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Proinflammatorische und prothrombotische Effekte doppelsträngiger DNA am vaskulären Endothel

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1. INHALTSVERZEICHNIS

2. ABKÜRZUNGSVERZEICHNIS

AIM-2	Absent in melanoma 2
ANCA	Anti-neutrophil cytoplasmic antibody
ANOVA	Analysis of variance
BSA	Bovine serum albumine
CD40	Cluster of differentiation 40
cGAS	cGAMP synthase
CRP	C-reactive protein
DAI	DNA-dependent activator of IRFs
DAMP	Danger associated molecular patterns
DAPI	4′,6-Diamidin-2-phenylindol
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EC	Endothelial cell
EDHF	Epithelial derived hyperpolarizing factors
EBV	Ebstein-Barr virus
ELISA	Enzyme-Linked Immunosorbent Assay
FI	Factor I; Fibrinogen
FII	Factor II; Prothrombin
FIII	Factor III; Tissue factor
FV	Factor V
FVII	Factor VII
FIX	Factor IX; Christmas factor
FX	Factor X; Stuart-Prower-Factor
FXIII	Factor XIII
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GPIa	Glycoprotein I a
GPIb	Glycoprotein I b
GPIIb	Glycoprotein II b
GPIIIa	Glycoprotein III a
GPVI	Glycoprotein VI
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HMEC	Human microvascular endothelial cell
HMGB1	High mobility group box 1
HSV-1	Herpes simplex virus 1
huDNA	Human genomic DNA
HUVEC	Human umbilical vein cell
ICAM-1	Intercellular adhesion molecules
IFI16	Interferon-γ-inducible protein16
IκBα	Inhibitor of κBα
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
INF-β	Interferon beta
IP-10	Interferon gamma-induced protein 10; CXCL10
IRF3	Interferon regulatory factor 3
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1; CCL2
MDA5	Melanoma Differentiation-Associated protein 5

miRNA	Micro RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium cholride
NaHCO3	Sodium hydrogen carbonate
NaHPO4	Sodium hydrogen phosphate
NALP3	NACHT, LRR and PYD domains-containing protein 3
NET	Neutrophil extracellular trap
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
p38	P38 mitogen-activated protein kinases
PAF	Platelet-activating factor
PAI-1	Plasminogen activator inhibitor
pAKT	Phosphorylated protein kinase, strain AK, Thymoma
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmocytoid dendritic cells
Poly(dA:dT)	Poly(deoxyadenylic-deoxythymidylic)
PRR	Pattern recognition receptors
pTBK1	Phosphorylated TANK-binding kinase 1
RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SEM	Standard error of the mean
siRNA	Small interfering RNA
SLE	Systemic lupus erythematodes
TF	Tissue factor; FIII
TFPI	Tissue factor pathway inhibitor
THBD	Thrombomodulin
TLR3	Toll-Like Receptor 3
TLR9	Toll-Like Receptor 9
TNFα	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
TRAF6	TNF receptor-associated factor 6
VCAM-1	Vascular cell adhesion protein 1
vWF	Von-Willebrand-factor

„Wir wissen nicht, dass die Entzündung als solche an die Gerinnung gebunden ist; im Gegentheil hat sich herausgestellt, dass die Lehre von den Stasen auf vielfachen Missverständnissen beruht. Es kann die Entzündung unzweifelhaft bestehen bei einem vollkommen offenen Strome des Blutes innerhalb der Gefässe des afficirten Theiles. Lassen wir also die Entzündung bei Seite und halten wir uns einfach an die Gerinnung des Blutes, an die Bildung des Gerinnsels (Thrombus), so scheint es am bequemsten, diesen Vorgang in dem Ausdrucke Thrombose zusammen zu fassen“ (1).

Rudolf Virchow, 1862

3. EINLEITUNG

Freie doppelsträngige DNA (dsDNA) kann durch das angeborene Immunsystem wahrgenommen werden und mittels Bindung an DNA-Rezeptoren eine unspezifische Immunantwort initiieren (2, 3). Doppelsträngige DNA kann im Zusammenhang mit verschiedenen Pathologien, einhergehend mit steriler oder infektiöser Inflammation, im Kreislaufsystem vorkommen (4). Sie wird akut bei Sepsis und Trauma, sowie bei chronischen Infektionen, Autoimmunprozessen oder Tumorerkrankungen freigesetzt (5). Insbesondere bei viralen Infektionen mit DNA-Viren spielt die doppelsträngige DNA als immunstimulatorisches Agens eine zentrale Rolle, z. B. bei Hepatitis B-Virusinfektionen (6). Diese vielfältigen Erkrankungen können mehrere Organsysteme betreffen und sich als Systemerkrankungen manifestieren (7). Die Schwere des systemischen Krankheitsverlaufs resultiert unter anderem aus der Gefäßbeteiligung, wie beispielsweise in Form einer (akuten) Vaskulitis (8). Diese Krankheiten können mit ischämischen Organschäden einhergehen, aber auch thrombotische Komplikationen verursachen und dabei mit tödlichen Thromboembolien vergesellschaftet sein (9, 10). Zudem tragen möglicherweise virale Infektionen mit DNA- und RNA-Viren direkt zu der Entstehung und Entwicklung von Atherosklerose, der am weitesten verbreiteten chronischen Gefäßerkrankung mit thromboembolischen Komplikationen, bei (11, 12).
Die Pathogenese der Gefäßbeteiligung und Thromboembolie durch dsDNA wird vornehmlich durch Immunzellen und sekundäre Immuneffekte vermittelt, da Endothelzellen nicht die primären Zielzellen dsDNA-vermittelter Entzündung sind. Dennoch könnte das Endothel durch eigenständiges Erkennen von dsDNA bei der Immunaktivierung und der inflammatorischen Antwort beteiligt sein, zumal Endothelzellen eine Schlüsselrolle im Verlauf der Krankheitsmanifestationen von Vaskulitis oder Atherosklerose spielen (13, 14).
Während die Effekte von dsDNA auf Immunzellen in den letzten Jahren umfassend untersucht wurden (2, 15, 16), bleiben die Auswirkungen und ihre pathophysiologische Bedeutung auf Zellen, welche nicht primär dem Immunsystem zugeordnet werden, wie zum Beispiel den Endothelzellen, weitgehend unbeleuchtet. Unsere Ausgangshypothese

war daher, dass das vaskuläre Endothel einen eigenen Anteil an der Entstehung der beschriebenen Krankheiten und deren Krankheitsfolgen hat. In den vorliegenden Arbeiten wurde demnach die übergeordnete Fragestellung behandelt, inwiefern dsDNA proinflammatorische und prothrombotische Effekte auf das vaskuläre Endothel ausübt, sowie welche Rezeptoren und Mechanismen des angeborenen Immunsystems hierbei eine Rolle spielen könnten. Zunächst lag der Fokus auf der Untersuchung der inflammatorischen Reaktion und im Verlauf wurden die Aspekte der Interaktion von Endothel und Blutgerinnung herausgearbeitet.
Es ist dennoch davon auszugehen, dass die inflammatorischen Wechselwirkungen zwischen Immunsystem und Endothelzellen komplex sowie multikausal sind. Gleichwohl sollte in diesen Studien ein direkter Effekt von dsDNA auf die Endothelzellfunktion untersucht werden. Hierzu analysierten wir in funktionellen Experimenten die Entzündungsreaktion sowie die Beeinflussung der Blutgerinnung durch Endothelzellen, sowohl auf Thrombozytenadhäsion als auch auf die plasmatische Gerinnung.
Ausgangspunkt der Experimente beider Arbeiten waren humane Endothelzellen, welche primär isoliert oder als Zelllinie kultiviert und entsprechend der verschiedenen Teilaspekte analysiert wurden (17, 18).

3.1 Das Endothel, Aktivierung und Dysfunktion

Das Endothel bildet die primäre, physikalische Barriere zwischen Blut und Geweben, und ist verschiedensten Einflüssen aus dem Kreislaufsystem unmittelbar ausgesetzt. Es bildet einen vitalen Zellverbund welcher den Austausch von Nährstoffen und biologisch aktiven Molekülen beeinflusst und dient, als innerste Membran des Kreislaufsystems, der Aufrechterhaltung der selektiven Gefäßwandpermeabilität (13). Diese selektive Permeabilität wird einerseits durch interzelluläre Verbindungs- und Signalproteine und andererseits durch spezifische Rezeptoren und Kanäle vermittelt (19, 20). Die Endothelzellen spielen durch die Produktion des Vasodilatators Stickstoffmonoxid (NO) eine entscheidende Rolle bei der Regulation des Gefäßtonus der glatten Muskelzellen der Media (21). Das Endothel steuert unter anderem die Hämostase und reagiert auf inflammatorische Aktivierung mit einer Modulation der Entzündungsantwort (22). Hierzu synthetisieren Endothelzellen Moleküle welche den Blutfluss durch die Inhibierung der im Blut gelösten Gerinnungsfaktoren gewährleisten. Endothelzellen sezernieren bzw. präsentieren beispielsweise *Tissue factor pathway inhibitor* (TFPI) oder Thrombomodulin (THBD) an der Zelloberfläche und verhindern dadurch die Spontangerinnung des Blutes (14). Auch Thrombozyten werden durch die Freisetzung von Stickstoffmonoxid (NO), Prostazyklin (PGI_2) und weiteren sogenannten *endothelial derived hyperpolarizing factors* (EDHF) inaktiviert und deren Anhaften vermindert (23). Endothelzellen eigenen sich daher gut um direkte Wechselbeziehungen zwischen Gefäßwand und Blutbestandteilen zu untersuchen.

Das Endothel interagiert mit verschiedenen Komponenten des Immunsystems und erfüllt immunologische Aufgaben (24): durch die Barrierefunktion als physikalische Schranke mit selektiver Permeabilität, durch Erkennung inflammatorischer Signale, die Regulation des Gefäßtonus und damit der Blutversorgung, sowie der Expression von Zell-Adhäsionsmolekülen zur Leukodiapedese und der Sekretion von Zytokinen (21, 25). Die Aktivierung der Endothelzellen hat hierfür die Expression von Adhäsionsmolekülen (v. a.

E/P-Selectin, VCAM-1, ICAM-1) zur Folge und insbesondere im venösen System erfolgt hierdurch die Rekrutierung und Extravasation von Immunzellen (25, 26). Für die Bearbeitung unserer Fragestellung war daher relevant, ob die Stimulation mit dsDNA eine Expression dieser Zelladhäsionsmoleküle an der Oberfläche auslösen und damit die aktive Rekrutierung von Immunzellen zum Endothel befördern kann. Wir konnten in unseren Ausgangsexperimenten eine unspezifische Aktivierung des Endothels mit Hochregulation proinflammatorischer Zytokine und Chemokine, sowie spezifisch den Nachweis der Hochregulation und Expression von ICAM-1 und VCAM-1 an der Zelloberfläche humaner Endothelzellen zeigen (18).

Inflammatorische Prozesse des Endothels führen zu einer Aktivierung und Dysfunktion des funktionellen und grundlegenden Gleichgewichts der Zellen (27, 28). Die Destabilisierung der physiologischen Barrierefunktion, beispielsweise ausgelöst durch Infektionen oder sterile Inflammation, erhöht die Vulnerabilität des Endothels gegenüber intravasaler Stressmoleküle. Chronische Entzündungen können zu einer dauernden Endothelaktivierung führen und in einer langfristigen Beeinträchtigung der Endothelzellfunktionen resultieren (29). Solch eine endotheliale Aktivierung und Dysfunktion ist Teil vieler entzündlicher Systemerkrankungen. Pathologien wie Vaskulitiden und Atherosklerose haben hier ihren Ausgangspunkt (30, 31). Wir konnten in unseren Arbeiten zeigen, dass Endothelzellen Rezeptoren des angeborenen Immunsystems exprimieren und in der Lage sind dsDNA zu erkennen und eine Entzündungsantwort hervorrufen (18). Hier könnte eine Verbindung zwischen chronisch inflammatorischen und infektiösen Erkrankungen, sowie dem Auftreten thromboembolischer Komplikationen liegen, da diese endotheliale Aktivierung einen proinflammatorischen und prothrombotischen Zustand befördern kann (14, 32). Aktivierte Endothelzellen begünstigen Blutgerinnung und Thromboseneigung indem sie einerseits die Expression antithrombotischer Proteine, wie Thrombomodulin (THBH), gewebespezifischen Plasminogenaktivator (t-PA) und den endothelialen Protein C Rezeptor herabregulieren (33-35). Andererseits exprimieren sie vermehrt prokoagulative Moleküle wie den antifibrinolytischen Plasminogen-Aktivator-Inhibitor-1 (PAI-1) und Gewebefaktor (FIII, *Tissue factor*, TF) (14). Gewebefaktor wird von Endothelzellen unter physiologischen Bedingungen lediglich geringfügig exprimiert (13). Jedoch kann bei verschiedenen infektiösen oder inflammatorischen Stimuli wie LPS oder IL-1β und TNFα die Expression von TF beträchtlich hochreguliert werden, welches einen prothrombotischen Zustand zusätzlich verstärken kann (14, 36, 37). Nach arterieller Thrombose sind zudem ein erhöhter Plasmaspiegel von *Tissue factor* nachweisbar und innerhalb arteriosklerotischer Plaques zeigt sich ein verstärktes Vorkommen des Gewebefaktors (38, 39). Plasminogen-Aktivator-Inhibitor-1 (PAI-1) wird von Endothelzellen synthetisiert und inhibiert den gewebespezifischen Plasminogenaktivator (tPA) und verhindert damit die Fibrinolyse und befördert die Bildung stabiler Thromben. PAI-1 ist bei kardiovaskulären Erkrankungen im Blut erhöht (40, 41) und an der Entstehung arteriosklerotischer Thromben beim metabolischen Syndrom beteiligt (42). Außerdem könnten erhöhte PAI-I-Plasmaspiegel bei einer chronischen Hepatitis C Infektion zu der Genese von Vaskulitiden bzw. Herz-Kreislauf-Ereignissen beitragen (43). Darüber hinaus können Endothelzellen durch die Freisetzung von prokoagulatorischen Mikropartikeln die Hämostase und Thromboserisiko fördern (44). Tatsächlich konnten wir in unseren Experimenten zeigen, dass die verwendeten Endothelzellen auf die Stimulation mit dsDNA mit einer Hochregulation und Freisetzung der prothrombotischen Moleküle TF und PAI-I reagieren (17). Die funktionelle Relevanz auf die Hämostase wurde in nachfolgenden Experimenten untersucht.

3.2 Blutgerinnung: Hämostase und Thrombose

Die Hämostase konstituiert sich aus zwei Teilvorgängen: der zellulären bzw. primären Hämostase und der plasmatischen bzw. sekundären Hämostase (45, 46). Diese Teilvorgänge laufen parallel und ineinandergreifend durch Aktivierung von Thrombozyten und plasmatischen Gerinnngsfaktoren ab und werden durch verschiedene hemmende bzw. –auflösende Faktoren (Protein C/S, THBD, ATIII, tPA etc.) beeinflusst (47, 48). Die Aktivierung der Thrombozyten erfolgt unter anderem durch endothelialen Von-Willebrand-Faktor, während der *Tissue factor*, wie oben beschrieben, eine entscheidende Rolle in der Aktivierung der plasmatischen Gerinnung innehat. Wir konnten zeigen, dass dsDNA zu einer verstärkten Expression von endothelialem Von-Willebrand-Faktor an der Zelloberfläche führt. Weiterhin konnten wir nachweisen, dass es sowohl unter statischen, als auch unter dynamischen Bedingungen zu einer verstärkten Endothelzell-Thrombozyten-Interaktion nach Stimulation mit dsDNA kommt und funktionell mehr Thrombozyten am Endothel anhaften. Dies könnte durch eine verstärkte Oberflächenexpression des Von-Willbrand-Faktors durch dsDNA vermittelt sein und zu dem Auftreten von thrombotischen Komplikationen bei entsprechenden Pathologien beitragen (17).
Systemische Entzündungsprozesse können mit Veränderungen der Hämostase, insbesondere in Richtung einer Thromboseneigung bzw. verstärkten Blutkoagulation einhergehen (49): bei schweren akuten Entzündungen bzw. Infektionen sind Erkrankungen wie die disseminierte intravasale Koagulopathie (50) oder die thrombotisch-thrombozytopenische Purpura mit thromboembolischen und hämorrhagischen Ereignissen bekannt (51). Auch chronische Entzündungen und Infektionen beeinflussen die Hämostaseaktivität und begünstigen bzw. bedingen die Entstehung von thromboembolischen Ereignissen (52-54). Wir konnten nachweisen, dass die Stimulation mit doppelsträngiger DNA zu einer Hochregulation der prothrombotischen Moleküle *Tissue factor* und PAI-I auf Endothelzellen führt und in einem endothelzellabhängigen Gerinnungsexperiment zu einer beschleunigten Blutgerinnung *in vitro* führt (17).

Die Thrombose ist ein pathologisches Ereignis der intravasalen Bildung eines Blutgerinnsels. Rudolf Virchow hat ihre Entstehung schon im 19. Jahrhundert in drei Grundannahmen zusammengefasst; der Virchow Trias: herabgesetzte Strömungsgeschwindigkeit des Blutes, Gefäßwandveränderungen und Veränderungen der Blutzusammensetzung (55). Arterielle und venöse Thrombose haben in ihrer Entstehung teils verschiedene, teils überlappenden Mechanismen (56). Die arterielle Thrombose entsteht meist durch Gefäßwandveränderung (z. B. im Sinne eines rupturierten arteriosklerotischen Plaques) und durch die Aktivierung von Thrombozyten an freigesetztem subendothelialen Kollagen (57). Demgegenüber ist eine herabgesetzte Strömungsgeschwindigkeit und die Aktivierung der Plasmakoagulation ausschlaggebend bei der Entstehung venöser Thromben (58). Die Zusammensetzung des Blutes mit prokoagulatorischer Folge verändert sich insbesondere auch bei Tumorerkrankungen sowie inflammatorischen Zuständen (59). Die arterielle Thrombose manifestiert sich folgenreich an den Koronarartieren bzw. den hirnversorgenden Gefäßen wo sie zu Ischämien wie Myokardinfarkt und Schlaganfall führen kann (60). Venöse Thrombosen treten überwiegend in den Extremitäten auf und können durch die Embolisation in die Lunge eine lebensgefährliche Komplikation verursachen (61). Hierzu konnten wir

demonstrieren, dass es nach Stimulation mit dsDNA zu einer verstärkten Thrombusbildung in einem *in vivo* Experiment sowohl im arteriellen, als auch im venösen Gefäßsystem und zu einem beschleunigten Verschluss der Gefäße kommt (17).
Eine Verbindung zwischen Thrombose und angeborenem Immunsystem zeigen neuere Forschungsergebnisse, welche die Interaktion aus Blutgerinnung, Thrombozyten und Immunmediatoren als Immunothrombose zusammenfassen. Hierbei führt eine reaktive Thrombosierung, welche bei lokaler Infektion entsteht, zur Reduzierung der Blutzufuhr, welches ein Ausbreiten der Erreger verhindert (62).

