Inherited human IRAK-1 deficiency selectively impairs TLR signaling in fibroblasts

Erika Della Mina[a,b], Alessandro Borghesi[c,d], Hao Zhou[e,1], Salim Bougarn[f,1], Sabri Boughorbel[f,1], Laura Israel[a,b], Ilaria Meloni[g], Maya Chrabieh[a,b], Yun Ling[a,b], Yuval Itan[h], Alessandra Renieri[g,i], Iolanda Mazzucchelli[d,j], Sabrina Basso[k], Piero Pavone[l], Raffaele Falsaperla[l], Roberto Ciccone[m], Rosa Maria Cerbo[c], Mauro Stronati[c,d], Capucine Picard[a,b,n,o], Orsetta Zuffardi[m], Laurent Abel[a,b,h], Damien Chaussabel[f,2], Nico Marr[f,2], Xiaoxia Li[e,2], Jean-Laurent Casanova[a,b,h,n,p,3,4] & Anne Puel[a,b,h,3,4]

Supporting information appendix contents:

SI Figures

Figure S1. Chest X rays
Chest X rays showing a bilateral (paramediastinal and perihilar), non-uniform increase in lung parenchyma density consistent with aspiration pneumonia.

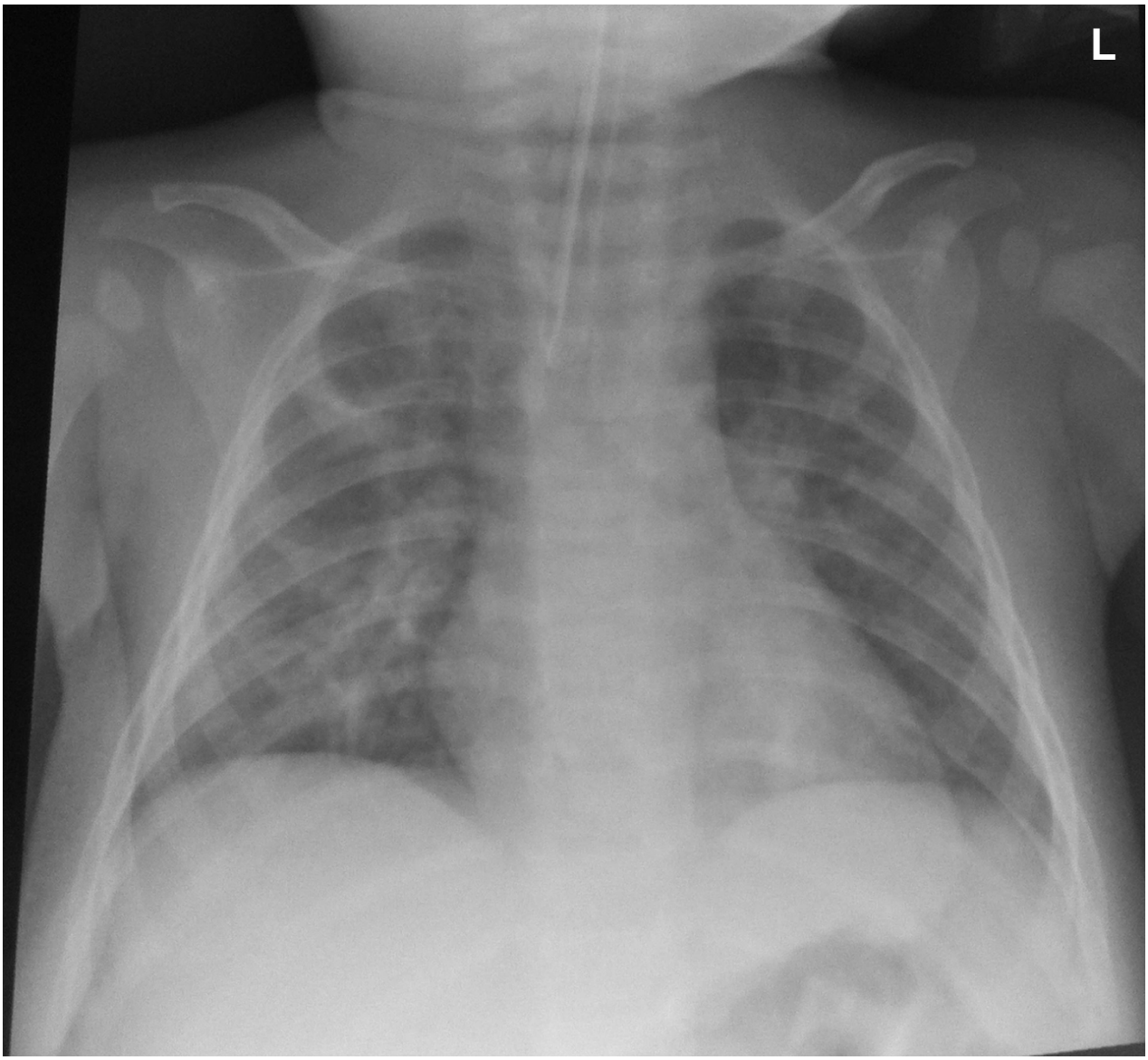


Figure S2. TLR4 silencing in control SV40-fibroblasts

A. *TLR4* (left) and *TLR2* (right) mRNA levels in healthy control's SV40-fibroblasts after transfection with si-*TLR4* or a nonsense siRNA (scrambled).

B. *IL8* mRNA levels in SV40-fibroblasts from a healthy control transfected with siRNAs targeting TLR4 (si-*TLR4*) or a nonsense siRNA (scrambled) for 48 hours, left unstimulated (NS) or stimulated with PAM-2 (10 μg/ml), LPS (10 μg/ml) or MPLA (1 μg/ml) for 4 hours.

C. IL-8 secretion by SV40-fibroblasts from a healthy control transfected with si-*TLR4* or with a nonsense siRNA (scrambled) for 48 hours, left unstimulated (NS) or stimulated with PAM-2 (10 μg/ml), LPS (10 μg/ml) or MPLA (1 μg/ml) for 18 hours.

The values shown (means ± SEM) were obtained in three independent experiments.

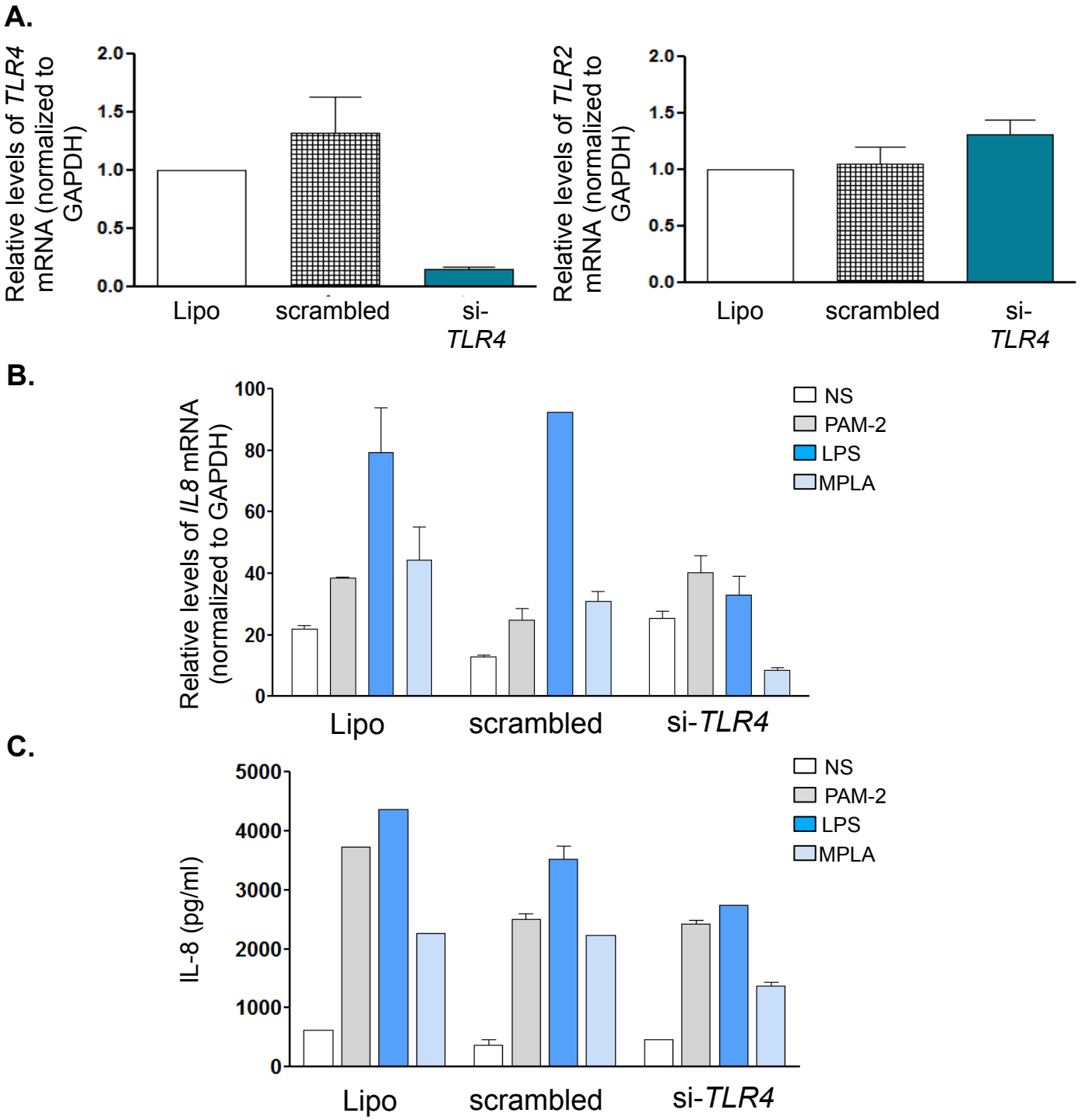


Figure S3. NF-κB translocation in response to TLR2, TLR4 and IL-1R agonists

A., B. NF-κB translocation, assessed by EMSA, in SV40-fibroblasts from a healthy control (C), the IRAK-1-deficient patient (P), and the IRAK-4-deficient patient (panel A) and MECP2-deficient and MyD88-deficient patients (panel B), following stimulation with PAM-2 (10 μg/ml), LPS (10 μg/ml), MPLA (1 μg/ml) and TNF-α (20 ng/ml) for 45 minutes.

C. NF-κB translocation, assessed by EMSA, in SV40-fibroblasts from MyD88- ($MyD88^{-/-}$) and MECP2-deficient ($MECP2^{-/Y}$) patients, following stimulation with IL-1β (10 ng/ml) and TNF-α (20 ng/ml) for 20 minutes.

Similar results were obtained in three independent experiments.

