Persistent colonization of non-lymphoid tissue-resident macrophages by Stenotrophomonas maltophilia

Takahashi, Ichiro; Hosomi, Koji; Nagatake, Takahiro; Tobou, Hirokazu; Yamamoto, Daiki; Hayashi, Ikue; Kurashima, Yosuke; ... Abe, Akio; Kunisawa, Jun; Kiyono, Hiroshi

International Immunology, Vol. 32, No. 2, pp. 133–141
doi:10.1093/intimm/dxz071
Advance Access publication 20 October 2019

Persistent colonization of non-lymphoid tissue-resident macrophages by *Stenotrophomonas maltophilia*

Ichiro Takahashi[1,2], Koji Hosomi[2], Takahiro Nagatake[2], Hirokazu Toubou[1], Daiki Yamamoto[1], Ikue Hayashi[1], Yosuke Kurashima[3], Shintaro Sato[3], Naoko Shibata[3,8], Yoshiyuki Goto[3,9], Fumito Maruyama[4,10], Ichiro Nakagawa[4], Asaomi Kuwae[5], Akio Abe[5], Jun Kunisawa[2,3,6,7] and **Hiroshi Kiyono[3]**

[1]Department of Mucosal Immunology, Graduate School of Biomedical and Health Science, Hiroshima University, Hiroshima 734-8553, Japan
[2]Laboratory of Vaccine Materials, Center for Vaccine and Adjuvant Research and Laboratory of Gut Environmental System, National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki-Osaka 567-0085, Japan
[3]International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
[4]Department of Microbiology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan
[5]Laboratory of Bacterial Infection, Kitasato Institute for Life Sciences, Kitasato University, Tokyo 108-8641, Japan
[6]Graduate School of Medicine, Graduate School of Pharmaceutical Sciences, and Graduate School of Dentistry, Osaka University, Suita-Osaka 565-0871, Japan
[7]Graduate School of Medicine, Kobe University, Kobe-Hyogo 650-0017, Japan

[8]Present address: School of Advanced Science and Engineering, Waseda University, Tokyo 169-8555, Japan
[9]Present address: Division of Molecular Immunology, Medical Mycology Research Center, Chiba University, Chiba 206-8673, Japan
[10]Present address: Microbial Genomics and Ecology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8530, Japan

Correspondence to: J. Kunisawa; E-mail: kunisawa@nibiohn.go.jp

Received 24 September 2019, *editorial decision* 6 October 2019; *accepted* 17 October 2019

Abstract

Accumulating evidence has revealed that lymphoid tissue-resident commensal bacteria (e.g. *Alcaligenes* spp.) survive within dendritic cells. We extended our previous study by investigating microbes that persistently colonize colonic macrophages. 16S rRNA-based metagenome analysis using DNA purified from murine colonic macrophages revealed the presence of *Stenotrophomonas maltophilia*. The *in situ* intracellular colonization by *S. maltophilia* was recapitulated *in vitro* by using bone marrow-derived macrophages (BMDMs). Co-culture of BMDMs with clinically isolated *S. maltophilia* led to increased mitochondrial respiration and robust IL-10 production. We further identified a 25-kDa protein encoded by the gene assigned as *smlt2713* (recently renamed as SMLT_RS12935) and secreted by *S. maltophilia* as the factor responsible for enhanced IL-10 production by BMDMs. IL-10 production is critical for maintenance of the symbiotic condition, because intracellular colonization by *S. maltophilia* was impaired in IL-10-deficient BMDMs, and *smlt2713*-deficient *S. maltophilia* failed to persistently colonize IL-10-competent BMDMs. These findings indicate a novel commensal network between colonic macrophages and *S. maltophilia* that is mediated by IL-10 and *smlt2713*.

Keywords: colitis, gut-resident macrophage, IL-10, intracellular commensal bacteria, symbiotic factor smlt2713

Introduction

The gut microbiota drives the maturation and function of the immune system (1, 2), including the production of secretory IgA and development of intra-epithelial lymphocytes in the small intestine (3, 4). Accumulating evidence has revealed that particular genera of commensal bacteria control the differentiation of specific T-cell populations. For example, segmented filamentous bacteria induce the differentiation of T_h17 cells (5), and clostridial strains induce regulatory T cells (6).

Although these previous studies mainly focused on the commensal bacteria in the intestinal lumen or mucus layers, genome-based bacterial analysis of intestinal tissues revealed *Alcaligenes* spp. as symbiotic resident bacteria of Peyer's patches (PPs), a key gut-associated lymphoid tissue in the small intestine (7–9). Orally administered *Alcaligenes* spp. is exclusively taken up by M cells in the PPs of gnotobiote mice (7). Furthermore, histologic analysis showed that *Alcaligenes* spp. expressing green fluorescent protein occurred predominantly within $CD11c^+$ dendritic cells (DCs) at the subepithelial dome region of PPs (10). Furthermore, *Alcaligenes* spp. enhance IgA production through up-regulation of expression of IL-6 and TGF-β from DCs in PPs (7). This anatomic containment is regulated by IL-22-producing innate lymphoid cells, and the depletion of this cell population resulted in systemic dissemination of the bacterium. Consequently, *Alcaligenes*-specific systemic IgG immune responses were associated with Crohn's disease and progressive hepatitis C virus infection in patients (8), suggesting that the presence of the microbe within gut-associated lymphoid tissue is indispensable for local as well as systemic homeostatic conditions in the hosts.

The intestinal lamina propria contains a heterogeneous mixture of CX_3CR_1-expressing mononuclear phagocytes, that is, macrophages (11, 12). Macrophages that reside in the colonic lamina propria (c-LP) actively contribute to host defense and barrier integrity, are highly phagocytic and constitutively secrete interleukin (IL)-10, which promotes the maintenance of $FoxP3^+$ regulatory T cells (13, 14). Therefore, we wanted to explore the microbial signals that regulate the anti-inflammatory characteristics of gut-resident macrophages important for the establishment of tissue homeostasis and immunologic tolerance.

In this regard, we found that *Stenotrophomonas maltophilia* is a persistent colonizer within non-lymphoid mucosal tissue-resident macrophages and helps to maintain homeostatic conditions in the colon through the induction of IL-10.

Methods

Mice

C57BL/6JJcl, BALB/cAJcl and C.B-17 *scid* mice (female; age, 6–8 weeks) were purchased from CLEA Japan (Tokyo, Japan). $IL\text{-}10^{-/-}$ mice (C57BL/6 background; female; age, 6 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our laboratory. Animal studies were conducted under protocols approved by Hiroshima University Committee (#28-146), the Animal Research Committee of the Institute of Medical Science, the University of Tokyo (#PA15-75) and National Institutes for Biomedical Innovation, Health, and Nutrition Committee (#DS27-47) on the Use and Care of Animals.

