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Beneficial Microbes, December 2011; 2(4): 283-293

Probiotic bacterial strains differentially modulate macrophage cytokine production in a strain-dependent and cell subset-specific manner

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Received: 19 August 2011 / Accepted: 15 November 2011
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Abstract

Gut mucosal macrophages play a pivotal role in driving mucosal immune responses, resulting in either activation of inflammatory immune responses to pathogenic challenge or tolerance to beneficial luminal contents such as food and commensal bacteria. Macrophage responses elicited are dependent on tissue environment and the resulting cell subset, where homeostatic macrophages resemble the M2 macrophage subset and inflammatory macrophages resemble M1s. Probiotics can modulate macrophage function with outcome dependent on subset present. Using a THP-1 monocyte cell line-derived model of $CD14^{high/low}$ M1 and M2 macrophages, the aim of this study was to investigate the immunomodulatory effects of a panel of heat-killed probiotic bacteria and their secreted proteins on the subset-specific inflammatory marker profile of TNFα, IL-6 and NFκB. M1 and M2 cells were generated by differentiation of monocyte stable transfectants for high and low CD14 expression with phorbol 12-myristate 13-acetate and vitamin D_3, respectively, where the resulting $CD14^{lo}$ M2 and $CD14^{hi}$ M1s mimicked homeostatic and inflammatory mucosal macrophages. Subsets were stimulated by enteropathic lipopolysaccharides in the presence or absence of heat-killed (HK) or secreted proteins (SP) from a panel of probiotic bacteria. Regulation of cytokine expression was measured by ELISA and NFκB activity by reporter assay. HK probiotics suppress $CD14^{lo}$ and augment $CD14^{hi}$ M1 and M2 production of TNFα whereas SPs augmented $CD14^{hi}$ M1 TNFα and were generally suppressive in the other subtypes. M2 macrophage IL-6 production was suppressed by both HK and SPs and differentially regulated in $CD14^{lo}$ and $CD14^{hi}$ M1s. NFκB activation failed to parallel the regulatory profiles for TNFα and IL-6 which is suggestive of probiotic bacteria exerting their regulatory effects on these cytokines in an NFκB-independent manner. In conclusion, HK and SP probiotics differentially regulate macrophage cytokines and NFκB activation in a subset-dependent manner and suggest a cautionary approach to probiotic treatment of mucosal inflammation.

Keywords: macrophage, probiotics, cytokines, inflammation

1.Introduction

The gut mucosal immune system is a major site of defence against potential pathogenic organisms that gain entry into the human body via the gastrointestinal tract (GIT). By its very nature, the GIT is full of microbes, their antigens and potential antigens released by the chemical breakdown of food. The mucosal immune system of the gut, in response to this microbe and food-antigen-rich environment has developed sophisticated mechanisms by which it can selectively taste luminal contents and respond to these signals; either by initiation of an immune inflammatory response or by a homeostatic mechanism resulting in tolerance or non-responsiveness of the immune system, whereby we tolerate useful products in the gut. This mechanism is referred to as oral tolerance, allowing the host to gain benefit from food/nutrients and beneficial commensal organisms.

These commensal microbes present in the gut afford health benefits through a variety of ways which include provision of metabolites which regulate host nutrition, epithelial

cell turnover and compete with pathogenic organisms for nutrient sources and binding sites on epithelial cells, thus preventing pathogenic invasion/infection. More recently, these organisms have been observed to modulate gut mucosal immune function which has been the focus of much intense investigation. In addition, the 'topping-up' of these 'friendly' bacteria by probiotics has been established to modulate immune function and confer health benefit to allergies, inflammatory pathologies and cancer.

Integral to modulation of gut mucosal responses are the macrophage cells present in the lamina propria of the mucosal layer. Gut mucosal macrophages are generally hyporesponsive or tolerised in the homeostatic healthy functioning gut: hyporesponsive to nutrients, food antigens and commensals whereas retaining the ability to be activated and elicit an inflammatory immune response upon pathogenic challenge. These macrophages are generally representative of the M2 macrophage subset, they express scavenger receptors (mannose receptor MR, CD13, CD36), anti-inflammatory/regulatory cytokines (interleukin (IL)-10 and transforming growth factor, TGFβ) and exhibit a reduced responsiveness to pathogen associated molecular patterns (PAMPs), i.e. macrophages fail to express CD14 and selected Toll-like receptors (TLRs); but in addition, the expression of the co-stimulatory molecules CD80/CD86 and the IgA FcR, CD89 are also reduced. These $CD14^{lo}$ M2-like macrophages, when in homeostatic conditions, are predominantly regulatory/anti-inflammatory and display a phagocytic phenotype characterised by the high expression of scavenger receptors (Platt and Mowat, 2008; Smith *et al.*, 2001; Smythies *et al.*, 2005). Macrophage effector phenotype, however, is partially governed by the local environment. Thus, in an inflammatory environment, gut mucosal macrophages exhibit a pro-inflammatory M1-like macrophage subset effector phanotype, characterised by the predominance of pro-inflammatory cytokine production, expression of high levels of CD14 ($CD14^{hi}$) and expression of co-stimulatory molecules CD80 and CD86 (Segura *et al.*, 2002; Zareie *et al.*, 2001). It is not clear, however, whether these different macrophage effector phenotypes result as a consequence of plasticity of one subset of gut macrophage cell or by recruitment and activation of a defined monocyte/macrophage subset from the peripheral circulation.

Probiotic bacteria are immunomodulatory and can exert their effects on a wide array of immune and mucosal cells including T-cells, B-cells, natural killer (NK) cells (Takeda *et al.*, 2006), dendritic cells (Foligne *et al.*, 2007a), monocytes/macrophages and epithelial cells (Zhang *et al.*, 2005). Dependent on cell type and strain of probiotic bacteria used, these immunomodulatory effects can manifest themselves as immune activatory, deviatory or regulatory/suppressive. Considering relative abundance and functionality in the gut mucosa, the potential role of probiotics in modulating macrophage function has been relatively sparsely investigated. Lactic acid bacteria (LAB), such as *Lactobacillus rhamnosus*, have been documented to modulate macrophage function by both suppressing and enhancing IL-12 production (Foligne *et al.*, 2007b; Ichikawa *et al.*, 2007; Shida *et al.*, 2006), which will impact on Th_1 development and activation, hence cell mediated immunity to intracellular resident pathogens. These LAB have also been described to suppress mucosal tumour necrosis factor (TNFα) during inflammation (Borruel *et al.*, 2002) and to augment the anti-inflammatory cytokine, IL-10 (Foligne *et al.*, 2007b). In addition, the secreted protein extract from *Lactobacillus plantarum* inhibits NFκB activity (Petrof *et al.*, 2009); such observations have suggested a suppressive role for *L. plantarum* and other probiotic bacterial species on NFκB-dependent inflammatory cytokines such as IL-1γ, IL-6, IL-8, monocyte chemotactic peptide-1 (MCP-1), IL-12 and TNFα. Thus, probiotic bacteria have been demonstrated to exert their immunomodulatory effects through both bacterial cell-associated mechanisms and through soluble secreted proteins (Frick *et al.*, 2007; Sanchez *et al.*, 2009; Yan *et al.*, 2007) and metabolites such as short chain fatty acids (Foey, 2011).

