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A Novel Anti-inflammatory Phenotype Transformed by Repetitive Low-dose Lipopolysaccharide in Primary Peritoneal Tissue-resident Macrophages

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Abstract. *Background/Aim: Our previous studies suggested that oral administration of lipopolysaccharide (LPS) regulates the progression of various diseases via transformation of tissue-resident macrophages (MΦ). Recently, we characterized microglia transformed by repetitive low-dose LPS treatment (REPELL-microglia) in vitro, and this response was similar to that observed in response to oral administration of LPS in vivo. Here, we examined the characteristics of peritoneal tissue-resident MΦ (pMΦ) transformed by repetitive low-dose LPS treatment (REPELL-pMΦ). Materials and Methods: Primary pMΦ were treated with low-dose LPS (1 ng/ml) three times; subsequently, phagocytic activity and gene expression were evaluated. Results: REPELL-pMΦ exhibited high phagocytic activity and elevated expression of Arg1, Gipr, Gdnf, and Fpr2. The gene expression profiles observed in REPELL-pMΦ were distinct from those of REPELL-microglia. Conclusion: REPELL-pMΦ have the potential to promote clearance of xenobiotics and to suppress inflammation. The present study also demonstrates the diversity of tissue-resident MΦ transformation that reflect their tissue origin.*

Lipopolysaccharide (LPS) is a physiologically active outer membrane component of Gram-negative bacteria. Macrophages (MΦ) play a significant role in innate immunity and express Toll-like receptor 4 (TLR4), a receptor for LPS (1).

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Although intravenous and/or intraperitoneal administration of LPS typically induces inflammation, we have demonstrated that oral administration of LPS can ameliorate signs and symptoms associated with various pathological conditions, including Alzheimer's disease, arteriosclerosis, and cancer (2-5). Because oral administration of LPS enhances the phagocytic activity of tissue-resident MΦ (2, 6), it has been suggested that oral LPS-mediated transformation of tissue-resident MΦ may be an important mechanism with respect to health and homeostasis maintenance. Recently, we developed a new *in vitro* model of repetitive administration of low-dose LPS (1 ng/ml) based on our estimates of the LPS concentration in vivo following oral administration. Using this model for microglia, which are brain tissue-resident MΦ, we characterized microglia transformed by repetitive low-dose LPS treatment (REPELL-microglia) (7). The results of this study revealed that REPELL-microglia generated *in vitro* exhibited high phagocytic activity accompanied with the elevated expression of pro-inflammatory, anti-inflammatory, and neuroprotective factors to an extent similar to the responses of microglia transformed by oral administration of LPS *in vivo*. Moreover, the results from this previous study also demonstrated that low- and high-dose LPS (1 and 100 ng/ml, respectively) induced distinct microglial phenotypes. These findings demonstrated the importance of characterizing unique MΦ transformation upon low-dose LPS treatment for understanding the effect of oral LPS administration on health.

However, it is not clear whether repetitive low-dose LPS has an effect on tissue-resident MΦ other than microglia. As the specific phenotypes of tissue-resident MΦ are different depending on the organ or tissue of origin (8), it was assumed that REPELL-MΦ characteristics are diverse and that their responses might depend on their tissues of origin. Since our previous results revealed that oral administration of LPS enhanced phagocytic activity of pMΦ (6), we focused on peritoneal MΦ (pMΦ) as a cell type that can be distinguished

from microglia in this study. Numerous studies have characterized the transformation of pMΦ by high-dose LPS treatment (100 ng/ml) as a potent inflammation model. For example, pMΦ are transformed by single high-dose LPS into a pro-inflammatory phenotype that produces pro-inflammatory cytokines including interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) whereas pMΦ are transformed by repetitive high-dose LPS into anti-inflammatory phenotype in which pro-inflammatory cytokine production is suppressed and anti-inflammatory cytokines such as IL-10 and transforming growth factor β1 (TGF-β) are produced (9-12). Although the characteristics of pMΦ transformed by high-dose LPS have been investigated, studies designed to elucidate the characteristics of pMΦ transformed by the repetitive low-dose LPS treatment (REPELL-pMΦ) have not been conducted.

In the present study, we characterized the responses of REPELL-pMΦ *in vitro* to further characterize tissue-resident MΦ transformation by repetitive low-dose LPS treatment. Among our findings, REPELL-pMΦ were found to exhibit high phagocytic activity and elevated expression of anti-inflammatory genes *Arg1, Gipr,* and *Gdnf*, as well as phagocytosis/antitumor gene *Fpr2*.

