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Expression of therapeutic proteins after delivery of chemically modified mRNA in mice

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Current viral vectors for gene therapy[1–3] are associated with serious safety concerns, including leukemogenesis[4], and nonviral vectors are limited by low gene transfer efficiency[5]. Here we investigate the therapeutic utility of chemically modified mRNA as an alternative to DNA-based gene therapy. A combination of nucleotide modifications abrogates mRNA interaction with Toll-like receptor (TLR)3, TLR7, TLR8 and retinoid-inducible gene I (RIG-I), resulting in low immunogenicity and higher stability in mice. A single intramuscular injection of modified murine erythropoietin mRNA raises the average hematocrit in mice from 51.5% to 64.2% after 28 days. In a mouse model of a lethal congenital lung disease caused by a lack of surfactant protein B (SP-B), twice weekly local application of an aerosol of modified SP-B mRNA to the lung restored 71% of the wild-type SP-B expression, and treated mice survived until the predetermined end of the study after 28 days.

Although mRNA is a potential therapeutic in a variety of medical indications, ranging from hereditary or acquired metabolic diseases to regenerative medicine, clinical applications have so far been limited to therapeutic cancer vaccination due to the strong immunogenicity and the limited stability of conventional mRNAs[6].

To investigate whether chemical modification can facilitate *in vivo* delivery of mRNA, we first tested red fluorescent protein (RFP) mRNA that was modified to increase stability and to avoid the activation of innate immunity commonly associated with mRNA transcribed *in vitro*[7]. We found that replacement of only 25% of uridine and cytidine with 2-thiouridine and 5-methyl-cytidine synergistically decreased mRNA binding to pattern recognition receptors, such as TLR3, TLR7, TLR8 and RIG-I, in human peripheral blood mononuclear cells (PBMCs) (**Fig. 1a**). These modifications substantially decreased activation of the innate immune system *in vitro* and *in vivo* (**Fig. 1b** and **Supplementary Fig. 1a**) and concomitantly increased the stability of the mRNA, allowing for prolonged, high-level cellular RFP expression in > 80% of cultured human and mouse alveolar type II epithelial cells (**Fig. 1c**,**d** and **Supplementary Fig. 1b**,**c**), as demonstrated by flow cytometry and cytokine enzyme-linked immunosorbent assays (ELISA) of cell culture supernatants and mouse blood sera.

We then explored whether modified mRNA has similar effects in a physiologically functional model *in vivo* using mouse erythropoietin (mEpo). Epo is a hormone produced by the peritubular capillary endothelial cells in the kidney and liver, regulating red blood cell production. Epo is used as a therapeutic in people who require dialysis[8] and has been shown to be beneficial in certain neurological diseases like schizophrenia[9]. Again, we demonstrated that double modification of mEpo mRNA significantly reduced binding to pattern recognition receptors and decreased activation of the immune system *in vivo* compared to unmodified RNA (**Fig. 2a**,**b**). Fourteen days after intramuscular administration of double-modified mRNA, mEpo levels were 4.8- and 4.4-fold higher compared to untreated mice or mice injected with unmodified mEpo mRNA, respectively ($P = 0.014$) (**Fig. 2c**,**d**). Concomitantly, the hematocrit of mice injected with dual-modified mEpo mRNA increased to 64.2 ± 5.9% ($P = 0.021$) at day 28, which was significantly higher compared to unmodified mEpo mRNA (54.2 ± 0.5%; $P = 0.021$) and to untreated mice (51.5 ± 1%) (**Fig. 2c**,**d**).

Next, we determined whether the effects of modified mRNA treatment could be adapted for lung administration in wild-type mice. When dual-modified EGFPLuc mRNA (coding for a fusion protein of enhanced green fluorescent protein and luciferase) was directly introduced into the lungs using a syringe-based, high-pressure spraying technique (**Supplementary Fig. 2**), we observed robust and prolonged expression of the EGFPLuc construct. Expression levels were more than four orders of magnitude higher in mice treated with modified mRNA than in mice receiving unmodified EGFPLuc mRNA at day 5 after treatment (**Supplementary Fig. 1d**).

