研究紹介
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新しいC型肝炎の霊長類モデル

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【導入の文章を 100 ～ 150 字程度でお願いします】（例）科学の発達がその副産物として生み出した感染症の脅威に対抗し、同じ科学技術を駆使して私たちは霊長類モデル研究を行っています。

はじめに――伝染病制圧の幻想

20 世紀初頭まで、伝染病は人類の存在をも脅かす存在でした。しかし、科学の進歩はそれまでの予想をはるかに上回り、多くの医学研究の成果、とくに抗生物質の発見やワクチンの開発によって、伝染病の多くは致死的疾病ではなくなりました。たとえば、戦前まで不治の病とされた結核は、BCG ワクチンの普及により多くの人が感染から予防され、また感染が確認された人もストレプトマイシンをはじめとする抗生物質の投与で治療できるようになったのです。1980 年に WHO が宣言した天然痘の根絶は微生物学の最大の偉業であり、もはや伝染病は人類の脅威ではなくなったかに見えました。

しかし、科学の発達はそれにともなう副産物として新たな感染症の脅威を生み出しました。交通手段の発達による人間の移動の高速化と範囲の拡大、それによる野生動物との接触機会の増大、一方では非衛生的条件での輸血や注射などの医療行為が、エンデミック（風土病）であった病原微生物を世界中に拡散させたのです。その代表例が、AIDS ウイルスや C 型肝炎ウイルスの地球規模での感染拡大です。さらに、抗生物質の乱用は多剤耐性を獲得した病原性微生物の拡大という問題を招いています。

ここでは C 型肝炎に焦点を当て、これまでの経緯と現状、および当研究室でおこなっている霊長類モデル研究について紹介したいと思います。

C 型肝炎――原因ウイルスの発見と現状

戦後のわが国では肝炎患者が急増し、原因究明が急務となりました。1970 年代に A 型と B 型の肝炎ウイルスが発見され、検査法を確立してスクリーニングを実施したにもかかわらず、なお半数の肝炎（非 A 非 B 型肝炎）の原因は謎に包まれていました。

1989 年、カイロン社のチョウらによって C 型肝炎ウイルス（Hepatitis C virus：HCV）が同定され、慢性肝炎のじつに 70％が HCV 感染によるものであることが判明しました。これを契機に、各種肝炎ウイルスの抗体検査、ウイルス遺伝子検査法が確立され、輸血後肝炎の発生は急速に減少していったのです。一方、1980 年代のウイルス汚染フィブリノゲン製剤（いわゆる薬害肝炎）問題が 2002 年ごろからマスコミで大きく取り上げられ、薬害エイズ問題と並んで重大な社会問題に発展したことは記憶に新しいと思います。

C 型肝炎は、慢性肝炎、肝硬変を経て肝癌に進展する、きわめて治しにくい疾患です。国内ではおよそ 200 万人が、全世界では 1 億 7 千万人以上が HCV キャリアであると報告されています。先進国では、多少の差はありますが平均的な HCV の罹患率は 1 ～ 2％にとどまっています。一方、発展途上国では、未検査のドナーからの輸血、汚染した医療機具の再利用や不適切な滅菌操作などの医原性感染、薬物注射の不正使用などによって、HCV キャリアは依然として増加しています。感染拡大を抑えるため HCV 予防ワクチンの開発が急がれていますが、まだ開発途上の段階にあり、実用化までには相当の時間を要すると思われます。現在、慢性 C 型肝炎の最も有効な治療法としてはペグ化 IFN とリバビリンの併用療法がおこなわれています。この治療法は慢性 C 型肝炎患者の約 50％にしか効果がなく、残り 50％の患者さんは依然として肝硬変や肝癌のリスクを免れない状況におかれています。

C 型肝炎研究のきっかけ

私は当時、米国メリーランド州にある国立アレルギー感染症研究所（National Institute of Allergy and Infectious Diseases）の研究員として、エイズウイルス遺伝子の病原性にかかわるメカニズム解明にとりくんでいました。縁あって国立感染症研究所・筑波医学実験用霊長類センターに異動することが決まっていましたが、ある日、友人宅で偶然見た日本のテレビ番組（フジテレビ「検証 C 型肝炎」の録画ビデオ）に大きなショックを受けました。というのは、1980 年代のある期間、血友病患者用の血液製剤に大量の感染性 HCV が混入していたこと、それが米国の刑務所囚人やドラッグ患者の血液を使用して製造されていたこと、ウイルス感染の危険性を認識していたにもかかわらず製薬メーカーは速やかな対応をとらず放置していたことなど、衝撃的な事実が報道されていたのです。

それより前に薬害エイズが社会的問題となっていましたが、製薬メーカーが営利追求を優先し、厚労省がそれを黙認するという問題が、またも露見した形になりました。この事件を知ってたいへんショックを受けるとともに、厚労省の研究機関である国立感染症研究所に所属することになっていた私は、C 型肝炎研究で自分が何か貢献できないものかと考えました。

HCVの動物モデル

通常のワクチン開発では、対象病原体を実験感染させたモデル動物に候補ワクチンを接種し、有効性や安全性を評価します。しかし、HCVは宿主域が狭く、ヒトとチンパンジー以外の動物には感染・発症しないため、マウスやラット、実験用サル類は評価系として使用できません。他方、チンパンジーは絶滅が危惧されている類人猿であることや倫理的理由から、日本を含む多くの国で実験動物として使用することが禁止されています。こうした理由で、HCVワクチンの開発研究はなかなか進まないのです。また、HCVは通常の感染症と異なり、免疫応答を巧妙に回避し長期にわたって持続感染するという特徴をもっています。この免疫回避のメカニズムについても未解明の点が多く残されていますが、その最大の理由も、ウイルス感染とそれに引きつづいて起こる免疫応答を評価できる動物モデルがないことです。

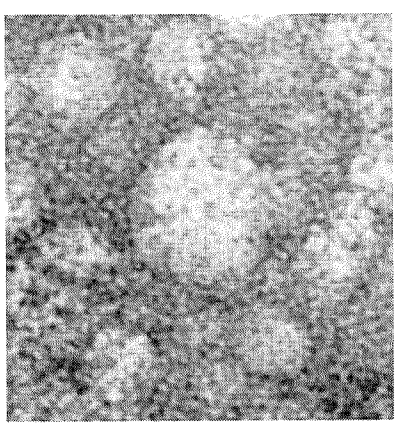


図1 GBV-Bウイルス粒子の電子顕微鏡写真

こうした状況を踏まえ、サル類の肝炎ウイルスでHCVに最も近縁なGBウイルス-B（GBV-B、図1）を用いたモデル動物システム研究に着手することにしました。GBV-BはHCVと同じフラビウイルス科ヘパシウイルス属に分類されており、タマリン、マーモセットなどの新世界ザル（図2）に感染し増殖します。ちなみに新世界とはヨーロッパから見たアメリカ大陸のことで、これらのサルは中南米に広く生息しています。

GBV-Bをタマリンに実験感染すると、急速に増殖して1週間でウイルス血症を示すようになります。また、これとほぼ並行して軽度の肝炎症状を呈します。すなわち、肝炎を示すマーカーであるアラニンアミノトランスフェラーゼ（ALT）値が上昇するとともに、病理的には図3に示すように、肝臓における類洞（肝細胞板間の毛細管）の崩壊や拡張、肝細胞の変性やアポトーシスが観察されるようになります。ウイルス血症は約3か月間持続し、その後、ウイルスが消失するに及んで寛解となります。GBV-BはHCVと同様に、肝臓細胞で最もよく増殖します。興味深いことに、GBV-B感染ザルのウイルス分布を解析したところ、肝臓だけでなくリンパ・造血組織でも高いウイルス増殖を示すことが明らかになりました。HCVも同様に多組織指向性（pleiotropism）を示すこと、それが肝臓以外での疾患の原因となっていることから、GBV-BはHCVと同様の特性をもったウイルスであると考えられます。

GBV-Bによる長期持続感染・慢性肝炎モデルへ

HCVの場合、急性感染者の70％が持続感染に移行することが報告されています。また、以前のチンパンジーへのHCV接種実験では、約3分の1が持続感染になったと報告されています。この長期にわたる持続的HCV感染によって、慢性肝炎→肝硬変→肝細胞癌と病態が進行することから、そのメカニズム解析には持続感染するモデル動物が必要です。

さきほど述べたように、GBV-BはHCVに類似したウイルスであり、その感染モデルはヒトでは困難なHCV感染初期における病態や免疫応答の解明に有用であると考えられています。しかし、このモデルには解決すべき大きな問題があります。タマリンに実験感染させたGBV-Bのほとんどは、すでに述べたように約3か月間のウイルス血症のあとは消失してしまい、長期持続感染は起こらないのです。これまでにわずか3例の持続感染例が報告されていますが、それも長くて2年程度でウイルスが消失してしまい、慢性肝炎症状は持続しませんでした。こうした理由で、この動物モデルのC型肝炎研究における有用性については懐疑的だったのです。

私たちは、GBV-Bの分子クローン由来ウイルスをマーモセットに感染させ、一定レベルの血中ウイルス量を3年以上維持する長期持続感染系の作出に成功しました。じつは、感染初期ではタマリンと比較してウイルス増殖効率が思わしくなく、ALT値もほとんど上昇しなかったので、正直なところ失敗だと思っていたのです。ところが、実験開始後1年、2年と経過しても血中ウイルスが継続的に検出され、想定外の嬉しい結果につながりました。このような個体ではALT値がHCV患者と同様に上昇していることから、慢性肝炎を呈していると考

