Controlling the Cytokine Storm in Severe Bacterial Diarrhea with an Oral TLR4 Antagonist

Dilara Islam[1], Eric Lombardini[2], Nattaya Ruamsap[1],
Rawiwan Imerbsin[2], Patchariya Khantapura[1], Ian Teo[3,4,5],
Pimmnapar Neesanant[1], Siriphan Gonwong[1], Kosol Yongvanitchit[1],
Brett E Swierczewski[1], Carl J Mason[1], Sunil Shaunak[3,4,5,6,7]

Short title: Cytokine Storm & Oral TLR4 Antagonist

Departments of Enteric Diseases[1] & Veterinary Medicine[2],
Armed Forces Research Institute of Medical Sciences (AFRIMS)
315/6 Rajvithi Road, Bangkok 10400, Thailand.

Departments of Medicine[3], Infectious Diseases[4], Immunity[5], Pathology[6] & Chemistry[7],
Imperial College London at Hammersmith campus, Ducane Road, London, W12 ONN, UK.

Corresponding author:

Professor Sunil Shaunak MD, PhD

Faculty of Medicine, Imperial College London, Hammersmith Hospital,

Du Cane Road, London, W12 0NN, UK

Telephone: +44 20 8383 2301

Email: s.shaunak@imperial.ac.uk

Key words

Bacterial diarrhea, Inflammation, TLR-4, Cytokines

Abbreviations

AFRIMS – Armed Forces Research Institute of Medical Sciences

CFUs – colony forming units

cGMP – Good Manufacturing Process

DG – dendrimer glucosamine

FDA – Food & Drugs Administration

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GLP – Good Laboratory Practice

LPS – lipopolysaccharide

NHP – non-human primate

PETIM – polypropyletherimine

RT-PCR – reverse transcriptase polymerase chain reaction

TGF-β – Transforming Growth Factor-β

Summary

Shigella dysenteriae causes the most severe of all infectious diarrheas and colitis. We infected rhesus macaques orally and also treated them orally with a small and non-absorbable polypropyletherimine dendrimer glucosamine (DG) that is a Toll Like Receptor-4 antagonist. Antibiotics were not given for this life threatening infection. Six days later, the clinical score for diarrhea, mucus and blood was 54% lower, colon IL-8 and IL-6 were both 77% lower, and colon neutrophil infiltration was 75% less. Strikingly, vasculitis did not occur and tissue fibrin thrombi were reduced by 67%. There was no clinical toxicity or adverse effect of DG on systemic immunity. This is the first report in nonhuman primates of the therapeutic efficacy of a small and orally bioavailable TLR antagonist in severe infection. Our results show that an oral TLR4 antagonist can enable controlled resolution of the infection-related-inflammatory response and can also prevent neutrophil-mediated gut wall necrosis in severe infectious diarrheas.

Introduction

Global antimicrobial resistance means that alternatives to antibiotics are urgently needed. Invasive gut Gram negative bacteria (e.g. Enteropathogenic/Enterohemorrhagic *Escherichia coli*, non-typhoidal *Salmonella*, *Shigella*)[1-3] and necrotizing enterocolitis in infants[4-5] cause life threatening disease by stimulating intense TLR4-MD-2-LPS cytokine-induced and neutrophil-mediated gut wall damage[6]. This provides pathogens with a substantial growth advantage and enhances their tissue invasion by destabilising tight junctions, destroying gut mucosa, and allowing transepithelial neutrophil migration into the gut lumen[7].

Clinical trials targeting infection-related-inflammation fail because of inadequate mouse disease models for testing drugs[8]. This is because mice are 6 log orders of magnitude more resistant to lipopolysaccharide-induced septic shock than humans[8]. In severe Shigellosis however, human disease pathogenesis has been well defined[9,10] and we and others have recently described clinically validated NHP models for vaccine and drug testing[11-13]. Cytokine-mediated-inflammation leads to excessive neutrophil infiltration and vasculitis of the colon[9-11,14,15]. Clinically, this presents as bloody diarrhea. Lack of effective vaccines for these severe infective diarrheas and the accelerating global antimicrobial resistance crises make this a military and WHO public health priority area.

This led us to propose that blocking the MYD88 pathway of the TLR4-MD-2-LPS-mediated cytokine storm without interfering with the TRIF/TRAM pathway should prevent gut wall damage[16-21]. We have previously modelled and synthesised and tested a 13.6 kDa polyamidoamine (PAMAM) dendrimer glucosamine and a 75% smaller 3.3 kDa polypropyletherimine (PETIM) dendrimer glucosamine in a rabbit closed intestinal loop model of *Shigella flexneri* infection. No antibiotics were given. For these *in vivo* studies, the abdominal wall was opened and 5 cm intestinal loops of gut created into which *Shigella flexneri* bacteria and DG were injected. The abdomen was then closed and the rabbits fasted and monitored for 18 hours before autopsy. We showed a large reduction in colon IL-6 and IL-8 and in *Shigella* invasion of tissues, with complete gut wall protection[22-24].

Small dendrimer drugs are showing increasing promise as new polyvalent medicines in several animal models of infection and inflammation[21]. PETIM-DG (DG) is a cGMP synthesised and analytically characterised generation 3 partially glycosylated dendrimer[21-23]. It blocks the production of pro-inflammatory cytokines by interfering with the electrostatic

binding of: (i) the 4′phosphate on the di-glucosamine of LPS to Ser118 on MD-2; (ii) LPS to Lys91 on MD-2; (iii) the subsequent binding of TLR4 to Tyr102 on MD-2[22]. Importantly, the human MD-2 residues 118, 91 & 102 are conserved in rhesus macaque and this means that NHPs are as sensitive as humans to LPS[22,26]. DG is not toxic when given orally, intraperitoneally or intravenously to mice and rabbits in the dose range 0.1-175 mg/kg[23].

We now describe the results of a cGMP and GLP prospectively randomised study that fulfils FDA requirements for testing DG in rhesus macaques infected with 2 x 10^9 CFUs of toxin producing *Shigella dysenteriae* Type 1. This bacterial pathogen causes the most severe of all infection and toxin-related diarrhea and colitis. Antibiotics were not used. DG has no antibacterial activity against either Gram negative or Gram positive bacteria.

Materials and Methods

Pharmaceutical grade synthesis of cGMP grade DG and its detailed analytical chemistry were performed at GlycoSyn New Zealand[23]. The GLP NHP study and tissue analysis was performed at AFRIMS, Bangkok, Thailand[12]. NHP sample size was based upon our previous studies[12,23].

Nonhuman primates

Adult rhesus macaques (*Macaca mulatta*; male & female, 5-15 years, weight 6.5-12 kg) of Indian origin were used. Animals were born and housed at AFRIMS and maintained according to the Guide for the Care and Use of Laboratory Animals, NRC, 2011. They were

fed monkey feed (083G, Perfect Companion Co Thailand), 15-20 biscuits daily and 1-2 treat tablets weekly. Fresh fruits and vegetables and hyper-chlorinated water were also provided.

Study ethics

The NHP studies were performed under Institutional Animal Care and Use Committee (IACUC) protocol #PN 13-05 "Defining the therapeutic efficacy of polypropyletherimine dendrimer glucosamine, a non-antibiotic based drug, in rhesus monkeys after *Shigella dysenteriae* infection" which was reviewed and approved by the AFRIMS IACUC & Biosafety Review Committee. The AFRIMS-Primate Research Committee is fully accredited by the Association for Assessment & Accreditation of Laboratory Animal Care International. Studies were conducted in compliance with the Animal Welfare Act and all other US federal statutes and regulations.

NHP pre-study screening

All NHPs had negative serologic test results for SIV, SRV, STLV-1 and were also negative for tuberculosis. On day -35 before *Shigella dysenteriae* Type 1 infection, 40 NHPs were moved to a separate wing and treated with azithromycin for 5 days as a prophylactic measure. Rectal swabs and stool swabs were taken to confirm the absence of enteric pathogens. On day -14, the AFRIMS biostatistician randomized a subgroup of 18 NHPs using the nQuery Advisor v. 6.01 procedure (i.e. random subset of cases and basic randomization list), and they were moved to an Animal Biological Safety Level 3 suite in individual cages. Prior to day 1 (defined as the day of infection), any NHP that did not meet all of the inclusion criteria was replaced. On day -40 and again on day -14, NHPs were screened by physical exam, complete

blood count and chemistry, and stool culture with a rectal swab, and gingival swab for enteric pathogens including *Shigella, Salmonella* & *Campylobacter* spp., and plasma anti-*S. dysenteriae* Type 1 lipopolysaccharide specific antibody (IgA, IgG & IgM) titers. Although it is common for NHPs to have subclinical *Campylobacter* spp. in their gut, only those NHPs that were free of diarrhea and *Campylobacter* spp. on day -14 were allowed to progress; 14 NHPs finally entered the infection/treatment part of the study.

