mcr-1 Gene Expression Modulates the Inflammatory Response of Human Macrophages to *Escherichia coli*

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ABSTRACT MCR-1 is a plasmid-encoded phosphoethanolamine transferase able to modify the lipid A structure. It confers resistance to colistin and was isolated from human, animal, and environmental strains of *Enterobacteriaceae*, raising serious global health concerns. In this paper, we used recombinant *mcr-1*-expressing *Escherichia coli* to study the impact of MCR-1 products on *E. coli*-induced activation of inflammatory pathways in activated THP-1 cells, which was used as a model of human macrophages. We found that infection with recombinant *mcr-1*-expressing *E. coli* significantly modulated p38-MAPK and Jun N-terminal protein kinase (JNK) activation and pNF- κ B nuclear translocation as well as the expression of genes for the relevant proinflammatory cytokines tumor necrosis factor alpha (TNF- α), interleukin-12 (IL-12), and IL-1 β compared with *mcr-1*-negative strains. Caspase-1 activity and IL-1 β secretion were significantly less activated by *mcr-1*-positive *E. coli* strains than the *mcr-1*-negative parental strain. Similar results were obtained with clinical isolates of *mcr-1*-positive *E. coli*, suggesting that, in addition to colistin resistance, the expression of *mcr-1* allows the escape of early host innate defenses and may promote bacterial survival.

KEYWORDS MCR-1, macrophages, cytokines, inflammation

Biochemical modification of the lipid A component of the lipopolysaccharide (LPS) of Gram-negative bacteria may serve as a strategy for promoting bacterial survival inside the host by providing resistance to components of the innate immune system (e.g., antimicrobial peptides) and helping to evade recognition by Toll-like receptor 4 (TLR4) (1). The most frequent modification of LPS is the addition of phosphoethanolamine (pET) or 4-deoxyaminoarabinose residues to the phosphate moieties of the lipid A molecule, which decrease the negative charge of lipid A and prevent the binding of cationic antimicrobial peptides, including colistin, interfering with their bactericidal activity (1). In some cases, these changes may also help Gram-negative pathogens to circumvent detection and clearance from the host organism by evading the detection by TLR4 (2, 3).

In *Escherichia coli*, modification of lipid A can occur by the upregulation of endogenous LPS modification systems (e.g., *pmrHFIJKLM*) following activation of two-component regulatory systems which control their expression (e.g., PmrA-PmrB) (4, 5) or by the acquisition of exogenous lipid A-modifying enzymes, such as those encoded by transferable *mcr*-type genes via horizontal gene transfer (6). The *mcr*-type genes, encoding pET transferases, have been recently identified in human, animal, and environmental isolates of *Enterobacteriaceae* and have attracted remarkable attention since

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they can be responsible for transferable resistance to colistin, a last-resort antibiotic for multidrug-resistant Gram-negative infections. Among *mcr* genes, *mcr-1* and related variants are the most diffused worldwide (6).

The colonization by MCR-1-producing *Enterobacteriaceae* of healthy individuals and patients has been reported in many countries where *mcr-1*-positive *E. coli* are largely disseminated (6–8), and in some cases, colonization is unrelated to the clinical use of colistin (9). This resistance determinant is also emerging in high-risk clones of *E. coli* as sequence type 131 (ST131) (6, 10), further increasing global health concerns.

The functional consequences of *mcr-1* expression on microbe-host interactions have not yet been clarified. High-level *mcr-1* mutants showed reduced fitness and attenuated virulence in a *Galleria mellonella* infection model compared with their parent strains (11), and pure modified LPS from MCR-1 strains is less active than wild-type LPS in inducing the production of tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) in human macrophages (11), suggesting that *mcr-1*-related lipid A modification may interfere with host defenses. Besides the activation of surface Toll-like receptors (TLRs), bacterial ligands are able to activate inflammatory pathways even in the cytosolic compartment. In particular, the activation of caspase-1 in the context of an inflammasome complex induces the production of procytokines (IL-1 β and IL-18) and of gasdermin D that, once cleaved, forms membrane pores that lead to cytokine release (12).

