

Supplemental figures and legends

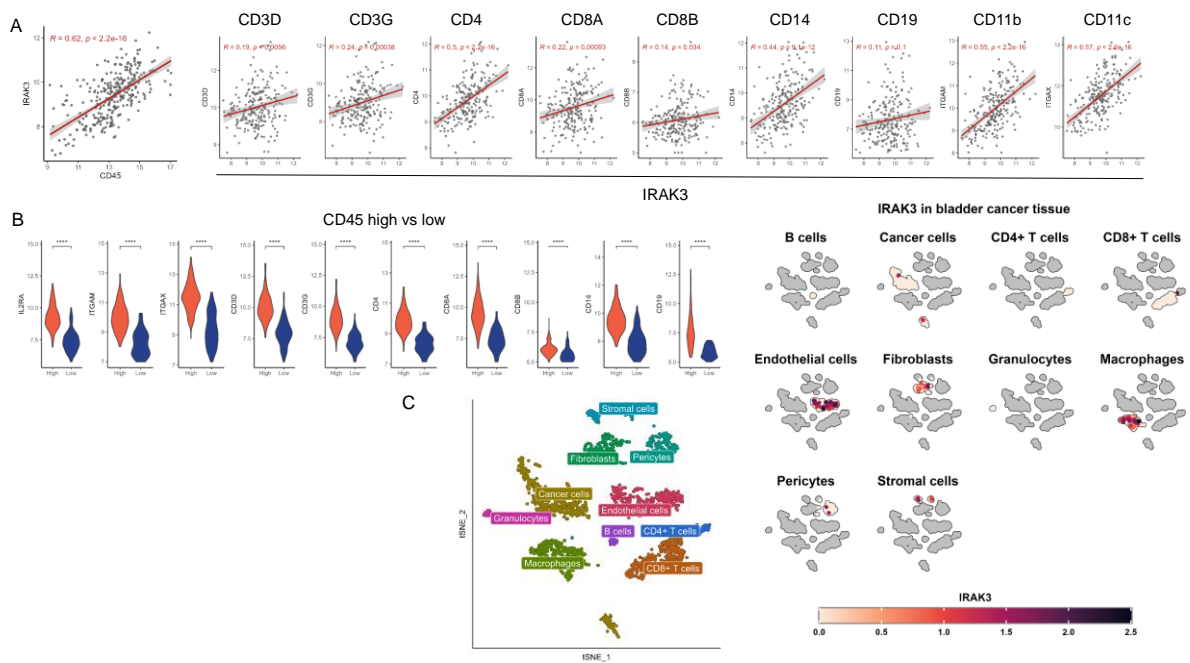


Figure S1. Supplementary figure to Figure 1. **A)** Correlation between *IRAK3* mRNA expression and lineage-specific genes in the IMvigor210 cohort. **B)** Expression of immune related genes in *CD45* high and low patients. **C)** Expression of *IRAK3* mRNA at the single cell level in bladder tumors using a public scRNAseq dataset.

