Electronic Supplementary Material (ESI) for Nanoscale.

Pristine Graphene Induces Innate Immune Training

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Generation of Murine Bone Marrow-Derived Dendritic Cells

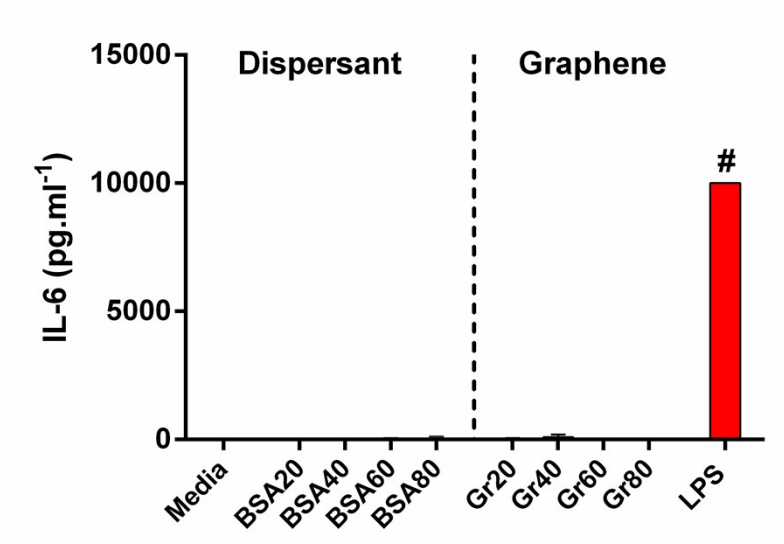
Murine bone marrow-derived immune cells were generated as described previously by our group.[1] Briefly, bone marrow cells were isolated from tibiae and femora of mice. Cells were grown in RPMI 1640 medium (Biosera) supplemented with 8% ultra-low endotoxin heat-inactivated foetal bovine serum (FBS) (Biosera), 2 mM L-glutamine (Gibco), 50 $U \cdot ml^{-1}$ penicillin (Gibco), 50 $\mu g \cdot ml^{-1}$ streptomycin (Gibco) and 20 $ng \cdot ml^{-1}$ of recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF) (Prepotech). On day 10, the loosely adherent cells were harvested and plated at a density of 0.625 x 10^6 $cells \cdot ml^{-1}$ and incubated with the appropriate stimuli as indicated in figure legends. Supernatants were collected and analyzed for cytokine secretion.

Fluorescence microscopy analysis

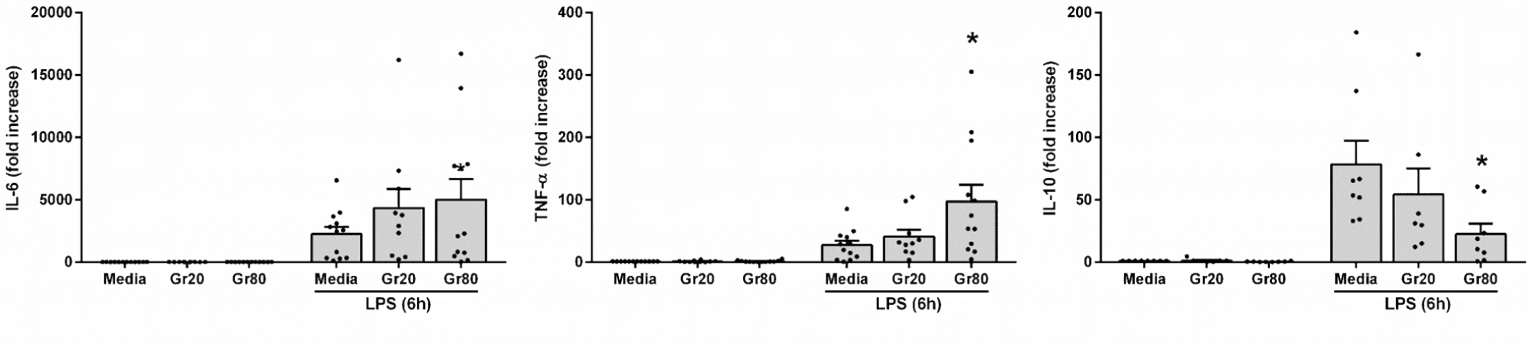
Day 6 BMDMs from wild-type C57BL/6 mice (1.2 × 10^6 cells/well) were plated on 12 wells plate and cultured overnight and on the second day, cells were incubated with graphene samples. After 24 h cells were washed with 1 mL warm PBS to remove the stimuli and incubated in complete DMEM supplemented with 15% of L929 cell line conditioned medium. The medium was renewed on day 3. On day 6, supernatants were discarded and the cells were washed three times with PBS. Afterwards, mitochondria were stained with MitoTracker green (200 nM) and Hoescht for nuclear staining, for 40 min. Cells were washed in PBS and analyzed on an Olympus BX51 Fluorescence Microscope with LSM 5 software.

