Mycobacterium avium subsp. *Paratuberculosis* (MAP) as a Modifying Factor in Crohn's Disease

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Background: Crohn's disease (CD) is a multifactorial syndrome with genetic and environmental contributions. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been frequently isolated from mucosal tissues of patients with CD but the cellular immune response to this bacterium has been poorly described. Our aim was to examine the influence of MAP on T-cell proliferation and cytokine responses in patients with inflammatory bowel disease (IBD).

Methods: Peripheral blood mononuclear cells (PBMCs) and mesenteric lymph node cells (MLNCs) were obtained from IBD patients and non-IBD controls. PBMC T-cell proliferation in response to MAP was determined using CFSE labeling and flow cytometry. The specificity of cytokine responses to MAP was controlled by parallel exposure to *Listeria monocytogenes* (LM) or *Salmonella typhimurium* (ST).

Results: Coincubation of PBMCs with MAP induced significantly more T-cell proliferation ($P < 0.0001$) in PBMCs isolated from CD patients compared to PBMCs obtained from ulcerative colitis (UC) patients or healthy volunteers. In addition, PBMCs from CD patients secreted significantly higher ($P < 0.05$) levels of tumor necrosis factor-alpha (TNF-α; 2302 ± 230 pg/mL) and interleukin (IL)-10 (299 ± 48 pg/mL) in response to MAP compared to UC patients (TNF-α: 1219 ± 411 pg/mL; IL-10: 125 ± 19 pg/mL) and controls (TNF-α: 1447 ± 173 pg/mL; IL-10: 127 ± 12 pg/mL). No difference in cytokine responses was observed in response to LM or ST. MLNCs from both CD and UC patients secreted significantly more TNF-α and IL-8 in response to MAP compared to MLNCs from non-IBD control patients.

Conclusions: Increased proliferation of T cells and an altered cytokine response suggest that prior exposure to MAP and engagement of the immune system is common in patients with CD. This does not imply causation but does support further examination of this bacterium as an environmental modifying factor.

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Key Words: mycobacterium, paratuberculosis, Crohn's disease, cytokine, inflammatory bowel disease

Increasing evidence suggests that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is widespread within the environment in most developed countries, and this may include exposure through the food chain.[1–4] Patients with Crohn's disease (CD) seem to have higher rates of detection of MAP from blood or mucosal tissues, albeit these findings are still controversial.[5–7] For most patients with CD, it is difficult to reconcile a putative etiologic MAP infection with several characteristic features of the disease. In addition, increased detection of bacteria within mucosal tissues of patients with CD is not specific to MAP, with other bacteria, notably enteroadherent strains of *Escherichia coli,* detected with some consistency.[8–10] However, given the heterogeneity of CD, MAP and other microbes could, at least theoretically, modify the host immune response in susceptible individuals and indirectly contribute to the pathogenesis of the disease. While many investigators have attempted to identify the presence of MAP either using molecular approaches or by culturing samples from patients with CD, a more important question may relate to whether there is evidence for previous exposure and engagement of the immune system by MAP in CD. Since serologic techniques have been inconsistent or inconclusive,[11–14] we examined the cellular and cytokine response to MAP. Our data clearly demonstrates that CD patients have an enhanced T-cell proliferative response to MAP associated with an altered cytokine profile. This study does not suggest a causal effect for MAP but does support the findings of others that altered immune reactivity to microbes and their components is a common feature associated with CD.[15–19]

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MATERIALS AND METHODS

Study Population

Patients with inflammatory bowel disease (IBD) were randomly recruited from the IBD clinic at Cork University Hospital, Cork, Ireland. Healthy volunteers were recruited randomly from the laboratory and the general public. Peripheral blood was obtained from patients with CD (n = 45, age range 17–76, mean age: 36.5 years, 13 women and 32 men), ulcerative colitis (UC) (n = 22, age range 15–84, mean age: 38.7 years, 13 women and 9 men), and healthy volunteers (n = 44, age range 21–48, mean age: 31.5 years, 20 women and 24 men). The disease activity of patients with CD or UC was determined by the Crohn's Disease activity index (CDAI)[36] and the clinical activity index (CAI) of Rachmilewitz,[37] respectively. Patients with a CDAI greater than 150 or a CAI greater than 4 were excluded. Of the CD patients, 31 had only small bowel involvement, 11 had colonic disease, of which 2 also had perianal involvement, and 3 had mixed small and large bowel involvement. Mean duration of disease was 9.5 years. Fourteen patients were on 6-mercaptopurine (6-MP), 14 were on aminosalicylates, 5 were on both, and 12 did not take any medications for their disease. Of the UC patients, 2 were on 6-MP, 12 were on aminosalicylates, 4 were on both, and 4 did not take any medications for their disease. Mean duration of disease was 7.1 years. None of the IBD patients were on steroids or anti-TNF treatment at the time of this study.

