT1/3. IL-26 stimulation increases the mRNA expression of IL-6, IL-8 and TNF-α but decreases cell proliferation. In inflamed colonic lesions of patients with Crohn's disease and ulcerative colitis, we found an elevated IL-26 mRNA expression that correlated highly with the IL-8 expression. However, there was no difference in the IL-26 mRNA expression in liver biopsy samples of patients with viral hepatitis in comparison to non-viral hepatitis and cholestatic liver disease. In comparison to the strong antiviral effect of IFN-α, IL-26 had no therapeutically relevant influence on the expression of the antiviral proteins MxA and 2',5'-OAS and the hepatitis C virus RNA replication rate *in vitro*.

Conclusion: Intestinal and hepatic cells express the functional IL-26 receptor complex. IL-26 modulates IEC proliferation and proinflammatory gene expression and its expression is up-regulated in inflammatory bowel disease indicating a role for this cytokine system in the innate host cell response during intestinal inflammation.

Keywords: IL-10 like cytokines, IL-26, Crohn's disease, ulcerative colitis, inflammatory bowel disease, intestinal epithelial cell, viral infection, HCV

Introduction

Interleukin (IL)-26, initially named AK155, has been originally identified by subtraction hybridization coupled with representational difference analysis as a gene upregulated in human T cells following infection with herpesvirus saimiri (HVS), with the capacity to transform these cells in culture [1]. The IL-26 protein has 24.7% amino acid identity and 47% amino acid similarity to human IL-10. IL-26 is a member of the IL-10 like cytokine family composed of cytokines with limited homology to IL-10 including IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29. IL-28A/B and IL-29 are also known as interferon-λs. IL-26 signals through a novel receptor pair consisting of the transmembrane proteins IL-20R1 and IL-10R2 [2]. In addition to cytokine specific ligand binding chains, IL-22, IL-26, IL-28A/B and IL-29 use a common second receptor chain (IL-10R2) for assembling their active receptor complexes and signaling.

The IL-26 gene is located on chromosome 12q14 + 3 in a cluster where IFN-γ and IL-22 are also positioned. IL-26 forms homodimers similarly to interleukin-10 [1]. Under basal conditions, IL-26 mRNA could not be detected in any cell line by Northern blot analysis [1]. Upon infection of T cells with various strains of HVS, expression was induced [1]. However, IL-26 mRNA could be detected in unstimulated fresh peripheral blood cells of healthy donors by RT-PCR analysis [1]. Although the IL-10 transcript was detected in most cell lines of T or B cell lineage, the IL-26 transcript was rather specific for T cells. It was determined that IL-26 mRNA is specifically overexpressed by T cells after HVS transformation. In another study, under basal conditions no IL-26 mRNA could be detected in monocytes, NK cells, and B and T cells [3]. The expression could be induced only in NK cells and T cells upon stimulation with IL-2/IL-12 and anti-CD3 monoclonal antibody, respectively [3]. This induction of IL-26 mRNA was observed specifically in activated memory cells (CD4+; CD45RO+) and during polarization toward type 1 T helper (Th) cells [3]. The observations that IL-26 is induced by

viral infection and in Th1 cells upon stimulation indicate that this gene may play an immune-protective role, particularly in viral infection.

Recent findings demonstrate that a heterodimer consisting of IL-20R1 and IL-10R2 functions as IL-26 receptor [2, 4]. This receptor complex is particularly expressed in epithelial cells such as keratinocytes and colonic epithelial cells [4]. However, there is currently very limited information on the biological functions of IL-26. Interestingly, both IL-22 and IL-26 have been identified outside of mammals and their organization and synteny demonstrate that this cluster of cytokines is well conserved during evolution [5]. However, in contrast to IL-22, no murine IL-26 homologue has been described so far, limiting the experimental opportunities to study the phenotypic consequences of IL-26 gene knockout and IL-26 mediated functions in murine models *in vivo*. Moreover, there are no studies on the effects of IL-26 in human disease published so far.

Given the IL-26 receptor expression in colonic epithelial cells [4] and our recent findings on the important role of other IL-10 like cytokines such as IL-22, IL-28A and IL-29 in the regulation of intestinal epithelial cells (IEC) [6, 7] and hepatic cells [8-10], we analyzed in this study signal transduction pathways and specific biological functions mediated by IL-26 in IEC and hepatic cells. Recently, we demonstrated that IL-22 and IL-28A, two other IL-10 like cytokines sharing the IL-10R2 subunit with IL-26 for signaling, are up-regulated in intestinal inflammation and viral infection, respectively [6, 7, 10]. Therefore, we focused in this study on the role of IL-26 in intestinal and hepatic inflammation and viral infection.

Materials and Methods

Reagents

Antibodies against pSTAT1 were from BD Transduction Laboratories (Franklin Lakes, NY) and antibodies against pSTAT3 were from Upstate Biotechnology (Lake Placid, NY). Antibodies against STAT1 and STAT3 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated extracellular signal-regulated kinase (ERK)-1/2 (Thr183/Tyr185), phosphorylated stress-activated protein kinase (c-Jun N-terminal kinase) SAPK/JNK (Thr183/Tyr185), and phospho-Akt (Ser473) were purchased from Cell Signaling (Beverly, MA). Anti-ERK-1/2, anti-SAPK/JNK, and anti-Akt antibodies were also from Cell Signaling. Horseradish peroxidase linked anti-rabbit secondary antibody was purchased from Amersham (Arlington Heights, IL). Recombinant human IL-26 was obtained from R&D Systems (Minneapolis, MN). IFN-α was from Peprotech (Rocky Hill, NJ).

Cell culture

The human colorectal cancer derived IEC lines SW480, SW620, Caco-2, HT-29, HCT116, T84 and DLD-1 and the hepatoma cell lines HepG2, Hep3B and Huh-7 were obtained from American Type Culture Collection (Rockville, MD). Primary hepatocytes from human donors were isolated using a modified two-step EGTA/collagenase perfusion procedure and cultured as previously described [11]. Cell were grown in Dulbecco's modified Eagle medium (DMEM) containing 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum (FCS) from PAA (Pasching, Austria) in a humidified 5% CO_2 atmosphere at 37°C. Huh-7 cells containing subgenomic HCV replicons I389luc-ubi-neo/NS3-3/5.1 (Huh 5-2) were described previously [12]. G418 (Geneticin; Life Technologies) was added at a final concentration of 250 μg/ml to this cell line.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as previously described [13]. Briefly, total RNA was isolated using Trizol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD). Two μg of DNase-treated total RNA were reverse transcribed using Qiagen Omniscript RT kit. To control for genomic contamination, an identical parallel PCR reaction was performed containing starting material that had not been reverse transcribed. The following conditions were used for semi-quantitative PCRs: 25-36 cycles (depending on the specific PCR) of denaturing at 95˚C for 45 sec, annealing at 61˚C for 45 sec, extension at 72˚C for 45 sec. The primers for the PCR reactions are shown in Table 1. The PCR products were subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. Densitometric analysis was performed using ImageJ 1.36 software from the NIH.

