Murine mammary carcinoma cells and CD11c+ dendritic cells elicit distinct responses to lipopolysaccharide and exhibit differential expression of genes required for TLR4 signaling

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Abstract

Although TLR are often studied on DC because of their ability to bridge innate and adaptive defenses, TLR are also expressed by epithelial cells. Because the majority of cancers are carcinomas, and thus of epithelial origin, we wanted to know whether a carcinoma and DC responded similarly to a TLR agonist. We found the mammary carcinoma 4T1 and CD11c+ DC both secreted proinflammatory chemokines in response to the TLR4 agonist lipopolysaccharide (LPS). However a clear dichotomy existed. DC, but not 4T1 secreted IL-1β, TNF-α, and upregulated CD80 and CD86 expression following LPS treatment. A potential reason for differential responsiveness was that DC expressed greater levels of TLR4, CD14, Myd88, and TRAM. Despite the low level of TLR signaling proteins, the carcinoma were able to elicit a range of responses contingent upon the source, dose, length, and frequency of TLR agonist treatment. Thus, carcinoma and DC are distinctly responsive to LPS.

Keywords

Dendritic cells; Tumor cells; Toll-like receptors; LPS

1. Introduction

TLR have a domain of leucine-rich repeats, a toll/interleukin 1 (IL-1) receptor (TIR) domain, and exist either on the cell surface or in intracellular compartments of white blood cells, fibroblasts, and epithelial cells [1,2]. Different TLR generally recognize pathogens through pathogen-associated molecular patterns (PAMPs). For example, TLR4 binds lipopolysaccharide (LPS), a key cell wall component of gram-negative bacteria; TLR3 recognizes double-stranded RNA from viruses; and TLR5 detects bacterial flagellin [3]. These receptors may function alone such as with TLR5 for flagellin, together such as with TLR2/TLR6 for diacyl lipopeptide recognition, or with additional proteins such as with TLR4 in association with MD-2 (Ly96), and CD14 for LPS recognition [1,3]. Downstream of these receptors signaling cascades converge, promoting expression of inflammatory cytokines and type I interferons (IFN) the purpose of which are to assist in initiating a response against a pathogen [1–3]. Indeed, TLR signaling has been implicated in host protection by initiating

antibacterial and antiviral responses [4,5]. However, some detrimental effects have also been associated with TLR signaling such as with atherosclerosis and colitis [6,7]. As a result, there is a concerted effort to delineate the extent to which different cells respond to different TLR agonists.

Since DC are capable of bridging innate and adaptive defenses there is great interest in characterizing how TLR agonists impact the capabilities of these cells. Toward this aim investigators have explored how TLR agonists directly impact DC. For instance, Messmer et al., [8] reported that LPS was capable of inducing DC maturation resulting in MHC Class II, CD80, and CD86 expression. Shen et al., [9] explored the signaling cascade induced in DC by LPS, and reported that Myd88 dependent and independent signaling was required. Additionally, investigators have explored how TLR agonist treated DC impact subsequent adaptive immune responses. For example, Wiethe et al., [10] reported that LPS and anti-CD40 treated DC, but not TNF-α treated DC, resulted in a Th1 response that assisted in controlling a *Leishmania major* infection, and Sato and Iwasaki [11] reported that an effector T cell response to herpes virus required TLR on stromal cells as well as DC. Because DC are differentially responsive to multiple distinct TLR agonists, there is also a growing interest in deciphering the cooperation that exists among TLR signaling cascades [12–15]. Thus, much remains to be learned with regard to DC and TLR.

Since epithelial cells express TLR, are responsive to TLR agonists [16–18], and since most tumors are epithelial in origin [19], there is great interest in characterizing how TLR agonists impact the capabilities of these cells. For instance, TLR agonists have been reported to slow tumor growth or progression [20,21]. Hirabayashi et al., [20] reported that a TLR3 agonist could slow the growth of several different breast cancer cell lines in vitro, and there are a number of instances in which TLR7 and TLR8 agonists were beneficial in fighting cancer in vitro and in vivo [21]. On the other hand, in some cases TLR agonists may augment tumor growth or progression. For instance, LPS has been reported to influence β1 integrin expression [22], stimulate endothelial sprouting [23], and signaling through TLR4 has been shown to increase motility of melanoma cells in vitro [24]. Such studies suggest that TLR agonists may influence angiogenesis or metastasis. Because of these findings, and because TLR agonist treated DC are also being explored to fight cancer [25], we were interested in comparing the responsiveness of DC and tumor cells to a TLR agonist.

For these reasons we began comparing responsiveness of CD11c^{+} bone marrow derived DC and 4T1 murine mammary carcinoma cells to LPS. We found that genes encoding multiple TLR were expressed in each type of cell, and that both types of cells secreted the proinflammatory chemokines CCL2 and CXCL1 in response to LPS. Yet, DC alone secreted IL-1β, TNF-α, and upregulated CD80 and CD86 expression following LPS treatment. Analysis of gene expression revealed that *TLR4* and *CD14*, which are utilized for LPS recognition, and *Myd88* and *TRAM*, which are used immediately downstream of the LPS receptors, were expressed at greater levels by DC. In an attempt to determine whether 4T1 was capable of eliciting a greater response to LPS we explored varying treatment conditions. Indeed, despite low levels of TLR signaling proteins, 4T1 was able to make a range of LPS dependent responses. For instance, treatment with LPS from *E. coli* serotype 0128:B12 enhanced tumor growth rate while treatment with LPS from *E. coli* serotype 026:B6 decreased tumor growth rate. Yet, LPS from *E. coli* serotype 026:B6 was capable of enhancing tumor growth if treatment continued for 72 rather than 24 hours. Collectively, these data demonstrate that responses to TLR agonists are multifactorial in nature depending upon not only the level of TLR signaling proteins, but also the source of agonist, dose, length, and frequency of treatment.