We also investigated the therapeutic potential of modified mRNA in the lung, using a mouse model of a hereditary disease, congenital surfactant protein B (SP-B) deficiency. SP-B enhances the spreading, adsorption and stability of surfactant lipids required for the reduction of surface tension in the alveolus[10]. Congenital SP-B deficiency is a rare, lethal condition leading to death soon after birth[11]. It is refractory to surfactant replacement, and lung transplantation is the only

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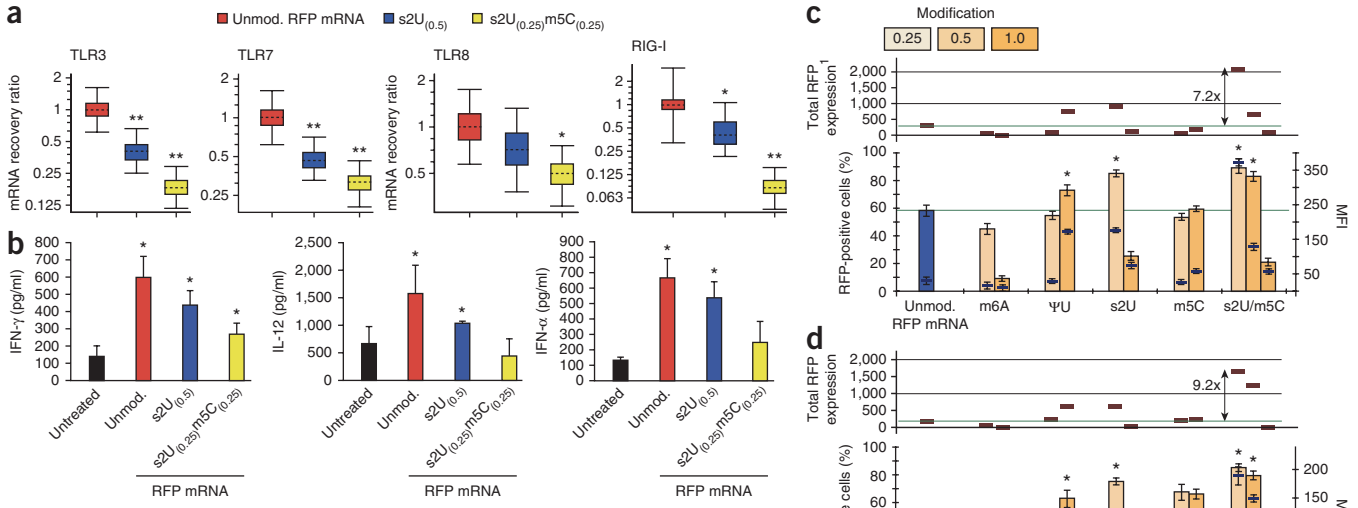

Figure 1 Modification of mRNA enhances transgene expression and decreases immune responses by reducing immunoreceptor binding. (**a**) RNA immunoprecipitation (RIP). PBMCs were transfected with 5 μg red fluorescent protein (RFP) mRNA, and the recovery ratios were determined by RIP using TLR3, TLR7, TLR8 and RIG-I specific antibodies. Boxes represent medians ± IQR (interquartile range). Whiskers represent the minimum and maximum observations. *, $P < 0.05$, **, $P < 0.01$ versus the unmodified RFP group. (**b**) Modifications of RFP mRNA inhibit immune responses *in vivo* after intravenous administration. Data represent the mean ± s.e.m. after 24 h ($n = 4$ each). $s2U_{(0.5)}$, modified mRNA with 50% 2-thiouridine (s2U) incorporation; $s2U_{(0.25)}m5C_{(0.25)}$, modified mRNA with 25% s2U and 25% 5-methylcytidine (m5C) incorporation. (**c**,**d**) A549 cells (**c**) and MLE12 cells (**d**) were transfected with 200 ng RFP mRNA. Transfection efficiency (blue and orange bars) and RFP mean fluorescence intensity (MFI) (small blue bars) were determined by flow cytometry (medians ± IQR are shown). *, $P < 0.05$ versus the unmodified RFP group. Total RFP expression (small red bars) was calculated as proportion of RFP-positive cells multiplied by MFI, given as arbitrary units. All experiments were performed twice in duplicates. Data represent the mean ± s.e.m.

therapeutic intervention currently available[12]. We chose a conditional knockout mouse model for SP-B deficiency in which the mouse SP-B cDNA is expressed under the control of exogenous doxycycline in SP-$B^{-/-}$ knockout mice[13]. After removal of doxycycline, SP-$B^{-/-}$ mice treated with EGFPLuc mRNA showed symptoms of acute respiratory distress leading to death within 3.7 ± 0.3 d (**Fig. 3a**,**b**). By contrast, temporary treatment with two doses of SP-B mRNA and subsequent cessation prolonged the average life span to 9.4 ± 0.3 d ($P < 0.001$)

