

# Systemic antibody responses to gut commensal bacteria during chronic HIV-1 infection

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Revised 1 March 2011

Accepted 2 March 2011

Published Online First

21 April 2011

## ABSTRACT

**Background** Human systemic antibody responses to commensal microbiota are not well characterised during health and disease. Of particular interest is the analysis of their potential modulation caused by chronic HIV-1 infection which is associated with sustained enteropathy and systemic B cell disturbances reflected by impaired B cell responses and chronic B cell hyperactivity. The mechanisms underlying B cell hyperactivation and the specificities of the resulting hypergammaglobulinaemia are only poorly understood.

**Methods** By a technique referred to as live bacterial FACS (fluorescence-activated cell sorting), the present study investigated systemic antibody responses to several gut and skin commensal bacteria as well as *Candida albicans* in longitudinal plasma and serum samples from healthy donors, chronic HIV-1-infected individuals with or without diarrhoea and patients with inflammatory bowel disease (IBD).

**Results** The data show that systemic antibody responses to the commensal microbiota were abundantly present in humans and remained remarkably stable over years. Overall systemic antibody responses to gut commensal bacteria were not affected during chronic HIV-1 infection, with titres decreasing when normalised to elevated plasma immunoglobulin G (IgG) levels found in patients with HIV. In contrast, increases in the titres of high affinity antimicrobiota antibodies were detected in patients with IBD, demonstrating that conditions with known increased intestinal permeability and aberrant mutualism can induce changes in antibody titres observed in these assays.

**Conclusion** Neither HIV-associated enteropathy nor B cell dysfunction impact on the high-affinity systemic antibody responses to gut commensal bacteria. HIV-associated hypergammaglobulinaemia is therefore unlikely to be driven by induction of antimicrobiota antibodies.

## INTRODUCTION

The human intestinal tract is inhabited by commensal bacteria which outnumber the cells of the human body by a factor of 10. More than 500 different bacterial species are believed to reside in the human gut, with a majority not amenable to in vitro culture.<sup>1 2</sup> This microflora is known to play a pivotal role in human health and disease. Unique adaptations of the intestinal immune system are crucial to maintain homeostasis with the resident

## Significance of this study

### What is already known about this subject?

- Enteropathy, B cell dysfunction and hypergammaglobulinaemia are characteristics of chronic HIV-1 infection.
- How enteropathy and B cell dysfunction impact on antibody responses to gut commensal bacteria in HIV-1 infection is unknown.
- The mechanisms underlying hypergammaglobulinaemia during HIV-1 infection are not defined. Antigen-driven stimulation could not be excluded to date.

### What are the new findings?

- HIV enteropathy and B cell dysfunction do not impact on antibody responses to gut commensal bacteria even in late-stage HIV-1 infection as normal levels of the gut commensal-specific antibody response are maintained throughout the course of infection.
- Severe enteropathy in patients suffering from inflammatory bowel disease (IBD) is associated with increased systemic antibody response to certain gut commensal bacteria.
- In patients with HIV, gut commensal antigens may be excluded as contributing to hypergammaglobulinaemia in an antigen-dependent manner.

microbiota.<sup>3</sup> Disturbances of this delicate balance due to external or internal factors are known to result in enteropathy such as during inflammatory bowel disease (IBD). They are also reported to be induced by infectious agents such as HIV-1.<sup>4</sup>

In fact, HIV-1 infection is tightly linked to the gastrointestinal tract, which serves as a major site of viral replication.<sup>5 6</sup> Intestinal CD4 T cells are a preferential target of the virus and are massively depleted during early infection.<sup>7–9</sup> HIV enteropathy has long been described<sup>10</sup> and is associated with gastrointestinal tract inflammation,<sup>11</sup> malabsorption,<sup>10</sup> diarrhoea,<sup>12</sup> increased intestinal permeability<sup>13 14</sup> and translocation of microbial products.<sup>5 15 16</sup> The underlying mechanisms contributing to enteropathy may be direct viral effects<sup>4</sup> and/or an indirect consequence of local immune activation and inflammation.<sup>17 18</sup>

## Significance of this study

**How might it impact on clinical practice in the foreseeable future?**

- ▶ In contrast to laboratory mice, the normal human population displays considerable immunoglobulin G (IgG) priming specific for their intestinal microbiota, indicating an altered host–microbiota relationship in humans and in mice.
- ▶ Enteropathy associated with inflammatory bowel disease, but not enteropathy associated with HIV, results in elevated IgG titres specific for some members of the intestinal microbiota, indicating that elevated microbiota-specific IgG is a variable phenomenon depending on the nature of the enteropathy and immune status of the individual.
- ▶ Patients with HIV maintain normal antibody responses to persistent antigens and intestinal bacteria despite drastic loss of CD4 T cells and B cell hyperactivation which may underlie the limited prevalence of microbiota-derived sepsis in patients with AIDS.
- ▶ The presence of comparable titres of systemic antibodies specific for intestinal bacteria in healthy individuals and HIV-infected patients does not support the hypothesis that gut microbial antigens drive HIV-associated hypergammaglobulinaemia and it thus remains a further challenge to identify its causality.

Commensal bacteria-specific antibodies play a critical role in maintaining intestinal balance and fighting systemic dissemination of bacteria.<sup>19</sup> We show here that specific antibody responses to commensal gut bacteria are abundantly available in humans on a systemic level.

B cell hyperactivity resulting in hypergammaglobulinaemia and B cell dysfunction, reflected by poor responsiveness to vaccination, are hallmarks of HIV-1 pathogenesis.<sup>20–24</sup> The exact mechanisms underlying these B cell disturbances, however, remain to be elucidated. These B cell disturbances have also been explicitly characterised in the HIV-infected gut by reports of intestinal B cell hyperactivity, early destruction of gastrointestinal germinal centres and specific blocking of the intrafollicular but not extrafollicular immunoglobulin (Ig) switch enzyme activation-induced deaminase.<sup>25–27</sup>

In this context of B cell dysfunction and hypergammaglobulinaemia we investigated whether HIV enteropathy, and therefore increased microbial exposure, produced measurable increases in the titres of high and low affinity antimicrobial antibody responses to commensal bacteria. Using a flow cytometric approach referred to as ‘live bacterial FACS’ (fluorescence-activated cell sorting)<sup>28</sup> as well as ELISA we studied longitudinal, systemic antibody responses to commensal gut bacteria in chronic HIV-infected individuals and patients with IBD. In contrast to patients with IBD who exhibited increased systemic antibody responses against several gut commensals, antibody responses to commensal gut bacteria were not altered during chronic HIV-1 infection and were also very well maintained in late stages of the disease at levels comparable with healthy controls.

**MATERIALS AND METHODS****Study individuals****Antiretroviral therapy (ART)-naive HIV study group (n=79)**

All HIV-1-infected patients were enrolled in the Swiss HIV Cohort Study (SHCS; <http://www.shcs.ch>). Plasma and serum

samples were obtained from the SHCS sample repository. Specifically, in this long-term (started in 1988) Swiss multicentre study, among other symptoms, diarrhoea and AIDS-defining enteric pathogens are systematically recorded together with clinical, general, virological and immunological laboratory parameters.<sup>29</sup> Study group characteristics are listed in table 1.

**IBD study group (n=29)**

Plasma samples were obtained from the University Hospital Bern. Patients were subgrouped into mild/active (n=12) and active (n=7) Crohn’s disease (CD) as well as mild (n=7) and active/hard (n=3) ulcerative colitis (UC). Study group characteristics are listed in table 2.

**Healthy donors (n=29+19)**

As it has been described that IgG antibody titres to antigens derived from certain gut-related bacteria increase with age,<sup>30</sup> we obtained plasma and serum samples for both disease cohorts from age-matched cohorts of healthy individuals (n=29 for the HIV control cohort and n=19 for the IBD control cohort).

Ethics committee approval and written informed consent from all study subjects were obtained according to the guidelines of the University Hospital Zurich and the University Hospital Bern.

**Bacteria and *Candida albicans***

*Escherichia coli*, *Klebsiella pneumoniae* and *Enterococcus faecalis* were primary isolates from stool samples of healthy individuals. *Staphylococcus epidermidis* and *Propionibacterium acnes* were primary human isolates from clinical specimens. Isolation and typisation were done by the Institute of Medical Microbiology, University of Zurich. *Bacteroides fragilis*, *Bacteroides thetaiotaomicron* and *E coli* Nissle (Mutaflor) were from the American Type Culture Collection. *B fragilis* and *B thetaiotaomicron* were grown anaerobically. *Legionella pneumophila* strain JR32 is also known as WT Philadelphia-1. *Bradyrhizobium japonicum* was a gift from Dr Hauke Hennecke, Institute of Microbiology, ETH Zurich. *C albicans* strain SC5314 is a well characterised clinical isolate.<sup>31</sup>

**Live bacterial FACS**

Live bacterial FACS was performed similarly to as previously described.<sup>28</sup> Details are described in supplementary figure 1 and the supplementary materials and methods.