Quantitative PCR

Real-time PCR was performed with a Rotorgene RG-3000 cycler (Corbett Research, Sydney, Australia) using the Quantitect SYBR Green PCR Kit from Qiagen (Hilden, Germany) following the manufacturer's guidelines. Oligonucleotide primers were designed not to amplify genomic DNA, according to the published sequences (Table 1). Each mRNA expression was normalized to beta-actin expression in the respective cDNA preparation.

Signal transduction experiments, gel electrophoresis and immunoblotting

The signal transduction experiments were performed in overnight serum-starved IEC lines as indicated. Cells were stimulated with 100 ng/mL IL-26, unless indicated otherwise. This concentration was based on pilot experiments demonstrating a significantly higher effect of 100 ng/mL IL-26 for the activation of certain kinases than lower concentrations. Cells were solubilized in lysis buffer [6] and lysates were cleared by centrifugation at 10,000 g for 20

minutes. The protein concentration of each sample was quantified by the Bradford method. Immunoblotting was performed as previously described [14].

Enzyme-Linked Immunosorbent Assay (ELISA)

BD OptEIA Human IL-8 Elisa Kit II (BD Biosciences, Bedford, MA) was used for quantification of IL-8 release according to the manufacturer's instructions.

Cell proliferation assay

HT-29 cells were seeded onto 96 well plates at a density of 5000 cells/well and grown for one day. After starvation in serum-free medium overnight, the cells were incubated with or without IL-26 (10 and 100 ng/mL) in medium containing 0.1% FCS for 48 hours. Cell proliferation was determined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. Relative means ± SEM from 3 independent experiments are shown.

Apoptosis assays

Apoptosis assays were performed as described previously [15]. For induction of TRAIL-mediated apoptosis, TRAIL at a concentration of 100 ng/ml was used, and for CD95-mediated cell death ligand specific anti-APO-1 mAb at concentrations of 500 and 1000 ng/mL was used. Cells were incubated for 24 hours with the respective ligands and with or without IL-26 (10 or 100 ng/ml). Cells were harvested and were lysed in a hypotonic lysis buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 50 µg/ml of propidium iodide and were incubated at 4°C overnight. The nuclei were then analyzed for DNA content by flow cytometry.

Anti-HCV assays

Anti-HCV assays were performed by A. Kaul and R. Bartenschlager (University of Heidelberg, Germany) as described recently [12, 16, 17]. The effect of IL-26 on HCV replication was determined in HCV expressing replicon systems including Huh 5-2 and Huh-7-Renilla cells as previously described [12]. A full-length chimeric genome between HCV strains J6 and JFH1 was generated and modified as described recently [16] to obtain a bicistronic genome carrying a luciferase reporter gene (Jc1-Luc) which was used for infection of Huh-7-Lunet and naïve Huh-7 cells. The method of virus production was described recently [17]. In all experiments, IFN-α served as positive control.

Colonic biopsies

Biopsies were taken from patients with Crohn's disease and ulcerative colitis undergoing diagnostic colonoscopy. The study was approved by the Ethics Committee of the Medical Faculty of the University of Munich. All participating subjects gave written, informed consent prior to biopsy sampling. From each patient four biopsies were collected: two from macroscopically non-inflamed sites and two from macroscopically inflamed mucosa. IL-26 and IL-8 mRNA levels were measured in each individual biopsy. For quantification, the average IL-26 and IL-8 mRNA expression of the two non-inflamed biopsies was compared to the average expression in the two inflamed biopsies. For calculation of the correlation coefficient, for each patient IL-26 mRNA expression was correlated to IL-8 mRNA expression in the four individual biopsies.

Human liver biopsy tissue sampling

Human liver biopsy tissue was taken from patients undergoing diagnostic liver biopsy for medical reasons such as staging of chronic hepatitis C. All participating subjects gave written, informed consent prior to liver biopsy. The study was approved by the Ethics Committee of

the Medical Faculty of the University of Munich and adhered to the principles of the Declaration of Helsinki. A 3 mm long segment of the biopsy cylinder was immediately stored in Trizol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD). cDNA was isolated as described previously [13].

Statistical Analysis

Statistical analysis was performed using two-tailed Student's t-test. P levels < 0.05 were considered as significant.

Results

IEC and hepatic cells express the IL-26 receptor complex

To utilize a cell system to study this ligand-receptor system, we first analyzed if the IL-26 receptor complex consisting of IL-10R2 and IL-20R1 is expressed in IEC and in hepatic cell lines. RT-PCR for IL-10R2 and IL-20R1 mRNA was performed in several IEC cell lines (HT-29, SW480, SW620, HCT116, Caco-2, T84, DLD-1) and hepatoma derived cell lines (Huh-7, HepG2, Hep3B) as well as Huh-7 cells expressing subgenomic HCV replicons (Huh 5-2) and Huh-7 cells cured from HCV by IFN-α and IFN-γ treatment (Huh-7-Lunet) and primary human hepatic cells isolated from two different donor livers. RT-PCR analysis demonstrated IL-10R2 and IL-20R1 mRNA expression in all IEC (Fig. 1A) and hepatic cell lines and primary hepatocytes (Fig. 1B) tested, with the exception of lacking IL-20R1 mRNA expression in HepG2 cells.

IL-26 induces STAT1/3-, ERK-1/2-, SAPK/JNK-1/2 and Akt phosphorylation

Next, we studied if the IL-26 receptor complex expressed by IEC and hepatic cells is functional by analyzing major intracellular signaling pathways. Since previous studies reported activation of STAT signaling by IL-26 [2, 4], we performed Western Blot analysis and investigated the influence of IL-26 on phosphorylation levels of STAT1 and STAT3 in IEC. In comparison to basal STAT1 phosphorylation in unstimulated cells, a strong STAT1 tyrosine phosphorylation was observed upon IL-26 stimulation starting 5 minutes after cytokine stimulation (Fig. 2A). IL-26 also induced tyrosine phosphorylation of STAT3 which was strongest after 10 minutes of stimulation (Fig. 2B). Similarly, STAT1 and STAT3 activation was observed in Hep3B cells following IL-26 stimulation (data not shown). In contrast, in HepG2 cells, which lack the IL-20R1 subunit, no STAT activation was detected after IL-26 stimulation demonstrating that the IL-20R1 subunit is essential for IL-26 signaling

(data not shown). In addition, ERK-1/2 MAP kinases (Fig. 2C) and – to a lesser degree – SAPK/JNK kinases (Fig. 2D) were transiently activated by 100 ng/mL IL-26. Moreover, IL-26 induced phosphorylation of Akt (Fig. 2E). The maximum of ERK-, SAPK/JNK- and Akt activation was 15 minutes after cytokine stimulation, while the maximal STAT1/3 phosphorylation levels were already reached 10 minutes after IL-26 stimulation.

