PROBIOTIC BACTERIAL DNA INDUCES INTERLEUKIN-10 PRODUCTION BY HUMAN DENDRITIC CELLS VIA TOLL-LIKE RECEPTOR-9

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INTRODUCTION

Interactions between the host and the microflora play a crucial role in mucosal immune homeostasis. A disturbance of this flora, or the host response to it, contributes to the pathogenesis of inflammatory bowel diseases (IBD) (Sartor, 2001). This has led to therapeutic approaches to modify the microflora with probiotic bacteria (Campieri and Gionchetti, 1999; Gionchetti et al., 2005; Hart et al., 2003). One such a probiotic preparation is VSL#3 (VSL#3 Pharmaceuticals, Ft. Lauderdale, FL, USA), a highly concentrated mixture of eight live bacterial strains (4 lactobacilli, 3 bifidobacteria and 1 Streptococcus thermophilus). VSL#3 is effective in the treatment of IBD in placebo-controlled clinical trials (Gionchetti et al., 2000; Gionchetti et al., 2003; Mimura et al., 2004).

Irradiated VSL#3 bacteria are as effective in ameliorating experimental colitis as the viable organisms. In addition, Toll-like receptor 9 (TLR9) signalling mediated the resolution of intestinal inflammation, as the beneficial effects of the VSL#3 probiotics were abolished in the presence of chloroquine, an inhibitor of TLR9 signalling, or in TLR9 deficient mice (Rachmilewitz et al., 2004). Since bacterial DNA, which contains unmethylated CpG dinucleotides (CpG motifs), activates innate immunity by binding to TLR9 (Hemmi et al., 2000; Krieg, 2002), these observations suggest that probiotic bacteria may operate via the effects of bacterial DNA on TLR9.

Dendritic cells (DC) recognize and respond to bacterial products and express pattern recognition receptors, including TLR9. DC activate naïve T cells and shape the developing T cell response into a Th1, Th2 or Th3/Tr1 response (Banchereau and Steinman, 1998). In part, these different outcomes are influenced by exposure of DC to microbial products. Production of interleukin (IL)-10 by DC may favour...
the generation of regulatory T cells and IgA-producing B cells (Akbari et al., 2001), while production of IL-12 by DC polarizes a proinflammatory Th1 response (Iwasaki and Kelsall, 1999). The regulatory role of DC is of particular importance in the intestine where the mucosal immune system is in close association with the external environment (Stagg et al., 2003).

The amount of bacterial DNA present within the human intestine is unknown, but it is likely that DC sample bacterial DNA, as they do other bacterial products, either indirectly via M cells or directly by reaching between epithelial cells into the gut lumen (Rescigno et al., 2001). Microbial products, including bacterial DNA, have been detected in the spleen (Rachmilewitz et al., 2004) and in the draining lymph nodes in both experimental colitis and in healthy humans (O’Boyle et al., 1998), suggesting that microbial products may have systemic as well as local immune effects.

Given the regulatory role of DC, we hypothesized that the beneficial effects of probiotic bacterial DNA are due, at least in part, to their interaction with DC. The aim of this study was to assess the effects of bacterial DNA derived from the clinically-effective probiotic preparation VSL#3 on human DC activation, cytokine production and stimulatory capacity.

MATERIALS AND METHODS

Bacterial and viral preparations

The VSL#3 (VSL#3 Pharmaceuticals, Ft. Lauderdale, FL, USA) bacterial strains, B. infantis B107, B. breve BBSF, B. longum BL04, L. acidophilus LA14, L. delbrueckii subsp. bulgaricus LB31, L. casei LC10, L. plantarum LPT, and, Streptococcus salivarius subsp. thermophilus TA061 (designated as S. thermophilus throughout this paper), were grown anaerobically (Anaerobic System, Mod. 2028, Forma Scientific Co., USA) at 37°C; Lactobacillus and Bifidobacterium strains in MRS medium (Difco, USA) supplemented with 0.05% L-cysteine and Streptococcus thermophilus in M17 medium (Difco, USA). Herpes simplex virus strain HFEM (HSV-1) was grown in Vero cells and inactivated at 60°C for 60 min. In addition, DNA derived from Escherichia coli strain B (Sigma, Saint Louis, USA) was used.

