

Production of a p65^{fl/fl}/LysMCre mouse model with dysfunctional NF- κ B signaling in bone marrow-derived macrophages

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Abstract

Here, we describe the production and characterization of a novel p65^{fl/fl}/LysMCre mouse model, which lacks canonical nuclear factor-kappaB member RelA/p65 (indicated as p65 hereafter) in bone marrow-derived macrophages. Cultured bone marrow-derived macrophages that lack p65 protein reveal NF- κ B signaling deficiencies, a reduction in phagocytic ability, and reduced ability to produce nitrites. Despite abnormal bone marrow-derived macrophage function, p65^{fl/fl}/LysMCre mice do not exhibit differences in naïve systemic immune profiles or colony forming units and time to death following *Salmonella* infection as compared to controls. Additionally, p65^{fl/fl}/LysMCre mice, especially females, display splenomegaly, but no other obvious physical or behavioral differences as compared to control animals. As bone marrow-derived macrophages from this transgenic model are almost completely devoid of canonical nuclear factor-kappaB pathway member p65, this model has the potential for being very useful in investigating bone marrow-derived macrophage NF- κ B signaling in diverse biological and biomedical studies.

Keywords

Animal model, bone marrow-derived macrophage, cytokine, nuclear factor-kappaB, innate immunity

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Introduction

The nuclear factor-kappaB (NF- κ B) pathway is a family of five highly conserved transcription factors (RelA/p65 (indicated as p65 hereafter), RelB, cRel, p50, and p52) that work together to promote a variety of functions, including cell survival and immune system response.^{1,2} There are two major NF- κ B pathways: canonical and non-canonical. The canonical pathway is activated by ligands such as lipopolysaccharide (LPS) and TNF, and functions through activation of the IKK complex (IKK β , IKK α , and IKK γ /NEMO). Active IKK β phosphorylates the inhibitor of kappaB alpha (I κ B α), which leads to its ubiquitination and subsequent proteasomal degradation.^{3,4} I κ B α normally inhibits NF- κ B family members p65/p50 by sequestration in the cytoplasm. Thus, NF- κ B pathway activation and I κ B α degradation leads to the release of the p65/p50 heterodimer, which then enters the nucleus and promotes transcription of a large number of target genes involved in cell survival and immune function.⁵ The non-canonical NF- κ B pathway, on the other hand, is activated by ligands such as RANKL and LT β , which activates an IKK α homodimer to cause proteolytic cleavage of p100

to p52. The p52/RelB transcription factor heterodimer can then upregulate target genes involved with B cell homeostasis and differentiation.¹ NF- κ B signaling is critical for many immune functions including the activation of macrophages and the production and regulation of cytokines.^{6–8} To better understand the role that the canonical NF- κ B signaling pathway plays in myeloid cells, the canonical pathway was targeted for deletion in our model.

As full cellular p65 deletion is not compatible with life, due to massive liver apoptosis during embryogenesis,^{9,10} it

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is necessary to create conditional p65 knockout models. Other conditional p65 knockout models have demonstrated important, positive, roles for the NF- κ B pathway in diverse areas such as in hematopoietic stem cell function when p65 is deleted in the hematopoietic compartment.¹¹ Mice with cardiomyocyte-specific deletion of p65 were protected from ischemia reperfusion injury through altered intracellular calcium homeostasis, suggesting a negative role for NF- κ B.¹² Furthermore, a conditional knockout model of p65 in adipocytes suggests a dual role for NF- κ B in regulating inflammation in adipose tissue, where lean conditions prompted pro-inflammation activity and obese conditions prompted anti-inflammation activity.¹³ Another study describes a doxycycline inducible system, where NF- κ B can be inhibited or activated in colony stimulating factor 1 receptor expressing cells.¹⁴ It was found that during a short window of time following injection of circulating mammary tumor cells, activation of NF- κ B in macrophages induced an M1 phenotype in the macrophages, which resulted in reduced lung tumor metastases.¹⁴ This antitumor effect was no longer seen two days post tumor cell injection, indicating a dual role of macrophage NF- κ B in this metastasis model.

The LysMCre animal model was initially created and characterized in 1999,¹⁵ and has since been used extensively in various fields of biomedical research.^{16–18} As NF- κ B has been shown to play important roles in macrophage function,^{19–21} we designed an animal model that lacks p65 in bone marrow-derived macrophages (BMDM). Here, we describe a conditional animal model made by crossing the LysMCre model to a p65^{fl/fl} animal,²² thus producing p65^{fl/fl}/LysMCre mice which have very efficient knockdown of p65 protein in BMDMs. The efficient knockdown of p65 protein was shown to lead to altered canonical NF- κ B signaling, reduced phagocytosis ability, and reduced nitrite production in BMDMs. Moreover, p65^{fl/fl}/LysMCre mice exhibited similar circulating immune cell profiles and comparable immune response to *Salmonella* infection.

Here, we have specifically developed and characterized the novel p65^{fl/fl}/LysMCre mouse model to study the role of BMDM NF- κ B signaling in immune system function. As aberrant expression of NF- κ B is associated with numerous diseases including autoimmune disease, neurodegenerative diseases, and many cancers, this model will provide an avenue to not only study basic immune function, but to also study many different diseases in the context of p65 loss in BMDMs.

Materials and methods

p65^{fl/fl}/LysMCre mouse production

p65^{fl/fl} mice were previously generated in the Baldwin laboratory.²² Briefly, the p65 gene in these animals was

manipulated to contain two LoxP sites, one near exon 4, and the other in the intron between exons 8 and 9. B6.129P2-Lyz2^{tm1(cre)lfo}/J animals,¹⁵ referred to as LysMCre from here on, were purchased from The Jackson Laboratory (stock #004781). These mice contain Cre enzyme under the control of the LysM promoter, which drives Cre recombinase expression in cells of the myeloid lineage. p65^{fl/fl} mice were bred to LysMCre mice for several generations to generate a fully transgenic p65^{fl/fl}/LysMCre mouse. Genotypes were confirmed via PCR using the following primers: Lys Mutant Primer 5' - CCCAGAAATGCCAGATTACG - 3', Lys Common Primer 5' - CTTGGGCTGCCAGAATTTCTC - 3', Lys Wild Type Primer 5' - TTACAGTCGGCCAGGCTGAC - 3'. Reactions included WT and common, or mutant and common primers. LysMCre PCR reaction as follows: 94°C for 3 min, 94°C for 30 s, 62°C 1 for minute, 72°C for 1 min, go to step 2 and repeat for 34 cycles, 72°C for 2 min. p65^{fl/fl} PCR primers used are: 5' CGACTTTG GGTGGAGGGTTACAGAAGGC 3' and 5' TGGTCT GGATTCGCTGGCTAATGGC 3'. The p65^{fl/fl} PCR reaction is: 95°C for 2 min, 95°C for 30 s, 65°C for 30 s, 72°C for 45 s, go to step 2 and repeat for 34 cycles, 72°C for 5 min.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the IACUC of Augusta University (#2014-0691) and the University of North Carolina at Chapel Hill (#18-175.0). All animals were housed under barrier conditions.

Bone marrow-derived macrophage isolation

Animals between the ages of 1 and 5 months were euthanized according to IACUC protocol. Hind leg femurs and tibias were dissected and cleaned of muscle. The ends of the bones were removed, and the bone marrow was flushed out using sterile 1xPBS/2%FBS and a syringe with a 27 gage needle. The marrow was resuspended via pipetting, filtered with a 70 μ m cell strainer, and cells were plated in macrophage media: DMEM (Fisher 11995-065) containing 10% FBS and 20% L929 conditioned media.²³ L929 conditioned media, containing M-CSF, was obtained by growing L929 cells (ATCC) to confluency for 1 week and collecting the culture media.²³ Cultured bone marrow monocytes were grown in the macrophage media for 1 week to allow differentiation prior to use. During the differentiation week, cells were washed with 1xPBS and media was changed twice, starting on day 3, post-harvest.

Tail vein blood collection

Mice were warmed by lamp for 5 min prior to restraining in the tailveiner restraint. Using a syringe and 30-gauge

needle, one of the lateral tail veins was punctured. Between 30 μ l and 70 μ l of blood was collected using a heparinized micro-hematocrit capillary tube (22-362566 Fisherbrand) and was then transferred into K₂ EDTA spray-coated vacutainer tubes (02-683-99C BD). Depending on the volume of blood, 300 to 500 μ l of pre-warmed 1 \times RBC Lysis Buffer (420301 Biologend) was pipetted into the blood samples and then all samples were incubated at room temperature for 5 min. 3.5 ml of PBS/2% FBS was added, and samples were centrifuged at 300 g for 5 min at 4°C. The supernatants were aspirated, and pellets were resuspended in 500 μ l PBS/2% FBS before proceeding to antibody staining and flow cytometry analysis.

Flow cytometry

BMDM samples were fixed using the eBioscience™ Intracellular Fixation Buffer (88-8824-00 Invitrogen). Samples were analyzed, with the NovoCyte Quanteon (Georgia Cancer Center's Flow Cytometry Shared Resource Laboratory), for myeloid cell (CD11b+) and macrophage (CD11b+, F4/80+) populations. Zombie Violet (423113 Biologend) was used as a viability dye. Antibody conjugations were as follows: CD11b-PE-Cyanine7 (25-0112-82 Invitrogen) and F4/80-FITC (123108 Biologend).

