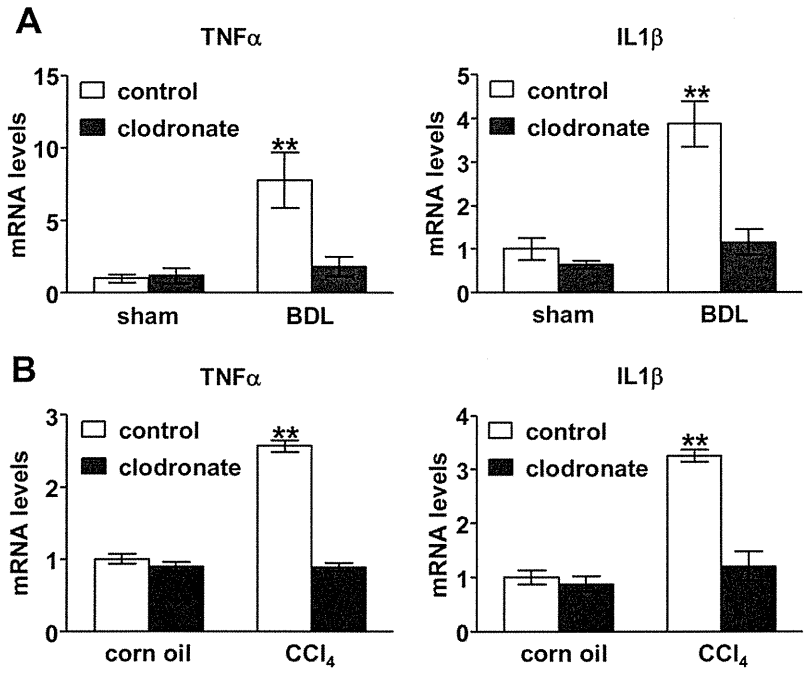
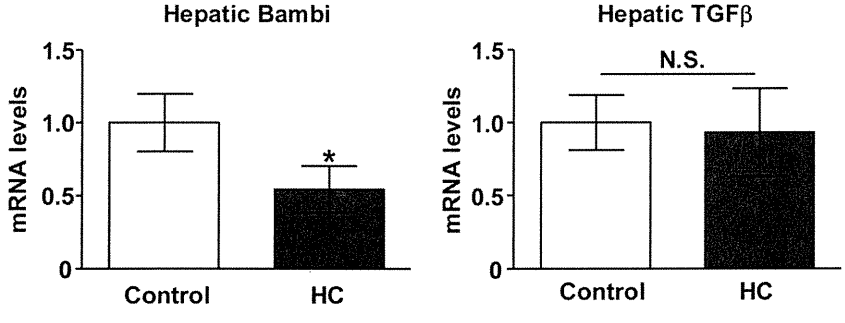
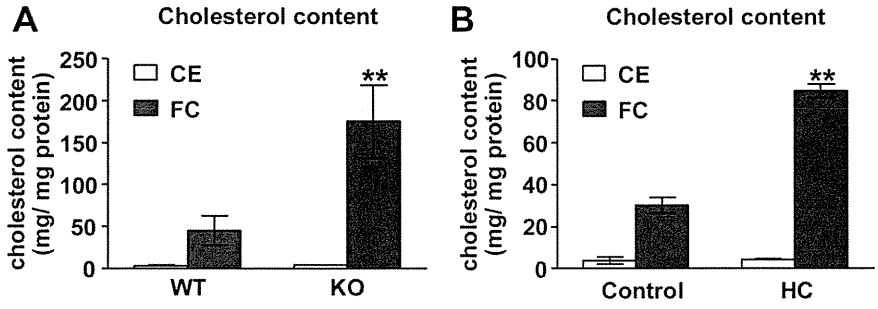


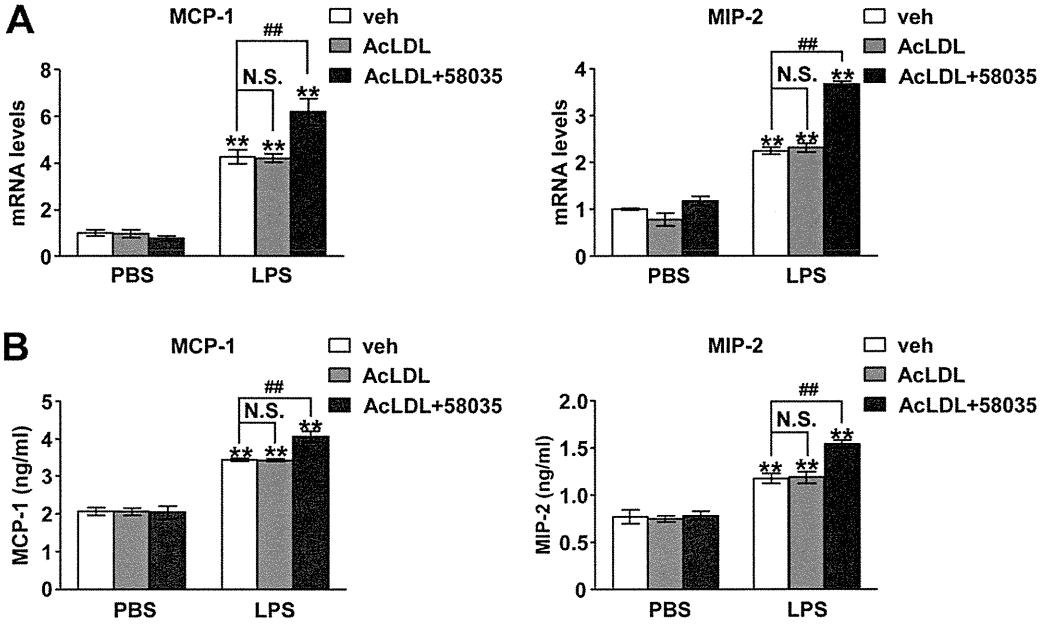
Supplementary Figure 5. Treatment with liposomal clodronate reversed BDL- and CCl_4-induced increase of TNFα and interleukin (IL)-1β mRNA expression in liver. WT C57BL/6 mice were injected with liposomal chlodronate (200 μL/mouse, intravenously) or vehicle. Thereafter, animals were subjected to (*A*) BDL or (*B*) CCl_4 intoxication to induce liver fibrosis. Hepatic expression of TNFα and IL-1β mRNA was shown. $^{**}P < .01$ compared with the (*A*) control diet–sham-operated group or the (*B*) control diet–corn oil group.



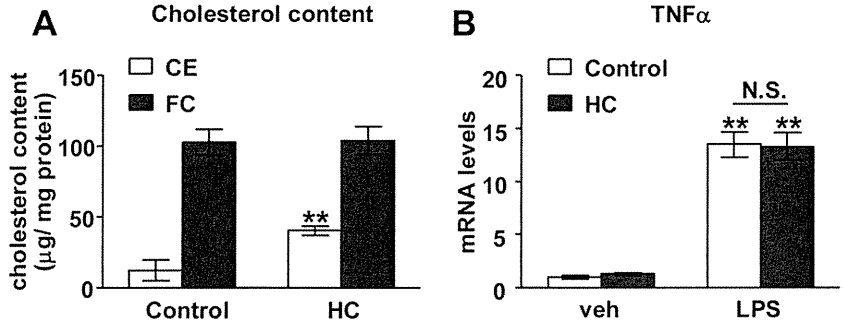
Supplementary Figure 6. Effects of the HC diet on hepatic expression of Bambi and TGFβ mRNA. Hepatic expression of Bambi and TGFβ mRNA (N = 3/group) after being fed a control or an HC diet for 4 weeks. $^{*}P < .05$ vs control diet group.



Supplementary Figure 7. Quantification of cellular FC and CE in late endosomes/lysosomes in HSCs. With late endosomes/lysosomes in WT HSCs, the mean (±SD) TC content was 48.46 ± 33.57 mg/mg cell protein. In late endosomes/lysosomes in NPC1 KO HSCs, the mean (±SD) TC content was increased significantly to 178.68 ± 81.39 mg/mg cell protein. Similarly, with late endosomes/lysosomes in HSCs from the control diet group, the mean (±SD) TC content was 33.82 ± 7.34 mg/mg cell protein. In late endosomes/lysosomes in HSCs from the HC diet group, the mean (±SD) TC content was increased significantly to 89.08 ± 6.57 mg/mg cell protein. (*A*) Quantification of FC and CE in late endosomes/lysosomes in HSCs from WT mice or NPC1-deficient mice. $^{**}P < .01$ vs WT mouse group. (*B*) The FC and CE levels in late endosomes/lysosomes in HSCs from control diet–fed mice or HC diet–fed mice. $^{**}P < .01$ vs control-diet group.



Supplementary Figure 8. FC, but not CE, promotes LPS-induced TLR4 signal transduction in HSCs. Paik et al[3] showed that LPS acts directly through TLR4 and then activates nuclear factor-κB to induce the production of inflammatory cytokines, including interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1), in human activated HSCs. Seki et al[4] also showed a strong up-regulation of MCP-1 and macrophage inflammatory protein-2 (MIP-2: mouse homologue of human IL-8) mRNA in mouse HSCs after LPS stimulation. Based on these reports, we evaluated the LPS responsiveness of HSCs loaded with FC using 2 highly quantitative methods: real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) of TLR4-induced inflammatory cytokines such as MCP-1 and MIP-2. (*A*) Expression of MCP-1 and MIP-2 mRNA in primary HSC cultures. HSCs were incubated with vehicle, AcLDL (50 μg/mL), or AcLDL plus compound 58035 (10 μg/mL) for 16 hours, and then treated with LPS (100 ng/mL) or not for 6 hours (N = 5–7/group). (*B*) Secreted MCP-1 and MIP-2 were quantified by ELISA. HSCs were incubated with vehicle, AcLDL, or AcLDL plus compound 58035 for 16 hours, and then treated with LPS or not for 24 hours (N = 5/group). $^{**}P < .01$ vs the corresponding culture without LPS treatment in each group. $^{\#\#}P < .01$ vs the LPS-treated control culture.



Supplementary Figure 9. Effects of the HC diet on cholesterol contents in Kupffer cells. We determined the level of cholesterol inside Kupffer cells. With Kupffer cells from the control diet group, the mean (±SD) TC content was 115.03 ± 22.61 μg/mg cell protein. In Kupffer cells from the HC diet group, the mean (±SD) TC content was increased significantly to 144.11 ± 38.83 μg/mg cell protein. (*A*) Quantification of cellular FC and CE in Kupffer cells from control diet–fed or HC diet–fed mice. Cholesterol concentrations were expressed as micrograms per milligrams of cellular proteins (N = 4/group). $^{**}P < .01$ vs the control diet group. (*B*) Expression of TNFα mRNA in primary Kupffer cell cultures (N = 5/group). Kupffer cells isolated from control diet–fed or HC diet–fed mice were treated with LPS (100 ng/mL) for 6 hours. $^{**}P < .01$ vs the control diet–control culture.

Supplementary Table 1. Effects of the HC Diet on Body Weight, Liver Weight, and Serum Lipid Levels

	Sham				BDL			
	Control diet		HC diet		Control diet		HC diet	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Body weight, g	28.2	0.3	27.2	0.6	27.5	2.3	21.8	0.3
Liver weight, g	1.4	0.1	1.2	0.1	1.7	0.2	2.1	0.2
Serum TG level, mg/dL	130.2	4.5	125.4	6.9	72.1	8.6	75.5	15.6
Serum TC level, mg/dL	103.2	9.8	135.0[a]	3.0	150.7	22.6	407.0[b]	17.7
Serum glucose level, mg/dL	116.6	5.4	120.8	17.6	85.3	26.7	103.0	23.0
	Corn oil				CCl_4			
Body weight, g	23.8	0.5	24.3	0.9	23.2	0.2	24.3	0.6
Liver weight, g	1.1	0.0	1.2	0.1	1.1	0.1	1.2	0.0
Serum TG level, mg/dL	229.4	19.6	173.8	20.6	170.8	16.9	193.5	12.0
Serum TC level, mg/dL	120.0	4.5	138.5[c]	9.0	118.0	4.1	143.8[d]	8.2
Serum glucose level, mg/dL	102.2	9.3	88.3	5.9	86.3	6.9	103.5	12.1

SEM, standard error of the mean.
[a]$P < .05$ vs the control diet-sham-operated group.
[b]$P < .01$ vs the control diet-BDL-operated group.
[c]$P < .05$ vs the control diet-corn oil group.
[d]$P < .05$ vs the control diet-CCl_4 group.

