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14. ABSTRACT Previous studies have shown that MDSC block adaptive anti-tumor immunity by producing high levels of oxidizing agents such as reactive oxygen species (ROS), nitric oxide (NO), and peroxynitrite. Despite high levels of the toxic oxidizing agents, MDSC survive and suppress anti-tumor immunity. We hypothesize that MDSC survival is mediated by the antioxidant-regulating transcription factor Nrf2. To test this hypothesis, BALB/c wild type and Nrf2 ^{-/-} mice were injected with 4T1 mammary carcinoma cells and assayed weekly for percentage of MDSC in the blood and for MDSC levels of ROS and glutathione, MDSC production of H ₂ O ₂ and suppressive activity, and MDSC apoptosis. Mice were also followed for survival. Nrf2 ^{-/-} MDSC had more ROS and less glutathione than wild type MDSC, indicating that Nrf2 ^{-/-} MDSC were more oxidatively stressed. Nrf2 ^{-/-} MDSC were more apoptotic than wild type MDSC. Tumor-bearing Nrf2 ^{-/-} and wild type mice accumulated MDSC in the blood with increasing tumor-burden. However, tumor-bearing Nrf2 ^{-/-} mice had less MDSC than wild type mice for a given sized primary tumor suggesting that MDSC apoptose more quickly in Nrf2 ^{-/-} mice. Tumor-bearing Nrf2 ^{-/-} mice lived longer than wild type mice demonstrating that Nrf2 contributes to tumor progression. Nrf2 ^{-/-} MDSC produce less H ₂ O ₂ and were less suppressive than wild type MDSC. These data are consistent with our hypothesis that Nrf2 regulates MDSC survival and suppressive activity, resulting in fewer and less suppressive MDSC in Nrf2 ^{-/-} mice, thereby increasing anti-tumor immunity against metastatic disease.					
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Introduction

Immune suppression is a major obstacle to breast cancer immunotherapy. A primary reason that immunotherapy is not effective is due Myeloid-Derived Suppressor Cells (MDSC). MDSC are a heterogeneous population of immature myeloid cells that accumulate in the blood, secondary lymphoid organs, and in primary and metastatic tumors in tumor-bearing individuals. MDSC are characterized by the surface markers Gr1 and CD11b in mice, and CD33 and CD11b in humans [1-3]. A variety of endogenous factors including vascular endothelial growth factor (VEGF) [4], prostaglandin E2 (PGE2) [5], IL-1 β [6, 7], IL-6 [8], S100A8/A9 [9, 10], the complement component C5a [11], and endotoxin [12] induce the accumulation of MDSC. MDSC block adaptive anti-tumor immunity by inhibiting the activation of CD4⁺ and CD8⁺ T lymphocytes [2, 13, 14]. MDSC also produce IL-10, which polarize macrophages to a tumor prototyping phenotype [15, 16]. A primary mechanism of MDSC-mediated suppression of T cells is by MDSC production of short-lived oxidants such as reactive oxygen species (ROS), nitric oxide, and peroxynitrite [17]. These reactive oxidizing agents are vital for T cell repression and for maintaining the inflammatory tumor microenvironment [18]. However, MDSC survive despite their high levels of these non-discriminatory toxic radicals. I hypothesize that MDSC withstand these oxidizing agents due to the transcriptional regulator Nrf2. Nrf2 is stabilized by the same factors that induce MDSC accumulation and suppression. Cells that are resistant to oxidative stress express the transcription factor NF-E2 related factor 2 (Nrf2). Nrf2 is considered a “good” transcription factor that when activated, induces the expression of protective and survival genes for antioxidant responses, phase II detoxification enzymes, and a plethora of other genes. These genes are grouped based on function and include genes for detoxification, antioxidant response, transcription, growth, defense and inflammatory response, signaling, and others [19, 20]. Nrf2 regulates genes controlled by the anti-oxidant response element (ARE) [21, 22] that are responsible for antioxidant responses, including glutathione synthesis genes such as GCL (Glutamate-Cysteine Ligase), and cystine transport genes x_CT and 4F2 [19, 23]. Cystine transport and Nrf2 may contribute to MDSC survival. We have previously shown that MDSC sequester cysteine [24]. This sequestration may facilitate MDSC resistance to toxic radicals since importation of cystine (via the x_C⁻ cystine/glutamate antiporter) and its reduction to cysteine are rate-limiting for the synthesis of the antioxidant glutathione (GSH) in MDSC. Nrf2 is a major transcriptional regulator of x_C⁻ and GSH synthesis genes [19, 20, 23]. Nrf2 is activated by the same oxidative radicals that MDSC use to facilitate immune suppression. Nrf2 protects cells against inflammation and is stabilized in response to inflammation, hypoxia, and other factors that are known inducers of MDSC. Since Nrf2 regulates antioxidant response and apoptosis, I hypothesize that Nrf2 regulates MDSC survival by protecting MDSC from oxidative stress. To test this hypothesis, I will be utilizing tumor-bearing Nrf2 deficient and wild type mice and comparing MDSC function and apoptotic rate in addition to monitoring these mice for survival and metastatic disease.

