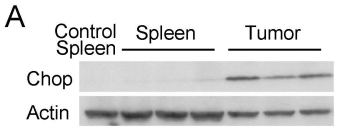
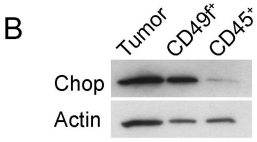
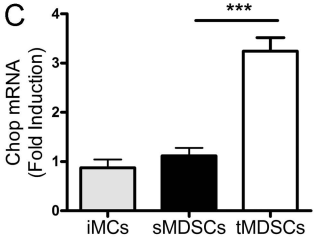
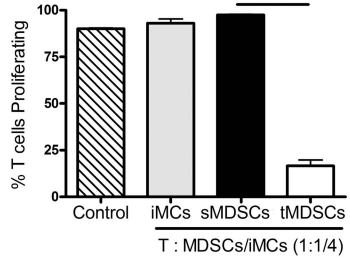
Figure S1



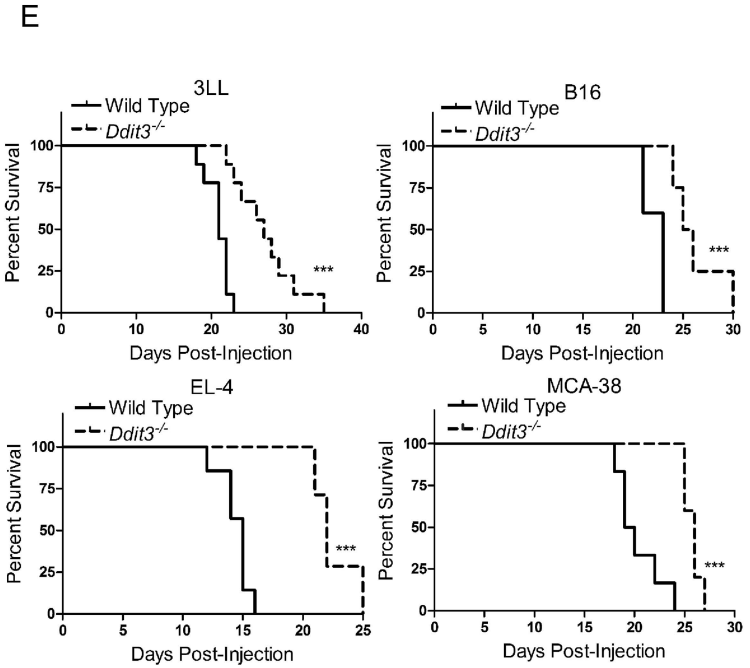


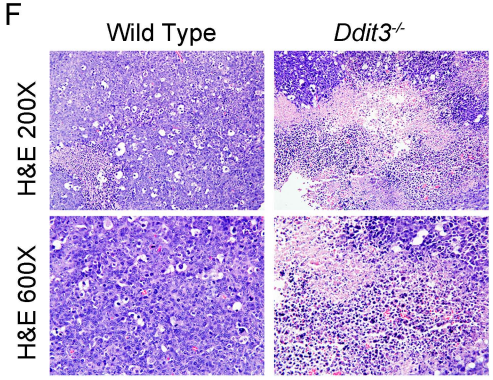


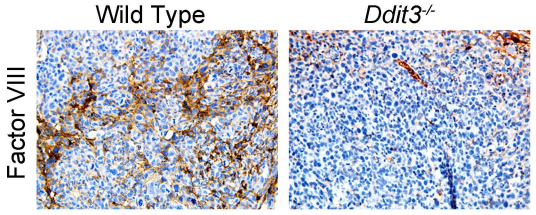


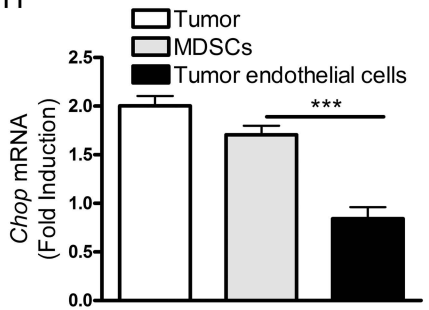
D

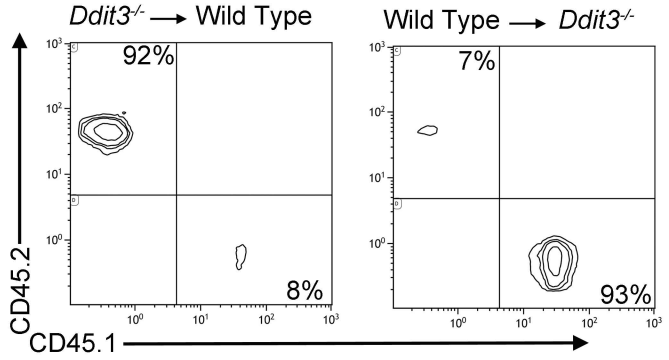
Sample	Tumor CD33		Tumor Chop
	Section 1*	Section 2*	Section 1/2**
1	2	0	-
2	54	35	+
3	2	0	+++
4	37	42	+
5	10	12	-
6	1	3	+++
7	7	28	+
8	0	0	++
9	0	0	+++
10	5	7	+
11	8	6	+
12	0	0	++
13	12	52	-
14	0	0	+
15	14	11	-
16	48	60	+
17	20	22	++
18	17	14	+
19	5	33	++
20	0	0	+
21	17	15	+
22	0	13	++
23	10	9	++
24	2	0	++











