<u>SUPPLEMENTAL MATERIALS</u>
Hypoxia is a dominant remodeler of the effector T cell surface proteome relative to activation and regulatory T cell suppression
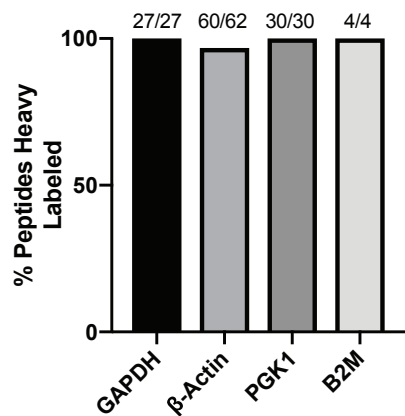James R. Byrnes, Amy M. Weeks, Eric Shifrut, Julia Carnevale, Lisa Kirkemo, Alan Ashworth, Alexander Marson, and James A. Wells

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SUPPLEMENTAL FIGURES

Figure S1. Expansion of T cells in SILAC media effectively labels proteins. Representative data from one CD8^{+} T cell labeling experiment following 2 weeks of expansion in heavy SILAC media. Cells were lysed, proteins trypsinized, peptides desalted, and analyzed with LC-MS/MS. Numbers above bars indicate the number of heavy labeled peptides identified for each protein out of the total number of peptides for that protein.

A)

B)
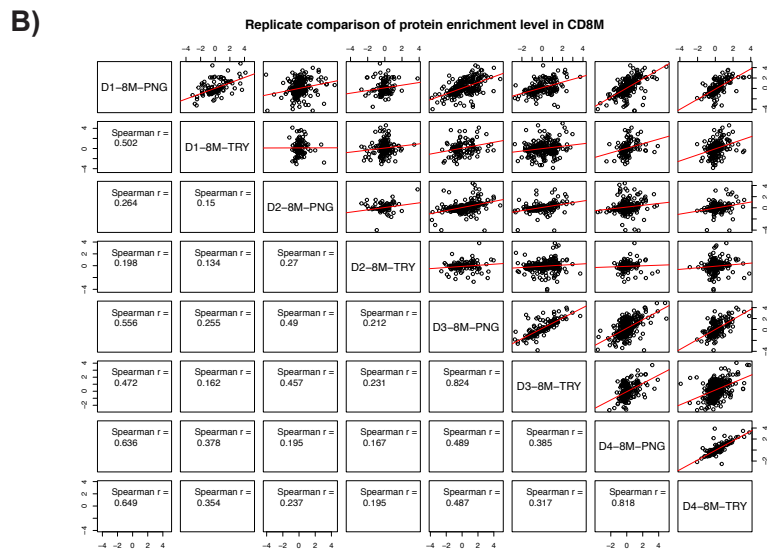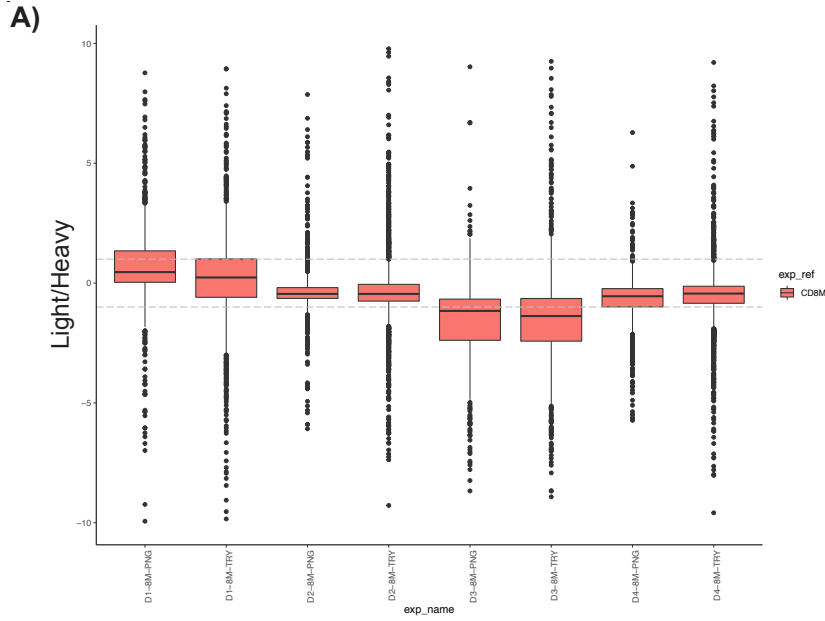

