Figure S1

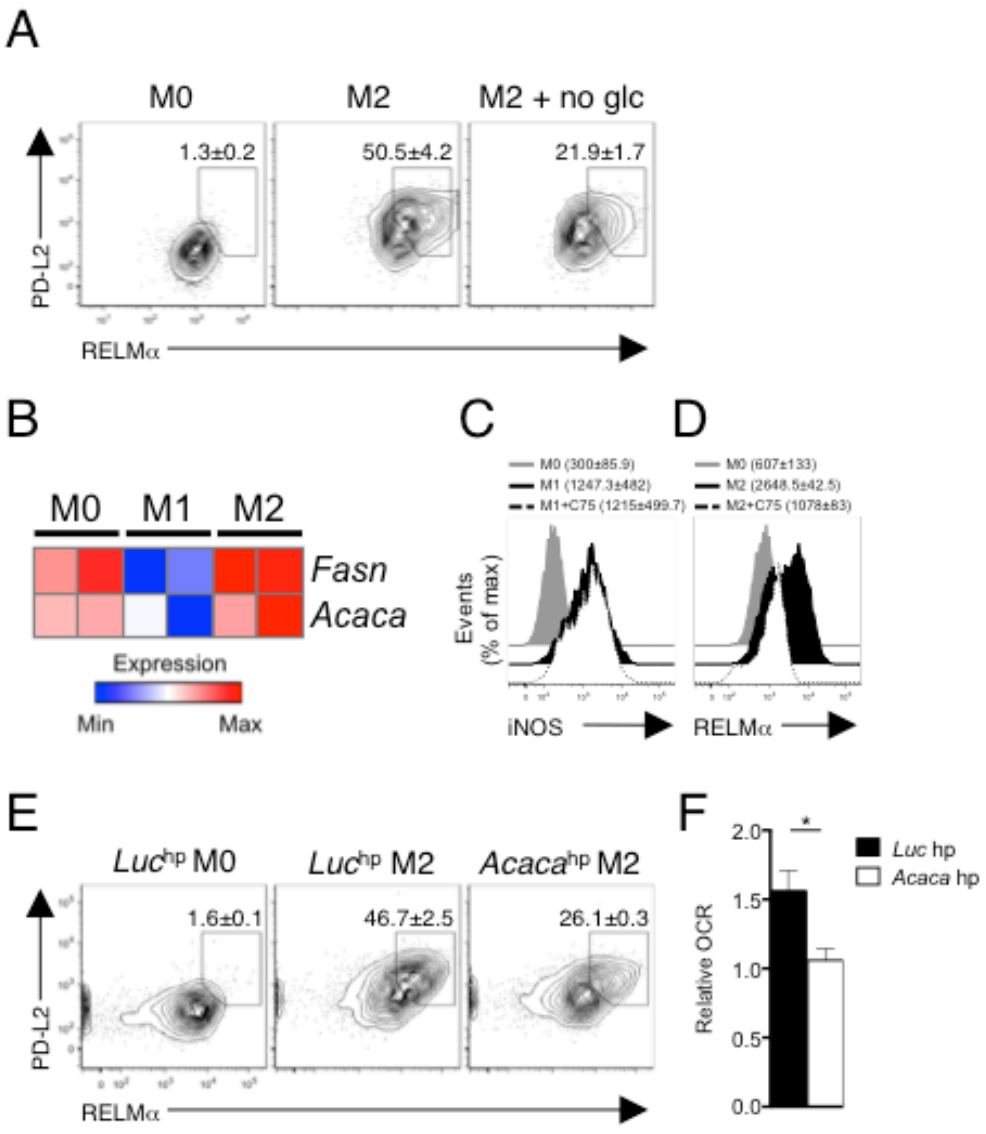
A
M0
M2
M2 + no glc
1.3±0.2
50.5±4.2
21.9±1.7
PD-L2
RELMα
B
M0
M1
M2
Fasn
Acaca
Expression
Min
Max
C
M0 (300±85.9)
M1 (1247.3±482)
M1+C75 (1215±499.7)
Events (% of max)
iNOS
D
M0 (607±133)
M2 (2648.5±42.5)
M2+C75 (1078±83)
RELMα
E
Luchp M0
Luchp M2
Acacahp M2
1.6±0.1
46.7±2.5
26.1±0.3
PD-L2
RELMα
F
*
Luc hp
Acaca hp
Relative OCR
2.0
1.5
1.0
0.5
0.0

