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CD38-Expressing Myeloid-Derived Suppressor Cells Promote Tumor Growth in a Murine Model of Esophageal Cancer

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1 **Abstract**

2 Myeloid derived suppressor cells (MDSCs) are an immunosuppressive
3 population of immature myeloid cells found in advanced stage cancer patients
4 and mouse tumor models. Production of inducible nitric oxide synthase (iNOS)
5 and arginase, as well as other suppressive mechanisms, allow MDSCs to
6 suppress T cell-mediated tumor clearance and foster tumor progression. Using
7 an unbiased global gene expression approach in conditional p120-catenin
8 knockout mice (*L2-cre;p120ctn^{fl/fl}*), a model of oral-esophageal cancer, we have
9 identified CD38 as playing a vital role in MDSC biology, previously
10 unknown. CD38 belongs to the ADP-ribosyl cyclase family and possesses both
11 ectoenzyme and receptor functions. It has been described to function in
12 lymphoid and early myeloid cell differentiation, cell activation and neutrophil
13 chemotaxis. We find that CD38 expression in MDSCs is evident in other mouse
14 tumor models of esophageal carcinogenesis, and CD38^{high} MDSCs are more
15 immature than MDSCs lacking CD38 expression, suggesting a potential role for
16 CD38 in the maturation halt found in MDSC populations. CD38^{high} MDSCs also
17 possess a greater capacity to suppress activated T cells, and promote tumor
18 growth to a greater degree than CD38^{low} MDSCs, likely as a result of increased
19 iNOS production. Additionally, we have identified novel tumor-derived factors,
20 specifically IL-6, IGFBP-3 and CXCL16, which induce CD38 expression by
21 MDSCs *ex vivo*. Finally, we have detected an expansion of CD38-positive
22 MDSCs in peripheral blood of advanced stage cancer patients and validated
23 targeting CD38 *in vivo* as a novel approach to cancer therapy.

1 **Introduction**

2 The immune system (both innate and adaptive) plays an essential role in
3 limiting tumor growth, and therefore, tumor progression requires escape from
4 immune surveillance. One mechanism that allows for tumor escape is the
5 activation and expansion of immunosuppressive cell populations, including but
6 not limited to, regulatory T cells (Tregs) and myeloid derived suppressor cells
7 (MDSCs) (1), the latter also referred to as immature myeloid cells (IMCs). Certain
8 therapeutics have demonstrated potential efficacy against MDSCs (2); however,
9 the need for more selective anti-MDSC therapeutics remains.

10 MDSCs have been observed in a number of mouse tumor models and
11 represent a heterogeneous population of immature monocytes and granulocytes
12 that are identified by their CD11b⁺Gr1⁺ phenotype in mice (3). In human disease,
13 the first immature myeloid cell population with immunosuppressive capacity was
14 described in head and neck cancer (4), and since then MDSCs have been
15 documented in cancers of the esophagus, stomach, pancreas, lung, kidney,
16 colon, skin, prostate, and breast (5–10). The immunophenotype of human
17 MDSCs varies (11), however, their immunosuppressive mechanisms match
18 those found in murine CD11b⁺Gr1⁺ MDSC populations.

19 MDSCs induce immune suppression primarily through inhibition of T cell-
20 mediated tumor clearance (3), but can also promote inhibition of NK cells (12)
21 and activation of Tregs (13). Arginase-1 (ARG1) and inducible nitric oxide
22 synthase-2 (iNOS) provide the bulk of the enzymatic activity required for MDSCs
23 to suppress T cell proliferation and activation of (3). ARG1 deprives T cells of

1 arginine by converting L-arginine into urea and L-ornithine, thereby reducing
2 expression of CD3 ζ chain, which renders T cells unable to respond to activation
3 signals (14). iNOS inhibits T cell function by a variety of mechanisms, including
4 inhibition of JAK3/STAT5 signaling (15), MHC Class II expression (16) and
5 induction of apoptosis (17).

6 CD38 expression is a common characteristic to several
7 immunosuppressive cell types. Foxp3⁺CD25⁺CD4⁺ Tregs expressing high CD38
8 levels possess a greater immunosuppressive activity than CD38^{low} Tregs (18).
9 CD38⁺CD8⁺ T cells suppress proliferation of CD4⁺ effector T cells, which requires
10 IFN γ secretion and cell-to-cell contact (19). Similarly, CD19⁺CD24^{hi}CD38^{hi} B
11 cells inhibit differentiation of T helper 1 cells in an IL-10 dependent manner, and
12 their dysfunction may play a role in autoimmune disorders such as systemic
13 lupus erythematosus (20).

14 CD38 is a member of the ribosyl cyclase family and is expressed on the
15 surface of diverse immune cells, including B cells, T cells, NK cells and myeloid
16 cells (21). CD38 possesses independent ectoenzyme and receptor functions.
17 As an ectozyme, CD38 catalyzes synthesis and hydrolysis of cyclic ADP-ribose
18 (cADPR), converting NAD⁺ to ADP-ribose (ADPR), as well as cADPR into ADPR
19 (21,22). Furthermore, at acidic pH, CD38 catalyzes synthesis and hydrolysis of
20 nicotinic acid adenine dinucleotide phosphate (NAADP) (21,22). Both reactions
21 are essential for calcium signaling, specifically for mobilization of intracellular
22 Ca²⁺ (22). Receptor activity of CD38 has been documented in multiple immune
23 cell types, where it is dependent on localization to the lipid rafts and association

1 with professional signaling complexes (21). In both mouse and human myeloid
2 cells, ligation of CD38 receptor leads to suppressed growth and survival resulting
3 in loss of the most differentiated immune populations (23).

4 In this study we have identified CD38 as a novel marker for MDSCs that
5 possess greater immunosuppressive capacity, thereby promoting tumor growth
6 *in vivo*. We have identified a mechanistic role for CD38 in promoting expansion
7 of the monocytic MDSC population, as well as in regulating expression of the
8 effector molecule iNOS by these cells. Additionally, we have established for the
9 first time that several cytokines, specifically IFN γ , TNF α , IGFBP-3, CXCL16 and
10 IL-6, are capable of inducing CD38 expression in MDSCs. Finally, we have
11 demonstrated that administration of an anti-CD38 monoclonal antibody slows
12 disease progression in tumor-bearing mice. As we have detected an expansion
13 of CD38-positive MDSC-like population in peripheral blood of advanced-stage
14 cancer patients, this study introduces the concept of anti-CD38 monoclonal
15 antibody therapy for potential treatment of certain solid tumors.

16

1 **Materials and Methods**

2 *Generation of MDSCs*

3 All animal studies were approved by the Institutional Animal Care and Use
4 Committee (IACUC) at the University of Pennsylvania. Mice were housed under
5 a 12-hour light/dark cycle and fed ad libitum. We have described the *L2-
6 Cre;p120ctn^{ff}* mouse model of oral-esophageal cancer previously (24). We also
7 used syngeneic subcutaneous transplantation models utilizing the HNM007 and
8 AKR ESCC cell lines in either C57BL/6J (Jackson Labs) or *Cd38^{-/-}* mice (gift from
9 Dr. Eduardo Chini). For generation of MDSCs, *L2-Cre;p120ctn^{ff}* mice were aged
10 until signs of preneoplasia and neoplasia were evident; subcutaneous tumor-
11 bearing mice were aged until tumors reached a volume of 0.8cm³. Spleens and
12 bone marrow were harvested upon euthanasia for MDSC isolation.

