SUPPLEMENTAL DATA

INTERLEUKIN-10 FACILITATES BOTH CHOLESTEROL UPTAKE AND EFFLUX IN MACROPHAGES

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Files contained herein:

SI Figure 1, 2 and 3

SI Materials and Methods

SI FIGURE LEGENDS

SI Fig.1. The induction of apoAI-mediated cholesterol efflux from macrophages by PPARγ agonists. [^{3}H]cholesterol-loaded Raw264.7 cells were treated with GW9662 (10 μM), IL10 (10 ng/ml), PGJ2 (5 μM), rosiglitazone (5 μM) or vehicle (Ctl) and subsequently incubated with medium with or without apoAI (20 μg/ml). ApoAI-induced [^{3}H]cholesterol efflux was measured as described. Results are the mean ± SEM of 3 independent experiments. Statistically significant difference from control are indicated (**, $p<0.01$; *, $P<0.05$).

SI Fig. 2. PPARγ agonists induce CD36 expression. (A) Raw264.7 cells were incubated with either medium alone, PGJ2, GW9662, IL10, or GW9662 plus IL10 (GW9662 was administrated 15 min before IL10 treatment) for 24hr. The results are representative of 3 independent experiments and values are expressed as fold change in abundance (± SEM). (B) Raw264.7 cells were treated for 24 h with either medium alone, 15d-PGJ2 (5 μM), rosiglitazone (5 μM) or IL10 (10 ng/ml) in the presence of AcLDL (50 μg/ml) as indicated. Real-time PCR analyses for CD36 mRNA levels in macrophages were conducted as described in Methods. The results are representative of 3 independent experiments and values are expressed as fold change in abundance (± SEM).

SI. Fig. 3. IL10 inhibits apoptosis in lipid-laden cells. Resting macrophages (Raw264.7 cells) were treated with or without IL10 for 24 hr in the presence of AcLDL (A) before immunocytochemistry for cleaved caspase 3 assay. In addition, lipid-laden Raw264.7

cells obtained by incubating cells with AcLDL for 36 hr were treated with medium for 24 h in the presence or absence of IL10 before apoptosis analysis (B). Expression of cleaved caspase-3 was determined as described in "Methods". Cells were counterstained with Hoechst 33258 for detection of nuclei and the expression of cleaved caspase-3 was observed under a fluorescence microscope.

EXPERIMENTAL PROCEDURES

Antibodies and reagents: Recombinant mouse interleukin-10, lipoprotein deficient serum (human), and ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Cat # S7101) were purchased from Chemicon International Inc (Temecula, CA). Acetylated low density lipoprotein (AcLDL), Acetylated low density lipoprotein labeled with 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-AcLDL), and apolipoprotein AI (apoAI) were purchased from Biomedical Technologies Inc. (Stoughton, MA). Rabbit anti-human CD36 (Cat# 100011), rosiglitazone and Cholesterol Assay Kit (Cat#10007640) were obtained from Cayman Chemical (Ann Arbor, MI). Rabbit polyclonal anti-ABCA1 (Cat# NB400-105) was from Novus Biologicals, Inc. (Littleton, CO). Rat anti-mouse CD204 (scavenger receptor type I/II) monoclonal antibody was from AbD Serotec **(**Raleigh, NC). Rabbit Anti-actin (I-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western Lightning Chemiluminescence Reagent Plus (Cat # NEL104) was purchased from PerkinElmer Life Science (Boston, MA). Restore Western blot stripping buffer was from PIERCE (Rockford, IL). Superscript III reverse transcriptase, NuPAGE Novex 4-12% bis-Tris Gel, RNase Out recombnant ribonuclease inhibitor, Alexa Fluor546-conjugated

