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**Rolle der angeborenen Immunantwort in bakteriellen und sterilen
Entzündungen**

Habilitationsschrift

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Cell. 2014 Jan 30;156(3):456-68. doi: 10.1016/j.cell.2014.01.006. (Schiwon, Weisheit et al., 2014).

2. Ly6C(low) and not Ly6C(high) macrophages accumulate first in the heart in a model of murine pressure-overload.

Weisheit C, Zhang Y, Faron A, Köpke O, Weisheit G, Steinsträsser A, Frede S, Meyer R, Boehm O, Hoeft A, Kurts C, Baumgarten G.

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3. CX3CR1 is a prerequisite for the development of cardiac hypertrophy and left ventricular dysfunction in mice upon transverse aortic constriction.

Weisheit CK, Kleiner JL, Rodrigo MB, Niepmann ST, Zimmer S, Dürr GD, Coburn M, Kurts C, Frede S, Eichhorn L.

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4. Characterization of transverse aortic constriction in mice based on the specific recruitment of leukocytes to the hypertrophic myocardium and the aorta ascendens.

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Meinem Mann Gunnar

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Abkürzungsverzeichnis

BNP:	<i>Brain natriuretic peptide</i>
CD:	<i>Cluster of Differentiation</i>
CO ₂ :	Kohlendioxid
DAMP:	<i>Damage- associated molecular patterns</i>
DC:	Dendritische Zellen
FACS:	<i>Fluorescence Activated Cell Sorting</i>
FKS:	Fetales Kälberserum
FP:	FACS- Puffer
GFP:	<i>Green fluorescent protein</i>
ICAM- 1:	<i>Intracellular adhesion molecule- 1</i>
IL:	Interleukin
Mac-1:	<i>Macrophage antigen- 1</i>
MCP-1/ -3:	<i>Monocyte chemoattractant protein- 1/ -3</i>
MDP:	<i>Macrophage/ Dendritic Cell Progenitor</i>
MHC:	<i>Major histocompatibility complex</i>
PAMP:	Pathogen- assoziierte molekulare Muster
PBS:	<i>Phosphate buffered saline</i>
PFA:	Paraformaldehyd
PMN:	Polymorphkernie Leukozyten, entspricht neutrophilen Granulozyten
PRR:	<i>Pattern recognition receptor</i>
TAC:	<i>Tranverse aortic constriction</i>
TGFβ:	<i>Transforming growth factor β</i>
TLR:	<i>Toll-like receptor</i>
TNF-α:	Tumornekrosefaktor α
VEGF:	<i>Vascular endothelial frowth Factor</i>

1 Einleitung

1.1 Das Immunsystem

Der menschliche Organismus ist durch eine Reihe verschiedener Effektorzellen und Moleküle, die zusammen das Immunsystem bilden, vor Krankheitserregern und den Schäden, die sie verursachen, sowie vor anderen schädlichen Substanzen wie etwa Toxinen geschützt. Um einen Organismus effektiv vor Krankheiten zu schützen, muss das Immunsystem eine Vielzahl an Aufgaben bewältigen. Zum einen muss das Vorhandensein einer Infektion immunologisch erkannt werden. Zum anderen muss die Infektion eingedämmt und wenn möglich vollständig abgewehrt werden. Gleichzeitig sollte das Immunsystem fein-justierend reagieren, damit es dem Körper keinen Schaden zufügt. Die Immunregulation ist daher ein wichtiges Merkmal aller Immunantworten. Darüber hinaus sorgt das Immunsystem bei vielen Erkrankungen dafür, dass das Individuum vor einem erneuten Auftreten der Krankheit geschützt ist, wenn derselbe Krankheitserreger die Ursache darstellt. Grundlage hierfür ist das einzigartige Merkmal des adaptiven Immunsystems, dass es ein immunologisches Gedächtnis entwickeln kann (Murphy et al., 2009).

Die immunologische Kompetenz von Organismen setzt sich grundsätzlich aus der angeborenen und der adaptiven (spezifischen oder erworbenen) Immunabwehr zusammen. Beide Teile sind eng miteinander verknüpft. Die angeborene Immunabwehr ist auch ohne Antigenkontakt bereits funktionsfähig und kann in ihrer Wirksamkeit durch den Einfluss der adaptiven Immunabwehr gesteigert werden. Sie besteht vor allem aus Makrophagen, neutrophilen Granulozyten und natürlichen Killerzellen, während die Hauptvertreter der adaptiven Immunantwort B- und T-Zellen sind. Sowohl die Zellen der angeborenen als auch der adaptiven Immunantwort entwickeln sich aus hämatopoetischen Stammzellen im Knochenmark. Aus diesen pluripotenten Stammzellen gehen alle Blutzelltypen hervor. Die gemeinsame myeloide Vorläuferzelle ist die Vorstufe der Makrophagen, Granulozyten, Mastzellen und dendritischen Zellen, sowie der Megakaryocyten und roten Blutkörperchen (Alberts et al., 2007; Murphy et al., 2009).

Das adaptive Immunsystem

Die adaptive Immunität ist evolutionsbiologisch relativ spät entstanden. Die Komplexität der Vorgänge im adaptiven Immunsystems ist hoch, da zum Beispiel der Mechanismus der Herstellung von Rezeptormolekülen im adaptiven Immunsystem eine große Variabilität und Neuordnung von Rezeptorgensegmenten voraussetzt. Mit Hilfe dieser großen Variabilität kann das adaptive Immunsystem eine spezifische Erkennung von fremden Antigenen ermöglichen, ein immunologisches Infektionsgedächtnis entwickeln und pathogenspezifische Adapterproteine bereitstellen. Die adaptive Immunantwort ist jedoch auch für Allergien, Autoimmunität und die Abstoßung von Gewebetransplantaten verantwortlich. B- und T-Lymphozyten vermitteln die adaptive Immunabwehr, die durch eine hohe Spezifität und die Ausbildung eines immunologischen Gedächtnisses charakterisiert ist. Sie lässt sich noch weiter in die zelluläre und die humorale Immunantwort unterteilen. Die zellvermittelte Immunantwort erfolgt hauptsächlich durch die T-Lymphozyten, die in zwei große Gruppen eingeteilt werden können: die T-Helferzellen, die Zytokine sezernieren und die zytotoxischen Lymphozyten, die durch spezielle Mechanismen andere Zellen abtöten können. Das humorale Immunsystem basiert auf den von B-Lymphozyten ausgeschütteten Antikörpern, die frei zirkulieren und körperfremde Strukturen wie Bakterien oder Toxine markieren und inaktivieren (Alberts et al., 2007; Murphy et al., 2009). Da in dieser Arbeit der Fokus auf der Antwort des angeborenen Immunsystems liegt, bleibt die Auseinandersetzung mit dem adaptiven Teil des Immunsystems verkürzt.

Das angeborene Immunsystem

Das angeborene Immunsystem ist aus evolutionärer Sicht ein stark konserviertes Abwehrsystem des Wirts, dessen Merkmale zwischen Pflanzen, Wirbellosen und Säugetieren geteilt werden (Gasteiger et al., 2017). Drei wichtige phagozytierende Zellarten gehören zu diesem Teil des Immunsystems: Makrophagen, neutrophile Granulozyten und dendritische Zellen (DC). Die Zellen des angeborenen Immunsystems entwickeln sich im Knochenmark aus einer gemeinsamen myeloiden Vorläuferzelle. Zusätzlich zu ihrer Fähigkeit, Partikel phagozytieren zu können, sezernieren die Zellen des angeborenen Immunsystems chemische Botenstoffe wie Chemokine und Zytokine, die das Entzündungsgeschehen maßgeblich mitbeeinflussen können (Sokol and Luster,

2015). Die Zellen des angeborenen Immunsystems besitzen ein Rezeptorrepertoire, sogenannte Mustererkennungsrezeptoren (*Pattern Recognition Receptors*, PRR), die spezifische Pathogen-assoziierte molekulare Muster (PAMP) erkennen. Die Interaktion zwischen PRR und PAMPs steuert die weiterführende Immunreaktion, die unter anderem durch die Freisetzung proinflammatorischer Zytokine charakterisiert ist (Kawai and Akira, 2010; Kumar et al., 2009).

Neben der unmittelbaren Abwehr von Pathogenen, ist das angeborene Immunsystem mit seinen Zellen an der Entwicklung eines immunologischen Gedächtnisses durch das adaptive Immunsystem beteiligt (Iwasaki and Medzhitov, 2015). Hier zeigt sich eine Überschneidung zwischen den antigenpräsentierenden Zellen des angeborenen Immunsystems und den T-Zellen des adaptiven Immunsystems.

Wenn es einem Mikroorganismus gelingt, die angeborene Abwehr des Wirts zu umgehen oder auszuschalten, einen lokalen Infektionsherd zu bilden und sich in einer Weise zu vermehren, dass eine weitere Ausbreitung im Körper möglich ist, entsteht eine Erkrankung. Die Ausbreitung von Krankheitserregern wird häufig von einer Entzündungsreaktion begleitet, die aus der Freisetzung von Signalmolekülen, Zytokinen und Chemokinen und der Rekrutierung weiterer Effektorzellen aus der Zirkulation besteht (Alberts et al., 2007; Murphy et al., 2009).

Monozyten spiegeln mit ihrem 10%igen Anteil im menschlichen Blut ihre Aufgabe als wichtige Akteure des angeborenen Immunsystems wider (Geissmann et al., 2010a). Im Blut und in der Milz stellen Monozyten das Reservoir dar, aus welchem diese Zellen insbesondere im Rahmen von Entzündungsprozessen in das betroffene Gewebe auswandern und dort zu Makrophagen oder sogenannten inflammatorischen dendritischen Zellen ausdifferenzieren (Robbins and Swirski, 2010). Vor allem durch ihre phagozytotische Aktivität und die Sekretion einer Vielzahl von Zytokinen sind sie in der Lage Entzündungsprozesse zu beeinflussen. In unterschiedlichen Geweben zeigen Monozyten/Makrophagen funktionelle und phänotypische Unterschiede. Ihre weitere Differenzierung und ihre Migration stehen in direktem Zusammenhang mit dem sie umgebenden Milieu (Murray and Wynn, 2011).

Im Wesentlichen werden in der Maus wie im Menschen auch entsprechend der spezifischen Antigenmuster zwei Monozyten- und Makrophagenpopulationen definiert:

Gr1⁺/Ly6C^{high}CCR2^{high}CX3CR1^{low} Monozyten und GR1⁻/Ly6C^{low} CCR2⁻CX3CR1^{high} Monozyten (Libby et al., 2013). In einer Reihe von Infektionsmodellen konnte gezeigt werden, dass Gr1⁺/Ly6C^{high} Monozyten sich zu unterschiedlichen Makrophagen- und DC-Populationen entwickeln können, die Inflamationsprozesse vermitteln (Geissmann et al., 2010b). Sie werden daher auch als inflammatorische Monozyten bezeichnet und zu einem frühen Zeitpunkt der Entzündung rekrutiert (Nahrendorf et al., 2010). GR1⁻/Ly6C^{low}-Monozyten befinden sich vor allem in den Blutgefäßen, wo sie eine patrollierende Funktion innehaben und für die Phagozytose von potentiellen Pathogenen zuständig sind (Auffray et al., 2007). Im Rahmen von Gewebeschäden und Entzündungsprozessen sind sie in der Lage, die Blutgefäße zu verlassen und CX3CR1-Fraktalkin-abhängig rasch das betreffende Gewebe zu erreichen und ausdifferenziert die Immunantwort zu steuern (Auffray et al., 2007).

Die in dieser kumulativen Habilitation zusammengeführten Arbeiten verdeutlichen, dass die Aufrechterhaltung der Entzündungsreaktion im Gewebe, sowohl in der bakteriellen Infektion, als auch in der sterilen kardialen Inflammation, auf die Rekrutierung von angeborenen Immunzellen durch die Freisetzung von Signalmolekülen wie Zytokinen und Chemokinen angewiesen ist. Außerdem lassen sich die Herausforderungen im Verständnis der komplexen, teilweise von Gewebe zu Gewebe variierenden Koordination der Immunantwort nachvollziehen (Muller, 2013). Die in diesen Arbeiten untersuchten Prozesse der Immunzellaktivierung, -migration und -rekrutierung bieten interessante Ansätze für die Etablierung immunmodulatorischer Therapien. Im weiteren Einleitungstext werde ich daher auf die Immunantwort der bakteriellen Infektion und der sterilen kardialen Inflammation näher eingehen.

1.2 Bakteriell induzierte Entzündungsreaktionen am Beispiel der Harnwegsinfektion

Harnwegsinfektionen zählen zu den häufigsten ambulant erworbenen Infektionen. Mit Ausnahme eines Anstiegs bei jungen Frauen im Alter von 14 bis 24 Jahren nimmt die Prävalenz von Harnwegsinfektionen mit dem Alter zu. Zwischen 50% und 60% der erwachsenen Frauen werden mindestens eine Harnwegsinfektion in ihrem Leben erleiden, und fast 10% der postmenopausalen Frauen geben an, im Vorjahr eine Harnwegsinfektion erlitten zu haben (Medina and Castillo-Pino, 2019). In den USA gibt es

als Folge von kompliziert verlaufenden Harnwegsinfektionen, jährlich schätzungsweise 250.000 Fälle von Pyelonephritis, mit einer höheren Häufigkeit bei Frauen. Bei Frauen im Alter von 18 bis 49 Jahren beträgt die geschätzte Inzidenz von Pyelonephritis 28/10.000; 7% der Fälle erfordern eine Krankenhauseinweisung (Medina and Castillo-Pino, 2019). Uropathogene *Escherichia coli* (UPEC) Bakterien werden am häufigsten als Erreger identifiziert und eine Infektion mit UPEC erhöht die Wahrscheinlichkeit rezidivierender Infektionen (Murray et al., 2021).

Der Harntrakt umfasst die Nieren, die Harnleiter, die Blase und die Harnröhre und wird mit Ausnahme der Harnröhre größtenteils als steril bewertet. Der Schutz vor einer mikrobiellen Besiedlung wird durch verschiedene lösliche Faktoren, die in den Urin sezerniert werden, und durch anatomische Barrieren wie Uroplakine-Plaques und eine Schicht aus hydratisiertem Schleim unterstützt. Darüber hinaus ist der Harntrakt mit Epithelzellen und verschiedenen residenten Immunzellen ausgekleidet, die zusätzlich vor einer Infektion schützen. Diese Barrieren sollen verhindern, dass Krankheitserreger in die Harnwege gelangen und eine anhaltende Infektion aufbauen (Abraham and Miao, 2015). Die ableitenden Harnwege sind besonders anfällig für Infektionen, da sich die Harnröhrenöffnung in unmittelbarer Nähe zum Ende des Magen-Darm-Trakts befindet. Die dem Lumen zugewandte Schleimhautoberfläche der Blase ist von mehreren Epithelzellschichten, dem sogenannten Urothel, ausgekleidet. Diese drei bis sechs Zellschichten sind durch *Tight-Junctions* verbunden und bilden ein undurchdringliches Gewebe. Die Zellen, die in direktem Kontakt mit dem Lumen stehen, werden Deckepithelzellen genannt und sind von Uroplakin-Plaques bedeckt, die die physikalische Barrierefunktion gegenüber Flüssigkeiten, Toxinen und Mikroorganismen weiter stärken (Hickling et al., 2015; Lacerda Mariano and Ingersoll, 2020).

Das angeborene Immunsystem der Harnwege besteht aus verschiedenen residenten und rekrutierten Zellen, die eine Vielzahl von PRRs exprimieren – wie Toll-like Rezeptor 2 (TLR2), TLR4, TLR5 und TLR11 – die eine frühzeitige Erkennung der Krankheitserreger ermöglichen und diese Signale so weiterleiten, dass eine schnelle und robuste proinflammatorische Immunantwort aktiviert wird (Abraham and Miao, 2015).

Das Immunsystem der Blase ist bereits gut charakterisiert und das Vorhandensein einer Vielzahl an angeborenen Immunzellen deutet auf die Anpassung an die besonderen

Gegebenheiten durch häufige bakterielle Kontakte hin. Es besteht überwiegend aus phagozytierenden antigenpräsentierenden Zellen, wie dendritischen Zellen und Makrophagen. Weitere residente Immunzellen des Blasengewebes sind CD4⁺ und $\gamma\delta$ ⁺-T-Zellen, NK-Zellen, Monozyten, Eosinophile und Mastzellen (Mora-Bau et al., 2015). Phagozyten wie neutrophile Granulozyten und Makrophagen spielen eine wesentliche Rolle in der Immunantwort gegen bakterielle Infektionen. Makrophagen machen fast 40% aller CD45⁺-Immunzellen in der Blase von Mäusen aus. Makrophagen sind eine wichtige Quelle für die Freisetzung von Chemokinen wie CXCL1 und CXCL2, die Neutrophile bei bakteriellen Infektionen anlocken (De Filippo et al., 2013). Um die Auflösung der Entzündungsreaktion zu fördern, phagozytieren Makrophagen nicht nur Bakterien selber, sondern auch die apoptotischen Neutrophilen (Bratton and Henson, 2011). Im Gewebe finden sich Makrophagen unterschiedlichen Ursprungs. Es gibt residente Makrophagen, die das Gewebe überwachen und eingewanderte Monozyten, die zu Makrophagen differenzieren können (Bratton and Henson, 2011). Bei Infektionen werden aus dem Knochenmark CCL2/CCR2-abhängig Monozyten freigesetzt, die in das entzündete Gewebe einwandern und die Entzündungsreaktion unterhalten. Im Rahmen von bakteriellen Infektionen produzieren Makrophagen proinflammatorische Zytokine, darunter Tumornekrosefaktor (TNF) (Engel et al., 2008). Auch Neutrophile und Epithelzellen können TNF produzieren (Roulis et al., 2011; Theilgaard-Mönch et al., 2005), hierbei ist jedoch unklar, ob diese TNF-Quellen die bakterielle *Clearance* der phagozytierenden Immunzellen beeinflussen können. Unter einer TNF-Inhibitorthherapie, wie sie bei Autoimmunerkrankungen eingesetzt wird, treten gehäuft bakterielle Infektionen auf, auch die Inzidenz für bakterielle Harnwegsinfektionen steigt (Teixeira et al., 2016). Es ist bekannt, dass TNFs einen direkten Stimulus für den respiratorischen *Burst* der Neutrophilen darstellen, außerdem verstärken sie die Zelladhäsion und hemmen die Neutrophilenmigration (Ferrante, 1992).

Die Abwehr gegen bakterielle Harnwegsinfektionen hängt von den Neutrophilen ab, die von CXCR2-Liganden angelockt werden und sowohl die endotheliale als auch die epitheliale Migration vermitteln (De Filippo et al., 2013; Ley et al., 2007). Neutrophile sind die ersten Immunzellen, die bei einer Harnwegsinfektion in die Blase rekrutiert werden, und sie spielen eine vorherrschende Rolle bei der bakteriellen *Clearance*. Sie werden von verschiedenen chemotaktischen Stoffen an den Infektionsherd gelockt, die von

oberflächlichen Blasenepithelzellen, Makrophagen und Mastzellen nach Aktivierung von PRRs durch verschiedene bakterielle Produkte freigesetzt werden. In Mausmodellen von Harnwegsinfektionen konnten Neutrophile bereits zwei Stunden nach der Infektion im Urin nachgewiesen werden, und ihre Anzahl erreichte sechs Stunden nach Infektionen ihren *Peak* (Agace et al., 1995). Die Anzahl der Neutrophilen korreliert mit der bakteriellen Belastung und mit abnehmender Bakterienzahl nimmt auch die Zahl der Neutrophilen ab (Abraham and Miao, 2015). Aufgrund der Freisetzung reaktiver Sauerstoffspezies und anderer zytotoxischer Produkte sind aktivierte Neutrophile für eine erhebliche Toxizität gegenüber dem umgebenden Blasengewebe verantwortlich. Tatsächlich verursachen übermäßige Reaktionen der Neutrophilen – einschließlich der erhöhten Expression von Cyclooxygenase 2 (COX2; auch bekannt als PTGS2) – eine entzündliche Schädigung des Blasengewebes und prädisponieren die Blase für anhaltende Infektionen (Hannan et al., 2014). Neutrophile zirkulieren im Blut und migrieren durch das Gefäßendothel in das infizierte Gewebe (Kamenski et al., 2012; Kolaczowska and Kubes, 2013). Um Bakterien im Blasenlumen zu erreichen, durchqueren aktivierte Neutrophile alle Schichten des Blasenepithels. Eine endotheliale Neutrophilenmigration erfordert Liganden der Chemokin-Rezeptoren CXCR1 und CXCR2, zu denen CXCL1, CXCL2 und CXCL6 gehören. Die Mechanismen der endothelialen Migration wurden bereits ausführlich untersucht (de Jong et al., 2019). Die Wanderung von Neutrophilen in epitheliales Gewebe, das die Grenzflächen von Barriereorganen nach außen darstellt (ein Beispiel stellen die Harnorgane dar) und einen bevorzugten Eintrittsort für Bakterien darstellt, wurde bisher hingegen wenig untersucht. Um Bakterien im Blasenepithel abzufangen, müssen Neutrophile die Basalmembran in Richtung Blase durchqueren. Die Basalmembran besteht hauptsächlich aus Kollagen IV (LeBleu et al., 2007), das von Metallomatrix-Proteinasen abgebaut werden kann (MMPs). Einige der MMPs können auch durch Chemokine reguliert werden, die z.B. von Immunzellen sezerniert werden.

1.3 Die sterile Entzündungsreaktion am Beispiel der links ventrikulären Hypertrophie

Herz-Kreislauf-Erkrankungen zählen weltweit zu den häufigsten Todesursachen. Trotz zahlreicher Therapieoptionen steigen Morbidität und Mortalität auf der Grundlage kardialer Erkrankungen weiter an (Balakumar and Jagadeesh, 2010). Besonders die

verschiedenen Formen von hämodynamischem Stress wie arterielle Hypertonie, Klappenvitien z. B. Aortenklappenstenosen sowie Myokardinfarkte, können durch eine chronische Erhöhung des intraventrikulären Drucks oder des Volumens den Herzmuskel langfristig schädigen. Es kommt zu einer pathologischen Hypertrophiereaktion des Myokards und konsekutiv zu einer Minderversorgung der Herzmuskelzellen mit Sauerstoff. Diese Konstellation kann zur myokardialen Dekompensation führen, die mit dem Untergang von Myozyten und der Fibrosierung des Muskelgewebes einhergeht und schließlich zum Herzversagen und zum Tode führt (Diwan and Dorn, 2007; Dorn, 2007). Das erwachsene menschliche Herz enthält ungefähr 4–5 Milliarden Kardiomyozyten. Da das Myokard über eine vernachlässigbare endogene Regenerationsfähigkeit verfügt, führt der Verlust von Kardiomyozyten zur Bildung einer Narbe. Die Gewebereparatur hängt von der Entzündungsreaktion ab, die dazu dient, das betroffene Gewebe von toten Zellen und Matrixtrümmern zu befreien aber auch wichtige molekulare Signale für die Aktivierung von reparativen Zellen zu liefern (Frangogiannis, 2012). Die Plastizität der Extrazellulärmatrix ist hierbei von entscheidender Bedeutung. Mit der Zeit führt unter anderem die Aktivierung von kollagenproduzierenden Fibroblasten zu einer Umstrukturierung der myokardialen Extrazellulärmatrix. Mit dem Gewebeumbau und vor allem der vermehrten Ablagerung von Kollagen Typ I geht eine zunehmende Versteifung des Myokards und somit eine weniger effektive Herzaktion, abnehmende Kontraktionskraft und Relaxationsgeschwindigkeit einher (Kania et al., 2009).

Untersuchungsergebnisse verdeutlichen, dass das kardiale Immunsystem auf einzigartige Weise an den physiologischen und pathophysiologischen Stress sowohl im gesunden Zustand als auch nach schweren Schädigungen angepasst ist (Epelman et al., 2015). Trotz der Vielfalt an pathologischen Reizen, die zu einer linksventrikulären Hypertrophie führen können, gibt es zahlreiche Gemeinsamkeiten in der darauffolgenden Reaktion des Herzgewebes. Neben einer Steigerung der Kardiomyozytenmasse, einer Sarkomerumlagerung und vermehrter extrazellulärer Matrixablagerungen, wurden insbesondere die lokale Entzündungsreaktion und die Immunzellaktivierung als gemeinsame Merkmale erkannt. Früher wurde die linksventrikuläre Hypertrophie als einfache Reaktion auf eine erhöhte Arbeitsbelastung angesehen. Heute ist bekannt, dass der Entwicklung dieser Erkrankung ein komplexer inflammatorischer und regenerativer

Prozess zugrunde liegt, der alle im Herzen vorhandene Zellarten und deren Wechselwirkungen mit zirkulierenden Zellen umfasst.

Das Herz enthält eine heterogene Population an Makrophagen, die sowohl im gesunden als auch im geschädigten Herzgewebe von Mensch und Maus vorhanden sind (Azzawi et al., 2005). Ähnlich wie Gewebemakrophagen in Gehirn und Leber, werden die meisten Makrophagen im Herzen aus embryonalem Dottersackgewebe und fetalen Lebervorläuferzellen gebildet. Residente Makrophagengruppen werden durch lokale Proliferation und in einem geringeren Maße auch durch die Rekrutierung von Monozyten aus der Zirkulation regeneriert (Epelman et al., 2014). Dies steht im Einklang mit Erkenntnissen, die zeigen, dass die meisten residenten Makrophagengruppen durch lokale Proliferation regeneriert werden (Frieler and Mortensen, 2015). Im Falle einer Gewebeschädigung werden zusätzlich Monozyten aus dem Blut rekrutiert und differenzieren im Gewebe zu Makrophagen (Hashimoto et al., 2013). Im Herzgewebe finden sich überwiegend nicht-inflammatorische gewebeständige Makrophagen, die den Oberflächenmarker Ly6C gar nicht oder nur sehr spärlich exprimieren (Pinto et al., 2012). Im Kontext einer Immunantwort nach einer Myokardschädigung ändert sich ihr Differenzierungsvermögen. Aus dem Blut einwandernde Monozyten werden zum Ort der kardialen Schädigung rekrutiert (Dutta and Nahrendorf, 2015). Nach einem Myokardinfarkt hat sich gezeigt, dass monozytäre Zellen nicht nur aus dem Knochenmark freigesetzt werden und einwandern, sondern auch aus Reservoirs in der Milz (Swirski et al., 2009).

Die Zellen des angeborenen Immunsystems erkennen über spezifische PRRs, und hier speziell über *Toll-like Rezeptoren* (TLR), Moleküle, die im Rahmen einer kardialen Gewebeschädigung freigesetzt werden, sog. *Damage associated molecular patterns* (DAMPs). Diese DAMPs können extrazelluläre Matrixkomponenten, Hitzeschockproteine oder mitochondriale DNA sein, die bei Gewebeverletzungen freigesetzt werden und das Immunsystem über TLRs aktivieren (Ehrentraut et al., 2015). Bei Mäusen kann eine DAMP-induzierte, sterile Schädigung durch eine druckinduzierte kardiale Hypertrophie oder einen Myokardinfarkt durch Hemmung der TLR4-Signalkaskade drastisch abgeschwächt werden (Mann, 2011) wodurch die Relevanz dieser Rezeptoren, die lange Jahre nur für die Erkennung von bakteriellen und viralen Pathogenen bekannt waren, im

Kontext steriler Entzündungsprozesse belegt wird. Studien zeigen, dass die Zellen des angeborenen Immunsystems eine erhebliche Rolle bei der Entstehung und Entwicklung der linksventrikulären Hypertrophie und dem weiteren Gewebeumbau spielen (Frieler and Mortensen, 2015). Aktuelle Studien zur Einzelzell-RNA-Sequenzierungsanalysen haben gezeigt, dass die Mehrheit der angeborenen Immunzellen, einschließlich Mastzellen, Monozyten und Makrophagen, Neutrophiler, dendritischer Zellen, Eosinophiler und natürlicher Killer-T Zellen bei Mäusen durch eine linksventrikuläre Drucküberlastung umfassend aktiviert werden (Martini et al., 2019).

Für die kardiale Immunantwort wesentlich ist auch die Induktion von Chemokinrezeptoren (Frangogiannis, 2007). Chemokine liefern hier wichtige Signale für die Rekrutierung und Extravasation von Leukozyten-Populationen in das Myokard. Sowohl CC-Chemokine, die als potente chemotaktische Stoffe für mononukleäre Zellen fungieren, als auch ELR-Motiv enthaltende CXC-Chemokine, die Neutrophile anziehen, werden im Herzgewebe nach einem Myokardinfarkt deutlich hochreguliert. Durch Interaktionen mit Chemokinrezeptoren, die von Leukozyten-Subpopulationen exprimiert werden, bestimmt das Chemokin-Expressionsprofil im geschädigten Myokard die Zusammensetzung des leukozytären Infiltrats. Neutrophile werden sehr früh nach einer Gewebeschädigung ins Myokard rekrutiert, es folgen proinflammatorische Monozyten und Lymphozyten (Nahrendorf et al., 2007).

Die Transmigration von Leukozyten in das geschädigte Myokard erfordert die Interaktion mit aktivierten Endothelzellen und verschiedenen Adhäsionsmolekülen. Die einzelnen Schritte bei der Emigration von Leukozyten werden durch Interaktionen zwischen spezifischen Molekülen auf Leukozyten und ihren Gegenrezeptoren auf Endothelzellen gesteuert. Der Kontakt von Chemokinen mit Chemokinrezeptoren auf den Monozyten überträgt Signale innerhalb der Zelle, die zur Aktivierung von Leukozyten-Integrinen führen. Integrine sind eine Familie von heterodimeren Adhäsionsrezeptoren, deren Aktivierung zu einer Konformationsänderung führt, die die Bindung der Liganden an extrazelluläre Matrixproteine begünstigt (Hynes, 1992).

Die meisten Leukozyten besitzen Integrine der $\beta 2$ -(CD18)-Familie, deren Liganden von Endothelzellen exprimiert werden. Insbesondere binden sie an die interzellulären Adhäsionsmoleküle 1 und 2 (ICAM-1 und ICAM-2) (Meerschaert and Furie, 1995). Nach

der Aktivierung binden Leukozyten-Integrine fest an ihre Liganden auf Endothelzellen, sodass die Leukozyten auf dem Endothel arretieren. Hierbei spielt PECAM-1 (CD31), das auf verschiedenen Immunzellen und besonders stark auf Endothelzellen exprimiert wird, eine Rolle und hilft Leukozyten bei der transendothelialen Migration.

Adhärente Leukozyten wandern zu nahegelegenen Endothelgrenzen, um sich auf die Extravasation vorzubereiten. Die intraluminale Wanderung wird durch eine Untergruppe von Leukozyten-Integrinen und Endothelzell-Adhäsionsmolekülen vermittelt u.a. Mac-1 (CD11b/CD18), das mit ICAM-1 interagiert und PECAM-1 (Muller, 2013; Privratsky and Newman, 2014). Im Kontext der Monozytenmigration und –adhäsion bei kardialen Entzündungsprozessen spielt zudem ein besonderer Chemokinrezeptor, der Fraktalkinrezeptor, CX3CR1, eine wichtige Rolle. Die Interaktion von CX3CR1 mit seinem Liganden Fraktalkin (CX3CL1), ist für die Rekrutierung von CX3CR1⁺ Monozyten von großer Bedeutung (Imai et al., 1997). Darüber hinaus kann die Oberflächenexpression von CX3CR1 herangezogen werden, um zwischen verschiedenen Monozytenpopulationen in der Maus zu unterscheiden. Man unterscheidet auch im Herzgewebe zwei Arten von Makrophagen, auch diese Makrophagenpopulationen werden anhand der Expression von CX3CR1 und des Chemokinrezeptor Typ 2 (CCR2) unterschieden. Differenziert werden die Makrophagen mit Hilfe der Ly6C-Expression in proinflammatorische Ly6C^{high} (CCR2^{high}, Cx3CR1^{low}) und residente Ly6C^{low} (CCR2^{low}, CX3CR1^{high}). Eine chronische Drucküberlastung induziert eine mononukleäre Zellreaktion, die durch konstant hohe Zahlen an Ly6C^{low} CX3CR1^{high} Makrophagen gekennzeichnet ist (Weisheit et al., 2014).

Dendritische Zellen stellen eine weitere Untergruppe der Leukozyten dar, die an der Immunantwort im Herzen beteiligt ist. DCs sind professionelle antigenpräsentierende Zellen, die die angeborene und adaptive Immunität miteinander verbinden. DCs weisen ein ähnliches Oberflächenmarkerprofil auf wie Makrophagen (u.a. Expression von CD11b, F4/80, MHCII) was ihre spezifische Bestimmung und Funktion oftmals schwierig gestaltet (Lee et al., 2018). CD11c wird auf murinen DCs stark exprimiert und kann unter der Berücksichtigung, dass auch einige Makrophagenpopulationen CD11c exprimieren können, als DC Marker eingesetzt werden (Wang et al., 2017). Im gesunden Zustand entspricht der Anteil der residenten DCs etwa 1% der gesamten kardialen Leukozyten

(Clemente-Casares et al., 2017). Als Antwort auf einen Myokardinfarkt und in der linksventrikulären Hypertrophie infiltrierten DCs das geschädigte Herzgewebe, internalisieren die lokal freigesetzten Antigene und wandern in die drainierenden Lymphknoten, wo sie die Peptidfragmente präsentierten und T-Zellen stimulierten (Santos-Zas et al., 2018). In einem Modell der viralen Myokarditis konnten auch im Herzen DCs in CD103⁺- und CD11b⁺-Subpopulationen unterteilt werden, jedoch ist die Funktion dieser Untergruppen in der Immunantwort des Herzens bisher nur wenig beleuchtet (Clemente-Casares et al., 2017).

Die Freisetzung von Zytokinen nach einer Schädigung des Myokards hat ebenfalls einen Einfluss auf das kardiovaskuläre System. Zum Beispiel induzieren einige inflammatorische Zytokine wie TNF- α , IL-1 β und IL-6 direkt eine Herzhypertrophie und korrelieren mit der Schwere der Hypertrophie (Xiao et al., 2020). TNF- α spielt eine zentrale Rolle bei der Initiierung und Aufrechterhaltung der proinflammatorischen Zytokinkaskade, die wiederum die Produktion anderer Zytokine wie IL-1 β und IL-6 stimuliert (Mann, 2015).

1.4 Zielsetzung

Die Reaktion des Immunsystems in der bakteriellen Infektion und der sterilen Myokardschädigung weist viele Gemeinsamkeiten auf. Das Verständnis für die Grundlagen und die Parallelen der angeborenen Immunantwort in der Infektion und der sterilen Inflammation erlauben die gezielte Suche nach immunmodulatorischen Therapieansätzen.

Das angeborene Immunsystem spielt in der Bekämpfung einer bakteriellen Infektion eine wesentliche Rolle, indem die Phagozyten die eingewanderten Bakterien erkennen, eliminieren, weitere Immunzellen aktivieren und abgestorbene Zellen entfernen (Schiwon, Weisheit et al., 2014). Durch die detaillierten Analysen des Inflammationsprozesses in der Harnwegsinfektion wurden Schäden erkannt, die die Immunantwort über die eigentliche Bakterienphagozytose hinaus verursacht, weil freigesetzte Effektormoleküle auch die residenten Gewebezellen angreifen. Die gleichen angeborenen Immunzellen, die diesen Prozess in der bakteriellen Harnwegsinfektion unterhalten, sorgen auch im Herzgewebe für die Aufrechterhaltung der Inflammation und tragen entscheidend zum Prozess der Narbenbildung und des Remodellings bei (Weisheit et al., 2014). Von wissenschaftlicher Relevanz ist in diesem Kontext die Frage, wie und wodurch die Entzündungsprozesse aufrechterhalten werden und ob man diese regulieren kann, um eine Schädigung von gesundem Gewebe zu minimieren.

