Dysregulated humoral immunity to nontyphoidal *Salmonella* in HIV-infected African adults

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Supporting Online Material

Materials and Methods

Study population and sera

Patient details are given in Table S1. Ethical approval was granted by the College of Medicine Research and Ethics Committee of the University of Malawi. No participant had a previous history of known *Salmonella* infection. Serum was separated within 2 hours and stored in aliquots at -80ºC. Antibody-deficient serum was from Malawian children lacking anti-*Salmonella* antibody and from antibody-deficient adults. Concentrated human normal immunoglobulin was obtained from Baxter.

Salmonella strains

Four invasive Malawian *S.* Typhimurium strains were used. A23753 and D23580 (which has been sequenced at the Wellcome Trust Sanger Institute, UK (S1, S2)), have standard sensitivity to serum killing as previously described (S3) and are representative of approximately 90% of current invasive Malawian *S.* Typhimurium strains investigated (S1, S3). A19520 and D19774, have increased sensitivity to serum killing and are representative of approximately 10% of invasive Malawian *S.* Typhimurium isolates. A23753 and A19520 were isolated from HIV-infected adults and D23580 and D19774 were from children. One invasive Malawian *S.* Enteritidis strain, D24954, was used that had been isolated from a bacteremic child.

Salmonella serum bactericidal assays

Briefly, 10 μl viable *Salmonellae* in log-growth phase was added to 90 μl undiluted serum (final *Salmonella* concentration 1 x 10^6 cfu/ml) and incubated at 37 C with number of viable *Salmonellae* determined after 45, 90 and 180 minutes. For inhibition assays, control HIV-uninfected serum was mixed with HIV-infected inhibitory serum/serum fraction/antibody preparation/phosphate buffered saline (PBS) at a final volume of 90 μl at a 50:50 ratio unless specified otherwise. Likewise, for assays to determine the specificity of anti-*Salmonella* bactericidal antibody, antibody-deficient serum was mixed with serum/antibody preparation/PBS at a 50:50 ratio.

Salmonella-specific IgG/IgM and C3/C5b-9 complement deposition by flow cytometry and functional complement assays

Briefly, 5 μl *Salmonellae* in log-growth phase was mixed with 45 μl 10% serum for antibody determination, or undiluted serum for complement deposition (final *Salmonella* concentration 2 x 10^8/ml). FITC-conjugated anti-IgG/IgM/C3 antibody or mouse anti-C5b-9 antibody followed by FITC-conjugated anti-mouse immunoglobulin antibody were used for detection prior to flow cytometric analysis on a FACSCalibur instrument (Becton Dickinson). The C5b-9 antibody recognizes a neo-epitope on the MAC which only forms when the MAC assembles. Hemolytic complement function was determined by radial immunodiffusion (Binding Site).

Confocal microscopy

Salmonellae incubated in serum and labeled with FITC-conjugated anti-IgG or C3 (above) were fixed with acetone on Superfrost Plus charged microscope sides (Leica), mounted in Prolonged-Gold DAPI (Invitrogen) and viewed under oil immersion 100x objective lens with an Axiovert 100M confocal microscope (Zeiss).

Immunophenotyping.

Lymphocyte cell subset enumeration was performed by standard four-color flow cytometry using a FACSCalibur instrument.

Total immunoglobulins

Total serum IgG, IgA and IgM were determined by turbidimetry on an Hitachi P800 Modular Analyzer using Tina-quant kits (Roche).

Size-fractionation of serum

This was performed using ultrafiltration spin columns (Vivascience) with 30, 100 and 300 kDa size-exclusion membranes.

Flagellin synthesis and purification

E. coli BL21 expressing his-tagged protein were lysed and the protein isolated by metal-affinity chromatography before immune-purification using an anti-flagellin monoclonal antibody (S4).

LPS

TLR-grade smooth LPS from *S*. Typhimurium, *S*. Enteritidis and *S*. Minnesota, and *Salmonella* lipid A and rough Rb, Rc, Rd and Re forms of *Salmonella* LPS were obtained from Alexis Biochemicals.

ELISA for anti-_Salmonella_ LPS, flagellin and outer membrane protein antibodies

S. Typhimurium LPS, flagellin or outer membrane proteins were coated onto plates at 5 μg/ml. Primary antibodies were added in step-wise dilutions. Alkaline-phosphatase-linked secondary antibodies (Southern Biotech) were added and enzyme activity detected using SigmaFast tablets (S4).

Fluorescent-bead-based immunoassay for anti-*Salmonella* LPS antibodies
S. Typhimurium LPS was conjugated to fluorescent micro-beads (Bio-Rad) via a poly-L-lysine linker. Beads were incubated with serum at a 1:100 dilution and PE-conjugated anti-human IgG (Southern Biotech) used as the secondary antibody. Antibody-binding to beads was analyzed using a Luminex flow-cytometric instrument (Bio-Rad).

Purification of total IgG from serum
This was performed as previously described (S3) using Streptococcal Protein G HiTrap Columns (GE Healthcare Bio-Sciences).

Generation of mutants of *S*. Typhimurium D23580
The *flgBCD* genes of *S*. Typhimurium D23580 were disrupted using the red recombinase method (S5) to generate mutants that did not elaborate flagella. Construction of an *ompR* mutation in *S*. Typhimurium has been described previously (S6). The *ΔompR*::kan^r was transferred to *S*. Typhimurium strain D23580 by generalized transduction using the P22 bacteriophage. The rough *galE* mutant of D23580 lacking long-chain LPS was previously described (S3).

Preparation of *S*. Typhimurium outer membrane proteins
Briefly, bacterial cells were harvested by centrifugation and washed and resuspended in 10 mM Tris-HCl (pH 7.4) containing protease inhibitors. The cells were lysed using a French pressure cell at 20,000 pounds per square inch, and cell envelopes were harvested by centrifugation. The outer membrane fraction was extracted with 2% (v/v) Triton X-100 and harvested by centrifugation before undergoing extensive washing in 10 mM Tris-HCl (pH 7.4) to remove the detergent (S7).

Preabsorption of serum antibodies
This was performed by preincubating serum with 1, 10, 100 or 200 μg/ml final concentration of *Salmonella* LPS/LPS component, flagellin or outer membrane proteins for 30 minutes at room temperature immediately prior to conducting serum bactericidal assays.

Affinity chromatography purification of anti-*S*. Typhimurium LPS and outer membrane protein antibodies
S. Typhimurium LPS and outer membrane proteins was coupled to 1 ml N-hydroxysuccinimide-activated sepharose columns (NHS-HiTrap, GE Healthcare) at 4^{o}C overnight. The column was extensively washed using an Äkta Purifier (GE Healthcare) and 10 ml of patient serum passed over the column. Bound antibody was eluted with a pH gradient from 7.4 to 1.8 using a citric acid buffer. The eluting protein was directly buffer exchanged using an in-line column (HiPrep 26/10, GE Healthcare) pre-equilibrated with PBS. Protein containing fractions were pooled and concentrated by ultrafiltration.

Preparation of *S*. Typhimurium porins and mouse immunizations

OMP F, C and D porins from *S*. Typhimurium were generated by repeated detergent extraction and characterized as previously described (S6). Animal experiments were performed with appropriate Home Office and local approval. Mice (C57BL/6J) were immunized with 20 μg *S*. Typhimurium porins in PBS and boosted with 20 μg porins 14 days later with sera isolated after a further 7 days.

Serum LPS concentrations

Serum was diluted 1:5 with endotoxin-free water and heat-inactivated for 5 minutes at 70 C to eliminate endogenous protease activity. LPS titer was then measured by Limulus Amebocyte Lysate assay (Lonza) according to the manufacturer's instructions.

Statistical methods

The Spearman approach was used for estimation of correlation. Comparisons of data from different groups of blood or sera were performed using the Mann-Whitney U test. Comparisons of data from experiments using different manipulations of *Salmonella* killing conditions were made using Student's t test.

