Lentiviral Delivery of RNAi for *In Vivo* Lineage-Specific Modulation of Gene Expression in Mouse Lung Macrophages

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Although RNA interference (RNAi) has become a ubiquitous laboratory tool since its discovery 12 years ago, *in vivo* delivery to selected cell types remains a major technical challenge. Here, we report the use of lentiviral vectors for long-term *in vivo* delivery of RNAi selectively to resident alveolar macrophages (AMs), key immune effector cells in the lung. We demonstrate the therapeutic potential of this approach by RNAi-based downregulation of p65 (RelA), a component of the pro-inflammatory transcriptional regulator, nuclear factor κB (NF-κB) and a key participant in lung disease pathogenesis. *In vivo* RNAi delivery results in decreased induction of NF-κB and downstream neutrophilic chemokines in transduced AMs as well as attenuated lung neutrophilia following stimulation with lipopolysaccharide (LPS). Through concurrent delivery of a novel lentiviral reporter vector (lenti-NF-κB-luc-GFP) we track *in vivo* expression of NF-κB target genes in real time, a critical step towards extending RNAi-based therapy to longstanding lung diseases. Application of this system reveals that resident AMs persist in the airspaces of mice following the resolution of LPS-induced inflammation, thus allowing these localized cells to be used as effective vehicles for prolonged RNAi delivery in disease settings.

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INTRODUCTION

Although manipulating *in vivo* gene expression using RNAi technology has been a major goal for medical researchers, the ability to do so in a cell type-specific fashion that avoids off-target knockdown remains elusive. The lung is an attractive target organ for RNAi-based therapy owing to its air interface and unique architecture that offers its entire epithelial surface as a potential target for inhaled delivery. Previous work harnessing RNAi for treatment of acute pulmonary infections has shown promise[1–4] but target gene knockdown has been transient and not restricted to specific lung lineages.

Specificity of RNAi expression is strongly associated with mechanism of delivery. Drawing on approaches pioneered for delivery of traditional gene therapy, a variety of methodologies have been used to deliver small interfering RNAs (siRNAs) to cells or tissues with varying degrees of success. These approaches have included introduction of siRNA directly into a target tissue[5–7] or addition of a ligand to a coating or stabilizing molecule to help provide specificity of delivery.[8,9] To achieve more prolonged expression, viral vectors have been used to deliver short hairpin RNAs (shRNAs) under the control of Pol II or Pol III promoters. Lentiviral vectors in particular have been used to knock down gene expression by direct injection into target tissues.[10–13] The ability to induce lineage-specific expression in a complex tissue such as the lung, however, has proven challenging. To improve upon existing technology and extend RNAi-based therapy to chronic lung diseases, a delivery system is needed that can target specific lung cell types to achieve sustainable, verifiable knockdown.

We recently reported a lentivirus-based method for manipulating gene expression specifically in alveolar macrophages (AMs).[14] Here, we adapt this approach to deliver shRNAs to this key immune effector cell in the lung. After establishing successful knockdown of a reporter gene *in vivo*, we apply the method to downregulate expression of a gene central to the inflammatory response in the lung, the p65 (RelA) subunit of the transcription factor, nuclear factor κB (NF-κB). To confirm successful, sustained p65 knockdown in target cells, we introduce a novel lentivirus-based approach to track transcription factor activity in live animals and demonstrate that shRNA-treated animals exhibit diminished activation of NF-κB, knockdown of downstream chemokines, and decreased neutrophil influx in response to intratracheally instilled lipopolysaccharide (LPS). Unexpectedly, our studies revealed that lentivirally transduced resident AMs persist in the airspaces following the resolution of NF-κB inflammatory activation, suggesting these cells as convenient vehicles for selective and sustained delivery of RNAi to the lung parenchyma.

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RESULTS

Lentivirally delivered RNAi results in the knockdown of a stable reporter gene in lung macrophages *in vivo*

To test lentiviral delivery of shRNA, we transduced a 293T cell line containing a single copy of the enhanced green fluorescence protein (eGFP) reporter gene with a lentiviral vector, lenti-DsRed-shGFP (a modified form of the published vector pLVTHM),[15] expressing the DsRed fluorescent protein together with an shRNA sequence known to knock down expression of GFP[16] (**Figure 1a**). With increasing multiplicity of infection, we observed a dose-dependent decrement in GFP mean fluorescence intensity and protein production consistent with knockdown of the transgene (**Figure 1 b,c**). No GFP knockdown was observed in 293T cells transduced by a control vector expressing a scrambled version of the shGFP sequence (lenti-DsRed-scramble).

Using our previously published method for specific transduction of AMs *in vivo*,[14] we next used intratracheal (IT) instillation of lenti-DsRed-shGFP or lenti-DsRed-scramble vectors (**Figure 1d**; 5×10^7 virions/mouse) to test these constructs in transgenic mice that ubiquitously express the GFP reporter gene under the control of the β actin promoter (β-actin-GFP mice). Four weeks after IT lentivirus, transduced (DsRed$^+$) AMs lavaged from mice treated with lenti-DsRed-shGFP had significantly lower levels of GFP expression as quantified by mean fluorescence intensity, suggesting that we could achieve knockdown of *in vivo* gene expression. Our previous work has demonstrated that cells transduced by the IT lentivirus method are resident AMs with surface marker profile: CD45$^+$, F4/80$^+$ CD3$^-$, B220$^-$, Ter119$^-$, CD11c$^+$, CD11b dim/$^-$.[14] Although no epithelial or endothelial cells are transduced using this approach (based on tropism of the vesicular stomatitis virus G viral envelope),[14,17–20] we have found that lentiviral transduction of a subset of lung CD11b$^+$/CD11c$^+$ dendritic cells does occur.[21]

Adaptation of lentiviral platform for manipulation of p65 gene expression

The transcription factor NF-κB has been implicated in a variety of lung diseases, including pneumonia, acute lung injury, emphysema, and malignancy.[22–25] Although these studies suggest NF-κB signaling as an intriguing target for knockdown in the lung, non-selective or global inhibition of NF-κB can be highly toxic, as evidenced by embryonic lethality of the p65 knockout mouse in contrast with lineage-specific p65 knockouts.[26–28] Because intratracheally instilled lentivirus allows us to specifically transduce resident AMs,[14] we reasoned that selective knockdown of NF-κB signaling in the lung might be achievable using this method. We therefore modified our RNAi-expressing vectors to knock down p65, the NF-κB family member known to be most active in the lung.[29] We cloned a previously published shRNA sequence known to target p65[30] or a scrambled version of this sequence into pLVTHM to create the vectors lenti-GFP-shp65 and lenti-GFP-scramble (**Figure 2a**).[15] To test the efficacy of lenti-GFP-shp65 *in vitro*, we assessed the effects of LPS stimulation on Raw 264.7 macrophages following transduction with lenti-GFP-shp65. Fifteen passages after lentiviral transduction of Raw cells, shp65 expression resulted in persistent knockdown of p65 mRNA and reduced NF-κB-dependent cytokine secretion at baseline and