16S rRNA-based metagenome analysis

Bacterial DNAs within intestinal macrophages were extracted according to the standard protocol for the QIAamp Metagenome Kit (Qiagen, Hilden, Germany). In brief, intestinal mononuclear cells (MNCs) were isolated from PPs, colonic patches (CPs) and c-LP of BALB/c mice (SPF, CLEA Japan, female, 7-week-old), which were digested by using Liberase TL (Roche Sigma-Aldrich, St Louis, MO, USA) and DNase I (Roche Sigma-Aldrich) and subsequently underwent Percoll (GE Healthcare, Chicago, IL, USA) discontinuous gradient centrifugation to remove non-cellular debris. In brief, physiological 100% Percoll was made by combining 9 parts Percoll with 1 part 10× phosphate buffered saline (PBS). The cell pellets were suspended in RPMI 1640 (Nakalai Tesque, Kyoto, Japan) with 2% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) and 30% Percoll and the resulting suspension layered over RPMI 1640 with 70% Percoll in a 15-ml Biologix centrifuge tube (Shawnee Mission, KS, USA). Centrifugation at 2000 rpm for 20 min at room temperature was done and the 30%/70% interface layer was collected and washed twice with RPMI 1640 containing 2% fetal bovine serum. The MNCs then were loaded onto a MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany) for purification of $CD11b^+$ cells.

Intestinal $CD11b^+$ cells were lysed by using buffer AHL, which was provided in the kit (Qiagen). Host nucleic acids were degraded by using Benzonase (Roche Sigma-Aldrich), which then was degraded by using proteinase K (Roche Sigma-Aldrich). Subsequently, bacterial cells were chemically and mechanically disrupted and microbial DNAs were purified by using a QIAamp UCP column (Qiagen).

Microbial DNA samples isolated from MACS-purified $CD11b^+$ cells were PCR-amplified in 2× KAPA Hi Fi Hot Start Ready Mix (Kapa Biosystems, Sigma-Aldrich) containing primers specific for the V3–V5 region of the 16S rRNA-encoding gene (*Escherichia coli* positions 334–939; F334: 5′-CCAG ACTCCTACGGGAGGCAGC-3′; R939: 5′-CTTGTGCGGG CCCCCGTCAATTC-3′) and subsequently re-amplified by using primers specific for the V3–V4 regions (*E. coli* positions 341–785; F341: 5′-CCTACGGGNGGCWGCAG-3′; R785: 5′-GACTACHVGGGTATCTAATCC-3′) (15). Nextera XT Index 1 primers (N7XX) from the Nextera XT Index Kit (Illumina, San Diego, CA, USA) were used to attach dual indices and Illumina sequencing adapters to the PCR products. AMPure XP beads (Beckman Coulter Genomics, Brea, CA, USA) were used for library cleanup. Each concentrated final DNA library was diluted to 4 nM in Resuspension Buffer (Illumina), pooled to a final concentration of 6 pM and denatured in 0.2 M NaOH. Paired-end sequencing was done on the MiSeq Desktop Sequencer (Illumina) for 602 cycles, producing a total of ~225 000 reads per sample. Sequences were then trimmed and classified by using the Quantitative Insights into Microbial Ecology (QIIME version 1.8) tool kit. Operational taxonomic units (OTUs) were picked at 97% sequence identity, and a representative sequence was chosen for each OTU by selecting the most abundant sequence in that OTU. These representative sequences were aligned by using PyNAST, and taxonomy was assigned by using the Ribosomal Database Project (RDP) Classifier and Basic Local Alignment Search Tool (BLAST) (16).

Bacteria

Stenotrophomonas maltophilia strains 13636 and 31559 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). *Stenotrophomonas maltophilia* strain 13636 (NCTC10258) was originally isolated from human

cerebrospinal fluid, whereas *S. maltophilia* strain 31559 was isolated from Japanese soil. *Escherichia coli* strain K12 derivative RIMD0509006 was purchased from the Pathogen Microbe Repository Units (Research Institute for Microbial Diseases, Osaka University, Japan). *Stenotrophomonas maltophilia* and *E. coli* strains were cultured in M9 minimal medium (Life Technologies, Thermo Fischer Scientific, Carlsbad, CA, USA) supplemented with 20 mM glucose and D,L-methionine (50 μg ml^{-1}).

To construct the *S. maltophilia smlt2713*-deficient mutant, we PCR-amplified the 1.7-kb DNA fragment containing *smlt2713* and its flanking region by using the primers B1-*smlt2713* (B1-*smlt2713*: 5′-AAAAAGCAGGCTGGTCTG CAACATCGGCAAC-3′) and B2-*smlt2713* (5′-AGAAAGCT GGGTCGGAACCAGGTGGCATACA-3′) with *S. maltophilia* strain 13636 genomic DNA as the template (Supplementary Figure 2). The resulting fragment was cloned into pDONR221 to obtain pDONR221-*smlt2713* by means of adaptor PCR amplification and site-specific recombination techniques by using the Gateway cloning system (Invitrogen, Thermo Fischer Scientific). By using pDONR221-*smlt2713* circular DNA as the template, inverse PCR amplification using primers R1-*smlt2713* (5′-CGGGATCCGGCGAGCACCGAACG GGAGA-3′) and R2-*smlt2713* (5′-CGGGATCCTACGACACC GCCTTCGGCGG-3′) was performed. The resulting fragment was digested by using *Bam*HI and self-ligated. The plasmid thus obtained was designated as pDORN221-*⊿smlt2713*. pDORN221-*⊿smlt2713* was mixed with a positive suicide vector, pABB-CRS2, to obtain pABB-*⊿smlt2713*, by using the Gateway cloning system (Invitrogen, Thermo Fischer Scientific). The plasmid was introduced into *E. coli* SM10λ *pir* and then transconjugated into the *S. maltophilia* ATCC13636 wild-type strain. The resulting mutant strain was designated as *S. maltophilia* ⊿smlt2713.

Generation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDMs) were prepared as described previously (17). In brief, bone marrow cells were aseptically isolated from mouse femora and cultured (5×10^5 cells per ml) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2 ng ml^{-1} macrophage colony-stimulating factor (Peprotech, Rocky Hill, NJ, USA) in a temperature-responsive 60-mm culture dish (RepCell, CellSeed, Tokyo, Japan); cells were incubated at 37°C in humidified 5% CO_2 for 6–10 days for differentiation into macrophages. During this differentiation period, half of the volume of the spent culture medium was replaced with fresh medium every 3 days. When the differentiated macrophages in the plate reached confluency, they were harvested and redistributed (5×10^5 cells per ml) onto a 35-mm dish (μ-Dish, ibidi, Grafelfing, Germany) appropriate for high-end microscopic observation.