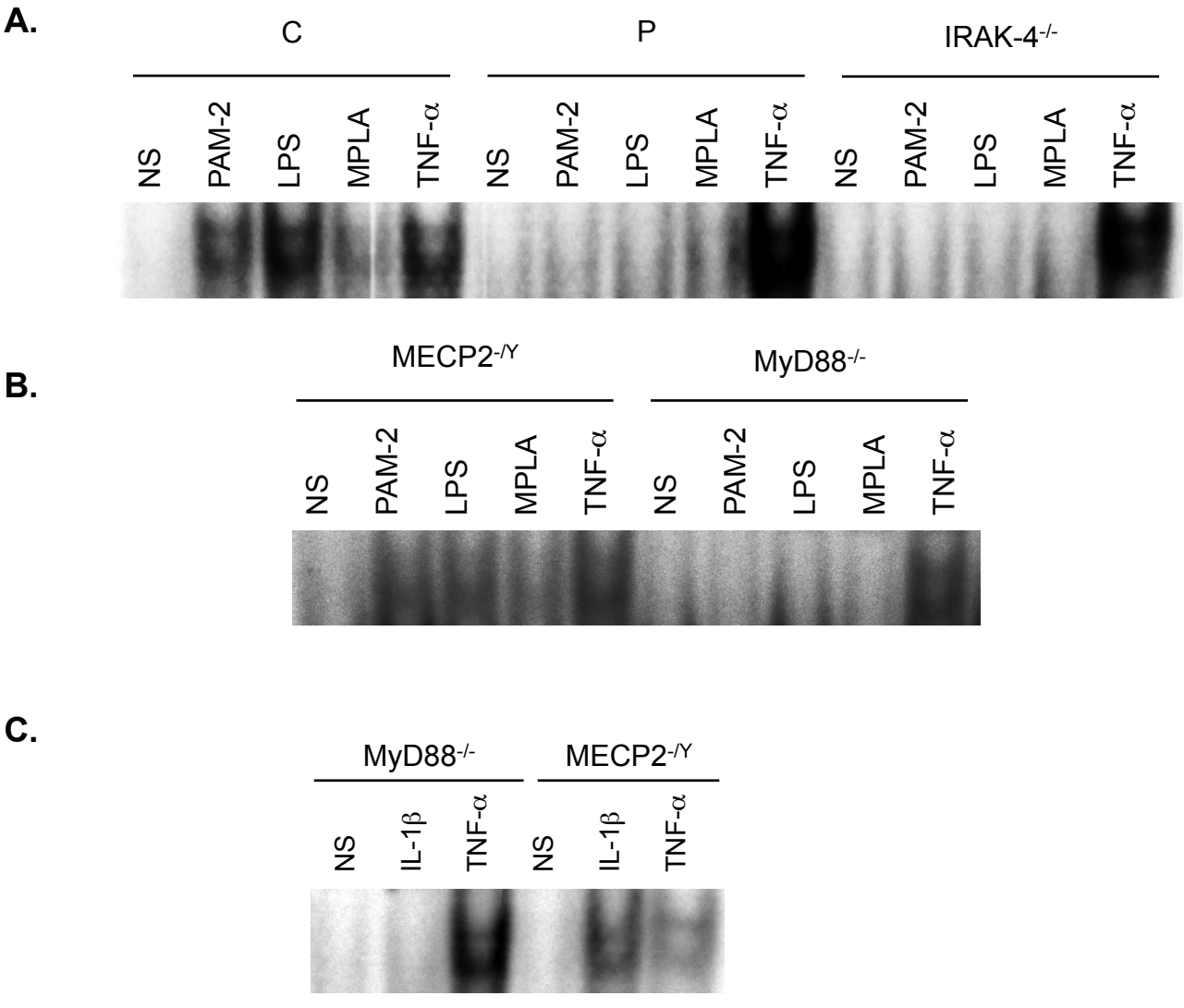


Figure S4. IL-6 production by SV40-fibroblasts

A., B. IL-6 secretion by SV-40-fibroblasts from healthy controls (n=5), the IRAK-1-deficient patient (P), and IRAK-4-, MyD88-, and MECP2-deficient patients (IRAK-4-/-, MyD88-/-, MECP2-/Y, respectively), left unstimulated or stimulated with PAM-3 (10 μg/ml), PAM-2 (10 μg/ml), FSL-1 (1 μg/ml), LTA (10 μg/ml), LPS (10 μg/ml), MPLA (1 μg/ml), poly(I:C) (panel A) and with IL-1β (10 ng/ml) (panel B), or TNF-α (20 ng/ml) as a positive control (panels A and B).

The values shown (means ± SEM) were obtained in three independent experiments.

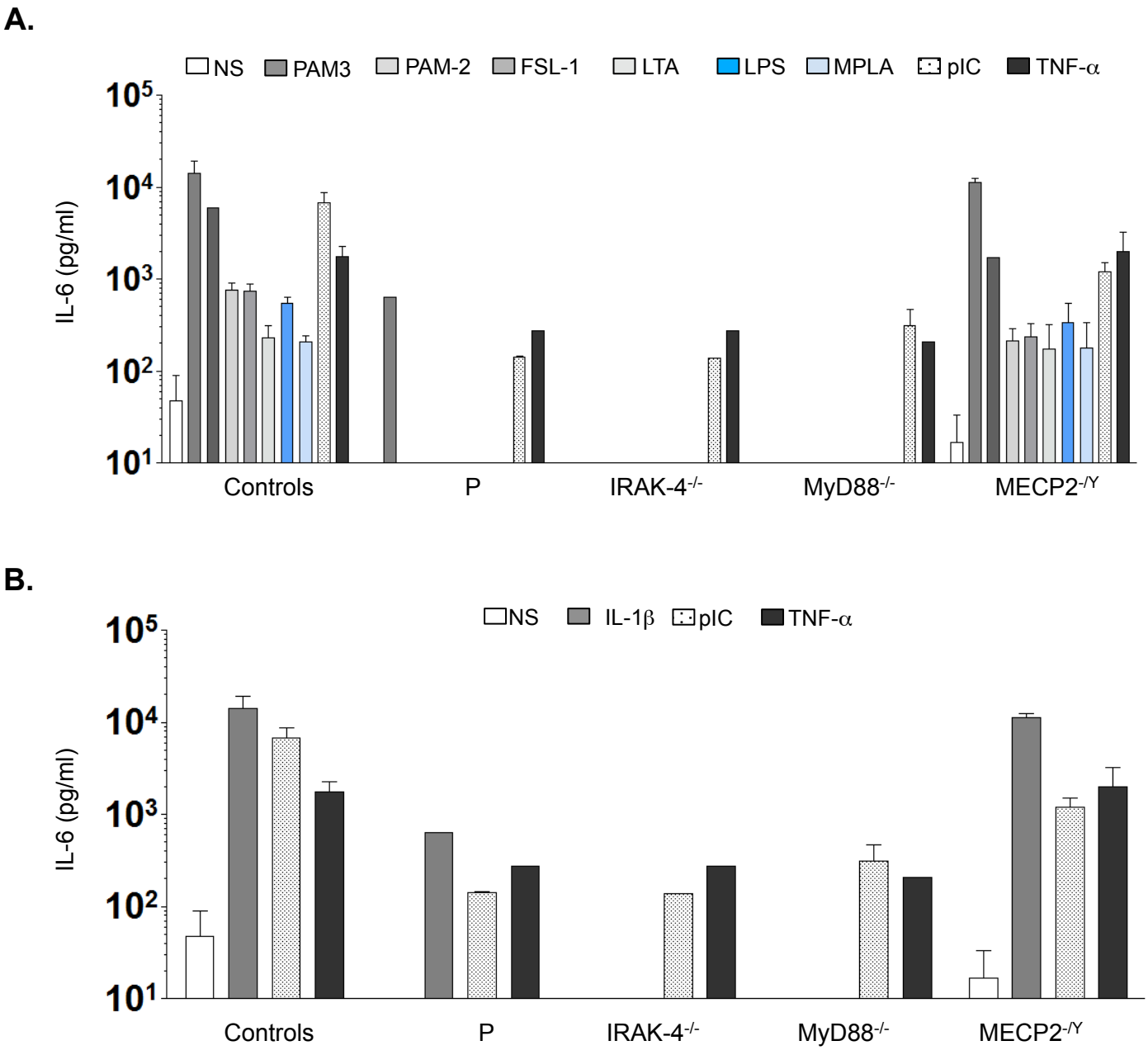


Figure S5. Cytokine production by primary fibroblasts

A. IL-6 secretion by primary fibroblasts from healthy controls (*n*=3), the IRAK-1-deficient patient (P), and IRAK-4-, MyD88-, and MECP2-deficient patients (IRAK-4$^{-/-}$, MyD88$^{-/-}$, MECP2$^{-/Y}$, respectively), left unstimulated or stimulated with PAM-2 (10 μg/ml), FSL-1 (1 μg/ml), LTA (10 μg/ml), LPS (10 μg/ml), MPLA (1 μg/ml), pIC (25 μg/ml), or TNF-α (20 ng/ml) as a positive control.

B. IL-6 secretion by primary fibroblasts from healthy controls (*n*=3), the IRAK-1-deficient patient (P), and IRAK-4-, MyD88-, and MECP2-deficient patients (IRAK-4$^{-/-}$, MyD88$^{-/-}$, MECP2$^{-/Y}$, respectively), left unstimulated or stimulated with IL-1β (10 ng/ml), pIC (25 μg/ml), or TNF-α (20 ng/ml) as a positive control. The values shown (means ± SEM) were obtained in three independent experiments.

