

Development and characterisation of a novel, megakaryocyte NF- κ B reporter cell line for investigating inflammatory responses

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ORIGINAL ARTICLE

Development and characterization of a novel, megakaryocyte NF- κ B reporter cell line for investigating inflammatory responses

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Abstract

Background: Because of the difficulties in acquiring large numbers of megakaryocytes, the impact of inflammatory responses on these cells and their ability to produce fully functional platelets under various pathological conditions has not been investigated in detail.

Objectives: The primary objective of this study is to develop and functionally characterize a novel megakaryocyte nuclear factor κ B (NF- κ B) reporter cell line to determine the effects of various inflammatory molecules on megakaryocytes and their signalling pathways.

Methods: A Meg-01-NF- κ B-GFP-Luc (Meg-01R) cell line was developed by inserting a reporter NF- κ B-GFP-Luc cassette into normal Meg-01 cells to produce luciferase following activation of NF- κ B to enable easy detection of pro-inflammatory and reparative signalling.

Results and conclusions: Meg-01 and Meg-01R cells have comparable characteristics, including the expression of both GPIIb α and integrin β_3 . Meg-01R cells responded to various inflammatory molecules as measured by NF- κ B-dependent bioluminescence. For example, inflammatory molecules such as tumor necrosis factor- α and Pam3CSK4 increased NF- κ B activity, whereas an antimicrobial peptide, LL37, reduced its activity. Meg-01R cells were also found to be sensitive to inhibitors (IMD0354 and C87) of inflammatory pathways. Notably, Meg-01R cells were able to respond to lipopolysaccharide (LPS; non-ultrapure), although it was not able to react to ultrapure LPS because of the lack of sufficient TLR4 molecules on their surface. For the first time, we report the development and characterization of a novel megakaryocyte NF- κ B reporter cell line (Meg-01R) as a robust tool to study the inflammatory responses/signalling of megakaryocytes upon stimulation with a broad range of inflammatory molecules that can affect NF- κ B activity.

Darius Widera and Sakthivel Vaiyapuri contributed equally.

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KEYWORDSFormyl peptide receptor, inflammation, megakaryocyte, NF- κ B, Toll-like receptor 4**1 | INTRODUCTION**

Platelets (small circulating blood cells) are known for their ability to regulate hemostatic, innate immune, and inflammatory responses.^{1,2} A large number of studies have focused on elucidating the roles of platelets in the regulation of innate immune/inflammatory responses and the molecular mechanisms responsible for these activities.^{1,3-5} Furthermore, platelets are recognized as having prominent roles in the adaptive immune system.^{1,2} However, only a relatively small number of studies have focussed on determining the impact on megakaryocytes (MKs), the precursors of platelets, on immune/inflammatory responses.^{6,7} This is an under researched area, although the reactivity and proteome of platelets produced during inflammation are modified to accustom the pathological conditions.⁸⁻¹⁰

MKs differentiate from hematopoietic stem cells (the progenitor cells from which innate immune cells such as neutrophils and monocytes are also derived), produce large quantities of proteins, and possess an extended invaginated membrane system for packaging into numerous platelets.⁷ MKs in the bone marrow produce platelets by extending proplatelets into sinusoids, where the shear flow of blood leads to budding of platelets into the bloodstream.^{7,11} Platelets are also produced in the mouse lungs, where MKs may interact with pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs); however, whether this function translates into humans remains unclear.¹² The environment surrounding MKs may alter the functions of platelets, for example, producing platelets that are more pro-aggregatory or aggressive toward pathogens, although the molecular mechanisms behind these actions are still poorly understood.⁸⁻¹⁰ Studies have shown that in mice, lipopolysaccharide (LPS) from Gram-negative bacteria causes an increase in circulating platelet count.⁸ Mice lacking functional Toll-like receptor (TLR)-4 (the receptor for LPS) have a significant reduction in the number of circulating platelets.⁸ Furthermore, some studies suggest that LPS treatment increases platelet production from MKs, as well as MK ploidy,^{13,14} although this may be due to the release of several factors from macrophages.¹⁵ Hence, determining the impact of inflammatory responses on MKs and subsequent platelet production will aid in better understanding of the significance of MKs and platelets in the regulation of thromboinflammation in various pathophysiological scenarios.

Meg-01 is a megakaryoblastic cell line frequently used as a surrogate for elucidating signalling pathways and functions in MKs because of the difficulties associated with acquiring large numbers of primary MKs.¹⁶⁻¹⁹ TLR2 is closely related to TLR4 and it signals through the same MyD88-dependent pathway. A previous study has reported that the treatment of Meg-01 cells and murine MKs with a TLR2 agonist, Pam3CSK4, resulted in increased MK ploidy, activated nuclear factor- κ B (NF- κ B), and altered protein

Essentials

- An easily detectable readout in megakaryocyte cell lines will enhance inflammatory research in these cells.
- Here, we report the development and characterization of a novel megakaryocyte NF- κ B-reporter cell line (Meg-01R).
- Multiple inflammatory molecules modulate NF- κ B activity in Meg-01R cells.
- Meg-01R cells respond to small molecule inhibitors such as IMD0354 and C87 that are known to inhibit NF- κ B activity upon stimulation with TNF α .

expression¹⁶; these results suggest that the MyD88-dependent pathway is likely to be active in MKs. Because of the lack of a reliable system to determine the role of MKs in the regulation of inflammatory responses, in this study we developed and characterized a robust, Meg-01-NF κ B-Luc-GFP (Meg-01R) reporter cell line for investigating the effect of inflammatory molecules on an MK-like cell line.

2 | MATERIALS AND METHODS

The raw data that support the findings of this study are available upon reasonable request.

2.1 | Cell culture

Meg-01 cells were grown in RPMI-1640 media supplemented with 10% (v/v) foetal calf serum (FCS) and 2 mM L-glutamine (Sigma-Aldrich). Cells were kept at 37°C in a humidified 5% CO₂ incubator. Every 2 to 3 days, the cells were removed by scraping, counted, and resuspended at a concentration of 2.5 × 10⁵ cells/mL in a vented suspension flask (Sarstedt). The media for Meg-01R cells was supplemented with 1 μ g/mL puromycin for selection (Apollo Scientific). All experiments were conducted in the absence of antibiotics and antimycotics.

THP-1 cells were grown in RPMI-1640 media supplemented with 10% (v/v) FCS, 0.2% (v/v) penicillin/streptomycin mix, and 0.4% (v/v) amphotericin B (Sigma-Aldrich). These cells were kept in a humidified 5% CO₂ incubator at 37°C.

HEK-293 cells were grown in high glucose DMEM containing 10% (v/v) FCS and 2 mM L-glutamine in a humidified 5% CO₂ incubator at 37°C.

Doubling time was calculated using Meg-01 and Meg-01R cells cultured in parallel under normal growth conditions in normal growth media using the formula:

$$\text{Doubling time} = \frac{\text{Duration (h)} \times \log_{10}(2)}{\log_{10}([\text{final}]) - \log_{10}([\text{initial}])}$$

2.2 | Immunoblotting

Meg-01 and THP-1 lysates were immunoblotted following a previously established protocol.²⁰⁻²² Further details are described in Appendix S1.

2.3 | RT-PCR

Total RNA was extracted from Meg-01 and Meg-01R cells and underwent RT-PCR to determine the presence of transcripts for MyD88. Further details are included in Appendix S1.

2.4 | XTT assay

Cell viability assays XTT assays were conducted according to manufacturer's instructions (Sigma-Aldrich). Further details are described in Appendix S1.