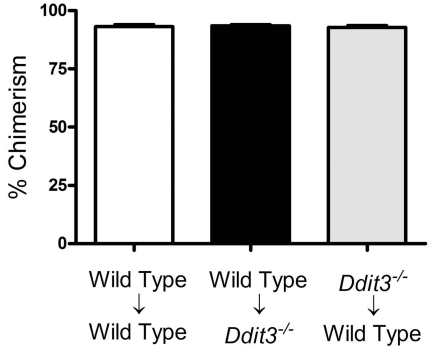


Figure S2

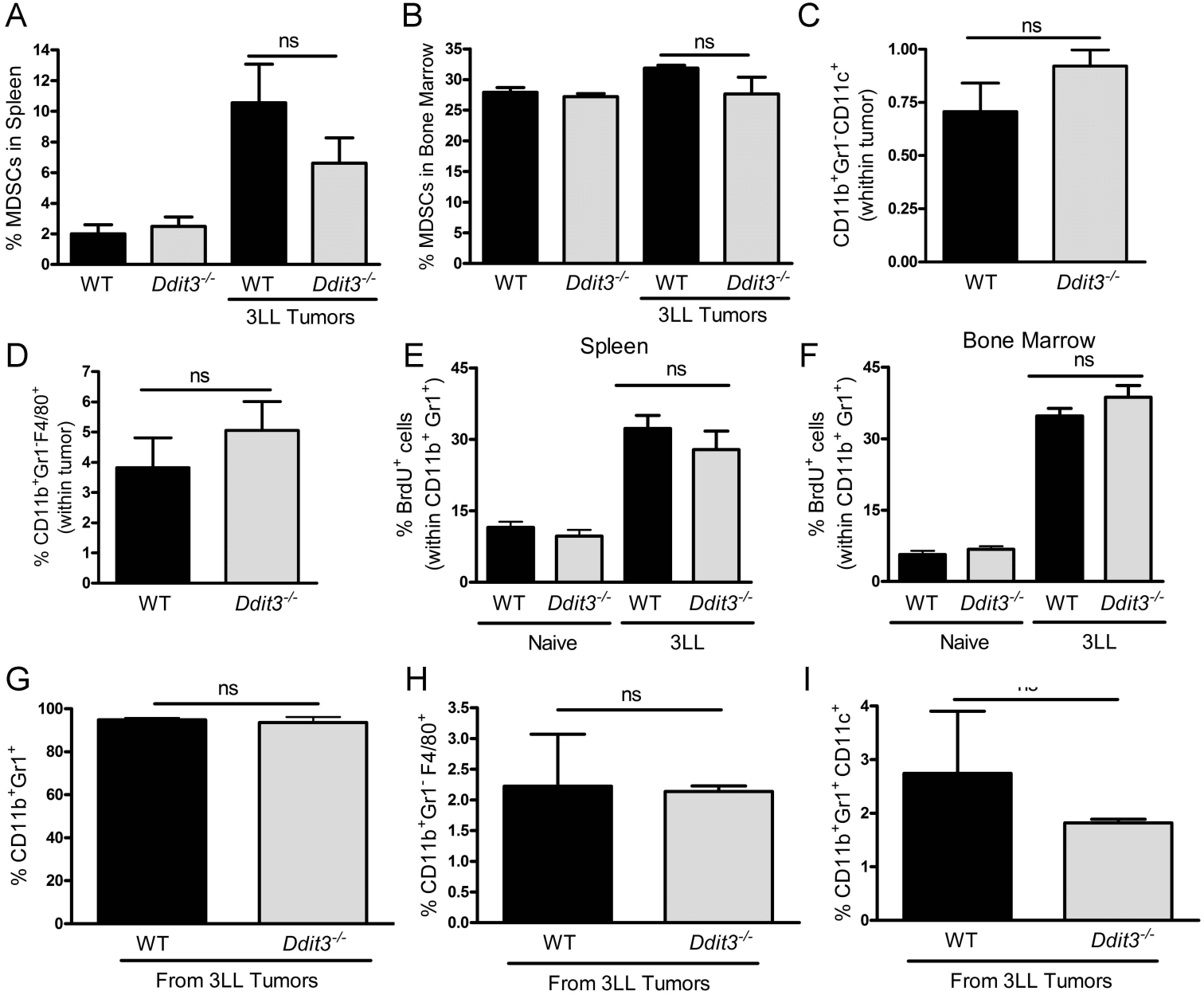

A
% MDSCs in Spleen
ns
WT
Ddit3-/-
WT
Ddit3-/-
3LL Tumors
B
% MDSCs in Bone Marrow
ns
WT
Ddit3-/-
WT
Ddit3-/-
3LL Tumors
C
CD11b+Gr1-CD11c+ (whithin tumor)
ns
WT
Ddit3-/-
D
% CD11b+Gr1-F4/80+ (within tumor)
ns
WT
Ddit3-/-
E
Spleen
% BrdU+ cells (within CD11b+ Gr1+)
ns
WT
Ddit3-/-
WT
Ddit3-/-
Naive
3LL
F
Bone Marrow
% BrdU+ cells (within CD11b+ Gr1+)
ns
WT
Ddit3-/-
WT
Ddit3-/-
Naive
3LL
G
% CD11b+Gr1+
ns
WT
Ddit3-/-
From 3LL Tumors
H
% CD11b+Gr1- F4/80+
ns
WT
Ddit3-/-
From 3LL Tumors
I
% CD11b+Gr1+ CD11c+
WT
Ddit3-/-
From 3LL Tumors

