することが難しい。この問題を解決する新基準としてirRCが米国において提唱され，癌免疫治療の治験に採用されている。irRCにおいては，4週間以上の間隔をおいた2回の画像検査において最低25%以上の腫瘍量の増加が認められた場合にはじめてirPDと判断されるため，従来のRECIST基準では有効性が認められず排除されていた症例の観察も可能であることが報告されている。

4)治験薬およびその投与方法

SVN-2B注1.0mg：1バイアル中に生理食塩水1mLあたり1.0mgのSVN-2Bを含有する注射剤

STI-01：1バイアル中にIFN-β 3×10^6IUを含有

SVN-2B：SVN-2B注1.0mg，1.0mLと乳化用の添加剤モンタナイド0.8mLを混合して乳化。調整した投与用薬液1.8mLを2ヵ所(1ヵ所0.9mL)に分けて原則14日ごとに皮下投与する。

STI-01：STI-01 3×10^6IUを1.0mLの生理食塩水に溶解し，2ヵ所に分けてSVN-2Bと同部位に皮下投与する。最初の8週間は原則7日ごとに投与し，8週目の投与以降は原則14日ごと，32週目の投与以降は原則28日ごとにSVN-2BまたはSVN-2B(プラセボ)と同じ日に投与する。

5)主要評価項目

無増悪生存期間とする。

6)副次的評価項目

免疫学的効果として，①SVN-2Bペプチド特異的CTL数(テトラマー解析)，②SVN-2Bペプチド特異的CTL活性(ELISPOT解析)を評価。

7)治験実施予定期間

2013年10月～2016年12月(登録期間：24ヵ月)。

8)対象

対象の選択基準，および除外基準は表2，3に示す。

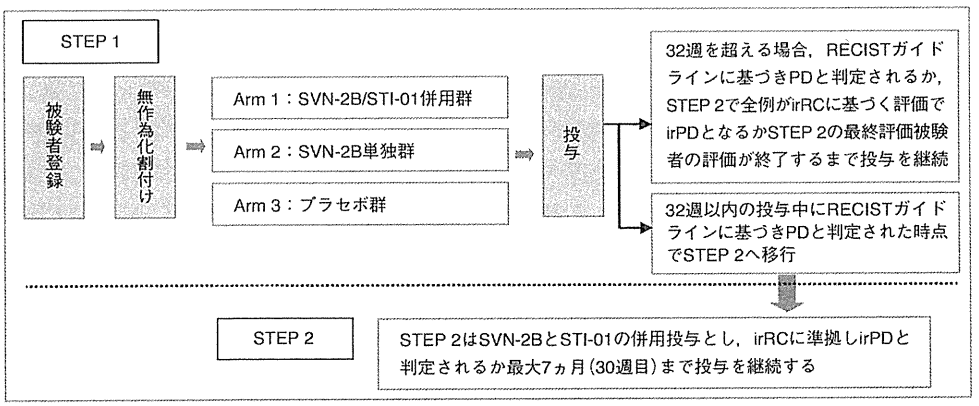

図3 本治験のデザイン

表2 選択基準

1) 組織学的に膵臓原発腺癌，膵臓腺管癌と確定診断された患者。
2) 癌細胞にサバイビン蛋白質の発現が確認された患者。
3) 以下の基準をすべて満たす患者。 a) 根治手術が不可能である(遠隔転移例，再発例，局所進行例等)患者。 b) ゲムシタビンまたはテガフール・ギメラシル・オテラシルカリウム配合剤に対し不応例，不耐容例の患者。 C) ゲムシタビンまたはS-1のいずれかしか投与していない場合，投与していない薬剤の投与不適応患者または投与を拒否した患者。
4) 前観察期のCTまたはMRIでRECISTに基づく測定可能評価病変がある患者。
5) HLA遺伝子型がHLA-A*2402である患者。
6) Eastern Cooperative Oncology Group(ECOG)Performance Statusが0または1の患者。
7) 登録前30日以内に重篤な臓器不全がないことが確認された患者(好中球≧1,500/μL，ヘモグロビン値≧8.0g/dL，血小板数≧$75\times10^3/\mu$L，血清クレアチニン値≦正常上限値の1.5倍，血清総ビリルビン値≦正常上限値の3倍，AST，ALT≦正常上限値の3倍)。
8) 同意取得時の年齢が20歳以上85歳以下の患者。
9) 本治験内容について十分な説明を受け，本人の文書による同意が得られている患者。

表3 除外基準

1）登録前90日以内のHIV抗体検査で陽性の患者。
2）NYHA分類ⅢまたはⅣの心疾患，登録180日以内の心筋梗塞の既往，治療を要する不整脈，活動性の狭心症，または重度の閉塞性肺疾患を有するなど，本治験治療に支障を来す疾患を有する患者。
3）コントロール不能な糖尿病または高血圧の患者。
4）ドレナージが必要な胸水，心嚢水または腹水を有する患者（登録時点においてドレーン抜去後，増悪がないと判断された患者は許容する）。
5）症状を伴う脳転移を有する患者。
6）活動性の重複癌を有する患者。
7）自己免疫疾患またはその疑いがある患者。
8）CRP値が15.0以上の重度な炎症疾患が疑われる患者。
9）間質性肺炎の既往歴のある患者。
10）生命予後に影響を及ぼすような他の疾患に罹患している患者。
11）過去に癌に対する免疫細胞療法を受けた患者。
12）登録時に以下のいずれかの前治療または処置を行い，治療または処置の終了時から以下の期間が経過していない患者。 a）手術療法，放射線療法　21日間 b）化学療法（分子標的薬を含む）　14日間 c）内分泌療法，免疫療法（BRM療法を含む）　14日間 d）輸血，造血因子製剤　14日間 e）免疫抑制剤　28日間 f）他の治験薬，未承認薬　28日間
13）小柴胡湯，ワルファリン，テオフィリンを投与中の患者。
14）ステロイド剤の全身投与（経口，静注）を必要としている患者。ただし，治療に伴う急性アレルギー症状の処置用に投与した場合を除く。
15）過去に重大な薬物アレルギーの既往がある患者。
16）ウシ由来物質に対し，過敏症の既往歴のある患者。
17）ワクチン等生物学的製剤に対し，過敏症の既往歴のある患者。
18）重度の精神病または精神症状を合併しており治験への参加が困難と判断される患者。
19）妊娠している，もしくは授乳中である患者。治験期間中に被験者本人およびパートナーの妊娠を希望する患者。効果の高い避妊法を行えない患者。
20）その他，治験責任医師または治験分担医師が不適切と判定した患者。

表4 投与期間，最終観察時/中止時スケジュール（0～32週目）

Week	0	1	2	3	4	5	6	7	8	10	12	14	16	18	20	22	24	26	28	30	32	中止時[e]
Day	1	8	15	22	29	36	43	50	57	71	85	99	113	127	141	155	169	183	197	211	225	
許容範囲（日）	−3～1	±3	±3	±3	±3	±3	±3	±3	±3	±7	±7	±7	±7	±7	±7	±7	±7	±7	±7	±7	±7	
治験薬投与 SVN-2B[a]	●		●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●[d]	
治験薬投与 STI-01[b]	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●[d]	
バイタルサイン，PS	●	●	●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●
血液学的検査	●	●	●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●
血液生化学的検査	●	●	●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●
尿検査	●	●	●		●		●		●	●	●	●	●	●	●	●	●	●	●	●	●	●
画像評価[c]									●			●			●			●			●	▲[e]
腫瘍マーカー[c]									●			●			●			●			●	▲[e]
免疫学的評価（CTL数，CTL活性）									●			▲			●			▲			●	▲
有害事象	←																					→
併用薬・併用療法	←																					→

●：必須，▲：可能な限り実施

a）SVN-2Bは，原則14日ごとに投与する。許容範囲は±7日とし，4日以上の間隔を空けること。ただし，祝日，休診等の理由により投与が定期的に行えない場合は，連日投与を避け，この限りではない。

b）STI-01は，最初の8週目まで原則7日ごとに1回投与する。その後，原則14日ごとにSVN-2BまたはSVN-2B（プラセボ）と同じ日に投与する。許容範囲は8週目までは±3日とし，2日以上の間隔を空けること。その後の許容範囲は±7日とし，4日以上の間隔を空けること。ただし，祝日，休診等の理由により投与が定期的に行えない場合は，連日投与を避け，この限りではない。

c）画像評価，腫瘍マーカー，免疫学的評価（CTL数、CTL活性）の許容範囲は，8週目が±7日，14，20，26週目，32週目が±14日とする。

d）STEP2においては，治験薬の投与を行わない。

e）画像評価，腫瘍マーカーの結果により，治験薬投与中止を決定した場合，投与中止を決定した画像評価および腫瘍マーカーの検査を中止時の検査とすることができる。

HLA-A*2402は日本人では最も多い遺伝子型ではあるが，60%に過ぎず，遺伝子型が合わないため，治験薬投与前に3分の1以上が脱落となる。また，膵癌は組織学的に確定診断がされない(できない)状況で化学療法が施行されている場合があるため，EUS-FNA等の侵襲的検査を選択基準を満たすために追加しなければならない場合がある。

目標症例数は71例(プラセボ投与群：15例，SVN-2B投与群:28例，SVN-2B/STI-01併用投与群:28例)である。

9)治験スケジュール

表4に示す。初回の効果判定は，8週後となる。

本治験の問題点と展望

現在，治験開始より約9ヵ月が経過した。治験実施症例数は目標数の3分の1を超えた。最初は札幌医科大学第一外科および東京大学医科学研究所附属病院外科で実施されたが，2014年8月より神奈川県立がんセンターでも実施予定である。

この臨床試験の最も大きな問題は，プラセボ投与が8週間にも及ぶ点であろう。膵臓癌は進行が早く，8週間の間に，急速に病状が進行する例もあり，救済策であるSTEP 2への移行が行うことができない症例も散見される。

しかし，本治験によりサバイビンワクチンの有効性が証明されれば，今後既存の化学療法との併用や，膵臓癌以外の固形癌に対する使用において有効な治療となる可能性が期待できる。

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REVIEW ARTICLE

Beyond dsRNA: Toll-like receptor 3 signalling in RNA-induced immune responses

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The innate immune system recognizes pathogen- and damage-associated molecular patterns using pattern-recognition receptors that activate a wide range of signalling cascades to maintain host homoeostasis against infection and inflammation. Endosomal TLR3 (Toll-like receptor 3), a type I transmembrane protein, senses RNAs derived from cells with viral infection or sterile tissue damage, leading to the induction of type I interferon and cytokine production, as well as dendritic cell maturation. It has been accepted that TLR3 recognizes perfect dsRNA, but little has been addressed experimentally with regard to the structural features of virus- or host-derived RNAs that activate TLR3. Recently, a TLR3 agonist was identified, which was a virus-derived 'structured' RNA with incomplete stem structures. Both dsRNA and structured RNA are similarly internalized through clathrin- and raftlin-dependent endocytosis and delivered to endosomal TLR3. The dsRNA uptake machinery, in addition to TLR3, is critical for extracellular viral RNA-induced immune responses. A wide spectrum of TLR3 ligand structures beyond dsRNA and their delivery systems provide new insights into the physiological role of TLR3 in virus- or host-derived RNA-induced immune responses. In the present paper, we focus on the system for extracellular recognition of RNA and its delivery to TLR3.

Key words: dendritic cell, dsRNA, endocytosis, innate immunity, structured RNA, Toll-like receptor (TLR), type I interferon, uptake receptor, viral infection.

