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Pristine graphene induces innate immune training

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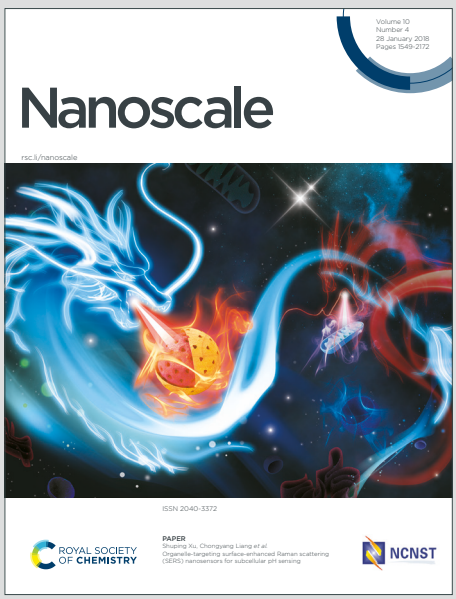

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ARTICLE

Pristine Graphene Induces Innate Immune Training

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Graphene-based materials are of increasing interest for their potential use in biomedical applications. However, there is a need to gain a deeper understanding of how graphene modulates biological responses before moving towards clinical application. Innate immune training is a recently described phenomenon whereby cells of the innate immune system are capable of being programmed to generate an increased non-specific response upon subsequent challenge. This has been well established in the case of certain microbes and microbial products. However, little is known about the capacity of particulate materials, such as pristine graphene (pGr), to promote innate immune training. Here we report for the first time that while stimulation with pGr alone does not directly induce cytokine secretion by bone-marrow derived macrophages (BMDMs)m, it programs them for enhanced secretion of proinflammatory cytokines (IL-6, TNF-α) and a concomitant decrease in production of the regulatory cytokine, IL-10 after Toll-like receptor (TLR) ligand stimulation. This capacity of pGr to program cells for enhanced inflammatory responses could be overcome if the nanomaterial is incorporated in a collagen matrix. Our findings thus demonstrate the potential of graphene to modulate innate immunity over long timescales and have strong implications for the design and biomedical use of pGr-based materials.

Introduction

The use of biomaterials to modulate the immune system is a fast growing area.[1] The unique physicochemical properties of graphene-based materials (GBMs)[2, 3] have recently spurred research interest into their biomedical applications. However, before clinical evaluation, novel biomaterials need to be been extensively studied with regard to their toxicity and immunomodulatory properties. Recently, we developed a convenient method to obtain endotoxin-free pristine graphene (pGr)[4] and reported how short-term incubation does not affect the viability, cytokine secretion or the activation status of bone-marrow derived dendritic cells (BMDCs) or macrophages. Until relatively recently, the innate immune response was considered as being rapid, non-specific, but also short-lived and incapable of developing memory. This dogma has been challenged by an increasing body of evidence suggesting that a subset of innate immune cells including macrophages, monocytes and natural killer cells can acquire memory characteristics after initial exposure, through epigenetic reprograming, enabling non-specific protection against subsequent challenge.[5-7] This concept differs from the classic adaptive immunological memory and has been named trained immunity. Following on from compelling evidence that Bacille Calmette-Guérin (BCG) vaccine protected mice not only against *Mycobacterium tuberculosis*, but also unrelated pathogens,[8] studies showed that vaccination with BCG also provided heterologous protection in humans.[9] Similarly, after injection with a non-lethal dose of *Candida albicans*, mice were protected against reinfection with a lethal dose of the same pathogen and importantly, with the bacterium, *Staphylococcus aureus*.[10] This protection was shown to be T-cell independent, but required epigenetic and metabolic reprogramming in a dectin-1/raf-1 dependent manner.

Innate immune training has also been demonstrated in response to endogenous factors including oxidised low-density lipoprotein (ox-LDL)[11, 12] and monosodium urate (MSU) crystals.[13] Bekkering and co-workers showed that ox-LDL is able to induce persisting proatherogenic modifications in monocytes, resulting in enhanced secretion of proinflammatory cytokines.[11]

Given the long-term effects of innate training, this is a phenomenon with enormous implications for the clinical use of biomaterials. Therefore, the aim of this work was to investigate the ability of endotoxin-free pristine graphene to train innate immune cells. If nanomaterials can induce a sustained innate immune 'memory', this could be exploited in the rational design of novel biomaterials.