Blood samples from six 15 week-old LysMCre control and six 15 week-old p65^{fl/fl}/LysMCre mice were processed (described in the tail vein blood collection method) and analyzed with the NovoCyte Quanteon (Georgia Cancer Center's Flow Cytometry Shared Resource Laboratory), for various cell populations: Macrophages (CD11b+, F4/80+); G-MDSCs (CD11b+, Ly-6G+); M-MDSCs (CD11b+, Ly-6C+); T Cells (CD3+); T Helper cells (CD3+, CD4+); Cytotoxic T cells (CD3+, CD8 α +). DAPI (564907 BD) was used as a viability dye. Antibody conjugations were as follows: CD11b-PE-Cyanine7 (25-0112-82 Invitrogen), F4/80-FITC (123108 Biologend), Ly-6G-APC-Cyanine7 (127624 Biologend), Ly-6C-PE (12-5932-82 Invitrogen), CD3-PE (12-0032-82 Invitrogen), CD4-APC (17-0042-82 Invitrogen), CD8 α -FITC (11-0081-82 Invitrogen).

Western blotting

Tissues/cells were lysed in RIPA buffer containing protease (Roche #11697498001) and phosphatase (Sigma #P0044-1ML) inhibitors, and lysates were run on 4-12% precast protein gels (Invitrogen #NP0231Box). Standard immunoblotting methods were followed. Antibodies used for western blotting include: p-p65 (Cell Signaling Technologies #3033), total p65 (Cell Signaling Technologies #8242), GAPDH (Santa Cruz #25778), and β -actin (Cell Signaling Technologies #4970).

Immunofluorescent staining

BMDMs were plated in 6-well (1.0×10^6 cells/well) cell culture plates containing cover glasses and allowed to adhere overnight. Wells were washed 1 \times with PBS and media was refreshed. Adherent cells were stimulated with LPS (50 ng/ml) for 3 h and were then washed once with PBS and fixed with 4% formaldehyde for 10 min at room temperature. Fixative was removed from wells and cells were washed 3 \times with PBS prior to immunostaining. Cells were incubated with PBS containing 5% goat serum and 0.3% triton x-100 for 60 min at room temperature followed by monoclonal rabbit anti-mouse NF- κ B p65 (1:400 dilution) (Cell Signaling Technologies #8242) overnight at 4°C. Cells were washed 3 \times with PBS and then incubated with goat anti-rabbit Alexa Fluor 555 (1:300 dilution) (Cell Signaling Technologies #4413) for 60 min at room temperature, washed again, and mounted in ProLong Gold medium (Thermofisher #P36930). Cells were examined using a Zeiss Axiovert 200 fluorescence microscope and captured and analyzed with a Zeiss AxioCam MRm camera and Axiovision software, respectively.

Peritoneal macrophage isolation

Animals were euthanized according to IACUC approved protocols and cold 1xPBS/5 mM EDTA was injected into the peritoneal cavity via syringe with a 27 gage needle. The peritoneal area was gently massaged and the PBS/EDTA was collected via syringe and 25 gage needle.²⁴ Cells from six LysMCre control mice were combined, and cells from six p65^{fl/fl}/LysMCre mice were combined. The isolated cells were plated for 4 h, after which, floating cells were aspirated. The remaining, adherent cells were then further selected for macrophages by using CD11b+ microbead/magnetic column selection (Miltenyi #130-093-636). Purified peritoneal macrophages were then processed for western blotting.

qPCR analysis

BMDMs grown in culture for 1 week (described above) were treated with 100 ng/ml LPS (Invitrogen #tlrl-eblps) or vehicle control for 4 h or 50 ng/ml LPS and 150 U/ml IFN γ for 24 h. Cells were harvested, and RNA was isolated using RNeasy Mini Plus Kit (Qiagen #74134). cDNA was then produced using the SuperScript II First-Strand Synthesis System (Invitrogen 11904018). TaqMan primer/probe systems (ABI/Life/Fisher) and 2 \times Master Mix (Roche 06402682001) were used for quantitative PCR. NF- κ B target genes IL-6 (Mm00446190_m1), IL-1 β (Mm00434228_m1), and TNF α (Mm00443258_m1) were normalized to GusB (Mm01197698_m1) expression. For polarization studies, iNOS (Mm00440502_m1), IL-12 (Mm01288989_m1), and IL-10 (Mm00439614) were

normalized to GAPDH (Mm9999915_g1). Each sample was run in triplicate and statistical analysis was determined using GraphPad Prism software.

Cytokine array

Mouse Cytokine Array Panel A (R&D Systems ARY006) was used to detect cytokines secreted into the media by LysMCre and $p65^{fl/fl}$ /LysMCre BMDMs. 1×10^6 BMDMs were seeded into 6 well cell culture plates with 4 ml of media, which was collected for the array after 24 h. R&D systems directions were followed, and the film was exposed for 1.5 h. ImageJ software was used to analyze the spots, and GraphPad Prism software was used for graphing and statistical analysis (unpaired t test).

Salmonella infection and splenic cell population analysis

LysMCre and $p65^{fl/fl}$ /LysMCre mice were infected (IP) with 700 CFUs of *Salmonella typhimurium* bacteria (4–5 mice each group) or sterile PBS (2 mice each group). Spleens and mediastinal lymph nodes were collected after 48 h and analyzed for bacterial colony forming units. Data is representative of two experiments. GraphPad Prism software was used for graphing and statistical analysis.

Splenomegaly analysis

Non-infected 7 week old LysMCre and $p65^{fl/fl}$ /LysMCre mice were examined for splenomegaly. Following euthanasia, spleens were dissected and weighed. GraphPad Prism software was used for graphing and statistical analysis (unpaired t test). A minimum of 6 mice were used per group.

Phagocytosis assay

Per directions, (Vybrant Phagocytosis Assay Kit -Thermo/Fisher #V6694), 50,000 LysMCre control or $p65^{fl/fl}$ /LysMCre BMDMs were plated into a 96 well tissue culture treated plate (Costar 3610). The BMDMs were allowed to settle overnight, and were then treated with 150U of IFN γ (Fisher PMC4031) for 6 h, or vehicle control. The media was then removed, cells were washed, and 0.2 μ g of LPS was added for 18 h. The fluorescent *E. coli* particles were added to the cells and incubated for 2 h to allow for phagocytosis to occur. Fluorescence activity was determined using a BioTek Synergy HT plate reader, analyzing at 480 nm excitation and 520 nm emission conditions. Each group, including positive and negative controls, was run in quadruplicate, and statistical analysis was determined using GraphPad Prism software (two-tailed student's t test).

Nitric oxide assay

Day 6 BMDM were plated in a 24 well tissue culture plate at a concentration of 3×10^5 cells per well, and were allowed to adhere overnight. Cells were washed with 1 \times PBS and 500ul of culture media was added to each well. BMDMs were treated with either vehicle control (sterile water), 50 ng/ml LPS for 24 h, 150 U/ml IFN γ for 24 h, 50 ng/ml LPS and 150 U/ml IFN γ for 24 h, or 150 U/ml IFN γ for 6 h followed by 50 ng/ml LPS for 18 h. Supernatants were collected and analyzed for nitric oxide response, per directions, using the QuantiChrom Nitric Oxide Assay Kit (BioAssay Systems D2NO-100). Colorimetric activity was determined using a BioTek Synergy HT plate reader with analysis at 540 nm. Each group, including positive and negative controls, was run in quadruplicate, and statistical analysis was determined using GraphPad Prism software.

Results

$p65^{fl/fl}$ /LysMCre bone marrow cells can differentiate into macrophages

$p65^{fl/fl}$ animals, previously generated in the Baldwin lab,²² were crossed with LysMCre mice¹⁵ purchased from The Jackson Laboratory. LysMCre mice contain Cre enzyme, which has been placed under the control of the LysM promoter, thus driving Cre recombinase expression in cells of the myeloid lineage.¹⁵ $p65^{fl/fl}$ mice were bred to LysMCre mice for several generations to generate fully homozygous $p65^{fl/fl}$ /LysMCre mice (Figure 1a), which were confirmed by PCR based genotyping. It was first determined via flow cytometry that bone marrow cultured monocytes from age matched control and $p65^{fl/fl}$ /LysMCre mice would yield a high purity, and that these monocytes could differentiate into macrophages (CD11b+/F4/80+) (Figure 1b). In fact, bone marrow from LysMCre and $p65^{fl/fl}$ /LysMCre mice produced equivalent percentages of bone marrow-derived macrophage (BMDM) populations following 1 week in culture. Cells were allowed to differentiate for one week and were stained with the CD11b monocyte marker (Figure 1b top panel), and CD11b and F4/80 antibodies to detect macrophages (Figure 1b bottom panel). There were no significant differences in percentages of CD11b+ myeloid cells (~99%) or CD11b+/F4/80+ macrophage populations (~93%) between control and $p65$ deficient BMDMs (Figure 1b).

