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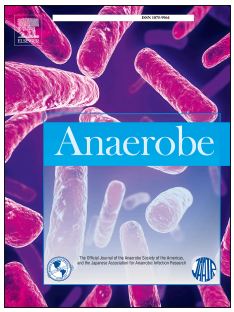

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The gut bacterium and pathobiont *Bacteroides vulgatus* activates NF-κB in a human gut epithelial cell line in a strain and growth phase dependent manner

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Short title: *B. vulgatus* modulates NF-κB in a strain and growth phase dependent manner

Abstract

The gut microbiota is increasingly implicated in the pathogenesis of Crohn's disease (CD) and ulcerative colitis (UC) although the identity of the bacteria that underpin these diseases has remained elusive. The pathobiont *Bacteroides vulgatus* has been associated with both diseases although relatively little is known about how its growth and functional activity might drive the host inflammatory response. We identified an ATP Binding Cassette (ABC) export system and lipoprotein in *B. vulgatus* ATCC 8482 and *B. vulgatus* PC510 that displayed significant sequence similarity to an NF-κB immunomodulatory regulon previously identified on a CD-derived metagenomic fosmid clone. Interestingly, the ABC export system was specifically enriched in CD subjects suggesting that it may be important for colonization and persistence in the CD gut environment. Both *B. vulgatus* ATCC 8482 and PC510 activated NF-κB in a strain and growth phase specific manner in a HT-29/kb-seap-25 enterocyte like cell line. *B. vulgatus* ATCC 8482 also activated NF-κB in Caco-2-NF-κ*Bluc* enterocyte like and LS174T-NF-κB*luc* goblet cell like cell lines and induced NF-κB-p65 subunit nuclear translocation and IL-6, IL-8, CXCL-10 and MCP-1 gene expression. Despite this, NF-κB activation was not coincident with maximal expression of the ABC exporter or lipoprotein in *B. vulgatus* PC510 suggesting that the regulon may be necessary but not sufficient for the immunomodulatory effects.

Introduction

Inflammatory Bowel Disease (IBD) including the two major disease subtypes ulcerative colitis (UC) and Crohn's disease (CD) are characterized by episodic and disabling inflammation of the gastrointestinal tract. Although the etiology of IBD is still undefined, the gut microbiota are now widely believed to contribute to the initiation and recurrence of the disease [1]. For instance, germ free animal models of IBD do not develop disease until

colonized by gut bacteria [2, 3]. Second, healthy mice develop colitis following fecal transfer from colitogenic mice, revealing that disease is transmissible via the microbiota [4]. Third, antibiotic treatment can be therapeutic in both humans and animal models of IBD [5-7] and some studies report that fecal transfers can successfully treat UC and CD in human subjects [8, 9]. Taken together these studies suggest that the gut microbiota is a key driver of IBD however the identity of the bacteria and/or bacterial factors underpinning active disease remains largely unknown.

Cross sectional studies have revealed that the gut microbiota profile differs between healthy and new onset IBD subjects [10-12] and that the gut microbiota undergoes dramatic structural alterations that are coincident with the onset of disease [13-15]. However, it is increasingly accepted that not all gut microbiota population shifts contribute to disease pathogenesis and that commensal bacteria vary significantly in their ability to initiate and sustain gut inflammation. *Bacteroides vulgatus* is one of the most abundant bacteria in the human gut where it is widely considered to be a pathobiont – a symbiont that is capable of causing pathology in response to host and/or environmental triggers. Consistent with this, recent research has revealed that *B. vulgatus* is solely responsible for driving small intestinal injury in specific pathogen free (SPF) mice lacking the CD relevant intracellular bacterial sensor *nod2* [7]. Similarly, strains of *B. vulgatus* or *Bacteroides thetaiotaomicron* induce severe colitis in SPF mice bearing genetic susceptibilities for host immunity (IL10 and SMAD3 pathways) or gut barrier function (core 1-derived *O*-glycan biosynthesis) that are relevant to human CD and UC, but interestingly, do not cause disease in mice not bearing these susceptibilities [5, 16].

B. vulgatus is characterized by important intraspecies variations with individual isolates differing in their ability to induce [5, 17] or protect against inflammation [18-20] in animal models of IBD. Notably, *B. vulgatus* is capable of invading colonic epithelial cells [21] and inducing pro-inflammatory cytokines [17, 21, 22], and tissue adherent strains are enriched in UC subjects [23] further suggesting a role for this bacterium in the pathogenesis of IBD. We recently described a CD-derived metagenomic fosmid clone encoding a putative ATP Binding Cassette (ABC) exporter and lipoprotein that activates the NF-kappa B (NF-κB) signalling pathway in a HT-29 gut epithelial enterocyte like cell line [24]. The NF-κB pathway is central to the pathogenesis of IBD and is widely targeted in an effort to decrease the frequency and severity of inflammatory episodes, and prevent progression of bowel damage [25]. In this study, we show that the ABC exporter and lipoprotein are highly conserved in *B. vulgatus* and specifically enriched in CD subjects. Interestingly, *B. vulgatus* strains ATCC8482 and PC510 activate NF-κB signaling in a strain specific and growth-phase dependent manner suggesting that a switch to an inflammogenic state may be part of the growth-related stress responses of the bacterium.

Materials and Methods

Genomic analyses of *B. vulgatus*. *B. vulgatus* isolates were identified in the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and Joint Genome Institute Integrated Microbial Genomes & Microbiomes (IMG/M, https://img.jgi.doe.gov/cgi-bin/mer/main.cgi) databases. The publicly available genome sequences of *B. vulgatus* were downloaded from the National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) database. The genome sequences of *B. vulgatus* ATCC 8482 (NCBI accession number: NC_009614), *B. vulgatus* PC510 (NCBI accession number: NZ_ADKO00000000), *B. vulgatus* CL09T03C04 (NCBI accession number:

NZ_AGXZ00000000), *B. vulgatus* 3775 SR(B) 19 (NCBI accession number: NZ_JNHJ00000000), *B. vulgatus* 3775 SL(B) 10 (iv) (NCBI accession number: NZ_JNHI00000000), *B. vulgatus* 3975 RP4 (NCBI accession number: NZ_JNHM00000000), *B. vulgatus* dnLKV7 (NCBI accession number: NZ_ASSN00000000), *B. vulgatus* 2789STDY5834842 (NCBI accession number: NZ_CYZI00000000), *B. vulgatus* 2789STDY5834897 (NCBI accession number: NZ_CZAN00000000), *B. vulgatus* 2789STDY5834944 (NCBI accession number: NZ_CZBK00000000), *Bacteroides* sp. 3_1_40A (NCBI accession number: NZ_ACRT00000000), *Bacteroides* sp. 4_3_47FAA (NCBI accession number: ACDR02000000), *B. vulgatus* mpk (NCBI accession number: NZ_CP013020) and *B. vulgatus* NLAE-zl-G202 (NCBI accession number: NZ_FOBA00000000) were downloaded from the NCBI. The extent of gene synteny in the genomic regions encoding the ABC export system and lipoprotein was assessed using Mauve [26] with genome sequences comprised of multiple contigs first aligned against the reference *B. vulgatus* ATCC 8482 genome sequence using the Mauve *Move Contigs* function.

