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Dominance of *E. coli* phagocytosis over LPS in the inflammatory response of microglia

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ABSTRACT

CNS bacterial infections are prevalent in neonates, the immune-compromised and elderly. During peripheral infections, macrophages employ multiple pattern recognition receptors (PRRs) to respond to pathogens, but less is known about brain microglia. We assessed microglial expression of PRRs, compared responses to whole *E. coli* and LPS, and tested the hypothesis that bacteria modulate the response to LPS. LPS increased the microglial phagocytic capacity, and changed expression of CD14, CR3, Fcgr1, Fcgr3a, TLR4, MARCO, MHCII, NOD2, TLR9 and SR-A, differently from stimulation with whole *E. coli*. Importantly, when added with LPS, *E. coli* dominated the microglial responses for 11/13 genes examined.

1. Introduction

Bacterial meningitis and inflammation are associated with high morbidity and mortality in infants, the immuno-compromised and the elderly (Russo and Johnson, 2003), and can cause white matter injury and cerebral palsy (Sherwin and Fern, 2005). Newborns are especially at risk, owing to increased exposure to Gram-negative *Escherichia coli* bacteria, an immature immune system and an incomplete blood brain barrier (BBB). Outside the CNS, invading micro-organisms encounter innate immune cells, which bear 'pattern recognition receptors' (PRRs) that can interact with 'pathogen-associated molecular patterns' (PAMPs), including lipopolysaccharide (LPS), peptidoglycan, flagellin, lipopeptides and unmethylated CpG DNA (Henderson et al., 1996). Within the CNS of healthy adults, the endogenous innate immune cell (microglial cell) is in a surveillance state with down-regulated effector functions, but microglia activate rapidly after cerebral or systemic infections (recently reviewed in Brodsky and Medzhitov, 2009; Carpentier et al., 2008; Kielian, 2009; Ransohoff and Perry, 2009). Less is known about the state of microglia in neonates or their responses to bacteria.

In peripheral macrophages, PRR engagement evokes release of cytokines and chemokines that promote an inflammatory response; the microbe is identified as foreign and dangerous, and phagocytosis is facilitated. After exposure to *E. coli*, macrophages produce reactive oxygen and nitrogen species and pro-inflammatory cytokines, and then the response must be modulated to co-ordinate resolution and repair (reviewed in Martinez et al., 2009; Ransohoff and Perry, 2009). High NO levels can facilitate bacterial invasion by increasing the BBB permeability (Buster et al., 1995), and bacterial PAMPs can also induce IL-10 and suppress immune responses, thus increasing bacterial survival (Brodsky and Medzhitov, 2009; Henderson et al., 1996; Wilson et al., 1998). In addition, although LPS normally induces fever to more effectively fight bacteria, this febrile response is reduced near birth and in neonates (reviewed in Mouihate et al., 2008), which is expected to exacerbate the infection.

It is important to consider that during *E. coli* infections, infiltrating macrophages and microglia within the CNS potentially encounter multiple bacterial PAMPs; however, the most commonly studied is LPS, which acts through Toll-like receptor 4 (TLR4). LPS has been applied to cultured neonatal microglia in numerous studies, but surprisingly little is known about interactions of microglia with whole *E. coli*. *E. coli* injected into the neonatal rat brain can be rapidly and efficiently phagocytosed (Kaur et al., 2004), and it was recently reported that TLR stimulation can increase *E. coli* phagocytosis and TNF-α production (Ribes et al., 2009).

Here, we asked whether the same inflammatory response is evoked in microglia exposed to whole *E. coli* and isolated LPS, and tested the hypotheses that bacterial phagocytosis is altered by the microglial activation state, and that *E. coli* can modulate the inflammatory response to LPS. Prior to stimulation, microglia expressed low levels of PRRs and inflammatory mediators. *E. coli* and LPS evoked different responses for CD14, CR3, Fcgr1, Fcgr3a, TLR4, MARCO, MHCII, NOD2, TLR9 and SR-A expression, and for production

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of NO and IL-10. Nevertheless, both stimuli employed p38 MAPK activation for induction of the inflammatory mediators, NO, TNF-α and IL-10. Importantly, when added with LPS, *E. coli* dominated the microglial response for 11/13 PRRs and inflammatory genes examined (iNOS, CR3 did not reach significance). These results implicate multiple PAMPs and PRRs in microglial responses to bacteria; hence, the overall CNS inflammatory outcome is expected to be more complex than LPS-mediated signaling through TLR4 alone.

2. Materials and methods

2.1. Microglial cultures and labeling

Microglia were isolated from brains of 1 day-old Sprague–Dawley rats (Charles River, St-Constant, Quebec, Canada), as previously described (Fordyce et al., 2005; Kaushal et al., 2007). Rat pups were sacrificed by cervical dislocation in accordance with guidelines from the Canadian Institutes of Health Research and the University Health Network. The cerebellum and surrounding meninges were removed, and the remaining brain tissue was mashed through a stainless steel sieve (100 mesh; Tissue Grinder Kit #CD-1; Sigma; Oakville, Canada), and then centrifuged (12 min, 1000 g), re-suspended and seeded into flasks with minimum essential medium (MEM; Invitrogen, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS; Wisent, St-Bruno, QC) and 100 μM gentamycin (Invitrogen). Two days later, cellular debris, non-adherent cells, and supernatant were removed and fresh medium was added. Microglia were harvested after 7–10 days by shaking flasks on an orbital shaker for 3–4 h (70 rpm, 37 °C, 5% CO_2). At this time, the supernatant was harvested, centrifuged (10 min, 1000 rpm), and the microglial pellet was re-suspended in MEM with reduced serum (2% FBS) to maintain a more resting state. This procedure yielded 99–100% microglia, as shown by labeling with FITC-conjugated tomato lectin (1:500; Sigma; Fig. 1A), isolectin B4, 'ionized Ca^{2+} binding adaptor-1'(Iba-1) or OX-42 antibodies (Kaushal et al., 2007; Ohana et al., 2009). For immunohistochemistry, microglia were fixed for 30 min in 4% para-formaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100, and then labeled overnight with rabbit polyclonal Iba-1 antibody (1:1000; Wako, Osaka, Japan), in blocking solution (PBS, containing 2% donkey serum). After washing (3×, 10 min each), microglia were labeled (2 h, room temperature) with a Cy3-conjugated secondary antibody (1:500; JacksonImmuno, West Grove, PA) and for 10 min with the nuclear stain, 4′,6′-diamino-2-phenylindole (DAPI; 5 μg/ml; Invitrogen). Photomicrographs were made with a Zeiss Axioplan 2 epifluorescence microscope and Axiocam digital camera (Zeiss, Thornwood, NY).