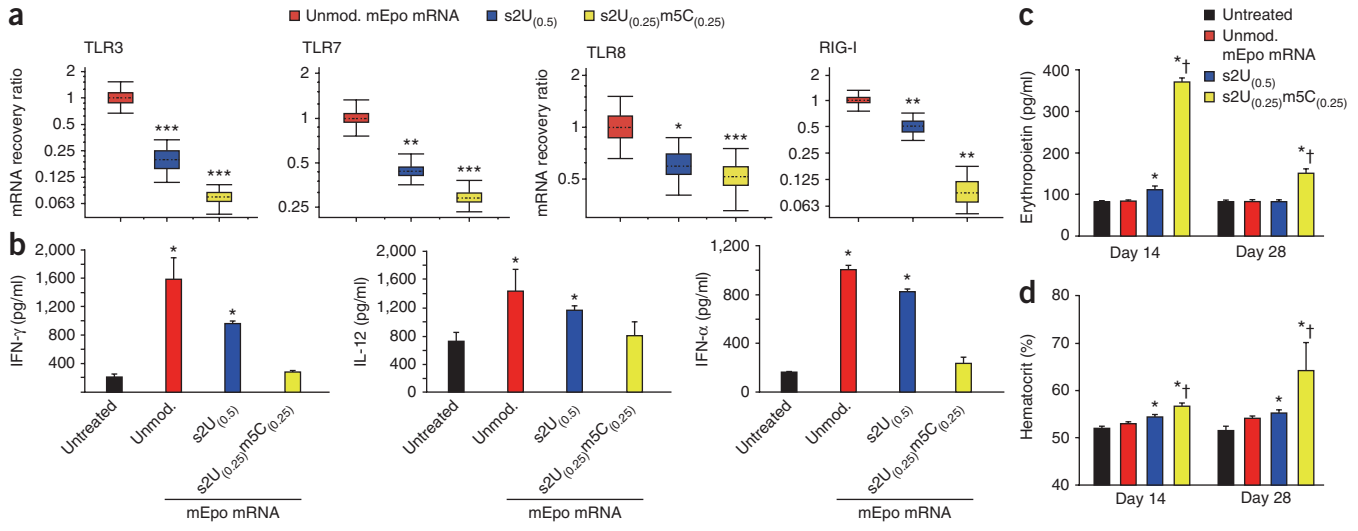

Figure 2 Dual-modification of mEpo mRNA reduces binding to innate immune receptors, prevents immune responses and prolongs transgene expression *in vivo*. (**a**) Human PBMCs were transfected with 5 μg of unmodified and modified mEpo mRNA, and the recovery ratios were determined by RIP using TLR3, TLR7, TLR8 and RIG-I specific antibodies. Boxes represent medians ± IQR. Whiskers represent the minimum and maximum observations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the unmodified mEpo mRNA group. (**b**) 5 μg unmodified and modified mEpo mRNA were injected into mice intramuscularly ($n = 4$ each) and cytokines were measured after 8 h in serum. (**c**,**d**) 5 μg of unmodified and modified mEpo were injected into mice intramuscularly ($n = 4$ each). (**c**) At days 14 and 28 mEpo was quantified in the serum with ELISA. (**d**) Hematocrit was determined in whole blood of the mice of the same experiment. Data represent mean ± s.e.m. *, $P < 0.05$ versus the untreated group of a respective time point; †, $P < 0.05$ versus the unmodified mEpo group of a respective time point.

