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Role of Vitamin D, Folate, and Cobalamin Deficiency in Mycobacterium avium Paratuberculosis Infection and Inflammation

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ROLE OF VITAMIN D, FOLATE, AND COBALAMIN DEFICIENCY IN *MYCOBACTERIUM AVIUM PARATUBERCULOSIS* INFECTION AND INFLAMMATION

by

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ABSTRACT

Vitamin D, folate, and cobalamin (vitamin B_{12}) are crucial micronutrients in human physiology that are necessary for healthy calcium, phosphorus, and single-carbon metabolism. Recent studies have indicated that these vitamins also affect the inflammatory response in ways unrelated to their well-characterized deficiencies. Accordingly, analysis of their effect on chronic inflammatory diseases like Crohn's disease (CD) is warranted. This investigation examines the effects of vitamin deficiency on macrophages and intestinal epithelial cells upon exposure to *Mycobacterium avium paratuberculosis* (MAP,) a pathogen capable of triggering CD, to model the inflammatory response in clinical CD patients. ELISA analysis of CD patient plasma established that MAP-positive patients have lower folate, vitamin B_{12}, and active vitamin D (calcitriol) than MAP-negative patients. Next, we investigated the effects of folate and vitamin B_{12} deprivation on macrophages to assess inflammatory cytokine expression, oxidative stress, and macrophage apoptosis. We determined that folate and B_{12} deprivation exacerbates inflammation while preventing infected macrophages from successfully undergoing apoptosis, whereas supplementation reversed these effects. Then, we examined the role of vitamin D in regulating cathelicidin expression during MAP infection. MAP infection blocked the conversion of inactive vitamin D (calcifediol) to calcitriol, thereby interrupting the expression of the antimicrobial peptide cathelicidin. Calcitriol treatment restored cathelicidin production, reduced inflammation and bacterial viability, and reduced oxidative stress in co-cultured macrophages, Furthermore, cathelicidin knockdown abolished calcitriol's beneficent effects. These studies detail the importance of vitamin availability for healthy immune functionality. The attenuation of inflammation during MAP infection further indicates that CD patients, who are at elevated risk

of vitamin deficiency, may benefit from supplementation or clinical screening for low vitamin levels.

To my parents, who knew how to keep me grounded and sane even when I didn't.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA: Analysis of variance

BHMT: Betaine homocysteine methyltransferase

CAMP: Cathelicidin Antimicrobial Peptide

Ccl3: Chemokine (C-C motif) ligand 3

CD: Crohn's disease

CD4+: Cluster of differentiation 4-positive

CD8+: Cluster of differentiation 8-positive

cDNA: complementary DNA

CI: Confidence interval

CYP27A1: Cytochrome P450 Family 27 Subfamily A Member 1

CYP27B1: Cytochrome P450 Family 27 Subfamily B Member 1

DAPI: 4′,6-diamidino-2-phenylindole

DHE: Dihydroethidium

DHF: Dihydrofolate

DHFR: Dihydrofolate reductase

ELISA: Enzyme-linked immunosorbent assay

EMEM: Eagle's Minimum Essential Medium

ER: Endoplasmic reticulum

FTHFS: Formyltetrahydrofolate synthase

ERK1/2: Extracellular signal-regulated protein kinases 1 and 2

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Gly: Glycine

Hcy: Homocysteine

IBD: Inflammatory Bowel Disease

IF: Intrinsic factor

IgA: Immunoglobulin A

IL-1β: Interleukin 1-beta

IL-6: Interleukin 6

IL-10: Interleukin 10

IRES: Internal ribosome entry site

JNK: C-Jun N-terminal kinase

LPS: Lipopolysaccharide

Lyz1/2: Lysozyme 1 and 2

MAP: *Mycobacterium avium* subsp. *tuberculosis*

MAT: Methionine adenosyltransferase

Met: Methionine

MS: Methionine synthase

MTases: Methyltransferases

Mtb: *Mycobacterium tuberculosis*

MTHFD: Methylenetetrahydrofolate dehydrogenase

MTHFR: Methylenetetrahydrofolate reductase

NADP: Nicotinamide adenine dinucleotide phosphate

NK cell: Natural killer cells

NLRP3: NLR family pyrin domain containing 3

NOS: Nitric oxide synthase

NOX-1: NADPH oxidase 1

PMA: Phorbol 12-myristate 13-acetate

PTH: Parathyroid hormone

RA: Rheumatoid arthritis

RT-qPCR: Reverse transcription quantitative real-time PCR

RXR: Retinoid X receptor

SAM: S-adenosyl-L-methionine

SAH: S-adenosyl homocysteine

Ser: Serine

SHMT: Serine hydroxymethyltransferase

siRNA: Short interfering RNA

STAT1/3: Signal transducer and activator of transcription 1 and 3

THF: Tetrahydrofolate

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor alpha

TNXIP: Thioredoxin interacting protein

TS: Thymidylate synthase

UC: Ulcerative colitis

VDR: Vitamin D receptor

CHAPTER 1: BACKGROUND

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Crohn's Disease and Mycobacterium avium subsp. paratuberculosis

Crohn's disease (CD) is a form of inflammatory bowel disease (IBD) characterized by asymmetrical, segmental, transmural inflammation in the gastrointestinal tract [1]. Most CD affects the terminal ileum and colon, but inflammation has also been recorded in other segments ranging from the mouth to the anus [1,2]. Common symptoms include abdominal pain, diarrhea, weight loss, and fever [1]. CD is most prevalent in North America and Europe, but countries in the process of industrialization are experiencing rises in CD incidence yearly [3]. CD flareups are associated with gut dysbiosis and reduction of anti-inflammatory microbial pathways in gut flora [4,5]. While CD is multifactorial and involves genetic, environmental, and microbial involvement, some bacteria are capable of triggering CD inflammation in susceptible patients [1,6]. Among these bacteria is *Mycobacterium avium paratuberculosis* (MAP,) a slow-growing, obligate intracellular pathogen and relative of *Mycobacterium tuberculosis* (Mtb) [6]. In ruminants, MAP causes Johne's disease, a chronic wasting disease transmitted through infected milk [7]. Assessing the mechanisms by which MAP induces CD, why some patients do not develop CD upon MAP exposure, and how MAP-induced CD differs from MAP-free CD is an ongoing research priority.

Prolonged or mismanaged CD flareups can result in complications that require surgical correction; these include strictures, fistulas, and abscesses [2]. During a stricture, fibrosis of the intestinal lining constricts the flow of fecal matter through the intestine, which leads to ballooning and pressure in the preceding intestinal segment [8]. Fistulas, by contrast, cause tissue remodeling in the gut that leads to the intestinal lining budding out from the intestine, sometimes causing an abscess [9]. Surgical resection of intestines afflicted with these complications is effective; however, patients who require surgery once frequently develop further complications necessitating additional surgeries [10]. Furthermore, CD patients are at elevated risk of gastrointestinal malignancies, anemia, and vitamin deficiency, particularly vitamin D, folate, and vitamin B_{12} deficiency [1,11-13]. Proactive management is, therefore, key to inducing and maintaining CD remission [2].

Current first-line treatment for moderate to severe CD utilizes biologic drugs, typically anti-TNF-α monoclonal antibodies to alleviate pro-inflammatory cytokine-mediated inflammation; however, this approach has serious shortcomings [14]. Approximately 50% of patients fail to respond to anti-TNF-α treatment, with more eventually losing responsiveness; furthermore, prolonged blockage of TNF-α function sensitizes patients to granulomatous diseases like tuberculosis [14,15]. Therefore, new treatment paradigms are necessary for CD maintenance. One possible therapeutic approach for CD treatment is nutritional support to compensate for malnutrition caused by malabsorption; small studies have shown that enteral nutrition can help maintain remission induced by anti-inflammatory pharmaceuticals [16]. The following review will discuss links between vitamins commonly deficient in CD patients and inflammation.

Vitamin D and Inflammation

In contrast to most vitamins, vitamin D does not mediate physiological effects through cofactor activity [17]. Rather, vitamin D is an endogenously synthesized steroidal hormone with several provitamin forms [17]. 7-dehydrocholesterol is converted upon exposure to ultraviolet radiation to cholecalciferol (D_3), the most common dietary form of vitamin D, which is subsequently hydroxylated at the 25' position to yield calcifediol [18]. Calcifediol remains in the bloodstream as the primary circulating form of vitamin D until it is hydroxylated at the 1' position by CYP27B1 to yield calcitriol, the active form of vitamin D [19]. Calcitriol binds to the vitamin D receptor (VDR,) which heterodimerizes with the retinoid X receptor (RXR) and translocates to the nucleus to function as a transcription factor [18]. While vitamin D-mediated transcription is best known for its central role in bone mineralization and calcium and phosphorus homeostasis, recent research has established a role for calcitriol signaling in the innate immune system and inflammation [20,21]. In macrophages, calcitriol is a necessary signal for Toll-like receptor (TLR)-mediated upregulation of cathelicidin production, thereby connecting nutrient intake with the anti-microbial immune response [22,23]. As CD patients are at elevated risk of vitamin D deficiency, patient macrophages may have impaired bacterial clearance capacity during periods of vitamin D deprivation, resulting in exacerbating disease pathology [24]. Vitamin D supplementation as a component of CD therapy, however, is a complex approach that requires further investigation [25].

Methyl Donors and Inflammation

The methionine cycle plays a crucial role not merely in the regulation of the essential methionine but also in the generation of S-adenosyl methionine (SAM), a ubiquitous cofactor necessary for methyltransferase reactions. Folate (vitamin B9) and cobalamin (vitamin B_{12}) are cofactors necessary for successful methionine regeneration from homocysteine, a vascular risk factor, and for maintaining an abundant level of intracellular SAM. Deficiency in SAM or its precursors leads to dysregulation of crucial methylation and cellular dysfunction. In this review, we discuss the metabolic pathways responsible for generating methionine and SAM, and the consequences of SAM deficiency in gastrointestinal tissue. We also discuss the effects of folate and B_{12} deficiency in correlation with SAM depletion in clinical studies, animal models, and cell culture systems. The observations collated in the following section highlight the complex role of methionine and SAM in human physiology and disease.

Folate, B_{12}, and the Methionine Cycle

Folate and B_{12}

Folate, also known as vitamin B_9, is a crucial vitamin in humans isolated from spinach in 1941. The name folate was derived from the Latin word for leaf, *folium* [26]. Folate supplementation soon showed similar effects as yeast and liver extracts in the prevention of megalocytic anemia [27]. Leafy vegetables and citrus fruits are high in natural folates, and the synthetic form of folate, folic acid, has been mandated as a grain supplement by the US Food and Drug Administration since 1998 [26,28,29]. Probiotic intestinal bacteria also have been known to synthesize folate and secrete it into the environment [30]. In mouse models, this phenomenon has

been shown to alleviate colitis [31]. Deficiency of folate leads to impaired red blood cell generation, resulting in macrocytic anemia; during pregnancy, increased folate consumption is required to avoid neural tube defects in the fetus [32,33]. Polymorphisms in genes related to folate metabolism such as *MTHFR* and *MTHFD* (encoding methylenetetrahydrofolate reductase and dehydrogenase, respectively) have been correlated with complications and spontaneous abortion during pregnancy, though sometimes only when there are compound mutations [34,35]. For some patients, a varied diet is insufficient to avoid folate deficiency: chronic malabsorption or improper folate storage can be caused by alcoholism, inflammatory bowel disease, celiac disease, and tropical sprue [36-40]. Folate comprises a pteridine ring, *para*-aminobenzoic acid, and at least one glutamic acid (Glu) residue. Glu residue count is highly variable, and the synthetic vitamin folic acid contains only one [41,42]. In the case of polyglutamic folates, the additional Glu residues are removed by the intestinal mucosa prior to release into circulation [43].

Both natural folates and synthetic folic acid undergo similar metabolic processing before their function as a coenzyme is realized: they are first absorbed by the upper small intestine by a carrier protein in the epithelium [44]. Absorbed folates are converted to dihydrofolate (DHF) and tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR) [45]. Upon conversion to THF, folate can participate as a cofactor in single-carbon metabolism. THF serves as a transient carrier of methyl groups via a two-enzyme process. Serine hydroxymethyltrasferase (SHMT) catalyzes the conversion of THF and serine to glycine and 5,10-methylenetetrehydrofolate, using vitamin B6 as a cofactor [46]. Methylenetetrahydrofolate reductase (MTHFR) then converts the latter product into L-5-methyltetrahydrofolate [47]. However, this is not the only method of

processing folate: 5,10-methylenetetrahydrofolate can be used by thymidylate synthase (TS) to convert deoxyuridine monophosphate (dU) to deoxythymidine monophosphate (dT), yielding DHF in the process as reviewed by Wilson and Mertes [48]. In addition to this metabolic product, formyltetrahydrofolate synthetase (FTHFS) fuses a free formic acid with THF to generate 10-formyltetrahydrofolate, an essential precursor in purine biosynthesis [49,50]. Alternatively, 5,10-methylenetetrahydrofolate can be processed via methylenetetrahydrofolate dehydrogenase (MTHFD) to generate the same metabolite [51]. For this review, the term 'folate' will refer to folates before their metabolism into a coenzyme (as they exist both naturally and synthetically in the diet) and their coenzyme forms. A schematic representation of folate metabolism is shown in Figure 1.

Vitamin B_{12} consists of a corrin ring with a central cobalt molecule: attached to the cobalt atom from above is a variable ligand distinguishing bioactive and dietary forms of the vitamin, while below is a ribose-3-phosphate-dimethylbenzimidazole ligand [52-54]. Some species in the human intestinal microbiota have been found to synthesize B_{12}, though it is not believed that gut bacteria serve as a significant source of B_{12} in humans [55]. Like folate, it requires biochemical modification before it can participate in metabolism. Human biochemistry makes use of adenosylcobalamin and methylcobalamin, which are generated from dietary hydroxocobalamin and cyanocobalamin. Unlike folate, only two reactions in normal metabolism require vitamin B_{12} as a cofactor: the conversion of methylmalonyl-CoA to succinyl-CoA in the mitochondrion and the conversion of homocysteine to methionine in the cytoplasm. A more thorough review has been conducted by Allen and colleagues [56]. Despite this limited biochemical utility, B_{12} is essential, and deficiency results in pernicious anemia due to impaired red blood cell development

[57]. This effect is sometimes masked by folate's beneficial effect on erythropoiesis and can lead to misdiagnosis—a dangerous situation, as prolonged B_{12} deficiency also has deleterious effects on myelination and nervous system development [58-60]. Infants are particularly susceptible to neurological degeneration from B_{12} deficiency, and failure to restore normal levels can result in severe damage [61-63].

The metabolism of cobalamin, known as vitamin B_{12}, is thoroughly intertwined with folate. B_{12} intestinal uptake is mediated by intrinsic factor (IF), a protein secreted by parietal cells in the stomach [64]. The IF- B_{12} complex is then taken up by the cubam receptor in the terminal ileum and transported through circulation via haptocorrin or transcobalamin protein carriers [65,66]. The metabolism of cobalamin, known as vitamin B_{12}, is thoroughly intertwined with folate. Intrinsic factor (IF), a protein secreted by parietal cells in the stomach, mediates B_{12} intestinal uptake [64]. The IF- B_{12} complex is then taken up by the cubam receptor in the terminal ileum, released into serum by MRP1, and transported through circulation via haptocorrin or transcobalamin protein carriers [65-67]. Most cobalamin in serum is bound to haptocorrin; however, most cells cannot absorb the haptocorrin- B_{12} complex [68]. Instead, the ubiquitously expressed transcobalamin receptor (TCblR) mediates uptake of the transcobalamin-B_{12} complex into the cell via endocytosis [69,70]. Upon lysosomal degradation, the receptor is destroyed, and B_{12} is released: the proteins ABCD4 and LMBD1 are necessary for translocation across the lysosomal membrane [71,72]. MMACHC, also called CblC, receives the translocated B_{12} and catalyzes the removal of alkyl or cyanide ligands [73,74]. It coordinates with MMADHC, alternatively named CblD, an enzyme that facilitates cob(II)alamin oxidation to aquocobalamin [75]. The CblC/CblD complex interacts with methionine synthase (MS) and

methionine synthase reductase (MSR) to ensure efficient cofactor delivery to its associated enzyme [76]. In addition, the enzyme methylmalonyl-CoA mutase in mitochondria requires adenosylcobalamin to function; however, the mechanism by which mitochondria take up cytosolic B_{12} remains unclear. One study in *C. elegans* suggests that an ABCG family protein may mediate membrane transport, but no mitochondrial membrane proteins have been identified in humans so far [77].

Any malfunction in this multi-factorial and complex process could lead to interruption in B_{12} uptake, causing deficiencies and ultimately an increased risk of developing disease. For example, autoantibodies against parietal cells or IF mutation may result in reduced IF secretion, which leads to B_{12} malabsorption and deficiency [78,79]. Similarly, damage to the ileum due to surgery or chronic inflammation, as seen in Crohn's disease, causes impairment in B_{12} uptake and increases the risk of developing additional symptoms and complications [11,80]. Metformin use in type 2 diabetes mellitus has also been correlated with decreased serum B_{12}; however, the mechanism underlying this phenomenon is unclear [81,82]. Impaired intracellular B_{12} metabolism may likewise lead to metabolic dysfunction [83]. Accumulation of unabsorbed B_{12} causes bacterial overgrowth and inflammation [84]. Vegetarians, mainly vegans, are also susceptible to vitamin B_{12} deficiency due to a different cause: vitamin B_{12} is primarily found in animal products and is poorly represented in plants [85-87]. As such, nutritional supplements are sometimes indicated in this patient group; a more comprehensive review of B_{12} deficiencies in differing vegetarian diets has been performed by Pawlak and colleagues [88].

Folate and B_{12} belong to a class of vitamins known as methyl donors, a descriptor they share with choline and betaine, as summarized by Zeisel [89]. Methyl donors are so named

because of their importance in single carbon metabolism, by which methyl groups are transferred from sources such as serine, glycine, and choline to a variety of other compounds, including proteins, RNA, DNA, and intermediate metabolites. The targets for methylation been extensively reviewed elsewhere [90,91]. This process is accomplished via the folate and methionine cycles. L-5-methyltetrahydrofolate donates the methyl group attached to its 5' carbon to B_{12} and subsequently homocysteine, converting it into methionine in a reaction catalyzed by methionine synthase (MS). This reaction yields tetrahydrofolate, which can participate in formyl and methyl group metabolism as described previously. Folate and B_{12} insufficiency leads to impeded methionine regeneration [92]. Methionine synthase's continued function depends on the availability of methionine synthase reductase, an associated protein that reduces the nonfunctional Cb(II) ion of B_{12} to Cb (I), ensuring continuous cofactor function [93]. The importance of folate in fetal neural tube development has already been mentioned, but general methyl donor depletion in early life has been found to alter both long-term neurological changes in mice and intestinal development in rats [94-96]. When these findings are combined with research on the necessity of methyl donors for developing B cells, a picture of cell growth and differentiation emerges, highly dependent on methyl donor availability and methionine metabolism [97].

