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ORIGINAL RESEARCH

Biphasic function of TLR3 adjuvant on tumor and spleen dendritic cells promotes tumor T cell infiltration and regression in a vaccine therapy

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ABSTRACT

Successful cancer immunotherapy necessitates T cell proliferation and infiltration into tumor without exhaustion, a process closely links optimal maturation of dendritic cells (DC), and adjuvant promotes this process as an essential prerequisite. Poly(I:C) has contributed to adjuvant immunotherapy that evokes an antitumor response through the Toll-loke receptor 3 (TLR3)/TICAM-1 pathway in DC. However, the mechanism whereby Poly(I:C) acts on DC for T cell proliferation and migration remains undetermined. Subcutaneous injection of Poly(I:C) regressed implant tumors (WT1-C1498 or OVA-EG7) in C57BL/6 mice, which coincided with tumor-infiltration of CD8$^+$ T cells. Epitope-specific cytotoxic T lymphocytes (CTLs) were increased in spleen by challenge with Poly(I:C)+Db126 WT-1 peptide but not Poly(I:C) alone, suggesting the need of an exogenous Ag density for cross-priming. In tumor, CXCR3 ligands were upregulated by Poly(I:C), which facilitated recruitment of CTL to the tumor. Thus, Poly(I:C) acts on splenic CD8α^+ DC to cross-prime T cells and on intratumor cells to attract CTLs. Besides CD8$^+$ T cell cross-priming, T cell recruitment into tumor was significantly dampened in $Batf3^{-/-}$ mice, reflecting the importance of tumor $Batf3$-dependent DC rather than macrophages in T cell recruitment. Poly(I:C)-induced XCR1^{hi} CD8α^+ DC with high TLR3 levels were markedly decreased in $Batf3^{-/-}$ mice, which hampered the production of IL-12 and IL-12-mediated CD4$^+$/CD8$^+$ T cell proliferation. Subcutaneous administration of Poly(I:C) and adoptive transfer of wild-type CD8α^+ DC largely recovered antitumor response in those $Batf3^{-/-}$ mice. Collectively, Poly(I:C) tunes up proper maturation of CD8α^+ DC to establish TLR3-mediated IL-12 function and cross-presentation in spleen and lymphocyte-attractive antitumor microenvironment in tumor.

Abbreviations: APC, antigen-presenting cells; BATF, basic leucine zipper transcription factor ATF-like; CFSE, carboxy-fluorescein succinimidyl ester; CTL, cytotoxic T lymphocyte; DAMP, damage-associated molecular pattern; DC, dendritic cell; IFN, type I interferon; MAVS, mitochondrial antiviral signal; MDSC, myeloid-derived suppressor cells; NK, natural killer; OVA, ovalbumin; PRR, pattern-recognition receptor; TAA, tumor-associated antigens; TAM, tumor-associated macrophages; TICAM-1, Toll-IL-1R homology domain-containing adaptor molecule 1; TLR, toll-like receptor; WT1, Wilms's tumor 1

Introduction

The essence of immunotherapy for cancer is to promote tumoricidal potential of lymphocytes in tumor. Dendritic cells (DC) play a key role in validating effector cells, antitumor NK cells[1] and CTLs, through their maturation,[2] and adjuvants are deeply involved in this process.[3] DC particularly participate in proliferation of CTL by the cross-presentation of tumor-associated antigens (TAAs) to lymphocytes. The DC priming of T cells conceptually occurs in draining lymph node (DLN)/spleen in line with complicated orchestration of molecular and cellular interactions.[3] On the other hand, a variety of myeloid cells are eventually involved in the regulation of this process. Tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC) as well as tumor-infiltrating DC are implicated in tumor reprogramming and progression through undefined events including epigenetic modification.[4,5] They are also adjuvant-sensitive having pattern-sensing receptors in tumor. Immune modulation by adjuvant immunotherapy works under these complicated conditions.[6] Thus, we analyzed what happens in spleen and tumor when adjuvant is subcutaneously (s.c.) injected with TAA in mouse tumor-implant models.

Immune adjuvants represent unusual pattern molecules originating from foreign microbes or denatured host products, which usually accompany microbial infection or tissue damage.[7] There are many kinds of pattern-recognition receptors

(PRRs), a signal which functions as a strong inducer of maturation of antigen-presenting cells (APCs).[3,7] Tumor regression is an outcome of the total function of adjuvant immunotherapy. There appear to be multiple cross-presentation-inducing pathways in DC, and multifarious pattern-sensing signals in innate immunity facilitate DC-mediated proliferation of TAA-specific CTL.[2] DC are a group of APCs consisting of many subsets. CD8α^+ DC are particularly important in mice for cross-presentation of exogenous Ags in various infections because both Ags and adjuvant are simultaneously provided by other cells infected with microbes to induce CD8α^+ DC maturation.[8] Tumors with TAA but no adjuvancy have evolved to circumvent the host immune system. CD8α^+ DC are the equivalent of human CD141$^+$ CD11c$^+$ DC,[9-12] both of which express high levels of TLR3,[10,11] and induce efficient cross-priming of CTLs. A desirable quality of an adjuvant is to provide a second signal to DC that upregulates MHC and positive co-stimulators, orchestrates the cytokine network, and contributes to lymphocyte-mediated tumor killing.[13]

Here, we employed Poly(I:C) as an adjuvant. Poly(I:C) has used for long time in immunotherapy in human and mice.[14,15] Poly(I:C) satisfies the above total quality,[16,17] but how its function is culminated in administration of Ag has not been well addressed yet. However, due to its endotoxin-like toxicity, only limited doses are used for clinical tests in vaccine immunotherapy in human.[18] Mice are relatively resistant to its toxicity, but sometimes accompanied with severe adverse effects including cytokinemia. Poly(I:C) acts as a ligand for endosomal TLR3 as well as many cytoplasmic RNA sensors in DC.[16] When Poly(I:C) is i.p. injected in mice, inflammatory cytokines and type I interferons (IFNs) are drastically increased in blood plasma.[19] Using the TLR3-specific agonist, we have verified that cross-presentation is induced through the TLR3/TICAM-1 pathway, with minimal participation of IFN/cytokines by the MDA5/MAVS pathway in CD8α^+ DC.[20] Ultimately, tumor efficiently regresses by treatment with Ag + TLR3 adjuvant in mice without cytokinemia.

It is getting clear that Poly(I:C)-based treatment includes a complex inflammatory response that promotes upregulation in checkpoint molecules (i.e. PD-1 and PD-L1) by tumor cells or stromal cells (including DC) in tumor microenvironment.[21] Tumor-associated DC directly participate in proliferation of CTL inside the tumor.[22-24] Myeloid cells in tumor are also targets for Poly(I:C) in adjuvant therapy.[25] Less-inflammatory TLR3 adjuvant would more benefit for combination therapy with checkpoint blockades than Poly(I:C) in tumor microenvironment having T and tumor cells with PD-1/PD-L1 to further bolster therapeutic efficacy (which will be shown with mouse models elsewhere), in the context that checkpoint blockades exhibit high therapeutic potential to some tumor types in mouse and clinical tests.[26,27] In vaccine immunotherapy, however, tumor regress well in mice by treatment with exogenously added Poly(I:C) and Ag.[20,28] What is the role of RNA adjuvant in T cell proliferation, tumor infiltration and tumor cell damage can be analyzed in mouse models.

BATF family proteins are pivotal transcription factors involved in the base for development and maturation for DC, which closely link the effective antitumor adjuvancy. Above all, $Batf3^{-/-}$ mice barely develop CD11c$^+$ DC and lose Ag-

presenting capacity of CD8α^+ and CD103$^+$ DC.[29,30] BATF3 acts cooperatively with IRF8 in the immune system, and is particularly associated with myeloid DC development.[31,32] $Batf3$ is minimally expressed in the common DC precursors,[33] while its expression is maximal in terminally differentiated CD8α^+ DC in the spleen and their equivalent cells in other tissues.[29,31] Since CD8α^+ DC are largely diminished in spleen in $Batf3^{-/-}$ mice, BATF3 may be involved in Poly(I:C)-mediated DC maturation and IL-12 production via the TICAM-1 pathway.

Here, we found that Poly(I:C) bi-modally modulates CD8α^+ DC in spleen and tumor for cross-priming of CTL and facilitating intratumor CTL infiltration, respectively, to establish antitumor microenvironment.

Results

Batf3 is required for CTL-mediated tumor regression by Poly(I:C)

WT1-C1498 cells, stably expressing WT1,[34] were implanted to the back of mice and treated with Poly(I:C) alone 5 and 12 d after implantation. WT1-C1498 tumor regressed in response to Poly(I:C) in wild-type mice (Fig. 1A), and this Poly(I:C) effect was abolished if CTL were depleted (Fig. 1B). Strikingly, $Batf3$ knockout completely abrogated the Poly(I:C) antitumor effect in C57BL/6 mice (Fig. 1C). NK cells were barely involved in the Poly(I:C)-induced tumor regression (Fig. 1D), but CD8$^+$ T cells infiltrated the tumors in wild type, but not in $Batf3^{-/-}$ mice (Fig. 1E).

The results indicated that Poly(I:C) induces WT1-directed CTL to regress C1498 tumor. However, the reason remained unknown why the CTL recognizing the Db126 epitope (RMFPNAPYL)[34] with the highest avidity to the MHC H-2D^b was barely detected in this setting (Nakajima et al., unpublished data). Then, we challenged Db126 peptide + Poly(I:C) to mice bearing WT1-C1498 tumor. The splenocytes were restimulated with the WT1 peptide *ex vivo* in order to detect specific CTL against WT1 tetramer (Fig. 1F). Specific CTL with tumor shrinkage was significantly detected upon early challenge with Poly(I:C) + Db126 in wild-type mice followed by restimulation (Fig. 1F). In $Batf3^{-/-}$ mice, neither tumor shrinkage nor specific CTL was detected by Poly(I:C) + peptide challenge under the panel F conditions (Fig. 1G). There appears a parallelism between the frequencies of Db126-specific CTLs and tumor regression, and a requirement of BATF3 for Poly(I:C)-dependent optimal antitumor responses in the WT1-C1498 model.