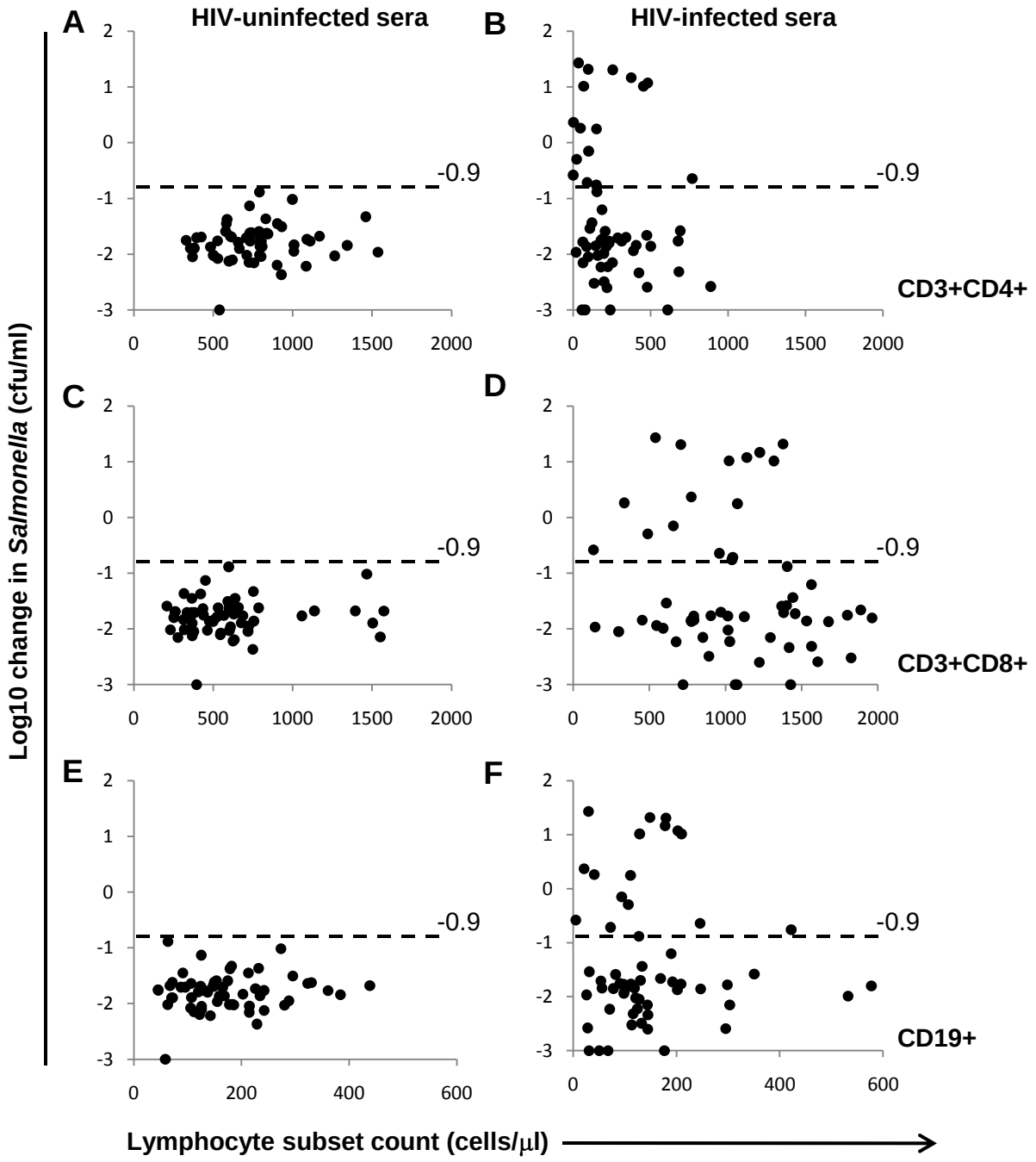

Fig. S1 Killing of NTS by sera from HIV-infected and HIV-uninfected African adults compared with peripheral lymphocyte subset counts. Killing of *S.* Typhimurium A23753 at 180 minutes by HIV-uninfected sera (**A**, **C** and **E**) and HIV-infected sera (**B**, **D** and **F**) compared with (**A** and **B**) CD4+ T lymphocyte count (CD3+CD4+ lymphocytes, cells/μl), (**C** and **D**) CD8+ lymphocyte cell count (CD3+CD8+ lymphocytes, cells/μl), and (**E** and **F**) B lymphocyte count (CD19+ lymphocytes, cells/μl). Horizontal dashed line indicates threshold for impaired killing of *S.* Typhimurium A23753 (-0.9 log10 change in *Salmonellae* cfu/ml). Mann-Whitney U test for cell counts in HIV-infected blood with impaired serum killing compared with HIV-infected blood with normal killing: for CD4+ T cells P=0.05, difference in medians 96 cells/μl, 95%CI -1.1 to 199; for CD8+ T cells P=0.09, difference in medians 270 cells/μl, 95%CI -31 to 579; for B cells P=0.88, difference in medians 3 cells/ml, 95%CI -59 to 64.

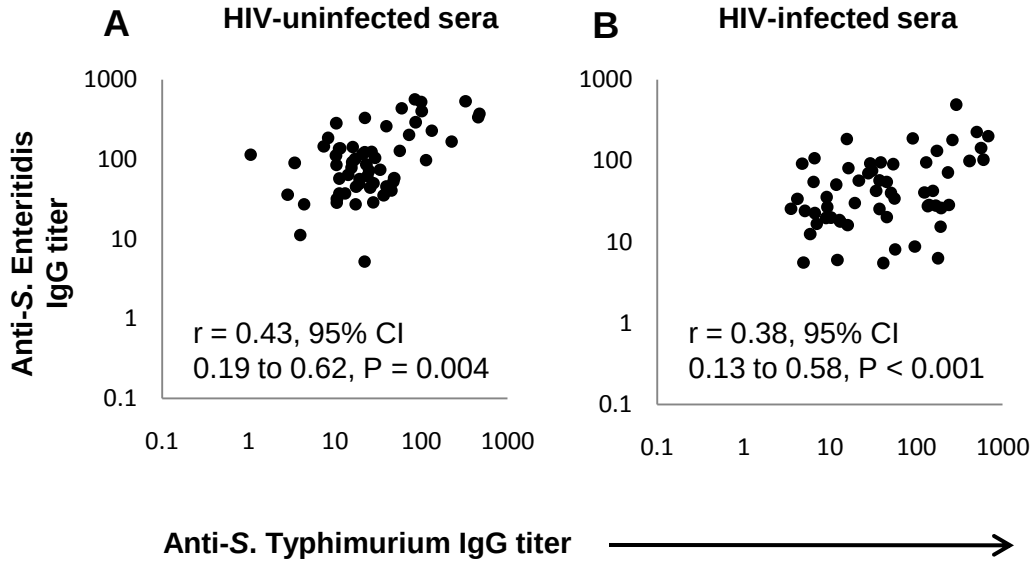

Fig. S2 Titers of anti-*S*. Typhimurium A23753 IgG compared with anti-*S*. Enteritidis D24954 IgG in (**A**) HIV-uninfected (n=58) and (**B**) HIV-infected (n=58) sera. Each point represents data for serum from one individual. r values are Spearman correlation coefficients.

