nature nanotechnology

ARTICLES

PUBLISHED ONLINE: 14 JUNE 2009 | DOI: 10.1038/NNANO.2009.151

Mechanisms for how inhaled multiwalled carbon nanotubes suppress systemic immune function in mice

L. A. Mitchell[1,2], F. T. Lauer[2], S. W. Burchiel[2] and J. D. McDonald[1]*

The potential health effects of inhaling carbon nanotubes are important because of possible exposures in occupational settings. Previously, we have shown mice that have inhaled multiwalled carbon nanotubes have suppressed systemic immune function. Here, we show the mechanisms for this immune suppression. Mice were exposed to 0, 0.3 or 1 mg m^{-3} multiwalled carbon nanotubes for 6 h per day for 14 consecutive days in whole-body inhalation chambers. Only those exposed to a dose of 1 mg m^{-3} presented suppressed immune function; this involved activation of cyclooxygenase enzymes in the spleen in response to a signal from the lungs. Spleen cells from exposed animals partially recovered their immune function when treated with ibuprofen, a drug that blocks the formation of cyclooxygenase enzymes. Knockout mice without cyclooxygenase enzymes were not affected when exposed to multiwalled carbon nanotubes, further confirming the importance of this enzyme in suppression. Proteins from the lungs of exposed mice suppressed the immune function of spleen cells from normal mice, but not those from knockout mice. Our findings suggest that signals from the lung can activate signals in the spleen to suppress the immune function of exposed mice.

Many products are already on the market that use or claim to use nanotechnology in their manufacturing processes (www.nanotechproject.org). Although these products in their final form may not pose a risk to the general public, individuals are likely to be exposed to nanocomponents in an occupational setting during production and processing. Among manufactured nanomaterials, carbon nanotubes have been widely studied, but there is an increasing level of concern in the toxicology community regarding the health hazards these materials may pose.

The potential health effects of carbon nanotubes following inhalation is of great interest because of their commercial applications and demonstrated potential for pulmonary exposure in the workplace. Some researchers have shown that nanotubes cause pulmonary damage following intratracheal instillation, including granuloma formation, inflammation and cellular damage[1–5]. However, others have reported that multiwalled carbon nanotube (MWNT) inhalation exposure produces a different spectrum of toxicities from that reported following intratracheal instillation exposures. Studies conducted in our laboratory[6] found that MWNTs dispersed evenly throughout the lung through inhalation and caused few pulmonary effects. Furthermore, Li and colleagues[7] performed a direct comparison of intratracheal instillation and inhalation exposures and found that intratracheally instilled MWNTs caused lung lesions in mice that were not observed after inhalation exposures with the same material at similar doses.

Although we did not observe overt lung toxicity in response to MWNT inhalation, we discovered systemic immune effects. These results are novel to the field and suggest that in order to study the biocompatibility of nanomaterials it is necessary to study extrapulmonary sites for damage and dysfunction. Interestingly, recent studies by Deng and colleagues[8] have demonstrated that MWNTs do not enter systemic circulation following pulmonary exposure. We are therefore interested in the mechanisms of lung signalling to systemic tissues that might explain the immunosuppression that has been measured in the spleen.

The purpose of this work was to elucidate the mechanism of systemic immunosuppression produced by MWNT inhalation. Our results reveal that activation of cyclooxygenase enzymes by MWNTs following inhalation exposure is a critical element in the suppression of systemic humoral immunity. The mechanism of action appears to involve activation and release of TGFß following alveolar macrophage phagocytosis of MWNT particles. These data show, for the first time, that inhaled MWNTs can activate the release of TGFß in the lung, which can have a direct effect on prostaglandin production in spleen cells, leading to immune suppression.

Results

Inhaled MWNTs decrease T-cell-dependent antibody response. T-cell-dependent antibody response was investigated to confirm previous observations that inhalation exposures up to 1 mg m^{-3} cause systemic immune suppression. Inhaled MWNTs caused a dose-dependent decrease in antibody formation in response to antigen challenge with sheep red blood cells (SRBCs), with a dose of 1 mg m^{-3} being the most sensitive exposure concentration (see Supplementary Fig. S1a). To determine whether this suppression was due to decreases in lymphocyte subpopulations, we used antibodies to known cell surface markers for cytotoxic T-cells (CD8), T-helper cells (CD4), natural killer cells (NK), macrophages (MAC), B-cells (CD19) and total T-cells (CD3). Lymphocyte subpopulations were not altered following exposure to inhaled MWNTs (see Supplementary Fig. S1b). An additional set of animals were exposed for 14 days to control air or 1 mg m^{-3} MWNTs then held for a 30-day washout period. T-cell-dependent antibody formation and T-cell proliferation in response to mitogen continued to be suppressed 30 days post exposure (see Supplementary Fig. S2a,b).