Antigen-specific CTL induction by Poly(I:C) is decreased in $Batf3^{-/-}$ mice

The EG7 implant tumor is a good model for Poly(I:C) therapy *in vivo*: TLR3/TICAM-1 pathway in DC is crucial for expansion of antigen-specific CTL.[28] OVA is an artificial Ag that contains multivalent CD8$^+$ and various CD4$^+$ epitopes. OVA and/or Poly(I:C) were s.c. injected into wild-type or $Batf3^{-/-}$ mice implanting EG7 tumor, and tumor growth was measured. Tumor growth retardation was observed in this model in response to Poly(I:C) + OVA (Fig. 2A left) concomitant with a marked increase of specific CTLs (Fig. 2A right). Notably, Poly
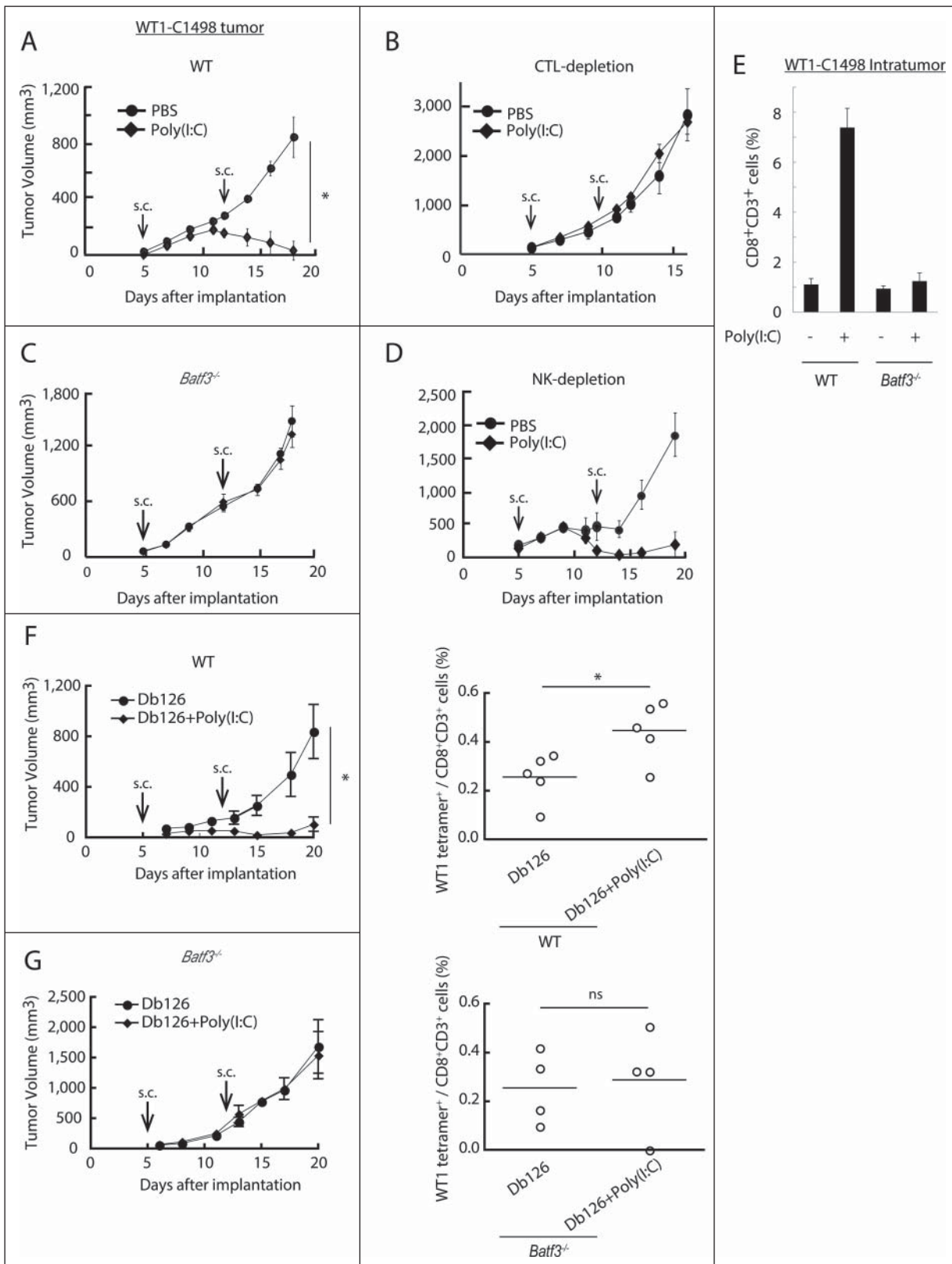
Figure 1. Poly(I:C) induces BATF3-mediated WT1-C1498 tumor regression. WT1-C1498 tumor was implanted to wild-type or *Batf3*^{−/−} mice (C57BL/6J) and Poly(I:C) was administered around tumor at day 5 and 12 after tumor implantation (A, C). The tumor volume was measured every 2 to 3 d. For depleting CTLs or NK cells, ascites containing anti-CD8α or anti-NK1.1 antibody was i.p. injected the day before Poly(I:C) therapy (B, D). WT1-C1498 tumor was harvested at day 19 as in panels A and C, and the proportion of tumor-infiltrating CD8^{+} T cells was evaluated by flow cytometer (E). Db126 peptide wrapped in DOTAP with or without Poly(I:C) were administered to tumor-bearing wild-type mice at day 5 and 12 after tumor implantation. At day 20, splenocytes were harvested and cultured in complete medium in the presence of Db126 (5 μg/mL) for 5 d. At day 25, the proportion of WT1-specific CD8^{+} T cells was evaluated (F). At day 21, splenocytes of *Batf3*^{−/−} mice treated as in panel F were harvested. The cells were re-stimulated with Db126 and the proportion of WT1-specific CD8^{+} T cells was evaluated as in panel F (G). Arrows show the day of Poly(I:C) administration. Error bars show ± SEM; n = 3 to 4 per group. Student's t-test was performed to analyze statistical significance. *p < 0.05. ns; not significant. The results are the representatives of more than two independent experiments.
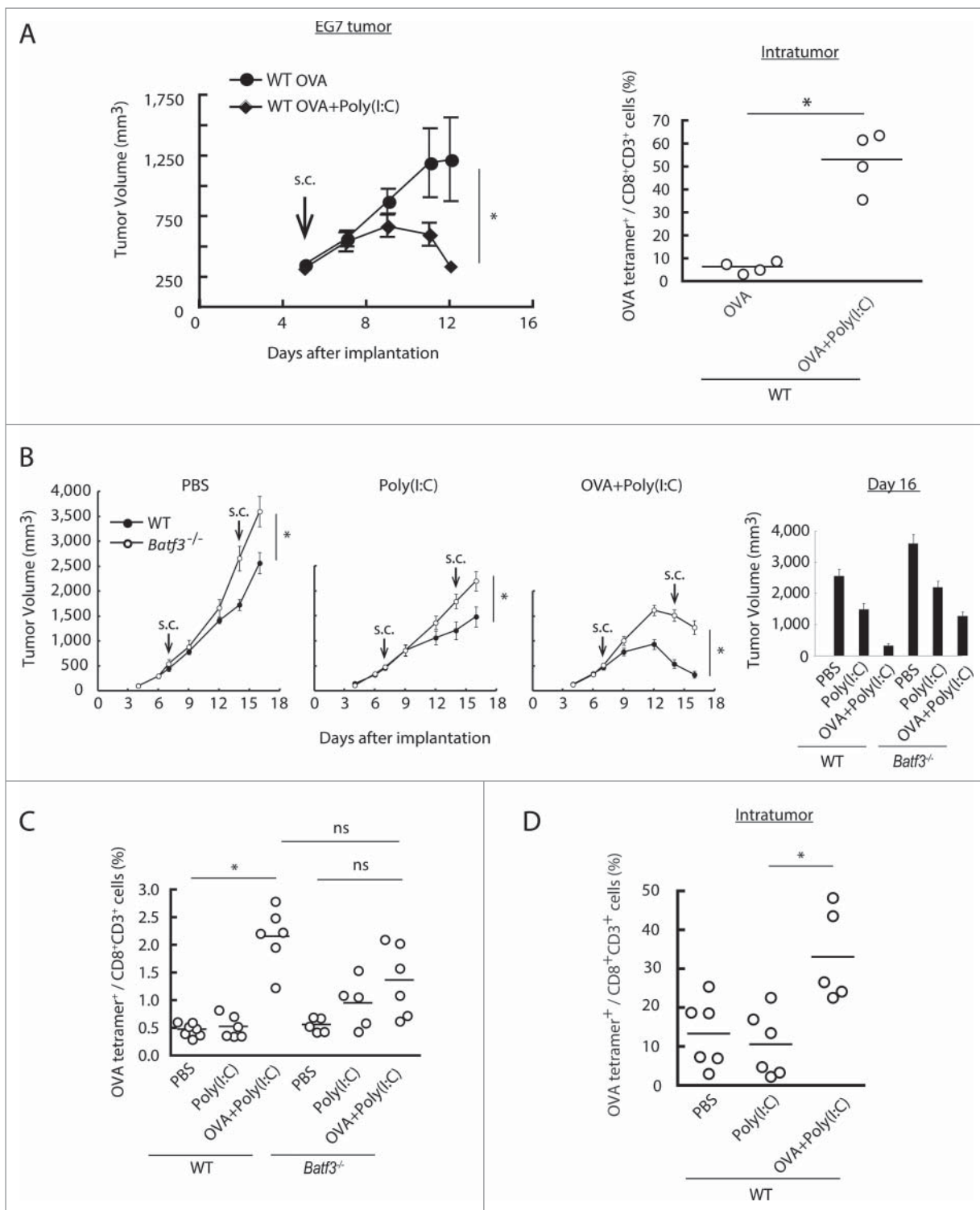
Figure 2. Combined administration of antigen and Poly(I:C) induces EG7 tumor regression. Wild-type mice were inoculated with EG7 tumor and had Poly(I:C) and OVA therapy day 5 after tumor implantation. At day 12, the proportion of tumor-infiltrating OVA-specific CD8+ T cells was evaluated (A). EG7 was implanted to wild-type mice or $Batf3^{-/-}$ mice and Poly(I:C) with or without OVA was administered around the tumor at day 7 and 14. PBS was used as control (B). At day 16, the proportions of OVA-specific CD8+ T cells in spleen were evaluated by flow cytometer (C). EG7-bearing wild-type mice had Poly(I:C) and OVA therapy at day 7, and at day 15, the proportion of tumor-infiltrating OVA-specific CD8+ T cells was evaluated (D). Error bars show ± SEM; n = 4 to 8 per group. Student's t-test (A, B) and Kluskal–Wallis test with Dunn's multiple comparison test (C, D) were performed to analyze statistical significance. $^*p < 0.05$, ns; not significant. Three similar experiments were performed and the results are the representative one.

