G-CSF-mediated inhibition of JNK is a key mechanism for \textit{Lactobacillus rhamnosus}-induced suppression of TNF production in macrophages

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Summary

\textit{Lactobacillus rhamnosus} is a human commensal with known immunomodulatory properties. To date the mechanism of these immunomodulatory effects is not well understood. To unravel the immunomodulatory signalling mechanism, we investigated the effects of two strains of \textit{L. rhamnosus}, \textit{L. rhamnosus} GG and GR-1, in modulating production of tumour necrosis factor-\(\alpha\) (TNF) in human mononuclear cell line THP-1 and mouse macrophages. Live \textit{L. rhamnosus} GG and GR-1 or their spent culture supernatant induced minute-amounts of TNF production but large quantities of granulocyte-colony stimulating factor (G-CSF) in macrophages compared with those induced by pathogenic \textit{Escherichia coli} GR-12 and \textit{Enterococcus faecalis}. By using neutralizing antibodies and G-CSF receptor knockout mice, we demonstrated that G-CSF secreted from \textit{L. rhamnosus} GG- and GR-1-exposed macrophages suppressed TNF production induced by \textit{E. coli}- or lipopolysaccharide-activated macrophages through a paracrine route. The suppression of TNF production by G-CSF was mediated through activation of STAT3 and subsequent inhibition of c-Jun-N-terminal kinases (JNKs). The inhibition of JNK activation required STAT3\(\alpha\)-mediated de novo protein synthesis. This demonstrates a novel role of G-CSF in \textit{L. rhamnosus}-triggered anti-inflammatory effects and its mechanism in the suppression of TNF production in macrophages.

Introduction

\textit{Lactobacillus} is a common constituent of the indigenous microbiota in the human intestinal and urogenital tracts (Reuter, 2001) and strains of this genus have been used as probiotics, defined as ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (Reid et al., 2003). Although lactobacilli, and probiotics in general, have been extensively studied for their role in ameliorating various inflammatory ailments (Madsen et al., 1999; O’Mahony et al., 2001; Borruel et al., 2002; Dieleman et al., 2003; McCarthy et al., 2003; Montrose and Floch, 2005), the mechanism(s) involved in their anti-inflammatory effects is not yet well understood. Studies over the last several years have revealed potential anti-inflammatory mechanisms of commensal microbes at the level of mucosal epithelial cells, where the microbes directly modulate production of inflammatory cytokines through inhibiting key signalling molecule nuclear factor \(\kappa\)B (NF-\(\kappa\)B) (Neish et al., 2000; Kelly et al., 2004) and proteasomes (Petrof et al., 2004). Recent studies have also indicated that the anti-inflammatory effects of probiotics are not limited to the level of intestinal mucosa but modulate the host immune responses at distant sites (Noverr and Huffnagle, 2004). The extra-intestinal anti-inflammatory effects produced by oral or systemic (subcutaneous) administrations of probiotics have been demonstrated in various inflammatory, autoimmune and atopic diseases including arthritis, dermatitis and allergies (Kato et al., 1998; Baharav et al., 2004; Miraglia del Giudice and De Luca, 2004; Noverr and Huffnagle, 2004; Sheil et al., 2004).

Most microbes harbour pathogen-associated molecular patterns and induce inflammatory cytokines through activating pattern recognition receptors. Probiotics are inefficient in inducing pro-inflammatory cytokines but can effectively modulate host immune responses, possibly through immunomodulating autocrines and/or paracrines (Madsen et al., 1999; Pena and Versalovic, 2003; Sheil et al., 2004).
Several microbes and parasites modulate immune responses and inflammation process to gain access to the host and proliferate. For example, the parasitic helminths potently stimulate IL-10 production and induce an anti-inflammatory environment in the host (Hartmann and Lucius, 2003). Protozoan parasite Toxoplasma gondii inhibits nitric oxide (NO) production and induces anti-inflammatory responses through transforming growth factor-β1 (TGF-β1) (Seabra et al., 2004). Mycobacterium inhibits interferon-γ (IFN-γ) production and response in macrophages through IL-6 secreted from Mycobacterium-infected macrophages (Nagabhushanam et al., 2003). Although various strains of probiotics induce anti-inflammatory effects in vitro and in experimental animal models, the mediators and mechanisms responsible for their effects have not yet been explored (Borruel et al., 2002; Morita et al., 2002; McCarthy et al., 2003; Pena and Versalovic, 2003; Sheil et al., 2004; Pena et al., 2005).

Macrophages are the major source of various cytokines and well populated in the normal intestinal lamina propria close to inter- and sub-epithelial layers and Peyer's patches, directly interacting with penetrating commensal bacteria (Sansone, 2004). Direct interaction between commensal bacteria and the large number of infiltrating peripheral monocytes at the onset of or during active intestinal inflammation has been shown to be involved in pathogenesis of inflammatory diseases (Mahida, 2000; Saniabadi et al., 2003; Watanabe et al., 2003; Welte et al., 2003). Therefore, immune responses and cytokines released by macrophages in response to probiotics are particularly crucial for understanding the mechanism(s) of their immunomodulating effects on the host. In this study, we examined an immunomodulating mechanism of two well-studied strains of Lactobacillus rhamnosus, L. rhamnosus GG and GR-1 in macrophages. L. rhamnosus GG (GG), which was isolated from the stool of a healthy individual, is one of the most studied probiotic strains and its role in ameliorating inflammatory ailments has been demonstrated in various animal disease models and human patients (Madsen et al., 1999; Gorbach, 2002; Guandalini, 2002; Doron et al., 2005; Montrose and Floch, 2005). L. rhamnosus GR-1 (GR-1) is an urogenital isolate that possesses a number of properties considered important for urogenital probiotics (Reid and Bruce, 2001; Gardner et al., 2002). This study showed that both GG and GR-1 potently induced production of granulocyte-colony stimulating factor (G-CSF), which was a crucial mediator for suppressing tumour necrosis factor-α (TNF) production through activating signal transducer and activator of transcription (STAT)-3 and subsequently inhibiting activation of c-Jun-N-terminal kinases (JNKs) in macrophages. These results define G-CSF as a key mediator through which probiotics, particularly L. rhamnosus, elicit immunomodulating effects on the host.

