Mitochondria respiratory chain (RC), consisting of five multisubunit complexes, is crucial for cellular energy production, reactive oxygen species generation, and regulation of apoptosis. Recently, a few mitochondrial proteins have been reported to be essential for innate immunity, but the function of mitochondrial RC in innate immunity is largely unknown. By knock-out of GRIM-19, a newly identified subunit protein of mitochondrial complex I, in mice, we found that heterogeneous mice (GRIM-19<sup>+/−</sup>) are prone to spontaneous urinary tract infection with reduced bacterial killing ability and proinflammatory cytokine production. These cells also have decreased intracellular killing ability against <i>S. saprophyticus</i>. The defects for this probably occur in the fusion of bacteria to lysosome, but not in the bacterial engulfment and macrophage migration. In addition, production of proinflammatory cytokine migration. In addition, production of proinflammatory cytokine production. Due to low cytokine production, the inflammatory response caused by in vivo bacterial challenge in the bladders of GRIM-19<sup>+/−</sup> mice is compromised. This study provides genetic evidence for a critical role of mitochondrial RC in innate immunity.

The mammalian innate immune system is critical for the early detection of the invading viral and bacterial pathogens and initiating cellular host defense responses. Detection of the microbes by host cells relies on several families of pattern recognition proteins. Four main families of pattern recognition proteins have been characterized: the transmembrane Toll-like receptors (TLRs),<sup>6</sup> cytosolic nucleotide oligomerization domain (NOD)-like receptors (NLRs),<sup>6</sup> C-type lectin receptors (CLRs), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (1). These pattern recognition proteins recognize the molecular markers of invaders, such as lipopolysaccharide (LPS), dsRNA, and unmethylated CpG DNA, signal to downstream pathways, including NF-κB, JNK, p38, and ERK, and initiate the production of nuclear-encoded pro-immune cytokines (2).

Mitochondria are organelles that control the “life and death” of a cell in most eukaryotes (3). As a powerhouse of the cell, one of the most important functions of mitochondria is to generate cellular energy (ATP) through oxidative phosphorylation. Mitochondrial respiratory chain (RC), localized in the inner membrane of mitochondria, consists of five multisubunit complexes (complexes I–V), which catalyze oxidative phosphorylation by transferring electrons from reducing substrates (NADH–FADH<sub>2</sub>) to molecular oxygen. The electron transport generates an electrochemical proton gradient across the mitochondrial inner membrane, which drives ATP synthesis by ATP synthase (complex V) (4, 5). During oxidative phosphorylation, reactive oxygen species (ROS) are also generated, which function as a signaling molecule and influence many cellular processes.
functions (6, 7). In addition, mitochondria also play important roles in the regulation of apoptosis and maintenance of intracellular Ca\(^{2+}\) homeostasis (8, 9). However, as the major functional components of mitochondria, whether mitochondrial RC directly regulates innate immunity is largely unknown.

GRIM-19 (genes associated with retinoid-IFN-induced mortality-19) was originally identified as an apoptosis-related gene in human cancer cell lines (10). We and others have recently demonstrated that GRIM-19 is a nuclear-encoded subunit of mitochondrial complex I (11–13). Strikingly, deletion of GRIM-19 alone totally destroys the assembly and enzymatic activity of complex I, although complex I contains 46 subunits in mammals (14). Knock-out of GRIM-19 in mouse causes early embryonic lethality (12), and knockdown of GRIM-19 in Xenopus leads to failure of early heart development (15).

In this study, we observed that mice with heterozygous GRIM-19 gene (GRIM-19\(^{+/-}\)) are prone to bacterial infection in their urinary tracts. Macrophages derived from these mice with a reduced mitochondrial complex I activity and enhanced ROS level display a decreased bacterial killing ability and production of selective proinflammatory cytokines. These results provide genetic evidence for the role of mitochondrial RC in innate immunity.

