Supporting Information

Identification of immune-responsive gene 1 (IRG1) as a novel target of A20

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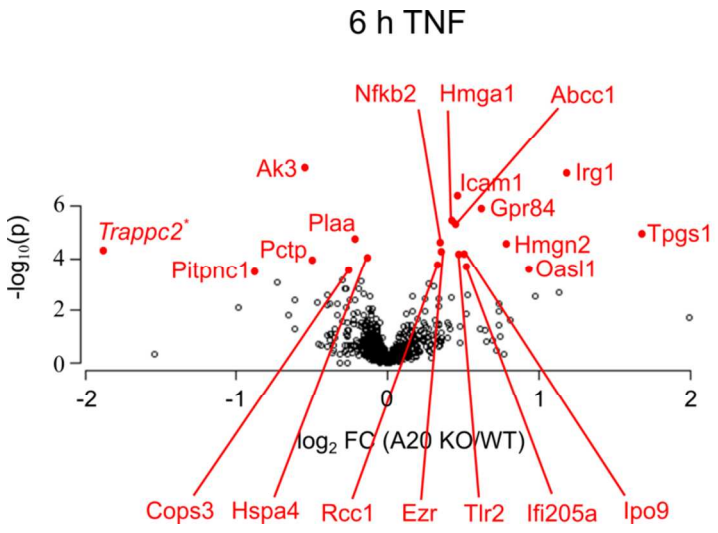


Figure S-1. Volcano plot visualizing protein regulation comparing the proteomes of wild-type (WT) and A20 deficient (KO) BMDMs following 6 h of TNF treatment. The x-axis shows the $\log_2$ fold change of the protein abundances between A20 KO and WT BMDMs and the y-axis shows the $-\log_{10}$ of the p-value. Significantly