別紙4

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
丸山　征郎	炎症のトポロジーへの血球関与のスペクトラム	丸山　征郎	別冊BIO Clinica 慢性炎症と疾患	北隆館	東京都	2013	9-13
伊藤　隆史	PAMPｓ/DAMPｓのセンサー細胞としてのマクロファージ・樹状細胞	丸山　征郎	別冊BIO Clinica 慢性炎症と疾患	北隆館	東京都	2013	84-89

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shrestha C, Ito T, Kawahara KI, Shrestha B, Yamakuchi M, Hashiguchi T, Maruyama I.	Saturated fatty acid palmitate induces extracellular release of histone H3: A possible mechanistic basis for high-fat diet-induced inflammation and thrombosis.	Biochem Biophys Res Commun.	437	573-578	2013
Sarikaphuti A, Nararatwanchai T, Hashiguchi T, Ito T, Thaworanunta S, Kikuchi K, Oyama Y, Maruyama I, Tancharoen S.	Preventive effects of Morus alba L. anthocyanins on diabetes in Zucker diabetic fatty rats.	Exp Ther Med.	6	689-695	2013
Takenouchi K, Shrestha B, Yamakuchi M, Yoshinaga N Arimura N, Kawaguchi H, Nagasato T, Feil R, Kawahara KI, Sakamoto T, Maruyama I, Hashiguchi T	Upregulation of non-β Cell- derived Vascular Endothelial Growth Factor A Increases Small Clusters of Insulin producing Cells in the Pancreas.	Experimental and Clinical Endocrinology & Diabetes	in press	in press	2014
伊藤 隆史	メタボリックストレスと自然炎症	Thrombosis Medicine	4 (1)	52-55	2014

丸山 征郎	東洋医学における血管観	血管医学	15 (1)	87-91	2014
丸山 征郎	生体のシステムから観た漢方の有効性とその作用機序	日本東洋医学会専門医会誌（ＤＶＤ）			2014
丸山 征郎	より大いなるヒトの生存の原理と戦略を俯瞰する －漢方薬の論理と効果－	Kampo Square	In press		2014

IV.　研究成果の刊行物・別刷

総論 炎症のトポロジーへの血球関与のスペクトラム

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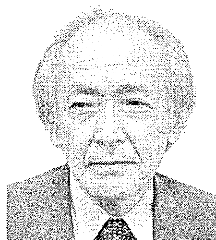

丸山 征郎
1972年，鹿児島大学医学部卒業後，米国セントルイス市ワシントン大学（1982～85年）にて血管内皮細胞の研究（トロンボモジュリンの純化精製，構造と機能などに従事。帰国後遺伝子クローニング，遺伝子組み替え体の臨床展開に従事。
研究テーマ：PAMPs/DAMPsと生体反応
趣味：チェロ（練習中），ジョギング，フルマラソン

Key words：topology of inflammation, DAMPs, PAMPs, inflammasome

はじめに

これまで炎症は，紅腫熱疼（＋機能障害），すなわち紅く腫れて熱感を帯びて痛み，そして機能障害をきたす病態概念で把握され，治療の標的とされてきた。しかし実は，炎症の「場」こそ，免疫応答や止血や治癒修復の**プラットフォーム**でもあり，生体にとって，侵襲に対する防御と修復にむかう必須のプロセスでもある。問題は過剰，あるいは時空と程度を逸脱した炎症が確かに生命活動の維持にとって負の要素が大きく，これは治療の対象となるのである。このように炎症は，痛みや腫脹という負の要素とトレードオフしつつ，感染防御，修復などのプラスの面を発揮しているのである。

本特集の意図は，炎症の位相，トポロジーが時間的，空間的に逸脱して，慢性化，急性転化，急性増悪，あるいは遠隔臓器波及に至るという病態について，特に血球の関与という点を念頭に置きつつ解説展開するということである。

1．炎症のダイナミズム：PAMPsとDAMPsによって構築される生体防御系

1）ダイナミックホメオスタシス

我々は始終何らかの侵襲に曝されている。これらの侵襲のうち，外因性のものは多くは病原微生物由来の分子やそのフラグメントであり，これらはPathogen-Associated Molecular Patterns（PAMPs）と呼ばれる。一方，体内でも細胞や組織が崩壊して生成される分子類のなかで，一種の異物として作用する分子類がある。それらの因子はDamage-Associated Molecular Patters（DAMPs）と呼ばれる。PAMPsもDAMPsも細胞膜上あるいは細胞内に発現しているPattern Recognition Receptors（PRRs）によって認識されて炎症を惹起する[1)]（図1）。PAMPs，DAMPsは細胞内NF-κBの活性化を引き起こし，IL-18，IL-1βの前駆体（pro-IL-18，pro-IL-1β）を産生，細胞内に蓄積する[2)]（図2）。しかしこれらのサイトカインは，プロ型の状態では細胞外には放出されず，セカンド刺激として各インフラマソーム（inflammasome）

Topology of inflammation and spectrum of blood cell contributions：Ikuro Maruyama, Systems Biology in Thromboregulation (Cooperation with Medipolis Research Institute), Kagoshima University Graduate School of Medical and Dental Sciences

