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BENCHMARK

Comparison of CRISPR and adenovirus-mediated Myd88 knockdown in RAW 264.7 cells and responses to lipopolysaccharide stimulation

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Abbreviations used: Ad-GFP, cells transduced with ad-GFP; Ad-MKD, cells transduced with ad (RGD)-GFP-m-MyD88-shRNA; ANOVA, analysis of variance; BSA, albumin solution from bovine solution; CAR, coxsackieviral-adenovirus receptor; Cas9, CRISPR associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; CrMKD, CRISPR MyD88 knockdown cells; CrROSA, CRISPR ROSA control cells; DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IL, interleukin; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response 88; NFκB, nuclear factor kappa light chain enhancer of activated B cells; NSAIDs, non-steroidal anti-inflammatory drugs; PAM, protospacer adjacent motif; RGD, arginine, glycine, and aspartate; TNF-α, tumor necrosis factor alpha

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ABSTRACT

Genomic manipulation offers the possibility for novel therapies in lieu of medical interventions in use today. The ability to genetically restore missing inflammatory genes will have a monumental impact on our current immunotherapy treatments. This study compared the efficacy of two different genetic manipulation techniques: clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) transfection to adenoviral transduction to determine which method would provide the most transient and stable knockdown of myeloid differentiation primary response 88 (MyD88). MyD88 is a major regulator of nuclear factor kappa light chain enhancer of activated B cells (NFκB) pathway in Raw 264.7 macrophages. Following genetic manipulation, cells were treated for 24 h with Lipopolysaccharide (LPS) to stimulate the inflammatory pathway. Confirmation of knockdown was determined by western immunoblotting and quantification of band density. Both CRISPR/Cas9 and adenoviral transduction produced similar knockdown efficiency (~64% and 60%, respectively) in MyD88 protein 48 h post adenoviral transduction. NFκB phosphorylation was increased in CRISPR/Cas9-mediated MyD88 knockdown and control cells, but not in adenovirus-mediated MyD88 knockdown cells, following LPS administration. CRISPR/Cas9-mediated MyD88 knockdown macrophages treated with LPS for 24 h showed a 65% reduction in tumor necrosis factor alpha (TNFα) secretion, and a 67% reduction in interleukin-10 (IL-10) secretion when compared to LPS-stimulated control cells ($P \leq 0.01$ for both). LPS did not stimulate TNFα or IL-10 secretion in adenovirus-mediated control or MyD88 knockdown cells. These data demonstrate that Raw 264.7 macrophages maintain responsiveness to inflammatory stimuli following CRISPR/Cas9-mediated reductions in MyD88, but not following adenovirus-mediated MyD88 knockdown.

Keywords: adenovirus, CRISPR/Cas9, inflammation, MyD88

INTRODUCTION

Current methods to modulate the inflammatory response include the use of NSAIDs, antibiotics, immune system suppressors, and retroviral based gene therapies [1-4]. Of these methods, NSAIDs are the most widely used and readily available therapy [4]. Despite the effectiveness of NSAIDs at attenuating inflammation and related pain, they have been linked to negative health outcomes such as hospitalization due to heart failure and risk of gastrointestinal complications such as bleeding [5]. More recently, adenoviral transduction and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) transfections have become popular tools for genomic manipulation. Continuous alteration of both vectors has significantly improved their ability to transfect cells. These modifications have enabled

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scientists to explore the use of these vectors to reduce the severity of diseases, reduce the ability for viruses to enter human cells (preventing wild type reversion), and to minimize off target side effects. [6-9]. Modulating the inflammatory response through molecular targeting versus current pharmacological approaches has the potential to change the way inflammatory diseases are being treated.