2.2. Phagocytosis of E. coli by neonatal rat microglia

For fluorescence imaging, 10^5 microglia were plated on 15 mm glass coverslips in 12-well plates; and for all other assays, 5×10^4 microglia/well were added to 96-well plates (both from VWR, Mississauga, ON) and incubated overnight before use. A 100 μl suspension of FITC-conjugated, heat-killed *E. coli* K12 bacteria (Invitrogen) in MEM with 2% FBS was added to each well and incubated for 1 h (37 °C, 5% CO_2). Then, 50 μl Trypan blue was added to quench the fluorescence of any *E. coli* that had not been phagocytosed (Hed et al., 1987). To quantify phagocytosis, fluorescence intensity (relative fluorescence units, RFU) was measured in each well at 480 nm excitation and 520 nm emission using a monochromatic plate reader (SPECTRAmax Gemini EM, Molecular Devices, Sunnyvale, CA). Phagocytosis was inhibited with 10 μM cytochalasin D, which interferes with actin polymerization.

In separate experiments, microglia were treated for 24 h with the following stimuli: 10 ng/ml lipopolysaccharide (LPS) from *E. coli* 0055:B5 (Alexis Biochemicals, San Diego, CA) or from *E. coli* K-235 (Sigma), 10 ng/ml IFNγ (Sigma), or the combination of LPS + IFNγ. We used a microglia:bacteria ratio (~1:150) that is consistent with a recent *in vitro* study of microglial infection by *E. coli* (Ribes et al., 2009). The 24 h incubation was chosen as the time at which there is significant microglial nitric oxide production after LPS treatment (Dello Russo et al., 2004; Nakamura et al., 1999). It was important to compare whole K12 *E. coli* to K-235 LPS, which is derived from a related strain, because the biological activity of LPS varies between different source strains of *E. coli* (see Discussion). The concentration of LPS we used (10 ng/ml) provides a reasonable comparison because there are several nanograms of LPS per million Gram-negative bacteria (Troelstra et al., 1999), and up to 100 ng/ml was not toxic to rat microglia (Fordyce et al., 2005; Kaushal et al., 2007) or macrophages (Wu et al., 2009). Previous studies have used LPS concentrations from 10 ng/ml to 10 μg/ml, and IFNγ from 10 to 100 ng/ml, but we found that the highest doses of either compound were cytotoxic in 24 h assays. Microglia toxicity from high LPS concentrations has been reported (von Zahn et al., 1997).

2.3. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Gene transcript levels were monitored using qRT-PCR on primary microglia (≥99% pure) as we have recently described (Ducharme et al., 2007; Kaushal et al., 2007; Ohana et al., 2009). 'Primer3Output' (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design gene-specific primers (Table 1). Total RNA was extracted using the TRIzol method (Invitrogen), followed by RNeasy Mini Kit (QIAGEN, Mississauga, ON) for further purification. A two-step reaction was performed according to the manufacturer's instructions (Invitrogen). In brief, total RNA (0.8 μg) was reverse transcribed in 20 μl volume using 200 U of SuperScriptII RNase reverse transcriptase, with 0.5 mM dNTPs and 0.5 μM oligo dT (Invitrogen). Amplification was performed on an ABI PRISM 7700 Sequence Detection System (PE Biosystems, Foster City, CA) at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 56 °C for 20 s. 'No-template' and 'no-amplification' controls were included for each gene, and melt curves showed a single peak, confirming specific amplification. The threshold cycle (C_T) for each gene was determined and normalized against the housekeeping gene, hypoxanthine guanine phosphoribosyl transferase (HPRT1).

2.4. Production of NO, TNF-α, IL-10, and role of p38 MAPK

Microglia were plated at 5×10^4 cells/well in 96-well plates and exposed for 24 h to *E. coli* K-12, LPS, IFNγ, or LPS + IFNγ, as described above. For each treatment, an average RFU was obtained from 3 wells of cells cultured from one animal, and multiple *n*s were obtained using cultures from different animals.

Nitric oxide production was measured as nitrite, using the colorimetric Griess assay according to the manufacturer's protocol (Invitrogen). That is, 100 μl of supernatant from each well was mixed with 10 μl 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 10 μl 1% sulfanilic acid (10 min, room temperature), and absorbance at 570 nm was quantified using a spectrophotometric plate reader (EL311SX, Bio-Tek Instruments, VT). Nitrite concentrations were determined by interpolation on a standard curve.

TNF-α and IL-10 were measured using enzyme-linked immunosorbent assays (ELISAs), as follows. High affinity 96-well microtitre plates (NUNC, Thermo Fischer, Rochester, NY) were coated with capture antibody (anti-TNF-α, ebioscience; anti-IL-10, BD Biosciences) and stored overnight at 4 °C. The plates were blocked for 2 h with heat-inactivated fetal bovine serum, and then standard solutions or sample supernatants were added, followed by 1 h incubation with a biotinylated secondary antibody and an Avidin–HRP detection enzyme. The enzymatic reaction was stopped with