$p65^{fl/fl}$ /LysMCre BMDMs lack p65 protein

We next confirmed via western blotting whether BMDM from mice lacked p65 protein. Protein analysis revealed that 7 day old $p65^{fl/fl}$ /LysMCre BMDMs lack p-p65 and have dramatically reduced total p65, as compared to

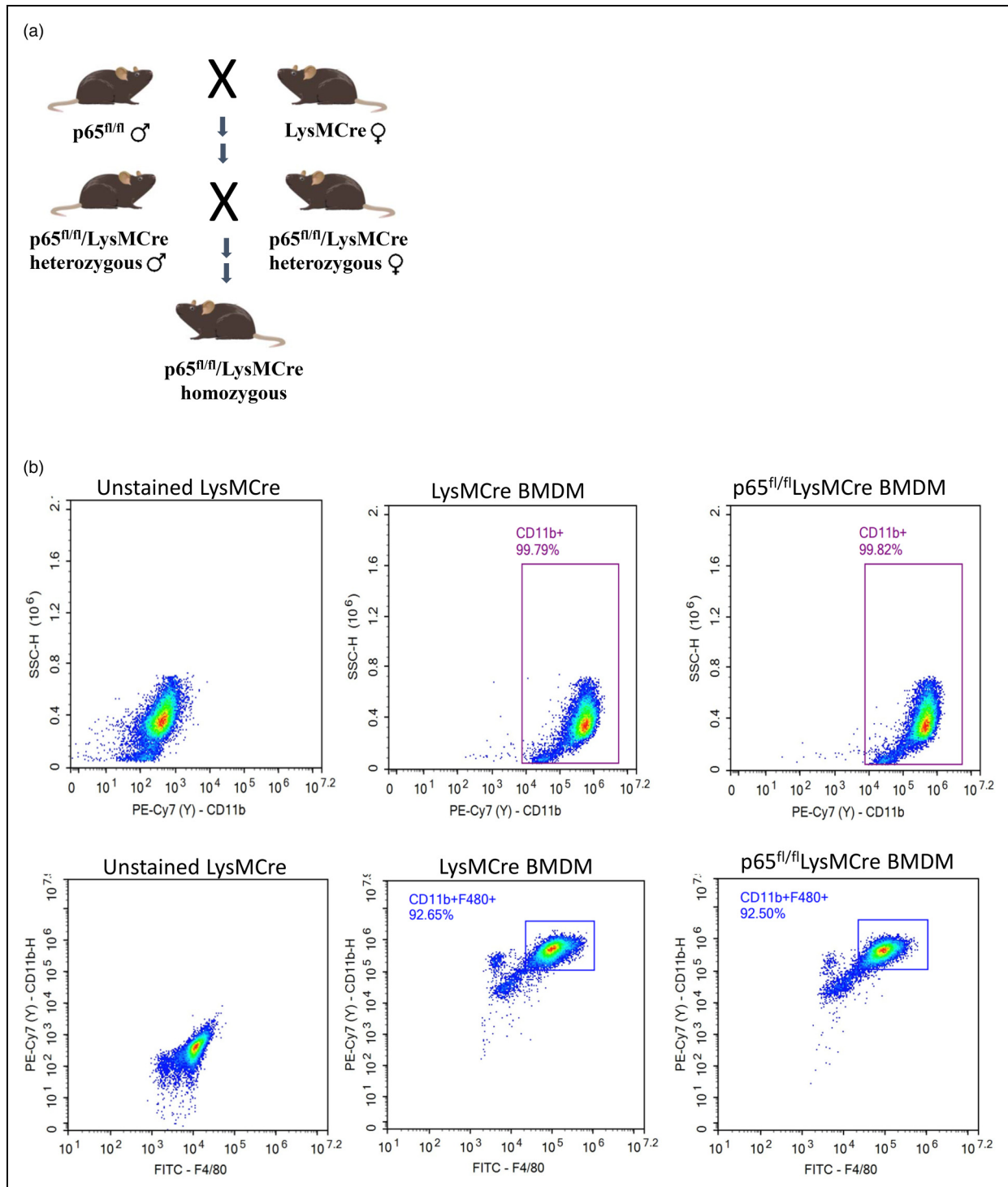


Figure 1. $p65^{fl/fl}/LysMCre$ model and bone marrow differentiation. Model of breeding schematic used to create homozygous $p65^{fl/fl}/LysMCre$ mice (A). Bone marrow from LysMCre and $p65^{fl/fl}/LysMCre$ mice was harvested and cultured for 1 week to test monocyte purity via CD11b+ staining (B top panel) and ability to differentiate into mature macrophages (CD11b+/F4/80+) (B bottom panel).

LysMCre and $p65^{fl/fl}$ control mice (Figure 2a, left panel and Figure 2b). This has been consistently seen in BMDMs

mice, and over multiple generations. However, it was found that peritoneal macrophages still retained residual amounts of p65 protein, indicating a leaky Cre system

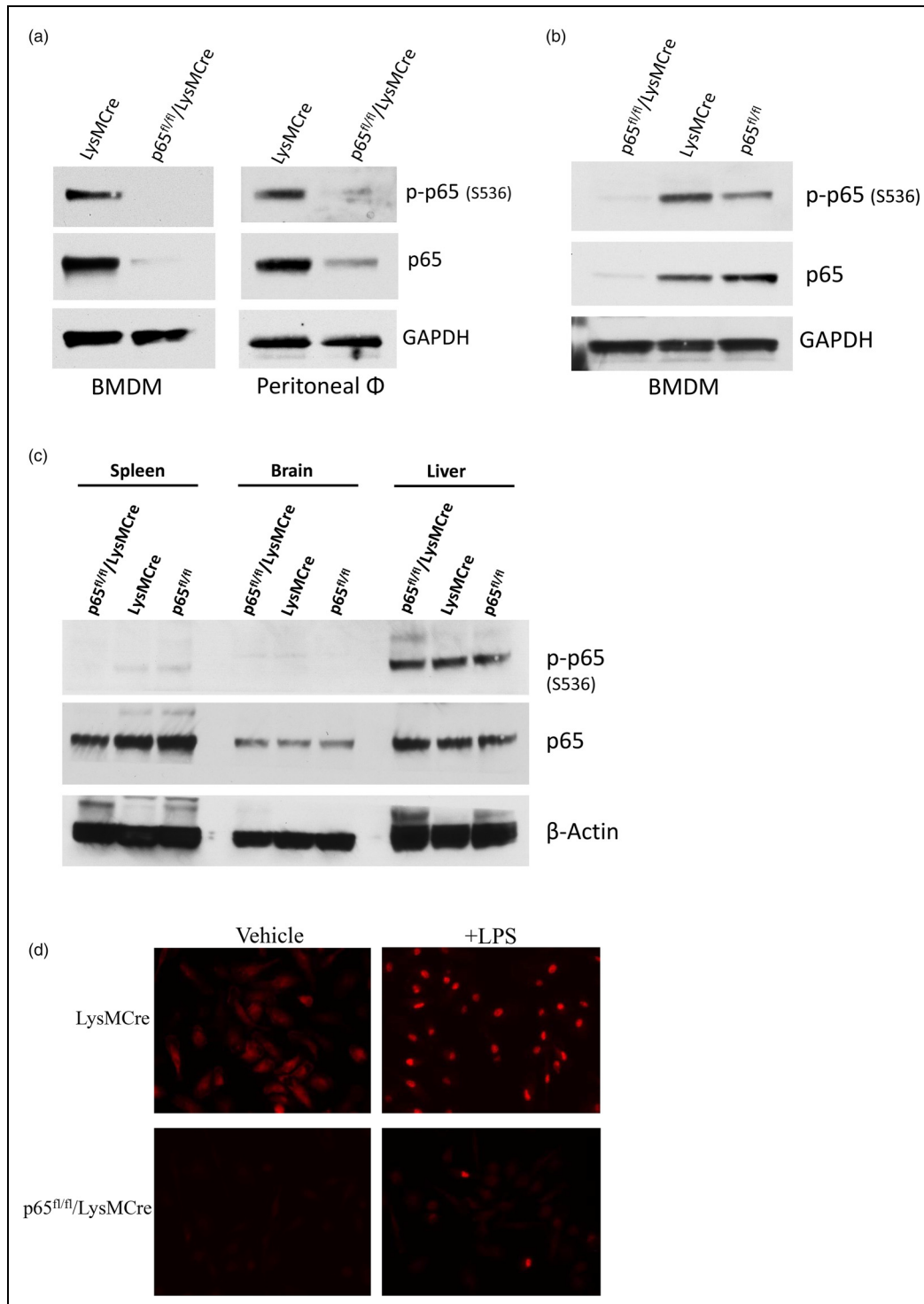


Figure 2. p65^{fl/fl}/LysMCre BMDMs lack p65 protein. p65^{fl/fl}/LysMCre BMDMs have very low to no p-p65 and total p65 protein (A left panel and B), while p65^{fl/fl}/LysMCre peritoneal macrophages have some residual p65 protein (A right panel). p65 total protein levels in spleen, brain, and liver of p65^{fl/fl}/LysMCre, LysMCre control, or p65^{fl/fl} control mice (C). LysMCre control or p65^{fl/fl}/LysMCre BMDMs were stained for p65 following treatment with 100 ng/ml LPS or vehicle control. Images on the right show nuclear localization of p65 following LPS treatment (10 \times images) (D).

(Figure 2a, right panel). This is not surprising, as the original LysMCre model was described as having less than 100% knockdown in mature macrophages.¹⁵

To determine any differences in phosphorylated or total p65 protein levels in various tissues, spleen, brain, and liver was analyzed via western blotting (Figure 2c). p65/p-p65 protein levels did not vary much between the brain and liver from control and the p65^{fl/fl}/LysMCre mice, but there was a small reduction in p65 protein between the p65^{fl/fl}/LysMCre spleen and control spleens (16.5% reduction when normalized to β -actin). No p-p65 protein was detected in the p65^{fl/fl}/LysMCre spleen, while a very small amount was present in the control spleens (Figure 2c).

