LETTERS

Phagocyte-derived catecholamines enhance acute inflammatory injury

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It is becoming increasingly clear that the autonomic nervous system and the immune system demonstrate cross-talk during inflammation by means of sympathetic and parasympathetic pathways[1,2]. We investigated whether phagocytes are capable of *de novo* production of catecholamines, suggesting an autocrine/paracrine self-regulatory mechanism by catecholamines during inflammation, as has been described for lymphocytes[3]. Here we show that exposure of phagocytes to lipopolysaccharide led to a release of catecholamines and an induction of catecholamine-generating and degrading enzymes, indicating the presence of the complete intracellular machinery for the generation, release and inactivation of catecholamines. To assess the importance of these findings *in vivo*, we chose two models of acute lung injury. Blockade of α_2-adrenoreceptors or catecholamine-generating enzymes greatly suppressed lung inflammation, whereas the opposite was the case either for an α_2-adrenoreceptor agonist or for inhibition of catecholamine-degrading enzymes. We were able to exclude T cells or sympathetic nerve endings as sources of the injury-modulating catecholamines. Our studies identify phagocytes as a new source of catecholamines, which enhance the inflammatory response.

Although *tumor*, *rubor*, *dolor* and *calor* (the Latin terms for swelling, redness, pain and heat) are classical features of acute inflammation, the proximal trigger of these responses is neuronal in origin. Similarly, the systemic acute-phase response to infection involves neurone-dependent responses (fever and activation of the hormonal stress response), which are regulated mainly by the hypothalamus[2]. Innate immunity and inflammation are the first lines of defence[4], with initial responses involving non-specific cellular and humoral pathways, resulting in mediator release[1]. Immune and pro-inflammatory mediators that are subsequently released rapidly activate neuronal responses that amplify local immune and inflammatory responses designed to contain pathogens and trigger systemic neuroendocrine and regional neural responses that seek to return the system to a homeostatic state[2]. One of the key pathways involved in the neuroendocrine–immune modulating network is the autonomic nervous system. Recently, vagal parasympathetic signalling has been shown to have an important regulatory function in inflammation through cholinergic receptors on phagocytic cells[1,5,6]. Functional interplay of the adrenergic nervous system with the immune/inflammatory system may counterbalance effects of the parasympathetic nervous system. The presence and synthesis of catecholamines in lymphocytes were first described more than a decade ago[3,7]. Catecholamines can thereby modulate the proliferation, differentiation and apoptosis of lymphocytes and the production of cytokines through adrenoreceptors expressed on T and B cells[8–12]. To investigate whether macrophages and neutrophils (polymorphonuclear cells; PMNs) might be able to generate and release catecholamines, as has been suggested[13,14], cells were incubated with bacterial lipopolysaccharide (LPS) and levels of noradrenaline and adrenaline in cell supernatants were determined. As shown in Fig. 1a–d, noradrenaline and adrenaline levels increased significantly in cell supernatants 15 min after exposure of macrophages and PMNs to LPS, decreasing at 1 h and 2 h, and then increasing by 4 h. Experiments were repeated with human PMNs exposed to the potent complement anaphylatoxin C5a (10 nM), with virtually identical results (data not shown). On the basis of these findings, we conducted experiments to evaluate the presence of two key enzymes involved in catecholamine synthesis in macrophages and PMNs after stimulation with LPS. Tyrosine hydroxylase (TH) is known to be the rate-limiting step in catecholamine synthesis, whereas dopamine β-hydroxylase (DBH) accounts for the final step of noradrenaline synthesis, converting dopamine to noradrenaline[15]. Macrophages or blood PMNs were incubated for 15 min and 4 h with 30 ng ml^{-1} LPS. Thereafter, messenger RNA expression for catecholamine-generating enzymes was analysed by real-time polymerase chain reaction (PCR). Low baseline mRNA levels for both key enzymes (TH and DBH) were detected in unstimulated macrophages. At 4 h after cell exposure to LPS, mRNAs for both enzymes were significantly upregulated (Fig. 1e, f). PMNs expressed low constitutive levels of mRNA for TH and DBH, but both were clearly upregulated 4 h after the addition of LPS (Fig. 1g, h). The actions of catecholamines can be terminated in three ways: by reuptake into nerve terminals, by diffusion into extracellular fluids or by metabolic transformation. Two enzymes are essential in the metabolic inactivation of catecholamines: monoamine oxidase (MAO) and catechol *O*-methyltransferase (COMT). Unstimulated macrophages contained mRNA for both COMT and MAO-A (Fig. 2a, b). After exposure of cells to LPS, the quantity of mRNA for COMT decreased whereas that for MAO-A increased. In accordance with this, when proteins were analysed by western blotting, exposure to LPS caused a decrease in macrophage COMT (Fig. 2c), whereas the same intervention brought about higher levels of MAO-A protein (Fig. 2d). Similar patterns were found in PMNs. Incubation with LPS decreased the level of mRNA for COMT (Fig. 2e) and increased that of mRNA for MAO-A (Fig. 2f). COMT protein amounts decreased when PMNs were exposed to LPS (Fig. 2g); the opposite was true for MAO-A (Fig. 2h). During COMT-mediated *O*-methylation of catecholamines, *S*-adenosylmethionine (SAM) serves as the methyl donor and is subsequently converted to *S*-adenosyl-L-homocysteine (SAH) after donation of the methyl group to the substrate. It has been known for many years that elevated SAH levels can serve as a strong inhibitor of the COMT-mediated *O*-methylation metabolism of catechols[16,17]. Increased phagocyte