For experiments involving mesenteric lymph nodes, the study population involved patients undergoing colectomy or small bowel resection. Mesenteric lymph nodes were obtained from patients with active CD (n = 6, age range 31–44, mean age: 38.2 years, 4 women and 2 men), UC (n = 6, age range 21–53, mean age 32.8 years, 1 woman and 5 men), and non-IBD patients (n = 7: age range 58–87, mean 70.7 years, 5 women and 2 men). Of the non-IBD patients, 6 patients underwent surgery for colon cancer and 1 for multiple colonic polyps.

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Ten mL of blood was collected in sterile EDTA-containing vacutainers from each participant by venipuncture. The blood was mixed with an equal volume of PBS (phosphate-buffered saline with $CaCl_2$ and $MgCl_2$; pH 7.2) (Gibco, Grand Island, NY) phosphate and carefully layered over 20 mL of histopaque (Sigma, St. Louis, MO) and centrifuged for 400g for 30 minutes. The "buffy coat" was removed from the interface and washed twice by centrifugation (10 min at 300g followed by 10 min at 250g) using culture medium (DMEM+4500 mg/L Glucose [Gibco] + 10% fetal calf serum [FCS] [Sigma] + 1% penicillin-streptomycin [Gibco]). PBMCs were resuspended in complete media at 1×10^6 cells/mL.

Mesenteric Lymph Node Cell (MLNC) Isolation

Mesenteric lymph nodes (MLNs) were isolated as previously described.[20] Briefly, 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. Single cell suspensions were generated from MLN by gentle extrusion of the tissue through a 180 μ 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 and resuspended at 1×10^6 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. These mononuclear cells are termed mesenteric lymph node cells (MLNCs).

Bacterial Strains

Mycobacterium avium subspecies *paratuberculosis* (MAP) ATCC43019 (American Type Culture Collection, Manassas, VA) was grown from frozen stocks initially in 10 mL Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with 10% Middlebrook OADC (oleic acid-albumin-dextrose-catalase) enrichment media (Becton Dickinson, San Jose, CA), 2 mL/L 80% glycerol, and 2 mg/mL mycobactin J (Synbiotics, Kansas City, MO). Cultures were incubated at 37°C in a shaking aerobic incubator. Following 2 weeks of incubation, 10 mL of culture was subcultured for a month into 100 mL of Middlebrook 7H9 broth supplemented with OADC and mycobactin J. MAP cultures were regularly tested for viability by Ziehl-Nielssen acid fast staining and tested for contamination by streaking serial dilutions on tryptic soy agar (TSA) (Merck, Darmstadt, Germany). The appearance of any colonies, following 24 hours incubation at 37°C, indicated contamination.

Salmonella typhimurium (ST) was kindly provided by R. Curtiss III (Washington University, St Louis, MO) was stored in 50% glycerol at −70°C. Prior to use, ST was cultured at 37°C in tryptic soy broth (TSB) (Merck) for 18 hours under aerobic conditions.

Listeria monocytogenes (LM) was grown in Brain-Heart Infusion broth (BHI, Oxoid, Cambridge, UK) aerobically at 37°C for 18 hours.

The concentrations of all bacteria used were estimated by measuring the absorbance at 600 nm and extrapolating bacterial numbers from a previously generated standard curve of OD versus colony-forming units (cfu). Prior to use the bacteria were centrifuged, washed twice, and resuspended in complete media at 1×10^6, 1×10^7, and 1×10^8 cfu/mL.

T-cell Proliferation Assay

PBMCs from healthy volunteers, CD patients, and UC patients were washed with PBS and stained with 5 μM Carboxy Fluoroscein Succinimidyl Ester (CFSE) (CellTrace CFSE Cell Proliferation Kit; Invitrogen) dye for 10 minutes in darkness with occasional shaking. PBMCs were washed with warm DMEM containing 10% FCS and 1% penicillin-streptomycin and resuspended at 1×10^6 cells/mL in 24-well plates. Duplicate wells were exposed to MAP at a bacteria-to-cell ratio of 1:1, 10:1, and 100:1. Phytohemagglutinin (PHA-M, Sigma) was used as a positive control at 5 μg/mL. PBMCs remained unstimulated as negative controls. All cells were incubated for 4 days at 37°C to allow for proliferation. In order to specifically measure T-cell proliferation, PBMCs were washed in PBS and T-cells were stained with APC-CD3 (BD Biosciences, San Jose, CA) for 30 minutes at 4°C. The cells were analyzed by flow cytometry. Proliferation was measured as the percentage of CD3+ve cells that had decreased CFSE staining as evident from the multiple peaks indicating multiple rounds of cell division.

