Supporting Information

Anti-Leukemia Effect Associated with Down-Regulated CD47 and Up-Regulated Calreticulin by Stimulated-Macrophages in Co-Culture

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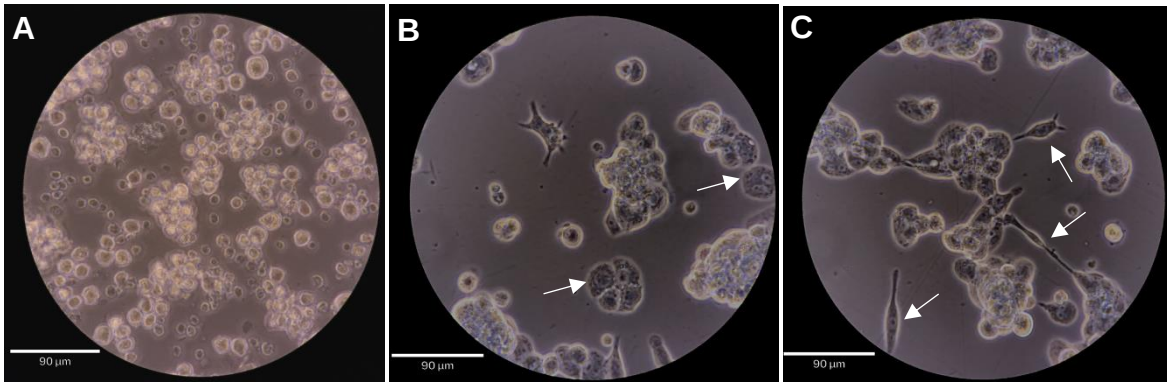
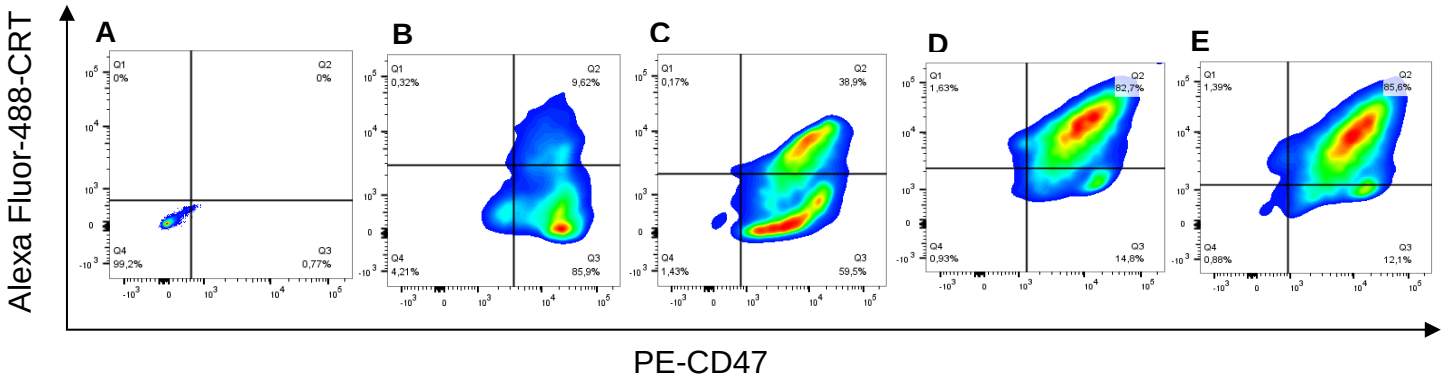
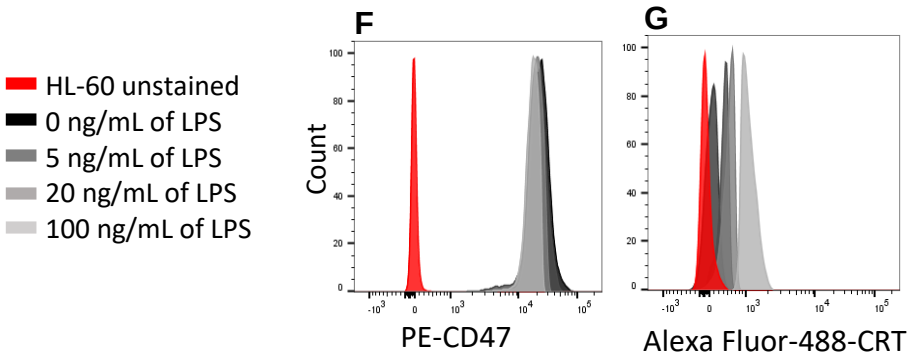
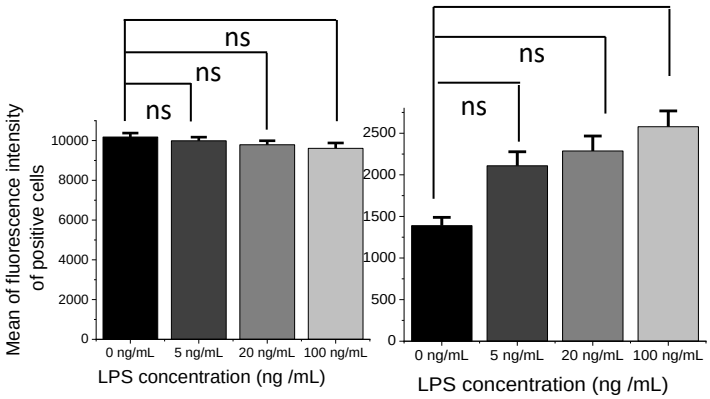


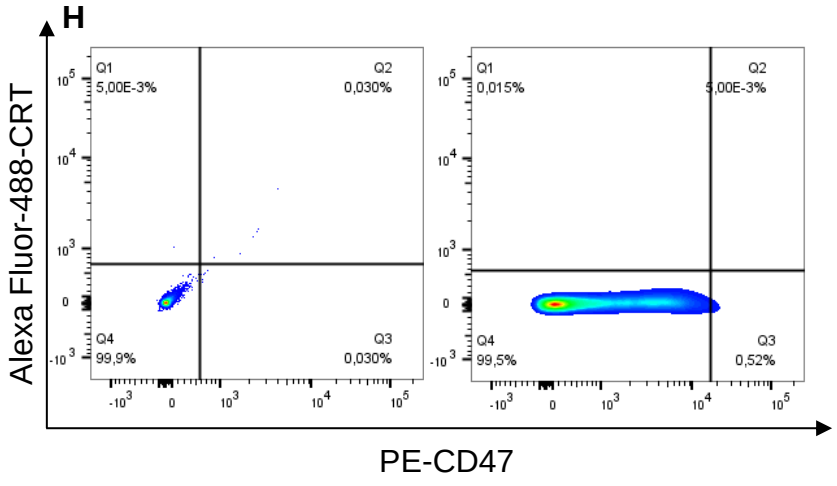
Figure S1. THP-1 cells phase contrast images before and after PMA treatment. THP-1 cells exhibit macrophage like phenotype, including changing in size and shape after PMA treatment. **A**, THP-1 before PMA treatment. Cells appear to be suspension in nature, growing in clumps. **B**, THP-1 after 4 hour of PMA incubation. Cells appear to be more adherent, bigger in size (white arrow). **C**, THP-1 cells after 24 hour of PMA treatment. Cells appear to look like macrophages (spindle in shape, white arrow) and bigger in size. Moreover, cells appear to be more internally complex in structure.

THP-1 were incubated with 5 ng/mL of PMA and incubated for 24 hours. Images were taken after 4 hour and 24 hour of incubation. All images were taken at 40 X objective lenses. The scale is 90 µm in all images.


