A CROSS-SECTIONAL STUDY ON THE EFFECT OF HIV VIRION AND
BACTERIAL LPS ON MEMORY B CELL APOPTOSIS

by

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*We also certify that written approval has been obtained for any proprietary material contained therein.
Dedicated to my dear husband Zhuang Wan whose patience and encouragement have always given me great support
I very much appreciate Dr. PingFu Fu for his support and guidance.

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A Cross-sectional Study on the Effect of HIV Virion and Bacterial LPS on Memory B Cell Apoptosis

Abstract

by

WEI JIANG

The gut mucosal barrier is disrupted in HIV disease, resulting in increased systemic exposure to microbial products such as Lipopolysaccharide (LPS). The consequences of a permeable gut barrier in HIV infection are not fully understood. This study is designed to investigate the predictors for enhanced apoptotic memory B cells (mB) in HIV disease. The primary hypothesis is that microbial LPS and HIV virions synergistically induce memory B cell apoptosis, and this effect is through Fas/Fas ligand (FasL) cell death pathway. The Secondary hypothesis is that the interaction of plasma level of FasL and Fas expression on mB predicts the percentage of mB apoptosis. A cross-sectional study including 20 HIV-negative controls and 20 HIV-infected donors was conducted in the present project. Fas expression and annexin V binding were tested on mB by flow cytometer; and plasma FasL measured by ELISA. The association of Fas expression and plasma level of FasL on mB death was tested by spearman correlation test and a linear regression model (Least-Square regression). In vitro, LPS and HIV virions cooperated to induce mB death in peripheral mononuclear cell cultures from HIV-negative control donors. Blocking Fas/FasL pathway prevented mB death induced by HIV and LPS. Fas surface expression on mB was increased by synergistic effect of HIV and LPS, and the induction of mB death was significantly related to its Fas surface expression. In vivo, a linear regression
model was used to test the predictors of the outcome, indicating that plasma level of FasL, Fas geometric mean expression on mB, and their interaction were associated with mB death. Both plasma FasL and Fas geometric mean expression were positively related to the percentage of mB death by Spearman correlation test. In HIV disease, it is important to inhibit HIV replication and microbial translocation to prevent B cell depletion. By determining the mechanisms of B cell depletion and perturbations in HIV disease, it may be possible to design interventions that will improve immune responses to vaccines, reduce selected opportunistic infections and perhaps slow disease progression by restoring the immunologic barrier that protects against microbial translocation.
INTRODUCTION

HIV epidemiology

Human immunodeficiency virus (HIV) is a lentivirus that attacks human immune system, and causes acquired immunodeficiency syndrome (AIDS). The incidence of HIV in the United States is approximately 56,300 people each year, with prevalence of HIV infection in the US of about 1,100,000 people (1, 2). Approximately 18,000 people with AIDS die each year in the US. There are four major routes of HIV transmission including sex, needles, breast feeding and from mother to her baby at birth (3, 4). However, until now there is not drug available to completely eliminate HIV virus.

AIDS is a life-threatening condition that can lead to opportunistic infections, cardiovascular and metabolic diseases and cancers. HIV infection leads to the disease of AIDS approximately two to ten years after infection. There are around two to ten years from HIV infection to AIDS. Antiretroviral treatment greatly reduces the mortality and the morbidity of HIV infection (3, 4). Antiretroviral treatment limits virus under detectable in peripheral blood, other tissues such as lymph nodes, brain and tears still contain variant levels of virus.

Innate and adaptive immunities

There are two types of immune responses in the human system, the innate and adaptive immune responses. The innate immune system has the function to immediately and non-specifically respond to invading pathogens, including inflammation, complement system, and cellular innate responses. For example, dendritic cells produce interferon-alpha in response to virus infection, and
interferon-alpha has the function of killing virus (5). When innate immunity cannot eliminate pathogens, adaptive immunity plays a role to control infection. Adaptive immune response defends the host from infection by a specific manner. This means that certain cells of adaptive immune system recognize and respond to specific pathogens, and have specific memories, next time these memory cells can attack the same invading pathogens with a fast and strong responses. Innate immunity defenses are immediate and short lasting. Adaptive immunity defenses mount often 1-2 weeks and last longer (5).