RNA Analysis

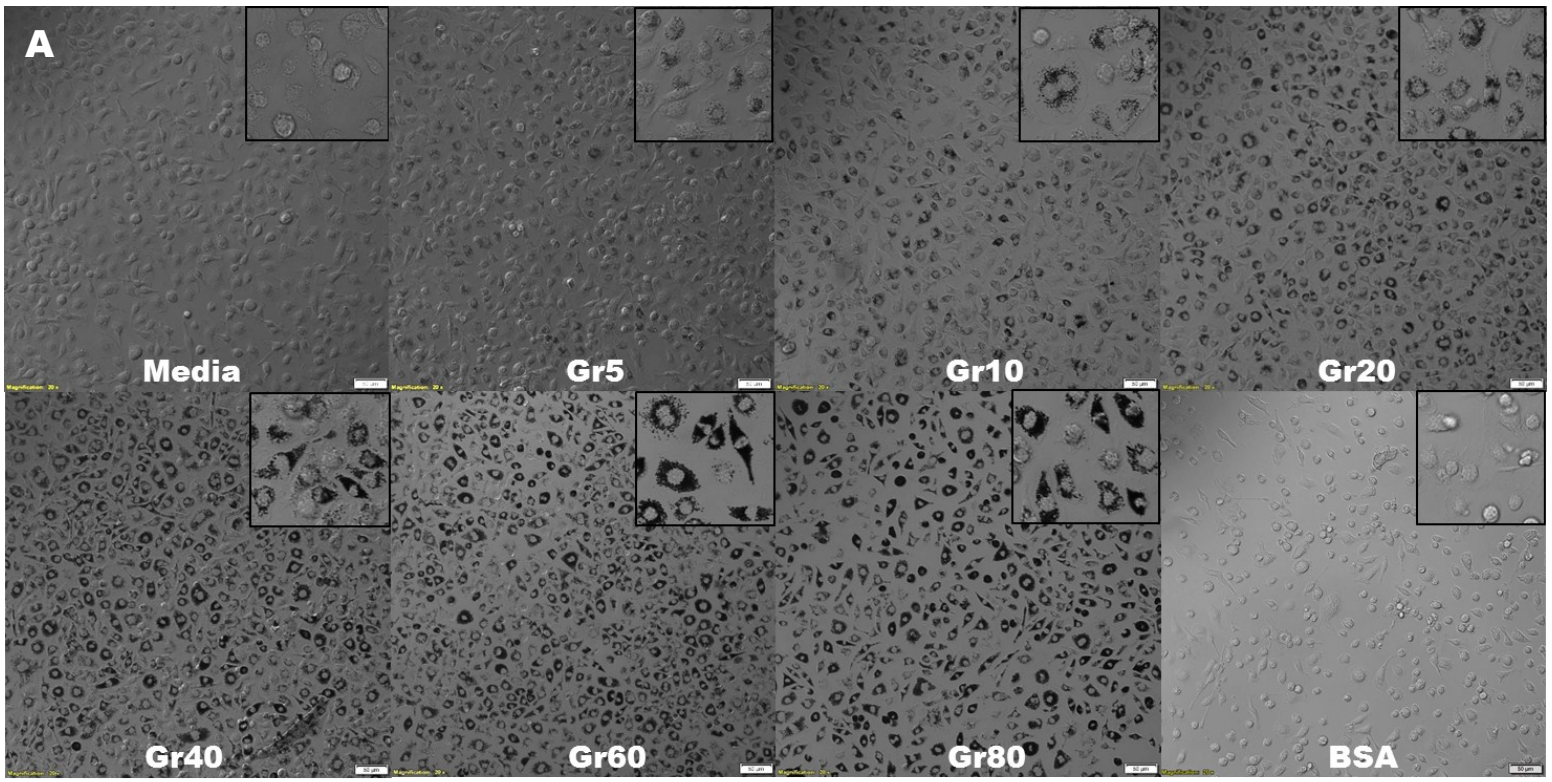
RNA was collected with a High Pure RNA Isolation Kit (Roche Life Science) and used as per manufacturer's instructions. The quality and quantity of the RNA was assessed by measuring the ratio of absorbance at 260 nm and 280 nm using a Nanodrop 2000 Spectrometer (Thermo Scientific). Similar amounts of RNA were reverse transcribed into complementary DNA (cDNA) with an M-MLV reverse transcriptase, RNase H minus, point mutant (Promega). Quantitative polymerase chain reaction was performed using KAPA SYBR FAST qPCR Kit Master Mix (2X) (Sigma). Samples were run on a QuantStudio 3 system. Dissociation curve analysis was performed after a completed qPCR to exclude non-specific products. The following primers were used (5´→3´). β-actin forward, TCC AGC CTT TCT TGG T, β-actin reverse, GCA CTG TGT TGG CAT AGA GGT C, IL-6 forward AGT CCG GAG AGG AGA CTT CA, IL-6 reverse GCC ATT GCA CAA CTC TTT TCT, IL-10 forward AGG CGC TGT CAT CGA TTT CTC, IL-10 reverse GAC ACC TTG GTC TTG GAG CTT AT, TNF-α forward TCC CCA AAG GGA TGA GAA GTT, TNF-α reverse TTG CTA CGA CGT GGG CTA C, iNOS forward TCC TGG ACA TTA CGA CCC CT , iNOS reverse CTC TGA GGG CTG ACA CAA GG, H2-Ab1 forward ACG GTG TGC AGA CAC AAC TA, H2-Ab1 reverse ACG ACA TTG GGC TGT TCA AG, Chil3 forward AAG CTC TCC AGA AGC AAT CC, Chil3 reverse AGA AGA ATT GCC AGA CCT GTG A. RNA expression was normalized to β-actin expression from the corresponding sample (Ct_{gene}–$Ct_{\beta\text{-actin}}$=ΔCt). Furthermore, ΔCt from relevant untreated control samples were subtracted from the ΔCt of each sample ($\Delta Ct_{treatment}$-ΔCt_{ctrl} =$\Delta\Delta Ct_{treatment}$). Fold induction was calculated as $2^{(-\Delta\Delta Ct)}$.