Figure S2. Replicate comparison for CD8+ activation dataset. (A) Box plots showing heavy and light distributions before normalization for both tryptic and PNGase fractions from each donor. (B) Protein-level correlations between each fraction for each donor. Spearman correlation *r* is indicated. Data were visualized using the custom R script as described in the Experimental Procedures.
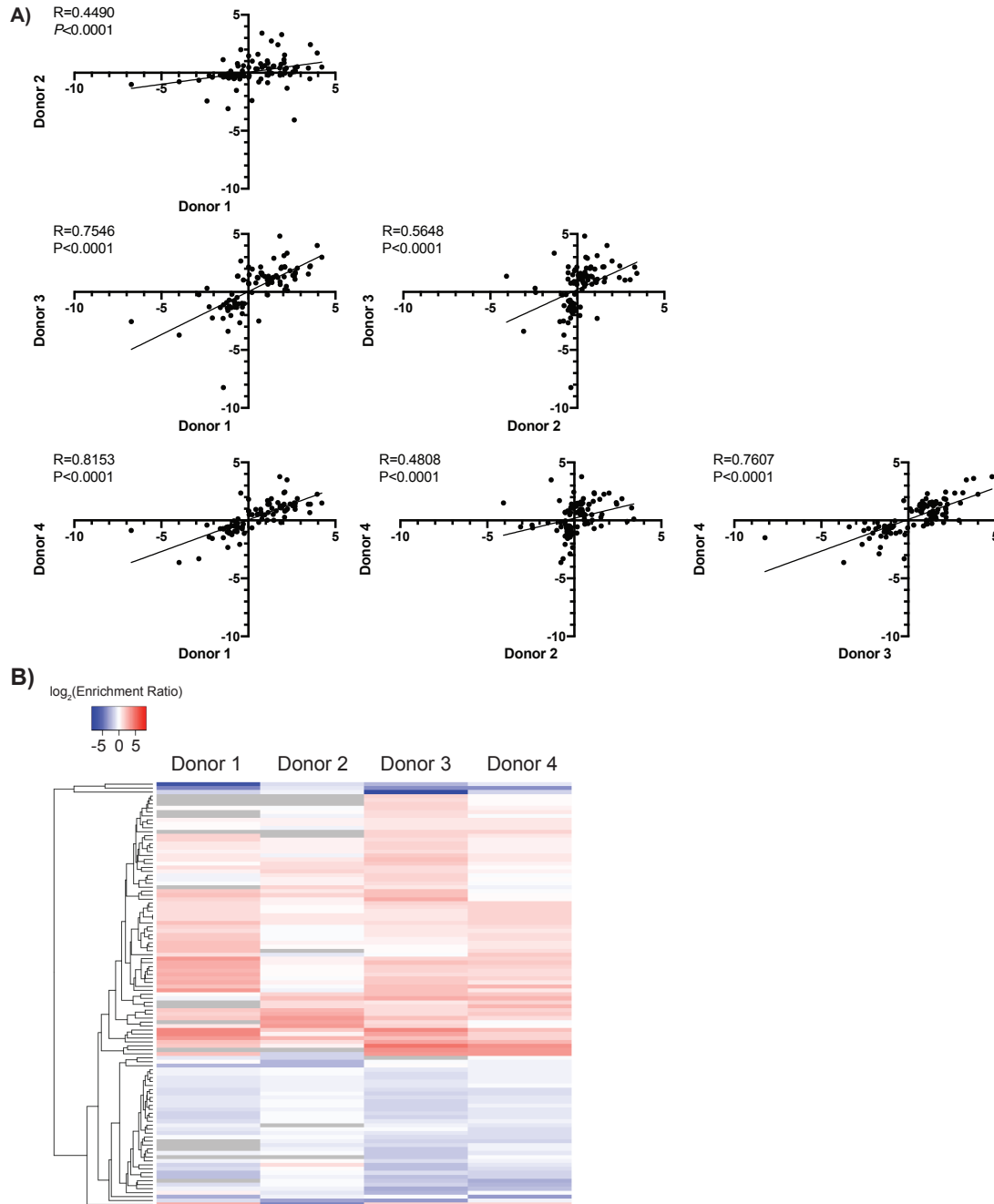
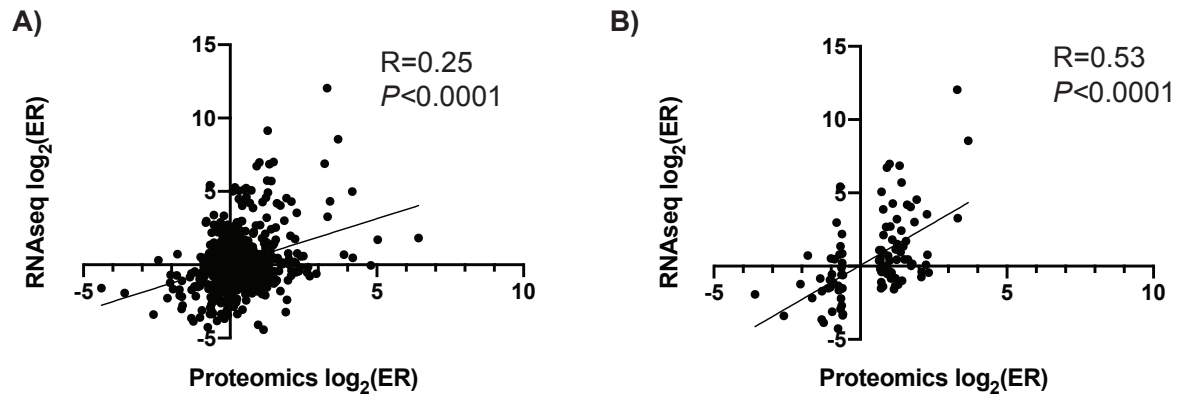**Figure S3. CD8⁺ activation in monoculture donor comparisons.** (A) Spearman correlations comparing SILAC ratios of all significantly-altered proteins identified when analyzing compiled data from N=4 donors. (B) Heatmap showing SILAC ratio for significantly-altered proteins for each donor. Grey boxes indicate the protein was not identified in cells from that donor.
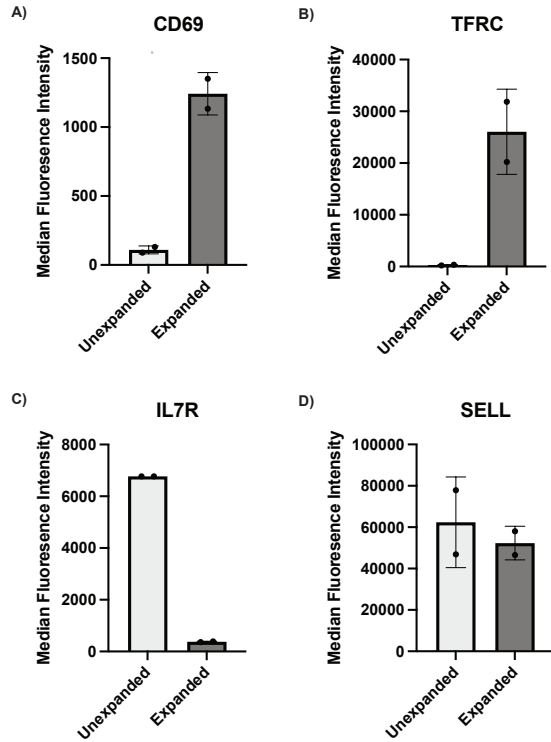