**EXPERIMENTAL PROCEDURES**

**Animals**—The mouse protocol was approved by the Biological Resource Centre Institutional Animal Care and Use Committee, Biopolis, Singapore. GRIM-19\(^{+/-}\) mice (C57BL/SW strain) were generated as described previously (12). 2–5 WT and GRIM-19\(^{+/-}\) mice from the same sex/age groups were mixed and bred in the same cage to eliminate the effect of environment on the phenotypes.

**Chemicals and Reagents**—Monodansylcadaverine (MDC), LPS, and muramyl dipeptide (MDP) were purchased from Sigma-Aldrich. Monoclonal antibodies against NDUFS3 and actin were purchased from Molecular Probes and Sigma-Aldrich, respectively. Antibody against mouse GRIM-19 was generated as described previously (12).

**Differentiation and Cell Culture for Bone Marrow-derived Macrophages (BMDMs)**—Bone marrow from mouse tibia and femur was harvested by flushing with EMEM-10 supplemented with 0.2 g/liter NaHCO\(_3\), 15 mM Hepes, pH 7.5, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin/streptomycin (Invitrogen). After centrifugation at 500 g for 10 min, bone marrow cells were resuspended in macrophage differentiation medium, which consists of 70% EMEM-10 and 30% L929 cell conditioned medium containing macrophage colony-stimulating factor. Bone marrow cells (2 \(
\times
\) \(10^5\)) were cultured in 10 ml of macrophage differentiation medium at 37 °C for 7 days. 10 ml of fresh macrophage differentiation medium was added at day 4. The adherent macrophages were detached from culture dishes by treatment with 1 mg/ml dispase (Sigma-Aldrich) followed by scraping with a sterile cell scraper. The resuspended cells were then directly used for migration assay or seeded on cell culture plates for other experiments.

**Bacteria Challenge on Macrophages**—BMDMs from WT and GRIM-19\(^{+/-}\) mice were cultured in macrophage differentiation medium and seeded in 6-well plates (2 \(
\times
\) \(10^5\) cells/well). Bacteria were cultured in LB medium at 37 °C until A\(_{560}\) of 4.2 (≈1 \(
\times
\) \(10^8\) bacteria/ml). Bacteria were then collected by centrifugation and resuspended in cell challenge medium (DMEM supplemented with 2 mM glutamine, 2 mM sodium pyruvate, and 10% FBS). After a brief wash of BMDMs with cell challenge medium, bacteria (multiplicity of infection = 50) were added onto the monolayer of BMDMs. The plates were incubated at 37 °C for 30 min to allow phagocytosis. Cells were then washed with and cultured in cell challenge medium containing 50 μg/ml gentamycin before being harvested for experiments. For measuring intracellular bacterial survival rate, macrophages were lysed in 1% Triton at the indicated time points after phagocytosis. The macrophage lysates containing internalized bacteria were diluted in LB medium and plated on an LB plate to determine output colony-forming unit (cfu). For MDC staining, BMDMs were grown on coverslips and treated with bacteria in the same manner as described above. After phagocytosis for 2 h, cells were incubated with 5 μM MDC for 15 min and then fixed, permeabilized, and stained with 0.1 μg/ml propidium iodide (PI) for 2 min. The MDC and PI staining were examined under an epifluorescence microscope.

16 S rDNA-based *Bacterial Identification*—Mouse urine was directly aspirated from bladder through a sterile needle and syringe. The urine was serially diluted in phosphate-buffered saline (PBS) and spread on yeast extract broth (YE) plate, which is LB supplemented with 5 g/liter yeast extract and 5 g/liter sucrose. After incubation at 37 °C overnight, the number of different colonies was counted, and the cfu/ml was determined. Each colony was characterized by 16 S rDNA sequencing. Briefly, the rDNAs from different colonies were amplified by primer F (5’-TAGCGAGTGGCCGACGGGTTG-3’) and primer R (5’-CCATGGTGTCAGGCGGTGTTG-3’). The 1.3-kb PCR products were cloned into the pDrive vector and sequenced using primer S (5’-TACGGGAAGCAGCATGTGGGAATA-3’). The 16 S rDNAs homologies were determined by using the BLAST tool in the Entrez Nucleotide database.