Aim 1: Determine if Nrf2 regulates MDSC survival by testing Nrf2^{-/-} and wild type MDSC for apoptotic marker expression *in vivo* and the rate of apoptosis *in vitro*.

Aim 2: Determine if Nrf2 regulates tumor-bearer survival and MDSC suppressive activity.

Aim 3: Determine if blocking cystine transport into MDSC while providing T cells with cysteine is a therapy for reducing MDSC-mediated immune suppression and delaying the growth of primary and metastatic mammary carcinomas.

Completion of these aims will determine if Nrf2 is a critical regulator of MDSC function and survival. New insight into Nrf2 modulating MDSC activity will provide future avenues for targeting MDSC as an adjuvant to cancer immunotherapy.

Body

Aim 1- In Progress

MDSC are functional immune suppressors despite their exposure to constant oxidative stress. Blood MDSC have high levels of ROS and tumor-infiltrating MDSC produce even more ROS (Previous data). Tumor-infiltrating MDSC are also exposed to even more oxidative stress due to the poorly vascularized and hypoxic tumor microenvironment and by ROS produced directly by tumor cells. Despite high levels of oxidative stress, MDSC are functionally suppressive and do not apoptose. Previously I had shown that MDSC from Nrf2^{-/-} MDSC are more oxidatively stressed than wild type MDSC. I hypothesize that MDSC resist apoptosis from oxidative stress by the activity of Nrf2. If MDSC lack Nrf2, then they would be more susceptible to apoptosis from oxidative stress. For all of the following experiments, mice were injected with 7000 4T1 tumor cells in the mammary fat pad. Mice were assayed weekly for tumor size and percentage of Gr1⁺CD11b⁺ MDSC in blood by flow cytometry.

Nrf2^{-/-} MDSC are more apoptotic than wild-type MDSC. A higher percentage of freshly isolated MDSC from Nrf2^{-/-} mice are apoptotic vs. MDSC from wild type mice (Figure 1A). In addition, wild type 4T1 tumor-bearing mice contain more MDSC in their blood vs. Nrf2^{-/-} mice (Figure 1B). These data suggest that Nrf2 expression protects MDSC from apoptosis, consistent with my hypothesis that MDSC resist apoptosis from oxidative stress by up-regulating Nrf2.

Aim 2- In Progress

Previously I had shown that tumor-bearing Nrf2^{-/-} mice live significantly longer than tumor-bearing wild type mice. Since 4T1 metastasis is the cause of death in 4T1 tumor-bearing animals [25], 4T1-bearing Nrf2^{-/-} animals may live longer due to enhanced resistance to metastasis. Resistance to metastasis requires a competent immune system [26]. MDSC are functional immune suppressors and tumor-bearer survival is negatively correlated with MDSC suppressive activity [6]. I hypothesize that Nrf2^{-/-} MDSC are less suppressive than wild type MDSC. If Nrf2^{-/-} MDSC are less suppressive, then there would be less immune suppression and could potentially allow for tumor-bearing Nrf2^{-/-} mice to survive longer because they are more resistant to metastatic disease compared to tumor-bearing wild type animals. The following data support this hypothesis. For all of the following experiments, mice were injected with 10⁵ 4T1 tumor cells in the mammary fat pad. MDSC purity for all experiments was determined by flow cytometry and was greater than 90%.

