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Supplemental Information

Obesity and Insulin Resistance Promote Atherosclerosis through an IFNγ-Regulated Macrophage Protein Network

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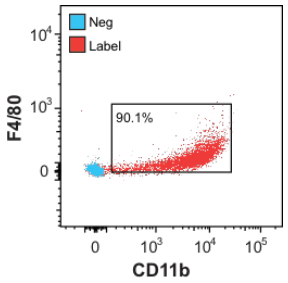

Fig. S1. Peritoneal macrophage purity (related to all figures). Wild-type or *Ifngr1-/-* C57BL6 mice or *Ldlr-/-* mice fed the various diets were injected with thioglycolate in the peritoneal cavity, and elicited peritoneal macrophages were isolated five days after injection. Peritoneal macrophage purity was confirmed by flow cytometry using antibodies raised against murine F4/80 and CD11b. A representative image is shown.

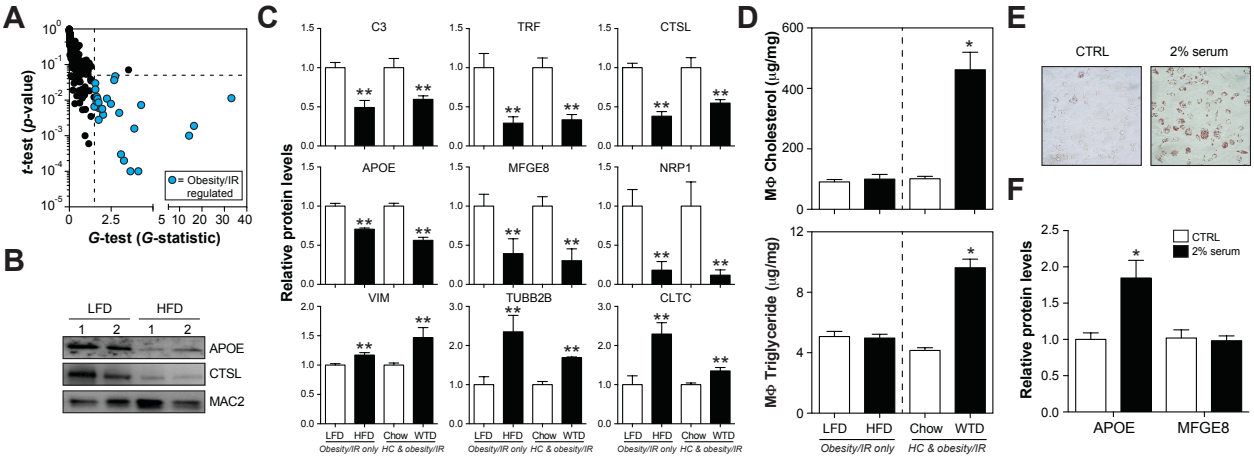

Fig. S2. Obesity/IR targets the MSRN, a pro-atherogenic macrophage protein network (related to Fig. 1). For the '*obesity/IR only*' model, *wt* C57BL/6 mice were fed a low-fat (LFD) or high-fat diet (HFD) for 9 weeks. For the '*HC & obesity/IR*' model, *Ldlr-/-* mice were fed a chow or western-type diet (WTD) for 12 weeks. *Panel A*: Proteomics analysis of elicited peritoneal macrophages in the *obesity/IR only* model. Differentially expressed proteins (blue circles) were identified based on the *G*-test ($G>1.5$) and *t*-test ($p<0.05$). *Panel B*: Immunoblotting validation for changes in protein abundance for APOE and CTSL. MAC2 was used as a control. *Panel C*: Regulation of the 9 MSRN proteins. *Panel D*: Peritoneal macrophages cholesterol and triglyceride levels. *Panels E-F*: Peritoneal macrophages were treated with and without 2% serum from WTD-fed *Ldlr-/-* mice. Cholesterol loading was confirmed by Oil-red-O staining (*Panel E*) and media APOE and MFGE8 were quantified (*Panel F*). Results show that *in vitro* cholesterol loading does not replicate the MSRN protein changes observed in WTD-fed *Ldlr-/-* mice. Results are mean ± SEM. *, $p<0.05$ (*t*-test). **, $G>1.5$ (*G*-test) and $p<0.05$ (*t*-test). n=2-5 mice/group.

