TICAM-1 via the RIP homotypic interaction motif domain in the C-terminal region and acts as an NF-κB inducer and apoptosis mediator in TICAM-1-mediated signalling [70–72]. TRAF6 has also been implicated in NF-κB activation by TICAM-1 in a cell-type-dependent manner [64,73].

TLR3–TICAM-1-mediated signalling is negatively regulated by a fifth TIR adaptor protein SARM [74]. SARM and TICAM-1 have been shown to interact and SARM strongly suppresses NF-κB activation, as well as IRF-3 activation by TICAM-1. Moreover, deubiquitinating enzyme A (DUBA) negatively regulates TLR3-mediated type I IFN production. DUBA selectively cleaves the Lys63-linked polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream-signalling molecules [75]. In addition, the ubiquitin-modifying enzyme A20 inhibits TICAM-1-mediated NF-κB activation by deubiquitinating TRAF6 [76]. However, the precise mechanisms by which TRAF3 and TRAF6 are ubiquitinated and their interaction with downstream-signalling molecules are unknown.

Antiviral function of TLR3

The role of TLR3 in viral infection is complex (Table 1). Studies in TLR3-deficient ($TLR3^{-/-}$) mice showed that the immune response to different viruses, including lymphocytic choriomeningitis virus (an ambisense RNA virus), vesicular stomatitis virus (a negative-stranded RNA virus), murine cytomegarovirus (MCMV, a dsDNA virus) and reovirus (a dsRNA virus), was unaffected in these mutant mice compared with wild-type mice [77].

By contrast, Hardarson *et al.* [78] reported that TLR3 is important in host defense against encephalomyocarditis virus (EMCV, a positive sense ssRNA virus belonging to the *Picornaviridae* family). When mice were inoculated intraperitoneally with 50 plaque-forming units EMCV, $TLR3^{-/-}$ mice were more susceptible to EMCV infection and had a significantly high viral load in the heart compared with wild-type mice. Opposing to these data, Kato *et al.* [24] showed that MDA5 but not TLR3 plays an important role in host defense against EMCV infection, when mice were infected with 100 plaque-forming units EMCV intraperitoneally. It is unclear why these different results were obtained from similar EMCV infection studies.

Table 1. The role of TLR3 in antiviral responses

	References
Protection	
Flaviviridae [+, ss]	
West Nile virus (WNV)	[84]
Picornaviridae [+, ss]	
Encephalomyocarditis virus (EMCV)	[78]
Poliovirus	[79,80]
Coxsackievirus group B serotype 3 (CVB3)	[82]
Herpesviridae [dsDNA]	
Murine cytomegarovirus (MCMV)	[90]
Herpes simplex virus 1 (HSV-1)	[101]
Deterioration	
Flaviviridae [+, ss]	
West Nile virus (WNV)	[83]
Orthomyxoviridae [−, ss]	
Influenza A virus (IAV)	[88]
Bunyaviridae [−, ss]	
Phlebovirus	[89]

More recently, the essential role of the TLR3–TICAM-1 pathway in protection from poliovirus infection, a virus belonging to the *Picornaviridae* family, has been demonstrated [79,80]. Poliovirus receptor (PVR)-transgenic/TICAM-1-deficient mice are more susceptible than PVR-transgenic mice to intraperitoneal or intravenous inoculation with a low titre of poliovirus [79,80]. Forty-eight hours after infection, virus titres in serum dramatically increased and mortality greatly decreased compared with PVR-transgenic or PVR-transgenic/IPS-1 (RLR adaptor)-deficient mice. It is well known that in cultured mammalian cells, poliovirus infection results in inhibition of cellular protein synthesis so-called 'shut-off' event [81]. Therefore, mRNA upregulation of RIG-I and MDA5 by type I IFN does not link to protein synthesis at an early stage of virus infection. Thus,

it appears that the inhibitory effects of viral multiplication on host cells depend on the TLR3–TICAM-1 pathway, but not the RLR–IPS-1 pathway.

In addition, Negishi *et al.* [82] showed that $TLR3^{-/-}$ mice are more vulnerable to coxsackievirus group B serotype 3 (CVB3, a virus belonging to the *Picornaviridae* family) than wild-type mice, in terms of higher mortality and acute myocarditis. The expression of IL-12p40, IL-1β and IFN-γ mRNAs, but not IFN-β mRNA, was impaired in the hearts of CVB3-infected TLR3-deficient mice compared with those of wild-type mice infected with CVB3. By contrast, expression of TLR3 by transgene protects mice from lethal CVB3 infection and hepatitis even in the absence of type I IFN signalling. Antibody blocking studies revealed that TLR3–TICAM-1-dependent type II IFN (IFN-γ) production is critical for host defense against CVB3 infection [82].

Remarkably, Wang *et al.* [83] demonstrated that TLR3 is involved in the viral pathogenesis of West Nile virus (WNV, a positive-stranded RNA virus). $TLR3^{-/-}$ mice showed impaired cytokine production and enhanced viral loads in the periphery, whereas in the brain, the viral load, inflammatory responses and neuropathology were reduced compared with wild-type mice [83]. TLR3-mediated peripheral inflammatory cytokine production is critical for disruption of the blood–brain barrier, which facilitates viral entry into the brain causing lethal encephalitis. Therefore, $TLR3^{-/-}$ mice are more resistant to lethal WNV infection. In contrast, Daffis *et al.* [84] reported the protective role of TLR3 in sublethal WNV infection. The absence of TLR3 enhances WNV mortality in mice and increases viral burden in the brain after inoculation with the pathogenic New York strain of WNV, although there are little differences in WNV-specific antibody responses, $CD8^+$ T-cell activation, blood–brain barrier permeability and IFN-α/β induction in draining lymph nodes and serum, between wild-type and $TLR3^{-/-}$ mice [84]. The reason why TLR3 shows the opposite function against WNV infection remains to be determined.

In other RNA viral infections such as respiratory syncytial virus, IAV and phlebovirus (all negative-stranded RNA viruses), TLR3-dependent inflammatory cytokine and chemokine production also appears to affect virus-induced pathology and host survival [85–89]. $TLR3^{-/-}$ mice infected with IAV exhibited reduced inflammatory mediators, leading to increased survival [88]. It is notable that experimental conditions using high viral doses may lead to the over-production of inflammatory cytokines and chemokines. However, what type of TLR3-expressing cells that respond to virus-derived dsRNA *in vivo* has not been shown in these studies.

Cellular immunity induced by the TLR3–TICAM-1 pathway

In addition to type I IFNs, CTLs and NK cells are also principal effector cells in antiviral immunity. The contribution of TLR3 to antiviral responses has been shown in MCMV infection [90], during which virus clearance is partly dependent on NK cell activation. $TLR3^{-/-}$ mice are hypersusceptible to MCMV infection. Cytokine (type I IFN, IL-12p40 and IFN-γ) production, and NK cell and NKT cell activation are impaired in $TLR3^{-/-}$ mice compared with wild-type mice.

Selective TLR3 expression in myeloid DCs but not in pDCs raises the possibility that TLR3 also plays a key role in the antiviral response by induction of adaptive immune responses rather than primary IFN-α/β production (Table 2). Myeloid DCs are the most effective professional antigen-presenting cells, possessing several antigen processing and transporting pathways [91,92]. One of the most notable features of myeloid DCs is the cross-presentation of exogenous antigens to $CD8^+$ T cells. This pathway is important for effective host CTL induction against viruses that do not directly infect DCs. Among the myeloid DC subsets, the splenic $CD8\alpha^+$ DC subset in mice and the $CD141(BDCA3)^+DNGR\text{-}1(CLEC9A)^+$ DC subset in humans highly express TLR3 and display a superior capacity for cross-presenting apoptotic and necrotic cell antigens after TLR3 stimulation [93–97]. Using TLR3-deficient mice, Schultz *et al.* [98] clearly showed that TLR3 plays an important role in cross-priming. Mouse $CD8\alpha^+$ DCs are activated by phagocytosis of apoptotic bodies from virally infected cells or cells containing poly(I:C) in a TLR3-dependent manner. Furthermore, immunization with virally infected cells or cells containing poly(I:C), both carrying ovalbumin antigen, induces ovalbumin-specific $CD8^+$ T-cell responses, which are largely dependent on TLR3-expressing DCs [98]. In many cases, virally infected cells produce IFN-α/β which activates DCs to

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Table 2. Expression of nucleic acid-sensing TLRs in DC subsets

	DC subset	TLR3	TLR7	TLR8	TLR9	References
Human	Myeloid DC					
	MoDC	+	−	+	−	[31–35]
	$CD11c^+CD1c^+$DC	+	−	+	−	[34,35,94,95]
	$CD141^+CLEC9A^+$DC	++	−	+	−	[94,95]
	Plasmacytoid DC	−	+	−	+	[34,35]
Mouse	Myeloid DC					
	BMDC	+	−	−	+	[95]
	$CD8\alpha^+$ DC	++	−	−	+	[93,95]
	Plasmacytoid DC	−	+	−	+	[93,95]

MoDC, monocyte-derived immature dendritic cells; BMDC, bone marrow-derived DC.

promote $CD8^+$ T-cell cross-priming [99]. Thus, both TLR3- and IFN-α/β-mediated signalling are likely implicated in licensing DCs for the cross-priming of $CD8^+$ T cells.

In humans, Ebihara *et al.* [100] demonstrated the role of TLR3, expressed in myeloid DCs, in the immune response to HCV infection. The JFH1 strain of HCV does not directly infect or stimulate myeloid DCs to activate T cells and NK cells, but instead the phagocytosis of HCV-infected apoptotic cells that contain HCV-derived dsRNA and their interaction with the TLR3 pathway in myeloid DCs, plays a critical role in DC maturation and activation of T and NK cells [100]. In addition, Jongbloed *et al.* [94] reported that $CD141^+$ DCs are able to cross-present viral antigens from human cytomegalovirus-infected necrotic fibroblasts. Physiologically, TLR3 in a DC subset specialized for antigen presentation appears to encounter viral dsRNAs in the endosome by uptake of apoptotic or necrotic virus-infected cells and signals for cross-presentation of viral antigens. Furthermore, a dominant-negative TLR3 allele was found in children with herpes simplex virus 1 (HSV-1) encephalitis [101]. TLR3 is expressed in the central nervous system, where it is required to control HSV-1. Interestingly, recent paper demonstrated that mouse $CD8\alpha^+$ DCs and human $CD141^+$ DCs are major producers of IFN-λ in response to poly(I:C), which depends on TLR3 [102]. Thus, TLR3 plays a role in the antiviral response, dependent on the viral genome structure, the route of virus entry into cells, the TLR3-expressing cell type that encounters viral dsRNA, and the properties of the host anti-viral effector functions.