Bacterial debris fractions. Mid log cultures were collected by centrifugation and sonicated (Branson Sonifier W-250, Heinemann, Germany) at a power level 5-6 at 30% duty for 5 min. Sonicates were centrifuged and bacterial debris fractions collected.

Bacterial DNA preparation. Genomic DNA was isolated from pure cultures of the probiotic bacteria as previously described (Lammers et al., 2003). Concentration and purity of all DNA preparations were determined by measuring OD260 absorbance and OD280 ratio, respectively. Only DNAs with an OD260/280 ratio > 1.8 were used. LPS content was less than 0.01 U of endotoxin µg⁻¹ of DNA as determined using the Limulus amebocyte assay (QCL-1000, BioWhittaker, USA). Bacterial DNA fractions were boiled for 10 min prior to each experiment.

Whole blood cultures

Blood was collected from consenting healthy volunteer donors into lithium heparin. Whole blood (50 µl) was incubated with medium alone (50 µl RPMI-1640 Dutch Modification, Sigma-Aldrich, UK) and with bacterial DNA preparations (35 µg/ml) for 4 h or 24 h at 37°C in 5% CO₂ to a final volume of 100 µl (North et al., 1996; Panoskalitsis et al., 2003; Sewell et al., 1997). As controls, the non-bacterial salmon sperm DNA (Roche, Italy) (35 µg/ml) and heat-inactivated HSV-1 (equivalent to 1.5 x 10⁶ pfu/ml) were used. Experiments were performed in duplicate and were repeated 3 to 8 times.

Dendritic cells

Enriched DC were prepared from non-adherent peripheral blood mononuclear cells (PBMC) that had been separated on Ficoll-Paque (Amersham Biosciences, UK). PBMC were cultured overnight in complete medium (RPMI-1640 Dutch Modification supplemented with 10% FCS, 2 mM L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin) at 37°C in 5% CO₂. DC-rich low-density cells were separated on hypertonic metrizamide (14.5% w/v) (Sigma, Saint Louis, USA) and incubated at 37°C in 5% CO₂ with bacterial DNA preparations and controls. Experiments were performed in duplicate. The data presented represent mean values obtained in 3 to 9 experiments.

Table 1. Monoclonal antibodies

<table>
<thead>
<tr>
<th>Antigen/fluorochrome (clone):</th>
<th>Purchased from:</th>
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</thead>
<tbody>
<tr>
<td>CD11c-FITC (KB90)</td>
<td>Dako, Cambridgeshire, UK</td>
</tr>
<tr>
<td>Goat-anti-mouse-FITC F(ab)²</td>
<td>Immunotech, UK</td>
</tr>
<tr>
<td>CD3-PC5 (UCHT-1)</td>
<td>Serotec, UK</td>
</tr>
<tr>
<td>CD14-PC5 (MIP9)</td>
<td>BD Biosciences Pharmingen, UK</td>
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<tr>
<td>CD16-PC5 (B73.1)</td>
<td></td>
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<tr>
<td>CD19-PC5 (4G7)</td>
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<tr>
<td>CD56-PC5 (N901)</td>
<td></td>
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<tr>
<td>CD8-PC5 (B9.11)</td>
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<tr>
<td>CD34-CyChrome (581)</td>
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<tr>
<td>CD40-PE (LOB7/6)</td>
<td></td>
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<tr>
<td>IL-10-PE (clone JES3-9D7)</td>
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<tr>
<td>IL-12-PE (clone C11.5)</td>
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<tr>
<td>HLA-DR-APC (G46-6)</td>
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<tr>
<td>CD3-FTC (UCHT1)</td>
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<tr>
<td>CD69-PE (FN50)</td>
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<tr>
<td>CD8-FTC/PE/APC (SK1)</td>
<td></td>
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<tr>
<td>CCR7 (2H4)</td>
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<tr>
<td>Rat anti-mouse IgM-biotin (R6-60.2)</td>
<td></td>
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<tr>
<td>Streptavidin-PE</td>
<td></td>
</tr>
<tr>
<td>TLR9 (26G593)</td>
<td>Imgenex, San Diego, USA</td>
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</table>
Intracellular cytokine production by dendritic cells

Whole blood was cultured with the different stimuli for 4 h and intracellular cytokine production by DC subsets assessed using a modification of the method described by North et al (North et al., 1996). In brief, paired cultures were established with monensin (3 mmol/l) added to one culture of each pair and medium alone added to the second culture. At the end of the incubation, blood was labelled for 15 min with antibodies (Table 1) for identification of DC subsets.

