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Therapeutische Immunmodulation der Interaktion dendritischer Zellen und CD4+ T Zellen bei Ischämie-Reperfusion der Leber

Dissertation

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München den 09.11.2018

Dominik Funken

Inhalt

Abkürzungsverzeichnis

I/R	ischemia/reperfusion
DC	dendritic cell
CD	cluster of differentiation
DAMP	danger associated molecular pattern
MHC	main histocompatibility complex
RIP	receptor-interacting protein
MLKL	mixed lineage kinase domain-like pseudokinase
IL	interleukin
TNFα	tumor necrosis factor α
NF-κβ	nuclear factor κβ

Originalarbeiten

Beide Publikationen widmen sich detailliert der komplexen Thematik der hepatischen Ischämie-Reperfusion (I/R).

Unter dem Titel „In situ targeting of dendritic cells sets tolerogenic environment and ameliorates CD4+ T cell response in the postischemic liver “ wurde in der Zeitschrift „The FASEB Journal by the Federation of American Societies for Experimental Biology“ über die Interaktion CD4+ T Lymphozyten mit hepatischen dendritischen Zellen und die protektive Modulation dieser Interaktion in der postischämischen Leber berichtet.
Alle Artikel unterliegen einem Gutachterprozess, der Impact Factor 2017 lag bei 5,5 Punkten.

Unter dem Titel „RIP-1 dependent programmed necrosis is negatively regulated by caspases during hepatic ischemia-reperfusion“ wurde in der Zeitschrift „SHOCK: Injury, Inflammation, and Sepsis: Laboratory and Clinical Approaches“ über die Rolle der Nekroptose in der postischämischen Leber berichtet.
Alle Artikel unterliegen einem Gutachterprozess, der Impact Factor 2015/2016 lag bei 3,05 Punkten.

Einleitung

Der Ischämie-Reperfusions-Schaden stellt nach wie vor eines der zentralen Probleme der Leberchirurgie dar. Das Krankheitsbild entsteht durch die Wiederherstellung der Durchblutung der Leber nach einer Ischämie-Periode. Auf Grund dieses scheinbaren Widerspruchs wird es auch als Reperfusionsparadox bezeichnet.

Pathophysiologisch wird eine komplexe Kaskade aus Ischämie bedingtem Zelltod, Freisetzung schädigender Sauerstoffradikale und der Ausbildung einer sterilen Entzündungsreaktion in Gang gesetzt. Die Schädigung des Gefäßendothels begünstigt die Adhäsion und Aktivierung verschiedener Immunzellen und führt zur Ausbildung eines Ödems im Gewebe. Durch den steigenden Gewebedruck und der damit verbundenen Ausbreitung der Ischämie unterhält die Reaktion sich selbst und verursacht zusätzliche Gewebeschäden.

Frühere Untersuchungen konnten zeigen, dass obwohl sowohl CD4 als auch CD8 positive T Lymphozyten in der postischämischen Leber akkumulieren, nur die Blockade CD4 positiver T Zellen einen protektiven Effekt auf die Leber hat[1, 2].

Die Aktivierung von T Zellen durch professionelle antigenpräsentierende Zellen bildet die Grundlage der zellulären Immunantwort, die sich gegen körperfremde Pathogene richtet. Neben Pathogenen können jedoch auch körpereigene Antigene präsentiert werden. Im Rahmen ausgeprägter Nekrosen oder einem stark oxidierenden Milieu, wie einem (post)ischämischen Organ, kommt es zur ausgeprägten Freisetzung dieser vormals intrazellulär gelegenen Zellbestandteile. Als sogenannte danger associated molecular patterns (DAMPs) stellen sie für das Immunsystem ein starkes Warn- und Aktivierungssignal dar. Die phagozytierenden Zellen des angeborenen Immunsystems nehmen diese auf und initiieren die zelluläre Immunantwort. Die Folge ist eine sterile Entzündungsreaktion des Gewebes[3].

Die wichtigsten Vertreter der professionellen antigenpräsentierenden Zellen in der Leber stellen die Kupfferschen Sternzellen und die hepatischen dendritischen Zellen (DCs) dar[4]. Da gezeigt werden konnte, dass Kupffer-Zellen mit CD4+ T Zellen interagieren, diese jedoch nicht aktivieren, liegt der Fokus dieser Arbeit auf der Interaktion der CD4+ T Zellen mit den dendritischen Zellen der Leber.

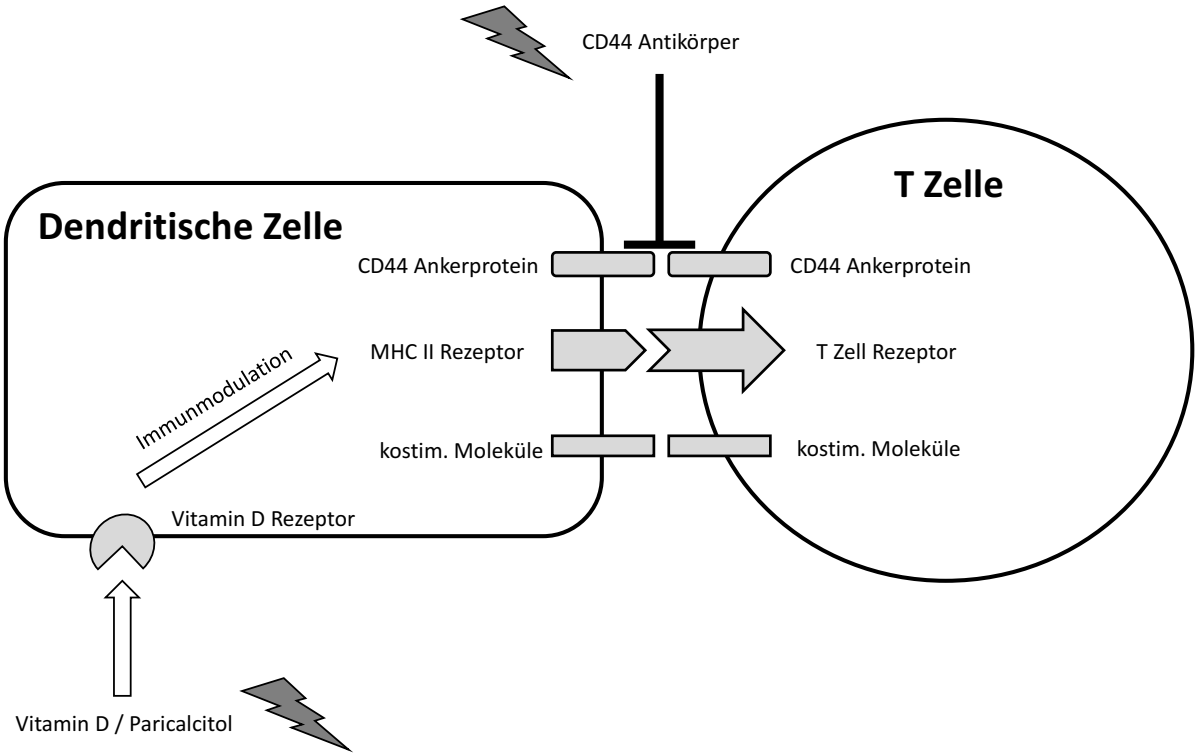

Abbildung 1: Zelluläre Interaktion und therapeutische Interventionen: Schematische Darstellung der Interaktion zwischen einer dendritischen Zelle und einer T Zelle. Mit Blitzen markiert sind die beiden Ansatzpunkte der untersuchten blockierenden (CD44) und modulierenden (Paricalcitol) therapeutischen Interventionen.

Während ausgeprägter Sauerstoffmangel zu einer Ischämie bedingten Nekrose des Gewebes führt, regen starke Zellschädigungen oder zytotoxische Immunzellen den kontrollierten Zelltod, die Apoptose, an. Dieser programmierte Zelltod wird durch die Aktivierung von sogenannten Todes-Rezeptoren initiiert und durch spezielle Enzyme, die Caspasen, vermittelt[5, 6]. Die Hauptaktivatoren der Todes-Rezeptoren stellen Zellbotenstoffe der Tumornekrosefaktor-Familie dar, die unter anderem von aktivierten T-Lymphozyten und dendritischen Zellen freigesetzt werden[6]. Bei den klassischen zytotoxischen T-Zellen handelt es sich um CD8 positive Zellen. Diese zellvermittelte Immunantwort wird durch CD4 positive T-Helferzellen vom Typ 1 unterhalten, die ihrerseits von professionellen antigenpräsentierenden Zellen aktiviert werden.

Neben der Apoptose kann die Aktivierung der Todes-Rezeptoren auch zur Nekroptose führen[3]. Bei dieser Sonderform des Zelltodes handelt es sich um eine rezeptorvermittelte Nekrose, die anders als die Apoptose zum Selbstverdau der Zelle führt. Dabei kommt es wie bei der unkontrollierten Form des Zelltods, der Nekrose, zur Freisetzung von DAMPs. Vermittelt wird diese Form des programmierten Zelluntergangs durch Proteine der RIP-Kinasen Familie[7, 8].

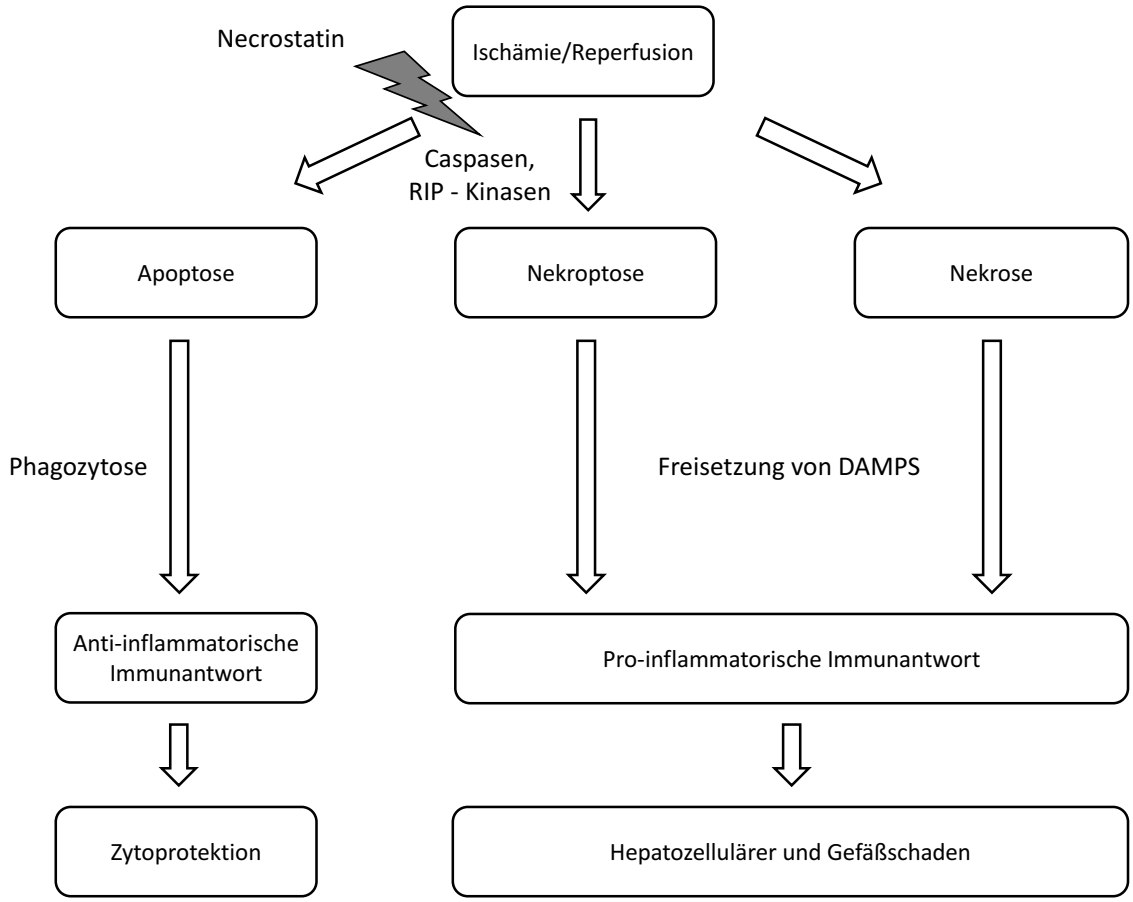

Abbildung 2: Zelltodarten und Intervention: Mögliche Arten des Zelltodes im Rahmen des Ischämie-Reperfusionsschadens. Der Blitz markiert den Ansatzpunkt der untersuchten therapeutischen Intervention mit Hilfe des Nekroptose-Inhibitors Necrostatin-1. DAMPS = danger associated molecular patterns.

Zielsetzung

Das Ziel der vorliegenden Arbeit war es, die Rolle der hepatischen dendritischen Zellen bei der Aktivierung der CD4+ T Lymphozyten während des Ischämie-Reperfusionsschadens der Leber zu untersuchen.
Wir untersuchten die Hypothesen, dass i) die dendritischen Zellen der Leber mit CD4+ T Lymphozyten interagieren und diese aktivieren; ii) die Modulation dieser Interaktion bzw. die Generierung tolerogener dendritischer Zellen die Rekrutierung von T Lymphozyten in die postischämische Leber und konsekutiv den Ischämie-Reperfusionsschaden verringert und iii) die Blockade der besagten Interaktion zu einer weiteren Reduktion des Schadens führt.

Im den zweiten Studie untersuchten wir die Rolle der programmierten Nekroptose während des hepatischen Ischämie-Reperfusionsschadens. Wir testeten die Hypothese, dass die Blockade dieser Art des Zelltods die entstehende Nekrose und die begleitende Inflammationsreaktion in der postischämischen Leber verringert.

Material und Methoden

Beide zentralen Fragestellungen der Arbeit wurden im Tiermodell an der Maus untersucht. In der Leber wurde mit einem Mikroclip eine reversible warme Ischämie für 90 Minuten erzeugt, gefolgt von einer Reperfusionszeit von 30 bis 240 Minuten. Zentrale arterielle und venöse Katheter gewährleisteten eine kontinuierliche invasive Blutdruckmessung, eine intravenöse Volumentherapie und die Applikation von Medikamenten, Farbstoffen und *ex vivo* gefärbten Immunzellen[9].

Die Akkumulation und Interaktion von Leukozyten allgemein, sowie spezifisch von CD4+ T Lymphozyten und dendritischen Zellen der Leber wurde mittels intravitaler Fluoreszenz- und Multiphotonen-Mikroskopie dargestellt. Weiterführende Fragestellungen wurden mittels anschließender konfokaler Mikroskopie der Leber *ex vivo* adressiert.

Als weitere Schadensparameter wurden das sinusoidale Perfusionsdefizit mittels intravitaler Fluoreszenzmikroskopie und die hepatischen Transaminasen aus dem Serum der Tiere bestimmt. Die Lebern der Versuchstiere wurden histopathologisch aufgearbeitet, und Ausmaß, sowie Schweregrad des hepatozellulären Schadens untersucht.