3.3 Gefäßveränderung durch akute und chronische Inflammation: Atherosklerose und Vaskulitis

Die Atherosklerose ist eine chronisch entzündliche Systemerkrankung der Arterien deren Folge chronische Herz-Kreislauf-Krankheiten wie pAVK, KHK und Myokardinfarkt, sowie zerebrovaskuläre Erkrankungen sind (63). Ihr Kennzeichen ist die langsam voranschreitende Degeneration der (arteriellen) Gefäße mit einer Verhärtung und Verdickung der Arterienwände durch subendotheliale Akkumulation von Lipiden (64, 65). Neben nicht-modifizierbaren (Alter, Geschlecht, etc.) und beeinflussbaren Krankheitsfaktoren wie Ernährung und risikobehafteter Lebensweise, können auch systemische Entzündungsprozesse verschiedenen Ursprungs in die Pathogenese der Arteriosklerose involviert sein (53). Sowohl sterile Entzündungen bei chronisch verlaufenden Autoimmunerkrankungen, als auch chronische Infektionen, teilweise durch DNA-Viren, scheinen an einer Endotheldysfunktion mit Entzündungsreaktion beteiligt zu sein und begünstigen dabei möglicherweise (frühzeitig) die Entstehung der Atherosklerose (8, 66, 67). Von der chronisch degenerativen Inflammation der Arteriosklerose wird die Vaskulitis als akute Gefäßentzündung pathophysiologisch unterschieden. Beide Entitäten treten unabhängig voneinander auf, jedoch kann in einigen Fällen der Arteriosklerose eine akute Vaskulitis vorangestellt sein (68, 69).

Vaskulitiden, Entzündungen der Gefäße, sind primäre oder sekundäre (auto-) immunologische Erkrankungen, welche durch die Infiltration der Gefäßwand mit aktivierten Leukozyten charakterisiert sind. Dadurch wird eine konsekutive Entzündungsreaktion hervorgerufen (70). Die verschiedenen Subtypen werden anhand der beteiligten Gefäße klassifiziert. Gemein ist allen Formen der Vaskulitis, dass die Entzündung zu einer reaktiven Schädigung der Gefäßwand führen kann, welche durch Einengungen des Lumens oder Blutungen in einer Unterversorgung des nachfolgenden Organs resultiert und Ischämie oder Nekrose als mögliche Folge haben (71, 72). Bei Virusinfektionen treten am häufigsten Polyarteritis nodosa und kryoglobulinämische Vaskulitis auf (71). Da die Pathogenese dieser viralen Vaskulitiden bisher nicht ausreichend verstanden wurden, wollten wir untersuchen, inwiefern Endothelzellen als innerste Schicht des Gefäßsystems in die Krankheitsentstehung involviert sind. Hierzu wurde primär die Frage bearbeitet ob und in welchem Ausmaß humane Endothelzellen Rezeptoren des angeborenen Immunsystems exprimieren und Virus-DNA oder endogene DNA erkennen können. Außerdem sollte untersucht werden ob durch doppelsträngige DNA, sowohl vom Menschen als auch von einem synthetischen dsDNA-Analogon, eine Entzündungsantwort auf den Endothelzellen hervorgerufen werden kann.

3.4 Doppelsträngige DNA

Die DNA ist Bestandteil aller kernhaltigen Zellen des Menschen und liegt im Normal- bzw. Ruhezustand als Doppelstrang (ds) vor (73). Als Träger der Erbinformation hat sie eine elementare genetische Funktion während des Zellzyklus. Darüber hinaus haben doppelsträngige DNA und ihre Bausteine Nukleinsäuren auch in der Pathogenese von Erkrankungen als Stimulatoren des Immunsystems eine zusätzliche Bedeutung (74): tritt DNA außerhalb ihrer üblichen Zellkompartimente auf, kann sie als Signalmolekül wirken, welches Gefahr z. B. durch Zellschädigung vermittelt (4). DNA wird damit den *damage associated molecular pattern* zugerechnet – körpereigene Moleküle die eine nicht-infektiöse Entzündungsantwort auslösen können (4). Tritt die DNA extrazellulär auf, beispielsweise im Kreislaufsystem, kann sie als immunstimulatorisches Signalmolekül wirken (75). In den vorliegenden Studien verwendeten wir einerseits das bewährte synthetische doppelsträngige DNA-Analogen poly(dA:dT), sowie humane genomische doppelsträngige DNA für die Stimulation der Endothelzellen.
DNA kann freigesetzt werden, wenn bei der physiologischen Apoptose, also dem programmierten Zelltod, die anfallenden Zellinhalte nur defizitäre aufgeräumt werden (4). Auch bei einem pathologischen Zelluntergang im Sinne einer Nekrose werden Zellinhalte mitsamt der DNA freigegeben, dies geschieht auch bei Ischämien, Inflammation und Tumorerkrankungen (76). Schließlich kann auch im Rahmen der NETose von neutrophilen Granulozyten dsDNA freigesetzt werden (77). NETose bezeichnet einen Vorgang der neutrophilen Granulozyten bei dem sich die Granula des Zytoplasmas auflösen, die Zelle rupturiert und DNA sowie granulären Proteinen (u. a. nukleäre Histone und antimikrobielle Moleküle) in den extrazellulären Raum übertreten (78, 79). Hierbei aggregiert die chromosomale DNA der neutrophilen Granulozyten zu sogenannten NETs, fadenförmigen Strukturen die sich zu einem dreidimensionalen Netzwerk formieren (15, 80).
Fremde dsDNA kann von Pathogenen während infektiöser Erkrankungen, wie beispielsweise chronischer Hepatitis B-Infektion, einigen Herpesvirus-Infektionen und weiteren Virusinfektionen in das Kreislaufsystem und/oder intrazellulär freigesetzt werden (15, 81).
Wenn die dsDNA durch einen der beschriebenen Mechanismen freigesetzt wurde, kann sie durch Interaktion mit nukleinsäuren-bindenden Proteinen in Zellen bzw. in Zellkompartimente aufgenommen werden (4). In erster Linie findet diese Aufnahme in Immunzellen statt, da diese die größte Dichte von entsprechenden Rezeptoren haben. Die Bindung an diese sog. *Pattern recognition receptors* (PRR) kann eine Aktivierung des angeborenen Immunsystems hervorrufen (82).

3.5 Angeborene Immunität und dsDNA: TLR9, RIG-I und LL-37

Immunzellen sind mit einer großen Anzahl von Signalrezeptoren ausgestattet, welche an pathogene, fremde oder körpereigene Substanzen binden und von diesen aktiviert werden (2). Diese sogenannten *Pattern recognition receptors* (PRR) erkennen Aktivatoren des angeborenen Immunsystems (83). Körperfremde Pathogene werden anhand spezifischer mikrobieller Strukturmotive, den sogenannten *Pathogen associated*

molecular patterns (PAMP) erkannt: z. B. bakterielle Kohlenhydrate (LPS, Mannose), Nukleinsäuren (DNA, RNA), Peptide (Flagellin), Peptidoglykane oder Komponenten der Zellwand (Chitin) etc. (84). Auch köpereigene Moleküle, sogenannte *Danger associated molecular patterns* (DAMP) (z.B. HMGB1, ProteinS100 ATP und RNA/DNA), können durch die PRR erkannt werden und eine inflammatorische Signalkaskade auslösen (16, 85). Wichtige Vertreten der PRR die DNA erkennen sind *Toll-Like-Receptor 9* (TLR9), *Absent in melanoma* (AIM-2), *DNA-dependent activator of IRFs* (DAI) (86), *Retinoic acid inducible gene I* (RIG-I) nach Transformation von DNA in RNA über Polymerase III (2, 87), *Interferon-γ-inducible protein16* (IFI16) (74) und *cGAMP synthase* (cGAS) (88). Diese Rezeptoren werden vornehmlich auf Immunzellen exprimiert und ihre Funktion wurde hier umfangreich charakterisiert. Jedoch können auch andere, dem Immunsystem nicht im engeren Sinne zugeordnete Zellen, diese Nukleinsäure-Rezeptoren exprimieren und damit immunologische Funktionen im Sinne einer unspezifischen Immunantwort übernehmen (89). Die im Folgenden beschriebenen Elemente des Immunsystems wurden in der Arbeit näher untersucht und sollen daher zum Verständnis kurz eingeführt werden.

Toll-like Rezeptoren sind Bestandteile des angeborenen Immunsystems und gehören zu den sogenannten *Pattern recognition receptors* (PRR). Einige Toll-like Rezeptoren befinden sich an der Zelloberfläche, andere intrazellulär (90). Toll-like Rezeptor 9 (TLR9) befindet sich intrazellulär an der Membran von Endosomen bzw. Endolysosomen und erkennt nicht-methylierte CpG DNA-Motive, welche hauptsächlich in Bakterien und Viren und seltener beim Menschen vorkommen (91). Gleichwohl kann TLR9 mit geringerer Affinität auch endogene dsDNA erkennen, sobald diese in das Endolysosom transportiert wurde (2, 92). Die nachgeschalteten Signalkaskaden von TLR9 führen zu einer proinflammatorischen Zytokinproduktion mit Interferonen, Interleukinen und TNFα. Wie vorbeschrieben, wird auch TLR9 als Rezeptor des angeborenen Immunsystems nicht nur in Immunzellen, sondern auch anderen Zellen exprimiert, so auch in Endothelzellen (93). Wir konnten in unserer Arbeit zeigen, dass die verwendeten humanen Endothelzellen TLR9 exprimieren und die proinflammatorische Reaktion des Endothels teilweise TLR9 abhängig war (18). Wir konnten unter anderem eine verstärkte Expression von Interferonen, Interleukinen und TNFα nachweisen.

Auch RIG-I ist ein intrazellulärer, zytosolischer Rezeptor des angeborenen Immunsystems, welcher in der Erkennung von dsRNA eine wichtige Rolle spielt (87). Als Teil der RIG-I like Rezeptoren (MDA5, LGP2) und damit *Pattern recognition receptors* ist er in der spezifischen Erkennung von RNA Viren (beispielsweise Influenza, HCV, Flavivirus) von Bedeutung (3). Durch weitere nachgeschaltete Moleküle wird eine antivirale Inflammationskaskade unter Beteiligung der Transkriptionsfaktoren NFκB und IRF3/7 aktiviert und es werden Zytokine bzw. Interferone produziert (94). Zudem konnte gezeigt werden, dass RIG-I auch dsDNA durch Transkription von dsDNA in dsRNA via RNA-Polymerase III erkennen und eine Interferon-Antwort induzieren kann (87). Außerdem gibt es zunehmend Beweise, dass durch RIG-I auch DNA-Viren wie EBV, Adenovirus, HSV-1 und ggf. auch HBV erkannt werden (95).
Ein Einfluss von RIG-I auf die endotheliale Gerinnung konnte durch die Modulation von pro- und antithrombotischen Proteinen TF, tPA und PAI-1 gezeigt werden (96, 97). Die Ergebnisse unserer Arbeiten zeigen, dass dsDNA zu einer Hochregulation dieses Rezeptors führt (17). Weiterhin konnten wir zeigen, dass nach knock-down von RIG-I mittels siRNA der prothrombotische Effekt in unserem von Endothelzellen abhängigen Gerinnungs-Assay teilweise reversibel war.

Um die Frage zu bearbeiten inwiefern die DNA in die Endothelzellen transportiert werden könnte, beschäftigten wir uns in der Ausgangsstudie mit dem Cathelicidin LL-37. Cathelicidine sind antimikrobielle Peptide die eine relevante Komponente des angeborenen Immunsystems bilden – beim Menschen ist LL-37 bekannt (98).
LL-37 findet sich in sekundären Granula von neutrophilen Granulozyten und Epithelzellen (99). Diese können bei Entzündung oder Aktivierung LL-37 freisetzen, unter anderem durch *neutrophil extracellular traps* (NETs) (100). Diese sind extrazelluläre Fasern aus der nukleären DNA neutrophiler Granulozyten welche Pathogene binden und durch verschiedene antimikrobielle Proteine und Enzyme unschädlich machen (101).
LL-37 kann an dsDNA binden (102). Zusätzlich wurde gezeigt, dass LL-37 extrazelluläre DNA im Zusammenhang mit Autoimmunerkrankungen in endosomale Kompartimente von plasmazytoide dendritischen Zellen und Monozyten transportieren kann und damit möglicherweise als Transporterprotein für dsDNA in das intrazelluläre Kompartiment dient (103, 104). Bei Patienten mit antineutrophilen cytoplasmatischen Antikörpern (ANCA)- assoziierten Vaskulitiden, sowie bei Hepatitis B-Virus (HBV) und Hepatitis C-Virus (HCV) Infektionen wurden erhöhte Serumspiegel von LL-37 gemessen (105, 106). Wir konnten zeigen, dass LL-37 zu einer Internalisierung der DNA in die Endothelzellen führt; jedoch weiterhin die proinflammatorische Antwort abschwächt, sodass hier die Ergebnisse möglicherweise auf einen protektiven Effekt hindeuten.

Zusammenfassend konnten wir mit beiden Forschungsarbeiten zeigen, dass sowohl virale, als auch genomische doppelsträngige DNA einen direkten pathophysiologischen Einfluss auf das vaskuläre Endothel ausübt und einen proinflammatorischen und prothrombotischen Zustand begünstigt. Wir konnten darlegen, dass Endothelzellen Komponenten der Immunreaktion produzieren und exprimieren, welche an dieser Reaktion beteiligt sein könnten. Außerdem ergaben sich Hinweise auf einen möglichen Aufnahmemechanismus der DNA in die Endothelzellen.
Diese Ergebnisse stehen im Einklang mit den zunehmenden Erkenntnissen, dass Zellen die nicht primär dem Immunsystem zugerechnet werden, wie die Endothelzellen, vielfältige immunologische Funktionen innehaben und eine wichtige Rolle in der Erkennung und Bekämpfung von exogenen und endogenen molekularen Strukturen (PAMP, DAMP) spielen. Diese Wechselbeziehung zwischen inflammatorischen Erkrankungen, angeborenem Immunsystem und thromboembolischen Ereignissen zeigt eine neue Verbindung zwischen Inflammation und Gefäßsystem auf. Diese könnte relevant für die Prävention und Therapie von Patienten mit kardiovaskulären Komplikationen bei chronischen Infektions- und Autoimmunerkrankungen von Bedeutung sein.

3.6 Beiträge des Doktoranden zu den Publikationen

Beitrag des Doktoranden zu Gaitzsch et al. (geteilte Erstautorschaft):

Joachim Pircher und Markus Wörnle konzipierten die Arbeit. Erik Gaitzsch und Joachim Pircher haben gemeinsam das Manuskript geschrieben. Erik Gaitzsch und Joachim Pircher planten und führten die meisten Experimente durch, analysierten die Ergebnisse und vollzogen die Datenauswertung. Der Doktorand Erik Gaitzsch war dabei vor allem für die *in vitro* Methoden zuständig, kultivierte und isolierte die Endothelzellen, führte die verschiedenen Stimulationen durch und erstellte die molekular- und proteinbiologischen Experimente. Thomas Czermak und Joachim Pircher führten die *in vivo* Experimente durch. Joachim Pircher und Markus Wörnle betreuten und unterstützen das Projekt.

Beitrag des Doktoranden zu Merkle et al. (Ko-Autorenschaft):

Monika Merkle und Markus Wörnle konzipierten die Arbeit und schrieben das Manuskript. Erik Gaitzsch führte einen Teil der *in vitro* Experimente in Endothelzellen durch (Zellkultur, Zell-Transfektion, FACS, Western blot, Immunfluoreszenz).

4.1 ZUSAMMENFASSUNG

Doppelsträngige DNA (dsDNA) induziert einen proinflammatorischen und prothrombotischen Phänotyp am vaskulären Endothel.
In den vorliegenden Arbeiten wurde ein direkter proinflammatorischer und prothrombotischer Effekt von dsDNA auf menschliche Endothelzellen untersucht und die funktionellen Auswirkungen auf Entzündungsantwort und Gerinnungsanpassung analysiert.

DsDNA kann außerhalb von Zellen ein Gefahrensignal und Aktivator des angeborenen Immunsystems sein (2). Sowohl bei Erkrankungen mit infektiöser als auch steriler Inflammation kann dsDNA im Blut vorhanden und nachweisbar sein (4). Bei viralen Infektionen, Autoimmunerkrankungen oder Gewebeschädigung kann sie durch das Binden an intrazelluläre *Pattern recognition receptors* (PRR) eine immunologische Antwort hervorrufen (3, 6).
Während die Effekte von dsDNA auf Immunzellen in den letzten Jahren umfassend untersucht wurden, bleiben die Auswirkungen und ihre pathophysiologische Bedeutung für Zellen, welche nicht primär dem Immunsystem zugeordnet werden, weitgehend unbeleuchtet.

DsDNA spielt eine zentrale Rolle bei viralen Infektionen mit DNA-Viren, wie z.B. Hepatitis B-Infektionen (6). Diese Erkrankungen sind mit einem verstärkten Vorkommen von dsDNA im Kreislaufsystem verbunden und können sich als Systemerkrankung, mit verschiedenen Organbeteiligungen manifestieren – einschließlich des Gefäßsystems (7). Im Krankheitsverlauf kann es zu lebensbedrohlichen Komplikationen kommen, unter anderem hervorgerufen durch Vaskulitiden, aber auch durch thromboembolische Komplikationen wie Schlaganfall und Herzinfarkt (8, 10). Darüber hinaus gibt es Hinweise, dass virale Erreger bzw. Infektionen eine Rolle in der Pathogenese der Atherosklerose spielen (12).
Diese Dissertation behandelt die Frage, inwiefern dsDNA proinflammatorische und prothrombotische Effekte auf das vaskuläre Endothel ausübt und welche Rezeptoren und Mechanismen des angeborenen Immunsystems dabei eine eigenständige Rolle spielen könnten.

Hierzu wurden Endothelzellen *in vitro* mit dem synthetischen DNA-Analogon poly(dA:dT) und genomischer DNA des Menschen (huDNA) transfiziert. Die Experimente wurden mit humanen mikrovaskulären Endothelzellen (HMEC), einer immortalisierten Zelllinie, durchgeführt. Zusätzlich wurden einige Schlüsselversuche in primären, selbstisolierten Endothelzellen aus humanen Umbilikalvenen (HUVEC) durchgeführt. Es wurden verschiedene molekularbiologische und proteinbiochemische Methoden wie RT-PCR, Western blot, ELISA, FACS, Immunfluoreszenzmikroskopie und Knockdown-Experimente mittels siRNA durchgeführt. Ein Krankheitsmodell wurde mit HBV-DNA enthaltenden Immunopräzipitaten von einem mit Polyarteritis nodosa erkrankten Patienten simuliert um herauszufinden ob virale Immunkomplexe und die darin enthaltene DNA eine ähnliche Wirkung wie die synthetische dsDNA auf das Endothel haben. Um die funktionelle Rolle von dsDNA im Zusammenhang mit einer Gerinnungsneigung genauer zu beleuchten erfolgten *in vivo* Experimente an Gefäßen des M. cremaster von Mäusen.