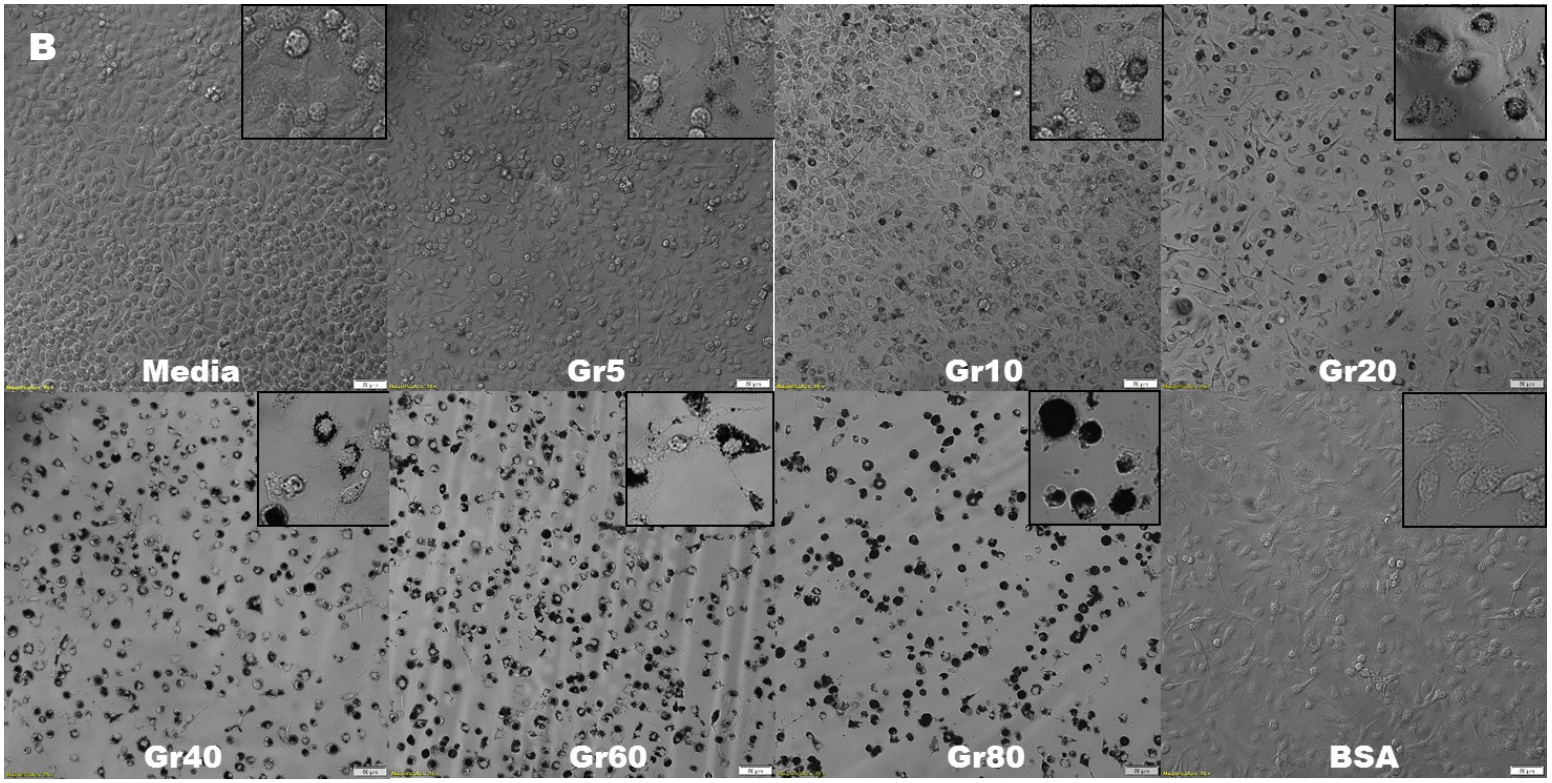


Supplemental Figure 1. BMDCs (0.625×10^6 cells·mL^{-1}) from C57BL/6 mice were stimulated with increasing concentrations (20, 40, 60, 80 µg·mL^{-1}) of pGr and respective exfoliation dispersant. Supernatants were collected 24 h later and tested for IL-6 by ELISA (#- Above the limit of detection).



Supplemental Figure 2. BMDMs (0.8×10^6 cells·ml^{-1}) from C57BL/6 mice were treated with media or pGr (20 µg·ml^{-1} or 80 µg·ml^{-1}) for 24 h and then washed. After 6 days cells were treated with either media or LPS (50 ng·ml^{-1}) for 6 h. Quantitative PCR analysis of IL-6, TNF-α and IL-10 mRNA expression. Messenger RNA levels calculated with respect to β-actin and fold increase calculated relative to untreated cells. Results are expressed as the mean ± SEM. Data are representative of twelve (IL-6, TNF-α) or eight (IL-10) independent experiments (∗ $p < 0.05$).