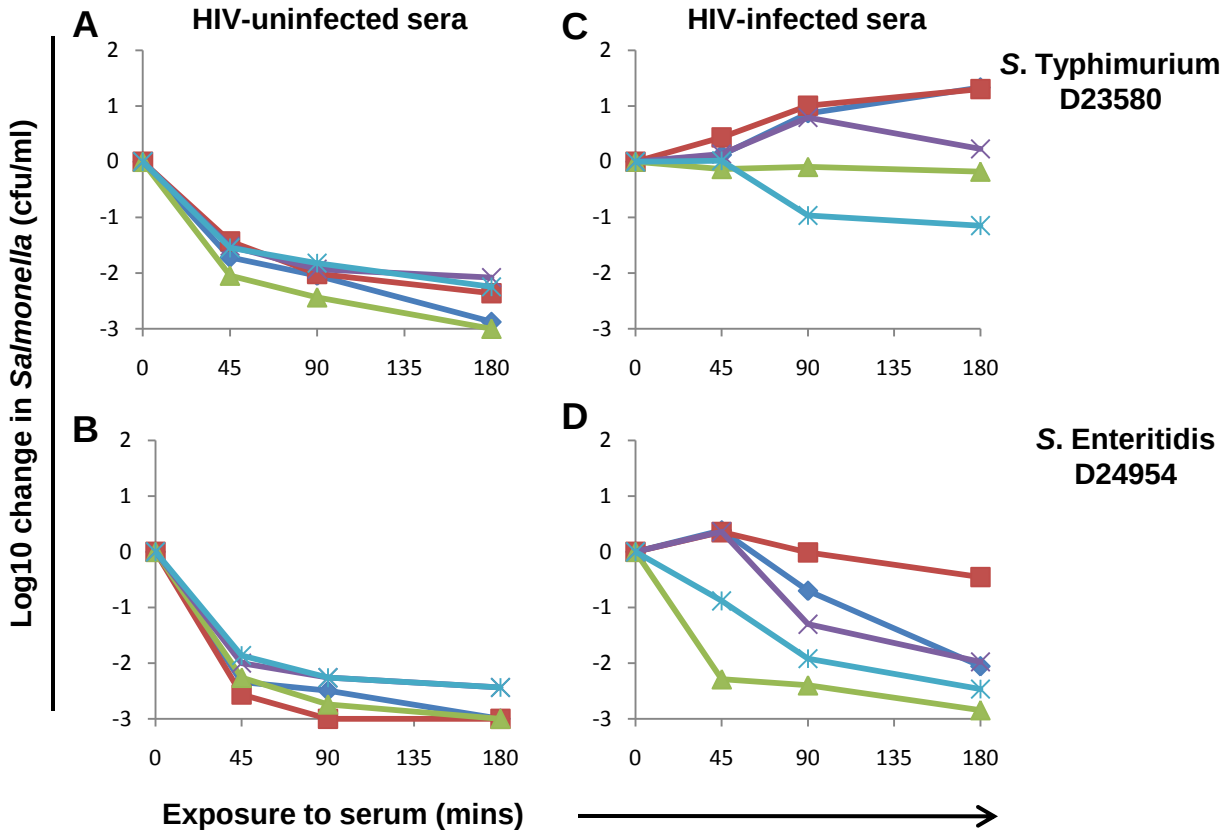

Fig. S3 Killing of *Salmonella enterica* serovars Typhimurium and Enteritidis by HIV-infected and HIV-uninfected sera. Killing of (**A** and **C**) *S*. Typhimurium isolate D23580, and (**B** and **D**) *S*. Enteritidis isolate D24954 by matched sera at 45, 90 and 180 minutes. Negative values correspond with a decrease in viable *Salmonella*e compared with the initial concentration. (**A** and **B**) sera from HIV-uninfected subjects (n=5). (**C** and **D**) sera from HIV-infected subjects (n=5). Anti-*S*. Enteritidis IgG titers correlate with impairment of killing of *S*. Enteritidis for the five HIV-infected sera shown in (**D**) (r=0.90, 95% CI 0.09 to 0.99, P=0.04). Each line represents data for serum from one individual.

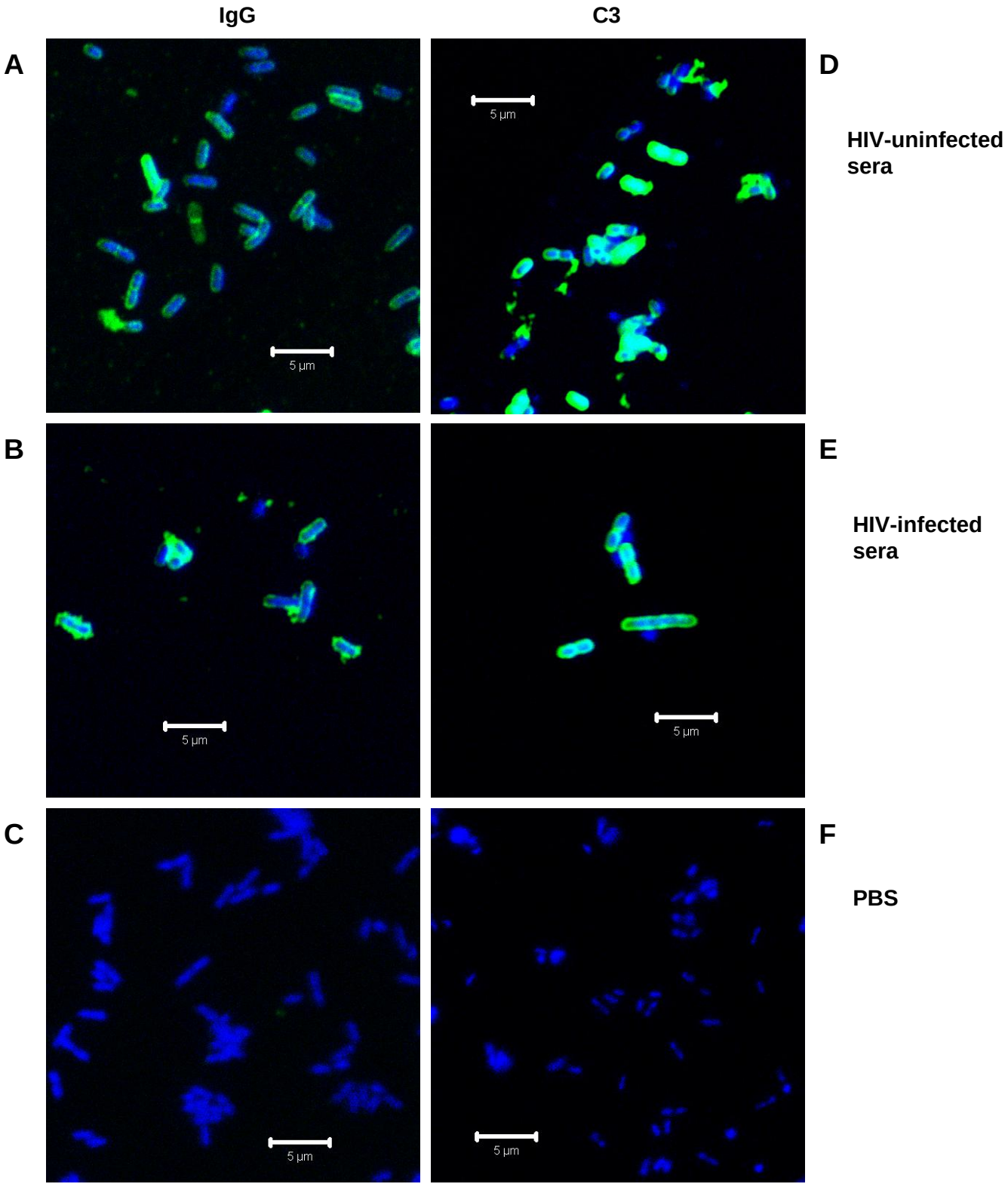

Fig. S4 (**A**, **B** and **C**) Binding of IgG and (**D**, **E** and **F**) deposition of C3 on *S*. Typhimurium D23580 by confocal microscopy. *Salmonellae* were incubated in (**A** and **D**) HIV-uninfected and (**B** and **E**) HIV-infected sera and (**C** and **F**) PBS for 20 minutes. *Salmonellae* appear blue (DAPI) and IgG and C3 are green (FITC-conjugated anti-human IgG and anti-human C3 antibodies).