Study design

The characteristics of the master cell bank of the *S. dysenteriae* Type 1 1617 strain[25] used were confirmed by (1) slide agglutination test to show it agglutinated with *S. dysenteriae* Type 1 specific sera; (2) plaque assay to show that it invaded intracellularly by forming hollow plaques in LLC-MK2 cells; (3) stability assay to show that it was stable by having >80% of single well isolated virulent colonies present after 3 consecutive sub-cultures on congo red agar plates. Our previous studies have determined that the optimal challenge dose for an 80% attack rate of Shigellosis is 2×10^9 CFUs[12]. On day 1, NHPs were challenged with *S. dysenteriae* at 4 hours after the first dose of DG. The bacterial suspension was given directly into the stomach using a pediatric nasogastric tube in a dose of 2×10^9 CFU in 20 ml sterile PBS and after 20 ml bicarbonate buffer to neutralise stomach acid. DG was given (dissolved in sterile water at 6 mg/kg) once daily, and into the stomach using a nasogastric tube after 20 ml bicarbonate buffer on days 1, 2, 3, 4 & 5. Control NHPs received 20 ml sterile water into the stomach via a nasogastric tube and after 20 ml bicarbonate buffer. After each dose of DG, NHPs were monitored for 1 hour for any adverse events. Stool was collected twice daily for culture & PCR. Isolation of the *S. dysenteriae* Type 1 1617 challenge strain from stool was confirmed

by slide agglutination and real-time PCR for *ipaH*. Stool *S. dysenteriae* CFU was not quantified**.**

The primers and probes used were:-

(1) *ipaH*-U1 (f)CCTTTTCCGCGTTCCTTGA

& *ipaH*-L1 (r)CGGAATCCGGAGGTATTGC.

ipaH-P1 probe VIC-CGCCTTTCCGATACCGTCTCTGCA-TAMRA.

(2) OOK1 (f)AGGAATCCATTTGTGTACCAAATGA

& OOK2 (r)AACATGAGCACATTGGAATTTTAGC.

PGAL-P probe VIC-TGAAAGTGGGAAATGTGTACTAGATCCAAAACAAGA-TAMRA (internal control).

The vet (RI) was allowed to rehydrate NHPs by subcutaneous infusion with water, sodium & potassium if they became clinically dehydrated. No antibiotics were given after infection with *S. dysenteriae*. Neither *Shigella* infected nor DG treated NHPs lost >5% body weight. On day 6, NHPs had a whole body autopsy with all tissue samples collected within 30 min of death.

NHP monitoring

They were scored daily for presence {1} or absence {0} of each of diarrhea, mucus and blood in stool to define the % clinical score. Hemoglobin, white blood cell count, cell type differential, platelets, urea, creatinine, albumin, bilirubin, alanine aminotransferase and γ-glutamyltransferase were monitored daily.

Oral dosing of DG

Studies have shown a gut transit time of ~16 h so a once daily oral dosing regimen was used. Three of the NHPs treated had a low body weight of 7.13 ± 0.39 kg/NHP; they received a total study DG dose of 214 ± 12 mg/NHP. They are referred to as the DG (214 mg) group. Three of the NHPs treated had a high body weight of 11.13 ± 0.72 kg/NHP; they received a total study DG dose of 334 ± 21 mg/NHP. They are referred to as the DG (334 mg) group.

Quantitative mRNA RT-PCR

Dissected tissue was collected in RNALater and disrupted with a bead beater. RNA was extracted using an RNeasy mini kit (Qiagen). Quantitative one step real-time RT-PCR[27-29] was performed for IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-γ, FoxP3 & TGF-β using the primer pairs and probes shown below. GAPDH was used as the housekeeping gene[23]. Positive control recombinant plasmids were provided by Francois Villinger (Emory University). Probes were 5'-labelled with FAM (6-carboxyfluorescein) and 3'-labelled with Black Hole Quencher except for GADPH which was 5'-labelled with Cal Flour Orange 560 and 3'-labelled with Black Hole Quencher. Each tissue sample was analysed twice.

Primer pairs & probes (5' to 3') used for qRT-PCR:

IL-1β: (f)GAGCAACAAGTGGTGTTCTCCA & (r)TCTTTCAACACGCAGGACAGG. Probe CAAAATACCTGTGGCCTTGGGCCTCAA.

IL-6: (f)TGGCTGAAAAAGATGGATGCT & (r)TTGCTCCTCACTACTCTCAAACCT. Probe TGATTTTCACCAGGCAAGTGTCCTCATTG[28].

IL-8 [CXCL8}: (f)TGGCTCTCTTGGCAGCCT & (r)TTGGGGTGGAAAGGTTTGGA.

Probe TCTGTGAAGGTGCAGTTTTGCCAAGG[30].

IL-10: (f)GAGAACCACGACCCAGACAT & (r)ATTCTTCACCTGCTCCACGGC.

Probe CCTGAGGGTCTTCAGATTCTCCCCCAG[31].

TNF-α: (f)GGCTCAGGCAGTCAGATCATC & (r)GCTTGAGGGTTTGCTACAACATG.

Probe TCGAACCCCAAGTGACAAGCCTGTAGC[28].

IFN-γ: (f)GAAAAGCTGACCAATTATTCGGTAA &

(r)AGCCATCACTTGGATGAGTTCA.

Probe TGACTCGAATGTCCAACGCAAAGCAGTA[28].

FoxP3: (f)CACCTGGCTGGGAAAATGG & (r)GCAGGAGCCCTTGTCAGATG.

Probe CACTGACCAAGGCTTCATCTGTGGCAT[32].

TGF-β: (f)GTATATTGACTTCCGCAAGGACCT & (r)TGTCCAGGCTCCAAATGTAGG.

Probe AGGGCTACCATGCCAACTTCTGCCTG.

GAPDH: (f)GCACCACCAACTGCTTAGCAC & (r)TCTTCTGGGTGGCAGTGATG.

Probe TCGTGGAAGGACTCATGACCACAGTCC[28].

Histology

Tissue samples were collected in 10% neutral buffered formalin, processed routinely, and stained with H&E. All slides were coded and interpreted and scored blind by EL [a Board certified Diplomate of the American College of Veterinary Pathologists] for:- (A) Tissue inflammation[33] {within normal limits = 0; minimal = 1; mild = 2; moderate = 3; marked = 4; severe = 5}. (B) Cell type infiltrate [i.e. neutrophil, lymphocyte, plasma cells, monocyte, eosinophil][34] absent {= 0} or present {= 1}. (C) Vasculitis absent {= 0} or present {= 1}. (D) Fibrin thrombi[35] absent {= 0} or present {= 1}.

Flow cytometry

Blood was collected into BD Vacutainer CPT cell preparation tubes with sodium citrate and into BD Vacutainer Na-heparin tubes. Plasma and buffy coat layers were harvested and frozen. Surface staining was performed for CD3 (APC mouse anti-human CD3 [clone SP34-2] #557597); CD4 (FITC mouse anti-human CD4 [clone M-T477] #556615); CD8 (PerCP mouse anti-human CD8 [clone SK1] #347314); CD14 (FITC mouse anti-human CD14 [clone M5E2] #557153); Ki67 (FITC mouse anti-human Ki-67 [clone B56] #561283) (BD Pharmingen).

Intracellular staining was performed for TNF-α (PE mouse anti-human TNF-α [clone MAb11] #557068); IL-1β (PE mouse anti-human IL-1β [clone AS10] #340516); IL-6 (PE rat anti-human IL-6 [clone MQ2-6A3] #551473); IL-8 (PE mouse anti-human IL-8 [clone G256-8] #554720, BD Pharmingen) before and after stimulation with PMA (50 ng/ml), ionomycin (5 µg/ml) and GolgiPlug containing Brefeldin-A (10 µg/ml).

Chopped tissue from each of ascending colon, transverse colon and descending colon, and from rectum and mesenteric lymph nodes was treated with EDTA-DTT, digested with collagenase, and the cell suspension collected enriched for lymphocytes. Surface staining was performed for CD3, CD4, CD8, Ki67 and TCR-γ/δ (FITC mouse anti-human TCR-γ/δ [clone B1] #559878, BD Pharmingen). A flow cytometer-FACSCalibur (Becton-Dickinson) equipped with automated FACS Loader & CellQuest Software was used for data acquisition and analysis as previously described[36].

Data and statistical analyses

Data was analysed using Graphad Prism 6 software and a 2-tailed non-parametric Mann-Whitney test. Results are shown as mean ± SEM with * *P<0.05*, ** *P<0.01*, ***P<0.001*.

Of the 6 rhesus macaques infected and treated with placebo, one was excluded because it was not infected:- (1) never required rehydration; (2) after 5 days was still eating biscuits {i.e. no anorexia}; (3) stool normal in form and consistency; (4) neither blood nor mucus present in stool; and at autopsy, (4) entire gastrointestinal tract normal; (5) background inflammation only on histology of jejunum, ileum, colon & rectum; (6) IL-8, IL-6 and IL-1β in ascending, transverse and descending colon, and rectum, were the same as in normal NHPs.

Death from fulminant dysentery (i.e. bloody diarrhea) is well recognised in the rhesus macaque model of *Shigella* infection[12,14,37]. One of the NHPs infected and treated with DG was excluded because:- (1) developed severe septic shock soon after infection; (2) required early and considerable subcutaneous fluid rehydration; (3) autopsy was one day early because of clinical deterioration and distress; and at autopsy, (4) a Waterhouse-Friderichsen syndrome was suggested by the histology in association with an enlarged, cavitating & necrotising adrenal gland[38,39]; (v) severe and segmental necro-hemorrhagic jejunitis, ileitis and typhlitis; (vi) diffuse, severe, neutrophilic colitis with focal necrotizing vasculitis; (vii) massive colon cytokine storm with IL-8 of 1,200% higher and IL-6 of 1,120% higher and IL-1β of 300% higher than in *Shigella* infection control.

