MicroRNAs (miRNAs) play critical roles in various biological processes, including cell proliferation, development and host defence. However, the molecular mechanism for miRNAs in regulating bacterial-induced inflammation remains largely unclear. Here, we report that miR-301b augments pro-inflammatory response during pulmonary infection, and caffeine suppresses the effect of miR-301b and thereby augments respiratory immunity. LPS treatment or *Pseudomonas aeruginosa* infection induces miR-301b expression via a TLR4/MyD88/NF-κB pathway. Importantly, caffeine decreases miR-301b expression through negative regulation of the cAMP/PKA/NF-κB axis. Further, c-Myb is identified as a target of miR-301b, which positively modulates anti-inflammatory cytokines IL-4 and TGF-β1, but negatively regulates pro-inflammatory cytokines MIP-1α and IL-17A. Moreover, repression of miR-301b results in increased transcription of c-Myb and elevated levels of neutrophil infiltration, thereby alleviating infectious symptoms in mice. These findings reveal miR-301b as a new controller of inflammatory response by repressing c-Myb function to inhibit the anti-inflammatory response to bacterial infection, representing a novel mechanism for balancing inflammation.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that regulate gene expression at post-transcriptional levels. The literature suggests that a single miRNA can play multiple roles in influencing divergent pathways by binding to different target mRNAs at 3'-untranslated regions (UTR). miRNAs have been implicated previously in many cellular processes, including but not limited to development, cellular proliferation, apoptosis and necrosis. More recently, findings have indicated that miRNAs may modulate immune responses at different stages, including the production of cytokines/chemokines, the expression of adhesion and co-stimulatory molecules and the release of exosomes. Several toll-like receptors (TLRs)/NF-κB-responsive miRNAs are shown to be abundantly expressed, and may be tailored to harness the TLRs/NF-κB pathway by interfering with their target gene expression. miRNAs may also provide feedback regulation of NF-κB signalling to maintain homeostasis. Importantly, a number of miRNAs have been implicated in downregulating inflammatory responses, helping to control overzealous inflammation during host defence against pathogens and other processes both in vitro and in vivo.

As a metabolic stimulant, caffeine (CAF) enhances performance by reducing physical fatigue and drowsiness. CAF exerts either positive or negative effects on neurons or other cells beneficial for patients with Parkinson’s disease and cancer. CAF has also been tested for the treatment of bronchopulmonary dysplasia in premature infants. However, excessive ingestion of CAF may lead to psychiatric diseases, including mild anxiety, jitteriness and insomnia. Previous studies have revealed that CAF activates the innate immune response in tumour models or suppresses tumour-necrosis factor (TNF)-α via the cyclic AMP/protein kinase A (cAMP/PKA) pathway in vitro. CAF could also negatively affect carcinogenesis through the regulation of DNA damage and repair.

More recently, CAF has been linked to regulation of miRNAs in cardiovascular diseases, but has not been linked to lung diseases. In particular, CAF has not been demonstrated to participate in the respiratory inflammatory response against microorganisms.

We aimed to explore the roles of CAF in host defence and in particular defined a regulatory mechanism exerted by a novel miRNA—miR-301b—in pulmonary infection. Using miScript PCR System Screening, we analysed the expression of a set of miRNAs in alveolar macrophages following CAF treatment. miR-301b, miR-301a and miR-15a are significantly suppressed, and only miR-301b is determined to be responsive and plays roles in regulating inflammation during bacterial infection. We observed less neutrophil infiltration following *Pseudomonas aeruginosa* infection at early times in the lungs of miR-301b-mimics-transfected mice. Neutrophils are normally associated with a heightened inflammatory response and exacerbated lung injury in acute infection models. We then dissected the molecular mechanism by which miR-301b impacts neutrophil infiltration and the inflammatory response. Our results indicate that CAF negatively regulates the inflammatory response in bacterial infection by inhibiting miR-301b and augmenting the expression of the miR-301b target c-Myb.

