Mesenchymal stem cells (MSCs) and macrophages are fundamental components of the stem cell niche and function coordinately to regulate haematopoietic stem cell self-renewal and mobilization. Recent studies indicate that mitophagy and healthy mitochondrial function are critical to the survival of stem cells, but how these processes are regulated in MSCs is unknown. Here we show that MSCs manage intracellular oxidative stress by targeting depolarized mitochondria to the plasma membrane via arrestin domain-containing protein 1-mediated microvesicles. The vesicles are then engulfed and re-utilized via a process involving fusion by macrophages, resulting in enhanced bioenergetics. Furthermore, we show that MSCs simultaneously shed micro RNA-containing exosomes that inhibit macrophage activation by suppressing Toll-like receptor signalling, thereby de-sensitizing macrophages to the ingested mitochondria. Collectively, these studies mechanistically link mitophagy and MSC survival with macrophage function, thereby providing a physiologically relevant context for the innate immunomodulatory activity of MSCs.
MSCs reproduce some of the beneficial effects of intact cells\textsuperscript{4,5}. Studies indicating that conditioned medium from cultured MSCs release extracellular vesicles including exosomes (50–100 nm in diameter) and microvesicles (MVs; 0.1–1 μm in diameter) into the extracellular space\textsuperscript{6–11} and that MSC-derived exosomes protect mice from myocardial or renal ischaemia, and pulmonary arterial hypertension\textsuperscript{12–15}. While the isolation of exosomes requires differential ultracentrifugation, MVs can be isolated from cell culture supernatant by low-speed centrifugation\textsuperscript{16–19}. The role of MVs in MSC biology is largely unknown.

MSCs reside within the bone marrow stem cell niche and regulate haematopoietic stem cell (HSC) maintenance via cross-talk with macrophages\textsuperscript{20–25}. The bone marrow niche represents a potent\textsuperscript{2} environment for studies investigating MSCs, as it is rich in factors secreted by MSCs that contribute to their therapeutic potency\textsuperscript{1–3}. More recent studies indicate that cells also shed extracellular vesicles. These vesicles are enriched in the extracellular space\textsuperscript{6–11} and that MSC-derived exosomes protect mice from myocardial or renal ischaemia, and pulmonary arterial hypertension\textsuperscript{12–15}.

So far, studies have focused on the isolation and characterization of exosomes. However, the isolation of MVs requires differential ultracentrifugation. Thus, MVs can be isolated from cell culture supernatant by low-speed centrifugation\textsuperscript{16–19}. The role of MVs in MSC biology is largely unknown.

We hypothesized that oxidative stress and mitochondrial dysfunction induce cell survival mechanism in MSCs that include mitochondrial transfer, thereby providing a physiological relevant explanation for this behaviour. To test this hypothesis, we studied mitophagy in MSCs and the role of macrophages in this process since these two cell types are in close proximity within the HSC niche\textsuperscript{20,24,25}. We demonstrate that under standard culture conditions MSCs undergo mitophagy and use arrestin domain-containing protein 1-mediated MVs (ARMMs) to unload mitochondria, which are engulfed by macrophages and re-utilized to increase bioenergetics. Moreover, we show that MSCs tolerate macrophages to mitochondrial transfer by shedding exosomes that modulate Toll-like receptor (TLR) expression and inflammatory signalling via transfer of regulatory microRNAs both in vitro and in an in vivo model of lung injury.
associated proteins’ tumour suppressor gene 101 (TSG101) and arrestin domain-containing protein 1 (ARRDC1)\(^{11,41}\) (Supplementary Fig. 1D). Collectively, these results indicate that MSCs employ the release of ARMMs to extrude mitochondria at their cell surface. Moreover, MSCs exhibited marked increases in apoptosis when treated with Bafilomycin A1 or low...
concentrations (3–5 mM) of chloroquine, which block the mitophagy flux, indicating that this process is critical for MSC survival (Supplementary Movie 2).

Next, we co-cultured GFP-labelled human MSCs from above with primary human or mouse macrophages. Live cell imaging revealed that macrophages nibble the plasma membrane of MSCs, establishing cell contact at areas where membrane blebs are enriched in RFP-labelled vesicles, which are subsequently stripped by the macrophage (Fig. 2e–h and Supplementary Movie 3). This activity was also observed between mouse macrophages and primary human MSCs (Fig. 3a and Supplementary Movies 4 and 5) but was not evident when macrophages were co-cultured with mouse or human fibroblasts (Supplementary Fig. 2). In a subsequent experiment, we co-cultured the macrophage cell line RAW 264.7 with human MSCs containing RFP-labelled mitochondria (10:1 ratio) for 4 h and recovered macrophages using FACS after staining with antibodies that recognize macrophage epitopes (that is, F4/80) not expressed by MSCs. Sorted macrophages were cultured for up to 2 weeks in RPMI media, which do not support MSC expansion and survival. Fluorescent microscopy of these macrophages revealed clear evidence of cell-associated RFP derived from human MSCs (Fig. 3b). To confirm these findings, we demonstrated using PCR amplification that these macrophages expressed the mitochondrial specific transcript human cytochrome c oxidase I (MT-COX I), which was confirmed on the basis of the restriction fragment pattern obtained after digestion of the PCR product with Bfa1 (Fig. 3b). This PCR product was not detected in mouse macrophages because of limited sequence homology between the two genes but was detected in human MSC-derived MVs as expected (Fig. 3c and Supplementary Fig. 3A). Lastly, we co-cultured Cy5-labelled human MSCs with macrophages that were pre-incubated with or without dextran sulfate (100 μg ml⁻¹), a nonspecific inhibitor of phagocytosis. Live cell imaging showed phagocytosis of MVs by macrophages over a period of 18 min, and confocal microscopy confirmed that the engulfed Cy5-labelled vesicles resided within the cell body of the macrophage (Supplementary Movie 6). However, MV uptake was blocked in macrophages pre-treated with dextran sulfate as evidenced by the accumulation of Cy5-labelled MVs on the macrophage surface (Supplementary Movie 7).

