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Killing of *Escherichia coli* by Crohn's Disease Monocyte-derived Macrophages and Its Enhancement by Hydroxychloroquine and Vitamin D

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Background: Crohn's disease (CD) is associated with defective innate immunity, including impaired neutrophil chemotaxis, and mucosal invasion by bacteria, particularly adherent and invasive *Escherichia coli* that replicate inside macrophage phagolysosomes. We compared CD and healthy control (HC) macrophages for their abilities to kill *E. coli* and generate neutrophil chemoattractants and also assessed the effects of hydroxychloroquine (HCQ) and vitamin D on killing of phagocytosed *E. coli*.

Methods: Peripheral blood monocyte-derived macrophages from CD and HC were compared for bacterial killing and generation of neutrophil chemoattractants in response to CD-derived *E. coli*. *Escherichia coli* replication was also assessed in the presence and absence of HCQ, alone and with antibiotics, and vitamin D.

Results: Monocyte-derived macrophages from patients with CD were similar to HC in allowing replication of phagocytosed CD-derived *E. coli*: HM605 {CD: N = 10, mean fold replication in 3 hr = 1.08 (95% confidence interval [CI], 0.39–1.78); HC: N = 9, 1.50 (95% CI, 1.02–1.97); *P* = 0.15} and also in generation of neutrophil chemoattractants in response to *E. coli* (mean fold chemotaxis relative to control: CD = 2.55 [95% CI, 2.31–2.80]; HC = 2.65 [95% CI, 2.46–2.85], *P* = 0.42). HCQ and 1,25 OH₂-vitamin D₃ both caused dose-dependent inhibition of intramacrophage *E. coli* replication 3-hour postinfection; HCQ: 73.9% inhibition (*P* < 0.001) at 1 μg/mL, accompanied by raised intraphagosomal pH, and 1,25 OH₂-vitamin D₃: 80.7% inhibition (*P* < 0.05) at 80 nM. HCQ had synergistic effects with doxycycline and ciprofloxacin.

Conclusions: CD and HC macrophages perform similarly in allowing replication of phagocytosed *E. coli* and generating neutrophil chemoattractants. Replication of phagocytosed *E. coli* was substantially decreased by HCQ and vitamin D. These warrant further therapeutic trials in CD in combination with relevant antibiotics.

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Key Words: phagolysosomes, chemotaxis, ciprofloxacin, doxycycline

There is growing consensus that Crohn's disease (CD) results from an altered relationship between the immune system and intestinal bacteria.¹ Changes in the fecal microbiota, particularly

decreased diversity,² are also found in animal models of gut inflammation,³ but the mucosa-associated microbiota shows more specific changes, even in inactive disease. These include

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decreased colonization by the probiotic *Faecalibacterium prausnitzii* and increase in *Escherichia coli*.⁴⁻⁶ Similar changes have been found in the treatment-naïve mucosa-associated microbiome in pediatric CD.⁷

CD mucosa-associated *E. coli* commonly adhere to and invade epithelial cell-lines and are therefore termed adherent-invasive *E. coli* (AIEC). Although originally isolated from CD ileal mucosa, they are also found in the colon.^{5,7} AIEC taken up by macrophages are initially destroyed within autophagosomes,⁸ but some escape this process and replicate within the acidic environment of phagolysosomes.⁹ Phagocytosed AIEC induce granuloma formation in vitro^{10,11} and in animal models.¹² This, and the common presence of *E. coli* DNA in CD-associated granulomas,¹¹ suggests a possible pathogenic role. AIEC are thought to translocate through microfold cells (M cells) that account for about 5% of the “dome” epithelium overlying Peyer’s patches in the distal ileum, and lymphoid follicles in the colon, the sites of the earliest lesions seen in CD.^{13,14} CD AIEC commonly express long polar fimbriae essential for M-cell translocation.^{6,15}

Patients with CD commonly have polymorphisms in genes (*NOD2/CARD15*, *IRGM*, and *ATG16L1*) linked to pathogen recognition and autophagy relevant to killing of bacteria within macrophages.¹⁶ CD is also associated with defective recruitment of neutrophils to sites of dermal¹⁷ and mucosal¹⁸ wounding and intradermally injected killed *E. coli*.¹⁹ Chemotaxis of CD neutrophils is normal when studied ex vivo,²⁰⁻²² and it has been suggested that delayed chemotaxis in vivo results from defective macrophage chemokine release.¹⁹ The delay in neutrophil chemotaxis could then contribute to increased bacterial uptake by macrophages.

CD peripheral blood monocytes have normal ability to kill Gram-positive bacteria,²³ but there has been no published study of their killing of Gram-negative bacteria. This is important not only because of the possible relevance of Gram-negatives such as *E. coli* in CD pathogenesis but also because macrophages have distinct mechanisms for killing of Gram-negative organisms. This includes interaction with a signaling lymphocyte-activation molecule involved in activation of NADPH oxidase within phagosomes.²⁴

Proof that intramacrophage *E. coli* replication may have a pathogenic role in CD will depend on resolution of the disease in response to treatments that target these *E. coli*. Antibiotic therapy has to date been only modestly effective,²⁵ possibly either because single antibiotics have been used, with multidrug resistance seen in 61.5% of AIEC,²⁶ or because some antibiotics, particularly penicillins and gentamicin, are unable to kill bacteria within macrophage vesicles.²⁷

Macrophages are relatively ineffective at killing bacteria in comparison with neutrophils.²⁸ In Q-fever and Whipple’s disease, where bacteria, *Coxiella burnetii* and *Tropheryma whipplei*, respectively, replicate inside macrophage phagosomes, addition of hydroxychloroquine (HCQ) to antibiotics has greatly improved eradication rates.²⁹⁻³¹ Bacteria, including AIEC, that replicate within macrophage vesicles typically require an acid

environment.⁹ HCQ is a mild base that is concentrated within intracellular vesicles thus raising their pH.³¹

Vitamin D deficiency has been implicated in conditions such as multiple sclerosis and CD that are more common in countries furthest from the equator.³² Vitamin D is important for normal function of the innate immune system including autophagy and a controlled trial of vitamin D supplementation in CD, regardless of their initial vitamin D status, only just failed ($P = 0.06$) to significantly reduce relapse.³³

Here, we have assessed the ability of CD monocyte-derived macrophages (MDM) to kill *E. coli* and to generate a neutrophil chemotactic response. We then assessed the ability of vitamin D and HCQ, at clinically achievable concentrations, to enhance macrophage killing of phagocytosed *E. coli*.

METHODS

Ethical Considerations

Ethical approval was obtained from the National Research Ethics Service Committee North-West England (study approval number 09/H1010/64) to take peripheral venous blood after informed consent from patients with CD recruited from the Royal Liverpool University Hospital and healthy controls (HC) recruited from Liverpool hospital and University staff.

