VII 宿主応答とその制御
ウイルス感染に対する細胞応答・

ウイルス感染に対するインターフェロン応答機構

成田 亮・米山光俊・藤田尚志

抗ウイルス自然免疫は，ウイルス感染に応答して活性化され，I型インターフェロン（IFN）や炎症性サイトカインなどの産生を誘導し，細胞に抗ウイルス活性をもたらす．この自然免疫におけるウイルス感染認識機構は長い間謎に包まれていた．しかし，TLR，そして近年RIG-Iが同定され，これらの解析の結果，その全体像が急速に明らかとなりつつある．本稿では，I型IFN遺伝子の発現制御機構と，自然免疫における細胞内外のウイルス感染認識機構について解説する．

▶▶KEY WORDS：自然免疫　I型IFN　RIG-I　MDA5　TLR

■はじめに■

われわれの生活は常にウイルス感染の脅威にさらされている．HIV, SARSウイルス，トリインフルエンザウイルス，そして最近ではノロウイルスと，ウイルス感染症は社会的にも大きな問題となっている．近年の分子生物学や細胞生物学の進歩により，ウイルス感染症の原因や発症機構が明らかとなってくるとともに，それに対抗するための免疫についての研究も目覚しい発展を遂げている．

われわれ人間をはじめ高等動物は，自然免疫と獲得免疫という2つの免疫系をもち，これらを協調的に働かせることによって，ウイルスや細菌の感染に対する生体防御を行なっている．獲得免疫は抗原特異的で非常に強力な免疫応答ではあるが，その発動には時間がかかることから，速やかに誘導される自然免疫が感染初期において非常に重要な役割を担っている．自然免疫系は，ウイルスや細菌の感染を認識して炎症性サイトカインやI型インターフェロン（IFN）の産生を誘導し，最前線の生体防御を行なう．なかでもI型IFNはウイルス感染に応答して一過的に分泌され，周囲の細胞に抗ウイルス活性をもたらすとともに，MHC蛋白質の発現を誘導し樹状細胞の成熟を促進するなど獲得免疫の調節も行なうことから，ウイルスに対する防御機構として中心的な役割を果たしているといえる．

しかし，自然免疫系がどのようにウイルス感染を認識し，I型IFN遺伝子の発現誘導を行なうのかは，長い間謎のままであった．その後の解析で，TLR（toll-like receptor），そして近年RIG-I（retinoic acid inducible gene-I）が同定されたことにより，自然免疫系のシグナル経路の詳細が急速に明らかとなってきた．

本稿では，I型IFN遺伝子の発現制御機構とウイルス感染に応答した自然免疫系のシグナル経路について，最新の知見を交えながら解説する．

I. I型IFN系

IFNは抗ウイルス活性をもつサイトカインであり，複数のIFN-αと単一のIFN-βからなるI型，IFN-γからなるII型，そしてIFN-λからなるIII型に分類される．自然免疫において中心的な役割を果たしているのはI型IFNであることから，本稿ではI型IFN中心に解説する．したがって以下，本稿でIFNとはI型IFNのことを指す．

ウイルスなどの感染が起こると，IFN遺伝子の転写が誘導され，細胞は一過的にIFNを分泌する．分泌されたIFNは産生細胞のみならず，体液を通じて未感染細胞のIFN受容体に結合し，その細胞内ドメインに存在しているチロシンキナーゼJak1，Tyk2を活性化することで，STAT1（signal transducers and activators of transcription 1），STAT2の特定のチロシン残基をリン酸化する．

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Mechanism of virus-induced expression of type I interferon genes

リン酸化されたSTAT1，STAT2はDNA結合能をもつIRF（IFN regulatory factor）-9と会合することで活性型3量体ISGF3（interferon stimulated gene fanctor 3）を形成し，核に移行して多様なIFN誘導遺伝子（IFN-stimulated gene；ISG）の活性化を行なう．そのなかには2′-5′オリゴ(A)合成酵素，二重鎖RNAによって活性化されるプロテインキナーゼPKR（protein kinase dsRNA dependent）などのウイルス増殖抑制作用をもつ蛋白質の遺伝子群が含まれ，それらの作用により細胞に抗ウイルス作用，増殖抑制作用などがもたらされる[1]（図1）．

IFN-β遺伝子のプロモーター領域にはATF/c-Jun，IRF，NF-κBの結合配列が並んで存在する．これら3種の転写因子はエンハンセオソームとよばれる転写複合体を形成し，IFN-β遺伝子の転写を強く誘導する．一方でIFN-α遺伝子のプロモーター領域にはIRFの結合配列が存在し，その発現制御にかかわる[2]（図1）．

IRFファミリーはI型IFNを制御する転写因子として発見され，IRF-1～IRF-9までの9種類からなる．そのなかでIRF-3とIRF-7は相同性が高く，I型IFNの発現制御を行なうという共通の機能をもつ[3]．IRF-3はほとんどの細胞において構成的に発現しているが，一方でISGであるIRF-7は定常状態における発現レベルは低く，IFN受容体，Jak-STAT経路を介して誘導される．このことから，IRF-7は主として2次的なIFN産生を誘導するものであり，IRF-3がI型IFN遺伝子の転写に必須の因子であると考えられていた．しかし，ノックアウトマウスを用いた近年の解析結果から，IRF-7がI型IFNの発現制御に必須のマスター因子として機能するという説が提唱されている[4]．定常状態におけるIRF-3とIRF-7は，ともに不活性型の単量体で存在しているが，C末端側の特定のセリン残基がリン酸化を受けることにより活性化され，2量体を形成する[5,6]．2量体化したIRF-3は核内に移行してCBP/p300と結合したのち，IFN遺伝子のプロモーター領域に結合し転写を誘導する．一方でIRF-7ホモ2量体は，必ずしもCBP/p300と強固な結合をすることはないと考えられている．

II. TLRによる細胞外ウイルス感染認識機構

TLR（Toll-like receptor）はおもに樹状細胞（dendritic cell；DC）やマクロファージといった免疫系の細胞の膜上に発現している膜蛋白質であり，細胞外の病原体成分を認識する受容体として働く分子である．ヒトでは現在10種類のTLRが報告されており，各TLRは異なる病原体成分（pathogen-associated molecular pattern；PAMP）を認識し，異なるシグナル経路で自然免疫系を活性化する．TLRは細胞外領域に存在するロイシンリッチリピートを

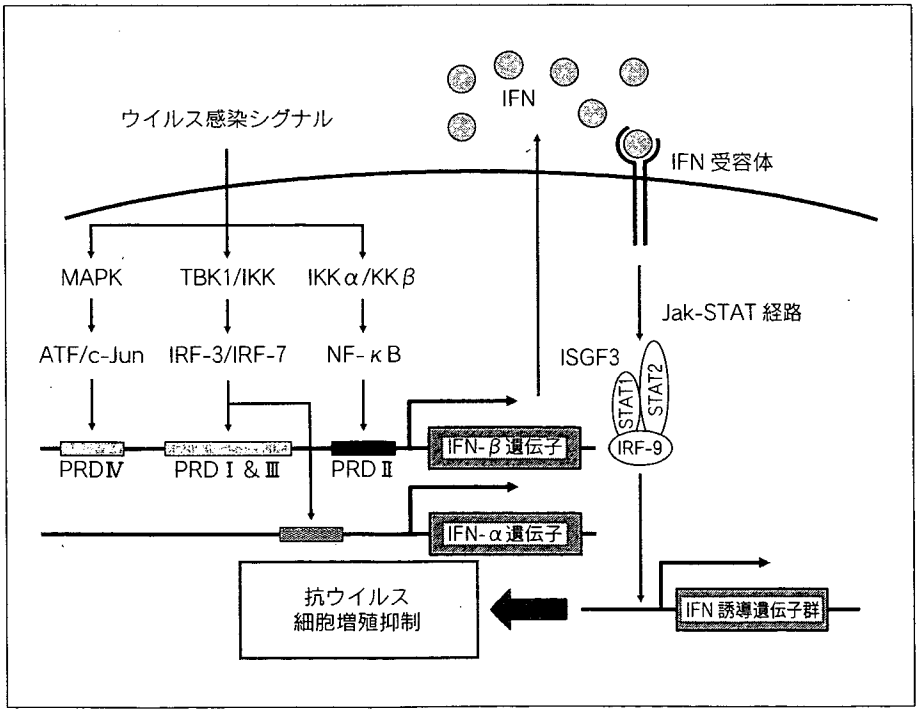

図1　I型IFN系
ウイルス感染に応答して，IRF-3などの転写因子が対応するキナーゼによって活性化され，IFNの転写を誘導する．誘導されたIFNは細胞外に分泌され，感染細胞だけでなく周囲の未感染細胞にも働きかける．IFN受容体にIFNが結合すると，Jak-STAT経路によりIFN誘導遺伝子群が誘導され，細胞に抗ウイルス活性および増殖抑制作用をもたらす．

介してPAMPの認識を行ない，細胞内領域のTIR（Toll/IL-1 receptor）ドメインを介して，同じくTIRドメインをもつアダプター分子MyD88（myeloid differentiation factor 88），TRIF（TIR-domain-containing adaptor protein-inducing IFN-β），TRAM（TRIF-related adaptor molecule），TIRAP（TIR-associated protein）などと結合し，シグナル伝達を行なうことで炎症性サイトカインやI型IFNの産生を誘導する．このうち，ウイルスの核酸をPAMPとして認識するのはTLR3，TLR7/8，TLR9であり，それぞれ二重鎖RNA（dsRNA），poly(U)などの1本鎖RNA（ssRNA），非メチル化DNA（CpG DNA）を認識する．また，他のTLRが細胞膜に局在しているのに対して，この3つTLRはおもにエンドソームに局在しており，エンドサイトーシスによって取り込まれたPAMP，とくに核酸構造を認識する．TLR2とTLR4は種々の細菌の構成成分を認識するだけでなく，単純ヘルペスウイルス1（herpes simplex virus-1）や呼吸器多核体ウイルス（respiratory syncytial virus）などのウイルスの非核酸成分の認識にもかかわることが知られている．また，TLR4はエンドトキシンを認識してIFNの産生に関与することが知られている[7]．ここでは，ウイルス核酸を認識しIFNの産生を誘導するTLR3，TLR7/8，およびTLR9について，そのIFN誘導機構を解説する．

TLR3は，ウイルスの複製の際に生じるdsRNAおよびpoly(I：C)を認識し，IFNの産生を誘導する．アダプター分子であるTRIFはTLR3と会合し，TRAF3（TNFR-associated factor 3）とNAP1［NF-κB-activating kinase (NAK)-associated protein 1］を介して[8,9]，IKK（IκB kinase）ファミリーであるTBK1（TANK binding kinase 1）およびIKKiを活性化する．活性化されたTBK1とIKKiによりIRF-3とIRF-7がリン酸化され，2量体を形成し核内へ移行してIFN遺伝子の転写を誘導する（図2）．

一方，TLR7/8とTLR9は，それぞれssRNAとCpG DNAを認識する．これらはおもにpDC（plasmacytoid DC）に発現しており，pDCによる大量のIFN-αの産生に関与している[10]．TRIFを介してシグナルを伝達するTLR3とは異なり，TLR7/8とTLR9はアダプター分子MyD88と直接会合して下流にシグナルを伝達する．IRF-7はpDCにおけるI型IFNの産生誘導に非常に重要な役割を担っており，リガンドによる刺激を受け取ると，MyD88，IRAK-4，IRAK-1，TRAF6，TRAF3およびIRF-7は複合体を形成し，TLRのTIRドメインと会合する[11,12]．IRF-7は，IKKαによりリン酸化されホモ2量体を形成し，核内に移行してI型IFN遺伝子の転写を行なう[13]（図2）．また，ユビキチンE3リガーゼであるTRAF6によるIRF-7のユビキチン化が，IRF-7の活性化に必須であることが報告されているが，そのメカニズムは明らかとなっていない[11]．