Figure S2

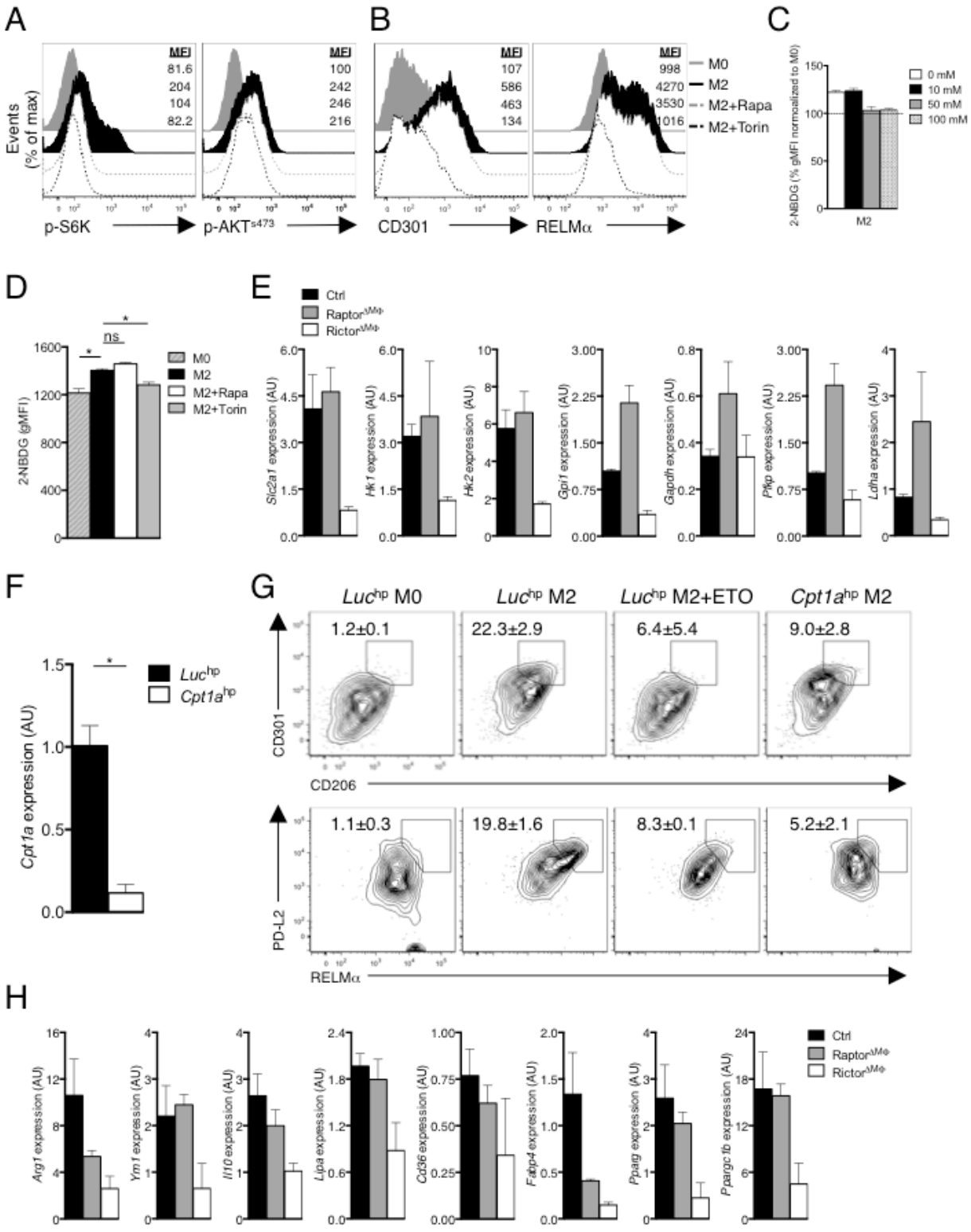
A
B
C
Events (% of max)
MFI
81.6
204
104
82.2
100
242
246
216
107
586
463
134
998
4270
3530
1016
M0
M2
M2+Rapa
M2+Torin
p-S6K
p-AKTs473
CD301
RELMα
2-NBDG (% gMFI normalized to M0)
0 mM
10 mM
50 mM
100 mM
M2
D
E
2-NBDG (gMFI)
ns
M0
M2
M2+Rapa
M2+Torin
Ctrl
RaptorΔMΦ
RictorΔMΦ
Slc2a1 expression (AU)
Hk1 expression (AU)
Hk2 expression (AU)
Gpi1 expression (AU)
Gapdh expression (AU)
Pfkp expression (AU)
Ldha expression (AU)
F
G
Cpt1a expression (AU)
Luchp
Cpt1ahp
Luchp M0
Luchp M2
Luchp M2+ETO
Cpt1ahp M2
1.2±0.1
22.3±2.9
6.4±5.4
9.0±2.8
CD301
CD206
1.1±0.3
19.8±1.6
8.3±0.1
5.2±2.1
PD-L2
RELMα
H
Arg1 expression (AU)
Ym1 expression (AU)
Il10 expression (AU)
Lipa expression (AU)
Cd36 expression (AU)
Fabp4 expression (AU)
Pparg expression (AU)
Ppargc1b expression (AU)
Ctrl
RaptorΔMΦ
RictorΔMΦ

