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## Nitric Oxide Production by Myeloid Derived Suppressor Cells Plays a Role in Impairing Fc Receptor-Mediated Natural Killer Cell Function.

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### Abstract

**Purpose:** Monoclonal antibodies (mAb) are used to treat solid and hematological malignancies, and work in part through Fc receptors (FcR) on natural killer cells (NK). However, FcR mediated functions of NK cells from cancer patients are significantly impaired. Identifying the mechanisms of this dysfunction and impaired response to mAb therapy could lead to combination therapies and enhance mAb therapy.

**Experimental Design:** Co-cultures of autologous NK cells and MDSC from cancer patients were used to study the effect of MDSC on NK cell FcR mediated functions including antibody dependent cellular cytotoxicity, cytokine production, and signal transduction *in vitro*. Mouse

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Conflict of interest

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breast cancer models were utilized to study the effect of MDSC on antibody therapy *in vivo* and test the efficacy of combination therapies including a mAb and a MDSC targeting agent.

**Results:** Cancer patient MDSC were found to significantly inhibit NK cell FcR mediated functions including ADCC, cytokine production, and signal transduction in a contact independent manner. In addition, adoptive transfer of MDSC abolished the efficacy of mAb therapy in a mouse model of pancreatic cancer. Inhibition of iNOS restored NK cell functions and signal transduction. Finally, non-specific elimination of MDSC or inhibition of iNOS *in vivo* significantly improved the efficacy of mAb therapy in a mouse model of breast cancer.

**Conclusions:** MDSC antagonize NK cell FcR mediated function and signal transduction leading to impaired response to mAb therapy in part through nitric oxide production. Thus, elimination of MDSC or inhibition of nitric oxide production offers a strategy to improve mAb therapy.

### Keywords

MDSC; NK; Nitric oxide; immunotherapy; Fc receptor

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### Introduction

Natural killer (NK) cells are large granular lymphocytes that participate in innate immune responses against virus infected and neoplastic cells via receptors for major histocompatibility antigens, markers of cellular stress (NKG2D) and immunoglobulin G (FcR) (1). NK cells are unique in that they constitutively express only a low-affinity, activating FcR (FcγRIIIa or CD16), enabling them to recognize antibody (Ab)-coated targets (1,2). FcR-activated NK cells can kill antibody coated targets and secrete cytokines such as IFN-γ and chemokines (e.g., RANTES and MIP-1α) that inhibit tumor cell proliferation, enhance antigen presentation and promote the chemotaxis of T cells (3). These properties of FcR-activated NK cells are an important component of the response to mAb therapy (4).

NK cells are an important component of immune surveillance against the development of malignancies and the control of established tumors. NK cell infiltration of established tumors is associated with improved disease prognosis (5,6). However, NK cells from cancer patients have reduced cytotoxic function and cytokine production (7). The cause of this dysfunction is not fully understood, but has been attributed to factors secreted by or expressed on the surface of tumor cells (8,9). Only a few studies have examined the role of immune suppressive cells in mediating NK cell dysfunction (10,11) in the setting of cancer.

MDSC are immature myeloid cells with immunosuppressive properties that expand in response to tumor and stroma-derived factors (12). The frequency of circulating MDSC correlates with tumor burden and has prognostic value (13). In mice, MDSC are identified by expression of Gr-1 and CD11b and in humans they are characterized as CD33<sup>+</sup>/CD11b<sup>+</sup>/HLA-DR<sup>low/neg</sup> (12). MDSC inhibit T-cells by a number of mechanisms including the production of free radicals, expression of amino acid catabolizing enzymes and the secretion of suppressive cytokines (14). Studies in murine models indicate that disruption of MDSC function can improve anti-tumor immune responses and impair tumor growth (15). Given

the ability of MDSC to suppress anti-tumor immune responses, they have received significant interest as a potential biomarker and therapeutic target (16).

Studies have shown that MDSC impair NK cell cytokine production and MHC I dependent cytotoxicity through contact dependent mechanisms (e.g. checkpoint ligand expression and reactive oxygen species production) and contact independent mechanisms (arginase and TGF- $\beta$  production) (10,11,17–21). However, the impact of MDSC on NK cell FcR-mediated function is unknown. Given the prominence of antibody therapy in the treatment of cancer, elucidation of the effects of MDSC on FcR-mediated NK cell function could have important clinical implications. In particular, this approach could identify novel mechanisms of resistance to mAb therapy.

It was hypothesized that MDSC could inhibit FcR-mediated NK cell functions and antagonize monoclonal antibody therapy. Utilizing MDSC and autologous NK cells from cancer patients and murine models of mAb therapy we show that MDSC nitric oxide (NO) production inhibits NK cell FcR-mediated signal transduction and downstream effector functions including ADCC, cytokine production and anti-tumor activity *in vivo*. To our knowledge, this is the first report to demonstrate that MDSC-derived NO can antagonize NK cell FcR function and blunt the response to antibody therapy.

## Results

### MDSC Inhibit NK Cell Cytotoxicity and Antibody Dependent Cellular Cytotoxicity

Given the ability of MDSC to interfere with anti-tumor immune responses it was hypothesized that MDSC could inhibit the antibody dependent cellular cytotoxicity (ADCC) function of NK cells. Autologous MDSC inhibited MHC I dependent NK cell cytotoxicity (K562 cytotoxicity assay), which was dose dependent while PBMC had no effect (Figure S1A and S1B). The effect of MDSC on NK cell ADCC was tested next. MDSC isolated from the peripheral blood of patients with melanoma (Figure 1A and S1C), head and neck squamous cell carcinoma (HNSCC, Figure 1B), and breast cancer (Figure 1C) all inhibited NK cell ADCC function. MDSC inhibition of NK cell ADCC reached statistical significance in the study of four melanoma patients while PBMC had no effect (Figure 1A and S1C). Again, the ability of MDSC to inhibit NK cell ADCC was dose dependent (Figure S1D). A phenotype of CD33<sup>+</sup>, CD11b<sup>+</sup>, and HLA-DR<sup>low</sup> was used to identify MDSC from melanoma patient blood draws. Before isolation the frequency of MDSC was 33.15% amongst total PBMC and after isolation the frequency was 76% representing a 2.3 fold enrichment (Figure S2A and S2B). Isolated MDSC were able to suppress T cell proliferation (Figure S2C).

### MDSC Inhibit Cytokine Production by FcR-Activated NK Cells

NK cells are an important source of cytokines, such as IFN- $\gamma$ . Our group has shown *in vitro* and in phase I clinical trials that co-stimulation of NK cells via the Fc $\gamma$ RIIIa and cytokines is a potent stimulus for the production of IFN- $\gamma$  and chemokines such as RANTES and MIP-1 $\alpha$  (22). Therefore, the effect of MDSC on NK cell cytokine production was examined. Co-culture of autologous MDSC and NK cells from melanoma patients significantly

inhibited the production of IFN- $\gamma$ , whereas PBMC did not (Figure 1D,  $p < 0.05$  and Figure S3A). This held for FcR-stimulated NK cells cultured with IL-12 (Figure S3B). MDSC inhibition of IFN- $\gamma$  production was dose dependent, and a time course experiment showed this effect was observable at 24 hours with maximal inhibition at 48 hours (Figure S3C and S3D). Co-culture of NK cells with autologous MDSC also significantly decreased the production of MIP-1 $\alpha$  (Figure S3E,  $p < 0.01$ ).

### MDSC inhibit FcR Mediated Signal Transduction

Erk activation is critical to NK cell FcR mediated effector functions and natural cytotoxicity (K562 killing). Given the impairment of these NK cell functions in the presence of MDSC it was hypothesized that impaired Erk activation could lead to reduced NK cell FcR-mediated functions following co-culture with MDSC (23). NK cells were stimulated via the FcR using the 3G8 anti-CD16 antibody and a cross-linking F(ab')<sub>2</sub> fragment. Measurement of p-Erk levels in CD56<sup>+</sup> NK cells showed that co-culture of melanoma patient NK cells and MDSC resulted in a 40% decrease in p-Erk levels (Figure 1E,  $p < 0.05$  and representative dot plot Figure S4). When NK cells were physically separated from MDSC levels of p-Erk in response to FcR stimulation were inhibited by an average of 28.3% (Figure 1F,  $p < 0.05$ ). When these cells were in direct contact, there was a small increase in the level of inhibition in comparison to the contact independent condition (Figure 1F). This result suggests that MDSC inhibition of NK cell FcR-mediated signal transduction relies on diffusible substances with the potential for an additional contact dependent mechanism to play a role.