To track the in vivo transfer of mitochondria, we systemically administered RFP-labelled human MSCs into C57BL/6 mice expressing a GFP reporter under control of the endothelial specific Tie2 promoter. At 24 h post injection, GFP-labelled endothelial cells, epithelial cells and macrophages that contained RFP-labelled mitochondria were visible (Supplementary Fig. 3B). Bfa1 digestion of mouse lung DNA following intravenous administration of human MSCs, exosomes or MVs yielded a pattern of restriction similar to those observed in RAW 264.7 macrophages (Fig. 3d). To follow the fate of viable human MSCs in the mouse lung, we measured the abundance of human-specific GAPDH transcripts via reverse transcriptase–PCR (RT–PCR)⁴². Human GAPDH mRNA was not detected in the lung tissue of untreated mice but was detected at 3 days post injection of human MSCs or human fibroblasts (Fig. 3d). However, expression rapidly declined and was no longer evident by 14 or 28 days post transplant, consistent with the clearance rate of cells from lung tissue. Expression of human COXI mRNA in mouse lung mirrored that of human GAPDH following injection of human
fibroblasts and was detected at 3 days but not 14 or 28 days post transplant. In contrast, human COXI transcripts were detected up to 28 days post injection of human MSCs, indicating that mouse lung tissue retained mtDNA long after the disappearance of viable human MSCs (Fig. 3d). Thus, MSC-derived vesicles constitute an effective mechanism to transfer mtDNA into the mouse lung.

MSC extracellular vesicles enhance macrophage energetics. To study the effect of MVs on macrophage bioenergetics, we analysed oxygen consumption rates (OCRs) using the SeaHorse technology. Human macrophages exhibit higher basal OCR than human MSCs or human fibroblasts (Fig. 4a). Co-culture of macrophages with human MSCs (Mac + hMSC) or MSC-derived exosomes (Mac + Exo) but not human fibroblasts (Mac + Fibro) significantly (analysis of variance (ANOVA) followed by Student–Neuman Keuls (SNK) post-hoc pairwise comparisons) increased their OCR, suggesting that MSCs or MSC-derived exosomes alter macrophage bioenergetics (Fig. 4a). Next, we repeated these measurements after treatment of cells with

Figure 3 | MSC transfer mitochondria to macrophages and lung tissues. (a) Top panel is DIC fluorescent overlay at time 0 of primary human MSCs infected with Organelle Lights to label mitochondria (green) and co-cultured with mouse (RAW 264.7) macrophages. Lower panels, time sequence at 45 min intervals showing transfer of green-labelled mitochondria from the a MSC to a macrophage (red arrow, see Supplementary Movie 4 for transfer of mitochondria in filamentous form, and Supplementary Movie 5 in which GFP signal is compensated to allow the tracking of the transferred mitochondria into macrophages). (b) Left panel, photomicrograph of FACS-sorted mouse macrophages that were co-cultured with mitochondria-labelled (RFP) human MSCs clearly show retention of RFP label. Right panel, electrophoretic pattern of human COX I PCR product treated with or without Bfa1 after amplification from the indicated cell sources. (c) MSC-derived exosomes and MVs express the Bfa1-sensitive 228-bp COX I mtDNA PCR product detected in human MSCs (b). (d) Left panel, electrophoretic pattern of Bfa1-digested human COX1 PCR product amplified from mouse lung DNA isolated 14 days after the intravenous administration of human MSCs, human MSC-derived MVs or exosomes. Right panel, human GAPDH and human COX1 relative expression levels quantified by RT–PCR in mouse lung (3–28 days) after a single (intratracheal (IT) or intravenous (IV)) injection of human MSCs, human MSC-derived exosomes or human fibroblasts. *P < 0.001, #P < 0.001 by ANOVA compared with untreated mouse lung. Plotted values (mean ± s.e.m.) are from experiments repeated four times. Scale bars, 20 μ.

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oligomycin A, an inhibitor of ATP synthase, which is required for the oxidative phosphorylation of ADP to ATP. These conditions differentiate ATP-linked respiration from the proton leak. Macrophages exhibited a higher level of proton leak as compared with human MSCs and fibroblasts, and proton leak was significantly (ANOVA followed by SNK post hoc pairwise comparisons) reduced following co-culture with human Mac\(^+\)Exo but not Mac\(^+\)Fibro (Fig. 4a). Co-culture with human MSCs (Mac\(^+\)hMSC) also significantly (ANOVA followed by SNK post hoc pairwise comparisons) reduced proton leak in macrophages.

We also repeated the OCR measurements following treatment of cells with the uncoupling agent carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) to determine how cells respond to an increase in ATP demand. All three cell types responded to FCCP treatment with increased OCR, and the magnitude of the response was greater in macrophages as compared with human MSCs and fibroblasts. Moreover, OCR was significantly increased in FCCP-treated macrophages following co-culture with human MSCs (Mac\(^+\)hMSC) or human Mac\(^+\)Exo but not Mac\(^+\)Fibro (Fig. 4a).