S-Adenosyl Methionine

S-adenosyl methionine is a modified form of the essential amino acid methionine, where an adenosyl group is covalently connected to the sulfur to generate a sulfonium ion. It serves as a universal methyl donor for a class of enzymes known as methyltransferases (MTases,) which

catalyze the transfer of methyl groups to biomolecules like DNA, RNA, protein, and other metabolites requiring, as reviewed previously [90,91]. SAM is synthesized from methionine and ATP via the enzyme methionine adenosyltransferase (MAT), also called S-adenosyl methionine synthase [98]. SAM is a crucial precursor to the anti-inflammatory polyamines spermidine and spermine [99]. Removal of the methyl group by MTases converts SAM to S-adenosyl homocysteine (SAH), subsequently degraded into adenosine and homocysteine by SAH hydrolase. This reaction can be inhibited by adenosine dialdehyde and similar compounds, small-molecule SAH analogs. These compounds have been used as indirect methyltransferase inhibitors by halting the cycle at this point, consequently impeding methionine regeneration and causing SAH buildup, as SAH is a methyltransferase inhibitor [100].

Following hydrolysis, homocysteine remains in the body as an intermediate metabolite and nonessential amino acid with several potential fates. It can be converted to homocysteine thiolactone with Met-tRNA synthetase and joined to proteins via oxidation with thiol groups [101]. Homocysteine can also be processed by cystathionine-β synthase to yield cystathionine, which is subsequently converted to cysteine via cystathionine-γ lyase using vitamin B6 as a cofactor. The process is referred to as the trans-sulfuration pathway, and it is crucial to the successful removal of SAH, a potent MTase inhibitor [102]. Alternatively, it can be remethylated to methionine via one of two pathways. Previously we mentioned vitamin B_{12}'s cofactor activity in coordinating the removal of a methyl group from 5-methyltetrahydrofolate in converting homocysteine to methionine. In addition to this mechanism, betaine homocysteine methyltransferase (BHMT) can regenerate methionine from homocysteine by removing a methyl group from betaine, a derivative of the methyl donor and neurotransmitter choline [103]. This

process has been summarized in Figure 2. Interestingly, the enzymes in the pathway are also used for the metabolism of selenocysteine and selenomethionine, which are structurally similar to their sulfur-containing counterparts but far less common in the body [104,105].

Defects in methionine and SAM metabolism, either genetic or resulting from low levels of folate or B_{12}, result in the buildup of one or more metabolites, associated with deleterious effects in the body. Of these, hyperhomocysteinemia, defined as excessively high serum homocysteine levels, has been associated with both global and tissue-specific inflammation, as well as deleterious effects on the vasculature and bone [106-111]. At another stage of the methionine cycle, hypermethionemia, or excessive levels of methionine, is also observed to alter cell proliferation: when artificially induced in culture, activated T cells divide more rapidly [112]. SAM can also serve as a precursor to purine nucleotides; as such, it can be administered as part of a combination therapy to patients with congenital abnormalities in purine biosynthesis to alleviate disease progression and symptoms [113].

Folate, B_{12}, and SAM: Links to Tissue-Specific Inflammation

The Gastrointestinal Tract

Folate deficiency in IBS patients is neither surprising nor unprecedented. Studies as early as 1968 describe low serum folate in groups of Crohn's patients, and a recent study found folate-associated metabolic pathways perturbed in CD and ulcerative colitis (UC) [114,115]. As such, folate metabolism has not gone unnoticed as a therapeutic target. In a pediatric IBD cohort dosed for one month with folate supplements, researchers found changes in micronuclei, nucleoplasmic bridge formation, and apoptosis in enterocytes and peripheral blood lymphocytes. These results

were segregated by IBD typing: CD patients showed decreased signs of chromosomal damage with folate supplementation, while UC patients showed increased signs of it [116]. Folic acid supplementation reduced the incidence of side effects of methotrexate, an immunosuppressive drug and folate analog used to treat CD and rheumatoid arthritis [117,118]. A review of meta-analyses regarding environmental risk factors identified high folate levels as protective against the development of IBD—this suggests that the action of folate in the gut is not necessarily a reaction against the symptoms of IBD but may have a prophylactic effect [119]. Genetic factors controlling folate metabolism have been correlated with increased risk of IBD: the substitution of A2756 to G *MTR*, the gene encoding methionine synthase, is particularly notable for corresponding with IBD in a 2009 meta-analysis [120]. Examination of a mechanistic basis for this phenomenon is still not clearly understood.

Folate bioavailability has shown distinct effects on host-microflora interactions. Folate biosynthetic pathways are downregulated in intestinal bacteria during a CD relapse, an observation which pairs with the finding that folate-producing probiotic bacteria alleviate the inflammatory effects of chemically induced colitis in mice [31,121]. These phenomena are observed in other animal models as well: methyl donor-enriched diets in a mouse model of CD alter the expression of genes involved with colonization by adherent-invasive E. coli: the surface marker used by the pathogen to adhere to the epithelial lining was downregulated, as was calprotectin, an inflammatory marker, and IgA secretion. By contrast, antibacterial genes such as Lyz1 and Lyz2 were upregulated [122]. This study builds upon earlier findings in guinea pigs indicating that folate-deficient diets in early life sensitize animals to *Shigella* infection [123]. The connection between folate, B_{12}, and the gut microbiome has been shown to work in both

directions: *H. pylori* infection decreases gut uptake of folate and B_{12}, leading to deficiency and pathogenic hyperhomocysteinemia [124].

These observations are not limited to folate: metabolic studies have shown perturbations in methionine metabolism and branched-chain amino acid oxidation in IBD, both pathways in which B_{12} is a crucial cofactor [115]. Furthermore, mutations in transcobalamin II, a protein responsible for B_{12} transport in serum, are associated with UC [125]. In a Swiss cohort of IBD that included CD, UC, and indeterminate colitis, B_{12} deficiency was associated with higher CD activity, stenosis, nephrolithiasis, and other complications [126]. Mouse models of colitis offer a complex picture: B_{12} deficiency leads to increased microbial dysbiosis in the gut flora and decreased enteric tissue damage. This effect may be caused by depletion of B_{12}-dependent CD8+ T cells and NK cells in the gut, resulting in a minimized short-term inflammatory response to dysbiosis [127,128]. These results, however, have not been replicated in humans. Notably, B_{12} ligands were shown to have drastically different effects on inflammation in chemically induced murine colitis: cyanocobalamin appeared to worsen pathogenesis and inflammation, while methylcobalamin ameliorated them [129]. Conversely, methyl donor depletion in rats was shown to aggravate chemically induced colitis and lead to ER stress; it is uncertain precisely which compound's absence was responsible for the effects observed [130].

Several studies have noted that perturbations in SAM availability and homocysteine recycling correlate or cause worsening IBD symptoms or complications. Independent of methyl donor levels, hyperhomocysteinemia has been correlated with osteoporosis in CD patients in both univariate and multivariate analyses. By contrast, folate deficiency was only correlated in univariate analysis [131]. In one study, vitamin B_{12} and SAM restriction independently led to

endoplasmic reticulum stress mediated by SIRT1 reduction, which exacerbated colitis in rats [130]. In this species, induced colitis in conjunction with methyl donor deficiency correlates with hepatic inflammation and macrovesicular steatosis, where decreases in folate, B_{12}, and the SAM/SAH ratio are closely correlated with inflammatory markers [132]. A murine model of colitis included SAM in a study of antioxidants as colitis treatment; SAM reduced serum amyloid A and TNF-α, improved reduced glutathione in circulation, and restored colonic length [133]. Unfortunately, there is a dearth of information assessing SAM's effect on clinical IBD patients. While one study has noted an inverse relationship between SAM levels and IBD diagnosis or activity, clinical data is necessary to determine whether SAM supplementation ameliorates symptomatic disease and reduces complications [134]. Should clinical data confirm the prior findings, then mechanistic studies determining SAM's effect will be warranted.

It is important not to overstate the significance of these findings; data is still mixed on the clinical effects of methyl donor deficiency, particularly in the long term. In the same Swiss study which found associations between B_{12} deficiency and CD complications, folate-normal patients had an increased occurrence of osteoporosis and fistula formation. Differences in treatment may explain this trend: patients treated with steroids, anti-TNF agents, or antibiotics had lower rates of folate deficiency but may have had more severe symptoms that necessitated such interventions [126]. A recent meta-analysis found no statistical decrease of serum folate levels in CD patients compared with healthy controls, though there was statistical significance in with IBD overall. The same analysis found that B_{12} concentrations were only significantly reduced in studies on Asian populations [38]. This finding is particularly noteworthy given a contradictory study which found folate and B_{12} deficiency specifically prevalent in CD patients compared with UC

controls (22.2% and 15.6% prevalence compared with 4.3% and 2.8% prevalence, respectively) though indeterminate IBD was not studied [12]. Not all studies have correlated low serum folate or B_{12} with elevated homocysteine levels in CD, questioning the link between these nutrients and metabolic imbalance in IBD [135-137].

The above findings are indicative of a pathogenic role for folate and B_{12} deficiency in IBD. The precise interaction between these nutrients and IBD appears to segregate by IBD subtype, with some conditions worsening in UC but not CD or vice versa. Overall, worsening inflammation appears to be correlated with low folate and B_{12}; however, future studies are warned to be wary of the applicability of animal models to human conditions. Furthermore, researchers investigating a mechanistic link between folate, B_{12}, homocysteine, and SAM are strongly recommended to confirm the link between low methyl donors, high homocysteine, and low SAM in their patient sets.

Systemic Inflammation

Methyl donor availability, particularly folate and B_{12}, alters various body-wide inflammatory conditions in chronic and acute disease models. In some models, a mechanism has been established; in others, only the characterization of the effect in specific tissues has been confirmed. Besides its metabolic role as a cofactor, vitamin B_{12} protects against septic shock by scavenging nitrous oxide reactive oxygen species, which modulates both their inflammatory signaling effect and the damage incurred by the cell producing them. This antioxidative role is substantiated by the observation that supraphysiological doses of B_{12} have differential effects on NOS expression based on the organ in question in a model of toxic shock [138]. Furthermore, in

a study analyzing the effects of vegetarian and omnivorous diets on B_{12} level and inflammatory status in diabetic patients, Lee et al. found that higher B_{12} levels in both dietary groupings were associated with lower IL-6 levels and increased catalase activity. However, the vegetarian group was more prone to deficiency [139].

Folate and B_{12} exert a significant effect on systemic inflammation via their canonical control of plasma homocysteine levels. Elevated homocysteine levels in serum have been found to mediate vascular inflammation by inducing cathepsin V expression and consequently the nuclear translocation of ERK1/2 [107]. This effect may be partially mediated by oxidative stress; treatment with selenium reverses the deleterious effects of homocysteine on endothelial cells and neuronal cells [140,141]. Sodium selenite has also been shown to reduce pathogenic clotting [142]. In addition, homocysteine stimulation results in NLRP3 inflammasome activation and resulting cellular stress via TXNIP—this mechanism contributes to homocysteine's role as a risk factor in renal failure [143]. The failure to maintain low homocysteine results in increased oxidative stress, particularly in patients with chronic infection, sometimes with dangerous long-term effects; for example, sustained hyperhomocysteinemia can lead to pregnancy complications in hepatitis E patients [110].

Altogether, there is evidence that maintaining appropriate folate and B_{12} levels modulates systemic inflammation via an antioxidant effect and reduction of homocysteine, which is a risk factor in vascular disease.

Immune Cells

A substantial body of evidence discussing the role of methyl donors in inflammation continues to be gathered. Folate deficiency in RAW264.7 macrophages correlates with increased pro-inflammatory cytokine secretion in vitro [144]. Correlating with this observation is the finding that folate supplementation in cultured macrophages mitigates inflammation upon stimulation with LPS [145]. Notably, B_{12} deficiency has been shown to reduce phagocytosis and bactericidal activity in neutrophils collected from human peripheral blood, suggesting a role for B_{12} in leukocyte function [146]. Homocysteine has also been shown to augment inflammatory cytokine expression in vivo by affecting histone methylation in macrophages [147]. Aberrant B cell development has been observed in murine models of methyl donor depletion, with fewer B cells emerging from the pre-pro stage compared with controls [97].

Prior studies have noted relationships between SAM metabolism changes and differential function in various immune cell types, particularly T cells. CD8+ T cells have shown aberrant functionality in methionine-deficient cancer microenvironments, primarily manifesting as reduced cytokine production and increased apoptosis [148]. In CD4+ T cells, methionine adenosyltransferase is found to be inhibited by ethanol, sensitizing them to apoptosis and potentially explaining one factor of alcohol's immunosuppressive properties. Survival after methionine adenosyltransferase inhibition is rescued by SAM supplementation [149]. This finding is of particular interest to patients with congenital hypermethionemia, typically due to mutations in methionine adenosyltransferase [150]. However, there are caveats with these observations, as other investigations have found that excessive methionine and methionine sulfoxide dosing has been correlated with M1 macrophage polarization and increased production

of pro-inflammatory cytokines such as IL-6 and TNF-α [151,152]. This suggests that the effects of methionine and methionine sulfoxide excess may be a confounding factor in the investigation of SAM deficiency and hyperhomocysteinemia. On this point, mechanistic studies like those undertaken with hyperhomocysteinemia are warranted. Exploring in vivo effects based on observations in vitro requires substantial care; systemic inflammation can increase if T cell survival is biased towards pro-inflammatory subsets, and high methionine diets have been shown to predispose rats to Th17 cell polarization [112,153].

The findings cited above suggest an essential role for methyl donors and SAM in immune cell survival and function, modulating inflammation when there is an adequate amount of methyl donors. When this improves pathogen removal, the overall effect might be termed beneficial. However, in conditions involving an excessive immune response, increased survival of immune cells may lead to persistence of inflammation. Further study may clarify the utility of these ambiguous findings.

The Nervous System

While folate is well known for its importance in preventing neural tube defects in utero, its effects on the nervous system do not end after birth. For example, folic acid supplementation has been shown to mitigate inflammation in Alzheimer's disease, decreasing serum TNF-α levels and slightly improving mental state in patients [154]. Folate's importance to the nervous system is further implied by the association of *MTHFR* polymorphisms with a predisposition to migraine, which disappeared upon folate supplementation in a clinical trial by Di Rosa et al.

[155]. Echoing these findings are associations between *MTHFR*, *MTR*, *MTHFD1,* and oxidative stress in patients with some types of neurodegenerative disease [156].

B_{12} has been implicated in neuroprotection in various models of disease. Postmortem sampling of prefrontal human cortexes indicated that B_{12} status was decreased with age. The same study simultaneously found decreased methylcobalamin levels and methionine synthase activity in the brains of autistic patients [157]. A clinical trial of methylcobalamin supplementation showed moderate effects against diabetic neuropathy [158]. Neuroprotective effects of B_{12} were also found in rat models of both epilepsy and bacterial meningitis, where B_{12} levels reduced oxidative stress, inflammatory cytokine expression, and hippocampal damage [159,160]. In mice with methionine-high and B6, folate, and B_{12}-low diets, microhemorrhages and amyloid- plaque buildup was observed in neural tissue, corresponding with increased inflammatory cytokine expression, emphasizing the protective effect of methyl donors and the dangerous impact of homocysteine [161,162]. B complex supplementation was shown to have a beneficial effect on peripheral nerve repair: B vitamin injections reduced inflammatory cytokine expression, polarized macrophages towards an M2 phenotype, and induced an anti-inflammatory phenotype in Schwann cells following injury [163]. The fact that B_{12} is implicated in neuroprotection in both the central and peripheral aspects of the nervous system suggests that neural tissue has a particular dependence on vitamin B_{12} that is still being elucidated, with a specific interest in a putative role for injury prevention and repair.

Independent of folate and B_{12}, SAM supplementation has shown striking effects on neuroinflammation and pathogenesis. Conversely, high levels of homocysteine are a risk factor for neurological disease [106]. SAM was reduced in the cerebrospinal fluid of Alzheimer's

disease (AD) patients, while homocysteine is notably elevated in AD patient plasma [108]. This reduction correlates with observations in 1995 of hypomethylation in the amyloid-β gene of an AD patient, in addition to observations of global methylation changes in the prefrontal cortexes of 12 AD patients compared with controls [164,165]. Notably, amyloid-β plaque buildup is reduced by SAM dosage in mouse models of AD [166]. Following ischemic stroke, a correlation between hyperhomocysteinemia and exaggerated STAT3 activation has been found in microglia; it also exacerbated long-term tissue damage [106,167]. At times, the distinction between methyl donor treatment and SAM treatment is not a well-defined one: vitamin B_{12} injection has shown efficacy at reducing hippocampal inflammation in a rat model of bacterial meningitis, and one of the mechanisms the authors identified as protective was increased availability of SAM, leading to increased methylation of CpG islands in the promotor of *Ccl3* [159]. Interestingly, methionine restriction in helper T cells has been found to reduce neuroinflammation by preventing T-cell proliferation and differentiation into pro-inflammatory subsets in autoimmune diseases like multiple sclerosis [112]. This observation highlights the continuing need for further study on global SAM effects in disease models, not merely tissue or cell-specific studies.

From these findings, we conclude there is strong evidence for a beneficial role for folate, B_{12}, and SAM in modulating neuroinflammation. These findings have been corroborated by investigators examining various types of neuroinflammation; the beneficent effects are promising for clinicians who are interested in prophylactic treatments for at-risk patients. We encourage further mechanistic studies on this topic to elucidate why the nervous system, in particular, has shown responsiveness to this approach, and which compound or treatment is most consistently beneficial.

The Liver

Liver disease has been correlated with decreased methyl donor availability in vivo and in vitro. The trend especially evident in chronic hepatitis C infection. Egyptian hepatitis C and liver cirrhosis patients have been found to possess low serum folate and elevated plasma homocysteine; furthermore, folate levels positively corresponded with platelet count, indicating low thrombocytopenia [168]. In chronic hepatitis C patients, SAM treatment improved response to pegylated interferon and ribavirin therapy by altering the methylation status of STAT1 in cultured cells. This modification led to an enhanced downstream signaling effect which enhanced the antiviral state of the treated cells [169]. Improved interferon and ribavirin therapy responses were also found with vitamin B_{12} supplementation, though the authors ascribe this finding to B_{12}-mediated IRES inhibition independent of B_{12}'s methyl donor activity [170]. The liver has been shown to have a particular sensitivity to methyl donor deficiency during induced colitis in one rat model, suggesting that these nutrients may have a protective role against hepatotoxic inflammation in other tissues [132].

Outside the strict paradigm of infection, SAM dosing has been found to reduce ethanol-induced apoptosis in primary hepatocytes. Interestingly, the authors found no indication that it altered JNK activity in the proapoptotic signaling cascade and have postulated that an antioxidant effect is responsible for the phenomenon [171]. The importance of SAM in hepatocyte survival in response to inflammatory oxidative stress was highlighted in a murine model of hepatitis, where SAM depletion led to liver failure and death [172].

