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

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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 mDCs 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- κ B 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, TAMs and MDSCs 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 TAMs.

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. 1A). The polyI:C-dependent hemorrhagic necrosis did not occur in TNF- α ^{-/-} mice (Fig. 1A). Histological

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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. 1A). 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. 1B). The retardation of tumor growth by polyI:C was also impaired in TNF- α ^{-/-} mice (Fig. 1B), 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. 1C) 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. 1D). 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. S2A), 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. S4A 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. S4C). 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. 2A). 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. 2B). 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. 2C 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-1⁻ Mfs in 3LL Tumor Produce 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. 3A). 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. S5A). Moreover, F4/80⁺/Gr-1⁻ cells were found to be the principal contributors to polyI:C-mediated TNF- α production (Fig. 3B 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 MDSCs or splenic Mfs, 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. 4A 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. 4C), 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. 4D). 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. S2B and C). The MC38 tumor contained the F4/80⁺/CD11b⁺/Gr1⁻ cells, as in the 3LL tumor (Fig. S5B).

IFN- β did not enhance rTNF- α -mediated 3LL killing efficacy (Fig. S7A), 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. S7B). 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. S7C). Accordingly, the TNF- α level was not up-regulated in tumor and serum in polyI:C-stimulated IFNAR^{-/-} mice (Fig. S7D). 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 cytotoxicity 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 Mfs. 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. S8A). 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. S8A and B). IPS-1 or

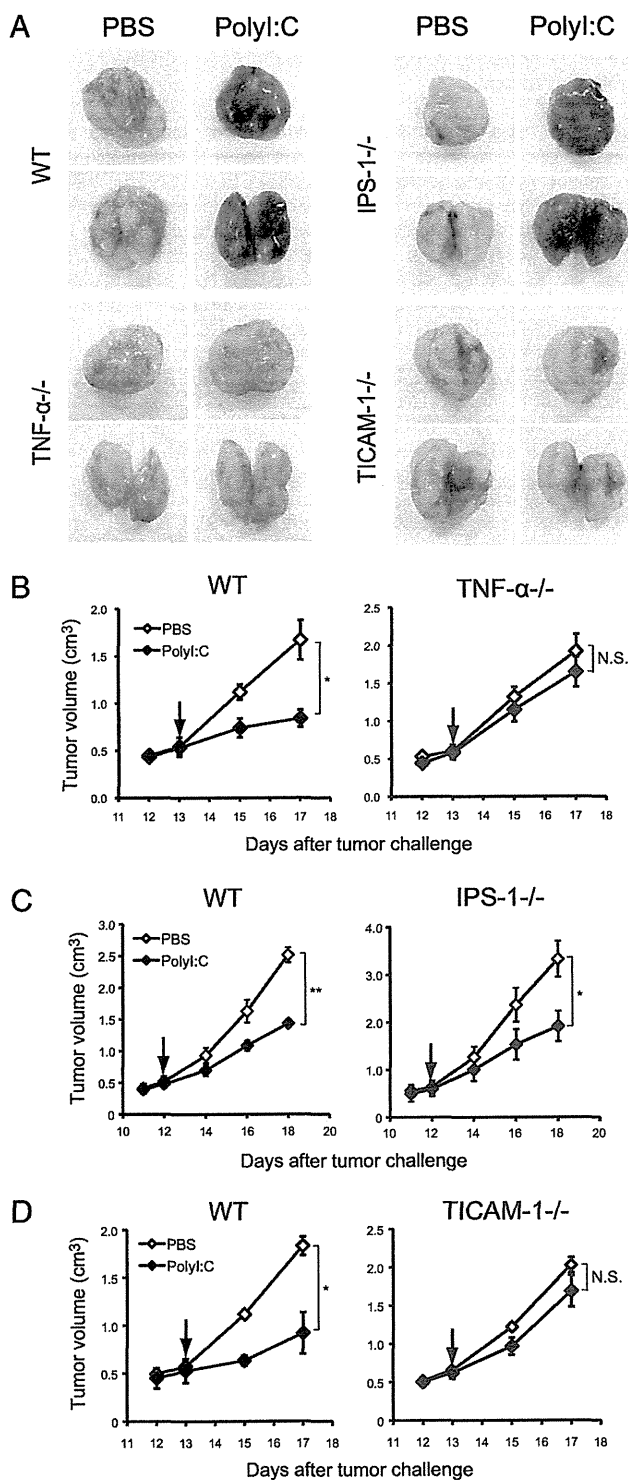


Fig. 1. Antitumor activity of polyI:C against 3LL tumor cells is mediated by the TICAM-1 pathway in vivo. (A) Representative photographs of 3LL tumors excised from WT, $TNF-\alpha^{-/-}$, $TICAM-1^{-/-}$, and $IPS-1^{-/-}$ mice. Whole tumor (Upper) and bisected tumor (Lower) are shown. (B–D) On day 0, 3LL tumor cells (3×10^5) 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 \pm 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 $TNF-\alpha$ tumoricidal effect against 3LL tumors. PolyI:C did not directly exert a cytotoxic effect on 3LL tumor cells (Fig. S8C).

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 $NF-\kappa B$ and IRF-3, which drive expression of $TNF-\alpha$ and $IFN-\beta$, respectively. PolyI:C-induced $TNF-\alpha$ 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 $IFN-\beta$ in these cells after polyI:C stimulation. Compared with $F4/80^+$ cells from WT mice, $IFN-\beta$ expression and production was barely decreased in $IPS-1^{-/-}$ $F4/80^+$ cells, but largely impaired in $TICAM-1^{-/-}$ $F4/80^+$ cells (Fig. S9A) 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. S9A), whereas M2 Mf-associated genes were barely affected by TICAM-1 disruption or polyI:C stimulation (Fig. S9B).

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 TAMs (Fig. S10D). We further examined whether $IFN-\beta$ 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 $IFN-\beta$ 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 $TNF-\alpha$ 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 $TNF-\alpha$ 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 $IL-1\beta$ at 4 h after in vitro polyI:C treatment, as were mRNA levels of $IFN-\beta$ and $TNF-\alpha$ 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. 5C). The polyI:C-induced M1 markers and $IL-10$ expression that were up-regulated 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 TLR3 stimulation (33). In our study, polyI:C stimulation increased *Jmjd3* mRNA in $F4/80^+$ cells

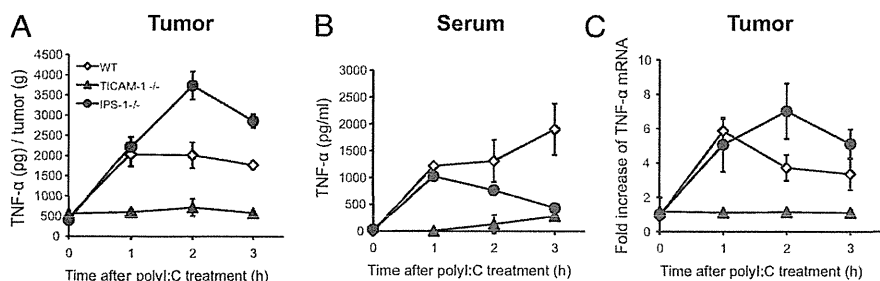


Fig. 2. TNF- α production in tumor and serum of polyI:C-injected 3LL tumor-bearing mice. Mice bearing 3LL tumor were i.p. injected with 200 μ g polyI:C. Tumor (A) and serum (B) were collected at 0, 1, 2, and 3 h after polyI:C injection, and TNF- α concentration was determined by ELISA. TNF- α level in 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 \pm SD. A representative experiment of two with similar outcomes is shown.