13

14 *Flow cytometry and cell sorting*

15 Single cell suspensions were prepared from mouse bone marrow or spleen by
16 mechanical disruption. Red blood cells were lysed, and the remaining leukocytes
17 were washed with PBS, and resuspended in PBS + 2% FBS. For analysis of
18 patient blood samples, peripheral blood mononuclear cells (PBMC) were
19 separated using gradient centrifugation. Samples were analyzed on a
20 FACScalibur (BD) or LSRII (BD). Cell sorting for multiple markers was
21 performed on a FACSAriaII (BD). Data were analyzed using FlowJo (Treestar).

1 Peripheral blood from previously untreated, advanced stage HNC patients was
2 obtained with informed consent under University of Pennsylvania IRB protocol
3 #417200 or Philadelphia VA Medical Center protocol #01090.

4

5 *Histology*

6 Subcutaneous tumors were fixed in buffered formalin solution, paraffin-
7 embedded and stained with hematoxylin and eosin (H&E). CD11b⁺Gr1⁺,
8 CD11b⁺Gr1⁺CD38^{low}, and CD11b⁺Gr1⁺CD38^{high} cells were sorted by flow
9 cytometry. Cytospin preparations were stained using the Hema 3 system (Fisher
10 Scientific).

11

12 *T cell suppression*

13 CD11b⁺Gr1⁺, CD11b⁺Gr1⁺CD38^{low}, and CD11b⁺Gr1⁺CD38^{high} cell populations
14 were sorted by flow cytometry. Antigen-specific CD8⁺ T cell suppression was
15 tested as described previously (24).

16

17 *Ex vivo MDSC differentiation*

18 Generation of MDSCs from bone marrow has been described previously (25).
19 Cytokine concentrations used: 0.1 ng/ml (GM-CSF and IL-4), 10 ng/ml (TNF α
20 and IFN γ), and 100 ng/ml (IL-6, CXCL16 and IGFBP-3). HNM007 or AKR
21 conditioned medium (CM) were used at 50% v/v. Anti-CD38 monoclonal antibody
22 and IgG2a isotype control were used at 10ug/ml.

23

1 *Colony formation and cell recovery assays*

2 Isolation of MDSCs from tumor-bearing *L2-cre;p120^{-/-}* mice by magnetic cell
3 sorting was described previously (24). 200,000 cells were seeded in each 35 mm
4 plate containing 1 ml of methylcellulose-based medium containing factors that
5 promote growth of granulocyte-macrophage progenitors (M3534; Stem Cell
6 Technologies). Anti-CD38 monoclonal antibody and IgG2a isotype control were
7 used at 10ug/mL. Colonies were counted after 7 days. For recovery assays,
8 5×10^5 MDSCs were seeded in complete RPMI 1640 medium supplemented with
9 antibodies; cells were quantified by Trypan exclusion using a Countess
10 automated cell counter (Invitrogen).

11

12 *Cytokine array*

13 Media from *ex vivo* differentiation cultures were collected and snap-frozen after 1
14 or 5 days of culture. Mouse cytokine array C3 kit was used according to the
15 manufacturer's protocol. Results were quantified using the ImageJ protein array
16 analyzer and normalized to positive controls to allow for comparison of relative
17 expression levels.

18

19 *ESCC/MDSC co-transplantation and anti-CD38 therapeutic study*

20 C57BL/6J recipient mice from Jackson Labs were injected subcutaneously with
21 a mixture of 2.5×10^5 syngeneic HNM007 tumor cells with either 2.5×10^5 CD38^{low}
22 or CD38^{hi} MDSCs obtained from HNM007 tumor-bearing C57BL/6J mice.
23 Recipient mice injected with 2.5×10^5 syngeneic HNM007 tumor cells alone

1 served as controls. For antibody treatment experiments, anti-CD38 monoclonal
2 antibody or IgG2a isotype control antibody were administered intraperitoneally
3 every 48 hours starting on day 5 post-injection. Measurements were taken every
4 2-3 days once tumors became palpable.

5

6 *Statistical analysis*

7 The Student's t test was used to whether there is significant difference between
8 two experimental groups ($p \leq 0.05$ was considered statistically significant).

9

10 Additional details can be found in Supplementary Materials and Methods.

1 **Results**

2 *Myeloid-derived suppressor cells from tumor-bearing L2-Cre;p120^{ff} mice exhibit*
3 *elevated CD38 expression*

4 We have previously demonstrated that MDSCs play a fundamental role in
5 tumor initiation and progression in a spontaneous genetic mouse model of ESCC
6 (*L2-Cre;p120^{ff}*; referred to hereafter as *p120^{-/-}*) (24). Here we sought to identify
7 genes associated with an immature myeloid phenotype that contribute to the
8 tumor promoting activities of MDSCs, thereby providing a platform to elucidate
9 underlying molecular mechanisms. To that end, we performed microarray
10 analysis of splenic MDSCs from 6-8 month old tumor-bearing *p120^{-/-}* mice and
11 age-matched littermate controls (Supplementary Fig.1). Among the 964 genes
12 showing differential expression between the two groups (Figure 1A), we identified
13 *Cd38* (ranked fifth highest among all genes tested (Supplementary Table 1)) as a
14 candidate gene of interest, as it has roles in both innate and adaptive immunity in
15 mice and humans, including, but not limited to chemotaxis of murine and human
16 neutrophils (26,27), early myeloid differentiation (23) and lymphoid cell activation
17 (28). We validated enhanced *Cd38* mRNA and protein expression in MDSCs
18 from tumor-bearing mice as compared to those isolated from control mice (Fig.
19 1B-D). We also observed increased CD38 in splenic MDSCs isolated from *L2-*
20 *IL1 β* mice, a model of Barrett's esophagus and esophageal adenocarcinoma (29)
21 (Supplementary Fig. 2).

22

1 *CD38 expression correlates with ESCC progression and expansion of monocytic*
2 *MDSC population*

3 To determine the kinetics of CD38 expression in MDSCs, we analyzed
4 splenic CD11b⁺Gr1⁺ populations from non-diseased (8 weeks) and tumor-bearing
5 (6-8 months) *p120*^{-/-} mice, as well as control mice. CD11b⁺Gr1⁺ cells were
6 slightly more abundant in spleens of non-diseased *p120*^{-/-} mice and markedly
7 elevated in spleens of tumor-bearing *p120*^{-/-} mice, compared to control mice (Fig
8 2A). CD38 expression was markedly increased only in splenic MDSCs from
9 tumor-bearing *p120*^{-/-} mice (Fig. 2B), while a more mature subset of myeloid cells
10 (CD11b⁺Gr1⁻) exhibited no change in CD38 levels (Fig 2B).

11 We next tested two murine ESCC cell lines (AKR (30) and HNM007 (31))
12 for their ability to generate MDSCs *in vivo* using a syngeneic transplant model.
13 We observed dramatically increased CD38 levels in all myeloid populations from
14 spleens of HNM007 tumor-bearing mice, yet in AKR tumor-bearing mice CD38
15 levels were overall lower (Fig. 2C, Supplementary Fig. 3). Interestingly, while
16 both cell lines induced expansion of myeloid populations in spleens of tumor-
17 bearing mice, it was significantly more pronounced ($p < 0.0009$) in HNM007 tumor-
18 bearing mice (Fig. 2D). Furthermore, we observed differences in distribution of
19 granulocytic and monocytic MDSCs (G-MDSC and M-MDSC, respectively), as
20 well as mature monocytes (Fig. 2D). G-MDSCs (CD11b⁺Ly6G⁺) were less
21 abundant ($p < 0.02$) in HNM007 tumor-bearing mice, compared to AKR. There
22 also was a trend of M-MDSC (CD11b⁺Ly6C⁺) expansion, accompanied by a
23 significant increase in mature monocytes (CD11b⁺Ly6C⁻Ly6G⁻) in HNM007,

1 compared to AKR tumor-bearing and control mice ($p < 0.02$). These findings
2 suggest that CD38 may be relevant to M-MDSC expansion in tumor-bearing
3 mice.

