

The anti-inflammatory role of granulocyte colony-stimulating factor in macrophage-dendritic cell crosstalk after *Lactobacillus rhamnosus* GR-1 exposure

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RECEIVED AUGUST 9, 2010; REVISED FEBRUARY 9, 2011; ACCEPTED FEBRUARY 11, 2011. DOI: 10.1189/jlb.0810445

ABSTRACT

MΦs are important sensory cells of the innate immune system and regulate immune responses through releasing different combinations of cytokines. In this study, we examined whether cytokines released by MΦs in response to the probiotic bacterial strain GR-1 modulate the responses of DCs. The cytokine profile released by GR-1-treated MΦs was characterized by low levels of TNF- α , GM-CSF, IL-6, and IL-12 but very high levels of G-CSF. GR-1 CM did not induce expression of the shared p40 subunit of IL-12 and IL-23 and costimulatory molecules CD80 or CD86 or increase T cell stimulatory capacity in DCs. However, in G-CSFR-deficient DCs or after antibody-mediated neutralization of G-CSF, GR-1 CM induced IL-12/23 p40 production significantly, indicating that G-CSF within the GR-1 CM inhibits IL-12/23 p40 production induced by other CM components. GR-1 CM and rG-CSF also inhibited LPS-induced IL-12 production at the mRNA and protein levels. The inhibition of IL-12 production by G-CSF was at least in part mediated through inhibition of JNK activation. Finally, splenic DCs of GR-1-injected mice produced less IL-12/23 p40 than those of PBS-injected mice in response to LPS *ex vivo*, and this was at least partially dependent on exposure to GR-1-induced G-CSF *in vivo*. Altogether, these results suggest that G-CSF modulates the IL-12/23 p40 response of DCs in the context of the probiotic GR-1 through MΦ-DC crosstalk. *J. Leukoc. Biol.* 89: 907–915; 2011.

Abbreviations: C-RPMI=complete RPMI, CM=conditioned media, CNT CM=nontreated (control) conditioned media, *E. coli* CM=condition media of *Escherichia coli* GR-12-treated macrophage, G-CSFR-/-=G-CSF receptor deficient, G-CSFR+/-=G-CSF receptor heterozygous deficient, or G-CSFR+/+=G-CSF receptor wild-type, GR-1=*Lactobacillus rhamnosus* GR-1, GR-1 CM=conditioned media of *Lactobacillus rhamnosus* GR-1-treated macrophage, IBD=inflammatory bowel disease, LTA=lipoteichoic acid, MΦ=macrophage, qPCR=quantitative PCR, Treg=regulatory T cell

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Probiotics are defined as “live microorganisms which confer beneficial health effects on the host when administered in adequate doses” [1, 2]. Such beneficial effects may vary considerably depending on the probiotic organism examined. These effects may include exclusion of harmful microbes by occupying an ecological niche, producing antimicrobial compounds, stimulating the host’s antimicrobial immune defenses, or modulating inflammatory responses [3, 4]. The immunomodulatory effects of probiotic bacterial strains have garnered much interest for their application in treating inflammatory diseases, such as arthritis and IBD. Different strains are likely to induce varied immune responses, such as the stimulation of distinct cytokine profiles, which can then have antimicrobial or anti-inflammatory effects on the host immune system [5]. Anti-inflammatory effects may be mediated through the release of cytokines, such as IL-10 or TGF- β , or the stimulation of Treg responses [6]. These effects could be harnessed therapeutically to manipulate immune responses; however, detailed knowledge of the effects of specific strains on immune cells is needed to precisely predict the effects of these strains on the host [7].

DCs are key APCs, capable of acquiring antigens in the periphery and migrating to the LNs, where they can present antigenic peptides to activate naïve T cells. The activation of DCs by signals such as bacterial products or cytokines is thought to affect the type of T cell responses they initiate. The interaction of probiotic bacteria with DCs may be part of their observed immunomodulatory effects; therefore, investigation of these interactions has been undertaken with varying results [6, 8–11]. In addition to direct effects on DCs, the local cytokine milieu produced by probiotic, bacteria-exposed MΦs, containing factors such as IL-12, IL-4, IL-6, IL-23, or IL-10, may affect

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the activation of DCs and in turn, skew T cell responses toward Th1, Th2, Th17, or Treg, respectively.

G-CSF is a pleiotropic cytokine best known for its role in promoting the production and function of neutrophils [12]. In addition, systemic, anti-inflammatory effects of this cytokine have been observed, which are thought to be mediated through modulation of T cells and DCs [13]. As rG-CSF is approved as a therapeutic agent for several disease states, such as immune reconstitution after chemotherapy-induced neutropenia and the mobilization of bone marrow stem cells into the blood prior to harvesting for stem cell transplant, valuable information regarding in vivo responses to this cytokine has been attained [14].

Previously, we have shown that the probiotic strain GR-1 elicits the release of G-CSF from MΦs and that the paracrine effects of G-CSF on neighboring MΦs suppress subsequent inflammatory reactions [15]. GR-1 treatment increased G-CSF expression in isolated human intestinal lamina propria cells from a subset of patients tested, and lower G-CSF responses were observed in cells from those afflicted with IBD [16]. Previous studies by others [17, 18] have shown that systemic administration of rG-CSF has beneficial effects on IBD, implying that G-CSF may play a regulatory role in the intestine, perhaps through anti-inflammatory effects on key immune cells such as MΦs and DCs. To further understand the role that G-CSF may play in the immunoregulatory effects of probiotics, we examined the effects of *Lactobacillus*-induced G-CSF on DCs. We found that G-CSF produced by GR-1-exposed MΦs has anti-inflammatory effects on DCs in terms of modulating IL-12/23 p40 production, at least in part through inhibiting the phosphorylation of JNK. As MΦs are highly populated in the lamina propria of the intestine [19], MΦ-derived cytokines could influence the function of DCs that have transiently migrated to the lamina propria, where they acquire antigen for presentation locally or in the LNs [20]. Therefore, these results give new insight into the role of G-CSF and MΦ–DC crosstalk in the immunomodulatory effects of probiotic strain GR-1.

MATERIALS AND METHODS

Mice

C57BL/6 and Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). G-CSFR^{−/−} mice [21], backcrossed onto the C57BL/6 background, were obtained originally from Daniel C. Link (Washington University Medical School, St. Louis, MO, USA) and maintained in the Animal Care and Veterinary Services facility at the University of Western Ontario (Canada) under conventional conditions. C57BL/6 and G-CSFR^{−/−} were crossed, and G-CSFR^{+/−} offspring were then crossed to generate G-CSFR^{+/+} and G-CSFR^{−/−} littermates. Matched pairs of littermates were used whenever possible. Male and female mice, aged 4–10 weeks, were used in all experimental procedures. All experimental protocols were approved by the University of Western Ontario Animal Use Subcommittee, which follows the regulations of the Animals for Research Act (Ontario, Canada) and the Canadian Council on Animal Care.