INTRODUCTION

The immune system has developed a strategy for maintaining host homoeostasis through its interaction with environmental microbes. An array of PRRs (pattern-recognition receptors) in the innate immune system recognizes PAMPs (pathogen-associated molecular patterns) and induces anti-microbial immune responses [1]. Endosomal TLRs (Toll-like receptors) 3, 7, 8 and 9 serve as sensors of exogenous nucleic acids, whereas cytoplasmic RLRs (RIG-I-like receptors), AIM2-like receptors and DDX family members recognize intracellular viral nucleic acids [2,3]. The compartmentalization of PRRs is important for sensing both extra- and intra-cellular PAMPs and transmitting signals via distinct adaptor molecules.

Among the nucleic acid-sensing TLRs, TLR3 that recognizes dsRNA has a unique expression profile and subcellular localization [4,5]. It is expressed in immune cells, including myeloid DCs (dendritic cells) and macrophages, and in non-immune cells such as fibroblasts, epithelial cells and neurons [5–7]. TLR3 localizes to the early endosome in myeloid DCs [8], whereas macrophages, fibroblasts and some epithelial cell lines express TLR3 both on the cell surface and in the early endosome [5,9]. Although TLR3s on the cell surface participate in dsRNA recognition [5], TLR3-mediated signalling is initiated from endosomal compartments in either cell type [8].

In the case of TLR3, virus-derived dsRNA and poly(I:C) (polyriboinosinic:polyribocytidylic acid), a synthetic dsRNA, were first identified as TLR3 ligands [4,5]. dsRNA exists as a viral genome or is generated in the cytosol during replication of positive-strand RNA viruses and DNA viruses [10]. Thus TLR3 appears to sense extracellular viral dsRNA released from infected cells and activates antiviral immunity [11]. Indeed, TLR3 mediates a protective response against positive-strand RNA virus infection, including PV (poliovirus), coxsackievirus group B serotype 3 and encephalomyocarditis virus, and DNA virus infection such as herpes simplex virus 1 and murine cytomegalovirus (Table 1) [12–19]. On the other hand, detrimental effects of TLR3 in host immunity to some RNA and DNA viruses also have been demonstrated [20–23]. Notably, TLR3-mediated signalling exacerbates negative-strand RNA virus infection, in which dsRNA is barely detectable [22,23]. In addition, RNA released from damaged cells or mRNA is also recognized by TLR3 [24,25]. However, little is known about which RNA molecules or structures activate TLR3 during infection or inflammation. We identified recently a structural unit that can activate TLR3; surprisingly, this 'structured' RNA recognized by TLR3 contains an incomplete stem with bulge and internal loops, but sufficiently induces type I IFNs (interferons) and pro-inflammatory cytokines in both human and mouse cells [26]. Hence the spectrum of TLR3 ligand structures appeared to be beyond the canonical dsRNA. The results offer new insights into the physiological role of TLR3 in virus- or host-derived RNA-induced immune responses. In the present review, we focus on exRNA (extracellular RNA) recognition and signalling by TLR3.

Abbreviations: AP-1, activator protein-1; CTL, cytotoxic T-cell; DC, dendritic cell; ECD, ectodomain; exRNA, extracellular RNA; HEK, human embryonic kidney; IFN, interferon; IL, interleukin; iPSC, induced pluripotent stem cell; IRF-3, IFN regulatory factor-3; LRR, leucine-rich repeat; LRR-CT, LRR C-terminal; LRR-NT, LRR N-terminal; MDA5, melanoma differentiation-associated gene 5; NF-κB, nuclear factor κB; NK, natural killer; ODN, oligodeoxynucleotide; PAMP, pathogen-associated molecular pattern; poly(I:C), polyriboinosinic:polyribocytidylic acid; PRR, pattern-recognition receptor; PV, poliovirus; RIG-I, retinoic acid inducible gene-I; RLR, retinoic acid inducible gene-I-like receptor; TICAM-1, Toll–IL-1 receptor domain-containing adaptor molecule-1; TIR, Toll–IL-1 receptor; TLR, Toll-like receptor.

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Table 1 Role of TLR3 in viral infections

Viral genome	Protection	Deterioration
(+) ssRNA	Poliovirus [12,13], coxsackievirus group B serotype 3 [14] and encephalomyocarditis virus [15]	West Nile virus [20]
dsRNA	Rotavirus [16]	
dsDNA	Herpes simplex virus 1 [17,18] and murine cytomegalovirus [19]	Vaccinia virus [21]
(−) ssRNA		Influenza A virus [22] and phlebovirus [23]

Table 2 Representative TLR3 ligands identified by *in vivo* or *in vitro* experiments using reporter assay and TLR3-deficient mouse DC/macrophage stimulation

Ab, antibody; PBMC, peripheral blood mononuclear cell.

RNA ligands for TLR3	Details	*In vitro* assay	Reference(s)
Exogenous RNA			
Viral dsRNA	Reovirus genome dsRNA	Mouse DC	[4]
Viral mRNA	*In vitro* transcribed HIV gag mRNA	HEK-293/TLR3	[24]
Viral structured RNA	*In vitro* transcribed PV RNA	Mouse DC and HEK-293/TLR3	[26]
Bacterial RNA	*Escherichia coli* total RNA	HEK-293/TLR3	[73]
	dsRNA of lactic acid bacteria	Mouse DC	[74]
Endogenous RNA			
RNA from necrotic cells	RNA from necrotic neutrophils	Macrophage	[25]
	UVB-irradiated U1 RNA (small nuclear RNA)	Human PBMC	[42]
Synthetic dsRNA	Poly(I:C)	HEK-293/TLR3 and Ab inhibition	[5]
	Poly(I:C_{12}U)	Mouse DC	[75]
In vitro transcribed dsRNA	Measles virus cDNA	HEK-293/TLR3	[31]
	pFastBac-CP*r*ME plasmid	Mouse DC and HEK-293/TLR3	[37,41]

RECOGNITION OF dsRNA BY TLR3

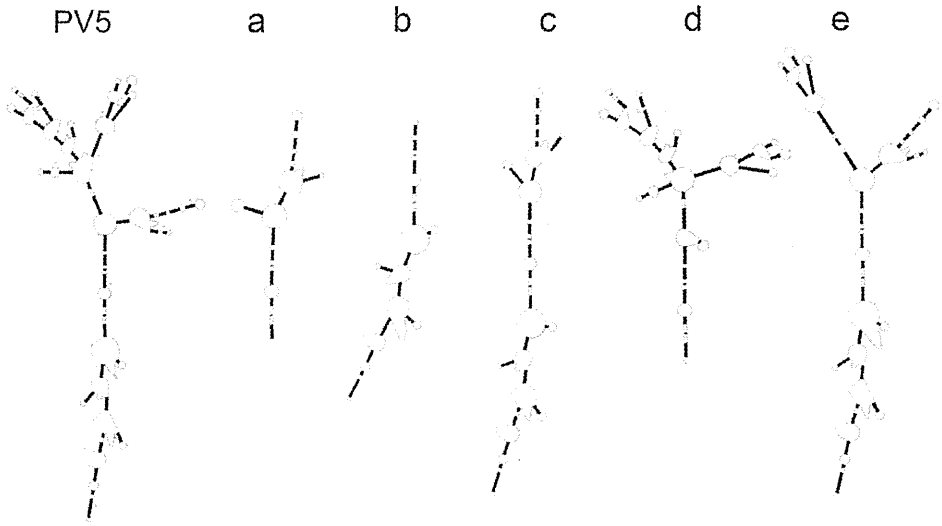
TLR3 recognizes viral or *in vitro* transcribed dsRNA in a sequence-independent manner and mediates downstream signalling via TICAM-1 (TIR domain-containing adaptor molecule-1; also known as TRIF) [27,28]. 5′-Triphosphorylation of dsRNA is dispensable for TLR3 recognition, differing from the dsRNA recognition mode of RIG-I (retinoic acid inducible gene-I) [29,30]. Furthermore, 2′-hydroxy groups are essential for TLR3 activation by poly(I:C), because 2′-O-methyl or 2′-fluoro modification of cytidylic acid abolishes the TLR3 activating ability of the I/C duplex [31].

TLR3 consists of an ECD (ectodomain) containing 23 LRRs (leucine-rich repeats) and the LRR-NT (LRR N-terminal) and LRR-CT (LRR C-terminal) regions, the transmembrane domain, the cytoplasmic linker region and the TIR (Toll–IL-1 receptor) domain [32]. Crystallized human TLR3 ECD is a horseshoe-shaped solenoid assembled from 23 LRRs, of which one face is largely masked by carbohydrate, whereas the other is unglycosylated [33,34]. The N-terminal histidine residues (His^{39} in LRR-NT, His^{60} in LRR1 and His^{108} in LRR3) and the C-terminal His^{539} and Asn^{541} in LRR20 of TLR3 ECD are indispensable for dsRNA binding [33–36]. The histidine residues are protonated at endosomal pH (~pH 6.0), generating an ionic attraction with the negatively charged phosphate backbone of dsRNA. Leonard et al. [37] showed that the TLR3 ECD binds as a dimer to a 40–50 bp length of dsRNA and that multiple TLR3 ECD dimers bind to long dsRNA strands. Binding affinities increase with both buffer acidity and dsRNA length. Structural analysis of the mouse TLR3 ECD–46-bp dsRNA complex revealed that dsRNA interacts with both an N- and a C-terminal-binding site on the glycan-free surface of each TLR3 ECD, which are on opposite sides of the dsRNA [38]. The ribose-phosphate backbone is the major determinant of binding, accounting for sequence-independent dsRNA binding to TLR3. In addition, the two LRR-CT regions come together, which is essential for stable receptor–ligand complex formation and facilitates the dimerization of the cytoplasmic TIR domain [39]. Indeed, a TLR3 mutant lacking LRR21 is constitutively active, probably because of ligand-independent dimer formation due to the altered configuration of the C-terminal TLR3 ECD structure [40].

Although a biochemical study showed that a dsRNA of 40–50 bp in length forms a stable complex with dimeric TLR3 ECD under acidic conditions (pH 5.5) [36], a dsRNA of >90 bp in length is required for TLR3-mediated cytokine production and DC maturation when added to mouse DCs [41]. Given that a dsRNA of >90 bp in length is required for stable complex formation with TLR3 at the pH within the early endosome (~pH 6.0–6.5), and that TLR3 localizes to the early endosome, TLR3 oligomerization in the early endosome is essential for downstream signalling.

RECOGNITION OF VIRUS- OR HOST-DERIVED RNA BY TLR3

Several reports suggest that TLR3 recognizes RNA molecules other than dsRNA (Table 2). In negative-sense RNA virus infections, such as influenza A virus and phlebovirus, which generate little dsRNA as intermediate replication products, TLR3-mediated inflammatory cytokine and chemokine production affects virus-induced pathology and host survival [22,23]. In addition, Karikó et al. [24] reported that *in vitro* transcribed HIV gag mRNA complexed with lipofectin activates TLR3. Cavassani et al. [25] also demonstrated that mouse macrophages responded to RNA from sterile necrotic neutrophils in a TLR3-dependent manner. However, which RNA molecules or structures of the virus- or host-derived RNAs activate TLR3 is unknown. A recent study showed that RNA from UVB-irradiated keratinocytes induces cytokine production in normal



	PV5	a	b	c	d	e
length (nt)	630	191	250	367	380	469
stability: FBS- free medium	+	-	+	+	+	+
stability: FBS-containing medium	+	-	+	+	-	+
IFN-β promoter activity in HEK293 cells (FBS-free medium)	+	-	-	+	-	+
IFN-β production in mouse splenic DC (FBS-free medium)	++	-	+	++	+	++

Figure 1 RNA structures recognized by TLR3

PV5 and its derivatives (RNA a–e) were transcribed *in vitro* using PV cDNA as a template. Upper panel, secondary structure of PV5 and its derivatives (RNA a–e) predicted by the Mfold software. Thick lines indicate dsRNA regions (1–11 bp). RNAs were incubated in FBS-free or -containing medium at 37 °C for 30 min. The degradability of RNAs was assessed by electrophoresis on agarose gel. The TLR3-activating ability of RNAs was assessed by IFN-β promoter reporter assay with HEK-293 cells transiently expressing human TLR3 and IFN-β production from splenic DCs isolated from wild-type and TLR3-deficient mice in FBS-free medium [26]. All RNAs failed to induce IFN-β production in splenic DCs isolated from TLR3-deficient mice. IFN-β production in mouse splenic DCs. +, <150 pg/ml; + +, >150 pg/ml.

human epidermal keratinocytes via TLR3 [42]. UVB-damaged small nuclear RNAs, including U1 RNA (165 nt in length) were the determinants of TLR3 activation, but the precise mechanism underlying how UVB-damaged U1 RNA activates TLR3 is unknown.