Application of the TLR3 ligand to adjuvant vaccine therapy

Selective expression of TLR3 in myeloid DCs, especially human $CD141^+$ DCs and mouse $CD8\alpha^+$ DC subsets, is the advantage in employing TLR3 ligands as adjuvant. In addition to the TLR3-dependent CTL activation described above, DC-mediated NK cell activation is also important for the adjuvancy of TLR3 ligands. Akazawa *et al.* [103] showed that the TLR3–TICAM-1 pathway is essential for poly(I:C)-induced NK-cell-mediated tumour regression in a syngeneic mouse tumour implant model. Remarkably, production of IFN-α is not impaired in TICAM-$1^{-/-}$ mice compared with wild-type mice after *in vivo* poly(I:C) injection or *in vitro* bone marrow-derived DC (BMDC) stimulation, whereas IL-12 production is completely dependent on TICAM-1, consistent with other reports [22,104]. Furthermore, NK cell activation requires cell–cell contact with BMDCs preactivated by poly(I:C) but not IFN-α or IL-12. Thus, the TLR3–TICAM-1 pathway in myeloid DCs facilitates the DC–NK cell interaction following NK cell activation. TICAM-1–IRF3-dependent expression of a novel molecule, namely IRF-3-dependent NK-activating molecule (INAM), in myeloid DCs is required for NK activation [104]. Poly(I:C)-induced MDA5-dependent myeloid DC activation is also implicated in NK cell activation [105,106].

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However, several issues remain unresolved including a suitable transport system for TLR3 ligands. Poly(I:C) injected intraperitoneally in mice activates both TLR3 and MDA5, indicating that extracellular poly(I:C) is delivered to endosomal TLR3 and further to cytosolic MDA5 in murine cells. A recent study demonstrated that CD14 enhances poly(I:C)-mediated TLR3 activation in bone marrow-derived macrophages by directly binding to poly(I:C) and mediating cellular uptake of poly(I:C) [107]. The internalized poly(I:C) then colocalizes with CD14 and TLR3. Since the extracellular domain of CD14 consists of LRRs [108], CD14 may associate with TLR3 and transfer poly(I:C) to TLR3 in macrophage endosomes. In the case of CD14-negative myeloid DCs, extracellular dsRNA must be internalized with the putative uptake receptor. Indeed, it has been demonstrated by our group and others that poly(I:C) is internalized into human monocyte-derived immature DCs and mouse BMDCs via clathrin-dependent endocytosis, and B- and C-type oligodeoxynucleotides share the uptake receptor with poly(I:C) [109]. Notably, among various synthetic dsRNAs, poly(I:C) is preferentially internalized and activates TLR3 in myeloid DCs. By contrast, *in vitro*-transcribed dsRNAs of various lengths (50–1000 bp) cannot be internalized into myeloid DCs [110]. Thus, uptake of TLR3 ligands largely depends on the dsRNA structure recognized by the uptake receptor expressed on myeloid DCs.

The dsRNA structure and the targeting approach of dsRNA to the endosomal TLR3 in the appropriate DC subset, are important factors involved in generating innate and adaptive immune responses by TLR3 ligands. Gowen *et al.* [111] showed that poly(I:C_{12}U) induces IFN-β in a TLR3-dependent and MDA5-independent manner, and exhibits protective anti-viral effects in mice. Identification of the putative dsRNA uptake receptor is crucial for analysing the intracellular transport of dsRNA. Furthermore, clarification of the differences between the RIG-I/MDA5-mediated and TLR3–TICAM-1-mediated signalling pathways is important for assessment of dsRNA-induced immune responses.

Concluding remarks

The protective role of TLR3 in virus infection is now becoming clear from experiments using an infectious mouse model or TLR3-transgenic mice. Since both CVB3 and poliovirus belong to the *Picornaviridae* family, along with encephalomyocarditis virus that is recognized by MDA5, distinct virus properties rather than virus genome structure appear to determine which RNA sensors act in antiviral defense in host cells. The molecular mechanism behind the anti-viral function of TLR3 *in vivo* and the identification of TLR3–TICAM-1-mediated signalling cascades distinct from those of RIG-I/MDA5, are important factors for understanding the role of RNA-sensors in the host defense system. In addition, characterization of a new myeloid DC subset that expresses a high level of TLR3 and has a high capacity to present antigen from apoptotic and necrotic cells after TLR3 activation, may provide insight into the role of TLR3 in the activation of NK cells and CTLs in viral infection. This, in turn, may advance the development of TLR3-related vaccine adjuvants effective against tumours and/or infectious diseases.

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Toll-like receptor 3 signaling converts tumor-supporting myeloid cells to tumoricidal effectors

Hiroaki Shime[a], Misako Matsumoto[a], Hiroyuki Oshiumi[a], Shinya Tanaka[b], Akio Nakane[c], Yoichiro Iwakura[d], Hideaki Tahara[e], Norimitsu Inoue[f], and Tsukasa Seya[a,1]

[a]Department of Microbiology and Immunology, and [b]Department of Cancer Pathology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638, Japan; [c]Department of Microbiology and Immunology, Graduate School of Medicine, Hirosaki University, Zaifu-cho, Hirosaki 036-8562, Japan; [d]Laboratory of Molecular Pathogenesis, Center for Experimental Medicine and Systems Biology, and [e]Department of Surgery and Bioengineering, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; and [f]Department of Molecular Genetics, Osaka Medical Center for Cancer, Nakamichi, Higashinari-ku, Osaka 537-8511, Japan

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Smoldering inflammation often increases the risk of progression for malignant tumors and simultaneously matures myeloid dendritic cells (mDCs) for cell-mediated immunity. PolyI:C, a dsRNA analog, is reported to induce inflammation and potent antitumor immune responses via the Toll-like receptor 3/Toll–IL-1 receptor domain-containing adaptor molecule 1 (TICAM-1) and melanoma differentiation-associated protein 5/IFN-β promoter stimulator 1 (IPS-1) pathways in ~~mDC~~ to drive activation of natural killer cells and cytotoxic T lymphocytes. Here, we found that i.p. or s.c. injection of polyI:C to Lewis lung carcinoma tumor-implant mice resulted in tumor regression by converting tumor-supporting macrophages (Mfs) to tumor suppressors. F4/80$^+$/Gr1$^-$ Mfs infiltrating the tumor respond to polyI:C to rapidly produce inflammatory cytokines and thereafter accelerate M1 polarization. TNF-α was increased within 1 h in both tumor and serum upon polyI:C injection into tumor-bearing mice, followed by tumor hemorrhagic necrosis and growth suppression. These tumor responses were abolished in TNF-α$^{-/-}$ mice. Furthermore, F4/80$^+$ Mfs in tumors extracted from polyI:C-injected mice sustained Lewis lung carcinoma cytotoxic activity, and this activity was partly abrogated by anti–TNF-α Ab. Genes for supporting M1 polarization were subsequently up-regulated in the tumor-infiltrating Mfs. These responses were completely abrogated in TICAM-1$^{-/-}$ mice, and unaffected in myeloid differentiation factor 88$^{-/-}$ and IPS-1$^{-/-}$ mice. Thus, the TICAM-1 pathway is not only important to mature mDCs for cross-priming and natural killer cell activation in the induction of tumor immunity, but also critically engaged in tumor suppression by converting tumor-supporting Mfs to those with tumoricidal properties.

Toll-like receptor | tumor-associated macrophages | TRIF

Inflammation followed by bacterial and viral infections triggers a high risk of cancer and promotes tumor development and progression (1, 2). Long-term use of anti-inflammatory drugs has been shown to reduce—if not eliminate—the risk of cancer, as demonstrated by a clinical study of aspirin and colorectal cancer occurrence (3). Inflammatory cytokines facilitate tumor progression and metastasis in most cases. Innate immune response and the following cellular events are closely concerned with the formation of the tumor microenvironment (4, 5).

By contrast, inflammation induced by microbial preparations was applied to patients with cancer for therapeutic potential as Coley vaccine with some success. A viral replication product, dsRNA and its analog polyI:C (6, 7), induced acute inflammation, and has been expected to be a promising therapeutic agent against cancer. Although polyI:C exerts life-threatening cytokinemia (8), trials for its clinical use as an adjuvant continued because of its high therapeutic potential (9, 10). Pathogen-associated molecular patterns (PAMPs) and host cell factors induced secondary to PAMP–host cell interaction act as a double-edged sword in cancer prognosis and require understanding their multifarious functional properties in the tumor environment.

Recent advances in the study of innate immunity show how polyI:C suppresses tumor progression (11). PolyI:C is a synthetic compound that serves as an agonist for pattern-recognition receptors (PRRs), Toll-like receptor 3 (TLR3), and melanoma differentiation-associated protein 5 (MDA5) (12–14). Although TLR3 and MDA5 signals are characterized as myeloid differentiation factor 88 (MyD88) independent (15, 16), they have immune effector-inducing properties (12–14, 17). TLR3 couples with the Toll–IL-1 receptor domain-containing adaptor molecule 1 (TICAM-1, also known as TRIF), and MDA5 couples with the IFN-β promoter stimulator 1 (IPS-1, also known as Cardif, MAVS, or VISA) (11, 15). Possible functions for the TICAM-1 and IPS-1 signaling pathways have been investigated by using gene-disrupted mice (15); although they activate the same downstream transcription factors NF-κB and IFN regulatory factor 3 (IRF-3) (15, 18), they appear to distinctly modulate myeloid dendritic cells (mDCs) and macrophages (Mfs) to drive effector lymphocytes (19, 20).

Tumor microenvironments frequently involve myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and immature mDCs (1, 21). These myeloid cells express PRR through which they are functionally activated. Once the inflammation process is triggered, immature mDCs turn mature so that they are capable of antigen cross-presentation and able to activate immune effector cells, which would act to protect the host system and damage the undesirable tumor cells (22). However, ~~TAM~~ and ~~MDSC~~ play a major role in establishing a favorable environment for tumor cell development by suppressing antitumor immunity and recruiting host immune cells to support tumor cell survival, motility, and invasion (23–25). Although these myeloid cell scenarios have been studied with interest, how the PRR signal in these myeloid cells links regulation of tumor progression has yet to be elucidated.

Here we show that TICAM-1 but not IPS-1 signal in tumor-infiltrating Mfs is engaged in conversion of the TAM-like Mfs to tumoricidal effectors. We investigated the molecular mechanisms in Mfs underlying the phenotype switch from tumor supporting to tumor suppressing by treating cells with polyI:C and found that the TICAM-1–inducing TNF-α and M1 polarization are crucial for eliciting tumoricidal activity in ~~TAM~~.