IL-26 decreases proliferation but does not influence apoptosis in IEC

Recently, we demonstrated that activation of ERK MAP kinases, Akt and STAT proteins following stimulation with chemokines or IL-22 mediates IEC proliferation [6, 18, 19], while STAT activation following IL-28A and IL-29 stimulation mediates antiproliferative signals in IEC [7]. Therefore, we analyzed the effect of IL-26 on IEC proliferation in the following experiments. Similar to IL-28A and IL-29 [7], IL-26 had a modest antiproliferative effect on the IEC. Compared to cytokine-free medium stimulated controls, IEC proliferation was diminished by 22% following stimulation with 10 and 100 ng/mL IL-26 (Fig. 3, p=0.05). However, IL-26 did not influence apoptosis induced by TRAIL or by anti-APO-1 treatment in HT-29 cells (data not shown).

IL-26 increases expression of proinflammatory cytokines

After demonstrating that the IL-26 receptor complex is functional in IEC, we next examined transcriptional targets of this cytokine. Previously we identified SOCS-3 as a STAT1/3-dependent gene [20]. Therefore, we analyzed SOCS-3 mRNA levels in IEC stimulated with IL-26. As shown in Fig. 4A and 4B, IL-26 treatment induced SOCS-3 mRNA expression already after one hour of stimulation. Next, we analyzed the role of IL-26 on the transcriptional regulation of the proinflammatory cytokines TNF-α, IL-8 and IL-6, three major inflammatory mediators in IECs, as downstream read-out of IL-26-mediated gene expression. The mRNA expression of all three cytokines was increased (TNF-α: 5.9-fold; IL-8: 2.8-fold, IL-6: 4.4-

fold) as measured by semi-quantitative PCR (Fig. 4A and 4B). Similarly, we measured a 3.7-fold increased IL-8 protein release by ELISA following IL-26 stimulation (Fig. 4C).

IL-26 mRNA expression is increased in the inflamed colonic mucosa of patients with inflammatory bowel disease (IBD)

Since we established that IL-26 up-regulates the expression of proinflammatory cytokines in IEC, we next analyzed by real-time PCR its expression in intestinal inflammation *in vivo.* This analysis included a total of 88 biopsy samples taken from 22 different IBD patients (Crohn's disease: n=12; ulcerative colitis: n=10). Ileal and colonic biopsies were taken from sites with endoscopically (macroscopic) inflamed colonic mucosa and were compared with those of endoscopically non-inflamed colonic mucosa taken from the same 22 patients. In patients with Crohn's disease, the colonic IL-8 mRNA expression, which was determined as a standard inflammatory marker, was 5.9-fold higher in the inflamed biopsy samples compared to uninflamed biopsies ($p<0.05$, Fig. 5A). Similarly, we measured increased IL-26 mRNA expression in inflamed colonic biopsies compared to non-inflamed colonic tissue (increase between 1.1 and 15.0-fold; Fig. 5A). Although there was a high correlation between IL-8 and IL-26 mRNA expression in patients with ulcerative colitis ($r=0.79$) and IL-8 expression was significantly increased up to 8.1-fold in the inflamed tissue ($p=0.02$; Fig. 5B), the difference of IL-26 mRNA expression in inflamed and non-inflamed biopsies did not reach statistical significance ($p=0.08$) in patients with ulcerative colitis.

IL-26 mRNA is expressed in human liver disease but is not up-regulated in viral hepatitis compared to non-viral hepatobiliary disease and has no antiviral properties against HCV

The IL-26 gene is located on chromosome 12q14 + 3 in a gene cluster where the IFN-γ gene is located. Moreover, its expression is up-regulated following HVS infection [1]. Based on these findings and given the antiviral properties, which we recently demonstrated for other IL-10

related cytokines [7, 9, 10], we next analyzed if IL-26 regulates the gene expression of antiviral proteins. While we demonstrated that IL-26 up-regulates the mRNA expression of IRF-7 6.4- and 5.0-fold, of IFN-α 4.4- and 3.4-fold, and of IFN-β 3.8- and 1.8-fold, in the IEC line HT-29 and the hepatic cell line Huh-7, respectively (Fig. 6), it had no direct effect on the gene transcription of the antiviral proteins 2',5'-OAS and MxA in these cells (data not shown). Consistently, in comparison to IFN-α, IL-26 had no significant effect on HCV RNA replication as measured in cell-based replication systems (A. Kaul and R. Bartenschlager, personal communication).

To analyze if IL-26 mRNA is regulated in viral infection *in vivo*, we collected liver biopsy tissue from patients with HCV infection and compared the IL-26 mRNA expression in these samples with biopsy tissue taken from patients with non-viral hepatobiliary disease. The biopsy tissues were taken from patients undergoing diagnostic liver biopsy for medical reasons such as staging of chronic hepatitis C; therefore and for ethical reasons, no normal controls were included in this analysis. This analysis included liver biopsy tissue from patients with hepatic steatosis (n=3), primary biliary cirrhosis (PBC; n=2), primary sclerosing cholangitis (PSC; n=2), autoimmune hepatitis (n=2), HCV (n=9) and patients with hepatitis of unknown origin (n=7). IL-26 mRNA expression was detectable in all liver biopsy samples (Fig. 7A). However, IL-26 mRNA expression levels measured by quantitative PCR in the HCV samples (mean expression relative to actin: 0.37) did not differ from that in biopsy samples taken from patients with non-viral hepatobiliary disease (steatosis hepatitis: 0.30, p=0.559; PBC: 0.18, p=0.085; PSC: 0.46, p=0.825; autoimmune hepatitis: 0.24, p=0.420; hepatitis of unknown origin: 0.65, p=0.236). Moreover, there was no significant correlation between IL-26 mRNA levels and the serum levels for C-reactive protein (CRP), Glutamate-Pyruvate Transaminase (GPT), Glutamate-Oxalate Transaminase (GOT), Gamma-glutamyl transferase (GGT), alkaline phosphatase and bilirubin (Fig. 7B and data not shown).