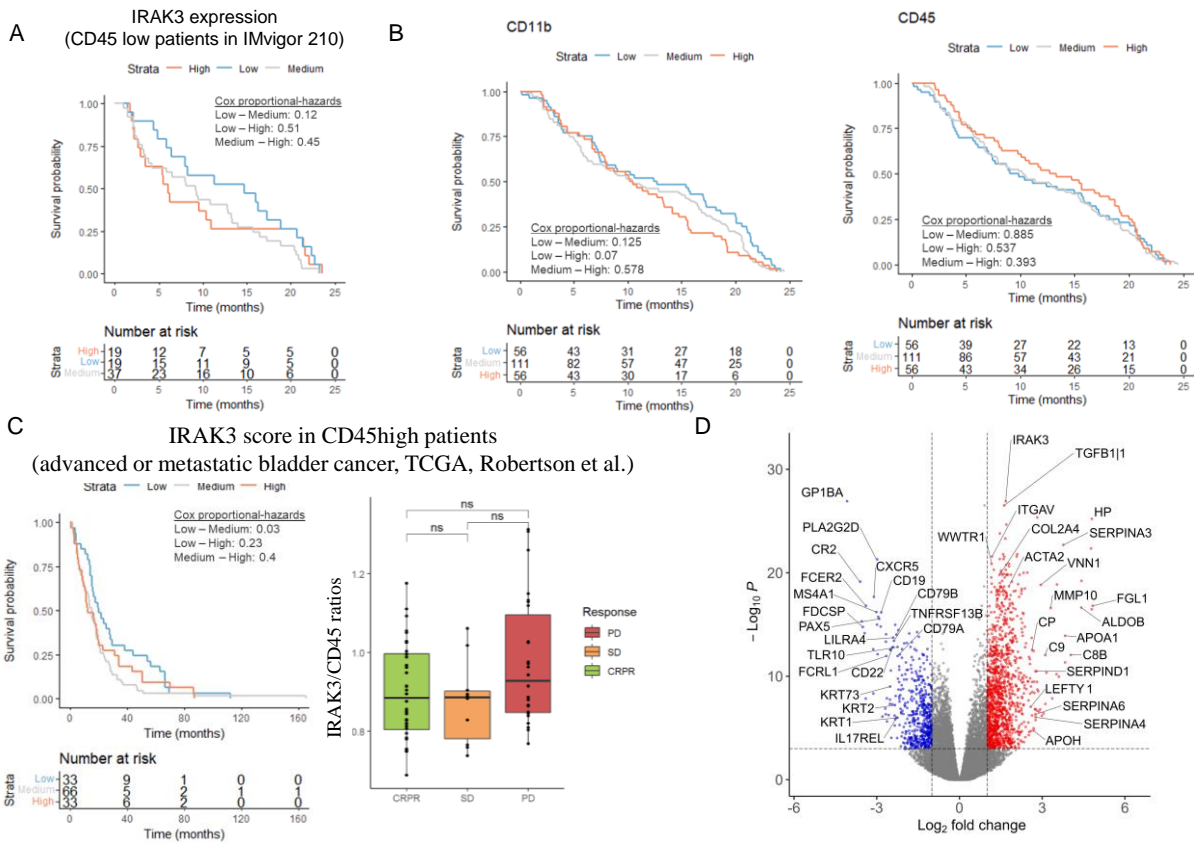


Figure S2. Supplementary figure to Figure 1. **A)** Patient survival analysis using pre-therapy *IRAK3* mRNA expression in *CD45* low patients of the IMvigor210 cohort. **B)** Patient survival analysis using pre-therapy *CD11b* or *CD45* expression in *CD45* high patients of the IMvigor210 cohort. **C)** Patient survival and response analysis using *IRAK3* mRNA expression in *CD45* high advanced or metastatic bladder cancer patients using a dataset from TCGA. Survival analysis was done using a Kaplan-Meier curve and fitted with a Cox proportional hazards regression model. **D)** Volcano plot to demonstrate up- and down-regulated genes when comparing *IRAK3* high and *IRAK3* low patients in the IMvigor210 trial. Differences were calculated using the Wald test from DESeq2.