4

5 *CD38^{high} MDSCs possess greater immunosuppressive and tumor-promoting*
6 *capacity than CD38^{low} MDSCs*

7 Since the CD38^{high} MDSC population expands in tumor-bearing mice, we
8 hypothesized that CD38^{high} MDSCs possess greater immunosuppressive
9 potential than CD38^{low} MDSCs. To test this, we sorted CD38^{high} and CD38^{low}
10 MDSCs from HNM007 tumor-bearing mice and assessed their capacity to
11 suppress OT-1 T cell growth following stimulation with cognate antigen. CD38^{high}
12 MDSCs demonstrated significantly greater T cell suppressive capacity, compared
13 to their CD38^{low} counterparts (Fig. 3A), at 2:1 OT-1 to MDSC ratio, while a trend
14 of increased suppression was observed at 1:1 and 4:1 ratios.

15 Next we evaluated the impact of co-injection of CD38^{high} MDSCs with
16 HNM007 cells on tumor growth. Tumor volumes in CD38^{high} group were
17 significantly larger than CD38^{low} tumors on days 6 and 10 (Fig. 3B), and larger
18 than control HNM007 tumors on days 8, 10 and 13 (Fig. 3B). No difference in
19 size was detected between the CD38^{low} and control HNM007 tumors.
20 Furthermore, CD38^{high}-injected tumors were characterized by increased necrosis
21 and inflammatory infiltrate, compared to controls (Fig. 3C, D). These results
22 suggest that CD38^{high} MDSCs may possess greater tumor-promoting capacity
23 than CD38^{low} MDSCs *in vivo*.

1 Next we investigated whether CD38 is required for the
2 immunosuppressive function of MDSCs by analyzing the capacity of MDSCs
3 from *Cd38^{-/-}* and *Cd38^{+/+}* (*wt*) mice bearing HNM007 tumors to suppress OT-1 T
4 cell proliferation. Interestingly, *Cd38^{-/-}* MDSCs exhibited significantly reduced
5 immunosuppressive capacity at 1:1 and 4:1 OT-1 to MDSC ratios (Fig. 3E).

6

7 *CD38^{high} MDSCs are phenotypically different from the CD38^{low} subset*

8 Next we analyzed CD38^{high} and CD38^{low} splenic MDSCs from tumor-
9 bearing *p120^{-/-}* mice via microarray (Supplementary Fig.4) and detected
10 differential expression of 498 genes (Fig. 4A, Supplementary Table 2). Among
11 genes with the greatest increase in expression, was inducible nitric oxide
12 synthase (*iNos*). qPCR analysis further revealed that *iNos* expression was
13 significantly elevated in CD38^{high} MDSCs compared to CD38^{low} MDSCs, while
14 expression of arginase 1 (*Arg1*) and NADPH oxidase subunit (*Nox2*), two
15 additional mediators of MDSC suppressive function, was comparable in the
16 subpopulations (Fig. 4B). iNOS protein expression was also validated in
17 CD38^{high} MDSCs (Fig 4C). Since *iNos* is a target of NFκB transactivation (32), we
18 evaluated phospho-NFκB levels in CD38^{high} and CD38^{low} MDSCs and found
19 elevated phospho-NFκB (p65) levels in the CD38^{high} population (Fig. 4C). To test
20 whether iNOS contributes to the increased immunosuppressive capacity of
21 CD38^{high} MDSCs, we used an iNOS inhibitor (L-NMMA), and found that it
22 completely abrogated OT-1 T cell suppression mediated by CD38^{high} MDSCs
23 (Fig. 4D). Finally, the CD38 inhibitor AraF-NAD (33) partially rescued OT-1 T cell

1 proliferation (Fig. 4E), suggesting that CD38 enzymatic activity is required for
2 immunosuppressive capacity of CD38^{high} MDSCs. Furthermore, iNOS expression
3 was decreased in MDSCs isolated from the spleens of HNM007 tumor-bearing
4 *Cd38*^{-/-} (Fig. 4F).

5 Morphological assessment of sorted CD38^{low} and CD38^{high} MDSCs
6 revealed that the CD38^{high} population consists of more immature cells, such as
7 promyelocytes (~10%), myelocytes (5-10%) and metamyelocytes (5-10%), and
8 band cells (~70%), whereas the CD38^{low} population consists of band cells
9 (<10%) and mature neutrophils (>90%) (Fig. 4G), demonstrating that CD38^{high}
10 MDSCs are morphologically more immature than CD38^{low} MDSCs.

11

12 *IFN* γ , *TNF* α , *CXCL16*, *IGFBP-3* and *IL-6* induce CD38 expression

13 Since we found that MDSCs from HNM007 tumor-bearing mice have
14 increased CD38 expression, compared to AKR tumors (Fig. 2C), we sought to
15 understand signaling pathways underlying this phenotype. We performed *ex vivo*
16 bone marrow differentiation assays using GM-CSF, IL-4 (both required for
17 CD11b⁺Gr1⁺ generation from bone marrow progenitors (25)) and conditioned
18 media (CM) from either HNM007 or AKR cells. Only HNM007 CM induced CD38
19 expression (Fig. 5A). Since IFN γ and TNF α are key components of the pro-
20 inflammatory milieu and are known activators of CD38 transcription (34), we
21 used these cytokines in *ex vivo* differentiation assays. Interestingly, both factors,
22 individually or in combination, induced CD38 expression in CD11b⁺Gr1⁺ cells
23 (Fig. 5A). A cytokine array using CM from *ex vivo* differentiation experiments

1 revealed several factors, including CXCL16 and IGFBP-3 that were present at
2 higher levels in HNM007 cultures as compared to AKR cultures (Fig.5B). In
3 addition, the pro-inflammatory cytokine IL-6, a predicted activator of CD38
4 transcription (34), was elevated in HNM007 cultures, albeit not as dramatically as
5 CXCL16 or IGFBP-3 (Fig. 5B). Next we investigated the capacity of recombinant
6 IL-6, CXCL16 and IGFBP-3 to increase CD38 expression *ex vivo*. Interestingly,
7 addition of IL-6, CXCL16 and IGFBP-3 in combination induced CD38 expression
8 in AKR CM cultures (Fig. 5C).

