Differential cytokine response from dendritic cells to commensal and pathogenic bacteria in different lymphoid compartments in humans

Liam O’Mahony*, Louise O’Callaghan*, Jane McCarthy*, David Shilling†, Paul Scully*, Shomik Sibartie*, Eamon Kavanagh†, William O. Kirwan†, Henry Paul Redmond†, John Kevin Collins*, Fergus Shanahan*.

Alimentary Pharmabiotic Centre* & Dept. of Surgery†, University College Cork, National University of Ireland, Cork.

Running head: Cytokine responses in different lymphoid tissues

Correspondence to:
Prof. Fergus Shanahan, Tel: 353-21-4901226
Alimentary Pharmabiotic Centre, Fax: 353-21-4345300
Department of Medicine, E-mail: F.Shanahan@ucc.ie
Cork University Hospital,
Cork, Ireland.
Abstract

Background: The resident host microflora condition and prime the immune system. However, systemic and mucosal immune responses to bacteria may be divergent.

Aim: To compare, in vitro, cytokine production by human mononuclear and dendritic cells from mesenteric lymph nodes (MLN) and peripheral blood mononuclear cells (PBMCs) to defined microbial stimuli.

Methods: Mononuclear cells and dendritic cells, isolated from the MLN (n=10) and peripheral blood (n=12) of patients with active colitis, were incubated in vitro with the probiotic bacteria, Lactobacillus salivarius UCC118 or Bifidobacterium infantis 35624, or the pathogenic organism Salmonella typhimurium UK1. IL-12, TNF-α, TGF-β and IL-10 cytokine levels were quantified by ELISA.

Results: PBMCs and PBMC-derived DCs secreted TNF-α in response to the lactobacillus, bifidobacteria and salmonella strains, while MLNCs and MLN derived DCs secreted TNF-α only in response to salmonella challenge. Cells from the systemic compartment secreted IL-12 following co-incubation with salmonella or lactobacilli, while MLN derived cells produced IL-12 only in response to salmonella. PBMCs secreted IL-10 in response to the bifidobacterium strain, but not in response to the lactobacillus or salmonella strain. However, MLNC secreted IL-10 in response to bifidobacteria and lactobacilli, but not in response to salmonella.

Conclusion: Commensal bacteria induced regulatory cytokine production by MLNCs while pathogenic bacteria induce Th1 polarising cytokines. Commensal-pathogen divergence in cytokine responses are more marked in cells isolated from the mucosal immune system compared to peripheral blood mononuclear cells.

Key words: Human, Mucosa, Inflammation, Dendritic cells, Cytokines.
Introduction

Scientific understanding of the gastrointestinal flora and its interaction with the host immune response has benefited recently from a convergence of interest from disparate traditional research disciplines. This reawakening of interest in intestinal bacteria has been due to several factors, including the discovery of *Helicobacter pylori* as a cause of peptic ulcer disease and gastric cancer, the role of the indigenous flora in the pathogenesis of inflammatory bowel disease, the development of culture-independent molecular techniques to study enteric bacteria, and the increasing recognition of the role of commensal flora in the development and fine tuning of the mucosal immune response (2, 16, 25, 27, 28). Early studies with gnotobiotic mice which demonstrated the importance of bacterial colonisation in promoting the development of the immune response have been complemented by modern molecular techniques, including laser dissection microscopy, to identify the molecular signals and transduction pathways underpinning the regulatory influence of the enteric flora on the structure and function of the mucosal immune system (7, 8, 20).

While the role of the intestinal epithelium as a sensor of the microbial environment within the gut is well established (15) and differential epithelial responses to commensal and pathogenic signals have been shown, regional specialisation of responses by gut-associated lymphoid cells to bacterial signals compared with systemic immune responses have received less attention. Although tissue-specific specialisation of immunologic responses to pathogens, including region-specific dendritic cell (DC) subsets, has been demonstrated in mice (11), evidence of this in humans is more sparse (31). Therefore, we performed a comparative study of cytokine responses from lymphoid populations including
isolated DCs from the human mesenteric lymph node and peripheral blood. The results confirm a divergence of cytokine responses to commensal lactobacilli and bifidobacteria in comparison with pathogenic salmonella and demonstrate different patterns of response in the two lymphoid compartments.
Materials and Methods

