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Supplemental Information

IRF5 and *IRF5* Disease-Risk Variants Increase Glycolysis and Human M1 Macrophage Polarization by Regulating Proximal Signaling and Akt2 Activation

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Figure S1

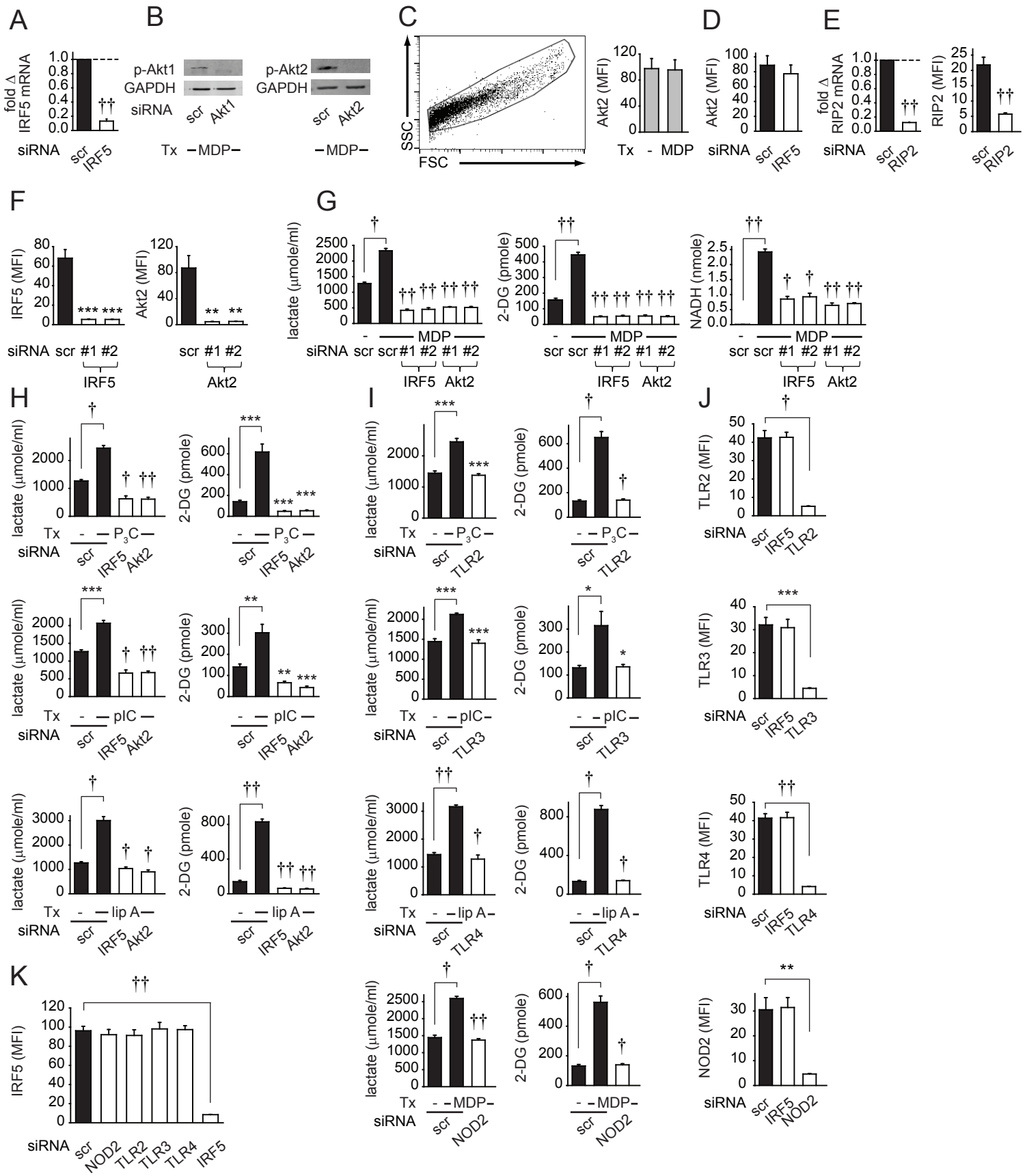
A
B
C
D
E
F
G
H
I
J
K
fold Δ IRF5 mRNA
siRNA
scr
IRF5
p-Akt1
GAPDH
p-Akt2
Akt1
Akt2
Tx
MDP
SSC
FSC
Akt2 (MFI)
fold Δ RIP2 mRNA
RIP2
RIP2 (MFI)
IRF5 (MFI)
lactate (μmole/ml)
2-DG (pmole)
NADH (nmole)
#1
#2
P3C
pIC
lip A
TLR2
TLR3
TLR4
NOD2
TLR2 (MFI)
TLR3 (MFI)
TLR4 (MFI)
NOD2 (MFI)