To examine the effect of MSC or exosomes on macrophage bioenergetics under conditions of altered homeostasis, we exposed macrophages to silica particles. Silica exposure results in a burst of mtROS production as evidenced by changes in MitoSOX Red fluorescence intensity; however, this effect is largely mitigated in macrophages incubated with human MSCs. Oligomycin A and FCCP were used to differentiate ATP-linked respiration from the proton leak. Macrophages were co-cultured with or without human MSCs or fibroblasts (1:10 ratio) or treated with human MSC-derived exosomes (40 μg per protein) in the presence or absence of Oligomycin A and FCCP to differentiate ATP-linked respiration from the proton leak. Plotted data (mean ± s.e.m.) were performed using six replicates per sample and repeated three times. (b) Pseudocoloured photomicrographs (0-240 min) of MitoSOX Red-stained macrophages that were non-stimulated (upper panel), or treated with silica (20 μg cm\(^{-2}\), lower panel) or silica plus human MSC-derived exosomes (added 10 min after silica, middle panel). Scale bars, 50 μ. (c) Time course of MitoSOX Red emission by human macrophages treated as in b. Figure is representative of five exposures (nine stages positions per test and 6 cells per stage). (d) OCR as in a of silica-exposed macrophages treated with or without human MSCs, human MSC-derived exosomes or human fibroblasts. Plotted values (mean ± s.e.m.) are from experiments repeated three times, *P < 0.05 as compared to control, #P < 0.05 as compared to silica treated macrophages, as determined by Student’s t-test.

Figure 4 | MSCs enhance macrophage bioenergetics. (a) Mitochondrial respiration of human macrophages, human MSCs or human fibroblasts was measured as OCR using the XF technology. Macrophages were co-cultured with or without human MSCs or fibroblasts (1:10 ratio) or treated with human MSC-derived exosomes (40 μg per protein) in the presence or absence of Oligomycin A and FCCP to differentiate ATP-linked respiration from the proton leak. Plotted data (mean ± s.e.m.) were performed using six replicates per sample and repeated three times.
MSC-derived exosomes (Fig. 4b,c). Silica exposure also decreased macrophage OCR, but this decrease was reversed by co-culture with human MSCs or human MSC-derived exosomes but not with human fibroblasts (Fig. 4d). The fact that transfer of partially depolarized mitochondria from MSCs to macrophages enhances that macrophage bioenergetics appears paradoxical. However, loss of mitochondrial membrane potential as a result of MSC expansion is not absolute as mitochondria exhibit residual membrane potential as evidenced by the concentration of JC-1 aggregates (Fig. 1c). This indicates that the mitochondrial membrane is not collapsed and the mitochondria are still capable of undergoing fusion. To determine whether these mitochondria are recycled in macrophages by fusion, we co-cultured human MSCs with macrophages after labelling cells with two different MitoTracker dyes (Red and Green)43. Live cell imaging clearly demonstrated the transfer and subsequent fusion (yellow colour in merged images) of RFP-labelled, human MSC-derived mitochondria with GFP-labelled mitochondria within human macrophages (Fig. 5). These data indicate that under oxidative infection, macrophages were harvested and co-incubated with the human MSCs for 2h. Images were collected using an inverted Nikon TiE fluorescent microscope equipped with a C2 oil immersion optic and NIS Elements Software. Organelle Lights were excited using a Lumencor diode-pumped light engine and detected using an ORCA-Flash4.0 SCMOS camera. (a,b) DIC images of two separate fields within the same dish. (c) A zoomed image of the outlined section within b (scale bars, 20 μ). The fluorescence-based images for each field appear in the panels below the DIC images, with a showing human MSC mitochondria (red); b showing macrophage mitochondria (green); c showing human MSC mitochondria (red) and macrophage mitochondria (green); d showing human MSC mitochondria (red); and e showing the overlay with yellow indicative of colocalization of human MSC and macrophage mitochondria. Not every macrophage was shown to take up human MSC mitochondria (a,d,g,j).

MSC-derived exosomes are enriched in microRNAs. Exosomes transfer RNAs between cells8. We hypothesized that this process may be exploited by MSCs to tolerize macrophages against mitochondrial transfer. To explore this possibility, we analysed the RNA content of human MSC-derived exosomes. Using microRNA microarray analysis, we identified 156 (45 increased; 111 decreased) microRNAs that differed (log2 > 1.0, P < 0.05 (ANOVA followed by Holm–Sidak post hoc pairwise comparisons) in abundance between exosomes compared with their parent MSCs. The 10 microRNAs that exhibited the greatest increase included miR451a (316-fold), miR1202 (45-fold), miR630 (40-fold) and miR638 (28-fold), while microRNAs that exhibited the greatest decrease in exosomes and were enriched in MSCs included miR125b (148-fold) and miR21 (91-fold; Fig. 6a,b). This pattern of microRNA expression was conserved in MSC-derived exosomes obtained from five human donors (Fig. 6c,d).