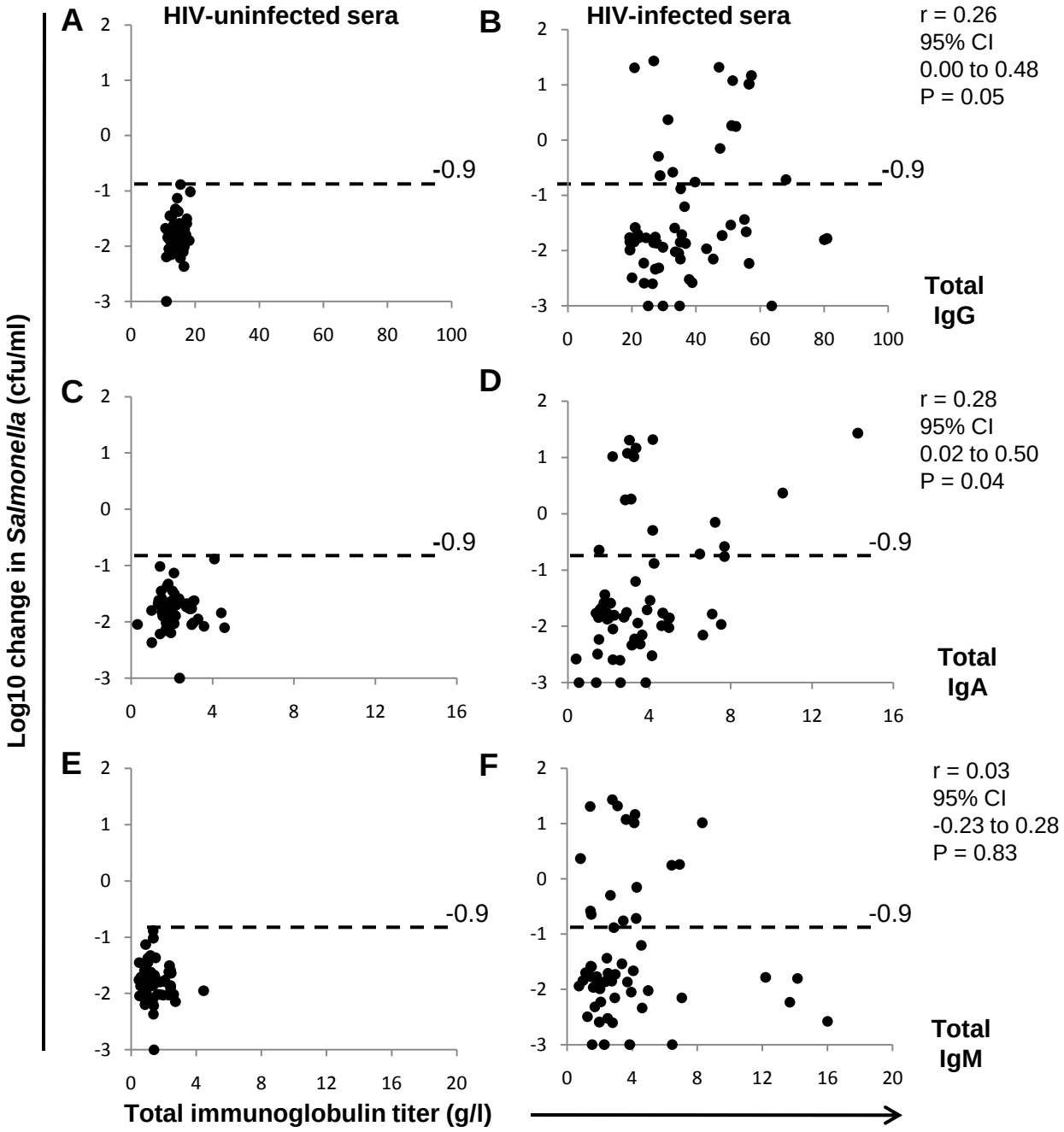

Fig. S5 Killing of NTS by HIV-infected and HIV-uninfected sera compared with total serum immunoglobulin concentrations. Killing of *S.* Typhimurium A23753 at 180 minutes by (**A, C** and **E**) HIV-uninfected sera and (**B**, **D** and **F**) HIV-infected sera compared with (**A** and **B**) total IgG, (**C** and **D**) total IgA and (**E** and **F**) total IgM concentrations (g/l). Horizontal dashed line indicates threshold for impaired killing of *Salmonella* A23753. r values are Spearman correlation coefficients.

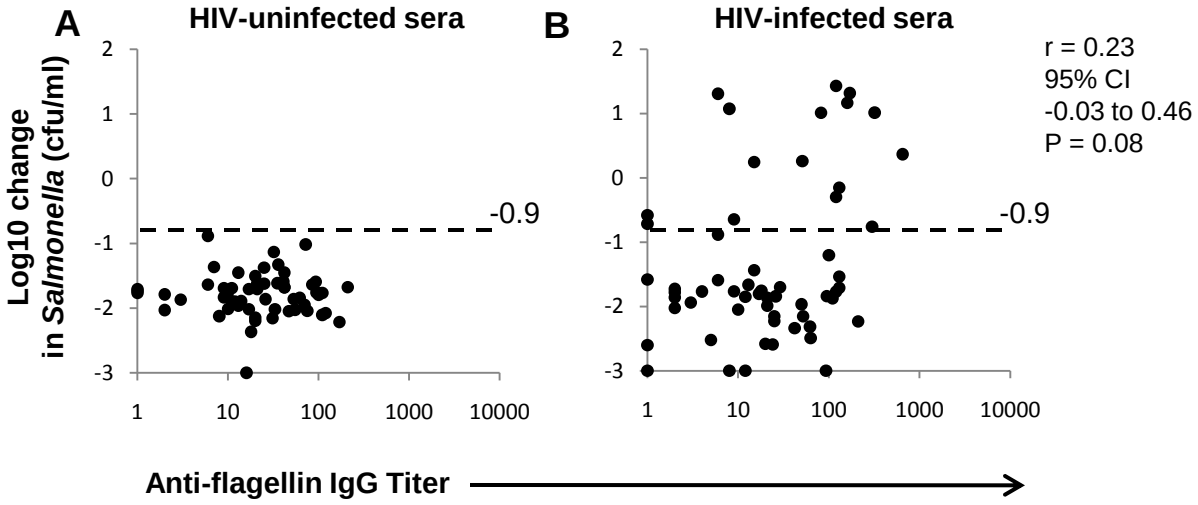

Fig. S6 Killing of NTS by HIV-infected and HIV-uninfected sera compared with serum *Salmonella* flagellin-specific IgG titer. Killing of *S*. Typhimurium A23753 at 180 minutes by HIV-uninfected sera (**A**) and HIV-infected sera (**B**) compared with *S*. Typhimurium flagellin IgG titer determined by ELISA. Horizontal dashed line indicates threshold for impaired killing of *S*. Typhimurium A23753. r value is Spearman correlation coefficient. Median IgG titer was the same in HIV-infected and HIV-uninfected sera (Mann-Whitney P=0.96, difference in medians 0.0 units, 95% CI -9.0 to 10).

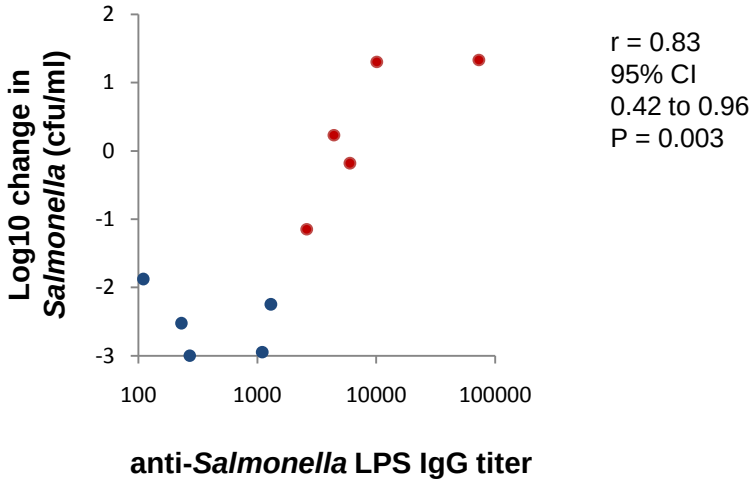

Fig. S7 Killing of NTS by HIV-infected and HIV-uninfected sera compared with anti-*Salmonella* LPS IgG titer measured by fluorescent-bead-based immunoassay. Killing of *S*. Typhimurium D23580 at 180 minutes by HIV-infected sera (red, n=5) and HIV-uninfected sera (blue, n=5) compared with anti-*S*. Typhimurium LPS IgG titer (arbitrary units). r value is Spearman correlation coefficient.

