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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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, H2Kb-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 (Mφ), which is mediated by pattern-recognition receptors (PRRs) for the recognition of microbial patterns.1 Antigen (Ag) presentation and upregulation of NK cell-activating ligands are major events induced in DC/Mφ 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/Mφ 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.4,5 Type I IFN and the IFNAR pathway in DC and other cells reportedly evoke and amplify T cell immunity.6,7 TLR7 resides exclusively in plasmacytoid DC whereas TLR3 mainly exists in

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myeloid DC/MF and epithelial cells. They are localized on the membrane of the endosome and deliver the signal via their adaptors, MyD88 and TICAM-1. RIG-I and MDA5 are ubiquitously distributed to a variety of mouse cells and signal the presence of cytoplasmic viral products through IFN-1. Thus, TRLR and RIG-I/MDA5 are candidates associated with DC maturation to drive effector cells. Indeed, viral dsRNA analog, poly(IC), is a representative ligand for TRLR and MDA5 and induces polyIC-mediated DC-NK reciprocal activation. These are also true in human DC. 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 DCs’ ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation. DC cross-presentation leads to the pathway partly sharing with that for endogenous Ag presentation. This DCs’ ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation. DC cross-presentation leads to the pathway partly sharing with that for endogenous Ag presentation. This DCs’ ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation. DC cross-presentation leads to the pathway partly sharing with that for endogenous Ag presentation. This DCs’ ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation. DC cross-presentation leads to the pathway partly sharing with that for endogenous Ag presentation. This DCs’ ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation. DC cross-presentation leads to the pathway partly sharing with that for endogenous Ag presentation. This DCs’ ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation. DC cross-presentation leads to the pathway partly sharing with that for endogenous Ag presentation. This DCs’ ability to deliver exogenous Ag to the pathway for MHC Class I-restricted Ag presentation has been described as cross-presentation. DC cross-presentation leads to the pathway partly sharing with that for endogenous Ag presentation.
An activating marker. Twenty-four hours after the last polyI:C + EG7 acc. c. treatment, cells were harvested from the LN excised (Fig. 3A). FACs profiles of total cells from each mosaic 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 or not 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−/− or DKO mice and IFNγ−/− mice (Fig. 4A). Proliferation of OVA-specific CD8+ T cells were severely suppressed in TICAM-1−/− and IPS-1−/− mice (Fig. 4A), suggesting that polyI:C-mediated cross-priming of CD8 T cells largely depends on the TLR3-IPS-1 pathway followed by IFNγ 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 IFNγ 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 analysis of WT and IPS-1−/− CD8 T cells but not of TICAM-1−/−, TICAM-1/IPS-1 DKO, and IPS-1−/− mice in the i.v. setting. Since TICAM-1 is the adaptor for TLR3 as well as cytoplasmic helicases, 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. The results were reproduced in additional experiments using more mice (Fig. 4D and E).
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-12 or IPS-12 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-12 and IPS-12 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-12 BMDC (Fig. S6A). The levels of these genes were hardly affected in IPS-12 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 CD3ε+CD8α+ 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).

|         | WT   | TICAM-1−/− | IPS-1−/− | DKO |
|---------|------|------------|----------|-----|
| PBS     | 7.37 | 7.46       | 7.11     | 8.99|
| EGF     | 8.59 | 8.51       | 7.15     | 6.06|
| EGF+HPC | 45.3 | 13.0       | 46.4     | 14.3|

**Table 1:**

|         | WT   | TICAM-1−/− | IPS-1−/− | DKO |
|---------|------|------------|----------|-----|
| PBS     | 0    | 0          | 0        | 0   |
| EGF     | 0    | 0          | 0        | 0   |
| EGF+HPC | 0    | 0          | 0        | 0   |

**Figure 3:** CD8 T cells in the draining LN 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 CD3ε+CD8α+ 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 7 days, splenocytes were harvested and stained with CD8a 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 collaborative 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 CD8^a^ 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, CD8^a^ and CD8^a^ 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, CD8^a^ and CD8^a^ 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 IFN-γ production (C). IFN-γ was measured by CBA assay. The data shown are representative of two independent experiments. Error bars show SD.

**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. 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 CD8^a^ DC TLR3 is stimulated in the spleen. Besides myeloid cells, however, some tumor cell lines express TLR3 and dsRNA targeting tumor cells may affect the growth rate of tumors, where the receptor-interacting protein (RIP) pathway is involved downstream of TICAM-1. Here we showed evidence that polyI:C injection facilitates maturation of TLR3-positive CD8^a^ 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 CD8^a^ 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...
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.

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 Alexa488Anti-TLR3 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.

TLR3 and MDA5 are main sensors for dsRNA and differentially distributed in myeloid, epithelial and neuronal cells,33 whereas MDA5 is ubiquitously expressed including non-mycloid stromal cells.6 Several reports suggested that i.v. injection of polyI:C predominantly stimulates 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.26,27 Both TLR3 and MDA5 link to the IRF-3/7-activating kinases leading to the production of IFNα.5,6 Once IFNαβ are released, IFNAR senses it to amplify the Type I IFN production,18 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.11 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−/− 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
polyIC (Alum-containing) is employed as an adjuvant for CD8α-DC to test proliferation of anti-OVA CTL.22 A question is why TICAM-1 is dominant to IPS-1 for response to exogenously-added polyIC 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,23 which affects DC subset-specific induction of cellular immune response. The high TLR3 expression (parity surface-expressed) is situated in CD8α-DC before polyIC stimulation, which is critical for the properties of from F4/80+Mφ and presumably BMDC of low TLR3 expression. The polyIC uptake machinery 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 + polyIC.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.21 These results on CD8α-DC encourage us to develop dRNA adjuvant immunotherapy supporting TAA soluble vaccines for cancer applicable to humans, which possess the counterpart of CD8α-DC.