2.5 | Lentiviral transduction

Meg-01R cells were generated as described previously.^{23,24} Briefly, HEK-293 cells were transfected with pGreenFire-NFκB-Puro vector (System Biosciences) for viral production before transduction of Meg-01 cells. Viral production proceeded for 48 hours. Viral particles were isolated from HEK-293 cells by removing the cells with centrifugation for 10 minutes at 1000g (4°C). The supernatant was filtered through a 0.45-μm diameter filter and then centrifuged at 100 000g for 1 hour at 4°C. The viral pellet was resuspended in phosphate-buffered saline (PBS) and left to precipitate overnight at 4°C. To aid transduction of Meg-01 cells, 5 μg/mL of polybrene was added to the PBS-virus suspension and incubated with 3.75×10^6 Meg-01 cells for 30 minutes at 4°C. Following transduction, normal cell culture medium (RPMI-1640 with 10% FCS and 2 mM L-glutamine) was added, and the cells were left for 96 hours before the addition of 1 μg/mL puromycin for the selection of transduced cells. Dead cells were removed via an extra centrifugation step (300g for 10 minutes) in PBS.

2.6 | Flow cytometry

Meg-01R cells were washed in PBS, centrifuged at 300g for 5 minutes, and resuspended in sterile-filtered 0.2% formyl saline (0.9%

[w/v] NaCl and 0.2% [v/v] formaldehyde) for 10 minutes at room temperature. Following fixation, cells were blocked with 10% (v/v) FcR blocking reagent (Miltenyi Biotec) in PBS (for nonpermeabilized samples) or 0.02% (v/v) polybutylene terephthalate (for permeabilized samples; PBS containing Triton X-100) for 30 minutes at room temperature.

For GPIbα (CD42b) and integrin β₃ (CD61) staining, cells were incubated with 1/50 dilution of anti-CD42b (SP219) rabbit antibody or 1/50 dilution of anti-CD61 (CRC54) mouse antibody (Abcam) in PEB buffer (PBS with 2 mM ethylenediaminetetraacetic acid and 0.5% [w/v] bovine serum albumin) for 20 minutes at room temperature. Cells were then washed in PEB and incubated with anti-rabbit Alexa Fluor 647 or anti-mouse Alexa Fluor 647 (ThermoFisher Scientific) respectively for 20 minutes in the dark. Finally, cells were diluted 1/10 in PEB and analyzed by flow cytometry (Accuri C6 Sampler Plus, BD Biosciences). The significant increase in background fluorescence in Meg-01R cells was subtracted to enable accurate comparison of receptor levels.

For TLR4 staining, cells were incubated with 1/10 dilution of anti-TLR4 CD284-PE (HTA125) antibody or its isotype IgG2a-PE control (Miltenyi Biotec) in PEB in the dark at room temperature. Samples were then washed twice with PEB before analysis of the samples by flow cytometry (Accuri C6 Sampler Plus, BD Biosciences).

2.7 | Luciferase assay

The luciferase assay performed in this study was based on previous experiments.²³ Briefly, 3.75×10^5 Meg-01R cells were seeded into a 24-well suspension plate in starvation media (RPMI-1640 with 2 mM L-glutamine) and left at 37°C in a 5% CO₂ incubator for 4 hours. Treatments were introduced to cells in RPMI-1640 containing 20% (v/v) FCS and 2 mM L-glutamine. For experiments involving inhibitors (BML-111, IMD0354, C87, or 1,8-cineole), Meg-01R cells were incubated with the inhibitor during and after the starvation period. Following induction, cells were incubated for 24 hours. Meg-01R cells were removed and any remaining adherent cells were rinsed off with media and collected. Meg-01R cells were washed before resuspension in cell lysis buffer (Promega). Each sample was agitated for 2 hours and then centrifuged at 5000g for 5 minutes at room temperature. NF-κB-mediated luciferase activity was measured by the addition of luciferin (Promega) and the level of luminescence at all wavelengths was recorded using a SpectraMax iD3 multimode microplate reader (Molecular Devices).

2.8 | Confocal microscopy

For each condition, 1×10^6 Meg-01 cells were stained in suspension. Cells were fixed in 4% (w/v) paraformaldehyde for 10 minutes before blocking with FcR blocking reagent in PBS (nonpermeabilized) or 0.02% polybutylene terephthalate (permeabilized). 1/100 dilution of mouse

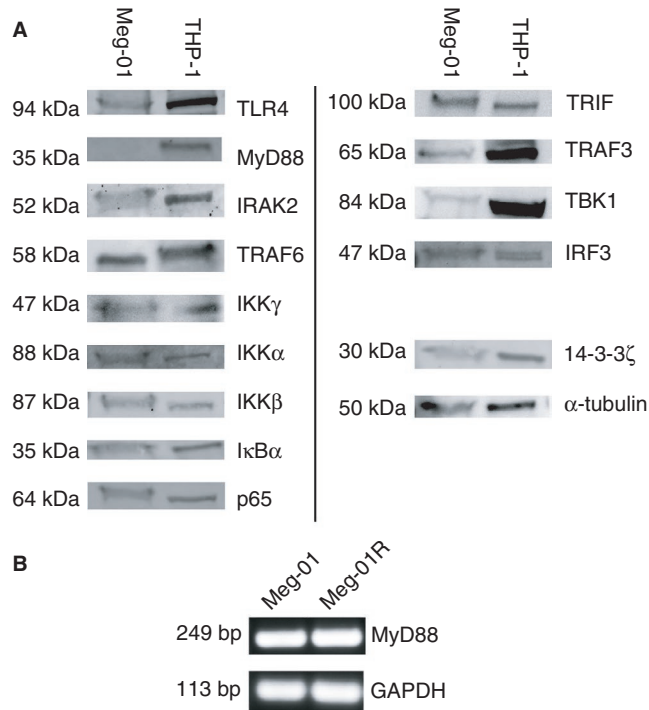


FIGURE 1 Presence of proteins involved in Toll-like receptor 4 signalling pathways in Meg-01 cells. A, Whole cell Meg-01 and THP-1 lysates were investigated via immunoblotting for the expression of TLR4, MyD88, IRAK2, TRAF6, IKK γ , IKK α , IKK β , I κ B α , p65, TRIF, TRAF3, TBK1, and IRF3. 14-3-3 ζ and α -tubulin were detected as loading controls. Proteins were detected either via fluorescence or enhanced chemiluminescence based on the secondary antibodies used. B, RT-PCR was used to investigate the presence of mRNA for MyD88 and GAPDH in both Meg-01 and Meg-01-NF κ B-Luc-GFP reporter (Meg-01R) cells

anti-TLR4 antibody (Abcam) was used to detect TLR4 after incubation with cells for 1 hour at room temperature. A 1/300 dilution of Alexa Fluor 555-conjugated goat anti-mouse IgG antibody (ThermoFisher Scientific) was used to visualize binding of the anti-TLR4 antibody to its target. Finally, the nuclei of Meg-01 cells were stained with 1/2000 4',6'-diamidino-2-phenylindole before resuspension in Mowiol 4-88 (Sigma-Aldrich) and mounting on a microscope slide. Slides were then imaged using a Nikon A1R confocal microscope and images were processed using Nikon NIS-Elements software and ImageJ.