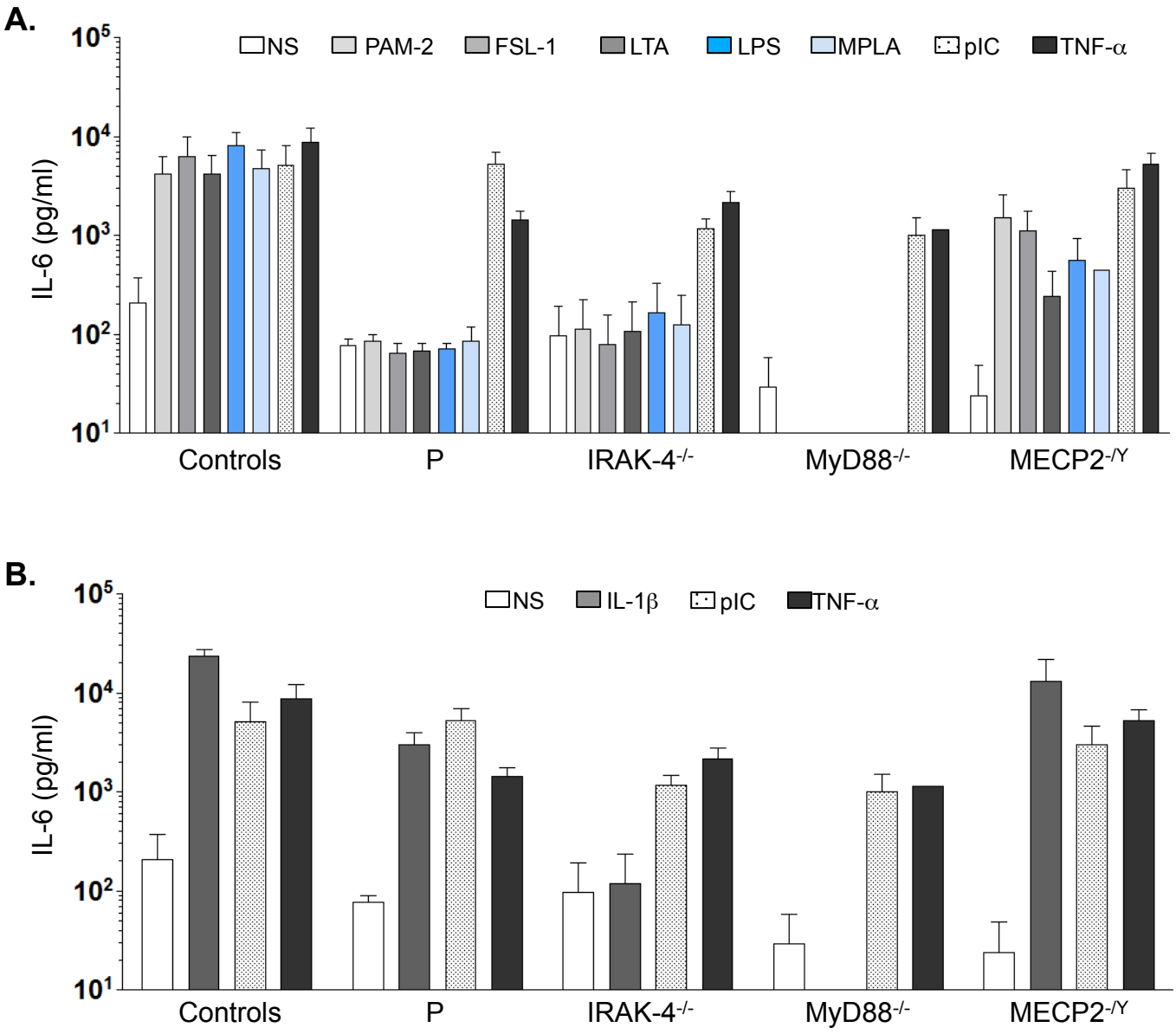


Figure S6. Impaired responses of fibroblasts to IL-1β

A. IRAK-1 protein degradation, phosphorylation of the IKKα/β, P65 and IκB-α proteins, and IκB-α protein degradation in SV40-fibroblasts from a healthy control (C), and the IRAK-1-deficient (P) and IRAK-4-deficient (IRAK-4$^{-/-}$) patients, left unstimulated or stimulated with IL-1β (10 ng/ml) for different periods of time (min), analyzed by western blotting.

B. IκB-α protein degradation in SV40-fibroblasts from a healthy control (C) and the IRAK-1-deficient patient (P), IRAK-4- and MyD88-deficient patients (IRAK-4$^{-/-}$, MyD88$^{-/-}$, respectively), left unstimulated or stimulated with IL-1β (left panel) or TNF-α (right panel) for different periods of time (min), analyzed by western blotting. The data for each cell line are represented as a ratio of the mean amount of IκB-α protein observed in three independent experiments to the mean amount of tubulin protein observed in the same experiments; all data are normalized with respect to the IκB-α/tubulin ratio for unstimulated SV40-fibroblasts from the healthy control.

Similar results were obtained in three independent experiments.

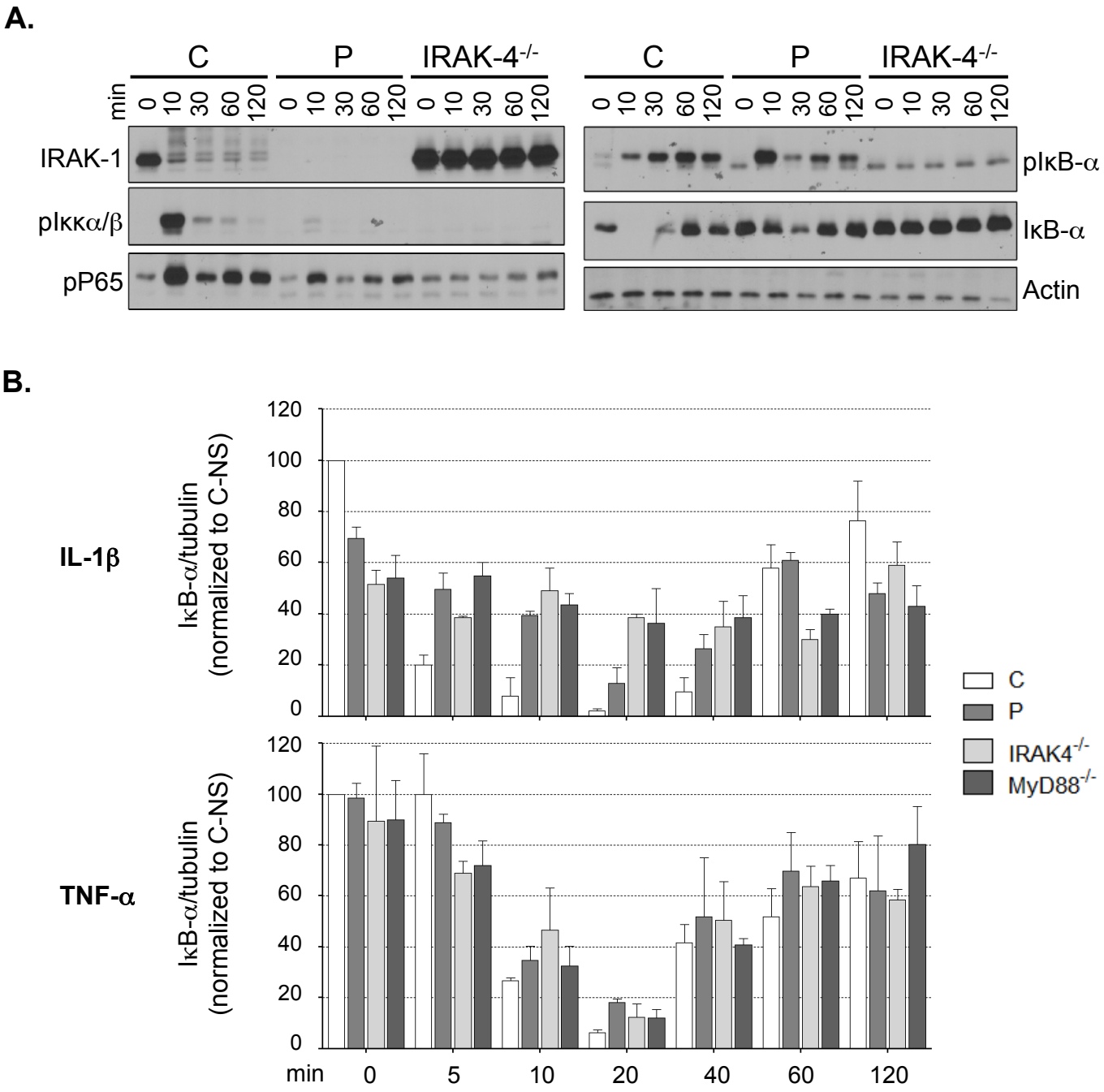
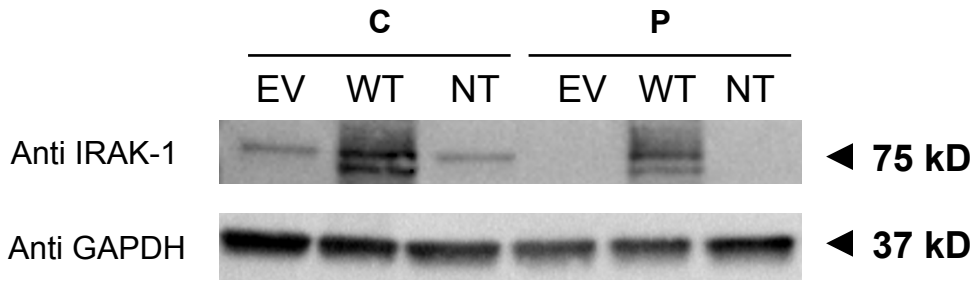


Figure S7. Complementation of the patient's cells

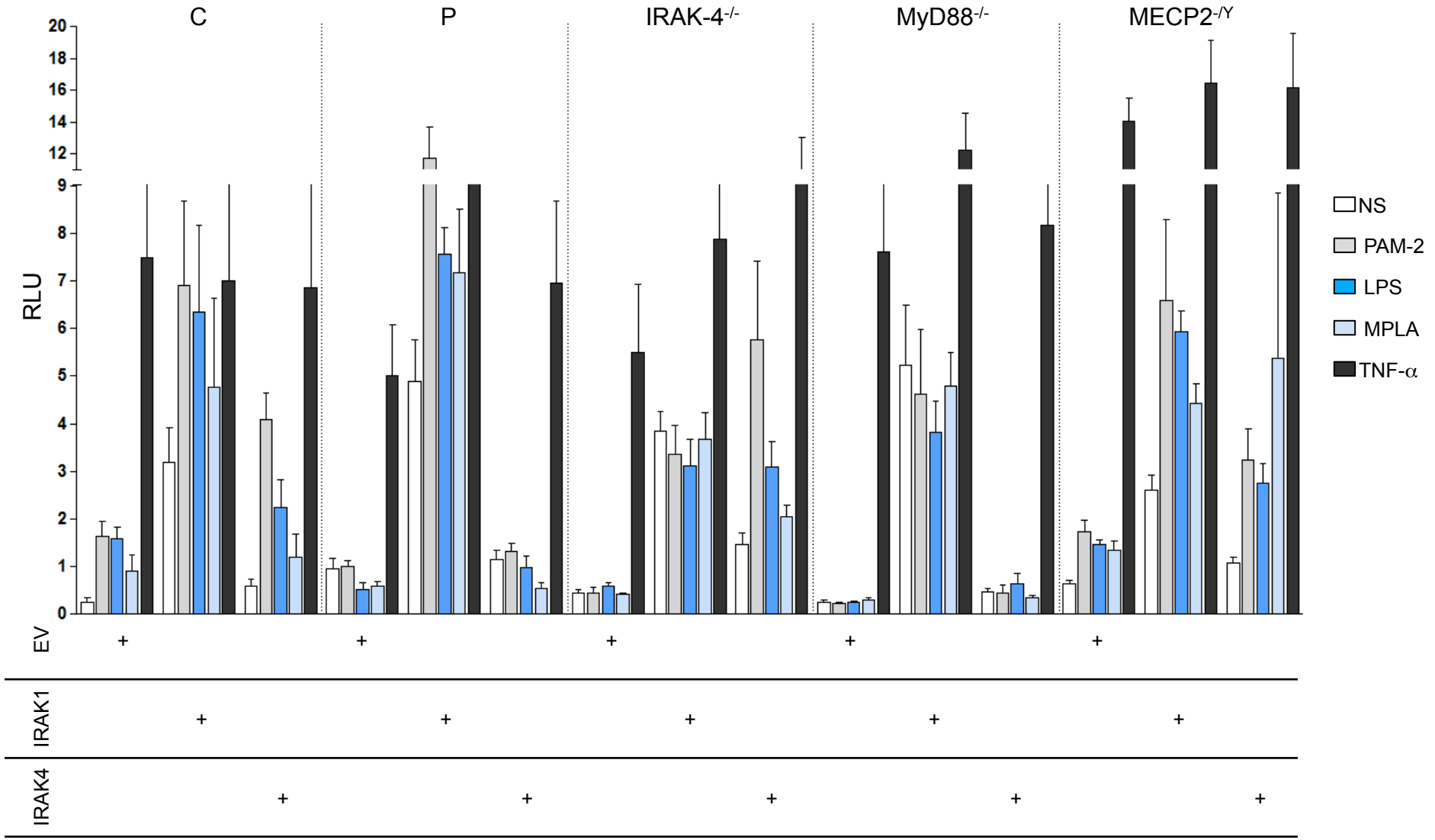
A. Western-blot analysis of IRAK-1 protein levels in total cell extracts from SV40-fibroblasts derived from a healthy control (C) and the IRAK-1-deficient patient (P) transiently transfected for 24 hours with pcDNA3.1-empty (EV), pcDNA3.1-*IRAK1* WT (WT), or left non-transfected (NT). Similar results were obtained in three independent experiments.

