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Supplemental Material to:

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The interaction between murine melanoma and the immune system reveals that prolonged responses predispose for autoimmunity

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Supplementary Materials and Methods

Tumor models

In vitro. B16F10 melanoma (ATCC) was maintained, injected, and monitored as previously described (1). B16F10 derivative cell lines generated from wild type C57BL/6 or DEREG mice were maintained in complete DMEM media. Tumor cell lines were expanded and frozen down within 1^{st} -2^{nd} passage. All cell lines generated were tested for mycoplasma and were confirmed to be negative. Analysis of B16F10 derivative cell lines was performed on 3^{rd} – 5^{th} passage cells.

In vivo. Groups of wild type C57BL/6, and DEREG mice were treated with PBS alone or DTA (500 ng/mouse) (Sigma Aldrich) on days -2, 5, 12, and 19 relative to tumor inoculation, unless otherwise indicated. PBS- or DT-treated mice were then monitored and measured for tumor growth.

Flow cytometry

Tumor infiltrating leukocytes (TILs). Established B16F10 tumors were excised from DEREG mice on the days indicated. Tumors excised from mice were digested, and then used for flow cytometry analysis as previously described (1). For intracellular staining of IFN-γ, and granzyme B, TILs were stimulated in vitro with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence of Golgi Plug (BD Biosciences) for 4 h, and then subjected to surface staining as aforementioned. Surface-stained TILs were then fixed and permeabilised using BD Cytofix/Cytoperm^{TM} (BD Biosciences) according to the manufacturer's protocol, and then stained with anti-mouse IFN-γ (XMG1.2), granzyme B (GB11), and respective isotype antibodies. Cells were acquired on the

BD FACSCANTO II (BD Biosciences) and analysis was carried out using FlowJo (Tree Star).

Tumor cell lines. Tumor cell lines were stained with anti-mouse CD119 (2E2), pan Rae1, CD155 (4.24.1), CD112 (502-57), NKp46-Fc chimera, ICAM1 (3E2), CD70 (FR70), CD80 (16-10A1), DR5 (MD5-1), TRAIL (N2B2), FasL (MFL3), PD-L1 (MIH5), PD-L2 (TY25), CD31 (MEC13.3), CD62L (MEL-14), CD62P (RB40.34), H-2K^b (AF6-88.5), H-2D^b (28-14-8), and respective isotype antibodies (eBioscience, BD Pharmingen, R&D Systems, BioLegend, and Abcam) on ice. 7-AAD (BD Pharmingen) was added immediately before flow cytometry analysis. Cells were acquired on the BD FACSCANTO II (BD Biosciences) and analysis was carried out using FlowJo (Tree Star).

Statistical analysis

Statistical analyses were carried out using Graph Pad Prism software. Significant differences in tumor growth were determined by a Mann–Whitney test. Significant differences in cell subsets were determined by an unpaired T test. Values of $P < 0.05$ were considered significant.

References

1. Ngiow SF, von Scheidt B, Akiba H, Yagita H, Teng MW, Smyth MJ. Anti-TIM3 antibody promotes T cell IFN-gamma-mediated antitumor immunity and suppresses established tumors. Cancer Res. 2011;71:3540-51.

Supplementary Figures

Supplementary Figure 1. B16F10 TILs in PBS- or DT-treated DEREG mice. Groups of C57BL/6 DEREG mice (n=4-7) were inoculated subcutaneously with the B16F10 melanoma cell line (5 x 10^4) on day 0. On days -2, 5, and 12, mice were injected intraperitoneally with either DT (500 ng) or PBS. On day 18-20, tumors were excised and FACS analyses on TILs were performed. Representative FACS plot for frequencies of (A) CD45.2$^+$ cells, (B) NK cells and total T cells, (C) CD4$^+$ T cells, (D) CD8$^+$ T cells, (E) Tregs, (F) Vβ11$^+$ cells, (G) Vβ13$^+$ cells, gated on (B-D) CD45.2$^+$ cells, (E) CD4$^+$ T cells, or (F-G) CD8$^+$ T cells are shown.

Supplementary Figure 2. Immune effector cell activation status of B16F10 TILs in PBS- or DT-treated DEREG mice. Groups of C57BL/6 DEREG mice (n=4-11) were inoculated subcutaneously with the B16F10 melanoma cell line (5 x 10^4) on day 0. On days -2, 5, and 12, mice were injected intraperitoneally with either DT (500 ng) or PBS. On day 18-20, tumors were excised and FACS analyses on TILs were performed. Representative FACS plot for (A-B) TCRβ level, (C-D) CD44 and CD62L expression, (E-F) IFN-γ, (G-H) granzyme B, (I-J) Ki67, gated on (A, C, E, G, and I) CD8$^+$ T cells, or (B, D, F, H, and J) CD4$^+$ T cells are shown.