Figure S3

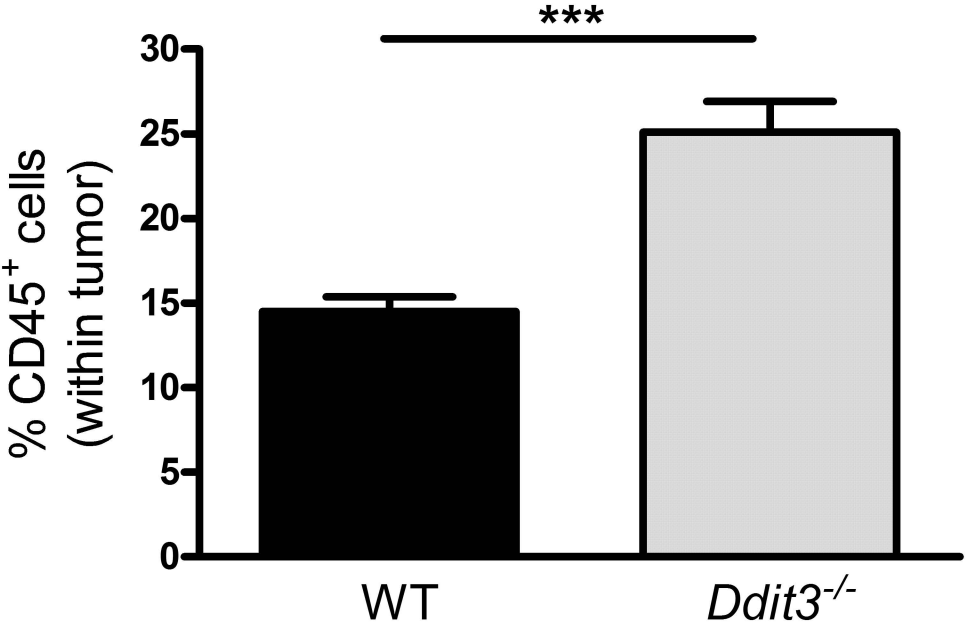


Figure S4

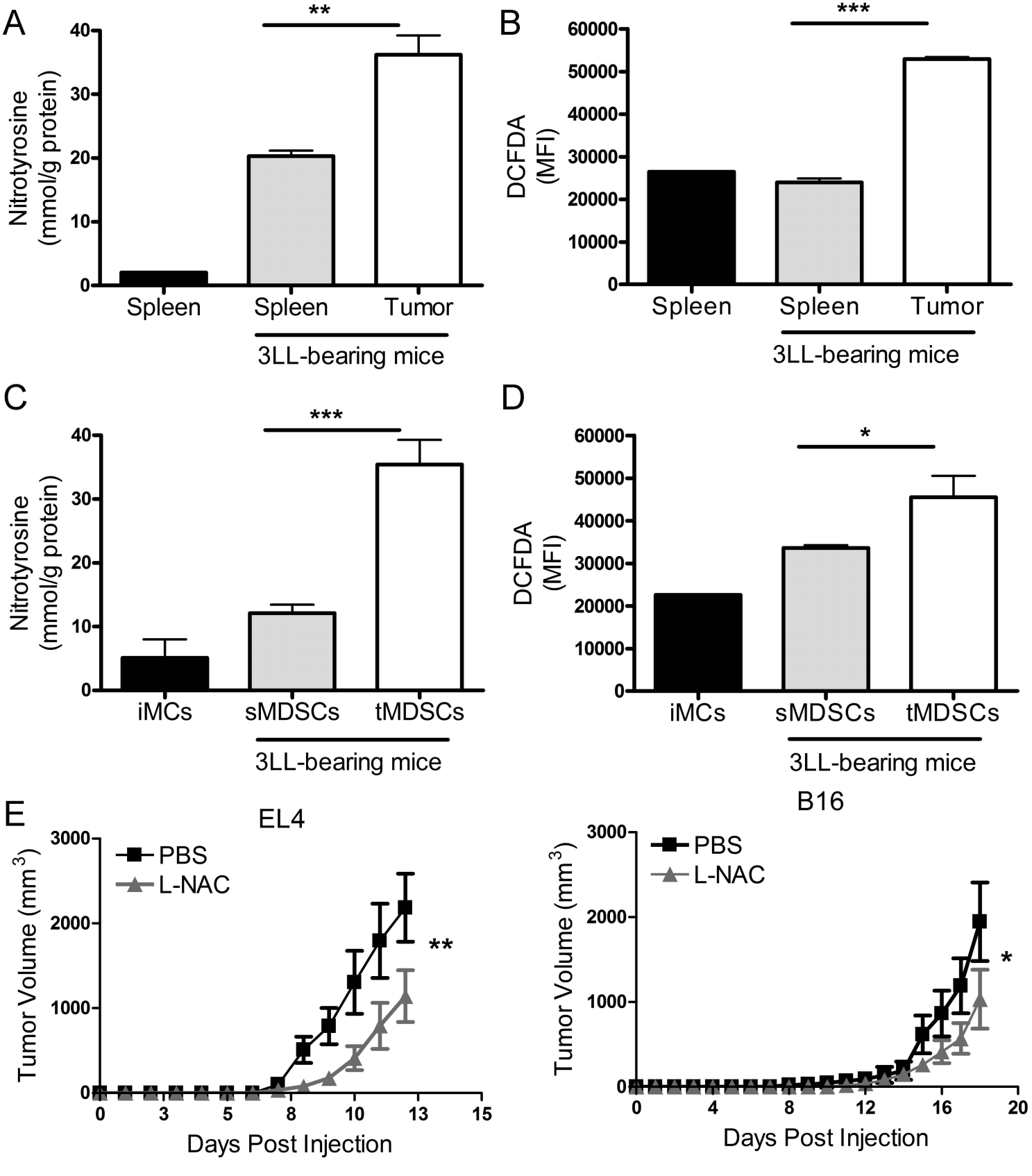