regulated proteins (FDR < 0.05) between the A20 KO and WT BMDMs are represented with red dots and indicated with their gene name. Gene names in italics and indicated with an asterisk point to unreliable estimates as the quantification was based on only one peptide in one or more conditions.

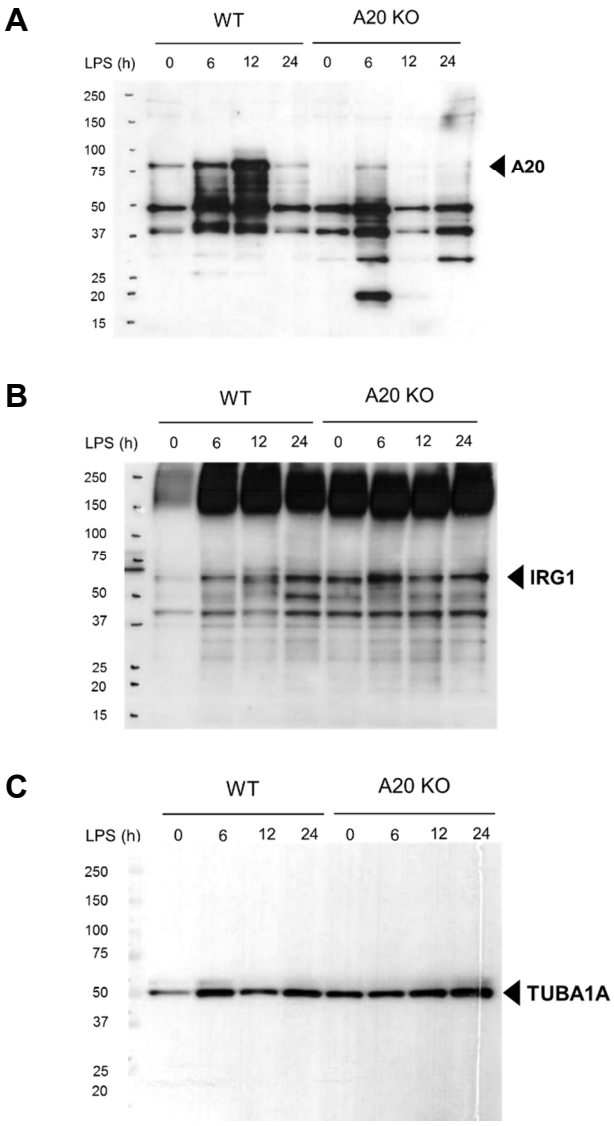
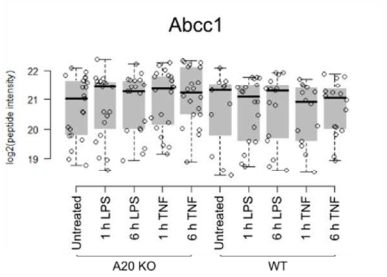
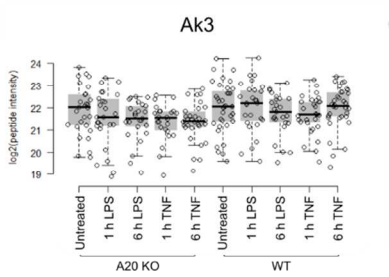
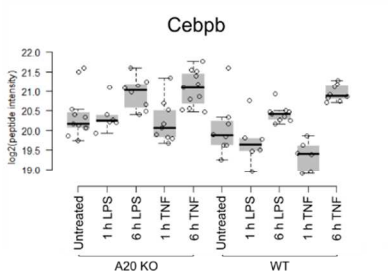
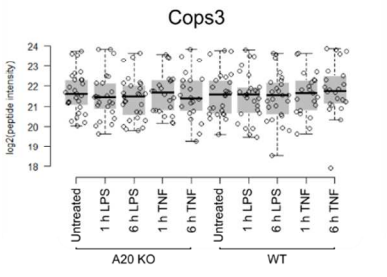


