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LACTOBACILLUS CASEI STRAIN SHIROTA SELECTIVELY MODULATES MACROPHAGE SUBSET CYTOKINE PRODUCTION

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ABSTRACT: ***Probiotics confer health benefits through many mechanisms including modulation of the gut immune system. Gut macrophages regulate immune homeostasis, mounting tolerogenic responses to food and commensal bacteria or immune inflammatory responses to pathogens. Local environment and macrophage subset determine immune response and tolerance, associated with an M2-like phenotype and inflammatory activation with an M1-like phenotype. Subset predominance will determine immunomodulatory effects of probiotic species such as Lactobacillus casei strain Shirota (LcS). The aim of this study was to investigate differential regulatory effects of LcS on M1 and M2 macrophage subsets. PMA or vitamin D_3 differentiated THP-1 human monocytic cells were used to investigate heat-killed LcS and secreted protein immunoregulation of M1 and M2 cytokine production, respectively. Additionally, regulation of $CD14^{lo}$ M2 and $CD14^{hi}$ M1 function was investigated. Cytokine expression was measured by ELISA and NFkB activity by reporter assay. Both HK-LcS and SP-LcS augmented IL-1β, suppressed IL-6 and differentially regulated TNFα and IL-8, dependent on macrophage subset. HK-LcS and LcS-SP augmented $CD14^{hi}$ M1 TNFα whereas suppressed $CD14^{lo}$ M2 IL-6 and $CD14^{hi}$ M1 NFkB. In conclusion, LcS differentially regulates macrophage cytokines and NFkB activation, is subset-dependent and suggests a cautionary approach to probiotic treatment of mucosal inflammation.***

KEY WORDS: Cytokines, Inflammation, Macrophage, Monocyte, Probiotics

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INTRODUCTION

The benefits of maintaining a healthy commensal microbiota has long since been recognised in the probiotic field, where supplementation by ingestion of lactic acid bacteria (LAB) facilitates a balanced microbial environment beneficial to the host. More recently, a health benefit of probiotics in the treatment of allergies has suggested that these probiotic supplements have a pivotal role to play in modulating the host's immune system. Probiotic investigations have indeed demonstrated LAB to modulate immune responses elicited by T, B, NK cells and monocyte/macrophages. These effects however, serve to confuse as they often display contradictory findings from study to study. This is partly attributable to immune cell phenotype being studied, its environment and the strain of probiotic being used. The Yakult bacteria, *Lactobacillus casei* strain Shirota (LcS) has been documented to be immune activator by inducing high levels of IL-6, TNFα and IL-12 (Christensen et al., 2002; Matsuguchi et al., 2003). In contrast, Borruel et al., 2003 found LcS to suppress the production of TNFα by human intestinal mucosa cells, preventing inflammatory effects. LcS has also been demonstrated to facilitate the development of Th_1 cells through the induction of IL-12 (Shida et al., 1998) and to augment NK cell cytotoxicity (Takeda et al., 2006). Thus, in the case of LcS, modulation of immune responses can be both regulatory/anti-inflammatory and activatory/pro-inflammatory. The same ambiguity exists when investigating another LAB, L. *Rhamnosus*, which has been shown to promote development of regulatory DCs (Foligne et al., 2007a), suppress epithelial cell production of IL-8 (Zhang et al., 2005), induce IL-12 production (Shida et al., 2006; Ichikawa et al., 2007) and to increase NK cell numbers (Gill et al., 2001). Again, as with LcS, *L. Rhamnosus* exhibits both immune-activatory and -regulatory function. The desired effect of the probiotic is determined by cell phenotype, recognition receptor expression and stimuli present in the local environment.

Commensal bacteria play an important role in gut mucosal development and function (Rhee et al., 2004). Inappropriate recognition of conserved commensal pathogen associated

molecular patterns (PAMPs) by their respective pattern recognition receptors (PRRs), however, could trigger a damaging inflammatory immune response. As a result, it is vital that recognition of bacterial PAMPs is tightly regulated allowing the host to tolerate commensal bacteria and, at the same time, maintaining responsiveness to pathogens. The balance between luminal contents such as commensal and pathogenic bacteria, present in the intestinal microflora, is vital for mucosal fate: tolerance (immune non-responsiveness) or immunity (Artis, 2008). The gastrointestinal tract (GIT) has developed subtle modifications; integral to this tolerance/activation decision and prevention of inappropriate immune responses to commensal bacteria (Schenk and Mueller, 2007), suppressing TLR, endogenous signal inhibitor and compartmentalisation of TLR expression as well as up-regulation of expression and activity upon dangerous insult.

Priming of gut mucosal immune responses requires the commensal and probiotic bacteria to instruct cells of the underlying immune tissue. One of the most pivotal cells in this immune interaction is the macrophage, residing in the lamina propria. Macrophage phenotypes determine the functional outcome in the gut mucosa. Indeed, macrophages generally belong to two distinct subsets- M1 pro-inflammatory or M2 anti-inflammatory/regulatory macrophages (Mosser and Edwards, 2008; Stein et al., 1992). M1 macrophages play an important role in eradication of invading pathogens by the production of pro-inflammatory cytokines, hydrolytic enzymes and reactive oxygen intermediates. Thus, the function of M1 macrophages is vital during pathogenic infection; upon clearance of pathogen however, it is essential to suppress this function as chronic activation may result in destructive inflammatory immunopathology. M2 macrophages, on the other hand, mediate immunoregulatory function and exhibit an anti-inflammatory effector function through expression of anti-inflammatory cytokines such as IL-10 and phagocytic scavenger receptors such as the mannose receptor (MR). Due to the differences in effector function of these subsets, they are both capable of cross-regulating each other. The relative proportion of these subsets determines overall immune fate.