Transient transfection of the smlt2713 gene into BMDMs

The *smlt2713* gene, cloned from *S. maltophilia* strain ATCC13636 chromosomal DNA by using the primers B1-*smlt2713* and B2-*smlt2713*, was sub-cloned into pFN22K (Promega, Madison, WI, USA). BMDMs were transiently transfected using Lipofectamine3000 (Invitrogen, Thermo Fischer Scientific) according to the standard protocol provided with the reagent.

Microscopic analysis of intracellular colonization of BMDMs by S. maltophilia

For the observation for the intracellular bacterial colonization of BMDMs, bacteria (*S. maltophilia* strain 13636, *smlt2713*-deficient mutant or 31559 or *E. coli* K12) were added to the BMDM culture at a multiplicity of infection of 50 and then incubated at 37°C for 2 h. In some experiments regarding the examination for the role of exogenous IL-10 in the maintenance of microbial colonization, murine recombinant IL-10 (5 ng ml^{-1}, BioLegend, San Diego, CA, USA) was added to the BMDM culture 16 h before the microbial infection. Extracellular bacteria were thoroughly washed away by using PBS, and BMDMs were incubated for another 4 h in DMEM supplemented with 100 μg ml^{-1} gentamicin (Fujifilm Wako, Osaka, Japan). The dish was then washed three times by using PBS, after which cells were fixed with 4% paraformaldehyde in PBS containing 3.5% sucrose for 15 min at room temperature, permeabilized with 0.1% Triton X-100 (Nakalai Tesques) in PBS for 10 min and then incubated with DAPI (Invitrogen, Thermo Fischer Scientific) for 5 min at room temperature to label bacterial and cellular DNA. Confocal images were acquired by using a laser scanning microscope (Fluoview FV1000, Olympus, Tokyo, Japan, or LSM5, Carl Zeiss, Oberkochen, Germany).

Transmission electron microscopy

For electron microscopic observation, BMDMs infected with bacteria were processed *in situ* for fixation, dehydration and embedding in the culture dish. The microbe-infected BMDMs were prefixed with 2% glutaraldehyde in 0.1 M PBS (pH 7.4) for 60 min at 4°C and then post-fixed with 1% osmium dissolved in 0.1 M PBS for 60 min at 4°C. After dehydration through graded ethanol, cells were embedded in Epon 812 resin (TAAB Laboratories Equipment, Aldermaston, UK). After polymerization, the hardened resin layer containing the embedded BMDMs was separated from the culture dish. Under a light microscope, a representative area was selected, trimmed and glued to a resin stub for sectioning. Ultrathin sections were cut by using a diamond knife, collected on single-hole grids and stained with uranyl acetate and lead citrate. Cells of interest were viewed under a transmission electron microscope (JEM-1230S, JEOL, Tokyo, Japan) at 80 kV.

Measurement of mitochondrial oxygen consumption

The oxygen consumption rate was examined by using an XF24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA). BMDMs (5×10^4 cells) were seeded into each well of an XF24 cell-culture microplate. *Stenotrophomonas maltophilia* strains 13636 and 31559 were added to the BMDM culture at a multiplicity of infection of 10, and then incubated at 37°C for 2 h. Extracellular bacteria were thoroughly washed away by using PBS, and the BMDMs were incubated in DMEM supplemented with 100 μg ml^{-1} gentamicin (Fujifilm Wako) for 4 h. After incubation, the XF24 cell culture plate was rinsed gently with PBS and pre-equilibrated for

1 h in unbuffered DMEM supplemented with 25 mM glucose. Compounds injected during the assay and their final concentrations were 1.5 μM oligomycin (inhibitor of ATP synthase), 1 μM FCCP (proton-uncoupling agent) and 0.5 μM rotenone + 0.5 μM antimycin A (inhibitors of the mitochondrial respiration complex). XFe Wave software (Seahorse Bioscience, North Billerica, MA, USA) was used to analyze the results.

Cytokine enzyme-linked immunosorbent assay

Cytokine release into the culture supernatants of BMDMs was measured through enzyme-linked immunosorbent assays (ELISAs) by using DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

Proteomic analysis of S. maltophilia exported proteins by matrix assisted laser desorption/inization–time of flight mass spectrometry

CentriPrep (Merck Millipore, Burlington, MA, USA) concentrates from *S. maltophilia* strain ATCC13636 culture supernatants were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis and visualized through Coomassie Brilliant Blue (CBB) staining. The protein band of interest, which had a molecular mass of 25 kDa, was excised from the gel and underwent in-gel tryptic digestion as follows. After being washed with 25 mM ammonium bicarbonate containing 50% acetonitrile, the gel piece underwent an additional reduction and alkylation step involving incubation in 10 mM dithiothreitol at 56°C for 1 h followed by treatment with 55 mM iodoacetamide in the dark at room temperature for 45 min. Finally, the gel piece was thoroughly washed with 25 mM ammonium bicarbonate containing 50% acetonitrile and subsequently dried in a vacuum centrifuge. The dried gel was swollen in 50 mM ammonium bicarbonate digestion buffer containing 10 μg ml^{-1} trypsin (Promega). After 30 min of incubation on ice, excess digestion buffer was removed. Digestion of the protein in the swollen gel was performed overnight at 37°C. The peptides were recovered through step extraction with 5% trifluoroacetic acid containing 50% acetonitrile. The resulting peptide extracts were pooled and concentrated by using a vacuum centrifuge. The peptides were desalted with Zip-tip C18 (Merck Millipore) before mass spectroscopy. Equal volumes of peptide and matrix solutions were mixed and crystallized on the sample plate. The matrix solution consisted of saturated α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid. The mass spectra were obtained on a Biflex IV MALDI-TOF mass spectrometer (Bruker, Billerica, MA, USA) operated in reflectron positive mode. The spectra were processed by using FlexAnalysis 3.0 and Bio-Tools 3.0 software (Bruker). Protein identification was performed by using the MASCOT software to search the NCBInr database, with Eubacteria as the taxonomy query.

Bacterial viability assay

BMDMs were cultured in 12-well culture plates (1×10^6 cells per well). *Stenotrophomonas maltophilia* strain 13636 or the *smlt2713*-deficient *S. maltophilia* mutant was added to the BMDM cultures at a multiplicity of infection of 10 and then incubated at 37°C for 2 h. After infection, extracellular bacteria were thoroughly washed away by using PBS, and the BMDMs were incubated for additional times (2, 48 or 96 h) in DMEM supplemented with 100 μg ml^{-1} gentamicin (Fujifilm Wako). After the appropriate time, the infected BMDMs were lysed in sterile distilled water and serial dilutions of the lysates were plated on selective agar plates (phenol red-based medium containing 1% maltose and 32 mg l^{-1} imipenem) (17) and cultured at 30°C for 24 h. The numbers of viable intracellular *S. maltophilia* were determined.

Data accession

The sequencing data generated in this study have been submitted to the DDBJ under the accession no. DRA0008371.