9

10 *Cross-linking of CD38 by an agonistic antibody impairs expansion and survival of*
11 *CD11b⁺Gr1⁺ cells in vitro and suppresses tumor growth in vivo*

12 To test whether cross-linking of CD38 with a monoclonal antibody has an
13 effect on MDSC function(s), MDSCs from spleens of tumor-bearing *p120^{-/-}* mice
14 were cultured in methylcellulose-based medium in the presence of an anti-CD38
15 monoclonal antibody (NIM-R5) or isotype control (IgG2a). Addition of anti-CD38
16 antibody inhibited growth of colonies from splenic MDSCs, and the effect of anti-
17 CD38 antibody remained unchanged regardless of whether splenocytes were
18 pre-sorted (Fig. 6A and 6B), demonstrating that the anti-CD38 antibody inhibits
19 MDSC proliferation and survival *in vitro*. In suspension culture, sorted MDSCs
20 survive only a few days, but their survival was further reduced in the presence of
21 anti-CD38 antibody (Fig. 6C). We also tested whether CD38 cross-linking
22 inhibits accumulation of CD11b⁺Gr1⁺CD38^{high} cells *ex vivo* in the presence of
23 HNM007 CM. Using an additional anti-CD38 antibody (clone 90), we observed a

1 dose-dependent decrease in CD38 expression within the CD11b⁺Gr1⁺ population
2 (Fig. 6D). Given that the proportion of CD11b⁺Gr1⁺ cells within the culture
3 remained consistent (25-30%; data not shown), these data demonstrate that the
4 CD11b⁺Gr1⁺CD38^{high} population is likely depleted as a result of CD38 cross-
5 linking. Lastly, anti-CD38 antibody treatment resulted in decreased tumor growth
6 rate *in vivo* in a subcutaneous HNM007 transplant ESCC model as compared to
7 isotype control (Fig. 6E). In aggregate, these data demonstrate the importance of
8 CD38 for MDSC-mediated ESCC progression and suggest targeting CD38 as an
9 approach to ESCC therapy.

10

11 *CD38 is expressed on human MDSC-like cell population that is expanded in*
12 *peripheral blood of advanced-stage cancer patients*

13 To determine whether our findings may be relevant to human cancers, we
14 analyzed CD38 expression in the CD15^{hi}CD33^{lo} population of PBMCs from
15 advanced stage head and neck cancer and non-small cell lung cancer patients
16 and healthy donors. In contrast to our observations in mice, we found that CD38
17 expression levels were unchanged in CD15^{hi}CD33^{lo} PBMCs from cancer
18 patients, compared to healthy donors (Supplementary Fig.7). However, this
19 population was significantly expanded from 0.5% of total PBMCs in healthy
20 donors to up to 17% in cancer patients (Fig.7).

21

1 Discussion

2 Using spontaneous genetic and syngeneic transplant tumor models, as
3 well as an *ex vivo* differentiation model, we have established for the first time that
4 tumor-derived signals drive expansion of monocytic MDSCs by inducing CD38
5 expression. Expansion of the CD11b⁺Gr1⁺CD38^{high} cell population occurs after
6 initial splenic MDSC accumulation is evident, which likely indicates a requirement
7 of threshold levels of tumor-derived signals for induction of CD38 by MDSCs
8 (Fig. 5D). Interestingly, two different ESCC cell lines exhibited differential
9 capacities to induce expansion of CD38^{high} MDSCs, thereby suggesting that the
10 tumor cells are responsible for promoting CD38 expression on MDSCs. Based
11 upon our *ex vivo* studies, the tumor-derived signals may act directly on immature
12 myeloid cell populations present in hematopoietic tissues to promote CD38
13 expression. Furthermore, our data suggest that the tumor-derived signals do not
14 promote enhanced proliferation of CD38^{high} MDSCs (RB1 pathway was activated
15 in CD38^{high} MDSCs (Supplementary Fig. 5)), but provide these cells with
16 increased survival potential.

17 Herein, we demonstrate that CD38^{high} MDSCs are halted at an earlier
18 differentiation stage compared to CD38^{low} MDSCs. Binding of cognate ligand by
19 CD38 can contribute directly to the differentiation halt (23), which suggests that
20 CD38 signaling may contribute to the maintenance of undifferentiated state
21 observed in CD38^{high} MDSCs. Although CD38 has been demonstrated to bind
22 CD31 (21), we do not know if this interaction contributes to the observed
23 properties of CD38^{high} MDSCs.

1 CD38^{high} MDSCs express elevated iNOS levels compared to CD38^{low}
2 MDSCs, and iNOS is required for T cell suppression by CD38^{high} MDSCs.
3 Interestingly, CD38 can induce iNOS upregulation in murine activated microglia
4 (resident monocytes of the brain) (35). Furthermore, *Cd38*^{-/-} mice produce less
5 tumor-associated microglia in a syngeneic transplant model of glioma (36).
6 Strikingly, we have found that in *Cd38*^{-/-} mice, subcutaneous ESCC tumors
7 induce a less pronounced expansion of M-MDSCs, regardless of the cell line
8 used to generate tumors (Supplementary Fig. 6). These findings support the
9 premise that CD38 promotes expansion of M-MDSCs, as well as elevated iNOS
10 expression. We also observed increased phospho-NFκB levels in
11 CD11b⁺Gr1⁺CD38^{high} cells. This is consistent with observations made in murine
12 B cells, where CD38 ligation activates NFκB (37). Furthermore, NFκB-mediated
13 activation of iNOS has been described in LPS-stimulated macrophages (38),
14 highlighting the possibility that elevated phospho-NFκB levels in CD38^{high}
15 MDSCs may contribute to increased expression of iNOS observed in these cells.

16 Several factors are likely to be responsible for activating CD38 expression,
17 including IFNγ, TNFα (34), as well as IL-6, IGFBP3 and CXCL16. We have
18 demonstrated that IFNγ and TNFα induce bone marrow-derived CD11b⁺Gr1⁺
19 cells to express CD38 *ex vivo*. As both IFNγ and TNFα are often produced
20 during chronic inflammation, they may be primary inducers of CD38 expression
21 (Fig. 5D). In fact, TNFα inhibition can impair immunosuppressive capacity of
22 MDSCs and induce differentiation in a murine model of chronic inflammation,
23 while MDSCs from *Tnf*^{-/-} mice have reduced iNOS levels (39).

1 Our finding of a CXCL16 and IGFBP-3-mediated response in MDSCs has
2 not been described previously. However, CXCL16 expression can be promoted
3 by IFN γ and TNF α (40), the two most potent inducers of CD38 expression in our
4 *ex vivo* system. Interestingly, IGFBP-3 has been shown to increase intracellular
5 Ca²⁺ levels *in vitro* (41). Ca²⁺ signaling, which can be mediated by ectoenzymatic
6 activity of CD38 (34), is important for multiple immunomodulatory processes
7 (42,43). Therefore, it is possible that in MDSCs IGFBP-3 can be modulating Ca²⁺
8 mobilization by increasing CD38 expression.

9 IL-6 is a major regulator of STAT3 signaling, which is essential for
10 establishment of immunosuppressive microenvironment within the tumor (44). In
11 MDSCs, STAT3 activation enhances production of the S100A8/A9 pro-
12 inflammatory proteins, which also contribute to maintenance of a low
13 differentiation or immature state (45). These data are in agreement with our
14 observation that IL-6 can promote CD38 expression on MDSCs generated *ex*
15 *vivo*, since CD38^{high} MDSCs are less differentiated than CD38^{low} MDSCs (Fig.
16 4B).

17 Herein, we demonstrate the efficacy of anti-CD38 monoclonal antibody
18 treatment *in vitro* and *in vivo*. Moreover, we report CD38 expression by human
19 MDSCs; therefore, anti-CD38 therapy may represent a novel approach to
20 targeting this immunosuppressive population in cancer treatment strategies.
21 Furthermore, since CD38^{high} Tregs possess enhanced suppressive potential
22 compared to CD38^{low} Tregs (18,19), anti-CD38 therapy may present the
23 advantage of targeting several immunosuppressive cell types at the same time.