2. Materials and methods

2.1. Cells and mice

4T1 murine mammary carcinoma cells were maintained in complete RPMI (cRPMI) (RPMI 1640, Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Lonza), glutamine (2 mM, Lonza), penicillin (100 U/mL, Lonza), streptomycin (100 μg/mL, Lonza), nonessential amino acids (Sigma, St. Lois, MO), 2-mercaptoethanol (5×10^{-5} M, Sigma), and sodium pyruvate (1 mM, Lonza). For in vivo analysis of TLR agonist treated tumor cells 1×10^6, 1×10^5, or 5×10^4 cells were cultured with or without TLR agonists in 75 cm^2 culture flasks for 24, 48, or 72 hours respectively. To determine whether frequency of treatments influenced tumor growth rate the cells were either treated one time with LPS or every 24 hours. After 24–72 hours the cells were harvested, counted, washed three times in sterile saline, and adjusted to 5×10^5 cells/ml. Next, 100 μl of cells were injected into the left hind flank of mice. Tumors were measured every 2 to 3 days and the volumes calculated (L x W^2/2). Mice were housed in a thoren caging system (Thoren Caging Systems Inc., Hazelton, PA). Food and water were provided *ad libitum*. All mice were used in accordance with an Institutional Animal Care and Use Committee approved protocol that followed the guidelines for ethical conduct in care and use of animals.

DC were generated from male OTII transgenic mice as previously described [12]. Briefly, bone marrow from the femurs and tibias were cultured at 1×10^6 cells/ml in cRPMI with GM-CSF (20 ng/ml, Peprotech, Rocky Hill, NJ) and IL-4 (10 ng/ml, Peprotech) with one ml/well in 24-well tissue culture dishes (Costar, Fisher). The media with GM-CSF and IL-4 was replaced every 2 to 3 days. Following 7 to 8 days, CD11c$^+$ DC were purified using CD11c microbeads and MS positive selection columns (Miltenyi Biotec, Auburn, CA). CD4$^+$ T cells were purified from spleens of male OTII mice using CD4 microbeads and magnetic cell separation (Miltenyi Biotec). Purity of the cells, which was $\geq 95\%$, was verified by flow cytometry.

2.2. TLR agonists

For TLR2 and TLR4 agonists peptidoglycan (PGN) from *Staphylococcus aureus* (Sigma), and LPS from *Escherichia coli* serotype 026:B6 (Sigma), *Escherichia coli* serotype 0128:B12 (Sigma), *Pseudomonas aeruginosa* serotype 10 (Sigma), *Salmonella enteritidis* (Sigma), or *Salmonella typhimurium* (Sigma) were used. Stocks were prepared in Hank's Balanced Salt Solution (HBSS, Lonza), aliquoted, and stored at −20°C in sterile microcentrifuge tubes. PGN was used at a final concentration of 5μg/ml, and LPS was used at a final concentration of 1, 10, or 100 ng/ml. An equal volume of HBSS was used as a negative control.

2.3. DC antigen presentation

Cytokine release and proliferation assays were used to determine whether DC were competent antigen presenting cells. For this purpose 100 μL of CD11c$^+$ DC at 2×10^6 cells/ml were added to 96-well flat bottom plates (Falcon). The DC were pulsed with 0.001, 0.01, 0.1, or 1.0 μM ovalbumin (OVA) peptide (ISQAVHAAHAEINEAGR, Sigma), or an equivalent volume of HBSS, and incubated for 2 hours at 37°C, 5% CO$_2$. Next, 100 μL of OVA specific CD4$^+$ T cells from OTII mice at a concentration of 1×10^6 cells/ml were added to each well and the cells were incubated at 37°C, 5% CO$_2$ for 48 hours. Eighteen hours before analysis the cells were pulsed with 110 μM 5-bromo-2′-deoxy-uridine (BrdU, Roche Diagnostics, Mannheim, Germany).

To assess cytokine release, supernatants were harvested after 48 hours, centrifuged at 450 x g to remove particulate materials, and stored at −20°C for ELISA. The samples were assayed for IFN-γ using a Quantikine Sandwich ELISA (R&D Systems, Minneapolis, MN).

Proliferation was assessed using a non-radioactive proliferation assay according to manufacturer's instructions (Roche Diagnostics). Briefly, the 96-well plates containing the cells were centrifuged for 8 minutes at 450 x g, and allowed to dry for 2 hours at 60°C. The cells were fixed with 200 μL of precooled (−20°C) fixative/well and washed 3 times with 200 μL of wash medium (phosphate buffered saline (PBS) + 10% bovine serum albumin (BSA))/ well. The cells were then incubated with 100 μL of nucleases/well for 30 min. at 37°C, washed 3 times with 200 μL of wash medium/well, and 100 μL of anti-BrdU-POD was added to each well. Following a 30 min. incubation at 37°C, and 3 washes with 200 μL wash medium/well, 100 μL peroxidase substrate was added to each well and absorbance was read at 405 nm (reference wavelength 490 nm) at 30 min. on a microplate reader (Phenix Research Products, Hayward, CA).

2.4. TLR reverse transcriptase-PCR analysis

TLR expression by 4T1 and CD11c$^+$ DC was determined by RT-PCR. For analysis, mRNA was isolated (Oligotex direct mRNA micro kit, Qiagen, Valencia, CA) from 5×10^5 to 1×10^6 cells. All of the mRNA was precipitated, resuspended in 25 μl diethyl pyrocarbonate (Sigma) treated H_2O and used for cDNA synthesis. Complementary DNA was made with random primers (0.5 μg, Promega, Madison, WI), dithiothreitol (2 mM, Promega), dNTP (0.2 mM each, Promega) and M-MLV reverse transcriptase (200 units, Promega) in a 1 hour reaction at 42°C. An aliquot (3 μl) of cDNA was amplified in a reaction with 1.25 mM $MgCl_2$ (Promega), dNTP (0.2 mM each, Promega), specific primers (20 μM), and GoTaq Flexi DNA Polymerase (2.5 units, Promega). The reaction conditions consisted of 35 cycles of 94° C for 15 sec., 59°C for 30 sec., and 74°C for 45 sec., on a MJ Research thermocycler (MJ Research, Waltham, MA). The TLR specific primers, which have been previously described [12], were synthesized by Integrated DNA Technologies (Coralville, IA) and analyzed for specificity with the NCBI Blast Program. PCR products were analyzed on a 2% agarose gel using the Alpha Innotech Gel Documentation System (Alpha Innotech Corp., San Leandro, CA).

2.5. ELISA

To determine whether TLR agonist treatment induced secretion of proinflammatory mediators, chemokine and cytokine specific ELISAs were used. For this purpose, 1 ml of 4T1 or CD11c$^+$ DC at 1×10^6 cells/ml were added to each well in 24-well tissue culture plates and treated with HBSS, LPS, or PGN as described above. After 24 hours, supernatants were harvested, centrifuged at $450 \times g$ to remove particulate materials, and stored at −20°C. For analysis, samples were assayed for CCL2, CCL5, CXCL1, IL-1β, and TNF-α using Quantikine Sandwich ELISAs (R&D Systems).