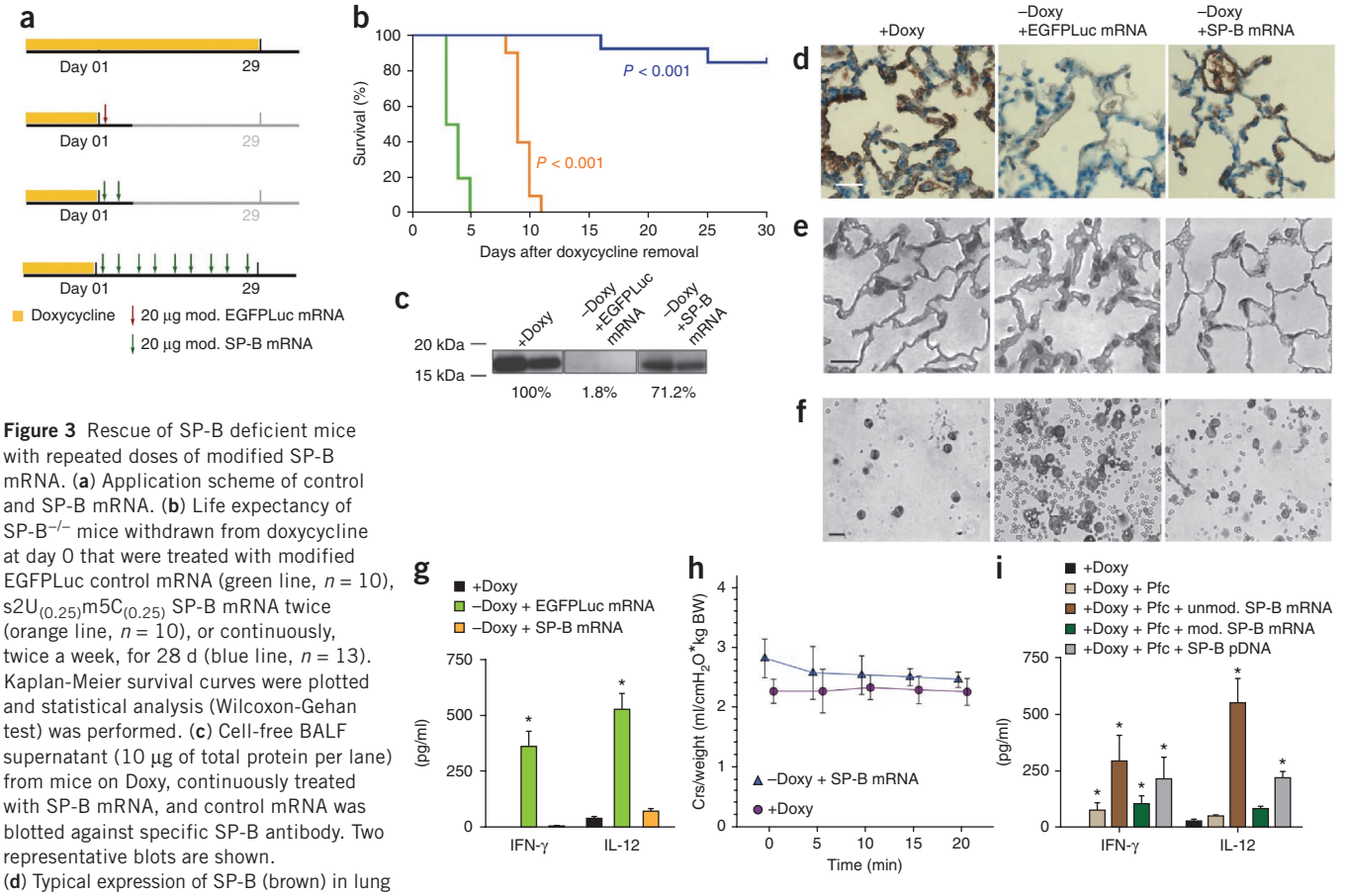

Figure 3 Rescue of SP-B deficient mice with repeated doses of modified SP-B mRNA. (**a**) Application scheme of control and SP-B mRNA. (**b**) Life expectancy of SP-B$^{-/-}$ mice withdrawn from doxycycline at day 0 that were treated with modified EGFPLuc control mRNA (green line, $n = 10$), s2U$_{(0.25)}$m5C$_{(0.25)}$ SP-B mRNA twice (orange line, $n = 10$), or continuously, twice a week, for 28 d (blue line, $n = 13$). Kaplan-Meier survival curves were plotted and statistical analysis (Wilcoxon-Gehan test) was performed. (**c**) Cell-free BALF supernatant (10 µg of total protein per lane) from mice on Doxy, continuously treated with SP-B mRNA, and control mRNA was blotted against specific SP-B antibody. Two representative blots are shown. (**d**) Typical expression of SP-B (brown) in lung tissue from mice described in **c**. Scale bar, 20 µm. (**e**,**f**) Typical lung histology and BALF cytospin preparations (stained with May-Grünwald/Giemsa) of the same mice described in **c**. Scale bar, 20 µm. (**g**) Cytokine levels at the time of necropsy were quantified in mice BALF by ELISA (mean ± s.e.m.); *$P < 0.05$ versus the untreated group. (**h**) Lung compliance over time. (**i**) Early cytokine levels were quantified in mice BALF by ELISA 8 h after administration (mean ± s.e.m.); 20 µg (50 µl) unmodified, s2U$_{(0.25)}$m5C$_{(0.25)}$ SP-B mRNA and SP-B pDNA was delivered to the mice lungs ($n = 4$ each); Doxy, doxycycline; Pfc, perfluorocarbon. *, $P < 0.05$ versus the untreated group.

