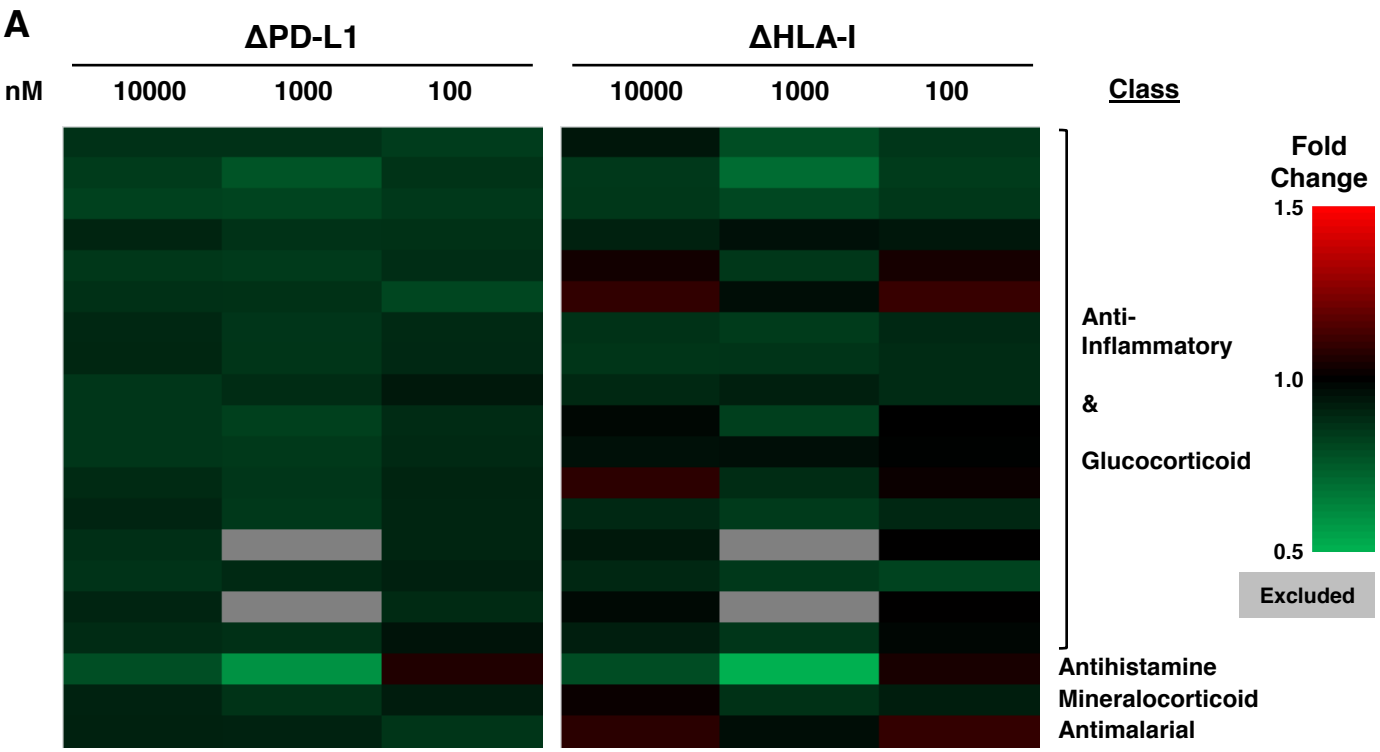
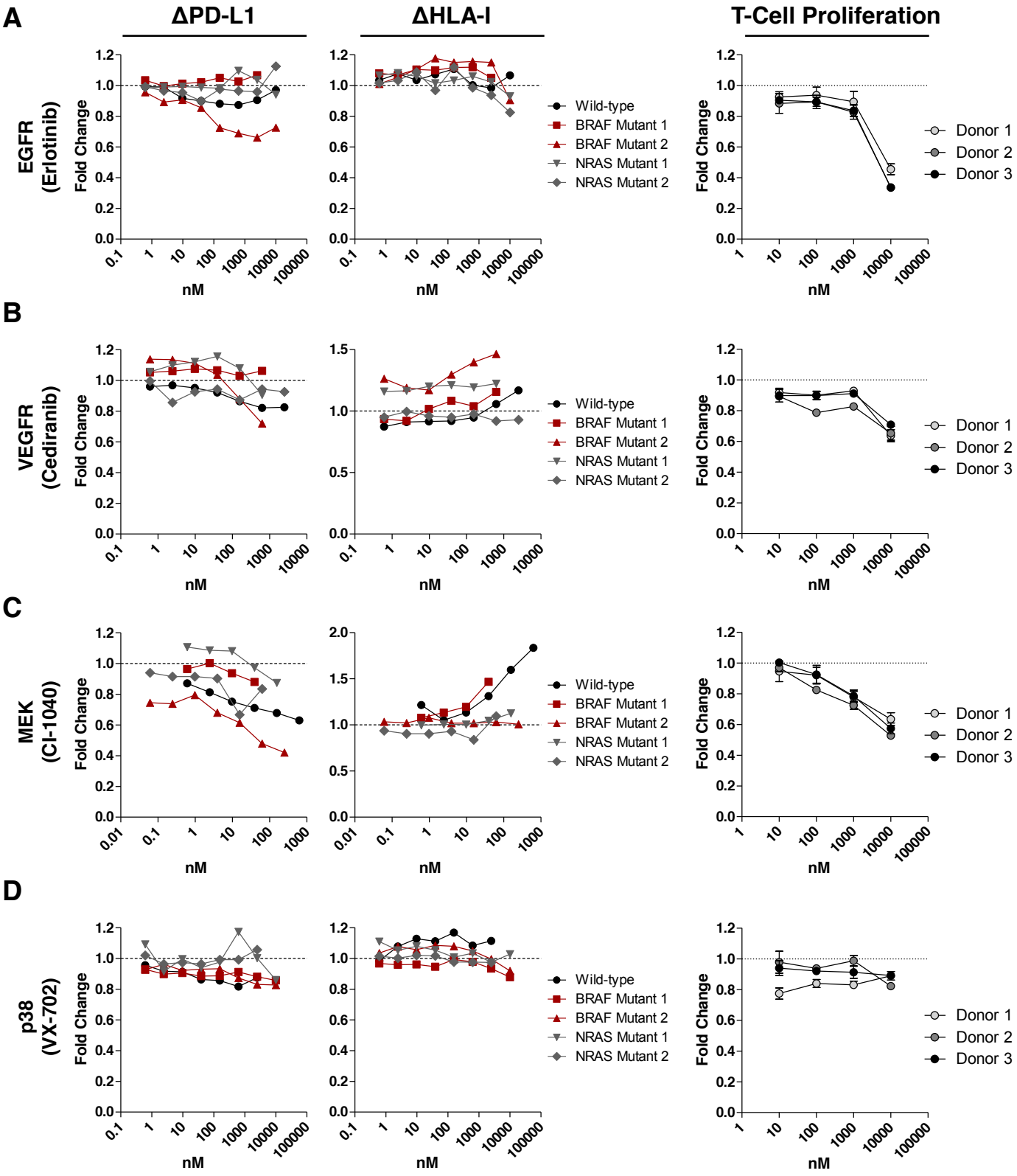
Supplementary Figures S1-S24

“A multikinase and DNA-PK inhibitor combination immunomodulates melanomas, suppresses tumor progression, and enhances immunotherapies”

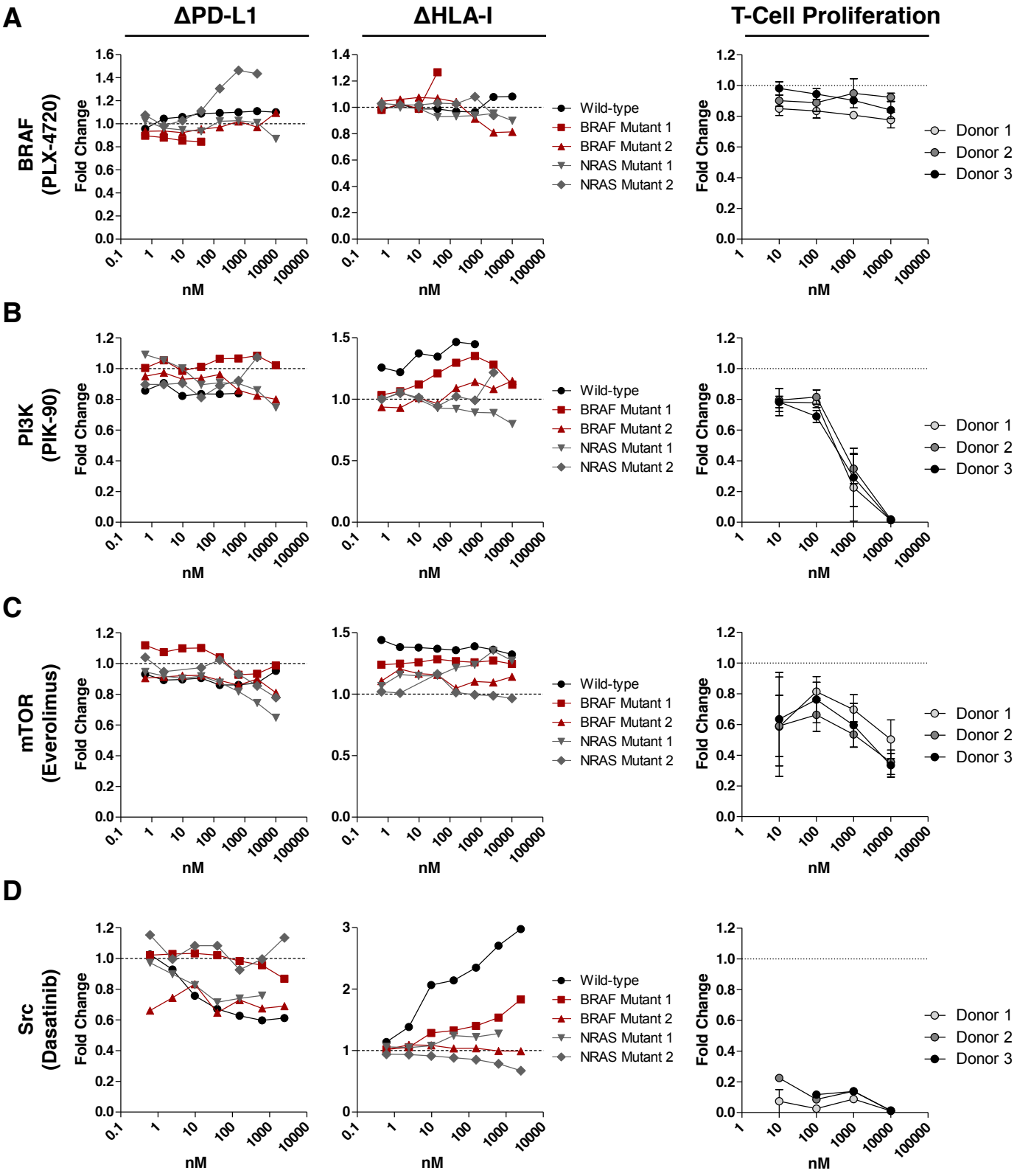
Tsai, et al.