**Results**

miR-301b expression is altered by CAF both in vitro and in vivo. CAF is implicated in infection and inflammation. To search for miRNAs that may be regulated by CAF, we used MH-S cells to perform array-based miRNA profiling. Because CAF treatment at higher concentrations (>3 mM) for 24 h affected cell viability (Fig. 1a), a safer concentration (1 mM) was used in subsequent
Figure 1 | CAF decreases miR-301b, 301a and 15a expression. a, MH-S cell viability was examined by MTT assay after treatment with CAF at different concentrations for 24 h. b, Expression of miRNAs was measured using the miScript PCR assay in MH-S cells with or without CAF treatment (1 mM, 24 h).

We found that inhibition of p38 and NF-κB suppressed LPS-induced miR-301b expression (Fig. 2d). These findings indicate that miR-301b expression may be regulated by a TLR4/p38/NF-κB axis.

To pinpoint the mechanistic feature of this signalling axis, we used cell culture models and, surprisingly, observed that CAF inhibited LPS-induced miR-301b expression in vitro (Fig. 2e). Pre-incubation with CAF for 24 h led to the strongest inhibition in miR-301b in MH-12, MH-S and BMDM cells (Supplementary Fig. 2b). CAF has previously been reported to indirectly preserve cAMP by inhibiting phosphodiesterase, an enzyme that degrades cAMP, thus activating downstream PKA24. Next, cells were pretreated with Sp-8-Br (activator of cAMP-dependent PkA) and Rp-8-Br (PKA inhibitor), respectively25. Sp-8-Br was found to inhibit LPS-induced miR-301b expression (Fig. 2f). Phosphorylation of PKA and cAMP-response element binding protein (CREB-1) was increased upon CAF treatment, but no further increase occurred upon LPS treatment (Supplementary Fig. 2c). A previous report has revealed that PKA activating agents inhibit NF-κB-dependent reporter gene expression26. Although LPS increased the phosphorylation of p65 (p-p65), CAF decreased p-p65, as assessed by immunoblotting (Supplementary Fig. 2c). We found that CREB-1 was translocated into the nucleus following CAF treatment, suggesting distinct roles of CREB and NF-κB as downstream transcription factors of PkA (Supplementary Fig. 2d). Supplementary Fig. 2e,f shows that p65 knockdown inhibited miR-301b expression, while knockdown of PkA or CREB-1 had no effects on miR-301b expression after LPS challenge. Additionally, PkA knockdown was found to restore the suppressed miR-301b expression after CAF treatment (Supplementary Fig. 2g). Together, our data suggest that CAF regulates miR-301b expression through a cAMP signalling axis.

miR-301b modulates bacterium-induced inflammatory responses in vivo. Both mRNA and IL-4, IL-6, TNF-α and TGF-β1 protein levels were found to increase following LPS treatment, as
Figure 2 | CAF inhibits LPS-induced miR-301b expression by regulating the cAMP/PKA/CREM/NF-κB axis. a, LPS (100 ng ml\(^{-1}\)) induces miR-301b expression in MH-S, MLE-12 and BMDM cells in a time-dependent manner as determined by qRT-PCR. b, Northern blot showing the expression of miR-301b, 15a and 301a in cells treated with LPS (100 ng ml\(^{-1}\), 6 h). c, qRT-PCR of LPS-induced miR-301b expression in BMDM from TLR4\(^{-/-}\) and TLR2\(^{-/-}\) mice, or in MH-S and MLE-12 cells with TLR4, MyD88 or TRIF knockdown. d, Cells were cultured for 6 h with LPS in combination with FR180204 (0.3 μM), SB203580 (0.5 μM), SP600125 (90 nM) and SN50 (10 μM), miR-301b was analysed by qRT-PCR. e, After CAF pretreatment for 24 h, LPS (100 ng ml\(^{-1}\)) was added for another 6 h incubation and miR-301b was detected by qRT-PCR. f, Cells were treated with LPS in combination with Sp-8-Br (2.5 μM) and Rp-8-Br (4 μM) for 6 h and miR-301b was analysed by qRT-PCR. Data in a-f are shown as means + s.d. from triplicates. Data in b are representative of three independent experiments. *P < 0.05, **P < 0.01. One-way ANOVA with Tukey’s post hoc.