A
B
C
D
E
Alexa Fluor-488-CRT
PE-CD47
Q1 0%
Q2 0%
Q4 99,2%
Q3 0,77%
Q1 0,32%
Q2 9,62%
Q4 4,21%
Q3 85,9%
Q1 0,17%
Q2 38,9%
Q4 1,43%
Q3 59,5%
Q1 1,63%
Q2 82,7%
Q4 0,93%
Q3 14,8%
Q1 1,39%
Q2 85,6%
Q4 0,88%
Q3 12,1%


HL-60 unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS
F
G
Count
PE-CD47
Alexa Fluor-488-CRT


ns
ns
ns
Mean of fluorescence intensity of positive cells
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
LPS concentration (ng /mL)


H
Alexa Fluor-488-CRT
PE-CD47
Q1 5,00E-3%
Q2 0,030%
Q4 99,9%
Q3 0,030%
Q1 0,015%
Q2 5,00E-3%
Q4 99,5%
Q3 0,52%

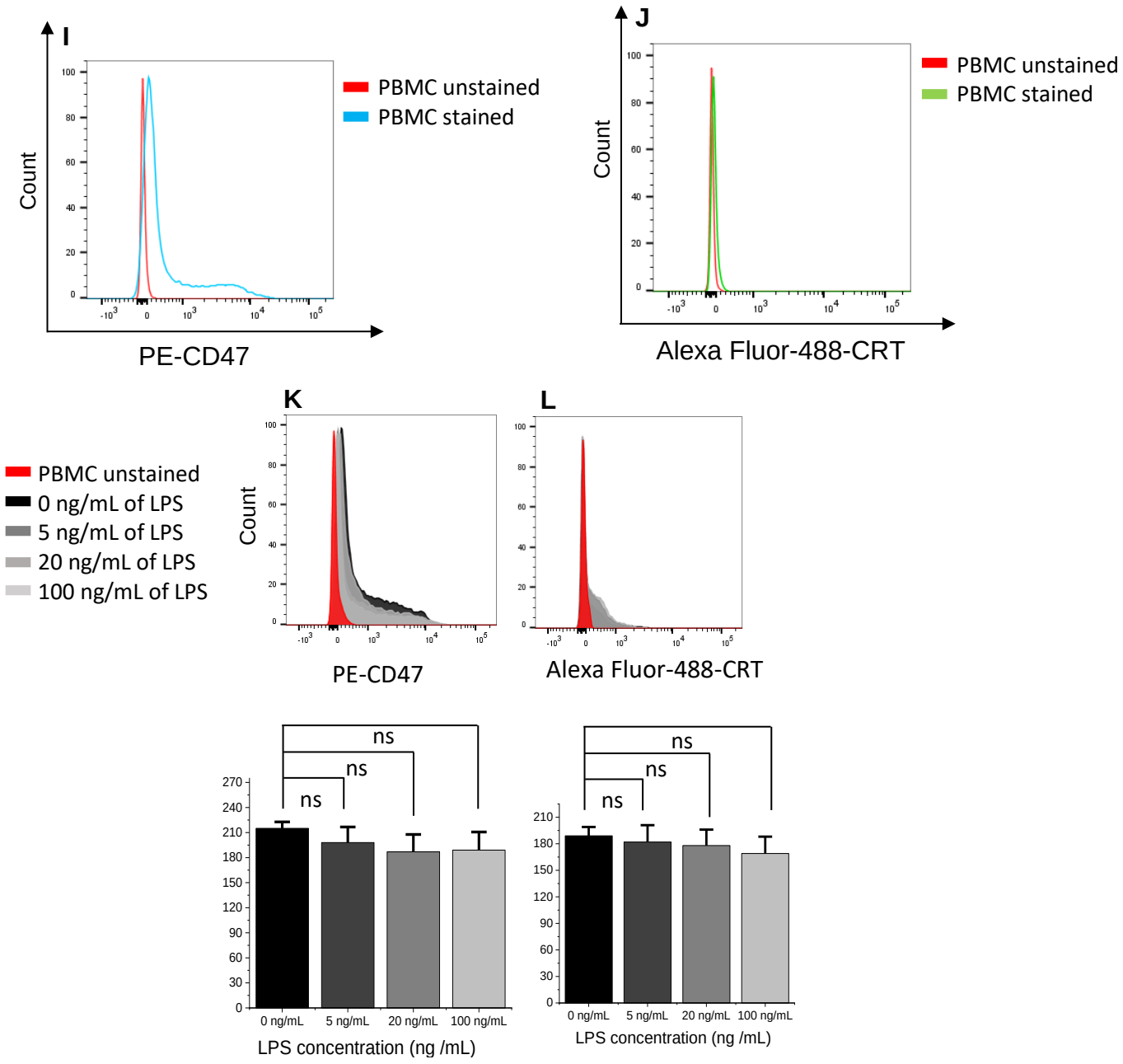


Figure S2. Flow cytometric analysis of CD47 and CRT in HL 60 and PBMC after co-culturing with THP-1 macrophages and without co-culture. **A**, Side scatter plots of HL60 cells unstained; **B-E,** HL60 cells stained with PE-anti-CD47 and Alexa-Fluor-488 anti-CRT antibodies at 0 ng/mL **B**, 5 ng/mL **C**, 20 ng/mL **D**, and 100 ng/mL **E** of LPS after co-cultured with THP-1 macrophages. **F** and **G**, Flow cytometric analysis of CD47 (F), and CRT (G), in HL-60 without co-culture. **H**, Side scatter plots of PBMC cells unstained and stained with anti-CD47 and anti-CRT antibodies after co-culture with THP-1 macrophages at 0 ng/mL of LPS. **I** and **J**, Histogram of the data in **A** for unstained PBMC and CD47 and CRT stained PBMC, respectively. **K** and **L**, Flow cytometric analysis of CD47 (F), and CRT (G), in PBMC without co-culture. Values in the graphs are shown as means ± SEM of three trials of duplicate samples. The statistical significance was determined by one way ANOVA (OriginPro 2019). ns: non-significant, $P > 0.05$.