Abundance of *B. vulgatus* genes in metagenomes. Metagenomic sequence and gene abundance data was downloaded from the Integrated Gene Catalogue (IGC) database, ([27], http://meta.genomics.cn/metagene/meta/dataTools). *B. vulgatus* PC510 coding determining sequences (CDS) were compared to IGC database using BLAST [28]. The abundance of *B. vulgatus* affiliated bacteria in the metagenomic datasets was assessed using the *hsp60* housekeeping gene [29]. The *B. vulgatus* gene counts for *hsp60*, the ABC export system and lipoprotein was assessed similarly with the best BLAST hits covering ≥ 90% of the query nucleotide sequence and having ≥ 98% sequence identity selected for further analysis and then used as a proxy to determine the respective gene counts in the 141 metagenomes. Raw gene counts were divided by the total metagenomic gene read count and then multiplied by

the total gene count of the smallest metagenome. Normalized gene read count was then $\log_2$ transformed and the counts between Healthy (H, n=59), CD (n=13) and UC (n=69) were compared between groups using a Wilcoxon test based on the underlying assumption that the $\log_2$ gene counts were not normally distributed. The *p*-values were adjusted for multiple testing using the "false discovery rate" method with $p_{adj} \leq 0.05$ considered significant.

Culture and growth experiments. *B. vulgatus* ATCC8482 [30] and PC510 [31] were cultured in Brain Heart Infusion (BHI, Difco™) broth supplemented with 10 $mg.L^{-1}$ of haemin, or LYHBHI medium [32], and each medium was buffered with mineral solutions 2 and 3 [33], under anaerobic (95% CO_2:5% H_2 headspace gasses) conditions. Bacterial cultures were routinely manipulated within a Coy vinyl anaerobic chamber with an oxygen free carbon dioxide/hydrogen (95% CO_2:5% H_2) atmosphere. Bacterial growth was measured by spectrophotometry (OD_{600nm}) using a SPECTRONIC 20D+ Spectrophotometer (ThermoFisher, Sydney).

Measurement of *B. vulgatus* immunomodulatory activities. The immunomodulatory potential of the *B. vulgatus* culture extracts was assessed using the HT-29/kb-seap-25 NF-κB enterocyte like reporter cell line [24]. Briefly, the HT-29/kb-seap-25 cell line is stably transfected with a secreted alkaline phosphatase (SEAP) reporter gene under the control of NF-κB binding elements and it also expresses a subset of TLRs (e.g. TLR3, TLR4 and TLR5) that are functionally responsive to their cognate ligands [24, 34]. We examined the immunomodulatory potential of the *B. vulgatus* strains following growth in LYHBHI medium. Briefly, three independent LYHBHI broth cultures were established from individual colonies of *B. vulgatus* ATCC8482 or PC510 (n=3 independent biological replicates per strain) and after overnight growth as described above, each individual culture

was used to inoculate 5 tubes of LYHBHI broth (n=15, consisting of n=3 independent biological replicates per strain with n=5 technical replicate for each biological replicate). The OD_{600nm} of these cultures was monitored longitudinally and a single tube from each independent series was sacrificed at early exponential, mid-exponential, early stationary and late stationary phase of growth. Cell free culture supernatant was prepared by centrifugation at 16,000g for 5 minutes and the supernatant fraction was then UV sterilized, filtered through a 0.2 μm filter and stored at -80^{o}C.

A 96-well microtiter plate was seeded with 50,000 cells per well and cultured for 24 hours. Cell free culture bacterial supernatants were added to the cultured cells (10% v/v) and the mixtures were incubated for a further 24 hours at 37^{o}C. The amount of secreted SEAP activity was quantified by *in situ* spectrophotometry (OD_{655nm}) with the QUANTI-Blue™ reagent, as directed by the manufacturer (Invivogen™) and average SEAP activity was calculated from the three independent biological replicates. The activation of NF-κB by either un-inoculated LYHBHI bacterial medium and by the NF-κB activator TNFα was also assessed. Statistically significant increases in NF-κB activation were identified using a t-test and accounting for sample variance with a $p<0.05$ considered to be significant.

We also assessed *B. vulgatus* ATCC 8482 immunomodulatory activity using our Caco-2-NF-kB*luc* enterocyte like and LS174T-NF-kB*luc* goblet cell like reporter cell lines. Briefly, Caco-2 and LS174T cells were treated with transduction medium (DMEM supplemented with 10% v/v Foetal Bovine Serum and 1% v/v Glutamax™ (Gibco) supplemented with 6μg.ml^{-1} polybrene) and 2 x 10^4 colony forming units of NF-κB Firefly Luciferase reporter lentivirus (Cellomics Technology), and centrifuged at 1200 g for 90 min at 32°C. The

transduction medium was replaced after 24 hours with complete growth medium and transduced cells were recovered following puromycin selection (2.5 $\mu g.ml^{-1}$) for 48 hours.

For the Caco-2-NF-kB*luc* and LS174T-NF-kB*luc* immunomodulatory assays, *B. vulgatus* ATCC 8482 culture supernatants were prepared essentially as described above except that two independent biological experiments were performed. Next, 96-well microtiter plates were seeded with 20,000 Caco-2-NF-kB*luc* or LS174T-NF-kB*luc* reporter cells per well in complete medium (DMEM supplemented with 1% v/v Glutamax™ (Gibco), and 5% or 10% v/v Foetal Bovine Serum respectively) and cultured for 24 hours. Then, cell free bacterial supernatants (10% v/v in complete media) was added to the cultured cells and incubated for 6 hours at 37°C. The activation of NF-κB was assessed using the Pierce™ Firefly Luc One-Step Glow Assay Kit (ThermoFisher Scientific) according to the manufacturer's instructions.

Nuclear translocation immunofluorescence assays. A 12 well-plate was seeded with 20,000 Caco-2 cells per well and cultured overnight at 37°C. Cell-free late stationary culture bacterial supernatant (10% v/v) was added to the cultured cells and incubated for 1 hour. Then, the cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilised using 0.1% Triton X-100 for 5 minutes. Cells were labelled with mouse anti-NF-κB p65 antibody (Cell Signaling Technology Inc.) for 1 hour, followed by Alexa Fluor 488 anti-mouse secondary antibody. The cells were counterstained with 4′,6-diamidino-2-phenylindole and visualized with an Olympus Confocal Microscope at 60 x magnification.