(Fig. 5B). 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⁺ Mfs.

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/He.] 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- κ B activation secondary to polyI:C stimulation, and indeed their signals converge at the I κ B 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

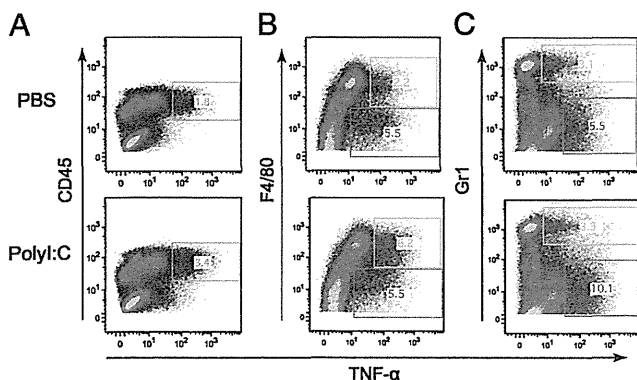


Fig. 3. F4/80⁺ cells are responsible for the polyI:C-induced elevation of TNF- α production in tumor. Mice bearing 3LL tumors were i.p. injected with 200 μ g polyI:C. TNF- α -producing cells in tumors of polyI:C- or PBS-injected mice were examined by immunohistochemical staining and flow cytometry to 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).

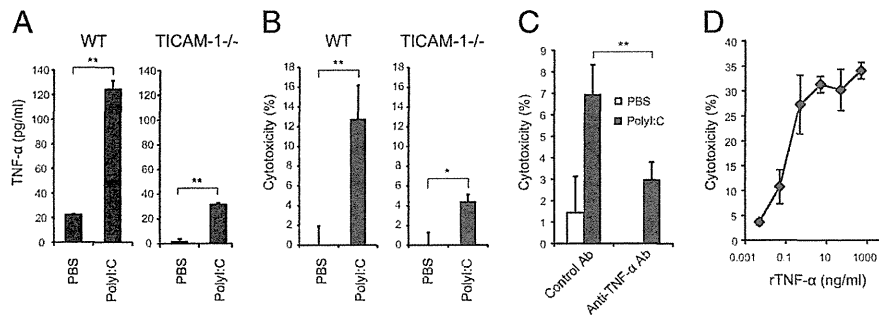


Fig. 4. PolyI:C enhances TNF- α production and cytotoxicity of F4/80⁺ cells in tumor. PolyI:C (200 μ g) or PBS was i.p. injected into 3LL tumor-bearing WT mice. After 30 min, F4/80⁺ cells isolated from tumor were cultured for 24 h and TNF- α concentration in the conditioned medium was determined by ELISA (A). In parallel, the cytotoxicity of tumor-infiltrating F4/80⁺ cells against 3LL tumor cells was measured by ⁵¹Cr-release assay (B). Anti-TNF- α neutralization antibody or control antibody was added (10 μ g/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 ⁵¹Cr-labeled 3LL tumor cell culture at various concentrations. After 20 h, cytotoxicity was measured; $n = 3$. Data are shown as average \pm 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- κ B 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

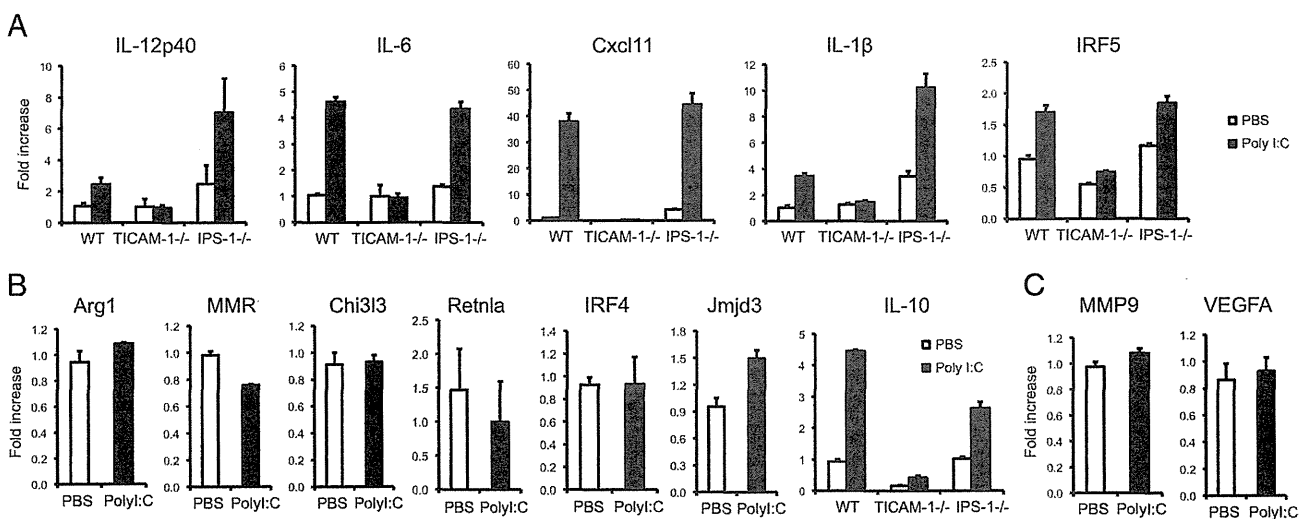


Fig. 5. PolyI:C induces M1 polarization of TAMs. F4/80⁺ cells were isolated from 3LL tumor and stimulated with polyI:C (50 μ g/mL) for 4 h. Total RNA was extracted and used to analyze the transcript expression levels of M1 (A) and M2 (B and C) markers; $n = 3$. Data are shown as average \pm SD. A representative 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% CO₂ 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*.

ACKNOWLEDGMENTS. We thank Dr. T. Taniguchi (University of Tokyo) and D. M. Segal (EIB/NCI, Bethesda, MD) for kindly providing us with IRF-3/7 double KO mice and mAb against mouse TLR3. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (MEXT), "the Carcinogenic Spiral" a MEXT Grant-in-Project, and the Ministry of Health, Labor, and Welfare of Japan, the Takeda Foundation, and the Akiyama Foundation, and the Waxman Foundation.