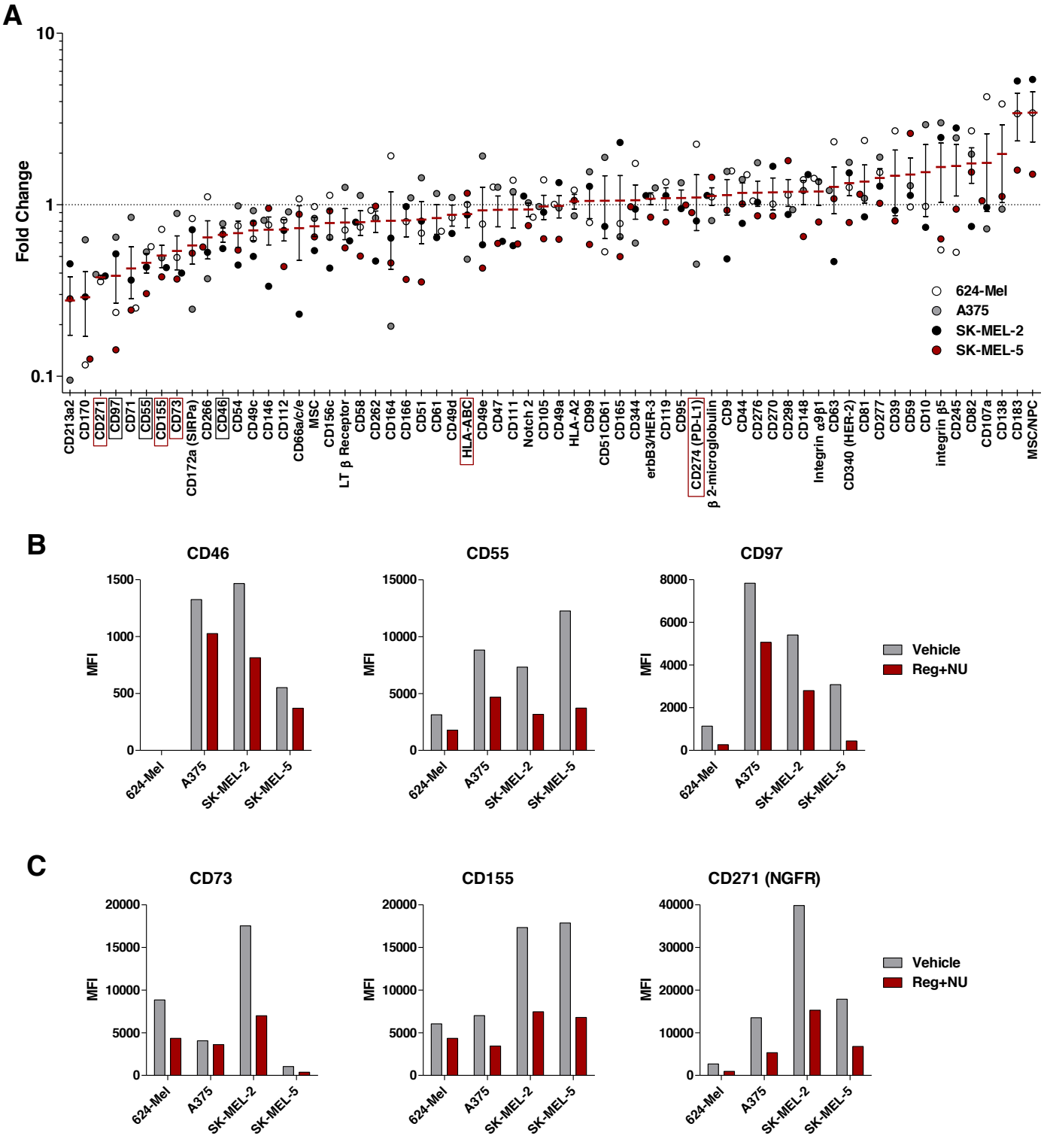
Supplementary Fig. S1: SC200 library HTS candidate drugs. (**A**) C8161 melanoma cells were treated with the indicated concentrations of various drugs. Changes in PD-L1 and HLA-I expression were assessed by flow cytometry after 48 hours of treatment with 1,120 compounds in the SC200 library compared to vehicle-treated cells. Compounds which on average across the three doses reduced PD-L1 expression by at least 10% are shown in a heat map. The class of each compound is indicated on the right. HTS was performed once.



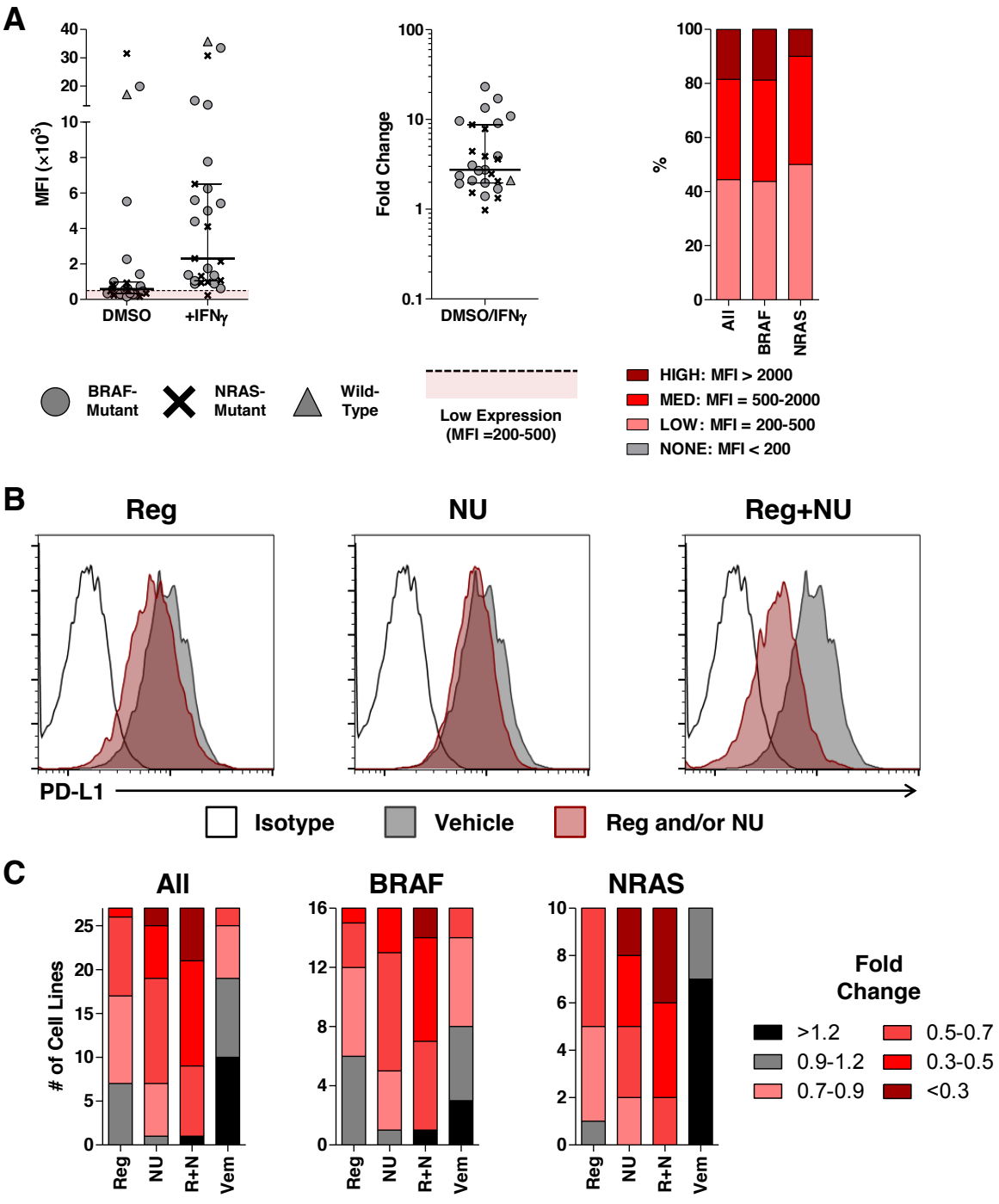
Supplementary Fig. S2: Effects of EGFR, VEGFR, MEK, and p38 candidate inhibitors on PD-L1 and HLA-I in a melanoma panel and on T-cell proliferation. (**A-D**) Changes in PD-L1 (left) and HLA-I (middle) expression were assessed after 48 hours of treatment with compounds selected from initial screening of the L1100 library in one wild-type, two BRAF-mutant, and two NRAS-mutant melanoma cell lines. Changes in T-cell proliferation (right) were assessed in three PBMC donors after five days of culture with varying concentrations of the candidate compounds along with anti-CD3 (OKT3, 50 ng/ml) and IL2 (100 U/ml) by ^{3}H-thymidine incorporation. Drug targets and names are indicated on the left. Multiple compounds from each class of drugs were assessed and results from representative compounds are shown. Data from T-cell proliferation represents mean ± SD. Experiments were performed once.



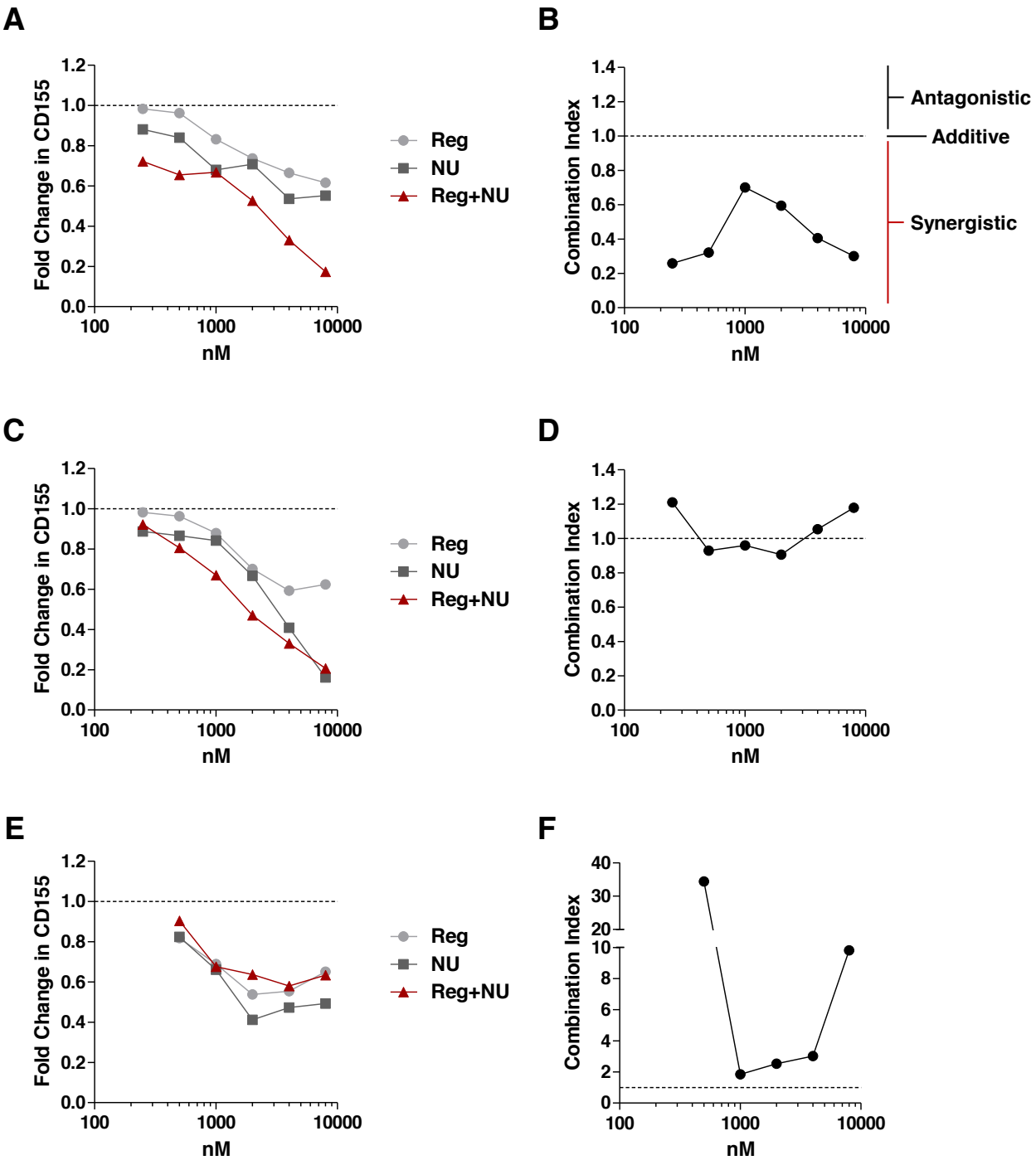
Supplementary Fig. S3: Effects of BRAF, PI3K, mTOR, and Src candidate inhibitors on PD-L1 and HLA-I in a melanoma panel and on T-cell proliferation. (**A-D**) Changes in PD-L1 (left) and HLA-I (middle) expression were assessed after 48 hours of treatment with compounds selected from initial screening of the L1100 library in one wild-type, two BRAF-mutant, and two NRAS-mutant melanoma cell lines. Changes in T-cell proliferation (right) were assessed in three PBMC donors after five days of culture with varying concentrations of the candidate compounds along with anti-CD3 (OKT3, 50 ng/ml) and IL2 (100 U/ml) by ^{3}H-thymidine incorporation. Drug targets and names are indicated on the left. Multiple compounds from each class of drugs were assessed and results from representative compounds are shown. Data from T-cell proliferation represents mean ± SD. Experiments were performed once.