### Inhibition of Nitric Oxide Production Enhances NK cell FcR Mediated Function.

MDSC can promote immune suppression through several contact independent mechanisms including expression of amino acid catabolizing enzymes, immune suppressive cytokines, and production of nitric oxide (NO). To investigate the role of these factors in suppressing FcR-mediated NK cell function, mice bearing 4T1 tumors were treated with neutralizing anti-IL-10 (24) or anti-TGF- $\beta$  (25) antibodies, or inhibitors targeting 2,3-indolamine dioxygenase (IDO) (26), arginase (27), or inducible nitric oxide synthase (iNOS). NK cells were isolated from the spleen and used in ADCC assays against trastuzumab-coated CT26 cells expressing human HER2. Only inhibition of iNOS and arginase rescued NK cell ADCC activity (Figure 2A–C). Arginase and iNOS both use arginine as a substrate and MDSC express high levels of both enzymes. This suggests that the arginase/iNOS arginine catabolism pathway in MDSC plays an important role in regulating NK cell function, and that manipulation of either pathway could impact NK cell function. The arginase inhibitor produced a reduction in splenic MDSC frequency suggesting that the enhanced NK function in this group could reflect reduced MDSC accumulation (Figure S5A–C). Alternatively, as both arginase and iNOS utilize arginine as a common substrate, and arginine availability has been linked to NK cell function, inhibition of either enzyme could improve NK cell function. If this was the case one could speculate that simultaneous inhibition of both enzymes would dramatically rescue NK cell function. However, when this was tested the inhibition of both enzymes was no more effective at rescuing NK cell function than inhibition of either enzyme alone (Figure S5D). Together, these results suggest that the NO-arginase arginine catabolism pathway plays an important role in the regulation of NK cell FcR-mediated functions and that iNOS is an important mediator of MDSC inhibition of NK

cell function. However, since it can be hard to draw firm conclusions from negative data obtained using neutralizing antibodies and chemical inhibitors, we concede that factors in addition to N.O. could still play an important role in the inhibitory effects of MDSC.

### **Production of Nitric Oxide by MDSC Is Involved in the Suppression of NK Cell Function**

To investigate the role of NO in MDSC inhibition of FcR-mediated NK cell functions, *NOS2* expression by MDSC was measured. MDSC expressed high levels of *NOS2* compared to NK and T cells (Figure S6A). NO production by MDSC was detected by intracellular flow cytometry using a NO-sensitive dye 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM) (Figure S6B), and the iNOS inhibitor L-NIL significantly inhibited NO production as measured by the Greiss reagent (Figure S6C,  $p < 0.05$ ).

It was hypothesized that L-NIL could rescue FcR-mediated NK cell functions in the presence of MDSC. Melanoma patient NK cells and MDSC were co-cultured in the presence or absence of L-NIL, and L-NIL was found to rescue NK cell ADCC (Figure 2D,  $p < 0.01$ ). These effects were also observed when examining the natural cytotoxicity function of NK cells (Figure S6D). Similarly, L-NIL rescued IFN- $\gamma$  production and Erk activation in the presence of MDSC (Figures 2E and 2F,  $p < 0.05$ ). When NK cells and MDSC were separated by a permeable membrane L-NIL still rescued the generation of p-Erk supporting a predominant contact independent mechanism (Figure S6E).

### **Nitric Oxide Is a Negative Regulator of NK Cell Function**

To examine the effect of NO on NK cell function, NK cells from normal donors were incubated with a NO donor, S-Nitroso-N-Acetyl-Penicillamine (SNAP, 0.1 mM) or DMSO overnight in the presence or absence of IL-12. SNAP significantly inhibited the ability of NK cells to lyse cetuximab-coated HT-29 colon cancer cells (Figure 3A and 3B,  $p < 0.001$ ). Similarly, SNAP significantly reduced the production of IFN- $\gamma$  (regardless of the presence of IL-12) (Figure 3C,  $p < 0.05$  and Figure S7A). SNAP also inhibited Erk activation in FcR-stimulated NK cells (Figure 3D,  $p < 0.05$ ). Importantly, SNAP was not toxic to NK cells as the viability of SNAP-treated NK cells was  $> 90\%$  as measured by flow cytometry and trypan blue exclusion (Figure 3E, 3F and Figure S7B). In addition, SNAP did not affect the total number of NK cells remaining after treatment (Figure S7C). Finally, SNAP produced levels of NO that were equivalent to that produced by human MDSC (data not shown). Together, these results suggest that NO can be a negative regulator of NK cell FcR function.

### **Exposure to Nitric Oxide Leads to Nitration of Proteins in NK Cells**

Given the ability of MDSC to inhibit Erk activation in NK cells and the ability of L-NIL to rescue this function it was hypothesized that impaired Erk activation could be caused by nitration of signaling molecules downstream of the FcR. To investigate this, NK cells were treated with SNAP or DMSO, probed with anti-nitrotyrosine (NT) and anti-CD16 (FcR) antibodies and analyzed by flow cytometry. SNAP treatment resulted in significant nitration of tyrosine residues on CD16<sup>+</sup> NK cells compared to DMSO (Figure 4A,  $p < 0.05$ ). Co-culture of MDSC with NK cells also caused a significant increase in the tyrosine nitration of CD16<sup>+</sup> NK cells (Figure 4B,  $p < 0.01$ ). Representative flow cytometry dot plots showing the

change in nitrotyrosine positive NK cells in the above two experiments is provided in Figure S8. Treatment of human NK cells with SNAP or co-culture with MDSC had no effect on CD16 expression (data not shown). Next, nitrotyrosine residues were immuno-precipitated from DMSO or SNAP-treated NK cells and probed for Erk. Immunoblot analysis showed that Erk was nitrated in SNAP treated NK cells (Figure 4C). This suggests that MDSC derived NO results in the nitration of tyrosine residues on signaling proteins like Erk as one mechanism by which MDSC inhibit FcR-mediated NK cell functions. With respect to the production of NO by murine MDSC, our group has previously used immunohistochemistry to show that large amounts of NO are produced by MDSC present in the spleens of tumor-bearing mice (28). Also, it has been shown in the literature that murine MDSC produce significant quantities of NO (12,29).

### Depletion of MDSC Improves NK Cell ADCC Function in an Animal Model

Chemotherapeutic agents 5-fluorouracil (5-FU) and gemcitabine deplete MDSC in tumor bearing mice (30). Therefore we investigated whether the elimination of MDSC from tumor bearing mice could augment NK cell function. NK cells were isolated from the spleen of mice with 4T1 mammary carcinomas 24 hours after treatment with 5-FU (50 mg/kg), gemcitabine (80 mg/kg) or PBS and used in an ADCC assay against trastuzumab coated CT26-HER2 cells. NK cells from non-tumor bearing mice demonstrated cytotoxic activity in the range of 40% at the 25:1 E:T ratio, whereas NK cells from the PBS group exhibited cytotoxic activity of just 10%. Gemcitabine and 5-FU eliminated MDSC from the spleens of tumor bearing animals and this was associated with NK cell ADCC function in the range of 30% lysis (Figure 5A and 5B and Figure S9A and S9B). In contrast to other reports, treatment with an anti-Gr-1 antibody only modestly reduced MDSC frequency and this correlated with a slight increase in NK cell ADCC function (Figure S9C and S9D). Together, these results demonstrate that MDSC in the spleen can impair NK cell function and their elimination can augment NK cell ADCC.

### Inhibition of NO *In Vivo* Enhances NK Cell-Mediated ADCC Activity

Next, it was investigated if inhibition of iNOS could augment NK cell function *in vivo*. Mice bearing 4T1 tumors were treated daily with PBS or the iNOS inhibitor L-NIL (20 mg/kg) for one week (31). NK cells isolated from the spleen were used in an ADCC assay against trastuzumab-coated CT26-HER2 cells. Treatment with L-NIL significantly improved the ADCC function of NK cells (Figure 5C–5D,  $p < 0.05$ ). As seen in figure S9E, there was minimal difference in the frequency of MDSC between the PBS and NIL-treated groups.

Nitric oxide generated by cancer cells has been postulated to dampen anti-tumor immune responses (32,33). To delineate the role of tumor versus MDSC-derived NO, the above experiment was repeated using *Nos2*<sup>-/-</sup> deficient mice. NK cells from B16F10 tumor-bearing *Nos2*<sup>-/-</sup> mice demonstrated normal ADCC activity compared to NK cells from wild type B16F10 tumor-bearing mice (Figure 5E). These results imply that host-derived and not tumor-derived NO is involved in the regulation of MDSC cell function in this tumor model.

### Depletion of MDSC Augments Trastuzumab Therapy *In Vivo*

Monoclonal antibodies (mAb) like trastuzumab are widely used in the treatment of cancer and there is evidence that NK cells participate in their efficacy (34,35). Therefore, it was hypothesized that MDSC could antagonize mAb therapy. To this end, we employed a well-characterized EMT6 mouse model of mammary carcinoma that overexpresses human HER2 (EMT6-HER2) and responds to Ab therapy in an NK cell-dependent manner (36). Importantly, EMT6-HER2 cells show similar growth kinetics as EMT6 parental cells and drive the expansion of MDSC (unpublished data). Once tumors were palpable (~50 mm<sup>3</sup>) mice were treated with PBS/IgG, 5-FU (MDSC-depleting agent), trastuzumab or 5-FU followed by trastuzumab. Tumors of mice receiving 5-FU alone or 5-FU in combination with trastuzumab exhibited a significant decrease in MDSC (Figure S10A and S10B). There was only a modest reduction in tumor volume in mice treated with trastuzumab compared to the PBS/IgG-treated control group as might be expected in a model in which the MDSC population is so prominent. Mice treated with 5-FU showed a reduction in tumor volume compared to the PBS/IgG-treated group. Importantly, 5-FU markedly enhanced the effectiveness of trastuzumab compared to either agent alone (Figure 6A,  $p < 0.01$  and  $p < 0.001$ , respectively). It is possible that 5-FU had a direct antitumor effect in this tumor model, but IHC staining for Ki67 and caspase3 showed no difference in proliferation or apoptosis between these treatment groups (Figure S11). These results are consistent with previous studies showing 5-FU did not induce apoptosis of tumor cells or affect the prevalence of immune cells (NK and T cells) within tumors (37). We propose that one major effect of 5-FU on tumor growth is due to its actions on the MDSC compartment.

