SUPPLEMENTARY INFORMATION

8 Supplementary figures

Tumor-infiltrating dendritic cells suppress nucleic acid-mediated innate immune responses through TIM-3-HMGB1 interactions

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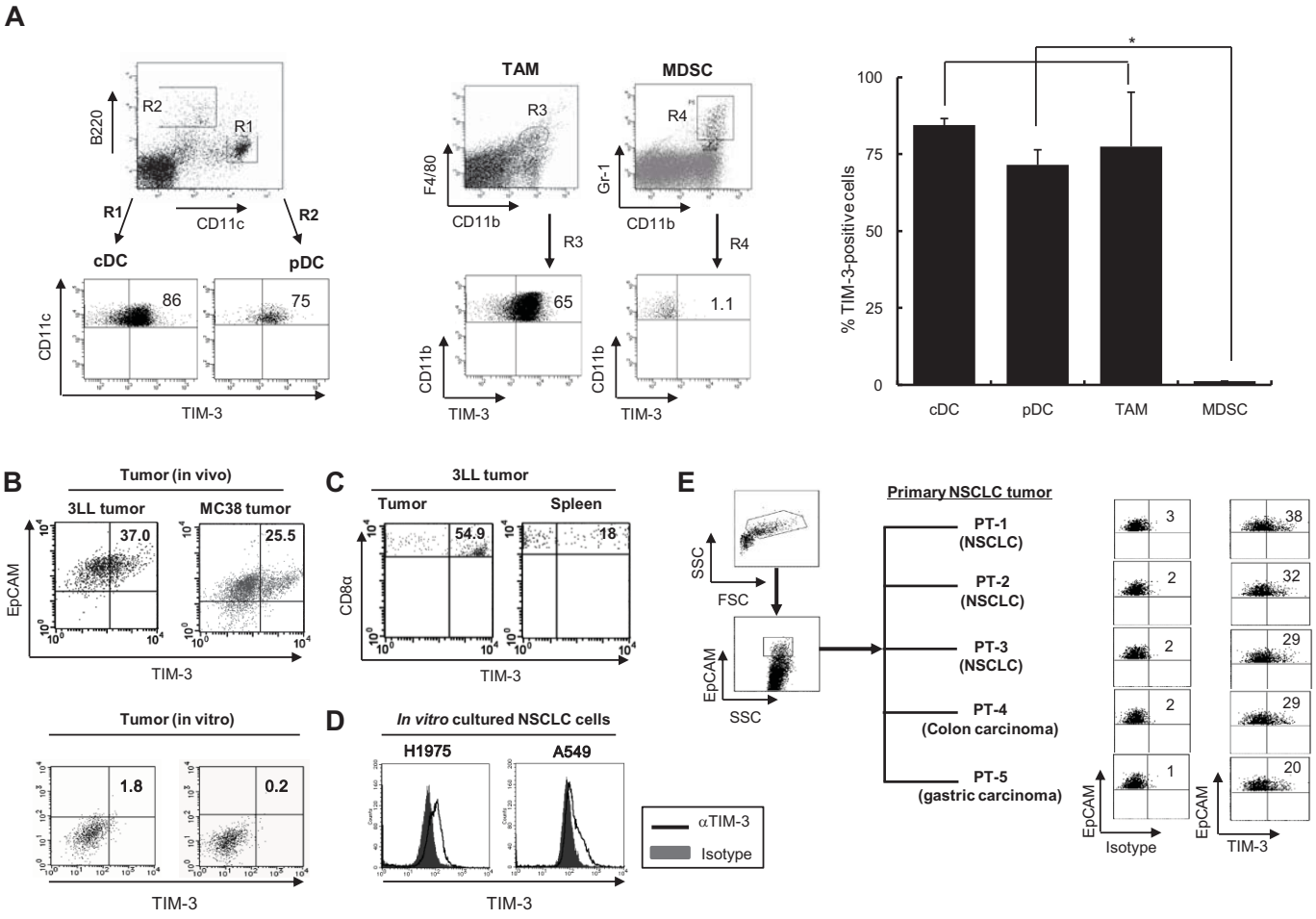
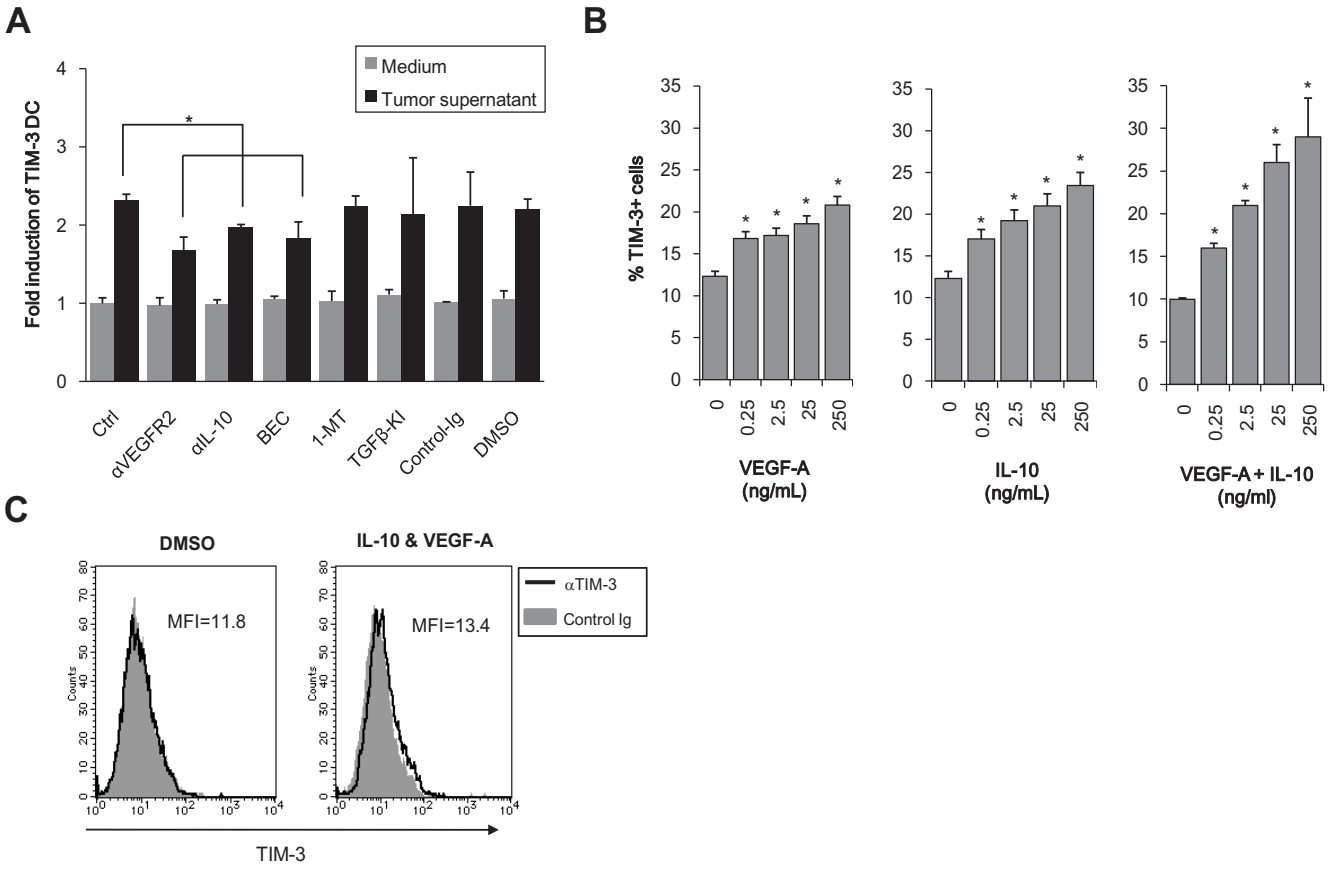