Figure S1 legend

Fig. S1, related to Fig. 1. IRF5 regulates glycolytic pathway genes and glycolysis following stimulation of multiple PRRs. (A) MDMs (n=4 donors) were transfected with IRF5 siRNA. Fold mRNA expression compared to scrambled siRNA-transfected cells for each donor+SEM. **(B)** MDMs were transfected with the indicated siRNA, treated with 100μg/ml MDP for 15 min, and Akt1 and Akt2 phosphorylation was assessed by western blot. Total GAPDH expression from whole cell lysates served as a loading control. **(C)** MDMs (n=6, similar results seen in an additional n=4) were treated with 100μg/ml MDP for 15min. Shown is gating strategy and summarized flow cytometry data for Akt2 (clone D6G4, Cell Signaling Technology) MFI+SEM**.** **(D)** MDMs (n=4) were transfected with IRF5 siRNA. Summarized flow cytometry data for Akt2 MFI+SEM. **(E)** MDMs were transfected with RIP2 siRNA. Fold mRNA (n=4) and protein (clone 251-RIG-G, BD Biosciences), as assessed by flow cytometry (n=8) compared to scrambled siRNA-transfected cells for each donor + SEM. **(F-G)** MDMs (n=4) were transfected with IRF5 (two different siRNAs: J-011706-05; J-011706-06, Dharmacon) or Akt2 (two different siRNAs: J-003001-10; J-003001-11, Dharmacon) siRNA. **(F)** Summarized flow cytometry data for IRF5 or Akt2 MFI+SEM. **(G)** Transfected cells were treated with 100μg/ml MDP for 24h, and then assessed for lactate production, glucose uptake and hexokinase activity. **(H-K)** MDMs (n=4) were transfected with the indicated siRNA. **(H-I)** Cells were then treated with 10μg/ml Pam_3Cys (TLR2 ligand), 100μg/ml poly I:C (TLR3 ligand) or 0.1μg/ml lipid A (TLR4 ligand) for 24h and assessed for lactate production and glucose uptake, or left untreated and assessed for the expression of **(J)** the indicated PRR (TLR2, TLR3, TLR4, eBiosciences; NOD2, Cayman Chemical) or **(K)** IRF5 by flow cytometry. Mean+SEM. Significance is shown relative to PRR ligand-treated, scrambled siRNA-transfected cells or as indicated. Tx, treatment; scr, scrambled. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1x10^{-4}$; ††, $p<1x10^{-5}$.