Die genaue Charakterisierung der beteiligten Immunzellen erfolgte mittels Durchflusszytometrie. Dabei wurden hepatische dendritische Zellen und T Lymphozyten aus den Lebern nicht mikroskopierter Versuchstiere isoliert und untersucht.

Die Interaktion zwischen CD4+ T Lymphozyten und hepatischen dendritischen Zellen wurde durch die Blockade des Oberflächenmoleküls CD44 mit einem monoklonalen Antikörper unterbunden[10]. Um tolerogene dendritische Zellen *in situ* zu generieren und die Interaktion *in vivo* therapeutisch zu beeinflussen, wurde das Vitamin D Analogon Paricalcitol verwendet[11].

Der Nachweis von Proteinen der Caspase- und RIP-Kinase-Familie, als aktive Nekroptose-Mediatoren, erfolgte mittels Western Blot. Außerdem wurde die Expression einer weiteren Proteinkinase, der MLKL, als Substrat des RIP-Kinase Nekroptosewegs ebenfalls per Western Blot analysiert.

Zur Inhibierung der RIP1-Kinase wurde Necrostatin-1 verwendet[12, 13].

Ergebnisse

Die vorliegenden Studien demonstrieren, dass es in der postischämischen Leber zu einem massiven sinusoidalen Perfusionsausfall und einer korrelierenden Erhöhung der Transaminasen im Serum kommt. In der Histologie zeigten sich große, konfluierende Areale geschädigter Zellen und teils flächenhafte Nekrosen der Hepatozyten.

Die Rekrutierung von Leukozyten in das geschädigte Gewebe zeigte einen signifikanten Anstieg im Vergleich zu scheinoperierten Tieren. Besonders ausgeprägt war dieser Effekt bei CD4+ T Lymphozyten zu beobachten.

In früheren Studien konnte gezeigt werden, dass diese T Zellen in der postischämischen Leber akkumulieren und in das Parenchym auswandern. T Zellen, die am sinusoidalen Endothel adherieren, interagieren außerdem mit Thrombozyten, anderen Leukozyten und hepatischen Kupffer-Zellen. Nach der Extravasation in das Leberparenchym interagieren sie zudem mit den hepatischen Sternzellen. CD4+ T Zellen haben so maßgeblichen Einfluss auf das Ausmaß der Entzündungsreaktion und des hepatozellulären Schadens.

Obwohl die einzelnen Interaktionen gezielt therapeutisch moduliert oder blockiert werden konnten, blieb der Mechanismus der T Zell-Aktivierung bislang unklar und damit nicht therapeutisch adressierbar.

Im Hauptteil der vorliegenden Arbeit wurde erstmals *in vivo* gezeigt, dass die CD4+ Lymphozyten direkt in der postischämischen Leber mit hepatischen dendritischen Zellen interagieren. Nach der vollständigen Blockade dieser Interaktion akkumulierten signifikant weniger T Lymphozyten in der Leber. Das Ausmaß des hepatozellulären Schadens lag dennoch auf dem Niveau der unbehandelten Kontrollgruppe, die sinusoidale Perfusion verschlechterte sich sogar.

In der zweiten Interventionsgruppe wurde die Interaktion der dendritischen Zellen mit den T Zellen nicht vollständig blockiert, sondern protektiv moduliert. Dazu wurde diese Versuchsgruppe mit einem synthetischen Vitamin D Analogon behandelt. Die immunmodulatorische Wirkung von Vitamin D ist gut bekannt und in zahlreichen Arbeiten beschrieben. Die therapeutische Anwendbarkeit von reinem Vitamin D wird jedoch durch die spezifischen Nebenwirkungen, insbesondere die Folgen der entstehenden Hypercalciämie limitiert. Synthetisches Vitamin D hat eine deutliche höhere Potenz als natürliches Vitamin D - im Fall des in der Studie verwendeten Paricalcitol um den Faktor 25. Dadurch war es möglich, bei deutlich geringerer Dosierung eine wesentlich höhere immunmodulatorische Wirkung zu erzielen.

Dieser Effekt wurde genutzt, um direkt *in situ*, in der Leber, die Ausreifung hepatischer dendritischer Zellen in Folge des Ischämie-bedingten Stresses zu verhindern. Die dendritischen Zellen der Leber befinden sich natürlicherweise in einem sehr unreifen Zustand. Ihre Fähigkeit Antigene zu präsentieren ist dadurch nur schwach ausgeprägt. Kostimulatorische Moleküle wie CD80 und CD86, die zur Aktivierung von T Zellen benötigt werden, sind nur in geringer Zahl auf der Zelloberfläche vorhanden. Unreife dendritische Zellen fördern die Selbst-Toleranz, während reife und aktivierte dendritische Zellen in der Lage sind, starke Immunantworten auszulösen. Als Reaktion auf eine Ischämie oder andere

Gefahrensignale reifen dendritische Zellen aus und entwickeln ihre maximale Kapazität zur Präsentation von prozessierten Antigenen und zur Aktivierung von T Lymphozyten. Die Aktivierung des Vitamin D Rezeptors hält die dendritischen Zellen in einem unreifen Zustand und generiert somit potentiell tolerogene Zellen.
In der Durchflusszytometrie bestätigte sich die verminderte Expression von kostimulatorischen Molekülen auf den leberständigen dendritischen Zellen nach der Behandlung mit Paricalcitol.
Weiterhin konnten wir zeigen, dass es sich bei den interagierenden Zellen um konventionelle dendritische Zellen aus dem Knochenmark handelt. Die sich aus eingewanderten Monozyten des Blutes entwickelnden inflammatorischen dendritischen Zellen wurden im aseptischen Krankheitsbild des Ischämie-Reperfusionsschadens nicht gefunden.
Die Reduktion der Anzahl der in das Leberparenchym ausgewanderten T Lymphozyten betraf sowohl CD4+ als auch CD8+ T Zellen. Auffallend war weiterhin der geringere Aktivierungszustand der CD4+ T Lymphozyten und das deutlich seltenere Vorkommen des Th1 Subtyps. Dieser Typ von T-Helferzellen produziert typischerweise Interferon γ, IL-2 und TNFα und trägt damit sowohl zur Apoptose von geschädigten Zellen, als auch zur Aktivierung von zytotoxischen T Zellen bei.
Passend zur verminderten Aktivierung der beteiligten Immunzellen konnte nach der Vorbehandlung mit synthetischem Vitamin D ein signifikanter hepatoprotektiver Effekt beobachtet werden. Alle Schadensparameter zeigten sich deutlich reduziert, die sinusoidale Perfusion war im Vergleich zu den scheinoperierten Tieren nicht eingeschränkt. In der postischämischen Leber akkumulierten deutlich weniger T Lymphozyten, die weiterhin eine verringerte Produktion von TNFα zeigten. In den dendritischen Zellen der Leber konnte deutlich mehr tolerogenes IL-10 nachgewiesen werden, als in den unbehandelten Tieren der Kontrollgruppe.
Nach der vollständigen Blockade der Interaktion von T Zellen und dendritischen Zellen konnte eine weitere Reduktion der Anzahl im Lebergewebe akkumulierende T Lymphozyten beobachtet werden. Gleichzeitig kam es jedoch zu einem deutlich ausgeprägteren hepatozellulären Schaden.
Wurde sowohl synthetisches Vitamin D als auch der blockierende monoklonale Antikörper gegen CD44 appliziert, hob die Blockade der zellulären Interaktion den protektiven Effekt des Paricalcitol vollständig auf. Diese Beobachtungen unterstreichen den protektiven Effekt der tolerogenen dendritischen Zellen auf die T Zell Antwort.
Die Ergebnisse dieser Studie sind im `FASEB Journal` veröffentlicht[14].

In der zweiten Studie untersuchten wir die Rolle der programmierten Nekroptose während des hepatischen Ischämie-Reperfusionsschadens. Dazu wurde der Inhibitor Necrostatin-1 verwendet. Die Behandlung mit Necrostatin-1 zur Verhinderung des Nekroptose-Zelltods beeinflusste den Organschaden und die Rekrutierung von Immunzellen dabei nicht signifikant. Die Western Blot Untersuchungen zeigten, dass RIP-1 als Nekroptose-Mediator in der Leber scheinoperierter Tiere vorhanden ist. In den postischämischen Lebern der Tiere der Kontroll- und Interventionsgruppen war

RIP-1 jedoch nicht mehr nachweisbar. Das Substrat des RIP-Signalweges lag nach I/R unverändert in den Hepatozyten vor und zeigte keine verminderte Expression. Das Fehlen von RIP-1 wurde somit nicht als Folge eines erhöhten Verbrauchs gewertet. Stattdessen konnte in den postischämischen Hepatozyten vermehrt Caspase-3 nachgewiesen werden. Caspase-3 ist in der Lage RIP-1 zu schneiden und damit unwirksam zu machen. Es kann somit davon ausgegangen werden, dass als Folge der Zellischämie die Aktivität von Caspase-3 als Teil des Apoptose Signalweges zunimmt. Dadurch wird RIP-1 abgebaut und die Nekroptose als programmierte Nekrose herunter reguliert. Als bevorzugter Zelltod geschädigter Hepatozyten, die nicht auf Grund zu großer Zellschäden direkt nekrotisch werden, muss somit die Apoptose gelten.
Die Applikation von Necrostatin-1 beeinflusste die hepatischen Schadensparameter und die sterile Entzündungsreaktion in der postischämischen Leber nicht.
Über diesen Teil der Dissertation wurde in der Zeitschrift `Shock` berichtet[15].

Eigenanteil an den vorgelegten Arbeiten

Über den Hauptteil der Dissertation wurde unter dem Titel „In situ targeting of dendritic cells sets tolerogenic environment and ameliorates CD4+ T cell response in the postischemic liver “ berichtet.
Bei dieser Studie hat der Doktorand den Großteil der Experimente und der Datenauswertung eigenständig durchgeführt. Er hat das Konzept der Studie mitentwickelt und die Publikation in der Zeitschrift ‚The FASEB Journal’ unter der Anleitung von Prof. Dr. Andrej Khandoga verfasst.

In der zweiten Studie mit dem Titel „RIP-1 dependent programmed necrosis is negatively regulated by caspases during hepatic ischemia-reperfusion“ hat der Doktorand die Experimente gemeinsam mit dem promovierten Mitbetreuer Dr. Dirk Rosentreter durchgeführt und ausgewertet. An der Konzeption der Studie und der Publikation in der Zeitschrift ‚SHOCK` war er mit beteiligt.

Zusammenfassung

Der Ischämie-Reperfusionsschaden stellt nach wie vor die häufigste Ursache für eine Leberdysfunktion oder ein Organversagen nach großen Leberoperationen oder der Organtransplantation dar. Das Krankheitsbild resultiert aus einer komplexen Kaskade unterschiedlicher pathophysiologischer Prozesse auf subzellulärer, zellulärer und Organebene. Strategien um die negativen Effekte der primären Ischämie und der anschließenden Reperfusion zu mindern oder therapeutisch zu beeinflussen, sind daher Gegenstand zahlreicher experimenteller und klinischer Studien.
Ein potentielles, jedoch ambivalentes therapeutisches Ziel stellen die CD4+ T Lymphozyten dar, die in großer Zahl in der postischämischen Leber akkumulieren. Gemäß ihrer unterschiedlichen pro- und anti-inflammatorischen Wirkung werden über die verschiedenen Untergruppen von CD4+ T-Helferzellen protektive beziehungsweise aggravierende Effekte im Ischämie-Reperfusionsschaden der Leber berichtet.
Als effektivste protektive Strategie hat sich dabei die gezielte Adressierung des Th1 Subtyps erwiesen. Th1 CD4 Lymphozyten wirken über die Sekretion von insbesondere Interferon γ und TNFα pro-apoptotisch und pro-inflammatorisch.
In der ersten Arbeit wird gezeigt, dass die gezielte Erhaltung der tolerogenen Eigenschaften ungereifter hepatischer dendritischer Zellen die Anzahl der in das postischämische Leberparenchym auswandernden CD4+ deutlich verringert. Der Th1 Subtyp ist von diesem Effekt am deutlichsten betroffen und erklärt den signifikanten protektiven Effekt der Intervention.
Weiterhin wird in der vorliegenden Arbeit erstmals in vivo gezeigt, dass CD4+ T Lymphozyten direkt in der Leber mit hepatischen dendritischen Zellen interagieren. Die Blockade dieser Interaktion hebt den protektiven Effekt der Immunmodulation durch synthetisches Vitamin D vollständig auf.
Dieser Effekt ist insofern überraschend, als dass in früheren Arbeiten gezeigt werden konnte, dass die Blockade des MHCII Moleküls als antigenpräsentierendes Molekül und direktes Gegenstück zum T Zell-Rezeptor der T Lymphozyten in der jeweils untersuchten Phase der frühen Reperfusion bis vier Stunden keinen signifikanten protektiven Effekt hat. Eine potentielle Erklärung für diese Beobachtung liegt in der Kinetik der Aktivierung dendritischer Zellen. Die Hochregulation der Expression und Produktion von Molekülen wie Zytokinen und Oberflächenmarkern benötigt im Mittel drei bis vier Stunden bevor ein signifikanter Anstieg zu verzeichnen ist. Dendritische Zellen sind eine der wenigen Zellarten, die sowohl das tolerogene Zytokin IL-10, als auch den nukleären Transkriptionsfaktor NF-$\kappa\beta$ konstitutiv exprimieren. Weiterführende Untersuchungen deuten darauf hin, dass die dendritischen Zellen über diese Eigenschaft bereits in der frühen Reperfusionsphase die Orchestrierung der körpereigenen Immunantwort - auch im nicht-allogenen Setting der Ischämie-Reperfusion - übernehmen. Sie stellen damit ein ausgesprochen vielversprechendes Ziel dar.
In der vorliegenden Arbeit demonstriert der Autor, wie diese Strategie erfolgreich angewandt und durch Immunmodulation ein relevanter protektiver Effekt auf die postischämische Leber erzielt werden kann. Die komplette Blockade der Interaktion

von dendritischen Zellen mit CD4+ T Lymphozyten stellt hingegen weder mechanistisch noch hinsichtlich des gesamttherapeutischen Nutzens eine effektive Strategie dar. Sie zeigt jedoch eindrucksvoll die Reversibilität der protektiven Immunmodulation.

Neben der Immunmodulation sind zuletzt die verschiedenen Mechanismen des Zelltodes wieder in den Fokus der Reperfusionsforschung gerückt. Es konnte gezeigt werden, dass die Kinase RIP1 eine zentrale Rolle im Nekroptose-Weg spielt und durch Kinaseinhibitoren pharmakologischer Intervention zugänglich ist. In den Organen Herz, Niere und Gehirn konnte in verschiedenen Studien beobachtet werden, dass der Kinase-Inhibitor Necrostatin-1 die RIP-1 Kinase in den postischämischen Organen erfolgreich inhibieren und den nekroptotischen Zelluntergang in den genannten Organen signifikant reduzieren können. In der zweiten vorliegenden Studie zeigt der Autor, dass RIP1 in der postischämischen Leber überraschend nicht exprimiert wird. Gleichzeitig sind die Proteinkinasen der Caspase-Familie deutlich hochreguliert und werden vermehrt exprimiert. Diese, den Zellzyklus und die Apoptose regulierenden Kinasen sind in der Lage RIP-1 abzubauen und Nekroptose zu verhindern. Die Daten zeigen, dass diese natürliche Unterdrückung des Nekroptose-Wegs nicht nur in gesunden Zellen, sondern auch in den postischämischen Hepatozyten stattfindet und damit nicht als potentieller therapeutischer Ansatz genutzt werden kann.

