New insights into the Shwachman-Diamond Syndrome-related haematological disorder: hyper-activation of mTOR and STAT3 in leukocytes.

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SUPPLEMENTARY INFORMATION

Supplementary Figure legends

Supplementary figure S1

SBDS protein expression in LCLs.

Western blot analysis to evaluate SBDS protein expression in LCLs. LY52 and LY53: LCLs derived from healthy control subjects. LY190, LY193, LY198: LCLs obtained from SDS patients carrying genotype 258+2T>C / 183-184TA>CT.

Supplementary figure S2

Western Blot analysis of mTOR S727 phosphorylation in LCLs.

Increase of S2448 phosphorylated mTOR in SDS. **a)** western blotting analysis using the polyclonal rabbit antibody recognizing the S2448 phosphorylated form of mTOR (arrowed). **b)** representative western blotting analysis of protein extracts from a control subject (on the left) and a SDS patient (on the right) and summary of the relative content of the S2448 phosphorylated mTOR in control (open box) and SDS-derived LCLs (black box). Data are mean ± SD of two independent experiments performed in duplicate. Student's t test has been reported. **c)** representative western blotting analysis of protein extracts from SDS LCLs culture in the absence or in the presence of IL-6, with (+) or without (-) 350 nM rapamycin. **d)** summary of the relative content of the S2448

phosphorylated mTOR in control (open box) and SDS patients (black box). Data represent the % values relative to untreated cells. The S2448 phosphorylated mTOR is presented in control (open box) and SDS-derived LCLs induced (+) or not (-) with IL-6. Data are mean ± SD of two independent experiments performed in duplicate. Student's t test has been reported.

Supplementary figure S3

Effect of SBDS gene silencing in LCLs on mTOR S2448 phosphorylation and role of ERK1/2 MAPK. a) LCLs derived from healthy donors were transiently transfected with 2 different specific siRNA sequences (siRNA 1 and siRNA 2) for SBDS, or with PE-conjugated siRNA sequence, or with scrambled sequence as control in the presence of cationic liposomal vector for 24 hours. mTOR S2448 phosphorylation was detected using PathScan phospho-mTOR ELISA kit. Data are mean ± SEM of 5 independent experiments performed in 3 different healthy control cell lines, in duplicate. **b)** Effect of pre-incubation of ERK inhibitor U0126 (10 μM) in SDS-derived LCLs for 1 hour before stimulation with IL-6 (10 ng/ml) for further 15 min. Data are mean ± SEM of 6 independent experiments performed in 3 different healthy control cell lines, in duplicate. Wilcoxon signed-rank test has been calculated.

Supplementary figure S4

Flow cytometric analysis of mTOR S2448 phosphorylation in primary leukocytes. Representative experiment conducted in leukocytes derived from peripheral blood of a SDS patient carrying genotype 258+2T>C/183-184TA>CT compared to a healthy control subject. **a)** morphological distribution of CD45 expressing leukocytes in healthy donor derived peripheral blood. **b)** morphological distribution of CD45 expressing leukocytes in SDS patient derived peripheral blood. PMNs, Monocytes and Lymphocytes are gated. **c,d)** B cell (CD19+) region isolated from lymphocytes regions of panels A and B, respectively, plotted on CD3 versus CD19 dotplot . **e)** mTOR S2448 phosphorylation observed in B cells in the presence or in the absence (UT) of IL-6 stimulation (10 ng/ml) for 15 min. **f)** mTOR S2448 phosphorylation observed in PMNs in the presence or in the absence of IL-6 stimulation (10 ng/ml) for 15 min. **g)** mTOR S2448

phosphorylation observed in Monocytes in the presence or in the absence of IL-6 stimulation (10 ng/ml) for 15 min.

Supplementary figure S5

Effect of rapamycin on mTOR S2448 phosphorylation in primary leukocytes.

Primary leukocytes derived from two patients carrying genotype 258+2T>C / 183-184TA>CT and from two healthy control subjects were incubated in the presence or in the absence of rapamycin for 1 hour before IL-6 stimulation (10 ng/ml) for further 15 min and analyzed by flow cytometry. **a,b)** MFI of mTOR S2448 phosphorylation measured in primary B cells derived from healthy control and SDS patient, respectively. **c,d)** MFI of mTOR S2448 phosphorylation measured in primary PMNs derived from healthy control and SDS patient, respectively. **e,f)** MFI of mTOR S2448 phosphorylation measured in primary monocytes derived from healthy control and SDS patient, respectively. Data are mean ± SEM of two independent experiments performed in LCLs obtained from three different SDS patients and compared to three different healthy donors. Student's t test has been calculated.