Figure S2

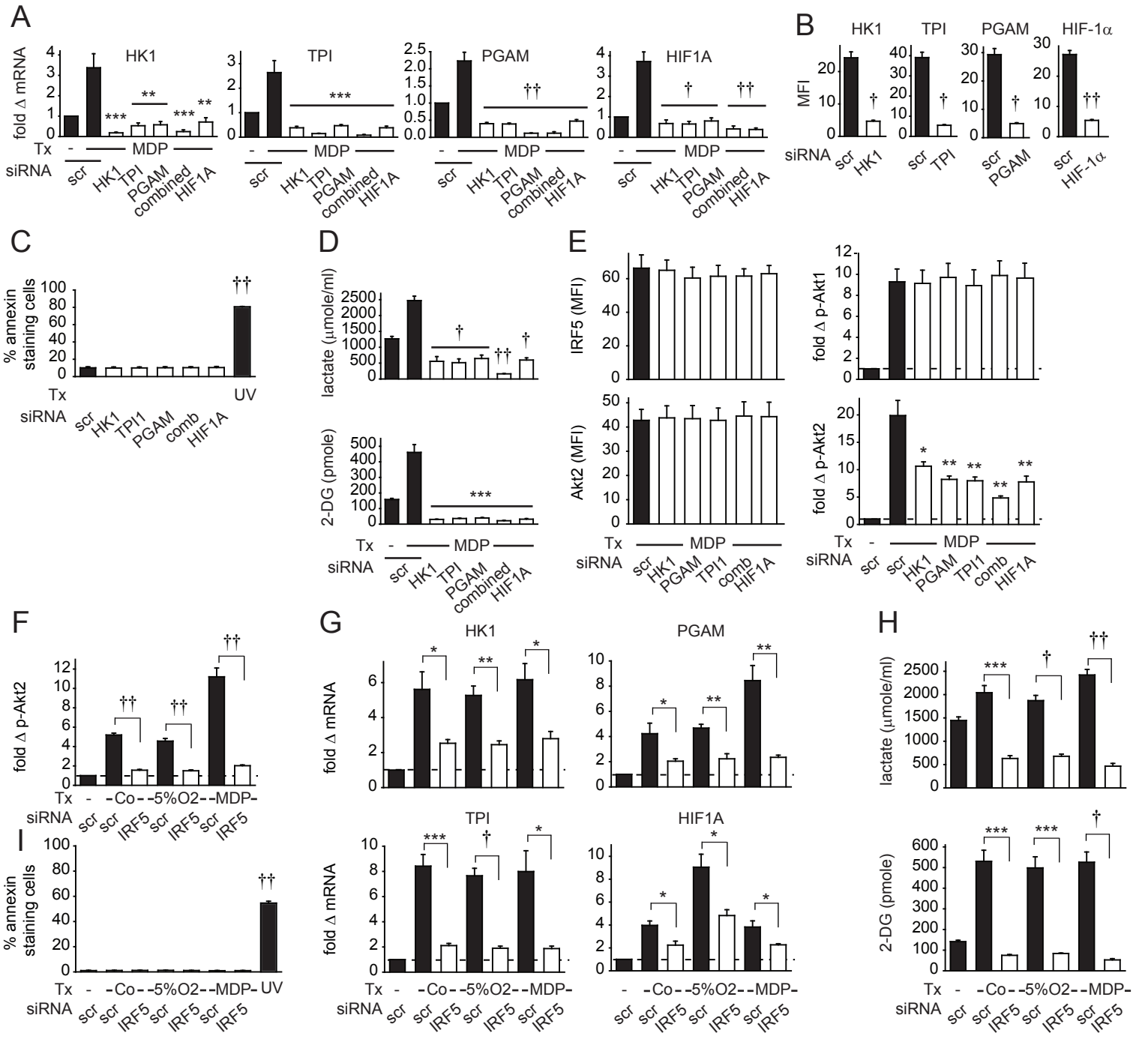

Fig. S2, related to Fig. 1. Hypoxia regulates glycolysis in an IRF5-dependent manner, glycolytic genes and HIF1A regulate glycolysis, and these genes cross-regulate each other's expression. MDMs were transfected with HK1, TPI, and PGAM siRNA, alone or in combination, or with HIF1A siRNA, then treated with 100μg/ml MDP and assessed for: **(A)** mRNA at 4h (n=8 donors), **(B)** protein expression for HK1 (Cell Signaling Technology), TPI (Millipore), PGAM (Cell Signaling Technology) and HIF-1α (Cell Signaling Technology) at 24h (n=8), **(C)** cell death (expressed as percent annexin V^+ cells, BD Biosciences) at 24h (n=4); UV stimulation at 50-100 J/m^2 is shown as a positive control, **(D)** lactate production and glucose uptake at 24h (n=4), and **(E)** expression of the indicated proteins by flow cytometry at 15 min (n=4). For **(A,D,E)** similar results were seen in an additional n=4**,** and significance is compared to scrambled siRNA-transfected, MDP-treated MDMs. **(F-I)** MDMs were transfected with IRF5 siRNA, and treated with 100μM $CoCl_2$ (Sigma-Aldrich) or cultured at 5% O2 and assessed for: **(F)** phospho-Akt2 induction at 15min (n=8, similar results seen in n=4), **(G)** mRNA expression at 4h compared to untreated, scrambled siRNA-transfected cells for each donor+SEM (n=4), **(H)** lactate production and glucose uptake at 24h (n=4) or **(I)** cell death at 24h (n=8). Controls included treatment with 100μg/ml MDP. Tx, treatment; scr, scrambled. *, $p<0.01$; **, $p<0.01$; ***, $p<0.001$; †, $p<1x10^{-4}$; ††, $p<1x10^{-5}$.