Results

Clinical outcome & score

In two NHPs, all of the procedures were performed and they were treated with water for injection only; they are the normal group. Six NHPs were infected with *S. dysenteriae* and they were treated with water for injection only; they are the *Shigella* group. Six NHPs were infected with *S. dysenteriae* and they were treated with oral DG once daily for 5 days; they are the DG group. Autopsy was on day 6. The total study dose of DG was either 214 ± 12 mg/NHP (n = 3) or 334 ± 21 mg/NHP (n = 3) (*P=0.02*).

Time to positive *Shigella* stool culture/PCR was 1.6 ± 0.2 days for all three groups. Infection led to a white cell count of 11.6 ± 1.2 compared to 7.9 ± 1.0 x 10^3/µl (*P=0.04*) in the normal group. Blood albumin fell from 4.36 ± 0.07 (normal group) to 2.76 ± 0.3 g/dl (*Shigella* group) (*P=0.002*) because primates develop a protein losing enteropathy[40]. No clinical toxicity of DG was seen, and DG had no effect on any hematological or biochemical parameter (data not shown).

Colon histopathology score

Treatment with DG (334 mg) for 5 days reduced the clinical score for diarrhea, mucus and blood on day 6 by 54% (**Figure 1a**). At the time of autopsy, the colon was macroscopically assessed for evidence of acute gut wall tissue injury; representative examples are shown in **Figure 2**. All histological tissue sections were scored blind by EL (a Board certified Diplomate of the American College of Veterinary Pathologists). DG reduced the colon

inflammation score by 71% (**Figure 1b**). This was due to a 75% reduction in gut wall infiltration by neutrophils with no significant change in either monocyte or lymphocyte infiltration (**Figure 1c** & **3**). The notable absence of tissue necrosis with DG treatment suggests that *Shigella's* reprogramming of neutrophils to die by necrosis (with inflammation) instead of apoptosis (without inflammation) did not occur. This enabled controlled resolution of the infection-related-inflammatory response[23,41-44].

Fibrin thrombi and vasculitis represent progressively more severe degrees of blood vessel-associated tissue injury from infection-related-inflammation[45,46] (**Supplementary Figure 1**). Strikingly, vasculitis did not occur with DG treatment (**Figure 1d** & **3**) and fibrin thrombi were reduced by 67%; they occurred rarely and with less severity in ileum only[35]. In addition, Peyer's patches in the ileum remained intact with no damage to their surface mucosa with DG treatment (**Figure 1-d** & **Supplementary Figure 2**).

Colon cytokines & tissue mediators

Quantitative mRNA RT-PCR of ascending, transverse and descending colon (pooled data) showed that DG (334 mg) reduced IL-8 by 77%, IL-6 by 77% and IL-1β by 61%. IFN-γ fell by 47.5% but did not reach statistical significance (*P=0.06*). TNF-α (inflammatory), IL-10 (anti-inflammatory), FoxP3 and TGF-β (T_{reg} cells[23]) did not change with infection or DG treatment (**Figure 4** & **Supplementary Table 1**). IL-4 was usually below the limit of detection[47] (data not shown).

Rectum cytokines & tissue mediators

In rectum, DG (334 mg) reduced IL-8 by 90%, IL-1β by 60% and IFN-γ by 52%. IL-6 did not change with either infection or DG treatment. TNF-α (inflammatory), IL-10 (anti-inflammatory), FoxP3 and TGF-β (T_{reg} cells[23]) did not change with either infection or DG treatment (**Figure 5** & **Supplementary Table 2**).

The diarrheal content of stool cytokines peaks on day 5 after infection and was measured for IL-8, IL-1β, IL-6, TNF-α and INF-γ proteins using the FlowMetrixTM System (Luminex Corp., Texas) as described[12]. IL-8 and IL-1β proteins were significantly increased in the diarrheal content after infection, and they were also significantly reduced by DG (334 mg) treatment. There was no change in IL-6, TNF-α or INF-γ proteins in the diarrheal content with either infection or after DG treatment.

Colon FACS analysis

FACS analysis showed that DG (334 mg) did not change either the % CD3+CD4+ lymphocytes or the % CD3+CD8+ lymphocytes in colon (**Figure 6A**).

The rhesus macaque is also a reliable human correlate for studying colon intraepithelial lymphocytes; i.e. γδ T-cell receptor (TCR) cells[33]. They play an important role in: (i) responding to bacteria and intestinal injury; and (ii) promoting epithelial healing and restoration of gut barrier function[48,49]. FACS analysis showed that the % γδ TCR cells in colon did not change from normal (9.0 ± 0.77 %) with either *Shigella* infection (11.77 ± 1.41

%) or DG (334 mg) treatment (6.75 ± 1.0 %); this range is consistent with a report for colon γδ TCR cells in normal rhesus macaques[33].

Expression of the Ki-67 nucleoprotein occurs only during the G1/S/G2/M phase of the cell cycle and is strictly associated with cell proliferation[50]. Importantly, DG (334 mg) treatment reduced the Ki67 activation of both CD3+CD4+ lymphocytes and CD3+CD8+ lymphocytes in colon. For CD3+CD4+ lymphocytes, Ki67 activation fell by 52% from 2.23 ± 0.3% (*Shigella* group) to 1.08 ± 0.04% (DG group) *(P=0.03)*. For CD3+CD8+ lymphocytes, Ki67 activation fell by 67% from 1.44 ± 0.29% (*Shigella* group) to 0.47 ± 0.06% (DG group) *(P=0.03)* (**Figure 6a**).

Blood FACS analysis

DG (334 mg) had no effect on blood lymphocyte TNF-α production (**Figure 6b**). DG (334 mg) also had no effect on blood monocyte TNF-α, IL-1β, IL-6, IL-8 production after *in vitro* stimulation with PMA/ionomycin/Brefeldin-A (**Figure 6c**). DG (214 mg) had no effect on any of the above (data not shown).

Mesenteric lymph node cytokines & tissue mediators

Gut mesenteric lymph nodes in NHPs are very similar to human[33]. Neither infection nor DG altered cytokines and tissue mediators in gut mesenteric lymph nodes. This confirmed: (i) the colon and rectum localization of the infection-related cytokine storm in NHPs; and (ii) the gut wall limited activity of DG (**Supplementary Table 3**). DG (214 mg) had no effect on any chemokine/cytokine/tissue mediator at any site (data not shown).

Mesenteric lymph node FACS analysis

Neither infection nor DG (i.e. 214 mg and 334 mg dosing) changed the % CD3+CD4+ lymphocytes or the % CD3+CD8+ lymphocytes in lymph nodes (**Figure 7a**). After *in vitro* stimulation with PMA/ionomycin/Brefeldin-A, neither *Shigella* infection nor DG treatment changed the % TNF-α producing CD3+CD4+ lymphocytes or the CD3+CD8+ lymphocytes in lymph nodes (**Figure 7b**).

Discussion

Here we show that in the most severe form of Gram negative bacterial diarrhea and colitis, the oral TLR-4 antagonist DG protected the rhesus macaque colon and rectum from pro-inflammatory cytokine-induced and neutrophil-mediated vasculitis and gut wall necrosis. Colon CD4+ and CD8+ lymphocyte activation was also reduced by DG. Importantly, the systemic innate immune cytokine response in both gut lymph nodes and blood remained intact and undisturbed.

By performing stool cultures twice daily after the administration of 2×10^9 CFUs of *Shigella dysenteriae* Type 1 1617 into the stomach, we were able to show that the time to the first positive stool for *Shigella* was 1.6 ± 0.2 days for all three groups studied. This infectious diarrhea was then allowed to progress for another 4 days. The only medical intervention was to ensure adequate hydration with subcutaneous infusion of water, sodium and potassium if the NHPs stopped drinking; anorexia is common in severe sepsis. This study is likely to be the closest to a study in humans of an oral TLR4 immuno-modulator in severe sepsis that

could be performed because a human clinical trials ethics committee would not allow the exclusion of antibiotics.

During the first two days of any severe bacterial infection, the immediate innate immune response tries to eradicate bacteria from the gut at the cost of mucosal destruction. All pathogenic Gram negative bacteria stimulate local tissue macrophages to produce IL-6 and IL-1β via TLR-4[1,6]; the gut associated lymphoid tissue contains the largest body pool of macrophages[51]. This leads to the recruitment of IL-8 and IL-1β producing blood monocytes into the gut by day 2-3, and of IL-8 producing blood neutrophils by day 3 after infection[52]. Monocytes and neutrophils have their own independent programs for recruitment into tissues[53].

Our new finding of a large reduction of IL-8, IL-6, IL-1β and IFN-γ in the colon and rectum of NHPs (**Figures 4-5**) is consistent with DG blocking the cell surface TLR4-MYD88 early cytokine pathway of the TLR4-MD-2-LPS receptor complex while sparing the endosomal TLR4-TRIF/TRAM late cytokine and β-interferon/CD86 pathway[16-21]. Maintaining TRIF signalling ensures an adequate gut pro-inflammatory cytokine response for recruiting the neutrophils required to prevent the spread of pathogenic bacteria into tissues and blood. It is important to remember that activation of the TRIF pathway of TLR4 results in a mild rather than a severe pro-inflammatory cytokine response[19]. TRIF signalling also ensures that mucosal antimicrobial peptide production (i.e. natural antibiotics) continues with these peptides acting to control local bacterial growth and replication[23,54,55]. Furthermore, stimulation of TRIF leads to sequential expression of IFN-β and IL-27p28 in macrophages (in response to gut commensal bacteria) with STAT1 activation also ensuring maintenance of gut wall Th17 cells[56].