Supplementary Figure 3. Foxp3+ Treg depletion enhances intratumor IFN-γ$^+$ and granzyme B$^+$ T cells. Groups of C57BL/6 DEREG mice (n=8-11) were inoculated subcutaneously with the B16F10 melanoma cell line (5 x 10^4) on day 0. On days -2, 5, and 12, mice were injected intraperitoneally with either DT (500 ng) or PBS. On day 18, tumors were excised and TILs were stimulated with PMA and

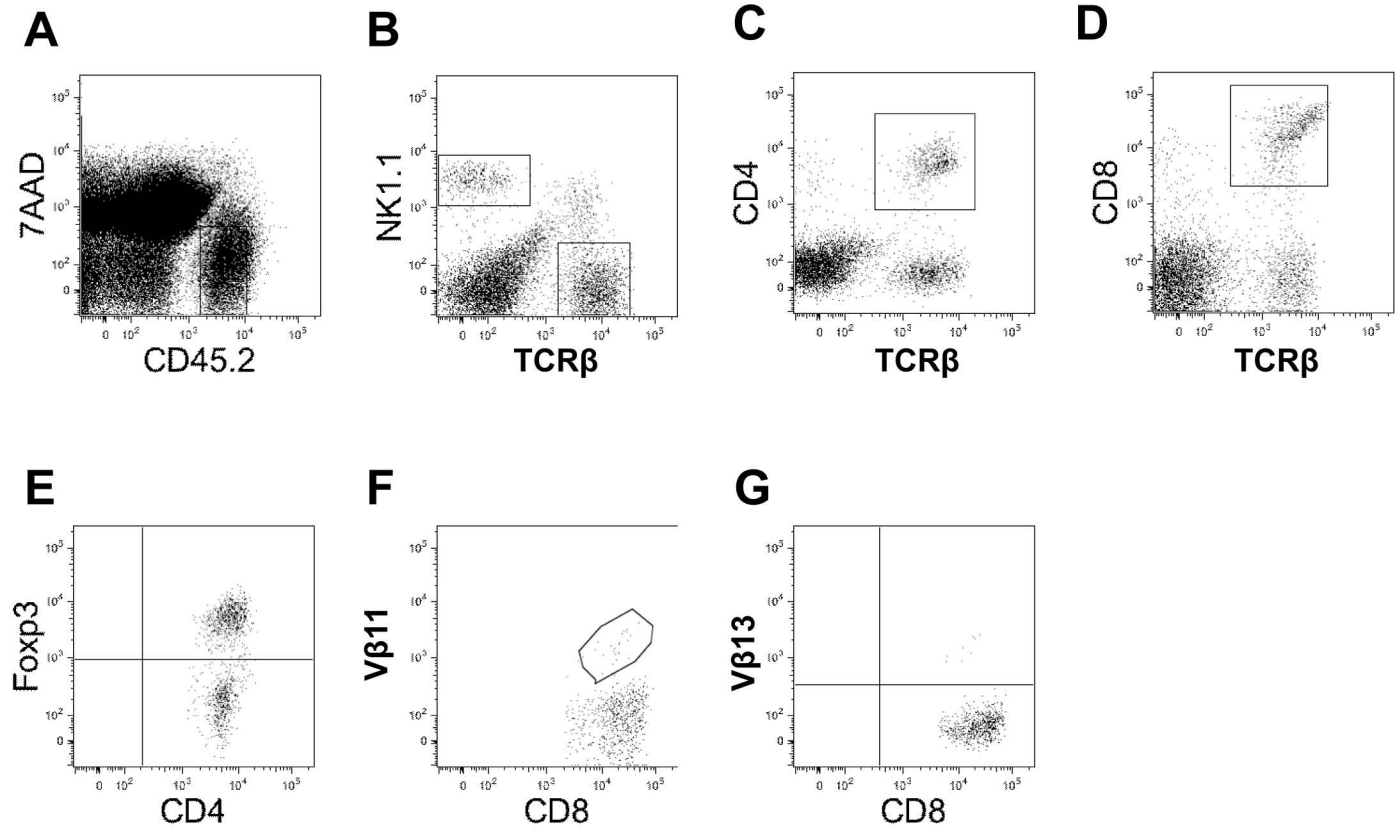
ionomycin *in vitro* for 4 h. FACS analyses on PMA and ionomycin-stimulated TILs were performed. Frequencies of (A) IFN-γ^+ cells, (B) granzyme B^+ cells, gated on (*left panel*) $CD8^+$ T cells, or (*right panel*) $CD4^+$ T cells of B16F10 TILs from PBS- or DT-treated mice are shown. Each symbol represents a single mouse. Statistical differences in frequencies of (A) IFN-γ^+ cells, or (B) granzyme B^+ cells, of respective $CD8^+$ T cells and $CD4^+$ T cells between PBS- and DT-treated mice were determined by an unpaired T test (***, $P < 0.001$). Representative FACS plot for (C and D) IFN-γ, (E and F) granzyme B, gated on (A, C and E) $CD8^+$ T cells, or (D and F) $CD4^+$ T cells are shown.