(**Fig. 3a**,**b**), demonstrating that continuous treatment of modified mRNA is both necessary and sufficient for survival. In a second experimental group, SP-B mRNA administration twice weekly for 4 consecutive weeks (**Fig. 3a**) protected the mice from respiratory failure and prolonged their average life span to 26.2 ± 2.7d ($P < 0.001$) (**Fig. 3b**) until the predefined endpoint of the study.

Successful expression of SP-B in the lungs was confirmed by semiquantitative western blot analysis (**Fig. 3c**) and immunostaining (**Fig. 3d**). SP-B mRNA–treated conditional SP-B$^{-/-}$ mice expressed 71.2 ± 8.3% of the amount of SP-B found in mice receiving doxycycline, whereas in mice treated with EGFPLuc mRNA levels dropped to 1.8 ± 0.4% (**Fig. 3c**). Lung histology was close to normal in mice that were treated with modified SP-B mRNA (**Fig. 3e**), whereas lungs of mice that received EGFPLuc control mRNA exhibited thickened alveolar walls (6.8 ± 1.8 µm compared to 3.9 ± 1.3 µm with SP-B mRNA and 3.5 ± 0.7 µm with doxycycline), cellular infiltration and interstitial edema after 4 d. This was accompanied by congestion and increased numbers of macrophages and neutrophils (442,172 ± 77,990 cells/ml broncho-alveolar lavage fluid (BALF) compared to 107,951 ± 45,255 cells/ml BALF with SP-B mRNA) (**Fig. 3f**). Furthermore, high numbers of inflammatory cytokines were observed in BALF, which was largely prevented in SP-B mRNA–treated mice (**Fig. 3g**) in which normal lung function was maintained, similar to that in SP-B$^{-/-}$ mice receiving doxycycline (**Fig. 3h** and **Supplementary Fig. 3**).

The effects of mRNA modifications on the immune response to SP-B mRNA could be detected as early as 8 h after administration of SP-B mRNA: unmodified SP-B mRNA elicited strong cytokine responses whereas application of dual-modified SP-B mRNA showed only marginally increased cytokine levels, lower than those seen after application of SP-B plasmid DNA, which was used as another control (**Fig. 3i**).

In summary, these results demonstrate the therapeutic potential of repeated administration of modified mRNA. We have shown the therapeutic efficacy of modified mRNA in a mouse model of a fatal human lung disease caused by the absence of SP-B. Modified SP-B mRNA mediates therapeutic expression levels of SP-B protein in the lungs and prevents inflammation, respiratory failure and death. We have also shown that intramuscular administration of modified mRNA can lead to systemic secretion of functional proteins. Delivery of modified mRNA provides temporary, fine-tunable expression, minimal immune activation and the possibility of repeated dosing, while avoiding the risk of tumor formation caused by integration into the host genome that is associated with viral gene therapy vectors. These attributes may circumvent certain obstacles limiting gene therapeutic approaches.

Transcript replacement therapy could be a promising approach for the treatment of inherited genetic disorders for which other treatment options are limited or unavailable. Moreover, administration of chemically modified mRNA may have potential in the field of regenerative medicine including previously demonstrated reprogramming of stem cells[14] and local application for the treatment of degenerative diseases. Further studies addressing size limitations, achievable protein levels, as well as studies into the molecular mechanisms of mRNA entry into the cell, are needed. As with gene delivery techniques, translation of mRNA therapy to the clinic will require strategies to: (i) further improve mRNA activity, (ii) promote tissue-specific mRNA expression and (iii) optimize large-scale production of mRNA for clinical use. However, current advances in tailor-made targeted delivery systems[15,16] together with *de novo* mRNA design[17] and recent progress with up-scaling of mRNA production[18] have already begun to address these challenges.

METHODS

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

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

M.S.D.K. designed research, conceived and performed experiments, and wrote the manuscript. G.H., A.W.F. and S.H.-J. conceived and performed animal experiments. M.K.A. performed animal experiments and cloning. M.H. contributed to animal experiments. G.N. performed cloning. M.I. and A.S. performed *in vitro* experiments. L.E.M., D.H., M.G. and R.H. contributed materials and support in drafting the manuscript. I.B. performed histology. C.R. and J.R. designed and supervised the research and composed and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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ONLINE METHODS

Animal procedures were permitted by the local ethics committee and performed according to the German law "Guidelines of protection of animal life."