B., C. SV40-fibroblasts derived from a healthy control (C), the IRAK-1-deficient patient (P) and IRAK-4-, MyD88-, and MECP2-deficient patients (labeled IRAK-4$^{-/-}$, MyD88$^{-/-}$, MECP2$^{-/Y}$, respectively) were transiently transfected with the *Renilla*, NF-kBLuc, pcDNA3.1-empty (EV), pcDNA3.1-IRAK1 WT, or pcDNA3.1-IRAK4 WT vectors, with the Lipofectamine LTX kit. Twenty-four hours after transfection, SV40-fibroblasts were left untreated or were stimulated with PAM-2 (10 μg/ml), LPS (10 μg/ml), MPLA (1 μg/ml) (panel **B**), or with IL-1β (10 ng/ml) (panel **C**), and TNF-α (20 ng/ml) as a positive control (panels **B** and **C**) for 24 hours and then harvested. Reporter gene activities were measured and values were normalized for transfection efficiency on the basis of *Renilla* luciferase expression. The values shown (means ± SEM) were obtained in three independent experiments.

A.



B.


C
P
IRAK-4-/-
MyD88-/-
MECP2-/Y
RLU
20
18
16
14
12
9
8
7
6
5
4
3
2
1
0
NS
PAM-2
LPS
MPLA
TNF-α
EV
IRAK1
IRAK4
+

C.

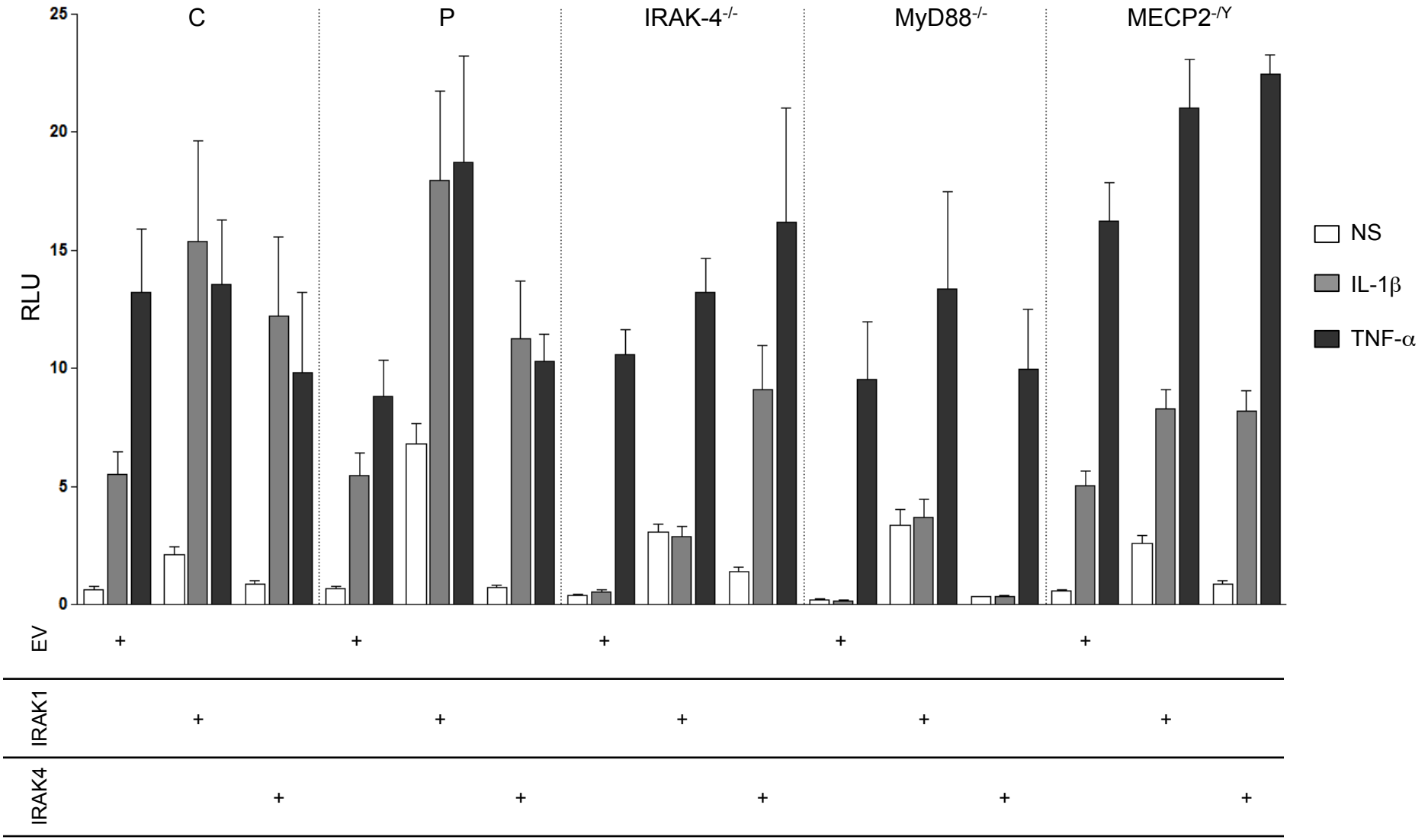

C
P
IRAK-4-/-
MyD88-/-
MECP2-/Y
RLU
25
20
15
10
5
0
NS
IL-1β
TNF-α
EV
IRAK1
IRAK4
+

Figure S7. Complementation of the patient's cells

D., E. SV40-fibroblasts from a healthy control, the IRAK-1-deficient patient (P), and from IRAK-4- or MyD88-deficient patients (IRAK-4$^{-/-}$, MyD88$^{-/-}$, MECP2$^{-/Y}$, respectively), were not transfected (NT) or were transiently transfected with pcDNA3.1-empty (EV) or with pcDNA3.1-IRAK1 wt (WT) for 24 hours. Cells were left untreated or were stimulated with PAM-2 (10 μg/ml), LPS (10 μg/ml), MPLA (1 μg/ml) (panel **D**) or with IL-1β (10 ng/ml) (panel **E**) and TNF-α (20 ng/ml) as a positive control (panels **D** and **E**), for 24 hours, and then harvested. IL-8 production was measured by ELISA.

The values shown (means ± SEM) were obtained in three independent experiments.

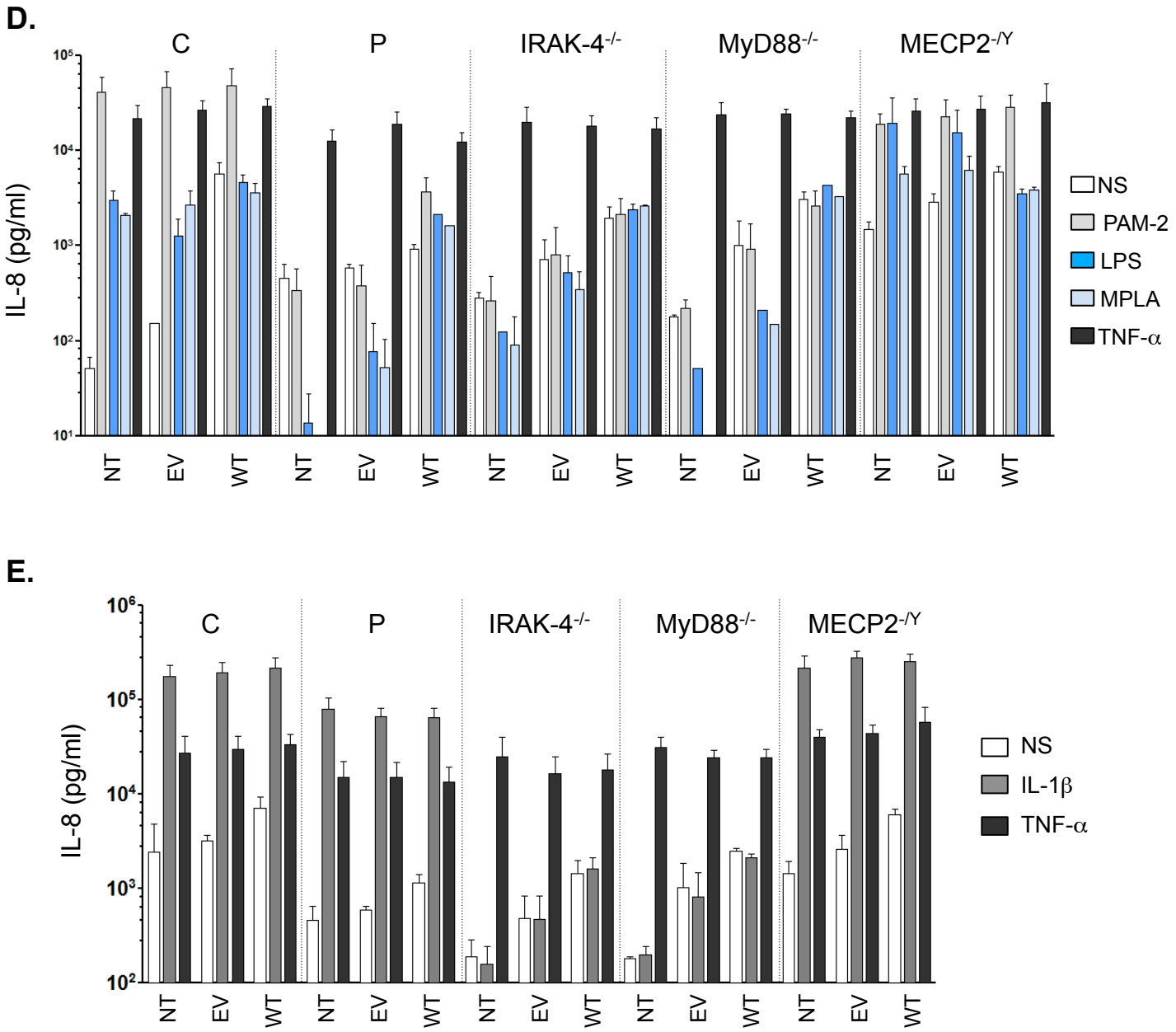


Figure S8. *IRAK1* silencing in control fibroblasts

A. *IRAK1* (left), *IRAK2* (center), and *IRAK4* (right) mRNA levels in SV40-fibroblasts from a healthy control transfected with siRNAs targeting *IRAK1* (si-*IRAK1*) or with a nonsense siRNA (scrambled). The values shown (means ± SEM) were obtained in three independent experiments.

B. IRAK-1 protein levels in SV40-fibroblasts from a healthy control transfected with siRNAs targeting *IRAK1* (si-*IRAK1*) or a nonsense siRNA (scrambled). Similar results were obtained in three independent experiments.