Figure S3

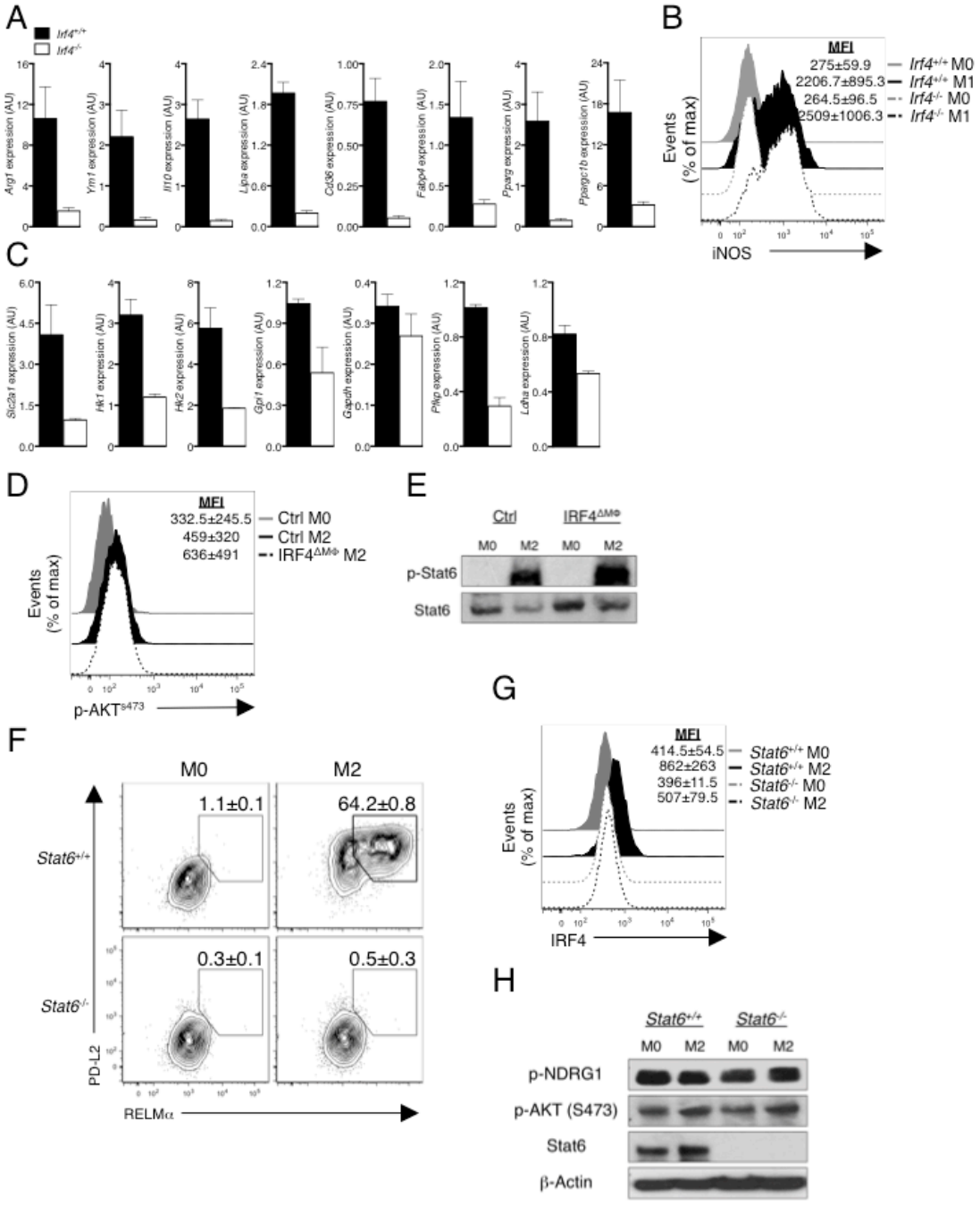
A
Irf4+/+
Irf4-/-
Arg1 expression (AU)
Ym1 expression (AU)
Il10 expression (AU)
Lipa expression (AU)
Cd36 expression (AU)
Fabp4 expression (AU)
Pparg expression (AU)
Ppargc1b expression (AU)
B
MFI
275±59.9 Irf4+/+ M0
2206.7±895.3 Irf4+/+ M1
264.5±96.5 Irf4-/- M0
2509±1006.3 Irf4-/- M1
Events (% of max)
iNOS
C
Slc2a1 expression (AU)
Hk1 expression (AU)
Hk2 expression (AU)
Gpi1 expression (AU)
Gapdh expression (AU)
Pfkp expression (AU)
Ldha expression (AU)
D
MFI
332.5±245.5 Ctrl M0
459±320 Ctrl M2
636±491 IRF4ΔMΦ M2
Events (% of max)
p-AKTs473
E
Ctrl
IRF4ΔMΦ
M0 M2 M0 M2
p-Stat6
Stat6
F
M0
M2
1.1±0.1
64.2±0.8
Stat6+/+
0.3±0.1
0.5±0.3
Stat6-/-
PD-L2
RELMα
G
MFI
414.5±54.5 Stat6+/+ M0
862±263 Stat6+/+ M2
396±11.5 Stat6-/- M0
507±79.5 Stat6-/- M2
Events (% of max)
IRF4
H
Stat6+/+
Stat6-/-
M0 M2 M0 M2
p-NDRG1
p-AKT (S473)
Stat6
β-Actin