determined by qRT-PCR and enzyme-linked immunosorbent assay (ELISA) (Supplementary Fig. 3a,b). To assess the regulatory mechanism in inflammation regulation, chemically synthesized mimics (301b-m) or inhibitors (301b-i) were transfected into cells. Having verified the transfection efficiencies, we studied the effects of these mimics or inhibitors on inflammatory cytokines compared to negative controls (NS-m or NS-i) (Supplementary Fig. 4a). Anti-inflammatory cytokines (IL-4 and TGF-β1) induced by LPS treatment were inhibited by 301b-m but increased by 301b-i compared to negative controls, as determined by qRT-PCR or ELISA (Supplementary Fig. 3a,b). However, the pro-inflammatory cytokines (IL-6 and TNF-α) were not changed significantly by manipulating miR-301b (Supplementary Fig. 3a,b). Additionally, the protein levels of IL-4 and TGF-β1 in MLE-12 cells were also lower in cells overexpressing 301b-m than those in control cells, whereas 301b-i increased these anti-inflammatory cytokines, as assessed by western blotting (Supplementary Fig. 4b). We next found that the conditioned medium of MLE-12 cells (treated with miR-301-m) decreased the migration capabilities of MH-S cells by ~30% using Boyden chamber assay (Supplementary Fig. 4c). The viability of MH-S cells was not affected by 301b-m transfection (Supplementary Fig. 4d), but it did decrease their phagocytosis ability (Supplementary Fig. 4e,f). Collectively, these findings suggest that miR-301b may selectively downregulate the expression of anti-inflammatory genes as well as phagocytic activities.

To determine whether the in vitro phenomenon occurs similarly in vivo and can be expanded to other bacteria, we established acute respiratory infections in cells and mice with different bacteria, P. aeruginosa (Pa), Klebsiella pneumoniae (Kp) and Streptococcus pneumoniae (Sp). These bacterial infections all induced miR-301b expression both in vitro and in vivo, but did not induce miR-15a and miR-301a (Supplementary Fig. 5a). To further study the physiological impact of miR-301b, systemic administration of miR-301b was performed to determine bacterium-induced gene expression in vivo. The expression of miR-301b in the lungs was consistent with in vitro data (Supplementary Fig. 5b). At 24 h after bacterial infection, IL-4 and TGF-β1 in bronchoalveolar lavage (BAL) were decreased by 301b-m, but IL-6 and TNF-α were not affected (Supplementary Fig. 5c). The lungs of mice infected with Pa were immunoblotted to detect IL-4 and TGF-β1, which produced similar results to the ELISA data (Fig. 3a). Interestingly, 301b-m injection resulted in higher bacterial burdens than in the NS-m group, while the effects of 301b-i were opposite those of the mimics, confirming the physiological relevance in pulmonary infection (Fig. 3b and Supplementary Fig. 5d). To determine whether miR-301b contributes to inflammatory cell infiltration, we performed haematoxylin and eosin (H&E) staining and found that 301b-m overexpression markedly inhibited neutrophil recruitment upon bacterial infection (Fig. 3c). More severe lung injury was observed in 301b-m-treated mice, whereas less lung injury was observed in 301b-i-treated mice after Pa
infection (Fig. 3e and Supplementary Fig. 5e). The 301b-m was found to inhibit neutrophil recruitment following Pa infection (Fig. 3e). In addition, myeloperoxidase (MPO) assay demonstrated that neutrophil infiltration was decreased in 301b-m transfected mice (Fig. 3g). Neutrophil mobilization and recruitment to the lung is usually associated with release of chemokines/cytokines to BAL. We next performed an ELISA to detect MIP-1α and IL-17A, which showed similar reductions (Fig. 3h). These findings are consistent with in vitro data about phagocyte migration, suggesting that phagocyte recruitment and phagocytosis may be impacted by enhanced miR-301b levels. The inhibitory effects of miR-301b on host defence may thus be due to effects on neutrophil recruitment to the lung.

