Supplementary Data for

Retinoic acid synthesis deficiency fosters the generation of polymorphonuclear myeloid-derived suppressor cells in colorectal cancer

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Supplementary Figures

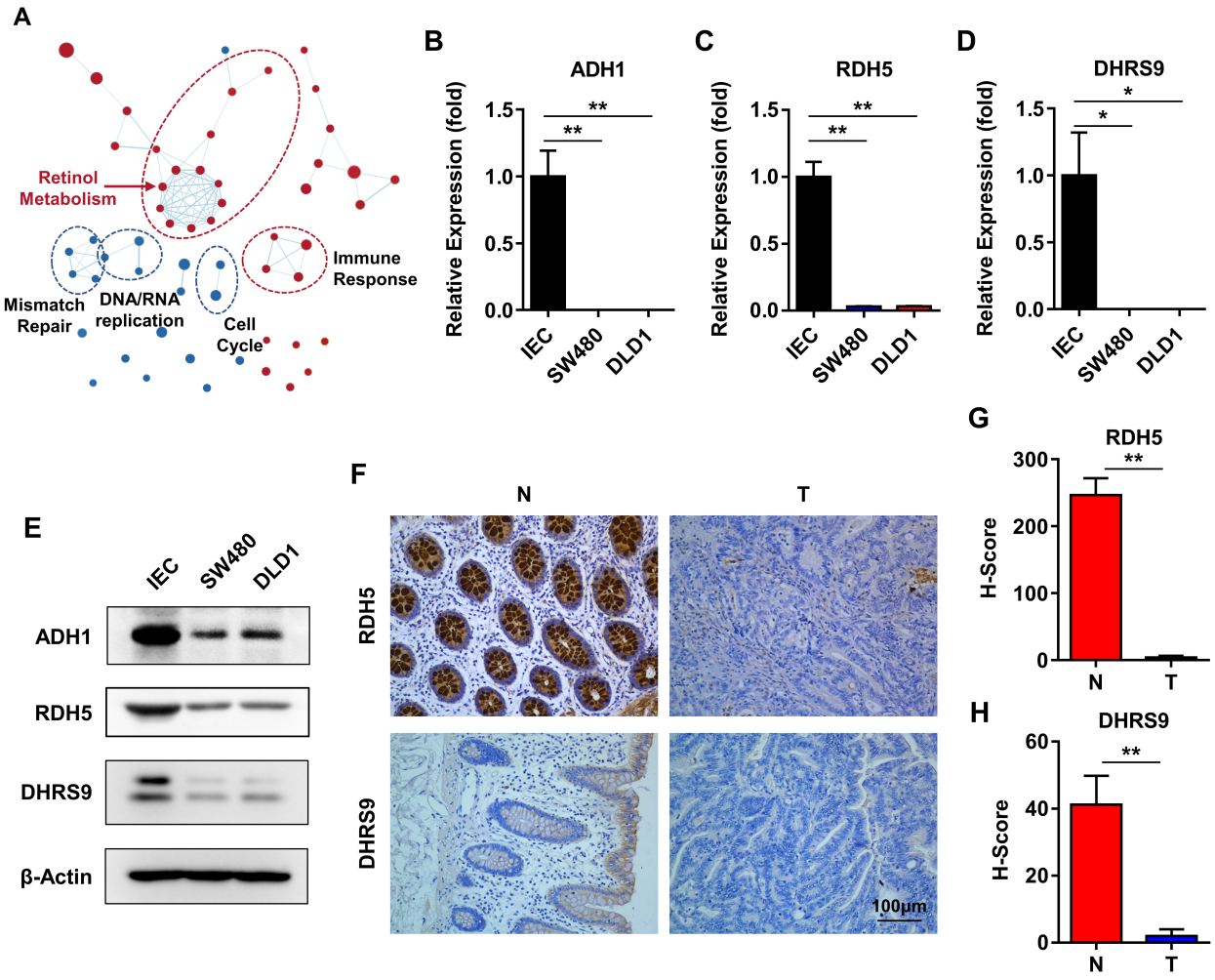


Figure S1.

Retinol metabolism is significantly downregulated in colorectal cancer. (**A**) The gene set c2.cp.kegg.v5.2.symbols from the Molecular Signatures Database-MsigDB was applied for GSEA analysis between CRC tissues (blue) and adjacent colonic tissues expression (red). Cytoscape and Enrichment map were used for visualization of the GSEA results (p value cutoff: 0.1). Enrichment results were mapped as a network of gene sets. Nodes represent enriched gene sets, which were grouped by their similarities according to the related gene sets. (**B - D**) Relative expression of ADH1 (B), RDH5 (C) and DHRS9 (D) in intestinal epithelial cells (IEC) and CRC cell line SW480 and DLD1 were determined by quantitative RT-PCR. Data represents three samples and are shown

as means ± SEM. *, $P < 0.05$, **, $P < 0.01$. (**E**) Immunoblots for ADH1, RDH5 and DHRS9 in IEC and CRC cell line SW480 and DLD1. (**F - H**) IHC staining showed RDH5 and DHRS9 expression in human CRC tissue (F). RDH5 (G) and DHRS9 (H) expression on tumor (T) and adjacent non-tumor tissues (N) from five patients were scored and summarized as means ± SEM. **, $P < 0.01$.