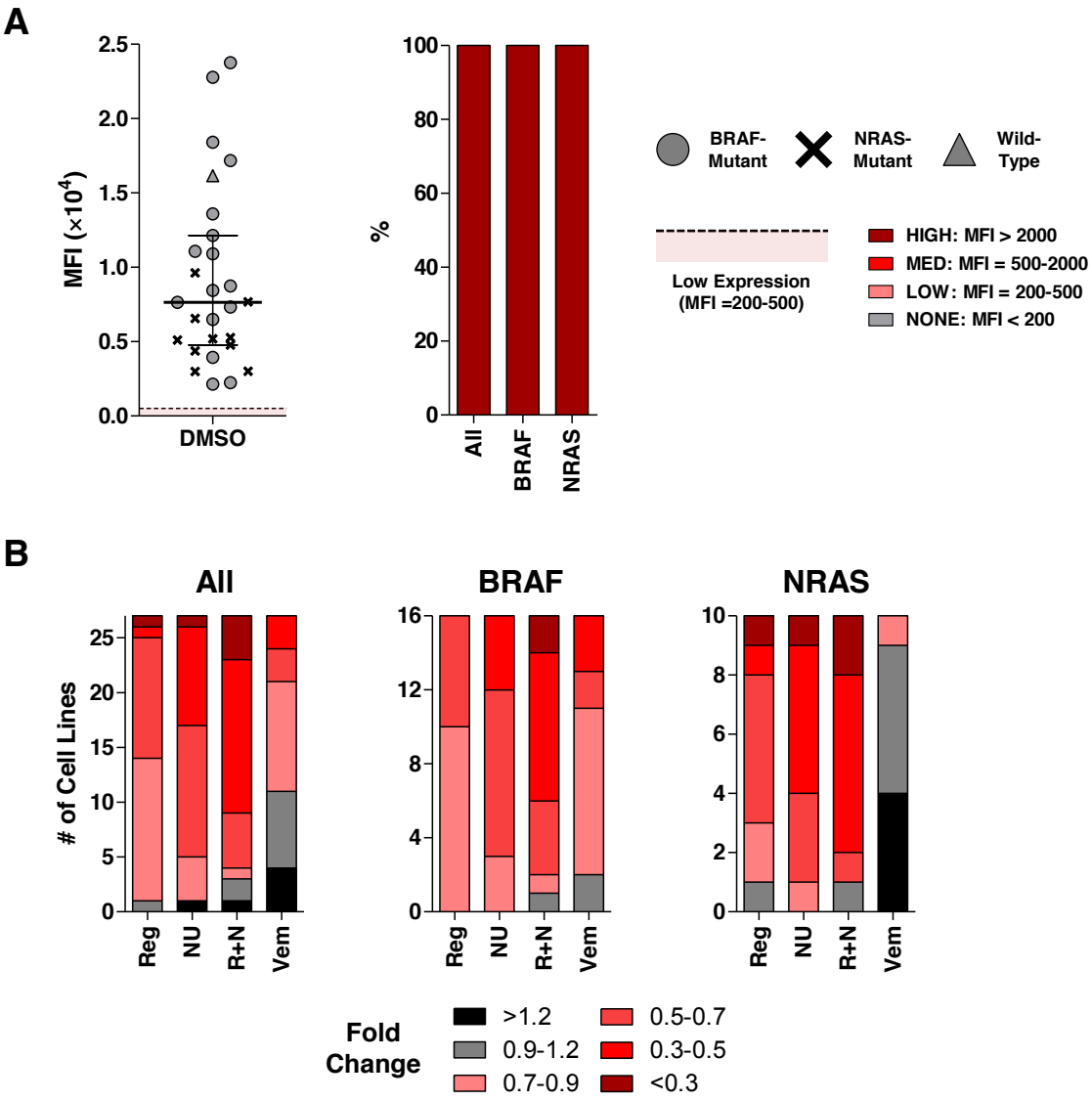
Supplementary Fig. S4: Immunomodulatory molecules are up- and down-regulated in melanoma by combination Reg plus NU treatment. (**A**) Four melanoma cell lines were treated with combination Reg (2 μM) and NU (1 μM) for 48 hours and expression of 332 cell surface markers was assessed by flow cytometry compared to vehicle-treated cells. Only molecules expressed (as defined by MFI > 200) by at least three of the four cell lines are shown. Mean ± SEM are shown. Complement regulators and immune-related molecules that were further examined are indicated by black and red boxes, respectively. (**B**) MFIs of complement regulators CD46, CD55 and CD97 in cells treated with vehicle or Reg (2 μM) plus NU (1 μM). (**C**) MFIs of immunomodulatory proteins CD73, CD155 and CD271 in cells treated with vehicle or Reg (2 μM) plus NU (1 μM). Expression array was performed once and changes in selected molecules were extensively confirmed in subsequent experiments.



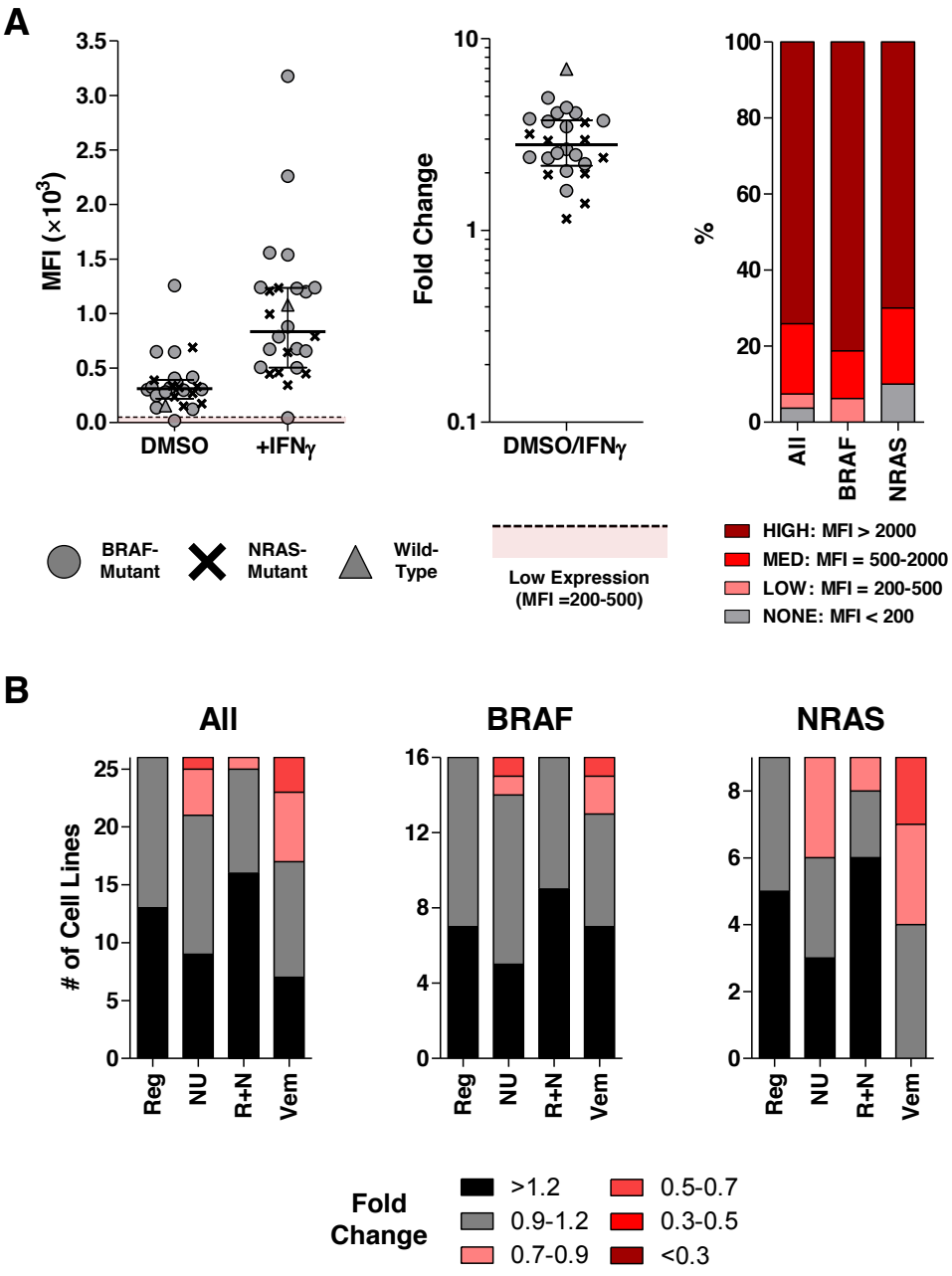
Supplementary Fig. S5: Expression, representative examples of drug-induced changes, and stratification of magnitudes of change in PD-L1. (**A**) MFIs of PD-L1 from vehicle-treated melanoma cell lines (left), fold induction of PD-L1 expression assessed by comparing vehicle-treated cells with cells stimulated with 20 U/ml of IFNγ (middle), and stratified PD-L1 expression levels (right). For expression, cells were stratified into groups designed none, low, medium, and high. No expression (none) was defined as an MFI below 200. Low expression was defined as MFIs between 200-500. Medium expression was defined as MFIs between 500-2000. High expression was defined as an MFI above 2000. Low expression is also indicated in the left panels in shaded areas. Results show median and interquartile range (where applicable) and are representative of at least two independent experiments. (**B**) G-361 melanoma cells were treated with 4 μM Reg (left), 2 μM NU (middle), or combination Reg (4 μM) and NU (2 μM) (right), and expression of PD-L1 was assessed by flow cytometry. Histograms from technical triplicates are shown and are representative of two independent measurements. (**C**) Magnitudes of change in PD-L1 in cells treated with Reg (4 μM), NU (2 μM), Vem (1 μM) or a combination of Reg and NU for 48 hours. All cell lines (left) were stratified by fold change into six groups as indicated by the legend or separated into lines harboring BRAF mutations (middle) or NRAS mutations (right). Results are representative of at least two independent measurements.



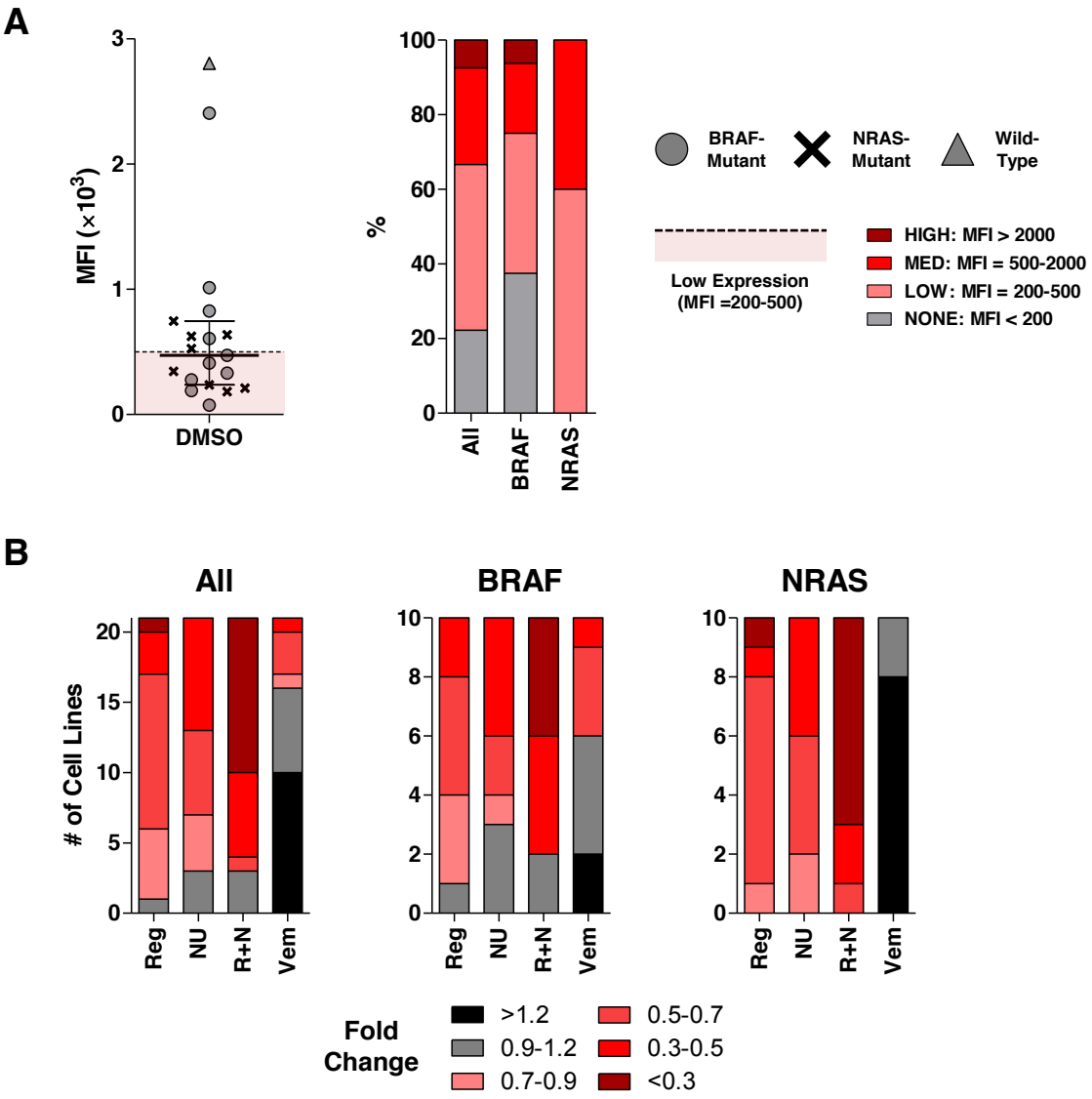
Supplementary Fig. S6: Representative examples of synergistic, additive, and antagonistic effects. Examples of synergistic (**A-B**), additive (**C-D**), and antagonistic (**E-F**) effects are shown. **A**, **C**, and **E** depict changes in CD155 in various melanoma cell lines treated with Reg and/or NU. Corresponding combination index (CI) values from Reg plus NU combination treatment are shown in **B**, **D**, and **F**. CI values were calculated using the Chou-Talalay method. CI < 1 indicates synergy, CI = 1 indicates additive effects, and CI > 1 indicates antagonism. X-axes represent concentrations of Reg. NU concentrations are half of those shown. Results are representative of at least two independent measurements.