Quantitative reverse transcriptase PCR (qRT-PCR) assays. Samples of the early exponential, mid-exponential, early stationary and late stationary growth cultures were also used for RNA extraction and measurement of the expression of the *B. vulgatus* PC510

CUU_1434 (lipoprotein$_{Bv510}$) and CUU_3451 (permease$_{Bv510}$) and the *B. vulgatus* ATCC8482 BVU_2810 (lipoprotein$_{Bv8482}$) and BVU_3172 (permease$_{Bv8482}$) by qRT-PCR. Primers targeting both lipoprotein$_{Bv510}$ and lipoprotein$_{Bv8482}$ (P_f 5′ CTAATAGTAACTATGTGATTG; P_r 5′ CTGTCTTTGGTTGCAGTTTCC), permease$_{Bv510}$ and permease$_{Bv8482}$ (P_f 5′ GCCTGGCGCTTCTTAATGCG; P_r 5′ TACGCTATGTGCATCCACCG) and total bacteria [35] were used for relative quantitation using the Q-gene software application [36]. Total RNA was recovered from the cell pellets using the QIAGEN RNeasey® Mini Kit, except that the cells were lysed with acid washed beads using a BioSpec Mini-Beadbeater-16 Homogenizer at maximum speed for 1 minute; followed by on-column DNase digestion as recommended by the manufacturers. The presence of any residual DNA was assessed by PCR, using primers that target the 16S rRNA gene (27F and 1492R, [37]) and once confirmed to be DNA free, RNA quality, quantity and integrity was assessed using an Agilent 2100 Bioanalyzer. Next, cDNA was produced with the SuperScript® III Double-Strand cDNA Synthesis Kit (Life Technologies™). Real time PCR analysis was carried out using SYBR Green Master Mix (Applied Biosystems®) and an ABI Prism 7700 (Applied Biosystems®). Gene expression of the lipoprotein/permease was expressed as the ratio of lipoprotein/permease gene copy number (GCN) per 16S rRNA GCN.

To quantify the effect of *B. vulgatus* ATCC 8482 late stationary phase culture supernatants on NF-κB regulated cytokine gene expression a 12-well plate was seeded with 20,000 Caco-2 cells per well and cultured for two weeks to generate a confluent monolayer. The cells were then treated with late stationary phase *B. vulgatus* culture supernatants (10% v/v) for 6 hours at 37°C. Total RNA was recovered from the cells using an RNeasy® Mini Kit (QIAGEN) and cDNA was synthesised using an iScript™ Reverse Transcription Supermix for RT-qPCR kit (Bio-Rad), as per the manufacturer's instructions. Real time quantitative PCR was carried

out using SYBR Green Master Mix (Applied Biosystems®) and the primers for IL-6 (P_f 5′ CGCCTTCGGTCCAGTTG; P_r 5′ ATGCAGGTACAGCGTACAGT), IL-8 (P_f 5′ ACTCCAAACCTTTCCACCC; P_r 5′ CCCTCTTCAAAAACTTCTCCAC), CXCL10 (P_f 5′ AGCAGAGGAACCTCCAGTCT; P_r 5′ TGTGGTCCATCCTTGGAAGC) and MCP-1 (P_f 5′ AGTCTCTGCCGCCCTTCT; P_r 5′ GTGACTGGGGCATTGATTG). Real-time RT-PCR threshold values (Ct) were normalised to the GAPDH housekeeping gene (P_f 5′ TGCACCACCACCTGCTTAGC; P_r 5′ GGCATGGACTGTGGTCATGAG) and used to calculate the fold change in gene expression relative to unstimulated cells.

Quantification of short chained fatty acid (SCFA) production. A 2 ml culture of *B. vulgatus* PC510 or ATCC8482 was centrifuged at 15,000 g for 15 minutes and 800 µl of cell free supernatant was transferred in duplicate to a fresh microfuge tube. Then 80 µl of an orthophosphoric acid/internal standard solution (20% meta-phosphoric acid/0·24% 4-methyl valeric acid) was added and the sample was mixed thoroughly. The concentrations of acetate, and propionate were determined as previously described with 4-methyl valerate used as an internal standard [38]. Succinate concentration was determined by ion exchange HPLC using a Dionex UltiMate® 3000 fitted with a Dionex Acclaim Organic Acid Analytical Column at 30ºC using a 50 mM NaH_2PO_4 pH 2.7 mobile phase at 0.6 ml.min^{-1} for 7 minutes. The succinate peak was identified and quantified using the Chromeleon software (Dionex).

Results

A putative NF-κB immunomodulatory regulon is highly conserved in *B. vulgatus*. Lakhdari et al., [24] recently identified a metagenomic fosmid clone (52B7) derived from a CD subject that exerts NF-κB modulatory activity in an *E. coli* host. The cloned DNA from 52B7 is predicted to be derived from a *Bacteroides* sp., and activity is attributable to a

permease component of an ABC transport efflux system and a distally encoded lipoprotein. We identified similar genes in both *B. vulgatus* ATCC8482 (89% amino acid similarity to BVU_2810 (lipoprotein$_{Bv8482}$) and 99% amino acid similarity to BVU_3172 (permease$_{Bv8482}$) and a recent fecal isolate recovered from a healthy Australian subject *B. vulgatus* PC510 (92% amino acid similarity to CUU_1434 (lipoprotein$_{Bv510}$) and 99% amino acid similarity to CUU_3451 (permease$_{Bv510}$)) and confirmed that the ABC export system is highly conserved in these strains (Fig 1). Further analysis revealed that the ABC export system is conserved across the 12 other publicly available genome sequences of *B. vulgatus* isolates recovered from the human (*B. vulgatus* CL09T03C04, *B. vulgatus* 3775 SR(B) 19, *B. vulgatus* 3775 SL(B) 10(iv), *B. vulgatus* 3975 RP4, *B. vulgatus* dnLKV7, *B. vulgatus* 2789STDY5834842, *B. vulgatus* 2789STDY5834897, *B. vulgatus* 2789STDY5834944, *Bacteroides* sp. 3_1_40A, *Bacteroides* sp. 4_3_47FAA), mouse (*B. vulgatus* mpk) and goat (*B. vulgatus* NLAE-zl-G202) gut. The permease$_{Bv510}$ is conserved in closely related *Bacteroides* spp. (e.g. *Bacteroides dorei* CL02T00C15 (97% amino acid similarity to permease$_{Bv510}$) and *Bacteroides massiliensis* dnLKV3 (86% amino acid similarity to permease$_{Bv510}$) and more distantly related *Bacteroides* spp. (e.g. *Bacteroides fragilis* str. 3725 D9 ii (63% amino acid similarity to permease$_{Bv510}$), *Bacteroides uniformis* 3978 T3 i (53% amino acid similarity to permease$_{Bv510}$)). As expected, these strains encode ABC export systems although these are characterized by variations in gene organization suggesting that they may fulfil different functional roles (Fig 1). In contrast, the lipoprotein is less highly conserved in *B. vulgatus* strains (n=9 of 14 publicly available *B. vulgatus* isolate genomes) and is also found in select *B. dorei* strains but not more distantly related *Bacteroides* spp.