In Vitro Cell Cytokine Production

PBMCs or MLNCs were seeded at 1×10^6 cells/mL in duplicate conditions in 96-well plates (Costar, Schiphol-Rijk, Netherlands). The cells were stimulated for 72 hours with MAP, ST, or LM at 3 different bacterial concentrations: 1×10^6, 1×10^7, and 1×10^8 cfu/mL. Unstimulated cells were present to assess spontaneous levels of cytokine secretion. Plates were incubated in a 5% CO_2 and 37°C humidified atmosphere, after which supernatants were harvested for cytokine analysis. Cytokine production was measured using cytometric bead arrays and flow cytometry according to the manufacturer's instructions (BD CBA Flex Sets; BD Biosciences). Cytokines measured included interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ).

Statistical Analysis

Results were analyzed using SigmaStat software 3.0. Mann–Whitney Rank sum test, Kruskal–Wallis analysis of variance (ANOVA) on ranks with Holm–Sidak posttest, paired, and unpaired *t*-tests were used to assess for any difference among the groups. Values are illustrated as means $\pm$ SEM. Statistically significant differences were accepted at $P < 0.05$.

Ethical Considerations

This study was approved by the Ethics Committee of Cork University Hospital, and informed consent was obtained from all volunteers.

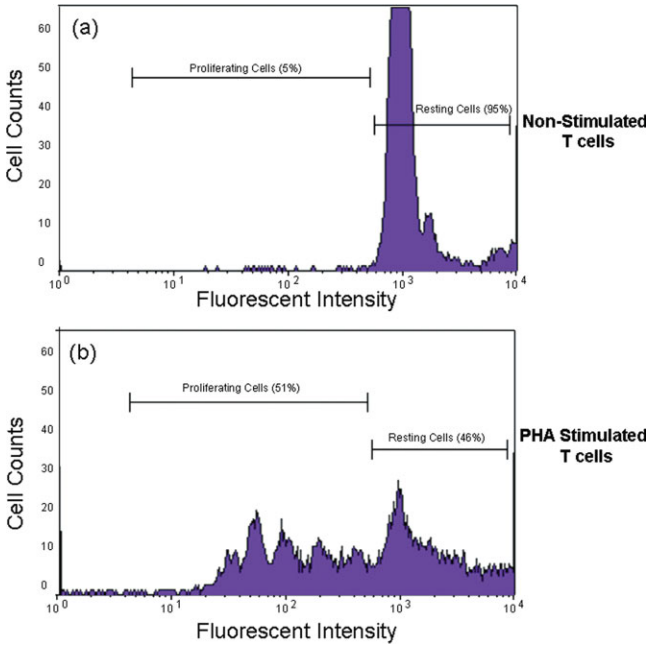

FIGURE 1. CFSE staining of T-cell proliferation. Representative examples are illustrated of CFSE-stained T-cells in unstimulated (a) and PHA-stimulated (b) PBMCs following 96 hours incubation. Resting and proliferating cells are indicated on the histograms. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS

MAP Influence on T-cell Proliferation

In vitro coincubation with MAP or PHA resulted in the proliferation of T cells, compared to nonstimulated negative control PBMC cultures, for all patient groups. A representative example is illustrated in Figure 1. There was a significant increase in the number of proliferating T cells in response to MAP in PBMCs isolated from CD patients when compared to PBMCs from the UC patients and healthy volunteers (Fig. 2a, $P < 0.05$). There was no significant difference in T-cell proliferation after exposure to MAP between PBMCs isolated from UC patients and healthy volunteers ($P = 0.211$). Prior BCG vaccination was not a contributing factor to this difference in T-cell proliferation, as similar rates were observed regardless of vaccination history. In order to eliminate the possibility that T cells from CD patients have an enhanced proliferative response independent of the antigen being tested, we assessed T-cell proliferation following exposure to PHA. In contrast to the result with MAP, T cells from CD patients proliferated significantly less in response to PHA compared to T cells from healthy volunteers (Fig. 2b). We observed a marked inverse relationship between MAP-induced T-cell proliferation and PHA-induced T-cell proliferation (Fig. 2c).