Figure S3

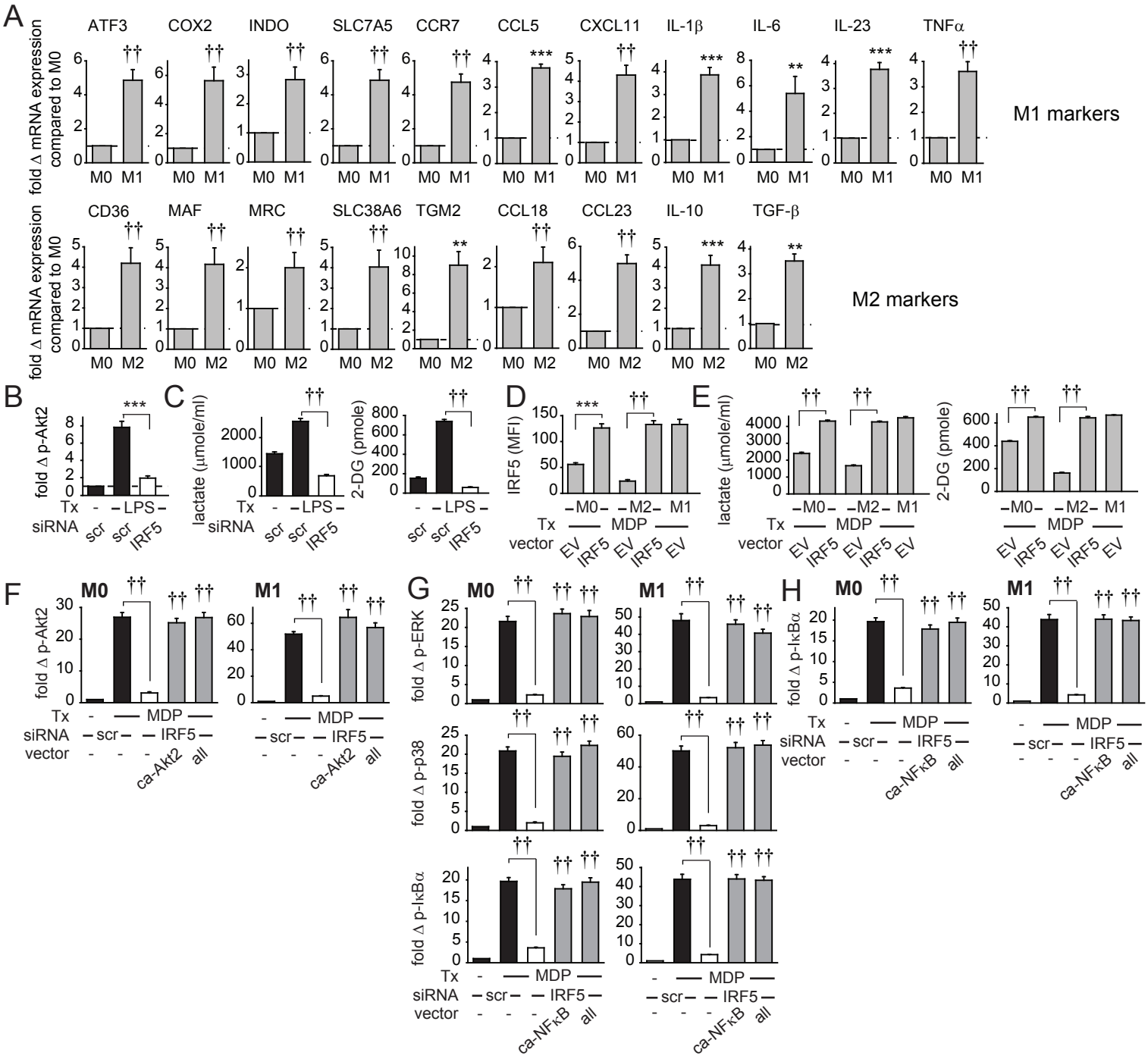

Fig. S3, related to Fig. 3. IRF5 is required for glycolysis upon LPS treatment and increasing IRF5 in M0 and M2 macrophages increases glycolysis. **(A)** MDMs (n=8 donors) were left non-polarized (M0) or polarized to M1 or M2 macrophages. Summarized data are represented as fold mRNA induction of M1 or M2 markers normalized to non-polarized (M0) MDMs for each donor+SEM. **(B-C)** MDMs (n=4) were transfected with IRF5 siRNA, treated with 0.1μg/ml LPS and assessed for: **(B)** Akt2 phosphorylation at 15min, and **(C)** lactate production and glucose uptake at 24h. **(D-E)** MDMs (n=4) were transfected with empty vector (EV) or an IRF5-expressing vector for 24h. Cells were left non-polarized (M0) or polarized to M1 or M2 macrophages and then treated with 100μg/ml MDP for 24h and assessed for: **(D)** IRF5 protein expression by flow cytometry, or **(E)** lactate production and glucose uptake. **(F-H)** MDMs (n=4) were transfected with IRF5 siRNA±EV, constitutively active Akt2 (ca-Akt2), a combination of constitutively active ERK, p38, and JNK kinases (ca-MAPK), constitutively active NFκB (ca-NFκB), or all vectors in combination ('all') and left non-polarized or polarized to M1 for 24h. Cells were then treated with 100μg/ml MDP for 15min and assessed by phospho-flow cytometry. Summary graph for M0 and M1 cells with fold phospho-protein induction+SEM. Note that M0 and M1 macrophage phospho-protein 'y' axes are on different scales due to higher levels of M1 macrophage signaling. Significance is relative to MDP-treated, EV-, IRF5 siRNA-transfected cells or as indicated. Tx, treatment; scr, scrambled; **, $p<0.01$; ***, $p<0.001$; ††, $p<1x10^{-5}$.