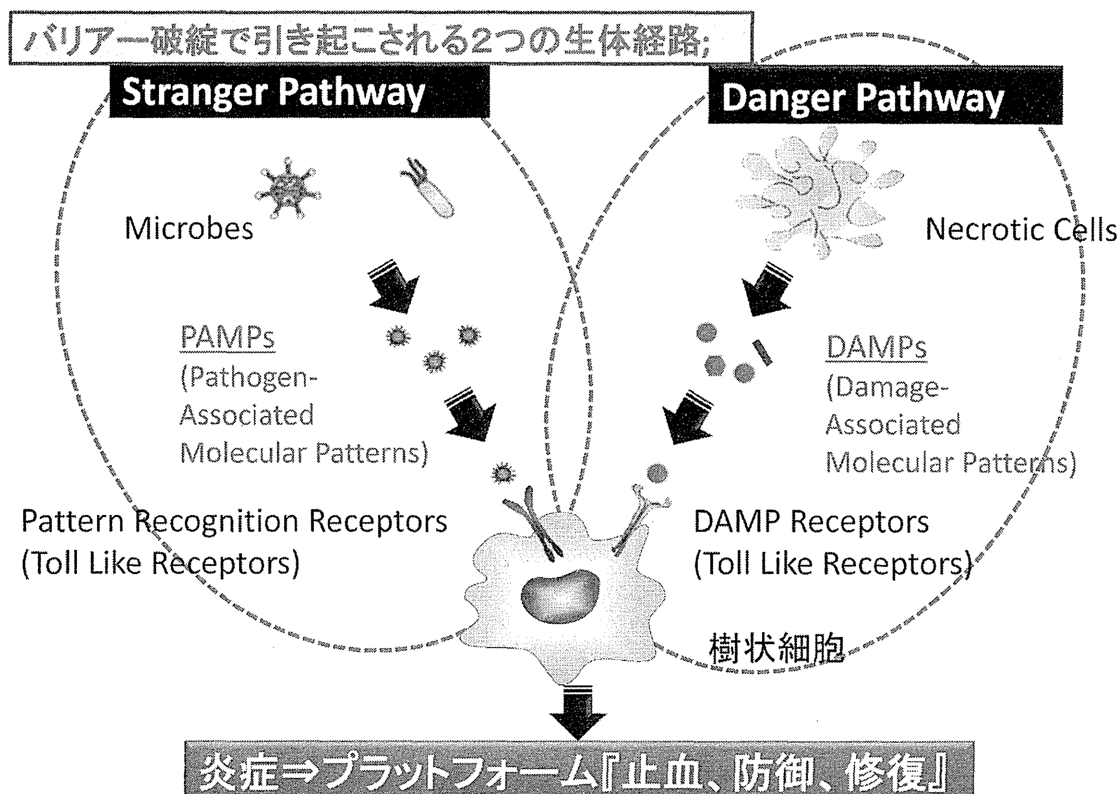

図1 PAMPs, DAMPsとPattern Recognition Receptors (PRRs) による侵襲応答

によってprocaspase-1がcaspase-1に活性化されて初めて, pro-IL-18, pro-IL-1βが成熟型となり, 細胞外に放出される。細胞外ではIL-1β, IL-18はリンパ球, NK細胞を集簇させ, IFN-γをはじめとする炎症性サイトカインの産生を促し, かくて当該部位で炎症が惹起される[3]。このようにPAMPsやDAMPsに曝される機会の多い非免疫組織の表皮や各臓器の表皮では, pro-IL-1β, Pro-IL-18を作って準備状態とし, 次の刺激（セカンドアタック）で速やかにIL-1β, IL-18として細胞外に遊離し, 当該侵襲部位に白血球類を呼び寄せて, 生体防御的免疫系を構築する。このようにPAMPs, DAMPsは生体内細胞にあまねく発現しているPRRs-inflammasomeを介して炎症・免疫, 止血・修復のスクランブル状態としておくことで, 中枢・末梢の神経系, 自律神経系, 内分泌系とは異なったダイナミックでしなやかな応答態勢を構築しているといえよう（図2)。

2) ダイナミックホメオステーシスから急性転化, 急性増悪, 遠隔臓器合併症へ

PAMPs-PRRs経路あるいはDAMPs-PRRs経路の片方からのシグナルで準備状態（pro-IL-1β, pro-IL18の産生）を作り, セカンド刺激で, 一挙に成熟型のIL-1β, IL-18放出を起こすという仕組みは, 緊急の生体防御に有利

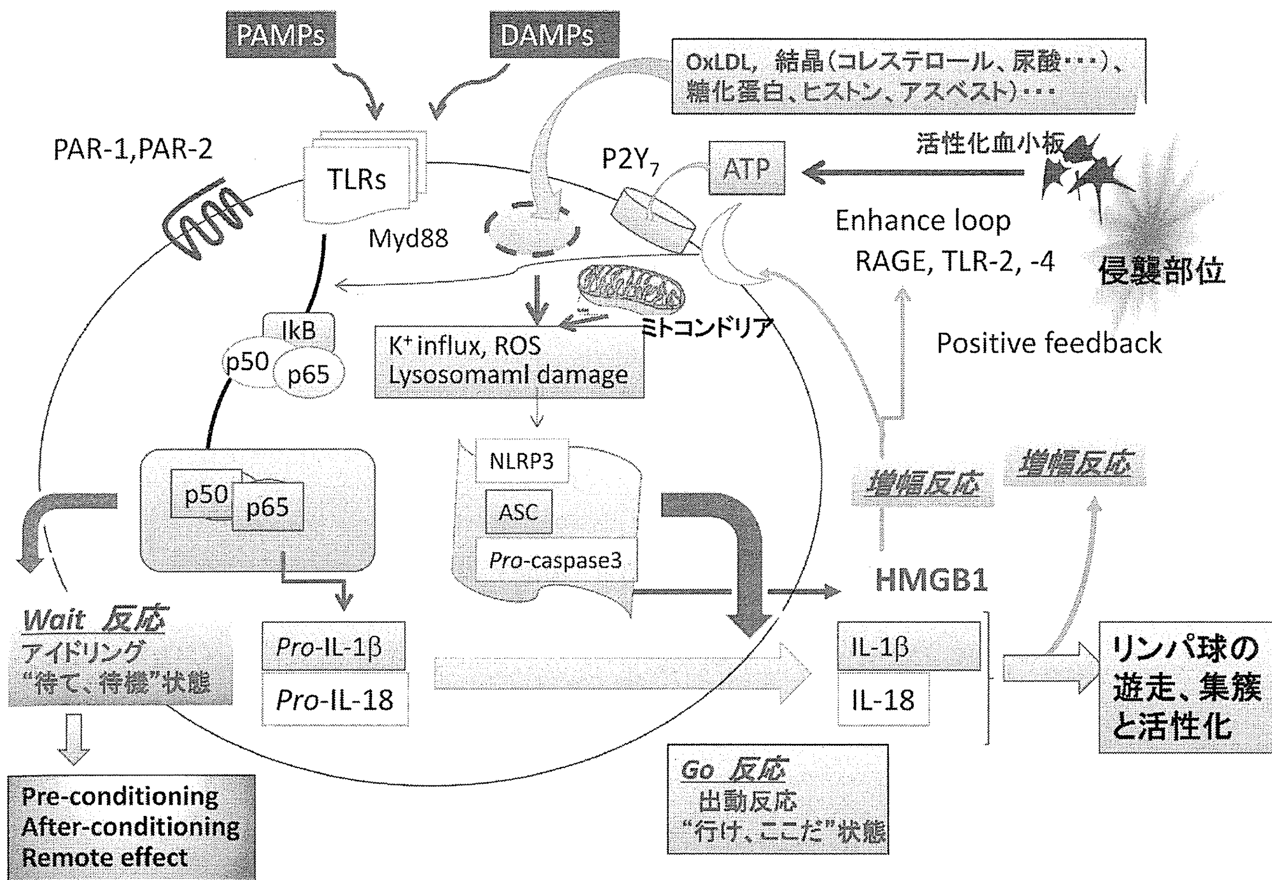

図２ PAMPs/DAMPs シグナリングと活性化血小板由来の ATP による反応の時間的，空間的増幅と拡散

である(緊急—増幅型応答)。しかし一方では，これは諸刃の刃でもある。すなわち DAMPs（あるいは PAMPs）により，インフラマソーム，あるいは NF-κB 活性化状態となっている白血球が循環している状態で，セカンド刺激が入ってくると，一挙に炎症が惹起されることになる。例えば肺や腎，消化管—肝臓など，外に開かれた臓器において PAMPs の侵襲を受けると，その臓器では一挙に炎症が発症する。いわば“出会いがしら”のアクシデンタルな病態で，間質性肺炎の急性増悪や，なんらかの基礎疾患がある状態，老化などでの肺炎や急性転化などがこれにあたる。さらにこれが全身性に波及すると，Systemic Inflammatory Response Syndrome（SIRS）や播種性血管内凝固症候群(DIC)[4]などの病態に発展する。

2．反応の最上流としての骨髄

1）骨髄幹細胞はリンパ管をパトロールして，障害部位で活性化，成熟化する

骨髄幹細胞は骨髄で産生されると，静脈系⇒リンパ管内を流れてパトロールしており，組織臓器の侵襲障害部位で活性化されて分化し，“その現地”で増殖して，修復に働く[5]。このように骨髄幹細胞は，骨髄からスタート