(I:C)/OVA-mediated tumor shrinkage was abrogated in $Batf3^{-/-}$ mice (Fig. 2B). 16 d after the tumor implantation with twice therapeutic injection, we found $Batf3$ expression profoundly linked to Poly(I:C)-mediated growth retardation of

implant EG7 (Fig. 2B right). Basal tumor growth was slightly accelerated in $Batf3^{-/-}$ mice compared to wild-type mice irrespective of Poly(I:C) therapy. 9 d after the Poly(I:C)/OVA injection, OVA(SL8)-specific proliferation of CTL in the spleen

was measured by OVA tetramer (Fig. 2C). OVA-specific CTLs were obviously expanded in the Poly(I:C)/OVA group in spleen by treatment with Poly(I:C) + OVA (Fig. 2C), whereas they were barely detectable in the PBS- or Poly(I:C)-treated groups in wild-type mice (Fig. 2C), suggesting the indispensability of exogenous Ag+Poly(I:C) for effective CTL induction. Notably, Tetramer-positive CTL were also increased in tumor in response to OVA+Poly(I:C) in wild-type mice (Fig. 2D). A similar tendency was observed in $Batf3^{-/-}$ mice but tetramer-positive T cells were mildly increased in response to Poly(I:C) for unknown reason. Less Ag-specific CTL proliferation was observed in $Batf3^{-/-}$ mice in response to OVA+Poly(I:C) (Fig. 2C).

CD8α^+ DC were increased in the spleen and DLN in wild-type C57BL/6 mice in response to Poly(I:C)/OVA (Fig. S1A,B, C). This incremental response of CD8α^+ DC was partially abolished in $Batf3^{-/-}$ mice. However, the CD8α^+ DC population is reportedly heterologous, not reflecting bona fide CD8α^+ DC.[30,33] We have gated with XCR1[9] and CLEC9A in addition to CD8α and CD11c (Fig. S1A). This CD8α^+ DC population was completely diminished and never increased by Poly(I:C)+ OVA in $Batf3^{-/-}$ mice (Fig. S1C). The XCR1^{hi}/CLEC9A$^+$ CD8α^+ DC became highly mobile in wild-type mice, soon leaving away from the spleen and DLN in response to Poly(I:C)/ OVA (Fig. S1C). Notably, this DC fraction was completely abolished in the CD8α^+ DC subset in $Batf3^{-/-}$ mice, suggesting the presence of quantitative difference of bona fide CD8α^+ DC in $Batf3^{-/-}$ mice. Wild-type and $Batf3^{-/-}$ CD8α^+ DC exhibited a little cell death resistance in response to Poly(I:C), which was independent of the reported RIP3 pathway and caspases[35] (Fig. S2A,B).

Induction of T cell cross-priming in CD8α^+ DC by OVA and Poly(I:C)

In vivo OVA-tetramer-specific CTLs were scarcely detected in spleen in tumor-unloading wild-type mice by stimulation with Poly(I:C) alone, but became detectable in mice with Poly(I:C)/ OVA (Fig. 3A). This Poly(I:C)/OVA-mediated CTL induction was completely abrogated in $Batf3^{-/-}$ mice, unlike the tumor-bearing model (Fig. 2C). Thus, some tumor factors in concert with Poly(I:C) + OVA facilitate mild induction of tetramer-positive T cells in $Batf3^{-/-}$ mice. OVA tetramer-positive CD8$^+$ T cells barely proliferated in tumor by Poly(I:C) alone treatment (Fig. 2D), although total CD8$^+$ T cells were increased in tumor when only Poly(I:C) was s.c. administered in mice.

In vitro cross-priming efficacy of CD8α^+ DC was tested using OT-1 T cells: CD8α^+ DC were isolated from the spleens of wild-type, $Batf3^{-/-}$ or $Tlr3^{-/-}$ mice and co-cultured with OT-1 cells in the presence of OVA and Poly(I:C). Without Poly (I:C) stimulation, none of the CD8α^+ DC derived from wild-type, $Batf3^{-/-}$ and $Tlr3^{-/-}$ mice cross-primed OT-1 cells at a low concentration of OVA (10 μg/mL), and almost equally cross-primed OT-1 at a high concentration of OVA (100 μg/ mL) (Fig. 3B). In the presence of Poly(I:C), however, OT-1 cells co-cultured with wild-type CD8α^+ DC robustly proliferated, while OT-1 cells co-cultured with $Batf3^{-/-}$ or $Tlr3^{-/-}$ CD8α^+ DC barely proliferated at low concentrations of OVA (Fig. 3B). Yet, the cross-priming induced by Poly(I:C) minimally

remained in $Batf3^{-/-}$ CD8α^+ DC. The IFNγ levels in the co-culture with 10 μg/mL OVA paralleled the proliferation of OT-1 (Fig. 3C). These data indicates that the basic level of cross-priming ability in CD8α^+ DC is almost the same in wild-type, $Batf3^{-/-}$ and $Tlr3^{-/-}$ genotypes under a high level of Ag input and that BATF3 supplies a basic condition which enables CD8α^+ DC to cross-prime OT-1 cells when Poly(I:C) stimulates TLR3/TICAM-1 signaling. Notably, the Ag level is critical for induction of cross-priming: Poly(I:C) is particularly required as a trigger for cross-priming under a low level of Ag input.

TLR3 expression and IL-12p40 production are decreased in $Batf3^{-/-}$ CD8α^+ DC

TLR3 and the cytoplasmic RNA sensors RIG-I/MDA5 share the Poly(I:C) signal for cytokine- and type I IFN-induction.[36] We tested the steady-state levels of these genes in $Batf3^{-/-}$ CD8α^+ DC from C57BL/6 mice. The expression of *Tlr3* was decreased, *Ddx58* (RIG-I gene) was increased, and *Ifih1* (MDA5 gene) and signal adaptors, *Ticam1* (TRIF) and *Mavs* (IPS-1), were unaffected by *Batf3* knockout in CD8α^+ DC compared to wild-type CD8α^+ DC (Fig. 4A). The protein expression of TLR3 in $Batf3^{-/-}$ CD8α^+ DC was also decreased to half compared to wild-type CD8α^+ DC (Fig. 4B), suggesting the predominance of the RIG-I pathway in Poly(I:C) sensing in $Batf3^{-/-}$ CD8α^+ DC. Of note, no significant upregulation of *Batf3* was observed in Poly(I:C)-stimulated CD8α^+ DC (Fig. S3), where TLR3 participated in Poly(I:C)-dependent IFN-β induction, but not in *Batf3* induction.

Next, we attempted to determine whether TLR3 signaling worked sufficiently in $Batf3^{-/-}$ CD8α^+ DC in response to Poly (I:C). CD8α^+ DC isolated from spleens of wild-type, $Batf3^{-/-}$ and $Tlr3^{-/-}$ mice were treated with Poly(I:C) and the mRNA were collected for determination of inducible mediators (Table S1). Ultimately, type I IFNs, *Ifna2* and *Ifnb*, and type III IFN, *Il28*, were induced by Poly(I:C) in $Batf3^{-/-}$ CD8α^+ DC comparable to wild-type CD8α^+ DC (Fig. 4C). Poly(I:C)-derived *Ccl5* and *Il15* were unaffected while *Il12b* was completely abolished in CD8α^+ DC by *Batf3* knockout (Fig. 4C). Although the RIG-I pathway may compensate for cytokine/chemokine production (Fig. 4A), Poly(I:C)-derived RIG-I upregulation failed to recover the IL-12p40 level. The RIG-I dominance in Poly(I:C) therapy might explain the remaining CTL induction in $Batf3^{-/-}$ mice with tumor (Fig. 2C).

We next tested whether IL-12p40 repression occurs in Poly (I:C)-injected $Batf3^{-/-}$ mice *in vivo*. When Poly(I:C) was s.c. injected to mice, IL-12p40 was abundantly produced by Poly(I: C) in wild-type, but not in $Batf3^{-/-}$ mice (Fig. 4D). IL-12p70 was actually produced in splenic CD8α^+ DC in wild-type mice but not $Batf3^{-/-}$ mice (Fig. 4E). In line with the IL-12p40 expression, we found the peak of *Batf3* signal in the enhancer region of TLR3 in accordance with those of p300, H3K27ac, and H3K4me1 by chip-sequence analysis (Fig. S4A). There was significant *Batf3* signal in the 5′-UT region of IL-12p40, which might represent the direct regulation of IL-12p40 by *Batf3* (Fig. S4B). No marked changes of the expression levels of membrane molecules, *Tnfsf9, Tnfsf10* and *Cd40*, were observed in
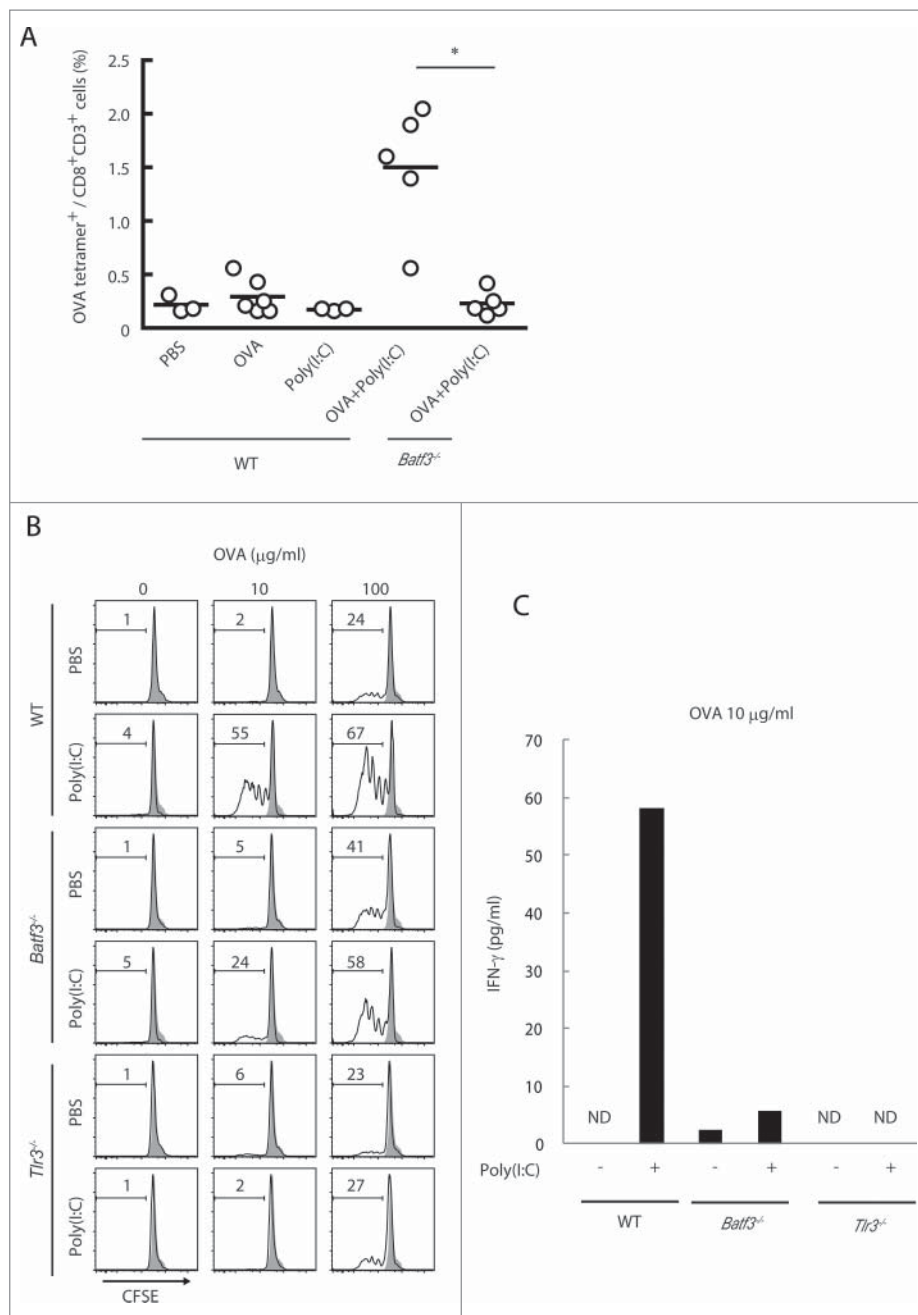
Figure 3. The cross-priming is partially reduced in *Batf3*^{−/−} mice. Poly(I:C) and OVA were administered to wild-type and *Batf3*^{−/−} mice with no tumor-loading. After 7 d, the proportion of OVA-specific CD8^{+} T cells in spleen was evaluated with tetramer by flow cytometer (A). CD8α^{+} DCs isolated from spleens of wild-type, *Batf3*^{−/−} and *Tlr3*^{−/−} mice were incubated with Poly(I:C) and OVA for 4 h and then co-cultured with CFSE-labeled OT-1 cells. After 60 h, the antigen-specific OT-1 proliferation was evaluated by diminution of CFSE (B) and IFNγ in the culture supernatant (C). Shadow histograms show wild-type specimen treated with PBS without OVA. Student's t-test was performed to analyze statistical significance. $^*p < 0.05$. More than three similar experiments were performed and the results are the representative one.