Gene therapy using adenoviral vectors (**Fig. S1**) is not a novel concept. Adenoviral vectors have been used to promote the overexpression of wild type p53 in cancer cells that contain the mutant version of this gene to undergo apoptosis [9,10]. Unfortunately, adenoviral vectors require high viral titer to promote a phenotypic response, and these titer loads are highly immunogenic and toxic (causing a potential cytokine storm) [11]. In addition to the immunogenicity associated with viral load, adenovirus production is very labor intensive [9]. While some studies have found doses and schedules that limit toxicity, incorporation of target genes and their expression has failed to occur in cells outside of the injection site area [6]. Additionally, many doses have elicited a severe inflammatory response [11]. Given the limitations with adenoviral use both *in vitro* and *in vivo*, CRISPR/Cas9 may represent a more feasible vector for gene delivery. Like adenovirus, there have been several *in vitro* and *in vivo* studies that have successfully utilized CRISPR/Cas9 to suppress various viral infections and cancers (**Fig. S2**) [12,13]. In addition to being easy to customize and produce, CRISPR/Cas9 allows scientists to target multiple loci simultaneously [14]. However, in certain instances, protospacer adjacent motif (PAM) sequences may not be readily available due to differences in DNA folding and off-target cleaving [14,15]. Despite this limitation, CRISPR/Cas9 genome manipulation has been successful in several animal studies and has the potential to be a powerful therapy in the treatment of cancer and other devastating diseases [16,17]. However, to date, no studies have directly compared the efficacy of adenoviral and CRISPR vectors on the same cellular signaling pathway.

The purpose of this study was to investigate the capabilities, limitations, and outcomes of adenoviral transduction and CRISPR/Cas9 transfection in RAW 264.7 macrophages. As a precursor to more mature macrophage cell lines, RAW 264.7 cells serve as a suitable transfection platform and gatekeeper to the inflammatory response. Of particular interest for modulation in this cell line was the nuclear factor kappa light chain enhancer of activated B cells (NFκB) signaling pathway, due to the many inflammatory processes this signaling pathway regulates [18]. Previous research has identified myeloid differentiation primary response 88 (MyD88) as a key adapter protein in this pathway (**Fig. 1A**) [19]. Reductions in MyD88 are expected to alter both pro-inflammatory signaling, mediated by Tumor necrosis factor alpha (TNFα), and anti-inflammatory signaling mediated by interleukin 10 (IL-10). Given the differences between CRISPR/Cas9 transfection and adenoviral transduction discussed above, we hypothesized that CRISPR/Cas9 transfection would provide a more stable, long term, and effective genomic transformation compared to adenoviral transduction in RAW 264.7 macrophages.

MATERIALS AND METHODS

Cell line and reagents

The RAW 264.7 macrophage cell line was purchased from American Type Culture Collection (TIB-71; ATCC; Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM; 4.5 g/L glucose, 110 mg/L sodium pyruvate, and L-Glutamine; 11995065), phosphate buffered saline (10010023), fetal bovine serum (FBS; A3160402), penicillin-streptomycin (15070063), Halt™ protease & phosphatase inhibitor cocktail (100X; 78446), Novex™ Tris-Glycine Transfer buffer (25X; LC3675), Novex™ Tris-Glycine SDS Running Buffer (10X; LC2675), Novex™ 4%–20% Tris-Glycine Mini Gels (WedgeWell™ format, 15-well; XP04205BOX), and SuperSignal™ West Pico PLUS Chemiluminescent Substrate (34578) were purchased from ThermoFisher Scientific (Waltham, MA). Albumin solution from bovine serum (BSA; A3059) was purchased from Sigma-Aldrich (St. Louis, MO). Western immunoblotting antibodies for Vinculin (13901S), MyD88 (D80F5), and CD80 (54521) were purchased from Cell Signaling Technology (Danvers, MA). Cell Lysis buffer (10X; 9803), Tris-Buffered Saline with Tween20 (TBST-25X; 9997S), and LPS were also purchased from Cell Signaling Technology.

Cell culture and cell stimulation

Raw 264.7 cells were thawed, passaged, and grown according to Hobbs *et al*., [20]. Antibiotic free DMEM containing 10% FBS was used as growth media as well as for CRISPR/Cas9 transfection. Macrophages were serum starved in antibiotic free DMEM containing < 0.01% BSA at 37°C and 5% CO_2 for 24 h. Adenoviral transductions were performed using antibiotic free DMEM containing < 0.01% BSA. Following starvation, cells were treated with LPS (10 ng/ml), and harvested for whole cell protein lysate and conditioned growth media at 24 h post-LPS treatment. Time points were determined based on prior detailed LPS time course studies [20] (**Fig. 2**).