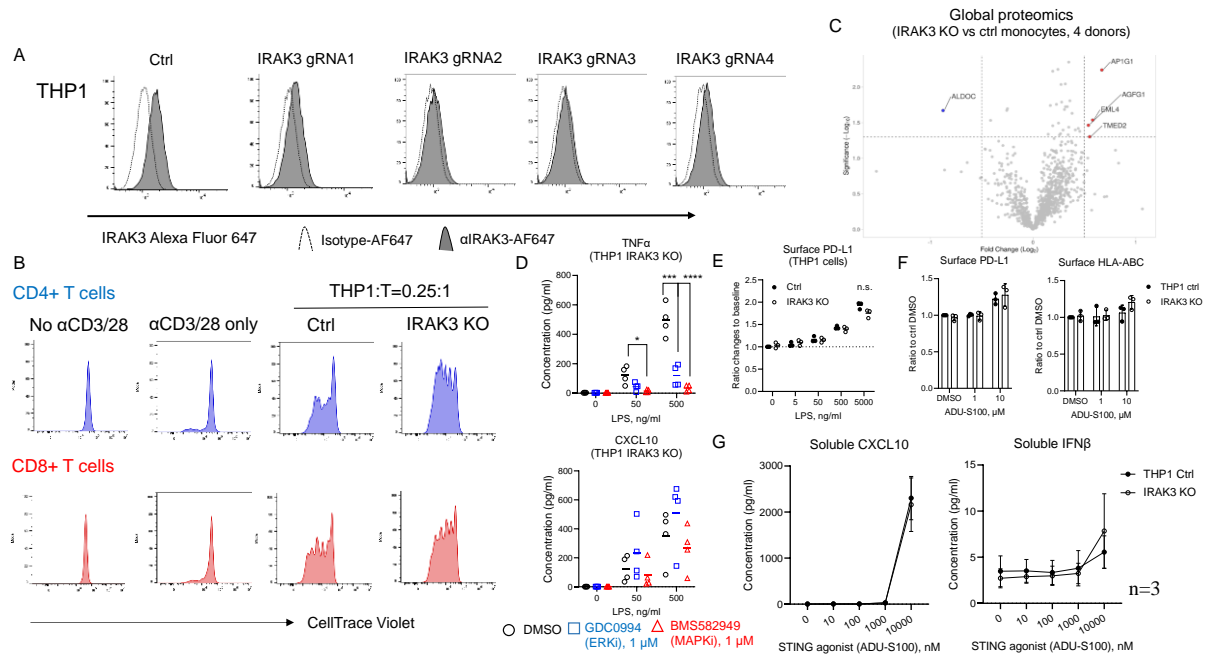


Figure S3. Supplementary figure to Figure 2. **A)** Comparison of 4 gRNAs targeting the *IRAK3* gene in human THP1 cells. Representative histogram of 3 experiments. **B)** Representative histograms to demonstrate T cell proliferation in the THP1:T cell co-culture assay. **C)** Volcano plot for global protein quantification upon *IRAK3* protein deletion in primary human monocytes from 3 individual donors. Log₂ fold changes and Log₁₀ P values in KO vs control monocytes for each protein were plotted. **D)** *IRAK3* deficient THP1 cells were treated with 1 μM pharmacological inhibitor against MAPK (BMS582949) or ERK (GDC0994) or DMSO for 2 hours, followed by activation using LPS as indicated in the graphs. Supernatants were harvested after 5 hours and cytokines were analyzed using Legendplex (4 biological replicates, unpaired T tests, *:P<0.05, ***:P<0.001, ****:P<0.0001). **E)** Surface PD-L1 expression on control or *IRAK3* KO THP1 cells after LPS treatment for 24 hours and normalized to the non-stimulated controls, 3 biological replicates. **F)** Surface expression of PD-L1 or HLA-ABC (n=3, normalized to DMSO controls) or **G)** release of CXCL10, IFNβ (n=3) after treatment with a STING agonist, ADU-S100 for 24 hours. Statistical analyses were performed using unpaired T tests in all experiments.

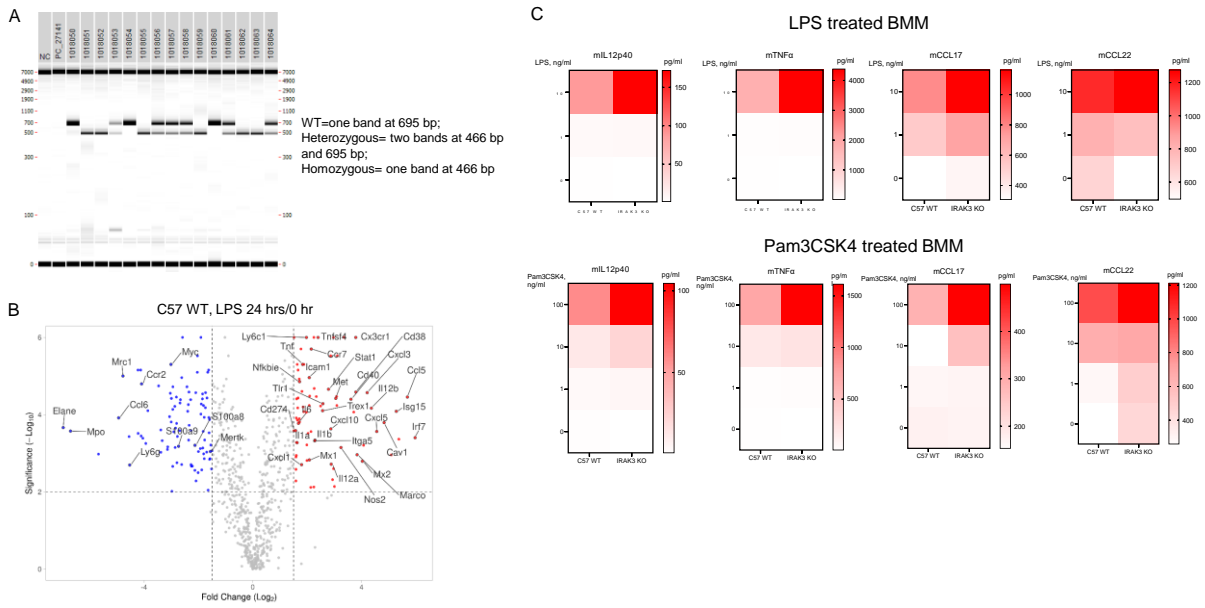


Figure S4. Supplementary figure to Figure 5. A) Genotype results for wild-type (*WT*), heterozygous and homozygous *IRAK3 KO* mice. **B)** Volcano plot to demonstrate differentially expressed mRNAs upon LPS treatment in bone-marrow derived macrophages (BMM) in *WT* mice ($n=3$) after 24 hours (Log_2 fold changes versus Log_{10} P values, unpaired T tests). **C)** Release of soluble factors from *WT* or *IRAK3 KO* BMM cells in response to LPS or Pam3CSK4 from at least 4 animals in each group.