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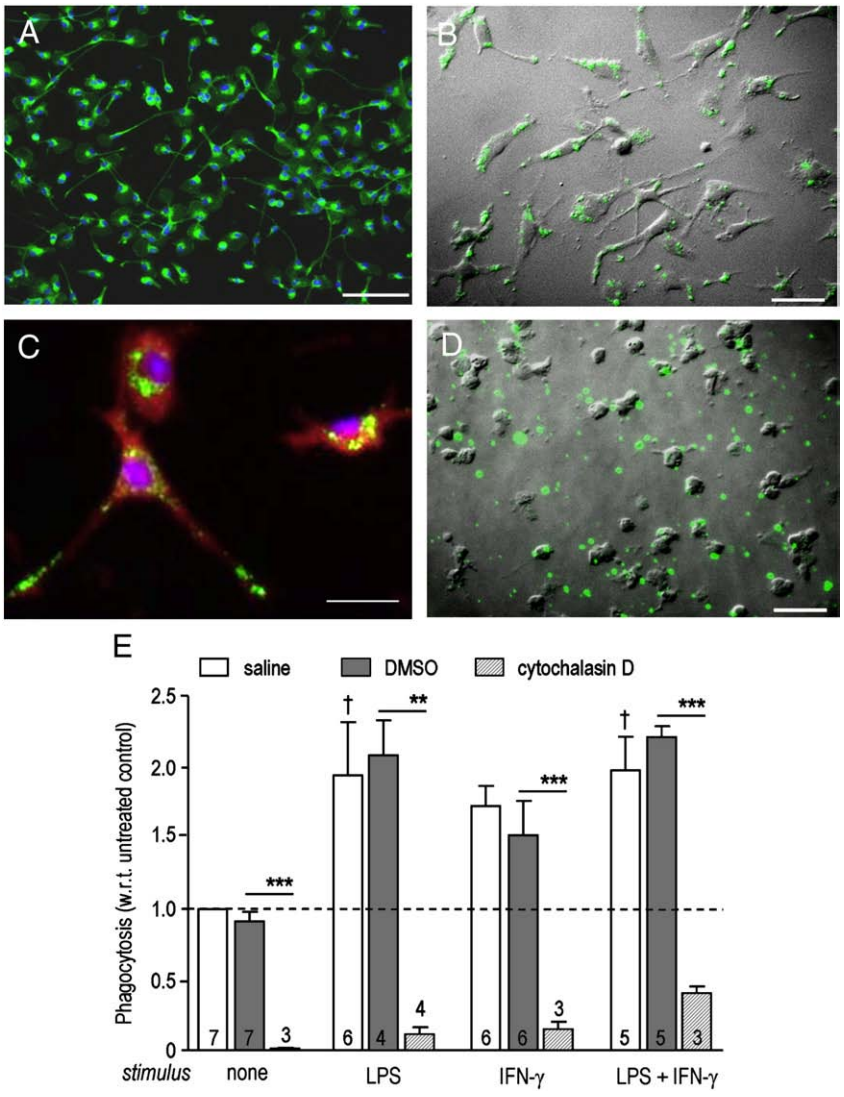

Fig. 1. Phagocytosis of *E. coli* bacteria is increased by bacterial LPS. A. A representative confocal image of untreated microglia shows that rat microglial cultures were essentially pure. All cells were labeled with the nuclear stain, DAPI, and microglia were labeled with FITC-conjugated tomato lectin; scale bar, 100 μm. B. Combined fluorescence and differential interference contrast (DIC) images after a 1 h exposure to FITC-conjugated *E. coli* K12 bacteria in minimal essential medium (MEM) with 2% FBS (scale bar, 50 μm). C. A higher magnification fluorescence image (same treatment as in B) shows phagocytosed *E. coli* (green) inside Iba-1-labeled microglia (red; Cy3-conjugated secondary antibody); nuclei were labeled with DAPI (scale bar, 20 μm). D. Combined fluorescence and DIC image of microglia that had been pre-treated with the actin-polymerization inhibitor, cytochalasin D (10 μM) before exposure to *E. coli* (scale bar, 50 μm). E. Quantification of *E. coli* phagocytosis; measured with a plate reader and expressed as relative fluorescence units (RFU; see Methods). The fluorescence signal of bacteria phagocytosed in 1 h by untreated microglia (dashed line) was used to normalize the values from microglia that had been pre-treated for 24 h with 10 ng/ml lipopolysaccharide (LPS) from the *E. coli* 0055:B5 strain, or 10 ng/ml IFNγ, or a combination of LPS + IFNγ. Significant differences are indicated as: $^{\dagger}p<0.05$ with respect to controls; and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ for treatments. Each *n* value (indicated on the bars) represents the average of 3 replicate experiments on microglia from *n* separate litters of rats.

sulfuric acid and the reaction product was measured at 450 nm absorbance, with a λ correction at 570 nm, using the plate reader (as above). Each treatment was averaged from 3 wells and compared with a standard curve to quantify the concentrations of TNF-α and IL-10; detection limits were 15 pg/ml for TNF-α and 20 pg/ml IL-10.

For some experiments, microglia were treated with the p38 MAP kinase inhibitor, SB202190 (2 μM; Calbiochem; San Diego, CA). SB202190 inhibits p38a/SAPKa with an IC_{50} of 50 nM (Manthey et al., 1998); thus, full inhibition is expected for the 2 μM concentration used.

2.5. Statistical analyses

Graphical data are presented as mean ± SEM, with significance levels indicated as: one symbol († * ◇ ▲), $p<0.05$; two symbols, $p<0.01$; three symbols, $p<0.001$. Analyses used 1-way ANOVA, with post-hoc tests: Dunnett to compare controls versus several treatments, and Bonferroni to compare pairs of means (Graphpad Prism 5 software, La Jolla, CA).

3. Results

3.1. Phagocytosis of E. coli is increased by bacterial LPS

First, we addressed whether prior activation of microglia affects their ability to phagocytose Gram-negative *E. coli* bacteria. Three stimulus paradigms were compared with untreated microglia: lipopolysaccharide (LPS), interferon-γ (IFNγ), and LPS + IFNγ. The microglial cultures were essentially pure (Fig. 1A), as all cells (DAPI-stained nuclei) were labeled with tomato lectin. Under the culturing conditions used for phagocytosis studies, most unstimulated microglia had one or more long processes and a fan-like lamellipodium (Fig. 1A) and were highly mobile (not shown). Microglia phagocytosed *E. coli* K12 bacteria, retracted their lamellipodia and acquired an

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Table 1
Primers used for quantitative real-time RT-PCR (qRT-PCR).

Gene name	Accession #	Primer sequence
HPRT-1 (housekeeping)	NM_012583.2	FP: CAGTACAGCCCCAAAATGGT
		RP: CAAGGGCATATCCAACAACA
IL-10	NM_012854	FP: CCTTGGGAAGCAACTGAAAC
		RP: GATGAGGGCAAGTGAAAGGA
iNOS	NM_012611	FP: GCTACGCCTTCAACACCAA
		RP: GCTTGTCACCACCAGCAGT
TNF-α	NM_012675	FP: GCCCACGTCGTAGCAAAC
		RP: GCAGCCTTGTCCCTTGAA
TLR4	NM_019178	FP: TGCTCAGACATGGCAGTTTC
		RP: GCGATACAATTCGACCTGCT
CD14	NM_021744	FP: ATTGCCCAAGCACACTCAC
		RP: GTCCTTTCTCGCCCAACTC
CR3	NM_012711	FP: TGCTGAGACTGGAGGCAAC
		RP: CTCCCCAGCATCCTTGTTT
Fcgr1	NM_001100836	FP: CAAGGGCTGAAAGGGAAGA
		RP: TCTGATGACTGGGGACCAA
Fcgr3a	NM_207603	FP: CAAAAGGCTGTGGTGAACCT
		RP: GACATAGTTGGCGTCCTGGT
MARCO	NM_001109011	FP: CTCCTGGGGCTCTCACAAT
		RP: CTGGGAACACACGGATGAC
MHCII	AJ554214	FP: CCAACACCCTCATCTGCTTT
		RP: AAGCCATCTTGTGGAAGGAA
NOD2	NM_001106172	FP: GACCAAACCTCTGTCCCTGA
		RP: CAAGGAGGAACTGGAAGACG
TLR9	NM_198131	FP: GCATGGCTACCTTTGCTGA
		RP: GCCTTATCGAACACCACGA
SR-A	XM_573919	FP: GTGCTTTGGGAGAGAATCGT
		RP: ACAAGTGACCCCAGCATCTT