Gut mucosal macrophages are central to deciding immune responsiveness to luminal antigens and bacteria as activatory or tolerogenic. This is determined by the cell subsets and molecules initiated by their local environment. Macrophages generally express a wide range of PRRs; intestinal macrophages, on the other hand, are both functionally and phenotypically different from blood-derived monocyte/macrophages. In the healthy gut, tolerant to luminal contents, intestinal macrophages express MHC II and display a regulatory/anti-inflammatory phenotype characterised by phagocytic function, scavenger receptor expression (MR, CD13, CD36), anti-inflammatory/regulatory cytokine expression (TGFβ and IL-10), reduced responsiveness to PAMPs (low or absent expression of CD14 and TLRs) and fail to express co-stimulatory molecules (CD80 and CD86); a phenotype resembling the M2 macrophage subset (Smith et al., 2001; Smythies, 2005; Platt and Mowat, 2008), important in immune regulation and resolution of inflammation by secreting anti-inflammatory cytokines (Mahida, 2000). Pathogenic insult or dysfunctional mucosal barrier results in inappropriate presentation of luminal antigens and a breakdown in tolerance leading to a localised immune activation/inflammation. Breakdown in tolerance to commensal bacteria is suggested to contribute to the pathogenesis of the inflammatory bowel disease, Crohn's disease. In this inflammatory pathology, lamina propria macrophages display a $CD14^{hi}$ phenotype (Segura et al., 2002) an increased TLR responsiveness and expression of the pro-inflammatory cytokines IL-1β, IL-8, IL-12 and TNFα (Zareie et al., 2001). This functional phenotype resembles that of the M1 macrophage subset. Thus, the macrophage subset present in the lamina propria is central to determining immune fate as tolerising or immune activatory/pro-inflammatory. Macrophage subset, differentiation status and resulting effector function of gut mucosal macrophages is dependent on environmental factors encountered. As such, gut mucosal macrophages can be primed towards the M2 anti-inflammatory/regulatory phenotype in healthy mucosa or towards a M1 pro-inflammatory phenotype during mucosal breakdown and immunopathology. This dichotomy in macrophage effector phenotype partially explains the differential regulation of adaptive immune responses between regulation, cell-mediated immunity or humoral immunity where M1 cells effect either a Th_1 or a Th_{17}-driven response and M2 cells effect a Th_2-driven response (Mills et al., 2000) or a Treg response.

Probiotic bacteria have been established to modulate macrophage effector function. LAB have been demonstrated to modulate macrophage function by either augmenting or suppressing IL-12 production (Shida et al., 2006; Ichikawa et al., 2007; Foligne et al., 2007b), suppression of TNFα in inflamed mucosa (Borruel et al., 2002) and, when administered at high levels, augment IL-10 (Foligne et al., 2007b). These modulatory effects have been described for whole bacterial cells with subtle differential effects being described for heat-killed versus live bacteria. These observations have lead investigators to study the active immunomodulatory compartments to probiotic bacteria: cell-associated or cell-secreted. The cell wall-derived component of polysaccharide/PGN complex was demonstrated to suppress LPS-induced IL-6 production by peripheral blood mononuclear cells (Matsumoto et al., 2005). In addition, when considering the immunomodulatory effects of these bacteria, secreted metabolites are likely to, and have been described to, play an important role in modulating the host immune response. Indeed, the conditioned medium/secreted protein extract obtained from the culture of *Lactobacillus plantarum* inhibits NFkB activity (Petrof et al., 2009), as such will have a profound effect on NFkB-dependent immune functions such as macrophage production of the inflammatory

cytokines; IL-1β, IL-6, IL-8, MCP-1, IL-12 and TNFα.

The dynamic balance between M1 and M2 macrophages is suggested to be critical in determining the functional effector fate of the gut mucosal immune system, immune activation or induction of tolerance. Integral to this immune fate is the activation stimuli available to the macrophage, the form of the probiotic modulator i.e. whole cell or secreted functional molecules and the functional phenotype of the macrophage being investigated. Thus far, no data exists focussing on the effects of both probiotic bacterial cells and secreted proteins on the immunomodulation of functional behaviour of distinct macrophage subsets, representative of regulatory/homeostatic gut mucosal macrophages and infiltrating inflammatory macrophages. Using a cell-line based model of high and low CD14 expressing macrophages resembling mucosal resident homeoststic- and infiltrating inflammatory macrophages, the aim of this study was to investigate the immunomodulatory capacity of secreted soluble proteins and cell-associated LcS on macrophage subset cytokine phenotype.

MATERIALS and METHODS

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

Lactobacillus casei strain Shirota (LcS) was obtained from commercially available Yakult probiotic drink (Yakult UK). LcS was cultured aerobically in deMan Rogosa Sharp (MRS) broth at 37°C. LcS was cultured in sterile broth culture for 18 hours until stationary phase was achieved. Bacterial cells were harvested by centrifugation at 5,000g for 10 minutes, washed in sterile saline solution and numbers adjusted to give an OD_{600} reading of 1.2. This turbidity reading was determined by cfu counting standards plated on MRS agar to equate to a count of $1x10^9$ cfu/ml. LcS was adjusted to a stock density of $1x10^{10}$ cfu/ml and heat killed at 90°C for 120 minutes (HK-LcS) according to the protocol of Young et al., 2004. Cell death was confirmed by plating of the bacterial sample on MRS agar for a minimum of 18 hours. In addition, secreted protein was extracted by precipitation of proteins secreted into growth supernatant by using chilled trichloroacetic acid (TCA) according to the protocol of Sanchez et al., 2009. Protein content was verified by SDS-PAGE and quantified by calibration to a commercially available Bradford protein stain. Secreted protein (LcS-SP) was adjusted by dilution to a stock concentration of 1mg/ml.

Monocyte and macrophage culture

The human monocytic cell line, THP-1, was obtained from ECACC and routinely used for this study between passages 7 and 35. THP-1 cells were maintained in R10 medium composed of RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 2mM L-glutamine, $100Uml^{-1}$ penicillin and $100\mu gml^{-1}$ streptomycin (Lonza, Wokingham, UK). THP-1 NFkB reporter cell lines, THP-1Blue ($CD14^{lo}$) and THP-1Blue-CD14 ($CD14^{hi}$) were maintained in R10 medium in the presence of the selection antibiotics, $200\mu gml^{-1}$ zeocin or $200\mu gml^{-1}$ zeocin and $10\mu gml^{-1}$ blastocidin, respectively (Autogen Bioclear, Calne, UK). Cells were plated out to a final density of $1x10^5$ cells/100µl/well in R10 medium in 96 flat-bottomed well tissue culture plates (monocyte cultures). Pro-inflammatory (M1-like) $CD14^{hi}$ macrophages and anti-inflammatory (M2-like) $CD14^{lo}$ macrophages were generated by differentiation of these monocytes in the presence of 25ng/ml PMA for 3 days or 10nM 1,25-$(OH)_2$-vitamin D_3 (Sigma-Aldrich, Poole, UK), for 7 days, respectively (Daignealt et al., 2010).