1 Recently, an anti-CD38 monoclonal antibody (Daratumumab) was shown to be
2 efficient in treatment of multiple myeloma in pre-clinical studies (46). A similar
3 approach may induce ablation of MDSCs in patients with advanced stage solid
4 cancers, and thus, may be suitable as an adjuvant to conventional therapies. The
5 expression pattern of CD38 in a broad range of cell types can raise a concern
6 about potential adverse effects of anti-CD38 therapy (47), however, early clinical
7 studies of Daratumumab in multiple myeloma have demonstrated an acceptable
8 safety profile, suggesting that an appropriate dosage and treatment schedule
9 allow for minimizing of the effects of targeting CD38 in normal tissue (48).

10 MDSCs contribute to the T cell suppression repertoire found in cancer,
11 which merits further investigation as a prospective therapeutic target (49). In this
12 study, we have identified CD38 as being suitable for potential MDSC targeting
13 and useful in identification of potentially immunosuppressive MDSC populations.
14 Thus, anti-CD38 monoclonal antibody therapy (46) may hold potential for
15 targeting CD38-expressing MDSCs (50) in patients with certain types of cancer.

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9

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Figure 1. CD38 is significantly upregulated in CD11b⁺Gr1⁺ cells from tumor-bearing *p120*^{-/-} mice. (A) Heatmap illustrating the results of a microarray analysis performed using CD11b⁺Gr1⁺ cells sorted from the spleens of 6 tumor-bearing *p120*^{-/-} mice and 3 pooled samples from healthy littermate controls (n=9). Increased expression of the *Cd38* gene and protein in CD11b⁺Gr1⁺ cells from tumor-bearing mice was confirmed by (B) qPCR (*p=0.007) and (C) FACS (n= 3; *p=0.009). (D) Frequencies of CD38⁺ cells (*p=0.003).

Figure 2. CD38 expression increases in monocytic myeloid cells with disease progression. (A) Splenocytes from healthy control, non-diseased (ND) *p120*^{-/-} and tumor-bearing (TB) *p120*^{-/-} mice were analyzed by FACS for CD38 expression on myeloid cell populations. (B) Histograms comparing CD38-FITC fluorescence levels on two cell subsets from control, *p120*^{-/-} non-diseased and *p120*^{-/-} tumor-bearing mice. (C) Splenocytes from control non-diseased and AKR or HNM007 subcutaneous tumor-bearing C57BL/6 mice analyzed by FACS. Histograms compare CD38 expression levels in listed subpopulations from control and tumor-bearing mice. (D) Splenocytes from control and tumor-bearing mice were analyzed by FACS for distribution of CD11b, Ly6C, Ly6G and CD38 antigens. Pie charts demonstrate the frequencies of lymphoid (CD11b⁻) and myeloid (CD11b⁺) cell populations in spleens of control and tumor-bearing mice with the myeloid population further broken down into Ly6C⁺, Ly6G⁺ and Ly6C⁻ Ly6G⁻ subsets (n=3 per group).

Figure 3. CD38^{High} MDSCs are more immunosuppressive and promote tumor growth more efficiently than the CD38^{Low} MDSCs. (A) CD38^{high} and CD38^{low} MDSCs from tumor-bearing *p120*^{-/-} mice were used in a T cell suppression assay (n=3; *p=0.0007). (B) C57BL/6 mice were injected with HNM007 cells in combination with MDSCs (CD38^{High} or CD38^{Low}) or alone (n=5 per group). Tumor volumes were compared between the CD38^{High} and CD38^{Low} groups (*p=0.004 and 0.03), and between CD38^{High} and control HNM007 tumors (** p=0.01, 0.003 and 0.01). (C) Representative H&E and CD45 immunohistochemistry of CD38^{High}-injected, CD38^{Low} or control tumors. (D) Tumors were scored for abundance of necrotic areas and inflammatory infiltrate on the scale 0-4. (E) Splenic MDSCs from HNM007 tumor-bearing *Cd38*^{-/-} or *wt* mice were used in a T cell suppression assay (*p=0.003 and 0.04).

Figure 4. CD38^{High} MDSCs are phenotypically different from the CD38^{Low} subset. (A) Heatmap illustrating the results of a microarray analysis performed using CD38^{High} and CD38^{Low} CD11b⁺Gr1⁺ cells sorted from spleens of 4 tumor-bearing *p120*^{-/-} mice. (B) qPCR analysis of *iNos*, *Arg1* and *Nox2* gene expression (*p=9x10⁻⁸). (C) Western blot analysis of iNOS and phospho-NFκB protein levels in CD38^{High} and CD38^{Low} MDSCs. (D) iNOS inhibitor (L-NMMA) and (E) CD38 inhibitor (AraF-NAD) were tested in a T cell suppression assay (*p=0.004 and 0.04, respectively). (F) Expression levels of iNOS in splenic MDSCs from tumor-bearing *Cd38*^{-/-} or *wt* mice were assessed by FACS. (G) Cytospin preparations from CD38^{high} and CD38^{Low} MDSCs.

Figure 5. IFN γ , TNF α , IGFBP-3, CXCL16 and IL-6 induce CD38 expression and impair myeloid cell differentiation. (A) CD38 expression in CD11b⁺Gr1⁺ cells from *ex vivo* differentiation cultures was tested by FACS. Results are presented as mean fluorescence intensity (MFI) (n=3; * p \leq 0.0001, **p=2.5x10⁻⁵). (B) Cytokine array performed with media from *ex vivo* differentiation cultures (24 or 120-hour). Each cytokine tested in duplicate. Difference in normalized expression between HNM007 and AKR groups is shown. (C) *Ex vivo* differentiation as in (A) with the addition of cytokines to the AKR conditioned media (n=3; *p<0.05, ** p<0.005). (E) In mice, early stages of cancer initiation and progression lead to MDSC expansion. Tumor progression leads to amplified signals (such as cytokines) reaching MDSCs, which induces a differentiation halt and expansion of CD38^{High} monocytic MDSCs with enhanced immunosuppressive capacity (mediated by iNOS, which produces nitric oxide (NO)).

Figure 6. Cross-linking of CD38 by an agonistic antibody impairs expansion and survival of CD11b⁺Gr1⁺ cells *in vitro* and suppresses tumor growth *in vivo*. (A) Representative images from methylcellulose cultures of CD11b⁺Gr1⁺ cells treated with anti-CD38 monoclonal antibody (NIMR-5) or isotype control (IgG2a), after 5 days of culture. (B) Number of colonies formed following 7 days of culture (n=3; *p=4x10⁻⁵). (C) CD11b⁺Gr1⁺ cells were cultured in RPMI with anti-CD38 or isotype control antibody, and counted at indicated time points. (n=6

per group; * $p < 5 \times 10^{-7}$, ** $p < 2 \times 10^{-7}$, *** $p < 0.0005$). (D) *Ex vivo* differentiation performed with HNM007 conditioned medium and anti-CD38 agonist (NIM-R5) or isotype control (IgG2a) antibody. CD38 expression (using the clone 90 antibody) on the surface of CD45⁺7-AAD⁻CD11b⁺Gr1⁺ was measured by FACS (n=3 per group; * $p < 0.003$, ** $p < 0.0005$). (E) HNM007 tumor growth kinetics in C57BL/6 mice treated with anti-CD38 (NIM-R5) or isotype control (IgG2a) antibody (start of treatment is marked by an arrow, n=6 per group; * $p = 0.005$, 0.005 and 0.04).