**Toll-like receptor and its ligands**
Toll-like receptor (TLR) families are responsible for the recognition of pathogen-associated molecular patterns (PAMPs) expressed by pathogens and distinguishable from host molecules. TLRs recognize structurally conserved molecules derived from microbes. PAMPs activation links to the innate immune system, results in the production of proinflammatory cytokines and the expression of antimicrobial genes. Activation of PAMPs also plays a key role in shaping adaptive immune response. There are more than 10 families of TLR ligands found until now; these TLR ligands include DNA of bacteria, RNA of virus and lipopolysaccharide (LPS) produced by most Gram-negative bacteria and Gram-positive bacteria. TLR ligands have the function of maintaining normal immune function, however, too much TLR ligands may result in immune activation and cause disease pathogenesis. A certain and low level of TLR ligands in human body is a consequence of living in a nonsterile environment, however, systemic heightened level of TLR ligands are believed to be a consequence of the impaired mucosal integrity, especially in the gut (6, 7).
TLR ligands can be found in bacteria, fungi and also viruses and provide, via TLR binding, early recognition of microbial invasion. TLR1, TLR2, and TLR6 are triggered by peptidoglycan and other microbial products, TLR3 by double-stranded RNA, TLR4 by lipopolysaccharide (LPS), TLR5 by flagellin, TLR7 and TLR8 by imidazoquinolines, and TLR9 by unmethylated CpG DNA (8). All TLRs, except TLR3, activate the adaptor molecule (MyD88)-dependent pathways that culminates in the activation of nuclear factor–kB (NF-kB) transcription factors, as well as the mitogen-activated protein kinases (MAPKs) extracellular signal–regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) (8-11). These transcription factors function in concert to promote inflammatory responses (e.g. IL-6, IL-10 and TNF-a) (12-14). HIV itself is also functional as TLR ligands since RNA sequences derived from the HIV genome are capable of signaling through TLR 7 and TLR 8 (15).

**Human B lymphocytes and TLRs.**

Human B lymphocytes produce antibodies against invading pathogens that are key to induce humoral immune response. Human B cells express high levels of TLR1, TLR6, TLR9 and TLR10, intermediate levels of TLR7, and low levels of TLR2 and TLR4 (16). Most B cell studies focus on TLR9, the main TLR on human B cells, which binds to bacterial DNA (17-21). It has been demonstrated recently that switched and IgM-positive memory B cells constitutively express Toll-like receptor 9 (TLR9) (22), and it has been suggested that the repeated stimulation through unmethylated CpG may function to continuously and specifically restimulate B cells and maintain serologic memory in the absence of traditional
protein antigens (23). In the model, serum IgG antibodies constitute the specific memory (with B cell receptor signals) built up by previous experience and vaccination, and IgM antibodies may represent first-line memory, indispensable against pathogens never encountered before and T cell independent (TI) antigens. On the other hand, naïve B cells are also reported to proliferate and produce IgM (not IgG) in response to CpG ODN (TLR9 ligand) alone from our previous work and others (19, 24). Furthermore, IgG class switch DNA recombination is induced among human naïve B cells in response to CpG ODN and IL-10 (25). Thus, TLRs may play a role in the autoimmune diseases those are present in HIV infection. However, the role of TLRs in autoimmune diseases is not clear and is controversial (26, 27). Importantly, TLR ligands enhance antigen-specific B cell responses through B cell receptor (BCR) stimulation (28). However, it remains to be demonstrated whether TLRs are important in specific recall reactions induced by either infection or vaccination (TLR ligands as adjuvants).