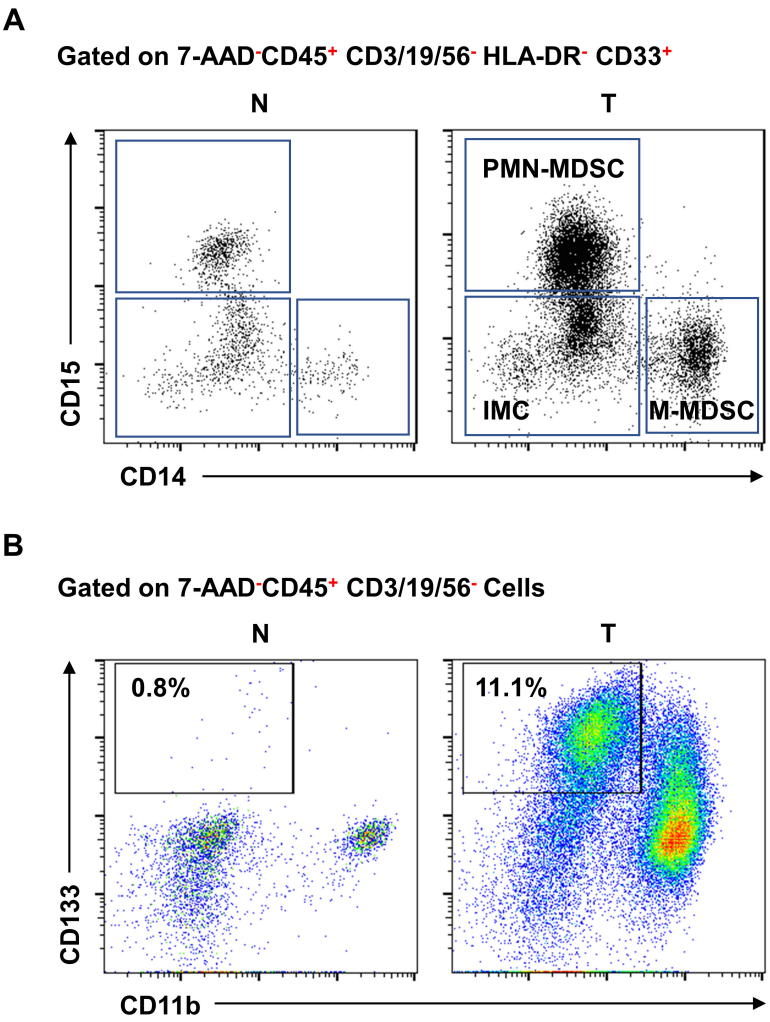


Figure S2.

PMN-MDSC and myeloid precursors were accumulated in CRC tissues. (**A**) Mononuclear cells were isolated from paired tumor (T) and adjacent non-tumor (N) tissues by Ficoll density gradient centrifugation. Flow cytometry analysis identified myeloid subsets in 7-AAD$^-$ CD45$^+$ CD3/19/56$^-$ HLA-DR$^-$ CD33$^+$ tumor-infiltrating suppressive myeloid cells. M-MDSC, monocytic MDSC, PMN-MDSC, polymorphonuclear MDSC, IMC, immature myeloid cell. (**B**) Myeloid precursors in 7-AAD$^-$ CD45$^+$ CD3/19/56$^-$ mononuclear cells was gated by CD133 and CD11b.