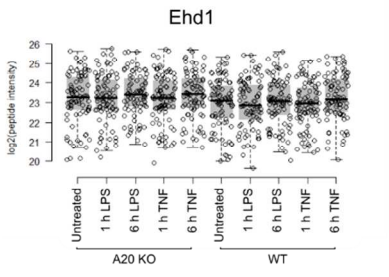
Figure S-2. Uncropped Western blots of Figure 3. Western blot analysis showing expression of A20 (A), IRG1 (B) and Tubulin (C) in WT and A20 KO BMDMs treated or not with 10 μg/ml LPS for the indicated time points.

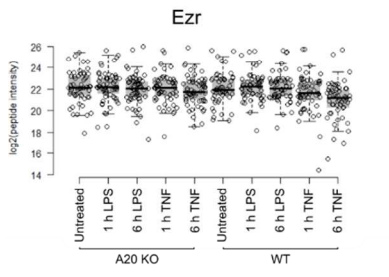

Abcc1
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

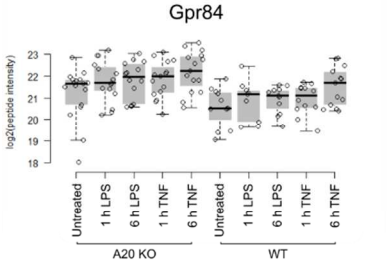

Ak3
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

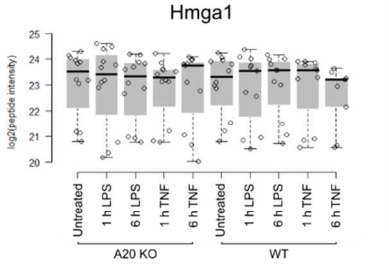

Cebpb
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