2.9 | Statistical analysis

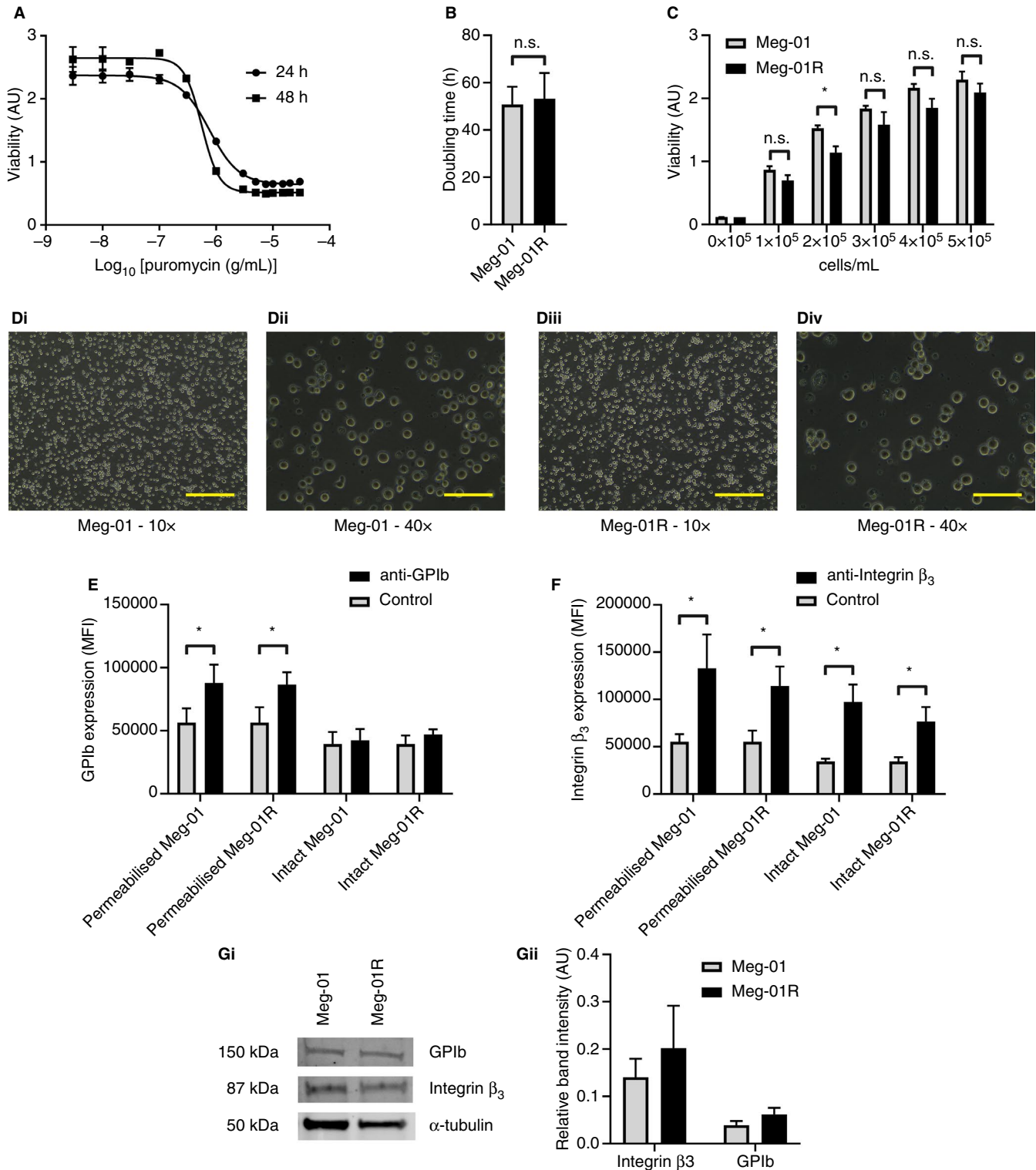
Logarithmic dose-response curves for puromycin toxicity were generated using a four-parameter curve with variable slope. For cell doubling time, an unpaired Student's *t* test was used to compare the mean values. For grouped data, analysis was performed using multiple *t* tests with the type I error rate controlled for using the Holm-Sidak method. For experiments containing multiple comparisons, data were analyzed using a one-way analysis of variance (ANOVA) with Bonferroni's post-hoc test. All statistical analysis was conducted using GraphPad Prism 8 (GraphPad). Data are represented as mean \pm standard deviation.

3 | RESULTS

3.1 | Meg-01 cells express various signalling proteins involved in TLR4 pathways

Platelets are reported to contain all the signalling proteins that are involved in MyD88-dependent and MyD88-independent signalling pathways.²⁵ Here, to determine whether Meg-01 cells are capable of inducing MyD88-dependent and -independent signalling upon ligation of inflammatory molecules such as LPS with TLR4, immunoblotting analysis was performed using Meg-01 cell lysates to confirm the presence of various signalling proteins. Meg-01 cell lysate was tested concurrently with an equivalent mass of THP-1 cell (a monocyte cell line capable of responding to LPS²⁶) lysate as a positive control. As expected, THP-1 cells express notable levels of TLR4, MyD88, IRAK2, TRAF6, IKK γ , IKK α , IKK β , I κ B α , p65, TRIF, TRAF3, TBK1, IRF3, and control proteins 14-3-3 ζ and α -tubulin (Figure 1A). Although all of these proteins were detectable in Meg-01 cells, surprisingly we could not detect MyD88 via this method. However, MyD88 mRNA was detectable via RT-PCR using specific primers for MyD88 (Figure 1B). This discrepancy may be due to the expression level of this protein being below the detection threshold of the immunoblotting technique or antibody sensitivity. Notably, a previous study¹⁶ has shown that Meg-01 cells are able to respond Pam3CSK4; therefore, MyD88-dependent signalling is likely to be active in these cells.

FIGURE 2 Characterization of Meg-01 and Meg-01R cells. A, Meg-01 cells were treated with puromycin and their viability was tested by an XTT assay to determine the susceptibility of naïve cells. Data represent mean \pm SD after 24 or 48 hours' incubation of 5×10^4 cells with puromycin ($n = 3$). B, The growth rate of Meg-01 and Meg-01R cells was compared to determine if the transduction process hindered growth. Data was analyzed using a Student's *t* test ($n = 3$). C, An XTT assay using different cell concentrations was performed to determine if the transduction process altered cell viability. Data were analyzed using multiple *t* tests corrected using the Holm-Sidak method ($n = 3$). D, The morphology of Meg-01 at 10 \times (i) and 40 \times (ii) and Meg-01R cells at 10 \times (iii) and 40 \times (iv) were compared using light microscopy. Scale bar represents 400 μ m at 10 \times and 100 μ m at 40 \times . Images shown are representative of five regions of interest taken from three separate flasks. Expression of GPIb (E) and integrin β_3 (F), intracellularly and on the surface of Meg-01 and Meg-01R cells, were compared to determine if the transduction process had modified the expression of specific receptors in MKs. Data were analyzed using multiple *t* tests comparing controls to antibody and Meg-01 to Meg-01R corrected via the Holm-Sidak method ($n = 4$ and $n = 3$, respectively). Furthermore, whole cell lysates were investigated via immunoblot analysis (representative blots shown in Gi) for the presence of GPIb and integrin β_3 (Gii). Data were analyzed using multiple *t* tests to compare Meg-01 to Meg-01R and corrected via the Holm-Sidak method ($n = 3$). For clarity, nonsignificant differences are not shown. The *P* values ($*P < .05$) are as calculated by GraphPad Prism



3.2 | Development of a Meg-01R (NF- κ B) reporter cell line

Viral particles containing the NF- κ B reporter sequence were generated from HEK-293 cells to develop an NF- κ B reporter Meg-01 cell line (Meg-01R), to aid in determination of the role of MK-like cells

during inflammation. A commercially available luciferase gene was used to produce a nonendogenous protein whose activity is easily detectable.²⁷ Successful transduction was confirmed by stimulating Meg-01R cells with tumor necrosis factor- α (TNF- α) and subsequently treating cell lysates with luciferin to measure the level of NF- κ B activity.

The optimal concentration of puromycin required to eradicate ~80% of nontransfected Meg-01 cells was determined using an XTT assay (Figure 2A). The results demonstrate that puromycin was capable of inducing cell death both over 24- and 48-hour time scales with a half maximal effective concentration of 760 and 560 ng/mL, respectively. Based on these results, a concentration of 1 μ g/mL puromycin was used to select transduced cells, which contain a puromycin-resistance gene, in subsequent experiments.