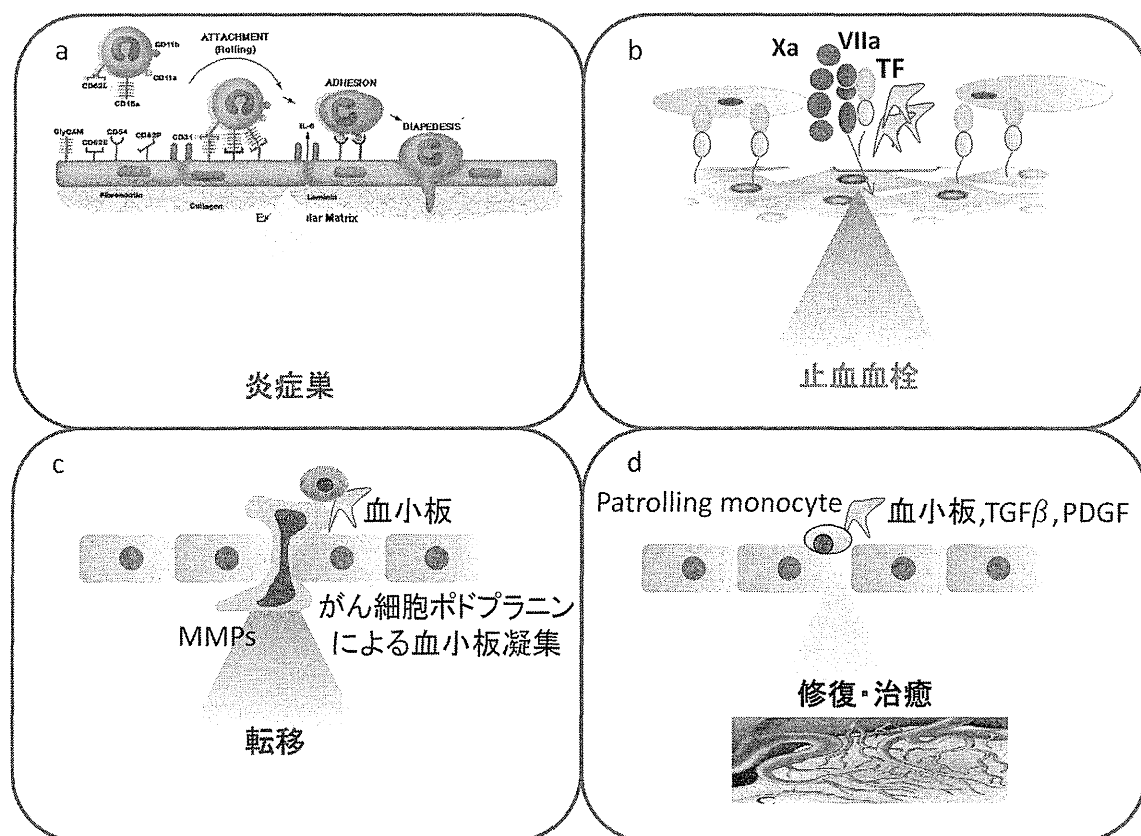

図3 “流れる組織”としての血液とパトローリング単球（d）

して，全身を循環し，異状部位の認識と修復に働いているという意味では，“流れる前駆体組織”ともいえよう。

2）途中下車は末梢が決める

血管の中には前述の多機能性骨髄幹細胞のほかに，末梢の血管網の異状部位を認識するパトローリング単球も流れており[6]，異状部位の認識と修復にあたる（図3）。例えば血管損傷が生ずると，その部位を速やかに認識して，止血と免疫に預かる。一方，損傷血管部位では，周辺の活性化内皮細胞のWeibel-Paldade小体から巨大分子型のvon Willebrand因子（vWF）が放出され，これが露出したコラーゲンに結合し，このコラーゲン・vWFに対して血小板が粘着・凝集してくる。この血管損傷部位には組織因子も露出しており，これに活性化凝固第Ⅶ因子が結合して凝固の外因系のトリガーが引かれる。このプロセスは“止血機転”とそこを足場にした修復反応に他ならないが，生成された血栓の表面には，活性化内皮細胞や単球からマイクロパーティクルが放出されて，血小板や血栓表面に結合して止血・炎症反応を促進する[7]。このように“流れる組織”としての末梢血幹細胞ほか血球がどこで“下車”して，止血・修復・炎症・免

系応答を発揮するかは，末梢が決めることになる。すなわち末梢のディマンドである。

3．マイクロベジウル類とNETsが創る新たなホメオスターシス

血球をはじめ，生体内の種々の細胞がアポトーシス，あるいは活性化されると，マイクロパーティクルをはじめとする種々のマイクロベジクルを放出する[8)]。このマイクロビジクルは，サイズ，生成過程，内蔵物によって，いくつかに分類されているが[9)]，生体の諸過程を制御する新たな細胞生物学の様式であることが判明してきて，現在，炎症やがんなどとの関連が精力的に研究されている。

一方，血小板は単球や好中球とハイブリッドを形成して接着性を高める一方，好中球からの核を放出させることも判明してきた。これをNeutrophile Extracellular Traps（NETs）と称するが，NETsの中には好中球由来のDNAはDAMPsとしてのヒストンや，HMGB1，elastaseなどが含まれており，殺菌や炎症，血栓の形成などに大きな役割を果たすことが注目されている[10)]。

おわりに

血球，あるいはその断片であるマイクロベジクル，逆に好中球と血小板のハイブリッド，NETsなどは新たな炎症像を提示しつつある。本特集では，血球と炎症の関係について，それぞれのパートのフロントランナーに関して解説してもらった。

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13 PAMPs/DAMPsのセンサー細胞としてのマクロファージ・樹状細胞

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研究テーマ：炎症と血栓
趣味：テニス

Key words：PAMPs，DAMPs，パターン認識受容体

Abstract

獲得免疫細胞が非自己抗原に出会ったとしても，それだけでは免疫応答は作動しない。自然免疫細胞による補助刺激シグナルが必要である。自然免疫細胞は獲得免疫細胞のような抗原特異的受容体は持ち合わせていないが，パターン認識受容体でもって感染や組織損傷の兆候を察知し，補助刺激分子を発現するようになる（図1）。感染の兆候は，外来微生物に共通で特有の構成成分（Pathogen-associated Molecular Patterns：PAMPs）という形でパターン認識され，組織損傷の兆候は，損傷に伴って細胞外へと放出される細胞内分子（Damage-associated Molecular Patterns：DAMPs）という形でパターン認識される。この自然免疫細胞によるパターン認識が，それに続く獲得免疫応答のスイッチを入れているのである。

はじめに
―自然免疫細胞の監視対象―

「免疫細胞は何を監視しているのか？」という問いは免疫学の中心テーマである。Burnetはクローン選択説において，監視対象が「非自己」であることを提唱した。あらゆる抗原レパートリーの中で，自己に反応するリンパ球が発生初期段階で取り除かれ，非自己に対する監視体制を作り出しているのである。しかしながら，免疫細胞が非自己抗原に出会ったとしても，それがありふれた無害な抗原であれば，免疫応答は作動しない。Janewayは，非自己に対する獲得免疫応答のスイッチは，自然免疫系が「感染」の兆候を察知した際に「オン」になると考え，監視対象は「感染性の非自己」であることを提唱した。その一方で，Matzingerは Janeway説では移植免疫，腫瘍免疫，自己免疫などを説明できないことに注目し，非自己に対する獲得免疫応答のスイッチは，自然免疫系が「危機的状況」を察知した際に「オン」になると考え，監視対象は「宿主の細胞が被ったダメージ」であることを提唱した。外来微生物であっても，共生的で無害であれば免疫応答は生じないし，非感染性の腫瘍であっても，細胞壊死を伴えば免疫応答は生じうる，という考えである[1,2]。実際には，Janeway説の「感染の兆候」は，外来微生物に共通で特有の構成成分（PAMPs）という形で認識され，Matzinger説の「危機的状況」は，ダメージの結果として細胞外に漏れ出てくる細胞内成分（DAMPs）という形で認識される。自然免疫細胞はパターン認識受容体というセンサーを発現していて，PAMPsおよびDAMPsの存在を常に監視しているのである（図1）。

PAMPs/DAMPs are subjects to surveillance by innate immune cells：Takashi Ito，Systems Biology in Thromboregulation (Cooperation with Medipolis Research Institute), Kagoshima University Graduate School of Medical and Dental Sciences