These probiotic bacteria share common molecules (or pathogen associated molecular patterns, PAMPs) with pathogenic bacteria, which are recognised by pattern recognition receptors (PRRs) expressed by cells of the gut mucosa. Just how the immune system recognises beneficial commensal or probiotic microbes from pathogens is a subject of intense research efforts. One potential way by which the host differentiates between good and bad has been suggested to be mediated by the PRR, nucleotide oligomerisation domain-2 (NOD2); indeed several probiotic LAB strains have been shown to be recognised by this receptor (Hasegawa *et al.*, 2006). NOD2 is an intracellular receptor which recognises muramyl dipeptide moieties derived from peptidoglycan (Girardin *et al.*, 2003); the location of this receptor suggests that either the whole probiotic bacterium or parts of, are required to gain entry inside the cell by means of phagocytosis. This in itself, is suggestive of phagocytically competent cells such as the M2 macrophage subset and the potential role of scavenger receptors which predominate on the same cell type. Such a mechanism may be important in macrophage discrimination between commensals and pathogens, preventing inappropriate recognition of commensal PAMPs by their PRRs and the ensuing destructive inflammatory immune responses; thus mediating tolerance vs. immune activation decisions. Indeed, probiotic bacteria have been demonstrated to modulate phagocytosis; whereby being stimulatory to phagocytosis in healthy subjects and conversely, suppressive in allergic patients (Isolauri *et al.*, 2001). Thus, recognition of PAMPs by PRRs such as TLRs and NODs, the phagocytic capability and functional phenotype of the macrophage determine responsiveness to probiotics as immuno-suppressive, regulatory or inflammatory.

Gut mucosal macrophages have been characterised as $CD14^{lo/absent}$, $TLR^{lo/absent}$ M2-like phenotype in healthy mucosa whereas $CD14^{hi}$, TLR^{hi} M1-like phenotype in inflammatory mucosa (Smith *et al.*, 2001; Smythies *et al.*, 2005; Zareie *et al.*, 2001). Thus, CD14 has been suggested as an indicator molecule of tolerogenic or inflammatory mucosal macrophages. CD14 is a multifunctional receptor; it facilitates innate responses to infectious non-self molecules as well as interacting with apoptotic self molecules, serving as a scavenger for apoptotic cells. It has been described to recognise the bacterial PAMPs; lipopolysaccharide (LPS), peptidoglycan, mycobacterial lipoarabinomannan and streptococcal cell wall polysaccharides (Pugin *et al.*, 1994; Soell *et al.*, 1995; Weidemann *et al.*, 1997; Wright *et al.*, 1990), thus having the capability to recognise and bind components of both Gram negative and Gram positive bacteria and serving as a co-receptor for TLR2 and TLR4, hence driving inflammatory immune responses directed at non-self bacterial components. On the other hand however, CD14 has been demonstrated to recognise apoptotic cells for clearance by phagocytosis (Devitt *et al.*, 1998; Pradhan *et al.*, 1997). Such a clearance mechanism results in a regulatory, anti-inflammatory response (Fadok *et al.*, 1998). Probiotic modulation of these functionally distinct macrophage subsets present in the gut mucosa will be determined by CD14 expression and the receptors that it associates with, and the downstream effector signalling pathways.

The signal pathway transcription factor, NFκB plays a dominant role in inflammatory responses. It has been well established to regulate macrophage inflammatory cytokine production such as TNFα, IL-6, IL-8 and IL-1β (Bondeson *et al.*, 1999). In addition, the role of NFκB in regulating macrophage phenotype has been investigated where inhibition of NFκB by adenovirus overexpression of the inhibitor, IκB, changed macrophage phenotype to a dominant anti-inflammatory phenotype (Wilson *et al.*, 2005). This was extended, whereby an anti-inflammatory role was described for IKKβ by the inhibition of classical, M1-like macrophage responses (Fong *et al.*, 2008). Surprisingly, the long term inhibition of IKKβ rendered mice more susceptible to IL-1β-associated endotoxin-induced shock. Inhibition of IKKβ, hence NFκB activation and NFκB-dependent inhibition of caspase-1 resulted in augmentation of IL-1β, demonstrating NFκB to play a negative regulatory role in IL-1β secretion (Greten *et al.*, 2007). Thus, it is likely that macrophage phenotype, hence inflammatory phenotype, is integrally associated with NFκB subunit association and activation. Several studies have suggested that probiotics (both bacterial cell-associated and bacterial-free fractions) modulate NFκB activation, whereby VSL#3 suppressed NFκB activation in intestinal epithelial cells (Petrof *et al.*, 2004, 2009) and the same cells in a murine model of colitis (Marteau *et al.*, 2004). It remains to be investigated whether probiotic modulation of distinct macrophage subsets and their effector cytokines is mediated through manipulation of NFκB-dependent mechanisms.

Current understanding of probiotic modulation of macrophage-mediated immune responses of distinct effector subsets relevant to mucosal homeostatic and inflammatory pathological environments is relatively poorly understood. Probiotic modulation of macrophage effector cytokines is, at best, confusing and often contradictory; observations being determined by cell source, level of differentiation, bacterial strain, bacterial preparation, stimulus used and local environment. Using a stably transfected NFκB-reporter cell line model of $CD14^{lo}$/$CD14^{hi}$ mucosal resident homeostatic- and infiltrating inflammatory-macrophages, the aim of this study was to investigate the relationship between a range of potentially immunoregulatory panel of probiotics (both bacterial cell and secreted protein preparations) and their effects on macrophage subset NFκB activation and corresponding cytokine effector phenotype, of relevance to mucosal macrophages found in the gastrointestinal tract.

