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TLR3/TICAM-1 signaling converts tumor-supporting myeloid cells to tumoricidal effectors

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Running title: **PolyI:C induces antitumor Mf**

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HS, MM, TS, designed research; HS, HO, **ST**, preformed research; HO, AN, **YI**, provided KO mice; **HT**, provided cell lines; MM, NI, analyzed data; HS, TS wrote the paper.

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Abstract

Smoldering inflammation often increases the risk of progression for malignant tumors and simultaneously matures myeloid dendritic cells (mDC) for cell-mediated immunity. PolyI:C, a dsRNA analog, is reported to induce inflammation and potent antitumor immune responses via the TLR3/TICAM-1 and MDA5/IPS-1 pathways in mDC to drive activation of NK cells and cytotoxic T lymphocytes. Here, we found that intraperitoneal or subcutaneous injection of polyI:C to 3LL tumor-implant mice resulted in tumor regression by converting tumor-supporting macrophages (Mf) to tumor-suppressors. F4/80⁺/Gr1⁻ Mf infiltrating the tumor are responded to polyI:C to rapidly produce inflammatory cytokines and thereafter accelerate M1 polarization. TNF- α was increased within 1 hour 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⁺ Mf in tumors extracted from polyI:C-injected mice sustained 3LL 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 Mf. These responses were completely abrogated in TICAM-1^{-/-} mice, and unaffected in MyD88^{-/-} and IPS-1^{-/-} mice. Thus, the TICAM-1 pathway is not only important to mature mDC for cross-priming and NK cell activation in the induction of tumor immunity, but also critically engaged in tumor suppression by converting tumor-supporting Mf to those with tumoricidal properties.

Key words: Toll-like receptor 3, TICAM-1 (TRIF), tumor-infiltrating macrophages, polyI:C, TNF-alpha

¥body

Introduction

Inflammation followed by bacterial and viral infections triggers high risk of cancer and promote tumor development and progression (1,2). Long-term use of anti-inflammatory drugs has been shown to reduce the risk of cancer, if not all, 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 following cellular events are closely concerned with the formation of 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, double-stranded (ds)RNA 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 still remain 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 the understanding their multifarious functional properties in tumor environment.

Recent advance on the study of innate immunity demonstrates how polyI:C suppress tumor progression (11). PolyI:C is a synthetic compound, that serves as an agonist for pattern-recognition receptors (PRRs), Toll-like receptor (TLR)3 and melanoma differentiation-associated protein (MDA)5 (12-14). Although TLR3 and MDA5 signals are characterized as MyD88-independent (16,17), they have immune-effector-inducing properties (12-15). TLR3 couples with the Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule (TICAM)-1 (also known as TRIF), and MDA5 couples with the interferon (IFN) β promoter stimulator (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 nuclear factor (NF)- κ B and interferon regulatory factor (IRF)-3 (15,18), they appear to distinctly modulate myeloid dendritic cells (mDC) and macrophages (Mf) to drive effector lymphocytes (19,20).

Tumor microenvironment frequently involves myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM), and immature myeloid dendritic cells (mDCs) (1,21). They 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). On the other hand, 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 Mf is engaged in conversion of the TAM-like Mf to tumoricidal effectors. We investigated the molecular mechanisms in Mf underlying the phenotype switch from tumor-supporting to tumor-suppressing by treating cells with polyI:C and found that the TICAM-1-inducing tumor necrosis factor (TNF)- α and M1 polarization are crucial for eliciting tumoricidal activity in TAM.

Results

***In vivo* effect of polyI:C on implant 3LL tumor**

Intraperitoneal (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 (Figure 1A). The polyI:C-dependent hemorrhagic necrosis did not occur in TNF- α -/- mice (Figure 1A). Histological and immunohistochemical analysis revealed vascular damage in the necrotic lesion, where disruption of vascular endothelial cells was indicated by fragmented CD31⁺ marker (Figure 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 (Figure 1A). The results suggest that polyI:C is a reagent that induces 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 wild-type (WT) C57BL6 mice. polyI:C, when

i.p. injected, resulted in tumor growth retardation (Figure 1B). The retardation of tumor growth by polyI:C was also impaired in TNF- α -/- mice (Figure 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-/- (Figure 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 (Figure 1D). The size differences of the implanted tumors became significant within two days after polyI:C treatment, suggesting that the molecular effector for tumor regression is induced early and its up-stream is TICAM-1. Similar results were obtained with MC38 implant tumor (Figure S2A), which is TNF- α -sensitive and MHC class I-positive (Table S1, 26).