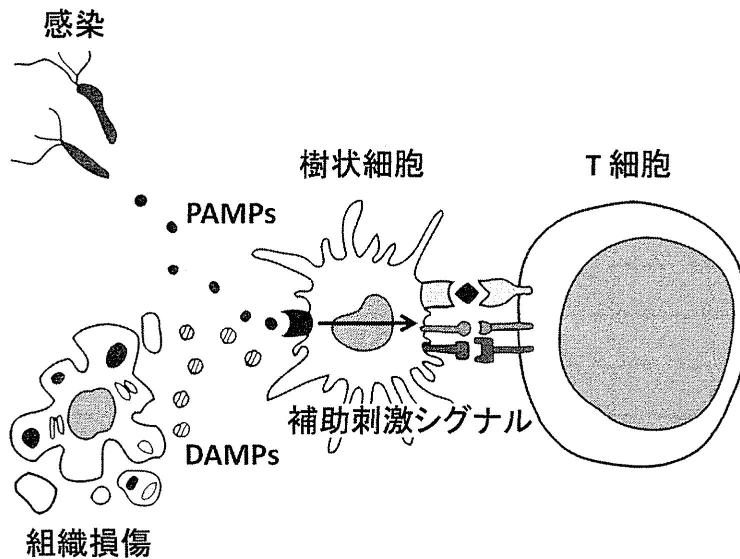

図1　獲得免疫応答に必要な抗原提示と補助刺激

表1　代表的なPAMPs/DAMPsと宿主免疫細胞の受容体

起源	PAMPs	受容体	起源	DAMPs	受容体
細菌	エンドトキシン	TLR4	核	HMGB1	RAGE, TLRs
	リポプロテイン	TLR2		ヒストン	TLR2, 4
	フラジェリン	TLR5		DNA	TLR9
	CpG-DNA	TLR9		RNA	TLR3
	RNA	NLRP3	ミトコンドリア	fMLP	FPR1
ウイルス	dsRNA	TLR3, RIG		DNA	TLR9
	ssRNA	TLR8	細胞質 他	尿酸	NLRP3
	CpG-DNA	TLR9		ATP	NLRP3
真菌	β-グルカン	Dectin		S100	RAGE

PAMPs/DAMPsとして，現在，数百種類の分子が報告されており，それらは数十種類のパターン認識受容体によって察知される（表1）。具体的には，グラム陰性菌の細胞壁の構成成分であるエンドトキシン(Lipopolysaccharide：LPS)，鞭毛の構成成分であるフラジェリン，ウイルスの二重鎖RNA(dsRNA)，真菌のβ-グルカンなどがPAMPsにあたり，宿主の自然免疫細胞は，Toll様受容体（Toll-like Receptors：TLRs）などのパターン認識受容体でPAMPsの存在を監視している。一方，DAMPsとしては，宿主細胞の核

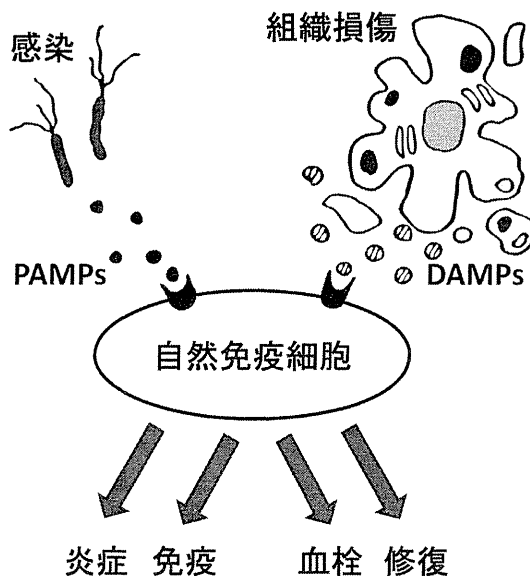

図２　PAMPs/DAMPs 刺激と細胞応答

内タンパク質である High-mobility Group Box 1（HMGB1），ミトコンドリアに含まれているホルミルペプチド（fMLP），エネルギー通貨であるアデノシン三リン酸（ATP），核酸最終代謝産物である尿酸などが知られていて，これらの分子が細胞外に漏出すると，細胞損傷の兆候として自然免疫細胞に察知される。

1．DAMPs の役割

HMGB1 や尿酸結晶などの DAMPs は，前述の通り，損傷を受けた細胞から放出されると，樹状細胞を活性化して補助刺激分子の発現を促し，獲得免疫応答を誘導する[2,3]。このように，DAMPs は危機的状況を周囲に知らしめるための警鐘（アラーム）としての役割を担っていて，アラーミンとも呼ばれる。アラーミンの役割は，補助刺激分子の発現誘導だけにとどまらない。好中球，単球，マクロファージ，樹状細胞などの自然免疫細胞を組織損傷部位に呼び寄せ，さらにはこれらの細胞を活性化して炎症性サイトカインの発現を誘導する[2,4−6]。また，幹細胞を組織損傷部位に呼び寄せ，創傷治癒にも寄与している[7]。このように，アラーミン（DAMPs）は組織損傷後の炎症，免疫，創傷治癒を誘導する分子として，生体防御に重要な役割を担っている（図２）。

2．PAMPs/DAMPs とインフラマソーム

尿酸結晶や ATP などの DAMPs は，自然免疫細胞のインフラマソームを活性化する（図３）。インフラマソームは炎症性サイトカイン IL-1β および IL-18 を活性化して分泌するのに必須の細胞内分子装置である[8]。LPS などの PAMPs 刺激を受けた自然免疫細胞は，IL-1β および IL-18 の前駆体（pro-IL-1β および pro-IL-18）を産生するが，これらはすぐに分泌されずに待機状態にある。そこに，尿

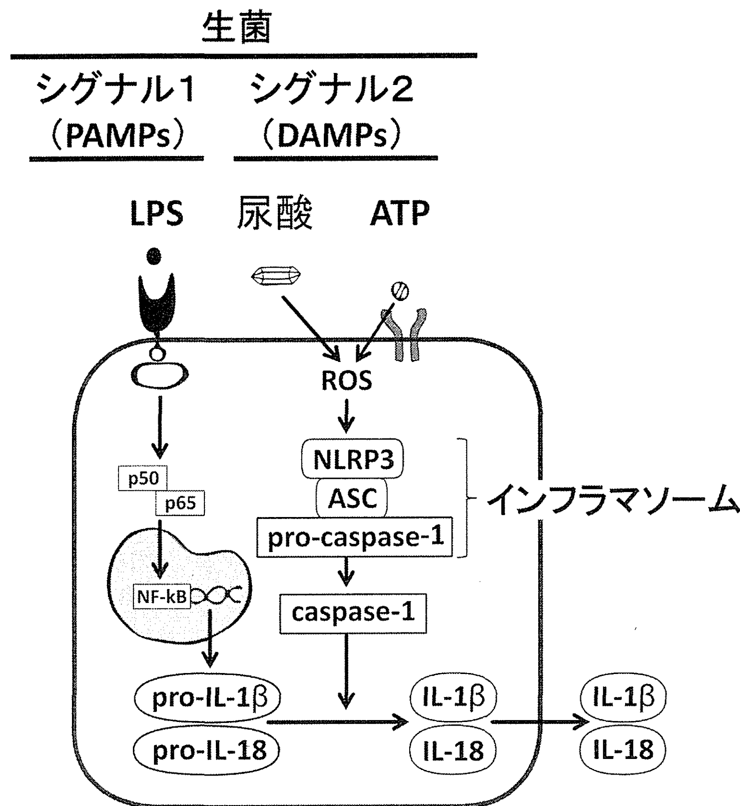

図3 IL-1βの分泌に必要な2つのシグナル

酸結晶やATPなどのDAMPs刺激が加わると，インフラマソームが活性化され，pro-IL-1βおよびpro-IL-18はそれぞれ活性型のIL-1βおよびIL-18に転換され，細胞外に分泌される（図3)。このように，IL-1βおよびIL-18の分泌は2ヒット方式を採用している。すでに死滅した菌であってもPAMPsは保有しているため，PAMPsのみを察知した場合には，自然免疫細胞は戦闘準備状態で待機し，続いて組織損傷を察知した場合に，炎症反応の引き金を引くのだと考えられる。こうすることで，不必要な場面での過剰な炎症反応を防いでいるのであろう。一方，生きた菌を察知した場合には，それ単独でIL-1βおよびIL-18の活性化と分泌が誘導される[9)]。この際には，生菌が有しているpoly-A tailのないメッセンジャーRNAがインフラマソームの活性化に重要な役割を果たしている。生菌を察知した場合には，たとえ組織損傷がなくても2ヒット分の刺激が入り，感染防御体制を迅速に整えていると考えられる。