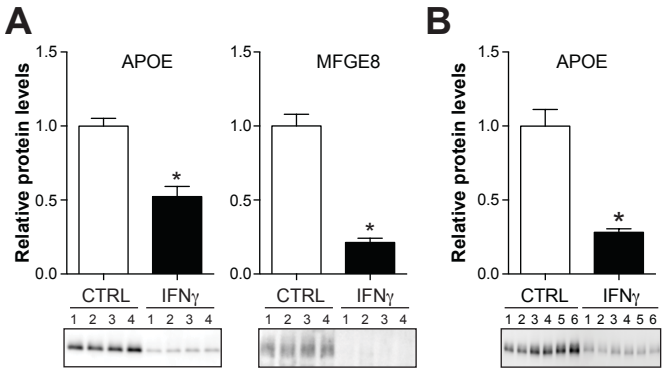

Fig. S3. IFNγ targets MSRN proteins in many types of macrophages (related to Fig. 3). APOE and/or MFGE8 protein levels in conditioned media collected from murine bone marrow-derived macrophages (*Panel A*) or human monocyte-derived macrophages (*Panel B*) treated with vehicle (CTRL) or IFNγ (12 ng/mL) for 24h. Results are mean ± SEM. *, $p<0.05$ Student's *t*-test. n=4-6 biological replicates/group.

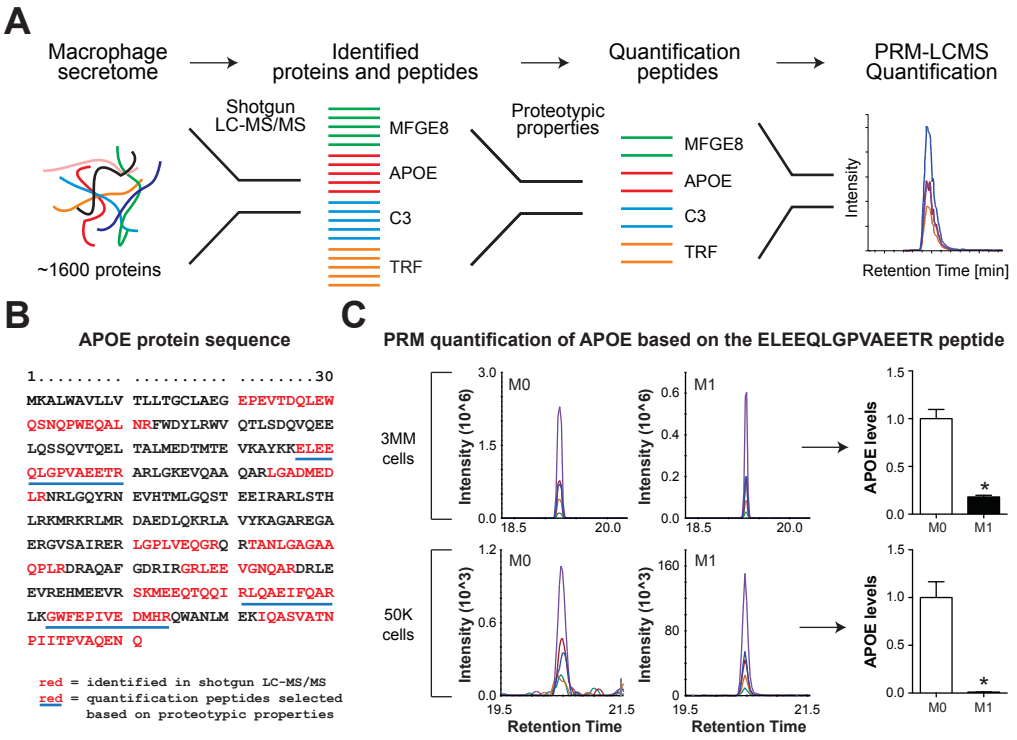

Fig. S4. A parallel reaction monitoring method for quantifying MSRN proteins in limited numbers of macrophages (related to Fig. 3). *Panel A*: Development of a targeted proteomics assay for quantification of MSRN proteins. MSRN proteins and identified peptides were selected from shotgun proteomics analysis and further evaluated for proteotypic properties using public resources. Best peptides (up to 5 per protein where available) were selected and further investigated using Parallel Reaction Monitoring LCMS in samples from unstimulated (M0) or classically activated (M1) BMDMs. Two conditioned were used: 3 million macrophages were cultured, proteins in the conditioned media were precipitated, and 0.2 µg was injected, or 50,000 macrophages were cultured and conditioned media were analyzed directly. *Panel A*: Workflow diagram. *Panel B*: Protein sequence of APOE indicating peptides selected for PRM quantification. *Panel C*: Quantification of APOE based on the ELEEQLGPVAEETR peptide in 3million or 50,000 M0 and M1 macrophages.