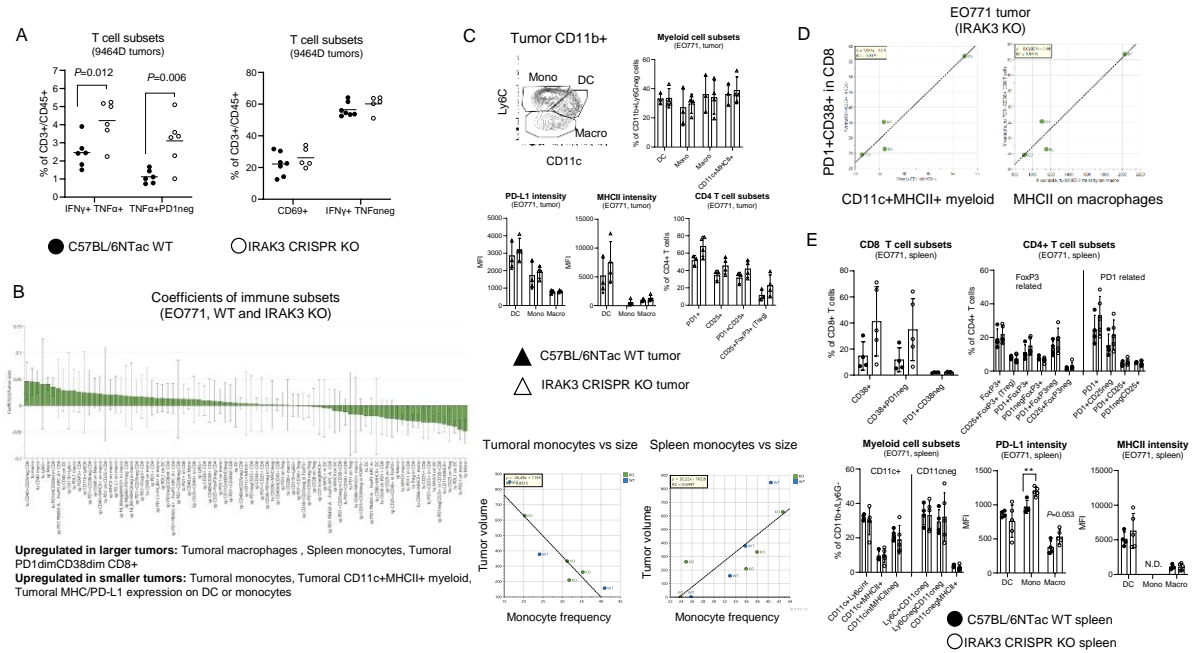


Figure S5. Supplementary figure to Figure 6. **A)** Percentages of T cell subsets expressing TNFA, IFNG, CD69 or PD-1 were shown in 9464D tumors implanted to *WT* or *IRAK3 KO* mice. Values from individual mouse were shown in the graphs and statistical analysis was done using unpaired T tests. **B)** Multi-variant analysis to identify immunological changes due to tumor volumes using the SIMCA software. **C)** Immunological changes in EO771 tumors from *WT* (n=3) or *IRAK3 KO* mice (n=4). **D)** Correlation between PD-1+CD38+ cytotoxic T cells and myeloid cell activation in EO771 tumors from *IRAK3 KO* mice (n=4). **E)** Immunological changes in spleens from EO771-bearing *WT* (n=4) or *IRAK3 KO* (n=5) mice. Age-matched female C57BL/6NTac or *IRAK3 KO* mice were used, unpaired T tests, **:P<0.01.