RESEARCH PAPER

OncoImmunology 1:5, 581–592; August 2012; © 2012 Landes Bioscience

Cross-priming for antitumor CTL induced by soluble Ag + polyI:C depends on the TICAM-1 pathway in mouse CD11c$^+$/CD8α^+ dendritic cells

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Keywords: cross-presentation, dendritic cell, TLR3, TICAM-1 (TRIF), tumoricidal CTL

Abbreviations: APC, antigen-presenting cells; CTL, cytotoxic T lymphocytes; DAMP, damage-associated molecular pattern; DC, dendritic cells; IFN, interferon; IPS-1, IFNβ promoter stimulator-1; MDA5, melanoma differentiation associated gene 5; Mf, macrophages; NK, natural killer; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptors; PV, poliovirus; RIG-I, retinoic acid inducible gene-1; SL8, an OVA tetramer; TICAM-1, Toll-IL-1 receptor homology domain-containing molecule-1; TLR, Toll-like receptor; WT, wild-type

PolyI:C is a nucleotide pattern molecule that induces cross-presentation of foreign Ag in myeloid dendritic cells (DC) and MHC Class I-dependent proliferation of cytotoxic T lymphocytes (CTL). DC (BM or spleen CD8α$^+$) have sensors for dsRNA including polyI:C to signal facilitating cross-presentation. Endosomal TLR3 and cytoplasmic RIG-I/MDA5 are reportedly responsible for polyI:C sensing and presumed to deliver signal for cross-presentation via TICAM-1 (TRIF) and IPS-1 (MAVS, Cardif, VISA) adaptors, respectively. In fact, when tumor-associated Ag (TAA) was simultaneously taken up with polyI:C in DC, the DC cross-primed CTL specific to the TAA in a syngenic mouse model. Here we tested which of the TICAM-1 or IPS-1 pathway participate in cross-presentation of tumor-associated soluble Ag and retardation of tumor growth in the setting with a syngeneic tumor implant system, EG7/C57BL6, and exogenously challenged soluble Ag (EG7 lysate) and polyI:C. When EG7 lysate and polyI:C were subcutaneously injected in tumor-bearing mice, EG7 tumor growth retardation was observed in wild-type and to a lesser extent IPS-1$^{-/-}$ mice, but not TICAM-1$^{-/-}$ mice. IRF-3/7 were essential but IPS-1 and type I IFN were minimally involved in the polyI:C-mediated CTL proliferation. Although both TICAM-1 and IPS-1 contributed to CD86/CD40 upregulation in CD8α$^+$ DC, H2K^b-SL8 tetramer and OT-1 proliferation assays indicated that OVA-recognizing CD8 T cells predominantly proliferated in vivo through TICAM-1 and CD8α$^+$ DC is crucial in ex vivo analysis. Ultimately, tumor regresses > 8 d post polyI:C administration. The results infer that soluble tumor Ag induces tumor growth retardation, i.e., therapeutic potential, if the TICAM-1 signal coincidentally occurs in CD8α$^+$ DC around the tumor.

Introduction

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are two major effectors for antitumor cellular immunity. These effectors are driven through activation of dendritic cells (DC) and/or macrophages (Mf), which is mediated by pattern-recognition receptors (PRRs) for the recognition of microbial patterns.[1,2] Antigen (Ag) presentation and upregulation of NK cell-activating ligands are major events induced in DC/Mf in response to PRRs, which link to evoking CTL- and NK-antitumor immunity, respectively. The immune-potentiating function of specific components of the classical adjuvants are largely attributable to the ligand activity of PRRs (CpG DNA/TLR9, polyI:C/TLR3, monophosphoryl lipid (MPL) A/TLR4, Pam2/TLR2, etc.).[3] That is, the DC/Mf competent to drive effectors are generated through PRR signal in inflammatory nest where affected cells and recruited immune cells encounter exogenous or endogenous PRR ligands. Since studying the functional properties of PRRs in tumor immunity is on the way using a variety of possible ligands and cell biological analyses, immune responses reflecting the total adjuvant potential around Ag-presenting cells (APC) in local inflammatory nests are not always elucidated even in mice.

RNA-sensing PRR pathways, including TLR3-TICAM-1, TLR7-MyD88 and RIG-I/MDA5-IPS-1 participate in driving Type I IFN induction and cellular immunity in DC subsets.[1,4,5] Type I IFN and the IFNAR pathway in DC and other cells reportedly evoke and amplify T cell immunity.[5,6] TLR7 resides exclusively in plasmacytoid DC[7] whereas TLR3 mainly exists in

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Submitted: 02/04/12; Revised: 03/02/12; Accepted: 03/02/12
http://dx.doi.org/10.4161/onci.19893

myeloid DC/Mf and epithelial cells.[8] They are localized on the membrane of the endosome and deliver the signal via their adaptors, MyD88 and TICAM-1.[7,8] RIG-I and MDA5 are ubiquitously distributed to a variety of mouse cells and signal the presence of cytoplasmic viral products through IPS-1.[9] Thus, TLR3 and RIG-I/MDA5 are candidates associated with DC maturation to drive effector cells.[10] Indeed, viral dsRNA analog, polyI:C, is a representative ligand for TLR3 and MDA5 and induces polyI:C-mediated DC-NK reciprocal activation.[11,12] These are also true in human DC.[13]

The point of this study is by which pathway antitumor CTL are induced for tumor regression in a mouse tumor-implant model. It has been postulated that DC present exogenous tumor Ag to the MHC Class I-restricted Ag-presentation pathway and proliferate CD8 T cells specific to the extrinsic Ag. When tumor cells provide soluble and insoluble exogenous Ag, this Class I Ag presentation occurs mostly TAP/proteasome-dependent, suggesting the pathway partly sharing with that for endogenous Ag presentation. This DC's ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation.[14] DC cross-presentation leads to the cross-priming and proliferation of Ag-specific CD8 T cells in vivo and in vitro.[14-18] A variety of PAMP[15,16] and intrinsic DAMP[17] as well as other factors including Type I IFN,[5,18] $CD4^+$ T cells[19] and NKT cells[20] augment cross-priming in tumor-bearing mice. However, by what molecular mechanism polyI:C enhances CTL induction in tumor-bearing mice remains largely unsettled.

Here, we made an EG7 tumor-implant mouse system and treated the mice with s.c.-injected ovalbumin (OVA)-containing cell lysates (Ag) and polyI:C. Spleen $CD8\alpha^+$ DC turn CTL-inducible when stimulated with Ag and polyI:C. In either case of s.c., i.p., or i.v. injection of polyI:C, the TLR3/TICAM-1 pathway predominantly participates in $CD8\alpha^+$ DC cross-priming and antitumor CTL induction. Earlier studies using non-tumor models, suggested that both TLR3 and MDA5 appeared to participate in polyI:C-dependent CTL induction.[21] TLR3 is predominantly involved in primary Ag response and Th1 skewing,[22] while MDA5 participates in secondary Ag response.[23] Importance of TLR3 in induction of cross-priming was first suggested by Schulz et al., who used OVA/polyI:C-loaded or virus-infected xenogenic (Vero) cells and mouse DC.[16] Here we demonstrate that the antitumor polyI:C activity is sustained by the TICAM-1 pathway in any route of injection in tumor-implant mice: antitumor CTL responses are mostly abrogated in TICAM-$1^{-/-}$ but not IPS-$1^{-/-}$ mice.

Results

Properties of EG7 tumor with high MHC in tumor-loading mice. The properties of the EG7 line we used are consistent with those reported previously.[24,25] It expressed high MHC Class I (H2-Kb) and no Qa-1b or Rae-1 (**Fig. S1**). The expression levels of these proteins were barely changed before and after implantation of EG7 cells into mice. Cell viability was not affected by in vitro stimulation with polyI:C only (**Fig. S1B**). However, a batch-to-batch difference of cell viability may have affected the rate of tumor growth in each mouse tumor-implant experiment.

$CD8^+$ T cells are responsible for tumor retardation by polyI:C. EG7 cells (2×10^6) were inoculated into the back of C57BL/6 (WT), and the indicated reagents were subcutaneously (s.c.) injected around the EG7 tumor (**Fig. 1A**). Growth retardation of tumor was observed by treatment with polyI:C or polyI:C plus EG7 lysate (**Fig. 1A**). EG7 lysate only had no effect on tumor regression. When $CD8\beta^+$ T cells were depleted before EG7 lysate/polyI:C treatment, polyI:C-mediated tumor growth suppression was cancelled (**Fig. 1A**), suggesting the participation of CD8 T cells in tumor growth suppression. The therapeutic potential of polyI:C appeared to be more reproducible in the presence of EG7 lysate than in the absence, judged from the increases of activated $CD8^+$ T cells (**Fig. 1B**) and cytotoxic activity (**Fig. 1C**) of LN T cells isolated from the mice sacrificed after the last therapy. Yet, the EG7 Ag could be more or less supplied from the implant tumor. $NK1.1^+$ cells did not participate in this EG7 tumor regression in this setting (data not shown).

Since EG7 lysate contains OVA, OVA-specific T cells in draining LN and spleen of the WT mice were counted by tetramer assay after the last therapy (**Fig. S2A and B**). The numbers of tetramer-positive cells were prominently increased in LN and spleen in mice with EG7 lysate and polyI:C. We confirmed the importance of simultaneous administration of Ag plus polyI:C for OVA-specific CTL induction as in **Figure S2C**, where pure Ag (OVA) was used instead of EG7 lysate for immunotherapy. The polyI:C adjuvant function appeared to be more efficient in the mixture of pure Ag than in polyI:C alone. Tumor regression (**Fig. S2C**) and OVA-specific CTL induction (**Fig. S2D**) were clearly observed in this additional experiment. To obtain reproducible data, we employed the EG7 lysate/polyI:C combination therapy as follows.

IFN-inducing pathways are involved in PolyI:C-derived EG7 growth retardation. We next inoculated EG7 cells (2×10^6) into the back of C57BL/6 (WT), TICAM-$1^{-/-}$, IPS-$1^{-/-}$, or TICAM-1/IPS-1 double-deficient (DKO) mice (**Fig. 2**). We s.c. administered EG7 lysate with or without polyI:C around the tumor. The EG7 lysate was the soluble fraction of EG7 which removed insoluble debris by centrifugation. The EG7 lysate contained unprecipitated micro-debris and soluble Ag. No other emulsified reagent was added for immunization. Thus, the adjuvant function of polyI:C per se is reflected in the tumor growth, although polyI:C had to be injected into mice twice a week. Retardation of tumor growth was observed $>$ 8 d after immunization with EG7 lysate + polyI:C in WT mice, though no growth retardation without polyI:C (**Fig. 2A**). The polyI:C-mediated tumor growth suppression was largely abrogated in TICAM-$1^{-/-}$ (**Fig. 2B**) and to a lesser extent in IPS-$1^{-/-}$ mice (**Fig. 2C**), and completely in TICAM-1/IPS-1 DKO mice (**Fig. 2D**). Hence, TICAM-1 plays an important role in inducing polyI:C-mediated tumor growth retardation in the s.c. setting we employed.