PolyI:C is a reagent that induces NK cell activation in MHC class I-negative tumors (12) and 3LL cells are actually 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 (Figure 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 enzyme-linked immunosorbent assay (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 (Figure S4A-B). Interferon (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 (Figure 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 post polyI:C i.p. injection (Figure 2A). The serum TNF- α levels in both were rapidly up-regulated within 1 h post polyI:C injection, although in WT the levels continued to increase but in IPS-1^{-/-} mice gradually decreased (Figure 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 (Figure 2C). The TNF- α mRNA levels peaked between 1 and 2 h after polyI:C injection, while 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-positive cells within the tumor.

F4/80⁺/Gr-1⁻ Mf in 3LL tumor produces TNF- α leading to tumor damage

We next investigated the cell types that had infiltrated into 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 (Figure 3A). The major population of those CD45⁺ cells was determined to be of CD11b⁺ myeloid-lineage cells that co-expressed either 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 (Figure S5A). Moreover, F4/80⁺/Gr-1⁻ cells were found to be the principal contributors to polyI:C-mediated TNF- α production (Figure 3B-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 non-producing 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 S2). Thus,**

the source of the TNF- α -producing cells in tumor is likely F4/80⁺ Mf with a TAM-like feature.

We harvested F4/80⁺ cells from tumor samples extracted from WT and TICAM-1^{-/-} mice at 30 min post polyI:C injection. These cells were used in *in vitro* experiments to verify the TNF- α -producing abilities and 3LL cytotoxicity properties (Figure 4A, B). WT F4/80⁺ Mf 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 (Figure 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 (Figure 4D). **TNF- α -producing ability was also observed in F4/80⁺ cells from implant tumor of MC38, B16D8, or EL4, and only MC38 tumor was remediable by TICAM-1-derived TNF- α (Figure S2B,C). MC38 tumor contained the F4/80⁺/CD11b⁺/Gr1⁻ cells as in 3LL tumor (Figure S5B).**

IFN- β did not enhance rTNF- α -mediated 3LL killing efficacy (Figure 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 IFNAR^{-/-} mice (Figure S7B). qPCR 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⁺ Mf of IFNAR^{-/-} mice (Figure S7C). Accordingly, the TNF- α level was not up-regulated in tumor and serum in polyI:C-stimulated IFNAR^{-/-} mice (Figure S7D). Thus, basal induction of type I IFN serves as a critical factor for TLR3 function in tumor F4/80⁺ Mf to produce TNF- α *in vivo*.** The 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⁺ Mf harvested from 3LL tumors in WT, TICAM-1^{-/-}, IPS-1^{-/-} and TLR3^{-/-} mice were stimulated with

polyI:C (Figure S8A). Both TNF- α release and 3LL cytotoxic abilities of polyI:C-stimulated F4/80⁺ Mf were specifically abrogated by the absence of TICAM-1 and TLR3 (Figure S8A, B). IPS-1 or MyD88 in F4/80⁺ Mf had no or minimal effect on the TNF- α tumoricidal effect against 3LL tumors. PolyI:C did not directly exert cytotoxic effect on 3LL tumor cells (Figure 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- κ B and IRF-3, that 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. As compared to 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 (Figure S9A) as other cytokines tested. M1 Mf-associated cytokines/chemokines were generally reduced in TICAM-1^{-/-} F4/80⁺ cells compared to WT and IPS-1^{-/-} cells >4 h after polyI:C stimulation (Figure S9A), while M2 Mf-associated genes were barely affected by TICAM-1 disruption or polyI:C stimulation (Figure S9B).

