Localized hypoxia links ER stress to lung fibrosis through induction of C/EBP homologous protein

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Supplemental Data

Supplemental Figure 1. CHOP levels in inducible transgenic mice expressing L188Q surfactant protein C (L188Q SFTPC) treated with bleomycin compared to wild type mice. L188Q SFTPC and WT controls were treated with doxycycline (2 g/l) for 1 week followed by intratracheal injection of bleomycin (0.08 units) and lungs were harvested 3 weeks later. Untreated WT mice were used as an additional control. Western blot for CHOP from lung tissue lysates is shown along with β-actin as loading control.

Supplemental Figure 2. Gene expression profiling of EpCAM positive epithelial cells isolated from wild type and CHOP deficient mice in the repetitive bleomycin model. WT and $CHOP^{-/-}$ mice were intratracheally injected with 6 doses of bleomycin (0.04 units) at intervals of 2 weeks and lungs were harvested 2 weeks after the last dose. EpCAM positive epithelial cells were isolated from the lungs of bleomycin-treated (Rep Bleo) mice and control (untreated) WT mice. Total RNA was extracted from the isolated EpCAM positive cells and used for RNA-sequencing. n=3-4 mice in each group. **(A)** Pie-charts showing differentially regulated genes (fold change>2, p value<0.05, and FDR<0.05) between groups. The subset of genes differentially regulated between the WT + Rep Bleo group and WT Untreated group was examined in epithelial cells from the $CHOP^{-/-}$ + Rep Bleo group. **(B)** Plots showing expression of representative ER stress genes. Comparisons between groups were made using unpaired, two-tailed Student's t-test. *p<0.05 compared to WT Untreated. **(C)** Differentially regulated GO pathways in $CHOP^{-/-}$ + Rep Bleo compared to WT + Rep Bleo.

Supplemental Figure 3. Immune/inflammatory cells identified in lungs of wild type and CHOP deficient mice following repetitive bleomycin treatment. Mice were intratracheally injected with 6 doses of bleomycin (0.04 units) at intervals of 2 weeks and lungs were harvested 2 weeks after the last dose for flow cytometry analysis. **(A-B)** Gating strategy and quantification

of myeloid cell subsets obtained from single cell suspensions of lung tissue. Viable ($DAPI^-$) $CD45^+$ cells were gated and percentages of alveolar macrophages ($CD103^-CD11c^+$), interstitial macrophages ($CD103^-CD11c^-CD11b^+Ly6G^-$), dendritic cells ($CD103^+CD11c^+$), and neutrophils ($CD103^-CD11c^-Ly6G^+CD11b^+$) were determined. (**C-D**) Gating strategy and quantitation of lymphocytes. Viable ($DAPI^-$) $CD45^+$ cells were gated and percentages of B cells ($CD19^+$) and T cells ($CD3^+$) were determined. Comparison between groups was made using unpaired, two-tailed Student's t-test. *=$p<0.05$ compared to WT.

<u>Supplemental Figure 4.</u> Dual immunofluorescence between pimonidazole and markers of type I AECs, fibroblasts, endothelial cells, or macrophages in the repetitive dose bleomycin model. (A-D) Dual immunofluorescence for pimonidazole (red) and markers for type I AECs (T1α), fibroblasts (S100A4), endothelial cells (CD34), or macrophages (F4/80) (all green) was performed on lung sections of mice treated in the repetitive bleomycin model. Nuclei were counterstained with DAPI (blue).

<u>Supplemental Figure. 5.</u> Dual immunofluorescence between pimonidazole and markers of different cell types in lungs of mice treated with bleomycin followed by exposure to hypoxia (14% O2). Dual immunofluorescence (IF) for pimonidazole (red) and cell specific markers (green) was performed in lungs of mice treated with intratracheal bleomycin (0.04 units) followed 7 days later by exposure to 14% O_2 for an additional 14 days or maintained in 21% O_2. Additional controls were only exposed to 14% O_2 for 2 weeks or were maintained in 21% O_2. (**A**) Dual IF is shown for type II AECs (pro-SPC). Nuclei were counterstained with DAPI (blue). White arrows point to cells with co-localization (yellow) of pimonidazole (red) and pro-SPC (green). (**B**) Quantification of the percentage of pro-SPC positive cells which were also positive for pimonidazole per high power field (HPF). Comparison between groups was made using unpaired, two-tailed Student's t-test. *=$p<0.05$ compared to single bleo + 21% O_2. Dual IF is

also shown for **(C)** type I AECs (T1α), **(D)** endothelial cells (CD34), **(E)** macrophages (F4/80), and **(F)** fibroblasts (S100A4). Nuclei were counterstained with DAPI (blue).

<u>Supplemental Figure 6.</u> Immune/inflammatory cells and M1/M2 markers in macrophages from lungs of wild type and CHOP deficient mice following single dose IT bleomycin treatment with or without subsequent exposure to hypoxia (14% O_2) and harvested at day 21 post-bleomycin. WT and $CHOP^{-/-}$ mice were treated with intratracheal bleomycin (0.04 units) followed 7 days later by exposure to 14% O_2 for an additional 14 days or maintained in 21% O_2. Lungs were harvested at 21 days post-bleomycin and immune/inflammatory cells were analyzed by flow cytometry. **(A)** Percentage of viable CD45+ cells in the lungs identified as alveolar macrophages ($CD103^-CD11c^+$), interstitial macrophages ($CD103^-CD11c^-CD11b^+Ly6G^-$), dendritic cells ($CD103^+CD11c^+$), or neutrophils ($CD103^-CD11c^-Ly6G^+CD11b^+$). **(B)** Percentage of viable $CD45^+$ cells in the lungs identified as B cells ($CD19^+$) and T cells ($CD3^+$). (**C**) WT and $CHOP^{-/-}$ mice were treated with intratracheal bleomycin (0.04 units) followed 7 days later by exposure to 14% O_2 for an additional 14 days or maintained in 21% O_2. Lungs were harvested at 21 days post-bleomycin and macrophages were isolated from single cell suspensions by adherence to plastic and mRNA was isolated for qPCR quantification of M1 macrophage markers (IL-6, TNFα, iNOS, and IL-12) and M2 macrophage markers (Arginase, IL-10, Ym1, Mannose Receptor). GAPDH was used for normalization. Comparisons between groups were made using one way ANOVA with Tukey's post-hoc test. *=$p<0.05$ compared to WT +14% O_2.