Figure S4

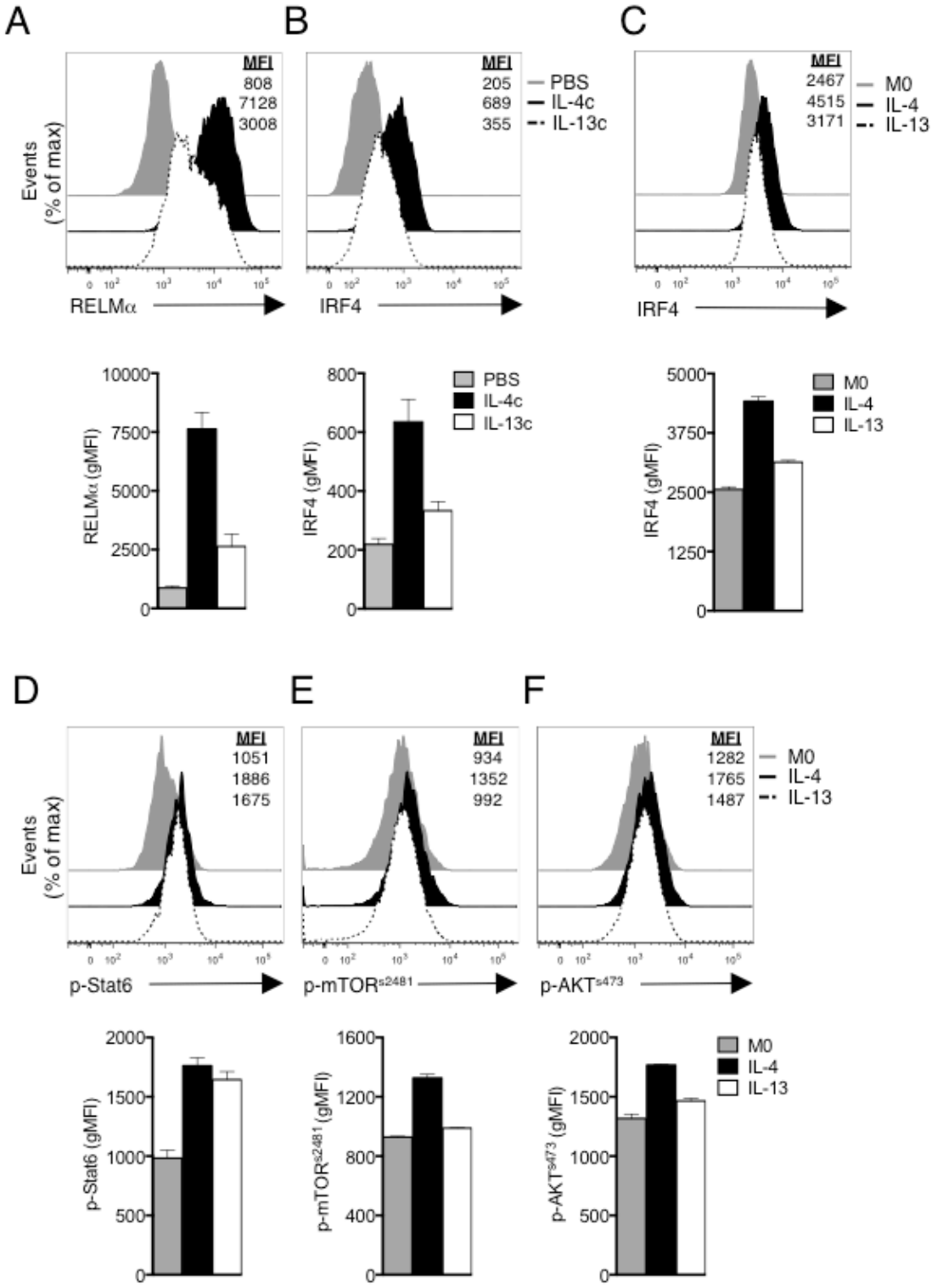
A
B
C
MFI
808
7128
3008
205
689
355
2467
4515
3171
PBS
IL-4c
IL-13c
M0
IL-4
IL-13
Events (% of max)
RELMα
IRF4
RELMα (gMFI)
IRF4 (gMFI)
D
E
F
1051
1886
1675
934
1352
992
1282
1765
1487
p-Stat6
p-mTOR^{s2481}
p-AKT^{s473}
p-Stat6 (gMFI)
p-mTOR^{s2481} (gMFI)
p-AKT^{s473} (gMFI)

Figure S5

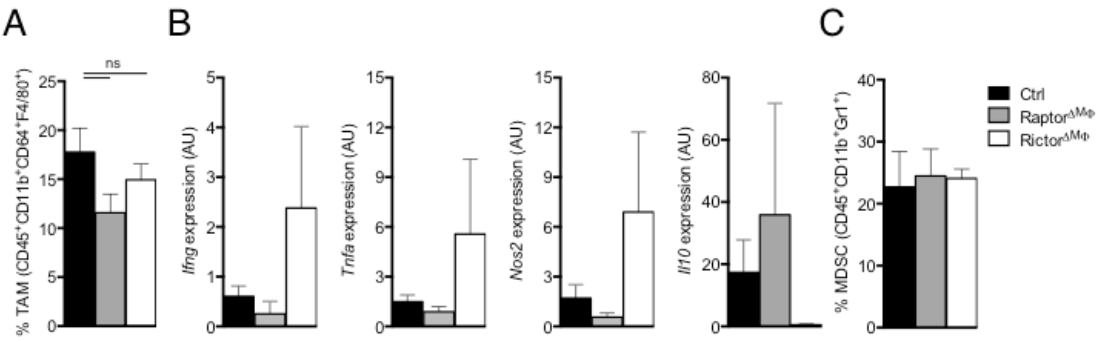
A
B
C
ns
% TAM (CD45+CD11b+CD64+F4/80+)
Ifng expression (AU)
Tnfa expression (AU)
Nos2 expression (AU)
Il10 expression (AU)
% MDSC (CD45+CD11b+Gr1+)
Ctrl
RaptorΔMΦ
RictorΔMΦ