c-Myb is a target of miR-301b. c-Myb possesses a conserved miR-301b seed sequence in its 3′ UTR with a high mirSVR score (Fig. 4a) and has been demonstrated to be a critical player in immune responses. We experimentally confirmed the prediction and showed that 301b-m inhibited the activity of a luciferase reporter containing c-Myb 3′ UTR, while the activity of luciferase reporters containing the mutant 3′ UTR of c-Myb was not significantly altered (Fig. 4b and Supplementary Table 3). Furthermore, c-Myb mRNA abundance in BMDM from mice transfected with 301b-m was lower than that in NS-m group (Fig. 4c). Similarly, 301b-m decreased c-Myb mRNA levels (Fig. 4d). Moreover, in vivo data showed that bacterial infection resulted in decreased c-Myb abundance, which was further decreased by 301b-m, but this decrease could be reversed by 301b-i (Fig. 4e). These data collectively identify c-Myb as a bona fide target of miR-301b.

c-Myb negatively modulates infection-induced anti-inflammatory responses. To determine whether c-Myb plays a role in infection-induced inflammatory responses, we knocked down c-Myb in MH-S and MLE-12 cells (Fig. 5a). Interestingly, c-Myb mRNA decreased upon LPS stimulation, which is negatively correlated with miR-301b (Fig. 5a). Anti-inflammatory cytokines (IL-4 and TGF-β1) were partially reduced (∼50–60%) by c-Myb knockdown (Fig. 5a,b). However, pro-inflammatory cytokines (IL-6 and TNF-α) did not change after c-Myb was knocked down (Fig. 5c). Similarly, anti-inflammatory cytokines were inhibited in BAL fluids of c-Myb knockdown mice after bacterial (Pa, Kp or Sp) infection (Supplementary Fig. 6a). However, IL-6 or TNF-α
release was not altered by c-Myb knockdown (Supplementary Fig. 6a). Bacterial burdens were higher in c-Myb knockdown mice locally and systemically than in control mice (Supplementary Fig. 6a). Bacterial burdens were higher in c-Myb knockdown mice than in control mice (Fig. 5d,e). MPO assays and ELISA also showed decreased MPO activity and anti-inflammatory responses in c-Myb knockdown mice, which confirmed the above observations (Fig. 5f,g). Overall, the results imply that c-Myb is a functional target of miR-301b in bacterial-induced inflammatory responses.

CAF decreases the susceptibility to bacterial infection in vivo. To define the direct role of CAF in bacterial invasion, we set out to elucidate how CAF administration improves cytokine production. We observed that CAF significantly decreased miR-301b levels and partially increased c-Myb mRNA levels within several hours either before or after infection (Supplementary Fig. 7a). Interestingly, 6 h CAF pretreatment followed by bacterial infection for 24 h led to the strongest miR-301b inhibition and highest c-Myb mRNA level in the tested times (Supplementary Fig. 7a). To verify whether CAF has a broad role in inflammatory responses, we used primary human alveolar macrophages to recapitulate the above results. CAF was found to prevent LPS-induced p65 nuclear translocation, but CREB-1 nuclear translocation was only changed following CAF treatment (Supplementary Fig. 8a). The miRNA database shows that hsa-miR-301b has two high scoring binding sites with human MYB mRNA (Supplementary Fig. 8b). We found that both hsa-miR-301b and hsa-miR-301a were decreased by CAF, but only hsa-miR-301b was increased by LPS stimulation (Supplementary Fig. 8c). Similar results were obtained in primary human alveolar macrophages, as seen in murine cells, showing that CAF increased MYB expression and enhanced LPS-induced IL-4 production (Supplementary Fig. 8d).