Red cells were lysed with 500 µl Optilys C (Beckman Coulter, UK) for 15 min at room temperature. Remaining cells were fixed with Leucoperm A (100 µl) and permeabilised with Leucoperm B (100 µl, Serotec). Fluorochrome-conjugated cytokine-specific antibody was then added for 30 min. Cells were washed and resuspended in 1% paraformaldehyde. The data presented were obtained in 3 experiments.

Surface expression of CD40, CCR7 and TLR9 on dendritic cells

Following stimulation of whole blood and/or enriched-DC populations for 4 h, cultures were labelled for identification of DC and with anti-CD40, anti-CCR7, anti-TLR9 or their isotype-matched controls. CD40, CCR7 or TLR9 surface expression on DC from DNA-treated cultures was compared expression on DC from medium cultures using WinList Version 5.0 software (Verity, USA).

Blocking of signalling via TLR9

To test the role of TLR9 in stimulation of DC by bacterial DNA, enriched-DC populations were stimulated with DNA derived from B. infantis in the presence of chloroquine (0.1, 1 or 10 µM), an antagonist of immune activation by synthetic oligodeoxynucleotides (Macfarlane and Manzel, 1998) or anti-TLR9 antibodies (5 µg/ml). In addition, DC were stimulated in the presence of an appropriate isotype-matched control antibody.

Flow cytometry

Data were acquired uncompensated using a FACScalibur (Becton Dickinson) flow cytometer. Compensation was applied to the data using WinList Version 5.0 flow-cytometry software (Verity, USA). Ongoing cytokine production was assessed by comparison of samples incubated with monensin with those incubated without monensin, as previously described (Hart et al., 2004; Hart et al., 2005) using enhanced normalised subtraction as described in detail by Panoskaltsis et al (Panoskaltsis et al., 2003). This technique allows positive cells to be resolved in situations where distribution histograms overlap. The use of the same antibody to label cells from both monensin-treated and untreated cultures gives this technique a high degree of sensitivity for detecting small changes in antibody binding. Absolute cell counts were determined by reference to counts of Flow-Count fluorospheres acquired simultaneously.

Enzyme-linked immunosorbent assay (ELISA)

Whole blood and enriched DC were cultured with the stimuli for different time periods, supernatants collected, stored at -70°C until assayed for IL-10, IL-12 and IFN-α by ELISA using paired antibody kits (Endogen, UK). Absorbance values were read at 450 nm on an ELISA plate reader. The detection limits of the assays were 36 pg/ml for IL-10, 31 pg/ml for IL-12 and 15.8 pg/ml for IFN-α.

Mixed lymphocyte reaction

DC-rich low density cells were prepared and exposed to medium alone, bacterial debris fractions and bacterial DNA for 4 h. Naïve CD4+ T-cells were prepared from PBMC by negative selection (purity > 87%) using StemSep kits (StemCell Technologies Inc., Canada). The antibody cocktail used to separate human naïve CD4+ T cells consists of anti-CD8, CD14, CD16, CD19, CD56, glycophorin A and biotinylated anti-human CD45-RO. Enriched DC (100 to 3000 per well) were cultured with allogeneic naïve CD4+ T-cells (5 x 10^4 per well) in 20 ml hanging drop Terasaki plates for 4 days at 37°C in humidified 5% CO2. Cells were pulsed with 1 µg/ml [3H] thymidine (specific activity: 2 Ci/mmol) for 2 h and then transferred by blotting onto filter discs. Thymidine uptake was measured on a Phosphoimager (Molecular Dynamics, Amersham Pharmacia Biotec, Bucks, United Kingdom). The experiments were performed 3 times.

Statistical analysis

Two-tailed t tests were used to compare proportions and absolute numbers of cytokine-positive cells. Data were paired where appropriate. Proliferation was compared using Two Way Repeated Measured ANOVA (pairwise multiple comparison procedures according to Holm-Sidak method). Values of p < 0.05 were regarded as significant.

RESULTS

Bacterial DNA of both probiotic strains and E. coli upregulate CD40, CCR7 and TLR9 expression on DC

Blood DC were identified as an HLA-DR+ lineage (CD3+, CD14+, CD16+, CD19+, CD34+, CD56+) population. Within this gate, CD11c+ (myeloid) and CD11c+ (plasmacytoid) DC populations were distinguished (Hart et al., 2004).