2.6. Flow cytometry

Flow cytometry was used to evaluate whether LPS modulated costimulatory molecule expression. For this purpose, 5×10^5 4T1 or CD11c$^+$ DC that were treated with HBSS or LPS for 24 hours at 37°C, 5% CO_2 were harvested, resuspended in labeling buffer ($1 \times$ PBS, 0.5% BSA, 2 mM EDTA), and phenotyped for CD80 (FITC) and CD86 (PE) expression. The antibodies were purchased from BD Pharmingen (San Diego, CA). Prior to antibody labeling, cells were incubated with Fc block (BD Pharmingen) for 15 min. on ice, washed, centrifuged, and resuspended in 1 mL labeling buffer. The cells were then aliquoted with 1 μg of antibody or isotype control, and incubated for 30 min. on ice. Following a wash with labeling buffer, the cells were fixed with 3.7% formaldehyde (Sigma) for 10 min. on ice, washed again, and resuspended in 0.5 ml HBSS. The samples were sent for analysis to the Cell Science Core Facility at Penn State College of Medicine, Hershey Medical Center (Hershey, PA).

2.7. Gene array analysis

Gene arrays were used to examine gene expression profiles of 4T1 and DC before and after LPS exposure. For this purpose Oligo GEArray Mouse Toll-like Receptor Signaling Pathway Microarrays (SuperArray Bioscience Corp., Frederick, MD) were used. First, total RNA was isolated from 1×10^6 to 3×10^6 4T1 or CD11c$^+$ DC that were treated with HBSS or LPS for 24 hours at 37°C, 5% CO_2 using the ArrayGrade Total RNA Isolation Kit (SuperArray Bioscience Corp.). The 260/280 for all RNA samples was at least 1.80. Identical amounts of total RNA (1.6–2.5 μg) isolated from the cells were used to make complementary DNA, and subsequently complementary RNA (cRNA) was synthesized, labeled, and purified using the TrueLabeling-AMP 2.0 kit (SuperArray Bioscience Corp.) according to manufacturer's instructions. The labeling reaction using 10 mM biotinylated-UTP (Perkin Elmer, Waltham, MA) was carried out for 4 hours. Purified biotinylated cRNA samples had a 260/280 of at least 1.80.

Hybridizations were performed according to manufacturer's instructions (SuperArray Bioscience Corp.) on 4 arrays at a time. Briefly, following a 2 hour pre-hybridization, 2 μg of biotinylated cRNA isolated from 4T1 +/− LPS or DC +/− LPS was added to 0.75 mL hybridization solution, and added to the arrays which were left to hybridize overnight at 60°C with 5 rpm continuous rotation in a hybridization oven (VWR, Bristol, CT). Following 15 min. washes with wash solution I (0.1 M SSC, 0.05 M SDS) and then wash solution II (0.005 M SSC, 0.025 M SDS), the arrays were cooled to room temperature, developed with streptavidin alkaline phosphatase, and chemiluminescence was captured using a CCD camera (Alpha Innotech). AlphaEase FluroChem 8800 software (Alpha Innotech) was used to record the developing arrays and for densitometric analysis. All data were normalized to the housekeeping gene *GAPDH*.

2.8. Quantitative reverse transcriptase PCR

Expression of select genes were analyzed by QRT-PCR. First, total RNA was isolated from 1×10^6 to 3×10^6 4T1 or CD11c$^+$ DC using the ArrayGrade Total RNA Isolation Kit (SuperArray Bioscience Corp.). The 260/280 for all RNA samples was at least 1.80. Messenger RNA was enriched from total RNA using a mRNA isolation kit (Invitrogen, Carlsbad, CA). All of the mRNA was precipitated, resuspended in 25 μl DEPC treated H_2O, and used for cDNA synthesis. Complementary DNA was generated as described above in a 1 hour reaction at 42°C. An aliquot (1 μl) of cDNA was amplified in a reaction with 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, Richmond, CA), and 200 nM gene specific primers. Each reaction was run in duplicate or triplicate. The reaction conditions consisted of 40 cycles of a two-step PCR reaction with 94°C for 10 sec., and 68°C for 30 sec., on an iQ5 Real Time PCR Detection System (Bio-RAD). Gene specific primers used included gapdh left 5′-ctt ccg tgt tcc tac ccc caa tgt-3′, gapdh right 5′-gcc tgc ttc acc acc ttc ttg atg t-3′, TLR4 left 5′-agt gcc ccg ctt tca cct ctg-3′, TLR4 right 5′-caa taa cct tcc ggc tct tgt gga-3′, TRAM left 5′-tca ggc cgg gga tcg ttt tc-3′, TRAM right 5′-cga ccc att gac cgc atc gt-3′, MyD88 left 5′-cct gac ccc act cgc agt ttg t-3′, MyD88 right 5′-tgc gcg act tca gct cct tca-3′, TIRAP left 5′-ttg cac cat acc cct gct gtc c-3′, TIRAP right 5′-aaa agc ctc cgt cct tgc ctc t-3′, CD14 left 5′-tgt cgt ggg caa caa ggg atg-3′, CD14 right 5′-aag gtg gag agg gca ggg aag a-3′. The primers were synthesized by Integrated DNA Technologies (Coralville, IA) and analyzed for specificity with the NCBI Blast Program. Standard curves were used to examine efficiency and reproducibility of each reaction, and melt curves were used to validate amplification of single products. The housekeeping gene *GAPDH* was used to establish relative expression ($\Delta\Delta C_T$).

2.9. Western blot

To analyze expression of TLR signaling proteins 5×10^6 tumor cells and CD11c$^+$ DC were washed 3 times with 10 mL ice-cold PBS, and transferred to microcentrifuge tubes. The cells

were resuspended in 150 μL of buffer A (10 mM Hepes (Sigma), 10 mM KCl (Sigma), 0.1 mM EDTA (Sigma) and 0.1 mM EGTA (Sigma)) supplemented with the protease inhibitors aprotinin, leupeptin, chymostatin, and pefabloc (Roche Molecular Biochemicals, Indianapolis, IN) and placed on ice. Following a 15 min. incubation, 10 μL of 10% Nonidet P-40 (Sigma) was added. The samples were vortexed for 10 sec., and centrifuged at 15,000 x g at 4°C for 1 minute. NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) and dithiothreitol (1x, New England Biolabs, Ipswich, MA) were added and the samples were stored at −20°C. SDS PAGE gels (12.5%, Invitrogen) were loaded with 15 μL of proteins, electrophoresed, and transferred to Invitrolon polyvinylidene difluoride (PVDF) membranes (Invitrogen). The membranes were blocked at 4°C in PBS with 5% powdered milk and 0.05% Tween 20 (Sigma) overnight. Primary antibodies (10 μg) specific for actin, TLR4, Myd88, TRAM, CD14, or Tirap (Santa Cruz Biotechnology, Santa Cruz, CA) were added, and the blots were incubated at room temperature for 3 hours. After washing 2 times with blocking buffer, a horse-radish-peroxidase-conjugated secondary antibody (Santa Cruz) was added and the blots were incubated for 1 hour at room temperature. Following 4 washes, proteins were visualized by enhanced chemiluminesence on an Alpha Innotech Gel Documentation System.