To elucidate differences or similarities between the transduced Meg-01R and standard Meg-01 cells, the doubling time of these cells was compared. The growth rate of Meg-01 (50.7 ± 4.35 hours) and Meg-01R (53.2 ± 6.28 hours) cells cultured parallelly was not significantly different (Figure 2B). Moreover, their viability was determined using an XTT assay and it did not show any significant differences after 6 hours except a slight reduction in the viability of Meg-01R cells observed when they were seeded at 2×10^5 cells/mL (Figure 2C). This slight reduction may be explained by the presence of 1 μ g/mL puromycin throughout

the experiment to prevent nontransfected cells from interfering with the results. To investigate whether the transduction altered the cellular morphology, light microscopy was used to analyze Meg-01 and Meg-01R cells. Meg-01 cells at both 10 \times (Figure 2Di) and 40 \times (Figure 2Dii) exhibited a spherical morphology when cultivated in suspension; the same morphology was observed in Meg-01R cells (Figure 2Diii-2Div). Furthermore, the expression of specific cell receptors was investigated by flow cytometry. GPIIb α was detectable in permeabilized and intact cells of both Meg-01 and Meg-01R at similar levels (Figure 2E). Moreover, integrin β_3 was detectable in both permeabilized and intact Meg-01 and Meg-01R cells at similar levels (Figure 2F).¹⁹ To corroborate these data, GPIIb α and integrin β_3 expression levels were analyzed using immunoblots, and they were found to not be significantly different in Meg-01 and Meg-01R cell lysates (Figure 2G). Together, these data suggest that the transduction in Meg-01R cells did not affect the proliferation, growth, and major characteristics of these cells compared with Meg-01.

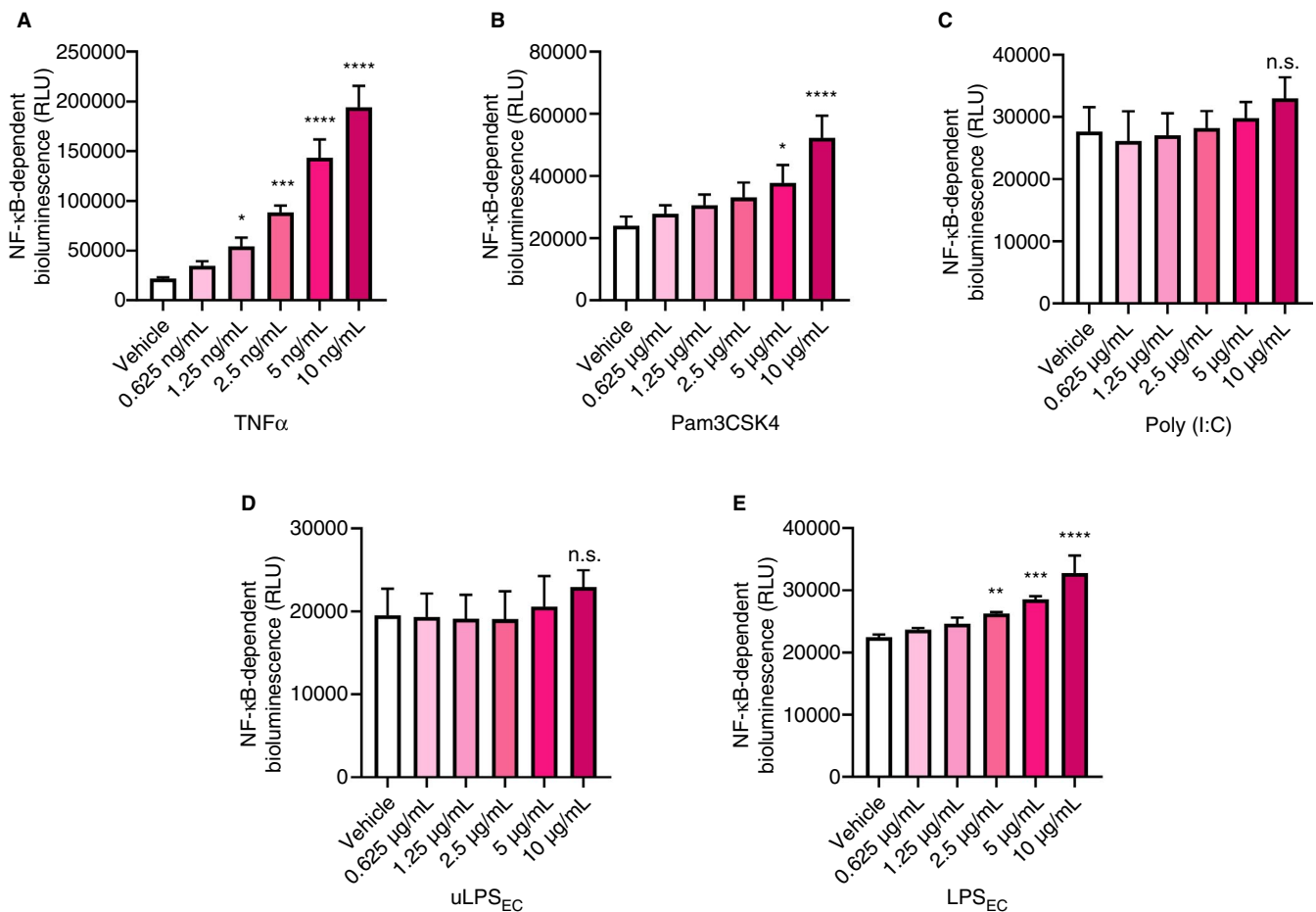


FIGURE 3 Impact of inflammatory molecules on NF- κ B activity in Meg-01R cells. Meg-01R cells were incubated with TNF- α (A), or a TLR1/2 agonist Pam3CSK4 (B) for 24 hours and the level of NF- κ B activity was recorded. Furthermore, NF- κ B activity was recorded in Meg-01R cells challenged with various concentrations of a TLR3 agonist, Poly(I:C) (C), or ultrapure (D) or non-ultrapure (E) versions of the TLR4 agonist LPS_{EC} for 24 hours. The level of NF- κ B activity was measured by quantifying the level of luminescence. Data represent mean \pm standard deviation ($n = 3$) and statistical significance was calculated using a one-way ANOVA with Bonferroni post hoc test. The P values (* $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$) are as calculated by GraphPad Prism