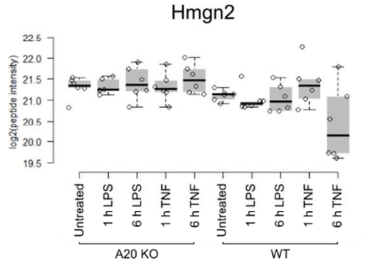

Cops3
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

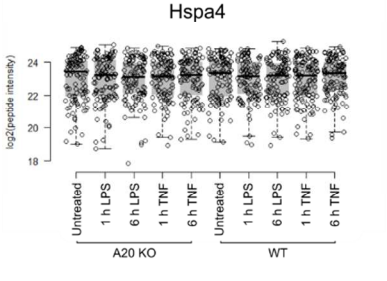

Ehd1
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

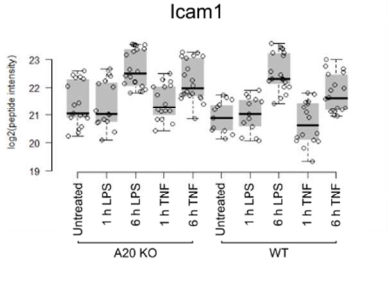

Ezr
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

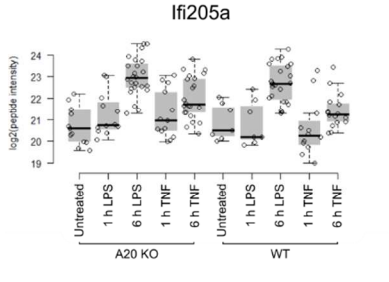

Gpr84
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

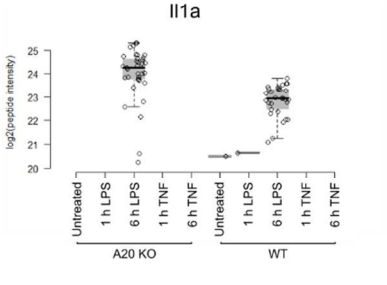

Hmga1
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

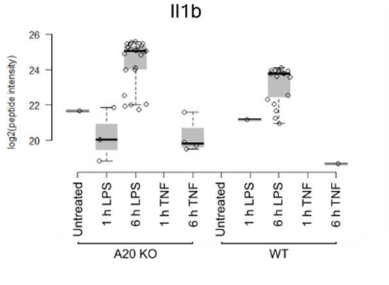