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production of catecholamines might therefore lead to an increased turnover of COMT, increasing levels of the powerful feedback inhibitor SAH and ultimately causing the downregulation of cellular mRNA and protein levels of COMT (Fig. 2). Collectively, these data indicate that LPS-stimulated macrophages and PMNs express catecholamine-generating and degrading enzymes and release noradrenaline and adrenaline after exposure to LPS *in vitro*. Thus, phagocytes possess the complete cellular machinery to generate catecholamines *de novo* and to release and inactivate them.

To evaluate the relevance of these findings *in vivo*, we used two different models of acute lung injury (induced by LPS and by IgG immune complex) to study the effects of adrenoceptor blockade in the setting of a single-organ injury. Adrenoceptors were blocked *in vivo* by using the appropriate antagonists in the two different models of lung inflammatory injury (Fig. 3a, c). The antagonists were delivered intraperitoneally. Vascular leakage of albumin into lungs, which correlates with lung injury, was measured after the deposition of IgG immune complexes (IC) or LPS in the lung. α_1-Adrenoceptors were blocked by prazosin, whereas α_2-adrenoceptors were blocked with RX 821002. β_1-Adrenoceptors were blocked by metoprolol, and β_2-adrenoceptor blockade was induced by ICI 118551. Only blockade of the α_2-adrenoceptors significantly reduced the intensity of the albumin leak into lung in the two models of injury, whereas blockade of other receptors neither intensified nor reduced the level of lung injury (Fig. 3a, c). Blockade of the β_2-adrenoceptors with different doses of ICI 118551 (10–20 mg kg^{-1}) failed to alter injury intensity significantly (data not shown). To clarify the significance of the α_2-adrenoceptors in our models of lung injury, an α_2-adrenergic agonist (UK 14304) was used to assess effects on lung injury. As shown in Fig. 3b, d, the severity of lung damage increased twofold and fourfold, respectively, when induction of IgG or LPS lung injury was followed immediately by intraperitoneal administration of UK 14304, whereas the α_2-adrenoceptor inhibitor RX 821002 substantially suppressed lung injury in each of the two models. The effects of these interventions were consistent with the morphological evaluation of lungs (Fig. 3e–k). Although the levels of lung injury with LPS and IC (positive controls) were comparable (Fig. 3f, i, respectively), animals treated with UK 14304 showed clearly increased evidence of injury as reflected in increased PMN numbers, fibrin and haemorrhage in the interstitial and alveolar compartments (Fig. 3g, j). In sharp contrast, blockade of the α_2-adrenoceptor by RX 821002 reduced the intensity of inflammation and injury (Fig. 4h, k) in comparison with positive controls, indicating a key function for the