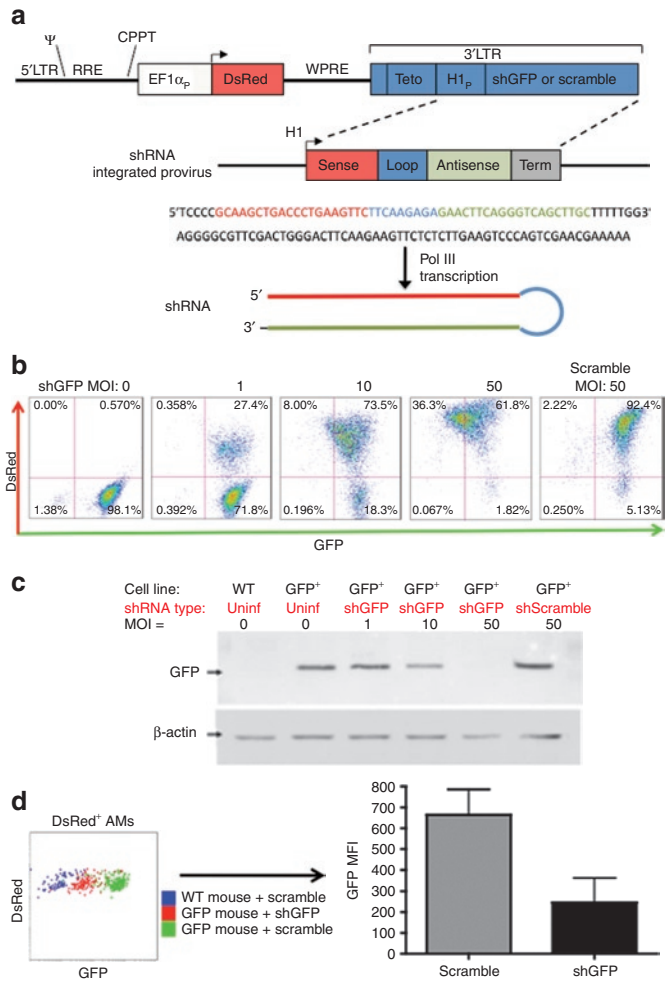

Figure 1 *In vitro* and *in vivo* testing of lentiviral vectors for RNAi delivery. (**a**) Schematic of the lenti-DsRed-shGFP vector designed for delivery of a shRNA sequence targeting enhanced green fluorescence protein (eGFP). In addition to constitutive expression of DsRed under control of the elongation factor 1 α (EF1α) promoter, a 20 bp sense sequence depicted in red targets eGFP and is followed by a linking loop sequence (blue) and then by the complementary, antisense sequence (green). A scrambled version of the same sequence was used as a control. The coding sequence is flanked by RNA Pol III transcriptional start (CCCC) and stop (TTTTT) sequences and expression is driven by the H1 promoter. An element (teto) precedes the H1 promoter that allows tetracycline inducibility of the H1 promoter in the presence of additional constructs that express a repressor fusion protein, TTR-KRAB.[15] In the absence of any TTR-KRAB-expressing construct (as used here), the H1-shRNA construct constitutively drives shRNA expression. All vectors in this manuscript include the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to augment gene expression levels. (**b**) Lenti-DsRed-shGFP or lenti-DsRed-scramble were used to transduce a GFP-expressing 293 cell line. Increases in lentiviral dose (as quantified by increasing multiplicity of infection (MOI)) result in increased production of the short hairpin targeting GFP and corresponding decreases in GFP mean fluorescence intensity (MFI). (**c**) Western blot of cell lysates demonstrates an inverse correlation between lenti-DsRed-shGFP MOI and GFP protein levels. (**d**) Lenti-DsRed-shGFP or -scramble was delivered to transgenic β-actin-GFP mice by intratracheal instillation. Four weeks later, alveolar macrophages (AMs) were harvested by bronchoalveolar lavage (BAL). The GFP MFI of transduced (DsRed$^+$) AMs is lower in lenti-DsRed-shGFP treated mice than in mice receiving the scramble control vector. CPPT, central polypurine tract; LTR, long-terminal repeat; ψ, Psi packaging signal; RRE, Rev response element.