The point of our recent study was that TLR3 recognizes incomplete stem structures formed in viral ssRNA and induces innate immune signalling [26]. Analyses with *in vitro* transcribed PV-derived ssRNAs and dsRNAs revealed that some PV ssRNAs activate TLR3 extracellularly, but do not activate RLRs, in human and mouse cells. Stability and length of RNA are crucial factors for TLR3 activation in that case. Functional PV RNA, 630 nt in length (PV5), bound to TLR3 ECD with high affinity, and both the N- and C-terminal dsRNA-binding sites of TLR3 ECD are required for PV5-induced IFN-β promoter activation in HEK (human embryonic kidney)-293 cells that transiently express human TLR3 (Figure 1). Furthermore, PV5 was internalized into cells via clathrin- and raftlin-mediated endocytosis and co-localized with endosomal TLR3, as observed previously with poly(I:C) uptake [43,44]. The secondary structure of PV5 predicted by Mfold software showed that PV5 possess double-strand regions (<11 bp in length) arranged in tandem, which are segmented with bulge or internal loops (Figure 1). The TLR3-activating ability of PV5 was abolished with RNaseIII treatment, indicating that the RNA duplex in PV5 is required for both the stability and functionality for the TLR3 activation. Analyses of PV5-derived RNAs partly having PV5 secondary structure (RNAs a–e in Figure 1) showed that longer stem structure with bulge and internal loops typically shown in RNA model c is the core RNA structure required for TLR3 activation in PV5 (Figure 1). Considering that dsRNA forms an A-type nucleotide duplex with 11 bp per turn [45], and that seven contiguous base pairs are needed for rapid duplex formation of DNA and RNA [46], incomplete stem structures containing contiguous base pairs may be required for stability that facilitates TLR3 binding. A fascinating model has been proposed for TLR3 dimer formation, in which shorter RNA duplexes (21–30 bp) can form less stable complexes with two TLR3 molecules [36]. Thus appropriate length or topology of multiple incomplete stems is required for TLR3 oligomerization, leading to the production of type I IFNs and pro-inflammatory

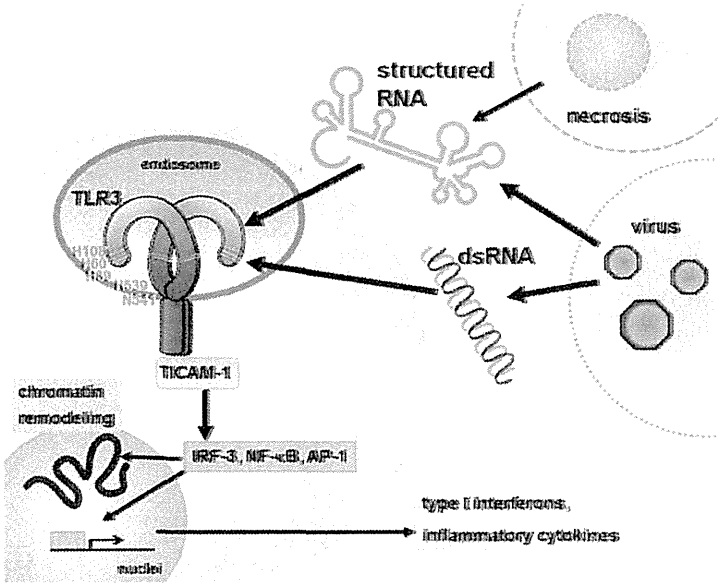


Figure 2 Model for dsRNA/structured RNA-induced TLR3-mediated immunity

Upon viral infection and sterile inflammation, virus- and host-derived RNAs are released from necrotic cells. In local environments, extracellular viral dsRNAs and virus/host-derived structured RNAs are rapidly taken up into cells via clathrin/raftlin-dependent endocytosis and delivered to endosomal TLR3. Once TLR3 is oligomerized by dsRNA/structured RNA, it recruits the adaptor protein TICAM-1 that activates the transcription factors, IRF-3, NF-κB and AP-1, leading to the production of type I IFNs and proinflammatory cytokines. The TLR3–TICAM-1 signal also induces chromatin modification in fibroblasts. In myeloid DCs, TLR3 activation triggers DC maturation capable of activating NK cells and CTLs. The key residues of TLR3, the N-terminal His^{99} in LRR-NT, His^{60} in LRR1, His^{108} in LRR3 and the C-terminal His^{539} and Asn^{541} in LRR20, which are involved in RNA binding are shown.

cytokines. RNA tertiary structure is also important for both the stability and activity of PV RNA.

Notably, mouse splenic DCs responded to shorter RNAs with mismatched duplexes that failed to activate human TLR3 expressed in epithelial cells, suggesting cell type- or species-specific RNA recognition by TLR3 (Figure 1). The precise mechanisms underlying this are currently unknown, but the high density of TLR3 expression and the potent phagocytic activity of mouse splenic DCs are advantageous for RNA-induced oligomerization of TLR3 and effective RNA uptake. In a study by Ewald et al. [47], mouse TLR3 was reported to undergo cathepsin-mediated proteolytic processing in the macrophage cell line RAW in a manner similar to that of mouse TLR9. Subsequent studies also demonstrated that human TLR3 ECD is cleaved at the loop exposed in LRR12 by cathepsins in a cell-type dependent manner [48,49], and the N- and C-terminal halves of human TLR3 remain associated after cleavage [49]. Requirement of proteolytic cleavage in TLR3 signalling appears to depend on cell type [49,50]. Potentially shorter structured RNAs may be recognized by protease-processed TLR3 in mouse DCs. Further studies are required to clarify the cell type- or species-dependent RNA recognition by TLR3.

UPTAKE OF exRNA

The ability of exRNAs to induce cellular responses primarily depends on the stability of these RNAs in the extracellular milieu and whether they are taken up into cells. dsRNA is resistant to degradation compared with ssRNA and, thus, viral dsRNA released from infected cells can be a potent activator of neighbouring virus-uninfected cells, leading to the induction of anti-viral states. Poly(I:C) is the most common dsRNA in both *in vitro* and *in vivo* studies to induce cellular responses, including type I IFN production and NK (natural killer) cell activation. Poly(I:C) is internalized into cells through clathrin-mediated endocytosis and delivered to endosomal TLR3 and to cytoplasmic MDA5 (melanoma differentiation-associated gene 5) [51]. Watanabe et al. [44] demonstrated that the cytoplasmic lipid raft protein raftlin is essential for poly(I:C) cellular uptake in human myeloid DCs and epithelial cells. In raftlin knockdown cells, surface-bound poly(I:C) neither enters the cells nor activates TLR3 and MDA5, indicating that cellular uptake is a pre-requisite for dsRNA-induced cellular responses. Upon poly(I:C) stimulation, raftlin translocates from the cytoplasm to the cell surface, where it associates with the clathrin–AP-2 (clathrin-associated adaptor protein-2) complex and induces cargo delivery. Interestingly, structured PV RNA is also internalized into cells via raftlin-mediated endocytosis and is delivered to endosomal TLR3 [26]. B- and C-type ODNs (oligodeoxynucleotides) that share the uptake receptor with poly(I:C) in humans inhibit cellular uptake of PV RNA [26,43,44,52]. Hence extracellular dsRNA/structured RNA and ODNs are recognized by a common uptake receptor and their internalization is regulated by raftlin. Mouse DCs express raftlin-2 in addition to raftlin, and raftlin knockdown does not affect poly(I:C) cellular uptake, suggesting that raftlin-2 functionally compensates for raftlin [44].

The uptake receptors for poly(I:C) have been identified by several groups. Lee et al. [53] reported that CD14 enhances poly(I:C)-induced TLR3 activation by mediating poly(I:C) uptake in mouse macrophages. Furthermore, the scavenger receptor class-A was identified as a cell surface receptor for dsRNA in human bronchial epithelial cells and mouse cells [54,55]. However, knockout of these molecules does not result in complete abrogation of poly(I:C)-induced TLR3 activation, indicating the presence of another uptake receptor. Indeed, human myeloid DCs do not express CD14 on the cell surface and an inhibitor for the scavenger receptor does not affect poly(I:C) uptake in human

myeloid DCs and epithelial cells [44]. Additionally, DEC-205 was identified as a receptor for ODNs in mouse DCs [56], but this is not the case of human DCs (M. Tatematsu and M. Matsumoto, unpublished work). Hence there must be several uptake receptors that participate in the cell entry of RNAs/DNAs in a cell type- and/or species-specific manner.

exRNA-INDUCED TLR3–TICAM-1 SIGNALLING

Following TLR3 oligomerization, TICAM-1 is recruited to the TLR3–TIR domain that activates the transcription factors, IRF-3 (IRN regulatory factor-3), NF-κB (nuclear factor κB) and AP-1, leading to the production of IFN-β and proinflammatory cytokines, as well as DC maturation [57] (Figure 2). exRNA-induced TLR3–TICAM-1-mediated signalling is classified into two categories; one that induces innate responses and the other that induces adaptive immune responses. The fibroblasts and epithelial cells that express TLR3, but not TLR7, -8 and -9, produce IFN-β and proinflammatory cytokines in response to viral dsRNA and structured RNA, which induce anti-viral states by inducing IFN-stimulated genes [26]. Host RNAs released from damaged cells could be taken up through raftlin-mediated endocytosis and activate TLR3, if they form functional structures as observed in PV RNA. Bernard et al. [42] showed that small nuclear RNAs derived from UV-damaged cells induced inflammation through activation of TLR3, but how these RNAs are delivered to endosomes and interact with TLR3 remains unknown.

Another important TLR3 signal is the induction of adaptive immune responses in myeloid DCs. TLR3 is highly expressed in the professional antigen-presenting DCs, including mouse CD8α^+ DCs and human BDCA3$^+$ DCs [58,59]. Myeloid DCs mature as a result of TLR3 activation through the expression of co-stimulatory molecules, NK-activating molecules including INAM (IRF-3-dependent NK-activating molecule) [60], and unidentified molecules involved in cross-presentation pathways, leading to the activation of NK cells and CTLs (cytotoxic T-cells) [61]. The TICAM-1–TBK1–IRF3 axis downstream of TLR3 is critical for gene induction involved in mouse DC-mediated NK/CTL activation [62,63]. In addition, mouse DCs produce the Th1-type cytokines, IFN-β and IL-12 (interleukin-12), via the TLR3–TICAM-1 pathway. This facilitates NK/CTL induction. Mouse DCs efficiently phagocytose the cell debris of virus-infected cells and mature through virus RNA-induced TLR3 activation [64].

The most intriguing finding is a link between TLR3 signals and epigenetic modifications [65]. Knockdown of TLR3 or TICAM-1 blocks the induction of human iPSCs (induced pluripotent stem cells) by retroviral reprogramming in human fibroblasts [65]. Poly(I:C)-induced TLR3 activation accelerates the development of iPSCs induced by the non-viral methods in fibroblasts. TLR3 activation leads to chromatin modification in fibroblasts by promoting genome-wide epigenetic alterations. These findings enable us to offer a new concept that RNA is an extracellular mediator that accounts for a broad range of TLR3–TICAM-1-mediated gene expression compared with other RNA-sensing receptors.