Bacterial Strains and Culture

Two representative CD mucosa-associated AIEC isolates were studied; HM605, a colonic isolate,⁵ and LF82, an ileal isolate.⁴ Both isolates replicate within phagolysosomes of murine and human MDM.^{9,27,34} Laboratory strain *E. coli* K12 (ATCC 29425; Manassas, VA) and *Staphylococcus aureus* Oxford stain (NCTC 6571; Public Health England; Porton Down, United Kingdom) were also tested. Isolates were grown overnight on Luria-Bertani (LB) agar at 37°C in air, washed 3 times in sterile phosphate-buffered saline pH 7.3 (Life Technologies; Paisley, United Kingdom), and resuspended to an optical density (OD_{550 nm}) equivalent to 10⁹ bacteria per milliliter.

Murine Macrophage Cell-line Culture

J774A.1 murine macrophages (91051511) obtained from the European Collection of Cell Cultures (Porton Down; United Kingdom) were cultured at 37°C in RPMI medium supplemented with 10% vol/vol fetal bovine serum, 2 mM glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin, in a humidified atmosphere of 5% CO₂ per 95% air. Macrophages were passaged by scraping twice weekly, up to passage 19.

Isolation of Human Peripheral Blood MDM and Neutrophils

Peripheral venous blood was taken after informed consent from 10 patients with CD recruited from the Royal Liverpool University Hospital and 10 HC recruited from Liverpool hospital and University staff. Tables, Supplemental Digital Content 1,

<http://links.lww.com/IBD/A836>, for inclusion/exclusion criteria (see Table 1, Supplemental Digital Content 1, <http://links.lww.com/IBD/A836>), baseline characteristics (equivalent apart from higher hsCRP in CD) (see Table 2, Supplemental Digital Content 1, <http://links.lww.com/IBD/A836>), and patient characteristics (see Table 3, Supplemental Digital Content 1, <http://links.lww.com/IBD/A836>). Five patients with CD had active disease (Harvey–Bradshaw Index > 4). None were receiving immunosuppressants, corticosteroids, or anti-tumor necrosis factor (TNF) therapy, and none had features of sepsis (median hsCRP = 5.9 mg/L; range, 1.04–18.3 mg/L) at the time of sampling. Blood (50 mL) was immediately heparinized using unfractionated heparin sodium 5 U/mL (Wockhardt UK Ltd, Wrexham; Wales), mixed 1:1 with phosphate-buffered saline, layered over Lymphoprep (Alere; Stockport, United Kingdom), and centrifuged at 800g for 20 minutes at room temperature. Mononuclear cells were aspirated, washed, resuspended in RPMI supplemented with 20 mM HEPES pH 7.4, 100 U/mL penicillin, and 100 µg/mL streptomycin, adjusted to 5×10^6 cells per milliliter and seeded into 100 mm Nunc culture dishes (VWR; Lutterworth, United Kingdom). After 2-hour incubation at 37°C, nonadherent cells were removed by washing and adherent monocytes differentiated into macrophages over 5 days.¹⁹

Neutrophils were isolated from heparinized venous blood using Polymorphprep (Alere) according to manufacturer's instructions and, after removal of red cells by hypotonic lysis, resuspended in RPMI with 20 mM HEPES pH 7.4, 0.5% vol/vol bovine serum albumin, 2 mM CaCl₂, and 2 mM MgCl₂.

Intramacrophage Bacterial Killing

Ability of human MDM and J774A.1 macrophages to kill phagocytosed bacteria was assessed by a gentamicin protection assay.²⁷ Macrophage monolayers were inoculated with bacteria at a multiplicity of infection of 25 (for MDM) or 10 (J774.A1). After 2-hour infection, macrophages were washed thrice with sterile phosphate-buffered saline and incubated for 1 hour in RPMI containing 10% vol/vol fetal bovine serum, 2 mM glutamine (for J774.A1) or in X-Vivo 15 (for MDMs), and 20 µg/mL gentamicin to kill adherent, noninternalized bacteria. Macrophages were washed and lysed with 1% vol/vol Triton X-100 for 5 minutes to release internalized bacteria. Bacteria colony-forming units were counted after overnight culture on LB agar. Parallel plates were cultured for a further 3 hours after 1-hour incubation with gentamicin (6 hr in total), again followed by lysis and overnight colony-forming units counting. Data were expressed as fold change of recovered intramacrophage bacteria at 6 hours relative to 3 hours.

Effects of HCQ, with and Without Antibiotics, and Vitamin D on Intramacrophage Survival of CD AIEC

Doxycycline and ciprofloxacin were tested at C_{max} (peak serum concentration achieved with standard oral dosing) and 10% C_{max}, based on published data.^{35,36} HCQ concentrations were

based on published steady state concentrations.³⁷ Concentrations of 1,25 OH₂-vitamin D₃ were those defined as deficient in human serum (<20 nM)^{32,38} and that required for optimal immune cell function (>80 nM).^{32,39}

Cytokine Enzyme-linked Immunosorbent Assays

Interleukin 6 (IL-6), IL-8, and TNF-α were quantified by enzyme-linked immunosorbent assay as per manufacturer's instructions (R&D systems; Abingdon, United Kingdom; IL-6, D6050; IL-8, D800C; TNF-α, DTA00C). Murine IL-6 and TNF-α released by J774A.1 macrophages were also determined by enzyme-linked immunosorbent assay (R&D systems; IL-6, M6000B; TNF-α, SMTA00B). OD_{450 nm} was measured using a Sunrise microplate reader (Tecan; Theale, United Kingdom).

Neutrophil Chemotaxis Assay

Neutrophil chemotaxis was quantified using a CytoSelect 96-Well Cell Migration Assay, 3 µm pore diameter (Cambridge Bioscience; Cambridge, United Kingdom) as per manufacturer's instructions. Chemoattractant in the lower chamber was X-Vivo 15 medium from 10⁵ MDM either uninfected or infected for 6 hours with *E. coli* HM605 or K12 (multiplicity of infection of 25). CyQuantGR fluorescence was measured using an F200 microplate reader (Tecan); excitation λ 485 nm, emission λ 535 nm.

Effect of HCQ on Macrophage Intraphagolysosome pH

J774A.1 macrophages cultured in 35 mm CELLview dishes (Greiner Bio-One; Stonehouse, United Kingdom) were washed and incubated in phenol red-free RPMI containing 10 µg/mL pHrodo-conjugated *E. coli* bioparticles and HCQ (0–10 µg/mL) for 3 hours at 37°C. Imaging was by a Zeiss LSM-510 meta-laser confocal microscope with atmosphere- and temperature-controlled stage (Carl Zeiss AG, Oberkochen, Germany). Ten random high-powered fields were imaged for each treatment, processed using LSM-5 software (Zeiss AG), and fluorescence intensity analyzed with AQM-6 (Kinetic Imaging; Nottingham, United Kingdom). A pH-calibration curve was constructed by incubation of pHrodo bioparticle-loaded macrophages with buffers (pH 4–7) containing ionophores nigericin (10 µM) and vanilomycin (10 µM) plus the vacuolar H⁺-ATPase inhibitor bafilomycin (0.1 µM).⁴⁰ NH₄Cl (2 mM) was used as an intraphagolysosome alkalinization control.