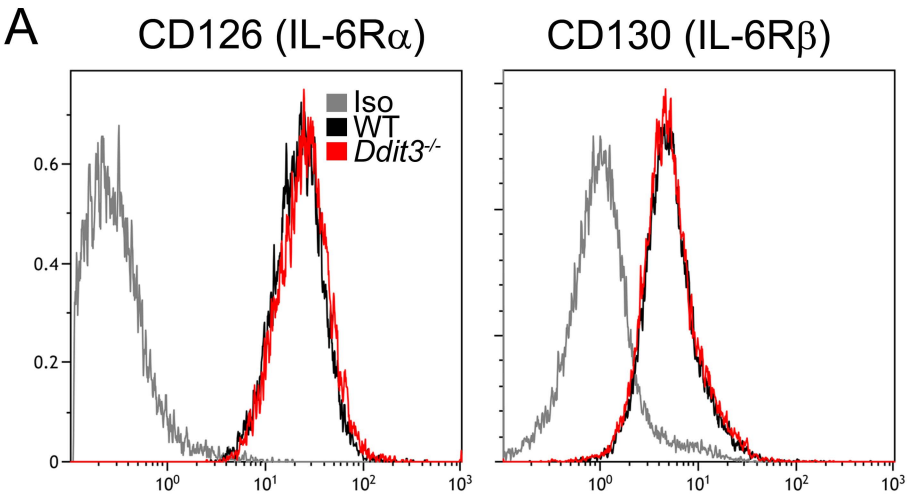
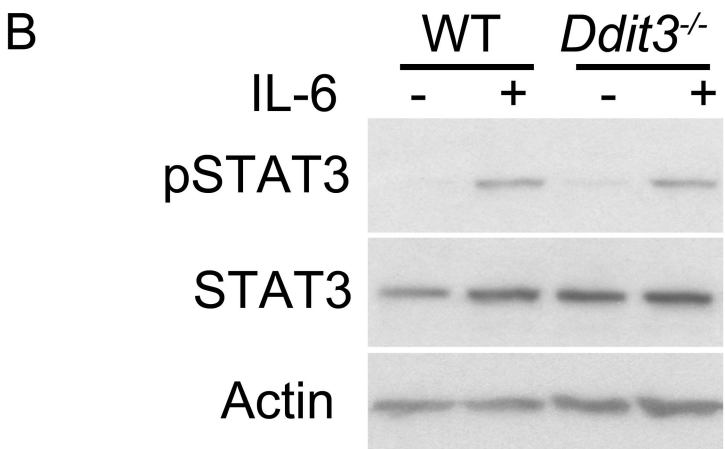