Most types of Mf are known to express TLR3 in mice (30). Messages and proteins for type I IFN induction were conserved in the F4/80⁺ tumor-infiltrating Mf (Figure S10A-C). However, the TLR3 mRNA level was low in M-CSF-derived Mf compared to TAM (Figure S10D). We further examined whether IFN- β production might also have relied on the TICAM-1 pathway in other types of Mf upon stimulation with polyI:C. In contrast to the F4/80⁺ cells isolated from tumor (Figure S11A,B), the IPS-1 pathway was indispensable for polyI:C-mediated IFN- β production in mouse peritoneal Mf (PEC) and M-CSF-induced bone marrow-derived Mf (M-CSF-BMDM) (Figure S11C,E). Yet, IPS-1 only slightly participated in polyI:C-mediated TNF- α production in these Mf subsets (Figure S11D,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 Mf.

PolyI:C influences polarization of tumor-associated macrophages

Plasticity is a characteristic feature of macrophages (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 Mf 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 (Figure 5A,B). The mRNA expressions were increased for M1 Mf markers IL-12p40, IL-6, CXCL11, and IL-1 β 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, as compared to 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 (Figure 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 (Figure 5A,B), reinforcing the results obtained with F4/80⁺ Mf isolated from 3LL tumors in mice injected with polyI:C (Figure S9A,B). It appears that TICAM-1 is responsible for the M1 polarization of F4/80⁺ Mf cells in tumor, but has no effect on the 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* (Figure 5A,B). Jmjd3, a histone H3K27 demethylase involved in IRF-4 expression, is reportedly induced by TLR stimulation (33). In our study, polyI:C stimulation induced an increase of Jmjd3 mRNA in F4/80⁺ cells (Figure 5B). These polyI:C-triggered M1 gene expression continued long in tumor-infiltrated Mf, a finding which may further explain the tumor-suppressing feature of these Mf 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⁺ Mf 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 which express minimal amounts of MHC class I/II and recruit a large amount of myeloid cells, F4/80⁺ Mf function to sustain the tumor in the surrounding microenvironment. This tumor-supporting environment can be disrupted by stimulation with RNA duplex through TICAM-1 signal and subsequent induction of mediators such as TNF- α . Thus, the TICAM-1 signal in tumor-infiltrating Mf plays a key role in TNF- α - and M1 shift-mediated tumor regression. The 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⁺ Mf 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 tumor regressed in patients with simultaneous virus infection (36,37), and that tumor growth was inhibited in tumor-bearing mice by polyI:C injection (6,7).

In contrast, polyI:C-stimulated PEC or bone marrow-derived Mf induce type I IFN via the IPS-1 pathway unlike the case of tumor-infiltrating F4/80⁺ Mf. 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 Mf leading to tumor regression. A marked finding is that the TLR3 protein level is high in tumor-infiltrating Mf compared to other sources of Mf (Figure S10). In addition, the IPS-1 pathway is unresponsive to polyI:C if the polyI:C is exogenously added to the tumor-infiltrating Mf without transfection reagents. The cytoplasmic dsRNA sensors normally work for IFN induction in tumor F4/80⁺ Mf, if the polyI:C is transfected into the cells. The TICAM-1-dependent TNF- α production by F4/80⁺ Mf (Figure S11D,F) is partly because the F4/80⁺ Mf express a high basal level of TLR3 and fail to take up extrinsic polyI:C into the cytoplasm. Of many subsets of Mf, these properties (38) are unique to the F4/80⁺ Mf.

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-Guerin (BCG) and endotoxin. Bio-assay 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 Mf 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 five to ten-fold less TNF- α than tumors in WT mice. This finding represented the first 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 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 since TLR4 can recruit TICAM-1 in addition to MyD88 (15). The lipid A derivative monophospholipid A (MPLA) 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 mDC (44). In both cases of TICAM-1 and MyD88 stimulation, tumor-infiltrating Mf facilitate the expression of many genes in addition to TNF- α . M2 phenotype of F4/80⁺ Mf or tumor-associated Mf 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 to polyI:C response, minute type I IFN of undefined source has to be provided to set the TLR3/TICAM-1 pathway, which may be primarily failed in IFNAR^{-/-} mice.** Cellular effectors, 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). On the other hand, distinct types of M2 cells differentiate when monocytes are stimulated with either 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 the first notion that tumor-infiltrating Mf is a target of dsRNA and converted from a tumor-supporter to a tumoricidal effector. Hence, the antitumor effect of dsRNA adjuvant is ultimately based on the liberation of type I IFN, functional maturation of mDC, and modulation of tumor-infiltrating Mf, where TICAM-1 is a crucial transducer in eliciting antitumor immunity.