Figure 7. CD38⁺ MDSC-like population is expanded in the peripheral blood of advanced-stage cancer patients. Histograms depict frequencies of CD38⁺CD15^{high}CD33^{low} cells in peripheral blood mononuclear cells (PBMC) from head and neck (HNC) and non-small cell lung (NSCLC) cancer patients and healthy donors.

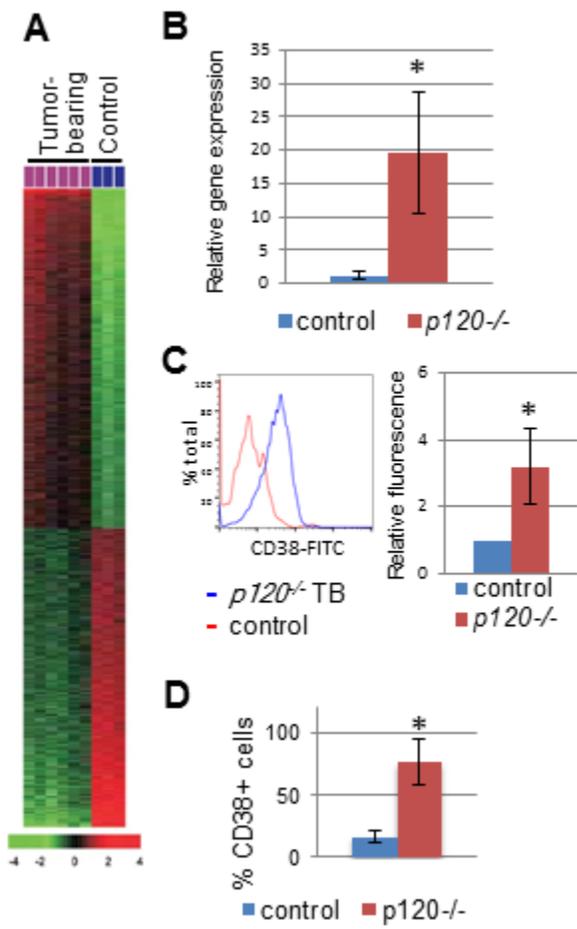


Figure 1

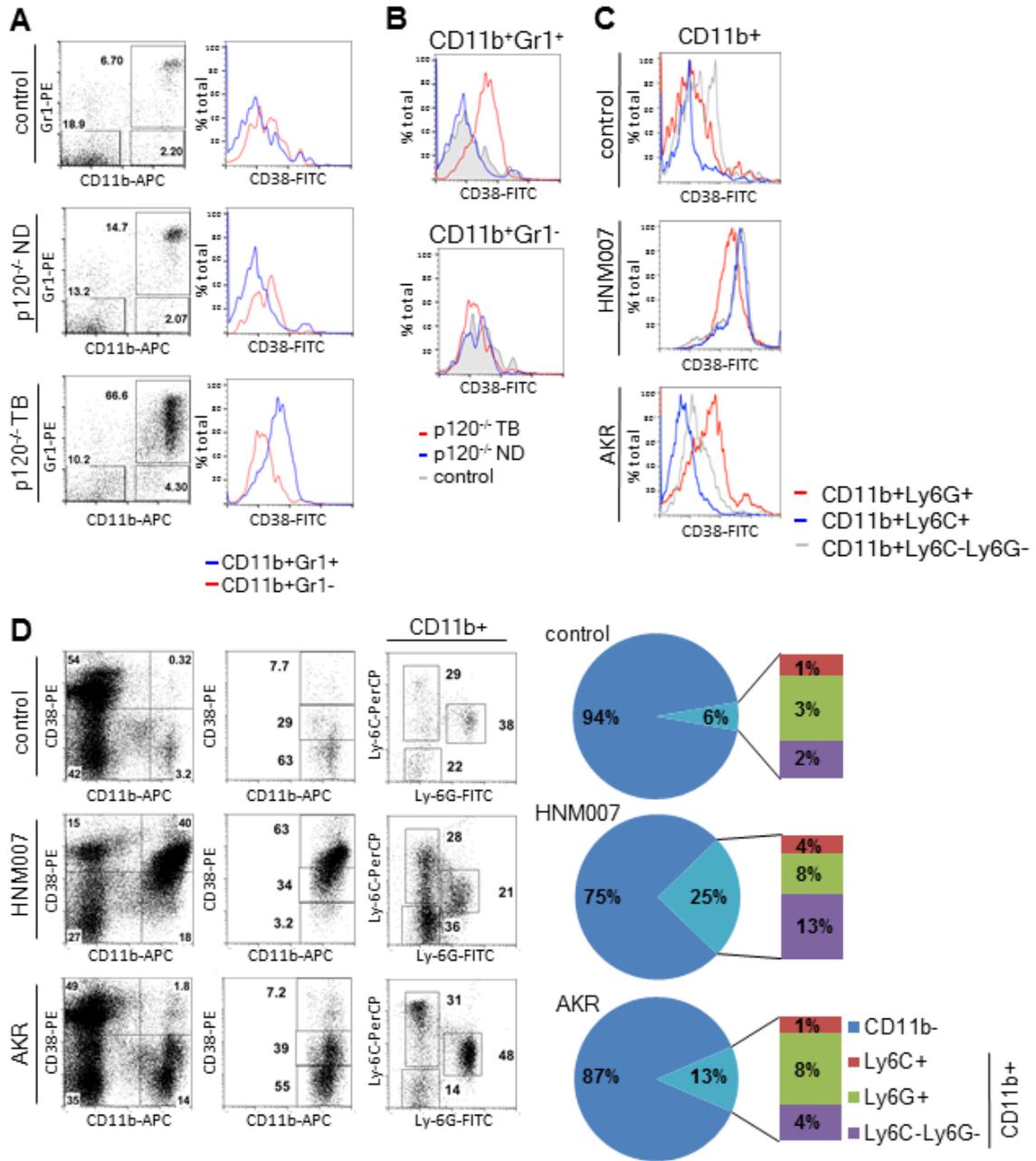


Figure 2

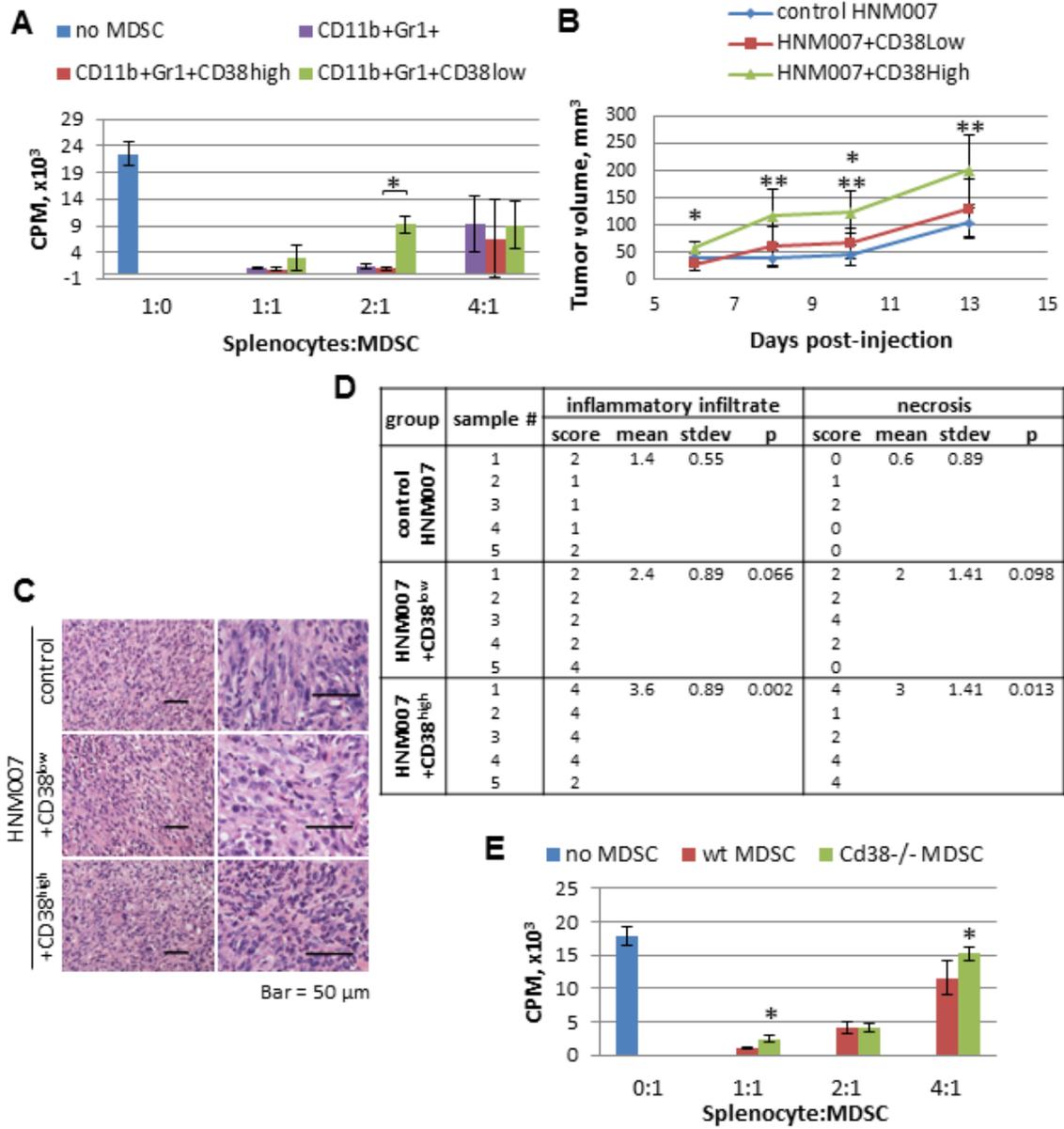