In total, the literature evidence suggests a beneficial role for SAM in the resolution of infection and the modulation of apoptosis in the liver. However, the findings are far from conclusive and only examine liver health from the paradigm of infection.

Other Tissues

Psoriasis, a chronic inflammatory condition primarily mediated by T cells, has been shown to correlate with hyperhomocysteinemia, and folic acid derivatives or topical B_{12} treatment provided mild attenuation of inflammation in initial trials. Clinical trial patients found a B_{12} containing cream superior to a standard hydrating cream for alleviating symptoms [173-175]. Atopic dermatitis patients also found improved relief of symptoms using B_{12} emollient cream compared to the unmodified control [176,177]. However, these effects are nutrient-specific; we could not find studies demonstrating folate as an effective intervention alone, though some utility has been noted for folate supplementation in conjunction with methotrexate treatment for psoriasis [178]. By contrast, vitiligo, a skin condition characterized by loss of melanocytes and skin pigmentation, was reduced or halted in a Swedish clinical trial that administered folic acid and intramuscular B_{12} [179].

Some studies have suggested a beneficial role for methyl donor supplementation in respiratory disease. Supplementation of cystic fibrosis patients with 5-methyltetrahydrofolate and vitamin B_{12} reduced red blood cell oxidative stress, even in patients who were folate and B_{12}-normal [180]. Beneficent effects of methyl donors on oxidative stress were echoed in a murine model of chronic asthma, where oxidative stress, tissue remodeling, and Th2 cytokine production were all ameliorated by SAM treatment [181]. Methyl donating nutrients have also been found to

have beneficial effects on the lungs. A Greek cohort of asthmatic girls was found to have an association between low serum folate and impeded lung function [182]. The observed antioxidative effects of betaine in the lungs in response to paraquat toxicity have been proposed to be mediated through liver-generated SAM [183]. Despite the beneficent effects previously described, SAM's role in the airways remains complex: SAM abundance can be scavenged by opportunistic pathogens like *Pneumocystis* species, leading to pathogenesis [184].

These results highlight the beneficial effects of methyl donors at epithelial tissues, which are consistently exposed to a broad category of microbes, some pathogenic. While more studies are indicated, the current results suggest a positive impact on immune regulation at these sites. It is too soon to conclude at this point that folate, B_{12}, and SAM have an exclusively or even generally positive impact on these conditions, but there is more than enough justification to examine the effects in more detail. Figure 3 highlights the beneficent effects of SAM supplementation in humans. Figur 4 notes the summed effects of methyl donor depletion or supplementation in human and rodent models.

Concluding Remarks

When one examines the effect of methyl donors on distinct tissue types, a distinct breakdown in effect by nutrient can be observed. In the gastrointestinal tract, folate, B_{12}, and SAM show marked anti-inflammatory effects on IBD, though SAM lacks support from clinical patients. Folate and B_{12} further exert beneficent effects on systemic inflammatory markers by reducing serum homocysteine, with an additional antioxidative effect in vitamin B_{12}. There is strong evidence that these vitamins also reduce neuroinflammation and promote neural tissue

survival, though further mechanistic studies are warranted to determine how this effect is mediated. While present, direct evidence for SAM's effect on systemic inflammation and neuroinflammation is comparatively lacking. Studies on immune cells also show anti-inflammatory effects upon folate and B_{12} dosage, with SAM enhancing cell survival. However, the pro-inflammatory effects of methionine excess make it challenging to determine whether SAM's antiapoptotic effect will translate to reduced inflammation in vivo. Vitamin B_{12} and SAM bioavailability have been shown to affect hepatic cell survival and infection response, though folate data is comparatively lacking in this tissue. In epithelial cells and airways, aberrant immune responses are ameliorated by folate and B_{12}, with SAM indicated as a likely mediator of their effects.

The findings collated in this review are striking not merely due to their variety, but the potency of their effects as well. Methionine metabolism and changes in S-adenosyl methionine levels are demonstrated to have wide-reaching and distinct effects on the same tissue type; similar phenomena are demonstrated for the methyl donating metabolites that control them. The complexity of the responses observed is a warning to those who might attempt therapies centered on methionine metabolism: when dealing with a metabolite crucial to many biological processes, it is necessary to tread carefully.

Despite these challenges, the state of the literature indicates that in some circumstances, folate, B_{12}, and S-adenosyl methionine are capable of pleiotropic and powerful effects on inflammation. Given their ubiquity in the body and the continuing problem of chronic inflammatory disease, any dietary factor capable of complementing or replacing an anti-inflammatory pharmaceutical is especially advantageous. Naturally, the contradictory and

complex findings necessitate further investigations to ensure SAM and methyl donor-based interventions are protective and do not exacerbate infection or inflammation. Building upon this work in the future may enhance the capacity of physicians to engage the health of their patients in ways that correct nutritional imbalances, mitigate chronic conditions, and improve quality of life.

Figures

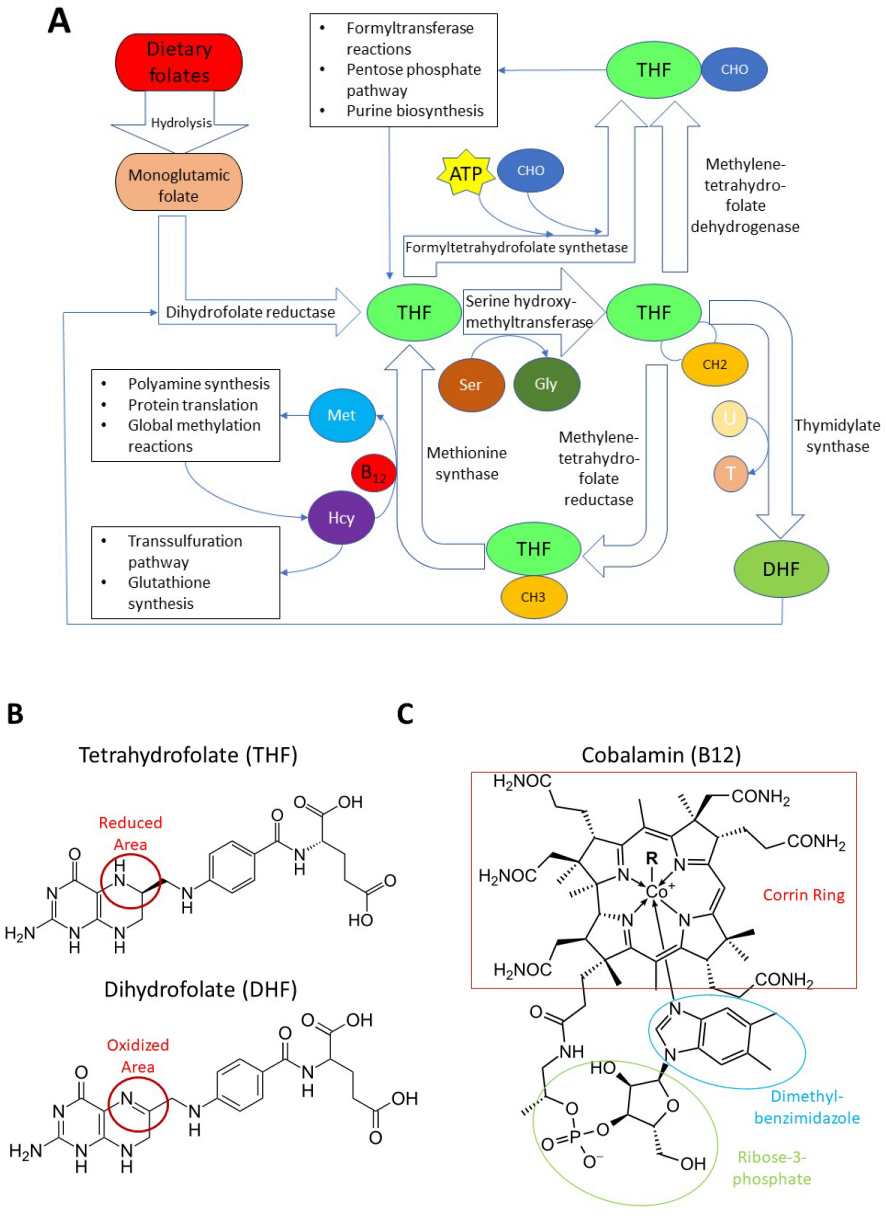

Figure 1: Outline of folate metabolism

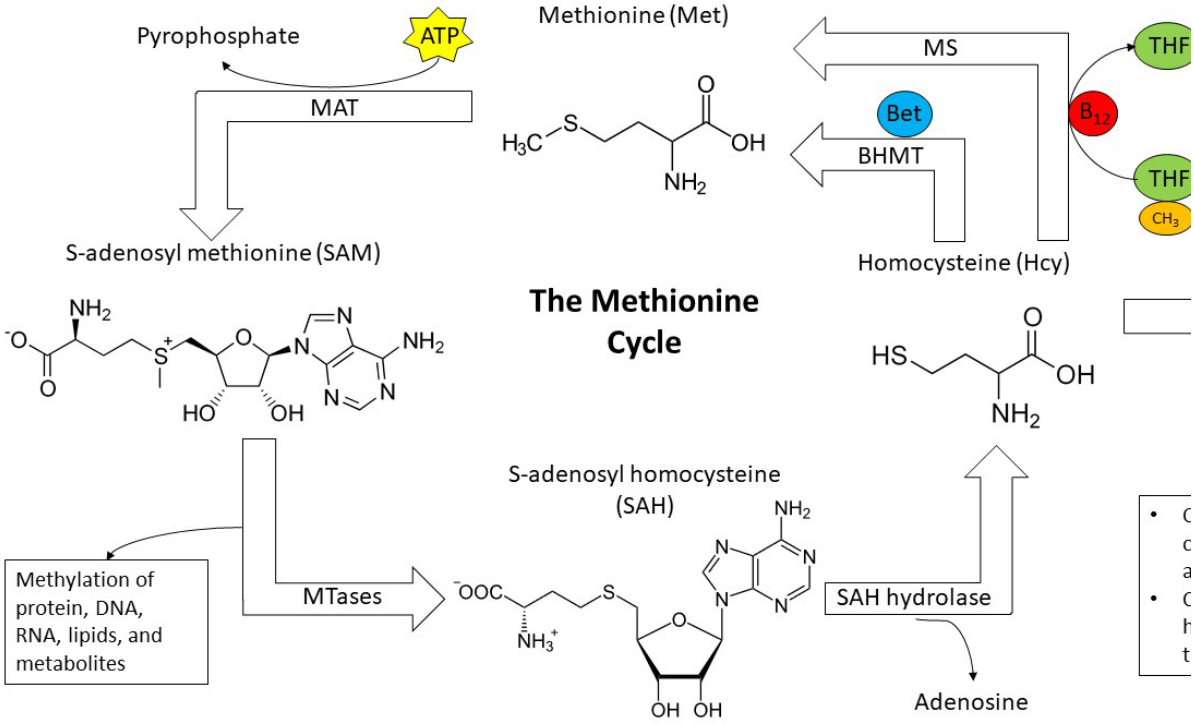

Figure 2: The methionine cycle.

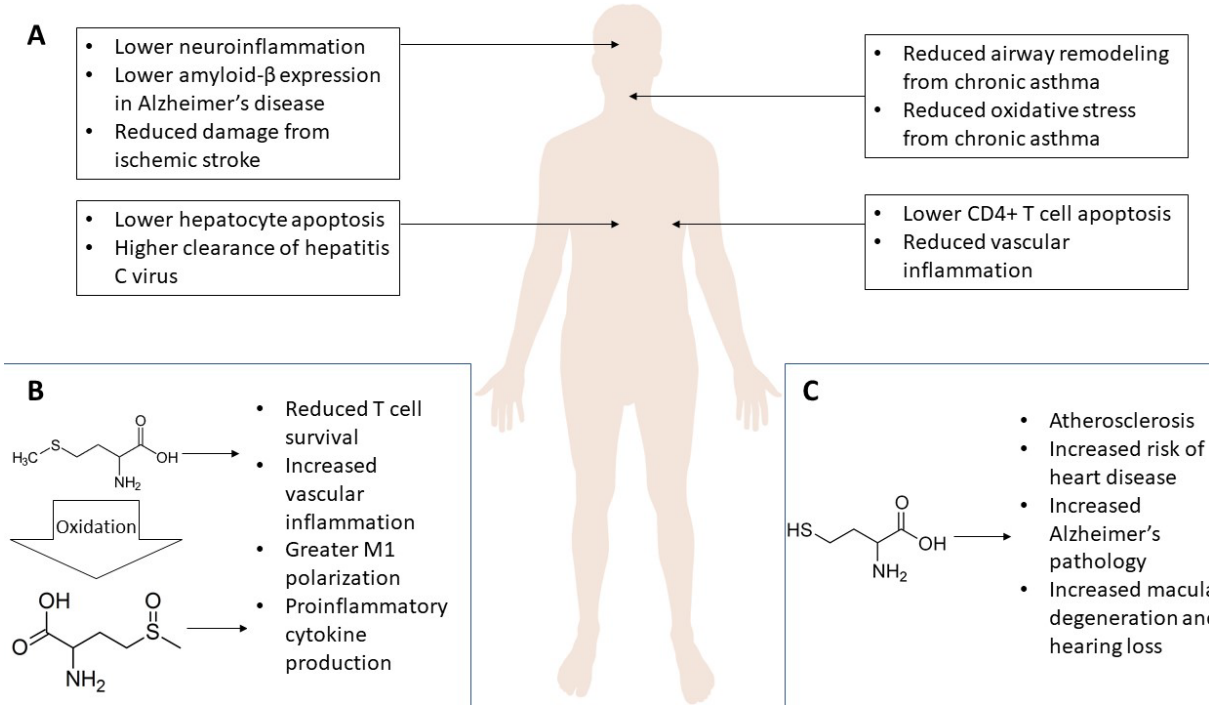

Figure 3: S-adenosyl methionine and inflammation.

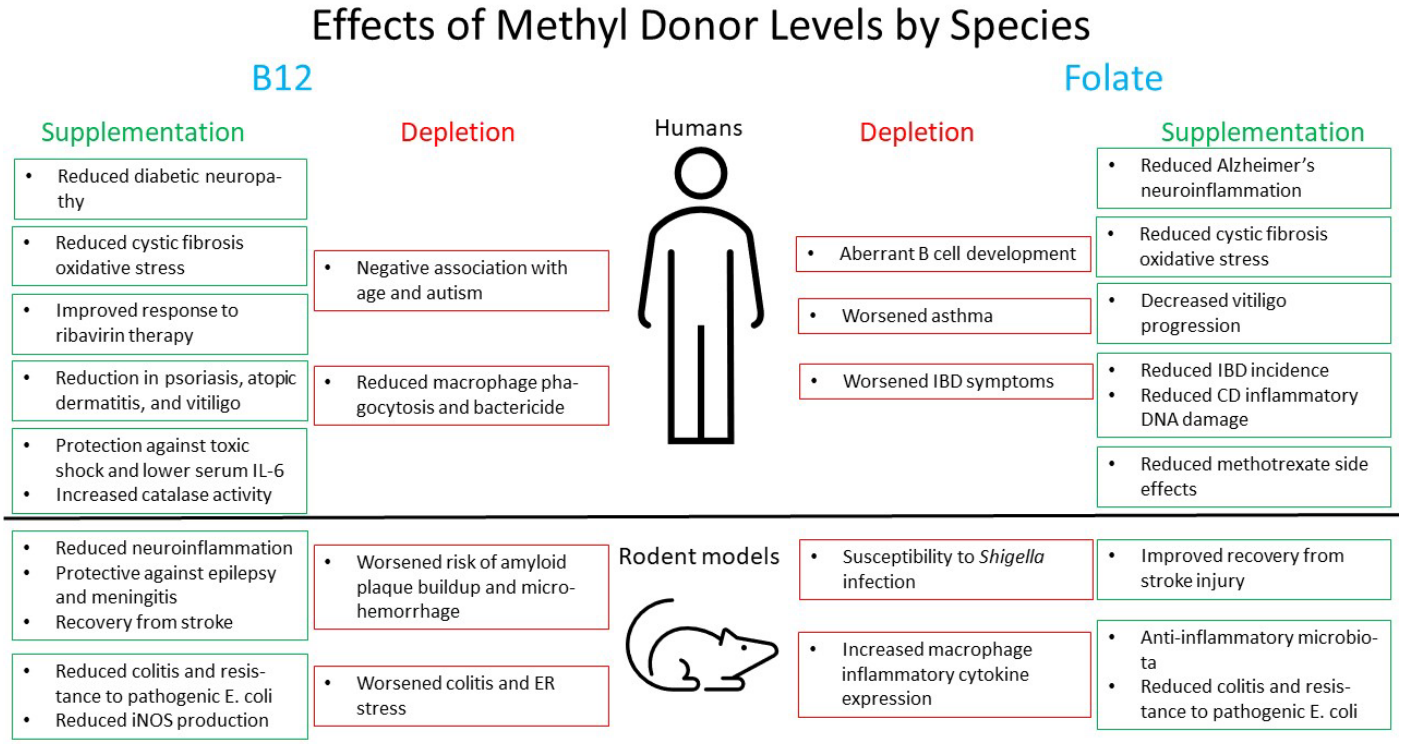

Figure 4: Overview of the effects of folate and B_{12} supplementation or depletion in humans and rodent models.

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CHAPTER 2: FOLATE AND VITAMIN B_{12} DEFICIENCY EXACERBATE INFLAMMATION DURING *MYCOBACTERIUM AVIUM PARATUBERCULOSIS* (MAP) INFECTION

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Introduction

Folate (vitamin B_9) and cobalamin (vitamin B_{12}) are essential cofactors in eukaryotic single-carbon metabolism [1,2]. Folate is naturally found in citrus fruits and leafy green vegetables; it is also supplemented in particular grains and synthesized by intestinal commensal bacteria such as *Bifidobacterium* strains [3-5]. In contrast, vitamin B_{12} is primarily found in animal products [6,7]. These two cofactors serve distinct roles in normal cellular metabolism, since folate transfer of methyl groups is necessary for the biosynthesis of purines, formyltransferase reactions, and the conversion of uridine to thymidylate [8-10]. Similarly, vitamin B_{12} is a cofactor for converting methylmalonyl-CoA to succinyl-CoA, thus proving a necessity for odd-chain fatty acid and amino acid metabolism [2]. Furthermore, the central cobalt moiety in the vitamin B_{12} corrin ring scavenges cyanide ions and reactive oxygen species (ROS) [11-14]. Folate and vitamin B_{12} overlap in function during the regeneration of methionine from homocysteine in a reaction catalyzed by methionine synthase [15]. During this reaction, L-methyltetrahydrofolate donates a methyl group to homocysteine using B_{12} as a cofactor [15]. The methionine generated this way can subsequently be incorporated into protein or adenosylated to

generate S-adenosyl methionine (SAM), which is the universal methyl donor and precursor to polyamine biosynthesis [16,17].