CONCLUSIONS

In plants, insects and nematodes, dsRNA-induced Dicer-mediated RNA interference is a powerful strategy for protection against viral infection [66–68]. Extracellular dsRNA is taken up into cells and systematically induces gene silencing [69,70]. In *Caenorhabditis elegans*, the membrane proteins SID-1 and SID-2 act as transporters of extracellular dsRNA, whose ability is dependent on the length of the dsRNA [71,72]. On the other hand, vertebrates have developed a wide range of anti-viral strategies, including an array of PRRs in the innate immune system, the IFN/cytokine system and the adaptive immune system. Extracellular dsRNAs are delivered to endosomal TLR3 that induce innate and adaptive anti-viral immunity. Additionally, structured RNAs with incomplete stem structures are recognized by both the dsRNA uptake receptor and TLR3, which may participate in the virus- or host-derived RNA-induced immune responses during infections or inflammation. The identification of the uptake receptor for dsRNAs and structured RNAs in human cells and also isolation of endogenous or exogenous TLR3-activating RNA molecules are important for improving our understanding of TLR3-mediated immunity.

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INAM Plays a Critical Role in IFN-γ Production by NK Cells Interacting with Polyinosinic-Polycytidylic Acid–Stimulated Accessory Cells

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Polyinosinic-polycytidylic acid strongly promotes the antitumor activity of NK cells via TLR3/Toll/IL-1R domain–containing adaptor molecule 1 and melanoma differentiation-associated protein-5/mitochondrial antiviral signaling protein pathways. Polyinosinic-polycytidylic acid acts on accessory cells such as dendritic cells (DCs) and macrophages (Mφs) to secondarily activate NK cells. In a previous study in this context, we identified a novel NK-activating molecule, named IFN regulatory factor 3–dependent NK-activating molecule (INAM), a tetraspanin-like membrane glycoprotein (also called Fam26F). In the current study, we generated INAM-deficient mice and investigated the in vivo function of INAM. We found that cytotoxicity against NK cell–sensitive tumor cell lines was barely decreased in *Inam*$^{-/-}$ mice, whereas the number of IFN-γ–producing cells was markedly decreased in the early phase. Notably, deficiency of INAM in NK and accessory cells, such as CD8α$^+$ conventional DCs and Mφs, led to a robust decrease in IFN-γ production. In conformity with this phenotype, INAM effectively suppressed lung metastasis of B16F10 melanoma cells, which is controlled by NK1.1$^+$ cells and IFN-γ. These results suggest that INAM plays a critical role in NK-CD8α$^+$ conventional DC (and Mφ) interaction leading to IFN-γ production from NK cells in vivo. INAM could therefore be a novel target molecule for cancer immunotherapy against IFN-γ–suppressible metastasis. *The Journal of Immunology*, **2014, 193: 5199–5207.**

Microbial components play a major role in activating innate and adaptive immune responses by triggering pattern recognition receptors. Nucleic acid adjuvants, including polyinosinic-polycytidylic acid (polyI:C) and unmethylated CpG dinucleotides, strongly promote Th1 immune responses against cancer and infected cells and induce type I IFN and other inflammatory cytokines (1, 2). PolyI:C strongly enhances priming and expansion of Ag-specific T cells and NK cells with dramatic regression of syngeneic implant tumors in mice (3–6). NK cells belong to group 1 innate lymphocytes (ILC1s) and control progression of several types of tumors and microbial infections (7). Although polyI:C (an analog of viral dsRNA) is a ligand for multiple receptors, including dsRNA-dependent protein kinase, retinoic acid–inducible gene-I, melanoma differentiation–associated protein-5 (MDA5), and TLR3, both of the pathways initiated by TLR3/Toll/IL-1R domain–containing adaptor molecule 1 (TICAM-1) and MDA5/mitochondrial antiviral signaling protein confer antitumor activity on NK cells in vivo (8, 9).

PolyI:C also directly and indirectly activates human NK cells and other ILC1s (10, 11). PolyI:C participates in secondary activation of murine NK cells through stimulation of accessory cells such as dendritic cells (DCs) and other myeloid cells (12–14). In these interactions, previous studies have shown that type I IFN and cell contact via IL-15 receptors play a critical role in accessory cell activation followed by NK activation (15). In contrast, our previous studies showed that polyI:C induced bone marrow–derived DC (BMDC)–mediated NK cell activation through the TLR3/TICAM-1/IFN regulatory factor 3 (IRF3) pathway, which promoted antitumor immunity by adoptive transfer in a type I IFN- and IL-15–independent manner (8, 16). As the key molecule for this NK–DC interaction, we identified a novel IRF3-inducible tetraspanin-like membrane glycoprotein, named IRF3-dependent NK-activating molecule (INAM). INAM expression was induced not only in myeloid DCs but also in NK cells by polyI:C stimulation in vivo. Transfection of INAM in both BMDC and NK cells cooperated in inducing IFN-γ production and cytotoxicity against the NK-sensitive B16D8 cell line.

To investigate the role of INAM in vivo, we generated INAM-deficient mice by the standard gene-targeting method. INAM expression was induced not only in NK cells and conventional DC

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Abbreviations used in this article: BMDC, bone marrow–derived DC; BST2, bone marrow stromal cell Ag 2; cDC, conventional DC; DC, dendritic cell; IFNAR1, IFN (α and β) receptor 1; ILC1, group 1 innate lymphocyte; INAM, IFN regulatory factor 3–dependent NK-activating molecule; IRF, IFN regulatory factor; Mφ, macrophage; MDA5, melanoma differentiation–associated protein-5; pDC, plasmacytoid DC; polyI:C, polyinosinic-polycytidylic acid; qPCR, quantitative real-time PCR; TICAM-1, Toll/IL-1R domain–containing adaptor molecule 1; WT, wild-type.

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(cDC) subsets but also in other immune cells including macrophages (Mφs) and plasmacytoid DCs (pDCs) by polyI:C stimulation. Cytotoxicity against NK cell–sensitive tumor cell lines was barely decreased in *Inam*$^{-/-}$ mice, whereas the number of IFN-γ–producing cells markedly decreased in the early phase. We also showed that CD8α$^+$ cDCs and Mφs facilitate secretion of IFN-γ from NK cells in response to polyI:C stimulation in vitro and in vivo. Notably, deficiency of INAM on NK and their accessory cells led to a robust decrease in IFN-γ production. Therefore, these results infer that INAM plays a critical role in the interaction of NK-CD8α$^+$ cDCs (and Mφs) leading to IFN-γ production from NK cells. In agreement with this suggested phenotype, INAM effectively suppressed lung metastasis of B16F10 melanoma cells by controlling activation of NK1.1$^+$ cells and IFN-γ. Taken together, these results provide the first demonstration, to our knowledge, that INAM plays a critical role in the interaction of NK–CD8α$^+$ cDCs, which allows NK cells to produce IFN-γ. We propose in this study that INAM is a novel target molecule for immunotherapy against IFN-γ–suppressible tumors.

Materials and Methods

Mice

All mice were backcrossed with C57BL/6 mice more than seven times before use. A C57BL/6 background *Inam* (*Fam26f*)–targeted embryonic stem cell line, JM8A3.N1 of FAM26F tm2a (European Conditional Mouse Mutagenesis Program) Wtsi, was purchased from the European Conditional Mouse Mutagenesis Program. Chimeric mice were generated by aggregation of the mutated embryonic stem cells at the 8 cell stage. To remove exon 2 of *Inam*, the *Inam* heterozygous mutants were crossed with Cre-transgenic mice. The *Inam* heterozygous mutants obtained were intercrossed to obtain *Inam* homozygous mutants. *Ticam-1*$^{-/-}$ and *Mavs*$^{-/-}$ mice were generated in our laboratory (8, 16). *Irf-3*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice were provided by Dr. T. Taniguchi (17). *Batf3*$^{-/-}$ C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (18). The *Batf3*$^{-/-}$ mice of C57BL/6 background [unlike 129 and BALB/c background (19)] lacked splenic CD8a$^+$ DCs as described previously (18) and evoked insufficient T cell functional response against extrinsic Ag and adjuvant (Azuma et al., submitted for publication). C57BL/6 background were purchased from CLEA Japan (Shizuoka, Japan). Experiments were performed with sex-matched mice at 8–14 wk of age. All mice were bred and maintained under specific pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine. Animal experimental protocols and guidelines were approved by the Animal Safety Center, Hokkaido University.

Semiquantitative RT-PCR and quantitative real-time PCR

Total RNA was extracted using TRIzol according to the manufacturer's instructions (Invitrogen). cDNA was generated by using the High Capacity cDNA Transcription Kit (ABI) with random primers according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using the Step One Real-Time PCR system (ABI). The primer sequences for qPCR analysis were 5′-CAACTGCAATGCCACGCTA-3′ and 5′-TCCAACCGAACACCTGAGACT-3′ for *Inam*; 5′-TTAACTGAGGCTGGCATTCATG-3′ and 5′-ACCTACACTGACACAGCCCAAA-3′ for *Il15*; 5′-GACAAAGAAAGCCGCCTCAA-3′ and 5′-ATGGCAGCCATTGTTCCTG-3′ for *Il18*; 5′-ACCGTGTTTACGAGGAACCCTA-3′ and 5′-GGTGAGAGCTGGCTGTTGAG-3′ for *Irf7*; 5′-GCCGAGACACAGGCAAAC-3′ and 5′-CCAGGGCTTGAGACACCTTC-3′ for bone marrow stromal cell Ag 2 (*Bst2*); and 5′-GCCTGGAGAAACCTGCCA-3′ and 5′-CCCTCAGATGCCTGCTTCA-3′ for *Gapdh*. The primer sequences for semi-qPCR analysis were 5′-CAACTGCAATGCCACGCTA-3′ and 5′-TCCAACCGAACACCTGAGACT-3′ for *Gapdh*.

Mφ depletion and stimulation using TLR agonists in vivo

To generate Mφ-depleted mice, mice were injected i.p. with 150 μl Clophosome-Clodronate Liposomes (FormuMax). For qPCR analysis of *Inam* induction using some TLR antagonists in Fig. 1E, mice were injected i.p. with 50 μg polyI:C (GE Bioscience), 50 μg Pam3CSK4 (Boehringer Ingelheim), 10 μg LPS (Sigma-Aldrich), 50 μg R837 (InvivoGen), and 50 μg CpG ODN1826 (InvivoGen). In other experiments, polyI:C was injected i.p. at a dose of 200 μg/mouse.

Cells

For isolation of DC subsets, Mφs and NK cells, spleens were treated with 400 Mandle U/ml collagenase D (Roche) at 37°C for 25 min in HBSS (Sigma-Aldrich). EDTA was added, and the cell suspension was incubated for an additional 5 min at 37°C. NK cells were purified from spleens by positive selection of DX5-positive cells with DX5 MACS beads (Miltenyi Biotec). CD8α$^+$ cDCs were purified using a CD8α$^+$ DC isolation kit and CD11c MACS beads (Miltenyi Biotec). CD8α$^-$ cDCs were purified with CD11c MACS beads (Miltenyi Biotec) from the negative fraction after CD8α$^+$ cDC separation. F4/80$^+$ Mφs were isolated using MACS-positive selection beads (Miltenyi Biotec) as described previously (13). pDC Ag-1$^+$ pDCs were isolated with pDC Ag-1 MACS beads (Miltenyi Biotec). All immune cells were purified from spleens by repeated positive selection to achieve high purity (90%). Leukocytes from the lung were prepared as previously reported (18). Mouse immune cells were cultured in RPMI 1640/10% FCS/55 μM 2-ME/10 mM HEPES. B16D8, B16F10, YAC-1, and RMA-S were cultured in RPMI 1640/10% FCS.