Supplementary figure S6

Flow cytometric analysis of STAT3 Y705 and S727 phosphorylation in primary leukocytes.

Representative experiment conducted in leukocytes derived from peripheral blood cells of a SDS patients carrying genotypes 258+2T>C / 183-184TA>CT compared to a healthy control subject. **a)** STAT3 phosphorylation observed in B cells in the presence or in the absence (UT) of IL-6 stimulation (10 ng/ml) for 15 min. **b)** STAT3 phosphorylation observed in PMNs in the presence or in the absence of IL-6 stimulation (10 ng/ml) for 15 min. **c)** STAT3 phosphorylation observed in Monocytes in the presence or in the absence of IL-6 stimulation (10 ng/ml) for 15 min.

Supplementary figure S7

Effect of rapamycin on STAT3 Y705 phosphorylation in primary leukocytes.

Primary leukocytes derived from two patients carrying genotype 258+2T>C / 183-184TA>CT and from two healthy control subjects were incubated in the presence or in the absence of rapamycin for 1 hour before IL-6 stimulation (10 ng/ml) for further 15 min and analyzed by flow cytometry. **a,b)** MFI of STAT3 Y705 phosphorylation measured in primary B cells derived from healthy control and SDS patient, respectively. **c,d)** MFI of STAT3 Y705 phosphorylation measured in primary PMNs derived from healthy control and SDS patient, respectively. **e,f)** MFI of STAT3 Y705 phosphorylation measured in primary monocytes derived from healthy control and SDS patient, respectively. Data are mean ± SEM of two independent experiments performed in LCLs obtained from three different SDS patients and compared to three different healthy donors. Student's t test has been calculated.

Supplementary figure S8

Effect of rapamycin on STAT3 S727 phosphorylation in primary leukocytes.

Primary leukocytes derived from two patients carrying genotype 258+2T>C / 183-184TA>CT and from two healthy control subjects were incubated in the presence or in the absence of rapamycin for 1 hour before IL-6 stimulation (10 ng/ml) for further 15 min and analyzed by flow cytometry. **a,b)** MFI of STAT3 S727 phosphorylation measured in primary B cells derived from healthy control and SDS patient, respectively. **c,d)** MFI of STAT3 S727 phosphorylation measured in primary PMNs derived from healthy control and SDS patient, respectively. **e,f)** MFI of STAT3 S727 phosphorylation measured in primary monocytes derived from healthy control and SDS patient, respectively. Data are mean ± SEM of two independent experiments performed in LCLs obtained from three different SDS patients and compared to three different healthy donors. Student's t test has been calculated.