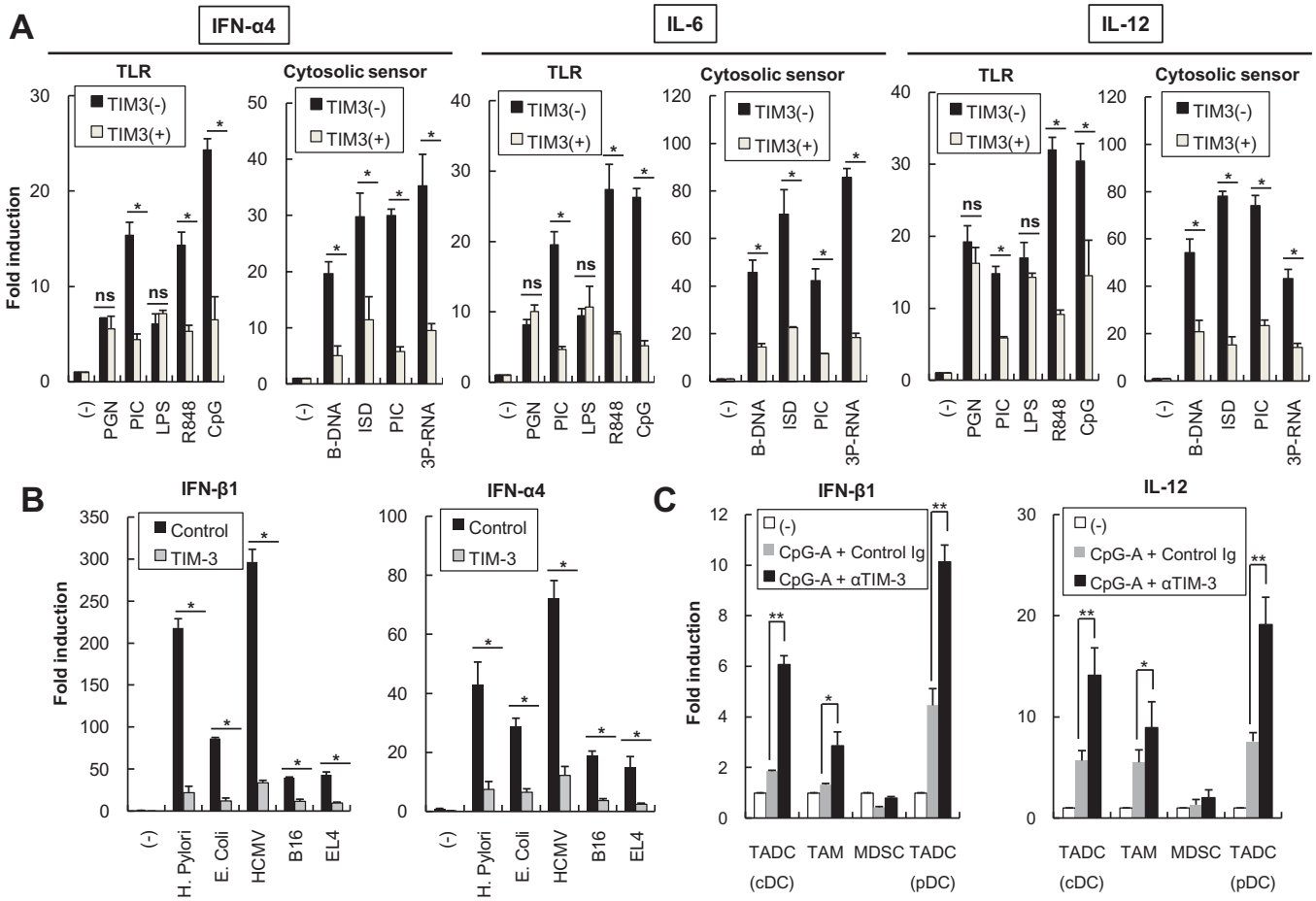
Supplementary Figure 1. TIM-3 expression in tumor cells and tumor infiltrating immune cells.