The ABC export system is enriched in CD metagenomes. Next, we determined the abundance of *B. vulgatus* affiliated bacteria and the ABC export system in H, CD and UC

subjects using the IGC database. The abundance of *B. vulgatus* affiliated bacteria was comparable in H, CD and UC metagenomes as assessed using *hsp60*. In contrast, except for CUU_3451 (putative permease), the genes encoding the putative ABC export system (CUU_3446 to CUU_3455) were significantly enriched in CD as compared to H metagenomes (Fig 2, $p_{adj} \leq 0.05$). Notably, these genes were also more abundant in CD as compared to UC metagenomes ($p_{adj} \leq 0.05$). Finally, the abundance of the lipoprotein (CUU_1434) was relatively low and similar in all the metagenomes analyzed (Fig 2, $p_{adj} \geq 0.05$).

***B. vulgatus* exhibits growth-phase dependent NF-κB immunomodulatory activity.** The NF-κB modulatory activity of 52B7 is primarily associated with the supernatant fraction with only weak activity associated with the cell lysate fraction [24]. Based on this observation we assessed the ability of *B. vulgatus* ATCC 8482 and *B. vulgatus* PC510 culture supernatants to modulate activation of NF-κB using the HT-29/kb-seap-25 reporter cell line [24]. Analysis of culture supernatants harvested from early exponential, mid-exponential, early stationary or late stationary growth phase revealed that both strains are capable of modulating NF-κB activation but that the modulatory effect is characterized by growth-phase dependent intraspecies variations (Fig 3A). Both strains exhibited immunomodulatory activity in stationary phase and in particular, the activity of *B. vulgatus* PC510 at early stationary phase was significantly higher than that of the LYHBHI medium or supernatant harvested from early exponential, mid-exponential or late stationary phase ($p<0.0001$) whereas the activity of *B. vulgatus* ATCC 8482 at late stationary phase was significantly higher than that of the LYHBHI medium or supernatant harvested from any of the preceding time points (Fig 3A, $p<0.0001$).

We further explored these findings using *B. vulgatus* ATCC 8482 and our Caco-2-NF-κB*luc* and LS174T-NF-κB*luc* reporter cell lines. As expected, only late stationary phase culture supernatants activated NF-κB in Caco-2-NF-κB*luc* (Figure 3B, $p<0.01$) and LS174T-NF-κB*luc* (Figure 3C, $p<0.05$). The extent of activation was modest using the LS174T cell line suggesting it has a higher threshold for activation than the HT-29 and Caco-2 cell lines possibly due to the higher level of mucin production. Consistent with this, the addition of 20% v/v culture supernatant from late stationary phase cultures resulted in significant increase in NF-κB activation in the LS174T-NF-κB*luc* cell line (Figure 3C, $p<0.0001$).

***B. vulgatus* ATCC 8482 late stationary phase culture supernatant induces NF-κB-p65 subunit nuclear translocation and cytokine expression.** We next examined the ability of *B. vulgatus* ATCC 8482 late stationary phase culture supernatant to induce NF-κB-p65 subunit nuclear translocation. As expected, IL-1β and *B. vulgatus* late stationary phase culture supernatant but not sterile culture medium resulted in NF-κB-p65 subunit nuclear translocation (Fig 4A). Consistent with this *B. vulgatus* late stationary phase culture supernatant induced expression of the NF-κB regulated cytokines IL-6, IL-8, CXCL-10 and MCP-1 (Fig 4B, $p<0.05$).

The *B. vulgatus* permease and lipoprotein are maximally expressed in late stationary phase. We next examined the expression of the permease and lipoprotein genes by qRT-PCR using matched *B. vulgatus* cells harvested from early exponential, mid-exponential, early stationary or late stationary phase cultures. Our analyses revealed that there was low level expression of the *B. vulgatus* ATCC8482 $\text{permease}_{\text{Bv8482}}$ and $\text{lipoprotein}_{\text{Bv8482}}$ in early exponential, mid-exponential and early stationary phase followed by a significant increase in expression from early to late stationary phase (Fig 5, $p<0.05$) which coincided with NF-κB

modulatory activity. The *B. vulgatus* PC510 permease$_{Bv510}$ was expressed at all-time points with a significant increase in expression occurring from early to late stationary phase (Fig 5, $p<0.05$). This was also associated with a gradual increase in the expression of the lipoprotein$_{Bv510}$ with a similar significant increase in expression occurring from early to late stationary phase (Fig 5, $p<0.05$) although this was not coincident with NF-κB modulatory activity.

***B. vulgatus* PC510 activates NF-κB independently of SCFA production.** *B. vulgatus* produces several short chain fatty acids (SCFA) that have previously been shown to modulate NF-κB activation [34]. Both *B. vulgatus* ATCC 8482 and *B. vulgatus* PC510 produced acetate, propionate, and succinate when grown in LYHBHI and there was a gradual accumulation of these SCFAs up to a maximum at late stationary phase (Table 1). However, as the culture supernatants are diluted approximately 10-fold for the immunomodulatory assays the concentrations of acetate and propionate are below the threshold necessary to induce NF-κB activation in HT-29 cells (≥6mM for acetate, ≥2mM for propionate [34]) at all-time points. In contrast, the concentration of succinate in late stationary phase is above the threshold necessary to induce NF-κB activation (≥6mM succinate [34]) although this was not coincident with NF-κB activation by *B. vulgatus* PC510 suggesting that the effect is mediated via a SCFA independent process.

Discussion

B. vulgatus is one of the most abundant and prevalent bacteria in the human gut [39-44] where it is considered to be a member of the healthy human core gut microbiota [45, 46]. While there have been contradictory reports on its relative abundance in CD (e.g. [47, 48]) and UC subjects (e.g. [11, 49] it appears that *B. vulgatus* is also capable of colonizing and