Methods

Mice and tumor cells. Inbred C57BL/6 wild-type (WT) mice were purchased from Clea Japan. TICAM1 $-/-$ and IPS-1 $-/-$ mice were generated in our laboratory and were maintained as described previously. **IRF-3/7 double KO mice were a gift from Dr. T. Taniguchi (Univ. Tokyo, Japan)**. TNF- α $-/-$ mice were kindly provided by Dr. A. Nakane (Hirosaki Univ. Japan) **and Y. Iwakura (Univ. Tokyo, Japan)**. Mice of 6-10 week of age were used in all experiments that were performed according to animal experimental

ethics committee guidelines of Hokkaido University. 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 (USA). 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 ID number 08-0290, “Analysis of anti-tumor immune response induced by the activation of innate immunity”.

Other detailed methods are provided in SI text.

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Figure legends

Figure 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- α $-/-$, TICAM-1 $-/-$, and IPS-1 $-/-$ mice. Whole tumor (upper panel) and bisected tumor (lower panel) are shown. (B-D) On day 0, 3LL tumor cells (3×10^6) were s.c. implanted into B6 WT (B-D), TNF- α $-/-$ (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 four days. Data are shown as tumor average size \pm standard error (SE). $n=3-4$ mice per group. * $p < 0.05$; ** $p < 0.001$. N.S.; not significant. A representative experiment out of two with similar outcome is shown.

Figure 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, 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 standard deviation (SD). A representative experiment out of two with similar outcome is shown.

Figure 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 out of two with similar outcome is shown. TNF- α $^+$ gating squares are shown by red and green.

Figure 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 were 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 out of three with similar outcome is shown.

Figure 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-C) markers. $n=3$. Data are shown as average \pm SD. A representative experiment out of two with similar outcome is shown.