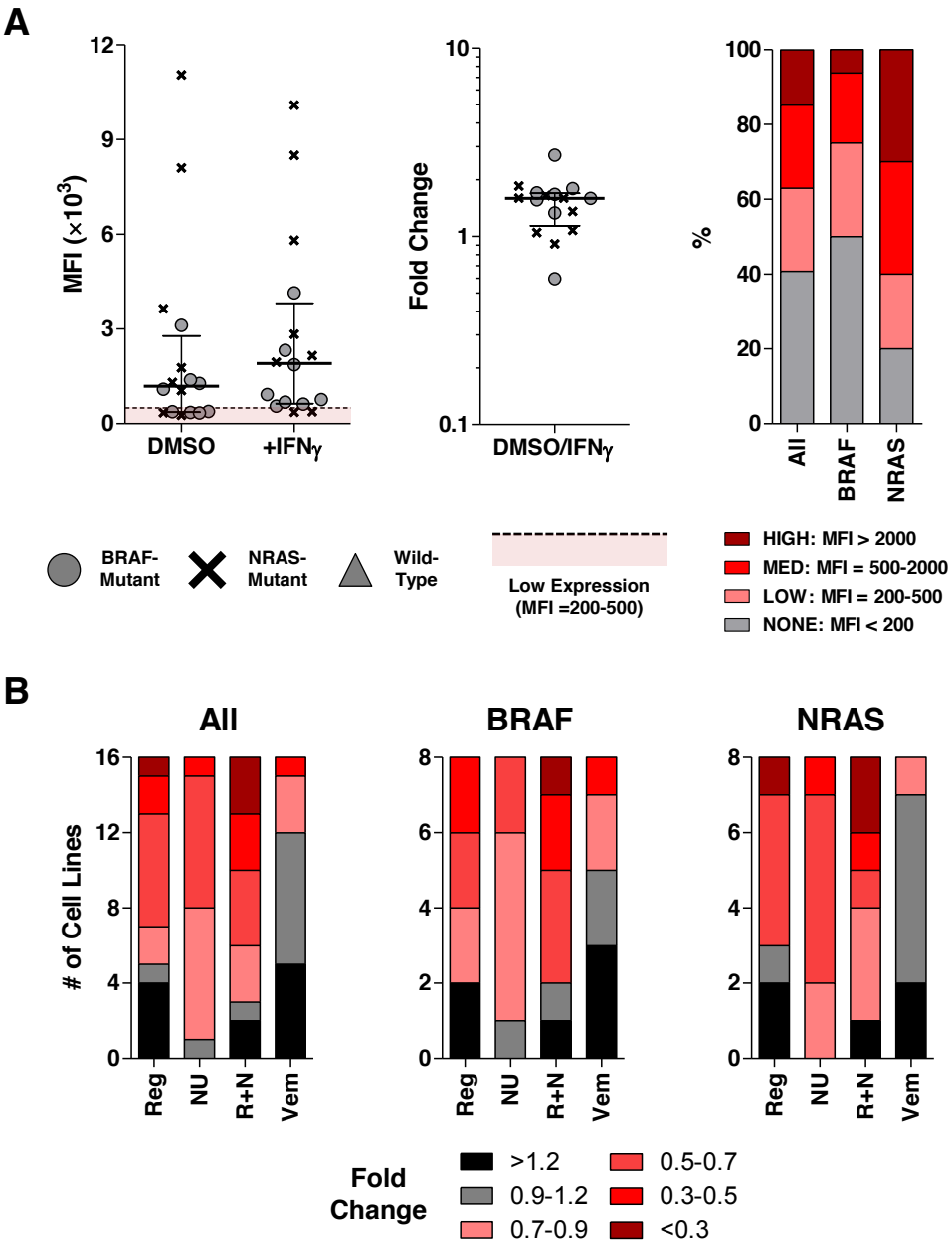
Supplementary Fig. S7: Expression and stratification of magnitudes of change of CD155. (**A**) MFIs of CD155 from vehicle-treated melanoma cell lines (left) and stratified PD-L1 expression levels (right). For expression, cells were stratified into groups designed none, low, medium, and high. No expression (none) was defined as an MFI below 200. Low expression was defined as MFIs between 200-500. Medium expression was defined as MFIs between 500-2000. High expression was defined as an MFI above 2000. Low expression is also indicated in the left panels in shaded areas. Results show median and interquartile range (where applicable) and are representative of at least two independent experiments. (**B**) Magnitudes of change in CD155 in cells treated with Reg (4 μM), NU (2 μM), Vem (1 μM) or a combination of Reg and NU for 48 hours. All cell lines (left) were stratified by fold change into six groups as indicated by the legend or separated into lines harboring BRAF mutations (middle) or NRAS mutations (right). Results are representative of at least two independent measurements.



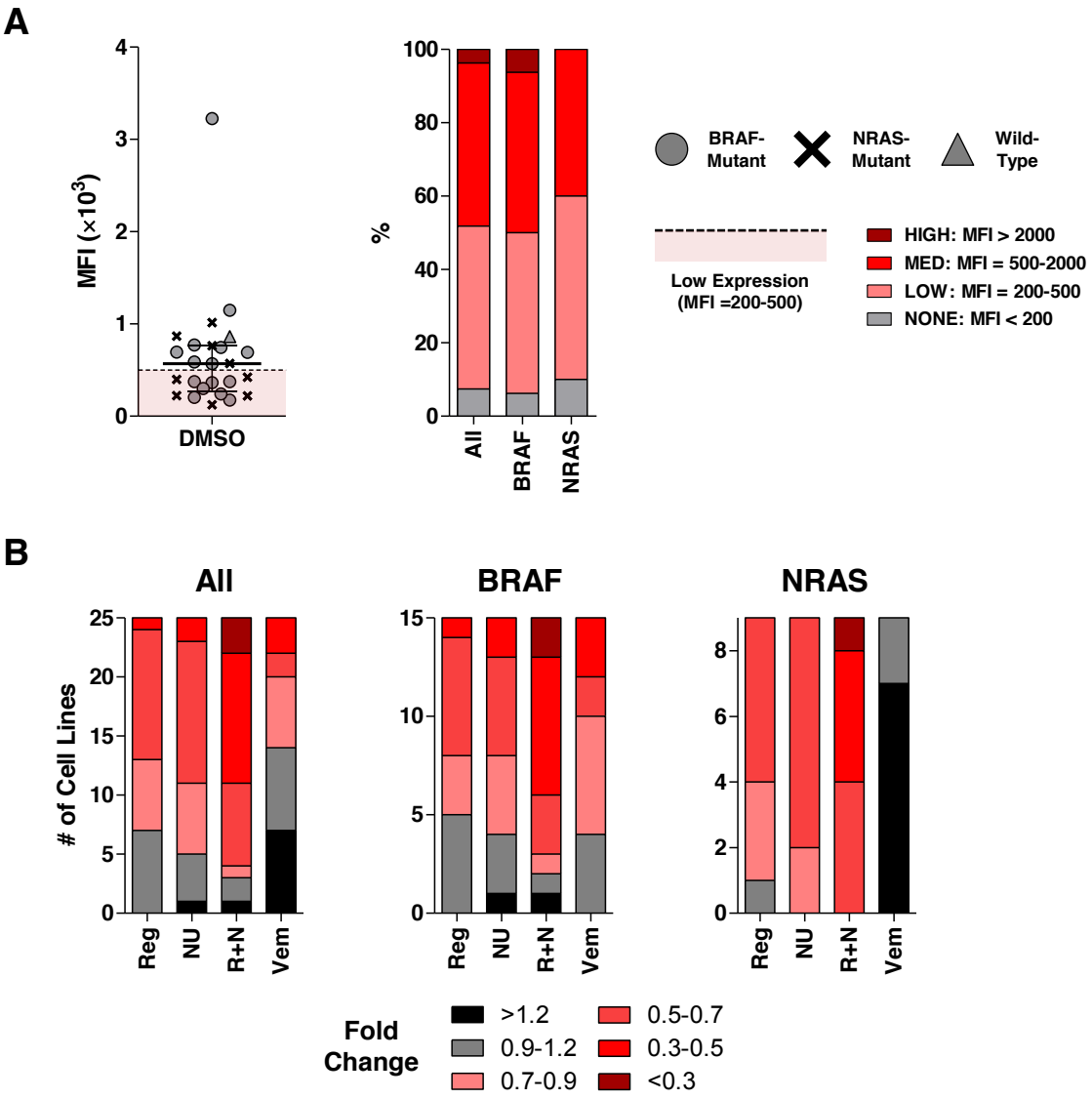
Supplementary Fig. S8: Expression and stratification of magnitudes of change of HLA-I. (**A**) MFIs of HLA-I from vehicle-treated melanoma cell lines (left), fold induction of HLA-I expression assessed by comparing vehicle-treated cells with cells stimulated with 20 U/ml of IFNγ (middle), and stratified HLA-I expression levels (right). For expression, cells were stratified into groups designed none, low, medium, and high. No expression (none) was defined as an MFI below 200. Low expression was defined as MFIs between 200-500. Medium expression was defined as MFIs between 500-2000. High expression was defined as an MFI above 2000. Low expression is also indicated in the left panels in shaded areas. Results show median and interquartile range (where applicable) and are representative of at least two independent experiments. (**B**) Magnitudes of change in HLA-I in cells treated with Reg (4 μM), NU (2 μM), Vem (1 μM) or a combination of Reg and NU for 48 hours. All cell lines (left) were stratified by fold change into six groups as indicated by the legend or separated into lines harboring BRAF mutations (middle) or NRAS mutations (right). Results are representative of at least two independent measurements.



Supplementary Fig. S9: Expression and stratification of magnitudes of change of CD73. (**A**) MFIs of CD73 from vehicle-treated melanoma cell lines (left) and stratified CD73 expression levels (right). For expression, cells were stratified into groups designed none, low, medium, and high. No expression (none) was defined as an MFI below 200. Low expression was defined as MFIs between 200-500. Medium expression was defined as MFIs between 500-2000. High expression was defined as an MFI above 2000. Low expression is also indicated in the left panels in shaded areas. Results show median and interquartile range (where applicable) and are representative of at least two independent experiments. (**B**) Magnitudes of change in CD73 in cells treated with Reg (4 μM), NU (2 μM), Vem (1 μM) or a combination of Reg and NU for 48 hours. All cell lines (left) were stratified by fold change into six groups as indicated by the legend or separated into lines harboring BRAF mutations (middle) or NRAS mutations (right). Results are representative of at least two independent measurements.



Supplementary Fig. S10: Expression and stratification of magnitudes of change of NGFR. (**A**) MFIs of NGFR from vehicle-treated melanoma cell lines (left), fold induction of NGFR expression assessed by comparing vehicle-treated cells with cells stimulated with 20 U/ml of IFNγ (middle), and stratified NGFR expression levels (right). For expression, cells were stratified into groups designed none, low, medium, and high. No expression (none) was defined as an MFI below 200. Low expression was defined as MFIs between 200-500. Medium expression was defined as MFIs between 500-2000. High expression was defined as an MFI above 2000. Low expression is also indicated in the left panels in shaded areas. Results show median and interquartile range (where applicable) and are representative of at least two independent experiments. (**B**) Magnitudes of change in NGFR in cells treated with Reg (4 μM), NU (2 μM), Vem (1 μM) or a combination of Reg and NU for 48 hours. All cell lines (left) were stratified by fold change into six groups as indicated by the legend or separated into lines harboring BRAF mutations (middle) or NRAS mutations (right). Results are representative of at least two independent measurements.



Supplementary Fig. S11: Expression and stratification of magnitudes of change of CD55. (**A**) MFIs of CD55 from vehicle-treated melanoma cell lines (left) and stratified CD55 expression levels (right). For expression, cells were stratified into groups designed none, low, medium, and high. No expression (none) was defined as an MFI below 200. Low expression was defined as MFIs between 200-500. Medium expression was defined as MFIs between 500-2000. High expression was defined as an MFI above 2000. Low expression is also indicated in the left panels in shaded areas. Results show median and interquartile range (where applicable) and are representative of at least two independent experiments. (**B**) Magnitudes of change in CD55 in cells treated with Reg (4 μM), NU (2 μM), Vem (1 μM) or a combination of Reg and NU for 48 hours. All cell lines (left) were stratified by fold change into six groups as indicated by the legend or separated into lines harboring BRAF mutations (middle) or NRAS mutations (right). Results are representative of at least two independent measurements.