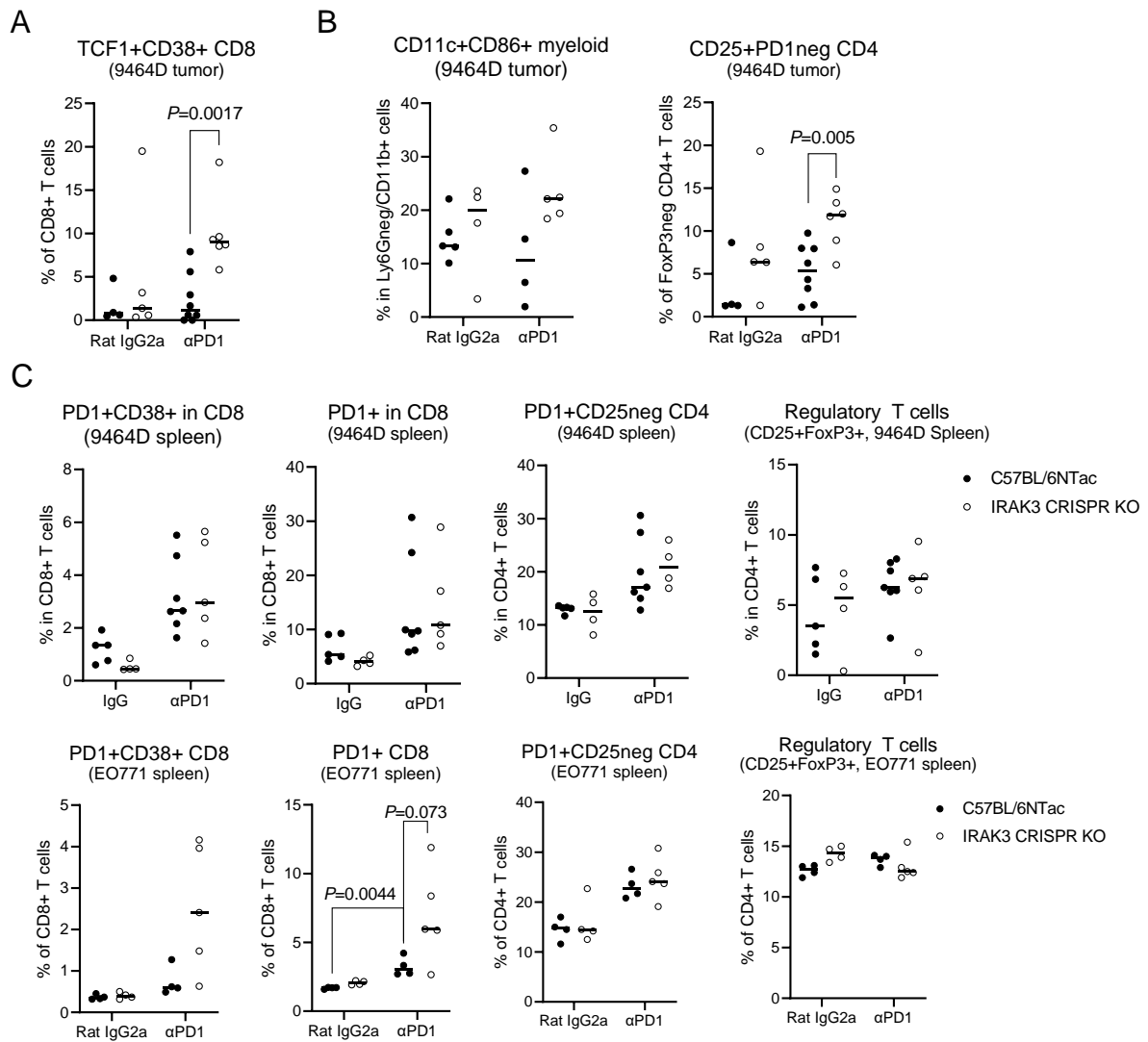


Figure S6. Supplementary figure to Figure 7. A) and B) Immunological changes in mice bearing the 9464D tumors from the 4 treatment groups. C) Immunological changes in spleens from *WT* or *IRAK3 KO* mice bearing 9464D or EO771 tumors after treatment of Rat IgG2a isotype or an anti-PD-1 antibody. Age-matched female C57BL/6NTac or *IRAK3 KO* mice were used. Number of animals were indicated in the plots. Statistical tests were done using unpaired T tests and P values were shown in the graphs.