CD8 T cell activation induced by the TICAM-1 pathway. CD8 T cell activation in the inguinal LN was tested with polyI:C + EG7 lysate in EG7 tumor-bearing mice using CD69 as

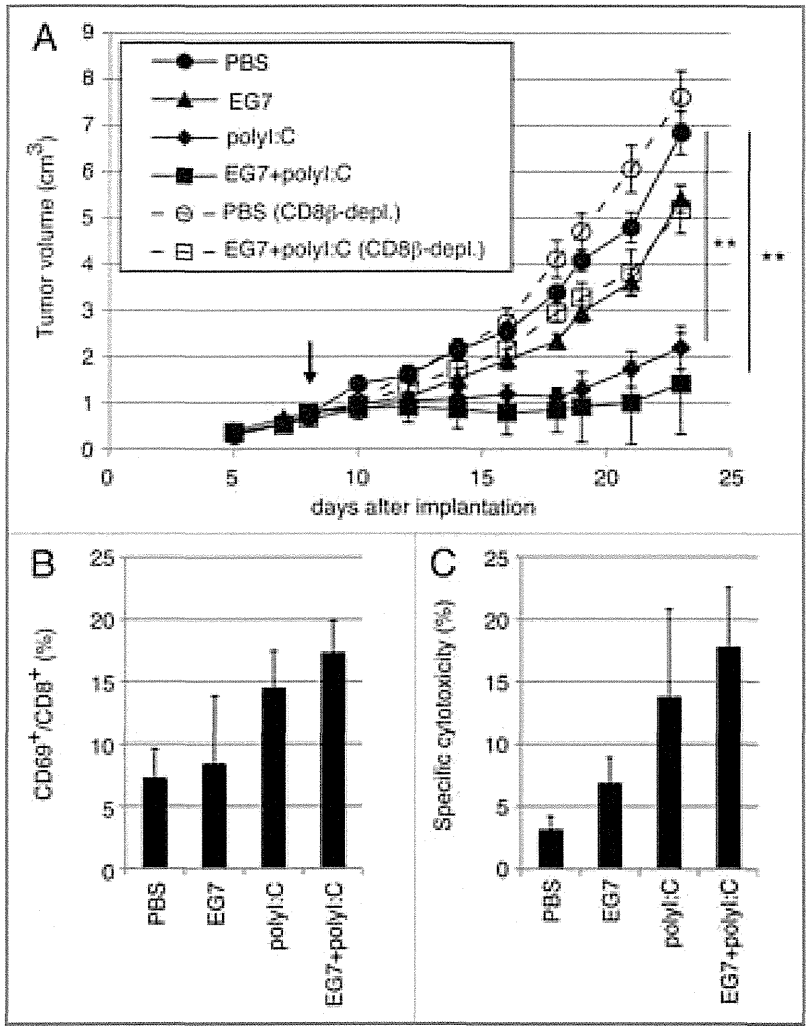

Figure 1. PolyI:C induces CTL-mediated tumor regression. (A) WT mice were challenged with EG7 cells and were treated with PBS (●), EG7 lysates (▲), polyI:C (♦) and EG7 lysates + polyI:C (■). The adjuvant therapy was started at the time indicated by the arrow and the indicated reagents injected twice per week. One of the two PBS groups (○) and one of the two EG7 lysates + polyI:C groups (□) were treated with anti-CD8β ascites in order to deplete $CD8^+$ T cells once a week. Each group had 3–5 mice. (B) Draining inguinal LNs were harvested 24 h after the last treatment and the proportion of CD69-expressing $CD8^+$ cells were counted. (C) LN cells were co-cultured with MMC-treated EG7 cells for 3 d and subjected to ^{51}Cr release assay to evaluate CTL activity. E/T = 50. All error bars used in this figure show ± SEM. Data are representative of two independent experiments. One-way analysis of variance (ANOVA) with Bonferroni's test was performed to analyze statistical significance. **, $p < 0.01$.

an activating marker. Twenty-four hours after the last polyI:C + EG7 sec.c. treatment, cells were harvested from the LN excised (**Fig. 3A**). FACS profiles of total cells from each mouse group are shown in **Fig. S3**. By combination therapy with EG7 lysate and polyI:C, T cells were activated in WT and IPS-$1^{-/-}$ mice, but the proportion of CD8 T cells was not affected by the therapy (**Fig. S4A**). Under the same conditions, T cells were barely activated in TICAM-$1^{-/-}$ mice in response to polyI:C (**Fig. 3A**). The proportion of $CD69^+$ cells are indicated in **Figure 3B**. IL-2 (**Fig. 3C**) and IFNγ (**Fig. S4B**) were highly induced in the WT and IPS-$1^{-/-}$ LN cells, while they were not induced in TICAM-$1^{-/-}$ or DKO cells. IFNγ levels were upregulated only in polyI:C-treated tumor-bearing mice, although the WT > IPS-$1^{-/-}$ profile for IFNγ production was reproducibly observed (**Fig. S4B**).

In vivo proliferation of CD8 T cells judged by tetramer assay and IFNγ induction. We next tested whether i.p. injection of polyI:C plus OVA induces CTL proliferation. PolyI:C and OVA were i.p. injected into mice and the polyI:C-dependent cross-priming of CD8 T cells were examined using the OVA tetramer assay. OVA-specific CD8 T cells were clonally proliferated in WT and IPS-$1^{-/-}$ mice, but not in TICAM-1/IPS-1 DKO and IRF-$3/7^{-/-}$ mice (**Fig. 4A**). Proliferation of OVA-specific CD8 T cells were severely suppressed in TICAM-$1^{-/-}$ mice (**Fig. 4A**), suggesting that polyI:C-mediated cross-priming of CD8 T cells largely depends on the TICAM-1 pathway followed by IRF-3/7 activation in the i.p. route. The results were reproduced in additional experiments using more mice (**Fig. 4B**) and $TLR3^{-/-}$ mice (**Fig. S5A and B**). The polyI:C cytokine response, where IFNα is IPS-1-dependent while IL-12p40 is TICAM-1-dependent, was also confirmed in serum level by polyI:C i.p. injection (**Fig. S5E**). Specific induction of IFNγ (**Fig. 4C**) was also observed in parallel with the results of **Figure 4A**.

Whether or not i.v. injection of polyI:C plus OVA induces Ag-specific CTL and cytotoxicity was next checked. OVA-specific OT-1 proliferation and cytotoxicity (**Fig. 4D and E**) were observed in in vivo analyses of WT and IPS-$1^{-/-}$ CD8 T cells but not of TICAM-$1^{-/-}$, TICAM-1/IPS-1 DKO, and IRF-$3/7^{-/-}$ mice in the i.v. setting.

Since TICAM-1 is the adaptor for TLR3 as well as cytoplasmic helicases,[24] we confirmed the level of cross-priming being decreased in $TLR3^{-/-}$ mice and an expected result was obtained (**Fig. S5A and B**). Furthermore, in $IFNAR^{-/-}$ mice, OVA-specific CTL induction was slightly reduced compared with that in WT mice, but higher than in TICAM-$1^{-/-}$ mice (**Fig. S5C and D**). Hence, in vivo cross-presentation induced by polyI:C mostly depends on the TLR3-TICAM-1 pathway followed by transcriptional regulation by IRF-3/7 in any administration route, and is further promoted by Type I IFN presumably produced by the stromal cells through the IPS-1 pathway.[26]

IPS-1 induces DC maturation but not cross-priming in vivo. Spleen DC maturation by i.v.-injected polyI:C was tested ex vivo using $CD8\alpha^+$ DC and $CD8\alpha^-$ DC isolated from WT or KO mice with no tumor as indicated in **Figure 5A**. The maturation markers CD86 and CD40 were upregulated on both $CD8\alpha^+$ and $CD8\alpha^-$

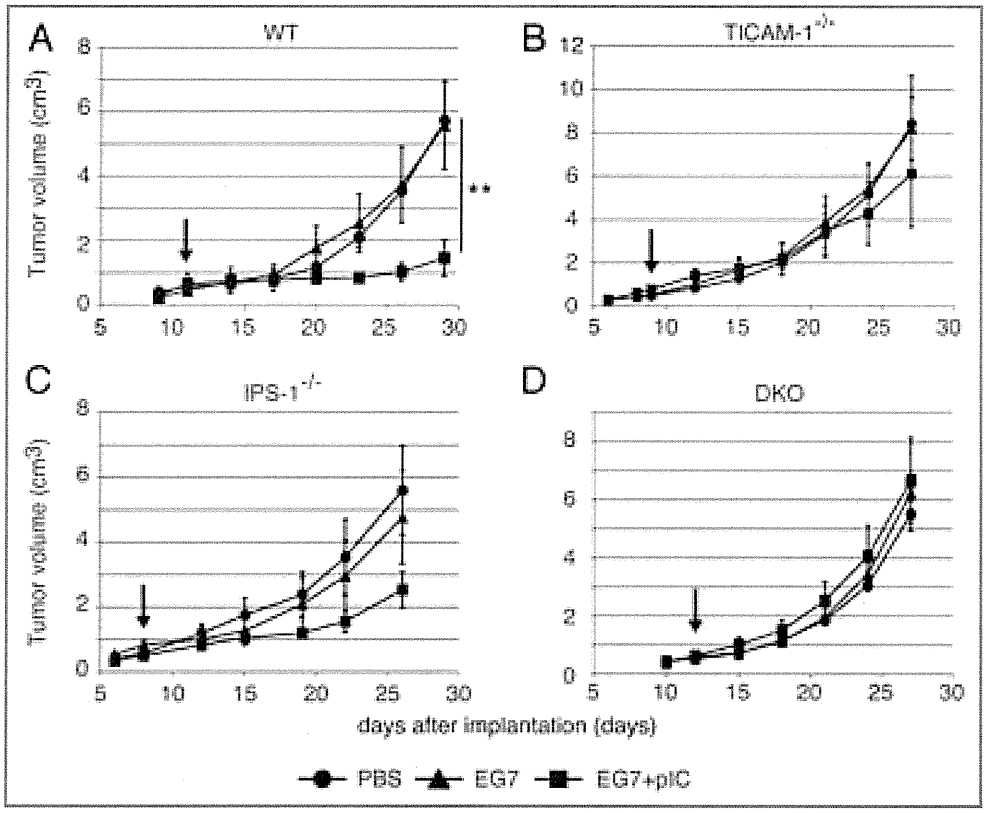

Figure 2. PolyI:C-induced tumor retardation is dependent on the TICAM-1 pathway. Antitumor effect of polyI:C on various KO mice were evaluated by using in vivo mouse tumor implant model. EG7 cells were inoculated to WT (A), TICAM-1$^{-/-}$ (B), IPS-1$^{-/-}$ (C) and DKO mice (D) on day 0. PBS (•), EG7 lysates (▲) or EG7 lysates + polyI:C (■) were s.c. administered around the tumor. The adjuvant therapies were started at the time indicated by the arrows and injected twice per week. Each group have 3–4 mice and error bar shows ± SEM. Data are representative of two independent experiments. **, $p < 0.01$

DC from WT mice when they were stimulated with OVA and polyI:C. Treatment of DC with OVA only did not induce upregulation of CD86 and CD40. Although the expression levels of CD86 and CD40 were a little less in CD8α$^+$ and CD8α$^-$ DC from TICAM-1$^{-/-}$ or IPS-1$^{-/-}$ mice than those from WT mice, both CD86 and CD40 were sufficiently upregulated even in the abrogation of either one pathway in polyI:C-injected mice. The CD86 and CD40 shifts were completely abolished in DKO mice (**Fig. 5A**). Thus, the TICAM-1 pathway participates in both potent co-stimulation and cross-priming, while the IPS-1 pathway mainly participates only in integral co-stimulation in myeloid DC.

We next assessed in vitro proliferation of OT-1 cells. CD8α$^+$ and CD8α$^-$ DC were prepared from PBS, polyI:C, OVA and OVA/polyI:C-treated mice, and mixed in vitro with CFSE-labeled OT-1 cells. WT, TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice were used for this study. OT-1 proliferation was observed with CD8α$^+$ DC but not CD8α$^-$ DC when OVA + polyI:C was injected (**Fig. 5B**). Furthermore, the OT-1 proliferation barely occurred in the mixture containing TICAM-1$^{-/-}$ CD8α$^+$ DC. Thus, OT-1 proliferation is triggered by the TICAM-1 pathway in CD8α$^+$ DC. Again, IPS-1 had almost no effect on OT-1 proliferation with CD8α$^+$ DC in this setting. In the mixture, IFNγ was produced in the supernatants of WT and IPS-1$^{-/-}$ CD8α$^+$ DC but not TICAM-1$^{-/-}$ DC by stimulation with OVA + polyI:C (**Fig. 5C**). No IFNγ was produced in the supernatants of CD8α$^-$ DC even from WT mice, which results are in parallel with those of OT-1 proliferation. In any case irrespective of tumor-bearing or not, Ag, polyI:C and the TICAM-1 pathway are mandatory for CD8α$^+$ DC to cross-prime and proliferate OVA-specific CD8 T cells.

We checked the TICAM-1- or IPS-1-specific gene expressions related to Type I IFN and MHC Class I presentation using genechip and qPCR (**Fig. S6**). PolyI:C-mediated upregulation of *Tap1*, *Tap2* and *Tapbp* messages diminished in TICAM-1$^{-/-}$ BMDC (**Fig. S6A**). The levels of these genes were hardly affected in IPS-1$^{-/-}$ BMDC (data not shown). PolyI:C-mediated upregulation was observed with MDA5 (*Ifih1*) in CD8α$^-$ and CD8α$^+$ DCs (**Fig. S6B**). Surprisingly, other factors including TLR3, TICAM-1 and MAVS messages were all downregulated in response to polyI:C in CD8α$^+$ DC (**Fig. S6B**), for the reason as yet unknown.