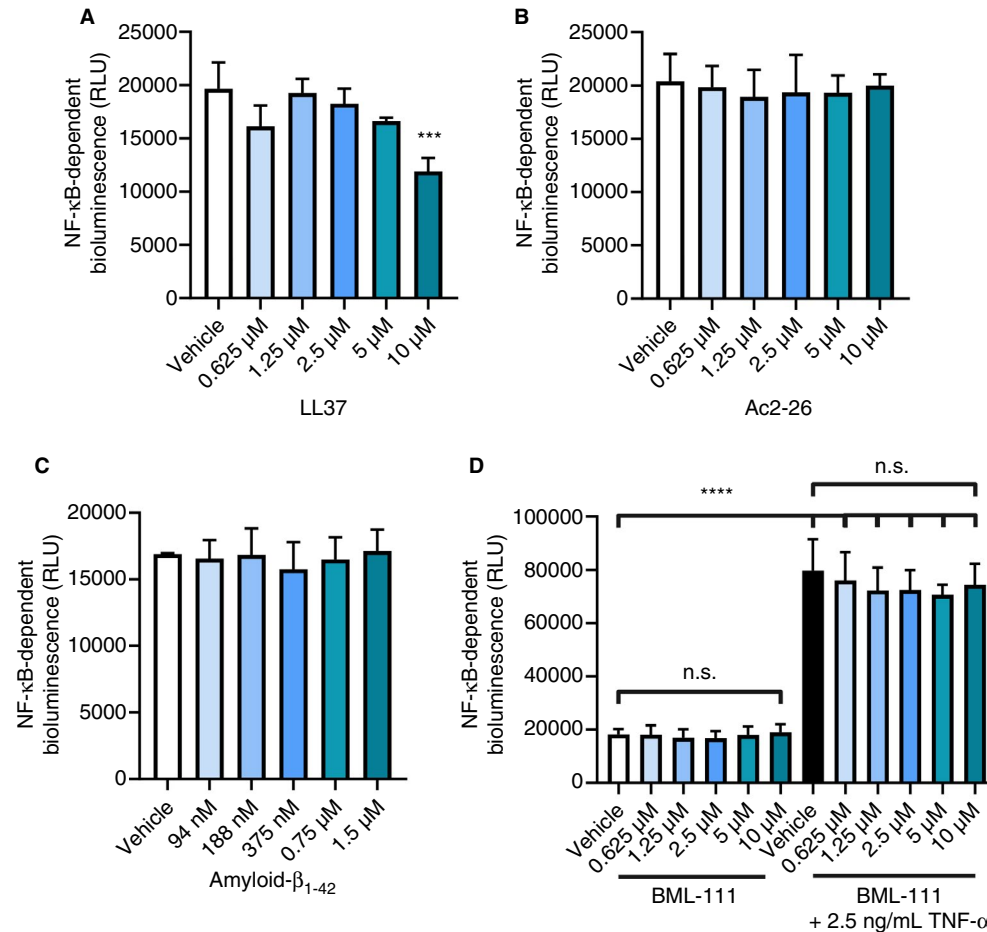


FIGURE 4 Effect of FPR2/ALX ligands on NF-κB activity of Meg-01R cells. Various ligands for FPR2/ALX were tested at different concentrations to determine their impact on NF-κB activity in Meg-01R cells over a 24-hour period. The FPR2/ALX agonist LL37 (A), the annexin A1-mimetic peptide, Ac2-26 (B), and amyloid-β (C) were tested in the absence of an inflammatory stimulus. A synthetic FPR2/ALX ligand, BML-111 (D) was tested (pretreated) in both the presence and absence of 2.5 ng/mL TNF-α. Data represent mean ± standard deviation (n = 3) and statistical significance was calculated using a one-way ANOVA with Bonferroni post hoc test. The P values (**P < .001 and ****P < .0001) are as calculated by GraphPad Prism

3.3 | Inflammatory molecules stimulate NF-κB activity in Meg-01R cells

To determine whether Meg-01R cells respond to various inflammatory molecules, luciferase assays to detect NF-κB-dependent bioluminescence were performed as a measure for NF-κB activity. Meg-01R cells were incubated with increasing concentrations of TNF-α (Figure 3A; a ligand for tumor necrosis factor receptor I and II [TNFRI/II], both of which are expressed on Meg-01 cells¹⁸), Pam3CSK4 (Figure 3B; a ligand for TLR1/2¹⁶), Poly(I:C) (Figure 3C; a ligand for TLR3²⁸), ultrapure LPS from *Escherichia coli* (uLPS_{EC} [a ligand for TLR4²⁰; Figure 3D]) and non-ultrapure LPS_{EC} (Figure 3E) 24 hours before lysis, and addition of luciferin to measure the NF-κB activity. The results suggest that TNF-α induced significant NF-κB-dependent bioluminescence in a concentration-dependent manner (from 1.25 to 10 ng/mL). Similarly, Pam3CSK4 increased NF-κB activity at 5 and 10 μg/mL. In contrast, Poly(I:C) did not significantly increase NF-κB activity at the concentrations tested.

Interestingly, although uLPS_{EC} was incapable of inducing NF-κB activity from Meg-01R cells, the non-ultrapure preparation tested was able to stimulate significant NF-κB activity. These results demonstrate that Meg-01R cells produce functional luciferase as a marker for NF-κB activity and successfully respond to various inflammatory molecules.

3.4 | FPR2/ALX ligands do not modulate NF-κB activity in Meg-01R cells

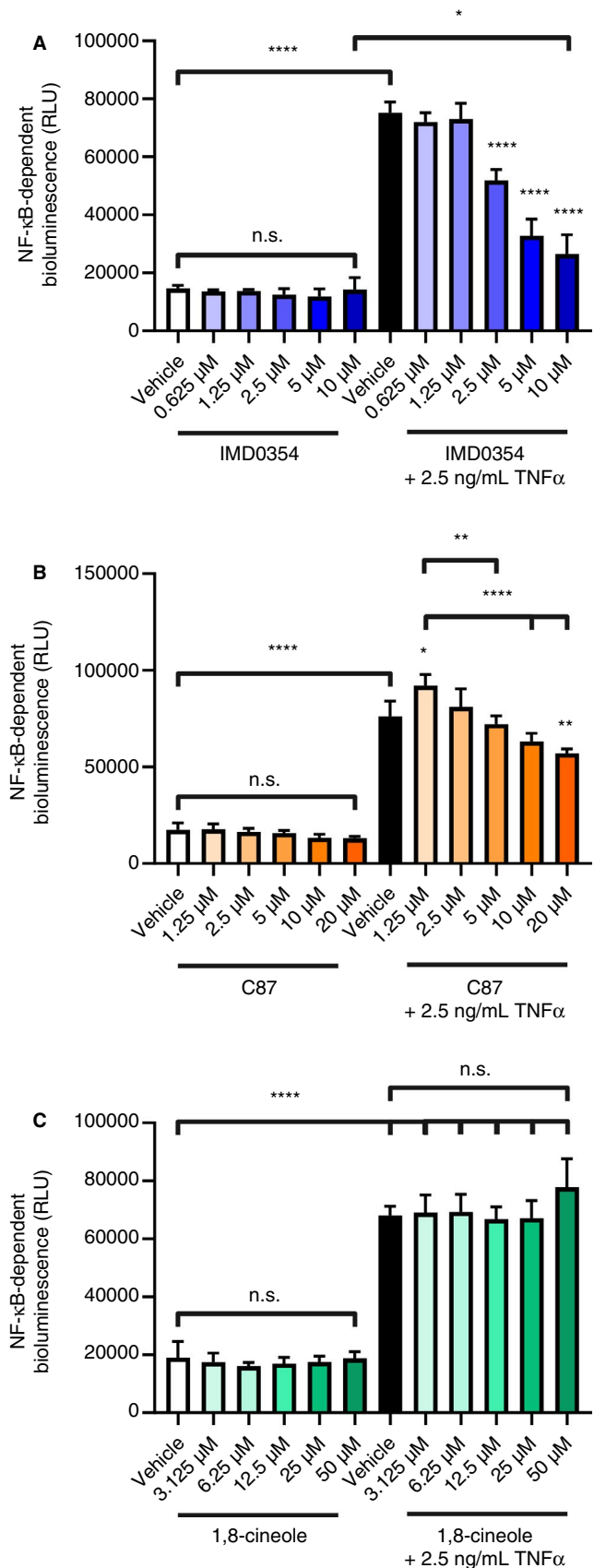
FPR2/ALX, a seven-transmembrane receptor that couples to G_i proteins, is involved in the regulation of the innate immune system and has been found to be expressed on platelets.²⁹⁻³² Because the ligands of FPR2/ALX perform both pro-inflammatory and reparative activities, their impact on modulating NF-κB activity was analyzed in Meg-01R cells. LL37 (an FPR2/ALX agonist that is known to activate platelets³¹), Ac2-26 (an annexin-1 mimetic

FIGURE 5 Impact of TNF- α pathway inhibitors on NF- κ B activity in Meg-01R cells. Different concentrations of an IKK β inhibitor, IMD0354 (A), a TNF- α antagonist C87 (B), and 1,8-cineole (C) were tested in both the presence and absence of 2.5 ng/mL TNF- α over a 24-hour period. Data represent mean \pm standard deviation ($n = 3$) and statistical significance (* $P < .05$, ** $P < .01$, and **** $P < .0001$) was calculated using a one-way ANOVA with Bonferroni post hoc test

peptide that acts as a pro-resolution mediator to control inflammation³³), and amyloid- β_{1-42} (reported to act via TLR2/4, FPR2/ALX, and RAGE^{23,34}) were tested in unstimulated Meg-01R cells. LL37 was able to significantly inhibit NF- κ B activity when used at a concentration of 10 μ M (Figure 4A), whereas Ac2-26 (Figure 4B) and amyloid- β_{1-42} (Figure 4C) were unable to modulate NF- κ B activity at any of the concentrations tested. Furthermore, BML-111 (a synthetic lipoxin A4 analogue and ligand for FPR2/ALX that is known as a pro-resolution mediator³⁵) was tested at a range of concentrations in both the presence and absence of 2.5 ng/mL TNF- α . In these experiments, TNF- α was able to significantly induce the NF- κ B activity over 24 hours. However, pretreatment of Meg-01R cells with BML-111 (Figure 4D) was unable to significantly modulate the NF- κ B activity in either TNF- α -stimulated or unstimulated Meg-01R cells. Further experiments would be necessary to determine the mechanisms that regulate the effects of LL37 in Meg-01R cells in line with the expression and significance of FPR2/ALX in Meg-01 cells.