Supplementary Table 1: Antibodies

Name	Clone	Application	Product information
Anti-human IRAK3	Rabbit IgG	WB	Cell Signaling Technology/4369
Anti-mouse IRAK3	Goat IgG	WB	Everest/EB08674
Anti-Rabbit IgG HRP-linked Antibody	Goat IgG	WB	Cell Signaling Technology/7074S
Anti-mouse IgG HRP-linked-Antibody	Horse IgG	WB	Cell Signaling Technology/7076S
Anti-human/mouse Vinculin	nVin-1, mouse IgG1	WB	Sigma Aldrich/V9131
Anti-goat IgG (H+L) Secondary Antibody, HRP	Rabbit IgG	WB	Invitrogen/81-1620
Anti-human IRAK3	Rabbit IgG	FACS	Atlas Antibodies/HPA043097
Rabbit polyclonal IgG	Rabbit IgG	FACS	R&D/AB-105-C
APC anti-human CD3	HIT3a, mouse IgG2a,k	FACS	Biolegend/300312
PE anti-human CD4	OKT4, mouse IgG2b,k	FACS	Biolegend/317410
PerCp-Cy5.5 anti-human CD8	RPA-T8, mouse IgG1,k	FACS	Biolegend/301032
PE-Cy7 anti-human CD56	HCD56, mouse IgG1,k	FACS	Biolegend/318318
Brilliant Violet 650 anti-human CD19	HIB19, mouse IgG1,k	FACS	Biolegend/302238
APC-Cy7 anti-human CD25	BC96, mouse IgG1,k	FACS	Biolegend/302614
PE-Dazzle anti-human CXCR3	G025H7, mouse IgG1, k	FACS	Biolegend/353736
FITC anti-human PD-L1	MIH2, mouse IgG1, k	FACS	Biolegend/393606
APC anti-human HLA-ABC	W6/32, mouse IgG2a, k	FACS	Biolegend/311410
PE-Cy7 anti-mouse CD206	C068C2, rat IgG2a, k	FACS	Biolegend/141720
Brilliant violet 650 anti-mouse I-A/I-E	M5/114.15.2, rat IgG2b, k	FACS	Biolegend/107641
PE-Dazzle 594 anti-mouse/human CD11b	M1/70, rat IgG2b, k	FACS	Biolegend/101256
APC-Cy7 anti-mouse Ly6G	1A8, rat IgG2a, k	FACS	Biolegend/127624
PerCp-Cy5.5 anti-mouse Ly6C	HK1.4, rat IgG2c, k	FACS	Biolegend/128012
PE anti-mouse CD11c	N418, Armenian hamster IgG	FACS	eBioscience/12-0114-83
Brilliant violet 421 anti-mouse PD-L1	10F.9G2, rat IgG2b, k	FACS	Biolegend/124315
Brilliant violet 605 anti-mouse CD86	GL-1, rat IgG2a, k	FACS	Biolegend/105037
FITC anti-mouse CD73	TY/11.8, rat IgG1, k	FACS	Biolegend/127219
APC anti-mouse CSF-1R	AFS98, rat IgG2a, k	FACS	Biolegend/135509
APC anti-mouse F4/80	BM8, rat IgG2a, k	FACS	Biolegend/123116
APC-Cy5.5 anti-mouse CD45.2	104, mouse IgG2a, k	FACS	ProSci/155-98-933-0.1
PE anti-mouse CD4	RM4-5, rat IgG2a, k	FACS	Biolegend/100512
PE-Cy7 anti-mouse CD25	PC61, rat IgG1, λ	FACS	Biolegend/102016
APC-Cy7 anti-mouse CD38	90, rat IgG2a, k	FACS	Biolegend/102728
Brilliant violet 421 anti-mouse PD1	29F.1A12, rat IgG2a, k	FACS	Biolegend/135221
PE-Dazzle 594 anti-mouse CD39	Duha59, rat IgG2a, k	FACS	Biolegend/143811
Brilliant violet 605 anti-mouse NKp46	29A1.4, rat IgG2a, k	FACS	Biolegend/137619
PerCP-Cy5.5 anti-mouse CD3	17A2, rat IgG2b, k	FACS	Biolegend/100218
APC anti-mouse FoxP3	FJK-16s, rat IgG2a k	FACS	eBioscience/17-5773-82
PE anti-mouse TCF1	C63D9/rabbit IgG	FACS	Cell Signaling/14456S
FITC anti-mouse IFNG	XMG1.2, rat IgG1, k	FACS	eBioscience/11-7311-81
PE anti-mouse TNFA	MP6-XT22, rat IgG1, k	FACS	eBioscience/12-7321-81
Alexa Fluor 700 anti-mouse CD107a	1D4B, rat IgG2a, k	FACS	Biolegend/121627

PE-Dazzle 594 anti-mouse CD69	H1.2F3, Hamster IgG	FACS	Biolegend/104535
ImmunoCult Human CD3/CD28 T Cell Activator	Antibody complex	In vitro stim	Stemcell/10971
Anti-mouse PD-1	RMP1-14, rat IgG2a, k	In vivo block	BioXcell/BE0146
Rat IgG2a isotype	2A3, rat IgG2a k	In vivo block	BioXcell/BE0089
Anti-mouse CSF-1R	AFS98, rat IgG2a, k	In vivo depletion	BioXcell/BE0213