Effect of TLR3-mediated IFN-inducing pathway on anti-tumor CTL induction. PolyI:C is a dsRNA analog capable of incorporating into the endosome and cytoplasm by exogenous administration in vitro.[27,28] However, no evidence has been proposed that polyI:C is internalized into the endosome of

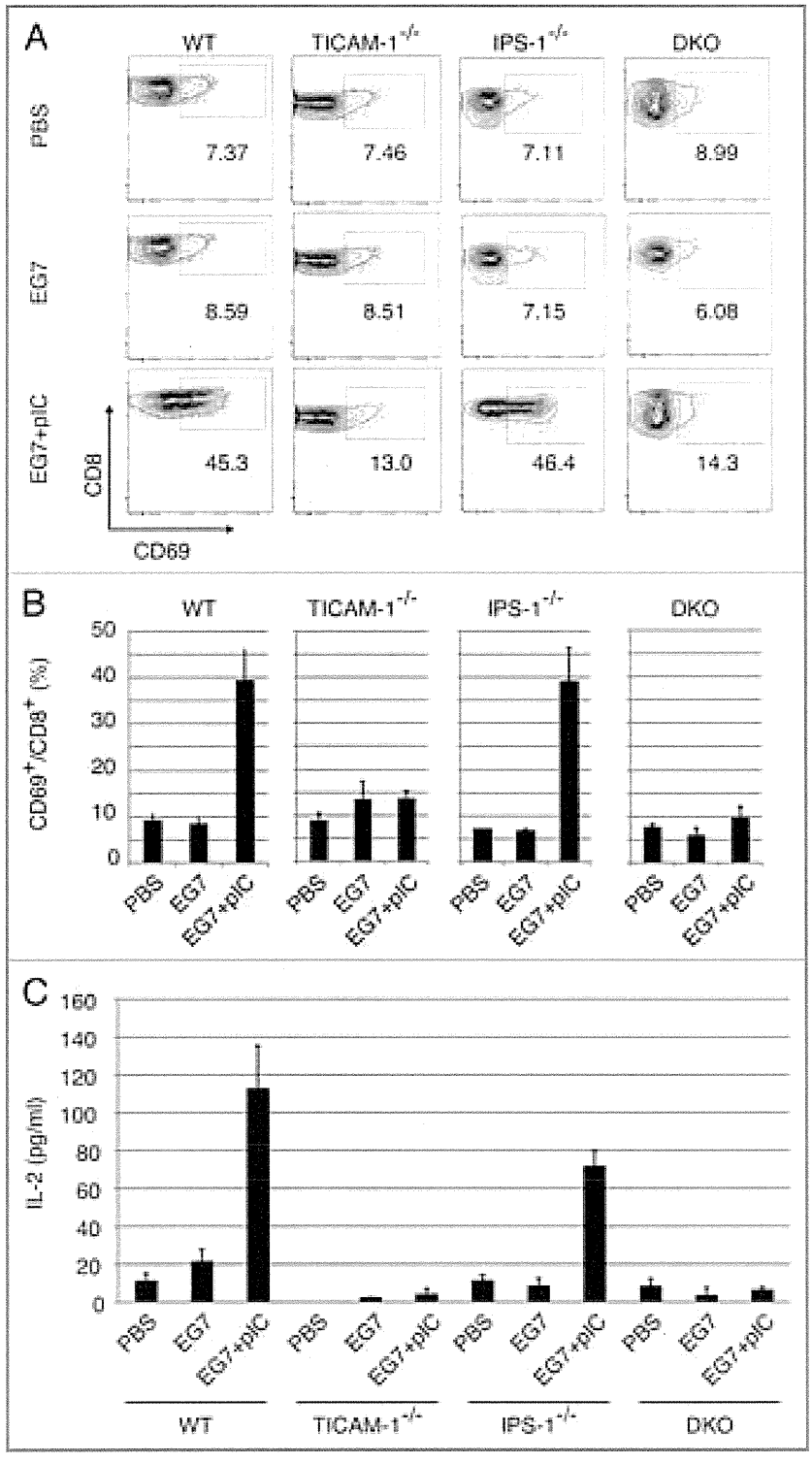

Figure 3. CD8 T cells in the draining LNs are activated through the TICAM-1 pathway by polyI:C. Draining inguinal LNs were harvested from tumor-bearing mice 24 h after the last treatment. LN cells were stained with CD3ε, CD8α and CD69, and the cells gated on $CD3\varepsilon^+CD8\alpha^+$ are shown (A). Spleen cells in each group of mice were stained separately, the CD8 levels in gated cells being variably distributed in FACS analyses. The average frequency of activated CD8 T cells defined by CD69 expression is shown (B). Alternatively, LN cells from the indicated mice were cultured for further 3 d in vitro and IL-2 production was measured by CBA assay (C).

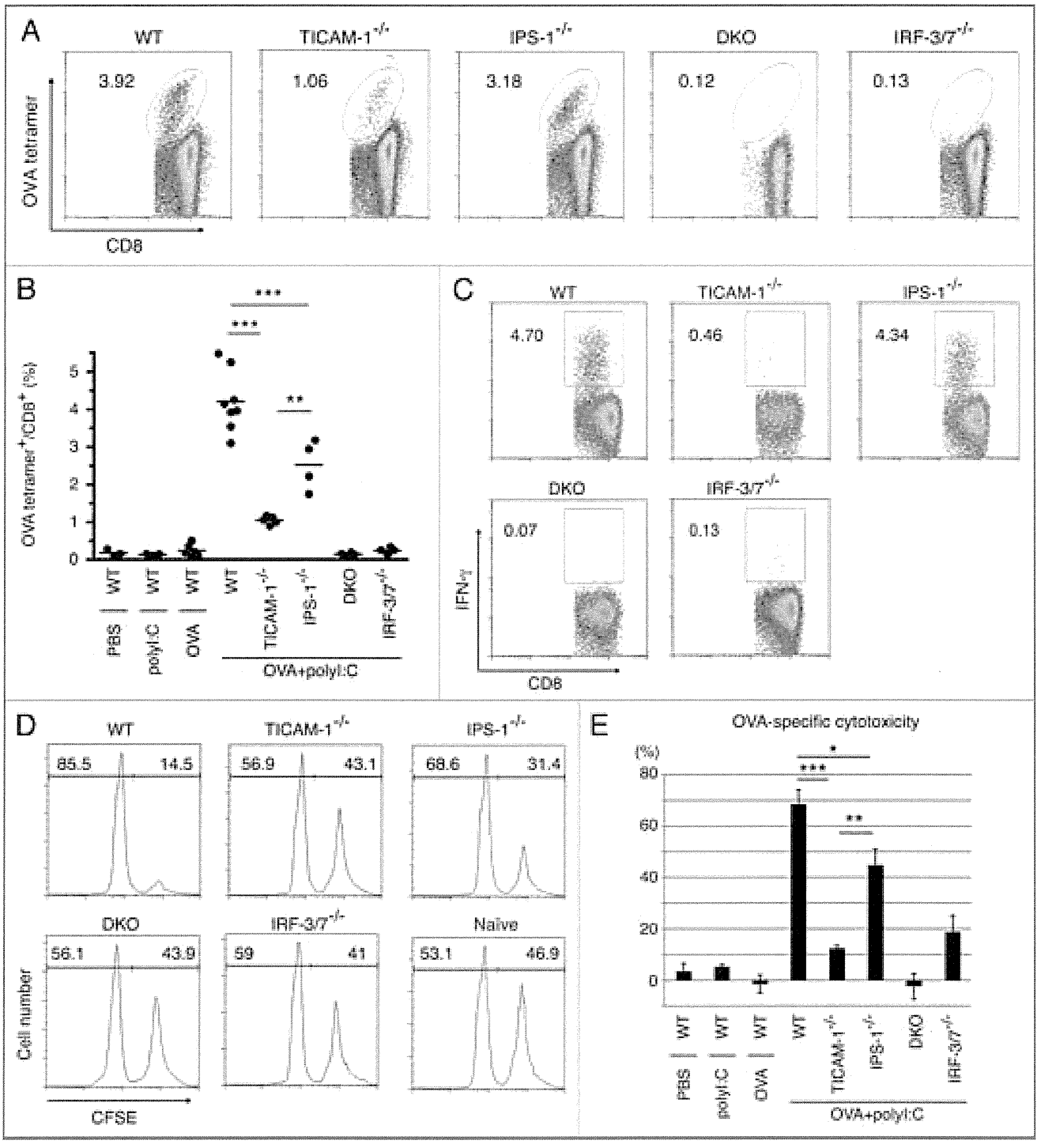

Figure 4. TICAM-1 and IRF-3/7 are essential for polyI:C-induced antigen-specific CTL expansion. WT, TICAM-1$^{-/-}$, IPS-1$^{-/-}$, TICAM-1/IPS-1 DKO and IRF-3/7$^{-/-}$ mice were i.p. administered with the combination of OVA and polyI:C. After 7days, splenocytes were harvested and stained with CD8α and OVA tetramer (A). The average percentages of OVA-specific CTL are shown (B). Alternatively, splenocytes were cultured in vitro in the presence of SL8 for 8 h and IFNγ production was measured by intracellular cytokine staining (C). To assess the killing activity, in vivo CTL assay was performed. The combinations of OVA and polyI:C were administered i.v. to each group of mice and 5 d later, cytotoxicity was measured (D). The data shown are collaborate or representative of at least three independent experiments. One-way analysis of variance (ANOVA) with Bonferroni's test was performed to analyze statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

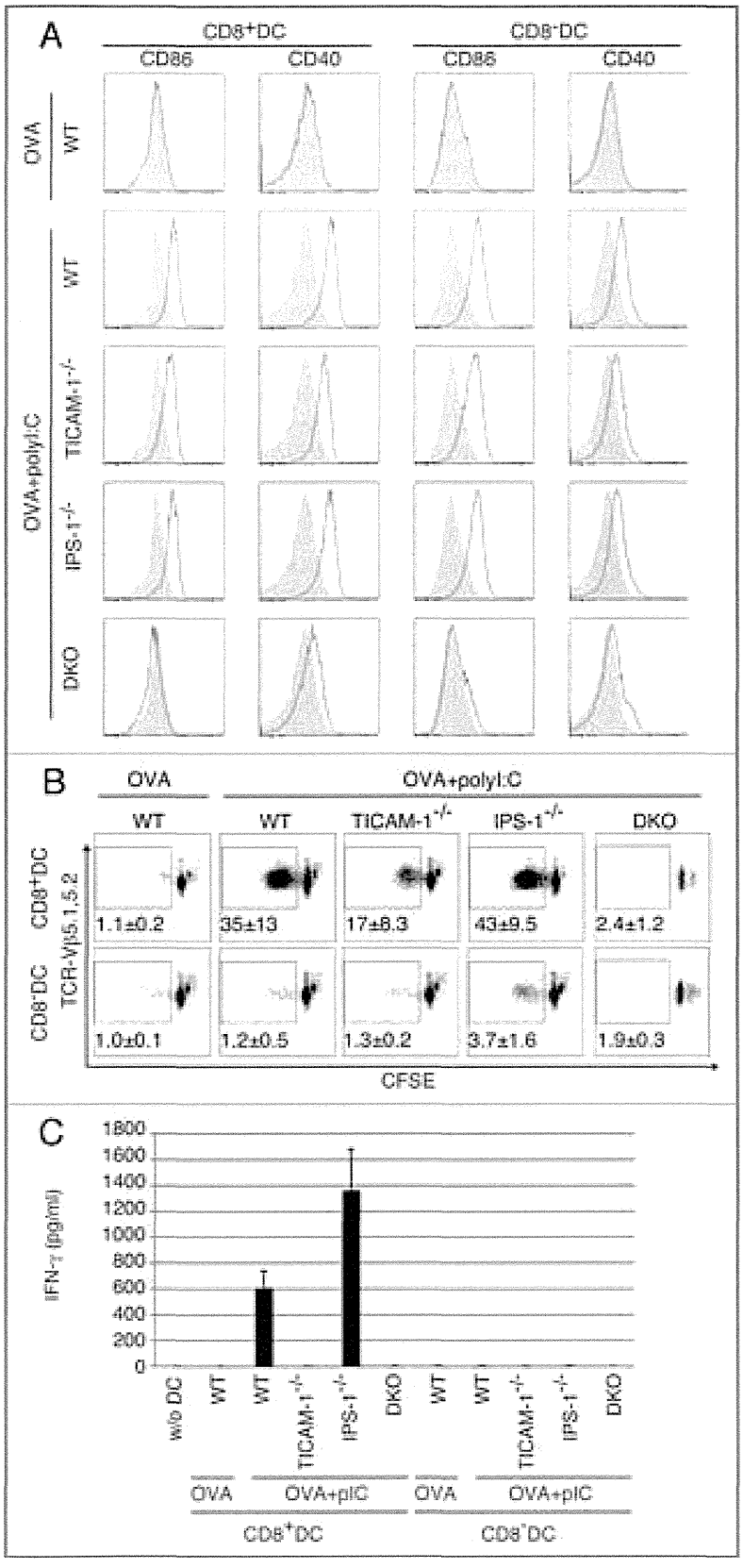

Figure 5. TICAM-1 in CD8α⁺ DC is more important than IPS-1 in polyI:C-induced cross-priming. OVA and polyI:C were administered i.v. and 4 h later, CD8α⁺ and CD8α⁻ DC were isolated from the spleen. CD86 and CD40 expressions were determined by FACS (A). Filled gray and black line show isotype control and target expression, respectively. Alternatively, CD8α⁺ and CD8α⁻ DC were co-cultured with CFSE-labeled RAG2$^{-/-}$/OT-1 T cells for 3 d. The cross-priming activity of each DC subset was determined with sequential dilution of CFSE (B) and IFNγ production (C). IFNγ was measured by CBA assay. The data shown are representative of two independent experiments. Err bar shows SD.