Activation of monocyte and macrophage cytokine production

Monocytes and macrophages were stimulated by the bacterial pathogen associated molecular pattern (PAMP); 100ng/ml *E. coli* strain K12 LPS (expressed in enteropathic GM-negative bacteria and detected by TLR4) and cultured for 18 hours (determined as optimal time period for expression of all the cytokines TNFα, IL-1β, IL-6 and IL-8) in a humidified environment at 37°C, 5% CO_2. Supernatants were harvested and stored at -20°C until required for assay by sandwich ELISA.

Regulatory effect of heat-killed and secreted protein of LcS

To investigate the potential regulation of monocyte and macrophage cytokine production and NFkB activation, HK-LcS and LcS-SP were added in culture to final concentrations of $3x10^8$ bacterial cells/ml and 3µg/ml respectively, as a pre-treatment for 18 hours prior to stimulation for 18 hours, in a humidified environment at 37°C, 5% CO_2, with the bacterial PAMP, K12-LPS at a concentration of 100ng/ml. As a control experiment, to demonstrate a physiologically relevant role for HK-LcS and LcS-SP, cytotoxicity assays (MTT and trypan blue exclusion) were carried out on both monocytes and macrophages, upto 10^9 and 100µg/ml, respectively. No significant reductions in viability were observed for the concentrations used in this study; viability was routinely >90%.

Cytokine measurement

Monocyte and macrophage production of the pro-inflammatory cytokines; TNFα, IL-1β, IL-6 and IL-8, were analysed by sandwich ELISA using commercially available capture and detection antibodies from R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK. Due to supernatant volume constraints, the $CD14^{hi}$ and $CD14^{lo}$ NFkB reporter transfectants were assayed for the pro-inflammatory cytokine, TNFα, and the dual pro- and anti-inflammatory cytokine, IL-6, in addition to NFkB activity (see below). Protocols were followed according to manufacturer's instructions and compared to standard curves, between the range of 7 to 5000pg/ml, using the recognised international standards available from NIBSC, Potter's Bar, UK. Colorimetric development was measured spectrophotometrically by an

OPTIMax tuneable microplate reader at 450nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA).

NFkB activity measurement

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

Statistical analysis

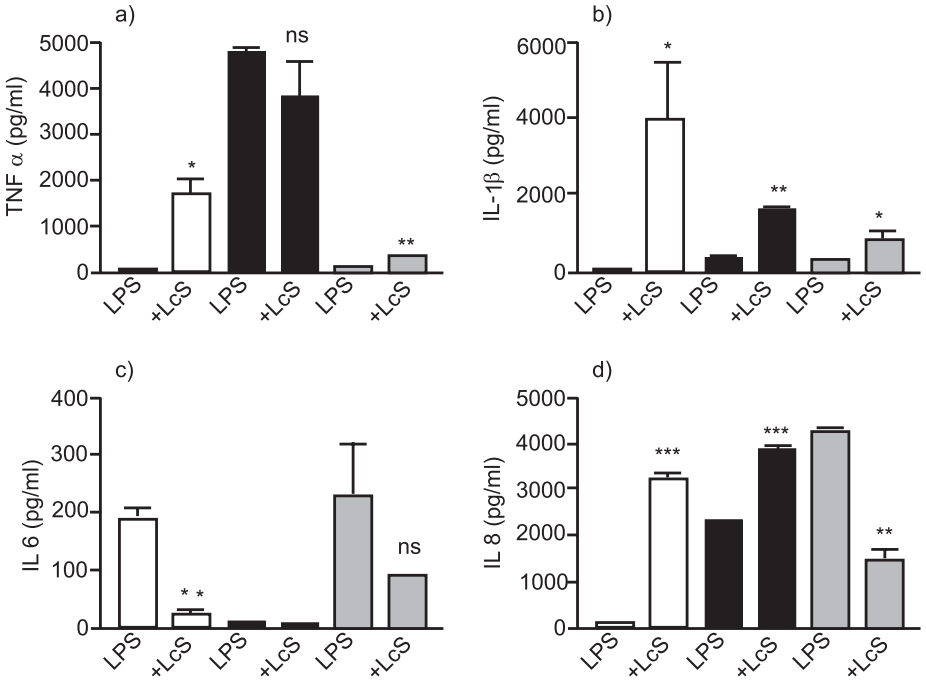
Measure of statistical significance was analysed by a paired Students' T test. Significance was set at $p<0.05$, where significant effects of either HK-LcS or LcS-SP compared to stimulus control were indicated as *$p<0.05$, **$p<0.01$ and ***$p<0.001$. NS denotes not significant.

RESULTS

Heat-killed LcS selectively modulates LPS-induced monocyte and macrophage cytokine production

LcS has been demonstrated to modulate innate immunity; this experiment was undertaken to establish whether heat-killed LcS (in the absence of any non-specific effects of lactic acid produced) exerted immunomodulatory effects on cytokine expression of THP-1-derived monocytes and the M1-like and M2-like subsets of macrophages. Results indicated that HK-LcS differentially regulates individual cytokine expression as well as monocytes, M1 and M2 macrophage subsets. HK-LcS augmented K12-LPS-induced monocyte and M2 TNFα production from 37± 2pg/ml to 1683±313pg/ml ($p=0.0118$) and 148±8pg/ml to 330±10pg/ml ($p=0.0012$), respectively. M1 macrophage production of TNFα was partially suppressed by KH-LcS (control 4733±116pg/ml to 3801±750pg/ml, $p=0.1264$) (Fig. 1a). Expression of IL-1β showed a similar trend to TNFα expression in the case of both monocytes and M2 macrophages. HK-LcS augmented LPS-induced monocyte IL-1β from 30±5pg/ml to 3978±1508pg/ml, $p=0.0455$ and M2 IL-1β from 317±58pg/ml to 844±187pg/ml, $p=0.036$. M1 macrophages, however, also exhibited an augmentation of IL-1β expression by HK-LcS from 352±57pg/ml to 1612±73pg/ml, $p=0.0034$) (Fig 1b). Monocyte and M2 macrophage IL-6 production displayed a differential regulation to TNFα and IL-1β, where HK-LcS suppressed LPS-induced IL-6 from control levels of 188±20pg/ml and 230±92pg/ml to 24±5pg/ml ($p=0.0071$) and 92±24pg/ml ($p=0.1217$) for monocytes and M2s respectively. Consistently, M1 macrophages expressed little or no IL-6 at the limits of detection for the sandwich ELISA (Fig. 1c). Finally, HK-LcS differentially regulated monocyte and macrophage IL-8 production. Monocyte and M1 LPS-induced IL-8 was augmented from control levels of 63±12pg/ml and 2277±12pg/ml to 3229±149pg/ml, $p=0.0007$ and 3888±52pg/ml, $p=0.0002$, respectively. M2 IL-8 production, on the other hand, was suppressed by HK-LcS from 4270±36pg/ml to 1462±225pg/ml, $p=0.0026$ (Fig. 1d).