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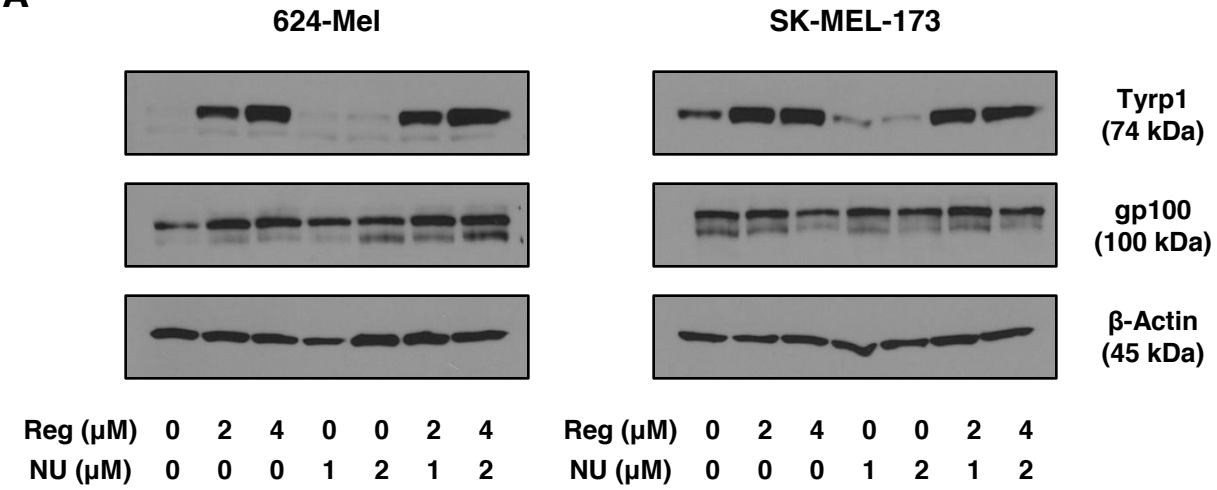
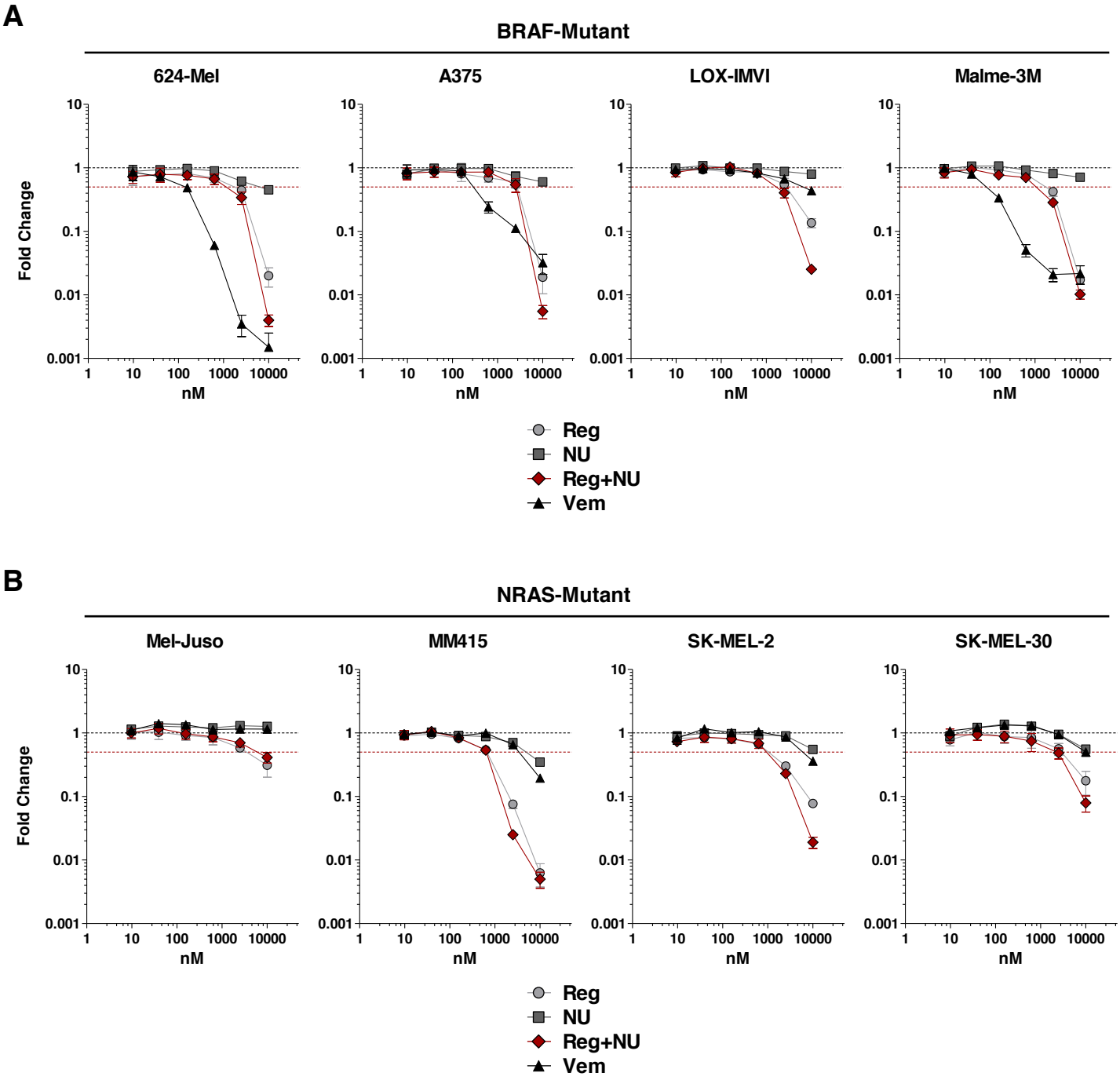
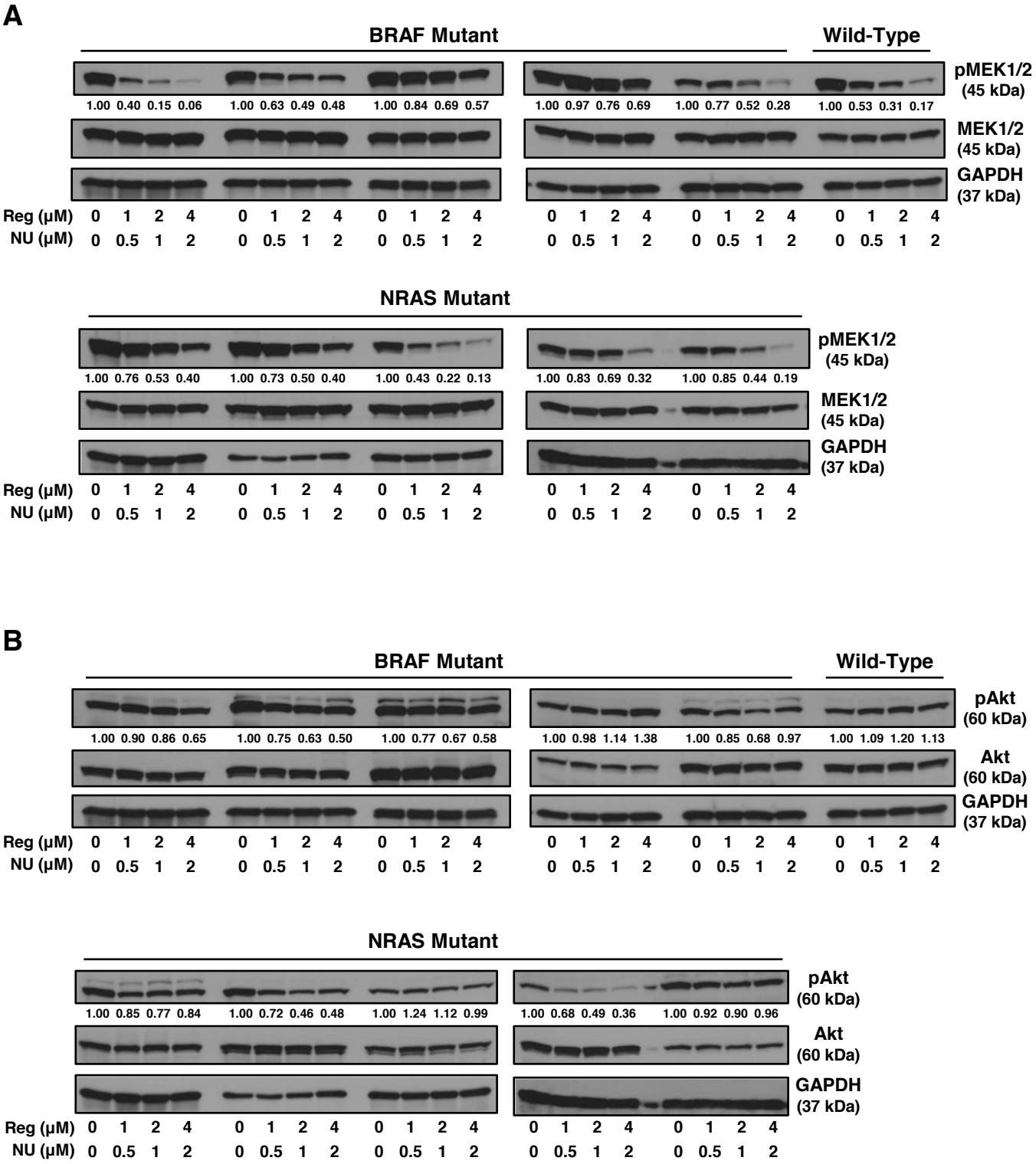


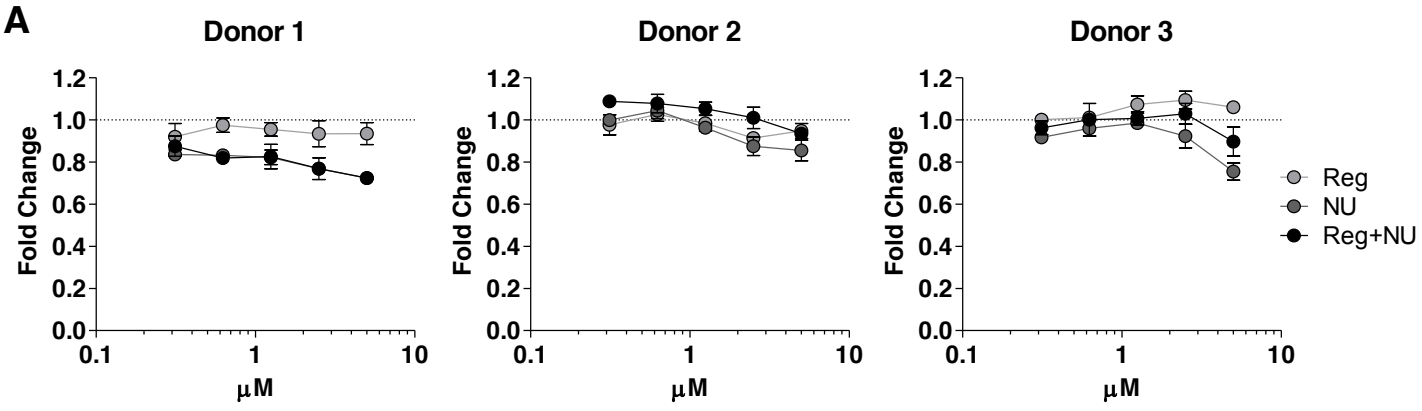
Supplementary Fig. S12: Reg increases melanoma antigen protein levels. (**A**) 624-Mel (BRAF-mutant) and SK-MEL-173 (NRAS-mutant) melanoma cell lines were treated for 48 hours with varying concentrations of Reg and/or NU. Protein levels of two melanoma antigens, gp100 and Tyrp1, were evaluated. Results are representative of two independent measurements.