Immunohistochemistry was also performed to verify that p65^{fl/fl}/LysMCre BMDMs lack p65 protein following lipopolysaccharide (LPS) stimulation (Figure 2d), as compared to LysMCre control BMDMs. LysMCre and p65^{fl/fl}/LysMCre BMDMs were treated with LPS or vehicle control for 4 h to stimulate canonical NF- κ B pathway activity, and cells were stained with p65. The staining clearly shows the lack of p65 staining in unstimulated p65^{fl/fl}/LysMCre BMDMs and drastically reduced nuclear localization of p65 upon LPS treatment (Figure 2d).

p65^{fl/fl}/LysMCre mice have altered canonical NF- κ B signaling

Canonical NF- κ B signaling pathway activity was next analyzed. LysMCre and p65^{fl/fl}/LysMCre BMDMs were treated with LPS or vehicle control for 4 h or 6 h to stimulate canonical NF- κ B pathway activity. IL-6, IL-1 β , and TNF α , all downstream target genes of canonical NF- κ B, were analyzed via qPCR to determine expression. LysMCre control BMDMs were able to efficiently upregulate the NF- κ B target genes after 4 h of LPS exposure, while the p65^{fl/fl}/LysMCre BMDMs had significantly reduced expression of IL-6, IL-1 β , and TNF α , indicating defective canonical NF- κ B signaling (Figure 3).

p65^{fl/fl}/LysMCre BMDMs secrete altered levels of select cytokines

To examine the effects of altered canonical signaling in the p65^{fl/fl}/LysMCre BMDMs on cytokine secretion, a cytokine profiler array was utilized. 24-h conditioned media from LysMCre or p65^{fl/fl}/LysMCre BMDMs was subjected to the Mouse Cytokine Array Panel A (R&D Systems) blot to determine whether there was a difference in cytokine secretion (Figure 4). While there was an unmeasurable amount of some cytokines analyzed in the panel (Supplemental Figure 1), LysMCre and p65^{fl/fl}/LysMCre BMDMs did release varying levels of multiple cytokines into their culture media. Significantly less CXCL10, TNF α , GM-CSF, and IL-1 α , and significantly more

TIMP-1 and M-CSF was secreted over 24 h by the p65^{fl/fl}/LysMCre BMDMs, as compared to control BMDMs (Figure 4). Some cytokines were detected but did not have significantly different levels between the two groups, including CXCL1, IL-16, IFN γ , and CCL2. This indicates that altered NF- κ B signaling in the p65^{fl/fl}/LysMCre BMDMs is in fact leading to altered secretion of some soluble factors.

p65^{fl/fl}/LysMCre BMDMs have altered innate immune function

A phagocytosis assay was used to determine whether the lack of NF- κ B signaling reduced phagocytosis efficiency in p65^{fl/fl}/LysMCre BMDMs. LysMCre control and p65^{fl/fl}/LysMCre BMDMs were plated into a 96 well plate, and were treated with vehicle control or with a pre-treatment of IFN γ for 6 h, followed by LPS treatment for 18 h to induce phagocytosis activity. The Vybrant assay used for this experiment provides fluorescein-labeled *E. coli* (K-12 strain) bio-particles, which should be efficiently engulfed by BMDMs. Macrophages that engulf these particles will fluoresce, which can be quantified using a plate reader. The p65^{fl/fl}/LysMCre BMDMs engulfed significantly fewer *E. coli* particles than the LysMCre control BMDMs in both the vehicle and IFN γ /LPS treated groups (Figure 5a). Interestingly, treatment of IFN γ /LPS had only negligible effects on phagocytic activity in either LysMCre control or p65^{fl/fl}/LysMCre BMDMs compared to vehicle treated BMDMs (Figure 5a).

LysMCre control and p65^{fl/fl}/LysMCre BMDMs were subjected to a nitrite production assay following 24 h of treatment with LPS, IFN γ , or LPS and IFN γ combined, to determine the ability of these cells to secrete nitric oxide, a common characteristic of stimulated macrophages that helps them kill pathogens.²⁵ The activity of this assay was based on the total amount of nitrite secreted into the media, as nitric oxide is a very short-lived molecule and nitrite is a much more stable metabolite of nitric oxide. The nitric oxide assay showed very little difference in nitrite levels among cells that had been stimulated by either LPS or IFN γ alone. However, cells that had been stimulated by LPS and IFN γ showed significant differences in the total amount of nitrites (Figure 5B). BMDMs lacking the canonical NF- κ B signaling pathway produced much lower concentrations of nitrates than the control macrophages, further indicating innate immune activity dysfunction.

p65 deficiency inhibits p65^{fl/fl}/LysMCre BMDMs from appropriately polarizing

As p65^{fl/fl}/LysMCre BMDMs showed reduced innate immune activity, we next determined whether these cells could polarize to an M1, classically activated, phenotype.

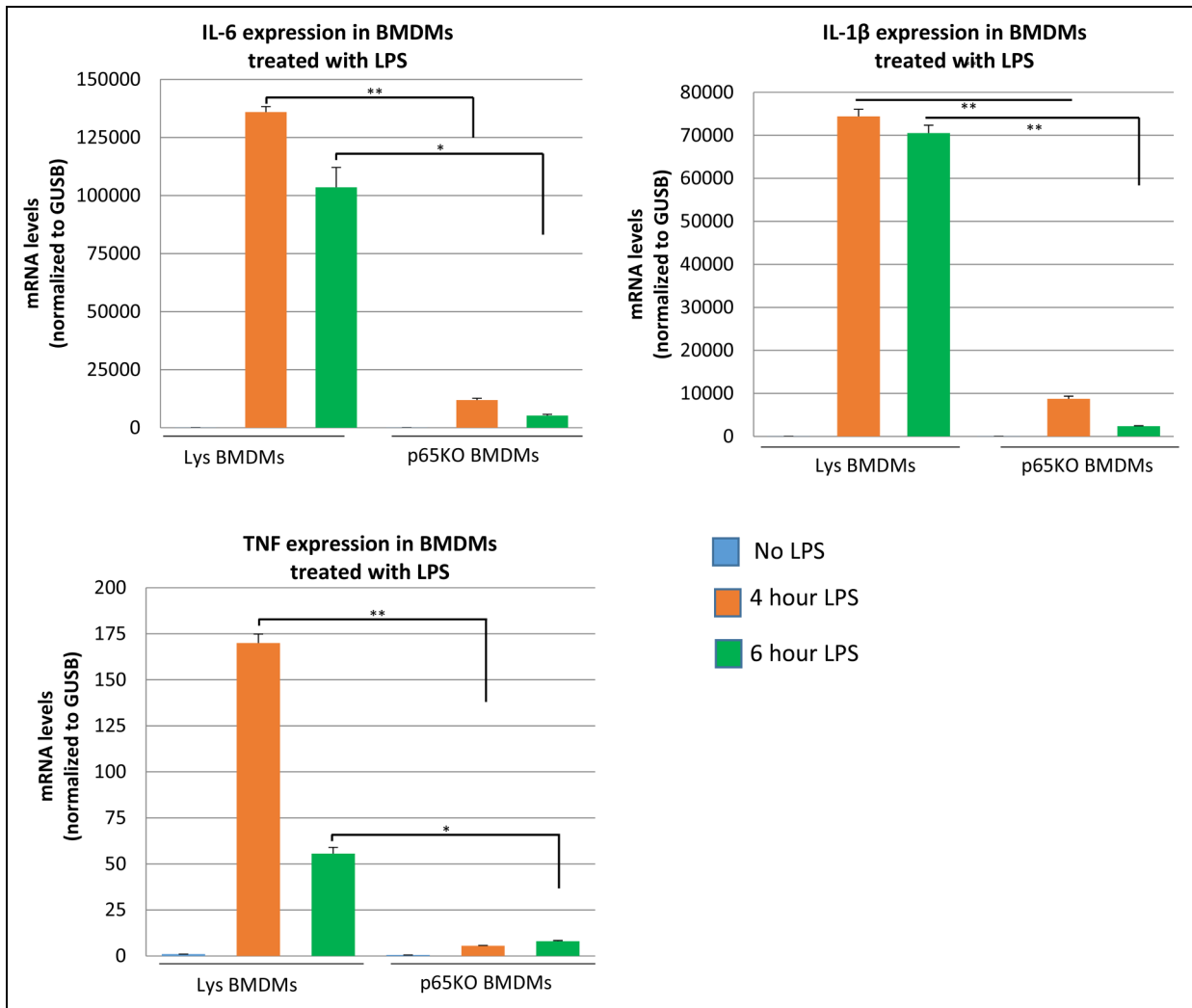


Figure 3. $p65^{fl/fl}/LysMCre$ BMDMs have deficient canonical NF- κ B signaling. LysMCre control or $p65^{fl/fl}/LysMCre$ BMDMs were treated for 4 or 6 h with 100 ng/ml LPS, and analyzed via RT-PCR for canonical NF- κ B target gene expression (IL-6, IL-1 β , and TNF). A two tailed Welch's t-test was used to calculate statistical significance: * $P \leq 0.0024$, ** $P \leq 0.0002$.

BMDMs from LysMCre control and $p65^{fl/fl}/LysMCre$ mice were treated with vehicle or a combination of 50 ng/ml LPS and 150 U/ml IFN γ for 24 h. qPCR analysis revealed that $p65^{fl/fl}/LysMCre$ BMDMs have a statistically significant reduction in iNOS and IL-12 mRNA expression, indicating a reduced ability to polarize to a pro-inflammatory (M1) phenotype (Figure 6a and 6b). $p65^{fl/fl}/LysMCre$ BMDMs also had a significant increase in IL-10 expression, indicating that the lack of p65 potentially pushes these cells into an alternatively polarized (M2-like) phenotype (Figure 6c).