Figure S6

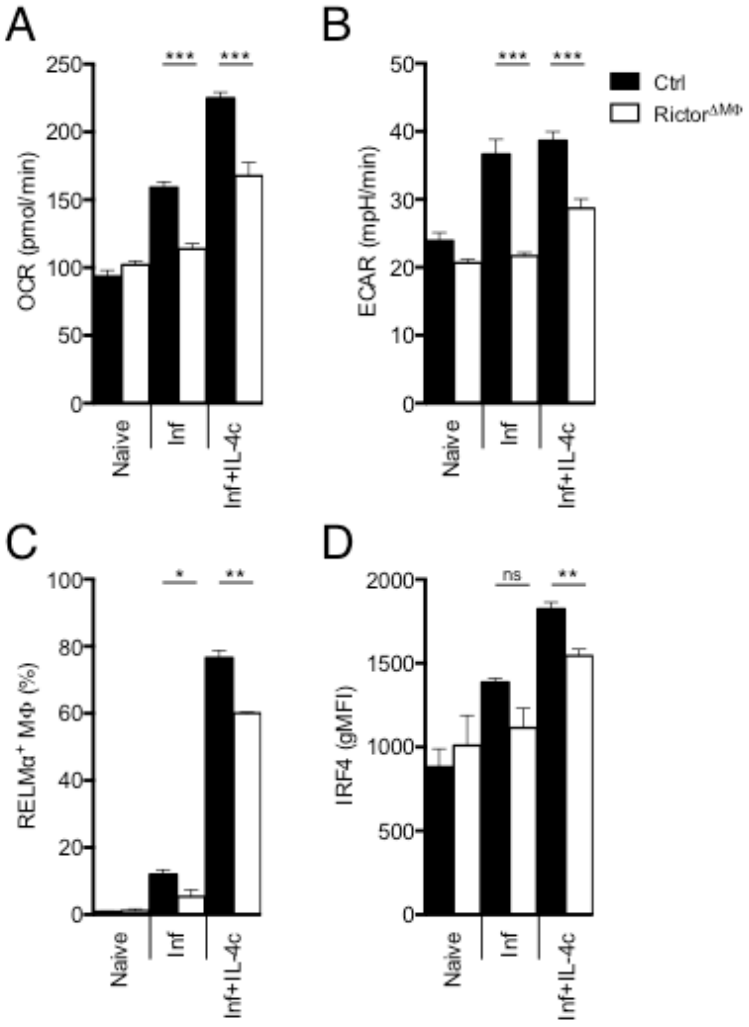
A
B
C
D
OCR (pmol/min)
ECAR (mpH/min)
RELMα+ MΦ (%)
IRF4 (gMFI)
Naive
Inf
Inf+IL-4c
Ctrl
Rictor^ΔMΦ
***
*
**
ns

Figure S1, related to Figure 1. Glucose withdrawal reduces M2 activation. (**A**) Expression of PD-L2 and RELMα from unstimulated macrophages (M0) or macrophages stimulated with IL-4 (M2) for 24 hr in the absence or presence of glucose, as indicated, assessed by flow cytometry. Data are from one experiment but values are mean % ± s.e.m. of data from two independent experiments. (**B**). Relative expression of *Fasn* and *Acaca* in M0, M1 and M2 macrophages. (**C**,**D**) Effects of inhibiting FASN with C75 on expression of iNOS in M1 macrophages and RELMα in M2 macrophages. Numbers are mean MFI values, ± s.e.m, of data from 2 independent experiments. (**E**) Effect of knocking down expression of *Acaca* using a retrovirally encoded *Acaca*-hpRNA on M2 activation as measured by PD-L2 and RELMα expression. Data are from one experiment but values are mean % ± s.e.m. of data from two independent experiments. (**F**) Effect of *Acaca*-hpRNA on M2 OXPHOS as measured by OCR. Data are mean ± s.e.m of technical replicates from one experiment, but representative of data from an additional experiment.

Figure S2 related to Figure 2. Chemical inhibition of mTORC2 decreases glucose uptake and activation of M2 macrophages. (**A**) Phosphorylation of S6K and AKT^{s473} by macrophages after culture for 24 hr in the absence (M0) or presence of IL-4 (M2) plus or minus rapamycin (Rapa; 20 nM) or Torin 1 (Torin; 100 nM), as measured by flow cytometry. (**B**) Expression of CD301 and RELMα in macrophages treated as in A, as measured by flow cytometry on M2 macrophages of adding increasing concentrations of glucose, as indicated in the key. (**D**) Effect on 2-NBDG s(**C**) 2-NBDG staining of macrophages treated as in A, as measured by flow cytometry. (**E**) Expression of genes encoding proteins involved in glycolysis in WT control (Ctrl), and *Raptor* and *Rictor*-deficient macrophages after culture with IL-4 for 3 hr as measured by qRT-PCR (expression normalized to WT M0 macrophages and presented in arbitrary units (AU)). (**F**) *Cpt1a* expression was suppressed by