Therefore, this study aims to compare the interaction of a *mcr-1*-positive *E. coli* MG1655 (MCRPEC) in PMA/THP-1 cells with respect to a *mcr-1*-negative MG1655 strain (MCRNEC). Additionally, the phosphorylation of NF- κ B (p65), p38-MAPK, and stressed-activated protein kinase/Jun N-terminal protein kinase (SAPK/JNK); the production of inflammatory cytokines; and the caspase-1 activity of infected macrophages were studied. Three clinical isolates of MCR-1-producing *E. coli* were used in the same experimental system, and their impact on macrophage functions was assessed (see Table S1 in the supplemental material).

RESULTS

Cloning and expression of the *mcr-1* gene. The *E. coli* MG1655 reference strain, representative of K-12 and being one of the first bacteria for which the whole-genome sequence has been obtained (13), was chosen as the bacterial host. The expression of the *mcr-1* gene in pLBil_*mcr-1*-carrying MG1655 conferred colistin resistance (MIC, 4 μ g/ml), compared with the colistin susceptible recipient strain carrying the empty vector pBCSk (MIC, ≤ 0.125 μ g/ml).

Endocytosis of MCRPEC and MCRNEC by PMA/THP-1 cells. PMA/THP-1 cells were cultured with MCRPEC or MCRNEC at different concentrations, and CFU counts in supernatants and cell lysates were recorded at different times. The results showed that >90% of bacterial cells of each strain were internalized after 1 hour. Table S2 in the supplemental material shows that the majority of internalized bacterial cells with MCRPEC or MCRNEC were killed after 2 hours (>99%). A slight increase in cells that survived MCRPEC compared to MCRNEC was observed after 2 and 4 hours (~1.6-fold).

Modulation of cytokine production by MCRPEC and MCRNEC in PMA/THP-1 cells. The expression of proinflammatory cytokine genes, such as TNF- α , IL-1 β , and IL-12 and of the anti-inflammatory cytokine IL-10 was evaluated by reverse transcription-quantitative PCR (RT-PCR). PMA/THP-1 cells were stimulated for 3 hours with MCRPEC or MCRNEC (50 multiplicity of infection [MOI]/cell) and afterward were incubated overnight in growth medium supplemented with antibiotics. PMA/THP-1 cells challenged with MCRPEC expressed smaller amounts of TNF- α , IL-1 β , and IL-12 mRNA than cells challenged with the isogenic MCRNEC strain (Figure 1a). The expression of the IL-10 gene was upregulated in cells with MCRPEC compared with those challenged with MCRNEC, but the differences did not reach statistical significance (Figure 1b). We also measured the concentrations of TNF- α , IL-1 β , IL-12, and IL-10 in the culture supernatants by immunoprecipitation assay. Despite the short time of stimulation with bacterial cells (3 hours), TNF- α , IL-1 β , and IL-10 were produced in measurable amounts and the results

FIG 1 Expression of proinflammatory cytokine genes by PMA/THP-1 cells. PMA/THP-1 cells were cultured in the presence or absence of MCRPEC, MCRNEC, or LPS as a positive control. Results represent the fold change mRNA compared with unstimulated cultures (mean $\pm$ SE of triplicate cultures). Data from three representative experiments are shown. Statistical analysis was performed with the Student's *t* test. A *P* value of <0.05 was considered statistically significant.

confirmed that PMA/THP-1 cultured with MCRPEC produced TNF- α and IL-1 β in smaller amounts than cells cultured with MCRNEC. In contrast, MCRPEC induced an increase in the production of IL-10 compared with MCRNEC (Figure 2b). These results suggested that the expression of the *mcr-1* gene is involved in the modulation of relevant inflammatory pathways in PMA/THP-1 cells.

To add further evidence to this hypothesis, three clinical isolates of *mcr-1*-positive *E. coli* isolates (FI-4551, FI-4531, and LC-17/15) were tested with PMA/THP-1 cells in the same experimental conditions used above. Figure 2 shows that all clinical isolates induced a lower production of IL-1 β and a higher production of IL-10 by PMA/THP-1 cells than MCRNEC. Interestingly, similar to MCRNEC, the clinical sample LC-17/15 had a higher production of TNF- α than MCRPEC and the other clinical samples. These results reinforce the hypothesis that *mcr-1* is involved in the escape of inflammatory pathways.