3．DAMPsと慢性炎症

DAMPsは組織損傷に伴って壊死細胞から放出される分子と考えられていたが，その後の研究で，細胞死に至るような強い傷害でなく，軽度のストレス下においても放出されうることが報告されている。ストレスが最小な状態を健常状態，最大の状態を細胞死と考え

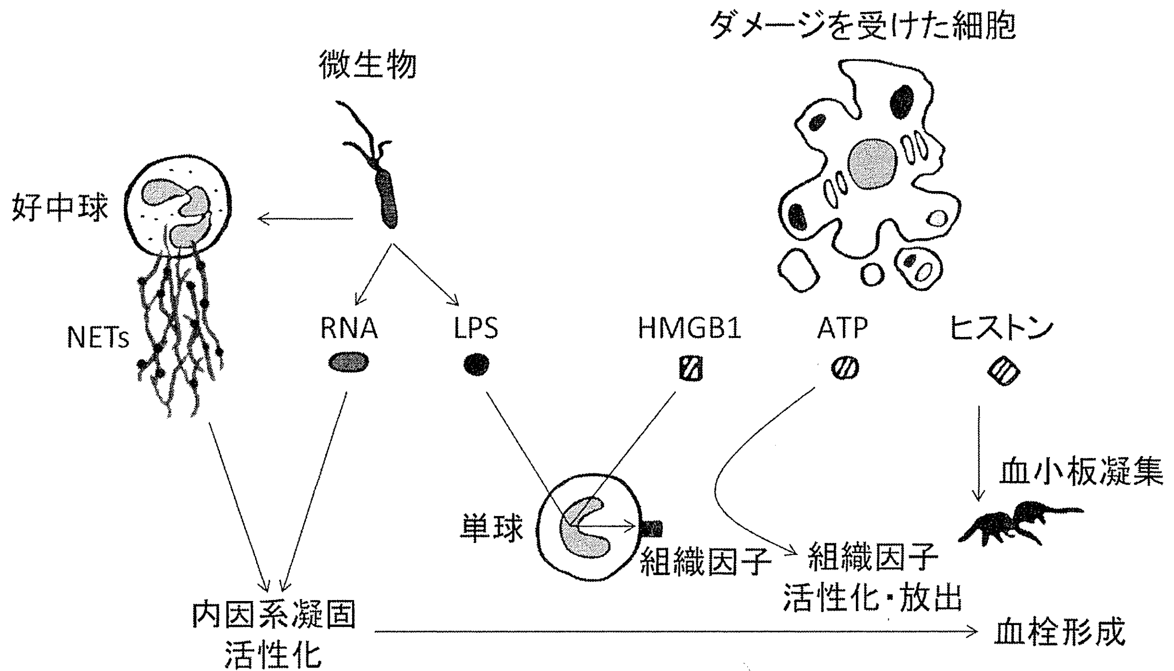

図４　PAMPs/DAMPsと血栓形成

た場合，この中間状態においては，細胞はストレスに適応し，恒常性を維持しようとする。このような状況は，para-inflammationもしくは自然炎症という概念で近年注目を集めていて，肥満，糖尿病，動脈硬化などの病態基盤となっている可能性が示唆されている[10]。高血糖状態では，ミトコンドリアにおける活性酸素種の産生が増加し，これによってインフラマソームが活性化されてIL-1βおよびIL-18の分泌が惹起される[11]。過剰なコレステロールは結晶化して血管壁に沈着し，インフラマソームの活性化を介して動脈硬化を進行させる[12]。また，高脂肪食の摂取や脂肪酸の負荷によるストレスで，核内タンパク質HMGB1やヒストンが細胞外に放出され，肥満や非アルコール性脂肪肝における炎症性変化，血栓傾向などに寄与している可能性が報告されている[13,14]。

4．PAMPs/DAMPsと血栓症

通常，PAMPsは上皮というバリアの外に存在し，DAMPsは細胞膜というバリアに囲まれて存在するため，血液成分もしくは血球がPAMPs/DAMPsと接することはない。しかしながら，このバリアが崩壊し，血液成分もしくは血球がPAMPs/DAMPsを察知すると，血栓形成の引き金が引かれる。単球はパターン認識受容体を介して，グラム陰性菌の細胞壁の構成成分であるLPS（PAMPs）や，細胞外に放出された核内タンパク質HMGB1（DAMPs）を感知すると，活性化して組織因子を発現するようになる[15]。さらに，細胞外に放出されたATP（DAMPs）は，単球表面の組織因子を活性型に転換し，外因系凝固反応を活性化する[16]。細菌由来のRNA（PAMPs），

宿主細胞由来のRNA（DAMPs），活性化した好中球が放出するNeutrophil Extracellular Traps（NETs）は，細胞外においては第XII因子を活性化する陰性荷電異物となり，内因系凝固反応を活性化する[17]。また，NETsの主要成分の1つであるヒストン（DAMPs）は，血小板を活性化する（図4）。このように，感染部位や組織損傷部位で血栓が形成されることは，局所で生じた混乱を中枢に持ち込ませないための意義があると考えられるが，同時に，血栓症としての病的側面も持ち合わせている[17]。急性冠症候群患者では，血栓中に細胞外HMGB1が検出され[18]，血清HMGB1値が予後予測因子となりうることが報告されている[19]。HMGB1高値が組織損傷の程度を反映した「結果」なのか，血栓症を惹起する「原因」となっているのか，更なる検討が必要である。

おわりに

PAMPs/DAMPsの概念は，免疫学の分野で提唱され，急性炎症の舞台で研究が進み，近年は慢性炎症との関連が注目を集めている。診断マーカーとしての意義を越え，PAMPs/DAMPsを制御することで炎症性疾患を治療しようとする試みが進められている。

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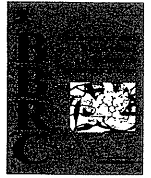

Saturated fatty acid palmitate induces extracellular release of histone H3: A possible mechanistic basis for high-fat diet-induced inflammation and thrombosis

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ABSTRACT

Chronic low-grade inflammation is a key contributor to high-fat diet (HFD)-related diseases, such as type 2 diabetes, non-alcoholic steatohepatitis, and atherosclerosis. The inflammation is characterized by infiltration of inflammatory cells, particularly macrophages, into obese adipose tissue. However, the molecular mechanisms by which a HFD induces low-grade inflammation are poorly understood. Here, we show that histone H3, a major protein component of chromatin, is released into the extracellular space when mice are fed a HFD or macrophages are stimulated with the saturated fatty acid palmitate. In a murine macrophage cell line, RAW 264.7, palmitate activated reactive oxygen species (ROS) production and JNK signaling. Inhibitors of these pathways dampened palmitate-induced histone H3 release, suggesting that the extracellular release of histone H3 was mediated, in part, through ROS and JNK signaling. Extracellular histone activated endothelial cells toexpress the adhesion molecules ICAM-1 and VCAM-1 and the procoagulant molecule tissue factor, which are known to contribute to inflammatory cell recruitment and thrombosis. These results suggest the possible contribution of extracellular histone to the pathogenesis of HFD-induced inflammation and thrombosis.

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1. Introduction

Overweight status and obesity are risk factors for the development of insulin resistance, type 2 diabetes mellitus, atherosclerosis, and fatty liver diseases [1], which are generally caused by increased consumption of high-fat foods with reduced physical activity [2]. Beside these metabolic disorders, obesity is also associated with prothrombotic complication with excess production of tissue factor and plasminogen activator inhibitor-1 [3,4]. Chronic low-grade inflammation is a key contributor to the initiation and development of obesity-related diseases [5], and is characterized by increased secretion of proinflammatory cytokines (TNF-α, IL-6) and decreased secretion of anti-inflammatory cytokines (adiponectin, IL-10) [6,7]. Macrophages are one of the major sources of the inflammatory responses [8], which are associated with high cell infiltration of expanding adipose tissue in obese individuals [7]. In addition to increased macrophage recruitment to adipose tissue, obesity polarizes anti-inflammatory macrophages (M2; alternatively activated macrophages) to proinflammatory macrophages (M1; classically activated macrophages) [9].