SUPPLEMENTARY FIGURES

Figure S1

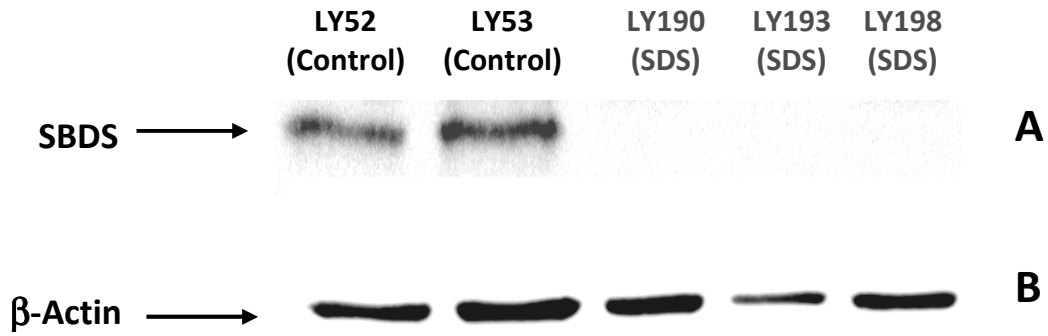


Figure S2

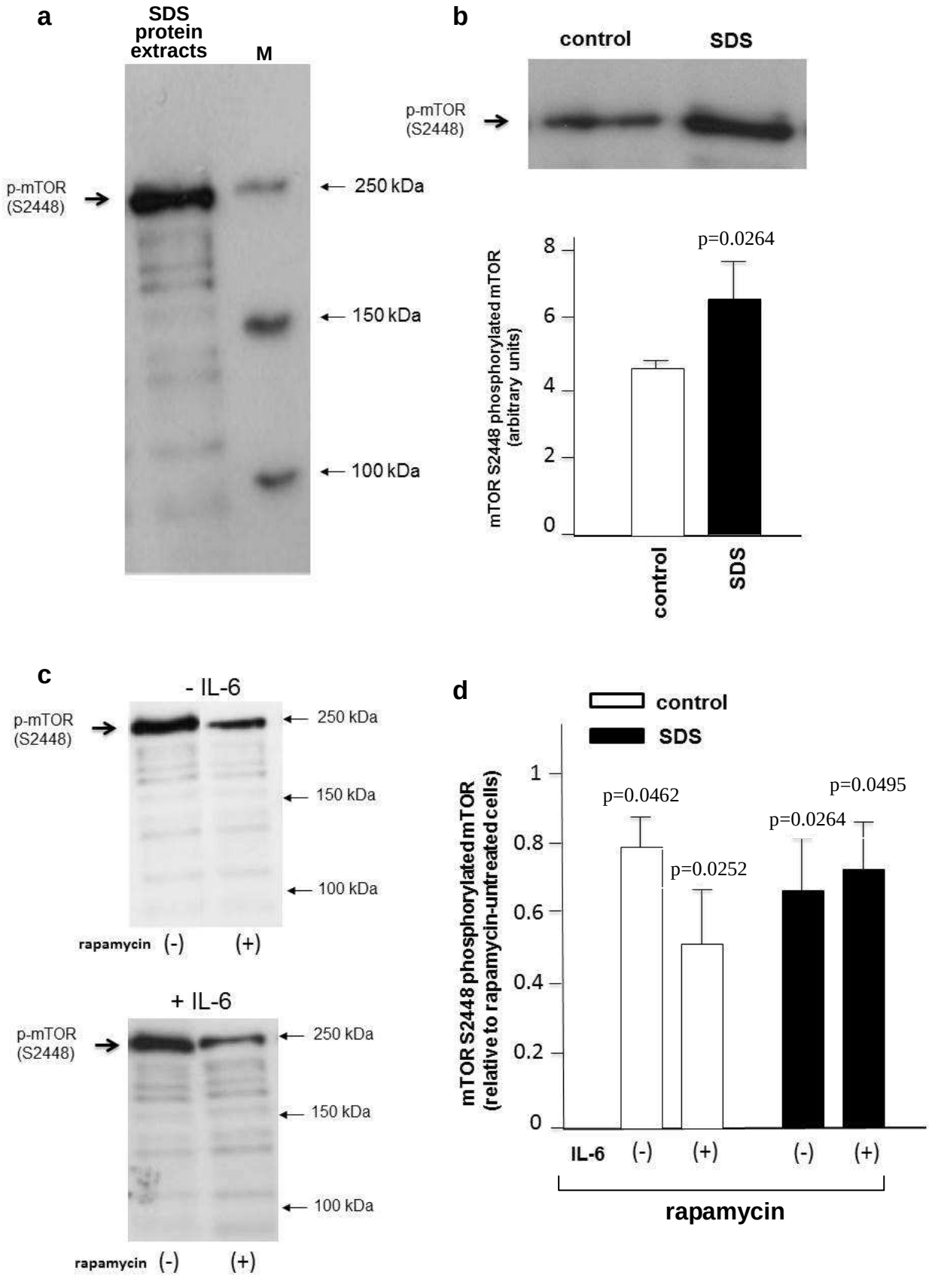

a
SDS protein extracts
M
p-mTOR (S2448)
250 kDa
150 kDa
100 kDa
b
control
SDS
p-mTOR (S2448)
mTOR S2448 phosphorylated mTOR (arbitrary units)
p=0.0264
c
- IL-6
p-mTOR (S2448)
250 kDa
150 kDa
100 kDa
rapamycin (-) (+)
+ IL-6
d
control
SDS
mTOR S2448 phosphorylated mTOR (relative to rapamycin-untreated cells)
p=0.0462
p=0.0252
p=0.0264
p=0.0495
IL-6 (-) (+) (-) (+)
rapamycin