In der ersten der vorliegenden Arbeiten (18) analysierten wir zunächst die Initiierung einer Entzündungsantwort der Endothelzellen. Wir konnten nachweisen, dass die Stimulation von humanen Endothelzellen mit poly(dA:dT) und genomischer DNA zu einer signifikanten Induktion von proinflammatorischen Zytokinen, Chemokinen und Adhäsionsmolekülen führt. Um einen Sensor zu isolieren, welcher für die Effekte verantwortlich sein könnte, untersuchten wir verschiedene Rezeptoren des Immunsystems, sogenannte *Pattern recognition receptors* (PRR) als mögliche Zielstruktur der dsDNA. Wir konnten dabei zeigen, dass Endothelzellen Rezeptoren des angeborenen Immunsystems exprimieren. Unsere Daten deuten darauf hin, dass der intrazelluläre Rezeptor TLR9 eine Schlüsselrolle in der endothelialen Immunaktivierung spielen könnte. Zusätzlich analysierten wir welcher Vorgang bei der Aufnahme von endogener DNA in Endothelzellen eine Rolle spielen könnte. dsDNA, gleich welcher Herkunft, ruft nur eine Entzündungsantwort hervor, wenn sie mit kationischen Lipiden (Lipofectamine) in die Endothelzellen transfiziert wurde. Die Transfektion war nötig um die Internalisierung der DNA in die Endothelzellen zu gewährleisten und den intrazellulären TLR9-Rezeptor aktivieren zu können. Als mögliches physiologisches Transportprotein zeigte sich das humane Cathelicidin LL-37. Wir konnten belegen, dass LL-37 an dsDNA bindet und eine Internalisierung von dsDNA nach gemeinsamer Stimulation mit LL-37 in Endothelzellen nachweisen.
Diese Ergebnisse zeigen, dass das vaskuläre Endothel intrazelluläre dsDNA wahrnehmen kann und dies eine Entzündungsantwort hervorruft. Diese Resultate könnten ein Hinweis auf die Entstehung und das Fortschreiten von Vaskulitiden bei viralen Infektionen und bei Autoimmunerkrankungen sein (18).

Im Rahmen der zweiten Arbeit (17) untersuchten wir prothrombotische Effekte der dsDNA am vaskulären Endothel. Die Transfektion des synthetischen dsDNA Analogons poly(dA:dT) in humane Endothelzellen führte zu einer Translokation der Transkriptionsfaktoren NFκB und IRF3 in den Zellkern im Sinne einer inflammatorischen Aktivierung. Des Weiteren führte dsDNA zur einer Hochregulation und Expression der prothrombotischen Moleküle *Tissue factor* und PAI-1. Dieser Effekt konnte mit viraler DNA aus Immunpräzipitaten eines Patienten mit Hepatitis B assoziierter Polyarteriitis nodosa reproduziert werden. PAI-I wurde durch poly(dA:dT) außerdem verstärkt von Endothelzellen in die Umgebung freigesetzt. Auch konnte eine kompensatorische Produktion von tPA beobachtet werden. Darüber hinaus ergab die Transfektion von dsDNA in Endothelzellen eine beschleunigte Initiierung der Blutgerinnung *in vitro*. Wir zeigten dies in einem endothelzellabhängigen Gerinnungsassay in Vollblut. Die prothrombotischen Effekte wurden nach Transfektion von poly(dA:dT) in Endothelzellen, genauso wie nach Transfektion von Hepatitis B Virus- DNA und humaner genomischer DNA nachgewiesen. Die Ergebnisse waren teilweise von dem intrazellulären Rezeptor RIG-I abhängig, welches in einem Knockdown-Experiment mittels siRNA nachgewiesen werden konnte. Zusätzlich führte dsDNA zur Oberflächenexpression von Von-Willebrand-Faktor, welches eine erhöhte Thrombozyten-Endothelinteraktion unter statischen Bedingungen und in einem Flusskammerassay unter Fluss zur Folge hatte.
Schließlich überführten wir die Ergebnisse in ein *in vivo* Modell um die prothrombotischen Effekte zu überprüfen. Die intraskrotale Injektion von dsDNA resultierte in einer beschleunigten Thrombusformation nach light/dye-induzierte Endothelschädigung in Arteriolen und Venolen des M. cremaster von Mäusen (17).

Zusammenfassend haben beide Studien zeigen können, dass virale und/oder endogene dsDNA einen proinflammatorischen und prothrombotischen Zustand am Gefäßendothel begünstigen und dass *Pattern recognition receptors* (PRR) des angeborenen Immunsystems in diesen Prozess der wechselseitigen Interaktion involviert zu sein scheinen. Diese Ergebnisse zeigen eine neue Verbindung zwischen endothelialer Inflammation und Gerinnungsaktivität auf, und könnten zu dem Verständnis der Entstehung von Vaskulitis und Thrombose unter dem Einfluss von dsDNA beitragen.

4.2 SUMMARY

Double stranded DNA induces a proinflammatory and prothrombotic phenotype in the human vascular endothelium.
In the work presented here, a direct proinflammatory and prothrombotic effect of dsDNA in human endothelial cells was shown and the functional implications on inflammation and coagulation were further analyzed.

Outside of the nucleus, double stranded DNA functions as a danger signal and is also a powerful activator of the innate immune system (2). DsDNA can appear in the bloodstream under conditions of infectious as well as sterile inflammation (4). Upon viral infections, autoimmune diseases or sterile tissue damage, dsDNA elicits an immunological response by binding to intracellular pattern recognition receptors (PRR) (3). The mentioned pathologies are associated with an increased occurrence of dsDNA in the circulatory system and higher cardiovascular risk (8, 10).
The impacts of dsDNA on immune cells have been broadly investigated. In contrast to this, fewer studies have examined the pathophysiological effects of dsDNA signaling on non-immune cells, such as the vascular endothelium.

DsDNA plays an important role in virus infections such as Hepatitis B, which frequently manifest as systemic disorders involving several organs and the vascular system (6). Morbidity and mortality can be attributed to vasculitis or thrombotic complications including fatal thromboembolic events, such as myocardial infarction and ischemic stroke (8, 10). Furthermore, virus infections are considered to contribute to the pathophysiology of atherosclerosis (12).
This thesis addresses the question of how double stranded DNA exerts proinflammatory and prothrombotic events on the vascular endothelium and which receptors and mechanisms of innate immune system might be involved.

Hence, *in vitro* endothelial cells were transfected with the synthetic DNA analog poly(dA:dT) and human genomic DNA (huDNA). The experiments were conducted in human microvascular endothelial cells (HMEC), an immortalized cell line. Additionally, key experiments were performed in primary endothelial cells from human umbilical veins (HUVEC), which were self-isolated. Different molecular and protein analysis methods such as RT-PCR, western blot, ELISA, FACS immunofluorescent microscopy and knockdown experiments via siRNA were applied. A disease model was developed using HBV-DNA containing immunoprecipitates from a patient diagnosed with polyarteriitis nodosa. To examine the functional role of dsDNA in the context of thrombosis, we performed *in vivo* experiments in vessels of the M. cremaster of mice.

In our first study (18), we analyzed the activation of known receptors of the innate immune system when inducing a proinflammatory response of endothelial cells (induced via what?). We were able to show that the stimulation of human endothelial cells with both poly(dA:dT) and human genomic DNA led to a significant induction of proinflammatory cytokines, chemokines and adhesion molecules. To isolate a sensor responsible for this response we analyzed different possible pattern recognition receptors of the immune system and could show that endothelial cells express innate immune receptors. Besides other possible candidates, our findings suggest TLR9 is a key player in this endothelial immune- activation.
Additionally, we investigated the mechanisms that might be responsible for the uptake of endogenous DNA into endothelial cells. We found that DNA, regardless of its origin, was able to exert its proinflammatory effects only if DNA nucleotides were transfected

with cationic lipids (lipofectamine). Transfection was necessary for the internalization of DNA into the endothelial cells in vitro and to activate the intracellular TLR9 receptor. Human cathelidin LL-37, appears to be a possible transporter for cellular uptake: we were able to show that LL-37 binds dsDNA and also to detect an internalization in endothelial cells after co-stimulation of dsDNA and LL-37.
In summary, these results show that the vascular endothelium is able to sense dsDNA and that dsDNA can cause an inflammatory response. These findings show a contributing pathway in development and progression of vasculitis related to viral infections or autoimmune diseases (18).

In the/ our other study, we analyzed the prothrombotic effects of double stranded DNA onto the vascular endothelium (17). As established in the previous study, dsDNA is an effective activator of the innate immune system because of its ability to bind intracellular pattern recognition receptors (e.g. TLR9) (during virus infections or sterile tissue damage). Transfection of cultured human endothelial cells with synthetic dsDNA led to nuclear translocation of transcription factors NFκB and IRF3 in an inflammatory reaction. Poly(dA:dT) induced the up- regulation of the prothrombotic molecules tissue factor and PAI-1. This result were reproduced with HBV-containing immunoprecipitates from the previously mentioned patient. Endothelial cells released increased amounts of PAI-I after stimulation with poly(dA:dT). Furthermore, transfection of dsDNA in endothelial cells resulted in accelerated blood clotting *in vitro*, which was shown in an endothelial cell dependent clotting assay in whole blood. These results where partly dependent on RIG-I signalling, as shown in a siRNA knock-down experiment. DsDNA also induced surface expression of von-Willebrand-factor, leading to increased platelet-endothelium-interactions in static condition and under flow in a flow chamber assay. Finally, the intra- scrotal application of dsDNA resulted in a faster thrombus formation upon light/dye-induced endothelial lesions in arterioles and venules of the M. cremaster of mice *in vivo* (17).

In conclusion, both studies could show that virus and endogenous dsDNA induce a proinflammatory and prothrombotic phenotype in the vascular endothelium. Pattern recognition receptors of the innate immune system seem to be involved in this response and its interactions. These results show a new connection between endothelial inflammation and coagulation activity, and might further contribute to the understanding of the development of vasculitis and thrombosis under the influence of dsDNA.

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LL37 inhibits the inflammatory endothelial response induced by viral or endogenous DNA

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LL37 inhibits the inflammatory endothelial response induced by viral or endogenous DNA

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ABSTRACT

In viral infection, morbidity and mortality often result from extrahepatic disease manifestations such as vasculitis. We hereby show that human microvascular endothelial cells express viral receptors of the innate immune system which are induced upon ligand engagement. Furthermore, stimulation of endothelial cells with the synthetic analog of viral DNA, poly (dA:dT), human DNA and hepatitis B virus-containing immunoprecipitates from a patient with polyarteritis nodosa induces an inflammatory response including the upregulation of adhesion molecules, which is mediated exclusively by TLR9 and involves an IRF3-dependent pathway. Thus, endothelial cells are able to actively participate in immune mediated vascular inflammation caused by viral infections. Furthermore, we provide evidence for the ability of LL37 to bind and internalize viral or endogenous DNA into non-immune cells. DNA nucleotides internalized by LL37 suppress the production of proinflammatory mediators suggesting a protective effect against direct responses to viral infection or circulating DNA-fragments of endogenous origin.

1. Introduction

Critical complications of viral infections often result from systemic disease manifestations such as vasculitis. The typical forms of viral disease associated vasculitis are polyarteritis nodosa and cryoglobulinemic vasculitis, which may occur in the course of an infection with the hepatitis B virus (HBV) respectively the hepatitis C virus (HCV) [1,2], although polyarteritis nodosa remains idiopathic in most cases. Both usually entail severe organ damages in the depending areas of supply of the involved blood vessels. Human immunodeficiency virus and parvovirus B 19 are also known to cause vasculitis. Even though several viruses are able to directly infect and replicate in endothelial cells [3–9], human microvascular endothelial cells do not classify as typical target cells in viral infection and the pathomechanism of viral-disease associated vasculitis is not well understood. We therefore hypothesized that viral receptors of the innate immune system, which recognize highly conserved pathogen associated molecular patterns and play a crucial role in the early response to viral infections [10], are expressed in endothelial cells and are able to mediate proinflammatory responses upon binding to nucleic acid fragments of viral origin.

Another disease entity characterized by the occurrence of vasculitis are autoimmune diseases including Systemic lupus erythematodes (SLE), which is equally mediated by DNA-fragments, but of endogenous origin [11]. Moreover, the onset or progression of several autoimmune disorders is triggered by viral infections.

Indeed, DNA has been shown to profoundly stimulate the immune response once internalized into cells [12]. The toll-like receptor 9 (TLR9) specifically recognizes non-methylated CpG sequences which are suppressed in human DNA. Nucleic acids not compartmentalized in endolysosomes can be recognized by DNA-dependent activator of IFN-regulatory factors (DAI) or inflammasomes as NACHT, LRR and pyrin-domain-containing protein 3 (NALP3) [12–14]. We show that human microvascular endothelial cells express viral receptors of the innate immune system and that ligand binding by viral or endogenous DNA induces an endothelial inflammatory response.

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To address the question how circulating DNA can be internalized into endothelial cells, we investigated the effects of another component of the innate immune system, the antimicrobial peptide human cathelicidin (LL37). LL37 is found in neutrophil extracellular traps (NETs) triggered by a variety of stimuli including microorganisms, proinflammatory mediators [15] and, very importantly, activated endothelial cells [16]. LL37 has been shown to transport extracellular DNA into endosomal compartments of plasmocytoid dendritic cells and monocytes in the course of autoimmune disease [17,18]. Significantly elevated serum levels of LL37 have been observed in patients affected by antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis [19], and in HBV and HCV infection [20].

2. Materials and methods

2.1. Cell culture of human HMEC and HUVEC

Human microvascular endothelial cells (HMEC) were provided by Ades et al. [21]. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously [22]. The procedure was approved by the university ethics review board. HMEC and HUVEC were cultured in M199 media supplemented with 10% fetal calf serum (FCS), 10% endothelial growth media (PromoCell, Germany) and 1% penicillin/streptomycin. For stimulation experiments, poly (dA:dT) or human genomic DNA was dissolved in cationic lipids (lipofectamine) or LL37 (10 μg/ml) (Innovagen, Sweden) and added to the cells as indicated; control samples were treated with lipofectamin alone. TLR9 inhibitor (ODN TTAGGG) (10 μg/ml) and negative control for ODN TTAGGG (10 μg/ml) were from InvivoGen (USA). For stimulation experiments with supernatants HMEC were transfected with cationic lipids and stimulated without or with poly (dA:dT) (5 μg/ml) or human genomic DNA (5 μg/ml), HMEC were washed with phosphate-buffered saline (PBS) and fresh medium was added for 4 h. The conditioned medium was added to untreated HMEC and stimulation experiments were performed for indicated time intervals. For each stimulation experiment controls were performed in parallel using culture medium alone.

2.2. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

RT-PCR analysis was done as described [23]. RT-PCR was performed on a Taqman ABI 7700 sequence detection system (PE Applied Biosystems, Germany). rRNA was used as reference gene. Taqman assay reagents or primers and probe were purchased from Applied Biosystems. Taqman gene expression assays or primers and probes are shown in Table 1.

2.3. FACS analysis

FACS analysis was performed as described [23]. Intracellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) was measured on the cell surface. Anti-ICAM-1 and anti-VCAM-1 antibody and respective negative controls were from BD Pharmingen (Germany).

2.4. Western blot

Western blot analysis was performed as previously described [24]. The following specific antibodies were used: ICAM-1 (Santa Cruz, USA); VCAM-1 (Cell Signaling, USA); phosphorylated interferon regulatory factor 3 (pIRF3) (Abcam, UK), phosphorylated TANK-binding kinase 1 (pTBK1) (Cell Signaling, USA), inhibitor of κBα (IκBα) (Cell Signaling, USA), phosphorylated protein kinase, strain AK, Thymoma (pAKT) (Cell Signaling, USA), TLR9 (Cell Signaling, USA), p38 (Cell Signaling, USA), TNF receptor-associated factor 6 (TRAF6) (Cell Signaling, USA).

2.5. ELISA

ELISA for interleukin 6 (IL-6), interleukin 8 (IL-8) and interleukin 1β (IL-1β) were performed on cell culture supernatants using commercial assay kits (Quantikine®, USA) and following providers instructions.

2.6. Knockdown of gene expression with short interfering RNA (siRNA)

Predesigned small interfering RNAs (siRNAs) specific for TLR9, absent in melanoma 2 (AIM2), DAI, NALP3, Toll-like receptor 3 (TLR3), retinoic acid inducible gene I (RIG-I) and melanoma differentiation antigen 5 (MDA5) were purchased from Ambion (Japan) [25]. Scrambled siRNA was used as the nonspecific negative control of siRNA.

2.7. Immunofluorescence experiments

For immunofluorescence experiments, HMEC were grown to confluence in 8-well microscope slides and stained as previously described [26]. After a 6 h treatment with poly (dA:dT) or human genomic DNA alone or transfected with lipofectamine or LL37, cells were washed with PBS followed by fixation and permeabilization in 100% methanol for 30 min. Fixed cells were washed again with PBS and blocked with 5% BSA in PBS for 1 h at room temperature. Cells were then incubated with first antibody (diluted 1:200 in blocking solution) for one hour at room temperature. The following first antibodies were used: rabbit IRF3 antibody (Cell Signaling Technology, USA), rabbit actin antibody (Santa Cruz Biotechnology, Germany), goat lamin A/C antibody (Santa Cruz Biotechnology, Germany). Afterwards, cells were washed three times with PBS and subsequently incubated with the secondary antibody (diluted 1:400 in blocking solution) and with DAPI (Invitrogen, UK) to visualize nuclei, for 30 min. The following secondary antibodies were used: Alexa Fluor 488 Chicken Anti-Rabbit IgG (Invitrogen, UK), Alexa Fluor 546 Donkey Anti-goat IgG (Invitrogen, UK).

2.8. Preparation of HBV-DNA

HBV-DNA containing immunoprecipitates were isolated from a patient with a HBV-associated polyarteritis nodosa with a high viral load during routine plasmapheresis treatment as described previously [25]. The concentration of HBV used for stimulation was 2.1×10^6geq/ml, confirmed by RT-PCR. For stimulation experiments, confluent HMEC in 6-well plates were used; once the virus was added, the plates were centrifuged at 1000 g for 45 min to allow efficient viral infection. Subsequent stimulation was performed as indicated. Written informed consent for the collection of plasma samples was obtained. The procedure was approved by the university ethics review board (Ethikkommission der Medizinischen Fakultät der Ludwig-Maximilians-Universität).

2.9. MTT proliferation assay

To assess the proliferative activity of HMEC, MTT assays were performed as described [27].

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Table 1
Taqman gene expression assays or primers and probes.

Gene	Gene name	Accession number	Assay ID	Context Seq AoD	Forward primer sequence 5′-3′	Reverse primer sequence 5′-3′	Probe sequence-5′-3′
TLR9	Toll-like receptor 9	NM_017442			CGGAGGCTGGATGTCAGC	GGAGTGGTCCACTGTCTTGAGG	AGCTGCGAGAGCTCAACCTTAGCGC
AIM2	Absent in melanoma 2	NM_004833.1	Hs00175457_m1	GATCAGGAGGCTGATCCCAAAGTTGTCA			
DAI	DNA-dependent activator of IFN-regulatory factors	NM_030776.2 NM_001160418.1 NM_001160419.2	Hs00229199_m1	ATTTACCGCCCAGAAGATTCTGGAA			
NALP3	NACHT, LRR and pyrin-domain-containing protein 3	NM_183395.2 NM_001079821.2 NM_001127462.2 NM_001127461.2	Hs00918082_m1	GAGCCGAAGTGGGGTTCAGATAATG			
TLR3	Toll-like receptor 3	NM_003265.2	Hs00152933_m1	TAGCAGTCATCCAACAGAATCATGAGACAG			
RIG-I	Retinoic acid-inducible gene 1	NM_014314.3	Hs00204833_m1	CCCTAGACCATGCAGGTTATTCTGGACTTT			
MDA5	Melanoma differentiation-associated protein 5	NM_022168.2	Hs01070332_m1	GCAGATGCAACCAGAGAAGATCCAT			
IL-6	Interleukin 6	NM_000600.2	Hs00174131_m1	TGGATTCAATGAGGAGACTTGCCTGGTGAA			
IL-8	Interleukin 8	NM_000584.2	Hs00174103_m1	GCTCTGTGTGAAGGTGCAGTTTTGCCAAG			
MCP-1	Monocyte chemotactic protein 1	NM_002982.3	Hs00234140_m1	TCAGCCAGATGCAATCAATGCCCCA			
RANTES	Regulated on activation, normal T cell expressed and secreted	NM_002985.2	Hs00174575_m1	TCCAACCCAGCAGTCGTCTTTGTCACCCG			
IP-10	Interferon gamma-induced protein 10	NM_001565.2	Hs00171042_m1	GTGGCATTCAAGGAGTACCTCTCTCTAGAAC			
IFN-α	Interferon alpha	NM_024013.2			CCTTCCTCCTGTCTGATGGA	ACTGGTTGCCATCAAACTCC	CAGACATGACTTTGGATTTCCCCAG
IFN-β	Interferon beta	NM_002176.2	Hs00277188_s1	TACCTCCGAAACTGAAGATCTCCTAGCCT			
ICAM-1	Intercellular adhesion molecule 1	NM_000201	Hs00164932_m1	TCACCGTGTACTGGACTCCAGAACG			
VCAM-1	Vascular cell adhesion molecule 1	NM_080682.1 NM_001078.2	Hs00365486_m1	GTTCAAGGAAGAGAAAACAACAAAGA			
CASP-1	Caspase 1	NM_001223.4 NM_001257118.1 NM_001127462.2 NM_001127461.2 NM_001243133.1 NM_004895.4	Hs00354836_m1	CCGCAAGGTTCGATTTTCATTTGAG			
IL-1β	Interleukin 1 beta	NM_000576.2	Hs00174097_m1	TGGAGCAACAAGTGGTGTTCTCCATG			
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	NM_002046.3	4310884E				
18S rRNA	18S ribosomal RNA	X03205.1	4310893E				

2.10. Statistical analysis

Values are provided as mean ± SD. Statistical analysis was performed by the ANOVA-analysis. Significant differences in expression levels are indicated for p values < 0.05 (*) or 0.01 (**), respectively.