Free fatty acid (FFA) levels are elevated in obese subjects through release from enlarged adipose tissue or reduced clearance [10]. FFA promotes inflammatory response signaling through Toll-like receptor (TLR) 4 and activates NF-κB and/or JNK signaling pathways that lead to insulin resistance, hepatic steatosis, and atherosclerosis [6,11–13]. Attenuation of these inflammatory responses, by targeting NF-κB or JNK or neutralizing TNF-α and IL-1β, improves insulin resistance and fatty liver diseases [14–16]. In addition to activation of TLR4, it has been reported that FFA activates NLRP3 inflammasomes, leading to activation of caspase 1 and secretion of IL-1β and IL-18 [16].

Although chronic low-grade inflammation plays a role in obesity-related complications [1,5], little is known about the molecular mechanism underlying its occurrence during HFD intake. A recent study showed that histones, which are nuclear proteins, function as endogenous danger signals or alarmins when they are released into the extracellular space during ischemia-reperfusion injury [17]. Extracellular histones can bind to different immune receptors, TLR2, TLR4, and TLR9, and contribute to leukocytosis, endothelial

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dysfunctions, organ failure, and even death [17–20]. In the present study, we show that HFD feeding or FFA treatment induces an active secretion of histone H3 into the extracellular space. This secretion is mediated, in part, through reactive oxygen species (ROS) production and the JNK signaling pathway. The extracellular histone then activates endothelial cells to express adhesion molecules, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and a procoagulant molecule, tissue factor. These findings suggest the possible contribution of extracellular histone to the pathogenesis of HFD-induced inflammation and thrombosis.

2. Materials and methods

2.1. Reagents

Sodium palmitate (P9767), FFA-free BSA (A6003), N-acetyl cysteine (NAC) (A9165), Bay-11 7082 and oleate (07501G) were obtained from Sigma–Aldrich (St Louis, MO, USA). SP600125 (FA-005) was purchased from SABioscience (Valencia, CA, Spain). SB203580 (559389) and U0126 (662005) were obtained from Calbiochem (Billerica, MA, USA). Anti-histone H3 antibodies, sc-8654 and ab1791, were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Abcam (Cambridge, MA, UK), respectively. Antibodies against p-SAPK/JNK, p-p42/44, p-p38, and p-p65 were purchased from Cell Signaling Technology (Beverly, MA, Canada).

2.2. Palmitate/BSA complex solution preparation

The palmitate/BSA solution was prepared as described previously [21] with slight modifications. Briefly, 100 mM palmitate solution was solubilized in 50% ethanol and then conjugated with 5% FFA-free BSA to achieve a final palmitate concentration of 5 mM. The conjugation was performed to increase the solubility of palmitate. The corresponding BSA solution with ethanol was used as a control. The solutions were prepared on the same day of the experiments.

2.3. Cell culture and treatment

A murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 100 U/ml penicillin–streptomycin, and 5 mg/L amphotericin. The cells were maintained at 37 °C under 5% CO_2. The cells were starved for 2 h in serum-free medium and then stimulated with palmitate/BSA (Pa–BSA) or BSA solution as indicated. Pretreatments with different inhibitors were carried out 1 h before cell stimulation.

2.4. Sample preparation for histone H3

The histone H3 levels in culture supernatants were analyzed by Western blotting as described previously [22]. Briefly, 1.6 ml of supernatant was incubated with heparin-Sepharose 6B beads (GE Healthcare Bio-Science, Piscataway, NJ) at 4 °C overnight, washed with PBS, and mixed with sample buffer for SDS–PAGE.

2.5. Western blotting

Cell lysates or prepared samples were separated by 10–15% SDS–PAGE, transferred to nitrocellulose membranes, blocked with 5% nonfat dry milk, and incubated with specific primary antibodies against histone H3 (1:500; Santa Cruz Biotechnology), p-SAPK/JNK (1:1000), p-p42/p44 (1:1000), p-p38 (1:1000), p-p65 (1:1000) and β-actin (1:1000; Santa Cruz Biotechnology) at 4 °C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:3000; MP Biomedicals, L.L.C, Irvine, CA, USA) followed by detection with an ECL detection system (Thermo Scientific, Waltham, MA, USA). The band intensities were measured using ImageJ 1.46 software (National Institutes of Health, Bethesda, MD, USA).

2.6. Immunocytochemistry

RAW 264.7 cells plated on four-chamber culture slides for 16 h were fixed with 2% paraformaldehyde, permeabilized with a mixture of ethanol and acetone (2:1) at −20 °C for 5 min, and blocked with 1% BSA in PBS containing 0.1% Triton X-100. The cells were then sequentially incubated with an anti-histone H3 antibody (1:250; Abcam) for 24 h at 4 °C, and an Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (A11070; Life Technologies, Carlsbad, CA, USA) for 1 h. The nuclei were stained with DAPI. The stained cells were visualized and photographed under a confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany).

2.7. Immunohistochemical staining

Immunohistochemical staining of histone H3 was performed as described previously [23] with slight modifications. Briefly, epididymal adipose tissue samples from male C57BL/6J mice fed a normal chow diet (crude fat: 4.6%, CLEA Japan Inc., Tokyo, Japan) or HFD-60 (crude fat: 35%, Oriental Yeast Co., Tokyo, Japan) for 12 weeks, were fixed in 10% formaldehyde neutral buffer, paraffin-embedded, and sectioned. The tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked in the presence of 0.3% hydrogen peroxide in methanol for 15 min. After antigen retrieval using an antigen-unmasking solution (Vector Laboratories Inc., Burlingame, CA, USA), the sections were blocked with 1% BSA in PBS containing 0.01% Tween 20 for 1 h. Subsequently, the sections were incubated with a rabbit anti-histone H3 antibody (1:800; Abcam) for 30 min at room temperature, followed by incubation with a secondary antibody for 30 min using a Histofine Simple Stain Mouse MAX-PO Kit (Nichirei Co., Tokyo, Japan). Finally, the sections were stained with DAB and counterstained with hematoxylin. Photographs were taken using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

2.8. ROS measurement

Intracellular ROS production was detected using the fluorescent probe H_2DCFDA (C400; Life Technologies) as described previously [24]. Briefly, RAW 264.7 cells treated with palmitate for 8 h were washed and incubated with 10 μM H_2DCFDA for 45 min at 37 °C. The fluorescence signals were analyzed using an Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA).

2.9. Total RNA isolation and quantitative real-time RT-PCR

Human umbilical vein endothelial cells (HUVECs) were treated with purified unfractionated calf thymus histone (Sigma–Aldrich, St. Louis, MO, USA) at concentrations of 0, 10 and 20 μg/ml for 4 h. Total RNA was extracted from HUVECs using an RNAqueous Total RNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol, and reverse-transcribed to cDNA. The mRNA expression levels of inflammatory genes were assessed with specific primers (Assay IDs: tissue factor, Hs01076029_m1; ICAM-1, Hs00164932_m1; VCAM-1, Hs003655486_m1; TLR 2, Hs00610101_m1 and TLR 4, Hs00370853_m1) as described previously [23]. Expression levels were calculated as the ratio of mRNA

level for a given gene relative to the mRNA level for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same cDNA sample.

2.10. Flow cytometric assay of tissue factor, ICAM-1 and VCAM-1

The cell surface expressions of tissue factor protein, ICAM-1 and VCAM-1 protein were assessed by flow cytometry as described previously [25] using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against human tissue factor (Sekisui Diagnostics), ICAM-1 (Beckman Coulter, Marseille, France) and VCAM-1 (BD Pharmingen).

2.11. Cell viability assay

Cell viability was assessed by MTT assays as described previously [22]. Briefly, 5×10^5 cells in 24-well plates were treated with different concentrations of palmitate (0–200 μM) for 16 h. The cells were then incubated with MTT solution for 3 h. The formazan product was solubilized in dimethyl sulfoxide, and the absorbances were measured at a wavelength of 570 nm with a reference wavelength of 630 nm.