Wild type MDSC produce more H₂O₂, and are more suppressive than Nrf2^{-/-} MDSC. MDSC produce H₂O₂ and reactive nitrogen species (RNS) such as nitric oxide and peroxynitrate which suppresses T cell activation [27]. Phorbol myristate acetate (PMA) stimulated wild type MDSC produce significantly more H₂O₂ than Nrf2^{-/-} MDSC (Figure 2). Unstimulated MDSC produced very low levels of H₂O₂. This result suggests that Nrf2 may regulate MDSC production of this immune suppressive molecule. Future experiments will be conducted on MDSC induced by different tumor models or derived from C57BL/6 mice to determine if Nrf2 regulates MDSC production of H₂O₂ universally or if this observation is restricted to 4T1-induced MDSC.

Wild type and Nrf2^{-/-} MDSC do not produce reactive nitrogen species (RNS). 4T1-induced MDSC from wild type or Nrf2^{-/-} mice did not produce RNS (Figure 3), suggesting that 4T1-induced MDSC do not use RNS to suppress T cell activation. Future experiments will utilize MDSC derived from other tumor models or from C57BL/6 wild type and Nrf2^{-/-} mice to determine if Nrf2 regulates RNS production in MDSC.

Wild type MDSC are more suppressive than Nrf2^{-/-} MDSC. Wild type MDSC suppressed CD4⁺ transgenic T cells significantly more than Nrf2^{-/-} MDSC (Figure 4). MDSC suppression of CD8⁺ T cells was

inconclusive (data not shown). Future experiments will focus on determining if Nrf2 enhances MDSC suppression of CD8⁺ transgenic T cells and confirm in additional tumor models whether Nrf2 regulates MDSC suppressive activity.

Aim 3-Completed

Aim 3 was completed during the 2010-2011 report period.

Key Research Accomplishments

Training Plan

Task 1: Meet yearly with my dissertation committee to review my experimental progress in the project. **(Completed to date)**

Task 2: Participate in weekly lab meetings, journal clubs, seminars, and talks with outside speakers. **(Completed to date)**

Task 3: Meet with my mentor weekly to discuss ongoing experiments. **(Completed to date)**

Task 4: Review manuscripts related to my proposal as suggested by my mentor. **(Completed to date)**

Task 5: Complete all necessary lab work to fulfill the objectives outlined in the research proposal. **(In progress)**

Task 6: Complete coursework required by the Biological Sciences Ph.D. program. **(Completed)**

Task 7: Pass oral examination on the background of my research, present and successfully defend my research during the comprehensive preliminary/qualifying exam to pass onto Ph.D. candidacy. **(Completed)**

Task 8: Present my research at a minimum of one national conference per year. **(Completed to date, but continuing on an annual basis)**

Task 9: Write up experimental results in a timely manner for publication in peer-reviewed journals.

Task 10: Collaborate with other students and investigators to fulfill my objectives. **(In progress)**

Task 11: Serve as a teaching assistant for two semesters. **(Completed)**

Task 12: Present a departmental seminar describing my completed thesis project, and defend my Ph.D. dissertation before my dissertation committee. **(completed one departmental seminar on work, in progress)**

Task 13: Locate a suitable post-doctoral position for continuation of my training.

Milestones and Deliverables:

1. Completion of my preliminary/qualifying exam. **(Completed)**
2. Completion of required coursework to fulfill the Biological Sciences Ph.D. program. **(Completed)**
3. Complete two semesters as a teaching assistant. **(Completed)**
4. Present my first oral presentation at a national conference.
5. Have my thesis research published in well-respected, peer reviewed journals.
6. Successfully defend my Ph.D. dissertation.
7. Obtain an appropriate and well-regarded post-doctoral position.

Task 1: Determine if Nrf2 regulates MDSC survival. (In Progress)

Task 1A: Determine the rate of cell death of Nrf2^{-/-} MDSC compared to wild type MDSC. **(Completed)**

Task 1B: Determine if Nrf2 regulates GSH levels and MDSC apoptosis in response to oxidative stress.

Task 1C: To determine if GSH regulates apoptosis in MDSC.

Task 1D: Determine if tumor MDSC more susceptible to apoptosis than blood MDSC.

Task 1E: Determine if Nrf2 protects MDSC from the oxidative tumor microenvironment.

Task 1F: Determine if Nrf2 protects MDSC from hypoxia.

Outcomes/Products/Deliverables: Nrf2 enhances MDSC resistance to apoptosis.

Task 2: Determine if Nrf2 regulates tumor-bearer survival and MDSC suppressive activity.