secondary antibody, and Bodipy 493/503 (Cat# D-3922) were purchased from Invitrogen (San Diego, CA). 15-Deoxy-Delta 12,14-prostaglandin J2 (15-d-PGJ2) was obtained from Biomol (Plymouth Meeting, PA). 1α,2α[N]-^{3}H-cholesterol (Cat # C8794), albumin from bovine serum (fatty acid free)(FAFB), PPARγ inhibitor GW9662 were purchased from Sigma-Aldrich (St. Louis, MO). Cleaved Caspase-3 (Asp175) antibody (Cat # 9661) was from Cell Signaling Technology (Danvers, MA). MSR $^{-/-}$ mice were purchased from Charles River Laboratories and CD36$^{-/-}$ mice were generously provided by Dr. K. J. Moore at the Massachusetts General Hospital. Mouse PPARγ $^{flox/flox}$ macrophages were kindly provided by Dr. C. H. Lee in Harvard School of Public Health.

Binding and uptake of AcLDL: To determine receptor-specific binding and uptake, fluorescence-labeled acetylated LDL (Dil-AcLDL) was used as described (1). Cells seeded on chamber slide (Nalge Nunc International) were treated with 10 ng/ml of IL10 for indicated time followed by incubation of Dil-AcLDL at 10 ug/ml in medium for 2 hr at 37°C. The media containing Dil-AcLDL was removed from culture and the cells were washed twice with probe-free media. The cells were visualized using standard rhodamine excitation:emission filter set. Fluorescence intensity was photographed using Nikon C1 Confocal System and quantified using Image J software.

Analysis of cellular cholesteryl ester contents and lipid accumulation: Macrophages were treated with or without 10 ng/mL IL10 for 8 hr or 24 hr. The cellular cholesterol content was determined by the enzymatic, fluorometric method, using the Cholesterol Assay Kit from Cayman Chemical. Briefly, the cellular lipids were extracted with hexane/isopropanol (3/2, v/v), dried under a nitrogen flush, and then dissolved in

isopropanol. For the determination of free cholesterol, the supernatant was added to enzyme mixtures containing cholesterol oxidase (2 U/mL). For the evaluation of total cholesterol, the supernatant was added to enzyme mixtures containing both cholesterol oxidase (2 U/mL) and cholesterol esterase (0.2 U/mL). The reaction mixtures for measuring free cholesterol and those for measuring total cholesterol were incubated at 37°C for 2 hours or 24 hours, respectively, followed by the addition of sodium hydroxide to terminate the reaction. Fluorescence intensity was measured using excitation at 540 nm and emission at 590 nm. The mass of cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol. After lipid extraction, the cellular protein was dissolved in sodium hydroxide, and the protein concentration was determined.

Flow cytometry: To detect the role of IL10 in lipid accumulation and foam cell formation, immunofluorescence flow cytometric analysis was performed using BODIPY 493/503 (Cat# D-3922) as described (2). In foam cell model 1, macrophages were treated with medium containing AcLDL (50 μg/ml) in the presence or absence of 10 ng/mL IL10 for 8 hr or 24 hr. In foam cell model 2, macrophages were laden with AcLDL (50 μg/ml) for 42 hr followed by IL10 treatment. The cells were washed with PBS and then scraped into 1 ml FACS buffer (PBS containing 0.1% BSA and 0.01% sodium azide). BODIPY stock (1 mg/ml in ethanol) was diluted to 10 μg/ml in PBS and was added directly to paraformadehyde-fixed cells. Cells were stained at room temperature for 20 minutes. After washing extensively, stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson). Data were analyzed with the Cell Quest program.

Accumulation of lipid droplets in macrophages and foam cell formation: For detecting lipid accumulation in macrophages, oil red O staining and BODIPY staining were performed as previously described (2,3). Macrophages were cultured in growth medium for incubation overnight on chamber slides (Nalge Nunc International). Cells were then treated as described above in "cell culture". For oil red O staining, neutral lipids were stained using 0.5% Oil-Red-O (Sigma) in isopropanol for 60 min. The Oil-Red-O-stained lipids were morphologically evaluated by microscopy. For BODIPY fluorescence staining of cholesterol ester, after incubation for indicated time, the cells were washed twice in ice-cold PBS, followed by paraformaldehyde fixation (2% in PBS) for 1hr at room temperature. The cells were stained with BODIPY working solution (10 μg/ml in PBS) for 2 hr at room temperature. The cells were rinsed twice with PBS and mounted on the slides. Confocal imaging was recorded in a LSM 510 META (Carl Zeiss Microimaging, Inc.) using an excitation wavelength of 488 nm and an emission wavelength from 505 to 635 nm.