retrovirus-mediated introduction of a shRNA targeting *Cpt1a* (*Cpt1a*^{hp}). Control cells were transduced with a control virus (*Luc*^{hp}). *Cpti1a* expression was measured by qRT-PCR. Expression is normalized to *Luc*^{hp} transduced macrophages and presented in arbitrary units (AU). (**G**) Expression of CD206, CD301, PD-L2 and RELMα in M0 and M2 *Luc*^{hp} transduced macrophages, in *Luc*^{hp} transduced M2 macrophages treated with etomoxir (ETO) and in *Cpt1a*^{hp} transduced M2 macrophages. (**H**) Expression of genes encoding a range of proteins that have been associated with M2 activation in (Ctrl), and *Raptor* and *Rictor*-deficient macrophages after culture with IL-4 for 3 hr as measured by qRT-PCR (expression normalized to WT M0 macrophages and presented in arbitrary units (AU)). In C-F, and H data are mean ± s.e.m. of technical replicates from one experiment. In A and G data are from pooled replicates within one experiments. In G numbers are mean % ± s.e.m. of data from two independent experiments. In D and F, * denotes $P < 0.05$ (Student's *t*-test). All data are from one experiment representative of two or more independent experiments.

Figure S3 related to Figure 5. Ablation of IRF4 inhibits IL-4 induced expression of metabolic genes and M2 indicator genes, but does not affect the activity of mTORC2 or Stat6. Bone marrow derived macrophages were collected from IRF4 WT (*Irf4*$^{+/+}$) and KO (*Irf4*$^{-/-}$) mice. (**A**) Expression of genes encoding a range of proteins that have been associated with M2 activation in *Irf4*$^{+/+}$ and *Irf4*$^{-/-}$ macrophages after culture with IL-4 for 3 hr as measured by qRT-PCR (expression normalized to WT M0 macrophages and presented in arbitrary units (AU)). (**B**) iNOS expression by macrophages cultured for 24 hr in the absence (M0) or presence of LPS+IFN-γ (M1), assessed by flow cytometry. (**C**) Expression of genes encoding proteins involved in glycolysis in in *Irf4*$^{+/+}$ and *Irf4*$^{-/-}$ macrophages after culture with IL-4 for 3 hr as measured by qRT-PCR (expression normalized to WT M0 macrophages

and presented in arbitrary units (AU)). (**D**) Phosphorylation of AKT^{s473} (p-AKT^{s473}) by IRF4 deficient macrophages after culture without or with IL-4 for 24 hr, assessed by flow cytometry. (**E**) Levels of Stat6 and phosphorylated Stat6 (p-Stat6) in IRF4 deficient macrophages after culture without (M0) or with IL-4 (M2) for 3 hr, assessed by immunoblotting. (**F**,**G**) Expression of PD-L2, RELMα and IRF4 in *Stat6*$^{+/+}$ and *Stat6*$^{-/-}$ macrophages after culture without (M0) or with IL-4 (M2) for 24 hr, assessed by flow cytometry. (**H**) Phosphorylation of NDRG1 (p-NDRG1), AKT^{s473} (p-AKT (S473)) and Stat6 in *Stat6*$^{+/+}$ and *Stat6*$^{-/-}$ macrophages without (M0) or with IL-4 (M2) for 3 hr, assessed by immunoblotting. β-Actin levels are shown as a loading control. In A and C, data are mean ± s.e.m. of technical replicates from one experiment. In B and D-H, data are from pooled replicates within one experiment. Numbers in B,D, F and G are mean % (F) or mean MFI (B,D,G) ± s.e.m. of data from two or more independent experiments. Data in E and H are from one experiment representative of two or more independent experiments.

Figure S4 related to Figure 5. Compared to IL-4, IL-13 is less effective at activating the mTORC2 pathway and inducing M2 activation.

(**A**) RELMα and (**B**) IRF4 expression in peritoneal macrophages from mice injected i.p. with equivalent microgram amounts of IL-4c or IL-13c, measured by flow cytometry. (**C**) IRF4 expression in bone marrow derived macrophages stimulated with equivalent microgram amounts of IL-4 or IL-13, measured by flow cytometry. (**D**) Phosphorylation of Stat6, (**E**) mTOR^{s2481}, (**F**) AKT^{s473} in bone marrow derived macrophages stimulated with IL-4 or IL-13, measured by flow cytometry. In each panel, histograms show data from one sample, and bar graphs show mean± s.e.m of technical replicates from one experiment . Data are from within one experiment, but are representative of data from two independent experiments.

Figure S5 related to Figure 6. Ablation of mTORC2 activity in macrophages shifts the tumor inflammatory profile. (A) Frequency of tumor-associated macrophages (TAM) from wild-type (Ctrl), *Raptor*$^{\Delta M\Phi}$ and *Rictor*$^{\Delta M\Phi}$ tumor-bearing animals on day 16 after inoculation. (**B**) Expression of gene encoding IFN-γ, TNFα, Nos2 and IL-10 in whole-tumor homogenates, as in panel A. (**C**) Frequency of myeloid-derived suppressor cells (MDSC), as in panel A. Data are mean ± s.e.m. of five to six individually analyzed mice/group in one experiment, and representative of two independent experiments.