Cell culture

To investigate potential interactions with NK–accessory cells, MACS-sorted accessory cells were cocultured with freshly isolated NK cells (accessory cells /NK = 1:2) with or without 20 μg/ml polyI:C for 24 h. In some coculture experiments using the transwell system, NK cells were added to 0.4-μm pore transwells (Corning) in the presence of polyI:C. Activation of NK cells was assessed by measuring the concentration of IFN-γ (ELISA; GE Healthcare) in the medium. For the IFN (α and β) receptor 1 (IFNAR1) blocking experiment, anti-IFNAR Ab at a final concentration of 10 μg/ml was added to the cultures before addition of polyI:C. For measurement of IL-12p40 and type I IFNs, we used ELISA kits purchased from BioLegend and PBL Biomedical Laboratories, respectively.

FACS analysis

For intracellular cytokine staining of NK cells, we isolated spleen or lung from polyI:C- or PBS-injected mice at each time point and harvested their leukocytes as described previously (18, 19). The leukocytes were incubated in medium with 10 μg/ml brefeldin A for 4 h. Cells were fixed and stained with a combination of anti-NK1.1 (PK136) and anti-CD3ε (145-2C11) Abs (BioLegend), followed by permeabilization and staining with anti-IFN-γ (XMG1.2) Ab (BioLegend), anti–granzyme B (NGZB) Ab (eBioscience), anti–TNF-α (MP6-XT22) Ab (BioLegend), anti–GM-CSF (MP1-22E9) Ab (BioLegend), or anti–IL-2 (JES6-5H4) Ab (BioLegend) using a BD Cytofix/Cytoperm Kit (BD Biosciences). For staining of the C terminus of INAM of each immune cell type, after treatment of anti-CD16/32 (no. 93), cell-surface molecules of splenocytes were stained with anti-CD3ε (145-2C11), anti-CD8α (53-6.7), anti-CD11c (N418), anti-NK1.1, anti-F4/80 (BM8), anti-Gr1 (RB6-8C5), anti-CD11b (M1/70), or anti-CD19 (MB19-1) Abs (BioLegend) or with anti-B220 (RA3-6B2) or anti-CD4 (L3T4) Abs (eBioscience). After staining of the cell surface, cells were fixed and permeabilized using a BD Cytofix/Cytoperm Kit (BD Biosciences) and then stained with an anti-INAM polyclonal Ab as described previously (16). To detect activating markers, NK receptors, and developmental markers, splenocytes were stained with anti-CD27 (LG.3A10), anti-CD25 (PC61), anti-NKp46 (29A1.4), anti-NKG2D (C7), anti-DNAM-1 (10E5), and anti-TRAIL (N2B2) Abs from BioLegend or anti-Fas (Jo2) from BD Biosciences. For detection of dead cells, samples were stained with ViaProbe from BD Biosciences. Samples were processed on an FACSCalibur flow cytometer and analyzed with FlowJo software (Tree Star).

Tumor inoculation and polyI:C treatment

PolyI:C therapy against mice with B16D8 tumor burden was described previously (8). B16F10 melanoma cells (2×10^5) were injected into wild-type (WT) or *Inam*$^{-/-}$ mice via the tail vein on day 0. PolyI:C was injected i.p. on days 1, 4, 7, and 10 at a dose of 200 μg/mouse. The control group was treated with PBS. All mice were killed 12 d after tumor inoculation. The lungs were excised and fixed in Mildform (Wako) for counting of surface colonies under a dissection microscope.

Statistical analysis

Statistical analyses were made with the Student *t* test for paired data. Statistical analyses were made with ANOVA in multiple comparisons. The *p* value of significant differences is reported.

Results

Generation of INAM-deficient mice

We designed a targeting vector to disrupt exon 2, which encodes the C-terminal transmembrane and cytoplasmic regions of INAM

(Fig. 1A). The heterozygosity and homozygosity of siblings were verified by Southern blot analysis (Fig. 1B). Mutant mice were born at the expected Mendelian ratio from *Inam*$^{-/-}$ and *Inam*$^{+/-}$ parents and showed normal healthy development under specific pathogen-free conditions (Fig. 1C). We also examined the composition of immune cells in the spleen and found no clear difference between WT and *Inam*$^{-/-}$ mice (Table I). Murine NK cells are divided into four subsets in their maturation stage based on the surface density of CD27 and CD11b: CD11b^{low}/CD27^{low}, CD11b^{low}/CD27^{high}, CD11b^{high}/CD27^{high}, and CD11b^{high} /CD27^{low} (20). We examined the composition of splenic NK cells in each maturation stage and found no clear difference between WT and *Inam*$^{-/-}$ mice (Supplemental Fig. 1A). A previous study showed that *Inam* mRNA is highly expressed in spleen and thymus under steady-state conditions

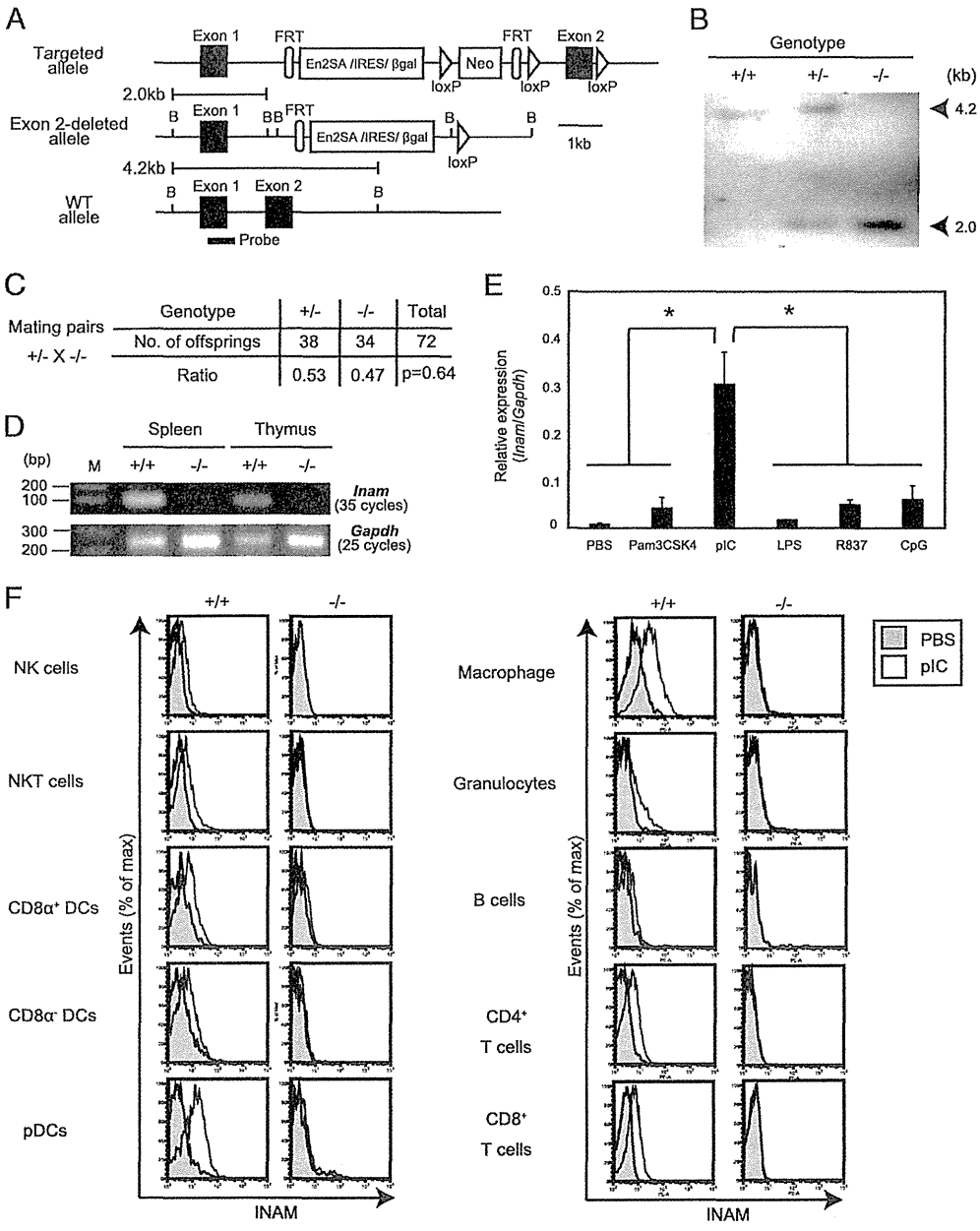

FIGURE 1. Generation of INAM-deficient mice. (**A**) Structure of the mouse *Inam*-targeted, *Inam*-disrupted, and WT allele. Closed boxes indicate the coding exon of Inam. A probe (602 bp) for Southern blot analysis was designed in exon 1. (**B**) Southern blot analysis of BamHI-digested genomic DNA isolated from WT (+/+), heterozygous mutant (+/−), and homozygous mutant (−/−) mice. (**C**) Genotype analyses of offspring from heterozygote intercrosses. The χ^2 goodness-of-fit test indicated that deviation from the Mendelian ratio was not statistically significant ($p > 0.1$). (**D**) RT-PCR analysis of spleen and thymus. Total RNA sets from spleen and thymus in WT (+/+) and *Inam*$^{-/-}$ (−/−) mice were extracted and subjected to RT-PCR to determine *Inam* expression. (**E**) *Inam* mRNA expression in response to TLR agonists. Total RNA were isolated from the spleens of mice in each group ($n = 3$) at 3 h after TLR agonist stimulation and subjected to quantitative PCR to determine to determine *Inam* expression. *$p < 0.05$ (**F**) INAM expression of immune cells. WT (+/+) and *Inam*$^{-/-}$ (−/−) mice were i.p. injected with 200 μg polyI:C (pIC) or PBS ($n = 2$). After 12 h, INAM expression of each immune cell type was analyzed by flow cytometry. Open histograms and shaded histograms indicate immune cells derived from the mice. Immune cells were classified as NK cells (CD3ε$^-$/NK1.1$^+$), NKT cells (CD3ε$^-$/NK1.1^{int}), B cells (CD19c$^+$/B220$^+$), CD8$^+$ T cells (CD3ε$^+$/CD8α$^+$), CD4$^+$ T cells (CD3ε$^+$/CD4α$^+$), classic CD8α$^-$ cDCs (CD11c^{high}/CD8α$^-$), classic CD8α$^+$ cDCs (CD11c^{high}/CD8α$^+$), pDCs (CD11c^{int}/B220$^+$), Mφs (CD11c$^{low\text{-}dim}$/CD11b$^{low\text{-}dim}$/F4/80$^+$), and granulocytes (CD11b^{high}/Gr-1$^+$). The data shown are representative of at least two independent experiments.

Table I. Development of hematopoietic cells in INAM-deficient mice

Cells	WT	*Inam*$^{-/-}$	Student *t* Test
CD4$^+$ T cells	16.9 ± 0.3	16.2 ± 2.2	$p = 0.69$
CD8$^+$ T cells	8.6 ± 0.5	8.0 ± 1.0	$p = 0.27$
B cells	55.6 ± 1.9	56.4 ± 3.5	$p = 0.65$
NK cells	1.2 ± 0.4	2.3 ± 0.7	$p = 0.22$
NKT cells	0.9 ± 0.1	0.76 ± 0.2	$p = 0.27$
pDCs	1.0 ± 0.1	1.0 ± 0.1	$p = 0.91$
CD8α$^+$ DCs	0.2 ± 0.01	0.3 ± 0.02	$p = 0.03$
CD8α$^-$ DCs	0.49 ± 0.03	0.8 ± 0.2	$p = 0.09$
Granulocytes	0.3 ± 0.04	1.0 ± 1.2	$p = 0.43$
Mφ	1.8 ± 0.6	2.2 ± 0.8	$p = 0.45$
Resident monocytes	0.4 ± 0.1	0.4 ± 0.1	$p = 0.96$
Inflammatory monocytes	0.2 ± 0.03	0.2 ± 0.2	$p = 0.82$

Data are percentages unless otherwise indicated.