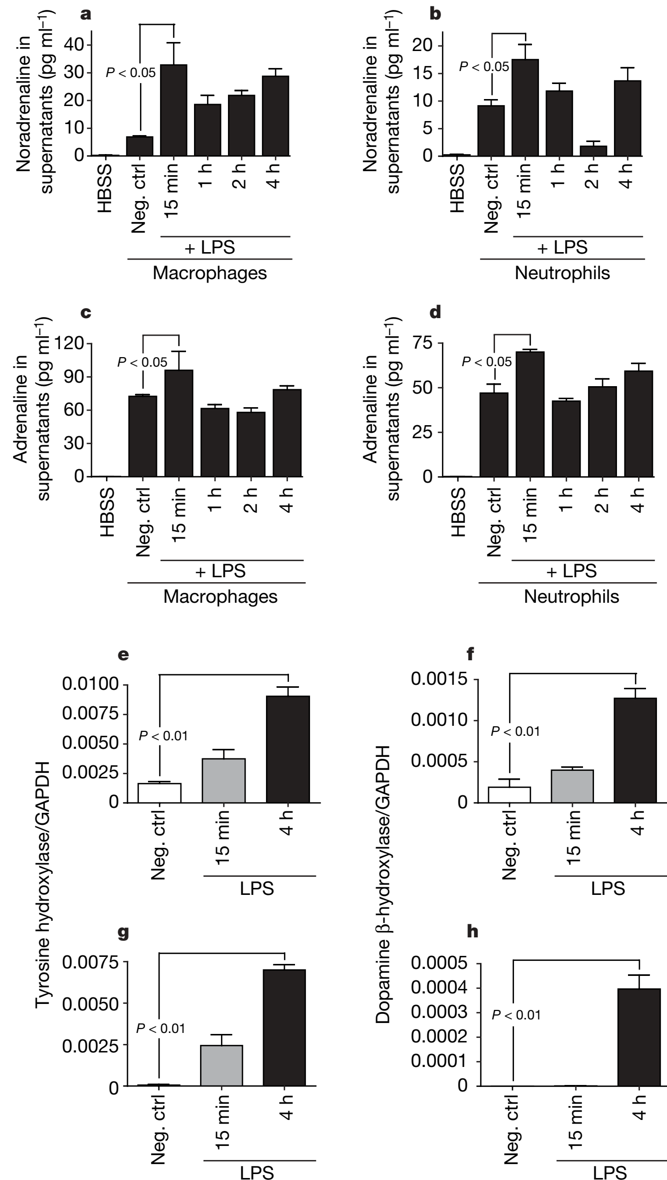

Figure 1 | Release of catecholamines from phagocytes and the presence of catecholamine-producing enzymes in phagocytes. a–d, After isolation, alveolar macrophages and blood PMNs were stimulated with 30 ng ml^{-1} of LPS *in vitro*. Cellular supernatant fluids were collected as a function of time thereafter and then analysed by enzyme-linked immunosorbent assay for noradrenaline (**a**, **b**) and adrenaline (**c**, **d**). HBSS served as negative control (neg. ctrl). **e–h**, After stimulation with 30 ng ml^{-1} of LPS, mRNA from alveolar macrophages (**e**, **f**) and neutrophils (**g**, **h**) was isolated and subjected to real-time PCR analysis for tyrosine hydroxylase and dopamine β-hydroxylase. Each bar represents $n \geq 6$ samples. All data are presented as means ± s.e.m.