Materials and Methods

Animals. Male C57BL/6 mice (n=18, 20-22 g) at 7 weeks of age were purchased from SLC, Inc., (Shizuoka, Japan) and were acclimated for 1 week prior to use in experiments. All mice (3-5 mice per cage) were maintained under specific pathogen-free conditions in a temperature- and humidity-controlled room under a 12-h light/dark cycle with unrestricted access to food and water. The animal experiments were reviewed and approved by the Animal Care and Use Committee of the Control of Innate Immunity TRA (Approval No.: 18-12). The experiment was conducted according to the Law for the Humane Treatment and Management of Animals Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Ministry of the Environment, Japan), the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions (Ministry of Education, Culture, Sports, Science and Technology, Japan), and the Guidelines for Proper Conduct of Animal Experiments (the Science Council of Japan). The health and well-being of animals were also assessed in accordance with the guidelines described above.

Cell culture and LPS treatment. Eight-week-old mice were euthanized by cervical dislocation under anesthesia with 4% isoflurane vapor. Resident peritoneal cells were collected by washing the peritoneal cavity with phosphate-buffered saline (PBS). Erythrocytes in each preparation were lysed with Red Blood Cell Lysis buffer (BioLegend, San Diego, CA, USA), and the remaining cells were washed with PBS. Primary pMΦ were enriched by adherence followed by extensive washing. Pooled pMΦ (2×10^6 cells/ml) were seeded in 12-well tissue culture plates (n=3) and cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Carlsbad, CA, USA), at 37˚C in 5% CO_2. Primary pMΦ were treated with LPS (1 ng/ml, purified LPS derived from *Pantoea agglomerans*, Macrophi Inc., Kagawa, Japan) or remained as untreated controls as described previously (7). The concentration of LPS was 1 ng/ml because our previous *in vivo* studies indicated that serum LPS levels were estimated to be at most 1 ng/ml, following oral administration of LPS at a concentration of 1 mg/kg/day (13). For repetitive treatment with LPS, cells received fresh medium containing LPS every 24 h for a total of three times. For single treatment with LPS, cells received fresh medium without LPS for the first 48 h and then received a one-time dose of LPS in fresh medium at that time point. Samples were collected at the indicated time points.

Cell proliferation assay. Twenty-four hours after the final LPS treatment, cell proliferation was assessed using the water-soluble tetrazolium salts (WST)-1 reagent according to the manufacturer's protocol (Takara Bio, Shiga, Japan). After primary pMΦ were incubated for 4 h in the presence of the WST-1 reagents, absorbance was measured at 450 nm using iMark microplate reader (BIO RAD, Hercules, CA, USA).

Phagocytosis assay. Phagocytic activity was measured by flow cytometry as previously described (14). Briefly, 24 h following the final LPS treatment (1 ng/ml), pMΦ were incubated for 3 h with fluorescent latex beads (Fluoresbrite YG Microspheres 2.0 μm; Polysciences, Warrington, PA, USA) at a cell/bead ratio of 1:5. Cells were washed to remove particles that had not been internalized and detached from the tissue culture plate with 0.25% trypsin treatment (Life Technologies). The extent of phagocytosis in 50,000 counted cells was assessed using a Beckman Coulter's Gallios flow cytometer and Kaluza software (Beckman Coulter, Indianapolis, IN, USA).

Quantitative RT-PCR. Quantitative RT-PCR was conducted as described previously (7). Four hours after the final LPS treatment, RNA was extracted from cells using RNeasy Mini Kit (QIAGEN, Hilden, Germany) and cDNA was synthesized by reverse transcription using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturers' instructions. Real-time PCR assay was conducted using 2 μl of cDNA as the template and 10 μl of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) using Stratagene Mx 3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). Primers used in this study are presented in Table I. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to GADPH. The thermal cycling conditions for PCR were 95˚C for 10 min for polymerase activation, followed by 45 cycles at 95˚C for 15 s for denaturation and 60˚C for 1 min for extension.

Statistical analysis. Statistical analysis was conducted using the GraphPad Prism 6.0 software package (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as mean±standard error (SE) of the mean. Differences between the groups were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test and a p-value <0.05 was considered to indicate significant differences. All experiments were conducted with at least two independent biological replicates.