A
Media
Gr5
Gr10
Gr20
Gr40
Gr60
Gr80
BSA


B
Media
Gr5
Gr10
Gr20
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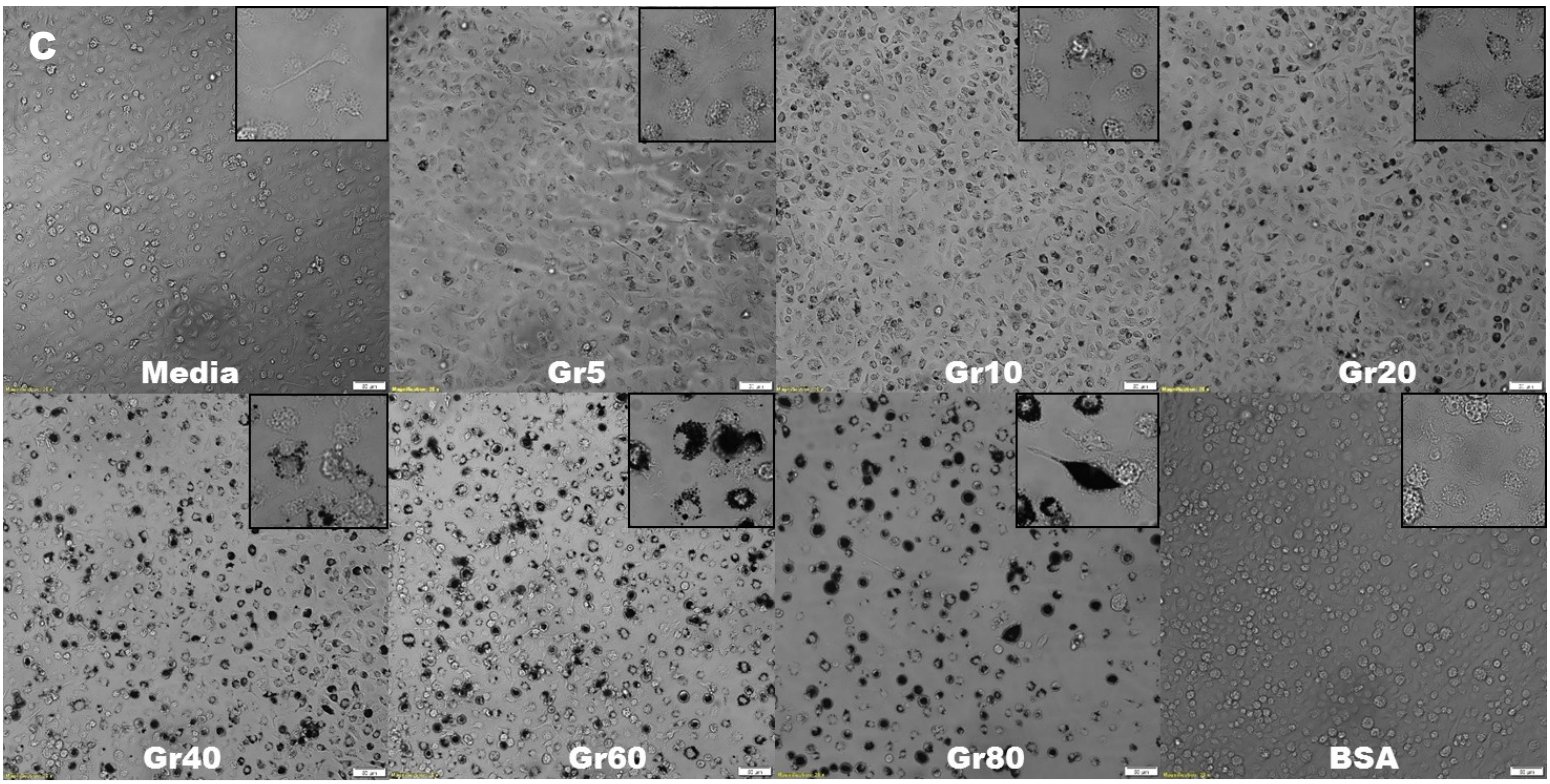
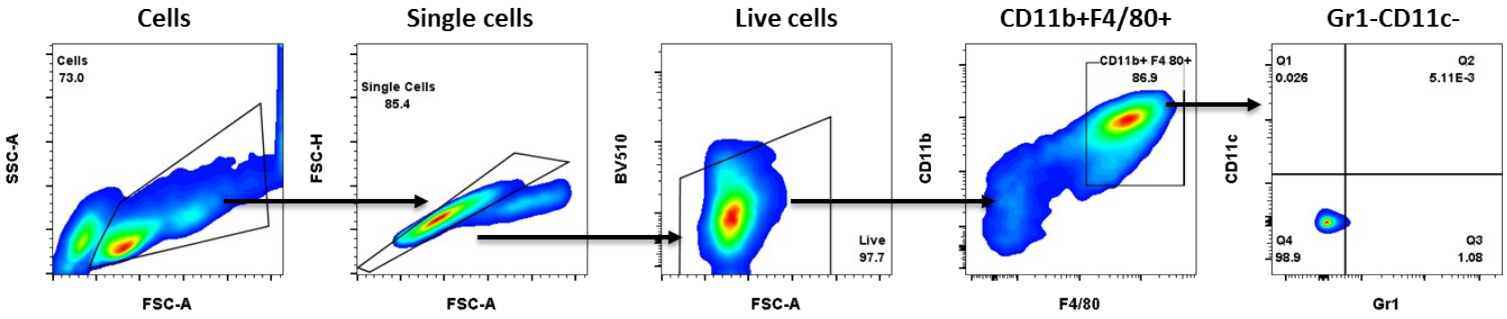
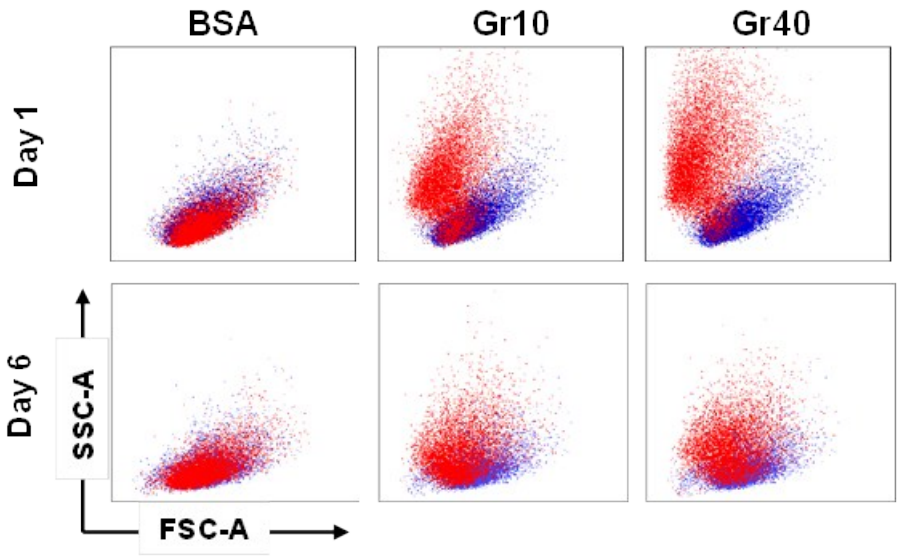
Gr80
BSA