**Figure S4. Expansion of T cells *in vitro* affects basal expression of certain T cell markers.** CD8^{+} T cells from N=2 donors were isolated and expanded *in vitro* as described in the Experimental Procedures. Surface expression of the indicated markers on unexpanded and expanded cells were compared using flow cytometry. Each dot represents data from one donor. Bars indicate mean, and error bars show standard deviation.
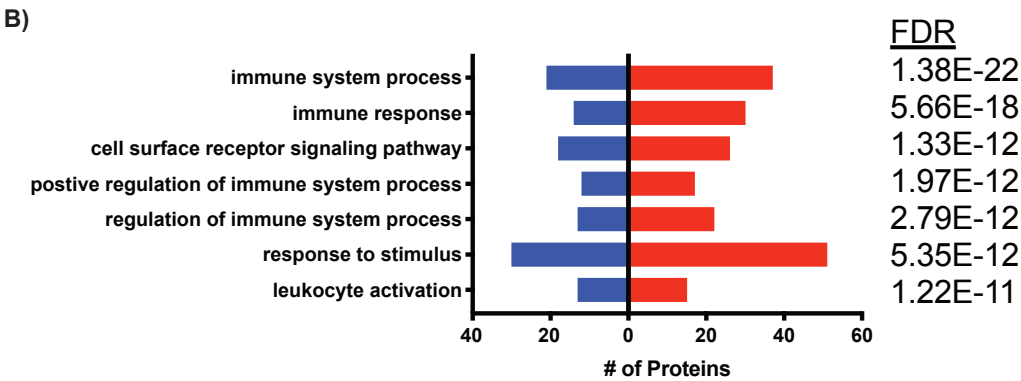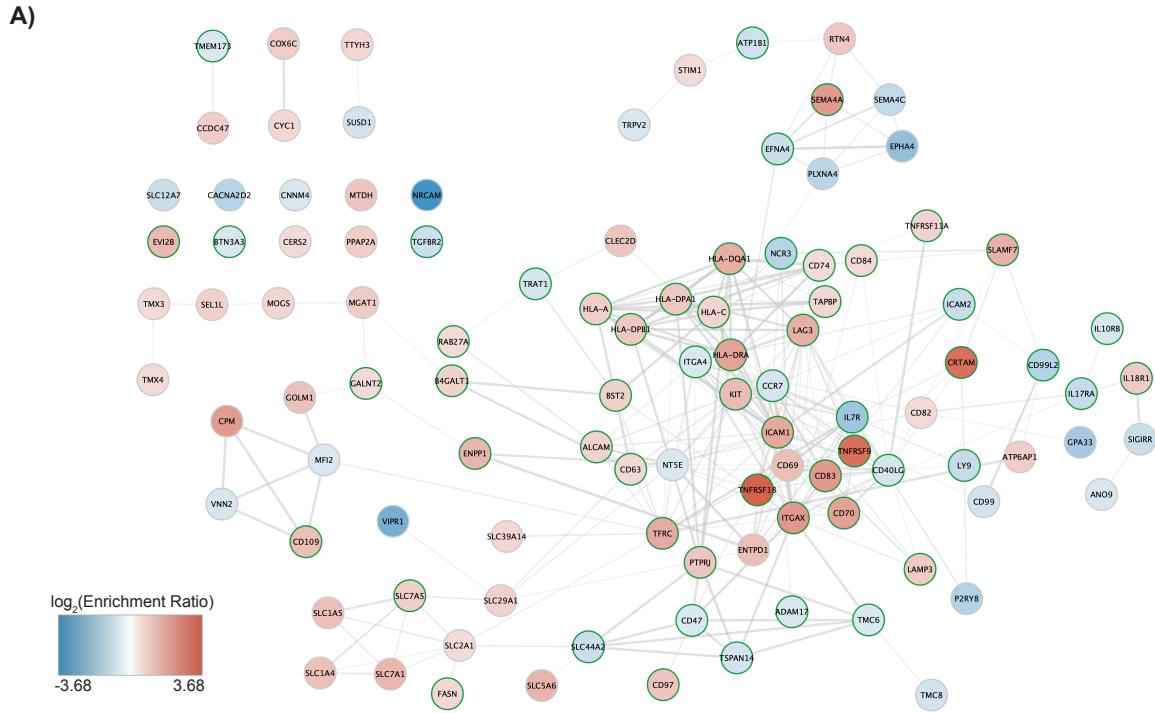
Figure S5. Network analysis of CD8⁺ activation in monoculture dataset. (A) STRING analysis of all significantly-altered proteins. Network is overlaid with a color gradient representing log₂(Enrichment Ratio) for each individual protein. Proteins with a gene ontology (GO) biological process annotation of "immune system process" are indicated with green borders. (B) Significantly-altered proteins were subjected to GO biological process pathway analysis using the STRING database. The number of proteins identified, the direction of regulation, and analysis FDR for each process are indicated.