<u>Supplemental Figure 7.</u> Immune/inflammatory cells and M1/M2 markers in macrophages from lungs of wild type and CHOP deficient mice following single dose IT bleomycin treatment with or without subsequent exposure to hypoxia (14% O_2) and harvested at day 10 post-bleomycin. WT and $CHOP^{-/-}$ mice were treated with intratracheal bleomycin (0.04 units) followed 7 days later by exposure to 14% O_2 for an additional 3 days or maintained in

21% O_2. Lungs were harvested at 10 days post-bleomycin and immune/inflammatory cells were analyzed by flow cytometry. (**A**) Percentage of viable CD45+ cells in the lungs identified as alveolar macrophages (CD103-CD11c+), interstitial macrophages (CD103-CD11c-CD11b+Ly6G-), dendritic cells (CD103+CD11c+), or neutrophils (CD103-CD11c-Ly6G+CD11b+). (**B**) Percentage of viable CD45+ cells in the lungs identified as B cells (CD19+) and T cells (CD3+). (**C**) WT and $CHOP^{-/-}$ mice were treated with intratracheal bleomycin (0.04 units) followed 7 days later by exposure to 14% O2 for an additional 3 days or maintained in 21% O2. Lungs were harvested at 10 days post-bleomycin and macrophages were isolated from single cell suspensions by adherence to plastic. mRNA was isolated for qPCR quantification of M1 macrophage markers (IL-6, TNFα, iNOS, and IL-12) and (B) M2 macrophage markers (Arginase, IL-10, Ym1, Mannose Receptor). GAPDH was used for normalization. Comparisons between groups were made using one way ANOVA with Tukey's post-hoc test. *=$p<0.05$ compared to WT + 14% O2.

Supplemental Figure 8. CHOP expression in macrophages isolated from mice in the bleomycin followed by hypoxia model. WT and $CHOP^{-/-}$ mice were treated with intratracheal bleomycin (0.04 units) followed 7 days later by exposure to 14% O_2 for an additional 14 days **(A)** or 3 days **(B)**. Control mice were maintained in 21% O_2. Lungs were harvested and macrophages were isolated from single cell suspensions by adherence in tissue culture plates. mRNA was isolated for qPCR quantification of CHOP and GAPDH was used for normalization. Comparisons between groups were made using one way ANOVA with Tukey's post-hoc test. NS= non-significant.

Supplemental Figure 9. Evaluation of HIF1α expression and fibrosis in epithelial HIF deficient mice and littermate controls in the bleomycin followed by hypoxia model. Mice with targeted deletion of HIF1/2 in lung epithelium ($HIF1/2^{\Delta/\Delta}$) and controls ($HIF1/2^{fl/fl}$) were

treated with intratracheal bleomycin (0.04 units) followed 7 days later by exposure to 14% O_2 for an additional 14 days or maintained in 21% O_2. Lungs were harvested at 21 days post-bleomycin. (**A**) Representative immunohistochemistry for HIF1α on lung sections. **(B)** Representative Masson's trichrome staining on lung sections. Scale bars: 800 μm.

Supplemental Figure 10. CHOP levels in epithelial HIF deficient mice and littermate controls in the bleomycin followed by hypoxia model. Western blot for CHOP from lung lysates obtained from HIF1/2$^{\Delta/\Delta}$ and HIF1/2$^{fl/fl}$ mice that were treated with intratracheal bleomycin (0.04 units) followed 7 days later by exposure to 14% O_2 for an additional 14 days or maintained in 21% O_2. Lung lysates from untreated HIF1/2$^{fl/fl}$ mice were used as a negative control. β-actin is shown as loading control.

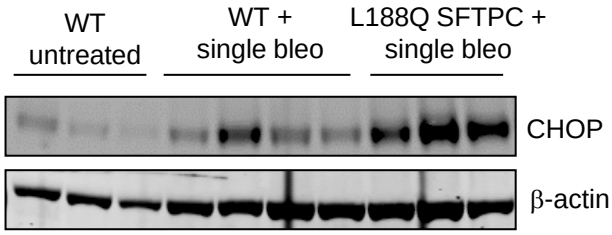
Supplemental Figure 11. siRNA mediated-knockdown of IRE1 and ATF4 followed by evaluation of CHOP in MLE12 cells exposed to hypoxia. MLE12 cells were transfected with IRE1, ATF4 or control non-targeted (NT) siRNA and exposed to hypoxia for 48 hours. Western blots for (**A**) IRE1, ATF4 and (**B**) CHOP. β-actin is shown as loading control.

Supplemental Figure 12. Efficiency of HIF1α knockdown by siRNA in MLE12 cells. MLE12 cells were transfected with HIF1α siRNA or control NT siRNA and exposed to hypoxia for 6 hours. qPCR for HIF1α normalized to RPL19. Groups were compared using unpaired, two-tailed Student's t-test. *=$p<0.05$ compared to NT siRNA + 1.5% O_2.

Supplemental Figure 13. Expression of pro-apoptotic mediators in CHOP siRNA or control siRNA treated AECs exposed to hypoxia. MLE12 cells were transfected with CHOP siRNA or NT control siRNA and exposed to hypoxia for 48 hours. Clustergram analysis of results from Mouse Apoptosis RT^2 Profiler PCR Array is shown. n = 3 samples per group.