3. Results

3.1. Effect of viral and human DNA on the expression of innate DNA and RNA recognition receptors

To test whether stimulation of HMEC with poly (dA:dT), a synthetic analog of viral DNA, influences the expression of innate viral nucleotide recognition receptors, HMEC were transfected with cationic lipids and stimulated without or with poly (dA:dT) (5 μg/ml) for different time intervals (3, 6, 9, 12, 24, 48 h). As it is likely that several links between viral DNA and RNA recognition systems exist [14] expression of both viral DNA and RNA recognition receptors was analyzed by RT-PCR (Fig. 1A, C–H). Basal expression levels of TLR9 were significantly increased by poly (dA:dT) from 9 to 48 h and expression of NALP3, TLR3, RIG-I and MDA5 was increased from 6 to 48 h. Basal expression of AIM2 and DAI was not detectable but was increased by poly (dA:dT) from 6 to 48 h stimulation time. In HMEC stimulated with poly (dA:dT) but not transfected with lipofectamine, expression of viral receptors remained unchanged (data not shown). A robust endothelial protein synthesis of TLR9 under basal and poly (dA:dT)-treated conditions for 12 and 24 h after transfection with lipofectamin was demonstrated by western blot (Fig. 1B). When HMEC were transfected with lipofectamin and stimulated without or with human genomic DNA (huDNA) (5 μg/ml) for different time intervals (12, 24 h), expression of TLR9 was increased after a stimulation time of 24 h and expression of AIM2, DAI, NALP3, TLR3, RIG-I and MDA5 after a stimulation time of 12 and 24 h (Fig. 1I–O).

3.2. Effect of viral and human DNA on the expression of cytokines and chemokines

Next we tested the effect of viral DNA on the endothelial expression of selected antiviral respectively proinflammatory chemokines and cytokines. HMEC were transfected with cationic lipids and stimulated without or with poly (dA:dT) (5 μg/ml) for different time intervals. Expression levels of IL-6, IL-8 and regulated on activation, normal T cell expressed and secreted (RANTES) were significantly enhanced over the entire stimulation time. Expression of monocyte chemoattractant protein-1 (MCP-1) was significantly increased by poly (dA:dT) from 6 to 24 h (Fig. 2A–D). In HMEC

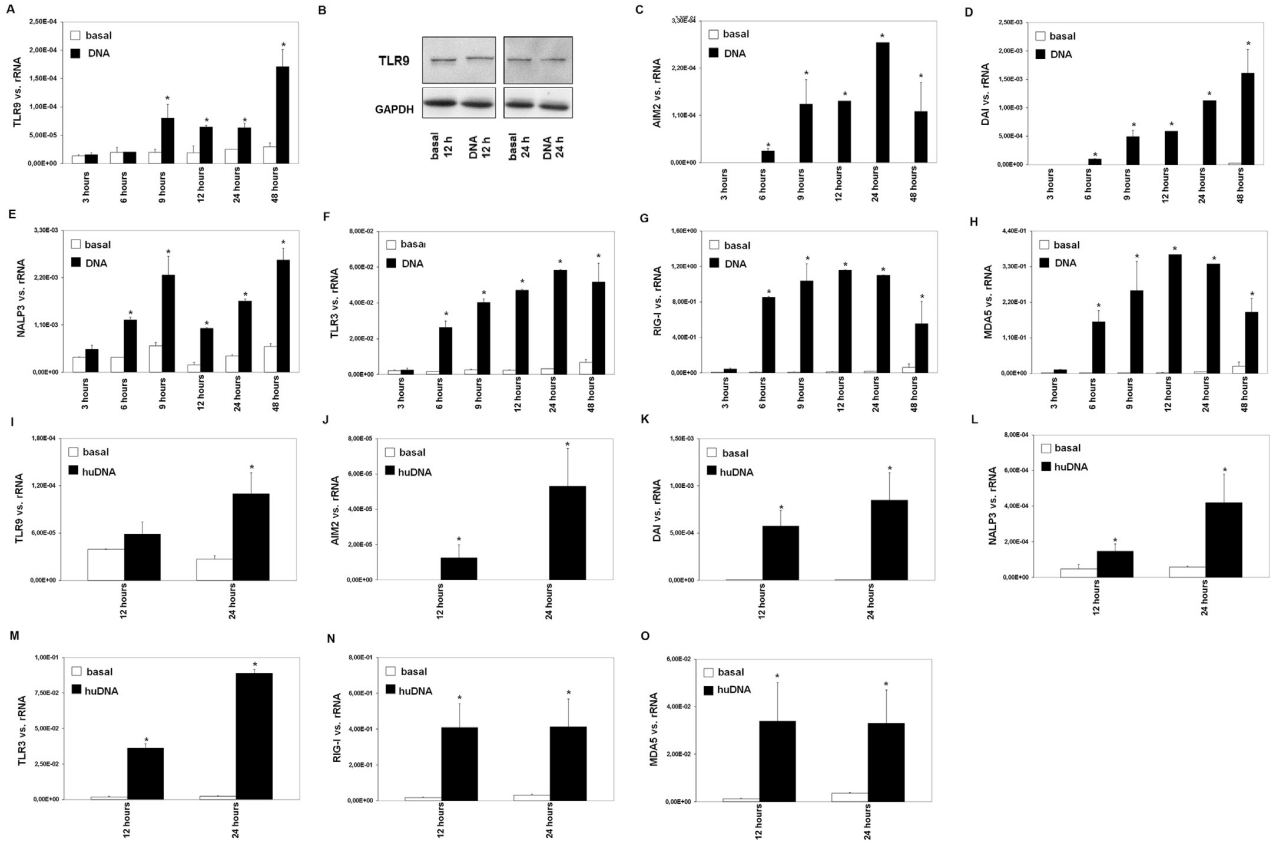

Fig. 1. Effect of viral and human DNA on the expression of innate DNA and RNA recognition receptors. HMEC were transfected with cationic lipids and stimulated without (basal) or with poly (dA:dT) (DNA) (5 μg/ml) for different time intervals (3, 6, 9, 12, 24, 48 h) and expression of TLR9 (A), AIM2 (C), DAI (D), NALP3 (E), TLR3 (F), RIG-I (G) and MDA5 (H) was analyzed by RT-PCR or stimulated without (basal) or with human genomic DNA (huDNA) (5 μg/ml) for different time intervals (12, 24 h) and expression of TLR9 (I), AIM2 (J), DAI (K), NALP3 (L), TLR3 (M), RIG-I (N) and MDA5 (O) was analyzed by RT-PCR. Results are given as means ± SEM of two experiments done in parallel for each condition and rRNA served as the reference gene. Comparable results were obtained in two series of independent experiments. (B) Protein synthesis of TLR9 was analyzed by western blot after transfection with cationic lipids under basal conditions or after stimulation with poly (dA:dT) (DNA) (5 μg/ml) for 12 and 24 h. Results shown are from a representative experiment performed in three series of independent experiments.

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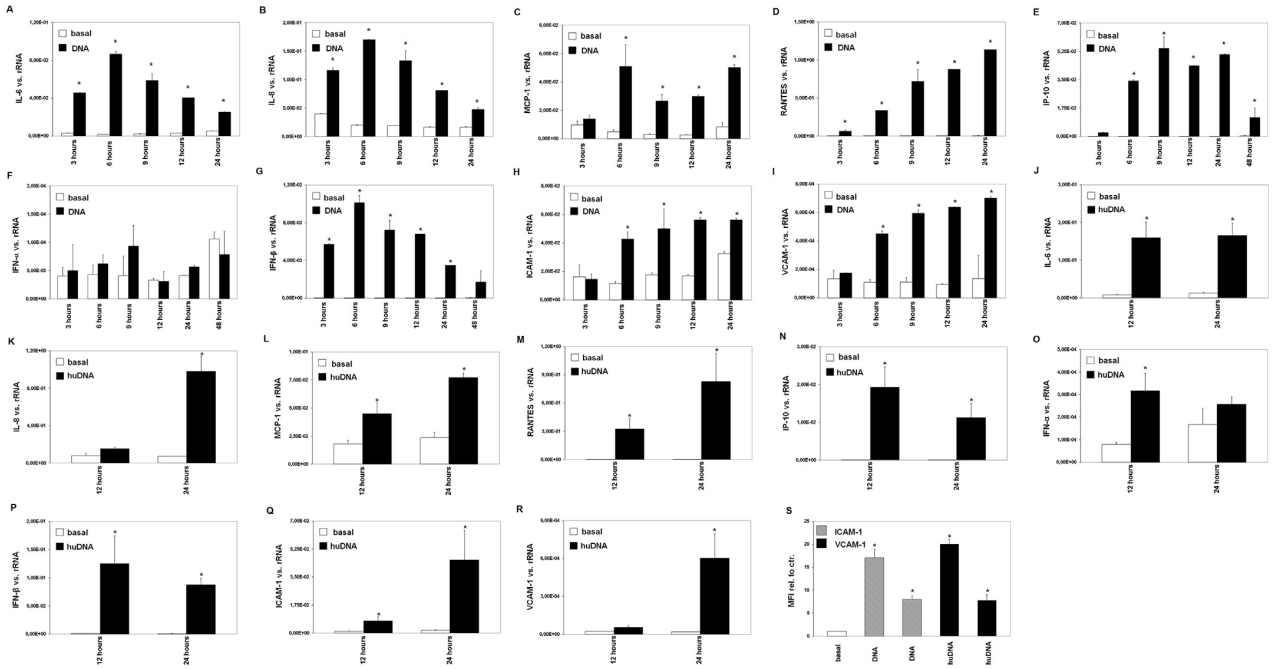

Fig. 2. Effect of viral and human DNA on the expression of cytokines, chemokines, type I interferons and adhesion molecules. HMEC were transfected with cationic lipids and stimulated without (basal) or with poly (dA:dT) (DNA) (5 μg/ml) for different time intervals (3, 6, 9, 12, 24 h) and expression of IL-6 (A), IL-8 (B), MCP-1 (C), RANTES (D), IP-10 (E), IFN-α (F), IFN-β (G), ICAM-1 (H) and VCAM-1 (I) was analyzed by RT-PCR or stimulated without (basal) or with human genomic DNA (huDNA) (5 μg/ml) for different time intervals (12, 24 h) and expression of IL-6(J), IL-8 (K), MCP-1 (L), RANTES (M), IP-10 (N), IFN-α (O), IFN-β (P), ICAM-1 (Q) and VCAM-1 (R) was analyzed by RT-PCR. Results are given as means ± SEM of two experiments done in parallel for each condition and rRNA served as the reference gene. Comparable results were obtained in two series of independent experiments. (S) Protein synthesis of ICAM-1 and VCAM-1 was analyzed by FACS in HUVEC after transfection with cationic lipids under basal conditions or after stimulation with poly (dA:dT) (DNA) (5 μg/ml) or human genomic DNA (huDNA) for 24 h. MFI: mean fluorescence intensity; rel. to ctr.: relative to control. Results shown are from a representative experiment performed in three series of independent experiments.

stimulated with poly (dA:dT) but not transfected with lipofectamine, expression of cytokines and chemokines remained unchanged (data not shown). When HMEC were transfected with cationic lipids and stimulated without or with huDNA for different time intervals (12, 24 h) expression of IL-6, MCP-1 and RANTES was increased after 12 and 24 h stimulation time, whereas the expression of IL-8 was only increased after 24 h of stimulation with huDNA (Fig. 2J–M). A correspondingly increased protein synthesis of cytokines and chemokines was demonstrated for the selected targets IL-6 and IL-8 by ELISA (data not shown).

3.3. Effect of viral and human DNA on the expression of interferon gamma-induced protein 10 (IP-10) and type I interferons

Upon poly (dA:dT) stimulation of HMEC after transfection with lipofectamine, a time-dependent increase in the expression of IP-10 was observed from 6 to 48 h. Basal expression of interferon-α (IFN-α) was low and not influenced by poly (dA:dT) stimulation, whereas expression of interferon-β (IFN-β) was significantly increased from 3 to 24 h stimulation time with a maximum expression after 6 h (Fig. 2E–G). When HMEC were stimulated with huDNA after transfection with lipofectamine for 12 and 24 h, the expression of IP-10, IFN-α and IFN-β was increased (Fig. 2N–P).

3.4. Effect of viral and human DNA on the expression of adhesion molecules

HMEC were transfected with lipofectamine and stimulated again without or with poly (dA:dT) (5 μg/ml) to test for the effect on the expression of adhesion molecules. Expression of both ICAM-1 and VCAM-1 was increased from 6 to 24 h poly (dA:dT) stimulation (Fig. 2H, I). huDNA was able to increase the expression of ICAM-1 and VCAM-1 as well (Fig. 2Q, R). An increase of protein synthesis of adhesion molecules ICAM-1 and VCAM-1 was confirmed by FACS after 24 h poly (dA:dT) or huDNA stimulation in HUVEC (Fig. 2S).

3.5. Effects of viral and human DNA on the endothelial expression of innate immune receptors, cytokines and chemokines are TLR9 dependent

To identify the viral receptors responsible for the poly (dA:dT) and huDNA induced changes in endothelial gene expression, HMEC were stimulated without or with poly (dA:dT) or huDNA for 12 h in the presence of the specific TLR9-inhibitor ODN TTAGGG. Both poly (dA:dT) and huDNA led to an increase in the expression of TLR9, AIM2, DAI, TLR3, RIG-I, MDA5, IL-6, MCP-1, RANTES, IP-10 and IFN-β when transfected with cationic lipids; stimulation with poly (dA:dT) or huDNA without cationic lipids remained without effect on their basal expression (data not shown). Except for TLR9, whose expression was not influenced by the TLR9-inhibitor, the poly (dA:dT)- and huDNA-dependent enhancement of the expression of all other genes was inhibited by the TLR9-inhibitor. Treatment with the TLR9-inhibitor alone had no effect on basal expression of targets tested. Equally, a non-functional TLR9-inhibitor used as negative control had no effect on the basal or the DNA-dependent increase in the expression of the tested genes (Supplemental Fig. 1).

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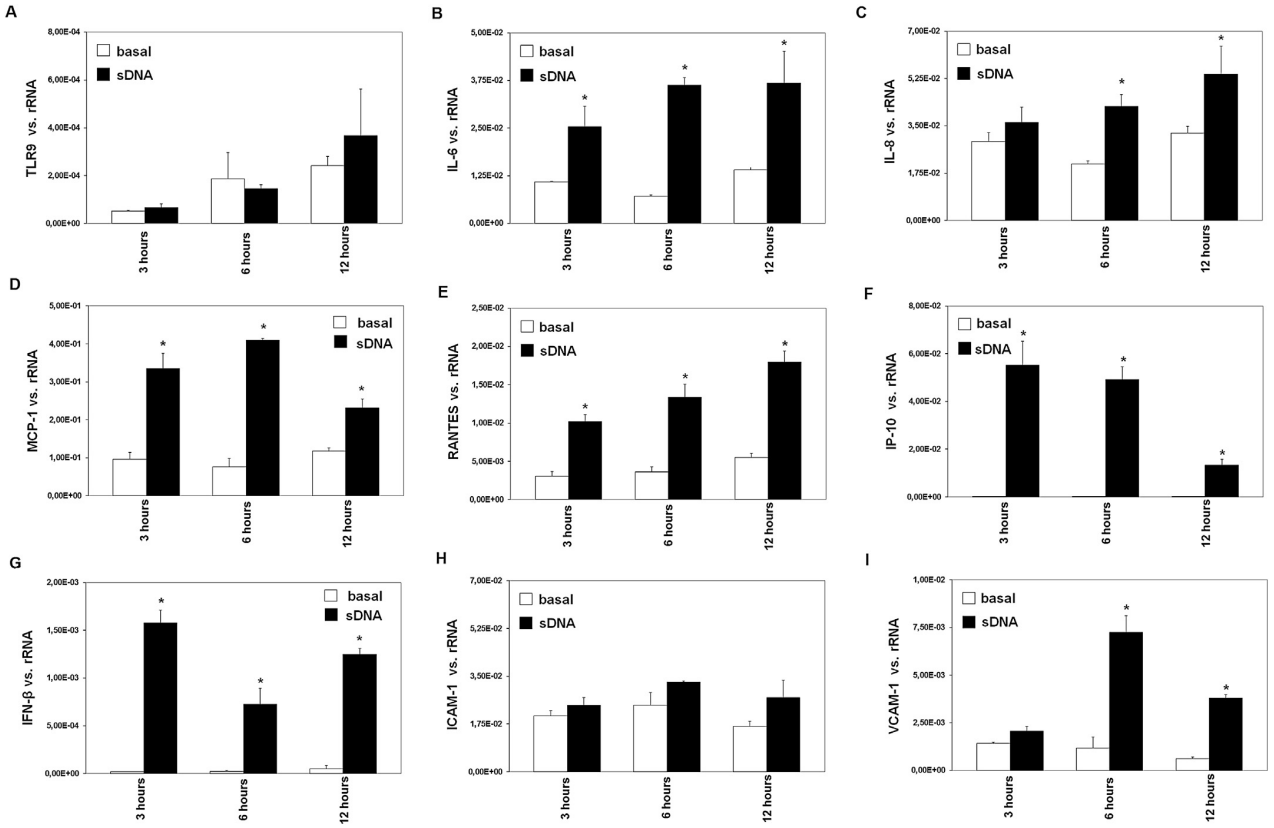

Fig. 3. Effect of endothelial-derived mediators on the expression of DNA receptor, cytokines, chemokines and adhesion molecules. HMEC were incubated for different time intervals (3, 6, 12 h) with supernatants (sDNA) of cells previously stimulated with poly (dA:dT) (5 μg/ml) for 6 h as described in Materials and Methods and expression of TLR9 (A), IL-6 (B), IL-8 (C), MCP-1 (D), RANTES (E), IP-10 (F), IFN-β (G), ICAM-1 (H) and VCAM-1 (I) was analyzed by RT-PCR.