**Complex I Spectrophotometric Enzyme Assay**—5 \(\times\) \(10^6\) BMDMs from WT and GRIM-19\(^{+/-}\) mice were challenged with *Staphylococcus saprophyticus* for 3 h. Mitochondria from BMDMs were isolated and subjected to NADH oxidation assay as described previously (13).

**RT-PCR and Real-time PCR**—RT-PCR was performed using the OneStep RT-PCR kit (Qiagen). For real-time PCR, RNA was first reverse-transcribed using the Expand reverse transcriptase from Roche Applied Science. Real-time PCR was then performed using the cDNA and SYBR Green PCR mix (Applied Biosystems) according to the manufacturer’s instructions. Primers for genes in real-time RT-PCR are: IL-10, forward 5’-AGTGGTGATGTGCCTGCTGG-3’, reverse 5’-GTGAGCACAGGTTGACAG-3’; interferon-γ (IFN-γ), forward 5’-GGCACTGTCATTCACAGGTA-3’, reverse 5’-CTTCAATCTTCGAGCT-3’; TNF-α, forward 5’-GCTGGTCCACTTGAATGA-3’, reverse 5’-GTGAGACAGTGGTTC-3’.
**Induction of Acute Cystitis**—Female WT and GRIM-19\(^{+/−}\) mice (40 weeks) were anesthetized with ketamine HCL (40 mg/kg) and xylazine (2.5 mg/kg). A 24-gauge polypropylene catheter (EXEL) was transurethrally inserted into bladder. After drainage of the remaining urine in the bladder, 150 μl of DMEM medium containing 1 × 10⁸ of *S. saprophyticus* was instilled into mouse bladder through a syringe (see Fig. 6A). The syringe was kept on the catheter, allowing the bladder mucus to soak in bacteria solution for 1 h. The catheter was then removed, and the mouse was allowed to void thereafter. Mice bladders were harvested 24 h after infection and sent for histological analysis.

**Histological Analysis and Gram Staining**—Mouse tissues were fixed in formalin and embedded in paraffin. Tissues were cut into 4-μm sections and stained with hematoxylin and eosin (H&E staining). Mouse penis slides were also stained for Gram-positive bacteria by Gram staining kit (Sigma-Aldrich) following the manufacturer’s instructions. Eosin was used for counterstaining in Gram staining.

**Cell Migration Assay**—Cell migration was quantified in triplicates using the CytoSelect\(^{TM}\) 96-well cell migration assay system (Cell Biolabs) according to the manufacturer’s instructions.

**Phagocytosis Assay**—Phagocytosis was quantified in triplicates using the CytoSelect\(^{TM}\) 96-well phagocytosis kit (Cell Biolabs) according to the manufacturer’s instructions.

**Measurement of ATP**—Total free cellular ATP was measured using bioluminescence assay kit HSII (Roche Applied Science) according to the manufacturer’s instructions.

**Flow Cytometric Analysis of ROS**—Fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFH-DA) (Molecular Probes), was used to determine the intracellular ROS levels. Cells were harvested and washed with PBS and then exposed to 5 μM H₂DCFH-DA at 37 °C for 1 h. ROS levels were analyzed by flow cytometry using a BD Bioscience FACScan machine.

**ELISA**—Mouse IL-6, IL-10, IL-12 and TNF-α were detected by ELISA using the ELISA MAX™ set from BioLegend according to the manufacturer’s instructions. Briefly, BMDMs from WT and GRIM-19\(^{+/−}\) mice were infected with *S. saprophyticus* or stimulated with LPS (10 ng/ml) with or without MDP (10 μg/ml) for 6 or 20 h. The cell culture medium was harvested and diluted before adding it into ELISA plates, which were coated with capture antibodies. The plates were kept at room temperature for 2 h to allow the cytokines in the medium to bind to capture antibodies. The cytokines were then detected by detection antibodies and quantified using a microplate reader at A₄₅₀ nm. Serially diluted cytokine standards were used to generate standard curve in each assay.