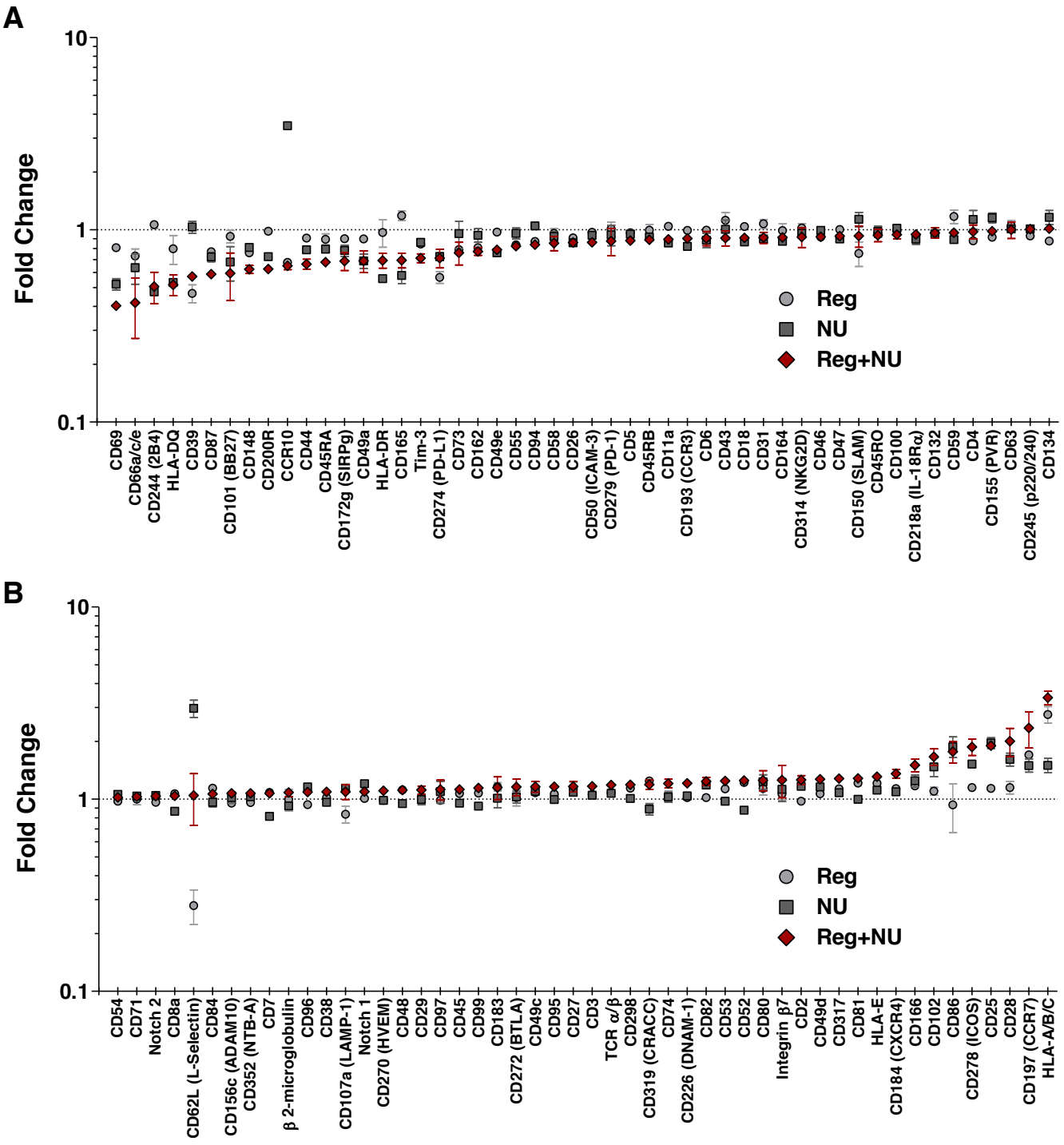
Supplementary Fig. S13: Reg and NU suppress melanoma proliferation. Melanoma cell lines were treated with varying concentrations of Reg, NU, Vem, or combination Reg plus NU for 48 hours. Proliferation was assessed by ^{3}H-thymidine incorporation and shown as fold change compared to vehicle-treated cells. Four representative BRAF-mutant cell lines (**A**) and four NRAS-mutant cell lines (**B**) are shown. Red dashed lines represent a fold change of 0.5. The concentrations for NU are half the values shown on the x-axes. All results show mean ± SD from one of two independent measurements.



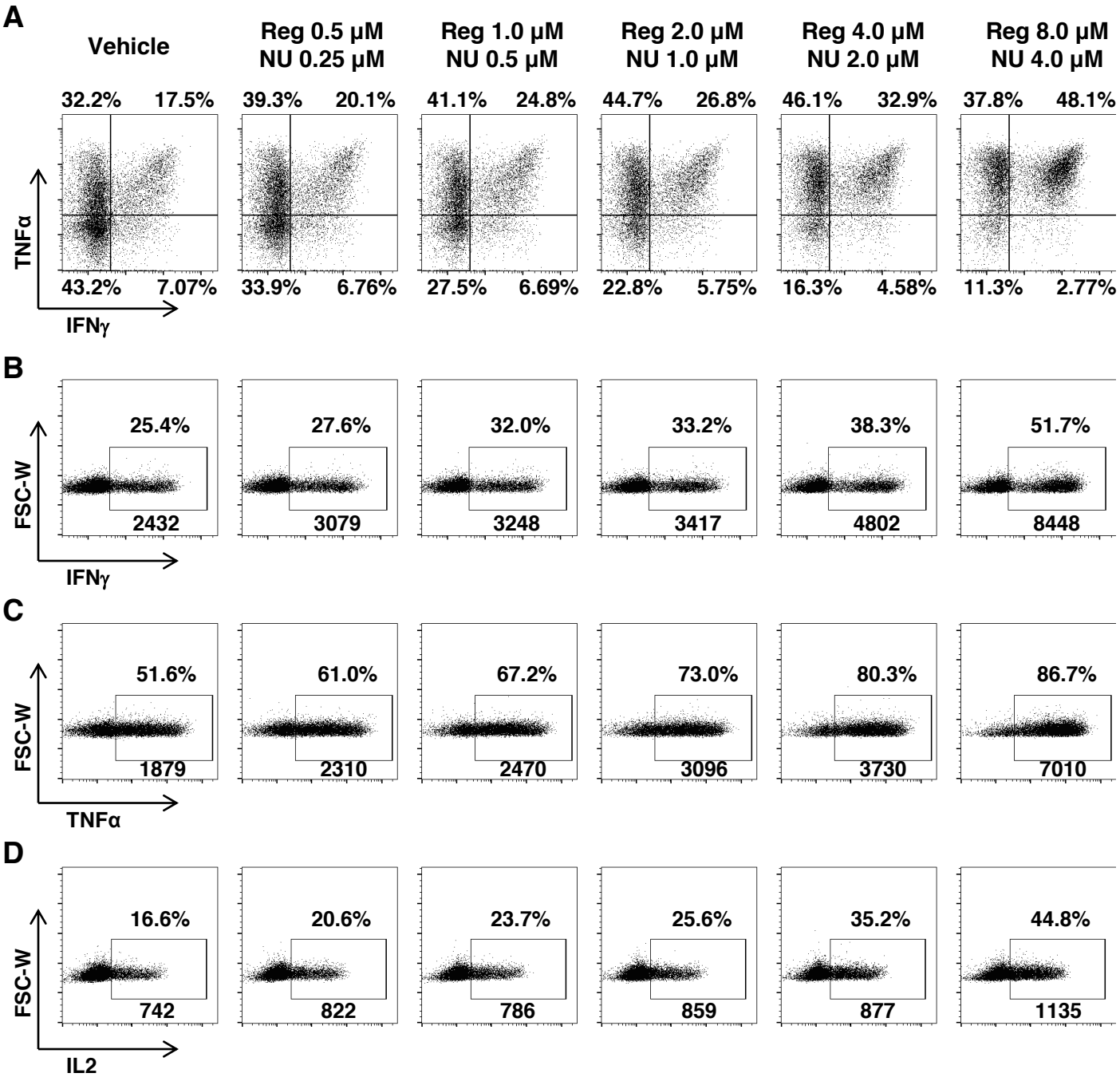
Supplemental Fig. S14: Reg and NU inhibit MEK and Akt phosphorylation. Eleven melanomas including five BRAF-mutant, five NRAS-mutant, and one wild-type cell line were treated for 24 hours with varying concentrations of combination Reg plus NU. MEK1/2 phosphorylation (**A**) and Akt phosphorylation (**B**) were evaluated in treated cells. Values represent densitometry-calculated fold change normalized to vehicle-treated cells. Results are representative of two independent measurements.



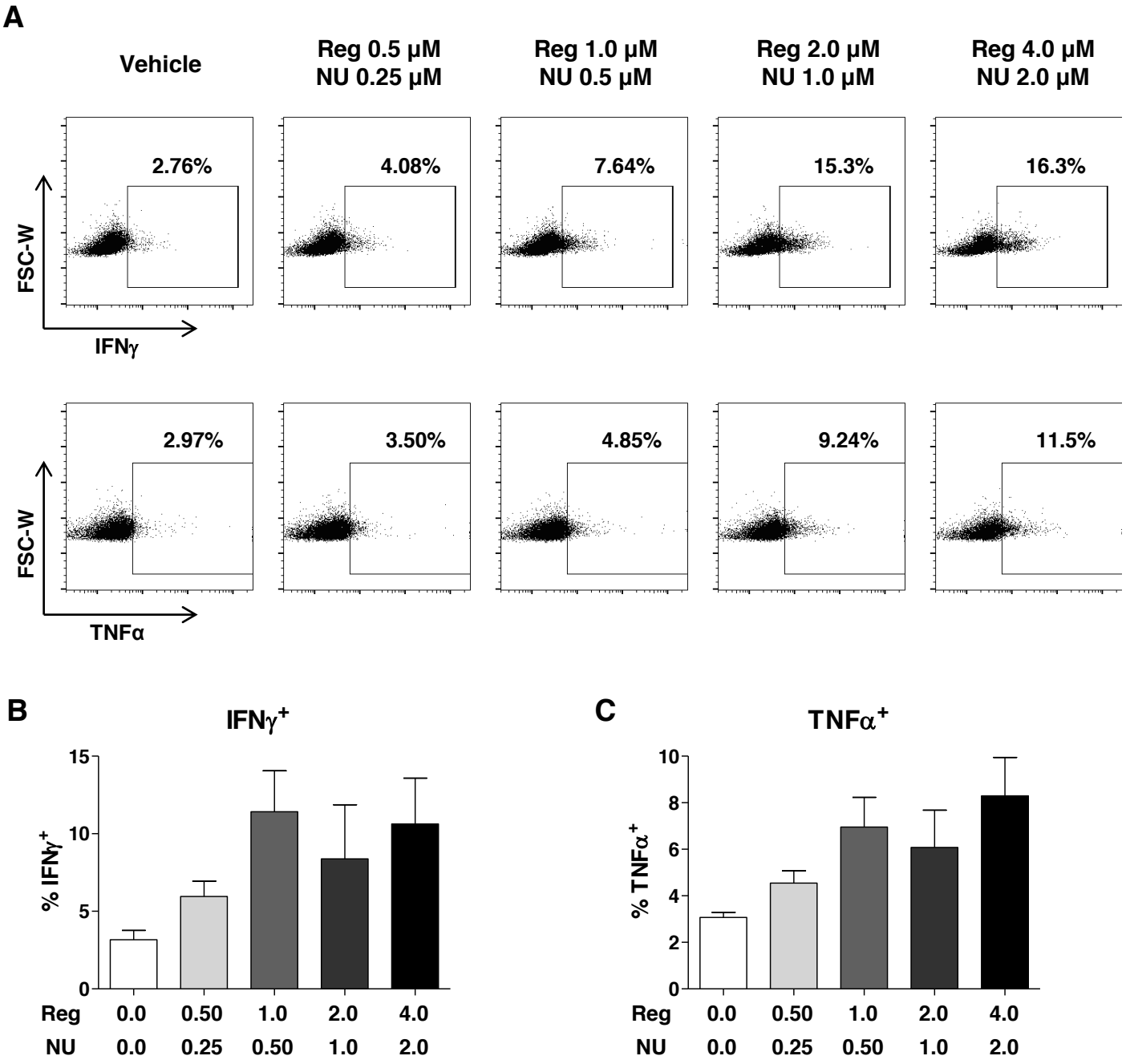
Supplementary Figure S15: T cells treated with Reg and NU proliferate normally. (**A**) PBMCs from three healthy human donors were cultured with varying concentrations of Reg and NU along with 20 ng/ml of anti-CD3 (OKT3) and 50 U/ml IL2 for five days. Proliferation was assessed by ^{3}H-thymidine incorporation and compared to vehicle-treated PBMCs. X-axes represent concentrations of Reg. NU concentrations are half of those shown. All results show mean ± SD. Results are representative of two independent experiments.



