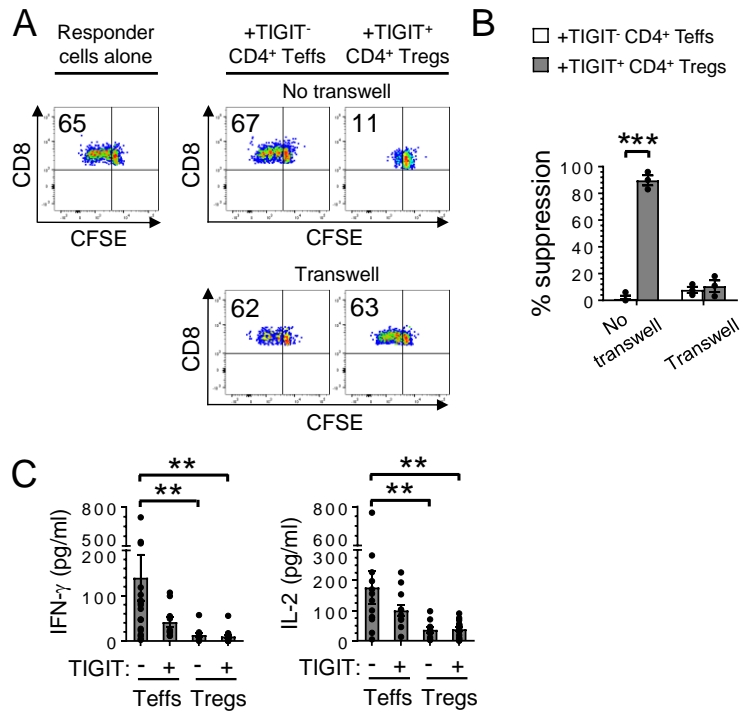
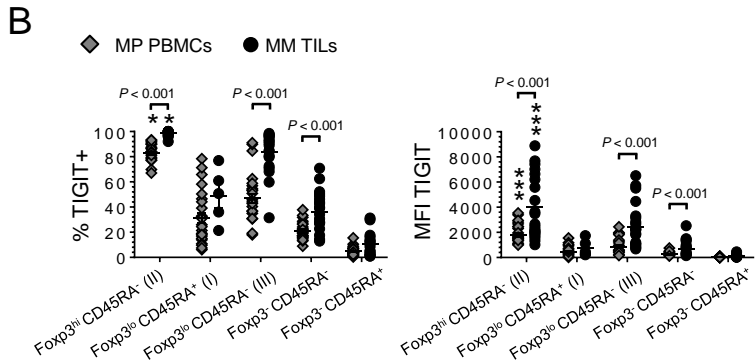
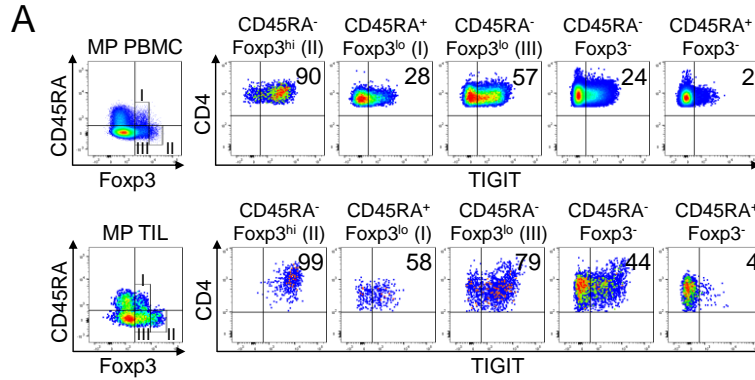


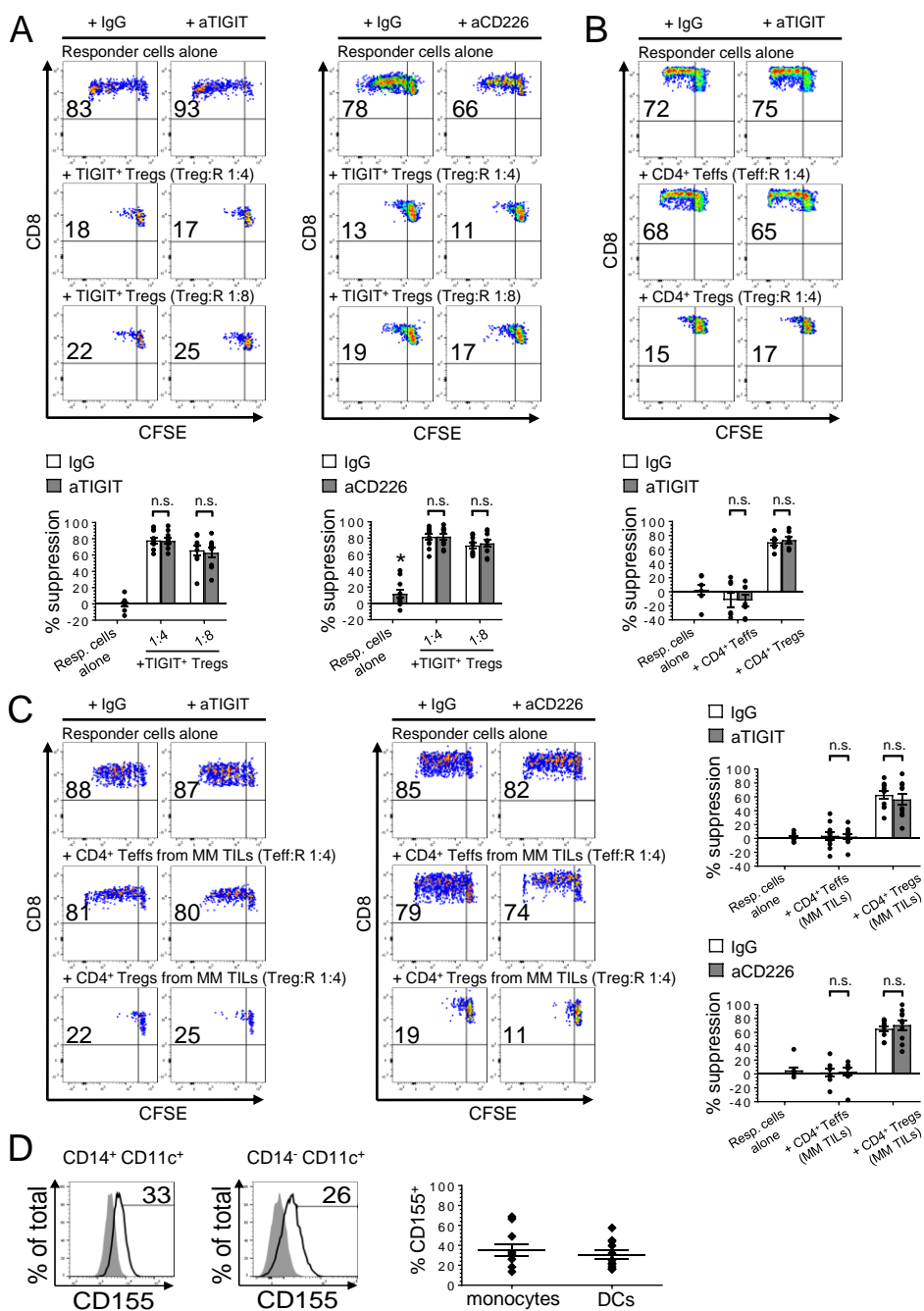
Supplemental Figure 1. Treg frequencies and TIGIT expression in T cell subsets in healthy donors and melanoma patients. (A) Summary data from all donors showing ex vivo frequencies of CD25^{hi} Foxp3⁺ CD4⁺ Tregs within total CD4⁺ T cells in PBMCs of healthy donors (HDs) and melanoma patients (MPs) and in metastatic melanoma (MM) tumor-infiltrating lymphocytes (TILs). *n* = 20. (B) Summary data showing ex vivo percentages and mean fluorescence (MFI) of TIGIT expression by total CD8⁺ T cells, total CD4⁺ T cells and CD25^{hi} Foxp3⁺ CD4⁺ Tregs in MM TILs (*n* = 12) Horizontal bars depict the mean percentages. Error bars indicate s.e.m. P values were obtained by unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.