2. Materials and methods

Bacterial culture and preparation of heat-killed and secreted protein extracts

Bifidobacterium breve strain NCIMB 8807 (BB), *L. rhamnosus* GG strain NCIMB 8824 (LR), *Lactobacillus salivarius* strain NCIMB 41606 (LS) and *L. plantarum* strain NCIMB 41605 (LP) were obtained from NCIMB (Aberdeen, UK). *Lactobacillus fermentum* strain MS15 (LF) was isolated from the crop of a chicken (Savvidou, 2009) and obtained from internal microbiology stocks at the University of Plymouth (UK). These probiotic bacterial species were cultured aerobically in De Man Rogosa Sharp (MRS) broth at 37 °C for 18 hours until stationary phase was achieved. Bacterial cells were harvested according to the method described in Habil *et al.* (2011). In brief, bacterial cells were harvested and washed in phosphate buffered saline and viable counts adjusted to a density of 1×10^9 cfu/ml. Probiotic bacteria were adjusted to 1×10^{10} cfu/ml and heat killed for 2 hours at 90 °C (HK) according to the protocol of Young *et al.* (2004). Cell death was confirmed by plating on MRS agar and incubation for a minimum of 18 hours. In addition, secreted protein (SP) was extracted by trichloroacetic acid precipitation of proteins secreted into growth supernatant according to the protocol of Sanchez *et al.* (2009). Protein content was verified by SDS-PAGE and quantified by calibration to Bradford protein assay. Secreted protein was adjusted to a stock concentration of 1 mg/ml.

Monocyte and macrophage culture

Transfectant human monocytic THP-1 NFκB reporter cell lines, THP-1Blue ($CD14^{lo}$) and THP-1Blue-CD14

($CD14^{hi}$) (Autogen Bioclear, Calne, UK) were routinely used for this study between passages 7 and 25 and maintained in R10 medium composed of RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin or 100 µg/ml streptomycin (Lonza, Wokingham, UK) in the presence of the selection antibiotics, 200 µg/ml zeocin or 200 µg/ml zeocin and 10 µg/ml blastocidin, respectively. Cells were plated out at 1×10^5 cells/100 µl/well in R10 medium in 96 flat-bottomed well tissue culture plates. Pro-inflammatory (M1-like) macrophages and anti-inflammatory (M2-like) macrophages were generated by differentiation of these monocytes in the presence of 25 ng/ml phorbol 12-myristate 13-acetate for 3 days or 10 nM 1,25-$(OH)_2$-vitamin D_3 (Sigma-Aldrich, Poole, UK), for 7 days, respectively (Daignealt *et al.*, 2010). The resulting $CD14^{lo}$ M2 and $CD14^{hi}$ M1 macrophage subsets were representative of homeostatic and inflammatory pathological mucosal macrophages, respectively. In addition, $CD14^{lo}$ M1 and $CD14^{hi}$ M2 subsets were also studied, acting as internal controls for observations of CD14 expression responses of the inflammatory pathological and homeostatic macrophages, and as potential intermediate subsets dependent on whether the macrophage subset is derived from recruited $CD14^{hi}$/$CD14^{lo}$ peripheral blood monocytes or as a consequence of environmental modification of resident mucosal macrophages.

Activation of macrophage cytokine production

Macrophages were stimulated by the bacterial PAMP; 100 ng/ml *Escherichia coli* strain K12 LPS (expressed in enteropathic Gram negative bacteria and detected by TLR4) and cultured for 18 hours (determined as optimal time period for secretion of the cytokines TNFα and IL-6) in a humidified environment at 37 °C, 5% CO_2, after which time supernatants were harvested and stored at -20 °C until required for assay by sandwich ELISA.

Regulatory effect of heat-killed and secreted protein probiotic preparations

To investigate probiotic regulation of macrophage cytokine production and NFκB activation, HK- and SP-probiotics were added in culture to final concentrations of 3×10^8 bacterial cells/ml and 3 µg/ml, respectively, as a pre-treatment for 18 hours prior to K12-LPS stimulation (100 ng/ml) for a further 18 hours in a humidified environment at 37 °C, 5% CO_2. To demonstrate a physiologically-relevant role for HK- and SP-probiotics, cytotoxicity assays (MTT and trypan blue exclusion) were carried out on both macrophages, up to 10^9 cells/ml and 100 µg/ml, respectively. No significant reductions in viability were observed for the concentrations used; viability was routinely >90%.

Cytokine measurement

Macrophage production of the inflammatory cytokines, TNFα and IL-6, were analysed by sandwich ELISA using commercially available capture and detection antibodies from BD-Pharmingen (Oxford, UK). Due to supernatant volume constraints, the $CD14^{hi}$ and $CD14^{lo}$ NFκB reporter transfectants were assayed for the pro-inflammatory cytokine, TNFα, and the dual pro- and anti-inflammatory cytokine, IL-6, and NFκB activity (see next paragraph). Protocols were followed according to manufacturer's instructions and compared to standard curves, using the recognised international standards available from NIBSC (Potter's Bar, UK). Colorimetric development was measured spectrophotometrically by an OPTIMax tuneable microplate reader at 450 nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA).

NFκB activity measurement

NFκB activity was measured by colorimetric reporter gene assay for secreted embryonic alkaline phosphatise (SEAP) associated with the stably-transfected reporter gene cell lines, THP-1Blue ($CD14^{lo}$) and THP-1Blue-CD14 ($CD14^{hi}$) (Autogen Bioclear). Briefly, at conclusion of the experiment, fresh supernatant was harvested and incubated with Quantiblue colorimetric reagent (Autogen Bioclear) for 30 minutes at 37 °C, 5% CO_2. Colorimetric development was measured by an OPTIMax tuneable microplate reader at 620 nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp.). Colour development was directly proportional to the reporter gene SEAP expression and NFκB activity.

Statistical analysis

Data were analysed using balanced analysis of variance (General Linear Model, Minitab version 16) followed by a multiple comparison test (LSD, least significant difference test). All means are presented with the appropriate standard error. Significance was set at the confidence intervals of 0.05, 0.01 and 0.005.

3. Results

Secreted protein and heat-killed probiotic strains selectively modulate macrophage subset TNFα production

Mucosal macrophage effector phenotype and functions differ from other tissue macrophages. They are generally hyporesponsive $CD14^{lo}$ when present in homeostatic, tolerogenic mucosa whereas under inflammatory conditions they are $CD14^{hi}$ and express inflammatory mediators. In an attempt to extrapolate earlier data to gut mucosal macrophages in inflammatory or tolerogenic conditions,

$CD14^{hi}$ and $CD14^{lo}$ stable transfectants were driven towards M1 and M2 macrophage subsets and stimulated by K12-LPS in the presence or absence of heat-killed or secreted protein preparations from a range of probiotic bacterial species.