Cell lines, bacteria, and reagents

Mouse bone marrow-derived, immortalized MΦs from C57BL/6 mice were obtained originally from Dr. Bharat Aggarwal [15, 22] (MD Anderson Cancer Center, University of Texas, Houston, TX, USA) and grown in RPMI

media (Sigma-Aldrich Canada, Oakville, ON, Canada), supplemented with 10% FBS (Sigma-Aldrich Canada), nonessential amino acids (Sigma-Aldrich Canada), and sodium pyruvate (Sigma-Aldrich Canada), and incubated at 37°C with 5% CO₂. GR-1 and *Lactobacillus reuteri* RC-14 (both obtained from Dr. Gregor Reid. The Canadian Research and Development Centre for Probiotics, Lawson Health Research Institute, London, ON, Canada) and *Lactobacillus rhamnosus* GG and *Lactobacillus casei* (both obtained from American Type Culture Collection, Manassas, VA, USA), were grown for 48 h anaerobically in de Man, Rogosa, and Sharpe media (Beckton Dickinson Canada, Mississauga, ON, Canada). *Escherichia coli* GR-12 was originally isolated from the urine of a patient with pyelonephritis [23] and grown overnight in LB broth under aerobic conditions. LPS derived from *E. coli* O111:B4 was purchased from List Biological Laboratories (Campbell, CA, USA). LTA, derived from *Staphylococcus aureus*, was obtained from Sigma-Aldrich Canada. Polymyxin B was obtained from Sigma-Aldrich Canada. rG-CSF and IFN-γ were purchased from R&D Systems (Minneapolis, MN, USA). Anti-CD3 antibody for the activation of T cells was obtained from eBioscience (San Diego, CA, USA).

Generation of MΦ CM

CM of bacteria-treated MΦs were prepared as described previously [15]. Bone marrow-derived, immortalized MΦs from C57BL/6 mice were plated at a concentration of 1 million cells/mL in antibiotic-free RPMI supplemented with 10% FBS, nonessential amino acids, and sodium pyruvate. MΦs were treated with 20 CFU bacteria/MΦ for 1–4 h at 37°C. Cells were then washed extensively with fresh media and incubated overnight (18 h) with fresh, supplemented RPMI containing penicillin and streptomycin (C-RPMI). The media were then filtered through a 0.2-μm sterile filter and frozen at −20 until use. To neutralize G-CSF activity within the CM, CM were treated for 1 h with 1 μg/mL anti-G-CSF (Cedarlane, Burlington, ON, Canada) at 37°C before addition of CM to the DC culture.

Generation and treatment of bone marrow-derived DCs

For the generation of DCs, bone marrow was flushed out of femurs and tibia obtained from G-CSFR^{+/+} and G-CSFR^{−/−} mice (aged 4–10 weeks) using a fine needle and PBS. Bone marrow cells were incubated in C-RPMI supplemented with GM-CSF and IL-4 (10 ng/mL each; PeproTech, Rocky Hill, NJ, USA) to stimulate the differentiation of DCs. Culture media were replaced with fresh media containing cytokines 2 or 3 days after initiation of the culture, and cells were used in experiments 5–7 days after culture initiation.

Adenovirus-mediated overexpression of MEK7

MEK7 adenovirus was obtained from Jiahui Han (The Scripps Research Institute, La Jolla, CA, USA). For transient overexpression of MEK7, DCs were infected with MEK7 or empty adenovirus at a multiplicity of infection of 100 in serum-free media for 1 h and then washed and incubated overnight in complete media before use in experiments the next day.

Surface, intracellular staining, and flow cytometry

For the analysis of CD80 and CD86 expression, DCs were stimulated overnight, harvested, and stained for 20 min on ice before reading on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For the analysis of intracellular expression of IL-12/23 p40, cells were stimulated for 6–18 h, as stated in the figure legends—the final 4 h in the presence of 10 μg/mL brefeldin A (BioLegend, San Diego, CA, USA)—and then fixed with 1% formaldehyde for 10 min before fluorochrome-linked antibody staining for 20 min in PBS containing 0.1% saponin and 5% FBS. Data acquisition and analysis were performed using CellQuest (Becton Dickinson). Additional data analysis was performed using FlowJo (Tree Star, Ashland, OR, USA). Fluorochrome-linked antibodies used for flow cytometry were obtained from eBioscience.

Cytokine determination by ELISA

IL-12 was measured in cell culture supernatants using a p70-specific ELISA from R&D Systems. G-CSF was measured using an ELISA from R&D Systems. IFN- γ , IL-17, IL-23 (anti-p19 capture, anti-p40 detection), TGF- β , TNF- α , GM-CSF, and IL-6 were measured using ELISA kits from eBioscience. For measurement of cytokine levels in mouse serum, blood was obtained by the saphenous vein-puncture method and allowed to clot prior to centrifugation and serum removal.

Real-time qPCR

Total RNA was prepared using Trizol reagent (Invitrogen Canada, Burlington, ON, Canada), and 1 μ g RNA was reverse-transcribed into cDNA using Oligo-dT priming and M-MuLV-RT (NEB Biosciences, Pickering, Ontario, Canada). Gene expression was then quantified using a Rotor-gene 3000 (Corbett Life Science, Concord, NSW, Australia) and EXPRESS Sybr GreenER qPCR mix (Invitrogen Canada). Expression levels were quantified using the two-standard curve method with *gapdh* as a normalizer. Primer sequences used were: *gapdh* 5'-GCATTGTGGAAGGGCTCATG-3' (forward), 5'-TTGCTGTTGAAGTCGACAGAG-3' (reverse); *il-12b* (the p40 subunit of IL-12) 5'-AGTCCCTTTGGTCCAGTGTG-3' (forward), 5'-AGCAGTAG-CAGTTCCTCCGTA-3' (reverse). Primers were obtained from Sigma-Aldrich Canada.