**Statistical Analysis**—The results were analyzed with the χ²-square test or Mann-Whitney test and Student’s t test. A p value of ≤ 0.05 was considered to be significant.

**RESULTS**

**GRIM-19\(^{+/−}\) Mice Are Prone to Urinary Tract Infection**—Although GRIM-19 homozygous knock-out mice (GRIM-19\(^{−/−}\)) died in the early stage of embryonic development, the heterozygous mice (GRIM-19\(^{+/−}\)) developed normally and were undistinguished from WT mice by appearance and behavior (12). However, we observed over 2 years that the heterozygous male mice were prone to spontaneous obstructed urinary tract infection (UTI). The infected mice usually presented with a swollen red penis and urinary retention caused by blockage of urethra (Fig. 1A). The urethra was completely obstructed by an eosinophilic and amorphous fibrinoid material that contained bacterial debris. The Gram stain showed the presence of Gram-positive bacteria (Fig. 1B). At 20 weeks of age, almost all WT mice were healthy (Fig. 1C, maroon bar). However, penis infection and urinary retention were observed in 20% of GRIM-19\(^{+/−}\) mice (purple bar). The rate of UTI increased as the mice grew older. At 80 weeks of age, 10% of WT mice developed UTI. In contrast, more than 60% of GRIM-19\(^{+/−}\) mice of the same age displayed UTI, which is much higher than that in the WT mice.

To identify the bacteria that caused UTI in the mice, we collected urine from bladders of seven GRIM-19\(^{+/−}\) mice with urine retention for bacterial culture. Homogenized penis tissue from one of the GRIM-19\(^{+/−}\) mice (number 7) was also included for bacterial culture in the same manner as the urine samples. After overnight culture, the bacterial numbers of different colonies were counted to determine the bacterial load in urine. The species of bacteria were identified by 16 S rDNA-based bacterial identification (Table 1). *S. saprophyticus* caused the majority of cases of UTI in mice. In some mice, *Staphylococcus xylosus*, *Escherichia coli*, and *Enterococcus faecalis* also contributed to the development of UTI. The cfu of *S. saprophyticus* in the urine culture was very high, ranging from 5.2 to 30.1 × 10⁶. *S. saprophyticus* is a coagulase-negative species of *Staphylococcus*. Studies showed that it is uniquely associated with UTIs. This suggests that *S. saprophyticus* is a major contributor to UTI in GRIM-19\(^{+/−}\) mice.
with uncomplicated UTI in humans. It is second to E. coli as the most frequent causative organism of uncomplicated UTIs in young women, although it can also cause UTI in males of all ages (16). Although the WT and GRIM-19 +/- mice were kept in the same cages, the infection rate and the bacterial load detected in urine of GRIM-19 +/- mice were much higher than those in the WT mice. These data suggest a compromised immunity against bacterial infection in GRIM-19 +/- mice.

**Bacterial Stimulation Increases GRIM-19 Expression and Mitochondrial Complex I Activity in the BMDMs**—To investigate whether GRIM-19/complex I is directly related to the bacterial infection, we first examined whether bacterial stimulation changed the gene expression of GRIM-19 in BMDMs from WT and GRIM-19 +/- mice. The GRIM-19 protein level increased significantly after stimulation with different Staphylococcus strains including Staphylococcus aureus, Staphylococcus epidermidis, and S. saprophyticus in the WT BMDMs as compared with the unstimulated cells (Fig. 2A). The basal level of GRIM-19 was lower in GRIM-19 +/- BMDMs, as expected, which was also increased after bacterial stimulation, but in much lower degree in comparison with the WT BMDMs (Fig. 2B). In addition to GRIM-19, the protein level of another complex I subunit, NDUFS3, was also increased after S. saprophyticus infection with little difference between the two cell types. Furthermore, bacterial infection significantly increased the complex I activity in both WT and GRIM-19 +/- BMDMs. The level of increase was ~2-fold in the WT cells as compared with GRIM-19 +/- cells (Fig. 2C). However, there is no statistical difference of ATP production between WT and GRIM-19 +/- BMDMs, although a trend of decreasing ATP level in GRIM-19 +/- BMDMs was observed (Fig. 2D). Last, the ROS was measured in the WT and GRIM-19 +/- BMDMs. A higher ROS level was observed in the GRIM-19 +/- BMDMs as compared with the WT BMDMs (Fig. 2E, left panel). Bacterial infection did not change the pattern (right panel).