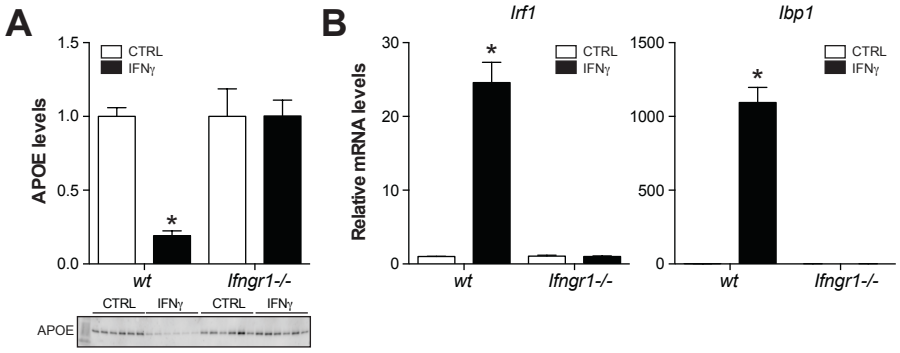

Fig. S5. IFNGR1 is required for IFNγ to target MSRN proteins and its target genes *in vitro* (related to Figs. 4-6). Wild-type (*wt*) and *Ifngr1-/-* macrophages were treated with vehicle (CTRL) or 12ng/mL IFNγ. *Panel A*: Media APOE levels. *Panel B*: mRNA levels of IFNγ-target genes. Results are mean ± SEM. *, $p<0.05$ Student's *t*-test. n=6 biological replicates/group.