FIGURE 1. Heat-killed LcS selectively modulates LPS-induced monocyte and macrophage cytokine production. THP-1 monocytes (open bars), M1 (bold) and M2 (shaded) macrophage subsets were stimulated by 100ng/ml *E.coli* K12 LPS in the presence or absence of $3x10^8$ cfu/ml heat-killed *Lactobacillus casei* strain Shirota (+LcS). M1 and M2 macrophages were generated by differentiating THP-1 monocytes with either 25ng/ml PMA for 3 days or 10nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. Cytokine production is expressed as the mean±SD in pg/ml for a) TNFα, b) IL-1β, c) IL-6 and d) IL-8. Data displayed is representative of triplicate samples of n=3 replicate experiments.



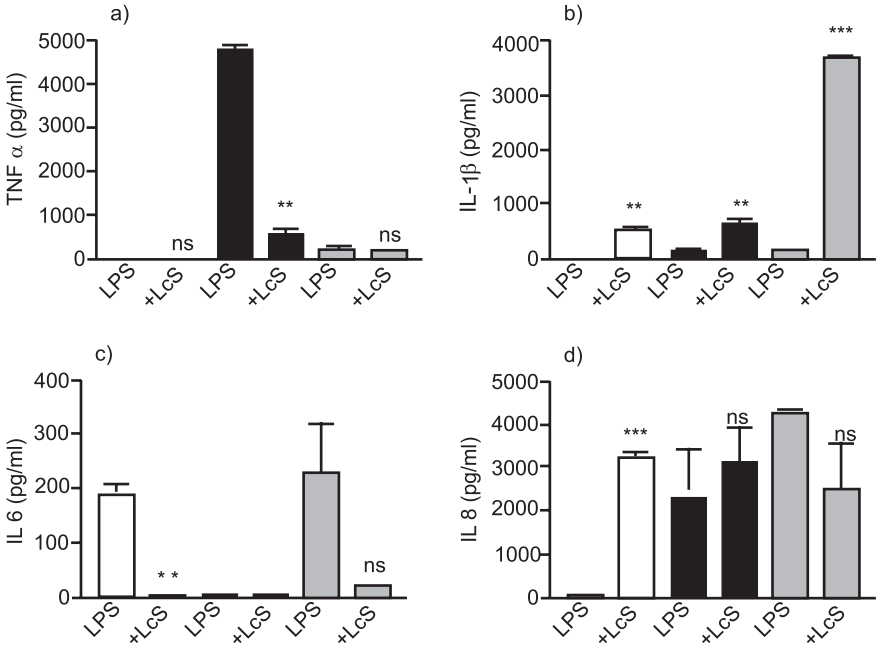
LcS secreted protein selectively modulates LPS-induced monocyte and macrophage production

The data above clearly demonstrates an immunoregulatory role for HK-LcS. This regulatory or modulatory role is understood to be associated with the whole bacteria, in particular, associated with the cell wall. Probiotic bacteria can produce metabolites which are immunomodulatory, such as short chain fatty acids but also likely to produce immunomodulatory proteins. This experiment was undertaken to establish whether protein secreted from LcS exerted any immunomodulatory effects on cytokine expression of monocytes and M1 and M2 macrophage subsets. Results indicated that LcS-SP differentially regulates individual cytokine expression as well as monocytes, M1 and M2 macrophage subsets. LcS-SP suppressed K12-LPS-induced monocyte and M1 TNFα production from 38± 6 pg/ml to 19±5 pg/ml (p=0.069) and 4733±116 pg/ml to 481±157 pg/ml (p=0.0011), respectively. M2 macrophage production of TNFα was not modulated by LcS-SP (control 145±118 pg/ml to 143±33pg/ml, p=0.986) (Fig. 2a). Expression of IL-1β showed a consistent augmentation response in monocytes, M1 and M2 macrophages. LcS-SP augmented LPS-induced monocyte IL-1β from 14±2 pg/ml to 501±62 pg/ml, p=0.0055; M1 macrophage IL-1β expression from 146±26pg/ml to 652±65 pg/ml, p=0.0024) and M2 IL-1β from 129±4pg/ml to 3673±36pg/ml, p<0.0001, (Fig 2b). Monocyte and M2 macrophage IL-6 production displayed a differential regulation to TNFα and IL-1β, where SP-LcS suppressed LPS-induced IL-6 from control levels of 188±20pg/ml and 230±92pg/ml to 5±2pg/ml (p=0.0046) and 25±15pg/ml (p=0.08) for monocytes and M2s respectively. Consistently, M1 macrophages expressed little or no IL-6 at the limits of detection for the sandwich ELISA (Fig. 2c). Finally, LcS-SP differentially regulated monocyte and macrophage IL-8 production. Monocyte LPS-induced IL-8 was augmented from control levels of 63±12 pg/ml to 3229±149 pg/ml, p=0.0007. M1 IL-8 production was partially augmented, although not significantly, from control level of 2277±1134 pg/ml to 3092±773 pg/ml, p=0.4331. M2 IL-8 production, on the other hand, was partially suppressed by LcS-SP from 4270±36 pg/ml to 2468±1080 pg/ml, p=0.1055 (Fig. 2d).