Influence of Intracellular Iron Availability on the Action of HCQ

To determine whether the antibacterial actions of HCQ were mediated through pH-mediated restriction of intramacrophage iron, macrophages were pretreated with either 10 µM ferric citrate, which exhibits pH-dependent solubility and release of free ferric iron, or 10 µM ferric nitrilotriacetic acid (FeNTA), which releases free ferric iron irrespective of pH.⁴¹

Genomic Analysis for CD-associated Polymorphisms in NOD2/CARD15, ATG16L1, and IRGM

Genomic DNA was extracted using a Nucleon BACC3 kit (Gen-Probe; Livingston, United Kingdom) from EDTA-treated peripheral venous blood taken from patients with CD and HC in whom macrophage function studies were also performed. DNA yield was determined using PicoGreen (Life Technologies), and samples normalized to 10 ng/ μ L. Single nucleotide polymorphisms were analyzed by TaqMan quantitative polymerase chain reaction for *NOD2/CARD15* (rs2066844, rs2066845, and rs2066847), *ATG16L1* (rs2241880), and *IRGM* (rs13361189).⁴²

Cytotoxicity Assay

Macrophage viability was assessed by measuring release of intracellular adenylate kinase into supernatants over 6 hours after drug treatments and/or bacterial infection using the ToxiLight bioassay kit as per manufacturer's instructions (Lonza; Slough, United Kingdom). Luminescence was measured on a Tecan F200 microplate reader.

Statistical Analysis

N indicates number of patients or independent experiments performed with cells seeded in triplicate for each experiment. Sample groups were assessed for normality and equality of variances. Comparison of CD patient data versus HC was by Mann-Whitney U test. Dose-response experiments were analyzed using Cuzick's test for trend, followed, where significant, by Dunnett's test. Other multiple treatment experiments were analyzed using one-way analysis of variance followed by selected pair-wise comparison of treatment means (StatsDirect; Sale, United Kingdom). Differences were considered significant when two-tailed, $P < 0.05$.

RESULTS

MDM from HC and Patients with CD Have Equivalent Ability to Kill Intracellular Bacteria

Neither CD nor HC peripheral blood MDM were very effective at killing *E. coli*. CD MDM allowed net replication of phagocytosed CD-derived *E. coli* HM605 (CD: N = 10 patients, mean fold replication over 3 hours = 1.08 [95% CI, 0.39–1.78]) as did HC MDM (N = 9, 1.50 [95% CI, 1.02–1.97]; $P = 0.15$; Mann-Whitney U test). Similar results were obtained for replication of phagocytosed *E. coli* K12 (CD: N = 10, mean fold replication over 3 hours = 0.54 [95% CI, 0.24–0.84]; HC: N = 10, 0.86 [95% CI, 0.47–1.26]; $P = 0.14$) and also for phagocytosed *S. aureus* (CD: N = 10, mean fold replication = 0.37 [95% CI, 0.18–0.55]; HC: N = 10, 0.48 [95% CI, 0.39–0.57]; $P = 0.09$) (Fig. 1). There was a nonsignificant trend toward increased intramacrophage replication of HM605 among patients with CD with active disease (mean fold replication = 1.74 [95% CI, 0.67–2.81]) compared with those with inactive disease (mean = 0.43 [95% CI, 0.0–0.87], $P = 0.10$), but replication of HM605 in patients with active disease was similar to that seen in HC (Fig. 2). No

correlation was seen between the total number of affected *NOD2/CARD15* and *ATG16L1* alleles and ability to kill intramacrophage HM605, but none of the patients with CD studied were homozygous for affected *NOD2/CARD15* or *IRGM* alleles, and only 1 was homozygous for affected *ATG16L1* (Fig. 2).

MDM from HC and Patients with CD Have Equivalent Ability to Generate Neutrophil Chemoattractants in Response to *E. coli*

HC and CD *E. coli*-infected MDM were more able to induce neutrophil chemotaxis than uninfected MDM but with no significant difference between CD (N = 10 patients) and HC (N = 7): HM605 (CD: mean fold change in neutrophil chemotaxis = 2.55 [95% CI, 2.31–2.80]; HC: 2.65 [95% CI, 2.46–2.85], $P = 0.42$ Mann-Whitney U test); K12 (CD: 2.20 [95% CI, 1.78–2.63]; HC: 2.23 [95% CI, 1.86–2.60], $P = 0.89$) (Fig. 3). No significant difference was seen in the chemotactic response generated by MDM derived from patients with active CD compared with those with inactive disease ($P = 0.60$) (see Table 3, Supplemental Digital Content 1, <http://links.lww.com/IBD/A836>).

Production of cytokines by human MDM in response to HM605 infection was also equivalent between CD (N = 10 patients) and HC (N = 7); TNF- α (CD: mean = 1915.3 pg/mL [95% CI, 1711.6–2119.0]; HC: mean = 1823.2 pg/mL [95% CI, 1590.6–2055.7], $P = 0.27$); IL-6 (CD: mean = 1720.1 pg/mL [95% CI, 1178.0–2262.3]; HC: mean = 1209.9 pg/mL [95% CI, 710.6–1709.1], $P = 0.23$); IL-8 (CD: mean = 6643.9 pg/mL [95% CI, 5065.5–8222.4]; HC: mean = 6639.0 pg/mL [95% CI, 4064.6–9213.4], $P = 0.89$).

HCQ Enhances Intramacrophage Killing of AIEC in Murine and Human Macrophages

HCQ induced a dose-dependent enhancement of intracellular killing of HM605 by J774A.1 macrophages ($P < 0.001$, Cuzick's test for trend). Net replication of *E. coli* (mean fold replication over 3 hr: 3.59 ± 0.35) occurred in the untreated control, but, at concentrations of HCQ at $\geq 2 \mu\text{g/mL}$, net killing of *E. coli* occurred (Fig. 4A). Similar results were obtained with nonhydroxylated chloroquine (see Fig, Supplemental Digital Content 2, <http://links.lww.com/IBD/A837>). HCQ also induced dose-dependent enhancement of intracellular killing of HM605 in human MDM ($P < 0.001$, Cuzick's test) (Fig. 4B). It should be noted that HCQ, up to $10 \mu\text{g/mL}$, showed no significant direct effect on growth of HM605 in broth over 7 hours monitored at OD_{600 nm} (Fig. 4C) and by colony-forming units on LB agar (see Fig, Supplemental Digital Content 3, <http://links.lww.com/IBD/A838>).

HCQ Raises Phagolysosomal pH and Alters Intracellular Iron Metabolism

Addition of HCQ to HM605-infected J774A.1 macrophages caused a dose-dependent increase in phagolysosomal pH (N = 6, $P < 0.005$, Cuzick's test). From a baseline of 5.21 ± 0.31 (mean \pm SEM), pH increased stepwise to 5.56 ± 0.36 at HCQ 5 $\mu\text{g/mL}$ and 7.25 ± 0.47 at 10 $\mu\text{g/mL}$ (Fig. 5).