(**A**) F4/80^{+}CD11b^{+} macrophages (TAM) (R3), CD11b^{+}Gr-1^{+} myeloid-derived suppressor cells (MDSC) (R4) and CD11c^{high} DC (cDC) (R1), and CD11c^{low} B220^{+} plasmacytoid DC (pDC) (R2) isolated from 3LL tumors were subjected to TIM-3 expression analysis by flow cytometry. Representative data (left) and the percentages of TIM-3^{+} cells (right) are shown. (**B, C**) TIM-3 expression was evaluated on EpCAM^{+} MC38 or 3LL tumor cells cultured *in vitro* or isolated from established tumors *in vivo* (**B**), or CD8^{+} T cells from 3LL tumor-bearing mice (**C**). (**D**) TIM-3 expression was evaluated on EpCAM^{+} epithelial tumor cells isolated from five cancer patients (PT-1 to PT-5). The percentages of TIM-3^{+} cells among EpCAM^{+} epithelial cells are shown in the right-upper quadrant of each dot plot. (**E**) TIM-3 expression was evaluated on the *in vitro*-cultured NSCLC cell lines (H1975 and A549) by flow cytometry. Similar results were obtained in three experiments.



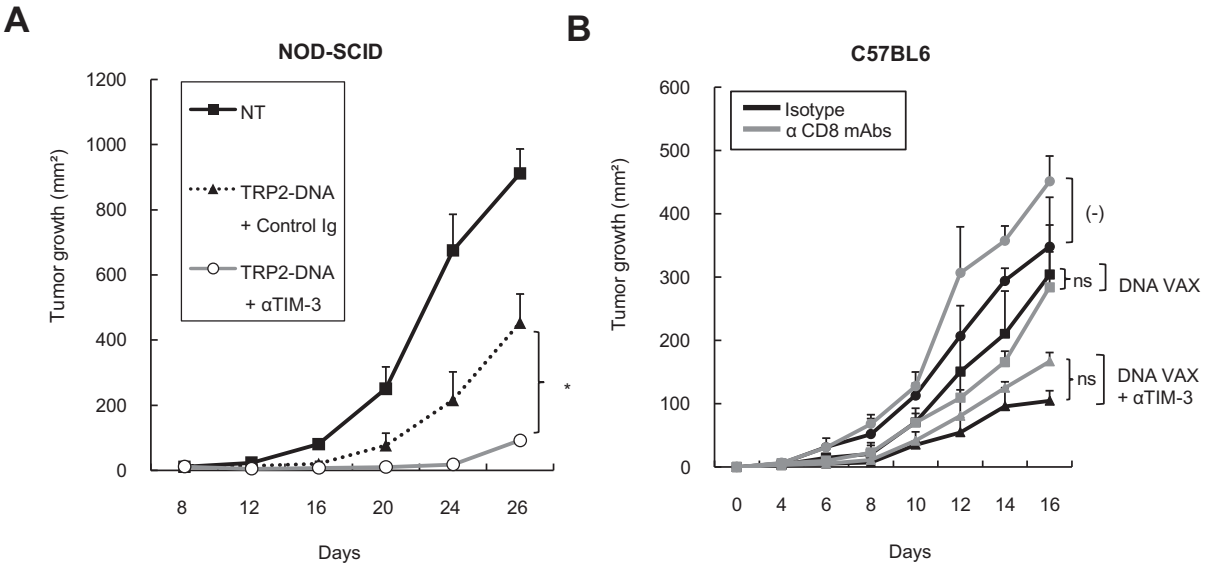
Supplementary Figure 2. TIM-3 induction by tumor microenvironmental factors.

(**A**) BMDC were treated with MC38 culture supernatants for 48 h. The culture supernatants were pretreated with blocking Ab for VEGF-R2 (αVEGFR2), IL-10 (αIL-10) or inhibitors for arginase-I (BEC), TGF-β1 receptor kinase (TGFβ-KI) or IDO (1-MT). TIM-3 expression on BMDC was evaluated by flow cytometry, and the fold induction of TIM-3$^+$ populations among CD11c$^+$ cells is shown. (B) BMDC were treated with rVEGF-A, IL-10 or both at the indicated concentrations. TIM-3 expression on BMDC was evaluated by flow cytometry and the fold induction of TIM-3$^+$ populations among CD11c$^+$ cells is shown. (**C**) MC38 or 3LL tumor cells were treated with VEGF-A and IL-10 for 48 h and the expression of TIM-3 was evaluated by flow cytometry.