Next, we focused on Pa infection to determine whether CAF has effects on inflammatory responses in a physiological context. IL-4 and TGF-β1 secretion in BAL fluids was found to be negatively correlated with miR-301b, but positively correlated with c-Myb, when mice were administrated with CAF at different times and IL-10 and TNF-α were not altered (Supplementary Fig. 9a). In addition, 24 h post Pa infection, the lungs (6 h pretreated with CAF group) showed similar results to the ELISA data (Fig. 6a). We next performed in vitro bacterial growth assays, demonstrating that CAF did not have obvious antibacterial effects (Fig. 6b). However, CAF was able to inhibit bacterial growth in vivo, particularly with 6 h CAF pretreatment (Fig. 6c). Furthermore, CAF significantly alleviated lung injury and increased neutrophil infiltration (Fig. 6d,e and Supplementary Fig. 10a,b). MPO assay showed a similar increase in neutrophil MPO activity (Fig. 6f). CAF was also found to increase MIP-1α and IL-17A levels in mouse BAL, while CAF alone has no function in the inflammatory response without infection (Supplementary Fig. 10c,d). We also noted no significant tissue injury in the liver and kidney (Supplementary Fig. 10e). However, infection induced lower c-Myb expression in all these tissues, whereas CAF counteracted this damaging effect by increasing c-Myb expression (Supplementary Fig. 10f). Lower CAF treatment did not induce significant neutrophil infiltration in BAL (Supplementary Fig. 10g) and had limited effects on infection mitigation, as reflected by bacterial burdens (Supplementary Fig. 10h). To determine a potential causal relationship, we established a gain-of-function setting using miR-301b mimics. After CAF treatment and subsequent infection, we found that CAF has no impact on levels of IL-4 and TGF-β1 in BAL (Fig. 6g).
Figure 5 | c-Myb plays a role in mediating infection-induced inflammatory responses. a, MHS-5 and MLE-12 cells were transfected with Ctrl siRNA or c-Myb siRNA for 24 h and were treated with LPS (100 ng ml\(^{-1}\)) for 6 h. mRNA of c-Myb, IL-4 and TGF-\(\beta\)1 was measured by qRT-PCR. b, c-Myb, IL-4 and TGF-\(\beta\)1 protein was assessed in MLE-12 cells from above by immunoblotting. c, mRNA levels of IL-6 and TNF-\(\alpha\) were determined in the MH-5 and MLE-12 cells by qRT-PCR. d, Mice (\(n = 3\)) were i.v. injected with Ctrl siRNA or c-Myb siRNA (50 \(\mu\)g per mouse, 24 h) and were infected with Pa (\(1 \times 10^7\) c.f.u.) for 24 h. Lungs were evaluated by H&E staining. Scale bar, 100 \(\mu\)m. e, Lungs were evaluated by immunostaining with a Ly6G antibody to show neutrophil infiltration. Scale bar, 50 \(\mu\)m. f, g, After treatment as above, MPO assay and ELISA were performed to determine MPO activities in lung homogenates or MIP-1\(\alpha\) and IL-17A levels in BAL. Data in \(\textbf{a-c}\) are means + s.d. from triplicates. Data in \(\textbf{b}\) are representative of triplicate samples. Data in \(\textbf{d-e}\) are representative of the three mice. Data in \(\textbf{f-g}\) are means + s.d. from three mice. *\(P < 0.05\), **\(P < 0.01\). One-way ANOVA with Tukey’s post hoc.

The overall findings reveal a central role of CAF and miR-301b in the bacteria-induced immune response. Modulation of miR-301b may be a novel strategy to regulate the c-Myb pathway by altering inflammation to reduce lung and other organ damage, and thus may ultimately be beneficial for patients with acute infection (Fig. 6i). We found ten other miRNAs (for example, miR-301a) with the potential to bind to the same or surrounding sites in c-Myb 3’UTR, which are very similar to miR-301b (Supplementary Fig. 12a). We used miR-301a, miR-302b (ref. 20) and miR-15a as controls to establish the specific role of miR-301b. Although both 301a-m and 302b-m showed the potential to target c-Myb 3’UTR, only 301a-m played a similar role in LPS-induced IL-4 release in MLE-12 cells (Supplementary Fig. 12b,c). Overall, the results confirmed that miR-301b serves as a novel regulator of inflammatory response following bacterial infection.