A)

RNAseq log$_2$(ER) vs Proteomics log$_2$(ER)

R=0.25
P<0.0001

B)

RNAseq log$_2$(ER) vs Proteomics log$_2$(ER)

R=0.53
P<0.0001

Figure S6. Correlation of CD8$^+$ activation proteomics data with RNAseq data from the DICE Database. Correlations for all proteins (A) and significantly-altered proteins (B) from the activation proteomics dataset with activation data from the DICE Database. Expression data from the DICE Database was averaged for all replicates, then a log$_2$(enrichment ratio [ER]) was calculated by dividing the expression signal for activated naïve CD8$^+$ cells by the signal for resting naïve CD8$^+$ cells. Only proteins found in both datasets are shown.

A)

B)
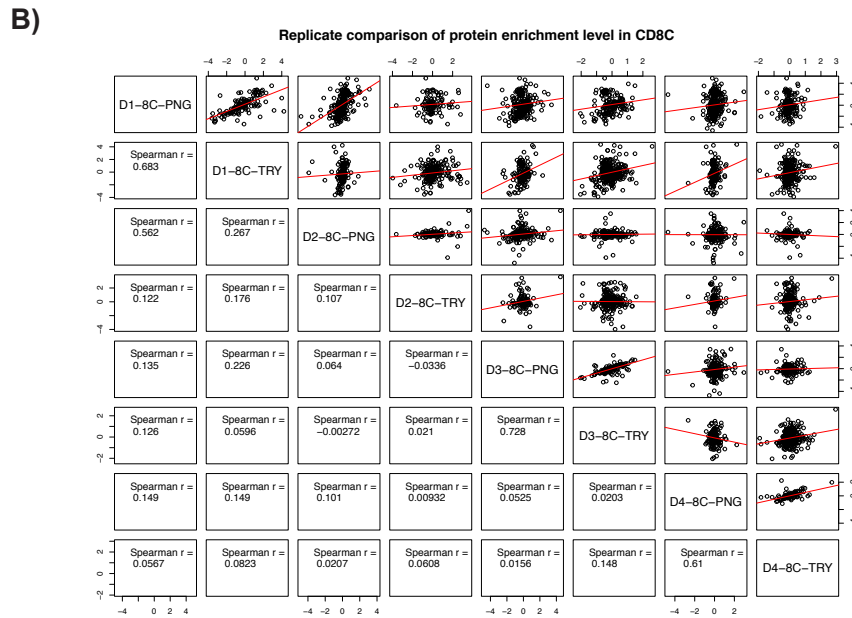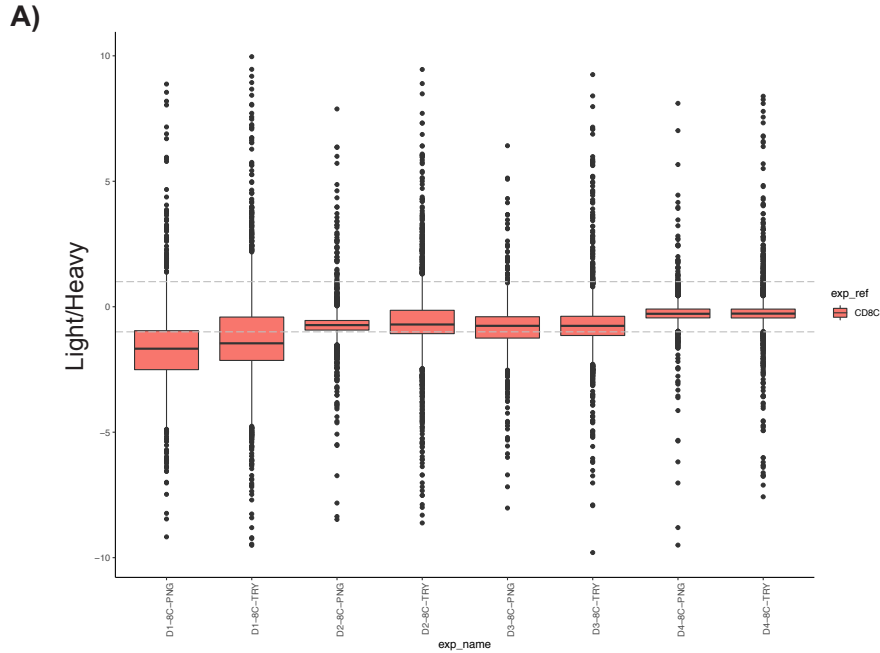
Figure S7. Replicate comparison for Treg co-culture dataset. (A) Box plots showing heavy and light distributions before normalization for both tryptic and PNGase fractions from each donor. (B) Protein-level correlations between each fraction for each donor. Spearman correlation *r* is indicated. Data were visualized using the custom R script as described in the Experimental Procedures.
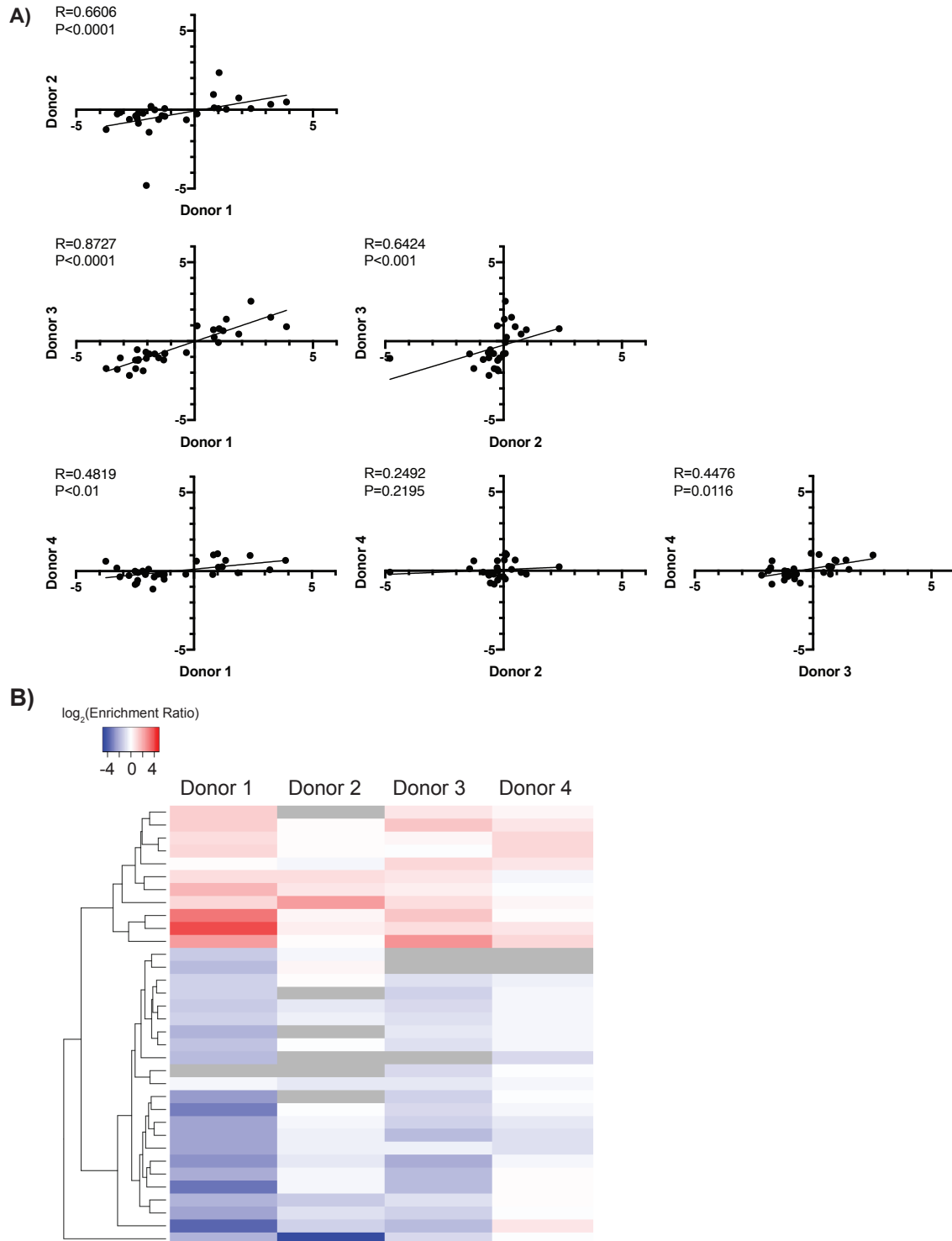
Figure S8. CD8$^+$ activation in Treg co-culture donor comparisons. (A) Spearman correlations comparing SILAC ratios of all significantly-altered proteins identified when analyzing compiled data from N=4 donors. (B) Heatmap showing SILAC ratio for significantly-altered proteins for each donor. Grey boxes indicate the protein was not identified in cells from that donor.

A)
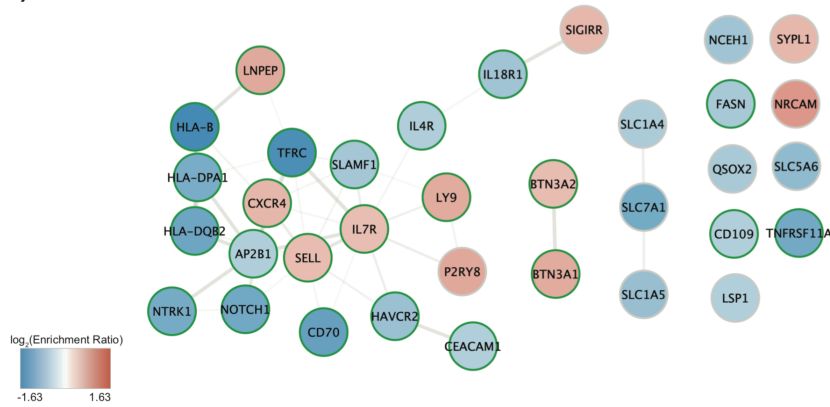
B)
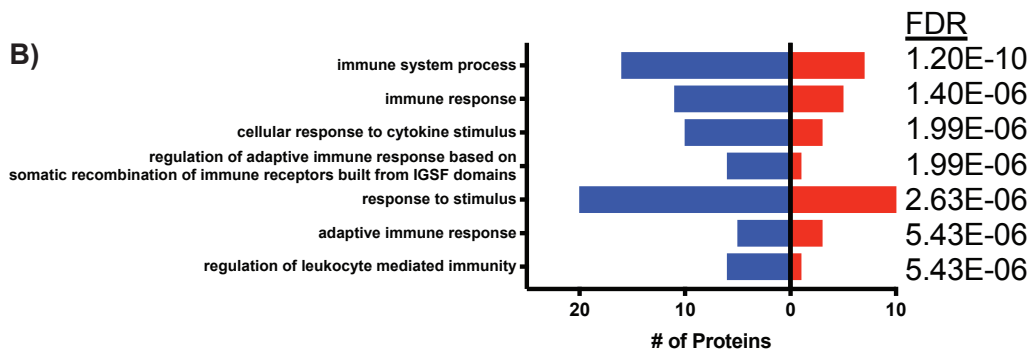

Figure S9. Network analysis of Treg co-culture dataset. (A) STRING analysis of all significantly-altered proteins. Network is overlaid with a color gradient representing log$_2$(Enrichment Ratio) for each individual protein. Proteins with a gene ontology (GO) biological process annotation of "immune system process" are indicated with green borders. (B) Significantly-altered proteins were subjected to GO biological process pathway analysis using the STRING database. The number of proteins identified, the direction of regulation, and analysis FDR for each process are indicated.