Results

Low-dose LPS is not cytotoxic to primary pMΦ. In order to evaluate cytotoxicity, primary pMΦ in culture were treated with low-dose LPS (1 ng/ml) three times, and proliferation

Table I. *List of primers used for quantitative RT-PCR.*

Gene		Primer
Arg1	Forward:	5'-CTCCAAGCCAAAGTCCTTAGAG-3'
	Reverse:	5'-AGGAGCTGTCATTAGGGACATC-3'
Ccl1	Forward:	5'-GGATGTTGACAGCAAGAGCA-3'
	Reverse:	5'-TAGTTGAGGCGCAGCTTTCT-3'
Fpr2	Forward:	5'-TCTACCATCTCCAGAGTTCTGTGG-3'
	Reverse:	5'-TTACATCTACCACAATGTGAACTA-3'
Gapdh	Forward:	5'-CGACTTCAACAGCAACTCCCACTCTTCC-3'
	Reverse:	5'-TGGGTGGTCCAGGGTTTCTTACTCCTT-3'
Gdnf	Forward:	5'-TGACTCCAATATGCCTGAAGATTATC-3'
	Reverse:	5'-AATGGTGGCTTGAATAAAATCCA-3'
Gipr	Forward:	5'-CCGCGCTTTTCGTCAT-3'
	Reverse:	5'-CCACCAAATGGCTTTGACTT-3'
Il10	Forward:	5'-GCTGGACAACATACTGCTAACC-3'
	Reverse:	5'-CCCAAGTAACCCTTAAAGTCCTG-3'
Il12b	Forward:	5'-ACAGCACCAGCTTCTTCATCAG-3'
	Reverse:	5'-TCTTCAAAGGCTTCATCTGCAA-3'
Il1b	Forward:	5'-GAAAGACGGCACACCCACCCT-3'
	Reverse:	5'-GCTCTGCTTGTGAGGTGCTGATGTA-3'
Il6	Forward:	5'-CCAGAGATACAAAGAAATGATGG-3'
	Reverse:	5'-ACTCCAGAAGACCAGAGGAAAT-3'
Nos2	Forward:	5'-GTTCTCAGCCCAACAATACAAGA-3'
	Reverse:	5'-GTGGACGGGTCGATGTCAC-3'
Ntf5	Forward:	5'-CAGCCGGGGAGCAGAGAA-3'
	Reverse:	5'- ACACCTGTCAACAGCAGCAC-3'
Tgfb1	Forward:	5'-GTCAGACATTCGGGAAGCAG-3'
	Reverse:	5'-GCGTATCAGTGGGGGTCA-3'
Tnfa	Forward:	5'-CTGTGAAGGGAATGGGTGTT-3'
	Reverse:	5'-GGTCACTGTCCCAGCATCTT-3'

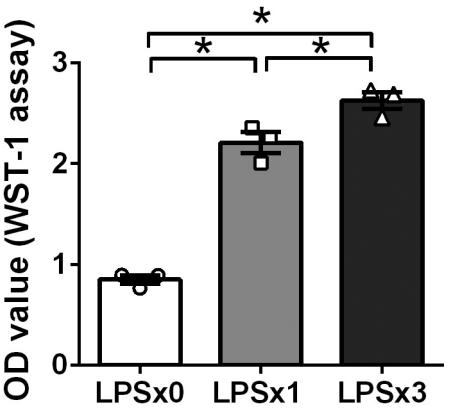

Figure 1. *Promotion of pMΦ proliferation by low-dose LPS. Primary pMΦ were treated with low-dose LPS (1 ng/ml) one or three times (n=3). Cell proliferation was measured by the WST-1 assay 24 h after the final LPS treatment. Mean±SE of each group are presented. Data are representative of two independent experiments. LPS ×0, no treatment; LPS ×1, single treatment with low-dose LPS; LPS ×3, treatment with low-dose LPS every 24 h for a total of three times. *p<0.05 for one-way ANOVA with Tukey's post-hoc correction for multiple comparisons.*

of REPELL-pMΦ was compared with that of pMΦ transformed by a single low-dose LPS (SINGLL-pMΦ) treatment using the WST-1 assay (Figure 1). The OD values were 0.85±0.03 for the untreated controls, 2.21±0.08 in response to single low-dose LPS treatment, and 2.63±0.07 in response to repetitive low-dose LPS treatment. These results demonstrated that primary pMΦ treated with low-dose LPS exhibited significantly enhanced proliferation compared with untreated controls. Moreover, REPELL-pMΦ proliferated at a higher rate (1.5-fold) than did SINGLL-pMΦ. Taken together, the results indicate that repetitive low-dose LPS is not cytotoxic, and contrarily, this strategy promotes proliferation of primary pMΦ *in vitro*.

Phagocytosis promoted by repetitive low-dose LPS treatment of primary pMΦ. To evaluate phagocytosis in response to LPS, the uptake of fluorescent latex beads was assessed after treatment with single or repetitive low-dose LPS (Figure 2A). Phagocytosis rate of REPELL-pMΦ was significantly enhanced (90.8±0.7%) compared with untreated controls (84.2±0.8%). Furthermore, the phagocytosis rate of REPELL-pMΦ was nearly as high as that of SINGLL-pMΦ (93.4±0.2%). To explore the mechanism underlying enhanced phagocytosis, mRNA expression of formyl peptide receptor 2 (FPR2) was analyzed in primary pMΦ treated with single or repetitive low-dose LPS (Figure 2B). The results demonstrated significant up-regulation of *Fpr2* expression in REPELL-pMΦ compared with untreated controls. Relative expression of *Fpr2* in the REPELL-pMΦ compared with the untreated controls was 7.09±0.99; this was comparable with the relative expression of *Fpr2* in SINGLL-pMΦ compared with the untreated controls (8.66±0.78). This result indicates that the expression of *Fpr2* in primary pMΦ is promoted by repetitive low-dose LPS treatment to an extent similar to what is observed in response to a single low-dose of LPS.