Ibuprofen partially rescues MWNT-induced immunosuppression. Gene expression for enzymes involved in the metabolism of arachidonic acid to prostaglandin H (PTGS2 or COX-2) and

[1]Lovelace Respiratory Research Institute, Albuquerque, New Mexico, USA, [2]University of New Mexico, Albuquerque, New Mexico, USA.
*e-mail: jmcdonal@lrri.org

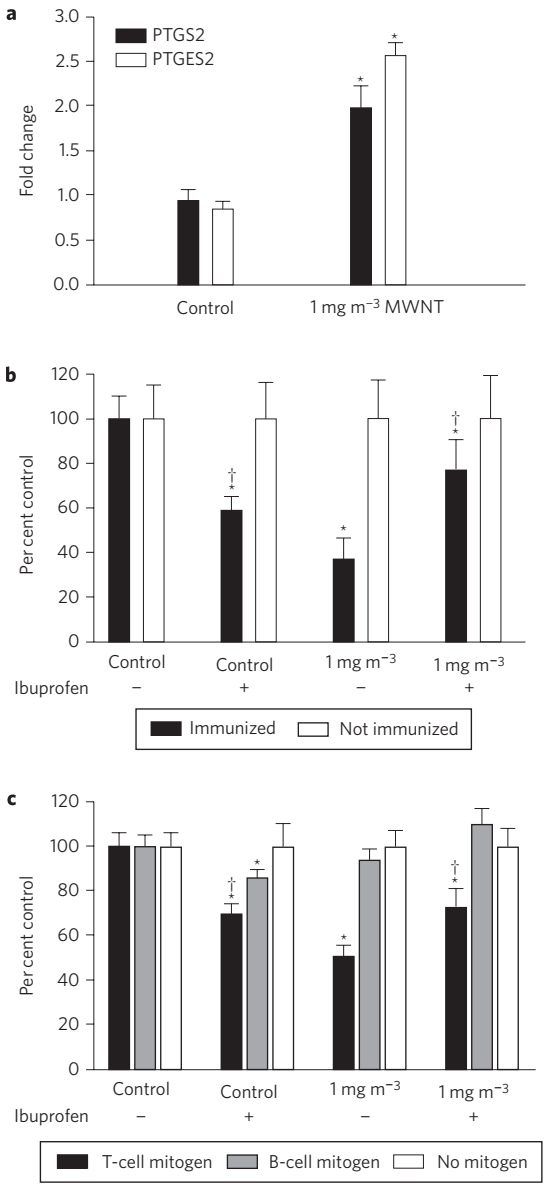

Figure 1 | Immunosuppression is partially rescued by treatment with ibuprofen. a, Gene expression of prostaglandin synthase 2 (PTGS2 or COX-2) and prostaglandin E synthase 2 (PTGES2) was upregulated in spleen cells from MWNT-exposed animals. *Statistically significant difference from control animals exposed to air ($P < 0.05$). **b**, Mice that received the cyclooxygenase antagonist ibuprofen in their drinking water exhibited partial rescue from MWNT-induced T-cell dysfunction. T-cell-dependent antibody response was decreased with 1 mg m^{-3} MWNT exposure. This suppression was attenuated with ibuprofen dosing. **c**, Mitogen-induced T-cell proliferation was reduced with 1 mg m^{-3} MWNT exposure. This reduction was partially rescued when mice were treated with ibuprofen. Error bars represent standard error of the mean for a given exposure group. *Statistically significant difference from control animals receiving non-treated water. †Statistically significant difference from 1 mg m^{-3} animals receiving non-treated water ($P < 0.05$).

conversion of prostaglandin H to prostaglandin E (PTGES2) were measured by real-time RT-PCR in spleen cells from animals exposed to 1 mg m^{-3} MWNTs or control air. Both prostaglandin synthase enzymes were upregulated in spleen cells following MWNT exposure (Fig. 1a). Conversely, gene expression in lung tissue from MWNT-exposed animals was not induced (see Supplementary Fig. S3). After measuring the induction of prostaglandin-associated enzymes using real-time RT-PCR, we decided to test the hypothesis that systemic activation of the cyclooxygenase pathway was involved in the observed immunosuppression by blocking COX-2 (PTGS2) using the pharmacologic inhibitor ibuprofen. Ibuprofen was successful in partially reversing MWNT-induced T-cell-dependent antibody suppression (Fig. 1b) and reducing T-cell proliferation (Fig. 1c). Animals in control chambers that received ibuprofen also had partially suppressed T-cell-dependent antibodies and T-cell proliferation, which led us to use the COX-2 knockout mouse to confirm these findings.

COX-2 knockout mice are unaffected by MWNT inhalation. When exposed to atmospheres containing 1 mg m^{-3} MWNTs, COX-2 knockout mice do not show decreased T-cell proliferation in response to mitogen, nor do they have a decreased antibody production when challenged with antigen. As shown in Fig. 2a and b, wild-type mice respond to MWNTs with suppressed T-cell proliferation and antibody response as seen before. In fact, immune function was slightly enhanced in COX-2 knockout mice. B-cell proliferation remained unaffected by these exposures.

BALF protein from exposed mice affects the immune function of splenocytes *in vitro*. Protein isolated from bronchoalveolar lavage fluid (BALF) of MWNT-exposed and control air-exposed mice was used to dose naive spleen cells *in vitro*. The naive spleen cells were collected from non-exposed wild-type and COX-2 knockout mice. T-cell-dependent antibody response was suppressed following exposure to BALF protein from MWNT-exposed animals compared to BALF protein from controls in spleen cells from wild-type but not COX-2 knockout mice (Fig. 2c). Furthermore, BALF protein from MWNT-exposed animals also caused significant reductions in T-cell proliferation in wild-type spleen cells, whereas T-cells from COX-2 knockout mice were unaffected (Fig. 2d).