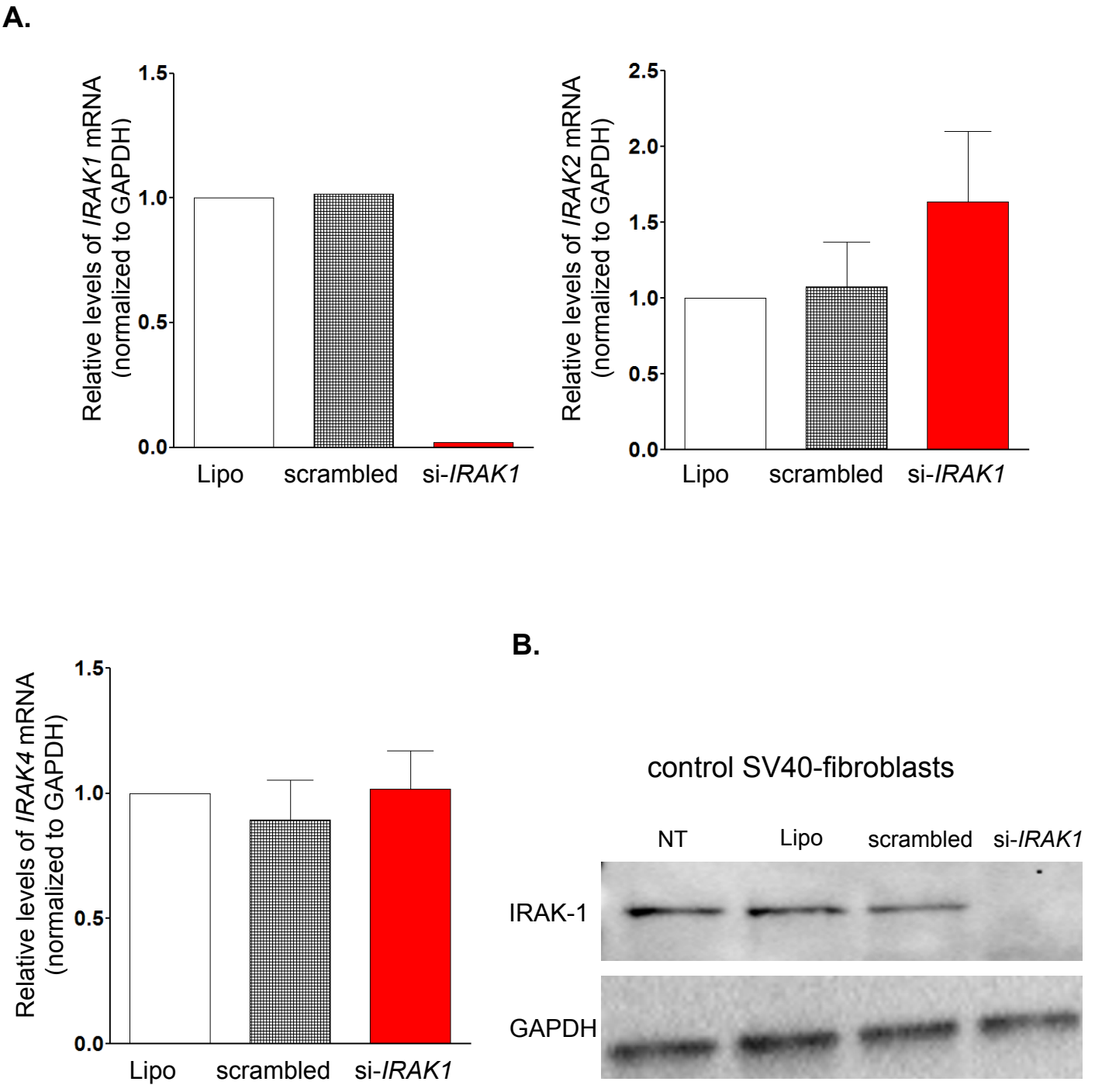


Figure S8. *IRAK1* silencing in control fibroblasts

C., D. *IL8* mRNA levels in SV40-fibroblasts from a healthy control transfected with siRNAs targeting *IRAK1* (si-*IRAK1*) or a nonsense siRNA (scrambled), unstimulated (NS) or stimulated with PAM-2 (10 μg/ml), LPS (10 μg/ml), MPLA (1 μg/ml) (panel **C**) or with IL-1β (10 ng/ml) (panel **D**) and TNF-α (20 ng/ml) as positive control (panels **C** and **D**).

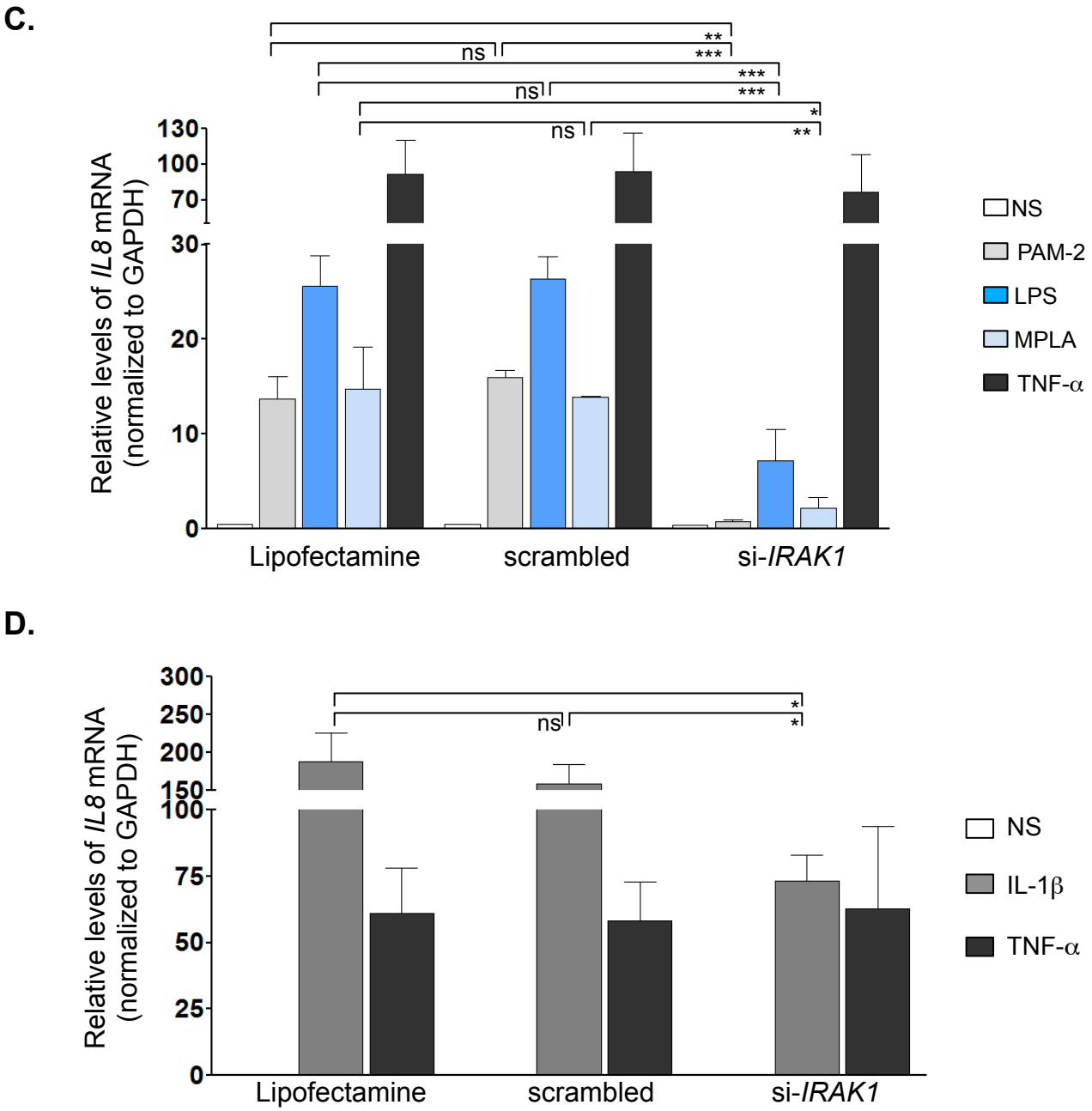


Figure S9. *IRAK2* silencing in the patient's fibroblasts

A., B. *IRAK2* (left), *IRAK1* (center) and *IRAK4* (right) mRNA levels in SV40-fibroblasts from healthy controls (panel **A**) and in SV40-fibroblasts from the patient (panel **B**) transfected with siRNAs targeting *IRAK2* (si-*IRAK2*) or with a nonsense siRNA (scrambled).

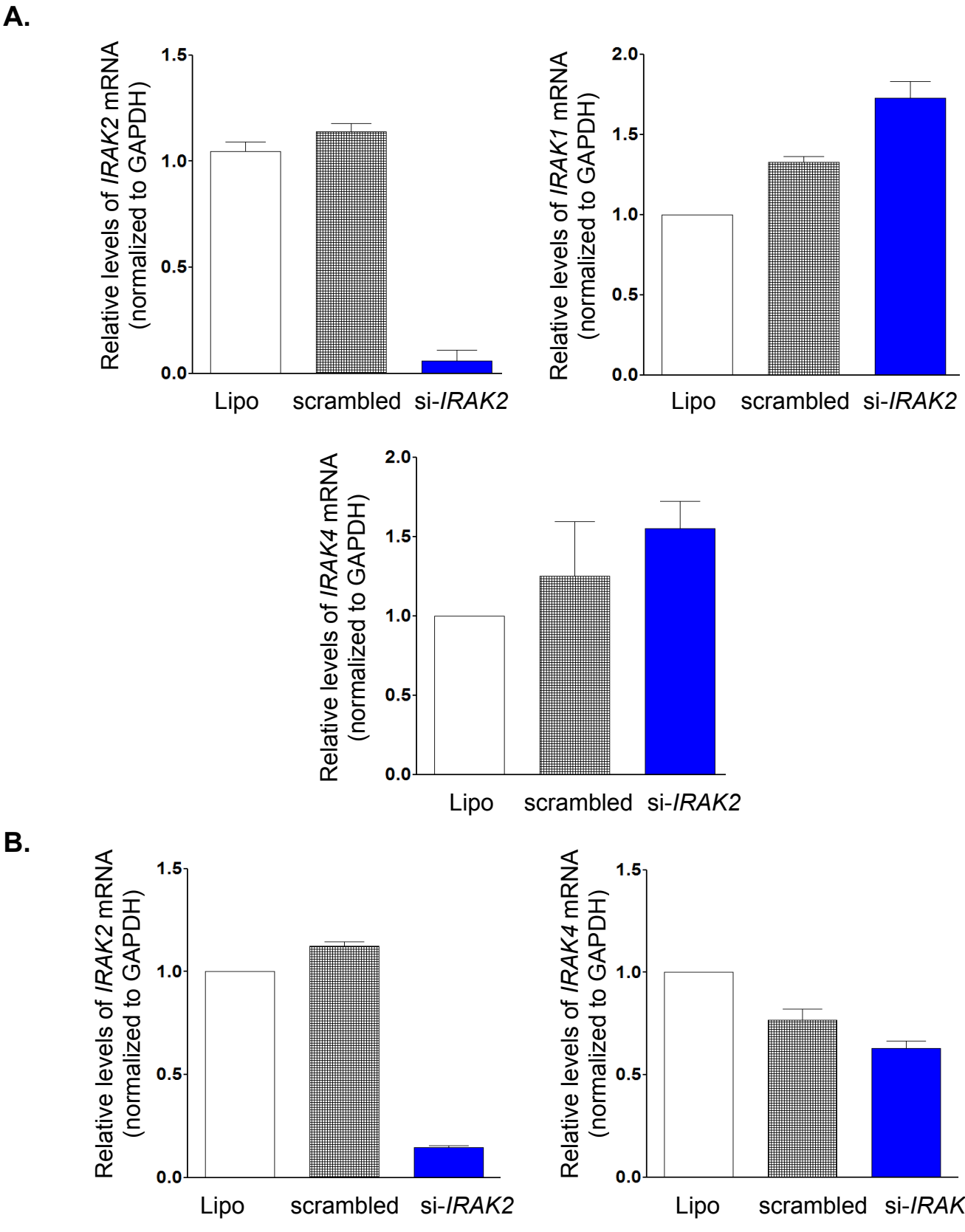


Figure S9. *IRAK2* silencing in the patient's fibroblasts

C., D. *IL8* mRNA levels (panel **C**) and IL-8 protein levels (panel **D**) in SV40-fibroblasts from a healthy control and from the IRAK-1-deficient patient, transfected with siRNAs targeting *IRAK2* (si-*IRAK2*) or with a nonsense siRNA (scrambled), then left unstimulated (NS) or stimulated with IL-1β (10 ng/ml) or TNF-α (20 ng/ml). The values shown (means ± SEM) were obtained in three independent experiments.