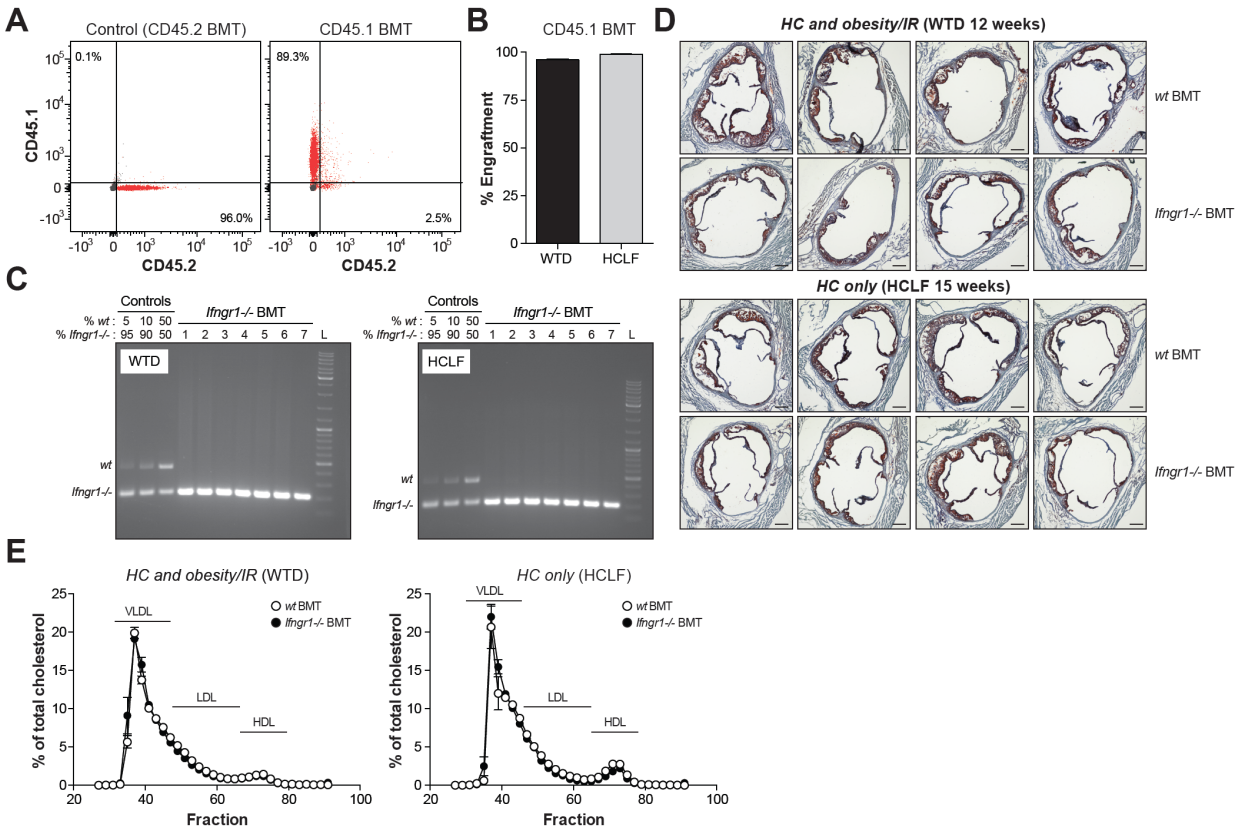

Fig. S6. Supplementary data for *wt* and *Ifngr1-/-* bone marrow transplants into *Ldlr-/-* mice (related to Fig. 6). *Ldlr-/-* mice transplanted with *wt* or *Ifngr1-/-* bone marrow cells were fed a WTD or HCLF diet for up to 15 weeks. *Panels A-B*: Engraftments for *wt* bone marrow transplants were assessed by flow cytometric analysis of the ratio of CD45.1 (donor) to CD45.2 (recipient) positive bone marrow cells. *Panel C*: Engraftments for *Ifgnr1-/-* bone marrow transplants were assessed by PCR analysis of the ratio of *Ifngr1-/-* (donor) and *wt* (recipient) in comparison to pre-defined mixtures of bone marrow from *wt* and *Ifngr1-/-* mice. Seven mice per dietary condition are shown as examples. All engraftments were judged to be >95%. *Panel D*: Cross-sections of the aortic root were stained with Oil-red-O, counterstained with hematoxylin and fast green, and lesion area was quantified. Four representative images are shown per group. Scale = 200 μm. *Panel E*: Plasma (100 μL) was fractionated by gel filtration using two Superose-6 columns in tandem, and cholesterol levels in each fraction were quantified using the Amplex Red Cholesterol Assay kit (Invitrogen). Results are mean ± SEM. n=5-12 mice/group.

Obesity/IR only Model						
Protein annotations			*wt* C57BL/6 mice HFD vs. LFD		*Ifngr1-/-* C57BL/6 mice HFD vs. LFD	
Uniprot	Entrez	Protein	G-test (HFD : LFD)	t-test	G-test (HFD : LFD)	t-test
P01027	12266	C3	-16.43	0.0019	0.57	0.4722
Q921I1	22041	TRF	-4.30	0.0073	0.00	0.9792
P06797	13039	CTSL	-4.13	0.0001	3.80	0.0070
P08226	11816	APOE	-3.66	0.0001	0.00	0.9646
P21956	17304	MFGE8	-2.69	0.0368	0.49	0.1593
P97333	18186	NRP1	-2.02	0.0039	0.49	0.0509
P20152	22352	VIM	1.50	0.0066	-0.50	0.0820
B2RSN3	73710	TUBB2B	1.56	0.0201	ND	ND
Q68FD5	67300	CLTC	2.49	0.0079	1.04	0.0035

HC +/- Obesity/IR Model						
Protein annotations			*Ldlr-/-* mice WTD vs. Chow		*Ldlr-/-* mice HCLF vs. Chow	
Uniprot	Entrez	Protein	G-test (WTD : Chow)	t-test	G-test (HCLF : Chow)	t-test
P01027	12266	C3	-9.80	0.0036	-0.01	0.8893
Q921I1	22041	TRF	-2.90	0.0082	0.31	0.2999
P06797	13039	CTSL	-4.46	0.0458	7.54	0.2722
P08226	11816	APOE	-12.72	0.0047	7.75	0.1559
P21956	17304	MFGE8	-3.03	0.0271	1.06	0.5212
P97333	18186	NRP1	-1.84	0.0013	0.85	0.4994
P20152	22352	VIM	3.57	0.0052	3.66	0.2052
B2RSN3	73710	TUBB2B	4.06	0.0169	0.78	0.4146
Q68FD5	67300	CLTC	2.63	0.0500	8.01	0.1054