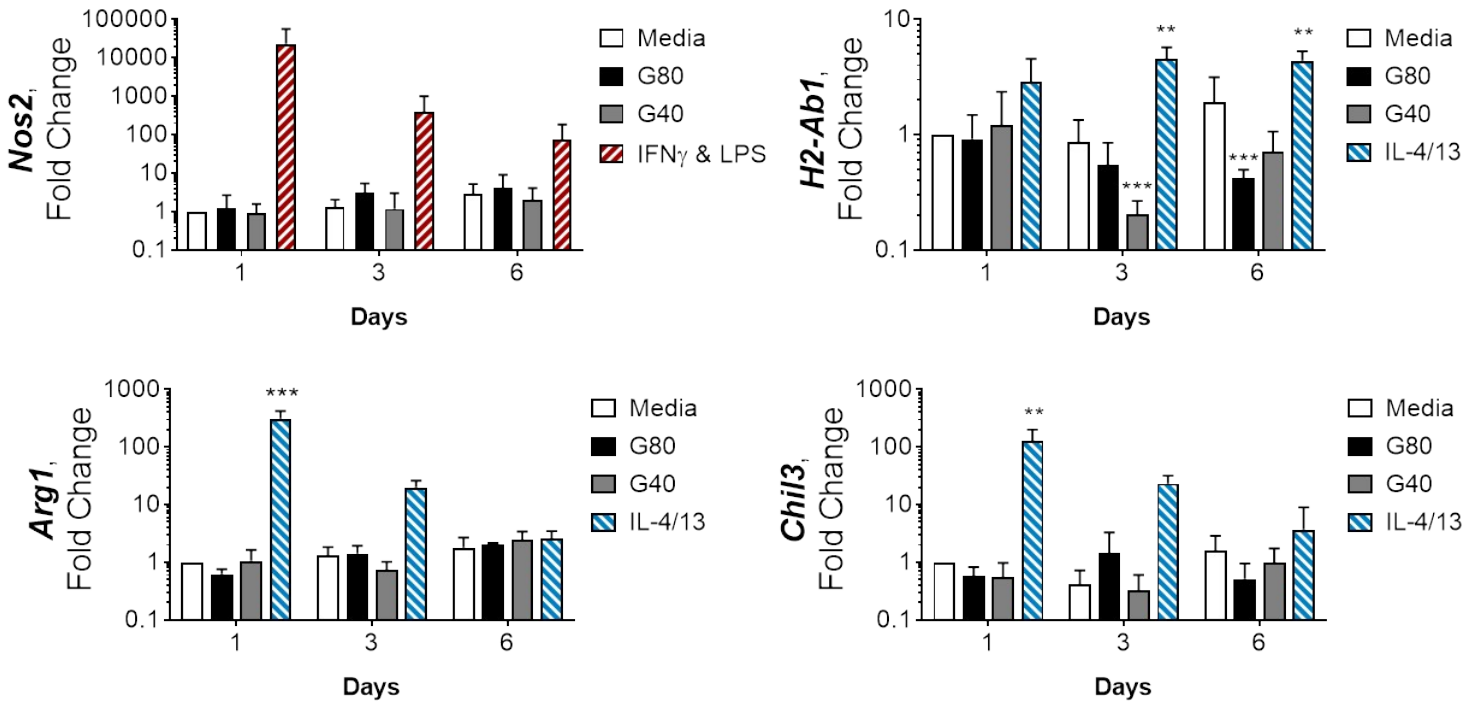
Supplemental Figure 3. Additional phase-contrast microscopy images of BMDMs from C57BL/6 mice (0.8×10^6 cells·ml^{-1}) incubated with media or increasing concentrations (5 to 80 µg·ml^{-1}) of pGr and respective exfoliation dispersant for 1 day (**A**), 3 days (**B**) and 6 days (**C**). Scale bar = 20 µm. Image x20; insert x60.