A
Nitrotyrosine (mmol/g protein)
**
Spleen
Spleen
Tumor
3LL-bearing mice
B
DCFDA (MFI)
***
Spleen
Spleen
Tumor
3LL-bearing mice
C
Nitrotyrosine (mmol/g protein)
***
iMCs
sMDSCs
tMDSCs
3LL-bearing mice
D
DCFDA (MFI)
*
iMCs
sMDSCs
tMDSCs
3LL-bearing mice
E
EL4
PBS
L-NAC
Tumor Volume (mm3)
**
Days Post Injection
B16
PBS
L-NAC
Tumor Volume (mm3)
*
Days Post Injection

Figure S5


A
CD126 (IL-6Rα)
CD130 (IL-6Rβ)
Iso
WT
Ddit3-/-
0.6
0.4
0.2
0
10^0
10^1
10^2
10^3


B
WT
Ddit3-/-
IL-6
-
+
-
+
pSTAT3
STAT3
Actin

Supplemental data

Supplemental Figure legends

Figure S1, related to Figure 1: Tumor-infiltrating MDSC preferentially expressed Chop and Chop deletion prolongs survival and impairs angiogenesis in tumor-bearing mice.

(A) Chop expression in tumor digests and splenocytes from 3LL tumor-bearing mice and spleens from control mice without tumors.

(B) Chop expression in ($CD49f^+$ $CD45^-$) tumor cells and tumor-infiltrating leukocytes ($CD49f^-$ $CD45^+$) sorted from tumor single cell suspensions 18 days after s.c. 3LL injection. Blots depict a representative experiment of 3 independent experiments.

(C) RNA expression of Chop and T cell suppression assay for MDSCs sorted from single cell suspensions of tumor (tMDSCs) or spleen (sMDSCs) of 3LL tumor-bearing mice and immature myeloid cells (iMCs) sorted from naïve spleens.

(D) Percentage of human colon adenocarcinoma biopsy samples positive for CD33 and Chop. * Number of $CD33^+$ cells per 4 fields of 40x (section/tissue core). ** -, negative reactivity; +, 1-30% cell positivity; ++, 31-60% cell positivity; +++, >61% cell positivity

(E) Survival of wild type or *Ddit3*$^{-/-}$ mice injected with s.c. 3LL lung carcinoma, B16 melanoma, EL-4 thymoma, or MCA-38 colon carcinoma. Tumor growth kinetics were measured by caliper until tumor volume reached a 3000 mm^3. Cumulative values of three independent experiments of 5 replicates per experiment per group.

(F) H&E staining of 3LL tumor cross-sections obtained 17 days after tumor injection depicting large areas of necrosis with stromal cell infiltrate in 3LL tumors isolated from *Ddit3*$^{-/-}$ mice.

(G) Tumor immunostaining of Factor VIII (von Willebrand Factor) with notably decreased expression in 3LL tumors from *Ddit3*$^{-/-}$ mice. Histology images depicted are representative tumors from a panel of 5 replicates from 3 independent experiments.

(H) Expression of Chop mRNA in whole tumor, CD11b^{+}Gr-1^{+} sorted MDSCs, and CD45^{-} CD11b^{-} CD31^{+} tumor endothelial cells. RNA expression experiments were performed on 3 replicates pooled from cells sorted from 3 independent experiments.

(I) Representative contour plot of 5 merged blood samples obtained from wild type CD45.1^{+} chimeric mice and *Ddit3*$^{-/-}$ CD45.2^{+} chimeric mice. Blood samples were stained for CD45.1^{+} and CD45.2^{+} to discriminate donor- from host-derived cells.

(J). Percentage efficiency from A was calculated as CD45^{+} group of interest/total positive CD45^{+} * 100% and recorded seven weeks after lethal radiation and bone marrow reconstitution.

Figure S2, related to Figure 2: Chop deletion does not alter accumulation or proliferation of MDSCs in bone marrow and spleen.

(A-B) Percentage of MDSCs (CD11b^{+} Gr1^{+} F4/80^{-} CD11c^{-}) in the spleen and bone marrow of tumor-free and 3LL tumor-bearing wild type and *Ddit3*$^{-/-}$ mice.

(C-D) Percentage of DCs (CD11b^{+} Gr1^{-} CD11c^{+} MHC-II^{+}) and tumor associated macrophages (CD11b^{+} Gr1^{-} F4/80^{+}) in 3LL tumors from wild type and *Ddit3*$^{-/-}$ mice. Plots depict a representative experiment of n=5 per group from 3 independent experiments.

(E-F) Percentage of proliferating MDSCs (CD11b^{+} Gr1^{+} BrdU^{+}) in the spleen and bone marrow of tumor-free and 3LL tumor-bearing wild type and *Ddit3*$^{-/-}$ mice.

(G-I) Percentage purity of MDSCs (CD11b^{+} Gr1^{+}), macrophages (CD11b^{+} Gr1^{-} F4/80^{+}), and MDSCs co-expressing CD11c^{+} after isolation of MDSCs from 3LL tumor digests.