Figure S3

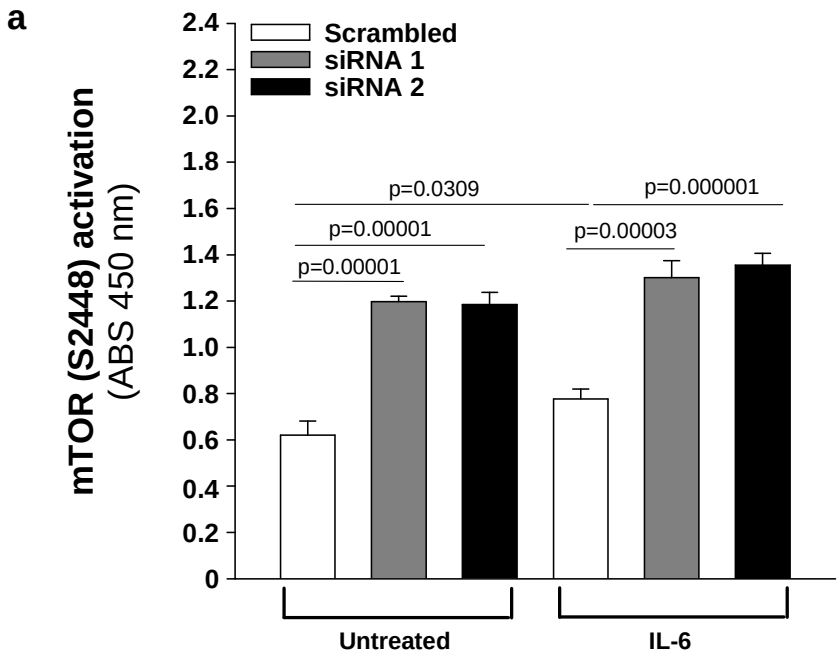
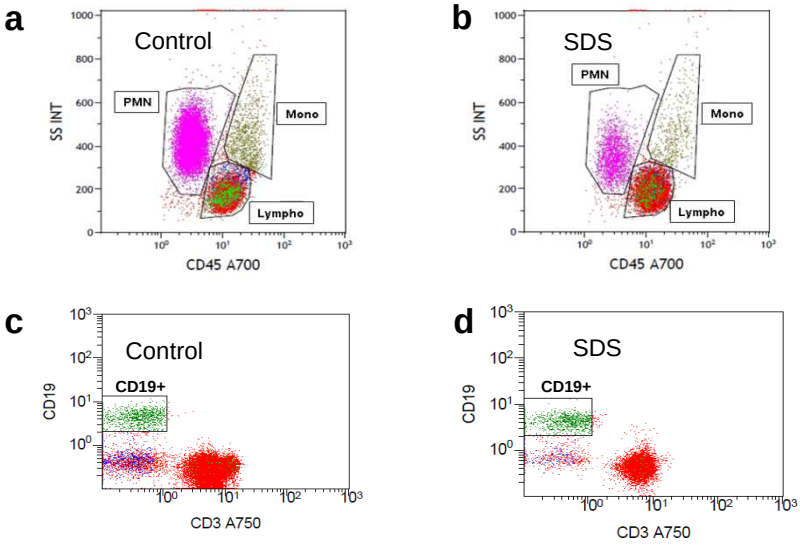
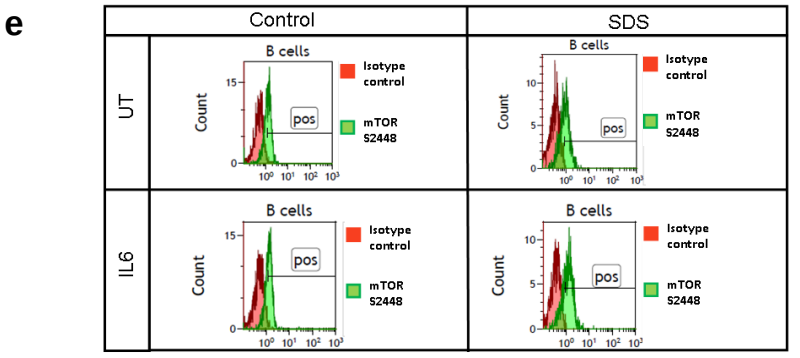
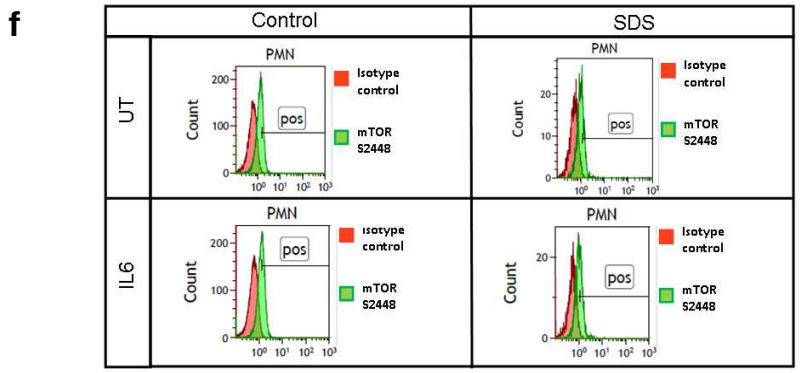

a
mTOR (S2448) activation
(ABS 450 nm)
Scrambled
siRNA 1
siRNA 2
p=0.0309
p=0.000001
p=0.00001
p=0.00003
p=0.00001
Untreated
IL-6
b
mTOR (S2448) activation
(ABS 450 nm)
Vehicle
U0126
p=0.00002
p=0.00060
p=0.00001
UT
IL6