(16). In our study, mRNA expression of *Inam* in these tissues was clearly absent in the *Inam*-null mouse (Fig. 1D). To assess the induction of *Inam* mRNA expression in response to TLR agonists in vivo, we performed qPCR analysis using spleens at 3 h after i.p. administration of those agonists or PBS. The levels of *Inam* mRNA expression was strongly induced by polyI:C, but not other TLR agonists (Fig. 1E). Hence, these data indicate that polyI:C is the strongest TLR agonist to induce *Inam* expression of the TLR agonists tested in vivo. To investigate the cellular distribution of INAM protein expression, we performed flow cytometric analysis using polyclonal Abs to mouse INAM after i.p. administration of polyI:C. The levels of INAM protein expression in these cells clearly reflected the absence of the mRNA (Fig. 1F). Flow cytometric analysis of spleen cells demonstrated that INAM expression was induced in all myeloid lineage cells, including DC subsets and NK cells. In particular, INAM expression was highly induced in pDCs and F4/80$^+$ Mφs.

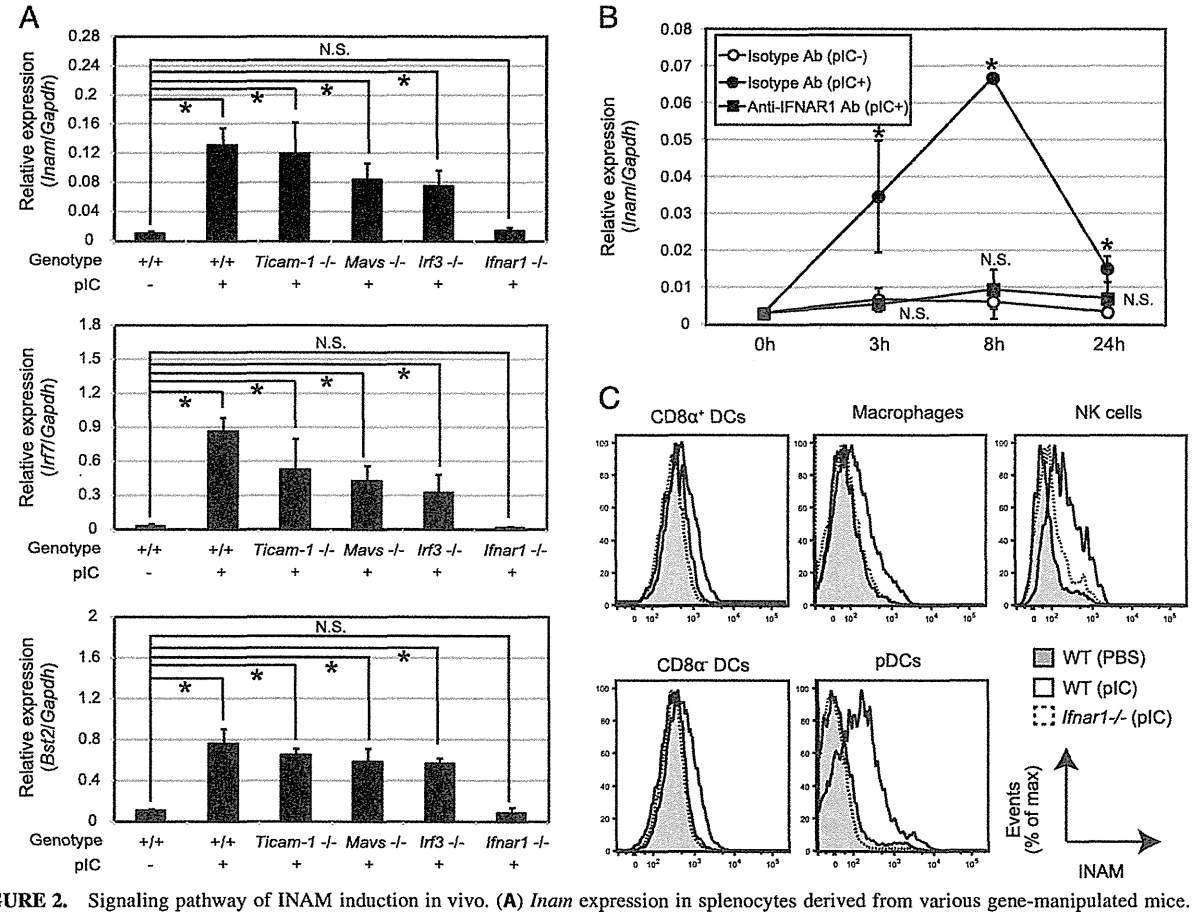

FIGURE 2. Signaling pathway of INAM induction in vivo. (**A**) *Inam* expression in splenocytes derived from various gene-manipulated mice. After 3 h, total RNA were isolated from the spleens of mice in each group ($n = 3$) and subjected to quantitative PCR to determine *Inam*, *Irf7*, and *Bst2* expression. (**B**) Type I IFN signaling is required for *Inam* expression of splenocytes derived from WT mice. Splenocytes ($n = 3$) were treated with polyI:C (pIC), IFNAR1-blocking Ab, or isotype control Ab for 0, 3, 8, and 24 h. (**C**) Type I IFN signaling is required for INAM expression of DC subsets, NK cells, and Mφs. WT and *Ifnar1*$^{-/-}$ mice were i.p. injected with 200 μg polyI:C or PBS ($n = 2$). After 12 h, INAM expression of each immune cell type was analyzed by flow cytometry. The data shown are representative of at least two independent experiments. Data are means ± SD of three independent samples. *$p < 0.05$.

Type I IFN signaling is required for INAM induction in vivo

The TLR3/TICAM-1 and MDA5/mitochondrial antiviral signaling protein pathways activate the transcription factor IRF3 in response to viral RNA. In BMDC, polyI:C (an analog of virus dsRNA) directly induces INAM expression via the TICAM-1/IRF3 pathway (16). Moreover, in the absence of pattern recognition receptor signals, IFN-α stimulation triggers INAM expression in BMDC. However, it is unclear which innate signal is required for its up-regulation in vivo. To understand the inducible pathway of *Inam* expression, we investigated its expression in spleen cells derived from various genetically manipulated mice. After polyI:C stimulation, *Inam* expression was completely undetectable in IFN (α and β) receptor 1 $(Ifnar1)^{-/-}$ mice, but not in $Ticam\text{-}1^{-/-}$ mice, a similar pattern of expression to that seen in type I IFN–inducible genes including *Irf7* and *Bst2* (Fig. 2A). Additionally, *Inam* expression was partially reduced in mice deficient in *Mavs* or *Irf3*, factors that are critical for producing type I IFN in response to polyI:C (3, 16). To assess the effect of type I IFN in WT mice, splenocytes were stimulated with polyI:C in the presence of anti-IFNAR1 Ab or isotype control Ab. Expression of *Inam* was transient, peaking at 8 h in the stimulated group in the presence of isotype control Ab (Fig. 2B). In contrast, blocking of the type I IFN receptor led to abrogation of *Inam* induction. In agreement with these results, INAM protein expression was completely undetectable in DC subsets, NK cells, and Mφs derived from IFNAR1-deficient mice (Fig. 2C). Hence, these data indicate that INAM expression depends on the IFNAR1 signaling pathway in vivo.

INAM is required for IFN-γ production through NK–accessory interaction

To identify the accessory cells directly responding to polyI:C and leading to IFN-γ production from NK cells, we performed an experiment on a coculture consisting of MACS-sorted splenic NK cells and myeloid immune cells including DC subsets and Mφs. Purified NK cells cultured in medium with or without polyI:C did not produce IFN-γ (Fig. 3A). In contrast, a high level of IFN-γ production was observed in the supernatant of NK cells cocultured with $CD8\alpha^+$ cDCs and Mφs in the presence of polyI:C, but not in pDCs and $CD8\alpha^-$ cDCs. In our reports, cell-to-cell contact is required for the interaction between NK cells and BMDC (8, 16). To confirm that the cell-to-cell contact is a prerequisite for the interaction between NK cells and splenic accessory cells, we performed coculture experiments using transwell system. As

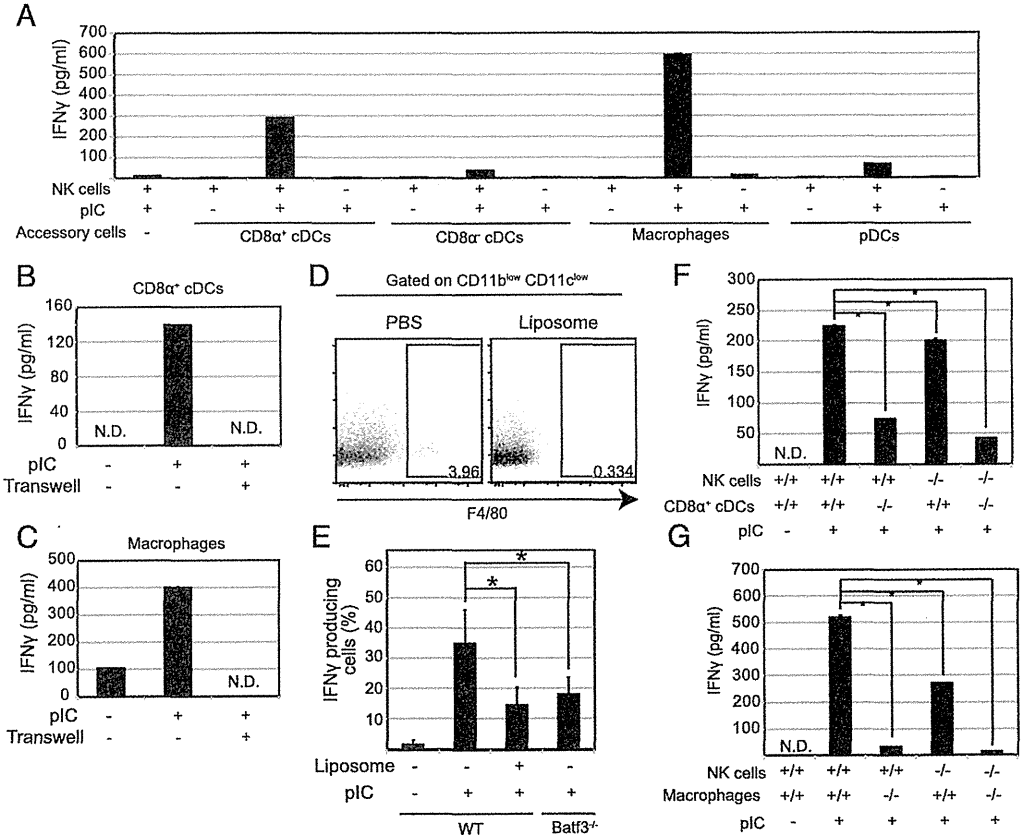

FIGURE 3. INAM-dependent NK cell activation in vitro. (**A**) IFN-γ production of NK cells via polyI:C (pIC)-stimulated DC subsets and Mφs. NK cells, DC subsets, and Mφs were enriched by MACS separation from WT and $Inam^{-/-}$ mice. (**B**) Cell-to-cell contact-dependent NK cell activation via $CD8\alpha^+$ cDCs. (**C**) Cell-to-cell contact-dependent NK cell activation via Mφs. NK cells were cocultured with DC subsets and Mφs in the presence of polyI:C (20 μg/ml) for 24 h. The concentrations of IFN-γ in the culture supernatants were measured by ELISA. (**D**) Mφ depletion with clodronate liposomes. WT mice were i.p. injected with clodronate liposomes (150 μl/mouse) to remove Mφs. After 24 h, the efficiency of Mφ depletion was measured by FACS analysis. (**E**) Production of IFN-γ by NK cells in WT, Mφ-depleted WT, and $Batf3^{-/-}$ mice. WT, Mφ-depleted WT, and $Batf3^{-/-}$ mice were i.p. injected with 200 μg polyI:C ($n = 3$). After 3 h, splenocytes were isolated, cultured with brefeldin A for an additional 4 h, and analyzed for intracellular content of IFN-γ by FACS, gating on $CD3\varepsilon^-$/$NK1.1^+$ cells. (**F**) INAM-dependent NK cell activation via $CD8\alpha^+$ cDCs. (**G**) INAM-dependent NK cell activation via Mφs. NK cells, $CD8\alpha^+$ cDCs, and Mφs were enriched via MACS separation from WT and $Inam^{-/-}$ mice. NK cells were cocultured with $CD8\alpha^+$ cDCs or Mφs in the presence of polyI:C (20 μg/ml) for 24 h. The concentrations of IFN-γ in the culture supernatants were measured by ELISA. The data shown are representative of at least two independent experiments. Data are means ± SD of three independent samples. $^*p < 0.05$.