Western blots

Total cell lysates were prepared using ice-cold lysis buffer containing 20 mM MOPS, 15 mM EGTA, 2 mM EDTA, 1 mM Na₃VO₄, 1 mM DTT, 75 mM β -glycerophosphate, 0.1 mM PMSF, 1 μ g/mL aprotinin, 10 μ g/mL pepstatin A, 1 μ g/mL leupeptin, and 1% Triton X-100. After incubation in lysis buffer on ice for 5 min, lysates were centrifuged for 15 min at 17,468 g to remove cell debris. SDS-PAGE loading buffer was then added to the cleared lysates (final concentrations: 12.5% glycerol, 31.25 mM Tris-HCl, 1% SDS, 0.02% bromophenol blue, and 1.25% β -ME). Samples were then boiled at 100°C for 5 min before resolving on SDS-PAGE gels using a Mini-Protean 3 apparatus (Bio-Rad, Mississauga, ON, Canada). Transfer to nitrocellulose membranes was performed using a Transblot SD semi-dry transfer system (Bio-Rad) at constant 18 V for 1 h. Membranes were blocked for 30 min using 5% w/v skim milk before incubation with primary antibodies. Staining was visualized using an ECL detection system (Pierce Biosciences, Fisher Canada, Nepean, ON, Canada). Band intensity quantification was performed using ImageJ (Rasband, W.S., *ImageJ*, U.S. National Institutes of Health, Bethesda, MD, USA, 1997–2009, <http://rsb.info.nih.gov/ij/>) [24], normalized to loading controls (β -actin or total protein of interest). Antibodies used for Western blotting were obtained from Cell Signaling Technology (NEB Biosciences).

Cytometric bead array analysis of phosphorylated JNK and p38

Phosphorylated JNK and p38 were measured in cell lysates using the cytometric bead array (Becton Dickinson), according to the manufacturer's instructions, with the following modification: cells were lysed using the lysis buffer listed in Western blots, above, prior to addition of the manufacturer's denaturation buffer and boiling at 100°C for 5 min. Quantification was carried out on a FACSCalibur (Becton Dickinson). Units of phosphorylated proteins were expressed relative to total protein measured by the Bradford assay (Bio-Rad).

Spleen cell isolation

Spleens were harvested and disrupted by gently forcing through a 40- μ m filter into PBS. RBCs were lysed using ammonium chloride potassium (ACK) lysis buffer (BioWhittaker, VWR International, Mississauga, ON, Canada). Splenic DCs were enriched by adherence to plastic culture dishes, followed by DC-specific analysis by gating on CD11c or CD80+ populations (see Supplemental Fig. 1). Cells were plated in C-RPMI and incubated at

37°C with 5% CO₂. Nonadherent cells were used in T cell stimulation assays.

Statistics and graphing

Statistical analysis was carried out using Prism 5.0c for Mac OS X (Graph-Pad Software, La Jolla, CA, USA). Two-tailed *t* tests and one-way ANOVA with Student Newman-Keuls post-test were performed as described in the figure legends. Pooled results of several independent experiments were used in all statistical analyses.

RESULTS

Probiotic *Lactobacillus* strains induce a distinct cytokine response in M Φ s

Previously, we have shown that M Φ s produce high levels of G-CSF and IL-10 but low levels of TNF- α in response to GR-1 and *Lactobacillus rhamnosus* strain GG in comparison with *E. coli* GR-12 and *Enterococcus faecalis* and that G-CSF induced by GR-1 has paracrine anti-inflammatory effects in M Φ s [15]. To further examine the strain specificity of this effect, M Φ s were treated with several bacterial strains, and the levels of TNF- α and G-CSF released into the culture media were compared by ELISA. Gram-negative *E. coli* strains GR-12 and Nissle 1917 induced robust production of TNF- α and G-CSF. However, GR-1 and strain GG and *Lactobacillus reuteri* RC-14 all showed preferential G-CSF production relative to TNF- α (Fig. 1A). *L. casei* showed the weakest induction of these cytokines among bacterial strains examined. We further examined the production of other cytokines in response to *E. coli* GR-12 and GR-1. The production of IL-6, IL-12, and GM-CSF was lower in GR-1-exposed than *E. coli* GR-12-exposed M Φ s (Fig. 1B); however, the production of TGF- β was slightly higher in GR-1-exposed than *E. coli* GR-12-exposed M Φ s. In summary, M Φ s preferentially produced remarkably high levels of

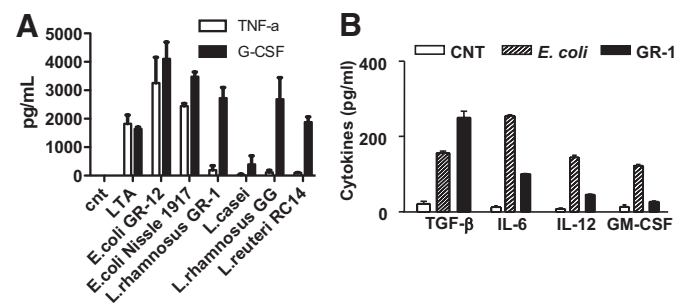


Figure 1. Live *Lactobacillus* strains preferentially induce high levels of anti-inflammatory cytokine production in M Φ s. Bone marrow-derived, immortalized M Φ s derived from C57BL/6 mice were treated with LTA (10 μ g/mL) or 50 CFU of the indicated bacteria, and cytokine release into the media was measured by ELISA at 4 h (for TNF- α) or 24 h (for G-CSF) poststimulation. *Lactobacillus* strains show preferential G-CSF production relative to TNF- α (A). GR-1 treatment induced lower levels of IL-6, IL-12, and GM-CSF compared with *E. coli* GR-12 but higher levels of TGF- β , all measured at 24 h (B). Data representative of at least three experiments. *L. casei*, *Lactobacillus casei*. cnt/CNT, Control.

G-CSF among several cytokines examined in response to *Lactobacillus* species.

Cytokines released by GR-1-treated MΦs do not induce inflammatory responses in DCs

The milieu of cytokines produced by MΦs exposed to different bacteria could affect DC activation. To examine how cytokines released by GR-1-exposed MΦs influence DC activation, DCs were treated with CNT CM, GR-1 CM, or *E. coli* CM in a 1:1 ratio with fresh culture media. *E. coli* CM enhanced intracellular expression of the shared p40 subunit of IL-12 and IL-23 (IL-12/23 p40; ~29% of cells vs. ~11% in control; **Fig. 2A**; $P < 0.01$; see Supplemental Fig. 2 for representative histograms). To determine if the increased response to the *E. coli* CM was a result of the presence of residual bacterial components in the media, *E. coli* CM was pretreated with Polymyxin B, which neutralizes the stimulatory effects of the Gram-negative bacteria cell wall component LPS. As shown in Supplemental Fig. 3, Polymyxin B (10 $\mu\text{g}/\text{mL}$) almost completely blocked expression of the IL-12/23 p40 stimulatory capacity of *E. coli* spent bacterial culture supernatant, verifying the effectiveness of Polymyxin B to neutralize the stimulatory capacity of *E. coli*-derived LPS that could be expected to be present in the *E. coli* CM. Polymyxin B decreased the IL-12/23 p40 response induced by *E. coli* CM to comparable levels as those induced by CNT CM (Fig. 2A), suggesting that the stimulatory effect of *E. coli* CM on DCs, over and above that of CNT CM itself, is largely a result of residual LPS from the *E. coli*. In contrast, *E. coli* CM-induced expression of the costimulatory mark-