Figure S4


a
Control
PMN
Mono
Lympho
SS INT
CD45 A700
b
SDS
PMN
Mono
Lympho
SS INT
CD45 A700
c
Control
CD19+
CD19
CD3 A750
d
SDS
CD19+
CD19
CD3 A750


e
Control
SDS
UT
IL6
B cells
Count
pos
Isotype control
mTOR S2448


f
Control
SDS
UT
IL6
PMN
Count
pos
Isotype control
mTOR S2448

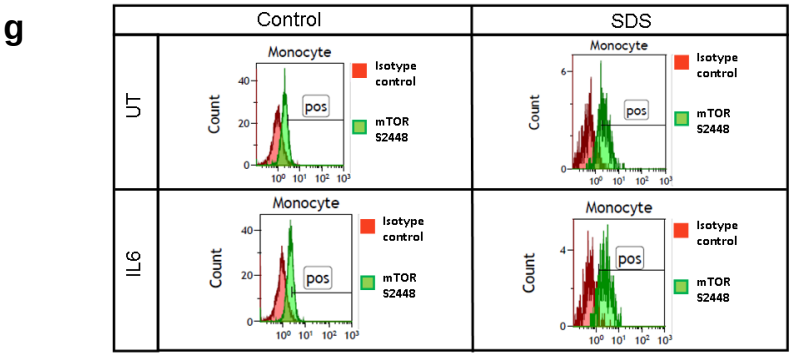

g
Control
SDS
UT
IL6
Monocyte
Count
pos
Isotype control
mTOR S2448

Figure S5

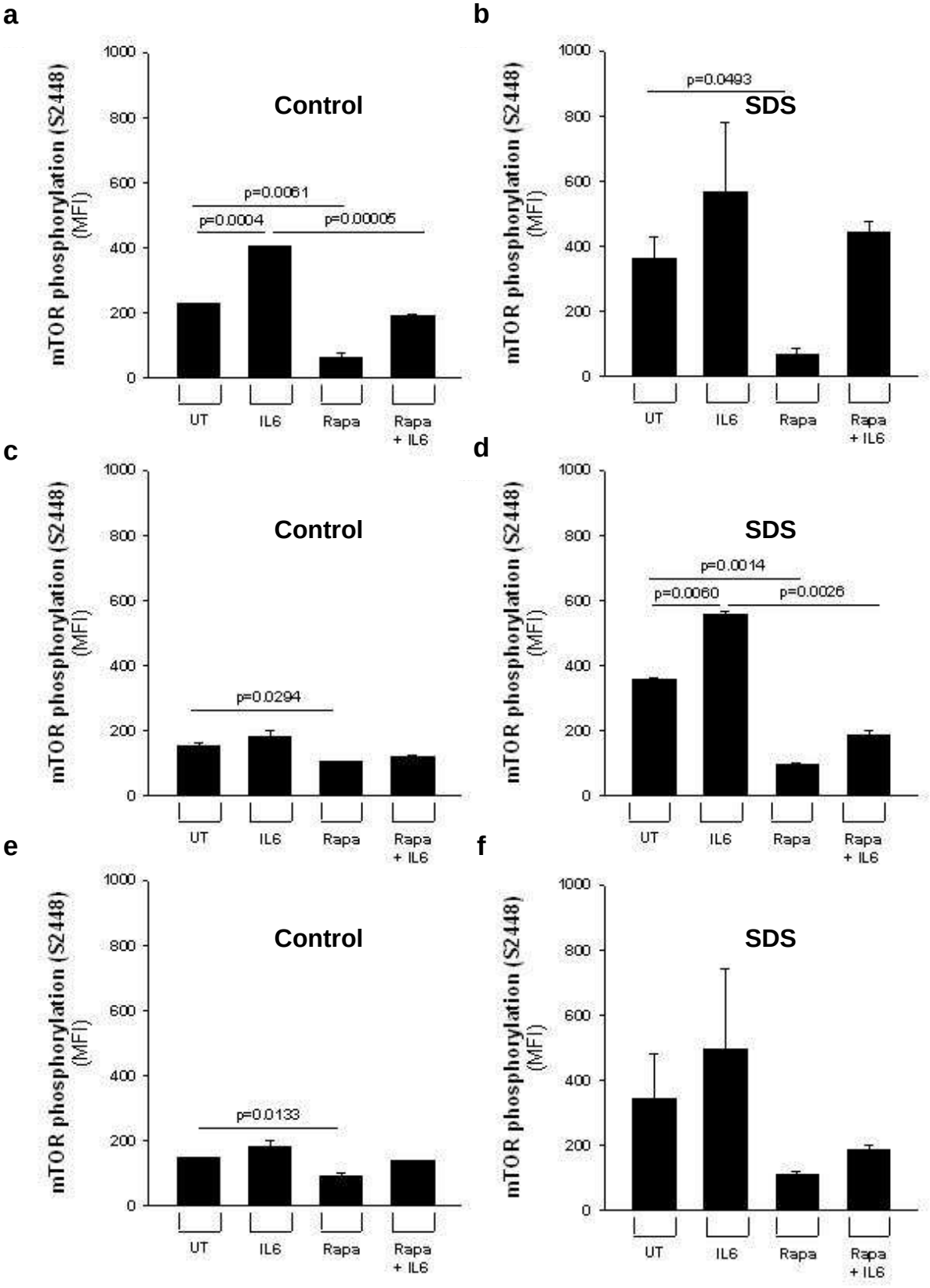

a
Control
mTOR phosphorylation (S2448) (MFI)
p=0.0061
p=0.0004
p=0.00005
UT
IL6
Rapa
Rapa + IL6
b
SDS
p=0.0493
c
Control
p=0.0294
d
SDS
p=0.0014
p=0.0060
p=0.0026
e
Control
p=0.0133
f
SDS