response to Poly(I:C) (Fig. S5). These data infer that BATF3 regulates the expression of TLR3, and then IL-12p40 production in CD8α^{+} DC in Poly(I:C) therapy.

Poly(I:C) endows CD8^{+} T cells the mobility to infiltrate in tumors by BATF3

In EG7 tumor specimens, CD8^{+} cells infiltrated the tumor in wild-type mice 9 d after the first Poly(I:C) or Poly(I:C)/OVA treatment (Fig. 5A,B), while CD8^{+} cells barely infiltrated in both peripheral and central area in the tumors in mice without

Poly(I:C) (Fig. 5B,C). Notably, no CD8^{+} cells entered into the tumor in *Batf3*^{−/−} mice in Poly(I:C) therapy (Fig. 5B,C). CD8^{+} T cells in tumors were counted for quantitative analysis (Fig. 5D), where Poly(I:C) treatment confers tumor-infiltrating mobility on CD8^{+} T cells and BATF3 is indispensable for this Poly(I:C) function.

How CTLs are recruited to the tumor by Poly(I:C) therapy is a next matter for analysis. EG7-bearing wild-type and *Batf3*^{−/−} mice were challenged with Poly(I:C)/OVA therapy and then the mRNA levels of chemokines/cytokines in tumor were assessed in wild-type and *Batf3*^{−/−} mice by RT-PCR.
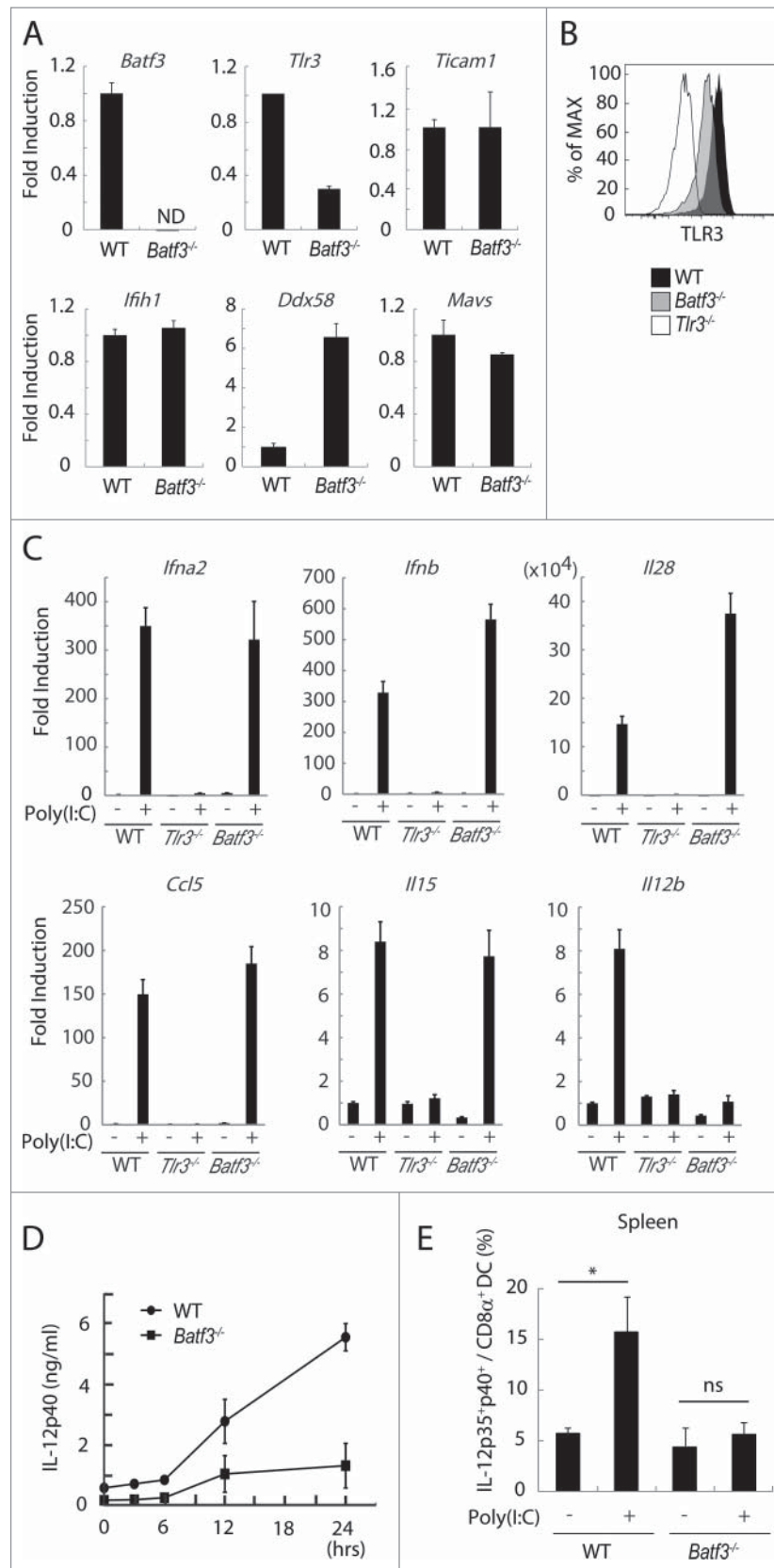
Figure 4. TLR3 and inducible IL-12 levels are decreased in CD8α⁺ DCs in Batf3⁻/⁻ mice. CD8α⁺ DCs in spleen were isolated from wild-type and Batf3⁻/⁻ mice and then the levels of mRNA were evaluated. The expression levels of the indicated genes were measured by qPCR (A). The level of TLR3 protein in CD8α⁺ DCs was assessed by flow cytometer (B). CD8α⁺ DCs were isolated from wild-type, Tlr3⁻/⁻ and Batf3⁻/⁻ mice, and stimulated with Poly(I:C). After 4 h, mRNA were collected and the expression levels of the indicated genes were evaluated (C). Poly(I:C) was s.c. administered to wild-type and Batf3⁻/⁻ mice. The blood serum was collected at the times indicated and the amount of IL-12p40 was measure by ELISA (D). Poly(I:C) was s.c. administered to wild-type and Batf3⁻/⁻ mice. 12 h later, splenocytes were harvested. Collected cells were cultured in the presence of Blefeldin A for 4 h. Then, the proportion of IL-12p35⁺ p40⁺ CD8α⁺ DCs (MHC class II⁺ CD11cʰⁱ) was evaluated by flow cytometer (E). Error bars show ± SEM; n = 4 to 6 per group. Kluska–Wallis test with Dunn's multiple comparison test were performed to analyze statistical significance. *p < 0.05, ns; not significant (E). The results are the representatives of three independent experiments.
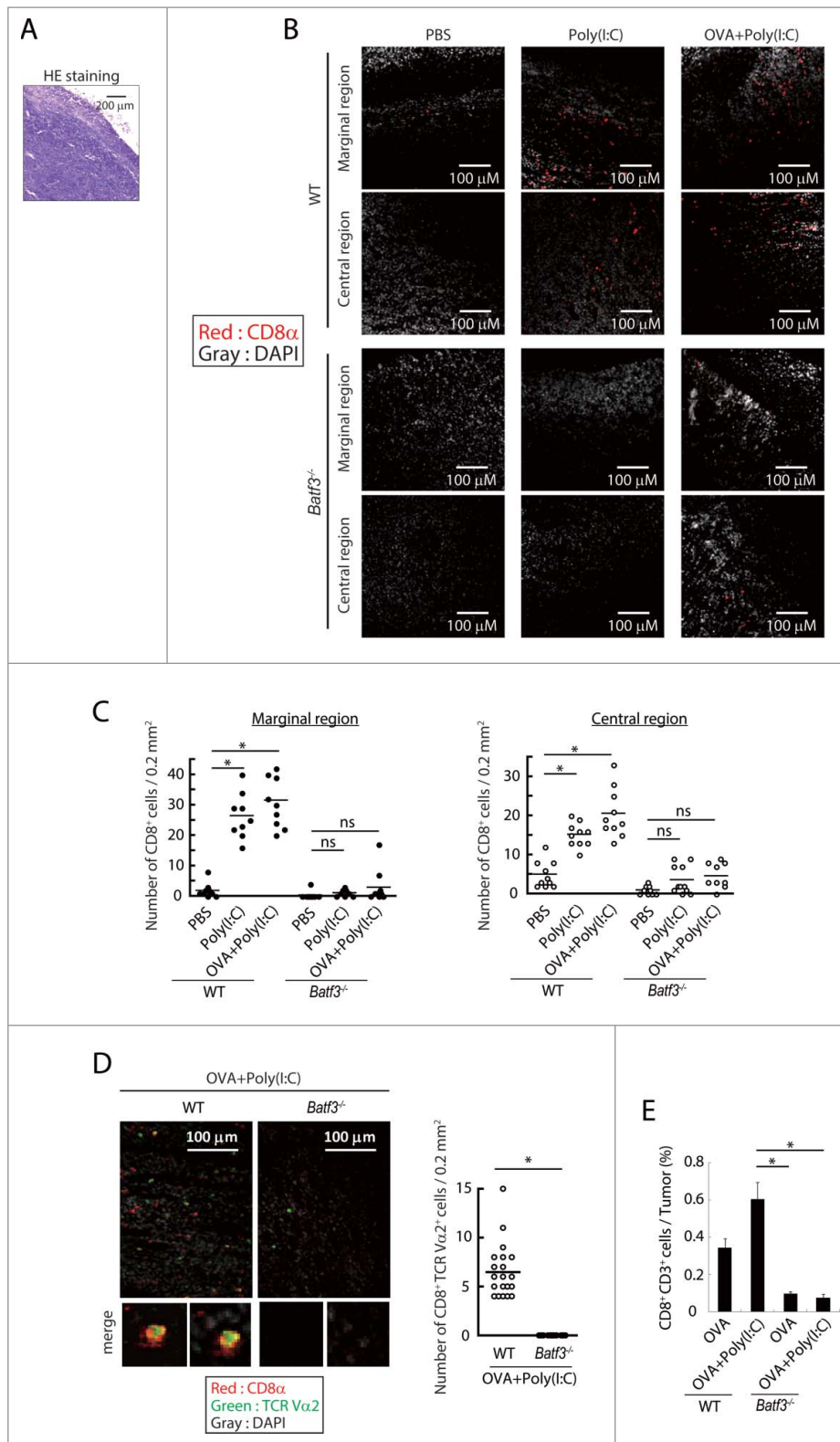
Figure 5. Poly(I:C)-induced CD8$^+$ cell infiltration into tumor tissues. EG7 tumor-bearing mice had Poly(I:C) + OVA therapy on day 7 and 14, and tumor tissues were excised at day 16. The tumor sections were stained with hematoxylin and eosin (A) or APC-anti CD8α antibody and DAPI (B, C). The confocal microscopy images ($\times$20 magnification) of a margin and a center of the tumor isolated from wild-type mice (Upper panels) or Batf3$^{-/-}$ mice (Lower panels) are shown (Red; CD8α, Gray; DAPI) (B). The numbers of CD8α^+ cells in 0.2 mm^2 of tissue sections were counted (C). Tumor-bearing mice had Poly(I:C) and OVA therapy at day 9 and tumor tissues were excised at day 15. Tumor sections were stained with APC-anti-CD8α , PE-anti-TCR Vα2 antibody and DAPI (D). The confocal microscopy images are shown (Red; CD8α, Green; TCR Vα2, Gray; DAPI) (Left panel). The numbers of CD8α^+ TCR Vα2$^+$ cells in 0.2 mm^2 of the tissue sections were counted (Right panel) (D). The proportion of tumor-infiltrating CD8$^+$ CD3$^+$ cells was evaluated by flow cytometer (E). The results are the representatives of each group (B, C, D). Error bars show $\pm$ SEM; n = 5 per group (E). Kluskal–Wallis test with Dunn's multiple comparison test (C, E) and Student's t-test (D) were performed to analyze statistical significance. $^*p < 0.05$, ns; not significant.