amoeboid morphology (Fig. 1B). Note that very few free bacteria remained outside the microglia, demonstrating the efficiency of phagocytosis by microglia plated on glass cover slips. The higher magnification image (Fig. 1C) shows *E. coli* inside both the processes and cell bodies of microglia, with particular concentration around the nuclei. Cytochalasin D inhibits the second phase of phagocytosis, which involves actin polymerization and formation of a phagocytic cup. After microglia were pre-treated with cytochalasin D, almost all *E. coli* remained outside the cells (Fig. 1D). For quantification (Fig. 1E), Trypan blue was added to quench the FITC fluorescence of any remaining adherent *E. coli* (see Materials and methods), and to show that the microglia were viable (no dye uptake). [NB: Statistical *p* values and numbers of independent experiments are indicated on all bar graphs.] Phagocytosis of *E. coli* was increased 1.9–2.3 fold after microglia were treated with LPS in saline or DMSO, and by 2–2.5 fold after combined LPS + IFNγ treatment. The ~1.7 fold change with IFNγ alone did not reach statistical significance. Regardless of the treatment paradigm, cytochalasin D inhibited phagocytosis; i.e., by 99% for untreated microglia, 94% after LPS, 91% after IFNγ, and 80% after combined LPS + IFNγ treatment. IFNγ was omitted for subsequent experiments because it can up-regulate expression of 'Fc fragment of IgG, low affinity receptors' (Fcγ) phagocytic receptors in rat microglia (Loughlin et al., 1992; Woodroofe et al., 1989), which would confound the questions being addressed.

3.2. Different microglial inflammatory profiles are evoked by whole *E. coli* and LPS

First, we asked whether responses to whole *E. coli* differ from isolated LPS. Because most studies using LPS do not consider its

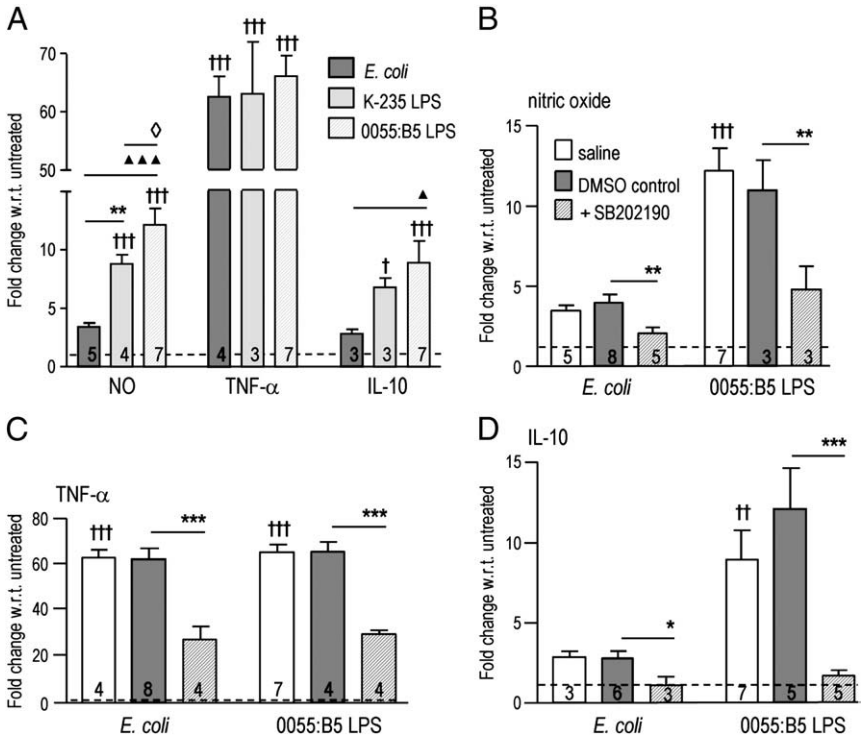

Fig. 2. Whole *E. coli* and LPS differentially up-regulate inflammatory molecules. A. Microglia were treated for 24 h with heat-killed *E. coli*, or with 10 ng/ml of lipopolysaccharide (LPS) from the related *E. coli* strain (K-235) or an unrelated strain (0055:B5). Each *n* value is from a separate litter of rats. Nitric oxide (NO) production (in μM) was measured with the Griess assay, and ELISAs were used to measure secreted TNF-α and IL-10 proteins (in pg/ml). The data were then normalized to the amounts of NO, TNF-α or IL-10 released from untreated microglia (dashed line). Comparisons indicated by the different symbols are: †, versus untreated controls; *, *E. coli* versus K-235 LPS; ▲, *E. coli* versus 0055:B5 LPS; ◇, comparisons between the two LPS strains. For all statistical tests, levels of significance are indicated as: one symbol, $p<0.05$; two symbols, $p<0.01$; three symbols, $p<0.001$. Each *n* value is shown on the bars and represents the average of 3 replicate experiments on microglia from *n* separate litters of rats. B–D. Role of p38 MAPK in inflammatory molecule production. Microglia were treated for 24 h with heat-killed *E. coli* or 10 ng/ml of LPS from the commonly used 0055:B5 strain. All values were normalized to the amounts produced by untreated microglia (dashed lines). The p38 MAPK inhibitor, SB202190 (2 μM), was compared with the DMSO solvent control. NO production (panel B), and TNF-α (panel C) and IL-10 (panel D) release were measured as above. Significant differences are expressed as $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. Each *n* value represents the average of 3 replicate experiments on microglia from *n* separate litters of rats.

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bacterial source, we compared LPS isolated from the most commonly used strain (*E. coli* strain 0055:B5) and strain K-235, which is closely related to the whole K12 *E. coli* bacteria used for the phagocytosis studies. Data in Fig. 2A show that whole *E. coli* evokes a different inflammatory profile from bacterial LPS. Four hallmark molecules (iNOS/NOS2, nitric oxide, TNF-α, IL-10) were analyzed that can distinguish between innate, humoral and classical modes of activation (Fernandez et al., 2009; Gordon, 2003) in microglia (Colton, 2009; Ransohoff and Perry, 2009). That is, innate activation induces reactive nitrogen and oxygen species, as well as both pro-inflammatory (e.g., TNF-α) and anti-inflammatory cytokines (e.g., IL-10). Classical activation does not induce anti-inflammatory cytokines, and humoral activation does not induce reactive nitrogen and oxygen species.