Results

In Vivo Effect of PolyI:C on Implant Lewis Lung Carcinoma Tumor. I.p. injection of polyI:C rapidly induced hemorrhagic necrosis in 3LL tumors implanted in WT mice, which was established >12 h after polyI:C treatment (Fig. 1*A*). The polyI:C-dependent hemorrhagic necrosis did not occur in TNF-α$^{-/-}$ mice (Fig. 1*A*). Histological

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[1]To whom correspondence should be addressed. E-mail: seya-tu@pop.med.hokudai.ac.jp.

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and immunohistochemical analysis revealed vascular damage in the necrotic lesion, where disruption of vascular endothelial cells was indicated by fragmented $CD31^+$ marker (Fig. S1). Although the polyI:C signal is delivered by TICAM-1 and IPS-1 adaptors (11, 13), the hemorrhagic necrosis was largely alleviated in TICAM-$1^{-/-}$ mice but not in IPS-$1^{-/-}$ mice (Fig. 1*A*). The results suggest that polyI:C is a reagent that induces Lewis lung carcinoma (3LL) hemorrhagic necrosis, and the TICAM-1 pathway and its products, including TNF-α, are preferentially involved in this response.

3LL implant tumors grew well in WT C57BL6 mice. PolyI:C, when i.p. injected, resulted in tumor growth retardation (Fig. 1*B*). The retardation of tumor growth by polyI:C was also impaired in TNF-$\alpha^{-/-}$ mice (Fig. 1*B*), suggesting that TNF-α is a critical effector for not only induction of hemorrhagic necrosis but also further 3LL tumor regression. To investigate the signaling pathway involved in the tumor growth retardation by polyI:C, we challenged WT, MyD88$^{-/-}$, TICAM-$1^{-/-}$, and IPS-$1^{-/-}$ mice with 3LL implantation and then treated the mice with i.p. injection of polyI:C. 3LL growth retardation was observed in both IPS-$1^{-/-}$ (Fig. 1*C*) and MyD88$^{-/-}$ mice, to a similar extent to WT mice. In contrast, polyI:C-dependent tumor growth retardation was abrogated in TICAM-$1^{-/-}$ mice (Fig. 1*D*). The size differences of the implanted tumors became significant within 2 d after polyI:C treatment, suggesting that the molecular effector for tumor regression is induced early and its upstream is TICAM-1. Similar results were obtained with MC38 implant tumor (Fig. S2*A*), which is TNF-α sensitive and MHC class I positive (Table S1) (26).

PolyI:C is a reagent that induces natural killer (NK) cell activation in MHC class I-negative tumors (12), and 3LL cells are class I negative and NK cell sensitive (Table S1) (27, 28). We tested whether NK cells activated by polyI:C damage the 3LL tumor in mice. Tumor growth was not affected by pretreatment of the mice with anti-NK1.1 Ab in this model (Fig. S3). Thus, NK cells, at least the $NK1.1^+$ cells, have a negligible ability to retard tumor growth in vivo.

PolyI:C Induces TNF-α Through the TICAM-1 Pathway in Mice. To test whether polyI:C treatment had elicited TNF-α production in vivo, we investigated the cytokine profiles of serum from polyI:C-stimulated WT and IPS-$1^{-/-}$ and TICAM-$1^{-/-}$ mice by ELISA. Prominent differences in TNF-α levels were observed in serum collected from polyI:C-injected WT and TICAM-$1^{-/-}$ mice. Serum TNF-α levels in WT and IPS-$1^{-/-}$ mice were significantly higher than that in TICAM-$1^{-/-}$ mice within 1 h after polyI:C injection (Fig. S4 *A* and *B*). IFN-β is a main output for polyI:C stimulation (11), and its production was decreased in TICAM-$1^{-/-}$ mice and totally abrogated in IPS-$1^{-/-}$ mice (Fig. S4*C*). Taken together, the data indicate that the TICAM-1 pathway was able to sustain a high TNF-α level in the early phase of polyI:C treatment, which is independent of IPS-1 and subsequent production of IFN-β.

TICAM-1^+ Cells in Tumor Produces TNF-α in Response to PolyI:C Stimulation. Using the 3LL implant WT, IPS-$1^{-/-}$, and TICAM-$1^{-/-}$ mouse models, we tested whether polyI:C-induced early TNF-α was responsible for the lately observed tumor regression. Time-course analyses of the polyI:C-induced TNF-α protein levels were performed by ELISA using serum samples and tumors extracted from the experimental mice. The tumor TNF-α levels in WT and IPS-$1^{-/-}$ mice increased at 2 h after polyI:C i.p. injection (Fig. 2*A*). The serum TNF-α levels in both were rapidly up-regulated within 1 h after polyI:C injection, although in WT the levels continued to increase but in IPS-$1^{-/-}$ mice gradually decreased (Fig. 2*B*). In TICAM-$1^{-/-}$ mice, however, no appreciable up-regulation of TNF-α protein was detected in either tumor or serum samples during the early time-course tested. To test whether the induced TNF-α protein was generated de novo in tumors, we examined the corresponding mRNA levels in excised tumors (Fig. 2*C* and Table S2). The TNF-α mRNA levels peaked between 1 and 2 h after polyI:C injection, whereas the TNF-α protein level was kept high at >2 h after polyI:C injection in tumor as well as serum. In the TICAM-$1^{-/-}$ mice, TNF-α production was largely abrogated in the tumor and serum samples, suggesting that TNF-α was mainly produced and secreted in response to polyI:C stimulation from the TLR3/TICAM-1^+ cells within the tumor.

$F4/80^+/Gr\text{-}1^-$ Mfs in 3LL Tumor Produces TNF-α Leading to Tumor Damage. We next investigated the cell types that had infiltrated the tumor by using various Mf markers in FACS analysis and tumor samples extracted at 1 h after polyI:C injection. We discovered that $CD45^+$ cells in the tumor produced TNF-α in response to polyI:C (Fig. 3*A*). The major population of those $CD45^+$ cells was determined to be of $CD11b^+$ myeloid-lineage cells that coexpressed $F4/80^+$, $Gr1^+$, or $CD11c^+$. A small population of $NK1.1^+$ cells was also detected. $CD4^+$ T cells, $CD8^+$ T cells, and B cells were rarely detected in these implant tumors (Fig. S5*A*). Moreover, $F4/80^+/Gr\text{-}1^-$ cells were found to be the principal contributors to polyI:C-mediated TNF-α production (Fig. 3 *B* and *C*). $F4/80^+$ cells in 3LL tumor highly expressed macrophage mannose receptor (MMR; CD206), a M2 macrophage marker, in contrast to splenic $F4/80^+CD11b^+$ cells. Both TNF-α–producing and –nonproducing $F4/80^+$ cell populations in 3LL tumor showed indistinguishable levels of CD206 (Fig. S6), and dissimilar to ~~MDSC~~ or splenic ~~Mf~~, as determined by the surface marker profiles (Table S3). Thus, the source of the TNF-α–producing cells in tumor is likely $F4/80^+$ Mfs with a TAM-like feature.

We harvested $F4/80^+$ cells from tumor samples extracted from WT and TICAM-$1^{-/-}$ mice at 30 min after polyI:C injection. These cells were used in in vitro experiments to verify the TNF-α–producing abilities and 3LL cytotoxicity properties (Fig. 4 *A* and *B*). WT $F4/80^+$ Mfs exhibited normal TNF-α–producing function and were able to kill 3LL cells upon exposure. This tumoricidal activity was ~50% neutralized by the addition of anti–TNF-α Ab (Fig. 4*C*), although incomplete inhibition by this mAb may reflect participation of other factors in TNF-α cytotoxicity. Furthermore, when active TNF-α protein (rTNF-α) was added exogenously to 3LL cell culture, the cytotoxic effects were still present and occurred in a dose-dependent manner (Fig. 4*D*). TNF-α–producing ability was also observed in $F4/80^+$ cells from implant tumor of MC38, B16D8, or EL4, and only the MC38 tumor was remediable by TICAM-1–derived TNF-α (Fig. S2 *B* and *C*). The MC38 tumor contained the $F4/80^+/CD11b^+/Gr1^-$ cells, as in the 3LL tumor (Fig. S5*B*).

IFN-β did not enhance rTNF-α–mediated 3LL killing efficacy (Fig. S7*A*), a finding that was consistent with previously published data (29). No effect of IRF3/7 on polyI:C-induced 3LL tumor regression in vivo was confirmed using IRF3/7 double-knockout mice. However, polyI:C-dependent tumor regression was abrogated in 3LL-bearing IFN-α/β receptor $(IFNAR)^{-/-}$ mice (Fig. S7*B*). Quantitative PCR analysis of cells from WT vs. IFNAR$^{-/-}$ tumor-bearing mice revealed that the TLR3 level was basally low and not up-regulated in response to polyI:C in tumor-infiltrating $F4/80^+$ Mfs of IFNAR$^{-/-}$ mice (Fig. S7*C*). Accordingly, the TNF-α level was not up-regulated in tumor and serum in polyI:C-stimulated IFNAR$^{-/-}$ mice (Fig. S7*D*). Thus, basal induction of type I IFN serves as a critical factor for TLR3 function in tumor $F4/80^+$ Mfs to produce TNF-α in vivo. These results suggest that the direct effector for 3LL cytolysis by polyI:C involves TNF-α, which is derived from TICAM-1 downstream independent of the IRF3/7 axis. Our results indicate that cytotoxic TNF-α is produced via a distinct route from initial type I IFN and downstream of TICAM-1 in $F4/80^+$ TAM-like ~~Mf~~. Type I IFN do not synergistically act with TNF-α on 3LL killing, but is required to complete the TLR3/TICAM-1 pathway.