Statistical analysis

Statistical comparisons were performed by using Student's *t*-test (Microsoft Excel 2010, Redmond, WA, USA); the *P* value was used to define statistical significance. ** is denoted as $P < 0.01$ against control.

Results

Presence of S. maltophilia in colonic macrophages

To assess whether microbes are present within non-lymphoid tissue-resident macrophages *in vivo*, we performed 16S rRNA-based metagenome analysis by using DNA isolated from $CD11b^+$ cells in various intestinal tissues (i.e. PPs, CPs and c-LP). The microbiota composition analysis of PPs $CD11b^+$ cells (Fig. 1) showed predominant abundance of *Firmicutes* at the phylum level (57% of *Lactobacillus* and 40% of segmented filamentous bacteria at the genus level), with trace abundances of *Bacteroidetes* (1.2%) and *Proteobacteria* (0.7%). In comparison to PPs, CP-derived $CD11b^+$ cells had higher abundance of *Bacteroidetes* (61%) and *Firmicutes* (35%) but lower abundance of the phylum *Proteobacteria* (2.5%). However, non-lymphoid tissue c-LP $CD11b^+$ cells demonstrated *Proteobacteria* (73%) followed by *Bacteroidetes* (21%) and phylum *Firmicutes* (3%) primarily. Of note, *Stenotrophomonas* spp. (including *S. maltophilia*) accounted for more than half (52%) of the microbes within the c-LP $CD11b^+$ cells. These results suggest that *Stenotrophomonas* spp. are constitutively present within c-LP macrophages.

In vitro characteristics of S. maltophilia-colonized macrophages

We next used an *in vitro* assay to examine the morphologic characteristics of *S. maltophilia*-harboring cells. To this end, we prepared BMDMs and cultured them with the clinically isolated *S. maltophilia* strain 13636 or the environmentally isolated *S. maltophilia* strain 31559. As a control microbe, we used *E. coli* strain K12 derivative RIMD0509006. Of these three strains, only *S. maltophilia* strain 13636 was able to enter BMDMs (Fig. 2A). In addition, transmission electron microscopic analysis confirmed the internalization of *S. maltophilia* strain 13636 within both the endosomal and cytosolic compartments of BMDMs (Fig. 2B).

We wanted to know whether this persistent colonization of macrophages by the microbe creates mutually

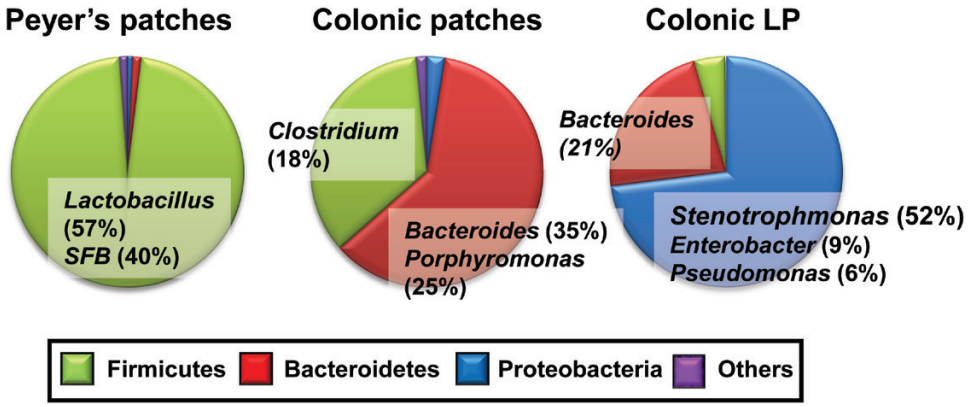

Fig. 1. Microbial distribution in the intestinal CD11b$^+$ macrophages of 8-week-old female BALB/c mice maintained under specific pathogen-free conditions. The microbial composition of CD11b$^+$ macrophages isolated from Peyer's patches, colonic patches and the colonic lamina propria (LP) was examined by using 16S rRNA-based metagenomic analysis. Three separate experiments were performed and yielded similar results; data from one representative experiment are shown. SFB, segmented filamentous bacteria.

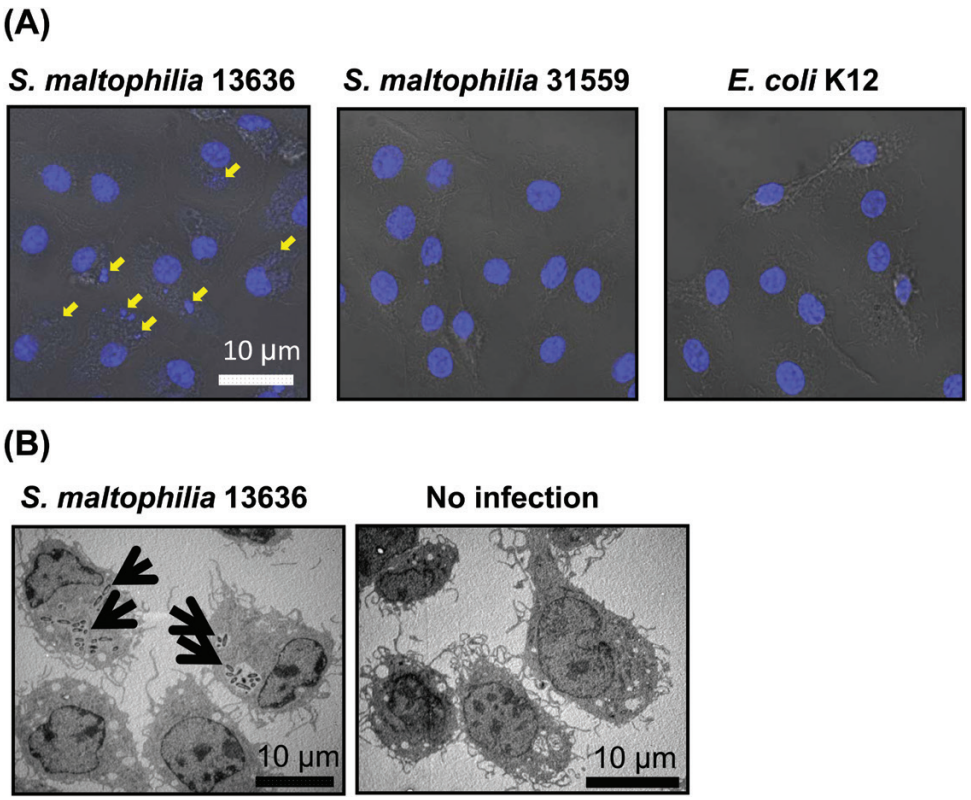

Fig. 2. (A) The intracellular colonization of BMDMs by *S. maltophilia* strains was examined through confocal microscopy. Mammalian and bacterial DNA was stained with DAPI. Arrows indicate *S. maltophilia* persistently colonized within BMDMs. (B) Intracellular colonization of BMDMs by *S. maltophilia* was further examined by using transmission electron microscopy. Arrows indicate *S. maltophilia* persistently colonized within endosomal and cytosol compartments of BMDMs. Three separate experiments were performed and yielded similar results; the data from one representative experiment are presented.