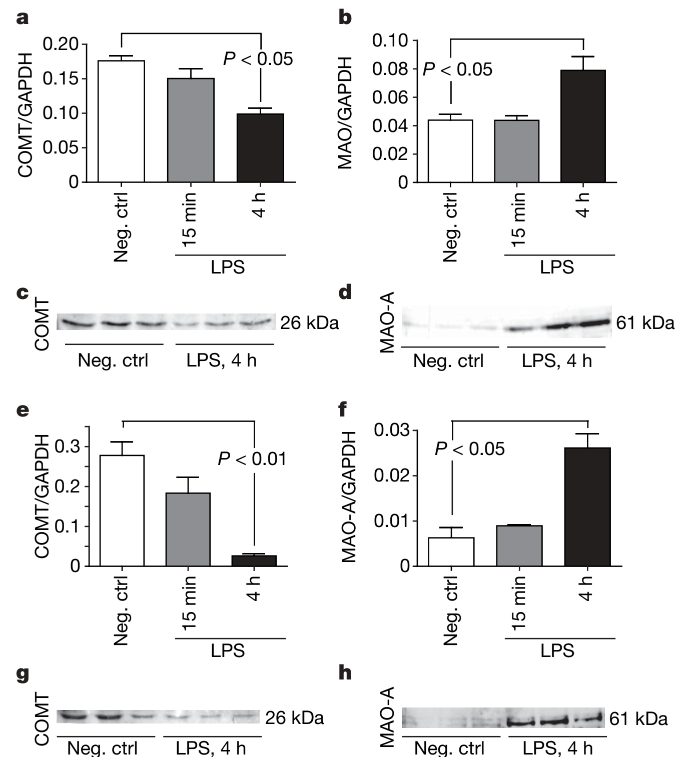

Figure 2 | Expression of catecholamine-inactivating enzymes in phagocytes. After isolation of macrophages and PMNs and stimulation *in vitro* with 30 ng of LPS, mRNA and protein from alveolar macrophages (**a–d**) and blood PMNs (**e–h**) were subjected to analysis by real-time PCR (**a**, **b**, **e**, **f**) and western blotting (**c**, **d**, **g**, **h**) for the catecholamine-inactivating enzymes COMT (**a**, **c**, **e**, **g**) or monoamine oxidase A (MAO-A) (**b**, **d**, **f**, **h**). Western blots were repeated at least three separate times. Representative blots are shown. Real-time PCR data are presented as means ± s.e.m.; $n \geq 5$ per bar. Neg. ctrl, negative control.

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α_2-adrenoceptor in these models of lung injury. α_2-Adrenoceptors belong to the G-protein-coupled, seven-transmembrane receptor family[18] and are currently divided into three subtypes: α_{2A}, α_{2B} and α_{2C} (ref. 19). In the present study we found that both alveolar macrophages and blood PMNs express all three subtypes of the α_2-adrenoceptors (Supplementary Fig. 1).

To exclude T cells or the sympathetic nerve endings as sources of the injury-modulating catecholamines, we achieved T-cell depletion with an antibody and sympathetic denervation by administering reserpine. Recovery of sympathetic function after administration of reserpine takes up to several weeks[20]. In the present study we harvested and exposed PMNs and alveolar macrophages to LPS 4 days after administration of reserpine *in vivo* to determine whether the production of catecholamines by phagocytes is impaired by reserpine. There was no difference in noradrenaline production between phagocytes from control and reserpine-treated animals (Fig. 4a). Accordingly, we induced lung injury in the presence or absence of α_2-adrenoceptor blockade in control animals and animals treated 4 days earlier with reserpine. Injury levels were significantly increased in reserpine-treated animals, whereas α_2-adrenoceptor blockade completely abolished vascular leakage (Fig. 4c). We repeated these experiments *in vitro* and *in vivo* 2 h after reserpine administration, with identical results (data not shown). As expected, adrenal gland homogenates from reserpine-treated animals had almost no detectable noradrenaline (Fig. 4b). In parallel experiments, mice were depleted of CD4 T cells and the intensity of albumin leakage was assessed. There was no change in the level of injury between T-cell-depleted and isotype-control-treated littermates. In the same animals, α_2-adrenoceptor blockade was protective (Fig. 4d). Bronchoalveolar lavage (BAL) fluid analysis was performed to determine whether the sympathetic nervous system and T cells were key sources of catecholamines in current models of lung injury. Peak levels of adrenaline occurred in BAL fluids 2 h after the initiation of immune-complex-mediated injury (Fig. 4e). In parallel, an adrenaline peak was found 3 h after the initiation of LPS-induced lung injury (data not shown). Other mice were depleted of PMNs, alveolar macrophages or T cells or were pharmacologically sympathectomized and subjected to lung injury. BAL fluids were obtained and measured 2 h after the initiation of injury. Whereas depletion of PMNs and alveolar macrophages significantly decreased the presence