Regulation of NF- κ B, p38-MAPK, and SAPK/JNK phosphorylation by MCRPEC and MCRNEC in PMA/THP-1 cells. The nuclear translocation of phosphorylated NF- κ B (p65) and AP-1 through the coordinate functions of TRAF6 on NF- κ B and p38-MAPK and SAPK/JNK phosphorylation on AP-1 (14, 15) represent a convergence point of many activated pattern recognition receptor (PRR) pathways and a crucial step for the transcription of cytokine genes.

To investigate whether MCRPEC differs from MCRNEC in the activation of these pathways, PMA/THP-1 cells were cultured in the presence of live MCRPEC or MCRNEC and the phosphorylation NF- κ B (p65), p38-MAPK, and SAPK/JNK was assessed by Western blot analysis.

Results of these experiments showed that phosphorylated-NF- κ B (P-NF- κ B; p65), P-p38-MAPK, and P-SAPK/JNK were significantly reduced in cells tested with MCRPEC

FIG 2 Production of TNF- α , IL-1 β , and IL-10 by PMA/THP-1 cells. PMA/THP-1 cells were cultured in the presence of live bacteria (50 MOI/cell) for 3 hours. Supernatants were collected after 16 hours and analyzed by the immunoplex assay. ANOVA one-way analysis revealed significant differences among cultures with the different strains. The Student's *t* test was used to compare cytokine production in cultures with MCRPEC and MCRNEC. A *P* value of ≤ 0.05 was considered statistically significant.

compared with those incubated with MCRNEC (Fig. 3), suggesting that the expression of the *mcr-1* gene may interfere with the ability of bacterial cells to activate proinflammatory pathways in PMA/THP-1 cells.

Modulation of Caspase-1 activation by MCRPEC and MCRNEC in PMA/THP-1 cells. To investigate whether MCRPEC activates caspase-1 differently from MCRNEC, we cultured PMA/THP-1 cells with live bacterial cells for 6 hours and measured the caspase-1 activation through a Caspase-Glo 1 inflammasome assay.

Results of these experiments showed that caspase-1 activation was significantly lower in cultures of PMA/THP-1 with MCRPEC than cultures with MCRNEC (Figure 4a), indicating that MCRPEC affects inflammasome activation. Since caspase-1 is involved in gasdermin D activation, membrane damage, and osmotic lysis of the cell (12), we assessed whether the presence of *mcr-1* in endocytosed bacteria may attenuate these phenomena. For this reason, THP-1 cells were cultured with MCRPEC or MCRNEC (as reported above), and we measured lactate dehydrogenase (LDH) activity in culture supernatants and the incorporation of a DNA fluorescent dye in order to detect the cellular membrane damage.

Figure 4b shows that LDH activity in supernatants of PMA/THP-1 cultured with MCRPEC was significantly lower than that released in supernatants of the cells cultured with MCRNEC. These results suggest that PMA/THP-1 cultured with MCRPEC incor-

FIG 3 NF- κ B, p38-MAPK, and SAPK/JNK phosphorylation by PMA/THP-1 cells. PMA/THP-1 cells were cultured for 20 minutes in the absence (US) or presence of MCRPEC, MCRNEC, or LPS as a positive control. Cells were analyzed by Western blot analysis. Data from one representative experiment out of three performed are shown. The bar graph shows the results of densitometric analysis of three different experiments. Data are expressed as mean fold increase $\pm$ SE of stimulated cultures over the unstimulated control. Statistical analysis was performed by Student's *t* test. A *P* value of ≤ 0.05 was considered statistically significant.

porated smaller amounts of DNA-binding dye than PMA/THP-1 cells infected with MCRNEC (see Fig. S1 in the supplemental material).