図2 左）アカテタマリン
右）コモンマーモセット

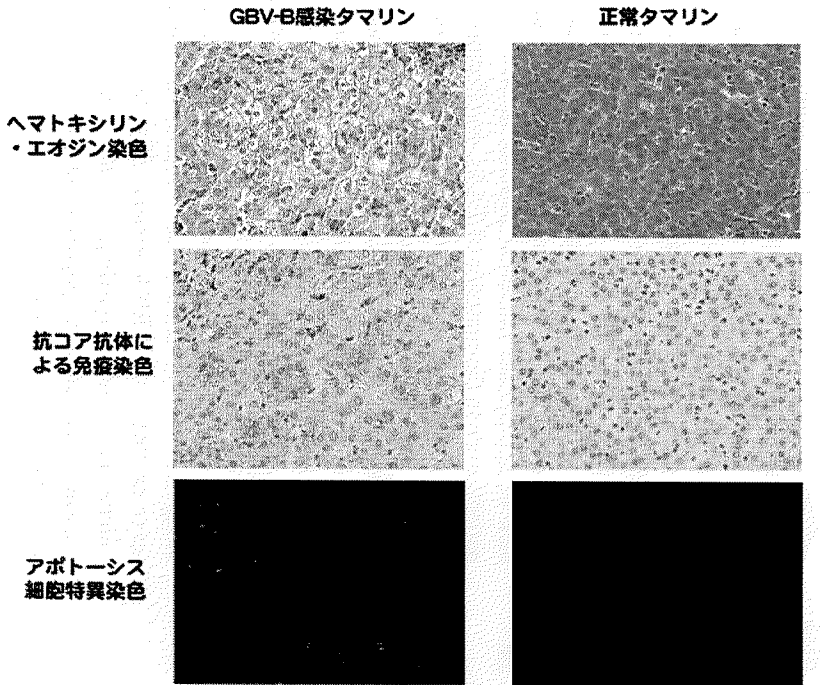


図3　GBV-B 感染タマリン肝臓の組織病理解析

えられます。でも、これで喜ぶのはまだ早い！　マーモセットでもヒトと同様の抗ウイルス免疫応答が本当に生じているのか？

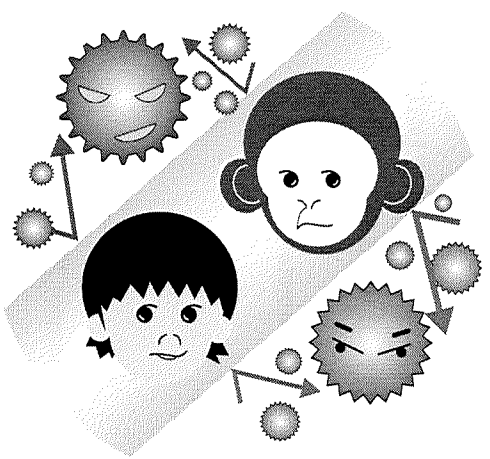
HCV と同様に、GBV-B も免疫応答を回避するようなアミノ酸置換をともなう遺伝子変異を起こすのか？　もしそうでないならば、まともな免疫応答が起こらないのでウイルスが増えているにすぎず、HCV の場合とは異なるのではないか、という疑問に答える必要がありました。

幸いにして、その答えは Yes でした。マーモセットは GBV-B に対する抗体や細胞障害性 T 細胞などの抗ウイルス免疫応答を示し、GBV-B は特異的なアミノ酸置換をともなう遺伝子変異を生じていることが確認されました。重要なのは、このアミノ酸置換に A から B、そして A に戻る場合（復帰変異）や、A から B、さらに C へと変異する場合（連続変異）がみられたことです。こうした変異は偶然では起こりえず、抗 HCV 免疫応答におけるエピトープ領域（抗体が結合する部分の配列）においてのみ認められます。つまり、生体側はアミノ酸 A を含むエピトープ領域を認識してウイルスを抑え込もうとしますが、ウイルス側の A から B への変異によって、このエピトープ認識ができなくなってしまいます。生体側はこれに対応して B 変異を認識するようになりますが、ウイルス側はさらに変異して免疫を回避する……というイタチごっこになるのです。以上のことから、GBV-B は HCV と同様に、宿主免疫応答を回避することで長期持続感染する能力をもち、その結果、感染宿主に慢性肝炎を生じさせることが明らかになったのです。

私たちは、この新たな C 型肝炎モデルを利用して、予防ワクチン開発や病態解明のための基礎的研究を推進していきたいと考えています。

おわりに

これまでの研究によって、GBV-B は遺伝子レベルだけでなく生体における動態や病原性などの面でも HCV に類似していることがわかりました。しかし、GBV-B の自然宿主やウイルス分布についての研究はまだほとんど進んでいません。中南米に生息するサルたちのウイルス疫学はたいへん興味深い研究テーマで、いつかチャレンジしてみたいと思います。こうしたさまざまな切り口から、ウイルスと宿主である霊長類のせめぎあいや共進化について、さらに研究を進めたいと考えています。



Cancer Immunol Immunother
DOI 10.1007/s00262-008-0652-9

REVIEW

The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer

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Received: 6 December 2008 / Accepted: 30 December 2008

Abstract Infection with RNA viruses presents a typical pattern of virus products, double-stranded RNA (dsRNA), and induces the maturation of antigen-presenting dendritic cell (mDC). There are several dsRNA sensors that are differentially distributed on the cell membrane and in the cytoplasm and are variably expressed depending on the cell type. Among these sensors, TLR3 links to the adaptor TICAM-1 (TRIF), which is characterized by its unique multipronged signaling cascades for cytokine/chemokine production, apoptosis and autophagy in both immune and tumor cells. In the context of mDC maturation, various cellular events are further induced in response to dsRNA; these include cross-priming followed by CD8+ CTL induction, NK activation and proliferation of CD4+ T cells including Th1, Th2, Treg and Th17 cells. In this review, we focus on the potential role of dsRNA in modulating the inflammatory milieu around mDCs and tumor-associated antigens to drive specific cellular effectors against the tumor.

Keywords Immunotherapy for cancer · RNA adjuvant · Toll-like receptor · TICAM-1 (TRIF) · Dendritic cells · Cellular effectors

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Published online: 31 January 2009

Introduction

Tumor progression often occurs during inflammation because cell growth is an event that is closely connected to both extrinsic and intrinsic inflammatory stimulation [1]. Many biological mediators such as cytokines and chemokines are involved in immune cell recruitment, which accelerates tumor development in an inflammatory milieu [2]. Immune-related cells are incorporated into the tumor matrix and evoke complicated immune responses against the tumor through cell–cell interactions. Ultimately, the antigen (Ag)-presenting cells (APC) mature as a result of the inflammatory stimuli and tumor-associated antigens (TAAs) and flow out to the regional lymph nodes where TAAs are presented to lymphocytes [3]. However, tumor remission does not occur frequently despite TAA presentation by APC [3, 4]. In contrast, most other infections facilitate myeloid dendritic cell (mDC) maturation [5] and provoke a robust immune response that contributes to pathogen eradication. If PRRs fail to be activated due to the lack of appropriate microbial patterns in APC of cancer patients even in the presence of TAAs, no effectors are generated for tumor targeting, thereby neither immune edition nor surveillance occurring against tumor.

Double-stranded (ds) RNA is a product of virus replication. A variety of RNA and DNA viruses generate replication-mediated dsRNA, polyU/UC or stem-loop structures [6], which serve as ligands for pattern-recognition receptors (PRRs). TLR3 [7], TLR22 [8], RIG-I/MDA5 [9], PKR [10], NALP3 [11, 12] and Dicer in the RNAi system [13] along with as yet unidentified receptors are believed to serve as PRRs for dsRNA sensing (Fig. 1a). These PRRs induce intracellular signaling cascades that regulate cell growth, differentiation, apoptosis and immune activation [6, 14]. Ultimately, dsRNA and its synthetic analog polyI:C

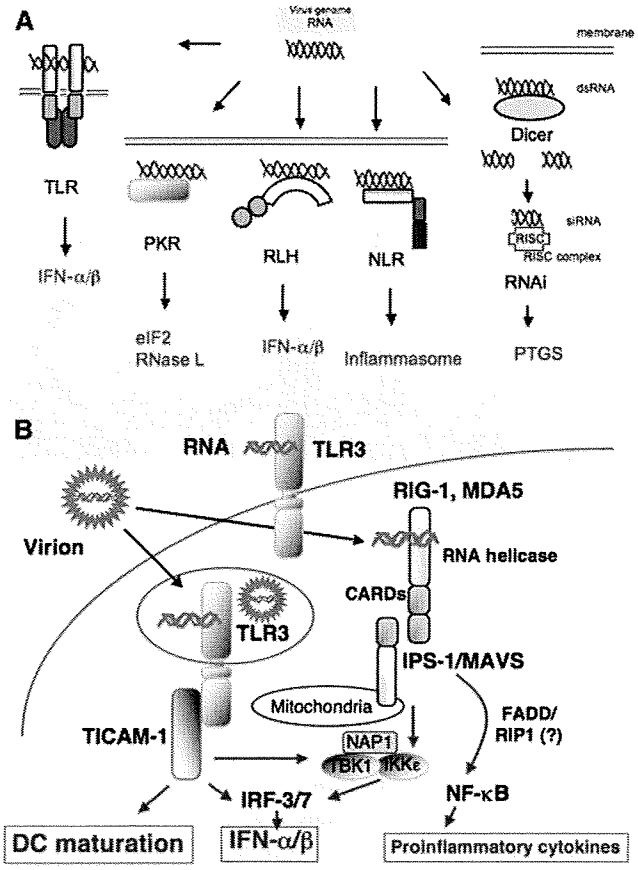

Fig. 1 dsRNA-sensing systems. **a** Double-stranded (ds)RNA are primarily generated during virus replication. Major dsRNA sensors in human cells are indicated. Dicer and RNA-recognizing helicases work in invertebrates as antiviral receptors, but in humans no evidence of these receptors for antiviral action has been proposed. How dsRNA selects a variety of RNA pattern sensors remains largely unknown. PTGS, post-transcriptional gene silencing. **b** TLR3 is mainly localized in the endosome of limited cell types, while RLH (RIG-I and MDA5) are ubiquitously distributed in the cytoplasm. Adaptor molecules, TICAM-1 and MAVS, are localized in the cytoplasm. Upon stimulation, TLR3 recruits TICAM-1 near the endosomal membrane, while MAVS recruits RLH on the mitochondrial membrane. The known outputs of TLR3 and RLH are indicated by *red*

exert a wide range of biological activities and can elicit immune responses. Since dsRNA-sensing PRRs are distributed across a variety of host cells in different combinations [6, 15], systemic inflammation occurs in various modes depending on the receptors and cell types involved in viral infection, virus vaccine inoculation or dsRNA administration for RNA therapy. An inflammatory environment promotes tumor growth and priming of dendritic cells. Many sterile and infectious RNAs induce inflammation.