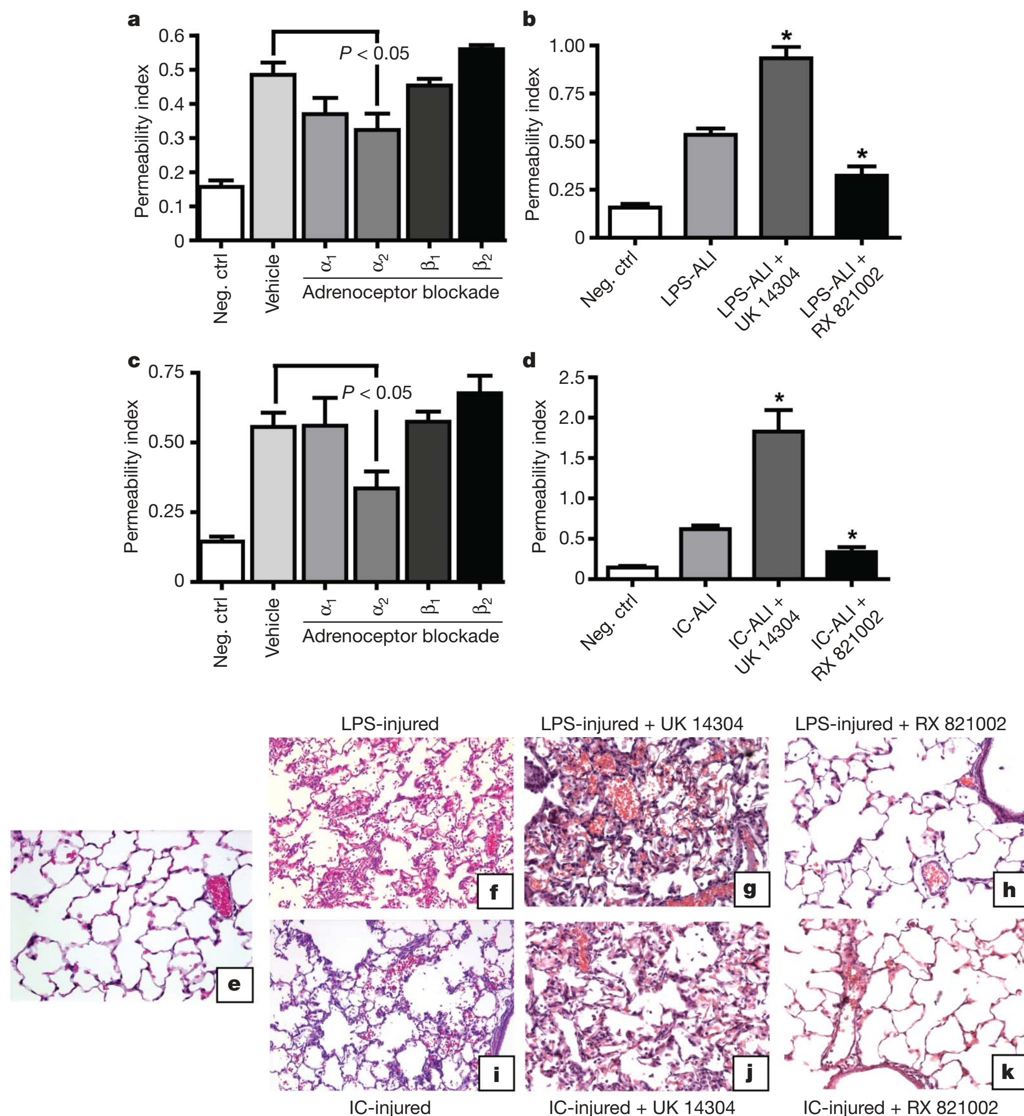

Figure 3 | The α_2-adrenoceptor mediates the severity of experimental acute lung injury. a–d, Acute lung injury (ALI) was induced by LPS (**a**, **b**) or IgG IC (**c**, **d**) in rats and injury severity was assessed by vascular leak of vascular ^{125}I-albumin. Adrenoceptors were blocked pharmacologically as described in the text. All data are presented as means ± s.e.m.; $n \geq 5$ per bar. Asterisks in **b** and **d** indicate $P < 0.01$ versus LPS-induced and IC-induced lung injury, respectively. **e–k**, Hematoxylin and eosin-stained lung sections. **e**, Uninjured lung. **f–h**, LPS-injured animals. **i–k**, IC-injured littermates. Sections are representative for $n \geq 3$ per group. Neg. ctrl, negative control.