Task 2A: Determine if Nrf2 regulates ROS, NO, and peroxynitrite production in MDSC. **(In Progress)**

Task 2B: Determine if Nrf2 regulates the suppressive activity of MDSC. **(In Progress)**

Task 2C: Determine if Nrf2 regulates MDSC accumulation and mammary tumor growth. **(Completed)**

Outcomes/Products/Deliverables: Nrf2 decreases MDSC oxidative stress and increases MDSC production of ROS, MDSC suppressive activity, and survival of tumor-bearing mice. Nrf2 increases MDSC accumulation in tumor-bearing mice. MDSC do not produce reactive nitrogen species.

Task 3: Determine if inhibition of MDSC sequestration of cysteine (via xCT) reduces MDSC accumulation, restores immune competence, delays metastatic disease, and increases survival time. **(Completed)**

Task 3A: Determine if SASP and NAC reduce MDSC production of ROS, NO, peroxynitrite, GSH levels, cystine transport, and reduce MDSC resistance to Fas-mediated apoptosis and suppressive activity. **(Completed)**

Task 3B: Determine if SASP and NAC affect MDSC accumulation and mammary tumor growth. **(Completed)**

Outcomes/Products/Deliverables: SASP reduces MDSC viability, GSH content, and cystine transport *in vitro*. There is no difference between inflammation-induced and conventional MDSC transport of cystine. SASP has no effect on tumor growth, metastatic disease, MDSC accumulation, or MDSC suppressive activity.

Reportable Outcomes

Milestones and Deliverables:

- Completed my preliminary/qualifying exam.
- Confirmed that Nrf2 regulates MDSC accumulation.

Presentations:

- Daniel W. Beury, Cassandra Nelson, Suzanne Ostrand-Rosenberg “Transcription Factor Nrf2 (NF-E2 Related Factor 2) Enhances Myeloid-Derived Suppressor Cell (MDSC) Accumulation and Tumor Progression” American Association of Immunologists 99th Annual Meeting. Boston, MA. May 4-8, 2012 (poster presentation)
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg “Communication among tumor-infiltrating immune cells enhances tumor-progression” UMBC Biological Sciences Departmental Seminar, Nov 28th, 2012 (oral presentation)
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg, “Anti-inflammatory effects of Myeloid-Derived Suppressor Cells and Macrophage crosstalk contribute to tumor progression” American Association for Cancer Research Annual Meeting. Washington, DC. April 6-10, 2013 (abstract submitted for oral and poster presentation)
- Daniel W. Beury, Katherine H. Parker, Suzanne Ostrand-Rosenberg, “Anti-inflammatory effects of Myeloid-Derived Suppressor Cells and Macrophage crosstalk contribute to tumor progression” American Association of Immunologists 100th Annual Meeting. Honolulu, Hawaii. May 3-7, 2013 (abstract submitted for oral and poster presentation)

Conclusions to Date

- It has been demonstrated that Nrf2 regulates oxidative stress in MDSC and MDSC apoptosis. Research to ascertain which proteins downstream of Nrf2 mediate MDSC oxidative stress and apoptosis would provide novel targets for future therapies aimed at reducing MDSC levels in tumor-bearing patients for enhancement of immunotherapeutic strategies of targeting cancer.
- It has been shown that Nrf2 increases MDSC accumulation in tumor-bearing animals. Nrf2 does not affect primary tumor growth, but does reduce survival in tumor-bearing animals. It has also been shown that Nrf2 enhances MDSC suppressive mechanisms and MDSC suppressive activity. Research to ascertain the mechanisms of Nrf2's pro-tumor activity and enhancement of MDSC suppressive activity would provide novel pathways to increase anti-tumor immunity.
- It has been demonstrated that inflammation enhances xC_C expression on MDSC, but higher xC_C expression does not enhance the ability of MDSC to transport cystine. *In vitro*, SASP inhibits cystine transport, reduces intracellular GSH, and increases cell death in MDSC. However, therapeutic administration of oral sulfasalazine to tumor-bearing animals has no effect on primary tumor growth, MDSC accumulation, metastatic disease, or MDSC suppressive activity. Therefore, SASP is a poor candidate for treatment of tumor-bearing individuals.