Vasculitis reflects the severity of neutrophil and *Shiga* toxin induced injury to the colon after infection, and is associated with high patient mortality[9,11,14,15,47]. The relative contribution of neutrophil lysosomal enzymes versus *Shiga* toxin to this tissue damage has been difficult to define precisely. However two published observations are noteworthy: (a) Fulminant and lethal bloody diarrhea can occur even when a Toxin negative mutant of *S. dysenteriae* Type 1 causes infection[14]; (b) In a rat model of mercuric chloride induced vasculitis of the colon, neutrophils were necessary for induction of vasculitis with the degree of vasculitis correlating with the number of neutrophils present[57]. In our study, the absence of tissue vasculitis combined with a 67% reduction in tissue fibrin thrombi with DG treatment (**Figures 1d** & **3**) indicated a substantial reduction in infection-related blood vessel damage, and correlated with a 75% reduction in colon neutrophil infiltration. This absence of tissue vasculitis with DG treatment was a surprising and unexpected result. Taken together, our observations suggest that the pro-inflammatory cytokine storm is primarily responsible for initiating the neutrophil-mediated pathology that leads to tissue vasculitis.

DG treatment did not alter the % CD3+CD4+ lymphocytes, CD3+CD8+ lymphocytes or γδ TCR cells in colon (**Figure 7a**). Ki-67+ proliferation of lymphocytes in the rectum of patients with Shigellosis has been described[44,50], and was also seen in the colon of our infected NHPs. Notably, DG treatment reduced Ki67 activation of CD4+ and CD8+ lymphocytes in colon by 52% and 67% respectively (**Figure 7a**). These results show that infection-induced and lymphocyte-associated inflammation of the colon was reduced by DG treatment.

The broad implication of our new findings with an oral TLR4 antagonist given once daily for just 5 days is to define an alternative strategy for protecting the gut epithelial barrier from

bacteria-induced and cytokine and neutrophil -mediated tissue damage. Such gut wall barrier breakdown is increasingly recognised as a major driving force in precipitating systemic sepsis-induced multi-organ failure[58]. Our results further support this growing recognition and also show that, in NHPs, IL-8 is as important as IL-6 in destroying the epithelial gut wall's integrity, promoting bacterial tissue invasion, and leading to severity of clinical disease seen. Our observations are also consistent with the dramatic increase in IL-8 that occurs in human shigellosis and point to this chemokine being the major orchestrator of gut mucosal inflammation in severe infectious diarrheal diseases[59]; this is consistent with the neutrophil recruiting and activating role of IL-8 in acutely infected tissues.

Taken together, this leads us to propose that non-absorbable oral TLR4 antagonists could be started at the same time as antibiotics in patients with symptoms and signs of infection to protect and preserve the gut wall barrier. The importance of maintaining the competence of a crucial body barrier (i.e. the gut wall) is that it minimizes the likelihood of bacterial tissue invasion. Strategies for orally based delivery of drugs to the colon are already well established using hard hypromellose capsules with pH sensitive methacrylic acid copolymer coatings (Eudragit)[60].

A direct consequence of the approach proposed would be significantly reduced systemic bacterial disease severity. This therapeutic approach should also mean much shorter courses of antibiotics; e.g. 3 days versus 14 days. This would impact immediately on the global antimicrobial crises, and without further increasing bacterial resistance.

Acknowledgements

SS, DI, IT & CJM conceived & designed the study. RI was the vet & EL the pathologist. DI, NR, PK, PN, SG, & KY performed the animal study & tissue analysis. The manuscript was written by SS, DI, EL, IT, BES & CJM with input from all authors. We are grateful for the technical support provided by Kesara Chumpolkulwong and Alongkorn Hanrujirakomjorn for veterinary procedures, Amnat Andang, Rachata Jecksaeng, Mana Saitasao and Siwakorn Sirisrisopa for husbandry, and Paphavee Lertsethtakarn, Sathit Pichyangkul, Ajchar Aksomboon, Nuanpan Khemnu, Kaewkanya Nakjarung and Sasikorn Silapong for assays.

This study was funded by NIAID under Inter-Agency Agreement Y1-AI-4906-03 and US Army Medical Research & Materiel Command and Imperial Biomedical Research Centre and Williams Trust grants to CJM, BES & SS.

Conflict of interest disclosure:

The authors declare no conflict of interest.

References

[1] Tsolis RM, Young GM, Solnick JV, Bäumler AJ. From bench to bedside: stealth of enteroinvasive pathogens. *Nat Rev Microbiol* 2008; **6:**883-92.

[2] LaRock DL, Chaudhary A, Miller SI. Salmonellae interactions with host processes. *Nat Rev Microbiol* 2015; **13:**191-205.

[3] Keestra-Gounder AM, Tsolis RM, Bäumler AJ. Now you see me, now you don't: the interaction of Salmonella with innate immune receptors. *Nat Rev Microbiol* 2015; **13:**206-16.

[4] Yazji I, Sodhi CP, Lee EK, Good M, Egan CE, Afrazi A *et al*. Endothelial TLR4 activation impairs intestinal microcirculatory perfusion in necrotizing enterocolitis via eNOS-NO-nitrite signalling. *Proc Natl Acad Sci* 2013; **110:**9451-6.

[5] Benkoe TM, Mechtler TP, Weninger M, Pones M, Rebhandl W, Kasper DC. Serum levels of IL-8 and gut-associated biomarkers in diagnosing necrotizing enterocolitis in preterm infants. *J Pediatr Surg* 2014; **49:**1446-51.

[6] Khan MA, Ma C, Knodler LA, Valdez Y, Rosenberger CM, Deng W *et al*. TLR4 contributes to colitis development but not to host defense during *Citrobacter rodentium* infection in mice. *Infect Immun* 2006; **74:**2522-36.

[7] Fournier BM, Oarkos CA. The role of neutrophils during intestinal inflammation. *Mucosal Immunol* 2012; **5:**354-66.

[8] Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W *et al*. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci* 2013; **110:**3507-12.

[9] Raqib R, Lindberg AA, Wretlind B, Bardhan PK, Andersson U, Andersson J. Persistence of local cytokine production in Shigellosis in acute and convalescent stages. *Infect Immun* 1995; **63:**289-96.

[10] Schnupf P, Sansonetti PJ. Quantitative RT-PCR profiling of the rabbit immune response: Assessment of acute *Shigella flexneri* infection. *PLoS One* 2012; **6,**e36446.

[11] Harendra de Silva DG, Mendis LN, Sheron N, Alexander GJ, Candy DC, Chart H *et al*. Concentrations of IL-6 and TNF in serum and stools of children with *Shigella dysenteriae* 1 infection. *Gut* 1993; **34:**194-8.

[12] Islam D, Ruamsap N, Khantapura P, Aksomboon A, Srijan A, Wongstitwilairoong B *et al*. Evaluation of an intragastric challenge model for *Shigella dysenteriae* 1 in rhesus monkeys (*Macaca mulatta*) for the pre-clinical assessment of *Shigella* vaccine formulations. *APMIS* 2012; **122:**463-75.

[13] Gregory M, Kaminski RW, Lugo-Roman LA, Galvez Carrillo H, Tilley DH, Baldeviano C *et al*. Development of an *Aotus nancymaae* model for Shigella vaccine immunogenicity and efficacy studies. *Infect Immun* 2014; **82:**2027-36.

[14] Fontaine A, Arondel J, Sansonetti PJ. Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a Tox-mutant of *Shigella dysenteriae* Type 1. *Infect Immun* 1988; **56:**3099-109.

[15] Raqib R, Sarker P, Bergman P, Ara G, Lindh M, Sack DA *et al.* Improved outcome in shigellosis associated with butyrate induction of an endogenous peptide antibiotic. *Proc Natl Acad Sci* 2006; **103:**9178-83.

[16] Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H *et al.* Role of adaptor TRIF in the MyD88-independent TLR signalling pathway. *Science* 2003; **301:**640-3.

[17] Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of TLR4 to the induction of IFN-β. *Nat Immunol* 2008; **9:**361-8.

[18] Watts C. Location, location, location: identifying the neighborhoods of LPS signaling. *Nat Immunol* 2008; **9:**343-5.

[19] Needham BD, Trent MS. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. *Nat Rev Microbiol* 2013; **11:**467-81.

[20] Shaunak S, Thomas S, Gianasi E, Godwin A, Jones E, Teo I *et al.* Polyvalent dendrimer glucosamine conjugates prevent scar tissue formation. *Nat Biotechnol* 2004; **22:**977-84.

[21] Shaunak S. Dendrimers drugs for infection & inflammation. *Biochem Biophys Res Commun* 2015 Jul 10. doi: 10.1016/j.bbrc.2015.07.033. [Epub ahead of print].

[22] Barata TS, Teo I, Brocchini S, Zloh M, Shaunak S. Partially glycosylated dendrimers block MD-2 and prevent TLR4-MD-2-LPS complex mediated cytokine responses. *PLoS Comput Biol 2011;* **7:**e1002095.