Bei sterilen Entzündungsreaktionen wie sie im Herzgewebe nach einer Schädigung durch eine chronische Überdruckbelastung auftreten, spielen die Mechanismen der angeborenen Immunantwort, die für die Eindämmung bakterieller Infektionen von sehr großer Bedeutung sind, wie Immunzellrekrutierung, Freisetzung von Effektormolekülen und Initiation des Geweberemodellings, ebenfalls eine entscheidende Rolle für den Verlauf der Erkrankung (Weisheit et al., 2014; Kleiner et al., 2021; Weisheit et al., 2021). Die strukturellen Umbauprozesse des Herzgewebes als Antwort auf eine chronische Druckbelastung folgen keinem passiv-degenerativen Heilungsprozess, sondern werden aktiv durch die angeborene Immunantwort beeinflusst mit einer entscheidenden Bedeutung für die Aufrechterhaltung der Herzfunktion (Weisheit et al., 2021).

2 Material und Methoden

Im folgenden Kapitel wird ein kurzer Überblick über die Materialien und Methoden gegeben, die für die Erstellung der vier Publikationen (Kleiner et al., 2021; Schiwon, Weisheit et al., 2014; Weisheit et al., 2014; Weisheit et al., 2021) eingesetzt wurden.

2.1. Materialien

Für die Analyse der Harnwegsinfektion wurden uropathogene E.coli Bakterien (UPEC) des Stamms 536 (O6:K15:H31), die von einem Patienten stammen verwendet (Berger et al., 1982). GFP exprimierende UPECs wurden von Daniel Engel generiert (Engel et al., 2006).

Zur Untersuchung der Relevanz von Monozyten und Makrophagen in der Entwicklung der Harnwegsinfektion wurden Chlodronat Liposomen über Nico van Rooijen aus Amsterdam bezogen. Entsprechenden Stammkonzentrationen und Dosierungsschemata finden sich in der entsprechenden Publikation. Alle anderen Materialien wurden von Sigma-Aldrich und Thermo Fisher Scientific bezogen, es sei denn, es findet sich eine andere Angabe in der entsprechenden Publikation (Schiwon, Weisheit et al., 2014).

2.2. Methoden

Tierhaltung

C57BL/6 Mäuse wurden von Janvier Labs oder von Charles River gekauft. Transgene Tiere wurden im Haus für Experimentelle Therapie der Universität Bonn gezüchtet. Alle Mäuse wurden unter spezifischen Pathogen-freien Konditionen gemäß FELASA Richtlinien gehalten und im Alter von 8 bis 12 Wochen in den Versuchen eingesetzt. Alle *in vivo* Experimente wurden vom LANUV Nordrhein-Westfalen bewilligt (Kleiner et al., 2021; Schiwon, Weisheit et al., 2014; Weisheit et al., 2014; Weisheit et al., 2021).

Anzucht der UPECS und Infektionsmodell

Die UPEC wurden über Nacht bei 37°C im Wärmeschrank unter Rotation in LB Medium kultiviert, UPECs wurden über Nacht unter statischen Bedingungen in LB-Medium kultiviert, bei 1200 g für 10 min zentrifugiert und in PBS auf eine Konzentration von 1×10^{10} CFU pro ml resuspendiert. Anästhesierte weibliche Mäuse im Alter von 8 bis 12 Wochen wurden durch die transurethrale Inokulation von 5×10^8 E. coli in die Blase mit

einem weichen Polyethylenkatheter (Außendurchmesser 0,6 mm) infiziert. Die Anzahl der Bakterien wurde quantifiziert, indem nach einer Nacht der Inkubation die Anzahl koloniebildender UPECs von homogenisierten Blasen auf CPS-ID-Platten (Biomérieux) ausgezählt wurden.

TAC Operation

Die transverse aortale Konstriktion als Modell für die Untersuchung der druckinduzierten linksventrikulären Hypertrophie wurde mit geschlossenem Brustkorb und so mikroinvasiv wie möglich durchgeführt, um das chirurgische Trauma bei der Durchführung des Eingriffs zu minimieren (Eichhorn et al., 2018). Die Mäuse wurden mit Isofluran (2 Vol%) anästhesiert und mit einer Intubationskanüle (AD 1,2 mm) intubiert. Die Atemfrequenz wurde auf 150/min mit Tidalvolumina von 8-10 ml/kg Körpergewicht unter Verwendung eines Kleintierbeatmungsgeräts (Harvard Apparatus; Holliston, MA) eingestellt. Ein 27 G Spacer wurde verwendet, um den Grad der Aortenverengung zu standardisieren. Sham-Tiere wurden intubiert und operiert ohne dass die Naht um die Aorta fest gezogen wurde. Die Mäuse wurden an Tag 3, 6 und 21 nach der Operation untersucht. Für die Qualitätskontrolle und die Aussage über die Hypertrophieentwicklung des Herzens wurde das Körpergewicht der Tiere bestimmt und mit dem Herzgewicht ins Verhältnis gesetzt, dem sog. Heart-weight/Body-weight-Index.

Durchflusszytometrie

Für den Verdau der Harnblasen erfolgte der enzymatische Verdau wie folgt: das Harnblasengewebe wurde mechanisch zerkleinert und eine Einzelzellsuspension hergestellt durch Verdau mit 1 mg/ml Collagenase IV und 100 mg/ml DNase I in FCS-haltigem RPMI für 30 min bei 37 °C unter milder Rotation (Schiwon, Weisheit et al., 2014).

Für den Verdau von Herzgewebe wurde die Einzelzellsuspensionen aus dem linken Ventrikel durch mechanisches Zerkleinern des Gewebes und anschließende Inkubation mit 1 mg/ml Collagenase II, 100 mg/ml Collagenase I, 500 U/ml Hyaluronidase und 50 U/ml DNase I für 45 min in RPMI bei 37 °C unter milder Rotation erzeugt. Für den Verdau von Milzgewebe erfolgte ebenfalls eine mechanische Zerkleinerung des Organs und ein anschließender Verdau mit 1 mg/ml Collagenase IV und 100 mg/ml DNase I in FCS-haltigem RPMI für 30 min bei 37 °C unter milder Rotation. Die Einzelzellsuspension wurde vor der Antikörperfärbung mit einem Lysepuffer für Erythrozyten inkubiert und gewaschen.

Blutproben wurden aus der V. cava der Tiere gesammelt und die Gerinnung wurde durch Zugabe einer 0,5 M EDTA-Lösung gestoppt. Die Blutproben wurden anschließend mit Lysepuffer für Erythrozyten inkubiert und gewaschen.

Vor der Antikörperfärbung erfolgte die Blockade der Fc-Rezeptoren. Die Zellen wurden dann mit einer titrierten Menge an Antikörpern inkubiert (die vollständigen Auflistungen der Antikörper finden sich in den jeweiligen Publikationen). Weitere Angaben zu Intrazellulärfärbungen, lebend-tot Färbungen und der Nutzung von BrdU für Proliferationsanalysen sind ebenfalls den Publikationen zu entnehmen (Kleiner et al., 2021; Schiwon, Weisheit et al., 2014; Weisheit et al., 2014; Weisheit et al., 2021). Die Messungen wurden mit einem FACSCanto II, LSR II und einer Fortessa (BD Biosciences) durchgeführt und die Daten-Analyse erfolgte mit der Flow-Jo-Software (Tristar).

Histologie

Die Durchführung der Immunfluoreszenzmikroskopie erfolgte an Kryoschnitten. Blasen und Herzen wurden in Tissue-Tec (Sakura) eingebettet und bei -80°C eingefroren. Die gefrorenen Blöcke wurden in 5 µm oder 30µm dicke Präparate geschnitten und auf Poly-L-Lysin-beschichteten Glasobjektträgern gelegt und mit Aceton fixiert.

Immunhistochemie bei Paraffinschnitten: Die Blasen und Herzen wurden in 10 % IHC Zink Fixativ (BD Bioscience) oder 4% Zink-PFA fixiert bevor das Gewebe in Paraffin eingebettet wurde. Die Schnittdicke entsprach hier 5 µm. Die Schnitte wurden mit Hämatoxylin und Eosin, Sirius Rot oder mit dem Neutrophilen-spezifischen Antikörper Ly6G gefärbt.

Die histologischen Präparate wurden mit einem IX71 Mikroskop (Olympus), einem Zeiss Axio Observer oder einem konfokalen Laser Scanning-Mikroskop LSM 510 Meta (Zeiss) und LSM780 (Zeiss) analysiert. Die Auswertung erfolgte mit CellR-Software (Olympus), der AIM und ZEN Software (Zeiss) und IMARIS (Bitplane). Eine vollständige Liste der verwendeten Antikörper findet sich in den entsprechenden Publikationen. Die Quantifizierung der Hypertrophie der Kardiomyozyten erfolgte mittels planimetrischer Messungen von querschnittenen Kardiomyozyten und einer verblindeten Auswertung.

Knochenmark-Chimären

Knochenmarkchimäre Tiere wurden durch Bestrahlen mit 9Gy erzeugt. Die Rekonstitution erfolgte durch die i.v. Injektion von 3×10^6 Spender-Zellen aus dem Knochenmark der Spendertieren. Es wurden regelmäßige Blutuntersuchungen zur Kontrolle der Rekonstitution durchgeführt.

Isolierung von Leukozyten

Die Blasen wurden nach der Isolation mit einem Skalpell aufgeschnitten und 30 min bei 37 °C mit 1,0 mg/ml Collagenase und 100 mg/ml DNase I in RPMI 1640 verdaut. Die Zellsuspensionen wurden durch ein 100 mm Nylongewebe filtriert und in RPMI 1640 Medium resuspendiert. Die Immunzellen aus dem Urin wurden gesammelt und zweimal mit PBS gewaschen und filtriert. Die Leukozyten aus dem Knochenmark wurden durch Spülen der Oberschenkelknochen mit PBS gewonnen.

Adoptiver Zelltransfer

Um aus Knochenmark gewonnene Ly6C⁺-Makrophagen adoptiv zu transferieren, wurde ein MACS-Isolierungsverfahren nach Herstellerangaben durchgeführt. Zum Zeitpunkt der Infektion wurden 1×10^6 Ly6C⁺-Makrophagen in die Empfänger-Mäuse intravenös injiziert. Für die Isolation von Neutrophilen wurde ebenfalls ein MACS Kit verwendet. Die Neutrophilen wurden vor dem adoptiven Transfer mit CFSE für 10 min bei 37 °C markiert. Es erfolgte die intravenöse Injektion von 2×10^6 Neutrophilen.

Chemokin- und Zytokinmessungen

Chemokin- und Zytokin-Analysen wurden mittels ELISA oder FlowCytomix Kits für eine Bead basierte Quantifizierung auf Proteinebene verwendet. Die jeweils für die einzelnen Versuche verwendeten Kits sind in den entsprechenden Publikationen detailliert aufgeführt.

In-vitro-Assays

Neutrophile wurden mittels MACS-Technik isoliert und in RPMI 1640-Medium mit 1% FCS kultiviert. Die Stimulation der Zellen erfolgte mit 10 ng/ml TNF und/oder 10 ng/ml CXCL2 für 6 Stunden. Nach der Inkubation wurden die Überstände gesammelt. Um die Phagozytose von UPECs und die antibakterielle Aktivität von Neutrophilen zu analysieren, wurden GFP-exprimierende UPECs mit Neutrophilen kokultiviert. Die mittlere Fluoreszenz

Intensität der Neutrophilen wurde als Marker für die Aufnahme von Bakterien und den intrazellulären Abbau bestimmt und mittels Durchflusszytometrie quantifiziert.

Analyse der MMP-9-Aktivität durch Zymographie

Die Durchführung der Zymografie erfolgte unter Nutzung von SDS-Gelen mit 0,1% Gelatine, durch die die Proteine bei 125 V für 2 Stunden wanderten. Danach wurde das Gel 1 Stunde in Renaturierungspuffer (Aqua, 25% Triton-X) bei RT inkubiert, bevor die Inkubation über Nacht in Entwicklungspuffer (0,5 M Tris HCL, 2 M NaCl, 50 mM CaCl₂, 0,2 % Brij-35, pH 7,5) bei 37 °C erfolgte. Das Gel wurde mit 0,5 % Coomassie für 45 min gefärbt und anschließend mit Entfärbepuffer (50 % Methanol, 40 % Aqua, 10 % Essigsäure) für 30 min behandelt. Die Gele wurden unter Verwendung eines Canon LIDE-Scanners digital analysiert und die Banden durch Densitometrie unter Verwendung der ImageJ-Software quantifiziert.

Herzdruck-Volumen-Messungen

Die Herzfunktion wurde unter Narkose mit einem spezifischen Katheter der Firma Millar gemäß dem Protokoll des Herstellers gemessen. Der Katheter wurde durch die rechte A. carotis eingeführt. Nach der Erfassung des systolischen Blutdrucks wurde der Katheter in die linke Herzkammer vorgeschoben. Die Herzfrequenz, das Schlagvolumen, das Herzzeitvolumen und die Auswurfraction wurden im Verlauf aufgezeichnet. Diese Daten wurden verblindet gespeichert und analysiert unter Verwendung der PVANTM-Software (Millar Instruments). Der Herzindex wurde unter Verwendung von Schlagvolumen und Körpergewicht der Tiere berechnet.

Real-Time-PCR

Die Gewebeproben wurden bei -80°C gelagert und in RNeasy Lysis Buffer mit 1% 2-beta-Mercaptoethanol homogenisiert. Die Aufreinigung der RNA erfolgte mit einem RNeasy mini kit (Qiagen). Die Quantifizierung der mRNA-Menge erfolgte mit einem Nanodrop. Die mRNA Expression wurde mit Hilfe eines TaqMan PCR Systems gemessen. Als interne Kontrolle wurde die 18S-ribosomale RNA amplifiziert. Die Normalisierung der Ergebnisse erfolgte mit der Delta-Delta-ct-Methode und die Ergebnisse sind als $2^{\Delta\Delta ct}$ dargestellt.

Statistische Auswertung

Zunächst erfolgte die Untersuchung auf eine Normalverteilung. Anschließend erfolgte die weitere Datenanalyse. Eine Analyse mittels *Student's t-Test* erfolgte immer dann, wenn zwei Gruppen miteinander verglichen wurden. Eine Analyse von mehr als zwei Gruppen erfolgte mittels *one-* oder *two-way* Varianzanalysen gefolgt von einer *posthoc* Analyse. Die statistischen Auswertungen erfolgten mittels Prism Software. $P < 0,05$ wurde als statistisch signifikant angesehen.

3 Ergebnisse

Zusammenfassung der Resultate der verwendeten Arbeiten und integrierte Diskussion im wissenschaftlichen Kontext.

3.1 Der Crosstalk zwischen Sentinel- und Helfer-Makrophagen ermöglicht Neutrophilen die Migration in das infizierte Uroepithel

Phagozytierende Zellen als Teil des angeborenen Immunsystems spielen eine entscheidende Rolle in der Bekämpfung bakterieller Infektionen. Hierbei ist insbesondere die Migration der Phagozyten in epitheliale Gewebe ein wichtiger Schritt, um an den Ort der Infektion vorzudringen. Weiterhin ist die Kommunikation der Immunzellen untereinander über die Freisetzung von Chemokinen und Zytokinen von hoher Relevanz für die Koordinierung der Immunantwort. An einem murinen Modell der bakteriellen Harnwegsinfektion wurde die Interaktion zwischen Phagozyten und die Rekrutierung der Zellen in das Harnblasenepithel untersucht. Durch den Einsatz von knochenmark-chimären Mäusen, adoptiven Zelltransfers und Migrationsassays konnte gezeigt werden, dass innerhalb der ersten vier Stunden nach Infektion mit uropathogenen *E. coli* Bakterien residente Ly6C⁻-Makrophagen die Infektion wahrnahmen und daraufhin Chemokine freisetzen, die die Rekrutierung von Neutrophilen und Ly6C⁺-Makrophagen aktivierten.

Unsere Analysen zeigten, dass insbesondere Neutrophile für die Phagozytose der Bakterien im Harnblasenepithel verantwortlich waren. In der Literatur wurde eine starke Häufung von Harnwegsinfektionen bei Patienten und Patientinnen beschrieben, die mit einem TNF-Inhibitor behandelt wurden ohne eine mechanistische Ursache zu benennen. Daraufhin untersuchten wir den Einfluss von TNF auf die Migrationsfunktion der Neutrophilen. Ein Fehlen von TNF führte in unserem Modell zu einer Inhibition der Migration von Neutrophilen, die ohne TNF nicht mehr bis in das infizierte Blasenepithel vordringen und die Bakterien phagozytieren konnten. Weiterführenden Versuche zeigten, dass TNF in der Blase hauptsächlich von Ly6C⁺-Makrophagen freigesetzt wurde und ein Fehlen dieser Zellen oder das Fehlen der TNF-Signalkaskade die transepitheliale Migration der Neutrophilen blockierte. Dieser Effekt wurde nicht direkt durch TNF, sondern durch das Chemokin CXCL2 vermittelt, das abhängig von TNF aus Ly6C⁻-Makrophagen in der Blase ausgeschüttet wurde (s. Abb. 6 A und B aus Schiwon, Weisheit et al. 2014,

S. 463). Immunfluoreszenzmikroskopische Bilder zeigten darüber hinaus, dass CXCL2 nicht nur in der Infektion in Ly6C⁺-Makrophagen in Vesikeln vorlag, sondern auch in der Homöostase. Genauso ließ sich CCL2, ein Chemokin für die Rekrutierung von Monozyten, in Vesikeln der residenten Ly6C⁺ Makrophagen nachweisen (s. Abb. 6 B und C aus Schiwon, Weisheit et al. 2014, S. 463). Dies erklärt die schnelle Freisetzung beider Chemokine im Inflammationsprozess. Durch die transurethrale Injektion von CXCL2 in die Blasen von infizierten Tnf^{-/-} Mäusen konnte die Blockade der transepithelialen Migration der Neutrophilen aufgehoben werden (s. Abb. 6 D und E aus Schiwon, Weisheit et al. 2014, S. 463). Nachdem die Rolle von CXCL2 als Schlüsselchemokin für die Migration der Neutrophilen in das Blasenepithel aufgedeckt werden konnte, nahmen wir die weitere mechanistische Aufklärung in den Blick und es zeigte sich, dass CXCL2 die Freisetzung von MMP9 durch die Neutrophilen aktivierte. Nur durch MMP9 konnten die Neutrophilen das Kollagen IV in der Basalmembran abbauen und in das infizierte Harnblasenepithel vordringen.

Crosstalk between Sentinel and Helper Macrophages Permits Neutrophil Migration into Infected Uroepithelium

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SUMMARY

The phagocytes of the innate immune system, macrophages and neutrophils, contribute to antibacterial defense, but their functional specialization and cooperation is unclear. Here, we report that three distinct phagocyte subsets play highly coordinated roles in bacterial urinary tract infection. Ly6C[−] macrophages acted as tissue-resident sentinels that attracted circulating neutrophils and Ly6C⁺ macrophages. Such Ly6C⁺ macrophages played a previously undescribed helper role: once recruited to the site of infection, they produced the cytokine TNF, which caused Ly6C[−] macrophages to secrete CXCL2. This chemokine activated matrix metalloproteinase-9 in neutrophils, allowing their entry into the uroepithelium to combat the bacteria. In summary, the sentinel macrophages elicit the powerful antibacterial functions of neutrophils only after confirmation by the helper macrophages, reminiscent of the licensing role of helper T cells in antiviral adaptive immunity. These findings identify helper macrophages and TNF as critical regulators in innate immunity against bacterial infections in epithelia.

INTRODUCTION

Phagocytes, such as neutrophils and macrophages, play an essential role in the innate cellular immune response against

bacterial infections. Neutrophils are particularly important; they clear bacteria by phagocytosis or kill them by secreting toxic compounds (Amulic et al., 2012). Neutrophils are bone-marrow-derived, circulate through the blood, and enter infected tissues by penetrating the vascular endothelium (Borregaard, 2010; Kolaczowska and Kubes, 2013; Nathan, 2006). Such endothelial neutrophil migration requires ligands of the chemokine receptors CXCR1 (Fan et al., 2007) and CXCR2 (Cacalano et al., 1994), which include CXCL1, CXCL2, and CXCL6 (previously designated CXCL5) (Ley et al., 2007; Soehnlein and Lindbom, 2010). The mechanisms underlying endothelial migration have been studied extensively (Chin and Parkos, 2006; Ley et al., 2007; Nathan, 2006; Nourshargh et al., 2010; Voisin et al., 2010; Wang et al., 2006), whereas those guiding neutrophils within tissues toward microbes are less well known.

Of particular importance is neutrophil migration into tissue epithelia, the interfaces of barrier organs like the urinary tract to the outside world, which represent a preferential entry site for bacteria (Anderson et al., 2003). To intercept them, neutrophils need to traverse the epithelial basement membrane. This basement membrane is composed primarily of collagen IV (Chin and Parkos, 2006; Ichiyasu et al., 2004; LeBleu et al., 2007), which can be cleaved by metalloproteinases (MMPs). MMPs are involved in infectious and malignant diseases of the urinary tract (Szarvas et al., 2011; Tan and Liu, 2012). Besides MMPs, chemokines are involved in crossing these membranes (Lämmermann et al., 2008; Shulman et al., 2009). Furthermore, in vitro studies suggest that some MMPs are regulated by chemokines (Chabot et al., 2006; Chakrabarti and Patel, 2005; Luca et al., 1997) and vice versa (Van den Steen et al., 2000). However, the role of chemokines, MMPs, and their

interaction for epithelial neutrophil migration in vivo are incompletely understood.

Macrophages are an important source of neutrophil-attracting chemokines in bacterial infections, such as CXCL1 and CXCL2 (Benoit et al., 2008; Kopydlowski et al., 1999; Mantovani et al., 2004; Mosser and Edwards, 2008). Macrophages possess intrinsic antibacterial activity, but this seems to be less critical for bacterial clearance than neutrophil effector functions (Dale et al., 2008; Kantari et al., 2008). After bacteria are cleared, macrophages phagocytize apoptotic neutrophils to promote resolution of inflammation (Bratton and Henson, 2011; Soehnlein and Lindbom, 2010). It is assumed that macrophages and neutrophils cooperate during infections (Kolaczowska and Kubes, 2013; Soehnlein and Lindbom, 2010), but a coherent model on how they communicate and share the anti-infectious tasks is presently lacking.

Virtually all tissues are populated by a dense network of tissue-resident macrophages, which have been proposed to partially originate from embryonic progenitors (Ginhoux et al., 2010; Schulz et al., 2012). In infections, the bone marrow releases in a CCL2/CCR2-dependent fashion inflammatory monocytes, which differentiate into further macrophages or into dendritic cells within tissues (Serbina and Pamer, 2006). Inflammatory macrophages can be distinguished from tissue-resident macrophages by the marker Ly6C, until it is downregulated after 1–2 days (Landsman et al., 2007; Plantinga et al., 2013). Little is known about the functional specializations of inflammatory Ly6C⁺ and resident Ly6C[−] macrophages.

Macrophages produce proinflammatory cytokines during bacterial infections, and, among these, tumor necrosis factor (TNF) is particularly important (Belge et al., 2002; Engel et al., 2008; Medzhitov, 2007; Mosser and Edwards, 2008). Also neutrophils and epithelial cells can produce TNF (Roulis et al., 2011; Theilgaard-Mönch et al., 2005), but it is unclear whether the cellular source of TNF impacts bacterial clearance. TNF is involved also in immune-mediated diseases, such as rheumatoid arthritis and inflammatory bowel disease, that respond well to TNF blockade therapy (Kountouras et al., 2005; Mpofu et al., 2005; Targan et al., 1997; Wright et al., 2010). However, bacterial infections often relapse during such inhibition, especially tuberculosis (Flynn et al., 1995; Keane et al., 2001) and the incidence of bacterial infections of the lung and the urinary tract infections is increased (Dixon et al., 2006; Listing et al., 2005; Strangfeld and Listing, 2006; Winthrop et al., 2009; Wright et al., 2010). This is often explained by the ability of TNF to stimulate phagocytosis and ROS production by neutrophils. The exact antibacterial role of TNF is incompletely understood.

Bacterial infections of the urinary tract (UTI) are among the most prevalent infections and affect more than 25% of the population in developed countries, especially young females (Foxman, 2002). They mainly result from uropathogenic *E. coli* (UPEC) ascending through the urethra into the bladder, where they first invade the uroepithelium (Mulvey et al., 1998). UPEC can persist intracellularly, and cause relapsing infections (Martinez et al., 2000), which is associated with an increased risk for bladder cancer (Parker et al., 2004). Their ascension into the kidney causes pyelonephritis, which can progress to renal failure (Kurts et al., 2013; Svensson et al., 2011). The defense against

UTI depends on neutrophils (Frendéus et al., 2000). These are attracted by CXCR2 ligands, which mediate both endothelial and epithelial migration (Godaly et al., 1997; Ley et al., 2007). The exact identity and the source of these ligands is unclear. The macrophage inhibitory factor (MIF) was recently described as a further CXCR2 ligand (Bernhagen et al., 2007; Santos et al., 2011). Its function in UTI is unclear. Also the roles of macrophages and of TNF in UTI are unknown.

Here, we studied the interplay of neutrophils and macrophages in a murine model of UTI of the bladder. We discovered that tissue-resident Ly6C[−] and proinflammatory Ly6C⁺ macrophages play distinct roles in antibacterial immunity. Their TNF-mediated crosstalk regulates neutrophil migration into the infected uroepithelium and facilitates optimal antibacterial defense. We propose a model of tri-cellular crosstalk in innate cellular immunity.

RESULTS

Kinetics and Location of Neutrophils and Macrophages in Urinary Tract Infection

We studied the roles of macrophages and neutrophils in a mouse model of UTI induced by transurethral instillation of UPECs. Bladders of uninfected mice mostly contained resident Ly6C[−] macrophages (Figures 1A and S1A available online). Already at 2 hr after infection, the numbers of neutrophils and Ly6C⁺ macrophages increased significantly within the infected bladder, whereas numbers of Ly6C[−] macrophages remained largely constant during the course of infection (Figure 1B). Maximal numbers of neutrophils and Ly6C⁺ macrophages were reached after 24 hr (Figure 1B). Hardly any of these cell types proliferated in the bladder (Figure S1B), suggesting that their rapid accumulation was due to recruitment from the circulation. Mainly neutrophils migrated into the uroepithelium (Figures 1C and 1D).

To study the importance of these phagocyte types for bacterial clearance, we determined uptake of UPEC expressing recombinant GFP. Almost 90% of the phagocytosed UPECs were detected within neutrophils 6 hr after infection (Figure 1E), supporting their central role in the defense against UTI (Frendéus et al., 2000). Ly6C[−] macrophages took up hardly any UPEC, and only some Ly6C⁺ macrophages did so after 24 hr, suggesting regulatory roles.

Distinct Functions of Ly6C⁺ and Ly6C[−] Macrophages in UTI

We speculated that Ly6C[−] or Ly6C⁺ macrophages might regulate the antibacterial defense by producing chemokines that attract the neutrophils (Soehnlein and Lindbom, 2010). Interestingly mainly the Ly6C[−] macrophages produced the neutrophil attractors MIF, CXCL1, CXCL2, and CXCL6 (Figures 2A and S2A). Ly6C⁺ macrophages hardly produced chemokines, but instead they were the main producers of TNF (Figures 2A and S2A), which is generally important in bacterial infections (Nathan, 2006). These observations suggested that Ly6C[−] and Ly6C⁺ macrophages might play distinct functions in UTI.

At this point, we wondered how well the Ly6C marker discriminates between these macrophages, since previous studies

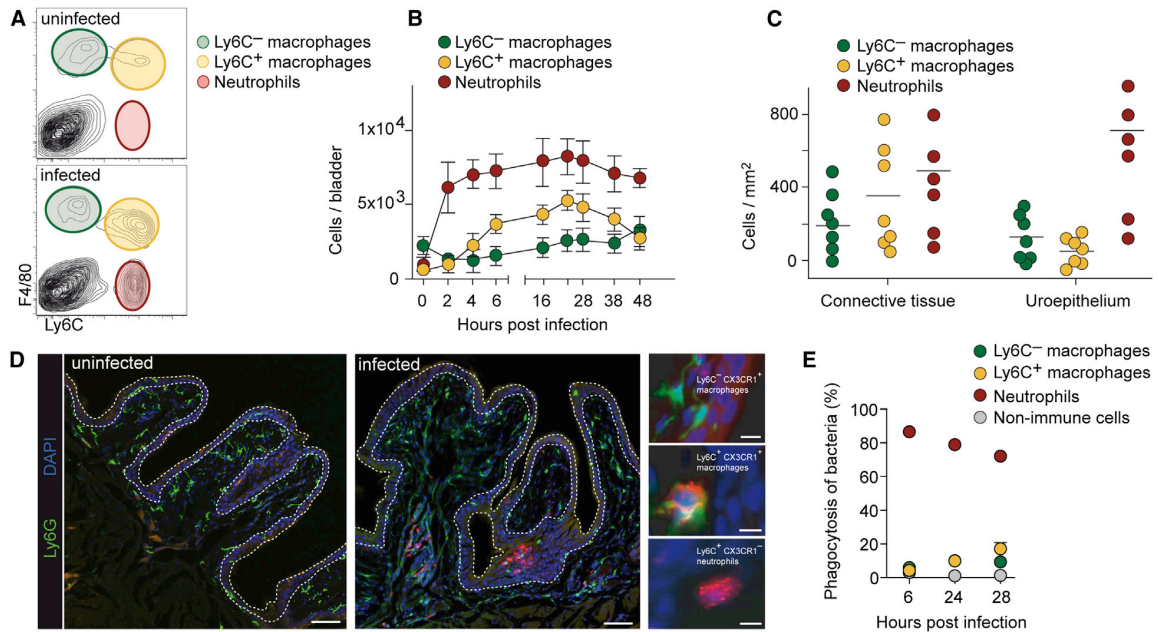


Figure 1. Recruitment and Positioning of Macrophages and Neutrophils in Bacterial UTI

(A) Flow-cytometric analysis of bladder homogenates of uninfected and infected mice (concatenated plots of five mice) 1 day after infection. Neutrophils (red), Ly6C⁺ (yellow) and Ly6C⁻ (green) macrophages were distinguished by their expression of Ly6C and F4/80 as indicated.

(B) Numbers of neutrophils, Ly6C⁺ and Ly6C⁻ macrophages in single-cell suspensions of bladders determined by flow cytometry.

(C) Number of neutrophils, Ly6C⁺ and Ly6C⁻ macrophages in bladder cryosections 24 hr after infection as shown in (D).

(D) Localization of neutrophils within the epithelium 1 day after infection. Cryosections from bladders of uninfected and infected *Cx3cr1⁺/GFP* mice, which express green fluorescent protein (GFP) in macrophages, were stained for Ly6C (red) and for cellular nuclei (DAPI-blue). The white dashed line highlights the uroepithelial border and the white bar indicates 200 μ m. The small images show representative stainings of the three phagocyte types. The white bar indicates 10 μ m.

(E) Contribution of neutrophils, macrophages and nonimmune cells (CD45⁻ cells) to phagocytosis of GFP-expressing UPECs, calculated by the formula ((mean fluorescence intensity of GFP⁺ cells in UPEC-GFP infected mice – mean fluorescence intensity of GFP⁺ cells in UPEC infected mice) $\times$ number of UPEC-GFP⁺ cells).

Data are means $\pm$ SEM and represent five (A and B) and three (C, D, and E) independent experiments in groups of five to seven mice. See also Figure S1.

showed that Ly6C⁺ monocytes downregulate this molecule after entering tissues (Landsman et al., 2007; Plantinga et al., 2013). When we transferred Ly6C⁺ monocytes, all of them expressed Ly6C in the infected bladder after 6 hr, almost 90% after 24 hr, and 70% after 48 hr (Figure S2B). To determine whether Ly6C⁻ macrophages upregulate this marker in UTI, we infected *Ccr2^{-/-}* mice, which lack Ly6C⁺ macrophages (Serbina and Pamer, 2006), and mice treated with clodronate liposomes, which selectively depletes Ly6C⁺ macrophages, but neither Ly6C⁻ macrophages (Figure S2C) nor neutrophils (Figure 2B). In both cases, no Ly6C⁺ macrophages were detected in the bladder 6 hr after infection (Figure S2D). Thus, Ly6C expression was mostly stable during the first 24 hr of the antibacterial immune response studied here, supporting the notion that Ly6C⁻ and Ly6C⁺ macrophages were functionally distinct macrophage types.

We next determined the roles of the chemokines produced by Ly6C⁻ macrophages by blocking them with neutralizing antibodies. CXCL1 was as critical for neutrophil recruitment as its receptor CXCR2 (Figure 2B). MIF contributed to such recruitment, whereas CXCL2 and CXCL6 were not required (Figure 2B).

Ly6C⁺ macrophage numbers were substantially reduced in *Ccr2^{-/-}* mice (Figure S2D), whose ligand CCL2 was produced by Ly6C⁻ macrophages as well (Figures 2A, S2A and S2E). CCL2 production by these macrophages was independent of the presence of Ly6C⁺ macrophages (Figure S2F), supporting the central role of resident Ly6C⁻ macrophages in the recruitment of circulating phagocytes. Bacterial clearance of the bladder was impaired in the absence of neutrophils (α CXCL1, α MIF, and *Cxcr2^{-/-}* mice), the main antibacterial effectors (Figure 2C) (Freundt et al., 2000). Notably, it was also impaired in mice lacking or depleted of Ly6C⁺ macrophages (Figure 2C). In summary, Ly6C⁻ macrophages produced the chemokines that attracted neutrophils and Ly6C⁺ macrophages into the infected bladder, and both recruited phagocyte types were important for antibacterial defense.

TNF from Ly6C⁺ Macrophages Permits Transepithelial Migration of Neutrophils

The importance of Ly6C⁺ macrophages was astonishing given that these cells hardly contributed to bacterial phagocytosis within the first day (Figure 1E). We speculated that their main

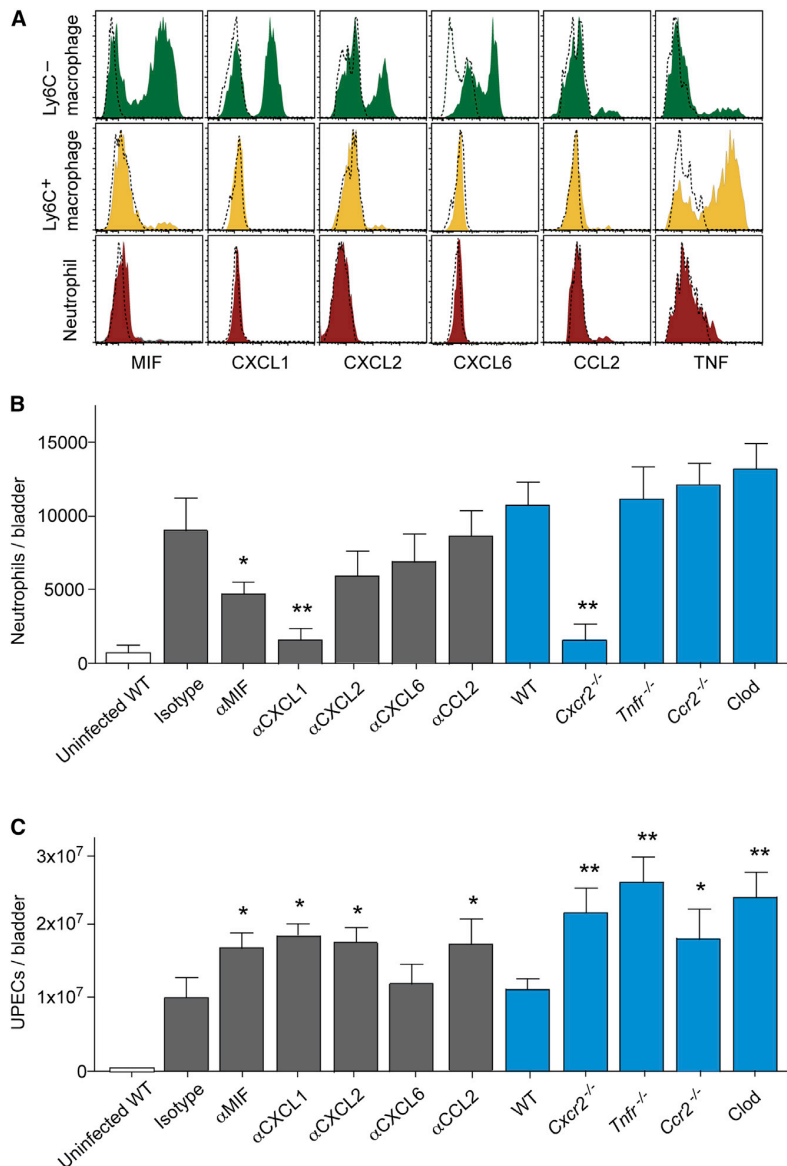


Figure 2. Chemokines Produced by Ly6C⁻ Macrophages Recruit Neutrophils into the Infected Bladder

(A) Intracellular flow-cytometric staining of Ly6C⁻ (green) and Ly6C⁺ (yellow) macrophages and neutrophils (red) 6 hr after infection for immune regulatory molecules (solid line) and the respective isotype control (dashed line).