### Inhibition of MDSC NO Production Enhances Trastuzumab Therapy *In Vivo*

Next the effect of NO inhibition on the response to trastuzumab was examined. EMT6-HER2 tumor bearing mice were treated with PBS/IgG, L-NIL, trastuzumab or L-NIL followed by trastuzumab. No difference in tumor volume was observed between the PBS/IgG and L-NIL treated groups, and trastuzumab modestly reduced tumor growth (Figure 6B). However, there was a significant decrease in tumor volume when mice received NIL in combination with trastuzumab ( $p < 0.005$ , Figure 6B). While there was a slight reduction of splenic MDSC in treatment groups compared to the control, the frequency of tumor infiltrating MDSC was similar indicating the observed differences in splenic MDSC frequency were likely driven by group differences in tumor volume (Figure S12). Importantly, the enhanced anti-tumor effect of the combination of trastuzumab and L-NIL was found to be NK cell dependent as depletion of NK cells with anti-asialo GM1 rescued tumor growth even in the setting of combination therapy (Figure 6C and S13A).

### Transfer of MDSC Inhibits mAb Therapy

To confirm the ability of MDSC to antagonize mAb therapy of cancer, a MDSC adoptive transfer experiment was performed. Athymic nude mice were inoculated with a pancreatic cancer cell line that overexpresses human HER1 (Panco2-EGFR). The parental and HER1 expressing cell lines grow at identical rates in this mouse strain and there is minimal expansion of MDSC (unpublished data). Following the establishment of tumors, mice were injected with PBS (mock injection), splenocytes from non-tumor bearing mice, or MDSC

from syngeneic mice bearing 4T1 mammary carcinoma tumors on day seven prior to the initiation of IgG or cetuximab therapy on day 9. A second round of cell injections was conducted on day 14 (schema shown in Figure S13B). Mice receiving splenocytes and cetuximab showed a significant reduction in tumor volume compared to the control group. In contrast, MDSC ablated the effect of cetuximab therapy (Figure 6D and 6E,  $p < 0.05$ ).

In order to elucidate the role of NO production in the inhibitory effects of transferred MDSC a similar study was conducted using MDSC from *nos2*<sup>-/-</sup> deficient mice. As before, injection of wild type MDSC abolished the anti-tumor effect of cetuximab therapy. However, *nos2*-deficient MDSC were less antagonistic of cetuximab (Figure S13C). Taken together, these results demonstrate that MDSC can dampen the anti-tumor response of mAb therapies and that nitric oxide production appears to play an important role in mediating this effect.

## Discussion

The current study provides evidence for a model in which MDSC inhibit FcR-mediated NK cell actions against antibody-coated tumor cells through the production of NO. The results suggest that nitration of NK cell proteins on tyrosine residues resulted in impaired signal transduction and activation of NK cells following FcR stimulation. Elimination of MDSC from tumor bearing hosts or inhibition of NO production restored NK cell effector functions and improved the anti-tumor effect of mAb therapy *in vivo*. Finally, transfer of wild-type MDSC significantly antagonized mAb therapy. The results reported provide a mechanism by which MDSC-derived NO leads to impaired FcR mediated NK cell function and reduced efficacy of mAb therapy for cancer.

NK cells play an important role in the response to mAb therapy through their ability to recognize and kill antibody coated tumor cells via engagement of the low affinity activating Fc $\gamma$ RIIIa (FcR or CD16) and inhibiting metastatic spread of cancer. As a result NK cells have received attention for their potential therapeutic and prognostic value (38). In both solid and hematological malignancies, alterations in the frequency, maturity, and expression of activating and inhibitory receptors by NK cells have been reported which correspond with impaired NK cell effector functions (39). While the mechanisms of this dysfunction are incompletely understood, reports have begun to explore these observations by analyzing the interaction between NK cells and cells with immune suppressive function including MDSC (10,11,18,19,21,40–42).

The present study provides evidence that MDSC impair NK cell FcR-mediated functions via the production of NO and the nitration of protein tyrosine residues. Our group has previously shown that MDSC-derived NO results in nitration of key tyrosine residues on the STAT1 transcription factor resulting in impaired NK and T cell response to interferon stimulation (28). Other groups have shown that MDSC-derived RNS can cause nitration of the T cell receptor, thus impairing its interaction with the peptide major histocompatibility complex (43). In addition, RNS have been shown to cause post-translational modification of chemokines resulting in impaired T cell trafficking within tumors (44). Finally, tumor-derived NO has also been shown to promote immune dysfunction. Co-culture of PBMCs with melanoma cell lines expressing high levels of *NOS1* resulted in tyrosine nitration of T



cells and monocytes. The present findings that MDSC-derived NO resulted in the nitration of Erk and impaired FcR-mediated NK cell function are the first to explore the effects of MDSC on the NK cell mediated response to anti-tumor mAb therapy. These results further support the ability of NO to mediate immune suppression and negatively impact mAb-based treatment of cancer.

NK cells also appear to have a role in preventing metastatic spread of cancer and in the control hematologic malignancies. Notably, there are reports showing that immune-suppressive MDSC are increased in patients with leukemia (45). Our results showing that MDSC can inhibit NK cell natural cytotoxicity suggest that MDSC could promote metastasis through impaired NK cell function. Indeed, investigators have shown that MDSC promote metastasis in the setting of breast cancer (46), but to date no report has implicated MDSC inhibition of NK cell function in this process.

Erk is rapidly phosphorylated in primary human NK cells following FcR stimulation and this is critical to NK cell cytokine production and antibody dependent cellular cytotoxicity (23,47). Erk phosphorylation in FcR stimulated NK cells was significantly inhibited by MDSC, but this could be rescued in the presence of an iNOS inhibitor. Treatment of NK cells with the NO donor SNAP resulted in the nitration of Erk on tyrosine residues suggesting that this is one mechanism by which MDSC inhibit Erk activation. Recently, it was identified that MDSC can inhibit p-Erk generation in NK cells through the inhibitory TIGIT receptor on NK cells (17). Analysis of NK cells and MDSC from patients used in this work confirmed expression of these molecules. However, the ability of the iNOS inhibitor L-NIL to rescue p-Erk generation in NK cells cultured in direct contact with MDSC suggests that TIGIT may play an alternative role in this system.

Few studies have investigated the relationship between NO and NK cell function. Earlier studies revealed that autocrine production of NO by NK cells could have a positive effect on NK cell function, and that human NK cells appear to express endothelial nitric oxide synthase but not inducible nitric oxide synthase (48,49). The amount of NO produced by activated NK cells is reported to be in the nanomolar range, which is substantially lower than the 2-20  $\mu$ M range of NO produced by MDSC. This suggests that exposure to higher levels of NO produced by MDSC is detrimental to NK cell activation and function.

In addition to producing NO, MDSC mediate immune suppression through expression of arginase and 2,3-indoleamine dioxygenase (IDO) and inhibitory cytokines (TGF- $\beta$  and IL-10) (12). Treatment of tumor bearing mice with neutralizing antibodies against TGF- $\beta$  and IL-10 or the IDO inhibitor 1-methyl-D-tryptophan did not improve NK cell ADCC. Nevertheless, based on studies in liver and lung cancer models, it is likely that these immune suppressive molecules can contribute to impaired NK cell function *in vivo* in other murine models and in humans (19). Notably, the arginase inhibitor nor-NOHA modestly improved NK cell ADCC function. This finding is consistent with reports showing that depletion of L-arginine results in impaired NK cell function (50). Arginase and iNOS utilize L-arginine as a substrate and MDSC are known to express high levels of arginase and iNOS (12). As a result, inhibition of either could increase the availability of arginine and decrease NO generation resulting in improved NK cell function.