**ELISA**

Details are described in supplementary figure 1 and the supplementary materials and methods.

**Total Ig levels**

Total IgA, IgG and IgM levels in plasma or serum samples were quantified by in-house ELISA. The following antibodies were used: goat antihuman IgA, IgG and IgM as unlabelled and horseradish peroxidase (HRP) conjugates (all SouthernBiotech, Allschwil, Switzerland). Purified human IgA, IgG and IgM (all Sigma, Buchs, Switzerland) were used for the standard.

**Soluble CD14 (sCD14), lipopolysaccharide (LPS) and endotoxin core IgM antibodies (EndoCabIgM)**

Commercially available ELISA kits were used to quantify sCD14 (R&D Systems, Schonenbuch, Switzerland) and EndoCabIgM (Hycultbiotech, Allschwil, Switzerland) according to the manufacturer’s instructions.

Plasma LPS was quantified with the commercially available LAL Endochrome kit (CharlesRiver, Allschwil, Switzerland) according to the manufacturer’s instructions for low range detection as previously described.<sup>32</sup>

## Intestinal microbiota

**Table 1** Study cohort characteristics (healthy donors and ART-naive HIV-infected individuals)

Study group	All HIV+	HIV+ (no diarrhoea)	HIV+ (with diarrhoea)	Healthy controls
n	72	33	39	29
Study period in days, median (range)	342 (55–3652)	2295 (468–3652)	198 (55–491)	216 (187–3287)
No. of samples per patient, median (range)	2 (2–6)	6 (3–6)	2 (2–3)	2 (2–6)
Sex (male/female)	56/16	23/10	33/6	17/12
Age at sampling, median (range)	35.8 (25.2–76)	38.3 (26.4–52.5)	33.2 (25.2–76)	39 (28–56)
CD4 count, median (range)	217 (1.5–760)	208 (110–350)	315 (1.5–760)	NA
pVL,* median (range)	5×10 <sup>4</sup> (61–5.2×10 <sup>5</sup> )	6×10 <sup>4</sup> (61–5.3×10 <sup>5</sup> )	1.9×10 <sup>4</sup> (1.2e3–1.5×10 <sup>5</sup> )	NA
CDC stage (first sample)				
A	60%	77%	49%	NA
B	32%	21%	41%	NA
C	8%	6%	10%	NA
CDC stage (last sample)				
A	32%	27%	36%	NA
B	40%	42%	38%	NA
C	28%	31%	26%	NA
HIV subtype				
B	97%	97%	97%	NA
C	1.50%	None	3%	NA
Recombinant	1.50%	3%	None	NA
Risk group				
MSM	47%	28%	64%	NA
IDU	39%	48%	31%	NA
HET	11%	21%	2.50%	NA
Blood†	1.40%	None	2.50%	NA
Unknown	1.40%	3%	None	NA
ART	None	None	None	NA
SMS/TMP	36%	33%	39%	NA

\*RNA copies/ml.

†Blood transfusion.

ART, highly active antiretroviral therapy; CDC, Centers for Disease Control; HET, heterosexual contact; IDU, intravenous drug use; MSM, men having sex with men; NA, not applicable; SMS/TMP, sulfamethoxazole/trimethoprim.

**Cytomegalovirus (CMV), polio, tetanus toxoid and gp120 IgG**

CMV IgG and IgM, polio IgG and tetanus toxoid IgG were quantified by commercially available ELISA kits (Immunolab, Kassel, Germany) according to the manufacturer's instructions.

Binding titres (IC<sub>50</sub>) of gp120 IgG were quantified by in-house ELISA with recombinant gp120 JR-FL as previously described.<sup>33</sup>

**Statistical analysis**

A detailed description of the statistical analysis can be found in the supplementary materials and methods.

**RESULTS****Live bacterial FACS allows the study of antibody responses to commensal bacteria with minimal cross-reactivity**

Live bacterial FACS has recently been introduced as a novel flow cytometric approach to study antibody responses against bacteria.<sup>28</sup> The underlying principle, as illustrated in figure 1A, is

that live bacteria are incubated with diluted plasma or serum samples and, if the sample contains specific antibodies against the respective bacteria, they will bind to the bacterial surface. Binding of these antibodies to the bacteria is then detected by fluorescence-labelled antihuman Ig antibodies. Fluorescently stained bacteria are subsequently analysed by flow cytometry. The intensity of the fluorescent signal relates to the concentration of specific antibodies in the tested serum or plasma sample and is calculated in reference to a standard plasma included in every assay (supplementary figure 1A).

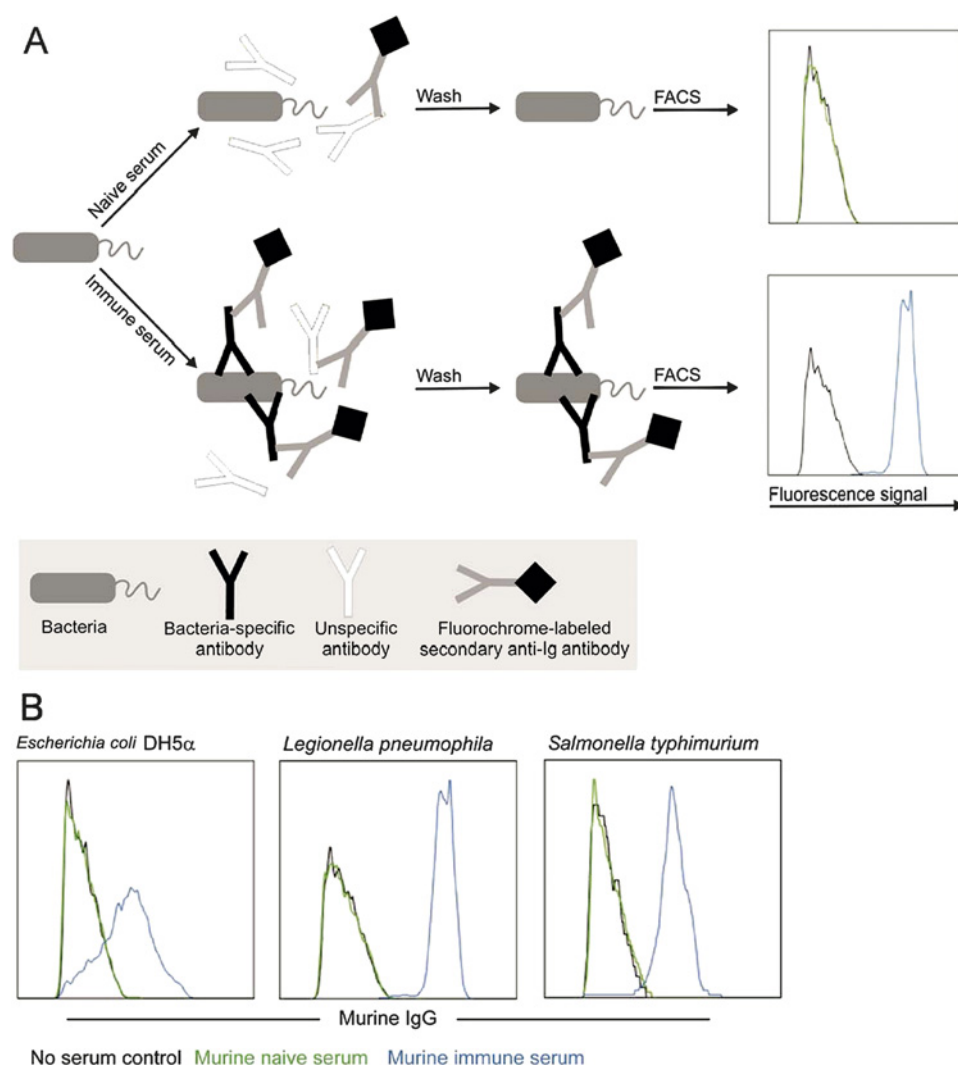
The use of live intact bacteria provides a high specificity and low cross-reactivity between different bacterial isolates as the membrane composition of bacteria is isolate specific, and highly cross-reactive antibodies against conserved intracellular bacterial proteins such as ribosomal proteins are excluded in this assay. The specificity of the technique, as previously reported,<sup>28</sup> was validated by staining of bacteria with mouse serum derived from

**Table 2** Study cohort characteristics (healthy donors and patients with IBD)

Study group	Crohn's disease	Ulcerative colitis	Healthy controls
Age at sampling, mean (range)	39 (20–61)	52 (34–70)	40 (26–58)
Sex (male/female)	11/7	8/2	8/16
CDAI score 0–600, mean (range)	114 (0–432)	NA	NA
Mayo score 0–6, mean (range)	NA	4 (0–6)	NA
Disease location	Ileal disease (n=5) Colonic disease (n=2) Small and large intestinal disease (n=11)	Colonic involvement (n=10)	NA
Complications (fissure, fistula, stenosis, stricture)	Uncomplicated (n=3) One or more complications (n=15)	Uncomplicated (n=10)	NA
Medication	Combinations of 5-ASA preparations, methotrexate, anti-TNF and/or corticosteroids	Combinations of 5-ASA preparations, methotrexate, mercaptopurines, ciclosporin, anti-TNF and/or corticosteroids	None

ASA, 5-aminosalicylic acid; CDAI, Crohn's disease activity index; IBD, inflammatory bowel disease; NA, not applicable; TNF, tumour necrosis factor.