Chemokines associated with CTL attraction (*Cxcl9*, *Cxcl10* and *Cxcl11*) and *Il12b* were upregulated in response to OVA+Poly(I:C) in the tumor in wild-type mice, in contrast to *Batf3⁻/⁻* mice (Fig. 6A). A similar tendency was observed in the protein levels of IL-12p40 (Fig. 6B) and CXCL10 (Fig. 6C) in the tumors harvested from wild-type vs. *Batf3⁻/⁻* mice. Since tumor cells do not secrete these mediators and macrophages are *Batf3*-independently activated in tumor (Figs. S6, 7), *Batf3*-dependent DCs in the tumor would be the main source of these mediators. In the same setting, *Ifng, Gzmb, Prf1, Il12rb1* and *Il12rb2* were upregulated in the tumor in response to Poly(I:C)

in wild-type, but not in *Batf3⁻/⁻* mice (Fig. 6D). CD8α⁺ DC was a dominant subset in intratumor DC compared with CD103⁺ DC, and increased after treatment with OVA+Poly(I:C) (Fig. 6E). The results infer that the CD8α⁺ DC in tumor modulate antitumor microenvironment to recruit CD8⁺ T cells, mostly of tumor-specific CTL, by Poly(I:C).

In this context, we checked the possibility that tumor microenvironment other than DC participated in the TLR3/Batf3-mediated T cell infiltration into the tumor. TLR3 levels were essentially low in EG7(OVA) and C1498(WT1) cells, and Poly(I:C) barely affected the expression levels of tumor cell TLR3
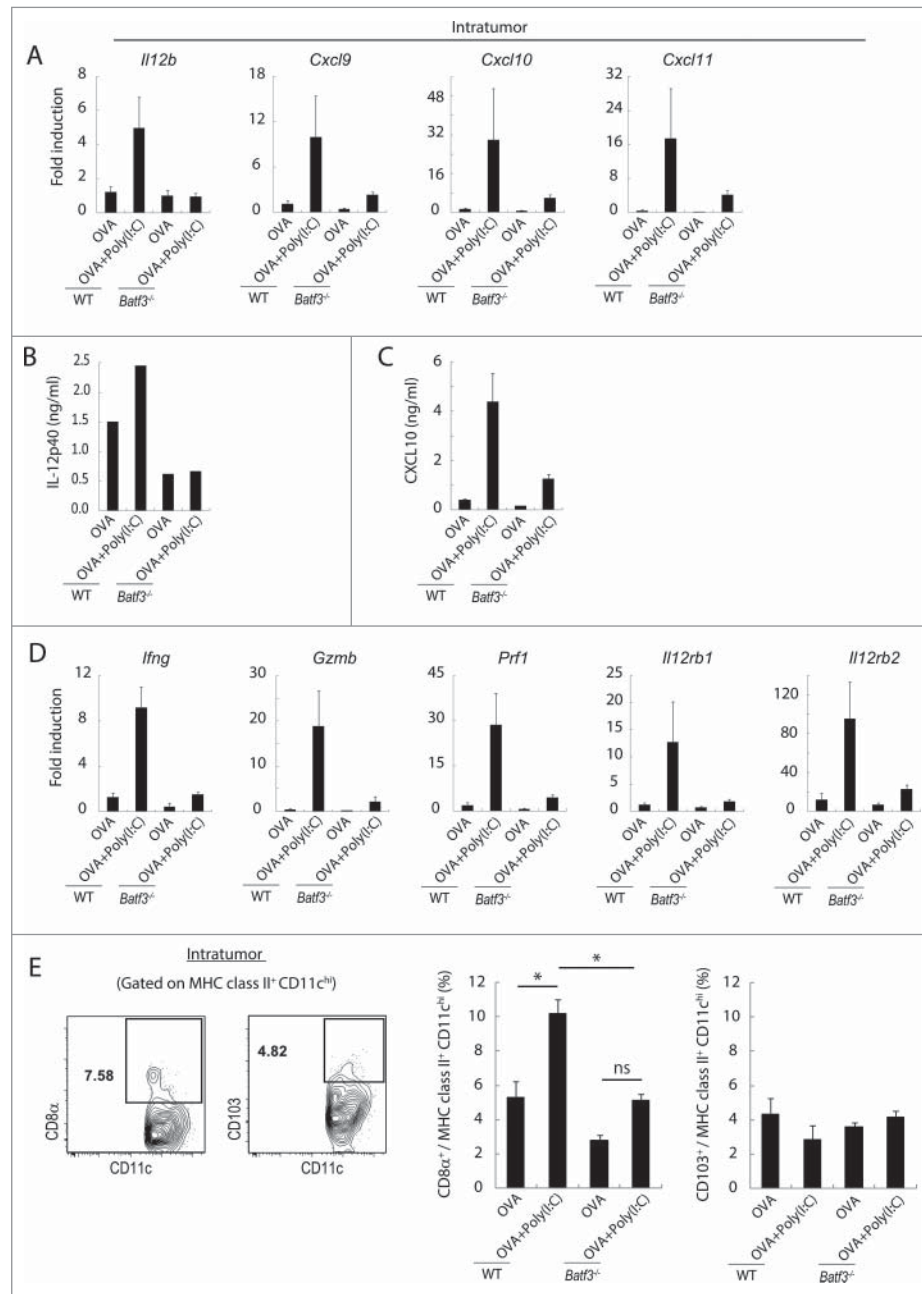

Figure 6. Poly(I:C) changes intratumoral gene expression patterns and IL-12 production in wild-type mice but not in *Batf3⁻/⁻* mice. EG7 tumor-bearing mice were challenged with Poly(I:C) and OVA at day 9 and 14 after tumor implantation. At day 15, tumor tissues were harvested and the levels of mRNA were evaluated (A, D). The quantity of the IL-12p40 and CXCL10 proteins in the fixed volume of tumor tissues was also evaluated by ELISA (B, C). The ratios of tumor-infiltrating CD8α⁺ DC and CD103⁺ DC subsets were evaluated by flow cytometer (E). Error bars show ± SEM; n = 4–7 per group. One-way analysis of variance (ANOVA) with Bonferroni's test was performed to analyze statistical significance (E). *$p < 0.05$, ns; not significant. The results are one of the two independent experiments. In panel B, one representative of each group is shown.