Lysmcre and $p65^{fl/fl}/LysMCre$ mice have similar systemic immune profiles

We next decided to characterize the systemic immune cell populations of the $p65^{fl/fl}/LysMCre$ mice. In order to assess

baseline differences between $p65^{fl/fl}/LysMCre$ mice and LysMCre control mice, blood serum from 6 age-matched LysMCre mice and 6 age-matched $p65^{fl/fl}/LysMCre$ mice was evaluated through flow cytometry. Collected blood samples were treated with RBC lysis buffer and stained with common immune cell marker antibodies. No significant differences were seen between the controls and p65 deficient mice in percentages of myeloid cells (CD11b+), macrophages (CD11b + F4/80+), granulocytic-MDSCs (CD11b + Ly-6G+), monocytic-MDSCs (CD11b + Ly-6C+), T cells (CD3+), T helper cells (CD3 + CD4+), and cytotoxic T cell (CD3 + CD8+) populations. However, there are noticeable trends, with fewer G-MDSCs and cytotoxic T cells, but more M-MDSCs in $p65^{fl/fl}/LysMCre$ mice (Figure 7). Flow cytometry histograms are in the supplemental data section (Supplemental Figure 2).

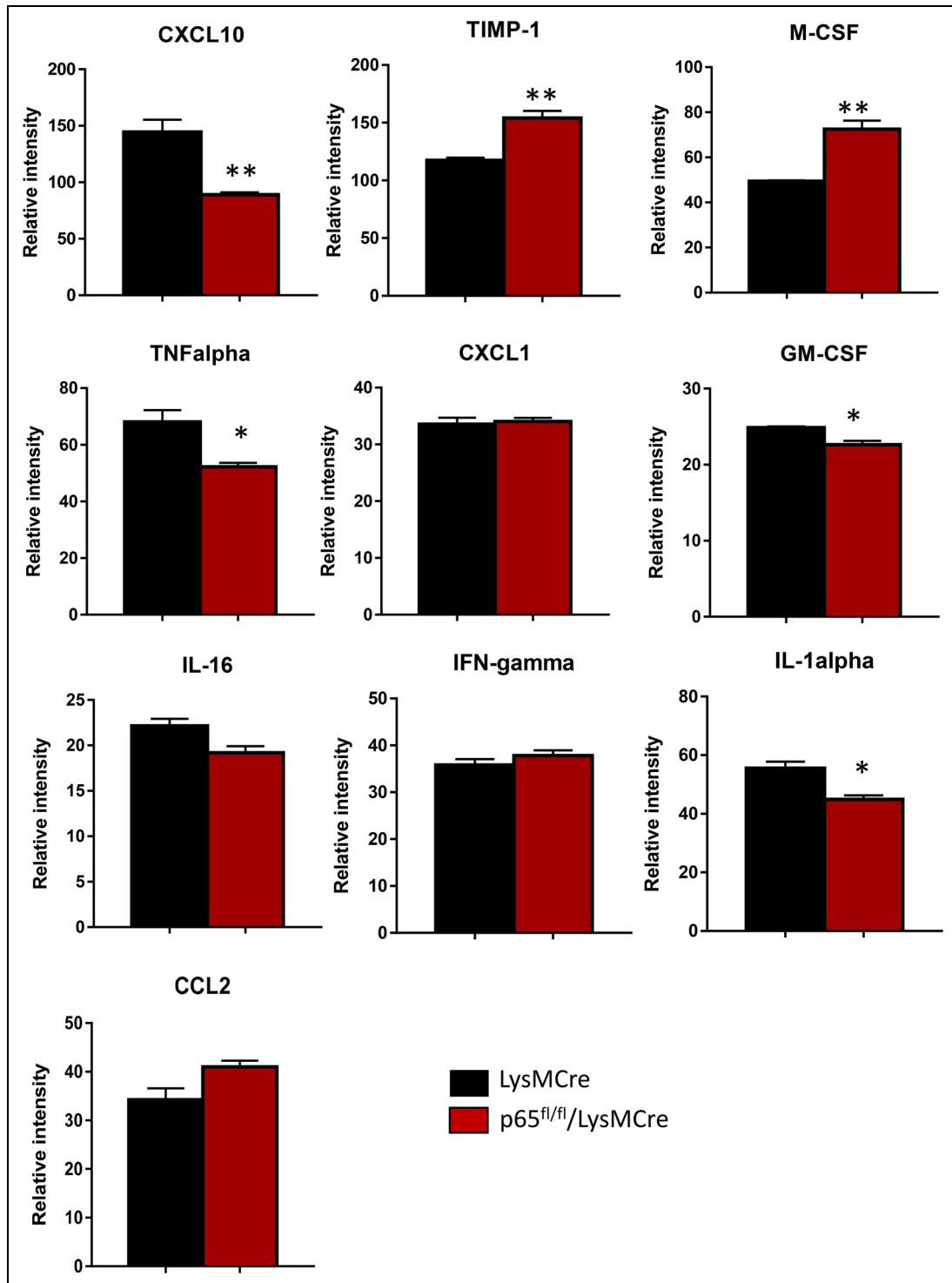


Figure 4. p65^{fl/fl}/LysMCre BMDMs secrete altered levels of selective factors. Cytokine array analysis reveals that p65^{fl/fl}/LysMCre BMDMs in culture secrete the following factors into the culture media at a significantly different levels than control BMDMs: CXCL10 (IP-10), TIMP-1, M-CSF, TNF-alpha, GM-CSF, and IL-1alpha. CXCL1, IL-16, IFN γ , and CCL2 secreted levels were detectable, but not statistically different. ** denotes p values of <0.02, * denotes p values of <0.04. The relative intensity of each spot was determined by ImageJ.

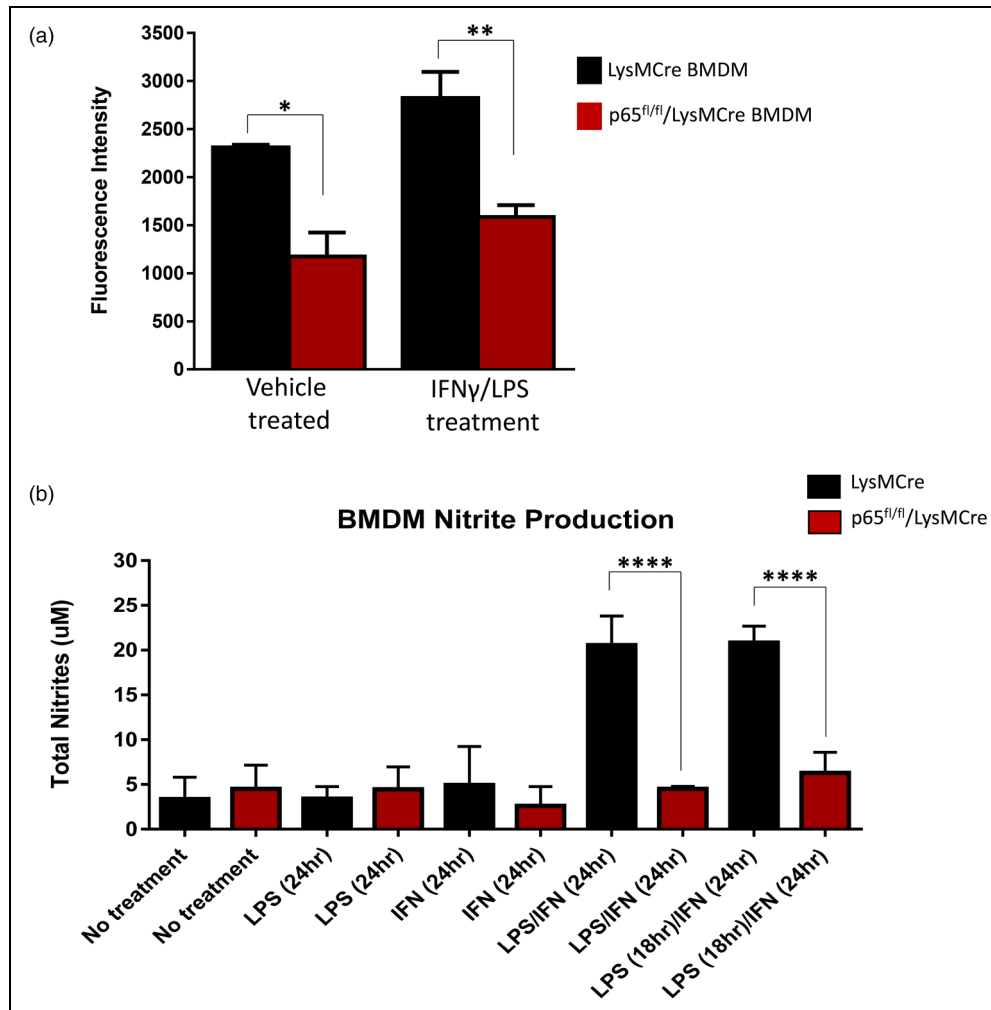


Figure 5. Cultured BMDMs that lack p65 signaling have reduced phagocytic activity and nitric oxide production. Cultured BMDMs were treated with 150U of IFN γ for 6 h and 0.2 μ g of LPS for 18 h or vehicle control, and allowed to phagocytose fluorescein-labeled *E. coli* particles. The number of particles engulfed corresponds to fluorescence intensity (A). BMDMs were treated with vehicle control, 50 ng/ml LPS for 24 h, 150 U/ml IFN γ for 24 h, 50 ng/ml LPS and 150 U/ml IFN γ for 24 h, or 150 U/ml IFN γ for 6 h followed by 50 ng/ml LPS for 18 h. Supernatants were then analyzed for nitric oxide response (B). *p < 0.02; ** p < 0.01; **** p < 0.0001.

p65^{fl/fl}/LysMCre mice do not show increased *Salmonella* burden following infection