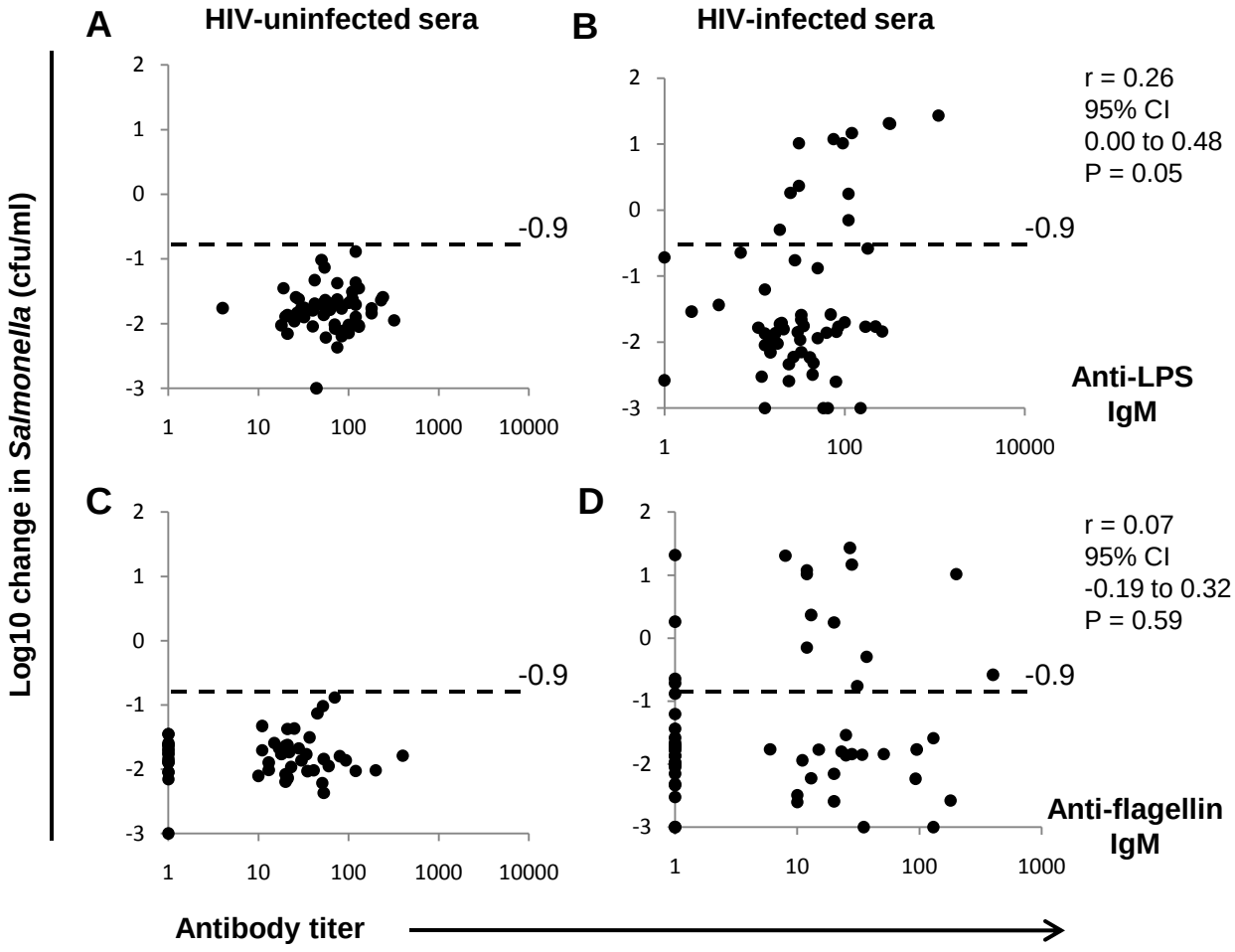

Fig. S8 Killing of NTS by sera from HIV-infected and HIV-uninfected sera compared with serum anti-*Salmonella* LPS and flagellin IgM titers. Killing of *S*. Typhimurium A23753 at 180 minutes by (**A** and **C**) HIV-uninfected sera and (**B** and **D**) HIV-infected sera compared with (**A** and **B**) anti-*S*. Typhimurium LPS IgM and (**C** and **D**) anti-*S*. Typhimurium flagellin IgM titers determined by ELISA. Horizontal dashed line indicates threshold for impaired killing of *S*. Typhimurium A23753. r values are Spearman correlation coefficients. Median IgM titers to LPS (Mann-Whitney P=0.005, difference in medians 21 units, 95% CI 7.0 to 37) and flagellin (Mann-Whitney P=0.09, difference in medians 3.0 units, 95% CI 0.0 to 13) were higher or not significantly different in HIV-uninfected sera compared with HIV-infected sera.

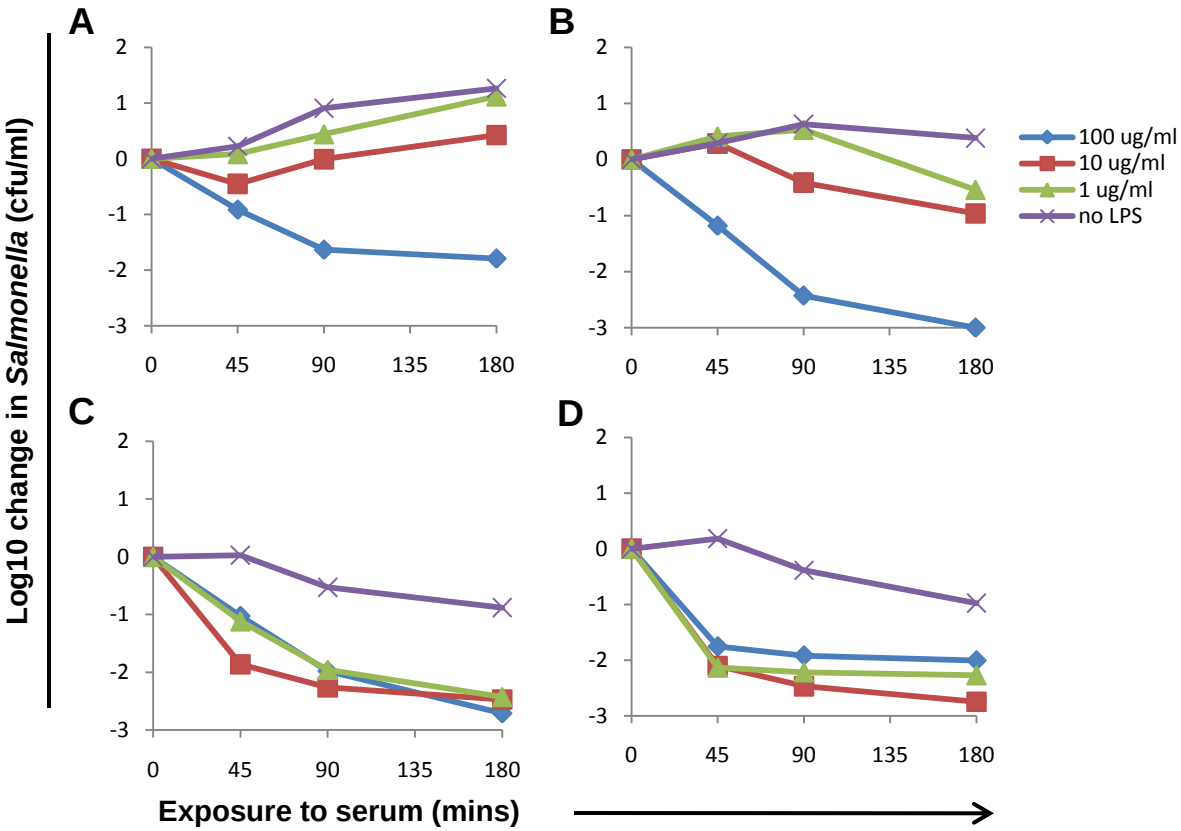

Fig. S9 Effect of antibody preabsorption with LPS on killing of NTS by HIV-infected sera. Effect of preabsorbing four HIV-infected sera (**A** – **D**) with 1, 10 and100 µg/ml *S.* Typhimurium LPS on serum killing of *S.* Typhimurium D23580. Each panel represents data using serum from one African adult.