Constructs for *in vitro* transcription. For *in vitro* transcription of red fluorescent (RFP) cDNA (678 bp), a SP6 promoter containing plasmid, pCS2+ DsRed.T4, was used. For *in vitro* transcription of human SP-B cDNA (1,146 bp), T7 promoter containing pVAX1 plasmid (Invitrogen) was used. We used a T7 promoter containing pST1-2β-globin UTR-A(120) construct, kindly provided by U. Sahin to generate the vector for *in vitro* transcription of EGFPLuc (2.4 kb) and mEpo (715 bp), which has been previously described in detail[17]. The constructs were cloned using standard molecular biology techniques.

Generation of (modified) mRNA. To generate templates for *in vitro* transcription, the pCS2$^+$ DsRed.T4, EGFPLuc and SP-B plasmids were linearized with XbaI, and pST1-A120-mEpo with PmeI, respectively. Linearized vector DNAs were purified with the NucleoSpin Extract II kit (Macherey-Nagel) and quantified spectrophotometrically. *In vitro* transcription was carried out with the mMESSAGE mMACHINE SP6 and T7 ultra kit (Ambion), respectively. The SP6 kit capped the mRNA with 7-methylGpppG, whereas the T7 kit attached the anti-reverse cap analog (ARCA; 7-methyl(3′-*O*-methyl)GpppG m^7G(5′)ppp(5′)G) in an ultra high-yield transcription reaction. To generate mRNA modifications, the following modified ribonucleic acid triphosphates were added to the reaction in the indicated ratios: 2-thiouridine-5′-triphosphate, 5-methylcytidine-5′-triphosphate, pseudouridine-5′-triphosphate and N^6-methyladenosine-5′-triphosphate (all from TriLink BioTechnologies and purity controlled by high-performance liquid chromatography and ^{31}P NMR). After *in vitro* transcription, pVAX1-SP-B and RFP plasmid–derived mRNA was enzymatically polyadenylated using the poly(A) tail kit (Ambion). The length of the poly(A) tails were estimated to be ~200-nt long. All capped mRNAs (RFP, mEpo, EGFPLuc and SP-B) were purified using MEGAclear Kit (Ambion) and analyzed for size and purity with Agilent RNA 6000 Nano Assay on a BioAnalyzer 2100 (Agilent Technologies).

Cell transfections. *Lung cell transfection.* Human and mouse alveolar type II epithelial cell lines, A549 and MLE12, respectively, were grown in minimal essential medium (Invitrogen), supplemented with 10% FCS, 1% penicillin-streptomycin and 0.5% gentamycin. One day before transfection, 80,000 cells/well were plated in 24-well plates. The cells (> 90% confluent) were transfected with 200 ng mRNA using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. After 4 h, the cells were washed with PBS and serum-containing medium was added. For long-term expression analyses, the cells were split regularly (if > 90% confluent).

Human peripheral blood mononuclear cell (PBMC) transfection. Human PBMCs cryopreserved in liquid nitrogen (CTL-Europe) were carefully thawed using CTL-Anti-Aggregate-Wash Supplement at 37 °C and by slowly adding sterile filtered RPMI-1640 (Invitrogen). For all described experiments a single characterized lot of PBMCs was used to permit reproduction of the data.

Flow cytometry. Flow cytometric analysis was conducted on A549 and MLE12 cells that were transfected with RFP mRNA as described above. The cells were detached from the plate surface with 0.25% trypsin-EDTA, washed three times with PBS and resuspended in PBS to measure fluorescence using a FACSCalibur (BD Biosciences). Transfection efficiencies were calculated from the percentage of the cell population that exceeded the fluorescence intensity of the control cells, which were treated with PBS alone. At least 2,500 gated cells per tube were counted. Data were analyzed with the Cellquest Pro software.

Cytokine detection. ELISA was carried out using human IL-8 and TNF-α kits (RayBio), mouse IFN-γ and IL-12 (P40/P70) kits (RayBio) and mouse IFN-α kit (RnD Systems).