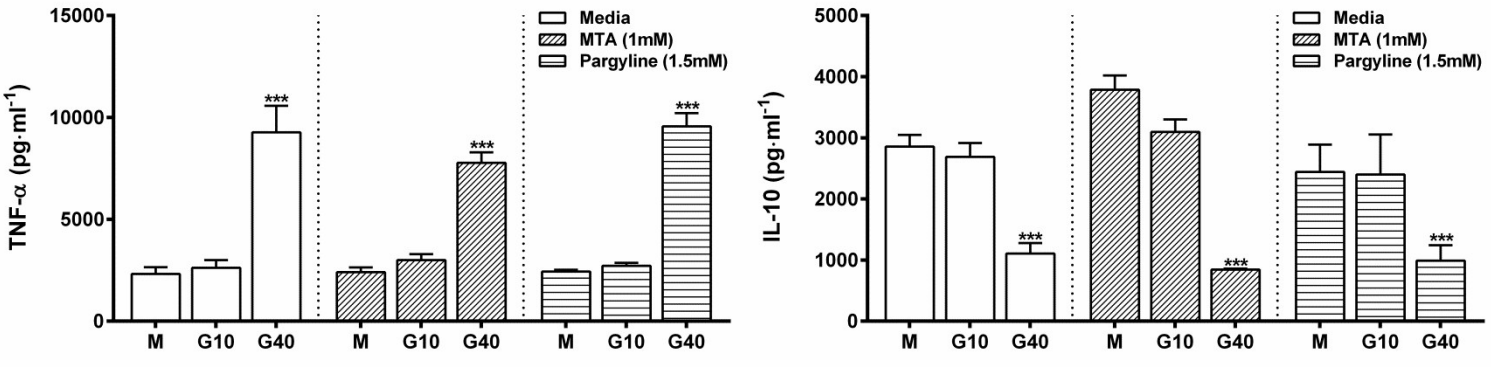
Supplemental Figure 4. Gating strategy.



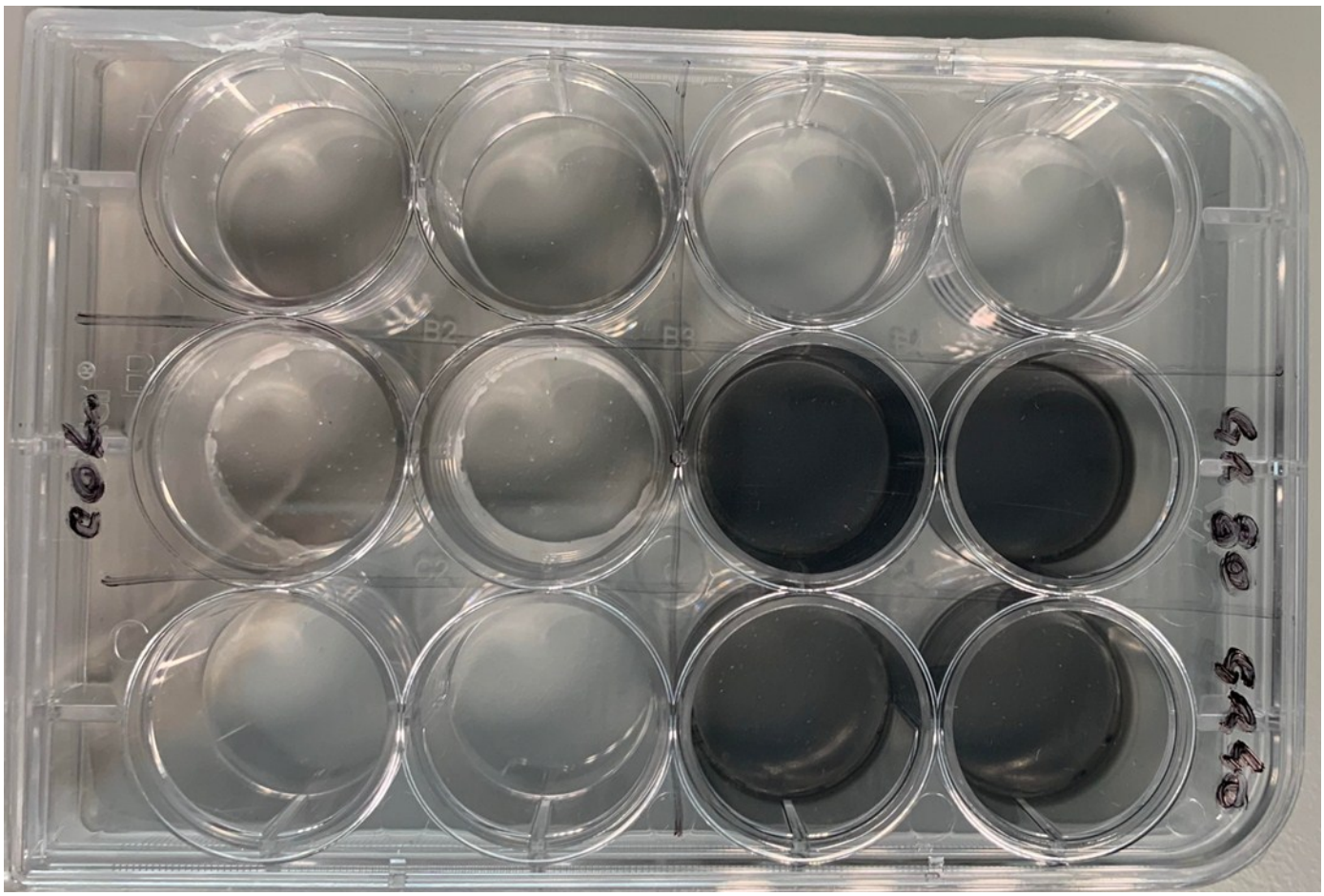
Supplemental Figure 5. BMDMs (0.8 x 10^6 cells·ml^{-1}) from C57BL/6 mice were treated with media, exfoliation solvent (BSA) or pGr (10, 40 µg·ml^{-1}) for 24 h. Cells were collected on day 1 and 6 and characterized by flow cytometry using a combination of markers (CD11b, CD11c, F4/80 and Gr1). Representative dot plots of macrophages analysed for changes in size and granularity by comparing forward and side scatter of different treatments (red) with untreated controls (blue). Representative of 3 independent experiments.