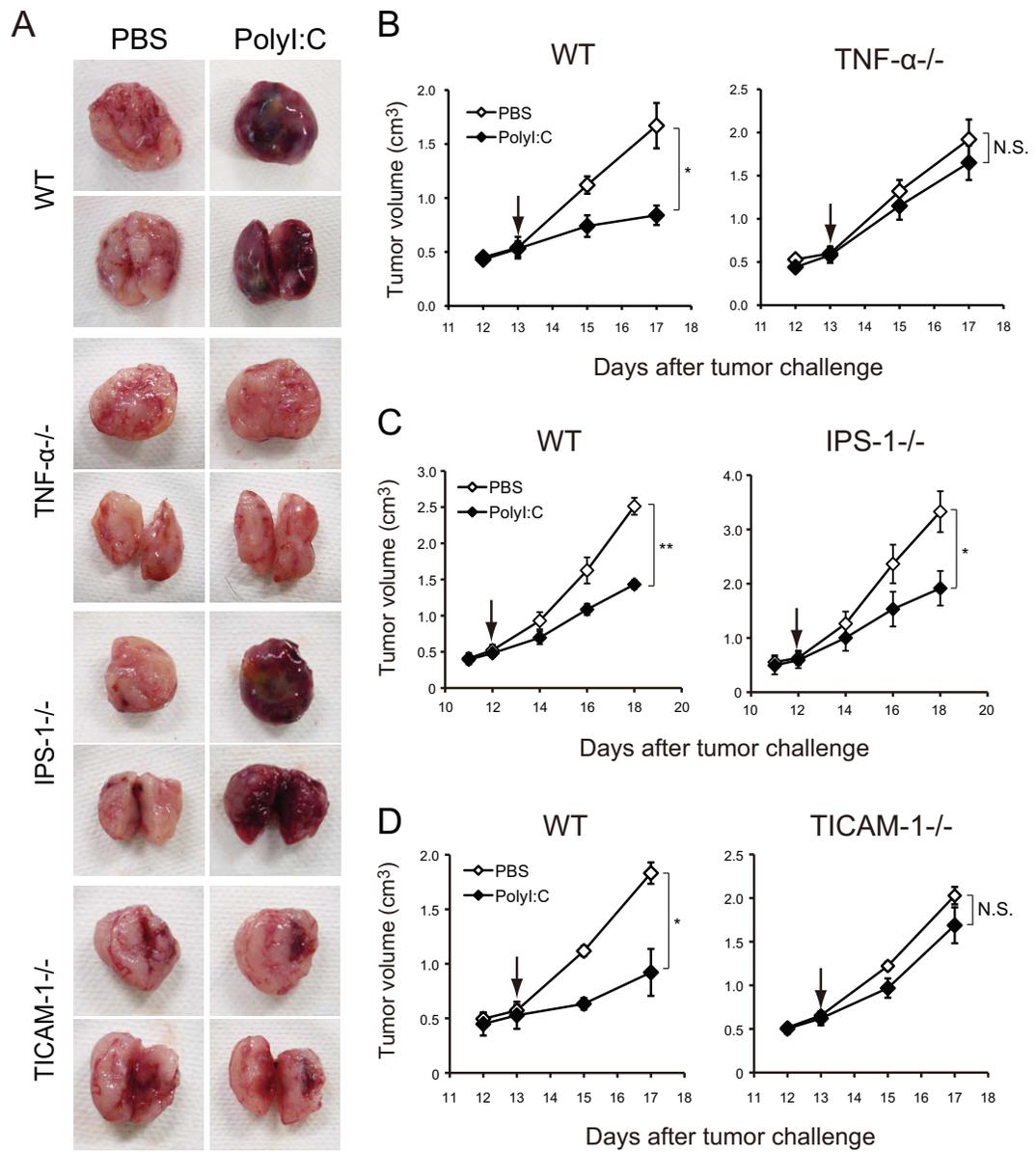


Figure 1. Shime et al.

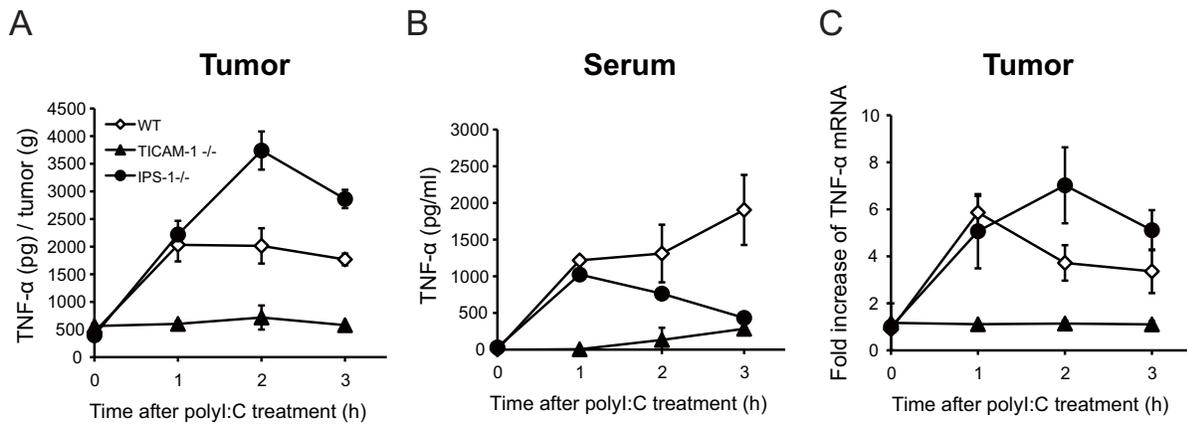


Figure 2. Shime et al.