Suppressed up-regulation of pro-inflammatory gene expression by repetitive low-dose LPS treatment of primary pMΦ. Expression of mRNAs encoding pro-inflammatory mediators IL-1β, IL-6, IL-12B, TNF-α, C-C motif chemokine ligand 1 (CCL1), and nitric oxide synthase 2 (NOS2) was analyzed in primary pMΦ treated with single or repetitive low-dose LPS (Figure 3). The results revealed significant up-regulation of genes encoding pro-inflammatory mediators in SINGLL-pMΦ compared with untreated controls, whereas expression of these genes was not up-regulated in REPELL-pMΦ. Relative expression of each of these mediators in SINGLL-pMΦ compared with the untreated controls was 660.2±6.3 for *Il1b*, 532.5±21.0 for *Il6*, 244.6±4.2 for *Il12b*, 61.7±4.3 for *Tnfa*, 5.8±0.4 for *Ccl1*, and 1690.2±628.8 for *Nos2*. Contrarily, relative expression of these inflammatory mediators in

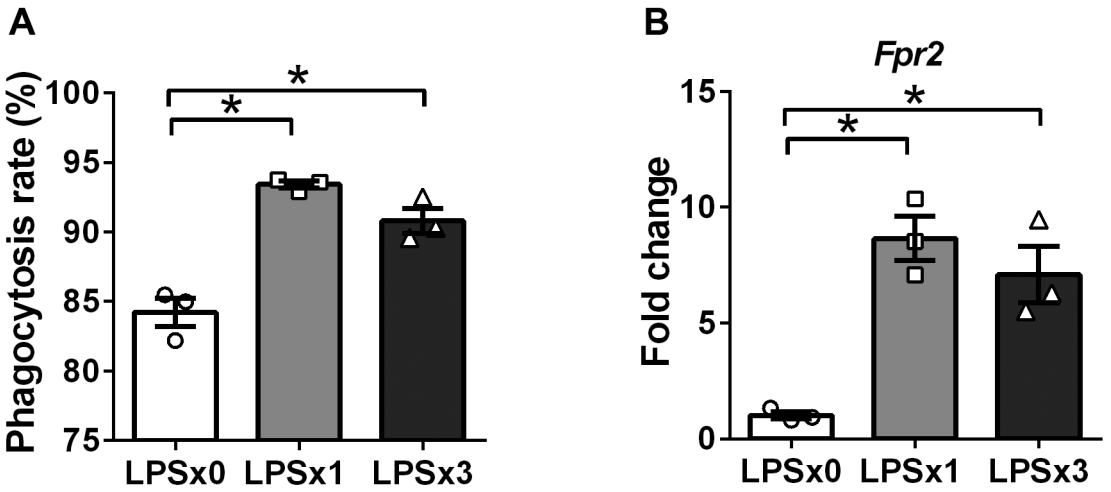

Figure 2. *Promotion of phagocytosis by repetitive low-dose LPS was comparable with that observed by single low-dose LPS in primary pMΦ. Primary pMΦ were treated with low-dose LPS (1 ng/ml) one or three times (n=3). (A) Primary pMΦ were treated with single or repetitive low-dose LPS. At 24 h after the final LPS treatment, cells were incubated with fluorescent latex beads for 3 h at a cell/bead ratio of 1:5. The phagocytosis rate (%) of 50,000 counted cells was assessed using a Beckman Coulter Gallios flow cytometer and Kaluza software. (B) Relative mRNA expression of Fpr2 was measured at 4 h after the final LPS treatment by real-time RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Data were normalized to the expression of GAPDH and presented as the relative fold change over untreated controls. Mean±SE of each group are presented. Data are representative of two independent experiments. LPS ×0, no treatment; LPS ×1, single treatment with low-dose LPS; LPS ×3, treatment with low-dose LPS every 24 h for a total of three times. *p<0.05 for one-way ANOVA with Tukey's post-hoc correction for multiple comparisons.*

REPELL-pMΦ compared with the untreated controls was 42.9±1.9 for *Il1b*, 3.9±0.1 for *Il6*, 0.8±0.1 for *Il12b*, 7.4±0.2 for *Tnfa*, 0.8±0.1 for *Ccl1*, and 91.5±5.6 for *Nos2*; these values were not significantly different from those of the untreated controls. Taken together, these results indicate that pro-inflammatory genes expression was not up-regulated by repetitive low-dose LPS treatment in primary pMΦ.