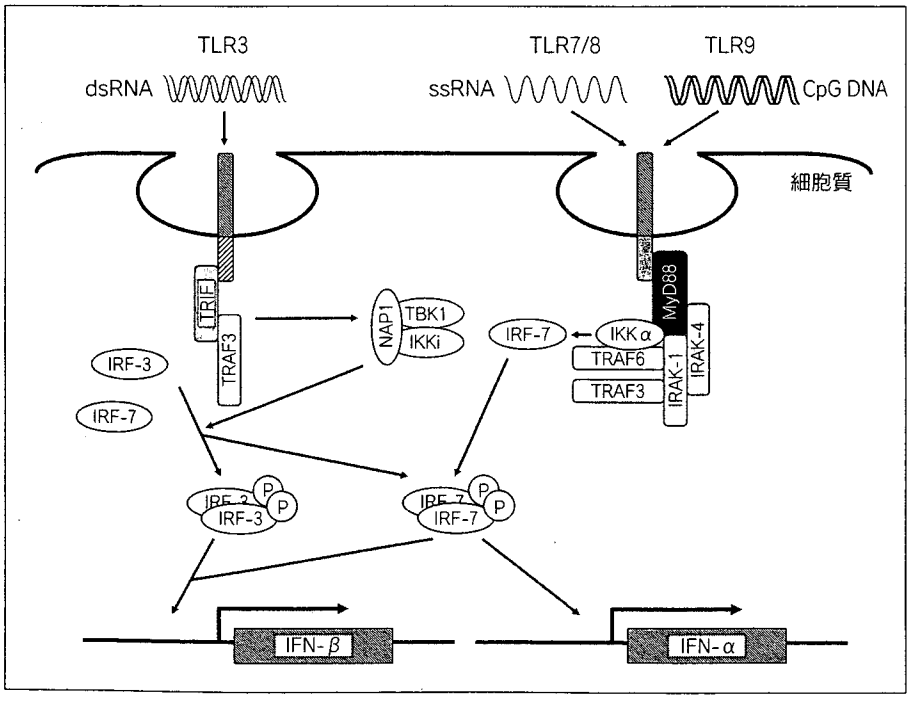

図2　TLRファミリーによるウイルス認識機構

TLR3は細胞外のdsRNAを認識し，アダプター分子であるTRIFを介してIFNの転写を誘導する．一方で，TLR7/8とTLR9はそれぞれssRNAとCpG DNAを認識し，アダプター分子であるMyD88を介してIFNの転写を誘導する．

III. RIG-Iファミリーによる細胞内ウイルス感染認識機構

前述のように，TLR3はpoly(I：C)やRNAウイルスのdsRNAを認識して，I型IFNの産生を誘導することが明らかとなった．しかし，TLR3はおもにエンドソームに局在していることから，細胞死などにより放出されたウイルス由来dsRNAを認識する受容体であり，細胞質内で複製された，もしくは感染により細胞質内に放出されたウイルスのdsRNAを認識することはできないと考えられる．また，TLR3ノックアウトマウスにおいて，SeV (Sendai virus)，NDV (Newcastle disease virus)，VSV (vesicular stomatitis virus) などのRNAウイルス感染に応答したI型IFNの産生能に変化がみられなかった[14]．以上のことから，細胞質内でウイルスのdsRNAを認識する受容体が存在すると考えられていた．

RNAヘリカーゼであるRIG-Iは，細胞質内でウイルス由来のdsRNAを認識する受容体として近年同定された[15]．RIG-IはC末端側にDExD/Hボックスを含むRNAヘリカーゼドメインを，N末端側にCARD (caspase recruitment domain) とよばれるドメインを2回くり返してもつという特徴的な構造をしている（図3）．siRNAを用いてRIG-Iの発現を抑制した結果，ウイルス感染によるIRF-3の2量体化およびIFN遺伝子などの発現が強く抑制された．また，RIG-Iを構成的に発現している細胞は，非常に強い抗ウイルス活性を示した．このことは，RIG-Iがウイルス感染に対する自然免疫誘導において非常に重要な役割を担っていることを意味する．RIG-Iを細胞に過剰発現させただけではシグナルの活性化は起こらず，ウイルス感染刺激によりシグナルは活性化される．一方で，CARDのみをもつRIG-I変異体を細胞に過剰発現させると，ウイルス感染刺激なしでIRF-3が活性化され，IFN-βの発現が誘導された．このことから，CARDを介して下流へシグナルが伝達されること，およびRNAヘリカーゼドメインを含むC末端領域が制御ドメインとして機能していることが示された．最近の報告で，RIG-IのC末端にリプレッサードメイン (repressor domain；RD) が存在し，RDを介した分子内会合によりRIG-Iを不活性型に保っていることが明らかとなった[16]．また，ATPaseであるRNAヘリカーゼドメインのATP結合部位に変異を導入したRIG-I K270Aは，細胞に過剰発現した結果ドミナントネガティブに働くことから，少なくともRIG-IがATPと結合することがウイルス感染に応答したシグナルの活性化に必須であることが判明した．

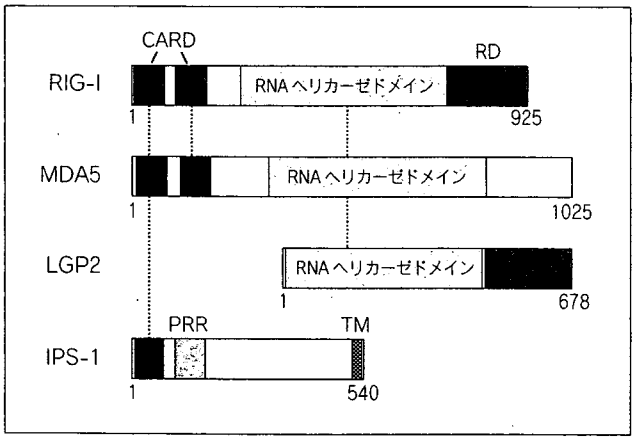

図3 RIG-Iファミリー分子とアダプター分子IPS-1

RIG-IとMDA5は非常によく似た構造をしており，N末端側にCARDが2つ存在し，C末端側にRNAヘリカーゼドメインが存在する．一方，LGP2はRNAヘリカーゼドメインをもつが，N末端側にCARDをもたない．またRIG-IとLGP2はそのC末端にRDが存在し，シグナルを抑制している．アダプター分子であるIPS-1は，N末端側にCARDとPRRを，C末端側にTMドメインを有しており，そのためIPS-1はミトコンドリア外膜に局在する．

以上のことから，分子内会合により通常不活性型で存在しているRIG-Iは，ウイルス由来dsRNAを認識するとそのATPase活性により立体構造を変化させ，CARDを介したシグナルを下流へ伝える，というモデルが提唱されている．また，最近の報告では，5′末端の三リン酸化されたssRNAが，RIG-Iに認識されIFN産生を誘導することが報告された[17,18]．このことは，RIG-Iが細胞質においてdsRNAだけでなく，ssRNAを認識する受容体としても働くことを示しており，非常に興味深い．

一方，RIG-Iと相同性を示すMDA5という分子が存在し，RIG-Iファミリー分子として報告された[19]．MDA5はN末端側にCARDを2回くり返してもち，C末端側にRNAヘリカーゼドメインをもつという，RIG-Iと非常によく似た構造をしており（図3），RIG-Iと同じくウイルス感染およびdsRNAを認識してIFNの産生を誘導する．さらに，ノックアウトマウスを用いた解析により，RIG-IとMDA5とでは認識するRNAの種類が異なることが示された[20]（図4）．RIG-Iは*in vitro*で転写されたdsRNAを認識し，MDA5はpoly(I：C)を認識する．また，認識するRNAウイルスもRIG-IとMDA5とで異なっており，RIG-IはNDV，SeVなどを認識するのに対して，MDA5

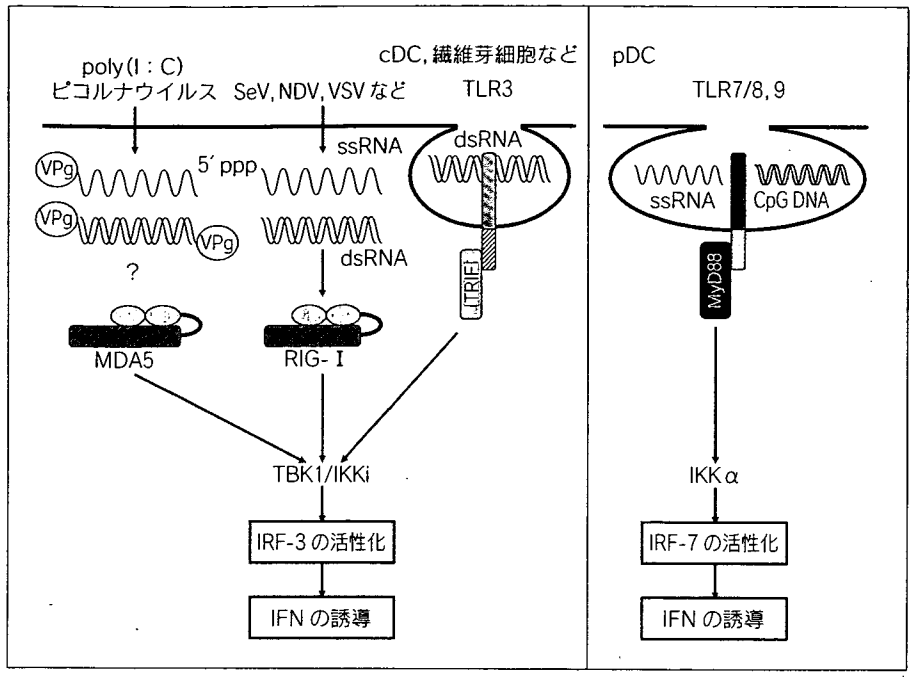

図 4　組織特異的なウイルス認識機構

細胞外のウイルス核酸を認識する TLR と細胞内で機能する RIG-I/MDA5 は，細胞の種類によって使い分けられ，自然免疫系において機能している．cDC や繊維芽細胞などの細胞では，おもに RIG-I/MDA5 と TLR3 によってウイルスが認識される．一方で，pDC においては TLR 7/8 および TLR9 が主として機能している．また，RIG-I と MDA5 で認識するウイルスが異なり，RIG-I は SeV や NDV などのウイルスを認識し，MDA5 はピコルナウイルスを認識する．

はピコルナウイルスを認識する．ピコルナウイルスの RNA の 5′末端は，VPg（virion protein, genome-linked）とよばれるピコルナウイルスの遺伝子のコードする蛋白質が結合しているため，RIG-I による 5′末端三リン酸の認識から逃れていると考えられる．では MDA5 は何を認識しているのか？　今後の解析に興味がもたれる．

RIG-I と MDA5 がアダプター分子である IPS-1（IFN-β promoter stimulator-1，別名：MAVS[21]，VISA[22]，Cardif[23]）と直接結合することで，そのシグナルは下流に伝達されることが明らかとなった[24]（図 5）．ノックアウトマウスを用いた解析により，IPS-1 は RIG-I/MDA5 を介する自然免疫において必須の役割を果たすアダプター分子であることが判明した[25,26]．IPS-1 は N 末端側に CARD をもち（図 3），活性化された RIG-I/MDA5 の CARD と会合することでシグナルを伝達する．また，IPS-1 の C 末端側には膜貫通ドメインが存在し，ミトコンドリア外

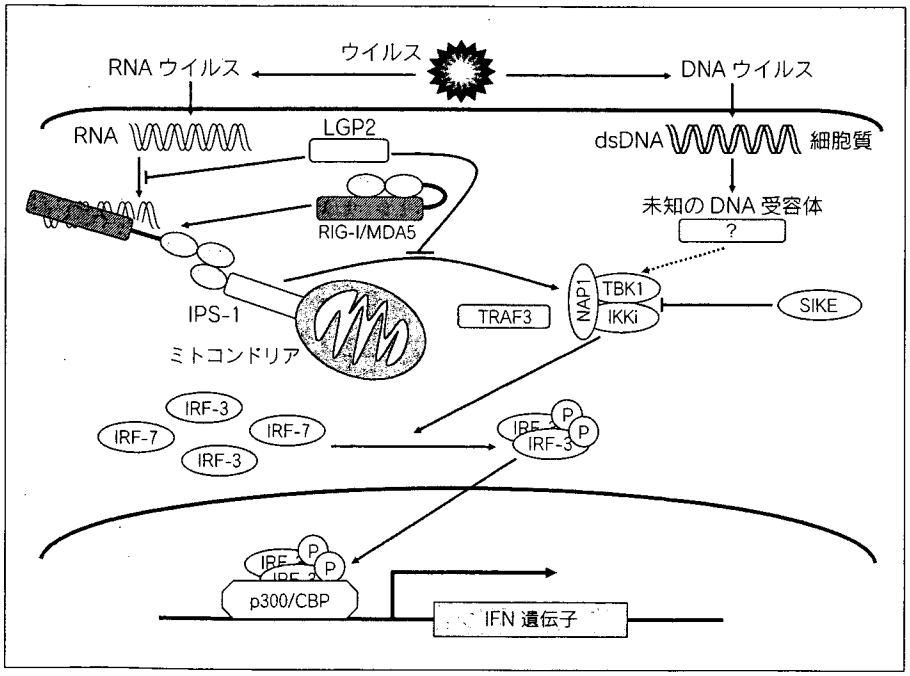

図 5　RIG-I ファミリーによる細胞内ウイルス認識機構

ウイルスの複製の過程で細胞質内に蓄積するウイルス RNA は，RIG-I/MDA5 によって認識され IPS-1 を介して IFN の産生を誘導する．LGP2 は RIG-I や IPS-1 と結合することにより，シグナルを抑制する因子として機能していると考えられる．細胞質内にトランスフェクションされた dsDNA は，TBK1/IKKi を介して I 型 IFN の転写を誘導するが，細胞質内で DNA 受容体として働く分子はいまだ明らかとなっていない．