Summary

Ischemia-reperfusion-injury still represents the most frequent cause of liver dysfunction or organ failure following major operation or organ transplantation. The disease pattern results from a complex cascade of different pathophysiological processes on the subcellular, cellular and organ level. Strategies to mitigate or therapeutically affect the negative effects of ischemia itself and subsequent reperfusion are therefore the subject of numerous experimental and clinical studies.

A potential but ambivalent therapeutical goal are CD4 + T lymphocytes, which accumulate in large numbers in the postischemic liver. According to their different pro- and anti-inflammatory capacities, protective or harmful effects upon hepatic ischemia-reperfusion-injury have been reported for the various subgroups of CD4 + T helper cells.

The most effective protective strategy so far has been addressing CD4+ T cells of the Th1 subtype. Th1 cells act pro-apoptotically and pro-inflammatory, mostly via the secretion of interferon γ and TNFα.

In the first study it is shown, that conserving the tolerogenic properties of immature hepatic dendritic cells significantly reduces the frequency of CD4+ T cells migrating into the parenchyma of the postischemic liver. The Th1 subtype is most affected by this effect, explaining the significant protective effect of the intervention.

Furthermore, it is shown for the first time *in vivo* that CD4 + T lymphocytes interact directly with hepatic dendritic cells in the liver. Blocking this interaction reverses the protective effect of immune modulation by synthetic vitamin D.

This effect is surprising, as it was shown in earlier studies, that blocking the MHCII molecule as antigen presenting molecule and direct counterpart of the T cell receptor on T lymphocytes did not have a significant protective effect during the investigated phase of early reperfusion up to four hours. This finding can be explained by the activation kinetics of dendritic cells. The upregulation of the expression and production of molecules such as cytokines and surface markers requires an average of three to four hours, before a significant increase can be observed. Dendritic cells are one of the few cell types that constitutively express both the tolerogenic cytokine IL-10 and the nuclear transcription factor NF-$\kappa\beta$. Further investigations indicate, that therefore dendritic cells orchestrate the immunological response already in early reperfusion phase - even in the non-allogenic setting of ischemia-reperfusion. Therefore, they represent a very promising target for further therapeutical approaches.

In the present work the author demonstrates the successfully implementation of this strategy, achieving a relevant protective effect on the postischemic liver via immunomodulation. Blocking the interaction of dendritic cells with CD4 + T lymphocytes completely, on the other hand, does not prove an effective strategy either mechanistically or in terms of overall therapeutic utility. However, it impressively demonstrates the reversibility of protective immunomodulation.

The mechanisms of cell death have recently regained importance in reperfusion research, after it has been shown that the kinase RIP1 plays a central role in the necroptotic pathway and is accessible to pharmacological intervention by kinase

inhibitors. In the organs of the heart, kidney and brain, various studies have shown that the kinase inhibitor necrostatin-1 can successfully inhibit RIP1 kinase in the postischemic organs and can significantly reduce the necroptotic cell death in the organs mentioned. In the second study, the author is able to show that RIP-1 surprisingly is not expressed in the postischemic liver. At the same time, the kinases of the caspase family are upregulated and show increased expression. These kinases regulate the cell-cycle, apoptotic cell death and are able to degrade RIP-1 and subsequently prevent necroptosis. The data shows that this natural suppression of the necroptosic pathway takes place not only in healthy cells but also in the postischemic hepatocytes and therefore cannot be used as a potential therapeutical approach.

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In situ targeting of dendritic cells sets tolerogenic environment and ameliorates CD4$^+$ T-cell response in the postischemic liver

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ABSTRACT: **CD4$^+$ T cells recruited to the liver play a key role in the pathogenesis of ischemia/reperfusion (I/R) injury. The mechanism of their activation during alloantigen-independent I/R is not completely understood. We hypothesized that liver-resident dendritic cells (DCs) interact with CD4$^+$ T cells in the postischemic liver and that modulation of DCs or T-cell–DC interactions attenuates liver inflammation. In mice, warm hepatic I/R (90/120–240 min) was induced. Tolerogenic DCs were generated *in situ* by pretreatment of animals with the vitamin D analog paricalcitol. A mAb-CD44 was used for blockade of CD4$^+$ T-cell–DC interactions. As shown by 2-photon *in vivo* microscopy as well as confocal microscopy, CD4$^+$ T cells were closely colocalized with DCs in the postischemic liver. Pretreatment with paricalcitol attenuated I/R-induced maturation of DCs (flow cytometry), CD4$^+$ T-cell recruitment into the liver (intravital microscopy), and hepatocellular/microvascular damage (intravital microscopy, alanine aminotransferase/aspartate aminotransferase, histology). However, interruption of T-cell–DC interaction increased proinflammatory DC maturation and even enhanced tissue damage. Simultaneous treatment with an anti-CD44mAb completely abolished the beneficial effect of paricalcitol on T-cell migration and tissue injury. Our study demonstrates for the first time that hepatic DCs interact with CD4$^+$ T cells in the postischemic liver *in vivo*; modulation of DCs and/or generation of tolerogenic DCs attenuates intrahepatic CD4$^+$ T-cell recruitment and reduces I/R injury; and interruption of CD44-dependent CD4$^+$ T-cell–DC interactions enhances tissue injury by preventing the modulatory effect of hepatic DCs on T cells, especially type 1 T helper effector cells. Thus, hepatic DCs are strongly involved in the promotion of CD4$^+$ T-cell–dependent postischemic liver inflammation.—Funken, D., Ishikawa-Ankerhold, H., Uhl, B., Lerchenberger, M., Rentsch, M., Mayr, D., Massberg, S., Werner, J., Khandoga, A. *In situ* targeting of dendritic cells sets tolerogenic environment and ameliorates CD4$^+$ T-cell response in the postischemic liver. FASEB J. 31, 000–000 (2017). www.fasebj.org**

KEY WORDS: **hepatic ischemia/reperfusion · transplantation · T lymphocytes · immunological tolerance · microvascular injury**

Ischemia/reperfusion (I/R) injury causes significant morbidity and mortality, and occurs during organ transplantation, resection, trauma, and septic and hemorrhagic shock. Hepatic I/R injury is the main reason for early organ failure after liver transplantation. I/R-induced injury is a complex cascade of events including cell death due to oxygen deprivation and adenosine triphosphate depletion during ischemia, but also activation of cells of the innate immune system resulting in inflammatory responses during reperfusion. The understanding of the processes leading to this destructive immune response is essential for developing effective interventions to prevent hepatocellular damage and liver failure after transplantation or major surgery.

Previous studies have shown that CD4$^+$, but not CD8$^+$ T cells play a major role during hepatic I/R injury (1, 2). CD4$^+$ T cells accumulate in the postischemic liver during the first 30 min of reperfusion. They activate the sinusoidal endothelium, increase I/R-induced platelet adherence and neutrophil migration *via* CD40-CD40L and CD28-B7-dependent pathways, and aggravate microvascular/hepatocellular injury (1). Furthermore, the early reduction of the absolute number of CD4$^+$ T cells has a protective

ABBREVIATIONS: ALT, alanine aminotransferase; APC, antigen-presenting cell; AST, aspartate aminotransferase; CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; eYFP, yellow fluorescent protein; I/R, ischemia/reperfusion; MHC II, major histocompatibility complex II; T_h1, type 1 T helper

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effect on hepatic I/R in rats (3) and mice (4, 5). Targeting cellular signaling cascades of $CD4^+$ T cells—T cell immunoglobulin and mucin domain 3—showed comparable protective results (6). T cell immunoglobulin and mucin domain 3, an immune checkpoint, is a cell surface protein that is specific for type 1 T helper (T_h1) cells, a $CD4^+$ T cell subtype involved in cell-mediated immunity. Usually the interaction between major histocompatibility complex II (MHC II) on dendritic cells (DCs) and the T-cell receptor on $CD4^+$ T cells leads to proliferation of antigen-specific T helper cells and their differentiation. However, there are no alloantigens in hepatic I/R and treatment with an MHC II antibody did not affect I/R injury in mice (1). This leads to the question of how $CD4^+$ T cells are activated during hepatic I/R. The liver contains various types of cells capable of presenting antigen to $CD4^+$ T cells (7). Liver sinusoidal endothelial cells and hepatic stellate cells can present antigens and could be found to contribute to hepatic microvascular damage through interactions with $CD4^+$ T cells (8, 9). Nevertheless, the most abundant liver resident antigen-presenting cells (APCs) are Kupffer cells and DCs. Both cell types have been shown to induce immune responses and immunologic tolerance in this context (10, 11). Indeed, Kupffer cells might aggravate recruitment of $CD4^+$ T cells in the postischemic liver independent of alloantigen by the release of reactive oxygen species, IL-6, and TNF-α (12). However, the role of DCs, which are the natural partner of T cells in initiating primary responses, remains unclear in the nonallogenic setting of liver I/R.

Our *in vivo* study therefore focuses on the role of DCs during hepatic I/R and the ability of these cells to trigger immune response and tolerance. Whereas mature and completely activated DCs are powerful inducers of immune responses, immature or insufficiently activated DCs are known to promote self-tolerance (13). Hepatic DCs typically are in a quite immature state; therefore, their inherent tolerogenic properties make them a potential *in situ* target. We hypothesized that DCs interact with $CD4^+$ T cells directly in the postischemic liver; modulation of hepatic DCs reduces $CD4^+$ T cell accumulation in the liver and attenuates hepatic I/R injury; and blockade of the interaction between hepatic DCs and $CD4^+$ T cells has a protective effect against hepatocellular damage in the postischemic liver.

MATERIALS AND METHODS

Animals

For fluorescence microscopy and flow cytometry experiments, 5- to 7-wk-old female C57BL/6 wild-type mice (Charles River Laboratories, Wilmington, MA, USA) were used. Two-photon and confocal microscopy were performed with C57BL/6 CD11c-eYFP mice (gift of R. Haas, Max von Pettenkofer Institute, University of Munich). These transgenic mice express the yellow fluorescent protein (eYFP) in the surface marker CD11c on common DCs (14). All experiments were carried out according to German legislation on the protection of animals.

Surgical procedure and interventions

The surgical procedure was performed as detailed elsewhere (15). Briefly, under inhalation anesthesia with isoflurane-N_2O in combination with buprenorphine (0.1 mg/kg body weight, s.c.), a polypropylene catheter was inserted into the left carotid artery in a retrograde direction for measurement of mean arterial pressure and application of fluorescence dyes. A warm (37°C) reversible ischemia of the left liver lobe was induced for 90 min by clamping the supplying nerve vessel bundle with a microclip. Sham-surgery animals (short clamping of the liver for 2–3 s, no ischemia) served as controls.

To generate tolerogenic DCs *in situ*, we used paricalcitol, a synthetic analog of 1,25-dihydroxyergocalciferol, the active form of vitamin D_2. It acts as an agonist for the vitamin D receptor, but with an up to 25-fold higher potential compared to vitamin D_2 itself (16, 17). To hamper the assumed interactions between DCs and $CD4^+$ T cells, a blocking nondepleting mAb against CD44 was used. CD44 is a multifunctional adhesion molecule that has been shown to be a costimulatory factor for T cell activation *in vitro* and *in vivo*. It is involved in early T-cell–DC interactions. Termeer *et al.* (18) reported that perturbation of CD44 on DC with specific antibodies interferes with early Ca^{2+} signaling events during the activation of $CD4^+$ T cells, resulting in T-cell apoptosis.

In the intervention groups, either paricalcitol (25 μg/kg body weight, dissolved in PBS; AbbVie, Ludwigshafen, Germany) or purified anti-CD44 mAb (rat IgG2b, 300 μg/mouse, low endotoxin, acid free; BioLegend, San Diego, CA, USA) was infused intraperitoneally 24 h before ischemia. In the I/R group treated with both medications, paricalcitol and anti-CD44 mAb were infused 24 and 23 h, respectively, before ischemia to avoid a potential interaction of both agents in the peritoneal cavity.

Experimental protocols

$CD4^+$ T cell migration and I/R injury

Intravital fluorescence microscopy and assessment of I/R injury were performed using C57BL/6 mice in 4 experimental groups: an I/R (90 min/120 min) group pretreated with PBS (350 μl) as vehicle, an I/R group pretreated with paricalcitol, an I/R group pretreated with CD44 mAb, and an I/R group pretreated both with paricalcitol and CD44 mAb ($n = 6$ for each group). A sham-surgery group acted as controls. As an additional control, we pretreated mice undergoing I/R with rat IgG2b (isotype-matched control for the anti-CD44 mAb; 300 μg/mouse; BioLegend), but we did not detect any difference from the I/R group treated with PBS as vehicle (data not shown). In another set of experiments, we verified the findings after a prolonged reperfusion time (240 min) in the same experimental groups ($n = 3$ each group).

Flow cytometry

To support the *in vivo* findings, flow cytometry was performed in the same 4 experimental groups (I/R 90/120 min, $n = 3$ in each group). Separate experiments were necessary for the fluorescence-activated cell sorting analysis because the fluorescence dyes used for intravital microscopy might influence flow cytometric measurements. Single-cell suspensions were used to analyze the accumulation of T cells in the liver parenchyma and to describe the maturation status of hepatic DCs.

Interaction of liver DCs and $CD4^+$ T cells in vivo

Interaction of hepatic DCs with $CD4^+$ T cells was assessed in an additional set of experiments using transgenic CD11c-eYFP mice. Intravital multiphoton imaging was carried out in a sham-surgery group, an I/R group pretreated with PBS as vehicle, and an I/R group pretreated with paricalcitol as previously described ($n = 3$ each group). The ischemia time was 90 min. The 2-photon

imaging was started after 60 min of reperfusion and lasted 60 min. At the end of the experiment (120 min of reperfusion), livers were collected and *ex vivo* confocal imaging was performed.

Intravital fluorescence microscopy

Intravital fluorescence microscopy was performed using a modified Leitz-Orthoplan microscope as previously described (15). For the intravital microscopic studies, $CD4^+$ T cells were isolated from spleens of syngeneic mice using a bead-based magnetic cell sorting system with anti-mouse CD4 beads according to the manufacturer's instructions. The isolation procedure did not lead to T-cell activation, and the purity of the $CD4^+$ T cells was routinely >95% as determined by flow cytometry. After the isolation procedure, ~94% of the $CD4^+$ T cells was viable. Isolated $CD4^+$ T cells were then labeled with the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE). A total of 1×10^7 CFSE-labeled $CD4^+$ T cells in a total volume of 200 μl PBS were slowly infused intraarterially *via* the carotid catheter into the aortic arch after 30 min of reperfusion. The mice under anesthesia were submitted to intravital microscopy, and the administered T cells were visualized immediately afterward and after the end of reperfusion time in 7 to 10 randomly chosen acini (9). Thereafter, the plasma marker FITC-conjugated dextran was infused, and sinusoidal perfusion was analyzed using an I2/3 filter block in sinusoids within 8 to 12 acini (15). Intravital microscopy was performed after 30 and 120 min of reperfusion and lasted ~15 min. T cells firmly attached to the endothelium for more than 20 s were counted as permanently adherent or extravasal cells and were quantified as the number of cells per acinus. The sinusoidal perfusion failure was calculated as the percentage of nonperfused sinusoids of all sinusoids visible per acinus.