Heat-killed LcS selectively modulates M1 and M2 macrophage subset production of inflammatory mediators: CD14-dependency

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 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 LcS. HK-LcS dramatically augmented LPS-induced TNFα production by $CD14^{hi}$ M1 cells (from control levels of 1161±148 pg/ml to 4507±367 pg/ml, p=0.0078) whereas weakly augmented TNFα production in $CD14^{lo}$ M1 cells (control 254±13 pg/ml to 397±50 pg/ml, p=0.0216) (Fig. 3a). M2 macrophages, on the other hand, produced low levels of TNFα; however, $CD14^{lo}$ M2 displayed suppression by HK-LcS (control 21±2 pg/ml to 14±1 pg/ml, p=0.0067) whereas $CD14^{hi}$ M2 TNFα production was slightly augmented (control 11±1pg/ml to 16±2pg/ml, p=0.07) (Fig. 3a). In contrast, HK-LcS failed to modulate LPS-induced IL-6 production by $CD14^{hi}$ M1 cells (from control levels of 62±8pg/ml to 70±18pg/ml, p=0.622) as well as

FIGURE 2. LcS secreted protein selectively modulates LPS-induced monocyte and macrophage cytokine production. THP-1 monocytes (open bars), M1 (bold) and M2 (shaded) macrophage subsets were stimulated by 100ng/ml *E.coli* K12 LPS in the presence or absence of 3mg/ml *Lactobacillus casei* strain Shirota secreted protein (+LcS). M1 and M2 macrophages were generated by differentiating THP-1 monocytes with either 25ng/ml PMA for 3 days or 10nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. Cytokine production is expressed as the mean±SD in pg/ml for a) TNFα, b) IL-1β, c) IL-6 and d) IL-8. Data displayed is representative of triplicate samples of n=3 replicate experiments.



$CD14^{lo}$ M1 cells (control 216±5pg/ml to 227±42pg/ml, p=0.734) (Fig. 3b). M2 macrophages, on the other hand, produced higher levels of IL-6 than M1 cells; however, $CD14^{lo}$ M2, representative of gut homeostatic mucosal macrophages, displayed a suppression by HK-LcS (control 547±52 pg/ml to 145±26 pg/ml, p=0.0014) additionally, $CD14^{hi}$ M2 IL-6 production was also suppressed (control 1548±286 pg/ml to 28±6 pg/ml, p=0.0111) (Fig. 3b). In addition, any regulation of pro-inflammatory cytokine expression was expected to be as a consequence of modulation of NFκB activity, as both TNFα and IL-6 are regulated by NFκB and exhibit binding consensus sequences in their respective promoter regions. These data are suggestive that LcS regulation of these pro-inflammatory cytokines is independent of NFkB activity, when comparing profiles between TNFα, IL-6 and NFkB. HK-LcS partially suppressed NFkB activity in both $CD14^{hi}$ and $CD14^{lo}$ M1 cells by 43% and 15% respectively. HK-LcS suppressed $CD14^{hi}$ M1 LPS-induced NFkB activity from control levels of 0.782±0.0076 to 0.442±0.032 arbitrary units, p=0.0017, whereas $CD14^{lo}$ M1 activity was suppressed from 0.258±0.017 to 0.220±0.0039, p=0.0347. In contrast, HK-LcS augmented LPS-induced NFkB activity in both $CD14^{hi}$ and $CD14^{lo}$ M2 macrophages by 155% and 46% respectively. HK-LcS augmented $CD14^{hi}$ M2 LPS-induced NFkB activity from control levels of 0.200±0.0004 to 0.509±0.039 arbitrary units, p=0.0051, whereas $CD14^{lo}$ M2 activity was augmented from 0.220±0.002 to 0.322±0.0061, p=0.0021 (Fig. 3c).

LcS secreted protein selectively modulates M1 and M2 macrophage subset production of inflammatory mediators: CD14-dependency

LcS-SP dramatically augmented LPS-induced TNFα production by $CD14^{hi}$ M1 cells (from control levels of 1161±148 pg/ml to 2971±199 pg/ml, p=0.012) whereas failed to modulate TNFα production in $CD14^{lo}$ M1 cells (control 254±13 pg/ml to 264±35 pg/ml, p=0.504) (Fig. 4a). M2 macrophages, on the other hand, produced low levels of TNFα; however, $CD14^{lo}$ M2 displayed a partial augmentation by LcS-SP (control 21±2 pg/ml to 30±6 pg/ml, p=0.067) whereas $CD14^{hi}$ M2 TNFα production failed to be modulated by LcS-SP (control 11±1pg/ml to 13±2pg/ml, p=0.321) (Fig. 4a). In contrast, LcS-SP suppressed LPS-induced IL-6 production by $CD14^{hi}$ M1 cells (from control levels of 62±8 pg/ml to 19±9 pg/ml, p=0.0002) whereas LcS-SP failed to modulate IL-6 production by $CD14^{lo}$ M1 cells (control 216±5 pg/ml to 222±37 pg/ml, p=0.840) (Fig. 4b). M2 macrophages, on the other hand, produced higher levels of IL-6 than M1 cells; however, $CD14^{lo}$ M2 displayed a suppression by LcS-SP (control 547±52pg/ml to 128±24pg/ml, p=0.0014) additionally, $CD14^{hi}$ M2 IL-6 production was also suppressed (control 1548±286 pg/ml to 99±39 pg/ml, p=0.0095) (Fig. 4b). Again, when comparing profiles between TNFα, IL-6 and NFkB, these data are suggestive that LcS regulation of these pro-inflammatory cytokines is independent of NFkB activity. LcS-SP partially suppressed NFkB activity in $CD14^{hi}$ by 40% whilst augmenting $CD14^{lo}$ M1 cells by 21%. LcS-SP suppressed $CD14^{hi}$ M1 LPS-induced NFkB activity from control levels of 0.782±0.0076 to 0.465±0.054 arbitrary units, p=0.0122, whereas $CD14^{lo}$ M1 activity was augmented from 0.258±0.017 to 0.312±0.005, p=0.0503. In contrast, LcS-SP partially augmented LPS-induced NFkB activity in both $CD14^{hi}$ and $CD14^{lo}$ M2 macrophages by 15% and 47% respectively. LcS-SP augmented $CD14^{hi}$ M2 LPS-induced NFkB activity from control levels of 0.200±0.0004 to 0.229±0.043 arbitrary units, p=0.353, whereas $CD14^{lo}$ M2 activity was augmented from 0.220±0.002 to 0.324±0.025, p=0.016 (Fig. 4c).

FIGURE 3. Heat-killed LcS selectively modulates M1 and M2 macrophage subset production of inflammatory mediators: CD14-dependency. $CD14^{hi}$ (bold) and $CD14^{lo}$ (shaded) M1 and M2 macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1-NFkB reporter monocytes with either 25ng/ml PMA for 3 days or 10nM 1,25-$(OH)_2$ vitamin D_3 for 7 days respectively. M1 and M2 macrophages were stimulated with 100ng/ml K12-LPS in the presence (+LcS) or absence of $3x10^8$ cfu/ml heat-killed *Lactobacillus casei* strain Shirota. Cytokine production is expressed as the mean±SD in pg/ml for a) TNFα and b) IL-6. NFkB activity (c) is expressed as the mean±SD in arbitrary absorbance units (A_{620nm}). Data displayed is representative of triplicate samples of n=3 replicate experiments.