膜に局在していることが明らかとなっている[21]．このミトコンドリアへの局在がシグナルの活性化に必須であることから，自然免疫におけるミトコンドリアの重要性が注目されている．RIG-I/MDA5と結合したIPS-1は，TBK1とIKKiを介してIRF-3とIRF-7を活性化し，I型IFNの産生を誘導する．また，IPS-1にはCARD近傍にPRR（proline-rich region）とよばれるドメインが存在する（図3）．IPS-1はPRRを介してTRAF3と結合することにより，TBK1/IKKiの活性化を行なっている[27]．さらに，C型肝炎ウイルス（hepatitis C virus；HCV）の非構造蛋白質NS3/4Aは，そのプロテアーゼ活性によりIPS-1をC末端側で切断することで，RIG-I/MDA5のシグナル経路特異的にIRF-3の活性化を阻害していることも明らかとなっている[23]．

これまで述べてきたとおり，ウイルスRNAの認識機構としてRIG-I/MDA5とTLRの2つのシステムが存在するわけであるが，細胞の種類によってこれらのシステムを使い分けていることが明らかとなっている[28]（図4）．RIG-Iノックアウトマウスを用いた解析の結果，大量にIFN-αを産生するpDCではTLRを，cDCや繊維芽細胞などではRIG-Iを介してI型IFNの産生を誘導していることが示されている．

RIG-Iファミリーには，RIG-I，MDA5のほかにLGP2という分子が存在する．LGP2はRNAヘリカーゼドメインをもっているが，RIG-IやMDA5と違い，N末端側にCARDをもっていない（図3）．LGP2を細胞に過剰発現させると，ウイルス感染に応答したIFN産生が強く抑制され，逆にsiRNAを用いて発現を抑制すると，IFN産生を増強した．また，LGP2のdsRNAに対する親和性は，RIG-IやMDA5よりも強いことが明らかとなったことから，RIG-I/MDA5と基質を競合することで負の調節因子として機能していることが示唆された[19]（図5）．しかし一方で，LGP2がIPS-1と結合してIKKiの活性化を阻害することで，IFN産生を抑制していることが最近報告された[29]．さらに，RIG-Iと同様にLGP2もC末端側にRDをもっており，RDを介してRIG-Iと結合することで，シグナルを抑制していることも報告されており[16]，LGP2による複雑なシグナル抑制メカニズムが存在するようである．いずれにせよ *in vitro* においては，LGP2はRIG-I/MDA5を介したシグナルを抑制することで，IFN産生シグナルの沈静化を行なうことを示しており，ノックアウトマウスなどを用いた今後の生理的な機能解析が期待される．

ではDNAウイルスの核酸は細胞内で認識されていないのだろうか？　最近，自己のDNAを含む二重鎖DNAがTLR9非依存的にIFN産生を誘導することが明らかとなった．細胞質内dsDNAはTLR，RIG-I/MDA5，IPS-1非依存的かつTBK1/IKKi依存的にI型IFNおよび炎症性サイトカインの産生を誘導することから[30,31]，細胞質内DNA受容体分子の存在が強く示唆されており，その同定に興味がもたれる（図5）．

■おわりに■

われわれは，高度に制御された免疫系を備えることで，さまざまなウイルスや細菌などの感染から身を守っている．一方，病原体も同時に進化を遂げ，宿主の免疫系を回避する機構を獲得することで生き残ってきた．RIG-I/MDA5の発見により，それまで未解明であった細胞内ウイルス感染認識機構の解析は急速に進み，その全体像が明らかとなりつつある．生体防御におけるシグナル経路とウイルスのもつ免疫回避機構の解明が進むことにより，感染疾患や免疫疾患などさまざまな疾患に対する創薬の開発や新たな治療法および予防法の確立につながるであろう．今後のさらなる解析に期待するとともに，免疫学の基礎研究が臨床に応用されることで，感染疾患や免疫疾患で苦しむ多くの方々の一助となることを願って止まない．

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VII 宿主応答とその制御
ウイルス感染に対する細胞応答・

センダイウイルス感染と宿主自然免疫

加藤 篤・清谷克寛

適応免疫に比べて自然免疫は，その防御作用によってどれほど多くの病原体が撃退されているのかを知る機会が少ない．感染による変化が訪れる前に病原体が除去されるからである．通常，センダイウイルスは，培養細胞でもマウス個体内でも旺盛に増殖する．ところが，アクセサリー蛋白質Vまたは C を改変したセンダイウイルスは，自然免疫に対する抵抗性を失い，V 改変ウイルスは感染１日目以降に，C 改変ウイルスは感染後直ちにマウス肺から排除される．V または C 蛋白質の宿主自然免疫への対抗方法をとおして，自然免疫の抗ウイルス作用の強さと未知の部分の力を紹介する．

▶▶KEY WORDS：センダイウイルス　アクセサリー蛋白質　自然免疫

■はじめに■

感染後しばらくして登場する抗体や細胞傷害性 T 細胞といった防御因子が抗原特異的であることに対して，感染早期に登場する防御因子は抗原非特異的に作用する．前者は適応免疫，後者は自然免疫とよばれている．自然免疫の防御因子としてインターフェロン（interferon；IFN）がよく知られている．IFN は，分子量 15～26 K の蛋白質の総称であり，Ⅰ型（α と β）とⅡ型（γ）がある．このうちウイルスなどの病原体が生体に侵入すると真っ先に登場するのは，Ⅰ型 IFN である．IFN-β は 1 種類，IFN-α は 15 種類以上の遺伝子がある．感染細胞から分泌されたⅠ型 IFN は直接病原体に作用するのではなく，周囲の細胞に RNA 依存性蛋白質リン酸化酵素（interferon-induced dsRNA-dependent protein kinase；PKR），2′,5′A 合成酵素および Mx 蛋白質などの発現を誘導し，細胞を抗ウイルス状態にすることで働く．一方，インターロイキン（interleukin；IL)-12，IL-6，腫瘍壊死因子（tumor necrosis factor-α；TNF-α）などのサイトカインも，病原体の侵入とともに分泌される．これらのサイトカインは近傍の細胞に構造上の変化を誘導し，細胞上に好中球や単球を接着させることにより感染の場を示す目印となる炎症巣形成に働き，病巣除去に貢献する．これらは総称して炎症性サイトカインとよばれている．Ⅰ型 IFN と炎症性サイトカインの発現はどちらも転写レベルで制御されているが，最近の研究ではその誘導経路間にクロストークが存在しているために，Ⅰ型 IFN と炎症性サイトカインは発現レベルで連携あるいは分担して感染初期防御反応を担っていると理解されている．

自然免疫機構によって「どのくらい多くの感染が対処されているのか？」ということはあまりよくわかっていない．なぜなら，そのような感染はごく初期に除去されてしまい，ほとんど症状や病理学的変化を残さないからである．ところが，近年自然免疫にかかわる因子や経路の実体が明らかにされ，それらを人為的に改変したノックアウトマウスがつくられるようになり，自然免疫の生体防御に果たす役割がいかに大きいかを知ることができることになった．

本稿ではセンダイウイルス（Sendai virus；SeV）を例に，ウイルスの側から自然免疫の重要性を紹介しよう．

Ⅰ．センダイウイルスとアクセサリー蛋白質

SeV は，1952 年に東北大学（仙台市）で，当時流行していた新生児肺炎に罹患した患者材料からマウスを用いて分離され，病因ウイルスとして発表された．ところが現在，これはウイルス分離の過程でマウス内在のウイルスが継代により偶然増幅されたものであり，SeV は新生児肺炎とは何ら関係ないウイルスであるとされている．し

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Sendai viral infection and host innate immunity

the FEBS Journal

Limited suppression of the interferon-β production by hepatitis C virus serine protease in cultured human hepatocytes

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Keywords
antiviral response; hepatitis C virus; innate immune response; interferon-β; serine protease

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Toll-like receptors and RNA helicase family members [retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene-5 (MDA5)] play important roles in the induction of interferon-β as a major event in innate immune responses after virus infection. TRIF (adaptor protein of Toll-like receptor 3)-mediated and Cardif (adaptor protein of RIG-I or MDA5)-mediated signaling pathways contribute rapid induction of interferon-β through the activation of interferon regulatory factor-3 (IRF-3). Previously, it has been reported that the hepatitis C virus NS3-4A serine protease blocks virus-induced activation of IRF-3 in the human hepatoma cell line HuH-7, and that NS3-4A cleaves TRIF and Cardif molecules, resulting in the interruption of antiviral signaling pathways. On the other hand, it has recently been reported that non-neoplastic human hepatocyte PH5CH8 cells retain robust TRIF- and Cardif-mediated pathways, unlike HuH-7 cells, which lack a TRIF-mediated pathway. In the present study, we further investigated the effect of NS3-4A on antiviral signaling pathways. Although we confirmed that PH5CH8 cells were much more effective than HuH-7 cells for the induction of interferon-β, we obtained the unexpected result that NS3-4A could not suppress the interferon-β production induced by the TRIF-mediated pathway, although it suppressed the Cardif-mediated pathway by cleaving Cardif at the Cys508 residue. Using PH5CH8, HeLa, and HuH-7-derived cells, we further showed that NS3-4A could not cleave TRIF, in disagreement with a previous report describing the cleavage of TRIF by NS3-4A. Taken together, our findings suggest that the blocking of the interferon production by NS3-4A is not sufficient in HCV-infected hepatocyte cells.

Persistent infection by hepatitis C virus (HCV) frequently causes chronic hepatitis [1,2], which progresses to liver cirrhosis and hepatocellular carcinoma [3,4]. This is a serious health problem because approximately 170 million people are currently infected with HCV worldwide [5]. To resolve the mechanism of persistent HCV infection, it will be necessary to better understand the virus life cycle and then to develop more effective anti-HCV reagents. HCV is an enveloped positive ssRNA (9.6 kb) virus belonging to the *Flaviviridae* family [6,7]. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid residues, which is cleaved co- and post-translationally into at least ten proteins in the order: core, envelope 1

Abbreviations
CARD, caspase recruitment domain; E1, envelope 1; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HEK293, human embryonic kidney 293; IFN, interferon; IRF-3, interferon regulatory factor 3; IKK-ε, inhibitor of κB kinase ε; MDA5, melanoma differentiation associated gene-5; MyD88, myeloid differentiation factor 88; NS2, nonstructural protein 2; RIG-I, retinoic acid-inducible gene I; siRNA, small interfering RNA; TBK, Tank-binding kinase 1; TLR, Toll-like receptor; TRIF, Toll-IL1 receptor domain-containing adaptor inducing IFN-β.

(E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and virally encoded serine protease located in the amino-terminal domain of NS3. Serine protease activity of NS3 requires NS4A, a protein consisting of 54 amino acid residues, to form a stable complex with the NS3 [8–10].

Virus-infected cells trigger the innate immune response by recognizing viral components, including DNA, ssRNA, dsRNA and glycoproteins. This response initiates signaling pathways leading to the induction of protective cellular genes, including type-I interferons [initially interferon (IFN)-β, and then IFN-α] and proinflammatory cytokines that directly limit viral replication. Within these signaling pathways, Toll-like receptors (TLRs) and RNA helicase family members play very important roles in the recognition of the viral components [11,12].