(Fig. S6A). Neither the relevant genes for lymphocyte attraction were induced in tumor cells in response to Poly(I:C) (Fig. S6B), nor occurred Poly(I:C)-mediated tumor cell death accordingly (Fig. S6C). Tumor-associated myeloid cells were situated in tumor with similar profiles in wild-type and $Batf3^{-/-}$ mice, and only minimally affected on Poly(I:C) treatment (Fig. S7). Thus, microenvironmental factors marginally regulate T cell infiltration into tumor.

Adoptively transferred CD8α+ DC is responsible for Poly(I:C)-induced tumor regression

Whether exogenously added CD8α+ DC work for tumor microenvironment in $Batf3^{-/-}$ mice is a question in this context. We next tested if adoptive transfer of wild-type CD8α+ DC can retard WT1-C1498 growth in Poly(I:C)-administered $Batf3^{-/-}$ mice. Tumor growth was significantly repressed by Poly(I:C) in the group of $Batf3^{-/-}$ mice which received transferred wild-type CD8α+ DC (left panel in Fig. 7A). $Batf3^{-/-}$ CD8α+ DC transferred in a similar way did not revert the tumor growth inhibition in this $Batf3^{-/-}$ mouse model (right panel in Fig. 7A). Control studies indicated that Poly(I:C) treatment did not induce tumor regression in $Batf3^{-/-}$ mice in the absence of additional IL-12p70 (left panel in Fig. 7B). Full tumor-repressive activity was induced by treatment with Poly(I:C) + IL-12p70 (Fig. 7B). IL-12 has been shown to enhance the rejection of many types of murine tumors,[37] but excess IL-12p70 alone exhibited minor potential for WT1 tumor regression (right panel in Fig. 7B). Ultimately, IL-12 induced by TLR3/TICAM-1 from CD8α+ DC is responsible for growth retardation of WT1-C1498 tumors in response to Poly(I:C) treatment, and BATF3 is indispensable for both spleen and tumor DC to evoke Poly(I:C)-derived antitumor effect. Notably, intratumor CD8+ T cells were replenished in response to Poly(I:C) + IL-12p70 treatment in $Batf3^{-/-}$ mice (Fig. 7C), and splenic CD8α+ DC density was unaffected in this setting (Fig. 7D).

CD4+ T cells promotes cross-priming by CD8α+ DC

Next, we confirmed whether IL-12p40 produced by CD8α+ DC is involved in effector induction in $Batf3^{-/-}$ mice. When IL-12 was i.p. injected to $Batf3^{-/-}$ mice together with Poly(I:C) and OVA, OVA-tetramer-positive cells were significantly increased in vivo compared to the group treated with Poly(I:C)/OVA without IL-12 (Fig. 8A). IFNγ production in OVA-specific CD8+ and CD4+ T cells was upregulated in wild-type, but not $Batf3^{-/-}$ mice in response to Poly(I:C) (Fig. 8B). IL-12-mediated IFNγ upregulation was assisted by CD4+ T cells in $Batf3^{-/-}$ mice (Fig. 8C), and this IFNγ upregulation was Poly(I:C)-dependent in wild-type mice, but marginal in $Batf3^{-/-}$ mice. CD4+ T cell-involved tumor regression is key in WT1 tumor regression. In vitro cross-priming-enhancing function of OT-II (CD4) cells was reproduced with $Batf3^{-/-}$ DC in the presence of IL-12 (Fig. S8A,B). The XCR1hi CD8α+ DC population, which were mostly in the TLR3hi CD8α+ DC subset, are responsible for production of IL-12 by Poly(I:C) stimulation in wild-type mice (Fig. S9). Hence, the scenario of TLR3 adjuvant is that expression of $Batf3$ governs TLR3-mediated IL-12p40

production in XCR1hi CD8α+ DC, which in turn activate CD4+ T cells and upregulate IFNγ; then, TICAM-1-mediated cross-priming of TAA-specific CD8+ T cell proliferation is triggered in XCR1hi CD8α+ DC in spleen for establishment of antitumor immunity. Simultaneously, TLR3-high DC release CXCR3 ligands in tumor to recruit the primed CD8+ T cells, resulting in tumor regression. The levels of PD-1 in T cells and PD-L1 in tumor cells are another matter to determine tumoricidal efficacy.

Discussion

Our study highlights the importance of TLR3 adjuvant in DC maturation for tumor-specific T cell proliferation and infiltration, which leads to tumor regression. It acts locally in either spleen/DLN or tumor. Poly(I:C) adjuvant not only elevates cross-priming ability of splenic DC, but also induces the liberation of IL-12, type I IFN, chemokines and other cytokines in tumor microenvironment through tumor DC. This is an essential event in T cell migration and exertion of tumoricidal activity in tumor. TLR3 rather than RIG-I/MDA5 participates in evoking Poly(I:C) antitumor immunity in CD8α+ DC, which distinguishes from IFN therapy and minimizes the adverse effect by systemic induction of cytokines/IFNs.[14,20] Other RNA adjuvants such as Poly(A:U) might be a candidate for antitumor adjuvant, but their functional properties remained undefined in terms of TLR3-specificity.[17,20,38] The point of this study was to clarify by what mechanism Poly(I:C) induces tumor growth retardation in tumor-bearing mice.

In our s.c.-injection setting, Ag and adjuvant limitedly act on both tumor and DLN/spleen. In DLN/spleen, TLR3 adjuvant alone treatment does not allow TAA-specific CTL proliferation, and treatment with both adjuvant and OVA Ag is required for tetramer-positive T cell proliferation in spleen. A number of studies have implicated extrinsic administration of Ag near DLN in Ag-specific CD8+ T cell proliferation,[13] and additional TLR3 adjuvant markedly enhances T cell proliferation even under low Ag input.[28] Indeed, in our setting only with Poly(I:C), tetramer-positive CD8+ T cells failed to proliferate in DLN/spleen, but plenty of CD8+ T cells migrate into tumor (Fig. 5).

Unless tumor permits T cell infiltration, the PD-1 Ab therapy is ineffective. As shown in melanoma PD-1 (or PD-L1) Ab therapy, metastatic tumors shrink if the CD8+ T cells infiltrate into the tumors.[39,40] We show here that Poly(I:C) has ability to provoke T cells to enter the tumor (Fig. 5). CD103+ DC in tumor may make CTL proliferate within tumor,[41,42] but this is unlikely in the vaccine therapy, where only a few CD103+ DC reside in the EG7-implant tumor (Fig. 6E) and OVA tetramer-positive CD8+ T cells barely proliferate in tumor by Poly(I:C) alone treatment (Fig. 2D), suggesting that these CD8+ T cells are recruited to tumor from spleen, where Ag-specific T cells markedly proliferate by s.c.-injected Poly(I:C) adjuvant + Ag (Fig. 2C).

Importance of Poly(I:C) stimulation in tumor is shown in Fig. 7A, where not only CD8α+ DC adoptive transfer but also additional Poly(I:C) is required for tumor regression. Actually, the ratio of CD8α+ subset/intratumor DCs is increased in response to Poly(I:C) and the Poly(I:C)-derived CXCL9, 10 and 11 are all upregulated in wild-type mice. These CXCR3 ligands
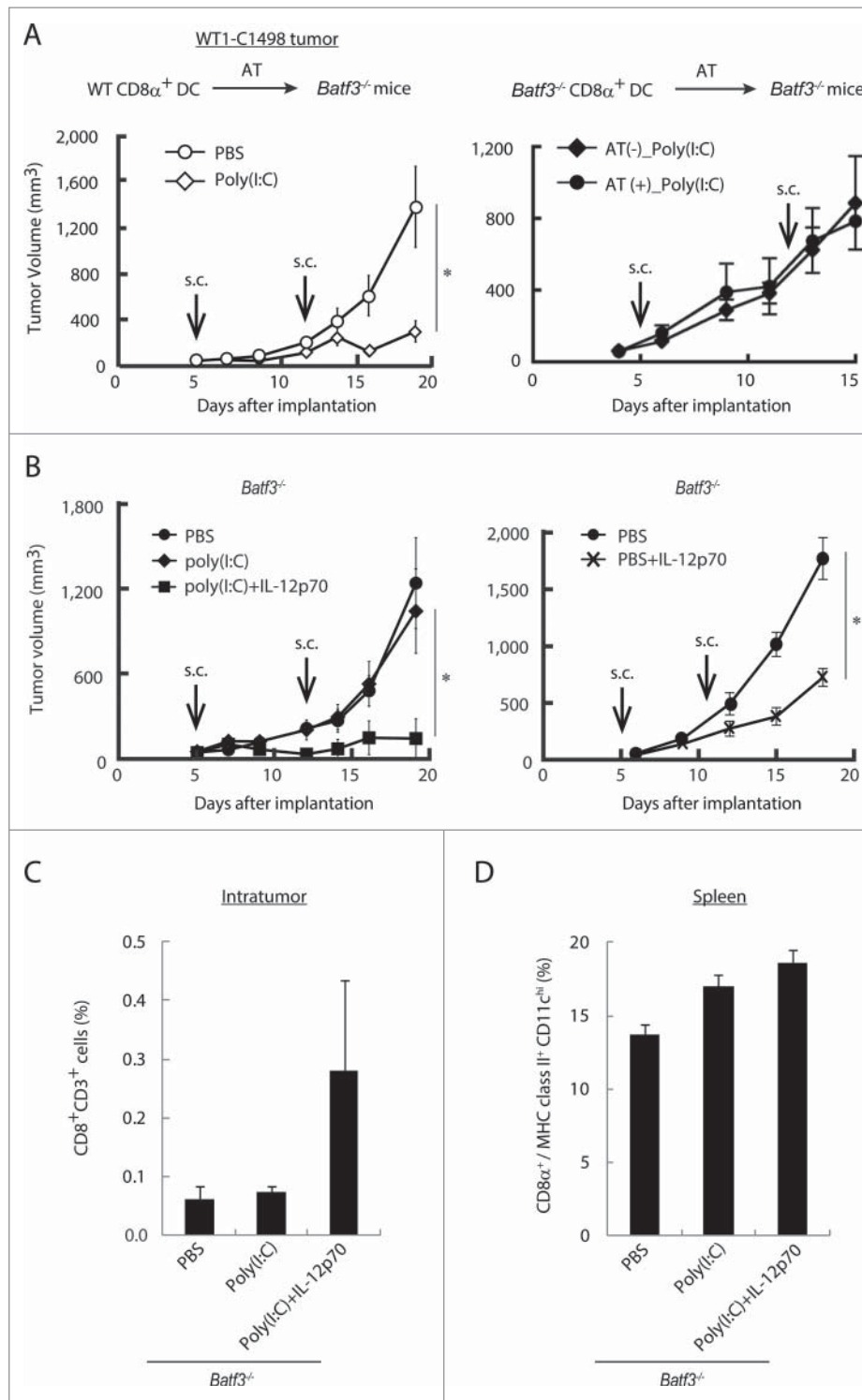
Figure 7. BATF3 plays a key role in CD8α^+ DCs for IL-12 production. CD8α^+ DCs were isolated from wild-type (Left panel) or *Batf3*$^{-/-}$ mice (Right panel) and i.v. transferred to WT1-C1498 tumor-bearing *Batf3*$^{-/-}$ mice at day 4 and 11 after tumor implantation. PBS or Poly(I:C) were administered to the recipient mice at day 5 and 12 (A). WT1-C1498 was implanted to *Batf3*$^{-/-}$ mice and the mice were treated with Poly(I:C) alone or Poly(I:C) and IL-12p70 (Left panel), PBS or IL-12p70 (Right panel) at day 5 and 12 (B). At day 13, tumor tissues were harvested and the proportion of tumor-infiltrating CD8$^+$ T cells was evaluated by flow cytometer (C). The spleen was also harvested and the proportion of CD8α^+ DCs was evaluated (D). Error bars show $\pm$ SEM; n = 3 to 5 per group. Kluskal–Wallis test with Dunn's multiple comparison test (the left B) and Student's t-test was performed (A, the right B) to analyze statistical significance. *$p < 0.05$. Two similar experiments were additionally performed and supported the results shown.