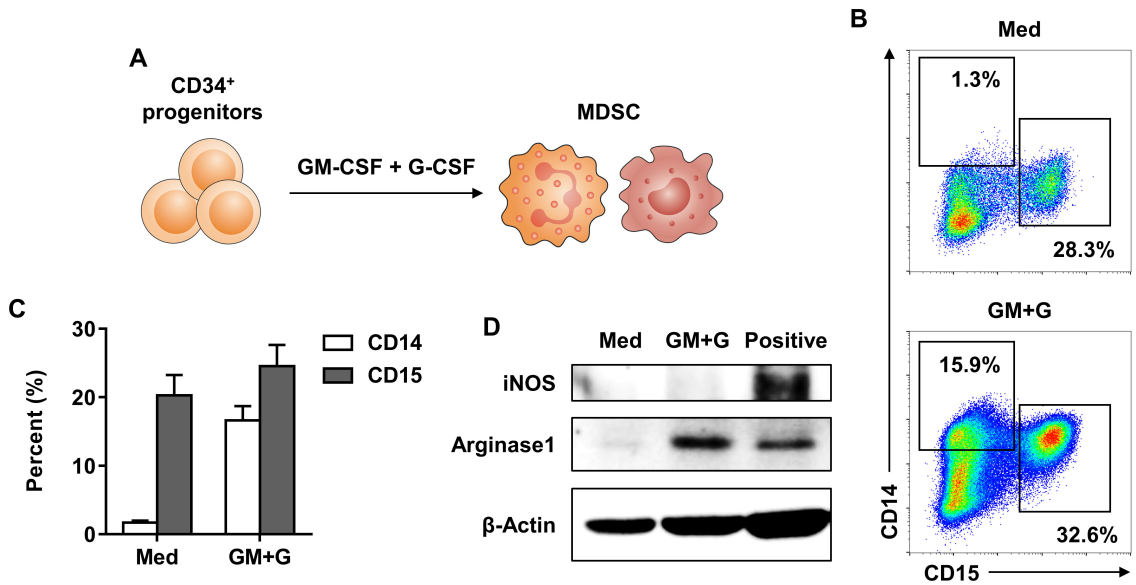


Figure S3.

GM-CSF and G-CSF could induce the generation of ARG1 expressing PMN-MDSC. (**A**) Schematic flow of *in vitro* model to induce MDSC. $CD34^+$ precursors were cultured with medium alone (Med) or combined cytokines (GM-CSF and G-CSF, GM+G) for 3 days. (**B** and **C**) The proportion of $CD14^+$ monocytic and $CD15^+$ polymorphonuclear cells induced in (A) was monitored by flow cytometry (B) and summarized as mean ± SEM in (C). (**D**) Immunoblot for ARG1 and iNOS expression in GM-CSF and G-CSF induced myeloid cells.