(B and C) Intravesical numbers of neutrophils (B) and UPECs (C) 6 hr after infection and treatment with antibodies (gray) or in knockout animals (blue). Clod = Clodronate liposomes.

Data are means ± SEM and represent three experiments in groups of three to five mice. *p < 0.05; **p < 0.01. See also Figure S2.

To clarify the role of TNF in UTI, we first asked whether it activates neutrophil effector functions. However, the in vitro ability of neutrophils to phagocytose UPEC and their antibacterial capacity were independent of TNF (Figure S3). The discrepancy between the requirement for TNF in vivo (Figures 2C and 3) and in vitro (Figure S3) suggested an effect on neutrophil migration. However, neutrophil recruitment into the whole bladder was not impaired in *Tnfr^{-/-}* mice (Figure 2B). Histological analysis revealed that despite their ability to enter the bladder, neutrophils failed to migrate into the bladder uroepithelium in *Tnfr^{-/-}* mice (Figures 4A to 4C), the site from where UPEC enter. Fewer neutrophils were detected also in the urine of *Tnfr^{-/-}* mice and in mice depleted of TNF-producing Ly6C⁺ macrophages (Figure 4D), consistent with their inability to enter and transmigrate through the uroepithelium into the bladder lumen.

When we reconstituted infected *Tnfr^{-/-}* mice either with recombinant TNF (Figure 4E) or with TNF-producing Ly6C⁺ macrophages (Figures 4E to 4G), neutrophils were able to enter the uroepithelium, verifying that Ly6C⁺ macrophages can permit epithelial neutrophil migration through TNF.

TNF Induces CXCL2 in Ly6C⁻ Macrophages

To determine whether migration of neutrophils into the uroepithelium required TNF to directly act on neutrophils, we adoptively transferred TNFR-competent and -deficient neutrophils into infected wild-type mice. TNFR-deficient neutrophils entered the bladder and its uroepithelium of infected wild-type mice as efficiently as TNFR-competent neutrophils (Figures 5A and 5B), indicating that TNFR on neutrophils was dispensable for both endothelial and epithelial migration. This differed from the situation in mice ubiquitously lacking TNFR, where endothelial

product, TNF might be involved (Figure 2A). Although this cytokine is generally important in bacterial infections (Nathan, 2006), its role in UTI is unknown.

When we infected *Tnfr^{-/-}* mice deficient for both TNF receptors, the bacterial load in their bladders was much higher than in wild-type controls (Figure 2C). In addition, the number of infected uroepithelial cells, which indicates persistence of UTI, was increased both in *Tnfr^{-/-}* mice and in the absence of the TNF-producing Ly6C⁺ macrophages (Figure 3A). Moreover, throughout a period of 6 weeks, more cystitis relapses of higher severity occurred (Figure 3B) and more mice developed pyelonephritis (Figure 3C). Thus, TNF is important to limit UTI.

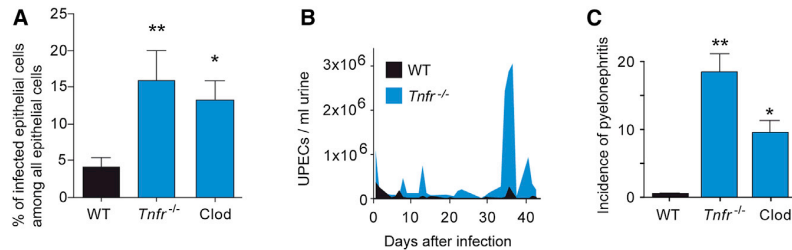


Figure 3. Aggravated Infection in Mice Lacking TNF Receptor or TNF-Producing Ly6C⁺ Macrophages

(A) Mice were infected with GFP-expressing UPECs and the percentage of infected GFP⁺ CD45-negative epithelial cells were determined one day after infection.

(B) Relapsing infections in wild-type (WT) and *Tnfr*^{-/-} mice analyzed by counting the number of UPECs within the urine.

(C) Incidence of pyelonephritis one day after infection in percent.

Data are means ± SEM and represent three independent experiments in groups of three to five mice. *p < 0.05; **p < 0.01.

(Figure 2B), but not epithelial migration (Figures 4A to 4C), was intact. We concluded that TNF acted on TNFR⁺ cells other than neutrophils.

To identify this cell, we generated bone marrow chimeras in which hematopoietic and/or nonhematopoietic cells lacked TNFR. When we infected these chimeras, transepithelial neutrophil migration required TNFR expression only by bone-marrow-derived cells, but not by tissue cells, such as stroma or epithelial cells (Figures 5C and 5D).

To clarify which bone-marrow-derived cell responded to TNF, we examined several molecules known to affect neutrophil migration and asked which were regulated in a TNF-dependent manner. We found that CXCR2, CXCL1, MIF, GM-CSF, IFN γ , IL-1 α , IL-4, IL-5, L-6, IL-17, CCL2, CCL3, CCL4, CCL5, CCL7, CD11b, CD47, CD49b, and ICAM-1 (Figures 6A and S4) were similarly expressed in bladders of infected *Tnfr*^{-/-} and wild-type mice. Only CXCL2 was markedly reduced in *Tnfr*^{-/-} mice (Figure 6A). This chemokine was required for the defense against UTI (Figure 2C), but not for neutrophil recruitment into the whole bladder (Figures 2B and 5A). However, like TNF (Figures 4A to 4C), CXCL2 is required for uroepithelial neutrophil migration (Hang et al., 1999), suggesting a causal connection. Indeed, secretion of CXCL2 was strongly reduced in the infected bladders of *Tnfr*^{-/-} and *Tnfr*^{-/-} mice, and of mice depleted of the main TNF producers, Ly6C⁺ macrophages (Figure 6A), indicating that TNF induced CXCL2. The cellular source of CXCL2 according to our flow cytometric analysis were Ly6C⁺ macrophages (Figures 2A and S2A). But as nonhematopoietic cells often escape flow cytometry, we also analyzed infected bladders by immunofluorescence microscopy. We failed to detect CXCL2 in uroepithelial cells at 3 hr after infection (Figures 6B and S5A), consistent with previous observations (Hang et al., 1999). By contrast, bladder macrophages stained brightly for intracellular CXCL2, not only in infected but also in uninfected animals (Figures 6B and S5B), indicating the existence of preformed intracellular pools. Macrophages also contained preformed CCL2 (Figures 6C and S5C), which can attract Ly6C⁺ macrophages. The rapid release of preformed pools can explain how these chemokines could be released within a few hours after urinary tract infection (Figure S5D). As only Ly6C⁺ macrophages produced CXCL2 in the infected bladder (Figures 2A and S2A), we concluded that TNF acted only on Ly6C⁺ macrophages, and not on neutrophils.

These results suggested that supplying CXCL2 should restore the in vivo migratory defect of neutrophils in the absence of TNF. To test this hypothesis, we transurethrally inoculated CXCL2 into the bladder of infected *Tnfr*^{-/-} mice. Indeed, under these conditions neutrophils entered the uroepithelium (Figures 6D, 6E, and S5E), confirming that CXCL2 acted downstream of TNF.

CXCL2 Permits Epithelial Neutrophil Migration by Inducing MMP-9

Next, we asked how CXCL2 enabled neutrophils to enter the uroepithelium. Epithelial basement membranes are comprised mainly of collagen IV, which is degraded by metalloproteinase 9 (MMP-9) (Van den Steen et al., 2002), and CXCR2 ligands have been shown to induce MMP-9. Histological sections of infected *Mmp*-9^{-/-} mice showed that neutrophils accumulated underneath the uroepithelium and failed to enter it (Figures 7A and S6A) or to transmigrate into the urine (Figure 7B). Furthermore, *Mmp*-9^{-/-} mice showed a markedly higher bacterial load in the urine (Figure 7C), indicating that MMP-9 is also required for defense against UTI. When we stained bladder cells for MMP-9 expression, almost 99% of the MMP-9⁺ cells were neutrophils (Figure S6B). To test whether neutrophil-intrinsic MMP-9 was important, we transferred MMP-9-competent neutrophils into infected *Mmp*-9^{-/-} mice, and MMP-9-deficient neutrophils into infected MMP-9-competent mice. Only MMP-9-competent neutrophils were detected within the uroepithelium (Figures 7D and S6C) indicating that neutrophil-intrinsic MMP-9 was necessary and sufficient for epithelial migration and explaining its requirement for antibacterial defense.

The migratory defect of MMP-9-deficient neutrophils was analogous to that in *Tnfr*^{-/-} mice (Figures 4A to 4D), suggesting a causal connection between TNF and MMP-9. We tested this by measuring secretion of MMP-9 by neutrophils in *Tnfr*^{-/-} and wild-type mice by zymography (Vandooren et al., 2013). Indeed, this secretion was reduced by 70%–80% in *Tnfr*^{-/-} and *Tnfr*^{-/-} mice (Figures 7E and S7A), whereas secretion of MMP-2 was unaffected (Figure S7B). A similar reduction was seen in mice depleted of Ly6C⁺ macrophages, the main TNF producers (Figures 7E and S7A), indicating that TNF from Ly6C⁺ macrophages caused the secretion of MMP-9. However, our finding that TNFR expression by neutrophils was dispensable for transepithelial migration (Figure 5B) implied that a downstream mediator, such as CXCL2, induced the secretion of

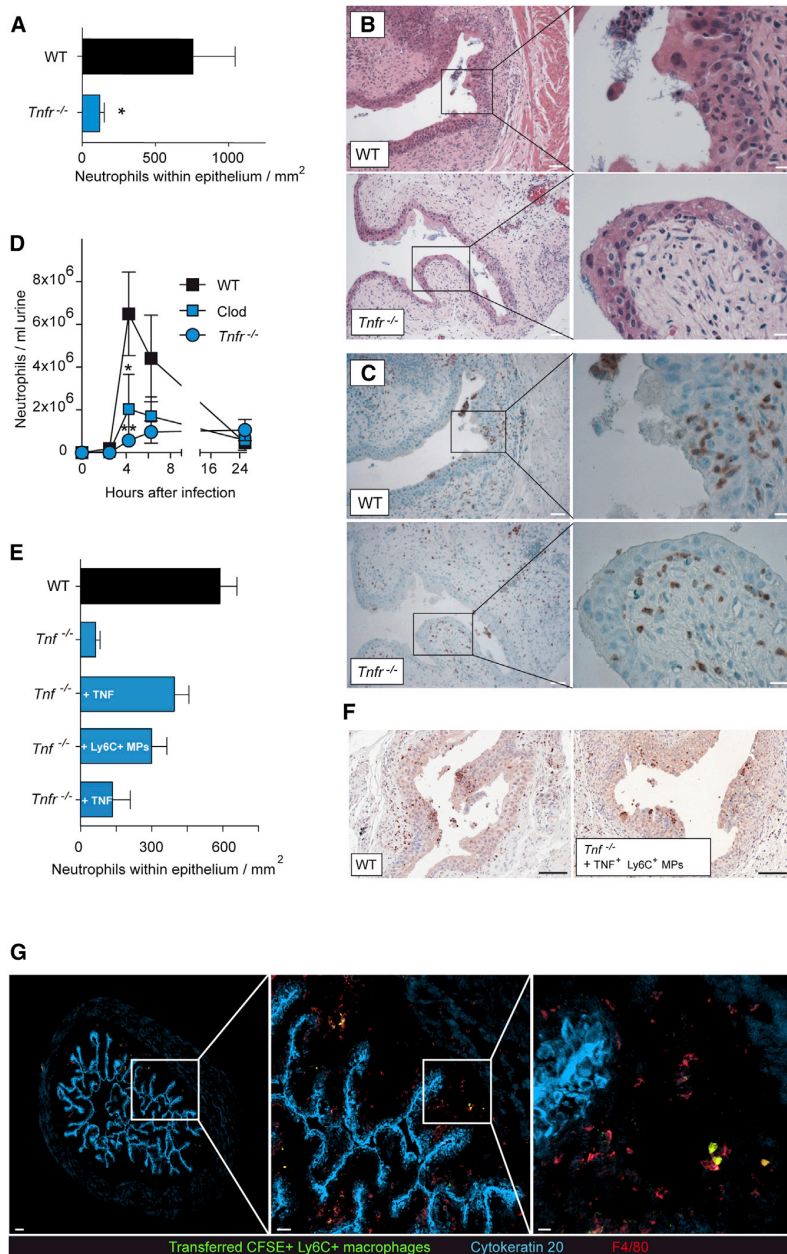


Figure 4. TNF Enables Neutrophils to Enter the Uroepithelium

(A–C) Analysis of transepithelial migration by histological enumeration of neutrophils 1 day after infection. Consecutive bladder sections of wild-type (WT) or *Tnfr*^{-/-} mice were either stained with HE (B) or Ly6G (C, brown), which specifically reveals neutrophils. The right panels show 4-fold magnifications of the inlay highlighted in the left column with a black box. White bars are 100 μ m (left) or 25 μ m (right). Quantitative analysis in (A). (D) Kinetics of neutrophil numbers in the urine of WT or *Tnfr*^{-/-} mice or after depleting Ly6C⁺ macrophages with clodronate liposomes (Clod) determined by flow cytometry.

(E–G) Restored neutrophil migration into the uroepithelium of *Tnfr*^{-/-} mice either 24 hr after i.v. transfer of 5×10^6 CFSE-labeled Ly6C⁺ monocytes (E–G) or after injection of 50 μ g TNF into the bladder (E). Neutrophil localization was revealed by myeloperoxidase staining of paraffin sections 1 day after infection. The transferred CFSE-labeled Ly6C⁺ monocytes (green) were counterstained for F4/80 (red) and the uroepithelium for cytokeratin 20 (blue). The bar graph indicates 300 μ m (F), 100 μ m (G, left), 30 μ m (G, middle), 10 μ m (G, right). Quantitative analysis in (E).

Data are means $\pm$ SEM and represent four (A–D) and two (E–G) independent experiments in groups of five to eight mice. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. See also Figure S3.

DISCUSSION

Taken together, our findings documented the following sequence of events during the first 4 hr of the phagocyte response against UTI: (1) resident Ly6C⁻ macrophages sensed the infection and produced chemokines that recruited neutrophils and Ly6C⁺ macrophages, (2) recruited Ly6C⁺ macrophages produced TNF in response to the infection, (3) TNF induced the release of preformed CXCL2 by Ly6C⁻ macrophages, (4) CXCL2 caused MMP-9 secretion by neutrophils, which allowed these cells to cross the epithelial basement membrane in order to combat the infection.

These results demonstrated that the antibacterial neutrophil response is coordinated by two macrophage subsets with distinct tasks: the Ly6C⁻ macrophages acted as tissue-resident sentinels and attracted circulating phagocytes by chemokines. The Ly6C⁺ macrophages did not directly participate in bacterial elimination but instead played a hitherto unknown helper role: in response to the infection, they licensed the Ly6C⁻ macrophages to send neutrophils into the frontline of infection, the uroepithelium.

The reliance on cues from helper cells is reminiscent of adaptive immunity. For example, dendritic cells can cross-prime

MMP-9 in neutrophils. We tested this by stimulating neutrophils in vitro with the different chemokines and measuring MMP-9 secretion. CXCL2 increased MMP-9 levels in wild-type neutrophils in a concentration-dependent manner, whereas neither CXCL1, CXCL6, MIF, CCL2, nor TNF was able to do so (Figure 7F). Furthermore, CXCL2, but not TNF, stimulated TNFR-deficient neutrophils to secrete MMP-9 (Figure S7C), confirming that the downstream mediator CXCL2, and not TNF, activated neutrophils to release MMP-9.

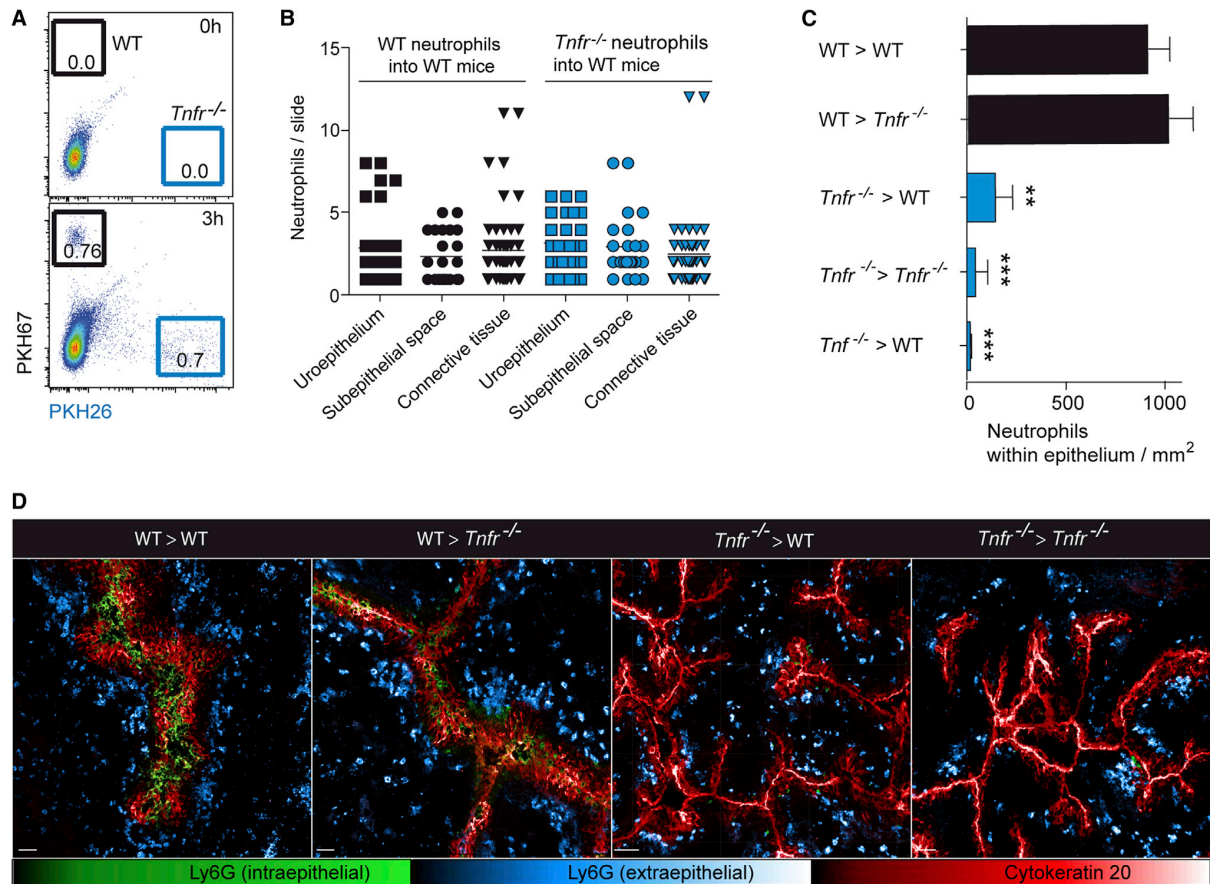


Figure 5. Trans epithelial Neutrophil Migration Requires TNFR Only on Bone-Marrow-Derived Cells

(A) Flow cytometric analysis of TNF-dependent transendothelial migration by transferring 3×10^6 PKH26-labeled *Tnfr*^{-/-} neutrophils together with the same number of PKH67-labeled WT neutrophils into infected WT animals.

(B) Bone-marrow-derived neutrophils were labeled by CFSE and adoptively transferred into infected mice. One day later the fluorescent neutrophils were localized in bladder cryosections stained for Ly6G.

(C and D) Irradiated *Tnfr*^{-/-} and wild-type (WT) were transplanted with bone marrow from *Tnfr*^{-/-}, *Tnfr*^{-/-} or WT mice. One day after infection, the localization of neutrophils was analyzed by immunofluorescence staining of bladder cryosections for Ly6G. The uroepithelium was labeled by cytokeratin 20 staining (red). Ly6G⁺ cells outside the uroepithelium are displayed as pseudocolor in blue, and those inside the uroepithelium in green. White bars are 100 μ m. Quantitative analysis is given in (C).

Data are means $\pm$ SEM and represent two (A and B) and three (C and D) independent experiments in groups of five to eight mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

cytotoxic CD8⁺ T lymphocytes (CTL) against viral infections only after having been licensed by CD4⁺ T helper cells or NKT cells (Castellino et al., 2006; Semmling et al., 2010; Smith et al., 2004). This involves the secretion of chemokines that bind CCR5 or CCR4, respectively, which attract the CTL toward the licensed DCs. Later, in infected tissues, the crosstalk between T helper cells and dendritic cells results in the production of CXCR3-specific chemokines that direct CTL toward virus-infected cells (Nakanishi et al., 2009). Our findings reveal an analogous role for the crosstalk between Ly6C⁻ and Ly6C⁺ macrophages in guiding neutrophils within bacterial infected tissues through coordinated chemokine production.

The chemokines that bind CXCR2 are often considered redundant. We found that Ly6C⁻ macrophages produced several such

chemokines, which played distinct chemotactic roles in regulating neutrophil migration in UTI: CXCL1, and to a lower extent MIF, caused endothelial migration, whereas CXCL2 by Ly6C⁻ macrophages played a nonredundant role in epithelial migration. Diverse signaling events by different ligands for the same receptor has been referred to as biased agonism and the underlying mechanisms are under intense investigation (Reiter et al., 2012). Biased agonism has been described for CCR5, CCR7, and CXCR3 ligands (Kenakin et al., 2012; Kohout et al., 2004; Scholten et al., 2012). We have observed that CXCL2 differs from other CXCR2 ligands by modulating phosphorylation of mitogen-activated protein kinase p38, but neither of Akt, ERK1/2 nor of JNK in neutrophils (D.R.E. and K.L., unpublished data), demonstrating biased agonism for CXCR2 ligands. p38

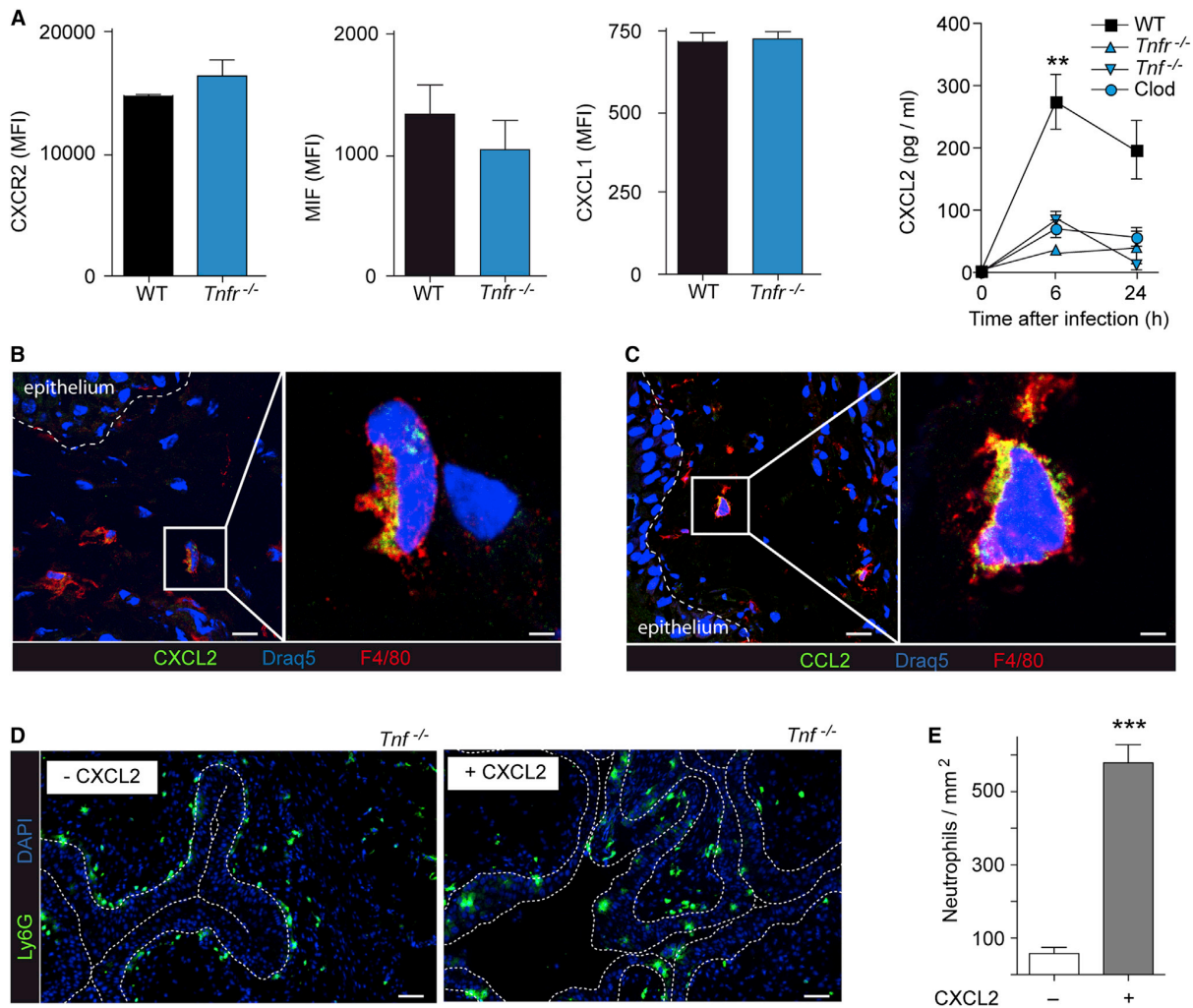


Figure 6. TNF-Dependent CXCL2 Enables Neutrophils to Enter the Uroepithelium

(A) Expression of CXCR2, MIF, CXCL1 and CXCL2 expression in WT and *Tnfr*^{-/-} mice 1 day after infection (First three bar graphs; MFI = Mean Fluorescence Intensity). CXCL2 expression was determined by ELISA in bladder homogenates of wild-type (WT), *Tnfr*^{-/-}, *Tnf*^{-/-}, and clodronate-liposome-treated animals (Clod) 6 and 24 hr after infection.

(B and C) Staining of cryosections of 3 hr infected bladders for macrophages by F4/80 (red) and CXCL2 (B) or CCL2 (C) in green. Nuclei were counterstained with DRAQ5 (blue). The white bar indicates 20 μ m (left) or 4 μ m (right).

(D) Restored migration of neutrophils into the uroepithelium by injection of CXCL2 into the bladder (+ CXCL2, right) into *Tnfr*^{-/-} mice infected one day before (Detailed quantitative analysis is shown in Figure S5E). The white dashed line highlights the uroepithelial border and the white bar indicates 100 μ m.

(E) Quantitative analysis of intraepithelial neutrophils as shown in (D).

Data in (A) and (E) are means $\pm$ SEM and represent three independent experiments in groups of five to eight mice. **p* < 0.05; ***p* < 0.01; ****p* < 0.001. See also Figures S4 and S5.

has been shown to induce neutrophil MMP-9 secretion in some (Dumitru et al., 2012), but not in other in vitro studies (Chakrabarti and Patel, 2005). Future studies are required to clarify whether CXCL2 modifies p38 signaling to induce MMP-9 secretion and epithelial neutrophil migration in vivo.

Epithelial immune cell migration is less well understood than endothelial migration (Kolaczowska and Kubek, 2013; Ley et al., 2007; Nourshargh et al., 2010), and our knowledge about

epithelial migration mostly stems from in vitro experiments, which do not mimic all aspects of the in vivo situation, especially barriers formed by basement membranes. The ability of CXCL2 to induce the secretion of MMP-9 (Chakrabarti and Patel, 2005) in the immediate vicinity of the epithelial basement membrane explains how neutrophils can penetrate collagen IV-rich epithelial basement barriers. These findings identify CXCL2 and MMP-9 as TNF-regulated gatekeepers for epithelial neutrophil migration.

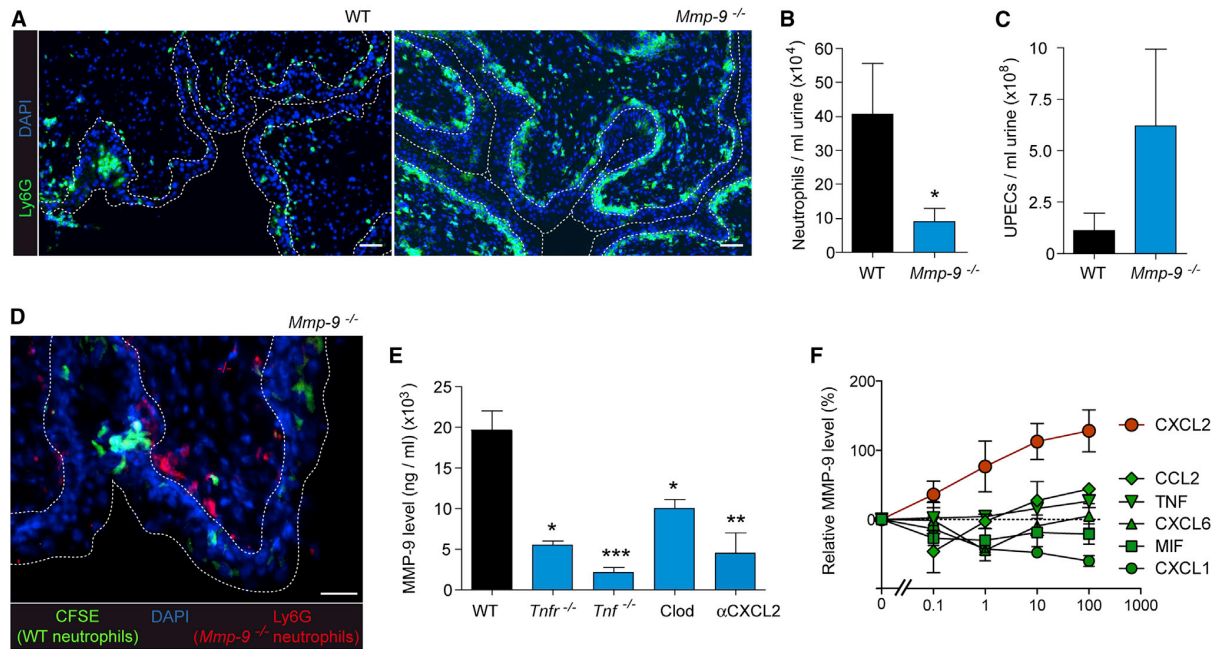


Figure 7. CXCL2-Induced MMP-9 Secretion Mediates the Entry of Neutrophils into the Uroepithelium

(A) Positioning of neutrophils in bladder sections of wild-type (WT) and *Mmp-9*^{-/-} mice one day after infection, revealed by staining with Ly6G Alexa488 (green). The white dashed line highlights the uroepithelial border and the white bar indicates 200 μ m (left) and 100 μ m (right). (Detailed quantitative analysis is shown in Figure S6A).

(B and C) Reduced numbers of neutrophils (B) and increased bacterial load (C) in the urine of *Mmp-9*^{-/-} mice over WT controls 1 day after infection.

(D) In contrast to endogenous neutrophils (stained with the red antibody Ly6G Alexa568) in *Mmp-9*^{-/-} mice, adoptively transferred MMP-9-competent neutrophils stained with CFSE (green) can enter the infected uroepithelium 1 day after infection. The white bar indicates 40 μ m.

(E) Analysis of MMP-9 levels by zymography (blots in Figure S7A) in one day infected murine bladders of WT, *Tnfr*^{-/-}, *Tnfr*^{-/-} + Clodronate Liposome and anti-CXCL2 treated mice.

(F) Relative MMP-9 secretion of bone-marrow-derived neutrophils 6 hr after stimulation by the indicated chemokine concentrations, determined by zymography. Data are means $\pm$ SEM and represent three independent experiments in groups of five to eight mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. See also Figures S6 and S7.

TNF is produced by recruited immune cells not only in UTI, but also in infections of other barrier organs, e.g., the lung (Chen et al., 2013) or the intestine (Platt and Mowat, 2008), consistent with its role as a licensing factor. Interestingly, TNF was produced by CCR2-dependent Ly6C⁺ DCs in listeriosis of the spleen (Serbina et al., 2003), whereas patrolling Ly6C⁻ monocytes recruited from the blood through CX₃CR1 did so in listeria peritonitis (Auffray et al., 2007), suggesting that immune cells other than Ly6C⁺ macrophages may play a helper role in different organ and/or pathogen contexts.

Consistent with its well-known central role in antibacterial defense (Nathan, 2006), pharmacologic TNF blockade in patients with rheumatoid arthritis (Dixon et al., 2006) or inflammatory bowel disease often causes relapses of tuberculosis infection (Flynn et al., 1995). Furthermore, such blockade is correlated with a higher incidence of pneumonia and, notably, of UTI (Strangfeld and Listing, 2006). Our findings in *Tnfr*^{-/-} mice can explain the latter association. Furthermore, these mice also showed more intraepithelial bacterial communities, which underlie the frequent relapses of bacterial cystitis. Our findings show that TNF is required for curtailing these communities. It re-

mains to be seen whether CXCL2 induction is important also in other TNF-dependent bacterial infections, for example listeriosis (Pfeffer et al., 1993) or tuberculosis (Flynn et al., 1995).

The ability of intravesically applied CXCL2 to restore transepithelial migration of TNF-deficient neutrophils might represent a new therapeutic concept in patients with relapsing UTI, especially after treatment with TNF blockers. Intravesical CXCL2 instillation might help eliminating bacteria and prevent their spreading into the kidney. Topical CXCL2 provision might also be effective in other infections that exacerbate during TNF inhibition therapy.

In conclusion, our study demonstrates that the innate immune response against bacterial infections requires the coordinated interaction between three phagocyte subsets with distinct tasks: (1) Ly6C⁻ macrophages as sentinels/coordinators, (2) Ly6C⁺ macrophages as helpers/advisors, (3) neutrophils as antibacterial effectors. The sentinels request a second opinion from the helpers, before unleashing the antibacterial effector cells into epithelial tissue. This mechanism may be relevant for barrier organs with an epithelial interface to the outside world, like the urogenital tract. The reliance on the coordination between

several immune cells may serve to reduce the risk of inadequate neutrophil responses that cause immunopathology.

EXPERIMENTAL PROCEDURES

Mice

Female mice between 8 and 12 weeks of age were maintained in specific pathogen-free condition and used in accordance with local governmental review boards (Bezirksregierung Köln, from 2008 Landesamt für Natur, Umwelt und Verbraucherschutz NRW in Recklinghausen, Germany). (See also [Extended Experimental Procedures](#)).

Reagents

Ly6C⁺ macrophages were depleted by intravenous injection of 250 μ l clodronate-liposome suspension into the lateral tail vein 6 hr prior to infection. For blocking experiments, 100 μ g of the antibodies were intravenously injected into wild-type mice 1 hr prior to infection (See also [Extended Experimental Procedures](#)). To restore neutrophil migration in *Tnf*^{-/-} mice, 50pg TNF or 2.5 μ g murine recombinant CXCL2 (AbD Serotec) was inoculated 1 hr before infection, 2, 4, 24, and 27 hr after infection and mice were sacrificed 30 hr after infection.

UPEC and UTI Model

The uropathogenic *E. coli* (UPEC) strain 536 (O6:K15:H31) originating from a patient suffering from urinary tract infection was used for induction of bladder infection (Berger et al., 1982). The generation of GFP-expressing UPECs (Engel et al., 2006) and the murine urinary tract infection model were described previously (Engel et al., 2008; Tittel et al., 2013; Tittel et al., 2011; Tittel et al., 2012). (See also [Extended Experimental Procedures](#))

Flow Cytometry

Single-cell suspensions were generated by digestion with 1 mg/ml collagenase IV (Sigma) and 100 μ g/ml DNaseI (Sigma) in FCS-containing RPMI for 30 min at 37°C under mild stirring. Fc receptors were blocked and cells were then incubated with titrated amount of antibodies (See the complete list of antibodies in the [Extended Experimental Procedures](#)). We performed flow cytometry on a FACSCanto II, LSR II and on a Fortessa (BD Biosciences) and analyzed the data with Flow-Jo software (Tristar).