IFN-β is induced by dsRNA, a common intermediate in many RNA virus infections, including HCV. The viral dsRNA as well as the synthetic dsRNA analogue poly(I-C) are recognized by TLR3, which is expressed on the cell surface or in endosome vesicles [13,14]. On the other hand, it has been shown that retinoic acid-induced gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) also recognize dsRNA molecules [15–17]. A recent study showed that MDA5 and RIG-I recognize different types of dsRNA: MDA5 recognizes poly(I-C), and RIG-I recognizes *in vitro* transcribed dsRNA [18]. Very recently, it was discovered that viral 5′-triphosphate RNA is the ligand for RIG-I [19,20]. Both MDA5 and RIG-I contain DexD/H-box helicase domains that serve as intracellular cytoplasmic dsRNA and 5′-triphosphate RNA receptors, respectively [15–20]. After dsRNA is recognized, the cytoplasmic domain of TLR3 recruits TIR-domain-containing adaptor inducing IFN-β (TRIF) through a myeloid differentiation factor 88 (MyD88)-independent pathway (TRIF-mediated pathway) [21–23]. In contrast, the caspase recruitment domains (CARDs) of MDA5 or RIG-I recruit the CARD adaptor inducing IFN-β, Cardif (also known as IPS-1, MAVS, or VISA), which was recently identified as an adaptor protein located in the outer membrane of mitochondria (this recruitment is known as the Cardif-mediated pathway) [24–27].

The TRIF- and Cardif-mediated signaling pathways rapidly induce IFN-β through the phosphorylation of multiple cellular factors, including IFN regulatory factor-3 (IRF-3) and kinases, including the Tank-binding kinase 1 (TBK-1) and inhibitor of κB kinase ε (IKK-ε) [28–31]. Although IRF-3 is located in the cytoplasm in an inactive state [28,29], phosphorylation (Ser385, 386, 396, 398, 402, 405, and Thr404) of IRF-3 by TBK-1 and IKK-ε induces dimerization and nuclear translocation of IRF-3, leading to transcriptional activation of IFN-β [28–31].

Recent studies have found that several RNA virus proteins could inhibit the early signaling activation (TRIF- and Cardif-mediated pathways) leading to IFN-β production [32,33]. Regarding HCV, Foy *et al.* [33] found that NS3-4A serine protease blocked HCV-induced activation of IRF-3 in the human hepatoma cell line HuH-7. Additional studies regarding this finding have shown that NS3-4A blocks the Cardif-mediated signaling pathway by cleaving the Cardif molecule and blocking downstream IFN-β activation [24,34,35], and that TBK-1, IKK-ε, and TRIF may also be targeted for cleaving by NS3-4A [36–38]. With respect to TRIF, NS3-4A was reported to cleave this molecule in both an *in vitro* experiment using a reticulocyte lysate system and an *in vivo* experiment using human embryonic kidney 293 (HEK293) and UNS3-4A-24 osteosarcoma cells [36]. These studies suggest that NS3-4A has the ability to inhibit both TRIF- and Cardif-mediated signaling pathways.

On the other hand, we recently demonstrated that HCV proteins exhibited conflicting effects on the IFN-β production in non-neoplastic human hepatocyte PH5CH8 cells [39,40]: Core and NS5B synergistically enhanced IFN-β expression and this enhancement was dependent on the RNA-dependent RNA polymerase activity of NS5B, but NS3-4A significantly inhibited the production of IFN-β induced by the combination of Core and NS5B. Furthermore, Li *et al.* [41] recently reported that PH5CH8 cells retained robust and functionally active TRIF- and Cardif-mediated signaling pathways, unlike HuH-7 cells, which lacked the TRIF-mediated pathway [41,42]. Therefore, using poly(I-C) as an inducer of IFN-β, we investigated the effects of NS3-4A on antiviral signaling pathways in PH5CH8 cells. Our results showed that the extracellular TLR3/TRIF signaling pathway was not blocked by NS3-4A because NS3-4A did not cleave TRIF, unlike in the previous study [36].

Results

Human hepatocyte PH5CH8 cells more readily activate IFN-β transcription in response to dsRNA compared to HuH-7 cells and their sublines

Recently, Li *et al.* [41] reported that PH5CH8 cells showed a better response to dsRNA, including IFN-β induction, than other human hepatoma cell lines (HuH-7, HepG2, and Hep3B). Therefore, using a dual

luciferase reporter assay, we first confirmed that PH5CH8 cells were much more effective at inducing IFN-β than HuH-7 cells and HuH-7-derived cell sublines (O [43], Oc [43], and OR6c [44]) that can support HCV RNA replication.

When the dsRNA analog, poly(I-C), was transfected into cells using a liposome-mediated procedure (intracellular dsRNA, T-pIC), PH5CH8 cells showed a more potent (> 25-fold) activation of the IFN-β gene promoter than HuH-7 and HuH-7-derived cell lines (Fig. 1A). Furthermore, when poly(I-C) was added to the culture medium (extracellular dsRNA; M-pIC), a significant elevation (12-fold) of the IFN-β gene promoter was observed in PH5CH8 cells only (Fig. 1B). These results were confirmed by quantitative RT-PCR analysis of endogenous IFN-β mRNA induction in cells treated with poly(I-C) (T-pIC, Fig. 1C; M-pIC, Fig. 1D). In both T-pIC and M-pIC treatments, the induction level of IFN-β mRNA was markedly higher in PH5CH8 cells than in O, Oc, OR6c, and HuH-7 cells (Fig. 1C,D). Next, we carried out quantitative RT-PCR analysis of TLR3, TRIF, RIG-I, MDA5, Cardif, and IRF-3 mRNAs to clarify their expression levels in the steady state and the effects of poly(I-C)

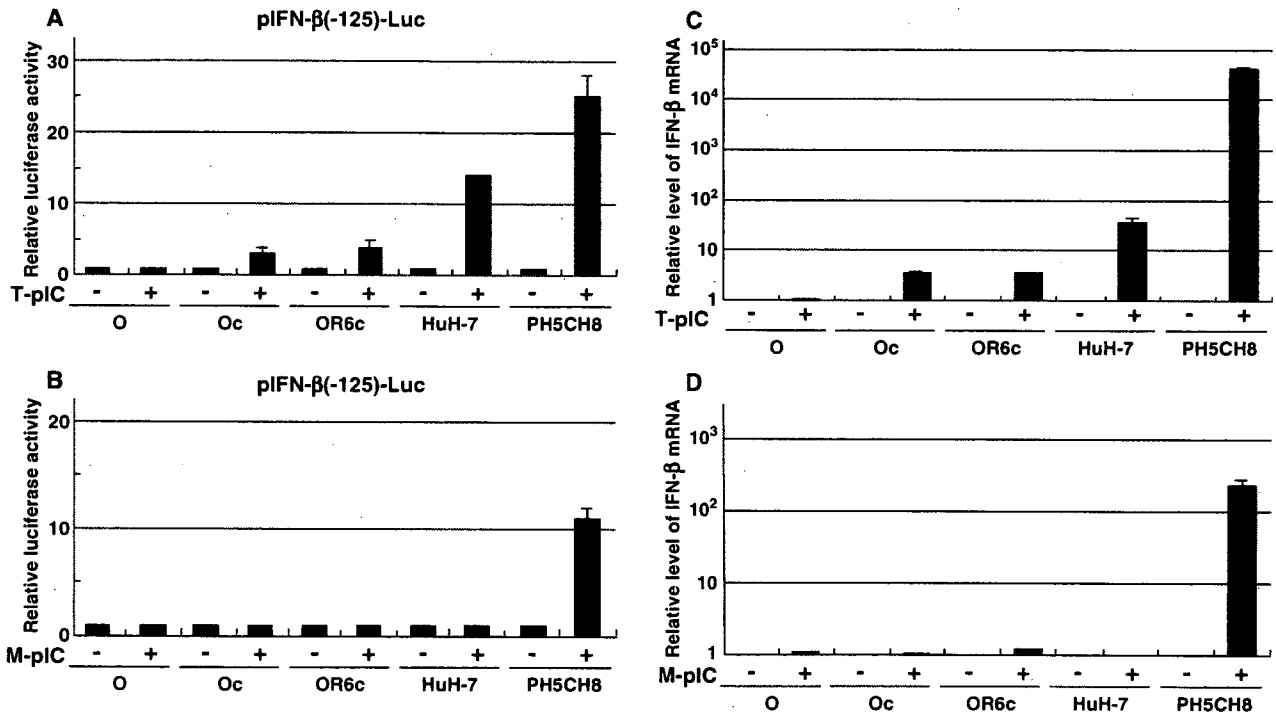

Fig. 1. PH5CH8 cells show high-level IFN-β production in response to dsRNA. (A) Dual luciferase reporter assay of the IFN-β gene promoter using the various cells treated with T-pIC. The following HuH-7-derived cell sublines were used: O, cloned cells [43] replicating genome-length HCV RNA; Oc, cured cells which were created by eliminating genome-length HCV RNA from the O cells by IFN treatment; and OR6c, cured cells which were created by eliminating genome-length HCV RNA from the cloned OR6 cells [44] by IFN treatment. Cells grown in 24-well plates were cotransfected with pIFN-β-(−125)-Luc and pRL-CMV (internal control reporter) and cultured for 42 h, and then poly(I-C) (1 μg) was transfected into the cells for 6 h before the reporter assay as described in the Experimental procedures. The relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). The lysate of cells without poly(I-C) treatment was used as a control. Data are the means ± SD from three independent experiments, each performed in triplicate. (B) Dual luciferase reporter assay of the IFN-β gene promoter using the various cells treated with M-pIC. The dual luciferase reporter assay was performed as described in (A) except that poly(I-C) was added to the medium (50 $\mu g \cdot mL^{-1}$) for 6 h before the reporter assay. (C) Quantitative RT-PCR analysis of IFN-β mRNA in various cells treated with T-pIC. Poly(I-C) (1 μg) was transfected into the cells for 6 h before the sampling for RNA preparation. Total RNA extracted from the cells was subjected to real-time LightCycler PCR analysis using the primer set of IFN-β (202 bp). Data are the means ± SD from three independent experiments. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of IFN-β mRNA concentration to that of GAPDH. The IFN-β mRNA levels were calculated relative to the level in the O cells treated with T-pIC, which was set at 1.0. (D) Quantitative RT-PCR analysis of IFN-β mRNA in various cells treated with M-pIC. Poly(I-C) was added to the medium (50 $\mu g \cdot mL^{-1}$) for 6 h before the sampling for RNA preparation. Quantitative RT-PCR analysis for IFN-β mRNA was performed as described in (C). The IFN-β mRNA level was calculated relative to the level in the O cells treated with M-pIC, which was set at 1.0.

treatment (T-pIC and M-pIC). In T-pIC treatment, RIG-I and MDA5 mRNAs were clearly induced in PH5CH8 and HuH-7 cells, and TLR3 mRNA was induced only in PH5CH8 cells. Moreover, there was no such induction in the other cell lines examined (supplementary Table S1). In M-pIC treatment, TLR3, RIG-I, and MDA5 were induced only in PH5CH8 cells (supplementary Table S1). The fact that these mRNAs were induced at substantial levels only in PH5CH8 cells treated with T-pIC or M-pIC suggests that the elevation of these mRNAs is mediated by the IFN-β induced by poly(I-C) treatment. In summary, these results revealed that PH5CH8 cells retain both the Cardif- and TRIF-mediated pathways for IFN-β production, whereas HuH-7 cells retain only the Cardif-mediated pathway, and that the HuH-7-derived cells lines used are lacking in both pathways for IFN-β production.

Parental PH5CH and PH5CH clones other than PH5CH8 also exhibit IFN-β response toward poly(I-C) treatment

PH5CH8 is one of eight cell lines that were previously cloned from parental PH5CH cells to examine HCV susceptibility *in vitro* [45]. Therefore, we used a dual luciferase assay to examine the effects of poly(I-C) treatment on the IFN-β gene promoter in PH5CH cells and these cloned cell lines. When T-pIC treatment was employed, the parental cells and all the cloned cell lines exhibited good IFN-β response, and the activation level in PH5CH2 and PH5CH6 cells was higher than that in PH5CH8 cells (Fig. 2A). However, when M-pIC treatment was used, the IFN-β response in the cloned cells and the parental cells was less than 50% of that in PH5CH8 cells (Fig. 2B). From these results, we concluded that PH5CH8 is the best cell line for the study of the dsRNA-induced antiviral signaling pathways.