Caspase-1 activation and LDH release were finally measured in cultures of PMA/THP-1 cells with clinical isolates of *mcr-1*-positive *E. coli* (Figure 4c and d). The activation of caspase-1 and the release of LDH were reduced in recombinant strains compared with MCRNEC as well as compared with the ATCC 25922 *E. coli* strain.

DISCUSSION

The dissemination of *mcr-1*-positive strains of *Enterobacteriaceae* suggests that *mcr-1*-mediated phenotypic changes might confer selective advantages to the bacterial

FIG 4 Caspase-1 activation and LDH release of PMA/THP-1 cells. (a) PMA/THP-1 cells were cultured in the presence of MCRPEC, MCRNEC, or LPS as a positive control for 6 hours. Caspase-1 activity is expressed as luminescence units (RLUs), as revealed by spectrophotometric analysis. (b) LDH activity in culture supernatants of PMA/THP-1 cells incubated with MCRPEC, MCRNEC, or LPS; data represent mean $\pm$ SE of triplicate cultures. (c and d) Caspase-1 activity and LDH production analysis in PMA/THP-1 cells cultured with isolated clinical samples. ANOVA one-way analysis revealed significant differences among cultures. Statistical analysis was performed by Student's *t* test. A *P* value of ≤ 0.05 was considered significant.

cells that go beyond the resistance to human cationic antimicrobial peptides (CAMPs) (1). Lipid A modifications can affect the stimulatory properties of LPS and the recognition of bacterial cells by cells of innate immunity. For example, the modification of pET to lipid A of *Neisseria gonorrhoeae* decreases the autophagy process activation in human macrophages, suggesting that it could represent a mechanism to escape the host immune system (16). The same modification in *Campylobacter jejuni* increases LPS recognition by human TLR-4 and also improves pathogen colonization, at least in a murine infection model (17). Modification in the lipid A structure in *Salmonella* strains did not cause a significant change in virulence (18). Therefore, it seems that the

functional consequences of pET addition to lipid A, in the context of host-pathogen interactions, depend on the bacterial species, the virulence of the strain, and its intrinsic ability to colonize the host.

For these reasons, the use of live bacterial cells, engineered to express the lipid A-modifying enzyme MCR-1, can provide information on host-pathogen interactions closer to reality than the use of single purified components. By using live bacterial cells to infect human macrophages, we observed that the expression of the *mcr-1* gene induces an immunomodulatory phenotype with a reduced expression of genes for the proinflammatory cytokines TNF- α , IL-1 β , and IL-12 which suggests the inhibition of M1 differentiation of macrophage cells (19) and the delay of pathogen clearance, as reported for different pathogens (20, 21).

Signaling activated by TLR usually converges in the nuclear translocation of NF- κ B and AP-1 following their phosphorylation by IRAK and p38-MAPK/JNK, respectively (14, 15, 22, 23). Interference with the activation of NF- κ B and/or p38-MAPK and/or SAPK/JNK represents a common mechanism for modulating the production of proinflammatory cytokines. Our data showed that infection with *mcr-1*-positive *E. coli* is able to modulate all of these pathways and therefore escape from innate recognition of its surface molecules.

The interference with cytosolic recognition of bacterial ligands, which leads to caspase-1 activation (24) is a further strategy used by pathogens to subvert innate immunity. Caspase-1 activation allows the release of mature IL-1 β and IL-18 (23, 25) through gasdermin-induced membrane pores. Activated caspase-1 can induce, through the same pathway, the osmotic lysis of the cell with the result of enhancing the inflammatory events at the infection site through the recruitment of phagocytes and other activated immune cells. Moreover, it has been reported that caspase-1 activity contributes to an increase in TLR-2 and TLR-4 signaling (26).

Our data suggest that the expression of the *mcr-1* gene also gives to *E. coli* cells the ability to escape recognition by cytosolic receptors and inflammasome activation. The recombinant *E. coli* strain (MCRPEC) and the 3 clinical isolates of *mcr-1*-positive *E. coli* modulate the inflammatory potential of THP-1 cells and limit the caspase-1 activation, the cellular membrane damage, and the massive release of inflammatory molecules compared with *E. coli* strains which do not express the *mcr-1* gene.