Several studies have shown that folate and B_{12} deficiencies are associated with exacerbated inflammation and damage in the brain, vasculature, immune system, liver, and gastrointestinal tract [18-27]. This phenomenon is partly due to their metabolic roles in regenerating methionine from homocysteine; homocysteine mediates inflammation via cathepsin V activation and TXNIP-induced NLRP3 inflammasome activation [28,29]. However, macrophages supplemented with folate in excess of standard cell culture concentrations display reduced pro-inflammatory gene expression [30]. These effects are also observed in vivo during macrophage-mediated neuroinflammation [31]. Therefore, folate and vitamin B_{12} may link hypovitaminosis and chronic inflammatory disease, particularly when the disease progression impedes vitamin uptake [21,32].

Crohn's disease (CD) is a chronic inflammatory bowel disease characterized by a pattern of relapse and remission and asymmetrical, segmental, and transmural inflammation [33]. CD patients are at elevated risk of malabsorptive vitamin deficiency due to chronic damage to the gastrointestinal wall [21,32,34,35]. Recently, a murine model of CD has indicated that a diet enriched in folate and cobalamin enhances the antibacterial response to pathogenic *E. coli* infection [36]. However, the clinical effect of folate and vitamin B_{12} supplementation on pathogens associated with CD needs further investigation.

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an obligate intracellular pathogen that is known as a causative agent in Johne's disease (JD), which is a chronic inflammatory disorder affecting the intestines of ruminants [37]. In genetically susceptible

patients, MAP infection induces CD, leading to chronic gastrointestinal inflammation that requires the use of immunosuppressive agents to manage disease symptoms [38-40]. Since standard CD therapy involves anti-TNF-α biologic drugs that fail to alleviate symptoms in roughly half of CD patients and exacerbate MAP infection in tissue culture, exploring alternative immunomodulatory interventions for this patient subset is an ongoing priority [41,42]. In this context, our present study analyzes the effects of folate and B_{12} supplementation or deprivation on MAP infection and the resultant inflammatory response.

Materials and Methods

Measurement of Plasma Folate and Vitamin B12

Plasma samples from peripheral blood (4.0 mL K_2-EDTA tube) were collected from 100 CD patients (CDAI ≥ 220 and ≤ 450). The presence of MAP was subsequently evaluated via *IS900* PCR as described earlier [43]. We randomly selected 35 MAP-positive and 35 MAP-negative CD patients for this study. Within the selected samples, we used the Human Folic Acid ELISA Kit (Competitive EIA) (LifeSpan BioSciences, Seattle, WA, USA), the 5-Methyltetrahydrofolate ELISA Kit (LifeSpan BioSciences, Seattle, WA, USA), and the Human vitamin B_{12} (VB12) ELISA Kit (Aviva Systems Biology, San Diego, CA, USA) to determine levels of folate and vitamin B12, respectively. This study was approved by the University of Central Florida Institutional Review Board # STUDY00003468. All samples were de-identified before handling.

Culture and Infection of Monocyte-Derived Macrophages

THP-1 immortalized monocyte-like cells (ATCC TIB-202) were cultured in custom formulated folate and B_{12}-free RPMI-1640 medium (ThermoFisher, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Sigma Life Science, St. Louis, MO, USA). Pure folic acid and 1 mg/mL vitamin B_{12} solution in methanol (Sigma Life Science, St. Louis, MO) were then added to the medium to generate media with differential B-vitamin status (Table 1). The cells were grown to confluency in treated cell culture flasks in a humidified 5% CO_2 incubator at 37 °C. A total of 2.0 mL of cell suspension was transferred to 12-well tissue culture plates with 1×10^5 cells per well. We then differentiated the cells into monocyte-derived macrophages using 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma Life Science, St. Louis, MO, USA), followed by 48 h of incubation at 37 °C. In MAP-positive treatment groups, monocyte-derived macrophages were infected with clinical MAP UCF4 (1×10^7 CFU/mL), followed by 24 h of incubation under the same conditions.

Measurement of IL-1β and TNF-α Expression in Cultured THP-1 Macrophages

RNA was isolated from each 2.0 mL sample of monocyte-derived macrophages following 24 h of infection with clinical MAP strain (UCF4). RNA was extracted using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer protocols. RNA concentrations were measured using NanoDrop (OD at 260 nm). RNA was then reverse-transcribed to cDNA cDNA was synthesized from 1000 ng of each RNA sample using 5.8 µL master mix made from the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) and then topped up to a total volume of 20 µL with RNase-free water, according to manufacturer

protocols. A thermal cycler (MyGene Series Peltier Thermal Cycler) was used to perform the reactions for 5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C. The cDNA samples were analyzed immediately by RT-qPCR analysis or stored at −20 °C. Gene expression was measured using specific primers for *GAPDH, TNF-α*, and *IL1β*, obtained from Bio-rad (Hercules, CA, USA), followed by quantitative reverse transcription PCR (RT-qPCR) analysis. For each sample, 5 µL of cDNA was mixed with 10 µL of PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA, USA), 1 µL primer mix, and 4 µL of DEPC-treated water. Samples were added in triplicate to a 96-well microamp RT-PCR reaction plate, and the experiment was run using the 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). GAPDH was the control used to obtain baseline CT readings. Relative mRNA expression levels were calculated using the equation (2^(− ΔΔCT.))

Quantification of THP-1 Macrophage Viability and Apoptosis during MAP Infection

THP-1 macrophages were cultured in 100 µL media on a 96 well opaque-sided plate. All cultured cells used the specialty RPMI 1640 formulations described previously. Macrophages were administered 50 ng/mL PMA and kept in a humidified incubator at 37° C and 5% CO_2 for 48 h. Macrophages were infected following this incubation with clinical MAP at 1×10^7 CFU/mL and maintained for 24 h. We then conducted one of two assays on the plated cells. First, we used the RealTime-Glo™ MT Cell Viability Assay (Promega, Madison, WI, USA), an ATP-based luminescence assay according to manufacturer protocols. Briefly, we mixed MT Cell Viability Substrate and NanoLuc® Enzyme to 2X concentrations in folate and B_{12}-free RPMI 1640 media and added 100 µL to each well. Cells were incubated with the reagent for 1 h, and

then luminescence was measured using the GloMax Navigator system GM-2000 (Promega, Madison, WI., USA) Next, we used the RealTime-Glo™ Apoptosis Assay (Promega, Madison, WI, USA) an annexin-V-based luminescence assay. Briefly, we combined 2X concentrations of Annexin NanoBiT® Substrate, $CaCl_2$, Annexin V-SmBiT®, and Annexin V-LgBiT in prewarmed folate and B_{12}-free RPMI 1640. 100 µL of the assay mixture were administered to each well and incubated for 20 min, and then the same instrument was used to measure luminescence. Both luminescence readings were analyzed to determine cell viability and apoptosis.

Culture and Treatment of Caco-2 Monolayers with Infected THP-1 Supernatant

The impact of vitamin concentration on cell death and oxidative stress was examined in an immortalized enterocyte-like cell line (Caco-2 ATCC HTB-37.) Cells were cultured in ATCC-formulated Eagle's Minimum Essential Medium (EMEM) supplemented with 20% FBS (ATCC, Manassas, VA, USA) and maintained at 37 °C in a humidified 5% CO_2 incubator. 3×10^5 cells were seeded in the base of clear-bottomed, opaque-sided 96-well plates with 200 µL media. The cells were allowed to differentiate for 14 days before the media was changed to the RPMI 1640 media with variable levels of folic acid and vitamin B_{12} (Table 1). On day 18, THP-1 macrophages were plated and differentiated for 48 h in RPMI 1640 media matching the vitamin concentrations of their paired Caco-2 monolayers. On day 20, the macrophages were infected with MAP as previously described. On day 21, supernatants were collected from each macrophage culture and centrifuged for 1 min at 8000 rcf to pellet debris and intact bacteria. Media was then removed from the Caco-2 wells and replaced with supernatant from infected macrophages with the same vitamin concentrations.

Quantification of Cytotoxicity and Oxidative Stress in Caco-2 Monolayers

After preparation of the Caco-2 monolayers and administration of the infected THP-1 macrophage supernatant, we used the LDH-Glo™ Cytotoxicity Assay and the NADP/NADPH-Glo™ Assay (Promega, Madison, WI, USA) to quantify plasma membrane damage and oxidative stress, respectively. Briefly, for the former assay, all components were combined in warm media and administered to intact, adherent cells inside their respective wells. The cells were incubated for 30 min, and luminescence was measured with the GloMax Navigator™ (Promega, Madison, WI, USA) For the latter assay, cells were lysed with 0.2 N NaOH solution with 1% DTAB, and the lysate was separated to be subjected to heat under acidic or basic conditions to decompose NADPH or $NADP^+$, respectively, according to manufacturer protocols. The heated lysate was then cooled to room temperature and administered the assay components. After a 30 min incubation, luminescence was again quantified with the GloMax™ Navigator. Cells were not reused between assays. All experimental groups were plated and assayed in triplicate.

Statistical Analysis

GraphPad Prism V.9.4.0 (GraphPad, La Jolla, CA, USA) was used for statistical analysis. First, the Kolmogorov–Smirnov normality test was used to assess normal distribution for all values. Following this analysis, we used Student's *t*-test was to assess significance between two groups of values. One-way ANOVA was used to assess significance in studies with multiple experimental groups, followed by Sidak's multiple comparisons test. All data are expressed as average ± SD of the mean, and the difference between treated samples vs. controls was

considered statistically significant at p-value < 0.05 and 95% confidence interval. All experiments save ELISAs of plasma samples were performed in triplicates unless noted otherwise.

Results

Folate and Vitamin B_{12} Are Reduced in MAP-Positive CD Patients

While previous studies have established that CD patients are at an elevated risk of folate and B_{12} deficiency, there is a lack of data on how MAP-positive and MAP-negative CD patient subsets compare. As such, we took plasma samples from patients previously confirmed to be MAP-negative or MAP-positive via *IS900* PCR and compared their folate and B_{12} levels via ELISA. We found that average MAP-positive plasma folate levels were significantly reduced to 14.48 ± 13.88 ng/mL from MAP-positive 24.15 ± 25.74 ng/mL [Figure 5A] In addition, there was a significant decrease in average vitamin B_{12} to 414.48 ± 94.60 pg/mL from 512.86 ± 129.12 pg/mL in MAP-positive versus MAP-negative patients [Figure 5B]. Since the plasma samples were collected from two subsets of CD patients, these results indicate that the risk of malabsorptive folate and B_{12} deficiency may be correlated particularly with MAP infection in CD patients.

THP-1 Macrophages Cultured in Folate and B_{12} Supplemented and Deficient Media Show Altered Cytokine Expression

Next, we considered how differing vitamin levels in cell culture media alter macrophage cytokine expression. To examine this phenomenon, we took folate and B_{12}-free RPMI 1640 and manually supplemented it with folate and B_{12} to yield folate-low media, folate-high media, B_{12}-

low media, B_{12}-high media, folate/B_{12}-low media, folate/B_{12}-high media, and ordinary RPMI 1640. After 48 h of PMA stimulation and differentiation in the depleted media, we infected them with clinical MAP for 24 h and examined relative cytokine mRNA. folate, B12, and folate/B_{12} deficiency increased TNF-α expression in uninfected macrophages by 1.63 ± 0.08-fold, 1.91 ± 0.17-fold, and 1.79 ± 0.19-fold, respectively; however, these effects were not statistically significant [Figure 6]. In infected macrophages, B_{12}-deficiency increased TNF-α expression from 20.37 ± 0.54-fold to 25.52 ± 1.17-fold [Figure 6]. However, no other vitamin deficiency, even concurrent folate/B12, significantly altered TNF-α expression. By contrast, folate, B12, and folate/B_{12} deficiency all had inhibitory effects on IL-1β expression in uninfected (2.31 ± 0.13, 2.38 ± 0.03, and 2.32 ± 0.07-fold, respectively) and infected macrophages (16.49 ± 0.16, 20.93 ± 0.17, and 17.26 ± 0.30-fold, respectively, compared with untreated 7.41 ± 0.38) [Figure 7].

We followed these experiments with another analysis to examine supplementation with folate and B12. Tenfold elevation of B_{12} in culture medium for infected macrophages reduced TNF-α expression from 12.49 ± 0.33 to 10.66 ± 0.39-fold, while folate had no significant effect (13.14 ± 0.50) [Figure 8]. Folate and B_{12} supplementation alone had no significant effect on TNF-α expression in uninfected macrophages (with a 0.89 ± 0.09 and a 1.06 ± 0.07-fold change, respectively) [Figure 8]. Interestingly, concurrent folate/B_{12} supplementation increased TNF-α expression by 1.34 ± 0.04-fold in uninfected macrophages but reduced it from 12.49 ± 0.33 to 11.31 ± 0.10 in infected macrophages [Figure 9]. Elevated folate reduced IL-1β expression from 1.00 ± 0.01 to 0.55 ± 0.10-fold in uninfected macrophages and from 10.75 ± 0.25 to 8.19 ± 0.11-fold in infected macrophages [Figure 9] Supplementation with B_{12} reduced IL-1β expression from 1.00 ± 0.04 to 0.80 ± 0.01-fold in uninfected macrophages and from 10.75 ± 0.25 to 9.39 ±

0.09-fold in infected macrophages [Figure 9]. Concurrent folate/B_{12} supplementation's effect is more uniform in IL-1β than TNF-α, which shows a reduction from 1.00 ± 0.04 to 0.69 ± 0.02-fold in uninfected macrophages and from 10.75 ± 0.25 to 7.00 ± 0.10-fold in infected macrophages [Figure 9]. These results indicate that folate and B_{12} supplementation have an anti-inflammatory effect on macrophage cytokine expression during MAP infection, with results varying by specific cytokine. By contrast, folate and B_{12} deprivation enhance the inflammatory response in identically treated cells.

MAP Infection Increases Apoptosis during Folate and B_{12} Supplementation and Decreases Apoptosis during Depletion

Apoptosis of infected cells is a critical immune mechanism for countering intracellular pathogens, and MAP consequently uses a suite of anti-apoptotic proteins to impede it [44]. Accordingly, we hypothesized that infected macrophages supplemented with folate and B_{12} would increase apoptosis to control the infection. We used two methods to assess the apoptosis of MAP-infected macrophages. First, we directly quantified apoptosis using annexin V luminescence. MAP infection increased apoptosis by 1.83 ± 0.40 fold compared to uninfected controls, as anticipated. Furthermore, folate-enriched media increased macrophage apoptosis during infection by 3.38 ± 0.07-fold, a significant increase compared to MAP infection in control media [Figure 10A]. An increase in apoptosis to 2.58 ± 0.14-fold was also observed in concurrent folate and B_{12} supplemented media [Figure 10A]. We did not observe a significant change in media supplemented with B_{12} alone. Depletion of folate, B12, and both folate and B_{12} had the inverse effect: annexin V luminescence decreased to 1.04 ± 0.08, 0.64 ± 0.12, and 0.45 ± 0.07-fold, respectively [Figure 10A].

We followed this experiment with an ATP-based viability assay. General macrophage viability after MAP infection declined to 0.80 ± 0.05 and 0.82 ± 0.02-fold in folate and concurrent folate/B_{12} supplemented media, corresponding with the observed increases in annexin V luminescence [Figure 10B]. Interestingly, there was no significant change in macrophage viability in the MAP-infected cells compared with the uninfected cells. Similarly, decreased annexin V in folate, B12, and folate/B_{12} deficient media did not correspond with a change in overall viability in these groups [Figure 10B]. However, there was a small but statistically significant decrease in overall macrophage viability in the folate/B_{12}-low group, from 1.00 ± 0.05-fold to 0.91 ± 0.04-fold.

Folate and Vitamin B_{12} Deficiency Individually Exacerbate Cytotoxicity in Caco-2 Monolayers

After assessing the effects of folate and vitamin deficiency on macrophage inflammation, we considered the impact of altered macrophage inflammation on enterocytes. We hypothesized that changes in macrophage survival and pro-inflammatory cytokine expression would have a detrimental impact on co-cultured Caco-2 cells. Accordingly, we differentiated Caco-2 cells into monolayers and maintained them in culture for 7 days in RPMI 1640 with altered vitamin levels. administered supernatant collected from macrophages cultured and infected in media with comparably altered vitamin levels. 24 h post-treatment, we examined LDH release and $NADP^+$/Total NADP in the monolayers.

Treating Caco-2 cells with supernatant from infected macrophages increased LDH luminescence 3.16 ± 0.95-fold compared with uninfected controls, though this effect did not reach statistical significance [Figure 11]. By contrast, administration of infected macrophage

supernatant in vitamin B_{12}-low conditions increased LDH luminescence 7.73 ± 1.01-fold, which was significant compared to both infected and uninfected cells in control media [Figure 11]. Similarly, folate-low media increased luminescence by 5.87 ± 1.78-fold compared with the control [Figure 11]. Interestingly, concurrent folate and vitamin B_{12} deprivation did not significantly alter LDH luminescence in culture. Treating Caco-2 cells with supernatant from infected macrophages did not significantly alter the percentage of NADPH compared with the control in any group, suggesting that oxidative stress did not mediate the damage observed in the LDH assay [Table 2].

Discussion

CD patients are at an elevated risk of malabsorptive folate and vitamin B_{12} deficiency [21,32]. Furthermore, attempts to avoid foods that patients believe to be triggers for CD relapse can lead to self-imposed dietary restriction and reduced nutrient intake [45]. Accordingly, periodic screenings for these vitamins are indicated for CD patients, particularly those who received ileal resection [40]. However, there is a dearth of information on the effects of folate and vitamin B_{12} supplementation on CD symptoms and inflammation. Furthermore, the state of the literature is murky on the effects of folate and B_{12} supplementation on common CD pathogens like MAP. Physicians with CD patients are left without guidance about the risks of prolonged folate and B_{12} deficiency in this patient subset and a potential tool to mitigate inflammation.

Gastrointestinal health and resilience against infection have been correlated with folate and vitamin B_{12} intake. Rat models of methyl donor deficiency indicate that methyl donor-low

diets impede bowel development and barrier function while aggravating induced colitis [18,46]. By contrast, folate-producing lactic acid bacteria have an anti-inflammatory effect on induced murine mucositis [47]. Additionally, murine diets supplemented with folate and other methyl-donating nutrients improved antimicrobial gene expression and resilience against adherent invasive *E. coli* in a mouse model of CD [36]. These findings concur with a study establishing that neonatal folate deprivation sensitizes adult guinea pigs to Shigella infection [48]. In humans, folate-associated metabolic pathways are perturbed in pediatric CD patients, and a review of meta-analyses for CD environmental risk factors indicated that high folate levels were protective [49,50]. Furthermore, low folate levels in CD were associated with increased CD activity in a Swiss cohort of patients [26].