The presence of immune suppressive cells like MDSC in the tumor microenvironment can be a major impediment to immune-based therapies (51). There are over a dozen mAb approved by the FDA for the treatment of cancer. These agents appear to function in part through the activation of NK cells (4). The finding that MDSC can inhibit FcR-mediated activation and function of NK cells suggests that eliminating MDSC or inhibiting their immune suppressive function could also enhance mAb therapy. Recently sunitinib was found to reduce the frequency of monocytic MDSC and their expression of arginase (52). In addition, imatinib and dasatinib were found to reduce the frequency of suppressive myeloid cells and increase the frequency of NK cells (53). Our group recently demonstrated that ibrutinib, a Bruton's tyrosine kinase inhibitor, reduced MDSC in tumor bearing mice and MDSC NO production (54). These results suggest that combining antibody therapy with kinase inhibitors active against MDSC could be an effective strategy. In general, strategies to deactivate or deplete MDSC and other immune suppressive cells could enhance the efficacy of immune-based therapies.

In summary, this report provides experimental evidence for an MDSC-mediated mechanism of action for impairment of FcR-mediated NK cell function. MDSC produce NO and are associated with inhibition of NK cell ADCC, cytokine production, FcR signal transduction and response to mAb therapy. These results suggest that mAb therapy in combination with agents targeting MDSC could be a successful therapeutic strategy and provide a rationale for the development of clinical trials to test such combinations.

## Material and Methods

### Cell lines.

EMT6, 4T1, K562, HT-29, and Raw 264.7 cells were purchased from ATCC and maintained under the recommended cell culture conditions. Panco2-EGFR<sup>+</sup> cells were generated as previously described (55). CT26-HER and EMT6-HER2 cells were gifted from the laboratory of Dr. P.T.P. Kaumaya (Ohio State University, Columbus, Ohio, USA). Cells were cultured in RPMI, IMDM, or DMEM with 10% FBS and 1% antibiotic-antimycotic.

### NK and MDSC Isolation

Peripheral blood from cancer patients was obtained at the Ohio State University Comprehensive Cancer Center under Institutional Review Board approved protocols (OSU IRB Nos. 1999C0348, 2004C0096 and 2010C0036, respectively). NK cells and MDSC were isolated by 30-minute incubation with the NK or myeloid cell enrichment cocktail (Stem Cell Technologies, Vancouver, BC) followed by Ficoll-Paque centrifugation (GE healthcare). For MDSC, cells were further selected using anti-HLA-DR magnetic beads for 15 minutes at 4°C and isolated using a MS-MACS column (54).

### ADCC Assay

Autologous NK cells and MDSC were co-cultured overnight with or without 2.5 mM L-NIL. Healthy donor NK cells were treated with DMSO or 0.1 mM SNAP (Sigma Aldrich) overnight. Target HT-29 tumor cells were labeled with <sup>51</sup>Cr and then coated with cetuximab or IgG. Target cells were added to NK cells, and following a 4 hr incubation supernatants

were harvested and quantified using a gamma counter. Mean percent cell lysis was determined as previously described (2).

## ELISA

96-well flat-bottom plates were coated with 100 µg/mL of polyclonal human IgG in cold PBS overnight at 4°C. NK cells, MDSC, or NK cells plus MDSC were plated at  $2 \times 10^5$  cells/well as previously described (23). Where indicated cells were treated with 2.5 mM of the iNOS inhibitor L-NIL. Cell-free supernatants were harvested and analyzed for levels of IFN- $\gamma$ , MIP-1 $\alpha$ , or TNF- $\alpha$  (ELISA; R&D Systems, Minneapolis, MN or Ebioscience) (23).

## Phospho-Erk Intracellular Staining

Autologous NK cells and MDSC were co-cultured overnight. Where indicated cells were also treated with 2.5 mM of L-NIL. The cells were incubated with the 3G8 anti-CD16 antibody (0.2 mg/mL) on ice for 30 minutes, and then cross-linked with a goat anti-mouse F(ab)<sub>2</sub> for 15 minutes. Cells were permeabilized, stained with phospho-Erk and CD56 antibodies and analyzed by FACS (23).

## Nitrotyrosine Staining

NK cells were treated with 0.1 mM S-Nitroso-N-acetylpenicillamine (SNAP) (56). Alternatively, autologous NK cells and MDSC were co-cultured overnight at a 1:1 ratio. Cells were washed with cold PBS, incubated in buffer A (Invitrogen) and permeabilized using cold methanol. Cells were stained with anti-CD16 and anti-nitrotyrosine antibodies in buffer B (Invitrogen) and analyzed by FACS.

## Immunoprecipitation

NK cells were treated with SNAP as described above. 20 µg of anti-nitrotyrosine beads (Millipore 16310) were added to 50 µg of protein lysate and incubated overnight at 4°C. The beads were washed with cold PBS and re-suspended in 2X Laemmli buffer and boiled. The sample was loaded on a SDS gel for protein expression by immunoblot analysis. The membrane was probed with an anti-Erk antibody (Cell Signaling) (28).

## Annexin V/ PI Staining

NK cells isolated from healthy donor leukopaks (American Red Cross) were treated with various concentrations of SNAP or DMSO. NK cells were stained with annexin V and propidium iodide (PI) (BD Pharmingen) (54).

## FACS Analysis

Human MDSC were stained with a panel of antibodies recognizing CD11b, CD33, HLA-DR, CD15, and CD14 (Beckman Coulter) as well as TIGIT ligands CD155 and CD112 (Biolegend) (13). NK cells were stained using antibodies against CD16 and CD56 (BD Pharmingen). Mouse MDSC were stained with antibodies against Gr-1 and CD11b (BD Biosciences) (28). Data was acquired using a BD-LSRII and the data was analyzed using the FlowJo.

### Nitrite Estimation

MDSC were cultured in 10% human AB (HAB) (Sigma Aldrich) media for 24 hours. Equal amounts of supernatant were mixed with modified Greiss reagent (Sigma Aldrich). Absorbance at 550 nm was measured using a microplate reader. Nitrite concentrations were determined using a standard curve (54).

### DAF-FM Staining

MDSC were stained with 2.5  $\mu$ M DAF-FM (Molecular probes, D-23841) for 30 minutes in serum free media. Cells were then washed three times with serum free media and incubated for an additional 20 minutes in RPMI complete media. Following the incubation, MDSC were washed and analyzed by FACS (57).

### Mouse NK Cell Isolation

Balb/c mice received single intraperitoneal injections of gemcitabine (80 mg/kg) or 5-FU (50 mg/kg). MDSC depletion was also accomplished by intraperitoneal injection of anti-Gr-1 (250  $\mu$ g) daily for 5 days. To inhibit MDSC function Balb/c mice received intraperitoneal injections of the following agents for 5 consecutive days: L-NIL (20 mg/kg), anti-TGF- $\beta$  Ab (200  $\mu$ g, BioXcell, clone 1D11.16.8) (25), anti-IL-10 Ab (250  $\mu$ g, BioXcell, clone JES5-2A5), the arginase inhibitor nor-NOHA (20 mg/kg) (27) or the IDO inhibitor, 1-Methyl-D-tryptophan via oral gavage (400 mg/kg, Sigma Aldrich) (26). Mice were sacrificed 24 hrs after the last treatment. NK cells were isolated from the spleen using a mouse NK cell enrichment kit (Stem Cell Technologies).

### Real Time PCR

Following TRIzol extraction (Invitrogen) and RNeasy purification (Qiagen), total RNA was quantitated and reverse transcribed. cDNA was used to measure gene expression by Real-Time PCR using pre-designed primer/probe sets and 2 $\times$  TaqMan Universal PCR Master Mix with 18s rRNA as an internal control (Applied Biosystems) (54).

### Tumor Studies

For *ex vivo* studies of the effect of depletion or inhibition of MDSC on NK cell function, 4-5 week old female Balb/c mice were injected with  $1 \times 10^5$  4T1 mammary carcinoma cells in the mammary fat pad. For *in vivo* studies of the effect of depletion or inhibition of MDSC function on the response to antibody therapy, Balb/c mice were injected with  $1 \times 10^6$  EMT6-HER2 mammary carcinoma cells in the mammary fat pad. MDSC transfer studies utilized immune-deficient athymic nude mice (FOXP1<sup>-/-</sup>) injected subcutaneously with  $1 \times 10^6$  Panco2-EGFR<sup>+</sup> pancreatic carcinoma cells. For the generation of MDSC in transfer studies, Balb/c mice were injected with  $1 \times 10^5$  4T1 mammary carcinoma cells while C57BL/6 or *Nos2*<sup>-/-</sup> B6.129P2-*Nos2*<sup>tm1/Lau</sup>/J mice were injected with  $1 \times 10^5$  B16F10 melanoma cells. MDSC were isolated from the spleen using a myeloid derived suppressor cell isolation kit (Miltenyi). Tumor volume was measured three times weekly using digital calipers. These studies were conducted under a protocol approved by Ohio State University's Institutional Animal Care and Use Committee (IACUC 2009A0179-R2).

## NK Cell Depletion

Three days prior to the start of treatment, a cohort of tumor-bearing mice were injected i.p. with 50  $\mu$ l anti-asialo GM1 polyclonal antibody (1 mg/ml) to deplete NK cells. NK depleted mice were administered the depleting antibody every 4 days until the end of the study. Depletion of NK cells was confirmed via flow cytometry by staining splenocytes from control and anti-asialo GM1 treated mice with an antibody against DX5.