beneficial microenvironments within the colonized cells. Recent studies have indicated that energy metabolism is changed when intracellular pathogens are excluded from macrophages (18). To address this question, we used a real-time extracellular flux analyzer to determine the oxygen consumption of *S. maltophilia*-colonized BMDMs. Intracellular colonization by *S. maltophilia* strain 13636 increased the spare mitochondrial oxygen consumption of BMDMs compared with that of uninfected (control) BMDMs, suggesting that BMDMs persistently colonized by *S. maltophilia* are similar to M2-type macrophages (Fig. 3A). In addition, BMDMs cultured with *S. maltophilia*

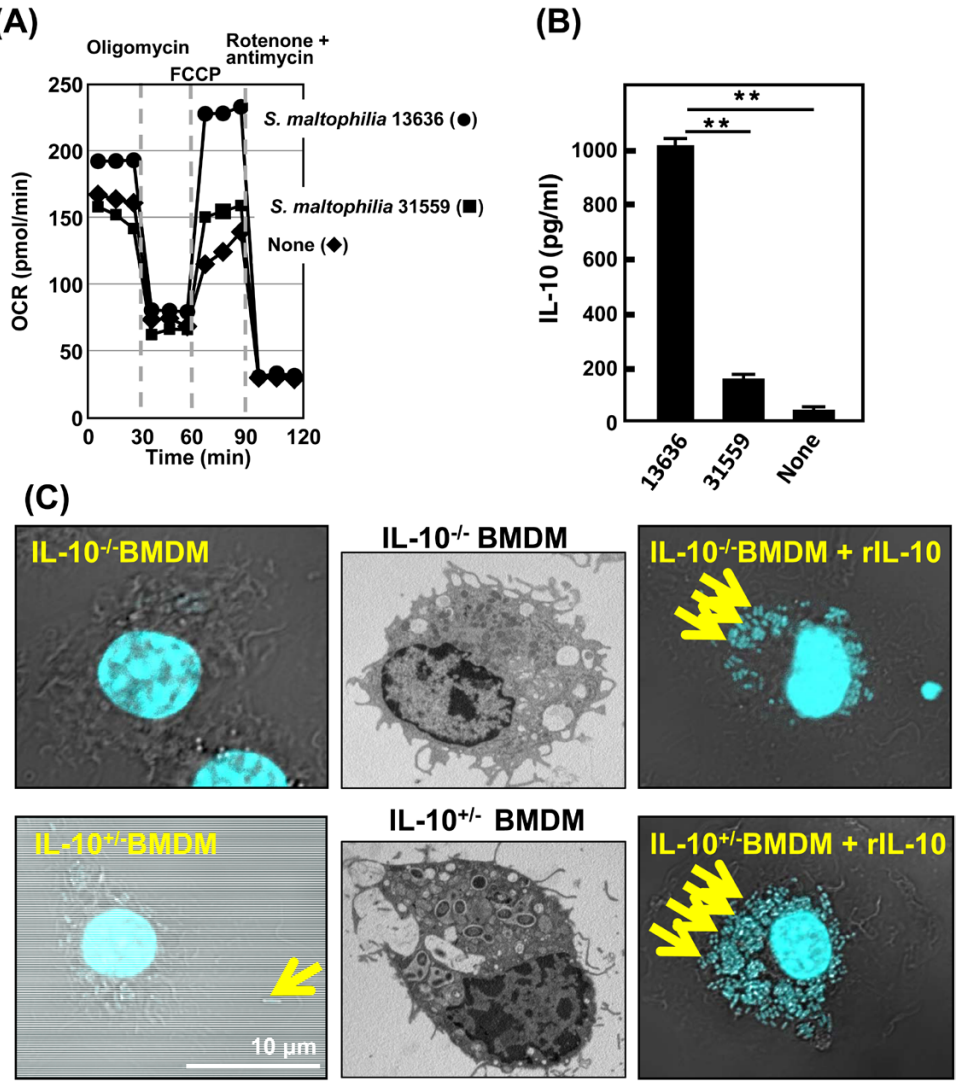

Fig. 3. (A) Real-time oxygen consumption rate (OCR) of BMDMs colonized by *S. maltophilia* was examined during sequential treatment with oligomycin (ATP synthase inhibitor), FCCP (proton uncoupling reagent that causes maximal oxygen consumption) and rotenone + antimycin A (mitochondrial respiration complex inhibitors). Three separate experiments were performed and yielded similar results; data from one representative experiment are presented. This graph indicates that mitochondrial fitness is required for persistent symbiotic colonization of *S. maltophilia* in BMDMs. (B) Robust IL-10 production by BMDMs harboring the clinically isolated *S. maltophilia* strain 13636. The amount of IL-10 released over 24 h into the culture supernatants of BMDMs infected with *S. maltophilia* strains was measured by an ELISA. Three separate experiments were performed and yielded similar results; data from one representative experiment are presented (mean ± 1 SD of triplicate measurements). $^{**}P < 0.01$. (C) Confocal and transmission electron microscopic analyses of intracellularly colonized *S. maltophilia* in IL-10-competent or -deficient BMDMs. In some experiments, BMDMs were pre-treated with recombinant IL-10 (rIL-10) before infection. These micrographs show that IL-10 is indispensable for intracellular colonization of BMDMs by *S. maltophilia*. Three separate experiments were performed and yielded similar results; data from one representative experiment are presented.

strain 13636 consistently showed robust IL-10 production, a typical M2 macrophage phenotype (Fig. 3B). In contrast, BMDMs cultured with the environmentally isolated *S. maltophilia* strain 31559 showed only marginal IL-10 production.

IL-10 is an anti-inflammatory cytokine that also is involved in the creation and maintenance of immunologically homeostatic environments in the intestine (19). In addition, colonic macrophages preferentially produce IL-10 in comparison to splenic macrophages (20). Because we noted enhanced IL-10 production by the BMDMs colonized with *S. maltophilia* strain 13636, we next examined the role of IL-10 in the maintenance of cytosolic colonization of *S. maltophilia* in macrophages. *Stenotrophomonas maltophilia* strain 13636 was unable to colonize IL-10-deficient BMDMs; however, the addition of exogenous IL-10 to the IL-10-deficient BMDMs effectively recovered their intracellular colonization. Furthermore, exogenous IL-10 addition enhanced the cytosolic habitation of *S. maltophilia* strain 13636 in wild-type (IL-10-competent) BMDMs (Fig. 3C). These findings collectively suggest that *S. maltophilia* strain 13636 induced robust IL-10 production from host macrophages, which supported the habitation of *S. maltophilia* strain 13636 in the cytoplasm of macrophages.