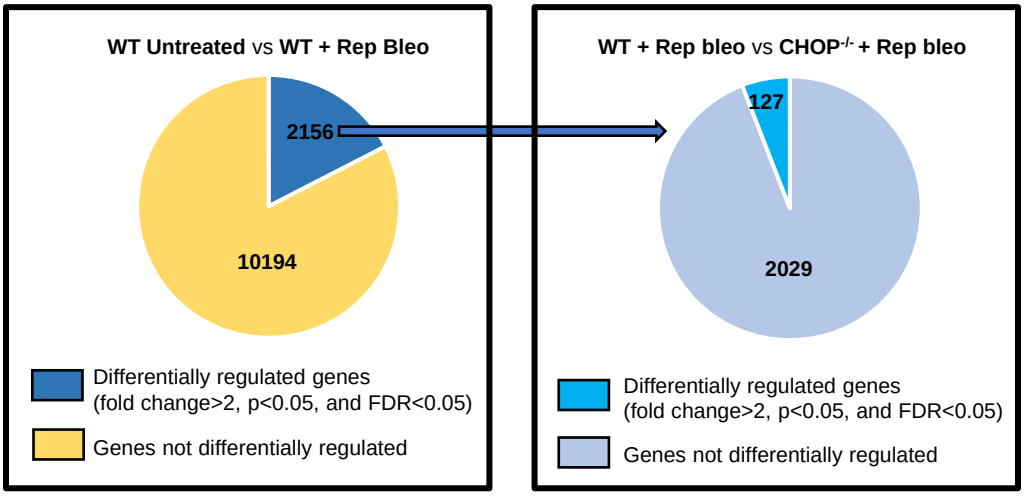
<u>Supplemental Figure 14.</u> Expression of CHOP-dependent apoptosis mediators in lungs of CHOP deficient mice treated with repetitive bleomycin. Wild type and $CHOP^{-/-}$ mice were studied using the repetitive bleomycin model and lungs were harvested 2 weeks after the last dose. qPCR for ATF5, GADD45A, and BNIP3L normalized to RPL19. Groups were compared using unpaired two-tailed Student's t-test. *=$p<0.05$.

Supplemental Figure 1



Supplemental Figure 2

A



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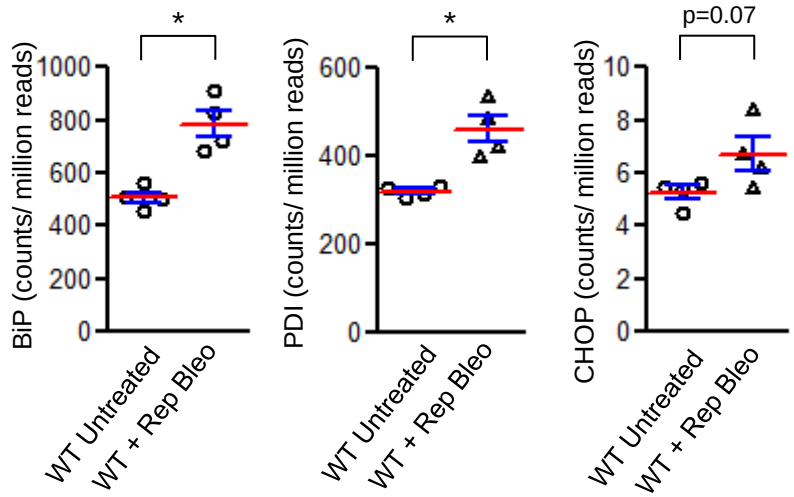
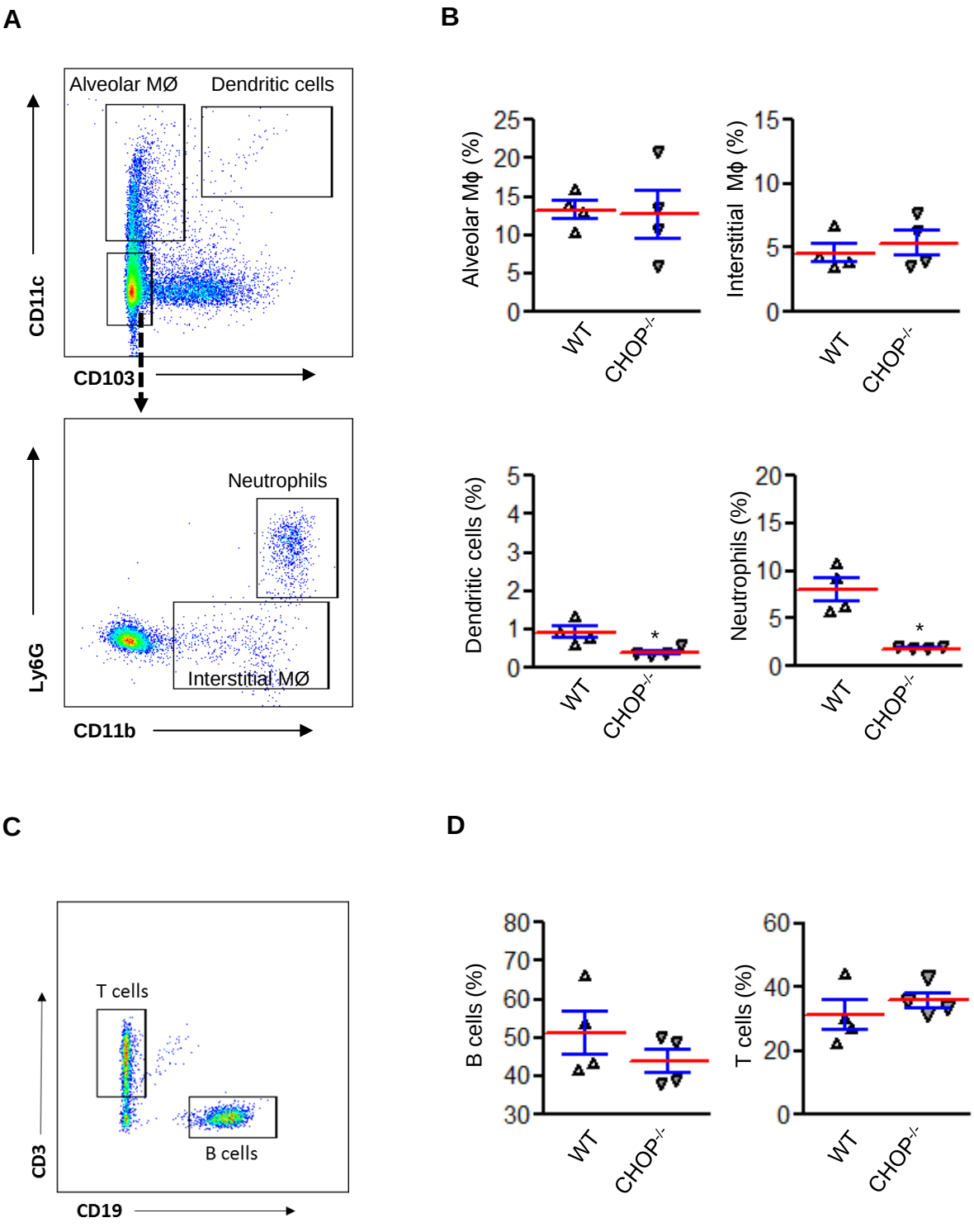