Multiphoton microscopy and laser scanning confocal microscopy

For multiphoton microscopy, the animals were injected intravenously with isolated $CD4^+$ T cells after 30 min of reperfusion. T cells were labeled *ex vivo* with eFluor 670 (eBioscience, San Diego, CA, USA) as previously described. We used a multiphoton LaVision Biotech (Bielefeld, Germany) TrimScope II system connected to an upright Olympus (Tokyo, Japan) microscope equipped with a Ti:Sa Chameleon Ultra II laser (Coherent) tunable in the range of 680 to 1080 nm and additionally an optical parametric oscillator compact to support the range of 1000 to 1600 nm and a ×16 water immersion objective (numerical aperture 0.8; Nikon, Tokyo, Japan). Single images were acquired from 80 to 100 μm depth, with a *z* interval of 4 μm. Nine hundred twenty nanometers was used as an excitation wavelength, with 1024 × 1024 pixels and detected by photomultiplier tubes (G6780-20; Hamamatsu Photonics, Hamamatsu, Japan). ImSpector Pro (LaVision Biotech) was used as acquisition software to generate images of intrahepatic DCs and $CD4^+$ T cell–labeled cells recruited to CD11c-eYFP liver mouse. An environmental box maintained a stable 37°C environment. Mice were anesthetized and mounted on a custom-built stage to visualize the liver tissue. After imaging, the murine liver was collected and fixed in 4% PFA for 24 h. After fixation, the liver samples were embedded in Tissue-Tek Cryo-Optimum Cutting Temperature (OCT) compound and stored at −80°C. Some of the sectioned samples were also stained with primary antibody CD3e anti-mouse purified clone 145-2c11 (eBioscience) and secondary antibody Alexa Fluor 594–goat anti-hamster IgG (A-21113; Thermo Fisher, Waltham, MA, USA), and mounted with fluorescence medium (Sigma-Aldrich, St. Louis, MO, USA) on the coverslip for T-cell visualization. Additional staining was performed for TNF-α (anti-mouse TNF-α PE, clone MP6-XT22, 12-7321-81; eBioscience), IL-10 (anti-mouse IL-10 FITC, clone JESS-16E3, 11-7101-81; eBioscience), NF-κB (anti-p65 (C-20; sc-327, rabbit polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and FoxP3 (unstained rat anti-mouse, anti-FoxP3 antibody (236A/E7, ab20034; Abcam, Cambridge, MA, USA). The slides were imaged using a LSM 880 laser scanning confocal microscope with Airyscan technology (Carl Zeiss GmbH, Jena, Germany).

Liver enzymes and histopathology

Clinical chemistry and routine pathology were additionally performed for all intravital fluorescence microscopy sets. All results were confirmed within experiments with identical treatment but without imaging. Blood samples were taken from the vena cava at the end of each experiment and centrifuged immediately at 2000 *g* for 10 min. The serum was formed into aliquots and stored at −80°C. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured at 37°C with an automated analyzer (AU 5800; Beckman Coulter, Brea, CA, USA) using standardized test systems (HiCo GOT and HiCo GPT; Roche Applied Sciences, Indianapolis, IN, USA).

For histopathology, liver samples of each experiment were fixed in 10% formalin for 24 h, embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin. To assess the severity of hepatocellular damage, we used a score allocating 0 to 3 points for no, low, intermediate, and high tissue damage, respectively. The criteria for tissue damage took into consideration sinusoidal congestion, hydropic degeneration (ballooning), and hepatocellular necrosis subdivided into single-cell and grouped-cell necrosis, necrosis in other than the centrilobular zone, and complete loss of hepatic microarchitecture.

Flow cytometric analysis

Flow cytometry was conducted with single-cell suspensions of naive, sham-surgery, and pretreated mice after I/R of 90/120 min (set 1, $n = 3$ each group) as well as after I/R of 90/240 min (set 2, $n = 3$ each group). Hepatocytes and hepatic nonparenchymal cells were isolated from the liver by the collagenase digestion method. Briefly, each liver was perfused *in situ via* the portal vein, initially with PBS solution and then with PBS containing 0.2% collagenase type II, with both solutions prewarmed to 37°C. The excised left liver lobe was incubated in collagenase solution for 20 min at 37°C and then homogenized to liberate nonparenchymal and parenchymal cells. The initial cell suspension was filtered through a 30 μm nylon mesh and treated with anti-mouse FcR blocking reagent to prevent nonspecific binding. Cell numbers were determined using a Coulter ACT 10 (Beckman Coulter). For each experiment, 3 samples containing 1×10^7 nucleated cells were prepared for further analysis. Eight-color (T cells), 7-color (DCs), and 6-color (inflammatory DCs) flow cytometry was conducted for each sample. The cells were washed and stained with fluorochrome-conjugated mAbs according to the manufacturer's instructions.

T cells were identified as $CD45^+CD3^+$ cells from lymphocyte gated $CD45^+$ cells and divided in $CD4^+$ and $CD8^+$ subsets. $CD4^+$ T cells where further analyzed for their expression of the surface markers CXCR3, CD25, and CD127. The $CD4^+CXCR3^+$ population was identified as T_h1 cells. The combination of $CD4^+CD25^+CD127^-$/low was used to check for regulatory T cells. All subpopulations of $CD4^+$ or $CD8^+$ T cells were analyzed for their expression of CD44 as a marker for maturation and regulatory properties (19).

Hepatic DCs were identified as $CD45^+CD11c^+F4/80^-$ cells (20). The exclusive marker mPDCA-1 was used to exclude

plasmacytoid DCs from our analysis. On the basis of their expression of CD11b, the liver DCs could be divided into CD11b low and CD11b high subpopulations. Both populations were analyzed for the expression of the costimulatory molecules CD80 and CD86 as markers for their maturation status and T-cell stimulation capacity.

To differentiate between common DCs and monocyte-derived inflammatory DCs in the setting of hepatic I/R, we investigated the distribution and maturation of these cell types by a third approach. The relevant population could be identified in an inflammatory peritonitis model (data not shown) *via* staining of CD45, CD11b, CD64, and FcγRIa, as proposed by Segura and Amigorena (21). Inflammatory DCs from the postischemic liver were analyzed for the expression of CD44 and CD86 to evaluate their ability to stimulate T cells.

Flow analysis was performed with a 10-color Gallios flow cytometer, and Kaluza software was used for data analysis (both from Beckman Coulter).

Reagents

Collagenase type II, CLS II, was purchased from Biochrom (Berlin, Germany). Immunomagnetic microbeads for mouse CD4 (L3T4) T cell isolation, FcR blocking reagent anti-mouse, VioBlue-, VioGreen-, VioBright FITC-, PE-, PerCP-, PerCP-Vio770-, PE-Vio770-, APC-, or APC-Vio770-conjugated mAbs directed against mouse CD3 (17A2), CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70.15.11.5), CD11c (N418), CD25 (7D4), CD44 (IM7.8.1), CD45 (30F11), CD64/FcγRI (REA286), CD80 (16-10A1), CD86 (PO3.3), CD127 (A7R 34), CD45R/B220 (RA3-6B2), CD183/CXCR3 (CXCR3-173), F4/80 (REA126), FceR1a (MAR-1), and mPDCA-1 (JF05-1C2.4.1) as well as appropriate immunoglobulin isotype control antibodies, were obtained from Miltenyi Biotec (San Diego, CA, USA). The fluorescent dyes for intravital microscopy were acquired from Molecular Probes (Eugene, OR, USA; CFSE, 5KM) and Sigma-Aldrich (FITC-conjugated dextran, MW 150,000, 0.1 ml, 5%).

Statistical analysis

ANOVA on ranks followed by Student–Newman–Keuls methods were used for the estimation of stochastic probability in intergroups comparison (SigmaPlot 12; Jandel Scientific, Erkrath, Germany). Means ± SEM are provided. A value of $P < 0.05$ was considered significant.

RESULTS

$CD4^+$ T cell recruitment

Migration of $CD4^+$ T cells was analyzed by intravital microscopy. In sham-surgery mice, only a few $CD4^+$ T cells (2 ± 1 per acinus) were found accumulated in hepatic sinusoids. In contrast, a significant increase in the number of adherent $CD4^+$ cells in liver sinusoids (8 ± 1 per acinus) was observed after 90 min of ischemia followed by 30 min (data not shown) and by 120 min of reperfusion (**Fig. 1**). Modulation of hepatic DCs by administration of paricalcitol significantly reduced the recruitment of $CD4^+$

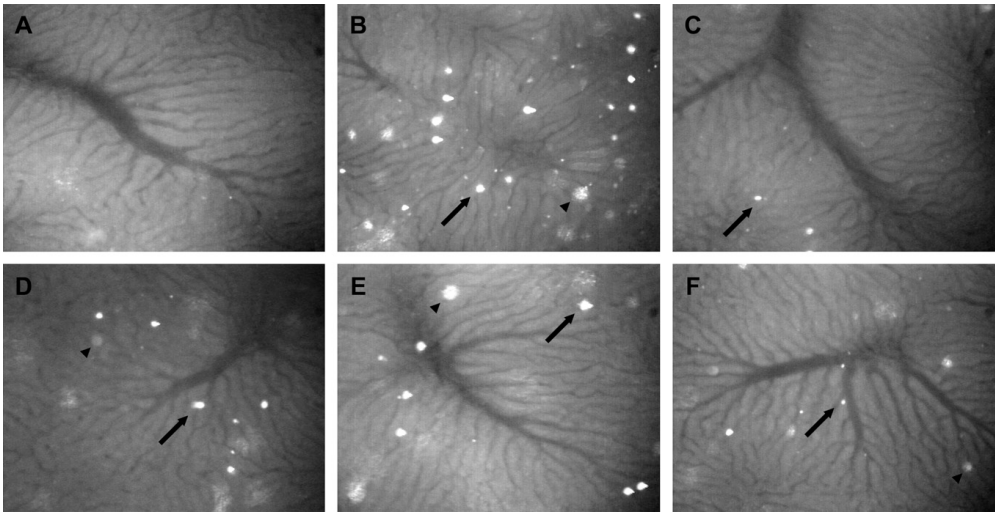

Figure 1. Recruitment of $CD4^+$ T cells. Microphotographs demonstrating CFSE-labeled $CD4^+$ T cells in hepatic microcirculation in sham-surgery mice (*A*) and in mice after I/R (90/120 min) visualized by intravital fluorescence microscopy in liver sinusoids per acinus. I/R induced $CD4^+$ T cell accumulation in hepatic sinusoids (*B*). Upon DC modulation with paricalcitol, T-cell recruitment was attenuated after 120 min (*C*) and 240 min (*D*) of reperfusion. Moreover, dual treatment with paricalcitol and anti-CD44 mAb (*E*), as well as anti-CD44 mAb alone (*F*), led to reduction of T-cell accumulation. Arrow depicts $CD4^+$ T cell intravascularly accumulated in sinusoid. Arrowhead indicates $CD4^+$ T cell localized out of focus in another tissue layer (not counted). Representative images are shown of dynamic process. Quantitative analysis was performed by video sequences of at least 1-min duration. Only T cells firmly attached to endothelium for more than 20 s were counted as permanently adherent or extravasal cells were quantified as number of cells per acinus. Original magnification, ×500; $n = 6$ animals per group.

T cells (5 ± 1 per acinus). Pretreatment of animals with anti-CD44 mAb in order to prevent interaction between $CD4^+$ T cells and DCs decreased postischemic $CD4^+$ T-cell accumulation even further than only paricalcitol treatment. However, simultaneous treatment with paricalcitol and anti-CD44 mAb recreated this result, presenting a distinctly lower number of adherent $CD4^+$ T cells. There was no difference between sole anti-CD44 treatment and paricalcitol in combination with anti-CD44 mAb. This finding demonstrates the reversibility of the effect of paricalcitol by a blocking antibody and emphasizes the modulating mechanism of action of vitamin D analogs.

Sinusoidal perfusion failure

I/R injury is associated with a severe deterioration of tissue perfusion leading to tissue hypoxia during reperfusion, known as the no-reflow phenomenon. Sinusoidal perfusion failure was determined as an established parameter of microvascular I/R injury using *in vivo* microscopy. In the I/R group treated with PBS solution as vehicle, ~15% of all sinusoids were not perfused (**Fig. 2**). In contrast, the perfusion failure was significantly improved in the paricalcitol-treated group compared to the vehicle-treated group. Treatment with anti-CD44 mAb resulted in greatly deteriorated vascular damage. In line with the T-cell recruitment data, the ameliorating effect of paricalcitol was completely reversed in the combined treatment group.

Histopathology

Hematoxylin and eosin staining of tissue samples was performed to assess the degree of liver damage. After I/R, multiple hydropic degenerated or necrotic cells were detected. In contrast, the paricalcitol-treated mice exhibiting only a few single-cell necroses showed no significant difference compared to the sham-surgery mice. Similar to the data on sinusoidal perfusion, a significantly higher damage level was observed upon CD44 blockade as well as upon double intervention with anti-CD44 mAb and paricalcitol, resulting in various grouped necrosis areas. The degree of histopathological damage was statistically even higher in this group than in the vehicle-treated I/R group (**Fig. 3**)

Liver enzyme activity

The serum activity of hepatic transaminases was measured as a marker of cell integrity quantifying the extent of hepatocellular tissue damage and necrotic injury. Hepatic I/R led to a marked elevation in liver enzyme activity (ALT 6994 ± 1467 U/L, AST 5744 ± 509 U/L; **Fig 4*A***). In line with the *in vivo* data regarding hepatic microcirculation, a considerable protective effect of paricalcitol on necrotic injury was detected, resulting in an ALT level of 1423 ± 301 U/L and an AST level of 1077 ± 422 U/L ($P < 0.05$). However, the protective effect was quite weak upon administration of anti-CD44 mAb. In addition, no significant differences were observed between the sole anti-CD44

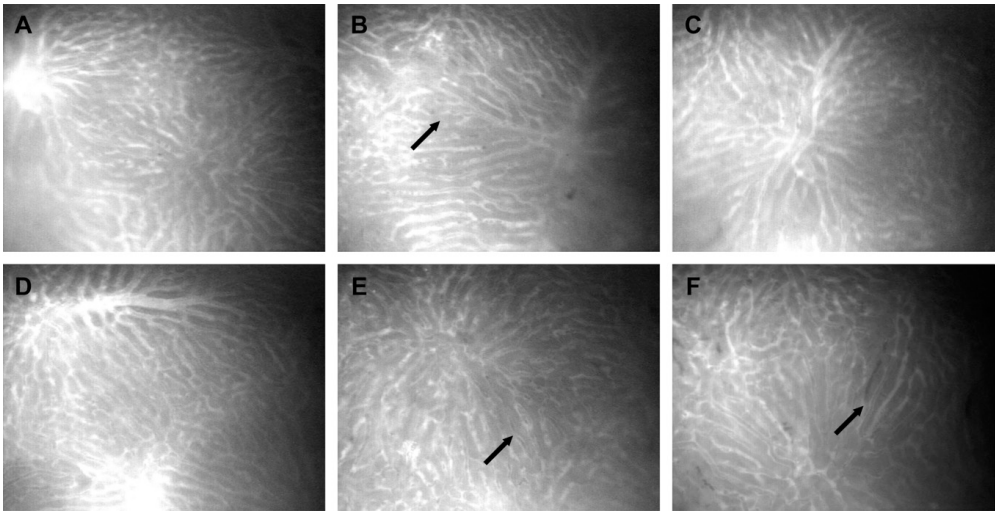

Figure 2. Sinusoidal perfusion failure. Sinusoidal perfusion failure (percentage of nonperfused sinusoids) was quantified by intravital microscopy as parameter of microvascular hepatic injury in sham-surgery mice (*A*) and mice after I/R (90 min/120 min). FITC-dextran was used as plasma marker. Hepatic I/R (*B*) induced severe perfusion failure, as shown by high amount of nonperfused sinusoids (arrow). Upon DC modulation with paricalcitol, T cell recruitment was attenuated after 120 min (*C*) and 240 min (*D*) of reperfusion. Administration of anti-CD44 mAb aggravated postischemic sinusoidal perfusion failure (*F*) and antagonized beneficial effect of paricalcitol in dual-treatment group (*E*). Original magnification, ×500; $n = 6$ animals per group.