Histology

Immunofluorescence microscopy in cryosections: we embedded bladders into Tissue-Tec (Sakura) and immediately froze it at -80°C. Frozen blocks were cut into 5 μ m sections and mounted on poly-L-lysine-coated glass slides (Menzel) and fixed them with Aceton. Immunohistochemistry in paraffin-sections: Bladders were fixed in 10% IHC Zinc Fixative (BD Bioscience). Afterward the tissue was embedded in paraffin, cut into 5 μ m sections and mounted on lysine-coated glass slides (Menzel). The sections were stained with hematoxylin and eosin or with the neutrophil-specific antibody Ly6G.

We analyzed slides using the CellR software (Olympus), the AIM and ZEN software (Zeiss) and IMARIS (Bitplane) on an IX71 microscope (Olympus), on a laser scanning confocal microscope LSM 510 Meta (Zeiss) and on a LSM 780 (Zeiss). A complete list of antibodies is provided in the [Extended Experimental Procedures](#).

Bone Marrow Chimeras and Adoptive Transfer of Leukocytes

Bone marrow chimeric animals were generated by irradiating mice with 9Gy and reconstitution with 5×10^6 donor BM cells injected into the tail vein. To adoptively transfer bone-marrow-derived macrophages and neutrophils, a MACS isolation procedure was established, which is described in detail in the [Extended Experimental Procedures](#).

Chemokine and Cytokine Analysis

The levels of CXCL2, MIF, CXCL1, CXCL5, CCL2, and CXCL2 in bladder homogenates were measured by ELISA (MM200, DY1978, MKC00B, MX000, MJE00, MM200 R&D Systems) according to the manufacturer's protocol. The expression of chemokines and cytokines in the infected bladder

was determined using the Th1/Th2 (BMS820FFRTU, eBioscience) and the chemokine Flow Cytomix Multiplex Kit (BMS821FF, eBioscience).

In Vitro Assays

Neutrophils were isolated as described in the [Extended Experimental Procedures](#) and cultured in RPMI 1640 media containing 1% FCS. To analyze the MMP-9 levels, cells were stimulated with 10 ng/ml TNF (eBioscience) and/or 10 ng/ml CXCL2 (AbD Serotec) for 6 hr. Afterward the supernatants were analyzed for MMP-9 level by zymography. (See also [Extended Experimental Procedures](#)).

Analysis of MMP-9 Activity by Zymography

Zymograms were performed as described previously (Vandooren et al., 2013). Briefly, proteins were separated on an SDS-gel containing 0.1% gelatin. After different incubation procedures (See [Extended Experimental Procedures](#)) the gel was stained with Coomassie and digitally analyzed using a Canon LIDE scanner. The bands were quantified by densitometry using ImageJ software. Alternatively, MMP9 activity was measured with the Biotrack MMP9 activity assay (RPN2634, GE Health Care).

Statistical Analysis

Results are expressed as mean $\pm$ s.e.m, *p < 0.05; **p < 0.01; ***p < 0.001. Comparisons were made using nonparametric Mann-Whitney or Kruskal-Wallis test for comparing two or more groups, respectively, in combination with Dunn's posttest using Graphpad Software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.01.006>.

AUTHOR CONTRIBUTION

M.S., C.W., L.F. and D.R.E. designed and performed the main experiments. S.G., A.D., S.T., J.-M.P., N.J.M., C.M.-S., K.L., T.Q., and M.F. performed further experiments. G.B., M.J.L., G.O., J.B., R.B., U.P., W. Kolanus, H.-J. G., N.G., W. Kastenmüller, and P.A.K. contributed essential analytic tools and edited the manuscript. C.K. and D.R.E. conceived and supervised the study, interpreted the results and wrote the paper. All authors discussed and interpreted the results.

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3.2 Ly6C^{low} und nicht Ly6C^{high} Makrophagen akkumulieren als erste Zellen im Herzen in einem murinen Modell der linksventrikulären Drucküberlastung

Der Prozess der Rekrutierung und Aktivierung von angeborenen und adaptiven Immunzellen könnte der Ausgangspunkt für immunmodulatorische Therapiestrategien zur Verbesserung des linksventrikulären Gewebeumbaus und der Herzfunktion sein. Mit Hilfe eines murinen Modells, der transversen aortalen Konstriktion (TAC), ließ sich in dieser Arbeit reproduzierbar und durch die Berechnung des *Heart-Weight/Body-Weight-Index* objektivierbar, eine linksventrikuläre Hypertrophie induzieren und die Immunantwort untersuchen. In der Entwicklung der linksventrikulären Hypertrophie kam es zu einer Akkumulation von Immunzellen als Ausdruck der Entzündungsreaktion. Insbesondere Makrophagen waren an Tag 6 nach TAC als dominierende Zellpopulation unter den Immunzellen zu beobachten, aber auch DCs und Neutrophile wurden im Herzgewebe nachgewiesen. Parallel hierzu war ein Anstieg der Monozyten im Blut zu beobachten. Ein wesentlicher Befund dieser Arbeit ist der Nachweis der Differenzierung der kardialen Makrophagen-Untergruppen in der Entwicklung der Hypertrophie (s. Abb. 3A aus Weisheit et al. 2014, S. 11). Hier zeigte sich eine starke Dominanz der Ly6C^{low}-Makrophagen an der Gesamtpopulation der kardialen Makrophagen (s. Abb. 3B aus Weisheit et al. 2014, S. 11). Die Anzahl an Ly6C^{low}-Makrophagen erreichte an Tag 6 nach TAC den höchsten Wert, während die Anzahl an Ly6C^{high}-Makrophagen kaum Schwankungen als Antwort auf die TAC zeigte (s. Abb. 3C, D aus Weisheit et al. 2014, S. 11). Um diese Beobachtungen besser bewerten zu können, wurde der Anteil der Ly6C^{low}- und Ly6C^{high}-Makrophagen zur Gruppe der gesamten CD45⁺-Immunzellen ins Verhältnis gesetzt (s. Abb. 3E aus Weisheit et al. 2014, S. 11) und es bestätigte sich die Beobachtung, dass Ly6C^{low}-Makrophagen über den gesamten beobachteten Verlauf der Hypertrophie-Entwicklung die dominante Makrophagengruppe waren. Weitere Untersuchungen zeigten, dass kardiale Ly6C^{low}-Makrophagen den Fraktalkinrezeptor CX3CR1 und die Adhäsionsmoleküle CD11b und CD31 auf der Oberfläche exprimierten und in direkter Nachbarschaft zu Endothelzellen im Myokard lagen. Die druckabhängig regulierte Expression von Adhäsionsmolekülen und CX3CR1 legt nahe, dass Ly6C^{low}-Monozyten/Makrophagen aus der Zirkulation an den Ort der Gewebeschädigung

rekrutiert wurden. Diese Erkenntnisse unterscheiden die Immunantwort nach TAC maßgeblich von derjenigen nach einem Myokardinfarkt, die von einem biphasischen Verlauf mit einer akuten pro-inflammatorischen mit $\text{Ly6C}^{\text{high}}$ -Makrophagen und Neutrophilen und einer sekundären *Remodelling*-Phase mit Ly6C^{low} -Makrophagen geprägt ist.

RESEARCH ARTICLE

Ly6C^{low} and Not Ly6C^{high} Macrophages Accumulate First in the Heart in a Model of Murine Pressure-Overload

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Abstract

Cardiac tissue remodeling in the course of chronic left ventricular hypertrophy requires phagocytes which degrade cellular debris, initiate and maintain tissue inflammation and reorganization. The dynamics of phagocytes in left ventricular hypertrophy have not been systematically studied. Here, we characterized the temporal accumulation of leukocytes in the cardiac immune response by flow cytometry and fluorescence microscopy at day 3, 6 and 21 following transverse aortic constriction (TAC). Cardiac hypertrophy due to chronic pressure overload causes cardiac immune response and inflammation represented by an increase of immune cells at all three time points among which neutrophils reached their maximum at day 3 and macrophages at day 6. The cardiac macrophage population consisted of both Ly6C^{low} and Ly6C^{high} macrophages. Ly6C^{low} macrophages were more abundant peaking at day 6 in response to pressure overload. During the development of cardiac hypertrophy the expression pattern of adhesion molecules was investigated by qRT-PCR and flow cytometry. CD11b, CX3CR1 and ICAM-1 determined by qRT-PCR in whole cardiac tissue were up-regulated in response to pressure overload at day 3 and 6. CD11b and CX3CR1 were significantly increased by TAC on the surface of Ly6C^{low} but not on Ly6C^{high} macrophages. Furthermore, ICAM-1 was up-regulated on cardiac endothelial cells. In fluorescence microscopy Ly6C^{low} macrophages could be observed attached to the intra- and extra-vascular vessel-wall. Taken together, TAC induced the expression of adhesion molecules, which may explain the accumulation of Ly6C^{low} macrophages in the cardiac tissue, where these cells

might contribute to cardiac inflammation and remodeling in response to pressure overload.

Introduction

Cardiovascular diseases are among the leading causes of death worldwide. Left ventricular (LV) hypertrophy is a main determinant of morbidity and mortality as it occurs in response to various stimuli, including systemic arterial hypertension, aortic stenosis, or remodeling of the myocardium after myocardial infarction (MI) [1]. Despite underestimation of its deleterious consequences, arterial hypertension leads to chronic pressure overload of the LV and initiates a remodeling process which changes tissue morphology and function. At the present time therapeutic approaches to modulate cardiac remodeling are still limited and presume an early diagnosis and consequent therapy of the initial stimuli [2]. Heart failure is the end stage disease of many, initially protective neurohumoral and inflammatory compensatory mechanisms which become harmful but in the course of disease [3, 4].

These mentioned compensatory mechanisms are accompanied by the activation of the cardiac immune response [5]. As the heart comprises more than just cardiac myocytes, e.g. endothelial cells, fibroblasts and a diversity of resident immune cells it becomes more and more essential to decode the cells' particular function and intercellular communication [6]. The complex pro- and anti-inflammatory response after MI has already been described as a monocyte-dependent process [7]. In addition to monocytes, neutrophils are rapidly recruited over the first 24 hours after MI to the site of injury and start degrading extracellular matrix components as well as phagocytosing dead cells. According to several surface markers it is possible to distinguish between resident and inflammatory macrophages and to describe a two phasic monocyte/macrophage response after MI [8]. The first phase is mainly affected by Ly6C^{high}, inflammatory monocytes/macrophages, while the second phase is dominated by the accumulation of Ly6C^{low}, tissue repairing monocytes/macrophages [9].

Current findings of Epelman et al. highlight that the immune system in the heart is uniquely adapted to the demands of physiological and pathological stress in steady state and after injury [10]. However, the dynamics of the immune response in the context of chronic pressure overload and consecutive LV tissue remodeling are incompletely understood. Our group recently verified the impact of MAC2⁺ cells in the development of cardiac hypertrophy but has not yet characterized the distinct macrophage subpopulations according to their surface expression of F4/80 and Ly6C following the definition of Geissman et al. [8, 11]. Recently using a murine model of urinary tract infection we found that F4/80⁺, Ly6C^{high} phagocytes showed pro-inflammatory helper macrophage functions by secreting TNF, while F4/80⁺, Ly6C^{low} phagocytes acted as sentinel macrophages

and displayed helper-cell functions by secreting CXCL2, allowing neutrophil transepithelial migration [12].

The process of recruitment and activation of innate and adaptive immune cells could provide the initial point for immune modulatory strategies to ameliorate LV remodelling and cardiac function. Recent studies highlight that uncontrolled, overshooting monocyte response can impair scar formation after myocardial infarction [13, 14]. Recruitment of monocytes and neutrophils from the circulatory system are important for the induction and maintenance of inflammatory processes. After myocardial infarction, recruited monocytes and macrophages play a critical role in cardiac tissue remodeling [15]. The migration of myeloid cells is controlled by specific chemoattractants, called chemokines, and the expression of adhesion molecules on the surface of immune cells and endothelial cells. Here, the expression of the integrin heterodimer CD11b, also known as Mac-1, on the surface of monocytes and granulocytes directly influences cell adhesion and transendothelial migration as it interacts with the intercellular adhesion molecule 1 (ICAM-1) on endothelial cells [16–18]. Moreover, platelet/endothelial-cell adhesion molecule 1 (PECAM1), also known as CD31 has been reported to be another important junctional molecule, for which leukocytes express ligands, that facilitates cell adhesion and extravasation [19, 20]. In that context, the chemokine receptor CX3CR1, which is also known as fractalkine receptor, contributes to cell recruitment during inflammation through chemotaxis and mediation of adhesion [21–23]. Moreover, CX3CR1 can be used to distinguish between different monocyte/macrophage populations, as Ly6C^{low} monocytes/macrophages express more CX3CR1 than Ly6C^{high} cells [24, 25]. In addition, CX3CR1 is required for monocyte crawling or patrolling in the lumen of the blood vessels [26]. In the kidney, the sensing of a Toll-like-receptor 7 dependent danger signals triggered the intravascular retention of Ly6C^{low} monocytes by the endothelium, requiring CX3CR1 expression by Ly6C^{low} monocytes and CD11b [27]. These results suggest that Ly6C^{low} monocytes behave as sentinels in the vessels, rarely extravasating in comparison to Ly6C^{high} monocytes following *Listeria* infections [26], surveying the integrity of the endothelium. Their impact in orchestrating immune responses under sterile conditions remained undescribed, yet [27].

Thus, we hypothesized that pressure overload attracts specific phagocytes necessary for remodelling the myocardium. Referring to the findings observed in response to MI we proposed temporally coordinated invasion of different types of monocytes. This may be explained by a dynamic expression of adhesion molecules on both partner endothelial cells as well as leukocytes.

Materials and Methods

Animal Handling and Care

Female C57BL/6 mice (20–22 g) between 8 and 12 weeks of age were purchased from Charles River or bred at the central animal facilities of the medical faculty of

Bonn (HET, House of Experimental Therapy). CX3CR1^{-/-} mice were purchased by Jackson Laboratory and bred with C57BL/6 mice to receive heterozygous CX3CR1^{+/-GFP} mice. All mice were maintained under specific pathogen-free condition in isolated, ventilated cages with free access to water and food.

Ethics Statement

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Treatment protocols were approved by the district government of Northrhine Westphalia, here the “State Office for protection of nature, environment and consumers” (LANUV Landesamt für Natur, Umwelt und Verbraucherschutz, Leibnizstraße 10, 45659 Recklinghausen, Germany), which is the responsible agency. The approval number is: 84–02.04.2011.A313. Animal experiments and handling were also supervised by the central animal facilities of the medical faculty Bonn (HET, House of Experimental Therapy, Sigmund-Freud Str. 25, 53127 Bonn, Germany). All surgical interventions were performed under anesthesia and analgesia as described below, and all efforts were made to minimize suffering.

Transverse aortic constriction (TAC)

The TAC model was used to generate pressure overload-induced heart failure. Mice were anaesthetized with isoflurane (2 vol%), intubated with a 22 G tube and ventilated 10 ml/kg BW, 120 breaths/min with a small animal ventilator, purchased from Harvard Apparatus (Holliston, MA). A 27 G needle was used to standardize the degree of aortic constriction. Sham control animals underwent intubation and surgery without aortic restriction. For analgesia, buprenorphine was administered (0.1 mg/kg subcutaneously) before surgical intervention started and 8h later. Mice were sacrificed 3, 6 and 21 days following surgical intervention.

Determination of Heart-Weight/body-Weight-Index

The body weight of each mouse was measured before sacrificing the animals. Weight of the whole heart was determined after carefully removing the organ from the murine thorax and washing out the blood with PBS. The index was calculated in mg/g.

Perfusion

For some experiments it was necessary to perfuse the circulation of the mice before harvesting the hearts. For this, a 23 G needle was placed in the right ventricle of the murine heart and the circulation was perfused with 37°C warm PBS lacking Ca²⁺ and Mg²⁺ for 3 min until the heart, lung and liver were pale.

Flow cytometry

Single cell suspensions from the LV were generated by mincing the tissue and 45 min digestion with 1 mg/ml Collagenase II, 100 mg/ml Collagenase I, 500 U/ml Hyaluronidase and 50 U/ml DNase I (all enzymes were purchased by Sigma, St. Louis, MO) in RPMI at 37°C under mild stirring. Blood samples were collected from the abdominal aorta and coagulation was stopped immediately by adding 0.5 M EDTA solution. Samples were next washed with red blood cell lysis buffer (eBioscience, San Diego, CA). Cells were incubated with CD16/CD32 (2.4G2, BD Bioscience, Franklin Lakes, NJ) antibody at 4°C for 5 min to block non-specific binding of immunoglobulin to the Fc receptors. Cells were stained in PBS lacking Ca²⁺ and Mg²⁺ but containing 0.1% FCS (PAA) and 0.5% sodium azide. Titrated amounts of the following labelled antibodies from eBiosciences (San Diego, CA), BD Biosciences (Franklin Lakes, NJ) and BioLegend (San Diego, CA) were used: CD45 (AFS98), F4/80 (BM-8), Gr1 (RB6-8C5), Ly6C (HK1.4), Ly6G (1A8), CD11b (M1/70), CD31 (MEC 13.3), MHCII/I-A/I-E (2G9), ICAM-1 (YN1/1.7.4) and corresponding isotype controls. Cells were incubated with the antibodies for 15 min at 4°C in the dark and were washed afterwards. The analysis of the mean fluorescence intensity (MFI) refers to the fluorescence intensity of each event in average and may be taken as an indicator for the density of an antigen on the investigated cells.

For intensified ICAM-1 detection surface staining was combined with intracellular staining. We incubated single cell suspension of the LV for 4 h with 1 µl/ml Golgi-Plug (BD Bioscience), incubated them with CD16/CD32 to block Fc receptors, stained for surface markers, fixed BD Cytofix/Cytoperm and permeabilized with BD-Perm/Wash-Buffer according to the manufacturer's protocol (BD Bioscience).

Absolute cell numbers were determined by adding fixed numbers of CaliBRITE APC-beads (BD Biosciences) before measurement as an internal reference. We performed flow-cytometry on a FACSCanto II, LSR II and on a Fortessa (BD Bioscience) and analyzed the data with Flow-Jo software (TreeStar, Ashland, OR). Gating was performed as follows: First all cells were determined via FSC-A and SSC-A, then single cells were defined via SSC-A and FSC-W. Further gating strategies are indicated in respective figure legends.

Histology

Immunofluorescence microscopy in cryosections: After overnight fixation in PFA4%, hearts were embedded in Tissue-Tec (Sakura) and immediately frozen at −80°C. Frozen blocks were cut into 30 µm sections and mounted on poly-L-lysine-coated glass slides (Menzel, Braunschweig, Germany). We used the following antibodies from eBiosciences and Bio Legend: CD31 (MEC 13.3), F4/80 (BM-8) and Ly6G (1A8). Furthermore we utilized AF488 labeled rabbit anti-GFP antibody purchased by Life technologies (Carlsbad, CA) and costained the slices with a donkey anti-rabbit antibody in AF647 (Life technologies) to minimize background fluorescence. Slices were analysed by immune fluorescence confocal

microscopes (LSM 510 Meta and LSM 780, Carl Zeiss Microscopy GmbH, Jena, Germany). Data was analyzed with Imaris-software (Bitplane AG Zurich, Switzerland).

Real Time PCR

Left ventricular tissue samples were stored at -80°C until homogenization in RNeasy Lysis Buffer (QIAGEN, Hilden, Germany) containing 1% 2- β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO) by drawing through a 21 G needle >10 times. Subsequently total RNA was purified using the RNeasy mini kit (QIAGEN), according to the manufacturer's instructions. CD11b, CX3CR1, ICAM-1 and 18s mRNA expression were quantified by mRNA RT-PCR using TaqMan PCR detection System (Life Technologies, Carlsbad, CA). Messenger-RNA concentration was quantified by absorbance spectroscopy (Nanodrop Products, Wilmington, DE). The 18S ribosomal RNA was amplified as an internal control. Amounts of specific cDNA were finally normalized to 18s using the Δct method, and results are depicted as $2^{\Delta\Delta\text{ct}}$ values, as described [28, 29].

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical tests performed included Student's t test and one-way ANOVA with Tukey's multiple-comparison test and were performed with Prism 4 (GraphPad Software, Inc. La Jolla, CA). P values of less than 0.05 were considered significant.

Results

TAC leads to cardiac inflammation

We studied the dynamics of phagocyte accumulation following cardiac hypertrophy using a murine model of TAC. Hereby, the determination of the Heart-Weight/Body-Weight-Index (HW/BW-Index) of the mice establishes the development of cardiac hypertrophy. The HW/BW-Index of TAC operated mice increased significantly on day 3 and 6 in comparison to sham controls and was still significantly elevated 3 weeks after TAC induction (Fig. 1A). The increased HW/BW-ratio is due to an increased heart weight because the body weight remained stable throughout the experimental duration (mean heart weight: $124.8 \text{ mg} \pm 12.85 \text{ mg/heart}$ in healthy animals versus $145.9 \text{ mg} \pm 16.11 \text{ mg}$, $164.4 \text{ mg} \pm 27.38$ and $170.8 \text{ mg} \pm 34.53 \text{ mg}$, 3, 6 and 21 days after TAC, respectively), indicating cardiac hypertrophy.

After preparation of the left ventricle (LV) and enzymatic digestion, the cardiac tissue suspension was stained for flow cytometry. To study the impact of immune cells in the process of cardiac remodeling, the single cell suspension was stained for the common leukocyte antigen CD45. Cardiac hypertrophy, provoked by TAC, significantly increased immune cell numbers in the LV tissue 3, 6 and 21 days following surgical intervention (Fig. 1B), indicating cardiac inflammation.

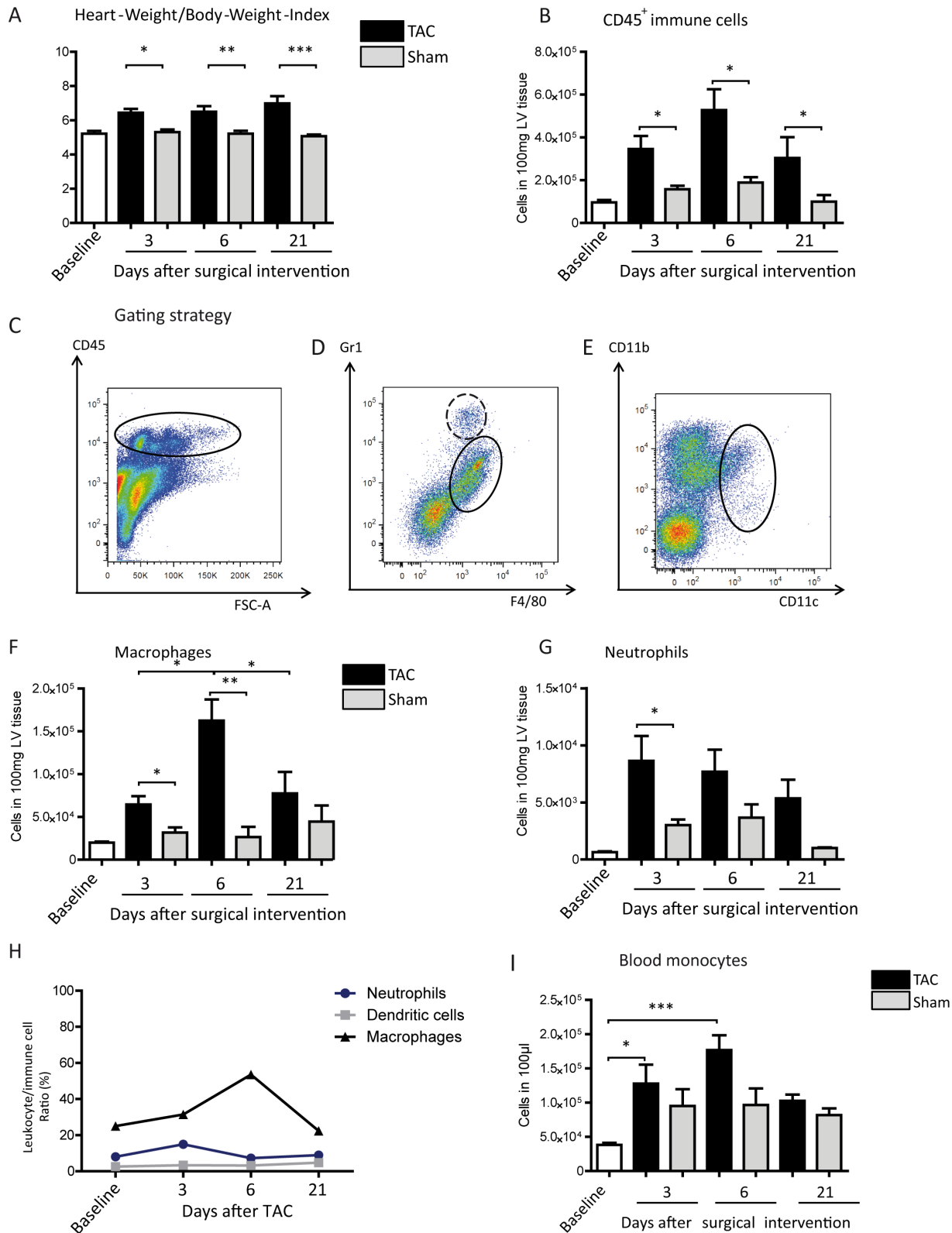


Figure 1. Morphological changes of the cardiac tissue in response to pressure overload. (A) Heart-Weight/Body-Weight-Index was calculated. Mean $\pm$ SEM, n: Baseline = 12 mice, 3d TAC = 11 mice, 3d Sham = 10 mice, 6d TAC = 12 mice, 6d Sham = 12 mice, 21d TAC = 10 mice, 21d Sham = 10 mice. (B) The number of immune cells in cardiac tissue was determined by flow cytometry using CD45 surface staining. (C–E) Gating strategy of cardiac tissue; concatenated plots of 3 healthy baseline mice. (C) Cardiac immune cells (plot on the left, black ellipse) were determined via CD45 staining, previously gating all single cells. (D) Neutrophils (plot in the middle, black dashed circle) and macrophages (plot in the middle, black ellipse) were distinguished regarding the expression of Gr1 and F4/80 as indicated, previously gating all CD45⁺ cells. (E) Dendritic cells (plot on the right, black ellipse) were determined by CD11b and CD11c staining, previously gating all CD45⁺ cells. (F, G) The number of macrophages and neutrophils was determined by flow cytometry analysis. (B, F, G) Mean $\pm$ SEM; n: Baseline = 10 mice, 3d TAC = 7 mice, 3d Sham = 7 mice, 6d TAC = 6 mice, 6d Sham = 4 mice, 21d TAC = 10 mice, 21d Sham = 10 mice. (H) Temporal development of leukocyte/immune cell ratio was determined for baseline animals as well as at 3, 6 and 21 days after TAC. Mean $\pm$ SEM; n PMN/macrophages: Baseline = 8 mice, 3d TAC = 5 mice, 6d TAC = 6 mice, 21d TAC = 5 mice; n DCs: Baseline = 4 mice, 3d TAC = 4 mice, 6d TAC = 8 mice, 21d TAC = 7 mice. (I) Flow cytometric quantification of monocytes in the blood. Mean $\pm$ SEM; n: Baseline = 10 mice, 3d TAC = 9 mice, 3d Sham = 7 mice, 6d TAC = 7 mice, 6d Sham = 4 mice, 21d TAC = 5 mice, 21d Sham = 4 mice. *P<0.05, **P<0.01, ***P<0.001.

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To determine the composition of immune cells, leukocytes were distinguished via surface staining for F4/80 (tissue macrophages), Ly6C (macrophage subpopulations), CD11c (dendritic cells) and Gr1 or Ly6G (neutrophils; see gating strategy in [Fig. 1C–E](#)). A significant increase in macrophages in the cardiac tissue was observed 3 days after TAC, which peaked at day 6 ([Fig. 1F](#)), whereas neutrophils were significantly increased at day 3 ([Fig. 1G](#)). At day 21 following TAC the number of macrophages and neutrophils in the heart were still elevated, although it did not reach the level of significance. Regarding the possible importance of leukocytes in the process of LV hypertrophy the proportion of macrophages, neutrophils and dendritic cells were examined in relation to all immune cells at the three time points and at baseline level. The phagocyte/immune cell ratio revealed that macrophages were the dominating cell population, representing more than half (mean: 53.55%) of the cardiac immune cells at day 6 following TAC, whereas the amount of neutrophils did not exceed 15% and the dendritic cells stayed below 5.4% and were not influenced by pressure overload ([Fig. 1H](#)). Moreover, whether or not a systemic reaction of the immune system to the stimulus of pressure overload occurred was examined. For this the number of monocytes in the circulation was ascertained at 3, 6 and 21 days after TAC ([Fig. 1I](#)). Although monocyte numbers appear to increase in response to surgical intervention, only the TAC-operated mice had significantly high numbers. Taken together these findings indicate that pressure overload led to cardiac inflammation as characterized by the accumulation of phagocytes.

Macrophages during LV remodeling

For in situ localization of immune cells cryosections of cardiac tissue were examined. As macrophages proved to be the most common immune cells in the flow cytometry analysis, cryosections were prepared from hearts of CX3CR1^{GFP/+} transgenic mice. In these mice one allele of the CX3CR1 gene, the monocyte/macrophage-specific receptor for the membrane-tethered chemokine fractalkine has been replaced by the gene encoding enhanced green fluorescent protein (GFP) which facilitates monocyte/macrophage identification in cardiac tissue [30]. Thereby, cardiac macrophages were observed mainly in the endocardial and myocardial region of the RV lumen, whereas these immune cells were less

abundant in the endocardial region of the LV lumen (Fig. 2 A–C). In intermediate fluorescence microscopical magnification of the endocardial and mid-myocardial regions of the RV lumen, the macrophages appeared to be evenly distributed (Fig. 2 D–F; 2G–I). A more detailed view revealed numerous macrophages in the vicinity of blood vessels (Fig. 2J–L).

To further elucidate cardiac macrophages they were classified according to their expression pattern of Ly6C (see gating strategy in Fig. 3A). Thus, cardiac Ly6C^{low} and Ly6C^{high} macrophages were quantified by flow cytometry after TAC or sham operation. Ly6C^{low} macrophages represent the dominant part of the whole F4/80⁺ cardiac macrophage population in steady state and at all-time points after surgical intervention (Fig. 3B). Cardiac Ly6C^{low} macrophages increased after TAC reaching a maximum at day 6 and remained elevated until day 21, (Fig. 3C) while the number of Ly6C^{high} macrophages did not alter significantly at any time-point (Fig. 3D). To gain better insight into the proportion of both macrophage subpopulations, the numbers of Ly6C^{low} and Ly6C^{high} macrophages were normalized to the total immune cells. This evaluation validates the transient up-regulation of Ly6C^{low} macrophages due to pressure overload and emphasizes the large differences between the amounts of the two groups of macrophages (Fig. 3E). Taken together our findings indicate that after TAC the Ly6C^{low} macrophages form the major cardiac immune cell population with a temporal peak at day 6.

Localization of phagocytes following TAC

To exclude an influence of circulating immune cells on the analysis of intracardial macrophages, we perfused the circulation of the mice with PBS prior to explanting the hearts. In these mice, the increase of F4/80⁺ macrophages following TAC exhibited the same general picture (Fig. 4A) as without perfusion (Fig. 1F). However, the TAC-dependent accumulation of macrophages in the cardiac tissue reached the level of significance only at day 6. A direct comparison of cardiac macrophage numbers prepared from perfused versus non-perfused animals revealed a significant decline due to perfusion significant only at day 6 (Fig. 4B).

Interestingly, the number of cardiac Ly6C^{low} macrophages was reduced by perfusion of the circulation, whereas the lower frequency of Ly6C^{high} macrophages was not influenced by perfusion (Fig. 4C, D). This could be taken as a sign for a specific adhesion of Ly6C^{low} macrophages to the cardiac vessel walls in response to pressure overload. In accompanying blood samples Ly6C^{low} and Ly6C^{high} monocytes were ascertained according to the Ly6C surface expression (gating strategy in Fig. 4E). Analyzed during the development of LV hypertrophy, the Ly6C^{low} monocytes did not differ between TAC- and sham-operated groups, whereas TAC seemed to increase Ly6C^{high} monocyte numbers compared to sham-operated controls in the circulation (Fig. 4G, H). However, this monocyte population did not reach the level of significance in comparison with the respective sham groups. But, accumulatively the Ly6C^{high} cell numbers across all time-points revealed a significant increase of these cells in the blood due to

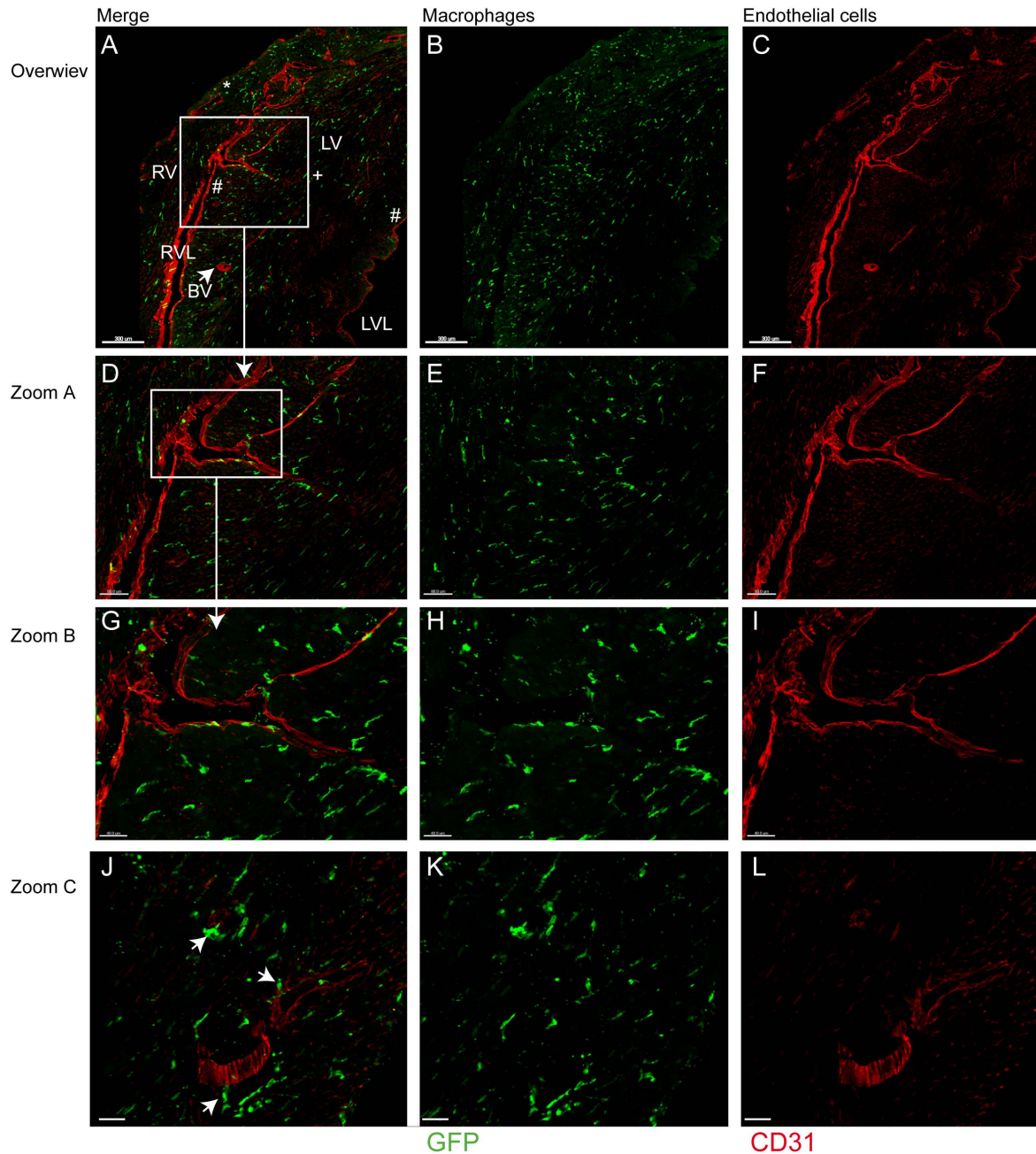


Figure 2. Macrophage localization in the heart determined by immunostaining. (A) Representative cardiac sections of CX3CR1^{GFP/+} mice 6 d after TAC, exhibiting macrophages in green, CD31 visualized in red by antibody staining. (A–C) Overview; * indicates the epicardial, + the mid-myocardial and # the endocardial regions; LV marks the left ventricle and RV the right ventricle. The arrow/BV marks one exemplary cardiac blood vessel. Scale bar: 300 µm. (D–F) Zoom in on the mid-myocardial and endocardial regions of the LV at the border to the RV. Scale bar: 80 µm; (G–I) Zoom in on the endocardial border zone of the LV and RV. Scale bar: 40 µm. (J–L) Zoom in on cardiac blood vessels in the mid-myocardial region of the LV, arrows indicate neighbouring macrophages. Scale bar: 35 µm.