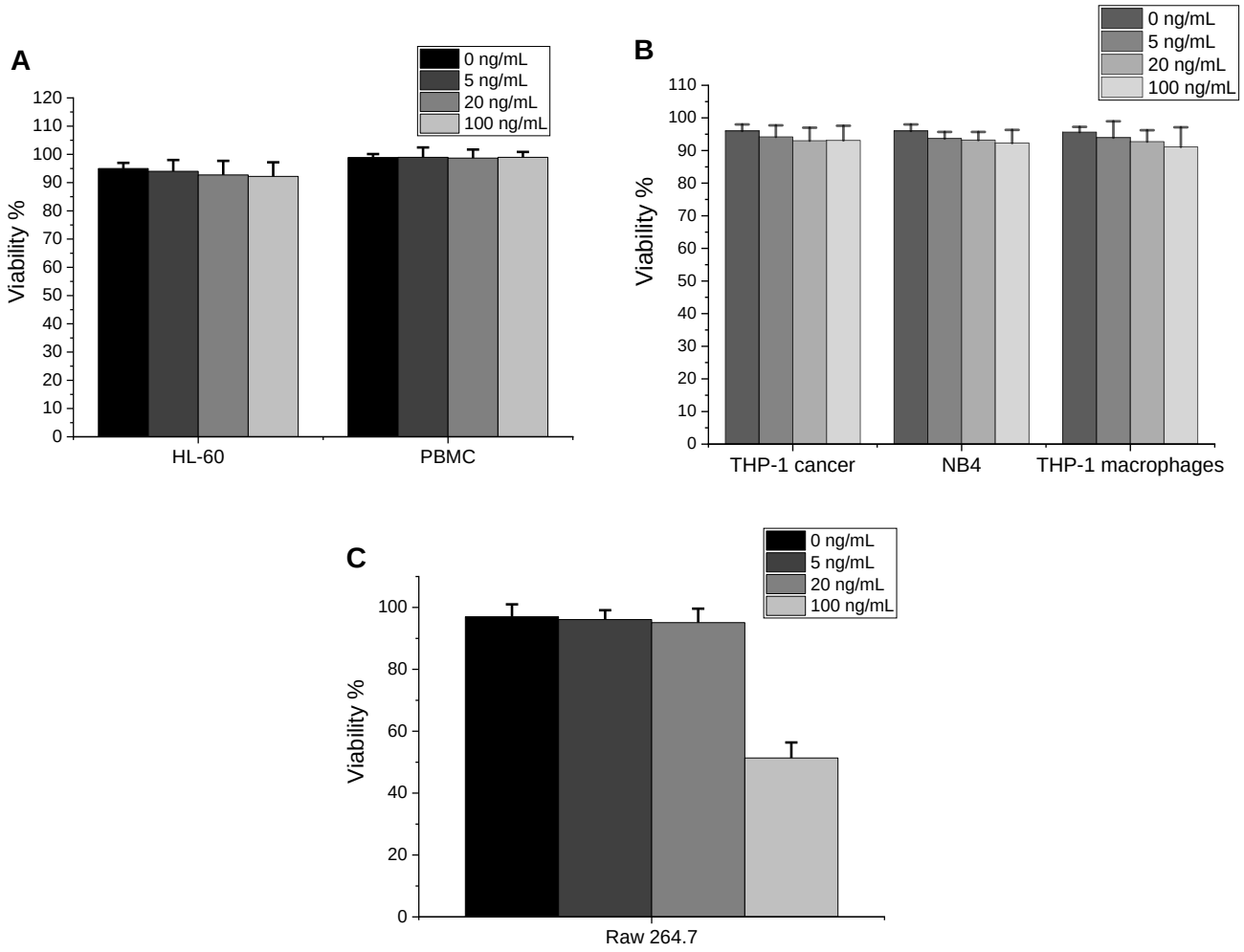


Figure S3. Viability of cancer and normal cells at different concentrations of LPS before co-culturing. Viability of HL-60 and PBMC cells **A**; THP-1 cancer, NB4, and THP-1 macrophages **B**; Raw 264.7 **C**. Cells were treated with 0, 5, 20, and 100 ng/mL of LPS and incubated overnight before flow cytometric analysis.

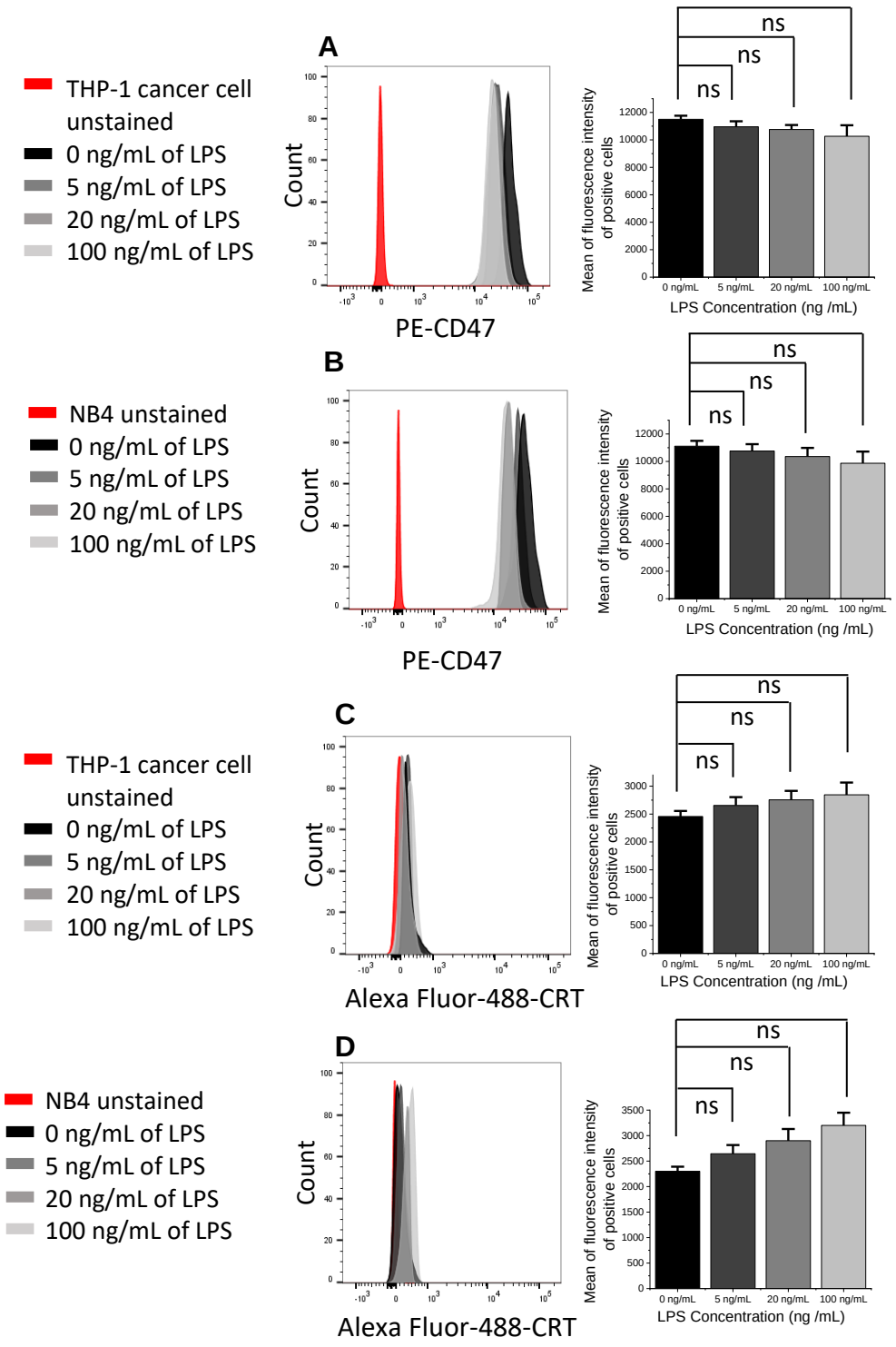
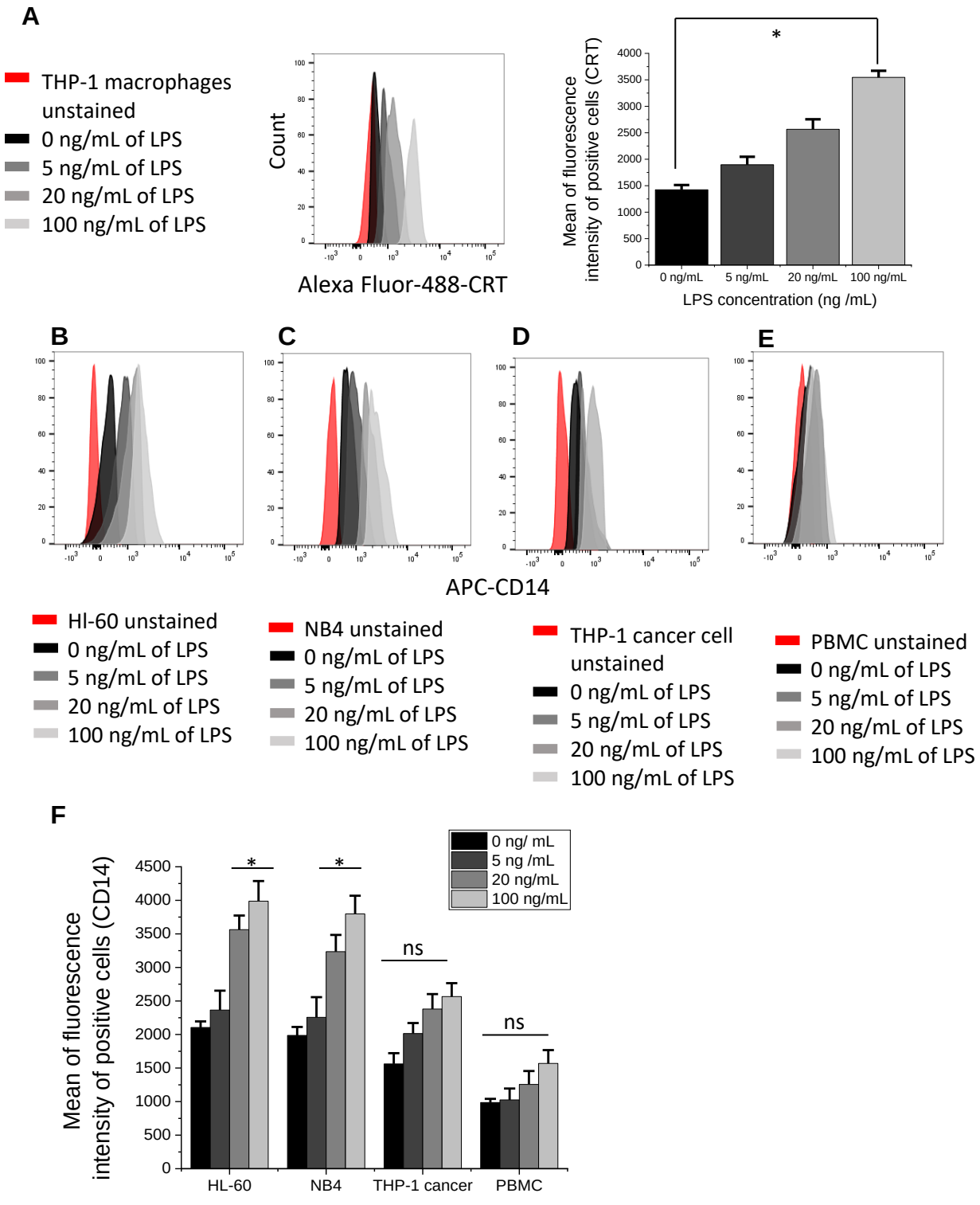