CRISPR/Cas9 transfection

Opti-MEM™ reduced serum medium (31985062), Lipofectamine™ CRISPRMAX™ (CMAX00003), TrueGuide™ tracrRNA (5 nmol; A35533), TrueGuide™ crRNA PC RosA26 (A35518), TrueGuide™ crRNA predefined (Assay Number CRISPR532905_CR; target sequence: GCATCCAACAAACTGCGAGT), and TrueCut™ CAS9 Protein V2 (A36499) were purchased from ThermoFisher Scientific (Waltham, MA). Macrophages were seeded at 30 × 10^4 cells/ml in 4 ml growth media and incubated for 6 h at 37°C and 5% CO_2. Annealing of gRNA and TracrRNA, as well as CRISPR/Cas9 transfection of either ROSA control vector or MyD88 knockdown vector was performed following incubation according to ThermoFisher Scientific standard protocol. Reagents volumes were adjusted to optimize transfection to cell line and seeding density. Transfected cells were expanded 72 post-transfection and allowed to grow an additional 96 h. When cells reached 85%–100% confluency, they were passaged and seeded into six-well tissue culture dishes at 12 × 10^4 cells/ml for experimentation.

Adenoviral transduction

Adenoviral sh-RNA pre-validated for MyD88 knockout (ad (RGD)-GFP-m-MyD88-shRNA (shADV-265270)) and an adenoviral sh-RNA vector tagged with GFP (ad)-GFP were purchased from Vector Biolabs (Malvern, PA). At the time cells previously transfected with CRISPR/Cas9 were seeded for experimentation, macrophages that were going to be transduced were seeded at 12 × 10^4 cells/ml in 4 ml of growth media. Cells were incubated for 16 h at 37°C and 5% CO_2. Following incubation, media was changed to serum and antibiotic free media, and transduction of either the adenoviral GFP control vector or MyD88 knockdown vector was performed according to Vector Biolabs standard

protocols using 1×10^7 PFU/ml of either control or MyD88 knockdown adenovirus vector. Note that cells underwent transduction 184 h after cells were transfected with CRISPR, as indicated in the previous section. Transduction and serum starvation prior to LPS treatment occurred simultaneously.

Western blotting

Cells were disrupted using Cell Lysis Buffer containing Halt™ protease and phosphatase inhibitor. Subsequent protein lysates were quantified using Bradford assay [21]. Western Blotting was done as previously described [22]. Membranes were probed for vinculin (124 kDa), p-NFκB (65 kDa), CD80 (50–75 kDa), and MyD88 (33 kDa). Band densities were quantified using NIH Image J (Version 1.60; NIH, Bethesda, MD, USA) [23]. MyD88 and p-NFκB expression were normalized to control vectors treated with LPS and vinculin (loading control). CD80 expression were normalized to untreated control vectors and vinculin.

Conditioned growth media collection and ELISAs

Mouse TNF-alpha Quantikine® ELISA kits (SMTA00B), Mouse IL-10 Quantikine® ELISA kits (SM1000B), and Mouse Quantikine® IL-6 ELISA kits (M600B) were purchased from Bio-Techne R&D Systems (Minneapolis, MN). Conditioned growth media was collected, stored, thawed, and prepared for ELISAs according to Hobbs *et al*. [20]. Assays were performed according to the manufacturer's protocols provided for each respective ELISA kit. Samples, standards, and assay diluent were loaded onto ELISA plates by hand and the rest of the assay, including incubations, were performed using the Dynex Technologies DS2® Automated ELISA System (Chantilly, VA). All values are presented as TNFα, IL-10, or IL-6 secretion (pg/ml) normalized to protein concentration (μg/μl).

Statistical analysis

All data are expressed as the mean ± standard deviation, representative of 3–5 independently-conducted experiments. The data for all figures were statistically analyzed with GraphPad Prism 8 software using a one-way ANOVA with Dunnett's multiple comparison post-test. Differences with $P < 0.05$ were considered significant.

RESULTS

CRISPR/Cas9 reduces MyD88 levels in LPS treated cells

To determine the efficiency of our vectors for each method, we first performed optimization experiments, which indicated that MyD88 knockdown was achieved 72 h after CRISPR mediated transfection, and maintained through five consecutive passages (**Fig. 1B**). Conversely, adenoviral knockdown required continual treatment to maintain a successful knockdown of MyD88. Exposure of reagents and high dosages of adenovirus resulted in the inability of cells to be passaged, as well as cytotoxicity 72 h following transduction (data not shown). Thus, a period 48 h following adenoviral transduction was chosen to balance cell viability with knockdown efficacy. At this time point, CRISPR/Cas9-transfected cells had been passaged twice and were 240 h post-transfection (**Fig. 2**). Analysis of knockdown efficiency revealed a ~60% and ~63% reduction in MYD88 protein levels in adenoviral and CRISPR/Cas9 untreated cells, respectively ($P \leq 0.001$ for both) (**Fig. 1C-1D**).