**Figure 1** (A) Live bacterial FACS (fluorescence-activated cell sorting) (fluorescence-activated cell sorting). Illustration of the assay design. (B) Specificity of the live bacterial FACS assay. Bacteria were incubated with naive murine serum (green histograms; 1/60 dilution) or serum from a mouse which had been immunised with the respective bacteria (blue histograms, *E coli*, 1/60 dilution; *L pneumophila* and *S typhimurium*, 1/450 dilution). Control bacteria were not incubated with any serum (black histograms).



naive mice or from mice previously immunised with the respective bacteria. Antibody binding could only be detected against the vaccinating bacterial strain, with no detectable cross-reactivity towards other bacteria. Serum from naive mice did not bind detectably to any of the bacterial strains tested (figure 1B). It has been previously reported for human antibody responses that cross-reactivity between different bacterial strains such as bacteroides and enterobacteria is very low.<sup>34</sup> Further, to inhibit potential cross-reactivity due to the presence of natural antibodies which are specific for certain galactose-containing carbohydrate epitopes, galactose was routinely added to the assay as has been described before.<sup>35</sup>

#### Antibody responses to commensal bacteria are abundantly detectable in humans and remain remarkably stable over years

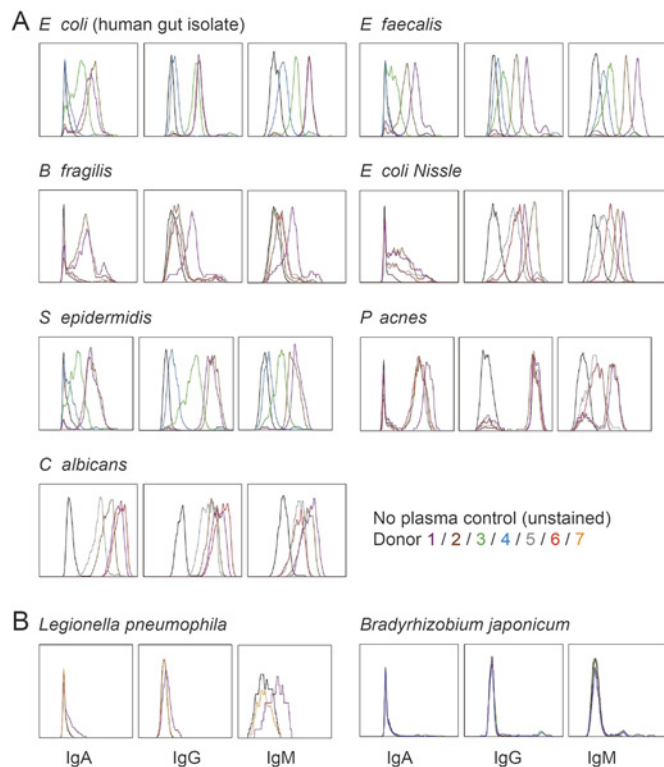
IgA, IgG and IgM responses against several human gut (primary human *E coli* isolate, *E coli* Nissle, *B fragilis* and primary human *E faecalis* isolate) and skin commensal bacteria (*S epidermidis* and *P acnes*) as well as *C albicans* as a human mucosa-associated commensal fungus were measured in human plasma or serum samples. In the majority of samples IgA, IgG and IgM responses were abundantly detectable against these bacteria and *C albicans* (figure 2A).

Importantly, to validate the specificity of the technique further, no antibody responses were detectable against the non-human-related soil bacterium *B japonicum* (figure 2B). No or only

very weak responses were detectable against the non-persistent human pathogen *L pneumophila* (figure 2B). This is consistent with previous reports which state <20% of a population to have antibodies against *L pneumophila*.<sup>36 37</sup>

Next, we analysed the stability of these antibody responses over time. IgA, IgG and IgM responses were compared from >20 healthy and 70 HIV-infected individuals from some of whom yearly samples were available in a time frame of up to 5–6 years. Thus, samples from several patients with HIV ranged from the early phase of infection until late stage and AIDS phase. The magnitude of responses differed up to 1000-fold between donors independent of HIV-1 infection, but remained remarkably stable over the years (figure 3). This might indicate limited changes in the microbial composition over time, individually different frequencies of certain commensal bacteria and/or different individual abilities to mount antibody responses against a certain bacterial strain.<sup>1 38</sup> The stability of responses was seen despite chronic, untreated progressing HIV-1 infection. In most patients, longitudinal samples followed the progression to AIDS, with CD4 counts dropping below 200 cells/mm<sup>3</sup>. Extensive statistical analysis confirmed that the commensal bacteria-specific antibody responses did not significantly change over time. No significant longitudinal correlation with CD4 counts, survival or total IgA, IgG and IgM levels was observed. In addition, the incidence of diarrhoea had no measurable impact on the antibody responses (data not shown).

## Intestinal microbiota



**Figure 2** Representative histograms of systemic human immunoglobulin (Ig) responses to bacteria and *C albicans* measured by live bacterial FACS analysis. (A) Human IgA, IgG and IgM responses to commensal bacteria and *C albicans* and (B) to the non-commensal bacterium *L pneumophila* and the soil bacterium *B japonicum*. Each plot depicts a histogram (black) of an unstained bacterial sample and several representative histograms (colours) from bacteria stained with plasma from different human donors (donors 1–7). The following plasma dilutions were used: *E coli*, 1/50; *E faecalis*, 1/50; *B fragilis*, 1/5; *E coli* Nissle, 1/5; *S epidermidis*, 1/50; *P acnes*, 1/50; *C albicans*, 1/45; *L pneumophila*, 1/50; *B japonicum*, 1/50.

Many bacteria are believed to express antigens which have blood group reactivity. Due to this ubiquitous AB-reactivity, individuals with blood group 0 might be suspected to have higher antibody responses against commensal bacteria. The healthy donors studied here did not show differences in the magnitude of responses against gut commensal bacteria between blood group 0 and the other blood groups (A/B/AB) (supplementary figure 2).<sup>39</sup>

### Antibody responses to gut commensal bacteria are not affected by chronic HIV-1 infection and do not contribute to hypergammaglobulinaemia

After verification that specific antibody responses to commensal bacteria can be abundantly detected in healthy individuals and are also remarkably stable over extended time periods in patients with HIV, live bacterial FACS was now applied to study antibody responses in HIV-infected individuals in more detail. To assess the impact of HIV-associated enteropathy and microbial translocation<sup>5 10 16</sup> in the context of HIV-associated hypergammaglobulinaemia,<sup>20</sup> we studied longitudinal plasma or serum samples of ART-naïve chronic HIV-infected individuals with respect to their antibody titres against a selection of commensal gut bacteria. As HIV enteropathy and gut permeability were reported to be most markedly increased during advanced HIV-1 infection,<sup>16</sup> we deliberately chose to study samples of patients with disease close to or in the AIDS phase

(median CD4 count: 217 cells/mm<sup>3</sup> (1.5–760)). Characteristics of the study cohort are listed in table 1.

Patient samples were grouped into two cohorts: (1) patients without reported incidence of diarrhoea and (2) patients with reported incidence of diarrhoea or related gastrointestinal symptoms. This grouping allowed us to analyse whether the incidence of diarrhoea further influences the antibody response to gut commensal bacteria. HIV-infected individuals were compared with a control study group of 29 HIV-negative age-matched healthy donors.