C

Differentially regulated GO pathways in 'CHOP$^{-/-}$ + Rep Bleo' compared to 'WT + Rep Bleo' that are relevant to fibrosis	
Pathways	**Genes**
Cellular response to growth factor stimulus	Mmrn2, Ltbp4, Acvrl1, Bmp6, Cdh5, Cldn5, Dll1, Edn1, Eng, Flt1, Fzd4, Ltbp1, Postn, Pde3a, Fgfbp3, Adgra2
Epithelial Cell Differentiation	Acvrl1, Bmp6, Cd34, Cdh5, Cldn5, Dll1, S1pr1, Eng, Notch4, Rbpj, Sox17, Sox18, Ppp1r16b, Podxl
Epithelium Development	Acvrl1, Aqp1, Cd34, Cdh5, Cldn5, S1pr1, Edn1, Eng, Flt1, Gja4, Notch4, Podxl, Sema3c, Sox17, Sox18, Ppp1r16b, Dact2, Podxl, Shank3, Bmp6
Cell Migration	Mmrn2, Arap3, Acvrl1, Aqp1, S1pr1, Edn1, Eng, Flt1, Gata2, Kit, Plat, Sema3c, Sox18, Nav1, Tie1, Sema3g, Gpm6a, Pkn3, Podxl, Cxcl3, Egfl7, Postn, Adgra2, Plvap, Sema7a, Sox17

Supplemental Figure 3



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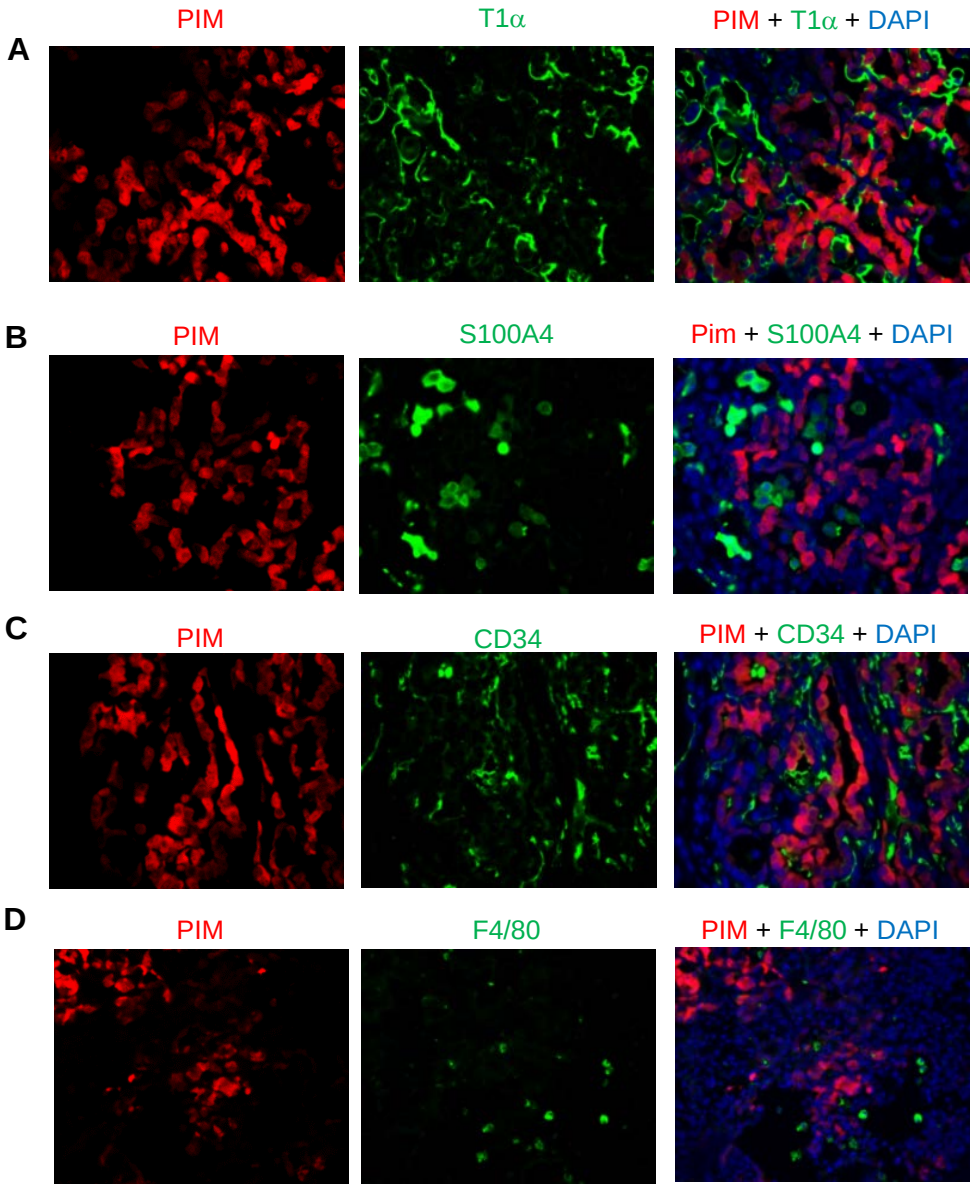