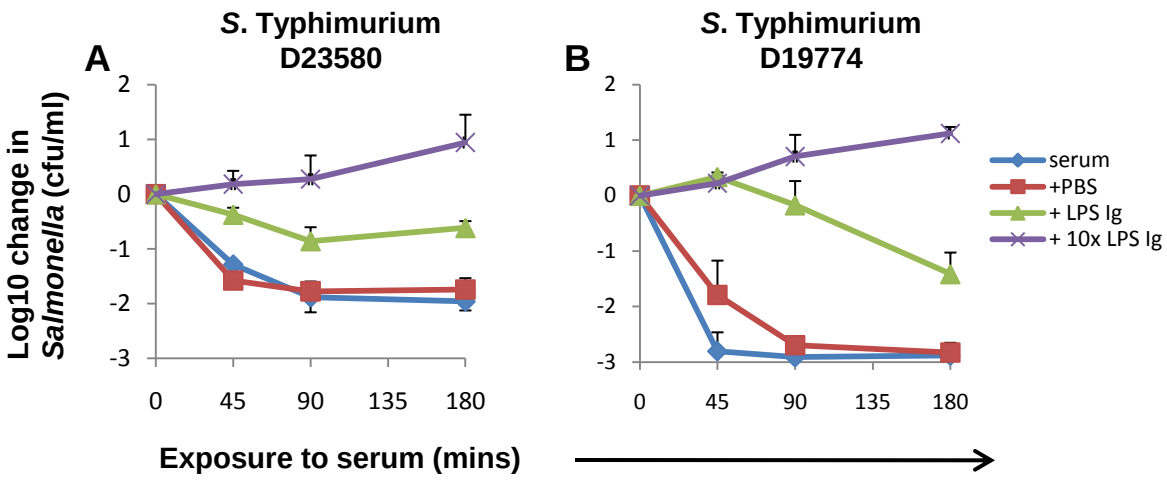

Fig. S10 Effect of addition of LPS-specific antibody extracted from HIV-uninfected serum on killing of NTS by autologous serum. Killing of (**A**) *S.* Typhimurium D23580 and (**B**) *S.* Typhimurium D19774 by 100% HIV-uninfected serum or 50% HIV-uninfected serum with 50% PBS or 50% anti-LPS antibody in PBS extracted from homologous serum at the same concentration as in source serum or 50% anti-LPS antibody in PBS extracted from homologous serum and concentrated to 10-fold the concentration in source serum. Data represent means +/- SD of 3 experiments.

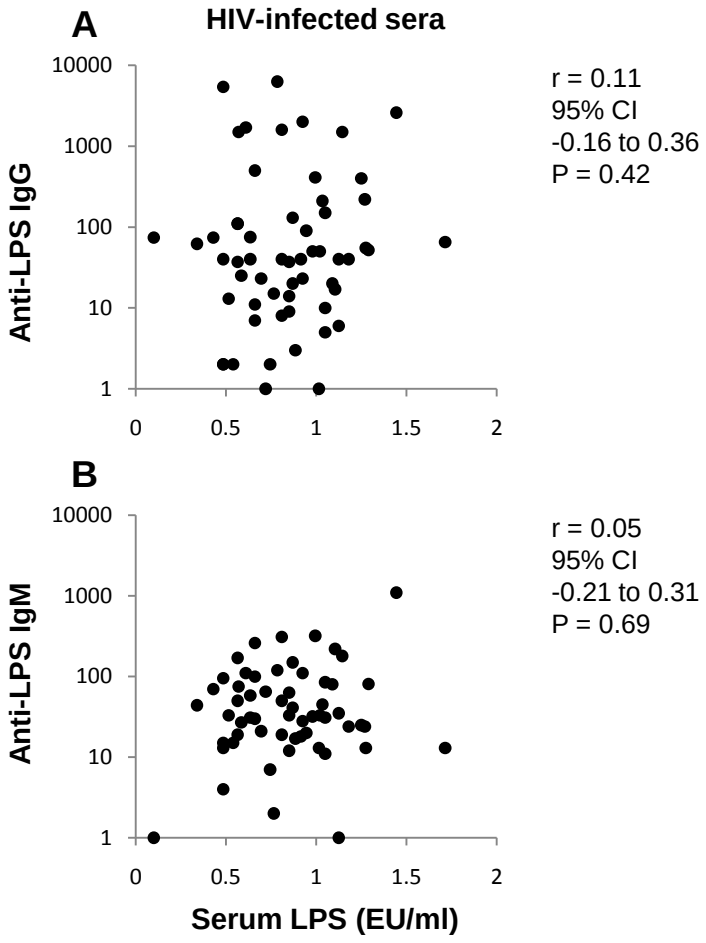

Fig. S11 LPS concentration compared with anti-*Salmonella* LPS antibody titers in HIV-infected sera. LPS concentration determined by Limulus Amebocyte Lysate assay compared with serum anti-*S.* Typhimurium LPS (**A**) IgG and (**B**) IgM titer in HIV-infected sera. r values are Spearman correlation coefficients. EU/ml = endotoxin units/ml.