MSC-derived exosomes inhibit TLR signalling in macrophages. Mitochondrial uptake can induce inflammation via activation of pattern recognition receptors34. Therefore, given the presence of mtDNA and microRNAs in MSC-derived MVs and exosomes, respectively, we hypothesized that exposure to these vesicles would tolerize macrophages to mitochondrial transfer by inducing changes in TLR expression. Subsequently, we profiled the expression of 84 TLR-associated transcripts in mouse macrophages. We contrasted these results with those observed in macrophages that were co-cultured with mouse or human MSCs, human MSC-derived exosomes or silica particles, which when phagocytized induce macrophage activation44. Co-culture of macrophages with MSC-derived exosomes induced nuclear translocation of the transcription factor NF-κB (Fig. 7a) resulting in significant changes (>2.5-fold increase or decrease) in expression of 50 of the 84 TLR-associated transcripts (Fig. 7b). For example, compared with silica-exposed macrophages those treated with exosomes exhibited significant (>2.5-fold) increases in transcripts associated with cytokine signalling including interleukin (IL)-1β, prostaglandin endoperoxide synthase 2 (PTGS2, aka COX2), granulocyte colony-stimulating...
factor 3 (CSF3), IL-10, chemokine (C–C motif) ligand 2 (CCL2, aka MCP-1), NF-κB-chemokine (C–X–C motif) ligand 10 (CXCL10), tumour necrosis factor (TNF) and reticuloendotheliosis oncogene (Rel; Fig. 7b). In contrast, transcripts encoding proteins involved in MyD88-dependent signalling (MyD88, TLR 1,4,5,7,8 and 9, IRAK1 and TRAF6), TRIF-dependent signalling (TLR adaptor molecule 1 (TICAM1) and TICAM2) and TLR-related signalling (CD80, CD86, IL-2, IL-12, Interferon gamma, PGLYRP1 and CSF2) were downregulated.

MSCs secrete PGE2 that acts on prostanoid receptors of LPS-stimulated macrophages to enhance their production of the anti-inflammatory cytokine IL-10 (ref. 45). However, this effect of MSCs was abrogated in macrophages from TLR4, MyD88, TNFR1 or COX2-deficient mice45. Consistent with these results, exosome treatment of non-stimulated macrophages augmented secretion of PGE2, TNF, IL-10 and IL-1-receptor antagonist (Fig. 7c), which may reprogramme macrophages3. These responses recapitulate those observed when macrophages are exposed to intact human or mouse MSCs, except that IL-6, CSF2 and IL-1 receptor 1 were increased more following exposure to mouse MSCs (Fig. 7b).

Subsequently, we treated TLR-signalling-deficient macrophages (TLR4–/–, TLR9–/–, MyD88–/–) or scavenger receptor-deficient macrophages (MARCO–/–) with MSC-derived exosomes. As shown in Fig. 7c, PGE2 production was similar following exosome treatment in all signalling-deficient macrophages as compared with wild-type cells from strain-matched C57BL/6J or BALB/CJ mice. In contrast, secretion of TNF and IL-10 was significantly (ANOVA followed by SNK post hoc pairwise comparisons) reduced in TLR4–/– and MYD88–/– macrophages as compared with wild-type cells following exosome treatment, and IL-10 secretion was also significantly reduced in macrophages from TLR9–/– mice (Fig. 7c). These data confirm the importance of TLRs and in particular MyD88-dependent pathways in mediating exosome-induced effects on macrophage function. Lastly, we showed that pre-incubation with dextran sulfate significantly (ANOVA followed by SNK post hoc pairwise comparisons)
Figure 7 | MSC-derived MVs inhibit TLR signalling in macrophages. (**a**) Upper panels, confocal microscopy showing intracellular localization of Cy5-labelled exosomes within macrophages 18 min post administration. Lower panel, nuclear localization of NF-κB in macrophages 2 h post administration of exosomes. Scale bars, 15 μm. (**b**) Partial heatmap illustrating mRNA levels of 84 TLR-associated transcripts in macrophages at 8 h post treatment with silica (20 mg cm⁻²), human or mouse MSCs (1:10 ratio) or human MSC-derived exosomes (40 μg protein). Transcript order is highest (top) to lowest (bottom), and each row represents a gene and each column a specific treatment. Red and green illustrates increased or decreased gene expression, respectively. Experiments were repeated four times. (**c**) Effect of exosome treatment on PGE2, TNF and IL-10 secretion in macrophages from the indicated mouse strains. Plotted data (mean ± s.e.m.) from experiments repeated five times. *P < 0.05 compared with native exosomes and indomethacin (Indo) as determined by ANOVA. (**d**) Upper panel, western blot illustrating the time-dependent effect of silica or human MSC-derived exosomes on expression of NF-κB, MyD88 and the indicated transcripts in macrophages at 8 h post treatment. Lower panel, fold change in expression of the indicated transcripts in macrophages RT–PCR demonstrating the negative regulation of TLR7 in macrophages. Pre-incubation of RAW 264.7 macrophages (Fig. 7c), while treatment of silica-exposed macrophages with exosomes ameliorated TLR7 induction following silica exposure (Fig. 7c). Pre-incubation of RAW 264.7 macrophages with indomethacin before treatment with native exosomes, or treatment with exosomes from DICER knockout MSCs significantly (ANOVA followed by SNK post hoc pairwise comparisons) reduced the observed effects on TLR mRNA expression (Fig. 7c) and reduced secretion of proteins such as TNF, MIP, MCP1, KC and IP-10 associated with macrophage activation (Supplementary Fig. 4B,C). The inhibitory effects of reduced the release of PGE2, TNF and IL-10 by exosome-treated macrophages, confirming the need for phagocytosis of MSC-derived vesicles in this process (Supplementary Fig. 4A).