2.12. Statistical analysis

All results are expressed as means ± SEM, and were statistically analyzed by one-way ANOVA or a *t*-test using Prism software. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Extranuclear release of histone H3 in HFD-fed mice

Histones are found in the nucleus of resting cells where they regulate transcription and are involved in chromatin remodeling [18,19]. Consistent with this, histone H3 was mainly localized in the cell nuclei in mice fed the normal chow (Fig. 1A, left panel). However, extranuclear histone H3 was increased in mice fed the HFD (Fig. 1A, right panel). An increase in extranuclear histone H3 was also observed in murine macrophage cell line RAW 264.7 cells treated with the saturated fatty acid palmitate (Fig. 1B). These results indicate that HFD feeding or saturated fatty acid treatment can induce translocation of histone H3 from the nucleus to the cytoplasm.

3.2. Saturated fatty acid palmitate induces extracellular release of histone H3 from RAW 264.7 cells

We treated RAW 264.7 cells with palmitate for time periods of 8–16 h and at increasing concentrations (0–200 μM) for 16 h. Palmitate induced extracellular release of histone H3 in 16 h (Fig. 2A) and in a dose-dependent manner (Fig. 2B). In contrast, the carrier protein BSA or unsaturated fatty acid oleate did not induce histone H3 release (Fig. 2C). The palmitate-induced histone H3 release was not caused by cell death (Fig. 2D), suggesting that active secretion, rather than passive leakage, may be involved in this process.

3.3. ROS and JNK signaling mediate palmitate-induced histone H3 release

Next, we examined the mechanism by which palmitate induces release of histone H3. In line with previous reports [16,26], palmitate induced ROS generation in RAW 264.7 cells (Fig. 3A). Treatment with NAC, a ROS inhibitor, attenuated ROS production (Fig. 3A) and inhibited palmitate-induced histone H3 release (Fig. 3B). These results indicate that palmitate-induced histone H3 release is mediated, in part, through ROS production. We further examined the signaling pathways for histone H3 release. As shown in Fig. 3C, palmitate increased the phosphorylation of p38, JNK, ERK1/2, and NF-κB p65. A selective inhibitor of JNK (SP600125) attenuated palmitate-induced histone H3 release,

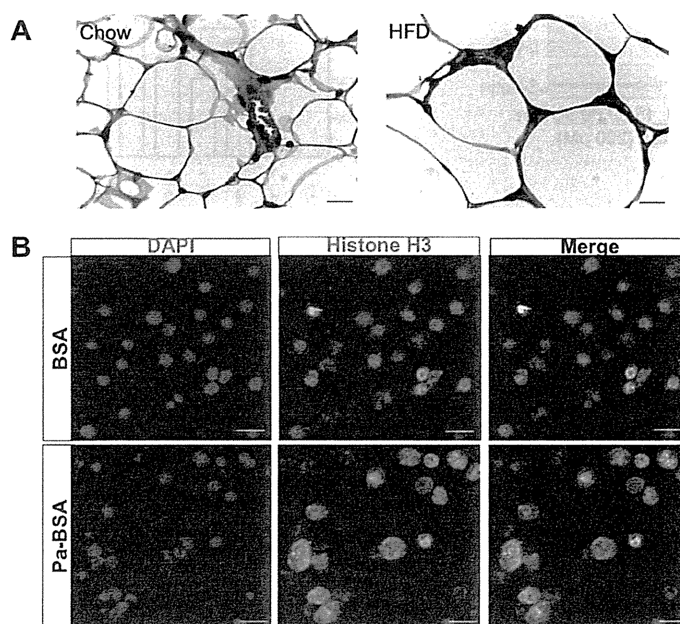

Fig. 1. Nuclear translocation of histone H3 *in vitro* and *in vivo*. (A) Representative photographs of immunostaining for histone H3 in adipose tissue from mice fed with a normal diet or HFD for 12 weeks. Original magnification: 40×; scale bar: 20 μm. (B) RAW 264.7 cells were plated on four-chamber culture slides and treated with 200 μM of BSA or palmitate/BSA (Pa-BSA) for 16 h. The cells were then immunostained for histone H3 and the nuclei were counterstained with DAPI. Original magnification: 20×; scale bar: 20 μm.

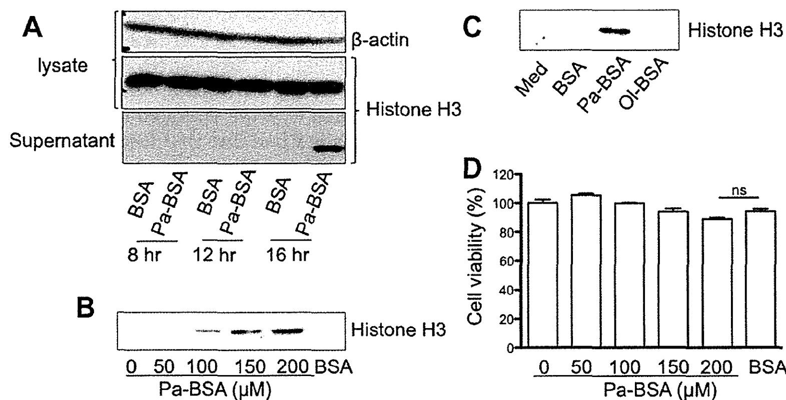

Fig. 2. Palmitate induces histone H3 release in time- and dose-dependent manners from RAW 264.7 cells. (A) RAW 264.7 cells were incubated with 200 μM of BSA or palmitate/BSA (Pa-BSA) for 8–16 h and histone H3 levels in supernatants and cell lysates were analyzed by Western blotting. (B) RAW 264.7 cells were incubated with increasing concentrations of Pa-BSA (0–200 μM) for 16 h and histone H3 levels in supernatants were analyzed by Western blotting. (C) RAW 264.7 cells were treated with 200 μM of BSA, Pa-BSA, or oleate/BSA (Ol-BSA) for 16 h, and histone H3 levels in supernatants were analyzed by Western blotting. (D) Cell viability was measured by MTT assays after 16 h of palmitate incubation.

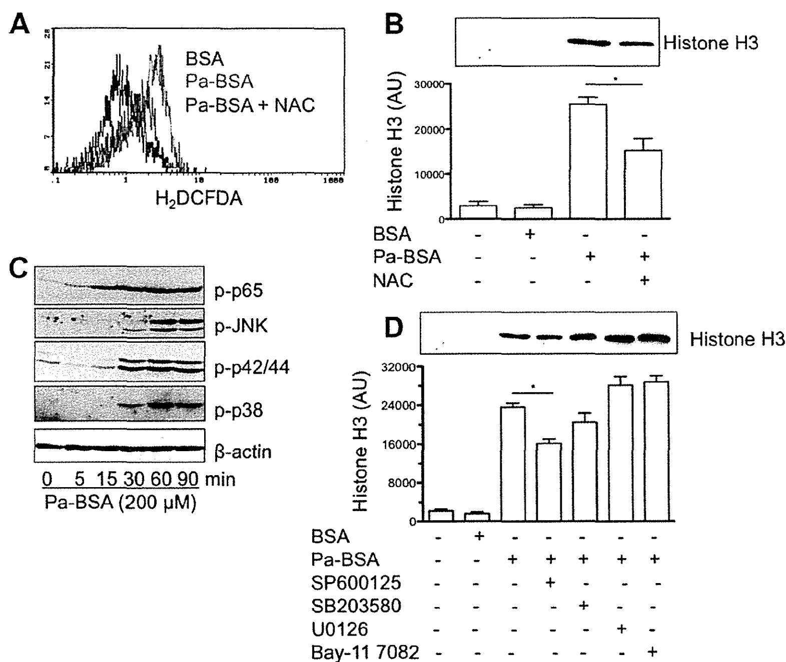

Fig. 3. Palmitate induces histone H3 through ROS and JNK signaling. (A) RAW 264.7 cells were treated with 200 μM of BSA or palmitate/BSA (Pa-BSA) for 8 h. Intracellular ROS production was measured using a fluorescent probe, H_2DCFDA. (B) RAW 264.7 cells were pretreated with NAC (5 mM) 1 h before palmitate stimulation. The histone H3 levels in the supernatants after 16 h were analyzed by Western blotting. (C) Immunoblotting for phospho-MAPKs and phospho-p65 in lysates of cells treated with palmitate (200 μM) for 0–90 min. (D) RAW 264.7 cells were pretreated with MAPK inhibitors (SB203580, SP600125, or U0126; 10 μM) and NF-κB inhibitors (10 μM) 1 h before cell stimulation. The histone H3 levels in the supernatants after 16 h were analyzed by Western blotting.

whereas a p38 inhibitor (SB203580), ERK1/2 inhibitor (U0126), and NF-κB inhibitor (Bay-11 7082) had no effects (Fig. 3B). These results indicate that palmitate-induced histone H3 release is mediated, in part, through the JNK signaling pathway.