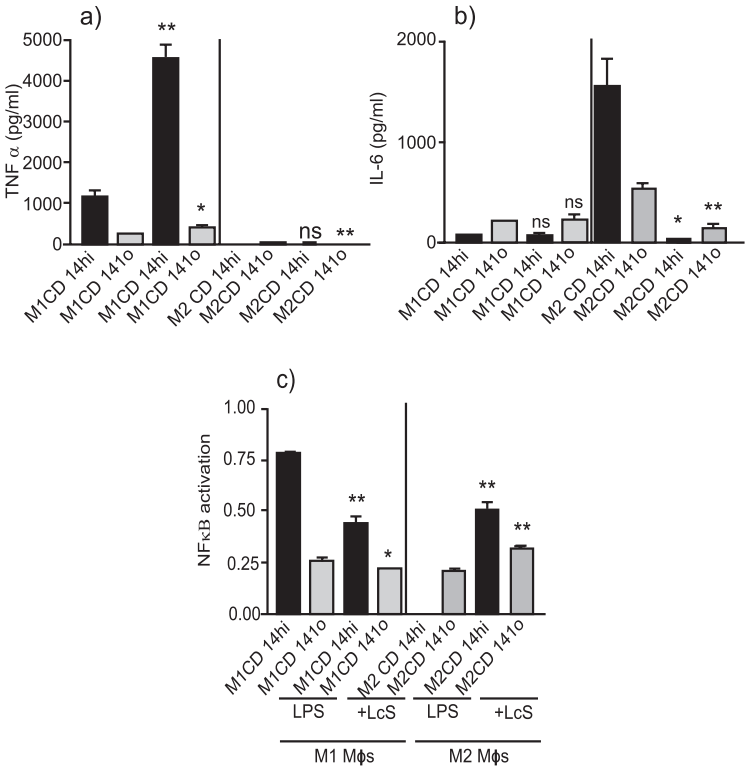
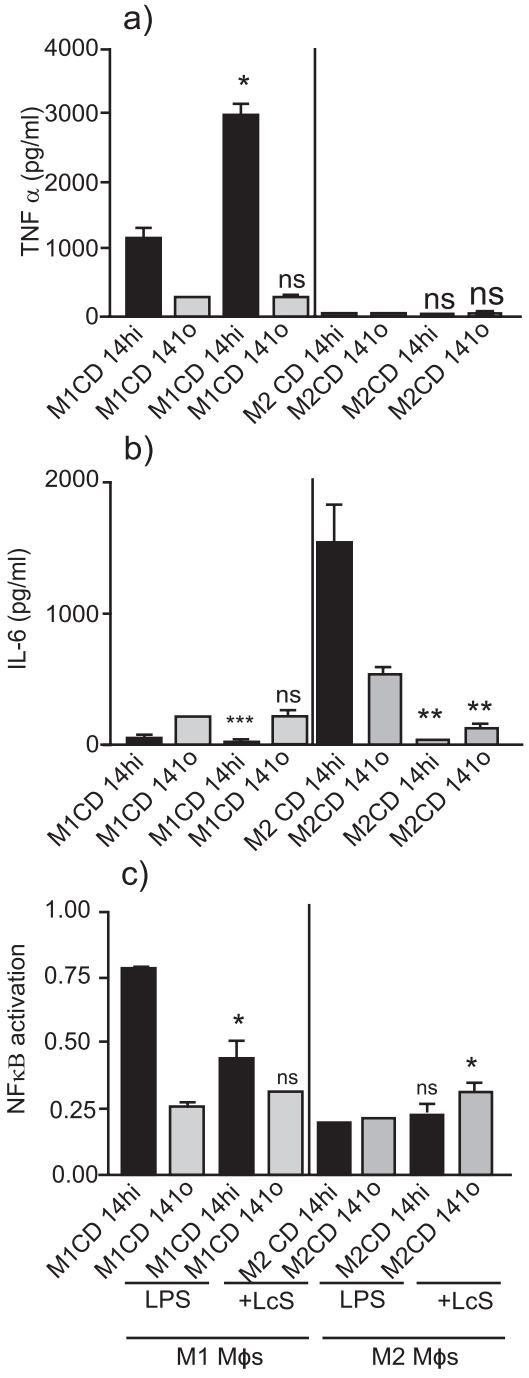


FIGURE 4. LcS secreted protein selectively modulates M1 and M2 macrophage subset production of inflammatory mediators: CD14-dependency. $CD14^{hi}$ (bold) and $CD14^{lo}$ (shaded) M1 and M2 macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1-NFkB reporter monocytes with either 25ng/ml PMA for 3 days or 10nM 1,25-$(OH)_2$ vitamin D_3 for 7 days respectively. M1 and M2 macrophages were stimulated with 100ng/ml K12-LPS in the presence (+LcS) or absence of 3μg/ml secreted protein from*Lactobacillus casei* strain Shirota. Cytokine production is expressed as the mean±SD in pg/ml for a) TNFα and b) IL-6. NFkB activity (c) is expressed as the mean±SD in arbitrary absorbance units (A_{620nm}). Data displayed is representative of triplicate samples of n=3 replicate experiments.