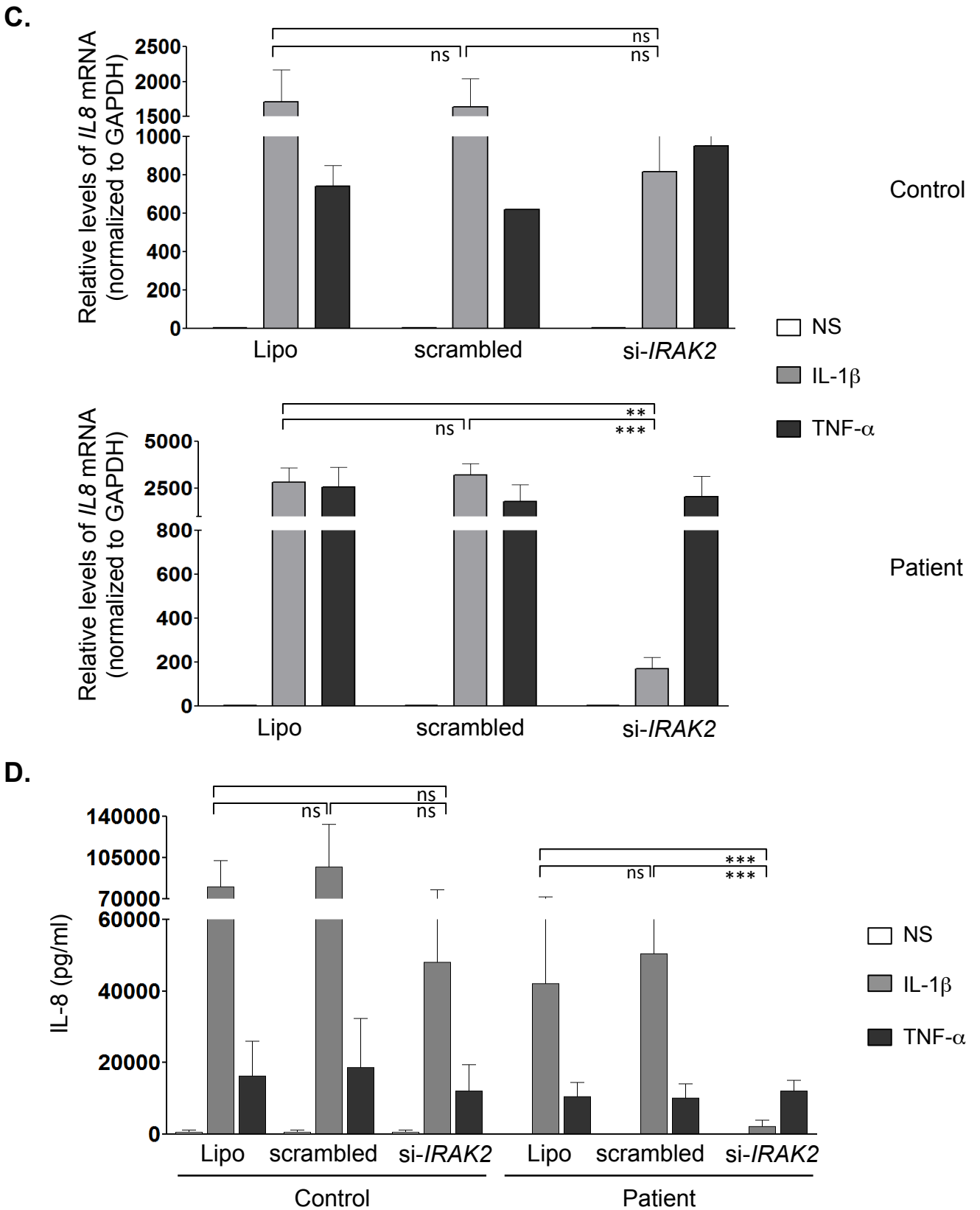


Figure S10. *IRAK1* and *IRAK2* silencing in control fibroblasts

A, B. *IRAK1* (left), *IRAK2* (center) and *IRAK4* (right) mRNA levels in SV40-fibroblasts from a healthy control (panel **A**), and in SV40-fibroblasts from the MECP2-deficient patient (panel **B**) transfected with siRNAs targeting *IRAK1* (si-*IRAK1*), *IRAK2* (si-*IRAK2*), both *IRAK1* and *IRAK2* (si-*IRAK1*+ si-*IRAK2*), or with a nonsense siRNA (scrambled).

The values shown (means ± SEM) were obtained in three independent experiments.

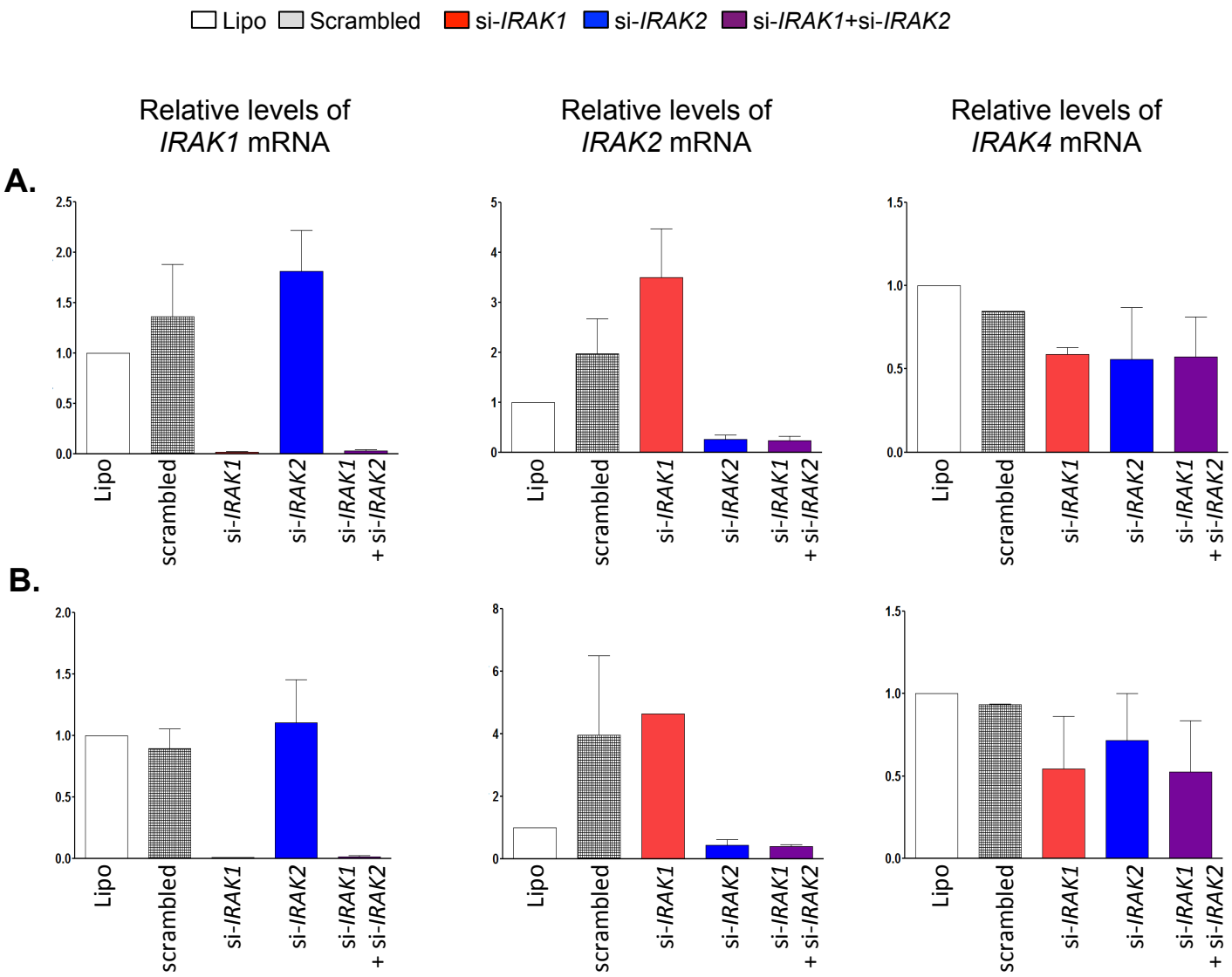
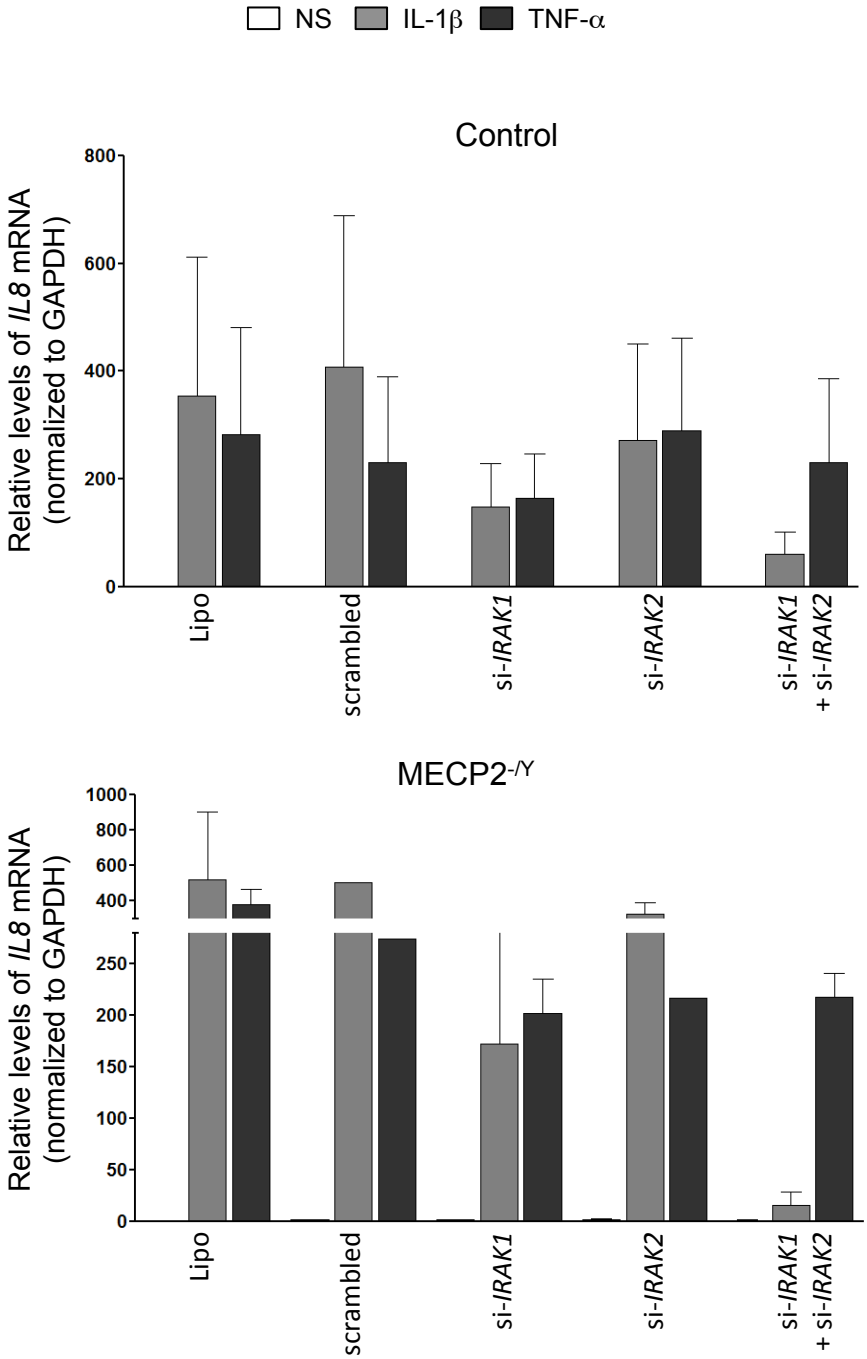