Results

Lactobacillus rhamnosus GR-1 and GG suppress Escherichia coli-induced inflammatory cytokines through a paracrine route in macrophages

We first examined how macrophages react to Lactobacilli by assessing production of TNF in response to two strains of live L. rhamnosus GG and GR-1 in C57BL/6 mouse bone marrow-derived immortalized macrophages (BMDIM). As shown in Fig. 1A, live L. rhamnosus GG and L. rhamnosus GR-1 are much less potent than pathogenic bacteria E. coli GR-12 and Enterococcus faecalis 1311 in inducing production of TNF. Treatments with bacterial spent culture supernatant (SCS) induced the similar profile of TNF production (data not shown). As macrophages are a main source of cytokines including anti-inflammatory cytokines, we hypothesized that GG- or GR-1-treated macrophages can indirectly modulate self or other macrophages through autocrine/paracrine routes. To examine their indirect immunomodulating effects, we obtained conditioned media (CM) from BMDIM treated with either E. coli, E. faecalis, Lactobacillus GG, or GR-1 and assessed their effects on TNF production induced by E. coli-SCS. As shown in Fig. 1B, pretreatment of CMs obtained from live GG- or GR-1-treated BMDIM (GR-1-CM or GG-CM respectively) strongly suppressed TNF production induced by E. coli-SCS. However, such effect was not detected when the pretreatment was less than 30 min (data not shown) and not by CMs prepared from E. coli- or E. faecalis-treated cells (Fig. 1B). CMs prepared from SCS of GG or GR-1 but not from heat-killed GG or GR-1 also suppressed the TNF production (data not shown). Although E. coli or E. faecalis induced large quantities of TNF in BMDIM (Fig. 1A), CMs prepared from E. faecalis- or E. coli-treated cells contained low amount of TNF (data not shown) and therefore, had little additive effects on subsequent TNF measurements (Fig. 1B). This was due to the facts that CMs were collected after 6 h of bacterial treatment and were further diluted with fresh culture media (1:1 ratio) before treating fresh cells. Production of TNF peaked at 5–6 h of E. coli-SCS treatment (data not shown). Real-time polymerase chain reaction (PCR) analysis further showed that GG-CM or GR-1-CM downregulated TNF mRNA expression induced by live E. coli in BMDIM (Fig. 1C). We also examined whether the inhibitory effect can be detected in human monocytic cell line THP-1 cells. As shown in Fig. 1D, CMs prepared from live GG- or GR-1-treated THP-1 cells also suppressed TNF production induced by E. coli-SCS in THP-1 cells.

Lactobacillus rhamnosus GG and GR-1 potently induce production of anti-inflammatory cytokines

To identify mediators that are involved in the TNF suppres-
sive effect of GG and GR-1, we examined the presence of inhibitory cytokines including IL-10 and G-CSF in CMs prepared from live *E. coli*- or GR-1-exposed BMDIM. IL-10 was detected up to 200–300 pg ml$^{-1}$ in all *E. coli*- or GR-1-exposed cells. Surprisingly, G-CSF was detected at levels up to 50–70 ng ml$^{-1}$ in GG- or GR-1-exposed BMDIM (Fig. 2A). *E. coli* induced ∼25 ng ml$^{-1}$ G-CSF, about half of the concentration induced by GG. Similar results were obtained from human THP-1 cells (data not shown). In consistent with protein production, live GG or GR-1 upregulated mRNA expression of IL-10 and G-CSF up to 6–8 and 17–20-fold, respectively, in BMDIM (Fig. 2B).

**G-CSF is the key inhibitory factor responsible for *L. rhamnosus*-induced suppression of TNF production**

Both IL-10 and G-CSF have been shown to have TNF suppressing effects on macrophages (Moore et al., 2001; Boneberg and Hartung, 2002; Nishiki et al., 2004). As GR-1 induced IL-10 and G-CSF production (Fig. 2), we examined whether GR-1-induced suppression of TNF production was mediated through IL-10 and/or G-CSF. As shown in Fig. 3A, lipopolysaccharide (LPS) induced TNF production in THP-1 cells, which was suppressed by CM prepared from GR-1-exposed THP-1 cells. Depletion of IL-10 by neutralizing antibodies from the GR-1-CM only marginally reversed the suppression of TNF production, whereas depletion of G-CSF reversed ∼95% of the suppression. As the neutralizing IL-10 antibody might not completely deplete IL-10 and small amounts of IL-10 could be enough to suppress TNF production, we further examined the role of IL-10 and G-CSF using primary peritoneal macrophages deficient in IL-10 or G-CSF-receptor (G-CSFR) respectively. Primary peritoneal macrophages isolated from wild-type (*C57BL/6*) or *C57BL/6* IL−10−/− mice were treated with live GR-1 to obtain conditioned media GR-1-CM$^w$ and GR-1-CM$^{IL−10−/−}$ respectively. Fresh primary peritoneal macrophages isolated from wild-type mice were treated with LPS (100 ng ml$^{-1}$) in the presence or absence of the CM pretreatment. As observed in BMDIM or THP-1 cells, both GR-1-CM$^w$ and GR-1-CM$^{IL−10−/−}$ suppressed TNF production in the similar extents (Fig. 3B), indicating that IL-10 is not required for the suppression. Involvement of G-CSF was further examined using G-CSFR$^{−/−}$ macrophages. Macrophages isolated from wild-type or G-CSFR$^{−/−}$ mice were treated with LPS (100 ng ml$^{-1}$) in the presence or absence of GR-1-CM$^w$ pretreatment and

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**Fig. 1.** Anti-inflammatory paracrine effects of *L. rhamnosus* GR-1 (GR-1) and GG (GG).