Adenoviral knockdown significantly reduces LPS-stimulated phosphorylation of NfκB compared to CRISPR knockdown

To analyze the downstream effects of MyD88 knockdown, expression of phosphorylated NFκB was examined 24 h following LPS stimulation. LPS-stimulated phosphorylation of NFκB was increased in cells transfected by the CRISPR/Cas9 method ($P < 0.01$); however, NFκB phosphorylation was unaffected under any condition in adenovirally-transduced macrophages (**Fig. 3A-3C**). LPS-stimulated NFκB phosphorylation was not reduced in CRISPR-mediated Myd88-deficient cells, suggesting that a MyD88-independent pathway may mediate NFκB phosphorylation under these conditions.

CRISPR significantly reduces cytokine secretion compared to adenovirus transduction

We next analyzed the ability of the methods to modulate secretion of pro-inflammatory TNFα, IL-6, and anti-inflammatory IL-10. TNFα, IL-10, and IL-6 secretion were assessed 24 h following LPS treatment. In response to LPS, the CRISPR/Cas9 method resulted in reduced secretion of TNFα, IL-10, and IL-6 by 65% ($P = 0.005$), 67% ($P = 0.011$), and 74% ($P = 0.037$) respectively, compared to control transfected cells. (**Fig. 4A**, **4C**, and **4E**). In cells transduced with adenovirus, MyD88 deficiency had no effect on LPS-stimulated TNFα, IL-10, or IL-6 secretion (**Fig. 4B**, **4D**, and **4F**). As a general marker of the inflammatory response, we also examined the expression of CD80. As **Figure 4G** shows, CD80 expression followed the general pattern of cytokine secretion, *i.e.*, LPS-stimulated CD80 expression was reduced in CRISPR-transfected cells, but not in adenovirally-transduced cells.

DISCUSSION

Scientists have been working for decades to understand the genetic defects responsible for inflammatory disease and the possibility of genetically manipulating DNA [24,25]. Genetic manipulation would allow for the removal of mutated genes and the insertion of the wild type gene, overexpression of target genes, or the ability to eliminate the gene completely [26,27]. The goal of this study was to compare the CRISPR/Cas9 and adenoviral gene delivery systems using MyD88 as a target molecule. To this end, we found that reduction of MyD88 was more effectively achieved and sustained using the CRISPR/Cas9 transfection system.

The RAW 264.7 macrophage cell line provides an excellent experimental system to compare the effects of adenoviral transduction and CRISPR transfection; indeed, they are widely used to study inflammatory processes, they are readily-available to the scientific community, and, as we show here, are receptive to multiple transfection techniques. Unpublished work in our laboratory has revealed that mouse bone marrow-derived macrophages are amenable to both CRISPR transfection and adenoviral transduction, suggesting that these cells may also be useful for efficacy comparisons between multiple genetic manipulation techniques. Additionally, both adenoviral transduction [28] and CRISPR transfection [29] have shown efficacy in murine Segment 3 (S3)

proximal tubal cells, although we are unaware of any discrete study that has directly compared the two methods in the same report. Conversely, we were unable to transduce primary human skeletal myoblasts with adenovirus (data not shown), although these cells had been shown to be successfully transduced with baculovirus [30]. Clearly, some cells types are more amenable than others for comparing genetic manipulation methodologies. While many cells can be transfected, the success of the transfection depends on multiple factors including cell viability, cell passage number, and membrane receptor: adjuvant interaction, and vector size.