These results were confirmed by in vitro assay, wherein the $F4/80^+$ Mfs harvested from 3LL tumors in WT, TICAM-$1^{-/-}$, IPS-$1^{-/-}$, and TLR3$^{-/-}$ mice were stimulated with polyI:C (Fig. S8*A*). Both TNF-α release and 3LL cytotoxic abilities of polyI:C-stimulated $F4/80^+$ Mfs were specifically abrogated by the absence of TICAM-1 and TLR3 (Fig. S8 *A* and *B*). IPS-1 or

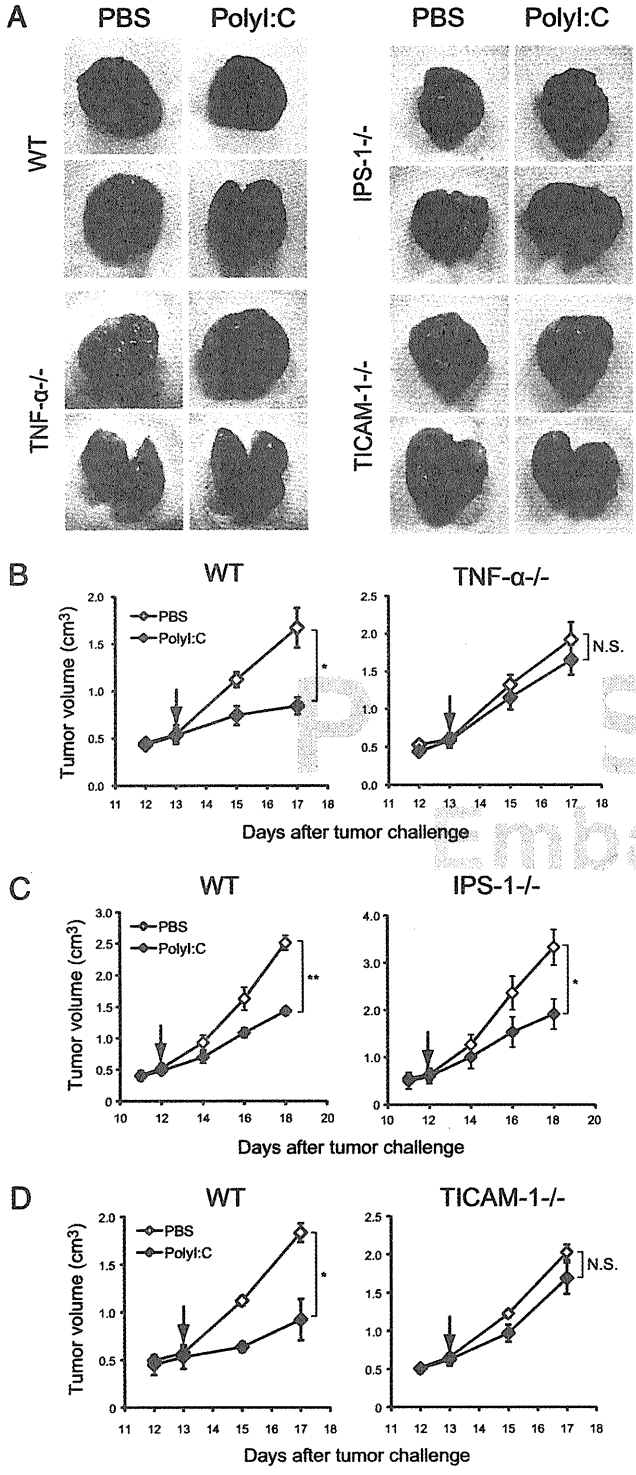

Fig. 1. Antitumor activity of polyI:C against 3LL tumor cells is mediated by the TICAM-1 pathway in vivo. (*A*) Representative photographs of 3LL tumors excised from WT, TNF-$\alpha^{-/-}$, TICAM-$1^{-/-}$, and IPS-$1^{-/-}$ mice. Whole tumor (*Upper*) and bisected tumor (*Lower*) are shown. (*B–D*) On day 0, 3LL tumor cells (3×10^6) were s.c. implanted into B6 WT (*B–D*), TNF-$\alpha^{-/-}$ (*B*), TICAM-$1^{-/-}$ (*C*), and IPS-$1^{-/-}$ (*D*) mice. PolyI:C i.p. injection was started on the day indicated by arrow, then repeated every 4 d. Data are shown as tumor average size ± SE; $n = 3$–4 mice per group. $^*P < 0.05$; $^{**}P < 0.001$. N.S., not significant. A representative experiment of two with similar outcomes is shown.

MyD88 in F4/80$^+$ Mfs had no or minimal effect on the TNF-α tumoricidal effect against 3LL tumors. PolyI:C did not directly exert a cytotoxic effect on 3LL tumor cells (Fig. S8*C*).

Role of the IPS-1 Pathway in F4/80$^+$ Cells. Both TICAM-1 and IPS-1 are known to converge their signals on transcription factors NF-κB and IRF-3, which drive expression of TNF-α and IFN-β, respectively. PolyI:C-induced TNF-α production was reduced in F4/80$^+$ cells extracted from tumors of TICAM-$1^{-/-}$ mice, but not in samples of IPS-$1^{-/-}$ mice. We examined the expression of IFN-β in these cells after polyI:C stimulation. Compared with F4/80$^+$ cells from WT mice, IFN-β expression and production was barely decreased in IPS-$1^{-/-}$ F4/80$^+$ cells, but largely impaired in TICAM-$1^{-/-}$ F4/80$^+$ cells (Fig. S9*A*) as other cytokines tested. M1 Mf-associated cytokines/chemokines were generally reduced in TICAM-$1^{-/-}$ F4/80$^+$ cells compared with WT and IPS-$1^{-/-}$ cells >4 h after polyI:C stimulation (Fig. S9*A*), whereas M2 Mf-associated genes were barely affected by TICAM-1 disruption or polyI:C stimulation (Fig. S9*B*).

Most types of Mfs are known to express TLR3 in mice (30). Messages and proteins for type I IFN induction were conserved in the F4/80$^+$ tumor-infiltrating Mfs (Fig. S10 *A–C*). However, the TLR3 mRNA level was low in macrophage colony-stimulating factor (M-CSF)–derived Mfs compared with ~~TAM~~ (Fig. S10*D*). We further examined whether IFN-β production might also have relied on the TICAM-1 pathway in other types of Mfs upon stimulation with polyI:C. In contrast to the F4/80$^+$ cells isolated from tumor (Fig. S11 *A* and *B*), the IPS-1 pathway was indispensable for polyI:C-mediated IFN-β production in mouse peritoneal Mfs and M-CSF–induced bone marrow-derived Mfs (Fig. S11 *C* and *E*). However, IPS-1 only slightly participated in polyI:C-mediated TNF-α production in these Mf subsets (Fig. S11 *D* and *F*). It appears then that the IPS-1 pathway is able to signal the presence of polyI:C and subsequently induce type I IFN. TICAM-1 is the protein that induces effective TNF-α in all subsets of Mfs.

PolyI:C Influences Polarization of TAMs. Plasticity is a characteristic feature of Mfs (25). Various factors and signals can influence polarization of Mf cells to induce the M1/M2 transition, which is accompanied by a substantial change in the Mf cell's expression profile of cytokines and chemokines. Previous studies have demonstrated that Mfs that have infiltrated into tumor are of the M2-polarized phenotype, which is known to contribute to tumor progression. To test the effects of polyI:C on the polarization of tumor-infiltrated Mf cells, we analyzed the gene expression profiles of these cells following in vitro polyI:C stimulation, and representative profiles were confirmed by quantitative PCR (Fig. 5 *A* and *B*). The mRNA expressions were increased for M1 Mf markers IL-12p40, IL-6, CXCL11, and IL-1β at 4 h after in vitro polyI:C treatment, as were mRNA levels of IFN-β and TNF-α and ex vivo results. The M2 Mf markers arginase-1 (*Arg1*), chitinase 3-like 3 (*Chi3l3*), and MMR (*Mrc1*) were unchanged, compared with unstimulated levels; however, the M2 Mf marker IL-10, a regulatory cytokine, was induced. In addition, there was no difference observed in the mRNA expression levels of MMP9 (*Mmp9*) and VEGFA (*Vegfa*), both of which are involved in tissue remodeling and angiogenesis events of tumor progression (Fig. 5*C*). The polyI:C-induced M1 markers and IL-10 expression that were upregulated in WT and IPS-$1^{-/-}$ F4/80$^+$ cells were found to be abrogated in TICAM-$1^{-/-}$ F4/80$^+$ cells (Fig. 5 *A* and *B*), reinforcing the results obtained with F4/80$^+$ Mfs isolated from 3LL tumors in mice injected with polyI:C (Fig. S9 *A* and *B*). It appears that TICAM-1 is responsible for the M1 polarization of F4/80$^+$ Mf cells in tumors, but has no effect on M2 markers. We further examined the expression of IRF-5 and IRF-4, which are considered the master regulators for M1 and M2 polarization, respectively (31, 32). As expected, polyI:C induced IRF-5 mRNA expression, but had no effect on IRF-4 mRNA expression in vitro (Fig. 5 *A* and *B*). Jmjd3, a histone H3K27 demethylase involved in IRF-4 expression, is reportedly induced by TLR stimulation (33). In our study, polyI:C stimulation increased Jmjd3 mRNA in F4/80$^+$ cells

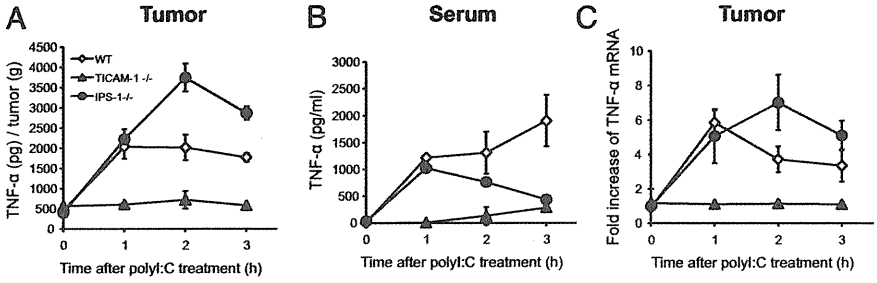

Fig. 2. TNF-α production in tumor and serum of polyI:C-injected 3LL tumor-bearing mice. Mice bearing 3LL tumor were i.p. injected with 200 μg polyI:C. Tumor (*A*) and serum (*B*) were collected at 0, 1, 2, and 3 h after polyI:C injection, and TNF-α concentration was determined by ELISA. TNF-α level in tumor is presented as [TNF-α protein (pg)/ tumor weight (g)]. (*C*) Tumors were isolated from polyI:C-injected tumor-bearing WT, TICAM-1$^{-/-}$, and IPS-1$^{-/-}$ mice, and TNF-α mRNA was measured by quantitative PCR; n = 3. Data are shown as average ± SD. A representative experiment of two with similar outcomes is shown.

(Fig. 5*B*). The polyI:C-triggered M1 gene expression continued long in tumor-infiltrated Mfs, a finding that may further explain the tumor-suppressing feature of these Mfs, in addition to the concern of early inducing TNF-α.