To test whether DNA derived from probiotic bacteria stimulated DC, expression of the activation marker CD40 and maturation marker CCR7 was determined at different doses of bacterial DNA (3.5 µg/ml, 35 µg/ml and 70 µg/ml). Bacterial DNA upregulated CD40 and CCR7

Probiotic bacterial DNA modulates dendritic cells
expression on both CD11c+ and CD11c- DC populations as evidenced by an increase in the proportion of DC expressing these markers and/or the intensity of marker expression on the positive cells (Fig. 1). Expression of CD40 and CCR7 was maximal at 35 µg/ml and this concentration was used in subsequent experiments. DNA from the probiotic B. infantis and from E. coli activated DC to a similar degree. There was no upregulation of CCR7 or CD40 on either DC population with salmon sperm DNA (data not shown).

FIGURE 1. CD40 and CCR7 expression on CD11c+ and CD11c- dendritic cells. CD40 and CCR7 expression on CD11c+ and CD11c- DC in the presence of B. infantis DNA or E. coli DNA at a concentration of 35 µg/ml for 4 h was assessed by comparison with the isotype control. The profiles for staining with antibody are shown and the shaded part of the histogram represents the proportion of cells staining positive for CD40 or CCR7 after subtraction of the isotype control using the Winlist algorithm. The intensity ratio (IR) represents the intensity of staining of the positive cells compared with the isotype staining. The intensity ratio (IR) is a ratio and not a linear measure and, therefore, apparently modest changes can reflect a large change in expression. Changes in expression are described both in percentage (%) of cells expressing the specific marker and in intensity ratio (IR), that is, the density of expression of the specific marker on the cell surface. The data are representative of 4 independent experiments.

DNA from probiotic bacteria stimulates production of IL-10 but not IL-12 or IFN-α in whole blood

We investigated the effects of bacterial DNA on cytokine production in whole blood. The mixture of DNA from VSL#3 bacterial strains, and DNA from all probiotic strains individually, induced high levels of IL-10. There was a trend towards higher IL-10 production in the presence of DNA from the bifidobacteria or S. thermophilus strains than in the presence of DNA from the other probiotic bacterial strains tested although this difference did not reach statistical significance. IL-10 levels in cultures stimulated with DNA from the intestinal pathogen E. coli, HSV-1 or non-bacterial DNA did not differ from levels in unstimulated cultures (Table 2).

IFN-α was detected in cultures stimulated with HSV-1 DNA from probiotic bacteria stimulates production of IL-10 but not IL-12 or IFN-α in whole blood

We investigated the effects of bacterial DNA on cytokine production in whole blood. The mixture of DNA from VSL#3 bacterial strains, and DNA from all probiotic but not probiotic bacterial DNA. Low levels of IFN-α were detected in the presence of DNA derived from E. coli (Fig. 2). Consistent with known kinetics of IFN-α production in response to viruses (Siegal et al., 1999), we found that IFN-α was detectable in HSV-1 stimulated
DNA from probiotic bacteria stimulates IL-10 production by myeloid dendritic cells

Initially, enriched-DC cultures were incubated with bacterial DNA for 16 h and cytokine expression was assessed by ELISA. Consistent with the results in whole blood, high levels of IL-10 were produced by enriched DC in the presence of DNA from all probiotic bacterial strains. DNA derived from the intestinal pathogenic bacteria *E. coli* did not induce significant IL-10 production (Table 3). None of the stimuli induced IL-12 or IFN-α by the enriched-DC population (data not shown).

To assess the contribution of the CD11c+ and CD11c- DC subsets to cytokine production in the presence of bacterial DNA, we assessed IL-10 and IL-12 production in whole blood by intracellular staining. A mix of DNA from the VSL#3 strains significantly increased the intensity of IL-10 staining in CD11c+ DC when compared with cells cultured in medium alone. Treatment with DNA from individual VSL#3 components (*L. bulgaricus*, *L. casei*, *B. infantis*, *B. longum* and *S. thermophilus*) also increased the level of IL-10 staining in CD11c+ DC. However, for the other individual VSL#3 strains, *L. acidophilus*, *L. plantarum* and *B. breve*, the enhancement in IL-10 staining narrowly failed to reach statistical significance. The proportion of CD11c+ DC making IL-10 was not significantly altered by any DNAs from probiotic bacteria but the number producing IL-10 was significantly increased by DNA from all probiotic strains except *B. longum* (data not shown). HSV-1 and salmon sperm DNA did not significantly affect any measure of IL-10 production by CD11c+ DC (Fig. 3). In contrast to the findings with CD11c+ DC, there was little effect of bacterial DNA on IL-10 production by CD11c- DC (Fig. 3).