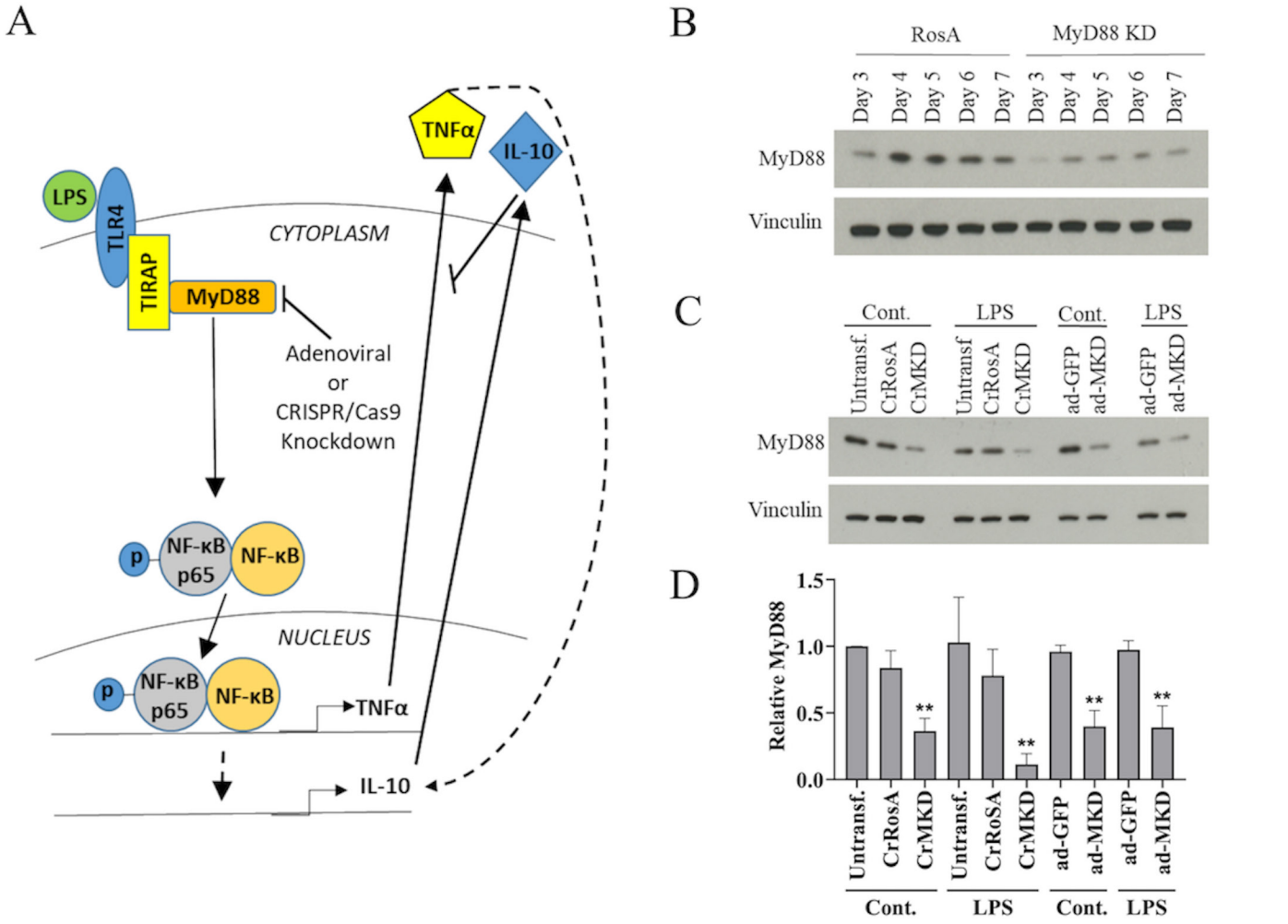

Figure 1. The efficacy of CRISPR/Cas9 transfection and adenoviral transduction in MyD88 knockdown. RAW 264.7 cells were selectively knocked down for MyD88 using either CRISPR/Cas9 transfection or adenoviral transduction. **A.** MyD88 mediated NF-κB pathway leads directly to TNFα secretion. In a negative feedback loop, TNFα production will lead to the production of IL-10, which reduces TNFα. **B.** Western blots of protein lysates of cells transfected with either CrRosA or CrMKD for 72 h and passaged for five consecutive days. Lysates were probed for MyD88 and Vinculin. **C.** Western blots of protein lysates 48 h post adenoviral transduction (216 h post CRISPR transfection) were probed for Vinculin (loading control) and MyD88. **D.** Quantification of (C) using Image J software. Presented as relative MyD88 expression normalized to untreated, untransfected MyD88 expression and vinculin (loading control). Data are presented at mean ± SD. Groups marked significant are in comparison to untransfected control group (Dunnet's multiple comparisons test; $^{**}P \leq 0.001$; $n = 4–5$). Untransfect. Cont. = 1.00; CrRosA Cont. = 0.84 ± 0.13; CrMKD Cont. = 0.36 ± 0.09; Untransf. LPS = 1.03 ± 0.34; CrRosA LPS = 0.78 ± 0.2; CrMKD LPS = 0.11 ± 0.08; ad-GFP Cont. = 0.96 ± 0.05; ad-MKD Cont. = 0.4 ± 0.12; ad-GFP LPS = 0.97 ± 0.07; ad-MKD LPS = 0.39 ± 0.16. CrRoSA-cells transfected with ROSA vector using CRISPR/CAS transfection; CrMKD-cells transfected with MyD88 knockdown vector using CRISPR/CAS transfection; ad-GFP-cells transduced with GFP vector using adenoviral transfection; ad-MKD-cells transduced with MyD88 knockdown vector using adenoviral transduction.