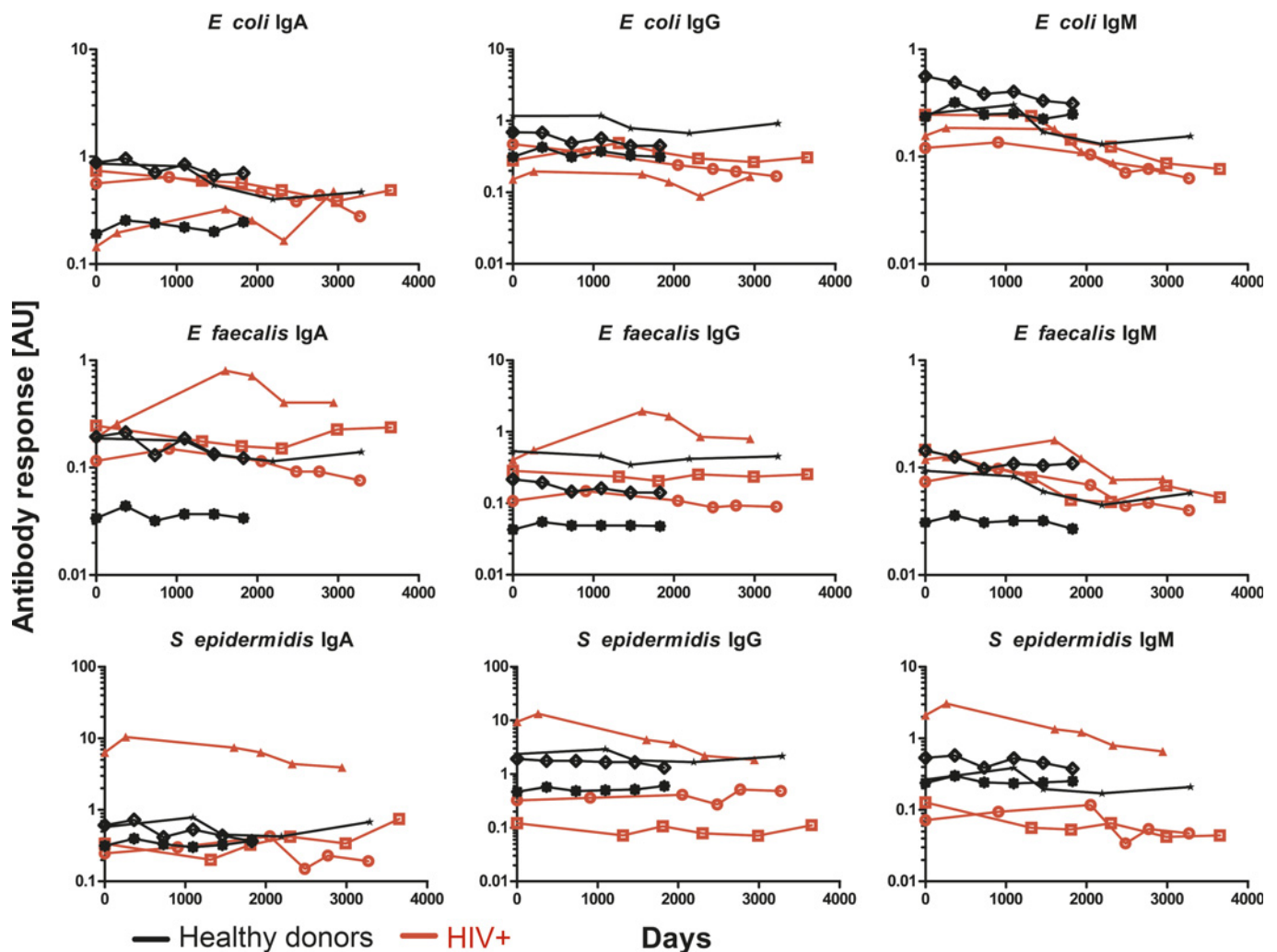
First, total IgA, IgG and IgM levels were analysed in plasma or serum. While total levels of IgA were not significantly different between HIV-infected and healthy donors, chronic HIV-1 infection was associated with markedly increased levels of total IgG and IgM in plasma or serum. No differences were observed between HIV-infected individuals with or without diarrhoea (figure 4).

We then addressed whether abnormal priming against the intestinal microbiota was responsible, or contributed to, hypergammaglobulinaemia by quantifying the longitudinal antibody responses against a selection of gut commensal bacteria including a primary human *E coli* isolate (Gram-negative facultative anaerobe bacteria), a primary human *E faecalis* isolate (Gram-positive aerobe and anaerobe bacteria), *E coli* Nissle, *B fragilis* (Gram-negative obligate anaerobe bacillus), *K pneumoniae* (Gram-negative facultative anaerobe bacteria) and *B thetaiotaomicron* (Gram-negative anaerobe bacteria), against *S epidermidis* and *P acnes* (skin commensal bacteria) as well as *C albicans* (opportunistic fungus). As mentioned above, extensive statistical analysis confirmed that the commensal bacteria-specific antibody responses did not significantly change over time. No significant longitudinal correlation with CD4 counts, survival or, importantly, total IgG and IgM levels was observed. In addition, the incidence of diarrhoea had no measurable impact on the antibody responses (data not shown).

Cross-sectional analysis revealed no differences in the antibody responses specific for all tested gut commensal bacteria per unit plasma volume (ie, the effective antibody concentration per defined plasma volume and not normalised to the total IgG level) between HIV-infected individuals and healthy donors, with the exception of the IgG responses specific for *E coli* Nissle and *K pneumoniae*, for which a slight decrease was observed when comparing HIV-infected individuals with or without diarrhoea with healthy controls (figure 5A). No differences were seen for IgA and IgM responses to the skin commensal bacteria. However, somewhat unexpectedly and surprisingly IgG responses per unit volume to *S epidermidis* and *P acnes* were reduced in the HIV-infected study group (figure 5C). None of the analysed responses differed significantly between HIV-infected individuals with or without diarrhoea.

*Candida*-specific IgG responses per unit volume were elevated in HIV-infected individuals compared with healthy controls (figure 5A), consistent with previous reports indicating that *C albicans* is a well-known AIDS-associated pathogen with higher colonisation prevalence in patients with AIDS.<sup>40 41</sup> All comparisons mentioned here were tested for significance by Student t test. We also compared the data by a non-parametric Wilcoxon rank sum test. Significant differences found by two-sided t test were confirmed by Wilcoxon rank sum test (supplementary table 1).

In conclusion, despite increased total IgG and IgM levels, antibody titres per unit volume of plasma to commensal gut bacteria were not increased during chronic, progressive HIV-1 infection. This suggests that the drastically increased total IgG

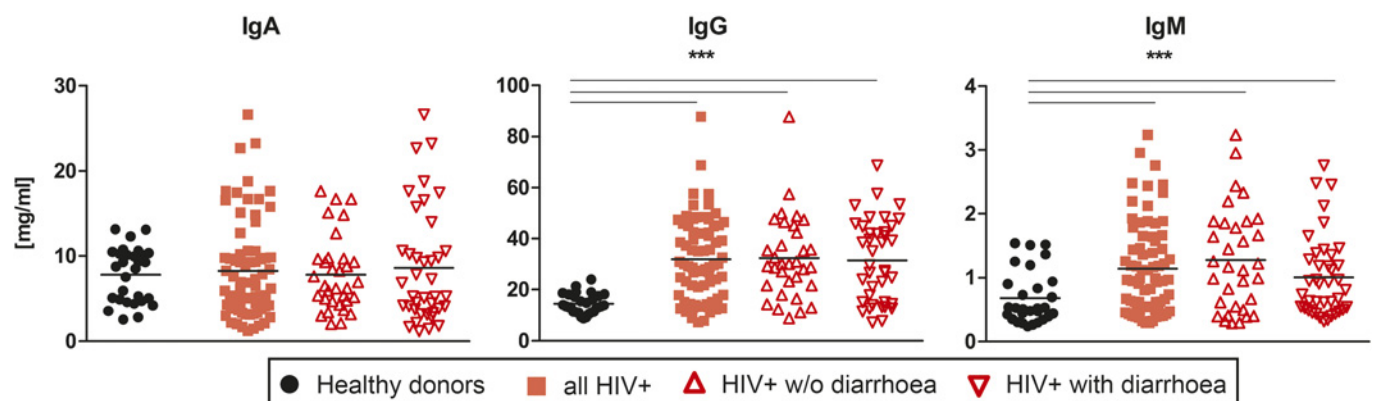


**Figure 3** Longitudinal antibody responses to commensal bacteria measured by live bacterial FACS analysis. Immunoglobulin A (IgA), IgG and IgM responses to *E coli*, *E faecalis* and *S epidermidis* were measured in longitudinal samples from three representative healthy donors (black lines) and three representative HIV-infected individuals (red lines). The HIV-infected individuals progressed to AIDS (CD4 count <200) within the depicted time range.

and IgM antibody levels in HIV-1 infection are not composed of high affinity antibodies specific for commensal bacteria and microbial translocation does not contribute to hypergammaglobulinaemia in an antigen-specific manner.