Study population

This study was approved by the Ethics Committee of Cork University Hospital and informed consent was obtained from all volunteers. The study population involved patients with inflammatory bowel disease undergoing colectomy or small bowel resection. Mesenteric lymph nodes (MLN) were obtained from ten patients with active IBD (n=10: age range 19-41 (mean 32.2), 3 women and 7 men: 5 patients with CD; 5 patients with UC). All patients were prescribed and taking systemic steroids (5'- amino salicylic acid). All patients required surgical section because of progressive disease and failure to respond to steroids. Peripheral blood mononuclear cells (PBMC) were obtained from patients with active inflammatory bowel disease (n=12). The age range was 20-51 (mean 28). Five women and seven men were studied, 5 patients with CD; 7 patients with UC. None of the MLN and PBMC donors were the same subject.

Mesenteric lymph node cell (MLNC) isolation

Mesenteric lymph nodes were selected for this study with careful regard to their anatomical location relative to areas of inflammation in the bowel. MLN selected directly drained an inflamed area of the bowel. From three patients within this study population, it was possible to obtain MLN draining segments of inflamed and non-inflamed bowel. Single cell suspensions were generated from MLN by gentle extrusion of the tissue through a 180µ mesh wire screen. Cells were washed and resuspended in DMEM (Dulbecco's modified eagle medium) containing 10% FCS (Invitrogen, Paisley, UK). Mononuclear cells were isolated by Ficoll-Hypaque density
centrifugation (3) and resuspended at 1x10^6 cells/ml in complete media - DMEM containing 25mM glucose, 10% FCS, 1% nonessential amino acids, 50U/ml penicillin and 50µg/ml streptomycin (Invitrogen, Paisley, UK). These mononuclear cells are termed mesenteric lymph node cells (MLNCs).

Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood was taken directly into sterile EDTA containing vacutainers. Mononuclear cells were isolated from blood by Ficoll-Hypaque density gradient centrifugation. Following a washing step, PBMCs were resuspended in complete media at 1 x 10^6 cells /ml.

Dendritic cell isolation

DCs from MLN and peripheral blood were isolated using identical procedures. Cells were resuspended at 5 x 10^7 cells/ ml in PBS (without Ca^{2+} or Mg^{2+}) with 4% FCS. For optimal recovery of DCs, 1mM EDTA was added to all media and cell suspensions were blocked with anti-CD32 antibodies (StemCell, Meylan, France). DCs were isolated from this cell suspension, according to the manufacturer's protocol, using a DC negative isolation kit (StemSep™ depletion cocktail, StemCell, Meylan, France). DCs were resuspended in Stemspan serum-free media (StemCell, Meylan, France) at 1x10^6 cells/ ml. Viability, determined by trypan blue exclusion, was consistently ≥ 98%. Purity of DC preparations were assessed using flow cytometry. Cells which were HLA-DR positive and CD3/CD14/CD16/CD19/CD20/CD56 negative were termed DCs. All antibodies were obtained from BD Biosciences, (Oxford, UK).
Bacterial strains

We have previously reported the selection criteria for isolation of *Lactobacillus salivarius* UCC118 (*L. salivarius*) and *Bifidobacterium infantis* 35624 (*B. infantis*) (5). *L. salivarius* and *B. infantis* were routinely cultured anaerobically for 24–48 hours in deMann, Rogosa and Sharpe medium, MRS, (Oxoid, Basingstoke, UK) and MRS supplemented with 0.05% cysteine (Sigma, Dublin, Ireland), respectively. *Salmonella typhimurium* UK1 (*S. typhimurium*) was cultured aerobically for 18–24 hours in tryptic soya broth (Oxoid, Basingstoke, UK). Bacterial cultures were harvested by centrifugation (3,000g x 15 mins), washed with PBS and subsequently diluted to final cell densities of 1x10^7, 1x10^5 and 1x10^3 colony forming units (cfu)/ ml in DMEM.