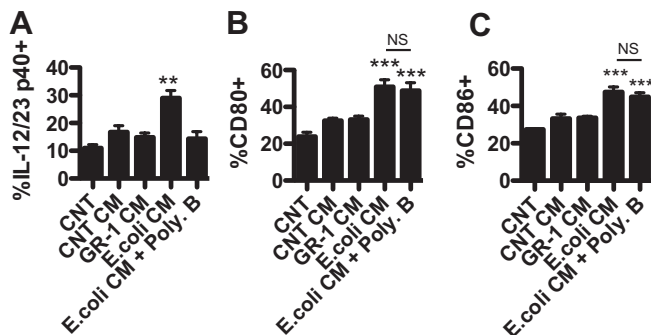


Figure 2. GR-1 CM does not induce an inflammatory phenotype in DCs, and *E. coli* CM induces high IL-12/23 p40 as a result of residual LPS and increases costimulatory marker levels independent of residual LPS. Bone marrow-derived DCs were treated overnight with CM in a 1:1 ratio with fresh media. Intracellular levels of IL-12/23p40 (A) and surface expression of costimulatory molecules CD80 (B) and CD86 (C) were measured by flow cytometry. GR-1 CM showed no significant induction of these molecules relative to CNT CM, and *E. coli* CM highly stimulated IL-12/23 p40 production and costimulatory molecule expression. The stimulatory effects of *E. coli* CM on IL-12/23 p40, but not CD80 or CD86, were LPS-dependent, as shown by the neutralization of LPS in the CM by Polymyxin B (Poly. B; 10 $\mu\text{g}/\text{mL}$; also see Polymyxin B control experiments in Supplemental Fig. 3). Bar graphs summarize data from at least three independent experiments. Representative histograms are shown in Supplemental Fig. 2. ** $P < 0.01$; *** $P < 0.001$; paired one-way ANOVA followed by Student Newman-Keuls post-test.

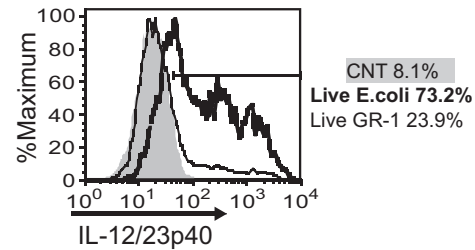


Figure 3. Live GR-1 weakly induces IL-12/23 p40 expression in DCs. Bone marrow-derived DCs were treated with 20 CFU of the indicated bacteria for 18 h—the final 6 h in the presence of brefeldin A. *E. coli*-treated cells show a large increase in intracellular IL-12/23 p40 expression, and in GR-1-treated cells, IL-12/23 p40 is induced to lower levels. Representative of three independent experiments.

ers CD80 (Fig. 2B) and CD86 (Fig. 2C) was not blocked by Polymyxin B, and the effect of *E. coli* spent bacterial culture supernatant was blocked >50% (Supplemental Fig. 3), suggesting LPS-independent stimulation of their expression by *E. coli* CM. GR-1 CM failed to induce IL-12/23 p40, CD80, or CD86 expression significantly (Fig. 2). Also, unlike *E. coli* CM-treated DCs, GR-1 CM-treated DCs did not induce T cell proliferation in a MLR relative to CNT CM (Supplemental Fig. 4A), nor did they induce IFN- γ and IL-17 production in syngeneic T cells stimulated with anti-CD3 (Supplemental Fig. 4B and C). Overall, these results demonstrated that GR-1 CM does not induce DC activation, and *E. coli* CM induces activation through LPS-dependent and -independent routes.

Live GR-1 weakly induces IL-12/23 p40 production in DCs relative to *E. coli* GR-12

As DCs appeared to be responding to residual bacterial products in the CM, we examined the direct effect of live GR-1 and *E. coli* GR-12 on bone marrow-derived DCs in vitro. Unlike live *E. coli* GR-12, which induced high levels of intracellular IL-12/23 p40 production, live GR-1 weakly induced production of the cytokine in DCs (Fig. 3). These results indicate that although live GR-1 is capable of stimulating IL-12/23p40 directly, it is less stimulatory than live *E. coli*.

G-CSF inhibits IL-12/23 p40 production in DCs in the context of GR-1 CM

As the induction of IL-12/23 p40 by *E. coli* CM was dependent on residual bacterial LPS present in the CM, residual GR-1 bacterial cell products could similarly be present in the GR-1 CM. However, although live GR-1 is able to at least weakly induce IL-12/23 p40 production in DCs (Fig. 3), GR-1 CM failed to induce any production of IL-12/23 p40 relative to CNT CM. This could be a result of the lack of a sufficient concentration of stimulatory components or the inhibition of the IL-12/23 p40 response by an anti-inflammatory cytokine(s) present in the GR-1 CM. To test if GR-1 CM contains an active component that could inhibit inflammatory cytokine production, IL-12 secretion stimulated by LPS was measured with or without pretreatment of DCs with GR-1 CM. Consistent with previous reports showing that the stimulation of IL-12 release re-