**Decreased Bacterial Killing Ability in GRIM-19 +/- BMDMs**—Upon bacterial infection, macrophages migrate toward the site of infection and engulf invading bacteria (phagocytosis). The ingested pathogens fuse with a lysosome to be digested. During this process, macrophages produce a number of cytokines and chemokines to trigger inflammation responses. To further dissect the function of GRIM-19 in the bacterial killing, we tested the effects of GRIM-19 reduction in these processes. GRIM-19 +/- BMDMs exhibited a normal migration (Fig. 3A) and phagocytosis (Fig. 3B). We also examined the fusion of bacterial phagosome with lysosome by staining the BMDMs with PI, a DNA dye used to visualize the engulfed bacteria, and MDC, used as a lysosome marker (17). After 3 h of infection, the majority of engulfed bacteria were fused with lysosome in the WT BMDMs, whereas the fusion occurred only partially in the GRIM-19 +/- BMDMs (Fig. 3C). The percentage of colocalization of bacteria (PI) with lysosome (MDC) is presented in Fig. 3D. The results indicated that GRIM-19 +/- BMDMs had a decreased capability to deliver the engulfed bacteria to lysosome. In agreement with this result, the bacterial killing ability, determined by the intracellularly living bacterial number after phagocytosis, was also reduced in the GRIM-19 +/- BMDMs as compared with the WT BMDMs (Fig. 3E). About 60% of S. saprophyticus was still alive inside GRIM-19 +/- BMDMs in comparison with 35% survival rate in the WT BMDMs after phagocytosis for 3 h. After 5 h, the survival rate of S. saprophyticus further decreased to 25% in the GRIM-19 +/- BMDMs as compared with 8.5% in the WT cells, so the survival rate is nearly three times higher in the GRIM-19 +/- than in the WT cells. This indicates that GRIM-19 +/- BMDMs kill bacteria less efficiently than the WT BMDMs.

**Cytokine Production in GRIM-19 +/- BMDMs**—Next, we tested various cytokine productions by ELISA in BMDMs after S. saprophyticus infection. Bacterial stimulation triggered dramatic increase of production of various cytokines, including IL-6, IL-10, IL-12, and TNF-α in both WT and GRIM-19 +/- BMDMs (Fig. 4, A–D). Although not much difference in the IL-10 and TNF-α production was observed between WT and GRIM 19 +/- BMDMs, GRIM 19 +/- BMDMs produced much less IL-12 and IL-6, to a lesser extent, than WT BMDMs upon bacterial stimulation. The cytokine production was also measured at the mRNA level by RT-PCR (Fig. 4E) and real-time RT-PCR (Fig. 4F). The results were similar. In addition to IL-6, IL-10, IL-12, and TNF-α, IL-1α, IFN-γ, and IFN-β were also up-regulated by S. saprophyticus infection in the WT and GRIM-19 +/- cells. The induction of IL-1α and IFN-γ mRNAs was lower in the GRIM 19 +/- BMDMs in comparison with levels in the WT BMDMs, but no obvious difference in IFN-β production was observed in both cell types (Fig. 4E). These data suggest that GRIM-19 knockdown specifically compromised production of certain cytokines in BMDMs upon S. saprophyticus infection.