The signaling pathways of PRRs are linked via adaptor proteins (Fig. 1b). The intra-cytoplasmic RNA sensors, RIG-I and MDA5, interact with MAVS (Cardif/IPS-1/VISA) on the outer membrane of mitochondria [16], and TLR3 resides in the endosome and interacts with TICAM-1 (TRIF) [17]. The signal selection systems of other dsRNA sensors are relatively less defined. Typically, stimulation of the TICAM-1 and MAVS pathways induces type I interferons (IFN) [18]. This is a reflection of the fact that the signaling cascades of both pathways converge upon the complex of the virus-activated kinase (VAK), i.e., NAP1/SINTBAD-IKKe/TBK1 [18, 19] (Fig. 2a). Other cellular responses, autophagy [20], proliferation [21] and apoptosis [22], are induced in cells stimulated with dsRNA (Fig. 2a). Study of the molecular mechanism of these responses is currently underway.

In mDCs, a variety of cellular effectors are driven in response to dsRNA. CD4 Th1, CD8 CTL, NK cells, regulatory T cells (Treg), and Th17 cells are activated/proliferated through dsRNA-stimulated mDCs [15]. Some inflammatory cytokines and chemokines, as well as IFN-inducible gene products are also up-regulated in mDCs. These effectors appear to be independently induced in a situation-dependent manner. However, the molecular mechanisms whereby these variable effectors are differentially induced by mDCs are unknown. We have determined that the TICAM-1 pathway in mDCs is involved in inducing all these effector cell types (Fig. 2b).

In this review, we focus on the TICAM-1 pathway in which cellular effectors are induced by mDCs. We also discuss the involvement of the TICAM-1 pathway in cancer progression and the therapeutic potential of TICAM-1 in antitumor immunotherapy.

TLR3 agonists in cancer immunotherapy

PolyI:C is a representative agonist for human and mouse TLR3 [23]. This compound is believed to be an analog of viral double-stranded RNA (dsRNA) and is a strong inducer of type I IFN in both humans and mice [24]. Initially, polyI:C was regarded as a PKR activator [25]. Later, it was determined that this compound is not only a TLR3 agonist, but also a stimulator of the cytoplasmic RNA sensor, MDA5 [26]. PolyI:C also activates RIG-I [26], but other viral RNA patterns, 5′-triphosphate [27, 28] and polyU/UC [29] may be natural ligands for RIG-I. Earlier, it was reported that polyI:C, which is capable of activating various PRRs, causes endotoxin-like cytokine stormsl; therefore, this compound was deemed to be too toxic for application in clinical therapy [30].

mDCs mature into APCs that drive cellular effectors (Fig. 3). TLR3 resides in the endosome of mDCs [17], senses dsRNA in the endosome, and relays signals to the TICAM-1 pathway, thereby leading to maturation of mDCs [6]. Thus, endosomal stimulation of TLR3 by ligands links to activation of mDCs (Fig. 1b). Certain dsRNA derivatives preferentially activate TLR3 rather than RLH receptors

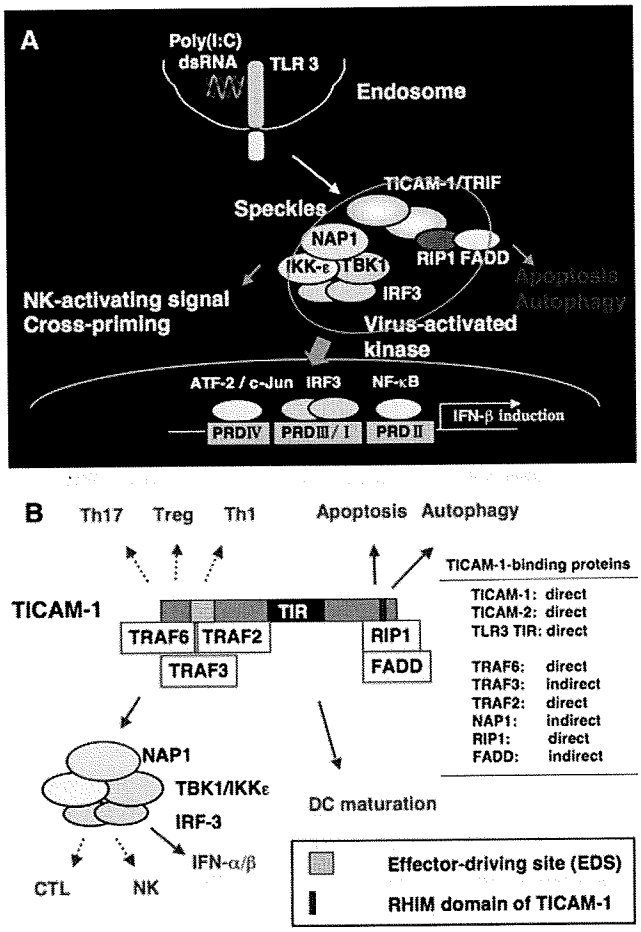

Fig. 2 An outline of the TICAM-1 pathway. **a** In human cells, TICAM-1 once detached from TLR3 serves as a signaling platform to induce apoptosis, autophagy, NK activation and cross-priming. TICAM-1 undergoes some modification secondary to complex formation with TLR3, forms multimer, and dissociated from TLR3 with unknown mechanism. The pathways for NK activation, CTL induction and autophagy are only partially identified, although the pathway for apoptosis is getting clarified. Although epithelial cells in bronchi, bile-duct and intestine express TLR3 on their surface membranes, it is undetermined whether surface-expressed TLR3 retains the cellular responses. **b** The N-terminal 'Effector-driving site (EDS)' recruits appropriate signal-transmitting molecules and matures mDCs leading to induction of effector cells, including NK and CTL. The C-terminal RHIM domain participates in signal transmission for apoptosis and autophagy. TICAM-1-binding proteins, either direct or indirect, are summarized in the inset table

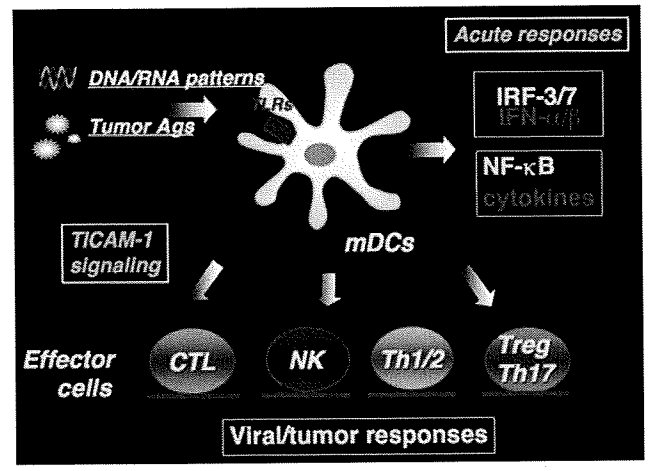

Fig. 3 Various effectors driven by the TICAM-1 pathway in mDCs. The effectors can be induced through the TICAM-1 pathway in mDCs are delineated in this figure. In an early phase of infection, cytokines and IFNs are released in response to microbial patterns. Later, the cellular effectors are induced secondary to activation of the TICAM-1 pathway in mDCs

when they are targeted to the endosome [6]. Synthetic or viral replication-induced RNA products with the stem or stem-loop structures possess mild TLR3-agonistic activity and have no toxic effect on mice. These modified RNA duplex signatures are potential TLR3 stimulants.

Although the natural ligands of TLR3 remain unknown, TLR3 recognizes RNA duplex. To date, it has been shown that polyI:C and the duplex signatures of RNA from many viruses and other synthetic RNAs can be recognized by TLR3. DOTAP and other lipofection agents can deliver RNA to the endosome where TLR3 is localized in mDCs [6]. TLR3 links to the adaptor TICAM-1 to induce IFN-β signaling. Whether or not TLR3 links to MyD88 in addition to TICAM-1 in mDCs remains unknown. However, based on the results from knock-out (KO) mice analyses, the contribution of the MyD88 pathway to the functioning of mDCs is minimal, if any [31].

TICAM-1 signaling

TICAM-1 is the largest of the four TLR adaptors identified so far [32]. It serves as a platform for the assembly of the TRAF family [33, 34] and TANK family [18, 19]. The N-terminal region of TICAM-1 [named Effector-driving site (EDS)] participates in the molecular recruitment (Fig. 2b). In contrast, RIP1 [35] and FADD [36] are recruited to its C-terminal region (Fig. 2b). A variety of cellular outputs were then developed [34, 37]. IFN-α/β, proinflammatory cytokines, ROS and K+ are induced in mDCs. Autophagy and apoptosis are evoked in cells other than mDCs. TICAM-1 modification and translocation lead to the formation of TICAM-1 homo-multimers in mDCs, which activate signal pathways leading to induction of cellular effectors, CTL, NK and CD4+ T cells [15]. The IFN-α/β-inducing pathway of TICAM-1 has been well characterized. Interferon regulatory factor (IRF)-3 and -7 are activated by virus-activated kinase (VAK) [38]. A similar pathway induces IL-1β, IL-6, TNF-α and IL-12p40 [38]. However, the pathways by which ROS are induced remains unknown. Recent reports suggest that LPS, a ligand that activates the TLR4-TICAM-1 pathway [39], induces the activation of

the inflammasome which may interfere with autophagy. This leads to incremental production of IL-1β as well as ROS [40]. Thus, entire pathways led by TICAM-1 remain to be characterized but the pathways appear to coordinately diverge to induce different effectors.

Two PRRs link the TICAM-1 adaptor in humans and mice. TLR3 directly couples with TICAM-1 [41, 42], whereas TLR4 recruits the TICAM-2 (TRAM)-TICAM-1 complex in human and mouse cells [39]. Once dsRNA is provided exogenously, it is taken up into the endosome where TLR3 is expressed [43]. When TLR3 is stimulated, TICAM-1 is recruited to the cytoplasmic TIR domain of TLRs and then dissociated from the receptor, leading to multimer formation [43, 44]. Multimeric TICAM-1 is capable of assembling TRAF family proteins (particularly TRAF2, 6 and 3) in the N-terminal region of TICAM-1 [33]. This ubiquitin E3 ligase complex binds VAK, consisting of NAP1 (or other TANK family proteins), IKKε and/or TBK1. VAK in turn activates IRF-3 and IRF-7 in the cytoplasm [38]. The phosphorylated IRFs translocate to the nucleus to activate the IFN-α/β promoters. The MAPK pathway may be activated through the N-terminal region of TICAM-1. On the other hand, the C-terminal portion of TICAM-1 recruits RIP1, which leads to the activation of IKKα/β and NK-κB [35]. These pathways sustain the production of inflammatory cytokines and type I IFNs. Although the TICAM-1 protein is maintained at low levels in normal cells, the mechanism by which this protein is regulated remains unknown.