Up-regulated expression of anti-inflammatory genes Arg1, Gipr and Gdnf expression by repetitive low-dose LPS treatment of primary pMΦ. Expression of mRNAs encoding arginase 1 (Arg-1), gastric inhibitory polypeptide receptor (GIPR), glial cell-derived neurotrophic factor (GDNF), neurotrophin 5 (NTF5), IL-10, and TGF-β was analyzed in primary pMΦ treated with single or repetitive low-dose LPS (Figure 4). The expression of *Arg1*, *Gipr*, and *Gdnf* was significantly up-regulated in REPELL-pMΦ compared with untreated controls and also with SINGLL-pMΦ. The relative expression of *Arg1* compared with untreated controls was 1.54±0.15 in REPELL-pMΦ in comparison with 0.33±0.01, representing down-regulation, in SINGLL-pMΦ cultures. The relative expression of *Gipr* in REPELL-pMΦ compared with untreated controls was 4.03±0.66; the relative expression in SINGLL-pMΦ compared with untreated controls was 1.19±0.31. The relative expression of *Gdnf* in REPELL-pMΦ compared with untreated controls was 9.85±0.88, whereas the relative expression of Gdnf in SINGLL-pMΦ cultures compared with untreated controls was 1.94±0.08. However, expression of *Il10*, *Tgfb1*, and *Ntf5* was significantly down-regulated in REPELL-pMΦ compared with untreated controls and with SINGLL-pMΦ. The Relative expression in REPELL-pMΦ compared with untreated controls was 0.32±0.01, 0.67±0.03, and 0.28±0.06 for *Il10*, *Tgfb1*, and *Ntf5*, respectively. Conversely, the relative expression in SINGLL-pMΦ compared with the untreated controls was 2.95±0.19, 1.02±0.06, and 0.83±0.06 for *Il10*, *Tgfb1*, and *Ntf5*, respectively. These results indicate that the expression of anti-inflammatory genes, including *Arg1*, *Gipr*, and *Gdnf*, was up-regulated by repetitive low-dose LPS treatment in primary pMΦ.

Discussion

Previous studies suggested a role for tissue-resident MΦ transformed by repetitive low-dose LPS in maintaining health and homeostasis *in vivo*. In our first study, we characterized REPELL-microglia (7). Here, our goal was to characterize REPELL-pMΦ in order to determine the larger impact of repetitive low-dose LPS on other tissue-resident MΦ and to explore diversity based on their tissue origin.

LPS is generally known as a pro-inflammatory inducer and has been described as cytotoxic at high concentrations (>100 ng/ml) (15). By contrast, treatment with repetitive low-dose LPS did not induce cytotoxicity, and this dosing strategy promoted pMΦ proliferation (Figure 1). This result suggests that treatment with low-dose LPS (1 ng/ml) is less cytotoxic than that with high-dose LPS.