349 persisting in the dysbiotic IBD gut environment. To better understand this dichotomy, we
350 examined the available *B. vulgatus* genome sequences and identified a putative regulon,
351 comprised of an ABC export system and lipoprotein, which is similar to an
352 immunomodulatory regulon previously identified on a CD derived metagenomic fosmid
353 clone. Furthermore, we determined that the ability of *B. vulgatus* to modulate NF-κB is
354 strain and growth phase dependent suggesting that this capability may be regulated in
355 response to environmental stressors affecting bacterial growth.
356
357 We previously demonstrated that spent culture supernatant prepared from an *E. coli* host
358 carrying 52B7 activated NF-κB in a gut epithelial cell line which is consistent with the
359 putative efflux function assigned to the ABC transport system [24]. Similarly, to fosmid
360 52B7 we detected NF-κB modulatory activity in spent culture supernatant prepared from two
361 independent strains of *B. vulgatus*. The extent of activation was less than previously reported
362 for *E. coli* carrying 52B7 although this might be related to the heterologous genetic
363 background of this host [24]. Activation of NF-κB was growth phase dependent and
364 interestingly the immunomodulatory activity of *B. vulgatus* PC510 was not coincident with
365 the maximum expression of the permease and lipoprotein, or with the production of several
366 SCFA known to affect NF-κB activity, suggesting that these factors may be necessary but not
367 sufficient for immunomodulatory activity. For instance, *B. vulgatus* ATCC 8482 also
368 produces an *N*-acyl-amide termed commendamide that activates NF-κB via a G-protein-
369 coupled receptor termed G2A (GPR132) that has been implicated in autoimmunity and
370 atherosclerosis [50]. While we observed a high degree of concordance between the
371 individual cell lines it is notable that previous studies reported that HT-29 cells were
372 unresponsive to *B. vulgatus* stimulation [34, 51]. However, this may not be surprising
373 considering that our results suggest that this activity is strain dependent and tightly associated

with cell growth. Critically, activation by *B. vulgatus* PC510 was detectable in early but not late stationary phase cultures suggesting that NF-κB modulation was likely due to secretion of a specific factor and not to the general release of microbiota-associated molecular patterns into the supernatant by stationary phase cultures.

It is well recognized that several gut pathogens initiate an inflammatory as it confers them with a competitive advantage in the gut environment. It remains to be determined whether specific *B. vulgatus* strains behave in a similar manner however in addition to a potential pathogenic role *B. vulgatus* may also activate NF-κB to establish a tolerogenic relationship with the host. For instance, a *B. vulgatus* strain isolated from a guinea pig with carrageenan induced colitis activated NF-κB in intestinal epithelial cells *in vitro* and *in vivo* [52, 53] however concomitant induction of TGF-β1 production by lamina propria mononuclear cells inhibited NF-κB recruitment to the promoter of the pro-inflammatory cytokine IL-6 in the intestinal epithelial cells [53]. Similarly, *B. vulgatus* mpk but not *E. coli* was able to induce a semi-mature lamina propria dendritic cell phenotype, which may prevent subsequent T-cell polarization, by triggering IL-6 secretion in the absence of pro-inflammatory cytokines such as IL-12 or TNFα [54]. IBD is characterized by a loss of tolerance to the gut microbiota and *B. vulgatus* may induce both pro and anti-inflammatory pathways *in vivo* to maintain a tolerogenic relationship with the host. It remains to be seen whether this capacity is characterized by intraspecies variations and how it might influence IBD risk.

The ability of individual bacterial strains to persist in the gut is supported by their capacity to rapidly respond and adjust their growth rate to changed environmental conditions. For *B. vulgatus* these adaptations may also be associated with effects on gene expression that affect its interaction(s) with the host and the inflammatory responses leading to IBD. Consistent

399 with this Sato et al., [23] revealed that the human gut is colonized by multiple strains of *B.*
400 *vulgatus* and that tissue adherent strains are enriched in UC subjects. Furthermore, Setoyama
401 et al., [55] previously demonstrated that *B. vulgatus* strains vary in their ability to drive
402 colitis. Both CD and UC are characterized by a high rate of discordance amongst
403 monozygotic twins (>60% for CD and >80% for UC [56]) and it is known that the gut
404 microbiota of monozygotic twins differs at the strain level [57]. This raises the intriguing
405 possibility that intraspecies variations in the strains colonizing healthy and IBD siblings could
406 influence disease risk.
407
408 Interestingly, while our data suggests that the ABC export system may not be sufficient for
409 NF-κB activation these genes are more abundant in CD associated metagenomes suggesting
410 that they are required for *B. vulgatus* to successfully colonize and persist in this gut
411 environment. Unfortunately, *B. vulgatus* is reported to be recalcitrant to genetic
412 characterization [58, 59] however a new approach termed metaparental mating [60] may
413 provide new opportunities to recover genetically tractable isolates thus enabling the
414 importance of the ABC export system for gut colonization to be assessed by reverse or
415 forward genetic methods. The genome sequences of *B. vulgatus* ATCC8482, PC510 and
416 mpk have been published [30, 31, 61], the sequences for several other human and animal
417 derived strains are available in the public databases and a further three genomes have been
418 sequenced but not yet publicly released (*B. vulgatus* RJ2H1 (IMG Submission ID:
419 2510065017), *B. vulgatus* RJ2L3 (IMG Submission ID: 2510065018) and *B. vulgatus* 274-
420 1D4 (IMG Genome ID: 2503754051)). Taken together, we anticipate that an integrated
421 approach combining functional and genomic dissections will provide new insights into the
422 gene repertoire necessary to support gut colonization and persistence, and influence host
423 health.

Conclusions

The immunomodulatory capacity of *B. vulgatus* is characterized by important intraspecies variations although whether there is a "switch" that transforms the bacterium into a pathogenic state remains to be determined. A deeper understanding of the intraspecies variations and triggers underpinning this switch may provide new insights into the pathogenesis of inflammatory bowel disease and enable a more sensitive diagnosis and/or management of disease.

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Author Contributions

PÓC, TW, SM, HB, JB and MM conceived and designed the experiments; PÓC and SM performed the (meta)genome analyses; PÓC and WJS grew *B. vulgatus* and prepared the samples for analysis; TW, RG, JB and PÓC performed the immunomodulatory experiments;

PÓC, TW, RG, SM, HB, JB and MM analyzed the data, and; PÓC wrote the manuscript with TW, RG, SM, HB, JB and MM.

Competing Interest

The authors declare no competing interest.