The alteration of these inflammatory pathways may therefore represent a common mechanism used by MCR-1-producing bacteria to prolong their survival within human hosts. A proper question is finally whether *mcr-1*-mediated lipid modifications are responsible for all the observed phenomena. Yang et al. used purified LPS extracted from *mcr-1* strains (11) to activate PMA/THP-1 cells and found a reduced production of TNF- α and IL-6, suggesting a reduced ability of modified LPS to activate inflammatory pathways. However, a recent proteomics and metabolomic profile of *mcr-1*-positive *E. coli* strains revealed the functional reduction of relevant metabolic processes, including the lipopolysaccharide biosynthesis (27). We could not rule out that, apart from lipid A modifications, the reduced synthesis of LPS could play a role in the observed modulation of proinflammatory events.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strain MG1655 from the Keio collection (28) was used for *mcr-1* expression, while *E. coli* DH10B was used as intermediate host to propagate the recombinant plasmid pLBII_ *mcr-1*. Three different clinical isolates of MCR-1-producing *E. coli* and *E. coli* ATCC 25922 were also included for comparison (Table S1) (7).

Bacteria were grown for 18 to 22 hours at 35 $\pm$ 1°C in cation-adjusted Mueller-Hinton (Becton, Dickinson and Company, MD, USA) broth under selective conditions (85 μ g/ml chloramphenicol) before incubation with cells.

Recombinant DNA methodology. The plasmid pLBII_ *mcr-1*, which was used for *mcr-1* expression experiments, was a pBC-SK(–) derivative (Agilent technologies, Santa Clara, CA) in which a Shine-Dalgarno sequence flanked by an NdeI site, separated by 4 bp, has been inserted downstream of the P_{LAC} promoter at the extremity of the vector polylinker (29). The pLBII_ *mcr-1* plasmid was constructed by amplification of the open reading frame *mcr-1* gene from strain FI-4451 (7), using the primers NdeI_MCR-1-F (5'-GGAATCCATATGATGCGAGCATACTTCTGTGG-3') and BamHI_MCR-1-R (5'-CGCGGATCCTCAGCGGATGAATGCGGTGC-3') (restriction sites are underlined). The resulting amplicon (1,642 bp) was digested with NdeI and BamHI and cloned into pLBII digested with the same enzymes. The authenticity of the

cloned fragments was confirmed by sequencing both strands at an external sequencing facility (Eurofins Genomics, Germany). The recombinant plasmid pLBIL_*mcr-1* was electroporated into competent cells as previously described (7, 30), and transformants were selected with chloramphenicol (85 µg/ml).

Endocytosis assay. The endocytosis assay was performed using *E. coli* strains MCRPEC and MCRNEC grown overnight in cation-adjusted Mueller-Hinton broth as described above. PMA/THP-1 cells were plated in 6 wells at 5×10^5 cells in 2 ml of medium and cultured with live bacteria (50 MOI/cell). Supernatant and lysates were collected at different times (1, 2, and 4 hours) and plated at different dilutions in selective medium for CFU determination. The lysates were obtained by treating the cells with 0.15% of Triton X-100 (Sigma-Aldrich, Milan, Italy) for 15 minutes at room temperature (RT).

Cell cultures and bacterial infection. The THP-1 cell line, derived from a human promonocytic leukemia patient, was obtained by ATCC (number TIB-202). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 5% L-glutamine at 37°C and 5% CO₂. For all the experiments, THP-1 cells were seeded in 24- to 96-well plates (5×10^5 cells/ml) in the presence of 20 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Milan, Italy) for 72 hours to allow differentiation. Then, the cells were washed and cultured for an additional 24 hours in fresh culture medium without PMA. These cells are referred as PMA/THP-1 cells.