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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. 1A 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. S8C). 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. S5A). 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. S5A).

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 ⁵¹Cr 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 ⁵¹Cr release was measured in each sample. Specific lysis was calculated by the following formula: cytotoxicity (%) = [(experimental release – spontaneous release)/(max release – spontaneous release)] \times 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 μ g/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 $\Delta\Delta C_t$ 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 μ m 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 μ m 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 μ L 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 (cm³) = (long diameter) \times (short diameter)² \times 0.4. PolyI:C (250 μ g/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 cm³ 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 $^{\circ}$ 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.
3. Smyth MJ, et al. (2004) NKG2D recognition and perforin effector function mediate effective cytokine immunotherapy of cancer. *J Exp Med* 200:1325–1335.
4. Remels L, Franssen 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.

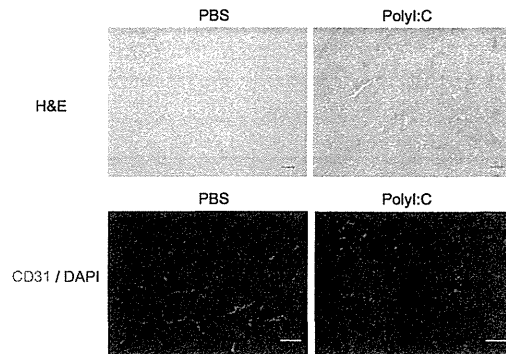


Fig. S1. Poly:I:C induces hemorrhagic necrosis of tumor. 3LL tumor-bearing mice were i.p. injected with 200 μ g poly:I:C and tumors were isolated 12 h later. Formalin-fixed tumors stained with H&E (*Upper*) and frozen sections stained with anti-CD31 antibody and DAPI nuclear stain (*Lower*). Original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

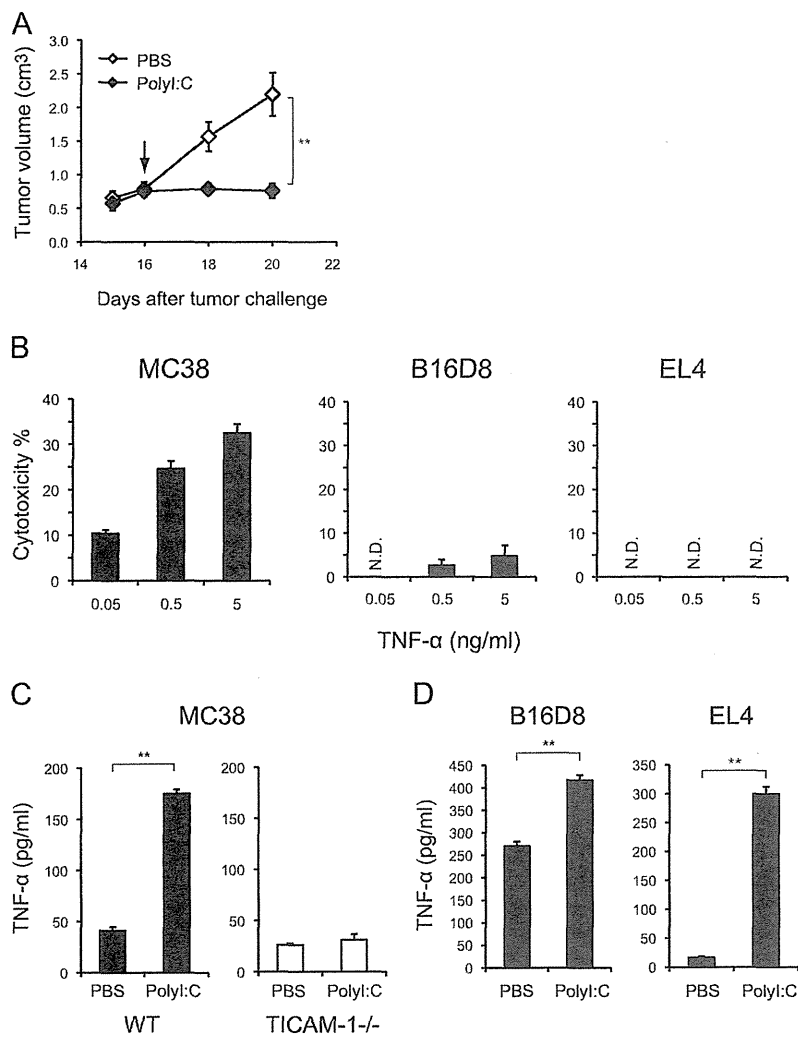


Fig. 52. PolyI:C induces TNF- α production by tumor-associated F4/80⁺ Mfs in various types of tumor. (A) MC38 cells (1×10^5) were s.c. implanted into C57BL/6 mice (day 0). PolyI:C (200 μ g) was i.p. injected on day 16. Data are shown as tumor average size \pm SE; $n = 3-4$ mice per group. (B) Sensitivity 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 μ g polyI: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 \pm SD. N.D., not detected. A representative experiment of two with similar outcomes is shown.

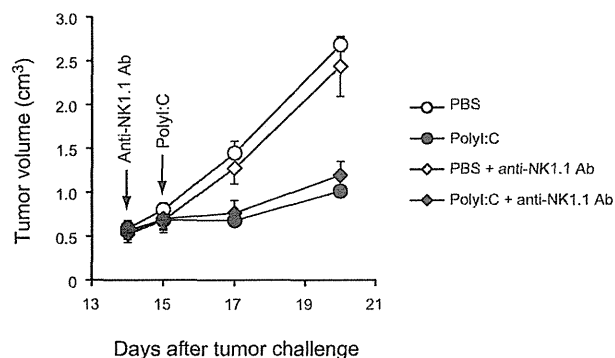


Fig. 53. NK cells are not essential for polyI:C-induced antitumor activity in vivo. 3LL tumor cells (3×10^6) were s.c. transplanted into C57BL/6 mice (day 0). NK cells were depleted by injection of anti-NK1.1 antibody (PK136) into 3LL tumor-bearing mice on day 14. All doses of antibody and treatment 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. PolyI:C (250 μ g) was i.p. injected on day 15 and the tumor volume was measured. Data shown are means \pm SE, $n = 3$. A representative experiment of two with similar outcomes is shown.