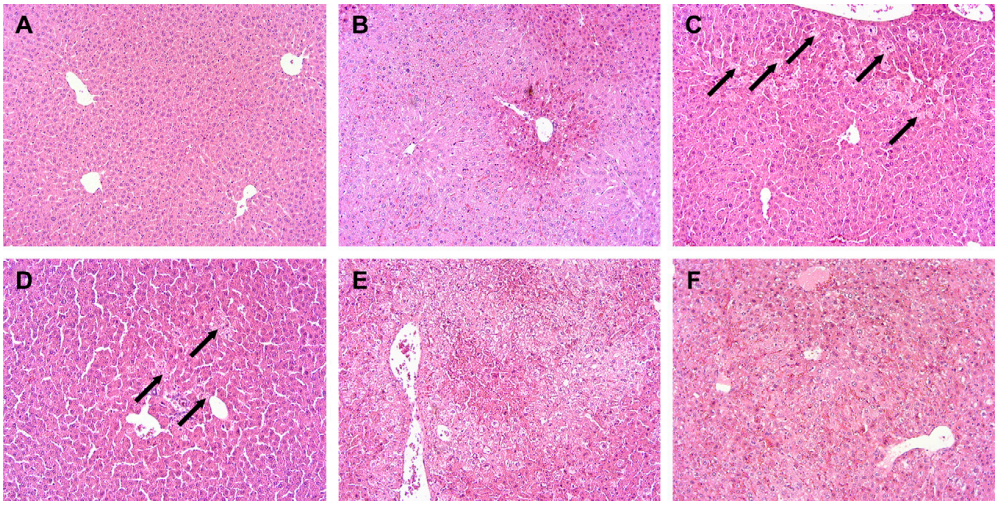

Figure 3. Histopathology. Liver damage was assessed in paraffin-fixed and hematoxylin and eosin–stained tissue sections. Massive cellular damage was found after I/R (90/120 min), as indicated by necrotic pericentral areas (*B*) compared to homogenous tissue structure of sham-surgery mice (*A*). In paricalcitol-treated group, only single-cell necrosis could be found (arrows) after 120 min (*C*) and 240 min (*D*) of reperfusion. Anti-CD44 mAb antagonized this protective effect in dual (with paricalcitol; *F*) and single (*E*) treatment group and showed similar damage levels as in vehicle-treated group with hemorrhagic and necrotic pericentral areas. Representative microphotographs from 6 experiments per group. Original magnification, ×200.

treatment and simultaneous treatment with paricalcitol and anti-CD44 mAb.

Flow cytometry

To confirm the data from intravital microscopy, we performed flow cytometric analysis of DCs and T cells accumulated in the liver parenchyma. Corresponding to the *in vivo* data, the frequency of T cells in the postischemic liver was significantly increased. This effect involved both $CD4^+$ and $CD8^+$ T cells. However, the $CD4^+$ population appeared to be affected more strongly by this tendency, although not statistically significantly (**Fig. 5**). Further analysis identified the biggest amount of $CD4^+$ T cells as $CXCR3^+$ T_h1 cells, indicating that T_h1 are the prevalent subtype of $CD4^+$ T cells during early reperfusion. In the paricalcitol-treated group, the overall extravasation of T cells tended to be lower than in the control group but did not reach statistical significance. The frequency of T_h1 cells, on the other hand, decreased sharply after immunomodulation. Combination therapy with paricalcitol and anti-CD44 mAb was found to reverse this effect and showed both increased frequencies of intrahepatic overall $CD4^+$ T cells and of T_h1 cells within this population. In all groups, no regulatory T cells could be found in our experiments (Supplemental Fig. 3).

Hepatic DCs were allocated into 2 subpopulations depending on their CD11b expression. CD11b high DCs were seen as proinflammatory. Both subpopulations were analyzed for their expression of costimulatory molecules CD80 and CD86. Liver I/R led to a significant activation of hepatic DCs, as seen in the increased expression of costimulatory molecules. Immunomodulation with paricalcitol did not alter the percentage of proinflammatory CD11b high DCs but reduced their maturation status. This modulatory effect was completely abolished after blocking CD44. Moreover, the blockade resulted in a significant increase of activated, proinflammatory CD11b high DCs. Treatment with anti-CD44 mAb alone showed similar results (**Fig. 6**).

In a third approach, we targeted inflammatory DCs of monocytic origin. Segura *et al.* (21) suggested the exclusive combination of CD64 and FCER1A to differentiate these cells against macrophage and myeloid DCs. In the analysis of this cell population, no difference between the control group and the intervention group was observed (data not shown).

These data, taken together, indicate that common hepatic DCs, but not plasmocytoid DCs or inflammatory DCs, can be modulated directly in the liver. Paricalcitol caused hepatic DCs to stay in a more immature state and to express less proinflammatory and costimulatory surface markers. Subsequently, fewer $CD4^+$ T cells accumulated in the postischemic liver. In the untreated liver, however, most $CD4^+$ T cells belonged to T_h1 cells; this subpopulation was considerably reduced under immunomodulation with paricalcitol. Anti-CD44 mAb abolished and reversed all of these effects, indicating a harmful effect of the complete blocking strategy.

Interactions between $CD4^+$ T cells and DCs

Fluorescence-labeled $CD4^+$ T cells were infused into mice expressing fluorescent CD11c-eYFP, a marker for DCs.

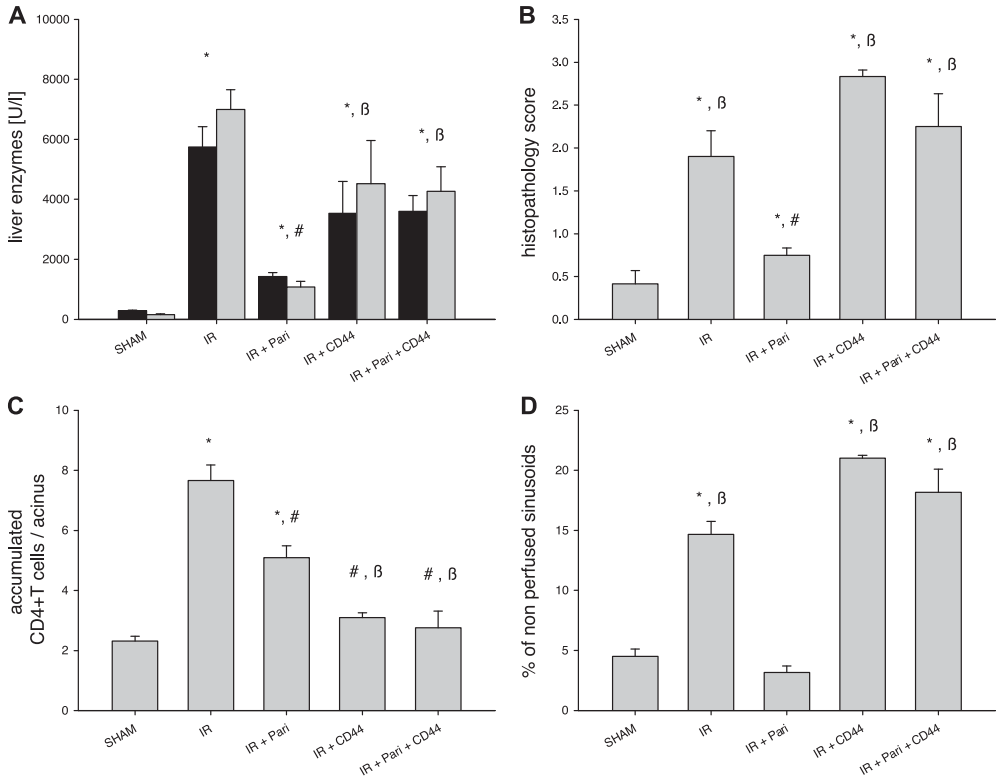

Figure 4. Quantitative analysis of $CD4^+$ T-cell migration and liver damage. Five groups were analyzed: sham-surgery group (control), vehicle-pretreated I/R (90 min/120 min) group, paricalcitol-pretreated I/R group (I/R + Pari), group pretreated with anti-CD44 mAb (I/R + CD44), and group receiving dual treatment with paricalcitol and anti-CD44 mAb (I/R + Pari + CD44). *A*) Bar graphs show serum activity of hepatic transaminases AST (black bars) and ALT (gray bars), which was determined to be a marker of hepatocellular necrotic injury. *B*) Hematoxylin and eosin–stained liver sections were analyzed semiquantitatively: 0 to 3 points were given for no, low, intermediate, and high tissue damage. $CD4^+$ T cell accumulation (*C*) and sinusoidal perfusion failure (*D*) were quantified by intravital microscopy. $n = 6$ animals per group. Data are shown as means ± SEM. $^*P < 0.05$ *vs.* sham-surgery group; $^{\#}P < 0.05$ *vs.* I/R group; $^{\beta}P < 0.05$ *vs.* I/R + Pari.

Both cell types were simultaneously visualized in the hepatic micovasculature *in vivo* using 2-photon microscopy. As shown in **Fig. 7**, $CD4^+$ T cells were found closely colocalized with DCs in the hepatic microvasculature during hepatic reperfusion. Such colocalization allows a direct interaction between both cell types. Three-dimensional reconstruction is provided in Supplemental Video 1.

After completing *in vivo* 2-photon microscopy, liver samples were taken and prepared for confocal laser scanning microscopy. Whereas no T-cell–DC interactions were observed in sham-surgery mice (data not shown), $CD4^+$ T cell were frequently colocalized with hepatic DCs after I/R (**Fig. 8***A*). Repeatedly, several $CD4^+$ T cells were found attached to a single DC, as demonstrated at an increased magnification in Fig. 8*B*. Modulation of hepatic DCs with paricalcitol attenuated $CD4^+$ T-cell–DC colocalizations in the postischemic liver (Fig. 8*C*, *D*).

To verify that the red staining in Fig. 8 really corresponded to T cells and was not simply fluorescence dye released from *ex vivo* stained cells during the preparation of tissue sections, we repeated the confocal imaging in the same tissue samples after additional immunostaining. Staining for the T-cell receptor surface marker CD3 and DAPI, a marker of nucleated cells, was conducted, and tissue sections were visualized by confocal microscopy. The results matched the *in vivo* data and clearly identified the cells colocalized to the hepatic DCs as T cells (Supplemental Fig. 1).

Additional staining for IL-10 and TNF-α was performed to show the functional properties of visualized $CD4^+$ T cells and hepatic DCs for the control and paricalcitol-treated groups. In the control group, no DCs producing tolerogenic IL-10 could be found, whereas most DCs in the pretreated livers stained positive for IL-10 (Supplemental Fig. 4). Correspondingly, $CD4^+$ T cells

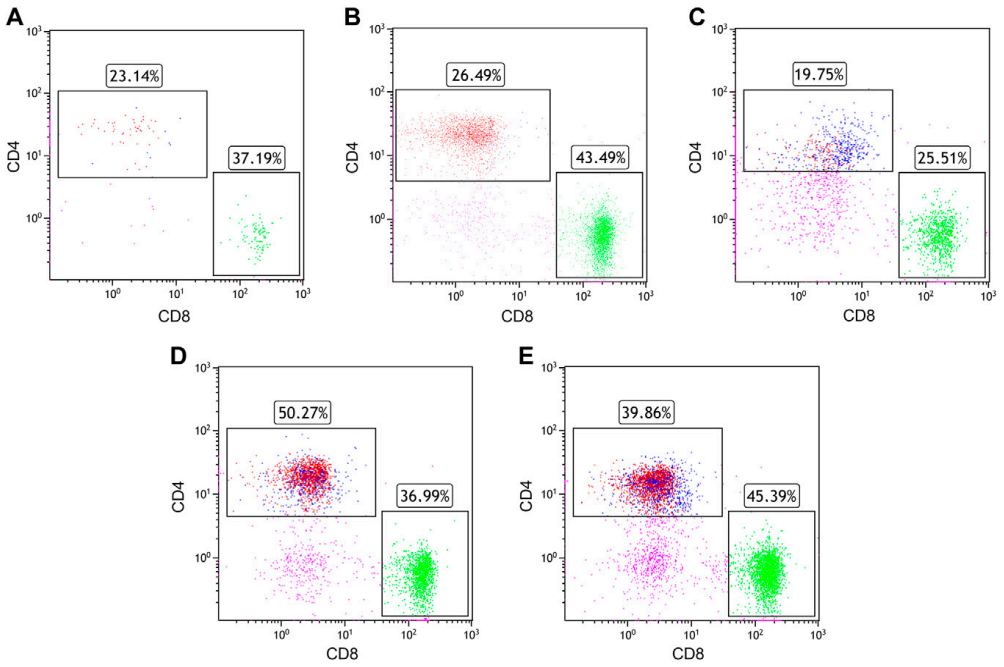

Figure 5. Flow cytometric analysis of T cells accumulated in liver. Livers were collagenase digested and analyzed by 8-color flow cytometry. Histograms show CD4 *vs.* CD8 staining of $CD45^+CD3^+$ lymphocyte gated cells. Hepatic I/R showed increased frequencies of $CD4^+$ and $CD8^+$ T cells (*B*) compared to sham-surgery animals (*A*). Treatment with paricalcitol (*C*) led to reduced numbers of T cells in both$^+$ and $CD8^+$ populations. Decrease of $CD4^+$ T cells was mainly due to reduced numbers of T_h1 T cells, shown as red subpopulation of $CD4^+$ T cells. This finding could not be found in any other treatment group. Blocking of CD44 antagonized this effect in single-treatment group (*E*) and in combined-treatment group (with paricalcitol; *D*). Both groups showed increased numbers of T cells accumulated in liver after I/R. Results are representative of 3 independent experiments per group.

colocalized with DCs in the control were found highly positive for proinflammatory TNF-α. In the intervention group, $CD4^+$ T cells showed reduced positivity for TNF-α (Supplemental Fig. 5).