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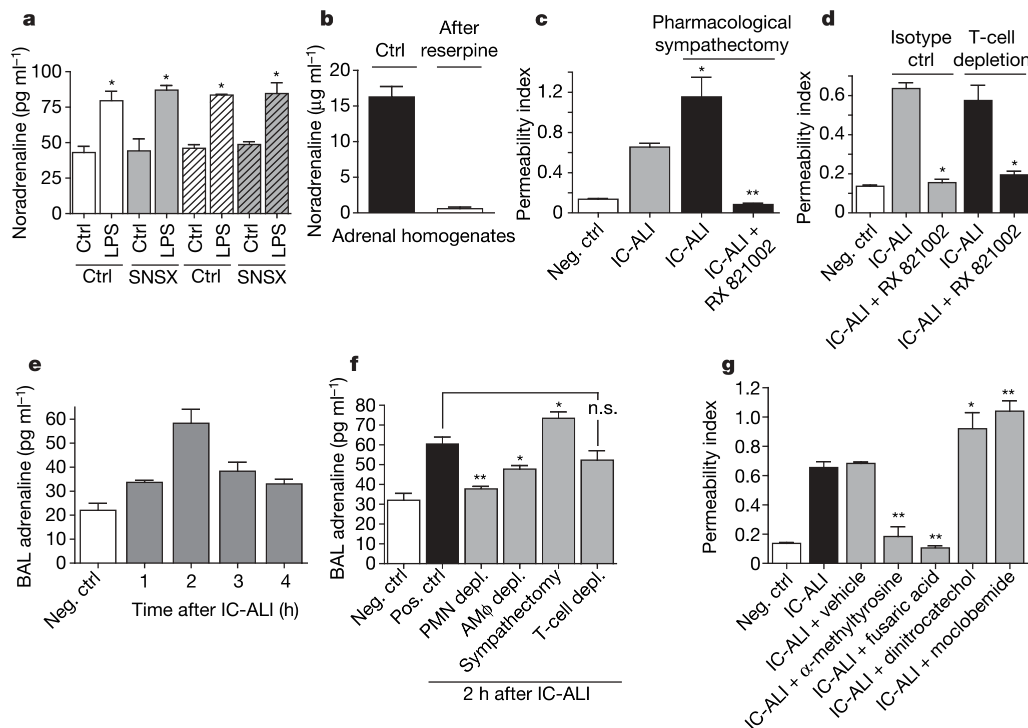

Figure 4 | Phagocyte-derived catecholamines mediate lung injury. **a**, After sympathectomy (SNSX), noradrenaline production in supernatants of LPS-stimulated phagocytes was assessed. Open bars, PMNs; hatched bars, alveolar macrophages. Asterisk, $P < 0.01$ versus respective control (ctrl). **b**, Sympathectomy was confirmed by adrenal gland homogenates. **c**, Severity of IC-induced acute lung injury (IC-ALI) in sympathectomized animals. Asterisk, $P < 0.05$ versus IC-ALI; two asterisks, $P < 0.01$ versus IC-ALI. **d**, Mice were T-cell depleted, and lung injury was compared with that in isotype-antibody-treated littermates. Asterisk, $P < 0.01$ versus IC-ALI. **e**, Adrenaline peak was determined in BAL fluids. **f**, Mice were depleted (depl.) of PMN, alveolar macrophages (AMΦ) or T cells or were sympathectomized, and adrenaline was measured 2 h after IC-ALI. Asterisk, $P < 0.05$ versus positive control (pos. ctrl); two asterisks, $P < 0.01$ versus positive control. Neg. ctrl, negative control. **g**, Severity of lung injury after inhibition of TH, DBH, COMT or MAO. Asterisk, $P < 0.05$ versus IC-ALI plus vehicle; two asterisks, $P < 0.01$ versus IC-ALI plus vehicle. Data are presented as means ± s.e.m.; $n \geq 5$ per bar.

of adrenaline in BAL fluids, reserpine-induced sympathectomy did not decrease adrenaline levels (Fig. 4f). These data indicate that the presence of adrenaline in BAL fluids requires PMNs and macrophages but not T cells. Phagocytes of adrenalectomized animals have higher baseline levels of TH, DBH and catecholamine secretion (M.A.F., D.R., R.P.L., A.J.C., D.E.D., B.A.N., F.S.Z., J.V.S., A. B. Lentsch, M.S.H.-L. and P.A.W., unpublished observations). It might be possible that the baseline production of catecholamines in phagocytes is increased as a compensatory endogenous mechanism to pharmacological sympathectomy. Last, we showed that pharmacological blockade of the catecholamine-generating TH (by using α-methyltyrosine) and DBH (by using fusaric acid) greatly diminished the inflammatory response, whereas inhibition of the catecholamine-degrading enzymes COMT (with dinitrocatechol) and MAO (with moclobemide) significantly increased lung injury as defined by the permeability index (Fig. 4g).