Figure S3, related to Figure 4: Increased leukocyte infiltration in tumor-bearing Chop deficient mice.

Percentage of $CD45^+$ cells in 3LL tumors from wild type and *Ddit3*$^{-/-}$ mice. Values graphed are a representative experiment from 3 replicates (n=15).

Figure S4, related to Figure 5: Antioxidant therapy with L-NAC slows tumor progression.

(A) Levels of nitrosylated protein in single cell lysates prepared from tumor digests and spleens of 3LL tumor-bearing mice and from naïve splenocytes.

(B) Production of ROS measured by DCFDA in single cell lysates prepared from tumor digests and spleens of 3LL tumor-bearing mice and from naïve splenocytes.

(C-D) Production of PNT and ROS were detected in tumor-MDSCs, splenic MDSCs (3LL-bearing mice) and iMCs (control spleen) as (A) and (B), respectively.

(E) Tumor growth in wild type mice s.c. injected with EL-4 or B16 treated daily with i.p. injection of PBS or L-NAC (1 mg/kg/day).

Figure S5, related to Figure 7: Chop deficiency does not affect IL-6 receptor expression or IL-6 signaling in MDSCs.

(A) Histograms of CD126 (left panel) and CD130 (right panel) after gating $CD11b^+$ $Gr1^+$ tumor MDSCs from wild type (black) and *Ddit3*$^{-/-}$ (red) tumors. Control isotype in grey. Histograms depicted are of n=5 replicates merged to one data file. Experiment depicted is representative of 3 independent experiments.

(B) Phosphorylation of STAT3 in wild type and *Ddit3*$^{-/-}$ MDSCs after 8 hours of culture with recombinant murine IL-6 (50 ng/mL).

Supplemental Experimental Procedures

Animals, Cell lines, and Reagents

C57BL/6 mice (6 to 8-wk-old female) were obtained from Harlan (Indianapolis, IN). OT-1, *Ddit3*$^{-/-}$, CD45.1, *Gp91*$^{phox-/-}$, and *Atf4*$^{+/-}$ mice were purchased from the Jackson Laboratories (Bar Harbor, ME). 3LL Lewis lung carcinoma, B16 melanoma, MCA-38 colon carcinoma, and EL-4 thymoma cells (American Type Culture Collection, Manassas, VA) were injected into the mice, as we described (Raber et al., 2013). Ovalbumin or IL-6-expressing 3LL cells (3LL-OVA, or 3LL-*IL-6*) were generated by transfection using Lipofectamine 2000 (Life Technologies) with vectors coding for cytosolic chicken ovalbumin (Yang et al., 2010) or IL-6 (Manderson et al., 2007), and harboring a neomycin resistance cassette (Addgene). 3LL clones were selected in medium supplemented with 500 µg/ml Geneticin. Bone marrow-derived MDSCs (BM-MDSCs) were generated by culturing BM cells for 3 days in the presence of G-CSF (100 ng/mL) and GM-CSF (20 ng/mL). For MDSCs depletion experiments, depleting antibody to Gr-1 (clone RB6-8C5, BioXcell) was administered i.p. 200 µg/dose on day 0 and every 4th day until tumor endpoint. For CD4^{+} or CD8^{+} T cell depletion studies, mice were pre-treated 24 hours before tumor injection with 400 µg anti-CD4 (clone GK1.5) or anti-CD8 (clone 53.6.72). Maintenance doses of the depleting antibodies were given twice a week. Cytotoxic effects of tumor MDSCs on 3LL cells *in vitro* were determined by non-radioactive cytotoxicity assay using LDH (Promega, Madison, WI). L-NAC (1 mg/kg/day) was injected i.p. starting at day 1 posttumor injection. Tumor volume was measured using calipers and calculated using the formula [(small diameter)2 x (large diameter) x 0.5]. Experiments using animals were approved by the LSU-IACUC and were performed following LSU animal care facility guidelines.

Antibodies

Purified antibodies against arginase I (clone 19) and iNOS (54/iNOS), fluorochrome conjugated antibodies against CD3ζ (G-3), CD8 (53-6.7), CD25 (PC61), Foxp3 (MF23), CD49f (GoH3), CD45.1 (A20), Gr-1 (RB6-8C5), CD11b (M1/70), CD11b (ICRF44), CD45.2 (104), Ly6G (1A8), Ly6C (AL-21), IFNγ (XMG1.2), MHCI (KH95), MHCII (AF6-120.1), CD11c (HL3), and 5-bromo-2-deoxyuridine (BrdU) labeling kit were obtained from Becton Dickinson Biosciences (BD Biosciences, San Jose, CA). Anti-β-actin antibody (AC-74) was obtained from Sigma-Aldrich (St. Louis, MO). Anti-C/EBPβ (C-19) and Chop (R-20) were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-eIF2α (eIF2α) was obtained from Life Technologies. Antibodies against peIF2α (E90), and p84 (5E10) were purchased from Abcam (Cambridge, MA). Antibodies against cleaved caspase 3 (5A1E), caspase 3 (8G10), p-STAT3 (3E2), STAT3 (124h6), and Atf4 (D4B8) were obtained from Cell Signaling (Beverly, MA). Anti-Chop (9C8) was obtained from Thermo Scientific. Antibodies against CD126 (D7715A7), CD130 (KPG130), IL-6 (MP5-20F3), CD33 (WM-53), HLA-DR (L243) were obtained from ebioscience, while anti-F4/80 (BM8) was purchased from Biolegend (San Diego, CA).