**Chronic immune activation in HIV infection**

HIV infection induces broad immune perturbation, including dysfunction and loss of CD4+ T cells and B cells (29). Although HIV virus infects CD4+ T cell, monocytes/macrophages and dendritic cells, the exact mechanisms of loss of CD4+ T cells, key cells to achieve adaptive humoral and cellular immune responses, are still not fully understood. In HIV infection, there is increasing consensus that immune activation is central to disease pathogenesis (30). Recent studies suggest that increased microbial products from the damaged gut in chronic HIV infection are at least partially responsible to chronic immune activation (31, 32). High levels of the Toll-like receptor 4 ligand,
lipopolysaccharide (LPS) and bacterial ribosomal 16S RNA are found in the plasma of individuals with chronic HIV infection. Levels of LPS and 16S rDNA are correlated with indices of immune activation and predict the magnitude of immune restoration after 48 weeks of antiretroviral treatment. The present study proposes that a similar (but somewhat more complex) mechanism may contribute to B cell perturbations in HIV infection (31, 32).

B cell perturbations in HIV disease

B cells are antibody-producing cells; antibodies have the function of killing pathogens. Perturbations of B cells will result in infection or autoimmune diseases. In HIV disease, there are three main B cell perturbations including polyclonal B cell activation, peripheral memory B cell depletion and impaired recall antibody responses (33-37). Although the lack of CD4+ T cell help may explain some of these deficiencies, there also appear to be intrinsic defects in B lymphocytes that can be demonstrated in functional assays that do not require T helper cells (38). The functional defects that have been described in B cells from HIV-infected persons include impaired proliferation responses (to B cell antigen receptor stimulation, CD40L and CpG ODNs), reduced antibody production following vaccination, B cell hyperactivation and hypergammaglobulinemia, and increased susceptibility to spontaneous apoptosis (39-41). B cell hyperactivation in HIV disease may lead to the increased susceptibility of these cells to apoptosis, and may also contribute to impaired immune responsiveness.

The effects of B cell depletion and impaired HIV-specific antibodies on SIV/HIV pathogenesis and disease progression are not clear and the results are
contradictory (42-46). However, B cells are depleted and functionally impaired in pathogenic SIV-infected rhesus macaques, but not in non-pathogenic African green monkeys (36, 47-50). Non-pathogenic SIV-infected animal models also do not demonstrate gut damage or increased systemic levels of microbial products (31, 49). B cell apoptosis is rare in non-pathogenic SIV-infected monkeys in the absence of gut enteropathy, B cell apoptosis is present in pathogenic SIV-infected monkeys with microbial translocation, suggesting an important hypothesis in the present study that HIV itself rarely induce B cell death unless co-cultured with TLR ligands.

B cells may also be activated and functionally impaired by HIV itself. It was demonstrated that B cells from HIV-infected viremic patients carry replication-competent virus on their surface through CD21, a complement receptor. It was also found that virus bound to B cells could efficiently infect activated CD4 T cells and cause B cell dysfunction (51, 52). There are other mechanisms involved in HIV-associated B cell dysfunction. HIV interacts with CXCR4 (53) on the B cell surface and induces B cell apoptosis. HIV-1 nef protein can activate and stimulate B cells to differentiate (54, 55) as reflected by polyclonal activation (hyperimmunoglobulinemia) in HIV infection (34, 56). In contrast, a recent study by Qiao et al shows that HIV nef protein directly inhibits B cell functional class switch (57). However, the mechanisms of HIV-associated B cell defects are not completely understood.

Microbial translocation may play an important role in HIV-associated B cell perturbations. Loss of memory B cells and reduced production of antigen-specific antibody are seen in the majority of chronic HIV infection even though the humoral
system is subject to repeated and long-term stimulation through TLR agonists released from the gut (31, 33, 34). This is not only due to desensitization since at the same time there is B cell polyclonal activation as reflected by increased total IgM and IgG levels (34, 56). Short-term exposure to TLR ligands (e.g. CpG ODNs) enhances immune responses and has adjuvant effects (19, 40, 58-60). However, chronic systemic exposure to microbial TLR ligands in HIV disease may have deleterious effects. Nevertheless, humoral immune dysfunction is present differently in HIV disease as reflected by enhanced ex vivo B cell apoptosis with reduced antigen-specific antibody production and polyclonal activation, as compared to other diseases occurring with microbial translocation (e.g. inflammatory bowel disease) where autoimmune disease appears to play an important role in immunopathogenesis and gut damage (61, 62). As neither CD4/B lymphopenia nor cell-mediated immune deficiency are recognized concomitants of untreated inflammatory bowel disease (63), it would appear that the virus maintains a central role in cellular and humoral immunodeficiency in HIV infection.