HC +/- Obesity/IR Model (Bone marrow transplantation)						
Protein annotations			*Ldlr-/-* mice WTD (*Ifngr1-/-* vs. *wt* BMT)		*Ldlr-/-* mice HCLF (*Ifngr1-/-* vs. *wt* BMT)	
Uniprot	Entrez	Protein	G-test (*-/-* : *wt*)	t-test	G-test (*-/-* : *wt*)	t-test
P01027	12266	C3	14.10	0.0190	0.29	0.6344
Q921I1	22041	TRF	0.19	0.1629	0.00	0.9837
P06797	13039	CTSL	0.50	0.1482	-0.25	0.1226
P08226	11816	APOE	7.37	0.0448	0.03	0.8749
P21956	17304	MFGE8	3.07	0.0073	0.52	0.2275
P97333	18186	NRP1	2.90	0.0030	0.38	0.1316
P20152	22352	VIM	0.00	0.9569	0.04	0.6143
B2RSN3	73710	TUBB2B	-0.52	0.0317	-0.24	0.1028
Q68FD5	67300	CLTC	-1.50	0.0240	0.02	0.6031

Table S1. Shotgun proteomics analysis of elicited peritoneal macrophages (related to Figs. 1, 4, 6). Proteomics analysis of the conditioned media collected from elicited peritoneal macrophages isolated from *wt* or *Ifngr1-/-* C57BL6 mice fed the LFD or HFD, from *Ldlr-/-* mice fed the chow, WTD, or HCLF diet, and from *Ldlr-/-* mice transplanted with *wt* or *Ifngr1-/-* bone marrow fed the WTD or HCLF diet. Proteomics data were analyzed by the G-test (G-statistic) and t-test (p-value). Differentially abundant proteins are shaded; red = up-regulated in 1st sample relative to the 2nd, green = down-regulated in the 1st sample relative to the 2nd. ND = not detected. n=3-5 mice/group.

Gene	Primer	Primer Sequence
Ifrngr1	Wild type	TCGCTTTCCAGCTGA
	Deficient	CTCGTGCTTTACGGTATCGC
	Common	CCACCTCAGCACTGTCTTCA
Srebp2	Forward	GTTGACCACGCTGAAGACAGA
	Reverse	CACCAGGGTTGGCACTTGAA
Abca1	Forward	GCTTGTTGGCCTCAGTTAAGG
	Reverse	GTAGCTCAGGCGTACAGAGAT
Sra1	Forward	TTCACTGGATGCAATCTCCAAG
	Reverse	CTGGACTTCTGCTGATACTTTG
Cd36	Forward	ATGGGCTGTGATCGGAACTG
	Reverse	GTCTTCCCAATAAGCATGTCTCC
Abcg1	Forward	GTGGATGAGGTTGAGACAGACC
	Reverse	CCTCGGGTACAGAGTAGGAAAG
Lxra	Forward	ACAGAGCTTCGTCCACAAAAG
	Reverse	GCGTGCTCCCTTGATGACA
Acta1	Forward	CCCAGACATCAGGGAGTAATGG
	Reverse	TCTATCGGATACTTCAGCGTCA
Cdh5	Forward	CCACTGCTTTGGGAGCCTT
	Reverse	GGCAGGTAGCATGTTGGGG
Cd11b	Forward	CCATGACCTTCCAAGAGAATGC
	Reverse	ACCGGCTTGTGCTGTAGTC
Irf1	Forward	GCTGGAGTTATGTCCCTTTCCATATC
	Reverse	GGACTCAGCAGCTCTACCCTACCT
Irf8	Forward	GCTGGTTCAGCTTTGTCTCC
	Reverse	GATCGAACAGATCGACAGCA
Ibp1	Forward	GTGTGGTAGAAGCCCACTATTGC
	Reverse	CCACATGAAAGGCCCAGTGTGC

Table S2. Primer Sequences for PCR (Related to Experimental Procedures).