To confirm the absence of regulatory T cells during early reperfusion, FoxP3 staining was carried out. No FoxP3-positive cells were detected in the control or intervention groups (Supplemental Fig. 3).

NF-κβ staining was performed to evaluate the regulation of this important regulator of cell death in hepatocytes and of functional properties of immune cells. We found that in the postischemic liver NF-κβ was mostly expressed by hepatocytes, whereas in the paricalcitol-treated group mostly hepatic DCs stained positive for the expression of NF-κβ (Supplemental Fig. 6).

DISCUSSION

Hepatic I/R is the most common cause for organ dysfunction and failure after major liver surgery and transplantation. Therefore, the development of strategies to minimize the negative effects of I/R is at the forefront of clinical and experimental studies (22). $CD4^+$ T cells contribute greatly to postischemic liver injury (1, 2).

Usually T cells are activated by interactions with APCs, especially DCs. First, we addressed the question of whether $CD4^+$ T cells interact with DCs in the postischemic liver. Thus, both cell types were visualized simultaneously using 2-photon *in vivo* microscopy. Two-photon laser scanning microscopy achieves excitation by using an objective lens to focus near-infrared light onto a small point within the sample. This ensures that the probability of a fluorophore absorbing 2 photons is highest at the focal plane. Other advantages include low scattering, high multichannel acquisition speed, and minimal phototoxicity (23–25). Here, we show for the first time that DCs interact with $CD4^+$ T cells directly in the postischemic liver. These findings might explain the early onset of $CD4^+$ T cell–driven tissue damage as well as the striking effect of DC targeting in the liver. Moreover, we observed that hepatic DCs participate in a dynamic interaction process with $CD4^+$ T cells, which also involves prolonged stable interactions. Moreover, these *in vivo* data were supported by the results of *ex vivo* confocal scanning microscopy. Together, these findings suggest that in both the allogenic setting of organ transplantation and during hepatic I/R

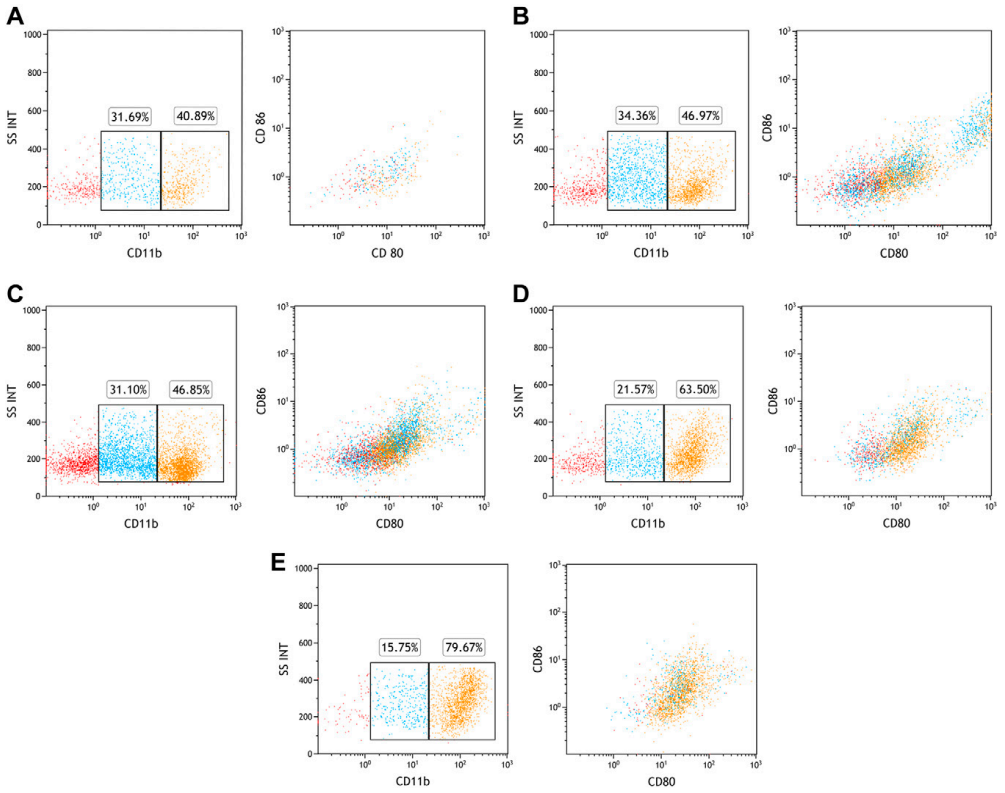

Figure 6. Flow cytometric analysis of hepatic DCs. Livers were collagenase digested and analyzed by 7-color flow cytometry. Histograms show allocation of $CD45^+CD11^+F4/80^-$ DCs into 2 subpopulations depending on their CD11b expression. CD11b high DCs (orange) were seen as proinflammatory. Both subpopulations were analyzed for activation markers CD80 and CD86. Hepatic I/R lead to notable activation of hepatic DCs (*B*) compared to sham-surgery animals (*A*). Immunomodulation with paricalcitol reduced activation status of hepatic DCs but did not alter percentage of proinflammatory DCs (*C*). Blocking CD44 abolishes modulatory effect of paricalcitol in simultaneous treatment group, resulting in increased numbers of activated, proinflammatory DCs (*D*). Treatment with anti-CD44 mAb alone shows similar results (*E*). Histograms are representative of 3 independent experiments per group.

injury, DCs might play a key role in initiating and orchestrating the T cell response. Previously, we have shown that blocking of MHC II molecules does not affect hepatic I/R injury (1). We therefore assume that Toll-like receptors binding to damage-associated molecular patterns replace alloantigens (26). These particles released by injured (hepatic) cells are known to unleash an inflammatory cascade, further amplifying tissue destruction. Our data match this perception, as $CD4^+$ T_h1 cells constitute a large proportion of intraparenchymal T cells in the postischemic liver (Supplemental Fig. 2). T_h1 cells are known to produce large amounts of proinflammatory TNF-α. Immunomodulation with paricalcitol reduces the TNF-α production (Supplemental Fig. 5). This finding is also consistent with comparable results upon specific targeting of cellular signaling cascades of $CD4^+$ T_h1 cells (6). Regulatory T cells, however, did not contribute to the amelioration of hepatic I/R injury during early reperfusion.

The next question we addressed in our study was whether a modulation of DCs by paricalcitol affects $CD4^+$ T-cell recruitment to the liver as well as hepatic I/R injury. The application of vitamin D as a regulatory effector of the immune system in general and of DCs in particular has been previously demonstrated (27, 28). In this context, vitamin D has been used to generate tolerogenic DCs (29–31). In order to maximize the beneficial effects of this therapeutic strategy, we evaluated highly potent synthetic vitamin D analogs as the most promising candidates. We chose paricalcitol because this drug is able to stimulate the vitamin D receptor pathway up to 25 times more strongly than vitamin D itself and because it is already in clinical use (32, 33). In addition, earlier studies reported a protective effect of this drug on I/R-induced acute kidney injury in the mouse (17).

We demonstrated that paricalcitol attenuates $CD4^+$ T-cell accumulation in the postischemic liver and reduces

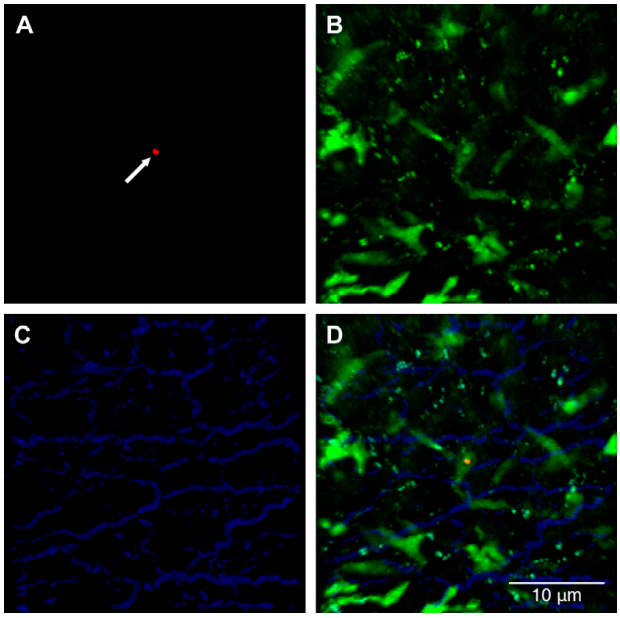

Figure 7. Two-photon microscopy. Representative images show $CD4^+$ T cells and hepatic DCs in postischemic mouse liver *in vivo*. *A*) $CD4^+$ T cell labeled with eFluor 670 (red) indicated by arrow. *B*) Hepatic DCs (green) visualized by expression of eYFP enhanced CD11c in transgenic C57BL/6-CD11c-eYFP mouse. *C*) Perfused liver sinusoids after plasma labeling with dextran cascade blue (blue). *D*) Merged image showing colocalization of $CD4^+$ T cells and DC. For better visualization of close interaction between both cells, 3-dimensional frame and reconstruction are presented in the Supplemental Data. Original magnification, ×16/0.8 water immersion objective. Representative microphotographs from 3 to 5 experiments per group.

microvascular and hepatocellular tissue damage. We showed that hepatic DCs are involved in the activation of $CD4^+$ T cells during hepatic reperfusion. The most potent stimulators of T cells are conventional myeloid DCs as important regulators of innate and adaptive immunity. They have been previously implicated in the regulation of inflammation and hepatic I/R injury, and it has been pointed out that the local liver microenvironment plays an important role in determining DC function during I/R injury (26, 34). Plasmacytoid DCs, on the other hand, do not interact with T cells but rather promote hepatic I/R injury, predominantly by induction of hepatocyte IRF-1 (35). Although inflammatory DCs are thought to stimulate antigen-specific T cells directly in inflamed tissues and to initiate memory responses in the tissue *via* stimulation of $CD4^+$ and $CD8^+$ tissue-resident memory T cells (21), they did not react to I/R. Therefore, plasmacytoid DCs and inflammatory DCs were excluded from further analysis.

Our flow cytometric data showed that upon warm ischemia, liver common DCs acquire a more mature phenotype, accompanied by the expression of enhanced numbers of costimulatory molecules. This finding applies to both subtypes of DCs found prevalently in the postischemic liver: CD11b high and CD11b low DCs. Notably, both subpopulations have been shown to produce large amounts of IL-12, directing $CD4^+$ T cell response in the T_h1 direction. Nevertheless, $CD11b^+$ high DCs have the most proinflammatory effect *via* secretion of IL-6 and TNF-α (20). After hepatic I/R, we registered a concomitant rise in numbers of both subtypes, but no shift toward one of either subpopulation. Flow cytometric analyses of these DC populations after pretreatment with paricalcitol similarly revealed no population shift, but we observed a reduced expression of costimulatory molecules on hepatic DCs in general. DCs have the most tolerogenic and antiinflammatory effect *via* secretion of IL-10. We showed that most hepatic DCs after immunomodulation express high amounts of IL-10 and correspondingly NF-κB, whereas other DCs are negative for both molecules (Supplemental Figs. 4 and 6). These findings suggest that paricalcitol allows the targeting of hepatic DCs *in situ*, directly in the liver. It prevents proinflammatory maturation and favors the generation of immature, regulatory DCs. This fact is of outstanding interest, not only for the nonallogenic setting of warm hepatic I/R but also for organ transplantation, as donor-derived intrahepatic DCs are usually not accessible for therapeutic approaches. After consideration of our *in vivo* microscopy data reporting a reduction in, but no complete loss of, $CD4^+$ T-cell accumulation to the postischemic liver, these findings emphasize the modulating, but not blocking, effect of paricalcitol. Neither DCs nor T cells are depleted or compromised in their inherent immunologic functions.

Furthermore, we answered the question whether blockade of the interaction between hepatic DCs with $CD4^+$ T cells leads to a protective effect against hepatocellular damage in the postischemic liver. We identified CD44 as a promising target, which triggers the interaction of hepatic DCs and $CD4^+$ T cells. CD44 is a multifunctional adhesion molecule that has been shown to be a costimulatory factor for T cell activation *in vitro* and *in vivo*. Perturbation of CD44 on DC interferes with early Ca^{2+} signaling events during the activation of $CD4^+$ T cells, resulting in T-cell apoptosis (18). We showed in this study that pretreatment with anti-CD44 mAb reduced the number of $CD4^+$ T cells

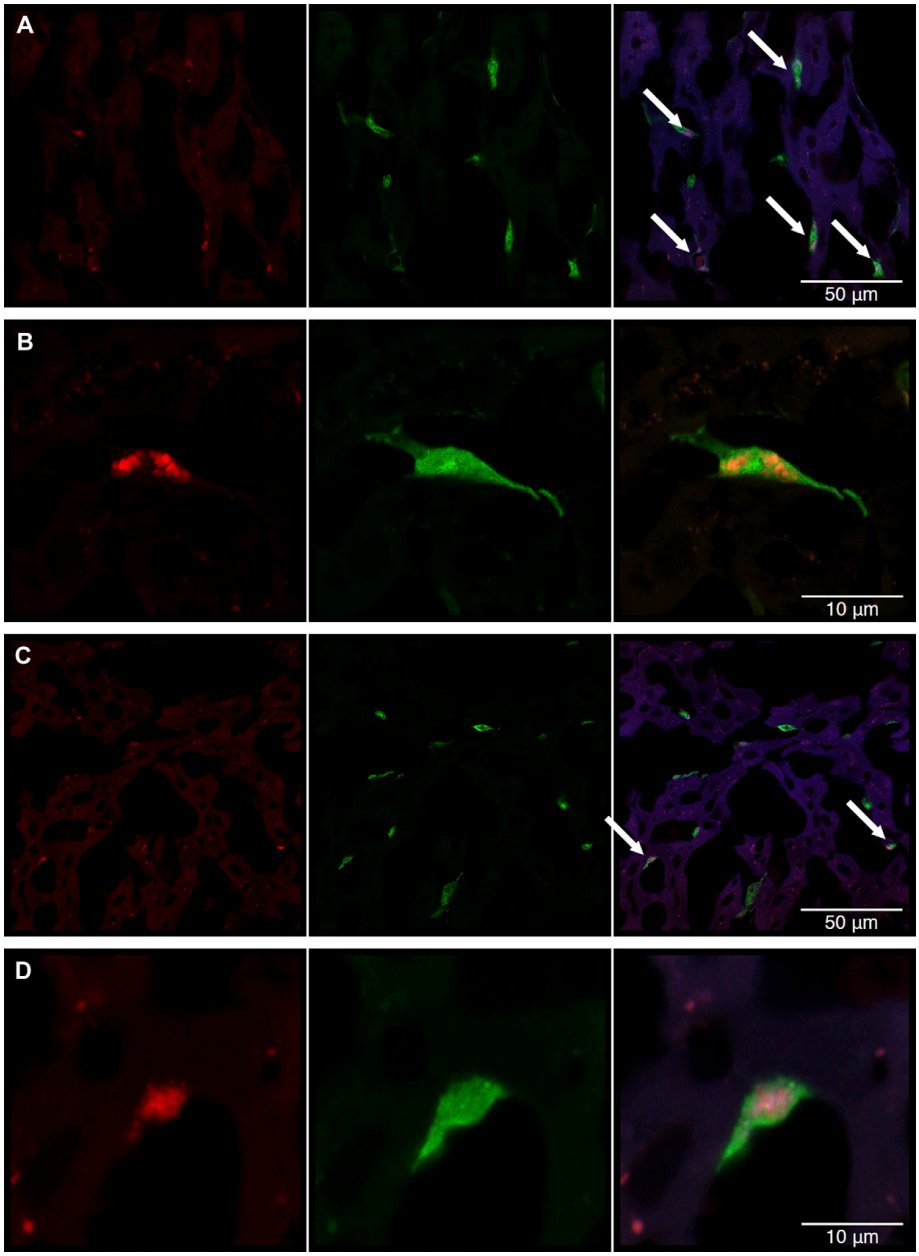

Figure 8. Confocal microscopy. After *in vivo* 2-photon microscopy, postischemic livers of treated and untreated transgenic C57BL/6 CD11c-eYFP mice were collected for *ex vivo* imaging by confocal laser scanning microscopy. Interactions between $CD4^+$ T cells labeled with eFluor 670 (red) and hepatic DCs (green) were visualized. Hepatocytes appear deep purple in merged images. *A*) $CD4^+$ T cell–DC colocalizations in postischemic liver. *B*) Same sample at higher magnification showing single DC interacting with several $CD4^+$ T cells. *C*, *D*) Two different magnifications showing that modulation of DCs using paricalcitol attenuates $CD4^+$ T-cell–DC colocalizations in postischemic liver. Original magnification, ×63/1.4 oil immersion objective. Arrows indicate $CD4^+$ T-cell–DC colocalizations. $n = 3$ each group. Representative microphotographs from 3 experiments per group.