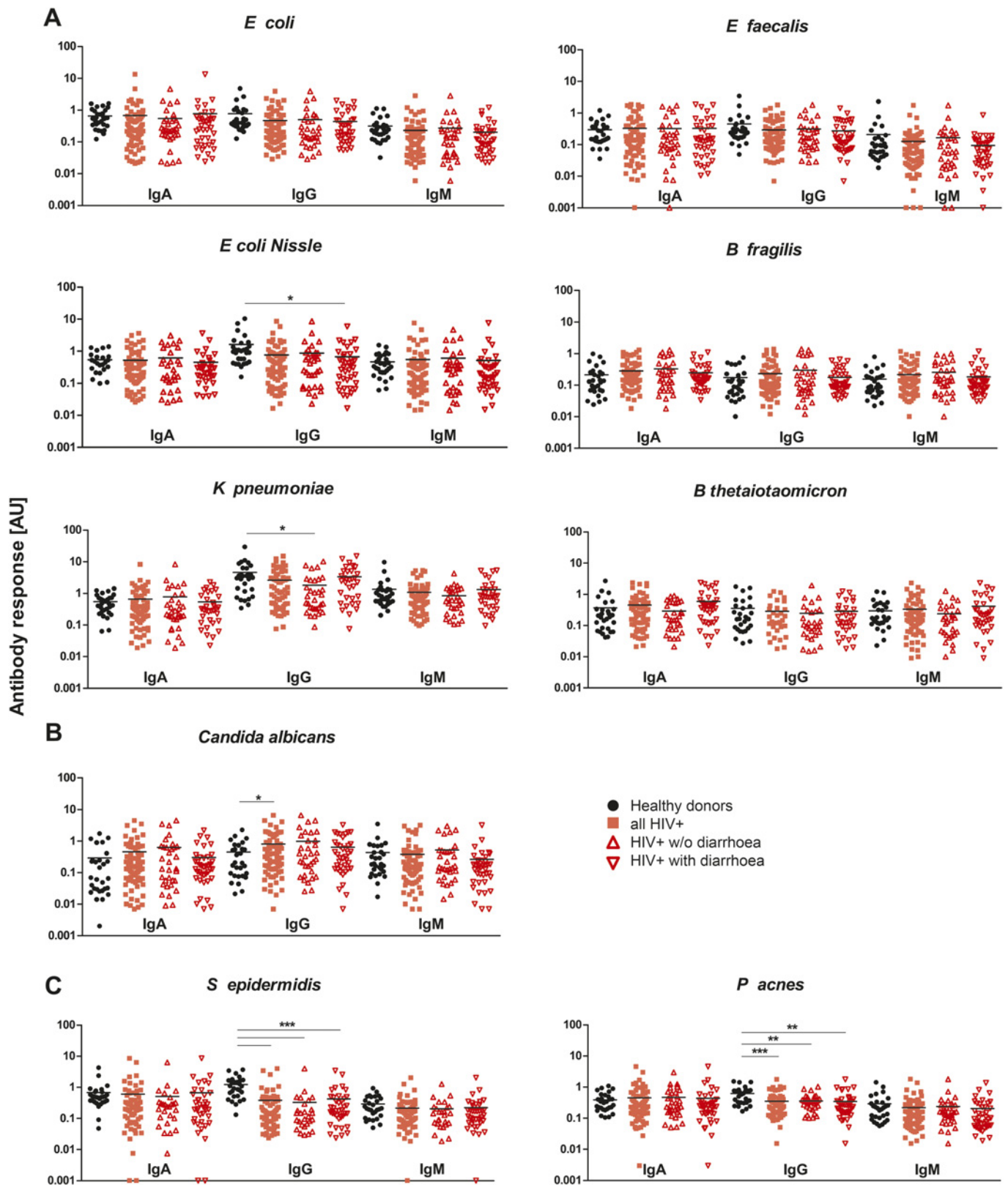
#### Patients with IBD show elevated systemic antibody titres against *E faecalis*, *K pneumonia* and *B thetaiotaomicron*

Since chronic HIV-1 infection, even in association with diarrhoea, showed no impact on the antibody responses to gut



**Figure 4** Total immunoglobulin A (IgA), IgG and IgM levels in healthy donors and HIV-infected individuals with or without diarrhoea. Total levels of IgA, IgG and IgM were quantified in serum or plasma samples. The median of multiple time points is shown for each donor. p Values (\*\*\*) were determined by Student t test. Statistical details are listed in supplementary table 1.

## Intestinal microbiota



**Figure 5** Antibody responses to commensal microflora during chronic HIV-1 infection measured by live bacterial FACS analysis. Immunoglobulin A (IgA), IgG and IgM responses to commensal gut bacteria (A), *C. albicans* (B) and commensal skin bacteria (C). The median of multiple time points is shown for each donor. p Values (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ) were determined by two-sided Student t test. Statistical details are listed in supplementary table 1.

commensal bacteria, antibody responses were now analysed in patients with IBD who are known to suffer from severe enteropathy, microbial translocation and a dysregulated microbiota.<sup>3</sup>

Elevated antibody responses to certain bacterial antigens (LPS, flagellin) have been described before for IBD, mainly in the context of CD.<sup>42–46</sup> However, the majority of these studies only

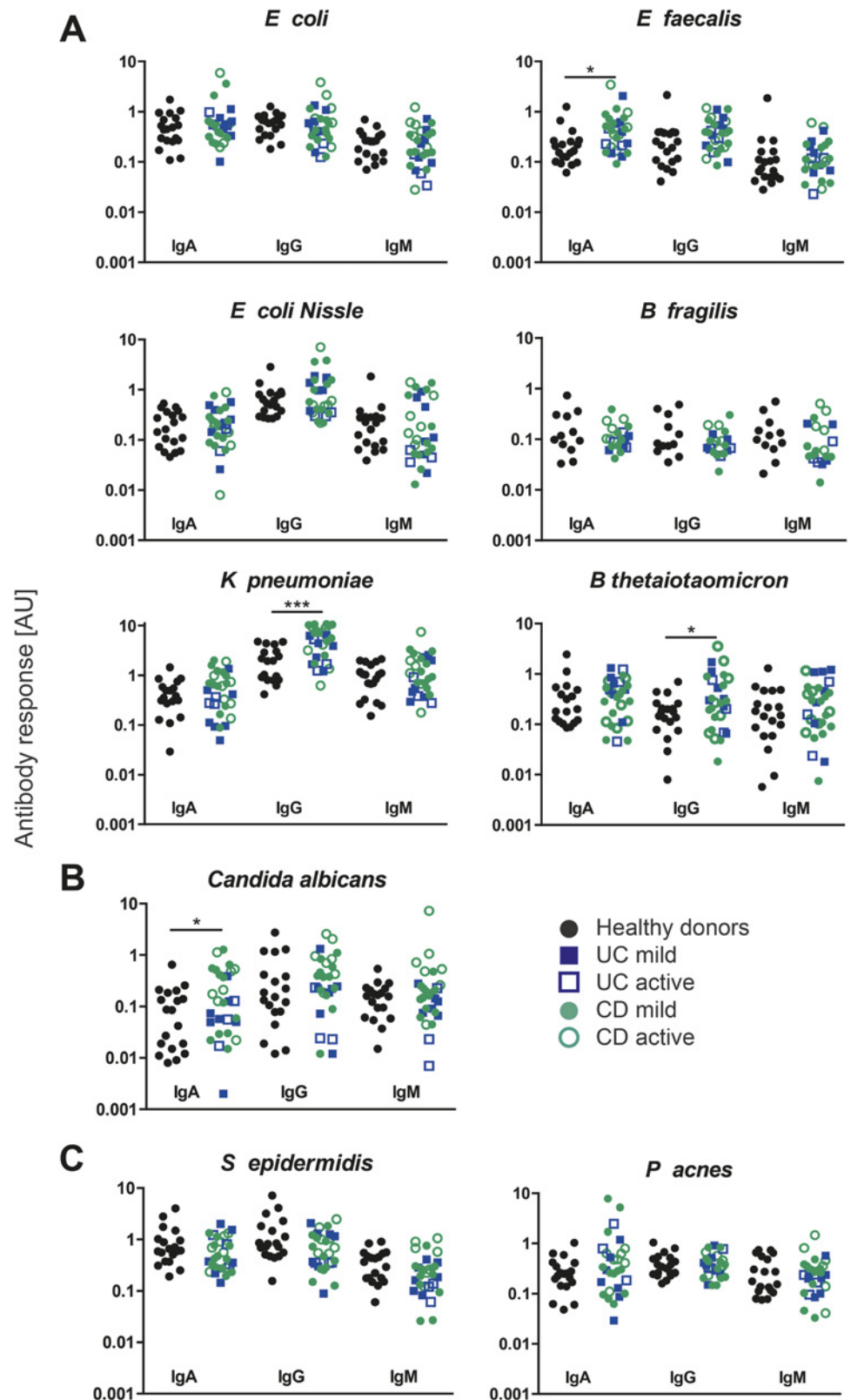
focused on certain bacterial products or bacterial lysates and we wished to analyse the antibody response to whole intact gut bacteria. This analysis should reveal whether (more severe) enteropathy in comparison with HIV-1 may impact on the antibody response to gut commensal bacteria.

Twenty-nine patients with IBD with different disease phenotypes (active or mild CD, active or mild UC) and 19

age-matched healthy donors were analysed. The characteristics of the study cohort are listed in table 2.