In vitro cell stimulations

All cells were seeded in 24 well tissue culture plates (Costar, Schiphol-Rijk, Netherlands) at 1x10^6 cells/ml. MLNCs were stimulated for 72 hours with *L. salivarius*, *B. infantis* or *S. typhimurium* at 3 different bacterial concentrations, 1x10^7 CFU, 1x10^5 CFU and 1x10^3 CFU. As 1x10^7 bacteria resulted in significant stimulation of cytokine production, this bacterial concentration was used in subsequent experiments. MLNCs isolated from non-inflamed bowel and PBMCs were stimulated for 72 hours with these bacteria at 1x10^7 cfu. MLN-derived and peripheral blood derived DCs were stimulated with *L. salivarius*, *B. infantis* or *S. typhimurium*. MLNC and PBMC cultures remained viable over the 72 hour culture period (>95% viable at 72 hours). Dendritic cell viability decreased rapidly after 24 hours incubation so all dendritic cell stimulations were terminated at 24 hours (mean viability at 24 hours >94%; mean viability at 72 hours = 31%). Non-stimulated cells
were present to assess spontaneous levels of cytokine secretion. Plates were incubated in a 5% CO₂ and 37°C humidified atmosphere after which supernatants were harvested for cytokine analysis. Cytokine production was measured, according to the manufacturer's instructions, using commercially available ELISA kits (R&D Systems, Abington, UK). Cytokines measured included TNF-α, IL-12 p40, IL-10 and TGF-β.

Intracellular cytokine staining

Following stimulation of PBMCs with *L. salivarius, B. infantis* or *S. typhimurium*, cells were examined by flow cytometry for intracellular cytokine expression. PBMC incubations were performed for 3, 6 and 12 hours in the presence of Golgistop (BD BioSciences). Cells were stained with FITC conjugated anti-CD3 antibodies for the identification of T cells while dendritic cells were identified as being CD3/CD14/CD16/CD19/CD20/CD56 negative (FITC conjugated) and Cy5-HLA-DR positive. Cells were fixed and permeabilised using a Cytofix/Cytoperm kit (BD BioSciences) followed by co-incubation with PE conjugated anti-IL-10 or anti-TNF-α antibodies. All antibodies were obtained from BD BioSciences. Flow cytometric analysis of the frequency of cytokine positive cells was performed using a BD FacsCalibur and Cell Quest Pro software.

Statistical Analysis

Results were analysed using ANOVA analysis. Values are illustrated as the mean ± standard error mean. Statistically significant differences in cytokine production between non-stimulated cells (control) and cells stimulated with bacteria were accepted at p< 0.05.
Results

Divergent immune responses of MLNCs to commensal and pathogenic bacteria

Strain-strain divergence in cytokine responses (i.e commensal bacteria versus pathogenic bacteria) was observed in MLNCs draining inflamed bowel (Figure 1). *L. salivarius* and *B. infantis* induced IL-10 production by MLNCs at the highest bacterial dose ($10^7$ CFU). *B. infantis* also significantly induced TGF-$\beta$ production while *L. salivarius* induced TGF-$\beta$ production did not reach statistical significance. *L. salivarius* and *B. infantis* did not induce TNF-$\alpha$ or IL-12. In contrast, *S. typhimurium* significantly induced IL-12 and TNF-$\alpha$ production by MLNCs but did not induce IL-10 or TGF-$\beta$.

To confirm that results were not skewed by the presence of inflammation in the resected specimens, MLNCs were isolated from two different drainage fields (from inflamed and non-inflamed segments), which were available in three patients with restricted disease distribution. The patterns of response of MLNCs to commensals and pathogens were similar irrespective of whether they were from sites draining inflamed or non-inflamed segments of bowel (Figure 2). Thus, the lactobacillus and bifidobacterium strains induced IL-10 and TGF-$\beta$ production while the salmonella induced IL-12 and TNF-$\alpha$ production. However, while the cytokine pattern was similar between MLNCs from both sites, the quantity of *S. typhimurium* stimulated IL-12 and TNF-$\alpha$ produced by MLNCs draining inflamed bowel was greater than the levels produced by MLNCs draining non-inflamed bowel.