M-pIC treatment activates IRF-3 through the TLR3/TRIF signaling pathway

To confirm that the TRIF-mediated pathway is activated in M-pIC treatment, and to determine if its activation is mediated by the TLR3 but not the TLR4 signaling pathway, we examined whether or not activation of IRF-3 by M-pIC treatment is specifically mediated by the TLR3 signaling pathway using TLR3-, TLR4-, and TRIF-specific small interfering RNA (siRNAs) [46,47]. Quantitative RT-PCR analysis revealed that the TLR3, TLR4, and TRIF mRNAs were drastically decreased (more than 70% reduction) in the

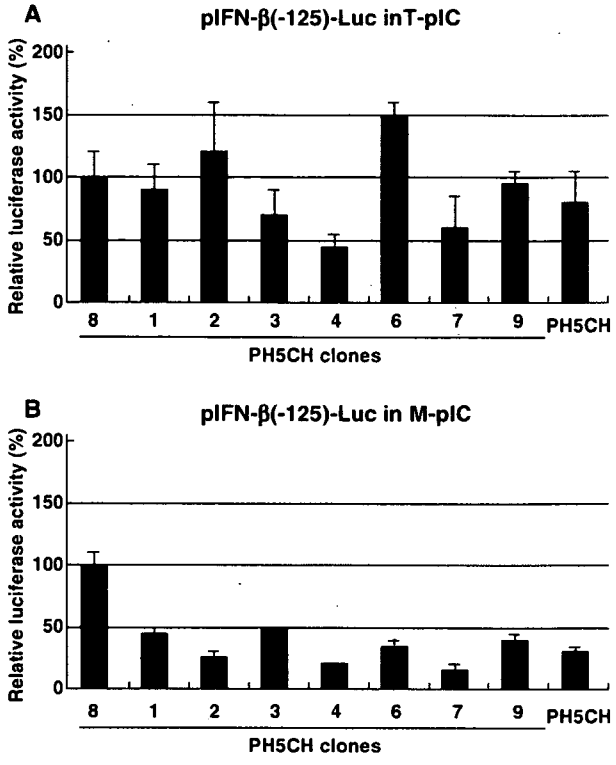

Fig. 2. IFN-β responses of parental PH5CH and PH5CH cloned cells by dsRNA treatment. (A) Dual luciferase reporter assay of the IFN-β gene promoter using parental PH5CH and PH5CH cloned cells treated with T-pIC. The T-pIC treatment and the dual luciferase reporter assay were performed as described in Fig. 1A. The IFN-β gene promoter activity level was calculated relative to the level in the PH5CH8 cells, which was set at 100. (B) Dual luciferase reporter assay of the IFN-β gene promoter using parental PH5CH and PH5CH cloned cells treated with M-pIC. The M-pIC treatment and the dual luciferase reporter assay were performed as described in Fig. 1B. The relative level of the IFN-β gene promoter activity was calculated as described in (A).

PH5CH8 cells transfected with TLR3, TLR4, and TRIF siRNAs, respectively, but not in the PH5CH8 cells transfected with the GL2 siRNA used as a control (Fig. 3A). We also confirmed that IRF-3 mRNA was not decreased in PH5CH8 cells transfected with any of these siRNAs (Fig. 3A). Under this condition, we performed a luciferase reporter assay using an IFN-β gene promoter in PH5CH8 cells treated with M-pIC. The activation of the IFN-β gene promoter was greatly suppressed (by more than 80%) in PH5CH8 cells transfected with TLR3 or TRIF siRNA, but not in the PH5CH8 cells transfected with GL2 or TLR4 siRNA (Fig. 3B). This result suggests that the activation of IRF-3 by M-pIC treatment is mediated by the TLR3/TRIF signaling pathway. We obtained further evidence by examining the status of the phosphorylation and dimerization of IRF-3. The results

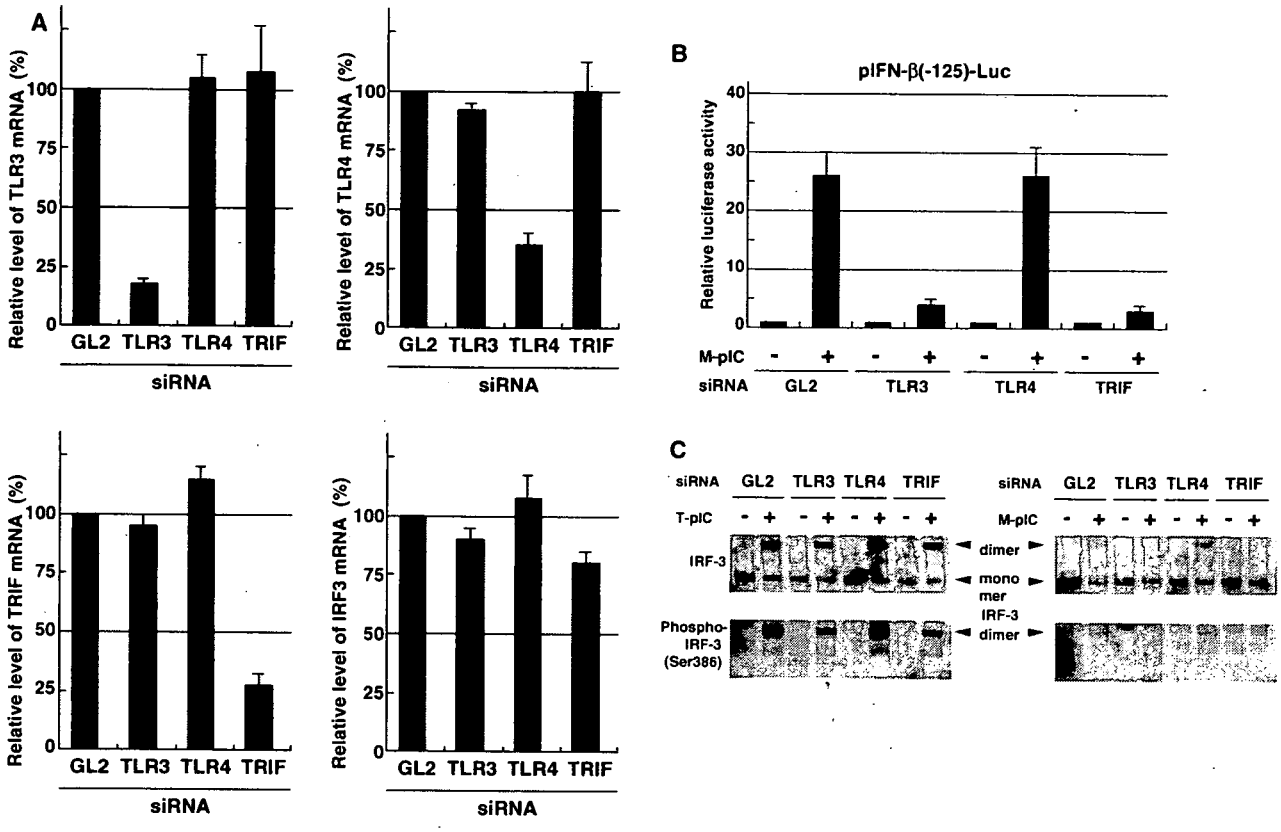

Fig. 3. Extracellular dsRNA treatment activates IRF-3 through the TLR3/TRIF signaling pathway in PH5CH8 cells. (A) Down-regulation of TLR3, TLR4, and TRIF mRNAs by transfection of TLR3, TLR4, and TRIF siRNAs, respectively. PH5CH8 cells were transfected with dsRNA duplexes targeting TLR3, TLR4, TRIF or luciferase GL2. After 3 days, the expression levels of TLR3, TLR4, TRIF, and IRF-3 mRNAs were determined by the quantitative RT-PCR as described previously [67]. (B) Dual luciferase reporter assay of the IFN-β gene promoter using siRNA-transfected PH5CH8 cells treated with M-pIC. The poly(I-C) treatment and the dual luciferase reporter assay were performed as described in Fig. 1. (C) Phosphorylation and dimerization analyses of IRF-3 in the siRNA-transfected PH5CH8 cells treated with poly(I-C). The poly(I-C) treatment was performed as described in Fig. 1. The lysate of cells transfected with GL2, TLR3, TLR4, or TRIF siRNA was prepared, and subjected to Native-PAGE as described in the Experimental procedures. The phosphorylation and dimerization of IRF-3 were analyzed by immunoblotting using anti-phospho-IRF-3 (Ser386) serum and anti-IRF-3 serum, respectively.

obtained by M-pIC treatment revealed that both the phosphorylation and dimerization of IRF-3 were almost completely abrogated in the cells transfected with TLR3 or TRIF siRNA, but not in those transfected with the GL2 and TLR4 siRNAs (Fig. 3C, right panel). Such a suppression of IRF-3 activation was not observed by T-pIC treatment (Fig. 3C, left panel), suggesting that the activation of IRF-3 by T-pIC treatment is mainly mediated by the Cardif-mediated signaling pathway [16].

HCV NS3-4A blocks the Cardif-mediated signaling pathway, but not the TRIF-mediated signaling pathway

Several studies [24,33,36,48–50] have demonstrated that NS3-4A blocks IFN-β induction by inhibiting the nuclear translocation of IRF-3 in HuH-7 cells harboring HCV replicons and HCV (JFH1 strain of genotype 2a)-infected HuH-7 cells. However, it has also been reported that HuH-7 cells possess weak or defective dsRNA-induced antiviral signaling pathways [41,42] (Fig. 1). Therefore, we examined whether or not NS3-4A can block the induction of IFN-β by poly(I-C) in PH5CH8 cells that retain dsRNA-induced signaling pathways. The results were quite different between T-pIC treatment and M-pIC treatment. First, in T-pIC treatment, the results showed that NS3-4As (the 1B-1 and HCV-O strains of genotype 1b) could drastically inhibit the enhancement of the IFN-β gene promoter activity, and that this suppressive effect of NS3-4A was dependent on its serine protease activity, because the NS3-4A/S1165A mutant lacking the serine protease activity did not exhibit the suppressive effect,