To determine the efficiency of our vectors, we analyzed the protein levels of MyD88 in RAW 264.7 cells after transduction or transfection. Our analysis revealed that both methods reduced MyD88 to similar levels (**Fig. 1C** and **1D**). Given this, it was expected that MyD88-dependent downstream signaling and cytokine secretion would be similar across the two methods. However, we found that cells transfected with CRISPR/Cas9 constructs responded to an LPS challenge, whereas cells transduced with adenovirus did not. This was surprising because a lack of MyD88 would be expected to perturb activation of associated pathways, irrespective of the method used to reduce its expression. One

possible explanation for this discrepancy concerns the persistent nature of CRISPR/Cas9 compared to adenovirus. While MyD88 protein levels were similar between the two methods at the time point examined, cells lacking MyD88 through CRISPR/Cas9 manipulation had lacked MyD88 for a longer period of time than had adenovirus-transduced cells. A second possibility regarding the discrepancy between the two methods concerns the adenovirus itself; indeed, adenovirally-transduced cells were refractory to LPS challenge for all outcomes measured, while CRISPR/Cas9 cells were not. This observation is generally consistent with previous *in vitro* and *in vivo* reports that utilized this vector in other cell types [6,11]. In any case, it is clear that CRISPR/Cas9-mediated MyD88 knockdown, but not adenoviral-mediated MyD88 knockdown, maintains the responsiveness of RAW 264.7 cells to inflammatory stimuli under the conditions reported here.

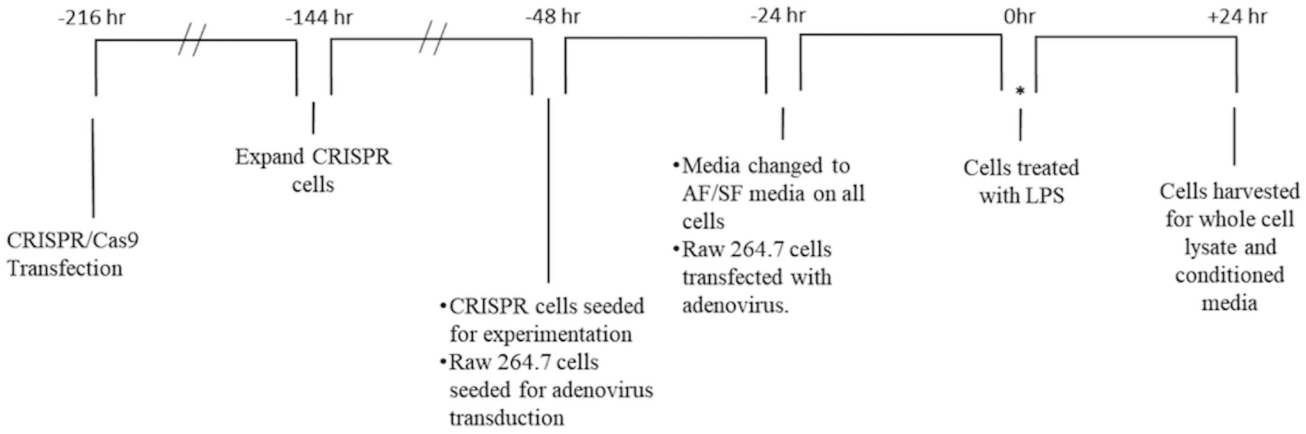

Figure 2. Timeline of experimental procedures. The point at which the cells were treated with LPS was designated as 0 h.