3.6. Effect of transfection with siRNA specific for AIM2, DAI, NALP3, TLR3, RIG-I and MDA5 on viral and human DNA-dependent expression of cytokines, chemokines, type I interferons and adhesion molecules

To identify further possible viral receptor responsible for poly (dA:dT) induced expression of inflammatory molecules, HMEC were transfected with siRNA specific for AIM2, DAI, NALP3, TLR3, RIG-I and MDA5 as described and stimulated without or with poly (dA:dT) (5 μg/ml) for 12 h after transfection with lipofectamine. The induction of the expression levels of IL-6, MCP-1, RANTES, IP-10, IFN-β, ICAM-1 and VCAM-1 as analyzed by RT-PCR was not blocked by the siRNAs or the negative control (Supplemental Fig. 2). As well, the huDNA-dependent expression of the aforementioned genes could not be blocked by the siRNAs used (data not shown). As the siRNAs available for TLR9 did not lead to a significant down-regulation of TLR9 protein, knockdown experiments with siRNA for TLR9 could not be performed.

3.7. Endothelial-derived mediators induce expression of DNA receptor, cytokines, chemokines and adhesion molecules

Next we tested whether the stimulation of HMEC with viral human DNA causes the secretion of mediators which could have an autocrine effect on the endothelial expression of factors that contribute to the initiation or progression of virus-associated vasculitis. HMEC were incubated for different time intervals (3, 6, 12 h) with supernatants of cells previously stimulated with poly (dA:dT) (5 μg/ml) for 6 h as described in Materials and Methods and expression of selected targets was analyzed by RT-PCR. Basal expression of TLR9 and ICAM-1 was not significantly influenced by treatment with conditioned medium. Basal expression of IL-6, MCP-1, RANTES, IP-10 and IFN-β was increased by stimulation with the supernatants from 3 to 12 h, expression of IL-8 and VCAM-1 was increased after 6 and 12 h (Fig. 3A–I). When HMEC were incubated for 6 h with supernatants of cells previously stimulated with huDNA (5 μg/ml) for 6 h, the expression levels of TLR9, IL-6, IL-8, MCP-1, RANTES, IP-10, IFN-β, ICAM-1 and VCAM-1 remained unchanged (data not shown).

3.8. Stimulation of HMEC with DNA did not lead to activation of the inflammasome pathway but is dependent on IRF3 activation

Our findings demonstrate an upregulation of several viral DNA- and RNA-recognition receptors, cytokines, chemokines, type I interferons and adhesion molecules in HMEC after exposure to DNA nucleotides. The subsequent recognition of DNA could finally lead to the assembly of inflammasome complexes that activate caspase-1, leading to the synthesis of interleukin 1β (IL-1β) and interleukin 18(IL-18); IL-1β is an important proinflammatory cytokine secreted upon assembly of inflammasome complexes after poly (dA:dT) stimulation by RT-PCR and ELISA [28]. Poly (dA:dT) stimulation after transfection with lipofectamine led to a time-dependent increase in mRNA expression of caspase-1 and IL-1β, but did not lead

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to an increased protein synthesis of IL-1β as measured by ELISA (data not shown). As a synthesis of active IL-1β protein is the final result of inflammasome activation, it is unlikely that the inflammasome pathway is involved in signal transduction upon DNA-stimulation of HMEC (Fig. 4A, B). To identify alternative signaling pathways, HMEC were transfected with lipofectamine and stimulated without or with poly (dA:dT) for different time intervals and western blots were performed as described. DNA stimulation increased the protein synthesis of interferon regulatory factor 3 (pIRF3), TANK-binding kinase 1 (pTBK1) and the mitogen-activated proteinkinase p38 after 3, 6 and 12 h, but decreased expression levels of the inhibitory protein of the transcription factor NF-κB (IκBα) and TNF-receptor associated factor 6 (TRAF6), whereas the expression of the proteinkinase B pAKT was not influenced by poly (dA:dT)-stimulation (Fig. 4C). We further confirmed the activation of IRF3 by DNA-nucleotides with immunohistochemical staining for pIRF3 in HMEC. Treatment with cationic lipids, LL37 or poly (dA:dT) alone did not influence the rate of IRF3 positive nuclei. When HMEC were stimulated with poly (dA:dT) or huDNA after transfection with cationic lipids or LL37, the percentage of IRF3 positive nuclei increased significantly (Fig. 4D).

3.9. LL37 prevents DNA-induced expression of cytokines and chemokines

As we observed that stimulation of endothelial cells with poly (dA:dT) or human genomic DNA could increase the expression of receptors of the innate immune system, cytokines, chemokines and adhesion molecules only in cells transfected with cationic lipids (lipofectamin), we analyzed the process of DNA-internalization by DAPI staining. HMEC were incubated for 6 h with lipofectamin, LL37, poly (dA:dT) or human genomic DNA alone, or with poly (dA:dT) and human genomic DNA after transfection with lipofectamin or LL37, and immunofluorescence staining for lamin A/C and beta-actin was performed as described in Materials and methods. When cells were stimulated with the analog of viral DNA or human endogenous DNA alone, no internalization of DNA fragments was observed. DNA nucleotides of viral or human origin were transferred into the cells only if endothelial cells were additionally treated with lipofectamin or LL37 (Fig. 4E).

Next HMEC were stimulated with poly (dA:dT) or human genomic DNA after transfection either with lipofectamin or LL37 for different time intervals and expression of the selected targets IL-6 and IFN-β was analyzed by RT-PCR. IL-6 and IFN-β were induced only when HMEC transfected with lipofectamin were stimulated with viral or endogenous DNA nucleotides. When HMEC were stimulated with DNA nucleotides and transfected with LL37 expression levels of IL-6 or IFN-β remained unchanged (Fig. 4F).

To test whether LL37 itself could have an effect on the expression of proinflammatory mediators, HMEC were incubated with LL37 for 1, 2 and 4 h and expression of the selected cytokine IL-6 was analyzed. LL37 increased expression of IL6 significantly after 1 and 2 h with a maximum after 1 h. The effect of LL37 on the activation of RNA-recognizing viral receptors is not uniform and seems to depend on the cell type tested [29,30]. When HMEC were stimulated with LL37 for 12 h, the basal expression of selected targets as IL-6, IL-8 and IFN-β was not influenced. However, stimulation with the TLR3-ligand poly (I:C), a synthetic analog of viral RNA, increased significantly the expression of IL-6, IL-8 and IFN-β, an effect which in the case of IL- and IL-8 was potentiated when HMEC were stimulated with poly (I:C) in combination with LL37. The poly (I:C)-induced expression of IFN-β was not further increased by additional LL37 treatment (Fig. 4G).

3.10. Effects of hepatitis B virus (HBV) containing immunoprecipitates on endothelial expression of cytokines, chemokines and proliferation

Next we tested whether HBV-containing immunoprecipitates from a patient with an HBV associated polyarteritis nodosa which were collected during plasmapheresis therapy have an effect on endothelial expression of proinflammatory mediators and whether this effect could be influenced by transfection with cationic lipids or LL37. When HMEC were stimulated with HBV-containing immunoprecipitates alone as described in Methods, the expression of the selected targets IL-6 and IL-8 was already increased after 3 h stimulation time. When HMEC were transfected with lipofectamin additionally to HBV stimulation, the expression of IL-6 and IL-8 was further but not significantly increased. The HBV-dependent induction of IL-6 and IL-8 was blocked by simultaneous transfection with LL37. We further examined the effect of DNA, HBV-containing immunoprecipitates and cationic lipids as well as LL37 on endothelial cell proliferation. Exposure of HMEC to different concentrations of poly (dA:dT) after transfection with cationic lipids had no effect on proliferation as determined by the MTT assay. In contrast, treatment with human genomic DNA or HBV resulted in a significant decrease in cell proliferation. When HMEC were treated with HBV without transfection with cationic lipids no difference in cell proliferation was observed compared to basal conditions (Fig. 5).

4. Discussion

While representing a relevant entity in terms of morbidity and mortality, the pathogenesis of viral-disease associated vasculitis so far is not well understood. Endothelial cells are crucial in the devolution of inflammatory processes ultimately leading to overt vasculitis and are located to come in direct contact with circulating pathogens, but microvascular endothelial cells do not qualify as primary target cells for viral infections. Accordingly, disease manifestations of viral infections such as vasculitis, glomerulopathies or thromboembolic disorders are supposed to be primarily immune-mediated and do not depend necessarily on a direct viral infection of cells. And indeed, not only the disease course usually parallels the one of related organ manifestations of autoimmune disorders, but the onset and progression of autoimmune disorders are frequently triggered by viral infections. We therefore hypothesized an activation of receptors of the innate immune system to be responsible for the induction of proinflammatory responses of endothelial cells and to thereby contribute to the development of overt vasculitis. We were able to show that stimulation of human endothelial cells with both poly (dA:dT), a synthetic analog of viral DNA, and human genomic DNA led to a significant induction of proinflammatory cytokines, chemokines and adhesion molecules. These data were confirmed and corroborated as to their clinical relevance by stimulation of endothelial cells with HBV-DNA containing immunoprecipitates. The DNA-induced effects were TLR9-dependent and not mediated by other DNA- or RNA-recognition receptors of the innate immune system. Downstream signaling was shown to encompass a significant increase in TBK-1 and the transcription factor IRF3, essential for the induction of interferons, whereas the induction of p38 and the suppression of the inhibitory IkBa would be expected to account for the mediation of the proinflammatory effects. A functional activation of the inflammasome pathway was to be excluded for the lack of IL-1β protein induction.

A common pathway in the initiation and progression of vasculitis related to both viral infections and autoimmune diseases that consists in an activation of viral receptors of the innate immune

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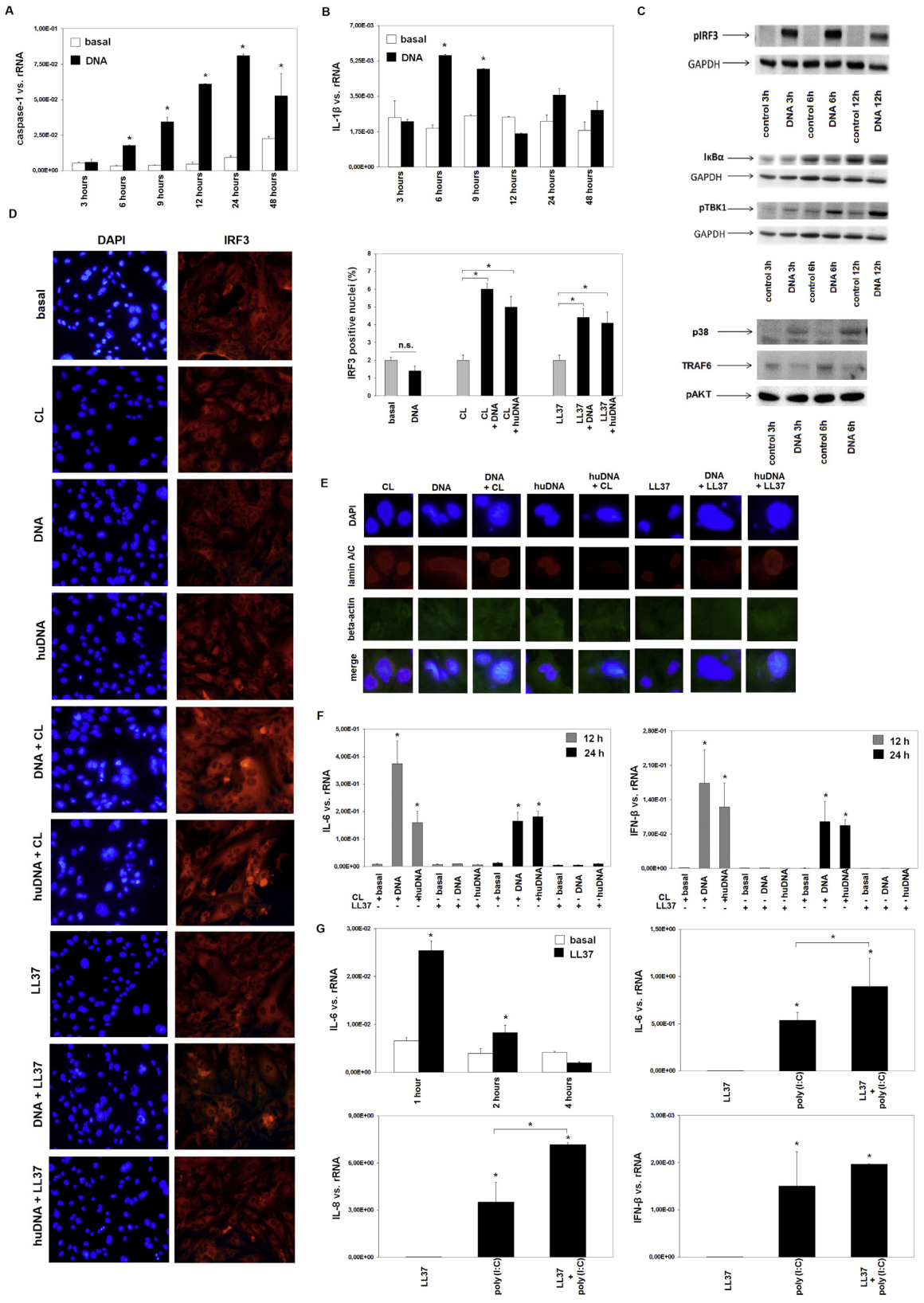

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system by viral or endogenous DNA and RNA nucleotides can thus be supposed. Our finding of a TRL9-dependent induction of proinflammatory cytokines, chemokines and adhesion molecules renders this hypothesis particularly interesting with regard to systemic lupus erythematodes (SLE), which is characterized by breakdown of tolerance to nuclear DNA antigens, immune complex deposition in tissues and multiorgan involvement [31]. Although the possible role of TLR9 in human SLE ultimately remains elusive [32], there are no data on possible alterations of endothelial TLR9 expression and its relevance for disease manifestations as vasculitis. Furthermore, the common hypothesis about SLE pathogenesis suggests that environmental triggers, and amongst them infectious agents, operate in the context of both susceptibility genes and epigenetic modifications, resulting in alterations in antigen presentation, lymphoid signaling, apoptosis and antigen clearance; herein, chronic activation of plasmocytoid dendritic cells (pDC) plays an essential role [33,34]. Given that TLR9 expressed in pDC is known to be required for an adequate response to DNA viruses [35], activation of TLR9 upon binding of viral DNA might represent the missing link able to clarify how viral infections can trigger autoimmune diseases. Another interesting observation with a possible impact on disease progression was the release of soluble mediators able to induce proinflammatory effects in an auto- and paracrine manner by DNA-stimulated endothelial cells; this effect was restricted to DNA of viral origin, though. Supposedly these mediators would be able to amplify primarily the local antiviral response, but also contribute to vascular inflammation including the recruitment of inflammatory cells and compromise the integrity of the endothelial barrier.

We found that DNA, irrespective of its origin, was able to exert its proinflammatory effects only if DNA nucleotides were transfected with cationic lipids (lipofectamine); obviously, transfection was necessary to allow for internalization of DNA into the endothelial cells in vitro and activate the intracellularly located receptor TLR9. To address the question how circulating DNA can be internalized into endothelial cells in vivo, we investigated the effects of another component of the innate immune system, the antimicrobial peptide LL37. Data elucidating the role of LL37 in viral infections are scarce. Though, it has been shown that epithelial cells are able to induce phenotypic maturation of pDCs; this process was associated with an increased expression of TLR9 in pDCs and of LL37 in epithelial cells, and epithelium-activated pDCs impaired the establishment of a productive HIV infection in two susceptible target cells [36]. Elevated serum levels of LL37 have been observed in patients affected by viral hepatitis B and C where they seemingly correlate with activity indices [20]. Furthermore, LL37 is amongst the most upregulated genes in blood cells from patients with SLE and correlate with high levels of expression of type I IFNs and IFN-induced genes. In addition, immune complexes isolated from patients with SLE contain LL37, and the presence of LL37 seems to be a prerequisite for the ability of DNA-specific antibodies to promote an FcγRIIA-mediated uptake of self-DNA into pDCs and trigger an endosomal TLR9 response [35]. This leads to a sustained activation of pDCs and type I interferon production, which in turn activates neutrophils and supports disease activity [11,31,35,37–40]. A cell type specific role of LL37 in complexing and delivering DNA into the cells between immune cells and non-immune cells is possible [41], but so far it was unknown whether LL37 is able to transport DNA into other cells than plasmocytoid dendritic cells and monocytes, leading to activation of TLR9 and the induction of type I interferons. Immunohistochemical staining showed that both lipofectamin and LL37 were able to internalize poly (dA:dT) or human DNA into the endothelial cells and to induce the transcription factor IRF3. Very notably, though, LL37 blocked DNA-induced expression of proinflammatory cytokines and type I interferons; correspondingly, stimulation with HBV-containing immunoprecipitates from a patient with polyarteritis nodosa failed to induce IL-6 and IL-8 in LL37 transfected endothelial cells. We therefore hypothesize an inhibitory effect of LL37 on inflammatory responses of endothelial cells upon stimulation with DNA which is located downstream to IRF3. Taking into consideration the known ability of LL37 to enhance or abrogate inflammatory signals depending on the cell type and the particular microenvironment [42], a possibly protective effect of LL37 in vasculitis related to both viral and autoimmune diseases merits further evaluation and potentially opens up alternative therapeutic strategies making use of LL37 as a target for immune modulation or even adopting LL37-inducing compounds.

5. Conclusions

We hereby provide evidence for a TLR9-dependent induction of proinflammatory cytokines, chemokines and adhesion molecules upon stimulation of human endothelial cells with poly (dA:dT), a synthetic analog of viral DNA, human genomic DNA and HBV-DNA containing immunoprecipitates. We infer a possible common pathway in the initiation and progression of vasculitis related to both viral infections and autoimmune diseases that consists in an activation of viral receptors of the innate immune system by viral or endogenous DNA and RNA nucleotides. In addition, activation of TLR9 in plasmocytoid dendritic cells upon binding of viral DNA might represent the missing link able to clarify how viral infections can trigger autoimmune diseases.

We also show for the first time that another component of the innate immune system, the antimicrobial peptide LL37, is able to internalize poly (dA:dT) or human DNA into endothelial cells and to block the DNA-induced expression of proinflammatory cytokines and type I interferons. This possibly protective effect of LL37 in vasculitis related to both viral and autoimmune diseases merits further evaluation and potentially opens up alternative therapeutic strategies.

Fig. 4. Effect of DNA nucleotides under different transfection conditions on signaling pathways, DNA internalization and expression of proinflammatory cytokines and chemokines. HMEC were transfected with cationic lipids and stimulated without (basal) or with poly (dA:dT) (5 μg/ml) (DNA) for different time intervals and mRNA expression of caspase-1 (A) and IL-1β (B) was analyzed by RT-PCR. Protein synthesis of IL-1β was analyzed by ELISA (data not shown). Results are given as means ± SEM of two experiments done in parallel for each condition and rRNA served as the reference gene. Comparable results were obtained in two series of independent experiments. (C) Protein expression of pIRF3, IκBα, pTBK1, p38, TRAF6 and pAKT was analyzed by western blot as described in Materials and Methods after transfection with cationic lipids and stimulation without (control) or with poly (dA:dT) (DNA) for different time intervals (3, 6, 12 h for pIRF3, IκBα and pTBK1; 3, 6 h for p38, TRAF6 and pAKT). Comparable results were obtained in two series of independent experiments. (D) HMEC were cultivated for 6 h under basal conditions or treated with cationic lipids (CL), poly (dA:dT) (DNA), human genomic DNA (huDNA), or LL37 alone or stimulated with poly (dA:dT) or human genomic DNA after transfection with cationic lipids or LL37; immunofluorescence was performed as described in Materials and methods. To measure the nuclear accumulation of IRF3, 100 cells from at least two independent fields were examined and the results are shown as the percentage of cells with nuclear IRF3 per 100 cells. (E) HMEC were treated as described above and immunofluorescence staining for DAPI, lamin A/C and beta-actin was performed as described in Materials and methods. (F) HMEC were cultivated under basal conditions or stimulated with poly (dA:dT) (DNA) or human genomic DNA (huDNA) for different time intervals (12, 24 h) either after transfection with lipofectamin (CL) or LL37. Expression of IL-6 and IFN-β was analyzed by RT-PCR. Results are given as means ± SEM of two experiments done in parallel for each condition and rRNA served as the reference gene. Comparable results were obtained in two series of independent experiments. (G) HMEC were stimulated with LL37 for different time intervals (1, 2, 4 h) and expression of IL-6 was analyzed by RT-PCR. HMEC were stimulated with LL37, poly (I:C) (5 μg/ml) or poly (I:C) in combination with LL37 for 12 h and expression of IFN-β was analyzed by RT-PCR. Results are given as means ± SEM of two experiments done in parallel for each condition and rRNA served as the reference gene. Comparable results were obtained in two series of independent experiments.