Figure S10. *IRAK1* and *IRAK2* silencing in control fibroblasts

C., D. *IL8* mRNA levels in SV40-fibroblasts from a healthy control (left panel) and from the MECP2-deficient patient (right panel) transfected with siRNAs targeting *IRAK1* (si-*IRAK1*), *IRAK2* (si-*IRAK2*), both *IRAK1* and *IRAK2* (si-*IRAK1*+si-*IRAK2*), or with a nonsense siRNA (scrambled), then left unstimulated (NS) or stimulated with IL-1β (10 ng/ml) (panel **C**) or with PAM-2 (10 μg/ml), LPS (10 μg/ml), MPLA (1 μg/ml) (panel **D**) and TNF-α (20 ng/ml) as a positive control (panels **C** and **D**).

The values shown (means ± SEM) were obtained in three independent experiments.

C.



D.

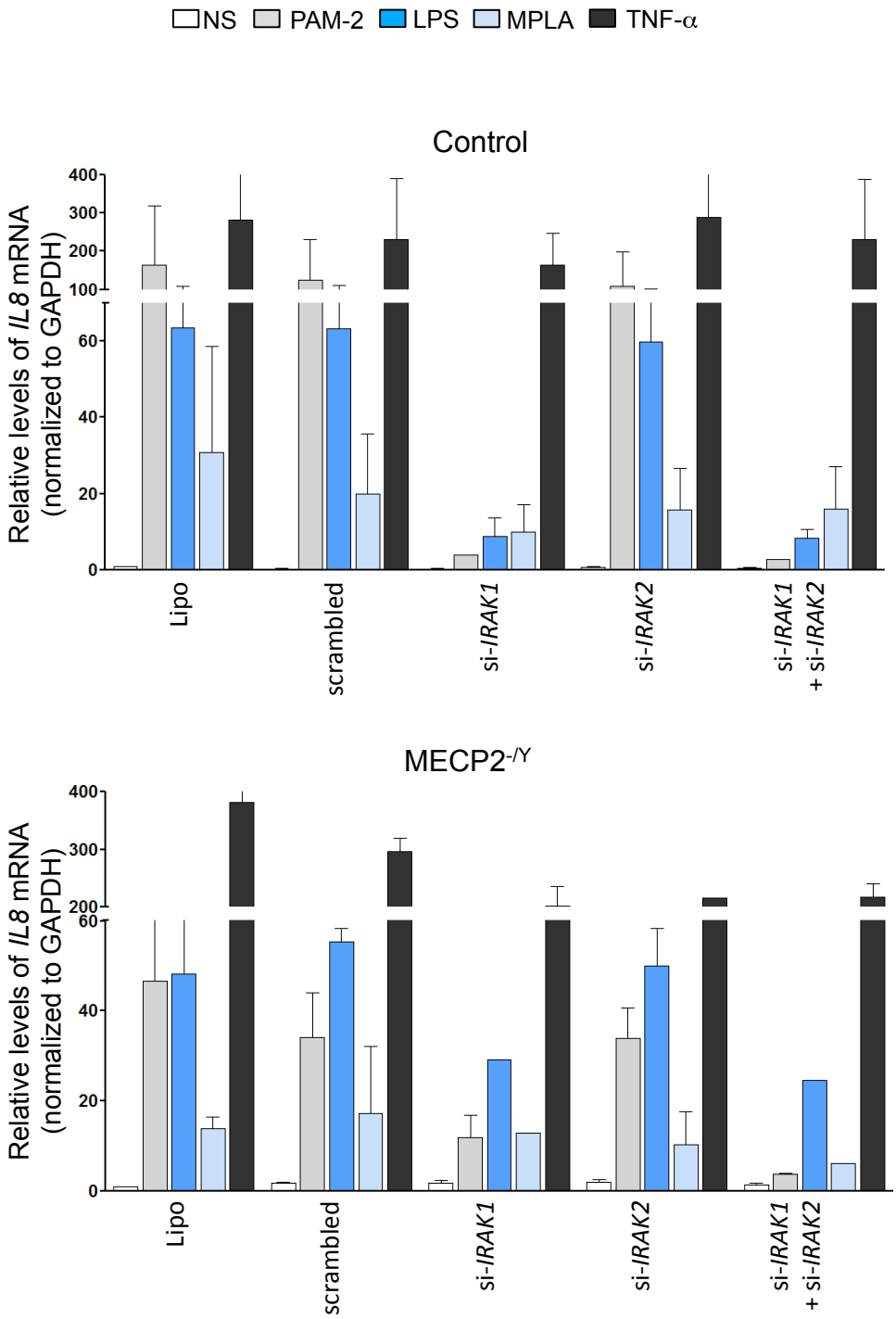

NS
PAM-2
LPS
MPLA
TNF-α
Control
Relative levels of IL8 mRNA (normalized to GAPDH)
400
300
200
100
60
40
20
0
Lipo
scrambled
si-IRAK1
si-IRAK2
si-IRAK1 + si-IRAK2
MECP2-/Y
Relative levels of IL8 mRNA (normalized to GAPDH)
400
300
200
60
40
20
0
Lipo
scrambled
si-IRAK1
si-IRAK2
si-IRAK1 + si-IRAK2

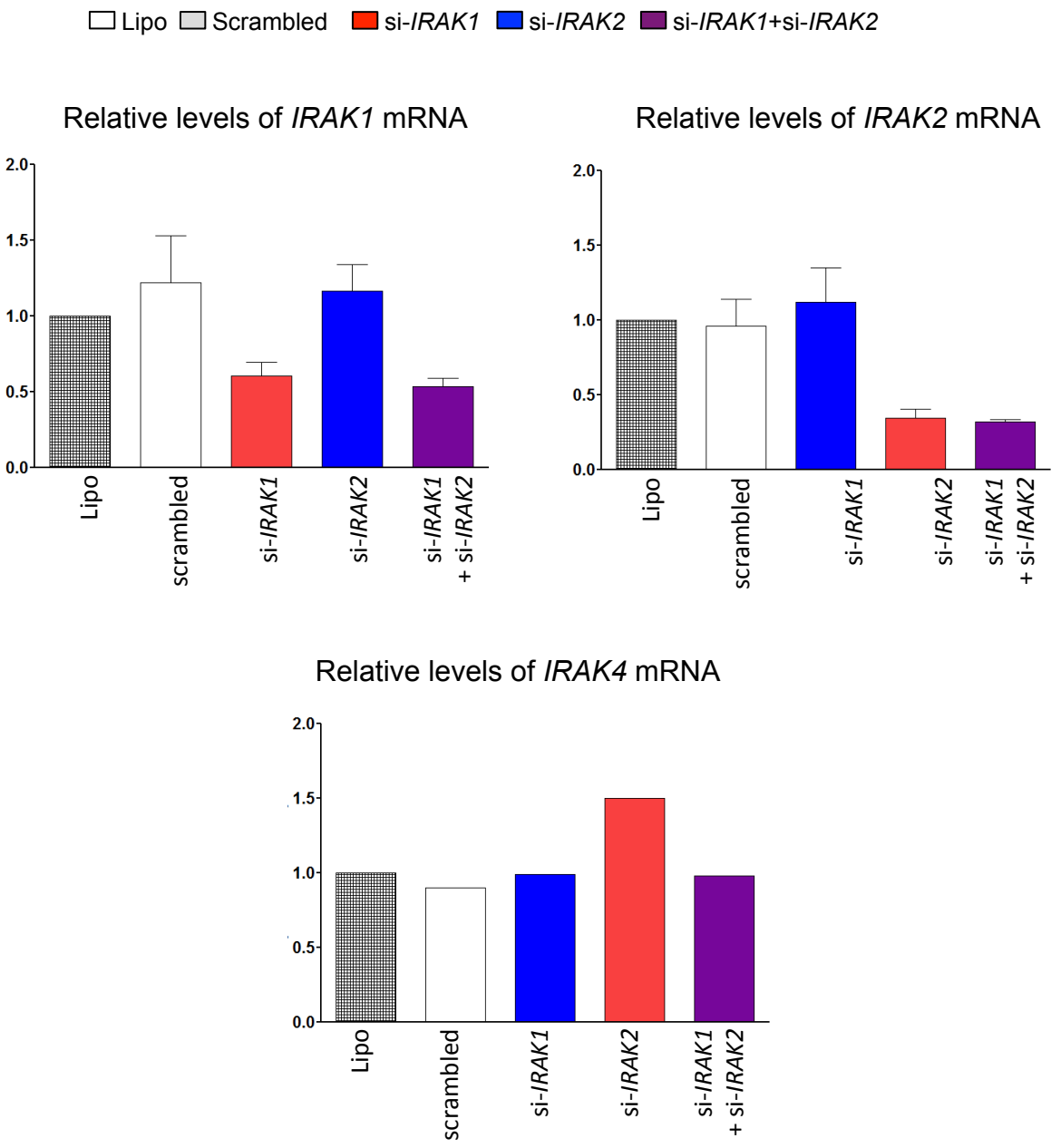
Figure S11. *IRAK1* and *IRAK2* silencing in control PBMCs

A. *IRAK1* (left), *IRAK2* (center) and *IRAK4* (right) mRNA levels in PBMCs from healthy controls transfected with siRNAs targeting *IRAK1* (si-*IRAK1*), *IRAK2* (si-*IRAK2*), both *IRAK1* and *IRAK2* (si-*IRAK1*+si-*IRAK2*), or with a nonsense siRNA (scrambled).