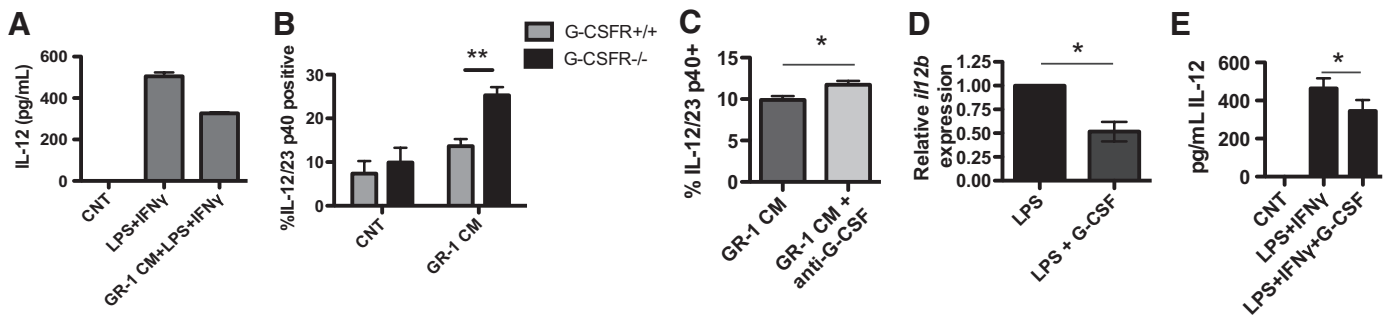


Figure 4. G-CSF inhibits IL-12/23 p40 production in DCs. DCs were pretreated for 15 min with GR-1 CM in a 1:1 ratio with fresh media and then stimulated with LPS (100 ng/mL) and IFN- γ (1000 U/mL), and IL-12 release into the media was measured 24 h later by ELISA. GR-1 CM inhibits LPS-induced IL-12 release (A). DCs were stimulated for 18 h with CM—the final 6 h in the presence of brefeldin A. Intracellular IL-12/23 p40 production was measured by flow cytometry. G-CSFR $^{-/-}$ DCs produce significantly higher levels of IL-12/23 p40 after GR-1 CM stimulation than WT DCs (B). Antibody-mediated neutralization of G-CSF within the GR-1 CM, by incubation of anti-G-CSF (1 μ g/mL) with GR-1 CM for 1 h prior to the addition of CM to the DC culture, also enhanced IL-12/23 p40 stimulation (C). DCs were pretreated for 45 min with rG-CSF (50 ng/mL) and then treated with LPS (100 ng/mL). IL-12 mRNA was measured by qPCR. rG-CSF inhibits *il12b* (p40 subunit of IL-12) mRNA transcription, as measured by real-time qPCR, 6 h after treatment (D). Data are shown relative to the positive control (LPS alone) and normalized to *gapdh*. No *il12b* expression was detected in nontreated cells. IL-12 protein (p70) was measured by ELISA in the culture supernatant of LPS (100 ng/mL) + IFN- γ (1000 U/mL)-treated DCs after 18 h of stimulation. rG-CSF inhibits IL-12 secretion (E). * $P < 0.05$; ** $P < 0.01$; unpaired *t* test (B) or paired *t* test (C–E), where samples from the same experiment are paired. Representative data of least two (A) or three (B–E) independent experiments. Data shown as mean + SEM.

quires IFN- γ in addition to LPS [25], LPS alone did not induce secretion of IL-12 from DCs, despite high induction of mRNA and intracellular protein levels (data not shown); therefore, LPS (100 ng/mL) plus IFN- γ (1000 U/mL) were used to induce IL-12 secretion. GR-1 CM inhibited IL-12 secretion from LPS + IFN- γ -treated DCs (Fig. 4A), indicating the presence of an anti-inflammatory component. As GR-1-exposed M Φ s secreted large amounts of G-CSF (Fig. 1 and ref. [14]), we examined the role of G-CSF in the inhibition of IL-12/23 using G-CSF-neutralizing antibodies as well as DCs generated from G-CSFR $^{-/-}$ mouse bone marrow, and G-CSFR $^{-/-}$ bone marrow cells differentiated into CD11c $^{+}$ DCs to a similar percentage as G-CSFR $^{+/+}$ cells, albeit at lower total numbers (Supplemental Fig. 5A and B; and data not shown). G-CSFR $^{-/-}$ CD11c $^{+}$ cells also showed similar expression of CD80 and CD86 (Supplemental Fig. 5C). G-CSFR $^{-/-}$ DCs showed enhanced IL-12/23 p40 production when treated with GR-1 CM alone (Fig. 4B; $P < 0.01$), suggesting that G-CSF, within the GR-1 CM, was able to suppress the IL-12/23 p40 production in response to a stimulatory component, possibly a GR-1-derived product present in the GR-1 CM. To confirm further the role of G-CSF, G-CSF in the GR-1 CM was neutralized using anti-G-CSF antibodies. GR-1 CM, pretreated with neutralizing anti-G-CSF antibodies, caused a slight but significant increase of IL-12/23p40 expression in WT DCs (Fig. 4C; $P < 0.05$). The minimal effect, relative to the enhanced IL-12/23 p40 production observed in G-CSFR $^{-/-}$ DCs, was possibly a result of an incomplete neutralization of the high levels of G-CSF present in the CM. To examine further the inhibitory effects of G-CSF on DC activation, DCs were treated with LPS (100 ng/mL), with or without a 45-min pretreatment with mouse rG-CSF (50 ng/mL). LPS-induced expression of *il12b* (encoding the p40 subunit of IL-12) at the mRNA level was reduced by $\sim 50\%$ after G-CSF pretreat-

ment (Fig. 4D; $P < 0.05$). rG-CSF also inhibited IFN- γ + LPS-induced IL-12 secretion by $\sim 30\%$ (Fig. 4E; $P < 0.01$). In contrast to the effects of G-CSF on IL-12 expression, rG-CSF did not inhibit LPS-induced CD80 or CD86 expression (Supplemental Fig. 6). Together, these results show that G-CSF, produced by GR-1-treated M Φ s, is capable of inhibiting the IL-12/23 response of DCs.

Phosphorylation of JNK is inhibited by rG-CSF, and reduced stimulation of JNK phosphorylation by GR-1 CM is related to reduced IL-12/23 p40 expression

As MAPKs and NF- κ B are key signaling components required for DC activation, we examined how these signaling molecules are regulated by GR-1 CM. As measured by Western blotting, *E. coli* CM and GR-1 CM induced tyrosine phosphorylation of p38, ERKs, and I κ B; however, tyrosine phosphorylation of JNKs was lower in GR-1 CM-treated than in *E. coli* CM-treated DCs (Fig. 5A). Cytometric bead array analysis, measuring levels of phosphorylated JNK (Fig. 5B) and p38 (Fig. 5C), revealed increased phosphorylation in response to *E. coli* CM (dependent on LPS present in the CM) but not GR-1 CM. Previously, we have shown that G-CSF inhibits LPS-induced TNF- α production in M Φ s, which is at least in part achieved through inhibiting JNK activation [15]. In DCs, JNK activation is also important in IL-12 production [26–28]. Thus, we examined whether G-CSF inhibits JNK phosphorylation in DCs. Indeed, G-CSF (≥ 10 ng/mL) was able to suppress LPS-induced phosphorylation of JNK in a dose-dependent manner (Fig. 5D). Relative band intensity analysis of four independent experiments showed a 50% average reduction in LPS-induced phosphorylation of JNK in DCs pretreated with 50 ng/mL G-CSF (Fig. 5E). As the reduced IL-12/23 p40 in GR-1 CM-stimulated DCs was at least partially dependent on G-CSF (Fig. 4B and C), and rG-CSF inhibits JNK (Fig. 5D and E), we examined