In contrast, MAVS, which is the adaptor molecule of RIG-I/MDA5 for signaling the presence of cytoplasmic dsRNA, also binds TRAF (3 and 6), TRADD and RIP1 in the outer mitochondrial membrane to activate VAK [45]. If this protein is cleaved at the C-terminus by the NS3/4A protease of HCV, it loses the ability to transduce signaling to VAK [46]. It also inactivated by proteolytic cleavage by caspase 1 [47].

The TICAM-1 pathway in cancer cells

Tumor cells induce autophagy via the TICAM-1 pathway [48]. PolyI:C is a compound that induces autophagy in tumor cells, and this reaction augments the activation of caspase 1 of the inflammasome that produces robust amounts of active IL-1β, IL-18 and IL-33 [49]. TICAM-1 KO cells lose the ability to undergo polyI:C-mediated inflammasome activation. This autophagy-augmenting activity is TICAM-1-dependent, and has been mapped to the N-terminal region of EDS.

Breast cancer cells undergo apoptosis upon treatment with polyI:C [50]. Intestinal epithelial cells of mice are injured upon intraperitoneal administration of polyI:C [51]. Previous studies have shown that TICAM-1-overexpressing cells induce apoptosis through a RIP/FADD/caspase 8-dependent pathway [52]. PKR may be additionally involved in dsRNA-derived apoptosis [53]. TLR3 as well as PKC-alpha plays a part in poly(I:C)-mediated tumor cell apoptosis [54]. In other reports, cell damage and apoptosis by polyI:C were not merely due to the TICAM-1 pathway, but were a consequence of the output secondary to other dsRNA-sensing pathways [22, 52–54].

Some tumor cell lines induce IL-6, IL-12p40, IL-1β, TNF-α and IL-8 in response to polyI:C. Of these, IL-12p40 induction is largely dependent on TICAM-1 [55]. Other cytokines partly depend on TICAM-1 and the MAVS pathway.

CTL and NK cell activation driven by mDCs

CTL is induced by TICAM-1

CTLs proliferate in response to Ags presented on MHC class I molecules in mDCs. Endogenous Ags, including proteins of viral origin, are presented on MHC class I molecules to induce MHC-restricted CTL in virus replication. Since dsRNA is produced along with Ag presentation in virus-infected mDCs, viral Ags are efficiently presented in a TAP-dependent manner under these circumstances, and pattern molecules, which are dsRNA molecules in this case, simultaneously stimulate mDCs. However, mDCs are not always infected with viruses and even when they are non-infected, they can present viral Ag in a TAP-independent manner [56]. In other word, when Ags and dsRNA are extrinsically taken up into mDCs, the cross-priming mechanism enables mDCs to present Ags on MHC class I molecules [56, 57]. Cross-priming is enhanced by the TICAM-1 pathway in mDCs (Fig. 2a), which efficiently induce Ag-specific CTL [58]. It is expected that similar dsRNA-mediated cross-priming occurs in mDCs that phagocytose TAA instead of viral Ags [3]. Activation of the pathway that induces CTL against TAAs may occur in mDCs and this may facilitate the regression of MHC-high tumors.

Based on increasing evidence obtained by deletion mutagenesis experiments, the N-terminal region of TICAM-1 is involved in the initiation of cross-priming in mDCs. The region contains the site required for TRAF-binding and VAK activation, and probably overlaps with EDS (Fig. 2b). However, induction of cross-priming is independent of IRF-3/7. Thus, occurrence of this event relies on the mechanism involving the molecules for VAK activation but is not dependent on the transcription factors IRF-3/7 [59].

NK cells are induced by TICAM-1

NK cell activation is reciprocally induced by dsRNA-stimulated mDCs (Fig. 4). The mDC-activated NK cells

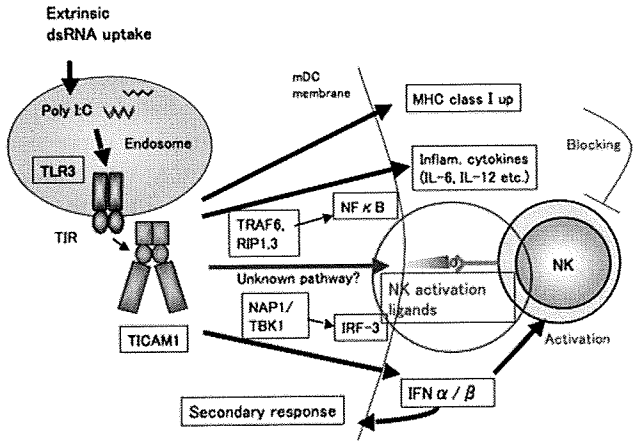

Fig. 4 Possible NK-inducing pathways against cancer. NK activation is an example of mDC output. For full activation, NK cells have to be supported by dendritic cells (myeloid DCs in this figure) that recognize pathogen-associated molecular patterns (PAMPs). In general, NK cells and dendritic cells are reciprocally activated by soluble signals and cell/cell contact. Since the tropism of the pathogen varies, the main NK activating players are determined by which sensor cells are attacked by the pathogens and induce innate signals for NK activation

effectively damage MHC-low tumor cells. The events involved in mDC-mediated NK activation upon stimulation with dsRNA remains unknown. TICAM-1 KO mDCs fail to activate NK cells and TICAM-1 KO NK cells fully restore IFN-γ induction and cytotoxic activity against NK target cells [60], suggesting that mDC TICAM-1 is essential for NK driving. Transwell experiments have revealed that cell–cell contact between mDCs and NK cells rather than with mDC-liberated mediators is crucial for mDC–NK activation [60]. The molecule responsible for NK activation must be expressed on the surface of mDCs in response to dsRNA stimuli and foster mDC–NK interaction (Fig. 4). Genechip analyses using TICAM-1 KO versus wild-type mDCs stimulated with polyI:C, have permitted the identification of several molecules as TICAM-1-dependent NK activation enhancers. NK activation followed by mDC maturation has a strong antitumor effect against MHC-low tumors. The TICAM-1 region required for NK driving in mDCs is the N-terminal region that includes the EDS of TICAM-1 (Fig. 2b). Induction of IRF-3, not IRF-7, is essential for this mDC-NK reciprocal activation [59].

Induction of Th, Treg and Th17 cells by mDCs

CD4+ Th cells

CD4+ Th cells play a pivotal role in skewing the immune responses against cancer. Th1 effector cells are critical for the maintenance of memory CD8+ T cells [61–63], while Th2 cells help B cells to produce various classes of immunoglobulins (Ig) [64, 65]. It is not completely clear as to how memory T cells are regulated by CD4+ T cells, but the importance of CD4+ T cells in the generation and expansion of CD8+ memory T cells has been reported [66]. Earlier data on CD4+ T cell functions should be interpreted cautiously since in those studies, the CD4+ Th populations frequently contained CD4+ regulatory T (Treg) and Th17 cells, and these contaminating cells acted in concert with CD4+ Th cells to modulate the development of CD8+ memory T cells. The possible roles of Treg and Th17 cells in tumor progression will be discussed later. In general, Treg cells suppress immune responses to induce immuno tolerance at tumor sites [67], while Th17 cells are evoked in conjunction with acute inflammation and are linked to smoldering inflammation around the tumor lesion to promote tumor incidence and growth [68]. The functions of CD4+ Th cells should be defined by discounting these Treg/Th17 effector functions.

The CD4+ Th cells consist of the Th1 and Th2 T cell subsets, based on their distinct cytokine secretion profiles. CD4+ Th1 cells produce cytokines IL-2 and IFN-γ. The latter is produced by Th0 (naive T) cells after IL-12 from mDCs stimulate the expression of Stat1 and subsequently that of T-bet, a master transcription factor in Th1 cells [69]. The TICAM-1 pathway in mDCs may contribute to Th1 polarization by preferentially inducing IL-12p40 [55, 60, 70]. CD4+ Th1 cells then provide cytokines for CD8+ T cells and synergistic activation of mDCs, which are essential for CD8+ T cell proliferation and function [71]. IL12p40 is a cytokine that is induced by VAK, which connects with the N- and C-terminal regions of TICAM-1 (Fig. 2b).

In contrast, some TLR ligands may promote the differentiation of CD4+ Th2 cells. IL-4 produced by basophils, eosinophils and NKT cells initiates Stat6 signaling, leading to the expression of GATA-3, which is a master transcription factor in Th2 cells [71]. Participation of TICAM-1 in Th2 polarization has been reported [72] but not confirmed by another group [73]. Several attempts have been made to establish CD4+ T cell clones from tumor-infiltrating T cells. The results indicated that most CD4+ T cell clones are Th1 effectors that secrete IFN-γ and IL-12, but not IL-4 [74].

Th17 cells

IL-17-producing T (Th17) cells are a distinct lineage within the general category of CD4+ Th cells, and secrete a unique set of cytokines, i.e., IL-17 [75, 76]. TGF-β and IL-6 produced by tumor cells, Treg cells and APCs activate the TGF-β and Stat3 signaling pathways, leading to the expression of RORγt, a critical transcription factor for Th17 cells [77]. Th17 cells were first identified as a new CD4+ T cell subset consisting of self-reactive CD4+ Th1 cells. These

cells were later associated with the pathogenesis of many autoimmune diseases [75, 76]. The role of Th17 cells in cancer is less defined than that of Th1 cells. Nonetheless, both IL-17 and IL-23 have been identified in cancer tissues [78], suggesting that Th17 cells together with proinflammatory cytokines may provide an environment favorable for cancer development or invasion. We recently showed that elevated lactic acid in cancer tissues and macrophages in response to TLR stimuli play a key role in IL-23 induction in mDCs or tumor-associated macrophages and help inducing Th17 cells in cancerous environments [79]. Thus, the induction of both IL-23 and IL12p40 by TICAM-1 may be crucial for Th17 stimulation in mDCs. Th17-mediated development of autoimmune disease is constrained by TICAM-1-dependent type I IFN production and its downstream signaling pathway [80]. However, the TICAM-1 region in mDCs that participates in Th17 development is unknown. Th17 cells might play certain roles in tumor progression.