The bacterial infection assay was performed using PMA/THP-1 cells cultured in the presence of live *E. coli* strains (20 or 50 MOI/cell) in medium without antibiotics (3 hours). Afterward, bacteria were removed, and the cells were incubated for additional times in medium supplemented with 1% of penicillin and 1% streptomycin. LPS was used as internal standard control in all the experiments at the concentration of 200 ng/ml. Additionally, in order to evaluate the possible fitness reduction of MCRPEC strains, the growth curves of both MCRNEC and MCRPEC bacterial strains in RPMI medium were evaluated and no significant differences were detected (data not shown).

RNA extraction and RT-PCR. RNA from each stimulated sample was obtained through TRIzol extraction (TRIzol reagent; Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and quantified using Nanodrop (Thermo Fisher, Waltham, MA, USA). A total of 2 µg of RNA was retrotranscribed using EuroScript Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (RNase H⁻) (EuroClone Pero, Italy). A total of 25 ng of cDNA from each sample was amplified by using the QuantiNova SYBR green PCR kit (Qiagen, Hilden, Germany), and the reaction was performed with the 7900HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression of genes for relevant proinflammatory cytokines was quantified using primers reported in Table S3 in the supplemental material.

Western blot analysis. To investigate the activation of NF-κB, p38-MAPK, and SAPK/JNK, 1×10^6 PMA/THP-1 cells were cultured for 20 minutes with live MCRPEC or MCRNEC at 50 MOI/cell. Cells were then lysed with RIPA buffer in the presence of phosphatase inhibitor cocktail 2 and 3, protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy), and centrifuged at $12,000 \times g$ for 15 minutes. The concentration of the proteins was determined by bicinchoninic acid (BCA) assay (EuroClone Pero, Italy). For each line, 40 µg of proteins was loaded on SDS-PAGE and blotted on nitrocellulose filters (Bio-Rad, Hercules, CA, USA). Membranes were stained with rabbit anti-caspase-1 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-IL-1β, rabbit anti-phosphorylated NF-κB (p65), rabbit anti-phospho-p38-MAPK, rabbit anti-phosphorylated SAPK/JNK (Cell Signaling Technology), and mouse anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by staining with anti-rabbit IgG (H+L) DyLight800 or anti-mouse IgG (H+L) DyLight 650 (Thermo Fisher, Waltham, MA, USA) as indicated (31). The reactions were visualized and acquired by the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Immunoplex assay. The concentrations of IL-1β, IL-10, IL-12 (p70), and TNF-α were determined in culture supernatants using the human cytokine/chemokine magnetic bead panel (Millipore, Bedford, MA, USA) and Luminex MagPix apparatus according to the manufacturer's recommendations.

DNA-binding dye assay. DNA damage was analyzed exploiting the RealTime-Glo annexin V apoptosis and necrosis assay (catalog number JA1011; Promega, Madison, WI, USA). Briefly, 5×10^4 PMA/THP-1 cells were seeded in 96 wells in 50 µl of medium without antibiotics and incubated with live bacteria (50 MOI/cell) for 24 hours at 37°C with 5% of CO₂. The incorporation of DNA-binding dye was recorded at different times (0, 3, 6, and 24 hours) using a Victor instrument to measure the fluorescence (relative fluorescence unit [RFU]).

Caspase-1 assay. Caspase-1 activity was quantified using Caspase-Glo 1 inflammasome assay (catalog number G9951; Promega, Madison, WI, USA) following the manufacturer's instructions. For this assay, 4×10^4 PMA/THP-1 cells were plated in 50 µl of growth medium without antibiotics and incubated for 6 hours with live bacteria of MCRPEC and MCRNEC (50 MOI/cell) at 37°C with 5% of CO₂. The amount of caspase-1 produced in PMA/THP-1 cells was measured in terms of luminescence (RLU) recorded using a Victor instrument.

LDH assay. LDH activity released from damaged cells was revealed in culture supernatants using the cytotoxicity detection kit plus (LDH) (Sigma-Aldrich, Milan, Italy) according to manufacturer's instructions.

Statistical analysis. Numerical data were expressed as mean and standard error, as indicated in the legend. We applied one-way analysis of variance (ANOVA) for multiple comparisons and the Student's *t* test for paired samples. The statistical significance was defined as a *P* value of <0.05. For the statistical analysis, we used R software version 3.6.2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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