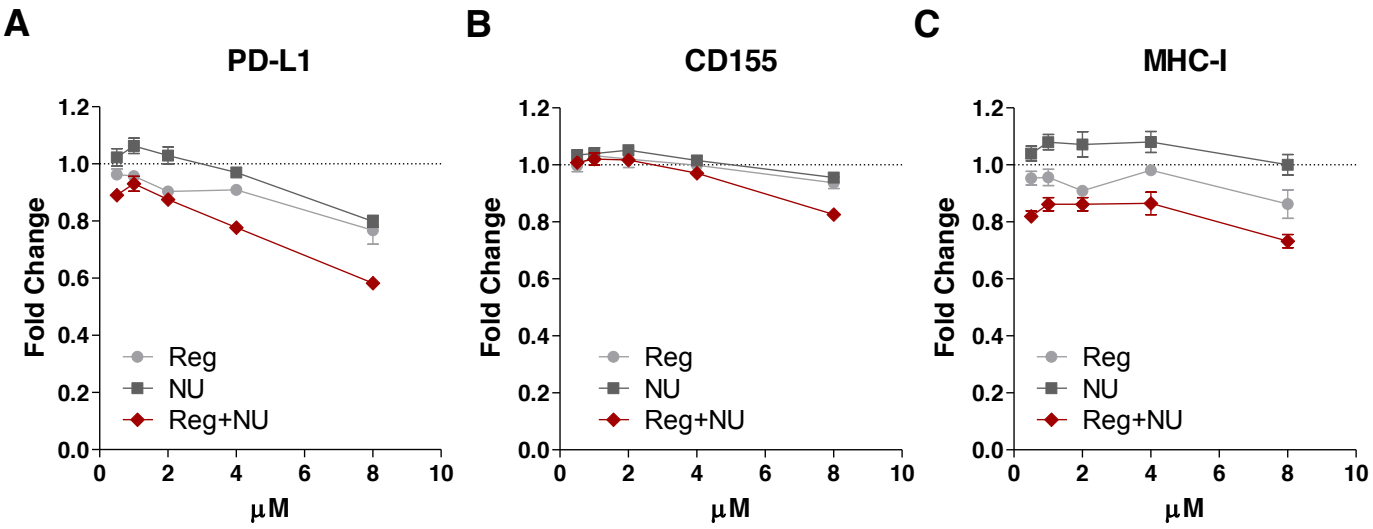
Supplementary Fig S16: Immunophenotyping of CD8⁺ T cells treated with Reg and/or NU. Human PBMCs (*n* = 3 donors) were cultured with Reg (2 μM) and/or NU (1 μM) for five days along with anti-CD3 (OKT3, 20ng/ml) and IL2 (50 U/ml). Changes in the expression of the indicated markers are shown for CD8⁺ gated populations. Molecules were sorted by fold change and separated into two groups (**A-B**). All results show mean ± SEM. Data is representative of two independent measurements.



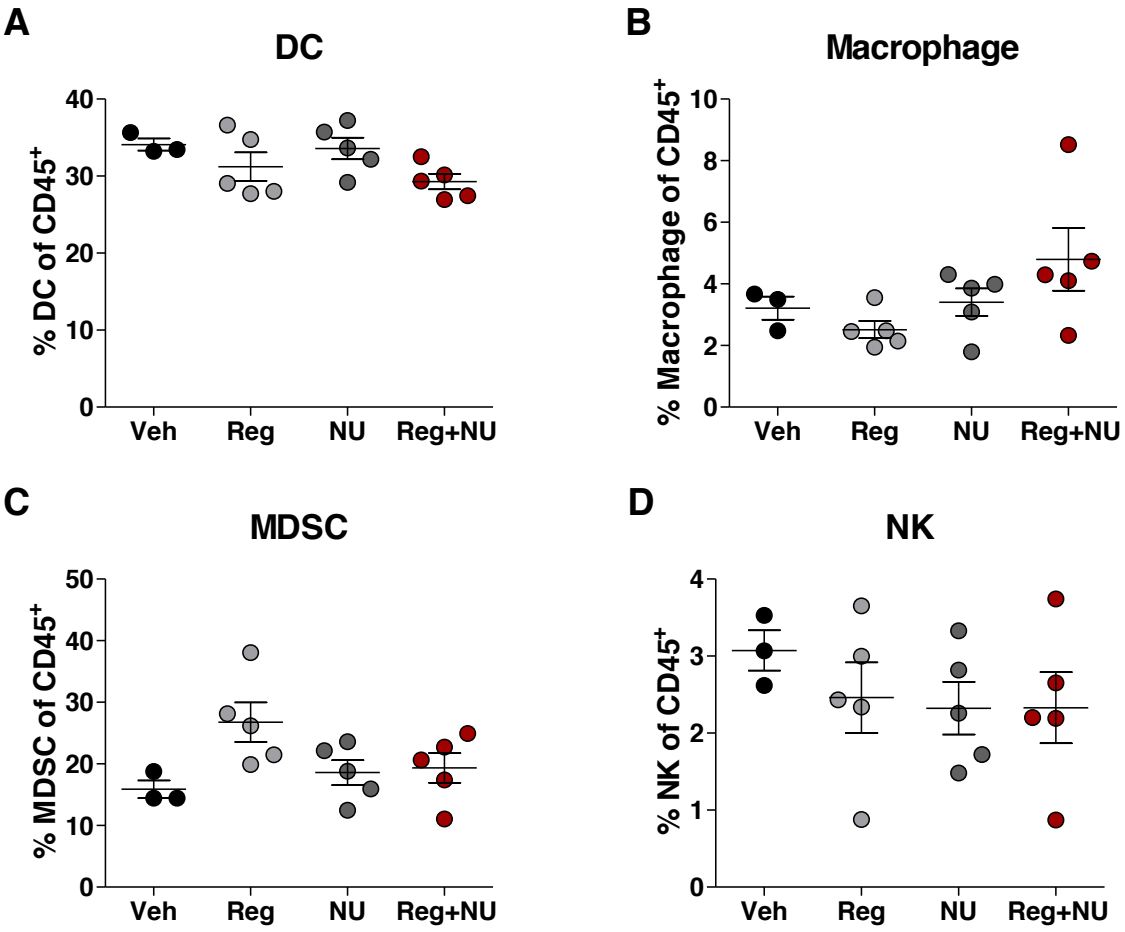
Supplementary Fig S17: Representative examples of increased cytokine production in Reg and NU combination treated CD8⁺ T cells stimulated with anti-CD3, PMA, and ionomycin. A-D) Human PBMCs were cultured with anti-CD3 (OKT3, 100 ng/ml), IL2 (100 U/ml), and varying concentrations of Reg and NU for 72 hours. Subsequently, T cells were reactivated with PMA (10 ng/ml) and ionomycin (0.5 µg/ml) for 6 hours with brefeldin A. Representative examples of IFNγ, TNFα, and IL2 positive $CD8^+$ cells from one PBMC donor are shown. In **B-D**, values below gates show MFIs for the gated population. Results are representative of independent measurements performed in three PBMC donors. Data supplements Fig. 4D.



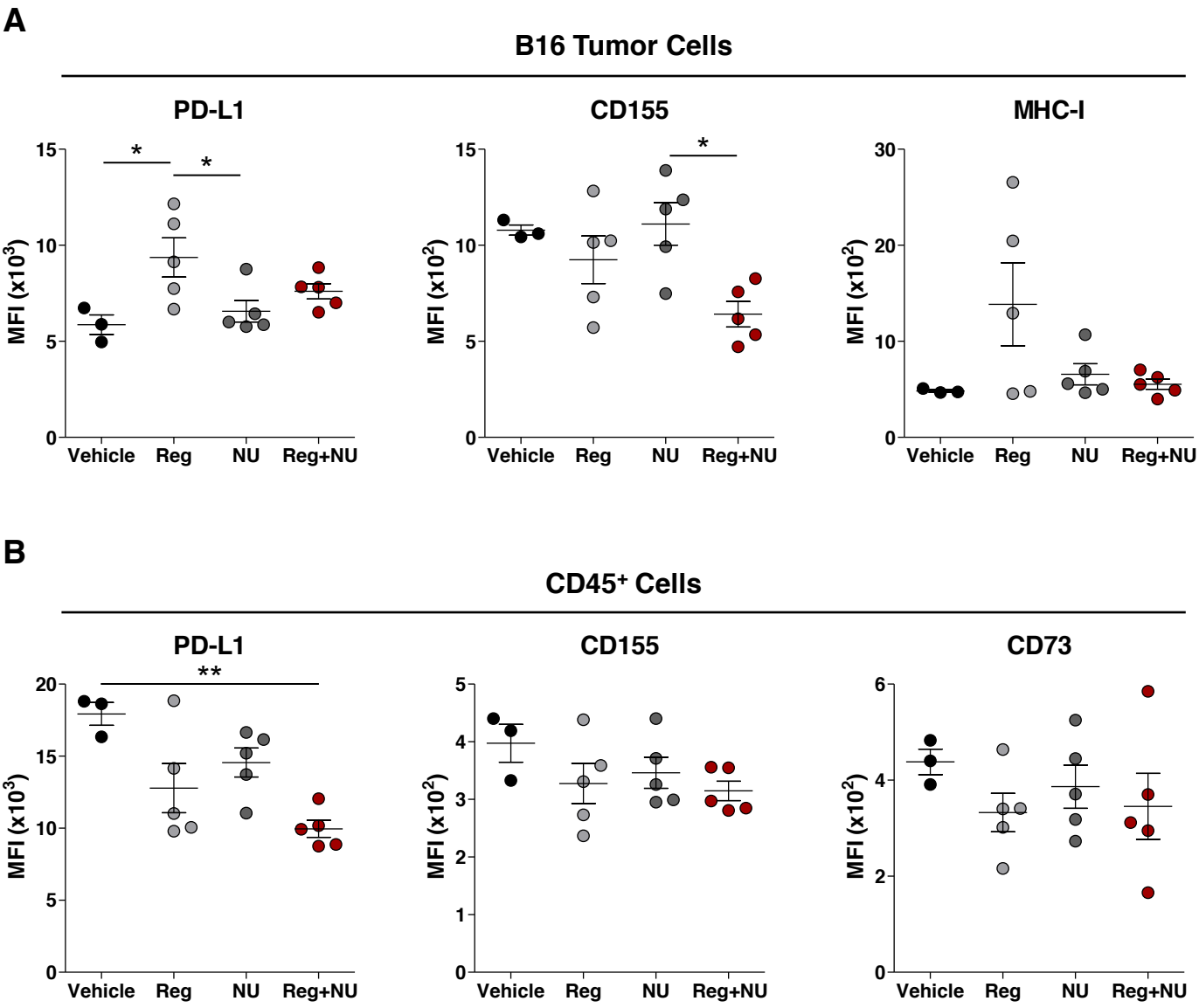
Supplementary Fig S18: Cytokine production is increased in anti-CD3-stimulated CD8⁺ T cells treated with combination Reg and NU. Human PBMCs were cultured with anti-CD3 (OKT3, 100 ng/ml), IL2 (100 U/ml), and varying concentrations of Reg and NU for 72 hours. (**A**) Representative examples of IFNγ and TNFα positive CD8⁺ cells. (**B**) Percent IFNγ positive cells. (**C**) Percent TNFα positive cells. **B-C** are gated on CD8⁺ cells and show mean ± SD. Results are from one PBMC donor and are representative of three independent measurements with different PBMC donors.



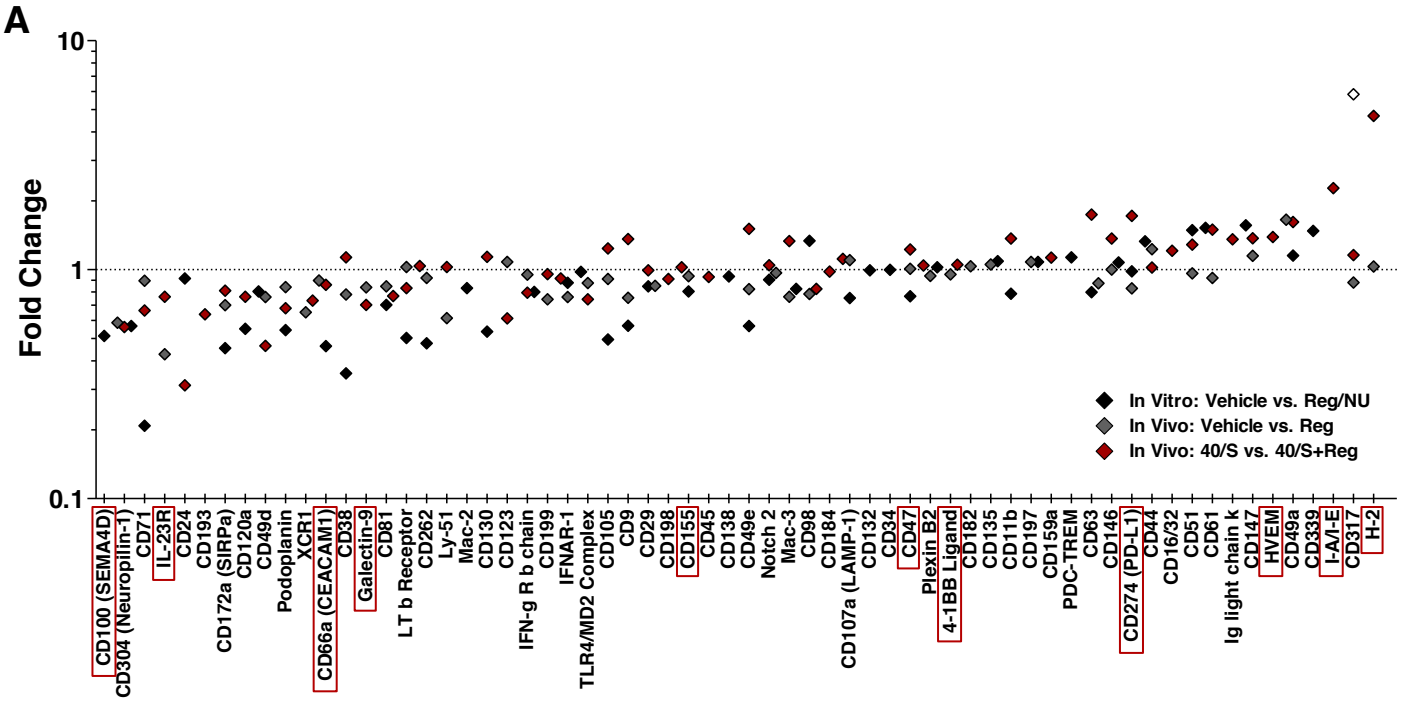
Supplementary Fig S19: Reg and NU reduce PD-L1 on B16. B16-F1 cells were pretreated with IFNγ for six hours to induce PD-L1 and MHC-I expression and subsequently treated with varying concentrations of Reg and/or NU for 24 hours. Expression of PD-L1 (**A**), CD155 (**B**), and MHC-I (**C**) were measured using flow cytometry and compared to vehicle-treated cells. The concentrations for NU are half the values shown on the x-axes. All results show mean ± SD and are representative of two independent measurements.