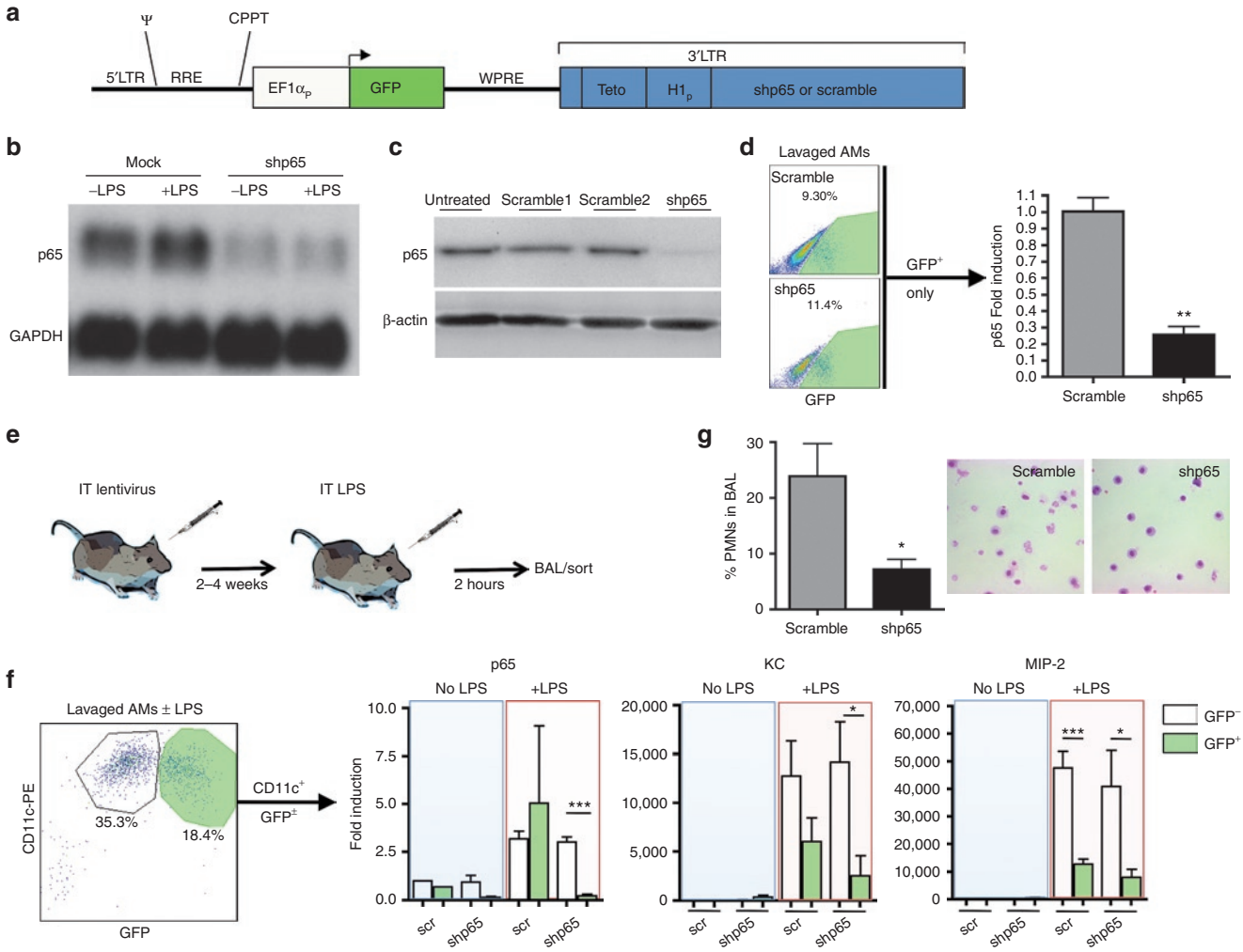

Figure 2 Lenti-mediated p65 knockdown *in vitro* and *in vivo*. (**a**) Schematic of lenti-GFP-shp65. The vector depicted in Figure 1a, above, has been modified to target the NF-κB subunit, p65 (RelA). A scrambled version of the p65 targeting sequence was used as a control (lenti-GFP-scramble). (**b**) Northern blot quantification of mRNA from lenti-GFP-shp65 transduced Raw 264.7 cells (or mock-transduced controls) in the absence or presence of stimulation with lipopolysaccharide (LPS). (**c**) Decreased p65 protein levels are seen by western blot analysis of clonal populations of lenti-GFP-shp65 treated Raw 264.7 cells when compared with untreated or lenti-GFP-scramble treated controls. (**d**) p65 mRNA levels in alveolar macrophages (AMs) harvested from mice treated with lenti-GFP-shp65 or -scramble. Two weeks after intratracheal (IT) lentivirus, there is less p65 message in sorted, GFP$^+$ AMs from lenti-GFP-shp65 treated mice than in AMs from mice treated with control vector (fluorescence activated cell sorter (FACS) plots depict representative data, $n = 3$ per group). (**e**) Schematic outlining experimental approach to quantify NF-κB dependent gene expression in AMs following lentiviral delivery of shp65 and subsequent LPS. (**f**) Transduced (GFP$^+$) or nontransduced (GFP$^-$), CD11c$^+$ cells were sorted and gene expression analyzed by quantitative PCR. p65 knockdown after treatment with lenti-GFP-shp65 persisted in the setting of LPS stimulation. Downstream expression of the chemokines KC and MIP-2 were also diminished. Knockdown was restricted to transduced (GFP$^+$) cells. (FACS plot depicts representative data, $n = 6$ per LPS-treated lentiviral condition). (**g**) Four weeks after IT lentivirus, fewer neutrophils were found in BAL cytospins from mice treated with lenti-GFP-shp65 than in lenti-GFP-scramble treated controls following IT LPS ($n = 10$ per group). * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. ψ, Psi packaging signal; GFP, green fluorescence protein.

in the presence of LPS stimulation (Figure 2b; **Supplementary Figure S1**). Western blots of lysates from clonal populations of Raw 264.7 macrophages similarly found less p65 protein in lenti-GFP-shp65 transduced clones than in those transduced with lenti-GFP-scramble (Figure 2c).

Lentivirus-based knockdown of p65 *in vivo*

We next examined whether lenti-GFP-shp65 treatment would result in knockdown of NF-κB activation *in vivo*. We administered lenti-GFP-shp65 to recipient mice and harvested AMs by bronchoalveolar lavage (BAL) 2 weeks later. After sorting to isolate transduced (GFP$^+$) AMs, p65 mRNA levels were measured by quantitative real-time PCR. p65 mRNA was significantly reduced in transduced (GFP$^+$) AMs lavaged from lenti-GFP-shp65 treated animals in comparison with those from scramble-treated controls (Figure 2d).

To test whether p65 mRNA knockdown was occurring only in transduced AMs or was spreading to all resident (CD11c$^+$) lung AMs, we next quantified p65 mRNA levels, comparing sorted transduced AMs (GFP$^+$) to nontransduced (GFP$^-$) AMs from groups of mice treated with the control versus shp65 expressing vectors. A subset of mice from each group were also exposed to subsequent IT LPS 2 hours before macrophage harvest to screen for any alteration in downstream NF-κB-dependent signaling

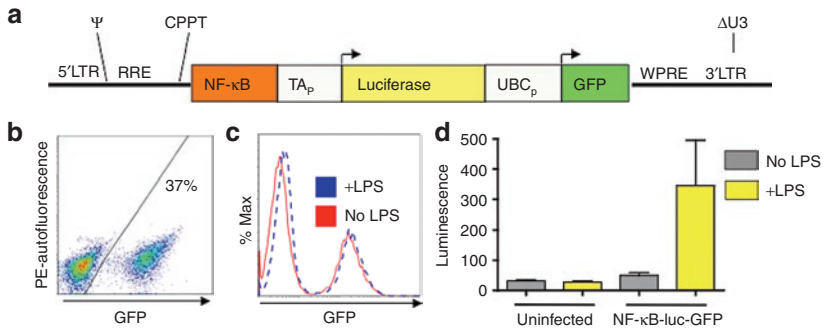

Figure 3 *In vitro* **testing of a lentiviral NF-κB reporter vector.** (**a**) Schematic of lenti-NF-κB-luc-GFP: four copies of the NF-κB consensus sequence precede the minimal thymidine kinase promoter (TAp) of the herpes simplex virus. Promoter activity is turned on in the presence of NF-κB activation, driving expression of the firefly luciferase reporter gene. GFP is constitutively expressed by the ubiquitin C (UBC) promoter, allowing tracking of transduced cells by flow cytometry. (**b**) Analysis of lenti-NF-κB-luc-GFP treated Raw 264.7 cells by flow cytometry demonstrates that 37% of cells are transduced (GFP$^+$). (**c**) GFP mean fluorescence intensity (MFI) of transduced Raw cells does not increase with NF-κB activation after lipopolysaccharide (LPS) stimulation. (**d**) Luminescence quantification in Raw 264.7 cells transduced with lenti-NF-κB-luc-GFP overnight followed by stimulation with LPS (100 ng/ml). Increased luminescence is generated by LPS stimulated cells compared with uninfected or unstimulated cells. Data represent seven independent experiments. ψ, Psi packaging signal; GFP, green fluorescence protein; NF-κB, nuclear factor κB.