Discussion

IL-26 is a novel member of the IL-10 like cytokine family and shares with IL-10 the IL-10R2 subunit for signaling. While IL-10 has anti-inflammatory properties, we here demonstrate that IL-26 has proinflammatory functions in IEC and is up-regulated in IBD but is also expressed in hepatic inflammation. Recently, our group demonstrated also proinflammatory functions for IL-22, another IL-10 like cytokine in IEC [6]. Similar to IL-22 [6], IL-26 up-regulates the gene expression of proinflammatory cytokines in IEC. In addition, we demonstrate functional IL-26 receptor expression in IEC. IL-26 activates ERK and SAPK/JNK MAP kinases, Akt and STAT proteins in IEC. In contrast, cell lines such as HepG2, which do not express the IL-20R1 subunit, are not responsive to IL-26 treatment.

It has been shown that activated T cells are a major source of IL-26 production [1, 3]. Depending on the T cell source of cytokine production, cytokines have been differentiated in Th1 and Th2 cytokines. Previous studies demonstrated an induction of IL-26 production particularly in Th1 cells [3]. Crohn's disease is considered to represent a Th1 and Th17 mediated intestinal inflammation, while ulcerative colitis has, at least to some degree, features of a Th2 mediated colitis [21, 22]. Consistent with this observation, in our study the relative up-regulation of IL-26 expression (compared to uninflamed tissue) was more pronounced in inflamed lesions of patients with Crohn's disease compared to patients with ulcerative colitis (3.52- vs. 2.61-fold).

In addition, we demonstrate that IL-26 induced STAT1/3 activation results in increased SOCS-3 mRNA expression. This confirms our previous studies demonstrating that SOCS-3 is an immediate early transcriptional target of STAT1/3 [6, 20]. Interestingly, in Crohn's disease increased colonic STAT1 phosphorylation and SOCS-3 protein levels were found [23] supporting our findings of a proinflammatory role for IL-26 in Crohn's disease. Another study demonstrated in a murine colitis model that SOCS-3 plays a negative regulatory role in STAT3 activation and intestinal inflammation [24]. In addition, we recently demonstrated that

signaling of other IL-10 like cytokines such as IL-22 and the Lambda-interferons IL-28A and IL-29 is abrogated by increased expression of SOCS proteins in hepatic cell lines [8, 9, 25] and neuroendocrine cells [26] suggesting that signaling of IL-10-like cytokines is regulated by SOCS proteins. Interestingly, we found a similar mechanism for the signaling mediated by interferon-α [27] and interferon-β [28]. Moreover, similar to our studies with IL-28A and IL-29 [7, 26, 29, 30], two other IL-10 related cytokines, IL-26 mediates antiproliferative effects in IEC.

Initial studies demonstrated that IL-26 expression is up-regulated following HVS infection [1]. Based on this finding and given the antiviral properties, which we demonstrated for other IL-10 related cytokines [7], we analyzed the role of IL-26 in viral infection. However, in contrast to IFN-α, IL-26 had no therapeutically relevant effect on the mRNA expression of the antiviral proteins MxA and 2',5'-OAS and on HCV replication *in vitro*. Moreover, the IL-26 mRNA expression in liver biopsy samples taken from patients with HCV infection was not significantly different from that of patients with non-viral hepatobiliary disease which is different to the high expression observed for other IL-10 like cytokines such as IL-28A following viral infection [7, 10].

However, since IL-26 is over-expressed by HVS-transformed human T cells, IL-26 might play a role as an autocrine factor in lymphocyte transformation by HVS or in the natural γ-herpesvirus infection which involves a close interplay between different cell types such as epithelial cells and lymphocytes. This resembles the role of the γ1-herpesvirus Epstein-Barr virus (EBV) in B-cell transformation. Although EBV expresses an own viral IL-10 variant, which binds the normal IL-10 receptor [31], the EBV IL-10 homologue is not necessary for transformation [32]. EBV transformation induces B lymphocytes to produce human IL-10. However, latently infected EBV-transformed B cells were shown to specifically over-express a novel IL-12 p40-related cytokine, named EBV-induced gene 3 (EBI3). EBI3 forms part of the hetero-dimeric IL-27 molecule that induces the proliferation of naive CD4+T cells [33-35]. Interestingly, expression levels of the EBI3 have also been reported to be elevated in

ulcerative colitis as compared to Crohn's disease [36]. In analogy to IL-27, the role of IL-26 for lymphocyte transformation, which plays a crucial role in the pathogenesis of IBD, remains to be elucidated. Interestingly, an association between EBV infection and IBD has been proposed [37]. IL-26 producing T cells are likely to play an important role in intestinal inflammation since we demonstrate here that IL-26 up-regulates the gene transcription of several proinflammatory cytokines such as IL-6, IL-8 and TNF-α in IEC, while its own expression is up-regulated in IBD.

In summary, this is the first report on the role of IL-26 in human gastrointestinal and liver disease. Here, we demonstrate that IEC and hepatic cells express the functional IL-26 receptor complex. IL-26 activates STAT1/3, Akt, ERK- and SAPK/JNK MAP kinases and up-regulates the expression of proinflammatory cytokines and of SOCS3 mRNA. In addition, IL-26 mRNA expression is up-regulated in inflamed colonic lesions of IBD patients. IL-26 has no therapeutically relevant effect on the gene expression of the antiviral proteins 2',5'-OAS and MxA and HCV replication *in vitro*. In conclusion, our data indicate a role for this novel cytokine system in promoting proinflammatory gene transcription suggesting a role in intestinal inflammation.

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Licence statement

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Tables

Table 1

IL-10R2 forward:	5'-GGCTGAATTTGCAGATGAGCA-3'
IL-10R2 reverse:	5'-GAAGACCGAGGCCATGAGG-3'
IL-20R1 forward:	5'-TACACCCCTCAGCTCCAAGACT-3'
IL-20R1 reverse:	5'-GAAGGAATACACAGCCTGCCAG-3'
IL-26 forward:	5'-GGCAGAAATTGAGCCACTGT-3'
IL-26 reverse:	5'- TCCAGTTCACTGATGGCTTTG-3'
IL-6 forward:	5'-AAAGAGGCACTGGCAGAAAA -3'
IL-6 reverse:	5'-GAGGTGCCCATGCTACATTT -3'
IL-8 forward:	5'-ATGACTTCCAAGCTGGCCGTGGCT-3'
IL-8 reverse:	5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'
TNF-α forward:	5'-ATGAGCACTGAAAGCATG-3'
TNF-α reverse:	5'-TCACAGGGCAATGATCCC-3'
SOCS-3 forward:	5'-TTCTGATCCGCGACAGCTC-3'
SOCS-3 reverse:	5'-TGCAGAGAGAAGCTGCCCC-3'
MxA forward:	5'-AGATCCAGGACCAGCTGAGCCTGT-3'
MxA reverse:	5'-GTGGAACTCGTGTCGGAGTCTGGTA-3'
IRF-7 forward:	5'-TGGTCCTGGTGAAGCTGGAA-3'
IRF-7 reverse:	5'-GCTCCATAAGGAAGCACTCG-3'
IFN-α forward:	5'-TTTCTCCTGCCTGAAGGACAGAC-3'