Untreated microglia produced relatively low levels of inflammatory mediators (Figs. 2–4). Data in Fig. 2A show that the initial NO level (2.21 ± 0.65 μM) was increased by all three stimuli; however, the response to phagocytosis of whole *E. coli* bacteria (3.4 fold) was smaller than after treatment with LPS. NO increased by 8.8 fold after treatment with the related K-235 strain, and by 12.5 fold after treatment with the 0055:B5 strain. Secreted IL-10 protein was low in untreated microglia (23.3 ± 4.3 pg/ml of supernatant); it increased by 6.8 fold after treatment with K-235 LPS and by 8.9 fold after 0055:B5 LPS. Again, LPS was more effective; IL-10 was not increased after phagocytosis of *E. coli*. The low responses to *E. coli* cannot be attributed to having less than 10 ng/ml LPS on the intact bacteria because TNF-α was induced to a similar degree by all three treatments. That is, TNF-α protein production/secretion was low in untreated microglia (117 ± 6.14 pg/ml of supernatant), and increased by 66 fold after exposure to *E. coli* and 63 fold after treatment with either the K-235 or 0055:B5 LPS strains.

We next asked if p38 MAPK is involved in microglial production of the same three inflammatory mediators, despite the differences in response to LPS and whole *E. coli*. For this experiment, we exploited the commonly used 0055:B5 LPS strain to facilitate comparisons with published studies. Results in Fig. 2B–D show that p38 MAPK contributes to both the LPS- and *E. coli*-induced responses. Production of all three inflammatory mediators was reduced by a saturating concentration of the p38 MAPK inhibitor, SB202190, but there were differences in the amount remaining. [Note: There were no differences between controls using saline versus DMSO.] NO production was increased 3.4 fold by *E. coli*, and SB202190 reduced this induction nearly to the baseline (Fig. 2B). LPS increased NO by 12 fold, and SB202190 reduced this induction to ~5-fold above baseline. TNF-α production (Fig. 2C) was increased 63 fold by *E. coli* and 66 fold by LPS; SB202190 reduced the induction by 58% and 57% after *E. coli* and LPS, respectively. However, substantial TNF-α remained; 25–30 fold above the baseline level seen in unstimulated microglia. Thus, despite the difference in response to *E. coli* and LPS for NO (but not TNF-α),

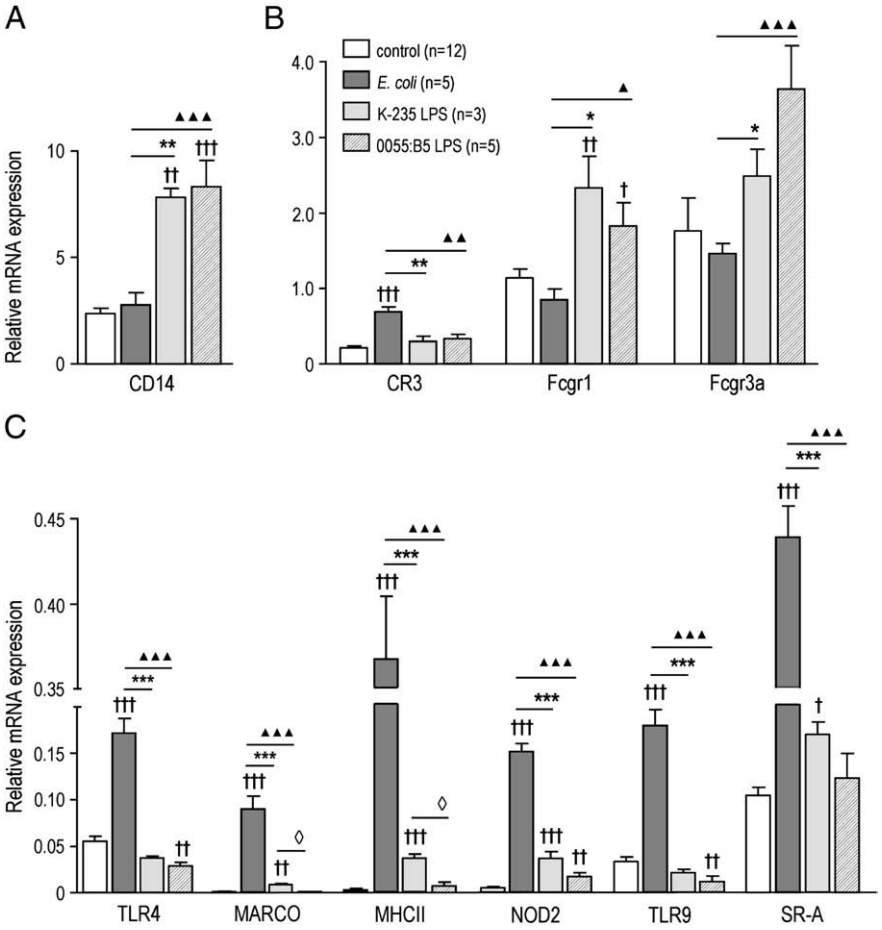

Fig. 3. *E. coli* and LPS differentially affect expression of receptors involved in pathogen recognition and phagocytosis. Quantitative real-time RT-PCR was used to monitor relative mRNA expression, with levels normalized to the housekeeping gene, HPRT-1. Microglia were treated for 24 h with heat-killed *E. coli*, or with 10 ng/ml of LPS from the related *E. coli* strain (K-235) or the 0055:B5 strain. The panels are organized according to levels of transcript expression after treatments; relatively high (A), medium (B) and low (C). Significant differences are indicated as: $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ with respect to untreated controls; $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$ for *E. coli* versus K-235 LPS; and $^{\diamond}p<0.05$ for *E. coli* versus 0055:B5 LPS. Each *n* value (shown on the key) represents the average of 3 replicate experiments on microglia from *n* separate litters of rats. Abbreviations are: cluster of differentiation 14 (CD14); complement receptor 3 (CR3); receptors for the Fcγ portion of immunoglobulins (Fcgr1, Fcgr3a); Toll-like receptors 4 and 9 (TLR4, TLR9); macrophage receptor with collagenous structure (MARCO); major histocompatibility complex II (MHCII); nucleotide binding and oligomerization domain receptor 2 (NOD2); scavenger receptor A (SR-A).