RNA extraction and analysis: Sequence-specific PCR primers: SR-I (Forward: 5'TTA AAG GTG ATC GGG GAC AAA 3'; reverse: 5' CAA CCA GTC GAA CTG TCT TAA G 3'); SR-II (Forward: 5'TTA AAG GTG ATC GGG GAC AAA 3'; Reverse: 5'AGC TGA TCT TAA AAG GGT CTT G 3'); CD36 (Forward: 5'CAA GCT CCT TGG CAT GGT AGA 3'; Reverse: 5' TGG ATT TGC AAG CAC AAT ATG AA 3'); ICAM-1 (Forward: 5' TGG AAG CTG TTT GAG CTG AG 3'; Reverse: 5' CAC ACT CTC CGG AAACGA AT 3'); TNFα (Forward: 5' CTG TAG CCC ACG TCG TAG C 3'; Reverse: 5' GGT TGT CTT TGA GAT CCA TGC 3'); MMP-9 (Forward: 5' TTC TGG CAC ACG CCT TTC 3'; Reverse: 5' CCA TAG TAA GTG GGG ATC ACG 3'); ABCA1

(Forward: CCC AGA GCA AAA AGC GAC TC 3'; Reverse: 5' GGT CAT CAT CAC TTT GGT CCT TG 3'); GAPDH (Forward:5' GGC AAA TTC AAC GGC ACA GT 3' ; Reverse: 5' CGC TCC TGG AAG ATG GTG AT 3'); β-actin (Forward: 5'CTA AGG CCA ACC GTG AAA AG; Reverse: ACC AGA GGC ATA CAG GGA CA).

Western-blot analysis: Cells were lysed in SDS sample buffer and 40μg of protein run on NuPAGE Novex 4-12% bis-Tris Gel and transferred to PVDF membrane. Blots were blocked in 5% non-fat dry milk in TBS containing 1% Tween-20 (TBS-T), and incubated with primary antibody (rabbit anti-human CD36, rat anti-mouse CD204 and rabbit anti ABCA1) at 4^{o}C overnight. Blots were washed in TBS-T, incubated with appropriate HRP-conjugated secondary antibody (1:10,000 dilution) for 45 min and developed with Western Lightning Chemiluminescence Reagent Plus. The membranes were stripped with Restore Western blot stripping buffer and were reprobed with an anti-actin antibody and quantified by densitometry.

Cholesterol efflux: For apoAI-independent cholesterol efflux assay, efflux medium [DMEM/glutamine/0.2% FAFB, ± IL10 (10 ng/ml) with PGJ2 (5μM), or GW9662 (10 μM)] without apoAI was added in one set of wells. For apoAI-dependent cholesterol efflux assay, efflux medium mentioned above with 20 μg/ml of apoAI was added in another set of wells. After incubation for 2, 4, 6, 8 or 10 hr, 100 μl of media was removed and transferred to 1.5 ml Eppendorf tube. Cell debris was spun down at full speed for 5 min. The supernatant was gently transferred to 5 ml counting fluid to measure radioactivity as effluxed cholesterol. At the end of the experiment, 0.5 ml of 0.1 N NaOH was added to lyse the cells and incubated for 5 hr at room temperature. 100 μl of cell

lysis was transferred to 5 ml counting fluid to measure radioactivity. ApoAI-induced [^{3}H]cholesterol efflux was measured as the fraction of total radiolabeled cholesterol appearing in the medium in the presence of apoAI after subtraction of values for apoAI-free medium. ApoAI-mediated efflux was determined using the following formula: (1) % efflux = efflux/total labeled cholesterol (2) apoAI specific efflux= % efflux (with apoAI) - % efflux (without apoAI) (3) % increase in apoAI mediated efflux = %(apoAI specific efflux)/ % efflux (without apoAI).