Treg cells

CD4+ Treg cells have been identified as a small subset of the T cell population. Several subpopulations of Treg cells have been reported. Naturally occurring CD4+/CD25+ Treg cells together with other CD4+ Treg cells, including CD4+/CD25− Treg, Tγ-1 and/or Th3 cells, are involved in T cells regulation [81]. Tγ-1 cells secrete IFN-γ and IL-10, while Th3 cells secrete high levels of TGF-β, IL-4 and IL-10. Foxp3 has been shown to be a specific marker of CD4+ Treg cells in both mice and humans [82, 83]. Its expression is highly restricted to the subset of Treg cells and is correlated with immunosuppressor activity, irrespective of CD25 expression.

CD4+ Treg cells can suppress host immune responses to a great extent and induce self-tolerance. Thus, despite their protective role in autoimmune diseases, these cells have inhibitory effects on cancer immunotherapy and anti-infectious responses [84]. That is, malignant tumors tend to progress more rapidly in a Treg-dominant environment. Recent studies have shown that the proportion of CD4+/CD25+ Treg cells was elevated in the total CD4+ T cell population in several different human cancers, including lung, breast and ovarian tumors [85, 86]. Ag-specific CD4+ Treg cells are situated at tumor sites, and these cells suppress the proliferation of naïve CD4+ Th cells upon activation by tumor-specific Ags [87]. TLR8 regulates CD4+ Treg function by sensing RNA in Treg cells: adoptive transfer of TLR8 ligand-stimulated Treg cells into tumor-bearing mice enhanced antitumor immunity [88]. Other TLR signaling may be associated with T and mDC functions that are suppressed by tumor-infiltrating $\gamma\delta$ T cells [89]. Naturally occurring Treg cells require the TICAM-1 pathway in Treg and mDCs for migration to inflamed nests (Fig. 2b), where the MyD88 pathway would restrain their suppressive functions [90]. CD8(+) DEC-205/CD205(+) DCs, but not the CD8(−) DCs, induce functional Foxp3(+) Treg from Foxp3(−) precursors in the presence of low doses of Ag [91]. Subsequent inflammatory Th1-type immunity is modulated by induced Treg cells, which also require the TICAM-1 pathway in mDCs [92]. Treg cells infiltrate the tumor mass and exert immunosuppressive effects that promote tumor progression.

Regulation of TICAM-1 as well as the MyD88 pathway in mDCs may down-regulate Treg in cancer patients [93]. Treg induction is sustained by mDCs with lower maturation stage [94] and what region of TICAM-1 participates in Treg induction remains unknown.

Extrinsic versus intrinsic inflammation for danger signal

PAMPs usually trigger initial or early inflammation around tumors and immune cells in an extrinsic fashion. When tumor cells are damaged through extrinsic inflammation, the destructed cells release cytosolic and nuclear constituents. Inflammation is also promoted by these intrinsic nuclear products including HMGB1, uric acids, S100 proteins, cathelicidins, ATP/adenosine and other nucleosomal proteins [95–98]. These molecules are derivatives of nucleic acids or often have DNA/RNA-binding domains. RNA, DNA and other nucleic acids of host origin also act as danger signals [1, 99]. They are released from damaged host cells or tumors and cause long-lasting inflammation [1, 99]. Recently, they have been named danger-associated molecular pattern (DAMP) or alarmin. Since tumor cells frequently undergo cell death by either apoptosis or necrosis, many cytosolic or nuclear factors are liberated from tumor nests. Tumor progression and reciprocal inflammation involve complicated episodes. We could promote tumor damage followed by DAMP liberation by radiation and/or chemotherapy [100, 101]. Recent reviews infer that electrochemotherapy (ECT) and CpG ODN administration to cancer patients synergistically induce a significant increase of the local effect and a systemic T-dependent antitumor response [100], and that some chemotherapeutic agents with immunostimulatory capacity may facilitate establishing combined chemo-immunotherapy strategies [101]. We should like to clarify these tumor-associated events and responses at a molecular level in order to develop appropriate strategies for the regulation for immune systems in cancer patients. Elucidation of the nucleic acid-recognition systems is essentially required for this purpose. Fundamental issues presented here would hopefully be useful for the development of cancer immunotherapy.

Perspectives

Cancer is a condition in which many immune-related cells form a network in concert with tumor cells. Immune aberrance is an alternative result of tumor growth. APCs and tumor cells exhibit a tight response to innate immune stimulation to alter the balance of tumor tolerance [102].

Cancer stem cells are believed to generate sibling cancer cells. These stem cells are usually vulnerable to irradiation, and their maintenance relies heavily on the gene repair system. It is not known what kinds of RNA sensors and their signaling pathways these stem cells are equipped with [103]. The events that occur in tumor and immune systems upon stem cell modulation by RNA require further study.

Vascular endothelial cells in solid tumors are cytogenetically abnormal. Unlike normal endothelial cells which remain diploid in long-term culture, the aneuploidy of tumor endothelial cells is exacerbated in culture. Tumor-associated endothelial cells upregulate many genes including the epidermal growth factor receptor (EGFR) gene. Accordingly, these cells are highly sensitive to EGF. Endothelial cells usually have a stock of surface-expressed TLR3, which can sense a small RNA duplex structure [104]. Targeting of tumor endothelial cells by immune effector cells may be a possible therapeutic strategy in anti-angiogenic therapy.

In immunological terms, our trials were aimed at elucidating the mechanisms by which mDCs select the mode of activation for various effectors. Results from studies on the dsRNA recognition system, indicated that the properties of PAMP and repertoires of host receptors critically affect these processes. Another issue is how most effective effector cells are induced in a case-dependent manner for tumor remission in patients. This review provides guidelines for the development of specific effector cells by selecting dsRNA receptors. Current knowledge on the TICAM-1 pathway could be directly applied to cancer immunotherapy.

Acknowledgments We are grateful to our laboratory members for their critical discussions. This work was supported in part by CREST, JST (Japan Science and Technology Corporation), and by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan, and by the Takeda Science Foundation, Uehara memorial Foundation, Northtec Foundation, Akiyama Foundation, Yakult foundation and Mitsubishi Foundation. Financial supports by the Sapporo Biocluster "Bio-S" the Knowledge Cluster Initiative of the MEXT, and the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT are gratefully acknowledged.

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Functional evolution of the TICAM-1 pathway for extrinsic RNA sensing

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Acknowledgements
We thank Drs A. Matsuo, T. Tsujita, A. Ishii, M. Shingai, M. Sasai, and K. Funami in our laboratory for their valuable discussions. This work was supported in part by CREST, JST (Japan Science and Technology Corporation), and by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan, and by the Takeda Science Foundation, Uehara memorial Foundation, Northtec Foundation, Akiyama Foundation and Mitsubishi Foundation. Financial supports by the Sapporo Biocluster 'Bio-S' the Knowledge Cluster Initiative of the MEXT, and the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT are gratefully acknowledged.

Immunological Reviews 2009
Vol. 227: 44–53

Immunological Reviews
0105-2896

Summary: The type I interferon (IFN) is a host defense factor against microbial pathogens in vertebrates. In mammals, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) in the cytoplasm are regarded as sensors for double-stranded RNA (dsRNA) and trigger IFN regulatory factor-3 (IRF-3) activation followed by type I IFN induction through the mitochondrial antiviral signaling (MAVS) adapter. This intrinsic pathway appears to link the main protective responses against RNA virus infection in mammals. On the other hand, human Toll-like receptor 3 (TLR3) is localized in the endosomal membrane or cell surface and signals the presence of extrinsic dsRNA. In response to RNA stimulation, TLR3 recruits the Toll-interleukin 1 receptor domain (TIR)-containing adapter molecule 1 (TICAM-1) adapter and induces IRF-3 activation followed by IFN-β promoter activation. Human TLR3 is localized limitedly extent in myeloid dendritic cells, fibroblasts, and epithelial cells. The TICAM-1 and cytoplasmic MAVS pathways converge at the IRF-3-activating kinase in human cells. The reason for the involvement of this extrinsic mode of IFN-inducing pathways in the dsRNA response remains unknown. In fish, two TLRs, i.e. endoplasmic TLR3 and cell surface TLR22, participate in teleost IFN production without the activation of IRF-3. TLR22 is distinct from mammalian TLR3 in terms of cellular localization, ligand selection, and tissue distribution. TLR22 may be a functional substitute for human cell surface TLR3 and may serve as a surveillance molecule for detecting dsRNA virus infection and alerting the immune system for antiviral protection in fish. In this review, we discuss the fundamentals of the extrinsic dsRNA recognition system, which has evolved to induce cellular effectors to cope with dsRNA virus infection across different vertebrate species.

Keywords: *Toll-like receptor, evolution, dsRNA recognition, TICAM-1 (TRIF)*

Introduction

Invading pathogens express specific pattern molecules and are recognized by host pattern recognition receptors (PRRs) (1, 2), representatives of which are Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RNA helicases [retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), etc.]. These receptors signal the presence of microbial patterns in myeloid dendritic cells (mDCs) and thus induce potent activation of the systemic host defense response (3). Recent studies on pattern receptors of

the innate immune system have increased our understanding of how mDCs mature through infection and subsequently orchestrate cellular immunity (4, 5). These molecules also serve as adjuvants for the induction of antigen-specific acquired immunity. TLRs, RIG-I-like helicases (RLHs), and NLRs are major targets for investigating the induction of robust acquired immune responses upon pathogen stimulation. These studies have been conducted using gene-disrupted mice and in *in vitro* human systems.