IgA, IgG and IgM responses to a human primary *E coli* isolate, *E coli* Nissle, primary *E faecalis*, *B fragilis*, *S epidermidis*, *K pneumoniae*, *B thetaiotaomicron*, *P acnes* and *C albicans* were analysed. IgA responses to *E faecalis*, IgG responses to *K pneumoniae* and *B thetaiotaomicron* as well as IgA responses to *C albicans* were

**Figure 6** Antibody responses to commensal microflora in patients with inflammatory bowel disease (IBD) measured by live bacterial FACS analysis. Immunoglobulin A (IgA), IgG and IgM responses to commensal gut bacteria (A), *C albicans* (B) and commensal skin bacteria (C). One time point per donor was analysed and is depicted. p Values (\* $p < 0.05$ ) were determined by two-sided Student t test. Statistical details are listed in supplementary table 1. CD, Crohn's disease; UC, ulcerative colitis.





## Intestinal microbiota

elevated in patients with IBD (figure 6). There was also a clear trend for elevated *E faecalis*-specific IgG responses but no differences for IgM titres. Antibody responses to *E coli*, *B fragilis*, *S epidermidis* and *P acnes* were not statistically significant between patients with IBD and healthy donors or when IBD disease phenotypes were analysed separately. Elevated systemic antibody titres specific for *E faecalis*, *K pneumoniae*, *B thetaio-taomicron* and *C albicans* indicate that enteropathy is commonly associated with increased antimicrobiota priming and suggests that the inability to detect such priming in HIV-associated enteropathy may be associated with poor ability to mount new T cell-dependent antibody responses in patients with HIV or lower levels of exposure to gut microbiota compared to patients with IBD.

### Low affinity antibodies against gut commensal bacteria are comparable between patients with HIV and healthy controls

As the live bacterial FACS technology assay is mainly able to detect high affinity bacterial antibodies we complemented our analysis with ELISAs which also quantify low affinity antibodies. We therefore compared two ELISA approaches with respect to their specificity of detecting bacteria-specific antibody responses, using either bacterial lysates or intact bacteria for coating of ELISA plates. Based on the absence of systemic antibody responses specific for the soil bacterium *B japonicum* when assayed by live bacterial FACS (figure 2B), we used lysates of *B japonicum* or whole *B japonicum* along with comparable preparations of *E coli* in ELISAs to evaluate the specificity of the respective coating procedures. Serum IgG antibodies of healthy donors exhibited variable degrees of binding to both lysates of *B japonicum* and whole bacteria (supplementary figure 3), indicating that both coating procedures resulted in considerable cross-reactivity, most probably due to exposure of conserved bacterial proteins in the ELISA. As coating of whole bacteria seems more comparable with live bacterial FACS analysis, we used this procedure for the quantification of systemic antibody responses to *K pneumoniae*, *E faecalis* and *E coli* in patients with HIV, patients with IBD and the respective healthy control groups. Optical density values from these ELISA measurements were related to a standard plasma included in every assay to determine an arbitrary titre for every sample (supplementary

figure 1B). Consistent with the results obtained with live bacterial FACS analysis, systemic IgG titres specific for *K pneumoniae* and *E faecalis* were significantly increased in patients with IBD. In contrast, no significant differences were observed between patients with HIV and healthy controls for all three tested bacteria (figure 7), further supporting the notion that HIV-associated hypergammaglobulinaemia is not driven by specificities for commensal gut bacteria.

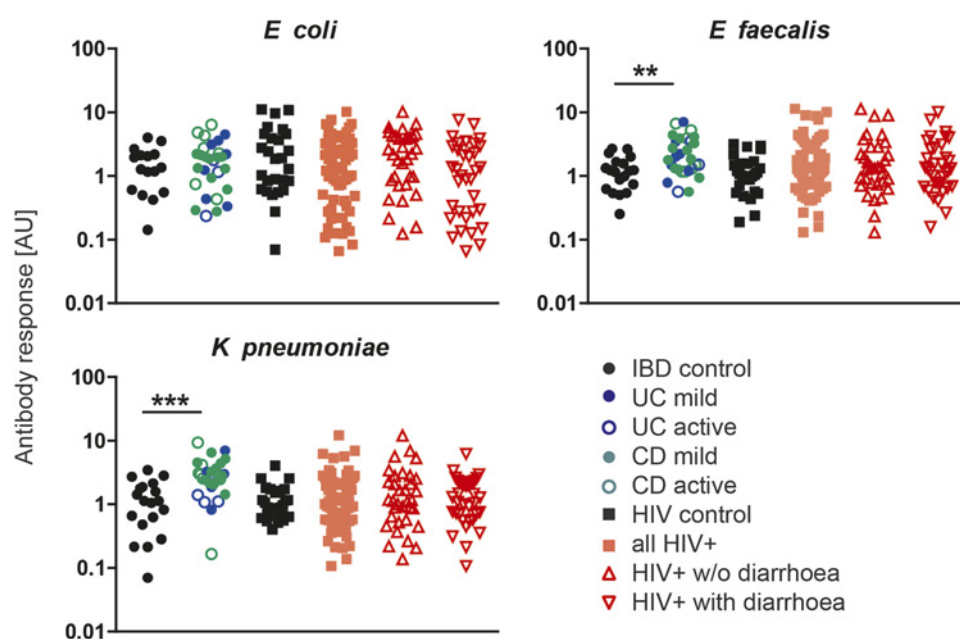
### Plasma levels of sCD14, but not of LPS and EndoCabIgM, are altered during chronic HIV-1 infection

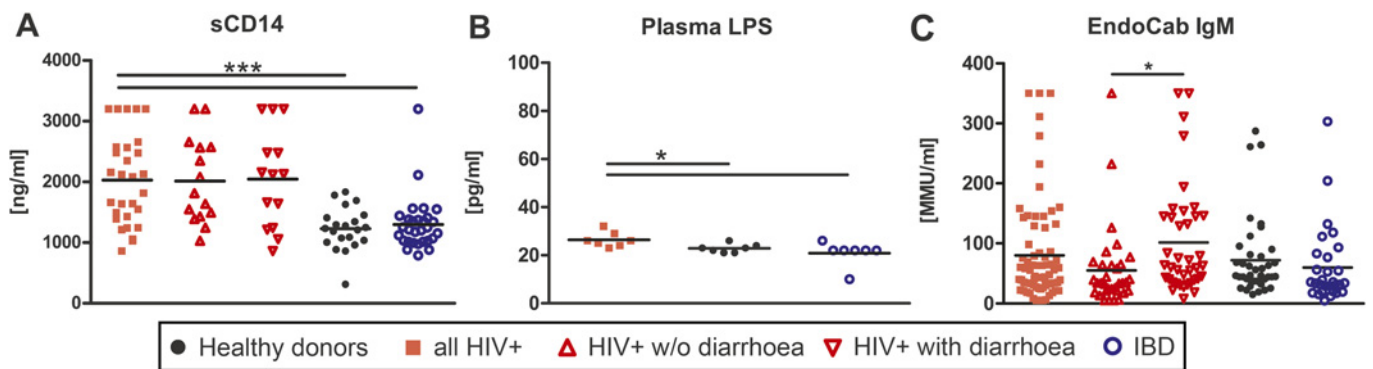
Since antibody responses to commensal bacteria do not seem to reflect enteropathy and microbial translocation in patients with HIV, other parameters associated with microbial translocation were analysed to assess the level of enteropathy in the different study groups. Plasma levels of sCD14, LPS and EndoCabIgM have recently been introduced as markers of microbial translocation during HIV-1 infection.<sup>5 16</sup> Plasma levels of sCD14 were analysed in the HIV-infected individuals with or without diarrhoea, healthy donors and patients with IBD (figure 8A). In agreement with previous reports, sCD14 was significantly elevated in HIV-infected individuals. No differences were observed between individuals with or without diarrhoea. In line with a previous report, sCD14 was not elevated in patients with IBD.<sup>47</sup>

The plasma samples with the highest levels of sCD14 were subsequently analysed for LPS content (figure 8B). Neither patients with HIV nor those with IBD showed markedly increased levels of LPS. Levels of LPS were minimally increased in patients with HIV, but the measured concentrations (20–40 pg/ml) were much below the previously published reports of >100–200 pg/ml.<sup>16</sup> Endotoxaemia has also been described in IBD, especially in the context of CD, but is also reported to be very transient.<sup>34 42 47 48</sup>

EndoCabIgM were analysed in HIV-infected individuals with or without diarrhoea as well as patients with IBD and healthy donors (figure 8C). Only the HIV-infected individuals with diarrhoea showed significantly higher levels of EndoCabIgM, but only when compared with the HIV-infected individuals without diarrhoea. No significant differences in EndoCabIgM were found between the other study groups.

**Figure 7** Antibody responses to gut commensal bacteria measured by ELISA. Immunoglobulin G (IgG) responses to three gut commensal bacteria were measured in patients with HIV and those with inflammatory bowel disease (IBD) and the respective matched healthy control groups by ELISA. For patients with HIV and those with IBD one time point per donor was analysed. p Values (\*p<0.05) were determined by two-sided Student t test. CD, Crohn's disease; UC, ulcerative colitis.





