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

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O’Mahony, Liam, Louise O’Callaghan, Jane McCarthy, David Shilling, Paul Scully, Shomik Sibertie, Eamon Kavanagh, William O. Kirwan, Henry Paul Redmond, John Kevin Collins, and Fergus Shanahan. Differential cytokine response from dendritic cells to commensal bacteria in response to the mucosa; inflammation isolated from the mucosal immune system compared with PBMCs. Pathogen divergence in cytokine responses is more marked in cells bacteria induce T cell helper 1-polarizing cytokines. Commensal-IL-10 in response to the cells produced IL-12 only in response to coincubation with Lactobacillus salivarius. PBMCs secreted IL-12 after derived DCs secreted TNF-α, whereas MLN cells secreted IL-12 after coincubation with Salmonella typhimurium. Interleukin (IL)-12, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and IL-10 cytokine levels were quantified by ELISA. PBMCs and PBMC-derived DCs secreted TNF-α in response to the Lactobacillus, Bifidobacteria, and Salmonella strains, whereas MLN cells and MLN-derived DCs secreted TNF-α only in response to Salmonella challenge. Cells from the systemic compartment secreted IL-12 after in vitro cytokine production by MLN cells, whereas pathogenic bacteria induce T cell help 1-polarizing cytokines. Commensal-pathogen divergence in cytokine responses is more marked in cells isolated from the mucosal immune system compared with PBMCs.

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the bowel. MLNs 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 noninflamed bowel. Single cell suspensions were generated from MLNs by gentle extrusion of the tissue through a 180-μm mesh wire screen.

Cells were washed and resuspended in DMEM containing 10% fetal calf serum (FCS; Invitrogen; Paisley, UK). Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation (3) and resuspended at 10^6 cells/ml in complete media-DMEM containing 25 mM glucose, 10% FCS, 1% nonessential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). These mononuclear cells are termed MLN cells (MLNCs).

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

DC isolation. DCs from the MLN and peripheral blood were isolated using identical procedures. Cells were resuspended at 5 × 10^7 cells/ml in PBS (without Ca^2+ or Mg^2+) with 4% FCS. For optimal recovery of DCs, 1 mM 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). DCs were resuspended in Stemspan serum-free media (StemCell) at 10^6 cells/ml. Viability, determined by trypan blue exclusion, was consistently ≥98%. The purity of DC preparations was assessed using flow cytometry. Cells that were human leukocyte antigen (HLA)-DR positive and CD3/CD14/CD16/CD19/CD56 negative were termed DCs. All antibodies were obtained from BD Biosciences (Oxford, UK).

Bacterial strains. We (5) have previously reported the selection criteria for isolation of Lactobacillus salivarius UCC118 and Bifidobacterium infantis 35624. L. salivarius and B. infantis were routinely cultured anaerobically for 24–48 h in deMann, Rogosa, and Sharpe medium (MRS; Oxoid; Basingstoke, UK) and MRS supplemented with 0.05% cysteine (Sigma; Dublin, Ireland), respectively. Salmonella typhimurium UK1 was cultured aerobically for 18–24 h in tryptic soya broth (Oxoid). Bacterial cultures were harvested by centrifugation (3,000 g × 15 min), washed with PBS, and subsequently diluted to final cell densities of 10^7, 10^5, and 10^3 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 10^6 cells/ml. MLNCs were stimulated for 72 h with L. salivarius, B. infantis, or S. typhimurium. Results are expressed as mean cytokine levels ± SE after stimulation with 0, 1 × 10^3, 1 × 10^5, or 1 × 10^7 bacterial cells [colony-forming units (cfu)/ml]; n = 10 MLNC stimulations. *Significant difference between nonstimulated cells (control) compared with cells stimulated with bacteria (P ≤ 0.05).
Nonstimulated cells were present to assess spontaneous levels of cytokine production. Bacteria at 24 h (mean viability at 24 h) and then rapidly after a 24-h incubation, so all DC stimulations were terminated at 72 h (95% viable at 72 h). DC viability decreased rapidly after a 24-h incubation, so all DC stimulations were terminated at 72 h (mean viability at 24 h >94%; mean viability at 72 h = 31%). Nonstimulated 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 tumor necrosis factor (TNF)-α, interleukin (IL)-12 p40, IL-10, and transforming growth factor (TGF)-β.

Intracellular cytokine staining. After 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 h in the presence of Golgistop (BD BioSciences). Cells were stained with FITC-conjugated anti-CD3 antibodies for the identification of T cells, whereas DCs were identified as being CD3/CD14/CD16/CD19/CD80/CD86/CD56 negative (FITC conjugated) and Cyc5-HLA-DR positive. Cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD BioSciences) followed by coincubation with phycoerythrin-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 FACS Calibur and Cell Quest Pro software.