Supplementary Table 2: other reagents

Name	Application	Product information
Lymphoprep	Cell isolation	StemCell/07851-07861
SepMate tubes	Cell isolation	StemCell/85450
CD14+ positive selection kit	Cell isolation	StemCell/17858
CD3+ positive selection kit	Cell isolation	StemCell/17851
Red blood cell lysis buffer	Cell isolation	Biologend/420301
Neon transfection system kit	CRISPR KO	Invitrogen/MPK10025
Alt-R® S.p. Cas9 Nuclease V3	CRISPR KO	IDT/1081058
TracrRNA	CRISPR KO	IDT/1072534
ID TE buffer	CRISPR KO	IDT/11-01-02-02
Nuclease free duplex buffer	CRISPR KO	IDT/11-01-03-01
Aqua fixable live/dead marker	FACS	Invitrogen by Thermo Fisher Scientific/L34966A
CellTrace violet	FACS	Invitrogen by Thermo Fisher Scientific/C34557
Zenon dye	FACS	Invitrogen/Z25408
FoxP3 staining buffer set	FACS	eBioscience/00-5523-00
Fixation/Permeabilization Kit	FACS	BD Biosciences/554714
DNase I	Tissue digest	Thermo Scientific/90083
Collagenase IV	Tissue digest	Abnova/P5275.100 MG
Tumor dissociation kit, mouse	Tissue digest	Miltenyi Biotech/130-096-730
GentleMacs C-tubes	Tissue digest	Miltenyi Biotech/130-093-237
MACS smartstrainers	Tissue digest	Miltenyi Biotech/130-110-916
IMDM medium	Cell culture	Gibco/12440-053
Heat inactivated FBS	Cell culture	Gibco/10500-064
Penicillin-streptomycin	Cell culture	Gibco/15140-122
PBS	Cell culture	Gibco /20012-027
MycoAlert mycoplasma detection kit	Cell culture	Lonza/LT07-218
Proteome profiler human phospho-kinase array	Phospho proteins	R&D systems/ARY003C
Lysis buffer 6	Phospho proteins	R&D systems/895561
pCREB (S133) Duoset IC ELISA	Phospho proteins	R&D systems/DYC2510
pHSP27 (S78/82) Duoset IC ELISA	Phospho proteins	R&D systems/DYC2314
WB gels (NuPAGE 4-12% Bis-Tris Gel)	WB	Invitrogen by Thermo Fisher Scientific/NP0321BOX
Protein Ladder	WB	Thermo Scientific/26619
iBlot 2NC Regular Stacks	WB	Invitrogen by Thermo Fisher Scientific/IB23001
MOPS SDS Running Buffer (20x)	WB	Novex by life technologies/NP0001
Transfer Buffer (20x)	WB	Novex by life technologies/NP0006-1
LDL Sample buffer (4x)	WB	Novex by life technologies/B0007
Skim milk powder	WB	OXOID/LP0033
Ponceaus S solution for electrophoresis (0.2%)	WB	Serva/33427.01
SuperSignal West Pico substrate	WB	Thermo Scientific/34580
SuperSignal West Femto substrate	WB	Thermo Scientific/34095
RIPA Buffer	WB	Thermo Scientific/89900
BCA Protein Assay Kit	WB	Thermo Scientific/23225
Protease and Phosphatase Inhibitor Cocktail	WB	Thermo Scientific/78446
rhGM-CSF	Functional	Peptotech/300-03
rmGM-CSF	Functional	Invitrogen/RP-8620
LPS	Functional	Novus Biologicals/NBP2-25295
R848	Functional	Adipogen Life Sciences/AG-CR1-3582
Pam3CSK4	Functional	Tocris/4633
ADU-S100 ammonium salt STING agonist	Functional	MedChemExpress/HY-12885B
Ravoxertinib-GDC-0994 (ERKi)	Functional	Selleck Chemicals/S7554-5MG

BMS-582949 (MAPKi)	Functional	Selleck Chemicals/S8124-1MG
DNase I	qPCR	Thermo Scientific/EN0521
RNAeasy Mini Kit	qPCR	Qiagen/74104
RNA Clean and Concentrator Kit	qPCR	Zymo Research/R1018
SsoAdvanced Universal SYBR Green Supermix	qPCR	Bio-Rad/1725271
SYBR Safe	qPCR	Invitrogen/S33102

Supplementary Table 3: sequences

Name	Application	Sequence
Hu IRAK3 crRNA1	CRISPR	<i>CTCCCTTGGCACATTCTGAAT</i>
Hu IRAK3 crRNA2	CRISPR	<i>AACATTATCCACGGTGACAT</i>
Hu IRAK3 crRNA3	CRISPR	<i>TCACCCAAACATACTAGAGT</i>
Hu IRAK3 crRNA4	CRISPR	<i>CAGAGCTCTCCGAGCAGCGC</i>
Mo IRAK3 gRNA1-Reverse	CRISPR	<i>GGTTGTGAGACCGGACTCCCTGG</i>
Mo IRAK3 gRNA2-Forward	CRISPR	<i>CAGTTGAGCTCGATTAGCCCTGG</i>
Carrier DNA	CRISPR	<i>CCAGCAGAACACCCCCATCGGCGACGGCCCCGTG CTGCTGCCCCGACAACCACTACCTGAGCACCCAGTC CGCCCTGAGCAAAGACCCCAACGAGA</i>
Mouse IRAK3 Forward Primer	qPCR	<i>AGCATGCGTGACAGAGAAAAC</i>
Mouse IRAK3 Reverse Primer	qPCR	<i>CTCTGGAAGCTGATAGGGGT</i>
Mouse β -actin Forward Primer	qPCR	<i>ATGACGATATCGCTGCGCTGGT</i>
Mouse β -actin Reverse Primer	qPCR	<i>CCTCGTACCCACATAGGAGTC</i>

Supplementary data

Supplementary data 1. Normalized mRNA counts for the mouse bone-marrow derived cells from 3 age-matched female *WT* and *IRAK3 KO* mice with or without LPS stimulation. Related to Figure 5C, 5D and Figure S4B.

Supplementary data 2. Quantification of proteome in control or *IRAK3 KO* primary human monocytes after 45 minutes LPS stimulation from 4 donors. Data related to Figure 2F, 2G and S3C.