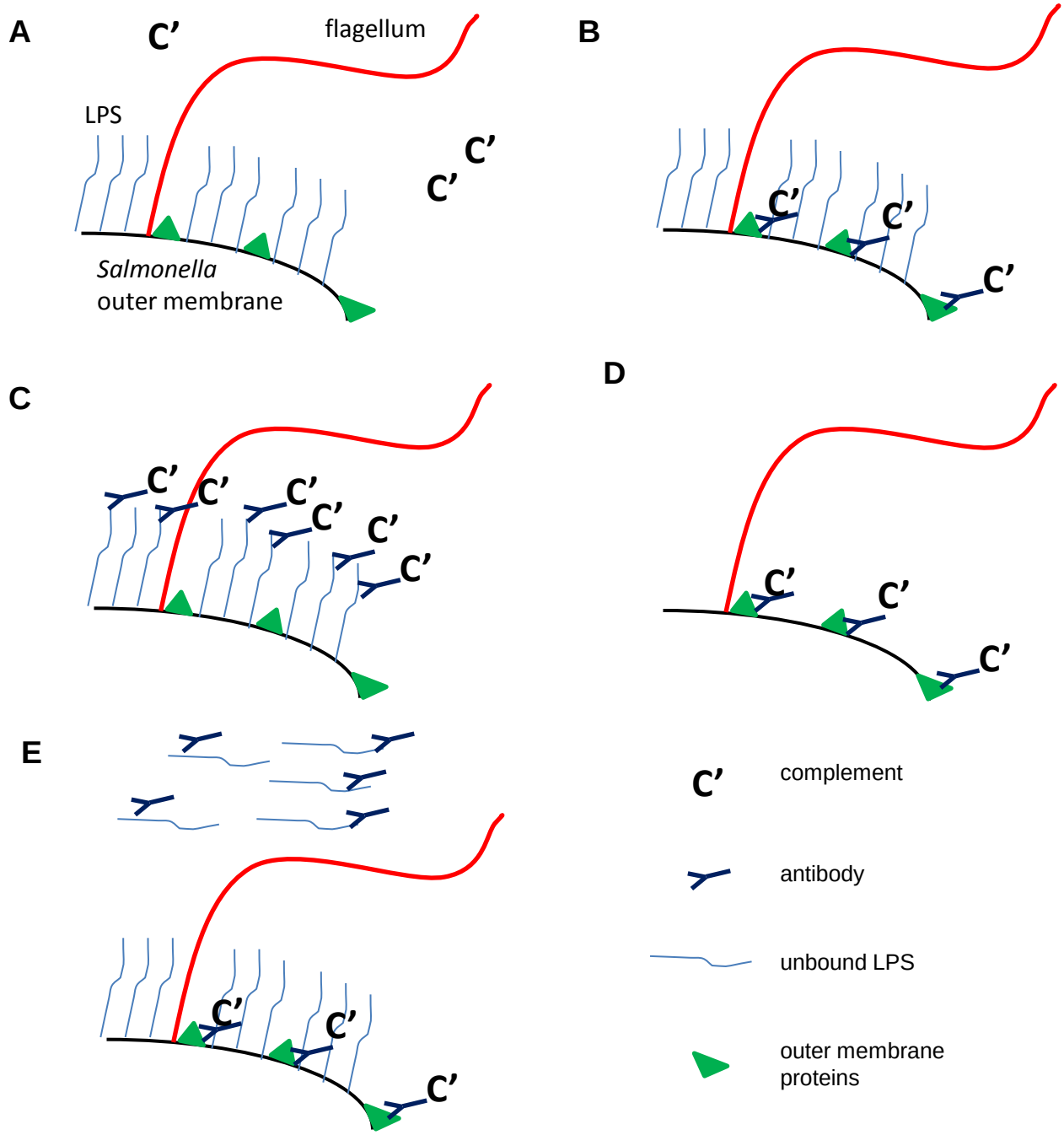

Fig. S12 African serum killing of NTS by anti-outer membrane protein antibodies and inhibition of killing by anti-LPS antibodies. (**A**) In young African children who lack anti-*Salmonella* antibodies (15) and in sera from African adults that has been preabsorbed with *Salmonella* outer membrane proteins, complement-mediated killing of NTS does not occur. (**B**) Acquisition of bactericidal antibody in the first two years of life (15) and supplementation of antibody-deficient serum with anti-outer membrane protein antibodies enables complement-mediated killing of *Salmonella*. We propose that this occurs through targeting complement deposition to the *Salmonella* outer membrane. (**C**) Anti-LPS antibodies present at high titers in the serum of some HIV-infected African adults or added to HIV-uninfected serum result in impaired *Salmonella* killing. We propose that this occurs through diversion of complement deposition away from the *Salmonella* surface or impeded access of antibody and complement to the outer membrane. (**D**) HIV-infected sera and antibody-deficient sera are able to kill *Salmonellae* that lack intact LPS. (**E**) Preabsorption of anti-LPS antibodies enables HIV-infected sera to kill *Salmonella*.

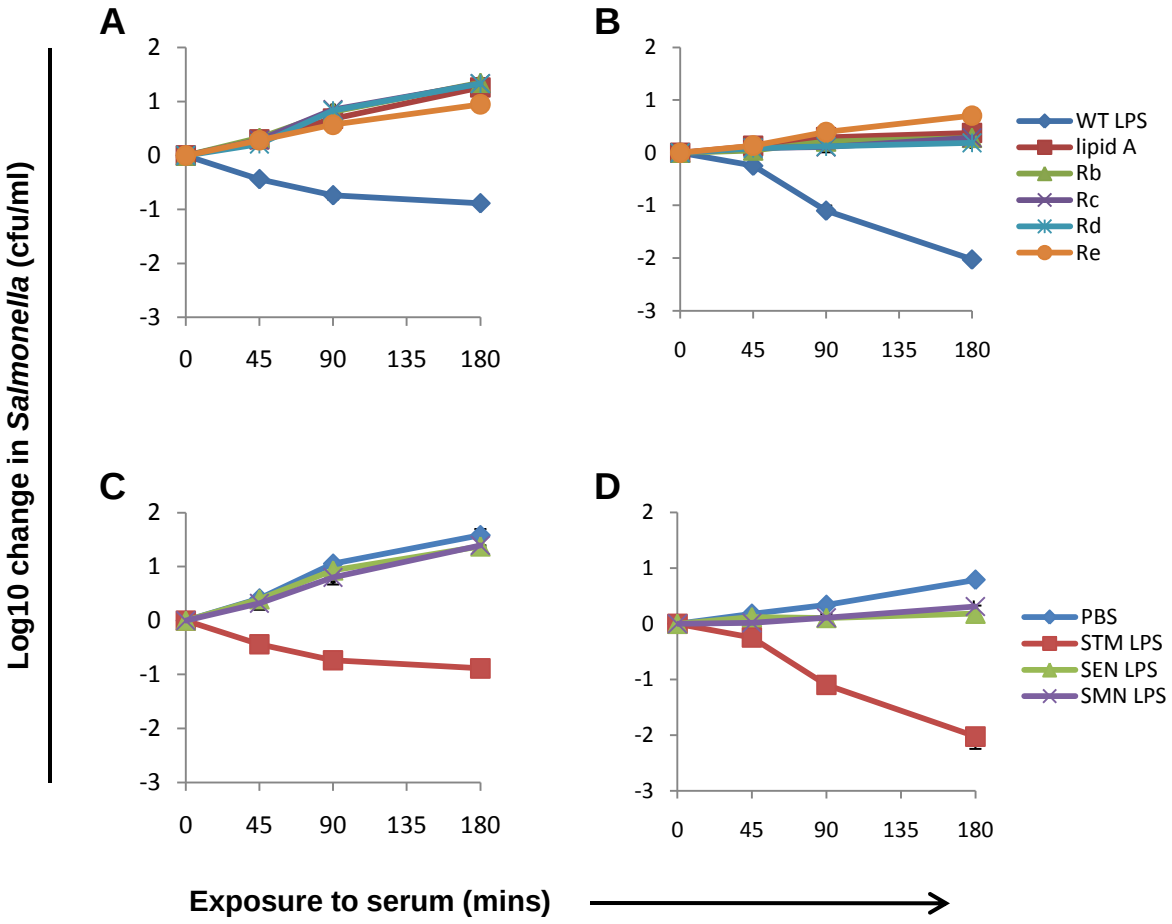

Fig. S13 Effect of preabsorbing HIV-infected sera with different components of *Salmonella* LPS and LPS from different *Salmonella* serovars on killing of *S*. Typhimurium D23580. (**A** and **B**) Effect of preabsorbing two inhibitory HIV-infected sera with *Salmonella* lipid A and LPS from wild type and five rough *Salmonella* mutants with truncations in the core oligosaccharide regions, Rb, Rc, Rd and Re on killing of D23580. (**C** and **D**) Effect of preabsorbing two inhibitory HIV-infected sera with LPS from *Salmonella* serovars Typhimurium (STM), Enteritidis (SEN) and Minnesota (SMN) on killing of D23580. Data represent means +/- SD of 3 experiments.

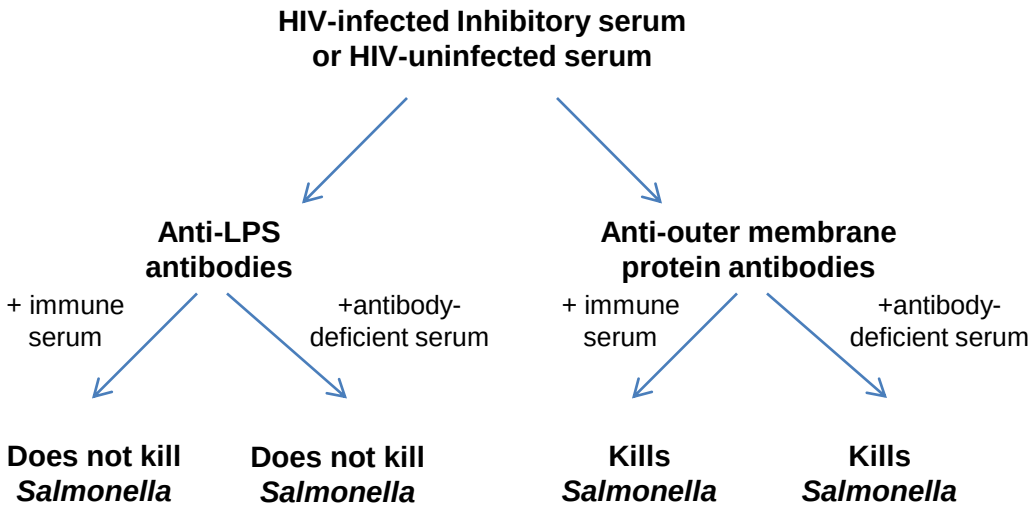

Fig. S14 Our experiments with affinity-purified LPS antibodies and outer membrane protein antibodies from African sera demonstrate presence of bactericidal and inhibitory anti-*S.* Typhimurium antibodies in African HIV-infected and HIV-uninfected sera.