Discussion

We have revealed a critical role of miR-301b in affecting inflammatory response against infection, which is negatively regulated by CAF. Using both in vitro and in vivo infection models, we show that CAF downregulates miR-301b, thereby increasing neutrophil...
infiltration and anti-inflammatory cytokines (IL-4 and TGF-β1). Neutrophils are normally increased with increased lung injury.22 However, early polymorphonuclear (PMN) may be beneficial for bacterial clearance, but in late phases may not be beneficial, as excessive PMN accumulation can result in life-threatening tissue injury.21,23 miR-301b may inhibit the initial recruitment of neutrophils for the immune response against bacterial infection. We also found that increased cAMP downregulates NF-κB but does not affect TNF-α upon infection. This might be because TNF-α release is predominantly related to MAPK signaling following bacterial infection.26,29 This pro-inflammatory response is so strong that it is difficult to differentiate CAF effects from the drastic response to bacterial infection. We have demonstrated that the expression of miR-301b after bacterial infection is dependent on the transcriptional activity of NF-κB. Chemical inhibitors of NF-κB or siRNA interference of the NF-κB component (p65) markedly decrease infection-induced miR-301b. Previous reports have

**Figure 6 | CAF and 301b-i decrease susceptibility and mortality after pulmonary bacterial infection.** a. Mice (n = 3) were infected with Pa (1 × 10^7 c.f.u.) for 24 h. CAF (50 mg kg^-1, i.v.) was injected at indicated times (pre-treatment strategy or post-infection treatment). Lungs were immunoblotted to detect c-Myb, IL-4 and TGF-β protein. b. The same amounts of PAO1 were cultured in LB liquid medium with carbenicillin (50 μg ml^-1) or different concentrations of CAF overnight. The same amounts of culture mediums were plated for c.f.u. counting. c. C.f.u. counting was also performed using lung homogenates from mice infected with PAO1 as above. d. Lungs from mice infected with Pa (24 h) and CAF (6 h pretreatment) were evaluated by H&E staining. Scale bar, 100 μm. e. Lungs were evaluated by immunostaining with a Ly6G antibody to show neutrophil infiltration. Scale bar, 50 μm. f. After treatment as above, MPO assays were performed to test MPO activity in lung homogenates. g. Mice were pretreated with miR-301b mimics or inhibitors. At 24 h later, mice were infected with Pa combined with CAF treatment as above. Inflammatory cytokines in BAL were assayed using ELISA. h. Kaplan-Meier survival curves (n = 12) of Pa-infected mice. Mice were i.v. injected with vehicle, NS-i or 301b-i (50 μg per mouse, 24 h), PBS or CAF (6 h pretreatment), the mice were challenged with Pa (1 × 10^7 c.f.u.). Survival was determined up to 7 days. Log-rank test. i. Schematic diagram showing how miR-301b is regulated and how it affects immune responses against bacterial infection. Data in a, d, e are representative of three mice. Data in b are means ± s.d. from triplicate. Data in c, f, g are means ± s.d. from three mice. *P < 0.05. One-way ANOVA with Tukey’s post hoc.
demonstrated that miR-301b promotes cell invasiveness by target-
ing TP63 in pancreatic carcinoma cells. However, no study has linked miR-301b to the regulation of inflammatory responses against infection. Our study has therefore extended the biological significance and function of miR-301b in the control of inflammation.

Earlier work has characterized miR-155, miR-1-146a and 132 as critical regulators in innate immunity and autoimmune inflammation. We have recently identified miR-302b as a regulator of the anti-

We have also shown that c-Myb plays crucial roles in regulating the balance between Th1 and Th2 cytokine production, which is critical for patients with severe infection and sepsis shock. In summary, we have identified a novel function of CAF in preferen-
tially regulating anti-inflammatory cytokine production by targeting IRAK4, thus inhibiting the pro-inflammatory response. This pathway may be tailored to alleviate sepsis-mediated tissue injury.

**Methods**

**Ethics statement.** Animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Dakota, School of Medicine (assurance no. A3917-01). Dissections and injections were performed under anesthesia, which was induced and maintained with ketamine hydrochloride and xylazine, and all efforts were made to minimize animal suffering.