Figure S4. Levels of CD47 and CRT on THP-1 and NB4 cancer cells without co-culture and after treatment of LPS. Flow cytometric analysis of CD47 in THP-1 cancer cells **A** and NB4 **B**; analysis of CRT in THP-1 cancer cells **C** and NB4 cells **D**, when cultured alone without THP-1 macrophages and treated with 0, 5, 20, and 100 ng/mL of LPS. Values in the graphs are shown as means ± SEM of three trials of duplicate samples each. The statistical significance was determined by one way ANOVA (OriginPro 2019). ns: non-significant, $P > 0.05$.


A
THP-1 macrophages unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS
Count
Alexa Fluor-488-CRT
Mean of fluorescence intensity of positive cells (CRT)
*
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
LPS concentration (ng /mL)
B
C
D
E
APC-CD14
Hl-60 unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS
NB4 unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS
THP-1 cancer cell unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS
PBMC unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS
F
0 ng/ mL
5 ng /mL
20 ng/mL
100 ng/mL
Mean of fluorescence intensity of positive cells (CD14)
*
*
ns
ns
HL-60
NB4
THP-1 cancer
PBMC

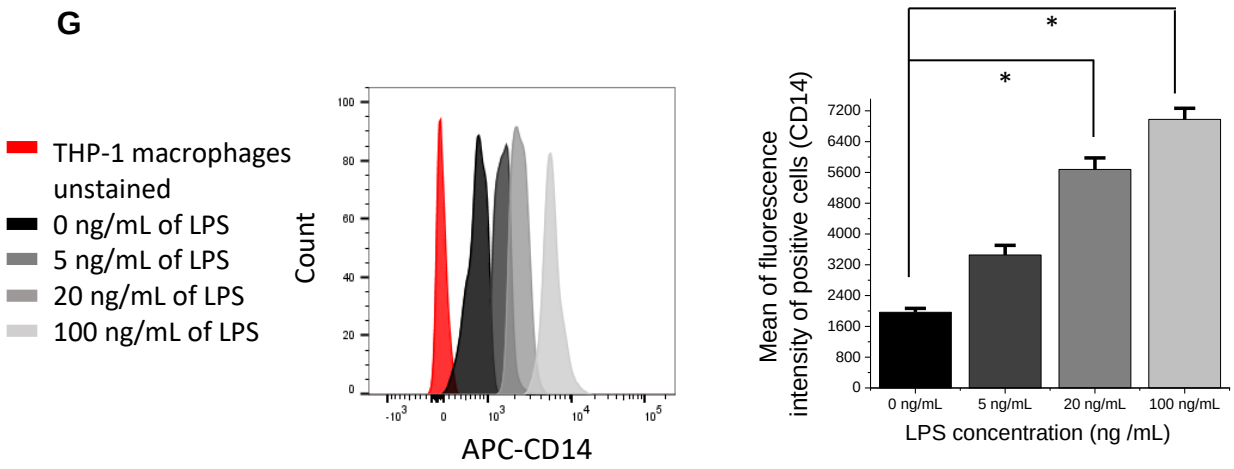
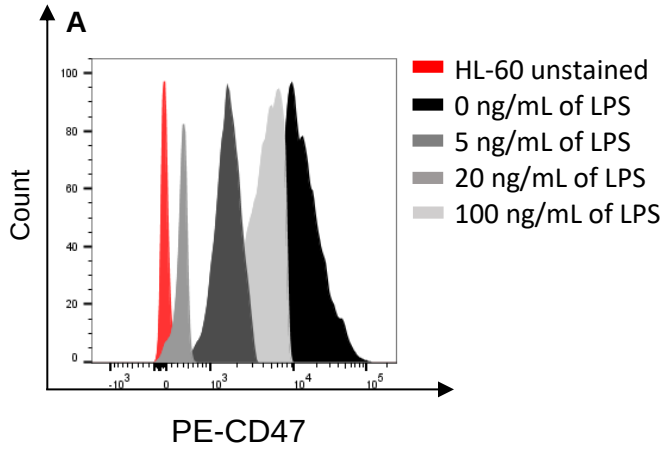
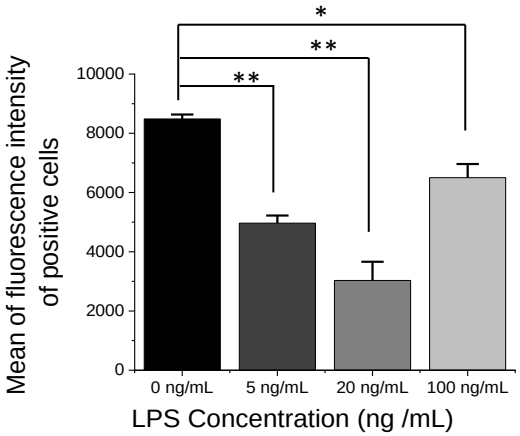
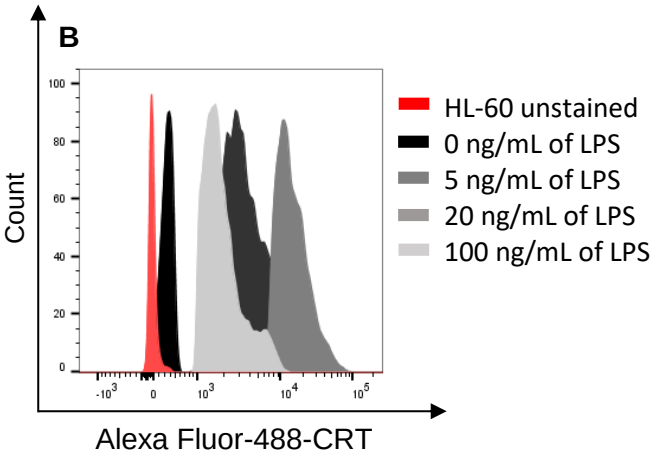
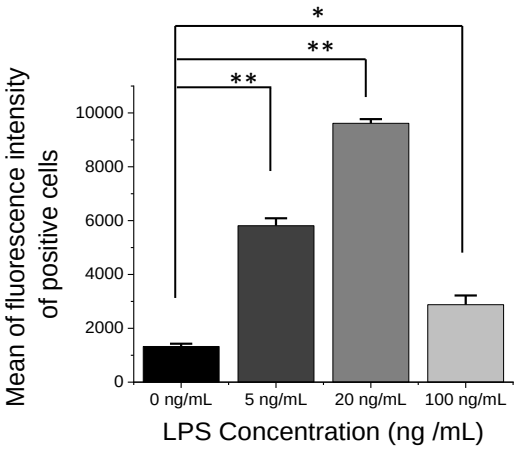