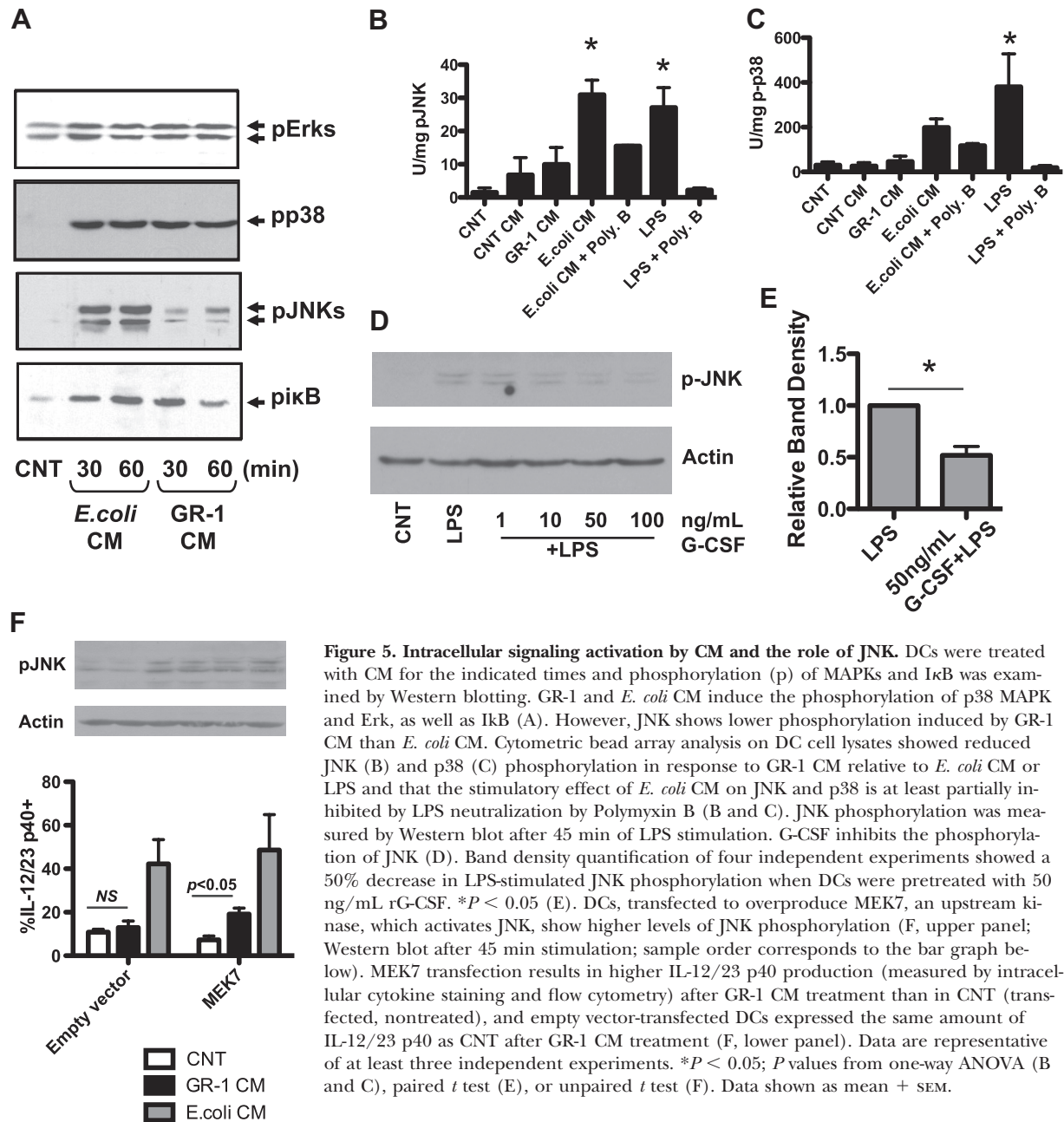


Figure 5. Intracellular signaling activation by CM and the role of JNK. DCs were treated with CM for the indicated times and phosphorylation (p) of MAPKs and I κ B was examined by Western blotting. GR-1 and *E. coli* CM induce the phosphorylation of p38 MAPK and Erk, as well as I κ B (A). However, JNK shows lower phosphorylation induced by GR-1 CM than *E. coli* CM. Cytometric bead array analysis on DC cell lysates showed reduced JNK (B) and p38 (C) phosphorylation in response to GR-1 CM relative to *E. coli* CM or LPS and that the stimulatory effect of *E. coli* CM on JNK and p38 is at least partially inhibited by LPS neutralization by Polymyxin B (B and C). JNK phosphorylation was measured by Western blot after 45 min of LPS stimulation. G-CSF inhibits the phosphorylation of JNK (D). Band density quantification of four independent experiments showed a 50% decrease in LPS-stimulated JNK phosphorylation when DCs were pretreated with 50 ng/mL rG-CSF. * $P < 0.05$ (E). DCs, transfected to overproduce MEK7, an upstream kinase, which activates JNK, show higher levels of JNK phosphorylation (F, upper panel; Western blot after 45 min stimulation; sample order corresponds to the bar graph below). MEK7 transfection results in higher IL-12/23 p40 production (measured by intracellular cytokine staining and flow cytometry) after GR-1 CM treatment than in CNT (transfected, nontreated), and empty vector-transfected DCs expressed the same amount of IL-12/23 p40 as CNT after GR-1 CM treatment (F, lower panel). Data are representative of at least three independent experiments. * $P < 0.05$; P values from one-way ANOVA (B and C), paired t test (E), or unpaired t test (F). Data shown as mean + SEM.

further the role of JNKs in IL-12/23 p40 production after CM treatment. MEK7 (a direct, upstream kinase for JNKs) was overexpressed using an adenoviral vector, and production of IL-12/23 p40 was examined after CM exposure. Viral-mediated overexpression of MEK7 caused phosphorylation of JNK (Fig. 5F, upper panel). Although DCs transfected with an empty vector produced the same amount of IL-12/23 p40 after CNT or GR-1 CM, DCs overexpressing MEK7 produced higher amounts of IL-12/23 p40 in response to GR-1 CM (Fig. 5F, lower panel; $P < 0.05$ vs. MEK7-transfected control), although still lower than those induced by *E. coli* CM. As G-CSF is abundant within the GR-1 CM (Fig. 1), inhibits IL-12/23p40 expression (Fig. 4), and can inhibit JNK activation (Fig. 5D and E),

these results suggest that the inhibition of JNK by G-CSF within the GR-1 CM is important for limiting IL-12/23 p40 expression.