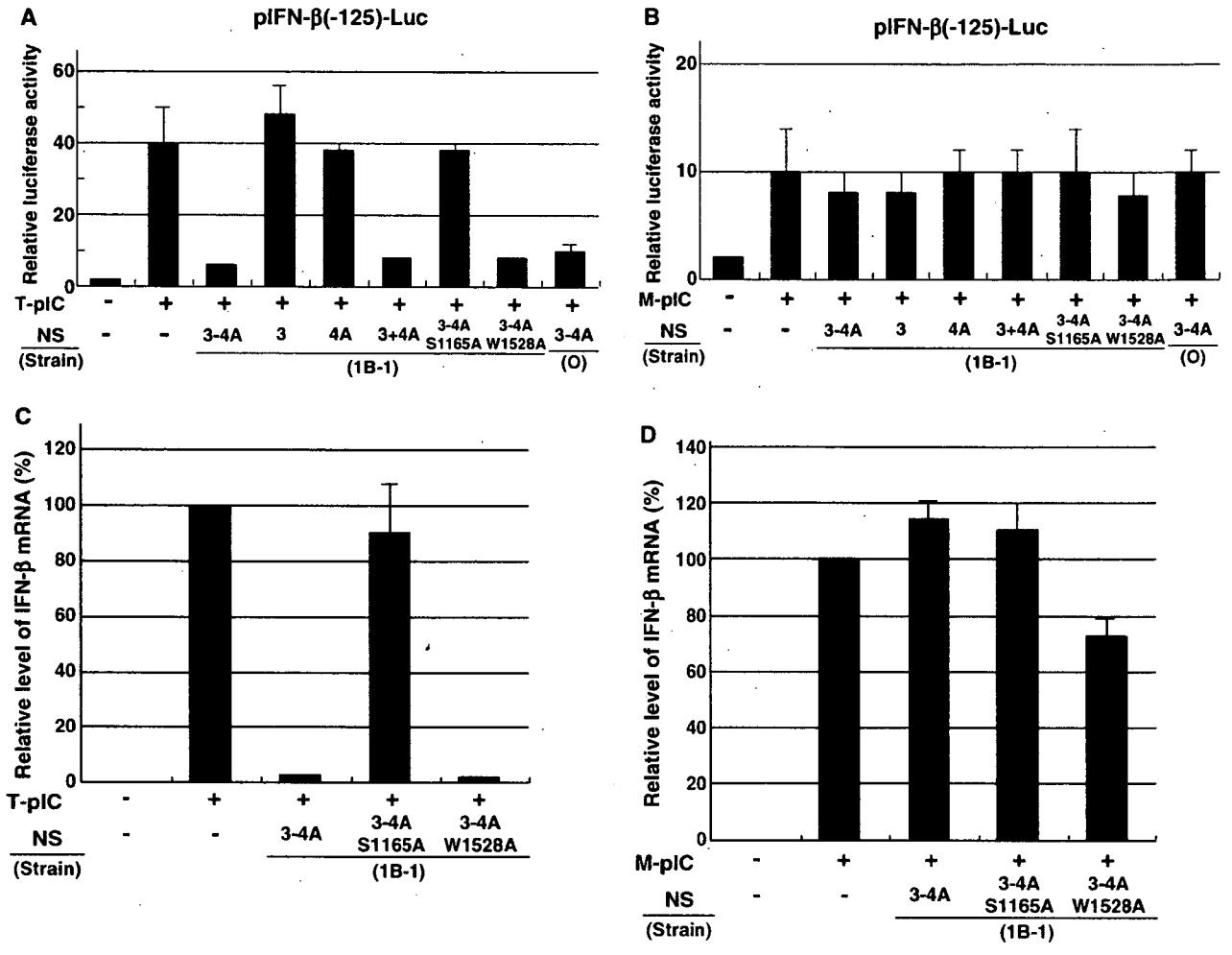

Fig. 4. NS3-4A blocked the Cardif-mediated signaling pathway, but not the TRIF-mediated signaling pathway. The poly(I-C) treatment, dual luciferase reporter assay, and quantitative RT-PCR analysis were performed as described in Fig. 1. The pCX4bsr expression vectors encoding NS3-4A, NS3, or NS4A from the 1B-1 strain and NS3-4A from the HCV-O strain were used for the transfection. The pCX4bsr expression vector encoding the NS3-4A/S1165A mutant (1B-1 strain) lacking serine protease activity or the NS3-4A/W1528A mutant (1B-1 strain) lacking RNA helicase activity was also used for the transfection. The lysate of PH5CH8 cells transfected with the pCX4bsr vector was used as a control (NS–). (A) Effect of NS3-4A on the IFN-β gene promoter activated by T-pIC treatment. (B) Effect of NS3-4A on the IFN-β gene promoter activated by M-pIC treatment. (C) Effect of NS3-4A on the IFN-β mRNA induction by T-pIC treatment. PH5CH8 cells stably expressing the NS3-4A or NS3-4A mutant (S1165A or W1528A) from the 1B-1 strain were subjected to T-pIC treatment. PH5CH8 cells infected with pCX4bsr retrovirus were used as a control (NS–). The IFN-β mRNA level was calculated relative to the level in the control PH5CH8 cells treated with T-pIC, which was set at 100. (D) Effect of NS3-4A on the IFN-β mRNA induction by M-pIC treatment. PH5CH8 cells that were the same as in (C) were subjected to M-pIC treatment. The IFN-β mRNA level was calculated relative to the level in the control PH5CH8 cells treated with M-pIC, which was set at 100.

although the NS3-4A/W1528A mutant lacking RNA helicase activity did (Fig. 4A). In addition, we confirmed that NS3 alone or NS4A alone did not exhibit the suppressive effect, but coexpression of NS3 and NS4A did, suggesting that the NS3/4A complex in *trans* [51] also can block IFN-β induction. In M-pIC treatment, however, we found that NS3-4As (strains 1B-1 and O) could not suppress the induction of the IFN-β gene promoter (Fig. 4B). Similar results were also obtained in the other cloned cell lines, PH5CH3 and PH5CH6 (data not shown), and in HeLa cells (supplementary Fig. S1). The results of the reporter assay were confirmed by quantitative RT-PCR analysis of endogenous IFN-β mRNA induced by T-pIC or M-pIC treatment in PH5CH8 cells. We found that the NS3-4A and NS3-4A/W1528A mutants, but not the NS3-4A/S1165A mutant, could suppress the induction of IFN-β mRNA following

T-pIC treatment (Fig. 4C), but none of these NS3-4As could suppress the induction of IFN-β mRNA following M-pIC treatment (Fig. 4D).

We next examined the effects of NS3-4A on the phosphorylation and dimerization of IRF-3 in PH5CH8 cells. We observed that both T-pIC and M-pIC treatments induced the phosphorylation at Ser386 and Ser396 of IRF-3, and formed the dimerization of IRF-3 (Fig. 5A,B, lanes 1 and 2), and that NS3-4A remarkably inhibited the phosphorylation and dimerization of IRF-3 in the cells treated with T-pIC, depending on its protease activity (Fig. 5A). However, the phosphorylation and dimerization of IRF-3 induced by M-pIC treatment was not inhibited by NS3-4A (Fig. 5B). From these results, we concluded that, in PH5CH8 cells, NS3-4A could not block the TRIF-mediated signaling pathway, although it could block the Cardif-mediated signaling pathway.

NS3-4A blocks the Cardif-mediated pathway by cleaving Cardif

NS3-4A is able to cleave the Cardif [24,34,35] and TRIF [36] molecules, resulting in the blocking of dsRNA-induced antiviral signaling pathways. However, our finding that IFN-β production was not suppressed by NS3-4A in cells treated with M-pIC seemed to contradict the finding of a previous study [36] in which NS3-4A-mediated cleavage of TRIF inhibited dsRNA-activated signaling through the TLR3 pathway. Therefore, we evaluated whether or not NS3-4A could impair the functional ability of

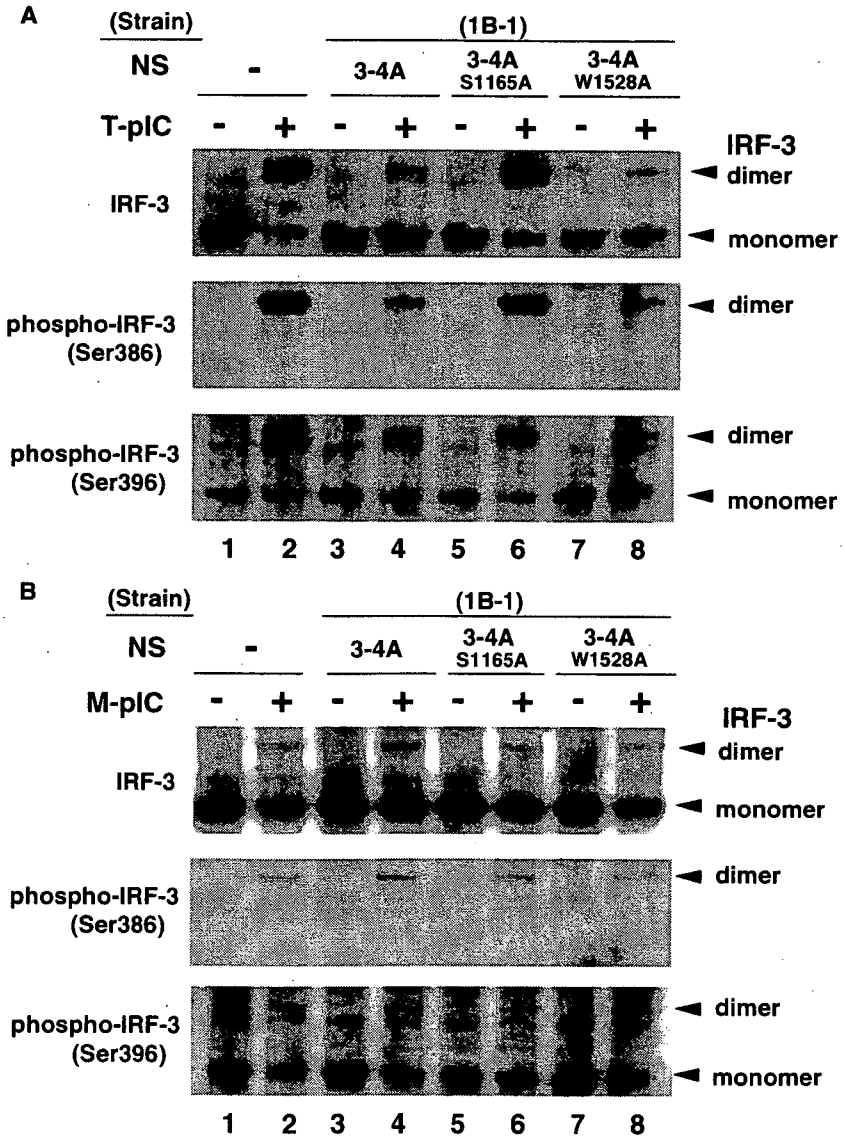

Fig. 5. Effect of NS3-4A on phosphorylation and dimerization of IRF-3 in PH5CH8 cells treated with intracellular or extracellular dsRNA. PH5CH8 cells that were the same as in Fig. 4C were used. The poly(I-C) treatment was performed as described in Fig. 1. (A) Effect of NS3-4A on phosphorylation and dimerization of IRF-3 in the PH5CH8 cells treated with T-pIC. The phosphorylation and dimerization analyses of IRF-3 were performed as described in Fig. 3C. Anti-phospho-IRF-3 (Ser396) serum was also used for the analysis. (B) Effects of NS3-4A on phosphorylation and dimerization of IRF-3 in the PH5CH8 cells treated with M-pIC. The phosphorylation and dimerization analyses of IRF-3 were performed as described in (A).

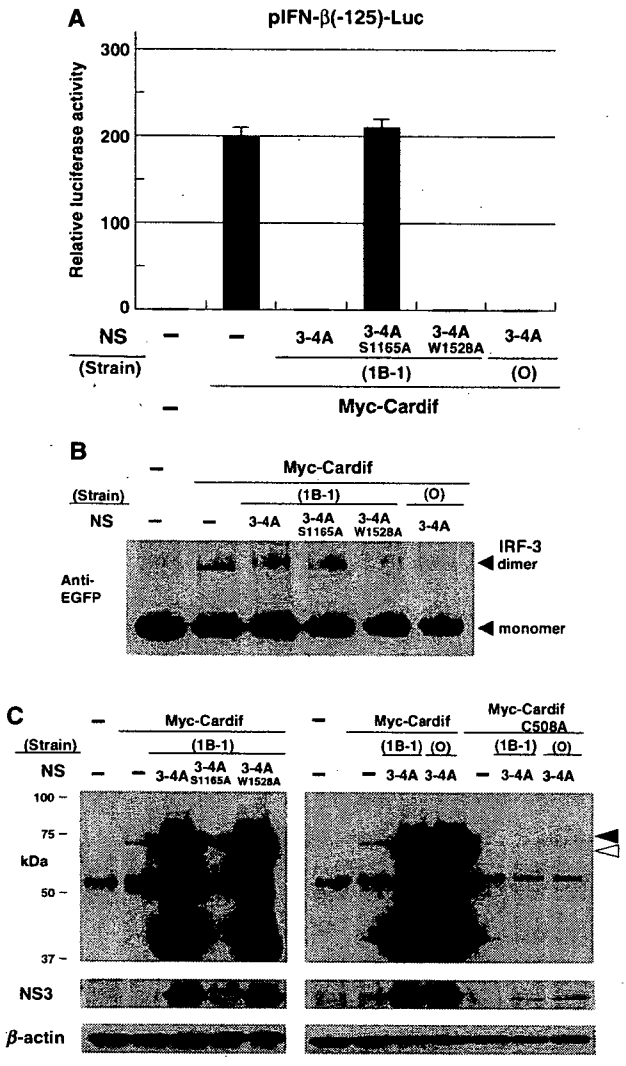

Fig. 6. NS3-4A blocks Cardif-mediated pathways by cleaving Cardif. (A) Effect of NS3-4A on the IFN-β gene promoter activated by the ectopic expression of Cardif in PH5CH8 cells. PH5CH8 cells were cotransfected with the pCX4bsr expression vector encoding NS3-4A or its mutant S1165A or W1528A, as described in Fig. 4, and the pCX4pur expression vector encoding myc-Cardif. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dual luciferase reporter assay was performed as described in Fig. 1A. (B) Effect of NS3-4A on IRF-3 dimerization induced by the ectopic expression of Cardif in PH5CH8 cells. The enhanced green fluorescent protein (EGFP)-IRF3 expression vector was used for the cotransfection in PH5CH8 cells with the myc-Cardif and NS3-4A (wild-type or its mutant S1165A or W1528A) expression vectors. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dimerization analysis of IRF-3 was preformed as described in Fig. 3C using anti-EGFP serum. (C) Cardif is cleaved by NS3-4A in PH5CH8 cells. PH5CH8 cells were cotransfected with the myc-Cardif (wild-type or its mutant C508A) and NS3-4A expression vectors (wild-type or its mutant S1165A or W1528A). Production of the myc-Cardif and NS3 in these cells was analyzed by immunoblotting using anti-myc and anti-NS3 sera, respectively. The PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors were used as a control (NS–). β-actin was used as a control for the amount of protein loaded per lane. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively.