**Mice.** TLR4−/− and TLR2−/− mice were provided by J. Sharma at the University of North Dakota. These mice were based on the C57BL/6J genetic background, and normal age- and sex-matched C57BL/6J mice were used as wild-type (WT) controls. Mice were kept and bred in the animal facility at the University of North Dakota. Animal experiments were performed with randomization. By using 12 mice for survival assays, this gave an effective power of better than 0.9 (when α = 0.1). Other experiments used at least three mice to achieve better statistical results.

**Cells.** MLE-12 and MH-S cells were obtained from ATCC and cultured in HiTES medium (MLE-12) and RPMI 1640 medium (MH-S) supplemented with 10% fetal bovine serum (HyClone Laboratories) and 100 U ml−1 of penicillin/streptomycin (Life Technologies) antibiotics in a 37 °C incubator with 5% CO2. The cell lines were authenticated by phenotypic analysis and were tested for mycoplasma. Mouse alveolar macrophage cells were isolated from the lung by BAL. In brief, the trachea was cannulated with a 20-gauge catheter, and 0.9 ml BAL buffer was instilled, flushed four times and retrieved. A total of 3.0 ml bronchoalveolar lavage fluid (BALF) was retrieved from each mouse and cytospin slides prepared with 0.5 ml BALF were analysed by H&E staining (Fisher) to enumerate leukocyte subtypes. The transcription factor c-Myb has been recognized as a proto-

**Infection experiments.** Pseudomonas aeruginosa strain PA01 WT was provided by J. Stone (Harvard Medical School). PA01-GFP strain was obtained from G. Pier (Brighton and Women’s Hospital, Harvard Medical School). Klebsiella pneumoniae strain Kp WT (ATCC 45816 serotype II) was provided by V. Miller (University of North Carolina). Streptococcus pneumoniae strain was obtained from ATCC (49619, Serotype 19F). LPS purified by phenol extraction from Es. coli serotype 0111:B4 was bought from Sigma. Caffeine was also bought from Sigma (Powder, ReagentPlus, #C0750).

After culturing in Luria-Bertani (LB) broth at 37 °C with vigorous shaking overnight, bacteria were centrifuged at 6,000 r.p.m. for 5 min and then resuspended in fresh LB broth to allow growth until the mid-logarithmic phase. The concentration of bacteria was counted by reading at an optical density of 0.5 OD at 600 nm. After infection at a multiplicity of infection (MOI) of 10:1 (bacteria:cells) cells were cultured in Luria-Bertani (LB) broth with a 100 U ml−1 of penicillin/streptomycin (Life Technologies) antibiotics in a 37 °C incubator with 5% CO2. The cell lines were authenticated by phenotypic analysis and were tested for mycoplasma. Mouse alveolar macrophage cells were isolated from the lung by BAL. In brief, the trachea was cannulated with a 20-gauge catheter, and 0.9 ml BAL buffer was instilled, flushed four times and retrieved. A total of 3.0 ml bronchoalveolar lavage fluid (BALF) was retrieved from each mouse and cytospin slides prepared with 0.5 ml BALF were analysed by H&E staining (Fisher) to enumerate leukocyte subtypes. The transcription factor c-Myb has been recognized as a proto-

**Transfection.** TLR4, MyD88, TRIF, PKA, p65, CREB-1 and c-Myb siRNA were bought from Santa Cruz Biotechnology. miRNA negative control (NS-m, CN-001000-01-05), miR-301a mimics (301a-m, C-310482-05), miR-301b mimics (301b-m, C-310669-05) miRNA inhibitor negative control (NS-i, C-310190-01) or miR-301b inhibitor (301b-i, IH-310775-04) were bought from Dharmacon. MH-S or MLE-12 cells were transfected, respectively, with miRNA or siRNA in serum-free medium using Fugene6 (Roche), Lipofectamine 2000 (Invitrogen), or Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. For co-immunoprecipitation, the cell lines were cotransfected with a HA-tagged construct as described above, downregulation of hyper-inflammation may be particularly beneficial for patients with severe infection and sepsis shock. In summary, we have identified a novel function of CAF in preferen-
tially regulating the anti-inflammatory response in bacterial infection through the repression of miR-301b expression. This pathway may be tailored to alleviate sepsis-mediated tissue injury.