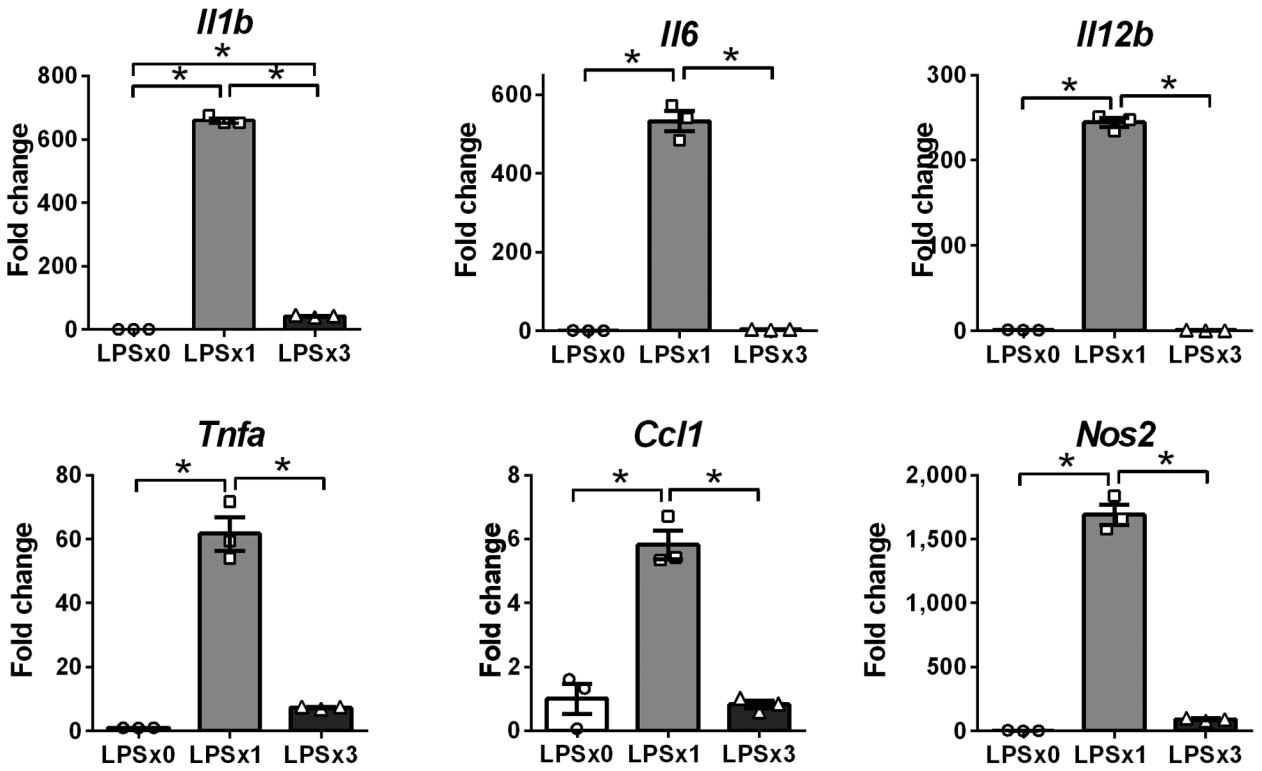

Figure 3. *Down-regulation of pro-inflammatory gene expression by repetitive low-dose LPS treatment of primary pMΦ. Primary pMΦ were treated with low-dose LPS (1 ng/ml) one or three times (n=3). Relative mRNA expression of pro-inflammatory mediators was measured at 4 h after the final LPS treatment by real-time RT-PCR using the $2^{-\Delta\Delta Ct}$ method 4 h after the final LPS treatment. Data were normalized to the expression of GAPDH and presented as the relative fold change over untreated controls. Mean±SE of each group are presented. Data are representative of two independent experiments. LPS ×0, no treatment; LPS ×1, single treatment with low-dose LPS; LPS ×3, treatment with low-dose LPS every 24 h for a total of three times. *p<0.05 for one-way ANOVA with Tukey's post-hoc correction for multiple comparisons.*

Phagocytosis assay revealed that REPELL-pMΦ exhibited high phagocytic activity which was comparable with that exhibited by SINGLL-pMΦ (Figure 2). This finding was associated with the up-regulated expression of the gene encoding FPR2. FPR2, encoded by the *Fpr2* gene, contributes to the resolution of inflammation by promoting phagocytosis (16, 17). This receptor may be involved in the enhanced phagocytic activity observed among REPELL-pMΦ. We previously demonstrated that oral administration of LPS resulted in enhanced phagocytosis of pMΦ *via* TLR4 (6), suggesting that FPR2 may be involved in this mechanism. Interestingly, results from a recent study indicated that FPR2 may exhibit antitumor activity (18), suggesting that REPELL-pMΦ may also exhibit tumor suppression activities. Because results from our previous study revealed that oral administration of LPS was associated with tumor suppression in a melanoma model developed by intraperitoneal injection of B16 cells (5), it is conceivable that FPR2 on pMΦ may serve to underscore the antitumor mechanism. However, phagocytosis mediated by pMΦ was suppressed by repetitive high-dose LPS treatment (11), and these findings are not consistent with enhanced phagocytic activity observed among REPELL-pMΦ in this study.

Quantitative RT-PCR analysis revealed that SINGLL-pMΦ exhibited up-regulated expression of pro-inflammatory genes including *Il1b, Il6, Il12b, Tnfa, Ccl1,* and *Nos2,* while the expression of these pro-inflammatory genes was suppressed in REPELL-pMΦ (Figure 3). This observation was consistent with that in previous reports which described pMΦ transformation by repetitive high-dose LPS treatment (9-12). In addition, we previously revealed that oral administration of LPS did not increase the secretion of pro-inflammatory mediators IL-6, TNF-α, and IL-12 from pMΦ (6); this finding is consistent with the suppression of pro-inflammatory gene expression observed in REPELL-pMΦ in this study. However, elevated expression levels of anti-inflammatory genes including *Arg1, Gipr* and *Gdnf* were observed in REPELL-pMΦ (Figure 4). Arg-1 is an arginine-metabolic enzyme encoded by *Arg1* gene, and plays a role in promoting anti-inflammatory responses by suppressing the production of nitric oxide from NOS2 (19). Likewise, GIPR is an incretin receptor encoded by the *Gipr* gene and several anti-