Hmgn2
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

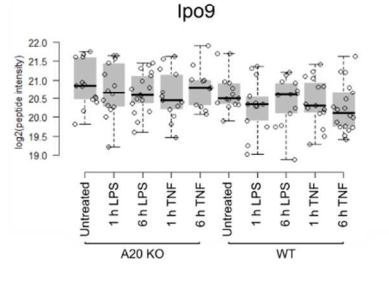

Hspa4
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT


Icam1
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT


Ifi205a
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT


Il1a
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT


Il1b
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT


Ipo9
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

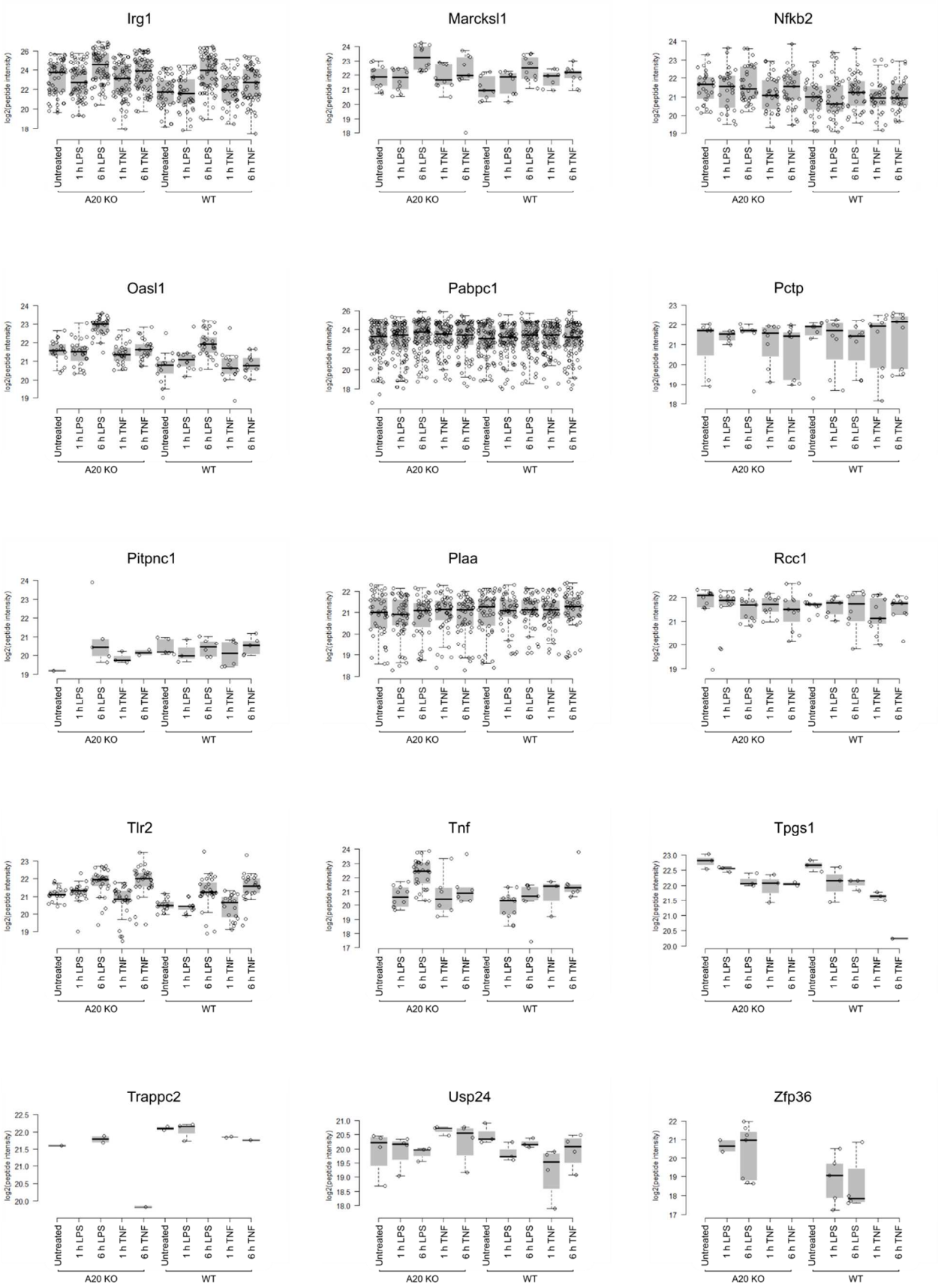
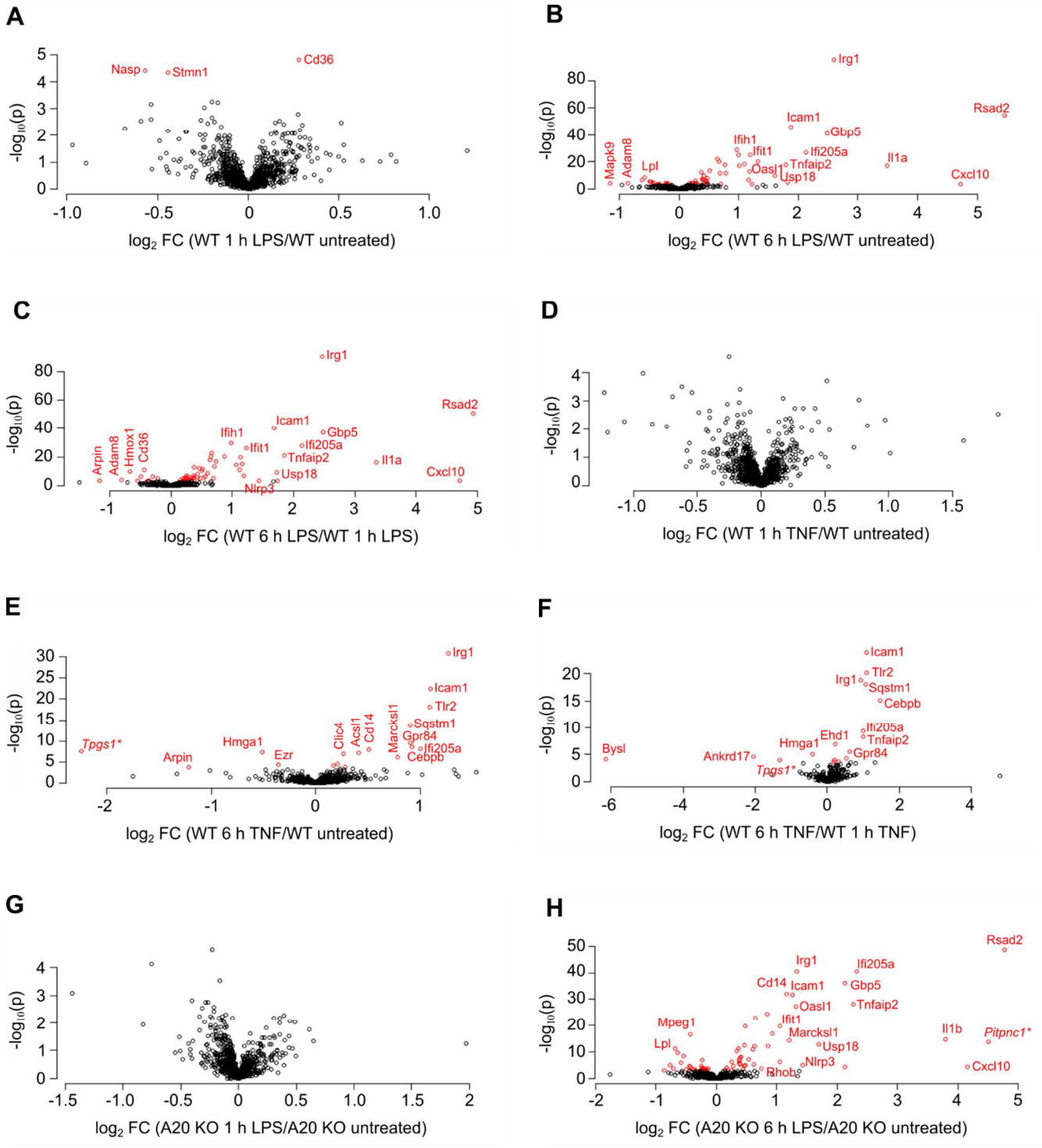