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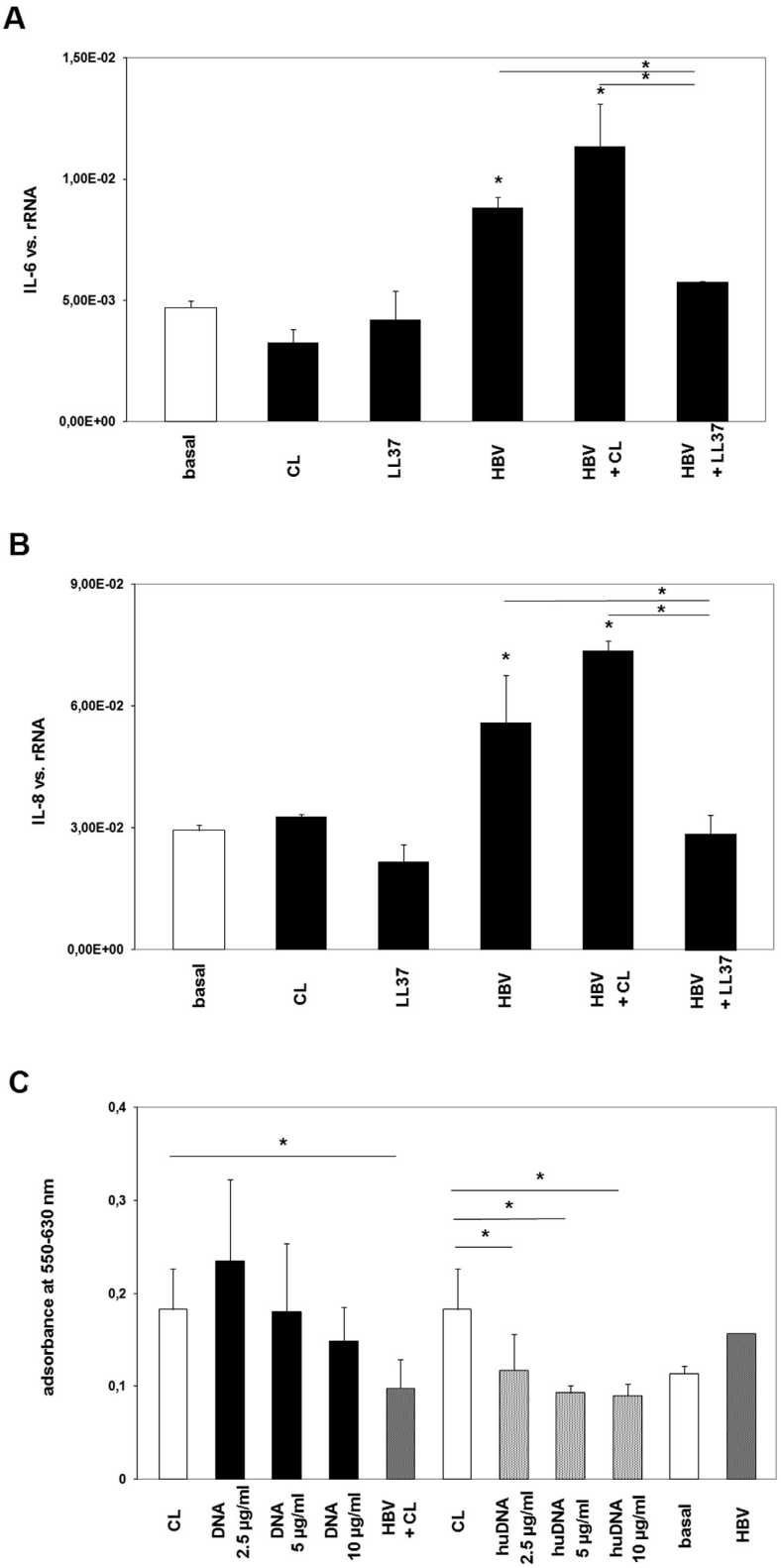

Fig. 5. Effect of Hepatitis B containing immunoprecipitates on the expression of cytokines, chemokines and proliferation of endothelial cells. HMEC were cultivated under basal conditions or treated with cationic lipids (CL), LL37, Hepatitis B containing immunoprecipitates alone (HBV) or HBV after transfection with cationic lipids for 3 h and expression of IL-6 (A) and IL-8 (B)was analyzed by RT-PCR. Results are given as means ± SEM of two experiments done in parallel for each condition and rRNA served as the reference gene. Comparable results were obtained in two series of independent experiments. (C) HMEC were transfected with cationic lipids (CL) and stimulated with different concentrations (2.5, 5, 10 μg/ml) of poly (dA:dT) (DNA), human genomic DNA (huDNA) or with Hepatitis B containing immunoprecipitates (HBV) or cultivated under basal conditions with or without HBV for 12 h. HMEC were analyzed for cell proliferation with the MTT assay as described in Material and Methods. Each bar represents the mean ± SEM of five parallel incubations for each condition. Comparable results were obtained in two series of independent experiments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2015.07.015.

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Double-stranded DNA induces a prothrombotic phenotype in the vascular endothelium

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Double-stranded DNA induces a prothrombotic phenotype in the vascular endothelium

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Double-stranded DNA (dsDNA) constitutes a potent activator of innate immunity, given its ability to bind intracellular pattern recognition receptors during viral infections or sterile tissue damage. While effects of dsDNA in immune cells have been extensively studied, dsDNA signalling and its pathophysiological implications in non-immune cells, such as the vascular endothelium, remain poorly understood. The aim of this study was to characterize prothrombotic effects of dsDNA in vascular endothelial cells. Transfection of cultured human endothelial cells with the synthetic dsDNA poly(dA:dT) induced upregulation of the prothrombotic molecules tissue factor and PAI-1, resulting in accelerated blood clotting *in vitro*, which was partly dependent on RIG-I signalling. Prothrombotic effects were also observed upon transfection of endothelial cells with hepatitis B virus DNA-containing immunoprecipitates as well human genomic DNA. In addition, dsDNA led to surface expression of von Willebrand factor resulting in increased platelet-endothelium-interactions under flow. Eventually, intrascrotal injection of dsDNA resulted in accelerated thrombus formation upon light/dye-induced endothelial injury in mouse cremaster arterioles and venules *in vivo*. In conclusion, we show that viral or endogenous dsDNA induces a prothrombotic phenotype in the vascular endothelium. These findings represent a novel link between pathogen- and danger-associated patterns within innate immunity and thrombosis.

The innate immune system constitutes a key response to both invading pathogens and sterile injury by recognition of pathogen associated- or danger associated molecular patterns (PAMPs or DAMPs, respectively). In this context lipopolysaccharides (LPS), peptidoglycans, high-mobility group protein-1 (HMGB1), double stranded DNA (dsDNA) and others are released into the circulation[1–3]. dsDNA is a powerful activator of the innate immune system[4] and acts via several so called pattern-recognition receptors such as TLR-9 (toll-like receptor 9), AIM2 (absent in melanoma 2), DAI (DNA-dependent activator of IRFs), RIG-I (after transformation of DNA by RNA polymerase III)[4,5], and most recently Interferon-γ-inducible protein16 (IFI16) and cGAMP synthase (cGAS) have been discovered and shown to recognize intracellular dsDNA[6]. While the dsDNA-mediated immune response has been extensively studied in immune cells, little is known so far about the pathophysiological relevance of dsDNA for the vascular endothelium.

dsDNA plays a central role in viral infections such as hepatitis B, which often manifest as systemic diseases involving several organs including the vascular system. Morbidity and mortality result in part from vasculitis[7,8] but also from thrombotic complications including fatal thrombo-embolic events, such as myocardial infarction and ischemic stroke[9,10]. Moreover viral infections are likely to play a role in the pathophysiology of atherosclerosis[11]. Even though not primarily considered part of the immune system, the endothelium as the inner layer of blood vessels plays a major role in host defense constituting an anatomical and functional barrier for pathogens to invade tissues. Additionally, the endothelium has essential function in suppressing inflammation and thrombosis by controlling vascular tone and function[12]. Endothelial inflammation leads to disruption of the haemostatic balance towards a prothrombotic state with increased risk of thrombo-embolic events[13]. Activated ECs are known

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to facilitate blood coagulation by down-regulation of antithrombotic proteins, such as thrombomodulin, tissue plasminogen activator or endothelial protein C receptor and by increased expression of procoagulant proteins such as the antifibrinolytic plasminogen activator inhibitor-1 (PAI-1) or tissue factor (TF)[14].

We and others have previously shown, that the vascular endothelium is able to sense intracellular dsDNA and can exert a strong inflammatory response[15,16]. In this study we investigated prothrombotic effects of dsDNA in the vascular endothelium.

Results

Double-stranded DNA led to nuclear translocation of transcription factors IRF3 and NF-κB. Human microvascular endothelial cells (HMEC) were treated with poly(dA:dT) 5 µg/mL with or without complexation with cationic lipids (Lipofectamine 2000) for 6 hours and stained with DAPI and anti-Lamin-antibody. Only poly(dA:dT) complexed with cationic lipids but not poly(dA:dT) alone led to uptake of dsDNA into the intracellular compartments (representative images in Fig. 1a). Transfection of endothelial cells with poly(dA:dT) led to nuclear translocation of transcription factors IRF3 and to a lesser extent of NF-κB as shown by immunofluorescent staining (representative images in Fig. 1b and c). To check integrity of the endothelial cell monolayer 8 hours after transfection with poly(dA:dT) bright field images were taken, which showed comparable intact monolayers in cells treated with poly(dA:dT) with or without cationic lipids (representative images in Fig. 1d).

Double-stranded DNA induces expression of prothrombotic genes in vascular endothelial cells. Next, we measured expression of pro- and antithrombotic genes by real-time PCR. Transfection of human microvascular endothelial cells (HMEC) with poly(dA:dT) (5 µg/mL) induced time-dependent expression of tissue factor as well as plasminogen activator inhibitor-1 (PAI-1) with a maximal relative increase at 6 or 12 hours, respectively (Fig. 2a and b). We also observe significantly increased expression of the fibrinolytic molecule tissue plasminogen activator (tPA) after 6 hours of cell transfection with poly(dA:dT) (Fig. 2c), while thrombomodulin (THBD) expression was slightly increased after 6 hours of transfection with poly(dA:dT) after an initial decrease after 3 hours (Fig. 2d).

Double stranded DNA induced prothrombotic proteins and accelerates endothelial dependent blood clotting *in vitro*. Next, upregulation of prothrombotic molecules tissue factor and PAI-1 was assessed on protein level. Tissue factor surface expression on the cell membrane was significantly increased after stimulation with poly(dA:dT) for 12 hours as assessed by flow cytometry (Fig. 3a). PAI-1 release by endothelial cells as measured by ELISA was significantly increased 12 hours after transfection with poly(dA:dT) but not after 6 hours as compared to respective time-matched controls. In contrast, PAI-1 release was not influenced after stimulation of endothelial cells with poly(dA:dT) alone, i.e. without cationic lipids (Fig. 3b).

In order to functionally analyze prothrombotic properties of endothelial cells, an endothelial dependent blood clotting assay was performed. Lysates of poly(dA:dT) transfected endothelial cells accelerated blood clotting time compared to time-matched control cells (Fig. 3c, left). Stimulation of whole blood with lysates of endothelial cells treated with poly(dA:dT) alone (i.e. without cationic lipids) had no effect on blood clotting time (Fig. 3c, right). Similar to cells transfected with the synthetic dsDNA analogue poly(dA:dT), lysates of endothelial cells transfected with human genomic DNA from peripheral human leukocytes also induced a significantly accelerated blood clotting compared to control cells after 12 hours (Fig. 3e). The prothrombotic effect of poly(dA:dT) after 12 hours was partly reversed after prior transfection of endothelial cells with siRNA silencing RIG-I receptor (Fig. 3d).

Double stranded DNA induces vWF upregulation and platelet tethering *in vitro*. Von Willebrand factor (vWF) surface expression and platelet adhesion were analyzed in primary human umbilical vein endothelial cells (HUVEC). Transfection of endothelial cells with poly(dA:dT) significantly increased surface expression of vWF after 12 hours as assessed by flow cytometry (Fig. 4a). To investigate the functional relevance of this observation, interactions between endothelial cells and platelets were examined in a model of static adhesion. Therefore endothelial cells pre-treated with poly(dA:dT) for 12 hours were then co-cultivated with freshly isolated platelets from healthy volunteers for 6 hours. Endothelial cells transfected with dsDNA showed significantly increased numbers of adherent platelets as compared to non-stimulated cells (Fig. 4b). In order to confirm our findings in a more physiological setting, we established a flow based assay of platelet endothelium interaction. Therefore freshly isolated human platelets were labeled with Calcein and flushed over cultured endothelial cells in a flow chamber simulating a vascular shear stress of 1 dyn/cm^2 and platelet-endothelial cell interactions were analyzed by immunofluorescence microscopy (Fig. 4c). Poly(dA:dT) transfected endothelial cells showed significantly increased amount of tethering platelets compared to non stimulated cells (Fig. 4d). Additionally the number of very slow rolling platelets was higher on poly(dA:dT) transfected endothelial cells, however (considering the high number of overall transfused platelets) the median velocity of platelets was not different in both groups (Fig. 4e).

Double stranded DNA accelerates microvascular thrombosis *in vivo*. To investigate the effects of double stranded DNA on thrombus formation *in vivo*, 10 µg poly(dA:dT) complexed with 10 µl of Lipofectamine or transfection reagent alone (control group) was injected into the scrotum of C57Bl/6 mice. Intravital microscopy of cremaster muscle vessels was performed 12 hours after injection and thrombus formation was induced by light/dye-injury after injection of FITC-labeled dextran. Vessel diameters did not differ significantly between both groups and were 53 +/− 5 µm vs. 48 +/− 6 µm in venules and 51 +/− 9 µm vs. 50 +/− 3 µm in arterioles control treated vs. dsDNA treated animals respectively. dsDNA stimulation significantly accelerated thrombus formation *in vivo* resulting in a reduced time until onset of thrombus formation after injury in arterioles (Fig. 5c)

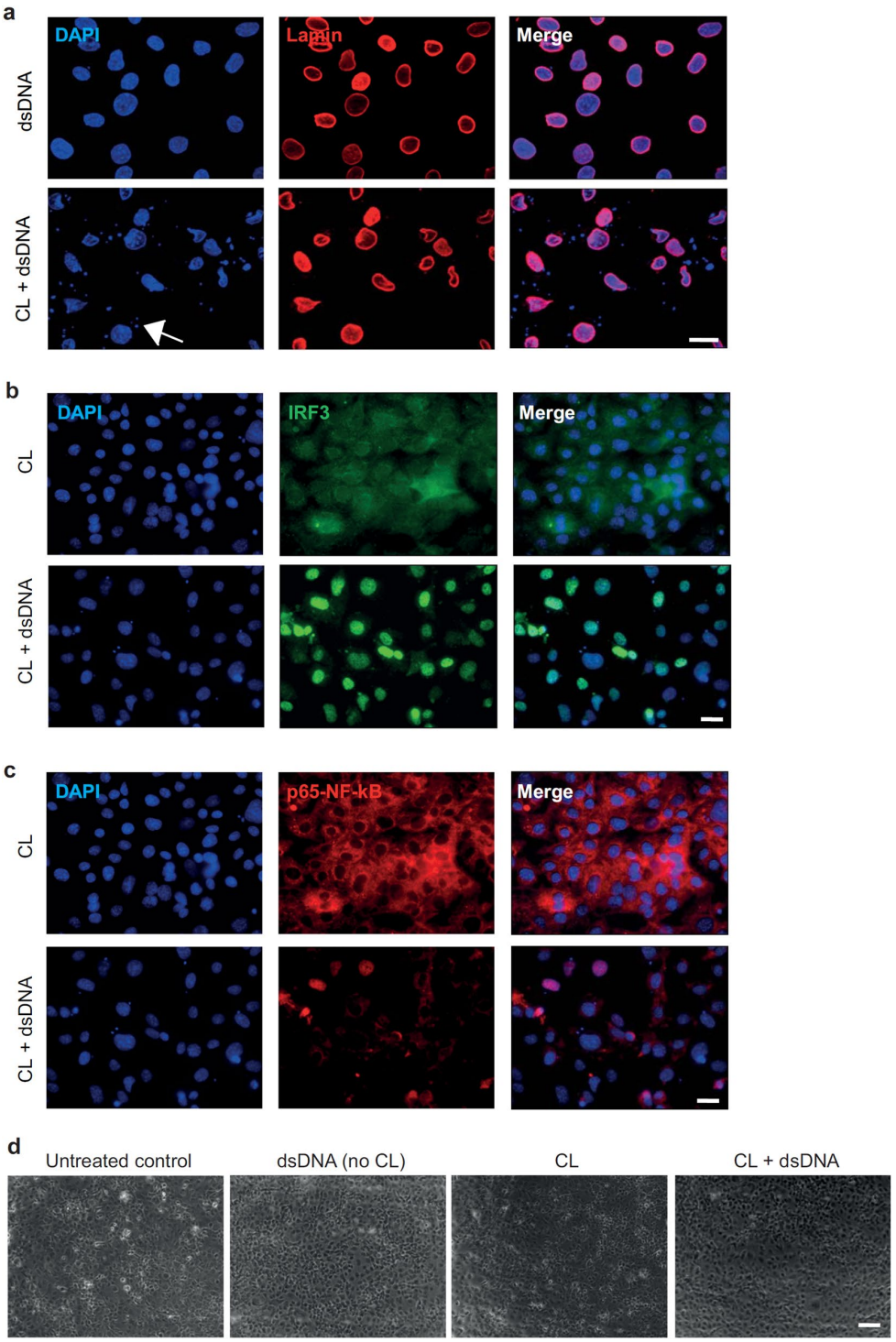

Figure 1. Double-stranded DNA led to nuclear translocation of transcription factors IRF3 and NF-κB. (**a**) Immunofluorescent staining of cultured endothelial cells with DAPI (blue) and anti-Lamin-antibody (red) showed transfection of poly(dA:dT) into the intracellular compartment after complex formation with cationic lipids. Scale bar is 10 µm. (**b**,**c**) Transfection of endothelial cells with poly(dA:dT) led to nuclear translocation of transcription factors IRF3 (**b**) and to a lesser extent of NF-κB (**c**). Cells were stained with antibodies against IRF3 (green) and p65-subunit of NF-κB (red). Scale bar is 10 µm. (**d**) Representative bright field images upon treatment of endothelial cells with poly(dA:dT) with or without cationic lipids. Scale bar is 100 µm.