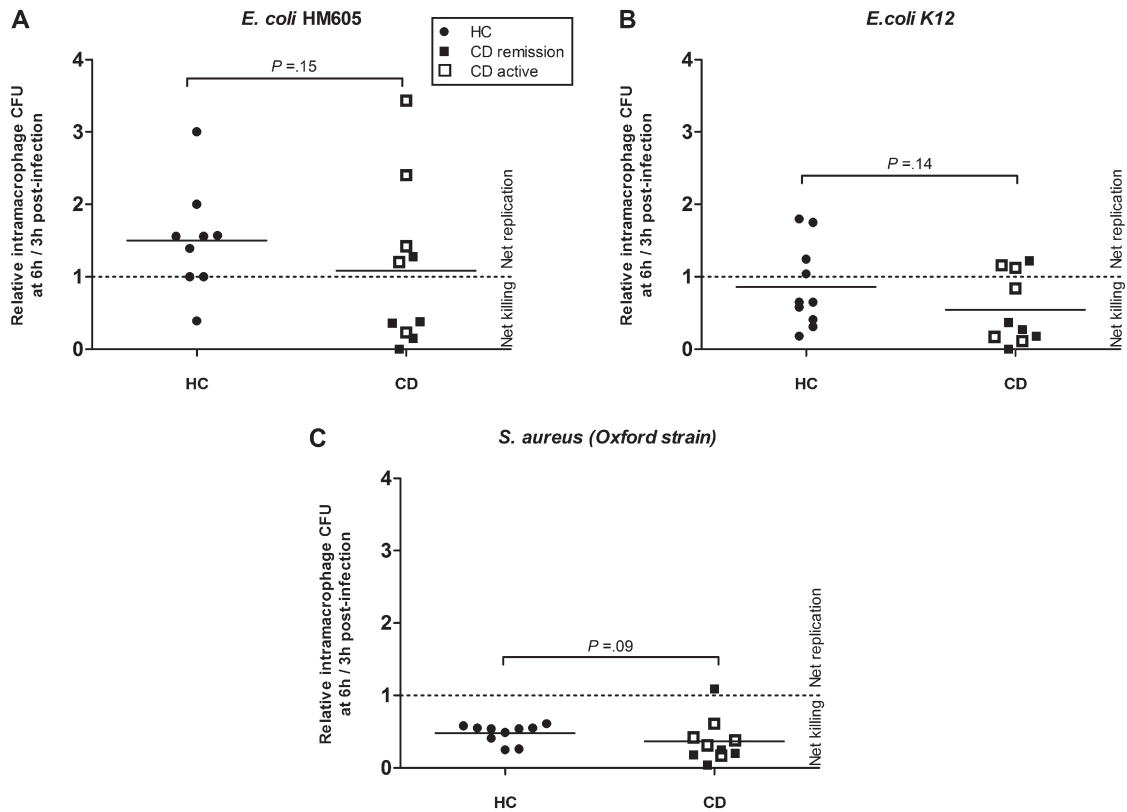


FIGURE 1. MDM isolated from patients with CD and HC possess similar ability to kill phagocytosed bacteria. No significant differences were seen between CD and HC in the killing of any of the bacteria tested; (A) AIEC HM605, (B) *E. coli* K12, and (C) *S. aureus*. Each point represents individual patient mean of triplicate samples, horizontal bar indicates overall mean.

Cotreatment with the iron substrates, ferric citrate (allowing pH-dependent release of ferric iron) and FeNTA (allowing pH-independent release of ferric iron irrespective of pH), only partially reversed the effect of HCQ, with a significant but partial effect seen only at 5 to 10 $\mu\text{g/mL}$ HCQ (Fig. 5).

A modest trend toward increased cathepsin G ($P = 0.03$, Cuzick's test) and macrophage elastase MMP12 ($P = 0.04$) activity was seen in whole cell lysates of HM605-infected macrophages treated with HCQ (see Data, Supplemental Digital Content 4, <http://links.lww.com/IBD/A839>).

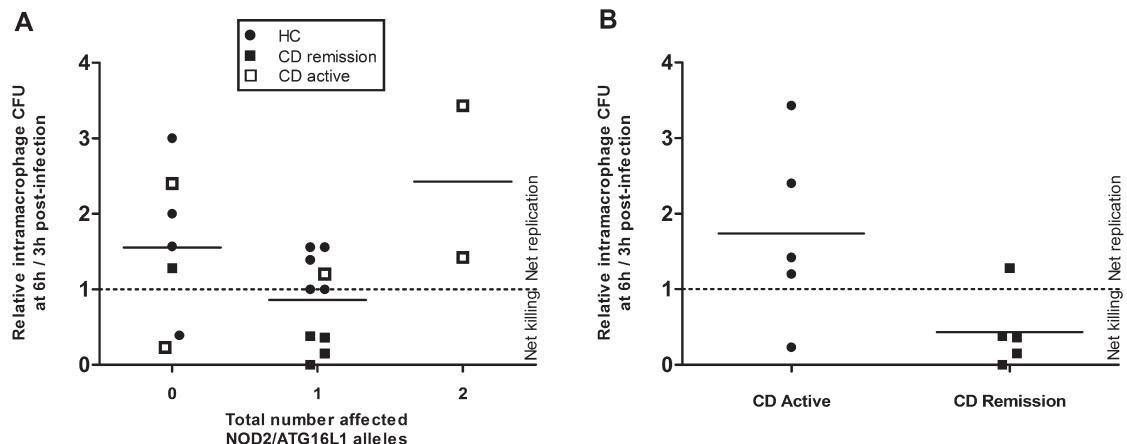


FIGURE 2. Presence of *NOD2/CARD15* and *ATG16L1* variants were not associated with altered ability to kill intramacrophage CD-derived *E. coli* HM605. Patient *NOD2/CARD15* and *ATG16L1* status reported as number of disease associated alleles. A, Killing of intramacrophage HM605 was unaffected by the total number of variant *NOD2* and *ATG16L1* alleles (possible range, 0–4: zero affected, N = 7; one affected, N = 10; two affected, N = 2) (zero versus one affected allele, $P = 0.18$; zero versus two affected alleles, $P = 0.65$; one versus two affected alleles, $P = 0.20$, analysis of variance). B, Effect of disease activity on killing of *E. coli* HM605 ($P = 0.10$, Mann-Whitney U test).