[23] Teo I, Toms SM, Marteyn B, Barata TS, Simpson P, Johnston KA *et al.* Preventing acute gut wall damage in infectious diarrhoeas with glycosylated dendrimers. *EMBO Mol Med* 2012; **4:**866-81.

[24] Kelleher D. Dendrimers branch out to support mucosal integrity. *EMBO Mol Med* 2012; **4:**860-2.

[25] Vongsawan AA, Kapatral V, Vaisvil B, Burd H, Serichantalergs O, Venkatesan MM *et al*. The genome of *Shigella dysenteriae* strain Sd1617 comparison to representative strains in evaluating pathogenesis. *FEMS Microbiol Lett* 2015; **362:**fnv011.

[26] Asquith M, Haberthur K, Brown M, Engelmann F, Murphy A, Al-Mahdi Z *et al*. Age-dependent changes in innate immune phenotype and function in rhesus macaques (Macaca mulatta). *Pathobiol Aging Age Relat Dis* 2012; **2**. doi: 10.3402/pba.v2i0.18052.

[27] Villinger F, Brar SS, Mayne A, Chikkala N, Ansari AA. Comparative sequence analysis of cytokine genes from human and nonhuman primates. *J Immunol* 1995; **155:**3946-54.

[28] Abel K, Alegria-Hartman MJ, Zanotto K, McChesney MB, Marthas ML, Miller CJ. Anatomic site and immune function correlate with relative cytokine mRNA expression levels in lymphoid tissues of normal rhesus macaques. *Cytokine* 2001; **16:**191-204.

[29] Jovanovic M, Rooney MS, Mertins P, Przybylski D, Chevrier N, Satija R *et al.* Immunogenetics. Dynamic profiling of the protein life cycle in response to pathogens. *Science* 2015; **347:**1259038.

[30] Verhoeven D, Sankaran S, Silvey M, Dandekar S. Antiviral therapy during primary SIV infection fails to prevent acute loss of CD4+ T cells in gut mucosa but enhances their rapid restoration through central memory T cells. *J Virol* 2008; **82:**4016-27.

[31] Benveniste O, Vaslin B, Le Grand R, Cheret A, Matheux F, Theodoro F *et al.* Comparative IL-2/IFN-γ and IL-4/IL-10 responses during acute infection of macaques inoculated with attenuated nef-truncated or pathogenic SICmac251 virus. *Proc Natl Acad Sci 1996;* **93:**3658-63.

[32] Gansuvd B, Asiedu CK, Goodwin J, Jargal U, Deckard LA, Andrades P *et al*. Expansion of CD4+CD25+ suppressive regulatory T cells from rhesus macaque peripheral blood by FN18/antihuman CD28-coated Dynal beads. *Hum Immunol* 2007; **68:**478-90.

[33] Veazey RS, Rosenzweig M, Shvetz DE, Pauley DR, DeMaria M, Chalifoux LV *et al*. Characterization of gut-associated lymphoid tissue (GALT) of normal rhesus macaques. *Clin Immunol Immunopathol* 1997; **82:**230-42.

[34] Sestak K, Merritt CK, Borda J, Saylor E, Schwamberger SR, Cogswell F *et al*. Infectious agent and immune response characteristics of chronic enterocolitis in captive rhesus macaques. *Infect Immun* 2003; **71:**4079-86.

[35] Rout WR, Formal SB, Giannella RA, Dammin GJ. Pathophysiology of *Shigella* diarrhea in the rhesus monkey: intestinal transport, morphological, and bacteriological studies. *Gastroenterology* 1975; **68:**270-8.

[36] Autissier P, Soulas C, Burdo TH, Williams KC. Immunophenotyping of lymphocyte, monocyte and dendritic cell subsets in normal rhesus macaques by 12-color flow cytometry. *J Immunol Meth.* 2010*;* **360:**119-28.

[37] Kotloff KL, Herrington DA, Hale TL, Newland JW, Van De Verg L, Cogan JP *et al.* Safety, immunogenicity, and efficacy in monkeys and humans of invasive *E. coli* K-12 hybrid vaccine candidates expressing *Shigella flexn*eri 2a somatic antigen. *Infect Immun* 1992; **60:**2218-24.

[38] Hukkanen RR, Liggitt HD, Murnane RD, Frevert CW. Systemic inflammatory response syndrome in non-human primates culminating in multiple organ failure, acute lung injury, and disseminated intravascular coagulation. *Toxicologic Pathol* 2009; **37:**799-804.

[39] Cary M, Kosanke S, White G. Spontaneous Waterhouse-Friderichsen syndrome in a gang-housed baboon. *J Med Primatol* 2001; **30:**185-7.

[40] Bennish ML, Salam MA, Wahed MA. Enteric protein loss during Shigellosis. Am J Gastroenterol 1993; **88:**53-7.

[41] François M, Le Cabec V, Dupont M-A, Sansonetti PJ, Maridonneau-Parini I. Induction of necrosis in human neutrophils by *Shigella flexneri* requires Type III secretion, IpaB and IpaC invasins, and actin polymerization. *Infect Immun* 2000; **68:**1289-96.

[42] Hathaway LJ, Griffin GE, Sansonetti PJ, Edgeworth JD. Human monocytes kill *Shigella flexneri* but then die by apoptosis associated with suppression of proinflammatory cytokine production. *Infect Immun* 2002; **70:**3833-42.

[43] Sabroe I, Dower SK, Whyte MK. The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. *Clin Infect Dis* 2005; **41:**S421-6.

[44] Raqib R, Moly PK, Sarker P, Qadri F, Alam NH, Mathan M *et al*. Persistence of mucosal mast cells and eosinophils in Shigella-infected children. *Infect Immun* 2003; **71:**2684-92.

[45] Carlson, J.A. The histological assessment of cutaneous vasculitis. *Histopathology* 2010; **56:**3-23.

[46] Heeringa P, Foucher P, Klok PA, Huitema MG, Tervaert JW, Weening JJ *et al*. Systemic injection of products of activated neutrophils and H_2O_2 in myeloperoxidase-immunized rats leads to necrotizing vasculitis in the lungs and gut. *Am J Pathol* 1997; **151:**131-40.

[47] Samandari T, Kotloff KL, Losonsky GA, Picking WD, Sansonetti PJ, Levine MM *et al*. Production of IFN-γ and IL-10 to Shigella invasins by mononuclear cells from volunteers orally inoculated with a Shiga toxin-deleted *Shigella dysenteriae* type 1 strain. *J Immunol* 2000; **164:**2221-32.

[48] Rakasz E, MacDougall AV, Zayas MT, Helgelund JL, Ruckward TJ, Hatfield G *et al*. γδ T cell receptor repertoire in blood and colonic mucosa of rhesus macaques. *J Med Primatol* 2000; **29:**387-96.

[49] Smith PM, Garrett WS. The gut microbiota and mucosal T cells. *Front Microbiol* 2011; **2:**111.

[50] Islam D, Veress B, Bardhan PK, Lindberg AA, Christensson B. In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis. *Infect Immun* 1997; **65:**739-49.

[51] Bain CC, Bravo-Blas A, Scott CL, Gomez Perdiguero E, Geissmann F, Henri S *et al*. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol* 2014; **15:**929-37.

[52] Kucharzik T, Hudson JT III, Lügering A, Abbas JA, Bettini M, Lake JG *et al*. Acute induction of human IL-8 production by intestinal epithelium triggers neutrophil infiltration without mucosal injury. *Gut* 2005; **54:**1565-72.

[53] Dal-Secco D, Wang J, Zeng Z, Kolaczkowska E, Wong CH, Petri B *et al*. A dynamic spectrum of monocytes arising from the in situ reprogramming of CCR2+ monocytes at a site of sterile injury. *J Exp Med* 2015; **212:**447-56.

[54] Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B *et al.* Downregulation of bactericidal peptides in enteric infections: A novel immune escape mechanism with bacterial DNA as a potential regulator. *Nat Med* 2001; **7:**180-5.

[55] Stockinger S, Duerr CU, Fulde M, Dolowschiak T, Pott J, Yang I *et al*. TRIF signaling drives homeostatic intestinal epithelial antimicrobial peptide expression. *J Immunol* 2014; **193:**4223-34.

[56] Kanagavelu S, Flores C, Termini JM, Riveron R, Romero L, Chung K *et al*. TIR-domain-containing adapter-inducing IFN-β (TRIF) regulates Th17-mediated intestinal immunopathology in colitis. *Mucosal Immunol* 2015; **8:**296-306.

[57] Qasim FJ, Mathieson PW, Sendo F, Thiru S, Oliveira DB. Role of neutrophils in the pathogenesis of experimental vasculitis. *Am J Pathol* 1996; **149:**81-9.

[58] Schulz K, Sommer O, Jargon D, Utzolino S, Clement HW, Strate T *et al.* Cytokine and radical inhibition in septic intestinal barrier failure. *J Surg Res* 2015; **193:**831-40.

[59] Pédron T, Thibault C, Sansonetti PJ. The invasive phenotype of *Shigella flexneri* directs a distinct gene expression pattern in the human intestinal epithelial cell line Caco-2. *J Biol Chem* 2003; **278:**33878-86.

[60] Hua S, Marks E, Schneider JJ, Keely S. Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: Selective targeting to diseased versus healthy tissue. *Nanomedicine* 2015; **11:**1117-32.

Figure Legends

Figure 1

Clinical score & colon histology score.