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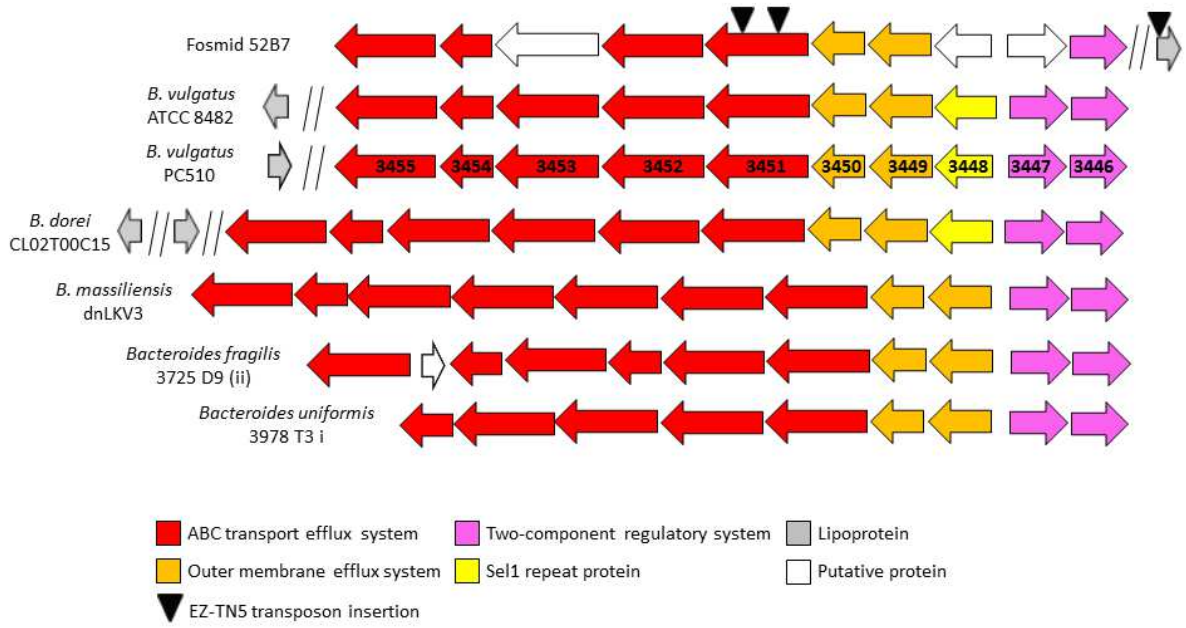


637

638 **Fig 1: Organization of the genes encoding the putative ABC export system and**

639 **lipoprotein from fosmid 52B7 and *Bacteroides* spp.** The locations of the transposon

640 insertions that abrogate NF-κB activation in fosmid 52B7 are indicated (closed inverted

641 triangles). The genes encoding the *B. vulgatus* PC510 ABC export system (CUU_3446 –

642 CUU_3455) are indicated. We also identified several genes (indicated as putative proteins)

643 that appear to contain premature translational stop codons in the putative ABC transport

644 efflux system of 52B7.

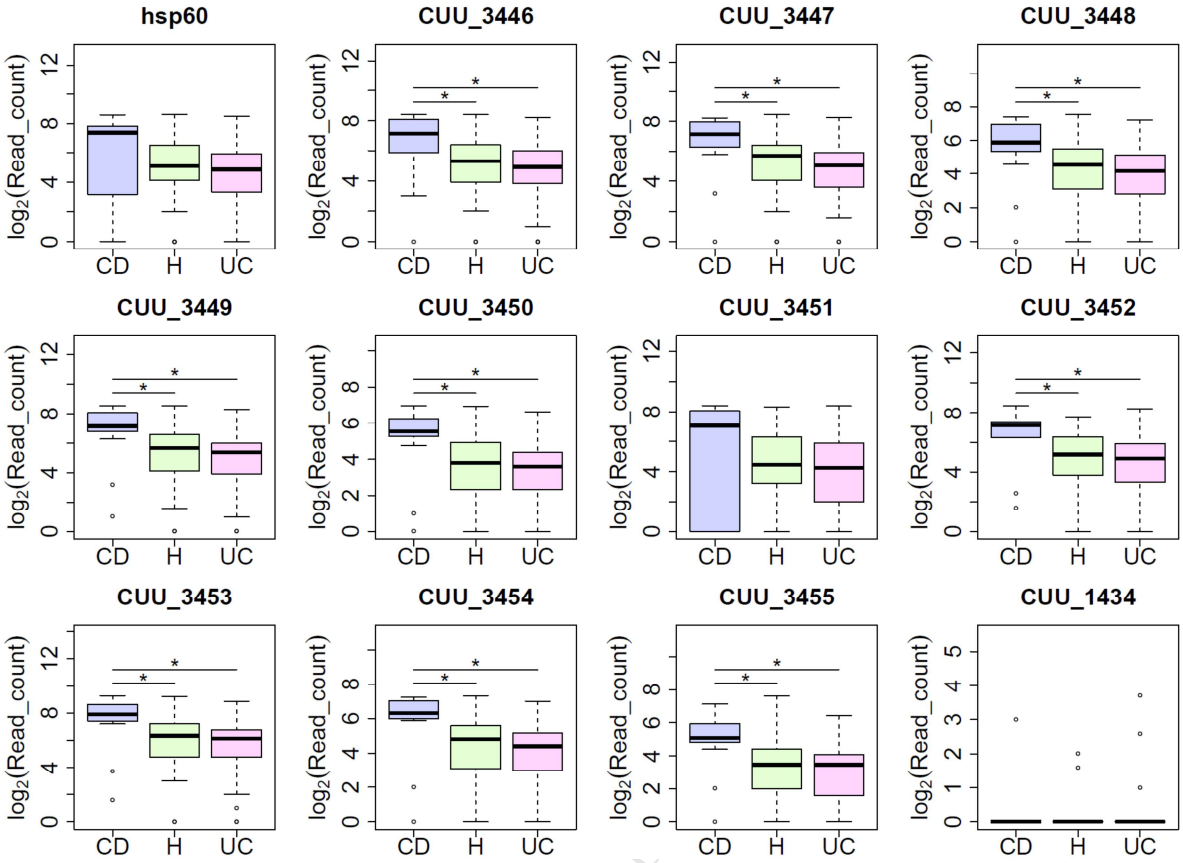
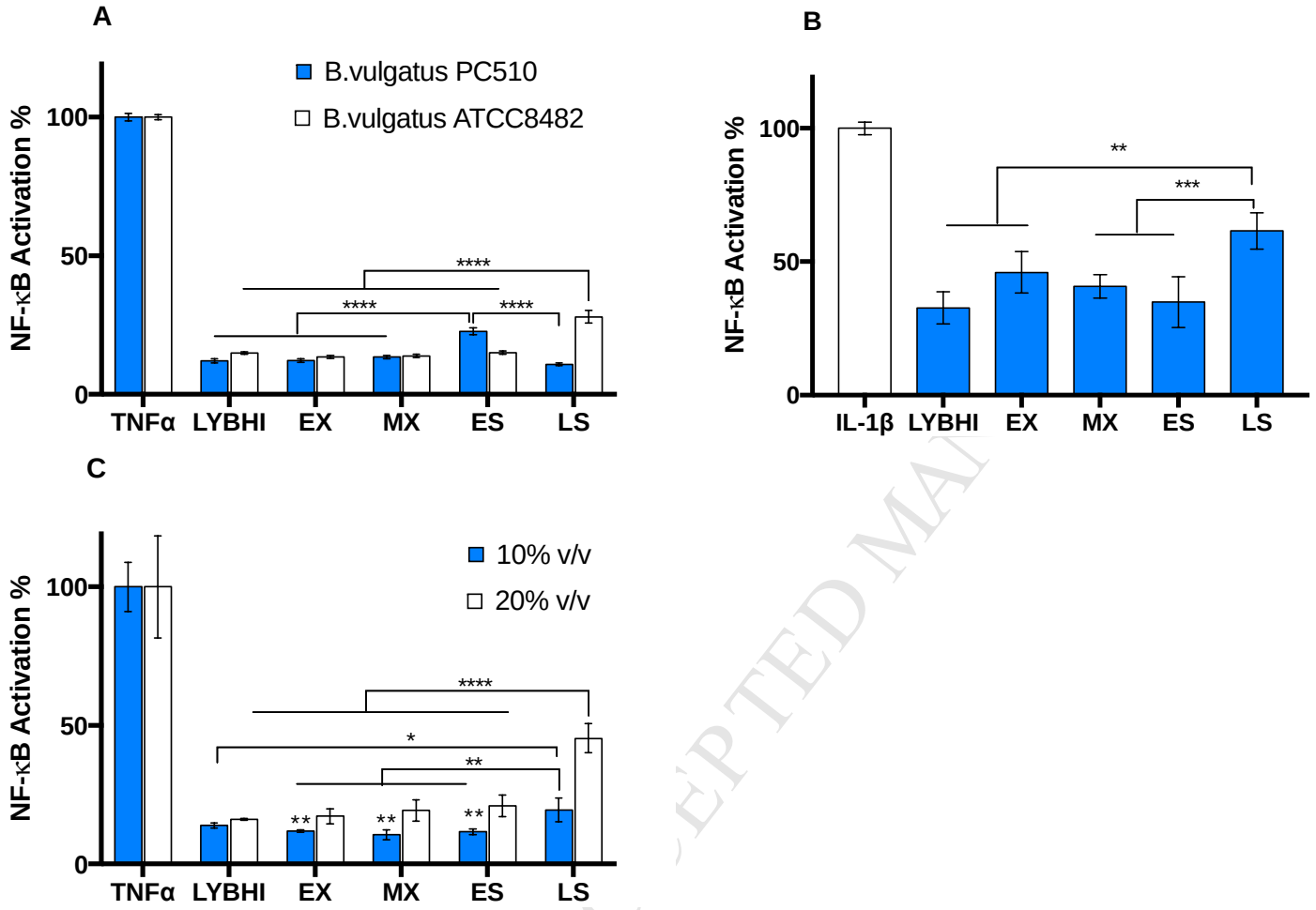