To establish a role for TGFß signalling from the lung we continued to use protein collected from BALF from exposed and unexposed mice to dose naive splenocytes in culture. Additionally, recombinant TGFß was used as a positive control for splenocyte immunosuppression and a treatment group was added that contained MWNT BALF protein as well as anti-TGFß (4 μg ml^{-1}) antibody to neutralize the signal. The neutralizing antibody was added at a high concentration to ensure that an adequate quantity was available to block the TGFß that was present in the lavage fluid. When splenocytes were cultured with MWNT BALF protein or TGFß they showed decreased T-cell-dependent antibody formation and T-cell proliferation (Fig. 3a,b) compared with splenocytes receiving BALF protein from animals exposed to control air. Suppressed T-cell-dependent antibody formation was partially rescued by adding anti-TGFß to MWNT BALF protein cultures (Fig. 3a) and decreased T-cell proliferation was completely attenuated by adding the neutralizing TGFß antibody (Fig. 3b).

BALF from MWNT-exposed animals was analysed for TGFß protein. Active TGFß was present at a significantly higher concentration (~26 pg ml^{-1} compared to ~16 pg ml^{-1} in control lavage fluid) in BALF from 1 mg m^{-3} MWNT-exposed animals compared to animals that received control air (Fig. 4a). Plasma from exposed animals was also analysed for TGFß levels. Although a trend was observed showing an increase in TGFß in the plasma of exposed mice, plasma levels were on the lower end of detection of the assay and so high background prevented detection of a statistically significant increase in this medium (data not shown). After 24-h exposures of naive splenocytes to exposed or unexposed BALF protein spleen cell supernatants were analysed for the prostaglandin E2 metabolite (13,14-dihydro-15-keto prostaglandin A2) and IL-10. The metabolite of PGE2 was significantly increased with exposure to

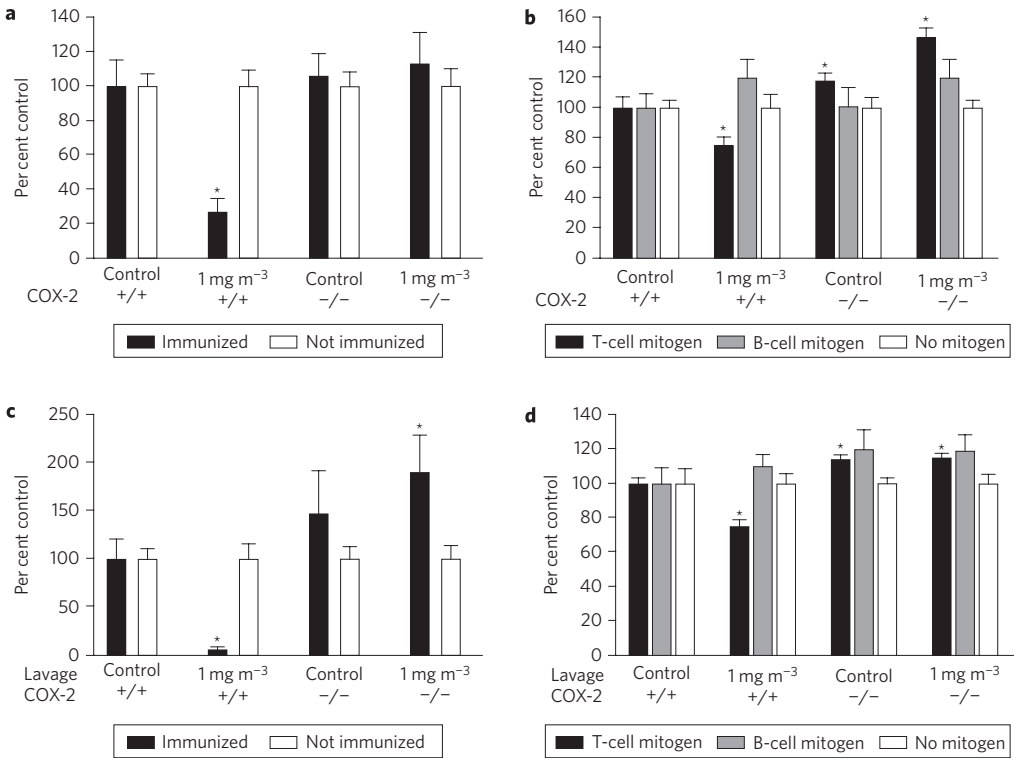

Figure 2 | MWNT-induced immunosuppression is dependent on activation of the cyclooxygenase pathway. a, Unlike wild-type littermates (COX-$2^{+/+}$), COX-$2^{-/-}$ mice did not show decreased antibody production in response to antigen after exposure to 1 mg m^{-3} MWNT. **b**, Following exposure to 1 mg m^{-3} MWNT, COX-$2^{-/-}$ mice did not express reduced T-cell proliferation as did COX-$2^{+/+}$ littermates. **c**, BALF protein was collected from COX-$2^{+/+}$ mice exposed to 1 mg m^{-3} MWNTs or control air. BALF protein from MWNT-exposed animals reduced antibody production in response to antigen in splenocytes collected from COX-$2^{+/+}$ mice. Antibody formation in splenocytes from COX-$2^{-/-}$ mice was not compromised following treatment with BALF protein from MWNT-exposed animals. **d**, T-cell proliferation decreased in splenocytes from COX-$2^{+/+}$ mice following exposure to BALF protein from MWNT-exposed animals. Splenocytes from COX-$2^{-/-}$ mice were not suppressed by BALF protein from MWNT-exposed mice. Error bars represent standard error of the mean for a given exposure group. *Statistically significant difference from control exposed wild-type mice ($P < 0.05$).