**Figure 8** Markers of microbial translocation during chronic HIV-1 infection compared with healthy donors and patients with inflammatory bowel disease (IBD). Plasma or serum samples were analysed for (A) soluble CD14 (sCD14), (B) lipopolysaccharide (LPS) or (C) endotoxin core immunoglobulin M antibodies (EndoCabIgM). p Values (\* $p < 0.05$ ; \*\*\* $p < 0.001$ ) were determined by two-sided Student t test.

In summary, despite marked upregulation of sCD14, other markers of microbial translocation were not substantially dysregulated in our study group of HIV-infected individuals.

#### Maintenance of other antibody responses during chronic HIV-1 infection

As so far the majority of commensal antibody responses studied here were not altered during chronic HIV-1 infection, other antibody responses were now evaluated in light of the reported HIV-associated B cell dysfunction. Antibody responses to novel antigens and booster vaccines such as tetanus toxoid have frequently been described to be impaired during chronic HIV-1 infection.<sup>22 24 49 50</sup> However, coherent information on antibody responses to persistent antigens (commensal bacteria, CMV) and vaccine antigens (polio), which were acquired before HIV-1 infection, is missing.

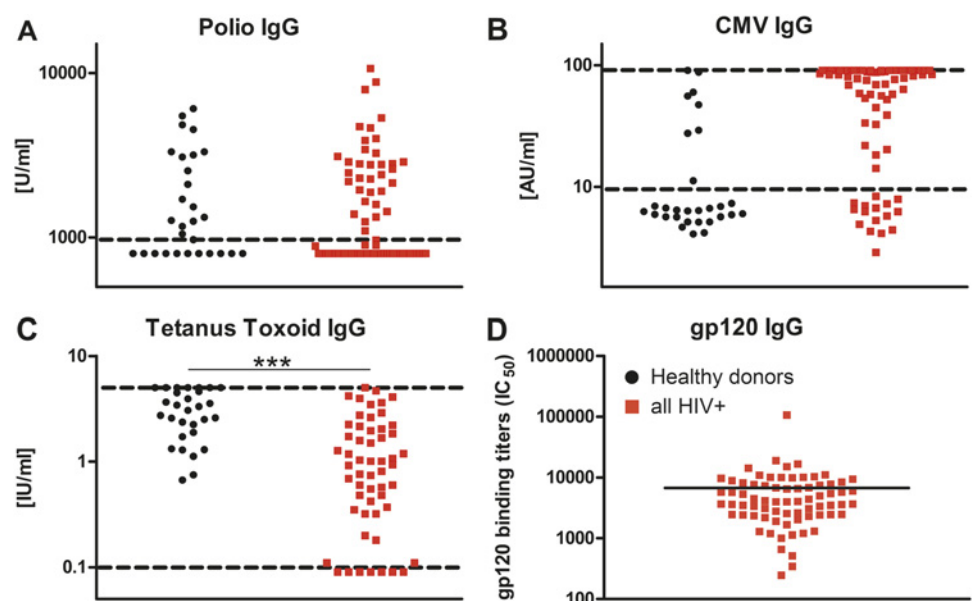
Polio vaccination is generally only performed during childhood, and re-exposure occurs only very rarely. This presumes that the polio-specific antibody response was acquired before HIV infection and was not re-boosted afterwards. Seropositivity (IgG) between the HIV-infected individuals and healthy donors studied here was nearly equal (47% vs 41%). Among the seropositive individuals, the levels of polio IgG did not differ significantly between the study groups (figure 9A). Patients

with HIV therefore successfully maintain specific serum antibody responses to viral vaccination antigens given before HIV infection.

As described previously, CMV seropositivity (IgG) was higher in the HIV-infected study group (78% vs 28%) (figure 9B). When CMV-specific IgG titres were quantified longitudinally, the magnitude of the response did not change with disease progression (no correlation with CD4 counts or significant decrease/increase over time, data not shown). CMV IgG levels were equally high or even higher in the HIV-infected individuals compared with the healthy donors. Overt CMV reactivation was unlikely because CMV IgM responses remained below the detection limit in all samples (data not shown). This is consistent with previous reports about increased or unchanged levels of CMV-specific antibodies during HIV-1 infection.<sup>51 52</sup>

Tetanus toxoid is an antigen which has generally been encountered before HIV-1 infection, but revaccination is required to maintain titres. The vaccination history of our study individuals was not known in detail. However, as boosting regimens with tetanus toxoid do not differ between healthy controls and HIV-infected patients, we assumed that the revaccination frequency would be similar in both study groups. Interestingly, in line with previous reports, IgG responses to tetanus toxoid were significantly lower in

**Figure 9** Maintenance of other antibody responses during chronic HIV-1 infection. Immunoglobulin G (IgG) responses to polio (A), cytomegalovirus (CMV) (B) and tetanus toxoid (C) in HIV-infected individuals compared with healthy donors. The dashed line indicates the detection limit of the assay. Samples below the detection limit are considered as seronegative. (D) IgG titres of HIV-infected individuals with specificity for the gp120 surface protein of the HIV-1 strain JR-FL. p Values (\*\*\* $p < 0.001$ ) were determined by two-sided Student t test.



HIV-1-infected individuals compared with healthy donors (figure 9C),<sup>23 50</sup> indicative of failure to respond effectively to boosting post-infection.

Finally, to assess the antibody response to a persistent antigen which is acquired concomitantly with HIV-1, the IgG antibody response to the HIV-1 envelope protein gp120 was analysed longitudinally. All tested HIV-infected individuals had an IgG response against the heterologous HIV JR-FL gp120 (figure 9D). No correlation between gp120 binding titres and markers of disease progression (CD4 counts and plasma viral load) was observed (data not shown). This is in line with a recent report showing that HIV-1 envelope-specific antibody responses are maintained even in advanced stages of the disease.<sup>53</sup>

In summary, the maintenance of antibody responses acquired before HIV-1 and/or to persistent antigens does not seem to be severely impaired during chronic infection. However, IgG responses to non-persistent tetanus toxoid antigen, which require revaccinations and CD4 T cell help also in healthy donors to be maintained, were lower during chronic HIV-1 infection, suggesting that the maintenance of antibody responses, also in situations with low CD4 T cell counts, is dependent on the nature (persistence, immunogenicity) of the antigen.

## DISCUSSION

The present study shows that systemic antibody responses to gut commensal bacteria are abundantly available in humans and the magnitude of responses to given bacteria may differ up to 1000-fold between individuals, but remains remarkably stable over years. Enteropathy associated with HIV has no or only very little effect on these systemic antibody responses when quantified per unit volume of the plasma in the concomitant presence of HIV-associated hypergammaglobulinaemia. Thus, patients with HIV, despite severe B cell disturbances, manage to maintain normal levels of antibody responses to gut commensal bacteria, even at very late stages of the infection.

The evident priming of systemic antibody responses to intestinal bacteria in humans at first appears to diverge from the notion of tight compartmentalisation between intestinal and systemic antibody responses as established in specific pathogen-free mice where systemic IgG responses to commensal bacteria are undetectable.<sup>28 54 55</sup> However, anticommensal microbe IgG responses are known to be induced and protective in the case of mice with deficiencies in innate immunity.<sup>28</sup> Living outside of the laboratory environment with common bouts of enteropathy throughout childhood and into adult life almost certainly underlies the observed human systemic IgG responses to gut commensal bacteria. Evidence of systemic antibody responses to commensal bacteria has been provided before,<sup>30 34 56</sup> but was never analysed with such specificity and depth in a large cohort of longitudinal samples as presented here.

As systemic priming to commensal bacteria is clearly ubiquitous in humans, we investigated how these responses might be affected by enteropathy. Chronic enteropathy in humans has frequently been described in the context of IBD, but is also known for HIV-1 infection.<sup>5 10</sup> Several studies associate HIV enteropathy with systemic translocation of microbial products such as LPS,<sup>16</sup> bacterial butyrate<sup>15</sup> and bacterial DNA.<sup>57</sup> Systemic translocation of LPS is suggested to drive chronic immune activation, which is a hallmark of HIV-1 infection and progression towards AIDS.<sup>16</sup> Further, HIV infection is also associated with severe B cell disturbances<sup>22</sup> as memory B cell responses are reported to be impaired, especially in the context of vaccination.<sup>23 24 49 50</sup> Conversely, B cell hyperactivity, observed in serum as well as in the intestinal tissue, and hypergammaglobulinaemia

of unknown antigen specificity are observed without clear evidence for the underlying mechanisms.<sup>23 25 58</sup> Although a role for an antigen-dependent mechanism underlying B cell hyperactivation could so far not be completely excluded, the vast majority of published reports indicate that polyclonal stimulation independent of B cell receptor (BCR) specificity may drive B cell hyperactivation and hypergammaglobulinaemia.<sup>22 23 59</sup> Alternatively, viral molecules and Toll-like receptor (TLR) ligands have been described to trigger (antigen-independent) polyclonal B cell activation. The HIV-1 envelope protein gp120 can interact with the complement receptor CD21 and the C-type lectin receptor DC-SIGN, which are both expressed on B cells.<sup>60 61</sup> These interactions have been associated with increased spontaneous Ig secretion and polyclonal B cell activation.<sup>60 62 63</sup> Similar to T cells, an indirect activatory effect via HIV-infected macrophages has been ascribed to Nef, but this contradicts a report of Nef-induced class switch inhibition.<sup>22 64</sup>