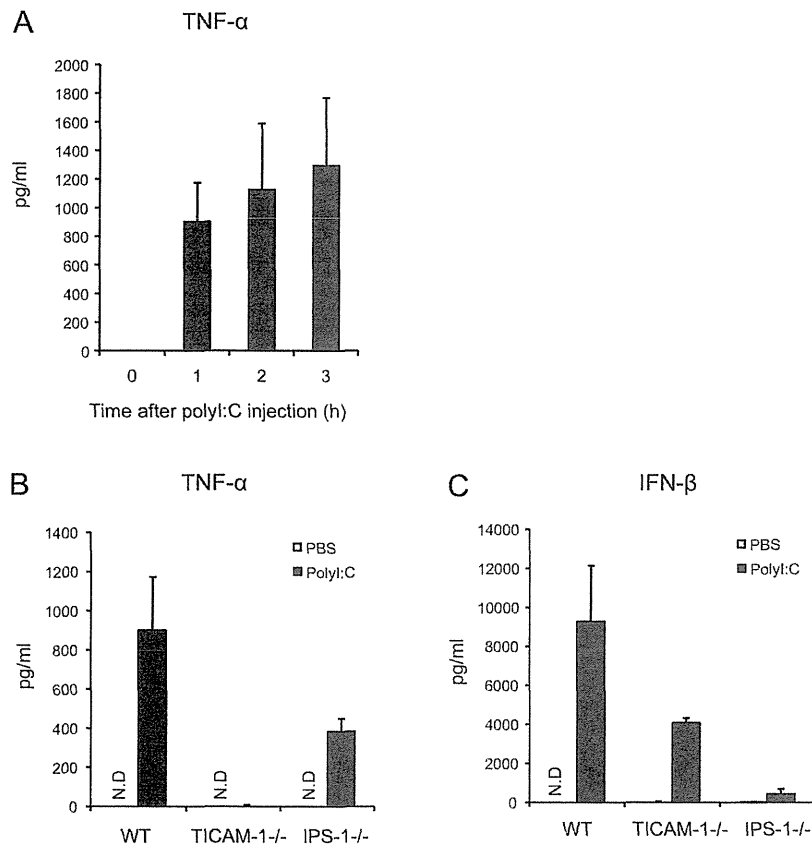


Fig. 54. Cytokine production in poly:I:C-treated mouse. (A) WT mice were injected i.p with 200 μ g poly:I:C. After 0, 1, 2, and 3 h, TNF- α concentration in serum was determined by ELISA. (B and C) WT, TICAM-1^{-/-}, and IPS-1^{-/-} mice were injected i.p with 200 μ g poly:I:C. After 1 h for TNF- α (B) and 4 h for IFN- β (C), serum cytokine levels were determined by ELISA. Data represents mean \pm SD ($n = 3$). N.D., not detected. A representative experiment of three with similar outcomes is shown.

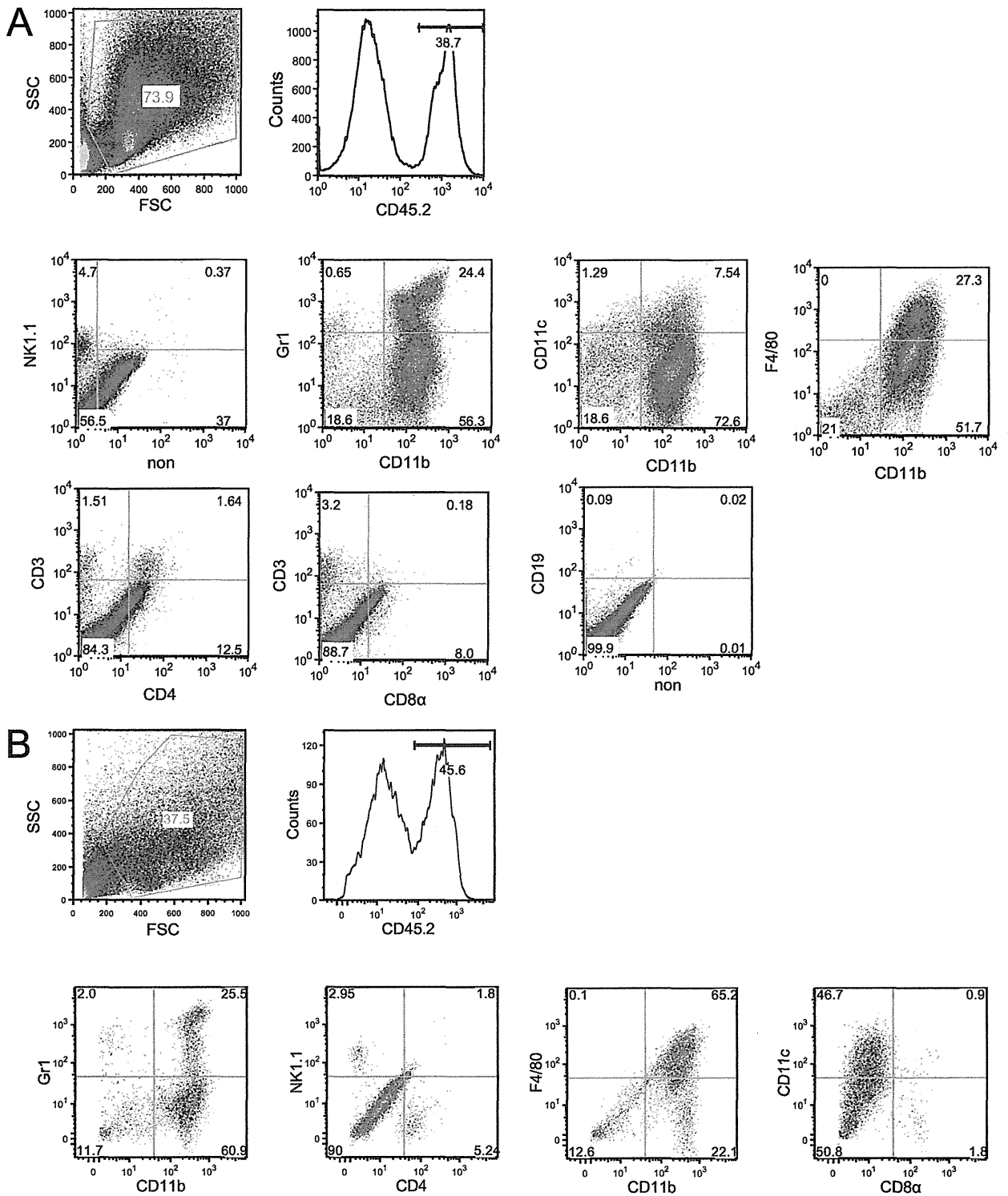
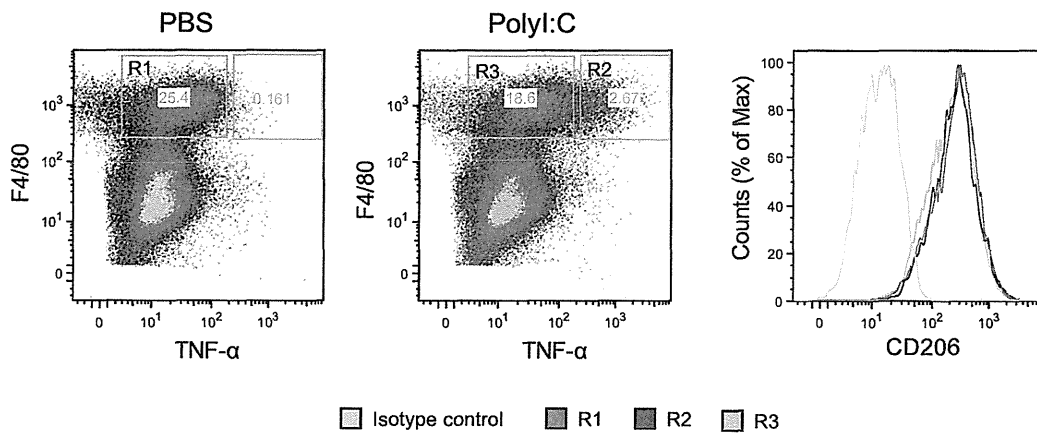


Fig. S5. Analysis of immune cells infiltrated into tumor. 3LL tumor cells (3×10^6) (A) or MC38 (1×10^6) (B) were transplanted s.c into B6 WT mice. After 2 wk, flow cytometric analysis was performed using freshly isolated whole tumor cell preparations in combination with staining of surface markers. CD45.2⁺ 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.