A. Production of TNF in non-treated (CNT) or live bacteria (10 cfu per cell)-treated bone marrow-derived immortalized macrophages (BMDIM) for 9 h was analysed from cell culture media.

B–D. Inhibition of TNF production (B and D) and mRNA expression (C) by *L. rhamnosus*-treated macrophage-conditioned medium. BMDIM (B and C) or human monocytic cell line THP-1 cells. Expression of TNF mRNA (C) was measured 4 h after treatment by real-time PCR analysis. Production of TNF was measured 9 h after treatments using bioassay as described previously (Poltorak et al., 1998). Data are expressed as means ± SD of three independent experiments.

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production of TNF was measured. As shown in Fig. 3C, TNF production in wild-type macrophages was potently suppressed by GR-1-CM. However, TNF production in LPS-activated G-CSFR−/− macrophages was not suppressed by GR-1-CM. We then examined whether the concentration of G-CSF present in GR-1-CM was high enough to elicit TNF suppressive effect in THP-1 cells using human recombinant G-CSF (hG-CSF). As shown in Fig. 3D, hG-CSF suppressed TNF production in a dose-dependent manner at a concentration range from 100 to 500 pg ml−1. These data supported a crucial role of G-CSF but not IL-10 in mediating TNF suppression induced by GR-1-CM in macrophages.

**GR-1-CM preferentially induces tyrosine phosphorylation of p38 MAPK and STAT3**

To investigate the mechanism of GR-1-CM-mediated TNF suppression, we first examined several signalling molecules known to be involved in anti-inflammatory pathways in macrophages. Among them, STAT-3 was strongly activated by GR-1-CM (Fig. 4A). GR-1-CM preferentially induced phosphorylation of STAT3 at tyrosine-705 (Y705), which is required for STAT3 activation, but not at serine-727 (S727). In contrast, *E. coli*-CM strongly induced phosphorylation of STAT3 at both tyrosine and serine residues. *E. coli*-CM also induced phosphorylation of STAT1 and STAT5 at Y-701 and Y-694 respectively. GR-1-CM slightly induced STAT5 Y-694 phosphorylation but STAT1 phosphorylation was not detected. Phosphorylation at S727 of STAT3 has been shown to be mediated through the mitogen-activated protein kinases (MAPK) ERK1 and ERK2 (Jain et al., 1998; Woetmann et al., 1999; Haq et al., 2002; Wierenga et al., 2003; Tian and An, 2004). Therefore, we examined whether GR-1-CM or *E. coli*-CM induced ERK activation. Indeed, *E. coli*-CM induced phosphorylation of ERK1 and ERK2 at their activation sites after 15 min of treatment, whereas GR-1-CM did not stimulate ERK1 and 2 phosphorylations (Fig. 4B). *E. coli*-CM also induced other MAPKs including JNKs and p38, but GR-1-CM induced only p38 MAPK phosphorylation (Fig. 4B).

**G-CSF is the factor responsible for JAK2-STAT3 pathway activation in GR-1-CM-treated macrophages**

To examine whether the STAT3 Y705 phosphorylation was induced by G-CSF or IL-10, we used human IL-10 or G-CSF neutralizing antibodies. As shown in Fig. 5A, phosphorylation of STAT3 at Y705 was not detected in G-CSF-depleted GR-1-CM, whereas IL-10-depletion did not affect the phosphorylation level. Similarly, in mouse macrophages, CM obtained from IL10-deficient peritoneal macrophages (GR-1-CMIL10−/−) still induced the STAT3 phosphorylation (Fig. 5B). We also used macrophages lacking G-CSF receptor (G-CSFR−/−) to examine the role of G-CSF. Figure 5C shows that GR-1-CM induced strong STAT3 Y705 phosphorylation but the phosphorylation was completely absent in G-CSFR−/− macrophages. In addition, STAT3 Y705 phosphorylation was induced by hG-CSF at a concentration of 100 pg ml−1 and maximally at 500 pg ml−1 (Fig. 5D). These concentrations were well correlated with the suppression level of TNF production (Fig. 3D). Although both IL-10 and G-CSF induces STAT3 Y705 phosphorylation, it was shown that G-CSF-induced STAT3 phosphorylation is mainly mediated through Janus kinase 2 (JAK2) and that the JAK2-specific inhibitor AG490 significantly blocks G-CSF-induced STAT3 activation (Hasegawa et al., 2003; Nishiki et al., 2004). On the other hand, IL-10-induced STAT3 phosphorylation is unaffected by AG490 (Donnelly et al., 1999; Nishiki et al., 2004). GR-1-CM-induced phosphorylation of STAT3 Y705 was also completely blocked by AG490 (Fig. 5E), which further supports the notion that G-CSF is the main factor involved in the activation of STAT3.
Fig. 3. G-CSF is required for the *L. rhamnosus* GR-1-CM-induced inhibition of TNF production in LPS-activated macrophages. 