The results of our investigation add to these findings by showing that folate and B_{12} alter inflammation during infection with a common CD pathogen. MAP-positive CD patients have significantly lower plasma folate and B_{12} than MAP-negative CD patients. We further show that pro-inflammatory cytokines IL-1β and TNF-α are significantly upregulated during folate and vitamin B_{12} deprivation in after MAP infection, while supplementation significantly reduces their expression. Macrophages in folate and B_{12}-deficient media are less likely to undergo apoptosis during MAP infection, suggesting this key mechanism to counteract intracellular pathogens is inhibited during vitamin deficiency. Folate supplementation, by contrast, increased apoptosis of infected macrophages. Finally, the treatment of enterocytes with folate-low or B_{12}-low supernatant from infected macrophages leads to increased LDH release without significant alterations in oxidative stress.

Our findings on folate and B_{12} in CD patient plasma cannot at this time be compared with normal clinical serum ranges for these vitamins, as the ELISAs used to quantify folate and vitamin B_{12} availability are not approved for clinical practice. However, these results indicate that even within a patient group at risk for folate and B_{12} deficiency, distinct subsets of folate and B_{12} availability can be characterized based on MAP infection. Furthermore, the data on cytokine availability indicate that folate and B_{12} levels affect macrophage-mediated inflammation after only 48 of h prophylactic exposure. Since monocyte-derived macrophages constitutively infiltrate the gut, these results suggest that clinical trials of folate and B_{12} supplementation may gradually impact inflammation patterns as older, vitamin-restricted cells are replaced [51]. Importantly, our work builds on the findings of Samblas and colleagues by showing that the anti-inflammatory effects of folate and B_{12} can be observed during an ongoing bacterial infection, not just after stimulation with a TLR agonist like LPS [30]. Since these findings are paired with improved apoptosis of infected macrophages, we propose that MAP-infected CD patients taking folate and B_{12} supplements may experience reduced inflammation upon the initial macrophage encounter with MAP. Altered inflammation may be followed by apoptosis of the pro-inflammatory, MAP-infected cells, resulting in the removal of both pathogen and inflammatory mediator.

Future studies are required to explore not merely how folate and B_{12} alter MAP infection in cell culture, but in animal and clinical studies. Our study centered on monocyte derived macrophages because prior studies have highlighted macrophages as a crucial innate immune mediator for both MAP destruction and proliferation [52]. However, we do not analyze other phagocytic immune cells or determine if the 48 h differentiation period is the only relevant

window to mediate folate and B_{12}'s anti-inflammatory effects. Furthermore, macrophages frequently interact with components of the adaptive immune system, thereby alter adaptive immunity. We do not explore how folate and B_{12} might affect this function to contribute to long-term inflammatory CD patterns. Accordingly, further investigation is warranted.

Figures

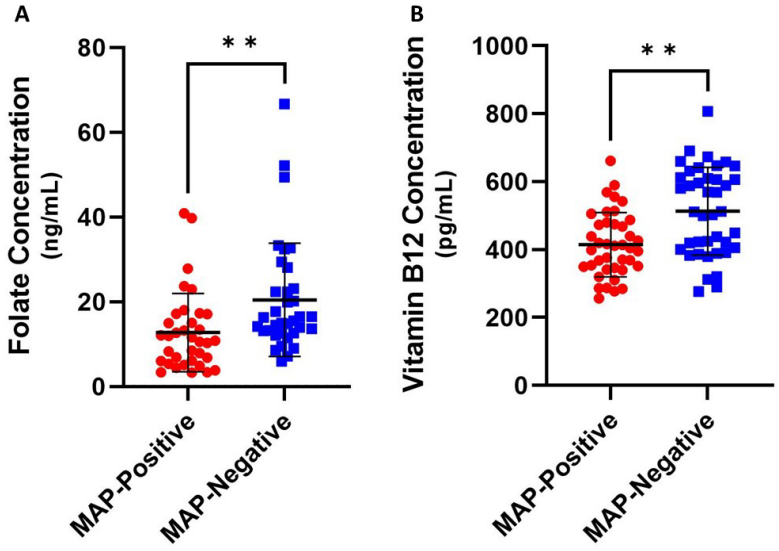

Figure 5: Plasma folate and B_{12} availability in MAP-negative and MAP-positive CD patients.

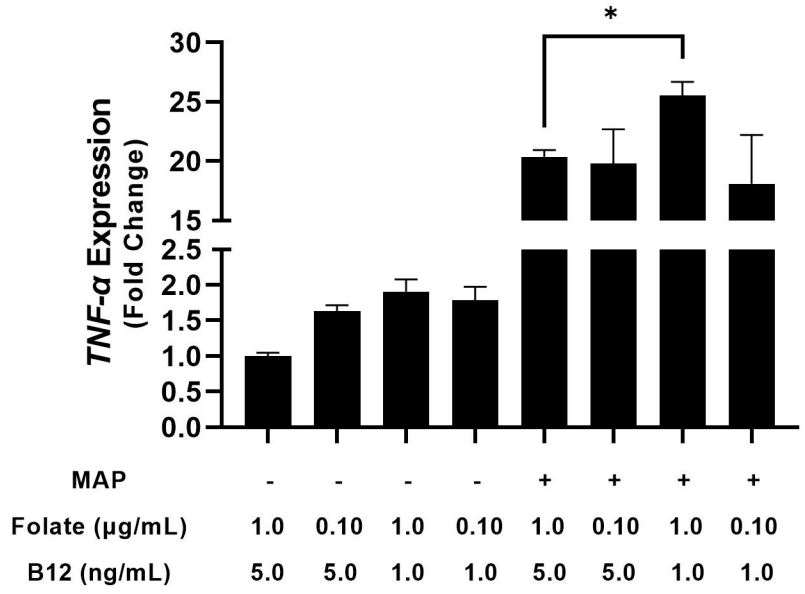

Figure 6: Effect of folate and B_{12} availability on TNF-α expression during MAP infection.

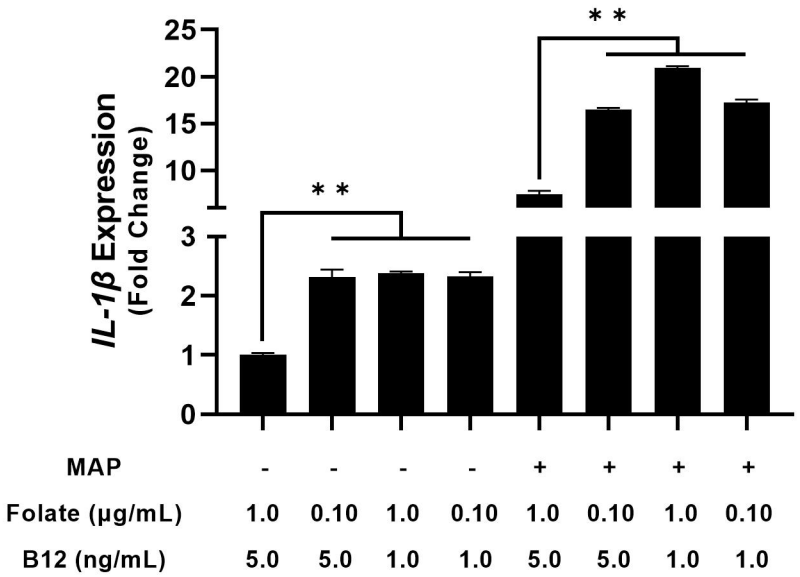

Figure 7: Effect of folate and B_{12} deficiency on IL-1β expression during MAP infection.

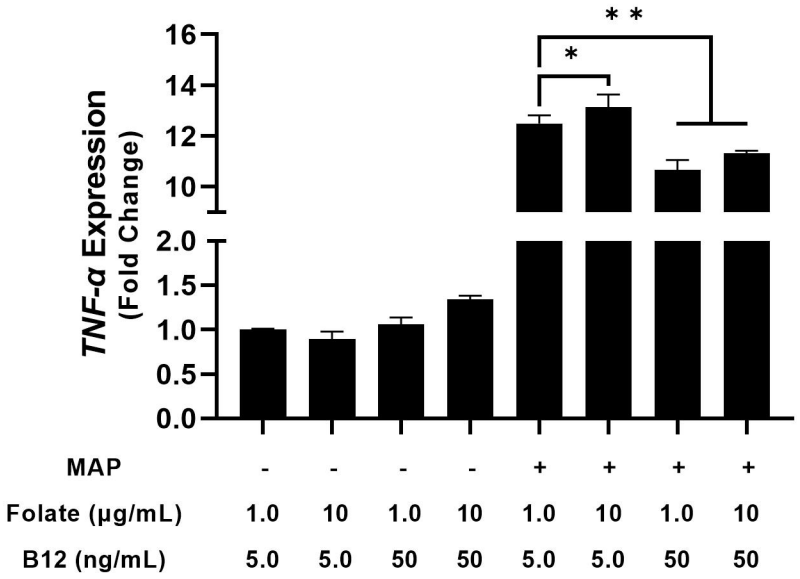

Figure 8: Effect of folate and B_{12} supplementation on TNF-α expression during MAP infection.

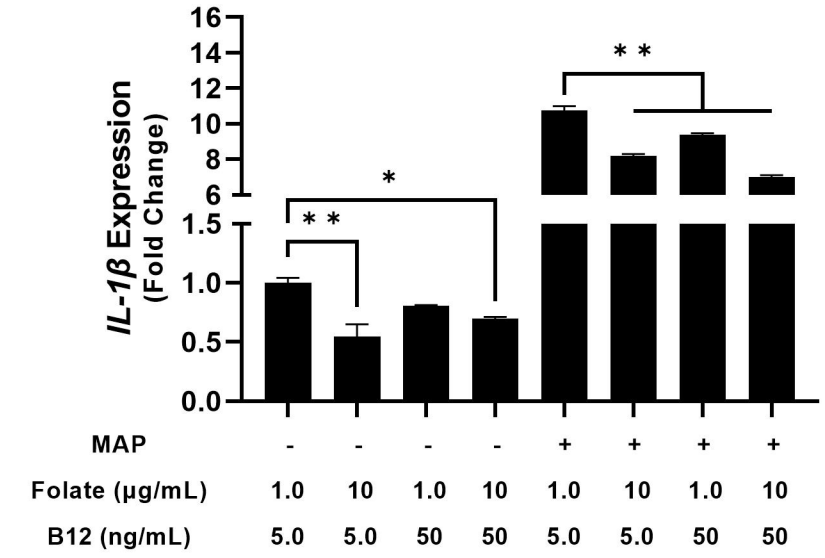

Figure 9: Effect of folate and B_{12} supplementation on IL-1β expression during MAP infection.

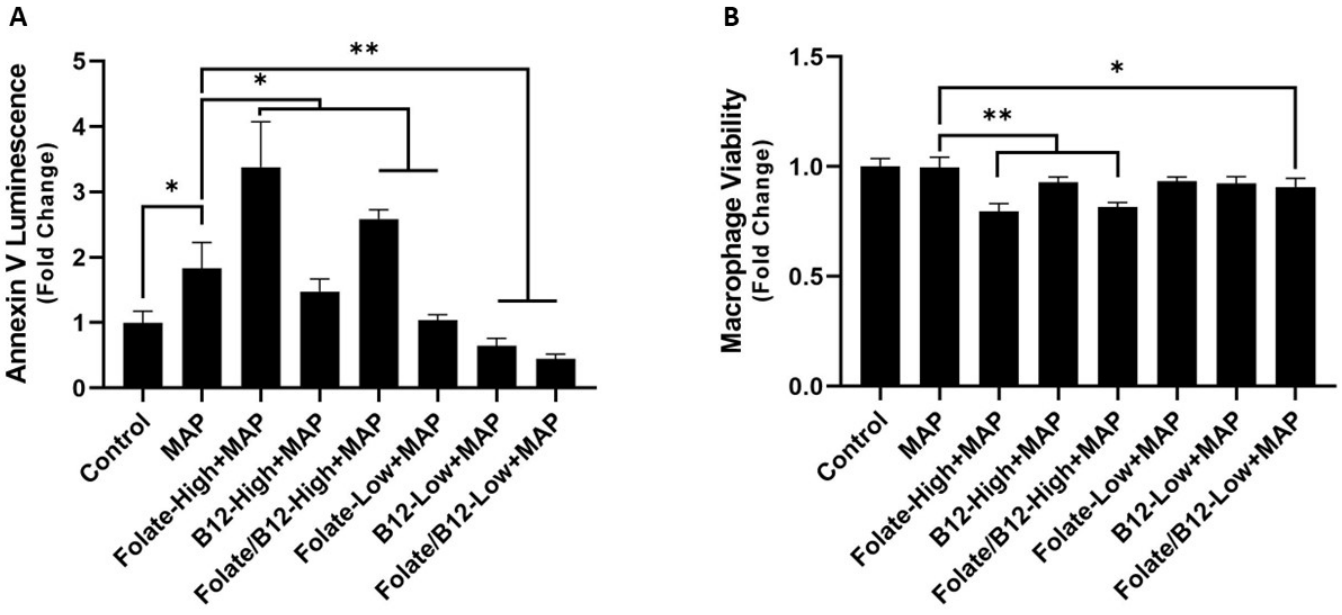

Figure 10: Effect of folate and B_{12} supplementation or depletion on macrophage apoptosis and viability.

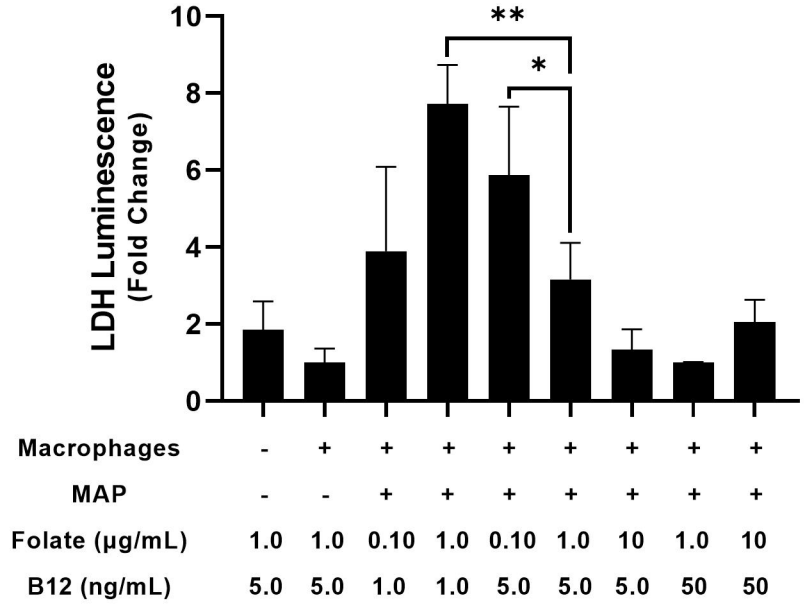

Figure 11: Effect of folate and vitamin B_{12} concentration on LDH release from Caco-2 monolayers after treatment with MAP-infected macrophage supernatant.

Tables

Table 1: Concentrations of folate and B_{12} in modified RPMI 1640 media for macrophage cell culture and infection.

Culture Condition	Folate (μg/mL)	B_{12} (ng/mL)
Control RPMI 1640	1.0	5.0
Folate-High	10.0	5.0
B_{12}-High	1.0	50.0
Folate + B_{12}-High	10.0	50.0
Folate-Low	0.10	5.0
B_{12}-Low	1.0	1.0
Folate + B_{12}-Low	0.10	1.0

Table 2: Effect of folate and vitamin B_{12} concentration on NADP release from Caco-2 monolayers after treatment with supernatant from infected macrophages.

Infection and Treatment	$NADP^+/(NADPH + NADP^+)*100 \pm SD$
Control (no infection)	6.23 ± 0.396
MAP infection (1×10^7 CFU/mL)	6.89 ± 0.761
Folate-low + MAP infection	7.74 ± 0.750
B_{12}-low + MAP infection	8.24 ± 0.657
Folate/B_{12}-low + MAP infection	7.07 ± 0.801
Folate-high + MAP infection	7.63 ± 1.04
B_{12}-high + MAP infection	6.59 ± 1.00
Folate/B_{12}-high + MAP	6.80 ± 0.716

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CHAPTER 3: CATHELICIDIN MEDIATES AN ANTI-INFLAMMATORY OF ACTIVE VITAMIN D (CALCITRIOL) DURING *M. PARATUBERCULOSIS* INFECTION

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Introduction

Vitamin D is a steroid hormone crucial to the efficient uptake and storage of calcium and phosphorus [1]. Most vitamin D are endogenously synthesized in human using exposure to ultraviolet radiation, which converts 7-dehydrocholesterol to an isomer of the pro-vitamin D_3, it then undergoes hydroxylation in the liver via the enzyme CYP27A1 to yield calcifediol or $25(OH)D_3$ [2]. Calcifediol makes up the majority of circulating vitamin D but displays minimal hormonal activity. When blood calcium or phosphate levels are low, the parathyroid gland detects the decline and releases parathyroid hormone (PTH) [3]. PTH acts upon the kidneys to stimulate the hydroxylation of $25(OH)D_3$ to $1,25(OH)_2D_3$, or calcitriol, using the enzyme CYP27B1 [2,3]. Interestingly, this enzyme can also be found in extra-renal tissues, including macrophages, where it regulates various intracranial events [4]. Calcitriol, the active form of vitamin D, is then carried through the circulation to tissues across the body [1]. As a fat-soluble hormone, it is capable of binding to the ubiquitously expressed vitamin D receptor (VDR), which heterodimerizes with the retinoid X receptor (RXR), then translocates to the nucleus, where it begins stimulating transcription of its target genes [1].

Among the genes enhanced by the VDR/RXR complex is *CAMP*, encoding Cathelicidin Antimicrobial Peptide [5]. The active form of cathelicidin is LL-37, which is a 37 residue-long peptide produced by macrophages in response to inflammation [6]. Like the defensin family, cathelicidin displays potent bactericidal and anti-inflammatory effects, through disruption of microbial membranes and conveying anti-inflammatory signals to immune cells [6]. Cathelicidin has shown notable beneficial effects even on persistent, long-term infections like tuberculosis and those found in inflammatory bowel disease (IBD) [7-11]. Its broad-spectrum effect on immunity makes cathelicidin a potential link between vitamin D and resistance to pathogens, even pathogens that are comparatively understudied; notably, its dependence on vitamin D signaling exposes cathelicidin to disruption when calcitriol is restricted [5,8]. Under ordinary circumstances, Toll-like receptors (TLRs) stimulation enhances transcription of the VDR and CYP27B1 in macrophages [7]. This signal allows the macrophage to enhance vitamin D-mediated cathelicidin production even without high circulating calcitriol [7,8]. However, previous work has shown that *Mycobacterium tuberculosis* (Mtb) lipoprotein LprE inhibits CYP27B1 and VDR upregulation, reducing cathelicidin production and enhancing bacterial survival [12]. This mechanism partially explains the persistence of tuberculosis within alveolar macrophages, and related bacteria might share a similar mechanism [12].