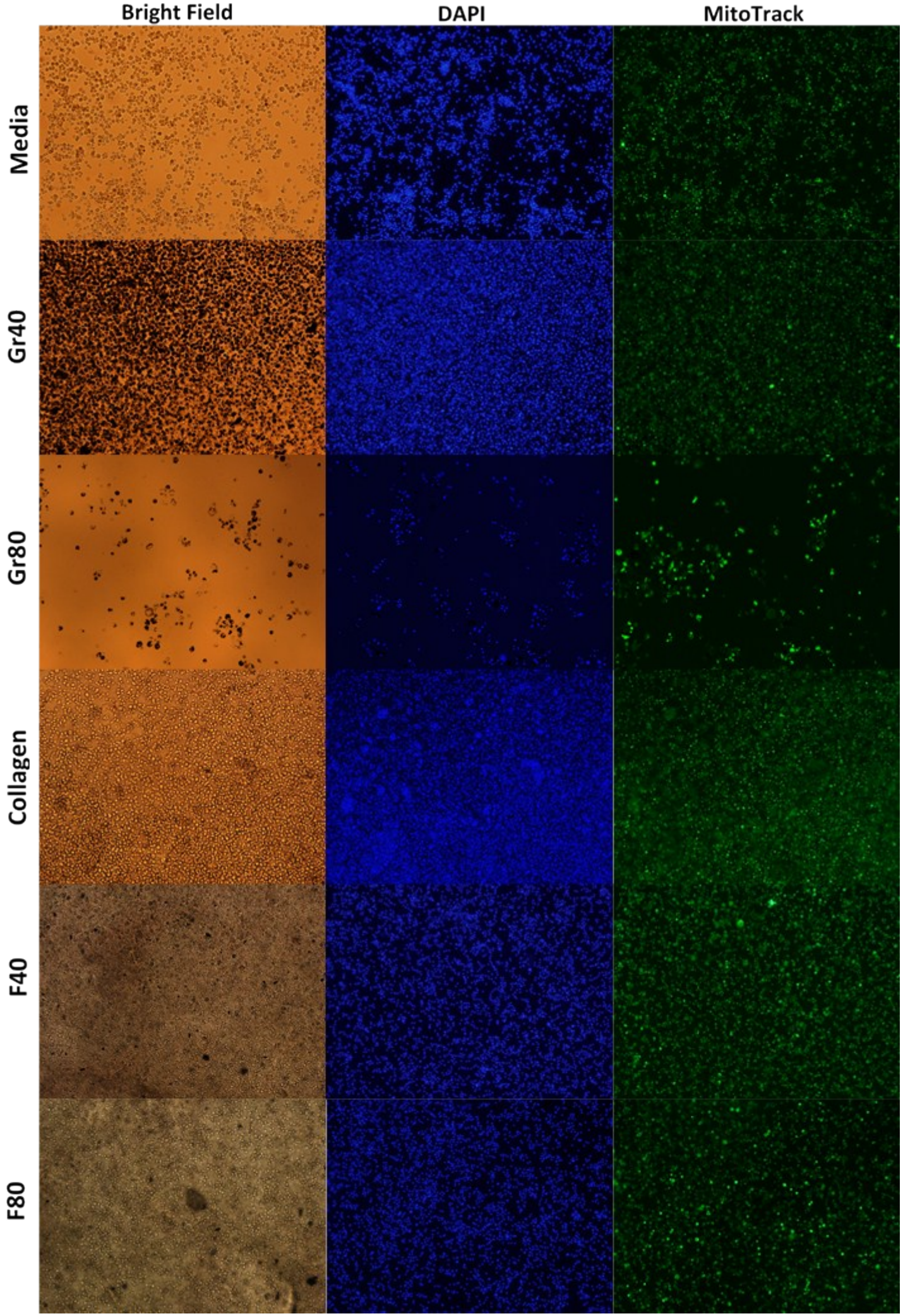
Supplemental Figure 6. BMDMs (0.8 x 10^6 cells·ml^{-1}) from C57BL/6 mice were treated with media or pGr (40 µg·ml^{-1} or 80 µg·ml^{-1}) for 24 h and then washed. Cells were collected on day 1, 3 and 6 and the expression of iNOS (Nos2), Arginase-1 (Arg1), Ym1 (chitinase-like 3, Chil3) and MHC II (H2-Ab1) was analysed by qPCR. Messenger RNA levels calculated with respect to β-actin and fold increase calculated relative to untreated cells. Results are expressed as the mean ± SEM. Data are representative of 3 independent experiments (vs media ** $p < 0.01$, *** $p < 0.001$).