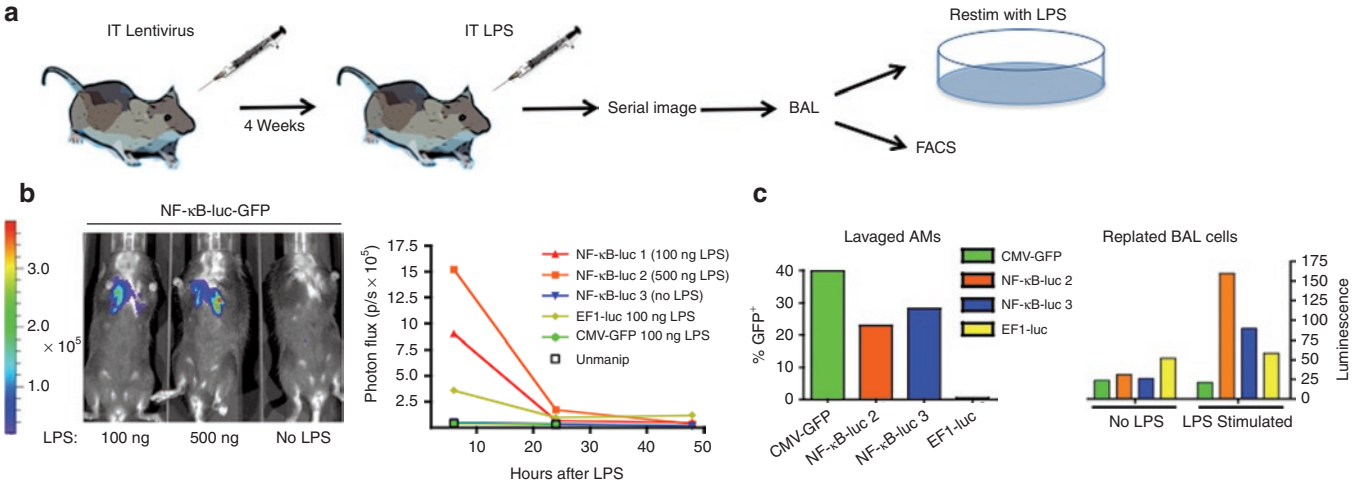

Figure 4 Reporter-based tracking of NF-κB activation in live animals. (**a**) Schematic outlining experiment to test lenti-NF-κB-luc-GFP *in vivo*. Experimental mice are administered intratracheal (IT) lentivirus followed 4 weeks later by IT lipopolysaccharide (LPS) before data collection. (**b**) *In vivo* imaging of thoracic photon flux in mice treated with LPS or control vehicle 4 weeks after IT lenti-NF-κB-luc-GFP. NF-κB-driven luciferase activity is detectable only in animals treated with both the reporter vector (designated mice NF-κB-luc 1, 2, or 3) and LPS and diminishes with time. (**c**) Analysis of lavaged alveolar macrophages from the experiment outlined in **a** and **b** reveals persistence of transduced (GFP$^+$) cells in the BAL 4 weeks after 100 ng IT LPS. A subset of BAL cells were cultured for 72 hours before exposure to either LPS (100 ng/ml) or control media. BAL cells from animals previously treated with lenti-NF-κB-luc-GFP demonstrate NF-κB-driven luciferase activity, regardless of whether or not they were previously exposed to LPS *in vivo*. NF-κB, nuclear factor κB; GFP, green fluorescence protein.

in GFP$^+$ versus GFP$^-$ AMs. (Figure 2e,f) We found that p65 knockdown selectively occurred in GFP$^+$ AMs and persisted in the setting of LPS-induced inflammation. In addition, decreased induction of the NF-κB-dependent chemokines KC and MIP-2, both chemotactic for neutrophils, was noted in transduced AMs in comparison with nontransduced AMs. p65 knockdown did not significantly affect expression of several other genes known to be NF-κB dependent, such as interleukin 1α (IL-1α) and tumor necrosis factor α (TNFα) (**Supplementary Figure S2**). Surprisingly, MIP-2 expression was also significantly diminished in AMs transduced with lenti-EF1α-GFP-scramble. This finding was not recapitulated at a later timepoint after lentiviral treatment, suggesting a transient, nonspecific effect of lentiviral transduction on gene expression that resolves with time (**Supplementary Figure S2**). The effect was only observed in NF-κB-dependent cytokine expression downstream of p65 and was not the result of nonspecific knockdown of the p65 mRNA target (Figure 2f).

To evaluate the suitability of lentivirus-based knockdown of p65 for long-term *in vivo* applications, we harvested AMs from treated mice at a later timepoint (4 weeks) following IT lentivirus (Figure 2e). In each of two repeat experiments ($n = 6$ per group and $n = 10$ per group), we found persistent knockdown of p65 message restricted to transduced (GFP$^+$) AMs 4 weeks after lentiviral treatment (**Supplementary Figure S2**). In all samples, knockdown of gene expression was restricted to lentivirally transduced AMs with no evidence of spillover to nontransduced AMs. Knockdown of p65 in the transduced subset of AMs also resulted in a decreased presence of neutrophils in the BAL of LPS-exposed mice, suggesting