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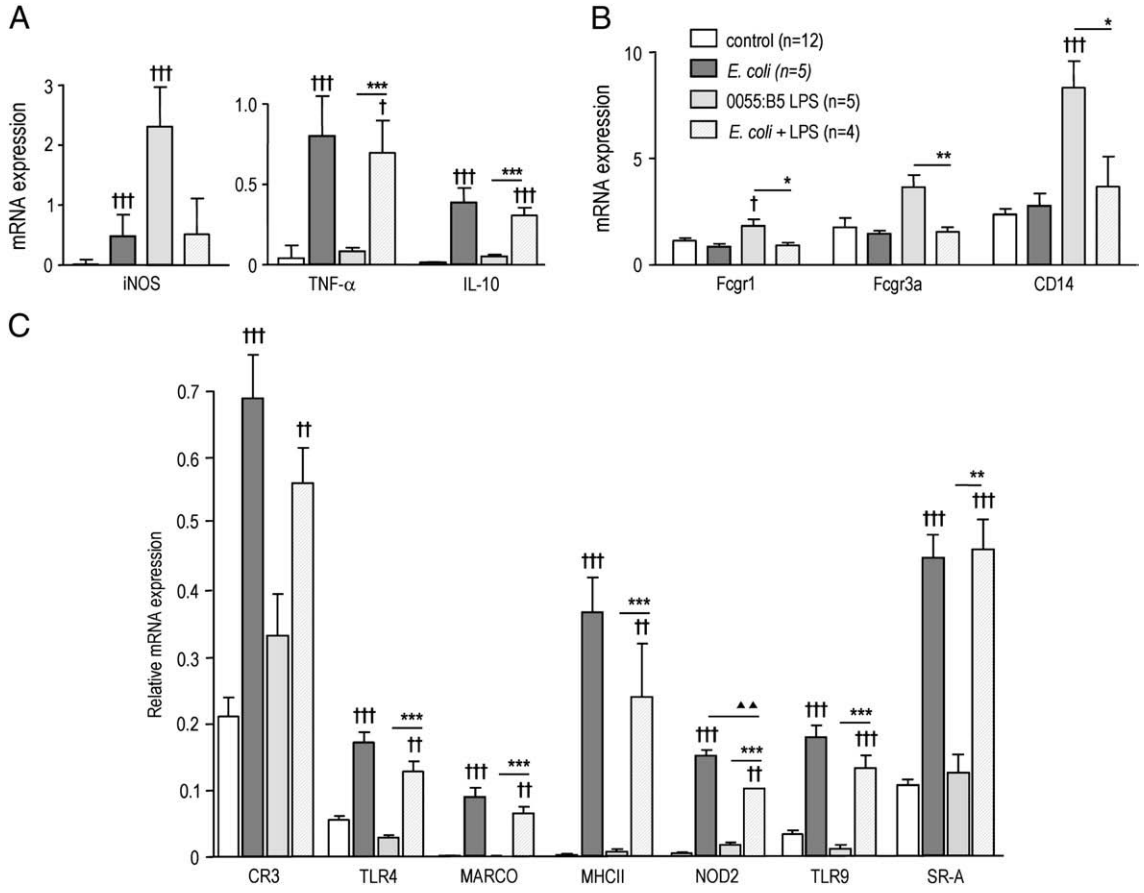

Fig. 4. Bacterial modulation of the microglial inflammatory response. Microglia were treated for 24 h with heat-killed *E. coli* or 10 ng/ml of the 0055:B5 LPS strain, with or without heat-killed *E. coli*. Relative mRNA expression was measured with quantitative real-time RT-PCR and normalized to the housekeeping gene, HPRT-1. Genes were grouped as pro- and anti-inflammatory mediators (A); and as higher-expressing (B) and lower-expressing receptors (C) involved in phagocytosis (iNOS, inducible nitric oxide synthase; other abbreviations are defined in Fig. 3 legend). Significant differences are indicated as: $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ with respect to untreated controls; $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$ for LPS versus LPS + *E. coli*; and $^{\blacktriangle\blacktriangle}$ $p<0.01$ for *E. coli* versus LPS + *E. coli*. Each *n* value (shown on the key) represents the average of 3 replicate experiments on microglia from *n* separate litters of rats.

both inflammatory mediators were reduced to a similar degree by inhibiting p38 MAPK. Inhibiting MAPK essentially abolished the induction of IL-10 (Fig. 2D); IL-10 was increased 2.9 fold by *E. coli* and 12 fold by LPS, and SB202190 reduced both to the baseline level. The remaining production of all three molecules apparently involved a different pathway, likely NFκB, which is known to contribute to microglial activation and cytokine production (Kielian, 2009; Selvaraj and Prasadarao, 2005).

3.3. Whole E. coli and LPS differentially regulate expression of receptors involved in pathogen recognition and phagocytosis

We next examined microglial expression of several molecules that are pattern recognition receptors (PRRs) for pathogens or are involved in phagocytosis. CD14 and TLR4 mediate LPS binding and signaling; CR3, Fcgr1 and Fcgr3a/CD16a ('Fc fragment of IgG, low affinity receptors'; also called FcγR1, FcγR3a) are cell-surface phagocytosis receptors; SR-A and MARCO are scavenger receptors involved in phagocytosis; TLR9 and NOD2 are intracellular receptors for degraded bacterial components; and MHCII mediates antigen presentation after phagocytic degradation. Results in Figs. 3 and 4 show that untreated microglia had low levels of iNOS, TNF-α, IL-10, MARCO, MHCII, NOD2 and TLR9, compared with the housekeeping gene (HPRT-1) or the levels induced by *E. coli* and LPS. In contrast, three receptors involved in LPS responses and phagocytosis (CD14, Fcgr1, and Fcgr3a) were constitutively expressed at high levels in untreated microglia; i.e., at levels comparable to or greater than HPRT-1. Fig. 3 also shows differences between microglial responses to *E. coli* phagocytosis and LPS treatment. *E. coli* did not affect CD14, Fcgr1 or Fcgr3a expression, but the first two were affected by one or both types of LPS. Specifically, the CD14 receptor, which was highly expressed in microglia regardless of the treatment (Fig. 3A), was increased by K-235 LPS (3.3 fold) and by 0055:B5 LPS (3.5 fold). Both Fcgr1 and Fcgr3a were well expressed in untreated microglia, but only Fcgr1 was up-regulated by LPS; i.e., 2.0 fold by K-235 LPS and 1.6 fold by 0055:B5 LPS. The converse pattern—greater up-regulation by *E. coli* than isolated LPS—was seen for the remaining 7 genes. Complement receptor 3 (CR3) expression was up-regulated by *E. coli* (3.3 fold) but not by either strain of LPS (Fig. 3B). Only *E. coli* up-regulated TLR4 (3.1 fold) and TLR9 (5.3 fold) (Fig. 3C). *E. coli* evoked much larger increases in MARCO (149 fold), MHCII (180 fold) and NOD2 (32 fold) than did LPS. There were minor differences between the two LPS strains; K-235 LPS up-regulated MARCO and MHCII by 14.5 and 18 fold, respectively while 0055:B5 LPS did not affect them. Again, to facilitate comparisons with the literature, the remaining experiments used the 0055:B5 LPS strain.