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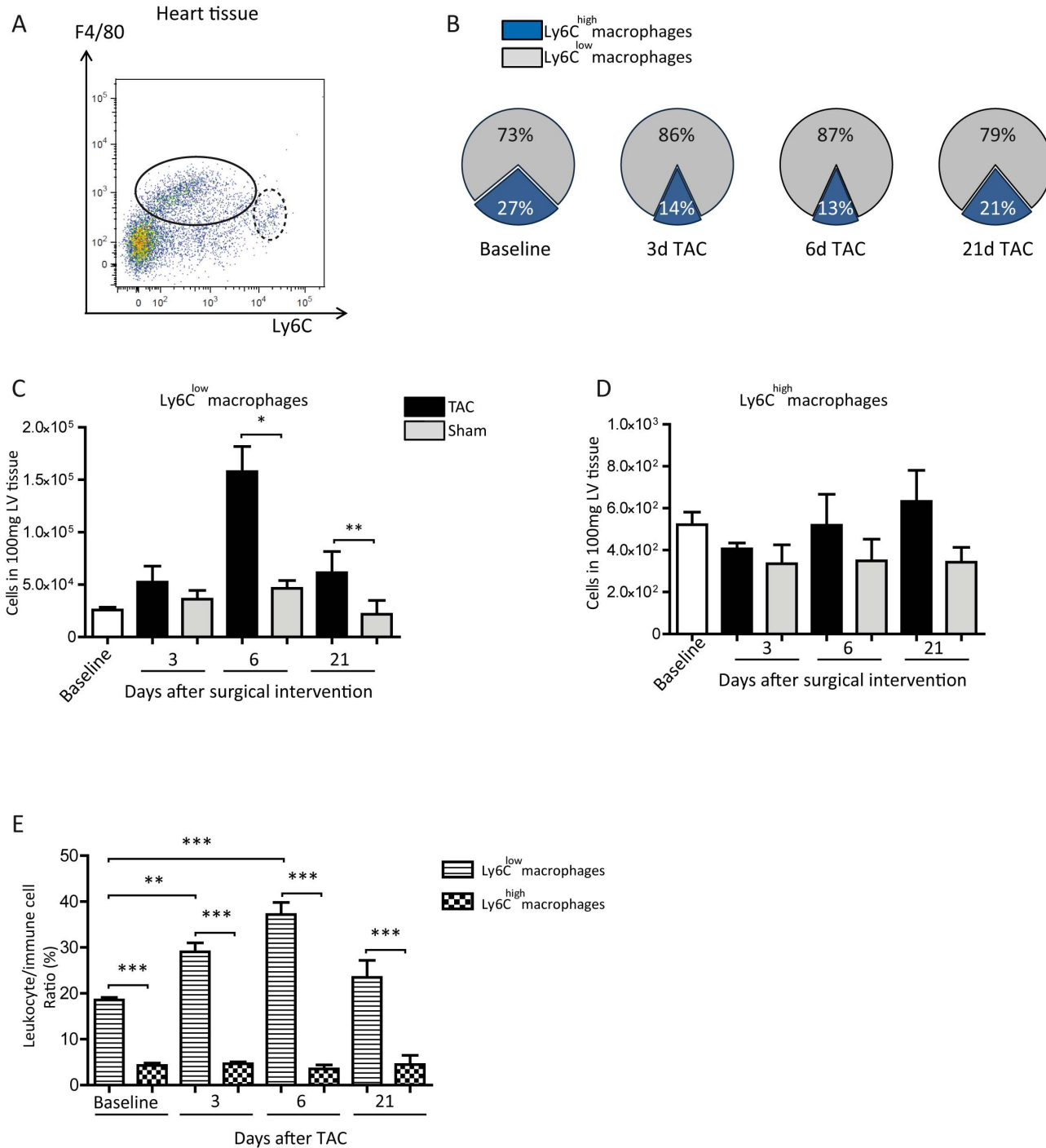


Figure 3. Cardiac phagocytes. (A) Gating strategy to determine the subpopulations of Ly6C^{low} (black ellipse) and Ly6C^{high} macrophages (black dashed ellipse) in the cardiac tissue via F4/80 and Ly6C staining, previously gating all immune cells; concatenated plots of 3 healthy baseline mice. (B) Pie graphs representing the relative proportion of Ly6C^{low} and Ly6C^{high} macrophages in relation to the whole population of cardiac macrophages. Graphs were drawn with Microsoft Excel 2010; results are shown as mean, n: Baseline = 10 mice, 3d TAC = 4 mice, 6d TAC = 4 mice, 21d TAC = 4 mice. (C, D) Flow cytometric quantification of Ly6C^{high} and Ly6C^{low} macrophages. (E) Flow cytometric quantification of the respective immune cell/macrophage ratio. (C, D, E) Mean $\pm$ SEM; n: Baseline = 10 mice, 3d TAC = 5 mice, 3d Sham = 4 mice, 6d TAC = 8 mice, 6d Sham = 4 mice, 21d TAC = 6 mice, 21d Sham = 7 mice. *P<0.05, **P<0.01, ***P<0.001.

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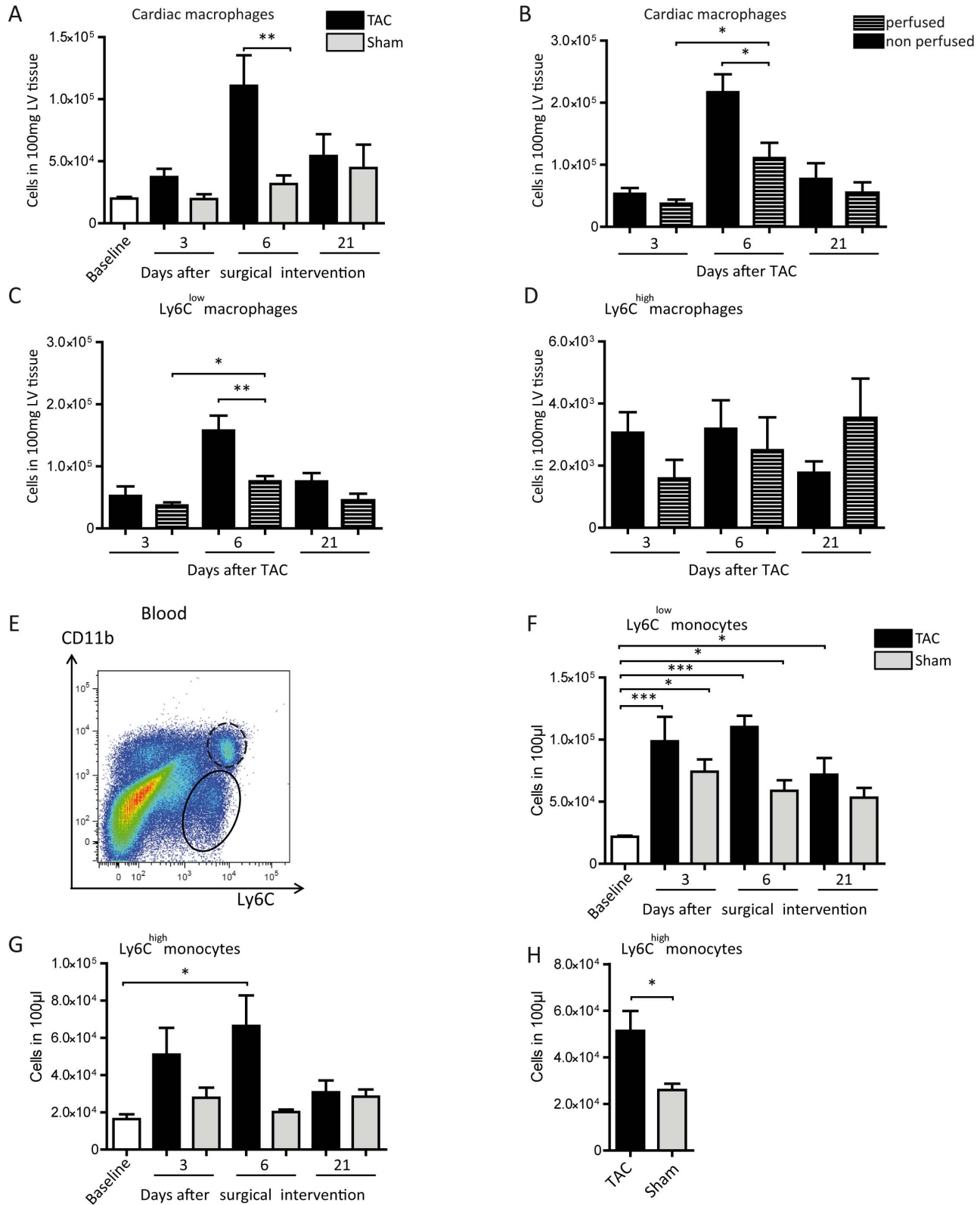


Figure 4. Flow cytometric quantification of macrophages in the heart and monocytes in the circulation. (A) Number of cardiac macrophages after PBS perfusion before explanting the hearts. Mean $\pm$ SEM; n: Baseline = 10 mice, 3d TAC = 6 mice, 3d Sham = 4 mice, 6d TAC = 8 mice, 6d Sham = 4 mice, 21d TAC = 6 mice, 21d Sham = 5 mice. (B) Number of cardiac macrophages with and without PBS perfusion before explanting the hearts. Mean $\pm$ SEM; n of perfused animals: 3d TAC = 6 mice, 6d TAC = 7 mice, 21d TAC = 6 mice; n of non-perfused animals: 3d TAC = 6 mice, 6d TAC = 5 mice, 21d TAC = 6 mice. (C, D) Quantification of cardiac Ly6C^{low} (C) and Ly6C^{high} (D) macrophages with and without PBS perfusion before explanting the hearts. Mean $\pm$ SEM; n of perfused animals: 3d TAC = 4 mice, 6d TAC = 8 mice, 21d TAC = 4 mice; n of non-perfused animals: 3d TAC = 4 mice, 6d TAC = 7 mice, 21d TAC = 4 mice. (E) Gating strategy to determine the subpopulations of Ly6C^{low} (black ellipse) and Ly6C^{high} (black dashed ellipse) monocytes in the blood via CD11b and Ly6C staining, previously gating all single cells; concatenated plots of 3 healthy baseline mice. (F, G) Quantification of Ly6C^{low} (F) and Ly6C^{high} (G) monocytes in the blood. Mean $\pm$ SEM; n: Baseline = 10 mice, 3d TAC = 7 mice, 3d Sham = 5 mice, 6d TAC = 7 mice, 6d Sham = 4 mice, 21d TAC = 5 mice, 21d Sham = 4 mice. (H) Accumulation of Ly6C^{high} monocyte numbers of all three time points after TAC- and sham-operation. Mean $\pm$ SEM; n: Baseline = 10 mice, TAC = 16 mice, sham = 12 mice. *P<0.05, **P<0.01, ***P<0.001.

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pressure overload (Fig. 4I). These findings strengthen our hypothesis that the observed accumulation of Ly6C^{low} macrophages in the heart after TAC is a local phenomenon and most likely due to the cardiac immune response in the process of left ventricular hypertrophy.

Expression of adhesion molecules

We hypothesized that the local attachment of Ly6C^{low} macrophages to the endothelium in response to pressure overload was due to up-regulation of adhesion molecules. Due to the accumulation of macrophages within the first week after TAC we focused on day 3 and 6. To test the above-mentioned hypothesis we analyzed whole LV tissue for expression of adhesion molecules by qRT-PCR. CD11b-, CX3CR1- and ICAM-1-mRNA expression were enhanced at day 3 and 6 after TAC (Fig. 5A–C), whereas CD31-mRNA expression was not altered compared to sham at the respective time points (data not shown).

To identify the responsible cell types for increased expression flow cytometry was applied and the mean fluorescence intensity (MFI) of the particular adhesion molecules was determined in conjunction with the particular cardiac cell population. On the surface of Ly6C^{low} macrophages from hypertrophied hearts MFI monitoring revealed a significant increase in CD11b fluorescence only at day 6 whereas CX3CR1 fluorescence was significantly elevated at both time points (Fig. 5D, E). Furthermore, CD31 fluorescence on Ly6C^{low} macrophages did not reach the level of significance at any time-point (Fig. 5F). In contrast, none of the determined antigens was altered on the surface of cardiac Ly6C^{high} macrophages in response to pressure overload (Fig. 5 G–I).

Based on the finding, that mRNA expression levels of ICAM-1 are regulated in the first week following TAC, we analyzed the expression of this adhesion molecule on the surface of cardiac endothelial cells which were identified as CD45-negative and CD31-positive cells (see gating strategy in Fig. 6A) [31]. Here, the MFI of the macrophage adhesion molecule CD31 was slightly elevated at days 3 and 6 after TAC, while ICAM-1 reached the level of significance at days 3 and 6 (Fig. 6B, C). Taken together, the corresponding adhesion molecules ICAM-1 and CD11b were elevated due to pressure overload on endothelial cells and Ly6C^{low} macrophages. This may facilitate the adhesion of Ly6C^{low} macrophages to the cardiac vessel wall.

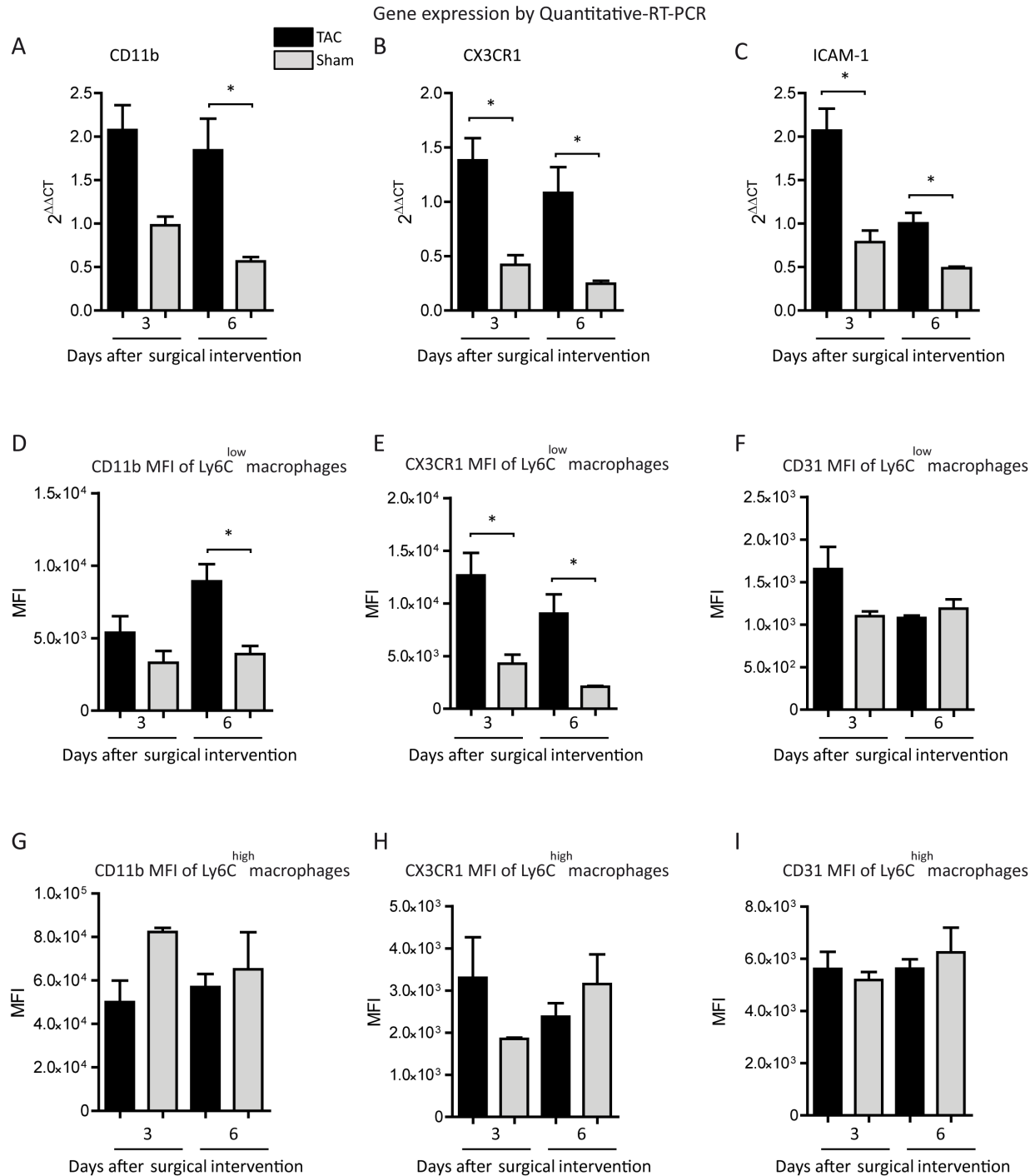


Figure 5. Analysis of adhesion molecules. (A–C) The mRNA-expression levels of adhesion molecules were quantified in the LV tissue of TAC- and sham-operated animals. The expression of the respective mRNA was normalized to healthy baseline controls. Mean $\pm$ SEM; n: 3d TAC = 6 mice, 3d Sham = 3 mice, 6d TAC = 6 mice, 6d Sham = 3 mice. (D–I) Flow cytometric delineation of the Mean Fluorescence Intensity (MFI) of CD11b (D, G), CX3CR1 (E, H) and CD31 (F, I) on the surface of cardiac Ly6C^{low} (E–G) and Ly6C^{high} (H–J) macrophages. Mean $\pm$ SEM; n: 3d TAC = 5 mice, 3d Sham = 3 mice, 6d TAC = 8 mice, 6d Sham = 3 mice. *P<0.05.

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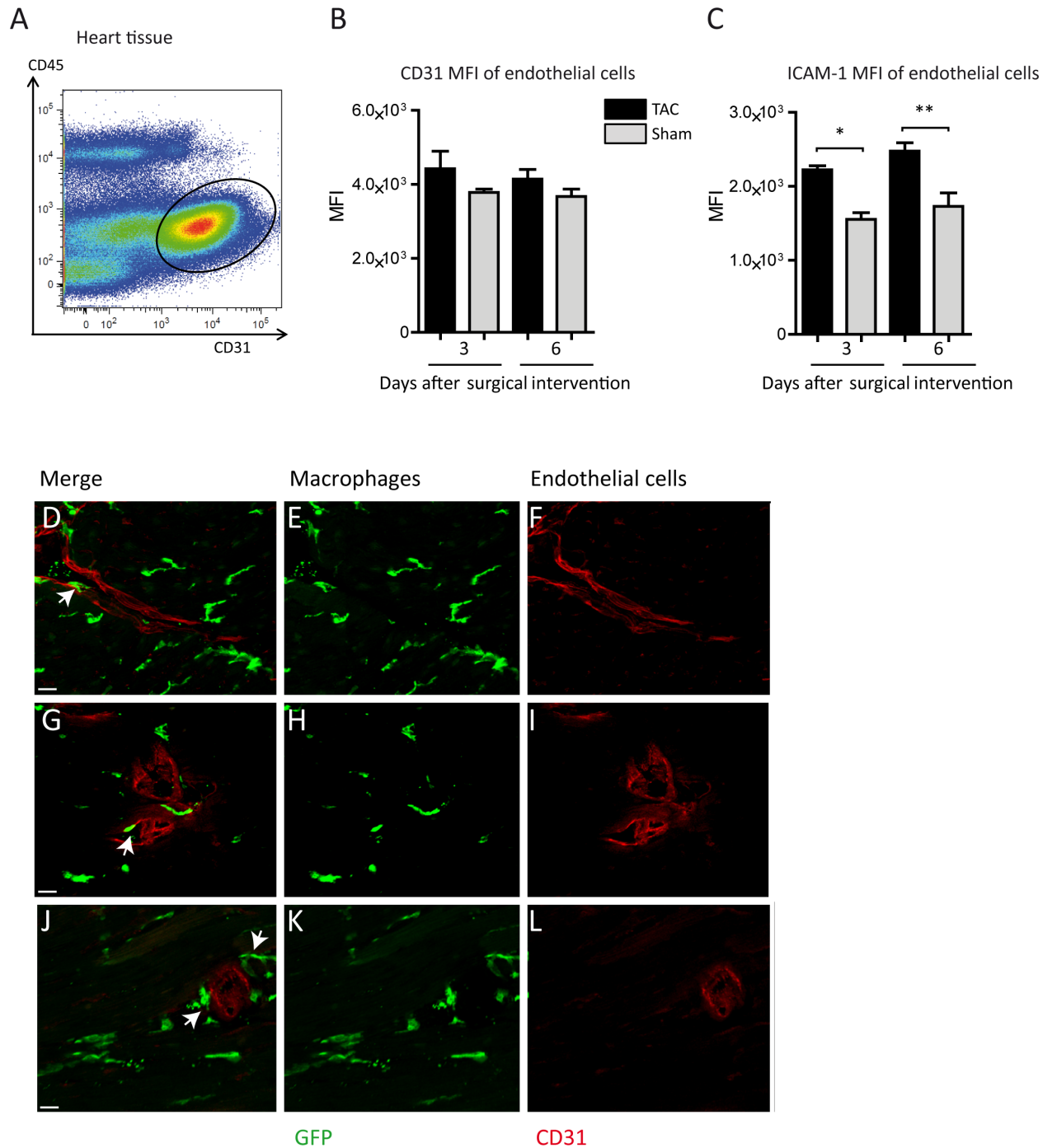


Figure 6. Macrophage adhesion in pressure overload. (A) Gating strategy to determine the endothelial cells among all single cells in the heart via CD45 and CD31 staining (black ellipse), previously gating all single cells; concatenated plots of 5 healthy baseline mice. (B, C) Flow cytometric delineation of CD31- and ICAM-1-MFI of cardiac endothelial cells. Mean $\pm$ SEM; n: 3d TAC = 5 mice, 3d Sham = 3 mice, 6d TAC = 8 mice, 6d Sham = 3 mice * $P < 0.05$, ** $P < 0.01$. (D–L) Representative cardiac sections of CX3CR1GFP/+ mice 6 d after TAC. Monocytes/macrophages marked by GFP fluorescence in green, CD31 visualized in red by antibody staining. Arrows indicate monocytes/macrophages at different steps of recruitment: adhering to the intravascular endothelium (D–F), migrating through the endothelium (G–I) and directly neighboring the blood vessels (J–L). Scale bars: 30 μ m (D–L).

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To further validate this conclusion monocytes and macrophages in the vicinity of cardiac blood vessels were visualised. Thereby the different steps of monocyte/macrophage recruitment to the cardiac tissue could be observed. GFP positive monocytes/macrophages attach to the vessel lumen migrate through the endothelium of small blood vessels and locate in close contact to the extravasal surface during pressure overload (Fig. 6D–L).

These findings highlight, that leukocyte adhesion happens in cooperation between immune cells and endothelium and that pressure overload provokes the expression of adhesion molecules, not only on macrophages, but also on endothelial cells.

Discussion

Our findings highlight the dynamic changes of the cardiac immune response during the development of LV hypertrophy, which were characterized by significantly increased immune cell numbers within the first 21 days after TAC. The increase in CD45⁺ cells was due to elevated macrophage numbers at day 3 and 6 after TAC, as well as to a raised number of neutrophils at day 3. In this context, we demonstrated for the first time that LV pressure overload induced the accumulation of Ly6C^{low} macrophages in the myocardium. This selection of immune cells is in concordance with the specific expression and interaction between CD11b on the surface of monocytes/macrophages and ICAM1 on endothelial cells.

The amount of TAC-induced cardiac hypertrophy in this study amounted to about 7.5 HW/BW at day 6 and 21 which is in agreement with the current literature [11, 32]. Acute pressure overload is accompanied by a transient inflammatory response, which was defined by increased expression of pro-inflammatory cytokines in the cardiac tissue [33]. To gain further insight into the dynamics of this inflammatory process it is necessary to specify the involved immune cells. In previous studies it was shown that the number of MAC2⁺ macrophages in the heart increases during development of cardiac hypertrophy [34]. Therefore, in this study special interest was devoted to monocytes/macrophages. According to the current literature [8, 12, 35], macrophage populations in cardiac tissue were defined by flow cytometry as CD45⁺, F4/80⁺, Ly6C^{high} or Ly6C^{low}. Tissue suspensions prepared from whole hearts for flow cytometry comprise immune cells from different sites (i.e., cells circulating in the blood stream or those residing in cardiac tissue and immune cells adhering to the border between the bloodstream and tissue). Previous studies, investigating cardiac tissue either analyzed perfused or non-perfused hearts [7, 32, 35, 36]. To differentiate between circulating, adhering and resident monocytes/macrophages we directly compared perfused and non-perfused hearts. Furthermore, we determined the amount of circulating monocytes in corresponding blood samples and evaluated the location of resident monocytes/macrophages by histology.

TAC induced a transient increase in F4/80⁺ monocytes/macrophages and Ly6G⁺ neutrophils. The dominant proportion of cardiac macrophages consisted of Ly6C^{low} cells in healthy baseline animals as well as in our model of cardiac hypertrophy. This cell population increased specifically in response to TAC, while Ly6C^{high} macrophages remained unaltered throughout the observation period. This was an unexpected finding as previous investigators reported that Ly6C^{high}/M1 macrophages dominated the immune response at day 3 in response to myocardial ischemia [7,9]. In case of MI Ly6C^{high}/M1 macrophages exhibit phagocytic, proteolytic and inflammatory functions, whereas Ly6C^{low}/M2 macrophages promote healing by myofibroblast accumulation, angiogenesis, and deposition of collagen [9]. However, MI leads to cellular necrosis ending in cellular degradation products inducing an acute severe inflammatory immune response within the first three days [7]. Attracted Ly6C^{high}/M1 macrophages seem to play a central role in clearing this cellular debris [37], which does not emerge in comparable amounts during pressure overload. According to our results TAC seems to cause a more chronic stress to the heart, substantiated by a slow prolonged remodeling process which may be dominated by Ly6C^{low}/M2 macrophages.

Perfusion of the circulation prior to explanting the heart reduced the number of cardiac macrophages, which continued to day 6 after TAC. In the perfused heart Ly6C^{low} monocytes/macrophages mirror the TAC-dependent temporal dynamics of the whole macrophage population determined in the cardiac tissue. Obviously the accumulation of macrophages in the heart appeared at a site which is accessible to the perfusion, indicating that these cells may be specifically attached to the endothelium. Attached cells could be demonstrated in histological preparations. It is important to mention that Ly6C^{low} monocytes/macrophages express more CX3CR1 than Ly6C^{high} cells. Therefore, the bright green cells in the histological sections can be referred to as Ly6C^{low} monocytes/macrophages [24,25].

Remarkably, the Ly6C^{high} monocyte fraction in the blood was elevated in response to pressure overload, whereas their Ly6C^{low} counterpart did not vary significantly in this compartment. To further explain the specific accumulation of Ly6C^{low} macrophages in the heart, the expression of selected adhesion molecules was monitored in cardiac tissue. CD11b-, ICAM-1- and CX3CR1-mRNA expression proved to be elevated following pressure overload. To specify the adhesion molecules, they were quantified and assigned to certain cell-types via flow cytometry. We found up-regulation of CD11b and CX3CR1 expression on the surface of Ly6C^{low} macrophages within the first week after TAC, which was not detectable on the Ly6C^{high} macrophages. Simultaneously ICAM-1 the ligand of CD11b was elevated on cardiac endothelial cells as shown by MFI determination. This can facilitate the attachment of Ly6C^{low} monocytes to the vessel wall and may contribute to the increased number of these cells compared to the Ly6C^{high} monocytes/macrophages. Moreover, the increased expression of CX3CR1 on the surface of Ly6C^{low} monocytes may further promote their adhesion to the endothelium, although CX3CL1-mRNA expression in whole LV

tissue was not influenced by TAC (results not shown). The specific up-regulation of the adhesion molecules has augmented the invasion of Ly6C^{low} monocytes/macrophages into the cardiac tissue during pressure overload. Interestingly, a CX3CR1 and CD11b dependent crawling of Ly6C^{low} monocytes at the luminal side of the endothelium has been recently described in the kidney [27], which may support our assumption. Although the increase in shear stress of the pre-stenotic vessels in our model can explain the up-regulation of ICAM-1 on the surface of endothelial cells, there is no obvious explanation for the changes on the surface of the Ly6C^{low} monocytes. Enhanced adhesion of monocytes from hypertensive patients has also been observed in an *in vitro* assay with human endothelial cells [38].

Moreover, our findings may contribute to understanding the role of Ly6C^{low} monocytes which have been described as specialized in surveying the endothelium [24,25] and to require CX3CR1 and CD11b expression for this function [27]. We report that Ly6C^{low} monocytes/macrophages up-regulate these adhesion molecules and accumulate at the site of inflammation but do not extravasate extensively. Instead, a major part seems to be locally attached to the pre-stenotic endothelium of the cardiac vasculature in response to pressure overload. These cells contribute to tissue inflammation in this condition, and may represent a promising therapeutic target to prevent subsequent organ damage.

Although, many studies have been performed to characterize the specific roles of leukocytes in the case of remodeling after a cardiac ischemic injury, the role of these immune cells in cardiac remodeling during pressure overload is less defined. Here we have shown that the temporal succession of the immune response during the development of cardiac hypertrophy clearly differs from that after MI. Among the immune cells involved Ly6C^{low} monocytes/macrophages dominated the population at any time-point during the development of LV hypertrophy. In contrast, Ly6C^{high} monocytes/macrophages were the most abundant cells at day three after infarction, while Ly6C^{low} monocytes invaded the heart later. Furthermore, we demonstrated a simultaneous up-regulation of corresponding adhesion molecules on the surface of Ly6C^{low} monocytes/macrophages, as well as on endothelial cells, which may explain the selective enrichment of these cells in the cardiac tissue. According to our results the performance of Ly6C^{low} monocytes/macrophages is suitable for mediating the remodeling during the development of cardiac hypertrophy.

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Author Contributions

Conceived and designed the experiments: CK CW GB AH. Performed the experiments: CW YZ AF OK GW AS. Analyzed the data: CW CK SF. Wrote the paper: CW CK OB RM GB.

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3.3 CX3CR1 stellt eine Voraussetzung für die Entwicklung von linksventrikulärer Hypertrophie und kardialer Dysfunktion bei Mäusen nach transverser aortaler Konstriktion dar

Die CX3CL1/CX3CR1-Achse vermittelt die Rekrutierung und Extravasation von CX3CR1-exprimierenden Untergruppen von Leukozyten. Zwei verschiedene Makrophagenpopulationen, pro-inflammatorische $\text{Ly6C}^{\text{high}}$ - ($\text{CCR2}^{\text{high}}$, $\text{Cx3CR1}^{\text{low}}$), und residente Ly6C^{low} - (CCR2^{low} , $\text{Cx3CR1}^{\text{high}}$) Zellen tragen zur Entzündung und Gewebereparatur im geschädigten Herzen bei. Bei einer chronischen Drucküberlastung, induziert durch eine TAC, wird eine Immunantwort induziert, die durch einen hohen Anteil an Ly6C^{low} $\text{Cx3CR1}^{\text{high}}$ -Makrophagen charakterisiert ist. Durch die Verwendung von CX3CR1-defizienten Mäusen ($\text{Cx3cr1}^{\text{GFP/GFP}}$) konnte in dieser Arbeit die Relevanz des Fraktalkinrezeptors in der Entwicklung der linksventrikulären Hypertrophie dargestellt werden. Der wegweisende Befund lag in der Beobachtung, dass ein Fehlen von CX3CR1 zu einer Kardioprotektion im Modell der TAC führt. Die CX3CR1-defizienten Mäuse bildeten keine linksventrikuläre Hypertrophie aus **Fehler! Verweisquelle konnte nicht gefunden werden.** (s. Abb. 2A aus Weisheit et al. 2021, S. 7) und zeigten keine Einschränkung der Pumpfunktion im Vergleich zu Wt-Mäusen. Dieser Befund spiegelte sich auch in weiterführenden molekularbiologischen Untersuchungen wider. Während Wt-Mäuse eine signifikant gesteigerte Aldolase Expression, als weiteren Hinweis auf eine Hypertrophie nach TAC zeigten, führte ein Fehlen von CX3CR1 zu einer konstant niedrigen Aldolase-Expression (s. Abb. 2B aus Weisheit et al. 2021, S. 7). Auch der Herzinsuffizienzmarker *brain natriuretic peptide* (BNP), wurde nicht vermehrt in $\text{Cx3cr1}^{\text{GFP/GFP}}$ Mäusen exprimiert im Vergleich zu Wt-Mäusen (s. Abb. 2C aus Weisheit et al. 2021, S. 7). Untersuchungen an Herzschnitten führten weiterhin zu der Beobachtung, dass in $\text{Cx3cr1}^{\text{GFP/GFP}}$ -Mäusen 21 Tage nach TAC keine deutliche Zunahme der Kardiomyozytengröße zu beobachten war, wohingegen in den Wt-Mäusen eine signifikante Zunahme gemessen werden konnte (s. Abb. 2D aus Weisheit et al. 2021, S. 7). Weiterführende Analysen zur Immunantwort in $\text{Cx3cr1}^{\text{GFP/GFP}}$ -Mäusen nach TAC verdeutlichten, dass eine frühe pro-inflammatorische Reaktion, charakterisiert durch die Zunahme von Neutrophilen und $\text{Ly6C}^{\text{high}}$ -Makrophagen, sowie den Anstieg von pro-inflammatorischen Zytokinen und CCL2 als Monozyten-chemoattraktives Molekül im

linken Ventrikel, den wesentlichsten Unterschied gegenüber den Wt-Tieren darstellten und eine mögliche Ursache für die Kardioprotektion der CX3CR1-defizienten Tiere sein könnten. Außerdem wurde in dieser Arbeit mit Hilfe von BrdU-Assays demonstriert, dass die zunehmende Anzahl an Leukozyten im linken Ventrikel nach TAC auf Rekrutierungsprozesse zurückzuführen ist und nicht auf lokale Proliferation. Diese Arbeit zeigt die Relevanz der Ly6C^{low} CX3CR1^{high}-Makrophagen in der Entwicklung der linksventrikulären Hypertrophie und einen kardioprotektiven Effekt durch das Fehlen von CX3CR1.

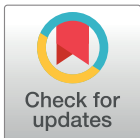
RESEARCH ARTICLE

CX3CR1 is a prerequisite for the development of cardiac hypertrophy and left ventricular dysfunction in mice upon transverse aortic constriction

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Abstract

The CX3CL1/CX3CR1 axis mediates recruitment and extravasation of CX3CR1-expressing subsets of leukocytes and plays a pivotal role in the inflammation-driven pathology of cardiovascular disease. The cardiac immune response differs depending on the underlying causes. This suggests that for the development of successful immunomodulatory therapy in heart failure due to chronic pressure overload induced left ventricular (LV) hypertrophy, the underlying immune patterns must be examined. Here, the authors demonstrate that Fractalkine-receptor CX3CR1 is a prerequisite for the development of cardiac hypertrophy and left ventricular dysfunction in a mouse model of transverse aortic constriction (TAC). The comparison of C57BL/6 mice with CX3CR1 deficient mice displayed reduced LV hypertrophy and preserved cardiac function in response to pressure overload in mice lacking CX3CR1. Moreover, the normal immune response following TAC induced pressure overload which is dominated by Ly6C^{low} macrophages changed to an early pro-inflammatory immune response driven by neutrophils, Ly6C^{high} macrophages and altered cytokine expression pattern in CX3CR1 deficient mice. In this early inflammatory phase of LV hypertrophy Ly6C^{high} monocytes infiltrated the heart in response to a C-C chemokine ligand 2 burst. CX3CR1 expression impacts the immune response in the development of LV hypertrophy and its absence has clear cardioprotective effects. Hence, suppression of CX3CR1 may be an important immunomodulatory therapeutic target to ameliorate pressure-overload induced heart failure.