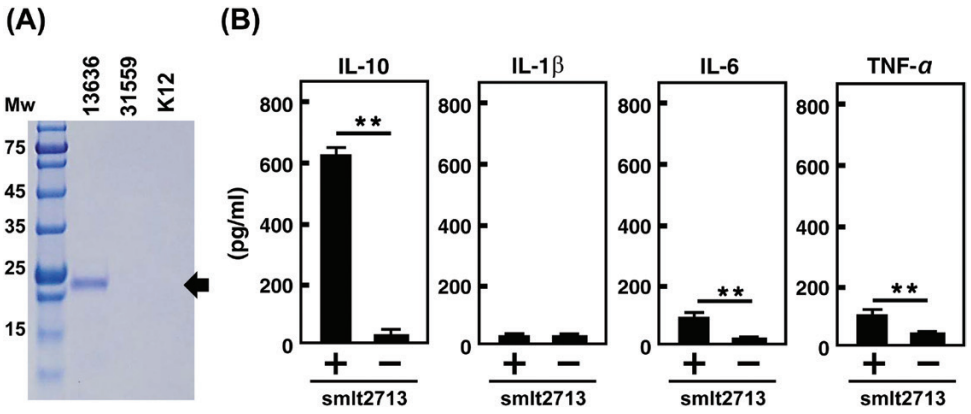

Fig. 4. (A) Identification of a 25-kDa protein uniquely secreted by the clinically isolated *S. maltophilia* strain 13636 cultured in synthetic M9 medium containing 20 mM glucose. Supernatants were prepared from *S. maltophilia* cultures that had been incubated for 96 h, concentrated with CentriPrep and separated through SDS–PAGE; the gel was stained with Coomassie Brilliant Blue. The migration of molecular mass standards (in kilodaltons) is indicated to the left of the gel image. The 25-kDa band was excised from the gel and analyzed by MALDI-TOF MS. This analysis revealed that the 25-kDa protein corresponds to the hypothetical protein encoded by the *smlt2713* gene of *S. maltophilia* strain K279a (Supplementary Figure 1). (B) Preferential IL-10 production by BMDMs transfected with the gene encoding the 25-kDa protein secreted by the clinically isolated *S. maltophilia* strain 13636. Cytokine release into the culture supernatants of BMDMs transiently transfected with *smlt2713* was measured by an ELISA. Three separate experiments were performed and yielded similar results; data from one representative experiment are presented (mean ± 1 SD of triplicate measurements). $^{**}P < 0.01$.

Identification of a symbiotic factor for persistent colonization by S. maltophilia strain 13636

We next aimed to identify the molecules in *S. maltophilia* strain 13636 responsible for their ability to live inside macrophages. Because microbe-derived effectors (symbiotic factors) for persistent colonization within macrophages are typically induced under nutritionally poor or acidic conditions (21, 22), we cultured the *S. maltophilia* strains in a chemically defined, nutritionally limited M9 medium that contained minimal amounts of glucose. We then concentrated the *S. maltophilia* culture supernatants and separated the components through SDS–PAGE, thus revealing that the clinical isolate, *S. maltophilia* strain 13636, secreted a 25-kDa protein that was absent from the products of the environmental isolate, strain 31559 (Fig. 4A). Furthermore, MALDI-TOF MS analysis revealed that this 25-kDa protein corresponded to the hypothetical protein encoded by *smlt2713* (recently renamed as SMLT_RS12935) in *S. maltophilia* strain K279a (Supplementary Figure 1).

To explore the effect of the hypothetical protein encoded by *smlt2713* on cytokine production, we expressed the gene in BMDMs. As in macrophages harboring *S. maltophilia* strain 13636, the expression of *smlt2713* induced the production of IL-10 in BMDMs without effects on other cytokines (e.g. IL-1β, IL-6 and TNF-α) (Fig. 4B).

Characteristics of BMDMs infected with an smlt2713-deficient strain of S. maltophilia

To examine the role of the smlt2713 protein in the persistent intracellular colonization of macrophages by *S. maltophilia* strain 13636, we constructed *smlt2713*-deficient *S. maltophilia* mutants (Supplementary Figure 2). The resulting *S. maltophilia smlt2713*-deficient strain failed to establish persistent intracellular colonization in BMDMs; the mutant strain instead aberrantly colonized endosomal vesicles and occasionally induced a morphology similar to pyroptotic cell death in the infected BMDMs (Fig. 5A). In addition, BMDMs colonized with *S. maltophilia smlt2713*-deficient strain showed reduced bacterial viability (Fig. 5B) and decreased anti-inflammatory cytokine IL-10 production in comparison to BMDMs colonized with the *smlt2713*-competent *S. maltophilia* strain 13636 (Fig. 5C). The necessity of IL-10 for intracellular survival of *S. maltophilia* was examined by using the *S. maltophilia smlt2713*-deficient strain. *Stenotrophomonas maltophilia smlt2713*-deficient strain aberrantly colonized endosomal vesicular compartments of wild-type BMDMs and did not establish cytosol colonization in the absence of exogenous IL-10; however, the addition of exogenous IL-10 to BMDMs effectively recovered their cytosol colonization (Supplementary Figure 3).

Effect of the BMDMs transduced with smlt2713 against enterocolitis

It was important to examine physiological contribution of *smlt2713 in vivo*. BMDMs transduced with *smlt2713* were subjected to the $CD4^{+}CD45RB^{high}$ T-cell-induced enterocolitis model (23). As shown in the Supplementary Figure 4, histologic analysis of the distal colon isolated from the $CD4^{+}CD45RB^{high}$ T-cell-reconstituted C.B-17 *scid* mice pretransferred with BMDMs transfected with the recombinant vector pFN22K encoding *smlt2713* ameliorated the inflammatory lesions such as disruption of epithelial layers and accumulation of MNCs into the lamina propria when compared with the control $CD4^{+}CD45RB^{high}$ T-cell-reconstituted C.B-17 *scid* mice.