accumulated in the postischemic liver. In contrast, the blockade $CD4^+$ T-cell–DC interactions led to significantly higher tissue damage. This finding is consistent with data from experiments upon complete depletion of the intrahepatic DCs (26). Moreover, the flow cytometric analysis revealed significant population shifts for both $CD4^+$ T cells and hepatic DCs. While in the untreated or immunomodulated liver proinflammatory CD11b high DCs made up around 40% of all hepatic DCs, their amount increased up to 70% in the anti-CD44-pretreated postischemic liver. Concomitantly, the amount of Th1 $CD4^+$ T cells increased in the same order of magnitude in these experiments.

Interestingly, upon the simultaneous treatment with paricalcitol and anti-CD44 mAb, the effect of paricalcitol was completely abolished, a finding that substantially corresponded to the data after sole anti-CD44 mAb treatment. We explain this result by the fact that, on the one hand, a complete blockade of DC–T-cell interaction effectively prevents an excessive response by Th1 cells. On the other hand, the regulatory component of the adaptive immune response is also blocked, preventing any form of endogenous damage control. It emphasizes the relevance of differentiating between the single subsets of both DCs and $CD4^+$ T cells as well as their functional properties. Moreover, these findings demonstrate the bilateral character of the interactions between DCs and T cells. Both cell populations seem capable of directing the immune response in a proinflammatory or tolerogenic direction, and both regulatory populations appear to be essential for the control of hepatic I/R injury.

In summary, our study demonstrates for the first time that hepatic DCs interact with $CD4^+$ T cells in the postischemic liver *in vivo*; that modulation of DCs and/or generation of tolerogenic DCs using paricalcitol attenuates intrahepatic $CD4^+$ T-cell recruitment and reduces T_h1-dependent I/R injury; and that it interrupts of CD44-dependent $CD4^+$ T-cell–DC interactions but enhances tissue injury probably by preventing IL-10-mediated control of T_h1 cell activation and increasing the amount of proinflammatory DCs. Thus, we suggest that hepatic DCs are strongly involved in the promotion of hepatic I/R injury and the modulation of the $CD4^+$ T cell response. These data provide significant new insights into the mechanisms that promote liver I/R injury and offer a potential therapeutic concept by targeting the vitamin D receptor pathway for amelioration of liver I/R injury. FJ

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AUTHOR CONTRIBUTIONS

D. Funken designed procedures and performed experiments, and wrote the article; H. Ishikawa-Ankerhold, B. Uhl, M. Lerchenberger, M. Rentsch, and D. Mayr designed procedures and performed experiments; S. Massberg and J. Werner created the study's concept and design; and A. Khandoga created the study's concept and design and wrote the article.

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RIP1-DEPENDENT PROGRAMMED NECROSIS IS NEGATIVELY REGULATED BY CASPASES DURING HEPATIC ISCHEMIA-REPERFUSION

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ABSTRACT—Programmed necrosis (necroptosis), a newly discovered form of cell death, is mediated by receptor-interacting protein 1 (RIP1) and plays a pivotal role after myocardial, renal, and cerebral ischemia-reperfusion (I/R). The relevance of necroptosis in the postischemic liver remains, however, unclear. The aim of this study was to analyze the role of programed necrosis during hepatic I/R. C57BL6 mice were subjected to warm hepatic I/R (90 min/240 min). The animals were pretreated with either the RIP1 inhibitor necrostatin-1 (Nec-1, 3.5 μg kg^{-1}) or vehicle (Nec-$1_{inactive}$, 3.5 μg kg^{-1}) administered systemically before ischemia. Sham-operated animals served as controls (n = 6 each group). The inflammatory response was evaluated by intravital microscopy. The hepatic transaminases alanine aminotransferase/aspartate aminotransferase in plasma as well as the activity of caspase-3 in tissue were determined as markers of hepatocellular injury. Leukocyte recruitment to the liver, sinusoidal perfusion failure, as well as the transaminase activities were strongly increased on I/R as compared with the sham-operated mice. Inhibition of the RIP1-dependent pathway with Nec-1, however, did not attenuate I/R-induced leukocyte migration, perfusion failure, and hepatocellular injury. Western blot analysis showed a baseline RIP1 expression in livers from sham-operated mice, whereas RIP1 expression was not detectable in both Nec-1–treated and vehicle-treated I/R group. Caspase-3 activity was significantly elevated after I/R in both postischemic groups. Our *in vivo* data show that RIP1-mediated necroptosis is not present in the postischemic liver and that I/R-induced caspase activation is associated with loss of RIP1 expression. Because caspases are able to cleave RIP1, we hypothesize that I/R-triggered caspase activation negatively regulates necroptosis and, thereby, determines apoptosis as a preferred route of cell death after hepatic I/R.

KEYWORDS—Postischemic liver injury, cell death, necroptosis, microcirculation, inflammation, leukocyte migration, perfusion failure

INTRODUCTION

Ischemia-reperfusion (I/R) injury causes significant morbidity and mortality and occurs during organ transplantation, resection, trauma, and septical as well ashemorrhagical shock. Hepatic I/R injury represents the main reason for early organ failure on liver transplantation. Ischemia-reperfusion–induced injury is a complex cascade of events including different mechanisms of cell death caused by oxygen deprivation and adenosine triphosphate depletion during ischemia but also by activation of cells of the innate immune system via inflammatory responses during reperfusion. The understanding of the processes leading to different forms of liver cell death is essential for the development of effective interventions to prevent hepatocellular damage and liver failure on transplantation or major surgery.

The different types of cell death on I/R include spontaneous and programmed cell death with or without initiating an inflammatory response. These types can be defined by morphological or biochemical behavior of the cell. *Apoptosis* is defined by its morphological criteria, including cell shrinkage, nuclear DNA fragmentation, and membrane blebbing (1). Phosphatidylserine exposure on apoptotic cells facilitates phagocytic uptake mainly by macrophages, leading to programmed cell death without causing an immune response (2). Apoptosis can be initiated by extracellular mechanisms and from any membrane-defined organelle in the cell. In contrast to apoptosis, during *necrosis*, the disruption of the cell membrane leads to a release of cytoplasm contents into the surrounding environment, finally resulting in inflammation (3). *Programmed necrosis*, also known as *necroptosis*, was first postulated in 1998. Necroptosis is a caspase-independent programmed cell death leading to cell swelling and membrane breakdown, resulting in morphology reminiscent of passive nonregulated necrosis, including an inflammatory response by the release of intracellular contents (4). It was shown that simultaneous caspase inhibition and tumor necrosis factor (TNF) stimulation in L929 fibrosarcoma cells undergo necrosis-like cell death (5, 6). Necroptosis is a strongly regulated process unlike necrosis and depends on the kinases receptor-interacting protein 1 (RIP1) and RIP3 (7, 8). Death receptors are able to initiate necroptosis under conditions that are insufficient to trigger apoptosis. Receptor-interacting protein 1 kinase activity and subsequent recruitment of RIP3 are necessary for the induction of necroptosis. Mixed lineage kinase domain-like protein (MLKL) was identified as a key component downstream of RIP3 in the execution of RIP1-mediated necroptosis *in vitro*. In contrast, activation of caspase-8 inhibits necroptosis

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by cleavage of RIP1. Necrostatin-1 (Nec-1) is a known specific kinase inhibitor of RIP1 and was shown to stop the initiation of necroptosis (9, 10).

Recent studies have shown a prominent role of necroptosis after cardiac, renal, and cerebral I/R (9, 11, 12). The relevance of programmed necrosis in the postischemic liver remains, however, unclear. In this study, we tested the hypothesis that, during hepatic I/R, blockade of the RIP-1–dependent pathway by using Nec-1 attenuates necrotic injury and inflammation *in vivo*.

MATERIALS AND METHODS

Animals

For experiments, 5- to 7-week-old female C57BL/6 wild-type mice (Charles River, Sulzfeld, Germany) were used. All experiments were carried out according to the German legislation on protection of animals.

Surgical procedure and experimental protocol

The surgical procedure was described elsewhere (13). Briefly, under inhalation anesthesia with isoflurane-N_2O, a polypropylene catheter was inserted into the left carotid artery in a retrograde direction for measurement of mean arterial pressure and application of fluorescence dyes, as described previously. A warm (37°C) reversible ischemia of the left liver lobe was induced for 90 min by clamping the supplying nerve vessel bundle using a microclip. Reperfusion time was 240 min in all experiments. A sham-operated group and two I/R groups were analyzed (n = 6 each): an I/R group treated with the inactive Nec-1 derivative Nec-$1_{inactive}$ (3.5 μg kg^{-1} body weight; Calbiochem, Darmstadt, Germany) and an I/R group treated with Nec-1 (3.5 μg kg^{-1} body weight; Sigma-Aldrich, Taufkirchen, Germany). Necrostatin-1 and Nec-$1_{inactive}$ were infused intra-arterially via the carotid catheter into the aortic arch 10 min before reperfusion.

Leukocyte (neutrophil)-endothelial cell interactions and sinusoidal perfusion failure

Intravital fluorescence microscopy was performed using a modified Leitz-Orthoplan microscope as described previously. Leukocytes were stained *in vivo* by rhodamine 6G (0.05%, 100 μL, i.a.; Sigma) and visualized in hepatic postsinusoidal venules using intravital fluorescence microscopy as described previously (14).

Thereafter, the plasma marker fluorescein isothiocyanate–conjugated Dextran (molecular weight, 150,000; 0.1 mL; 5%; Sigma) was infused, and sinusoidal perfusion was analyzed using an I2/3 filter block in sinusoids within six to nine acini. Intravital microscopy was started after 240 min of reperfusion and lasted approximately 20 min.

All videotaped images were quantitatively analyzed offline using Capimage software (Zeintl, Heidelberg, Germany). Rolling leukocytes were defined as cells crossing an imaginary perpendicular through the vessel at a velocity significantly lower than the centerline velocity in the microvessel. Their numbers are given as cells per second per vessel cross section. Leukocytes firmly attached to the endothelium for more than 20 s were counted as permanently adherent cells and were quantified as the number of cells per square millimeter of endothelial surface calculated from the diameter and length of the vessel segment observed. The sinusoidal perfusion failure was calculated as the percentage of nonperfused sinusoids of all sinusoids visible.

Liver enzymes

Blood samples were taken from the carotid artery at the end of the experiment, immediately centrifuged at 2,000*g* for 10 min, and stored at −80°C. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined at 37°C with an automated analyzer (Hitachi 917; Roche-Boehringer, Mannheim, Germany) using standardized test systems (HiCo GOT and HiCo GPT; Roche-Boehringer).

Caspase-3 activity

Frozen liver tissue was homogenized with 1 mL of ice-cold lysis buffer per 100 mg of liver tissue containing 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.1% CHAPS, and 0.1 mM EDTA. After filtration, the homogenates were centrifuged and the protein concentration of the supernatant was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Schwerte, Germany). Twenty micrograms of protein were used in a final volume of 200 μL in a 96-well plate with 50 μM of the selective substrate for caspase-3, caspase-7, and caspase-8 acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid 7-amino-4-methylcoumarin (Ac-DEVD-AMC; PeptaNova, Sandhausen, Germany) in the presence or absence of 10 μM of the specific caspase-3, caspase-7, and caspase-8 inhibitor acetyl-L-aspartyl-L-glutaminyl-L-threonyl-L-aspart-1-al (Ac-DEVD-CHO; PeptaNova) in substrate buffer including 50 mM HEPES, pH 7.5, 1% sucrose, 0.1 % CHAPS, and 10 mM dithiothreitol. The amount of the fluorescent AMC released was measured by fluorometry (Tecan Infinite F200, Männedorf, Switzerland) with 360 nm excitation and 430 nm emission filters. Data are expressed as change in fluorescence (ΔF) * min^{-1} * μg^{-1}.

Western blotting

Small pieces of liver tissue were homogenized in 1 mL of ice-cold lysis buffer (30 mM Tris/HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) including protease inhibitor cocktail. After 10-min incubation on ice and 10-min centrifugation (10,000*g*), the supernatant was collected. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific). Twenty micrograms of total lysates were run on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After blocking with 5% (wt vol^{-1}) milk powder in 1× PBS including 0.1% Tween, membranes were incubated with mouse-IgG2a-RIP1 (BD, Heidelberg, Germany), goat-IgG-MLKL (Santa Cruz Biotechnology, Dallas, Tex), or rabbit-IgG-GAPDH (Cell Signaling Technology, Beverly, Mass), followed by incubation with goat anti–rabbit IgG–horseradish peroxidase (Santa Cruz Biotechnology) and donkey anti–goat IgG–horseradish peroxidase (Santa Cruz Biotechnology). Signal was visualized with Wasabi software (Wasabi Systems, Norfolk, VA) and an Iluminizer (Hamamatsu Photonics, Hamamatsu, Japan) using chemiluminescence.