Even though systemic immune cell populations did not significantly vary between control and p65^{fl/fl}/LysMCre mice (Figure 7), it was determined that the p65^{fl/fl}/LysMCre mice consistently displayed splenomegaly (Figure 8a). Female p65^{fl/fl}/LysMCre mice seem to be even more susceptible to enlarged spleens than male p65^{fl/fl}/LysMCre mice of the same age (Figure 8a). Due to this observation, and because there was a clear decrease in the ability of p65^{fl/fl}/LysMCre BMDMs to engulf *E. coli* particles and produce nitrite products, it was next determined whether p65^{fl/fl}/LysMCre mice had increased vulnerability to bacterial infection as compared to controls. *Salmonella typhimurium* was chosen for this study as this pathogen targets

macrophages for intracellular infection. p65^{fl/fl}/LysMCre and LysMCre control mice were infected (IP) with 700 colony forming units (CFUs) of *Salmonella* bacteria or vehicle control, and mediastinal lymph nodes and spleens were harvested 48 h post infection to determine CFU burden. Results indicate that the p65^{fl/fl}/LysMCre mice did not have a significant difference in *Salmonella* burden, as compared to LysMCre control mice in either the mediastinal lymph nodes or spleens (Figure 8b). A higher dose (4×10^4 CFUs) of *Salmonella* was also injected (IP), with no difference in CFU burden between LysMCre control and p65^{fl/fl}/LysMCre mice after 48 h (data not shown). Latency to death following *Salmonella* infection was also assessed, and no difference was found between the two groups (data not shown).

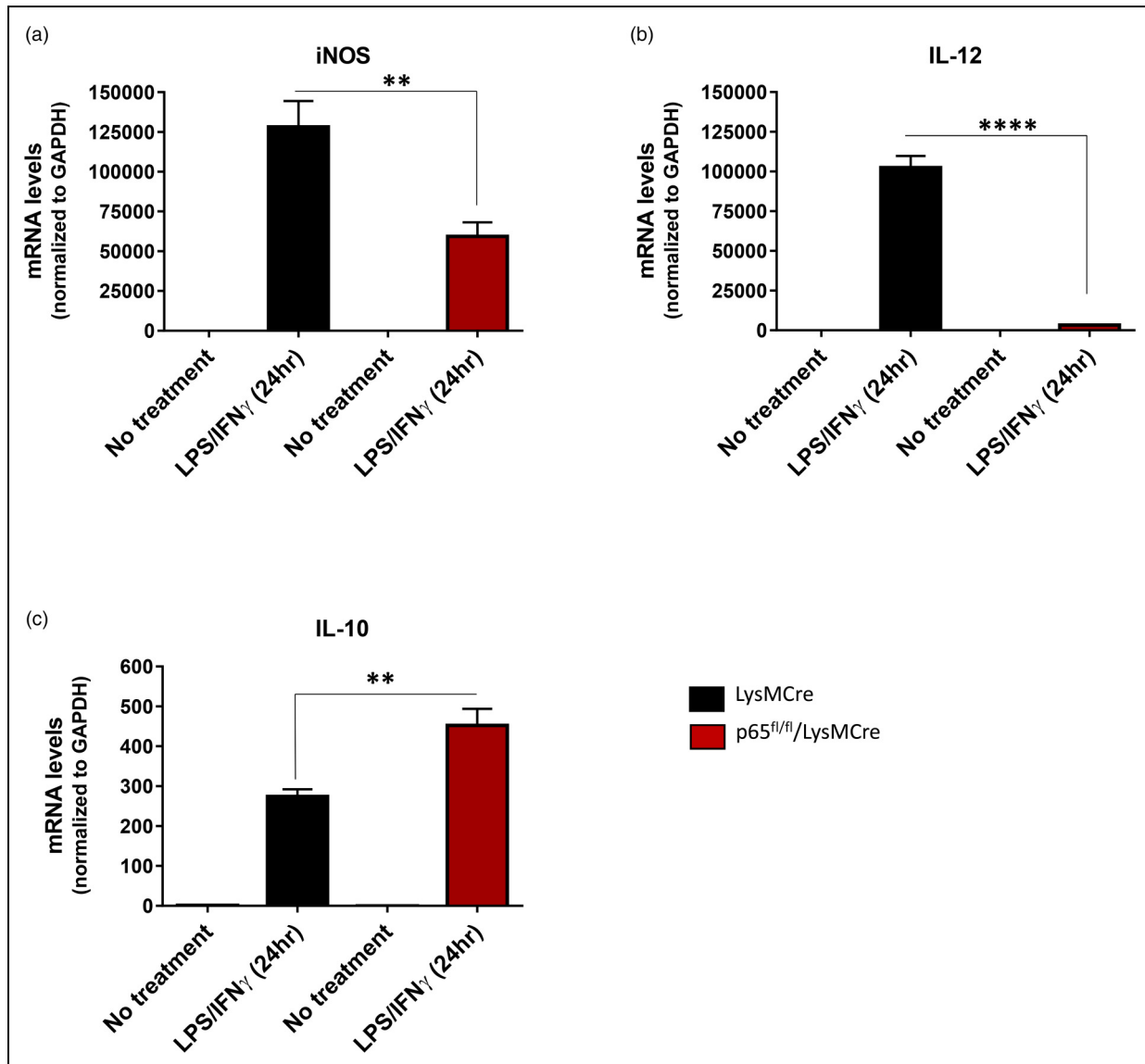


Figure 6. p65 deficiency inhibits p65^{fl/fl}/LysMCre BMDMs from appropriately polarizing. BMDMs were treated with vehicle control or a combination of 50 ng/ml LPS and 150 U/ml IFN γ for 24 h. Gene expression was analyzed for iNOS (A), IL-12 (B), and IL-10 (C). ** p value is less than or equal to 0.003; **** p value < 0.0001.

Discussion

Here, we describe the creation and characterization of an animal model that efficiently lacks canonical p65 in bone marrow-derived macrophages (BMDMs). We examined the effects of knocking down p65 in both BMDM expression and functionality, and on the ability of the p65^{fl/fl}/LysMCre mouse model to respond to *Salmonella* infection. The p65^{fl/fl}/LysMCre model was created by crossing the p65^{fl/fl} mouse model²² to the LysMCre mouse model.¹⁵ In theory, the LysMCre animal should provide deletion of floxed genes in all cells of the myeloid lineage, but in reality, full deletion is not usually seen in all myeloid cell types. Full myeloid deletion was not seen in the p65^{fl/fl}/

LysMCre model, as p65 is not fully knocked down in peritoneal macrophages, however, there is excellent p65 knockdown in BMDMs (Figure 2). Lack of full myeloid deletion is certainly a limitation of this model for in vivo assessment of total myeloid NF- κ B activity, however, this model still provides a useful method to determine the role of BMDM NF- κ B signaling in various in vitro and in vivo roles. This model is also simple to use compared to many conditional animal models because no additional inducing agent, such as tamoxifen administration, is needed to induce p65 deletion. While robust p65 knockdown was seen in male and female mice of all ages, mice around 3 months of age were frequently used for BMDM studies, as these primary