PAMPs (pathogen-associated molecular patterns) are bacterial products that bind to and activate TLRs localized at cell membranes or the cytosolic NLRs such as nucleotide oligomerization domain 2 (NOD2) and stimulate cytokine production (18). To examine the role of GRIM-19 in PAMP-stimulated cytokine production, we also treated WT and GRIM 19 +/- BMDMs with LPS (TLR-4 ligand) and MDP (NOD2 ligand). As shown in Fig. 4, A–D, LPS alone or in combination with MDP stimulated the production and secretion of cytokines such as IL-6, IL-10, IL-12, and TNF-α. These PAMPs induced the same pattern of cytokine production as the live S. saprophyticus. That was, less IL-12 and IL-6 were produced by treatment with LPS and MDP in GRIM-19 +/- BMDMs, and no difference was seen in the production of IL-10 and TNF-α in both BMDMs. These

**TABLE 1**

**Bacteria isolated from the mice with UTI**

| Sample | Bacteria stains (percentage) | Bacteria counts |
|--------|------------------------------|-----------------|
| Urine 1 | S. saprophyticus (100%) | 15.4 ± 10^6 cfu/ml |
| Urine 2 | S. saprophyticus (100%) | 7.1 ± 10^6 |
| Urine 3 | E. faecalis (100%) | 8.7 ± 10^6 |
| Urine 4 | S. saprophyticus (82%); S. xylosus (18%) | 17.5 ± 10^6 |
| Urine 5 | S. saprophyticus (95%); E. coli (5%) | 5.2 ± 10^6 |
| Urine 6 | S. saprophyticus (63%); E. faecalis (37%) | 30.1 ± 10^6 |
| Urine 7 | S. saprophyticus (100%); E. coli (3%); E. faecalis (27%) | 28.6 ± 10^6 |

S. saprophyticus, E. coli, and M. faecalis were the most common bacteria isolated from the urine sample. The bacterial load was significantly higher in the GRIM-19 +/- mice compared with the WT mice.
results suggest that GRIM-19 may affect cytokine production via TLR-4 and NOD2 pathways.

Mitochondrial RC Function Is Important for Cytokine Production in BMDMs—GRIM-19 is one of the subunits in mitochondrial complex I. To evaluate the role of the mitochondrial RC in bacterial infection-induced cytokine production, we treated the WT BMDMs with RC inhibitors. Rotenone, a specific inhibitor of complex I, decreased IL-1α, IL-12β, IFN-γ, and IL-10 production upon bacterial stimulation and had no effect on TNF-α production (Fig. 5A), which is similar to the levels in the GRIM-19+/− BMDMs. In comparison with the rotenone treatment, GRIM-19 knockdown has no effect on IL-10 production (Fig. 4). This could be due to a basal complex I activity that remained in GRIM-19+/− BMDMs in contrast to a total inhibitory effect by rotenone. Oligomycin, an inhibitor of mitochondrial ATP synthase (complex V), has a very similar effect on the BMDM cytokine production as rotenone (Fig. 5A). The role of mitochondrial RC on LPS- and MDP-induced cytokine production was also examined. Different concentrations of LPS and MDP induced various productions of cytokines (Fig. 5B). Rotenone inhibited the production of IL-12 family members (IL-12α, IL-12β, IL-23α, and IL-27), IL-6, IL-10, and...
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**FIGURE 3. Decreased bacterial killing ability in GRIM-19+/− BMDMs.** A and B, cell migration (A) and phagocytosis (B) of BMDMs were measured as described under "Experimental Procedures." OD, optical density; C, BMDMs from WT and GRIM-19+/− mice were infected with S. saprophyticus at multiplicity of infection 50 for 2 h followed by MDC staining. The cells were then fixed and stained with PI for bacterial visualization. The internalized bacteria (red particles in cells) and its colocalization with MDC staining (blue particles) were examined under fluorescence microscope. D, the statistical data showing the percentage of S. saprophyticus colocalized with positive MDC staining. E, BMDMs from WT and GRIM-19+/− mice were infected with S. saprophyticus at multiplicity of infection 50. BMDMs were lysed at the indicated time points after infection. The viable bacteria in cells were determined by serial dilution and growth on LB plate. Error bars in panels A, B, D, and E indicate S.D.