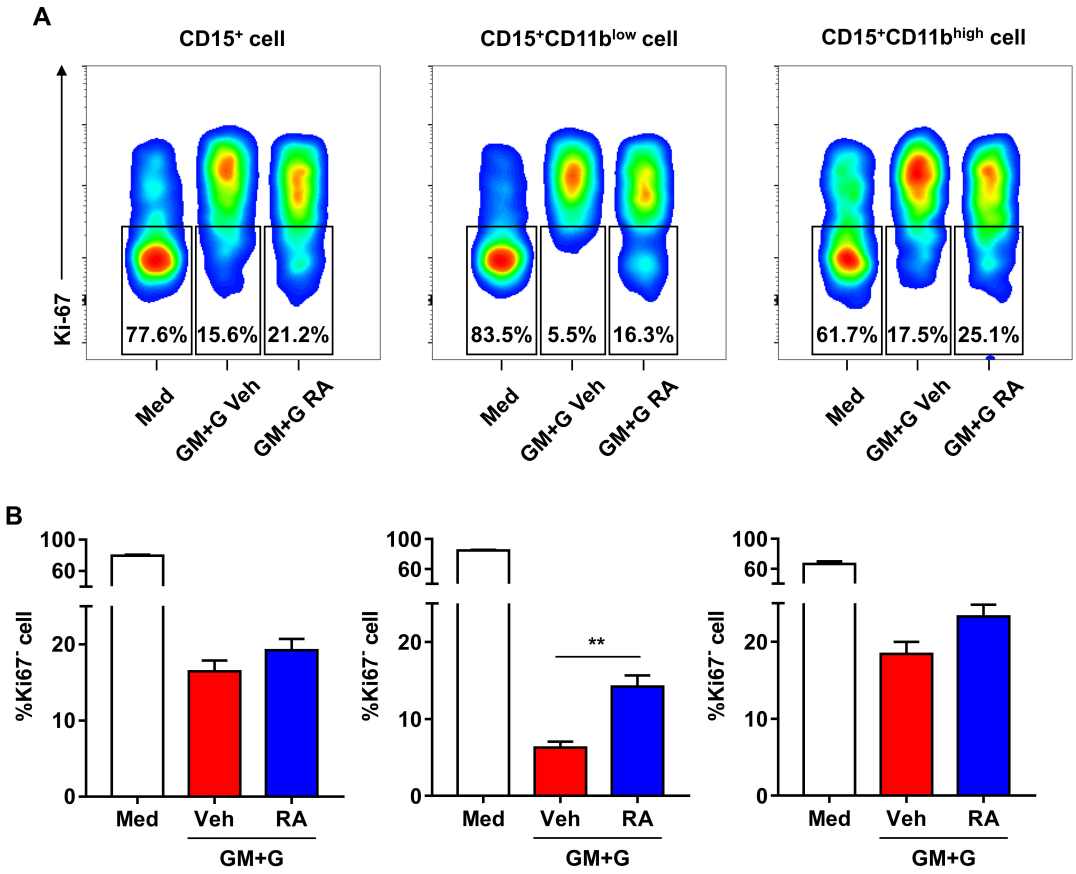
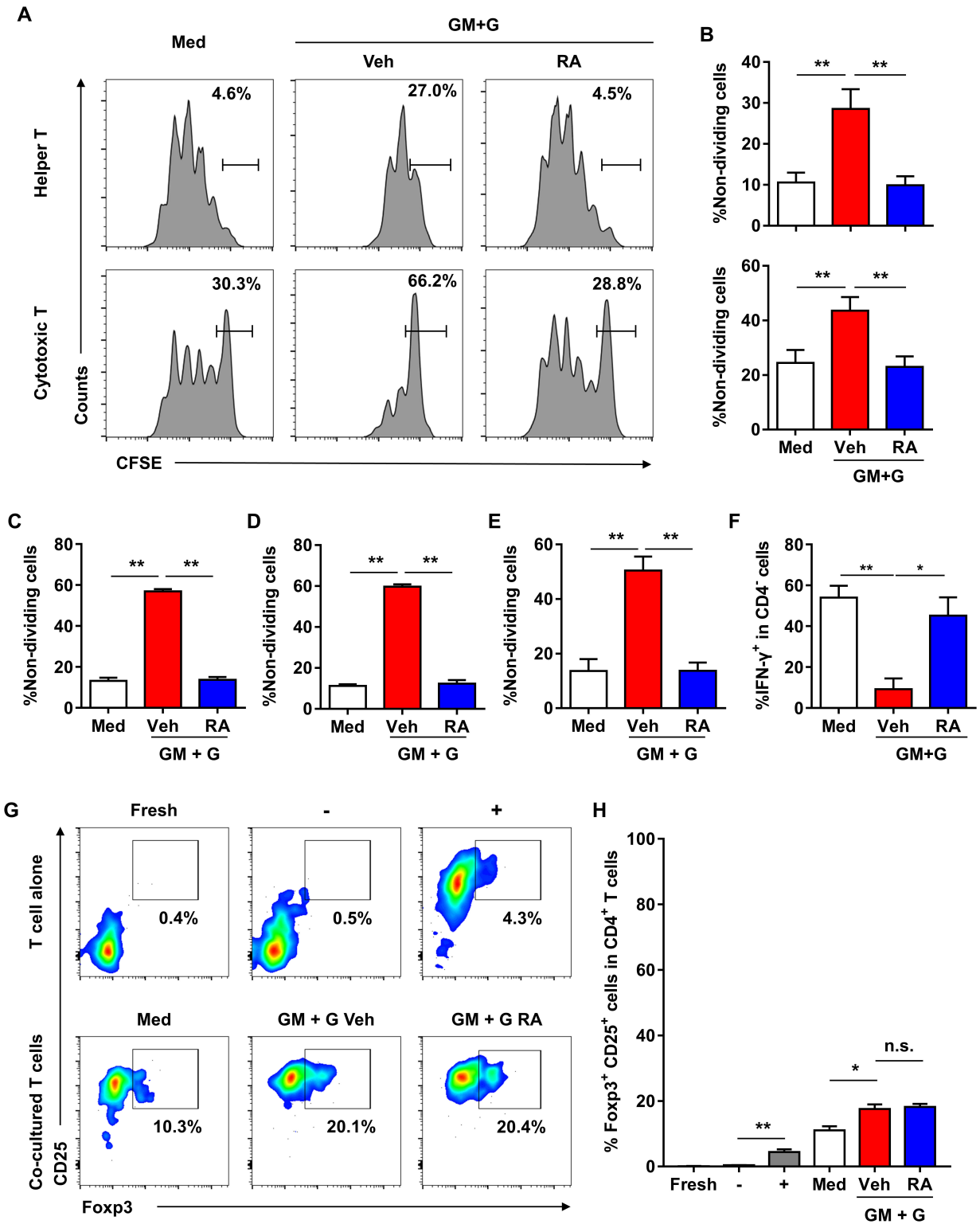


Figure S4.

Retinoic acid slightly impairs the proliferation of cytokine-induced myeloid cells. (**A**) $CD34^+$ precursors were cultured with medium alone (Med) or combined cytokines (GM+G) in the presence of vehicle (Veh) or retinoic acid (RA) for 3 days. The proliferation of $CD15^+$, $CD15^+CD11b^{low}$, and $CD15^+CD11b^{high}$ myeloid cells was monitored by flow cytometry. (**B**) The proportion of $Ki\text{-}67^-$ non-proliferating cells in each myeloid subset from four samples were summarized as mean ± SEM. **, $P < 0.01$.