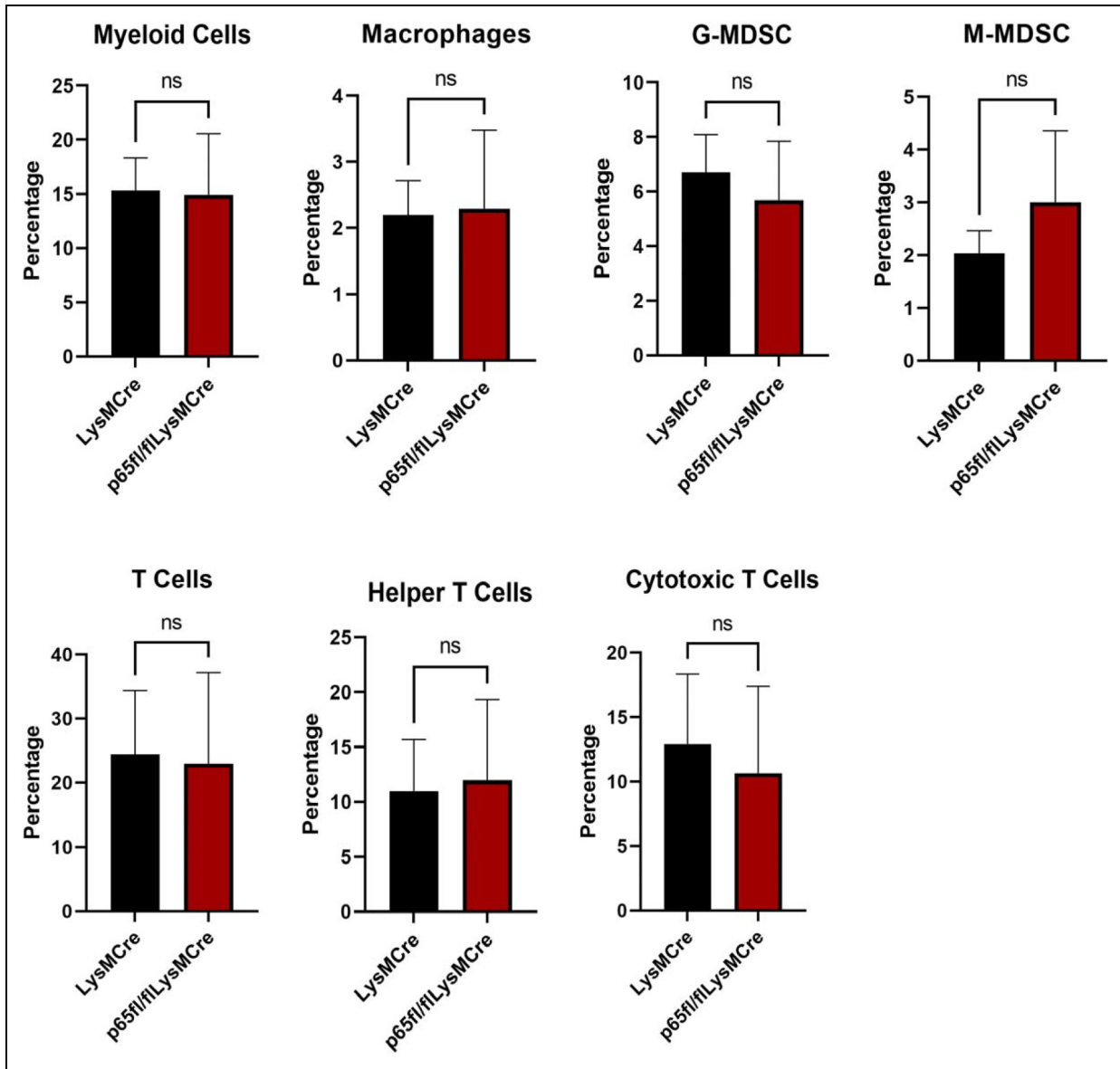


Figure 7. Systemic immune cell profile of p65^{fl/fl}/LysMCre mice. Blood samples (n = 6) were collected from LysMCre mice (black bars) and p65^{fl/fl}/LysMCre mice (red bars) and stained with a panel of antibodies to identify various immune cell populations: myeloid cells (CD11b+), macrophages (CD11b + F4/80+), granulocytic-MDSCs (CD11b + Ly-6G+), monocytic-MDSCs (CD11b + Ly-6C+), T cells (CD3+), T helper cells (CD3 + CD4+), and cytotoxic T cell (CD3 + CD8+). Live cells were determined by staining negative for DAPI.

cells grew best from animals of this age. As mice age, bones tend to become more brittle and it is harder to collect the bone marrow. Otherwise, no mice were excluded from these studies.

As part of the initial characterization of the p65^{fl/fl}/LysMCre mouse model, we examined p-p65/p65 protein levels in three different tissues; spleen, brain, and liver. As the spleen is the largest lymphatic organ and contains a large number of monocytes,²⁶ we were interested in seeing whether p-p65/p65 levels were altered in splenic tissue of the p65^{fl/fl}/LysMCre mice. In fact, we saw a slight decrease

in both total and p-p65 in the spleens of p65^{fl/fl}/LysMCre mice (Figure 2c). The resident macrophages of the central nervous system are microglia, which are derived from embryonic yolk sac progenitors²⁷ and not hematopoietic progenitors.²⁸ Thus, we did not expect much p-p65/p65 change in the brain tissue of the p65^{fl/fl}/LysMCre mice as compared to controls, which was demonstrated in the western blot (Figure 2c). The liver has a large population of resident macrophages called Kupffer cells, even during times of health.²⁹ As most Kupffer cells are also yolk sac-derived^{30,31} and not bone-marrow derived, we did not anticipate p-p65/

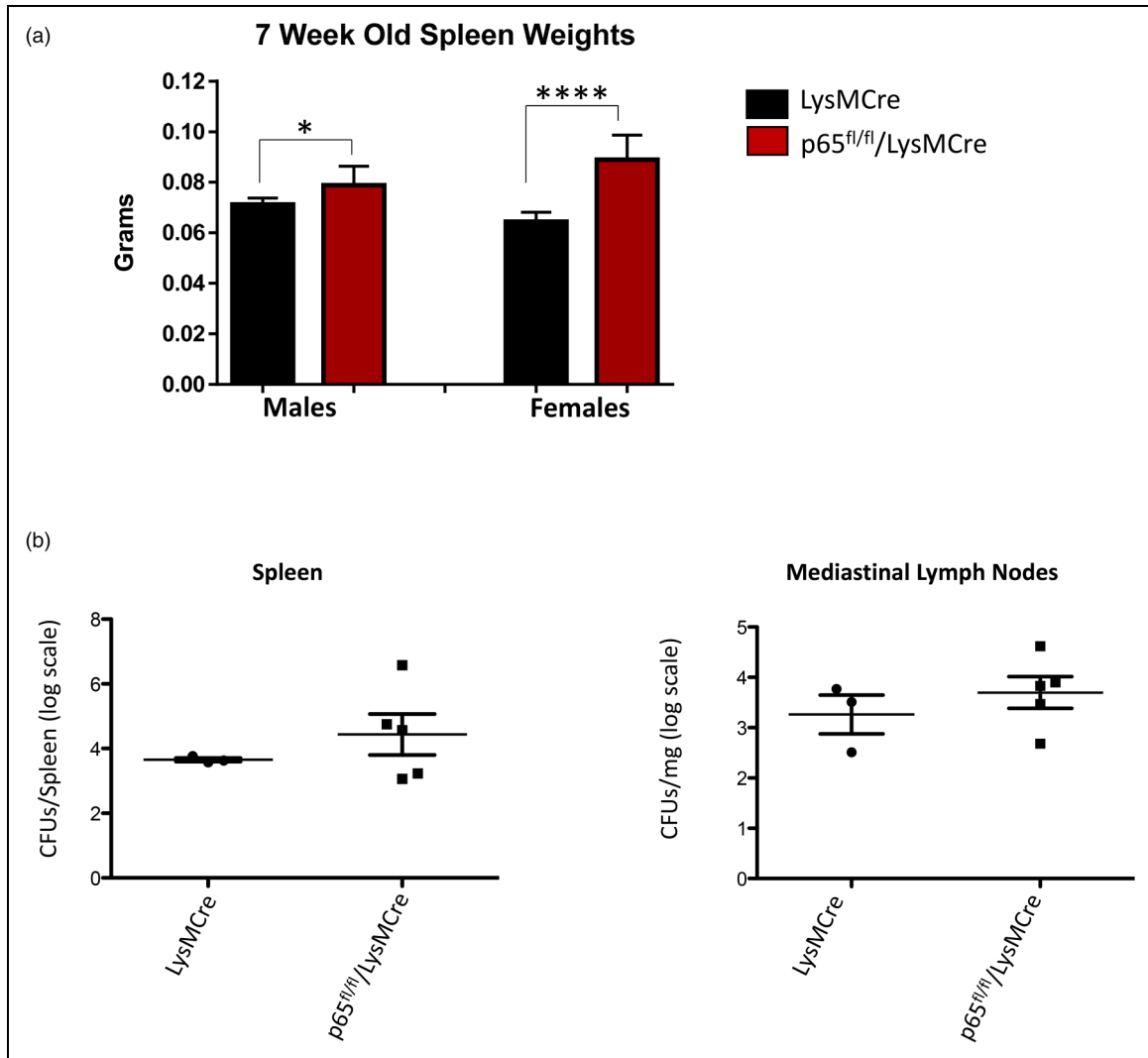


Figure 8. p65^{fl/fl}/LysMCre mice exhibit splenomegaly but do not show increased *Salmonella* burden following infection when compared to control mice. Spleens were weighed from 7 week old animals (A). Animals were injected with 700 CFUs of *Salmonella typhimurium* bacteria or vehicle control. CFUs of *Salmonella* in spleen and mediastinal lymph nodes were counted in control LysMCre and p65^{fl/fl}/LysMCre animals 48 h post infection (B). Data is representative of two experiments. * indicates a p value of <0.04, **** indicates a p value of <0.0001.

p65 differences, which was supported by the western data (Figure 2c).

Phospho-p65 and total p65 knockdown in p65^{fl/fl}/LysMCre BMDMs corresponded to a reduction in common canonical NF- κ B target gene expression levels (Figure 3). Changes in production and secretion of some cytokines was also seen in naïve (untreated) p65^{fl/fl}/LysMCre BMDMs, with less CXCL10, TNF α , GM-CSF, IL1 α , and more TIMP-1, and M-CSF being detected, as compared to LysMCre BMDMs (Figure 4). LPS treatment would probably increase the differences seen in secreted cytokines between the p65^{fl/fl}/LysMCre and control BMDMs. Phospho-p65 and total p65 protein levels were also examined in several organs in control and p65^{fl/fl}/LysMCre model mice, including the spleen, brain, and liver (Figure 2c). Phospho-p65 and total

p65 protein levels were slightly decreased in the spleens of p65^{fl/fl}/LysMCre mice, and the p65^{fl/fl}/LysMCre mice, especially females, exhibited splenomegaly (Figure 8a). Liver and brain p65 protein levels were very similar between p65^{fl/fl}/LysMCre model and control mice, indicating these organs are largely unaffected by LysMCre action.

Functionally, p65^{fl/fl}/LysMCre BMDMs were unable to perform innate immune functions as well as control BMDMs, as was seen in the reduced ability to engulf bacterial products and produce nitrites (Figure 5). It is well known that canonical NF- κ B plays a role in macrophage polarization,^{20,32–34} so expression of markers associated with classically activated M1 macrophages (iNOS, IL-12) and alternatively activated M2 macrophages (IL-10) was analyzed (Figure 6). The p65^{fl/fl}/LysMCre BMDMs

lacking p65 were skewed towards an alternative phenotype, which is logical based on their reduced ability to efficiently perform innate immune functions.