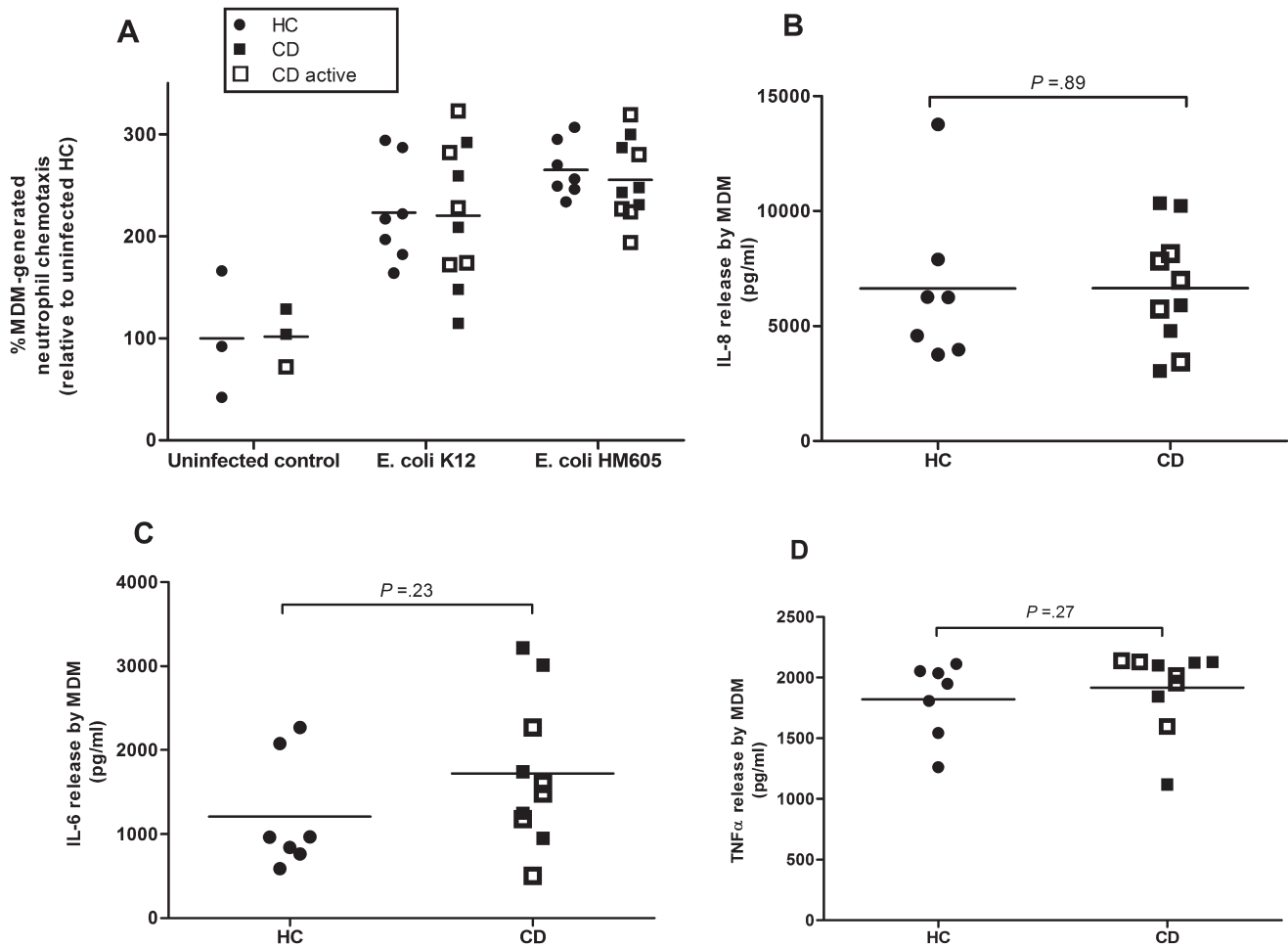


FIGURE 3. *E. coli*-infected MDM from patients with CD and HC have equivalent ability to generate neutrophil chemoattractants. A, MDM-induced neutrophil chemotaxis was equivalent between CD (N = 10) and HC (N = 9) whether uninfected or infected with *E. coli* (either AIEC HM605 or K12). Infected macrophages induced greater neutrophil chemotaxis than uninfected controls ($P < 0.001$; analysis of variance). MDM production of (B) IL-8, (C) IL-6, and (D) TNF- α in response to HM605 infection were also equivalent between CD (N = 10) and HC (N = 7). All assays performed in triplicate. P values by Mann-Whitney U test.

HCQ decreased intracellular respiratory burst as assessed by flow cytometric measurement of dihydrorhodamine fluorescence ($P < 0.05$, Cuzick's test) but did not affect total respiratory burst as assessed by lucigenin-mediated luminescence ($P = 0.73$, Cuzick's test); (see Data, Supplemental Digital Content 5, <http://links.lww.com/IBD/A840>). HCQ neither alter production of TNF- α ($P = 0.31$, Cuzick's test) nor IL-6 ($P = 0.79$) from HM605-infected J774A.1 murine macrophages (Fig, Supplemental Digital Content 6, <http://links.lww.com/IBD/A841>).

HCQ Enhances the Antimicrobial Effect of Doxycycline and Ciprofloxacin Against AIEC Replicating Within Macrophages

Doxycycline at 10% C_{max} was ineffective at killing intramacrophage HM605 ($89.01\% \pm 8.57\%$, mean% \pm SEM% survival relative to control, $P = 0.08$) and only achieved modest

decreases in viable bacteria at C_{max} ($75.5\% \pm 6.72\%$, $P < 0.01$). However, at both 10% C_{max} ($48.9\% \pm 5.36\%$, $P < 0.001$) and C_{max} (34.49 ± 4.71 , $P < 0.001$), cotreatment with HCQ plus doxycycline led to significant enhancement of bacterial killing relative to control and was also significantly more effective than antibiotic monotherapy ($P < 0.001$ at both 10% C_{max} and C_{max} ; N = 6). (Fig. 6).

Ciprofloxacin effectively killed intramacrophage HM605 at 10% C_{max} ($4.95\% \pm 0.92\%$, mean% \pm SEM% survival relative to control, $P < 0.001$) and led to near complete killing at C_{max} ($0.2\% \pm 0.0\%$, $P < 0.001$). Combination of HCQ with ciprofloxacin was more effective than antibiotic monotherapy at 10% C_{max} ($2.8\% \pm 0.82\%$, $P < 0.05$), but at C_{max} , no additional effect was seen ($0.3\% \pm 0.1\%$, $P = 0.86$) (N = 3). Similar results were obtained for killing of intramacrophage ileal CD AIEC LF82 (see Fig, Supplemental Digital Content 7, <http://links.lww.com/IBD/A842>).