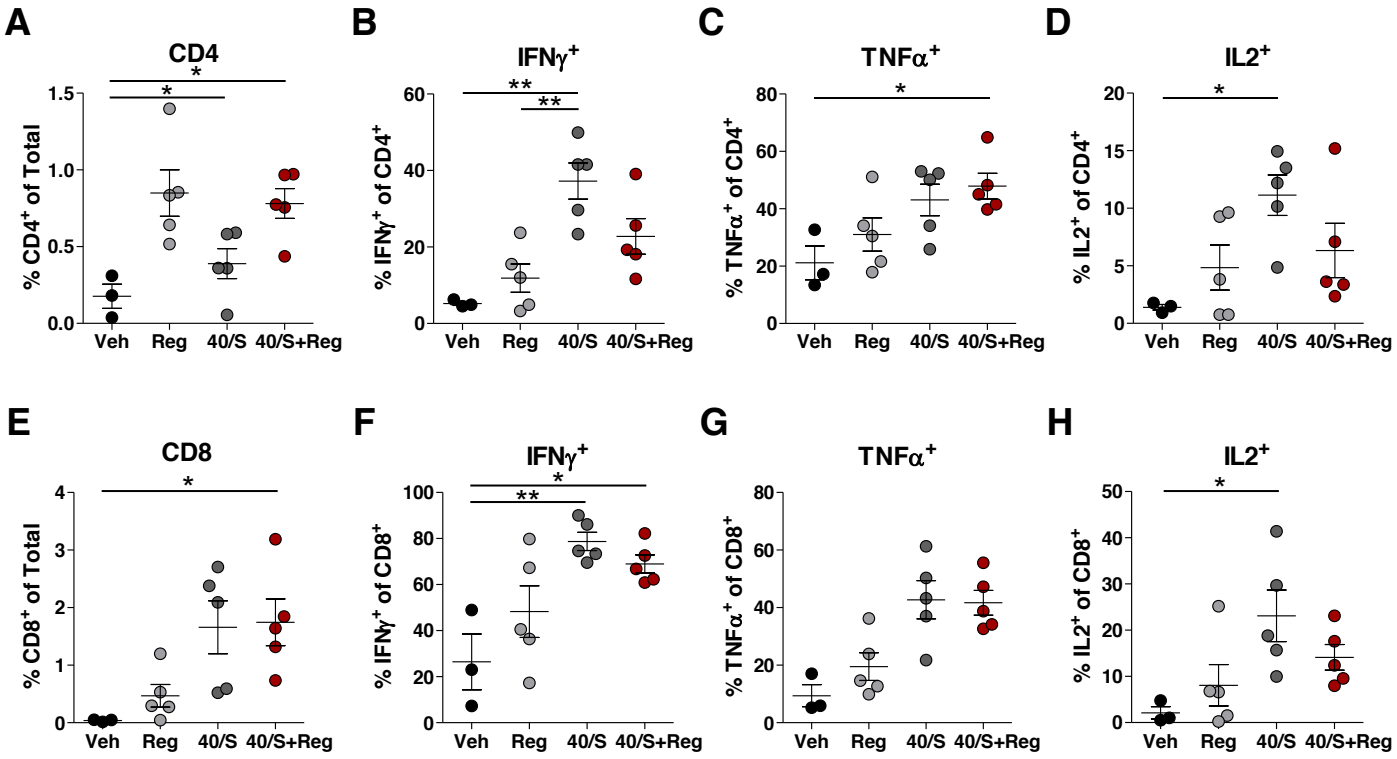
Supplementary Fig. S20: Dendritic cells, macrophages, myeloid-derived suppressor cells, and natural killer cells of the tumor microenvironment are not altered by Reg and NU. B16-F1 tumor-bearing mice were treated with Reg (5 mg/kg) and/or NU7026 (NU, 5 mg/kg) on days 8-12 post-tumor inoculation and assessed for various cell populations on day 13. Dendritic cells (DC, $CD45^+CD11c^+CD11b^-$) (**A**), macrophages ($CD45^+CD11b^+F4/80^+CD11c^-$) (**B**), myeloid-derived suppressor cells (MDSC, $CD45^+CD11b^+Gr\text{-}1^+$) (**C**), and natural killer cells (NK, $CD45^+NK1.1^+$) (**D**) were assessed. None of the groups were significantly different by one-way ANOVA with Tukey post-test. All results show mean ± SEM and are representative of two independent experiments.



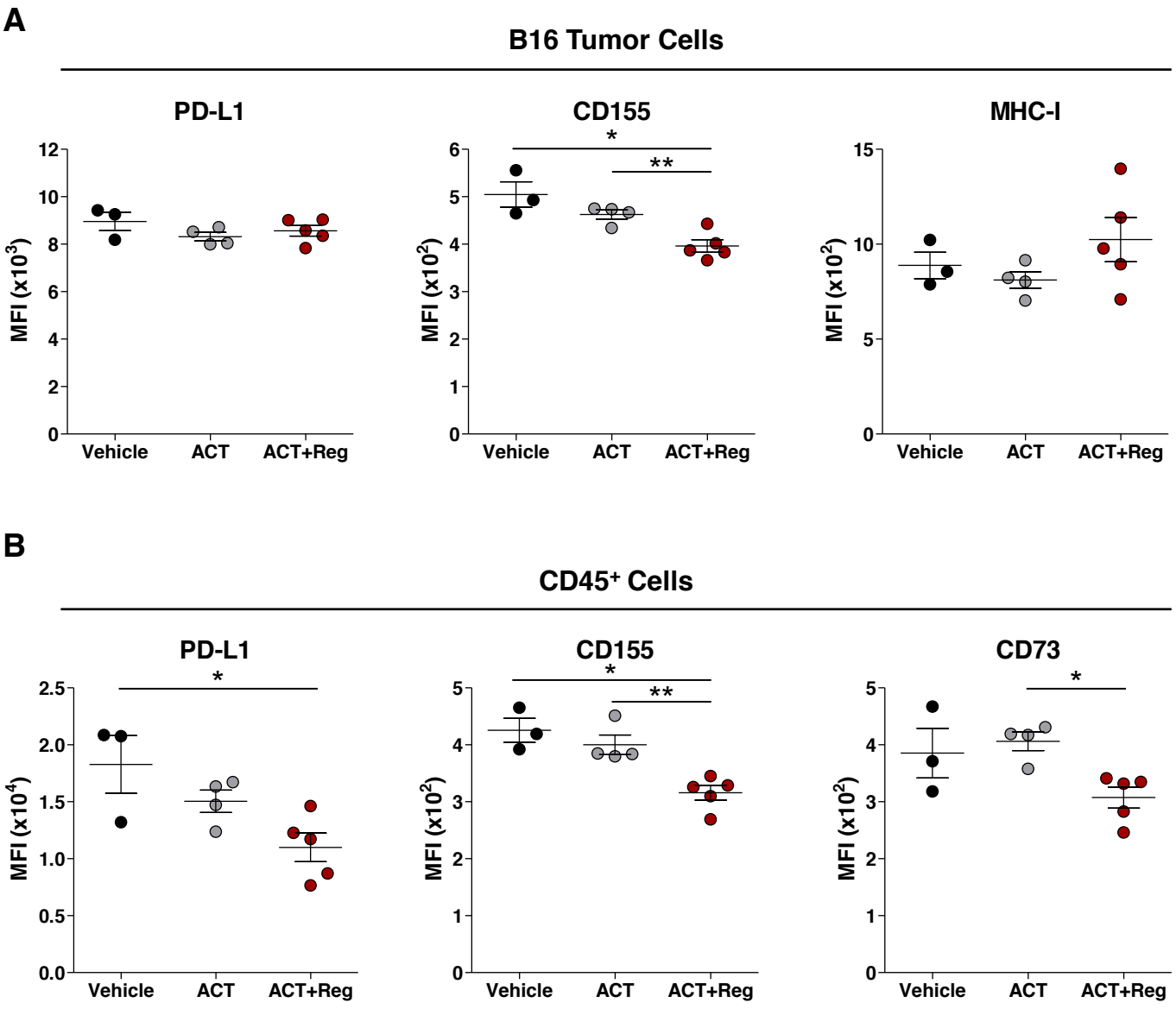
Supplementary Fig S21: PD-L1, CD155, and MHC-I are altered by Reg and NU7026 on B16 tumor cells and intratumoral leukocytes. B16-F1 tumor-bearing mice were treated with daily Reg (5 mg/kg) and/or NU7026 (NU, 5 mg/kg) on days 8-12 post-tumor inoculation and evaluated on day 13. PD-L1, CD155, and CD73 expression on $CD45^-$ B16 tumor cells (**A**) and $CD45^+$ leukocytes (**B**) were measured. Differences were assessed by one-way ANOVA with Tukey post-test. All results show mean ± SEM and are representative of two independent experiments. *, $P < 0.05$, **, $P < 0.01$.



Supplementary Fig. S22: Immunosuppressive molecules are altered *in vitro* and *in vivo* by Reg in B16. For symbols shown in black, B16-F1 cells were treated with combination Reg (4 μM) and NU (2 μM) for 48 hours and compared to vehicle-treated cells to assess fold change. For gray symbols, B16-F1 tumor-bearing mice were treated with Reg (5 mg/kg) on days 8-9, 11-14, and 16 post-tumor inoculation and compared to vehicle-treated mice on day 17 to assess fold change. Two mice per group were pooled. For red symbols, B16-F1 tumor-bearing mice were treated with combination anti-CD40 antibody (40) and c-di-GMP (STING agonist, S) on days 10 and 15 post-tumor inoculation, with or without Reg as above. Mice treated with the triple combination were compared to mice treated with anti-CD40 and c-di-GMP to assess fold change. Average expression was used from three mice per group. Results show 64 molecules that were expressed in at least one experimental setting. Missing symbols indicate that molecule expression was not detected. Potentially immunosuppressive or immunostimulatory molecules are boxed in red. Experiments were performed once.



Supplementary Fig. S23: Cytokine production in T cells from B16 tumors is increased with Reg, anti-CD40, STING agonist, and combination therapies. B16-F1 tumor-bearing mice were treated with Reg (5 mg/kg) with or without a combination of anti-CD40 antibody (40) and c-di-GMP (STING agonist, S). On day 17 post-tumor inoculation, leukocytes were isolated and stimulated with PMA and ionomycin for six hours. $CD4^+$ (**A**) and $CD8^+$ (**E**) T-cell densities were assessed and shown as a percentage of all cells. In addition, $CD4^+$ T cells producing IFNγ (**B**), TNFα (**C**), and IL2 (**D**) were measured. Identical cytokines were measured in $CD8^+$ T cells (**F-H**). Differences were assessed by one-way ANOVA with Tukey post-test. All results show mean ± SEM and experiments were performed once. *, $P < 0.05$, **, $P < 0.01$.



Supplementary Fig S24: Reg and ACT influence PD-L1, CD155, and CD73 on B16 tumor cells and intratumoral leukocytes. B16-F1 tumor-bearing mice were treated with Reg (5 mg/kg) on days 8-12 and ACT with pmel T cells on day 13 post-tumor inoculation. On day 17, PD-L1, CD155, and CD73 expression on $CD45^-$ B16 tumor cells (**A**) and $CD45^+$ leukocytes (**B**) were measured. Differences were assessed by one-way ANOVA with Tukey post-test. All results show mean ± SEM and experiments were performed once. *, $P < 0.05$, **, $P < 0.01$.