3.5 | Small molecule inhibitors reduce TNF- α -induced NF- κ B activity in Meg-01R cells

IMD0354, C87, and 1,8-cineole were reported as small molecule inhibitors that are able to affect TNF- α -induced NF- κ B activity in other cell types. IMD0354 acts as an inhibitor of IKK β to prevent the phosphorylation and subsequent degradation of I κ B.^{23,36} C87 acts as an antagonist to TNF- α by directly binding to TNF- α and thus has been proposed to disrupt the TNF- α -TNFRI/II complex.^{37,38} The 1,8-cineole is a plant-derived compound that has been shown to inhibit nuclear translocation of p65 and degradation of I κ B α .^{24,39} Here, these compounds were tested to determine their impact on TNF- α -mediated NF- κ B activity in Meg-01R cells. In the absence of TNF- α , IMD0354 (Figure 5A), C87 (Figure 5B), and 1,8-cineole (Figure 5C) do not alter the NF- κ B activity in Meg-01R cells. However, when cells were stimulated with 2.5 ng/mL TNF- α , IMD0354 significantly inhibited NF- κ B in a dose-dependent manner. C87 was also capable of inhibiting NF- κ B activity in Meg-01R cells at 20 μ M. Interestingly, C87 mildly potentiated TNF- α -induced NF- κ B activity at 1.25 μ M; however, this increase was not observed at higher concentrations. 1,8-cineole was, however, unable to inhibit TNF- α -induced NF- κ B activity in Meg-01R under the current settings used. These results demonstrate that in addition to the impact of inflammatory molecules, the inhibitory



potential of various small molecule inhibitors on NF- κ B activity can also be determined using Meg-01R cells.

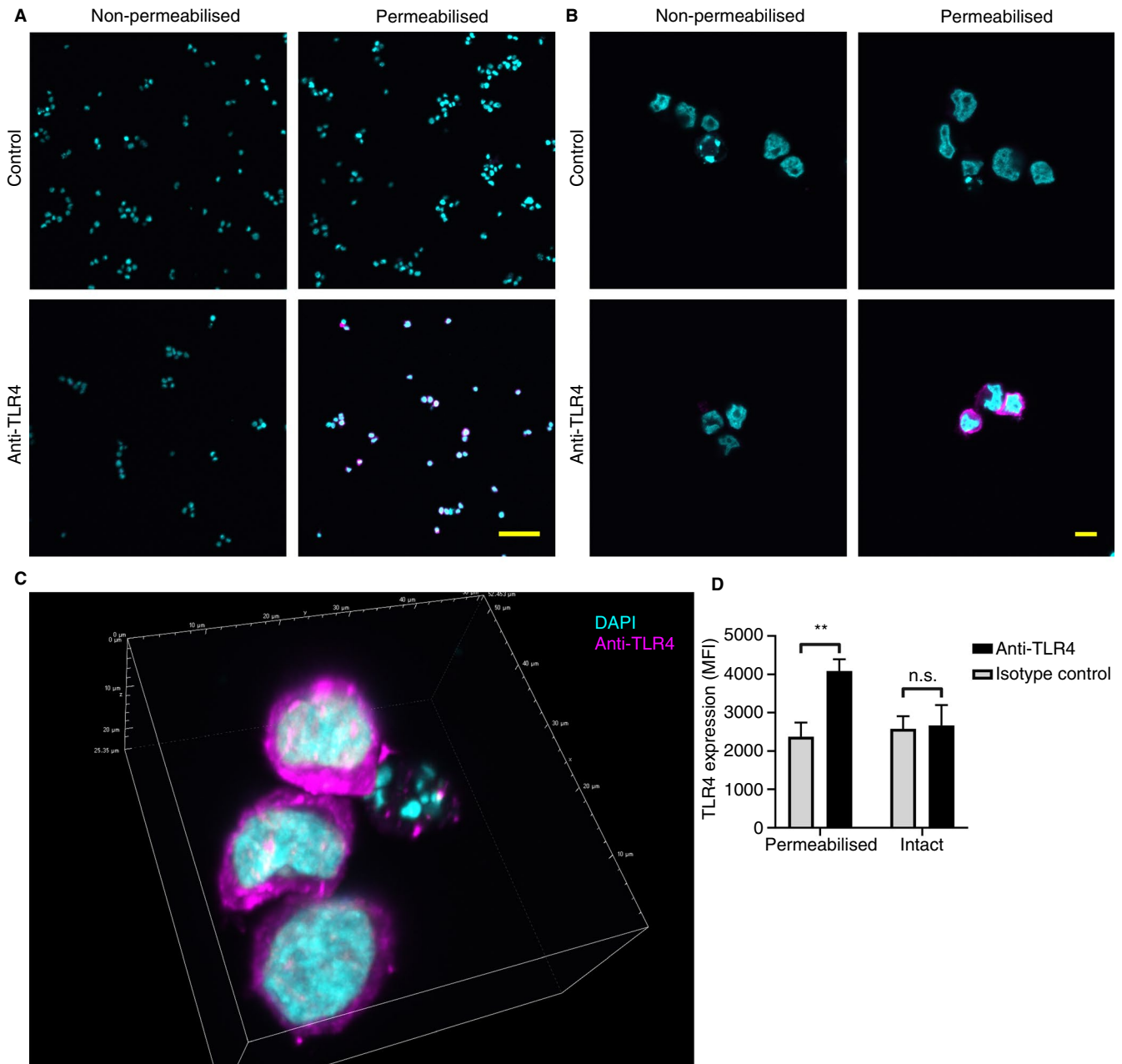


FIGURE 6 Expression of TLR4 in Meg-01 and Meg-01R cells. A, 20 \times magnification images of fluorescently labelled Meg-01 cells acquired via confocal microscopy. Images represent non-permeabilized negative control, permeabilized negative control, permeabilized cells with the anti-TLR4 antibody present, and non-permeabilized cells with an anti-TLR4 antibody present (clockwise from the top left). Scale bar shows 100 μ m. B, Same as A, but images were acquired with 100 \times magnification. Scale bar denotes 10 μ m. C, Three-dimensional reconstruction of Meg-01 cells taken with a 100 \times objective showing the distribution of TLR4 in the cytoplasm. In all images, nuclei have been stained with 4',6-diamidino-2-phenylindole (cyan) and the anti-TLR4 antibody is shown in magenta. D, Flow cytometry-based assays were used to quantify the level of TLR4 by quantifying antibody binding to both permeabilized and non-permeabilized Meg-01R cells. Median fluorescence intensity of TLR4 expression in non-permeabilized and permeabilized Meg-01R cells ($n = 3$) was calculated. Data represent mean \pm standard deviation and were analyzed using multiple t tests with the type I error rate corrected for using the Holm-Sidak method (** $P < .01$)

3.6 | TLR4 is predominantly present inside Meg-01/ Meg-01R cells

To further scrutinize the lack of Meg-01R response to the ultrapure preparation of LPS, the level of TLR4 expression on the surface and inside of Meg-01 and Meg-01R cells was examined. The results obtained using confocal microscopy demonstrate that

TLR4 is largely detectable inside Meg-01 cells as only the permeabilized cells show strong binding to anti-TLR4 antibodies (Figure 6A and 6B). The z-stack image in Figure 6C further demonstrates that TLR4 is expressed ubiquitously within the cytoplasm.