CD8α⁺ DC where TLR3 is expressed in vivo. Peritoneal (PEC) Mf and bone marrow-derived DC[22] usually phagocytoze polyI:C and deliver them into the endosome. In mouse CD8α⁺ DC direct internalization of polyI:C has remain unproven. Using labeled polyI:C and anti-mouse TLR3 mAb, 11F8,[22] we checked whether the exogenously-added polyI:C encountered with TLR3 in CD8α⁺ DC in vitro. TLR3 (green) was merged with TexasRed-polyI:C 30–120 min after polyI:C stimulation in the culture (**Fig. 6A**). The quantities of CD8α⁺ and CD8α⁻ DC where FITC-polyI:C was incorporated were determined by FACS analysis (**Fig. 6B**). Thus, the process by which polyI:C injected reaches the endosomal TLR3 is delineated in the CD8α⁺ DC.

Discussion

PolyI:C is an analog of virus dsRNA, and acts as a ligand for TLR3 and RIG-I/MDA5. PolyI:C has been utilized as an adjuvant for enhancement of antitumor immunity for a long time.[29] However, the mechanistic background of the therapeutic potentials of polyI:C against cancer has been poorly illustrated. It induces antitumor NK activation through DC-NK cell-to-cell interaction when CD8α⁺ DC TLR3 is stimulated in the spleen.[11] Besides myeloid cells, however, some tumor cell lines express TLR3 and dsRNA targeting tumor cells may affect the growth rate of tumors,[30] where the receptor-interacting protein (RIP) pathway is involved downstream of TICAM-1.[31] Here we showed evidence that polyI:C injection facilitates maturation of TLR3-positive CD8α⁺ DC (i.e., APC) to trigger CTL induction against exogenous soluble Ags including EG7 lysate or OVA. The TICAM-1 adaptor for TLR3 and IRF-3/7 are involved in the cross-presentation signal in CD8α⁺ DC, but the molecule/mechanism downstream of TICAM-1 that governs cross-presentation remains elusive. Since most of the tumor-associated Ags (TAA) are predicted to be liberated from tumor cells

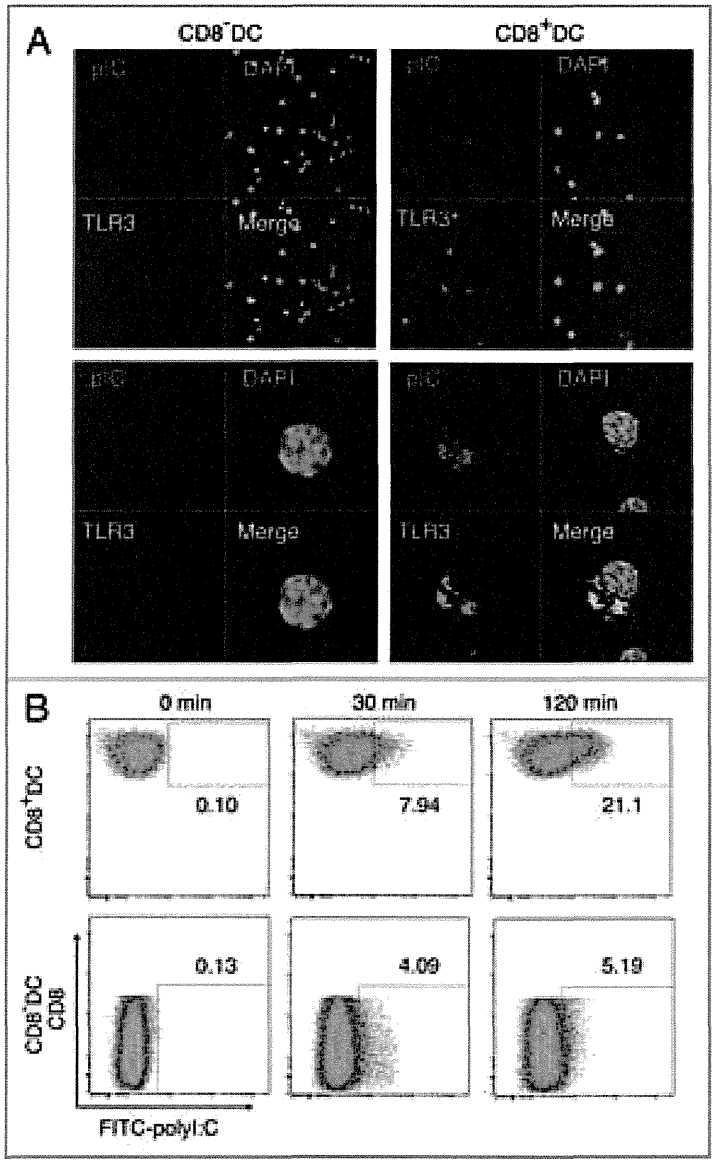

Figure 6. PolyI:C encounters TLR3 in CD8α⁺ DC. CD8α⁺ and CD8α⁻ DC were isolated by FACSAriaII and stimulated with 20 μg/ml TexasRed-polyI:C for 2 h. Then cells were stained with Alexa647-antiTLR3 and subjected to confocal microscopic analysis (A). Alternatively, splenic DC isolated by MACS were incubated with FITC-polyI:C for the time shown in figure and analyzed the degrees of polyI:C uptake by FACS (B). Data shown are the representative of three independent experiments.

as soluble Ags, the TICAM-1 pathway in CD8α⁺ DC would be crucial for driving of tumor-specific CTL around the tumor microenvironment. In any route of polyI:C injection, this is true as shown first in this study. Although TICAM-1 is an adaptor of other cytoplasmic sensors, DDX1, DDX21 and DHX36,[32] the antitumor CTL responses are merely relied on TLR3 of CD8α⁺DC in this system. Taken together with previous reports,[11,12] TICAM-1 signaling triggers not only NK activation but also CTL induction.

TLR3 and MDA5 are main sensors for dsRNA and differentially distributed in myeloid cells.[33,34] TLR3 is limitedly expressed in myeloid, epithelial and neuronal cells,[33] whereas MDA5 is ubiquitously expressed including non-myeloid stromal cells.[33] Several reports suggested that i.v. injection of polyI:C predominantly stimulate the stromal cells which express IFNAR,[26] thereby robust type I IFN are liberated from these cells to be a systemic response including cytokinemia and endotoxin-like shock.[35,36] Both TLR3 and MDA5 link to the IRF-3/7-activating kinases leading to the production of IFNα/β.[37,38] Once IFNα/β are released, IFNAR senses it to amplify the Type I IFN production,[39] and reportedly this amplification pathway involves cross-priming of CD8 T cells in viral infection.[18] Tumor progression or metastasis can be suppressed through the IFNAR pathway.[40] These scenarios may be right depending on the conditions employed. Our message is related to what signal pathway is fundamentally required for induction of antitumor CTL in DC. The CTL response is almost completely abrogated in TICAM-1$^{-/-}$ and IRF-3/7$^{-/-}$ mice, but largely remains in IPS-1$^{-/-}$ and IFNAR$^{-/-}$ mice when Ag and polyI:C are extrinsically administered. The results are reproducible in some other tumor-implant models (data not shown), and even in IFNAR$^{-/-}$ mice, TICAM-1-specific genes are upregulated to confer tumor cytotoxicity (**Fig. S6**, Azuma et al., unpublished data). In addition, the upregulation of these genes is independent of IPS-1 knockout in DC. Our results infer that the primary sensing of dsRNA in CD8α⁺ DC is competent to induce cross-presentation, which minimally involves the IPS-1 or IFNAR amplification pathway, at least at a low dose of polyI:C. Yet, subsequent induction of Type I IFN via the IFNAR may further amplify the cross-priming.[18,41] Further studies are needed as to which of the TICAM-1-inducible genes link to the cross-presentation in CD8α⁺ DC.

The main focus of this study was to identify the pathway for transversion of immature DC to the CTL-driving phenotype by co-administration of polyI:C with soluble Ag. The IPS-1 pathway, although barely participates in antitumor CTL driving, can upregulate CD40/CD86 co-stimulators on the membranes of splenic CD8α⁺ and CD8α⁻ DC in response to polyI:C, suggesting that MDA5 does function in the cytoplasm of splenic CD8α⁺ and CD8α⁻ DC to sense polyI:C. However, effective CTL induction happens only in CD8α⁺ DC when stimulated with polyI:C. CD8α⁺ DC express TLR3 but CD8α⁻ DC do not, and CD8α⁺ DC with no TLR3 fail to induce CTL, suggesting that integral co-stimulation by MDA5/IPS-1 is insufficient for DC to induce cross-priming of CD8 T cells: antitumor CTL are not induced until the TICAM-1 signal is provided in DC. At least, sole effect of the IPS-1 pathway and upregulation of co-stimulators on CD8α⁺ DC is limited for cross-priming and induction of antitumor CTL, which result partly reflects those in a previous report where IPS-1 and TICAM-1 harbor a similar potential for CD8 T cell proliferation when

polyI:C (Alum-containing) is employed as an adjuvant for CD8α+ DC to test proliferation of anti-OVA CTL.[21]

A question is why TICAM-1 is dominant to IPS-1 for response to exogenously-added polyI:C in CD8α+ DC. The answer is rooted in the difference of functional behavior between BMDC and CD8α+ DC. TLR3 levels are variable depending upon subsets of DC,[22] which affects DC subset-specific induction of cellular immune response. The high TLR3 expression (partly surface-expressed) is situated in CD8α+ DC before polyI:C stimulation, which is distinct from the properties of F4/80+ Mf and presumably BMDC of low TLR3 expression. The polyI:C-uptake machinery[15] appears to efficiently work in concert with the TLR3/TICAM-1 pathway in CD8α+ DC and this tendency is diminished when CD8α+ DC are pretreated with Alum + polyI:C.[21] Furthermore, there are functional discrepancies between CD8α+ splenic DC and GM-CSF-induced BMDC, which appears to reflect the difference of their TLR3 levels.[22] These results on CD8α+ DC encourage us to develop dsRNA adjuvant immunotherapy supporting TAA soluble vaccines for cancer applicable to humans, which possess the counterpart of CD8α+ DC.

There are two modes of dsRNA-mediated DC maturation, intrinsic and extrinsic modes that are governed by the IPS-1 and TICAM-1 pathways, respectively.[9,34] It is important to elucidate the in vivo qualitative difference in the two pathways in tumor-loading mice. TLR3+ DC/Mf are responsible for CTL driving via an extrinsic route in viral infection.[34] Previous data suggested that dsRNA in infectious cell debris, rather than viral dsRNA produced in the cytoplasm of Ag-presenting cells or autophagosome formation, contribute to fine tuning of DC maturation through extrinsic dsRNA recognition.[16] It is reported that dsRNA-containing debris are generated secondary to infection-mediated cell death,[41] and DC phagocytose by-stander dead cells. Likewise, soluble tumor Ags released from tumor cells usually are extrinsically taken up by APC in patients with cancer.[42] If CTL are successfully induced in therapeutic biotherapy targeted against cancer cells, this extrinsic TICAM-1 pathway must be involved in the therapeutic process.

Cross-presentation occurs in a TAP-dependent[43] and -independent fashions.[44,45] The peptides are transported by TAP into the endoplasmic reticulum (ER) and loaded onto MHC Class I for presentation at the cell surface. ER and phagosome might fuse each other for accelerating cross-presentation.[46] Another possibility is that cross-presentation occurs in early endosomes where TLR3 resides. This early endosome cross-presentation does not always depend on TAP[42-44] but requires TLR stimulation.[34] TLR4/MyD88 pathway is involved in the TAP-dependent early endosome model,[43] where recruitment of TAP to the early endosomes is an essential step for the cross-presentation of soluble Ag. These models together with our genechip analysis of polyI:C-stimulated BMDC suggested that some ER-associated proteins are upregulated in BMDC by polyI:C-TICAM-1 pathway. The results infer that the TLR3/TICAM-1 rather than the TLR4/MyD88 pathway more crucially participates in cross-presentation in response to dsRNA or viral stimuli and facilitates raising CTL antitumor immunity in APC.

