# Deletion of *C/EBP Homologous Protein* (Chop) in Tumor Stroma Mediates the Accumulation of Myeloid-Derived Suppressor Cells (MDSC) in Tumors

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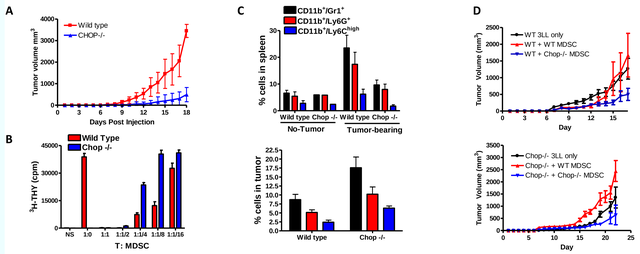
## Introduction

Interactions between malignant cells and tumor stroma play a major role in cancer development, growth, and metastasis. The presence of stromal myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment has been linked to a robust inhibition of anti-tumor immunity and subsequent enhancement of tumor progression. These MDSC-mediated immunosuppressive functions represent a major obstacle to the advancement of cancer immunotherapy. Though several mechanisms of MDSC-mediated immunosuppression have been described, these findings have not yet led the way to the development of successful therapies to block harmful MDSC function in cancer. No master mediators of MDSC immune regulatory activity in tumors have been identified to date.

Preliminary findings suggest that Chop, a stress sensor molecule associated with the induction of apoptosis, may play a major role in regulating the immunosuppressive functions of MDSCs.




***(A)*** *Deletion of stromal Chop prevents tumor growth.* ***(B)*** *Chop KO MDSCs are less suppressive of T cell proliferation than WT mice MDSCs.* ***(C)*** *MDSC accumulation in spleen and tumor of WT and Chop KO mice.* ***(D)*** *Co-administration of Chop KO MDSCs with cancer cells led to decreased tumor growth.*

In stromal Chop knockout mice, tumors grew more slowly (indicating enhanced host immunity and less immunosuppression by MDSCs) despite the fact that there was actually an unexpected increase in the number of MDSCs in the tumor. These findings suggest that Chop likely plays an important mediating role in the function and accumulation of these cells. However, the mechanisms by which Chop deletion in the stroma modulates MDSC accumulation remain completely unknown.

## Objective

- Determine the mechanisms by which stromal Chop deletion regulates the accumulation of MDSC in tumors.

## Preliminary Methods

- ***Model:*** Experiments were performed on wild type C57BL/6 mice and Chop -/- mice. Tumor growth was initiated by s.c. injection with 1 x $10^6$ Lewis lung carcinoma (3LL) cells.

- ***MDSC isolation:*** Whole tumors and spleens were excised and disrupted into a single cell suspension in $Ca^{++}$ and $Mg^{++}$ free PBS containing 2% Fetal Bovine Serum (FBS) and 1 mM EDTA. 1 x $10^6$ cells were aliquoted into sample tubes and MDSCs were collected using the EasySep Mouse MDSC Positive Selection Kit (StemCell Technologies).

## 1. BrdU Proliferation Assay

***MDSC proliferation at the tumor site is greater in Chop knockout mice than wild type mice***

- Healthy and 17 day tumor-bearing wild type C57BL/6 and Chop -/- mice were injected i.p. with 200 uL of a 10 mg/ml solution of BrdU in sterile 1x DPBS. 24 hours following BrdU injection, spleens and tumors were collected, stained for BrdU and the MDSC cell surface antigens CD11b and Gr1, and subsequently analyzed by flow cytometry.

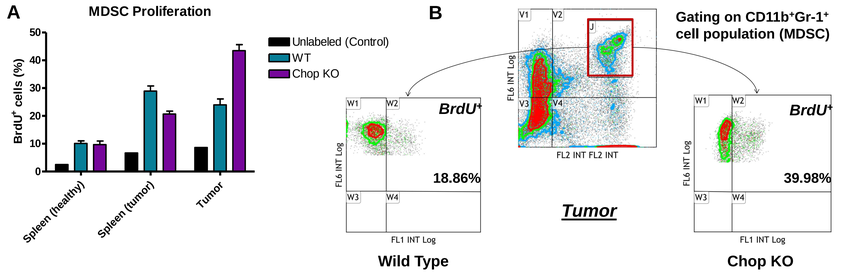



***Figure 1. (A)*** *Measurements of MDSC proliferation (BrdU⁺ incorporation) in spleens of healthy and tumor-bearing mice and at the tumor site. Proliferation was higher at the tumor site in Chop KO mice as compared to WT controls.* ***(B)*** *Representative BrdU incorporation experiment showing gating on CD11b⁺Gr1⁺ cells and BrdU positivity in WT and Chop KO tumor sites as determined by FACS.*

## 2. Apoptosis Analysis

***Stromal Chop deletion may lead to increased apoptosis of MDSCs at the tumor site***

- ***Apoptosis assay:*** Translocation of phosphatidylserine across the plasma membrane is an early marker of apoptosis. Annexin V, a $Ca^{++}$ dependent phospholipid-binding protein, has high affinity for PS and when labeled with a fluorochrome it can be used for the detection of apoptosis using flow cytometry. The presence of PS on the extracellular membrane leaflet was tested using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences).