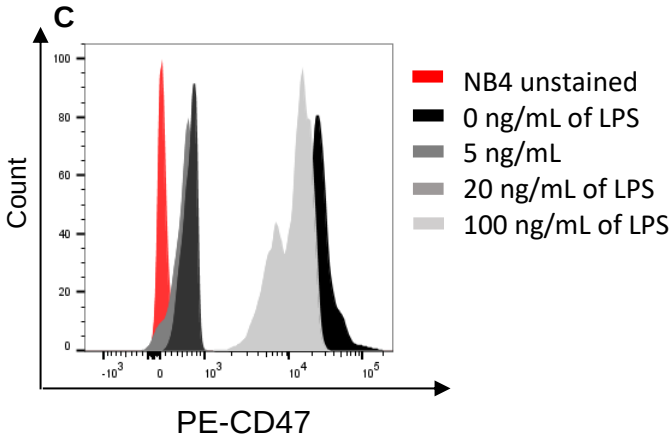
Figure S5. Levels of CRT on THP-1 macrophages and CD14 on leukemic, normal and THP-1 macrophage cells after treatment with different concentrations of LPS when cultured alone. **A**, CRT levels on THP-1 macrophages after treatment of 0, 5, 20, and 100 ng/mL of LPS in the absence of co-culture. **B-D**, flow cytometric analysis of CD14 level on HL-60 **B**, NB4 **C**, and THP-1 cancer cells **D**, and PBMC normal cells **E**, after treatment with 0, 5, 20, 100 ng/mL of LPS. **F**, a graph representing the mean of fluorescence intensity of CD14 levels on cancer and normal cells. **G,** CD14 levels on THP-1 macrophages after treatment with 0, 5, 20, 100 ng/mL of LPS when cultured alone. Values are shown as means ± SEM of three trials of duplicate samples each. The statistical significance between the different LPS concentrations in each group was determined by one way ANOVA (OriginPro 2019), * $P<0.05$, ns: non-significant

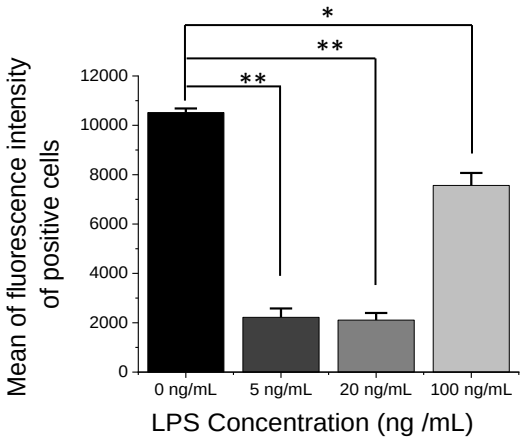

A
Count
PE-CD47
HL-60 unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS

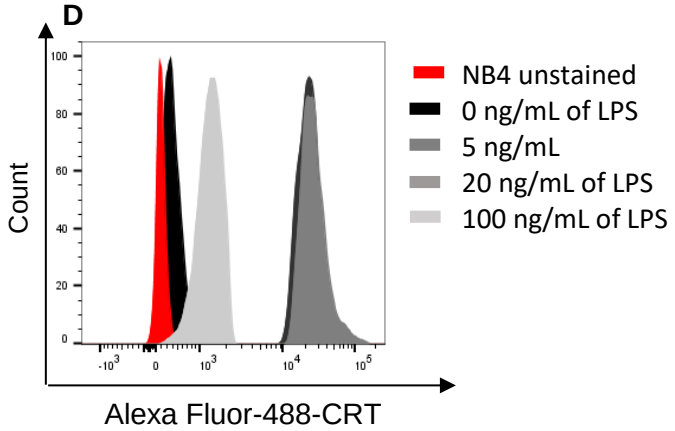

Mean of fluorescence intensity of positive cells
LPS Concentration (ng /mL)
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
*
**
**

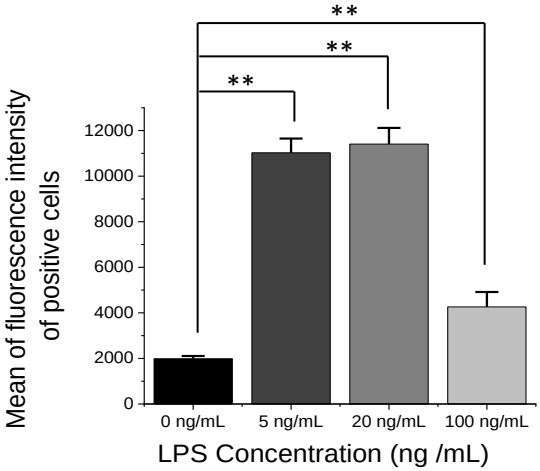

B
Count
Alexa Fluor-488-CRT
HL-60 unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS

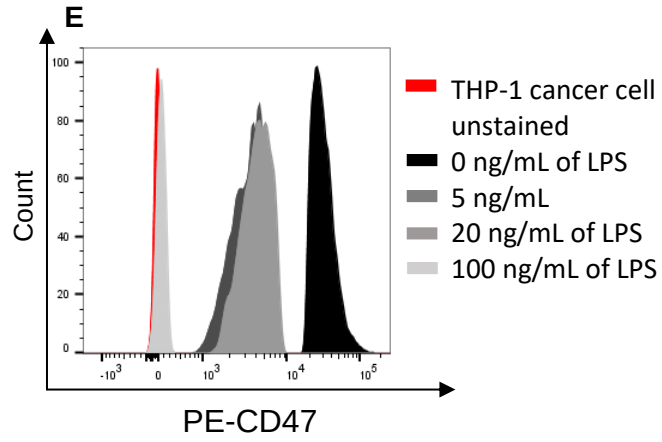

Mean of fluorescence intensity of positive cells
LPS Concentration (ng /mL)
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
*
**
**

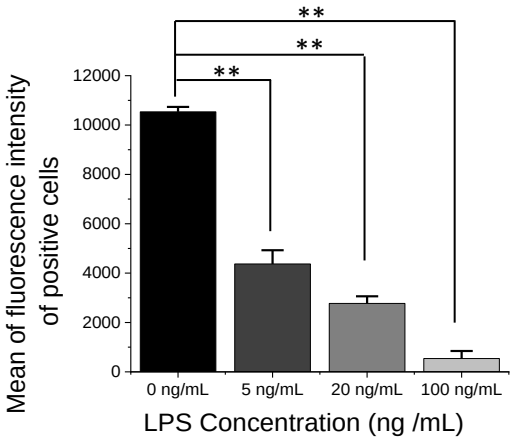

C
Count
PE-CD47
NB4 unstained
0 ng/mL of LPS
5 ng/mL
20 ng/mL of LPS
100 ng/mL of LPS

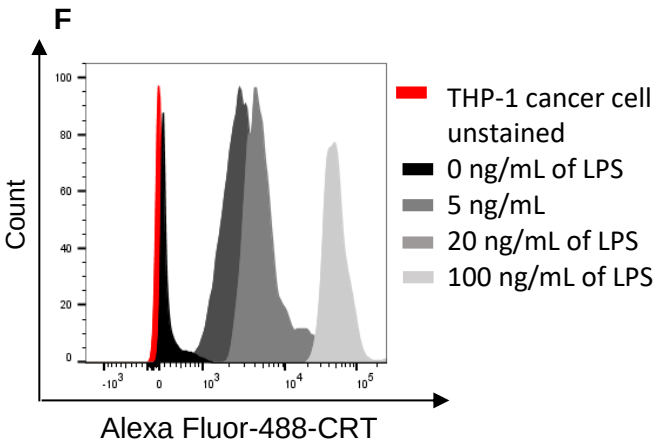

Mean of fluorescence intensity of positive cells
LPS Concentration (ng /mL)
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
*
**
**

