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 strategies 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.

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 of 1 x 10⁶ Lewis lung carcinoma (SLL) cells.
• MDSC isolation: Whole tumors and spleens were excised and disrupted into a single cell suspension in Ca⁺² and Mg⁺²-free PBS containing 2% Fetal Bovine Serum (FBS) and 1 mM EDTA. 1 x 10⁶ cells were aliquot into sample tubes and MDSCs were collected using the EasySep Mouse MDSC Positive Selection Kit (StemCell Technologies).
• Spleen Tumor
• 6
• Chop -/-

1. BrdU Proliferation Assay
Stromal Chop deletion leads to increased apoptosis of Gr1+ cells in the tumor site.
• BrdU proliferation assay
• Healthy and 17 day tumor-bearing wild type C57BL/6 and Chop -/- mice were injected i.p. with 200 µL of a 10 mg/ml solution of BrdU in sterile 1x PBS. 24 hours following BrdU injection, spleens and tumors were collected, stained for BrdU and the MDSC surface antigens CD11b and Gr1, and subsequently analyzed by flow cytometry.

2. Apoptosis Analysis
Stromal Chop deletion leads to increased apoptosis of Gr1+ cells in the tumor site.
• Apoptosis assay:
• Annexin V, a Ca⁺²-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-FTIC 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 -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 TNFR-II expression and the MDSC surface markers CD11b and Gr1 and analyzed with flow cytometry.

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

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 6 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 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 maintained, 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.

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References
1. Lewis lung carcinoma
2. Healthy and 17 day tumor-bearing wild type C57BL/6 and Chop -/- mice were injected i.p. with 200 µL of a 10 mg/ml solution of BrdU in sterile 1x PBS. 24 hours following BrdU injection, spleens and tumors were collected, stained for BrdU and the MDSC 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 showing gating on CD11b+ /Gr1+ cells and BrdU positively in WT and Chop KO tumor sites as determined by FACS.

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 WT tumor site.