3.4. Extracellular histone activates endothelial cells to express the adhesion molecules VCAM-1 and ICAM-1 and the procoagulant molecule tissue factor

Finally, we examined the effects of extracellular histone on the surrounding cells, including endothelial cells. Extracellular histone significantly increased the mRNA expressions of the adhesion molecules ICAM-1 and VCAM-1 and the procoagulant molecule tissue factor in endothelial cells in dose-dependent manners (Fig. 4A). Extracellular histone also increased the mRNA expression of TLR2, but not that of TLR4 (Fig. 4A), which were both reported to act as receptors for extracellular histones [18–20]. Furthermore, extracellularhistone induced the expression of ICAM-1, VCAM-1, and tissue factor proteins on the surface of endothelial cells (Fig. 4B). These results suggest a possible contribution of extracellular histones to the pathogenesis of HFD-induced inflammation and thrombosis.

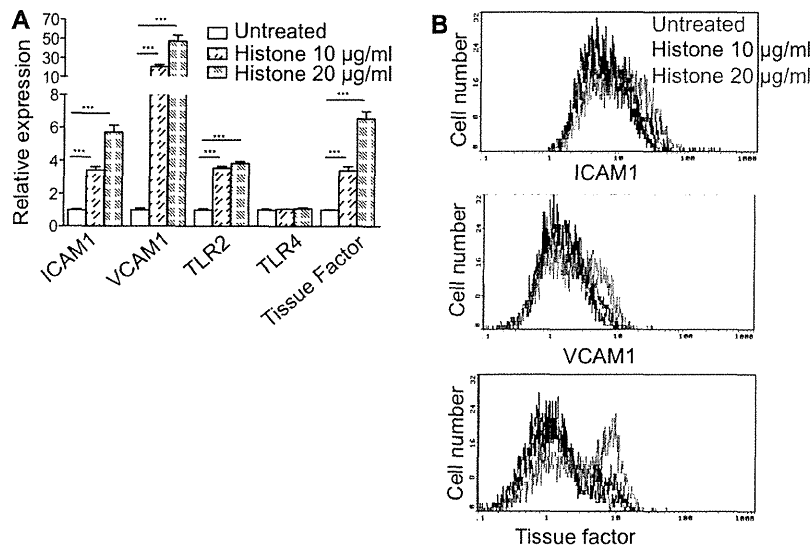

Fig. 4. Extracellular histone induces the expressions of the adhesion molecules ICAM-1 and VCAM-1 and the procoagulant molecule tissue factor in endothelial cells. (A) HUVECs were treated with histone at concentrations of 0, 10 and 20 μg/ml for 4 h, and the expressions of ICAM-1, VCAM-1, TLR2, TLR4 and tissue factor were analyzed by RT-PCR. (B) HUVECs were treated with histone for 6 h and measured for their surface expression of ICAM-1, VCAM-1 and tissue factor by flow cytometry.

4. Discussion

The present study has demonstrated that histone H3 is released into the extracellular space from macrophages treated with palmitate *in vitro* and adipose tissue in mice fed a HFD *in vivo*. The release of histone H3 is an active process in living cells, rather than passive leakage from dying cells. The extracellular histone then activates the endothelium to express cell adhesion molecules and procoagulant protein, tissue factor. These results suggest the possible contribution of extracellular histone to the pathogenesis of HFD-induced inflammation and thrombosis.

Histones are nuclear proteins that form hetero-octamers to wind up the double strands of DNA in nucleosomes and are involved in chromatin remodeling and gene transcription regulation [18,19]. Histones can be released into the extracellular space during sepsis, and the plasma concentration of histones can reach 70 μg/ml in the acute inflammatory condition [27]. In this study, we showed that histone H3 was also released into the extracellular space during chronic low-grade inflammation induced by HFD feeding or FFA treatment. Palmitate (C16:0) and oleate (C18:1) are most abundant saturated and unsaturated fatty acid, respectively, in HFD used in this study. Consistent with previous studies showing that palmitate induces expression of proinflammatory molecules [6,28,29], palmitate, but not oleate, induced histone H3 release in our *in vitro* study.

ROS are linked to multiple pathologies, such as cardiovascular diseases, diabetes, neurological disease, and cancer. The increased ROS levels during obesity deregulate the production of adipokines [30]. Consistent with previous data, we found that palmitate induced ROS generation from RAW 264.7 cells, and that treatment with NAC, a ROS inhibitor, inhibited the histone release. In addition to ROS, NF-κB and JNK, which are regulators of inflammation, have been reported to link obesity and metabolic diseases [12]. FFA activates both NF-κB and JNK signaling following activation of TLR4, leading to increased expressions of proinflammatory cytokines such as TNF-α and IL-6 [6]. Our results also demonstrated activation of NF-κB and MAPKs in time-dependent manners. Although palmitate activated these various pathways, incubation of the cells with ERK, p-38, and NF-κB inhibitors in presence of palmitate had no effects on the histone release. However, a JNK inhibitor inhibited the histone release. These results suggest that the JNK signaling pathway is involved in the histone release induced by palmitate.

A previous study showed that the JNK signaling pathway is activated by palmitate, and that inhibition of ROS reduces the activation of this signaling pathway and enhances insulin sensitivity in hepatocytes [26]. We treated cells with palmitate in the presence of NAC or a JNK inhibitor and measured the activation of JNK by Western blotting. The cells treated with the JNK inhibitor showed attenuated JNK activation, while NAC did not inhibit the JNK activation (data not shown). Our results show that the activation of JNK is not dependent on ROS and that both ROS and JNK activation partly contribute to the histone release induced by palmitate.

Finally, we performed *in vitro* experiments examining the effects of the extracellular histone. It has been reported that histones, which are released from dying cells during sepsis or inflammation, can activate TLR2/TLR4 and contribute to endothelial dysfunction [19]. ICAM-1 and VCAM-1 are upregulated during endothelial activation and involved in the interactions of inflammatory cells with the endothelium and the transmigration of these cells into adipose tissue [31]. We showed that the released histone induced the expressions of adhesion molecules ICAM-1 and VCAM-1 in endothelial cells and enhanced the expression of TLR2, but not TLR4. Besides these effects, the histone induced the expression of a procoagulant protein, tissue factor, from endothelial cells, which plays a crucial role in coagulation and thrombosis. A recent study showed that tissue factor promotes inflammation and diet-induced obesity through protease-activated receptor 2 (PAR2) [32].

In summary, our study has demonstrated that palmitate induces the release of histone H3 from activated macrophages, in part, through ROS generation and the JNK signaling pathway. The extracellular histone activates the endothelium and enhances proinflammatory and prothrombotic responses. Overall, we suggest that histones provide the cross-talk between obesity and inflammation. However, further studies are required to investigate the associations of histones with diet-induced obesity and inflammation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.117.

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