A 3LL tumor



B Spleen (naive mouse)

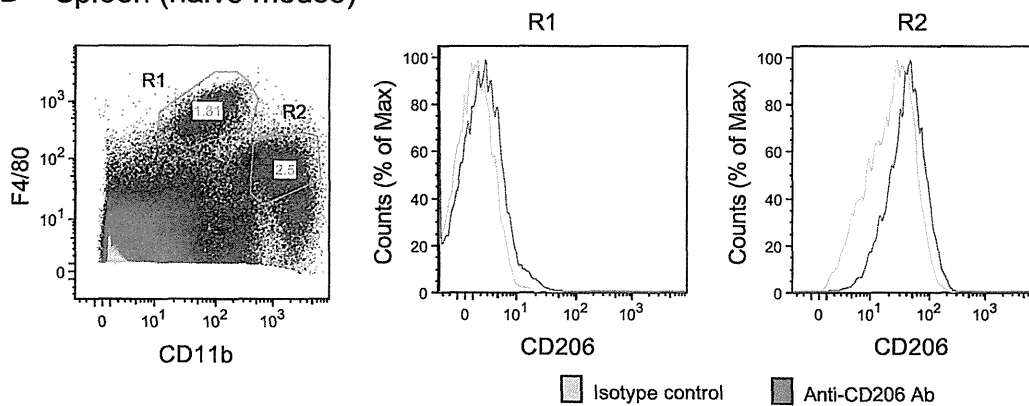


Fig. 56. Both TNF- α -producing and -nonproducing F4/80⁺ macrophages in 3LL tumor of polyI:C-injected mouse express CD206 (macrophage mannose receptor). (A) 3LL tumor-bearing mice were injected i.p with 200 μ g polyI:C. After 1 h, single-cell suspension of tumor was incubated in the presence of 10 μ g/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.

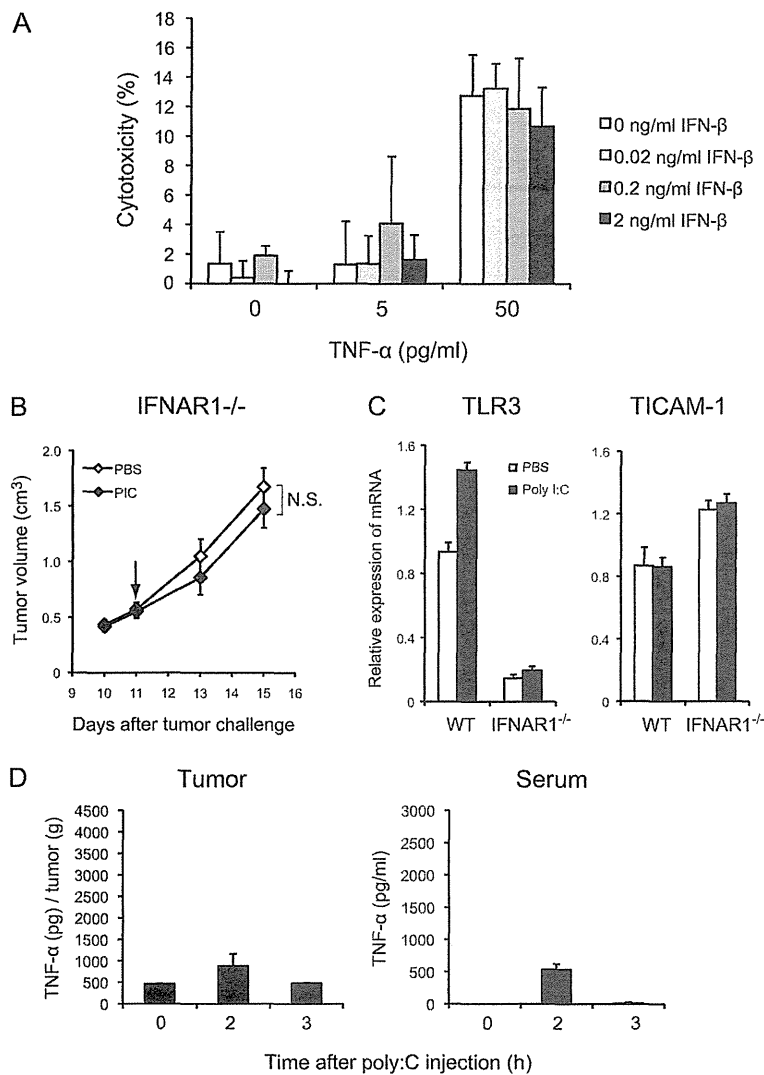


Fig. S7. Involvement of type I IFN signaling in 3LL tumor regression induced by poly:I:C. (A) Effect of IFN- β on cytotoxic activity of TNF- α against 3LL tumor cells. 3LL cells were incubated in the presence of 0, 5, and 50 pg/mL recombinant mouse TNF- α in combination with 0, 0.02, 0.2, and 2 ng/mL recombinant mouse IFN- β . Cytotoxicity was determined by ^{51}Cr release assay. (B) Disabling poly:I:C for 3LL tumor regression in IFN- α/β receptor (IFNAR1)^{-/-} mice. Poly:I:C was i.p. injected on day 11; $n = 3-4$ mice per group. Data are shown as average \pm 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 poly:I:C-stimulated IFNAR1^{-/-} mice. Mice bearing 3LL tumors were i.p. injected with 200 μg poly:I:C. Tumor (*Left*) and serum (*Right*) were collected at 0, 2, and 3 h after poly:I: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)].