Comparison between MLNC and PBMC cytokine responses
The cytokine response of cells derived from the mucosal immune compartment to commensal bacteria was not reflected in the response of cells derived from the systemic compartment (Figure 3). While *L. salivarius* significantly induced IL-12 and TNF-α production when co-incubated with PBMCs, these cytokines were not secreted by MLNCs. Similarly, *B. infantis* induced TNF-α production by PBMCs but not by MLNCs. In addition, differences were also evident with regulatory cytokines; *L. salivarius* induced IL-10 production by MLNCs but not by PBMCs. *B. infantis* induced IL-10 production by both MLNCs and PBMCs. TGF-β was not induced over control for any bacterial stimulated PBMC cultures (result not shown) while *B. infantis* induced TGF-β production in MLNC cultures. In contrast to the responses to commensals, *S. typhimurium* induced a similar cytokine profile for both MLNCs and PBMCs.

*Intracellular Cytokine Staining*

In stimulated PBMC cultures, both T cells and dendritic cells secreted IL-10 and TNF-α in response to co-incubation with each of the bacterial strains examined (Figure 4). However, it is evident from these histograms that a greater percentage of the dendritic cell population (HLA-DR positive) was manufacturing cytokine compared to T cells within the same cultures. This suggests that dendritic cells are responsible for the bulk of cytokine released into culture supernatants and measured using ELISA.

*Dendritic cell cytokine responses to microbial challenge*

Isolated DC preparations were consistently > 90% HLA-DR positive and CD3/CD14/CD16/CD19/CD20/CD56 negative (mean=93.2% +/- 2.9%). *L. salivarius*
induced IL-10, TNF-α and IL-12 secretion by peripheral blood derived DCs but only induced IL-10 secretion from MLN derived DCs (Figure 5). *B. infantis* induced IL-10 and TNF-α, but not IL-12, production by peripheral blood derived DCs. *B. infantis* stimulated the production of IL-10 alone by MLN derived DCs. *S. typhimurium* significantly induced IL-12 and TNF-α production by both MLN and peripheral blood derived DCs but only induced IL-10 secretion from PBMC derived DCs. It is important to note that the absolute levels of cytokines released from DCs was substantially less than that released from mononuclear cell populations. This may reflect the different culture periods for DCs (24 hours) and mononuclear populations (72 hours) but also suggests that individual DC subpopulations require co-operation with other mononuclear cells in order to achieve the optimal response to bacterial challenge.
Discussion

Discriminatory responses to pathogens and commensals are expected, irrespective of the lymphoid compartment and this is evident in the present study. The expected TH1 polarised cytokine response to salmonella was found for both the mesenteric lymph node and peripheral blood. However, in vitro responses to the commensal bacteria were divergent in the two lymphoid compartments. While significant levels of TNF-α were produced in response to the lactobacillus and bifidobacterium strains by peripheral blood mononuclear cells and by DCs derived from that source, mesenteric lymph node cells and their associated DCs did not elaborate this cytokine in the presence of the same microbial stimuli. In addition, the ratio of IL-12/IL10 production by cells from the periphery in response to L. salivarius is reversed in the mesenteric lymph node.

The murine mesenteric lymph node has been shown to restrict access of mucosal dendritic cells bearing commensal bacteria to the systemic immune compartment (17). However, dendritic cell cytokine responses to commensals and pathogens within the human mesenteric lymph node have not previously been explored. The human mesenteric lymph node is a rich repository of mucosal-associated lymphoid cells and has the advantage of enabling purification of DC and other cells without introducing the potentially confounding variable of artifactual distortion due to enzymatic tissue digestion which is required for cell isolation from the intestinal lamina propria (12, 13, 26). However, access to human mesenteric lymph nodes is limited and largely dependent on availability of surgically resected material from patients undergoing colectomy for inflammatory bowel disease or colorectal cancer. To avoid unnecessary confounding variables, the present study was
restricted to material from patients with inflammatory bowel disease because reports by us and by others of local immunosuppression in tumor-draining lymph nodes (24). Mesenteric nodes in the drainage field of gastrointestinal tumors are subject not only to the immunosuppressive effects of occult micrometastases that are undetected by conventional histology but are also exposed to immunosuppressive factors from the primary tumor. However, the divergent results between the mesenteric and peripheral lymphoid compartments were not disease-related as the samples from both sites were taken from patients with active disease. More importantly, we took advantage of the availability of lymph nodes draining inflamed and non-inflamed segments of colonic mucosa in the case of three individuals undergoing colectomy for sub-total colonic involvement with ulcerative colitis. The results showed that the pattern of cytokine responses to the different microbial stimuli were the same irrespective of whether the nodes were in the field of drainage of inflamed or non-inflamed colonic segments.