RNA immunoprecipitation (RIP). 1 × 10^6 human PBMCs (CTL-Europe) were transfected with 5 μg mRNA using 12.8 μl Lipofectamine 2000 in 1 ml OptiMEM I. After 4 h, the media was supplemented with 10% FCS. After 24 h the cell suspension was transferred into tubes and the cells were pelleted by centrifugation for 10 min at 350 r.c.f. Subsequently, a modified version of the ChIP-IT Express protocol (ActiveMotive) was used to perform the RIP. DEPC-treated water (Serva Electrophoresis) was used for preparing all necessary reagents. According to the ChIP-IT manual, fixation solution was added to the cells, followed by adding Glycine Stop-Fix solution and ice-cold 1× PBS and pelleting the cells at 4 °C. Then the cells were resuspended in lysis buffer, supplemented with protease inhibitors PIC and PMSF, and incubated on ice for 30 min. After centrifugation for 10 min at 2,400*g* at 4 °C, the supernatant was subjected to the capture reaction. The TLR-mRNA/RIG-mRNA complexes were captured overnight on magnetic beads in 8-well PCR strips as described in the ChIP-IT Express manual. Additionally, SUPERase RNase Inhibitor (Applied Biosystems/Ambion) was added to a final concentration of 1 U/μl. Anti-human TLR3 mouse IgG1, TLR7 rabbit IgG1, TLR8 mouse IgG1 (all from Imgenex) and RIG-1 rabbit IgG1 (ProSci Incorporated) were used as antibodies. After washing the magnetic beads, the TLR-mRNA/RIG-mRNA-antibody complexes were eluted, reverse cross-linked and treated with Proteinase K following the ChIP-IT Express protocol. Finally the eluted mRNA was subjected to reverse transcription and quantitative RT-PCR as described above.

***In vivo* bioluminescence.** D-luciferin substrate was dissolved in water, pH adjusted to 7, and the final volume adjusted to obtain a concentration of 30 mg/ml. This solution (50 μl) was applied to the nostrils of each anesthetized mouse and absorbed by sniffing (1.5 mg luciferin/per mouse). After 10 min, bioluminescence was measured with an IVIS 100 imaging system (Xenogen), as described previously[19], using the following camera settings: field of view 10, f1 f-stop, high-resolution binning and exposure times of 10 min. The signal in the lung region was quantified and analyzed by background subtraction using the Living Image Software version 2.50 (Xenogen).

Animal studies. Six- to 8-week-old female BALB/c mice (Charles River Laboratories) were maintained under specific-pathogen-free conditions and were kept with a 12 h/12 h light/dark cycle in individually ventilated cages, provided with food and water *ad libitum*. Animals were acclimatized for at least 7 d before the start of the experiments. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life. For all experiments except tail vein injection, the animals were anesthetized intraperitoneally with a mixture of medetomidine (0.5 mg/kg), midazolam (5 mg/kg) and fentanyl (50 μg/kg). After the particular experiment, the animals were administered an antidote dose subcutaneously that consisted of atipamezol (50 μg/kg), flumazenil (10 μg/kg) and naloxon (24 μg/kg). Blood for ELISA and hematocrit measurements was obtained by means of retrobulbar venous plexus puncture using microhematocrit capillaries (Marienfeld) at different time points.

Tail vein injection. We mixed 25 μg RFP mRNA with *in vivo* megafectin (MP Biomedicals Europe) in an mRNA/lipid ratio of 0.25 and enhancer-3, as recommended by the manufacturer. The integrity and particle size of the injected complexes were determined by dynamic light scattering using a ZetaPALS/Zeta potential analyzer (Brookhaven Instruments Corp). The mice were placed in a restrainer and 100 μl of the mRNA/megafectin solution (equivalent to 5 μg mRNA) were injected into the tail vein within 30s, using a 27-gauge needle and a 1 ml syringe.

Intramuscular injection. BALB/c mice were anesthetized and 50 μl of mEpo (5 μg) were injected into the tibialis anterior with a 29-gauge needle. Hematocrit values were measured on Automatic Hemoanalyser (Sysmex). Erythropoietin amounts were quantified in the prepared serum by ELISA (R&D Systems).

Intratracheal high pressure spraying. BALB/c and SP-B$^{-/-}$ mice[13] were anesthetized and suspended on a plate system (Hallowell EMC) at a 45° angle by the upper teeth. A modified cold-light otoscope Beta 200 (Heine Optotechnik) was used to provide optimal illumination of the trachea. A small spatula was used to open the lower jaw of the mouse and blunted forceps were used to help displace the tongue for maximal oropharyngeal exposure. A MicroSprayer

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Model IA-1C connected to a High Pressure Syringe Model FMJ-250 (both from PennCentury) was endotracheally inserted and consecutively 25 µl of Fluorinert FC-77 (Sigma) and either 25 µl luciferase mRNA (10 µg) or 50 µl SP-B mRNA solution (20 µg) were applied consecutively. The MicroSprayer tip was withdrawn after 5 s and the mouse was taken off the support after 5 min.