a result, IFN-γ production was completely blocked under transwell conditions (Fig. 3B, 3C). Therefore, NK cells are primed through contact with CD8α$^+$ cDCs and Mφs independent of soluble mediators. To directly test the contribution of CD8α$^+$ cDCs and Mφs to polyI:C-mediated NK cell activation in vivo, we analyzed *Batf3*$^{-/-}$ mice, which largely lack the CD8α$^+$ cDC population in the spleen of C57BL/6 mice (21), and Mφ-depleted mice generated by clodronate liposome injection (22, 23). Approximately 85% of Mφs were depleted at 24 h after clodronate liposome injection (Fig. 3D). Three hours after polyI:C stimulation, NK cell secretion of IFN-γ was partially decreased in *Batf3*$^{-/-}$ and Mφ-depleted mice (Fig. 3E). These results indicate that CD8α$^+$ cDCs and Mφs are responsible for secretion of IFN-γ from NK cells in response to polyI:C stimulation.

INAM acts on NK cells and BMDC to orchestrate NK–DC interaction triggered by polyI:C stimulation (16). To investigate the role of INAM in the interaction of NK-CD8α$^+$ cDC and NK-Mφ, we performed an experiment on a coculture of MACS-sorted splenic NK cells with their accessory cells isolated from WT and *Inam*$^{-/-}$ mice. Cocultures of NK cells and accessory cells lacking INAM showed that IFN-γ production from NK cells required INAM expression in either NK cells or accessory cells (Fig. 3F, 3G). Notably, deficiency of INAM in both NK and accessory cells led to a marked decrease in IFN-γ production. Taken together, these results suggest that INAM is required for cell–cell contact in both NK cells and accessory cells and early IFN-γ production by NK cells.

INAM plays a critical role in rapid IFN-γ production by NK cells in response to polyI:C in vivo

To investigate the role of INAM in polyI:C-mediated cytotoxicity of NK cells, we injected WT and *Inam*$^{-/-}$ mice with polyI:C. After 0, 3, and 24 h, we isolated splenic NK cells and measured cytotoxicity ex vivo. In the four NK-sensitive cell lines B16D8, RMA-S, B16F10, and YAC-1, we found no difference between WT and *Inam*$^{-/-}$ mice in the cytotoxic effect of NK cells against these cell lines (data not shown). Consistent with these results, cell numbers expressing granzyme B, known as a cytotoxic lymphocyte protease, barely differed between splenocytes of WT and *Inam*$^{-/-}$ mice (Fig. 4A). To determine the role of INAM in NK cell production of IFN-γ in response to polyI:C, we isolated splenocytes 0, 1, and 3 h after injecting WT and *Inam*$^{-/-}$ mice with polyI:C and determined the intracellular content of IFN-γ in NK cells. After 3 h, NK cells isolated from *Inam*$^{-/-}$ mice produced less IFN-γ than WT NK cells (Fig. 4B). Additionally, we also measured the numbers of other cytokine-producing cells, including GM-CSF, IL-2, and TNF-α, from NK cells at 3 h after polyI:C stimulation in WT and *Inam*$^{-/-}$ mice and confirmed no INAM dependence of the production of these cytokines (Supplemental Fig. 2A). Therefore, INAM specifically regulates IFN-γ through CD8α DC at least within this time frame. We also measured CD69 expression, known as an NK-activating marker at 0, 3, and 24 h after polyI:C stimulation. CD69 upregulation in response to polyI:C was partially impaired in NK cells from *Inam*$^{-/-}$ mice in comparison with those from WT mice 24 h after polyI:C stimulation (Fig. 4C). We found no clear difference between WT and *Inam*$^{-/-}$ mice in expression of CD27 or NK1.1, both of which evoke IFN-γ production through their interaction with the ligands, or in any other NK receptors at 0, 3, and 24 h after polyI:C injection (24) (Supplemental Fig. 1B). These results indicate that INAM-mediated NK activation is independent of incremental expression of these receptors. Previous reports suggested that proinflammatory cytokines including IL-12, IL-15, IL-18, and type I IFN play critical roles in the cytotoxicity and IFN-γ production of NK cells (15, 25, 26). To determine their expression at 0, 3, and 24 h

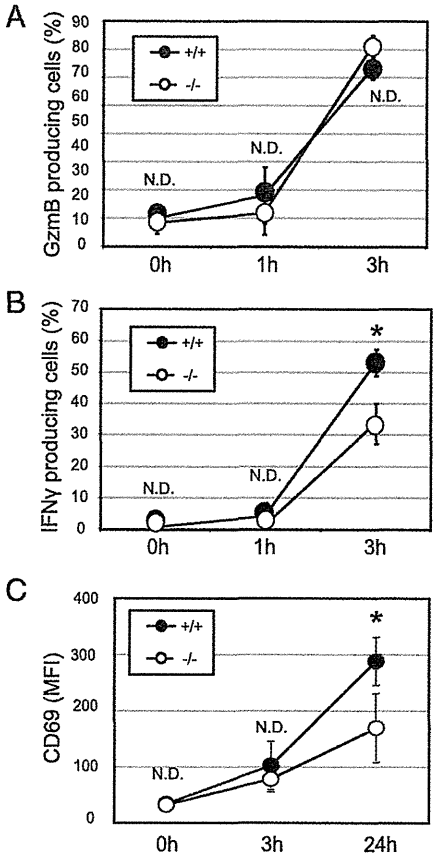

FIGURE 4. INAM-dependent NK cell activation in vivo. (**A**) Production of granzyme B (GzmB) by NK cells. (**B**) Production of IFN-γ by NK cells. WT (+/+) and *Inam*$^{-/-}$ (−/−) mice were i.p. injected with 200 μg polyI:C. After 0, 1, and 3 h, splenocytes were isolated, cultured with brefeldin A for an additional 4 h, and analyzed for intracellular content of IFN-γ and granzyme B by FACS, gating on CD3ε$^-$/NK1.1$^+$ cells (n = 3 or 4). (**C**) Expression of CD69 on the surface of NK cells. WT (+/+) and *Inam*$^{-/-}$ (−/−) mice were i.p. injected with 200 μg polyI:C or PBS. After 0, 3, and 24 h, CD69 expression was assayed by FACS, and the data were quantitatively analyzed using mean fluorescence intensity (MFI), gating on CD3ε$^-$/NK1.1$^+$ cells (n = 3). The data shown are representative of at least two independent experiments. Data are means ± SD of three independent samples. *$p < 0.05$.

after polyI:C stimulation, we performed ELISA and qPCR analysis of serum and spleen cells from WT and *Inam*$^{-/-}$ mice. However, protein levels of IL12p40, IFN-α, and IFN-β were not affected by *Inam* disruption in mice (Supplemental Fig. 2B). Additionally, mRNA expression of *Il-15* and *Il-18* genes was not decreased in *Inam*$^{-/-}$ mice (Supplemental Fig. 2C). These results suggest that INAM plays a critical role in the CD69 expression and rapid IFN-γ production, but not the cytotoxicity, of NK cells in response to polyI:C in a cytokine-independent manner.

INAM is required for the antimetastatic effect by polyI:C-based cancer immunotherapy

Malignant melanomas are one of the most important targets of NK-mediated cancer immunotherapy (27). In this study, we tested two types of polyI:C-based cancer immunotherapy model using B16D8 and B16F10 cell lines. NK cells show high cytotoxicity activity against B16D8 cells established in our laboratory as a subline of the B16 melanoma cell line (28). This subline was characterized by its low or virtually absent metastatic properties when injected s.c. into syngeneic C57BL/6 mice. In contrast, the B16F10 subline was characterized by its high metastatic capacity

especially into the lung (29). In this model, NK1.1$^+$ cells and IFN-γ have a critical role in the suppression of pulmonary metastases (30).

A mouse model with s.c.-implanted B16D8 and polyI:C therapy has been established in our laboratory (8). To investigate the function of INAM involved in tumor growth retardation mediated by polyI:C, we challenged WT and *Inam*$^{-/-}$ mice with B16D8 implantation and then treated the mice with i.p. injection of polyI:C. The rate of B16D8 growth retardation was indistinguishable between WT and *Inam*$^{-/-}$ mice (Supplemental Fig. 3), which was largely dependent on the antitumor effect of polyI:C. This result is consistent with the observation that there is no difference in tumoricidal activity against B16D8 between WT and *Inam*$^{-/-}$ mice. To determine the role of INAM in the production of IFN-γ by lung NK cells in response to polyI:C, we isolated leukocytes from the lung at 0, 3, and 6 h after administration of polyI:C to B16F10-injected WT and *Inam*$^{-/-}$ mice and determined the intracellular content of IFN-γ in NK cells (Fig. 5A). After 6 h, NK cells isolated from *Inam*$^{-/-}$ mice produced less IFN-γ than WT NK cells (Fig. 5B). To investigate the function of INAM involved in pulmonary metastases induced by polyI:C, we i.v. challenged WT and *Inam*$^{-/-}$ mice with B16F10 cells and then treated the mice by i.p. injection of polyI:C. After four rounds of polyI:C treatment, we counted tumor foci in the lung. Under unstimulated conditions, there was no difference in the number and size of tumor foci in the lungs between WT and *Inam*$^{-/-}$ mice (Fig. 5C). In WT mice, i.p. injection of polyI:C exerted a significant inhibition in the growth of pulmonary metastases in tumor-bearing mice compared with PBS controls (Fig. 5D). In contrast, the effect of polyI:C therapy for pulmonary metastases was partially abrogated in *Inam*$^{-/-}$ mice. These results demonstrate that INAM plays a critical role in IFN-γ production by lung NK cells in response to polyI:C and unequivocally exhibits antitumor function in polyI:C-based cancer immunotherapy against IFN-γ–sensitive tumors metastasized to the lung.

BMDC confer direct cytotoxic activity on NK cells by stimulation with RNA via INAM-dependent cell–cell contact (16). Then, NK cells kill tumor cells via effectors, such as TRAIL and granzyme B, secondary to upregulation of INAM. However, splenic DCs hardly induce direct NK cytotoxicity as shown in this study. In this study, *Inam*$^{-/-}$ mice studies revealed that DC/Mφ primed NK cells in vivo to induce IFN-γ that was a major effector for NK antimetastatic activity. Thus, taken together with the previous results that BMDCs induce NK cytotoxicity via INAM (16), INAM-involved DC–NK contact induces two arrays of NK tumoricidal activities, killer effector and IFN-γ producer, depending on the properties of DC subsets. The role of INAM in ILC activation will be a matter of future interest in this context.