TRIF as well as Cardif in PH5CH8 cells. First, we confirmed the effect of NS3-4A on the activation of the IFN-β gene promoter by the Cardif exogenously expressed in PH5CH8 cells. The results of the luciferase reporter assay revealed that NS3-4As (strains 1B-1 and HCV-O) completely suppressed the activation (200-fold induction) of the IFN-β gene promoter by Cardif, and that this suppression was dependent on the serine protease activity of NS3-4A (Fig. 6A). This result was supported by the results of the dimerization analysis of IRF-3 (Fig. 6B). Next, we confirmed that wild-type Cardif, but not the Cardif mutant (C508A located in the C-terminal region), was cleaved by the NS3-4As (strains 1B-1 and HCV-O), and that this cleavage was dependent on its serine protease activity (Fig. 6C). These results are in agreement with previous studies in which NS3-4A blocked the intracellular dsRNA signaling pathways through cleavage at the Cys508 residue of Cardif [24,34,35].

NS3-4A does not block the TRIF-mediated pathway because it lacks the ability to cleave TRIF

Because we demonstrated that NS3-4A blocked the intracellular dsRNA signaling pathways through cleavage of Cardif in PH5CH8 cells, we performed the same analysis regarding TRIF exogenously expressed in PH5CH8 cells. The results of the luciferase reporter assay using the IFN-β gene promoter revealed that NS3-4As (strains 1B-1 and HCV-O) could not suppress the activation (1000-fold induction) of the IFN-β gene promoter by TRIF (Fig. 7A). This result was also supported by the results of the dimerization analysis of IRF-3 (Fig. 7B). Furthermore, we demonstrated that the exogenously expressed TRIF was not cleaved by NS3-4As (strains 1B-1 and HCV-O) (Fig. 7C). These results indicate that NS3-4A could not block the TRIF-mediated signaling pathway, and suggest that NS3-4A did not suppress the M-pIC-induced production of IFN-β because NS3-4A did not have the ability to cleave TRIF.

To confirm the results obtained in PH5CH8 cells, we examined the status of Cardif and TRIF molecules expressed exogenously in the O cells replicating genome-length HCV-O RNA efficiently and their cured Oc cells. The results revealed that Cardif was cleaved in the O cells but not in the Oc cells (Fig. 8A,B), and that the cleavage of Cardif occurred

when NS3-4As (strains 1B-1 and HCV-O) were expressed in the Oc cells (Fig. 8B). From these results, we confirmed that NS3-4A could cleave Cardif in the O and Oc cells. In contrast, TRIF was not cleaved in either O or Oc cells (Fig. 8C). We further confirmed that TRIF was not cleaved in the O cells transfected with TLR3 siRNA, indicating that the resistance of TRIF to NS3-4A is not related to the presence of TLR3 (Fig. 8C). We also performed the same analysis using HeLa cells, and obtained results (supplementary Fig. S2) similar to those obtained in PH5CH8 cells (Figs 6C, 7C and 8). In addition, we observed that, like TRIF, exogenously expressed MDA5 and RIG-I were not cleaved by NS3-4A in PH5CH8 cells (data not shown). Taken together, the above results indicate that NS3-4A cleaves the Cardif molecule, resulting in interruption of the Cardif-mediated pathway, but NS3-4A is not able to cleave the TRIF molecule, and thus the TRIF-mediated pathway is not suppressed by NS3-4A.

Discussion

In the present study, we demonstrated that parental PH5CH cells and their clones retained both TRIF- and Cardif-mediated pathways as antiviral dsRNA signaling pathways, and confirmed that the PH5CH8 cell line was far more useful for the study of antiviral pathways than HuH-7 or the cell lines cloned from it. From the results of the present study and a previous study [41], we considered the possibility that immortalized hepatocyte cells possess the functional TRIF- and Cardif-mediated signaling pathways. Based on this assumption, we examined IFN-β production in three other immortalized human hepatocyte cell lines, NKNT-3 [52], IHH10.3 [53], and IHH12 [53], after treatment with poly(I-C). However, the results revealed that none of these immortalized cell lines responded to both M-pIC and T-pIC treatments. Therefore, we suggest that PH5CH and the cell lines cloned from it are uniquely suitable for the comprehensive study of antiviral TRIF- and Cardif-mediated signaling pathways.

We failed to obtain evidence that NS3-4A was able to cleave TRIF as reported by Li *et al.* [36]. In our study (Fig. 7C), there was no evidence of the cleavage of the TRIF molecule in NS3-4A-expressed PH5CH8

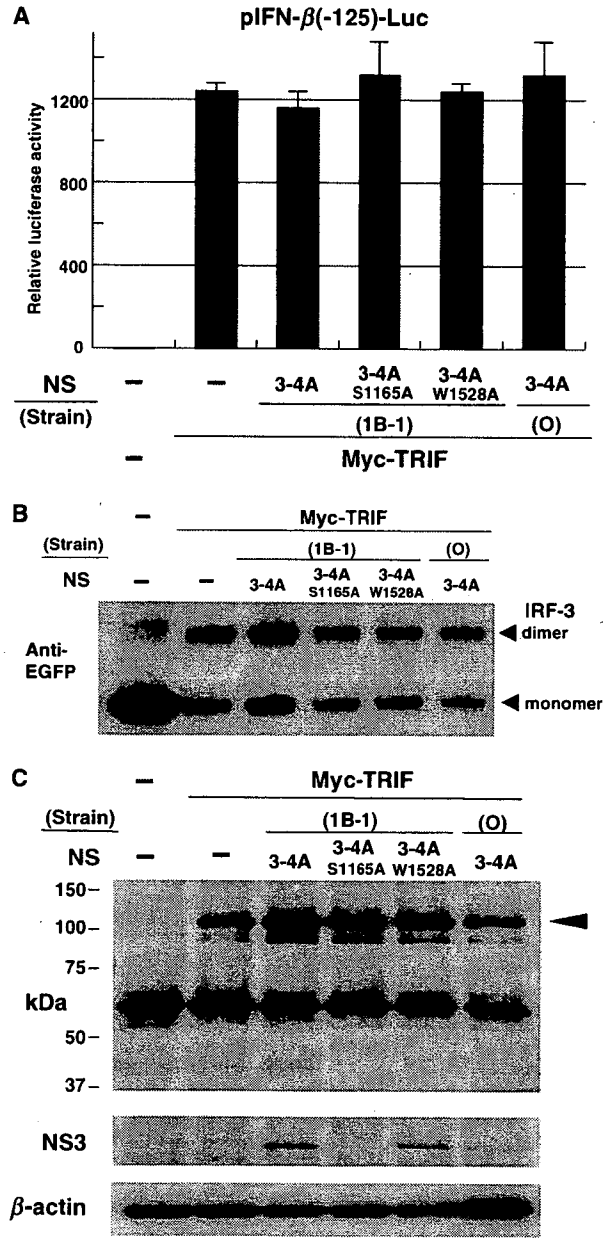

Fig. 7. NS3-4A does not block the TRIF-mediated pathway because it lacks the ability to cleave TRIF. (A) Effect of NS3-4A on the IFN-β gene promoter activated by the ectopic expression of TRIF in PH5CH8 cells. PH5CH8 cells were cotransfected with the pCX4bsr expression vector encoding NS3-4A or its mutant S1165A or W1528A, and the pCX4pur expression vector encoding myc-TRIF. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dual luciferase reporter assay was performed as described in Fig. 1A. (B) Effect of NS3-4A on IRF-3 dimerization induced by the ectopic expression of TRIF in PH5CH8 cells. The dimerization analysis of IRF-3 was performed as described in Fig. 6B except that the myc-TRIF expression vector was used in place of the myc-Cardif expression vector. (C) TRIF is not cleaved by NS3-4A in PH5CH8 cells. PH5CH8 cells were cotransfected with the myc-TRIF and NS3-4A (wild-type or its mutant S1165A or W1528A) expression vectors. Production of myc-TRIF and NS3 in these cells was analyzed by immunoblotting using anti-myc and anti-NS3 sera, respectively, as described in Fig. 6C. The black arrowhead indicates the noncleaved TRIF.

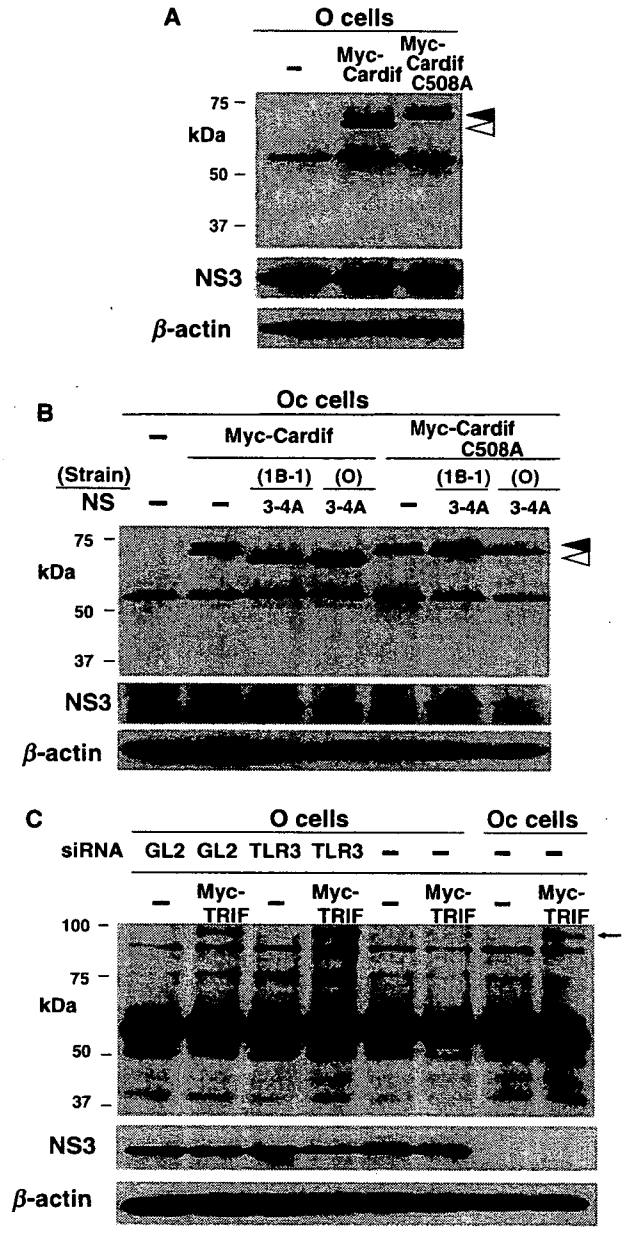

Fig. 8. TRIF is not cleaved in genome-length HCV RNA replicating cells. (A) Cardif is cleaved in the O cells replicating genome-length HCV-O RNA efficiently. The O cells were transfected with the myc-Cardif (wild-type or its mutant C508A) expression vector. Production of the myc-Cardif and NS3 in the O cells was analyzed by immunoblotting as described in Fig. 6C. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively. (B) Cardif is cleaved by NS3-4A in the cured Oc cells. The Oc cells were cotransfected with the myc-Cardif (wild-type or mutant C508A) and NS3-4A expression vectors. The production of the myc-Cardif and NS3 in these cells was analyzed by immunoblotting as described in Fig. 6C. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively. (C) TRIF is not cleaved in the O cells. The O and Oc cells were transfected with the myc-TRIF expression vector. The O cells transfected with GL2 or TLR3 siRNA were also used for the analysis. Production of myc-TRIF in these cells was analyzed by immunoblotting as described in Fig. 6C. The black arrowhead indicates the noncleaved TRIF.

cells. Nor did we observe any cleavage of TRIF by the NS3-4A expressed in the Oc cells, which exhibited almost no response to the T-pIC and M-pIC treatments (Figs 1 and 8C), or the HeLa cells, which exhibited a good response to the T-pIC and M-pIC treatments (supplementary Figs S1 and S2). We further observed that TRIF was not cleaved in the O cells, in which the HCV NS protein precursor was efficiently processed by NS3-4A (Fig. 8C). Regarding the cellular localization of NS3-4A, it has recently been reported that the localization of NS3-4A expressed transiently in HuH-7 cells was the same as that in genome-length HCV RNA replicating cells, and that NS3-4A was localized not only on the endoplasmic reticulum, but also on mitochondria [54]. From these findings, we suggest that NS3-4A is unable to cleave TRIF in cultured human cells.