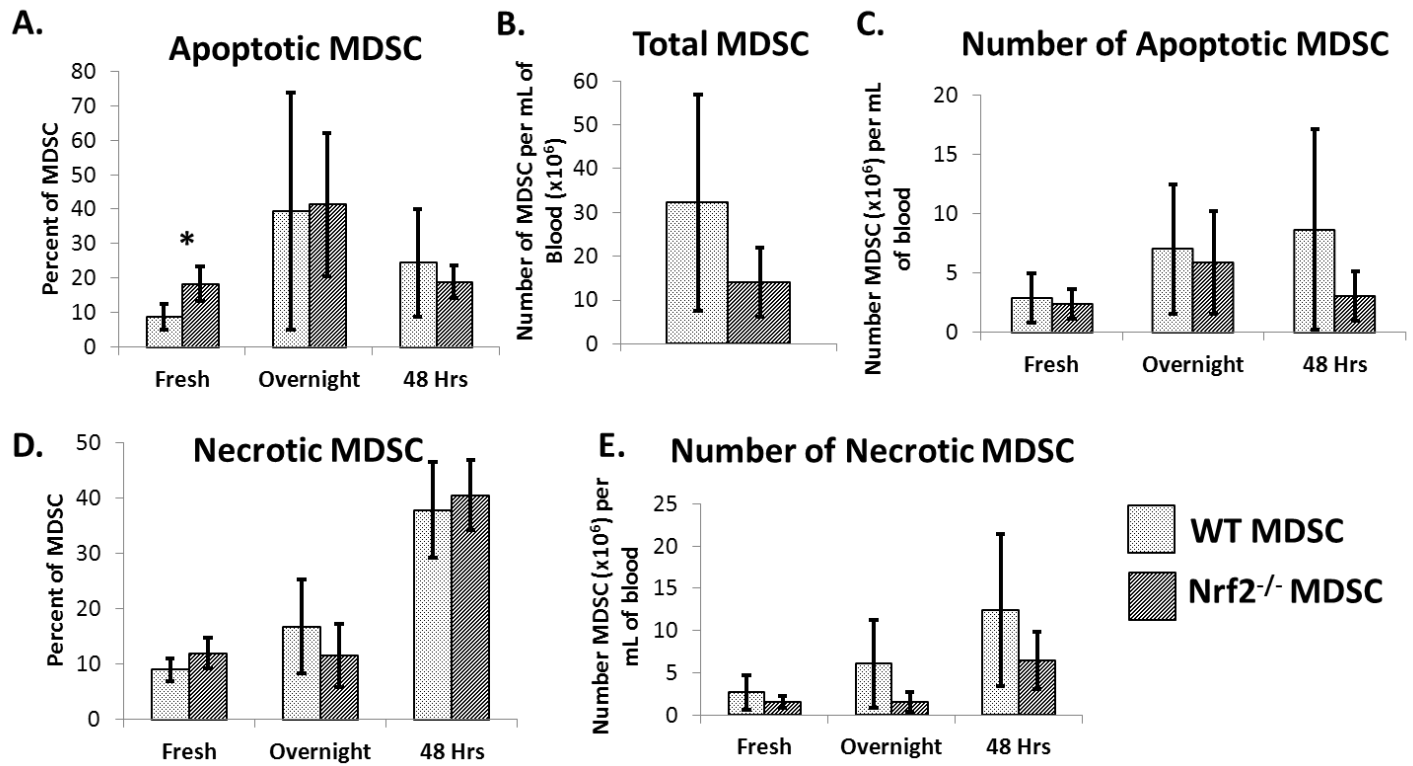
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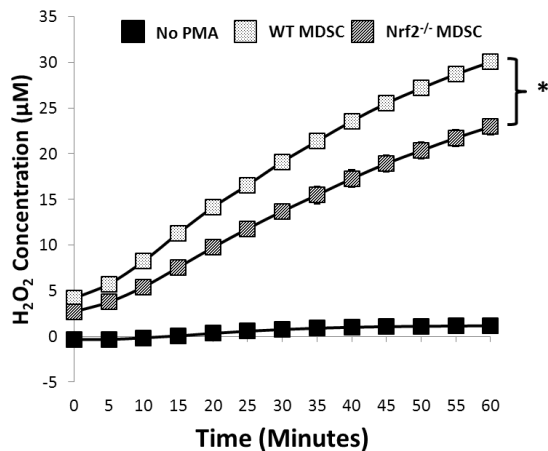
Supporting Data

Figure 1: *Nrf2*^{-/-} MDSC are more apoptotic than wild type MDSC.