A)

Cell Count

B)
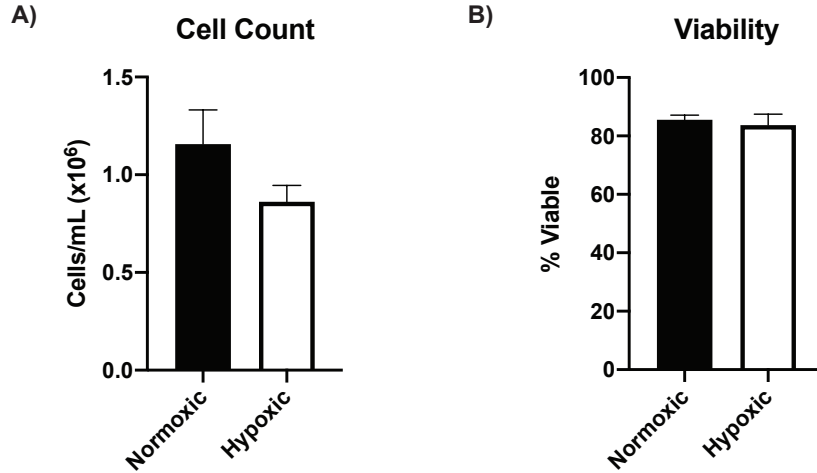
Viability

Figure S10. Effect of hypoxia on CD8+ T cell expansion and viability. Bar graphs showing cell counts (A) and viability (B) of CD8+ cultures after three days of activation in either normoxic or hypoxic conditions. Data represent mean -/+ standard error of the mean for N=3 biological replicates, each with two technical replicates.

A)

B)
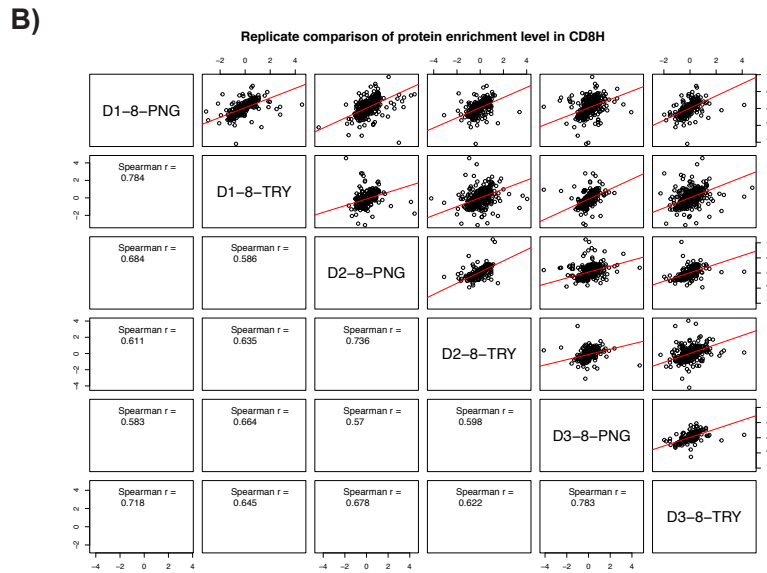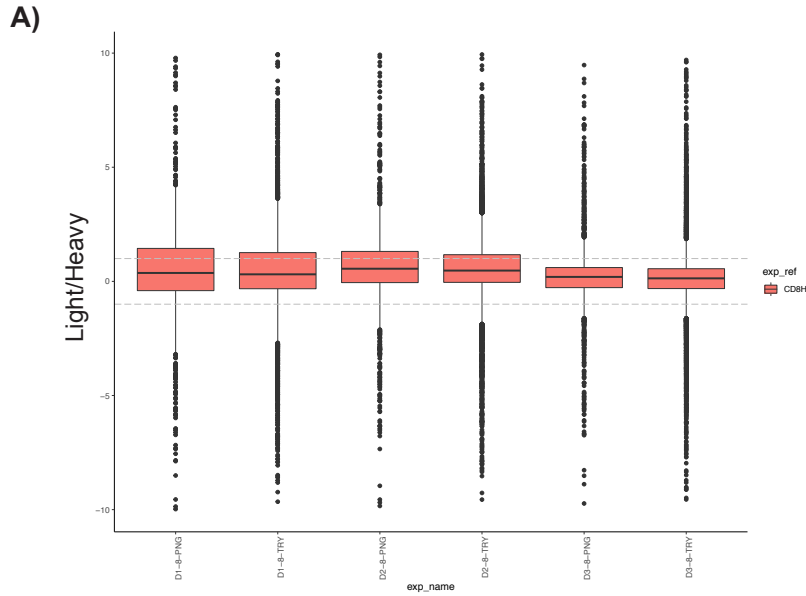