Figure S6 related to Figure 6. Inhibition of mTORC2 activity in macrophages suppresses the elimination of a primary *H. polygyrus* infection. (**A-B**) Basal OCR and ECAR of peritoneal macrophages isolated from WT (Ctrl) and *Rictor*$^{\Delta M\Phi}$ mice infected with *H. polygyrus* and 9 days later, given injection of PBS (Inf) or IL-4c (Inf+IL-4c), and with injection of IL-4c again on day 11, 13 and 15, followed by analysis on day 16 after infection. (**C**) Frequency of RELMα^{+} peritoneal macrophages from mice as in A. (**D**) Geometric mean fluorescence intensity (gMFI) of IRF4 staining IRF4 by peritoneal macrophages, as in A. Data are mean ± s.e.m. of three to five individually analyzed mice/group in one experiment, and representative of two independent experiments.

Experimental Procedures

Animals and *in vivo* experiments

C57BL/6J, B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J, *Rictor*$^{tm1.1klg}$/SjmJ, B6.Cg-*Rptor*$^{tm1.1Dmsa}$/J, B6.129S1-*irf4*^{tm1Rdf}/J (all purchased from Jackson Laboratory), B6 *Irf4*$^{-/-}$ and B6 *Stat6*$^{-/-}$ mice were bred and maintained in specific pathogen free conditions under protocols approved by the institutional animal care at Washington University School of Medicine, and were used at 8-10 weeks of age. For infection with *Heligmosomoides polygyrus bakeri (H. polygyrus)*, we followed protocols described in detail previously. Mice were infected orally by gavage with 200 infective L3 stage larvae. For quantification of parasite burden, intestines were removed from mice, opened longitudinally, and placed into a metal strainer on top of a 50 ml tube filled with phosphate buffered saline (PBS; Corning) for 4 h at 37°C. Parasites dropped through the filter into the tube and were recovered for counting on a dissecting microscope. For 2-deoxy-D-glucose (2-DG; Sigma) injection, day 7 *H. polygyrus* post-infected mice were received 2-DG (0.4 g per kg body weight) or PBS intraperitoneally for 3 hr, and peritoneal macrophages were harvested and analyzed afterward. For CSF1R blockade experiment, mice were injected i.p. with 500 µl of 3% thioglycollate (Sigma) immediately prior to IL-4 complex (IL-4c; containing 5 µg of IL-4, PeproTech, and 25 µg of anti-IL-4 clone 11B11; BioXcell) injection with 0.5 mg anti-CSF1R mAb (Clone AFS98; BioXcell) or control anti-rat IgG1 antibody (BioXcell). For experiments involving the injection of IL-4 and IL-13, mice received 5 µg of recombinant IL-4 or IL-13 (PeproTech) complexed to 25 µg of anti-IL-4 (clone 11B11; BioXcell) or anti-IL-13 (clone eBio13A; eBioscience), or 100 µl PBS vehicle control, i.p. on days 0 and 3, and peritoneal exudate cells were harvested on day 4. For tumor experiments, $2x10^5$ B16-OVA melanoma cells were suspended in 100 µl of PBS and then injected s.c. Tumors were excised from mice at day 16 post-implantation. Tumors were measured every other day, and at the end of the

experiment removed and minced in HBSS (Corning), and filtered through a 70 μm strainer to obtain single cell suspensions. Peritoneal exudate cells (PECs) were harvested from sacrificed naïve or infected mice by peritoneal lavage with 10 ml of sterile PBS containing 5% FBS (HyClone) per mouse. Total numbers of PECs and peritoneal macrophages were determined by cell counting in combination with multi-color flow cytometry. Bone marrow was flushed from the femur and tibia using RPMI containing 5% FBS and dissociated into a single cell suspension by repeated pipetting. Cells were maintained on ice until use or analysis.

Preparation of macrophages from bone marrow and macrophage activation

Bone marrow cells were grown in the presence of recombinant mouse M-CSF (20 ng/ml; PeproTech) in complete medium (RPMI 1640 containing 10 mM glucose, 2 mM L-glutamine, 100 U/mL of penicillin/streptomycin and 10% FBS) for 7 days. Day 7 macrophages were washed and variously stimulated with IL-4 (20 ng/ml; PeproTech), IL-13 (20 ng/ml; PeproTech) or lipopolysaccharide (LPS, 20 ng/ml; Sigma) plus IFN-γ (50 ng/ml; R&D Systems) in the presence or absence of 10 mM 2-deoxy-D-glucose (2-DG; Sigma), 50 μM UK5099 (Sigma), 20 nM rapamycin (Rapa; Millipore), 100 nM Torin 1 (Torin; R&D Systems), 20 μM LY294002 (PI3Ki; Calbiochem), 5 μM triciribine (AKTi; Cayman) or 20 μM C75 (Sigma), for 24 hr. For M-CSF withdrawal experiment, day 7 bone marrow derived macrophages were withdrawn M-CSF for 24 hr prior to the IL-4 stimulation for another 24 hr. Macrophages were then harvested and analyzed by FACS for expression of markers of the activation.

Retroviral transduction

Retroviral transduction of macrophages was accomplished using a protocol that we have used previously to transduce bone marrow derived dendritic cells (Everts et al., 2014). Sequences for luciferase and for *Mpc-1*, *Cpt1a* and *Acaca* short hairpin RNAs

(shRNAs) were obtained from Open Biosystems and cloned into MSCV-LMP retroviral vector, encoding huCD8 or green fluorescent protein (GFP) as a reporter. For overexpression, *Slc2a1* sequence (Glut1) was cloned into MSCV-IRES retroviral vector, encoding GFP as a reporter (Chang et al., 2015). Recombinant retroviruses were used for spin infection (2500 rpm, 2 hr) of day 3 bone marrow macrophage cultures. After 7 days in culture with M-CSF, macrophages were harvested and transduced macrophages were gated based on huCD8 or GFP expression.