Striking plasticity of DC responses to different microbial stimuli has previously been reported (9, 14). Cytokine responses by DCs may also vary for different species of bacteria within the same genus (4). Discriminatory cytokine responses to different microbes enables the host to interpret the local environment and sense danger. This signalling is mediated by a series of pattern recognition receptors including toll-like receptors and C-type lectins on the surface of DCs (1, 6, 19). The expression of these receptors is probably variant in different tissues. Another factor which may influence cytokine production in the different lymphoid compartments is the dose of bacteria exposed to the system. Variability of cytokine responses may also reflect heterogeneity within DC populations. Thus, murine DCs from the intestinal Peyer’s patch promote IL-10 rather than IL-12p40 responses, whereas the latter are more characteristic of splenic DCs (10). The results of the present study
with human mesenteric lymph node support the murine evidence for regional specialisation of DCs. Notwithstanding, the degree to which one may extrapolate across species is limited because normal murine responses to intestinal antigens are biased toward a Th2 profile whereas human mucosal immune responses appear to have a Th-1 bias (22).

Translocation of bacteria is an important issue in clinical medicine and translocation was traditionally measured by culturing bacteria from the mesenteric lymph node. More recently, mesenteric lymph nodes have been demonstrated to be important gatekeepers limiting bacterial access to the systemic circulation (17). The results of the present study have clinical implications because commensal bacteria, including those studied here, have been used as probiotics in animal models of inflammatory bowel disease (18, 23), and are in human clinical trials (www.proeuhealth.vtt.fi). Whether the numbers of bacteria used in our studies are clinically relevant is unknown but are likely to be relevant in the context of clinical conditions with increased gut permeability and a high rate of sepsis (e.g. IBD). Moreover, numbers of bacteria recovered from the periphery in experimentally infected animals are in the range of bacteria tested in this study (30). Lastly, we have previously demonstrated that probiotics administered parenterally have beneficial effects – therefore it is important to understand how the immune system perceives them in different locations (29). In this respect, it is noteworthy that while lactobacilli are known to stimulate IL-12 responses from human peripheral blood (21), the more relevant responses are probably those from gut-associated lymphoid tissue. In contrast to peripheral blood, regulatory cytokines such as IL-10 and TGF-β were produced in preference to TNF-α and IL-12 after exposure in vitro to lactobacilli and bifidobacteria.
In conclusion, regional specialisation of immune responses to microbial stimuli is evident in humans. In addition to polarised responses to commensals and pathogens, there are divergent responses in peripheral blood and mesenteric lymphoid cells to microbial challenge. This should be considered in the interpretation of immunological responses to commensal or probiotic organisms in humans.
Grants

The authors are supported in part by Science Foundation Ireland in the form of a centre grant (Alimentary Pharmabiotic Centre), by the Health Research Board (HRB) of Ireland, the Higher Education Authority (HEA) of Ireland, and the European Union (PROGID QLK-2000-00563).

Disclosures

The authors (LO’M, JKC, FS) have been affiliated with a multi-departmental university campus-based research company (Alimentary Health Ltd), which investigates host-flora interactions and the therapeutic manipulation of these interactions in various human and animal disorders. The content of this article was neither influenced nor constrained by this fact.
REFERENCES


Figure Legends.

Figure 1. MLNC cytokine profiles.
MLNC cytokine secretion is dependant on the type and dose of bacterial strain being tested. *L. salivarius* stimulated IL-10 production while *B. infantis* induced IL-10 and TGFβ secretion by MLNCs. In contrast, *S. typhimurium* induced IL-12 and TNFα production. Results are expressed as mean cytokine level +/- standard error following stimulation with 0, 1x10^3, 1x10^5 or 1x10^7 bacterial cells, n=10 MLNC stimulations.