## Statistical Analysis

Statistical differences between treatment groups were determined using an ANOVA model and Student's t-test. For murine tumor studies, a linear mixed model was employed to model longitudinal tumor volume for mice under each treatment. Comparisons were done at each time point and averaged across all time points using t-statistics. The Holm-Bonferroni method was used for adjusting raw p-values for multiple comparisons across treatment groups.

## Study Approval

Written informed consent was received from all the human participants prior to inclusion in the experimental studies under Institutional Review Board- approved protocols (OSU IRB Nos. 1999C0348, 2004C0096 and 2010C0036). All mouse studies were conducted under a protocol approved by Ohio State University's Institutional Animal Care and Use Committee (IACUC 2009A0179-R2).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgement

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### Translational Relevance

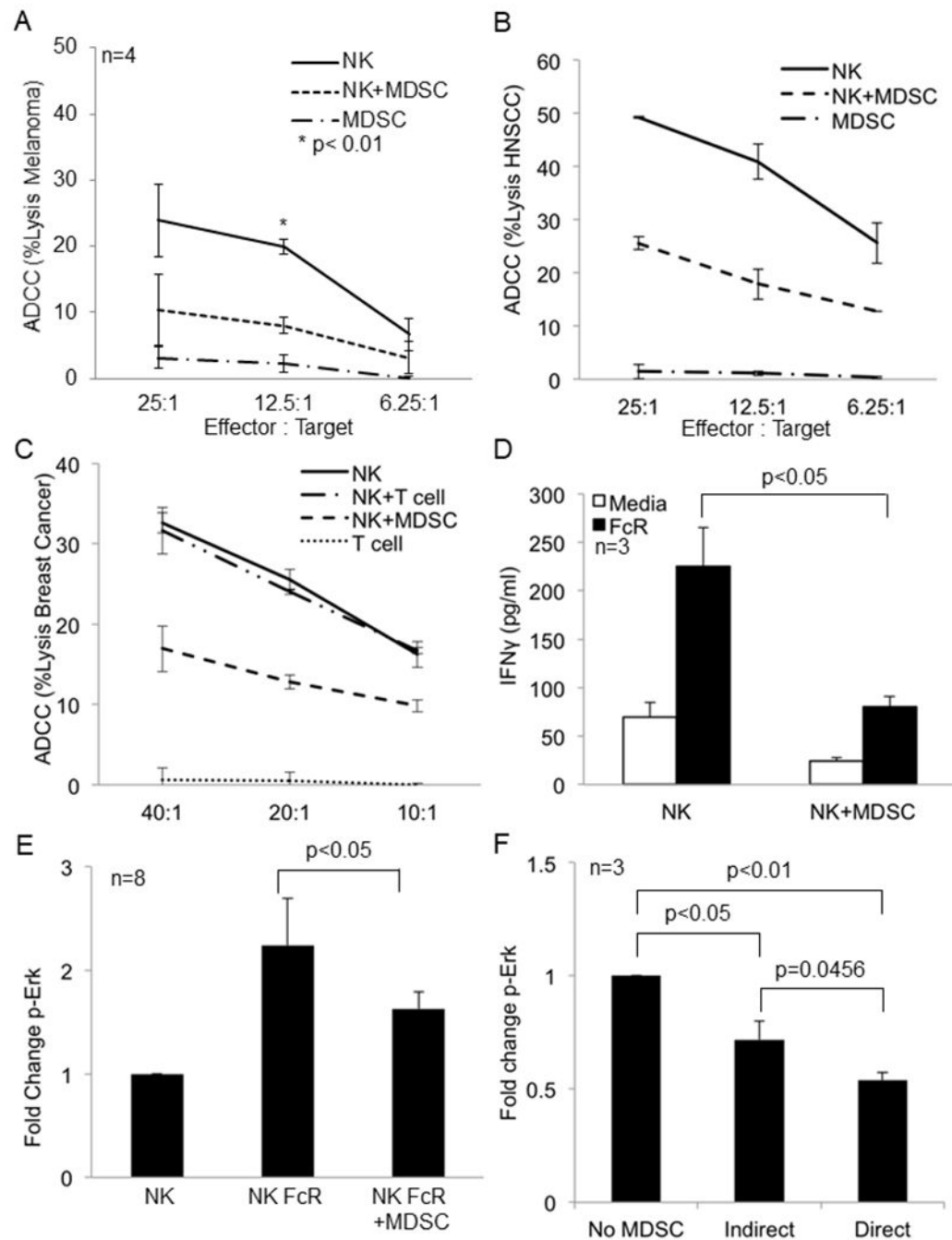
Monoclonal antibodies (mAb) are a mainstay in the current cancer therapeutics landscape with over a dozen FDA approved molecules used to treat solid and hematological malignancies. One of the mechanisms of action of mAbs is the activation of the innate immune system, including NK cells, through engagement of Fc receptors including FcR $\gamma$ IIIA. It is also well appreciated that cancer evolves to evade the immune system in part by promoting the expansion of immune suppressive cells such as myeloid derived suppressor cells (MDSC) which are known to antagonize immune based therapies. Understanding the impact MDSC have on FcR mediated NK cell functions and the response to mAb therapy could lead to the development of novel combination therapies that enhance the efficacy of mAb therapy in multiple disease settings by eliminating or antagonizing the immune suppressive function of MDSC.

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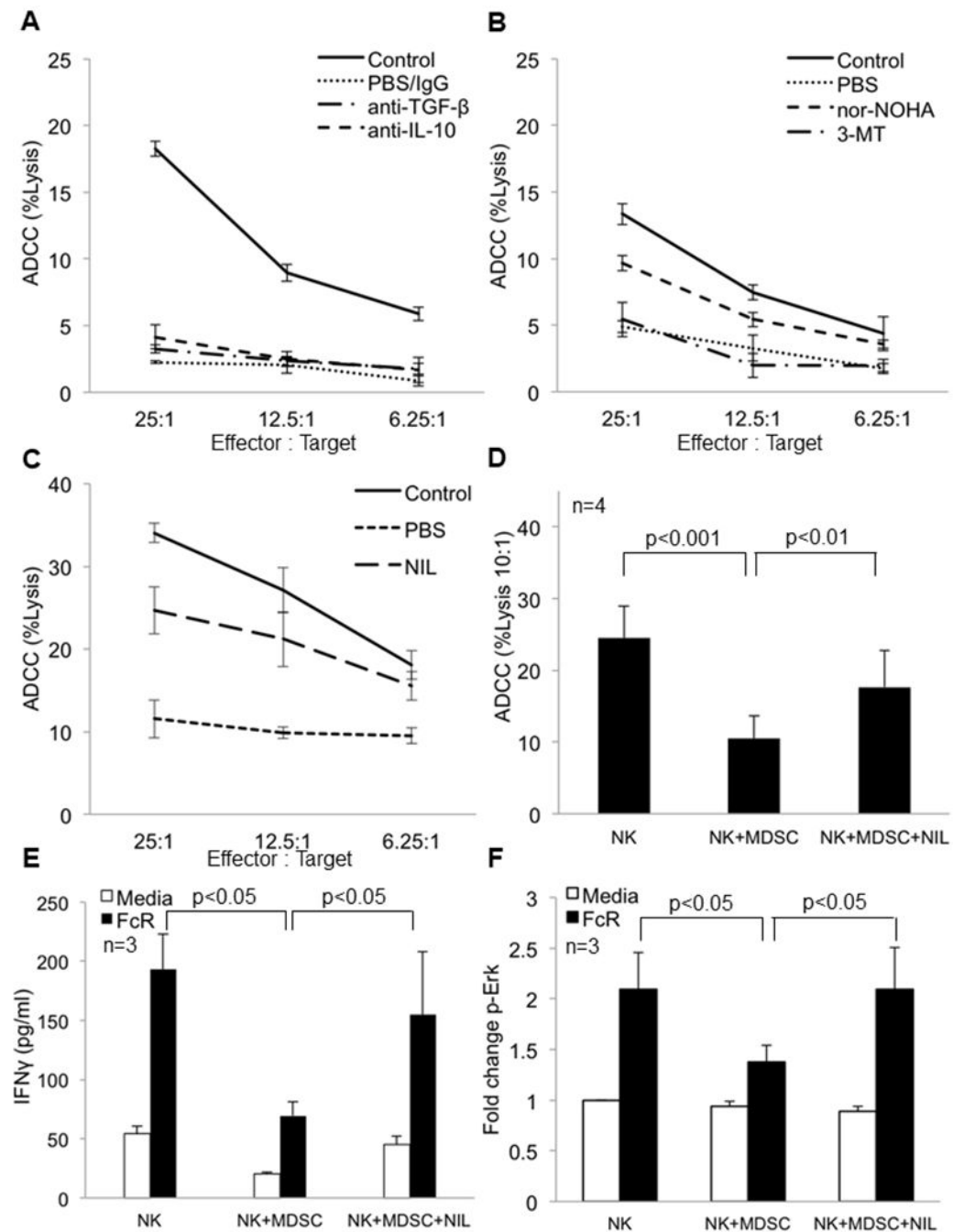
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**Figure 1: MDSC inhibit FcR mediated NK cell effector functions and signal transduction.**