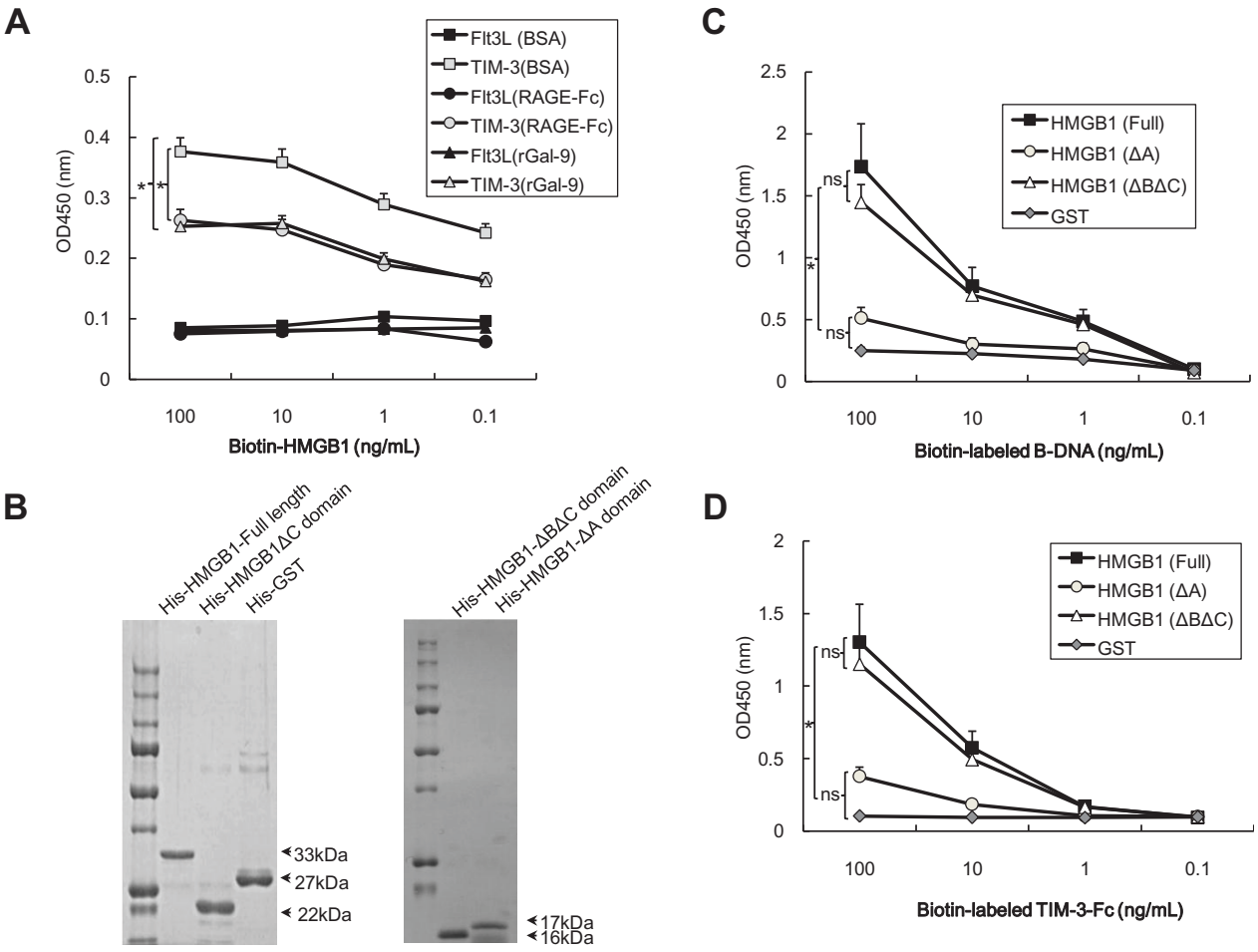
Supplementary Figure S3. TIM-3$^+$ myeloid cells suppress innate responses mediated by nucleic acids

(**A**) TIM-3$^+$ or TIM-3$^-$ BMDC were stimulated with various TLR ligands (PGN, poly(I:C), LPS, R848, CpG-ODN) or cytosolic sensors (poly(dA:dT) (B-DNA), interferon-stimulatory DNA (ISD), poly(I:C), 3p-RNA) for 8 h. The mRNA levels of IFN-α4, IL-6, and IL-12 were quantified by RT-PCR. (**B**) MEFs were transfected with TIM-3 or control vector for 24 h, followed by stimulation with genomic DNA isolated from bacteria (Helicobacter pylori and E. coli), virus (human cytomegalovirus (HCMV)) or tumors (B16-F10 and EL-4) for 8 h. The IFN-β1 and IFN-α4 levels were quantified by RT-PCR. (**C**) CD11c^{high} DC (TADC (cDC)), CD11c^{int}B220$^+$ DC (TADC (pDC)), F4/80$^+$CD11b$^+$ macrophages (TAM) or CD11b$^+$Gr-1$^+$ MDSC isolated from established 3LL tumors were pre-treated with anti-TIM-3 mAb or control Ig for 30 min followed by stimulation with CpG-A for 3 h. The IFN-β1 and IL-12 levels were quantified by RT-PCR.