3. Results

3.1. 4T1 and CD11c$^+$ DC express genes encoding TLR2 and TLR4

Prior to comparing the extent to which 4T1 tumor cells and bone marrow derived CD11c$^+$ DC differed in responsiveness to TLR agonists we needed to identify TLR expressed by both cell types. For this purpose we screened *TLR1* to *TLR9* expression by RT-PCR. Analysis of 4T1 tumor cells revealed that *TLR2*, *3*, *4*, *5*, and *9* were consistently expressed with *TLR2* and *TLR4* expressed at the greatest levels (Fig. 1A). To validate function prior to analyzing TLR gene expression by DC, we characterized MHC expression, and ability to present antigen. The DC expressed high levels of MHC Class I, MHC Class II (data not shown), and were capable of eliciting antigen specific T cell proliferation and cytokine secretion (Fig. 1B). For these assays CD4$^+$ T cells were purified from spleens of OTII mice, and showed dose dependent IFN-γ secretion and proliferation when cocultured with OVA peptide pulsed DC (Fig. 1B) indicating that the DC were competent antigen presenting cells. With regard to TLR expression, these DC showed consistent expression of *TLR2*, *4*, *7*, *8*, and *9* with *TLR2* and *TLR4* expressed at the greatest levels (Fig. 1A). Collectively, these data showed that 4T1 and CD11c$^+$ DC expressed multiple genes encoding TLR, and that *TLR2* and *TLR4* were expressed by both types of cells.

3.2. 4T1 and CD11c$^+$ DC respond to TLR2 and TLR4 agonists

Next, we evaluated the response of 4T1 and CD11c$^+$ DC to TLR2 and TLR4 agonists using PGN and LPS respectively. For this purpose 4T1 and DC were treated with PGN, LPS, or HBSS as a control for 24 hours, supernatants were harvested and assessed for inflammatory chemokine and cytokine levels by ELISA (Fig. 2A). Both types of cells secreted CXCL1 and CCL2 in response to PGN and LPS treatment, with CXCL1 secretion induced to the greatest extent. Baseline CXCL1 secretion by 4T1 was 352 pg/ml, and secretion increased to 2,132 pg/ml following PGN treatment, and 2,593 pg/ml following LPS treatment (Fig. 2A). Baseline CXCL1 secretion by DC was 179 pg/ml, and secretion increased to 1,622 pg/ml following PGN treatment, and 1,511 pg/ml following LPS treatment (Fig. 2A). With regard to CCL5 secretion, 4T1 exhibited a modest response (less than two fold increase) to PGN and LPS treatment, whereas DC did not increase CCL5 secretion in response to either agonist (Fig. 2A). With regard to inflammatory cytokines, DC secreted IL-1β and TNF-α following TLR agonist treatment, with TNF-α secretion induced to the greatest extent. Baseline TNF-α secretion by DC was 108 pg/ml, and secretion increased to 2,638 pg/ml following PGN treatment, and 2,558 pg/ml following LPS treatment (Fig. 2A). 4T1 secreted no detectable IL-1β or TNF-α in

response to the TLR agonists at any of the doses examined (PGN 0.5 to 50 μg/ml, LPS 1 to 100 ng/ml), or at any of the time points examined (24, 48, 96 hours, data not shown).

Since TLR agonists are known to induce DC maturation which concomitantly results in increased costimulatory molecule expression, we next asked whether CD80 and CD86 expression were modulated by TLR agonist treatment. For this purpose we looked at CD80 and CD86 expression on 4T1 and DC before and after treatment with LPS. 4T1 showed no detectable costimulatory molecule expression before or after LPS treatment, whereas DC upregulated both CD80 and CD86 expression following LPS treatment (Fig. 2B). Since 4T1 made a greater response to LPS than PGN, at least with respect to chemokine secretion, we did not assess whether PGN treatment upregulated expression of the costimulatory molecules. Thus, in response to TLR2 and TLR4 agonists, 4T1 secreted the chemokines CXCL1, CCL2 and CCL5, and DC secreted CXCL1, CCL2, IL-1β, TNF-α, and upregulated CD80 and CD86 expression. Collectively, these data revealed that although both 4T1 tumor cells and bone marrow derived CD11c^{+} DC were capable of responding to TLR agonists, they did so in distinctive manners.

3.3. Gene array analysis confirms 4T1 and CD11c^{+} DC are distinctly responsive to LPS

To further elucidate the differential responsiveness of tumor cells and DC to LPS we treated each cell type with the TLR agonist and used gene arrays to assess expression of genes important for TLR responses. We discuss only those genes that exhibited a greater than two-fold change in expression.

Overall, LPS modulated gene expression to a greater extent in DC than 4T1 (Tables 1 and 2). The DC response included an increase in expression of *CD80* and *CD86* (Table 1) correlating with the changes in protein expression shown by flow cytometry (Fig. 2B). With regard to inflammatory mediators, *CXCL10* (IP-10) exhibited the greatest induction of expression (21.8 fold induction), followed by *IL-12β* (p40 subunit, 14.0 fold induction), then *Clecsf9* (11.5 fold induction), *IL-6* (11.0 fold induction), and *IL-1β* (7.1 fold induction) (Table 1). Several genes that encode signaling proteins were also modulated by LPS including *IRF3*, *IRF7*, and *PRKR* with a 6.5, 24.9, and 4.8 fold induction of gene expression respectively (Table 1). *TLR3* was the only gene to show a reduction (3.4 fold) in expression by DC following LPS treatment (Table 1).