Figure S11. Replicate comparison for CD8$^+$ hypoxia dataset. (A) Box plots showing heavy and light distributions before normalization for both tryptic and PNGase fractions from each donor. (B) Protein-level correlations between each fraction for each donor. Spearman correlation *r* is indicated. Data were visualized using the custom R script as described in the Experimental Procedures.
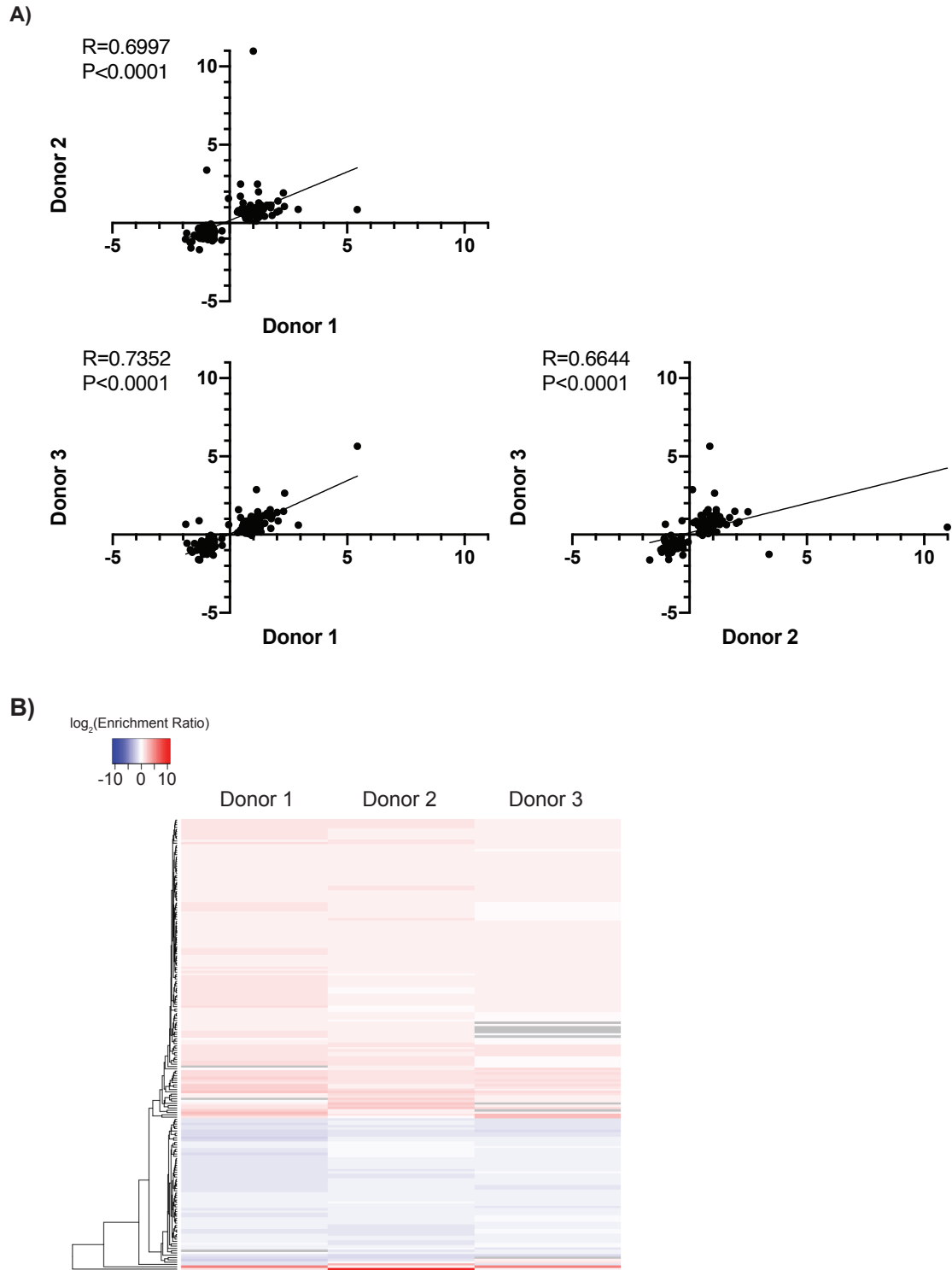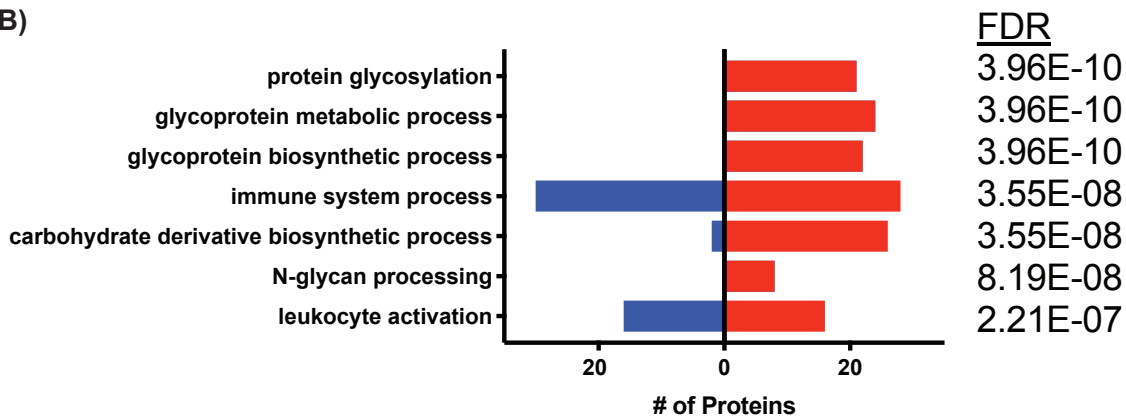
Figure S12. CD8+ activation in hypoxia donor comparisons. (A) Spearman correlations comparing SILAC ratios of all significantly-altered proteins identified when analyzing compiled data from N=3 donors. (B) Heatmap showing SILAC ratio for significantly-altered proteins for each donor. Grey boxes indicate the protein was not identified in cells from that donor.
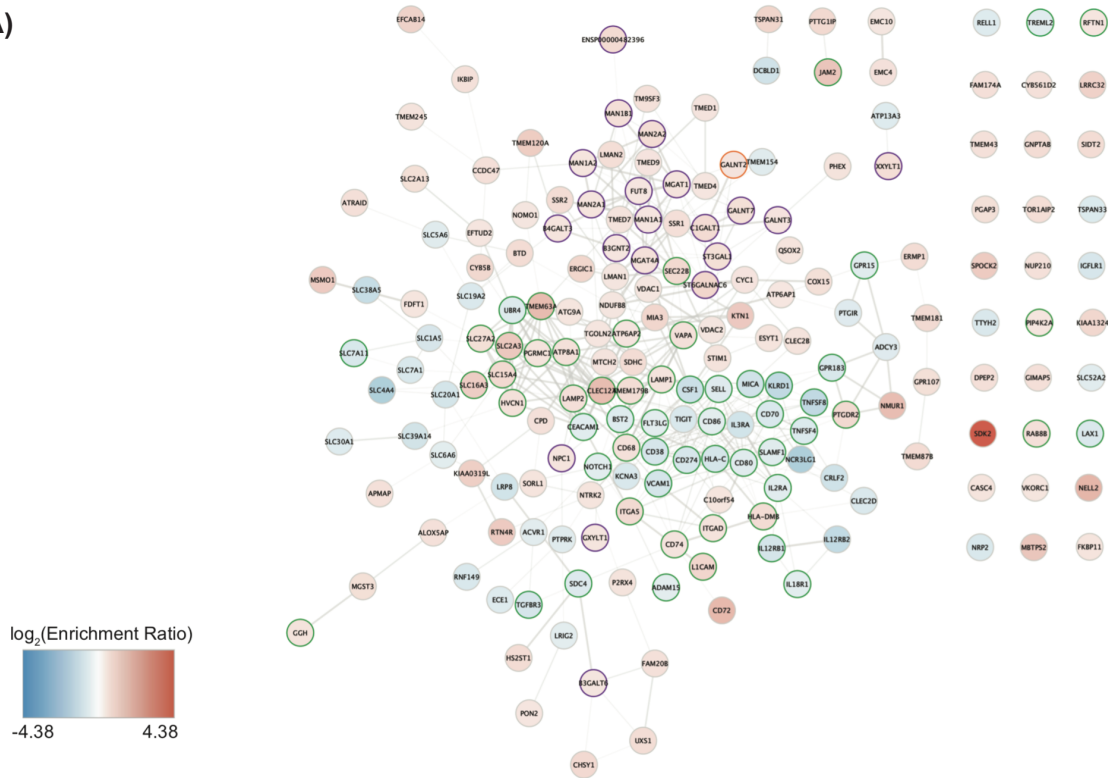
Figure S13. Network analysis of CD8⁺ hypoxia dataset. (A) STRING analysis of all significantly-altered proteins. Network is overlaid with a color gradient representing log₂(Enrichment Ratio) for each individual protein. Proteins with a gene ontology (GO) biological process annotation of "immune system process" are indicated with green borders and proteins with an annotation of "protein glycosylation" with purple borders. Proteins annotated for both processes are indicated with an orange border. (B) Significantly-altered proteins were subjected to GO biological process pathway analysis using the STRING database. The number of proteins identified, the direction of regulation, and analysis FDR for each process are indicated.