### Table 3. Effect of bacterial DNA on IL-10 production by enriched dendritic cell population.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IL-10 (pg/ml) ± SEM</th>
<th>P-value*</th>
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<tbody>
<tr>
<td>No stimulus</td>
<td>10 ± 3</td>
<td>8</td>
</tr>
<tr>
<td>Salmon sperm DNA</td>
<td>19 ± 6</td>
<td>3</td>
</tr>
<tr>
<td>HSV-1</td>
<td>16 ± 6</td>
<td>3</td>
</tr>
<tr>
<td><em>Escherichia coli</em> strain B DNA</td>
<td>0 ± 0</td>
<td>3</td>
</tr>
<tr>
<td>VSL#3 DNA mix</td>
<td>2170 ± 341</td>
<td>8</td>
</tr>
<tr>
<td><em>L. acidophilus</em> DNA</td>
<td>1107 ± 110</td>
<td>6</td>
</tr>
<tr>
<td><em>L. bulgaricus</em> DNA</td>
<td>401 ± 42</td>
<td></td>
</tr>
<tr>
<td><em>L. casei</em> DNA</td>
<td>571 ± 113</td>
<td>6</td>
</tr>
<tr>
<td><em>L. plantarum</em> DNA</td>
<td>557 ± 52</td>
<td>6</td>
</tr>
<tr>
<td><em>B. breve</em> DNA</td>
<td>1471 ± 398</td>
<td>6</td>
</tr>
<tr>
<td><em>B. infantis</em> DNA</td>
<td>1848 ± 310</td>
<td>6</td>
</tr>
<tr>
<td><em>B. longum</em> DNA</td>
<td>1584 ± 397</td>
<td>5</td>
</tr>
<tr>
<td><em>S. thermophilus</em> DNA</td>
<td>2279 ± 367</td>
<td>6</td>
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</tbody>
</table>

*a* Statistical significance of each stimulus was assessed relative to unstimulated control. 
*b* Bacterial strains in the VSL#3 mixture.
Neither the proportion of IL-10 positive cells nor the level of IL-10 was affected by bacterial DNA and only L. acidophilus and B. longum significantly increased the number of IL-10 producing CD11c+ DC (data not shown). Again, neither HSV-1 nor salmon sperm DNA altered IL-10 production. There was little or no IL-12 production by DC in the presence of the bacterial DNA, while IL-12 production was detected in the presence of LPS (data not shown).

**FIGURE 3. IL-10 production by DC in response to bacterial DNA.** The level of staining within the IL-10 positive DC population expressed as an intensity ratio of the linearized mean fluorescence of the positive population in the sample stained with anti-IL-10 to the linearized mean fluorescence of the total population in the sample stained with the isotype control reagent. The results represent the mean values and SEM of 3-7 independent experiments. Significant differences between DC in cultures stimulated with DNA and DC in non-stimulated (medium only) cultures are marked with (*) p < 0.05 for intensity ratio.

**IL-10 production by bacterial DNA-stimulated DC is TLR9 dependent**

To test the role of TLR9 in probiotic bacterial DNA-induced IL-10 production by DC, an anti-TLR9 or chloroquine was added to cultures stimulated with DNA derived from B. infantis. In addition, control wells with an appropriate isotype control were analysed.

Production of IL-10 by DC was partially but significantly inhibited by the two highest concentrations (1 or 10 mM) of chloroquine, showing a reduction of respectively 27.9 ± 14.2 % (p < 0.05) and 32.4 ± 8.1 % (p < 0.01) of the IL-10 levels detected in the B. infantis DNA cultures (data not shown). After blocking of TLR9, IL-10 production by DC was almost completely abrogated, corresponding to a reduction of 90.9 ± 5.2 % (p < 0.0001) of the IL-10 levels measured in the enriched-DC cultured with B. infantis DNA. Isotype-matched controls did not affect IL-10 production (Fig. 4).