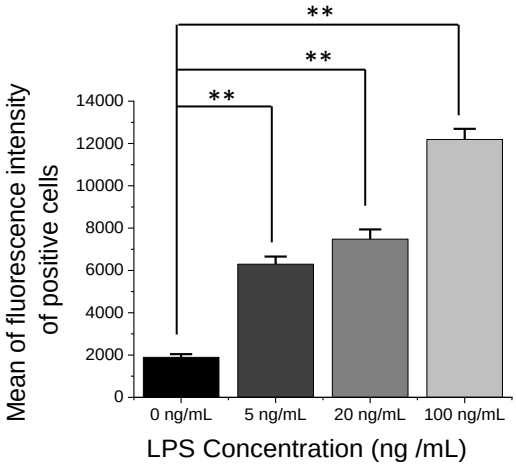

D
Count
Alexa Fluor-488-CRT
NB4 unstained
0 ng/mL of LPS
5 ng/mL
20 ng/mL of LPS
100 ng/mL of LPS

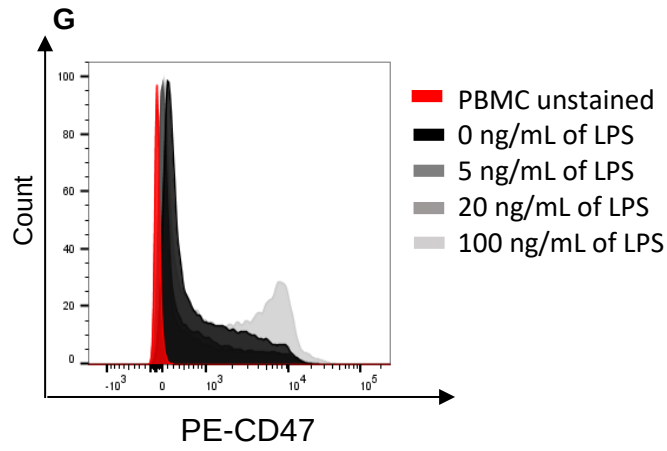

Mean of fluorescence intensity of positive cells
LPS Concentration (ng /mL)
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
**
**
**

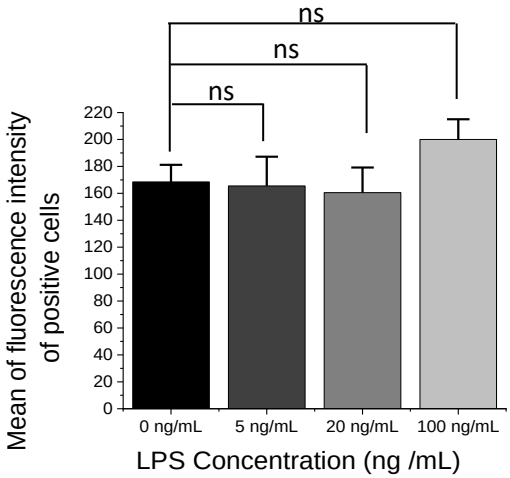

E
Count
PE-CD47
THP-1 cancer cell unstained
0 ng/mL of LPS
5 ng/mL
20 ng/mL of LPS
100 ng/mL of LPS

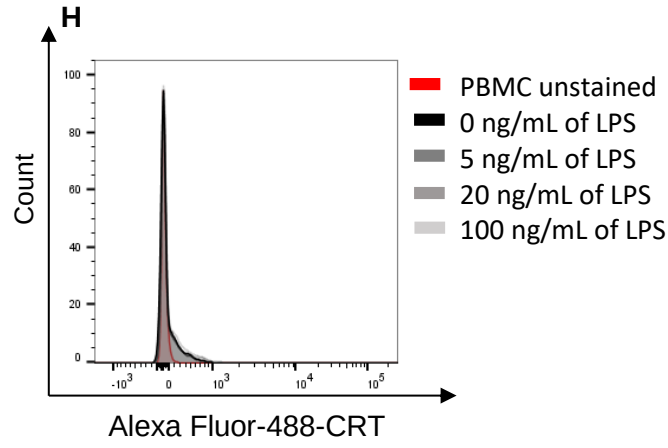

**
**
**
Mean of fluorescence intensity of positive cells
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
LPS Concentration (ng /mL)


F
Count
Alexa Fluor-488-CRT
THP-1 cancer cell unstained
0 ng/mL of LPS
5 ng/mL
20 ng/mL of LPS
100 ng/mL of LPS


**
**
**
Mean of fluorescence intensity of positive cells
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
LPS Concentration (ng /mL)


G
Count
PE-CD47
PBMC unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS


ns
ns
ns
Mean of fluorescence intensity of positive cells
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
LPS Concentration (ng /mL)


H
Count
Alexa Fluor-488-CRT
PBMC unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS

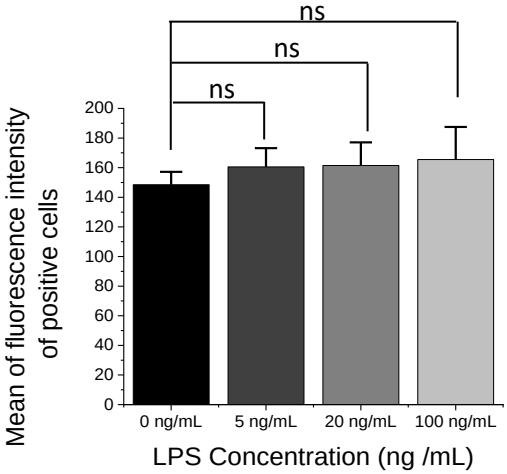
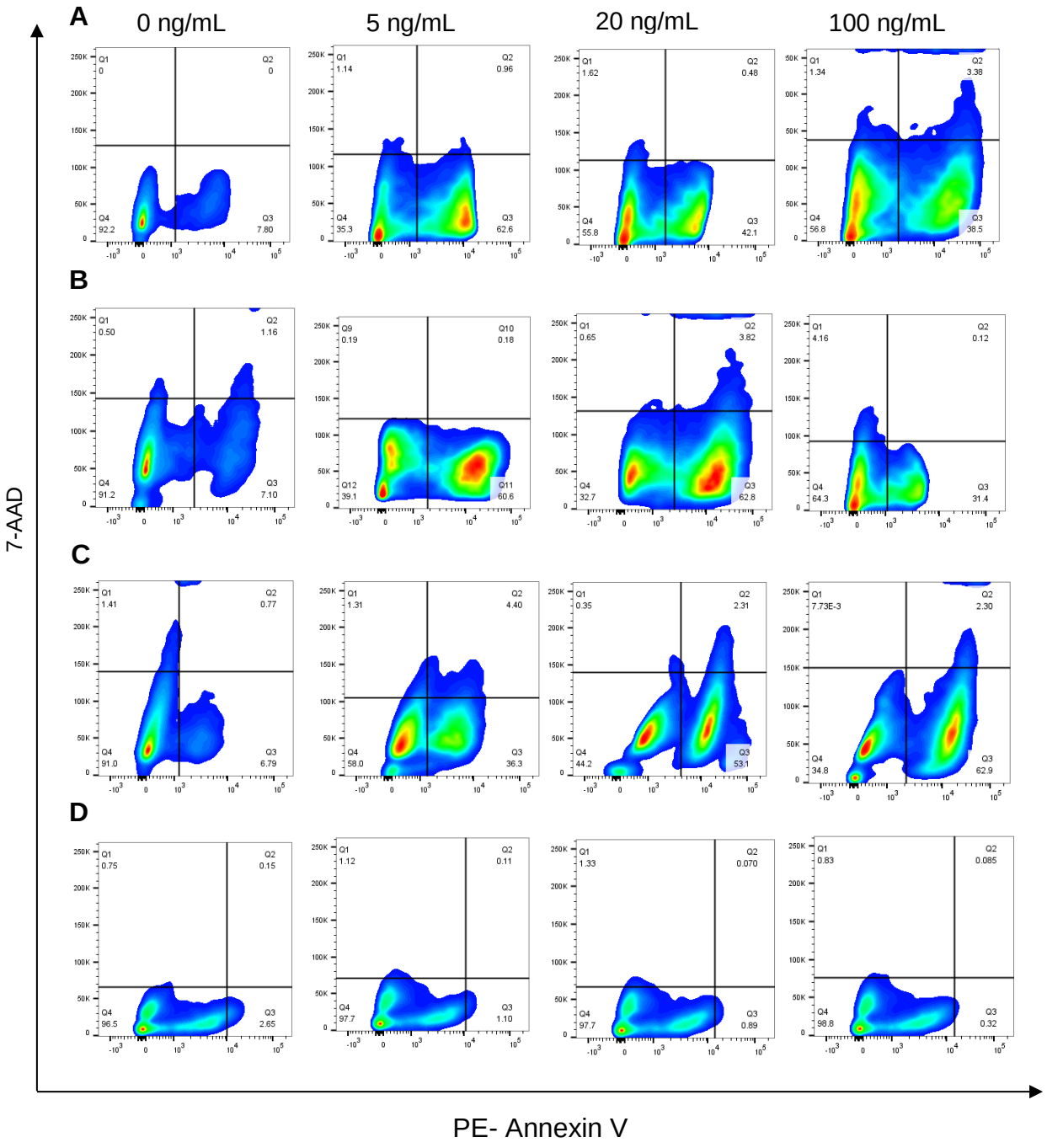