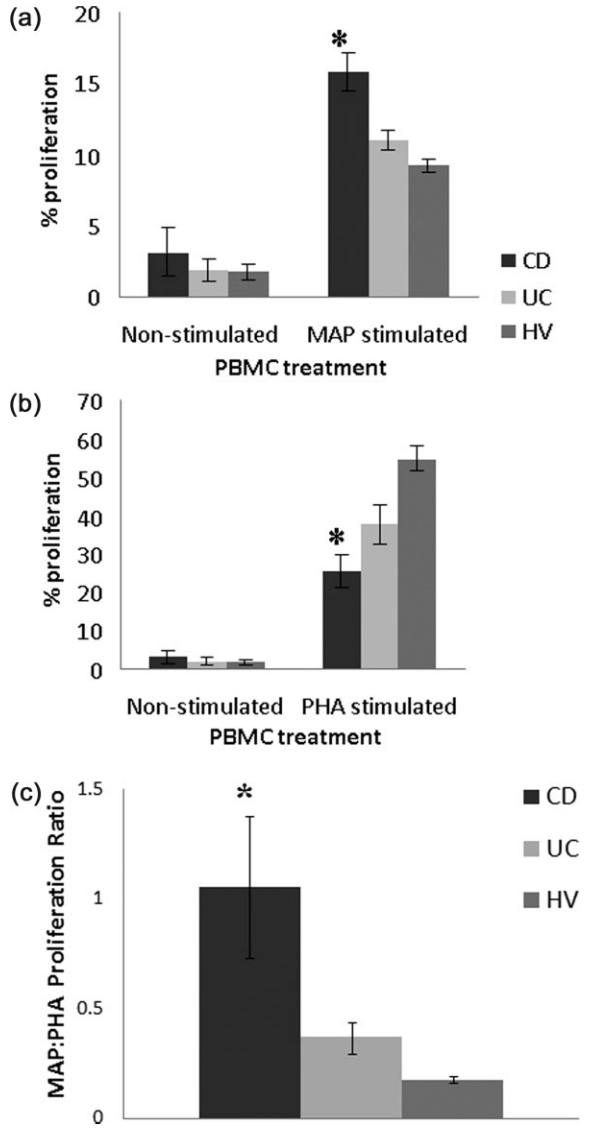

FIGURE 2. T-cell proliferation in response to MAP and PHA. (a) Coincubation with MAP resulted in a T-cell proliferative response from all individuals tested. However, more T cells responded to MAP in PBMCs from CD patients compared to T cells from the UC and healthy volunteer groups. No differences were observed in unstimulated cells from the 3 groups. Results are expressed as mean ± standard error for each group. *$P < 0.0001$ for CD patients ($n = 10$) versus healthy volunteers (HV; $n = 10$) and $P = 0.002$ for CD versus UC patients ($n = 10$). (b) In contrast to MAP, PHA stimulation resulted in a significantly lower T-cell proliferative response in PBMCs from CD patients compared to healthy volunteers. There was no statistically significant difference between the CD and UC ($n = 10$) T-cell response to PHA. Results are expressed as mean ± standard error for each group. *$P < 0.05$; CD patients ($n = 10$) versus HV ($n = 10$). (c) A marked inverse relationship between MAP-induced T-cell proliferation and PHA-induced T-cell proliferation was observed. Results are expressed as mean ± standard error for each group. *$P < 0.05$; CD patients ($n = 10$) versus HV ($n = 10$) and UC patients ($n = 10$).

PBMC Cytokine Response to Bacterial Strains

Following in vitro exposure to MAP, PBMCs secreted significant amounts of TNF-α in a dose-dependent manner (Fig. 3a). PBMCs from CD patients secreted significantly greater amounts of TNF-α in response to MAP