Irg1
Marcksl1
Nfkb2
Oasl1
Pabpc1
Pctp
Pitpnc1
Plaa
Rcc1
Tlr2
Tnf
Tpgs1
Trappc2
Usp24
Zfp36
log2(peptide intensity)
Untreated
1 h LPS
6 h LPS
1 h TNF
6 h TNF
A20 KO
WT

Figure S-3. Boxplots showing the $\log_2$ peptide intensities of the significantly regulated proteins. The y-axis shows the $\log_2$ intensity of the peptide and the x-axis shows the different conditions studied. Each data point represents a single peptide, the median of the peptide intensity is represented by a thick black line, the box itself comprises the interquartile range and the whiskers extend to the most extreme data points that lies within 1.5 times the interquartile range on each side[1]. The gene name is given for every protein.


A
Nasp
Stmn1
Cd36
-log10(p)
log2 FC (WT 1 h LPS/WT untreated)
B
Irg1
Rsad2
Icam1
Gbp5
Ifih1
Ifit1
Ifi205a
Il1a
Tnfaip2
Oasl1
Usp18
Mapk9
Adam8
Lpl
Cxcl10
log2 FC (WT 6 h LPS/WT untreated)
C
Irg1
Rsad2
Icam1
Ifih1
Gbp5
Ifit1
Ifi205a
Arpin
Adam8
Hmox1
Cd36
Tnfaip2
Il1a
Usp18
Cxcl10
Nlrp3
log2 FC (WT 6 h LPS/WT 1 h LPS)
D
log2 FC (WT 1 h TNF/WT untreated)
E
Irg1
Icam1
Tlr2
Sqstm1
Clic4
Acsl1
Cd14
Marcksl1
Gpr84
Ifi205a
Cebpb
Tpgs1*
Arpin
Hmga1
Ezr
log2 FC (WT 6 h TNF/WT untreated)
F
Icam1
Tlr2
Irg1
Sqstm1
Cebpb
Ifi205a
Tnfaip2
Ehd1
Hmga1
Gpr84
Bysl
Ankrd17
Tpgs1*
log2 FC (WT 6 h TNF/WT 1 h TNF)
G
log2 FC (A20 KO 1 h LPS/A20 KO untreated)
H
Rsad2
Irg1
Ifi205a
Cd14
Icam1
Gbp5
Oasl1
Tnfaip2
Mpeg1
Ifit1
Marcksl1
Il1b
Pitpnc1*
Lpl
Usp18
Nlrp3
Cxcl10
Rhob
log2 FC (A20 KO 6 h LPS/A20 KO untreated)