Although amino acid sequences (PSSTPC/SAHLT, cleavage at Cys372; the P6 residue is underlined) surrounding the NS3-4A *trans*-cleavage site in TRIF [36] resemble those (DLEVVT/STWVL for NS3-4A; DEMEEC/ASHLP for NS4A/4B; DCSTPC/SGSWL for NS4B/5A; EDVVCC/SMSYS for NS5A/5B; the P6 residue is underlined) in the NS proteins from the 1B-1 and HCV-O strains and that (EREVPC/HRPSP, cleavage at Cys508; the P6 residue is underlined) in Cardif, only the TRIF site lacks the acidic P6 residue that is conserved and important in viral cleavage sites [55]. Accordingly, we examined whether or not a TRIF mutant (P to E at the P6 residue) is cleaved by NS3-4A in PH5CH8 cells. However, no cleavage of the TRIF mutant was observed (unpublished data). To clarify why TRIF is not cleaved by NS3-4A, further analysis will be necessary.

Although the results obtained in the present study suggest that the suppression of IFN-β production by NS3-4A is limited in human hepatocyte cells, it has recently been reported [56] that HCV can block the dsRNA-induced signaling pathway via the NS3-4A-independent pathway in addition to the NS3-4A-dependent pathway. However, because HuH-7 cells infected with the HCV genotype 2a clone (JFH1) were used in that study, it is not clear whether or not the TRIF-mediated pathway is also inhibited by the NS3-4A-independent pathway. To clarify this point, it will be necessary to study an HCV infection system using human hepatocyte cells in which both the TRIF- and

Cardif-mediated pathways are functional, such as PH5CH8 cells.

We clearly demonstrated that Cardif was cleaved by NS3-4As of 1B-1 and HCV-O strains obtained from healthy HCV carriers [57]. Although we observed that this cleavage was dependent on the protease activity of NS3-4A (Fig. 6), the correlation between the inhibitory effect of NS3-4A on the Cardif-mediated signaling pathway and the protease activity of NS3-4A remains unclear. Furthermore, we have no evidence that all NS3-4As derived from patients with HCV are able to cleave the Cardif molecule. To clarify these issues, further comparative analysis among HCV strains obtained from patients with different hepatic disease conditions will be needed. In addition, in the present study, we observed that the bands corresponding to the cleaved Myc-Cardif became extremely intense in PH5CH8 cells (Fig. 6C). This phenomenon has been observed in previous studies [24,34,49]. Although these previous studies did not explain what caused this phenomenon, we speculate that the cleaved Myc-Cardif is transferred to the cytosolic (soluble) fraction, although noncleaved Myc-Cardif remains in the membrane (insoluble) fraction. To clarify the reason for this phenomenon, several experiments may be needed.

In summary, we show that NS3-4A could not cleave TRIF, but could cleave Cardif, in PH5CH8 cells possessing functional TRIF- and Cardif-mediated antiviral signaling pathways, and suggest that the disruption of the IFN-β production system by NS3-4A is not sufficient in HCV-infected hepatocyte cells. This information will be useful for understanding the roles of NS3-4A in persistent HCV infection.

Experimental procedures

Cell culture

Non-neoplastic human hepatocyte PH5CH-derived cloned cells, including PH5CH8 cells, which are susceptible to HCV infection and supportive of HCV replication [45], were maintained as described previously [58]. HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. The O cells replicating genome-length HCV RNA were cultured in DMEM with 10% fetal bovine serum and G418 (300 $\mu g \cdot mL^{-1}$; Geneticin, Invitrogen) as described previously [43]. The Oc and OR6c cured cells, which were created by eliminating genome-length HCV RNA from O cells [43] and OR6 cells [44] by IFN treatment, respectively, were also cultured in DMEM with 10% fetal bovine serum.

Construction of expression vectors

Retroviral vectors pCX4bsr and pCX4pur [59], which contain the resistance gene for blasticidin and puromycin, respectively, were used to construct the various expression vectors. pCX4bsr/NS3-4A(1B-1), pCX4bsr/NS3(1B-1) and pCX4bsr/NS4A(1B-1) were constructed according to the previously described method [60]. The DNA fragments encoding NS3-4A, NS3, and NS4A derived from the HCV 1B-1 strain belonging to genotype 1b (accession no. AB0802999) [61] were subcloned into the *Eco*RI and *Not*I sites of pCX4bsr. To construct pCX4bsr/NS3-4A(O), the DNA fragment encoding NS3-4A derived from the HCV-O strain belonging to genotype 1b [43] were also subcloned into the *Eco*RI and *Not*I sites of pCX4bsr. pCX4bsr/NS3-4A (1B-1)/S1165A and pCX4bsr/NS3-4A(1B-1)/W1528A were constructed by PCR mutagenesis with primers containing base alterations according to the previously described method [62]. To construct pCX4pur/myc-Cardif, the DNA fragment encoding Cardif (IPS-1/MAVS/VISA, accession no. DQ181928) was amplified from cDNAs obtained from PH5CH8 cells by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The primer sequences containing the *Sph*I (forward) and *Not*I (reverse) recognition sites for Cardif were designed to enable expression of the Cardif ORF. The obtained DNA fragment was subcloned into the *Sph*I and *Not*I sites of pCX4pur/myc, which can express myc-tagged protein, according to the previously described method [39]. To construct pCX4pur/myc-TRIF, the *Eco*RI-*Not*I fragment of pCXpur/myc-TRIF encoding myc-TRIF ORF [39] was subcloned into the *Eco*RI and *Not*I sites of pCX4pur. To construct pEGFP-C1/IRF-3, the DNA fragment encoding IRF-3 (accession no. NM_001571) was amplified by PCR as described above. The primer sequences containing the *Xho*I (forward) and *Hin*dIII (reverse) recognition sites for IRF-3 were designed to enable expression of the IRF-3 ORF. The obtained DNA fragment was subcloned into the *Xho*I and *Hin*dIII sites of pEGFP-C1 (Clontech, Mountain View, CA, USA), and the obtained pEGFP-C1/IRF-3 was used for IRF-3 dimerization analysis. The nucleotide sequences of these constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Poly(I-C) treatment

Poly(I-C) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was added to the medium at 50 $\mu g \cdot mL^{-1}$ (M-pIC), or 1 μg of poly(I-C) was complexed with Lipofectamine™ 2000 (Invitrogen) for transfection (T-pIC). Cells were assayed for poly(I-C)-induced responses 6 h after exposure by either route.

Luciferase reporter assay

For the dual luciferase assay, we used a firefly luciferase reporter vector, pIFN-β-(−125)-Luc [63], containing the IFN-β gene promoter region (−125 to +19). The reporter assay was carried out as previously described [40]. Briefly, a total of 0.3×10^5 cells were seeded in a 24-well plate, 24 h before transfection. Then, 0.1 μg firefly luciferase reporter vector, 0.2–0.4 μg HCV protein expression plasmid (pCX4bsr series), and 0.2 ng pRL-CMV (Promega, Madison, WI, USA) as an internal control reporter were transfected into the various cell lines. To maintain the efficiency of transfection, up to 0.4 μg of pCX4bsr was added instead of HCV protein expression vectors. In some cases, 20 ng of pCX4pur/myc-Cardif or pCX4pur/myc-TRIF were added as the effector plasmid. The cells were cultured for 48 h, and then a dual luciferase assay was performed according to the manufacturer's protocol (Promega). In some cases, the cells were cultured for 42 h and then poly(I-C) was added to the medium or transfected into the cells for 6 h before the reporter assay. Three independent triplicate transfection experiments were conduced to verify the reproducibility of the results. Relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG & G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

Western blot analysis

Preparation of cell lysates, SDS/PAGE, and immunoblotting were performed as described previously [64]. Anti-NS3 (Novocastra Laboratories, Newcastle, UK), anti-myc (PL14; Medical and Biological Laboratories, Nagoya, Japan) or anti-β-actin serum (AC-15; Sigma, St Louis, MO, USA) was used in this study as a primary antibody. Immunocomplexes were detected by a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA, USA).

IRF-3 dimerization analysis

Preparation of cell lysates and native-polyacrylamide gel electrophoresis were performed as described previously [65]. After the separation of proteins, immunoblotting was performed as described above. Anti-IRF3 serum (FL-425; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the detection of the endogenous IRF-3 dimerization. Anti-phospho-IRF-3 (Ser386) serum (IBL, Gunma, Japan) and anti-phospho-IRF-3 (Ser396) serum (Upstate Biotechnology, Lake Placid, NY, USA) were used for detection of the phosphorylated IRF-3. The dimerization of exogenous IRF-3 was detected by anti-EGFP monoclonal serum (JL-8; Clontech).

Preparation of PH5CH8 cells stably expressing HCV proteins

PH5CH8 cells were infected with retrovirus pCX4bsr encoding various HCV proteins, as described previously [64]. pCX4bsr/NS3-4A(1B-1), pCX4bsr/NS3-4A(1B-1)/S1165A, and pCX4bsr/NS3-4A(1B-1)/W1528A were used to obtain the PH5CH8 cells stably expressing NS3-4A(1B-1), the NS3-4A(1B-1)/S1165A mutant lacking the serine protease activity [51], and the NS3-4A(1B-1)/W1528A mutant lacking the helicase activity [66], respectively. At 2 days postinfection, PH5CH8 cells were changed with fresh medium containing blasticidin (20 $\mu g \cdot mL^{-1}$), and the culture was continued for 7 days to select the cells expressing HCV proteins.

Real-time LightCycler PCR

Total cellular RNA was extracted using an Isogen extraction kit (Nippon Gene, Toyama, Japan). Before reverse transcription, the RNA was treated with RNase-free DNase I (TaKaRa Bio, Ohtsu, Japan) to completely remove the genomic DNA as described previously [40]. Real-time LightCycler PCR was performed according to a method described previously [67]. The sequences of sense and antisense primers for TRIF (accession no. AB093555) were 5′-AAGCCATGATGAGCAACCTC-3′ and 5′-GTGTCC TGTTCCTTCCTCCAC-3′. The sequences of sense and antisense primers for RIG-I (accession no. NM_014314) were 5′-AATGAAAGATGCTCTGGATTACTTG-3′ and 5′-TTGTCTCTGGGTTTAAGTGGTACTC-3′. The sequences of sense and antisense primers for MDA5 (accession no. NM_022168) were 5′-AAGTCATTAGTAAA TTTCGCACTGG-3′ and 5′-TCATCTTCTCTCGGAAAT CATTAAC-3′. In addition, we used primer sets for IFN-β [40], TLR3 [39], TLR4 [39], Cardif [24] and GAPDH [40].

RNA interference

siRNA duplexes targeting the coding regions of human TLR3 [39], TLR4 (Dharmacon, Lafayette, CO, USA; catalog no. M-008088-00), TRIF (Dharmacon; catalog no. M-012833-00), and luciferase GL2 [68] (Dharmacon) as a control were chemically synthesized. PH5CH8 cells were transfected with the indicated siRNA duplex using OligofectAMINE (Invitrogen). Total RNA was extracted at 3 days after transfection, and real-time LightCycler PCR was performed to examine RNA-mediated interference efficiency as described above.

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