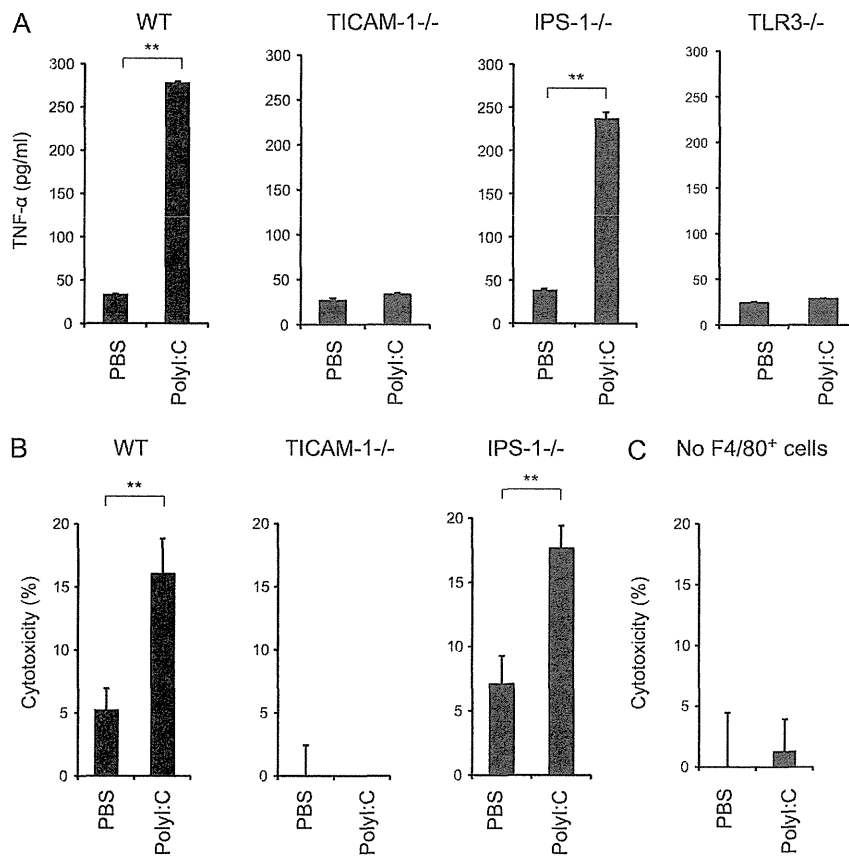


Fig. 58. In vitro poly:I:C-stimulated F4/80⁺ cells secrete TNF- α and have cytotoxic activity. (A) F4/80⁺ cells isolated from 3LL tumor were stimulated with poly:I:C (50 μ g/mL) in vitro. After 24 h, the conditioned medium was collected and TNF- α concentration was determined by ELISA. (B) F4/80⁺ cells isolated from tumor were mixed with ⁵¹Cr-labeled 3LL tumor cells in the presence or absence of poly:I:C (50 μ g/mL). After 20 h, radioactivity of the conditioned medium was measured. $E/T = 10$. (C) ⁵¹Cr-labeled 3LL tumor cells were incubated for 20 h in the presence or absence of poly:I:C (50 μ g/mL); $n = 3$. Data are shown as average \pm SD. ** $P < 0.001$. A representative experiment of three with similar outcomes is shown.

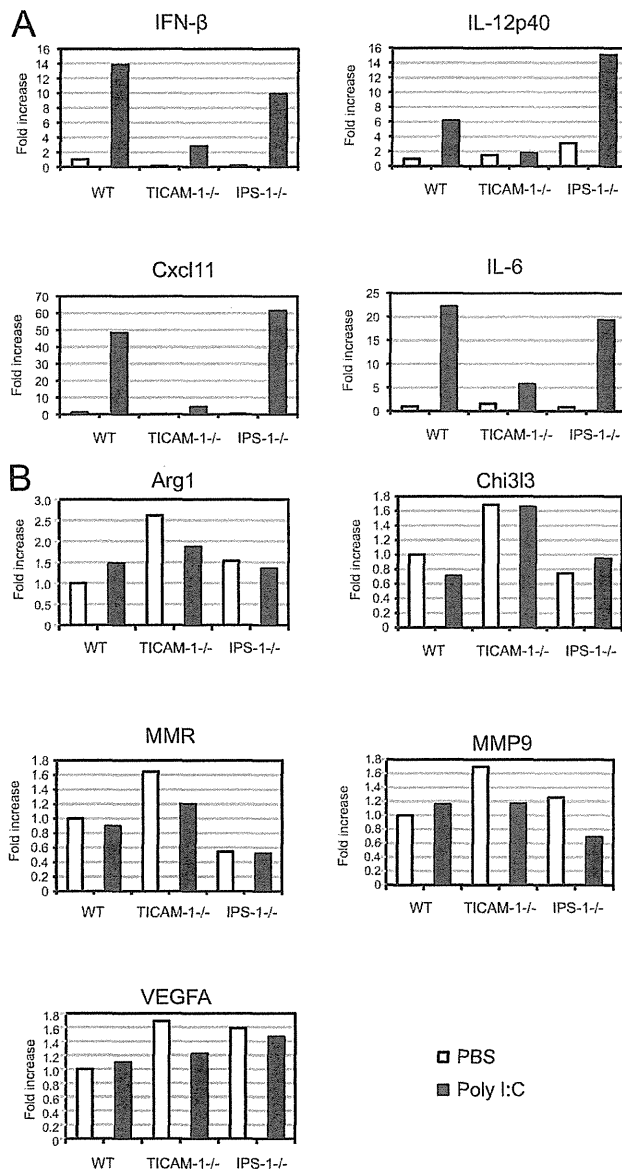


Fig. 59. PolyI:C induces the expression of M1 but not M2 macrophage-associated genes in tumor-infiltrated F4/80⁺ cells through the TICAM-1 pathway. 3LL tumor-bearing mice were i.p injected with 200 μg polyI:C. After 3 h, tumors pooled from two mice treated with polyI:C or PBS were mixed. F4/80⁺ cells were isolated from the mixed tumor, and the expression of (A) M1- and (B) M2-related genes was analyzed. A representative experiment of two with similar outcomes is shown.