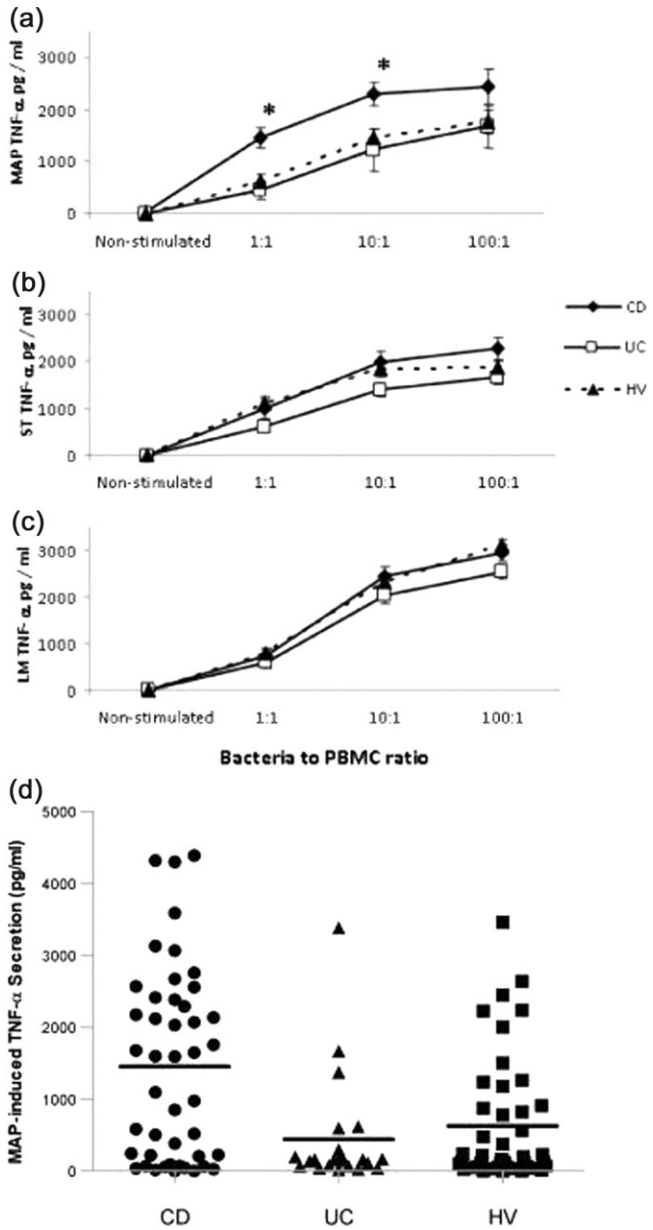

FIGURE 3. PBMC TNF-α response to bacteria. PBMCs from CD patients release significantly more TNF-α in response to MAP (a) but not in response to ST (b) or LM (c) as compared to PBMCs from UC patients or healthy individuals. Results are expressed as mean ± standard error for each bacterial concentration used. *$P < 0.05$; CD ($n = 45$) versus UC patients ($n = 22$) or HV ($n = 44$). (d) Scatterplot illustrating individual TNF-α responses upon stimulation with MAP (MAP-to-cell ratio of 1:1).

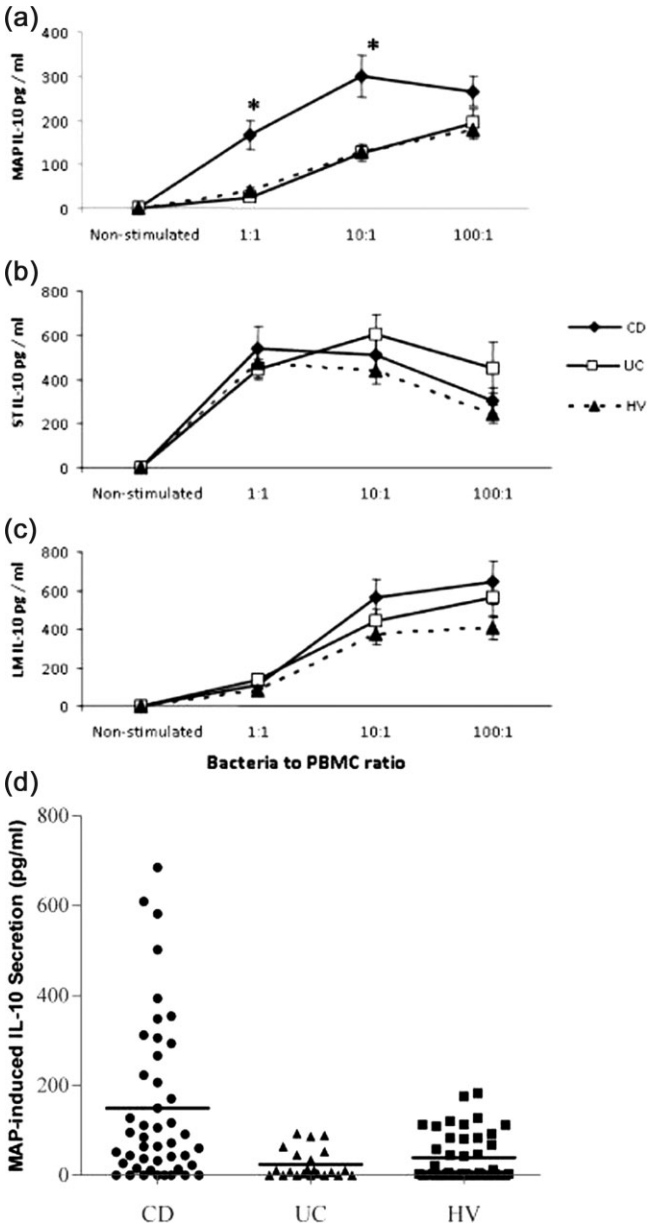

FIGURE 4. PBMC IL-10 response to bacteria. PBMCs from CD patients release significantly more IL-10 in response to MAP (a) but not in response to ST (b) or LM (c) as compared to PBMCs from UC patients or healthy individuals. Results are expressed as mean ± standard error for each bacterial concentration used. *$P < 0.05$; CD ($n = 45$) versus UC patients ($n = 22$) or HV ($n = 44$). (d) Scatterplot illustrating individual IL-10 responses upon stimulation with MAP (MAP-to-cell ratio of 1:1).

stimulation when compared to the level of TNF-α released by PBMCs from UC patients or healthy volunteers. This differential cytokine response was specific to MAP stimulation, as the PBMC TNF-α response to ST (Fig. 3b) and LM (Fig. 3c) was similar for CD patients, UC patients, and healthy volunteers. Although there was a wide variation in TNF-α responses, a common denominator could not be identified in high-responders (Fig. 3d).