3.4. Bacterial domination of the microglial inflammatory response

Results in Figs. 2 and 3 show different inflammatory responses of rat microglia to *E. coli* compared with LPS alone. Fig. 4 shows that phagocytosis of *E. coli* affects the microglial response to LPS. That is,

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compared with 0055:B5 LPS alone, phagocytosis of *E. coli* by LPS-stimulated microglia changed the mRNA expression of 11 out of 13 genes examined. The combined treatment increased expression of TNF-α and IL-10 (by 8.4 and 5.9 fold, respectively; Fig. 4A), TLR4 (4.5 fold), MHCII (37 fold), TLR9 (11.7 fold), MARCO (170 fold), SR-A (5.4 fold) and NOD2 (6 fold) (Fig. 4C). Three receptors (Fig. 4B) were decreased; i.e., CD14 was reduced by 56%, Fcgr1 by 49%, and Fcgr3a by 57%; while iNOS and CR3 did not differ. The striking consequence was that for 12/13 genes examined, whole *E. coli* dominated the microglial response; i.e., transcript expression was the same for *E. coli* alone as for *E. coli* + 0055:B5 LPS, regardless of how the cells responded to 0055:B5 LPS alone.

4. Discussion

There are several salient results in this first such study of microglia. (i) Untreated microglia constitutively expressed several pattern recognition receptors (PRRs), but had very low expression of several inflammatory mediators, and were thus initially in a relatively 'resting' state. (ii) Microglial phagocytic activity and expression of several PRRs and inflammatory molecules were differentially affected by whole K12 *E. coli* and LPS. This supports the hypothesis that inflammatory responses involve multiple microglial PRRs and bacterial pathogen-associated molecular patterns (PAMPs), rather than LPS and TLR4 alone. Nevertheless, there was a convergence of the two stimuli in one of the main signaling pathways for microglial activation because the increases in nitric oxide, TNF-α and IL-10 were abrogated by inhibiting p38 MAPK. (iii) Minor differences were seen in microglial responses to LPS derived from two different *E. coli* strains; i.e., the commonly used 0055:B5 strain and the K-235 strain, which is more closely related to the K12 *E. coli* strain used for phagocytosis. (iv) Some bacteria can modulate peripheral immune responses; thus, a key finding was that the responses of microglia to *E. coli* phagocytosis over-rode their responses to LPS when the stimuli were combined.

4.1. LPS increases microglial phagocytosis

In principle, phagocytosis in the CNS can involve complement-mediated (innate), Fcγ receptor-mediated (adaptive) or opsonin-independent mechanisms. Here, the likely pathway was innate phagocytosis; serum was present in the medium to provide opsonins and accessory factors, and the complement receptor, CR3, was constitutively expressed on microglia. Macrophages exposed to pathogens acquire an initial pro-inflammatory phenotype, with production of TNF-α, IL-1β, chemokines, proteases and redox proteins to incapacitate and remove the invader. In peripheral tissues, the killing phase is reinforced by classical activation triggered by IFNγ from T lymphocytes (Adams and Hamilton, 1987). Microglia can also produce IFNγ after interactions with PAMPs (Suzuki et al., 2005); thus, we first asked whether IFNγ or LPS affect their phagocytic capacity.

We found that LPS increased phagocytosis of *E. coli*; consistent with previous studies of murine microglia (abd-el-Basset and Fedoroff, 1995; Ribes et al., 2009) and macrophages (Doyle et al., 2004). Experimental design differences make it difficult to compare some studies. In one, there was no effect of LPS on phagocytosis by murine microglia (von Zahn et al., 1997), but the cells were cultured in suspension and latex beads were used as the target. A study reporting decreased phagocytosis by murine macrophages used red blood cells and 10 μg/ml LPS (Sundaram et al., 1993), a concentration that can evoke TLR4-independent activation (Qin et al., 2005) and kill microglia (von Zahn et al., 1997). The reported effects of IFNγ also vary, possibly depending on cell type. IFNγ has been reported to increase phagocytosis-related molecules, including Fcγ- and MHC class II receptors (Loughlin et al., 1992; Woodroofe et al., 1989), as well as TLR4, TNF-α, IL-1β and IL-6 (De Simoni et al., 1997; Minghetti et al., 1996). One study showed an increase in myelin phagocytosis by microglia, but a decrease by macrophages (Smith et al., 1998); we observed little effect of IFNγ on phagocytosis by rat microglia.

4.2. LPS and E. coli evoke different inflammatory profiles

In the healthy adult brain, microglia are in a suppressed immunological state (Ransohoff and Perry, 2009). While perinatal brain injury and inflammation have been extensively studied (reviewed in Dammann and O'Shea, 2008; Saliba and Henrot, 2001; Silverstein et al., 1997), less is known about the normal state of neonatal microglia. They are expected to be constitutively engaged in phagocytosis while helping define the brain architecture during development. Here, we found that neonatal rat microglia are phagocytosis-competent, and express several LPS binding and signaling molecules (CD14, and TLR4) and cell-surface phagocytosis receptors (CR3, Fcgr1, Fcrg3a, and SR-A), as well as TLR9, an intracellular receptor for degraded bacterial components. Their relatively quiescent inflammatory state is indicated by very low p38 MAPK activation (Fordyce et al., 2005; Kaushal et al., 2007), low production of NO, TNF-α and IL-10, and low expression of MHCII (important for antigen presentation), MARCO (a membrane phagocytosis receptor) and NOD2 (which detects intracellular bacterial degradation products).