Statistical analysis. Results were analyzed using ANOVA. Values are illustrated as means ± SE. Statistically significant differences in cytokine production between nonstimulated 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 vs. pathogenic bacteria) was observed in MLNC-draining inflamed bowels (Fig. 1). L. salivarius and B. infantis induced IL-10 production by MLNCs at the highest bacterial dose (10⁷ cfu). B. infantis also significantly induced TGF-β production, whereas L. salivarius induced TGF-β production did not reach statistical significance. L. salivarius and B. infantis did not induce TNF-α or IL-12. In contrast, S. typhimurium significantly induced IL-12 and TNF-α production by MLNCs but did not induce IL-10 or TGF-β.

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 noninflamed 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 noninflamed segments of bowel (Fig. 2). Thus the Lactobacillus and Bifidobacterium strains induced IL-10 and TGF-β production, whereas the Salmonella strain induced IL-12 and TNF-α production. However, whereas the cytokine pattern was similar between MLNCs from both sites, the quantity of S. typhimurium-stimulated IL-12 and TNF-α produced by the MLNC-draining inflamed bowel was greater than the levels produced by the MLNC-draining noninflamed 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. Whereas L. salivarius significantly induced IL-12 and
TNF-α production when coincubated 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 bacteria-stimulated PBMC cultures (result not shown), whereas 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 DCs secreted IL-10 and TNF-α in response to coincubation with each of the bacterial strains examined (Fig. 4). However, it is evident from these histograms that a greater percentage of the DC population (HLA-DR positive) was manufacturing cytokine compared with T cells within the same cultures. This suggests that DCs are responsible for the bulk of cytokines released into culture supernatants and measured using ELISA.

DC 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 (Fig. 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 were substantially less than those released from mononuclear cell populations. This may reflect the different culture periods for DCs (24 h) and mononuclear populations (72 h) but also suggests that individual DC subpopulations require cooperation with other mononuclear cells 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-polarized cytokine response to Salmonella was found for both the MLN and peripheral blood. However, in vitro responses to the commensal bacteria were divergent in the two lymphoid compartments. Whereas significant levels of TNF-α were produced in response to the Lactobacillus and Bifidobacterium strains by PBMCs and by DCs derived from that source, MLNCs and their associated DCs did not elaborate this cytokine in the presence of the same microbial stimuli. In addition, the ratio of IL-12 to IL-10 production by cells from the periphery in response to L. salivarius is reversed in the MLN.

The murine MLN has been shown to restrict access of mucosal DCs bearing commensal bacteria to the systemic immune compartment (17). However, DC cytokine responses to commensals and pathogens within the human MLN have not previously been explored. The human MLN is a rich repository of mucosa-associated lymphoid cells and has the advantage of enabling purification of DCs 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 MLNs is limited and largely dependent on availability of surgically resected material from patients undergoing colectomy for IBD or colorectal cancer. To avoid unnecessary confounding variables, the present study was restricted to material from patients with IBD because reports by us and 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 because the samples from both sites were taken from patients with active disease. More importantly, we took advantage of the availability of lymph node-draining inflamed and noninflamed segments of colonic mucosa in the case of three individuals undergoing colectomy for subtotal colonic involvement with UC. The results showed that the patterns 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 noninflamed colonic segments.

Striking plasticity of DC responses to different microbial stimuli has previously been reported (4a, 9). 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 signaling 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 that 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-12 p40 responses, whereas the latter are more characteristic of splenic DCs (10). The results of the present study with human MLN support the murine evidence for regional specialization 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 Th1 bias (22).

Translocation of bacteria is an important issue in clinical medicine, and translocation was traditionally measured by culturing bacteria from the MLN. More recently, MLNs 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 IBD (18, 23) and are in human clinical trials (www.proeuhaealth.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). Finally, we (29) have previously demonstrated that probiotics administered parenterally have beneficial effects; therefore, it is important to

Fig. 4. Intracellular cytokine staining. These representative histograms illustrate that T cells (CD3 positive; top) and dendritic cells [DCs; CD3/CD14/CD16/CD19/CD20/CD56 negative and human leukocyte antigen (HLA)-DR positive; bottom] stain for intracellular TNF-α and IL-10 after stimulation with L. salivarius, B. infantis, or S. typhimurium. The percentage of DCs positive for intracellular cytokines was greater than the percentage of cytokine-positive T cells. The percent values illustrated represent the percent positive for each cell type.
understand how the immune system perceives them in different locations. In this respect, it is noteworthy that although *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 cells were exposed in vitro to *Lactobacilli* and *Bifidobacteria*.

In conclusion, regional specialization of immune responses to microbial stimuli is evident in humans. In addition to polarized 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.

**DISCLOSURES**

L. O’Mahony, J. K. Collins, and F. Shanahan have been affiliated with a multidisciplinary university campus-based research company (Alimentary Health Limited), 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**