Additionally, the absence of TLR4 on the surface of Meg-01R cells was corroborated using a flow cytometry-based assay. Here, the binding of an anti-TLR4 antibody to the surface of Meg-01R cells was not

FIGURE 7 Effect of LPS-binding molecules CD14 and LL37 to induce LPS-mediated NF- κ B activity in Meg-01R cells. NF- κ B activity was measured in lysates from Meg-01R cells that were stimulated with uLPS_{EC} or uLPS_{SM} in the presence of 2 μ g/mL CD14 (A; n = 3), or 10 μ M LL37 following no co-incubation before addition (B; n = 6) or 15 minutes co-incubation (C; n = 5). Data represent mean \pm standard deviation and statistical significance was calculated using a one-way ANOVA with Bonferroni post hoc test. The P values (**P < .01 and ****P < .0001) are as calculated by GraphPad Prism

significantly different from the isotype controls (Figure 6D). Conversely, the binding of the anti-TLR4 antibody was significantly greater in permeabilized cells compared with their isotype control. These results demonstrate the reasons for the lack of response of Meg-01R cells to ultrapure LPS (detailed previously). Further experiments are required to determine whether activating Meg-01R cells with specific molecules (for example, valproic acid) might elevate the levels of TLR4 on the surface, which would then enable ligation with LPS molecules to exert inflammatory effects (in this case, NF- κ B activity).

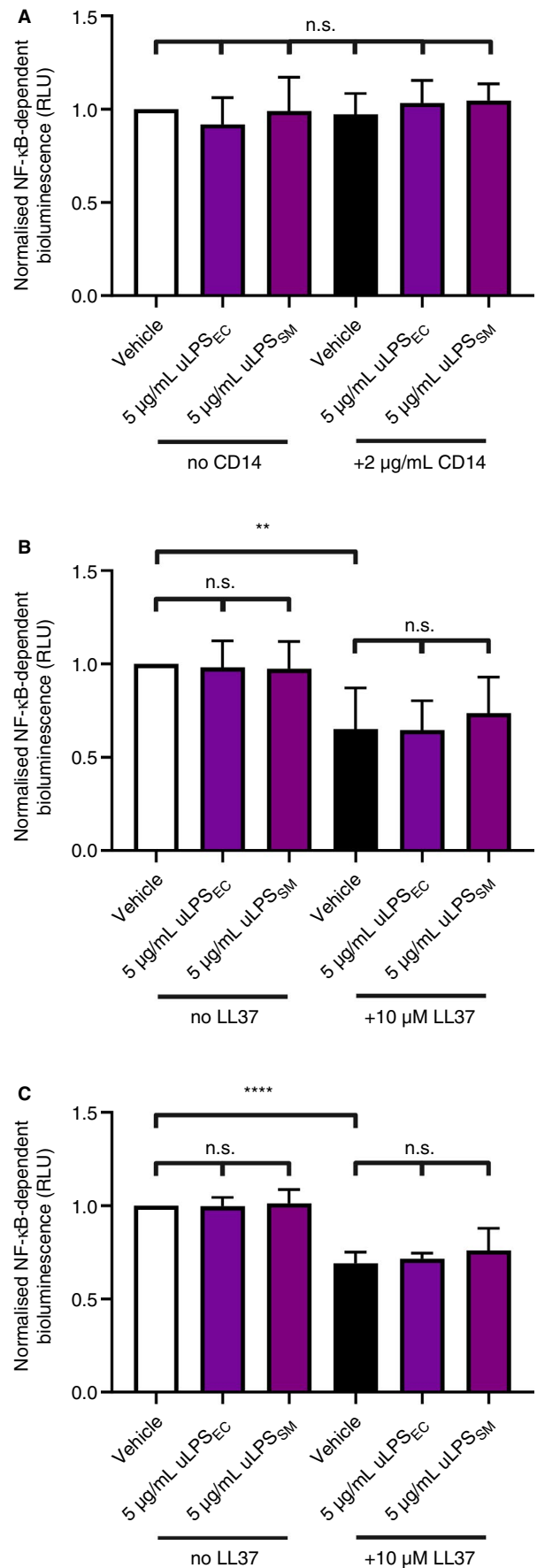
3.7 | CD14 and LL37 do not enable LPS to stimulate NF- κ B activity in Meg-01R cells

To determine if TLR4-induced NF- κ B activity could be promoted by cluster of differentiation 14 (CD14; a TLR4 co-receptor^{5,40,41}) or LL37 (a molecule capable of binding LPS⁴²⁻⁴⁴), the assays were performed in the presence and absence of these molecules along with LPS chemotypes. LL37 has been shown to be capable of transmitting LPS across the cell membrane of human epithelial cells.⁴³

Meg-01R cells were co-incubated with uLPS_{EC} or ultrapure *Salmonella minnesota* LPS (uLPS_{SM}) for 24 hours with/without CD14 or LL37. A physiologically relevant concentration of 2 μ g/mL was chosen for CD14.⁴⁵ The results show that CD14 did not significantly alter NF- κ B activity in Meg-01R cells on its own, and it was not capable of promoting uLPS_{EC} or uLPS_{SM} to stimulate NF- κ B activity (Figure 7A). Furthermore, LPS chemotypes did not induce any activity on their own. When LL37 and uLPS_{EC} or uLPS_{SM} were added simultaneously (Figure 7B) or following 15 minutes preincubation with LL37 (Figure 7C), there was no change in NF- κ B activity compared with LL37 alone (Figure 7C). These results demonstrate that CD14 and LL37 do not modulate LPS-induced NF- κ B activity.

4 | DISCUSSION

The role of MKs during inflammation is not fully understood. Thrombopoietin is known to stimulate the growth and development of MKs and it is removed from the plasma by platelets, which means that, during thrombocytopenia (which can be induced by inflammatory diseases such as sepsis⁵), high levels of thrombopoietin are detected in the plasma.^{7,46} Very few studies have been



conducted to examine the effect of PAMPs using MK cell lines but they demonstrate that NF- κ B can be activated.^{16,28} Mice lacking TLR4 have significantly lower platelet counts than their wild-type counterparts but intravenous injection of a sublethal dose of LPS (0.2 mg/kg) leads to a significant increase in platelet count regardless of whether TLR4 was present.⁸ Moreover, TLR2 and TLR4 have been shown to induce the production of interleukin-6 (IL-6), via NF- κ B, in CD34⁺ cells, which leads to increased MK maturation and platelet production.¹³ Furthermore, circulating platelets have been shown to have higher expression of TLR2 and TLR4 in particular disease states, which suggests that MKs can respond to inflammatory states to tailor the platelet phenotype to the altered pathological situation.^{8,10,47} Moreover, as platelets are anucleate cells and TLRs lead to activation of transcription factors, it has been suggested that platelet TLRs are relics from MKs or hemopoietic stem cells.⁶

TLR4 is unique among the TLR family of receptors in that it can signal via two distinct pathways.⁵ These pathways are known as the MyD88-dependent and the MyD88-independent pathways.⁴⁸⁻⁵⁰ TLR1, 2, 4, 5, 6, 7, 8, 9, and 10 can all activate the MyD88-dependent pathway, whereas TLR3 and TLR4 activate the MyD88-independent pathway.^{5,48,51} Here, we demonstrate that a plethora of proteins required for both MyD88-dependent signalling (TLR4, IRAK2, TRAF6, IKK γ , IKK α , IKK β , I κ B α , and p65) and MyD88-independent signalling (TLR4, TRIF, TRAF3, TBK1/NAK, and IRF3) are present in Meg-01 cells. Interestingly, MyD88 was detectable in the THP-1 cells used as a positive control but was not detectable in Meg-01 via immunoblotting. This may be due to the low expression level of MyD88 in Meg-01 cells and/or limitations of the antibodies used, as its presence was reported in platelets previously.²⁵ However, mRNA of MyD88 was prominently detected in both Meg-01 and Meg-01R cells, which suggests that this protein is indeed present in these cells at similar levels. One complication with identifying proteins in Meg-01 cells and MKs, however, is their ability to produce platelet-like particles or platelets, respectively, because it can be difficult to ascertain whether the proteins are present for signalling in the progenitor cell or merely produced for packaging into the progeny.