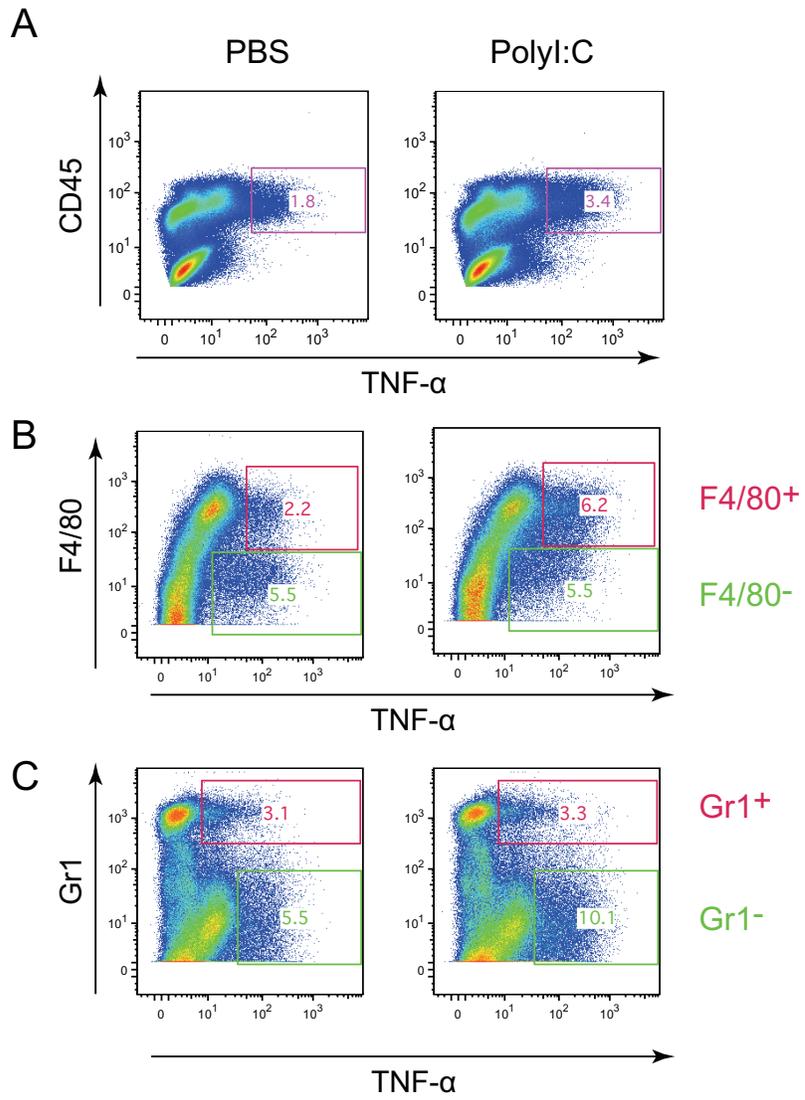


Figure 3. Shime et al.