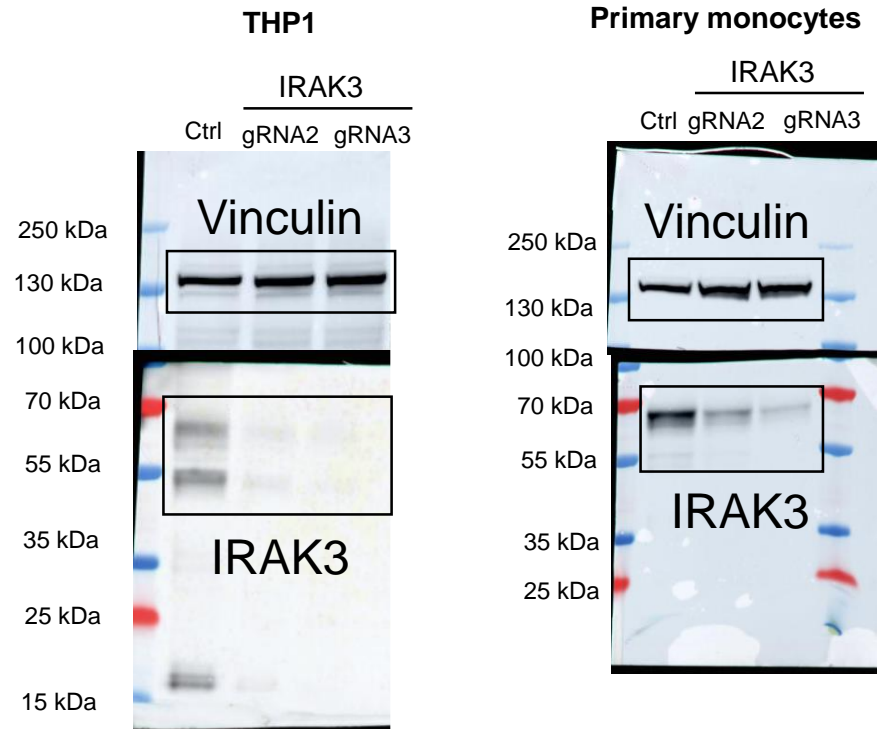
Supplementary data 3. Detection of phosphorylated peptides in control or *IRAK3 KO* primary human monocytes after 45 minutes LPS stimulation from 3 donors. Data related to Figure 3A.

Supplementary data 4. Analysis of the RNAseq data from the IMvigor210 trial. Data related to Figure 1 and S1 and S2.

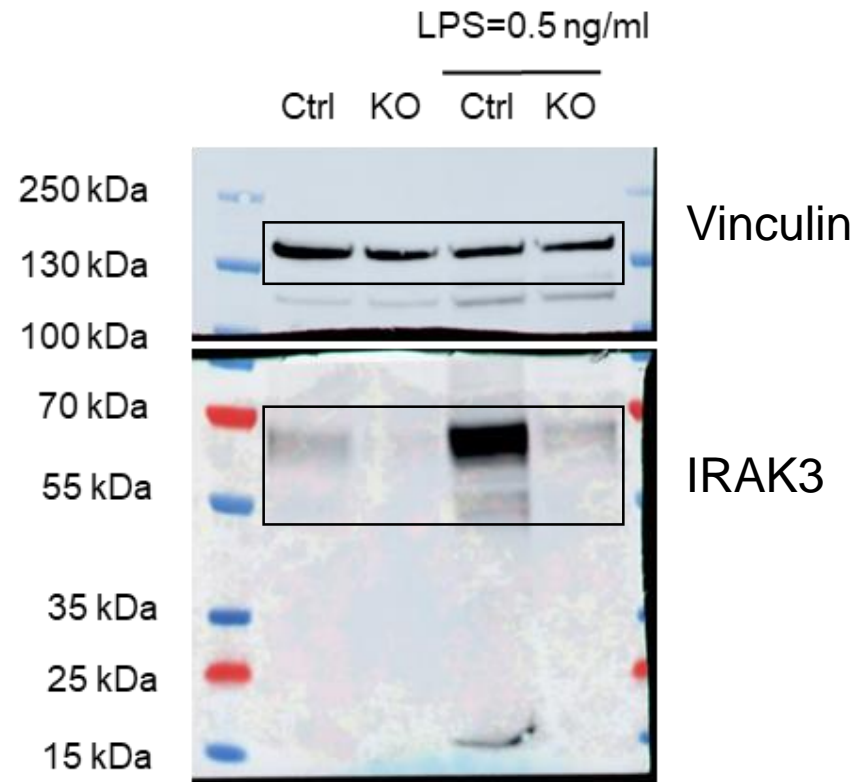
Uncropped images for western blotting

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Full unedited gel for Figure 2B



Full unedited gel for Figure 2C



Full unedited gel for Figure 5B