(a) NK cells from the peripheral blood of a melanoma patient were cultured alone or with autologous MDSC overnight and then used in a  $^{51}\text{Cr}$  release ADCC assay against cetuximab-coated HT-29 cells. Values represent the mean  $\pm$  SD from four independent experiments,  $p < 0.01$ . Significance was determined using the paired t-test. (b) Results from one ADCC assay as conducted in (a) using NK cells and autologous MDSC from a HNSCC patient. (c) Results from one ADCC assay as conducted in (a) using NK cells and autologous MDSC from a breast cancer patient. (d) Autologous NK cells and MDSC from

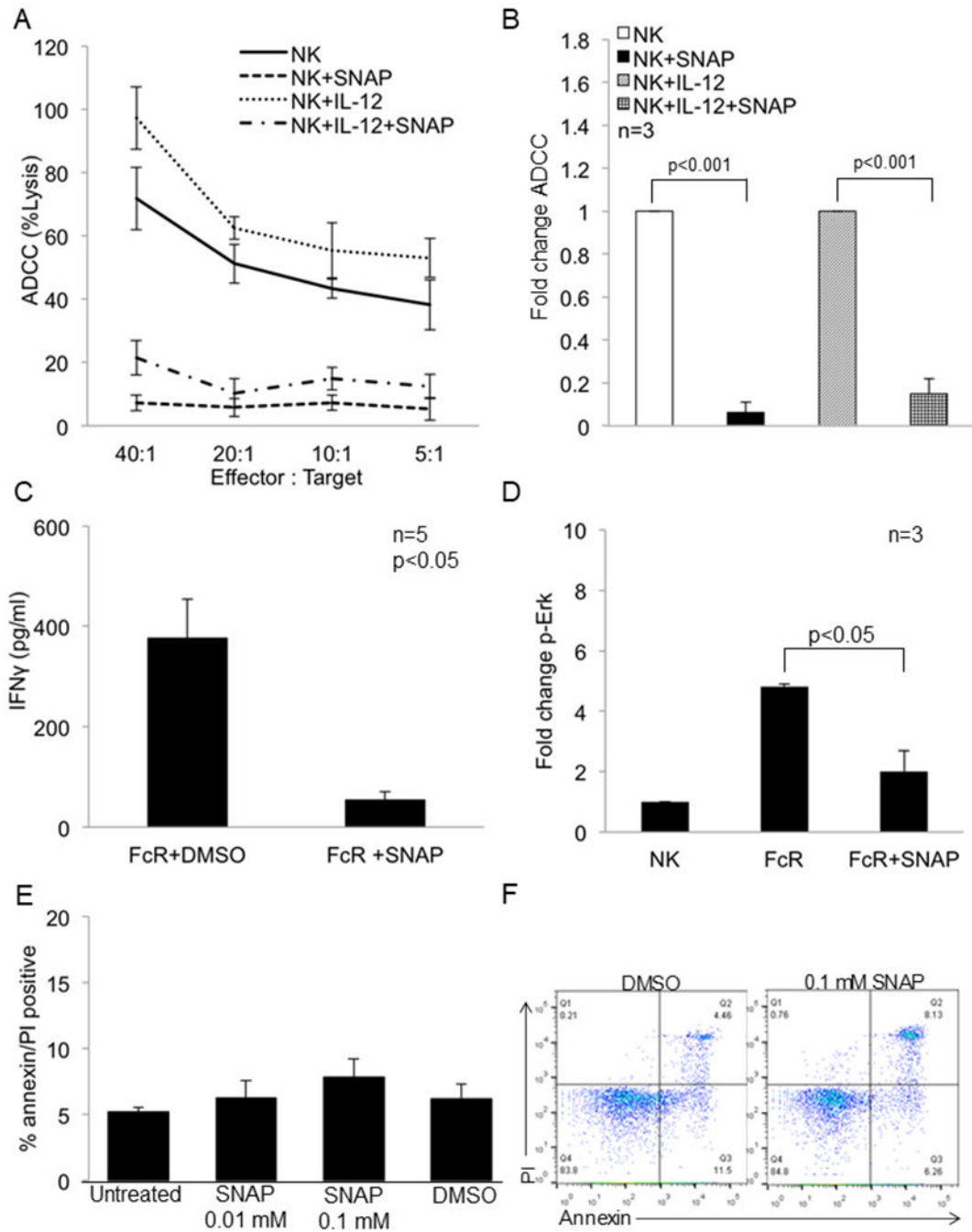
the peripheral blood of melanoma patients were co-cultured at a 1:1 ratio in 96 well plates coated with human IgG (100 µg/ml) or media (control). Supernatants were collected after 48 hrs and cytokine levels measured by ELISA. Quantification of data from three independent experiments, values shown are the mean ± SE,  $p < 0.05$ . Significance was determined using a paired t-test. (e) Quantification of changes in p-Erk levels in FcR activated CD56<sup>+</sup> NK cells measured by flow cytometry in the presence or absence of MDSC. Values are mean ± SE from eight independent experiments,  $p < 0.05$ . Significance was determined using a paired t-test. (f) NK cells were cultured alone, in direct contact with MDSC (Direct), or physically separated from MDSC by a permeable 0.4 µm Corning Transwell® membrane (Indirect) at a 1:1 ratio overnight. NK cells were then stimulated through the FcR using the anti-CD16 3G8 antibody and F(ab')<sub>2</sub> and levels of p-Erk determined as described above. Values are the mean ± SE from 3 independent experiments. Significance was determined using a paired t-test and Holms method. Representative flow cytometry dot plot for p-Erk is provided in Supplementary Figure 4.



**Figure 2: iNOS inhibition restores FcR mediated NK cell functions and signal transduction in the presence of MDSC.**

Female Balb/c mice were inoculated with  $1 \times 10^5$  4T1 tumor cells or were left un-injected (no tumor-control). Following the establishment of tumors, mice were treated daily with (a) intraperitoneal injections of IgG (250  $\mu$ g), anti-TGF- $\beta$  antibody (200  $\mu$ g), or anti-IL-10 antibody (250  $\mu$ g) for four consecutive days prior to NK cell isolation for the ADCC assay. The mice were sacrificed 24 hrs after the last treatment. Each group consists of pooled samples from spleens of 4-5 mice. Values represent mean  $\pm$  SD from one experiment. (b) Mice were treated with PBS (vehicle), arginase inhibitor nor-NOHA (20 mg/kg) i.p or the

IDO inhibitor 3-methyltryptophan (MT, 400 mg/kg) via oral gavage prior to NK cell isolation for the ADCC assay. (c) Mice were given intraperitoneal injections of PBS (vehicle) or iNOS inhibitor, L-NIL (20 mg/kg) for one week. NK cells isolated from the spleen were employed in a standard ADCC assay using trastuzumab-coated CT26-HER2 positive tumor cells as targets. (d) NK cells and MDSC from the peripheral blood of melanoma patients were co-cultured overnight at a 1:1 ratio with or without the nitric oxide inhibitor L-NIL (2.5 mM). ADCC function of NK cells displayed as the mean percent lysis of cetuximab-coated HT-29 tumor cells at the 10:1 effector to target ratio. Means  $\pm$  SE from four independent experiments are shown,  $p < 0.05$ . Significance was determined using a linear mixed model. Treatment of NK cells with L-NIL alone did not enhance ADCC activity (not shown). (e) Autologous NK cells and MDSC isolated from peripheral blood of melanoma patients were co-cultured in 96 well plates coated with human IgG or media with or without the iNOS inhibitor L-NIL (2.5 mM). Supernatants were harvested after 48 hrs and analyzed for levels of IFN $\gamma$  by ELISA. Values represent mean  $\pm$  SE from three independent experiments,  $p < 0.05$ . Significance was determined using a linear mixed model. (f) p-Erk expression in NK cells co-cultured overnight with MDSC in the presence or absence of L-NIL (2.5 mM). p-Erk levels are expressed as the average fold change  $\pm$  SE from three independent experiments,  $p < 0.05$ . Significance was determined using a linear mixed model.