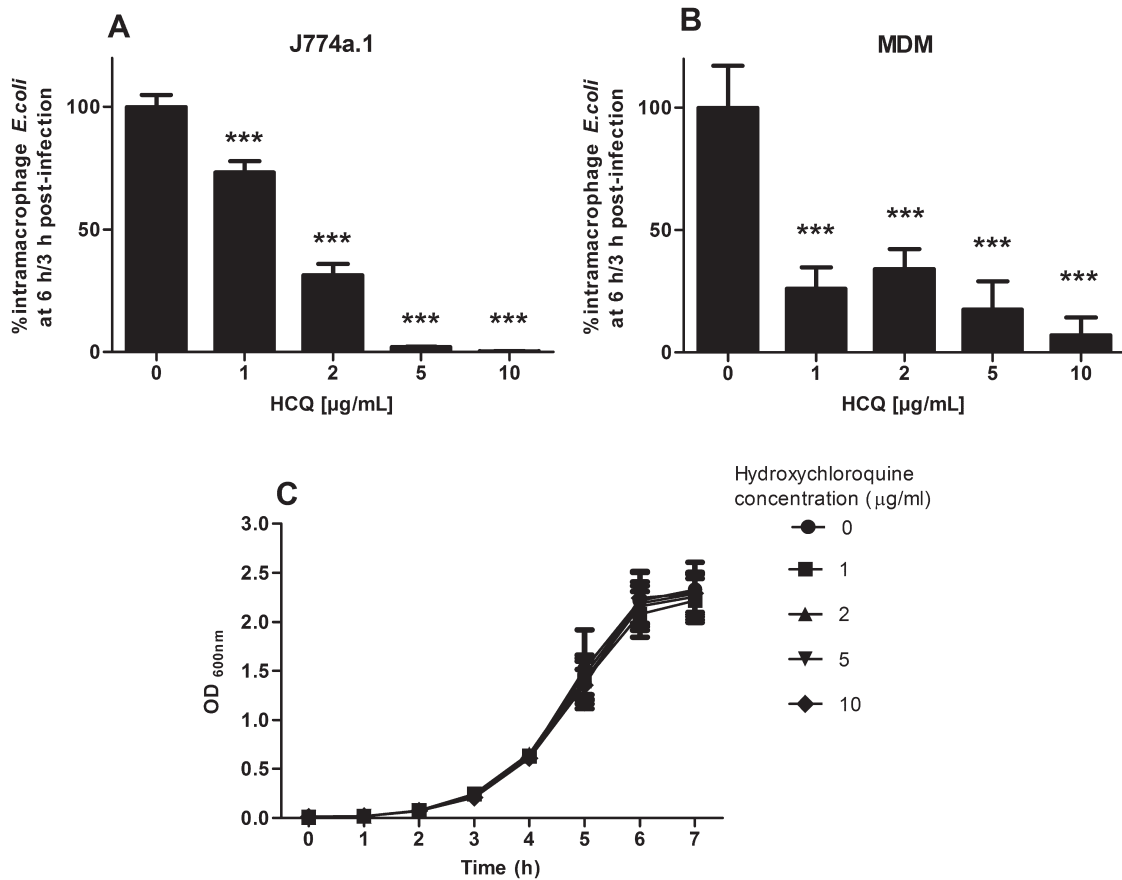


FIGURE 4. HCQ enhances intramacrophage killing of AIEC. HCQ treatment causes a dose-dependent decrease in survival of intramacrophage AIEC HM605 in (A) J774A.1 murine macrophages (N = 3, Cuzick's test for trend $P < 0.001$) and (B) healthy human MDM (N = 5, Cuzick's test; $P < 0.001$). *** $P < 0.001$, Dunnett's test versus control. C, HCQ did not have a direct bactericidal action as observed by growth in broth, measured at OD_{600 nm} (N = 3).

Vitamin D Enhances Killing of Intramacrophage AIEC

Intramacrophage HM605 survival was decreased by treatment with 1,25 OH₂-vitamin D₃ in a dose-dependent manner; in human MDM ($P = 0.012$, Cuzick's test) and in J774A.1 macrophages ($P < 0.001$); (Fig. 7). Cotreatment with 1,25 OH₂-vitamin D₃, modestly increased intracellular respiratory burst in J774A.1 macrophages relative to vehicle-treated control (N = 5, $P = 0.002$, Cuzick's test). No effect was seen on total (intracellular plus extracellular) respiratory burst (see Data, Supplemental Digital Content 5, <http://links.lww.com/IBD/A840>). No macrophage cytotoxicity was seen as assessed by released adenylate kinase in response to any of the drugs tested or vitamin D.

DISCUSSION

Here, we show that MDM from healthy individuals are ineffective at killing CD mucosally derived AIEC, but that CD MDM are similarly ineffective. Moreover, the CD MDM show no defect in generation of neutrophil chemoattractants in response to *E. coli*. Since *E. coli* replicating within macrophage phagolysosomes represent a plausible therapeutic target in CD, and are likely to be relatively resistant to antibiotic therapy, we investigated the

effects of HCQ and 1,25 OH₂-vitamin D₃ on killing of CD *E. coli* by macrophages. Both HCQ and 1,25 OH₂-vitamin D₃ are shown to greatly enhance the ability of macrophages to kill phagocytosed *E. coli*. The effect of HCQ is synergistic with antibiotics, suggesting a plausible new approach to therapy.

Defective neutrophil chemotaxis has been consistently reported in patients with CD. Neutrophils are usually plentiful in CD tissue sections, but it has been suggested that a delayed neutrophil response to an initial bacterial attack may result in bacteria being taken up by macrophages before the neutrophils arrive.¹⁸ These macrophages, being much less effective than neutrophils at killing phagocytosed bacteria, may then become chronically infected, resulting in granuloma formation. It has been suggested that the decreased in vivo neutrophil chemotaxis seen in CD may be due to a defective macrophage chemokine response to bacterial triggers.¹⁹ Studies have shown defective secretion of IL-8 by CD MDM in response to the NOD2 ligand muramyl dipeptide¹⁸ and into CD blister fluid.⁴³ In the latter study, blister fluid concentrations of IL-8, but not those of TNF- α or other cytokines, correlated with decreased chemotaxis. However, subsequent studies of CD MDM have shown decreased secretion of TNF- α and other cytokines but not IL-8.^{19,44} Here, CD peripheral