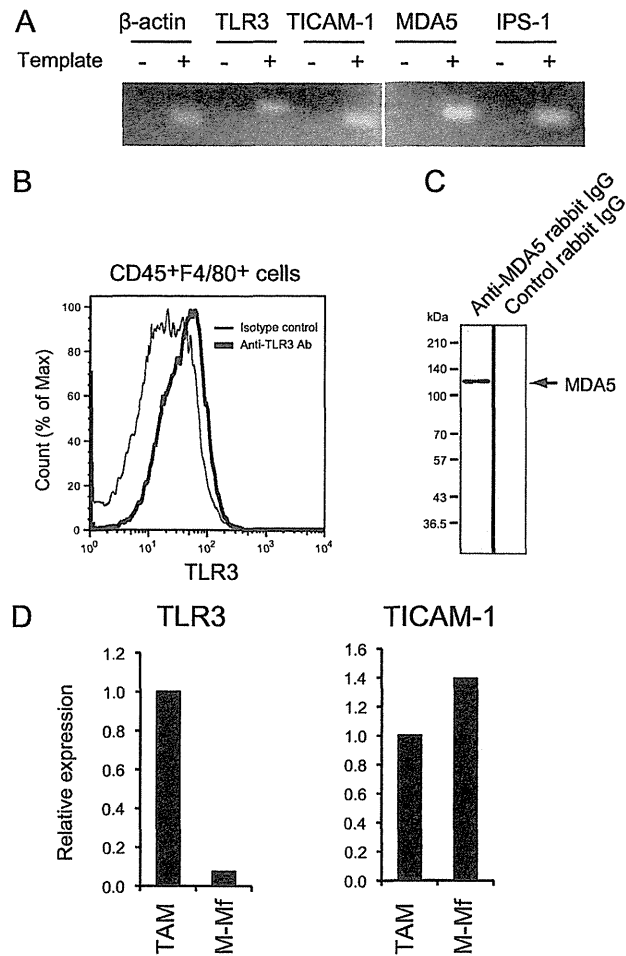


Fig. S10. Expression of TLR3 and MDA5 in 3LL tumor-associated F4/80⁺ cells. (A) mRNA expression of TLR3, TICAM-1, MDA5, and IPS-1 in 3LL tumor-associated F4/80⁺ cells. Total RNA (1 μg) of F4/80⁺ cells isolated from 3LL tumor was used as a template for RT-PCR analysis. (B) Single-cell suspension of 3LL tumor was stained with FITC-labeled anti-CD45 and PE-labeled anti-F4/80 antibody, followed by intracellular staining with Alexa 647-labeled anti-TLR3 antibody (11F8) or isotype control antibody (rat IgG2a). CD45⁺F4/80⁺ cells are shown. (C) Cytoplasmic extract of F4/80⁺ cells was subjected to SDS/PAGE and immunoblotted with rabbit anti-MDA5 antibody or control IgG purified from rabbit serum. (D) mRNA expression of TLR3 and TICAM-1 in F4/80⁺ tumor-associated macrophages (TAM) and macrophage colony-stimulating factor-induced bone marrow-derived macrophages (M-Mf).

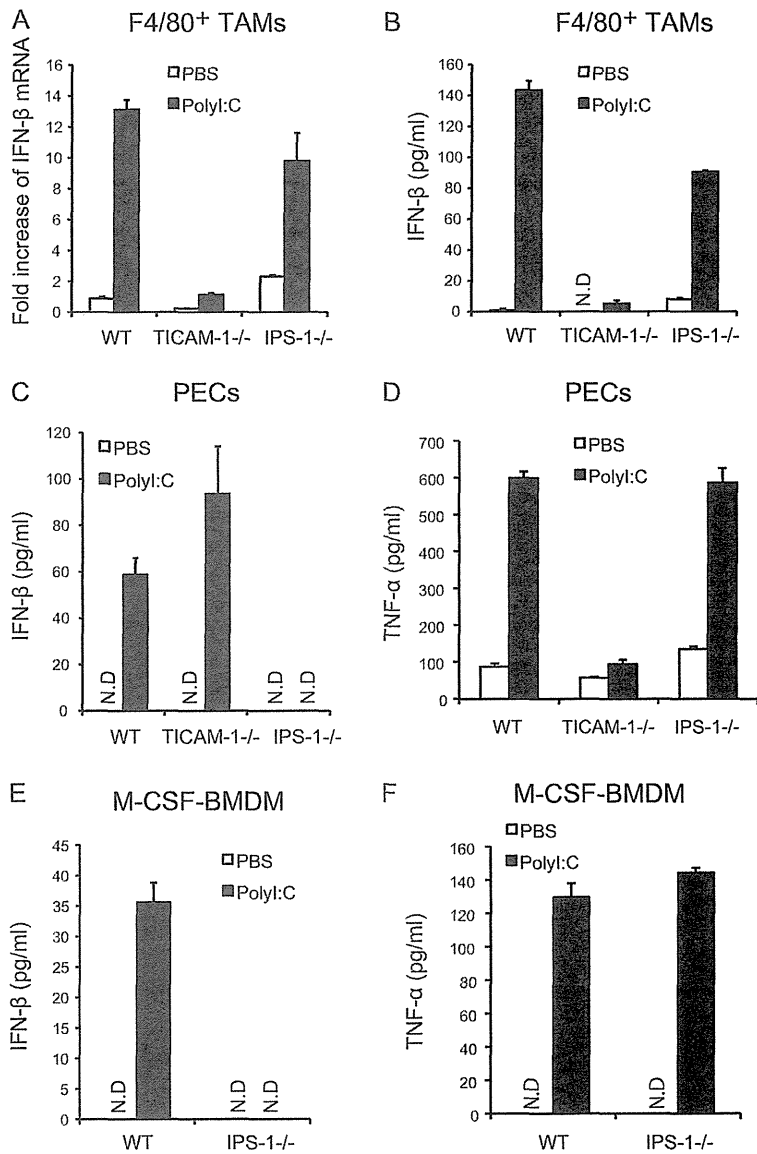


Fig. S11. In vitro stimulation with polyI:C increases the production of IFN-β and TNF-α by Mfs. (A and B) F4/80⁺ cells were isolated from 3LL tumor implanted in WT, TICAM-1^{-/-}, and IPS-1^{-/-} mice and stimulated with 50 μg/mL polyI:C. After 4 h, cells were harvested and IFN-β mRNA expression was analyzed by quantitative PCR analysis (A). After 20 h, IFN-β concentration in culture supernatant was determined by ELISA (B). (C and D) Peritoneal exudate cells (PECs) isolated from WT, TICAM-1^{-/-}, and IPS-1^{-/-} mouse were stimulated with 50 μg/mL polyI:C for 20 h. The concentrations of IFN-β (C) and TNF-α (D) in culture supernatant were determined by ELISA. (E and F) Macrophage colony-stimulating factor (M-CSF)-induced bone marrow-derived macrophages (BMDM) were prepared from WT and IPS-1^{-/-} mouse and cultured in the presence of 30% L929 supernatant containing M-CSF. After 6 d, adherent cells were harvested and stimulated with 50 μg/mL polyI:C for 20 h. The concentrations of IFN-β (E) and TNF-α (F) in culture supernatant were determined by ELISA. Data are shown as mean ± SD (n = 3). N.D., not detected. A representative experiment of two with similar outcomes is shown.

Table S1. Expression of various markers on 3LL and MC38 tumor cells

Surface marker	3LL	MC38
H2-K ^b	-	++
H2-D ^b	±	++
RAE1	++	Not determined
CD45	-	-

Expression of surface markers was analyzed by flow cytometry. Expression was evaluated by mean fluorescence shift: -, ~0.99; ±, 1~10; +, 11~100; ++, 101~.