Although multiple RNA sensors couple with TICAM-1 and signal to activate the Type I IFN-inducing pathway,[25] at least TLR3 in the CD8α+ DC are critical in CTL driving. CD8α+ DC are a high TLR3 expresser, while BMDC express TLR3 with only low levels.[22] CD8α- DC do not express it.[22] The Ag presentation and TLR3 levels in CD8α+ DC appear reciprocally correlated with the phagocytosing ability of DC. Although the TLR3 mRNA level is downregulated secondary to polyI:C response after maturation, this may not be related to the CD8α+ DC functions. Yet, polyI:C might interact with other cytoplasmic sensors for DC maturation,[32,47]

The route of administration and delivery methods may be important for culminate the polyI:C adjuvant function. The toxic problem has not overcome in the adjuvant therapy using polyI:C[35,36] and this is a critical matter for clinical introduction of dsRNA reagents to immunotherapy. The most problematic is the life-threatening shock induced by polyI:C. Recent advance of polyI:C study suggests that PEI-jet helps efficient uptake of polyI:C into peritoneal macrophages.[48] LC (poly-L-lysine and methylcellulose) has been used as a preservative to reduce the toxic effect of polyI:C.[49] Nanotechnological delivery of polyI:C results in efficient tumor regression.[50] There are many subsets of DC that can be defined by surface markers, and selecting an appropriate administration route can target a specific DC subset. The route for s.c. administration usually mature dermal/epidermal DC or Langerhans cells.[51,52] Some DC subsets with unique properties specialized to CTL induction would work in association with the route of polyI:C administration. Attempting to develop more harmless and efficient dsRNA derivatives will benefit for establishing human adjuvant immunotherapy for cancer.

Materials and Methods

Mice. TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice were made in our laboratory and backcrossed more than eight times to adapt C57BL/6 background.[12] IRF-3/7$^{-/-}$ and IFNAR$^{-/-}$ mice were kindly provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). TLR3$^{-/-}$ mice were kindly provided by S. Akira (Osaka University, Osaka, Japan). Rag2$^{-/-}$ and OT-1 mice were kindly provided from Drs N. Ishii (Tohoku University, Sendai, Japan). Rag2$^{-/-}$/OT-1 mice were bred in our laboratory. All mice were maintained under specific pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine. Animal experiments were performed according to the guidelines set by the animal safety center, Hokkaido University, Japan.

Cells. EG7 and C1498 cells were purchased from ATCC and cultured in RPMI1640/10% FCS/55 μM 2-ME/1 mM sodium pyruvate and RPMI1640/10% FCS/25 ng/ml 2-ME, respectively. Mouse splenocytes, OT-1 T cell, CD8α+ DC and CD8α- DC were harvested from the spleen and cultured in RPMI1640/10% FCS/55 μM 2-ME/10 mM HEPES.[41] B16D8 cells were cultured in RPMI/10% FCS as described previously.[12]

Reagents and antibodies. Ovalbumin (OVA) and polyI:C (polyI:C) were purchased from SIGMA and Amersham Biosciences, respectively. $OVA_{257-264}$ peptide (SIINFEKL: SL8)

and OVA ($H2K^b$-SL8) Tetramer were from MBL. Following Abs were purchased: anti-CD3ε (145-2C11), anti-CD8β (53-6.7), anti-CD11c (N418), anti-CD16/32 (93), anti-CD69 (H1.2F3) and anti-IFNγ(XMG1.2) Abs from BioLegend, anti-B220 (RA3-6B2), anti-CD4 (L3T4), anti-CD40 (1C10), anti-CD86 (GL1), and anti-MHC I-SL8 (25-D1.16) Abs from eBiosciences, anti-TCR-Vβ5.1/5.2 Ab and ViaProbe from BD Biosciences. The Rat anti-mouse TLR3 mAb (11F8) was kindly provided by David M. Segal (National Institute of Health, Bethesda, MD). To rule out LPS contamination, we treated OVA or other reagents with 200 μg/ml of Polymixin B for 30 min at 37°C before use. Texas Red- or FITC-labeled poly(I:C) was prepared using the 5' EndTag™ Nucleic Acid Labeling System (Vector Laboratories) according to the manufacturers instructions.

Tumor challenge and poly I:C therapy. Mice were shaved at the back and s.c. injected with 200 μl of 2×10^6 syngenic EG7 cells in PBS. Tumor volumes were measured at regular intervals by using a caliper. Tumor volume was calculated by using the formula: Tumor volume (cm^3) = (long diameter) × (short diameter)^2 × 0.4. A volume of 50 μl of a mixture consisting of the lysate of 2×10^5 EG7 cells with or without 50 μg of poly I:C (polyI:C) was s.c. injected around the tumor. We added no other emulsified reagent for immunization since we want to role out the conditional effect of the Ag/polyI:C. The treatments were started when the average of tumor volumes reached at 0.4–0.8 cm^3 and performed twice per week. EG7 lysate were prepared by three times freeze/thaw cycles (-140°C/37°C) in PBS, with removal of cell debris by centrifugation at 6,000 g for 10 min.[53] To deplete CD8 T cells, mice were i.p. injected with hybridoma ascites of anti-CD8β mAb. The dose of antibody and the treatment regimens were determined in preliminary studies by using the same lots of antibody used for the experiments. Depletion of the desired cell populations by this treatment was confirmed by FACS for the entire duration of the study.

Evaluation of T cell activity in tumor-bearing mice. Draining inguinal LN cells were harvested from tumor-bearing mice after 24 h from the last polyI:C treatment. The activity of T cells was evaluated by CD69 expression and IL-2/IFNγ production. These cells were stained with FITC-CD8α, PE-CD69, PerCP/Cy5.5-7AAD and APC-CD3ε. To check cytokine production, LN cells were cultured for 3 d in vitro in the presence or absence of EG7 lysates and IL-2 and IFNγ productions were determined by Cytokine Beads Array (CBA) assay (BD). To assess the cytotoxic activity of CTL, standard ^{51}Cr release assay was performed. For CTL expansion, 2.5×10^6 LN cells were co-cultured with 1.25×10^5 mitomycin C-treated EG7 cells in the presence of 10 U/ml IL-2 for 5 d. Then, LN cells were incubated with ^{51}Cr-labeled EG7 or C1498 cells for 4 h and determined cytotoxic activity. The cell-specific cytotoxicity was calculated with subtracting the cytotoxity for C1498 from for EG7 cells.

Antigen-specific T cell expansion in vivo. Mice were i.p. immunized with 1 mg of OVA and 150 μg of poly I:C. After 7 d, spleens were homogenized and stained with FITC-CD8α and PE-OVA Tetramer for detecting OVA-specific CD8 T cell populations. For intracellular cytokine detection, splenocytes were cultured with or without 100 nM OVA peptide (SIINFEKL; SL8) for 8 h and 10 μg/ml of Brefeldin A (Sigma-Aldrich) was added to the culture in the last 4 h. Then cells were stained with PE-anti-CD8α and fixed/permeabilized with Cytofix/Cytoperm (BD Biosciences) according to manufacturer's instruction. Then, fixed/permeabilized cells were further stained with APC-anti-IFNγ. Stained cells were analyzed with FACSCalibur (BD Biosciences) and FlowJo software (Tree Star).

In vivo CTL assay. The in vivo CTL assay was performed as described.[54] In brief, WT, TICAM-$1^{-/-}$, $MAVS^{-/-}$ and IRF-$3/7^{-/-}$ mice were i.v. administered with PBS, 10 μg of OVA or OVA with 50 μg of polyI:C. After 5 d, 2×10^7 target cells (see below) were i.v. injected to other irrelevant mice and 8 h later, the OVA-specific cytotoxicity was measured by FACSCalibur. Target cells were 1:1 mixture of 2 μM SL8-pulsed, 5 μM CFSE-labeled splenocytes and SL8-unpulesed, 0.5 μM CFSE-labeled splenocytes. OVA-specific cytotoxicity was calculated with a formula: {1-(Primed [$CFSE^{high}$(%)/$CFSE^{low}$(%)]/Unprimed [$CFSE^{high}$(%)/$CFSE^{low}$(%)]} × 100.

DC preparation. DCs were prepared from spleens of mice, as described previously.[55] In brief, collagenase-digested spleen cells were treated with ACK buffer and then washed with PBS twice. Then splenocytes were positively isolated with anti-CD11c MicroBeads. $CD11c^+$ cells were acquired routinely about ≥ 80% purity. Further, to highly purify $CD8\alpha^+$ and $CD8\alpha^-$ DCs, spleen DC were stained with FITC-CD8α, PE-B220, PE/Cy7-CD11c and PerCP5.5-7AAD. $CD8\alpha^+$ or $CD8\alpha^-$ $CD11c^+B220^-$ DCs were purified on FACSAriaII (BD). The purity of the cells was ≥ 98%.

OT-1 proliferation assay. Ten micrograms of OVA with or without 50 μg of polyI:C were i.v. injected to WT, TICAM-$1^{-/-}$, IPS-$1^{-/-}$ and DKO mice. After 4 h, $CD8\alpha^+$ or $CD8\alpha^-$ DC were purified from the spleen. 2.5×10^4 $CD8\alpha^+$ or $CD8\alpha^-$ DC were co-cultured with 5×10^4 1 μM CFSE-labeled Rag$2^{-/-}$/OT-1 T cells for 3 d in 96-well round bottom plate. These cells were stained with PE-anti-TCR-Vβ5.1,5.2 and APC-anti-CD3ε and T cell proliferation was analyzed by CFSE dilution using FACSCalibur. Additionally, IFNγ in the culture supernatant was measured by CBA assay.

Statistical analysis. P-values were calculated with one-way analysis of variance (ANOVA) with Bonferroni's test. Error bars represent the SD or SEM between samples.

Disclosure of Potenial Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We are grateful to Drs. T. Taniguchi (University Tokyo, Tokyo), N. Ishii (Tohoku University, Sendai) and D.M. Segel (NCI, Bethesda) for providing us with IRF-$3/7^{-/-}$ mice, OT-1 mice and anti-monse TLR3 mAb, respectively. Invaluable discussions about the peptide vaccine therapy with Dr. N. Satoh (Sapporo Medical

University, Sapporo) are gratefully acknowledged. We thank Drs H. Takaki, J. Kasamatsu, H.H. Aly, and H. Shime in our lab for their critical comments on this study.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research, MEXT) and the Ministry of Health, Labor, and Welfare of Japan, and by the Takeda and the Waxmann Foundations. Financial supports by a MEXT Grant-in-Project "The Carcinogenic Spiral" is gratefully acknowledged.

Supplemental Materials

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/oncoimmunology/article/19893/

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OncoImmunology 1:6, 917-923; September 2012; © 2012 Landes Bioscience

TLR3/TICAM-1 signaling in tumor cell RIP3-dependent necroptosis

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Keywords: interferon-inducing pathway, necroptosis, RIP signaling, TLR3, TICAM-1, TLR3, TRIF

Abbreviations: CTL, cytotoxic T lymphocyte; DAI, DNA-dependent activator of IFN-regulatory factors; DAMP, damage-associated molecular pattern; HMGB1, high-mobility group box 1; HSP, heat shock protein; mDC, myeloid dendritic cell; NK, natural killer; NLR, NOD-like receptor; PAMP, pathogen- associated molecular pattern; PRR, pattern-recognition receptor; RIP, receptor-interacting protein kinase; TICAM-1, Toll-IL-1-homology domain-containing adaptor molecule 1; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; TNFR1, TNFα receptor 1

The engagement of Toll-like receptor 3 (TLR3) leads to the oligomerization of the adaptor TICAM-1 (TRIF), which can induces either of three acute cellular responses, namely, cell survival coupled to Type I interferon production, or cell death, via apoptosis or necrosis. The specific response elicited by TLR3 determines the fate of affected cells, although the switching mechanism between the two cell death pathways in TLR3-stimulated cells remains molecularly unknown. Tumor necrosis factor α (TNFα)-mediated cell death can proceed via apoptosis or via a non-apoptotic pathway, termed necroptosis or programmed necrosis, which have been described in detail. Interestingly, death domain-containing kinases called receptor-interacting protein kinases (RIPs) are involved in the signaling pathways leading to these two cell death pathways. Formation of the RIP1/RIP3 complex (called necrosome) in the absence of caspase 8 activity is crucial for the induction of necroptosis in response to TNFα signaling. On the other hand, RIP1 is known to interact with the C-terminal domain of TICAM-1 and to modulate TLR3 signaling. In macrophages and perhaps tumor cell lines, RIP1/RIP3-mediated necroptotic cell death can ensue the administration of the TLR agonist polyI:C. If this involved the TLR3/TICAM-1 pathway, the innate sensing of viral dsRNA would be linked to cytopathic effects and to persistent inflammation, in turn favoring the release of damage-associated molecular patterns (DAMPs) in the microenvironment. Here, we review accumulating evidence pointing to the involvement of the TLR3/TICAM-1 axis in tumor cell necroptosis and the subsequent release of DAMPs.