**The loss of memory B cells may be related to increased susceptibility of these cells to apoptosis**

Spontaneous B cell apoptosis ex vivo as measured by binding of annexin V is increased in acute and chronic HIV infection (36, 64). Several cell death signaling pathways have been implicated in HIV infection, such as TNFα/TNFR, TRAIL and Fas/FasL (65-71). Moreover, studies by Susan Moir and others indicate that enhanced CD95/Fas expression on B cells in treatment-naïve HIV+ donors is related to B cell apoptosis by exogenous Fas ligand in vitro (41). Fas is expressed
at low levels on the surface of naïve B cells and enhanced levels in memory B cells (72, 73). In contrast with Fas express, the expression of Fas ligand is reported to be much more restricted and often requires cell activation. Monocytes or macrophages are capable of producing Fas ligand after activation by opsonizedzymosan or HIV infection in vitro (74, 75). Importantly, in vivo treatment of anti-Fas ligand Ab (RNOK203) reduces cell death in circulating B cells from SIV-infected individuals and increases antibody responses to viral proteins (76). Thus, a Fas/FasL-induced cell signal may be involved in B cell death in HIV infection. In the present study, the hypothesis is that, memory B cell apoptosis is induced by HIV virus and bacterial LPS, and this effect is mediated by Fas/Fas ligand cell death signals. To test the hypothesis, the synergistic effect of HIV viral load and microbial LPS on memory B cell apoptosis in vitro will be investigated and the association of the percentage of memory B cell death and Fas expression on memory B cells and plasma level of Fas ligand in vivo.

**Enhanced memory B cell apoptosis may result in impaired antibody responsiveness to vaccination in HIV infection.**

A remaining gap in knowledge is the effect of antiretroviral therapy on microbial translocation and B cell restoration. Data from previous studies have shown that the levels of LPS and 16s rDNA in plasma are significantly reduced after initiation of antiretroviral therapy, but never decrease to normal even among patients with restored normal CD4 counts (31). Consistent with this, B cell recovery was slower than CD4 T cell recovery after antiretroviral therapy and was also never restored to normal (77, 78). Although the data relating to HIV-specific IgA are conflicting, it remains clear that the majority of chronically HIV-infected individuals do not mount
vigorous HIV-specific IgA antibody responses either locally in mucosal sites or systemically (45, 79-82).

Although short-term administration of HAART may improve antibody responses (83), long-term administration is still unable to maintain protective levels of antibodies against vaccination antigens like measles, tetanus, influenza and pneumococcus (33, 84). We therefore hypothesize that low levels of microbial translocation and HIV RNA in patient plasma after HAART may contribute to the incomplete recovery of antibody responses. The present study was designed to be better understood the mechanisms of memory B cell apoptosis in HIV disease. This knowledge would be valuable to improve vaccine responsiveness, decrease opportunistic infections, and slow down disease progression.
SPECIFIC AIMS

Specific aim1: To determine the synergistic effect and mechanisms of the induction of memory B cell apoptosis by HIV and LPS in vitro.

The study hypothesis is that neither HIV alone nor LPS alone, but rather the combination of HIV and LPS together induce memory B cell death. This effect is through Fas/Fas ligand cell death pathway.

Specific aim2: The interaction of plasma levels of FasL and Fas expression on memory B cells predict the magnitude of memory B cell apoptosis enhanced in vivo in HIV disease.

The study hypothesize is that the in vitro findings from aim1 will reflect biologic mechanisms of memory B cell death in HIV infection in vivo. Therefore plasma levels of FasL and Fas expression on memory B cells will operate synergistically to induce memory B cell apoptosis. A linear regression model will be used to define the predictors of memory B cell death in a cross sectional study among HIV-negative controls and viremic HIV+ HAART-naïve donors.
STUDY METHODS

Study site
This study was conducted in Case Western Reserve University, University Hospital, Case Medical Center.