Figure S6

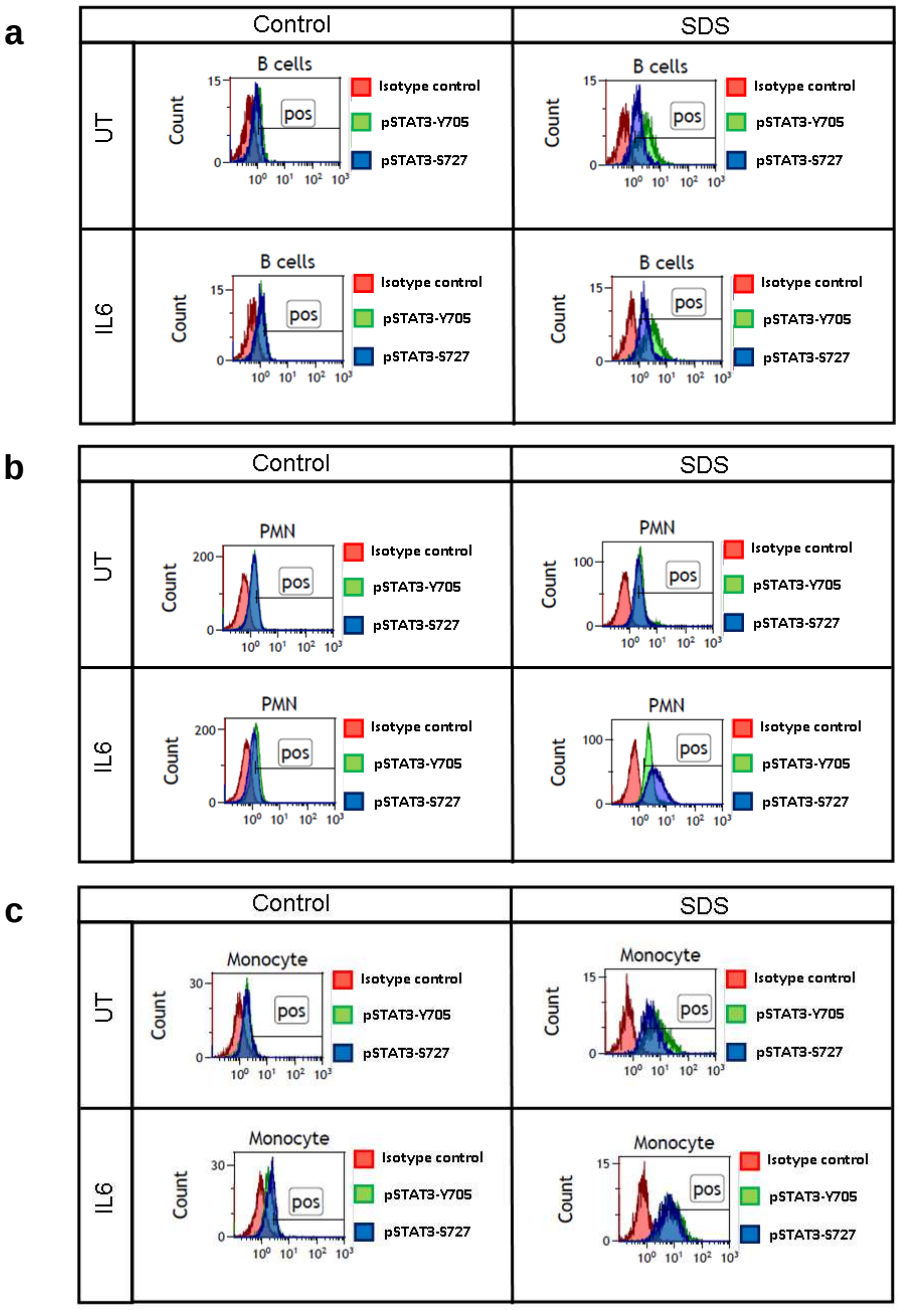

a
Control
SDS
UT
IL6
B cells
Count
pos
Isotype control
pSTAT3-Y705
pSTAT3-S727
b
PMN
c
Monocyte