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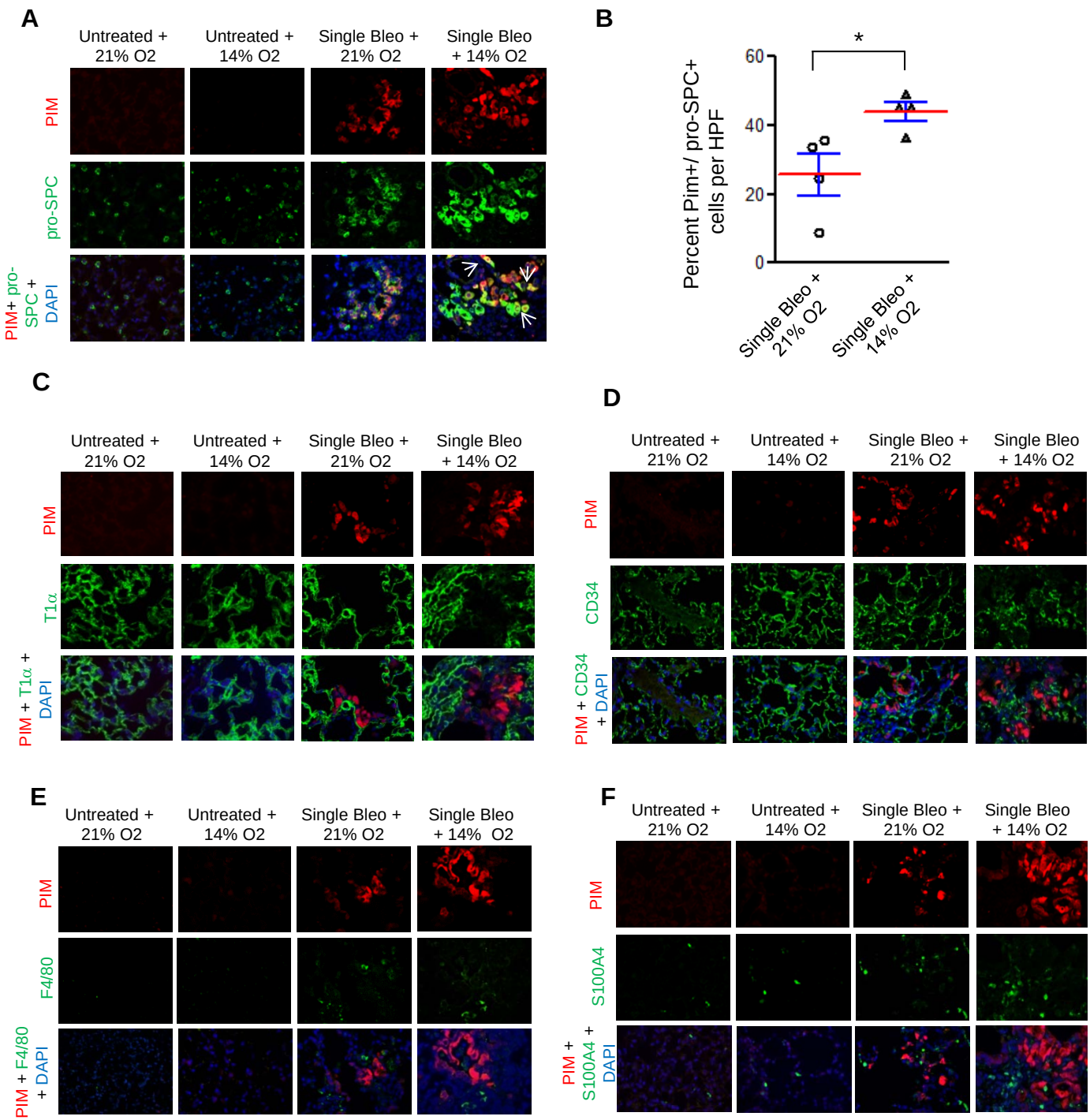