IFN-α reverse:	5’-CTCTGACAACCTCCCAGGCACA-3’
IFN-β forward:	5’-TGCTCTGGCACAACAGGTAG-3’
IFN-β reverse:	5’-GCTGCAGCTGCTTAATCTCC-3’.
2’,5’-OAS forward:	5’-ATTGACAGTGCTGTTAACATCATC-3’
2’,5’-OAS reverse:	5’-AGATCAATGAGCCCTGCATAAACC-3’
GAPDH forward:	5’-CGGAGTCAACGGATTTGGTCGTAT-3’
GAPDH reverse:	5’-AGCCTTCTCCATGGTGGTGAAGAC-3’

Table 1. PCR primers used for RT-PCR analysis.

Figure legends

Figure 1. IL-20R1 and IL-10R2 are expressed in IEC and hepatic cells. mRNA expression of IL-20R1 and IL-10R2 as analyzed by RT-PCR analysis of mRNA derived from (A) intestinal epithelial cell lines and (B) hepatic cell lines and primary hepatocytes as indicated.

Figure 2. IL-26 induces STAT1/3-, ERK-1/2-, SAPK/JNK-1/2 and Akt phosphorylation. Following stimulation of HT-29 cells with IL-26 (100 ng/mL), increased phosphorylation was observed for STAT1 (panel A), STAT3 (panel B), ERK-1/2 (panel C), SAPK/JNK (panel D) and Akt (panel D). One representative experiment (n=3) is shown.

Figure 3. IL-26 decreases cell proliferation. Compared to medium stimulated controls, 10 and 100 ng/mL IL-26 decreased proliferation of HT-29 cells (* $p=0.05$). Experiment was performed in triplicates.

Figure 4. IL-26 increases mRNA expression of proinflammatory cytokines and SOCS-3. (A) Up-regulation of IL-6, IL-8, TNF-α and SOCS-3 mRNA expression after stimulation of HT-29 cells with 100 ng/mL IL-26. (B) The mRNA expression levels were normalized to GAPDH mRNA levels using NIH ImageJ software version 1.36. mRNA expression at start of the experiment (0 hours) was arbitrarily set to 1.0. (C) IL-8 protein levels increase up to 3.7-fold and reach a maximum after 12 hours of IL-26 stimulation. IL-8 protein concentration was determined by ELISA.

Figure 5. IL-26 mRNA expression is up-regulated in inflammatory bowel disease. Relative IL-26 mRNA expression in inflamed and uninflamed colonic and ileal biopsies

correlated with IL-8 mRNA expression in Crohn's disease (panel A) and ulcerative colitis (panel B).

Figure 6. Regulation of antiviral genes by IL-26 in IEC and hepatic cell lines. (A) IL-26 (100 ng/ml) up-regulates the mRNA expression of IRF-7, IFN-α and IFN-β in HT-29 cells up to 6.4-, 4.4- and 3.8-fold, respectively. (B) In Huh-7 cells, IL-26 had a similar effect with an up-regulation of IRF-7, IFN-α and IFN-β mRNA expression of up to 5.0-, 3.4- and 1.8-fold, respectively, as measured by quantitative PCR. In both cell lines (HT-29 and Huh-7), IL-26 had no direct effect on the mRNA expression of the antiviral genes MxA and 2',5'-OAS (data not shown).

Figure 7. IL-26 is expressed in human hepatic and hepatobiliary inflammation. (A) Quantitative PCR analysis in patients with steatosis hepatis (n=3), cholestatic liver disease (PBC, n=2; PSC, n=2), autoimmune hepatitis (AIH, n=2), viral hepatitis (HCV, n=9) and hepatitis of unknown origin (n=7) revealed detectable IL-26 mRNA levels in all patients without significant differences between the different disease categories. mRNA expression is depicted relative to actin expression. (B) IL-26 mRNA expression levels did not correlate with GOT and alkaline phosphatase (aP) serum levels (correlation IL-26/GOT: r = -0.35 correlation IL-26/aP: r = -0.11).