**FIGURE 4. Effect of TLR9 signalling on IL-10 production by DC.** Cultures were stimulated with DNA derived from bifidobacteria and in some cultures an anti-TLR9 or isotype-matched control antibody was added. After 24 h of stimulation, IL-10 production by enriched-DC populations was measured by ELISA. The data show mean values and SEM of 5 independent experiments. Evaluation of significance was compared to DNA-stimulated cultures without addition of anti-TLR9 antibodies or isotype-matched control antibodies. *p < 0.0001.

Exposure to bacterial DNA reduces the stimulatory capacity of DC

We evaluated the effect of probiotic bacterial DNA on the stimulatory capacity of DC. Purified naïve T cells were activated in the presence of enriched DC that had been pretreated with medium alone, probiotic bacterial debris or probiotic bacterial DNA. Enriched DC were potent stimulators of allogeneic naïve CD4+ T-cells irrespective of pre-treatment with medium or bacterial debris fractions. However, T cells co-cultured with bacterial DNA-exposed DC showed significantly lower proliferation (Fig. 5).

IL-10 levels were significantly lower in co-cultures of T cells
with non-stimulated DC (610.8 ± 46.2 pg/ml) than in cocultures of T cells with bacterial debris- (3307.5 ± 192.1 pg/ml) and bacterial DNA-exposed DC (3140.6 ± 316.9 pg/ml). However, there was no difference in IL-10 levels between the bacterial debris-treated cultures and the bacterial DNA-treated cultures, indicating that IL-10 levels alone were not responsible for the lower T cell proliferation in response to bacterial DNA-exposed DC. Additional experiments by flow cytometry using annexin/propidium-iodide staining, and by electron microscopy, also suggested that lower T cell proliferation was not due to toxicity or apoptosis.

**FIGURE 5.** Effect of bacterial DNA-treated DC on T cell activation. DC-rich low density cells were prepared and exposed to medium alone, bacterial debris fractions or bacterial DNA and then used to stimulate naïve allogeneic CD4+ T-cells. After 4 days, T cell proliferation was determined by [3H]-thymidine incorporation. Data are representative of 3 independent experiments. *p < 0.05, **p < 0.01.

**DISCUSSION**

We have demonstrated that DNA derived from probiotic bacteria induces IL-10 production by human myeloid DC in a TLR9-dependent manner and inhibited the ability of DC to stimulate naïve T cells. The ability of probiotic bacterial DNA to induce an immunoregulatory cytokine profile in DC may contribute to the beneficial effects of probiotic bacteria in treating IBD.

In a whole blood assay DNA from individual VSL#3 strains, or a mixture of DNA from all the strains, stimulated the production of IL-10, but not IL-12 or IFN-α. DNA from E. coli activated DC but did not stimulate IL-10 production, indicating that induction of IL-10 is not a property of all bacterial DNA.

The second part of our study focused on interactions between bacterial DNA and DC. Bacterial DNA derived from all the individual VSL#3 strains enhanced IL-10 levels by myeloid DC. There was little evidence of IL-10 production by plasmacytoid DC in response to bacterial DNA but this was not due to a lack of activation since both CD40 and CCR7 were upregulated. In line with our data, Hofmann et al. (Hofmann et al., 2010) have recently identified myeloid DC as central mediators in the regulatory and protective immune response they observed in a murine model of colitis after CpG-ODN pretreatment.

In general, stimulation with DNA from probiotic bacteria increased the level of IL-10 within cytokine-secreting myeloid DC but did not increase the proportion of IL-10+ DC. This suggests that enhancement of IL-10 production by bacterial DNA is due to upregulation of production within cells already producing IL-10 rather than induction of production in additional cells. The number of IL-10 positive DC was increased in cultures stimulated with bacterial DNA and this may be attributable to the observed increase in cells with a DC phenotype in stimulated cultures. Thus, it is possible that stimulation with DNA may lead to the differentiation of new DC, perhaps inducing MHC class II on non-expressing precursors or causing loss of lineage markers from other differentiated populations.

The production of IL-10 by DC stimulated with bacterial DNA could be completely blocked with a monoclonal antibody to TLR9 and was partially inhibited by chloroquine, a biochemical antagonist of TLR signalling.