Alveolar macrophages and PMNs have a central function in the pathophysiology of IgG IC-induced lung injury[21]. The α_2-adrenoreceptor is expressed on alveolar macrophages[11], and modulation of the α_2-adrenoceptor regulates LPS-induced production of tumour necrosis factor (TNF)-α *in vitro* from alveolar macrophages[22]. We sought to determine whether *in vitro* blockade of the adrenoceptors, especially the α_2-adrenoreceptor, might be linked to the present findings *in vivo*. There was a variable inhibition *in vitro* of various macrophage-derived cytokines (TNF-α, interleukin-6 and interleukin-1β) and chemokines (cytokine-induced neutrophil chemoattractant (CINC)-1) by α_1-adrenoceptor, α_2-adrenoceptor, β_1-adrenoceptor or β_2-adrenoceptor blockade (Supplementary Table 1), with blockade of the α_2-adrenoreceptor achieving the most consistent suppression of mediators. Identical experiments with PMNs revealed similar patterns (data not shown).

Taken together, the results of this study indicate that both alveolar macrophages and blood PMNs are capable of the production of catecholamines *de novo* and of their release, implicating phagocytes as a previously unrecognized source of catecholamines. Enhanced production or decreased generation of catecholamines (with the use of inhibitors of catecholamine-generating or catecholamine-degrading enzymes) increases or decreases the intensity of inflammatory injury, respectively. Blockade of α_2-adrenoceptors *in vivo* greatly decreases the intensity of inflammatory injury. In many respects, the phagocytic system can be looked on as a diffusely expressed adrenergic organ.

METHODS SUMMARY

Animals and anaesthesia. All studies were conducted in accordance with the University of Michigan Committee on Use and Care of Animals. Specific pathogen-free male Long–Evans rats (300–325 g; Taconic) or C57BL/6 mice (Jackson Laboratory) were anaesthetized with isoflurane (Aerrane; Henry Schein).

Isolation of PMNs and alveolar macrophages. Whole blood was drawn into syringes containing anticoagulant citrate dextrose (ACD) (Baxter Health Care), in a volume ratio of 1:10. Neutrophils were isolated by using Ficoll-Paque gradient centrifugation (Pharmacia Biotech AB) followed by a dextran sedimentation step. After hypotonic lysis of residual red blood cells, neutrophils were resuspended in Hanks balanced salt solution (HBSS) containing Ca^{2+} and Mg^{2+}. Rat alveolar macrophages were isolated by repeatedly lavaging the lungs of anaesthetized healthy animals. After centrifugation of BAL fluids, cells were resuspended in HBSS containing Ca^{2+} and Mg^{2+}.

Stimulation of phagocytes by LPS. Macrophages or PMNs (3×10^6 or 5×10^6 in 1.0 ml) were isolated and incubated with 30 or 50 ng of LPS (Escherichia coli 0111:B4; Sigma Aldrich). Supernatants were obtained and frozen at −80 °C.

Enzyme-linked immunosorbent assay/enzyme immunoassay analysis of cell supernatants. Noradrenaline and adrenaline enzyme immunoassays were obtained from Rocky Mountain Diagnostics.

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Alveolitis induced by IgG IC and by LPS. IgG IC-induced alveolitis was performed as described previously[23,24]. For LPS-induced alveolitis, rats received 300 μg of LPS (Sigma Aldrich) intratracheally.
Morphological assessment of lung injury. At 4 h after IgG IC deposition or 6 h after LPS deposition, lungs were fixed by intratracheal instillation of 10 ml of PBS-buffered (pH 7.2) formalin (10%). The lungs were further fixed in a 10% buffered formalin solution for histological examination by tissue sectioning and staining with haematoxylin and eosin.
Statistical analysis. All values are expressed as means ± s.e.m. Data were analysed with ANOVA and a Student–Newman–Keuls test. Differences were considered significant at $P \leq 0.05$.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Western blot analysis of MAO-A, COMT, α_{2A}-adrenoceptors, α_{2B}-adrenoceptors and α_{2C}-adrenoceptors. After isolation and exposure to LPS, phagocytes were lysed with Laemmli buffer containing 5% mercaptoethanol. Samples were then separated in a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking (5% Tris-buffered saline pH 7.0 containing 0.1% Tween 20) and washing, membranes were then incubated overnight with primary antibodies against adrenoreceptors, MAO-A and COMT (Santa Cruz Biotechnology) and antibodies against GAPDH (Abcam) at 4 °C. Membranes were analysed by enhanced chemiluminescence.