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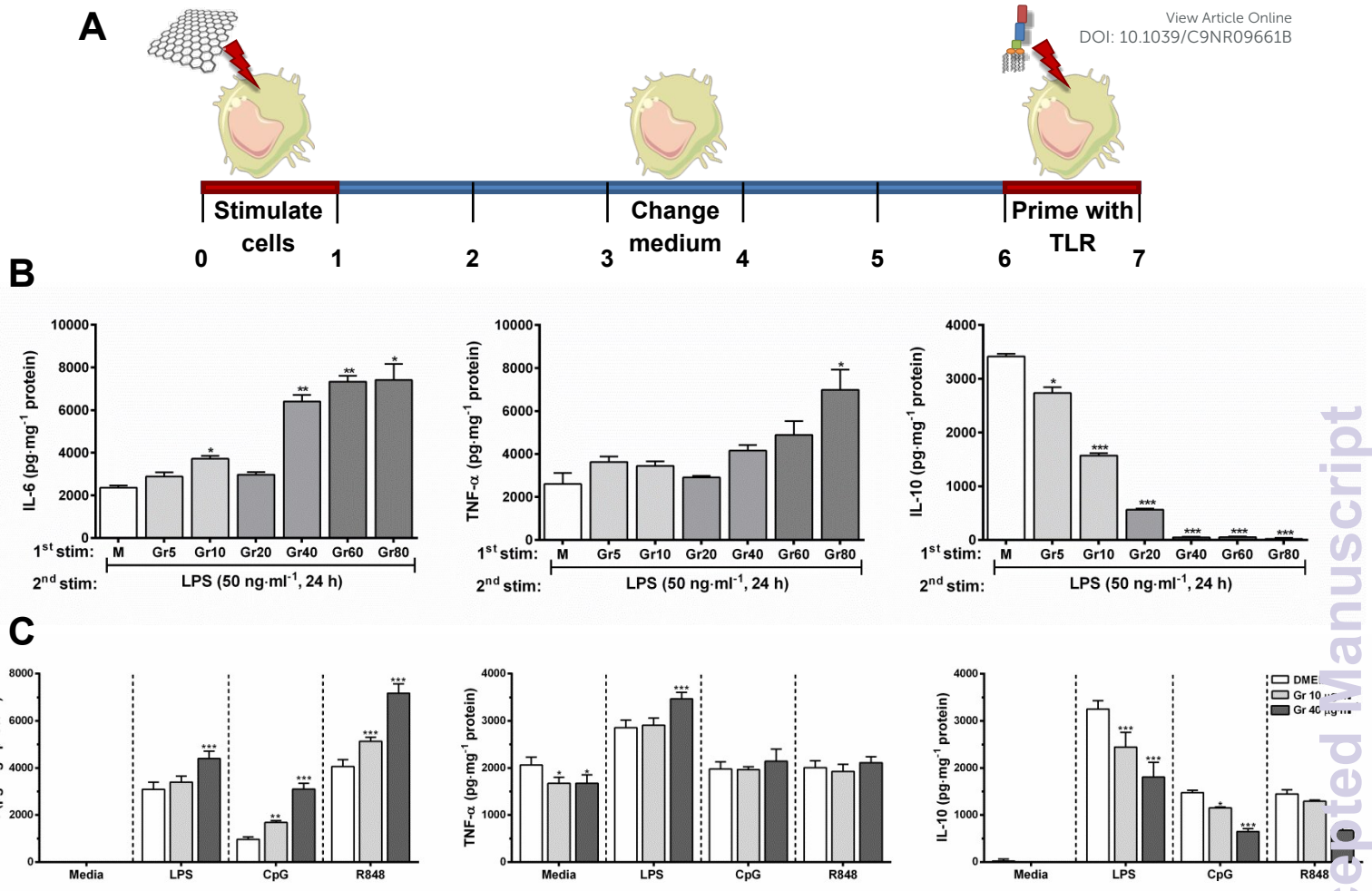

Figure 1. Pre-exposure of macrophages to endotoxin-free graphene enhances the production of pro-inflammatory cytokines following restimulation with a TLR-agonist. (**A**) Schematic of trained immunity protocol. (**B**) BMDMs (0.8 x 10^6 cells·ml^{-1}) from C57BL/6 mice were treated with medium or increasing concentrations of pGr (5-80 µg·ml^{-1}) for 24 h and then washed. On day 6, cells were treated with LPS (50 ng·ml^{-1}). (**C**) BMDMs (0.8 x 10^6 cells·ml^{-1}) from C57BL/6 mice were treated with medium or pGr (10 µg·ml^{-1} or 40 µg·ml^{-1}) for 24 h and then washed. After 6 days cells were treated with either medium, LPS (10 ng·ml^{-1}), CpG (10 µg·ml^{-1}) or R848 (0.5 µg·ml^{-1}). After 24 h, supernatants were tested for IL-6, TNF-α and IL-10 by ELISA. Results are mean cytokine concentrations (± SD) for triplicate samples. Data are representative of three independent experiments (vs Medium + TLR ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$).

Results and discussion

Cells of the innate system, including macrophages and monocytes, are key players in the response generated against infection and implanted materials.[1] While the ability of graphene to modulate macrophage responses acutely has been addressed,[4] we sought to investigate whether pristine graphene could train bone-marrow derived macrophages and thus mediate long-term innate effects. We previously reported the undesirable impact of endotoxin contamination on the ability of pGr to modulate the immune response,[4] hence we devised a protocol to produce endotoxin-free pristine graphene that does not trigger the secretion of interleukin (IL)-6 in bone-marrow derived dendritic cells for concentrations up to 80 µg·ml^{-1} (**Figure S1**). This allows us to study the capacity of pGr to induce innate training without the confounding results that might arise from the presence of foreign contaminants. Graphene samples used in this work were generated via a liquid exfoliation method, as previously described.[4] Characterization results from transmission electron microscopy (TEM) images showed few-layers graphene sheets with a mean length of 417 ± 20 nm.