A)

B)
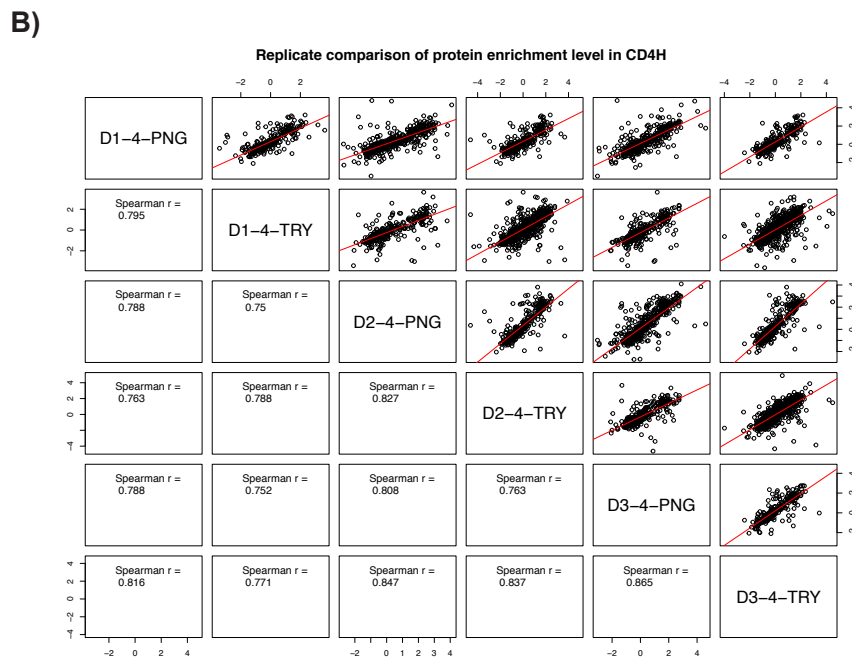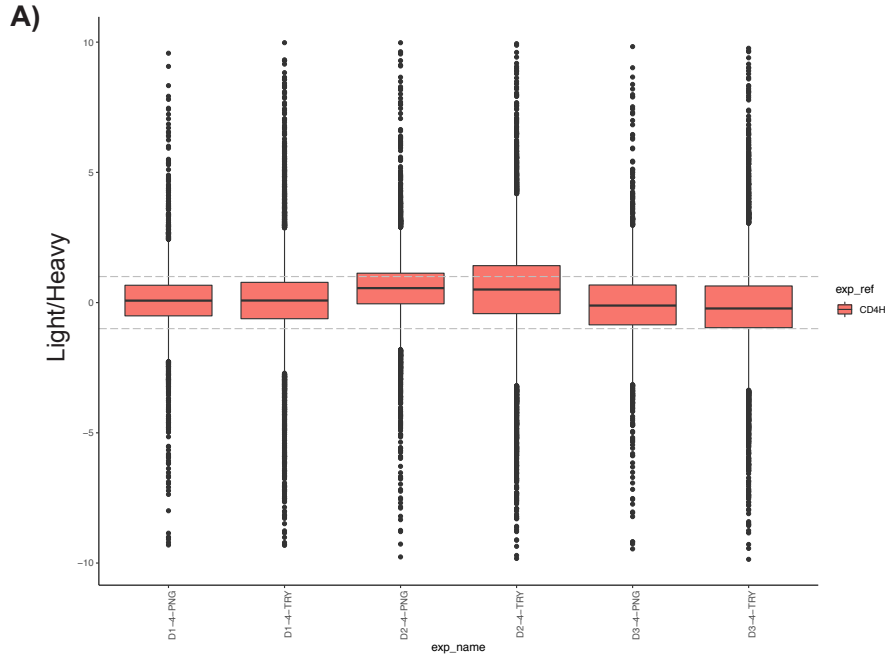
Figure S14. Replicate comparison for CD4+ hypoxia dataset. (A) Box plots showing heavy and light distributions before normalization for both tryptic and PNGase fractions from each donor. (B) Protein-level correlations between each fraction for each donor. Spearman correlation *r* is indicated. Data were produced using the custom R script as described in the Experimental Procedures.
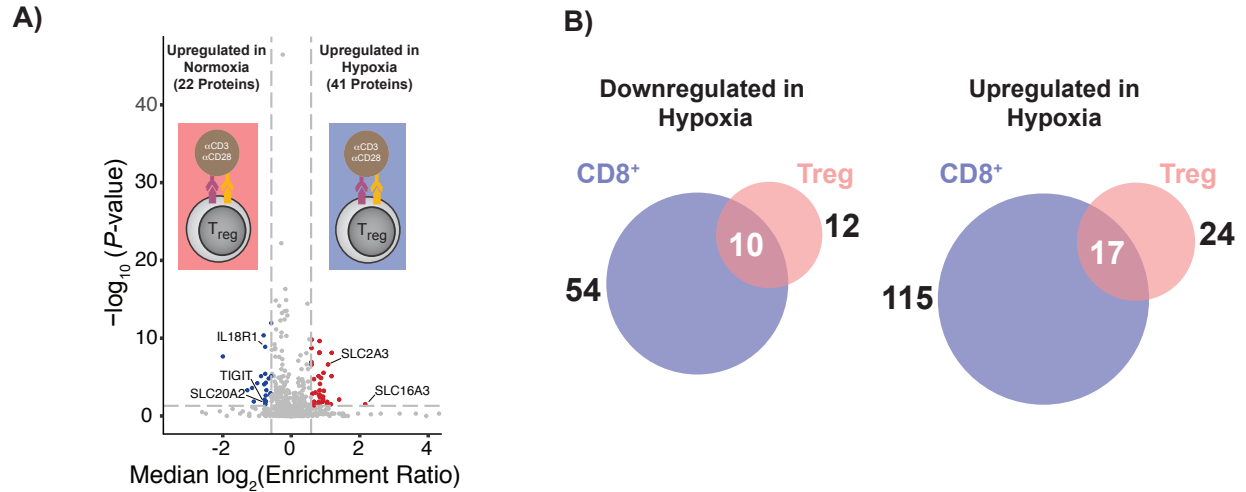
Figure S15. Effect of hypoxic culture on the Treg surfaceome. (A) Tregs were stimulated with anti-CD3/anti-CD28 beads in either normoxic (20% O_2) or hypoxic (1% O_2) conditions for three days. Volcano plot shows compiled results from N=2 donors. Proteins with a -/+1.5-fold change and $P<0.05$ were included in downstream analysis. Proteins significantly down- (blue) or upregulated (red) are indicated. (B) Venn diagrams showing proteins commonly down- or upregulated in hypoxia on both CD8+ T cells and Tregs.