Isolation of mRNA and detection of rat α_{2A}-adrenoreceptors, α_{2B}-adrenoreceptors, α_{2C}-adrenoreceptors, TH, DBH, MAO-A and COMT by quantitative real-time PCR analysis. Total RNA was extracted from phagocytes with Trizol reagent (Life Technologies) in accordance with the manufacturer's instructions. Reverse transcription was performed with 1 µg of RNA by using AMV Reverse Transcriptase (Roche). Real-time quantitative PCR was then performed with SYBR Green PCR Master Mix (Applied Biosystems). The following primer pairs were used: rat α_{2A} adrenoreceptor 5′-CTT GGC CCT CGA CGT GCT CTT TTG-3′ (forward) and 5′-CGG CGG CGC GGA ACA GG-3′ (reverse); rat α_{2B} adrenoreceptor, 5′-TCG GCC ATC ACC TTT CTC ATC CTT-3′ (forward) and 5′-GGG CGT CGG GGC GTT GGT C-3′ (reverse); rat α_{2C} adrenoreceptor, 5′-TGG CTC ATC TCG GCT GTC ATC TCC-3′ (forward) and 5′-TGT CCC CCT CGG CAC CCT CTC-3′ (reverse); rat TH, 5′-AGT CTG GCC TTC CGC GTG TTT CAA-3′ (forward) and 5′-GGG CGC TGG ATA CGA GAG GCA TAG-3′ (reverse); rat DBH, 5′-CTG CGA CCC CAA GGA TTA TG-3′ (forward) and 5′-CAG CAC GTG GCG ACA GTA GTT-3′ (reverse); rat MAO-A, 5′-GTT GAG CGG CTG ATC CAC TTT GT-3′ (forward) and 5′-CCG GCC TCT CCA GCT TCA CTC T-3′ (reverse); rat COMT, 5′-GAC GCG AAA GGC CAA ATC AT-3′ (forward) and 5′-GCT GCT GCT CCC TCT CAC ATA-3′ (reverse); rat GAPDH, 5′-CGG CAA GTT CAA CGG CAC AGT CA-3′ (forward) and 5′-CTT TCC AGA GGG GCC ATC CAC AG-3′ (reverse). Product sizes were 403 base pairs (bp) (α_{2A}-adrenoreceptor), 427 bp (α_{2B}-adrenoreceptor), 409 bp (α_{2C}-adrenoreceptor), 458 bp (TH), 439 bp (DBH), 451 bp (MAO-A), 435 bp (COMT) and 424 bp (GAPDH).

Drugs. All drugs (from Sigma Aldrich unless stated otherwise) were administered intraperitoneally: propane-1,2-diol (vehicle; 1 ml kg^{-1}), Prazosin (2 mg kg^{-1}), RX 821002 (10 mg kg^{-1}), metoprolol (15 mg kg^{-1}), ICI 118551 (10 mg kg^{-1}) and UK 14304 (2 mg kg^{-1}), α-methyltyrosine (200 mg kg^{-1}), fusaric acid (75 mg kg^{-1}), 3,5-dinitrocatechol (50 mg kg^{-1}) and moclobemide (20 mg kg^{-1}; Biotrend).

Depletion of CD4 T cells, neutrophils and alveolar macrophages. For depletion of CD4 T cells, C57BL/6 mice were treated with 0.2 mg of CD4-specific monoclonal antibody (GK1.5; BD Pharmingen) intraperitoneally 48 h before induction of lung injury. Alveolar macrophages and neutrophils were depleted as described previously[25,26].

Pharmacological sympathectomy. To achieve pharmacological sympathectomy, mice were injected intraperitoneally with reserpine (5 mg kg^{-1}). Four days after reserpine injection, acute lung injury was initiated as described above.

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