Discussion

In this study we demonstrated that the tumor-supporting properties of tumor-infiltrating F4/80$^+$ Mfs characterized by M2 markers are dynamic and able to shift to an M1-dominant state upon the particular signal provided by PRRs. In 3LL tumors that express minimal amounts of MHC class I/II and recruit a large amount of myeloid cells, F4/80$^+$ Mfs function to sustain the tumor in the surrounding microenvironment. This tumor-supporting environment can be disrupted by stimulation with an RNA duplex through a TICAM-1 signal and subsequent induction of mediators such as TNF-α. Thus, the TICAM-1 signal in tumor-infiltrating Mfs plays a key role in TNF-α and M1 shift-mediated tumor regression. These results were confirmed using another cell line, MC38 colon adenocarcinoma (34), although MC38 cells express MHC class I. B16D8 melanoma (12) and EL4 lymphoma (35) were resistant to TNF-α, but their F4/80$^+$ Mfs still possessed TNF-α–inducing potential by stimulation with polyI:C; their susceptibilities to polyI:C reportedly depend on other effectors (12, 35). These results may partly explain the reported findings that tumors regressed in patients with simultaneous virus infection (36, 37), and that tumor growth was inhibited by polyI:C injection in tumor-bearing mice (6, 7).

In contrast, polyI:C-stimulated PEC or bone marrow-derived Mfs induce type I IFN via the IPS-1 pathway unlike the case of tumor-infiltrating F4/80$^+$ Mfs. Nevertheless, all of these Mf subsets produce proinflammatory cytokines, including TNF-α, in a TICAM-1–dependent manner. Thus, the key question that arose was why predominant TICAM-1 dependence for polyI:C-mediated production of TNF-α occurred in F4/80$^+$ tumor-infiltrating Mfs leading to tumor regression. A marked finding is that the TLR3 protein level is high in tumor-infiltrating Mfs compared with other sources of Mfs (Fig. S10). In addition, the IPS-1 pathway is unresponsive to polyI:C if the polyI:C is exogenously added to the tumor-infiltrating Mfs without transfection reagents. The cytoplasmic dsRNA sensors normally work for IFN induction in tumor F4/80$^+$ Mfs if the polyI:C is transfected into the cells. TICAM-1–dependent TNF-α production by F4/80$^+$ Mfs (Fig. S11 *D* and *F*) occurs partly because F4/80$^+$ Mfs express a high basal level of TLR3 and fail to take up extrinsic polyI:C into the cytoplasm. Of many subsets of Mfs, these properties (38) are unique to the F4/80$^+$ Mf.

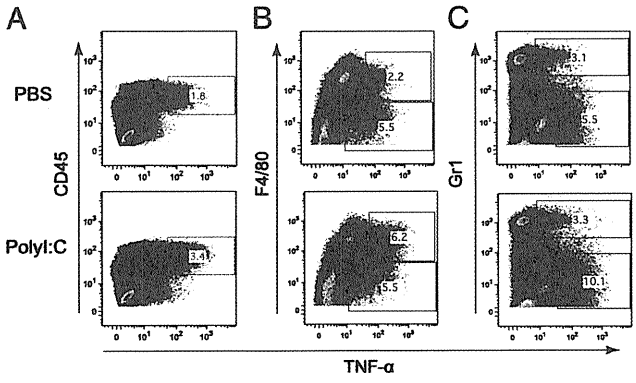

Fig. 3. F4/80$^+$ cells are responsible for the polyI:C-induced elevation of TNF-α production in tumor. Mice bearing 3LL tumors were i.p. injected with 200 μg polyI:C. TNF-α–producing cells in tumors of polyI:C- or PBS-injected mice were examined by immunohistochemical staining and flow cytometry to determine intracellular cytokine expression profiles of CD45$^+$ cells (*A*), F4/80$^+$ cells (*B*), and Gr1$^+$ cells (*C*). CD45$^+$ cells in tumor were gated and are shown in *B* and *C*. A representative experiment of two with similar outcomes is shown. TNF-α$^+$ gating squares are shown in red (positive) and green (negative).

Hemorrhagic necrosis and tumor size reduction are closely correlated with constitutive production of TNF-α (39, 40). The association of PRR-derived TNF-α and hemorrhagic necrosis of tumor has been described earlier. Carswell et al. (41) showed that TNF-α is robustly expressed in mouse serum following treatment with bacillus Calmette–Guérin and endotoxin. Bioassay of TNF-α as reflected by the degree of hemorrhagic necrosis of transplanted Meth A sarcoma in BALB/c mice led the authors to speculate that Mfs are responsible for TNF-α induction. Many years later, Dougherty et al. (42) identified the mechanism responsible for the TNF-α production associated with antitumor activity; macrophages isolated from tumors in mice with inactivating mutation in the TLR4 gene [Lps(d) in C3H/HeJ] expressed 5- to 10-fold less TNF-α than tumors in WT mice. This finding represents a unique recognition of a PRR contributing to the cancer phenotype. Subsequent studies determined that MyD88 is involved in the induction of TNF-α via TLR4 binding to its cognate ligand, lipid A endotoxin (15, 43). Because the TLR3 signal is independent of MyD88, this MyD88 concept is not applicable to the present study on polyI:C-dependent tumor regression.

Alternatively, endotoxin/lipid A may have activated TICAM-1 in previous reports on TLR4-derived TNF-α because TLR4 can recruit TICAM-1 in addition to MyD88 (15). The lipid A derivative monophospholipid A preferentially activates the TICAM-1 pathway of TLR4 (43). It is likely that TICAM-1 participates in TLR4-mediated tumor regression in addition to MyD88, although MyD88 is not involved in the polyI:C signaling. This point was further proven using TNF-α$^{-/-}$ mice: TICAM-1–derived TNF-α in F4/80$^+$ Mf cells has a critical role in the induction of tumor necrosis and regression by polyI:C. The results are consistent with the finding that both TICAM-1 and IPS-1 pathways are able to induce NF-κB activation secondary to polyI:C stimulation, and indeed their signals converge at the IκB kinase complex (18).

TICAM-1 is able to induce many of the IFN-inducible genes that MyD88 cannot in mDCs (44). In both cases of TICAM-1 and MyD88 stimulation, tumor-infiltrating Mfs facilitate the expression of many genes in addition to TNF-α. The M2 phenotype of F4/80$^+$ Mfs or tumor-associated Mfs is modified dependent on these additional factors. IFNAR facilitates polyI:C-mediated tumor regression in tumor-bearing mice, lack of which results in no induction of TLR3 (Fig. S7). Thus, preceding the polyI:C

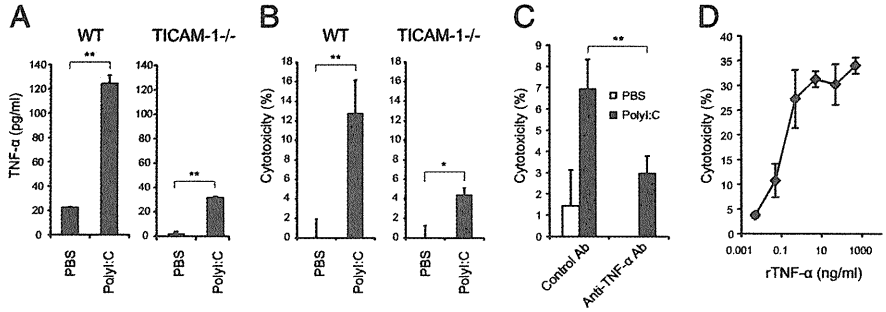

Fig. 4. Poly I:C enhances TNF-α production and cytotoxicity of F4/80$^+$ cells in tumor. Poly I:C (200 μg) or PBS was i.p. injected into 3LL tumor-bearing WT mice. After 30 min, F4/80$^+$ cells isolated from tumor were cultured for 24 h and TNF-α concentration in the conditioned medium was determined by ELISA (*A*). In parallel, the ~~cytotoxicity~~ of tumor-infiltrating F4/80$^+$ cells against 3LL tumor cells was measured by ^{51}Cr-release assay (*B*). Anti–TNF-α neutralization antibody or control antibody was added (10 μg/mL) to mixed culture of isolated tumor-infiltrating F4/80$^+$ cells and 3LL tumor cells (*C*). (*D*) Cytotoxic activity of TNF-α against 3LL tumor cells. Recombinant TNF-α was added to ^{51}Cr-labeled 3LL tumor cell culture at various concentrations. After 20 h, cytotoxicity was measured; $n = 3$. Data are shown as average ± SD. $*P < 0.05$, $**P < 0.001$. A representative experiment of three with similar outcomes is shown.

response, minute type I IFN of undefined source has to be provided to set the TLR3/TICAM-1 pathway, which may primarily fail in IFNAR$^{-/-}$ mice. Cellular effectors, cytotoxic T lymphocyte (CTL) and NK cells, are induced secondary to activation of IFN-inducible genes in a late phase of polyI:C-stimulated myeloid cells (45–47). The relationship among the TICAM-1–mediated type I IFN liberation, these late-phase effectors, and tumor regression remains an open question in this setting.

M1 Mf cells function to protect the host against tumors by producing large amounts of inflammatory cytokines and activating the immune response (48, 49). However, distinct types of M2 cells differentiate when monocytes are stimulated with IL-4 and IL-13 (M2a), immune complexes/TLR ligands (M2b), or IL-10 and glucocorticoids (M2c) (50). In our study, polyI:C stimulation led to incremental expression of the M1 Mf-related genes. In contrast, polyI:C stimulation was not associated with M2 polarization, except for IL-10. Other genes related to angiogenesis and extravasation were not affected by polyI:C treatment. Thus, polyI:C was able to induce the characteristic M1 conversion and, in turn, contribute to tumor regression. It is notable that TAM cells usually have defective and delayed NF-κB activation in response to different proinflammatory signals, such as expression of cytotoxic mediators NO, cytokines, TNF-α, and IL-12 (51–53). These observations are in apparent contrast with the function of other resident Mf species. This discrepancy may again reflect a dynamic change in the tumor microenvironment during tumor progression.

In line with our findings, virus infection has been observed to instigate tumor regression in patients with cancer (36, 54). Gene therapy for cancer patients using virus-derived vectors has proved effective in reducing tumors in clinic (36, 37). Administration of dsRNA elicits IFN induction, NK cell activation, and CTL proliferation for antitumor effectors in vivo (19, 55). This is a unique finding that tumor-infiltrating Mfs are a target of dsRNA and converted from tumor supporters to tumoricidal effectors. Hence, the antitumor effect of dsRNA adjuvant is ultimately based on the liberation of type I IFN, functional maturation of mDCs, and modulation of tumor-infiltrating Mfs, where TICAM-1 is a crucial transducer in eliciting antitumor immunity.