4T1 showed more modest changes in gene expression following LPS treatment. Only *CCL2* and *G-CSF* increased in expression (both 3 fold induction), whereas five genes showed reduced expressing following LPS treatment (Table 2). The reductions in gene expression were also modest with *Btk*, *IFN-α*, *Ly64*, *Mapk11,* and *TLR6* all exhibiting two-fold reductions (Table 2).

Next, we compared expression of genes downstream of Myd88 and TRIF. Both *IL-1β* and *IL-12β*, gene products downstream of Myd88, were expressed at greater levels by DC following LPS treatment; expression increased 7.1 and 14.0 fold respectively (Table 1). Both *IFN-β* and *CXCL10,* gene products downstream of TRIF, were expressed at greater levels by DC following LPS treatment; expression increased 2.8 and 21.8 fold respectively (Table 1). In contrast, these genes were not upregulated in 4T1 following LPS treatment. Thus, in response to LPS treatment, of the 113 genes assessed with the arrays two genes showed elevated expression and five genes showed reduced expression by 4T1, whereas eighteen genes showed elevated expression and one gene showed reduced expression by DC. Moreover, none of the genes were modulated in the same way by the tumor cells and DC. Collectively, these data confirmed that tumor cells and DC respond in distinct manners to LPS.

3.4. Comparison of TLR signaling potential between 4T1 and CD11c⁺ DC

To determine whether TLR signaling cascades differed between the tumor cells and DC we compared expression of genes involved in TLR signaling. Genes encoding surface receptors and co-receptors that were analyzed with the arrays, and known to be involved in LPS signaling, included *TLR4* and *CD14*. Based upon array analysis *TLR4* showed 3.5 x greater expression by DC; the integrated density values (IDV) for *TLR4* were 0.049 +/− 0.01 for DC, and 0.014 +/− 0.00 for 4T1 (Table 3). *CD14* showed 11.4 x greater expression by DC, the IDV for *CD14* were 0.182 +/− 0.09 for DC, and 0.016 +/− 0.00 for 4T1 (Table 3).

Within the Myd88 dependent pathway *TIRAP*, *Myd88*, *IRAK4*, *TRAF6*, and *TAK1* were analyzed. Although each gene was detected from both cell types, two of the genes (*TIRAP* and *TAK1*) were expressed at greater levels by the tumor cells, two (*Myd88* and *TRAF6*) were expressed at greater levels by DC, and one (*IRAK4*) was expressed at similar levels by both (Table 3). Downstream of TAK1 are the MAPK and NFκB pathways. Since the arrays included many different NFκB and MAPK family members we focused on members of these pathways that were expressed at the greatest levels by both cell types. For NFκB the genes encoding p50 and p65 subunits were expressed at the greatest levels by both 4T1 and DC, and DC showed slightly higher levels of expression of these genes (Table 3). For the MAPK members we compared a MKKK (*MEKK1*), a MKK (*MKK3*), and a MAPK (*MAPK11*). Although *MAPK11* was expressed at similar levels between the cell types, *MEKK1* showed greater expression by the DC, and *MKK3* was expressed at greater levels by the tumor cells (Table 3).

Within the Myd88 independent (TRIF dependent) pathway *TRAM*, *TRIF*, *TBK1*, and *IRF3* were analyzed. Although each gene was detected from both cell types, three of the genes (*TRIF*, *TBK1*, and *IRF3*) were expressed at greater levels by the tumor cells, and one (*TRAM*) was expressed at greater levels by DC (Table 3). Based upon array analysis *TRAM* showed 3.4 x greater expression by DC, the IDV for *TRAM* was 0.017 +/− 0.00 for DC, and 0.005 +/− 0.00 for 4T1 (Table 3). Collectively, these data revealed that *TLR4*, *CD14*, *Myd88*, and *TRAM*, genes encoding proteins important at the beginning of the TLR signaling pathway, were expressed at greater levels by DC.

3.5. Comparison of TLR signaling potential using QRT-PCR and western blot

In order to validate some of the differences that were evident with the arrays we used QRT-PCR and western blot. Results which were in agreement with the array data included greater expression of *TLR4*, *CD14*, *Myd88*, and *TRAM* by the DC, and greater expression of *TIRAP* by 4T1 (Fig. 3A). Western blot analysis revealed that the differences observed at the RNA level were also evident at the protein level (Fig. 3B). Collectively, these data showed that expression of genes and proteins important for LPS recognition and response differed between the DC and tumor cells, with many of the genes and proteins expressed at greater levels by the DC.

3.6. Tumor cells are capable of eliciting varying responses to LPS

Because it is well established that DC respond to TLR agonists, and because of the poor responsiveness (Table 2), and the low level of TLR signaling proteins expressed by 4T1 relative to DC (fig. 3), we felt compelled to further explore the extent to which TLR agonist could influence the tumor cells. Thus, to explore whether the tumor cells were capable of making a greater response to LPS we used different sources of LPS, and varied the doses, lengths, and frequencies of treatment. To begin with we treated 4T1 with LPS from *E. coli* serotype 0128:B12, *E. coli* serotype 026:B6, *Pseudomonas auruginosa*, *Salmonella enteritidis*, and *Salmonella typhimurium* (Fig. 4A). Surprisingly, the greatest differences were found between the tumors treated with LPS from two different serotypes of *E. coli*. While treatment with 1, 10, and 100 ng/ml of LPS from *E. coli* serotype 0128:B12 induced secretion of 928, 1,366, and

1,607 pg/ml of CCL2 respectively, treatment with LPS from E. coli serotype 026:B6 induced secretion of 444, 1,014, and 1,132 pg/ml of CCL2 respectively (Fig. 4A). Moreover, LPS from different gram (−) bacteria exerted differential effects on tumor growth rates. We compared the growth rates of tumors following treatment with 1, 10, and 100 ng/ml of the 5 different types of LPS (Fig. 4B and data not shown). Again the greatest differences were evident upon treatment with LPS from the 2 different serotypes of *E. coli*. Whereas treatment with 1ng/ml of LPS from *E. coli* serotype 0128:B12 caused a modest increase in tumor growth rate relative to untreated 4T1, treatment with 1 ng/ml of LPS from *E. coli* serotype 026:B6 resulted in a modest, but significant, decrease in tumor growth relative to untreated 4T1 (Fig. 4B). After 25 days of tumor growth control 4T1 tumors averaged 1,442 mm^3, tumors treated with LPS from *E. coli* serotype 0128:B12 averaged 1,614 mm^3, and tumors treated with LPS from *E. coli* serotype 026:B6 averaged 1,175 mm^3. The growth rates of tumors treated with the different serotypes of *E. coli* were significantly different at days 18 to 25. These data indicate that LPS from different gram (−) bacteria, as well as different serotypes of the same species, can induce different levels of chemokine secretion and differentially influence tumor growth rate.