There are two modes of dRNA-mediated DC maturation, intrinsic and extrinsic modes that are governed by the IPS-1 and TICAM-1 pathways, respectively.43-45 It is important to elucidate the in vivo qualitative difference in the two pathways in tumor-loading mice. TLR3+ DC/Mφ are responsible for CTL driving via an extrinsic route in viral infection.13 Previous data suggested that dRNA, in infectious cell debris, rather than viral dRNA produced in the cytoplasm of Ag-presenting cells or autophagosome formation, contribute to fine tuning of DC maturation through extrinsic dRNA recognition.46 It is reported that dRNA-containing debris are generated secondary to infection-mediated cell death,47 and DC phagocytose bystander dead cells. Likewise, soluble tumor Ags released from tumor cells usually are extrinsically taken up by APC in patients with cancer.48 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-dependent49 and -independent fashions.50,51 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.49 Another possibility is that cross-presentation occurs in early endosomes where TLR3 resides. This early endosome cross-presentation does not always depend on TAP49,51 but requires TLR stimulation.50 TLR4/MyD88 pathway is involved in the TAP-dependent early endosome model,52 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 polyIC-stimulated BMDC suggested that some ER-associated proteins are upregulated in BMDC, by polyIC-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 dRNA 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,20 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.21,22

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.21,22 This is a critical matter for clinical introduction of dRNA 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 peritumoral macrophages.44 LC (poly-L-lysine and methylcellulose) has been used as a preservative to reduce the toxic effect of polyI:C.44 Nanotechnological delivery of polyI:C results in efficient tumor regression.53 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.23,24 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 dRNA 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.22 IRF-3−/− 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 mM pyruvate and RPMI1640/10% FCS/25 mg/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 mM 2-ME/10 mM HEPES.54 B16D8 cells were cultured in RPMI100% FCS as described previously.55 Reagents and antibodies. Ovalbumin (OVA) and polyI:C (polylC) were purchased from SIGMA and Amersham Biosciences, respectively. OVA257-264 peptide (SIINFEKL: SL8)
and OVA (H2Kb-SL8) Tetramer were from MBL. Following Abs were purchased: anti-CD3 (145-2C11), anti-CD8 (53-6.7), anti-CD11c (N418), anti-CD16/32 (93), anti-CD69 (HI.10a) and anti-IFNγ (XMG1.2) Abs from BioLegend, anti-B220 (RA3-6B2), anti-CD4 (L3T4), anti-CD30 (1C10), anti-CD86 (GL1), and anti-MHC I LS8 (25-21.30.16) Abs from eBioscience, anti-TCR-Vβ1.5/1.2 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(1:C) was prepared using the 5’ EndTaq™ Nucleic Acid Labeling System (Vector Laboratories) according to the manufacturer’s instructions.

Tumor challenge and poly I:C therapy. Mice were shaved at the back and s.c. injected with 200 µl of 2 × 10⁶ syngeneic 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 (cm³) = (long diameter) × (short diameter)² ÷ 6. A volume of 50 µl of a mixture consisting of the lysate of 2 × 10⁶ EG7 cells with or without 50 µg of poly I:C (poly(I:C)) was s.c. injected around the tumor. We added no other emulsified reagent for immunization since we want to rule 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 cm³ 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-CD8+ 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 FACSS 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. Those cells were stained with FITC-CD8, PE-CD3, PerCP/Cy5.5, 7AAD and APC-CD3s. To check cytokine production, LN cells were cultured for 3 d in vitro in the presence of absence of EG7 lysates and IL-2 and IFNγ productions were determined by Cytokine Beads Array (CBA) assay (BD). To assess the cytotoxicity activity of CTL, standard 51Cr release assay was performed. For CTL expansion, 2.5 × 10⁵ mitomycin C-treated EG7 cells in the presence of 10 U/ml IL-2 for 5 d. Then, LN cells were incubated with 51Cr-labeled EG7 or C1498 cells for 4 h and determined cytotoxic activity. The cell-specific cytotoxicity was calculated with subtracting the cytotoxicity 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-CD8 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 (SINIFKL-SL8) for 4 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-CD8ε 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, Inc.).

In vivo CTL assay. The in vivo CTL assay was performed as described.54 In brief, WT, TICAM-1−/−, MAVS−/− and IRF-3−/− mice were i.v. administered with PBS, 10 µg of OVA or OVA with 50 µg of polyI:C. After 5 d, 2 × 10⁵ 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-unspeckled, 0.5 µM CFSE-labeled spleno- cytes. OVA-specific cytotoxicity was calculated with a formula: [1 - (Primed [CFSE±(%)]/Unprimed [CFSE±(%)]) × 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 CD8ε+ and CD8α+ DCs, spleen DC were stained with FITC-CD11c, PE-B220, PE-Cy7, CD11c and PerCP-Cy5.5. CD8α+ or CD8ε+ CD11c+B220 DCs were purified on FACS(AriaII) (BD). The purity of the cells was ≥ 98%.

OT-1 proliferation assay. Ten micromgrams of OVA with or without 50 µg of polyI:C were i.v. injected to WT, TICAM-1−/−, IFR-3−/− and DKO mice. After 4 h, CD8ε+ or CD8α+ DCs were purified from the spleen. 2.5 × 10⁵ CD8ε+ or CD8α+ DCs were co-cultured with 5 × 10⁴ 1 µM CFSE-labeled Rag2−/−OT-1 T cells for 3 d in 96-well round bottom plate. Those cells were stained with PE-anti-TCR-Vβ1.5.1.2 and APC-anti-CD3 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 Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials

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