Probiotic bacteria have been demonstrated to modulate innate immunity. This experiment was undertaken to establish whether heat-killed (in the absence of any non-specific effects of lactic acid produced) or secreted protein from a panel of established probiotic bacteria exerted immunomodulatory effects on the expression of the pro-inflammatory cytokine, TNFα, by THP-1-derived M1-like and M2-like macrophage subsets. Results indicated that HK- and SP-probiotic bacteria samples differentially regulated LPS-induced TNFα production by M1 and M2 macrophage subsets; modulation of TNFα being dependent on macrophage subset, CD14 expression and probiotic strain. The most obvious effect was observed for $CD14^{hi}$ macrophages, where in $CD14^{hi}$ M1s (representative of inflammatory infiltrating macrophages) heat killed probiotics augmented LPS-induced TNFα by ×4, ×3.2, ×3.6, ×4 and ×3.7 of control (1,161±148 pg/ml) and secreted proteins augmented by ×4, ×4.1, ×4.1, ×3 and ×3.5 for BB, LR, LF, LS and LP, respectively (Figure 1B). $CD14^{hi}$ M2s displayed a differential sensitivity to HK compared to SP (Figure 1D). HK obtained for the LABs all augmented TNFα production (control 14±1 pg/ml) resulting in levels ×4.9, ×12, ×3.6 and ×7 for LR, LF, LS and LP, respectively. SP extracts, in comparison, only weakly modulated cytokine production where LS suppressed production by 36% (control 14±1 pg/ml to 9±1 pg/ml) and LP augmented by 86% (control 14±1 pg/ml to 26±3 pg/ml). Generally, in the case of $CD14^{lo}$ macrophages, HK and SP probiotics partially suppressed LPS-induced TNFα production. In the case of $CD14^{lo}$ M1s, HK suppressed cytokine production (control levels of 1,454±94 pg/ml) by 51% (709±4 pg/ml), 19% (1,184±98 pg/ml), 40% (876±175 pg/ml) and 57% (628±316 pg/ml) for BB, LF, LS and LP, respectively. SP suppressed

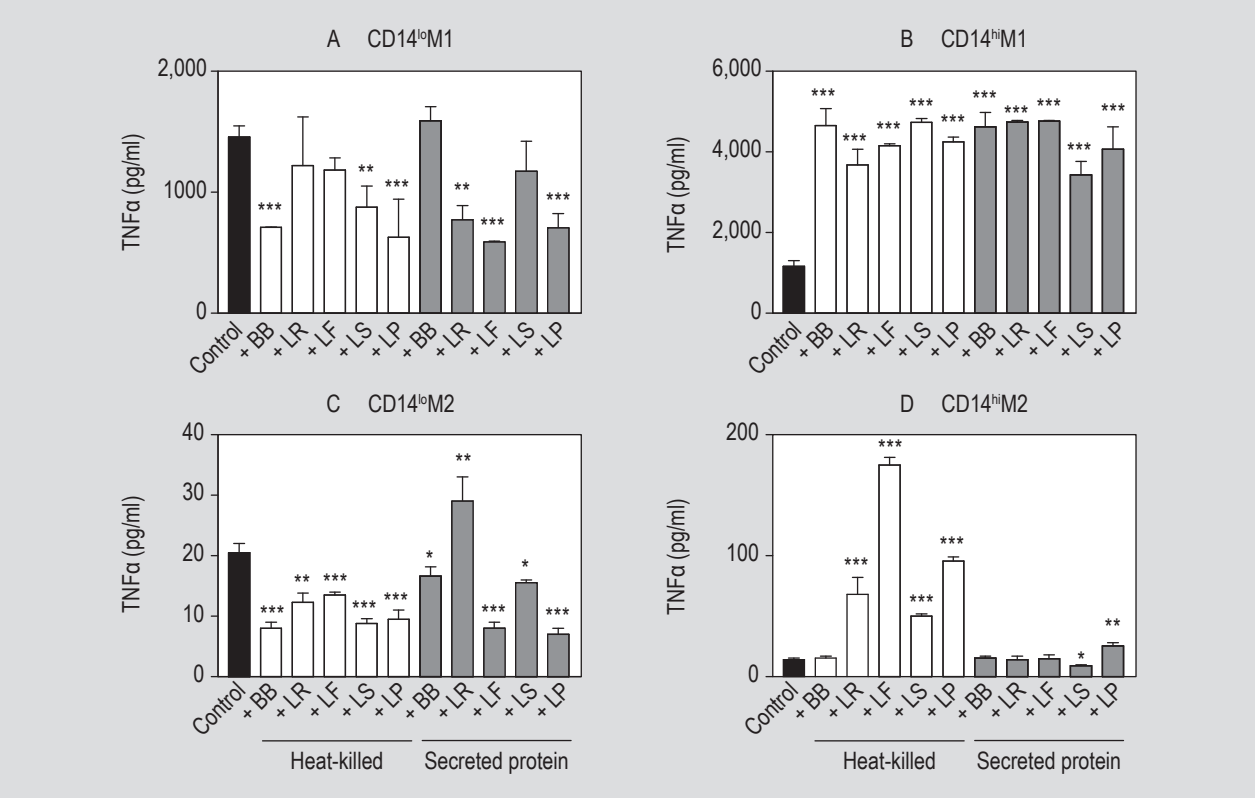

Figure 1. Secreted protein and heat-killed probiotic strains selectively modulate macrophage subset TNFα production. THP-1-derived $CD14^{hi}$ and $CD14^{lo}$ macrophage subsets were stimulated with 100 ng/ml *Escherichia coli* K12 lipopolysaccharides in the presence or absence of 3×10^8 cfu/ml heat-killed (HK) probiotic bacterial strains (*Bifidobacterium breve* (BB), *Lactobacillus rhamnosus* (LR), *Lactobacillus fermentum* (LF), *Lactobacillus salivarius* (LS) and *Lactobacillus plantarum* (LP)), depicted by clear bars, or 3 µg/ml secreted protein extracted from each of these probiotic strains (depicted by hatched bars). M1 and M2 macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1- NFκB reporter monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate for 3 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. TNFα pro-inflammatory cytokine production is expressed as the mean±SD in pg/ml for (A) $CD14^{lo}$M1, (B) $CD14^{hi}$ M1, (C) $CD14^{lo}$ M2, and (D) $CD14^{hi}$ M2 macrophage-like subsets. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as * $P<0.05$, ** $P<0.01$ and *** $P<0.005$.

by 47% (770±120 pg/ml), 59% (590± 6 pg/ml) and 52% (704±119 pg/ml) for LR, LF and LP. (Figure 1A). Finally, in the case of $CD14^{lo}$ M2 macrophages (representative of homeostatic healthy gut mucosal macrophages), HK suppressed TNFα (control 21±2 pg/ml) by 62%, 38%, 33%, 57% and 52% for BB, LR, LF, LS and LP. SP suppressed by 19%, 62%, 24% and 67% for BB, LF, LS and LP, respectively, whereas LR-SP augmented TNFα by 38% (Figure 1C).