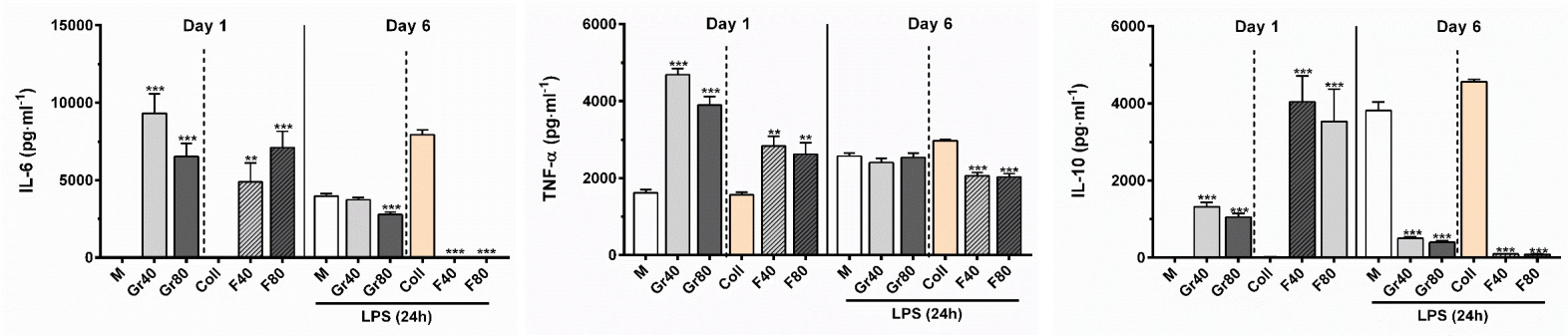
Supplemental Figure 7. BMDMs from C57BL/6 mice (0.8×10^6 cells·ml^{-1}) were pre-treated with the histone methyltransferase MTA (1 mM) or the histone demethylase inhibitor pargyline (1.5 mM) for 1 h and subsequently stimulated with pGr (0, 10, 40 µg·ml^{-1}). Twenty-four hours after, the cells washed and replenished with fresh media. After 6 days cells were treated with LPS (50 ng·ml^{-1}) for 24 h. Supernatants were tested for IL-10 and TNF-α by ELISA. Results are mean cytokine concentrations (± SEM) for three independent experiments (vs M $*$ $p < 0.05$).



Supplementary Figure 8. Photograph of the collagen films. Collagen alone (Coll); F40 (pGr 40 $\mu g \cdot ml^{-1}$); F80 (pGr 80 $\mu g \cdot ml^{-1}$).


Bright Field
DAPI
MitoTrack
Media
Gr40
Gr80
Collagen
F40
F80

Supplemental Figure 9. Additional fluorescence microscopy images of BMDMs after 6 days of training. BMDMs from C57BL/6 mice (0.8×10^6 cells·ml^{-1}) were treated with pGr (0, 40, 80 µg·ml^{-1}) or seeded on top of collagen scaffolds containing increasing concentrations of pGr (0, 40, 80 µg·ml^{-1}). Twenty-four hours after, the supernatants were collected, the cells washed and replenished with fresh media. After 6 days cells were treated with LPS (50 ng·ml^{-1}) for 24 h. Mitochondria were labelled with MitoTracker (green) and nucleus with DAPI (blue). Cells were analysed on an Olympus BX51 Fluorescence Microscope with LSM 5 software.



Supplemental Figure 10. BMDMs from C57BL/6 mice (0.8×10^6 cells·ml^{-1}) were treated with C-Gr (0, 40, 80 µg·ml^{-1}) or seeded on top of collagen scaffolds containing increasing concentrations of C-Gr (0, 40, 80 µg·ml^{-1}). Twenty-four hours after, the supernatants were collected, the cells washed and replenished with fresh media. After 6 days cells were treated with LPS (50 ng·ml^{-1}) for 24 h. Supernatants were tested for IL-6, TNF-α and IL-10 by ELISA. Results are mean cytokine concentrations (± SEM) for three independent experiments (M vs C-Gr/ Coll vs C-F $*$ $p < 0.05$, $**$ $p < 0.01$, $***$ $p < 0.001$).

REFERENCES

[1] Lebre, F.; Sridharan, R.; Sawkins, M. J.; Kelly, D. J.; O'Brien, F. J.; Lavelle, E. C. *Sci Rep* **2017**, *7*.