To examine the role of microRNAs in macrophage tolerization, we treated RAW 264.7 cells, which use TLRs to recruit autophagy proteins in phagosomes to degrade its cargo⁴⁶, with exosomes derived from human MSCs transfected with or without a short-hairpin RNA (shRNA) designed to inhibit DICER expression in the presence or absence of indomethacin, a cyclooxygenase inhibitor (Supplementary Fig. 4B). Treatment of naïve RAW 264.7 macrophages with native exosomes enhanced TNF and reduced TLRs and MyD88 mRNA expression over 24 h (Fig. 7c), while treatment of silica-exposed macrophages with exosomes ameliorated TLR7 induction following silica exposure (Fig. 7c). Pre-incubation of RAW 264.7 macrophages with indomethacin before treatment with native exosomes, or treatment with exosomes from DICER knockout MSCs significantly (ANOVA followed by SNK post hoc pairwise comparisons) reduced the observed effects on TLR mRNA expression (Fig. 7c) and reduced secretion of proteins such as TNF, MIP, MCP1, KC and IP-10 associated with macrophage activation (Supplementary Fig. 4B,C). The inhibitory effects of...
indomethacin were restricted to TLR4 and MyD88 mRNA, while the effects of DICER-deficient exosomes were of greater magnitude and also involved negative regulation of TLR 7 and 9 (Fig. 7c). Concomitant treatment with indomethacin and exosomes from DICER-deficient exosomes demonstrated additive effects (Fig. 7c).

Importantly, miR-451 is one of the most abundantly expressed microRNAs in MSC-derived exosomes, but its maturation occurs independent of DICER47. Therefore, its expression is not altered in exosomes from DICER knockdown MSCs. MiR-451 negatively regulates cytokine production in dendritic cells infected with influenza virus48. Consistent with these results, transfection of RAW 264.7 macrophages with a miR-451 mimic significantly (Student’s t-test) decreased TNF mRNA expression in non-stimulated macrophages, and inhibited mRNA expression and protein release in silica-exposed macrophages (Supplementary Fig. 4D). In contrast, treatment of cells with a miR-451 antagonist yielded the opposite result. These data confirm a role of exosome-derived microRNAs in regulating cytokine expression in macrophages.

**Discussion**

MSCs modulate macrophage function by a variety of mechanisms, and this crosstalk contributes to their anti-inflammatory activity but the physiological relevance of this crosstalk remains obscure particularly as it relates to the survival and function of MSCs. In this study, we report that during their *ex vivo* culture MSCs transfer partially depolarized mitochondria to macrophages as a pro-survival mechanism in response to oxidative stress and that these mitochondria are repurposed via a process involving fusion to increase macrophage bioenergetics. Moreover, we show that MSCs also desensitize macrophages to mitochondrial transfers by repressing TLR-signalling. Our data indicate that MSCs employ two different types of MVs to achieve these goals. MSCs load mitochondria in the cytoplasm into LC3 containing MVs that are recovered from cell culture media with low-speed centrifugation. These MVs express the ESCRT-I-associated proteins TSG101 and ARRD1 and are extruded from cells in ARMMS11, which bud outwards directly from the plasma membrane where they are identified by macrophages. MSCs also shed exosomes that modulate TLR signalling and cytokine secretion in macrophages, in part, by transfer of regulatory microRNAs.

Previous reports indicate that mitochondria transferred by MSCs improve the energetic activity of the alveolar epithelium of LPS-treated mice30–33 and animal models of rotenone-induced airway injury52. However, the beneficial effects of mitochondria were limited to acceptor cells almost completely deficient of mitochondrial function30,53. Therefore, it is unclear whether rescue is because of the transfer of mitochondria, mtDNA or release of other mediators by MSCs33. Importantly, the bone marrow niche contains few, if any, epithelium, so the physiological relevance of this is unclear.

Our data suggest that mitochondrial transfer by MSCs is not altruistic but rather may serve to enhance MSCs’ cell survival by unloading partially depolarized mitochondria. Elimination of depolarized mitochondria is a priority for MSCs that experience high mtROS generation when cultured under atmospheric oxygen tension28 since inhibitors of the mitophagy flux induce MSC apoptosis. Unexpectedly, MV-mediated mitochondrial transfer augments macrophage function by improving mitochondrial bioenergetics. As reported for the alveolar epithelial cells, recovery of the energetic function of macrophages is characterized by an increased ability to generate ATP under conditions in which the cells exhibit mitochondrial uncoupling or an enhanced proton leak, and involves protection of the macrophage by reducing mtROS generation. This outcome is consistent with data indicating that transfer of mitochondria, even if partially depolarized, is followed by fusion inside the acceptor macrophage. Notably, several studies have reported that transfer of only a few mitochondria is sufficient to rescue cells depleted of mtDNA by culture in ethidium bromide30,34,55. Furthermore, the current study confirms evidence that exosomes, which do not carry mitochondria, contain nucleic acids56, including mtDNA that can be transferred, long term, in *vivo* to the lung. Presence of mtDNA inside exosomes is not surprising as mtDNAs are dispersed throughout the mitochondrial network as histone-free nucleoids with an average size in mammals under 100 nm, and contain a single copy of mtDNA per nucleoid57. However, we cannot completely exclude the possibility that the exosome preparations could be contaminated by apoptotic bodies.