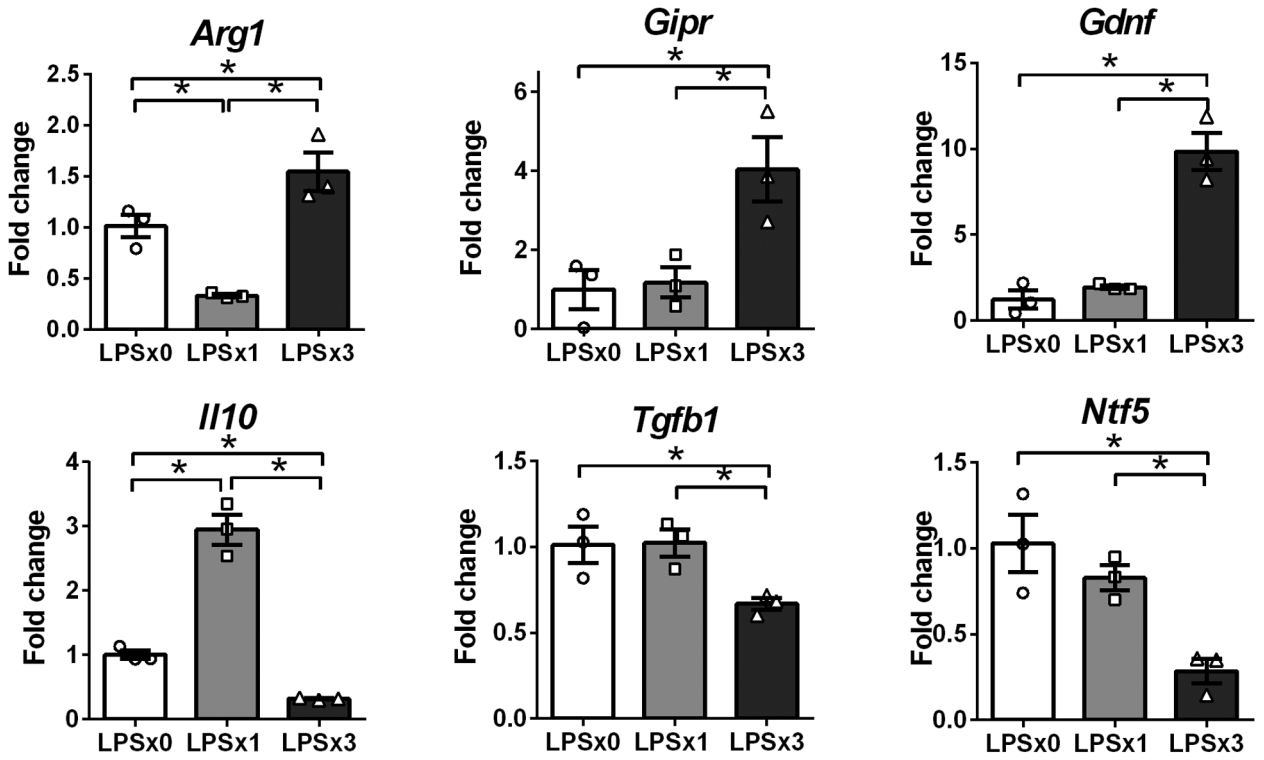

Figure 4. *Up-regulation of anti-inflammatory gene expression by repetitive low-dose LPS treatment of primary pMΦ. Primary pMΦ were treated with low-dose LPS (1 ng/ml) one or three times (n=3). Relative mRNA expression of anti-inflammatory molecules was measured at 4 h after the final LPS treatment by real-time RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Data were normalized to the expression of GAPDH and presented as the relative fold change over untreated controls. Mean±SE of each group are presented. Data are representative of two independent experiments. LPS ×0, no treatment; LPS ×1, single treatment with low-dose LPS; LPS ×3, treatment with low-dose LPS every 24 h for a total of three times. *p<0.05 for one-way ANOVA with Tukey's post-hoc correction for multiple comparisons.*

inflammatory effects have been attributed to this receptor, most notably with respect to pathologies associated with periodontal disease, arteriosclerosis, and obesity (20-22). GDNF is a neurotrophic factor encoded by the *Gdnf* gene and is involved in both anti-inflammation and neuroprotection (23, 24). Thus, REPELL-pMΦ may contribute to the maintenance of homeostasis *via* their profound anti-inflammatory effects. However, gene expression of *Il10, Tgfb*, and *Ntf5* was suppressed in REPELL-pMΦ compared with untreated controls and with SINGLL-pMΦ (Figure 4). These findings were inconsistent with previous reports of enhanced expression of *Il10*, *Arg1*, and *Tgfb1* in pMΦ transformed by repetitive high-dose LPS treatment (9).