So far, studies have primarily evaluated the short-term effects of pGr on cells of the innate system. We showed that pristine graphene alone does not trigger cytokine secretion in BMDCs and BMDMs or affect their activation state, after a 24 h exposure time.[4] However, the long-lasting effects of pGr on immune responses are poorly understood. To study the potential of pGr to train cells, we used an established *in vitro* training assay with murine bone-marrow derived macrophages. Cells were incubated with increasing concentrations of pGr (5-80 µg·ml^{-1}) for 24 h and rested for 5 days before stimulating with the TLR4-agonist LPS (50 ng·ml^{-1}) to simulate an encounter with a pathogen (**Figure 1A**). Pre-exposure of macrophages to pGr resulted in significantly enhanced secretion of the pro-inflammatory cytokines IL-6 and tumour necrosis factor (TNF)-α, and decreased secretion of the

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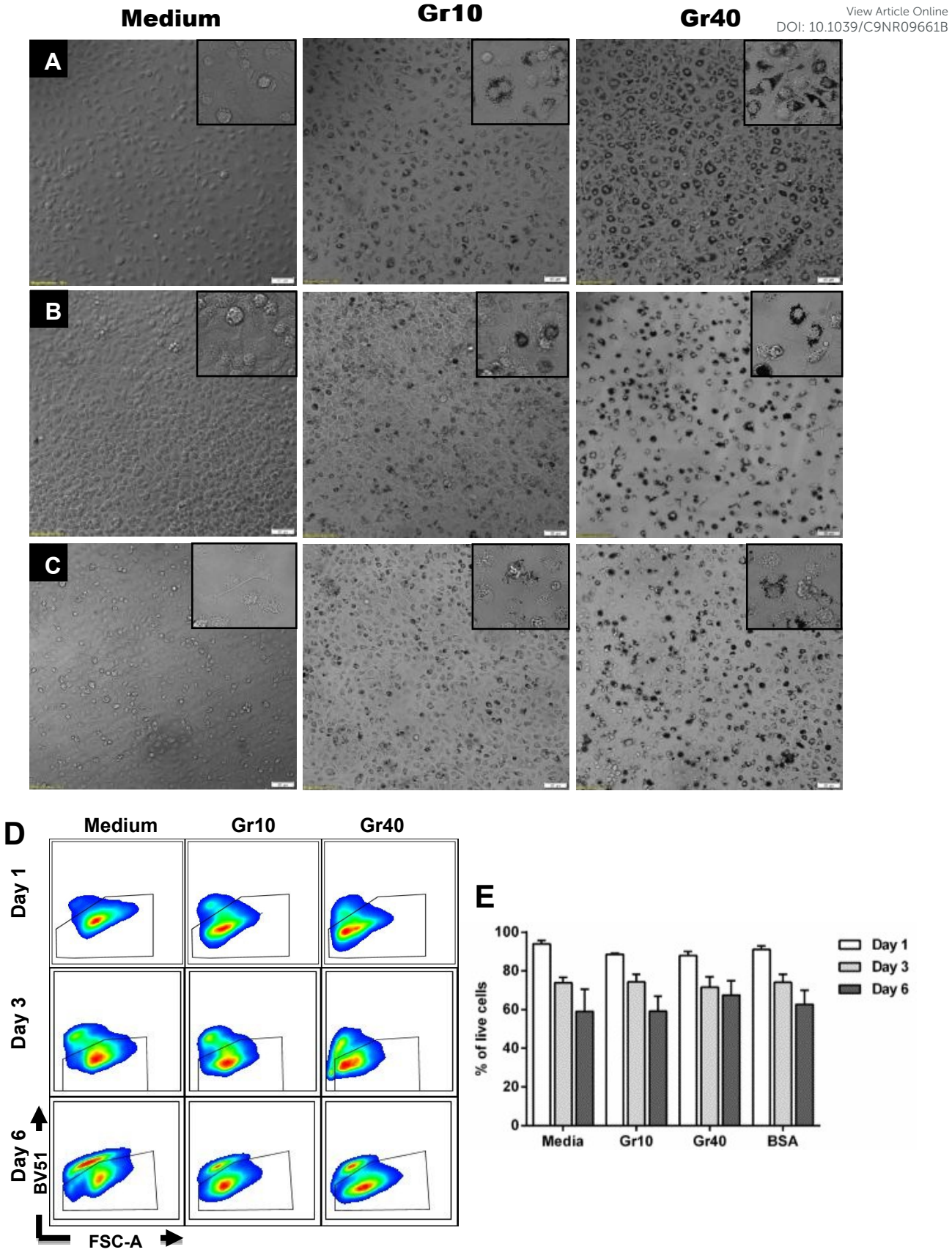

Figure 2. Bone-marrow derived macrophages can efficiently take up and retain pristine graphene, without affecting cellular viability. BMDMs (0.8 x 10^6 cells·ml^{-1}) from C57BL/6 mice were treated with medium exfoliation dispersant (BSA) or pGr (10 µg·ml^{-1} or 40 µg·ml^{-1}) for 24 h. Cells were collected on day 1, 3 and 6 and toxicity assessed by flow cytometry using a fixable viability stain (FVS-BV510). Representative phase-contrast microscopy images of BMDMs stimulated for 1 day (A), 3 days (B) and 6 days (C). Scale bar = 20 µm. Image x20; insert x60. (D) Representative dot plots. (E) Results are represented as percentage of cells/single cells. Error bars show mean ± SEM for three independent experiments (vs medium: NS $p \geq 0.05$).