TUNEL assay: Foam cells were obtained as described above. After culturing for 24 hr, DNA fragmentation in apoptotic cells was assessed by the TUNEL assay. To induce apoptosis, the cells were deprived of serum for 24 hours in the presence or absence of IL10 (10 ng/ml). Then TUNEL reactions were performed using ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Cat # S7101, Chemicon International), according to the manufacturer's instructions. Using a fluorescence microscope, TUNEL-positive macrophages were counted and apoptosis was evaluated for analysis.

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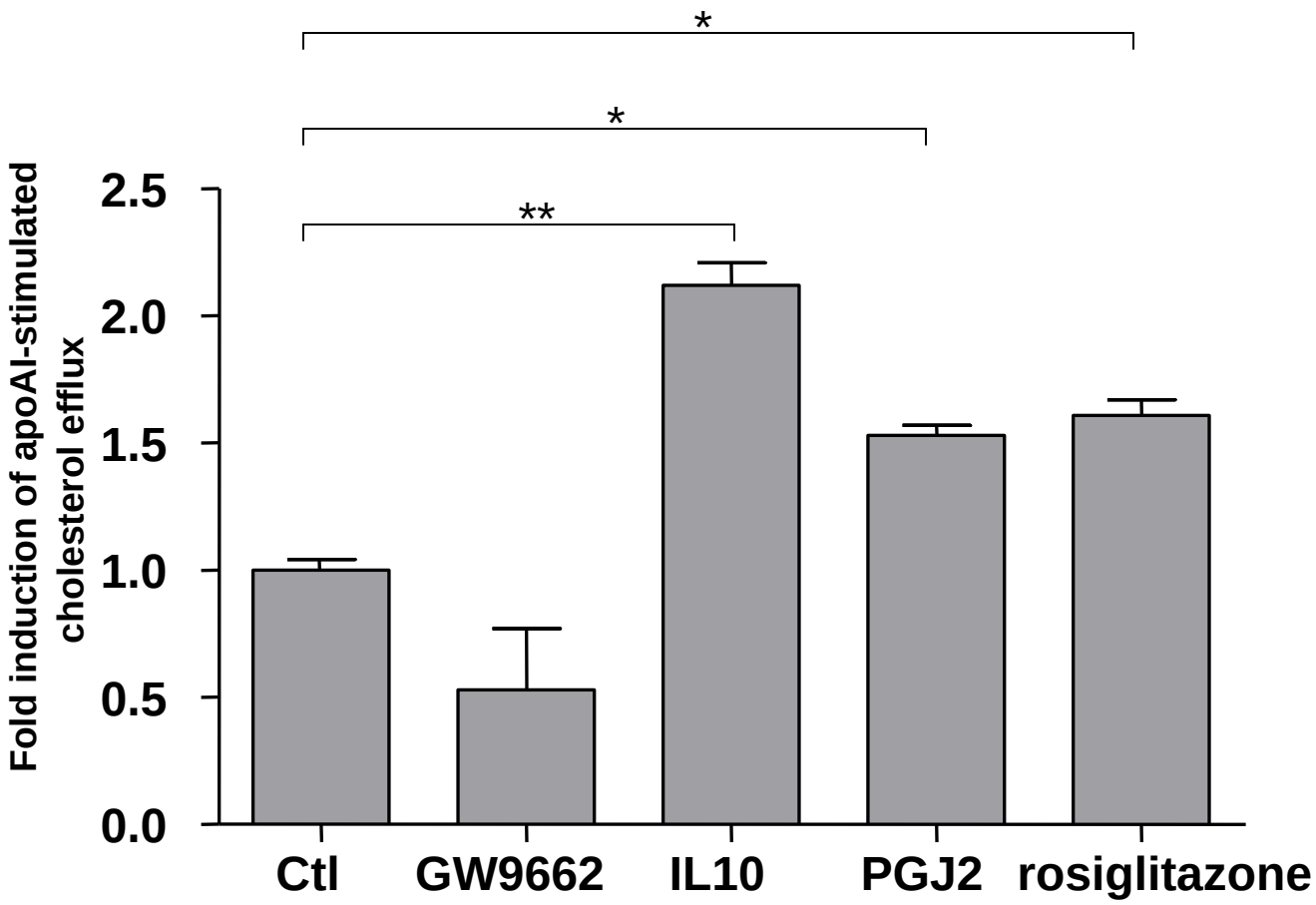
SI Figure 1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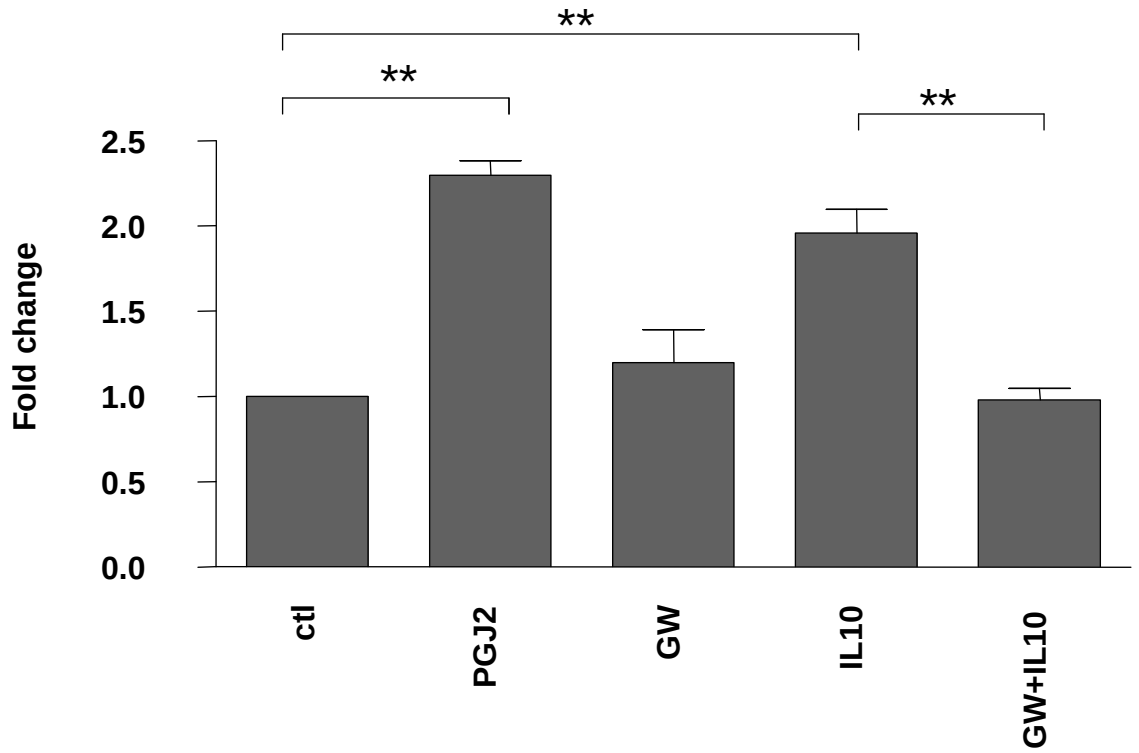
Fold induction of apoAI-stimulated cholesterol efflux
2.5
2.0
1.5
1.0
0.5
0.0
Ctl
GW9662
IL10
PGJ2
rosiglitazone