MWNT BALF protein and TGFß, as was IL-10. Addition of TGFß antibody to MWNT BALF protein cultures completely attenuated the increase in PGE2 metabolite and IL-10 in spleen cell cultures (Fig. 4b,c).

Discussion

Others[13] have conducted a risk assessment study that determined that a concentration of 53 μg m^{-3} of carbon nanotubes could be detected when a simulated occupational exposure scenario was carried out. An average human weighing 70 kg with a respiratory minute volume of 15.5 l min^{-1} working in this environment would have ~0.039 mg of MWNT deposited in the lung in an 8 h work day and a total of 0.55 mg deposited over 14 days. A quantity of 0.55 mg of material in the human lung (~70 m^2 surface area) is equal to ~0.008 mg m^{-2} lung surface area over 14 days. Mice only have ~600 cm^2 of lung surface area, so over 14 days at the 1 mg m^{-3} exposure concentration there was ~0.06 mg m^{-2} lung surface area deposited. It should therefore be noted that, based on a risk assessment model, the human calculated lung burden is 7.5-fold less than the burden the mice in this study received. With scaled-up production as a result of increasing demand as well as occupational exposures that persist for much longer than the 14-day paradigm used here, immune dysfunction is a concern for those who work in this industry.

This work shows that inhalation exposure to MWNTs can cause systemic immune suppression. These effects persist up to 30 days post exposure and are unrelated to lymphocyte population changes due to cell death. After initial findings that gene expression of prostaglandin synthase enzymes PTGS2 and PTGES2 were upregulated in the spleens of MWNT-exposed mice, follow-on studies were conducted to characterize this pathway. First, mice were exposed to MWNTs or control air while simultaneously receiving ibuprofen or vehicle in their drinking water. Ibuprofen, a non-steroidal anti-inflammatory drug, is a global cyclooxygenase antagonist, meaning it blocks COX-1 and COX-2. The ibuprofen study was successful in that dosing with ibuprofen partially rescued MWNT-induced immune suppression. Interestingly, control animals that received ibuprofen in their drinking water were also moderately suppressed. Because ibuprofen is a global COX inhibitor, we believe this suppression is due to modulation of COX-1. COX-1 is constitutively expressed while COX-2 is inducible. Without the induction of COX-2 by MWNTs, we hypothesize that ibuprofen is acting more on COX-1 and causing a detrimental effect that is unrelated to that seen in MWNT-induced suppression. To confirm the involvement of the COX-2 pathway in MWNT-induced immune suppression, we used COX-2 mice in our inhalation exposure system. COX-2 knockout or wild-type littermates were exposed to control air or 1 mg m^{-3} MWNT for 14 days. As expected, COX-2 knockout mice were not suppressed in response to MWNT exposure. Wild-type littermates showed decreased T-cell proliferation and antibody responsiveness following MWNT exposure. Previous data[6] have shown that IL-10 gene and protein expression is upregulated with MWNT inhalation. Although IL-10 is a known immunomodulatory cytokine that keeps the immune system in homeostasis, it is also induced by prostaglandin E2 (PGE2). Furthermore, PGE2 can affect the immune system without the help of IL-10 by blocking T-cell IL-2 autocrine activity[14–17].

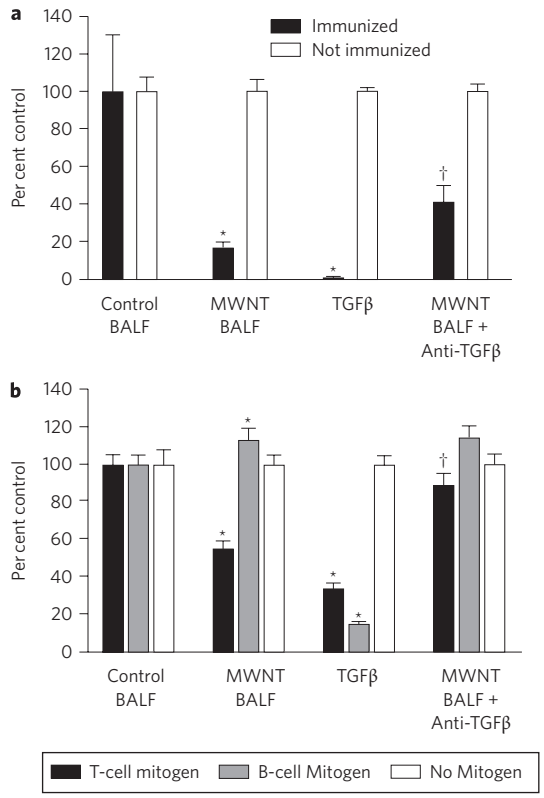

Figure 3 | Lavage fluid protein collected from MWNT-exposed (1 mg m^{-3}) animals is capable of inducing immune dysfunction. a, MWNT BALF protein and TGFß were effective in inducing decreased antibody production in naive splenocytes similar to *in vivo* exposures. Neutralizing anti-TGFß added to MWNT BALF protein cultures led to less antibody suppression, albeit not complete rescue. **b**, T-cell proliferation was completely restored when anti-TGFß was added to MWNT BALF protein treated media. *Statistically significant difference from control BALF. †Statistically significant difference from MWNT BALF. Error bars represent standard error of the mean for a given exposure group.