Mycobacterium avium subsp. *paratuberculosis* (MAP) is known to cause Johne's disease in ruminants, resulting in intestinal damage and chronic wasting [13]. Furthermore, in some genetically susceptible patients, MAP infection causes Crohn's disease (CD), an inflammatory bowel disease (IBD) characterized by asymmetrical, segmental, transmural inflammation with a relapsing-remitting pattern [13-16]. Similar to Mtb, MAP can infect macrophages and evade

immune system clearance to establish a persistent infection, which warranted the necessity of using antibiotics for MAP eradication among infected CD patients [17-20]. Therefore, we were intrigued to find out if MAP shares Mtb's method of evading immune detection by interfering with vitamin D signaling, which could be responsible for interference with *CAMP* expression and subsequent dysregulation of the intestinal microbiota in CD.

Additionally, therapeutic interventions of inactive vitamin D for IBD, which have so far shown mixed results for CD overall, might prove ineffective in MAP-infected patients and effective for MAP-uninfected patients. As such, it is necessary to determine whether MAP survival depends on interference with macrophage conversion of inactive calcifediol to active calcitriol, thereby inhibiting cathelicidin production and bacterial clearance. The objective of this study is to examine the effect of various forms of vitamin D and exogenous cathelicidin treatment on MAP infection and burden and subsequent macrophage-mediated inflammatory response. Our study clearly outlines a novel immunoevasive mechanism of MAP infection and reveals the importance of vitamin D signaling in eradicating infection in CD.

Materials and Methods

Measurement of Plasma Calcitriol and Cathelicidin in Clinical Samples

Plasma from peripheral blood samples (4.0 mL K_2-EDTA tube) was collected from 100 CD patients (CDAI ≥220 and ≤450). The status of MAP infection was subsequently determined via *IS900* PCR as described earlier [21], and then we randomly selected 40 MAP positive and 40 MAP negative CD patients for this study. We then used the Human Cathelicidin Antimicrobial Peptide ELISA Kit (MyBioSource, San Diego, CA) and the Calcitriol ELISA Kit (MyBioSource,

San Diego, CA) to determine the plasma levels of cathelicidin and calcitriol, respectively. This study was approved by the University of Central Florida Institutional Review Board # STUDY00003468. All samples were de-identified before handling.

Infection and Treatment of Monocyte-Derived Macrophages

The THP-1 cell line (ATCC TIB-202) was cultured in RPMI-1640 medium (ATCC 30-2001) with 10% fetal bovine serum (FBS; Sigma Life Science, St. Louis, MO). The cells were maintained in a humidified 5% CO_2 incubator at 37°C and grown to confluency in cell culture flasks. A total of 1.0 mL of cell suspension was transferred to 12-well tissue culture plates with $1x10^5$ cells per well. They were then differentiated into monocyte-derived macrophages using 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma Life Science, St. Louis, MO) followed by 48 hours of incubation at 37°C. Next, monocyte-derived macrophages were treated with 5 µg/mL lipopolysaccharide (LPS) or infected with clinical MAP UCF4 ($1x10^7$ CFU/mL), followed by 24 hours of incubation at the same conditions. When the macrophages were infected or stimulated with LPS, they also were dosed with 50 ng/mL vitamin D2, vitamin D3, calcifediol, or calcitriol, all purchased from Sigma Aldrich (St. Louis, MO), or 30 µg/mL LL-37 (Tocris Bioscience, Bristol, UK).

Measurement of CAMP, NOX-1, TNF-α, IL-1β, and IL-10 Expression in Treated Macrophages and Caco-2 Monolayers

RNA was isolated from each 1.0 mL sample of monocyte-derived macrophages following 24 hours of treatment with vitamin D or cathelicidin or from Caco-2 cells following 24 hours of co-culture with infected or treated macrophages. RNA was then reverse-transcribed to

cDNA, then gene expression was measured using specific primers for *GAPDH, CAMP, TNF-α, IL-1β,* and *IL-10* obtained from Bio-rad (Hercules, CA) followed by quantitative reverse transcription PCR (RT-qPCR) analysis. RNA was extracted using the RNeasy ® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer protocols. RNA concentrations were measured using NanoDrop (OD at 260 nm). Next, cDNA was synthesized from 1000 ng of each RNA sample using 5.8 µL master mix made from the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) and then topped up to a total volume of 20 µL with RNase-free water, according to manufacturer protocols. A thermal cycler (MyGene Series Peltier Thermal Cycler) was used to perform the reactions for 5 min at 25°C, 20 min at 46°C, and 1 min at 95°C. The cDNA samples were stored at -20°C or used immediately for RT-qPCR analysis. For each sample, 5 µL of cDNA was mixed with 10 µL of Fast SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA), 1 µL primer mix, and 4 µL of DEPC-treated water. Samples were added in triplicate to a 96-well microamp RT-PCR reaction plate, and the experiment was run using 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA). Deleted: repeated sentence. GAPDH was the control used to obtain baseline CT readings. Relative mRNA expression levels were calculated using the equation (2^(- ΔΔCT).

Measurement of CAMP, TNF-α, IL-1β, and IL-10 Protein Level in Treated Macrophages

Following 24 hours of infection and treatment with vitamin D forms or LL-37, monocyte-derived macrophages were pelleted by centrifugation at 2,500 rpm for 5 min at 4°C. The supernatants were saved, and TNF-α, IL-1β, and IL-10 protein levels were determined using the Ella automated immunoassay system (ProteinSimple, Santa Clara, CA). The Human Cathelicidin

Antimicrobial Peptide ELISA Kit (MyBioSource, San Diego, CA) was used to determine LL-37 levels following manufacturer's instructions.

Measurement of MAP Viability in MGIT Culture

We inoculated 1 mL BACTEC™ MGIT™ ParaTB medium (BD Diagnostics, Sparks, MD) with 10^7 CFU/mL MAP strain UCF4 as described earlier [22]. The media was then treated with LL-37 (Tocris Bioscience, Bristol, UK) and Halt™ Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL). The same amount of protease inhibitor cocktail and LL-37 was added to the media every 3 days to maintain a consistent concentration. Bacterial growth expressed in CFU/mL was quantified daily using the BACTEC™ MGIT™ 320 for 20 consecutive days. The medium contains a molecule which fluoresces in the presence of actively respiring mycobacteria, permitting automatic quantification of growth as described previously [19].

Measurement of MAP Viability in Infected Macrophages

We cultured THP-1 macrophages in 2 mL media as described previously. Following 24 hours of MAP infection and vitamin D/LL-37 treatment, the cultures were treated with 350 µL lysis buffer (Qiagen, Hilden, Germany) and incubated at room temperature for 15 minutes. Subsequently, 700 µL of each sample were transferred to a respective 1.5 mL microcentrifuge tube, and all samples were centrifuged for 1 minute at 8,000 rcf. The pellet was resuspended by gently vortexing, and 100 µL of each sample was mixed with 100 µL BacTiter-Glo Microbial Cell Viability Assay (Promega, Madison, WI) in a 96 well opaque-sided plate. Samples were incubated at room temperature on a shaker for 5 minutes, and luminescence was recorded using

the GloMax Navigator system GM-2000 (Promega, Madison, WI). Bacterial viability was analyzed from the generated luminescence.

Measurement of Calcitriol Production in Treated Macrophages

Following 24 hours of infection with MAP and treatment with 50 ng/mL calcifediol, THP-1 monocyte-derived macrophages were pelleted by centrifugation at 2,500 rpm for 5 min at 4°C. The supernatants were saved, and calcitriol levels were determined using the Calcitriol ELISA Kit (MyBioSource, San Diego, CA).

Knockdown of CAMP by siRNA Transfection

5 nmol of Silencer™ Pre-Designed siRNA (siRNA ID: 14402, ThermoFisher, Waltham, MA) specific to *CAMP* were diluted first in 50 uL nuclease-free H_2O. 3.3 µL of this stock were mixed with 30 µL Optimem media (Gibco, Waltham, MA) and further diluted in an additional 450 µL Optimem. 27 µL Lipofectamine reagent (Invitrogen, Carlsbad, CA) was then mixed with 450 µL Optimem and the resulting mixture was added to the 459 µL of diluted siRNA mix. 300 µL of the resulting transfection master mix was added to every 2 mL of media containing target cells, or 15 µL into 100 µL of a 96 well plate.

Co-culturing THP-1 Macrophages with Caco-2 Monolayers

The effects of calcitriol and cathelicidin on macrophage-mediated oxidative stress were examined in a human enterocyte-like cell line (Caco-2 ATCC HTB-37). Cells were routinely cultured in ATCC-formulated Eagle's Minimum Essential Medium (EMEM) supplemented with 20% FBS (ATCC, Manassas, VA) and maintained at 37°C in a humidified 5% CO_2 incubator.

Cells were grown in 12-well plates or microscope slides at a density of 3×10^5 cells per well until confluency and differentiation were reached in 21 days. On day 14, THP-1 macrophages were plated separately in co-culture wells. They were differentiated, infected with MAP, and treated with vitamin D within these wells as previously described. 24 hours following the infection, the co-culture wells were transferred to the 12-well plates containing Caco-2 cells to permit the free exchange of cytokines and other paracrine signals.

Visualizing Caco-2 Oxidative Stress via DHE Fluorescence Staining Assay

DHE fluorescence staining was performed on Caco-2 monolayers following 24 hours of co-culture with MAP-infected macrophages. First, monolayers were washed twice with cold PBS and then fixed with 4% paraformaldehyde (PFA) for 15 min. Monolayers were then washed twice with cold PBS and treated with 1 μM DHE stain (Sigma Aldrich, St. Louis, MO) for 25 min. Next, 60 μL VECTASHIELD Antifade Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) was used to co-stain nuclei. Slides were examined under Amscope IN480TC-FL-MF603 Fluorescence Microscope, where red staining indicates oxidative stress and blue staining represents nuclei. Captured images were analyzed by measuring average integrated density using NIH Image J 1.39o software, which was also used to generate merged images as we described earlier [23].

Measurement of Nicotinamide Adenine Dinucleotide Phosphate (NADPH)

Following 24 hours of co-culture with infected and treated macrophages, Caco-2 cells were lysed, and their levels of NADPH and total NADP were measured using the NADP/NADPH Assay Kit (Abcam, Cambridge, UK) according to manufacturer protocols.

Briefly, the cell lysates were halved, with one half heated for 30 min to degrade the oxidized NADP+ while leaving the NADPH untouched. Each lysate was then mixed with the kit developer in triplicate on a 96 well plate and left to incubate in the dark for 24 hours. NADP was then quantified for each well, with the heated lysate measuring the reduced NADPH as a fraction of total NADP.

Statistical Analysis

GraphPad Prism V.7.02 (GraphPad, La Jolla, CA, USA) was used for analyzing data statistics. The Kolmogorov–Smirnov normality test was used to test normal distribution for all values. Two-way analysis of variance (ANOVA) was used to assess significance among experiments, which was followed by Bonferroni correction test. Data are expressed as average ± SD of the mean, and the difference between treated samples vs. controls was considered statistically significant at a level of P-value < 0.05 and 95% confidence interval (CI). All experiments were performed in triplicates.

Results

Cathelicidin and Calcitriol Are Reduced in MAP-Infected CD Patients

We measured cathelicidin and calcitriol levels in 80 clinical plasma samples, 40 of which were MAP negative, 40 of which were MAP positive. We observed statistically significant reductions in plasma cathelicidin [Figure 12A] and plasma calcitriol [Figure 12B]. Cathelicidin in MAP-positive patients measures 155.55±49.77 ng/mL, increasing to 193.01±78.95 ng/mL in MAP-negative patients. The shift in calcitriol levels was more dramatic than cathelicidin; the

average calcitriol for MAP-positive patients was 51.48±31.04 pg/mL, but 272.36±94.77 pg/mL. This trend lends preliminary support to the hypothesis that MAP infection alters calcifediol hydroxylation.

Calcitriol Enhances CAMP Expression and Cathelicidin Production in THP-1 Macrophages

We next examined whether THP-1 macrophages respond to treatment with all forms of vitamin D or only calcitriol and calcifediol. Treatment with vitamin D2 and D3 did not significantly enhance *CAMP* expression compared with the control. Calcifediol treatment enhanced *CAMP* expression in uninfected cells by a factor of 3.29±0.15, and calcitriol enhanced expression by a factor of 5.24±0.08 [Figure 13A]. The effect of calcitriol increased in a dose-dependent manner. Treatment with 25 ng/mL calcitriol enhanced expression by only 2.79±0.07 fold, and 100 ng/mL yielded a 6.37±0.07-fold change [Figure 13B]. These phenomena change after MAP infection; MAP-infected cells had no significant *CAMP* enhancement upon treatment with calcifediol. However, calcitriol is still effective at increasing *CAMP* expression during MAP infection, increasing *CAMP* mRNA by a factor of 2.52±0.23 [Figure 13C]. These trends were later validated by ELISA, confirming that expression corresponds with LL-37 production [Figure 14].

Calcitriol and LL-37 Reduce Pro-Inflammatory Cytokine Expression in Infected Macrophages

To examine calcitriol's effect on MAP-induced inflammation, we infected THP-1 macrophages with MAP and treated them with different forms of vitamin D. Calcitriol was the only form of vitamin D which significantly reduced TNF-α and IL-1β expression [Figure 15A-B] and production [Table 3] compared to the untreated, infected cells. Furthermore, calcitriol

treatment partially rescued IL-10 expression [Figure 15C] and production [Table 3] in infected macrophages.

We observed similar effects with LL-37 treatment. MAP-infected macrophages showed a sharp decrease in TNF-α expression upon LL-37 treatment, a 5.27±0.23-fold increase reduced to 2.61±0.08 after treatment [Figure 16A]. IL-1β showed a similar decrease with LL-37, a 4.12±0.16-fold change reduced to 2.13±0.13-fold [Figure 16B]. IL-10, by contrast, increased in expression upon LL-37 treatment from 1.94±0.11-fold to 3.85±0.17-fold [Figure 16C]. These trends were then verified by measuring cytokine production levels. TNF-α was secreted into the supernatant at concentrations of 173.12±4.73 pg/mL upon MAP infection, but LL-37 treatment reduced it to 89.43±4.96 pg/mL. Likewise, IL-1β secretion dropped from 163.87±5.72 pg/mL to 82.23±4.39 pg/mL and IL-10 secretion increased from 69.18±3.69 pg/mL to 102.31±4.11 pg/mL [Table 4]. Interestingly, LPS-stimulated macrophages also decreased pro-inflammatory cytokine expression and increased IL-10 expression upon LL-37 treatment [Figure 17A-C]. These results were verified by measuring cytokine production levels [Table 4]. As such, LL-37 not only functions by clearing bacteria but can serve as an anti-inflammatory signal.

LL-37 Reduces MAP Viability in both Bacterial Culture and Macrophages

To verify that LL-37 reduces extracellular MAP viability, we inoculated five MGIT tubes with MAP and cultured them over the course of 20 days with differing concentrations of LL-37. We observed a concentration-dependent bacteriostatic effect of LL-37, by reducing both rate of growth and maximum bacterial load. At 50 ug/mL, MAP culture required an additional 3 days to

reach the stationary phase, and bacterial load at stationary phase was far lower than the untreated culture [Figure 18].

Additionally, we tested the effects of multiple vitamin D forms and LL-37 on bacterial viability in MAP-infected macrophages. There was no significant change upon treatment with the inactive forms of vitamin D. However, both LL-37 treatment and calcitriol treatment substantially reduced MAP viability from 2.92±0.45*10^4 CFU/mL to 1.07±0.41*10^4 CFU/mL and 1.24±0.52*10^4 CFU/mL, respectively [Figure 19].

Knockdown of *CAMP* Eliminates the Anti-Inflammatory Effect of Calcitriol during MAP Infection

We treated two groups of macrophages with 50 ng/mL calcifediol and infected one group with MAP. Following 24 hours of infection, we collected the supernatant and measured calcitriol level. The uninfected macrophages yielded 72.98±2.86 pg/mL calcitriol, while infected macrophages produced only 16.64±9.23 pg/mL [Figure 20A]. As such, the data indicate that MAP interferes with the conversion of calcifediol to calcitriol.

Furthermore, we transfected THP-1 macrophages with CAMP-siRNA to inhibit cathelicidin translation while leaving other VDR-controlled genes unaffected [Figure 8B]. We analyzed cytokine expression [Figure 21] and production [Table 5] in cells where *CAMP* was knocked down and calcitriol was present in the medium. *CAMP*-knockdown macrophages treated with calcitriol showed no significant reduction compared with untreated macrophages in TNF-α, IL-1β and IL-10 expression had no significant rescue. Accordingly, we conclude that during MAP infection, cathelicidin is necessary for calcitriol to mediate its anti-inflammatory effects.

Calcitriol and Cathelicidin Reduce Macrophage-Mediated Oxidative Stress on Co-cultured Caco-2 Monolayers.

To examine the tissue damage effect of MAP-infected macrophages on co-cultured Caco-2 monolayers, we used three methods to assess oxidative stress levels. First, co-cultured Caco-2 monolayers were stained with DHE, imaged, and the red DHE stain was quantified using imageJ software [Figure 22A]. Untreated MAP infection in co-cultured macrophages raised oxidative stress in the monolayer 14.78±0.71 fold compared with the control. Treatment with LL-37 or calcitriol reduced oxidative stress to 1.74±1.22 fold and 2.78±1.00 fold, respectively [Figure 22B].

We verified these results with analysis of *NOX-1* expression and NADPH/NADP assay in co-cultured Caco-2 monolayers. Expression of *NOX-1* was 5.09±0.09 fold higher when the co-cultured macrophages went untreated, but calcitriol treatment reduced it to 2.03±0.14 fold, and LL-37 reduced *NOX-1* expression to 2.51±0.16 [Figure 23A]. MAP infection in co-cultured macrophages caused a decline in NADPH/NADPt ratio to 44.51±3.81%, indicating that a highly oxidative intracellular environment was present. Treating MAP-infected macrophages with LL-37 or calcitriol rescued NADPH to 63.12±2.63% and 73.44±1.17% of total NADP, respectively [Figure 23B].

Discussion

Vitamin D deficiency is widespread in CD patients [24,25]. A recent meta-analysis found an inverse relationship between circulating vitamin D and CD severity [26]. Similarly, low vitamin D levels are inversely correlated with the likelihood of later surgical intervention in these patients [27]. From a therapeutic standpoint, vitamin D supplementation has shown promise in

reducing disease activity and inflammatory biomarkers [28]. However, little is known about how vitamin D is metabolized in patients with inflammatory bowel disease.

Vitamin D activation is necessary to mediate transcriptional changes [1]. It has been reported that vitamin D can directly inhibit the growth of bacteria following exposure to high doses, but the mechanism is unclear [29]. Subversion of the antibacterial response is a classical and potent way for mycobacteria to evade the host immune response and establish persistent infection [17,30]. Therefore, understanding how MAP alters the function of the macrophages in CD is crucial to explain why it is challenging to eradicate the infection in these patients. It is worth noting that Mtb possesses a host of mechanisms that assist in its survival within alveolar macrophages, many of which involve preventing phagolysosome fusion and halting apoptotic signals [31,32]. Interestingly, calcitriol has been shown to upregulate autophagy via cathelicidin, which leads to phagolysosome fusion and destruction of phagocytosed bacteria [33,34]. Similarly, a substantial body of work in cattle establishes MAP's adept evasion of the bovine immune system [17].