Figure 1

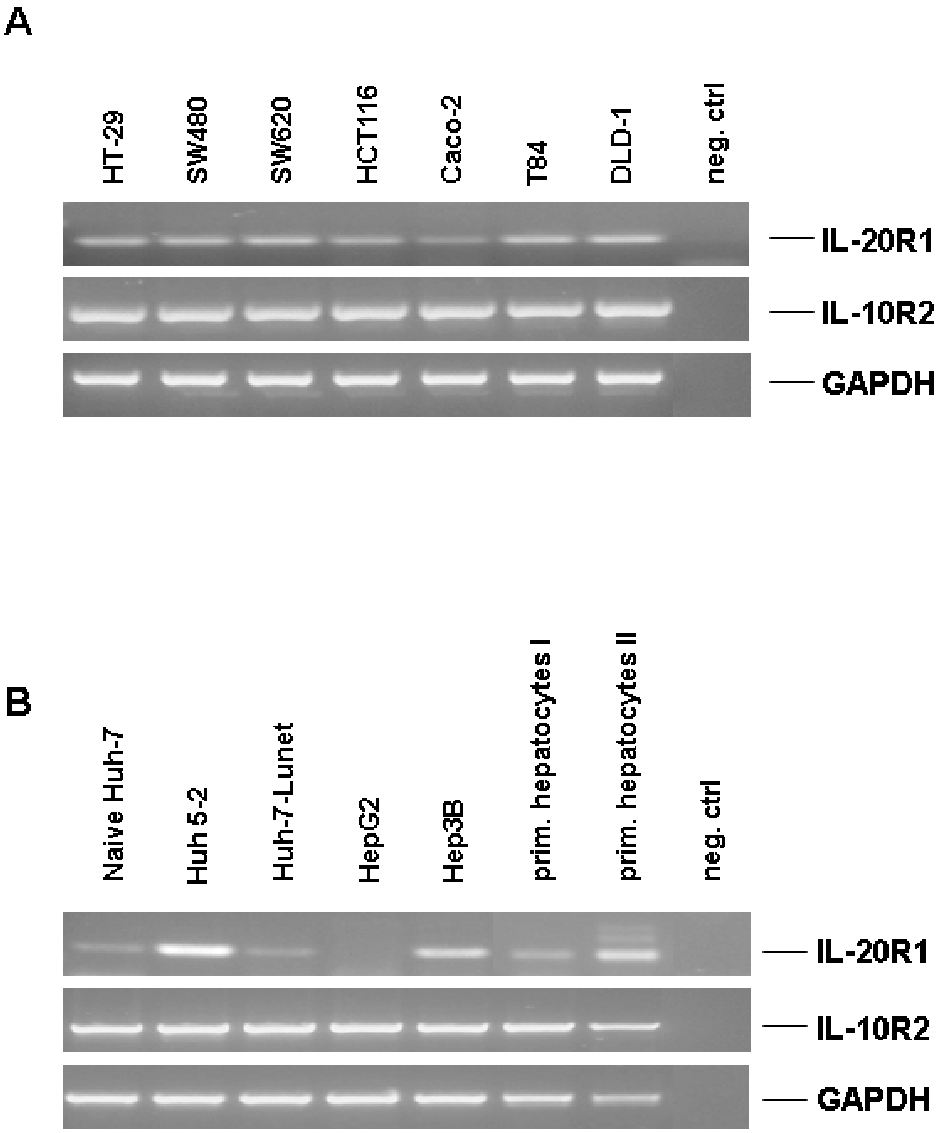

A
HT-29
SW480
SW620
HCT116
Caco-2
T84
DLD-1
neg. ctrl
IL-20R1
IL-10R2
GAPDH
B
Naive Huh-7
Huh 5-2
Huh-7-Lunet
HepG2
Hep3B
prim. hepatocytes I
prim. hepatocytes II
neg. ctrl
IL-20R1
IL-10R2
GAPDH

Figure 2

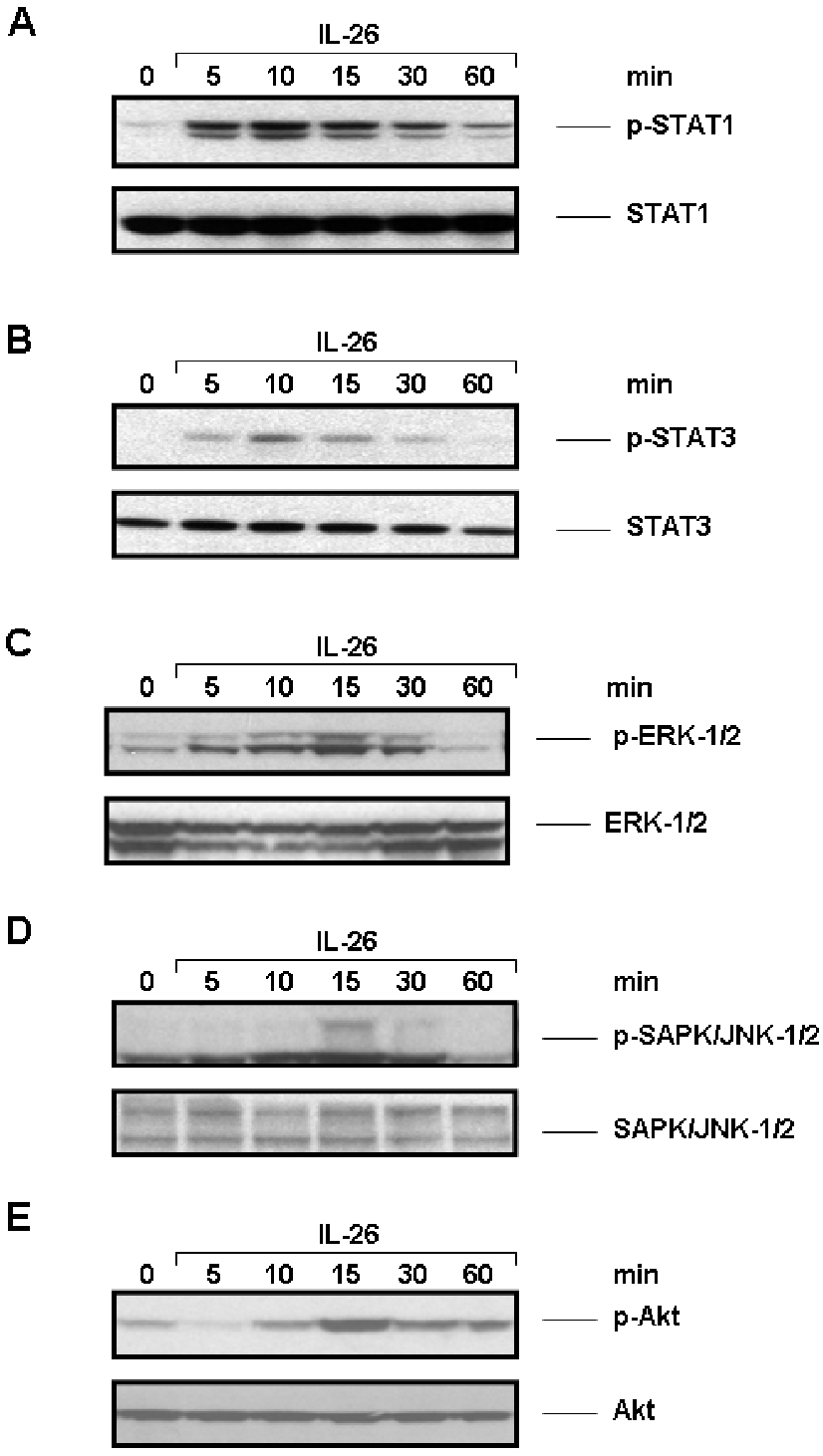

A
IL-26
0 5 10 15 30 60
min
p-STAT1
STAT1
B
IL-26
0 5 10 15 30 60
min
p-STAT3
STAT3
C
IL-26
0 5 10 15 30 60
min
p-ERK-1/2
ERK-1/2
D
IL-26
0 5 10 15 30 60
min
p-SAPK/JNK-1/2
SAPK/JNK-1/2
E
IL-26
0 5 10 15 30 60
min
p-Akt
Akt