Secreted protein and heat-killed probiotic strains selectively modulate macrophage subset IL-6 production

The data above clearly demonstrates an immunomodulatory role for both HK and SP probiotics with respect to the expression of the pro-inflammatory cytokine TNFα by pro-inflammatory (M1) and anti-inflammatory/regulatory (M2) macrophage subsets. These macrophage subsets have been described to express different cytokine profiles which underlie their effector function; one such differential cytokine which exhibits both pro-inflammatory and anti-inflammatory properties is IL-6. This experiment was undertaken to establish whether HK and SP probiotics exerted any selective immunomodulatory effects on the expression of IL-6 by M1 and M2 macrophage subsets. Results indicated that HK and SP differentially regulated IL-6 cytokine expression by M1 and M2 macrophage subsets. Both HK and SP preparations suppressed LPS-induced IL-6 production by M2 macrophages, potency of suppression being regulated by the level of CD14 expression. With respect to $CD14^{lo}$ M2 macrophages (representative of homeostatic healthy gut mucosal macrophages), HK suppressed IL-6 (control 547±52 pg/ml) by 58%, 58%, 34%, 98% and 91% for BB, LR, LF, LS and LP. SP suppressed by 86%, 19%, 73%, 94% and 92% for BB, LR, LF, LS and LP, respectively (Figure 2C). IL-6 production by $CD14^{hi}$ M2s was highly sensitive to suppression, where HK suppressed cytokine production (control 1,681±144 pg/ml) by 98%, 96%, 95%, 99% and

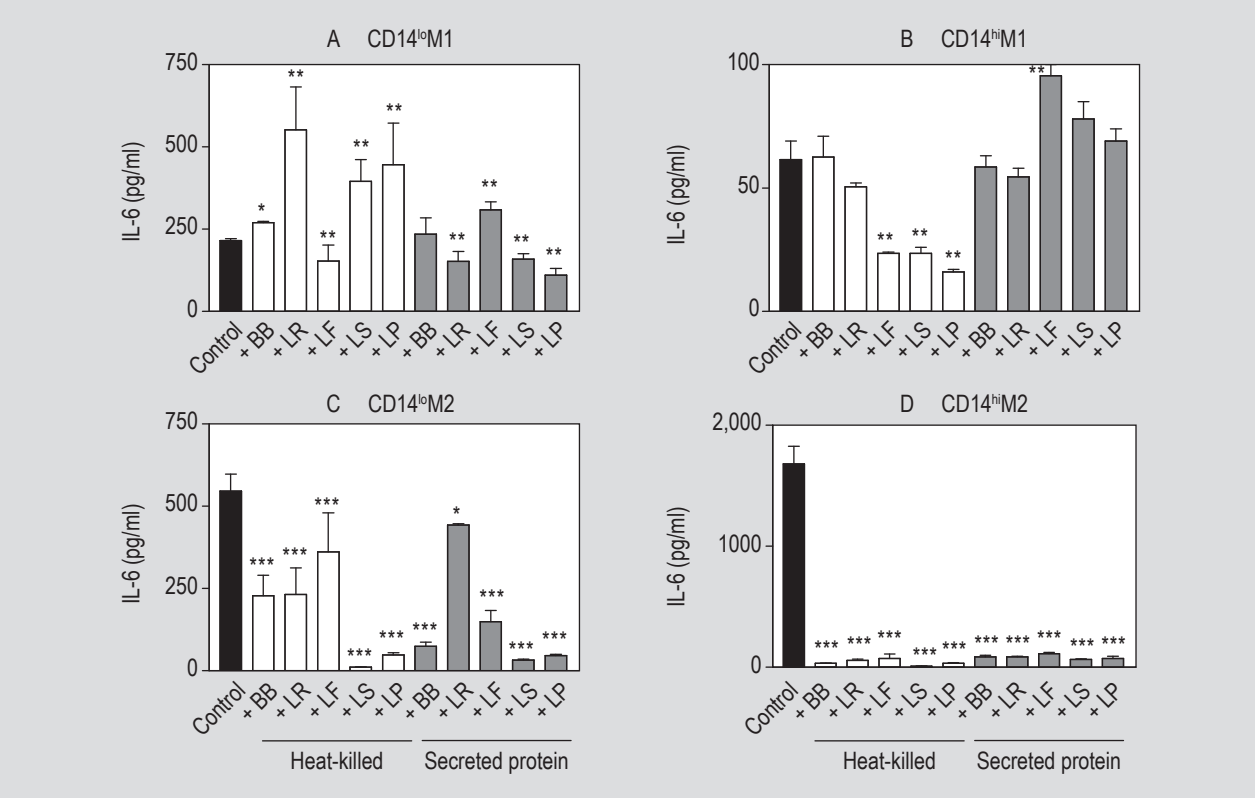

Figure 2. Secreted protein and heat-killed probiotic strains selectively modulate macrophage subset IL-6 production. THP-1-derived $CD14^{hi}$ and $CD14^{lo}$ macrophage subsets were stimulated with 100 ng/ml *Escherichia coli* K12 lipopolysaccharides in the presence or absence of 3×10^8 cfu/ml heat-killed (HK) probiotic bacterial strains (*Bifidobacterium breve* (BB), *Lactobacillus rhamnosus* (LR), *Lactobacillus fermentum* (LF), *Lactobacillus salivarius* (LS) and *Lactobacillus plantarum* (LP)), depicted by clear bars, or 3 µg/ml secreted protein extracted from each of these probiotic strains (depicted by hatched bars). M1 and M2 macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1-NFκB reporter monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate for 3 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. The production of the inflammatory mediator IL-6 is expressed as the mean±SD in pg/ml for (A) $CD14^{lo}$M1, (B) $CD14^{hi}$ M1, (C) $CD14^{lo}$ M2 and (D) $CD14^{hi}$ M2 macrophage-like subsets. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as * *P*<0.05, ** *P*<0.01 and * *P*<0.005.**

98% and SP suppressed by 95%, 95%, 93%, 96% and 95% for BB, LR, LF, LS and LP, respectively (Figure 2D). Pro-inflammatory M1 macrophages exhibit a different IL-6 regulatory profile upon exposure to HK and SP probiotics. $CD14^{lo}$ M1 macrophages HK-BB, HK-LR, HK-LS and HK-LP augment LPS-induced IL-6 (control 216±5 pg/ml) by ×1.3, ×2.6, ×1.8 and ×2.1, respectively, whereas HK-LF suppressed IL-6 production by 29% (Figure 2A). LR-SP, LS-SP and LP-SP suppressed IL-6 by 30%, 26% and 49%, LF-SP augmented cytokine production by ×1.4 and BB-SP failed to modulate LPS-induced IL-6 (Figure 2A). The $CD14^{hi}$ M1 subset (representative of infiltrating inflammatory mucosal macrophages) displayed an intriguing profile upon introduction of heat-killed or secreted protein of these probiotic strains. Heat-killed LF, LS and LP suppressed IL-6 (LPS control 62±8 pg/ml) by 61%, 61% and 74%, whereas the secreted protein from the same strains augmented production by 55%, 26% and 11%, respectively. HK-LR. and LR-SP suppressed IL-6 by 17% and 11%. Finally, *B. breve* failed to modulate LPS-induced IL-6 in these pro-inflammatory macrophages (Figure 2B).