IFN-γ, but not TNF-α. Together, these data suggest that the mitochondrial RC is important for the bacterially induced cytokine production.

**GRIM-19 Is Crucial for Bacteria-induced Inflammatory Response in Vivo—** Bacterial infection triggers inflammatory reaction, which is characterized by an increase in vascular permeability, tissue edema, and neutrophil infiltration. The infiltrating neutrophils kill invading pathogens at the site of infection. The inflammatory responses are accelerated by a variety of proinflammatory cytokines including IL-12 and IL-6. Thus, we checked whether reduction of GRIM-19 affects the bacteria-induced inflammatory response in vivo. We generated an acute bacterial cystitis model by injection of 1 × 10⁸ of S. saprophyticus into mouse bladder (Fig. 6A). The acute inflammatory response in bladder was examined by histological staining 24 h after intravesical instillation of S. saprophyticus. The severity of inflammation was graded as + to +++ by the level of tissue edema and neutrophil infiltration (Fig. 6B). As shown in Fig. 6C, the bacteria-induced inflammation was significantly more severe in the bladders from WT mice than those from GRIM-19+/− mice (p < 0.05). The data suggest that GRIM-19 is crucial for bacteria-induced inflammatory response in vivo.

**DISCUSSION**

Mitochondrial defects have been implicated in a wide variety of clinical problems and age-related diseases (19, 20). However, their function in immunity was largely unknown. Discovery of mitochondrial antiviral signaling (MAVS) being an essential gene for virus-induced innate immunity and a novel mitochondrial protein provides the first clue for a direct involvement of mitochondria in immunity. Subsequently, more mitochondrial proteins have been found to regulate antiviral immunity (21–23). Furthermore, one of such proteins, NLRX1, is implied to associate with a subunit of mitochondrial RC complex III, UQCRC2 (24). In addition, mitochondrial dynamics controlled by fusion/fission have also been reported to regulate antiviral pathways (25). These findings highlight novel roles of mitochondria in immunity. In this study, using GRIM-19+/− mice as a model, we provide the first genetic evidence demonstrating that mitochondrial respiratory chain plays an indispensable role in antibacterial immunity in mice.

We found that mice with heterozygous genotype of GRIM-19 succumbed to bacterial infection in their urinary tracts at least partially due to decreased bacterial killing ability. One of the possible defects is in the fusion of bacteria and lysosomes (Fig. 3). Autophagy is a process for delivery of cytosolic protein or organelles to lysosomes for degradation (26), which is involved in trapping and killing many intracellular pathogens (27–29). Thus, reduced bacteria and lysosome fusion could be due to impaired autophagy. To investigate this possibility, we checked the protein level of microtubule-associated protein 1 light chain 3 (LC3), a widely used autophagic marker. Indeed, basal level of LC3 expression was reduced in the GRIM-19+/− BMDMs. However, under our experimental conditions, bacterial challenge failed to further reduce LC3 level in the macrophages (data not shown). Thus, the involvement of autophagy in the mitochondria-mediated intracellular killing requires further investigation.

Bacterial challenge induces production of a number of cytokines that are important to regulate innate and adaptive immune response against bacteria. By microarray, we identified many cytokines whose production was reduced in the GRIM-19+/− BMDMs (data not shown). This was further confirmed by ELISA and RT-PCR (Fig. 4). We found that IL-12, IFN-γ, and IL-6 production was decreased in GRIM-19+/− BMDMs as compared with levels in WT BMDMs upon bacterial stimulation. Mitochondrial RC inhibitors, rotenone and oligomycin, also reduced production of those cytokines, which suggests that production of IL-12, IFN-γ, and IL-6 is mitochondrial RC-dependent. Pro-inflammatory cytokines, IL-12, IFN-γ, and IL-6, play important roles in triggering inflammatory response including vascular dilation and neutrophil infiltration. In agreement with this, the severe bacterial infection led to a very mild inflammatory response in GRIM-19+/− urinary mucosa (Fig. 1B), as well as in the in vivo bacterial challenge model (Fig. 6). Thus, the compromised production of pro-inflammatory cyto-
Kines may contribute to the immunodeficiency in GRIM-19−/− mice.