(**A**) DG (334 mg) reduced the total clinical score for diarrhea, mucus & blood/NHP by 54%.

(**B**) The right side Y-axis shows the % inflammation score/NHP after correcting for the % inflammation score/NHP being 52% in normal NHPs as shown on the left side Y-axis. DG (334 mg) reduced the colon inflammation score/NHP by 71%.

(**C**) The right side Y-axis shows the % neutrophil infiltrate/NHP after excluding the lymphocytes and monocytes[34] that are shown on the left side Y-axis. DG (334 mg) reduced the colon neutrophil infiltrate score/NHP by 75%. There was no change in the lymphocyte or monocyte infiltrate with either infection or after DG treatment.

(**D**) DG prevented vasculitis as shown on the left side Y-axis. The right side Y-axis shows the % fibrin thrombi score/NHP which was reduced by 67%.

Mean ± SEM with 2-tailed Mann-Whitney test (*$P<0.05$). AC ascending colon; TC transverse colon; DC descending colon.

Figure 2

Macroscopic pathology of colon.

(**A**) Mucosal surface of normal rhesus macaque colon.

(**B**) Mucosal surface of *Shigella dysenteriae* Type 1 infected colon at day 6 showing severe hemorrhagic gut wall damage.

(**C**) Mucosal surface of *Shigella dysenteriae* infected colon at day 6 after DG (334 mg) treatment showing minimal damage.

Figure 3

Microscopic histopathology of colon.

(**A**) Normal rhesus macaque colon with mild background lymphoplasmacytic infiltrate in the lamina propria. M = mucosa.

(**B**) *Shigella dystenteriae* Type 1 infected colon. Mucosa has herniated into sub-epithelial connective tissue and muscularis mucosae (arrow) and is infiltrated by many neutrophils. The herniated glands are abscessed (circles).

(**C**) *Shigella* (Sh) infected colon. Severe neutrophil infiltration of eroded and ulcerated mucosa (UM) with inflammatory infiltrate extending beyond the lamina propria and into the sub-epithelial connective tissue and muscularis mucosae. Medium sized arteries in the sub-mucosa have neutrophils infiltrating and disrupting the full thickness of their vessel walls; this is diagnostic of vasculitis (dotted oval).

(**D**) Sh infected colon + DG 334 mg treatment. There is mild infiltration only of the mucosa (M) by lymphocytes, monocytes and neutrophils.

H&E (x 200).

Figure 4

Cytokines in colon.

(**A-D**) DG (334 mg) reduced IL-8 by 77%, IL-6 by 77%, IL-1β by 61% and IFN-γ by 48% (*P=0.06*) compared to *Shigella* control on day 6. DG (214 mg) had no effect. Pooled results for the ascending colon, transverse colon and descending colon are shown.

Normal (■); *Shigella* (●); DG 214 mg (▼); DG 334 mg (▲). Scatter plot with mean ± SEM with 2-tailed Mann-Whitney test (**P<0.05, **P<0.01, ***P<0.001*).

Figure 5

Cytokines in rectum.

DG (334 mg) reduced IL-8 by 90%, IL-1β by 60%, and IFN-γ by 52% when compared to *Shigella* infection control. DG (214 mg) had no effect.

Normal rhesus macaque (■); *Shigella* infection control (●); DG 334 mg (▲). Scatter plot with mean ± SEM with 2-tailed Mann-Whitney test (**P<0.05, **P<0.01*).

Figure 6

Lymphocyte & monocyte function in colon & blood.

(**A**) DG (334 mg) did not change the % CD3+CD4+ lymphocytes or % CD3+CD8+ lymphocytes in colon but reduced their Ki67 activation on day 6.

(**B-C**) DG (334 mg) had no effect on the % TNF-α producing CD3+CD4+ lymphocytes and CD3+CD8+ lymphocytes or on the % TNF-α, IL-1β, IL-6, IL-8 producing CD14+ monocytes in blood after *in vitro* stimulation with PMA/ionomycin/Brefeldin-A [days 0 & 6].

Normal (■/□); *Shigella* (●/○); DG 214 mg (▼); DG 334 mg(▲/△). Mean ± SEM with 2-tailed Mann-Whitney test (*$P<0.05$).

Figure 7

Gut mesenteric lymph nodes.

(**A**) FACS analysis of CD3+CD4+ lymphocytes and CD3+CD8+ lymphocytes in gut mesenteric lymph nodes on day 6. Neither infection nor DG changed the % CD3+CD4+ lymphocytes or the % CD3+CD8+ lymphocytes in lymph nodes.

(**B**) FACS analysis of CD3+CD4+ lymphocytes and CD3+CD8+ lymphocytes in gut mesenteric lymph nodes after *in vitro* stimulation with PMA/ionomycin/Brefeldin-A. Neither infection nor DG treatment changed the % TNF-α producing CD3+CD4+ lymphocytes or CD3+CD8+ lymphocytes in lymph nodes.

Normal rhesus macaque (□/■); *Shigella* infection control (○/●); DG 214 mg (▼); DG 334 mg (△/▲). Mean ± SEM with 2-tailed Mann-Whitney test (*$P<0.05$).

Figure 1

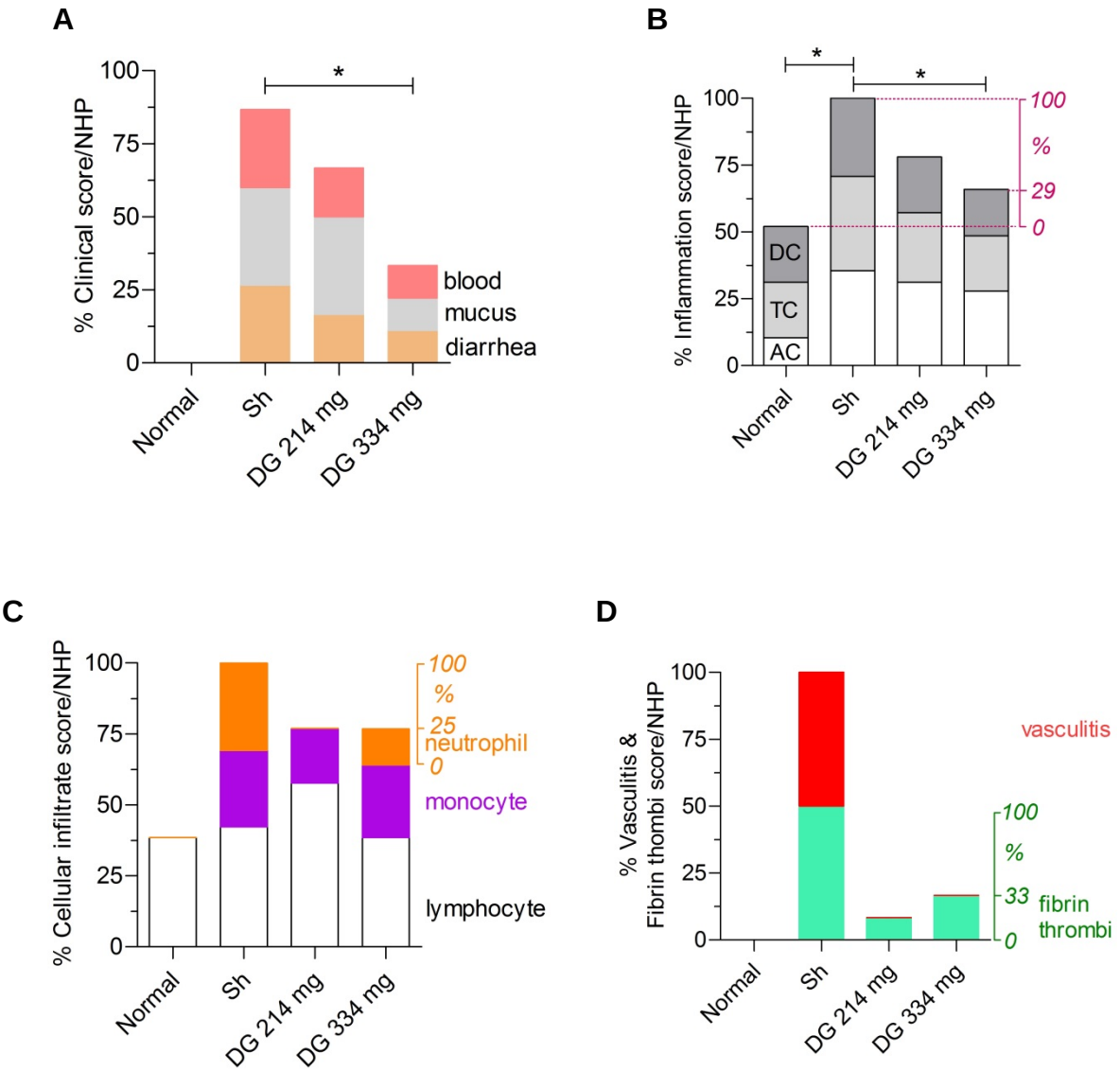
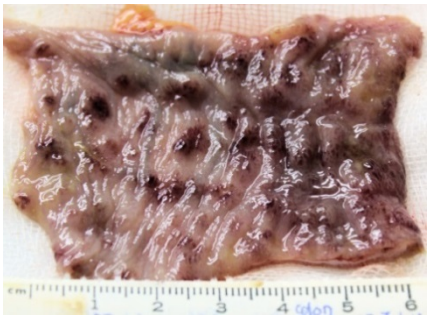