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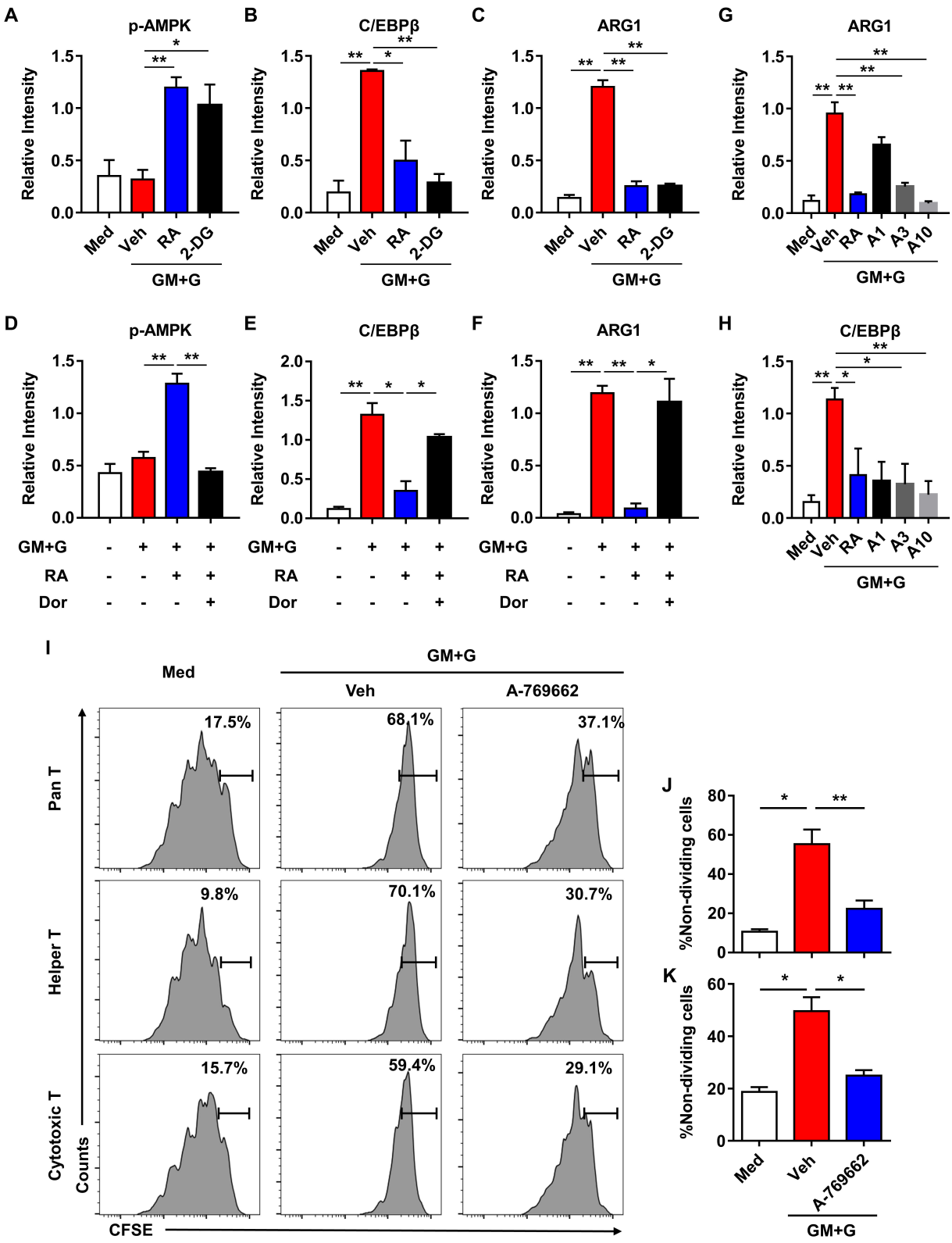
54 **Figure S5.**

55 **Retinoic acid could abrogate the suppressive function of MDSC.** (**A** and **B**) Myeloid cells