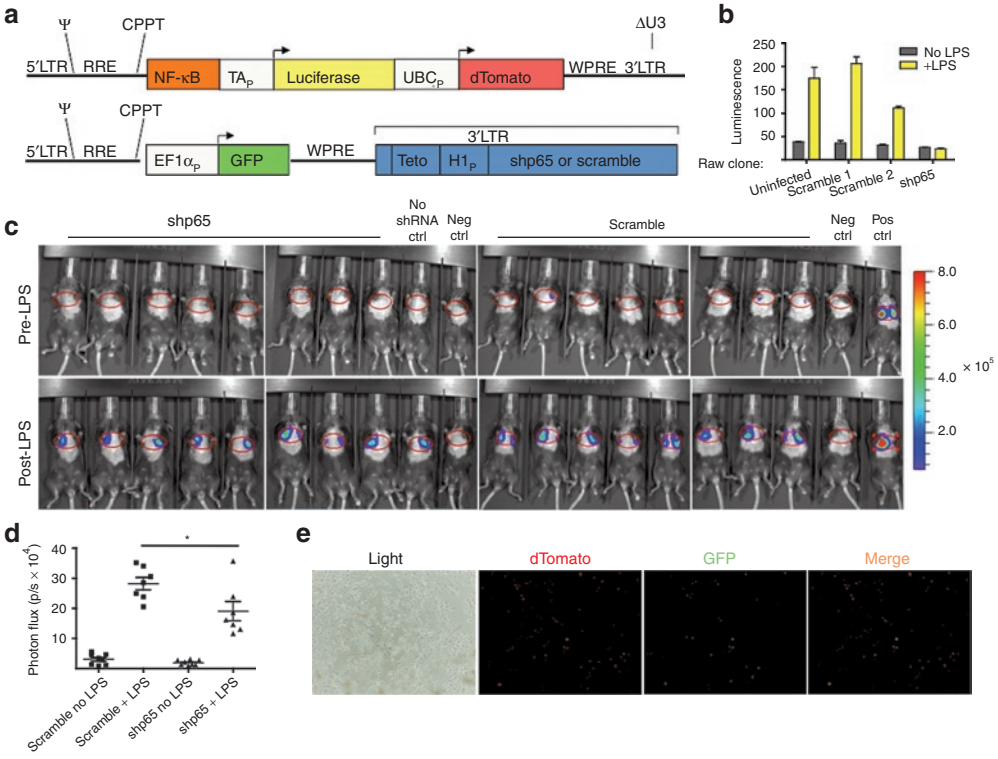

Figure 5 Reporter-based quantification of *in vivo* NF-κB knockdown. (**a**) Schematics of vectors combined in this experiment. The dTomato reporter has been cloned into the second position of lenti-NF-κB-luc-GFP to create lenti-NF-κB-luc-dTomato. This combination of reporters allows independent tracking of transduced cells in experiments combining this vector with lenti-GFP-shp65. (**b**) Clonal Raw 264.7 cells sorted after transduction with lenti-GFP-shp65 or lenti-GFP-scramble before transduction with the lenti-NF-κB-luc-GFP reporter vector. NF-κB-driven luminescence was measured in the presence or absence of lipopolysaccharide (LPS) stimulation. (**c**,**d**) C57BL/6J mice (n = 8 per group) received an intratracheal (IT) mixture of lenti-NF-κB-luc-dTomato and either lenti-GFP-shp65 or lenti-GFP-scramble. Two weeks after IT lenti treatment, a photon camera was used to quantify thoracic luciferase activity of recipient mice before and after IT LPS instillation. Less NF-κB-driven luciferase activity is measured in lenti-GFP-shp65 treated mice in response to IT LPS when compared with controls. (**e**) A subset of 50,000 lavaged BAL cells per animal was maintained in culture for 96 hours following *in vivo* LPS exposure. Representative images from one sample demonstrate BAL cells exhibiting both dTomato and GFP fluorescence after *in vivo* treatment with a mixture of two lentiviruses. *P < 0.05. ψ, Psi packaging signal; GFP, green fluorescence protein; Neg ctrl, negative control; NF-κB, nuclear factor κB; Pos ctrl, positive control.

a functionally relevant anti-inflammatory effect persisted 4 weeks after RNAi delivery (n = 10 per group; P = 0.02; **Figure 2g**).

Next, to compare our p65 knockdown results with complete ablation of p65, we used mice in which p65 is deleted following cre-mediated excision in myeloid cells including AMs (LysM-Cre x p65-floxed mice; hereafter p65$^{\Delta/\Delta 26}$). To assess the effects of relative p65 levels on global lung inflammatory signaling, we analyzed levels of 11 different cytokines in the BAL fluid (BALF) of both lenti-GFP-shp65 treated mice and p65$^{\Delta/\Delta}$ mice following LPS exposure. Because epithelial cells as well as AMs experience NF-κB activation mediated via LPS binding of toll-like receptor 4, we anticipated that AM-specific p65 downregulation or ablation might not be sufficient to affect global lung expression of inflammatory genes. As expected, we found no decrease in NF-κB dependent cytokine levels in the BALF of mice deficient in p65 resulting either from RNAi delivery (with lenti-GFP-shp65) or cre-mediated excision (p65$^{\Delta/\Delta}$) (data not shown).

Testing of a novel lentiviral system for detecting activation of NF-κB

Next, we sought to track in real time the kinetics of NF-κB activation in AMs *in vivo* as well as the effects of our RNAi delivery method on this kinetic. Traditional assays of NF-κB activation require harvesting the cells (*in vitro*) or animal (*in vivo*) of interest to determine whether nuclear translocation and DNA binding of NF-κB dimers has occurred, making longitudinal studies of individual animals impossible. To allow tracking of NF-κB activation *in vivo*, we created a novel lentiviral vector (lenti-NF-κB-luc-GFP, **Figure 3a**) containing four tandem copies of the NF-κB consensus sequence located upstream of the minimal TA promoter (TAp), the TATA box of the herpes simplex virus thymidine kinase (HSV-TK) promoter. Located downstream from TAp is the firefly luciferase reporter gene as well as a ubiquitously expressed GFP reporter under control of the constitutive mammalian ubiquitin C (UBC) promoter. This combination of reporter genes allows for independent tracking of transduced (GFP$^+$) cells by flow cytometry in addition to continuous, real-time assessment of NF-κB activation levels by luciferase expression.

We performed preliminary testing of lenti-NF-κB-luc-GFP in Raw 264.7 macrophages. After overnight infection, Raw macrophages were split before stimulation with either *E. Coli*-derived LPS (100 ng/ml), known to upregulate the NF-κB pathway through specific ligation of toll-like receptor 4, or standard media (negative control wells).[31] In the unstimulated state, lenti-NF-κB-luc-

GFP-transduced Raw macrophages had levels of luminescence that were detectable just above background, consistent with either minimal basal levels of NF-κB signaling in macrophages cultured on plastic or basal leak of the luciferase reporter (**Figure 3d**). When stimulated with LPS, NF-κB-luc-GFP-transduced cells alone exhibited a dramatic increase in luciferase activity indicating that NF-κB-driven luciferase production had occurred. A subset of cells was analyzed by flow cytometry to determine the transduction efficiency as illustrated in **Figure 3b**. The GFP mean fluorescence intensity of LPS stimulated and unstimulated cells was identical, indicating that UBC-driven gene expression from the second position was not affected by NF-κB binding activity (**Figure 3c**).