Next, we evaluated LPS from *E. coli* serotype 026:B6 further. While treatment with 1, 10, and 100 ng/ml of LPS resulted in a decreased growth rate relative to untreated 4T1 (Fig. 4C), differences among 4T1 treated with different doses of LPS were not significantly different. Yet varying length and frequency of treatment resulted in significant differences (Fig. 4D). While treatment with 1 ng/ml for 24 hours resulted in a modest, but significant decrease in tumor growth relative to untreated 4T1, the effect was lost if treatment continued for 72 hours (Fig. 4D). After 25 days of tumor growth the tumors that were treated with 1ng/ml of LPS for 24 hours averaged 1,175 mm^3, whereas the tumors that were treated for 72 hours averaged 1,439 mm^3, the same size as untreated 4T1. Moreover, if treatment occurred every 24 hours for 72 hours, a modest, but significant increase in tumor growth occurred (Fig. 4D). After 25 days of growth tumors that were treated with 1ng/ml of LPS every 24 hours for 72 hours averaged 1,789 mm^3. The differences between growth rates of tumors treated for 24 hours 1 time and 72 hours 3 times, and the differences between the growth rates of tumors treated for 72 hours 1 time and 72 hours 3 times were significantly different at days 18 to 25. Thus, whether a tumor shows increased or decreased growth following TLR agonist treatment depends upon not only the source of TLR agonist, but also the dose, length and frequency of treatment.

4. Discussion

While some studies have explored how different types of cells respond at the genetic level to TLR agonists [8,26,27], the ultimate aim of this study was to enhance the understanding of the way a tumor cell responds to a TLR agonist by comparing it to the way a professional antigen presenting cell responds to the same agonist, and by examining the response to varying treatment conditions. Although both types of cells were capable of secreting proinflammatory chemokines in response to LPS, there were stark differences in secretion of proinflammatory cytokines and expression of the costimulatory molecules CD80 and CD86. Gene expression analysis suggested that DC and 4T1 may exhibit differential responsiveness to LPS because of differences in TLR signaling capabilities; TLR4, CD14, Myd88, and TRAM were expressed at greater levels by DC than 4T1. However, beside the differential expression of these proteins, there were other differences between the DC and tumor cells revealed in this study.

Some of the genes that encode TLR signaling proteins (*TAK1*, *MKK3*, and *IRF3*) were expressed at greater levels by the tumor cells than by the DC. To determine whether expression of these genes increased in the DC following LPS treatment, which may facilitate LPS responsiveness, we compared expression before and after LPS treatment. Expression of *TAK1*, *MKK3*, and *IRF3* increased following treatment of DC with LPS (data not shown) suggesting that the lower baseline level of expression in the DC did not impair LPS

responsiveness. These data also suggest that the greater level of expression of these genes by the tumor cells did not negatively influence LPS responsiveness. On the contrary, there may be other reasons for the elevated expression of these genes by the tumor cells. For example, TGF-β is often expressed by cancer cells, and MKK3 and TAK1 are involved in the TGF-β signaling cascade [28,29]. Also, Neil and Schiemann [30] recently reported that TAK1 was required for the ability of TGF-β to foster tumor growth. Thus, elevated *MKK3* and *TAK1* expression may be a consequence of TGF-β expression and autocrine signaling by the tumor cells. *TIRAP* and *IRF3* are other examples of genes that were expressed at greater levels by the tumor cells, the results of which may be unrelated to TLR signaling. For instance, TIRAP is negatively regulated by caspase-1 which is also used for activating IL-1β and IL-18 [31]. Thus, low *TIRAP* expression by DC may be related to the expression of caspase-1 necessary for proinflammatory cytokine secretion by these cells. An inverse correlation between IRF3 and A20 expression has also been previously reported [32]. Thus, the greater level of *IRF3* expression by 4T1 may be related to the lower level of *A20* expression in these cells compared to the DC (data not shown). Accordingly, the greater levels of *TIRAP*, *TAK1*, *MKK3*, and *IRF3* expression by 4T1 compared to DC may be unrelated to LPS responsiveness. Moreover, there have been no reports that these proteins exert a negative influence on TLR signaling.

Although the tumor cells were less responsive than DC to LPS, CXCL1 and CCL5 secretion by the tumor cells in response to LPS may be indicative of Myd88 dependent and TRIF dependent responses respectively since secretion of CXCL1 has been reported to be Myd88 dependent, and CCL5 secretion has been reported to be TRIF dependent [4,33]. In particular, CXCL1 production by macrophages following treatment with LPS was dependent upon Myd88 [33], and CCL5 production by macrophages following exposure to *Pseudomonas aeruginosa* was dependent upon TRIF [4]. Another potential explanation for the differential responsiveness of these cells to LPS may be related to TGF-β expression by the tumor cells. Naiki et al., [34] reported that TGF-β can inhibit Myd88 expression. However, we have explored this possibility using siRNA targeting TGF-β and found that neither the level of Myd88 nor the tumor cell's response to LPS were altered (data not shown).

It is also necessary to investigate a wider range of treatment conditions. For instance, while we could detect mRNA encoding IL-6 after 24 hours of treatment with LPS, we could not detect IL-6 protein secretion until 96 hours later (data not shown). Yet, Huang et al., [35] reported that tumor cells secreted IL-6 after 24 hours of LPS treatment. In that study however, the authors used a different tumor cell line (MC26) and a higher dose of LPS (1,000 ng/ml) [35]. These data are reminiscent of some of the early studies with LPS in tumor settings that showed success or failure was dependent upon the dose, route, and timing of LPS delivery [36,37]. Thus, further evaluating a larger range of LPS doses and a time dependent analysis of gene expression would be valuable in deciphering the full extent of LPS responsiveness by the tumor cells. Such studies may also further elucidate why the tumor cells and DC respond differently to the same TLR agonist.