Introduction

Cell death is an important process for both development and homeostasis in multicellular organisms. The mode of cell death is closely associated with other biological responses occurring within the host, including inflammation. Cell death has been categorized as apoptotic or necrotic and, until recently, apoptosis had been considered as a synonym of programmed cell death.[1] Caspases are a family of cysteine proteases that mediate apoptotic cell death in response to ligands of death receptors, including tumor necrosis factor α (TNFα), FAS ligand (FASL) and TRAIL, as well as to intracellular damage, upon the induction of pro-apoptotic BH3-only members of the Bcl-2 family. However, it is now clear that apoptosis is not the only cellular mechanism that mediates programmed cell death. Necrotic cell death, which has traditionally been viewed as a form of passive cell death, may also be regulated, and in this case has been called necroptosis or programmed necrosis.[2] Necroptosis may be induced by TNFα receptor 1 (TNFR1) agonists, but also by innate pattern-recognition receptors (PRRs) such as Toll-like receptor (TLR) 3 and TLR4.[1,4] These two TLRs can recruit the adaptor TICAM-1 (also known as TRIF), leading to Type I interferon (IFN) signaling.[3] In line with this notion, the TLR3 ligand polyI:C (a synthetic double-stranded RNA, dsRNA) can activate either apoptosis or necrosis, depending on the cell lines tested. Cell death induced by the TLR3-TICAM-1 axis may therefore be executed through two distinct subroutines.[5] The mechanisms that dictate the cellular decision to undergo apoptosis or necroptosis in response to TLR3 signaling, as well as the mechanisms that mediate the execution of necroptosis, are the subject of intense investigation.

Toll-like receptors and other PRRs harbor the ability to specifically recognize microbal molecules, known as pathogen-associated molecular patterns (PAMPs).[6] PAMPs trigger the maturation of myeloid dendritic cells (mDCs) through the activation of TLR and/or other pathways, eventually eliciting cellular immunity.[7] In mDCs, nucleic acid-recognizing TLRs (i.e., TLR3, TLR7, TLR8 and TLR9) reside in endosomes and sense their ligands only when they are internalized.[8] The uptake of DNA or RNA of microbial origin therefore allows cross-presentation to T cells and the exposure of natural killer (NK) cell-activating ligands. Besides this extrinsic maturation route, it is known that the formation of autophagosomes may deliver cytoplasmic nucleic acids of viral origin to the endosome via autophagy.[9] In either route, TLR signaling links immunological events to pathological cell death.

Recently accumulated evidence suggests that TLRs serve as receptors not only for foreign PAMPs but also for cellular

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Submitted: 05/28/12; Accepted: 06/22/12
http://dx.doi.org/10.4161/onci.21244

Table 1. Host response to nucleic acids and other DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids(PAMP)	
cytosolic long dsRNA	MDA5
cytosolic 5'-PPP-RNA	RIG-I
endosomal >140 bp dsRNA	TLR3
nonmethylated CpG DNA	TLR9
cytosolic dsDNA	DNA sensors*
Self molecular patterns(DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4,**
S100 proteins	RAGE
Self nucleic acids (DAMP)	
Self DNA	DNA sensors*
Self mRNA	TLR3

*See **Table 2**; ** D40, CD91, Scavenger receptors etc.

Table 2. RNA·DNA recognition molecules in vertebrates

Receptors	Adaptors	Ligands	Induction of Type I IFN
		TLR family	
TLR3	TICAM-1	dsRNA, stem RNA	+
TLR7/8	MyD88	ssRNA	+
TLR22	TICAM-1	dsRNA	+
PKR	?	dsRNA	-
		RLR family	
RIG-I	MAVS	5'-PPP RNA, dsRNA	+
MDA5	MAVS	dsRNA (long)	+
		NLR family	
NALP3	ASC	dsRNA	+
NOD2	MAVS	ssRNA	+
		DDX family	
DDX1	TICAM-1	dsRNA	+
DDX21	TICAM-1	dsRNA	+
DHX36	TICAM-1	dsRNA	+
		DNA sensors	
TLR9	MyD88	CpG DNA	+
DAI	TBK1	dsDNA	+
Pol3/RIG-I	MAVS	dsDNA	+
IFI16	TBK1	dsDNA	+
DDX41	STING	dsDNA	+
DHX9	MyD88	dsDNA	+
DDX36	MyD88	dsDNA	+
ZAPS	?	dsDNA	+

constituents that are liberated from damaged or necrotic cells.[10] Thus, innate pattern-recognition is not only a mechanism for discriminating pathogens from the host, but also a means for inspecting cellular homeostasis. Molecules that, upon release from damaged/necrotic cells, activate the immune system are called damage-associated molecular patterns (DAMPs).[11] The most popular TLR adaptor MYD88 is known to contain death domains, and some reports have suggested that TLR signaling may be involved in cell death secondary to PAMP/DAMP-stimulation. Necroptotic or damaged cells may thus represent a result of TLR death signaling, and generate a functional complex consisting of sources of DAMPs as well as of the phagocytic response.[11,12]

DAMPs refer to intracellular molecules that acquire inflammation-inducing capacities when released from cells. DAMPs do not belong to the cytokine family but rather resemble PAMP in their functional properties, in particular with regard to mDC and macrophages. The functions of DAMPs may be associated with responses including regeneration and tumorigenesis. During the past 5 years, necroptotic cell death has been closely connected with innate immune responses involving pattern-sensing.[12,13] DAMPs include a large number of cytosolic or nuclear molecules (**Table 1**), as well as, surprisingly, self nucleic acids.[14] This implies that, like viral DNA and RNA, autologous nucleic acids can evoke inflammation. Here, we discuss the importance of the immune modulation induced by nucleic acids and necroptotic host cells.

Necroptosis: Programmed Necrosis Induced by TNFα

TNFα has been reported to induce two different types of cell death, apoptosis and necrosis, in a cell type-specific manner.[15,16] Through TNFR1, TNFα is implicated in NFκB activation and contributes to cell growth in many cancer cell lines. In parallel TNFα-induced hemorrhagic necrosis has been observed in several cancer cell lines, but the molecular mechanisms underlying these differential responses to TNFα remain poorly understood. Recently, several reports have suggested that the formation of a supracomplex containing the receptor-interacting protein kinase 1 (RIP1) and its homolog RIP3 (which has been named "necrosome") is responsible for the switch from apoptosis to necroptosis.[17,18] RIP1 and RIP3 can assemble only in the absence of functional caspase-8, indicating that this enzyme acts as a key protease for blocking the formation of the necrosome.[5,19] Many viral factors, as well as the genomic instability that frequently characterizes tumor cells, can compromise caspase-8 function, thereby facilitating the induction of necroptosis. Hence, TNFα can promote cell death by signaling through its receptors, including TNRF1 and downstream via RIP1/RIP3, although the output of TNFα signaling is ultimately determined by cell type.

Virus-Mediated Necroptosis

It is notable that a necrotic phenotype has been observed in polyI:C-stimulated bone marrow-derived murine macrophages and other cell lines.[13] TICAM-1 and RIP3 are involved in this process, suggesting the implication of the necrosome pathway in dsRNA-mediated cell death.[12,13] It has been reported that viral

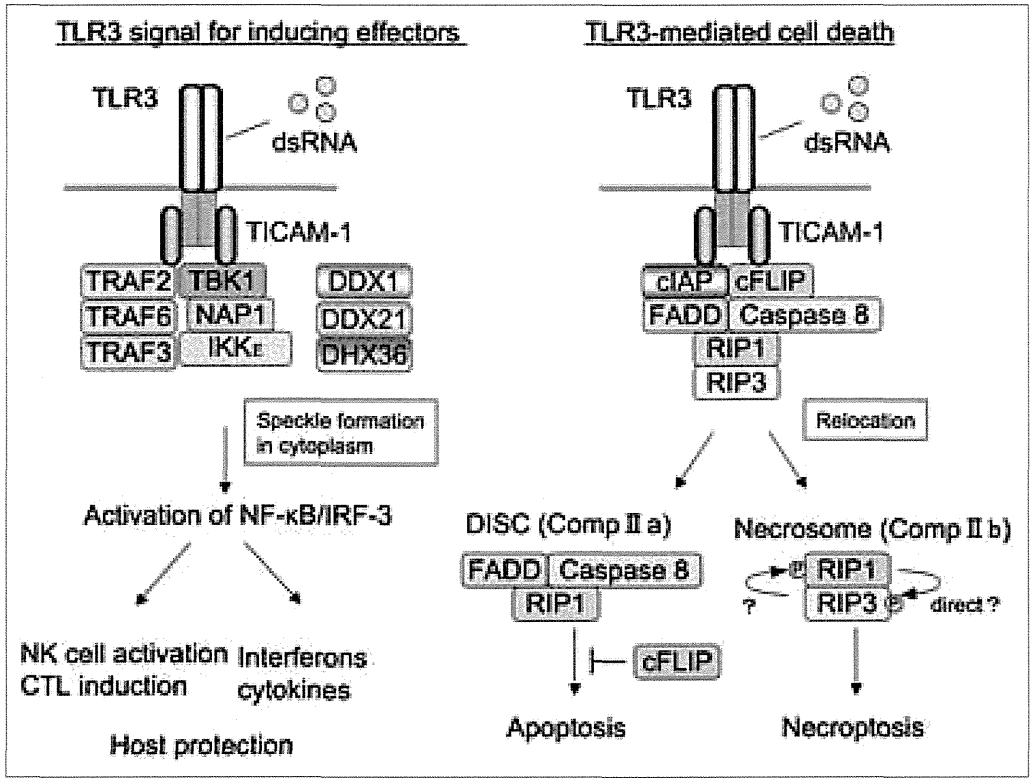

Figure 1. TLR3 signals inducing cell death or effector functions in myeloid cells. Cell survival (left panel) and cell death (right panel) signals are schematically depicted. TICAM-1 assembles in a supramolecular complex around oligomerized Toll-like receptor 3 (TLR3) in the endosome. The complex (named Speckle) then dissociates from TLR3, tranlocating to the cytoplasm. IRF-3 and NFκB are activated by Speckle, leading to their nuclear translocation and induction of Type I interferon (IFN) and inflammatory cytokines, respectively. In dendritic cells (DCs), natural killer (NK) cell-activating ligands and factors for cross-presentation are induced downstream of IRF-3/7 (left panel). In contrast, cell death signaling culminates in apoptosis and/or necrosis depending on downstream signal transducers (right panel). TLR3-dependent apoptosis has been reported in several cancer cell lines,[7] while TLR3-dependent necroptosis has been observed in mouse bone marrow-derived macrophages.[13] These events rely on RIP1/RIP3 activation, similar to those elicited upon ligation of the tumor necrosis factor α receptor 1 (TNFR1). Whether or not the translocation of the TICAM-1 complex is required for the cell death signaling, as well as the mechanisms determining either cytokine secretion or cell death, remain unknown.

dsRNA frequently induces apoptosis in infected cells, a process that in general is known as cytopathic effect.[20] TICAM-1 and RIPs, mainly RIP1, may also be involved in virus-derived necrotic cell death.[5,13] This is relatively rare compared with apoptosis since it occurs only when the viral genome encodes caspase-8 inhibitors.[19] Furthermore, this process requires viral dsRNA to be delivered from the cytosol to the endosomes (where TLR3 is situated) of infected cells. This may happen if the dsRNA is engulfed by autophagosomes, which ensure its transfer to endosomes. The possible involvement of another PRR that sense viral RNA, RIG-I/MDA5, in cell death as induced by viral infection cannot be always ruled out. TNFα can be produced downstream of the TLR3- and RIG-I-mediated RNA-sensing pathways and may induce necrotic cell death. Many RNA viruses trigger cell death,[20] but the factors determining the induction of necroptosis in virus-infected cells remain to be clarified.

DNA viruses can induce necroptosis via another mechanism, which involves the DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1/ZBP1).[21] DAI is a DNA sensor[22] and directly activates RIP3 in the absence of Type I IFN induction.[21] This said, the sensing of DNA in the cytoplasm of virus-infected cells is complex, and it may be that DAI is not the only molecule linked to such a necroptotic response. It is unknown whether RIP3-mediated necroptosis can be induced even if caspase-8 is blocked upon the recognition of viral DNA by DAI or via other mechanisms.[20] In fact, this type of virus-derived necrosis has been reported with DNA viruses that encode caspase inhibitors including vaccinia virus (VV), which encodes B13R/Spi2, poxvirus, encoding CrmA, the Kaposi sarcoma-associated herpesvirus (KSHV), encoding K13 and the molluscum contagiosum virus (MCV), which encodes MC159.[20,23] Generally speaking, the mode of cell death secondary to virus infection differ as a function of viral species. The physiological role of TLR3- and DAI-mediated necroptosis should therefore be analyzed in a virus-specific fashion.