Methods

Inbred C57BL/6 WT mice were purchased from CLEA Japan, Inc. TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice were generated in our laboratory and maintained as described previously. IRF-3/7 double-KO mice were a gift from T. Taniguchi

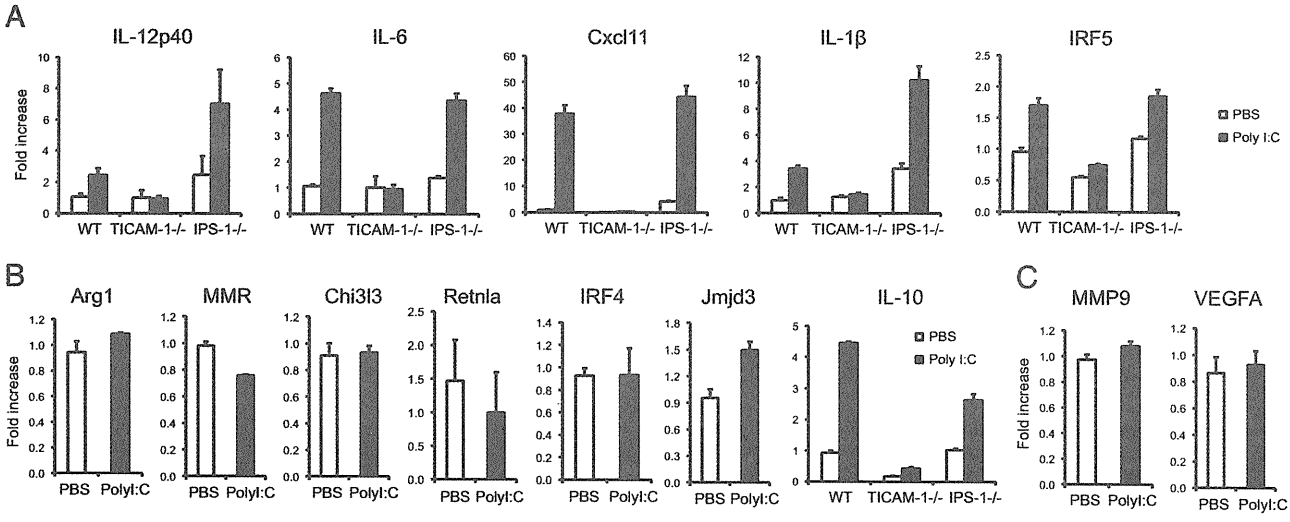

Fig. 5. Poly I:C induces M1 polarization of TAMs. F4/80$^+$ cells were isolated from 3LL tumor and stimulated with polyI:C (50 μg/mL) for 4 h. Total RNA was extracted and used to analyze the transcript expression levels of M1 (*A*) and M2 (*B* and *C*) markers; $n = 3$. Data are shown as average ± SD. A representative experiment of two with similar outcomes is shown.

(University of Tokyo, Tokyo, Japan). TNF-$\alpha^{-/-}$ mice were kindly provided by A. Nakane (Hirosaki University, Aomori, Japan) and Y. Iwakura (University of Tokyo). Mice 6–10 wk of age were used in all experiments. 3LL lung cancer cells were cultured at 37 °C under 5% CO_2 in RPMI containing 10% FCS, penicillin, and streptomycin. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments in the Animal Safety Center, Hokkaido University, Japan. All mice were used according to the guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, who approved this study as no. 08-0290, "Analysis of Anti-Tumor Immune Response Induced by the Activation of Innate Immunity."

Other detailed methods are provided in *SI Methods*.

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Supporting Information

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SI Methods

Reagents. PolyI:C was purchased from GE Healthcare, which was free from LPS contamination. TNF-α and IFN-β ELISA kit was purchased from eBioscience and PBL InterferonSource, respectively. Recombinant TNF-α was purchased from R&D Systems.

Tumor Cells and Tumor-Infiltrated Immune Cells. We first tested the amounts of macrophages (Mfs) in implant tumors formed in B6 mice. Mouse lymphoma (EL4), Lewis lung carcinoma (3LL), adenocarcinoma MC38, and melanoma (B16D8) lines grew well in the back of mice, and the Mf content was maximal in the 3LL tumor. MC38, a murine colon adenocarcinoma cell line, was a gift from S. A. Rosenberg (National Cancer Institute, Bethesda) (1). Hemorrhagic necrosis shown in Fig. 1*A* was typically induced in response to polyI:C in 3LL tumor. We then used the 3LL line for this study.

3LL cells were found to express very low amounts of detectable MHC class I or class II (Table S1), suggesting this cell type as a possible target for natural killer (NK) cells but not cytotoxic T lymphocytes (CTLs). 3LL cells were found to express appreciable amounts of the NKG2D ligand, retinoic acid-inducible gene 1, consistent with previous reports (Table S1) (2, 3). 3LL cells also expressed mRNA transcripts for Toll-like receptor 3 (TLR3), Toll–IL-1 receptor domain-containing adaptor molecule 1 (TICAM-1), IFN-β promoter stimulator 1 (IPS-1), and melanoma differentiation-associated protein 5 (MDA5). Exposure to polyI:C-stimulated peritoneal Mfs caused significant death of 3LL cells, which was likely an effect of liberated inflammatory cytokines such as TNF-α (4). Consistent with previously reported data about 3LL properties in vitro, the 3LL cells we used were not damaged by direct polyI:C treatment or exposure to 3LL-derived cytokines (Fig. S8*C*). When 3LL cells were implanted s.c. in mice, the resulting tumors were found to contain a high amount (>30%) of $CD45.2^+$ cells (Fig. S5*A*). The major population of those $CD45.2^+$ cells was determined to be of $CD11b^+$ myeloid lineage cells that coexpressed $F4/80^+$, $Gr1^+$, or $CD11c^+$. A small population of $NK1.1^+$ cells was also detected. $CD4^+$ T cells, $CD8^+$ T cells, and B cells were rarely detected in these implant tumors (Fig. S5*A*).

Cytotoxic Activity Assay. Mice bearing 3LL tumor were injected i.p. with polyI:C. Mice were killed and $F4/80^+$ cells were isolated from tumor by using MACS-positive selection beads (Miltenyi) as described previously. 3LL cells were labeled with ^{51}Cr for 3–5 h and then washed three times with the medium. $F4/80^+$ cells, and 3LL cells were cocultured at the indicated ratio. After 20 h, supernatants were harvested and ^{51}Cr release was measured in each sample. Specific lysis was calculated by the following formula: cytotoxicity (%) = [(experimental release − spontaneous release)/(max release − spontaneous release)] × 100.

Flow Cytometric Analysis. Mononuclear cells prepared from spleen and tumor were treated with anti-CD16/32 (no. 93) and stained with APC–anti-CD45.2 (no. 104), FITC–anti-CD11b (M1/70), PE–anti-GR1 (RB6-8C5), FITC–anti-CD11c (N418), PE– or APC–anti-F4/80 (BM8), PE–anti-NK1.1 (PK136), PE–anti-CD49b (DX5), PE–anti-CD3e (145-2C11), FITC–anti-CD4 (GK1.5), FITC–anti-CD8a (53-6.7), and PE- and anti-CD19 (MB19-1; eBioscience and Biolegend; Table S2). Samples were analyzed with FACSCalibur (BD Biosciences), and data analysis was performed using FlowJo software (Tree Star). For intracellular cytokine staining, we freshly isolated tumors from polyI:C or PBS-injected mice at 1 h and incubated the cells in the presence of 10 μg/mL Brefeldin A for 3 h. Cells were fixed and stained with the combination of anti-CD45.2 Ab and anti-F4/80 Ab or anti-Gr1 Ab, followed by permeabilizing and staining with anti-TNF Ab using BD Cytofix/Cytoperm Kit (BD Biosciences).

Quantitative PCR Analysis. Tumor samples were cut into small pieces and homogenized with TRIzol Reagent (Invitrogen). Total RNA was isolated according to the manufacturer's instruction. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) with a StepOne Real-Time PCR System (Applied Biosystems). Expression of the cytokine gene was normalized to the expression of *GAPDH*. We used primer pairs listed in Table S3. Data were analyzed by the ΔΔCt method.

ELISA and Cytokine Beads Assay. Tumor samples were cut into small pieces and homogenized with CelLytic MT Mammalian Tissue Lysis/Extraction Reagent (Sigma) supplemented with Complete Protease Inhibitor Mixture (Roche) on ice. Lysate was centrifuged to remove insoluble materials, and the supernatant was used for ELISA. Serum cytokine concentration was determined by ELISA or cytokine bead assays. Data were shown as TNF-α (pg) per weight of tumor (g).

Histochemistry and Immunohistochemistry. 3LL tumor was fixed with buffered 10% formalin overnight and embedded in paraffin wax, and sections 4 μm in thickness were stained with H&E. For immunohistochemistry, tumor was embedded in optimal cutting-temperature compound, and snap-frozen in liquid nitrogen. Cryosections 6 μm in thickness were air-dried for 60 min and fixed for 15 min with prechilled acetone and then incubated with FITC–anti-CD31 antibody (390; BioLegend). The sections were mounted in Prolong Gold Antifade Reagent with DAPI (Invitrogen). Images were obtained with a Leica LSM510 confocal laser-scanning microscope.

Tumor Challenge and PolyI:C Treatment. Mice were shaved at the back and injected s.c with 200 μL of 3×10^6 3LL cells in PBS(−). Tumor size was measured using a caliper. Tumor volume was calculated using the following formula: tumor volume (cm^3) = (long diameter) × (short diameter)^2 × 0.4. PolyI:C (250 μg/head) with no detectable LPS was injected i.p. as indicated. In some cases, polymixin B-treated polyI:C was used. When an average tumor volume of 0.5–0.8 cm^3 was reached, the treatment was started and repeated every 4 d.

Isolation of $F4/80^+$ Cells from Tumor. Tumors formed by 3LL cells were excised at 2 wk after transplantation and treated with 0.05 mg/mL Collagenase I (Sigma), 0.05 mg/mL Collagenase IV (Sigma), 0.025 mg/mL hyaluronidase (Sigma), and 0.01 mg/mL DNase I (Roche) in HBSS at 37 °C for 10 min. $F4/80^+$ cells were isolated by using biotinylated anti-F4/80 antibody (BM8) and Streptavidin MicroBeads (Miltenyi). We routinely prepared $F4/80^+$ cells at >90% purity from tumor.

1. Zitvogel L, et al. (1995) Cancer immunotherapy of established tumors with IL-12. Effective delivery by genetically engineered fibroblasts. *J Immunol* 155:1393–1403.
2. Masuda H, et al. (2002) High levels of RAE-1 isoforms on mouse tumor cell lines assessed by the anti-pan-Rae-1 polyclonal antibody confers tumor cell cytotoxicity on mouse NK cells. *Biochem Biophys Res Commun* 290:140–145.
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4. Remels L, Fransen L, Huygen K, De Baetselier P (1990) Poly I:C activated macrophages are tumoricidal for TNF-α-resistant 3LL tumor cells. *J Immunol* 144:4477–4486.