I κ B prevents binding of p65 (a member of the NF- κ B family of transcription factors) to its specific DNA promoter sequence but is degraded following phosphorylation by IKK β and ubiquitinated before its degradation.^{5,52,53} p65 is a transcriptional activator that enables transcription of inflammatory cytokines in response to PAMPs and DAMPs, such as ligands of TLRs and TNF- α .^{5,53,54} The MyD88-dependent pathway directly leads to NF- κ B activation brought about by I κ B α degradation; however, cross-talk between the two pathways mean that the MyD88-independent pathway can also induce NF- κ B activation.^{5,28}

Meg-01 cells and MKs are reported to express TLR1, 2, 3, 4, and 6 and Meg-01 cells are frequently used as a surrogate for elucidating signalling pathways in MKs.^{16-19,28,55-57} Because these receptors all can induce NF- κ B activation, we endeavored to develop a reporter cell line to determine the role of MKs in regulating inflammation.

Therefore, we transfected HEK-293 cells with a previously published and commercially available reporter plasmid with a puromycin-resistant gene (pGreenFire-NF κ B-Puro)²³ to produce lentiviral particles that can stably transfect other cell types with this reporter construct. Successfully transduced cells were selected with puromycin, a compound cytotoxic to mammalian cells. A concentration capable of killing ~80% of nontransduced Meg-01 cells was used to ensure that a pure population of Meg-01-GFP-Luc-NF κ B-Puro (Meg-01R) cells developed.

Initially, the Meg-01R cell line was compared with Meg-01 cells to determine whether the transduction process adversely affected the cells or changed their phenotype. Growth rate and morphology remained constant throughout the two cell types and only a minor defect in viability was detected, which may be due to the presence of puromycin. Furthermore, Meg-01R cells express specific functionally important proteins in the same compartments as Meg-01 cells. The Meg-01R cells were then characterized by stimulating them with a range of PAMPs and DAMPs. First, a DAMP, TNF- α was tested for its effects as Meg-01 cells have been reported to express both TNFR1 and TNFR2, two receptors that stimulate NF- κ B activation.^{18,54} Indeed, TNF- α was able to significantly activate NF- κ B to transcribe luciferase in a concentration-dependent manner as determined by the level of luciferase activity.

Pam3CSK4 is a synthetic ligand for the TLR1/2 heteroreceptor and it has previously been shown to induce phosphorylation of NF- κ B's p65 subunit and degradation of I κ B over the period of 1 hour.¹⁶ Phosphorylation of p65 was also shown to occur in murine MKs following 30 minutes of treatment with Pam3CSK4.¹⁶ In accordance with this previous study, Pam3CSK4 was able to induce luciferase production downstream of NF- κ B albeit not as strongly as the lowest concentration of TNF- α tested.

Poly(I:C) is a synthetic ligand for TLR3 and, although TLR3 couples to the MyD88-independent pathway, signalling downstream of TLR3 can lead to NF- κ B activation.²⁸ Poly(I:C) and Poly(A:U) (another synthetic TLR3 ligand) have been shown to induce I κ B degradation and phosphorylation of p65 in human CD34⁺ cells derived from umbilical blood.²⁸ In this study, Poly(I:C) was incapable of stimulating the NF- κ B activity at any of the concentrations tested. This discrepancy may be due to changes in the expression levels of specific receptors and characteristics observed during MK maturation.

The preparation of LPS_{EC} can alter the characteristics of the responses induced by this ligand therefore an ultrapure and a non-ultrapure version were both tested. This has been hypothesized to be due to the presence of bacterial contaminants in non-ultrapure preparations that can activate other pro-inflammatory receptors such as TLR2.^{20,51} The ultrapure version of LPS_{EC} was unable to activate NF- κ B; however, the non-ultrapure preparation was capable of inducing activity at a range of concentrations. This finding lends more weight to the hypothesis that many of the effects observed when using non-ultrapure LPS_{EC} may be induced by contaminants in the preparations.

Moreover, FPR2/ALX ligands were tested on Meg-01R cells to determine if they could affect NF- κ B activity in this cell type. The

agonist, LL37,³¹ was able to significantly inhibit NF- κ B activity on its own over a period of 24 hours in contrast to the annexin A1-mimetic, Ac2-26.³³ Amyloid- β_{1-42} is a neuropeptide involved in the progression of Alzheimer's disease. Indeed, it has been related to inflammation in the brain and is capable of inducing NF- κ B activity in a glioblastoma cell line.^{23,34} However, amyloid- β_{1-42} was incapable of activating NF- κ B at any of the tested concentrations in this study. This may be due to the concentrations tested being subthreshold. Interestingly, the synthetic lipoxin A4-analogue, BML-111 was incapable of modulating NF- κ B activity in the absence or presence of TNF- α .³⁵ However, this may be due to signalling via a different downstream pathway.

A potential application of this novel reporter cell line is for the identification of compounds that affect inflammatory responses in MKs during disease states in an NF- κ B-dependent manner.¹⁵ To evaluate this, Meg-01R cells were treated with an IKK β inhibitor, IMD0354,³⁶ a TNF- α antagonist, C87,³⁷ or 1,8-cineole for 24 hours in the presence or absence of TNF- α . Neither IMD0354 nor C87 had a significant effect on unstimulated Meg-01R cells; however, they could both significantly inhibit the increase in NF- κ B activity induced by TNF- α . Interestingly, the lowest concentration of C87 tested (1.25 μ M) potentiated the NF- κ B activity induced by TNF- α although the mechanism behind this is currently unknown. 1,8-cineole had no effect in either stimulated or unstimulated cells.

Meg-01 cells have previously been described to express TLR4 when the cells were fixed with methanol, a fixative known to be capable of permeabilizing cells.⁵⁶ To confirm the distribution of TLR4 in Meg-01 cells, they were fixed with 4% PFA (for immunocytochemistry) or 0.2% formyl saline (for flow cytometry) and then cells were left intact or permeabilized with PBS containing Triton-X100. Moreover, two different anti-TLR4 antibodies were used to confirm its presence depending on the detection method. With both methods, TLR4 was not detectable on the surface of Meg-01 cells; however, it was detectable intracellularly. Previous studies have suggested that MKs express TLR4 on their surface but this increases during MK maturation.⁵⁷

Although TLR3 is predominantly detectable intracellularly, poly(I:C) is capable of being internalized leading to its activation.²⁸ In contrast, LPS is not capable of crossing the cell membrane on its own.⁴³ LPS is internalized after binding to CD14-TLR4 to enable it to enter endosomes and induce MyD88-independent signalling.^{40,41} Furthermore, LL37 is known to be capable of binding LPS and other negatively charged molecules, usually in an anti-inflammatory capacity.^{42-44,58,59} Shaykhiev et al.⁴³ demonstrated, through the use of primary human bronchial epithelial cells and human pulmonary mucocarcinoma NCI-H292 cell line, that 20 μ g/mL LL37 can induce the uptake of LPS in epithelial cells over the course of 24 hours.⁴³ Importantly, this internalization was shown to be independent of TLR4 and dependent of caveolae and functional epidermal growth factor receptor.⁴³ Three treatments – 2 μ g/mL CD14, simultaneous addition of 10 μ M LL37 and LPS, addition of 10 μ M LL37 and LPS after 15 minutes of co-incubation – were tested to determine if these molecules could deliver LPS

to its target receptor. Two μ g/mL CD14 had no effect on NF- κ B activity in Meg-01R cells and was unable to promote LPS to induce luciferase synthesis. Furthermore, in both conditions, LL37 significantly inhibited NF- κ B activity; however, this was not modulated by the simultaneous addition of LPS, nor the co-incubation of LL37 and LPS. This may suggest that TLR4 in Meg-01R cells is not found in the endosome and therefore it may not stimulate MyD88-independent signalling nor interact with internalized LL37/LPS complexes.

In conclusion, here we developed a MK reporter cell line and demonstrated that MKs are responsive to a range of PAMPs and DAMPs, and their pathway inhibitors. Hence, this reporter cell line can be applied to screen a broad range of inflammatory molecules that act via NF- κ B to determine their functions in MKs.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Thomas M. Vallance, Darius Widera, and Sakthivel Vaiyapuri designed the experiments, analyzed the data, and wrote the manuscript. Thomas M. Vallance, Jonathan J. Sheard, Yiming Meng, and Enrico C. Torre performed the experiments and analyzed the data. Ketan Patel has provided advice and support for the design of experiments.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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