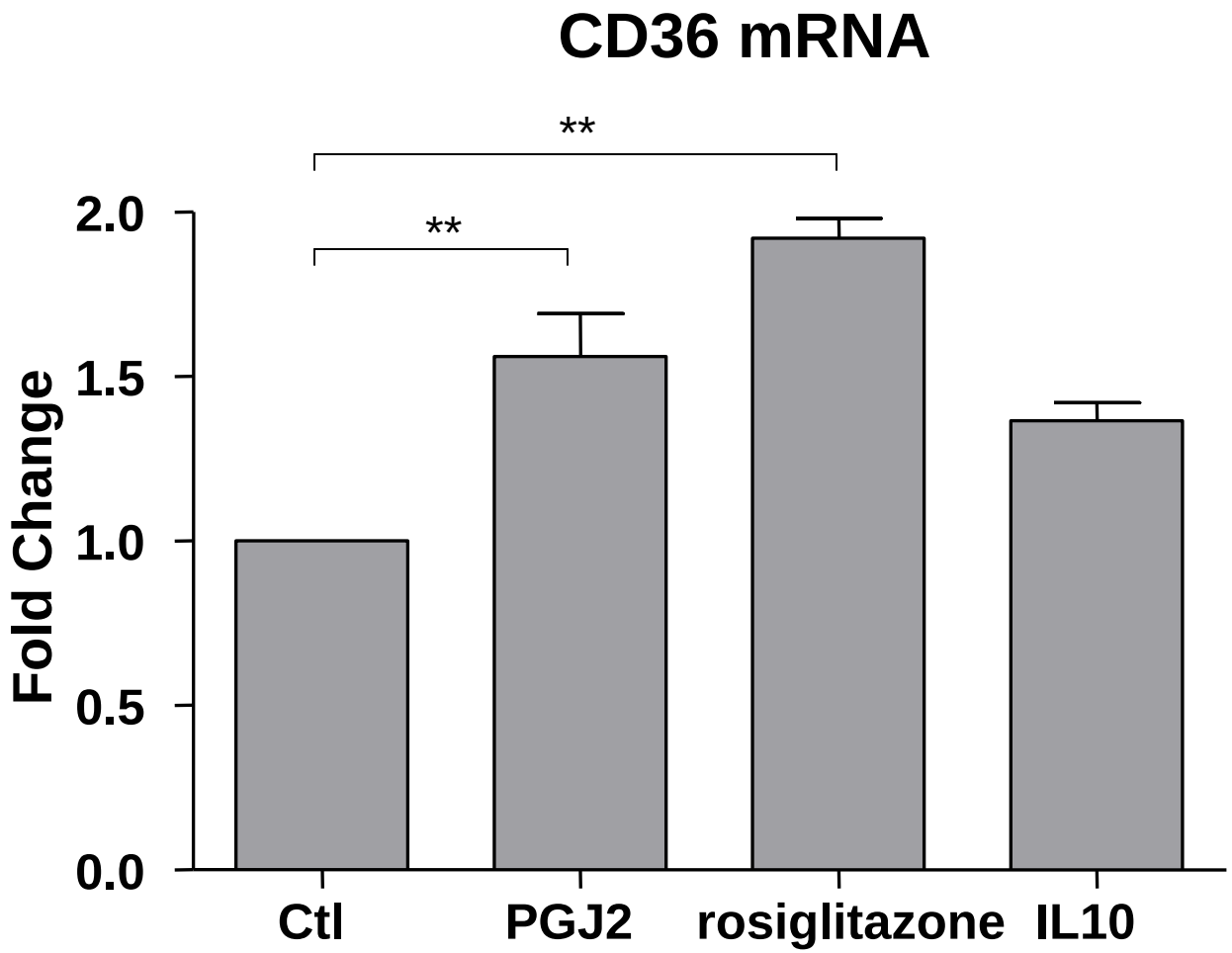
SI Figure 2

A



SI Figure 2

B


CD36 mRNA
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Fold Change
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