**Figure 3: The NO donor SNAP inhibits NK cell function and signal transduction.**

The nitric oxide donor SNAP inhibits NK cell FcR-mediated function and signal transduction. (a) Purified NK cells from normal donors were treated with DMSO or SNAP (0.1 mM) in the presence or absence of IL-12 (10 ng/ml) and then used in a  $^{51}\text{Cr}$  release ADCC assay against cetuximab-coated HT-29 cells. Representative results shown are from one of three independent experiments. (b) Fold change in ADCC activity of NK cells (20:1 effector target ratio) treated in a similar fashion as in (a) against cetuximab-coated HT-29 cells. The mean  $\pm$  SE from three independent experiments are shown,  $p < 0.001$ . Significance

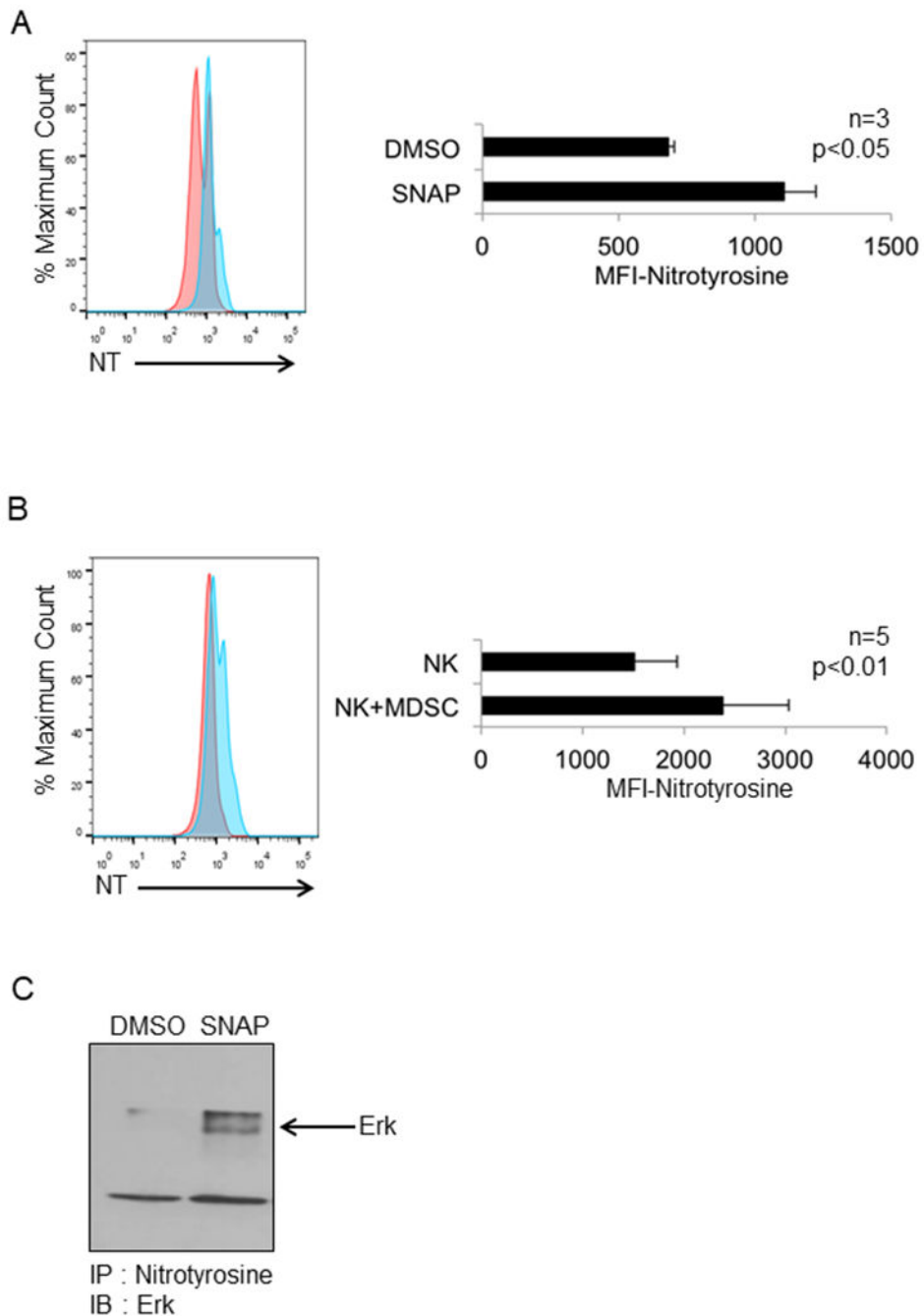
was determined using a t-test. (c) Levels of IFN- $\gamma$  production after 48 hrs measured by ELISA from healthy donor NK cells treated with DMSO or SNAP (0.1 mM) and activated with immobilized IgG. Values represent mean  $\pm$  SE from five independent experiments,  $p < 0.05$ . Significance was determined using a paired t-test. (d) Fold change in the expression of p-Erk in NK cells treated with DMSO or SNAP (0.1 mM) and then stimulated through the FcR via 3G8 antibody. Values represent mean  $\pm$  SE from three independent experiments,  $p < 0.05$ . Significance was determined using a paired t-test. (e) NK cells isolated from normal donors were treated with the indicated doses of SNAP or DMSO for 24 hrs. NK cells were then stained with annexin V/PI to determine the percentage of apoptotic cells. Values displayed are from three independent experiments. (f) NK cells isolated from normal donors were treated with the indicated doses of SNAP or DMSO for 24 hrs. NK cells were then stained with annexin V/PI to determine the percentage of apoptotic cells. A representative flow cytometric dot plot is provided for the quantification data in 3E.

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**Figure 4: Treatment of NK cells with the nitric oxide donor SNAP or co-culture with MDSC results in nitration of tyrosine residues in NK cells.** Purified NK cells from normal donors were treated with SNAP (0.01 mM) or DMSO (control). Following permeabilization, cells were stained with anti-CD16 and anti-nitrotyrosine antibodies and analyzed by flow cytometry. (a) Representative flow cytometry profile and quantification of nitrotyrosine levels in CD16<sup>+</sup> NK cells shown as mean fluorescence intensity (MFI) (right panel) from three independent experiments,  $p < 0.05$ , significance was determined using a paired t-test. (b) Autologous NK cells and MDSC isolated from peripheral blood of melanoma patients were co-cultured overnight at a ratio of



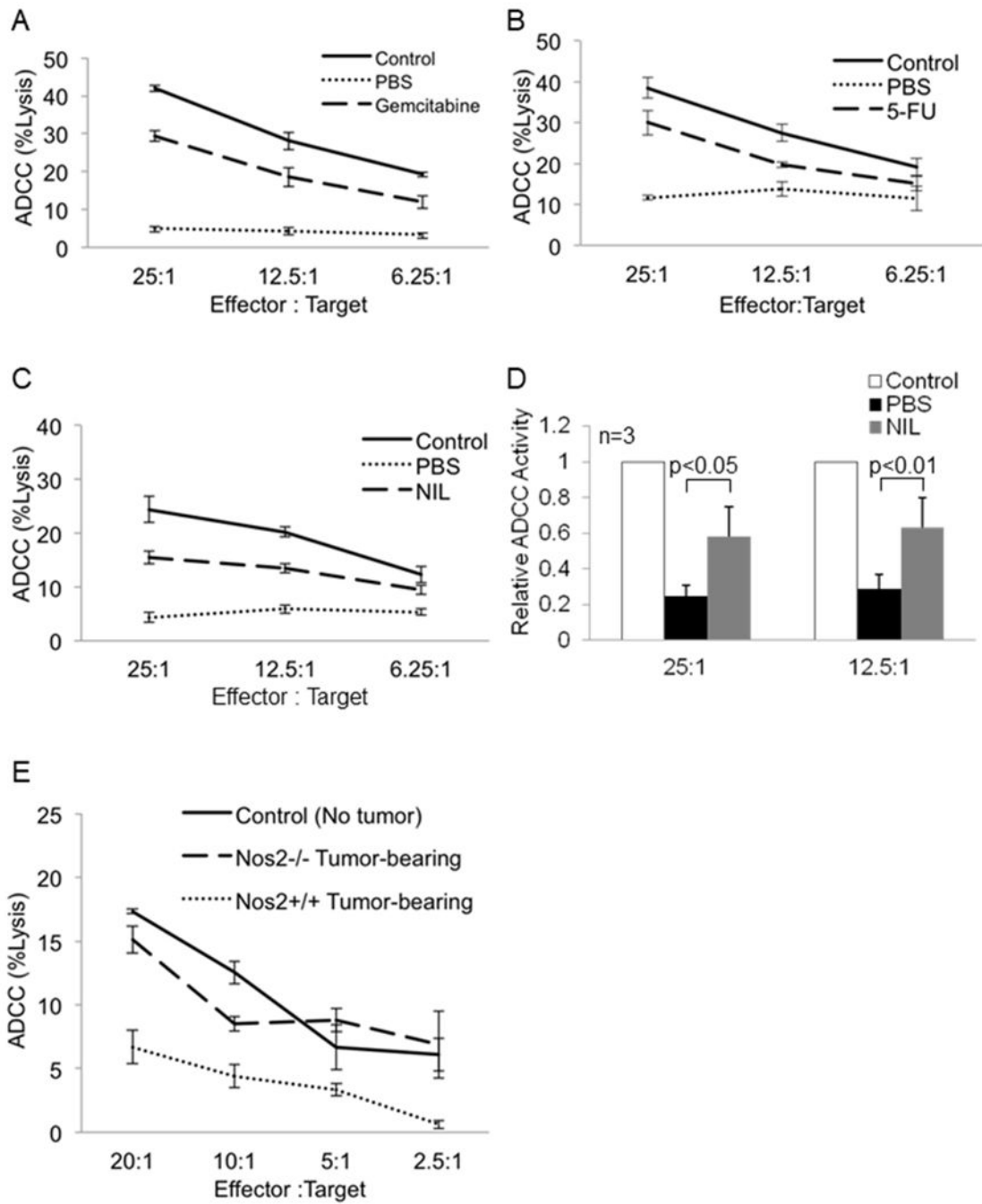
1:1 and then stained with anti-CD16 and anti-nitrotyrosine antibodies. Representative flow cytometry profile (left) and quantification of nitrotyrosine in CD16<sup>+</sup> NK cells as mean fluorescence intensity (MFI) (right) from five independent experiments,  $p < 0.05$ , significance was determined using a paired t-test. (c) Immunoblot showing the nitration of Erk protein. Purified NK cells from normal donors were treated with SNAP or DMSO (control), immunoprecipitated with anti-nitrotyrosine beads and probed with anti-Erk antibody.