In vivo, real-time imaging of NF-κB activation

Having determined that transduction with lenti-NF-κB-luc-GFP resulted in LPS-responsive luciferase activity, we proceeded to *in vivo* testing. We administered IT lenti-NF-κB-luc-GFP, or control lentiviruses constitutively expressing either GFP (negative control) or luciferase (positive control) to recipient C57BL/6 mice followed 4 weeks later by IT LPS (illustrated in **Figure 4a**). Negative control mice and mice treated with lenti-NF-κB-luc-GFP had no measurable background luciferase activity by photon flux imaging before IT LPS exposure (**Figure 4b**). In contrast, mice treated with lenti-NF-κB-luc-GFP followed by either 100 ng or 500 ng LPS exhibited luciferase activity localized to the thorax that was consistent with a dose response. We observed that luciferase activity in LPS-treated animals diminished with time, raising the possibility of either resolution of NF-κB activation or resident AM cell death following LPS stimulation. AMs have previously been presumed to have a short half life that may be further diminished by inflammatory activation suggesting rapid dropout of transduced AMs exposed to LPS might be expected.[32] More recent literature has instead reported that resident AMs persist in the alveolar space following LPS, but the issue remains controversial.[33] Because IT lentivirus tags resident AMs, we were able to address this question using an independent experimental approach. We performed BALs and harvested AMs from treated mice 4 weeks after LPS stimulation, a time point after decrement of our NF-κB-luciferase reporter signal. Even after treatment with significant doses of LPS, a high percentage of lavaged AMs were found to be GFP^+ suggesting that tagged, resident AMs persisted in the alveolar space following inflammatory injury (**Figure 4c**).

It occurred to us that AMs persisting in the alveolar space after LPS treatment might lack the capacity to respond to LPS. To explore this question further, subsets of AMs lavaged from each animal were cultured overnight before incubation in media with or without LPS. Recapitulating our *in vivo* findings, AMs from lenti-NF-κB-luc-GFP treated mice but not controls had increases in luminescence in response to LPS stimulation (**Figure 4c**). This result demonstrated that tagged AMs persisting after LPS stimulation were in fact capable of upregulating NF-κB activity in response to repeated LPS exposure.

Longitudinal tracking of NF-κB knockdown *in vivo*

Long term, verifiable downregulation of gene expression is desirable if RNAi is to be adapted as a treatment for chronic diseases. We therefore sought to determine whether we could track p65 knockdown over time in live mice. To determine whether our lenti-NF-κB reporter vector could detect shRNA-mediated downregulation of p65, we sorted single GFP^+ Raw 264.7 cells following transduction with lenti-GFP-shp65 or -scramble (**Figure 5a**). After expansion in culture, these clones were subsequently transduced with lenti-NF-κB-luc-GFP. After incubation in LPS-containing or control media, we observed abrogation of NF-κB-driven luciferase expression only in lenti-GFP-shp65-transduced cells (**Figure 5b**), suggesting that our reporter vector could be used to visualize p65 knockdown. To adapt this approach for use in live animals, we devised an experiment using mixtures of lenti-GFP-shp65 (or scramble) and an altered form of the lenti-NF-κB-luc-GFP vector described above, now with the red dTomato fluorochrome cloned into the second position (lenti-NF-κB-luc-dTomato; **Figure 5a**). In preparation for this experiment, we performed IT injections of mixtures of lentiviruses containing either a red or green fluorochrome demonstrating that significant percentages of AMs are transduced with both constructs when a mixture of two lentiviruses is administered via IT instillation (**Supplementary Figure S3**).

Two weeks after administering the IT lenti-GFP-shp65/lenti-NF-κB-luc-dTomato mixture to groups of C57BL/6 mice, we performed photon flux imaging before and 8 hours after administration of IT LPS. Consistent with our previous results, the lenti-GFP-shp65 and -scramble-treated groups had similar, low-level luciferase activity localized to the thorax before instillation of LPS (**Figure 5c,d**). Both groups were found to have significantly increased luciferase activity following IT LPS, as expected. However, the increase in luciferase activity following LPS was significantly attenuated in the lenti-GFP-shp65-treated group, consistent with diminished NF-κB activation in the setting of p65 knockdown. To confirm that diminished NF-κB driven luciferase levels resulted from p65 knockdown rather than dropout of transduced cells, we performed BALs on treated mice 8 hours after LPS stimulation. Fluorescence microscopy and flow cytometry revealed no difference in the percentage of AMs transduced with lenti-NF-κB-dTomato in the two experimental groups, indicating that cell death was not responsible for differences in NF-κB-driven luciferase activity (**Figure 5e**; **Supplementary Figure S4**). The presence of dTomato in this experiment resulted in lower than usual transduction percentages following LPS exposure, possibly resulting from toxic effects of the fluorochrome as has been reported.[34] Repeat experiments substituting lenti-NF-κB-luc-GFP resulted in higher transduction efficiency consistent with our typical results (data not shown).

DISCUSSION

In this article, we describe durable knockdown of NF-κB signaling in AMs, a key innate immune cell population in the lung. Our technique results in sustained downregulation of p65 in this long-lived lung resident cell-type, suggesting that it has the potential to alter the course of chronic inflammatory conditions in the lung. p65 knockdown, in turn, diminishes the LPS-induced influx of neutrophils to the lung demonstrating its functional significance.

We have previously reported that induction of AM inflammatory cytokines and neutrophil recruitment are dampened in

the absence of myeloid p65 during pneumococcal pneumonia.[28] The data presented here are consistent with our previous findings and suggest a role for AM NF-κB signaling in the inflammatory response in the lung that extends beyond pneumonia to include other types of lung injury as well.

The relative influence of specific lung cell types in response to inflammatory injury is a topic that has not yet been well addressed in the scientific literature. An intriguing question raised by our research is whether knockdown of p65 in a subset of AMs is sufficient to decrease NF-κB signaling in the entire lung. Because AMs are present in the alveolar space and secrete cytokines and other effector molecules directly into the epithelial lining fluid, they have the potential to influence gene expression in alveolar epithelial cells. Our previously published data indicate that AM p65 is critical for whole lung cytokine production early in pneumococcal pneumonia.[28] The literature as a whole suggests that both resident AMs and alveolar epithelial cell NF-κB signaling ultimately contribute to the inflammatory response in the lung.[35,36] Our data support this hypothesis, as neither knockdown nor complete deletion of AM p65 resulted in decreased epithelial lining fluid cytokine levels in our experiments. Additional studies using a cre-lox system to induce epithelial lineage-specific ablation of p65 signaling might help to further address this issue in the future.