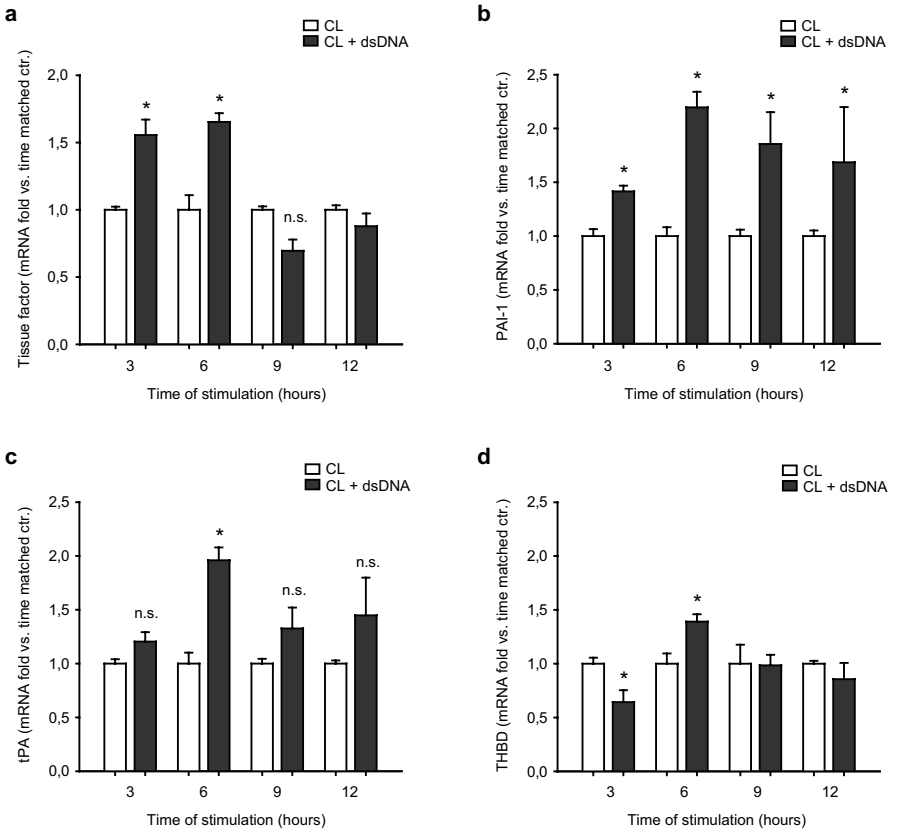

Figure 2. Double-stranded DNA induces expression of prothrombotic genes in vascular endothelial cells. (**a**,**b**) Expression of the prothrombotic molecules tissue factor (**a**) and Plasminogen activator inhibitor-1 (PAI-1, **b**) as assessed by RT-PCR upon transfection of vascular endothelial cells with poly(dA:dT). (**c**,**d**) Expression of the antithrombotic molecules tissue plasminogen activator (tPA, (**c**)) and thrombomodulin (THBD, (**d**)) upon transfection of vascular endothelial cells with poly(dA:dT); (*$P < 0.05$ vs. respective time-matched control, $n = 4$).

and eventually also in significantly reduced time until complete thrombotic vessel occlusion in both venules and arterioles (Fig. 5a and b, representative images in Fig. 5e).

Hepatitis B virus DNA-containing immunoprecipitates induce a prothrombotic phenotype. To translate our findings into a clinical context we investigated whether Hepatitis B virus DNA induces prothrombotic effects in the vascular endothelium. Therefore we transfected microvascular endothelial cells with hepatitis B virus (HBV)-containing immunoprecipitates, that were collected during plasmapheresis from a patient with HBV-associated polyarteritis nodosa. Similar to transfection with the synthetic analogue poly(dA:dT) HBV-containing immunoprecipitates exerted a prothrombotic phenotype in transfected endothelial cells resulting in upregulation of tissue factor already after 3 hours and upregulation of PAI-1 expression after 10 h as assessed by real-time PCR (Fig. 5a and b respectively, left images). HBV-DNA alone (i.e. without cationic lipids) had no effect on expression of tissue factor and PAI-1 expression compared to time-matched controls (Fig. 6a and b, respectively, right images).

Discussion

In this study, we show direct prothrombotic effects of intracellular double stranded DNA (dsDNA) in the vascular endothelium. dsDNA led to upregulation of the procoagulatory proteins tissue factor and PAI-1 and increased surface expression of vWF and eventually resulted in accelerated blood clotting *in vitro* and thrombus formation in a model of endothelial injury *in vivo*. Similar effects were observed after transfection of endothelial cells with hepatitis B virus DNA containing immunoprecipitates and with endogenous human DNA.

In previous work we showed that dsDNA, both from viral origin as well as endogenous DNA, can induce pro-inflammatory effects in endothelial cells resulting in upregulation of inflammatory cytokines such as IL-6, IL-8, MCP-1, RANTES, IP-10 and IFN-β, as well as the adhesion molecules ICAM-1 and VCAM-1 on human endothelial cells[15]. Furthermore, dsDNA has been described to induce TNFα release from endothelial cells and thereby promoting leukocyte adhesion[16]. Similar effects have also been observed in glomerular endothelial cells where dsDNA functionally increased albumin permeability[17].

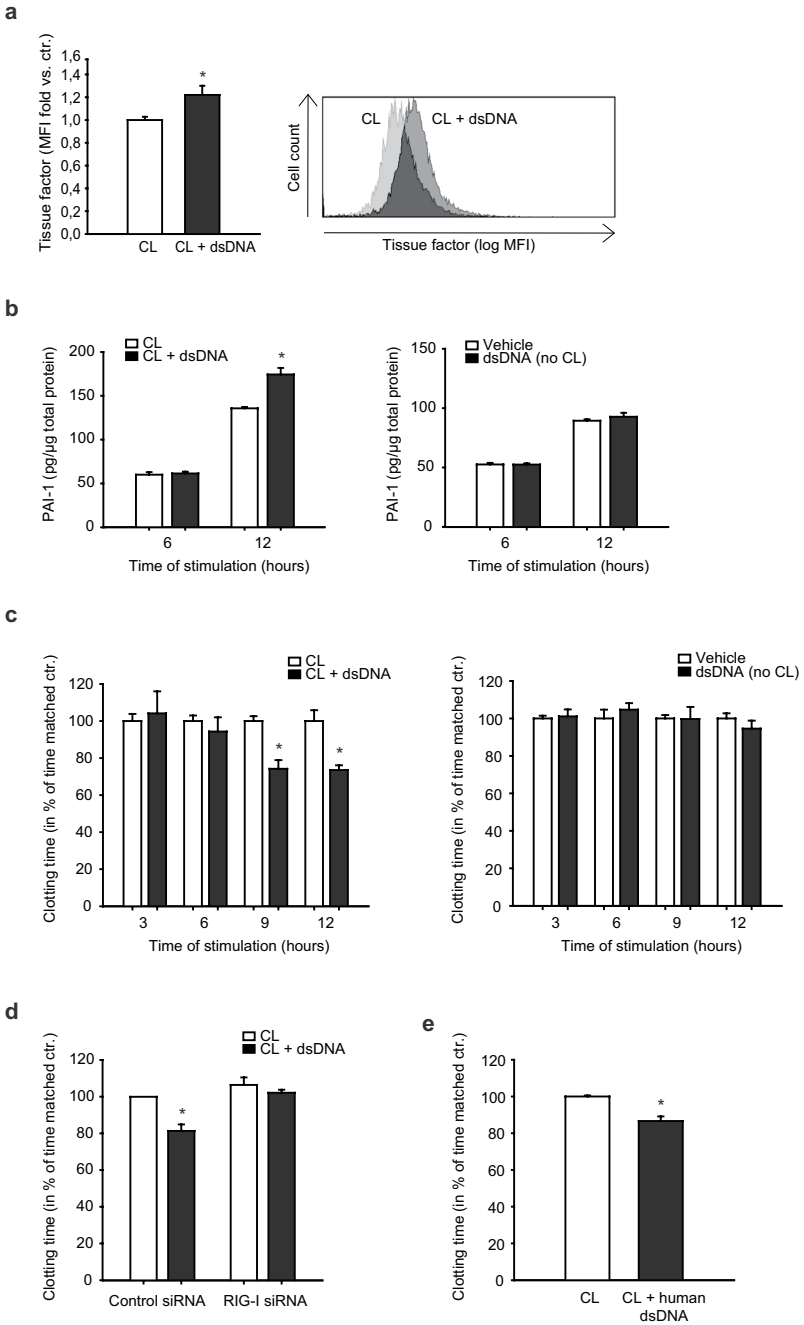

Figure 3. Double stranded DNA induces prothrombotic proteins and accelerates endothelial dependent blood clotting *in vitro*. (**a**) Tissue factor surface expression was assessed by flow cytometry 12 hours after transfection of endothelial cells with poly(dA:dT) (*$P < 0.05$ vs. control, $n = 8$, MFI mean fluorescence intensity). Representative histogram is shown on the right. (**b**) PAI-1 release by endothelial cells was measured by ELISA 6 and 12 hours after transfection with poly(dA:dT), PAI-1 release after stimulation of endothelial cells with poly(dA:dT) alone (i.e. without cationic lipids) is shown on the right (*$P < 0.05$ vs. time matched control, $n = 5$). (**c**) Stimulation of whole blood with endothelial cell lysates transfected with poly(dA:dT) accelerated blood clotting time compared to stimulation with untreated cell lysates as measured by thromboelastometry. Stimulation of whole blood with lysates of endothelial cells treated with poly(dA:dT) alone (i.e. without cationic lipids) had no effect on blood clotting time (right) (*$P < 0.05$ vs. time matched control, $n = 4$). (**d**) The prothrombotic effect of poly(dA:dT) after 12 hours was partly reversed by siRNA-silencing of RIG-I (*$P < 0.05$ vs. time matched control, $n = 4$). (**e**) A similar prothrombotic effect was observed 12 hours after transfection of endothelial cells with human genomic DNA (*$P < 0.05$ vs. time-matched control, $n = 4$).

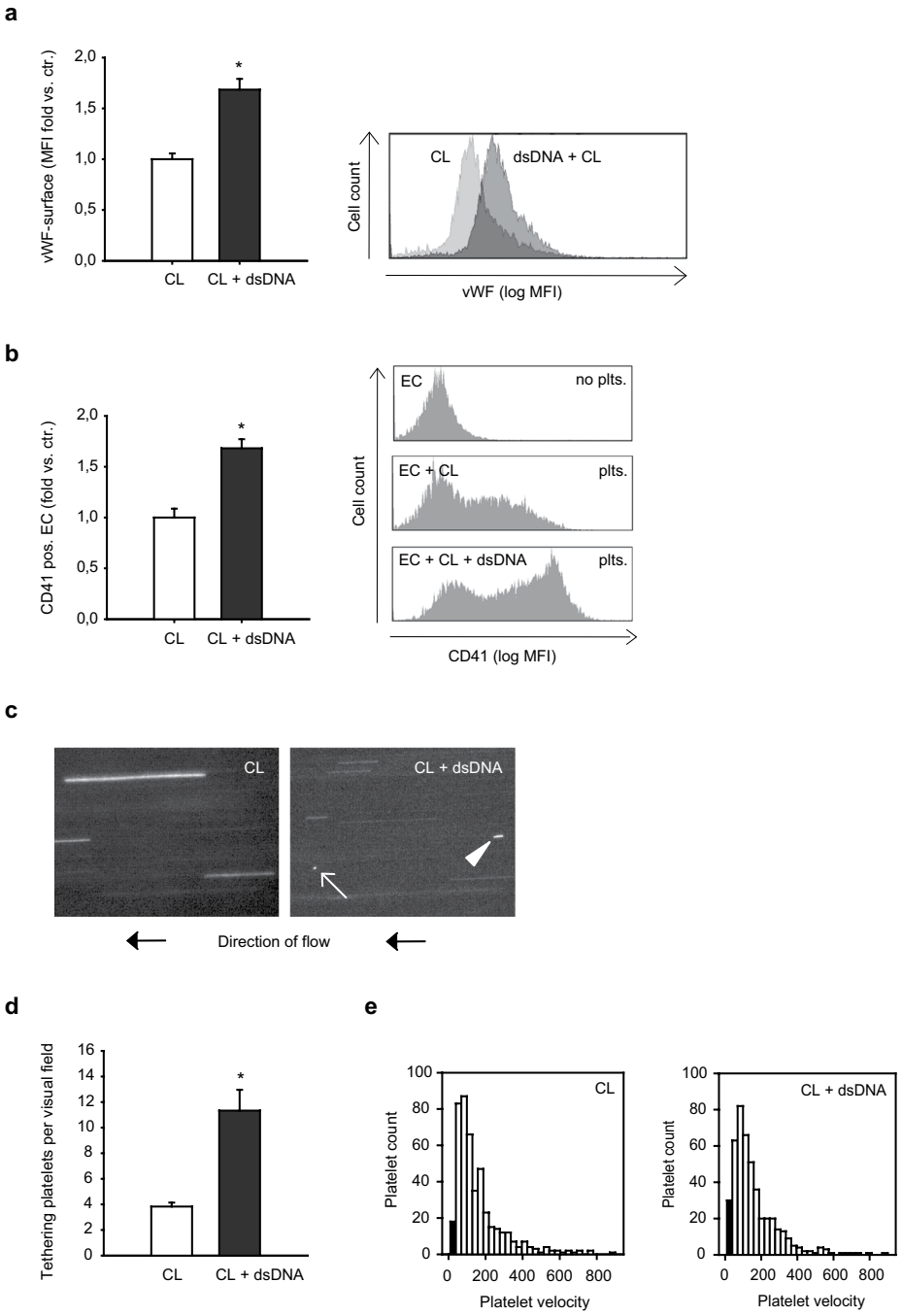

Figure 4. Double-stranded DNA induces vWF upregulation and platelet tethering *in vitro*. (**a**) Transfection of endothelial cells with poly(dA:dT) for 12 hours caused upregulation of von Willebrand factor on the endothelial cell surface (*$P < 0.05$ vs. control, $n = 6$; MFI mean fluorescence intensity). Representative histogram is shown on the right. (**b**) Co-cultivation of endothelial cells transfected with poly(dA:dT) for 12 hours and non-stimulated isolated platelets resulted in increased adhesion of platelets to endothelial cells, as assessed by FACS analysis (*$P < 0.05$, vs. control, $n = 6$). Representative histograms are shown on the right. (**c**) Representative snap shots of flow chamber assays with transfusion of fluorescently labeled platelets simulating vascular shear stress of 1 dyn/cm^2. Platelet tethering (white arrow) and very slow rolling platelets (white arrowhead) were analyzed after stimulation of endothelial cells with poly(dA:dT) for 12 hours. (**d**) Quantitative analysis showed increased number of tethering platelets (*$P < 0.05$ vs. control, $n = 9$) as well as (**e**) increased number of very slow rolling platelets (as displayed by the black columns in the histograms showing platelet velocity profiles) upon transfection with poly(dA:dT).

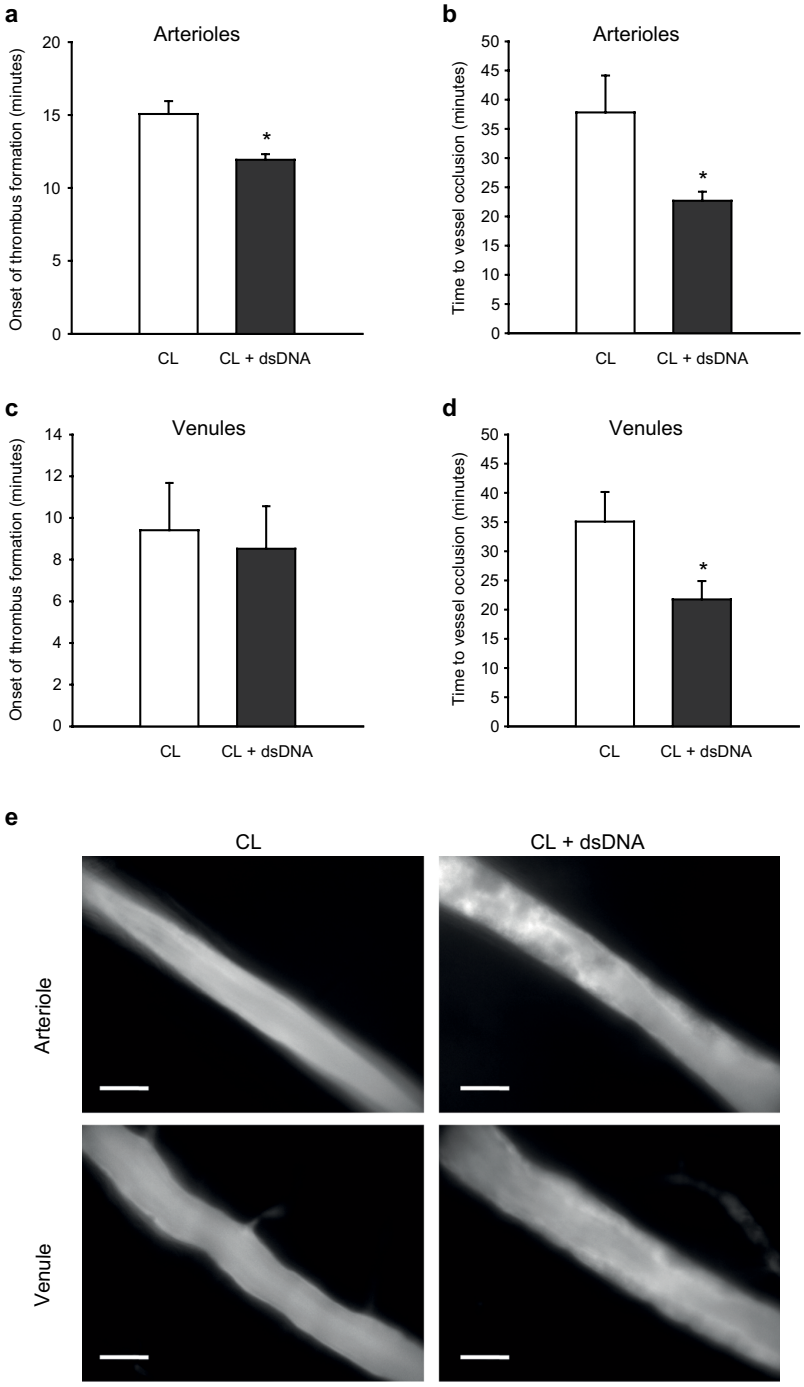

Figure 5. Double-stranded DNA accelerates microvascular thrombosis *in vivo*. Thrombus formation *in vivo* was investigated by phototoxic (light/dye-induced) vessel injury of microvessels in the mouse cremaster muscle. Intrascrotal injection of poly(dA:dT) (5 µg DNA 12 hours prior to the experiment) resulted in a more rapid onset of thrombus formation in arterioles (**a**) but not in venules (**c**). Time to complete vessel occlusion was significantly accelerated after poly(dA:dT) injection in both arterioles (**b**) and venules (**d**). (**e**) Representative images show thrombus formation in mouse cremaster arterioles (20 min after start of injury) and mouse cremaster venules (10 min after start of injury) with markedly increased thrombus formation in poly(dA:dT) treated animals (right pictures) as compared to control animals (left pictures). Scale bar is 50 µm. (*$P < 0.05$ vs. control, $n = 6$ animals each).