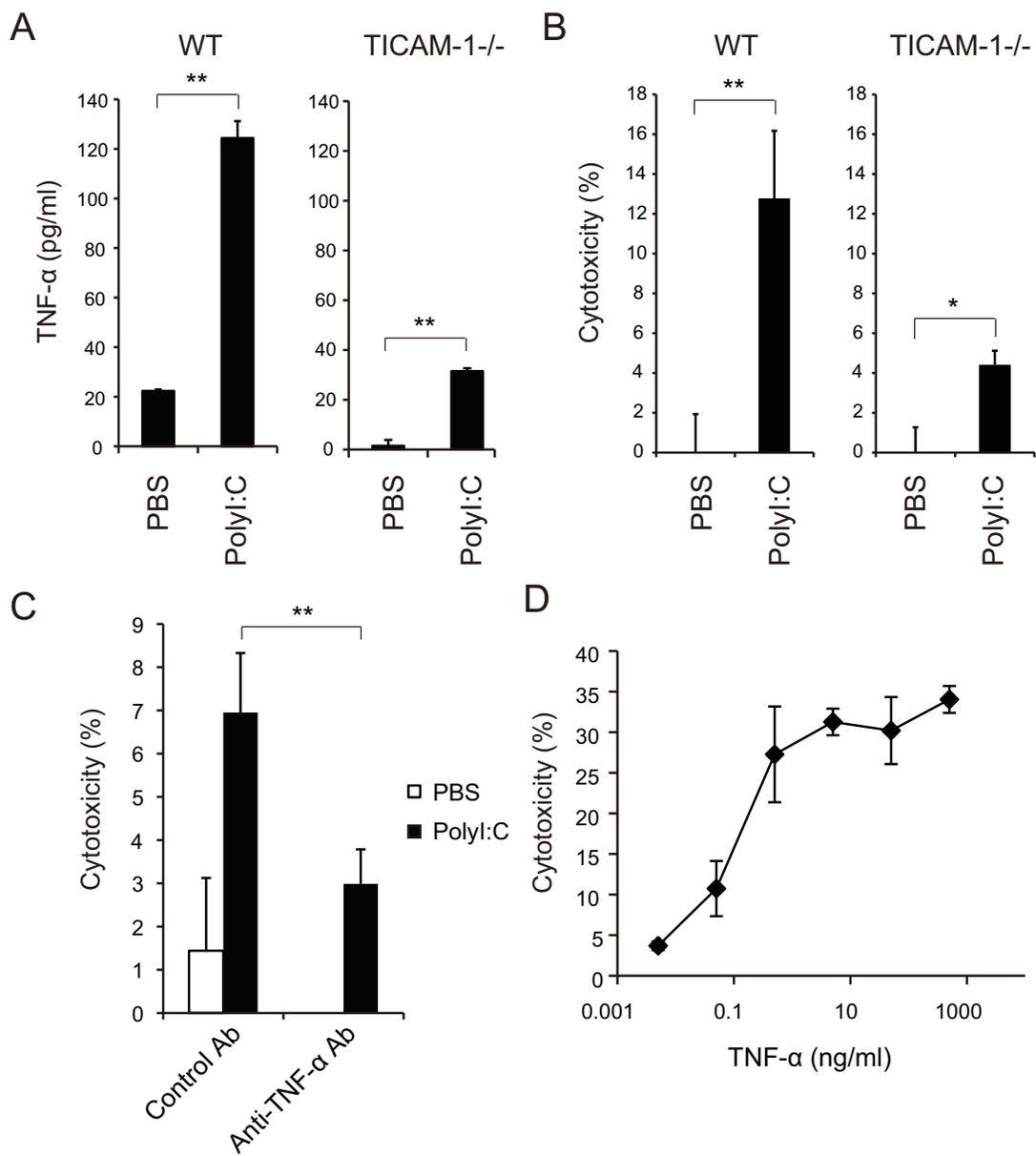


Fig. 4. Shime et al.