A)

B)
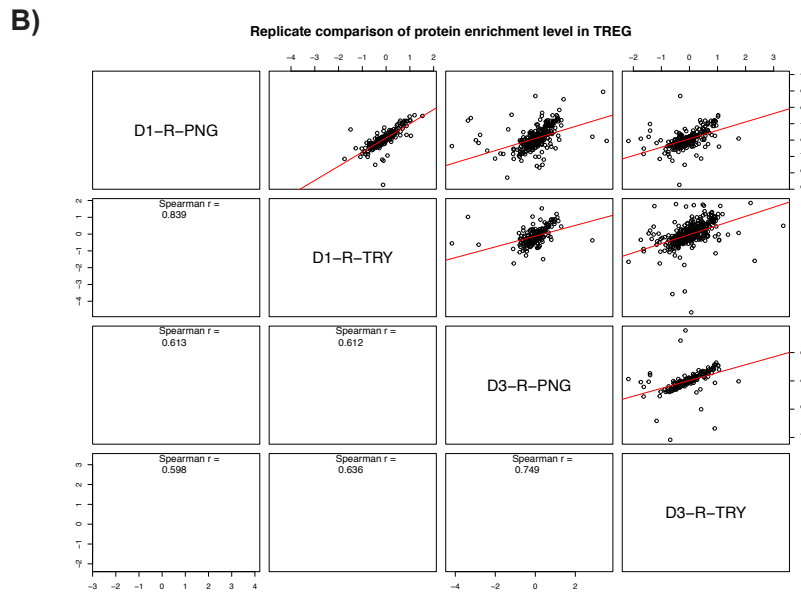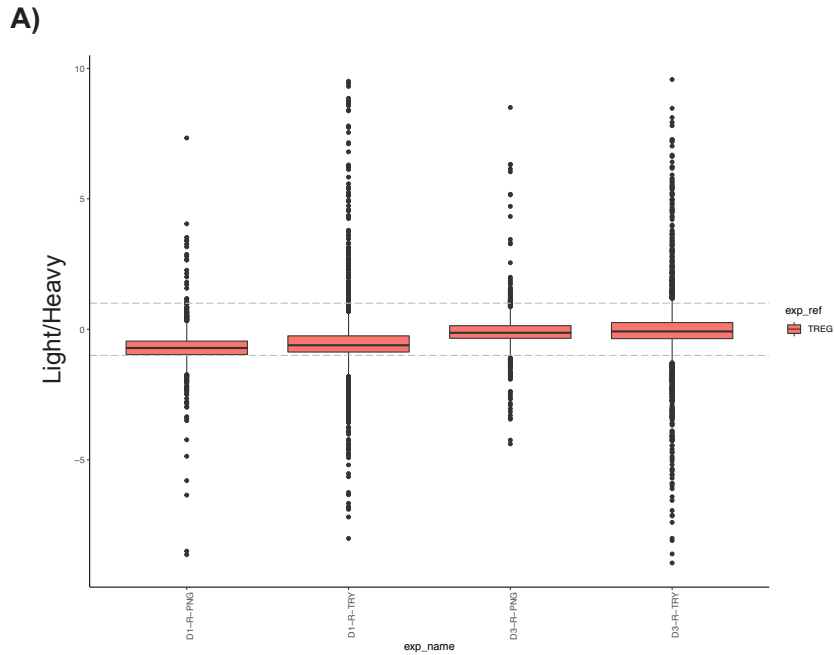
Figure S16. Replicate comparison for Treg hypoxia dataset. (A) Box plots showing heavy and light distributions before normalization for both tryptic and PNGase fractions from each donor. (B) Protein-level correlations between each fraction for each donor. Spearman correlation *r* is indicated. Data were produced using the custom R script as described in the Experimental Procedures.

SUPPLEMENTAL DATA FILE LEGENDS

Table S1. SILAC analysis output of CD8$^+$ activation in monoculture experiments. Excel sheet contains tabs with the raw output of our in-house analysis script showing SILAC enrichment ratio and *P*-value for identified proteins. Additional tabs show significantly (*P*<0.05, -/+ 1.5-fold change) up- and downregulated protein lists.

Table S2. SILAC analysis output of CD8$^+$ activation in Treg co-culture experiments. Excel sheet contains tabs with the raw output of our in-house analysis script showing SILAC enrichment ratio and *P*-value for identified proteins. Additional tabs show significantly (*P*<0.05, -/+ 1.5-fold change) up- and downregulated protein lists.

Table S3. SILAC analysis output of CD8$^+$ activation in hypoxia experiments. Excel sheet contains tabs with the raw output of our in-house analysis script showing SILAC enrichment ratio and *P*-value for identified proteins. Additional tabs show significantly (*P*<0.05, -/+ 1.5-fold change) up- and downregulated protein lists.

Table S4. SILAC analysis output of CD4$^+$ activation in hypoxia experiments. Excel sheet contains tabs with the raw output of our in-house analysis script showing SILAC enrichment ratio and *P*-value for identified proteins. Additional tabs show significantly (*P*<0.05, -/+ 1.5-fold change) up- and downregulated protein lists.

Table S5. SILAC analysis output of Treg activation in hypoxia experiments. Excel sheet contains tabs with the raw output of our in-house analysis script showing SILAC enrichment ratio and *P*-value for identified proteins. Additional tabs show significantly (*P*<0.05, -/+ 1.5-fold change) up- and downregulated protein lists.

Table S6. Identified peptide list for CD8$^+$ activation in monoculture experiments. Excel sheet with ProteinProspector outputs for each raw file used.

Table S7. Identified peptide list for CD8$^+$ activation in Treg co-culture experiments. Excel sheet with ProteinProspector outputs for each raw file used.

Table S8. Identified peptide list for CD8$^+$ activation in hypoxia experiments. Excel sheet with ProteinProspector outputs for each raw file used.

Table S9. Identified peptide list for CD4$^+$ activation in hypoxia experiments. Excel sheet with ProteinProspector outputs for each raw file used.

Table S10. Identified peptide list for Treg activation in hypoxia experiments. Excel sheet with ProteinProspector outputs for each raw file used.