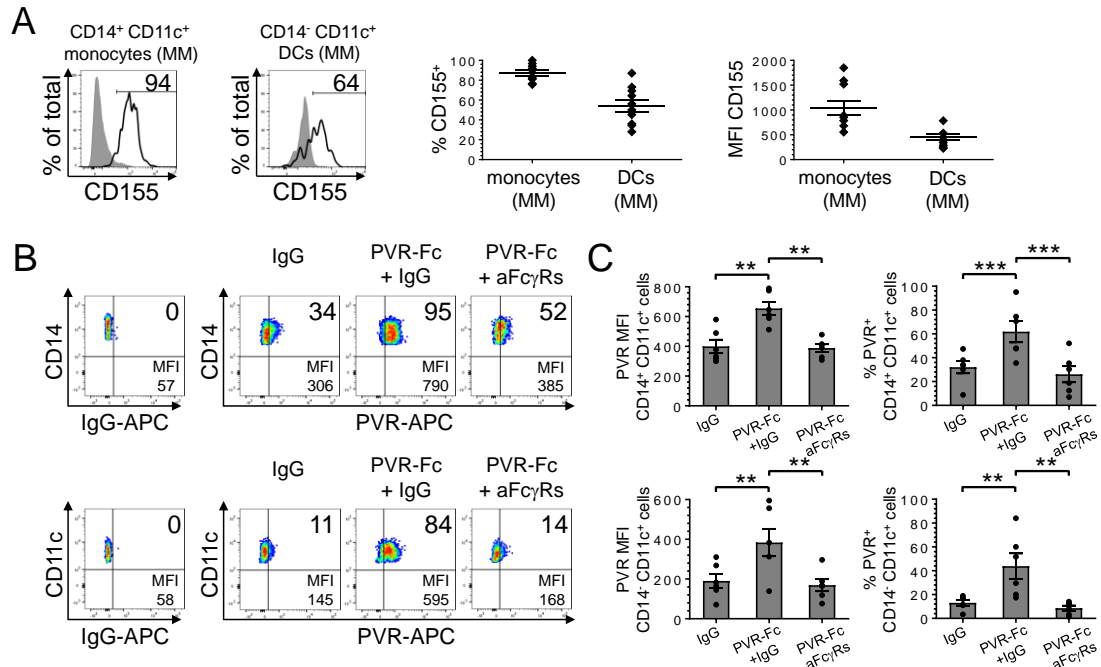
Supplemental Figure 2. TIGIT⁺ Treg suppression in transwell experiments and pro-inflammatory cytokine production by Tregs. (A and B) Flow cytometric analysis from one representative experiment (A) and summary data (B) showing the suppression of proliferation of responder CD8⁺ T cells that were stimulated for 6 days with autologous antigen presenting cells (APCs) and anti-CD3 mAbs in the presence of TIGIT⁻ CD4⁺ Teffs or TIGIT⁺ CD4⁺ Tregs. TIGIT⁻ CD25⁻ CD127^{-/+} CD4⁺ Teffs or TIGIT⁺ CD25^{hi} CD127⁻ CD4⁺ Tregs isolated from PBMCs of melanoma patients (MPs) were cultured either in direct contact with responder T cells in the bottom chamber of a transwell plate (*No transwell*) or in the top chamber of a transwell plate (*Transwell*) with allogeneic APCs and anti-CD3 mAbs ($n = 3$). P values were obtained by paired t-test. (C) Pooled data showing the production of IFN- γ and IL-2 by TIGIT⁻ and TIGIT⁺ CD25⁻ CD127^{-/+} CD4⁺ Teffs and CD25^{hi} CD127⁻ CD4⁺ Tregs isolated from PBMCs of MPs after a 6-day in vitro stimulation with aCD3/CD28 beads ($n = 20$). Results represent the mean of independent experiments. Error bars indicate s.e.m. P values were obtained by unpaired t-test. **P < 0.01; ***P < 0.001.



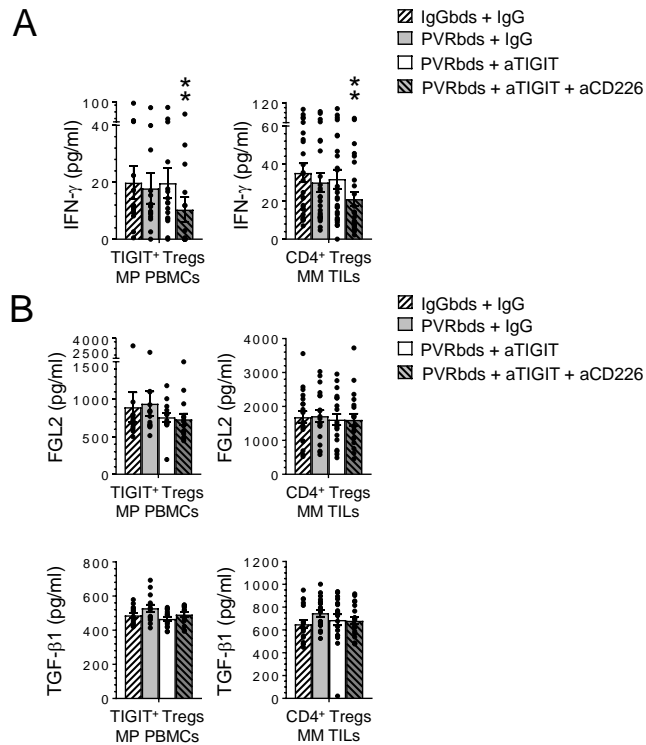
Supplemental Figure 3. TIGIT expression in Foxp3⁺ and Foxp3⁻ CD4⁺ T cell subsets in melanoma patients. (A and B) Flow cytometry dot plots from 2 different melanoma patients (MPs) (A) and summary data from all MPs (B) showing ex vivo TIGIT expression [% and mean fluorescence intensity (MFI)] by Foxp3^{hi} CD45RA⁻ activated CD4⁺ Tregs (Fr. II), Foxp3^{lo} CD45RA⁺ resting CD4⁺ Tregs (Fr. I) and non-suppressive Foxp3^{lo} CD45RA⁻ CD4⁺ T cells (Fr. III), as well as Foxp3⁻ CD45RA⁻ and Foxp3⁻ CD45RA⁺ CD4⁺ T cells in PBMCs and tumor-infiltrating lymphocytes (TILs) of MPs ($n = 20$). Horizontal bars depict the mean percentage or MFI. Error bars indicate s.e.m. P values were obtained by unpaired t-test and repeated-measures ANOVA followed by Tukey's test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



Supplemental Figure 4. Treg suppression upon TIGIT or CD226 blockade in melanoma patients. (A) Flow cytometric analysis from representative experiments and summary data showing the suppression of responder CD8⁺ T cell proliferation after a 6-day in vitro stimulation (IVS) with autologous antigen presenting cells and anti-CD3 mAbs in the absence or presence of TIGIT⁺ CD25^{hi} CD127⁻ CD4⁺ Tregs isolated from PBMCs of melanoma patients (MPs) and with blocking aTIGIT or aCD226 mAbs or IgG control mAbs ($n = 10$). **(B)** Flow cytometric analysis from one representative experiment and summary data showing the suppression of responder CD8⁺ T cell proliferation after a 6-day IVS as in A but in the absence or presence of total CD25⁻ CD127^{+/+} CD4⁺ Tefs or CD25^{hi} CD127⁻ CD4⁺ Tregs isolated from PBMCs of MPs and with blocking aTIGIT mAbs or IgG control mAbs ($n = 7$). **(C)** Flow cytometric analysis from one representative experiment and summary data showing the suppression of responder CD8⁺ T cell proliferation after a 6-day IVS as in A but in the absence or presence of CD25⁻ CD127^{+/+} CD4⁺ Tefs or CD25^{hi} CD127⁻ CD4⁺ Tregs isolated from metastatic melanoma (MM) tumor-infiltrating lymphocytes (TILs) and with blocking aTIGIT or aCD226 mAbs or IgG control mAbs ($n = 10$). The ratios of CD4⁺ T cells to responder CD8⁺ T cells are indicated. **(D)** Representative histograms and summary data showing PVR/CD155 expression on CD14⁺ CD11c⁺ monocytes and CD14⁻ CD11c⁺ DCs in non-CD3 cells from some samples used in stimulations of A, B and C ($n = 10$). Solid grey histogram indicates isotype staining. Results represent the mean of independent experiments. Error bars indicate s.e.m. P values were obtained by paired t-test. *n.s.*, non significant.



Supplemental Figure 5. PVR expression by circulating antigen presenting cells (APCs) upon treatment with human PVR-Fc and by APCs in the tumor microenvironment. (A) Representative histograms and summary data showing ex vivo PVR/CD155 expression on CD14⁺ CD11c⁺ monocytes and CD14⁻ CD11c⁺ DCs in metastatic melanoma (MM). *n* = 10. Solid grey histogram indicates isotype staining. (B-C) Flow cytometric analysis from one representative experiment (B) and summary data showing PVR expression [% and mean fluorescence intensity (MFI)] (C) on CD14⁺ CD11c⁺ monocytes and CD14⁻ CD11c⁺ DCs in PBMCs of donors after 2 hours of incubation in the presence of soluble PVR-Fc with or without blocking antibodies against Fcγ receptors CD16, CD32 and CD64 (aFcγRs) and/or IgG control mAbs. At the end of incubation, cells were stained with APC-labeled anti-PVR mAbs or APC-labeled IgG isotype control mAbs. *n* = 6. Results represent the mean of independent experiments. Error bars indicate s.e.m. P values were obtained by repeated-measures ANOVA followed by Tukey's test. ***P* < 0.01.



Supplemental Figure 6. Cytokine production by TIGIT⁺ Tregs from melanoma patients upon TIGIT and/or CD226 blockade and PVR binding. (A-B) Summary data showing the production of IFN- γ (**A**) and FGL2 and TGF- β 1 (**B**) by TIGIT⁺ CD25^{hi} CD127⁻ CD4⁺ Tregs isolated from PBMCs of melanoma patients (MPs) ($n = 15$ for IFN- γ , $n = 14$ for FGL2 and TGF- β 1) and total CD25^{hi} CD127⁻ CD4⁺ Tregs isolated from metastatic melanoma (MM) tumor-infiltrating lymphocytes (TILs) ($n = 27$ for IFN- γ , $n = 20$ for FGL2, $n = 19$ for TGF- β 1) after a 6-day IVS with aCD3/CD28/IgG or aCD3/CD28/PVR beads in the presence of aTIGIT and/or aCD226 blocking mAbs and/or IgG control mAbs. Error bars indicate s.e.m. P values were obtained by repeated-measures ANOVA followed by Tukey's test. *P < 0.05; **P < 0.01; ***P < 0.001.