A. THP-1 cells were exposed to LPS (100 ng ml$^{-1}$) without (CNT) or with pretreatment (45 min) of GR-1-CM for the time indicated. G-CSF- or IL-10-depleted GR-1-CMs from THP-1 cells were obtained by incubating human G-CSF (0.1 μg ml$^{-1}$) or IL-10 (0.3 μg ml$^{-1}$) neutralizing antibodies in GR-1-CM for 1 h.

B. Isolated primary mouse peritoneal macrophages were exposed to LPS with or without pretreatment with GR-1-CM prepared from wild-type or IL-10$^{-/-}$ peritoneal macrophages (GR-1-CM$^{wt}$ and GR-1-CM$^{IL10^{-/-}}$ respectively).

C. Mouse peritoneal macrophages were isolated from wild-type or G-CSFR$^{-/-}$ mice and exposed to LPS (100 ng ml$^{-1}$) with or without pretreatment with GR-1-CM prepared from wild-type primary macrophages.

D. THP-1 cells were treated with LPS (100 ng ml$^{-1}$) with or without various concentrations of recombinant human G-CSF in THP-1 cells. Production of TNF was measured 9 h after LPS-treatment as described previously (Poltorak et al., 1998). Data are expressed as means ± SD of three independent experiments.

Fig. 4. STAT3 Y-705 phosphorylation is induced by GR-1-CM. BMDIM were treated with GR-1-CM or *E. coli*-CM for the time indicated. Total cell lysates (35 μg per lane) were analysed for Western blotting using phospho-specific or anti-peptide antibodies against various protein kinases indicated. Results are representative of two independent experiments.
STAT3 activation is required for the suppression of TNF production by GR-1-CM

To examine whether STAT3 activation is required for GR-1-CM-mediated inhibition of TNF production, we first assessed the TNF suppressing effects of GR-1-CM in the presence or absence of different chemical inhibitors for JAKs and STAT3 in BMDIM. As shown in Fig. 6A, the suppressive effects of GR-1-CM on TNF production was almost completely abolished by membrane permeable STAT3 inhibitor peptide (STAT3i) and JAK2 inhibitor AG490, but not by JAK3 inhibitor, indicating that the GR-1-CM inhibitory effect required JAK2-mediated STAT3 activation. We further examined whether the phosphorylation at Y705 is crucial for the GR-1-CM suppressive effects using adenovirus-mediated overexpression of Y705F-mutated dominant negative human STAT3 (hSTAT3-DN). Y705 phosphorylation was previously shown to be necessary for dimerization formation and transcription activation (Shuai et al., 1994). We infected human monocytic THP-1 cells with either adenovirus encoding hSTAT3-DN or empty adenovirus vector (Ad-vector) as previously described by Williams et al. (Williams et al., 2004). THP-1 cells infected with either Ad-vector or DN-hSTAT3 induced TNF in response to E. coli-SCS (Fig. 6B). GR-1-CM suppressed TNF production in Ad-vector-infected cells, whereas in DN-hSTAT3-infected cells TNF production was not suppressed by GR-1-CM. These data indicate that STAT3 Y-705 phosphorylation is required for the suppression of TNF production.

STAT3-mediated inhibition of JNK activation by GR-1-CM is a key mechanism that suppresses TNF production in E. coli-activated macrophages

It has been shown that bacterial components such as LPS activate MAPKs, Akt (PKB) and NF-κB, which are key signalling molecules for regulating production of TNF (van den Blink et al., 2001; Ojaniemi et al., 2003). Therefore, we examined whether GR-1-CM pretreatment modulates activation of these signalling molecules using Western blot analysis. As shown in Fig. 7A, GR-1-CM did not affect
Fig. 6. STAT3 Y705 phosphorylation induced by G-CSF is required for the GR-1-CM-induced suppression of TNF production in E. coli-activated macrophages.

A. BMDIM were treated with none (CNT) or E. coli-SCS in the presence of GR-1-CM with or without 30 min pretreatment of inhibitors for JAK2 (AG490; 30 μM), JAK3 (JAK3i; 100 μM) and STAT3 (ST3i; 0.5 mM).

B. DN-hSTAT3 (Y705F) was overexpressed using an adenoviral vector in human monocytic THP-1 cells. Top panel shows overexpression of DN-hSTAT3 in DN-hSTAT3 adenovirus-transfected THP-1 cells. Endogenous STAT3 was almost undetectable in empty adenovirus-transfected cells under the same Western blotting conditions. The adenovirus empty vector or DN-hSTAT3-transfected THP-1 cells were treated with none (CNT), E. coli, E. coli + GR-1-CM or GR-1-CM. Production of TNF was measured 9 h after treatments as described in the legend to Fig. 1. Data are expressed as means ± SD of three independent experiments.

Fig. 7. Specific inhibition of JNKs by GR-1-CM is mediated through STAT3.

A. BMDIM were treated with none (CNT), E. coli-SCS with or without pretreatment (45 min) of GR-1-CM for the time indicated. GR-1-CM specifically inhibited tyrosine phosphorylation of JNKs without apparent inhibition of other signalling molecules.

B. Human monocytic THP-1 cells were treated with none (CNT), LPS 100 ng ml⁻¹ with or without pretreatment of GR-1-CM or GR-1-CM + AG490 (30 μM). Similarly, THP-1 cells transfected with adenovirus empty vector or DN-hSTAT3 (Y705F) were treated with LPS with or without pretreatment of GR-1-CM for 30 min. Total cell lysates (35 μg per lane) were analysed by Western blotting using phospho-specific or anti-peptide antibodies against the indicated proteins. Results are representative of three independent experiments.