are diminished in tumor in *Batf3*$^{-/-}$ mice, suggesting that *Batf3*-dependent DC rather than MDSC or TAM are the source of T cell-recruiting chemokines. Although the exact subsets of DC for T cell attraction to tumor are undetermined, the results reinforce the idea that Poly(I:C) acts on *Batf3*-positive DCs in tumor as well.

Recent reports demonstrated that checkpoint blocking of the effector step by PD-1 or PD-L1 Ab brought high tumoricidal
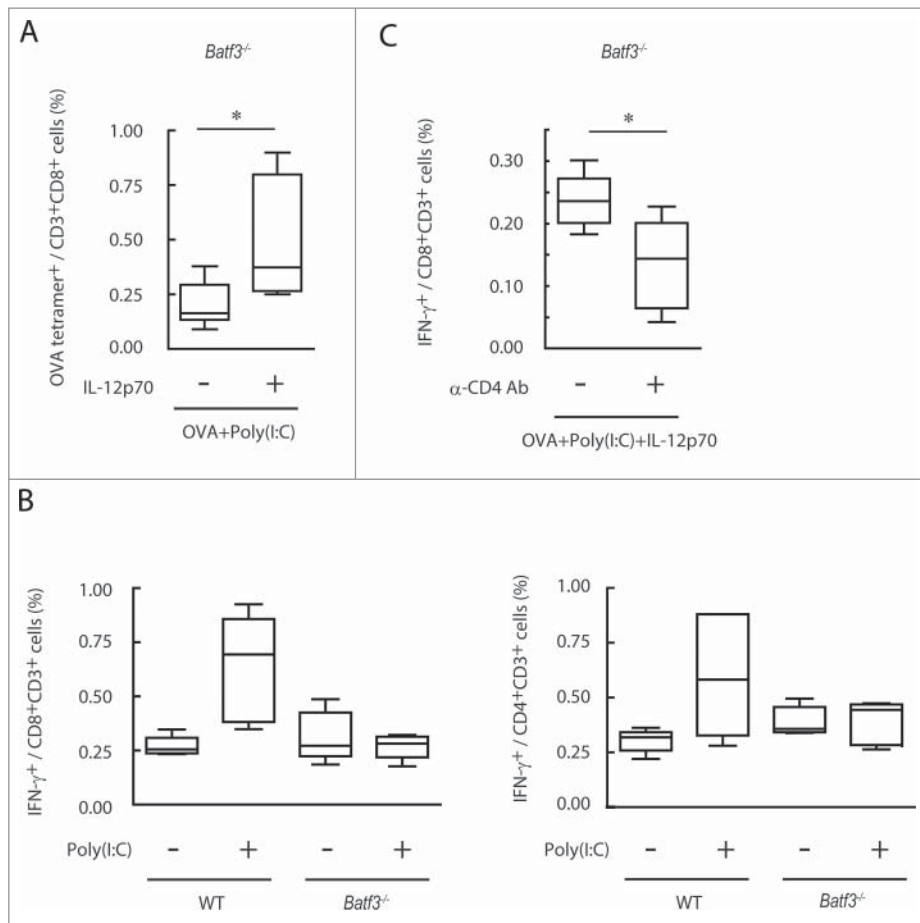
Figure 8. IL-12p70 induces CTL activation and CD4$^+$ T cell assist it in *Batf3*$^{-/-}$ mice. Poly(I:C) and OVA (1 mg) with or without IL-12p70 were i.p. administered to *Batf3*$^{-/-}$ mice. 7 d later, splenocytes were harvested and the proportion of OVA-tetramer$^+$ CD8$^+$ T cells was evaluated (A). Poly(I:C) and OVA (60 μg) were administered to wild-type or *Batf3*$^{-/-}$ mice at day 0 and 7. At day 14, the proportion of IFNγ^+ CD8$^+$ T and CD4$^+$ T cells in spleen were evaluated (B). Acsites containing anti-CD4 antibody were injected to *Batf3*$^{-/-}$ mice at day 0 and 7. 24 h later, Poly(I:C) and OVA (250 μg) with or without IL-12p70 were administered to *Batf3*$^{-/-}$ mice. At day 15, the proportion of IFNγ^+ CD8$^+$ T cells in spleen was evaluated (C). Error bars show $\pm$ SEM; n = 4 to 5 per group. Student's t-test (A, C) and Kluskal–Wallis test with Dunn's multiple comparison test (B) were performed to analyze statistical significance. *$p < 0.05$. Two similar experiments were performed and the results were supported.

activity to CTL in tumor site.[27] TLR3 adjuvant synergistically contributes to the PD-L1 therapy by promoting T cell entry into tumor (Kataoka, Takeda et al., submitted for publication). TLR3 adjuvant in DLN/spleen is also indispensable for T cell proliferation (priming step) by *Batf3*-dependent CD8α^+ DC in vaccine therapy since no tumor regression was observed by only adoptive transfer of *Batf3*$^{-/-}$ CD8α^+ DC subset (Fig. 7A). *Batf3*$^{-/-}$ CD8α^+ DC have moderate potential for T cell proliferation (presumably, due to high MDA5 expression) but not for TAA-specific T cell priming or IL-12 induction. CD8α^+ DC at least work in two distinct modes that sustain T cell priming in spleen and recruitment of effector T cells in tumor to regress tumor. Although tumor microenvironment works like lymphoid organ where intratumor DC might proliferate CTL in the tumor,[22] in our vaccine models CD8α^+ DC subset play a major role in recruiting CTL to tumor in response to Poly(I:C).

In our study, the Ag density is crucial for priming CTL. Tumor-specific CTL unable to determine by OVA tetramer (targeting other than OVA epitopes) might join Ag-specific T cell priming in spleen or tumor. It is also likely that OVA Ag are released from tumor cells to spread over spleen/DLN and taken up into CD169 macrophages or CD8α^+ DCs.[43]

IL-12 and chemokines are released from myeloid cells in tumor. It is accepted that macrophages and DC in tumor contribute to the formation of tumor microenvironment. IL-12 induces activation of T cells by IFNγ and PD-1 downregulation in CD8$^+$ T cells.[21] Transfer of CD8α^+ DC or moving CD8α/XCR1^{hi} DC both require Poly(I:C) stimulation for tumor reduction, suggesting that TLR3 signal is critical for the formation of tumor microenvironment to facilitate antitumor immunity. In fact, IL-12 administration with DC transfer results in full tumor regression (Fig. 7). What is the role of IL-12-derived CD4$^+$ T cells (Th1, Th17 or Treg) in the context of antitumor microenvironment yet remains to be further discussed.

Our next question was why *Batf3*$^{-/-}$ mice have lost the T cell priming and tumor-infiltrating abilities induced by Poly(I:C). *Batf3*$^{-/-}$ mice are >90% depletion of XCR1^{hi} CLEC9A$^+$ CD8α^+ DC in spleen but still possess them in DLN. *Batf3*$^{-/-}$ mice exhibit minor T cell priming in response to Poly(I:C) or Poly(I:C) + OVA (Fig. 2C), but no T cell infiltration into tumor (Fig. 5), which may represents the features of *Batf3*-independent DC. Alternatively, RIG-I-dependent cross-presentation happens in *Batf3*$^{-/-}$ CD8α^+ DC, which is less efficient than TLR3-dependent one.[44] There exist DC in epidermal or tumor

region that express high levels of RIG-I/MDA5, which harbor some ability to compensate cross-priming for *Batf3*^{−/−} CD8α[+] DC in response to Poly(I:C) or tumor-derived factors.[35] Thus, regarding T cell priming ability CXCR1^{hi}/CD8α[+] DC can be replaced with other DC subsets in spleen/DLN with high Ag input, but the ability of T cell infiltration into tumor is dependent on *Batf3*-positive DC in tumor (Fig. 6). In the EG7 tumor model, tumor-infiltrating CD8α^{+} DC rather than CD103^{+} DC may be more important to attract priming T cells from spleen.[45] Thus, the mode of T cell proliferation and recruitment to tumor is environment- or tumor-type-dependent, probably in conjunction with DC subsets.[41,46] In any case, *Batf3* is implicated in TLR3-dependent DC maturation by Poly(I:C) in both spleen/DLN and tumor.

Because OVA is an artificial Ag with nonself epitopes, we challenged C1498-bearing mice with WT-1 peptide (Db126) and compared with Poly(I:C) in this study. It becomes obvious that tumors with mutated Ags of TAA are effective targets for anti-PD-1 therapy than those with differentiated or testis-specific Ags.[47] WT-1 likely mimics the latter self-Ag, so that C1498 may represent anti-PD-1 therapy-resistant tumors, yet Poly(I:C) has some therapeutic efficacy toward C1498(WT1). As expected, Db126 + Poly(I:C) less induced tetramer-specific CTL than OVA + Poly(I:C), suggesting the importance of the quality of Ags in vaccine immunotherapy for cancer.[48,49] Even in the case with Db126, however, Poly(I:C) endorses proliferation of antitumor CTL in spleen and induces tumor shrinkage. Since C1498 tumor cells do not produce CXCR3 ligands by stimulation with Poly(I:C) *in vitro* (data not shown), recruiting CTL to tumor will be induced by intratumor DC in WT-1-expressing tumors. In several papers, CXCL10 (or other CXCR3 ligands) allows CXCR3^{+} CD8^{+} T cells to direct to inflammatory lesions.[50] Although myeloid cells produce CXCR3 ligands by some stimulations, *Batf3*^{−/−} DC and tumor stroma fail to produce CXCR3 ligands in response to Poly(I:C). Characterization of the tumor-resident DC (CD8α[+] DC in our model) is a matter of great interest in this scenario.