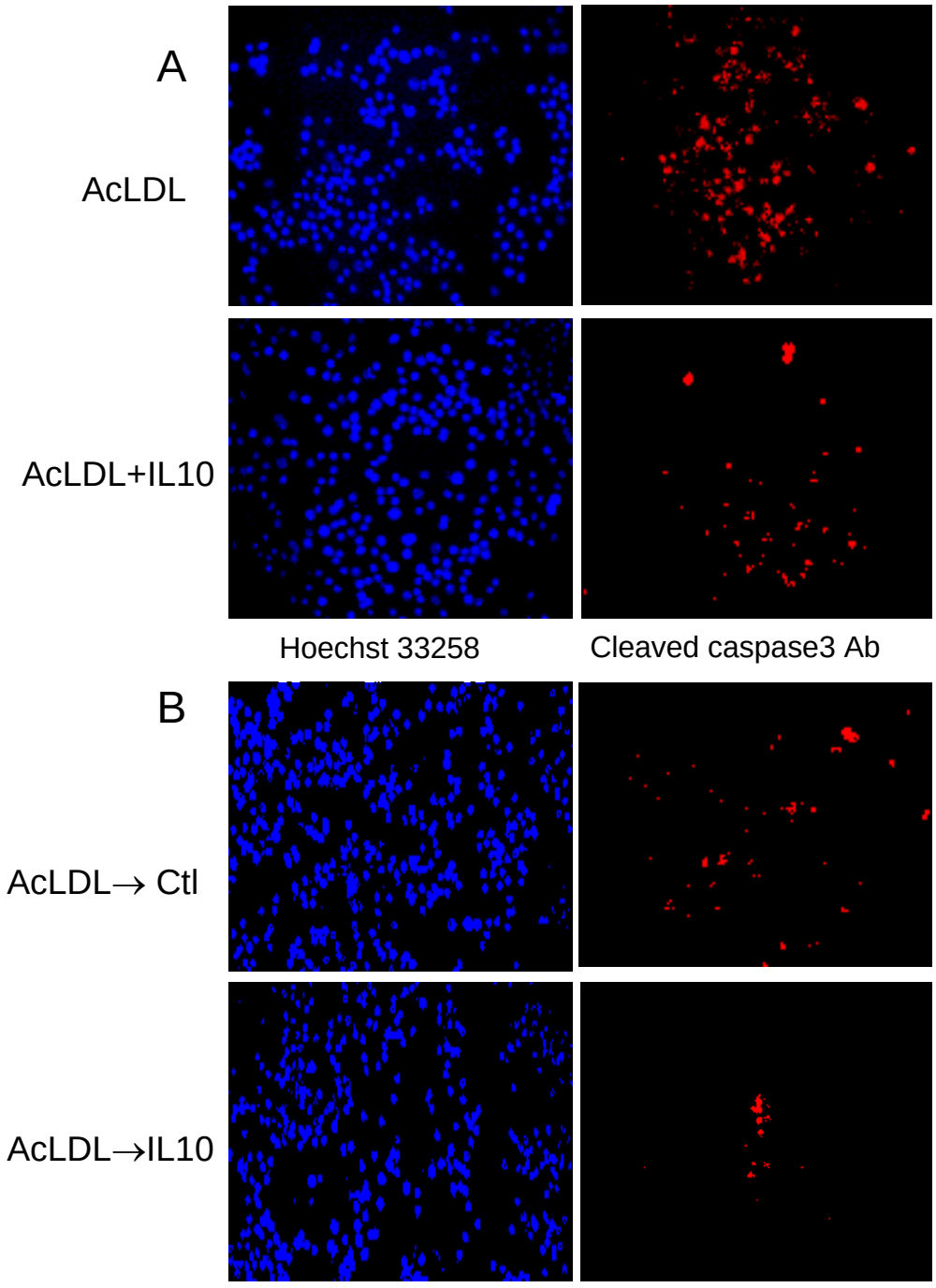
1.5
1.0
0.5
0.0
Ctl
PGJ2
rosiglitazone
IL10

SI Figure 3


A
AcLDL
AcLDL+IL10
Hoechst 33258
Cleaved caspase3 Ab
B
AcLDL→ Ctl
AcLDL→IL10