C and D. THP-1 cells were transfected with adenoviral empty vector or wild-type MKK7. C. Activation of JNK was analysed using Western blots and overexpression of MKK7 constitutively induced JNK phosphorylation. D. GR-1-CM failed to suppress TNF production in LPS-activated cells overexpressing MKK7. Data are expressed as means ± SD of three independent experiments.
degradation of IκB-α nor activation of Akt, ERK1 and ERK2 by E. coli-SCS. However, activation of p46- and p54-JNKs was completely blocked by GR-1-CM in BMDIM. Similar results were detected in THP-1 cells (data not shown). To further examine whether the JNK inhibition requires STAT3 Y705 phosphorylation, adenovirus-mediated overexpression of human DN-hSTAT3 and JAK2 inhibitor AG490 were used in THP-1 cells. As shown in Fig. 7B, LPS-induced phosphorylation of JNK was inhibited by GR-1-CM in THP-1 cells and adenoviral empty vector transfected THP-1 cells. The JNK inhibition was completely reversed by AG490 (30 μM) and DN-hSTAT3 overexpression (Fig. 7B). These data indicate that the inhibition of JNK activation by GR-1-CM is mediated through STAT3 activation.

Although STAT3 activation is required for inhibition of LPS-induced JNK activation, this does not necessarily indicate that JNK inhibition is involved in the suppression of TNF production. To confirm the role of JNK inhibition in the suppression of TNF production, we constitutively activated JNK using adenoviral overexpression of wild-type human MAPK kinase 7 (MKK7), a JNK-specific upstream kinase (Yang et al., 1998), in THP-1 cells. Figure 7C shows that overexpression of wild-type MKK7 constitutively induced JNK activation and the JNK activation was not affected by GR-1-CM treatment. We then measured whether suppression of TNF production by GR-1-CM was abolished in MKK7-transfected cells. As shown in Fig. 7D, TNF production induced by LPS was suppressed by GR-1-CM in Ad-vector transfected cells but not in MKK7 overexpressing cells. These results suggest that inhibition of JNK is a crucial step for GR-1-CM-mediated suppression of TNF production.

Inhibition of JNKs by STAT3 requires de novo protein synthesis

To examine whether inhibition of JNK activation by GR-1-CM requires transcriptional activation of STAT3, we analysed JNK inhibition in GR-1-CM-exposed THP-1 cells after pretreating cells with a translational inhibitor actinomycin D (20 μg ml⁻¹) for 30 min. As shown in Fig. 8A, the inhibitory effect of GR-1-CM on JNK activation was abolished by actinomycin D, indicating that de novo protein synthesis is required for the JNK inhibition. To further investigate the role of STAT3, we tested whether constitutively active STAT3α mimics the GR-1-CM effect on JNK activation and on TNF production. Expression of constitutively active human STAT3α-C, which forms homodimerization through cysteine–cysteine residues instead of phosphotyrosine–SH2 interactions (Bromberg et al., 1999), suppressed JNK activation induced by LPS (Fig. 8B). Production of TNF induced by LPS was also diminished about 40% in THP-1 cells constitutively

![Fig. 8](image_url)

**Fig. 8.** Inhibition of JNK by GR-1-CM requires STAT3α-mediated de novo protein synthesis.  
A. THP-1 cells were treated with GR-1-CM for 45 min with or without pretreatment with actinomycin D (20 μg ml⁻¹) for 30 min, and then exposed to none (CNT) or LPS (100 ng ml⁻¹) for 30 min.  
B. THP-1 cells constitutively expressing hSTAT3α-C were treated with LPS (100 ng ml⁻¹) for 30 min in the presence or absence of GR-1-CM pretreatment (45 min). Activation of p38 and JNKs was analysed by phospho-specific Western blotting. Results are representative of two independent experiments.  
C. Production of TNF in response to LPS (100 ng ml⁻¹) in wild-type or hSTAT3α-C-transfected THP-1 cells was analysed as described in the legend to Fig. 1. Data are expressed as means ± SD of three independent experiments.

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expressing STAT3\textsuperscript{c-C} (Fig. 8C). These results indicate that transcriptional activation of STAT3 is required for the inhibition of JNK activation and subsequent TNF production in GR-1-CM-treated macrophages.

Discussion

Probiotics such as strains of lactobacilli have been recognized as health beneficial microorganisms attenuating inflammation in the intestine (Madsen et al., 1999; O’Mahony et al., 2001; Borruel et al., 2002; Dieleman et al., 2003; McCarthy et al., 2003). In addition to local immunomodulatory roles, recent studies have also shown that Lactobacillus can modulate host inflammation away from the site of contact (Kato et al., 1998; Montrose and Floch, 2005) and systemic anti-inflammatory effects can also be achieved through subcutaneous applications of probiotics (Sheil et al., 2004). Macrophages are present in almost all tissues and modulate immune responses through releasing autocrine and paracrine factors. Under normal conditions, marked number of mononuclear cells infiltrate into the colonic mucosa without inducing inflammation (Sansonetti, 2004). In susceptible individuals, the immunologic tolerance of resident or newly infiltrated macrophages to intestinal microbes is lost and chronic inflammation ensues (Miettinen et al., 2000; Sanibadi et al., 2003; Watanabe et al., 2003; Weite et al., 2003). Probiotics such as L. rhamnosus have been shown to suppress the inflammatory responses in such conditions (Madsen et al., 1999; O’Mahony et al., 2001; Borruel et al., 2002; Dieleman et al., 2003; McCarthy et al., 2003; Montrose and Floch, 2005). In this study, we have shown that L. rhamnosus GG and GR-1 are inefficient in inducing pro-inflammatory cytokine TNF in macrophages (Fig. 1A), but rather preferentially induce secretion of anti-inflammatory cytokines such as IL-10 and G-CSF (Fig. 2).