Necroptosis in Inflammation

Apoptosis plays a major role in physiological contexts, while necrosis is very common under pathological conditions.[1] Necroptosis differs from accidental necrosis in its programmed nature, and differs from apoptosis in that necroptosis often stimulates inflammation. When virus-infected cells undergo apoptosis, they are removed by phagocytosis. Viral genomes, be they either

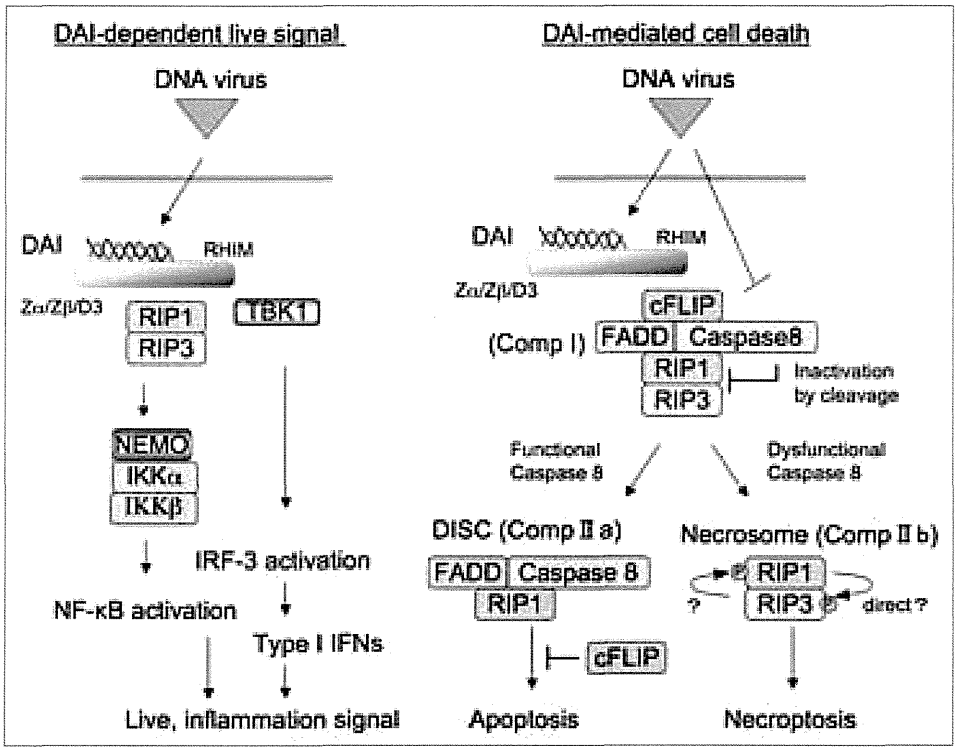

Figure 2. Necroptosis induced by the DAI pathway. Cell survival (left panel) and cell death (right panel) signals transmitted by the DNA-dependent activator of IFN-regulatory factors (DAI) are schematically depicted. Pro-survival signaling involves the activation of IRF-3 and NFκB to support antiviral responses (left panel). Type I IFNs and inflammatory cytokines are the main effectors induced by IRF-3/NFκB activation. In contrast, DAI activates RIP3 to induce necroptosis during viral infection, provided that caspases are inhibited. When viruses express caspase inhibitors, the RIP1/RIP3 necrosome plays a dominant role in the activation of cell death via necroptosis (right panel). If caspase-8 is active, RIP3 should get inactivated and apoptosis should be the dominant phenotype, though this scheme has not yet been experimentally confirmed. The mechanisms determining the choice between these two signaling pathways are unknown.

DNA- or RNA-based, are degraded in infected cells, thus being able neither to stimulate phagocytes including macrophages and DCs, nor to favor the liberation of DAMPs. In contrast, non-apoptotic cell death is accompanied by the release of DAMPs and viral products, resulting in the activation of macrophages,[13] as it occurs during chronic infection, in which viruses produce caspase inhibitors or render infected cells resistant to apoptosis.[24] A typical model of necroptosis evokes two effectors, namely, viral nucleic acids and DAMPs, to modulate immune and bystander cells of the host. In the context of necroptosis, these effectors allow for the amplification of inflammatory responses by myeloid phagocytes (mDCs and macrophages). These cells accumulate in inflammation as induced by persistent viral infection, and mediate the secondary release of cytokines and other biologically active molecules. In addition, viral factors can result in incipient inflammation, as observed in chronic infections by the hepatitis B or C virus.[24] This, in conjunction with viral nucleic acids and DAMPs, may modify the features of the infectious milieu. Further studies are needed to clarify the importance of viral nucleic acids and DAMPs in the context of virus-dependent chronic inflammation, as it may facilitate tumor progression.

Necroptosis and Oncogenesis

Accumulating evidence indicates that pro-inflammatory signals, including those following the activation of NFκB, are crucial for oncogenesis. Moreover, DAMPs have been associated with tumorigenesis as well as with antitumor immune responses.[25,26] Tumor progression is not always accompanied by viral infections, and it remains unclear whether DAMPs released from non-infected tumor cells are sufficient to support tumor growth. It has been reported that self mRNA acts as a TLR3 ligand[14] and that self DNA can stimulate host cell sensors.[22,27] Due to the uncomplete identification and functional characterization of DNA sensors and their signaling pathways, however, it is unknown whether host nucleic acids are potent inducers of inflammation as compared with viral RNA or unmethylated CpG DNA of bacterial origin. Moreover, the role of RNA sensors in the tumor microenvironment has not yet been clarified (**Table 2**).

DAMPs have recently been characterized at the molecular level[11] and representative DAMPs (**Table 1**) include HMGB1,[28] uric acid crystal,[10] S100 proteins,[29] naked actin[30,31] and heat-shock proteins (HSPs).[32] The functional features of DAMPs and the mechanisms whereby they provoke inflammation have been delineated,[11,28,29] and these studies have introduced the concept of "inflammasome" in the field of innate immunity.[33] Caspase-1 is activated upon the administration of NOD-like receptor (NLR) ligands, which include some DAMPs as well as inorganic PAMPs. Active caspase-1, together with the upregulation of the immature variants of IL-1 family proteins that ensues TLR stimulation, accelerates the robust release of IL-1β, IL-18 and IL-33.[34] There are many kinds of NLRs as well as TLRs, and the common pathways (including those centered around the adaptor ASC) can be activated by a variety of cytoplasmic DAMPs and PAMPs.[33,34] The cytoplasmic immature forms of the abovementioned cytokines are activated by limited caspase-1-mediated proteolysis, and then are secreted into the extracellular microenvironment.[34] Hence, IL-1 family proteins require two DAMPs/PAMP signals for their upregulation and activation.[35] Of note, the tumorigenic properties of asbestos and silica are in part attributable to the activation of the inflammasome, leading to the secreton of IL-1 family proteins. However, not all DAMPs operate as inflammasome activators, even in the broad sense of this term.

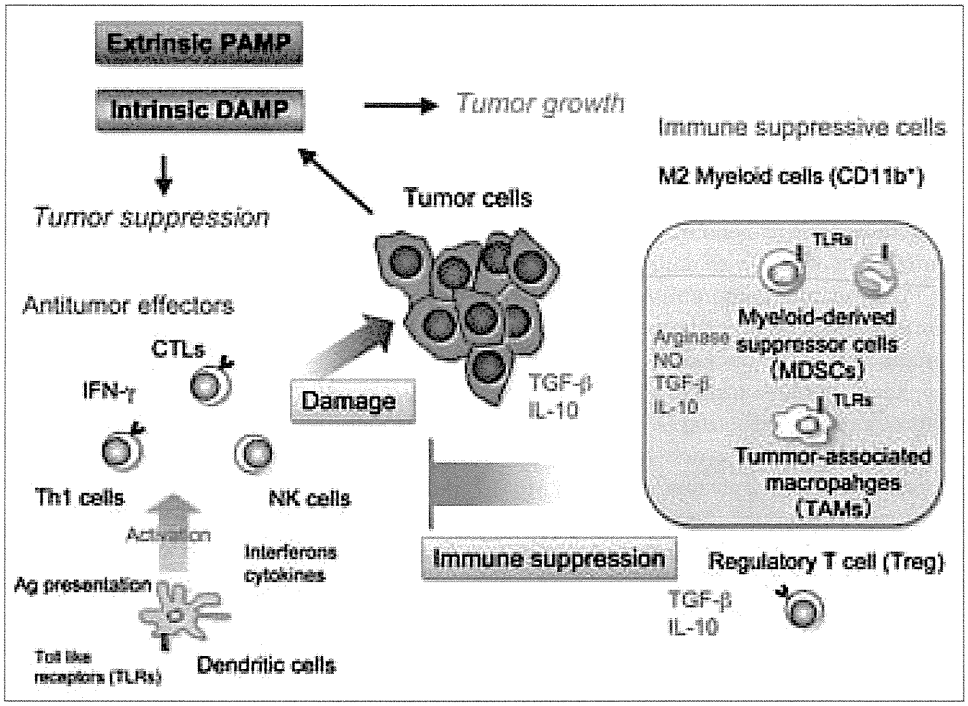

Figure 3. Inflammation provides the microenvironment for infection-related cancer. Immune cells infiltrating the tumor mass may modulate the local microenvironment upon the recognition of pathogen- or damage-associated molecular patterns (PAMP/DAMPs). Cancer cells undergoing necrosis liberate DAMPs and debris containing nucleic acids, which recruit immune cells stimulating an inflammatory response. In some cases, tumors benefit from the inflammatory response, while in other cases they regress following inflammation. The mechanisms determining this switch remain to be clarified.

Immune Response Elicited by the Phagocytosis of Dead Cells

Phagocytosis of dead cells involves not only cell clearance but also the initiation of an immune response. Dead cell antigens are rapidly presented on MHC Class II molecules after internalization by DCs, driving the recruitment and activation of various $CD4^+$ T cell subsets, including Th1, Th2, Th17 and regulatory T cells (Tregs) (**Fig. 1**). In the presence of a second co-stimulatory signal provided by TLRs, working as an adjuvant, DCs cross-present antigens on MHC Class I molecules to induce the proliferation of $CD8^+$ cytotoxic T lymphocytes (CTLs).[36] The presentation of exogenous antigens by DCs is therefore dependent on the presence of PAMPs/DAMPs.[36] Accordingly, necrotic debris appears to result in CTL cross-priming more efficiently than apoptotic bodies. Cross-presentation is enhanced by molecules such as Type I IFN and CD40, and by immune cells including $CD4^+$ T, NK and NKT cells. Hence, the use of adjuvants to affect many cell types of the immune system other than antigen-presenting cells, and a precise evaluation of the total cross-priming activity appear to be indispensable for the development of efficient adjuvant therapies.

The TLR3/TICAM-1 axis is best known as an inducer of cross-presentation in vivo.[37] The cross-presentation activity of the TLR3 ligands polyI:C and viral dsRNA was first described by Schulz et al. in 2005.[38] While the potency of polyI:C as an adjuvant has been reported by Steinman and colleagues,[37,39] the precise identity of the DAMPs participating in cross-presentation and possessing latent cross-priming (CTL-inducing) capacities has not yet been determined.

It is known that phagocytosis induces functional changes in mDCs and macrophages (**Fig. 2**): phagocytes are skewed toward a regulatory phenotype accompanied by the production of IL-10 and TGFβ during the phagocytosis of apoptotic cell debris, even in the presence of PAMP.[40,41] This suggests that material that cannot be taken up exerts different effects on mDCs than internalizable material during their phagocytic interactions. Phagocytes undergo cytoskeletal rearrangement when they take up cell debris, involving cell adhesion molecules that accelerate the interaction between the phagocyte membrane and cell debris. The opsonization of dead cells further enhances phagocytosis as well as the induction of an immune outcome.[42] Complement-mediated opsonization of dead cells strongly alters the functional properties of mDCs and macrophages.[43] Yet, it has been impossible to discriminate apoptotic and necroptotic cells based on this.[44] Thus, the mechanisms whereby necroptotic cells initiate an immune response upon phagocytosis by mDCs and macrophages, compared with apoptotic cells, remain largely uncharacterized. Elucidating the role of necroptotic cells and DAMPs as adjuvants for NK-cell activation and antigen presentation is highly relevant for antitumor therapy. Since the phagocytosis of dead cells by mDCs usually leads to the generation of tolerogenic mDCs, additional adjuvants appear to be required for mDCs to present tumor antigens in an immunogenic fashion, leading to the induction of an effective immune response against cancer.