Table S2. RT-PCR primers used in this study

	Forward primer (5'-3')	Reverse primer (5'-3')
IFN- β	CCAGCTCCAAGAAAGGACGA	CGCCCTGTAGGTGAGGTTGAT
IL-12p40	AATGTCTGCGTGCAAGCTCA	ATGCCCACTTGCTGCATGA
IL-6	GTGCATCATCGTTGTTTACATAAATC	CTGGGAAATCGTGGAAATGAG
TNF- α	AGGGATGAGAAGTCCCAAATG	GCTTGCTACTCGAATTTTGAGAAG
IL-1 β	TGACGGACCCCAAAGATGA	TGCTGCTGCGAGATTGAAG
IL-10	GGCGTGTATCGATTCTC	TGCTCCACTGCCTTGCTCTTA
Cxcl11	GGCTGCGACAAAGTTGAAGTGA	TCCTGGCACAGAGTTCTTATTGGAG
IRF4	AGCCAGCAGGTTTATAACTACA	CCTCGTGGGCCAAACGT
IRF5	GGTCAACGGGGAAAAGAACT	CATCCACCCTTCAGTGTACT
Jmjd3	CGAGTGGTTCGCGGTACAT	GAAGCGGTAACAGGAATATTGGA
Arg1	GGAATCTGCATGGGCAACCTGTGT	AGGGTCTACGTCTCGCAAGCCA
MMP9	CAAGTGGGACCATCATAACATCA	GATCATGTCTCGCGGCAAGT
VEGFA	GACATCTCCAGGAGTACC	TGCTGTAGGAAGCTCATCT
Chi3l3	TCACTACACACATGAGCAAGAC	CGGTTCTGAGGAGTAGAGACCA
Mrc1	CTCTGTTACAGCTATTGGACGC	CGGAATTTCTGGGATTGAGCTTC
Retnla	CCAATCCAGCTAACTATCCCTCC	ACCCAGTAGCAGTCATCCCA
GAPDH	GCCTGGAGAAACCTGCCA	CCCTCAGATGCCTGCTTCA

Table S3. Expression of surface markers on tumor-infiltrated F4/80⁺ cells

Marker	Expression*
I-Ab	+
H2-D ^b	+
H2-K ^b	+
CD80	++
CD86	++
CD40	±
CD11c	±
CD3	-
CD4	-
CD8 α	-
Gr1	+
B220	+
CD11b	+++
CD206 (MMR)	++

*Expression was evaluated by mean fluorescence shift: -, ~0.99; ±, 1 ~10; +, 11 ~100; ++, 101 ~1,000; +++, 1,001~.

The Toll-Like Receptor 3-Mediated Antiviral Response Is Important for Protection against Poliovirus Infection in Poliovirus Receptor Transgenic Mice

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RIG-I-like receptors and Toll-like receptors (TLRs) play important roles in the recognition of viral infections. However, how these molecules contribute to the defense against poliovirus (PV) infection remains unclear. We characterized the roles of these sensors in PV infection in transgenic mice expressing the PV receptor. We observed that alpha/beta interferon (IFN- α/β) production in response to PV infection occurred in an MDA5-dependent but RIG-I-independent manner in primary cultured kidney cells *in vitro*. These results suggest that, similar to the RNA of other picornaviruses, PV RNA is recognized by MDA5. However, serum IFN- α levels, the viral load in nonneural tissues, and mortality rates did not differ significantly between MDA5-deficient mice and wild-type mice. In contrast, we observed that serum IFN production was abrogated and that the viral load in nonneural tissues and mortality rates were both markedly higher in TIR domain-containing adaptor-inducing IFN- β (TRIF)-deficient and TLR3-deficient mice than in wild-type mice. The mortality rate of MyD88-deficient mice was slightly higher than that of wild-type mice. These results suggest that multiple pathways are involved in the antiviral response in mice and that the TLR3-TRIF-mediated signaling pathway plays an essential role in the antiviral response against PV infection.

Poliovirus (PV), which belongs to the genus *Enterovirus* in the family *Picornaviridae*, is the causative agent of poliomyelitis (38). The host range of PV is restricted to primates (18). This species' tropism is determined primarily by the cellular PV receptor (PVR; CD155), which gives the virus access to susceptible cells (14–16, 20). Mice are generally not susceptible to PV. However, transgenic mice expressing human PVR (PVR-tg mice) become susceptible to PV and develop a paralytic disease similar to human poliomyelitis after the administration of PV intravenously, intraperitoneally, intracerebrally, or intramuscularly but not orally (26, 40). PV shows a neurotropic phenotype in both humans and PVR-tg mice. PV preferentially replicates in neurons, especially in motor neurons in the anterior or ventral horn of the spinal cord and in the brainstem. However, the efficiency of PV replication is low in nonneural tissues (4, 25). We previously found that innate immune responses that are mediated by type I interferons (IFNs) play important roles in controlling viral replication in nonneural tissues and in the mortality rates of PVR-tg mice (19). In PVR-tg mice deficient in IFNAR1, PV efficiently replicates in nonneural tissues such as the liver, pancreas, and spleen, which are not normal targets of PV. IFNAR1-deficient mice die after the inoculation of a small amount of PV by peripheral routes. The results suggest that the type I IFN response forms an innate immune barrier that prevents PV replication in nonneural tissues and subsequent PV invasion of the central nervous system (CNS). This response therefore plays important roles in the tissue tropism and pathogenicity of PV (25).

The sensors that are involved in the production of type I IFNs in response to RNA viral infections have been recently identified and characterized (1, 46–48). The RIG-I-like receptors (RLRs) retinoic-acid-inducible gene 1 (RIG-I) and melanoma

differentiation-associated gene 5 (MDA5) are expressed in the cytoplasm of all cell types, with the exception of plasmacytoid dendritic cells (pDCs). RIG-I and MDA5 have RNA binding domains and differentially recognize specific characteristics of nonself viral RNAs (17, 22, 36, 37). In addition, RLRs have DExD/H box RNA helicase domains (51) that activate downstream signaling pathways resulting in the activation of IFN regulatory factor 3 (IRF-3) and IRF-7 (53). TLR3 and TLR7 are the sensors for viral double-stranded RNA (dsRNA) and single-stranded RNA, respectively (2, 8, 12). TLR3 is expressed in the endosome of macrophages and conventional dendritic cells (DCs) (28) but not in pDCs. TLR3 is also expressed in a variety of epithelial cells, including airway, uterine, corneal, vaginal, cervical, biliary, and intestinal epithelial cells, which may function as efficient barriers to infection. The TLR3-mediated signaling pathway is transmitted through Toll-interleukin-1 (IL-1) receptor (TIR)-containing adaptor molecule 1, which is also known as TIR domain-containing adaptor inducing IFN- β (TRIF), and finally results in the activation of IRF3 and IRF7 (13, 34, 51). TLR7 is specifically expressed in the endosome of pDCs and contributes to the production of a large amount of IFNs in response to many RNA virus infections (5, 7). TLR7 signaling is mediated by the adaptor molecule myeloid differentiation factor 88 (MyD88). These sensors do not contribute equally

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