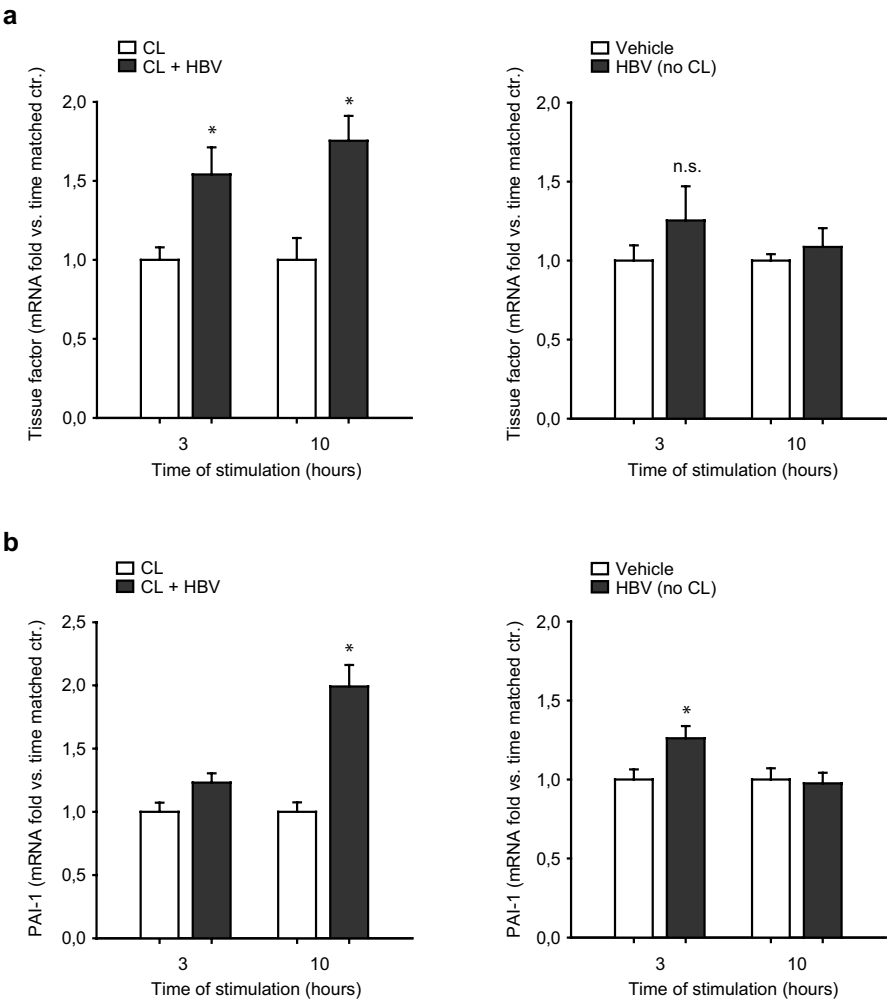

Figure 6. Hepatitis B virus DNA-containing immunoprecipitates induce a prothrombotic phenotype. Endothelial cells were transfected with HBV-DNA containing immunoprecipitates isolated from a patient with ongoing hepatitis B infection and associated polyarteritis nodosa with a high viral load. HBV-DNA containing immunoprecipitates resulted in upregulation of tissue factor starting 3 hours after transfection (**a**) as well as PAI-I at 10 hours after transfection (**b**) as analyzed by RT-PCR. Expression of tissue factor and PAI-1 after stimulation of endothelial cells with HBV-DNA alone (i.e. without cationic lipids) is shown on the right ((**a** and **b**), respectively). (*$P < 0.05$ vs. control).

In this study we show intracellular dsDNA leads to upregulation of tissue factor, a crucial protein in the activation of the extrinsic pathway of the coagulation cascade[18]. Tissue factor initiates the extrinsic pathway of the coagulation cascade and contributes to thrombus growth and stabilization[19]. Additionally, under certain pathophysiological conditions such as sickle cell disease or antiphospholipid syndrome tissue factor-positive endothelial microparticles have been observed, that can be recruited to the sites of vascular injury and contribute to increased thrombin generation[20,21]. Physiologically resting endothelial cells express, if at all, very little amounts of tissue factor, however, the protein is upregulated under inflammatory conditions[14,22–24]. Indeed, on a functional level we observed, that stimulation of endothelial cells with dsDNA resulted in accelerated blood clot formation in an endothelial-dependent *in vitro* whole blood clotting assay, which has been shown to be, at least in part, tissue factor dependent[25].

dsDNA furthermore induced expression of plasminogen activator inhibitor-1 (PAI-1), which is the major physiologic inhibitor of tissue-type plasminogen activator in plasma and thereby serves as an endogenous mechanism to prevent intravasal thrombosis[26]. PAI-1 levels are elevated in many diseases associated with increased risk of ischemic cardiovascular events[27,28] and have recently been shown to be relevant cardiovascular events in chronic hepatitis C infections[29].

Interestingly, we also observed changes in antithrombotic molecules. While thrombomodulin expression seems to be less influenced by intracellular dsDNA, we observe an upregulation of the fibrinolytic molecule tPA

at certain time points, indicating compensatory mechanisms. However, in our functional experiments dsDNA shows an overall prothrombotic effect, which could be explained by a relative stronger expression of the prothrombotic or antifibrinolytic molecules such as PAI-1, a strong prothrombotic counterpart to tPA[14].

Several molecular mechanisms seem to be involved in dsDNA signaling in endothelial cells. Consistent with findings of previous studies in similar cells we found intracellular dsDNA to induce nuclear translocation of the transcription factor IRF3 and to lesser extent also NF-κB[16]. These transcription factors can upregulate proinflammatory and prothrombotic genes. The list of possible receptors being involved in the intracellular sensing of dsDNA is long and further expanding[4–6]. Several of these receptors including AIM2, DAI, TLR9 or RIG-I are expressed in endothelial cells and intracellular dsDNA signaling is likely dependent on more than a single receptor and pathway[30]. In a previous study by our group we could see that upregulation of some inflammatory markers is dependent on TLR9 signaling[15]. Considering the slighter prothrombotic effect of dsDNA after transfection of endothelial cells with siRNA and dsDNA, it was partly reversed with siRNA silencing RIG-I receptor, indicating this receptor to be relevant for prothrombotic effects.

Besides pro-coagulatory effects, dsDNA in endothelial cells induced surface expression of von-Willebrand-factor (vWF) which can bind GPIb-receptor on platelets resulting in platelet-endothelium interactions. Indeed, platelet-endothelial cell-aggregates were significantly increased after prior stimulation of endothelial cells with dsDNA. Moreover, transfection of primary human endothelial cells cultivated in flow chambers led to increased platelet tethering under dynamic conditions. Platelet-endothelial interactions via vWF-GPIb or CD40-CD40L, even when remaining transient, have been shown to further activate the endothelium and thereby increase inflammatory effects resulting in thrombosis but also acceleration in development of atherosclerotic lesions[31,32].

Eventually, dsDNA stimulation led to increased thrombus formation upon light/dye-induced endothelial injury in mouse cremaster vessels *in vivo*. In this model the endothelium is not immediately disrupted, such as in the case of ferric chloride based injury models, but gradually exposed to increasing oxidative stress. Thus, *in vitro* observed effects of dsDNA are likely to contribute to the prothrombotic effects. However, additional effects including expression of other endothelial cell surface molecules, such as inflammatory adhesion molecules (e.g. ICAM-1, VCAM-1) or P-Selectin, but also redistribution or interplay between surface molecules may foster prothrombotic effects. Furthermore, other mechanisms such as modulation of endothelial miRNA expression, e.g. miR-126 might influence endothelial injury and consecutive vascular thrombosis[33]. While accelerated complete thrombotic vessel occlusion was consistent in arterioles and venules, the onset of thrombus formation was accelerated only in arterioles. Among the differences between mechanisms contributing to thrombosis in arterioles and venules lower shear rates as well as a considerable role of leukocytes in venules are the most important ones. vWF is particularly important for platelet adhesion at higher shear rates, which could explain significant differences in the onset of thrombus formation in arterioles but not in venules. Notably we observed increased surface expression of vWF *in vitro* upon stimulation of dsDNA which would support this hypothesis.

Poly (dA:dT) when transfected into cells mimics effects of viral DNA and has been used as an analog for viral DNA in previous studies by ourselves and others[15–17]. Viral infections, such as hepatitis B are often associated with vasculitis and subsequently thromboembolic complications[7,8,34]. We therefore used immunoprecipitates isolated from a patient with ongoing hepatitis B infection and associated systemic vasculitis, to stimulate primary endothelial cells. Indeed, with regard to slight differences in the kinetics, we do see a similar upregulation of prothrombotic genes as compared with synthetic dsDNA stimulation, suggesting a putative role of dsDNA triggered endothelial activation in viral infections. Our observations could help to elucidate the so far incompletely understood relation between viral infections and atherothrombotic diseases.

Apart from viral infections, dsDNA can be released into the bloodstream from damaged host cells in the context autoimmune diseases, tumor lysis or formation of neutrophil extracellular traps (NETs). We have previously shown that human genomic dsDNA can enter the intracellular compartment of endothelial cells under certain circumstances and induce an inflammatory response[15]. In this study we demonstrate that transfection of cultured endothelial cells with human genomic dsDNA accelerates blood clotting *in vitro*. The induced phenotype is therefore similar to the one observed after poly(dA:dT) treatment, however the extent of the prothrombotic effect observed was less pronounced as compared to stimulation with poly(dA:dT). This prothrombotic phenotype might thereby contribute to thrombo-embolic complications in inflammatory autoimmune disorders that are well known to be associated with an increased risk of atherothrombotic events[35–37].

In conclusion we could show for the first time a direct pathophysiological role of viral as well as genomic intracellular dsDNA in thrombosis and haemostasis by endothelial mediated mechanisms. Our results are in line with the growing evidence, that non-primary immune cells, such as endothelial cells, play an important role in the recognition and reaction to pathogen- and danger associated molecular patterns within the innate immune system. Ultimately, our findings represent a novel link in the increasingly recognized reciprocal connection between innate immunity and thrombotic disorders and therefore could be relevant for therapeutic decisions in patients with inflammatory and cardiovascular diseases.

Materials and Methods

Chemicals and Antibodies. Poly(dA:dT) was from Invivogen (Toulouse, France), human genomic DNA was from AMS Biotechnology (Milton Park, UK), Lipofectamine 2000 transfection agent was from Invitrogen (Carlsbad, USA), Accutase was from PAA (Cölbe, Germany), p65-NF-kB antibody and rabbit IRF3 antibody were from (Cell Signaling Technology, USA), goat lamin A/C antibody was from Santa Cruz Biotechnology (USA). DAPI, Alexa Fluor 488 Chicken Anti-Rabbit IgG, Alexa Fluor 546 Donkey Anti-goat IgG were all from Invitrogen (UK). Predesigned TaqMan primers for tissue factor, PAI-1, tPA, thrombomodulin and GAPDH were from Applied Biosystems (Carlsbad, California, USA), PAI-1 ELISA-Kit was from Abcam (UK), RIG-I-siRNA and negative control siRNA was from Ambion/ThermoFisher (USA). Calcein-AM was from Merck-Millipore

(Darmstadt, Germany), anti-human tissue factor-FITC- and anti-human vWF-FITC-antibodies and respective isotype-controls were from AbD Serotec (Oxford, UK), anti-human CD41-APC-antibody was from BD (USA). All other chemicals unless otherwise indicated in the method section were from Sigma Aldrich (Taufkirchen, Germany).

Isolation of hepatitis B virus-DNA (HBV-DNA) containing immunoprecipitates. HBV-DNA containing immunoprecipitates were isolated from a patient with a hepatitis B associated polyarteritis nodosa with a high viral load, during routine plasmapheresis treatment as described previously[15]. The concentration of HBV-DNA used for stimulation was 2.1×10^6 geq/mL as confirmed by real-time-PCR. Written informed consent for the collection of plasma samples was obtained and the procedure was approved by the university ethics review board.

Cell culture and stimulation of endothelial cells. Human microvascular endothelial cells (HMEC) were provided by Ades *et al.*[38], human umbilical vein endothelial cells (HUVECs) were isolated and cultivated as described previously[23]. Briefly, cells were cultured in M199 media supplemented with 10% fetal calf serum, 10% endothelial growth media (PromoCell, Heidelberg, Germany), and 1% penicillin/streptomycin. The procedure was approved by the university ethics review board. dsDNA stimulation was performed as previously described [5]. Briefly, 5 µg/mL poly(dA:dT), or for indicated experiments 5 µg/mL human genomic DNA or HBV-DNA containing immunoprecipitates were complexed with 1 µl of Lipofectamine 2000 in order to achieve intracellular transfection with dsDNA. For stimulation with HBV-containing immunoprecipitates, culture dishes with endothelial cells were additionally centrifuged at 1000 g for 45 min after addition of the immunoprecipitates in order to increase efficiency of viral infection. siRNA knockdown of RIG-I was performed as described previously[15].

Immunofluorescence microscopy. For immunofluorescence experiments, HMEC were grown to confluence in 8-well microscope µ-slides (Ibidi, Germany) and stained as previously described[39]. After treatment of cells as indicated cells were washed with PBS followed by fixation and permeabilization in 100% methanol or for 30 min. Fixed cells were washed again with PBS and blocked with 5% BSA in PBS for 1 h at room temperature. Cells were then incubated with first antibodies diluted 1:200 in blocking solution (goat lamin A/C antibody, mouse p65-NF-kB antibody, rabbit IRF3 antibody) for one hour at room temperature. Afterwards, cells were washed three times with PBS and subsequently incubated with the secondary antibody diluted 1:400 in blocking solution (Alexa Fluor 488 Chicken Anti-Rabbit IgG and Alexa Fluor 546 Donkey Anti-goat IgG) and with DAPI for 30 min. Cells were washed and kept in PBS for microscopy. Images were taken with an Axiovert 200 M microscope with ApoTome (Zeiss, Jena, Germany).

Real-time PCR. RNA isolation from endothelial cells and real-time polymerase chain reaction (PCR) were performed as described previously[23]. Commercially available pre-developed TaqMan reagents were used for the human target genes PAI-1, Tissue factor, tPA and thrombomodulin. GAPDH was used as reference housekeeping gene.

Flow cytometry. Tissue factor and von Willebrand factor were measured on endothelial cell surface by flow cytometry. Endothelial cells after indicated stimulation were washed and detached from dishes using accutase and stained with fluorescent antibodies or respective isotype controls for 30 minutes at 37 °C. Analysis was performed using a FACS Canto II flow cytometer (BD, USA).

ELISA. PAI-1 protein levels were measured in supernatants of cultured endothelial cells after indicated treatments by ELISA according to the manufacturer's protocol.

Blood donors. All blood donors were healthy volunteers, who had given written consent and had not taken any medications for at least 10 days. The investigation was carried out according to the principles of the Helsinki-Declaration.

Endothelial-dependent blood clotting assay. Endothelial cells were stimulated as indicated and then lysed with 15 mM n-octyl-D-glycopyranosidase in 0.1 M imidazol buffer; 20 µL of cell lysate and 20 µL of 200 mmol/L CaCl2 for re-calcification were added to 300 µL of citrated (3.13% sodium citrate) human whole blood from healthy volunteers, and clotting time was measured by thromboelastometry (ROTEG; Tem Innovations, Munich, Germany).

Platelet preparation. Platelet isolation was performed as previously described[40]. Platelet rich plasma was obtained by centrifugation of anticoagulated (3.13% sodium citrate) whole blood at 340 *g* for 15 minutes. After another centrifugation step at 600 *g* for 10 minutes in the presence of 2 ng/mL Prostaglandin, platelets were washed and resuspended in calcium-free modified Tyrode buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO3, 400 mmol/L Na2HPO4, 1 mmol/L MgCl2, 5 mmol/L *D*-glucose, and 5 mmol/L HEPES) and adjusted to the concentration required for the respective experiment. Platelet counts were obtained using a resistance particle counter (Coulter Z2, Beckman Coulter, Krefeld, Germany).

Platelet-endothelial cell-aggregates. Endothelial were cultivated and stimulated as indicated. After removing the transfection medium and several washing steps with PBS isolated washed platelets from healthy donors (prepared as described above) were co-cultivated with endothelial cells for 6 hours. Cells were then washed with PBS in order to remove non-adherent platelets and detached with accutase. Platelet-endothelial

cell-aggregates were measured by staining with anti-human CD41-APC antibody and quantified by flow cytometry.

Flow chamber assay. Endothelial cells were cultivated and stimulated as indicated in a µ-slide from IBIDI (Martinsried, Germany). Freshly isolated human platelets were labeled with Calcein (10 µM for 30 min) and perfused over the cultured endothelial cells at a shear rate of 1 dyn/cm^2. Live immune-fluorescence imaging was performed using a Zeiss Axiotech Vario microscope (Carl Zeiss, Oberkochen, Germany). Images were recorded with a digital camera (AxioCam HSm; Carl Zeiss). From the resulting length of the platelet trace in single images, velocities of single platelets were calculated by using the exposure time of each single picture. Platelet-endothelial cell-interaction was expressed by analysis of the amount of tethering platelets (platelets not moving in at least one single picture) as well as by analysis of frequency histograms consisting of all platelet velocities.

Animals. Animal experiments were performed in wildtype C57Bl/6 mice, which were purchased from Charles River (Sulzfeld, Germany). Surgical procedures were performed under short-term anesthesia induced by a single intraperitoneal injection of Midazolam 5 mg/kg (Ratiopharm, Germany), Fentanyl 0.05 mg/kg (CuraMED Pharma, Germany), and Medetomidinehydrochloride 0.5 mg/kg (Pfizer, Germany; produced by Orion Pharma, Finland) diluted in 0.9% NaCl. All experiments were conducted in accordance with the German animal protection law and approved by the district government of Upper Bavaria. The investigation conforms to the Directive 2010/63/EU of the European Parliament.

Intravital assessment of microvascular thrombosis. Microvascular thrombosis *in vivo* was investigated in arterioles and venules of the mouse cremaster muscle using a light/dye-injury model as previously described[41,42]. After anesthesia all surgical procedures were conducted on a thermo-controlled plexi-glass stage to maintain body temperature at 37 °C with cover slips for microscopy. Intravital fluorescence microscopy was performed using a modified microscope (Zeiss Axiotech Vario, Carl Zeiss Microscopy GmbH, Germany). Images were recorded with a digital camera (AxioCam HSm, Carl Zeiss Germany) and analyzed with "AxioVision" (Carl Zeiss Microscopy GmbH, Germany). After the surgical preparation of the cremaster muscle 1–2 arterioles and 1–2 venules per cremaster with a diameter of around 50 µm were chosen and FITC-dextran (average molecular weight 150,000 (Sigma-Aldrich, Germany)) in a concentration of 5% in PBS was given in a dose of 1 µl/g mouse via a tail vein catheter. Light with a wave length of 450–490 nm was used to excite the fluorescent dye inducing photochemical injury of the endothelial cell layer. Two end points were defined, first the onset of thrombus formation and second the time until total cessation of blood flow (occlusion time).

Statistical Analysis. Data were analyzed using Student *t*-test to compare normally distributed variables and Mann–Whitney *U* test when normal distribution was not given. All data are expressed as mean ± SEM. Differences were considered significant when the error probability was $P < 0.05$.

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Author Contributions

E.G. planned and performed most of the experiments, analyzed the data and took part in writing the manuscript. T.C., A.R., Y.H., M.B. and M.M. performed experiments. H.M. and C.S. revised the manuscript for intellectual content. M.W. and J.P. designed the study, analyzed the data, performed experiments and wrote the manuscript.

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Name, Vorname

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