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regulatory cytokine IL-10 following restimulation with LPS compared to non-trained cells (**Figure 1B**). Gene expression data suggests that the variation observed in cytokine production was a result of differential transcription (**Figure S2**). The highest concentration of pGr tested (80 μg·ml^{-1}) was capable of significantly enhancing expression of TNF-α while decreasing IL-10 expression after 6h when compared to the control. Our observations are in line with previous reports for microbial stimuli[10, 14] and endogenous inducers,[11, 12] but only hinted at for exogenous particulate materials.[14, 15] Schrum et al observed that not only *Plasmodium falciparum*–infected red blood cells but the parasite hemozoin crystals, were able to train peripheral blood mononuclear cells (PBMCs) resulting in increased pro-inflammatory cytokine secretion with a concomitant inhibition of IL-10 production after exposure to a secondary stimuli and this correlated with changes at the epigenetic level.[14]

To address whether pGr training for enhanced inflammatory responses was a general phenomenon and not restricted to restimulation with LPS, we performed similar training experiments using additional TLR-agonists. BMDMs were incubated with pGr (10 or 40 μg·ml^{-1}) or left untreated. On day 6, cells were re-stimulated with a lower dose of LPS (10 ng·ml^{-1}), the TLR9-agonist CpG (10 μg·ml^{-1}) or the TLR 7/8-agonist R848 (0.5 μg·ml^{-1}). Training with pGr resulted in a significant enhancement in IL-6 secretion accompanied by a decrease in IL-10 secretion for all three TLR agonists (**Figure 1C**). Interestingly, the effect observed for TNF-α with LPS-restimulation was lost for the rest of the TLRs tested, presumably due to differences patterns of expression generated by the various stimuli.[16, 17] Butcher and colleagues observed that, while several TLR-agonists had the capacity to affect gene expression and cytokine secretion in BMDMs, the expression patterns were more extensively related to the downstream activation of the nuclear factor- κB (NF-κB) via TLR4 activation, reflecting the dominant

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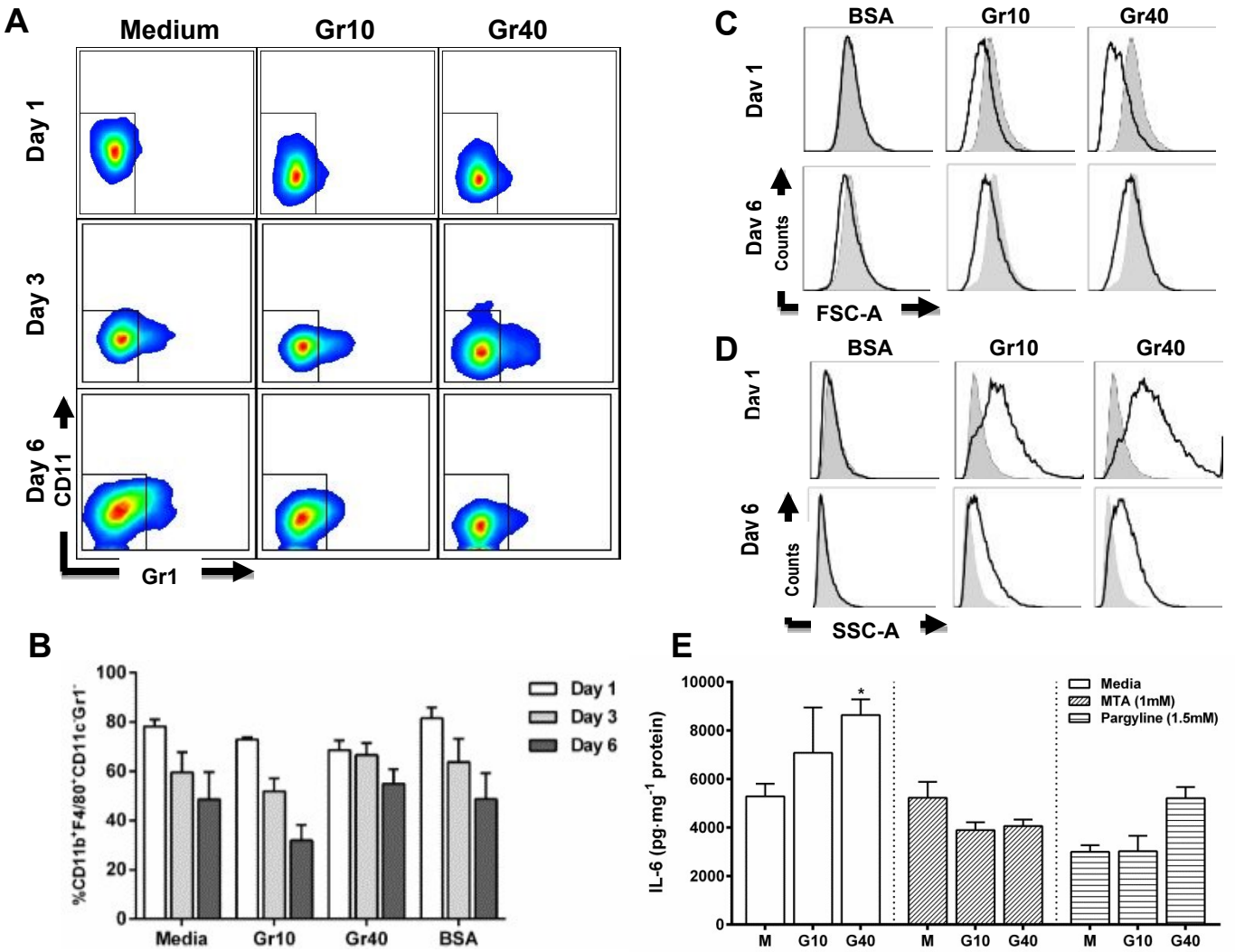