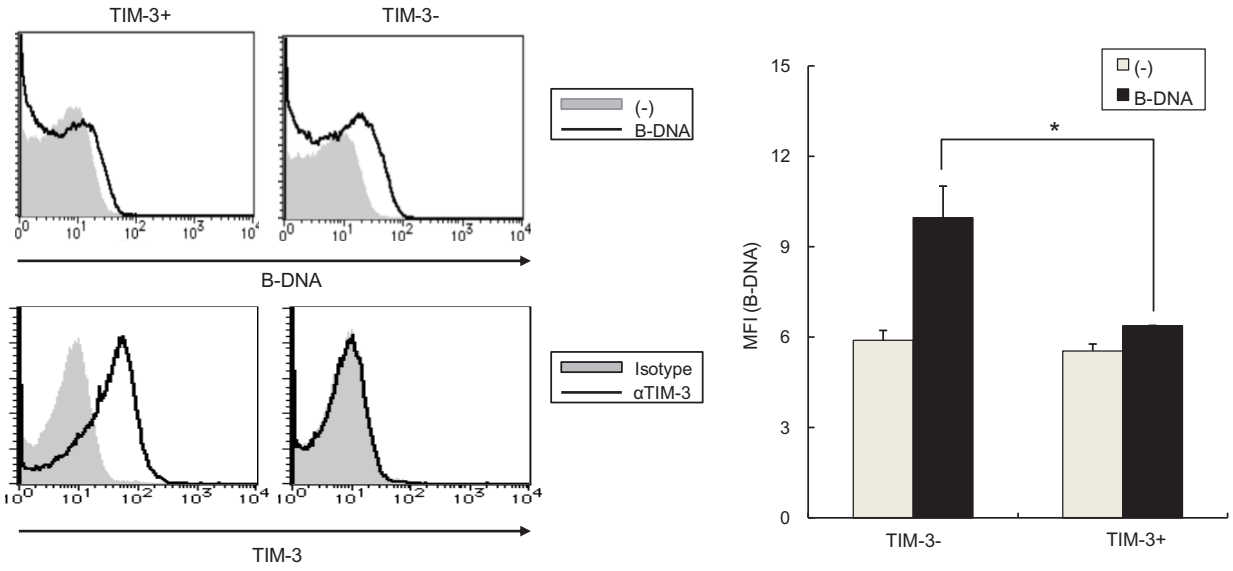
Supplementary Figure 4. Adaptive immunity is dispensable for antitumor responses mediated by anti-TIM-3 mAb.

(**A**) B16-F10 melanoma cells inoculated into NOD-SCID mice (n=4 per group) were treated with plasmid DNAs encoding TRP-2 (TRP2-DNA) in the presence of control Ig or anti-TIM-3 mAb (250 μg, i.p.) on days 8, 10 and 12 after tumor inoculation. (**B**) B16-F10 melanoma cells inoculated into wild-type C57BL/6 mice (n=4 per group) were treated with plasmid DNA alone or in combination with anti-TIM-3 mAb on days 8, 10 and 12 after tumor inoculation. In some groups, the mice were treated with anti-CD8 mAb (250 μg / mouse, ip) to deplete $CD8^+$ cells three days before tumor inoculation. Tumor growth was measured on the indicated days. NT: not treated.