In summary, we report here that REPELL-pMΦ are characterized by high phagocytic activity and elevated gene expression of *Arg1, Gip, Gdnf,* and *Fpr2*. Because the characteristics of REPELL-pMΦ were similar to those of pMΦ of mice orally administered with LPS, the *in vitro* REPELL-MΦ system may be used as a model to mimic the condition of oral administration of LPS. Some of the characteristics reported for REPELL-pMΦ were not in agreement with previous reports describing pMΦ transformed by repetitive high-dose LPS (9-12), suggesting diversity of pMΦ transformation depending on different doses of LPS as observed in REPELL-microglia (7). Because thioglycollate broth (TGC) promotes monocyte infiltration into the abdominal cavity and changes pMΦ population (25), it is also important to appreciate that changes of pMΦ population by TGC may also contribute to these discrepancies between the characteristics of REPELL-pMΦ and previous reports (9-12). Both of microglia and tissue-resident pMΦ are known to originate from the yolk sac (26, 27). For characterization of tissue-resident MΦ, pMΦ were collected from naïve mice without the administration of TGC. By contrast, in most previous reports, pMΦ were collected as peritoneal exudate cells after treatment with TGC (9, 10, 12). Since the pMΦ populations characterized in previous studies contain an estimated 50% infiltrating monocytes (25), REPELL-pMΦ in this study may represent characteristics of tissue-resident pMΦ rather than monocytes.

The gene expression profiles determined for REPELL-pMΦ in this study were different from those of REPELL-microglia, as presented in our previous study (7).

Table II. *Comparison of mRNA expression profiles: REPELL-pMΦ vs. REPELL-microglia.*

	vs. untreated		*vs.* SINGLL	
Gene	REPELL-pMΦ	REPELL-microglia	REPELL-pMΦ	REPELL-microglia
Il1b	→	→	↓	↓
Il6	→	→	↓	↓
Il12b	→	↑	↓	↓
Tnfa	→	↑	↓	↓
Ccl1	→	↑	↓	↑
Nos2	→	↑	↓	↑
Arg1	↑	↑	↑	↑
Gipr	↑	↑	↑	→
Gdnf	↑	↑	↑	↓
Il10	↓	↑	↓	→
Tgfb1	↓	→	↓	→
Ntf5	↓	↑	↓	↑
Fpr2	↑	→	→	↓

↑up-regulated; ↓down-regulated; →unchanged. Findings for REPELL-microglia were from our previous study (7).

Comparison of gene expression profiles between REPELL-pMΦ and REPELL-microglia are presented in Table II. Although some gene expression patterns were shared between REPELL-pMΦ and REPELL-microglia, profiles determined for *Ccl1*, *Nos2*, *Gdnf*, *Il10*, *Ntf5*, and *Fpr2* were clearly different between the two sets of resident macrophages. The comparison suggests that the tissue-resident MΦ are transformed by repetitive low-dose LPS to exhibit diverse phenotypes depending on their tissue origin. Indeed, this concept was supported by important studies focused on the diversity of tissue-resident MΦ phenotypes among various tissues and organs (8).

In conclusion, we characterized REPELL-pMΦ in this study and demonstrated that they exhibit high phagocytic activity and elevated expression of anti-inflammatory genes (*Arg1, Gipr,* and *Gdnf*) and phagocytosis associated gene (*Fpr2*). The results suggest that REPELL-pMΦ contribute to the maintenance of homeostasis *via* their impact on the clearance of xenobiotics and their role in the suppression of the inflammatory response. Further comparisons between REPELL-pMΦ and REPELL-microglia indicate that treatment with repetitive low-dose LPS induces differential transformation of tissue-resident MΦ depending on their tissue origin. The use of both *in vivo* and *in vitro/ex vivo* models of tissue-resident MΦ transformed by repetitive low-dose LPS will facilitate future human studies aimed at evaluating diverse MΦ transformation profiles and will provide important clues toward a better understanding of the homeostatic maintenance mechanisms promoted by oral administration of LPS.

Conflicts of Interest

HM, KY, MY, HI, and GS are employed by the Control of Innate Immunity, Technology Research Association. HI, CK, and GS are employed by Macrophi Inc. This does not alter our adherence to journal policies on sharing data and materials.

Authors' Contributions

HM, HI, and GS conceptualized the study and coordinated the experiments. HM, KY, and MY performed the experiments and HM performed data curation and formal analysis. HI and GS acquired the funding and administrated the project. HM wrote the manuscript supervised by HI, CK, and GS with contribution from all Authors.

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