Because p65^{fl/fl}/LysMCre BMDMs are defective in engulfment ability and production of nitrites (Figure 5), and due to the fact that p65^{fl/fl}/LysMCre animals exhibit splenomegaly (Figure 8a), we determined whether p65^{fl/fl}/LysMCre mice would be able to clear bacterial infection as efficiently as LysMCre control mice. *Salmonella typhimurium* was chosen for this study as this pathogen targets macrophages for intracellular infection, and it is one of the most commonly used model pathogens to study the macrophage/pathogen interaction. Our results demonstrate that while p65^{fl/fl}/LysMCre BMDMs are defective in innate immune functions, the p65^{fl/fl}/LysMCre mice have a normal enough overall immune system to combat *Salmonella* infection (Figure 8b). This result was somewhat surprising, as we figured the dysfunctional BMDMs in the p65^{fl/fl}/LysMCre animals would have negatively impacted their ability to fight bacterial infection. However, naïve p65^{fl/fl}/LysMCre mice do not show significant differences in blood leukocyte populations, as compared to controls (Figure 7), suggesting that the target myeloid cells that *Salmonella* would infect are present. It may be that in wild type mice, the many *Salmonella* type III secretion effectors that block NF- κ B signaling prevent a strong NF- κ B response in infected cells.³⁵ Thus, p65 depletion has no effect because the response is normally inhibited. It could be that *Salmonella* strains carrying deletions in specific effectors would have their attenuation restored in p65^{fl/fl}/LysMCre animals. It is still surprising that uninfected cells that are not intoxicated by these effectors do not require p65 to mount a normal response.

The p65^{fl/fl}/LysMCre model has very efficient knock-down of canonical NF- κ B p65 in BMDMs, which could render this model very useful in determining the role of canonical myeloid NF- κ B signaling in a wide variety of applications. To that end, our lab has successfully used this model in an immunology study³⁶ as well as in a glioblastoma study,³⁷ which implicates the importance of canonical NF- κ B signaling in peripheral macrophages in the growth and development of glioblastoma tumors. These studies underscore the idea that the p65^{fl/fl}/LysMCre model can be valuable not only in cell culture studies but also in a wide range of in vivo applications that implicate canonical NF- κ B signaling in BMDMs.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics statement

The authors attest that the work presented in this manuscript is original and has not been previously published. Strict adherence to the scientific research ethics code has been maintained. This work was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal protocols were approved by the IACUC of Augusta University and the University of North Carolina at Chapel Hill.

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Supplemental material

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References

- Bradford JW and Baldwin AS. IKK/Nuclear factor-kappaB and oncogenesis: roles in tumor-initiating cells and in the tumor microenvironment. *Adv Cancer Res* 2014; 121: 125–145.
- Hayden MS and Ghosh S. Signaling to NF-kappaB. *Genes Dev* 2004; 18: 2195–2224. Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review 2004/09/17. DOI: 10.1101/gad.1228704.
- Hoffmann A, Levchenko A, Scott ML, et al. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* 2002; 298: 1241–1245.
- Baldwin AS. Regulation of cell death and autophagy by IKK and NF-kappaB: critical mechanisms in immune function and cancer. *Immunol Rev* 2012; 246: 327–345.
- Oeckinghaus A and Ghosh S. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol* 2009; 1: a000034. 2010/01/13.
- Dorrington MG and Fraser IDC. NF-kappaB Signaling in macrophages: dynamics, crosstalk, and signal integration. *Front Immunol* 2019; 10: 705. 2019/04/27.

7. Liu T, Zhang L, Joo D, et al. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* 2017; 2: 17023-. 2017/11/22.
8. Mussbacher M, Derler M, Basilio J, et al. NF-kappaB in monocytes and macrophages - an inflammatory master regulator in multitasked immune cells. *Front Immunol* 2023; 14: 1134661. 2023/03/14.
9. Beg AA, Sha WC, Bronson RT, et al. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 1995; 376: 167–170.
10. Alcamo E, Hacohen N, Schulte LC, et al. Requirement for the NF-kappaB family member RelA in the development of secondary lymphoid organs. *J Exp Med* 2002; 195: 233–244.
11. Stein SJ and Baldwin AS. Deletion of the NF-kappaB subunit p65/RelA in the hematopoietic compartment leads to defects in hematopoietic stem cell function. *Blood* 2013; 121: 5015–5024.
12. Zhang XQ, Tang R, Li L, et al. Cardiomyocyte-specific p65 NF-kappaB deletion protects the injured heart by preservation of calcium handling. *Am J Physiol Heart Circ Physiol* 2013; 305: H1089–H1097. 2013/08/06.
13. Gao Z, Zhang J, Henagan TM, et al. P65 inactivation in adipocytes and macrophages attenuates adipose inflammatory response in lean but not in obese mice. *Am J Physiol Endocrinol Metab* 2015; 308: E496–E505. 2015/01/08.
14. Connelly L, Barham W, Onishko HM, et al. NF-kappaB activation within macrophages leads to an anti-tumor phenotype in a mammary tumor lung metastasis model. *Breast Cancer Res* 2011; 13: R83. 2011/09/03.
15. Clausen BE, Burkhardt C, Reith W, et al. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 1999; 8: 265–277.
16. Cheng TL, Lai CH, Shieh SJ, et al. Myeloid thrombomodulin lectin-like domain inhibits osteoclastogenesis and inflammatory bone loss. *Sci Rep* 2016; 6: 28340.
17. Anas AA, van Lieshout MH, Claushuis TA, et al. Lung epithelial MyD88 drives early pulmonary clearance of *Pseudomonas aeruginosa* by a flagellin dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* 2016; 311: L219–L228. DOI: 10.1152/ajplung.00078.2016
18. Lee LY, Harberg C, Matkowskyj KA, et al. Cell-Specific Over-activation of Nrf2-mediated Gene Expression in Myeloid Cells Decreases Hepatic Ischemia Reperfusion Injury. *Liver Transpl* 2016; 72. DOI: 10.1002/lt.24473
19. Hagemann T, Biswas SK, Lawrence T, et al. Regulation of macrophage function in tumors: the multifaceted role of NF-kappaB. *Blood* 2009; 113: 3139–3146.
20. Hagemann T, Lawrence T, McNeish I, et al. Re-educating” tumor-associated macrophages by targeting NF-kappaB. *J Exp Med* 2008; 205: 1261–1268.
21. Mancino A and Lawrence T. Nuclear factor-kappaB and tumor-associated macrophages. *Clin Cancer Res* 2010; 16: 784–789.
22. Steinbrecher KA, Harmel-Laws E, Sitcheran R, et al. Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility to inflammation. *J Immunol* 2008; 180: 2588–2599.
23. Weischenfeldt J and Porse B. Bone marrow-derived macrophages (BMM): isolation and applications. *CSH Protoc* 2008; 2008: pdb prot5080.
24. Zhang X, Goncalves R and Mosser DM. The isolation and characterization of murine macrophages. *Curr Protoc Immunol* 2008; Chapter 14, 14.1.1 -14.1.14: 1–18. DOI: 10.1002/0471142735.im1401s83.
25. Palmieri EM, McGinity C, Wink DA, et al. Nitric Oxide in Macrophage Immunometabolism: Hiding in Plain Sight. *Metabolites* 2020; 10: 1–34. 2020/10/30. DOI: 10.3390/metabo10110429.
26. Kim E, Yang J, Beltran CD, et al. Role of spleen-derived monocytes/macrophages in acute ischemic brain injury. *J Cereb Blood Flow Metab* 2014; 34: 1411–1419. 2014/05/29.
27. Hagemeyer N, Kierdorf K, Frenzel K, et al. Transcriptome-based profiling of yolk sac-derived macrophages reveals a role for Irf8 in macrophage maturation. *EMBO J* 2016; 35: 1730–1744. 2016/07/15.
28. Goldmann T, Wieghofer P, Muller PF, et al. A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. *Nat Neurosci* 2013; 16: 1618–1626. 2013/10/01.
29. Triantafyllou E, Woollard KJ, McPhail MJW, et al. The role of monocytes and macrophages in acute and acute-on-chronic liver failure. *Front Immunol* 2018; 9: 2948. 2019/01/09.
30. Li X, Hollingshead N, Lampert S, et al. A conserved pathway of transdifferentiation in murine Kupffer cells. *Eur J Immunol* 2021; 51: 2452–2463. 2021/07/30.
31. Ide S, Yahara Y, Kobayashi Y, et al. Yolk-sac-derived macrophages progressively expand in the mouse kidney with age. *Elife* 2020; 9: 1–12. 2020/04/18. DOI: 10.7554/eLife.51756.
32. Chiang CF, Chao TT, Su YF, et al. Metformin-treated cancer cells modulate macrophage polarization through AMPK-NF-kappaB signaling. *Oncotarget* 2017; 8: 20706–20718. 2017/02/06.
33. Porta C, Rimoldi M, Raes G, et al. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB. *Proc Natl Acad Sci U S A* 2009; 106: 14978–14983. 2009/08/27.
34. Shen J, Sun X, Pan B, et al. IL-17 induces macrophages to M2-like phenotype via NF-kappaB. *Cancer Manag Res* 2018; 10: 4217–4228. 2018/10/17.
35. Takemura M, Haneda T, Idei H, et al. A *Salmonella* type III effector, PipA, works in a different manner than the PipA family effectors GogA and GtgA. *PLoS One* 2021; 16: e0248975. 2021/03/19.
36. Sierra RA, Trillo-Tinoco J, Mohamed E, et al. Anti-Jagged immunotherapy inhibits MDSCs and overcomes tumor-induced tolerance. *Cancer Res* 2017; 77: 5628–5638.
37. Achyut BR. Canonical NFkappaB signaling in myeloid cells is required for the glioblastoma growth. 2017; 7: 13754. DOI: 10.1038/s41598-017-14079-4.