Another complicating factor in unraveling why DC and tumor cells are differentially responsive to LPS is the wide variety of DC and tumor cells which exist. While extending this type of investigation to other subsets of DC as well as other types of tumor cells would help determine the overall implications of our findings, there remains much to be done with the CD11c^{+} DC and 4T1 tumor cells used in this study. For instance, in order to validate a role for TLR4, CD14, Myd88, and TRAM in the differential responsiveness of the cells to LPS, siRNA can be used to drive down expression of these genes in DC, or the genes may be over expressed by the 4T1 tumor cells prior to monitoring responsiveness to LPS. Additionally, the differences we observed could be attributed to the different genetic backgrounds of the cells since 4T1 was derived from Balb/C3H mice, while DC were from OTII mice. Exploring whether the genetic background influences responsiveness to TLR agonists warrants investigation and may help

explain results obtained using TLR agonists in different model systems. Finally, if one is to fully understand the implications of TLR agonists in a tumor setting, analysis of the agonists must extend beyond different DC subsets, different types of tumor cells, and genetic backgrounds to include characterizing stromal cell responsiveness as well. The practicality of intratumoral delivery of TLR agonists will benefit from a clearer understanding of the ways in which these agonists impact different cell types. The more we understand about how these agonists differentially impact varying cell populations the better position we may be in for making more informed decisions about transitioning TLR agonists into a clinical setting.

Acknowledgments

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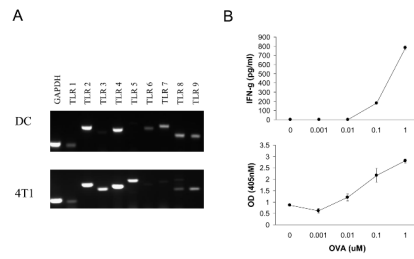
Fig. 1.
4T1 and CD11c⁺ DC express *TLR2* and *TLR4*. A. RT-PCR was used to assess *TLR1* to *TLR9* expression by 4T1 and CD11c⁺ DC. *GAPDH* was used as a positive control. B. Functional activity of CD11c⁺ DC. OVA specific CD4⁺ T cells were cultured with OVA peptide pulsed DC to evaluate the antigen presenting capability of the DC. T cell cytokine secretion and proliferation were assayed at 48 hours. The data show the average and standard deviation of triplicate wells from a representative of each experiment. All data are representative of three separate experiments.
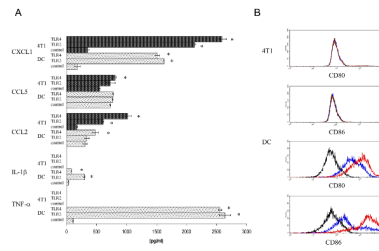
Fig. 2.
4T1 and CD11c^{+} DC are distinctly responsive to LPS. A. 4T1 and DC were treated with five µg/ml PGN (TLR2), ten ng/ml LPS (TLR4), or HBSS (control) for 24 hours. Supernatants were harvested and assayed for the indicated chemokines and cytokines by ELISA. The data are the average and standard error of three separate experiments. Where indicated (*) p< 0.05 using Student's *t*-test relative to control. B. 4T1 and DC were treated with ten ng/ml LPS (red), or HBSS (blue) for 24 hours and then labeled for CD80 and CD86 expression. The isotype control is shown in black. The histograms are representative of three separate experiments.
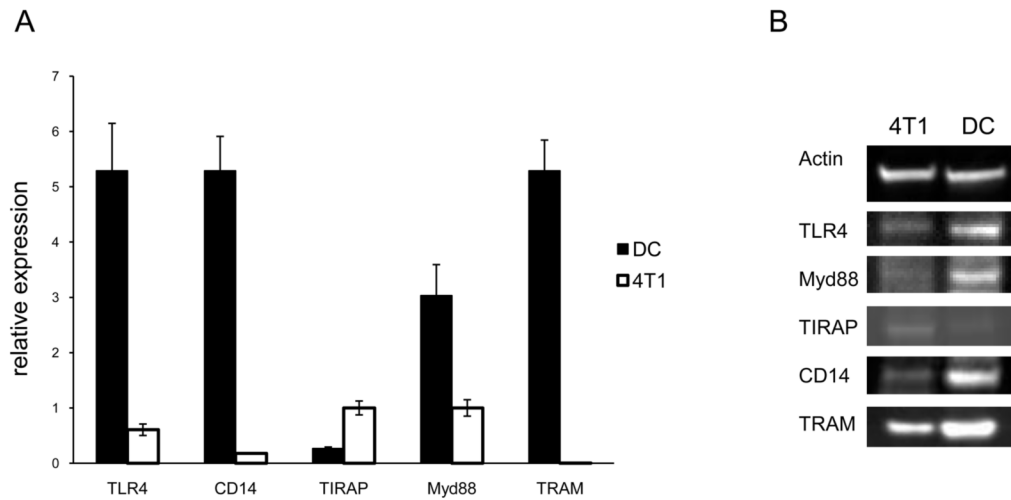
Fig. 3.
Comparison of TLR signaling potential using QRT-PCR and western blot. A. RNA was extracted from an equal number of 4T1 and DC for QRT-PCR. Relative expression was set to *GAPDH*. B. Proteins were isolated from an equal number of 4T1 and DC for western blot analysis. All data are representative of three separate experiments.
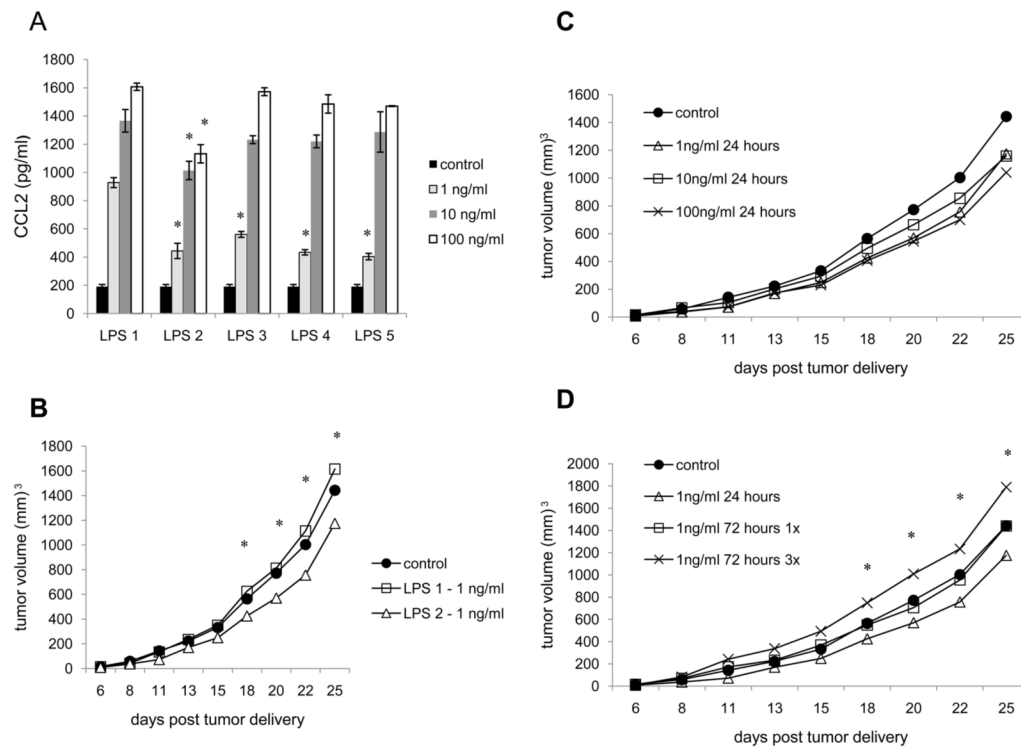
Fig. 4.
Responsiveness of tumor cells to LPS is dependent upon the source of agonist, dose, length, and frequency of treatment. A. 4T1 were treated with LPS from (1) *E. coli* serotype 0128:B12, (2) *E. coli* serotype 026:B6, (3) S*almonella enteritidis*, (4) *Salmonella typhimurium*, or (5) *Pseudomonas aeruginosa* and supernatants were harvested 24 hours later for chemokine analysis. B. 4T1 were treated with LPS from (1) *E. coli* serotype 0128:B12 or (2) *E. coli* serotype 026:B6 for 24 hours prior to injection into Balb/c mice. C and D. 4T1 were treated with LPS (2) from *E. coli* serotype 026:B6 prior injection into Balb/c mice. All data represent the average of three separate experiments. For in the vivo growth experiments 5 mice/group were used for each experiment. Where indicated (*) p< 0.05 using Student's *t*-test.