The effect of lenti-GFP-scramble on MIP-2 expression in transduced AMs at early timepoints raises the question of a nonspecific effect of lentiviral infection on the inflammatory response. The HIV-1 long-terminal repeat (LTR) promoter is known to contain NF-κB binding sites, which could conceivably affect NF-κB signaling in transduced cells. However, the bulk of the HIV genome including these and other transcription factor binding sites have been deleted from the pHAGE and pLVTHM vectors used in our experiments both for safety purposes and to minimize LTR-associated transcriptional interference.[37] An alternative potential explanation of this finding might be the induction of immunological tolerance caused by lentiviral infection of antigen presenting cells, such as AMs and dendritic cells, as featured in our experiments. Lentiviral infection is known to activate toll-like receptor signaling in transduced dendritic cells *in vitro* and *in vivo*.[38] Although another report did not find a subsequent tolerance to LPS stimulation of lenti-transduced bone marrow derived dendritic cells in culture,[39] important differences between our studies exist, including our *in vivo* approach, and the timing and duration of LPS stimulation. If confirmed, this finding could have broad implications for clinical application of lentiviral gene therapy.

In addition to the tools for manipulating and tracking AM gene expression described above, we report that resident AMs persist in the alveolar space even after significant inflammatory LPS injury, extending recently published observations on the prolonged life span of AMs in both the quiescent and inflamed lung.[14,33,40] This finding, together with our previous, similar observations in the setting of elastase- and acute cigarette smoke-induced lung inflammation demonstrate that resident AMs, in addition to possessing a long life span, are characterized by the capacity to withstand a broad variety of inflammatory insults. Together with their accessibility for gene transfer, these features suggest them as good potential targets for gene therapy designed to alter the course of inflammatory lung diseases.

Potential toxicity associated with constitutive shRNA expression is a concern that was partially addressed in this study but warrants further investigation. Although our results did not clearly demonstrate decreased survival of lenti-GFP-shp65-transduced AMs, this issue is particularly significant with a target molecule such as NF-κB that has many downstream targets and is known to affect cell survival in the setting of injury. Indeed, constitutive high-level expression of any shRNA molecule could render the basic cellular gene silencing apparatus unavailable for other microRNAs involved in natural cellular metabolism. We anticipate that the use of inducible promoter systems to allow temporal control of gene expression will help to address this issue.

In this manuscript, we describe a novel lentiviral vector allowing us to measure activation of the transcription factor NF-κB in live animals in real time. In contrast to conventional methods for measuring transcription factor activity, our vector allows for linear tracking of p65 activation in individual, live animals. This characteristic has many potential advantages, not the least of which is the need for fewer animals to perform measurements at multiple time points. In comparison with existing methods, we expect that this will be a valuable tool to investigators performing studies of this and other transcription factors *in vivo*.

Our studies demonstrate a technique for specific delivery of shRNA to resident AMs, key immune effector cells in the lung. This tool will provide a new approach to the study of AM function and could potentially be used to alter the course of lung diseases in which AMs have been implicated, such as chronic obstructive pulmonary disease, pulmonary alveolar proteinosis, tuberculosis, and others. Although the tropism of vesicular stomatitis virus G pseudotyped lentivirus for AMs rather than lung epithelium in our system results from the architecture of the polarized epithelial cell surface,[14,17,41,42] recently published literature suggests that lentiviruses can be engineered to achieve transduction of cell types displaying specific cell receptors, thus potentially allowing extension of this approach to other cell types.[43,44]

METHODS

Animal studies and luciferase imaging. C57BL/6J, transgenic chicken β-actin-GFP mice (The Jackson Laboratory, Bar Harbor, ME), or mice deficient in myeloid p65 (generated by crossing LysM-Cre mice with p65-floxed mice[26] to produce LysM-Cre^{tg+}/p65$^{loxP/loxP}$ (p65$^{\Delta/\Delta}$) and LysM-Cre^{tg-}/p65$^{loxP/loxP}$ (p65$^{F/F}$) littermates) were maintained in a pathogen-free facility. All animal studies were approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine.

Vectors and viral production. Replication incompetent lentiviruses used in the gene transfer experiments were created using a 5 plasmid transfection procedure as previously described.[45,46] Briefly, 293T cells were transfected with the pHAGE[14] or pLVTHM[15] backbone lentiviral vector together with four expression vectors encoding the packaging proteins Gag-Pol, Rev, Tat, and the G protein of the vesicular stomatitis virus. All viral supernatants were concentrated approximately 100-fold by ultracentrifugation. Titering of all vectors was performed by infection of FG293 cells as previously published.[44] Viral titers of approximately 5×10^8 to 5×10^9/ml were typically achieved using this protocol.

pLVTHM is a previously published lentiviral backbone expressing a GFP reporter gene under control of the ubiquitous EF1α promoter that contains cloning sites to insert shRNA molecules in the 3′ LTR after a Pol III promoter, H1. An element (teto) precedes the H1 promoter that allows tetracycline (tet)-inducibility of the H1 promoter in the presence of

additional constructs that express a repressor fusion protein, TTR-KRAB. In the absence of any TTR-KRAB-expressing construct (as used in the experiments described herein) the H1-shRNA construct constitutively drives shRNA expression. To adapt pLVTHM for use in GFP knockdown experiments, cDNA encoding a variant of the red fluorescent protein adapted from *Discosoma sp*. (DsRed-Express; Clontech, Mountain View, CA) was amplified by PCR with PmeI and SpeI restriction sites added to the 5′ and 3′ ends, respectively. This amplicon was cloned into the pLVTHM backbone downstream of the EF1α promoter by ligation to PmeI/SpeI cohesive ends. Previously published shRNA's targeting eGFP[16] or p65[30] were adapted for cloning into pLVTHM by the addition of MluI (5′) and ClaI (3′) restriction sites and were inserted into the 3′ LTR using standard directional cloning techniques. Scrambled versions of these shRNA sequences were generated using an online tool designed for this purpose (sirnawizard, Invitrogen, Carlsbad, CA); the vectors created using these sequences were used as negative control vectors in specified experiments.

The pHAGE lentiviral backbone is an optimized self-inactivating lentiviral LTR, nonreplicative vector derived from the original pHR'CMV-lacZ vector previously described by Naldini *et al.*[14,45,47] cDNA encoding the NF-κB enhancer sequence upstream of the minimal TA promoter of the herpes simplex virus followed by the firefly luciferase gene was amplified by PCR from plasmid NFκB-luciferase (Clontech) with SpeI and BamHI restriction sites attached to the 5′ and 3′ ends. This amplicon was cloned into the first position of the pHAGE backbone by ligation to SpeI/BamHI cohesive ends.

In vitro *transduction and LPS stimulation.* 293T or Raw 264.7 cells were transduced with lentivirus at varying multiplicities of infection as indicated in individual experiments. Cells were incubated overnight with lentiviral supernatant in the presence of 5 mcg/ml polybrene (Sigma, St Louis, MO). Cultured cells were harvested and fluorochrome expression was assessed by flow cytometry (BD FACScan; BD Biosciences, San Jose, CA, and FlowJo analysis software; Treestar, Ashland, OR). In some cases, cells were stained with propidium iodide to exclude dead cells. In experiments quantifying NF-κB signaling, Raw 264.7 cells were incubated with 100 ng/ml LPS (List Biological Laboratories, Campbell, CA) for 6 hours before analysis.