ns
ns
ns
Mean of fluorescence intensity of positive cells
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
LPS Concentration (ng /mL)

Figure S6. CD47 and CRT expression levels in cancer and normal cells after co-culturing with LPS activated Raw 264.7. Flow cytometric analysis of CD47 in HL-60 (**A**), NB4 (**C**), THP-1 (**E**), and PBMC (**G**) after co-culturing with Raw 264.7 murine macrophages; analysis of CRT in HL-60 (**B**), NB4 (**D**), THP-1 (**F**), and PBMC (**H**) after co-culturing with Raw 264.7 murine macrophages. The expression levels of CD47 and CRT in all cells mentioned above were assessed by incubating PE-conjugated anti human CD47 and Alexa Fluor® 488 conjugated anti human CRT with cancer cells and normal cells for 30 min at room temperature. Representative graphs indicate the mean of fluorescence intensity of positive counts of cancer or normal cells at 0, 5, 20, and 100 ng/mL of LPS used to activate Raw 264.7, 24 hour prior to flow cytometry analysis. Values in the graphs are shown as means ± SEM of three trials of duplicate samples. The statistical significance was determined by independent two-sample t-test (OriginPro 2019). * $P < 0.05$, ** $P < 0.01$ ns: non-significant, $P > 0.05$.


A
0 ng/mL
5 ng/mL
20 ng/mL
100 ng/mL
B
C
D
7-AAD
PE- Annexin V

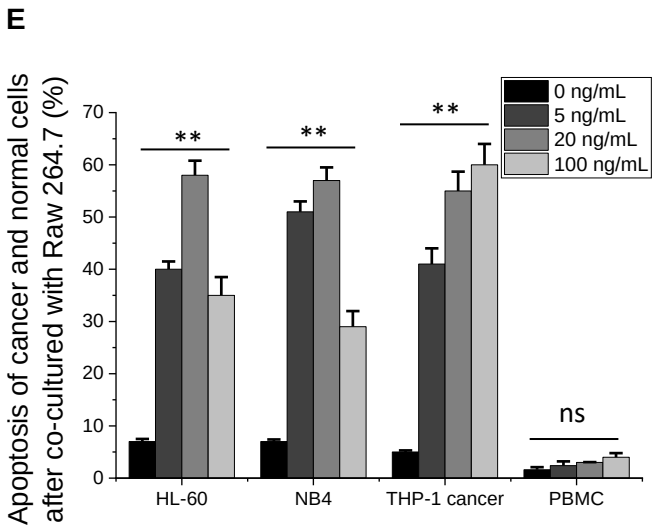
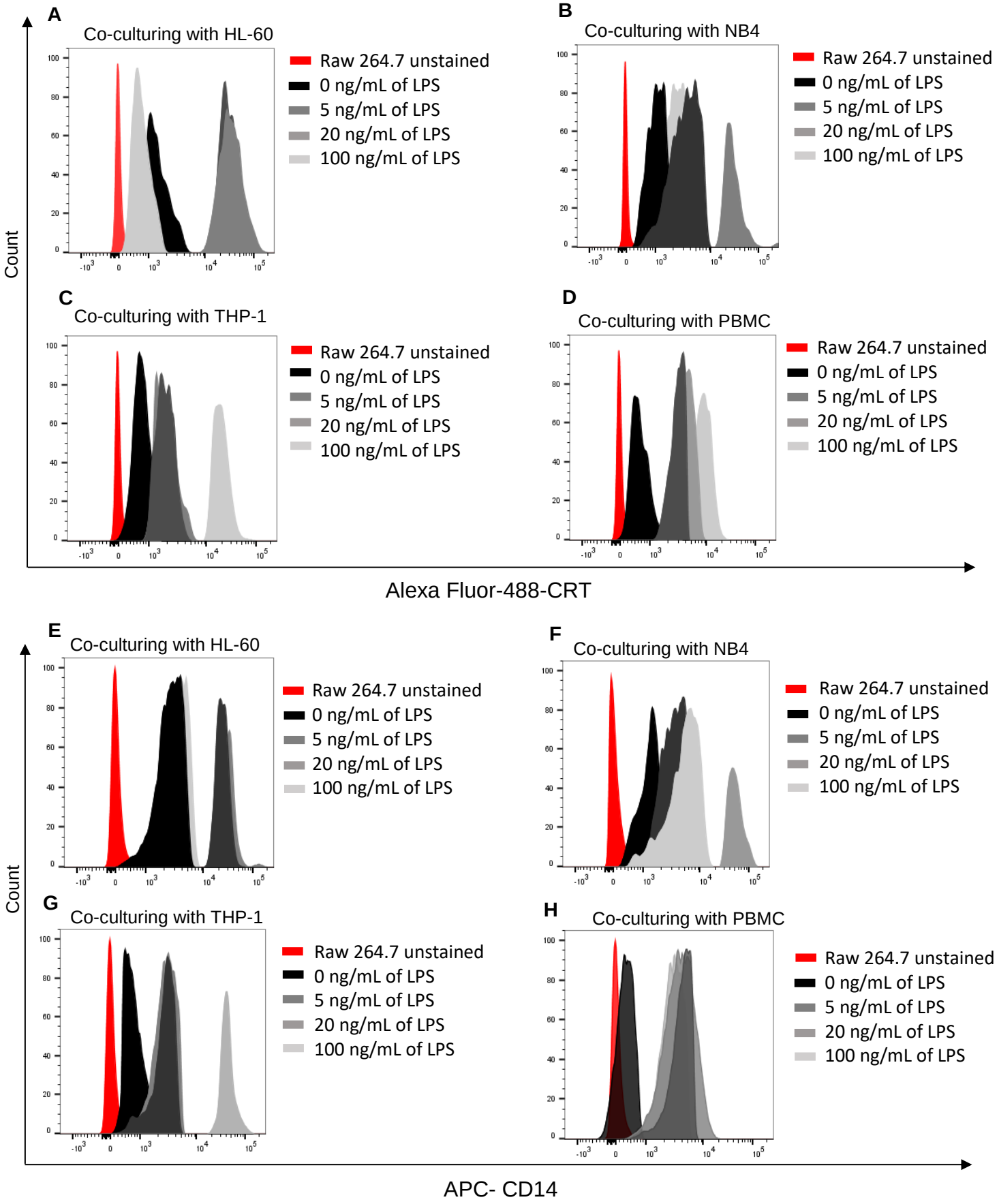