* (P≤ 0.05), significant difference between non-stimulated cells (control) to cells stimulated with bacteria.

(A) IL-12 (B) TNF-α (C) IL-10 and (D) TGF-β.

Figure 2. Inflammed versus non-inflammed MLNC.
Bacterial stimulated cytokine production by MLNC draining non-inflamed bowel (n=3) is similar to cytokine production by MLNC draining inflamed bowel (n=10) in response to stimulation with 1x10^7 *L. salivarius, B. infantis* or *S. typhimurium*. *L. salivarius* and *B. infantis* induced IL-10 and TGFβ production while *S. typhimurium* induced IL-12 and TNFα production. However, *S. typhimurium* induced greater amounts of TNFα and IL-12 production in MLNC draining inflamed versus non-inflamed bowel.

* (P≤ 0.05), significant difference between nonstimulated cells (control) to cells stimulated with bacteria.

(A) IL-12, (B) TNF-α, (C) IL-10 and (D) TGF-β.
Figure 3. MLNC and PBMC cytokine profiles.

Bacterial induced cytokine production by MLNCs (n=10) and PBMCs (n=12) were divergent. *L. salivarius* induced IL-12 and TNF-α production by PBMCs, which were not induced in MLNCs. In addition, *L. salivarius* did not induce IL-10 production by PBMCs. *B. infantis* induced TNFα production by PBMCs which was not induced in MLNCs. *S. typhimurium* induced a similar cytokine profile from both MLNCs and PBMCs.

Results are expressed as mean cytokine level +/- standard error following stimulation with 0 (non-stimulated) or 1x10⁷ bacterial cells.

* (P ≤ 0.05), significant difference between non-stimulated cells to cells stimulated with bacteria.

Figure 4. Intracellular cytokine staining.

These representative histograms illustrate that T cells (CD3 positive) and dendritic cells (CD3/CD14/CD16/CD19/CD20/CD56 negative and HLA-DR positive) stain for intracellular TNF-α and IL-10 following stimulation with *L. salivarius*, *B. infantis* or *S. typhimurium*. The percentage of dendritic cells positive for intracellular cytokine was greater than the percentage of cytokine positive T cells. The percent values illustrated represent the percent positive for each cell type.
Figure 5. Dendritic cell cytokine profiles.

Cytokine production by DCs, in response to commensal bacteria, depends on the site from which the DCs were isolated. *L. salivarius* and *B. infantis* induced TNF-α production by peripheral blood derived DCs (n=3) which was not induced in MLNC derived DCs (n=5). In addition, *L. salivarius* induced IL-12 production by PBMC derived DCs but not MLNC derived DCs. In contrast, *S. typhimurium* induced TNF-α and IL-12 production by both PBMC and MLNC derived DCs. IL-10 was secreted by PBMC and MLNC derived DCs upon co-incubation with *L. salivarius* and *B. infantis*. While PBMC derived DCs alone secreted IL-10 in response to *S. typhimurium*.

Results are expressed as mean cytokine level +/- standard error following stimulation with 0 (non-stimulated) or 1x10^7 bacterial cells.

* (P ≤ 0.05), significant difference between nonstimulated cells to cells stimulated with bacteria.
Figure 1.

A: IL-12 (pg/ml)

B: TNF-alpha (pg/ml)

C: IL-10 (pg/ml)

D: TGF-beta (pg/ml)

- L. salivarius
- B. infantis
- S. typhimurium

Bacterial dose (cfu/ml)
Figure 2.
Figure 3.

[Bar charts showing cytokine levels (IL-12, TNF-α, and IL-10) for different treatments (Non-stimulated, L. salivarius, B. infantis, S. typhimurium) in PBMC and MLNC samples. Significant differences are marked with an asterisk (*).]
Figure 4.

IgG  vs  TNFα

IgG  vs  CD3

HLA-DR

IgG  vs  IL10

IgG  vs  HLA-DR

CD3

IL10

HLA-DR
Figure 5.

[Graph showing cytokine production in response to stimulation with L. salivarius, B. infantis, and S. typhimurium in PBMC-DC and MLNC-DC cultures.]

- IL-12 pg/ml
- TNF-alpha pg/ml
- IL-10 pg/ml