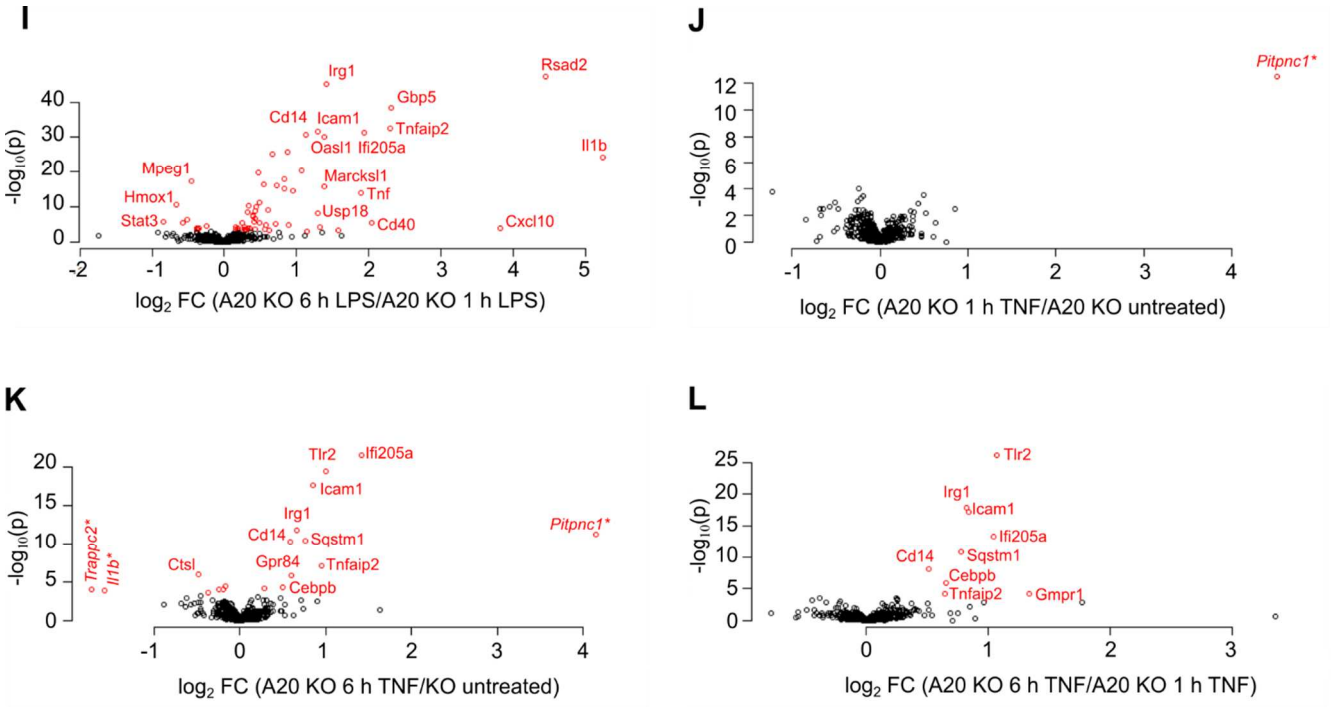


Figure S-4. Volcano plots visualizing protein regulation by comparing the proteomes upon LPS or TNF treatment. The x-axis shows the $\log_2$ fold change of the protein abundances between two treatment conditions and the y-axis shows the $-\log_{10}$ of the p-value. Significantly regulated proteins (FDR < 0.05) are represented with red dots and indicated with their gene name. Gene names in italics and indicated with an asterisk point to unreliable estimates as the quantification was based on only one peptide in one or more conditions. For reasons of readability, not all significantly regulated proteins are indicated. However, $\log_2$ fold changes and p-values for all proteins can be found in Table S-2.

Table S-1. MSqRob Annotation file. (Separate Excel file)

This is the annotation file used for the MSqRob analysis. The first column ("sample") contains the unique names of the samples, the second ("treat") and third ("mouse") column contains respectively the variables treatment and mouse.[1]

Table S-2. MSqRob Results table. (Separate Excel file)

The results table shows all proteins from the MsqRob analysis. Every sheet contains the results for the differential protein abundance between genotypes (A20 KO vs WT BMDMs) for a treatment condition (untreated, after 1 h of LPS treatment, after 6 h of LPS treatment, after 1 h of TNF treatment and after 6 h of TNF treatment) as well as the results for the differential protein abundances within treatments for each genotype. The first, second and third column respectively show the protein accession, the gene name and the protein name for each protein. The column "Log2 fold change" indicates the estimated log2 fold change between the proteomes of WT and KO A20 BMDMs. "Standard error" denotes the standard error on this log2 fold change. Degrees of freedom, T- and p-values are given for the corresponding moderated t-tests in their respective columns. "q value" is the smallest Benjamini-Hochberg false discovery rate (FDR) threshold at which the protein would be significant. The column "significant" indicates whether the protein is significant at the default 5% FDR threshold.[1]

Table S-3. List of known substrates and associated proteins of A20. Presented values are the A20/WT ratio values of protein abundances in this differential proteomics study. For every protein, the cell type and treatment used to validate the substrate or interaction are presented, as well as if it leads to degradation of the protein.