A similar PBMC cytokine response was observed regarding IL-10 secretion. IL-10 release in response to MAP stimulation was dose-dependent and PBMCs from CD patients secreted significantly greater amounts of IL-10 compared to IL-10 secretion from UC patients or healthy volunteers (Fig. 4a). In addition, the enhanced response was restricted to MAP as the IL-10 response to ST and LM was identical for PBMCs from all patient groups (Fig. 4b,c). Similar to TNF-α responses, there was a wide variation in individual IL-10 responses (Fig. 4d). Patients who had a high TNF-α response did not necessarily exhibit a high IL-10 response.

In contrast to the cytokine results presented above, the IFN-γ response to MAP was significantly elevated in PBMCs isolated from healthy volunteers compared to PBMCs derived from either IBD group (Fig. 5a). The IFN-γ response was also elevated in the healthy volunteer PBMC coincubations with ST or LM (Fig. 5b,c).

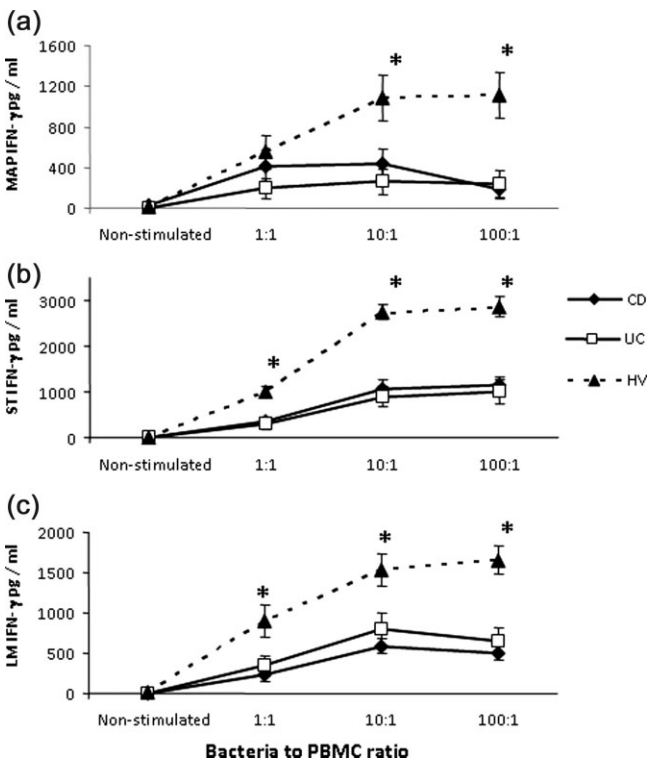

FIGURE 5. PBMC IFN-γ response to bacteria. PBMCs from CD and UC patients release significantly less IFN-γ than PBMCs from healthy volunteers (HV) upon stimulation with MAP (a), ST (b), and LM (c). Results are expressed as mean ± standard error for each bacterial concentration used. *$P < 0.05$; HV ($n = 44$) versus CD ($n = 45$) or UC patients ($n = 22$).

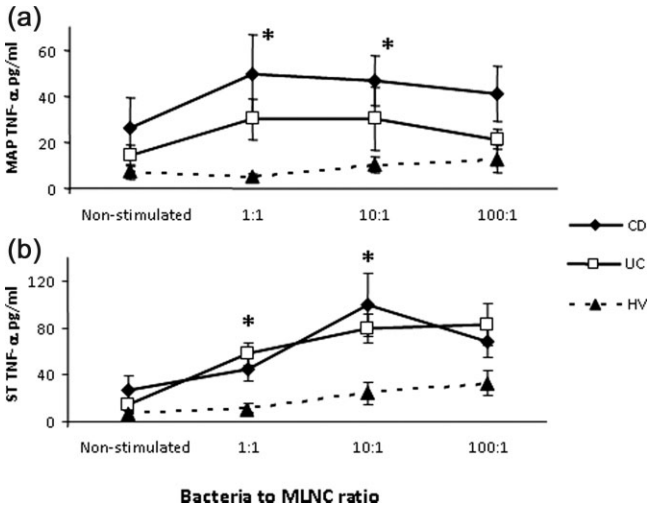

FIGURE 6. MLNC TNF-α secretion in response to bacteria. MLNCs from CD and UC patients secrete significantly more TNF-α than MLNCs from non-IBD controls in response to stimulation with MAP (a) or ST (b). There was no difference between the CD and UC groups. Results are expressed as mean ± standard error for each bacterial concentration used. *$P < 0.05$; CD ($n = 6$) or UC ($n = 6$) versus non-IBD controls (HV; $n = 7$).