Suppression of immune responses through inhibitory autocrine and paracrine factors is a common strategy of some pathogens including mycobacteria and parasitic nematodes. Pathogenic strains of Mycobacterium tuberculosis have been shown to induce IL-6 and inhibit the inflammatory responses of macrophages (Nagabhushanam et al., 2003). The IL-6 secreted by M. tuberculosis-infected macrophages inhibits the responses of uninfected adjacent macrophages to IFN-γ. Parasitic nematodes including helminths and filarial nematodes have been shown to drive the infected host to T\textsubscript{2} type responses through IL-10-dependent and -independent paracrine routes, resulting in downregulation of various inflammatory cytokines such as TNF and IL-12 production (Houston et al., 2000; Goodridge et al., 2001; Hartmann and Lucius, 2003; Wohleben et al., 2004). It is not yet known whether production of anti-inflammatory cytokines is necessary for the in vivo anti-inflammatory effects of Lactobacillus. Although IL-10 is a potent and well-studied anti-inflammatory cytokine (Moore et al., 2001), studies in IL-10 knockout mice indicate that production of IL-10 is dispensable for the anti-inflammatory effects of Lactobacillus (Madsen et al., 1999; Dieleman et al., 2003; McCarthy et al., 2003; Pena et al., 2005). This in vitro study showed that IL-10 was not required for the suppression of TNF production in macrophages (Fig. 3). We have found that live GG and GR-1 or their SCS induced G-CSF production at a range of 50–75 ng ml\textsuperscript{−1}, concentrations that were high enough to potently suppress LPS-induced TNF production (Fig. 3D). The suppression of TNF production was likely mediated through downregulation of TNF mRNA expression (Fig. 1C) and a paracrine effect of G-CSF, as G-CSFR\textsuperscript{−/−} macrophages produced similar amounts of TNF as wild-type cells (Fig. 3C). A previous study has shown that peak production of TNF (3–4 h of post-treatment) precedes the production of G-CSF, which peaks at 10–24 h after LPS stimulation in THP-1 cells (Hareng et al., 2003).

G-CSF is required for the differentiation of neutrophils in the bone marrow and at the same time elicits potent anti-inflammatory effects in monocytes and in septic mice (Gorgen et al., 1992; Boneberg and Hartung, 2002; Nishiki et al., 2004). Unlike IL-10, which has broader anti-inflammatory effects, G-CSF is unique in that it suppresses production of pro-inflammatory cytokines while simultaneously activating the antibacterial defence of neutrophils (Boneberg and Hartung, 2002). Therefore, suppression of inflammation through induction of G-CSF by probiotics may be particularly important in the intestine and urogenital tracts where antimicrobial activity has to be preserved.

Seven different human G-CSF receptor isoforms have been described, which are generated by alternative splicing; however, the physiologic roles of these isoforms and the expression pattern on various cell types are still unknown (Boneberg and Hartung, 2002). The dissociation constant for G-CSF with its receptor has been measured at approximately 60–80 pM (Nicola and Metcalf, 1985; Nicola et al., 1986). However, the biologic response is induced at a much lower concentration (half-maximal at 3 pM = 72 pg ml\textsuperscript{−1}) (Nicola and Metcalf, 1985), suggesting that only a low percentage of receptors need to be occupied for relevant signal transduction to occur. In human peripheral monocytes, inhibition of LPS-induced TNF production by G-CSF was detected at 10–500 ng ml\textsuperscript{−1} (Demetri and Griffin, 1991; Nishiki et al., 2004). This study showed that hG-CSF induced STAT3 activation and significantly inhibited LPS-induced TNF production at 300–500 pg ml\textsuperscript{−1} range, indicating that human monocytic THP-1 cells are more sensitive to G-CSF than peripheral monocytes. In fact, a subset of monocytic cells was noted to bind G-CSF at very low levels (Nicola and Metcalf, 1985), whereas THP-1 cells were shown to express high-affinity
G-CSF receptors (Kondo et al., 1991). It is not yet clear why E. coli-CM did not have TNF suppressing effects, even though G-CSF was still present to about half the quantity induced by GG or GR-1 (Fig. 2). Unlike GG- or GR-1-stimulated BMDM, E. coli harbouring LPS activates TLR4 and potently produces type I interferons, granulocyte macrophage-CSF and IL-6. Because these cytokines further enhance TNF production in activated macrophages (Cochran and Finch-Arietta, 1992; Hayes and Zoon, 1993), it is possible that the lack of the suppressive effects in E. coli-CM may be due to the counteracting enhancing effects induced by these factors.

G-CSF has shown to attenuate LPS-induced release of TNF-α, which is mainly mediated through activation of STAT3 (Boneberg and Hartung, 2002). In human monocytes, G-CSF activates STAT3 by inducing phosphorylation only at Y705 through activated JAK2, which is completely blocked by AG490 JAK2 inhibitor (Nishiki et al., 2004). IL-10 induces STAT3 phosphorylation at both Y705 and S725, which is not sensitive to AG490 JAK2 inhibitor (Nishiki et al., 2004). ERK1 and ERK2 are also strongly activated by IL-10 but not by G-CSF. The signaling mechanisms induced by GR-1-CM were very similar to those observed in G-CSF-mediated pathways. GR-1-CM induced phosphorylation at Y705 but not at S725 of STAT3 (Fig. 4A), the Y705 phosphorylation was blocked by AG490 (Fig. 5E) and ERK activation was not detected in GR-1-CM-treated cells (Fig. 4B). We further showed that GR-1-CM<sup>ΔG-10-12</sup> still induced STAT3 Y705 phosphorylation (Fig. 5B), whereas the phosphorylation was detected neither in macrophages treated with G-CSF-depleted GR-1-CM (Fig. 5A) nor in G-CSFR<sup>−/−</sup> macrophages treated with GR-1-CM (Fig. 5C). These data indicate that STAT3 activation by GR-1-CM was mainly mediated through G-CSF. Interestingly, the intracellular protozoan T. gondii also elicits potent TNF suppressing effects in macrophages, which is IL-10-independent but STAT3 activation-dependent (Butcher et al., 2005). Whether G-CSF also plays a role in the T. gondii-mediated anti-inflammatory effects has yet to be determined.