		A20 KO/WT ratio					Literature			
Protein	**Gene Name**	**-**	**1 h LPS**	**6 h LPS**	**1 h TNF**	**6 h TNF**	**Cell type ***	**Treatment ***	**Degradation**	**Ref.**
E3 ligase activity										
RIP1	RIPK1	1.01	1.00	0.98	0.99	0.95	MEF	TNF	yes	2
ASK1	ASK1	N/A	N/A	N/A	N/A	N/A	HEK293T	TNF	yes	3
DUB activity										
TRAF6	TRAF6	N/A	N/A	N/A	N/A	N/A	MEF	LPS, IL-17	no	4
TBK1	TBK1	0.98	0.98	1.09	0.96	0.95	MEF	poly(I:C)	no	5
IKKε	IKBKE	1.00	1.00	1.00	1.01	1.01	MEF	poly(I:C)	no	5
Caspase 8	CASP8	1.00	1.00	1.00	1.00	1.00	HEK293T	-	no	6
cIAP1	BIRC2	N/A	N/A	N/A	N/A	N/A	MEF	TNF, IL-1	no	7
cIAP2	BIRC3	N/A	N/A	N/A	N/A	N/A	MEF	TNF, IL-1	no	7
RIP3	RIPK3	0.98	0.99	1.02	1.02	1.00	MEF	TNF (and CHX and/or Z-VAD)	no	8
RIP2	RIPK2	N/A	N/A	N/A	N/A	N/A	BMDM	MDP	no	9
Other										
TRAF2	TRAF2	N/A	N/A	N/A	N/A	N/A	MEF	TNF, actinomysin D, TWEAK	yes	10
UBC13	UBE2N	1.00	1.00	1.00	1.00	1.00	MEF	IL-1	no	11
UBCH5C	UBE2D3	1.00	1.00	1.00	1.00	1.00	MEF	IL-1	no	11
NEMO	IKBKG	1.00	1.00	1.00	1.00	1.00	MEF	TNF	no	12-14
HOIP	RNF31	1.00	1.00	1.00	1.00	1.00	MEF	TNF	no	12-14
HOIL-1	RBCK1	N/A	N/A	N/A	N/A	N/A	MEF	TNF	no	12-14
SHARPIN	SHARPIN	N/A	N/A	N/A	N/A	N/A	MEF	TNF	no	12-14
NLRP3	NLRP3	1.28	1.59	1.47	1.85	1.67	BMDM	LPS and ATP and nigericin	no	15
STAT1	STAT1	1.04	0.99	1.01	0.98	1.03	BMDM	IFNγ	no	16

A20 ubiquitin-editing complex										
ABIN-1	TNIP1	N/A	N/A	N/A	N/A	N/A	HEK293T	-	no	[17]
ABIN-2	TNIP2	N/A	N/A	N/A	N/A	N/A	HEK293T	TNF, IL-1	no	[18]
ABIN-3	TNIP3	N/A	N/A	N/A	N/A	N/A	THP-1	TNF, LPS	no	[19]
TAX1BP1	TAX1BP1	N/A	N/A	N/A	N/A	N/A	MEF	TNF, IL-1	no	[20]
ITCH	ITCH	1.00	1.00	1.00	1.00	1.00	MEF	TNF, LPS	no	[21]
RNF11	RNF11	N/A	N/A	N/A	N/A	N/A	MEF	TNF, LPS	no	[22]

CHX: Cycloheximide; Z-VAD: pan-caspase inhibitor (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone); TNF–CHX–Z-VAD: 'cocktail' for caspase-independent necroptosis induction; MDP: Muramyl dipeptide; TWEAK: TNF-related weak inducer of apoposis; ATP: Adenosine triphosphate; ATP and nigericin: NLRP3 inflammasome stimuli; (*) If multiple cell lines and/or stimulation conditions were used in the referred study, the most relevant experiments for comparison with our study are represented in this table .

1. Goeminne, L. J. E.; Gevaert, K.; Clement, L., Experimental design and data-analysis in label-free quantitative LC/MS proteomics: A tutorial with MSqRob. *J Proteomics* **2017**.
2. Wertz, I. E.; O'Rourke, K. M.; Zhou, H.; Eby, M.; Aravind, L.; Seshagiri, S.; Wu, P.; Wiesmann, C.; Baker, R.; Boone, D. L.; Ma, A.; Koonin, E. V.; Dixit, V. M., De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature* **2004,** *430* (7000), 694-9.
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4. Boone, D. L.; Turer, E. E.; Lee, E. G.; Ahmad, R. C.; Wheeler, M. T.; Tsui, C.; Hurley, P.; Chien, M.; Chai, S.; Hitotsumatsu, O.; McNally, E.; Pickart, C.; Ma, A., The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nat Immunol* **2004,** *5* (10), 1052-60.
5. Parvatiyar, K.; Barber, G. N.; Harhaj, E. W., TAX1BP1 and A20 inhibit antiviral signaling by targeting TBK1-IKKi kinases. *J Biol Chem* **2010,** *285* (20), 14999-5009.
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