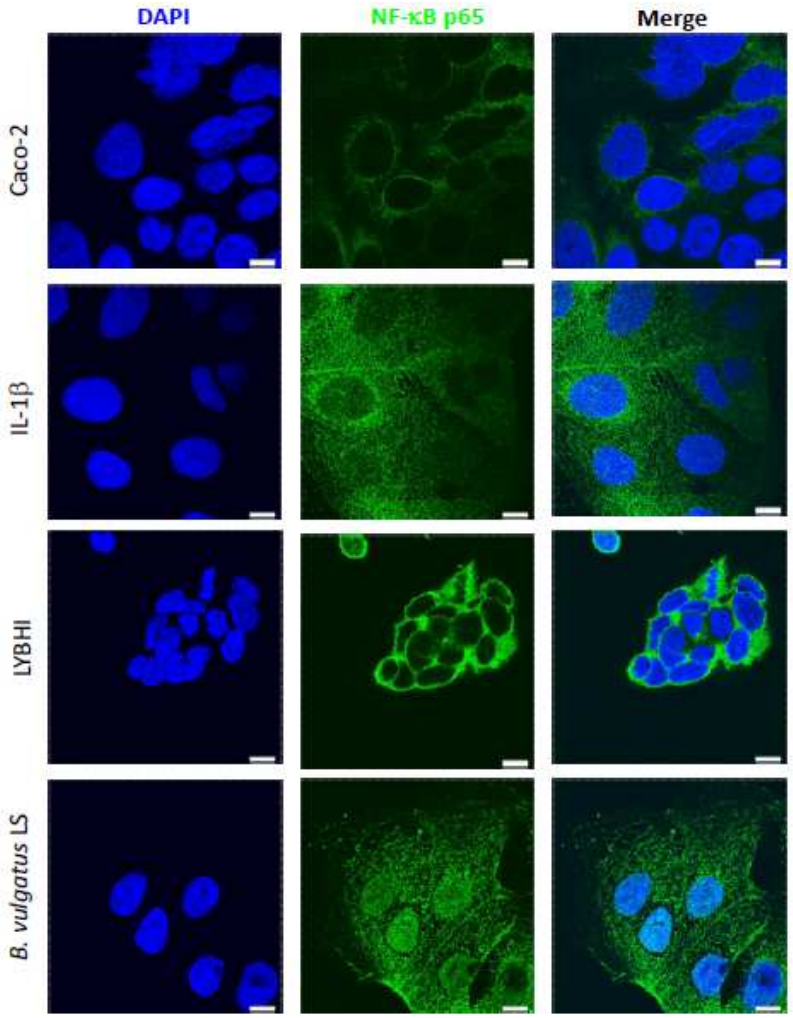
Fig 2: Abundance of *B. vulgatus* PC510 ABC export system in H, CD and UC subjects. Boxplot depicting the log2 transformed metagenomic read count of *hsp60*, and the genes encoding the ABC export system and lipoprotein in healthy (H), Crohn's disease (CD) and ulcerative colitis (UC) fecal metagenomes. * p_{adj}-value ≤0.05.

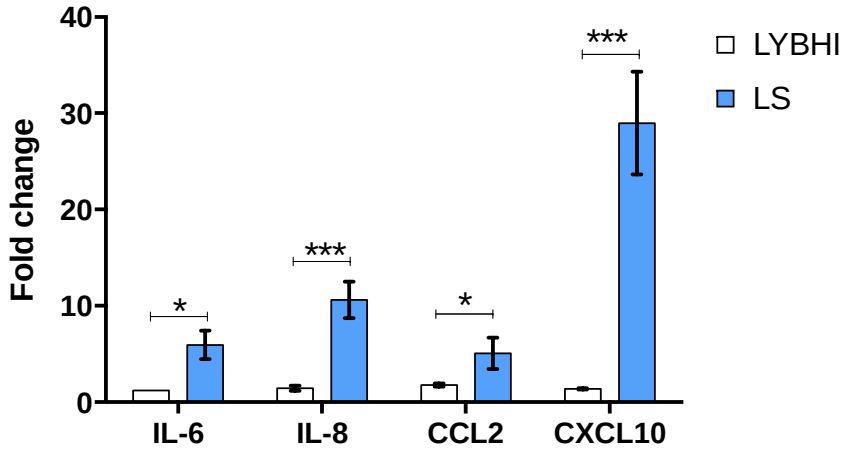


665 **Fig 3A: *B. vulgatus* mediated NF-κB modulation is characterized by intraspecies variations and growth phase dependency.** The effects