It has been reported that human cells induce interferon-β (IFN-β) in response to various RNA structures (6, 7). Double-stranded RNA (dsRNA) and its analog polyinosinic–polycytidylic acid (polyI:C) have been identified as potent immune stimulators of viral patterns and are recognized by PRRs. PRRs link cytoplasmic adapter molecules in these mammalian cells. Cytoplasmic RLH and membrane-associated TLRs that induce IFN-α/β involve the mitochondrial antiviral signaling (MAVS) (also known as IPS-1, Cardif, or VISA) or TICAM-1 [Toll-interleukin-1 receptor (IL-1R) (TIR) domain-containing adapter-inducing IFN-β (TRIF)] adapters, respectively, to converge the signal at IRF-3-activating kinases for IFN-β induction (4, 5, 8). IFN-β induction is IRF-3 dependent in mDCs and fibroblasts/epithelial cells (4, 5). By contrast, IFN-α/β is differentially induced in an IRF-7-dependent manner in plasmacytoid DCs (pDCs) (9). This allows activation of the myeloid differentiation factor 88 (MyD88) adapter protein and IKKα [inhibitor of nuclear factor (NF) κB (IκB) kinase α] kinase, which directly activates the IRF-7 transcription factor (10). However, the molecular assembly and mechanism involved in polyI:C-mediated activation of transcription factors still remain unclear in mice and humans.

Some PRRs preferentially recognize nucleic acid structures that are unique to infectious microbes. Type I IFN induction and cytotoxic T-lymphocyte (CTL)/natural killer (NK) cell activation are major outputs for RNA-sensing PRRs in mammalian cells (5, 11). A variety of RNA sensors in the cytoplasm or membranes are engaged in the detection of microbial RNA. These are expressed in a cell-type specific fashion and participate in IFN-α/β production in various cell types. However, the combinations of these receptors that induce cellular immunity still remain undetermined. It is generally accepted that RNA patterns that are exogenously provided or are produced in bystander cells are internalized by mDCs through phagocytosis and are then recognized by endosomal PRRs. By contrast, RNA patterns produced in the cytoplasm of infected cells are directly recognized by PRRs present in the cytoplasm (12). In this review, we adopted an evolutional approach to study TLRs present on the cell membrane and the recognition of the external dsRNA pattern that is specifically formed in other cells during virus replication.

Fish (teleost) have >20 TLRs that include orthologs of human TLRs and other TLRs unique to lower vertebrates living in water (13, 14). Teleost have orthologs of the IFN-inducing genes of mammals and PRRs for microbial pattern recognition. Teleost also have a TICAM-1 ortholog which has no TRAF-binding site but retains the RIP1-binding site (15, 16). Fish may have orthologs of RLH and NLRs. Hence, by comparing the mammalian PRR receptor/adapter system with that of fish, it is possible to examine the development of the innate recognition system during evolution. Molecular evolution by which the mammalian immune system has been established in the current form can be analyzed through the genomic information of vertebrate TLR systems. In this study, we cast insight into the functional properties of fish TLRs and adapters involved in IFN induction.

Recognition of RNA duplexes in vertebrates

Viral replication usually generates dsRNA in the cytoplasm of infected cells and signals to activate antiviral responses. dsRNA, stem-loop structure of RNA, 5′-uncapped triphosphate of RNA, and specific RNA sequences are rapidly recognized by PRRs in the cytoplasm (4, 5, 17), then implicated in host defense (Fig. 1). Many pattern-sensing receptors have been identified in mammals: PKR (dsRNA-dependent protein kinase), Dicer of the short interfering/microRNA system, RLHs including RIG-I, MDA5, and LGP2, and other helicases. These receptors are accompanied by adapters that transduce the dsRNA-sensing signal downstream. Other RNA-sensing molecules such as helicases may also be present in the cytoplasm to join a molecular assembly for foreign RNA detection. The synthesized dsRNAs are incorporated into these molecular complexes to prohibit RNA replication in virus-infected cells.

TLR3 is present in the early endosome and can recognize dsRNA delivered inside the endosomal membrane (18). TLR3 may not have a direct role in capturing dsRNA generated by virus replication in the cytoplasm, but it has an important role in trapping phagocytosed dsRNA (Fig. 2), which is usually wrapped in a membrane that originates from the infected cell (19). In comparison to the direct recognition system of dsRNA in the cytoplasm, this mode of RNA recognition is unique and sophisticated, concerning activation of cellular immunity. As RNA-sensing TLRs and RLH are conserved across vertebrates (20), we hypothesize

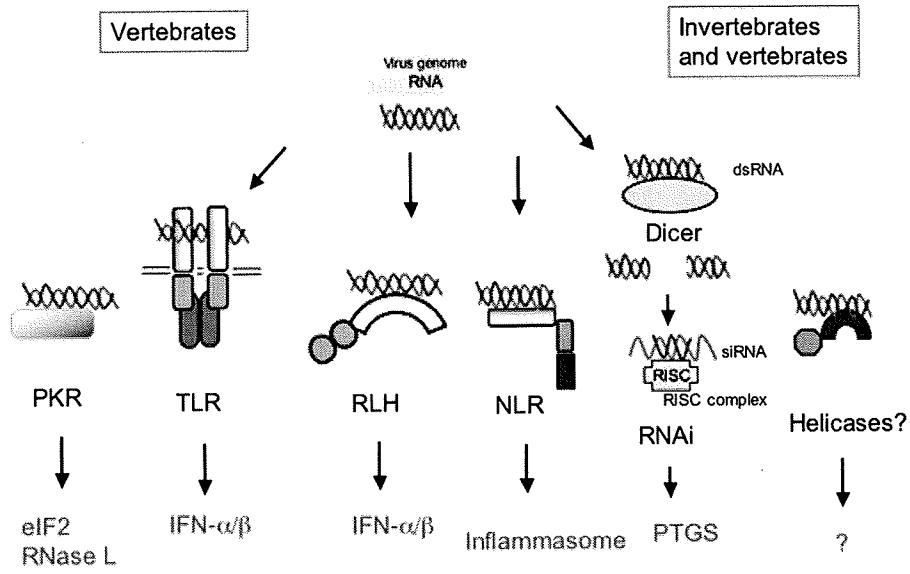


Fig. 1. Various RNA sensors in vertebrates. dsRNA are generated during virus replication. Major RNA sensors in vertebrate cells and their responses on stimulation with dsRNA are indicated. Dicer and RNA-recognizing helicases work even in invertebrates. How dsRNA selects a variety of RNA pattern sensors remains largely unknown. PTGS, post-transcriptional gene silencing.

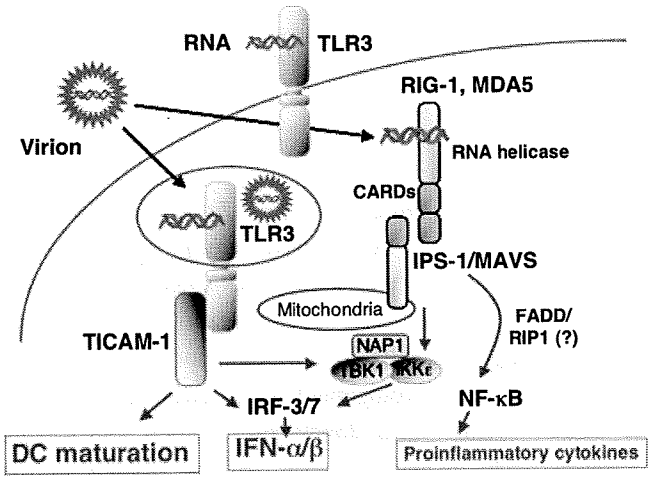
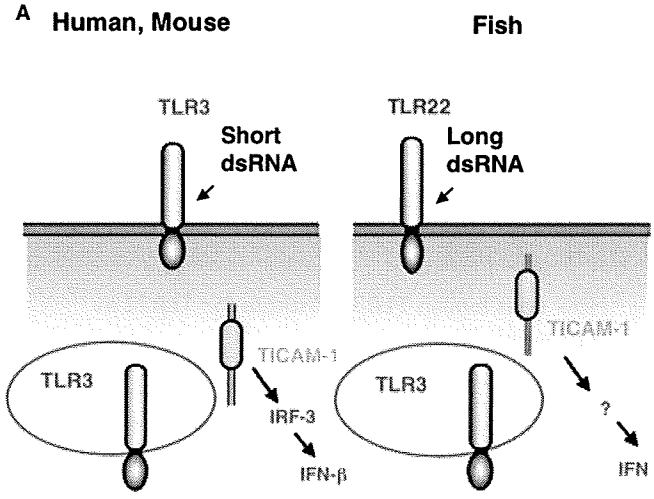


Fig. 2. Cell surface, endosomal and cytoplasmic recognition of dsRNA in mammalian cells. TLR3 is distributed either on the cell surface or in the endosome. Its distribution ratio depends on cell types. RLH (RIG-I and MDA5) reside in the cytoplasm. Adapter molecules, TICAM-1 and MAVS, are localized in the cytoplasm. Upon stimulation, TLR3 recruits TICAM-1 near the endosomal membrane, while MAVS recruits RLH on the mitochondrial membrane. The known outputs of TLR3 and RLH are indicated by red. TLR, Toll-like receptor; RIG, retinoic acid-inducible gene; RLH, RIG-I-like helicase; TICAM, Toll-interleukin 1 receptor domain-containing adapter molecule.

that the two distinct pathways of dsRNA recognition were established in a vertebrate ancestor ~500 Ma and that the two systems have been preserved in mammals (Fig. 2). We focus on the fish membrane-associated dsRNA recognition system and analyze it in terms of its physiological significance and functional feature and also from an evolutionary point of view. We also address the question of why vertebrates need the surface system for dsRNA recognition in addition to the cytoplasmic virus-sensing systems.