Activation of STAT3 was shown to be a key step for G-CSF-mediated suppression of TNF production (Nishiki et al., 2004); however, the molecular mechanism remains to be unraveled. In peripheral blood mononuclear cells (PBMC), G-CSF suppresses TNF production through post-translational silencing (Boneberg and Hartung, 2002) but in human monocytes TNF mRNA level is also diminished (Nishiki et al., 2004). STAT3 activation is also important for the IL-10-induced anti-inflammatory effects (Takeda et al., 1999; Lang et al., 2002); however, STAT3-independent anti-inflammatory effects were also reported (O’Farrell et al., 1998; Williams et al., 2004). Inhibition of p38 MAPK activation by IL-10 in murine macrophages was suggested as another mechanism for the suppression of TNF (Kontoyiannis et al., 2001); however, in human monocytes and macrophages no such inhibition was detected by IL-10 (Donnelly et al., 1999; Denys et al., 2002). In this study, we showed that GR-1-CM potently and specifically inhibited JNK activation induced by E. coli-SCS or LPS (Fig. 7A). The inhibition of JNK was dependent on STAT3-Y705 phosphorylation, as overexpression of DN-STAT3 (Y705F) completely reversed the JNK inhibition (Fig. 7B). In addition, constitutive expression of a dominant active form of STAT3Δα (STAT3Δα-C) abolished activation of JNK induced by LPS but had no effects on p38 activation (Fig. 8B). Altogether, these data indicate that JNK inhibition by GR-1-CM was mediated through STAT3 activation.

c-Jun-N-terminal kinase activation was shown to play a key role in TNF production (Swantek et al., 1997; Bennett et al., 2001; Manning and Davis, 2003). Our data suggest that inhibition of JNK activation by GR-1-CM is a key step for suppression of TNF production (Fig. 7C and D). How G-CSF-triggered STAT3 activation leads to JNK inhibition warrants further studies. Our present findings indicate that the attenuation of JNK activation by GR-1-CM or G-CSF was mediated through STAT3-dependent transcriptional activity.

In summary, this study identified a novel mechanism for the suppression of TNF by L. rhamnosus mediated through G-CSF paracrine effects in macrophages. G-CSF induced STAT3 activation, which in turn prevented JNK activation and consequently resulted in suppression of TNF production in LPS- or E. coli-activated macrophages.

**Experimental procedures**

**Cell cultures, bacteria and reagents**

Mouse bone marrow-derived immortalized macrophages (obtained from Dr B. Aggarwal; Houston, TX) and human THP-1 monocytes (ATCC, Manassas, VA) were grown in 75 cm<sup>2</sup> flasks (obtained from Dr B. Aggarwal; Houston, TX) and human THP-1 monocytes (ATCC, Manassas, VA) were grown in 75 cm<sup>2</sup> flasks containing complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (HyClone), 5000 U ml<sup>−1</sup> of penicillin, 5 mg ml<sup>−1</sup> of streptomycin (Invitrogen, Carlsbad, CA), 5 mM sodium pyruvate and 5 mM MEM non-essential amino acids at 5% CO<sub>2</sub>, 37°C. Lactobacillus spp. were grown anaerobically in deMan, Rogosa, Sharpe (MRS) broth (Becton Dickinson, Sparks, MD) for 48 h at 37°C. L. rhamnosus GR-1 was isolated and grown as previously described (Reid et al., 1988) and L. rhamnosus GG and E. coli GR-12 were obtained from ATCC. E. faecalis 1311 were from our strain collection. All bacteria were harvested by centrifugation (3000 g, 15 min) at the stationary growth phase and washed three times with phosphate buffered saline.
saline (PBS) and subsequently diluted to obtain final cell densities in RPMI 1640 medium without antibiotics. LPS from *E. coli* O111:B4 was purchased from List Biological Laboratories (Campbell, CA). Antibodies for phospho-specific (Thr180/Tyr182) p38, phospho-specific (Thr202/Tyr204) ERK1/2, Ser727) STAT3, (Tyr701) STAT1, (Tyr694) STAT5, phospho-specific (Thr183/Tyr185) SAPK/JNK and antibodies for their counterpart wild-type proteins were purchased from New England Biolabs (Beverley, MA), IL-10 and G-CSF neutralizing antibody was obtained from ebioscience (San Diego, CA) and R&D Systems (Minneapolis, MN) respectively.

**Primary peritoneal macrophages from wild-type C57BL/6, G-CSFR<sup>−/−</sup> and IL-10<sup>−/−</sup> mice**

Homozygote IL-10-deficient (C57BL/6-IL10<sup>tm1Cgn</sup>; IL-10<sup>−/−</sup>) and control strain (C57BL/6) mice were purchased from The Jackson Laboratory (Bar Harbor, MA). Homozygote G-CSFR-deficient (G-CSFR<sup>−/−</sup>) mice were provided by D.J. Link (Washington University Medical School, St Louis, MO). Primary peritoneal macrophages from these mice were obtained by normal saline lavage as described previously (Border, 1988). Briefly, mice received intraperitoneal injection of 3% thioglycollate (1 ml) and peritoneal macrophages were harvested 4 days post injection by lavage of the peritoneal cavity with normal saline. The cells were washed and incubated overnight before use.