Figure S4

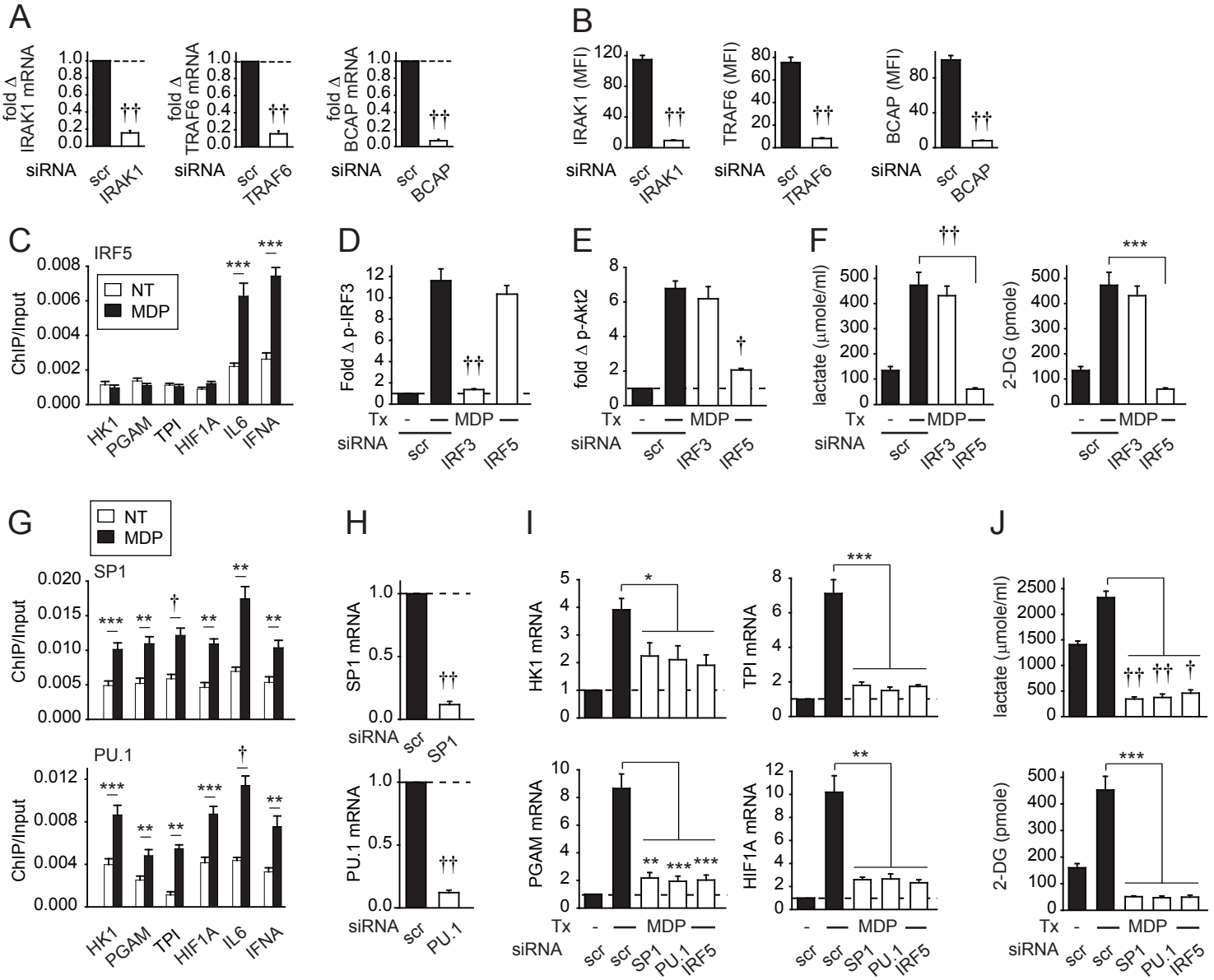

Fig. S4, related to Fig. 5. SP1 and PU.1 regulate NOD2-induced glycolysis. **(A-B)** MDMs were transfected with the indicated siRNA. **(A)** Fold mRNA (n=4 donors) and **(B)** IRAK1, TRAF6 and BCAP (R&D Systems) protein expression as assessed by flow cytometry (n=4) compared to scrambled siRNA-transfected cells for each donor + SEM. **(C)** MDMs (n=8) were treated with 100μg/ml MDP for 4h and binding of IRF5 (#ab2932, Abcam) to the indicated promoters was assessed by ChIP. *IL6* and *IFNA* promoters were included as positive controls. **(D-F)** MDMs (n=4) were transfected with IRF3 or IRF5 siRNA, treated with 100μg/ml MDP and assessed for: **(D)** IRF3 phosphorylation (clone D6O1M, Cell Signaling Technology) at 15min by flow cytometry, **(E)** Akt2 phosphorylation at 15 min, and **(F)** lactate production and glucose uptake at 24h. Mean+SEM. **(G)** MDMs (n=8) were treated with 100μg/ml MDP for 4h and binding of SP1 (#ab13370, Abcam) and PU.1 (#PA5-17505, Thermo Fisher Scientific) to the indicated promoters was assessed by ChIP. *IL6* and *IFNA* promoters were included as positive controls. **(H-J)** MDMs were transfected with SP1, PU.1 or IRF5 siRNA and assessed for: **(H)** mRNA expression (n=4), **(I)** mRNA expression following treatment with 100μg/ml MDP for 4h compared to untreated, scrambled siRNA-transfected cells for each donor (n=8), or **(J)** lactate production and glucose uptake (n=4) following treatment with 100μg/ml MDP for 24h. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1x10^{-4}$; ††, $p<1x10^{-5}$.