Accumulation of mtDNA that escapes mitophagy induces TLR 9-mediated inflammation that in the case of cardiac muscle is associated with heart failure55 and mice transplanted with cells harbouring allogeneic mtDNA trigger MyD88 responses to reject these cells56. Therefore, silencing TLR responses in macrophages is likely necessary to induce tolerance to transferred
Figure 8 | Human MSCs and their exosomes prevent the accumulation of Ly6C\(^{hi}\) monocytes in the lungs of silica-exposed mice. (a) Upper panel, absolute number of F4/80/CD11b- and Ly6C/CCR2-expressing cells in lung tissue of mice 72 h post administration of saline (50 µl), silica (0.2 g kg\(^{-1}\)) or silica plus human MSC-derived exosomes (\(\sim 3 \times 10^{11}\) exosomes containing 40 µg protein). \(^*P<0.05\) compared with saline by t-test). Lower panel, representative histograms of flow cytometric data analysed in a showing the phenotype and frequency of cells recovered from lung tissue after enzymatic digestion. (b) Multiplex ELISA of inflammatory (TNF, MCP1 and KC) and fibrotic (TGF\(\beta\) and IL-10) mediators secreted by cultured F4/80/CD11b/ and Ly6C/CCR2 cells from a. Plotted values (mean ± s.e.m.) are from experiments using \(N=5\) animals per group and repeated three times. \(^*P<0.05\) compared with saline, \(#P<0.001\) compared with silica-treated monocytes by ANOVA.
mitochondria. Consistent with this hypothesis, we demonstrate that uptake of MSC-derived exosomes represses TLR signalling in macrophages and the production of inflammatory mediators by targeting pathways (TLRs and NF-κB) central to inflammation.

Interestingly, microRNAs present in MSC-derived exosomes are highly conserved between human MSC donors. One such microRNA, miR-451, is highly abundant in exosomes but is expressed at low levels in macrophages and dendritic cells where it regulates cytokine production.58–60. Mir-451 is known to suppress TNF, and macrophage migration inhibitory factor, which inhibits the anti-inflammatory effects of glucocorticooids and negatively regulates p38 MAPK signalling to protect from diabetic nephropathy.48,58–60. Indeed, ectopic expression of a mir-451 mimic in macrophages inhibits TNF secretion in response to silica. Consistent with these findings, MSC-derived exosomes prevent the recruitment of Ly6C hi monocytes and reduces secretion of pro-fibrotic IL-10 and TGFβ by these cells in the lung of silica-exposed mice. Therefore, these data suggest that, as tested in vitro, immunomodulatory activities may have evolved, in part, as a mechanism by which MSCs survive oxidative stress.

Figure 9 | Human MSCs and their exosomes ameliorate experimental silicosis. (a) Photomicrographs of lung sections stained with haematoxylin and eosine from mice 28 days after intratracheal administration of silica (0.2 g kg⁻¹) alone or followed 3 days later with an intravenous injection of human MSCs, human MSC-derived exosomes (~3 × 10¹⁵ exosomes containing 40 μg protein) or human fibroblasts (scale bars, 500 μm). (b) Upper panel, photomicrographs of Diff-Quick-stained cytospins of BAL from mice in a. Lower panel, differential cell counts showing counts of total cells (left) and percentage of macrophages, lymphocytes, neutrophils and eosinophils (right panels). *P < 0.05 compared with control, †P < 0.05 compared with fibroblasts treated mice by Student’s t-test. (c) Hydroxyproline content of lung tissue from animals treated as in a. *P < 0.001 compared with saline by Student’s t-test, †P < 0.05 compared with silica by ANOVA. (d) Quantification of mouse TNF, IL-6, IL-10 and Col1α1 levels in lung tissue from mice in a at 14 and 28 d post treatment. Plotted values (mean ± s.e.m.) are representative of experiments using 15 animals per group and repeated three times. *P < 0.001 compared with saline by Student’s t-test, †P < 0.05 compared with silica by ANOVA.
and serendipitously confers on cells the ability to suppress inflammation, in lung injury models. Indeed, our data illustrate a physiological role for the innate immune regulatory activity of MSCs, and in doing so further highlights the important association between MSCs and macrophages in vivo.

Methods

Human MSC and cell lines. Human MSCs were harvested from small volume aspirates of the iliac crest bone marrow from five healthy adult volunteers by the Center for Preparation and Distribution of Adult Stem Cells formerly at the Tulane University School of Medicine (New Orleans, LA). The Institutional Review Board at the Tulane University approved the procedures involved in the procurement of these cells as previously described. Human MSCs were also provided by the National Center for Preparation and Distribution of Adult Stem Cells at the University of Minnesota. Adult normal human dermal or lung fibroblasts were commercially obtained from Lonza (Walkersville, MD). Mouse MSCs were isolated from FVB/NJ or C57BL/6J mice (The Jackson Laboratory) as previously described. All studies employing human or mouse MSCs were performed using low passage (1–4) populations. Primary mouse macrophages were harvested from the peritoneal cavity of C57BL/6 mice (The Jackson Laboratory), TLR4−/− (The Jackson Laboratory), TLR9−/− and MyD88−/− (−/−) (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University and Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Suita) and MARCO/−/− (−/−) (provided by Dr Andreas Holan at the University of Montana) strains as previously reported. Human monocytes were isolated from the peripheral blood of normal human volunteers (blood donors at local blood bank) and were differentiated into mature macrophages as previously described. Cells were cultured at specific densities as described in the text. The mouse macrophage cell lines IC21 and RAW 264.7 were purchased from the American Type Culture Collection (Rockville, MD) and were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% Penicillin G and 100 U/mL of penicillin G and 100 μg/mL of streptomycin and grown at 37°C in 5% CO2.