Discussion

In this study, we used 16S ribosomal RNA-based metagenome analysis to reveal persistent colonization of non-lymphoid c-LP $CD11b^{+}$ macrophages with *S. maltophilia*. In contrast,

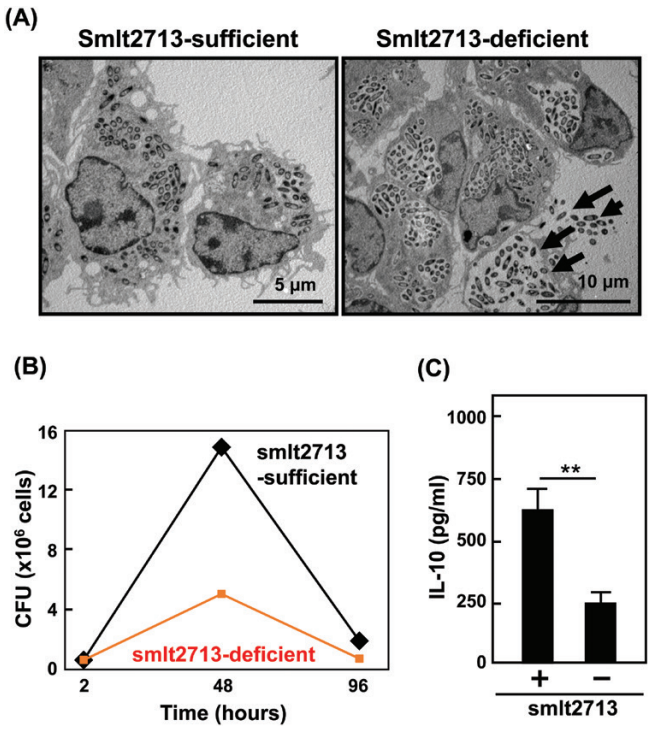

Fig. 5. (A) Intracellular colonization of BMDMs by *S. maltophilia* and the *smlt2713*-deficient mutant was examined through transmission electron microscopy. Arrows indicate pyroptotic cell disruption of BMDMs colonized with the *smlt2713*-deficient mutant. Three separate experiments were performed and yielded similar results; data from one representative experiment are presented. (B) Viability of intracellular *S. maltophilia* strains in BMDMs. Three separate experiments were performed and yielded similar results; data from one representative experiment are presented. (C) Reduced IL-10 production by BMDMs infected with the *smlt2713*-deficient mutant. IL-10 release over 24 h into the culture supernatants of BMDMs infected with *S. maltophilia* strains was measured by an ELISA. Three separate experiments were performed and yielded similar results; data from one representative experiment are presented (mean ± 1 SD of triplicate measurements). $^{**}P < 0.01$.

lymphoid tissue-resident CD11b^{+} macrophages were devoid of this organism. An *in vitro* study in which we recapitulated this persistent colonization by using bone marrow-derived CD11b^{+} macrophages and a clinically isolated *S. maltophilia* strain showed robust IL-10 production and mitochondrial oxygen consumption by the infected macrophages. These anti-inflammatory characteristics of the host macrophages may help to maintain the microbe within the cells.

We further noticed that these traits were mediated in part by the hypothetical protein annotated as smlt2713 and secreted from *S. maltophilia* strain 13636. Infection of BMDMs with *smlt2713*-deficient *S. maltophilia* showed disrupted colonization and decreased IL-10 production. In addition, our preliminary adoptive transfer experiments to assess the effects of *smlt2713*-transduced macrophages on CD45RB^{high} T-cell-induced colitis indicated that intracellular colonization of tissue-resident macrophages with *smlt2713*-competent *S. maltophilia* restores or maintains intestinal homeostasis.

Stenotrophomonas maltophilia is a ubiquitous bacterium that is found in a wide range of habitats, but it typically is associated with plants and promotes plant growth and health (24, 25). In addition, *S. maltophilia* can colonize extreme, man-made niches in hospitals, such as central venous catheters and dental suction systems. Both environmental and clinical isolates of *S. maltophilia* exhibit multiple antibiotic and stress resistance phenotypes (24, 25). For example, under nutritionally limiting conditions, *S. maltophilia* reportedly can change its morphology to a small colony variant (SCV), a slow-growing quasi-persistent population in host tissues (26, 27). Because SCVs result in down-regulation of the bacterial electron transport chain and energy production, they are better able to persist in hostile mammalian subcellular organelles, such as the phagocytic vacuoles of macrophages, and under acidified and nutritionally deficient conditions and are less sensitive to antibiotics than are their wild-type counterparts (27). Therefore, we speculate that the robust IL-10 production and enhanced mitochondrial oxygen consumption characteristic of *S. maltophilia*-colonized macrophages might alter the metabolic state of the intracellular microbes so that they are less active but still alive, like SCVs.

In a study based on 'IgA-bound bacterial flow cytometry coupled with 16S rRNA-based metagenome analysis' (IgA-Seq), Bunker *et al.* demonstrated *S. maltophilia* as a representative commensal bacterium in the luminal spaces of the small and large intestines (3). Macrophages in the PPs and CPs might degrade and process phagocytosed *S. maltophilia*, leading to the generation of *S. maltophilia*-specific IgA responses. In contrast, clonic and perhaps small-intestinal LP macrophages may allow the organism to persist in the endosomal and cytosolic compartments, utilizing the bacteria to create symbiotic microenvironments that can be advantageous for cellular activities, such as energy metabolism. These findings support the absence of *Stenotrophomonas* spp. as an intra-tissue symbiont of phagocytes resident in mucosa-associated lymphoid tissue such as PPs and CPs and the persistent presence of the bacterium in non-lymphoid tissue-resident macrophages in our 16S ribosomal RNA-based metagenome analysis.

In contrast, Naito *et al.* used 16S ribosomal RNA-based molecular analysis to reveal the presence of *S. maltophilia* in cryptic regions of the murine cecum and proximal colon (28). These investigators also noted that the microbe modulated the proliferation (i.e. induction of necroptosis of cryptic organoids) and differentiation (i.e. goblet cell differentiation) of cryptic epithelial progenitors (28). Therefore, *S. maltophilia* might be able to colonize diverse anatomic sites of the gastrointestinal tissues by using distinctive strategies (i.e. robust IL-10 production, enhanced mitochondrial oxygen consumption, *S. maltophilia*-specific IgA production, and modulation of cryptic epithelial proliferation and differentiation).

The 25-kDa hypothetical protein encoded by the *smlt2713* gene is annotated as a bacterial protein exported by the type II secretion (T2S) machinery (29). This T2S system reportedly mediates the transition of some environmental bacteria to mammalian pathobionts (29). For example, the T2S machinery of *Legionella pneumoniae*, which typically occupies a watery habitat, allows the bacterium to replicate and grow within human macrophages (30). In our current study, BMDMs transiently transfected with *smlt2713* showed enhanced production of the anti-inflammatory cytokine IL-10, and deficiency

of either *smlt2713* or IL-10 impaired the organism's ability to intracellularly colonize macrophages. Therefore, we speculate that the hypothetical protein encoded by *smlt2713* contributes to the persistent colonization of murine macrophages by *S. maltophilia* because of the protein's effects on IL-10 production, thereby creating and maintaining immunologically homeostatic conditions.

In conclusion, our study suggests that the symbiotic factor encoded by the *S. maltophilia smlt2713* gene might be exploited to mine other beneficial gut bacteria for improving gut-barrier integrity and alleviating inflammatory conditions at mucosa.