Figure S5

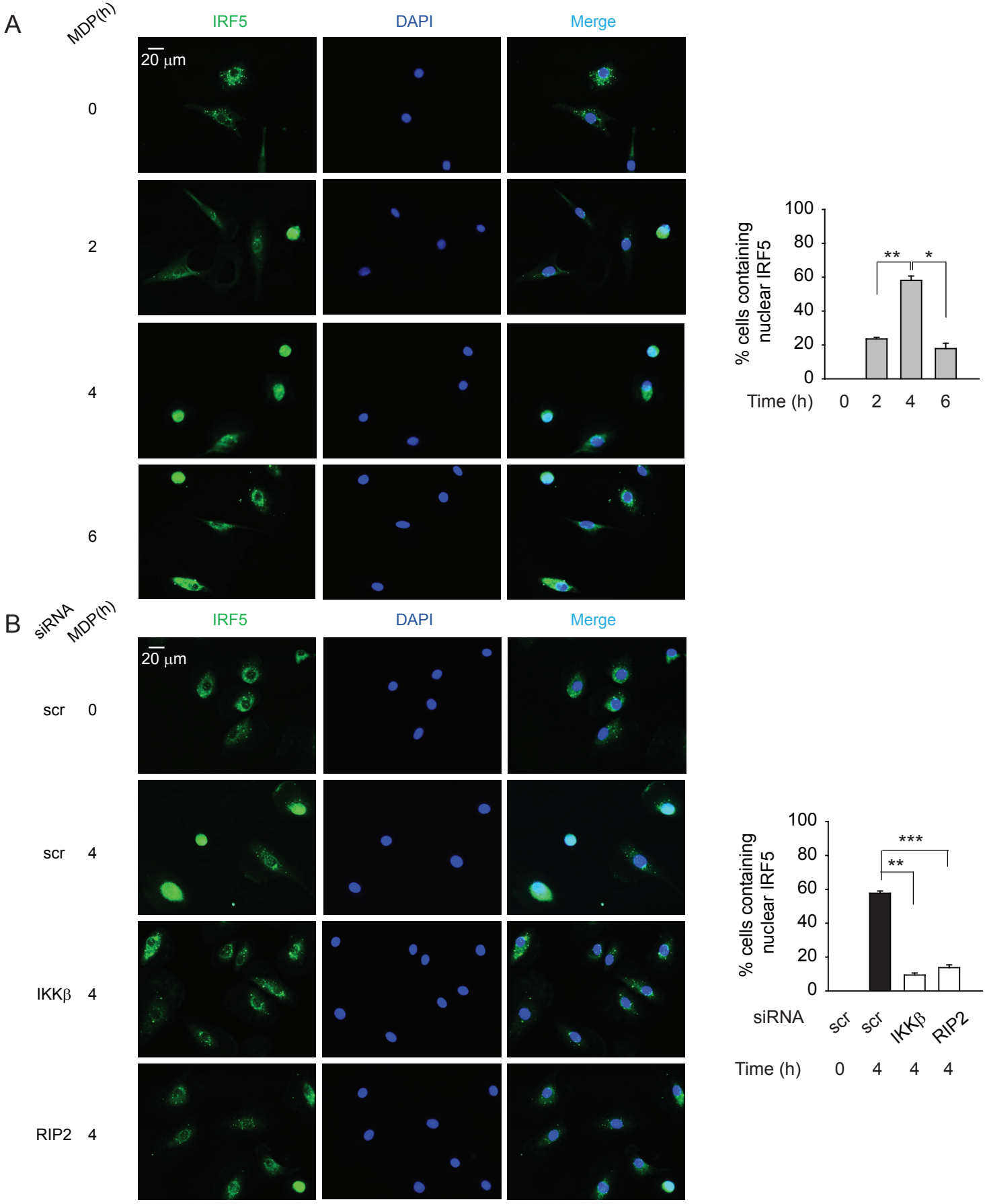

Fig. S5, related to Fig. 6. RIP2 and IKKβ are required for NOD2-mediated IRF5 nuclear translocation. **(A)** MDMs were treated with 100μg/ml MDP for the indicated times or **(B)** transfected with IKKβ or RIP2 siRNA, and then treated with 100μg/ml MDP for 4h and immunostained for IRF5 (green) and nucleus (DAPI; blue). Summary graphs indicate percent of cells demonstrating IRF5 nuclear translocation and represent three independent experiments. Mean+SEM. Scr, scrambled. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Figure S6