Materials and methods

Cells

WT1-C1498 cells were provided by H. Sugiyama (Osaka University, Osaka. Japan) (Nakajima et al., 2012), and EG7 cells were purchased from ATCC (Manassas, VA), where authentication was checked using short tandem repeat analysis. Cells were obtained during 2012–2014 and used within 6 mo after resuscitation. EG7 cells were cultured in RPMI 1640 (GIBCO, the catalog number: 11875–093) supplemented with 10 % heat-inactivated FBS (Thermo Scientific, SH30910.03), 55 μM 2-mercaptoethanol (GIBCO, 21985–023), 10 mM HEPES (GIBCO, 15630–080), 1 mM sodium pyruvate (GIBCO, 11360–070), 50 IU penicillin/50 μg/mL streptomycin (GIBCO, 15070–063) and 0.5 mg/mL G418 (Roche, 04 727 894 001).[51] WT1-C1498 cells were cultured in RPMI 1640 supplemented with 10 % heat-inactivated FBS, 55 μM 2-mercaptoethanol, 50 IU penicillin/50 μg/mL streptomycin and 0.5 mg/mL G418. Splenocytes were cultured in complete medium containing 10 % heat-inactivated FBS, 45 % RPMI 1640, 45 % AIM-V

(GIBCO, 12055–091), 1 × non-essential amino acid (GIBCO, 11140–050), 55 μM 2-mercaptoethanol and 50 IU penicillin/ 50 μg/mL streptomycin. 3 and 5 d later, recombinant mouse IL-2 (20 IU/mL) (Pepro Tech, 212–12) was added to the culture.[34]

Mice

Wild-type C57BL/6J mice were purchased from CLEA. *Batf3*^{−/−} mice were purchased from Jackson Laboratory (Bar Harbor, ME). *Rag2*^{−/−} and OT-1 mice, and *Tlr3*^{−/−} mice were kindly provided by N. Ishii (Tohoku University, Sendai, Japan) and S. Akira (Osaka University, Osaka, Japan), respectively. OT-II mice were kindly provided from Dr Kazuya Iwabuchi (Kitasato University, Kanagawa, Japan). All mice were backcrossed >8 times to C57BL/6 background and maintained under specific pathogen-free conditions in the animal faculty 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.

Reagents and antibodies

Poly(I:C) and EndogGade® Ovalbumin (EndoOVA) were purchased from GE healthcare Life Sciences (the catalog number: 27-4732-01) and Hyglos (321001), respectively. OVA$_{257-264}$ peptide (SIINFEKL: SL8), OVA$_{323-339}$ peptide (ISQAVHAAHAEINEAGR: OVA helper peptide), OVA (H2K[b]-SL8) Tetramer was purchased from MBL (TS-5001-P, TS-M703-P, TS-5001-1). WT1$_{126-134}$ peptide (RMFPNAPYL: Db126) and WT1 (H2D[b]-Db126) Tetramer were kindly provided by H. Sugiyama (Osaka University, Osaka. Japan). Brefeldin A and z-VAD-FMK (z-VaL-Ala-Asp fluoromethyl ketone) were purchased from Sigma-Aldrich (B6542-5MG, V116-2MG). Collagenase D and Annexin-V-Fluous Staining Kit were purchased from Roche (11 088 882 001, 1 988 549). Recombinant mouse IL-12p70 (577002) and following antibodies, anti-CD3 (Clone: 17A2, the catalog number: 100215), anti-CD8α (53–6.7, 100706 and 100729), anti-CD11b (M1/70, 101206), anti-CD11c (N418, 117317), anti-CD16/32 (93, 101302), anti-CD103 (2E7, 121405), anti-CLEC9A (7H11, 143503), anti-F4/80 (BM8, 123109), anti-Gr1 (RB6-8C5, 108407), anti-I-A[b] (KH74, 115305) and anti-IFNγ (XMG1.2, 505809), anti-IL-12/IL-23p40 (C15.6, 505203), anti-TLR3 (11F8, 141905), anti-XCR1 (ZET, 148205) were purchased from BioLegend. Mouse IFN gamma ELISA KIT (88–7314) and Abs, anti-CD4 (GK1.5, 12-0041-82), anti-IL-12p35 (4D10p35, 50-7352-80), anti-TCR Vα2 (B20.1, 12-5812-82) were from eBiosciences. ViaProbe was from BD Biosciences (555816). Mouse CXCL10/IP10/CRG-2 ELISA KIT was purchased from R and D Systems (515–87921). ProLong® Gold which contains DAPI was purchased from Life technologies (P36931).

Tumor challenge and Poly(I:C) therapy

Mice were s.c. injected with 1 × 10^{6} syngeneic WT1-C1498 or 2 × 10^{6} EG7 cells on the back.[28,34] The tumor volume was calculated by the formula: Tumor volume [mm[3]] = 0.52 × (long diameter [mm]) × (short diameter [mm])[2]. Poly(I:C) (50 μg) with or without IL-12p70 (100 ng) was s.c. injected around WT1-C1498 tumor at day 5 and 12 after tumor implantation.

Poly(I:C) and WT1$_{126-134}$ peptide (100 μg) were wrapped in 20 μg of DOTAP (Roche, 11202375001) for s.c. injection. In EG7 tumor, Poly(I:C) with or without OVA (100 μg) treatment was started when the tumor volume reached about 200–600 mm^3. CD8$^+$ T cells or NK cells were depleted with intraperitoneal (i.p.) administration of hybridoma ascites of anti-CD8α or anti-NK1.1 mAb to mice a day prior to Poly(I:C) treatment.[28,52] CD8α^+ DCs were isolated from spleens of wild-type or Batf3$^{-/-}$ mice for CD8α^+ DC-adoptive transfer by CD8$^+$ DC isolation kit (Miltenyi Biotec, 130-091-169). The purity of the cells was routinely ≥90 %. For adoptive transfer, 5 × 10^5 CD8α^+ DC was intravenously (i.v.) administered to Batf3$^{-/-}$ mice 24 h before the Poly(I:C) treatment.

Analysis of intratumoral microenvironment

WT1-C1498 and EG7 tumors were excised from tumor-bearing mice. For flow cytometer analysis, tumors were cut finely and treated with 0.05 mg/mL collagenase I (Sigma-Aldrich, C0130-100 MG), 0.05 mg/mL collagenase IV (Sigma-Aldrich, C5138-1G), 0.025 mg/mL hyaluronidase (Sigma-Aldrich, H6254-500MG) and 0.01 mg/mL DNase I (Roche, 10 104 159 001) in Hanks' balanced salt solution (Sigma-Aldrich, H9269-500ML) at 33°C for 10 min. Intratumor IL-12 and CXCL10 was measured by ELISA of the supernatant obtained from fixed volume of tumors. Tumor-infiltrating CD8$^+$ T cells and myeloid cells were analyzed by FACS AriaII (BD Biosciences). For the exclusion of false-positive, dead cells were excluded by removing of FSC^{lo} SSC^{hi} and 7-AAD$^+$ populations. Furthermore, the isotype control antibodies were used as negative controls. For microscopic analysis, 4% paraformaldehyde-fixed, frozen tumor sections of thickness of 10 μm were stained with hematoxylin (Sigma-Aldrich, H9627) and eosin (Merck, 1.15935.0025) or APC-anti-CD8α mAb, PE-anti-TCR-Vα2 and DAPI.[40] The stained specimens were analyzed under BZ-9000 (KEYENCE) or LSM510 META microscopy (Zeiss).

OT-1 proliferation assay

OT-1 T cells were prepared from spleens of Rag2$^{-/-}$/OT-1 mice by CD8$^+$ microbeads (Miltenyi Biotec, 130-049-401). OT-1 cells were labeled with 1 μM CFSE for 10 min at room temperature. 5 × 10^4 CD8α^+ DCs were seeded in a 96-well flat bottom plate in the presence of OVA and Poly(I:C) (50 μg/mL). The CD8α^+ DCs were incubated for 3 h and then co-cultured with CFSE-labeled 2.5 × 10^4 OT-1 cells. In some experiments, IL-12p70 (100 ng/mL) was added simultaneously with OT-1 cells to the wells with DC. After 60 h, these cells were stained with anti-TCR Vα2 and anti-CD8α and OT-1 proliferation was determined with diminution of CFSE by FACS AriaII. Additionally, IFNγ in the culture supernatant was measured by ELISA. In OT-II co-culture assay, 2.5 × 10^4 OT-II T cells were added to the conditioned wells with CD8α^+ DCs together with OT-1 cells.

Reverse transcription-PCR and real-time PCR

Total RNA was prepared using TRIzol Reagent (Ambion, 15596018) following the manufacturer's instructions. Reverse transcription-PCR was carried out using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4368813) according to the manufacturer's instructions. Real-time PCR was performed using a Step One real-time PCR system (Applied Biosystems). Sequences of primers in this study are shown in Table S1. Levels of target mRNAs were normalized to Gapdh and fold-induction of transcripts was calculated using the ddCT method relative to unstimulated cells.

Antigen-specific T cell expansion in vivo

Mice were i.p. immunized with OVA, Poly(I:C) (150 μg) and IL-12p70 (100 ng) once or twice at weekly intervals. For CD4$^+$ cell depletion, hybridoma ascites of anti-CD4 mAb ($\sim$100 μg) was i.p. injected to mice a day prior to immunization. Spleens were collected on day 7 after the last immunization, and Ag-specific CTL activation was analyzed by tetramer assay or intracellular IFNγ staining. In some cases, splenocytes were pulsed with SL8 (100 nM) (for OVA-specific CD8$^+$ T cells) or helper peptide (1 μM) (for CD4$^+$ T cells) for 6 h and Blefeldin A (10 μg/mL) was added during the last 4 h.

Statistical analysis

p values were calculated by the following statistical analysis. For the multiple comparisons, one-way analysis of variance (ANOVA) with Bonferroni's test or Kluskal–Wallis test with Dunn's multiple comparison test were performed. For the comparison between two groups, Student's t-test was performed. Error bar represent the SD or SEM between samples.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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