Discussion

In this study, we provide the first demonstration, to our knowledge, that INAM plays a critical role in the interactions of NK-CD8α$^+$ cDCs and Mφs leading to IFN-γ production from NK cells in vivo. Additionally, we also propose that INAM is a novel target molecule for cancer immunotherapy against IFN-γ–suppressible metastasis.

IFN-γ coordinates a diverse array of cellular programs via STAT1 activation, such as antimicrobial response, anti- or protumor response, production of proinflammatory cytokines, and induction of IRF1 (31). IRF1 activates a large number of secondary response genes, which carry out a range of immunomodulatory functions (32, 33). In secondary lymphoid organs including spleen and lymph nodes, NK cells are a dominant IFN-γ producer responding to polyI:C (5). IFN-γ primes Ag-specific CD4$^+$ and CD8$^+$ T cells and also activates other innate immune cells including Mφs (34–36). The TLR3-dependent IFN-γ signaling pathway is important in protecting the host from pathogenesis induced by Coxsackievirus group B serotype 3 infection, which leads to IFN-γ production from NK cells (37, 38). Hence, IFN-γ

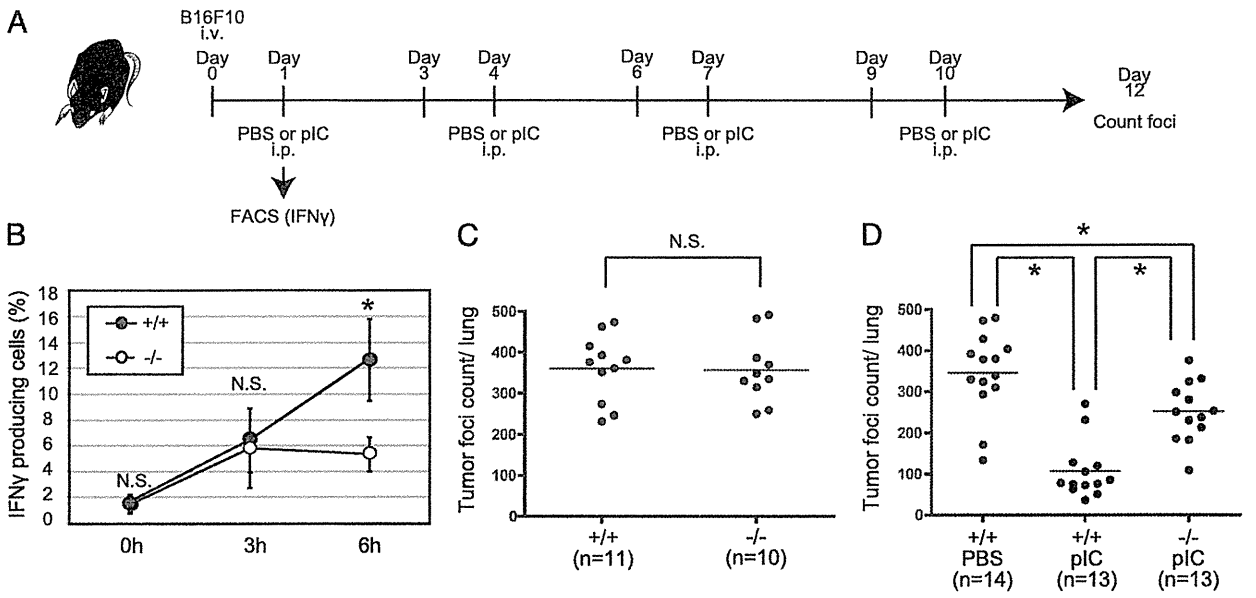

FIGURE 5. Antimetastatic activity of INAM against B16F10 melanoma. (**A**) The time schedule of polyI:C (pIC) treatment. (**B**) Production of IFN-γ by NK cells in the lung. After 24 h, WT and *Inam*$^{-/-}$ mice were i.p. injected with 200 μg polyI:C. Lung leukocytes were isolated and cultured with brefeldin A for an additional 4 h, and analyzed for frequency of NK cells and production of IFN-γ/granzyme B by FACS, gating on CD3ε$^-$/NK1.1$^+$ cells ($n = 3$ or 4). (**C**) Tumor foci counts in the lung of WT (+/+) and *Inam*$^{-/-}$ (−/−) mice under unstimulated conditions at day 12. (**D**) Tumor foci in the lung of WT (+/+) and *Inam*$^{-/-}$ (−/−) mice. WT (+/+) and *Inam*$^{-/-}$ (−/−) mice were i.v. injected with 2×10^5 B16F10 melanoma cells at day 0. At days 1, 4, 7, and 10, WT and *Inam*$^{-/-}$ mice were i.p. injected with 200 μg polyI:C. At day 12, the mice were sacrificed, and lungs were removed and fixed in 10% formalin solution to count surface colonies under a dissection microscope. The data shown are representative of at least two independent experiments. Data are means ± SD of three independent samples. *$p < 0.05$.

derived from NK cells controls innate and adaptive immunity, leading to a Th1 response.

In this study, we show that INAM evokes IFN-γ production by NK cells in the early phase by polyI:C stimulation (Figs. 4B, 5B). In a murine CMV infection model, IFN-γ is induced in NK cells by IL-12 and IL-18 produced by murine CMV–infected CD11b$^+$ cDCs, whereas these cytokines barely evoke any cytotoxic response in NK cells (39). In addition, IFN-γ production from NK cells is induced by anti-CD27 Ab stimulation, but again no cytotoxic response is triggered (24). Therefore, these reports indicate that NK cell cytotoxicity and IFN-γ production are independently controlled by different mechanisms. We found no clear difference between WT and *Inam*$^{-/-}$ mice in expression of these cell surface molecules and cytokines. Hence, the INAM-dependent IFN-γ production from NK cells is based on an as-yet-unknown mechanism(s) acting in a manner independent of these molecules.

CpG DNA is known to induce IFN-γ from NK cells, which is mediated through pDCs. TLR9 in pDCs responds to CpG, and the pDCs liberate IFN-α and TNF-α that participate in the induction of IFN-γ from NK cells (40). We checked induction of the Inam mRNA in spleen after stimulation with CpG in WT and *Inam*$^{-/-}$ mice (Fig. 1E). The levels of *Inam* mRNA as well as numbers of IFN-γ–producing cells were hardly increased in response to i.p. administration of CpG in WT as well as *Inam*$^{-/-}$ mice, suggesting no participation of INAM in CpG-induced NK cell IFN-γ production (data not shown). CpG participates in the activation of the TLR9 pathway in pDCs, but INAM in splenic cDCs and Mφs does not participate in CpG-mediated NK priming. The result is consistent with the fact that polyI:C is an agonist for TLR3 (but not for TLR9 predominantly expressed in pDCs), which is mainly expressed in CD8α$^+$ DCs, especially professional Ag-presenting CD141$^+$ and CD103$^+$ DCs in mice (41).

CD8α$^+$ cDCs directly recognize polyI:C via the TLR3/TICAM-1 pathway and promote IFN-γ production from NK cells in vitro (9). However, previous analysis of *Batf3*$^{-/-}$ mice indicated that absence of CD8α$^+$ cDCs resulted in weak NK cell activation, in agreement with our data (19). We also found that NK cell secretion of IFN-γ was partially decreased in mice depleted of Mφs by injection of clodronate liposomes (Fig. 3E). Notably, expression of INAM by both NK cells and accessory cells is required for early IFN-γ production through NK-CD8α$^+$ cDC and/or NK–Mφ interactions (Fig. 3F, 3G). The physiological role of these accessory cells in NK activation is poorly understood. However, our results indicate that CD8α$^+$ cDCs and Mφs facilitate early secretion of IFN-γ from NK cells in response to polyI:C and INAM plays a critical role in the interaction between NK cells and CD8α$^+$ cDCs and/or Mφs, leading to IFN-γ production.

IFN-γ exhibits both anti- and protumor activities (42). Systemic administration of polyI:C exerted a significant inhibitory effect on the growth of lung metastases in B16F10 melanoma-bearing mice (30, 42). Using this model, a previous study reported that NK1.1$^+$ cells and IFN-γ have a critical role in the protection of lung metastases (30). Previous studies demonstrated that the IFN-γ receptor expressed on host cells, but not on melanoma cells, is important for development of lung metastases (43–45). Hence, lung metastases are prevented by the IFN-γ–inducible immune response following NK cell activation. We show that INAM is involved in the IFN-γ production of lung NK cells in response to polyI:C stimulation and unequivocally exhibits antitumor functions in polyI:C-based cancer immunotherapy against IFN-γ–sensitive tumor foci in the lung (Fig. 5D). Therefore, we propose that INAM is a novel target molecule for cancer immunotherapy against IFN-γ–suppressible metastasis.

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Disclosures

The authors have no financial conflicts of interest.

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特集I　抗腫瘍免疫の抑制と活性化

dsRNAによるTAM, MDSCの腫瘍免疫抑制作用から活性化作用への変換*

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Key Words：dsRNA adjuvant, immune suppression, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs)

はじめに

がんでみられる免疫抑制細胞の増加は，がんの進展やがん免疫療法の治療効果に影響する．がん細胞の排除に働く抗がん免疫応答は，腫瘍随伴マクロファージ(tumor-associated macrophages；TAMs)やミエロイド由来サプレッサー細胞(myeloid-derived suppressor cells；MDSCs)に代表されるミエロイド(骨髄)系免疫細胞によって抑制され，がんの進展は促進される．一方，これらの免疫細胞の機能調節に基づいた新たな治療概念が提唱されている．本稿では，がんの進展におけるTAMsおよびMDSCsの役割について概説し，2本鎖RNA(double-stranded RNA；dsRNA)アジュバントによる自然免疫系シグナルの活性化が，TAMsとMDSCsの機能とがんの治療効果に与える影響について紹介する．

ミエロイド系免疫細胞による免疫抑制とがんの進展

がん細胞と免疫系との相互作用はがんの進展と密接に関係している[1]．がんの初期段階では，炎症反応が発がんを促進する．一方，がん化した細胞は自己に由来するため免疫原性は弱いものの，major histocompatibility complex (MHC) クラスI分子によるがん抗原(tumor antigen)の提示またはMHCクラスI分子の発現低下によって，細胞傷害性T細胞(cytotoxic T lymphocytes；CTLs)や樹状細胞(dendritic cells；DCs)，ナチュラルキラー(natural killer；NK)細胞の活性化がうまく誘導されると，これらの細胞の働きで体内から排除される．しかし，がんの進行に伴って，全身および腫瘍内微小環境(tumor microenvironment)で免疫抑制的な環境が徐々に構築される[2]．その結果，抗がん免疫応答は抑制され，がんの進展は促進する．免疫抑制的な環境は，がん細胞が産生する免疫抑制性因子やがん細胞によって誘導される制御性T細胞(regulatory T cells；Tregs)に加えて，免疫抑制活性を持つミエロイド系免疫細胞が増加することにより構築される．そのようなミエロイド系免疫細胞には，TAMsおよびMDSCsが含まれる．マウスの担がんモデルでTAMsやMDSCsを除去すると，免疫抑制が解除されるために，抑えられていた抗がん免疫応答が惹起され，腫瘍の成長は阻止される[3]．また，免疫抑制の影響は抗がん免疫応答にかかわる広範囲の免疫細胞に及ぶため，これらの細胞の存在はがんワクチンなどの免疫療法の妨げともなる．

以下に述べるように，TAMsやMDSCsは，腫

* dsRNA induces conversion of immunosuppressive TAMs and MDSCs to cells with antitumor activity.

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