56 induced with indicated conditions were washed and then co-cultured CFSE-labeled T cells at the

ratio 1:2 in the presence of coated anti-CD3 and soluble anti-CD28 antibody for 5 days. The proliferation of helper and cytotoxic T cells was examined by FACS to assess the suppressive capacity of myeloid cells (A). Data represents 11 samples and are shown as mean ± SEM in (B). **, $P < 0.01$. (**C** – **E**) Myeloid cells were also cocultured with CFSE-labeled T cells at the ratio 1:1 in the presence of coated anti-CD3 and soluble anti-CD28 antibodies for 5 days. The percentage of non-dividing T cells in pan (C), helper (D) and cytotoxic (E) T cells are shown as the means ± SEM (n = 4). **, $P < 0.01$. (**F**) Myeloid cells induced with indicated conditions were washed and then co-cultured with anti-CD3/CD28 antibody activated T cells for 5 days. The expression of IFN-γ in $CD4^-$ T cells was examined by FACS. Data from three independent experiments are summarized as mean ± SEM. *, $P < 0.05$. (**G**) Pan T cells were isolated (Fresh) and then cultured with (+) or without (-) anti CD3/28 stimulation or co-cultured with MDSC induced under indicated conditions with anti CD3/28 stimulation for 5 days. The percentage of regulatory T cells in $CD4^+$ T cells was determined by the co-expression of Foxp3 and CD25 through flow cytometry. (**H**) The percentage of regulatory CD4 T cells in (G) was summarized as mean ± SEM in (H) (n = 4). *, $P < 0.05$; **, $P < 0.01$.



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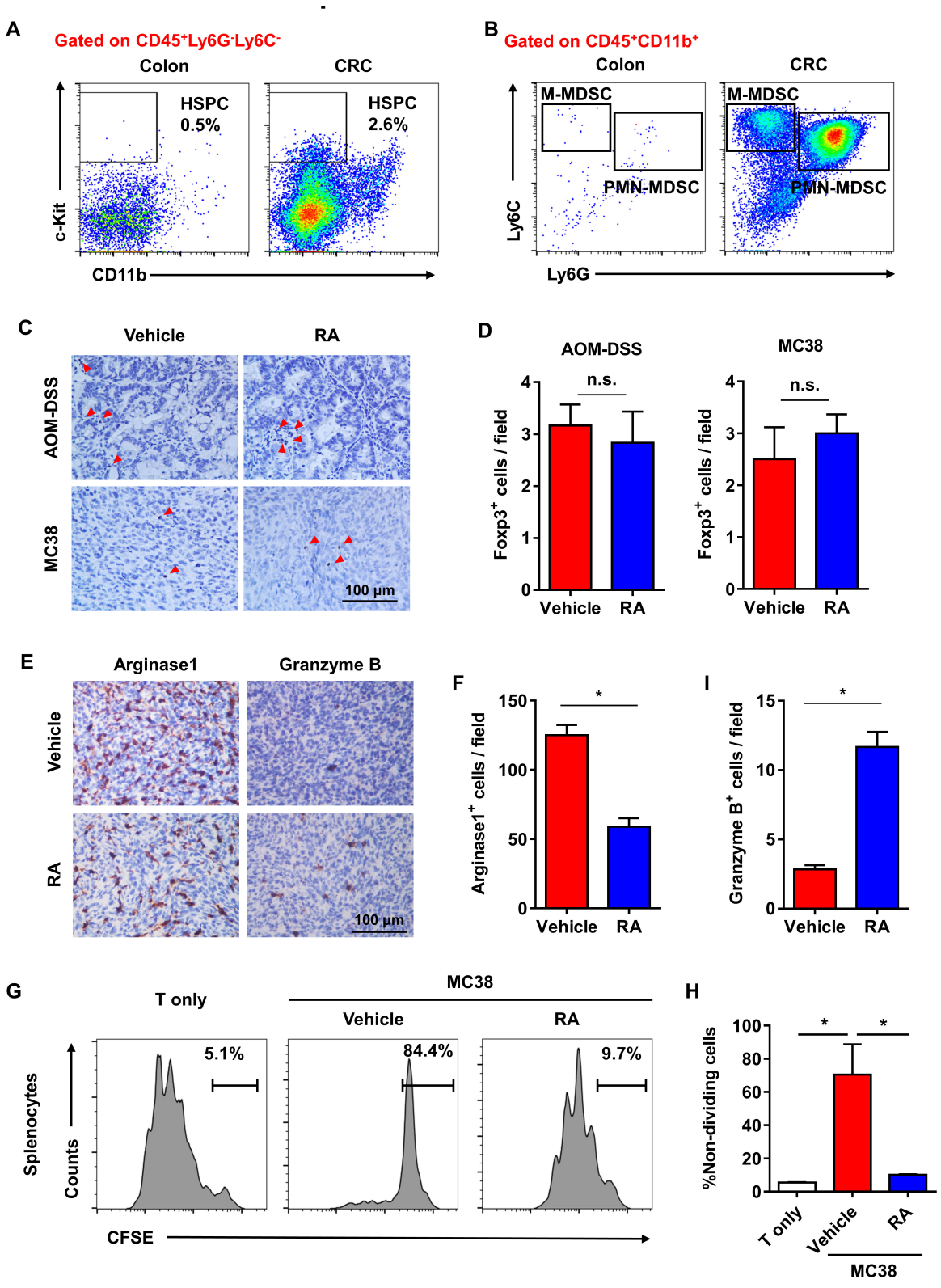
73 **Figure S6.**