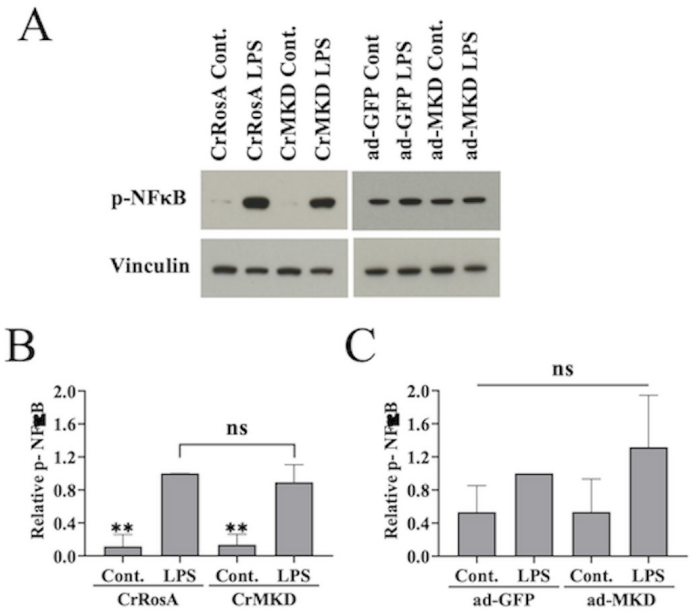

Figure 3. The effects of MyD88 knockdown on intermediary cytokines. Raw 264.7 cells were transfected with either adenovirus or CRISPR/ Cas9 and subsequently treated with LPS (10 ng/ml) for 24 h. Macrophages were harvested for whole cell protein. **A.** Western Blot film of whole cell lysate probed with either vinculin or p-NFκB. **B.** Western blot analysis of p-NFκB normalized to both vinculin and either CrRosA LPS or ad-GFP LPS. Data are represented as mean ± SD. Groups marked significant are in comparison to CrRosA LPS (B) or ad-GFP LPS (C) (Dunnet's multiple comparisons test; ns = significant; $^{**}P \leq 0.001$; $n = 4$). CrRosA Cont. = 0.12 ± 0.14; CrRosA LPS = 1.00; CrMKD Cont. = 0.14 ± 0.13; CrMKD LPS = 0.89 ± 0.21; ad-GFP Cont. = 0.53 ± 0.32; ad-GFP LPS = 1.00; ad-MKD Cont. = 0.54 ± 0.40; ad-MKD LPS = 1.32 ± 0.63.