Innate activation of macrophages (i.e., not requiring IFNγ) follows exposure to LPS, which acts through TLR4, or to bacteria, which express additional PAMPs. In the CNS, constitutively expressed TLR4 is thought to provide an early sensor of infection that can initiate and propagate activation signals within the parenchyma. LPS binds to CD14, which forms a complex with MD-2 and TLR4, signals through MyD88, activates NF-κB and p38 MAPK, and evokes production of pro-inflammatory molecules; e.g., TNF-α, iNOS, reactive oxygen and nitrogen species (recently reviewed in Kielian, 2009). We previously showed that p38 MAPK is an important link between LPS-mediated inflammation, neurotoxicity and potassium channels in rat microglia (Fordyce et al., 2005; Kaushal et al., 2007). Innate activation is usually followed by a regulated anti-inflammatory response, with increases in the anti-inflammatory cytokine, IL-10, and in expression of co-stimulatory molecules (e.g., CD40, and CD40 ligand), scavenger receptors (e.g., SR-A, and MARCO), mannose receptors and MHCII molecules, which promote endocytosis, phagocytosis and antigen presentation (Gordon, 2003; Martinez et al., 2009; Ransohoff and Perry, 2009). Our observation that production of NO, TNF-α and IL-10 was inhibited by SB202190 is especially interesting because p38 MAPK is being considered as a therapeutic target to reduce pro-inflammatory cytokine production during CNS inflammation (Borders et al., 2008).

We expected microglial responses to whole bacteria and LPS to differ, and thus characterized an inducible subset of 13 molecules related to bacterial recognition, phagocytosis and inflammatory responses. First, we compared two bacterial sources of LPS (0055:B5, and K-235) because, although rarely addressed, different strains can vary in biological activity. LPS is comprised of an inner and outer core, lipid A and the O-antigen, but can differ in the number of side chains on the O-antigen (Rietschel et al., 1994). We found that both LPS strains induced expression of several molecules characteristic of innate activation (iNOS/NO, TNF-α, IL-10, CD14, and NOD2). However, there were small differences in responses to the two LPS strains, and the K-235 strain was more consistent with innate activation in increasing expression of MHCII, MARCO and SR-A. The reduction in TLR4 by 0055:B5 LPS might represent endotoxin tolerance (Nomura et al., 2000; Sato et al., 2000); a process that could reduce inflammatory damage during meningitis.

In intact *E. coli*, the lipid A moiety of LPS is not exposed; instead, other bacterial PAMPs are thought to evoke phagocytosis-dependent

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signaling (Rietschel et al., 1994). Consistent with LPS being inactive in intact bacteria, we found that NO production and expression of Fcgr1, Fcgr3a and CD14 were unchanged after phagocytosis of *E. coli*. Few studies have examined responses of neonatal microglia to *E. coli*. We found that phagocytosis of *E. coli* by neonatal rat microglia increased production of TNF-α and expression of CR3, MHCII, TLR4, SR-A, NOD2 and TLR9. Consistent with some of our results, an *in vivo* study of *E. coli* phagocytosis in the neonatal rat brain showed increased CR3 and MHCII expression (Kaur et al., 2004). Some differences can be seen in related studies on macrophages, where whole *E. coli* induced TNF-α, IL-10 and NO (Cross et al., 2004; Paul-Clark et al., 2006). A microarray study of human macrophages showed similar genes were altered by LPS and *E. coli* but their magnitude and timing differed and the authors speculated that whole bacteria activate multiple TLRs (Nau et al., 2003). Our data support a model whereby free LPS acts on TLR4, while additional PAMPS on whole heat-killed *E. coli* act through distinct microglial PRRs to up-regulate receptors involved in phagocytosis and inflammation.

4.3. Bacterial modulation of the inflammatory response

Bacterial PAMPs can modulate the innate immune response (Brodsky and Medzhitov, 2009). For instance, the outer membrane protein, OmpA, can suppress production of pro-inflammatory cytokines by macrophages during early stages of *E. coli*-mediated meningitis (Selvaraj and Prasadarao, 2005). Here, we found that the microglial response to phagocytosis of *E. coli* predominated over the response to LPS for all genes examined; i.e., for the nine genes that increased (TNF-α, IL-10, CR3, TLR4, MARCO, MHCII, NOD2, TLR9, and SR-A) and the four that decreased (iNOS, CD14, Fcgr1, and Fcgr3a). The expression levels of 12/13 genes were the same after exposure to *E. coli* alone as for *E. coli* combined with LPS. Again, this supports the view that inflammatory gene transcription was dominated by bacterial PAMPS other than LPS.

Consequences of *E. coli*-induced gene changes will likely be context-dependent, as illustrated by the following examples. 1. Several of the up-regulated genes should facilitate phagocytosis, bacterial degradation and antigen presentation. For instance, CR3 mediates phagocytosis of complement-coated pathogens, MHCII is involved in antigen presentation, TLR9 recognizes CpG unmethylated DNA released by degraded bacteria (Hemmi et al., 2000), and NOD2 recognizes bacterial peptidoglycans released from the phagosome (Herskovits et al., 2007; Inohara et al., 2005). Because Fcgr3a/CD16a can inhibit phagocytosis and increase inflammation (Pinheiro da Silva et al., 2007); the observed decrease might be beneficial. 2. Outcomes of several gene changes are expected to be more complex. For instance, NO has antimicrobial actions and iNOS inhibitors can exaggerate macrophage responses to infections (Fang, 1997; MacMicking et al., 1997). In contrast, NO can weaken the BBB (Winkler et al., 2001; Yamauchi et al., 2007) and form cytotoxic peroxynitrite molecules that can kill neurons (Fordyce et al., 2005; Kaushal et al., 2007). TNF-α is important in defenses against pathogens, but high levels contribute to septic shock (Gardlund et al., 1995) and BBB weakening (Tsao et al., 2001). Decreased Fcgr1 is expected to reduce phagocytosis of antibody-coated pathogens after IgG extravasation when the BBB is incomplete in neonates or compromised during bacterial meningitis. 3. Some changes evoked by phagocytosis of *E. coli* likely reflect down-regulated pro-inflammatory responses or an alternative activation state (Colton, 2009; Ransohoff and Perry, 2009); e.g., decreased CD14, and increased scavenger receptors (SR-A, and MARCO). Increases in IL-10 have been reported after Gram-negative bacterial infections (Hessle et al., 2000) and after exposing microglia to LPS or microbes (Ledeboer et al., 2002). These changes are also significant because scavenger receptors can remove LPS (Van Amersfoort et al., 2003) and CD14 is a pattern recognition receptor required for LPS binding, recognition and delivery to TLR4.

Together, our results suggest that pro-inflammatory microglial responses to free LPS will be dampened by phagocytosis of Gram-negative bacteria. This has broader implications because several endogenous CNS molecules apparently act through TLR4, including fibrinogen, fibronectin, heparin sulfate, hyaluronan and heat-shock proteins (Kielian, 2009). While modulation of the response to bacteria might have evolved to reduce bystander damage during infections, it is also relevant to other CNS diseases/disorders with an inflammatory component; e.g., multiple sclerosis, stroke and trauma.

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