The exact molecular mechanisms of how GRIM-19 regulates cytokine productions are not fully understood. It has been reported that partial inhibition of oxidative phosphorylation by spontaneous mutation in the nuclear encoded subunits can increase ROS (30). As expected, we observed an enhanced ROS level in GRIM-19−/− BMDMs that compared with the WT cells (Fig. 2E). ROS, as a common second messenger for the induction of numerous cellular processes, has been well defined in antimicrobial immune signaling and phagocyte bactericidal activity. It regulates activation of key transcription factors such as NF-κB and activator protein-1 (AP-1) in control of macrophage effect or functions and cytokine production (1). It is therefore proposed that ROS might play a functional role linking GRIM-19 and antibacterial innate immunity. Hoetzenecker et al. (31) reported that depletion of glutathione, which is the primary intracellular ROS scavenger, abrogated LPS-induced IL-6 and IL-12β mRNA in mouse macrophages, and an antioxidant, N-acetylcysteine, fully reversed this suppression. Our

FIGURE 4. Decreased IL-12 production in GRIM-19−/− BMDMs. A–D, BMDMs from WT and GRIM-19−/− mice were infected with S. saprophyticus (SS) or stimulated with LPS alone (10 ng/ml) or with MDP (10 μg/ml) for 6 or 20 h. The cell culture medium was harvested to check the production of IL-6 (A), IL-12 (B), IL-10 (C), and TNF-α (D) by ELISA. E and F, BMDMs from WT and GRIM-19−/− mice were infected with S. saprophyticus for 6 h. Total RNA were isolated and used for measuring various cytokine gene expressions by RT-PCR (E) and real-time RT-PCR (F). Relative gene expression indicates the -folds of increase in comparison with the basal level expression of WT and GRIM-19−/− BMDMs without treatment in F. Error bars in panels A–D and F indicate S.D.
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observation of reduction of IL-6 and IL-12β cytokines with a concomitant rise of mitochondria-derived ROS in the GRIM-19+/− BMDMs is in agreement with these findings. The Jackson group (32) revealed a similar result in dendritic cells. They additionally investigated the underlying mechanism showing a negative regulation of ROS in IL-12 expression by constraining p38 MAPK activity. Another study, done by Khan et al. (33), reported that H2O2 activates calmodulin protein, which prevents the nuclear translocation of c-Rel transcription factor and blocks subsequent transactivation of IL-12β in macrophages. This study also showed that c-Rel plays a dominant role over p65 NF-κB in activating IL-12 transcription (33). Considering a minor effect on NF-κB activation stimulated by bacterial challenge in the GRIM-19+/− BMDMs (data not shown), it will be interesting to determine whether signaling factors such as c-Rel or p38 MAPK act as the downstream mediator of ROS in control of cytokine production in the GRIM-19+/− BMDMs in future studies.

NOD2 is a mammalian cytosolic pathogen recognition molecule, and its variants have been associated with risk for Crohn disease. Interestingly, Barnich et al. (34) reported that expression of GRIM-19 was significantly decreased in the involved area of colonic biopsies from Crohn disease and ulcerative colitis patients. This study also showed that expression of GRIM-19 in Caco-2 cells decreased, whereas down-regulation of GRIM-19 by siRNA increased, the invasive ability of Salmonella, suggesting a protective role of GRIM-19 in host against bacterial infection. Our finding of GRIM-19 function in bacterial infection is in agreement with these results. Because NOD-2 was found to associate with GRIM-19 in that study, it will be interesting to further investigate whether NOD-2 pathway is involved in GRIM-19-regulated mouse innate immunity.

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