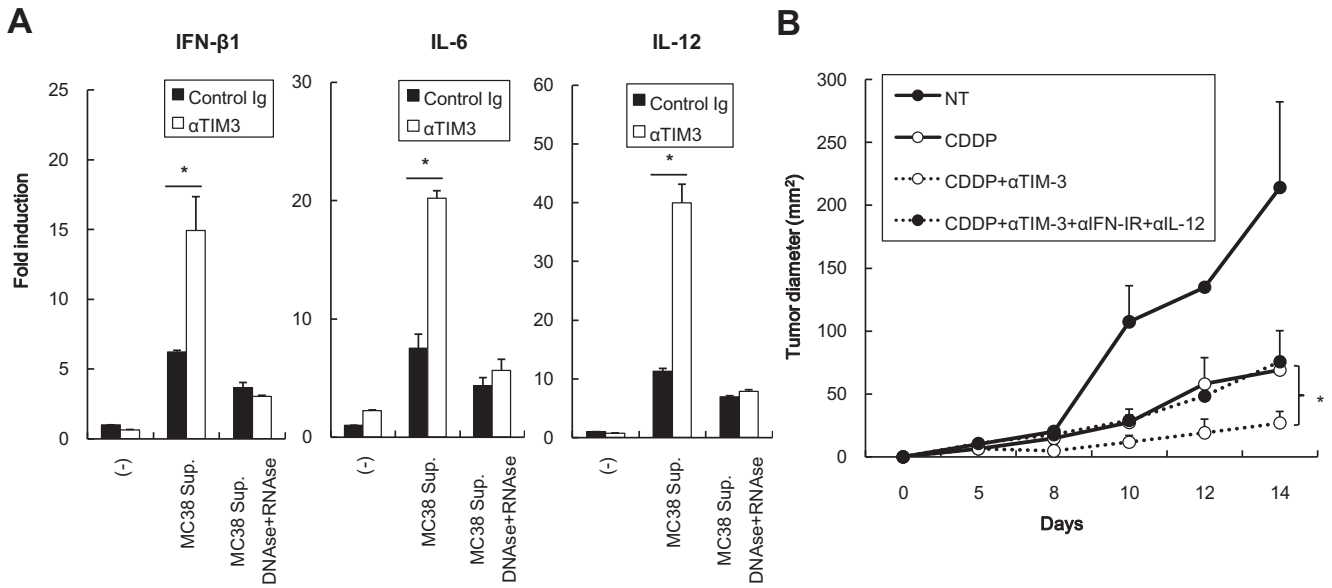
Supplementary Figure 5. TIM-3 binds to the A-Box domain in HMGB1.

(**A**) Fc-fusion proteins containing TIM-3 or Flt3L were coated onto plastic plates and biotin-labeled HMGB1 protein was added at the indicated concentrations in the presence of RAGE-Fc fusion protein (100 ng/mL), recombinant galectin-9 protein (10 ng/mL) or BSA (100 ng/mL). The binding of biotin-labeled DNA to HMGB1 was measured by colorimetric analyses (**B**) Generation of full-length or A, C, B/C-box-deleted HMGB1 proteins or control protein (GST). The purification of each protein was confirmed by Coomassie staining. (**C**) Full-length, ΔA, ΔBΔC-Box or GST (control) proteins (100 ng/mL) were coated onto plastic plates, and then biotin-labeled B-DNA or TIM-3-fc fusion proteins were added at the indicated concentrations. The binding of biotin-labeled molecules to the plate-bound proteins was measured by colorimetric analysis.