To monitor GFP reporter gene knockdown, a 293T cell line containing a single integrated copy of a GFP-expressing vector was generated as follows: 293T cells were transduced with a limiting dilution (multiplicity of infection = 0.1) of lentiviral vector encoding GFP. Single cells were sorted into individual wells of a 96 well plate and a single clone was picked and passaged for knockdown studies detailed in the text.

Quantification of* in vitro *gene expression. Protein levels of GFP (293 cell lysates) and p65 (Raw 264.7 cell lysates) were quantified by western blot using a rabbit polyclonal anti-GFP primary antibody (Abcam) and rabbit antihuman p65 primary antibody (Cell Signaling Technology, Danvers, MA), respectively. For analysis of p65 expression levels, total RNA was prepared with RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacture's protocol, followed by Northern analysis using[33] P-labeled cDNA probes for p65 and glyceraldehyde-3-phosphate dehydrogenase as previously described.[48] The p65 probe was generated by PCR amplification of mouse cDNA using primers 5′GCCTCATCCACATGAACTTGTGGG3′ and 5′ACCATGGTCTGGGCAAGGACTGGG3′. To evaluate NF-κB dependent cytokine levels in cell culture supernatants, we performed a mouse cytokine protein antibody-based assay (RayBiotech, Norcross, GA) as per the manufacturer's instructions. Luciferase expression levels in Raw 264.7 cells were quantified using the DualGlo luciferase assay system and Promega Glomax Multidetector plate reader (Promega, Fitchburg, WI).

In vivo transduction and LPS stimulation. In preparation for IT lentivirus or IT LPS injections, mice were anesthetized with isoflurane. Concentrated lentiviral supernatant (100 mcl) or LPS (either 20 ng or 100 ng in 100 mcl phosphate-buffered saline; List Biological Laboratories) was delivered via a blunt-ended, 18-gauge needle into the posterior oropharynx just above the tracheal entrance as previously described.[14] Aspiration of this fluid bolus upon the subsequent inhalation was confirmed in each recipient by investigator visualization. Viral supernatants were mixed with Lipofectamine 2,000 (5% final vol/vol; Invitrogen) before instillation to increase transduction efficiency.[14]

Quantification of in vivo gene expression. Two or 4 weeks after IT lenti-GFP-shp65 or -scramble, RNA was isolated from sorted, GFP^+ AMs using an RNeasy Micro Kit (Qiagen). RNA was reverse transcribed using TaqMan Reverse Transcription reagents (Applied Biosystems, Carlsbad, CA). Quantitative PCR of cDNA was performed using a StepOneReal Time PCR System and TaqMan primers (Applied Biosystems). Reactions were performed in duplicate using 1:20 diluted cDNA. mRNA expression levels were normalized to 18s rRNA and quantification of relative gene expression presented as fold change compared with the relevant baseline was calculated using the $2^{-\Delta\Delta CT}$ method.

Noninvasive bioluminescence imaging. Before imaging, mice were anesthetized with isofluorane and 150 mcl D-luciferin substrate (30 mg/ml; Xenogen, AlamedΔa, CA) was administered by intraperitoneal injection. A series of bioluminescent images were taken for up to 32 minutes after luciferin injection using a Xenogen IVIS 100 imager (Caliper Life Sciences, Hopkinton, MA). Photon output was quantified at the plateau of the time course using Living Image software (Xenogen) and expressed as photon flux (p/s).

BAL. After euthanasia, BAL was performed by cannulation of the trachea and instillation and aspiration of three consecutive 1,000 mcl aliquots of phosphate-buffered saline per mouse. BAL cells and fluid were separated by centrifugation. The first fraction of BALF was stored at −80 °C for subsequent analysis of cytokine levels. Cells from all 3 fractions were pooled for analysis by flow cytometry, cytospin, or for RNA extraction. Cytospin cell counts were performed in a blinded fashion with a minimum of 200 cells counted per sample; neutrophils were quantified as a percent of nucleated cells in the BAL.

Flow cytometry. Flow cytometry was performed on unstained 293 or BAL cells to quantify transduction efficiency (percentage of BAL cells GFP^+ or $DsRed^+$). In some cases, propidium iodide (2 mcg/ml; Molecular Probes, Eugene, OR) was added to cell samples to exclude dead cells from analysis. A gating algorithm was used to identify AMs within BAL cell populations as previously published.[14] All flow cytometry was performed on BD FACScan or BD LSR II (BD Biosciences) instruments. Cell sorting experiments were performed on a Moflo flow cytometer (DakoCytomation, Fort Collins, CO). Analysis of raw data was completed using FlowJo software (Treestar).

BALF cytokine profile analysis. BALF aliquots from mice exposed to lentiviruses and LPS were analyzed for levels of up to 11 cytokines by multiplex cytokine array (Millipore, Billerica, MA). Analysis was performed using a Qiagen LiquiChip 200 machine and analyzed using LiquiChip analyzer software (Qiagen). The cytokines assayed were IL-1α, IL-1β, IL-6, IL-10, KC, MCP-1, MIP1α, MIP1β, TNFα, GCSF, and LIX.

Statistics. The two-tailed Student's *t*-test was used to compare specified groups with the exception of the data displayed in **Supplementary Figure S1**, where the nonparametric Mann–Whitney test was applied. In all studies, differences between groups were considered statistically significant at $P < 0.05$. In the experiment described in **Figure 5**, one mouse in each group was excluded from post-LPS analysis after failure to aspirate intratracheally administered LPS. Error bars in all figures represent standard error of the mean (SEM).

SUPPLEMENTARY MATERIAL

Figure S1. NF-κB dependent cytokine production in is suppressed in lenti-GFP-shp65 transduced Raw 264.7 macrophages after stimulation with LPS.

Figure S2. Gene expression in GFP$^+$ or GFP$^-$ BAL cells 4 weeks after EF1α-GFP-shp65 or EF1α-GFP-scramble lenti and 2 hours after IT LPS 20 ng.

Figure S3. Fluorescence activated cell sorter analysis of lavaged AMs from a mouse treated with a mixture of red (CMV-dsRed) and green (NF-κB-luc-GFP) lentivirus.

Figure S4. Fluorescence activated cell sorter analysis of lavaged AMs from mice treated with mixtures of lenti-NF-κB-luc-dTomato and lenti-GFP-sh65 or – scramble and intratracheal LPS demonstrates similar percentages of double positive AMs between groups.

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