A
% Clinical score/NHP
100
75
50
25
0
*
blood
mucus
diarrhea
Normal
Sh
DG 214 mg
DG 334 mg
B
% Inflammation score/NHP
*
*
DC
TC
AC
100
%
29
0
C
% Cellular infiltrate score/NHP
100
%
25
0
neutrophil
monocyte
lymphocyte
D
% Vasculitis &
Fibrin thombi score/NHP
vasculitis
100
%
33
0
fibrin
thrombi

Figure 2

A



B



C

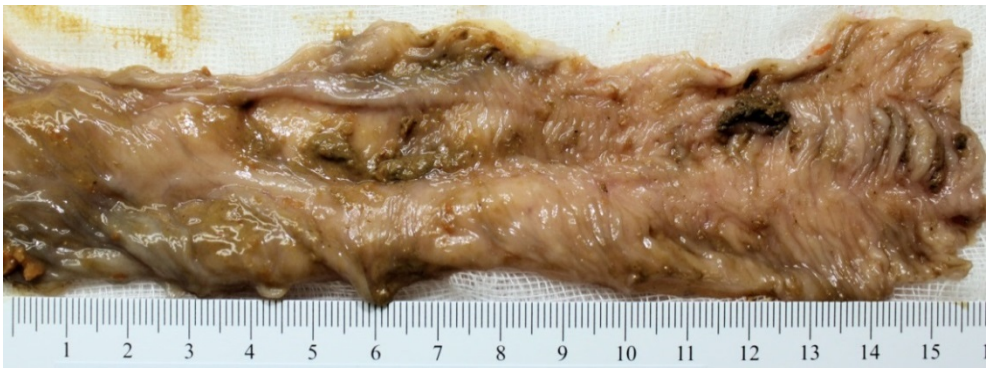
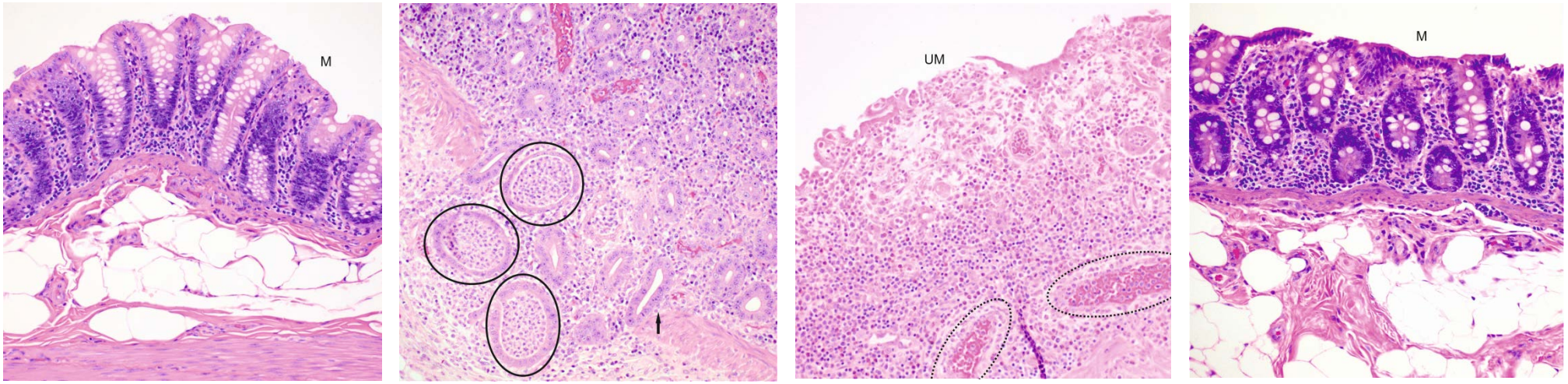


Figure 3

Normal NHP

Shigella dysenteriae (Sh) Type 1 infection

Sh + DG 334 mg



A **B** **C** **D**

Figure 4

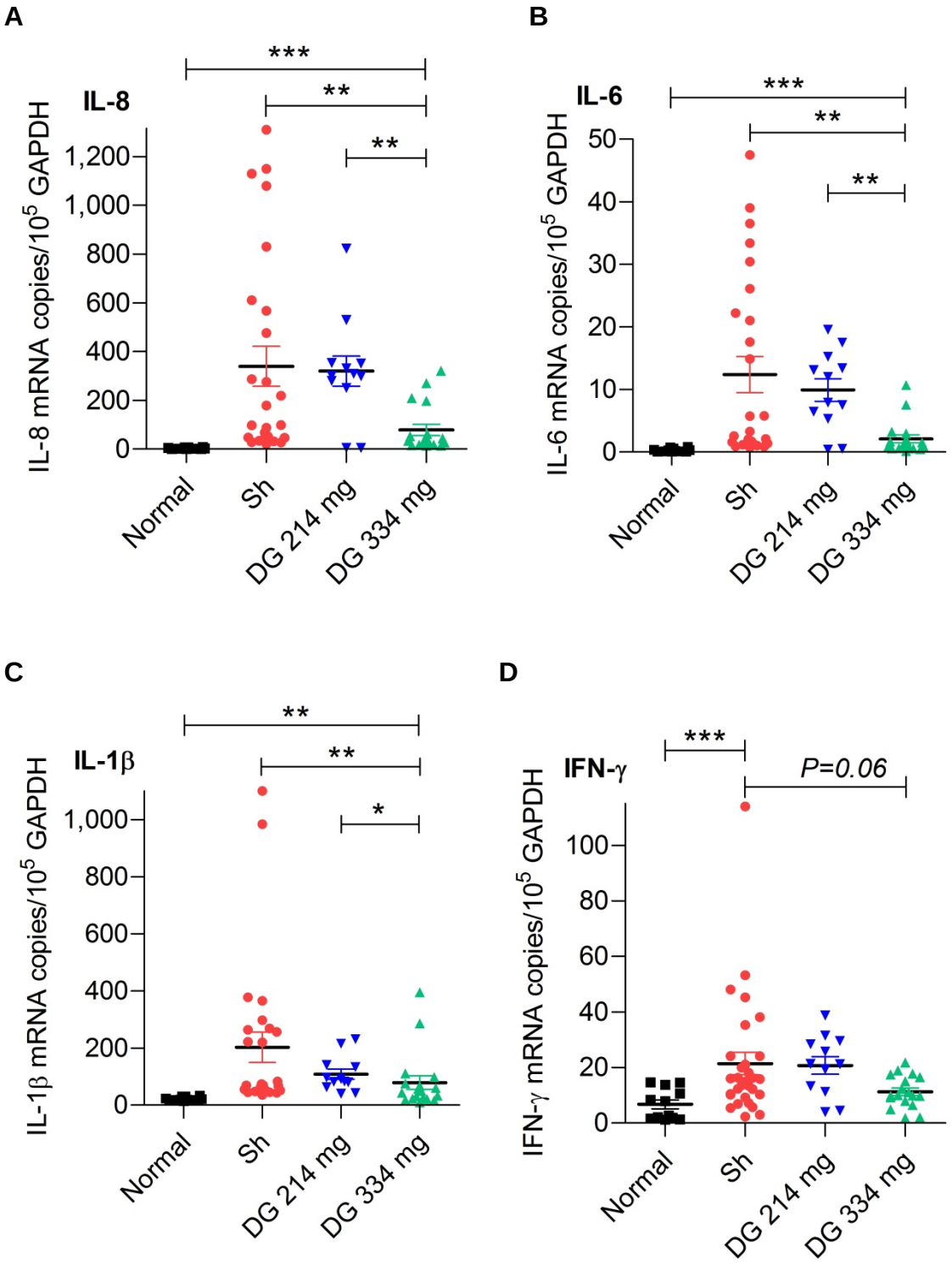

A
IL-8
IL-8 mRNA copies/10^5 GAPDH
1,200
1,000
800
600
400
200
0
***
**
**
Normal
Sh
DG 214 mg
DG 334 mg
B
IL-6
IL-6 mRNA copies/10^5 GAPDH
50
40
30
20
10
0
***
**
**
Normal
Sh
DG 214 mg
DG 334 mg
C
IL-1β
IL-1β mRNA copies/10^5 GAPDH
1,000
800
600
400
200
0
**
**
*
Normal
Sh
DG 214 mg
DG 334 mg
D
IFN-γ
IFN-γ mRNA copies/10^5 GAPDH
100
80
60
40
20
0
***
P=0.06
Normal
Sh
DG 214 mg
DG 334 mg