- ***Western blotting:*** Thirty micrograms of cell lysates were electrophoresed in 8–12% Tris-Glycine gels, transferred to PVDF membranes, and immunoblotted with specific antibodies against caspase 8, cleaved caspase 8, and β-Actin (Sigma). Membrane-bound immune complexes were detected by using Clarity ECL western substrate (Bio-Rad).

- ***TNFR-II expression quantification****:* Spleen and tumor cells from WT and Chop KO mice were disrupted into a single cell suspension and stained for TNFRII expression and the MDSC surface markers CD11b and Gr1 and analyzed with flow cytometry.

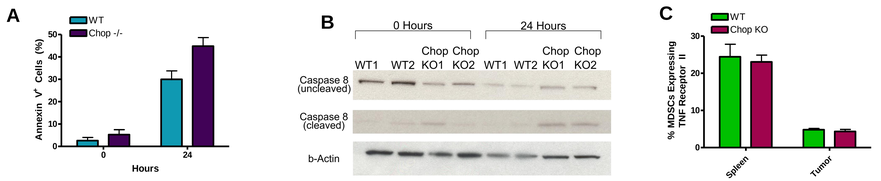
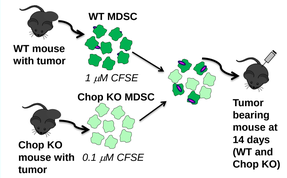



***Figure 2. (A)*** *Although Chop is generally regarded as a pro-apoptotic molecule, Chop KO MDSCs displayed more Annexin V⁺ cells than WT MDSCs, indicating that apoptosis may be occurring at a greater rate at the Chop KO tumor site than at the WT tumor site.* ***(B)*** *Kinetic blotting of caspase 8 apoptosis markers in WT and Chop KO tumor site MDSCs at 0 hours and 24 hours after collection.* ***(C)*** *Expression levels of TNF receptor II in WT and Chop KO spleens and tumors. No major differences were observed between Chop KO and WT MDSCs at the tumor site.*

## 3. Adoptive Transfer of MDSCs: Migration

***Stromal Chop deletion enhances MDSC accumulation at the tumor site***




Wild type and Chop KO Gr-1+ MDSCs were sorted from spleens of mice bearing 3LL tumors for 14 days. WT MDSCs were labeled with a high concentration of CFSE (1 μM), while Chop KO MDSCs were labeled with low CFSE (0.1 μM). WT mice bearing 3LL tumors for 14 days were injected i.v. with a 1:1 mix of the labeled MDSCs in 0.2 mL of PBS. 24 hours later, the ratio of bright:dull CFSE-labeled MDSC in spleens and tumors was calculated by flow cytometry after gating into Gr1+ cells.

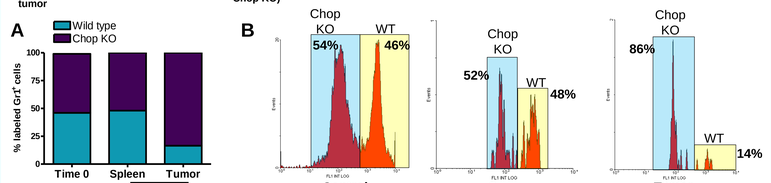



***Figure 3. (A)*** *Chop KO MDSCs were found in a higher concentration at the tumor site than WT MDSCs, indicating that they may display high affinity for this site.* ***(B)*** *Histogram comparison of CFSE-labeled WT and Chop KO MDSC migration in a control group versus the spleen and tumor site. Only one spleen sample contained measurable amounts of the labeled MDSCs.*

## Conclusions

- MDSC accumulation at stromal Chop KO tumor sites is primarily due to a marked increase in their rate of proliferation there and a higher affinity for this tissue.

- Though Chop is typically associated with the promotion of apoptosis, deletion of this molecule in the stroma did not lead to the reduction in apoptosis rates that we anticipated. Total levels of Annexin V⁺ cells were considerably higher in the Chop KO mice at both 0 and 24 hours. Cleaved caspase 8, a marker for apoptosis, was present in Chop KO MDSCs at time 0 and became more pronounced at 24 hours. Despite this potentially increased apoptosis, there seems to be a net accumulation of MDSCs at the tumor site of Chop KO mice due to their greatly elevated proliferation rates there.

- Decreased signaling through the tumor necrosis factor (TNF) receptor II has been associated with increased caspase-8 mediated apoptosis in MDSCs, but data regarding receptor expression levels are not widely available. The significantly higher expression levels in the spleen correspond to the lower rates of apoptosis seen there versus the tumor site, but expression in the tumor of Chop KO mice was not markedly lower than in WT as we had expected.

- Chop KO MDSCs also appear to display preferential migration to the tumor site relative to WT MDSCs, but this study bears repeating due to low cell counts.

Further studies are needed to identify details about the mechanisms underlying MDSC proliferation, apoptosis, and affinity at the tumor site. Identifying Chop as a potential master mediator of MDSC function and accumulation could lead to removing a major barrier to effective immunotherapy treatments.

## Acknowledgements

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