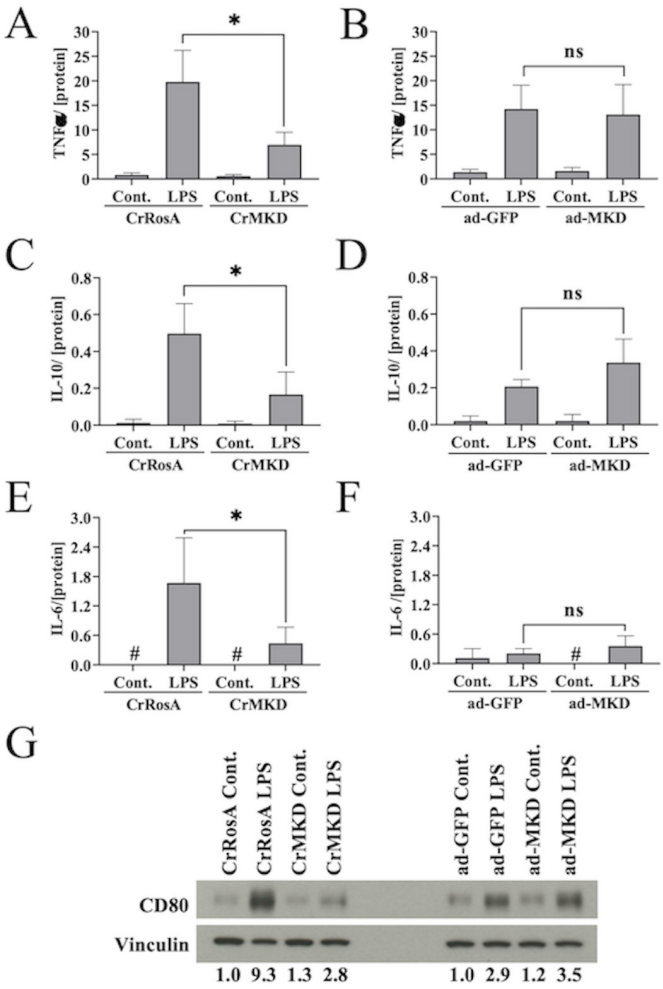

Figure 4. The effects of MyD88 knockdown on TNFα, IL-10, IL-6 and protein secretion as well as CD80 cytokine expression. A-F. RAW 264.7 cells were treated with LPS (10 ng/ml) for 24 h. ELISA analysis of secreted TNFα, IL-10, and IL-6 for cells transfected with CRISPR (A, C, and E, respectively), as well as for cells transduced with adenovirus (B, D, and F, respectively). All secretion values normalized to protein concentrations. **G.** Western blots of protein lysates 24 h post-LPS probed for CD80 and vinculin (loading control). Band densities of (G) quantified using Image J software and normalized to both vinculin and either CrRosA/ad-GFP LPS (indicated by the numbers below the blots; *n* = 1). Data are presented as mean ± SD. LPS-stimulated IL-10 and TNFα secretion of MyD88 knockdown cells were compared to their respective transfection controls treated with LPS. (Dunnet's multiple comparisons test; ns = not significant; # = no cytokine detected; *$P \leq 0.05$; *n* = 3). TNF-α secretion (A and B)-CrRosA Cont. = 0.85 ± 0.39; CrRosA LPS = 19.79 ± 6.42; CrMKD Cont. = 0.58 ± 0.31; CrMKD LPS = 6.95 ± 2.56 (*P* = 0.0049 *vs*. CrRosA LPS); ad-GFP Cont. = 1.37 ± 0.55; ad-GFP LPS = 14.23 ± 4.86; ad-MKD Cont. = 1.61 ± 0.73; ad-MKD LPS = 13.11 ± 6.11. IL-10 secretion (C and D)-CrRosA Cont. = 0.01 ± 0.02; CrRosA LPS = 0.50 ± 0.16; CrMKD Cont. = 0.01 ± 0.01; CrMKD LPS = 0.17 ± 0.12 (*P* = 0.011 *vs*. CrRosA LPS); ad-GFP Cont. = 0.02 ± 0.03; ad-GFP LPS = 0.21 ± 0.04; ad-MKD Cont. = 0.02 ± 0.04; ad-MKD LPS = 0.34 ± 0.13. IL-6 Secretion (E and F)-CrRosA Cont. = 0.00; CrRosA LPS = 1.67 ± 0.92; CrMKD Cont. = 0.00; CrMKD LPS = 0.44 ± 0.33 (*P* = 0.037 *vs*. CrRosA LPS); ad-GFP Cont. = 0.11 ± 0.19; ad-GFP LPS = 0.21 ± 0.10; ad-MKD Cont. = 0.00; ad-MKD LPS = 0.36 ± 0.21.

Earlier work that examined the effects of siRNA-mediated MyD88 reduction in RAW 264.7 cells demonstrated that siRNA significantly reduced MyD88 protein levels, but only within the first 24 h following transfection [20]. Like adenovirus transductions, siRNA transfections were also toxic to the cells and produced significant cell death over longer periods. Thus, when considering the optimal approach to gene knockdown in RAW 264.7 cells, researchers should consider that, across the three methods siRNA, CRISPR/Cas9, and adenovirus, the CRISPR/Cas9 vector is better-suited for relatively longer-term studies where knockdown must be achieved quickly and maintained for an extended period of time.

In summary, we found that the responsiveness of MyD88-deficient RAW 264.7 cells to an inflammatory stimulus differed, depending on whether MyD88 was reduced by CRISPR/Cas9 transfection or by adenoviral transduction. CRISPR/Cas9-transfected cells were responsive to LPS, and the lack of MyD88 was associated with reduced TNFα and IL-10 secretion. Conversely, adenovirally-transduced cells were unresponsive to LPS stimulation, indicating that adenoviral transduction confers a degree of resistance to LPS challenge under these conditions.

Given these observations, the CRISPR/Cas9 method may lead to a more systemic incorporation of the gene of interest and therefore, the intended changes, with fewer unexpected consequences in RAW 264.7 cells.

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Supplementary information

Figure S1. Adenoviral transduction mechanism.
Figure S2. CRISPR/CAS9 mechanism.
Supplementary information of this article can be found online at https://jbmethods.org/jbm/article/view/359.