**Measurement of miRNA and mRNA expression.** Total RNAs were isolated using TRIzol reagent (Invitrogen), following the manufacturer’s instructions. RNAs were eluted in RNase-free water and stored at −80 °C. For non-radioactive northern blot, DIG-modified miR-301b, miR-301a, miR-15a and sno202 probes were synthesized by Integrated DNA Technologies. Northern blot was performed following a published protocol described previously. Real-time PCR profiling of miRNA was performed using a SYBR Green-based, miScript PCR System (Qiagen). The
expression of other miRNAs was detected using Quantitect SYBR Green RT-PCR Kit (Qiagen). The separate well 2-ΔΔCt cycle threshold method was used to determine relative quantities of individual miRNAs or mRNA, and these were expressed as fold changes, respectively. GAPDH was used as control throughout the manuscript.

**Histological analysis.** Organs were fixed in 10% formalin by a routine histological procedure. BAL (5 μl) and blood were applied evenly on microscope slides. After H&E staining (Thermo Fisher), the numbers of neutrophils were counted using a light microscope. The formalin-fixed tissues were used for H&E staining or immunohistochemical staining to examine tissue damage or Ly6G expression post-treatment.

**Immunoblotting.** Samples taken from each cell or tissue of mice after experimental treatment were lysed with RIPA buffer (30 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol and complete cocktail (Life technologies) and phosphatases (Sigma)). Lysates were centrifuged at 14,000 g for 15 min, the supernatants were collected, and concentration was quantitated. The samples were boiled for 10 min and equal amounts were applied to 12% SDS gel for Immunoblotting.

**Transfection of HEK293 cells.** HEK293 cells (Invitrogen) were cultured in glass bottomed dishes (MatTek). Fluorescence images were obtained by a laser scanner (LSCM). The relative expression of miRNAs or mRNA, and these were expressed as fold changes, respectively. GAPDH was used as control throughout the manuscript.

**Cytokine profiling and assays.** Cytokine concentrations in the first 0.6 ml BAL fluid collected at the indicated times after infection were measured by standard ELISA kits following the manufacturer’s instructions ( Bioscience). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out following the manufacturer’s instructions. Lung tissues were homogenized and equal amounts of proteins were used for MPO assays.

**Confocal laser scanning microscopy.** Alveolar macrophage, MH-S and MLE-12 cells were cultured in glass bottomed dishes (MatTek). Fluorescence images were obtained using an LSM 510 Meta confocal microscope (Carl Zeiss Micro Imaging®). For immunostaining, the cells were fixed in 3.7% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS and incubated with blocking buffer containing 2% BSA for 30 min. Cells were incubated with primary antibodies at 1/500 dilution in blocking buffer for 1 h and washed three times. After incubation with appropriate fluorescent-conjugated secondary antibodies, the coverslips were mounted on slides with Vectashield mounting medium. The images were captured and processed using the software provided by the manufacturer. 

**Migration assay.** MLE-12 cells were transfected with NS-m or 30b-m and treated with LPS. The culture medium was collected and placed in the lower chamber. MH-S cells were centrifuged into the Boyden chamber with an 8 μm porous membrane (Corning) in 100 μl RPMI 1640 medium supplied with 1% serum and incubated at 37 °C for 4 h. Cells were removed from the upper side of membranes, and nuclei of migratory cells on the lower side of the membrane were stained with crystal violet. Migration of LPS-treated mice was accepted as significant at P < 0.05.

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**Author contributions**
X.L., S.H., C.H. and M.W conceived and designed the experiments. X.L., S.H., R.L. and X.Z. performed the experiments. X.L., S.H., R.L., X.Z., S.Z., M.Y., Y.Y. and Y.W. analysed the data. C.H. and M.W. contributed reagents, materials and analysis tools. X.L., S.H., C.H. and M.W. wrote the manuscript.

**Additional information**
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**Competing interests**
The authors declare no competing financial interests.