Bone Marrow Chimeras

Recipient mice lethally irradiated with 950 rads (2 rounds of 475 rads during the same day) were reconstituted with 1 x 10^7 bone marrow cells and 1 x 10^6 splenocytes from donor mice (Loinard et al., 2012). Chimeric engraftment was verified in peripheral blood 7 weeks after transplantation by monitoring the corresponding switch from $CD45.1^+$ cells into $CD45.2^+$ or from $CD45.2^+$ into

CD45.1^{+} using flow cytometry. A week later, mice were injected s.c. with 3LL tumor cells and tumor growth kinetics and MDSCs suppressive activity evaluated.

Tolerogenic effect of MDSCs

To determine the effect of Chop in the tolerogenic activity of MDSCs *in vivo*, we used a model previously described (Dolcetti et al., 2010). Briefly, CD8^{+} T cells (5 x 10^{6}) from CD45.2^{+} OT-1 mice were adoptively transferred via tail vein into CD45.1^{+} mice. Two days later MDSCs were sorted from 3LL tumor-bearing wild type or *Ddit3*$^{-/-}$ mice, pulsed with 2 μg/mL SIINFEKL for 1 hour, and 5 x 10^{6} MDSCs transferred i.v. into the mice previously injected with OT-1. The same day, mice received s.c. vaccination with 4 x 10^{6} DCs generated from bone marrow cells cultured in medium containing GM-CSF (20 ng/mL) and IL-4 (10 ng/mL) for 6 days. During the final 24 hours of culture, DCs were exposed to 2 μg/mL SIINFEKL and 1μg/mL LPS. Mice received a second injection with SIINFEKL-pulsed MDSCs 5 days later. Twelve days after the initial DCs immunization, draining lymph nodes were recovered and challenge with SIINFEKL for 24 hours, after which they were monitored for IFNγ production by Elispot (R & D systems).

Adoptive Cellular therapy

For T cell immunotherapy experiments, CD45.2^{+} wild type or *Ddit3*$^{-/-}$ mice were injected with 3LL-OVA cells (1 x 10^{6}) at day 0 or at stratified time points to achieve tumor of similar palpable volume. Mice then received adoptive transfer of 5 x 10^{6} CD45.1^{+} CD8^{+} OT-1 cells via tail vein injection. The following day, mice were vaccinated s.c. with 100 μg of SIINFEKL peptide in 0.2mL of PBS. Ten days later spleens and tumors were tested for transferred OT-1 cells and function.

Chromatin Immunoprecipitation

ChIP assays were performed using SimpleChip kits (Cell Signaling). Chromatin was prepared from 4 x 10^6 cells MDSCs positively selected from tumor and spleens of wild type and *Ddit3*$^{-/-}$ bearing 3LL tumors or splenic iMCs from control mice. Chromatin was immunoprecipitated with antibodies against Atf4, C/EBPβ, Histone H3, or rabbit IgG. Eluted and purified DNA was analyzed by qPCR with pre-validated primers against the Chop promoter (Atf4), IL-6 promoter (C/EBPβ) and Arginase I promoter (C/EBPβ) purchased from Qiagen.

Sorting of cells

Tumors were digested with DNAse and Liberase (Roche USA, Branchburg, NJ). Tumor digest were then used to isolated different tumor populations by flow cytometry. 3LL tumor cells were recovered by sorting CD49f$^+$ CD45$^-$ cells. Tumor leukocytes were gated as CD49f$^-$ CD45$^+$, and subdivided into the following: CD11b$^+$ Gr1$^+$, CD11b$^+$ Gr1$^-$, CD11b$^+$ CD11c$^+$, CD11b$^+$ F4/80$^+$, B220$^+$, and CD3$^+$. For functional assays, MDSCs were isolated as described (Rodriguez et al., 2005). Purity for each population ranged from 90%-99%, as measured by flow cytometry. In MDSCs co-injection experiments, 1 x 10^6 tumor-derived MDSCs were injected with 1 x 10^6 3LL cells s.c. For MDSCs adoptive transfer experiments, 3LL tumor-bearing control mice received control or *Ddit3*$^{-/-}$ tumor MDSCs (3 x 10^6 i.v.) on days 3 and 6 after tumor injection.