Previous work has shown that Mtb possesses at least one protein that subverts the vitamin D signaling pathway in macrophages, altering the antibacterial response [12]. Here, we present evidence that MAP is similarly capable of affecting vitamin D activation. Our analysis of clinical samples has shown that calcitriol and cathelicidin are both reduced in MAP-positive CD patients compared with MAP-negative CD patients. Moreover, we demonstrated that both calcifediol and calcitriol induce expression and production of cathelicidin in uninfected macrophages, but MAP infection alters calcifediol's inductive capacity. First, calcitriol treatment reduces pro-inflammatory cytokine expression and restores IL-10 production in MAP-infected macrophages.

In addition, LL-37 treatment displayed similar effects to calcitriol, and we verified that LL-37 has potent anti-microbial effects against MAP in both bacterial culture and infected macrophages. Consequently, *CAMP* knockdown removes the beneficial effects of calcitriol and cathelicidin on MAP infection, which validated the role of LL-37 as a mediator of calcitriol's anti-inflammatory signal in macrophages. Finally, we show that the anti-inflammatory effect of calcitriol and cathelicidin reduces MAP-induced oxidative stress in Caco-2 cells co-cultured with infected macrophages.

These findings strongly suggest that MAP and Mtb share a homologous mechanism that interferes with vitamin D signaling, which justifies further study on how MAP uniquely leads to CD pathogenesis. Additionally, our data highlight cathelicidin's key role in mediating vitamin D's anti-inflammatory properties and indicate that MAP substantially improves its viability by disrupting vitamin D signaling [Figure 24]. Likewise, the inductive effect of cathelicidin on co-cultured epithelial cells suggests that this effect may correspond with reduced oxidative stress in intestinal tissue.

Further studies may determine if MAP suppresses calcitriol production in the same way as Mtb via a lipoprotein-mediated disruption of TLR2 signaling [12]. However, the lack of a comprehensive genomic map of any MAP strain may hamper the bioinformatics approach to examine homology between the two species. Nevertheless, impeded stimulation of this pathway would be compelling evidence of a homologous protein and could then direct protein isolation and purification studies.

Outside the context of immunity, vitamin D is a crucial signal for maintaining bone homeostasis [1]. Since IBD patients are at increased risk of osteoporosis and other skeletal

abnormalities, an investigation into the mechanism by which IBD disrupts bone homeostasis is warranted [35]. Previous work in our laboratory has identified distinct changes in undercarboxylated osteocalcin, activated osteocalcin, and serum calcium levels in MAP-infected bovines and CD patients [36]. We have further noted a correlation between osteoporosis markers in the blood of rheumatoid arthritis (RA) patients, polymorphisms in the TNF-α gene and those of its receptor, and MAP infection [37]. The findings of this study are highly suggestive of a novel mechanism by which MAP might interfere with bone homeostasis. An aberrant, prolonged inflammatory response paired with impaired vitamin D activation may account for MAP's deleterious effect on CD and RA patients, where its presence would represent a subgroup at particular risk of osteoporosis. Consequently, testing for MAP DNA in RA and CD patients may prove valuable for clinicians.

From a therapeutic standpoint, MAP suppression of vitamin D activation suggests that the active form of vitamin D supplementation may prove more effective in MAP-infected CD patients since most vitamin D in circulation and commercial supplements is inactive [1]. This suggestion has some precedent in clinical trials with Mtb; despite vitamin D deficiency being a risk factor for tuberculosis, a course of supplementation with inactive vitamin D in Mongolian children had no significant impact on Mtb infection rates [38]. Furthermore, the fact that cathelicidin supplementation mirrors the effects of calcitriol on macrophage-mediated inflammation and enterocyte oxidative stress suggests that LL-37 could be a therapeutic option for the suppression of CD inflammatory symptoms. Accordingly, further studies of this phenomenon and vitamin D's effect on CD patients are warranted.

Figures

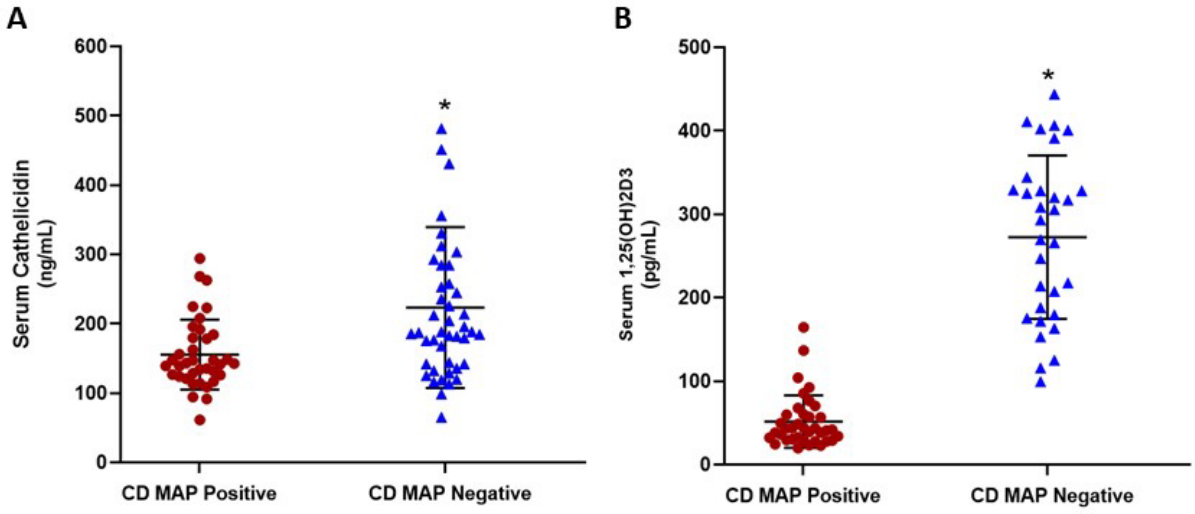

Figure 12: Plasma cathelicidin and calcitriol in MAP-positive and MAP-negative CD patients.

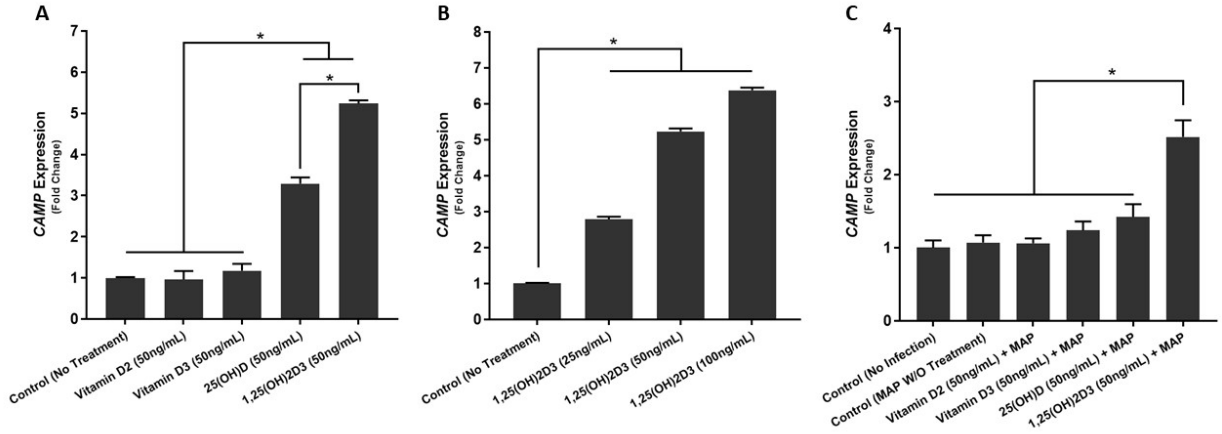

Figure 13: Effect of different forms of vitamin D and different concentrations of calcitriol on *CAMP* expression.

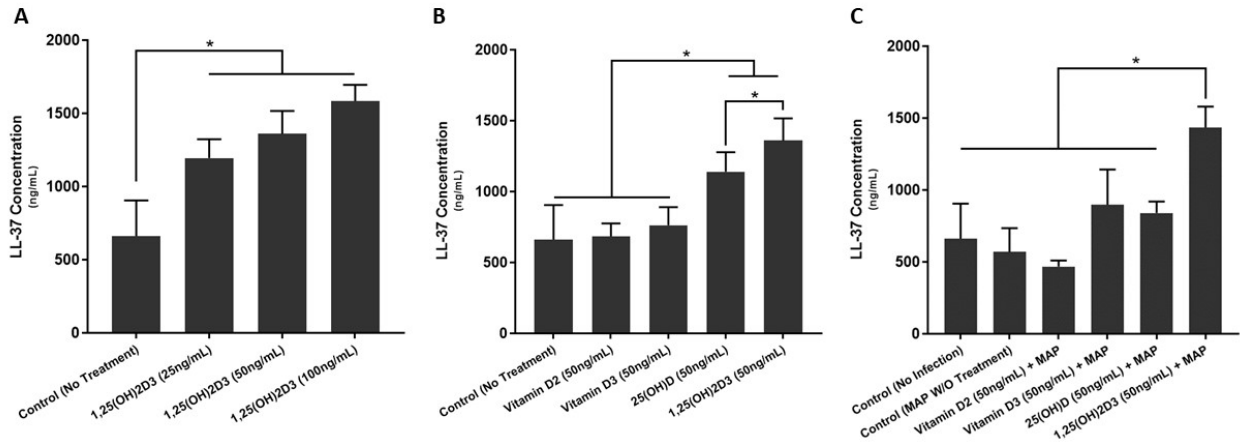

Figure 14: Effect of different forms of vitamin D and different concentrations of calcitriol on LL-37 production.

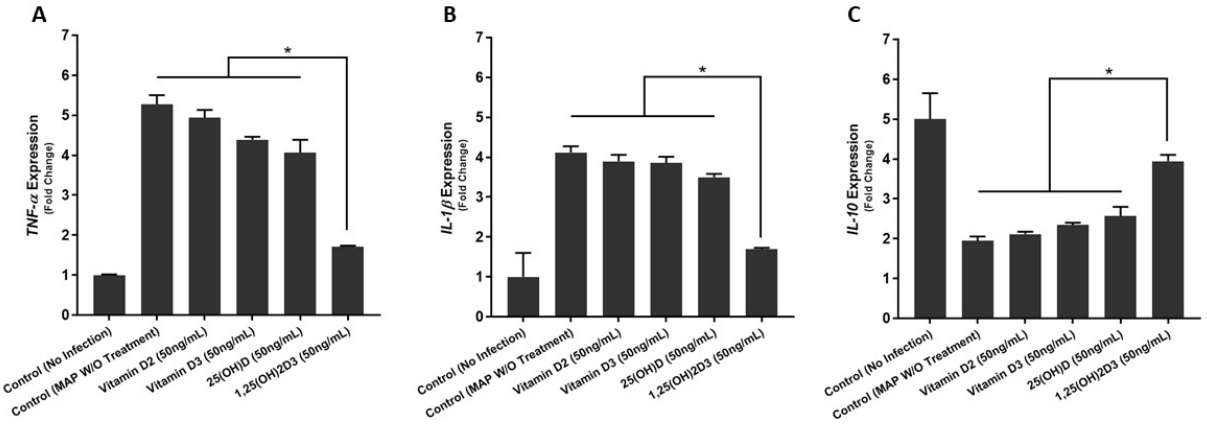

Figure 15: Effects of vitamin D treatment on cytokine expression in MAP-infected macrophages.

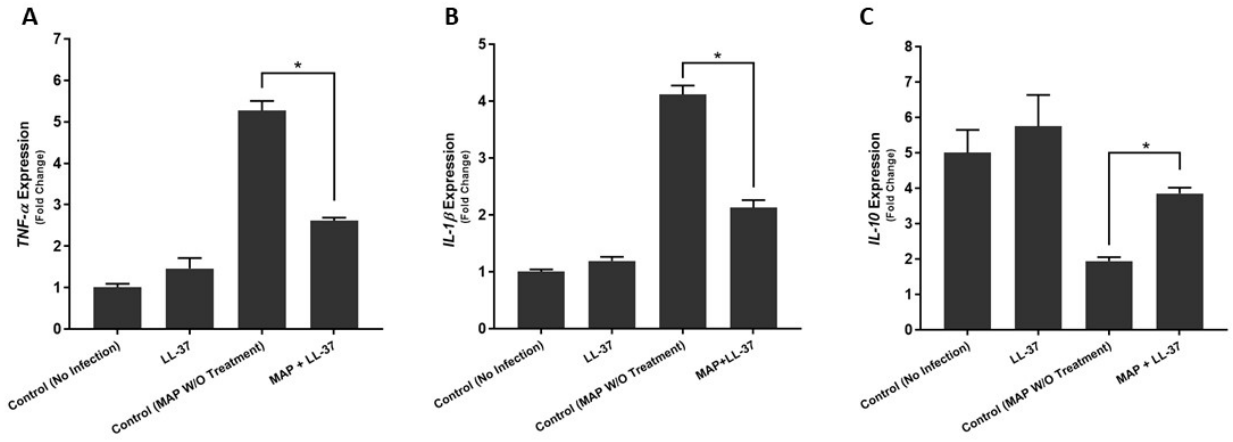

Figure 16: Effect of LL-37 treatment on cytokine expression in MAP-infected macrophages

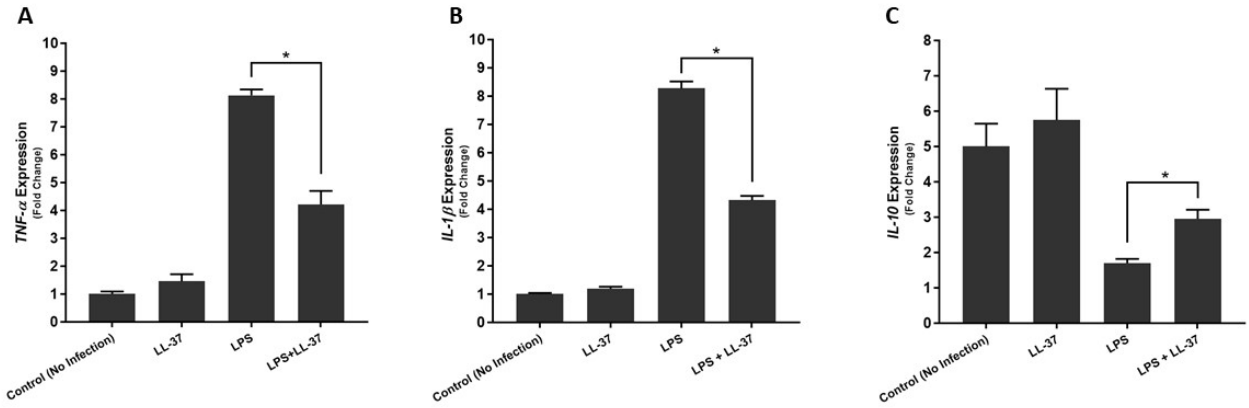

Figure 17: Effect of LL-37 treatment on cytokine expression in LPS stimulated macrophages.

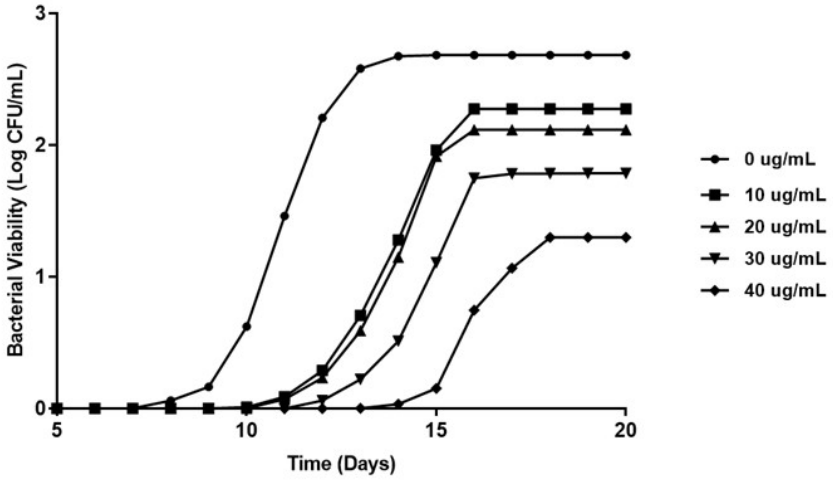

Figure 18: Direct effect of LL-37 treatment on MAP viability in MGIT culture.

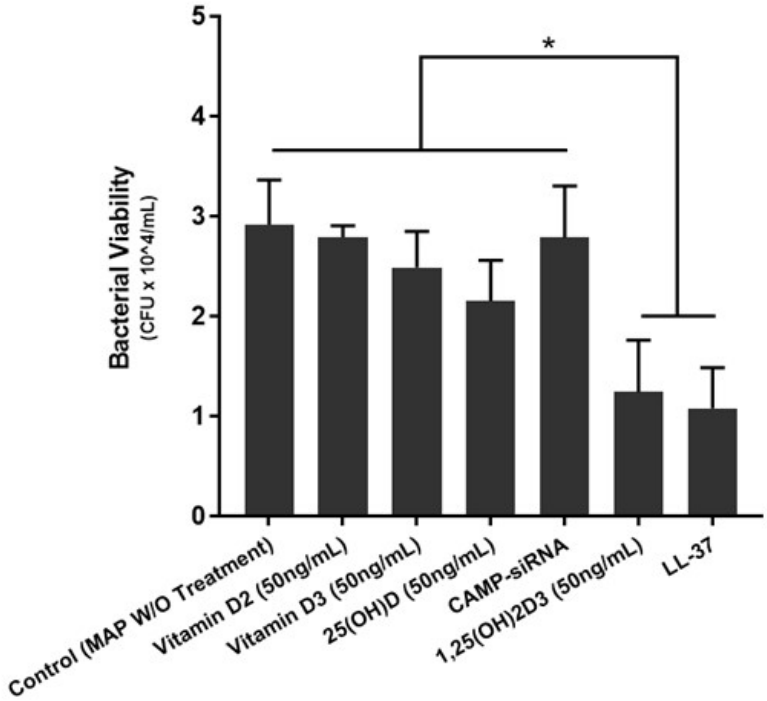

Figure 19: Effect of vitamin D and LL-37 on intracellular MAP viability.