A role for TLR stimulation of B cells has mainly been discussed in the context of serological maintenance of antibody titres,<sup>65</sup> but may well be applicable to hyperactivation and hypergammaglobulinaemia: memory B cells respond to TLR9, which recognises CpG DNA, and start to proliferate and secrete Ig independently of BCR triggering.<sup>65 66</sup> Naive B cells, however, are reported only to upregulate TLR9 upon activation by BCR triggering,<sup>65 66</sup> which may be relevant in the context of a proposed mechanism stating that polyclonal activation of naive B cells mainly contributes to hypergammaglobulinaemia.<sup>23</sup> It has indeed been shown that B cells from HIV-1-infected donors are responsive to stimulation by CpG DNA.<sup>58</sup> Increased levels of LPS, however, may be excluded as a direct contributor to TLR-mediated polyclonal B cell activation since B cells do not express TLR4 along with the majority of other TLRs.<sup>66 67</sup>

Furthermore, cytokines and involvement of T cell help have been described in the context of HIV-associated polyclonal B cell activation: T cell help provided to B cells via CD40/CD40L interaction is often discussed as a contributor to B cell activation, also in the absence of BCR triggering. It has been reported that during HIV-1 infection the frequency of T cells expressing CD40L and thus being able to provide co-stimulation to B cells is increased.<sup>68</sup> Similar to TLR9 stimulation, T cell help provided by activated CD4 T cells was shown to induce proliferation and Ig secretion by memory B cells.<sup>65 69</sup> Thus, in such a scenario the observed hypergammaglobulinaemia *in vivo* would be a secondary effect of the high level of activation of CD4 T cells.

The data presented in this study are consistent with an antigen-independent mechanism underlying HIV-associated B cell hyperactivation and hypergammaglobulinaemia—at least with respect to a selection of gut microbial antigens. Our data show that HIV-infected individuals, even in late stages of the disease, maintain normal levels of low and high affinity antibody responses towards gut commensal bacteria per unit volume of plasma. As observed for healthy donors, these levels remain remarkably stable over the whole course of infection and do not correlate with any parameters of disease progression. Even during episodes of diarrhoea, which are associated with enhanced intestinal permeability during HIV-1 infection,<sup>13</sup> no impact on systemic antibody responses was observed.

These data indicate that HIV enteropathy is not reflected in changes of systemic antibody responses specific for gut commensal bacteria—at least for the panel of gut bacteria which were used for analysis. Furthermore, our data indicate that HIV-associated B cell dysfunction does not completely obliterate the ability to produce and maintain systemic antibody responses. Indeed, increased antibody titres were observed for both CMV

and *Candida* and, similar to gut commensal bacteria, polio-specific antibody levels were comparable between HIV-infected individuals and healthy controls.

As HIV-associated enteropathy and B cell disturbances do not alter the antibody responses to gut commensal bacteria, we subsequently studied antibody responses in patients with IBD. IBD is associated with severe enteropathy, but is not clearly related to any major systemic immunodeficiency or B cell dysfunction. In patients with IBD we indeed observed elevated systemic antibody responses to a number of gut commensal bacteria, probably driven by increased exposure to intestinal commensal antigens in IBD. Although the underlying cause of UC or CD remains unclear, in the absence of overt T or B cell immunodeficiency, enteropathy is clearly associated with increased antimicrobiota antibody titres. This suggests that either the immunodeficiency or the nature of the enteropathy prevents excessive priming against the intestinal microbiota in HIV-infected individuals. It should be noted in this context that gut bacterial surface-specific antibody responses, at least in mice, seem to depend on CD4 T cell help,<sup>28 70</sup> and intestinal CD4 T cells are massively depleted in HIV-1 infection.<sup>7 8</sup> Owing to lack of faecal material or intestinal histology from the Swiss HIV cohort, we cannot exclude specific effects of HIV infection on local mucosal antibody responses as has been observed in the severe intestinal lesions in IBD.<sup>71</sup>

To quantify the extent of enteropathy-associated microbial translocation, we measured previously reported markers of bacterial translocation in both patients with HIV and those with IBD. Several studies have reported increased levels of LPS and sCD14 during chronic HIV-1 infection which have been attributed to HIV enteropathy and microbial translocation.<sup>5 16 72 73</sup> In addition, decreased levels of EndoCabIgM were associated with chronic LPS exposure and B cell dysfunction during chronic HIV-1 infection.<sup>16 74</sup>

Within our cohort, sCD14 was significantly elevated in the HIV-infected individuals but this was not associated with similar increases in plasma levels of LPS or alterations in EndoCabIgM levels. Two other studies in African cohorts and one study in a European cohort also failed to detect elevated levels of LPS in the plasma<sup>32 75 76</sup> or altered EndoCabIgM levels.<sup>76</sup> It could be speculated that regional differences or blood collection and storage methods may account for the unchanged levels of LPS and EndoCabIgM in European and African study groups. However, a new study in South African HIV-infected individuals also reported increased levels of plasma LPS compared with healthy controls.<sup>77</sup>

Recently, elevated levels of sCD14 during chronic HIV-1 infection have been attributed to increased LPS exposure.<sup>16 74</sup> However, it has previously also been shown that treatment of monocytes with the HIV-1 envelope protein gp120<sup>78</sup> or other stimuli<sup>79</sup> induces release of sCD14. These and our findings may implicate that increased levels of sCD14 during chronic HIV-1 infection may rather be a general marker of aberrant activation of monocytes and may not necessarily be a sole consequence of increased LPS exposure. This is also supported by the fact that sCD14 is generally not elevated in patients with IBD<sup>47</sup> despite some reports indicating transiently increased levels of LPS.<sup>34 42 47 48</sup>

Therefore, the absence of increased antimicrobiota IgA and IgG in HIV is likely to be due to mild or transient intestinal commensal translocation, as determined by the absence of strong serum markers of bacterial translocation, possibly also involving the ability of liver sinusoidal cells and Kupffer cells to effectively clear IgA-coated bacteria from the circulation<sup>80 81</sup> and

the intrinsic immunodeficiency in HIV failing to induce new antigen-specific antibody responses. This is in contrast to individuals with IBD, showing elevated systemic antibody titres to several gut commensal bacteria.

In conclusion, our data show that HIV-associated enteropathy is not associated with increased systemic antibody responses to a number of commensal gut bacteria, including bacterial species to which systemic antibody responses were clearly elevated in patients with IBD, and further that antigen-specific effects of the translocating microbiota are unlikely to contribute to hypergammaglobulinaemia.

#### Author footnote:

\*The members of the Swiss HIV Cohort Study and the Swiss Mother and Child HIV Study are: Barth J, Bategay M, Bernasconi E, Böni J (SNF-grant 315200-114148), Brazzola P, Bucher HC, Bürgisser P, Burton-Jeangros C, Calmy A, Cavassini M, Cheseaux JJ, Drack G, Dubs R, Duppenhaler A, Egger M, Elzi L, Fehr J, Fischer M, Flepp M, Francioli P (President of the SHCS), Furrer H, Fux CA, Gorgievski M, Grawe C, Günthard H, Gyr T, Hasse B, Hirsch HH, Hirschel B, Hösli I, Kahlert C, Kaiser L, Keiser O, Kind C, Klimkait T, Kovari H, Ledergerber B, Martinetti G, Martinez de Tejada B, Müller N, Nadal D, Pantaleo G, Posfay-Barbe K, Raio L, Rauch A, Regenass S, Rickenbach M, Rudin C (Chairman of the Mother & Child Substudy), Schmid P, Schultze D, Schöni-Affolter F, Schüpbach J, Speck R, Taffé P, Telenti A, Trkola A, Vernazza P, von Wyl V, Weber R, Wylér CA, Yerly S.

**Acknowledgements** We would like to thank Reinhard Zbinden (Institute of Medical Microbiology, University of Zurich) for providing bacterial isolates, Anna-Katharina Ehler (Institute of Medical Virology, University of Zurich) and Barbara Stecher (Institute of Microbiology, ETH Zurich) for experimental support, Herbert Kuster for help organising all the shipments. Furthermore, we thank all patients participating in the SHCS, all the staff in the SHCS clinical centres, the SHCS laboratories and the data centre.

**Funding** This work was supported by the Swiss National Science Foundation (grant # 310000-129751 to AO, grant # 130865 to HFG and grant # 315200-114148 to Roland Regoes), and by the SHCS project No 598. The SHCS is financed in the framework of the Swiss HIV Cohort Study, supported by the Swiss National Science Foundation (SNF grant #3345-062041).

**Competing interests** None.

**Ethics approval** This study was conducted with the approval of the University Hospital Zurich and the University Hospital Bern.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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