Experimental silicosis. Crystalline silica (z-quartz, average size, 1.7 μm) was obtained from US Silica Co. (Berkley Springs, WV) and was selected by sedimentation according to Stokes’ law, acid hydrolyzed and baked overnight (200°C, 16 h) to remove contaminating substances. Silica in distilled water (Millipore), pathogen-free female C57BL/6 mice (Charles River Laboratories, Kingston, NY) weighing 20–25 g were housed in pathogen-free cabinets. Animals were anesthetized via intraperitoneal administration of sodium pentobarbital (200 mg/kg; Henry Schein, Indianapolis, IN), exposed to silica (0.2 μg/g−1) or saline (control) and samples administered to mice at ten different concentrations (500,000 cells per mouse), or human MSC-derived exosomes (∼3 × 1011 exosomes containing 40 μg protein). Animals were euthanized by exsanguinations at 3, 14 and 28 days post exposure. All lung samples were harvested at a terminal anesthesia point, fixed and embedded in paraffin for histological analysis or snap-frozen in liquid nitrogen and stored at −80°C for RNA or collagen analysis. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Collagen deposition was measured with hydroxyproline assay as previously described. Briefly, dried left lungs were acid-hydrolyzed in 6 N HCl at 110°C under nitrogen gas for 16 h. After intravenous injection of IC21 cells, lungs were separated from the trachea and incubated in a 60°C water bath. Following three consecutive high-speed centrifugations, a 40% dilution in PBS of the supernatant was oxidized with chloramine-T and the reaction was stopped with perchloric acid. Finally, samples were boiled with 6 M HCl for 15 min. Hydroxyproline content in lung was calculated from a hydroxyproline standard curve.

Inflammatory cells were measured in BALF of mice 28 days after the treatment with silica or intervention with MSCs, MSC-derived exosomes or fibroblasts. Mice were killed with an overdose of sodium pentobarbital and the lungs lavaged with a single volume of saline. Recovered BALF was concentrated above 80%. BALF cells were counted using a Z1 Coulter Particle Counter (Beckman Coulter Inc., Fullerton, CA), and 50,000 cells were transferred to glass slides using a Shandon Cytospin 4 (Thermo Electron Corporation, Pittsburgh, PA) at 750 rpm for 5 min. After 3 days of drying, cells were stained using the Diff-Quick stain (Dade Behring, DE), and macrophages, neutrophils and lymphocytes were counted (400 cells).

Isolation of MVs. Purification of exosomes was performed as previously described. Briefly, MSCs were expanded in multilayer culture plates (Millipore) in medium depleted of serum-derived MVs/exosomes by overnight ultracentrifugation at 100,000 g or in serum-reduced MSC medium (Invitrogen) supplemented with bovine serum albumin (Sigma-Aldrich). Conditioned medium from 1010 MSCs was collected every 24 h and was subjected to successive centrifugations at 300 and 2,000 g (10 min each) to remove cells. The cell-free supernatant was then centrifuged at 10,000 g for 30 min to remove cell debris and 100,000g (Beckman Coulter Optima L-90K ultracentrifuge) at 4°C for 90 min. The 10,000 and 100,000 g pellets were washed and suspended in 100 μL of PBS. Total protein was isolated as was isolated by ethanol precipitation was used to perform electron microscopy, western blotting or in vivo administration.

Electron microscopy analysis was performed on MSC extracellular vesicles loaded on carbon-coated grids and fixed in 4% paraformaldehyde. Grids were

Measuring MSC-derived exosomes by nanoparticle tracking. Exosomes were diluted in particle-free PBS and measured using Nanoparticle Tracking Analysis as described. Briefly, videos were collected using a NanoSight LM101 system equipped with a 405-nm laser and a highly sensitive digital camera (OrcaFlas2.8, with camera shutter speed fixed at 30.01 ms and gain set to 500), and analysed with the NTA software (NANOView software version 2.3). Ambient temperature was recorded on the NTA software while the video was being recorded. To study exosomes associated with MSCs, single 1-ml fractions were collected and sucrose density was determined on an aliquot of each gradient with the use of a refractometer (Reichert) and the fractions re-suspended in 3 mL PBS, centrifuged for 70 min at 100,000 g 4°C and the pellet suspended in sample buffer before being analysed with NanoSight tracker to determine particle number as previously described, or SDS–PAGE and western blot.