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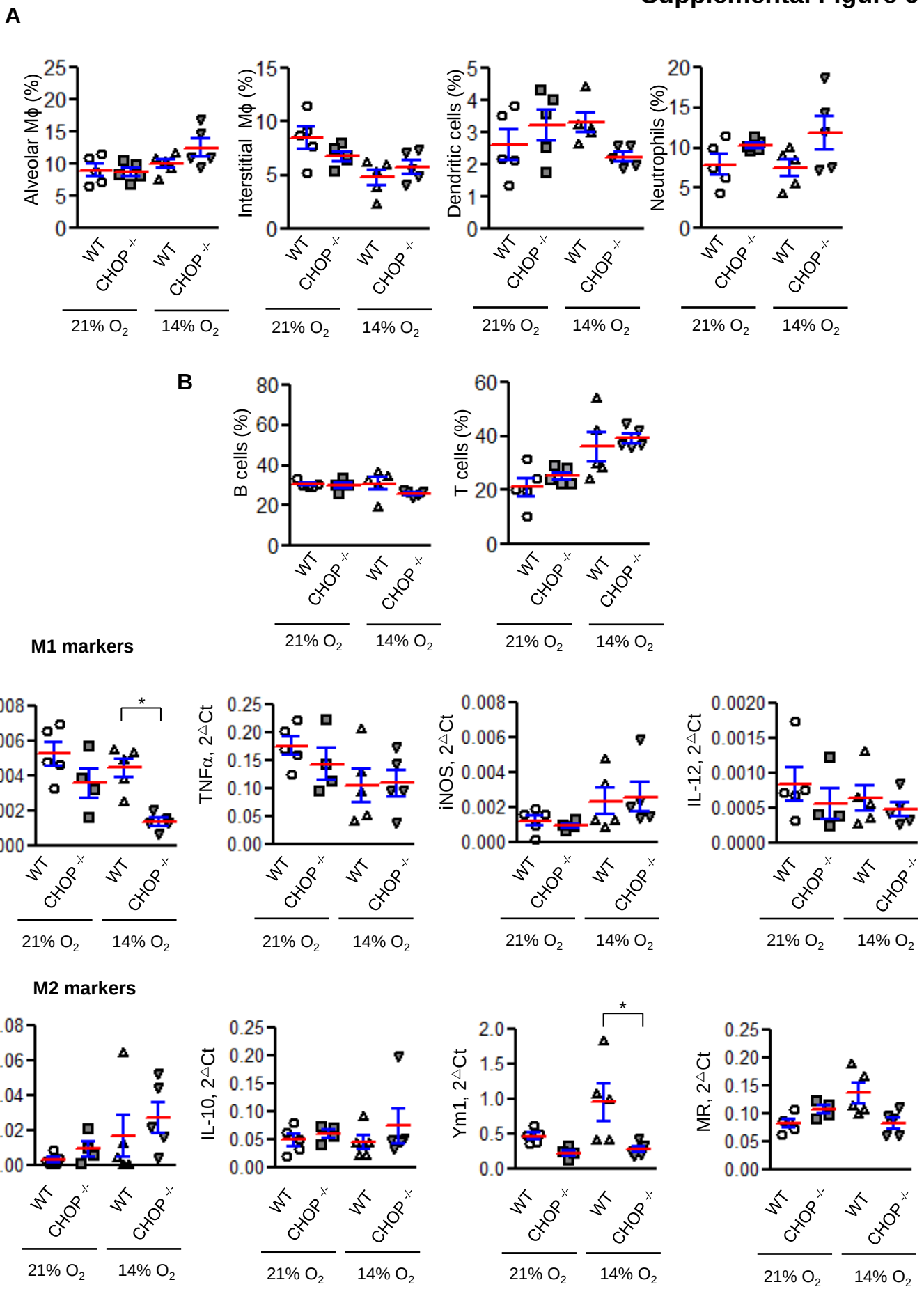

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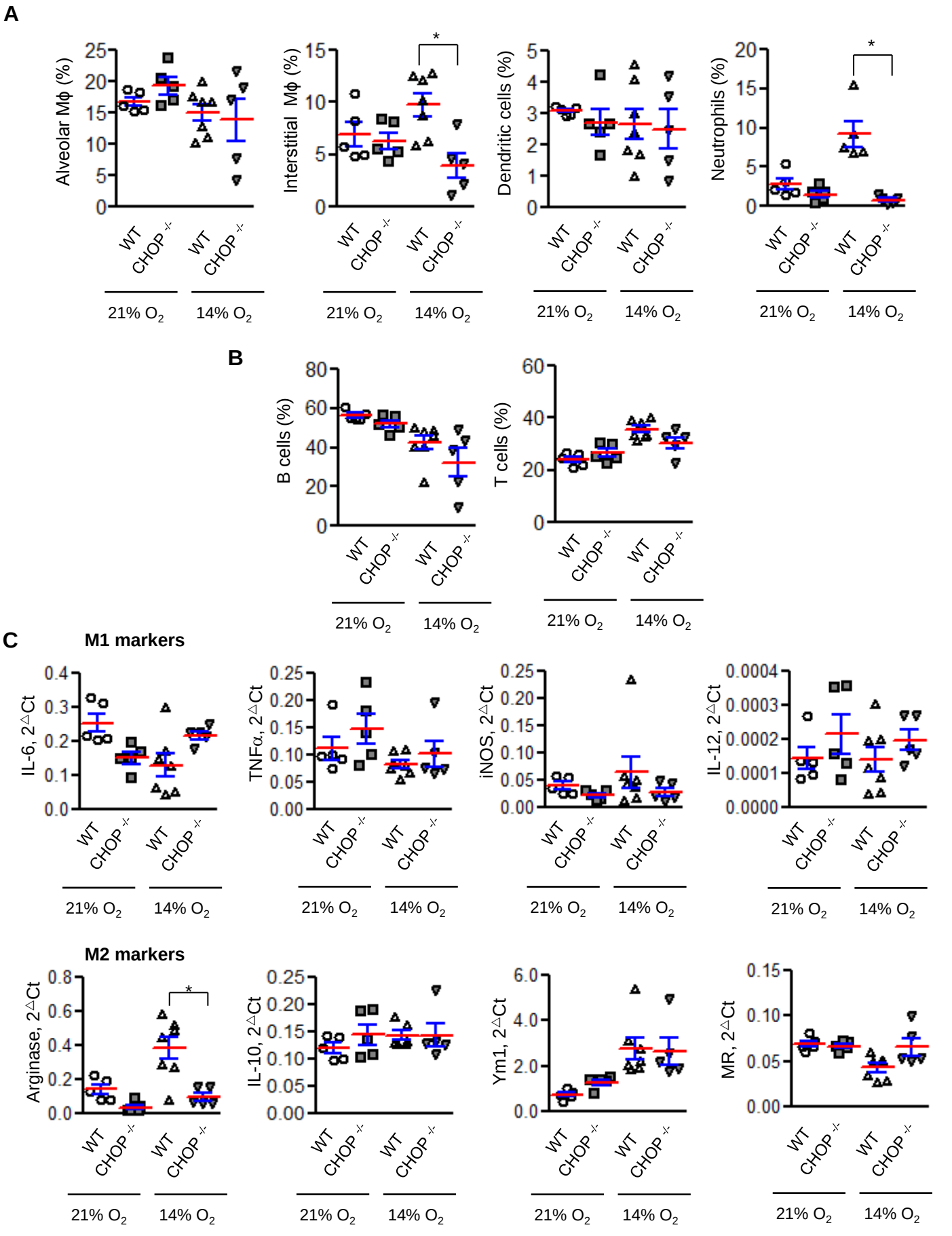