74 **AMPK activation could impair the suppressive capacity of MDSC.** (**A - C**) Relative expression

75 intensity of p-AMPK (A), C/EBPβ (B) and ARG1 (C) as in Figure 4D from three samples are

shown as the means ± SEM. *, $P < 0.05$, **, $P < 0.01$. (**D** - **F**) Relative expression intensity of p-AMPK (D), C/EBPβ (E) and ARG1 (F) as in Figure 4G from four samples are shown as the means ± SEM. *, $P < 0.05$, **, $P < 0.01$. (**G** and **H**) Relative intensity of ARG1 (G) and C/EBPβ (H) as in Figure 4H from three samples are shown as the means ± SEM. *, $P < 0.05$, **, $P < 0.01$. (**I**) Myeloid cells were induced with medium alone (Med) or cytokines (GM+G) in the presence of vehicle (Veh) or A-769662 for 3 days. These myeloid cells were washed and then co-cultured CFSE-labeled T cells in the presence of anti-CD3 and anti-CD28 antibody for 5 days. The proliferation of pan, helper and cytotoxic T cells was detected by FACS to assess the suppressive capacity of myeloid cells. (**J** and **K**) The proportion of non-dividing helper (J) and cytotoxic (K) T cells are shown as mean ± SEM. n = 3; *, $P < 0.05$; **, $P < 0.01$.



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88 **Figure S7.**

89 **Supplementation of retinoic acid could abrogate the function of MDSC in mouse.** (**A** and **B**)

90 Mononuclear cells were isolated from CRC tissues of AOM-DSS treated mouse or colon tissue of

healthy littermate. Hematopoietic stem and progenitor cells (HSPC) in $CD45^+Ly6G^-Ly6C^-$ cells was gated by c-Kit and CD11b (A). M-MDSC and PMN-MDSC subsets in $CD45^+CD11b^+$ cells were gated by Ly6G and Ly6C (B). (**C**) IHC staining detected foxp3 expression in tumor tissues from vehicle- or RA-treated mice. Red arrow, $foxp3^+$ cells. (**D**) Number of $foxp3^+$ cells in AOM-DSS induced CRC (left) and MC38 tumor (right) were quantified and summarized as the means ± SEM. n = 6/group. (**E**) Immunohistochemistry staining detected arginase 1 and granzyme B expression in MC38 tumor from vehicle or retinoic acid treated mice. (**F**) Number of arginase 1^+ cells in (E) were quantified and summarized as mean ± SEM. n = 6/group; *, $P < 0.05$. (**G** and **H**) Tumor-infiltrating myeloid cells from MC38 mice treated with vehicle or retinoic acid were purified and co-cultured with CFSE-labeled splenocytes for 3 days in the presence of anti-CD3 and anti-CD28 antibody. The proliferation of splenocytes was monitored by FACS (G). Data from three samples were summarized as mean ± SEM in (H). *, $P < 0.05$. (**I**) Number of granzyme B^+ cells in (E) were counted and shown as mean ± SEM. n = 6/group; *, $P < 0.05$.

Supplementary Table

Table S1. Summary of materials.

Materials	Manufactor	Cat. No.
1. Antibodies		
CD33-APC	Beckman Coulter	Cat#IM2471
CD45-Krome Orange	Beckman Coulter	Cat#A96416
CD3-PC5.5	eBioscience	Cat#45-0037
CD19-PC5.5	eBioscience	Cat#45-0199
CD56-PC5.5	eBioscience	Cat#35-0567
CD11b-PE-Cy7	Beckman Coulter	Cat#A54822
HLA-DR-PE-CF594	BD Pharmingen	Cat#562304
CD14-AF700	BD Pharmingen	Cat#557923
CD15-EF450	eBioscience	Cat#48-0159
CD15-FITC	eBioscience	Cat#11-0159
CD133/2-PE	Miltenyi Biotec	Cat#130-090-853
IFN-γ-EF450	eBioscience	Cat#48-7319-42
CD4-PE-Cy7	eBioscience	Cat#25-0049-42
Ki67-PE	BD Pharmingen	Cat#556027
CD45-BV605	BioLegend	Cat#103140
Ly6G-PE-CF594	BD Biosciences	Cat#562700
CD11b-PE-Cy7	BD Biosciences	Cat#561098