TLR9 is expressed differentially on freshly isolated leukocyte subsets, with TLR9 mRNA detectable in human B cells and plasmacytoid, but not myeloid, DC (Kadowaki et al., 2001; Krug et al., 2001). A possible explanation for TLR9 dependent IL-10 production by myeloid DC is, that the response of myeloid DC to bacterial DNA may result from direct interaction of bacterial DNA with TLR9 on plasmacytoid DC leading to indirect activation of surrounding cells. Krug et al. showed that myeloid DC respond to bacterial DNA stimulation only in the presence of plasmacytoid DC (Krug et al., 2001), demonstrating that the response of myeloid DC to bacterial antigens can be broader in the presence of other cells. Plasmacytoid DC were found to be activated by bacterial DNA in our experiments. It remains possible, however, that bacterial DNA activates myeloid DC by another, as yet unidentified, pathway. For instance, molecular exchange (Knight et al., 1998), possibly via exosomes, could enable myeloid DC to receive functional TLR9-bacterial DNA complexes from plasmacytoid DC (Andre et al., 2004; Stoovogel et al., 2002).

In our preliminary studies, TLR9 was not expressed on the surface of unstimulated DC but was expressed on approximately 50% of DC following stimulation with bacterial DNA. This receptor is reportedly expressed intracellularly in the endoplasmic reticulum (ER) of unstimulated DC (hamd-Nejad et al., 2002; Latz et al., 2004), suggesting that there may be redistribution of TLR9 to the cell surface following stimulation as has been described for the scavenger receptor CD68 (Kurushima et al., 2000). Eaton-Bassiri and colleagues have reported upregulation of TLR9 on the surface of PBMC following stimulation with bacteria (Eaton-Bassiri et al., 2004). Recently, Latz et al. suggested that TLR9 is likely to redistribute from the ER to the site of DNA uptake in endosomes directly beneath the cell surface and, consistent with our data, observed that in the presence of CpG DNA a small portion of TLR9 is detectable on the cell surface (Latz et al., 2004).
Certain viruses and CpG motifs induce TLR9-dependent secretion of pro-inflammatory cytokines including IFN-α and IL-12 (Akira et al., 2001; Krieg, 2002; Wagner, 2002). We observed neither of these cytokines in our cultures stimulated with probiotic DNA as opposed to high levels of IL-10. This is consistent with the reduced IL-12 and IL-6 production by bone marrow derived macrophages when stimulated with VSL#3 DNA compared with synthetic CpG oligodinucleotides (Rachmilewitz et al., 2004). A possible explanation is that the genome of probiotic bacterial DNA contains ‘inhibitory’ CpG motifs rather than ‘stimulatory’ CpG motifs, leading to a dominant anti-inflammatory immune response (Krieg et al., 1998).

DNA from VSL#3 significantly reduced the ability of mature DC to stimulate naïve CD4+ T cells. Cell death due to toxicity of DNA preparations or apoptosis were excluded and high IL-10 production appeared not to be the only factor responsible for the lower proliferative response, since DC exposed to bacterial debris fractions induced comparable high IL-10 production but did not result in lower CD4+ T cell proliferative responses.

The mechanisms which underlie the clinical action of VSL#3 remain to be defined. Here we demonstrate that DNA derived from probiotic bacteria exerts immunoregulatory effects. Physiologic bacterial lysis is likely to release significant amounts of bacterial DNA in the intestine and we suggest that this material contributes to immune homeostasis. In addition to immunoregulatory effects, probiotic bacteria have diverse biological activities, including reinforcement of barrier integrity, production of antimicrobial compounds and synthesis of short chain fatty acids (Guarner and Malagelada, 2003). Any of these activities, singularly or in combination, may be relevant in human gut physiology. VSL#3 has been shown to be beneficial in the IL-10 knockout mouse model of colitis (Madsen et al., 2001). However, this does not mean that probiotic-induced IL-10 plays no role in the control of intestinal inflammation in humans; there is abundant evidence in mice that mucosal delivery of IL-10, by bacterial, viral or immunological vectors, can control inflammation. One of the challenges now ahead in the study of probiotics is to define which activity, or more likely combination of activities, is responsible for efficacy in a particular clinical context.

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REFERENCES


Probiotic bacterial DNA modulates dendritic cells