Secreted protein and heat-killed probiotic strains selectively modulate macrophage subset NFκB activity

Both TNFα and IL-6 are regulated by NFκB and exhibit binding consensus sequences in their respective promoter regions. Any such regulation of the expression of these inflammatory cytokines by probiotic bacteria was expected to be as a consequence of modulation of NFκB activity. When comparing profiles between TNFα, IL-6 and NFκB, these data are suggestive that probiotic regulation of these pro-inflammatory cytokines is largely independent of NFκB activity. One clear observation was that the probiotic secreted protein augmented NFκB activation in $CD14^{lo}$ M1, $CD14^{lo}$ M2 and $CD14^{hi}$ M2 macrophages. SP only partially or failed to suppress NFκB activity upon LPS stimulation of $CD14^{hi}$ M1s (Figure 3B). SP augmented NFκB in $CD14^{lo}$

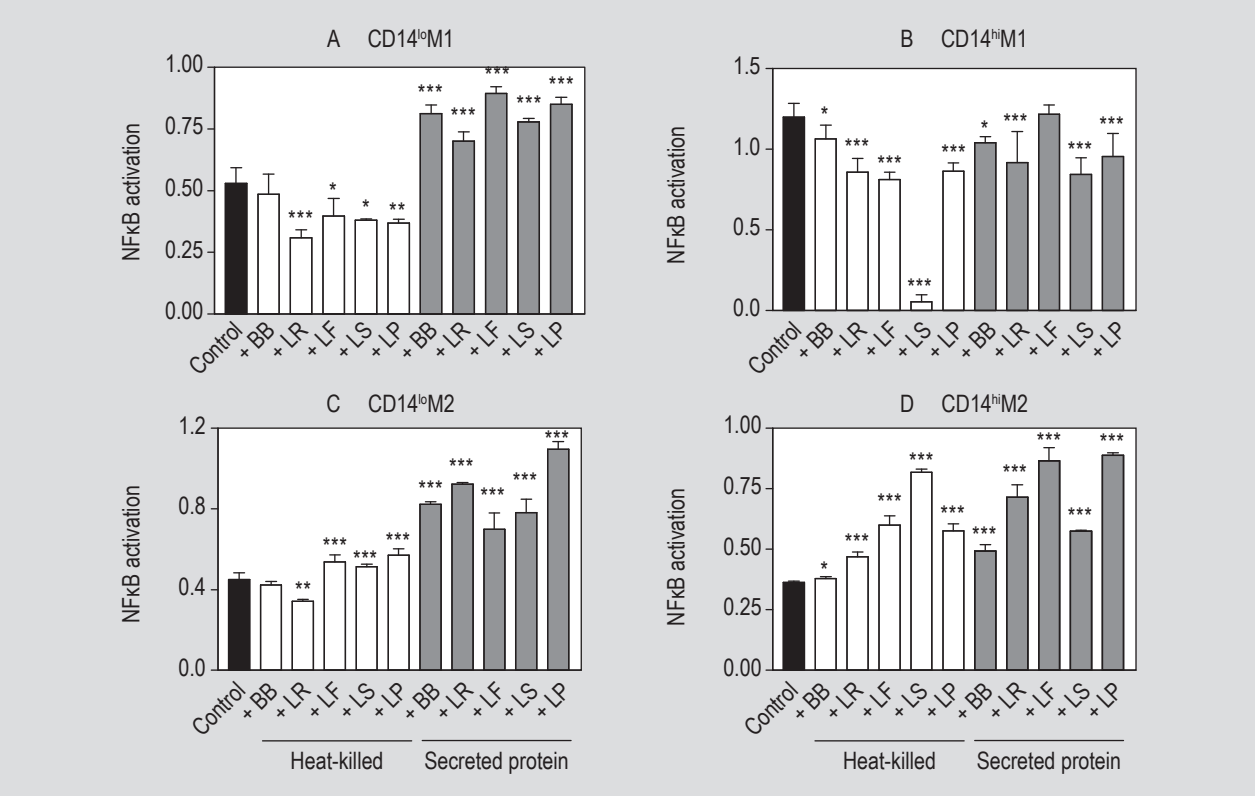

Figure 3. Secreted protein and heat-killed probiotic strains selectively modulate macrophage subset NFκB activity. THP-1-derived $CD14^{hi}$ and $CD14^{lo}$ macrophage subsets were stimulated with 100 ng/ml *Escherichia coli* K12 lipopolysaccharides in the presence or absence of 3×10^8 cfu/ml heat-killed (HK) probiotic bacterial strains (*Bifidobacterium breve* (BB), *Lactobacillus rhamnosus* (LR), *Lactobacillus fermentum* (LF), *Lactobacillus salivarius* (LS) and *Lactobacillus plantarum* (LP)), depicted by clear bars or 3 µg/ml secreted protein extracted from each of these probiotic strains (depicted by hatched bars). M1 and M2 macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1-NFκB reporter monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate for 3 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. NFκB reporter gene activity is expressed as the mean±SD in arbitrary absorbance units (A_{620nm}) for (A) $CD14^{lo}$M1, (B) $CD14^{hi}$ M1, (C) $CD14^{lo}$ M2 and (D) $CD14^{hi}$ M2 macrophage-like subsets. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as * *P*<0.05, ** *P*<0.01 and * *P*<0.005.**