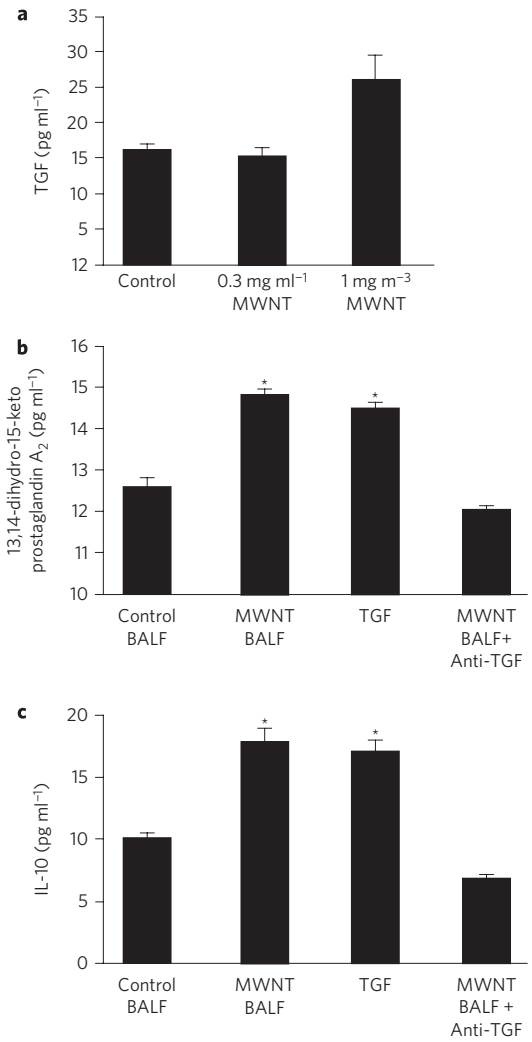

Figure 4 | MWNTs induce TGFß release in the lung that activates prostaglandin E2 and IL-10 expression in splenocytes. a, TGFß was analysed by ELISA in BALF from MWNT exposed and unexposed animals. BALF from animals that received 1 mg m^{-3} MWNT contained higher levels of TGFß than control animal BALF. **b**,**c**, After 24 h treatment with control or MWNT BALF protein, TGFß, or MWNT BALF protein + anti-TGFß antibody cells that were treated with MWNT BALF protein or TGFß secreted higher levels of prostaglandin E2 (as measured by its metabolite 13,14-dihydro-15-keto prostaglandin A2) (**b**) and the immunomodulatory cytokine IL-10 (**c**). MWNT BALF protein + anti-TGFß did not exhibit significant increases in prostaglandin metabolite or IL-10 in culture supernatants. *Signifies statistically significant difference from control BALF ($P < 0.05$), $n = 7$ mice exposed for BALF collection and $n = 7$ naive mouse spleens per group. Error bars represent standard error of the mean for a given exposure group.

A recent publication on the biodistribution of MWNTs has shown that translocation from the lung to the circulation is unlikely[8]. Additionally, histological analysis of the spleen following inhalation exposure did not reveal any signs of foreign material, whereas the lung was laden with black particulate matter. *In vitro* exposure of MWNT with freshly isolated murine splenocytes shows that very high concentrations of MWNT are required to induce immune alterations in culture. This indicates that the observed immunosuppressive effects seen *in vivo* are likely not due to MWNT entering the circulation and acting directly on the spleen cells, because concentrations that were efficacious in these *in vitro* studies were far higher than that calculated to deposit in the lung during inhalation studies. Also, the immunosuppressive profile of the *in vitro* work was not similar to that seen *in vivo* and was likely due to non-specific cell death from MWNTs lying on top of cell monolayers (see Supplementary Fig. S5). We believe that a signal, likely TGFß, is being released by the lung upon MWNT inhalation, resulting in systemic immunosuppression (see Fig. 5). To test this, we isolated protein from BALF from MWNT-exposed and unexposed mice and treated naive splenocytes (harvested from COX-2 wild-type or knockout mice) in culture. These studies showed that BALF protein from MWNT-exposed animals is capable of inducing suppressed T-cell proliferation and antibody formation in spleen cells isolated from COX-2 wild-type but not COX-2 knockout mice. BALF protein from control animals was not capable of inducing immunosuppression. The *in vitro* immunosuppression data indicate that a signalling mechanism exists in the lung that activates the cyclooxygenase pathway in the spleen, leading to T-cell dysfunction and decreased T-cell-dependent antibody formation.