Figure 3 Exposure to graphene alters macrophage morphology and trains cells for enhanced IL-6 production via epigenetic changes. BMDMs (0.8 x 10^6 cells·ml^{-1}) from C57BL/6 mice were treated with medium, exfoliation solvent (BSA) or pGr (10 μg·ml^{-1} or 40 μg·ml^{-1}) for 24 h. Cells were collected on day 1, 3 and 6 and characterized by flow cytometry using a combination of markers (CD11b, CD11c, F4/80 and Gr1). (A) Representative dot plots. (B) Results are represented as percentage of cells/single cells/live cells. Macrophages were analysed for changes in size and granularity by comparing forward (C) and side scatter (D), respectively, of different treatments (solid black line) with untreated controls (grey histograms). Error bars show mean ± SEM for three independent experiments (vs medium: NS $p \geq 0.05$). 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}). After 24 h, the cells washed and replenished with fresh medium. After 6 days cells were treated with LPS (50 ng·ml^{-1}) for 24 h. (E) Supernatants were tested for IL-6 by ELISA. Results are mean cytokine concentrations (± SEM) for three independent experiments (vs M * $p < 0.05$).

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role of this pathway.[16] Collectively, these data indicate that pGr-trained macrophages are reprogramed to respond with elevated inflammatory cytokine secretion to a broad range of stimuli. Our findings provide the first evidence that pristine graphene has the ability to induce trained immunity.

It has been reported that upon training with microbial factors, innate cells undergo functional as well as morphological changes.[5] Here, BMDMs incubated with pGr were visualized using phase contrast microscopy on day 1, 3 and 6, at 2 different magnifications. After 24 h of incubation with pGr, no significant change in cell numbers was observed for concentrations of up to 80 μg·ml^{-1}, compared to the non-treated control (**Figure 2, Figure S3**). Furthermore, there were no clear differences in the morphology of macrophages stimulated with graphene, which was visible inside cells. Upon training for 3 days, we start observing a decrease in cell numbers for higher graphene concentrations, accompanied by an increase in cells with round morphology, normally associated with a more inflammatory profile.[18] When cells were rested for 5 days after stimulation, we observed an overall decline in cell number compared to day 1, with no significant difference between pGr treated cells and controls

Given our earlier observations, this study next investigated whether pristine graphene affected cellular viability and phenotype. Cells were stimulated with pGr (10 to 40 μg·ml^{-1}) or respective exfoliation dispersant for 24 h, 3 days or 6 days. Cells were stained with a fixable viability stain (FVS-BV510) and fluorescence intensity measured by flow cytometry. As observed in **Figure 3A, B,** exposure to pGr did not significantly increase cell death at any of the time points assessed. Overall, our results indicate that the reprograming observed for graphene-stimulated macrophages was not mediated by cytotoxic effects.

BMDMs were characterized using a combination of surface markers (CD11b, F4/80, CD11c, Gr1) measured by flow cytometry[19] according to the strategy outlined in **Figure S4**. As before, no significant differences were observed between trained cells and the untrained controls in the overall percentage of CD11b$^+$F4/80$^+$Cd11c$^-$Gr1$^-$ cells at any of the 3 time points (**Figure 3A, B**). Surprisingly, there was a downregulation in the expression of macrophage-specific surface markers during the course of the training protocol, in particular F4/80. The basis for this phenomenon is still unclear, but some studies suggest that F4/80 expression levels in macrophages can vary depending on the activation status[20] or cellular adhesion[21]. This might help to explain the overall decrease in cell number observed on day 6 (Figure 2) which is not related to a significant drop in viability or to the presence of pGr.