M1s (LPS control level of 0.530±0.063 arbitrary units) by 53%, 32%, 69%, 47% and 60% (Figure 3A); $CD14^{lo}$ M2s (LPS control 0.427±0.009 arbitrary units) by 92%, 116%, 63%, 83% and 156% (Figure 3C) and $CD14^{hi}$ M2s (LPS control 0.363±0.005 arbitrary units) by 36%, 97%, 138%, 58% and 144% for BB, LR, LF, LS and LP secreted protein extracts, respectively (Figure 3D). With the exception of LF-SP, where no modulation of NFκB was observed, the probiotic SPs only partially suppressed $CD14^{hi}$ M1 NFκB (LPS control 1.198±0.084 arbitrary units) by 13%, 23%, 30% and 20% for BB, LR, LS and LP, respectively (Figure 3B). The heat-killed preparations seemed to partially suppress M1 NFκB activation whereas they augmented NFκB activation in the M2 macrophage subset. In both $CD14^{lo}$ M1 and $CD14^{hi}$ M1s, HK-BB failed to modulate NFκB activity. The HK-LABs suppressed NFKB: where in $CD14^{lo}$ M1s, LPS-induced NFκB activity was suppressed by 42%, 25%, 28% and 31% (Figure 3A) and in $CD14^{hi}$ M1s by 28%, 32%, 96% and 28% for LR, LF, LS and LP, respectively (Figure 3B). HK-LABs generally augmented LPS-induced NFκB activity in M2 macrophages, whereas the heat-killed preparation of *B. breve* failed to modulate NFκB activity. HK-probiotics augmented NFκB activity in $CD14^{hi}$ M2s by 29%, 65%, 125% and 59% for LR, LF, LS and LP, respectively (Figure 3D). Finally, in the case of $CD14^{lo}$ M2s, HKs from LF, LS and LP weakly modulated NFκB activity, augmenting by 26%, 20% and 33%, respectively (Figure 3C).

4. Discussion and conclusions

The probiotic bacterial stains (BB, LR, LF, LS and LP) differentially modulate macrophage production of the inflammatory mediator cytokines TNFα and IL-6. Immunomodulation of such macrophage-derived mediators is dependent on macrophage subset present in the mucosa, the CD14 expression and the format by which the probiotic is presented, i.e. bacterial cell wall associated (contact signal) or soluble secreted product (non contact-mediated signal).

Probiotic bacteria used in this study exhibited a strong pro-inflammatory effect on $CD14^{hi}$ M1 macrophages which mimic infiltrating, inflammatory mucosal macrophages. Both heat-killed (HK)- and secreted protein (SP)-preparations augmented LPS-induced TNFα production in these macrophages. In general, $CD14^{lo}$ M1 macrophage TNFα was partially suppressed by these probiotic preparations with the exception of HK-LR, BB-SP and LS-SP which failed to modulate TNFα. These contrasting data between $CD14^{hi}$ and $CD14^{lo}$ M1s highlight an important role for CD14 expression in probiotic immunomodulation. M2 macrophages were relatively poor producers of TNFα. $CD14^{lo}$ M2 cells however, which mimic homeostatic mucosal macrophages, show a partial probiotic modulation; all HK-strains suppressed LPS-induced TNFα, most SPs were suppressive apart from LR-SP which up-regulated TNFα. With respect to $CD14^{hi}$ M2 macrophage TNFα production, heat-killed LABs augmented LPS-induced TNFα whereas *B. breve* failed to modulate this cytokine. This modulatory activity would appear to be associated with the bacterial cell, as secreted protein failed to modulate pro-inflammatory TNFα in $CD14^{hi}$ M2 cells. IL-6 production was differentially modulated by HK and SP probiotic preparations. HK-LF, HK-LS and HK-LP suppressed $CD14^{hi}$ M1 macrophage IL-6 whereas the secreted protein from these three probiotics augmented IL-6. BB and LR failed to modulate this cytokine. $CD14^{lo}$ M1 macrophages produce a higher level of IL-6 compared to the $CD14^{hi}$ M1s. They also exhibit differential regulation by probiotics, in contrast to observations of modulation of $CD14^{hi}$ M1s; HK-LR, HK-LS and HK-LP augmented IL-6. M2 macrophages produce higher levels of IL-6 than M1s and both $CD14^{hi}$ and $CD14^{lo}$ M2 macrophage IL-6 production was suppressed by both HK- and SP- probiotics used in this study. $CD14^{hi}$ M2 macrophages were extremely sensitive to probiotic suppression of LPS-induced IL-6. For the first time in this study, *B. breve* demonstrated a clear immunoregulatory capacity, where both SP and HK extracts suppressed IL-6 in both $CD14^{hi}$ and $CD14^{lo}$ M2s. Thus, probiotics differentially modulate the pro-inflammatory cytokines TNFα and IL-6 produced by LPS-stimulated M1 and M2 macrophages.

LPS induction of monocyte/macrophage pro-inflammatory cytokine expression is well established to be NFκB -regulated (Bondeson *et al.*, 1999). Indeed, both IL-6 and TNFα are regulated by NFκB and express NFκB binding consensus sequences in their promoter regions. It was expected that NFκB activation would parallel probiotic regulation of TNFα and IL-6. In fact, the only profile that partially parallels NFκB activation is the regulation of IL-6 production by $CD14^{hi}$ M1s. M2 macrophage NFκB activation is partially augmented by these probiotic bacteria. Regulation of IL-6 however, resulted in the opposite effect, suppression, suggesting that probiotic modulation of IL-6 was NFκB -independent. In addition, probiotic augmentation of TNFα by pro-inflammatory $CD14^{hi}$ M1 macrophages was also NFκB -independent, as NFκB was either partially suppressed or unaltered. Modulation of $CD14^{hi}$ M2 TNFα did, however, parallel that of NFκB activation. Due to this poor level of association of NFκB with cytokine production, it is likely that other signalling pathways are involved in probiotic regulation. LPS activates mitogen activated protein kinases (MAPKs) (Foey *et al.,* 1998). These pathways are regulated by the probiotic bacterium *Lactobacillus reuteri* which suppressed macrophage TNFα production through the inhibition of the transcription factor, AP-1 (Lin *et al.*, 2008). AP-1 inhibition is suggestive that this probiotic may effect its regulation by suppression of the p38, p42/p44 ERK and JNK MAPKs. In addition to probiotics modulating MAPKs, cytokine production may be modulated directly via activation of other signal regulators or indirectly through the downstream activity of early-expressed cytokines.