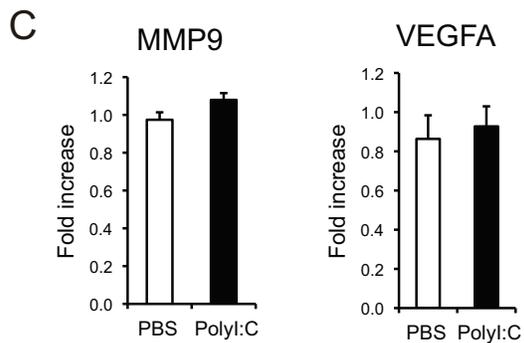
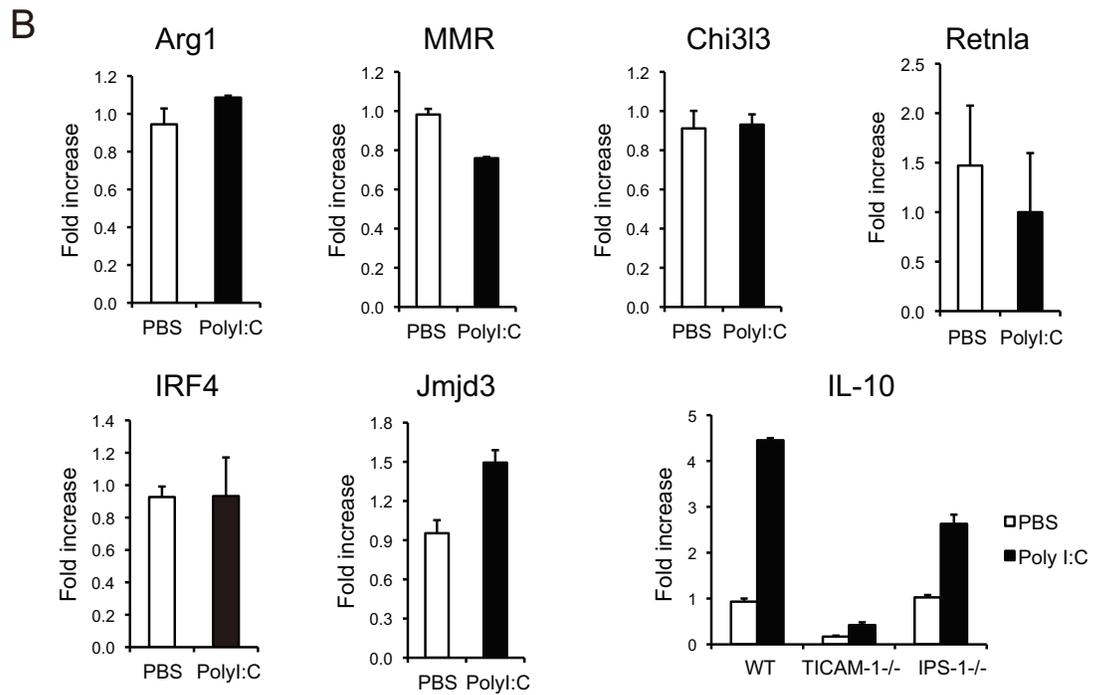
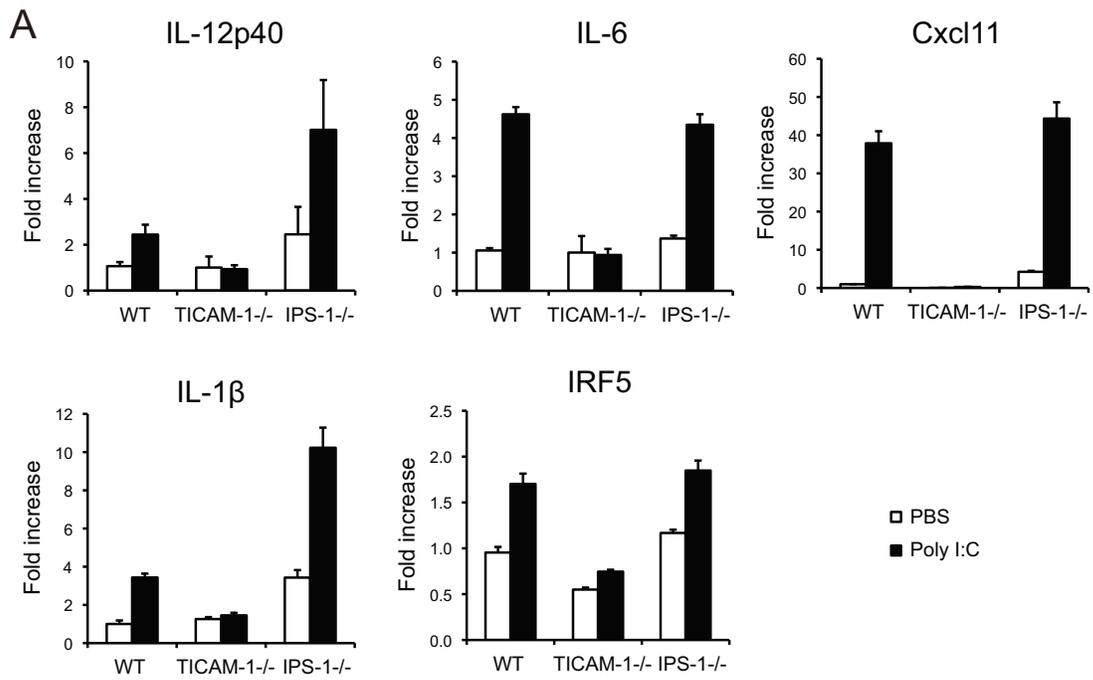


Figure 5. Shime et al.