We further compared the size and complexity of BMDMs treated with pGr and non-treated cells, based on forward and side-scatter analysis of CD11b$^+$F4/80$^+$Cd11c$^-$Gr1$^-$ cells (**Figure S5**). Incubation of macrophages with graphene, but not the exfoliation dispersant, led to a decrease in forward-scatter (**Figure 3C**) pointing to a decrease in size and an increase in side-scatter compared to untreated controls (**Figure 3D**), reflecting an increase in cellular granularity and complexity due to the presence of graphene flakes inside the cells. Interestingly, smaller size has been linked to a M1 inflammatory phenotype in macrophages[22]. In order to assess the impact of pGr on macrophage phenotype during training we analyzed the expression of iNOS (Nos2) as an M1 marker, Arginase-1 (Arg1) and Ym1 (chitinase-like 3, Chil3) as M2 markers as well as MHC II (H2-Ab1) as a general marker of activation. Except for a decrease in MHCII expression on days 3 and 6, no significant differences were observed in cells stimulated with pristine graphene compared to untrained cell (**Figure S6**), illustrating that graphene showed no signs of polarising the cells towards either phenotype

Trained immunity in macrophages results from epigenetic changes, such as DNA methylation or histone modifications[5, 23] that alter on gene transcription and modulate the function of innate immune cells. So far, we observed that long-term exposure to graphene does not significantly alter cell survival or induce changes in lineage-specific markers, but has a profound impact on how macrophages respond to a second unrelated stimulus. This prompted us to address if this effect was due to epigenetic reprograming induced by pGr. To study this, BMDMs were preincubated with the histone methyltransferase methylthioadenosine (MTA, 1 mM) or the histone demethylase inhibitor pargyline (1.5 mM) for 1 h before adding pGr (10, 40 μg·ml^{-1}) and the training protocol outlined in Fig. 1 was carried out. Inhibition of both pathways affected graphene´s ability to train macrophages to secrete IL-6 upon LPS priming (**Figure 3E**), although it had no impact on IL-10 and TNF-α protein levels (**Figure S7**). Changes in histone methylation status can lead to repressed or enhanced transcription depending on how many and where the methyl groups are.[24, 25] In this case, both histone methylation and, to a smaller extent, histone demethylation, seem to be involved in macrophage training. Differences observed between IL-6 and the other cytokines have been reported before,[14, 26] and may arise from the selective effect of specific inhibitors on particular genes. Overall, our data suggest that pGr is able to train macrophages via epigenetic reprograming.

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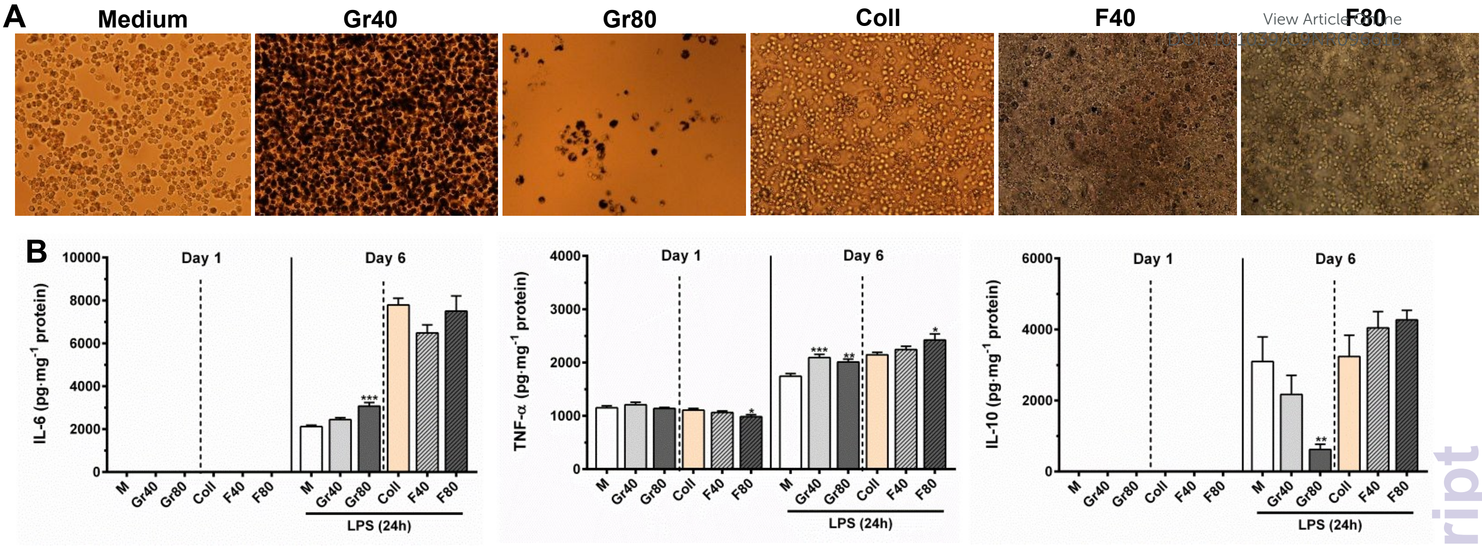