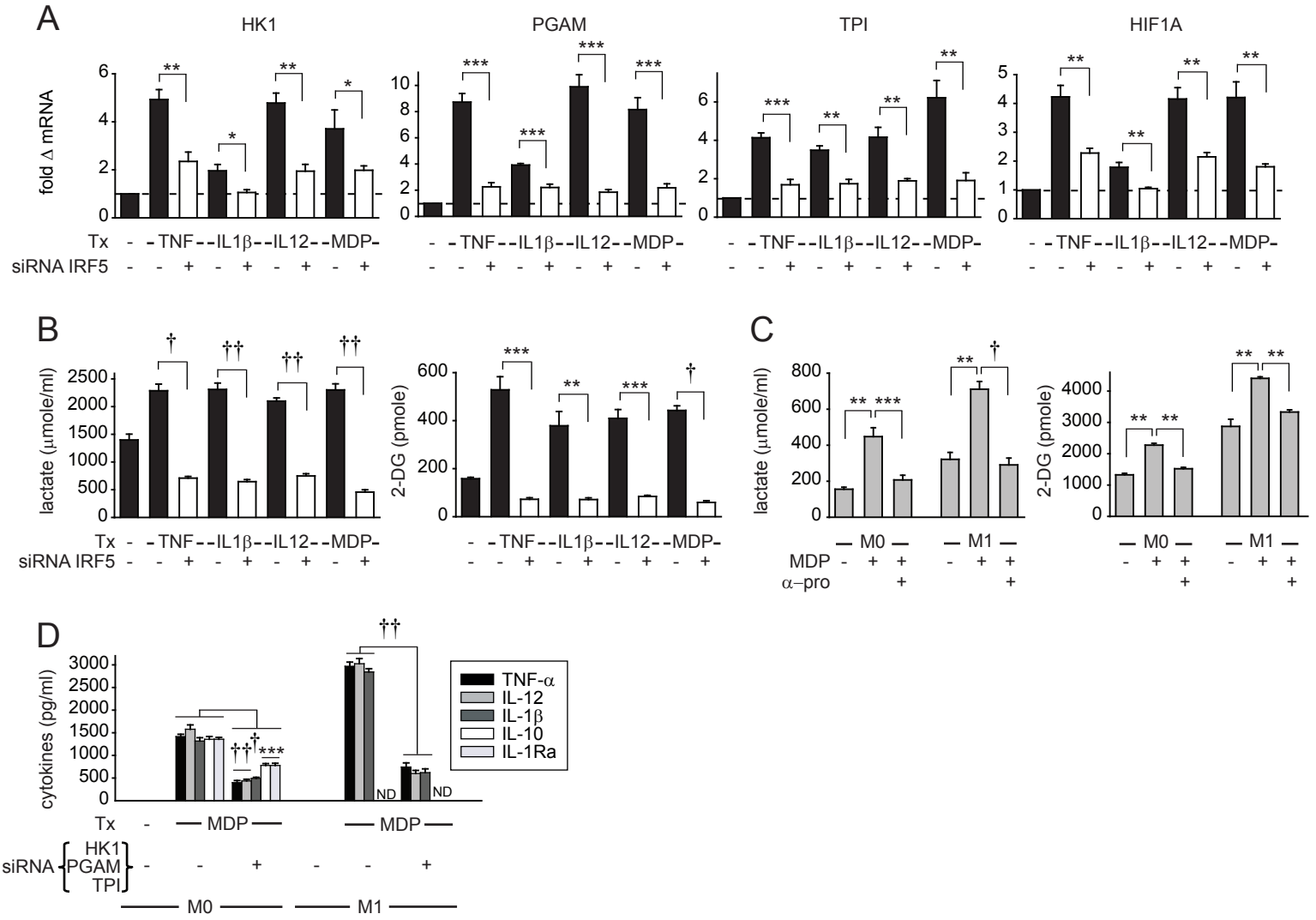

Fig. S6, related to Fig. 6. IRF5 is required for pro-inflammatory cytokine-induced glycolysis and glycolysis is required for optimal PRR-induced cytokines. (A-B) MDMs (n=4 donors) were transfected with IRF5 siRNA, and treated with 10ng/ml TNF-α, IL-1β or IL-12 (eBiosciences) and assessed for: **(A)** mRNA expression of indicated genes at 4h compared to untreated, scrambled siRNA-transfected cells for each donor+SEM, or **(B)** lactate production and glucose uptake at 24h. Controls included treatment with 100μg/ml MDP. **(C)** MDMs (n=4) were left non-polarized (M0) or polarized to M1 macrophages and incubated with neutralizing 1μg/ml anti-TNFR1 antibody (R&D), 0.5mg/ml IL-1Ra (blocks IL-1R signaling) (GenScript) and neutralizing 1μg/ml anti-IL-12Rβ1 (BD Bioscience) in combination for 1h ("α-pro"), then treated with 100μg/ml MDP and assessed for lactate production and glucose uptake at 24h. **(D)** MDMs (n=4) were transfected with combined HK1, TPI1 and PGAM siRNA for 24h, and left non-polarized (M0) or polarized to M1 macrophages and treated with 100μg/ml MDP for 24h. Cytokine secretion+SEM. Tx, treatment; scr, scrambled; ND, not detected. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1x10^{-4}$; ††, $p<1x10^{-5}$.