Metabolism assays

For real-time analysis of extracellular acidification rates (ECAR) and oxygen consumption rates (OCR), macrophages were analyzed using an XF-96 Extracellular Flux Analyzer (Seahorse Bioscience) as described in detail previously (Huang et al., 2014). Measurements were taken under basal conditions and following the sequential addition of 1 μM oligomycin, 1.5 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP), 200 μM etomoxir (ETO) and 100 nM rotenone plus 1 μM antimycin A (Rot/Ant) (all purchased from Sigma). The concentration of glucose in the medium was accessed with a Glucose Assay Kit according to the manufacturer's instructions (Eton Bioscience). ATP concentrations were determined with an ATP Determination Kit according to the manufacturer's instructions (Invitrogen). The level of triacylglycerols (TAG) in macrophages was determined from $1x10^6$ macrophages which were lysed in a 0.5% Tween 20 at 70°C for 5 min, then incubated with Triglyceride Reagent (Sigma) at 37°C for 30 min, and centrifuged at maximum speed for 3 min; after which supernatant was measured using absorbance at 540 nm after incubation with Free Glycerol Reagent (Sigma) at 37°C for 30 min. TAG levels were normalized to protein amounts in each sample using a BCA Protein Assay Kit (Pierce).

Flow Cytometry

Cells were kept on ice and blocked with 5 µg/ml of anti-CD16/32 (clone 93, eBioscience) before surface staining with antibodies to CD45 (clone 30-F11, eBioscience), CD11b (clone M1/70, eBioscience), F4/80 (clone BM8, eBioscience), CD64 (clone X54-5/7.1, BioLegend), CD301 (clone ER-MP23, AbD Serotec), PD-L2 (clone TY25, eBioscience) and Ly-6G&Ly-6C (Gr1; clone RB6-8C5, BD Biosciences). For intracellular staining, cells were fixed in Cytofix/Cytoperm (BD Biosciences), and stained with rabbit anti-RELMα (PeproTech), mouse anti-NOS2 (clone C-11, Santa Cruz Biotechnology), mouse anti-TNFα (clone MP6-XT22, eBioscience), mouse phospho-AKT Ser473 (p-AKT^{s473}; clone M89-61, BD Biosciences), mouse anti-phospho-Stat6 Tyr641 (p-Stat6; clone J71-733.58.11, BD Biosciences), rabbit anti-phospho-mTOR Ser2481 (p-mTOR^{s2481}; Cell Signaling) and anti-Glut1.RBD (METAFORA biosystems) for 30 min at 4°C in 1X Perm/Wash (BD Biosciences), followed by incubation with appropriate fluorochrome-conjugated anti-rabbit or anti-mouse IgG in the same buffer. For IRF4 and Ki67 staining, cells were fixed in Fixation/Permeabilization (eBioscience), and stained with mouse anti-IRF4 (clone 3E4, eBioscience) or mouse anti-Ki67 (clone B56, BD Biosciences) for 30 min at 4°C in 1X Permeabilization Buffer (eBioscience). The uptake of 2-NBDG (10 µg/ml; Invitrogen) over 2 hr of culturing macrophages in RPMI medium at 37°C was measured by flow cytometry. NBDG binding reflected the ability of cells to use glucose in as much as that it could be competed by glucose (Fig. S2C). All macrophage staining data shown represent cells that were gated as CD11b^{+}/F4/80^{+} or F4/80^{+}, and living based on LIVE/DEAD (Invitrogen) or 7-amino-actinomycin D (eBioscience) staining. Data were acquired on a FACSCanto II flow cytometer (BD Biosciences), and analyzed with FlowJo v.9.5.2 (Tree Star).

Cell fractionation and Western blotting

Cells were frozen and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100 and 0.1% SDS) with Protease Inhibitor Cocktail (Sigma) and phosphatase inhibitor Cocktail (Sigma). For mTOR and Stat6 western blotting, anti-phospho-p70 S6 kinase (p-S6K), anti-p70 S6 kinase (S6K), anti-phospho-NDRG1 Thr346 (p-NDRG1), anti-NDRG1, anti-p-AKT^{s473}, anti-AKT, anti-p-Stat6, anti-Stat6 and anti-β-actin (all purchased from Cell Signaling) were used at a dilution of 1:1000, followed by detection with peroxidase-linked antibody to rabbit antibody IgG (1:2000 dilution; Cell Signaling). Proteins were detected by ECL Western Blotting Detection Reagents (GE Healthcare). Images were analyzed using ImageJ (NIH).

Gene expression

For quantitative RT-PCR, total RNA isolation was used with TRIzol Reagent (Invitrogen), and single-strand cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The Taqman method was used for real-time PCR with primers from Applied Biosystems and an Applied Biosystems 7000 sequence-detection system. The expression of mRNA was normalized to that of mRNA encoding β-actin. Analysis of our reported RNAseq data (Huang et al., 2014) using GENE-E was used to generate data in Fig. 1B and S1B.

References

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