Figure 3

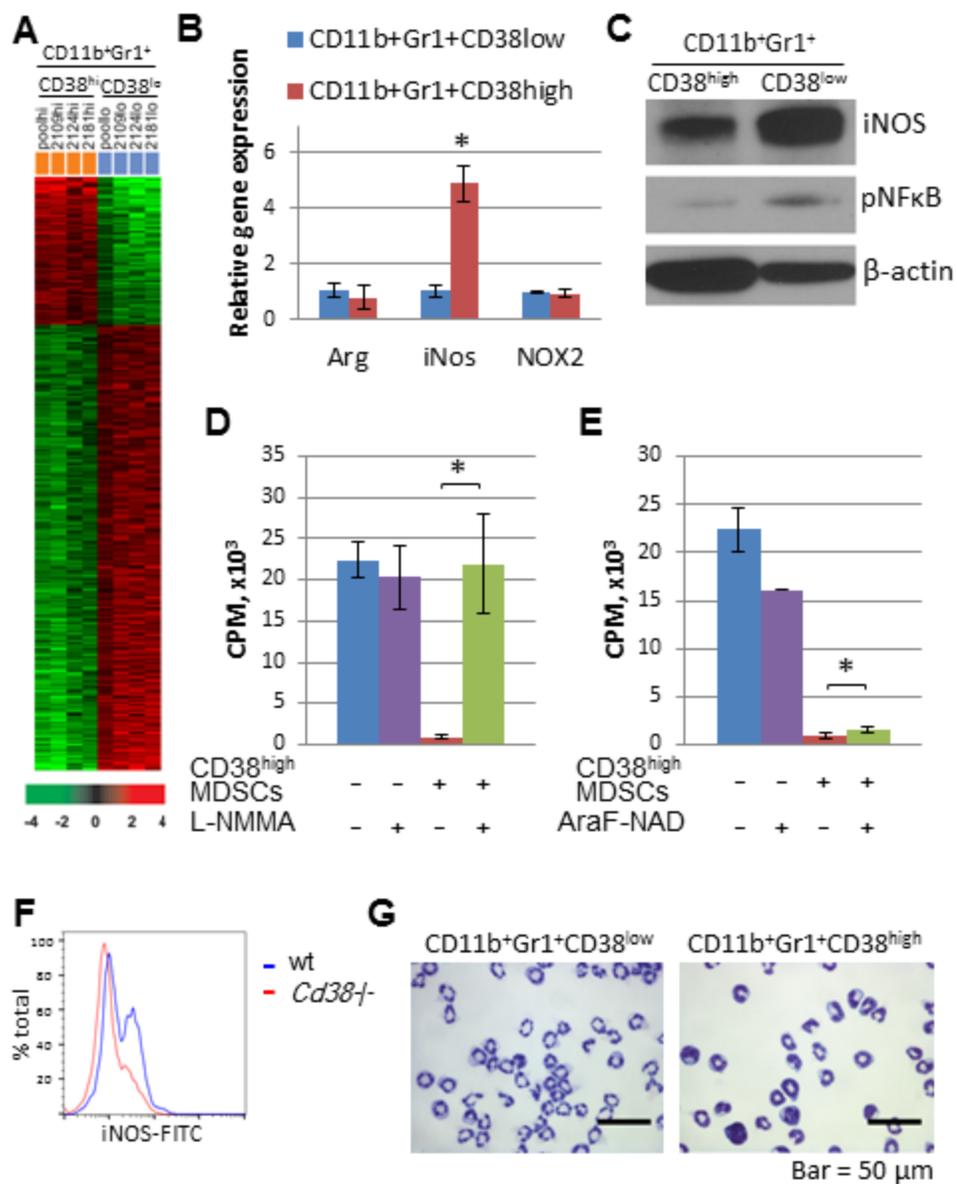


Figure 4

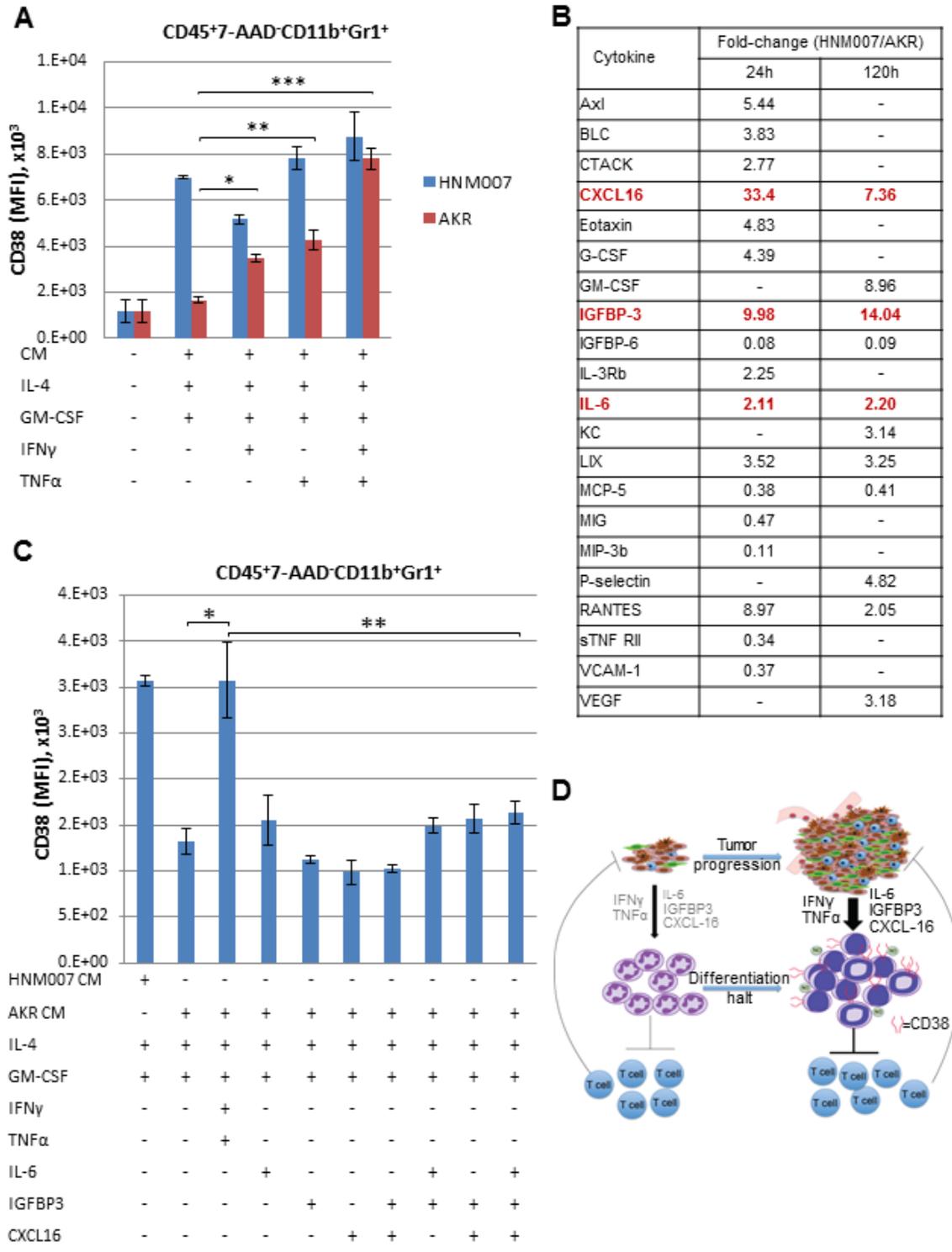


Figure 5

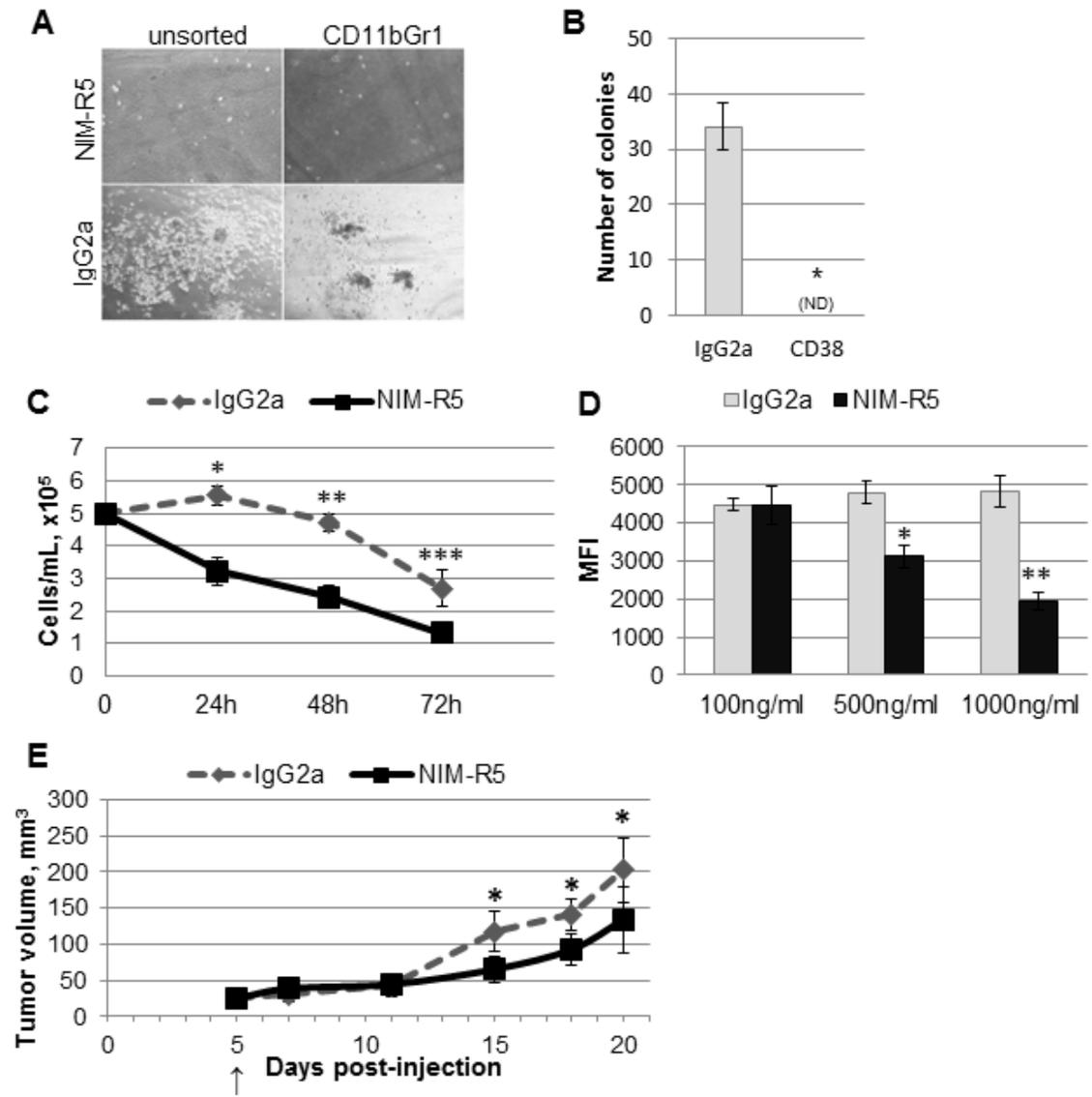


Figure 6

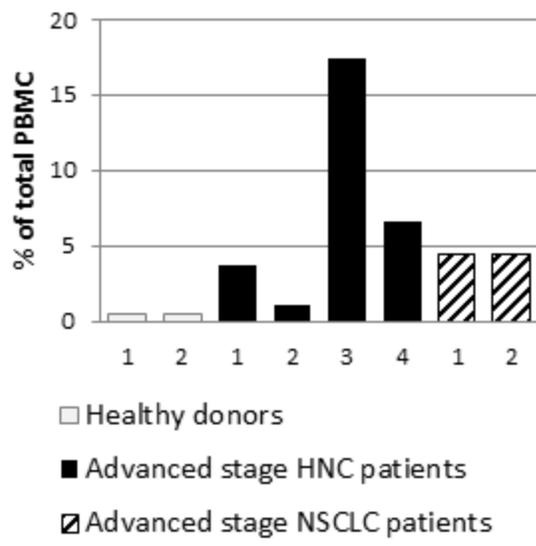


Figure 7