Lung function measurements. Homozygous SP-B$^{-/-}$ mice ± doxycycline ± modified mRNA were anesthetized as described above. To prevent spontaneous breathing, vecuronium-bromide (0.1 mg/kg) was injected intraperitoneally. Lung mechanical measurements were conducted as described previously[20]. Briefly, a blunt steel cannula (outer diameter: 1 mm) was inserted into the trachea through a tracheostomy. The piston pump respirator served as both respirator and measuring device (flexiVent, SAV). During tidal ventilation the respirator was set to a volume-controlled, pressure-limited ventilation mode (Vt = 10 µl/g; Pmax = 30 cmH_2O, PEEP 2–3 cmH_2O) at 2.5 Hz and 100% oxygen. The applied Vt was 8.4 ± 1.4 µl/g in animals receiving doxycycline and 8.9 ± 0.4 µl/g body weight in animals receiving mRNA and no doxycycline (difference not statistically significant). Dynamic mechanics of the respiratory system as well as lung input impedance were measured at 5-min intervals after a recruitment maneuver (two inflations to 15 µl/g over 1 s) to provide a standard volume history. Oscillatory measurement ventilation was halted at PEEP-level. To determine impedance of the respiratory system (Z_{rs}) by forced oscillations (FOT), a forcing signal consisting of an 8-s pseudorandom oscillatory signal was applied with an amplitude of 3 ml/g. The forcing signal contained frequencies between 1.75 to 19.6 Hz[21,22]. Data were collected at 256 Hz and analyzed within 4s windows with 66% overlap. Lung impedance data were displayed as resistance (real part) and reactance (imaginary part) of the respiratory system within the frequency domain. Lung impedance data (Z_{rs}) were partitioned, applying the constant phase model of the lung, as suggested[23]. In this model Zrs consists of an airway resistance (Rn), airway inertia (Inertia), tissue elastance (H_L) and tissue damping (G_L) according to the equation: $Z_{rs} = R_{aw} + j\omega I_{aw} + (G_L - jH_L)/\omega\alpha$ with ω being the angular frequency and α the frequency dependence of $Z_{rs} = (\alpha = (2/\pi) \tan^{-1} (1/\eta))$. Lung hysteresivity (eta = G_L/H_L) is a measure for lung tissue composition taking into account both tissue damping and tissue elastance[24,25]. For each measurement the fitting of the constant phase model is automatically tested. Fitting quality is displayed as coherence of determination (COD): data were rejected when COD < 0.85.

Surfactant protein analysis. Total protein content of the lavage supernatants was determined with the Biorad Protein Assay Kit (Biorad). Ten micrograms of total protein was separated under nonreducing conditions on NuPage 10% Bis-Tris gels using a NOVEX X-cell II Mini-Cell system (Novex). After electrophoresis the proteins were transferred onto a PVDF membrane (ImmobilonP) with a NuPage Blot module (Novex). Surfactant protein B (SP-B) was detected by the polyclonal rabbit antiserum directed against SP-B (c329, gift of W. Steinhilber, Altana), followed by enhanced chemiluminescence assay (Amersham Biosciences) with horseradish peroxidase conjugated goat anti-rabbit polyclonal anti-IgG (1:10,000; Dianova). Under these conditions the assay could detect about 2.5 ng of SP-B per lane[26]. A chemiluminescence detection system, DIANA III dev. 1.0.54 with the Aida Image analyzer (Ray test Isotopenmessgeräte) was used and the data were quantified with Quantity One 4.6.7 (Biorad).

Immunohistochemical staining. Fixed (3% paraformaldehyde) and paraffin-embedded sections were subjected to immunohistochemistry as recommended at http://www.abcam.com/technical. The slides were incubated with anti-human/anti-mouse SP-B antibody (1:4,000; c329, gift of W. Steinhilber, Altana) at 37 °C for 32 min. Antibody detection was performed by means of DAB ultraView (Ventana).

Statistics. Differences in mRNA expression between groups were analyzed by pair-wise fixed reallocation randomization tests with REST 2005 software[27]. Half-life values of bioluminescence decay were calculated with Prism 5.0. All other analyses were performed using the Wilcoxon-Mann-Whitney test with SPSS 15 (SPSS). Data are presented as the mean ± s.e.m. or as the median ± IQR (interquartile ranges) and $P < 0.05$ (two-tailed) was considered statistically significant.

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