Figure 3

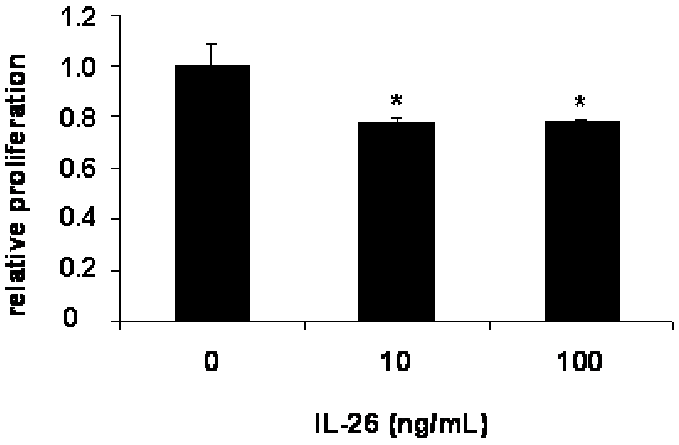


Figure 4

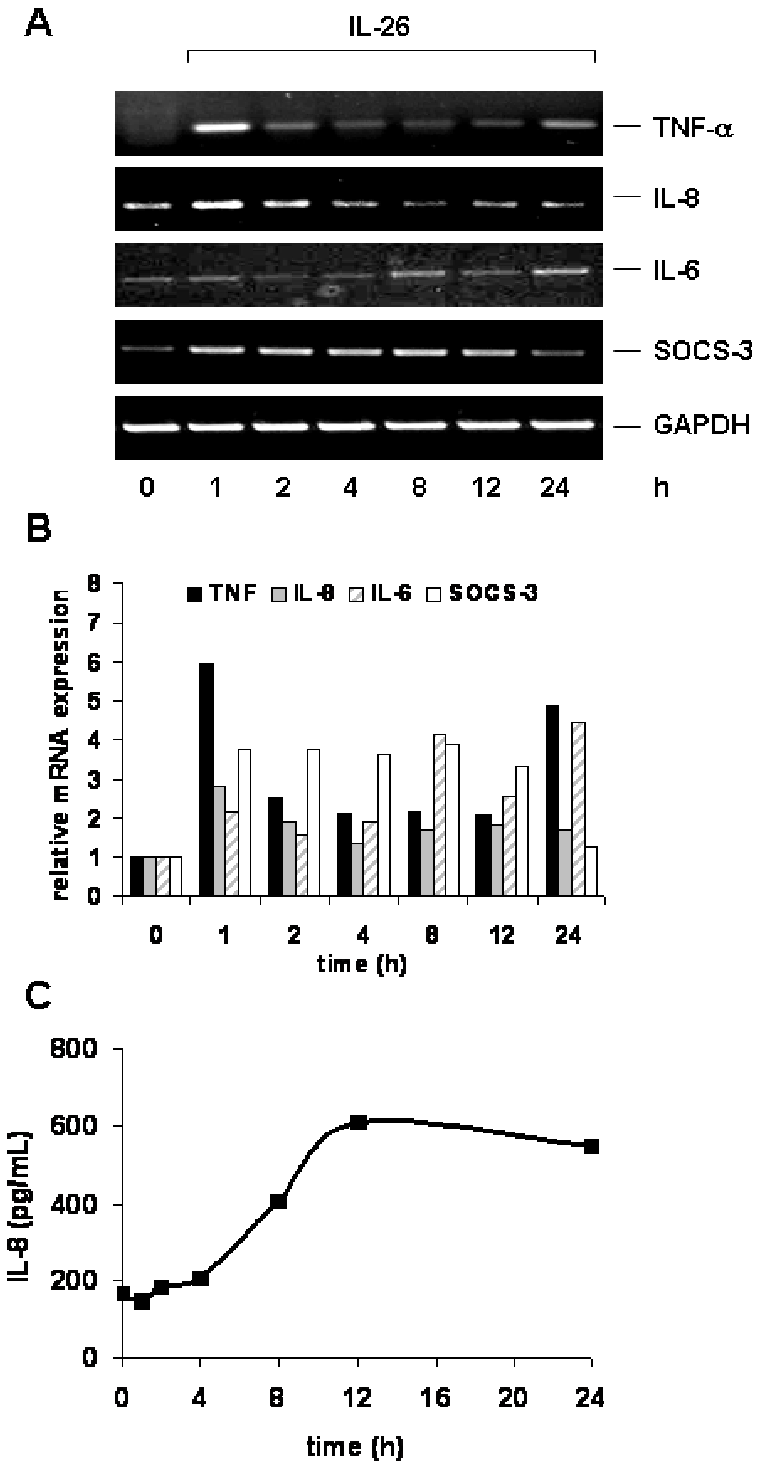
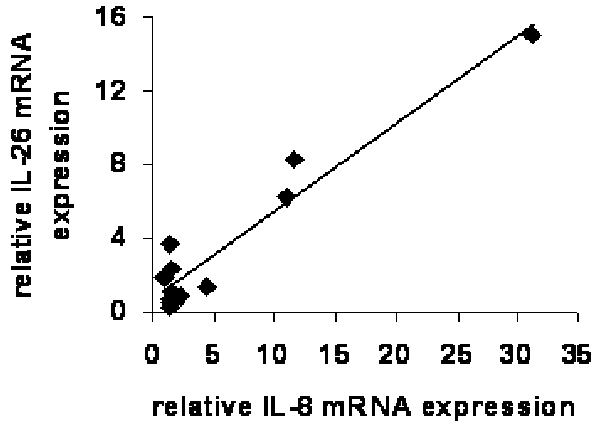

A
IL-26
TNF-α
IL-8
IL-6
SOCS-3
GAPDH
0 1 2 4 8 12 24 h
B
TNF IL-8 IL-6 SOCS-3
relative mRNA expression
0 1 2 3 4 5 6 7 8
0 1 2 4 8 12 24
time (h)
C
IL-8 (pg/mL)
0 200 400 600 800
0 4 8 12 16 20 24
time (h)

Figure 5

A


relative IL-26 mRNA expression
16
12
8
4
0
0
5
10
15
20
25
30
35
relative IL-8 mRNA expression

B

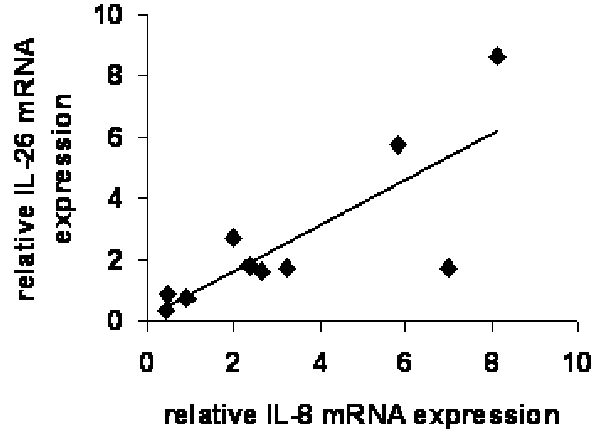

relative IL-26 mRNA expression
10
8
6
4
2
0
0
2
4
6
8
10
relative IL-8 mRNA expression