666 of the culture supernatants on NF-κB activation in the HT-29/kb-seap-25 cell line were measured by quantification of SEAP reporter gene

activity using the QUANTI-Blue™ reagent and average SEAP activity was calculated from three independent experiments. NF-κB reporter gene activation (NF-κB activity (OD_{655nm})) is expressed as the optical density at 655 nm (OD_{655nm}) as assessed following stimulation for 24 hours. Baseline activation of the reporter gene was assessed using un-inoculated bacterial culture medium (LYHBHI) and *B. vulgatus* PC510 (ES) and *B. vulgatus* ATCC8482 late stationary (LS) supernatants that exhibit significantly higher activity compared to the un-inoculated LYHBHI medium and other experimental time points (early exponential (EX), mid-exponential (MX), early stationary (ES)) are indicated ($p<0.001$). **B.** The effects of the culture supernatants on NF-κB activation in the Caco-2-NF-κB*luc* reporter cell line were measured using luciferase assay. Average NF-κB activation was accessed following 6 hours stimulation and baseline activation of the reporter gene was assessed using un-inoculated LYHBHI bacterial culture medium. *B. vulgatus* ATCC8482 late stationary (LS) culture supernatants exhibit significantly higher activity compared to the un-inoculated LYBHI medium on the Caco-2 reporter cell line ($p<0.05$). **C.** *B. vulgatus* ATCC8482 late stationary (LS) culture supernatants significantly increase NF-κB activation on LS174T-NF-□B*luc* reporter cell line in dose response manner (10% and 20% respectively) (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

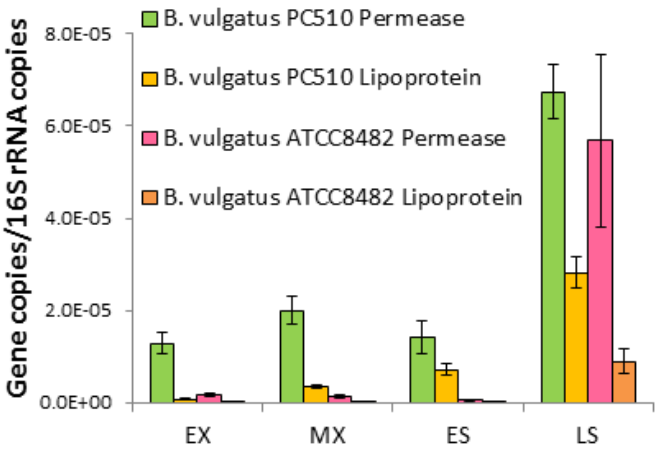




693 **Fig 4A.** ***B. vulgatus*** **ATCC8482 late stationary phase culture supernatant induces NF-κB-p65 subunit nuclear translocation.** Caco-2

694 cells were stimulated with either IL-1β, LYBHI medium or *B. vulgatus* ATCC 8482 late stationary culture supernatants for 1 hour. The cell

nuclei are indicated in blue while the NF-κB-p65 subunit is indicated in green. The cell nuclei in the central panels are largely black for unstimulated Caco-2 cells and cells stimulated with LYHBHI indicating little NF-κB-p65 nuclear translocation. In contrast, IL-1β and *B. vulgatus* LS culture supernatant results in NF-κB-p65 nuclear translocation as revealed by green staining of the nuclei. Scale bars represents 10μm. **B.** *B. vulgatus* ATCC8482 late stationary phase culture supernatant induce expression of NF-κB-p65 dependent cytokines. Caco-2 cell monolayers were treated with late stationary *B. vulgatus* ATCC8482 (10% v/v) culture supernatants or LYBHI medium for 6 hours. The expression of IL-6, IL-8, CCL2 and CXCL10 was assessed by quantitative RT-PCR. Data are normalized to GAPDH and presented as fold-change relative to unstimulated cells. *B. vulgatus* LS supernatant increased the expression of pro-inflammatory cytokines IL-6 ($p<0.05$), IL-8 ($p<0.001$), CCL2 ($p<0.05$) and CXCL10 (*$p<0.05$, ***$p<0.001$).



703

704 **Fig 5. Maximal expression of the *B. vulgatus* ATCC8482 but not PC510 lipoprotein and**
705 **permease genes is coincident with immunomodulatory activity at late stationary phase.**
706 The extent of gene expression (Gene copies/16S rRNA gene copies) is expressed as the ratio
707 of gene copies of the lipoprotein or permease per copy of the 16S rRNA gene. There is a
708 significant increase in gene expression following the transition from ES to LS phase
709 (p<0.05). Early exponential (EX), mid-exponential (MX), early stationary (ES) and late
710 stationary (LS).

	Early exponential phase		Mid exponential phase		Early stationary phase		Late stationary phase	
SCFA	*B. vulgatus*		*B. vulgatus*		*B. vulgatus*		*B. vulgatus*	
	8482	PC510	8482	PC510	8482	PC510	8482	PC510
AcOH	2.45±0.20	0.52±0.26	1.93±0.08	2.24±0.19*	3.72±0.13*	8.21±0.37*	11.53±0.19*	12.76±0.17*
Pr	0.22±0.09	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.78±0.08*	13.08±0.99*	9.00±0.12*
Suc	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.38±0.28	55.92±1.53*	167.61±2.34*	80.63±4.68*

Table 1. SCFA production (mM) by *B. vulgatus* ATCC8482 and *B. vulgatus* PC510 grown in LYHBHI. *Statistically significant (p<0.05) change in SCFA concentration compared to the previous timepoint of the same strain. AcOH – Acetate; Pr – Propionate; Suc – Succinate

Highlights for Review

- The pathobiont *Bacteroides vulgatus* has been implicated in the etiology of both Crohn's disease (CD) and ulcerative colitis although, relatively little is known about how its growth and functional activity might drive the host inflammatory response.
- We identified an ATP Binding Cassette (ABC) export system and lipoprotein in *B. vulgatus* ATCC 8482 and *B. vulgatus* PC510 that displayed significant sequence similarity to an NF-κB immunomodulatory regulon previously identified on a CD-derived metagenomic fosmid.
- The ABC export system was specifically enriched in CD subjects suggesting that it may be important for colonization and persistence in the CD gut environment.
- Both *B. vulgatus* ATCC 8482 and PC510 activated an NF-κB responsive reporter gene in gut epithelial enterocyte and goblet cell culture line in a strain and growth phase specific manner. Consistent with this, *B. vulgatus* ATCC 8482 also induced NF-κB-p65 subunit nuclear translocation and expression of IL-6, IL-8, CXCL-10 and MCP-1.
- However, NF-κB reporter gene activation was not coincident with maximal expression of the ABC exporter or lipoprotein in *B. vulgatus* PC510.