TGFß has been shown to have an immunoregulatory role in macrophage 'house cleaning'. During macrophage phagocytosis of apoptotic cells (efferocytosis) and other 'garbage' found in biological systems, macrophages will release anti-inflammatory mediators. Macrophages do this to counteract any potential release of products from dying cells and to prevent an unnecessary immunologic response. The main anti-inflammatory mediator released is TGFβ. TGFβ functions to prevent T-cell proliferation and suppress

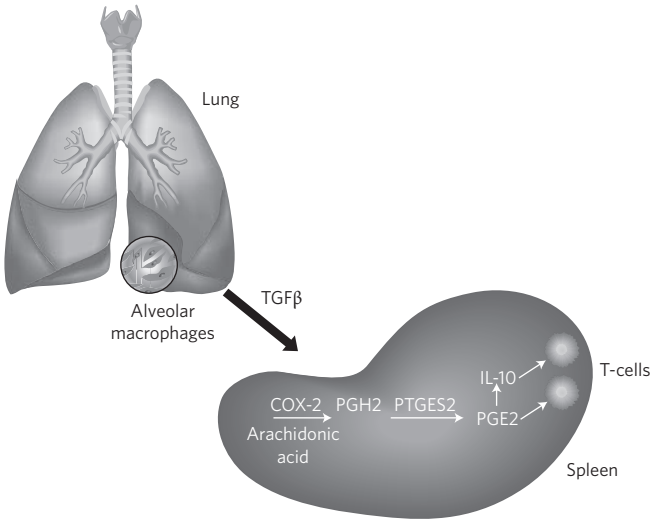

Figure 5 | Schematic of the proposed mechanism by which inhaled MWNTs induce systemic immune suppression.

Table 1 | Particle size data collected during animal exposures to MWNT aerosol.

	$dN/d\log D_p$ (no. cm^{-3})	$dM/d\log D_p$ (μg m^{-3})	Surface (nm^2 cm^{-3})
Median (nm)	86.4	590	134.2
Mean (nm)	94.5	579	146.3
Geometric standard deviation	1.73	2.39	1.57

Particle number size distribution ($dN/d\log D_p$ (no. cm^{-3})) as well as particle diameter based on particle mass ($dM/d\log D_p$ (μg m^{-3})) are described.

activation of macrophages. Furthermore, it has been shown that the anti-inflammatory cytokine TGFβ not only upregulates COX-2 but also increases arachidonic acid release[18].

Because TGFß was suspected to be the signalling mechanism from the lung to the spleen we measured TGFß concentrations in BALF from exposed and unexposed animals. Active TGFß was upregulated in BALF from MWNT-exposed mice. Additionally, upon *in vitro* treatment of naive splenocytes with MWNT-exposed BALF protein or recombinant TGFß, splenocytes exhibited suppressed T-cell-dependent antibody formation and T-cell proliferation as well as increased prostaglandin E2 metabolite and IL-10 in culture supernatants compared to those that received control-air-exposed BALF protein. The addition of a monoclonal antibody against TGFß to media treated with MWNT BALF protein was protective against PGE2 and IL-10 release and partially so against subsequent immunosuppression. One limitation of this work is the necessity for the development and use of a lung-specific TGFß knockout mouse to fully elucidate a role for TGFß signalling in MWNT-induced systemic immunosuppression.

The current studies have shown a role for TGFß secretion from the lung in signalling induction of the COX pathway and subsequent immunosuppression. We used a highly relevant inhalation exposure system to expose mice to inhaled MWNTs. We also used this exposure system to prime mouse lungs for isolation of MWNT-induced proteins that were used to directly expose naive splenocytes *in vitro*. The *in vitro* studies showed that TGFß is indispensable in PGE2 and IL-10 expression, but does not act alone in the induction of immunosuppression, as made evident by its only partial attenuation with TGFß blockage. Because COX-2 is necessary for MWNT-induced immune suppression, as seen in studies making use of COX-2 knockout mice, at least one additional signalling mechanism from the lung is inducing the COX pathway and resulting in arachidonic acid products other than PGE2 that are immunomodulatory. 15-deoxy-Δ14-prostaglandin J2 is a product of the cyclooxygenase pathway that is known to inhibit LPS-induced TNFα, macrophage inflammatory protein 2 and nitric oxide production[19,20]. Prostaglandin J2 acts through peroxisome proliferator activator receptor γ dependent or independent mechanisms to block the transcription of inflammatory genes. Prostaglandin J2 can also block IL-2 expression in T-lymphocytes[21]. PGJ2 is downstream of the prostaglandin D2 synthase that converts prostaglandin H2 to prostaglandin D2. Prostaglandin D2 is then metabolized to prostaglandin J2. Prostaglandin D2 is also known to induce lipoxygenase expression resulting in the anti-inflammatory molecule, lipoxin A4 (ref. 22).

These novel results suggest that TGFß is released from the lung upon inhalation exposure to low levels of MWNT. This cytokine activates the cyclooxygenase pathway in the spleen, leading to prostaglandin and IL-10 production and release, ultimately causing T-cell dysfunction and altered systemic immunity.