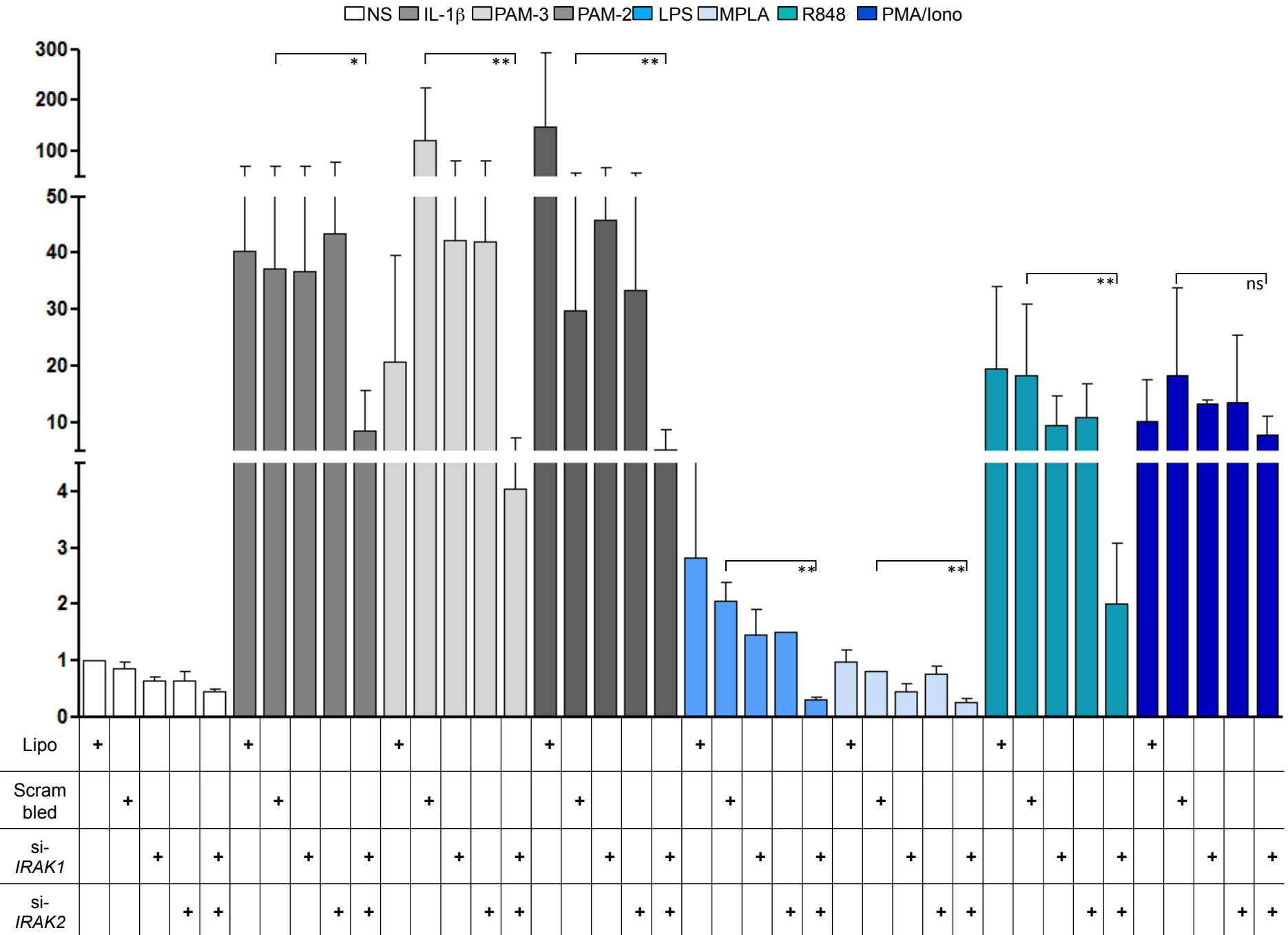
B. *IL8* mRNA levels in PBMCs from healthy controls transfected with siRNAs targeting *IRAK1* (si-*IRAK1*), *IRAK2* (si-*IRAK2*), both *IRAK1* and *IRAK2* (si-*IRAK1*+si-*IRAK2*), or with a nonsense siRNA (scrambled), then left unstimulated (NS) or stimulated with IL-1β (20 ng/ml), PAM-3 (1 μg/ml), PAM-2 (1 μg/ml), LPS (10 ng/ml), MPLA (1 μg/ml), R848 (1 μg/ml), and, as positive controls, PMA/ionomycin (10^{-7} M/10^{-5} M).

The values shown (means ± SEM) were obtained in two independent experiments analyzing PBMCs from three healthy controls each time.

A.



B.


NS
IL-1β
PAM-3
PAM-2
LPS
MPLA
R848
PMA/Iono
300
200
100
50
40
30
20
10
4
3
2
1
0
*
**
**
**
**
**
ns
Lipo
Scram bled
si-IRAK1
si-IRAK2

SI Tables

Table S1. Human IRAK-1-deficiency vs. IRAK-4 and MyD88 deficiencies

	Fibroblasts		Leukocytes	
	IRAK1$^{-/Y}$	**IRAK4$^{-/-}$, MyD88$^{-/-}$**	**IRAK1$^{-/Y}$**	**IRAK4$^{-/-}$, MyD88$^{-/-}$**
TLR 1/2	-	-	+	-
TLR 2/6	-	-	+	-
TLR 4	-	-	+	-
TLR 7	ND	ND	+	-
TLR 8	ND	ND	+	-
IL-1R	+	-	+	-

ND: not done

Table S2. IRAK deficiencies in mice

	IRAK1$^{-/Y}$	IRAK2$^{-/-}$	IRAK3$^{-/-}$	IRAK4$^{-/-}$, MyD88$^{-/-}$
***S. aureus* infection**	survival ↓	ND	ND	survival ↓
LPS septic shock	survival ↑	survival ↑	ND	survival ↑
Splenocytes				
TLR4	normal *IL1b*, *TNF mRNA but IL10* ↓	ND	ND	no proliferation
TLR2/6	ND	ND	ND	no proliferation
TLR7,8	ND	ND	ND	no proliferation
TLR9	ND	ND	ND	no proliferation
TLR3	ND	ND	ND	normal proliferation
Macrophages				
TLR 4	responses ↓*	↓ responses	responses ↑	↓ responses
MEFs				
TLR 2/6	responses ↓	responses ↓**	responses ↑	ND
TLR 4	responses ↓	responses ↓**	responses ↑	ND
IL-1R	responses ↓	responses ↓**	responses ↑	ND

ND: not done, *less impaired than Irak-1-deficient or Irak-4-deficient mice;
** less impaired than Irak-1-deficient or Irak-4-deficient mice

SI Materials and Methods

Array comparative genomic hybridization and breakpoint definition

Genome-wide array-CGH was performed with the Human Genome CGH Microarray Kit 4x180K (Agilent Technologies, California, USA), according to the manufacturer's protocol. We labeled 500 ng of DNA from the patient's whole blood and 500 ng of DNA from a pool of male genomic DNA (Promega Wisconsin, USA) with the Cy5 and Cy3-dUTP fluorochromes, respectively, with the Genomic DNA Labeling Kit PLUS (Agilent Technologies, California, USA). The labeled DNAs were then combined, denatured and pre-annealed with 5 ng of human Cot-1 DNA (Invitrogen, Life Technologies, USA). After 24 hours of hybridization at 65°C in a rotary incubator, images of the arrays were acquired with an Agilent scanner and analyzed with Feature Extraction software (v9.5, Agilent Technologies, California, USA). Copy number variations (CNVs) were identified with the aberration algorithms Z-Score and ADM-1, provided with CGH-Analytics software (v3.5). For breakpoint identification at base-pair level, four customized primer pairs were designed to bind on either side of the breakpoint region delimited by aCGH, and PCR was performed under the following conditions (95°C for 2 min, 35 cycles of 95°C for 30 s, 58-61°C for 30 s, and 68°C for 4 min and then 72°C for 5 min). PCR products were then subjected to electrophoresis in 1% agarose gels for purification and sequencing. Primers binding to the region deleted in the patient yielded no amplicon with DNA from the patient, but did amplify a DNA fragment from the control. We thus selected the forward primer of the pair yielding correct amplification of the proximal breakpoint, and the reverse primer used to amplify the distal breakpoint from the proband's DNA for PCR to amplify the full-length sequence and to refine the breakpoints.

Microarray analysis

Primary fibroblasts were used to seed culture plates at a density of $2.5x10^5$ cells/well. The plates were incubated overnight and the cells were then left unstimulated or stimulated for 6 hours with PAM-2 (10 μg/ml), FSL-1 (1 μg/ml), LPS (10 μg/ml), MPLA (1 μg/ml), IL-1β (10 ng/ml) or TNF-α (20 ng/ml). RNA was extracted with the ZR RNA MicroPrepZymo kit (ZR1061, Zymo Research, California, USA), according to the manufacturer's instructions. Total RNA was converted to cRNA with the Illumina® TotalPrepTM RNA Amplification Kit, and biotin-labeled cRNA was hybridized onto HumanHT-12 v4.0 Expression BeadChips with the HumanHT-12 v4.0 Expression BeadChip Kit (Illumina San Diego, CA, USA). Probe detection (P<0.01) and the mean intensity of the raw signal were analyzed by PCA, to check the reproducibility of the results. Randomly selected technical replicates were located very close together in the projection space. We also compared the number of probes detected and mean signal intensity between samples. We found no outliers or other evidence for technical artifacts. We minimized the potential 'batch effect' between sets, by applying a background correction and quantile normalization with the neqc function of the limma R package. The initial number of probes was 47323. A set of filters was applied to select genes considered substantially affected by stimulation in the patient vs healthy controls. For the first filter, we retained probes satisfying the requirements for detection (P<0.01) in at least 10% of all samples (n=19507). For each individual and each set of stimulation conditions, fold-changes in expression between stimulated and unstimulated conditions were calculated. For each set of conditions, transcripts were further filtered based on a minimal 1.5-fold change in expression (upregulated or downregulated), with a minimum absolute difference in expression of more than 150 relative to unstimulated samples. In a final filtering stage, probes satisfying the previous filters for two of the three healthy control

samples for each stimulation were retained and used in the relevant figures. No explicit statistical analysis was performed on differences in gene expression. The genes satisfying our filter criteria were further analyzed by Ingenuity Pathway Analysis (IPA) Software, Version 28820210 (QIAGEN). The microarray data used in this study have been deposited in NCBI's Gene Expression Omnibus (GEO) with the accession number GSE92466.

Western blotting and EMSA

Western blotting was performed with the rabbit mAb2697 for phospho-IKKα/β, the rabbit mAb2859 for phospho-IκBα, the rabbit mAb3033 for phospho-NF-κB-p65, and, as a loading control, the β-actin mouse mAb3700 (all from Cell Signaling). For EMSA, SV40-transformed fibroblasts, were stimulated 45 min after treatment, with PAM-2 (10 μg/ml, Invivogen) LPS (10 μg/ml, Sigma-Aldrich), MPLA (1 μg/ml, Invivogen) or TNFα (20 ng/ml, R&D Systems). Nuclear extracts were prepared and 10 μg of these extracts were incubated with a ^{32}P-labeled double-stranded NF-κB-specific oligonucleotide κBprobe (5'GATCATGGGGAATCCCCA-3' and 5'-GATCTGGGGATTCCCCAT-3').

Complementation and silencing in PBMCs

For the silencing of IRAK-1, IRAK-2 or both, PBMCs were transiently transfected for 3 days with 50 nM siRNA targeting *IRAK1* or *IRAK2* or a mixture of siRNAs targeting *IRAK1* and *IRAK2* (50 nM final concentration) or scrambled siRNA (50 nM) labeled with Viromer Green (Lipocalyx, Germany), according to the manufacturer's instructions. PBMCs were then stimulated for four hours, for the determination of *IL8, IRAK1, IRAK2,* and *IRAK4* mRNA levels.