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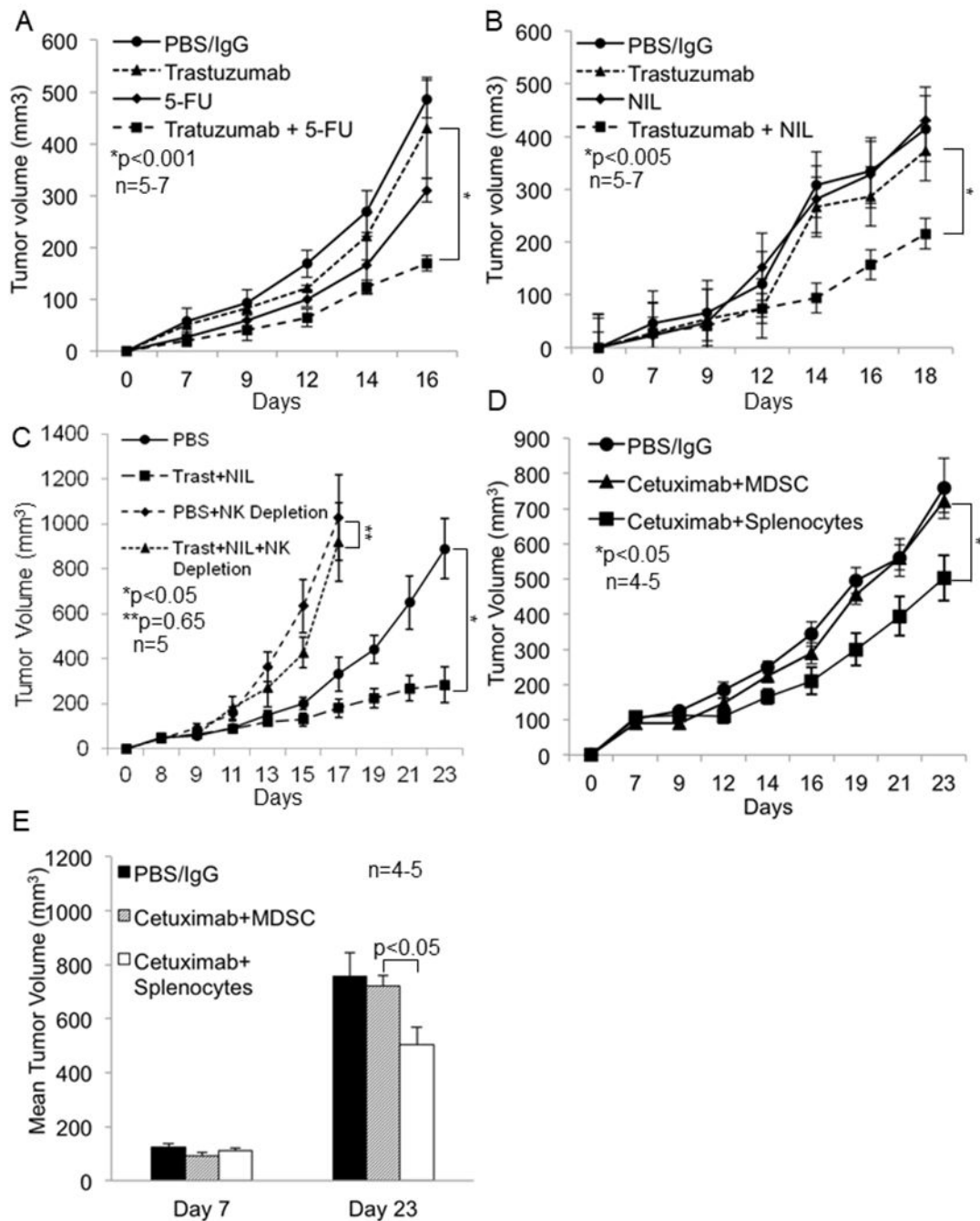
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**Figure 5: Depletion of MDSC or inhibition of nitric oxide production in mice augments NK cell-mediated ADCC activity.**

Female Balb/c mice were inoculated with  $1 \times 10^5$  4T1 tumor cells or were left un-injected (no tumor-control). The mice were sacrificed 24 hrs after treatment. NK cells isolated from the spleen were used in a standard ADCC assay using trastuzumab-coated CT26-HER2 positive tumor cells as targets. (a) Mice were treated with PBS (vehicle) or gemcitabine (80 mg/kg). Graph displays mean percent lysis by pooled NK cells from 4-5 mice per treatment group. One of two representative experiments is shown. (b) Mice were treated with PBS (vehicle) or 5-fluorouracil (50 mg/kg). Graph displays mean percent lysis by pooled NK cells from

4-5 mice per treatment group. One of two representative experiments is shown. (c) NK cells isolated from mice treated daily for one week with PBS (vehicle) or iNOS inhibitor, L-NIL (20 mg/kg) were used in a standard ADCC assay using trastuzumab-coated CT26-HER2 cells. Each group consists of pooled samples from spleens of 5 mice. (d) Fold change in NK cell ADCC activity from mice treated as in (c). Values displayed are the means  $\pm$  SE from three independent experiments,  $p < 0.05$  for the 25:1 and 12.5:1 effector target ratios. Data was log-transformed for testing group difference using a linear mixed effect model with random donor effect, and the p-value was calculated using Bonferroni method. (e) Female C57BL/6 (wild type) and *Nos2*<sup>-/-</sup> mice were inoculated with B16F10 tumor cells or left uninjected (control). NK cells were isolated from the spleen and used in a standard ADCC assay using trastuzumab coated CT26-HER2 tumor cells. Each group consists of pooled samples from spleens of 3-4 mice. Values represent mean  $\pm$  SD from one experiment.



**Figure 6: MDSC antagonize mAb therapy *in vivo* and MDSC depletion or inhibition of nitric oxide enhances the efficacy of mAb therapy.**

Female Balb/c mice were inoculated with  $1 \times 10^6$  EMT6-HER2 tumor cells in the mammary fat pad. Following the establishment of tumors (day 7), mice were treated once a week i.p. with PBS or 5-FU and thrice weekly with IgG or trastuzumab and tumor growth was measured three times a week using digital calipers. (a) Tumor growth in mice treated with PBS and IgG (10 mg/kg), 5-FU (50 mg/kg), trastuzumab (10 mg/kg) or the combination of trastuzumab plus 5-FU. Differences in tumor volumes were tested using a linear mixed model and Student's t-test. Values represent mean  $\pm$  SE,  $p < 0.001$ . (b) Tumor growth in mice

treated with PBS and IgG (10 mg/kg), L-NIL (20 mg/kg), trastuzumab (10 mg/kg) or combination of trastuzumab plus L-NIL. Each group consisted of 5-7 mice. Values represent mean  $\pm$  SE,  $p < 0.005$ , significance was determined using a linear mixed model and Student's t-test. (c) Depletion of NK cells with anti-asialo-GM1 abrogates the anti-tumor effect of trastuzumab plus NIL. Balb/c mice were inoculated with  $1 \times 10^6$  EMT6-HER2 tumor cells in the mammary fat pad. Three days prior to treatment, mice were administered PBS or 50  $\mu$ g/mouse of anti asialo GM1 polyclonal antibody to deplete NK cells. On day 8, mice were randomized and treated thrice weekly with PBS and IgG or trastuzumab (10 mg/kg) plus L-NIL (20 mg/kg). NK depleted mice were administered the depleting antibody every 4 days until the end of the study.  $n=5$  for each treatment group. (d) Athymic nude mice were inoculated with  $1 \times 10^6$  Panco2-EGFR tumor cells on day zero. After establishment of tumors mice were injected with PBS, splenocytes, or MDSC on day 7 prior to the initiation of treatment with IgG or cetuximab (0.5 mg/kg) on day 9. Mice were re-injected with PBS, splenocytes, or MDSC on day 14 and IgG or cetuximab treatment was continued. (d) Tumor growth curves of mice treated as described above. (e) Mean tumor volume in mice inoculated with Panco2-EGFR cells before (day 7) and after treatment (day 23). Each group consisted of 4-5 mice. Differences in tumor volumes were tested using a linear mixed model and Student's t-test. Values represent mean  $\pm$  SE,  $p < 0.001$ .