Supplementary Figure 4. Immunogenicity of B16F10 tumor cell lines derived from PBS- or DT-treated DEREG mice. Groups of C57BL/6 DEREG mice were inoculated subcutaneously with the melanoma cell line B16F10 (5×10^4) on day 0. On days -2, 5, 12, and 19, mice were injected intraperitoneally with either DT (500 ng) or PBS. Tumors from each group were excised when tumor size exceeded 120 mm^2 in size, and cell lines were then generated and stained for expression of respective markers as indicated. Representative histogram plots for the different markers on respective tumor cell lines (PBS, n= 3 cell lines; DT, n= 4-5 cell lines) are shown.

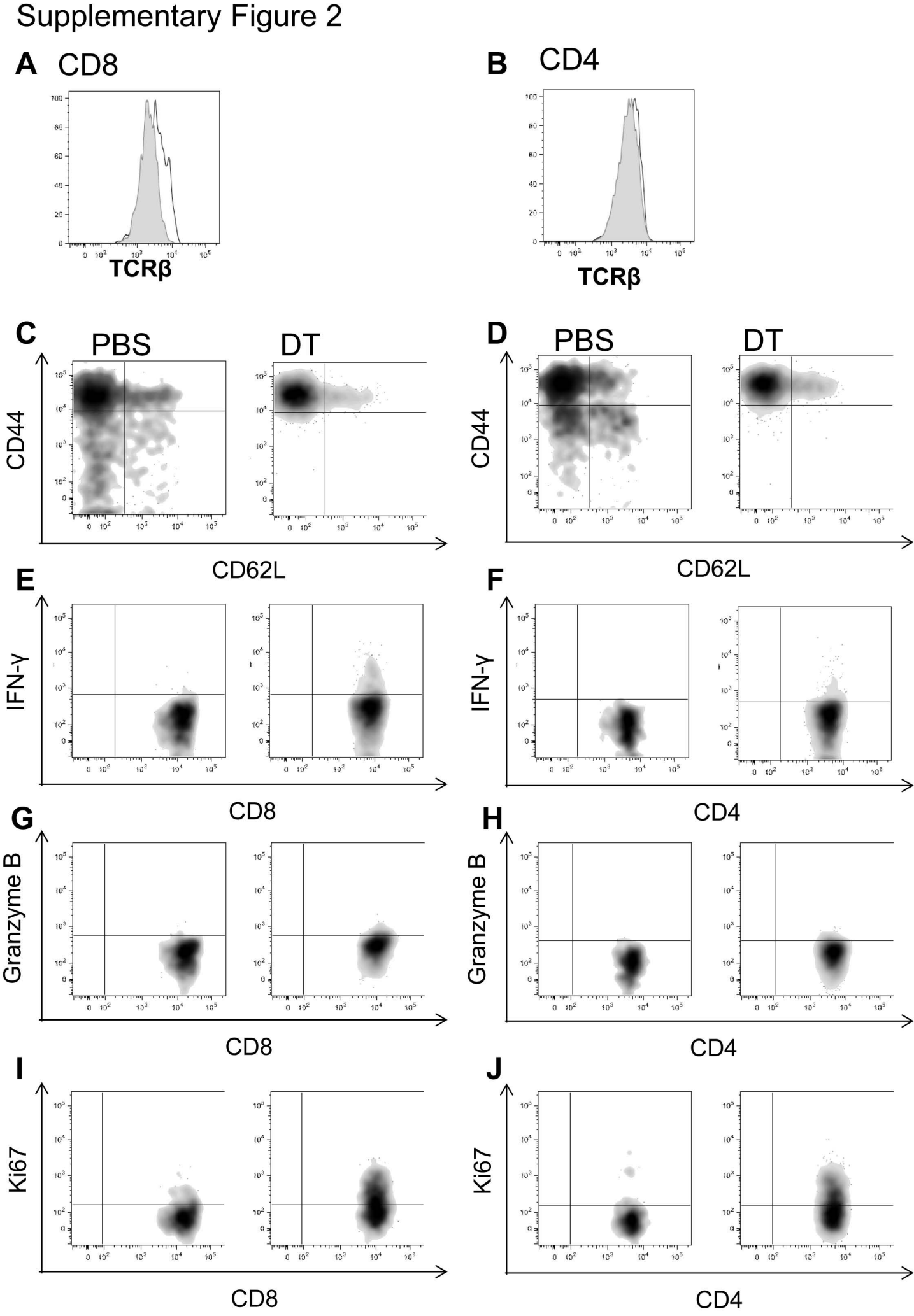
Supplementary Figure 5. DT does not directly affect B16F10 tumor growth nor does it induce up-regulation of tumor MHC-I expression. (A) Groups of C57BL/6 wild type mice (n =3-5) were inoculated subcutaneously with the melanoma cell line B16F10 (5×10^4) cells on day 0. On days -2, 5, and 12, mice were injected intraperitoneally with either DT (500 ng) or PBS. Mice were measured for tumor development and tumor size (mm^2) of mice is represented as the mean ± SEM. Data