1 Introduction

Left ventricular (LV) hypertrophy is a main determinant of morbidity and mortality as it occurs in response to various stimuli, including systemic arterial hypertension, aortic stenosis or remodeling of the myocardium after myocardial infarction (MI) [1]. Chronic pressure

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Abbreviations: BP, blood pressure; BrdU, bromodeoxyuridine; BW, body weight; CI, cardiac index; CO, cardiac output; EF, ejection fraction; FACS, fluorescence-activated cell sorting; HR, heart rate; IL, interleukin; LV, left ventricle; MCP1, monocyte chemoattractant protein 1; PV, pressure volume; SV, stroke volume of the left ventricle; TAC, transverse aortic constriction; Wt, wild type mice (C57BL/6).

overload initiates a remodeling process which changes tissue morphology and the function of the heart [2, 3].

Currently, therapeutic approaches to influence cardiac remodeling are still limited and presume an early diagnosis and consequent therapy of the underlying pathomechanisms [4]. Many, initially protective neurohumoral and inflammatory compensatory processes which become harmful in the course of disease culminate in heart failure as the final stage of the disease [5, 6]. In this light, understanding the underlying mechanisms of the cardiac immune response and its association with cardiac tissue remodeling during chronic pressure overload conditions are necessary for the development of new therapeutic strategies.

Cardiac macrophages are known to play a crucial role in cardiac tissue remodeling and healing after injury. They are derived from yolk sac and fetal monocyte progenitors and can be distinguished based on expression of the chemokine receptor, Chemokine Receptor Type 2 (CCR2). Studies have demonstrated that CCR2⁺ cardiac resident macrophages are derived from monocytes while CCR2[−] macrophages originate from the embryonic developmental stage [7]. In steady state, resident cardiac macrophages are replenished by local proliferation. However, following cardiac injury, cardiac macrophages are replaced by blood monocytes [8, 9].

Two major macrophage subsets differentiated in pro-inflammatory Ly6C^{high} (CCR2^{high}, Cx3CR1^{low}) and resident Ly6C^{low} (CCR2^{low}, CX3CR1^{high}) cells contribute to inflammation and tissue repair in the stressed heart. In chronic pressure overload a myeloid cell response is induced which is characterized by constant high numbers of Ly6C^{low} CX3CR1^{high} macrophages [10], in contrast to the biphasic macrophage response following myocardial infarction (MI) [11]. As to this, we were able to show, that Ly6C^{low} CX3CR1^{high} macrophages act as sentinels and display helper-cell functions, allowing neutrophil trans-epithelial migration in a murine urinary tract infection model [12], but little is known about the role of these cells in myocardial pressure overload.

The receptor CX3CR1 and its ligand fractalkine (CX3CL1) play an important role in mediating monocyte recruitment and adhesion. In case of inflammation CX3CR1 is required to mediate Ly6C^{low} monocyte patrolling and crawling and monocyte-adhesion from blood vessels [13]. In response to pressure overload, cardiac Ly6C^{low} macrophages express high surface levels of CX3CR1 and accumulate predominantly in LV tissue next to blood vessels [10]. In kidney ischemia/reperfusion injury CX3CR1 deficiency reduced the entry of monocytes into the kidney and attenuated symptoms and fibrosis [14].

Aim of this study was to examine the influence of CX3CR1 in the orchestration of the cardiac immune response in the development of LV hypertrophy.

2 Materials and methods

2.1 Mice

Female C57BL/6J mice aged 10 to 14 weeks were purchased from Charles River or bred at the central animal facilities of the medical faculty of Bonn (House of Experimental Therapy HET). *Cx3cr1*^{GFP/GFP} mice were originally purchased by Jackson Laboratory (Jackson Laboratory strain description B6.129P2(Cg)-Cx3cr1tm1Litt/J; stock No: 005582) and bred at the central animal facilities of the medical faculty of Bonn (HET). Mice were backcrossed at least 8 times to C57BL/6J background prior to experimental inclusion. All mice were kept under specific pathogen-free conditions in isolated, ventilated cages with free access to water and food. Mice were at rest for 1 wk in the animal facility. All animal experiments were approved by governmental ethics board at the Ministry of Nature, Environment and Consumer Protection of the German state of Northrhine Westphalia (LANUV Recklinghausen permit numbers 84–

02.04.2011.A313, 84–02.04.2016.A374) and were supervised by the central animal facilities of the medical faculty of Bonn (HET, Sigmund-Freud Str. 25 in Bonn, Germany). All surgical interventions were performed under anesthesia and analgesia as described below, and all efforts were made to minimize suffering of the mice.

2.2 Transverse aortic constriction (TAC)

We used a closed-chest model to minimize surgical trauma performing the intervention as minimally invasive as possible [15]. In short, mice were anaesthetized with isoflurane (2 Vol%), intubated with an intubation cannula (OD 1.2 mm). Respiratory rate was set to 150/min and tidal volumes to 8–10 mL/kg body weight using a small animal ventilator (Harvard Apparatus; Holliston, MA). A 27 G spacer was used to standardize the degree of aortic constriction. Sham animals underwent intubation and surgery except that the suture around the aorta was not tight. For postoperative pain management buprenorphine was administered (0.1 µg/g subcutaneously) every 8 h for the next three days after surgery. Mice were sacrificed 3, 6 and 21 days following surgical intervention by atlanto-occipital dislocation. The selection of the times of analysis is based on our preliminary results and mirrors the immune response following TAC composed of early and late inflammatory phase and remodelling phase [16]. We compared TAC vs. sham (control) groups and CX3CR1 deficient mice with C57BL/6J mice.

For additional illustration of the experimental setting and a schematic view of the aortic constriction model see [S1](#) and [S2](#) Figs.

2.3 Cardiac pressure–volume measurements

The cardiac function was measured under anesthesia with a 1.4 French pressure conductance catheter (SPR-839, Millar Instruments, Houston, TX) according to the manufacturer's protocol. The catheter was inserted through the right carotid artery. After measuring the systolic blood pressure (BP), the catheter was placed into the left ventricle. Heart rate (HR), stroke volume (SV), cardiac output (CO) and ejection fraction (EF) were recorded while the amount of anesthesia was reduced to 0.8 Vol% isoflurane. These data were stored and analyzed blinded using PVAN™ software (Millar Instruments). The cardiac index (CI) was calculated by using CO and the body weights. The effectiveness of our TAC intervention was proven by determining the systolic pressure in the carotid artery ([Table 1](#)).

2.4 Determination of heart-weight/body-weight-index

The body weight (BW) of each mouse was measured before sacrificing the animals. The heart weight (HW) of the whole heart (without heart-auricles, pericardium, and blood) was determined after carefully explanting the organ from the murine thorax and rinsing the organ thoroughly with PBS to remove any remaining blood. The index was calculated in mg HW / g BW.

2.5 Flow cytometry

Immune cells were isolated from blood and LV tissue as previously described [9]. Peripheral blood (100 µl) was collected in tubes with ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, MO). Erythrocytes were lysed with RBC lysis buffer (Thermo Fisher Scientific, Waltham, MA). Whole hearts were flushed with PBS, minced into small pieces using a single-edged blade, and digested in gently agitated RPMI medium with 1 mg/ml collagenase-2 (Sigma Aldrich) and 1 mg/ml DNase I (Sigma Aldrich) at 37°C for 60 min.

Unspecific Fc-receptor binding was blocked incubating the cells with antimouse CD16/32 (BD Biosciences, Franklin Lakes, NJ) for 10 min at 4°C. Cells were stained with antimouse

Table 1. CX3CR1 deficiency protects the heart from decompensation following TAC.

	A			B		
	BL6 sham n = 8	BL6 TAC [21d] n = 7		CX3CR1 ^{GFP/GFP} sham n = 6	CX3CR1 ^{GFP/GFP} TAC [21d] n = 7	
sBP carotis, mmHg	102 ± 20	167 ± 30	***	115 ± 24	167 ± 24	*
HR, beats/min	609 ± 25	594 ± 46	ns	603 ± 20	639 ± 16	ns
EF, %	39 ± 6	21 ± 10	**	36 ± 8	48 ± 4	ns; ###
CO, µl/min	13370 ± 1694	6054 ± 2326	****	12724 ± 2983	11348 ± 1694	ns; #
CI, µl/min/BW	548 ± 181	212 ± 87	****	459 ± 110	461 ± 62	ns; #
ESV, µl	35 ± 5	46 ± 11	ns	41 ± 7	21 ± 5	**; ###
EDV, µl	53 ± 5	51 ± 9	ns	57 ± 8	34 ± 6	***; ###
dPdt _{max} , mm Hg/sek	15377 ± 3328	9508 ± 2654	*	11024 ± 4783	12845 ± 2670	ns

Part A indicating C57BL/6 data, Part B CX3CR1^{GFP/GFP} data; values are mean ± SD.

*p < 0.05.

**p < 0.01,

*** P<0.001; transverse aortic constriction (TAC) versus respective sham, ns = not significant versus respective sham.

#p indicates TAC Wt vs. TAC CX3CR1^{GFP/GFP}; sBP carotis = systolic blood pressure in carotid artery; HR = heart rate; EF = ejection fraction; CO = cardiac output; CI = cardiac index; ESV = end-systolic volume; EDV = end-diastolic volume; dPdt_{max} is the maximal rate of rise of left ventricular pressure [21].

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fluorochrome-conjugated antibodies in panel-appropriate combinations in the dark for 20 min at 4°C with following antibodyclones from Thermo Fisher (Waltham, MA), BD Biosciences (Franklin Lakes, NJ) and BioLegend (San Diego, CA): CD45 (AFS98), F4/80 (BM-8), CD115 (c-fms), Ly6C (HK1.4), Ly6G (1A8), CD31 (MEC 13.3); LIVE/DEAD Fixable Dead Cell Stain Kit (Thermo Fisher). BrdU Assay was performed according to the manufacturer's protocol using the BD APC FlowKit (BD Bioscience). BrdU was administered 18 hours before analysis. We determined absolute cell numbers by adding fixed numbers of CaliBRITE APC-beads (6µm size) (BD Biosciences) before measurement as internal reference. Additionally, LV tissue and spleen were weighted before digestion and cell numbers were normalized to 100 mg tissue respectively. Flow cytometry was performed on a BD LSR Fortessa II and data were analyzed with Flow-Jo software (BD Bioscience); (Gating strategy S3 and S4 Figs).

2.6 Histology

Sirius red staining. Hearts were fixated in 4% zinc-paraformaldehyde and embedded in paraffin, and 5 µm sections from the site of the papillary muscle insertion were stained with picrosirius red (SR), as previously described [17]. Cardiomyocyte hypertrophy was assessed using area planimetry of cross-sectioned cardiomyocytes in collagen stained sections by manual cell count of at least 200 cells/slide [3]. Images were taken with a Zeiss Axio Observer and evaluated with Zen-Software.

2.7 Real time PCR

The LV tissue samples were stored at -80°C until homogenization in Trizol according to manufacturer instructions (Thermo Fisher). After reverse transcription of 2000 ng total RNA into cDNA (High capacity cDNA Reverse Transcription Kit; Thermo Fisher) Aldolase C (Mm01298116_g1, Amplicon Length 59), BNP (Mm01255770_g1, Amplicon length 68) and 18S (Mm02601777_g1, Amplicon Length 76) cDNA expressions were quantified by qPCR using TaqMan PCR detection System (Life Technologies, Carlsbad, CA). The 18S ribosomal RNA was amplified as internal control. The amounts of specific cDNA were normalized to 18S using the

Δct method and differences between treatment groups were calculated by using the $\Delta\Delta\text{ct}$ method. Results were depicted as $2^{-\Delta\Delta\text{ct}}$ values with respect to the indicated controls [18, 19].

2.8 Cytokine and chemokine measurement

For cytokine and chemokine analysis LV tissue samples were stored at -80°C until homogenization in RIPA Buffer normalized to 100 μg total Protein utilizing a BCA Protein assay Kit (Thermo Fisher) according to the manufacturer's protocol. Samples were employed according to the manufacturer's protocol. Quantikine ELISA-Kits for the detection of MCP-1, IL-6, IL-1 β and IL-10 (R&D Systems, Wiesbaden, Germany) were used.

2.9 Statistical analysis

Appropriate assumptions of data (e.g. normal distribution or similar variation between experimental groups) were examined before statistical tests were conducted. The number of experiments and the number of mice per group are provided in the figure legends. Student's t-tests were used whenever two groups were compared, and one-way or two-way analyses of variance followed by Tukey's test for multiple comparisons. The analysis was performed with Prism 8 (GraphPad Software, Inc. La Jolla, CA). The results are provided as mean $\pm$ SD unless noted otherwise; $p < 0.05$ was considered statistically significant.

3 Results

3.1 CX3CR1 signaling contributes to loss of cardiac function in pressure overload induced LV hypertrophy

To explore the specific role of CX3CR1^{high} Ly6C^{low} macrophages in the development of LV hypertrophy, *Cx3cr1*^{GFP/GFP} mice were subjected to a minimal-invasive mouse model of TAC [20]. Firstly, we evaluated the impact of CX3CR1 expression for the preservation of myocardial function in our model of LV hypertrophy and assessed functional parameters by pressure-volume (PV) measurements 21 days after TAC. In our recent studies, we found that 21 days after TAC the acute phase of cardiac remodeling is completed and allows to evaluate the effect of chronic pressure overload and LV hypertrophy on myocardial performance. Hence, in the current study we focused on 21 days after TAC. One major criterium for a good quality in TAC surgery and PV catheter performance is a constant systolic blood pressure, measured in the carotid artery in TAC mice. Our data highlight this high quality of TAC-surgery and PV catheter performance, as there is no difference between Wt and *Cx3cr1*^{GFP/GFP} TAC animals observable at day 21 (Table 1).

Our PV measurements revealed that CX3CR1 critically contributes to impaired cardiac function as it normally occurs in response to pressure overload. In Wt mice ejection fraction (EF) and cardiac output (CO) were significantly reduced 21 days after TAC compared to respective sham mice. In contrast, there was no reduction of EF and CO detectable in *Cx3cr1*^{GFP/GFP} mice (Fig 1A and 1B). Additionally, we defined the cardiac index (CO/body-weight) relating heart performance to the size of the corresponding animal, to exclude potential influences of animal weight to the results, and observed significantly better left ventricular function in *Cx3cr1*^{GFP/GFP} mice compared to Wt mice 21 days after TAC (Table 1). Merely, ESV (end-systolic volume) and EDV (end-diastolic volume), parameters related to systolic and diastolic cardiac function, were significantly reduced in response to pressure overload in *Cx3cr1*^{GFP/GFP} mice. These findings indicate that *Cx3cr1*^{GFP/GFP} mice react to TAC induced pressure overload but do not develop severe impairment of LV function and signs of heart failure.

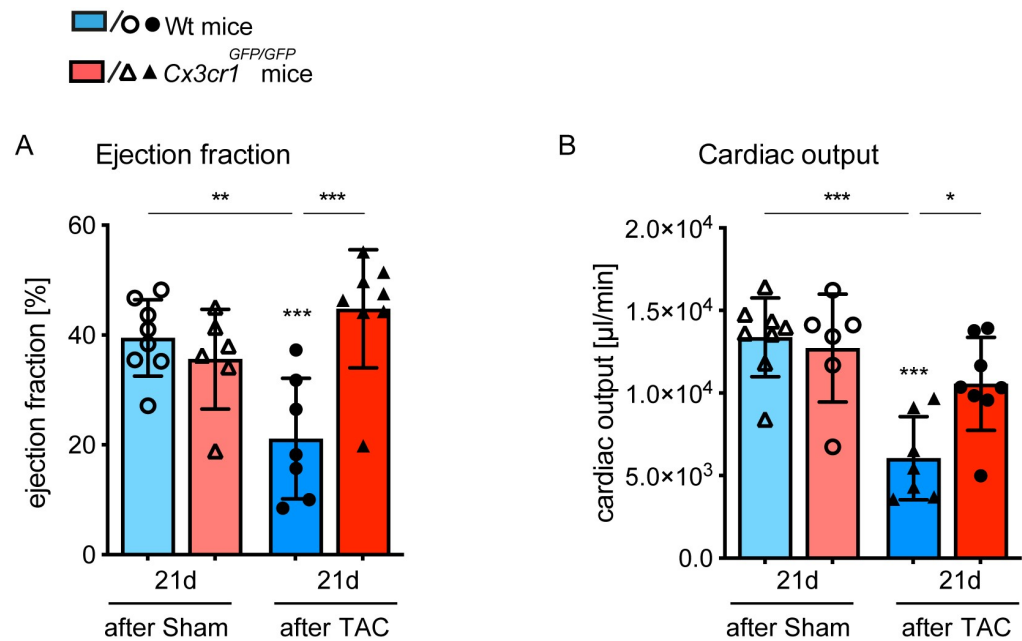


Fig 1. *Cx3cr1^{GFP/GFP}* mice exhibit a cardioprotective phenotype in response to chronic pressure overload. Using pressure volume measurement, the cardiac function of *Cx3cr1^{GFP/GFP}* mice and Wt mice was evaluated 21 days after TAC or sham operation. (A, B) Ejection fraction and cardiac output are depicted as plots, more information is provided in Table 1. N = 6–8 mice/group; * above individual columns indicate significant differences between TAC and respective sham group; *P < 0.05, **P < 0.01, *** P < 0.001.

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3.2 CX3CR1 signaling modulates the development of LV hypertrophy

Heart-Weight/Body-Weight-Index (HW/BW-Index) was calculated to evaluate the development of cardiac hypertrophy. Myocardial hypertrophy represents a cardioprotective mechanism in adaptation to pressure overload, when compared to CX3CR1 competent C57BL/6 mice (Wt), *Cx3cr1^{GFP/GFP}* mice demonstrated with significantly less hypertrophy which did not exceed sham levels at any timepoint (Fig 2A).

These results were confirmed by determination of aldolase expression in LV tissue, whose expression activity is known to correlate with the degree of hypertrophy [22]. While Wt animals showed a significant increase of aldolase expression 3 and 6 days after TAC, the expression in *Cx3cr1^{GFP/GFP}* mice did not change, corroborating the finding that cardiac hypertrophy is attenuated in the absence of CX3CR1 signaling (Fig 2B). Besides, we analyzed the kinetic of brain natriuretic peptide (BNP) which is predominantly produced by cardiac atria and ventricles in response to increased cardiac stretch. Levels of BNP are powerful predictors of ventricular dysfunction and mortality [23]. Pressure overload caused a significant increase in left ventricular BNP expression at day 6 after TAC in Wt animals compared to *Cx3cr1^{GFP/GFP}* mice and controls respectively. *Cx3cr1^{GFP/GFP}* mice also presented with a significant elevation of BNP expression at day 6 after TAC compared to controls (Fig 2C). At day 3 after TAC the elevation of BNP expression did not reach the level of significance.

We shed light on the cellular level and investigated cardiomyocyte size using cross-sectional area planimetry of Picrosirius Red-stained cardiomyocytes (Fig 2D): according to the above-mentioned data, it unraveled significantly enlarged cardiomyocytes in Wt animals 21 days following TAC intervention, whereas in *Cx3cr1^{GFP/GFP}* mice TAC did not cause any noteworthy

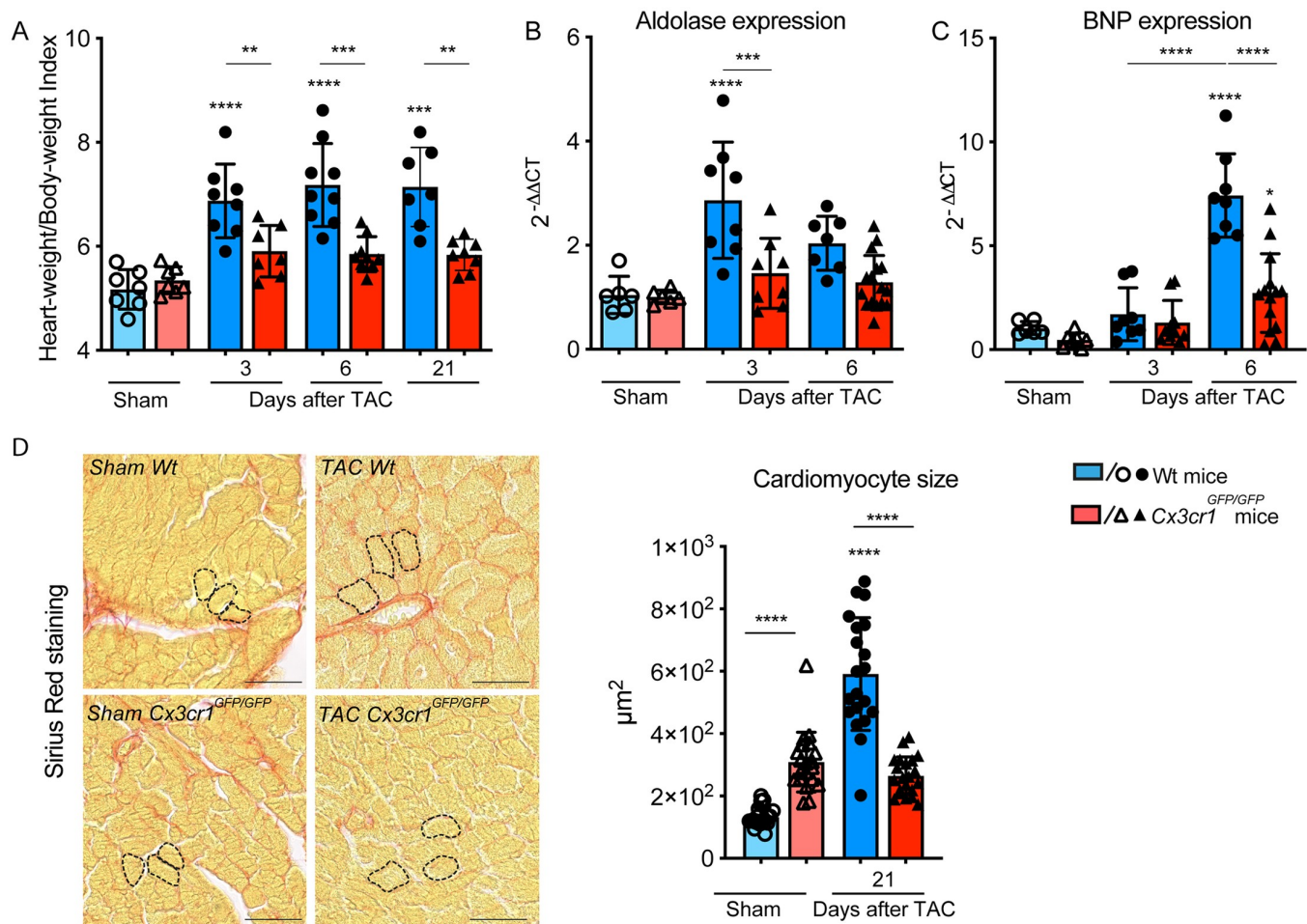


Fig 2. CX3CR1 expression modulates the development of LV hypertrophy in response to pressure overload. (A) Heart-Weight/Body-Weight-Index of Wt and *Cx3cr1*^{GFP/GFP} mice calculated 3, 6 and 21 days after transverse aortic constriction (TAC) and sham operation; n = 7–9 mice/group. (B) Aldolase mRNA expression pattern in LV tissue determined 3 and 6 days after TAC and sham operation in Wt and *Cx3cr1*^{GFP/GFP} mice; n = 6–14 mice/group. (C) BNP mRNA expression pattern in LV tissue determined 3 and 6 days after TAC and sham operation in Wt and *Cx3cr1*^{GFP/GFP} mice; n = 6–14 mice/group. (D) Representative sections of the left ventricle stained with sirious red of Wt and *Cx3cr1*^{GFP/GFP} mice 21 days after TAC or Sham operation to determine cardiomyocyte size. Dashed lines highlight exemplary individual cardiomyocytes; scale bar indicating 50μm. Quantification of cardiomyocyte size by area planimetry; n = 5–6 mice/group, 4 slides per mouse were randomly analyzed by manual cell count of at least 50 cells/slide and mean was used for statistical analysis. * above individual columns indicate significant differences between TAC and respective sham group; **P<0.01, ***P<0.001, ****p < 0.0001.

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increase in cardiomyocyte size (Fig 2D). Taken together, these findings support our hypothesis that development of myocardial hypertrophy is modulated by CX3CR1^{high} Ly6C^{low} macrophages.

3.3 CX3CR1 deficiency drives an early pro-inflammatory phenotype in the cardiac immune response

Time-points for the analysis of the immune response were chosen according to our recent findings highlighting that pressure overload induced a significant accumulation of CX3CR1^{high} Ly6C^{low} macrophages within the first week following TAC intervention [10]. We determined the influence of fractalkine receptor expression in the orchestration of the cardiac

immune response characterizing the reaction of the immune system in response to pressure overload in *Cx3cr1^{GFP/GFP}* mice starting with an overlook for monocyte dynamics in spleen and blood. In response to pressure overload, neither Wt, nor *Cx3cr1^{GFP/GFP}* mice displayed any significant changes in Ly6C^{low} macrophage number in the spleen compared to sham. But, comparing both genotypes, *Cx3cr1^{GFP/GFP}* mice present with significantly reduced Ly6C^{low} macrophage numbers indicating that the splenic reservoir of Ly6C^{low} macrophages is influenced by CX3CR1 signaling [24]. This situation is also reflected in the blood. The number of Ly6C^{low} monocytes is significantly reduced in *Cx3cr1^{GFP/GFP}* mice compared to Wt mice. However, CX3CR1 deficiency caused a significant increase in Ly6C^{high} monocyte numbers in the blood in response to pressure overload 3 days after TAC compared to Wt mice and respective sham (Fig 3B). Wt mice also presented with increased Ly6C^{high} monocyte numbers in response to TAC. But we observed a 3-fold increase of Ly6C^{high} monocytes in CX3CR1 deficient mice whereas in Wt mice the number of these cells increased only 1.7-fold.

In the myocardium, blocking of CX3CR1 expression disrupted the normal Ly6C^{low} macrophage driven immune response resulting in significantly increased Ly6C^{low} macrophage numbers only 6 days after TAC compared to the sham group and with considerably lower cell numbers compared to Wt (Fig 3C). Opposing to these findings, we observed increased numbers of Ly6C^{high} macrophages and neutrophils in the LV myocardium of *Cx3cr1^{GFP/GFP}* mice at day 3 and 6 after TAC compared to the respective Wt groups and sham control. The number of Ly6C^{high} macrophages in Wt mice was significantly elevated only at day 3 after TAC compared to sham controls and reached baseline level on day 6 whereas neutrophils did not increase significantly at any time-point (Fig 3D and 3E).

The immune reaction in the hypertrophic heart was further portrayed investigating the protein expression of cytokines and chemokines, known to mediate inflammation and leukocyte recruitment. Here, impaired CX3CR1 signaling caused an increase in pro-inflammatory cytokine levels in LV tissue. The protein concentration of IL-6 and IL1- β increased significantly in *Cx3cr1^{GFP/GFP}* mice compared to Wt and respective sham 3 and 6 days after TAC. In Wt mice the kinetics of IL-6 and IL1- β was less impressive, reaching the level of significance only at day 3 (IL-6) but on day 3 and 6 (IL1- β) after TAC in Wt mice compared to sham (Fig 3F and 3G).

Various molecules are involved in monocyte chemotaxis and attraction. Among these, CCL2 is modulating the recruitment of Ly6C^{high} CCR2⁺ monocytes, which is of interested with regard to Ly6C^{high} monocyte kinetics in *Cx3cr1^{GFP/GFP}* mice during pressure overload. Blocking of CX3CR1 signaling was accompanied by a significant increase in CCL2 levels 3 and 6 days after TAC compared to sham controls. Noteworthy, CCL2 concentration increased 4-fold on day 3 in *Cx3cr1^{GFP/GFP}* mice while Wt mice exhibited a weaker, but significant increase of CCL2 at the same time. While CCL2 concentration peaked on day 3 in *Cx3cr1^{GFP/GFP}* mice and decreased thereafter, it remained elevated in Wt mice after 6 days compared to respective sham (Fig 3H). Furthermore, the concentration of IL-10 protein in LV tissue was determined, as it is known to improve post MI repair and macrophage polarization [25]. A deficiency in CX3CR1 signaling influenced IL-10 concentration causing significantly lower IL-10 levels in the LV tissue of *Cx3cr1^{GFP/GFP}* mice compared to Wt mice 3 days after TAC. Though, IL-10 levels significantly increased in *Cx3cr1^{GFP/GFP}* mice and Wt animals at both time-points after TAC in comparison with the respective sham controls (Fig 3I).

In the next step, we investigated the origin of the increased Ly6C^{high} macrophage number in the heart tissue of *Cx3cr1^{GFP/GFP}* mice, which could be either driven by CCL2-CCR2 mediated recruitment from the circulation, or by local proliferation within the myocardium. Therefore, we examined local immune cell proliferation in response to pressure overload performing BrdU assays. However, we could not detect TAC induced differences in proliferation

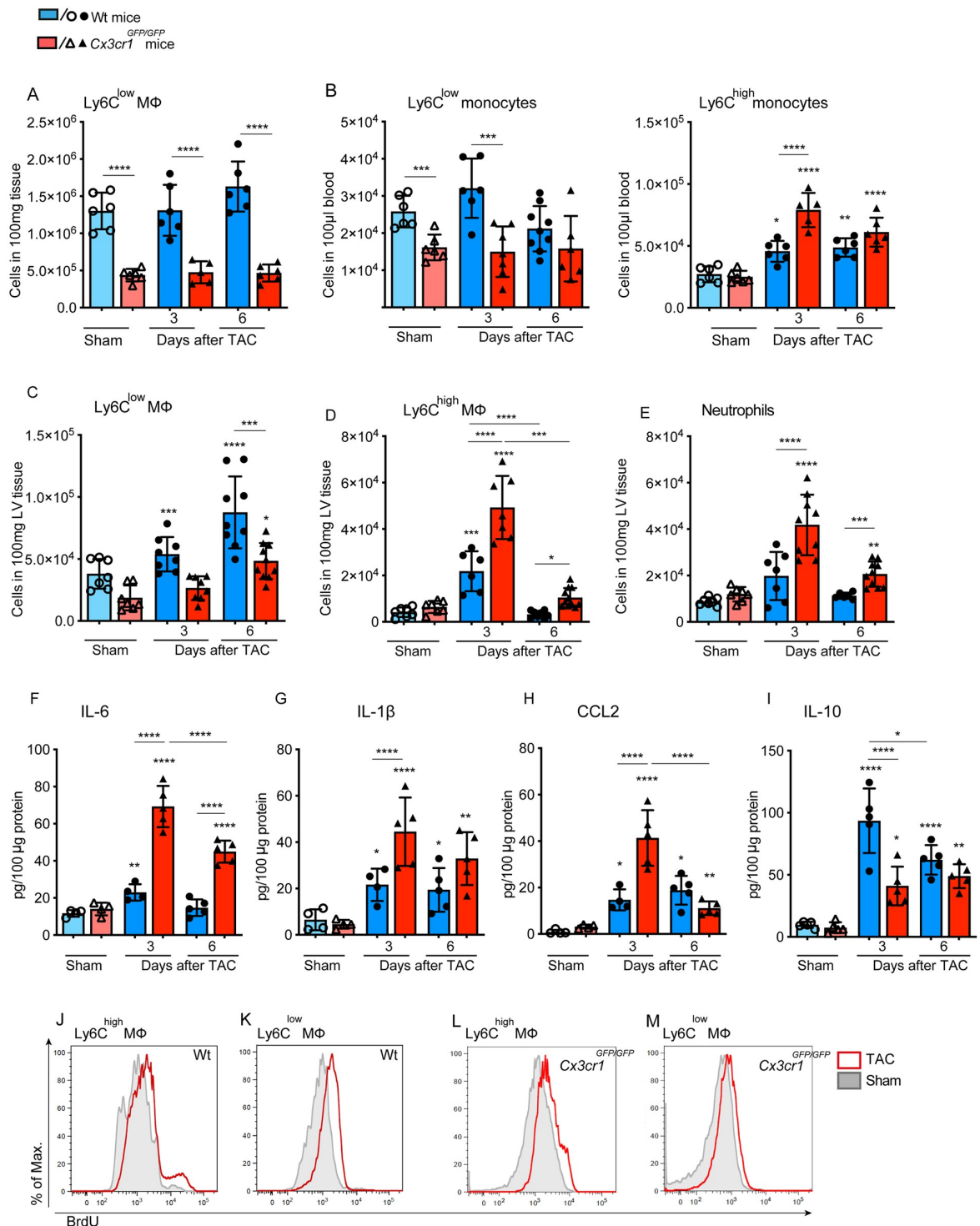


Fig 3. CX3CR1 deficiency drives the immune response to a pro-inflammatory phenotype during chronic pressure overload. Quantification of Ly6C^{low} F4/80⁺ macrophages in the spleen and of (B) Ly6C^{low} and Ly6C^{high} CD115⁺ monocytes in the blood of Wt and *Cx3cr1*^{GFP/GFP} mice determined by flow cytometry 3 and 6 days after TAC and sham operation. (C-E) Number of neutrophils, Ly6C^{low} and Ly6C^{high} macrophages in the LV tissue was determined by flow cytometry analysis in *Cx3cr1*^{GFP/GFP} and Wt mice 3 and 6 days after TAC and sham operation. The particular cell subsets were defined as follows: neutrophils as CD45⁺ F4/80⁻ Ly6G⁺; macrophages as CD45⁺ F4/80⁺ Ly6G⁻; Ly6C^{high} and Ly6C^{low}

macrophages were further discriminated according to the respective Ly6C surface expression; (F-I) Concentrations of the pro-inflammatory cytokines IL6 and IL-1 β , the chemokine MCP1 and IL-10 were determined in LV tissue homogenates at day 3 and 6 after surgical intervention in *Cx3cr1^{GFP/GFP}* and Wt mice using ELISA assays. (J-M) BrdU content of cardiac Ly6C^{high} and Ly6C^{low} macrophages was determined by flow cytometry analysis at day 3 after TAC. BrdU was administered 18h before analysis. Histograms are based on concatenated plots of 4 mice. N = 6–8 mice/group; * above individual columns indicate significant differences between TAC and respective sham group; *P < 0.05, **P < 0.01, *** P < 0.001, ****p < 0.0001.

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of neither Ly6C^{low}, nor Ly6C^{high} macrophages in the LV tissue of *Cx3cr1^{GFP/GFP}* mice and Wt mice (Fig 3J–3M).

Taken together, these data highlight that CX3CR1 expression balances the immune response in the heart. A lack of CX3CR1 dependent recruited cells drives the immune response towards an early pro-inflammatory phenotype.

4 Discussion

Chronic pressure overload caused by systemic arterial hypertension or aortic valve stenosis initiates processes which can adversely support tissue remodeling and cardiac function. Normally, myocardial hypertrophy represents a cardioprotective mechanism in adaptation to pressure overload. However, many inflammatory mechanisms become harmful in the course of this disease potentially culminating in terminal heart failure [5, 6]. We are aware that the cardiac immune response differs depending on the underlying causes. This suggests that development of new therapeutic strategies demands a thorough understanding of the underlying mechanisms of the cardiac immune response and its correlation to cardiac tissue remodeling.

In this study, we were able to demonstrate that fractalkine receptor CX3CR1 expression is essential in the development of LV hypertrophy and heart failure in response to pressure overload.