Ly6C-AF647	BioLegend	Cat#128009
CD8a-AF700	BD Biosciences	Cat#557959
c-Kit-BV421	BioLegend	Cat#105827
Functional grade anti-CD3 antibody	eBioscience	Cat#16-0037-85
Functional grade anti-CD28 antibody	eBioscience	Cat#16-0289-85
LEAF™ Purified anti-mouse CD3ε	BioLegend	Cat#100314
LEAF™ Purified anti-mouse CD28	BioLegend	Cat#102112
rabbit anti-arginase1 antibody	CST	Cat#93668S
rabbit anti-ADH1 antibody	Abcam	Cat#ab108203
rabbit anti-RDH5 antibody	Novus	Cat#NBP2-15097
rabbit anti-DHRS9 antibody	Novus	Cat#NBP1-89378
mouse anti RIG-I antibody	OriGene	Cat#TA506141
rabbit anti-human CD11b antibody	Invitrogen	Cat#PA5-29633
Anti-granzyme B antibody	Abcam	Cat#ab4059
anti-arginase1 antibody	CST	Cat#9819
anti-phospho-AMPK antibody	CST	Cat#2535T
anti-AMPK antibody	CST	Cat#2532S
anti-CEBP/β antibody	Santa Cruz	Cat#sc-7962
anti-β-actin antibody	CST	Cat#4967
HRP-linked anti-rabbit IgG antibody	CST	Cat#7074S
HRP-linked anti-mouse IgG antibody	CST	Cat#7076S
CD34 MicroBeads kit	Miltenyi	Cat#130-046-702

Pan T Cell Isolation Kit	Miltenyi	Cat#130-096-535
2. Chemicals		
Retinoic acid	Sigma-Aldrich	Cat#R2625
CFSE	eBioscience	Cat#65-0850-84
2-DG	Sigma-Aldrich	Cat#D8375
Dorsomorphin	APExBio	Cat#B3252-5
A769662	APExBio	Cat#A3963-10
AOM	Sigma	Cat#A5486
DSS	MP Biomedicals	Cat#0216011080
3. Recombinant Proteins		
SCF	R&D Systems	Cat#255-SC-050
FLT-3L	R&D Systems	Cat#308-FK-025
TPO	R&D Systems	Cat#288-TP-025
IL-3	R&D Systems	Cat#203-IL-010
GM-CSF	R&D Systems	Cat#215-GM-010
G-CSF	R&D Systems	Cat#214-CS-025
4. Commercial Assay Kits		
SurePrint G3 Human Gene Expression v3 8x60K kit	Agilent	Cat#G4851C

5X All-In-One RT MasterMix	ABM	Cat#G492
SYBR Green Real-time PCR MasterMix	TOYOBO	Cat#QPS-201
Dako REAL™ EnVision™ detection systems	Dako	Cat#K5007
Seahorse XF Glycolysis Stress Test Kit	Seahorse Bioscience	Cat#103020-100
5. Primers		
GCGGTGGACATCAACAAGG	Thermo Fisher	Human ADH1-F
AACATAACAGGGAAGCCATCAT	Thermo Fisher	Human ADH1-R
CTGTGACCAACCTGGAGAGTCT	Thermo Fisher	Human RDH5-F
GATGCGCTGTTGCATTTTCAGGT	Thermo Fisher	Human RDH5-R
CCAGAGAATGTCAAGAGGACTGC	Thermo Fisher	Human DHRS9-F
CTGTAGTCCTCTAGTGTCAGCC	Thermo Fisher	Human DHRS9-R
CGCCAAGTCCAGAACCATAG	Thermo Fisher	Human ARG1-F
AGGTCCCCATAATCCTTCACAT	Thermo Fisher	Human ARG1-R
ATTGCTCTTCCCCTCCGCT	Thermo Fisher	Human GLUT3-F
ATCCTCAAAAGTCCTGCCACG	Thermo Fisher	Human GLUT3-R
GCCTCCCACTGGGTTTTACCT	Thermo Fisher	Human HK3-F
TCCCGCAACAGACTCACGAC	Thermo Fisher	Human HK3-R
GGCAGGAGAATGTGCTGGTCAT	Thermo Fisher	Human PFKFB3-F
CATAAGCGACAGGCGTCAGTTTC	Thermo Fisher	Human PFKFB3-R
GGAATGAATGTTGCTGGTGTCT	Thermo Fisher	Human LDHA-F
AGTCCAATAGCCCAGGATGTG	Thermo Fisher	Human LDHA-R

AGAAGACCGTGGACAAGCACAG	Thermo Fisher	Human CEBPB-F
CTCCAGGACCTTGTGCTGCGT	Thermo Fisher	Human CEBPB-R
TCGATGCTCTTAGCTGAGTGTCC	Thermo Fisher	Human 18S-F
TGATCGTCTTCGAACCTCCG	Thermo Fisher	Human 18S-R

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