Materials and Methods

Animals. Male C57Bl/6 mice were purchased from Harlan Laboratories at approximately 8 weeks of age. Male wild-type B6;129P2 (COX-2$^{+/+}$) and B6;129P2-PTGS2^{tm1Unc}(referred to as COX-2 knockout or COX-2$^{-/-}$ mice) were purchased from Taconic at approximately 9 weeks of age. Mice were exposed for 6 h per day for 14 consecutive days to atmospheres containing 0.3 or 1 mg m^{-3} MWNTs or control air ($n = 7$ unless otherwise stated). We estimated the total amount of MWNTs deposited in the lung to be 0.15 mg kg^{-1} at 0.3 mg m^{-3} and 0.5 mg kg^{-1} at 1 mg m^{-3} (~3.75 and 12.5 μg total, respectively). Some animals were treated with ibuprofen as part of these studies. This was accomplished by diluting ibuprofen in drinking water at a concentration of 0.05 mg ml^{-1} along with 0.01 mg ml^{-1} cyclodextrin, ~8 mg kg^{-1} day^{-1} ibuprofen.

Exposure system and characterization. Dispersible MWNTs were purchased from Shenzhen Nanotech Port Co. Inhalation exposure atmospheres were produced by mechanical agitation/resuspension of MWNTs using a jet mill (Fluid Energy) coupled to a dry powder screw feeder (Scheck AccuRate)[12]. Aerodynamic particle size distribution was determined using a Mercer cascade impactor (In-Tox Products) operated at 2 l min^{-1}. Particle number size distribution was determined by a Fast Mobility Particle Sizer (FMPS; TSI Corp.) (see data in Table 1).

Spleen harvest and cell isolation. Spleens were harvested into sterile HBSS in sterile 15-ml centrifuge tubes on ice. Using sterile instruments, spleens were homogenized. Counts and viabilities were recorded and used for normalizing lymphocyte number during cell plating.

Flow cytometry. All reagents for this analysis were obtained from BD Biosciences unless otherwise indicated. Subsets of lymphocytes were characterized using antibodies against cell surface markers to specific cell types. Three custom cocktails (all rat anti-mouse) were ordered from BD Biosciences to identify six lymphocyte subpopulations: CD3 (all T-cells), CD4 (T-helper-cells), CD8 (cytotoxic T-cells), CD19 (B-cells), CD16 (natural killer cells) and Mac-1 (macrophages). Samples were analysed using a FACScalibur Flow Cytometer (Beckton Dickson). CD45-positive cells were gated and 10,000 gated events were acquired for each sample. CellQuest software was used to collect the data.

Jerne-Nordin plaque assay. Each sample was immunized with SRBCs in duplicate in 48-well tissue culture plates (Corning). SRBC-free media was used for non-immunized control wells for each sample. Cells were incubated for 4 days in an incubator. An 0.8% solution of agarose (SeaPlaque®, Cambrex) in 2× RPMI medium (Gibco), SRBCs, and spleen cells were incubated face down on custom slide trays at 37 °C for 1 h. Guinea pig complement (Colorado Serum) was diluted 1:20 in Dulbecco's PBS containing Ca^{2+}, and Mg^{2+} (Sigma) was added and then incubated an additional 2 h. SRBC lysis was quantified by counting plaques in the SRBC/agar lawn.

Mitogenesis. Concanavalin A (Sigma), a T-cell mitogen, was added to wells at a final concentration of 1 μg ml^{-1}. Lipopolysaccharide (Alexis), a B-cell mitogen, was added to wells at a final concentration of 10 μg ml^{-1}. Supplemented RPMI medium was added to 'no mitogen' control wells. Plates were placed in a 5% CO_2, 37 °C incubator for 48 h. Following incubation, the cells were pulsed with 1 μCi per well of ^{3}H-thymidine (MP Biomedicals) and incubated for an additional 18 h. Cells were lysed and harvested using a Brandel Model 24V cell harvester and collected on filter paper (Whatman) and counted on a liquid scintillation beta counter.

Real-time RT-PCR. RNA from spleen samples was isolated using RNeasy Qiagen Kit, and RNase-free sterile pellet pestles (Kimble/Kontes). A reverse transcription

step was performed on total RNA at a concentration of 8 ng μl^{-1} using cDNA archive kit (Applied Biosystems). cDNA was detected using Universal PCR master mix (Applied Biosystems) and TaqMan primer/probe sets (Applied Biosystems) for indicated genes.

ELISAs and EIA. TGFß and IL-10 ELISA kits were purchased from ebiosciences. Prostaglandin E2 metabolite EIA was purchased from Cayman Chemical.

Experiments using BALF to dose splenocytes in vitro. Naive splenocytes were exposed to BALF protein *in vitro* from exposed and unexposed animals. Total protein was quantified using a BioRad protein assay (BioRad). Total BALF protein was then added to *in vitro* treatment media at a concentration of 1 $\mu g\ ml^{-1}$ and naive splenocytes were assayed for T- and B-cell proliferation as well as T-cell-dependent antibody response as described above. Recombinant TGFß was used as a positive control (10 ng ml^{-1}) (R&D Systems). Anti-TGFß1 antibody (Sigma) was used (4 $\mu g\ ml^{-1}$) to neutralize MWNT BALF protein-induced immunosuppression. Splenocytes were also incubated for 24 h with BALF protein, TGFß, or MWNT BALF protein + anti-TGFß and culture supernatants were harvested for analysis of prostaglandin metabolite (prostaglandin A2) and IL-10 by ELISA. Detailed methods are provided in the Supplementary Information.