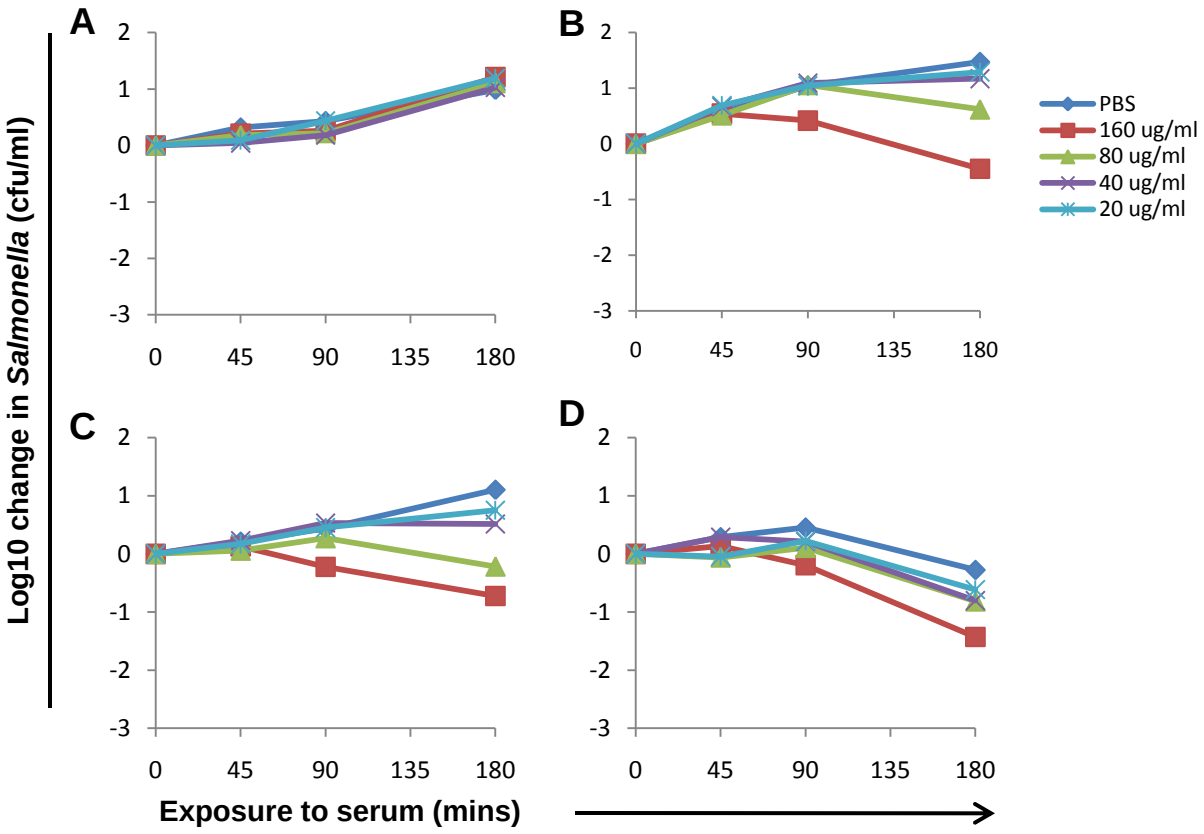

Fig. S15 Effect of normal human immunoglobulin on inhibition of killing of *Salmonella* by HIV-infected sera. Pooled normal immunoglobulin from HIV-uninfected donors at 160, 80, 40 and 20 mg/ml was mixed in equal volumes with serum from four HIV-infected Africans (**A** to **D**) and ability to kill *S.* Typhimurium D23580 investigated. Concentration of anti-LPS IgG in HIV-infected serum in panel **A** was over 10-fold greater than that in sera used in the three other panels. HIV-infected sera used for experiments in each panel correspond with those used for experiments represented in corresponding panels in Fig. S8.

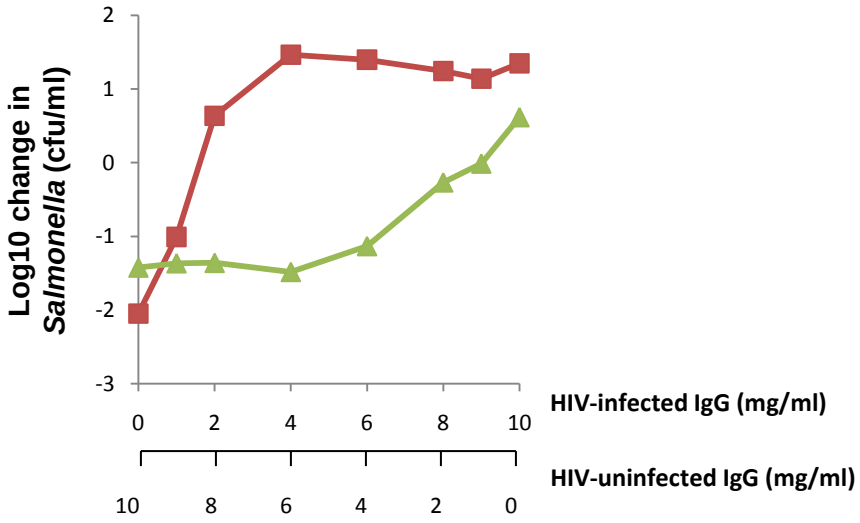

Fig. S16 Killing of NTS by antibody-deficient serum supplemented with total IgG purified from HIV-infected and HIV-uninfected serum. Killing of *S*. Typhimurium D23580 at 180 minutes. Supplemental IgG at 10 mg/ml consisting of different proportions of IgG from two HIV-infected African adults (one per line) and one HIV-uninfected African adult.

	HIV-uninfected	HIV-infected		
		Impaired NTS Killing	Normal NTS Killing	All
Number	58	16	42	58
Age (median and range)	27 (17-53)	34 (27-58)	33 (17-60)	33 (17-60)
Gender (male:female)	36:22	10:6	15:27	25:33
CD4 count cells/μl (median and range)	772 (329-1536)	130 (1-817)	237 (25-930)	221 (1-930)
Number with CD4 count <200 cells/ml	0	11 (69%)	13 (22%)	24 (41%)
Total hemolytic complement activity CH100 U/ml (median and range)	1012 (737-1801)	929 (552-1844)	1147 (682-2178)	1147 (552-2178)
Alternative pathway hemolytic complement activity % (median and range)	63 (30-121)	87 (48-166)	93 (40-257)	93 (40-257)

Table S1 Subject details. HIV-infected subjects were consecutive consenting patients at the antiretroviral clinic at QECH who fulfilled the criteria for taking part in the study. Most had been referred from local Voluntary Counseling and Testing Centers for assessment. Some had been diagnosed with HIV infection while in-patients at QECH, but all had recovered from their acute illness by the time of recruitment. HIV-uninfected subjects were hospital staff and healthy relatives of patients attending the hospital. Exclusion criteria were fever (≥38ºC), malaria parasitemia, pregnancy, acute illness and current medication including antiretroviral therapy and cotrimoxazole (except one patient on isoniazid and ethambutol continuation phase tuberculosis treatment and one patient on fluconazole continuation phase cryptococcal treatment). No subject had a history of *Salmonella* disease. Total hemolytic complement function was slightly lower in HIV-infected serum with reduced *Salmonella* killing compared to HIV-infected sera with normal *Salmonella* killing (Mann-Whitney P=0.04). Alternative pathway hemolytic complement function was the same in both groups (Mann-Whitney P=0.4).

Supplementary References

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