DISCUSSION

Lactobacillus casei strain Shirota selectively modulates pro-inflammatory cytokine production by M1 and M2 macrophage subsets. In addition, the format of LcS, whether as heat killed whole bacteria or secreted proteins from LcS bacterial cells, also displays a differential modulation of these macrophage effector phenotypes. HK-LcS differentially modulated cytokine expression that was dependent on degree of maturation and route of maturation of monocytic/macrophage cells. HK-LcS augmented monocyte LPS-induced TNFα, IL-1β and IL-8 whereas suppressed IL-6 production. M1 IL-1β and IL-8 production was augmented by HK-LcS whereas TNFα was suppressed. When investigating CD14-dependency, however, TNFα production was augmented in the pro-inflammatory infiltrating $CD14^{hi}$ M1 phenotype. In addition, HK-LcS suppressed NFkB activation in both $CD14^{hi}$ and $CD14^{lo}$ M1 macrophages. Neither IL-6 nor TNFα production was suppressed in $CD14^{hi}$ M1 macrophages. This is suggestive that HK-LcS modulatory effects on these cytokines are independent of NFkB signalling. With respect to the anti-inflammatory/regulatory macrophage subset, M2, TNFα and IL-1β production was augmented whereas IL-6 and IL-8 were suppressed. IL-6 production was suppressed in both $CD14^{hi}$ and $CD14^{lo}$ M2 macrophage subsets. Again, NFkB activation did not fully correlate with HK-LcS modulation of the pro-inflammatory cytokines; it did however, correlate with TNFα and IL-1β where an augmentation observed in TNFα and IL-1β was reproduced for NFkB activity. The augmentation of NFkB activity was observed in both $CD14^{hi}$ and $CD14^{lo}$ M2 macrophages. LcS-SP augmented LPS-induced monocyte IL-1β and IL-8 whereas suppressed TNFα and IL-6. Secreted protein augmented M1 production of IL-1β and IL-8 and suppressed M1 TNFα; in contrast, augmented $CD14^{hi}$ production of TNFα whereas $CD14^{hi}$ M1 NFkB activity was suppressed, $CD14^{lo}$ M1 cells were hardly modulated by LcS-SP with respect to both TNFα and NF-kB. IL-6 expression was suppressed by secreted protein in $CD14^{hi}$ M1s; no modulation of IL-6 was observed in $CD14^{lo}$ M1s. M2 macrophages exhibit a different modulation profile by secreted protein with respect to these pro-inflammatory cytokines. LcS-SP failed to modulate TNFα, augmented IL-1β whereas suppressed IL-6 and IL-8 production. Suppression of IL-6 production was observed for both $CD14^{hi}$ and $CD14^{lo}$ M2 macrophages. When considering NFkB activity, secreted protein partially augmented NFkB in both $CD14^{hi}$ and $CD14^{lo}$ M2 macrophages; this does not correlate to either modulation of TNFα or IL-6 but may play a role in regulation of the IL-1β response.

From preliminary investigation of the secreted protein extract, SDS-PAGE analysis showed the presence of 3 protein bands of varying molecular mass (81KD, 49KD and 26KD, data not shown). This extract could vary between probiotic strains and indeed, proteins secreted during distinct stages of

growth. Future investigations will attempt to identify the immunomodulatory protein(s) in the secreted protein extract. More recently, fractionation of a conditioned medium produced by *Lactobacillus rhamnosus* GG identified several protein bands of which, cell wall-associated hydrolase, serpin B1 and a transcriptional regulator could play a significant role in modulating host immunity (Sanchez et al., 2009). In addition, *Lactobacillus fermentum* and *L. rhamnosus* GG have been shown to secrete immunomodulatory proteins that regulate signalling pathways that drive pro-inflammatory cytokine production as well as effector function (Yan et al., 2007; Frick et al., 2007). In the case of *Lactobacillus casei* strain Shirota, heat-killed LcS exhibited similar immunomodulatory effects to that of secreted protein, suggesting that the biological effector function of LcS is shared between both the bacterial cell-associated fraction and secreted proteins. It would be fair to conclude that the Yakult strain is best used as a bacterial culture rather than substitution by a secreted, bioactive component.

Cytokines regulated in this study have been stimulated by LPS of the enteropathic bacterium, *E. coli* strain K12 which transduces its signal via TLR4 and induces cytokine expression through the mitogen activated protein kinase (MAPK) and NFkB signalling pathways. It has been established that some probiotic strains exert their effects through suppression of these pathways. Indeed, NFkB has been demonstrated to be inhibited by certain LAB (Petrof et al., 2004 and 2009). The findings of this study however, do not seem to support this suppressive role on NFkB; HK-LcS only partially suppressed M1 LPS-induced NFkB activity whereas IL-6 and TNFα production were not suppressed. In fact, TNFα produced by $CD14^{hi}$ M1 macrophages was augmented by HK-LcS; suggestive of NFkB having a suppressive role in TNFα production and/or HK-LcS modulates these pro-inflammatory cytokines in an NFkB-independent manner. M2 macrophage NFkB, like M1s, did not parallel cytokine expression; LcS augmented NFkB activity whereas suppressed IL-6 production. These data were repeated using LcS-SP. If LcS modulation of pro-inflammatory cytokine expression is independent of NFkB, two alternative pathways present themselves as integral to these LcS modulatory effects: NOD2 and MAPKs. NOD2, an intracellular pattern recognition receptor, has been demonstrated to recognise probiotic bacteria (Hasegawa et al., 2006). Specifically, it recognises a breakdown product of the bacterial cell wall component, PGN, namely muramyl dipeptide (Girardin et al., 2003). This receptor has been demonstrated to have both pro- and anti-inflammatory function, capable of activation or suppression of NFkB and MAPK activity. MAPKs have been shown to play a role in driving LPS-induced TNFα and IL-1β production (Foey et al., 1998); modulation of these cytokines by LcS may well target MAPK activity. Again, LPS induces these pro-inflammatory cytokines by activating transcription factors such as NFkB, cJun and ATF family members; LcS appears to modulate these responses in an NFkB-independent manner, then cJun and ATFs, hence JNK (Jun N-terminal Kinase) and p38 MAPKs may well represent the targets for modulation by LcS. Indeed, the probiotic *Lactobacillus reuteri* was demonstrated to suppress monocyte and macrophage TNFα production by inhibition of the MAPK-regulated transcription factors, cJun and AP-1 (Lin et al., 2008).

Probiotic bacteria are predominantly gram positive, characterised by external-facing lipoteichoic acid and exposed PGN which is accessible to host lysosomal enzymes. PGN has been described to induce macrophage production of TNFα, IL-1β and IL-6 (Gupta et al.,1995; Weidemann et al., 1994; Jin et al., 1998), all of which possess NFkB, AP-1 and CREB binding sites in their promoters. Interestingly, PGN activates AP-1 and CREB/ATF in M2-like vitamin D_3-differentiated THP-1 macrophages (Gupta et al., 1999). CREB/ATF-1 binds to the CRE (c-AMP response element) on these cytokine promoter regions; cAMP suppresses TNFα expression and, as such, binding of CREB/ATF-1 to the CRE may serve as a negative regulatory signal to pro-inflammatory cytokine production (Foey et al., 2003). In addition, cAMP has a positive effect on IL-10 production, thus it is possible that the immunomodulatory effects of probiotics may be dependent on the appropriate recognition of PGN, serving to both directly suppress pro-inflammatory cytokine expression and indirectly through induction of the anti-inflammatory cytokine, IL-10.