Figure 6

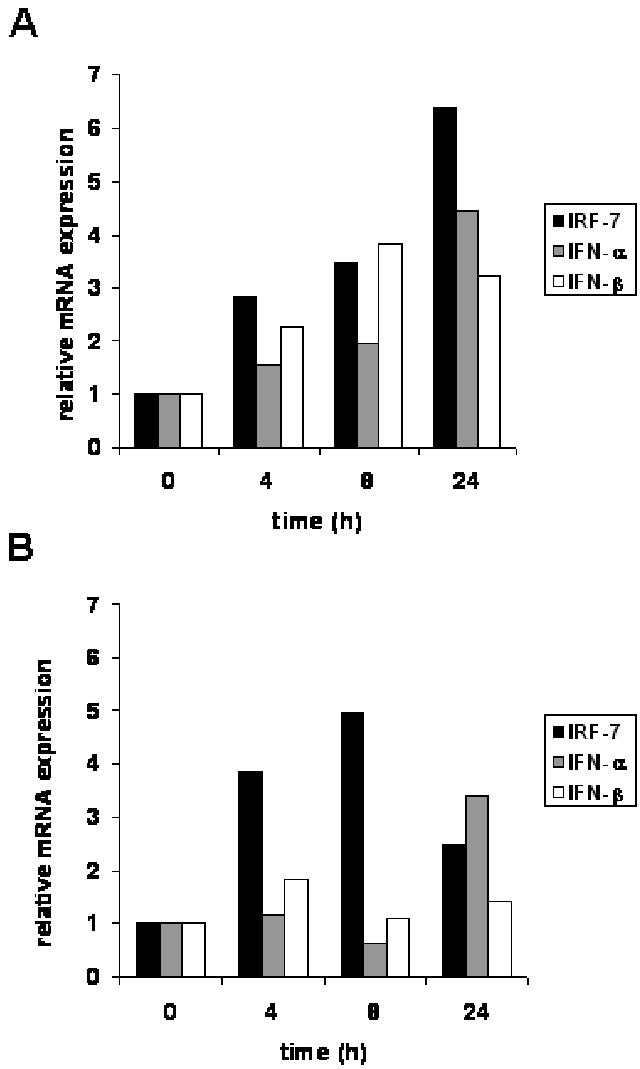
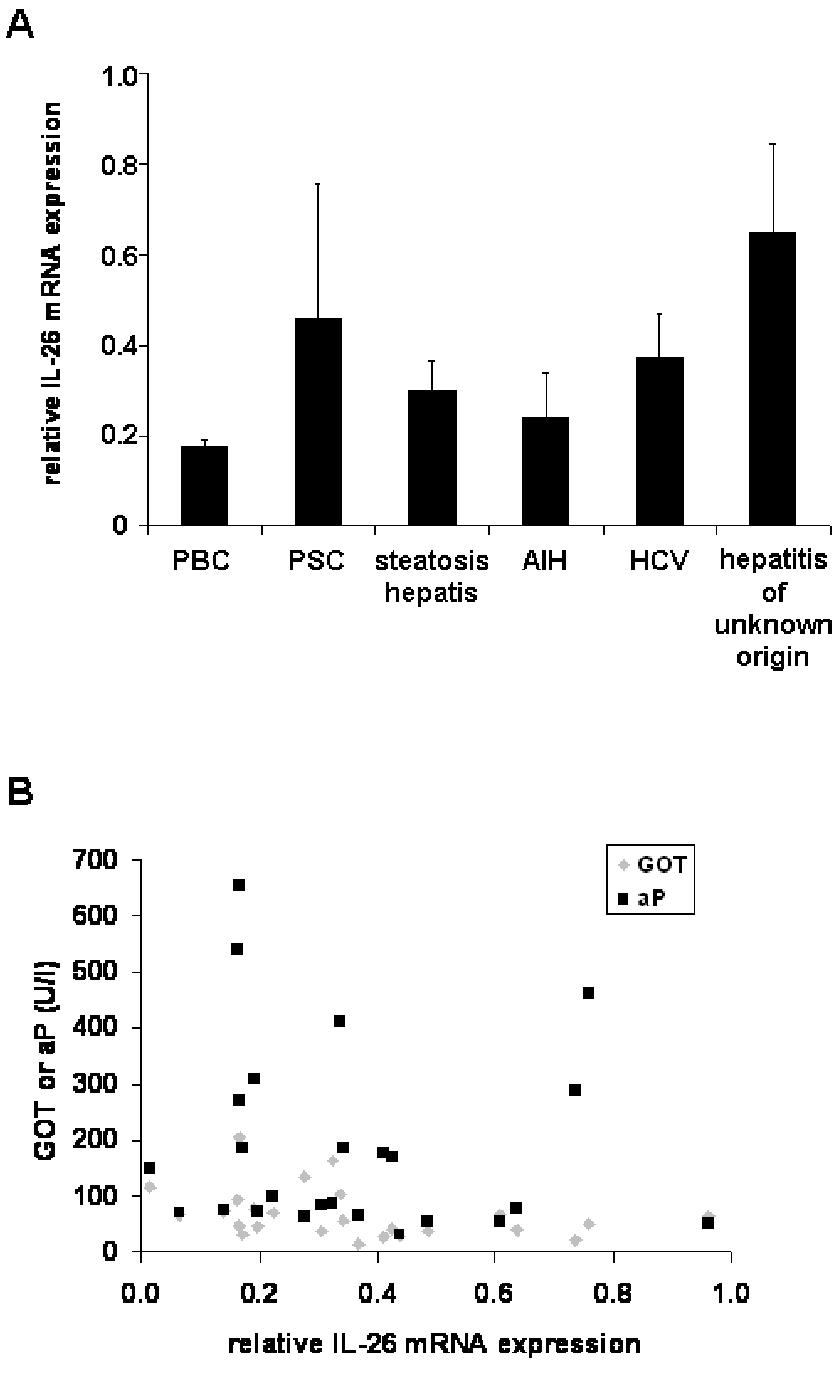

A
relative mRNA expression
0
1
2
3
4
5
6
7
0
4
8
24
time (h)
IRF-7
IFN-α
IFN-β
B
relative mRNA expression
0
1
2
3
4
5
6
7
0
4
8
24
time (h)
IRF-7
IFN-α
IFN-β

Figure 7


A
relative IL-26 mRNA expression
1.0
0.8
0.6
0.4
0.2
0
PBC
PSC
steatosis hepatis
AIH
HCV
hepatitis of unknown origin
B
GOT
aP
GOT or aP (U/l)
700
600
500
400
300
200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
relative IL-26 mRNA expression