Figure S7. Elimination of cancer cells by LPS activated Raw 264.7. Side scatter plots showing apoptosis rate (PE- Annexin V) of all cancer and normal cells after co-culturing with activated Raw 264.7 at 0, 5, 20, and 100 ng/mL of LPS respectively: **A,** HL-60; **B,** NB4; **C,** THP-1 cancer cells; **D,** PBMC normal cells. **E,** The percentage (%) of apoptosis of cancer and normal cells after co-culturing with Raw 264.7. Values in the graph are shown as means ± SEM of three trials of duplicate samples. The statistical significance was determined by one way ANOVA (OriginPro 2019). ** $P < 0.01$, ns: non-significant, $P > 0.05$. The side scatter plots shown here are one representative out of three trials.


A
Co-culturing with HL-60
B
Co-culturing with NB4
C
Co-culturing with THP-1
D
Co-culturing with PBMC
Raw 264.7 unstained
0 ng/mL of LPS
5 ng/mL of LPS
20 ng/mL of LPS
100 ng/mL of LPS
Count
Alexa Fluor-488-CRT
E
Co-culturing with HL-60
F
Co-culturing with NB4
G
Co-culturing with THP-1
H
Co-culturing with PBMC
Count
APC- CD14

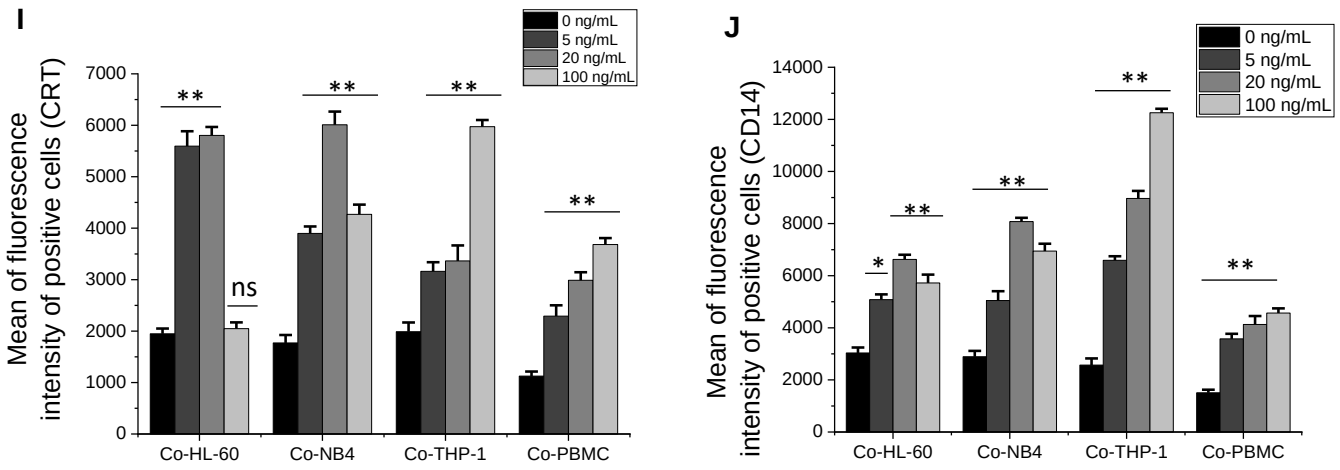
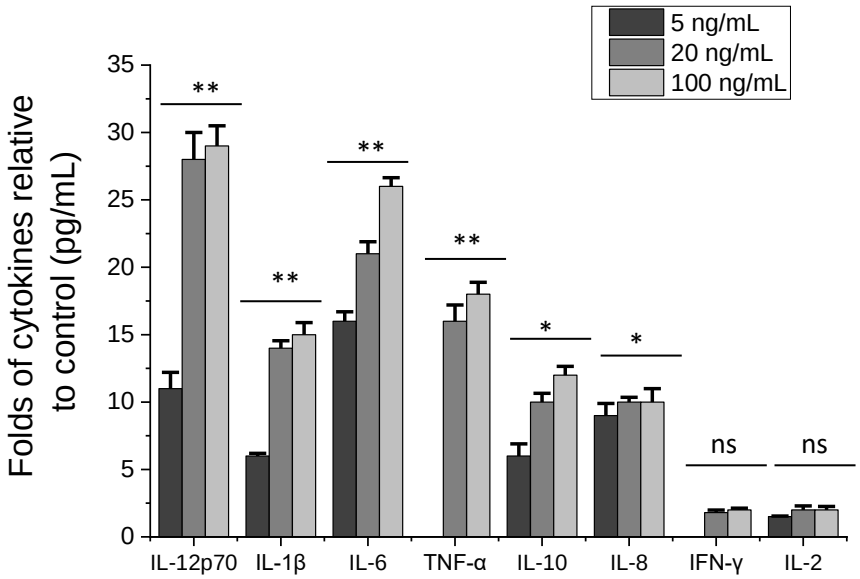


Figure S8. Increased levels of CRT in LPS-activated Raw 264.7 co-culturing with leukemic cell lines. Flow cytometric histograms showing CRT (**A-D**) and CD14 (E-H) levels in Raw 264.7 when activated with 0, 5, 20, 100 ng/mL of LPS and co-culturing with HL-60 (**A,E**), NB4 (**B,F**), THP-1 cancer cells (**C,G**) and PBMC normal cells (**D,H**). The expression levels of CRT were assessed by incubating Alexa Fluor® 488 conjugated anti human CRT and APC conjugated anti human CD14 with Raw 264.7 after co-culturing with each cell line mentioned above for 30 min at room temperature. Graphs representing CRT (**I**) and CD14 (**J**) values in Raw 264.7 after co-culturing with HL-60, NB4, THP-1 cancer cells, and PBMC normal cells. Values are shown as means ± SEM of three trials of duplicate samples each. The statistical significance was determined by one way ANOVA (OriginPro 2019).* $P < 0.05$, ** $P < 0.01$, ns: non-significant, $P > 0.05$.

A



B

	IL-12p70			IL-1β			IL-6			TNF-α			IL-10			IL-8			IFN-γ			IL-2		
LPS conc. ng/mL	5	20	100	5	20	100	5	20	100	5	20	100	5	20	100	5	20	100	5	20	100	5	20	100
Raw 264.7	++	+++ +	+++ +	+	++	++	++	+++	+++ +	++	+++	+++	+	++	++	+	++	++	-	-	+	-	+	+

Figure S9. Increased levels of M1 macrophage polarization markers after LPS activation of Raw 264.7. **A,** Levels of cytokines (pg/mL) productions relative to the control (0 ng/mL of LPS) of Raw 264.7 after activation of LPS at 0, 5, 20, and 100 ng/mL of LPS. **B,** The multiples of change of cytokines levels in Raw 264.7 relative to the control. The amount of cytokines secreted by macrophages (pg/mL) is presented by multiples here, and was calculated based on the amount secreted at each LPS concentration (ng/mL) relative to the amount secreted without activation (control, 0 ng/mL) of LPS. A cut off of 2 folds was determined to be the threshold and was determined according to the amount secreted relative to the control. −<2, + >2–9, ++ >10–17, +++>18–25, ++++>26–33. Values in the graphs are shown as means ± SEM of three trials of duplicate samples. The statistical significance was determined by one way ANOVA (OriginPro 2019).* $p < 0.05$, ** $P < 0.01$, ns: non-significant, $P > 0.05$.