Live GR-1 induces G-CSF production in vivo and suppresses IL-12/23 p40 production in LPS-challenged DCs

To examine the role of G-CSF in DC activation after GR-1 treatment in vivo, G-CSFR $^{+/+}$ and G-CSFR $^{-/-}$ mice were injected i.p. with live GR-1, and responses of DCs to LPS were examined ex vivo. I.p. injection of LPS induced high amounts of TNF- α [~ 500 pg/ml; $P < 0.05$ vs. PBS (vehicle control)], measured in the serum 1 h after injection (Fig. 6A). However,

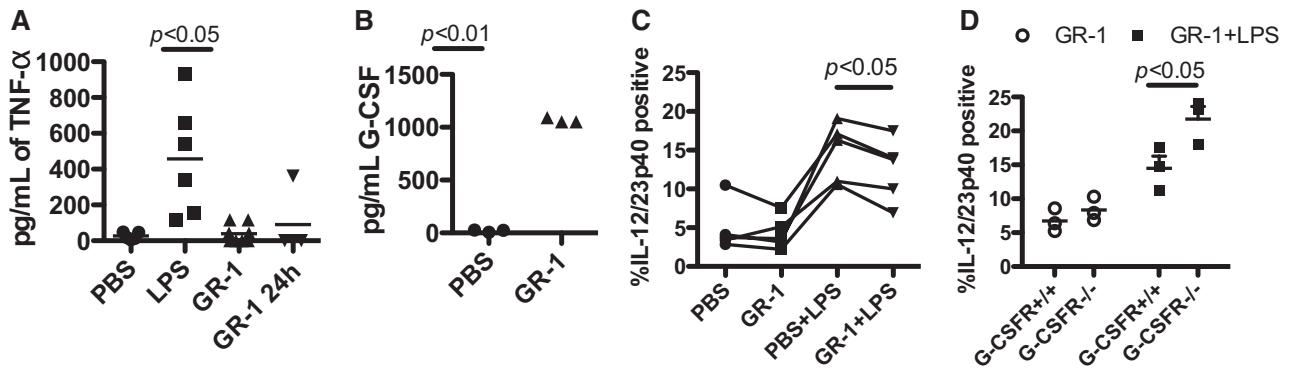


Figure 6. In vivo effect of GR-1-induced G-CSF on DCs. Mice were injected i.p. with 10^7 CFU GR-1 and analyzed 24 h later for circulating G-CSF levels by ELISA. Although TNF- α , measured in the blood 1 h after injection, was highly induced by LPS (1 μ g/g body weight), it was not induced by GR-1, 1 h or 24 h after injection (A). G-CSF was highly induced in the blood by GR-1 injection (24 h after injection; B). Splenocytes from mice injected with GR-1 were harvested 24 h after injection, and cells were treated ex vivo overnight with 1 μ g/mL LPS and analyzed for the IL-12/23 p40 subunit production by flow cytometry [DCs were enriched by adherence and gated on CD80 ($n=2$)- or CD11c ($n=3$)-positive cells; see gating example in Supplemental Fig. 1]. Injection with GR-1 inhibits LPS-induced IL-12/23 p40 production ex vivo. Lines connect pairs of PBS- and GR-1-injected mice from independent experiments (C). DCs in the spleens of GR-1-injected G-CSFR $^{-/-}$ responded with higher IL-12/23 p40 production after ex vivo stimulation (as in C) than G-CSFR $^{+/+}$ cells (all samples gated on CD80+; $n=3$; D). Data are representative of at least three experiments; P values are derived from paired t test (C) and unpaired t test (A, B, and D). Horizontal lines indicate group mean.

only low levels of TNF- α (20–50 pg/ml) were detected in GR-1-injected mice, 1 h and 24 h after injection. G-CSF was highly induced to over 1000 pg/mL, 24 h after live GR-1 injection (Fig. 6B; $P<0.01$ vs. PBS). These results show that a similar profile of TNF- α /G-CSF production is induced by GR-1 in vivo, as observed after in vitro treatment of M Φ (Fig. 1). We then examined how DCs exposed to GR-1-stimulated G-CSF in vivo respond to LPS challenge ex vivo. Spleens were removed 24 h after PBS or GR-1 injection and splenocytes isolated and plated in RPMI. DCs were enriched by removing nonadherent cells. Adherent cells were stimulated with LPS and analyzed for intracellular IL-12/23 p40 expression by flow cytometry with gating on the CD11c+ ($n=3$) or CD80+ cells ($n=2$; see Supplemental Fig. 1 for gating example). Consistent with the in vitro data reported here, splenic DCs (defined by plastic adherence and CD11c or CD80 expression), isolated from G-CSFR $^{+/+}$ mice 24 h after GR-1 injection when circulating G-CSF levels are high, showed less IL-12/23 p40 production in response to LPS treatment ex vivo than those from PBS-injected mice (Fig. 6C; $P<0.05$). However, splenic DCs from GR-1-injected G-CSFR $^{-/-}$ mice showed a significantly higher level of IL-12/23 p40 induction after ex vivo LPS stimulation than those from GR-1-treated WT mice (Fig. 6D; $P<0.05$). This indicates that in vivo, GR-1-induced G-CSF can have anti-inflammatory effects on DCs, consistent with the in vitro data reported above.

DISCUSSION

The mechanism of immune modulation by strains of probiotics is an active area of study that may have important therapeutic implications for inflammatory disorders. The role of crosstalk between M Φ s and DCs over the course of a response to probiotic bacteria remains to be fully explored. In this

study, we show that the probiotic GR-1 modulates DC function through preferentially inducing G-CSF production in M Φ s. GR-1 induced a cytokine profile in M Φ s characterized by high G-CSF and TGF- β but low levels of inflammatory cytokines such as TNF- α relative to those induced by *E. coli* (Fig. 1). The preferential stimulation of G-CSF over TNF- α was also observed for *L. rhamnosus* GG and *L. reuteri* RC-14, and *L. casei* showed little stimulatory effects on G-CSF or TNF- α production. As GR-1 showed high G-CSF induction, and the immunological effects of this strain are less well-studied than GG, it was selected for further investigation. The GR-1 CM did not induce the expression of IL-12/23 p40, costimulatory markers, allogeneic T cell expansion, or T cell cytokine production any higher than the CNT CM (Fig. 2 and Supplemental Fig. 4). However, GR-1 CM treatment of G-CSFR $^{-/-}$ DCs or the neutralization of G-CSF in the CM using polyclonal antibody revealed that the IL-12/23 p40-inducing capacity of factors within the CM, possibly residual bacterial products, was inhibited by G-CSF (Fig. 4B and C). Consistent with this, GR-1 CM inhibited LPS-induced IL-12 secretion (Fig. 4A), and rG-CSF also inhibited IL-12 expression in DCs in response to LPS (Fig. 4D and E). Our preliminary evidence also shows that G-CSF inhibits IL-12 expression induced in response to the Gram-positive cell wall component LTA (data not shown). The observed M Φ -DC crosstalk is likely to be important in situations where infiltrating DCs are transiently exposed to the cytokine microenvironment, set up by tissue resident M Φ s responding to bacterial stimulation, as may occur in the intestinal or urinary tract.