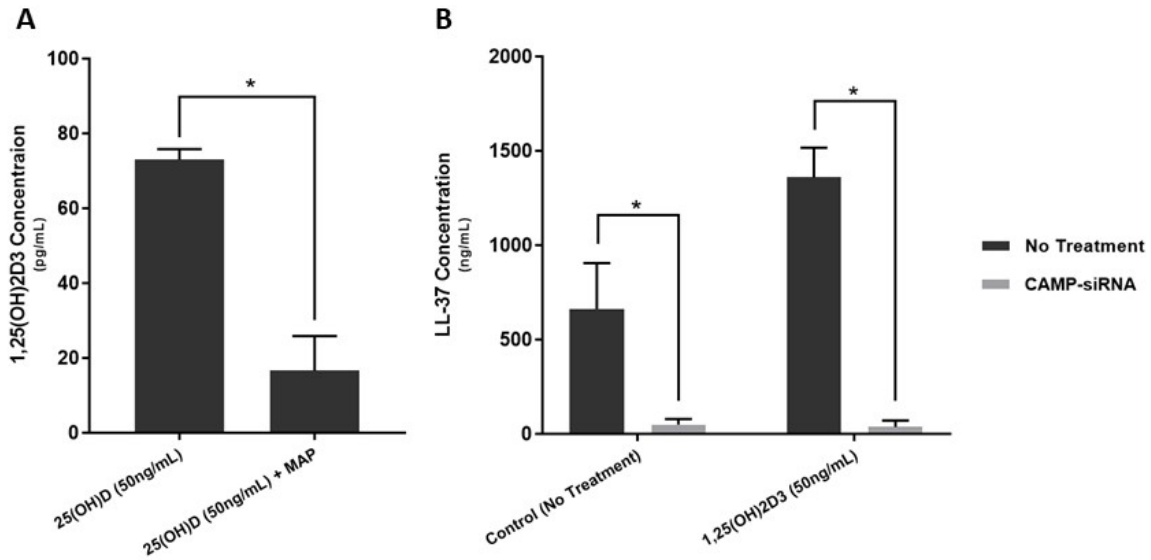

Figure 20: Disruption of calcitriol production after MAP infection and successful knockdown of *CAMP*.

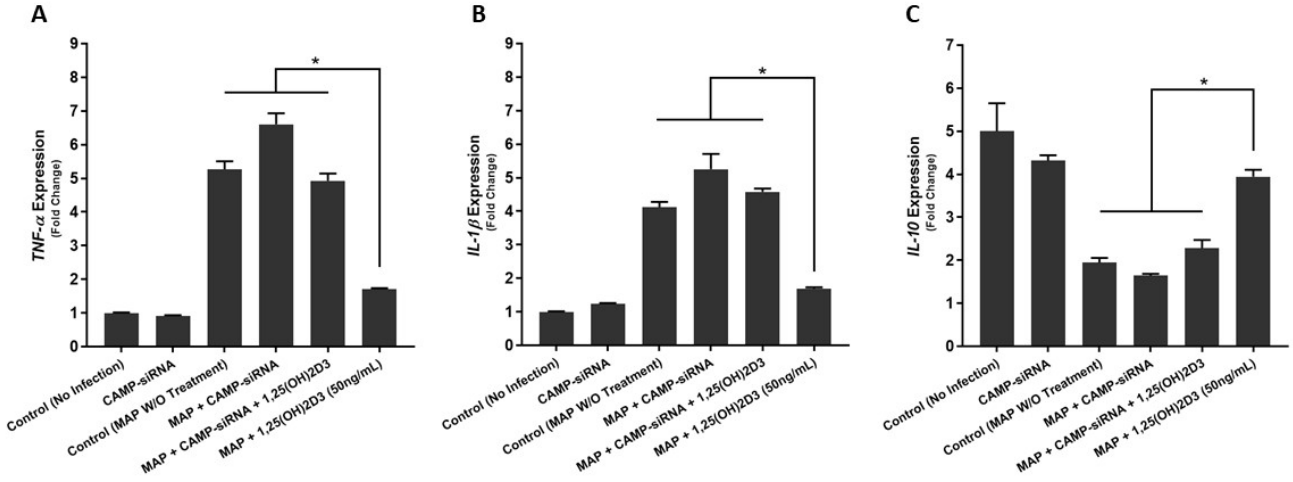

Figure 21: Effect of *CAMP*-siRNA transfection on cytokine expression in MAP-infected macrophages.

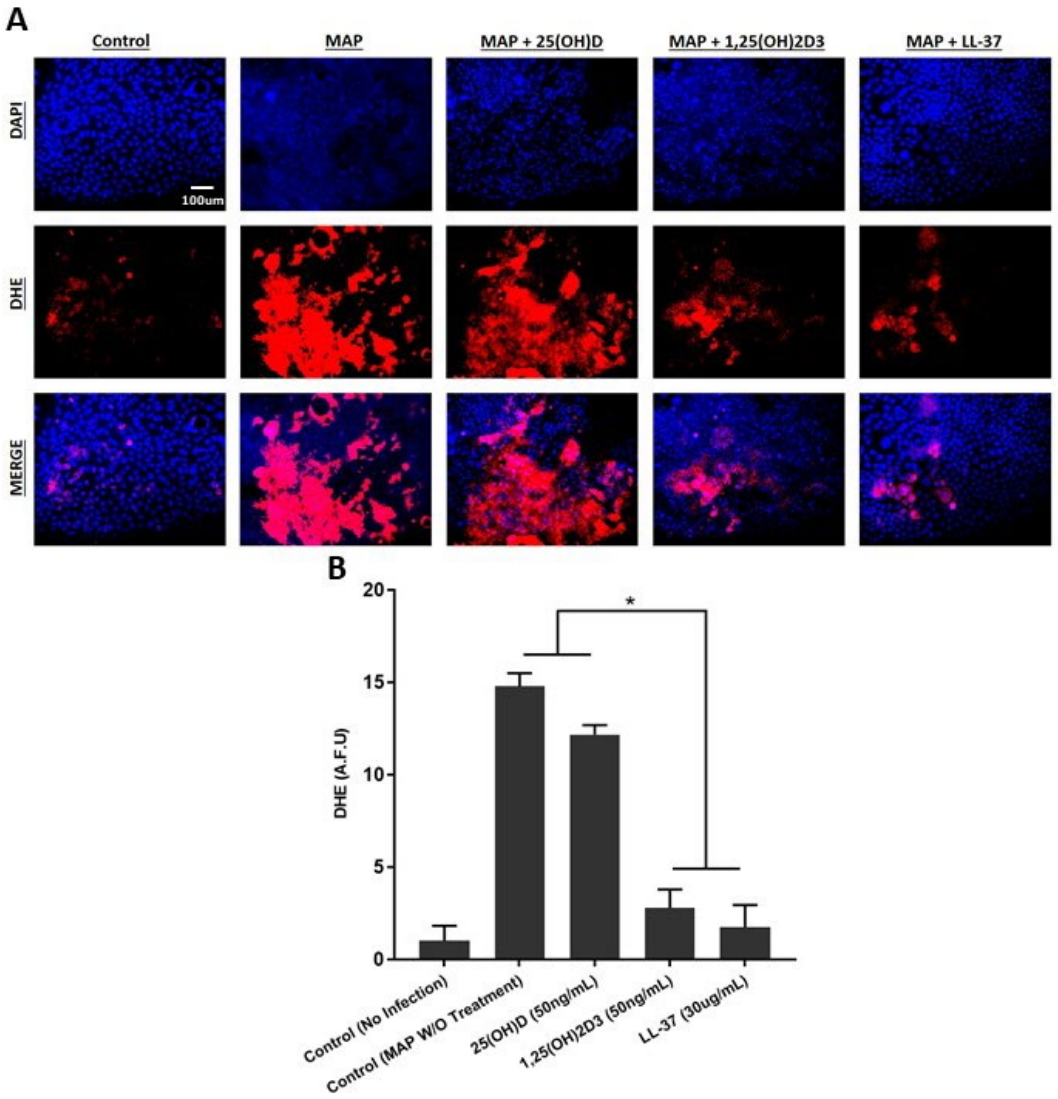

Figure 22: Impact of calcitriol and LL-37 treatment on oxidative stress in Caco-2 monolayers co-cultured with MAP-infected macrophages.

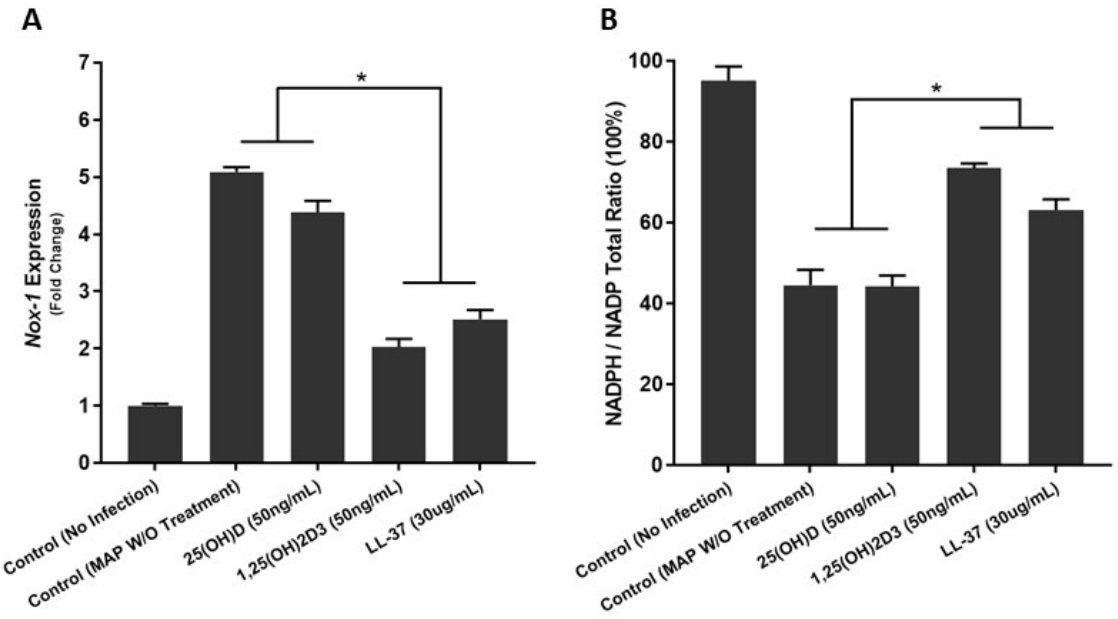

Figure 23: Quantitative analysis of oxidative stress levels in Caco-2 cells co-cultured with MAP-infected macrophages.

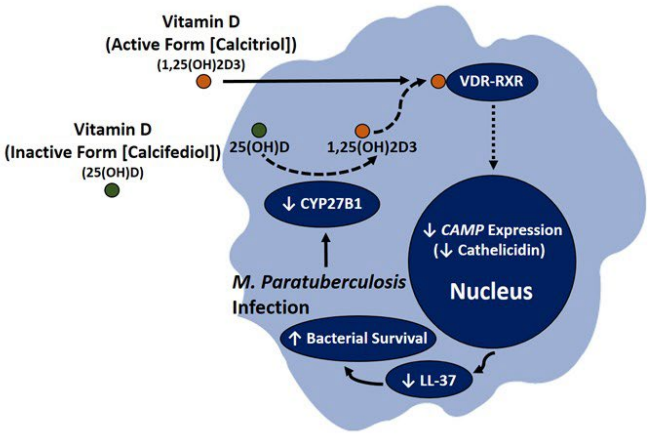

Figure 24: Cathelicidin mediates an anti-inflammatory role of calcitriol during MAP infection.

Tables

Table 3: Effect of vitamin D treatment on cytokine production in MAP-infected macrophages.

Infection and Treatment	TNF-α ± SD (pg/mL)	IL-1β ± SD (pg/mL)	IL-10 ± SD (pg/mL)
Control (no infection)	60.52 ± 1.75	56.13 ± 1.81	125.65 ± 6.11
Control (MAP W/O Treatment)	173.12 ± 4.73	163.87 ± 5.72	69.18 ± 3.97
MAP + Vitamin D2	157.84 ± 3.14	145.37 ± 6.17	73.14 ± 3.69
MAP + Vitamin D3	151.11 ± 5.44	140.61 ± 5.74	76.95 ± 5.26
MAP + 25(OH)D	136.21 ± 2.48	127.54 ± 3.84	82.12 ± 2.55
MAP + 1,25(OH)2D3	81.66 ± 2.79*	74.39 ± 3.72*	105.28 ± 4.58*

Table 4: Effects of LL-37 treatment on cytokine production in MAP-infected and LPS-stimulated macrophages.

Infection and Treatment	TNF-α ± SD (pg/mL)	IL-1β ± SD (pg/mL)	IL-10 ± SD (pg/mL)
Control (No Infection)	60.52 ± 1.75	56.13 ± 1.81	125.65 ± 6.11
LL-37	64.12 ± 2.83	61.42 ± 3.94	129.37 ± 3.82
Control (MAP W/O Treatment)	173.12 ± 4.73	163.87 ± 5.72	69.18 ± 3.69
MAP + LL-37	89.43 ± 4.96*	82.23 ± 4.39*	102.31 ± 4.11*
LPS Treatment	194.21 ± 3.51	183.04 ± 2.48	51.23 ± 1.56
LPS + LL-37	95.75 ± 2.64*	94.71 ± 1.75*	96.14 ± 6.41*

Table 5: Effect of *CAMP*-siRNA transfection on cytokine production in MAP-infected macrophages.

Infection and Treatment	TNF-α ± SD (pg/mL)	IL-1β ± SD (pg/mL)	IL-10 ± SD (pg/mL)
Control (No Infection)	60.52 ± 1.75	56.13 ± 1.81	125.65 ± 6.11
CAMP-siRNA	69.07 ± 3.65	61.91 ± 2.63	120.46 ± 3.97
Control (MAP W/O Treatment)	173.12 ± 4.73	163.87 ± 5.72	69.18 ± 3.69
MAP + CAMP-siRNA	182.67 ± 3.18	173.75 ± 4.57	75.62 ± 5.26
MAP + CAMP-siRNA + 1,25(OH)2D3	144.18 ± 4.03	132.86 ± 5.76	80.19 ± 2.55
MAP + 1,25(OH)2D3	81.66 ± 2.79*	74.39 ± 3.72*	105.28 ± 4.58*

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CHAPTER FOUR: CONCLUSIONS AND FUTURE DIRECTIONS

Crohn's disease affects approximately 214 of 100,000 Americans and 314 of 100,000 Europeans [1]. Furthermore, CD incidence is rising in geographic regions undergoing industrialization and development [2]. Since CD can seriously harm quality of life and lead to complications like strictures, fistulas, abscesses, and colon cancer, active therapeutic intervention is necessary to manage this emerging problem [3]. While in the past a variety of immunomodulatory drugs have been used to manage CD inflammation, the current first-line treatment for moderate to severe CD consists of biologic agents, typically anti-TNF-α antibodies [3,4]. However, this therapeutic approach has severe shortcomings: 50% of CD patients display no response or eventually lose responses to anti-TNF-α antibodies [4]. Prolonged use of anti-TNF-α antibodies also has deleterious effects on immune system function, sensitizing patients to tuberculosis and other granulomatous infections [5,6]. New treatment paradigms are therefore necessary to manage this growing global health issue.

CD patients are at an elevated risk of micronutrient deficiencies, including folate, vitamin B_{12}, and vitamin D [3]. However, the impact of vitamin deficiencies on MAP infection and CD inflammation remains poorly characterized. The investigation detailed herein illustrates the connection between folate, vitamin B_{12}, and vitamin D and macrophage-mediated inflammation during MAP infection. Initially, we demonstrate that MAP-positive CD patients have lower plasma folate and vitamin B_{12} levels than MAP-negative CD patients. Folate and B_{12} supplementation attenuate inflammatory cytokine expression in macrophages after MAP exposure; conversely, folate and B_{12} deficiency exacerbates macrophage inflammatory cytokine expression and oxidative stress in co-cultured enterocytes. Furthermore, folate and B_{12}

deficiencies impede apoptosis of infected macrophages, depriving the immune system of a crucial clearance mechanism for intracellular bacteria.

Similarly, our investigation indicates a compelling role for vitamin D in macrophage mediated inflammation and MAP infection. We demonstrate that MAP-positive CD patients have lower levels of plasma calcitriol than MAP-negative patients. Conversion of calcifediol to calcitriol, a necessary step for vitamin D signaling, is shown to be impeded by MAP infection in macrophages. Calcitriol deprivation removes a necessary signal for *CAMP* expression and cathelicidin production, which improves intracellular and extracellular MAP survival. Furthermore, cathelicidin treatment is an anti-inflammatory signal that reduces inflammatory cytokine release independent of its bactericidal properties. Calcifediol and calcitriol are similarly anti-inflammatory, but during MAP infection only calcifediol remains effective. We establish that cathelicidin is a necessary downstream signal for calcitriol to mediate its anti-inflammatory effects. *CAMP* knockdown abolishes calcitriol's effect on inflammatory cytokine expression. Finally, oxidative stress in intestinal epithelial cells co-cultured with MAP-infected macrophages is reduced by cathelicidin and calcitriol treatment, highlighting their relevance for CD tissue damage in the intestinal tract.

The findings detailed in this investigation open a rich avenue of future inquiry to develop future research. While MAP is present in approximately 50% of CD patients, it is uncertain whether folate and vitamin B_{12}'s effect on inflammation occurs with other CD-associated pathogens like adherent-invasive *E. coli* [7]. Furthermore, the effect of folate and B_{12} on other macrophage antimicrobial processes such as cathelicidin expression and phagocytosis remains uncharacterized. While we have described how macrophages respond in response to altered

folate and B_{12} availability, a mechanism by which these vitamins affect cell death and inflammatory cytokine expression remains unknown. Similarly, we confirmed that MAP infection blocks vitamin D activation in macrophages. However, the mechanism and putative virulence factor by which MAP mediates this effect remains uncharacterized. Our findings also suggest that cathelicidin and calcitriol may have beneficent effects on MAP-positive CD patients. Transition to *in vivo* models of MAP infection and Crohn's disease may illuminate if clinical trials are warranted.

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APPENDIX—CO-AUTHOR PERMISSION FOR RELEASE

We, the authors, give our permission to include data and materials described in Vaccaro et. al. 2021 (below) in the dissertation contents of Mr. Joseph A. Vaccaro for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: The Role of Methyl Donors of the Methionine Cycle in Gastrointestinal Infection and Inflammation.

Authors: Joseph A. Vaccaro and Saleh A. Naser

Journal: Healthcare (Basel), 10(1):61.

DOI: 10.3390/healthcare10010061.

Published: December 29, 2021

We, the authors, give our permission to include data and materials described in Vaccaro et. al. 2022 (below) in the dissertation contents of Mr. Joseph A. Vaccaro for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: Cathelicidin Mediates an Anti-Inflammatory Role of Active Vitamin D (Calcitriol) During *M. paratuberculosis* Infection.

Authors: Joseph A. Vaccaro, Ahmad Qasem, and Saleh A. Naser

Journal: Frontiers in Cellular and Infection Microbiology 12:875772

DOI: 10.3389/fcimb.2022.875772

Published: April 4, 2022

We, the authors, give our permission to include data and materials described in Vaccaro et. al. 2023 (below) in the dissertation contents of Mr. Joseph A. Vaccaro for Doctor of Philosophy in Biomedical Sciences at the University of Central Florida.

Article Title: Folate and Vitamin B_{12} Deficiency Exacerbate Inflammation during *Mycobacterium avium paratuberculosis* (MAP) Infection.

Authors: Joseph A. Vaccaro, Ahmad Qasem, and Saleh A. Naser

Journal: Nutrients, 15(2):261

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