Figure 5

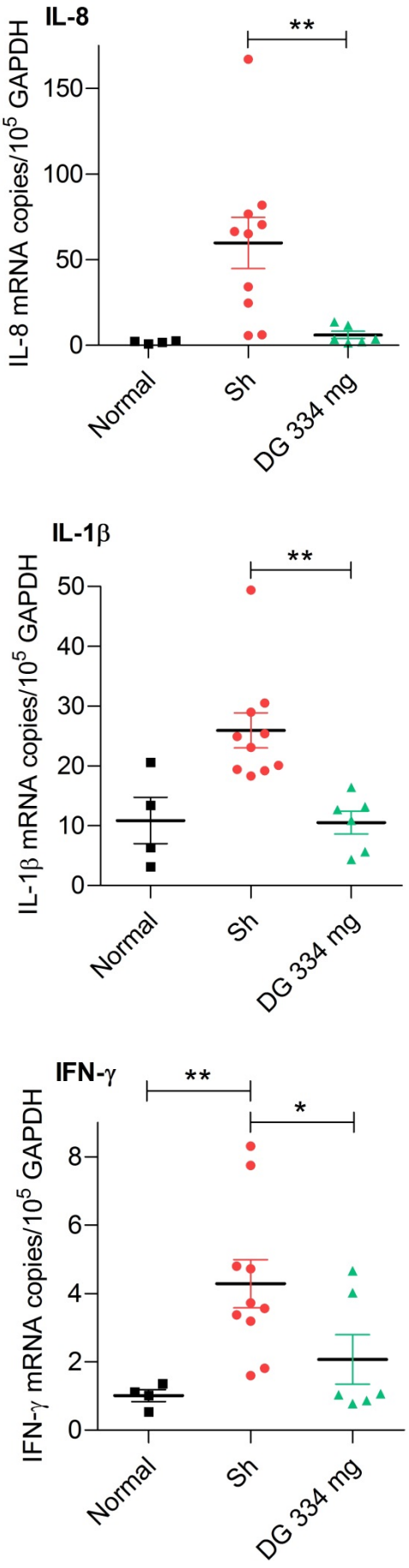
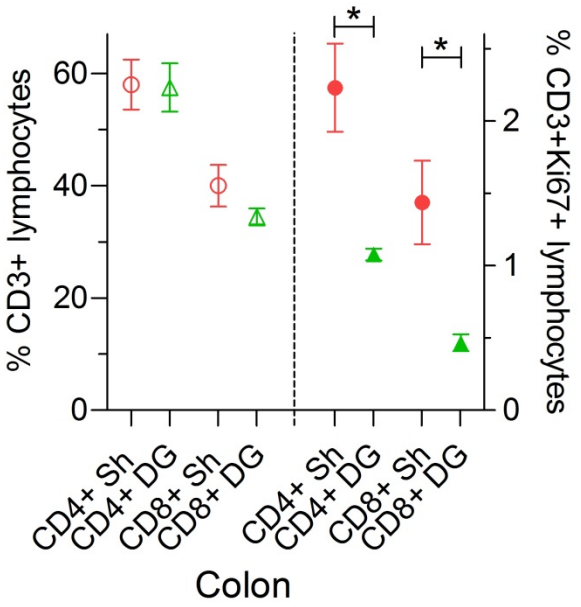
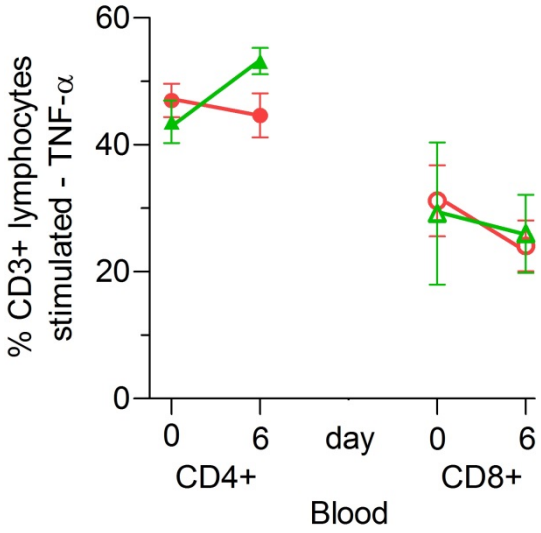

IL-8
IL-8 mRNA copies/10^5 GAPDH
**
150
100
50
0
Normal
Sh
DG 334 mg
IL-1β
IL-1β mRNA copies/10^5 GAPDH
**
50
40
30
20
10
0
Normal
Sh
DG 334 mg
IFN-γ
IFN-γ mRNA copies/10^5 GAPDH
**
*
8
6
4
2
0
Normal
Sh
DG 334 mg

Figure 6

A


% CD3+ lymphocytes
% CD3+Ki67+ lymphocytes
60
40
20
0
2
1
0
*
*
CD4+ Sh
CD4+ DG
CD8+ Sh
CD8+ DG
CD4+ Sh
CD4+ DG
CD8+ Sh
CD8+ DG
Colon

B


% CD3+ lymphocytes stimulated - TNF-α
60
40
20
0
0
6
day
0
6
CD4+
CD8+
Blood

C

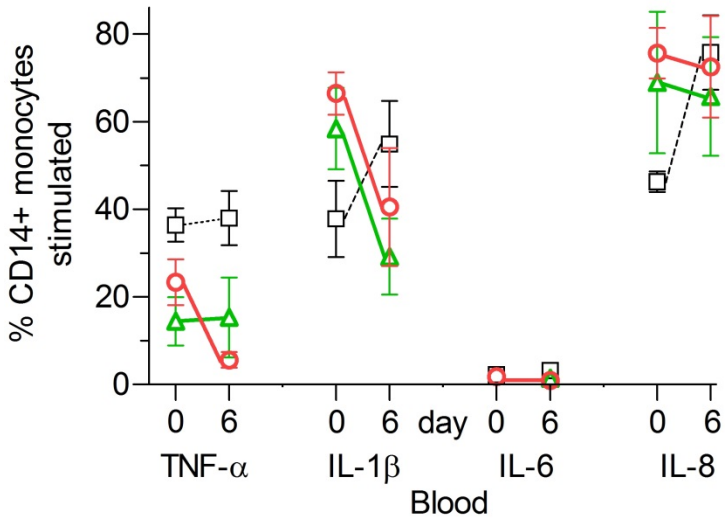
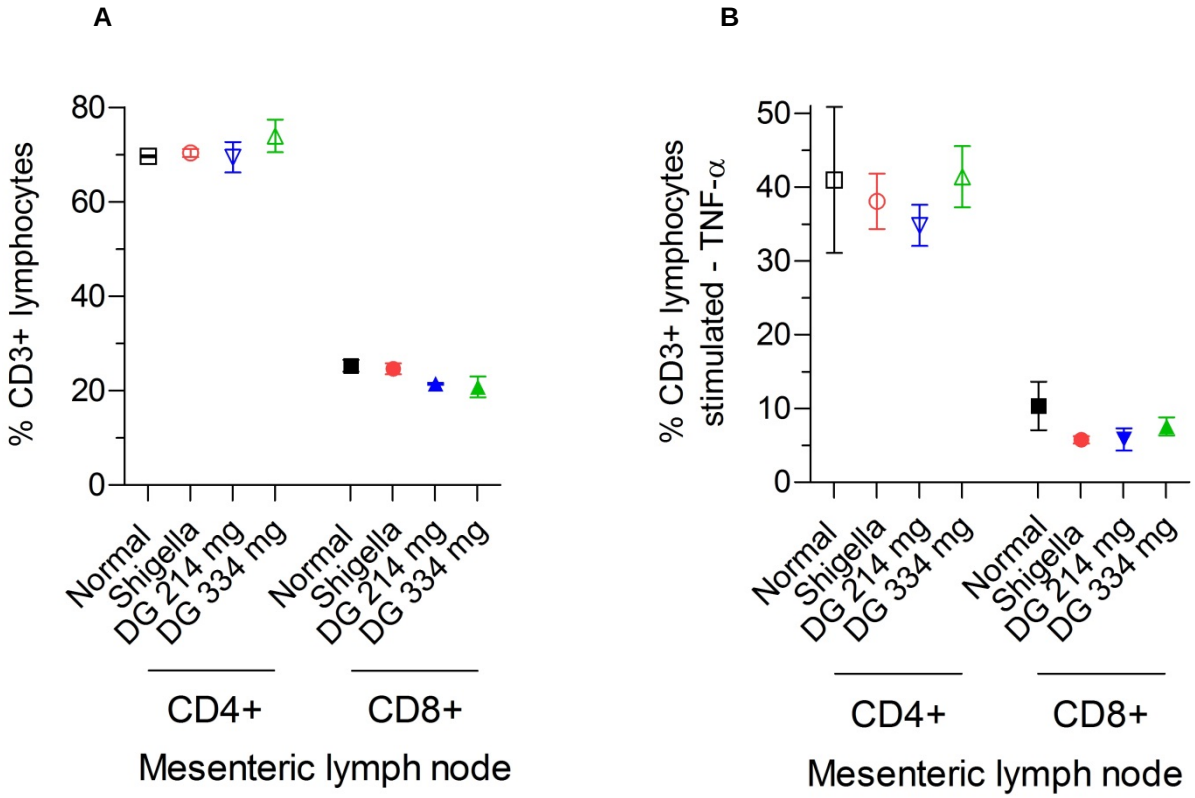

% CD14+ monocytes stimulated
80
60
40
20
0
0
6
0
6
day
0
6
0
6
TNF-α
IL-1β
IL-6
IL-8
Blood

Figure 7


A
% CD3+ lymphocytes
80
60
40
20
0
Normal
Shigella
DG 214 mg
DG 334 mg
Normal
Shigella
DG 214 mg
DG 334 mg
CD4+
CD8+
Mesenteric lymph node
B
% CD3+ lymphocytes stimulated - TNF-α
50
40
30
20
10
0
Normal
Shigella
DG 214 mg
DG 334 mg
Normal
Shigella
DG 214 mg
DG 334 mg
CD4+
CD8+
Mesenteric lymph node