Figure S7

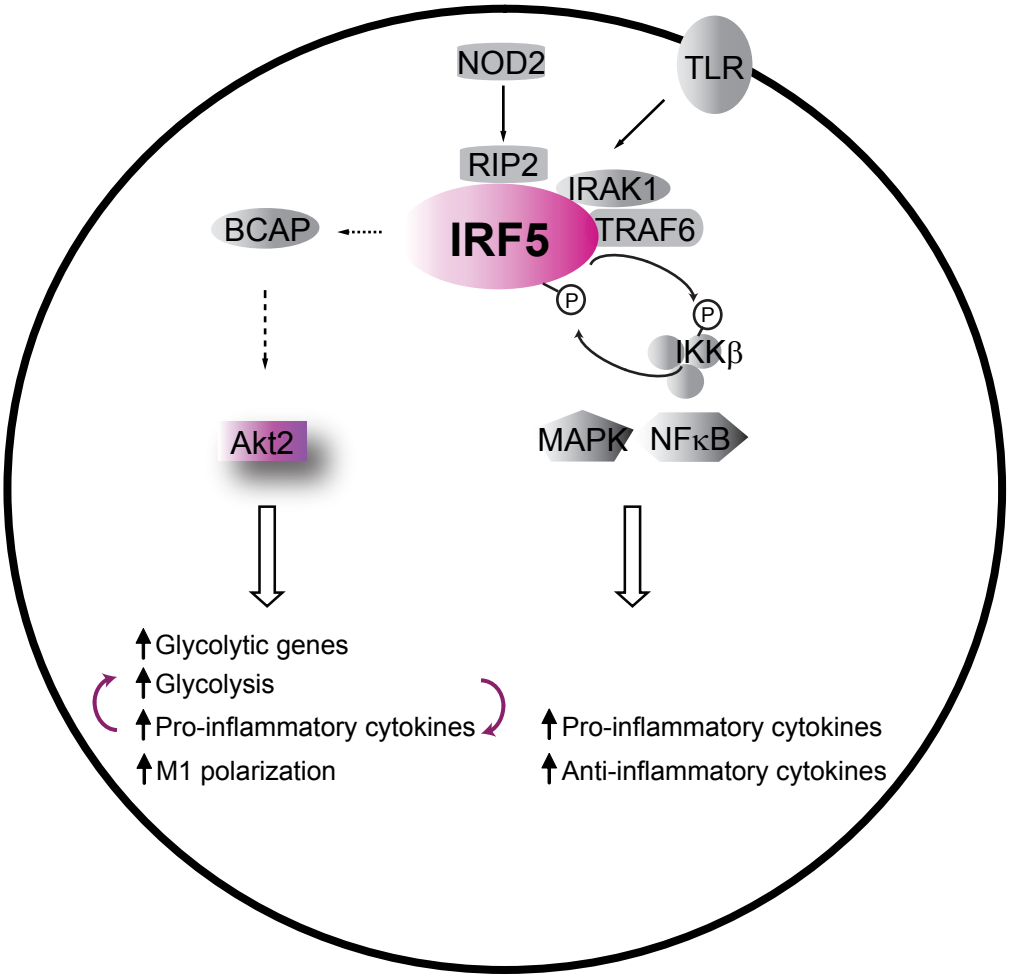

Fig. S7, related to Fig. 7. IRF5 activates Akt2 to regulate PRR-induced glycolysis and M1 polarization of macrophages. Stimulation of the PRR NOD2 in MDMs leads to IRF5 association with RIP2, IRAK1 and TRAF6. Each of these molecules is required for IRF5 serine phosphorylation. IRF5 then leads to activation of Akt2. Of note is that NOD2-induced Akt2 activation is BCAP-dependent, whereas MAPK and NFκB pathway activation is IRAK1/TRAF6-dependent. Moreover, IRF5 and IKKβ cross-regulate each other's phosphorylation. Akt2 activation is required for optimal upregulation of glycolytic gene expression, glycolysis, M1 polarization and pro-inflammatory cytokine secretion, but not anti-inflammatory cytokine secretion. In contrast, MAPK and NFκB activation increases the secretion of both pro- and anti-inflammatory cytokines. Furthermore, autocrine NOD2-induced pro-inflammatory cytokines and glycolysis cross-regulate each other. IRF5 also regulates PRR-induced glycolysis in mice in vivo. The dependency on IRF5 and Akt2 for optimal glycolysis extends to multiple PRRs. Macrophages from rs2004640/rs2280714 TT/TT disease-risk carriers express increased IRF5, and show increased NOD2-induced Akt2, MAPK and NFκB pathway signaling, and enhanced glycolysis and M1 polarization relative to GG/CC carriers.