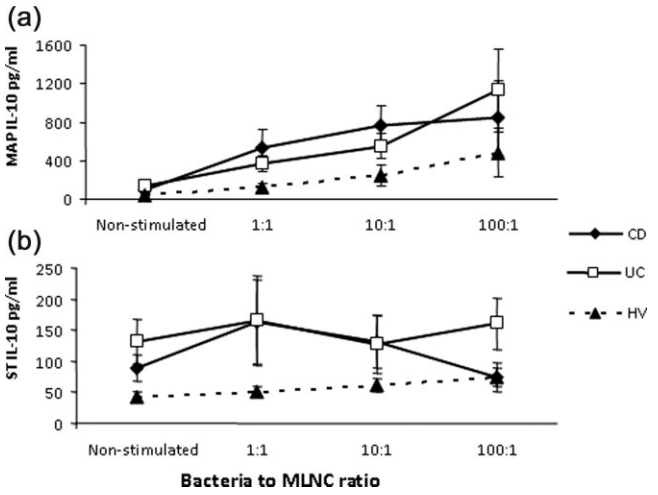

FIGURE 7. MLNC IL-10 response to bacteria. MLNCs from CD and UC patients secreted substantially more IL-10 than MLNCs from non-IBD controls in response to stimulation with MAP (a) or ST (b). However, these differences did not reach statistical significance. Results are expressed as mean ± standard error for each bacterial concentration used. CD ($n = 6$), UC ($n = 6$), and non-IBD controls (HV; $n = 7$).

Both CD and UC patients exhibited elevated levels of IL-6 secretion in response to MAP compared to healthy volunteers (results not shown). However, this difference was restricted to MAP, as the PBMC IL-6 response to ST or LM was identical for all groups. No differences in IL-2, IL-4, or IL-8 secretion by PBMCs in response to bacterial stimulation was observed between the 3 groups.

MLNC Cytokine Response to Bacteria

MLNCs from CD and UC patients secreted significantly more TNF-α than MLNCs isolated from non-IBD controls in response to stimulation with MAP or ST (Fig. 6a,b). Indeed, TNF-α secretion did not increase over baseline non-stimulated values for the bacterial stimulated MLNCs from non-IBD controls. There was no difference between the CD and UC groups. Similarly, the IL-10 response to MAP and ST was elevated in the MLNCs isolated from IBD patients compared to MLNCs obtained from non-IBD controls (Fig. 7a,b). Finally, MLNCs from both groups of IBD patients released significantly more IL-8 in response to MAP stimulation, but not ST, when compared to the MLNC response from non-IBD control patients (results not shown).

Effect of BCG Vaccination and Immunosuppressants on Cytokine Production

There were no significant differences in the levels of cytokines secreted in response to MAP by PBMCs from subjects who had prior BCG vaccination to those who did not. An example is presented in Table 1, whereby PBMC release of TNF-α in response to MAP (10:1 bacteria:PBMC) is not influenced by vaccination status. In addition, there was no significant difference in cytokine production between CD patients who were ($n = 19$) and those who were not ($n = 26$) on 6-MP.

DISCUSSION

This study demonstrates that T cells from patients with CD exhibit an enhanced proliferative response to

TABLE 1. Secretion of TNF-α in Response to MAP Is Not Influenced by Prior BCG Vaccination

	BCG –ve (TNF-α)	BCG +ve (TNF-α)	*P*-value
Crohn's disease *n*=45	*n*=26 (2226 ± 293 pg/mL)	*n*=19 (2407 ± 377 pg/mL)	0.702
Ulcerative colitis *n*=22	*n*=12 (1569 ± 732 pg/mL)	*n*=10 (799 ± 215 pg/mL)	0.364
Healthy controls *n*=44	*n*=19 (1350 ± 230 pg/mL)	*n*=25 (1521 ± 252 pg/mL)	0.631

MAP when compared to T cells from UC patients and healthy volunteers. This contrasts with a reduced response to the mitogen, PHA. In addition, secretion of TNF-α and IL-10 by peripheral blood mononuclear cells in response to MAP is also greater in patients with CD. The differential effects are unlikely to be a nonspecific or disease-related effect and are bacterial strain-dependent because the TNF-α and IL-10 response to ST and LM was similar for CD, UC, and healthy controls. The results are consistent with an increased frequency of MAP-reactive T cells due to prior exposure to MAP in patients with CD.

Both the innate and adaptive immune responses are important in the pathogenesis of CD.[21,22] One hypothesis is that impaired innate immunity could be a predisposing risk factor for the development of CD.[23] In contrast, enhanced innate responses in patients with CD to bacteria or bacterial components, such as flagellin, mediated by pattern-recognition receptor activation are also well described.[15,22,24] Activation of the innate system by MAP is primarily due to recognition by pattern recognition receptors such as TLR-2, TLR-4, and NOD2.[25] For example, inhibition of TLR-4 in human PBMCs resulted in reduced TNF-α and IL-10 production after stimulation with live MAP. In addition, PBMCs from individuals homozygous for the 3020insC NOD2 mutation showed a defective cytokine response after stimulation with MAP.[25] It is unlikely that the enhanced TNF-α response to MAP observed in our CD patient group is due simply to a change in pattern recognition receptor expression, as there was no difference in the TNF-α response to ST or LM, which also trigger the same receptors.[26,27] Thus, our results suggest that the adaptive immune response to MAP may be responsible for the enhanced cytokine production. In addition, the altered T-cell proliferative response to MAP in CD patients supports this hypothesis.