The CX3CL1/CX3CR1 axis mediates recruitment and extravasation of CX3CR1-expressing subsets of leukocytes and plays a pivotal role in the inflammation-driven pathology of cardiovascular disease. We investigated the role of CX3CR1 in the development of pressure overload induced LV hypertrophy examining *Cx3cr1^{GFP/GFP}* mice, which are deficient for CX3CR1 [26]. CX3CR1 is ubiquitously expressed on most tissue macrophages and DCs but does not play a major role for their ontogeny, homeostatic migration, or colonization of tissues with resident phagocytes [27]. Our latest results showed that a pressure overload induced immune response is characterized by the accumulation of Ly6C^{low} CX3CR1^{high} macrophages labeling the immune reaction in chronic cardiac impairment [10]. The expression of CX3CR1 on other, than immune cells in the heart was excluded by flow cytometry analysis of CD45^{negative} cells (S2 Fig). We can confirm that CX3CR1 deficient mice present significantly reduced numbers of Ly6C^{low} macrophages in the spleen and Ly6C^{low} monocytes in the blood compared with Wt animals [26]. In the heart of *Cx3cr1^{GFP/GFP}* mice, a low number of Ly6C^{low} macrophages were accompanied by increased numbers of pro-inflammatory neutrophils and Ly6C^{high} macrophages in response to pressure overload. We suppose that the accumulation of Ly6C^{high} macrophages in the heart is due to enhanced CCL2 driven recruitment of Ly6C^{high} CCR2⁺ monocytes from the circulation in the absence of CX3CR1. This presumption is corroborated by the measurement of increased CCL2 concentrations in the homogenates of LV tissue from CX3CR1 deficient mice. A similar phenotype was observed in mice deficient for Nr4a1, an obligate transcription factor for Ly6C^{low} monocytes, in a model of myocardial infarction. Absolute numbers of Ly6C^{high} and Ly6C^{low} macrophages were increased in the myocardium on day 7, and global macrophage transcription was skewed towards a pro-inflammatory phenotype [28]. We assume that the elevated number of Ly6C^{low} macrophages

in LV tissue at day 6 after TAC in CX3CR1 deficient mice points to a local conversion of recruited CCR2⁺ Ly6C^{high} monocytes to Ly6C^{low} macrophages. Further differentiation of monocyte derived macrophages in the heart can lead to a reduction in Ly6C expression and consequently to an increase in Ly6C^{low} macrophages in the tissue [28]. Our advanced flow cytometry approaches revealed that the source of Ly6C^{high} and Ly6C^{low} macrophages in the first week after TAC is not local proliferation.

The pro-inflammatory phenotype we observed in the early phase of hypertrophy development characterized by increasing pro-inflammatory immune cell numbers was supported by increased concentrations of the pro-inflammatory cytokines IL-6 and IL-1 β and a reduced expression of pro-remodeling/anti-inflammatory cytokine IL-10 in myocardial tissue. The reduced levels of IL10 in the heart of CX3CR1 deficient mice might be due to the low number of Ly6C^{low} macrophages we observed. Ly6C^{low} macrophages or M2 macrophages are known to release IL-10 in response to cardiac injury to suppress inflammation and activate profibrotic processes [25]. Our data highlights that the normal immune reaction in the development of LV hypertrophy is disrupted in the absence of CX3CR1 skewing the immune response toward pro-inflammation. The evaluation of inflammation as beneficial or harmful has to be correlated to the course of disease (chronic vs. acute), the respective organ and of course the point of time the results are generated. In this project we had a kinetic which allowed us to observe the acute response, the start of remodelling and a later remodelling time point. In the context of brain ischemia, CX3CR1 deficiency exhibited beneficial effects due to the limited recruitment of Ly6C^{low} iNOS⁺ monocytes [29]. In a mouse model of myocardial infarction, the treatment with anti-Fralkine-antibody turned out to be cardioprotective, reducing adverse tissue remodeling and macrophage migration [30]. More research is needed to clarify the cardioprotective effect of inflammation in the development of LV hypertrophy.

Our investigations focusing on the morphology of the heart in CX3CR1^{GFP/GFP} mice elucidated that the cardiomyocytes presented with abrogated hypertrophy following TAC, which was reflected by constant HW/BW-Index, reduced levels of Aldolase and BNP and unchanged left ventricular function. These findings emphasize the hypothesis that the absence of CX3CR1 supports better adaptation of the myocardium to stress.

Taken together, our data suggests that CX3CR1 deficiency decreases susceptibility of the myocardium to hypertrophic damage, therefore preserving myocardial function; the underlying mechanisms seem to involve increased and timely inflammatory response.

CX3CR1 expression impacts the immune response in the development of LV hypertrophy and its absence has clear cardioprotective effects. Hence, suppression of CX3CR1 may be an important immunomodulatory therapeutic target to ameliorate pressure-overload induced heart failure.

Supporting information

S1 Fig. Time scale of the experimental setting. Animals were analyzed 3, 6 and 21 days following surgical intervention. PV catheter measurement was performed at day 21. (DOCX)

S2 Fig. Schematic view of transverse aortic constriction. The lumen of the aortic arch is restricted with the help of a standardized spacer and fixed with a suture. (DOCX)

S3 Fig. Exemplary gating strategy of LV tissue. Gating of cells of interest was performed according to beads granularity and size (6 μ m) (A) excluding trash, myocytes and myocyte fragments. Firstly, single cells were gated excluding doublets (B). Next, living cells were

isolated (C). Living CD45+ immune cells (D) were differentiated according to Ly6G and F4/80 surface expression (E). Ly6G+ F4/80- neutrophils and F4/80+ macrophages were defined. Concatenated plots of 3 individual samples were used.
(DOCX)

S4 Fig. Exemplary gating to define Ly6C^{high} CX3CR1⁻ and Ly6C^{low} CX3CR1^{high} macrophages in the LV tissue. Concatenated plots of 3 individual samples.
(DOCX)

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3.4 Charakterisierung der transversen aortalen Konstriktion in Mäusen basierend auf der spezifischen Rekrutierung von Leukozyten in das hypertrophierte Myokard und die Aorta ascendens

In der vorliegenden Studie wurde die zeitliche Dynamik der angeborenen Immunantwort als Reaktion auf eine TAC in verschiedenen Geweben von C57BL/6- (Wt-) Mäusen untersucht, um die Rekrutierungsprozesse in das geschädigte Myokard besser verstehen zu können. In vorangegangenen Arbeiten konnte gezeigt werden, dass insbesondere die Akkumulation von Ly6C^{low} $\text{CX3CR1}^{\text{high}}$ -Makrophagen die Pathologie der linksventrikulären Hypertrophie beeinflusst. Inwiefern eine TAC eine charakteristische, von Leukozyten dominierte Immunantwort im Myokard, der *Aorta ascendens* und *Aorta descendens*, sowie Milz, Blut und drainierenden Lymphknoten provoziert, war ebenfalls Gegenstand dieser Arbeit. Die Quantifizierung der Immunzellen im Hochdruck- und Niederdruckbereich der Aorta 3, 6 und 21 Tage nach operativer Intervention zeigte einen direkten Einfluss auf die Leukozytenpopulationen im aufsteigenden Teil der Aorta, während in der Aorta descendens keine signifikanten Unterschiede in den einzelnen Leukozytenpopulationen zu beobachten waren (s. Abb. 2a-l aus Kleiner et al. 2021, S. 5). Die Zellzusammensetzung im Hochdruckteil der Aorta wies in der Dynamik der Immunantwort deutliche Parallelen zu den immunologischen Reaktionen im Myokard des linken Ventrikels auf. Insbesondere der Anteil der Neutrophilen und $\text{Ly6C}^{\text{high}}$ -Makrophagen erhöhte sich signifikant zu allen drei Zeitpunkten nach TAC im Vergleich zu den Kontrollen (s. Abb. 2b, c f und g aus Kleiner et al. 2021, S. 5). Weiterhin wurde durch die Analyse von Milzgewebe gezeigt, dass es nach einer Zunahme an Tag 3 nach TAC an Tag 6 zu einem Abfall der Makrophagen in der Milz kommt, der von einem Anstieg der Monozyten im Blut begleitet wird. Die frühe Akkumulation von Makrophagen an Tag 3 nach TAC in der Milz spiegelt die systemische Entzündung wider und der Abfall der Makrophagen im Vergleich zu Tag 3 nach TAC könnte für die Freisetzung der myeloiden Zellen aus der Milz in die Zirkulation sprechen. Die Analyse der DC-Dynamik im Myokard, Aortengewebe, Blut, Lymphknoten und Milz als Reaktion auf die TAC gab einen ersten Einblick in die Rolle von DCs in der Modulation von sterilen Entzündungen. Während in der *Aorta ascendens* der Anteil der DCs bereits an Tag 3 nach TAC ein Maximum erreicht, war dies im Myokard erst an Tag 6 der Fall. Außerdem präsentierten die DCs, die aus der

Aorta ascendens isoliert wurden, basierend auf der Untersuchung der CD86 MFI, ein deutlich höheres Aktivierungsniveau im Vergleich zu den DCs im Myokard. Dieser Befund verweist darauf, dass es durch die Entzündungsreaktion im Hochdruckteil der Aorta zu einer stärkeren DC Antwort nach TAC als im Myokard kam.

Research Article

Characterization of Transverse Aortic Constriction in Mice Based on the Specific Recruitment of Leukocytes to the Hypertrophic Myocardium and the Aorta Ascendens

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Transverse aortic constriction (TAC) is a model that mimics pressure overload-induced left ventricular (LV) hypertrophy in mice. Alterations in immune cell functionality can promote cardiac and vascular remodeling. In the present study, we characterized the time course in innate immune cell dynamics in response to TAC in the different tissues of mice. It was determined whether TAC induces a characteristic leukocyte-driven immune response in the myocardium, aorta ascendens and descendens, spleen, blood, and draining lymph nodes supported by cytokine-driven chemotaxis in mice at 3, 6, and 21 days following surgery. We used complex flow cytometry staining combinations to characterize the various innate immune cell subsets and a multiplex array to determine cytokine concentrations in the serum. The results of the current study indicated that leukocytes accumulate in the myocardium and aorta ascendens in response to TAC. The leukocyte dynamics in the myocardium were dominated by the Ly6C^{low} macrophages with an early accumulation, whereas the response in the aorta ascendens was characterized by a long-lasting proinflammatory phenotype driven by Ly6C^{high} macrophages, neutrophils, and activated DCs. In contrast to the high-pressure environment of the aorta ascendens, the tissue of the aorta descendens did not react to TAC with any leukocyte increase. The levels of proinflammatory cytokines in the blood were elevated in response to TAC, indicating a systemic reaction. Moreover, our findings strongly suggest that cardiac macrophages could origin from splenic pools and reach the site of the inflammation via the blood. Based on the current findings, it can be concluded that the high-pressure conditions in the aorta ascendens cause a characteristic immune response, dominated by the accumulation of leukocytes and the activation of DCs that varies in comparison to the immune cell dynamics in the myocardium and the aorta descendens.

1. Introduction

Chronic pressure overload induces left ventricular (LV) hypertrophy and consequently results in congestive heart failure. This process is driven by the immune response and is known to influence the outcome and quality of life. In sterile inflammations, the innate immune system is activated by danger-associated molecular patterns (DAMPs) that are released by injured or necrotic cells and damaged extracellular matrix. DAMPs can activate the response of leukocytes

via toll-like receptor signaling [1]. Our recent studies indicated that LV hypertrophy induced by pressure overload provokes an immune response which is dominated by anti-inflammatory macrophages [2]. These cardiac Ly6C^{low} CX3CR1⁺ macrophages contribute to myocardial hypertrophy and impaired heart function following pressure overload in a mouse model of transverse aortic constriction (TAC) [3].

As previously shown, our TAC model causes LV hypertrophy and heart failure in mice [3]. Moreover, we assume

that the narrowing of the aortic lumen provokes a high-pressure (in front of the constriction) and a low-pressure part (behind the constriction) of the aorta that are characterized by a specific leukocyte response. The TAC model can be used to analyze the high-pressure and low-pressure parts of the aortic tissue in the same animal. The aim of the present is to further investigate the local and systemic inflammatory response induced by TAC. Besides, the present study investigates not only the immune response in the myocardium but also the ongoing inflammatory processes in the aorta, spleen, lymph nodes, and blood.

The most important cells of the innate immune system involved in cardiac remodeling processes are monocytes/macrophages, neutrophils, and dendritic cells (DCs) [4]. Murine monocytes and macrophages can be classified by either the Ly6C^{low} or Ly6C^{high} surface expression in the patrolling monocytes or anti-inflammatory macrophages (Ly6C^{low} in mice and CD14^{low}CD16⁺ in humans), which survey the vascular lumen, clear cellular debris, and contribute to remodeling processes, as well as in the inflammatory monocytes and macrophages (Ly6C^{high} in mice and CD14^{high}CD16⁺ in humans), which secrete a variety of pro-inflammatory cytokines and maintain inflammation [5, 6]. Our previous work had shown that these two distinct macrophage subsets cross-talked and orchestrated the immune defense against bacterial urinary tract infection, by regulating neutrophil migration into the infected uroepithelium [7]. In the context of cardiovascular diseases, we elucidated the influence of pattern recognition receptors expressed by cardiac macrophages and circulating leukocytes to cardiac remodeling following myocardial infarction and pressure overload induced LV hypertrophy, using our TAC model [2, 3, 8, 9].

We recently published that cardiac macrophages do not replenish by local proliferation in response to pressure overload-induced LV hypertrophy but appear to be recruited via the chemotaxis of CCL2 to the site of the myocardial inflammation [3]. In this context, the source of the recruited monocytes has not been further investigated.

Under steady-state conditions, the resident cardiac DCs represent approximately 1% of the total cardiac leukocytes and can be divided into CD103⁺ and CD11b⁺ cells in a model of viral myocarditis [10]. In the context of MI, the DCs infiltrated the infarcted heart, internalized locally released cardiomyocyte-derived antigens, and migrated into the mediastinal lymph nodes, where they presented antigen-derived peptides and stimulated T cells [11].

In the present study, we focused on the dynamics of leukocyte accumulation in the myocardium as well as in the ascending and descending parts of the aorta, spleen, lymph nodes, and blood to characterize the potential remote immunologic effects of TAC-induced cardiac hypertrophy.

2. Materials and Methods

2.1. Mice. Female C57BL/6J mice aged 10–14 weeks were purchased from Charles River (Wilmington, MA, USA). The mice were kept under specific pathogen-free conditions in isolated, ventilated cages with free access to water and

food. All animal experiments were approved by the governmental ethics board at the Ministry of Nature, Environment and Consumer Protection of the German state of North Rhine Westphalia (LANUV Recklinghausen permit numbers 84-02.04.2011.A313, 84-02.04.2016.A374) and were supervised by the central animal facilities of the medical faculty of Bonn (HET, Venusberg-Campus 1, Bonn, Germany). All surgical interventions were performed under anesthesia and analgesia as described below, and all efforts were made to minimize the suffering of the mice.

2.2. Transverse Aortic Constriction (TAC). TAC was performed as published previously [2]. In short, mice were anesthetized with isoflurane (2 vol%) and intubated with an intubation cannula (OD 1.2 mm). The respiratory rate was set to 150/min and the tidal volumes to 8–10 ml/kg body weight using a small animal ventilator (Harvard Apparatus; Holliston, MA, USA). A 27 G spacer was used to standardize the degree of aortic constriction. The sham control animals underwent intubation and surgery except that the suture around the aorta was not tight. For postoperative pain management, buprenorphine was administered (0.1 µg/g subcutaneously) every 8 h for the next 3 days after surgery. The mice were sacrificed 3, 6, and 21 days following surgical intervention.

2.3. Flow Cytometry. The immune cells were isolated from the blood, spleen, lymph node, aorta, and LV tissue. Peripheral blood (100 µl) was collected in tubes with ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, MO, USA). The spleen tissue and lymph nodes were mechanically homogenized and filtered via 80 µm meshes. The spleen samples and blood were treated with RBC lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) to eliminate erythrocytes. Whole hearts were flushed with PBS, and the heart tissue and parts of the aorta were minced into small pieces using a single-edged blade and digested in gently agitated RPMI medium with 1 mg/ml collagenase 2 (Sigma Aldrich) and 1 mg/ml DNase I (Sigma Aldrich) at 37°C for 60 min.

Unspecific Fc-receptor binding was blocked incubating the cells with anti-mouse CD16/32 (BD Biosciences, Franklin Lakes, NJ, USA) for 10 min at 4°C. The cells were stained with anti-mouse fluorochrome-conjugated antibodies in panel-appropriate combinations in the dark for 20 min at 4°C with following antibody clones from Thermo Fisher (Waltham, MA, USA), (i.e., CD45 (30-F11), CD11b (ITGAM), CD115 (c-fms), F4/80 (BM-8), Ly6C (HK1.4), Ly6G (1A8)) and BioLegend (San Diego, CA, USA) (i.e., CD103 (2E7), CD11c (Cr4)). We performed live/dead staining using Hoechst 33258 in a dilution of 1 µl/100 ml (Thermo Fisher). Flow cytometry was performed on a BD Canto and a BD LSR Fortessa, and the data were analyzed using the Flow-Jo software (BD Bioscience).

2.4. Cytokine Measurement. The cytokine concentrations in the serum of the mice were measured using a custom-made FlowCytomix Multiple Analyte Detection System (Thermo Fisher, formerly eBioscience). This bead-based

immunoassay for the simultaneous detection of the cytokines in one sample was performed according to the manufacturer's instructions. As suggested by the manufacturer, 25 μ l of mouse serum was used. The data were evaluated using the FlowCytomix Pro Software.

2.5. Statistical Analysis. Appropriate assumptions of the data (e.g., normal distribution or similar variation between experimental groups) were examined before the statistical tests were conducted. Student's *t*-tests were performed whenever two groups were compared, whereas one-way or two-way analyses of variance followed by Tukey's test were performed for multiple comparisons. The analysis was performed using Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). The results were provided as mean $\pm$ standard deviation (SD) unless noted otherwise; a *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. The Effect of TAC on the Immune Responses in the Myocardium and the High-Pressure and Low-Pressure Parts of the Aorta. Our recent studies highlighted that LV hypertrophy caused by pressure overload induces a complex immune response in the myocardium [2]. Moreover, we focused on the immune response in the LV tissue and did not investigate the systemic or remote immune response. In the present study, we examined the migration pattern of immune cells to the ascending and descending parts of the aorta, draining lymph nodes, blood, spleen, and LV tissue in response to TAC, which has not been determined systematically in previous studies.

In this project, we performed TAC or sham surgery and analyzed the immune response in the course of the disease at 3, 6, and 21 days after intervention (Figure 1(a)). Our model of TAC induced LV hypertrophy was evaluated by calculating the heart-weight/body-weight-index of the mice. Hypertrophy of the hearts becomes significant at days 6 and 21 after TAC compared to the controls (Figure 1(b)). In addition to the myocardium, we investigated the immune cell dynamics in the ascending and descending parts of the aorta, draining lymph nodes, spleen, and blood, which allowed us to describe the migration and recruitment processes provoked by TAC (Figure 1(c)). The immune cell subsets and activation markers were determined via flow cytometry. Our gating strategy was based on the guidelines for the use of flow cytometry in immunology [12] and was optimized and refined with the help of our flow cytometry core facility (Figure 1(d) and Supplemental Figure 1).

The characterization of the immune cell composition in the myocardium indicated a significant increase in the proportion of immune cells at all three time points (Figure 2(a)). The percentage of neutrophils and Ly6C^{low} macrophages was significantly elevated at days 3 and 6 after TAC compared with the controls (Figures 2(b) and 2(d)). The Ly6C^{high} macrophages significantly accumulated only at day 3 after TAC compared to controls (Figure 2(c)). Next, it was determined whether and how our model of TAC-induced LV hypertrophy affected the high-pressure and

low-pressure parts of the aorta to determine specific immunological signatures.

Flow cytometry analyses performed at days 3, 6, and 21 after TAC or sham surgery revealed that TAC surgery directly influenced the immune reaction in the ascending aortic tissue compared with that in the descending part of the aorta. The immune response in the ascending part of the aorta was characterized by a significant increase in CD45⁺ immune cells at days 3, 6, and 21 after TAC compared to controls. The increase in CD45⁺ immune cells was significantly higher at day 6 compared to day 3 indicating that the inflammation increases in the first 6 days (Figure 2(e)). Especially neutrophils and Ly6C^{high} macrophages invaded the aortic tissue in the high-pressure environment, reaching the level of significance at all three time points in comparison with the sham animals (Figures 2(f) and 2(g)). The Ly6C^{low} macrophages significantly increased in response to TAC at days 3 and 6 compared with controls decreasing to sham level at day 21 (Figure 2(h)). In the LV tissue, the neutrophils and Ly6C^{low} macrophages dominated the immune response with significantly elevated cell proportions at days 3 and 6 after TAC compared with the control groups, a situation which was mirrored in the ascending part of the aorta. The Ly6C^{high} macrophage levels were significantly increased at day 3 only in the myocardium, whereas the reaction in the aorta ascendens lasted for 21 days (Figures 2(c) and 2(g)). In the descending part of the aorta, a significant increase in immune cells was observed only at day 6 after TAC compared with day 3 which we could not directly break down to any of the analyzed innate immune cell subsets (Figure 2(i)). The proportion of the different leukocyte subsets did not vary in response to TAC (Figures 2(j)–2(l)). The descending part of the aorta did not reflect the immune response of the myocardium but reacted to the surgical intervention and maybe changes in the blood flow f.e. turbulences that may result in an increase in immune cells in general.

3.2. TAC Provokes an Immune Response in Spleen and Blood. Certainly, monocytes and macrophages play important roles in the orchestration and maintenance of the cardiac inflammation in the development of pressure overload-induced LV hypertrophy. Our recent findings demonstrated that CCL2-driven chemotaxis plays an important role in the recruitment of cardiac macrophages to the site of cardiac injury [3]. The remote activation of the splenic immune response provoked by TAC has not been systematically studied yet. In post-MI inflammation and remodeling, it is already known that cardiac macrophages originate mostly from splenic pools and are transported via the blood to the site of the inflammation [13]. However, the immune response in MI and LV hypertrophy with a biphasic response after MI differs from the chronic response with dominating Ly6C^{low} macrophages in the myocardium after TAC [2]. Considering this, we determined whether the immune cell composition of the spleen and blood is affected from TAC, and whether the splenic myeloid cell pool might serve as a source to supply the cardiac immune response in our TAC model.

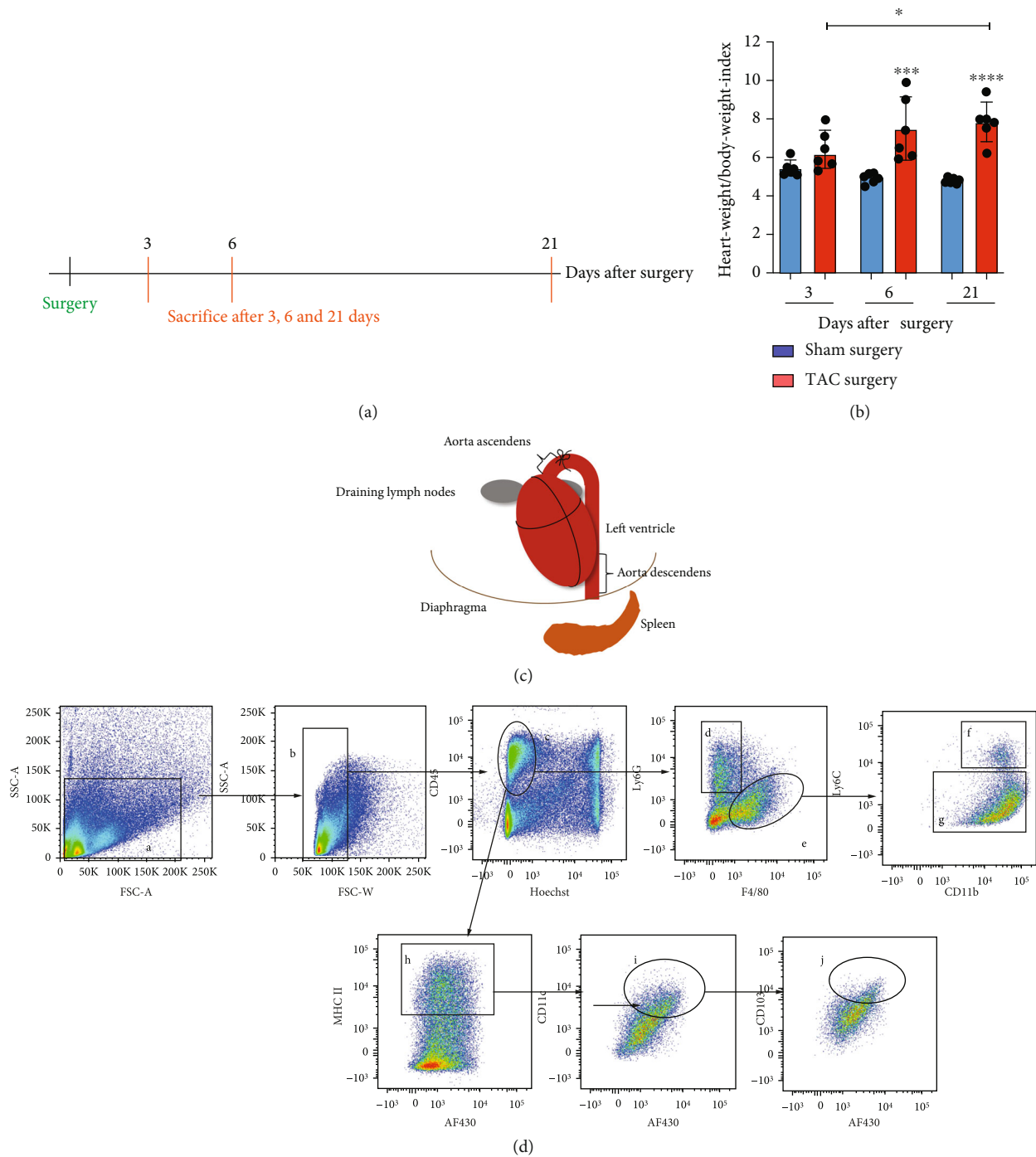


FIGURE 1: Methodological procedure and definition of the immune cell subsets. (a) We sacrificed the mice 3, 6, and 21 days after TAC or sham surgery. (b) Heart-weight/body-weight-index in mg/g was calculated 3, 6, and 21 days after TAC or sham surgery. (c) In addition to the LV tissue, we analyzed the tissue of the aorta ascendens, aorta descendens, spleen, blood, and heart draining lymph nodes. (d) The gating strategy for flow cytometry analyses was performed as illustrated for the tissue of the aorta ascendens. All cell-like events (a) were isolated by using the forward scatter area versus sideward scatter area. Doublets were excluded using forward scatter area versus sideward scatter width (b). We defined the living immune cells (c), using CD45 staining versus Hoechst staining. The CD45⁺ immune cells were further classified by staining for the surface markers Ly6G and F4/80 to determine neutrophils (d) and macrophages (e). Next, we checked for the Ly6C surface expression of the macrophage population as a function of CD11b to differentiate between the Ly6C^{high} (f) and the Ly6C^{low} macrophages (g). We analyzed the DC compartment based on the population of the CD45⁺ cells (c) and stained for MHC-II to identify MHC-II⁺ immune cells (h). We used CD11c as a function of the autofluorescence channel AF430 for the definition of classical dendritic cells (i). The cDCs were further discriminated according to the CD103 surface expression (j).

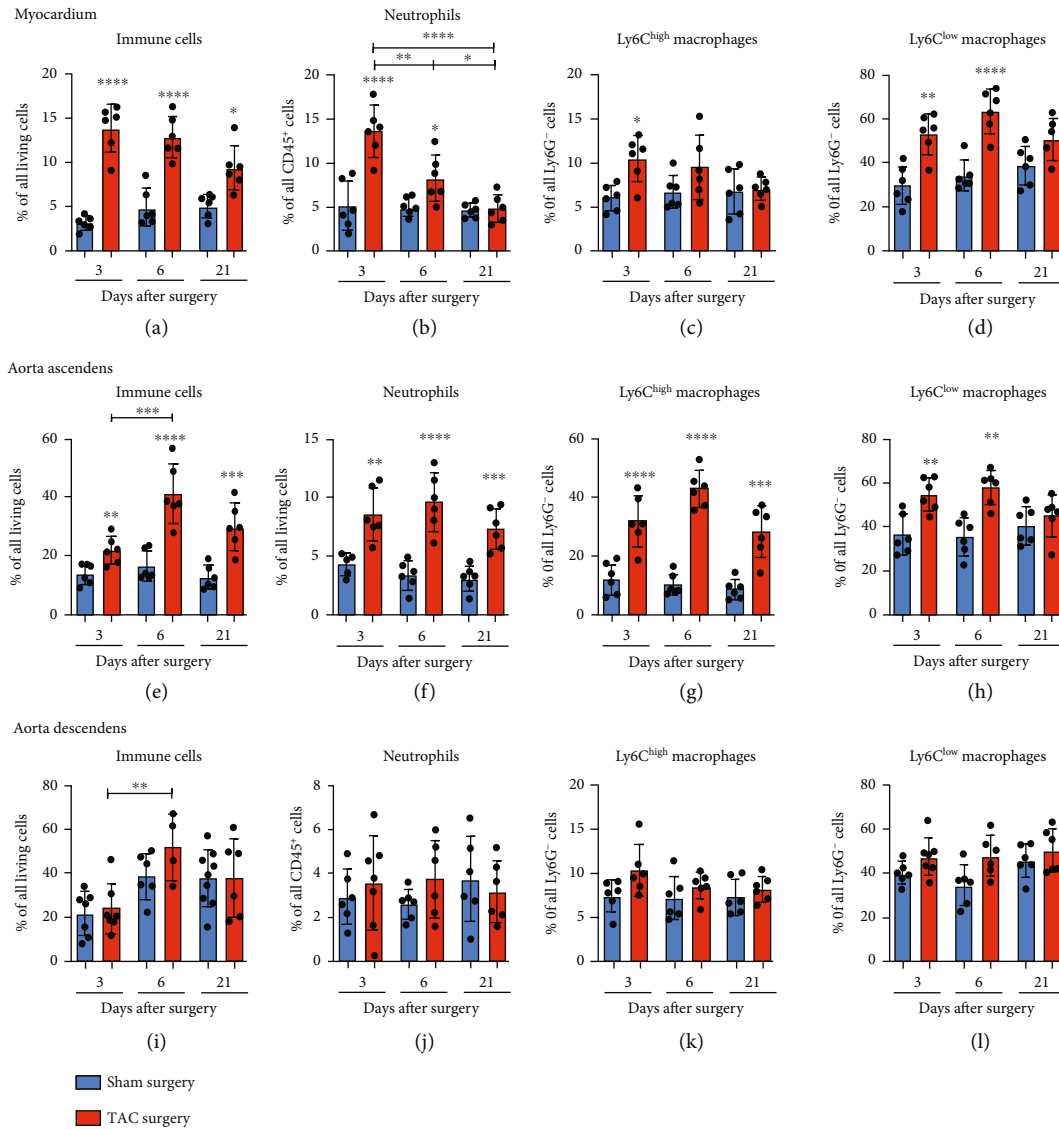


FIGURE 2: Innate immune response in the myocardium and the ascending and descending parts of the aorta. (a)–(l) The CD45⁺ immune cells, neutrophils, Ly6C^{low}, and Ly6C^{high} macrophages in the tissue of the LV, the aorta ascendens and descendens of the mice were quantified via flow cytometry. The particular cell subsets were defined as follows: neutrophils as CD45⁺ F4/80[−] Ly6G⁺ and macrophages as CD45⁺ F4/80⁺ Ly6G[−]. Moreover, the Ly6C^{high} and Ly6C^{low} macrophages were further discriminated according to the respective Ly6C surface expression. *The above individual columns indicate the significant differences between the TAC and respective sham group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

The results of the present study showed a decrease in splenic immune cells at day 6 after TAC compared with the sham group, not reaching the level of significance (Figure 3(a)). The kinetics of the Ly6C^{low} macrophages in the spleen showed a significant increase at day 3 compared with the sham controls and both other time points that were paralleled by an increase in DCs (Figures 3(d) and 3(e)). The first increase of splenic Ly6C^{low} macrophages was followed by a decline to sham level at days 6 and 21 after TAC (Figure 3(d)). The quantification of splenic Ly6C^{high} macrophages and neutrophils did not reveal any changes following TAC (Figures 3(b) and 3(c)). These findings showed that there is a remote reaction of the immune system in the spleen triggered by TAC.

From the spleen, the immune cells are transported via the circulation to the site of inflammation and injury. We suggested that TAC provoked changes in the amount of immune cells in the blood caused by the release of monocytes from the spleen for the recruitment of monocytes to the injured heart. Our flow cytometry-based quantification approach revealed a significant increase only in the population of the Ly6C^{low} monocytes at day 3 after TAC compared with the sham group (Figure 4(d)). No significant increase was detectable at days 6 and 21 after TAC. The reaction of neutrophils did not alter in response to TAC, and the observed changes in the immune cell subsets did not cause alterations of the total immune cell compartment (Figure 4(a)). In addition to the flow cytometry studies,

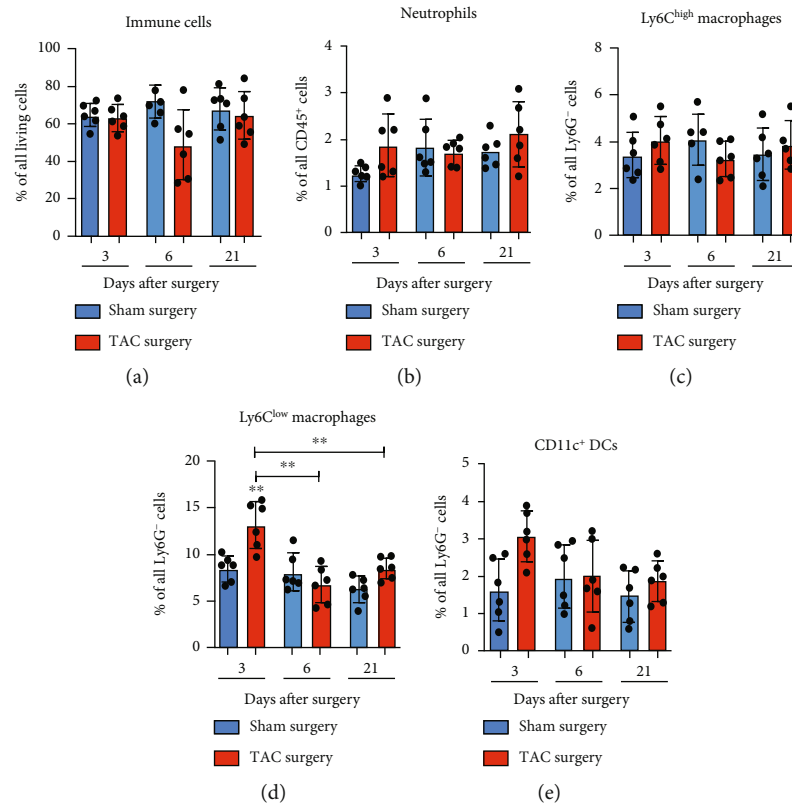


FIGURE 3: Innate immune response in the spleen. (a)–(e) The CD45⁺ immune cells, neutrophils, and Ly6C^{low} and Ly6C^{high} macrophages, and DCs in the spleen of the mice were quantified via flow cytometry. The particular cell subsets were defined as follows: neutrophils as CD45⁺ F4/80⁻ Ly6G⁺ and macrophages as CD45⁺ F4/80⁺ Ly6G⁻. Moreover, the Ly6C^{high} and Ly6C^{low} macrophages were further discriminated according to the respective Ly6C surface expression, and the DCs were defined as CD45⁺ F4/80⁻ Ly6G⁻ and CD11c⁺. *The above individual columns indicate the significant differences between the TAC and respective sham group; * $P < 0.05$, and ** $P < 0.01$.

we determined the levels of proinflammatory cytokines in the serum of TAC and sham mice as indicators of systemic inflammation. The results of the current study indicated a significant increase in IL-6 and IL-1b at day 3 after TAC compared with the control group (Figures 4(e) and 4(f)). The concentration of TNF- α was significantly elevated at days 3 and 6 in response to TAC compared with the sham group (Figure 4(g)). This data demonstrated a systemic immunological reaction reflected in the blood by an increase in proinflammatory cytokines.