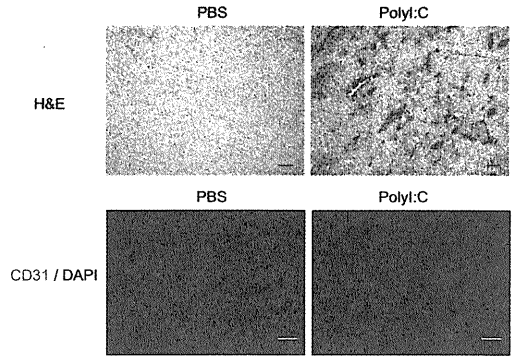

Fig. S1. Poly I:C induces hemorrhagic necrosis of tumor. 3LL tumor-bearing mice were i.p. injected with 200 μg poly I:C and tumors were isolated 12 h later. Formalin-fixed tumors stained with H&E (*Upper*) and frozen sections stained with anti-CD31 antibody and DAPI nuclear stain (*Lower*). Original magnification 10× for all panels. (Scale bars, 100 μm.) A representative experiment of three with similar outcomes is shown.

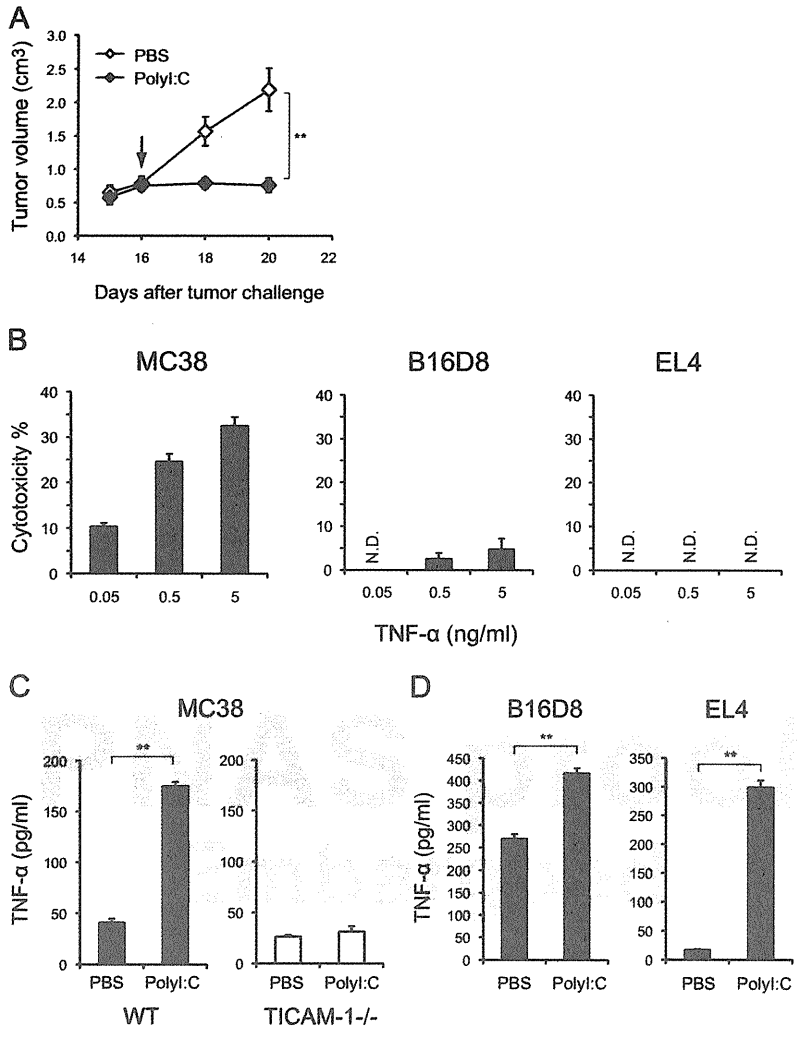

Fig. S2. Poly I:C induces TNF-α production by tumor-associated F4/80$^+$ Mfs in various types of tumor. (*A*) MC38 cells (1×10^5) were s.c implanted into C57BL/6 mice (day 0). Poly I:C (200 μg) was i.p. injected on day 16. Data are shown as tumor average size ± SE; $n = 3$–4 mice per group. (*B*) Sensitivity of MC38, B16D8, and EL4 cells to recombinant TNF-α. (*C* and *D*) MC38, B16D8, and EL4 tumor-bearing mice were i.p injected with 200 μg poly I:C. After 1 h, F4/80$^+$ cells were isolated from tumors and incubated for 24 h. TNF-α concentration in the conditioned medium was determined by ELISA; $n = 3$. Data are shown as average ± SD. N.D., not detected. A representative experiment of two with similar outcomes is shown.

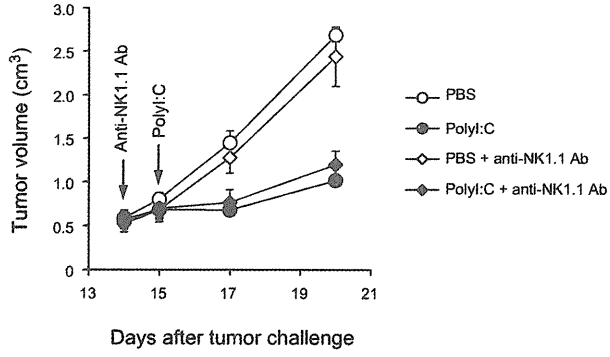

Fig. S3. NK cells are not essential for poly I:C-induced antitumor activity in vivo. 3LL tumor cells (3×10^6) were s.c transplanted into C57BL/6 mice (day 0). NK cells were depleted by injection of anti-NK1.1 antibody (PK136) into 3LL tumor-bearing mice on day 14. All doses of antibody and treatment regimens were determined in preliminary studies using the same lot of antibodies used for the experiments. Treatment was confirmed to deplete completely the desired cell populations for the entire duration of the study. Poly I:C (250 μg) was i.p injected on day 15 and the tumor volume was measured. Data shown are means ± SE, $n = 3$. A representative experiment of two with similar outcomes is shown.

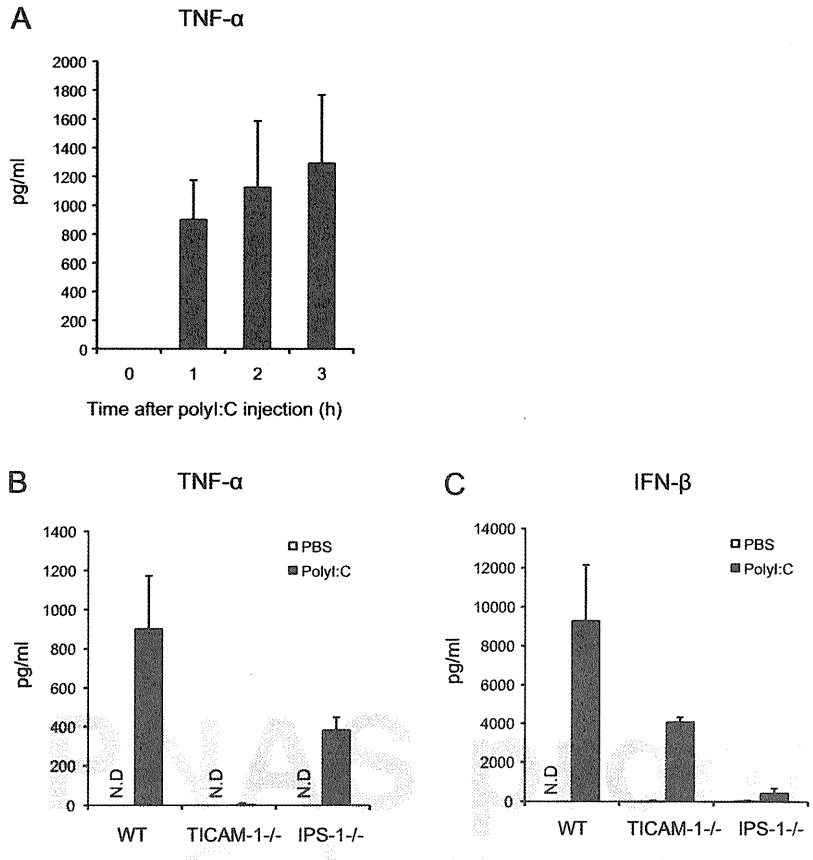

Fig. S4. Cytokine production in polyI:C-treated mouse. (*A*) WT mice were injected i.p with 200 μg polyI:C. After 0, 1, 2, and 3 h, TNF-α concentration in serum was determined by ELISA. (*B* and *C*) WT, TICAM-1$^{-/-}$, and IPS-1$^{-/-}$ mice were injected i.p with 200 μg polyI:C. After 1 h for TNF-α (*B*) and 4 h for IFN-β (*C*), serum cytokine levels were determined by ELISA. Data represents mean ± SD (n = 3). N.D., not detected. A representative experiment of three with similar outcomes is shown.