Statistical analysis

Analysis of variance on ranks followed by Student-Newman-Keuls methods was used for the estimation of stochastic probability in intergroup comparison (SigmaPlot 12; Jandel Scientific, Erkrath, Germany). Mean values ± SEM are given. Values of $P < 0.05$ were considered significant.

RESULTS

Leukocyte-endothelial cell interactions

Leukocyte (neutrophil)–endothelial cell interactions were analyzed in postsinusoidal venules as a sign of hepatic inflammation after hepatic I/R. Leukocytes were labeled by systemic application of rhodamine 6G. As shown in Figure 1, the numbers of rolling and firmly adherent leukocytes in postsinusoidal venules were very low in sham-operated mice, whereas they were dramatically enhanced after 90 min of ischemia followed by 240 min of reperfusion in mice treated with Nec-$1_{inactive}$ (21 ± 2 rolling leukocytes mm^{-1} * s and 215 ± 22 adherent leukocytes mm^{-2}) . Inhibition of RIP1 using Nec-1, however, did not reduce the postischemic inflammatory reaction because leukocyte migration did not significantly differ between I/R groups with Nec-1 and Nec-$1_{inactive}$.

Sinusoidal perfusion failure

Ischemia-reperfusion injury is associated with a severe deterioration of tissue perfusion leading to tissue hypoxia during reperfusion (“no-reflow phenomenon”). Sinusoidal perfusion failure was determined using *in vivo* microscopy as a recognized parameter of microvascular I/R injury. In the I/R group treated with Nec-$1_{inactive}$ as vehicle, about 20% of all sinusoids were not perfused (Fig. 2). In the Nec-1–treated I/R group, the perfusion failure was not improved compared with that in the vehicle-treated group.

Liver enzyme activity

The serum activity of hepatic transaminases was measured as a marker of cell integrity quantifying the extent of hepatocellular necrotic injury. Hepatic I/R (90 min/240 min) increased dramatically the activity of AST and ALT in the vehicle-treated group (AST, 9,758 ± 2,408 U/L; ALT, 2,072 ± 657 U/L) as compared with that in the sham-operated group. In line with the

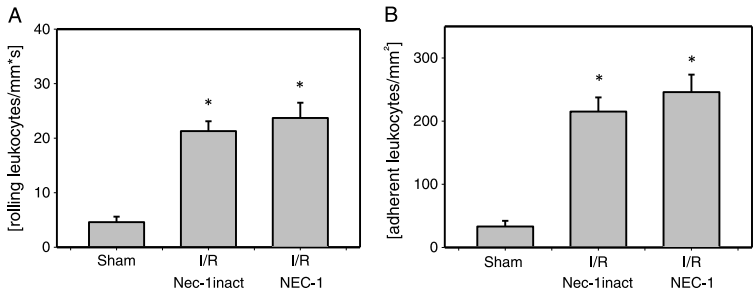

FIG. 1. **Leukocyte–endothelial cell interactions**. Leukocyte rolling and adherence were quantified using intravital microscopy in postsinusoidal venules of sham-operated mice, mice after I/R (90 min/240 min) treated with Nec-$1_{inactive}$, and mice after I/R treated with Nec-1. n = 6 animals per group, mean ± SEM, *$P < 0.05$ vs. sham-operated group.

data on hepatic microcirculation, no protective effect of Nec-1 on necrotic injury could be detected (Fig. 3).

Detection of RIP1 expression in freshly isolated liver tissues

To investigate the *in vivo* presence of the molecular machinery that has been demonstrated to be required for the execution of RIP1-mediated necroptosis, Western blot analyses were performed to assess the expression of RIP1 and MLKL in lysates of homogenized liver tissue. Receptor-interacting protein 1, the key mediator of necroptosis, was expressed in livers from sham-operated mice. In both postischemic groups, however, no RIP1 expression was detectable (Fig. 4). Mixed lineage kinase domain-like protein is critically involved in the execution of RIP3/RIP1-mediated necroptosis.

As shown by Western blot, MLKL was slightly expressed in murine livers but did not differ between the sham-operated mice and mice treated with either NEC-1 or Nec-$1_{inactive}$ (Fig. 4). These results together suggest that necroptosis is not present in the liver after I/R.

Caspase-3 activity

It is well known that activated caspase-8 triggers apoptosis via cleavage of caspase-3. In addition, it might attenuate necroptosis by cleavage of RIP1. The caspase-3 activity was determined in homogenates of liver tissue from all experimental groups. The caspase-3 activity was significantly increased in both I/R groups after 240 min of reperfusion (I/R + Nec-1, 1.8 ± 0.2 $\Delta F * min^{-1} * \mu g^{-1}$; I/R + Nec-$1_{inactive}$, 2 ± 0.3 $\Delta F * min^{-1} * \mu g^{-1}$) as compared with the sham-operated group. Treatment with Nec-1, however, did not affect caspase-3 activity, which remained on the same level as detected in the vehicle-treated I/R group (Fig. 5).

DISCUSSION

Hepatic I/R is the most common cause of organ dysfunction and failure after liver transplantation; therefore, strategies to minimize the negative effects of ischemia are now in the forefront of clinical and experimental studies (15). Different modes of cell death play a role in hepatic I/R injury. The ultimate goal of understanding mechanisms of cell death is to prevent tissue injury in hepatic I/R. For a successive therapy of hepatic I/R injury, it is important to know which mode of cell death: apoptosis, necrosis, or a mixed form of cell death is occurring. Ischemia-reperfusion is a complex process that results in hepatic cell death via a combination of necrosis and apoptosis (16). Apoptosis is the main form of cell death on hepatic I/R (17, 18). Necrotic cell death is also a prominent feature of I/R injury in the liver and is thought to be a much more inflammatory mode of cell death as compared with apoptosis. Because of the rupture of the plasma membrane, necrosis results in the release of cellular constituents in the extracellular environment, which can elicit an inflammatory response. Recently, there has been renewed interest in necrotic cell death in the context of I/R injury because RIP1 has been implicated in programmed necrosis and is modifiable by kinase inhibition. Necrostatin-1 is a well-known inhibitor of the kinase domain of RIP1 and thus in preventing necroptosis (9, 10). In cardiac, renal, and brain I/R injury, it has already been shown that the inhibition of RIP1 by Nec-1 attenuates necrotic cell death (9, 11, 12). However, the role of necroptosis in hepatic I/R injury has remained unclear. Tumor necrosis factor-α is the best trigger described for RIP1-mediated necroptosis (4) and also plays a major role in mediating hepatic I/R. It was shown that the inhibition of TNF-α protects against hepatic I/R injury in rats (19). Here, we tested the hypothesis that Nec-1 exerts a protective effect on hepatic I/R injury and attenuates the inflammatory response *in vivo*.

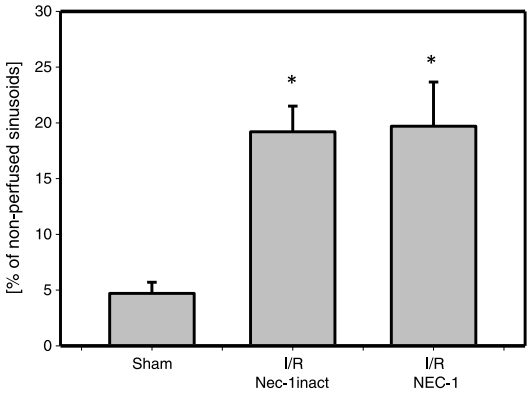

FIG. 2. **Sinusoidal perfusion failure**. Sinusoidal perfusion failure (= percentage of nonperfused sinusoids) was measured using intravital microscopy as a parameter of microvascular hepatic injury in sham-operated mice, mice after I/R (90 min/240 min) treated with Nec-$1_{inactive}$, and mice after I/R treated with Nec-1. n = 6 animals per group, mean ± SEM, *$P < 0.05$ vs. sham-operated group.

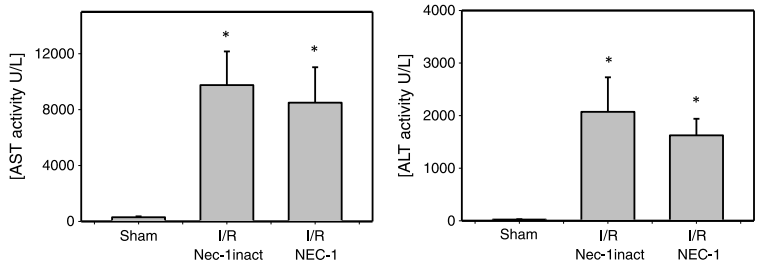

FIG. 3. **Liver enzyme activity**. Serum activity of the liver enzymes AST (black bars) and ALT (white bars) was determined as a marker of hepatocellular necrotic injury in sham-operated mice, mice after I/R (90 min/240 min) treated with Nec-$1_{inactive}$, and mice after I/R treated with Nec-1. n = 10 animals per group, mean ± SEM, $^{*}P < 0.05$ vs. sham-operated group.

Our results show that, unlike in other organs, Nec-1 has no protective effect on cell death induction in I/R injury in the liver. Plasma activity of the liver enzymes ALT and AST was used as a parameter of liver necrosis and strongly increased on 240 min of reperfusion compared with that in the sham group. However, treatment with Nec-1 did not alter ALT and AST levels in a significant manner and was even slightly increased.

A hallmark feature of hepatic inflammation during I/R is recruitment of various types of leukocytes to the afflicted site. Inflammatory stimuli activate endothelial cells to express adhesion molecules and chemokines that physically engage circulating leukocytes and promote their adhesion to the vessel wall. The initial interaction of neutrophils with hepatic endothelium (rolling) is mediated by selectins, whereas the firm adhesion is triggered by the interaction between beta2-integrins of leukocytes and intercellular adhesion molecule 1 on endothelial cells (20, 21). The next step is transendothelial and interstitial migration toward the stimuli from the damaged cells (22, 23). In our study, we analyzed recruitment of leukocytes in the hepatic microvasculature using intravital microscopy. We could observe a dramatic increase in the number of rolling and adherent leukocytes already after 240 min of reperfusion. However, treatment with Nec-1 did not affect leukocyte migration after 240 min. These results are in line with the data on hepatocellular injury.

Next, our data show that postischemic sinusoidal perfusion increases strongly on I/R. Hepatic microcirculatory perfusion failure is a determinant of liver dysfunction after I/R (24). The postischemic shutdown of the hepatic microcirculation is triggered by sinusoidal narrowing caused by endothelial cell edema, (25), stellate cell–mediated vasoconstriction (26, 27), or by activated Kupffer cells. In addition, inflammation- and injury-associated adherence of leukocytes in outflow venules may alter sinusoidal perfusion because of an increase of blood viscosity (28) and, hence, vascular resistance (29). Furthermore, perfusion failure in sinusoids is thought to be caused by sluggish blood flow, intravascular hemoconcentration, and procoagulant conditions (30). Administration of Nec-1, however, does not show any effect on sinusoidal perfusion failure.

A surprising result of our study is that the RIP1 is not expressed in the liver after I/R. Also, MLKL levels did not differ between the experimental groups. Necroptotic cell death is also naturally suppressed by caspase-mediated cleavage of RIP1. In this case, apoptosis seems to be the preferred route of cell death. In our studies, RIP1 was found completely absent on I/R, which was in correlation with the measured increase in caspase activity.

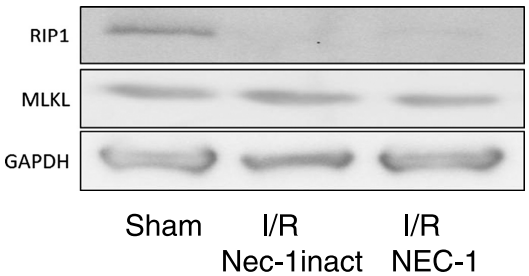

FIG. 4. **Liver expression of RIP1 and MLKL**. Representative Western blots showing RIP1 and MLKL expression in sham-operated mice, mice after I/R (90 min/240 min) treated with Nec-$1_{inactive}$, and mice after I/R treated with Nec-1. GAPDH was used as a loading control. See Figure, Supplemental Digital Content 1, at http://links.lww.com/SHK/A294.

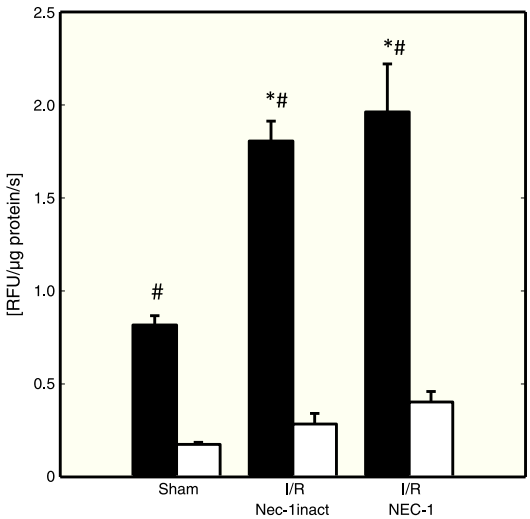

FIG. 5. **Caspase-3 activity in liver tissue**. Caspase-3 activity was determined in tissue homogenates of sham-operated mice, mice after I/R (90 min/240 min) treated with Nec-$1_{inactive}$, and in mice after I/R treated with Nec-1 (closed bars). Open bars show internal controls upon incubation of tissue homogenates from all groups with caspase inhibitor Ac-DEVD-CHO. n = 6 animals per group, mean ± SEM, $^{*}P < 0.05$ vs. sham-operated group, $^{\#}P < 0.05$ vs. internal controls.

Our results suggest that necroptosis is prevented by caspase activity in the liver. It was already shown in mice lacking Casp8 specifically in hepatocytes (Casp8Δhepa) that loss of Casp8 prevents proteolytic cleavage of RIP1 in hepatocytes (31).

Hepatic RIP1 expression was analyzed in several recent studies and can be altered by different agents and mechanisms. These studies have shown that RIP1 is upregulated during sepsis via Fas-L (32), during acetaminophen-induced liver failure via TNF-α (33), in gall bladder cancer cells (34), and is detectable during concavalin A–induced hepatitis (35). In contrast, ethanol intoxication did not affect hepatic RIP1 expression (36). Even though not in the liver, the study by Newton et al. (37) provides important information describing ubiquitination of RIP1 via the TNF receptor 1–dependent pathway within minutes after TNF-α stimulation *in vitro*. As is well understood, TNF-α is one of the key mediators of hepatic I/R released from Kupffer cells immediately after the onset of reperfusion. Thus, this study might give an additional explanation of the lack of RIP-1 expression in the postischemic liver.

Finally, it cannot be excluded that RIP3 alone may be able to execute programmed necrosis. However, there are no data in the literature about the role of RIP3 for necroptosis induction during hepatic I/R injury so far.

Our *in vivo* data show that i) RIP1-mediated necroptosis is not present in the postischemic liver and ii) I/R-induced caspase activation is associated with loss of RIP1 expression in the liver. Because caspases are able to cleave RIP1, we hypothesize that I/R-triggered caspase activation negatively regulates necroptosis and, thereby, determines apoptosis as a preferred route of cell death after hepatic I/R.

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Abbildungsverzeichnis

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