Probiotics are generally Gram-positive bacteria that express peptidoglycan in their cell wall. Peptidoglycan is recognised by the intracellular receptor NOD2 (Hasegawa *et al.*, 2006), which, through expression of short and long splice variants can positively or negatively regulate NFκB/MAPK-dependent pro-inflammatory cytokines (Girardin *et al.*, 2003; Rosentiel *et al.*, 2006). Peptidoglycan also activates the transcription factors AP-1 and CREB/ATF in M2-like macrophages (Gupta *et al.*, 1999). Several pro-inflammatory cytokine promotors possess CREB/ATF-binding sequences, indeed cAMP/CREB negatively regulates the pro-inflammatory cytokine TNFα, whilst, at the same time, inducing the expression of the anti-inflammatory cytokine IL-10 (Foey *et al.*, 2003). Thus, pro-inflammatory cytokines may be modulated indirectly by probiotic bacteria through the production of anti-inflammatory/regulatory cytokines. IL-10 may induce suppressor of cytokine signalling (SOCS) proteins, suppressing the activity and expression of several cytokines. One such example includes IFNγ, significant for activation and differentiation of pro-inflammatory M1 macrophages. Interestingly, similar to IL-10 regulation, IL-6, when acting as an anti-inflammatory mediator, induces SOCS (Xing *et al.*, 1998). This negative feedback mechanism may partially explain the differing probiotic regulation observed between IL-6 and TNFα production, in particular, for $CD14^{hi}$ M1 and M2 macrophages. Additionally, there is a reciprocal relationship between IL-6 and TNFα, in conditions where TNFα is augmented or highly expressed, IL-6 expression is low/suppressed. These regulatory processes suggest probiotic bacteria to modulate macrophage-driven responses by inducing endotoxin tolerance to PAMPs. In this study, tolerance may be initiated via chronic LPS stimulation or cross-tolerisation through NOD2, TLR2, TNF-R and IL-1βR signalling (Ferlito *et al.*, 2001; and reviewed in Biswas and Lopez-Collazo, 2009). This study suggests that regulation/tolerisation is likely to be dependent on environmental stimuli, macrophage lineage and CD14 expression. Future research will focus on the mechanisms of endotoxin tolerance, mediated by probiotics, facilitating a comprehensive mechanistic understanding of probiotic immunomodulatory function.

With respect to CD14 expression, $CD14^{hi}$ expression levels dramatically affected LPS-induced TNFα production, where the probiotic bacteria used generally augmented this pro-inflammatory cytokine in both M1 and M2 macrophages; modulation was less pronounced in $CD14^{lo}$ macrophages. CD14 has been described to be both pro-inflammatory, through oligomerisation with the LPS receptor TLR4, and anti-inflammatory through its action as a scavenger receptor for apoptotic cells. The relative suppression of TNFα produced by $CD14^{lo}$ macrophages would suggest that modulation is as a consequence of down-regulation of the CD14/TLR4/TLR2 pro-inflammatory complex rather than downstream suppressive function of IL-10 or TGFβ induced by the recognition and phagocytosis of apoptotic cells. M2 IL-6 production was suppressed by probiotics with a stronger suppression evident in $CD14^{hi}$ M2s. Regarding the fact that IL-6 is both pro- and anti-inflammatory, probiotic suppression of this cytokine may exhibit inflammatory and tolerogenic/suppressive function. Modulation of M1 IL-6 production was less clear and exhibited both suppressive and augmentation responses for both $CD14^{hi}$ and $CD14^{lo}$ which appeared to be strain selective and dependent on form of probiotic used to modulate the macrophage response.

Generally, the form of probiotic used (heat-killed or secreted protein) showed little difference in modulation of LPS-induced TNFα and IL-6. Two notable exceptions presented themselves for $CD14^{hi}$ M2 TNFα and $CD14^{hi}$ M1 IL-6 production: heat-killed LABs strongly augmented $CD14^{hi}$ M2 TNFα whereas secreted protein weakly modulated this cytokine. On the other hand, $CD14^{hi}$ M1 IL-6 was suppressed by HK-preparations of *L. fermentum, L. salivarius* and *L. plantarum* whereas their secreted proteins partially augmented IL-6. Heat-killed probiotics utilised, investigated the immunomodulatory capability of cell-associated factors whereas secretory protein preparation investigated the potential for secreted, soluble immunomodulators. Preliminary SDS-PAGE analysis has identified several proteins either consistent between probiotic strains or strain-specific (data not shown). A previous investigation on *L. rhamnosus* GG conditioned medium identified several potential immunoregulators including a cell-wall-associated hydrolase, sepin B1 and a transcriptional regulator (Sanchez *et al.*, 2009). Additionally, secreted immunomodulatory proteins have been suggested to modulate signalling pathways driving pro-inflammatory cytokine production and function (Frick *et al.*, 2007; Yan *et al.*, 2007). From this and other studies it can be concluded that beneficial immunomodulatory effects are associated with both bacterial cell-associated and secretable fractions, thus any future modulation of the immune system is best considering this, making use of both fractions provided by delivery of live probiotic strains either individually or in combination.

Cytokine production acts as a useful readout for probiotic immunomodulation. These bacteria are capable of both driving immune responses towards predominant Th_1 and Th_2 responses and suppressing such responses. This suggests probiotics to manipulate and redress immunopathological mechanisms: Th_1-driven pathologies such as CD, may benefit from probiotics that either suppress harmful immune reactions or induce type II cytokines (IL-10, IL-4, IL-13) and conversely, Th_2-driven pathologies such as ulcerative colitis, may benefit from immunosuppressive probiotics or those that induce type I cytokine expression (IFNγ, IL-12 and TNFα). Combinations of probiotics will allow development of disease-group-specific treatments (Th_1 or Th_2-driven) based on a thorough

understanding of the immunopathogenic mechanisms. In the context of mucosal macrophages, some probiotics may be inappropriate in inflammatory pathologies where $CD14^{hi}$ M1 subset cells predominate (Zareie *et al.*, 2001) as, according to findings in this study, probiotic treatment may enhance inflammation by augmentation of TNFα production. A cautionary approach to their usage is recommended. On the other hand, probiotic treatment of $CD14^{lo}$ M2s, resembling homeostatic/regulatory mucosal macrophages (Platt and Mowat, 2008; Smith *et al.*, 2001; Smythies *et al.*, 2005), fails to augment inflammatory cytokines and may well induce expression of the regulatory cytokines, IL-10 and TGFβ, resulting in tolerance/immune hyporesponsiveness.

In conclusion, probiotic modulation of macrophage inflammatory cytokines TNFα and IL-6 is largely NFκB-independent, but dependent on macrophage subset and the nature by which the probiotic is introduced. Probiotic strains used can differentially exert both immune activatory or suppressive functions. Future probiotic development will consider whether they are to be used prophylactically in healthy individuals or as a therapeutic treatment of defined pathological conditions, probiotic strain-specific effects, gut mucosal integrity and immune phenotype of mucosal macrophages – one step closer to personalised medicine!

Acknowledgements

This study was part funded by the Seale Hayne Educational Trust and the University of Plymouth VC's Research and Innovation Fellowship. Neama Habil is funded by the Ministry of Higher Education, Government of Iraq.

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