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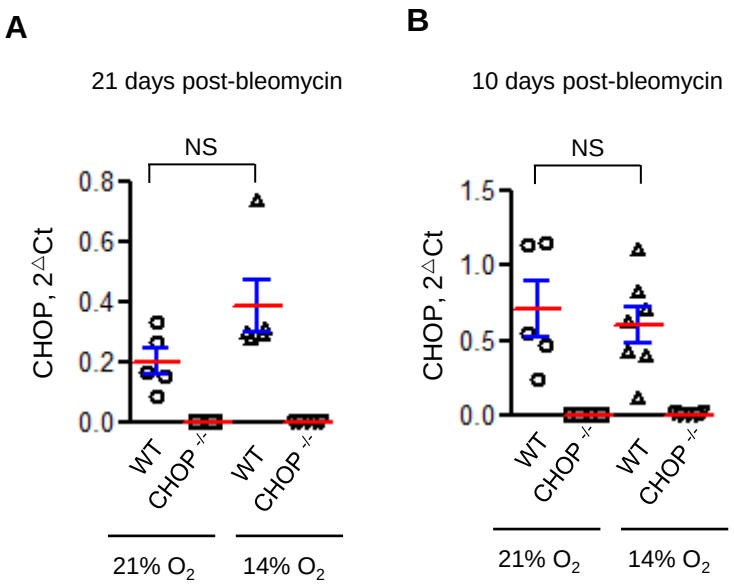

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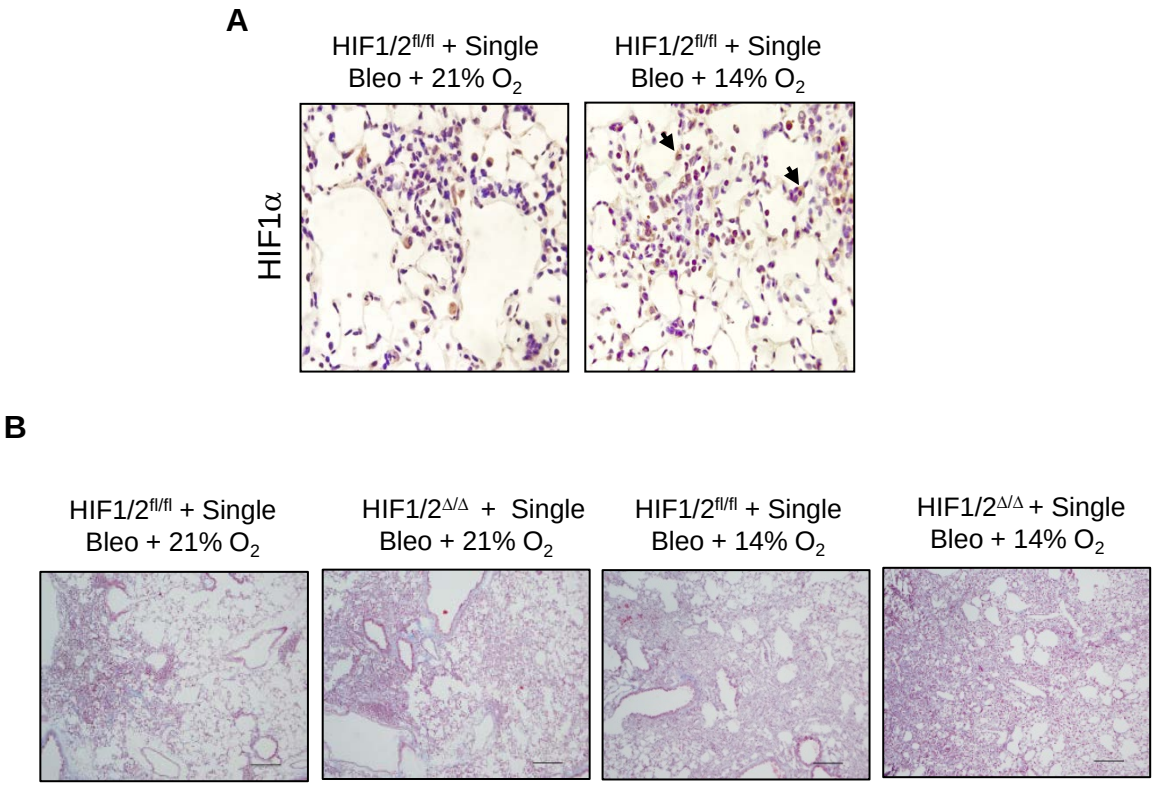

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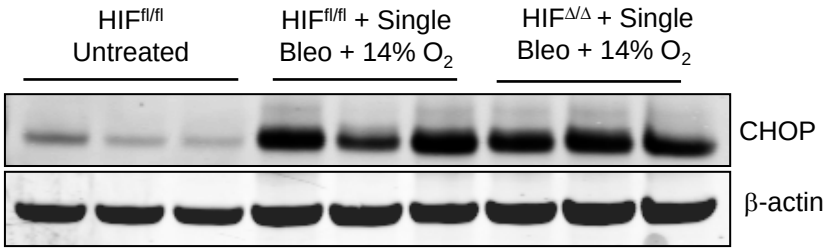