Received 12 March 2009; accepted 15 May 2009; published online 14 June 2009

References

1. Stern, S. T. & McNeil, S. E. Nanotechnology safety concerns revisited. *Toxicol. Sci.* **101,** 4–21 (2008).
2. Lam, C. W., James, J. T., McCluskey, R. & Hunter, R. L. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicol. Sci.* **77,** 126–134 (2004).
3. Muller, J. *et al.* Respiratory toxicity of multi-wall carbon nanotubes. *Toxicol. Appl. Pharmacol.* **207,** 221–231 (2005).
4. Shvedova, A. A. *et al.* Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **289,** L698–L708 (2005).
5. Warheit, D. B. *et al.* Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. *Toxicol. Sci.* **77,** 117–125 (2004).
6. Mitchell, L. A. *et al.* Pulmonary and systemic immune response to inhaled multiwalled carbon nanotubes. *Toxicol. Sci.* **100,** 203–214 (2007).
7. Li, J. G. *et al.* Comparative study of pathological lesions induced by multiwalled carbon nanotubes in lungs of mice by intratracheal instillation and inhalation. *Environ. Toxicol.* **22,** 415–421 (2007).
8. Deng, X. *et al.* Translocation and fate of multi-walled carbon nanotubes *in vivo*. *Carbon* **45,** 1419–1424 (2007).
9. Bide, R. W., Armour, S. J. & Yee, E. Allometric respiration/body mass data for animals to be used for estimates of inhalation toxicity to young adult humans. *J. Appl. Toxicol.* **20,** 273–290 (2000).
10. Miller, F. J. Dosimetry of particles in laboratory animals and humans in relationship to issues surrounding lung overload and human health risk assessment: a critical review. *Inhal. Toxicol.* **12,** 19–57 (2000).
11. Godwin, D. A., Wiley, C. J. & Felton, L. A. Using cyclodextrin complexation to enhance secondary photoprotection of topically applied ibuprofen. *Eur. J. Pharm. Biopharm.* **62,** 85–93 (2006).
12. Cheng, Y. S., Marshall, T. C., Henderson, R. F. & Newton, G. J. Use of a jet mill for dispersing dry powder for inhalation studies. *Am. Ind. Hyg. Assoc. J.* **46,** 449–454 (1985).
13. Maynard, A. D., Baron, P. A., Foley, M., Shvedova, A. A., Kisin, E. R. & Castranova, V. Exposure to carbon nanotube material: aerosol release during the handling of unrefined single-walled carbon nanotube material. *Toxicol. Environ. Health A* **67,** 87–107 (2004).
14. Beck-Speier, I. *et al.* Agglomerates of ultrafine particles of elemental carbon and TiO_2 induce generation of lipid mediators in alveolar macrophages. *Environ. Health Perspect.* **109** (Suppl. 4), 613–618 (2001).
15. Burchiel, S. W. PGI2 and PGD2 effects on cyclic AMP and human T-cell mitogenesis. *Prostaglandins Med.* **3,** 315–320 (1979).
16. Chemnitz, J. M. *et al.* Prostaglandin E2 impairs CD4+ T-cell activation by inhibition of lck: implications in Hodgkin's lymphoma. *Cancer Res.* **66,** 1114–1122 (2006).
17. Choudhry, M. A. *et al.* Role of NFAT and AP-1 in PGE2-mediated T-cell suppression in burn injury. *Shock* **18,** 212–216 (2002).
18. Freire-de-Lima, C. G. *et al.* Apoptotic cells, through transforming growth factor-beta, coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. *J. Biol. Chem.* **281,** 38376–38384 (2006).
19. Kim, W. J., Kim, J. H. & Jang, S. K. Anti-inflammatory lipid mediator 15d-PGJ2 inhibits translation through inactivation of elF4A. *EMBO J.* **26,** 5020–5032 (2007).
20. Porter, D. W. *et al.* PGJ2 inhibition of LPS-induced inflammatory mediator expression from rat alveolar macrophages. *J. Tox. Environ. Health* **70,** 1967–1976 (2007).
21. Fionda, C., Nappi, F., Piccoli, M., Frati, L., Santoni, A. & Cippetelli, M. PGJ2 negatively regulated rankl gene expression in activated T lymphocytes: Role of NFkB and early growth response transcription factors. *J. Immunol.* **178,** 4039–4050 (2007).
22. Rajakariar, R. *et al.* Hematopoietic prostaglandin D2 synthase controls the onset and resolution of acute inflammation through PGD2 and 15-deoxy 12, 14, PGJ2. *Proc. Natl Acad. Sci. USA* **104,** 20979–20984 (2007).

Acknowledgements

This work was supported by NIEHS (P30 ES-012072) and EPA (RD-83252701).

Author contributions

L.M. conceived and designed the experiments and wrote the article. L.M. and F.L. conducted the experiments. S.B. and J.M. contributed materials and analysis tools. All authors discussed the results and commented on the manuscript.

Additional information

Supplementary information accompanies this paper at www.nature.com/naturenanotechnology. Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/. Correspondence and requests for materials should be addressed to J.D.M.