shown are representative from two independent experiments. (B) Tumors from each group were excised when tumor size exceeded 120 mm^2 in size, and cell lines were then generated and stained for expression of H-$2K^b$ and H-$2D^b$.

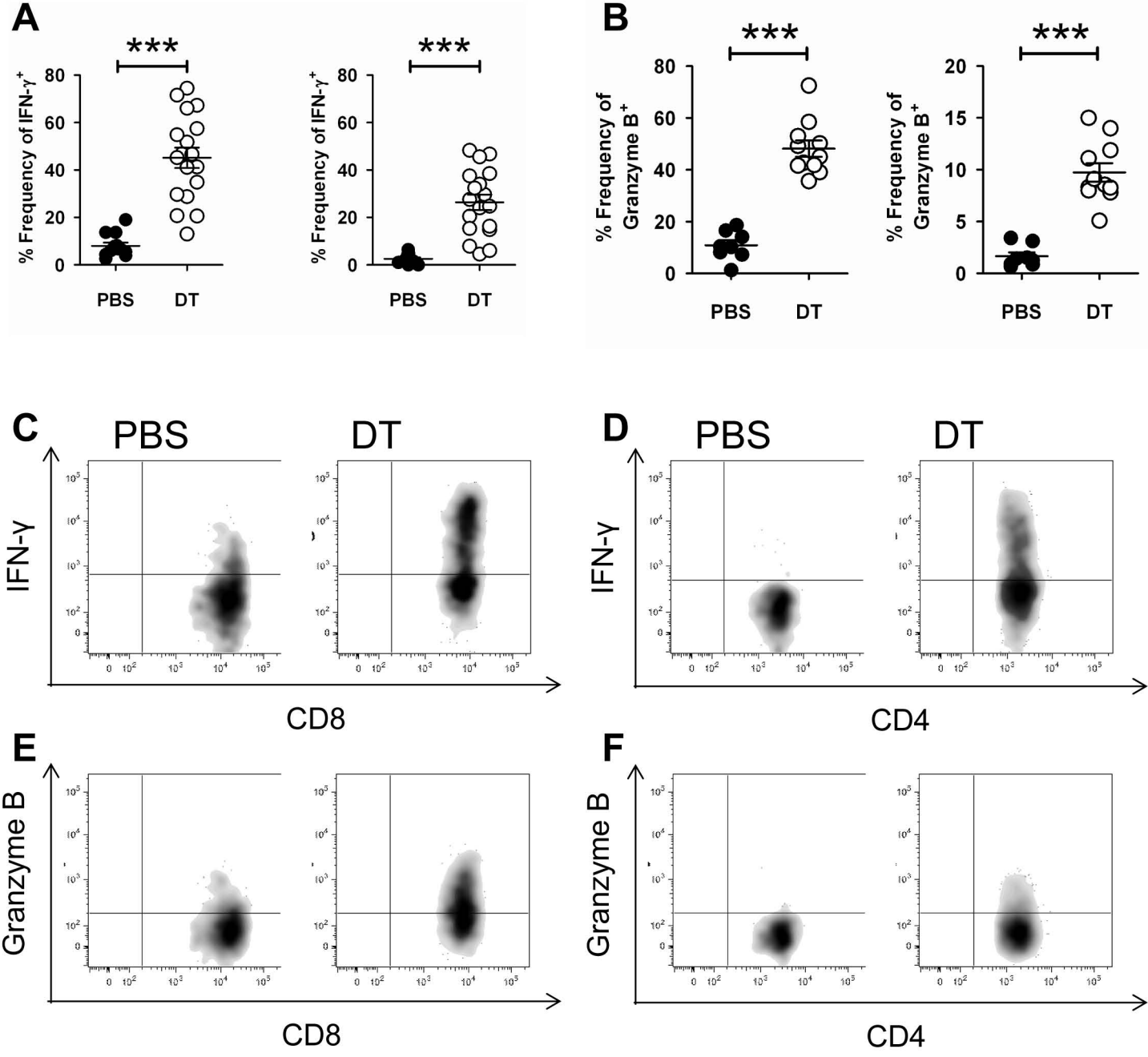
Supplementary Figure 1



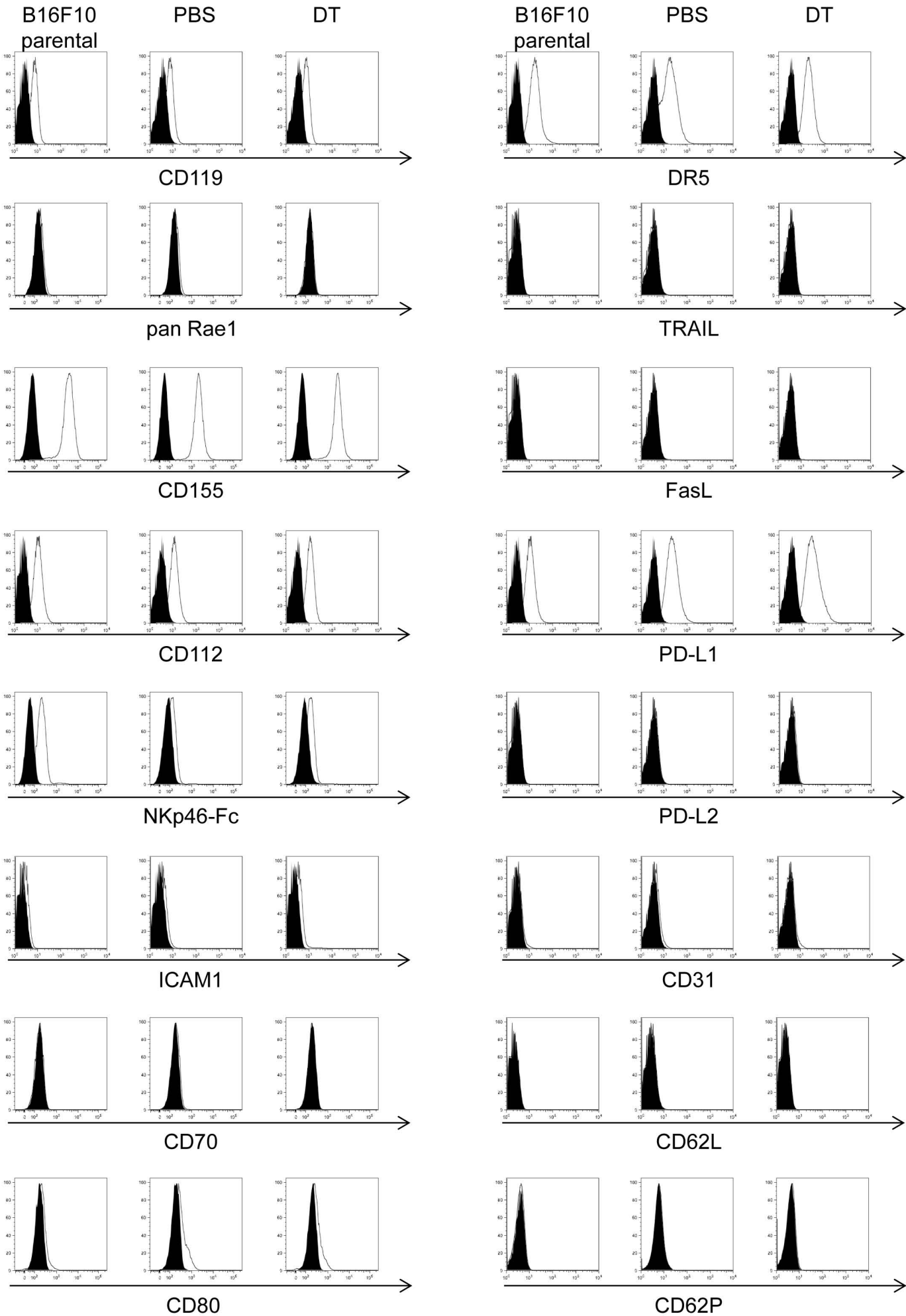
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5