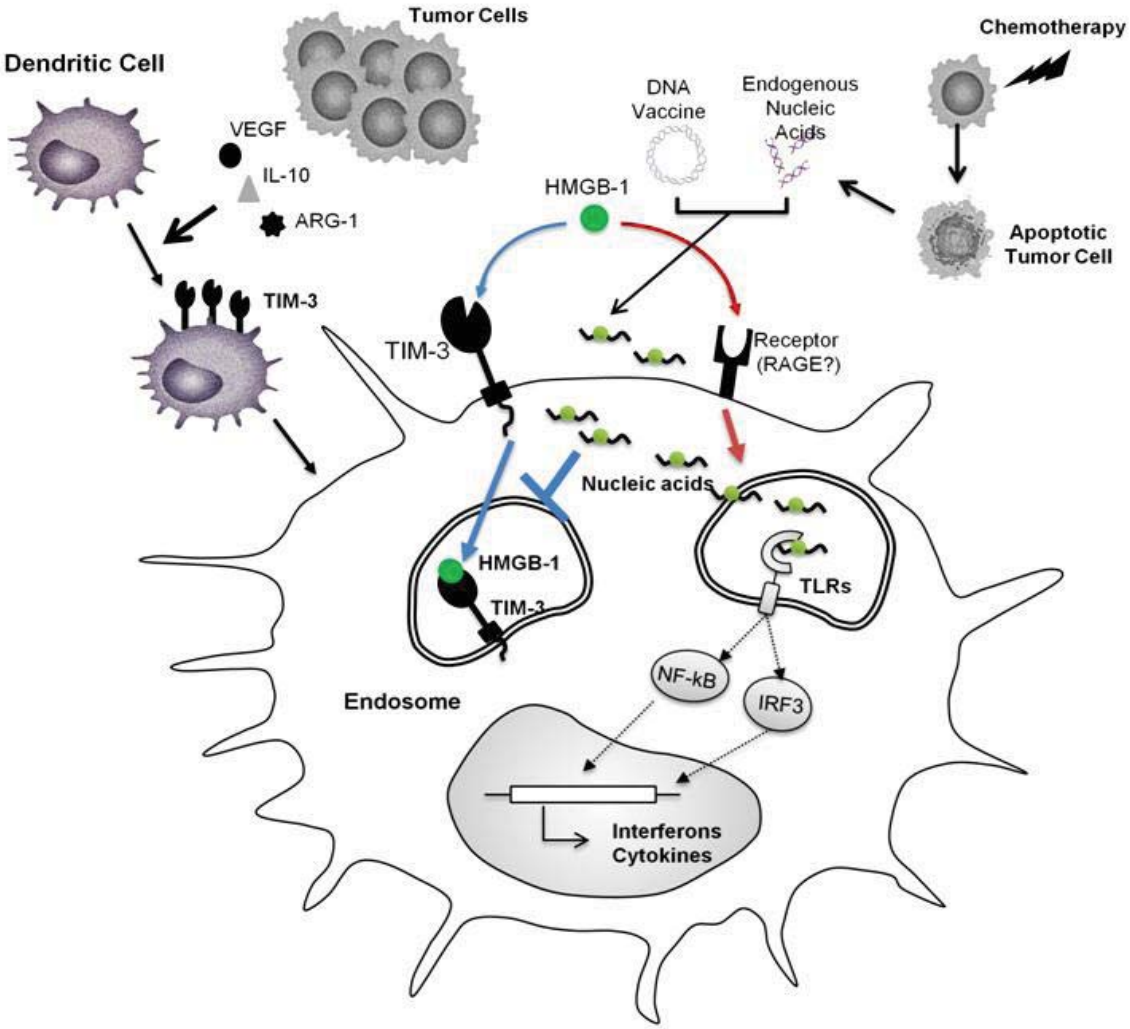
Supplementary Figure 6. Impaired uptake of B-DNA in TIM-3$^+$ endosomes

TIM-3$^+$ or TIM-3$^-$ BMDC were treated with HMGB1 in the presence of Alexa-flour 488-labeled B-DNA for 1 h. The endosomes isolated from these cells were fixed and stained with biotin-labeled anti-TIM-3 mAb and streptavidin-PE. Shown are histogram plots of representative experiments with the percentages of gated endosomes that were positive for B-DNA or TIM-3 indicated (Solid lines). Filled columns show unstained or secondary antibody-only stained controls. The average mean fluorescence intensity (MFI) of B-DNA in endosomes (n=3) was also shown (right panel).



Supplementary Figure 7. TIM-3 suppresses innate responses of nucleic acids released from dying tumor cells

(**A**) MC38 cells were treated with CDDP, and TIM-3^{+} BMDC were co-cultured with 10% supernatant of dying tumor cells in the presence of control Ig or anti-TIM-3 mAb. In some samples, dying tumor cells were treated with DNase and RNase before stimulation with tumor cell supernatant. The mRNA levels of IFN-β1, IL-6 and IL-12 in the cells were quantified by quantitative RT-PCR. (**B**) The DT-untreated CD11c-DTR mice were inoculated subcutaneously with MC38 cells (1 x 10^5/mice) and then treated with systemic CDDP in the presence of control Ig or anti-TIM-3 mAb. For some instances, the mice treated with CDDP and anti-TIM-3 mAb were treated with neutralizing Ab for type-I IFN receptor (αIFN-IR) and IL-12p40 (αIL-12). Non-treated mice serves as control (NT). The tumor growth was measured on the indicated days.



Supplementary Figure 8. TIM-3-mediated suppression of innate immune responses.

The mechanisms by which DC-derived TIM-3 impedes innate antitumor immune responses are shown. Although the release of nucleic acids from dying tumor cells upon cytotoxic therapies or DNA adjuvants boost innate immune responses mediated by TLR- and/or cytosolic sensor-mediated pattern recognition pathways, tumor microenvironments modulate DC to upregulate TIM-3, which interferes with HMGB-1-mediated transport of nucleic acids into endosomal vesicles and thus impedes downstream signals activating innate immune responses.