Surface recognition of dsRNA in mammals

We initiated a study on the functions of the membrane-associated dsRNA recognition receptor TLR3 in human cells. Stimulation of human fibroblasts/epithelial cells with polyI:C leads to the production of type I IFN. We have produced monoclonal antibodies (mAbs) against human TLR3 and obtained one which blocks polyI:C binding to TLR3, named the mAb TLR3.7 (21). The TLR3.7 mAb interferes with IFN-β production induced by exogenously added polyI:C in human fibroblasts/epithelial cells (18, 21). Hence, it appears that TLR3.7 mAb blocks the interaction between TLR3 and polyI:C on the cell surface by binding to TLR3. If this is the case, human TLR3 must be localized on the cell surface of the fibroblast to capture external dsRNA. This hypothesis was proven by results from fluorescence-assisted cell sorting (FACS) and imaging analyses (Fig. 3A). However, using the same mAb, human mDC TLR3 could not be detected on the surface (18) but was found to be localized in intracellular compartments, particularly endosome (Fig. 3A). mDCs respond to polyI:C to induce type I IFN in the early endosome (22, 23). In this case, how does endosomal TLR3 recognize polyI:C outside the cells? It is rational that there is a transporter that shuttles dsRNA from the cell surface to the endosome in mammals (5). The recognition of dsRNA by TLR3 on the cell surface is



B

IFN-inducing receptors in human and fish

Genes	Human	Fish
TLR3	+	+
RIG-I	+	--
TLR22	–	+
IFN	+	+

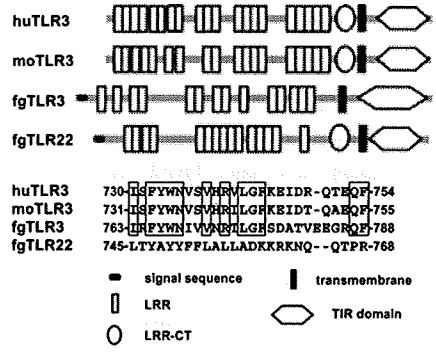


Fig. 3. Different TLRs cover surface dsRNA recognition in fish and mammals. (A) TLR3 and TLR22 in vertebrates. In human and mouse, TLR3 encompasses cell surface and endosomal RNA sensing and induces IRF-3 activation. In fish, two distinct gene products, TLR3 and TLR22, participate in dsRNA sensing. IFN is induced in an IRF-3-independent fashion. Although the structural information is not shown in the panel, mammalian TICAM-1 structurally differs from fish TICAM-1. IRF-3-activating kinase indirectly assembles in an N-terminal portion of mammalian TICAM-1 but not fish TICAM-1. A C-terminal portion contributes to IFN promoter activation in fish cells. (B). Difference of IFN-inducing receptors between human and fish. Upper table indicates that humans lack TLR22 while fish lack RIG-I, although both have IFN-inducible pathways. The structural differences among human (hu) TLR3, mouse (mo) TLR3, fgTLR3, and fgTLR22 are depicted in the lower panel. The primary structures of the linker region (a determinant of TLR3 localization) are shown below the structural models.

experimentally proven by using the mAb probe for determining the localization of human TLR3. However, the dsRNA shuttling system has not yet been proved.

If TLR3 participates in the induction of IFN-β in epithelial cells, its downstream molecules should activate IRF-3. Therefore, we searched for an adapter molecule that could directly interact with TLR3 and activate IRF-3; the molecule was identified by employing the yeast two-hybrid system. It was named TICAM-1 (24) and is now popularly known as TRIF (25).

Human TICAM-1 consists of an N-terminal region (1–234), a TIR domain (235–500), and a C-terminal region (501–680). The N-terminal region of TICAM-1 harbors tumor necrosis factor (TNF) receptor-associated factor (TRAF) family proteins (26, 27) and forms a complex containing IRF-3-activating kinases (28, 29). This kinase complex is crucial for activating the IFN-β promoter (28, 29) and inducing the activation of NK (5, 30) and CTL (12, 31) effector cells (*Fig.* 4). The C-terminal region of TICAM-1 can recruit receptor-interacting protein-1 (RIP-1), and this event is followed by the activation of other effectors (32). All these signaling events constitute the TICAM-1 pathway. Human and mouse TICAM-1 pathways involve mDC maturation, cytokine/chemokine induction, cross-presentation of exogenous antigens for proliferation of $CD8^+$ T cells (5, 12, 31, 33), NK cell activation (30, 34), and induction of autophagy and apoptosis (35). CD4+ regulatory T (Treg) cells and Th17 cells may be induced by mDCs matured through TICAM-1 signaling. TICAM-1 may act as a platform that recruits various signaling molecules for mDC output in mammals. However, one question that remains unanswered is whether the TICAM-1 pathway is conserved in lower vertebrates such as fish.

Surface recognition of dsRNA in fish

Fish [*Takifugu rubripes* (fg)] have ~20 TLRs and three TLR adapters, i.e. fgMyD88, fgTICAM-1, and fgTIRAP/Mal (36). By using the yeast two-hybrid analysis system, we found at least two TLRs that share the fgTICAM-1 adapter (37). The first report on fgTLRs (13) showed that fgTLR3 and fgTLR22 choose the fgTICAM-1 adapter in fish cells and induce fish type I IFN by recognizing dsRNA. fgTLR3 and fgTLR22 are quite different in their primary structures (*Fig.* 3B) and are classified into different clades by gene tree analysis (13, 37). However, both fgTLR3 and fgTLR22 directly bind to fgTICAM-1 in fish cells as well as in yeast. Confocal analysis has shown that fgTLR3 resides in the endoplasmic reticulum (ER) and recognizes relatively short dsRNA, whereas fgTLR22 recognizes long dsRNA present on the cell surface (37). The

properties of fgTLR3 and fgTLR22 are summarized in Fig. 3B. fgTLR22 is particular, as fgTLR22 preferentially recognizes long dsRNA, localizes exclusively to the cell surface, and is widely distributed across tissue/organs. In summary, two of the receptors that recognize dsRNA are also involved in the TICAM-1 pathway in fish. The fish TICAM-1 pathway leads to the activation of the IFN promoter.

The next question is how TICAM-1 is assembled by TLR22 to transmit the dsRNA recognition signal. Possible answers may lie in the structural difference between mammalian and teleost TICAM-1 (Fig. 3B). Over-expression of zebrafish (zf)TICAM-1 activates the zfIFN promoter, but zfTICAM-1 does not interact with zfTRAF6 (16). Results from genomic retrieval analysis suggest that zebrafish lacks IRF-3. The zfTICAM-1 N-terminal region does not contain the TRAF6-binding motif (that participates in IRF-3 activation), and the C-terminal region of zfTICAM-1 can adequately activate the zfIFN promoter. This observation suggests the involvement of RIP1-mediated NF-κB activation in zfIFN promoter activation (16, 37).

Human TICAM-1 stimulates IRF-3-mediated type I IFN induction by means of its N-terminal region (38, 39) (Fig. 4). Thus, fish TICAM-1 behaves like human TICAM-1; however, fish TICAM-1 does not employ IRF-3 to activate the IFN-β promoter (16, 40). Although the TICAM-1 pathway is conserved across both fish and humans, the molecular bases for IFN induction in response to extrinsic dsRNA differ in the two vertebrate species (Fig. 3). Our speculation is that although fish cells have an IFN output similar to that of human cells, the signal cascade that leads to IFN production is modally different. Teleost TICAM-1, which is structurally dissimilar to human TICAM-1 (36), might help in explaining the differential selection of the signal pathways.

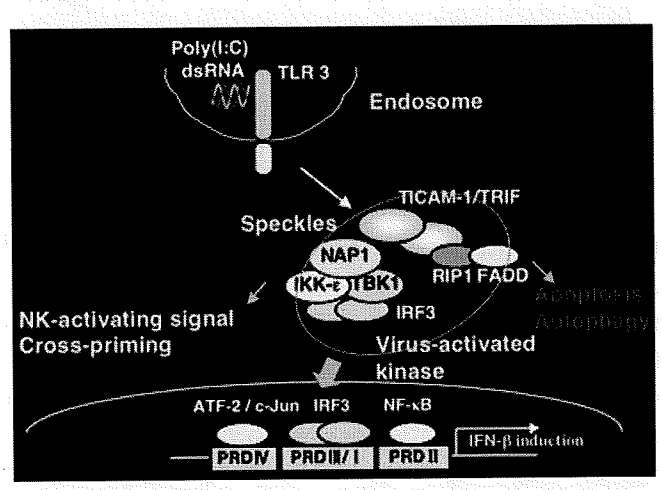


Fig. 4. TICAM-1 is dissociated from TLR3 to form a signaling unit, Speckle. In human cells, TICAM-1 once detached from TLR3 serves as a signaling platform to induce apoptosis, autophagy, NK activation, and cross-priming. TICAM-1 undergoes some modification secondary to complex formation with TLR3 and dissociated from TLR3 with unknown mechanism. The pathways for NK activation, CTL induction, and autophagy are not yet identified, although the pathway for apoptosis is getting clarified. It is undetermined whether surface-expressed TLR3 or TLR22 retain the cellular responses.

How does human TLR3 substitute for TLR22 in mammals?

The differences between TLR22 and TLR3 can be summarized as follows. Based on confocal microscopy and FACS analyses, over-expressed fgTLR22 is localized on the cell surface, while fgTLR3 resides in the ER and endosomes in fish cells (37). fgTLR22 is ubiquitously distributed over the organs/tissues of teleost, while human and fgTLR3 are present only in a limited cell repertoire. These two TLRs do not merge with each other or with fgTICAM-1 in resting cells. When stimulated with polyI:C, a part of the fgTLR22 population enters the cytoplasmic region to merge with fgTICAM-1 (37). Similarly upon stimulation, fgTLR3 is clustered and merges with fgTICAM-1 in the cytoplasm (37). Immunoprecipitation studies have supported their molecular interactions: fgTICAM-1 coprecipitates with fgTLR22 or fgTLR3 in human HEK293 cells. A reporter assay has shown that the dominant-negative form of fgTICAM-1 blocks the fgTLR22- and fgTLR3-mediated IFN promoter activation induced by endogenous fgTICAM-1 in RTG-2 (rainbow trout) cells. Thus, fish have a novel TICAM-1-coupling TLR, TLR22, which is clustered on the cell surface. Although mammals have lost TLR22, TLR3 is distributed on the surface membrane as well as in the endosomes only in some kinds of epithelial cells (41–44), and this appears as though TLR3 compensates for the loss of TLR22 in limited cell types.

We tested the physiological function of fgTLR22 and found that fgTLR22-expressing RTG-2 (rainbow trout) cells become resistant to virus infection (37). We used birnavirus, which is a representative dsRNA virus found in water. Cytopathic effect formation was observed in control cells that did not express fgTLR22, whereas it was barely detected in cells expressing fgTLR22. The level of TCID50 in the supernatant, which reflects virus replication in the cells, was high in the control cells and ~100-fold lower in fgTLR22-expressing cells. Virus RNA levels were suppressed in fgTLR22-expressing cells. Conversely, IFN mRNA was upregulated in virus-infected cells.