Figure S7

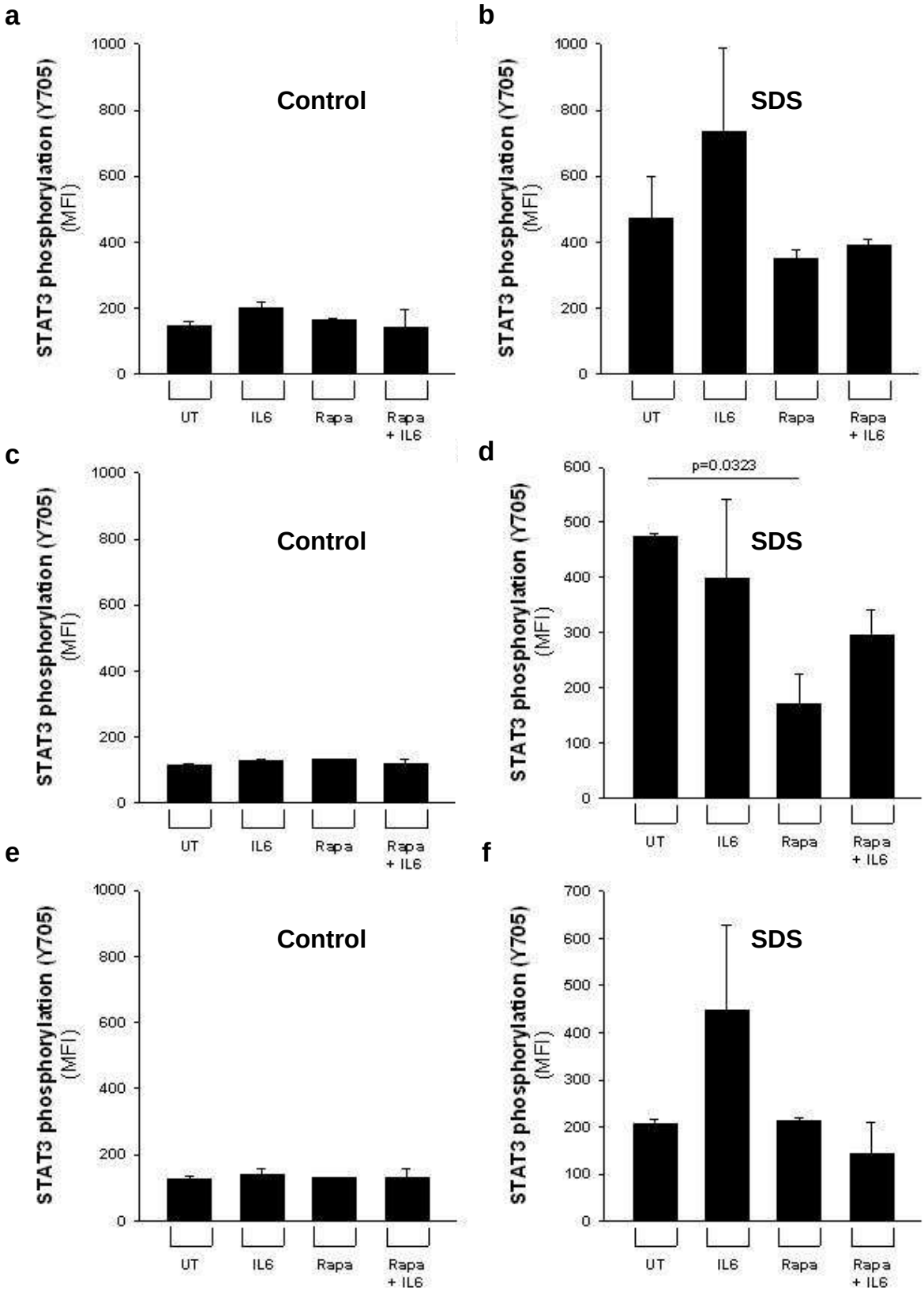
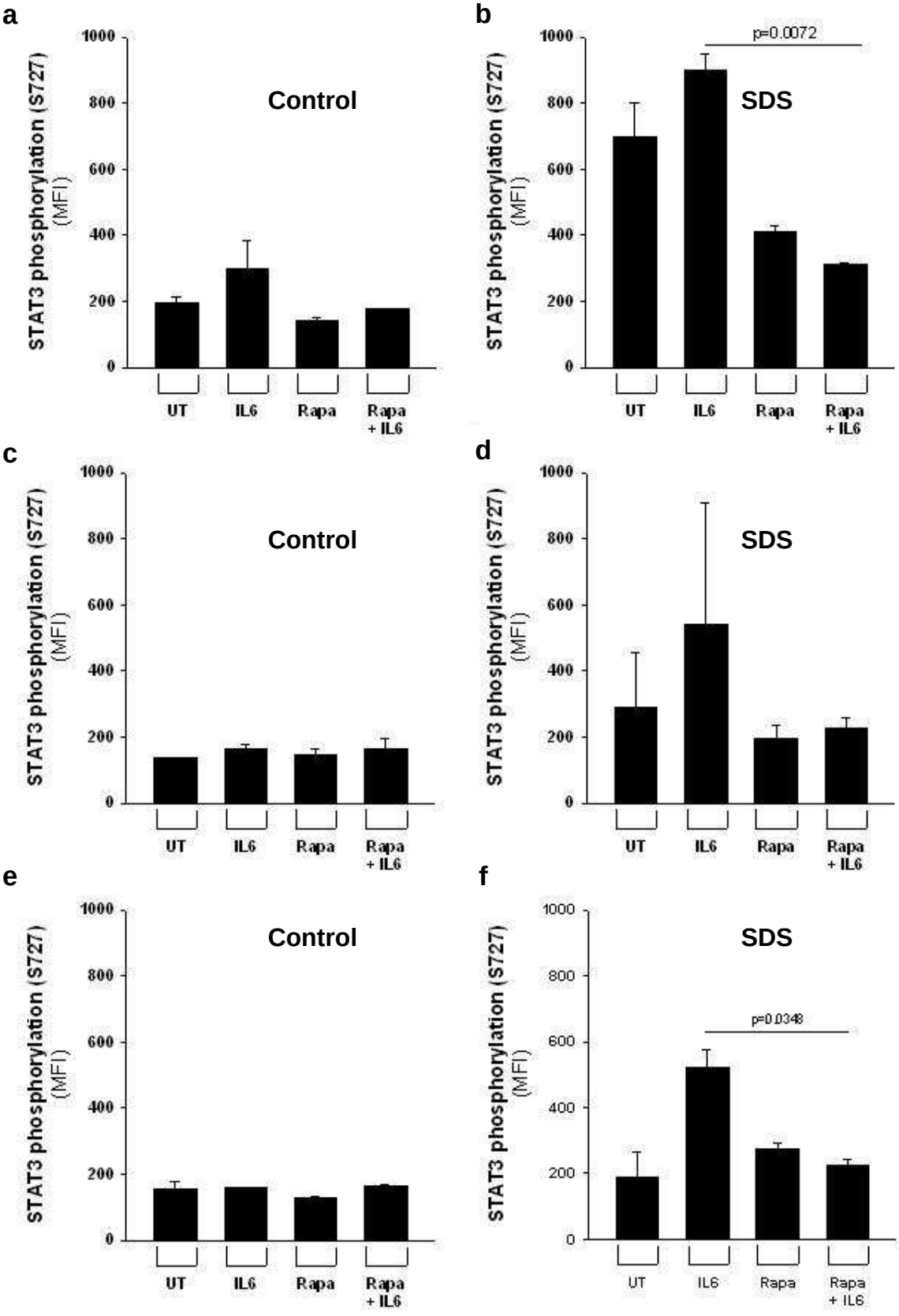

a
Control
STAT3 phosphorylation (Y705) (MFI)
1000
800
600
400
200
0
UT
IL6
Rapa
Rapa + IL6
b
SDS
c
Control
d
SDS
p=0.0323
e
Control
f
SDS
700

Figure S8


a
Control
STAT3 phosphorylation (S727) (MFI)
1000
800
600
400
200
0
UT
IL6
Rapa
Rapa + IL6
b
SDS
p=0.0072
c
Control
d
SDS
e
Control
f
SDS
p=0.0348