The analysis of draining lymph nodes with regard to changes in the leukocyte composition did not exhibit noteworthy changes in response to TAC (data not shown).

3.3. Dendritic Cells Are Activated in Response to TAC in the Myocardium and Aorta Ascendens. Recently, a published work demonstrated that TAC-induced LV hypertrophy is associated with the accumulation of CD11c⁺ MHC-II⁺ cells in the LV, spleen, and blood [14]. Based on these findings, we decided to take a closer look at both the DC activation pattern and subtypes in the myocardium, aorta ascendens and descendens, and draining lymph nodes.

Our analyses revealed that the percentage of MHC-II⁺ cells was significantly elevated in the myocardium 3 and 6 days after TAC compared with the respective control group

(Figure 5(a)). These MHC-II⁺ cells were further subdivided into classical DCs according to the CD11c surface expression. The amount of classical DCs (cDCs) significantly increased at day 6 compared with that at days 3 and 21 after TAC and the respective controls (Figure 5(b)). However, the activation of these cells in the myocardium, measured by CD86 MFI, did not show any changes in response to TAC (Figure 5(c)). Since the majority of the aortic CD11c⁺ MHC-II⁺ DCs showed no staining for E-cadherin ligand CD103, a small CD103⁺ DC population could be confined in the myocardium (Figure 5(d)). In our model, the CD103⁺ DCs increased at days 3 and 6 after TAC compared with the control groups. At day 21, the increase in CD103⁺ DCs returned to the initial level (Figure 5(d)).

In addition to the myocardium, we examined the DC response in the ascending, high-pressure part of the aorta. In response to TAC, we observed a significant increase in MHC-II⁺ cells at day 3 after TAC compared with the sham group (Figure 5(e)). The proportion of cDCs was significantly elevated at days 3 and 6 after TAC compared with the respective sham animals and presented with an earlier onset compared with our observations in the myocardium (Figure 5(f)). We determined a significant increase in the activation marker CD86 at day 3 after TAC compared with the sham group and with the TAC groups at

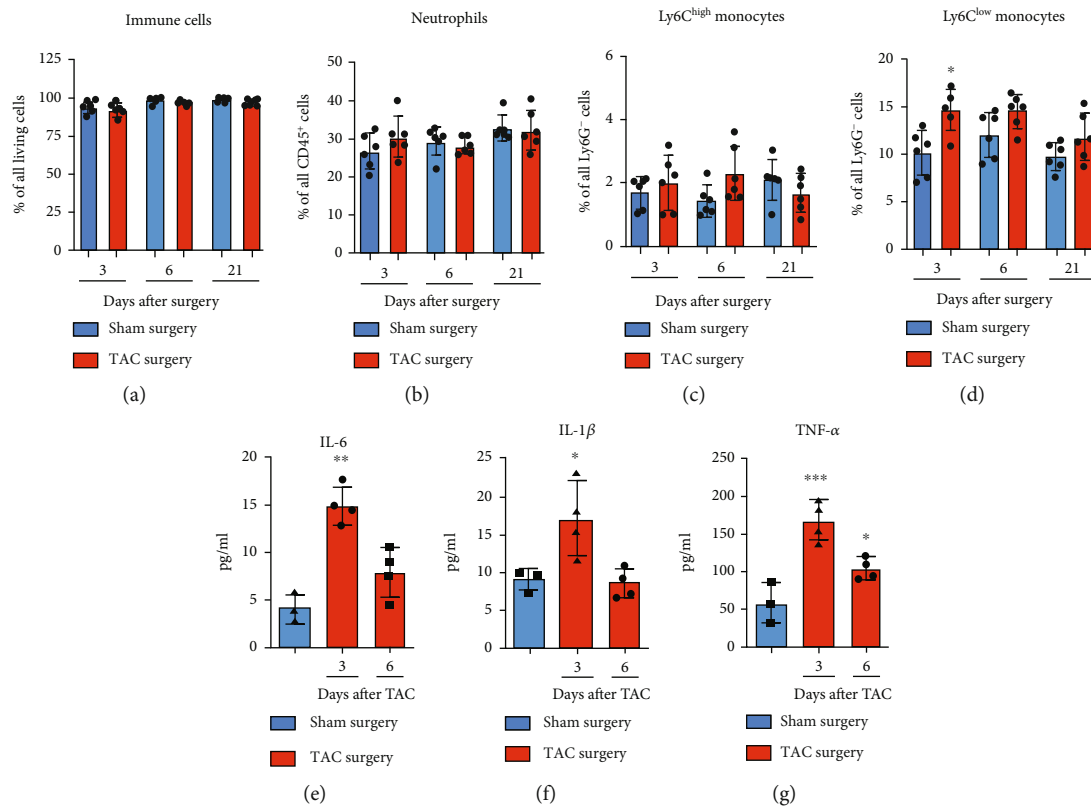


FIGURE 4: Innate immune cell quantification and cytokine levels in response to TAC in the blood. (a)–(d) The CD45⁺ immune cells, neutrophils, and Ly6C^{low} and Ly6C^{high} monocytes in the blood of the mice were quantified using flow cytometry. The particular cell subsets were defined as follows: neutrophils as CD45⁺ CD115[−] Ly6G⁺ and monocytes as CD45⁺ CD115⁺ Ly6G[−]. In addition, the Ly6C^{high} and Ly6C^{low} monocytes were further discriminated according to the respective Ly6C surface expression. (e)–(g) The cytokine levels of IL-6, IL-1β, and TNF-α in the serum of the mice were measured at days 3 and 6 after TAC or after day 3 after sham surgery. *The above individual columns indicate the significant differences between the TAC and respective sham group; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

days 6 and 21 in the aorta ascendens (Figure 5(g)). The CD86 MFI indicated a higher activity level of cDCs in the aorta ascendens after TAC (mean: 5501–8004) compared with that in the myocardium (mean: 3447–4414) (Figures 5(k), 5(g), and 5(o) and Supplementary Figure 2). The amount of CD103⁺ DCs was significantly increased in response to TAC at day 6 compared with the control group and the 21-day TAC group in the aorta ascendens (Figure 5(h)).

In the tissue of the aorta descendens, less changes were observed in the population of DCs. The findings of the present study showed a significant increase in MHC-II⁺ cells at day 6 after TAC compared with the sham group (Figure 5(i)). The kinetics of cDCs and DC86 MFI did not reach the level of significance at any time point (Figures 5(j) and 5(k)). The mean of the CD86 expression pattern did not exceed the mean of 3513 at day 6 after TAC, which represents a low activation level compared to the CD86 MFI measured on the surface of the cDCs in the aorta ascendens and the draining lymph nodes (Figures 5(c) and 5(g) and Supplementary Figure 2). However, we determined a significant increase in CD103⁺ DCs at day 3 after TAC compared with the sham group (Figure 5(l)).

The examination of the draining lymph nodes with focus on the DC dynamics in response to TAC revealed a significant increase in cDCs at day 3 after TAC compared with the respective control group and the 21-day TAC group (Figure 5(n)). CD86 MFI was significantly elevated at day 3 after TAC in comparison with the respective control group and presented with a higher activation level (mean: 6428 at day 6 to 8519 at day 3) compared with the myocardium and the aorta descendens (Figure 5(o)).

This finding indicated an influence of TAC on the population of DCs and the activation status as well as on the DC subsets in the myocardium, aorta ascendens and descendens, and draining lymph nodes.

4. Discussion

In the present project, we further investigated the immune response in the myocardium, aortic tissue, spleen, blood, and lymph nodes following TAC in mice. TAC is a commonly and frequently used animal model for the examination of pressure overload-induced LV hypertrophy. Relevant systemic immunological reactions following TAC

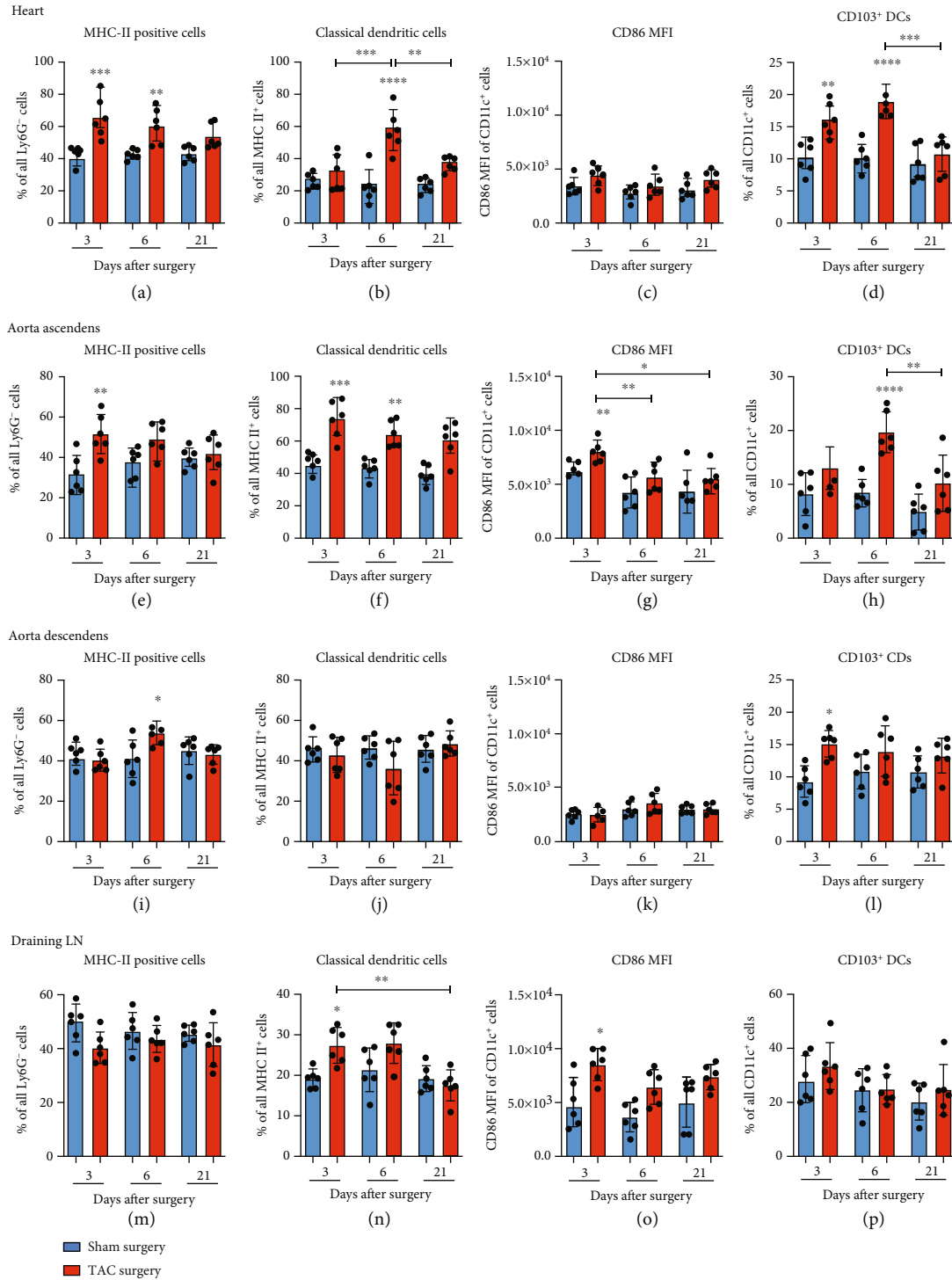


FIGURE 5: TAC provoked the response of the DC compartment in the myocardium, aorta ascendens, aorta descendens, and draining lymph nodes. (a)–(p) The total of the MHC-II⁺ cells was determined from the fraction of CD45⁺ Ly6G[−] cells. The population of cDCs was defined using the CD11c surface staining. CD86 MFI was calculated from the population of all cDCs. The population of CD103⁺ cDCs was determined based on the results of the CD103 surface staining. *The above individual columns indicate the significant differences between the TAC and respective sham group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

induction are of great interest in the field of cardiovascular research [15]. Alterations in the immune cell activity and quantity can promote cardiac remodeling and functional outcome [16]. Herein, we characterized the time course in

innate immune cell dynamics in response to TAC in the different tissues of mice. We investigated whether TAC induces a characteristic leukocyte-driven immune response in the myocardium, aorta ascendens and descendens, spleen,

blood, and draining lymph nodes supported by cytokine-driven chemotaxis in mice in the course of the disease.

The results of the current study indicated that the dynamics of immune cells and neutrophils and Ly6C^{high} and Ly6C^{low} macrophage accumulation in the myocardium were mirrored in the aorta ascendens which is directly affected by the increase in blood pressure after TAC. We observed significantly increased amount of leukocytes at all three time points following TAC in the myocardium and the tissue of the aorta ascendens, highlighting a chronic inflammation in a duration of 21 days. The TAC model allowed the simultaneous analysis of the aortic tissue in a high- and low-pressure environment in front of and behind the knot of the suture.

We assume that the early accumulation of macrophages at day 3 in the spleen reflects the systemic inflammation in response to TAC and the decline in macrophages compared to day 3 after TAC could mirror the release of these cells to the circulation. Unfortunately, our data did not indicate a significant increase in monocytes in the blood at day 6 after TAC. This might be because monocytes were recruited from the circulation maybe in the same amount as they are released from the spleen. This observation underlined the hypothesis that in LV hypertrophy the invading monocytes were released from the spleen and transported via the blood to the site of inflammation similar to the replenishment of monocytes following MI [13]. Our recently published findings demonstrated that the peak of inflammatory activity is at day 3 and is paralleled by the high levels of CCL2 in the myocardium, a chemokine, which is known to attract CCR2⁺ monocytes from the circulation [3]. Besides, we could already show by means of BrdU analyses that there is no local proliferation of macrophages in the myocardium in response to TAC [3]. This supports the idea that macrophages are recruited to the heart in the development of LV hypertrophy. Moreover, the present study investigated the systemic immune response expressed by the increased levels of proinflammatory cytokines in the serum of the mice. The findings that we already published were focused on elucidating the cytokine expression pattern in the myocardium following TAC and confirmed the newly acquired data [3]. We suggest that tissue-resident endothelial and immune cells account for the increase in proinflammatory cytokines and induce a systemic immune response to attract immune cells in response to TAC.

The analysis of the DC dynamics in the myocardium, aortic tissue, blood, lymph nodes, and spleen in response to TAC allows to determine the role of DCs in the modulation of sterile inflammations. Moreover, we identified the DCs based on the surface staining of F4/80, CD11c, MHC-II, and CD103. In the myocardium, the MHC-II⁺ CD11c⁺ DCs have accumulated following TAC and peaked at day 6, whereas in the aorta ascendens, the proportion of DCs significantly increased already at day 3, and the aortic DCs presented with a significantly higher activation level based on CD86 MFI in comparison with the DCs isolated in the myocardium and aorta descendens. This finding indicates that inflammation and the high-pressure environment in the ascending part of the aorta in our TAC model induced a

DC response that was more pronounced than that in the myocardium. The chronic remote inflammation induced in the heart provoked a later onset of the DC response with less activated DCs. We assume that the increase in DCs in the aorta ascendens, which is highly affected by the onset of high-pressure after TAC, contributed to the inflammation process maybe through the breakdown of vascular tolerance as in Kawasaki disease [17]. The finding confirming that CD86 expression was much higher in the DCs found in the aorta ascendens than in the myocardium or the aorta descendens supported the hypothesis that DCs are highly involved in vascular inflammation in response to TAC. The higher activation level of CD11c⁺ cDCs in control animals suggests a reaction to the sham intervention i.e. at day 3 in the sham group but might also indicate that CD86 expression is elevated under steady-state conditions in the lymph nodes. Additionally, in the group of the MHC II⁺ CD11c⁺ DCs, we observed 15% to 20% of CD103⁺ DCs after TAC in the myocardium and the aorta ascendens. CD103, a ligand for E-cadherin expressed by most epithelial cells and also a marker for CD11b⁺ DCs in many tissues, was shown to originate from the aortic sinus [18], a region which was directly affected by the TAC surgery in our model. In the draining lymph nodes, we observed a relevant increase in the population of cDCs and at day 3 an early elevation in cell activation. We hypothesize that the DC response in the draining lymph nodes played a critical role in the orchestration of the immune response following TAC in the aorta and the myocardium and contributed to T cell activation. T cells are known to promote nonischemic heart failure, and the specific antigen recognition of CD4 T cells appears to be crucial for the progression from compensated cardiac hypertrophy to heart failure [19].

Our analyses of the aorta descendens did not reveal any significant changes in the immune response following TAC which might be due to the fact that the tissue was not directly and even remotely affected.

The TAC model used in this project mirrors the situation of patients suffering from diseases such as chronic arterial hypertension or aortic valve stenosis that result in LV hypertrophy. It was considered that the acute onset of pressure overload in the TAC model did not perfectly reflect the patients' clinical course in which the pressure overload slowly evolves over the years leading to LV hypertrophy and heart failure. Despite the limitations of the current study, the model of TAC in mice has been extensively used to examine signaling pathways that contribute to adverse cardiac remodeling and hypertrophy in the context of pressure overload [15]. The observations collected in this study promote a model that can be used to compare high-pressure and low-pressure conditions in the aortic tissue in parallel in the same animal.

5. Conclusion

Based on the findings of the current study, it is likely that the monocytes that have accumulated in response to TAC in the myocardium originate from splenic pools and reach the site of the inflammation via the blood driven by chemotaxis. The

high-pressure conditions in the aorta ascendens cause a characteristic immune response, dominated by the accumulation of leukocytes that varies depending on the immune cell dynamics in the myocardium and the aorta descendens.

Abbreviations

DAMPs: Danger-associated molecular patterns
 DC: Dendritic cell
 FACS: Fluorescence-activated cell sorting
 IL: Interleukin
 LV: Left ventricle
 MFI: Mean Fluorescence Intensity
 MHC: Major histocompatibility complex
 MI: Myocardial infarction
 TAC: Transverse aortic constriction
 Wt: Wild-type mice (C57BL/6).

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary material.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

CKW, LE, JC, and SF conceived and designed the experiments. JLK, YZ, OK, and AF performed the experiments. CKW, JLK, SF, OK, and AF analyzed the data. CKW, JLK, SF, and MC wrote the manuscript.

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Supplementary Materials

Supplemental Figure 1: fluorescence minus one (FMO) controls of CD11c and CD103 staining in addition to Figure 1. Supplemental Figure 2: direct comparison of CD86 MFI of CD11c⁺ cells in heart, aorta ascendens, aorta descendens, and lymph node in addition to Figure 5. (*Supplementary Materials*)

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4 Diskussion

Die vier dargestellten experimentellen Arbeiten untersuchen die Gemeinsamkeiten und Unterschiede der angeborenen Immunantwort in der bakteriell induzierten und der sterilen Entzündung. Die Arbeiten nehmen insbesondere die Rolle der angeborenen Immunzellen in den Fokus und analysieren deren Dynamik und Rolle in der Induktion und Aufrechterhaltung der Inflammation in den beiden Kontexten. Durch die Übertragung der Befunde aus der bakteriellen Inflammation auf die sterile Inflammation lassen sich viele Parallelen aufzeigen, die für die Erforschung und Etablierung neuer immunmodulatorischer Therapiestrategien von Bedeutung sein können.

Die angeborene Immunantwort in der bakteriellen Infektion und der sterilen Myokardschädigung weist viele Übereinstimmungen auf. Die Immunzellen haben vielfältige Aufgaben in den unterschiedlichen Entzündungskontexten zu erfüllen. Gemeinsam ist ihnen die Fähigkeit, auf eine Vielzahl unterschiedlicher Signale zu reagieren (Meier-Schellersheim et al., 2019) und ihr Verhalten durch Kommunikation mit anderen Immun- und Nicht-Immun-Zellen anzupassen (Kumar and Roger, 2019; Leppkes et al., 2018). Für den Fall, dass ihre Effektorfunktion und ihre interzelluläre Kommunikation beeinträchtigt sind, kann die Bekämpfung von Krankheitserregern, die Tumoralabwehr oder der Abtransport abgestorbener Zellen gestört sein und hierdurch können sich verschiedene Infektionserkrankungen entwickeln (Meier-Schellersheim et al., 2019).

In der bakteriellen Harnwegsinfektion dokumentieren die Ergebnisse unserer Arbeit einen einzigartig aufeinander abgestimmten *Crosstalk* zwischen den beteiligten Immunzellen innerhalb der ersten 4 Stunden nach einer Infektion. Zunächst erkennen residente Ly6C⁻-Makrophagen die Infektion und setzen Chemokine frei, die Neutrophile und Ly6C⁺-Makrophagen anlocken. Die rekrutierten Ly6C⁺-Makrophagen produzieren als Reaktion auf die Infektion TNF. Dieses TNF wiederum induziert die Freisetzung von prä-formiertem CXCL2 durch Ly6C⁻-Makrophagen. CXCL2 löst im folgenden Schritt die Sekretion von MMP-9 durch Neutrophile aus. Erst durch die MMP9-Freisetzung sind die Neutrophilen in der Lage, die epitheliale Basalmembran zu durchqueren und an den Infektionsort zu gelangen, um die Bakterieninvasion einzudämmen. In dieser koordinierten Antwort des Immunsystems fungieren die Ly6C⁻-Makrophagen als residente Wächter und die Ly6C⁺-Makrophagen spielen eine bisher unbekannte Helferrolle, ähnlich der Funktion von CD4⁺-

T-Helferzellen (Smith et al., 2004). Als Reaktion auf die Infektion ermöglichen die Helfermakrophagen die Reaktion der Ly6C-Makrophagen, um Neutrophile koordiniert in das Uroepithel vordringen zu lassen. Dieser Ablauf schafft einen Schutz für das Gewebe, um unkontrollierte Zerstörungen durch Neutrophile, wie sie bei Traumapatienten durch eine überschießende ROS-Produktion beobachtet wurden, zu verhindern (Swain et al., 2002). Außerdem weisen unsere Ergebnisse die beiden Mediatoren CXCL2 und MMP9 als durch TNF regulierte Pfortner für die transepitheliale Neutrophilenmigration aus.

Beide Makrophagenpopulationen, die wir auch in der Harnwegsinfektion beobachten konnten, lassen sich im Herzgewebe in der Entwicklung der linksventrikulären Hypertrophie nachweisen. Allerdings bezeichneten wir sie anhand der Ly6C-Expression auf der Oberfläche und in Anlehnung an andere Publikationen aus dem Feld der kardiovaskulären Forschung als Ly6C^{low} und Ly6C^{high} (Nahrendorf et al., 2007). Unsere Ergebnisse aus dem TAC Modell tragen zum Verständnis der Rolle von Ly6C^{low} Makrophagen bei, die bisher hauptsächlich in der Funktion der residenten Gewebewächter gesehen wurden (Geissmann et al., 2010b), und im Licht unserer Ergebnisse als Schlüsselzellen in der Entwicklung der chronischen Drucküberlastung des linken Ventrikels betrachtet werden müssen. Die Lage der Ly6C^{low}-Monozyten/Makrophagen auf der luminalen Seite, adhärent zu Endothelzellen der kardialen Gefäße in Wt-Mäusen unterstützt die Auffassung, dass diese Zellen ebenfalls in der sterilen kardialen Inflammation eine Wächterfunktion ausüben.

Im Gegensatz zur Immunantwort nach einem Myokardinfarkt mit einer biphasischen Reaktion aus pro-inflammatorischen Ly6C^{high}-Markophagen und einer zweiten Gewebeheilungs-Phase mit der Akkumulation von Ly6C^{low}-Makrophagen (Nahrendorf et al., 2007), präsentiert sich die Immunantwort in der linksventrikulären Hypertrophie über den gesamten Verlauf mit einer Akkumulation von Ly6C^{low}-Makrophagen im Myokard. Diese Einphasigkeit spricht für eine chronische Entzündungsreaktion durch den protrahierten Verlauf der Hypertrophieentwicklung ähnlich wie bei der Wundheilung (Krzyszczuk et al., 2018). Nach einem Myokardinfarkt spielen die phagozytierenden Ly6C^{high}-Makrophagen eine zentrale Rolle bei der Beseitigung von Zelltrümmern (Frantz and Nahrendorf, 2014). Ein vergleichbar akuter Untergang von Myokardzellen mit dem Anfallen von Zelltrümmern, findet nach einer TAC nicht statt. Vielmehr handelt es sich in

diesem Modell um eine chronische Druckbelastung des Herzens, die auch zu einer chronischen, von Ly6C^{low} -Makrophagen dominierten Immunantwort führt.

Unsere Untersuchungen an CX3CR1-defizienten Mäusen konnten zeigen, dass das Fehlen der Ly6C^{low} -CX3CR1^{high}-Makrophagen im Myokard einen kardioprotektiven Effekt hat. Dieser Effekt wird am ehesten durch die starke pro-inflammatorische Immunantwort, die durch die Zunahme von Neutrophilen und $\text{Ly6C}^{\text{high}}$ -Makrophagen, sowie die Freisetzung von pro-inflammatorischen Zytokinen im Vergleich zu den Wt-Tieren charakterisiert ist, erklärt. Speziell im Bereich der Wundheilung verdeutlichen Studien den Zusammenhang zwischen Inflammation und Heilungsprozess. $\text{Ly6C}^{\text{high}}$ -Makrophagen produzieren pro-inflammatorische Zytokine wie TNF und IL-6, die das Anfangsstadium der Wundheilung erleichtern (Hesketh et al., 2017).

Im Bereich der Hirnischämie hat sich gezeigt, dass ein Fehlen von CX3CR1 durch eine verminderte Rekrutierung von Ly6C^{low} -iNOS⁺-Monozyten einen positiven Effekt auf den Erkrankungsverlauf hat (Donnelly et al., 2011). Diese Beobachtung wurde in einem Myokardinfarkt-Mausmodell bestätigt, in dem die Behandlung mit einem Fraktalkin-Antikörper einen kardioprotektiven Effekt durch die Reduktion des negativen Gewebeumbaus und der Makrophagenmigration aufwies (Gu et al., 2015). Daher scheint der Fraktalkinrezeptor in seiner Funktion in der Rekrutierung und Adhäsion von Monozyten ein sehr interessantes Ziel für eine immunmodulatorische Therapie darzustellen.

Meine Arbeiten verdeutlichen, dass die Aufrechterhaltung der Entzündungsreaktion im Gewebe, sowohl in der bakteriellen Infektion, als auch in der sterilen kardialen Inflammation, auf die Rekrutierung von Immunzellen durch die Freisetzung von Signalmolekülen wie Zytokinen und Chemokinen angewiesen ist (Muller, 2013). Nach TAC konnte keine lokale Proliferation von Makrophagen nachgewiesen werden, vielmehr wurde die steigende Anzahl an $\text{Ly6C}^{\text{high}}$ -Makrophagen in CX3CR1 defizienten Mäusen innerhalb der ersten Woche nach chirurgischer Intervention auf die CCL2 vermittelte Rekrutierung von CCR2⁺-Monozyten aus dem Blut zurückgeführt. Hier zeigt sich eine weitere Parallele zur bakteriellen Infektion, in der die Monozyten ebenfalls über CCL2 an den Infektionsherd gelockt wurden.

Im Myokardinfarkt-Mausmodell konnte nachgewiesen werden, dass Monozyten aus der Milz in das geschädigte Herzgewebe rekrutiert werden und, dass die Milz damit ein Reservoir für Monozyten darstellt, die schnell freigesetzt werden und ins Myokard migrieren können. Eine ähnliche Beobachtung ließ sich auch für das Mausmodell der TAC machen. Hier zeigte sich zunächst ein systemischer Nachweis der pro-inflammatorischen Zytokine IL-6, IL-1 β und TNF α im Blut und eine damit einhergehende Zunahme der Ly6C^{low}-Makrophagen in der Milz an Tag 3. Den systemischen Zeichen einer Inflammation mit Zunahme der Milz-Makrophagen folgte eine Abnahme der myeloiden Zellen im Milzgewebe an Tag 6 nach TAC, dem Zeitpunkt der maximalen Makrophagen Akkumulation im Myokard. Entgegen unserer Hypothese konnte kein paralleler Anstieg der Monozyten im Blut beobachtet werden. Dies könnte am ehesten auf die Homöostase aus der Freisetzung von Zellen aus der Milz und der Geweberekrutierung ins Myokard zurückgeführt werden.

Die Rolle der DCs als Teil der Leukozyten wurde erst in der vierten Arbeit detaillierter untersucht. Es ist bereits bekannt, dass eine Hypertonie DCs aktiviert (Abbas et al., 2015). Die Akkumulation in der *Aorta ascendens* an Tag 3, die mit einer hohen Aktivierung der DCs einhergeht und im Myokard an Tag 6 nach TAC, legt nahe, dass auch diese Zellen einen Beitrag zur sterilen Inflammation im Myokard leisten. Eine erhöhte Anzahl an DC-Spiegel sind auch bei stenotischen koronaren *Bypass*-Transplantaten zu beobachten (Cherian et al., 2001). Studien weisen darauf hin, dass Interaktionen zwischen DCs und T-Zellen an der progressiven Entwicklung von Atherosklerose und der Degeneration von Venentransplantaten nach kardiovaskulärem *Bypass* beteiligt sind und ein Transplantatversagen begünstigen (Dieterlen et al., 2016).

Der Vergleich und die Übertragbarkeit von immunologischen Phänomenen aus der bakteriellen Infektion und der sterilen kardialen Inflammation können einen wesentlichen Beitrag für das Verständnis der zugrundeliegenden pathophysiologischen Mechanismen liefern.

Ausblick: Die bisher gesammelte Expertise und die neuen Erkenntnisse zu den Abläufen der angeborenen Immunantwort in der Harnwegsinfektion und der sterilen kardialen Inflammation in der linksventrikulären Hypertrophie lassen sich synergistisch auch in anderen Erkrankungen anwenden. Die Relevanz von Monozyten/Makrophagen, die chemokinabhängige Rekrutierung von Leukozyten für die Aufrechterhaltung der Inflammation aber auch das Geweberemodelling nach einer Schädigung spielen in fast allen Krankheitsbildern eine große Rolle und entscheiden über die Erkrankungsschwere und den Verlauf. In der Aortenklappenstenose, einer Erkrankung, die sowohl bakteriell als auch steril durch degenerative Prozesse vorangetrieben werden kann, nutzen wir die Ergebnisse und Methoden meiner bisherigen Forschungsarbeiten dazu, die zugrundeliegenden immunologischen Mechanismen zu entschlüsseln und innovative immunmodulatorische Therapien entwickeln zu können.

5 Zusammenfassung

Die Reaktion des Immunsystems in der bakteriellen Infektion und der sterilen Myokardschädigung weist viele Gemeinsamkeiten auf. Das angeborene Immunsystem spielt in der Bekämpfung bakterieller Infektionen eine wesentliche Rolle, indem Phagozyten die eingewanderten Bakterien erkennen, eliminieren, weitere Immunzellen aktivieren und abgestorbene Zellen entfernen (Schiwon, Weisheit et al., 2014). Bei sterilen Entzündungsreaktionen wie sie im Herzgewebe nach einer Schädigung durch eine chronische Überdruckbelastung auftreten, spielen die Mechanismen der angeborenen Immunantwort, die für die Eindämmung bakterieller Infektionen von sehr großer Bedeutung sind, wie Immunzellrekrutierung, Freisetzung von Effektormolekülen und Initiation des Geweberemodellings, ebenfalls eine entscheidende Rolle für den Verlauf der Erkrankung (Weisheit et al., 2014; Kleiner et al., 2021; Weisheit et al., 2021).

Am Modell der bakteriellen Harnwegsinfektion konnte die komplexe Kommunikation zwischen zwei Makrophagengruppen zur Kontrolle der transepithelialen Migration von Neutrophilen an den Infektionsherd aufgeschlüsselt werden. Die Kommunikation der Immunzellen untereinander über TNF, CCL2 und CXCL2 ist hierbei von hoher Relevanz für das Gelingen der Infektionseindämmung.

Der Prozess der Rekrutierung und Aktivierung von Leukozyten bildet auch den Ausgangspunkt für die Entwicklung der druckinduzierten linksventrikulären Hypertrophie. Mit Hilfe eines murinen Modells, der transversen aortalen Konstriktion (TAC), ließ sich die Akkumulation von Leukozyten als Ausdruck der Entzündungsreaktion nachweisen. Hierbei spielten insbesondere die Ly6C^{low}-CX3CR1^{high}-Makrophagen eine wesentliche Rolle, deren Relevanz durch die Verwendung von CX3CR1-defizienten Mäusen untersucht werden konnte. Die Daten zeigen, dass ein Fehlen von CX3CR1 im TAC-Modell einen deutlichen kardioprotektiven Effekt erzeugt, der am ehesten durch die frühe pro-inflammatorische Immunreaktion vermittelt wird.

Die Analyse der zeitlichen Dynamik der angeborenen Immunantwort als Reaktion auf eine TAC in verschiedenen Geweben trug dazu bei, die Rekrutierungsprozesse in das geschädigte Myokard besser verstehen zu können. Die Quantifizierung von Monozyten/Makrophagen im Milzgewebe von Mäusen nach TAC legt den Schluss nahe, dass durch die Freisetzung pro-inflammatorischer Zytokine aus dem geschädigten

Myokard eine systemische Immunreaktion ausgelöst wird, die zunächst zu einem Anstieg der myeloiden Zellen in der Milz führt und im weiteren Verlauf zu einem deutlichen Abfall, was einer Freisetzung dieser Zellen ins Blut entsprechen könnte. Die freigesetzten Zellen werden in der Folge an den Inflammationsort im Herzen rekrutiert. Interessante neue Aspekte konnten auch zur Aktivierung und Akkumulation von DCs im Hochdruck- und Niederdruckbereich der Aorta nach TAC gesammelt werden. Die *Aorta ascendens* spiegelt hier eine wesentlich stärkere Immunreaktion wider, die in Teilen mit der Dynamik im Myokard vergleichbar ist.

Diese Arbeiten tragen durch den Einblick in die Grundlagen der angeborenen Immunantwort dazu bei, zukünftig nicht nur neue Therapieoptionen definieren zu können, sondern auch möglicherweise immunologische Biomarker zu identifizieren, die Auskunft über den Grund für das Persistieren einer Harnwegsinfektion geben, oder einen schnellen Progress der linksventrikulären Hypertrophie vorhersagen lassen.

6 Überlappung durch geteilte Autorenschaften

Die vorliegende Habilitationsschrift hat vier publizierte Originalarbeiten zur Grundlage. Drei der Arbeiten habe ich als Erstautorin (Schiwon, Weisheit et al., 2014; Weisheit et al., 2014; Weisheit et al., 2021) und eine der Arbeiten als Letztautorin veröffentlicht (Kleiner et al., 2021). Die Cell-Publikation von 2014 habe ich mit Frau Dr. Marzena Schiwon als geteilte Erstautorin zusammen veröffentlicht. Sie war zu dieser Zeit in der Arbeitsgruppe von Herrn Prof. Dr. med. Christian Kurts im Institut für Molekulare Medizin und Experimentelle Immunologie (IMMEI) tätig. Die Daten für die Publikation haben wir arbeitsteilig erhoben und gemeinsam in den Kontext für das Manuskript gesetzt. Durch mein Stipendium beim Else-Kröner-Forschungs-Kolleg Bonn konnte ich mich für zwei Jahre sehr intensiv in die Fragestellung des Zell-Zell-Crosstalks von Immunzellen einarbeiten und maßgebliche Versuche, die die Arbeit in diesem hochrangigen *Journal* publikationsfähig gemacht haben, durchführen. Meine Leistung lag hierbei unter anderem in der Durchführung der histologischen und durchflusszytometrischen Analysen, sowie der Etablierung und Durchführung der ELISA-Assays an den Maus-Harnblasen, um den zugrundeliegenden molekularbiologischen Mechanismus des *Cross-Talks* der Immunzellen aufzuklären. Der Arbeitsaufwand für diese hoch anspruchsvolle und komplexe Arbeit hat die Verteilung der Erstautorschaft auf zwei Mitarbeiterinnen erforderlich gemacht.

Eine Überlappung mit anderen Habilitationsschriften ist nicht gegeben.

7 Literaturverzeichnis

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