In humans, TLR3 is expressed in the endosomes and on the surface of epithelial cells/fibroblasts (18, 22). Expression of TLR3 on the cell surface membrane of human bronchial, bile-duct, and intestinal epithelial cells has also been reported

(41–44). Thus, surface-expressed human TLR3 appears to be a functional remnant of fish TLR22: TLR3 functions in the mucosal region wherein body fluids are continuously in contact with the flora. Because cell surface-associated dsRNA recognition is indispensable even in humans, TLR3 is expressed on human fibroblasts and epithelial cells. Likewise, TLR22 may be a functional substitute for human cell surface TLR3 and may act as a surveillance molecule for detecting dsRNA virus infection.

Evolution of the surface RNA recognition system in vertebrates

The results from bootstrap probability analysis indicate that TLR22 does not belong to the TLR3 family and is instead proximal to mouse TLR13, which has not been characterized as a dsRNA-recognizing TLR. Thus, two arms of the TICAM-1 pathway have evolved as dsRNA receptors in fish, and only TLR3 has been preserved in mammals (*Table 1*). Development of TLR22 instead of TLR3 may afford some advantage for protection against RNA viruses by augmenting the susceptibility of the local IFN response to long RNA duplexes.

We wanted to understand why teleosts require a cell surface RNA recognition system. Fish live in water and are exposed to many kinds of negative-stranded RNA viruses belonging to the Rhabdoviridae and dsRNA viruses (45, 46). Bacteria such as *Rhodovulum sulfidophilum* and perhaps other species are involved in the extracellular liberation of ribosomal and transfer RNAs into the sea (47). Thus, the sea may contain RNA viruses and RNA products of microbial origin. The sea is home to a unique and mysterious microbial environment. During evolution, vertebrates in water may have been protected from these pathogens by developing a set of RNA-sensing TLRs and an IFN system, which are distinct from those expressed in land animals. Our studies indicate that RNA sensing by TLRs protects fish from spreading or exacerbating infection. Land animals preserve the surface RNA recognition system to a limited extent in their epithelial ducts where the microbial environment is retained similar to that found in the sea.

Over-expressed teleost TLR22 protects host cells from infection with IPNV, which is a naked bisegmented dsRNA virus belonging to the family Birnaviridae (48). Birnaviruses have a single T = 13 *icosahedral* shell that is composed of 120 subunits, and these viruses lack the characteristic inner capsid. Aquatic birnaviruses are distributed worldwide, can infect a range of fish and shellfish species (45, 46), and are viral pathogens that cause diseases in fry and young fish. Although teleosts have the gene that encodes a putative ortholog of the cytoplasmic RNA sensor MDA5 (36, 49), IPNV efficiently infects teleost cells unless TLR22 is expressed in some population of cells. Thus, fish MDA5 is insufficient for protection against this type of dsRNA virus. Although all cells do not express TLR22, IFN seems to be sufficiently induced by TLR22-expressing cells to provide an antiviral environment in surrounding cells, resulting in host cell protection. However, the manner in which TLR22 detects the IPNV infection remains to be clarified. The necessity of TLR22 and its mode of dsRNA recognition in fish are of interest for further investigation.

Effector induction by endosomal TLR3 in mammals

We produced a TICAM-1 knockout (KO) mouse and tested the effector-inducing properties using the syngeneic tumor implant system of this mouse (30, 50). PolyI:C was intraperitoneally administered as the ligand for TLR3 stimulation. In this system, RLH may sense polyI:C similarly in TICAM-1 KO as well as in wildtype mice, but detectable phenotypes shouldreflect only the difference in TICAM-1 in mice. Mouse melanoma line B16

Table 1. Repertoire of pattern recognition receptors in vertebrates

	TLR																				
	1	2	3*	4	5	6	7	8	9	10	12	13	14	21	22*	MyD88	TICAM*	RIG-I	MDA-5	IPS-1	IFN
Human	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+
Mouse	+	+	+	+	+	+	+	+	+	*psd*	+†	+	–	–	–	+	+	+	+	+	+
Chicken	+	+	+	+	+	*psd*	+	–	–	–	–	–	+‡	+	–	+	+	–	+	*frg*	+
Xenopus	+	+	+	+	+	±	+	+	+	±	+	+	+	+	+	+	+	+	+	*frg*	+
Fugu	+	+	+	–	+	–	+	+	+	–	–	–	+	+	+	+	+	–	+	*frg*	+
Zebra	+	+	+	+	*frg*	–	*frg*	*frg*	+	–	+	–	+	*frg*	+	+	+	–	+	*frg*	+
Ascidia	~3?															–	–	–	–	–	–
Sea urchin	~300?															7	2	6?	6?	1?	–

psd, pseudogene; *frg*, fragment.
*TLR3, TLR22, and TICAM are IFN-inducing genes.
†Mouse TLR11.
‡Bird TLR15.
Ascidia and Sea urchin are invertebrate references.

[low major histocompatibility complex (MHC) expresser] and the C57BL/6 cell lines were used in this study.

The tumors grew well in wildtype mice. When polyI:C was administered intraperitoneally, tumor growth was retarded. Similar results were obtained with MyD88 KO, PKR KO, and IFN-β KO mice. PolyI:C-mediated tumor growth retardation was completely abrogated in TICAM-1 KO mice, suggesting that TICAM-1 is crucial for tumor-directed effector induction. IFN-β is an output of the activation of the TICAM-1 pathway, but it barely affects tumor regression. Retardation of tumor growth by polyI:C was completely abrogated in wildtype mice by depletion of NK1.1- or asialoGM-1-positive cells (30). Tumor growth suppression in response to polyI:C was normally observed in $CD8^+$ T-cell-depleted mice. Hence, NK/NKT cells, not CTLs, are effectors responsible for tumor regression in this mouse model with low MHC-expressing tumor. As polyI:C activates the TICAM-1 pathway, size reduction of the implant tumor reflects the potential of the effectors induced by the functioning of the TICAM-1 pathway (Fig. 5).

We next checked whether TICAM-1 in mDCs or other immune cells is important for tumor growth retardation. TICAM-1 was transfected into bone marrow-derived DCs (BMDCs), and these cells were adoptively transferred to mice with tumor burden. Tumor growth was significantly reduced in mice injected with TICAM-1-positive BMDCs but not in those injected with other BMDCs that did not express TICAM-1 (50). Thus, the mDC TICAM-1 pathway is involved in anti-tumor NK activation (30) (Fig. 5).

The TICAM-1 pathway activates transcription factors, IFN regulatory factor-3 (IRF-3), IRF-7, activator protein 1 (AP1), and NF-κB in mouse cells. The results from our *in vitro* NK assay suggest that IRF-3 largely participates in mDC-NK reciprocal activation (T. Ebihara, M. Matsumoto, T. Seya, unpublished data). Actually, polyI:C-mediated tumor growth retardation was abrogated in IRF-3 KO mice but not IRF-7 KO mice. Thus, in mDCs, induction of the molecules that drive NK activation would depend on IRF-3 activation.

We found that tumor-specific CTLs are induced by polyI:C when EG7 cells [a high MHC expresser with ovalbumin (OVA)] are employed as the implant tumor. Therefore, we checked the levels of the OVA epitope-responsive $CD8^+$ T cells, i.e. OT-1. BMDCs expressing TICAM-1 potentially induce T-cell proliferation and IFN-γ induction (Fig. 4). These T-cell responses are largely independent of IRF-3 or IRF-7 in mDCs (M. Azuma, T. Ebihara, M. Matsumoto, T. Seya, unpublished data). Thus, when implant tumor expresses high levels of MHC, CTLs driven through mDCs act as the main effector cell in mice (31). CTLs and NK cells are induced by distinct routes in mDCs (51, 52).

Cellular immune activation by mDCs depends on the situation of TLR3-adapter complex. Cytoplasmic activation of the

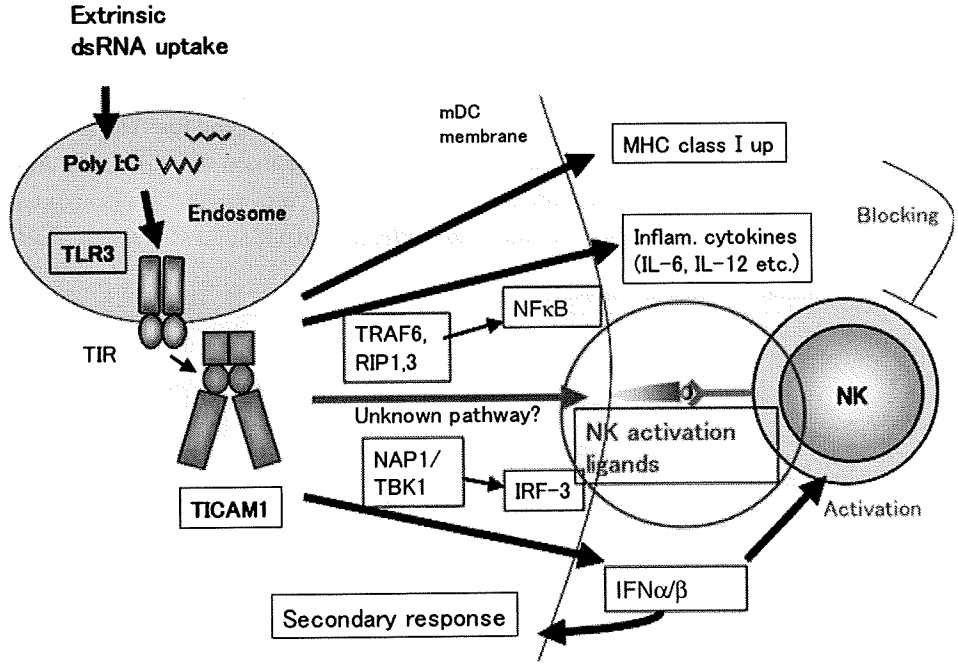


Fig. 5. Mechanism of mDC-NK reciprocal activation induced by dsRNA stimulation of mDCs. TICAM-1 has a crucial role in NK activation driven by polyI:C-stimulated mDCs in human cells. When TLR3 grasps the dsRNA signature in the endosome of mDCs, TICAM-1 in mDCs is activated to evoke a signal pathway reaching to the expression of NK-activating ligands. NK cell activation is then induced via mDC-NK contact. Some soluble factors may be important for NK activation in addition to the expression of NK-activating ligands.