A high level of TNF-α secretion in response to MAP is particularly important in patients with CD due to the potent response of patients to anti-TNF therapies. Our data are in agreement with another study that examined the association between CD, MAP, and TNF-α secretion. Significantly higher TNF-α concentrations were found in mucosal organ culture supernatants from CD compared to UC, irritable bowel syndrome, and controls. When TNF-α levels were correlated with the presence of MAP, significantly greater concentrations were only found in MAP-positive CD patients.[28]

Immunologic tolerance to bacterial antigens is primarily mediated by IL-10 and TGF-β, produced by regulatory T cells and dendritic cells.[24] Although IL-10 is considered an antiinflammatory cytokine, it could have a complicating role in relation to MAP. Neutralization of IL-10 has been shown to enable macrophage killing of MAP and increase the expression of TNF-α in in vitro cultured bovine monocyte-derived macrophages.[29] The enhanced IL-10 response to MAP observed in our study could contribute to the survival and immune escape of MAP, hence accounting for the increased detection rates in CD tissues.

The role of IFN-γ in IBD is uncertain, with studies variably reporting enhanced,[30,31] reduced,[32] or unaltered[33] levels of this cytokine in blood and mucosal tissues. In our study, PBMC release of IFN-γ was significantly lower in both the CD and the UC patients compared to healthy volunteers upon exposure to MAP, ST, and LM. It is intriguing to hypothesize that mucosal inflammation is associated with an inability to mount an appropriate IFN-γ response and thus combat intracellular bacteria. This could contribute to the increased frequency and persistence of mycobacteria or MAP and related organisms within tissues from IBD subjects. Our results are in agreement with a previous study demonstrating lower IFN-γ responses in response to mycobacterial antigens from whole blood and PBMC cultures from IBD patients.[13] IFN-γ has been suggested to exert a dual role, where low levels would contribute to physiological inflammation and higher levels would be associated with an uncontrolled inflammatory state.[34] Perhaps an innate inability to secrete adequate levels of IFN-γ, coupled with an exaggerated IL-10 and TNF-α response, could drive mucosal inflammation in susceptible individuals when infected with certain pathogenic intracellular microorganisms. Alternatively, MAP infection may directly suppress activation of the immune response. Supernatants from MAP cultures have been shown to inhibit *E. coli* killing by adherent human monocytes.[38] The authors of that study suggested that CD may occur due to an acquired defect in phagocyte function, driven at least in part by cell wall mannans shed by microbes (including MAP) within the gut. CD patients commonly express circulating anti-*Saccharomyces cerevisiae* antibodies (ASCA), which are reactive with mannan epitopes also expressed by MAP.[39,40] A prospective study is required to investigate the correlation between ASCA positivity, the presence of MAP, and an altered immune response in patients with CD.

IFN-γ release assays (IGRA) are increasingly being used for *Mycobacterium tuberculosis* screening prior to anti-TNF treatment in CD. These tests detect tuberculosis infection by measuring in vitro T-cell IFN-γ release in response to 2 *M. tuberculosis* antigens, ESAT-6 and CFP-10, that are absent from BCG vaccine and most nontuberculous mycobacteria.[41] Our results show reduced IFN-γ release in response to a range of bacteria (MAP, ST, and LM), suggesting that negative IGRA results in CD patients should be interpreted with caution.

We found that MLNC cytokine responses to MAP and ST were elevated in both the CD and UC patients compared to MLNCs from non-IBD controls. The mucosal cytokine response does not appear to be unique to MAP in these patients. First, this is consistent with a previous

report by us and may reflect nonspecific immune activation in lymph nodes draining an inflamed segment of mucosa.[20] We did find previously that MLNCs and MLN-derived dendritic cells secreted significant amounts of TNF-α but not IL-10 in response to ST challenge.[20] Second, our control MLNCs could give rise to the confounding factor of local immunosuppression in tumor-draining lymph nodes as reported by us and others.[35] 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.

In conclusion, while several investigators have reported increased rates of detection of MAP in CD, the results have been variable and inconsistent. Our data are consistent with previous exposure and immune engagement with MAP is more common in patients with CD. This does not imply a cause and effect relationship with the disease but the altered T-cell proliferation and cytokine responses may be a modifying variable if patients are continually or repeatedly exposed to MAP in the food chain.

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