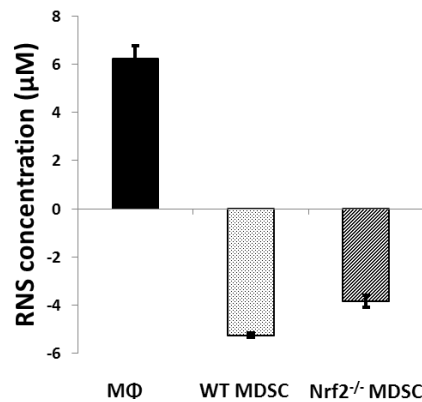
MDSC were isolated from the blood of six wild type and *Nrf2*^{-/-} tumor-bearing mice. The volume of blood collected was measured by pipetting. Cells were resuspended in 4T1 tumor cell-conditioned media to enhance cell viability, plated in untreated petri dishes, and incubated at 37°C for the indicated time. Cells were stained for Gr1 and CD11b and with Annexin V and PI and analyzed by flow cytometry. MDSC were identified as Gr1⁺CD11b⁺ cells and were classified as apoptotic (Annexin V⁺, PI⁻) or necrotic (Annexin V⁺, PI⁺). Data was analyzed for significance using a *t*-test (**p*<.05). A and D: The percentage of apoptotic (A) and necrotic (D) MDSC analyzed at the specified time points. B: The total number of MDSC per mL of blood as calculated by [(1mL/collected blood volume) x (number of cells collected) x (percentage of MDSC in blood)]. C and E: The total number of MDSC of apoptotic or necrotic MDSC per mL of blood was calculated by (total number of MDSC per mL of blood) x (percentage of apoptotic or necrotic MDSC). The total number of apoptotic (C) or necrotic (E) analyzed at the specified time points.

Figure 2: Wild type MDSC produce more H₂O₂ than Nrf2^{-/-} MDSC.



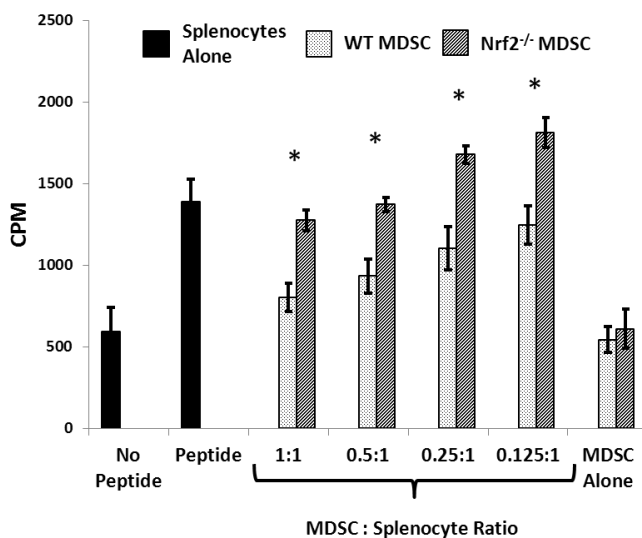
Wild type and Nrf2^{-/-} MDSC were plated and stimulated with PMA. Unstimulated wild type MDSC were included as a negative control. H₂O₂ production was measured over time using an Amplex Red Hydrogen Peroxide Assay (Invitrogen). Data were analyzed using Mann-Whitney test (*p=.007). Data is representative of one of two independent experiments.

Figure 3: MDSC do not produce reactive nitrogen species (RNS)



Macrophages (MΦ), wild type, and Nrf2^{-/-} MDSC were plated and stimulated for 16hrs with LPS and IFNγ. Supernatants were analyzed for RNS by a Griess test. Data is representative of one of two independent experiments.

Figure 4: Nrf2^{-/-} MDSC are not suppressive.



DO11.10 splenocytes were plated with or without wild type or Nrf2^{-/-} MDSC. Cells were stimulated with DO11.10 cognate peptide and after 3 days were pulsed for 24hrs with ³[H]-Thymidine and lysed on nylon membranes. T-cell activation was measured as a function of radio-label incorporation via scintillation counting. Wild type and Nrf2^{-/-} MDSC with splenocytes were analyzed by *t*-test. All Nrf2^{-/-} MDSC:Splenocyte ratios were significantly different than wild type counterparts (*p<.005).