Table 1

Genes modulated in CD11c$^+$ DC by LPS.

Gene Name	DC control	DC + LPS	Expression ratio[b]
CD80	0.017 +/− 0.01[a]	0.056 +/− 0.02	**3.4**
CD86	0.119 +/− 0.07	0.377 +/− 0.11	**3.2**
Clecsf9	0.011 +/− 0.00	0.128 +/− 0.04	**11.5**
CXCL10	0.007 +/− 0.00	0.156 +/− 0.16	**21.8**
IFN-β	0.007 +/− 0.00	0.020 +/− 0.01	**2.8**
IL-12α (p35)	0.026 +/− 0.02	0.058 +/− 0.02	**2.2**
IL-12β (p40)	0.059 +/− 0.04	0.824 +/− 0.04	**14.0**
IL-1α	0.005 +/− 0.00	0.024 +/− 0.01	**4.6**
IL-1β	0.124 +/− 0.08	0.875 +/− 0.08	**7.1**
IL-6	0.020 +/− 0.00	0.224 +/− 0.03	**11.0**
IRF3	0.006 +/− 0.00	0.036 +/− 0.03	**6.5**
IRF7	0.039 +/− 0.02	0.981 +/− 0.06	**24.9**
Ly96	0.073 +/− 0.04	0.148 +/− 0.05	**2.0**
IκB-ε	0.031 +/− 0.01	0.067 +/− 0.03	**2.2**
PRKR	0.012 +/− 0.00	0.056 +/− 0.02	**4.8**
PTGES	0.005 +/− 0.00	0.015 +/− 0.00	**2.9**
COX2	0.005 +/− 0.00	0.059 +/− 0.02	**11.5**
RIPK2	0.014 +/− 0.01	0.033 +/− 0.01	**2.4**
TLR3	0.019 +/− 0.03	0.006 +/− 0.00	**− 3.4**

[a] Average and standard error of the integrated density value (IDV) from three separate experiments.

[b] Ratio of the IDV from DC treated with LPS/control.

Table 2

Genes modulated in 4T1 tumor cells by LPS.

Gene Name	4T1 control	4T1 + LPS	Expression ratio[b]
CCL2	0.036 +/− 0.01[a]	0.117 +/− 0.03	**3.3**
G-CSF	0.087 +/− 0.02	0.301 +/− 0.06	**3.5**
Btk	0.009 +/− 0.01	0.004 +/− 0.00	**− 2.1**
IFN-α	0.009 +/− 0.01	0.004 +/− 0.00	**− 2.5**
Ly64	0.008 +/− 0.01	0.004 +/− 0.00	**− 2.2**
Mapk11	0.011 +/− 0.01	0.004 +/− 0.00	**− 2.7**
TLR6	0.008 +/− 0.00	0.004 +/− 0.00	**− 2.0**

[a] Average and standard error of the IDV from three separate experiments.

[b] Ratio of the IDV from 4T1 treated with LPS/control.

Table 3

Relative expression of genes involved in the TLR signaling cascade.

Gene Name	DC	4T1	Expression ratio[b]
TLR4	0.049 +/− 0.01[a]	0.014 +/− 0.00	3.5
CD14	0.182 +/− 0.09	0.016 +/− 0.00	11.4
TIRAP	0.018 +/− 0.00	0.093 +/− 0.02	0.2
Myd88	0.123 +/− 0.01	0.078 +/− 0.01	1.6
IRAK4	0.013 +/− 0.00	0.013 +/− 0.00	1.0
TRAF6	0.016 +/− 0.00	0.008 +/− 0.00	2.0
TAKI	0.003 +/− 0.00	0.007 +/− 0.00	0.4
NF-kBp50	0.191 +/− 0.02	0.109 +/− 0.01	1.8
NF-kBp65	0.049 +/− 0.01	0.030 +/− 0.00	1.6
MEKK1	0.114 +/− 0.02	0.047 +/− 0.01	2.4
MKK3	0.197 +/− 0.02	0.618 +/− 0.07	0.3
MAPK11	0.138 +/− 0.02	0.142 +/− 0.02	0.9
TRAM	0.017 +/− 0.00	0.005 +/− 0.00	3.4
TRIF	0.004 +/− 0.00	0.006 +/− 0.00	0.7
TBK1	0.045 +/− 0.00	0.051 +/− 0.01	0.9
IRF3	0.006 +/− 0.00	0.010 +/− 0.00	0.6

[a]Average and standard error of the integrated density value (IDV) from three separate experiments.

[b]Ratio of the IDV from DC/4T1.