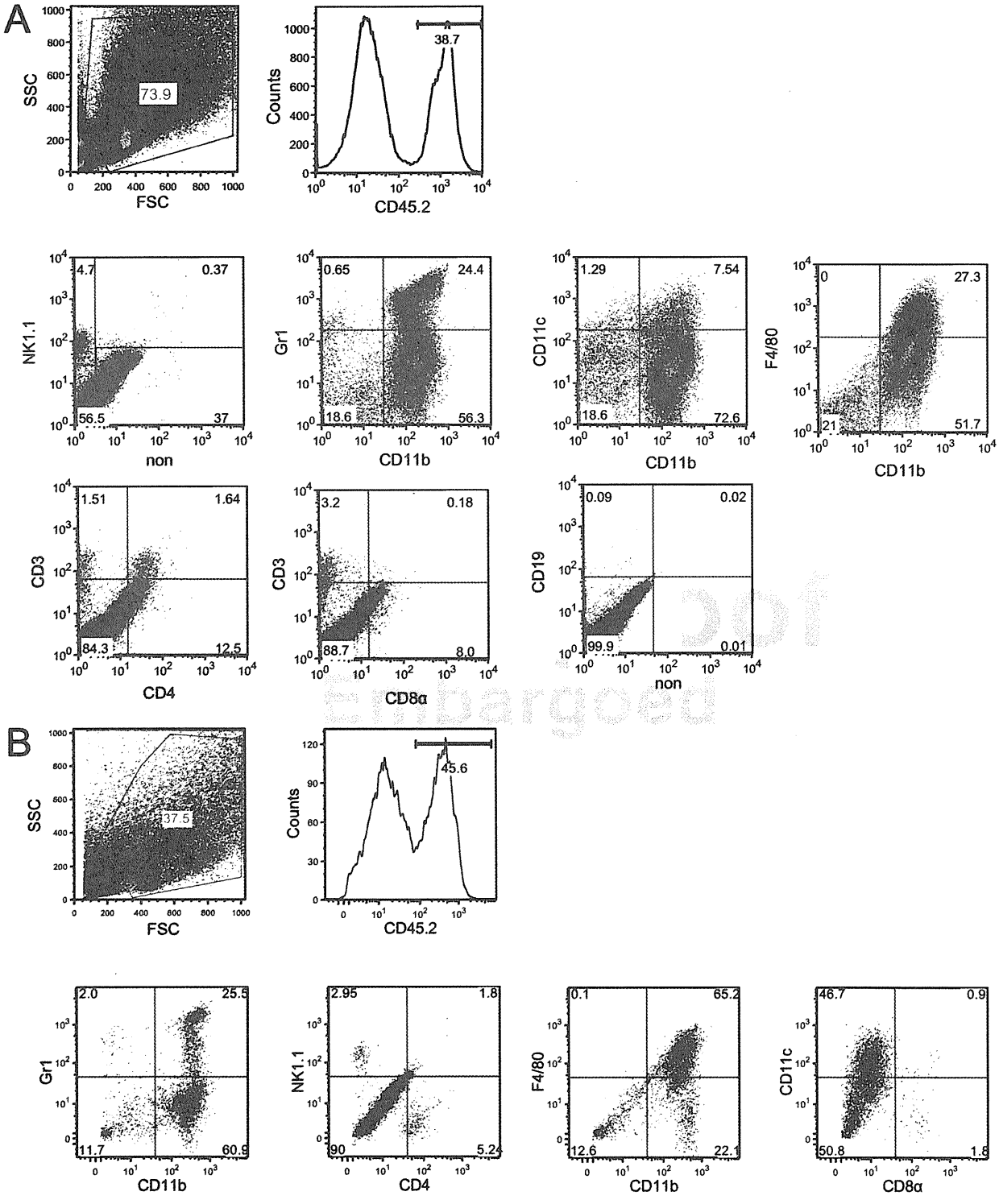

Fig. S5. Analysis of immune cells infiltrated into tumor. 3LL tumor cells (3×10^6) (*A*) or MC38 (1×10^6) (*B*) were transplanted s.c into B6 WT mice. After 2 wk, flow cytometric analysis was performed using freshly isolated whole tumor cell preparations in combination with staining of surface markers. $CD45.2^+$ cells were gated, and the expression of indicated surface markers was further analyzed. Numbers represent percentage of the gated and positive cells. A representative experiment of two with similar outcomes is shown. FSC, forward scatter; SSC, side scatter.

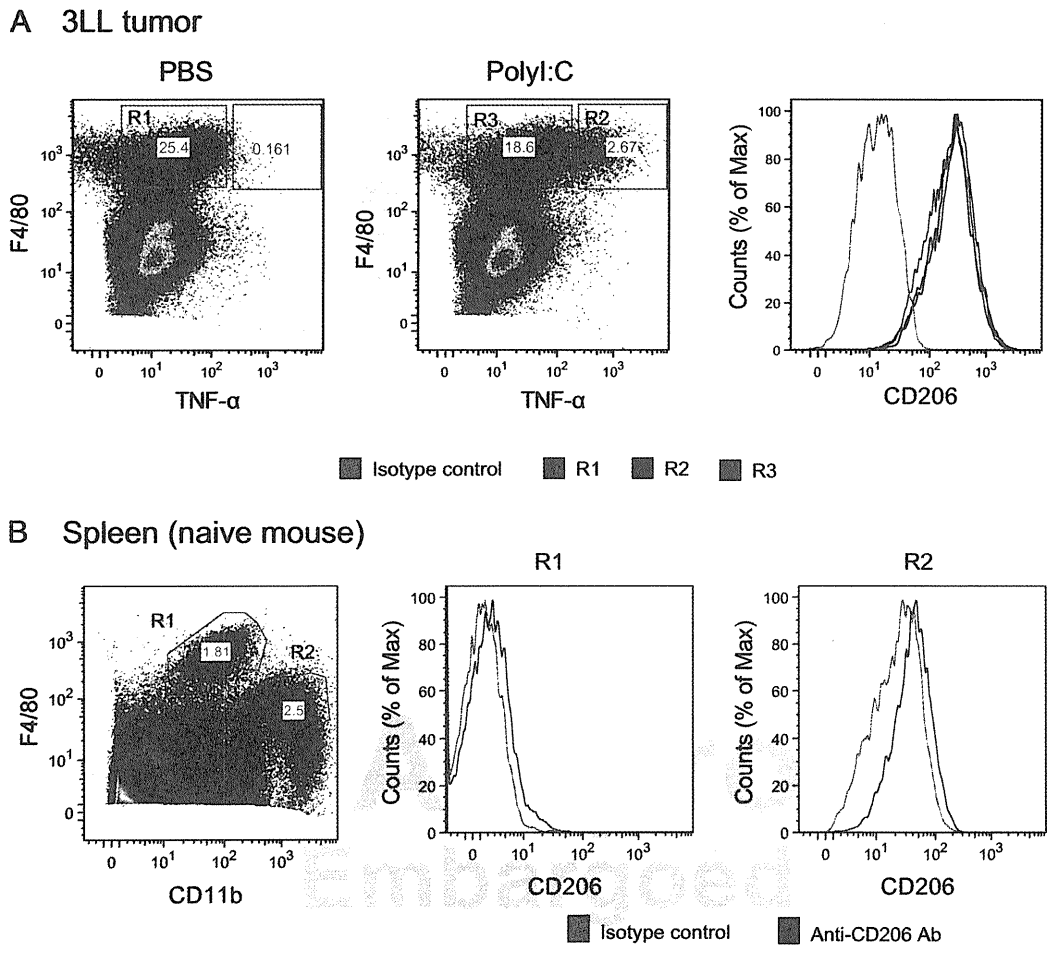

Fig. S6. Both TNF-α–producing and –nonproducing F4/80$^+$ macrophages in 3LL tumor of polyI:C-injected mouse express CD206 (macrophage mannose receptor). (*A*) 3LL tumor-bearing mice were injected i.p with 200 μg polyI:C. After 1 h, single-cell suspension of tumor was incubated in the presence of 10 μg/mL Brefeldin A for 3 h. Intracellular cytokine staining for TNF-α in CD45.2$^+$F4/80$^+$ cells was performed. R2, and R1 and R3 indicates TNF-α–producing and –nonproducing F4/80$^+$ cells, respectively. (*B*) CD206 expression in splenic F4/80$^+$CD11b$^+$ cells (R1 and R2) of naive mouse.

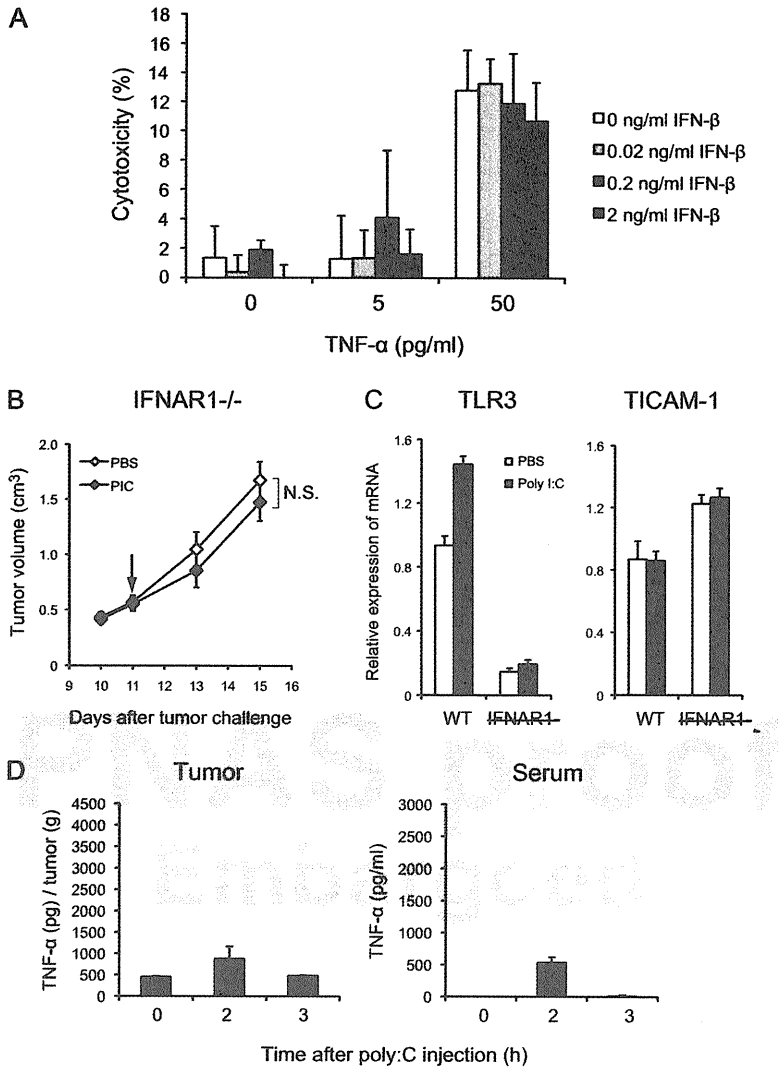

Fig. S7. Involvement of type I IFN signaling in 3LL tumor regression induced by polyI:C. (*A*) Effect of IFN-β on cytotoxic activity of TNF-α against 3LL tumor cells. 3LL cells were incubated in the presence of 0, 5, and 50 pg/mL recombinant mouse TNF-α in combination with 0, 0.02, 0.2, and 2 ng/mL recombinant mouse IFN-β. Cytotoxicity was determined by ^{51}Cr release assay. (*B*) Disabling polyI:C for 3LL tumor regression in IFN-α/β receptor $(IFNAR1)^{-/-}$ mice. PolyI:C was i.p injected on day 11; $n = 3$–4 mice per group. Data are shown as average ± SE. N.S., not significant. (*C*) Levels of the mRNA of TLR3 and TICAM-1 in 3LL tumor-associated $F4/80^{+}$ cells of WT or $IFNAR1^{-/-}$ mice. (*D*) TNF-α levels in tumor and serum in polyI:C-stimulated $IFNAR^{-/-}$ mice. Mice bearing 3LL tumors were i.p. injected with 200 μg polyI:C. Tumor (*Left*) and serum (*Right*) were collected at 0, 2, and 3 h after polyI:C injection, and TNF-α concentration was determined by ELISA. TNF-α level in tumor is presented as [TNF-α protein (pg)/tumor weight (g)].