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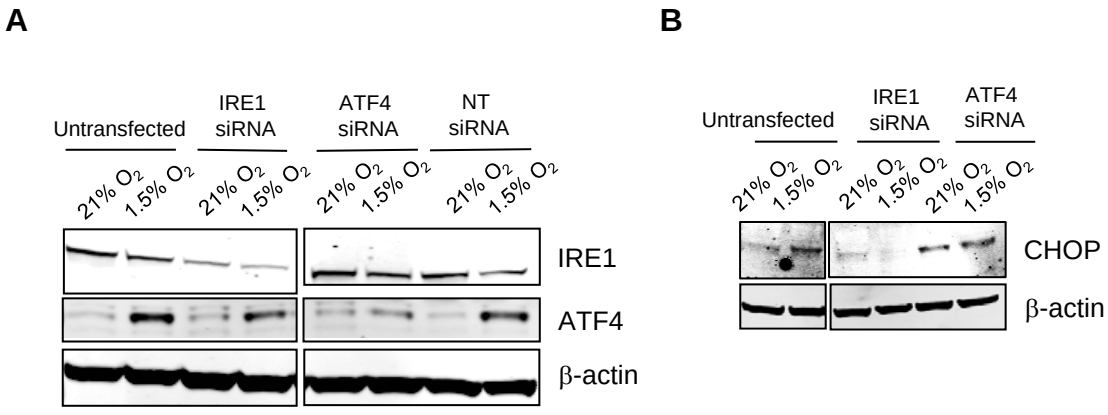

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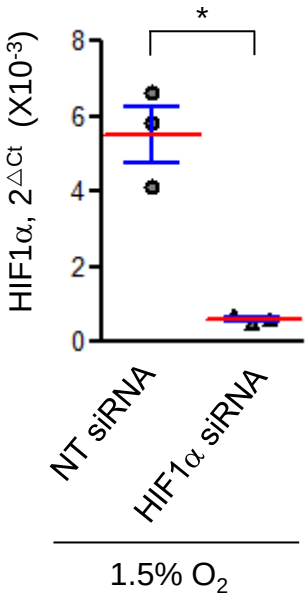
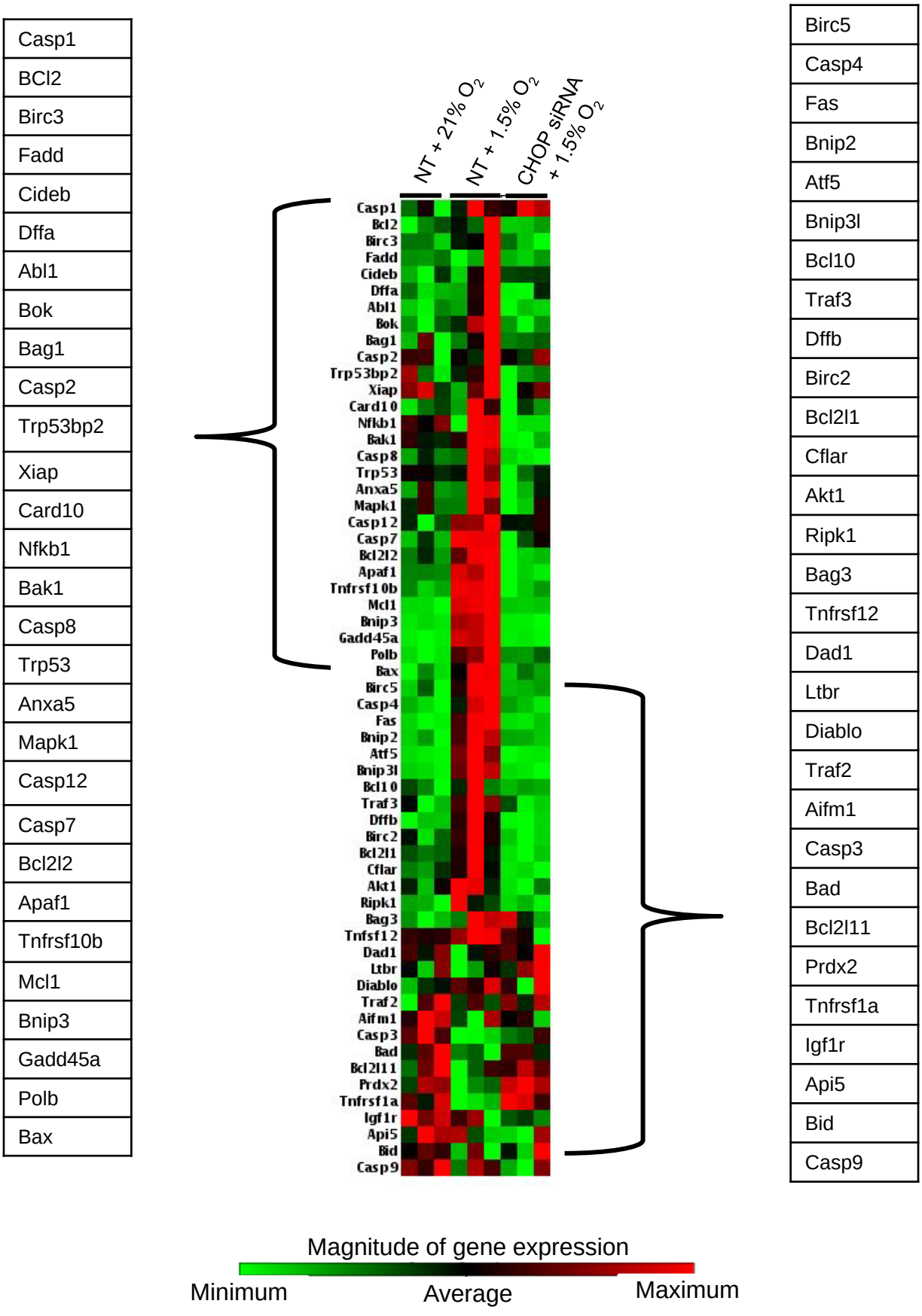


Supplemental Figure 13



Supplemental Figure 14