BM-MDSCs Models

Bone marrow-derived MDSCs (BM-MDSCs) were generated by culturing BM cells for 3 days in the presence of G-CSF (100 ng/mL) and GM-CSF (20 ng/mL). BM-MDSCs were exposed to

tumor derived stress factors by the addition of 40% 3LL tumor explants supernatant (TES) for 24 hours on day 3 of the BM-MDSCs protocol. TES was produced by from 3LL tumors digested at 17 days after injection. Digested tumors were depleted of red blood cells, plated at a 1 X 10^7 tumor cells/mL and cultured overnight. Supernatants were removed, centrifuged to remove non-adherent cells, syringe filtered to remove cell debris, and stored at -80C until use. L-NAC (2 mM), MnTBAP (100 μM), or PTIO (100 μM) were added to BM-MDSCs cultures on day 3.

T cell proliferation

T cell proliferation was measured using Carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies), as described (Raber et al., 2013). Data is expressed as the percentage of T cells proliferating as tested by the dilution of CFSE fluorescence compared with non-activated T cells.

Western Blot and ELISA

Cell lysates were electrophoresed in TrisGlycine gels, transferred to PVDF membranes, and immunoblotted with antibodies against peIF2α, eIF2α, Chop, C/EBPβ, arginase I, p84, p-STAT3, STAT3, and β-actin diluted at (all 1:500). Membrane-bound immune complexes were detected using ECL detection reagent (GE Healthcare). ELISA for nitrosylated protein (Millipore), and IL-6 (eBioscience) were performed on lysates from sorted MDSCs. Densitometry of C/EBPβ isoforms normalized to nuclear p84 was calculated using NIH Image J.

IL-6 production in MDSCs

For analysis of IL-6 production in MDSCs, tumor-bearing mice were injected i.p. with 0.25 mg of Brefeldin A for 6 hours, after which tumors were isolated and MDSCs tested for IL-6 by flow

cytometry. To assess integrity of the IL-6 signaling pathway in MDSCs, BM-MDSCs were developed using BM cells from wild type, and *Ddit3*$^{-/-}$ mice. On protocol day 3, mouse recombinant IL-6 (R&D Systems) was supplemented to the culture at 50 ng/mL for 8 hours.

MDSCs suppressive mechanism assays

Superoxide production was quantified in freshly isolated MDSCs using the Superoxide Anion Assay Kit (Sigma). Peroxynitrite levels were determined in tissue or MDSCs lysates using a Nitrotyrosine ELISA Assay (EMD Millipore).

C/EBPβ activity

Nuclear extracts from MDSCs were tested for C/EBPβ DNA binding activity using the TransAM C/EBPβ DNA-binding ELISA kit (Active Motif, Carlsbad, CA).

Antigen Presentation Assay

Exogenous presentation of antigen was determined using tumor MDSCs sorted from tumor-bearing control or *Ddit3*$^{-/-}$ mice. MDSCs were pulsed with SIINFEKL peptide (2 μg/mL for 3 hours), washed twice with PBS, then plated with naïve CFSE labeled OT-1 cells at decreasing dilutions (1:1/2 – 1:1/16, OT-1:MDSCs). OT-1 cell proliferation was evaluated after 72 hours of co-culture.

Quantitative PCR

Total RNA was isolated from tumor-derived MDSCs using TRIzol (Invitrogen, Life Technologies). RNA was then converted to cDNA using Bio-Rad iScript cDNA synthesis kit

(Bio-Rad, Hercules, CA). Quantitative PCR was performed on an Applied Biosystems thermocycler (7900 HT) using Bio-Rad SYBR green supermix with primers against murine Chop forward (GGAGCTGGAAGCCTGGTATG) reverse (GGATGTGCGTGTGACCTCTG), Atf4 forward (GCCTGACTCTGCTGCTTACA) reverse (CTTGCCTTACGGACCTCTTC), and C/EBPβ forward (GACAAGCTGAGCGACGAGTA) reverse (AGCTGCTCCACCTTCTTCT G). Relative expression was calculated using the delta-delta *Ct* method and normalized to the reference gene Actb forward (TGTGATGGTGGGAAT GGGTCAGAA) reverse (TGTGGTGCCAGATCTTCTCCATGT). For miRNA experiments, total RNA was isolated using TRIzol and converted to cDNA using miScript II RT Kit (Qiagen). Quantitative PCR was performed using miScript SYBR Green PCR Kit using primers against miR-142-3p and RNU6-2 (Qiagen).

Statistical analysis

Statistical analyses were carried in SAS 9.3 (SAS Institute, Cary, NC). Tests were conducted at 5% significance level. Continuous data were checked for unequal variances with the Brown-Forsythe and Levene tests. Percentage data were arcsine transformed and further checked for unequal variances. Experimental groups differences of endpoints were assessed by ANOVA with the Satterthwaite correction for unequal variances using the MIXED procedure. Means comparisons were carried out with the Tukey procedure for all comparisons or with the Dunnet procedure for comparisons with the control group.

Supplemental Reference List

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