**Generation of bacterial SCS and macrophage culture CM, and treatments**

Cultures of lactobacilli, *E. coli* GR-12 and *E. faecalis* 1311, were grown to an optical density of 1.5 at 600 nm (representing approximately 10<sup>9</sup> cells ml<sup>−1</sup>). Bacteria-free SCS was collected by centrifugation at 6000 × g for 10 min at 4°C. SCS was separated from cell pellet and passed through a 0.22 μm pore size filter unit (Nalgene, Rochester, NY). The BMDIM, primary peritoneal macrophages, or human monocytic THP-1 cells were grown in culture medium and approximately 2 × 10<sup>6</sup> cells were plated into six-well cell culture plates. Macrophages were then treated with bacterial SCS (1/20 dilution) or live bacteria (10 cfu per cell) in antibiotic-free culture medium. After 6 h of exposure, cells were washed with PBS and replaced with fresh cell culture medium with antibiotic to eliminate bacterial components. The overnight macrophage culture medium was used as CM. For assaying, the CM was further diluted with fresh culture medium (1:1) to supplement nutrients and used on freshly plated macrophages. For controls, overnight CM of macrophages without treatments was used.

**Cell lysates and immunoblotting**

Total cell lysate extraction and Western blot analysis were performed as previously described (Kim *et al*., 2001). Briefly, cells were lysed in ice-cold cell lysis buffer containing 20 mM MOPS, 15 mM EGTA, 2 mM EDTA, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 mM dithiothreitol, 75 mM β-glycerophosphate, 0.1 mM phenylmethylsulphonyl fluoride, 1 μg ml<sup>−1</sup> aprotin, 10 μg ml<sup>−1</sup> pepstatin A, 1 μg ml<sup>−1</sup> leupeptin and 1% Triton X-100 and then sonicated on ice. Cell extracts were obtained by centrifuging the homogenate at 18 000 × g for 10 min. These extracts were electrophoretically resolved in ready-made 10% SDS-polyacrylamide gels (Bio-Rad), followed by transfer onto nitrocellulose membranes. Membranes were subsequently blocked with 5% skim milk for 30 min, immunoblotted with antibodies and developed using an enhanced chemiluminescence detection system (ECL; Pierce Bioscience).

**Adenoviral (Ad) and retroviral viral infections**

A recombinant, replication-deficient, adenovirus vector encoding the human STAT3 Tyr<sup>705</sup>(r)Phe (AdSTAT3-DN) and an identical construct lacking the insert (Ad<sup>0</sup>) were provided by L. Williams (Imperial College, London, UK). MKK7 wild-type adenovirus was obtained from J. Han (The Scripps Research Institute, La Jolla, CA). The recombinant viruses were purified and concentrated as described previously (Williams *et al*., 2004). Human monocytic THP-1 cells were transiently infected with adenovirus at the stated multiplicity of infection (moi) for 1 h in serum-free medium. Cells were then washed and recultured in growth medium for 24 h. Puromycin resistance gene-containing retroviral vector constructs for dominant active human STAT3<sup>α</sup>-C were obtained from J.F. Bromberg (Memorial Sloan-Kettering Cancer Center, New York). Viruses were generated in Phexion Amphotropic producer cells using the calcium phosphate method of transfection (Miller and Buttimore, 1986). Viruses were produced at 32 °C and virus-containing medium was collected 36 h post transfection and filtered through a 0.45 μm filter. THP-1 cells were plated in six-well plates at a density of 5 × 10<sup>5</sup> cells per well. One round of retroviral infection was performed by replacing medium with 2 ml of retrovirus and retrovirus-infected cells were selected by culturing in media containing puromycin (10 μg ml<sup>−1</sup>). The retrovirus-infected cells were maintained in culture medium containing puromycin (5 μg ml<sup>−1</sup>) and then normal culture media 24 h before experiments.

**Cytokine analysis**

Production of IL-10 and G-CSF in macrophage cell culture supernatants was measured using quantitative enzyme-linked immunosorbent assay kits purchased from ebioscience and R&D Systems respectively. Standard curves for each cytokine were generated using purified recombinant proteins of known concentration provided by the manufacturer. TNF bioassays were used to measure TNF production as previously described (Poltorak *et al*., 1998).

**Quantitative real-time PCR**

Expression of TNF of gene transcripts in macrophages was quantified on the Rotor-Gene RG3000 quantitative multiplex PCR instrument using the Brilliant SYBR Green PCR Master Mix (Applied Biosystems). Total cellular RNA was isolated using TRIzol (Life Technologies) according to the manufacturer’s instructions. Briefly, 1 μg of total RNA was reverse transcribed by using oligo(dT) primers and the Superscript II reverse transcriptase system (Invitrogen) according to the manufacturer’s recommendations. Oligonucleotide primers were the following: for TNF, 5'-CTGGAAATATGCTCCCAGAAA-3' (5' primer) and 5'-CATTGGGAAACCTTCATCC-3' (3' primer); for IL-10, 5'-TGCTATGCTGCTG-3' (5' primer) and 5'-CATGGCCATAGCGCTG-3' (3' primer); for G-CSF, 5'-CCGATAGACCTGTCAG-3' (5' primer) and 5'-CATGGCCATAGCGCTG-3' (3' primer).
GAGA-3' (5' primer) and 5'-ACCCAGAATCCATGGCTCAA-3' (3' primer); for G3PDH, 5'-GCATTGGAAGGGCTAGT-3' (5' primer) and 5'-TTGCTTGAAGTCGCGAG-3' (3' primer).

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