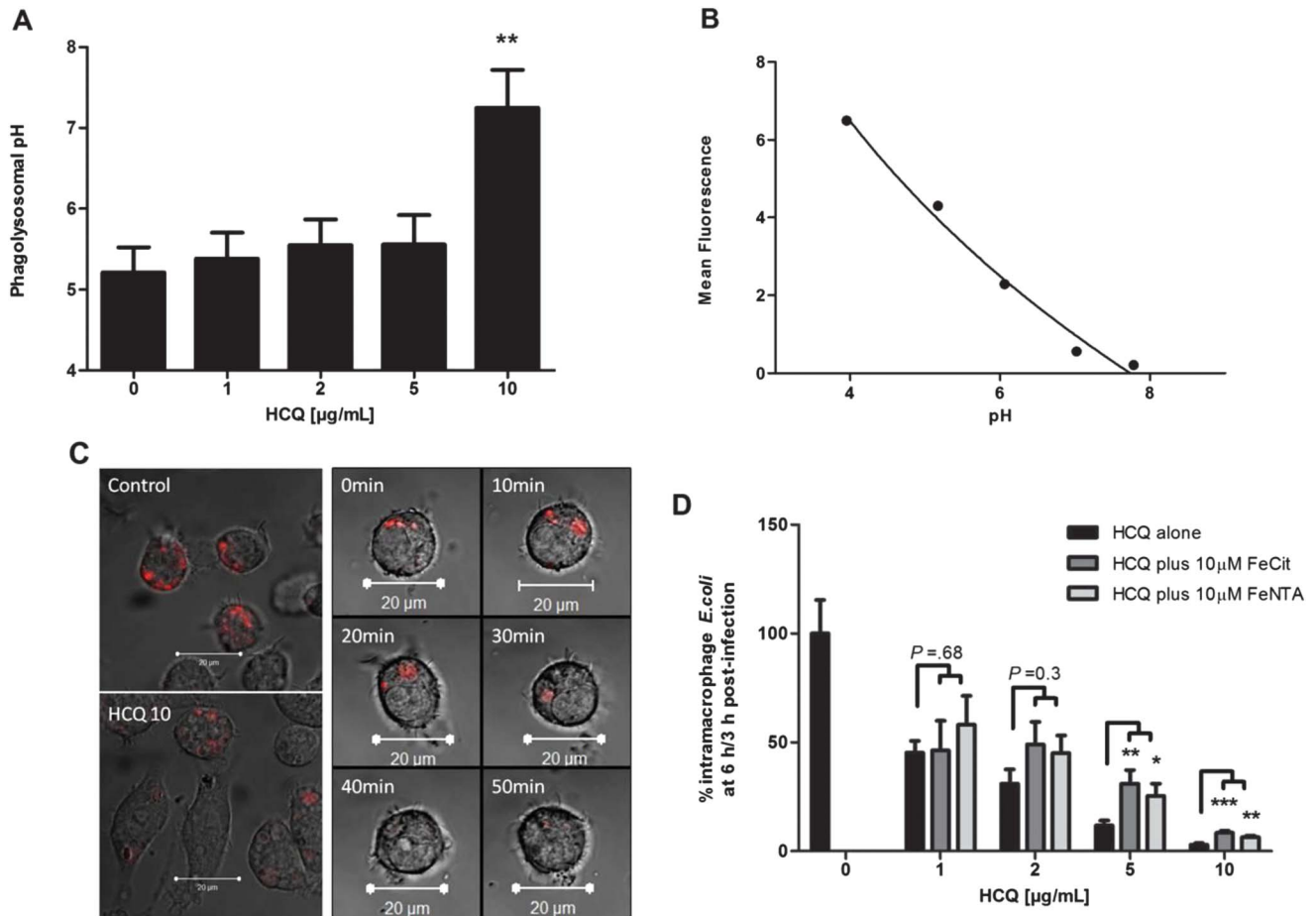


FIGURE 5. HCQ leads to alkalinization of macrophage phagolysosomes, and its effects on killing of phagocytosed *E. coli* are only partly reversed by iron supplementation. A, HCQ increased phagolysosomal pH in AIEC HM605-infected J774A.1 macrophages as assessed by confocal microscopy ($n = 6$, Cuzick's test for trend $P < 0.005$). $**P < 0.01$ Dunnett's test versus control. B, Representative standard curve of mean confocal-acquired fluorescence of pHrodo *E. coli* bioparticle-loaded macrophages with phagolysosomes equalized in calibration buffers at pH 4 to pH 7 ($n = 3$). C, Confocal images illustrating increased phagolysosomal pH with 10 $\mu\text{g/mL}$ HCQ (HCQ10) as indicated by decreased pHrodo fluorescence compared with untreated control (left panel). Onset of the change in pH occurs within 30 minutes (right panel). D, Iron supplementation only partially antagonizes the effect of HCQ on intracellular survival of AIEC HM605 in J774A.1 macrophages ($N = 3$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ analysis of variance).

blood MDM behaved similarly to those from HC in their ability to generate neutrophil chemoattractants in response to *E. coli*; their *E. coli*-induced secretion of IL-8, TNF- α , and other cytokines was also similar. A defective macrophage cytokine response has previously been reported in individuals who are homozygous or compound heterozygous for altered *NOD2/CARD15* alleles,^{18,44,45} but this genotype is only present in around 8% of CD and was not present in any patients in our study. The mechanism underlying the defective neutrophil chemotaxis seen in vivo in the majority of patients with CD is therefore still unclear, but the presence of circulating chemotaxis inhibitors remains a possibility.⁴⁶⁻⁴⁸

Peripheral blood MDM from patients with CD have previously been shown to be normal in their ability to kill phagocytosed *S. aureus*.²³ Given the different mechanisms involved in killing of Gram-negative organisms by macrophages, it was important also to assess the ability of CD macrophages in

killing AIEC. It is shown here that even healthy macrophages are ineffective at killing phagocytosed *E. coli* including AIEC but that CD macrophages are no worse. It has recently been shown that MDM from patients with CD, homozygous for the *ATG16L1* risk allele show impaired killing of phagocytosed AIEC when studied in the presence of phorbol ester to mimic inflammatory conditions.⁴⁹ Only 1 of the patients in this study was homozygous for the *ATG16L1* risk allele.

The mechanisms by which microbes evade intramacrophage killing include resistance to pH changes, prevention of phagosomal maturation, escape into the cytosol, and resistance to reactive oxygen species.^{50,51} This is typified by *C. burnetii*, the agent of Q-fever, which is adapted to survive at the acidic pH within macrophage phagolysosomes.^{52,53} Although CD AIEC isolates, such as LF82, are rapidly attacked through the autophagocytic pathway,⁸ some can escape autophagy and then survive and,