The cytokine profiles obtained upon stimulation are different when comparing M1 profiles to that of M2 macrophages. In addition, they demonstrate a differential regulation by LcS probiotic products. The cause of these differential responses is as a consequence of differential signalling molecules utilised during macrophage differentiation and subsequently, activation of these distinct subsets. Of particular interest were the preferential expression of IL-6 by M2 (anti-inflammatory) macrophages and the suppression of its production upon treatment with both HK-LcS and LcS-SP. In addition to its many characterised pro-inflammatory properties, IL-6 can also exhibit anti-inflammatory effects via its ability to induce suppressor of cytokine signalling proteins (SOCS) expression (Xing et al., 1998; reviewed in Heinrich et al., 2003). The regulation of SOCS and their downstream targets, STATs, plays an important role in macrophage subset differentiation and determination of effector responses. SOCS-3 is essential for function of classical M1 macrophages (Liu et al., 2008) and suppresses anti-inflammatory effectors that result from IL-10/IL-6 activation of M2 macrophages, thus controlling macrophage plasticity; knockout of SOCS3 results in an alternative M2-like phenotype with increased expression of arginase, MR, anti-inflammatory cytokines and SOCS-1. Thus, there is a reciprocal relationship between SOCS-3 and SOCS-1 regulation of STAT-3 and STAT-1-dependent genes expressed by the M2 and M1 subsets respectively. In addition to IL-6, another cytokine which is generally considered pro-inflammatory, IL-1β, also exhibits anti-inflammatory

properties; its response is probably mediated by its ability to induce IL-10 expression (Foey et al., 1998). LcS treatment of M2 macrophages augmented IL-1β production; these M2 cells generally express IL-10 that also signals through STAT-3. Conversely, pro-inflammatory TNFα, predominantly produced by the pro-inflammatory M1 macrophage phenotype, not only can induce IL-6 but can inhibit it's action via the induction of SOCS-3 expression (Bode et al., 1999). SOCS-3 induction will again affect macrophage plasticity by inhibiting STAT-3, hence reversal of M2 macrophages to a pro-inflammatory, M1 effector phenotype. Thus, probiotic modulation of cytokine effector responses mediated by SOCS/STAT interactions would have a dramatic effect on macrophage plasticity, hence effector phenotype.

The inflammatory cytokines investigated in this study (TNFα, IL-1β, IL-6 and IL-8) have all been described to be dependent on NF-kB activity (Bondeson et al., 1999). The fact that the LcS cytokine modulation profile did not always follow the NFkB activation profile suggests that there was a varying degree of NFkB-dependency, determined by CD14 expression and macrophage subset differentiation. In addition, although not directly modulating cytokine expression, NFkB may play an indirect role in cytokine expression through controlling macrophage polarisation/differentiation. The endogenous upstream activator of NFkB, IKKβ has been described to maintain the M2 macrophage phenotype, whereas deletion of IKKβ facilitated reversion to an M1 phenotype (Hagemann et al., 2008). The alternative NFkB p50 subset homodimer also controls macrophage plasticity; inhibition of p50 NFkB resulted in M2 macrophages reverting to an M1 phenotype through the restoration of STAT-1 activity (Hagemann et al., 2008; Porta et al., 2009). Taken together, there is a SOCS/STAT/NFkB/MAPK signalling axis integral to macrophage plasticity and cytokine effector phenotype. Modulation of macrophage-driven immune responses by probiotic bacteria will be dependent on regulation of this axis; the same probiotic may induce both immune activatory and immune regulatory responses, dependent on the macrophage phenotype being exhibited by the local gut mucosal macrophages responding to these bacteria.

Finally, macrophage subset expression of CD14 has a role to play in cytokine expression, NFkB activation and regulation by probiotic bacteria. HK-LcS and LcS-SP greatly augmented TNFα production by $CD14^{hi}$ M1 macrophages that exhibit a phenotype similar to infiltrating pro-inflammatory macrophages in the inflamed gut mucosa. No such dramatic up-regulation was observed either in $CD14^{lo}$ M1 or M2 macrophage subsets. This observation alone is suggestive of a cautionary approach to the use of probiotics in the case of patients with inflammatory disease that display an increased proportion of $CD14^{hi}$ M1-like macrophages infiltrating the gut mucosa. The use of such probiotics should only be considered once the macrophage effector phenotype has been characterised for the inflammatory pathology. Both HK-LcS and LcS-SP displayed little effect on both $CD14^{hi}$ and $CD14^{lo}$ M1 production of IL-6. M2 macrophages however, were dramatically regulated: LcS suppressed LPS-induced M2 production of IL-6, independent of CD14 expression. As discussed, IL-6 is both pro- and anti-inflammatory; its anti-inflammatory effects being mediated through STAT-3, a prominent transcription factor in regulating M2 macrophages. It is possible that LcS not only suppresses pro-inflammatory responses but also anti-inflammatory responses. The resulting effector outcome, with regards mucosal inflammation will then be determined by the net strength of regulatory/inflammatory mediators and their receptor-mediated responses. The most significant result obtained with respect to NFkB modulation was observed for both $CD14^{hi}$ M1 and $CD14^{hi}$ M2 macrophages when modulated by HK-LcS: suppressing M1 whereas enhanced M2 NFkB activity; this activity however, did not correlate with either TNFα or IL-6 production. This result is suggestive that these CD14-dependent observations for NF-kB activation were independent of the resulting LcS modulation of cytokine production and that other signalling pathways are important in LcS modulation of macrophage cytokine production.

In conclusion, signals induced by bacterial cell-associated and secreted protein products of the probiotic bacterium, *Lactobacillus casei* strain Shirota differentially regulate macrophage production of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL-8 as well as modulating the activity of the pro-/anti-inflammatory transcription factor, NFkB. Modulation of these cytokine profiles is determined by macrophage effector phenotype and the expression levels of CD14. The modulatory results for HK-LcS did not fully parallel those of LcS-SP, thus the cell-derived component is essential when considering design of probiotics to be used as a prophylactic in healthy individuals or for the treatment of pathological conditions. The future development of probiotics will take into consideration strain-specific effects and will be tailored to the desired effect on both the microbial population of the gut and the host's immune system.

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