To understand the molecular basis of the observed phenotypes of DCs, we investigated CM-induced activation of MAPK signaling pathways (Fig. 5A–C). GR-1 CM and *E. coli* CM were observed to induce Erk and I κ B phosphorylation. However, GR-1 CM consistently showed reduced stimulation of JNK in

DCs, an important kinase for IL-12 production and subsequent Th1 responses [26–28]. Also, forced activation of JNK by adenoviral expression of WT MEK7 enhanced IL-12/23 p40 production in GR-1 CM-exposed DCs (Fig. 5F). These data, together with data indicating that rG-CSF inhibited IL-12 production (Fig. 4D and E) and JNK phosphorylation (Fig. 5D and E), suggest an important role of JNK inhibition by G-CSF in suppressing IL-12/23 p40 production in LPS or GR-1-CM-exposed DCs.

Although rG-CSF inhibited LPS-induced IL-12 production (Fig. 4D and E), *E. coli* CM, containing high levels of G-CSF, still induced high levels of IL-12/23 p40. Although the induction of IL-12/23 p40 by *E. coli* CM is also dependent on LPS, it is possible that the concentration of LPS within the CM is high enough to overcome the inhibitory effect of G-CSF. Alternatively, the high level of other cytokines, such as TNF- α (Fig. 1), may modify the effect of G-CSF. Further work will be required to elucidate the effect of other cytokines on the inhibitory properties of G-CSF.

IL-12/23 p40 is the shared subunit of the cytokines IL-12 and IL-23, associated with the induction of Th1 and Th17 responses, respectively. The antibody used for intracellular staining in this study is specific for the p40 subunit; therefore, the measured increases could be indicative of the production of both cytokines. However, ELISAs specific for the cytokines showed an increase in the release of IL-12 (Fig. 4A and E) but not IL-23 (data not shown), suggesting that IL-12/23 p40, measured in the intracellular staining experiments, was mainly indicative of increased IL-12 production. However, as we showed previously that G-CSF can inhibit IL-23 expression in human blood monocytes [16], the inhibition of IL-23 by G-CSF in DCs cannot be ruled out.

To determine whether GR-1-induced G-CSF could also inhibit the inflammatory responses of DCs in vivo, we injected mice i.p. with live GR-1, and GR-1 induced production of high levels of G-CSF (Fig. 6B), as measured in the serum, but only low levels of TNF- α (Fig. 6A), similar to as is seen with the M Φ CM in vitro. DCs isolated from the spleens of GR-1-injected mice produced lower IL-12/23 p40 when stimulated with LPS ex vivo (Fig. 6C), and DCs of GR-1-injected G-CSFR $^{-/-}$ mice produced higher levels of IL-12/23 p40 than GR-1-injected G-CSFR $^{+/+}$ DCs after LPS treatment (Fig. 6D). Collectively, these results indicate that G-CSF, produced in the context of the response to GR-1 in vivo, can inhibit the inflammatory response of DCs. As direct treatment of DCs with live GR-1 only weakly induced IL-12/23 p40 relative to *E. coli* (Fig. 3), and GR-1 CM was able to inhibit LPS-induced IL-12 release by DCs (Fig. 4A), the overall effect of GR-1 in vivo on DCs may result from a combination of direct stimulation with GR-1 and the effects of M Φ -derived cytokines such as G-CSF.

Our results are in line with previous studies showing that G-CSF pretreatment in mice protects against an otherwise lethal dose of LPS, which is accompanied by a suppression of LPS-induced production of serum TNF- α , IL-12, and IL-18 [29, 30], and that T cells from G-CSF $^{-/-}$ mice produce large quantities of IFN- γ in a chronic infection model [31]. Similarly, in humans, G-CSF injection attenuates the inflammatory responses of blood cells by reducing production of TNF- α and

IFN- γ in response to ex vivo LPS challenge [32, 33] and mobilizes Th2-inducing DCs into the peripheral blood [34]. The effects of G-CSF on Th17 responses, in which IL-23 is an important player, have been controversial. Recent studies have suggested that G-CSF injection leads to an increase in Th17 in the blood [35, 36], and another study indicated reduced Th17 levels after G-CSF injection [37]. Although our studies into the effects of G-CSF on Th17 responses are ongoing, our unpublished data lend support to the inhibition of Th17 responses by G-CSF and show that this may occur through the effects of G-CSF on DCs.

Recently, we have shown that G-CSF is highly expressed in the isolated intestinal cells and tissues of mice and humans, and lower G-CSF levels are observed in IBD-afflicted human intestinal samples than those from non-IBD individuals [16]. As probiotics, including some *Lactobacillus* strains, have been shown to have the ability to modulate intestinal immunity, the ability of these bacteria to induce G-CSF production and the downstream effects of G-CSF on key immune cells may have important implications for the treatment of human diseases. Preliminary studies using G-CSF for the treatment of IBD have been successful in mouse colitis models [38, 40] and in human clinical trials [17, 41]. The role of G-CSF in the observed beneficial effects of probiotics in colitis remains to be explored, but it is likely that the immunomodulatory effects of G-CSF on DCs, as we have shown here, could contribute to the resolution of the destructive inflammatory response.

Interestingly, G-CSF has been shown to have inhibitory effects on human monocyte-derived DCs in the context of soluble factors released by pancreatic cancer cells [42]. Although the treatment times were considerably longer than those used in the present study, these results suggest the possibility that the anti-inflammatory effects of G-CSF, which may be beneficial in the case of some inflammatory diseases, may also be detrimental in certain cancers, where the immunosuppressive environment of the tumor must be overcome for the immune system to eliminate cancerous cells.

In conclusion, G-CSF, which is highly induced in M Φ s by GR-1, can modulate IL-12 production by DCs in vitro and in vivo. The identification of cytokine pathways regulating inflammatory responses in DCs and the crosstalk between M Φ s and DCs in the course of a response will prove valuable in the development of novel therapeutics to aid in the management of inflammatory disorders.

AUTHORSHIP

A.J.M. designed and performed experiments, analyzed data, and wrote the manuscript; S.S. and H.I.S. performed experiments; S.O.K. conceived of the study, secured funding, designed and supervised experiments, analyzed data, and wrote the manuscript.

ACKNOWLEDGMENTS

The study was supported by the Natural Sciences and Engineering Research of Canada (no. 312482-2008 RGPIN) to S.O.K. We thank Drs. Y. Fujio and J. Han for the adenovirus

vectors for Adeno-empty vector and MEK7, respectively, and Dr. D. J. Link for G-CSFR^{-/-} mice.

DISCLOSURE

There is no conflict of interest.

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KEY WORDS:

probiotics · cytokines · growth factors · immunomodulation