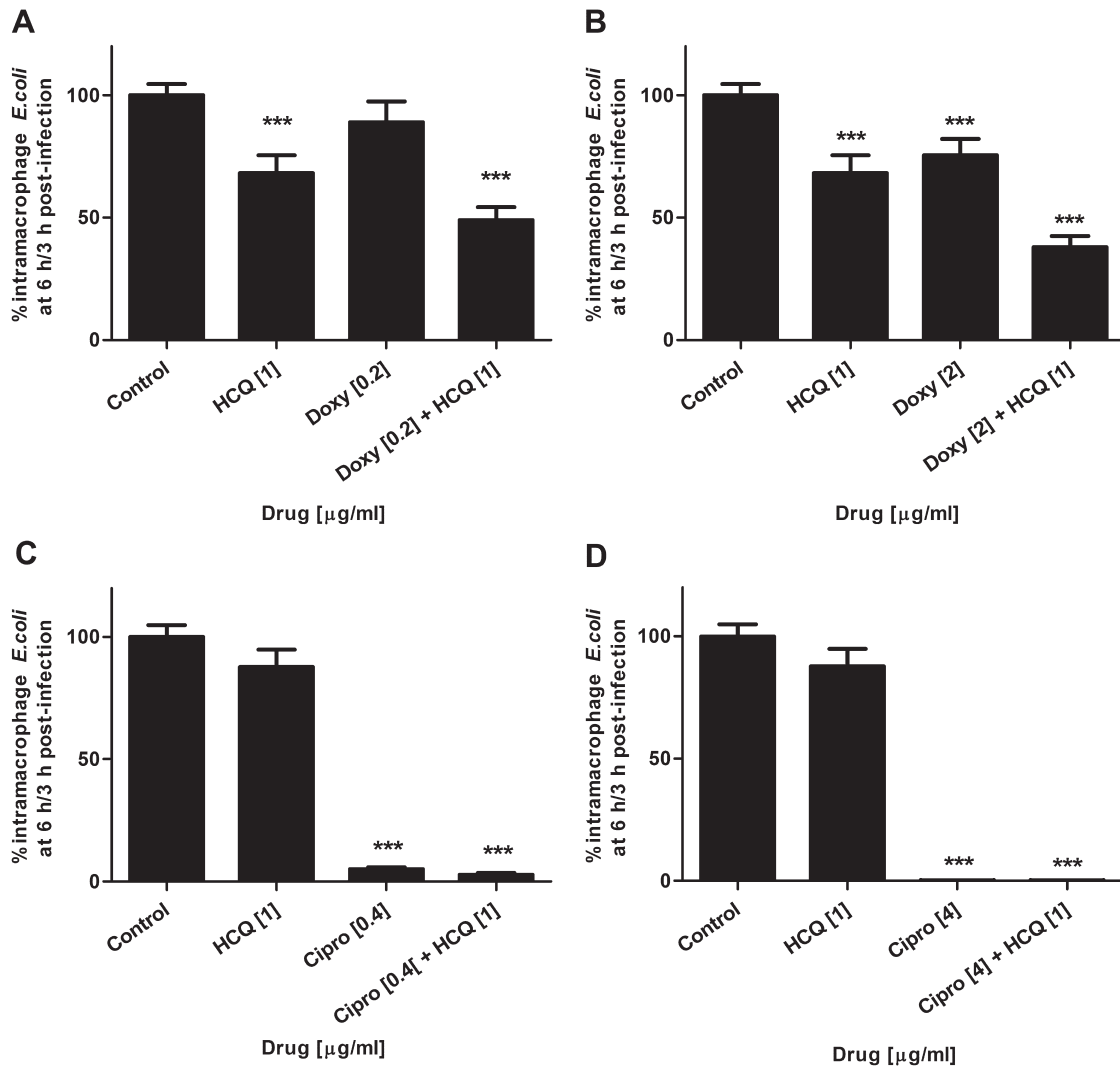


FIGURE 6. HCQ enhances antibiotic efficacy against intramacrophage AIEC. Combination therapy (antibiotic plus HCQ, 1 $\mu\text{g/mL}$) was more effective at killing AIEC HM605 within J774.A1 macrophages than antibiotic monotherapy at (A) 10% C_{max} doxycycline and (B) C_{max} doxycycline [2 $\mu\text{g/mL}$]; $N = 6$. Likewise, for (C) 10% C_{max} ciprofloxacin. D, No additional benefit was seen with ciprofloxacin at C_{max} [4 $\mu\text{g/mL}$]; $N = 3$. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.001$; analysis of variance.

like *C. burnetii*, replicate within mature phagolysosomes, which provide them with the acidic pH necessary for their replication.⁹ The survival of *C. burnetii* within macrophages is substantially decreased in vitro by HCQ treatment and this translated into clinical response in a randomized trial.⁵⁴ Similar effects have been seen for HCQ against *T. whipplei*, the causative organism of Whipple's disease and a combination of doxycycline and HCQ is now recommended as first-line therapy.⁵⁵ This study shows a marked effect of HCQ on survival of phagocytosed AIEC within macrophages, similar to that previously shown for chloroquine⁹ and probably mediated largely by increased intravacuolar pH.

We have shown here that HCQ, at concentrations that should be achievable in vivo, has a marked synergistic effect with antibiotics, such as ciprofloxacin and doxycycline that are effective against intracellular *E. coli*. HCQ has not proved effective when

used, mainly on the basis of its anti-inflammatory effects, as a single agent in CD,⁵⁶ although it has been successfully used in the treatment of chronic granulomatous disease, an inherited condition associated with defective neutrophil function, impaired NADPH oxidase free-radical production and granulomatous colitis that mimics CD.⁵⁷ Eradication of *E. coli* replicating within tissue macrophages, in analogy with the causative organisms of Whipple's disease and Q-fever, will almost certainly require that HCQ is combined with appropriate antibiotics. This approach has a proven safety record even with long-term treatment.⁵⁸

In view of the associations between CD and polymorphisms in genes such as *NOD2/CARD15*, *ATG16L1*, and *IGRM*, all of which impact on autophagy, it has been suggested that stimulation of autophagy would be a plausible therapeutic strategy.⁸ It might therefore seem counterintuitive to consider a drug such as HCQ

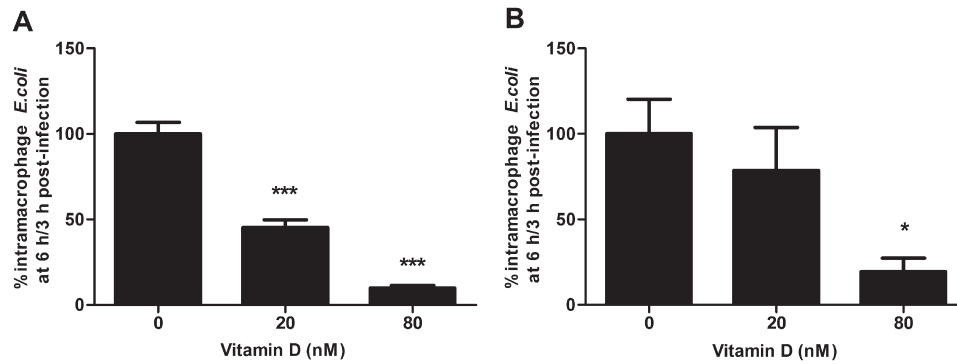


FIGURE 7. Vitamin D enhances intramacrophage killing of AIEC. 1,25 OH₂-vitamin D₃ caused dose-dependent decrease in intramacrophage survival of AIEC HM605 in (A) J774A.1 murine macrophages (N = 3, Cuzick's test for trend; $P < 0.001$) and (B) human MDM (N = 3, Cuzick's test; $P = 0.012$). * $P < 0.05$, *** $P < 0.001$ Dunnett's test versus control.

that, like other quinolone antimalarials, inhibits the late stages of autophagy, particularly fusion of autophagosomes with lysosomes.⁵⁹ This effect is dose-dependent, however, and it has been reported that chloroquine may even enhance fusion at lower concentrations.⁶⁰ In support of this, it has been shown to inhibit intramacrophage replication of *Mycobacterium tuberculosis* and to enhance the antituberculosis protectiveness of both isoniazid and 25 OH-vitamin D₃.⁶¹ Moreover CD-derived AIEC can replicate within phagolysosomes, having escaped autophagy,⁹ whereas rapamycin, which stimulates autophagy, becomes ineffective once intramacrophage replication of AIEC has become established.⁸

Vitamin D has been shown to be important to autophagy and other aspects of the innate immune response.^{32,39,62} We have shown here that vitamin D, also at concentrations readily achievable with oral supplementation, substantially enhances the ability of human MDM to kill phagocytosed *E. coli*. There is clear justification for further studies of vitamin D supplementation in CD.

It can currently still be argued that the increase in mucosally associated *E. coli* commonly found in CD may be a secondary phenomenon. Proof of the direct involvement of *E. coli* in CD pathogenesis now depends on showing that a therapy targeted against *E. coli* replicating within macrophages can induce remission in CD. A controlled trial of combination ciprofloxacin/doxycycline/HQC therapy in active CD has therefore been commenced (ClinicalTrials.gov NCT01783106).

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