Figure 4. Incorporation of graphene on a collagen films impairs its training abilities. BMDMs from C57BL/6 mice (0.8×10^6 cells·ml^{-1}) were treated with pGr (0, 40, 80 μg·ml^{-1}) or seeded on top of collagen films (F) containing increasing concentrations of pGr (0, 40, 80 μg·ml^{-1}). Twenty-four hours after, the supernatants were collected, the cells washed and replenished with fresh medium. After 6 days cells were treated with LPS (50 ng·ml^{-1}) for 24 h. (A) Representative microscopy images. (B) Supernatants were tested for IL-6, TNF-α and IL-10 by ELISA. Results are mean cytokine concentrations (± SEM) for two independent experiments (M vs pGr/ Coll vs pF * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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Since training of macrophages for elevated inflammatory responses could be potentially detrimental in the context of biomaterials, we investigated whether the incorporation of pristine graphene in a collagen substrate would modulate its ability to train macrophages. We engineered a collagen-based film containing either 40 or 80 μg·ml^{-1} of pGr (**Figure S8**), according to a previously reported method.[27] BMDMs were cultured on top of the collagen films using the same *in vitro* training protocol as before. As a control, we subjected cells to the same amount of pGr in suspension for 24 h and rested them for 5 days, before priming with the second stimulus (LPS, 50 ng·ml^{-1}), and measured responses after the initial incubation and after priming. Since it is well documented that macrophages can respond differently to specific physical cues (e.g. stiffness and topography),[28] we first examined the ability of macrophages to adhere and migrate within the collagen films. Fluorescence microscopy images revealed that BMDMs seeded onto collagen films were able to attach to the matrix and remain viable for the duration of the training assay (**Figure 4A, S9**), while there was a decrease in cell numbers for macrophages incubated with the highest concentration of pGr in suspension (80 μg·ml^{-1}).

To determine if the innate training effect reported for pGr might be modulated by incorporation into a matrix, next we measured cytokine production. After 24 h of exposure, BMDMs did not secrete IL-6 or IL-10 and secreted similar levels of baseline TNF-α for all the treatments (**Figure 4B**). As expected, training macrophages with pGr in suspension resulted in a stronger response to LPS restimulation, reflected in an increase in proinflammatory cytokine secretion, accompanied by a significant drop in IL-10 production. Remarkably, when graphene was incorporated in the collagen film, this effect was substantially attenuated; a complete recovery in IL-10 secretion and a small increase in TNF-α were observed for the film containing the highest amount of pGr (F80). It is important to stress that the baseline levels of cytokine secretion are different from cells seeded on tissue culture plastic or collagen matrix, which probably relates to differences in stiffness and/or topography.[29] However, the differences suggest that the epigenetic changes induced by pristine graphene in suspension can be attenuated if the material is incorporated into an extra cellular matrix film such as collagen. This is a key observation since graphene-based materials are being studied as promising materials in multiple biomedical areas,[27, 30, 31] and could impact the outcome of new therapies. Further longer-term and *in vivo* studies will be invaluable to address the overall impact of incorporating graphene into a matrix and fully assess the capacity of pGr to generate long-lasting effects *in vivo*, capable of affecting hematopoietic precursor cells, as some studies now suggest,[12, 32] and being transmitted to the next generation.

Of note, all these results are valid for endotoxin-free pristine graphene since we performed similar studies with contaminated pGr and obtained contrasting results (**Figure S10**). BMDMs exposed for 24 h to contaminated material, either in suspension or in a film, exhibited elevated secretion of all cytokines tested, as observed previously.[4] In contrast, responsiveness following LPS restimulation on day 6 was reduced, particularly in the case of the films. This is not surprising; it is well documented that overexposure to LPS can cause innate tolerance,[16, 33, 34] characterized by a diminished response to secondary stimuli. We hypothesize that one of the reasons behind the lack of evidence for the capacity of particulate materials to train innate cells is the use of contaminated material while performing the training protocol that can lead to vastly different outcomes.

Experimental section

Materials

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Cytidine-phosphate-guanosine oligonucleotide 1826 was supplied by Oligos etc. Lipopolysaccharide from *E. coli*, Serotype R515, Toll-like receptor grade was purchased from Enzo Life Sciences. R848 (Resiquimod) and Pam3CSK4 were obtained from InvivoGen. MTA and pargyline were supplied by Sigma.

Graphene Preparation

Endotoxin-free pristine graphene samples were prepared as described by Lebre et al.[4] Briefly, graphene dispersions were prepared by liquid phase exfoliation using a solution of 20 mg·ml^{-1} endotoxin-free BSA (Akron) in endotoxin-free water (Baxter Healthcare). Graphite powder (10 mg·ml^{-1}; Asbury Carbons, Grade 3763) was added to the exfoliation liquid and sonicated for 6 h via bath sonication (Fisherbrand FB11201 bath at 37 kHz). To recover the graphene sheets, samples were centrifuged for 90 min at 106 g. The concentration was determined from extinction spectroscopy (Perkin-Elmer Lambda 1050 UV-Vis spectrometer).