Live cell imaging and electron microscopy. Human or mouse MSCs (1 × 106) were infected with baculoviruses (BacMam2 delivery system, Life Technologies) encoding fluorescent proteins (Organelle Lights, Life Technologies) to target mitochondria (GFP) or LC3-associated with phagosomes (RFP) for 48 h at 37°C in a multiplicity of infection of 30 in accordance with the manufacturer’s specifications. Subsequently, 1 × 106 cells were plated into Mattek dishes and co-incubated with an equal amount of bone marrow-derived macrophages (C57BL/6), BALB/cJ and FVB(NJ) or macrophage cell lines (RAW 264.7, IC-2). MSCs were maintained alive using a Tokai Hit temperature-controlled humidified chamber at 37°C for periods of up to 72 h. During this time, multimode imaging events in five dimensions were obtained using high-speed confocal imaging on a Nikon Ti stand equipped with a 40× objective, an emission filter set and an Andor iXon electron-multiplying charge-coupled device camera. Images were collected using shuttered illumination in two colours with cubes specifically designed for GFP, ratiometric dyes or membrane/cyttoplasmic labels, and differential interference contrast modes. Image series were viewed as movies for qualitative assessment, or were imported to Metamorph for quantitative assessment of cell motion, protein expression and organelle transfer between MSCs and macrophages. To inhibit the mitophagic flux, double GFP and RFP-labeled MSCs were treated with 3 μM chloroquine (Invitrogen) and images taken every 5 min as described. In some experiments, MSCs were stained with Cy5 dye (Amersham) following the manufacturer’s instructions; MSC-conditioned medium was collected and extracellular MVs isolated as described above. Cy5-labeled vesicles were delivered under fluorescence microscopy to Mattek dishes with macroparticles from different mouse strains (C57BL/6, BALB/cJ, TLR4−/−, TLR9−/−, MyD88−/−, MARCO−/− and RAW 264.7) and phagocytosis followed as described above. To inhibit phagocytosis, macrophages were treated for 2 h with 100 μg/mL dextran sulfate (Sigma-Aldrich) before their exposure to Cy5-labeled vesicles.

Electron microscopy analysis was performed on MSC extracellular vesicles loaded on carbon-coated grids and fixed in 4% paraformaldehyde. Grids were
labelled with mouse or rabbit anti-human ATP synthase (ab54880). Grids were observed at 80 kV with a JEOL 1210 transmission electron microscope (TEM) with high-resolution AMT digital camera. Size of MVs was measured on five to seven different pictures using the TEM software.

RNA analysis. RNA was extracted using the RNeasy micro kit (Qiagen), and first-strand cDNA synthesized using SuperScript III (Invitrogen). Quantitative PCR was performed using the Taqman single-gene expression assay (Invitrogen) on an ABI 7900 Real Time PCR System (Applied Biosystems). The following oligonucleotide primers were used: NCOX-1 (Hs00392097_g1), IGADPH (Hs02596864_g1), mCOLLAGEN (Mm00472589_m1), mTNF (Mm00442582_m1), mIL-6 (Mm00446190_m1), mIL-10 (Mm00439614_m1), mMyD88 (Mm00440338_m1), mTLR9 (Mm00446193), mTLR7 (Mm00446590_m1) and mTLR4 (Mm00442733_m1). Mouse Toll-Like Receptor Signaling Pathway genes were measured using a mouse RT2 Profiler PCR Array System (SA Bioscience Version 4) as previously described. Alternatively, total RNA was isolated from five different human MSC donor populations or their exosomes using the Qiagen miRNeasy Mini Kit (PN 217004) according to the manufacturer’s instructions. Samples were then analysed using human microRNA microarrays from Agilent (Version 3, PN: G4470C) and data analysed using the Agilent GeneSpring GX II software. In some cases, array data were validated via quantitative RT–PCR.

miRNA from five human MSC donors was isolated using a human COXI, tRNA leucine and mitochondrial hypervariable regions I and II (HVRI) were sequenced to generate specific probes to allow for the documentation of fragment length polymorphism (FLP). We identified polymorphisms in the sequences of HVRI in each one of the five hMSC donors used in the current study and partial homology in human tRNA^Leu(UUR) probes between species that yielded a positive hybridization signal in mouse macrophages. Subsequently, we sequenced the COX gene from our five human MSC donors and designed COX-I-specific primers encompassing base pairs 64–293 that were conserved among the different human MSC populations. In Supplementary Fig. 4 we illustrate sequences spanning nucleotides 108–293 that exhibit little and no conserved among the different human MSC populations. In Supplementary Fig. 4 we illustrate sequences spanning nucleotides 108–293 that exhibit little and no conserved among the different human MSC populations. In Supplementary Fig. 4 we illustrate sequences spanning nucleotides 108–293 that exhibit little and no conserved among the different human MSC populations.

Dicer knockdown in human MSCs. Human MSCs (1 × 10⁶) were infected with a lentivirus encoding a Dicer-specific shRNA (Sigma-Aldrich) and reduced Dicer expression in selected cells was confirmed by western blotting using an anti-dicer antibody (H-212, Santa Cruz Biotechnology).

Statistics. Results are presented as mean ± s.e.m. from at least three experiments. Statistical differences between groups were analysed using one-way ANOVA followed by SNK test for post hoc pairwise comparisons (statview 4, Abacus Concepts Inc, Berkeley, CA). For microarray data, the significant differences were determined by one-way ANOVA with Holm–Sidak all pairwise multiple comparison procedure. The statistical significance of differences was set at P < 0.05.

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Author contributions
L.A.O. and D.G.P. conceived the project, supervised the studies and wrote the manuscript. M.D.G., J.N., E.S. and S.V.B. designed and conducted experiments. J.K. contributed to mouse and monocyte studies. N.K., Y.P.D., G.D.L. and G.D. contributed to RNA and microRNA studies. C.M.S.C., D.B.S. and S.C.W. conducted live image and electron microscopy studies. D.W.H.R. contributed to pathological assessment of mouse tissue. S.S. contributed to macrophage biogenesis studies. D.H.M. provided clinical-grade human MSCs.

Additional information
Accession codes: Microarray data have been deposited in the GEO NCBI database with accession code GSE71241.
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