Funding

This research was supported, at least in part, by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) and the Japan Society for the Promotion of Science (JSPS; kakenhi JP15H05790, JP18H02150, JP18H02674, JP17K09604, JP26670241 and JP26293111 to J.K.; JP19K10088 to I.T.; JP19K07617 to T.N.; and JP18K17997 to K.H.); the Japan Agency for Medical Research and Development (AMED; JP15gm0410004h0106 to I.T.; JP18ek0410032s0103, JP18ak0101068h0002, JP19gm1010006s0103 and 19ek0410062h0001 to J.K.); the grant for Joint Research Project of the Institute of Medical Science, the University of Tokyo (to J.K. and H.K.); Cross-ministerial Strategic Innovation Promotion Program (SIP to J.K.); the Canon Foundation (to J.K.); and the Ministry of Health, Labour, and Welfare of Japan (grant number JP19KA3001 to K.H.).

Conflicts of interest statement: the authors declared no conflicts of interest.

References

1 Honda, K. and Littman, D. R. 2016. The microbiota in adaptive immune homeostasis and disease. *Nature* 535:75.
2 Kurashima, Y. and Kiyono, H. 2017. Mucosal ecological network of epithelium and immune cells for gut homeostasis and tissue healing. *Annu. Rev. Immunol.* 35:119.
3 Bunker, J. J., Flynn, T. M., Koval, J. C. *et al.* 2015. Innate and adaptive humoral responses coat distinct commensal bacteria with immunoglobulin A. *Immunity* 43:541.
4 Cervantes-Barragan, L., Chai, J. N., Tianero, M. D. *et al.* 2017. *Lactobacillus reuteri* induces gut intraepithelial $CD4^+CD8\alpha\alpha^+$ T cells. *Science* 357:806.
5 Ivanov, I. I., Atarashi, K., Manel, N. *et al.* 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485.
6 Tanoue, T., Atarashi, K. and Honda, K. 2016. Development and maintenance of intestinal regulatory T cells. *Nat. Rev. Immunol.* 16:295.
7 Obata, T., Goto, Y., Kunisawa, J. *et al.* 2010. Indigenous opportunistic bacteria inhabit mammalian gut-associated lymphoid tissues and share a mucosal antibody-mediated symbiosis. *Proc. Natl Acad. Sci. USA* 107:7419.
8 Sonnenberg, G. F., Monticelli, L. A., Alenghat, T. *et al.* 2012. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science* 336:1321.
9 Fung, T. C., Bessman, N. J., Hepworth, M. R. *et al.* 2016. Lymphoid-tissue-resident commensal bacteria promote members of the IL-10 cytokine family to establish mutualism. *Immunity* 44:634.
10 Shibata, N., Kunisawa, J., Hosomi, K. *et al.* 2018. Lymphoid tissue-resident *Alcaligenes* LPS induces IgA production without excessive inflammatory responses via weak TLR4 agonist activity. *Mucosal Immunol.* 11:693.
11 De Schepper, S., Verheijden, S., Aguilera-Lizarraga, J. *et al.* 2018. Self-maintaining gut macrophages are essential for intestinal homeostasis. *Cell* 175:400.
12 Kayama, H., Ueda, Y., Sawa, Y. *et al.* 2012. Intestinal CX^3C chemokine receptor $1^{(high)}$ ($CX3CR1^{(high)}$) myeloid cells prevent T-cell-dependent colitis. *Proc. Natl Acad. Sci. USA* 109:5010.
13 Kim, M., Galan, C., Hill, A. A. *et al.* 2018. Critical role for the microbiota in CX^3CR1^+ intestinal mononuclear phagocyte regulation of intestinal T cell responses. *Immunity* 49:151.
14 Varol, C., Zigmond, E. and Jung, S. 2010. Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria. *Nat. Rev. Immunol.* 10:415.
15 Rudi, K., Skulberg, O. M., Larsen, F. and Jakobsen, K. S. 1997. Strain characterization and classification of oxyphotobacteria in clone cultures on the basis of 16S rRNA sequences from the variable regions V6, V7, and V8. *Appl. Environ. Microbiol.* 63:2593.
16 Maruyama, N., Maruyama, F., Takeuchi, Y., Aikawa, C., Izumi, Y. and Nakagawa, I. 2014. Intraindividual variation in core microbiota in peri-implantitis and periodontitis. *Sci. Rep.* 4:6602.
17 Kerr, K. G., Denton, M., Todd, N., Corps, C. M., Kumari, P. and Hawkey, P. M. 1996. A new selective differential medium for isolation of *Stenotrophomonas maltophilia*. *Eur. J. Clin. Microbiol. Infect. Dis.* 15:607.
18 Caputa, G., Castoldi, A. and Pearce, E. J. 2019. Metabolic adaptations of tissue-resident immune cells. *Nat. Immunol.* 20:793.
19 Saraiva, M. and O'Garra, A. 2010. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10:170.
20 Ueda, Y., Kayama, H., Jeon, S. G. *et al.* 2010. Commensal microbiota induce LPS hyporesponsiveness in colonic macrophages via the production of IL-10. *Int. Immunol.* 22:953.
21 Harms, A., Maisonneuve, E. and Gerdes, K. 2016. Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science* 354:aaf4268.
22 Helaine, S., Cheverton, A. M., Watson, K. G., Faure, L. M., Matthews, S. A. and Holden, D. W. 2014. Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. *Science* 343:204.
23 Leach, M. W., Bean, A. G., Mauze, S., Coffman, R. L. and Powrie, F. 1996. Inflammatory bowel disease in C.B-17 scid mice reconstituted with the $CD45RB^{high}$ subset of $CD4^+$ T cells. *Am. J. Pathol.* 148:1503.
24 An, S. Q. and Berg, G. 2018. *Stenotrophomonas maltophilia*. *Trends Microbiol.* 26:637.
25 Brooke, J. S. 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin. Microbiol. Rev.* 25:2.
26 Anderson, S. W., Stapp, J. R., Burns, J. L. and Qin, X. 2007. Characterization of small-colony-variant *Stenotrophomonas maltophilia* isolated from the sputum specimens of five patients with cystic fibrosis. *J. Clin. Microbiol.* 45:529.
27 Proctor, R. A., von Eiff, C., Kahl, B. C. *et al.* 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* 4:295.
28 Naito, T., Mulet, C., De Castro, C. *et al.* 2017. Lipopolysaccharide from crypt-specific core microbiota modulates the colonic epithelial proliferation-to-differentiation balance. *mBio* 8:e01680.
29 Cianciotto, N. P. and White, R. C. 2017. Expanding role of type II secretion in bacterial pathogenesis and beyond. *Infect. Immun.* 85:e00014-17.
30 White, R. C. and Cianciotto, N. P. 2016. Type II secretion is necessary for optimal association of the legionella-containing vacuole with macrophage Rab1B but enhances intracellular replication mainly by Rab1B-independent mechanisms. *Infect. Immun.* 84:3313.