Graphene Films Preparation

Endotoxin-free pristine graphene (40 and 80 µg·ml^{-1}) and collagen type I (Southern Lights Biomaterials; 0.5% w/v) were mixed in acetic acid solution (0.5 M) to create composite suspensions. These suspensions were then blended at 10 000 RPM until homogenous, and the film substrates were then prepared via room temperature evaporation on a tissue culture polystyrene surface.

Mice

Eight to 16-week-old wild-type C57BL/6 mice were bred in the Trinity Biomedical Sciences Institute Bioresources Unit. Animals were maintained according to the regulations of the European Union and the Irish Department of Health (Reference Number 091210). All animal studies were approved by the Trinity College Dublin Animal Research Ethics Committee.

Generation of Bone Marrow-Derived Macrophages

Murine bone marrow-derived macrophages were generated as described previously by our group.[4] Briefly, bone marrow cells were cultured for 6 days in Dulbecco's Modified Eagle's Medium (DMEM) - high glucose, supplemented with 8% ultra-low endotoxin heat-inactivated foetal bovine serum, 2 mM L-glutamine, 50 U·ml^{-1} penicillin, 50 µg·ml^{-1} streptomycin. Cells were plated on non-tissue cultured treated petri dishes (Corning) at a density of 1.0 × 10^6 cells·ml^{-1} supplemented with 25% of L929 cell line conditioned medium containing macrophage colony-stimulating factor (M-CSF). Fresh medium was added on day 3 and on day 6 adherent cells were collected.

In Vitro Training Model

Day 6 BMDMs were plated at a density of 0.8 x 10^6 cells·mL^{-1} supplemented with 15% of L929 cell line conditioned medium and left overnight before stimulation as indicated in figure legends. 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 re-stimulated with the appropriate stimuli according to figure legend. Cells were harvested for quantitative polymerase chain reaction (qPCR) at 6 h and supernatants were collected 24 h after and stored until further analysis.

Microscopy Analysis

Before imaging, BMDMs (1×10^6 cells·ml^{-1}) were seeded on culture dishes with glass bottom (Greiner) and left overnight. After that, cells were incubated with graphene samples according to the training model protocol, and visualized at specified time periods (1, 3 and 6 days). Cell morphology was analyzed by phase contrast imaging (Olympus IX81). Pictures were taken at x20 and x60 magnification.

Flow Cytometry Analysis

BMDMs (0.8 × 10^6 cells·ml^{-1}) were plated on non-tissue cultured treated petri dishes (Corning) and cultured overnight. On the second day, cells were incubated as described in the figure legends and harvested at specific time-points (1, 3 and 6 days). For analysis, BMDMs were placed on ice for 30 min before harvesting with volume of PBS-EDTA (5 mM) solution by gently pipetting up and down and transferring to flow cytometry tubes. Cells were incubated with Fixable Viability Stain 510 for at RT for 15 min. After washing with PBS, cells were stained with anti-mouse Fc Block, 15 minutes prior staining with CD11c-PE, F4/80-PerCP-Cy5.5, CD11b-PE-Cy7 and Gr1-APC-Cy7 anti-mouse antibodies (eBiosciences, Biolegend or BD Bioscience) for an additional 30 min at 4 ºC, washed with PBS and resuspended in flow cytometry buffer. Samples were acquired on a BD Canto II flow cytometer and the data analyzed using FlowJo software.

Cytokine Measurement

The concentration of the cytokines IL-6 and IL-10 was measured by ELISA using antibodies obtained from Biolegend. TNF-α concentrations were also determined by ELISA according to the manufacturer's instructions (R&D Systems). Cytokine values were normalized to cellular protein content.

Statistics

Cytokine concentrations measured by ELISA assays were subjected to ANOVA analyses. Where significant differences were found, the Dunnett's multiple comparisons post hoc test was performed. Statistical calculations were performed using the GraphPad Prism 6 software (GraphPad Software Inc.). In all cases, p-values ≤ 0.05 were considered statistically significant (∗ $p < 0.05$, ∗∗ $p < 0.01$ and ∗∗∗ $p < 0.001$).

Conclusions

In this study, we show for the first time that pGr exposure can prime cells of the innate system to respond more vigorously to

a second non-related stimulus, via epigenetic changes. By incorporating pristine graphene into a collagen matrix we were able to modulate the innate response generated after training. This calls for a reassessment of the impact of nanoparticulate material on the innate system and their capacity to induce long term and potentially deleterious epigenetic changes as well as further investigation into how the use of a natural polymer matrix can regulate this process.

Conflicts of interest

There are no conflicts to declare.

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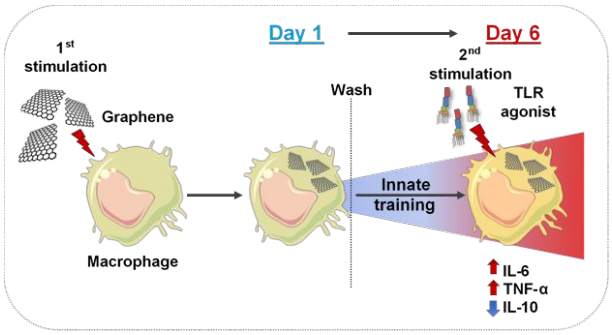

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