The study population
Twenty HIV-negative control subjects were recruited from the Case Western University Student and Employee Health Services; these controls matched by age and demographic profile. Twenty patient population consists of 43% Caucasian, 48% African-American, 4% Hispanic, 0.5% Asian, and 5% other. Approximately 80% of our patients are male. Patients were recruited from Case Western Reserve University, University Hospital.

Case selection
Inclusion criteria.

HIV+ donors
- HIV-1 infection, documented by any licensed rapid HIV test or HIV enzyme or chemiluminescence immunoassay (E/CIA) test kit at any time prior to study entry and confirmed by a licensed Western blot or a second antibody test by a method other than the initial rapid HIV and/or E/CIA, or by HIV-1 antigen, plasma HIV-1 viral load.
- Eligible HIV+ subjects will be not receiving antiretroviral therapy for at least 6 months.
- Peripheral CD4 counts are above 300 cells/ul, obtained within 30 days prior to study entry at any laboratory that has a CLIA certification or its equivalent.
- Laboratory values include Absolute neutrophil count (ANC) \(\geq 750/mm^3\), hemoglobin \(\geq 8.0\) g/dL
- Female study subjects with a negative serum or urine pregnancy test
- Platelet count \(\geq 50,000/mm^3\)
- No history of AIDS-related opportunistic infections
- Age \(\geq 21\) years
- Ability and willingness of subject or legal guardian/representative to provide informed consent.

**HIV-negative donors**

- HIV infection status is self-report
- Age \(\geq 21\) years
- Ability and willingness of subject or legal guardian/representative to provide informed consent

**Exclusion criteria**

- Pregnancy
- Use of immunomodulatory drugs in the last 3 months
- Cancer requiring treatment
- Significant anemia
- Severe illness
• Inability to provide informed consent

Protection of human subjects

The Institutional Review Board of Case Western Reserve University and University Hospital approved this study (01-98-55). Subjects were informed about the study and asked to sign an IRB approved informed consent/HIPAA document. Subjects were consented in a private exam room in the Family Medicine or the Special Immunology Unit (SIU) at University Hospitals Case Medical Center (UHCMC) by the research assistant/nurses or Researchers/Investigators on the staff trained in Human Subject Protections. Subjects were given time to read the consent, asked questions and considered the risks and/or benefits to participation in this research study prior to obtaining their signature. All subjects enrolled in the study were given a copy of their signed and dated informed consent document. This study presented no more than minimal risk or harm to the participants.

Protected health information was collected, including any information about health status, provision of health care, or payment for health care that can be linked to a specific individual. At the time of enrollment, study participants were given a unique identifier that were used to label their study samples. Identifying information, such as the participant's name, date of birth or medical record number was not recorded on any of the data collection forms. Personal identifiers were not entered into the study data set and were not used during analysis, summary, or reporting of the study findings.
All study materials were locked in the file cabinets in the principal investigator’s office. Documentation of personal identifiers including the participant’s unique identifiers was stored electronically in a password-protected file saved on a secure server maintained by the university. The principal investigator had undergone the Collaborative Institutional Training Initiative (CITI) training as well as the investigator-training curriculum provided by the university in the protection of human subjects.

**Statistical Analysis**

**Independent and dependent variables in the regression model**

Independent variables for the study included the percentage of Fas-positive memory B cells, Fas geometric mean expression on memory B cells, their interactions, age, gender, peripheral CD4 counts and HIV status. Dependent variables were the percentage of annexin V binding among memory B cells. The residuals and outliers were shown in the residual plots.

**Regression model building, checking and diagnosis**

- Model selection

  The purpose of choosing a model for a data set is to select variables that best describe the dependent variable. To avoid over fitting, model selection should be simplified (88).

  Linear regression describes the linear relationship between one variable and the other continuous variables; this includes one dependent variable
(Y) and the other predictors (X₁ to Xₖ). When the dependent variable and some predictive variables are related, it is possible that independent variables predict a dependent value with better than chance accuracy.

Linear regression provides the line that "best" fits the data. This line can then be used to examine how much changes of the predictors predict the outcome.

The regression equation is an algebraic representation of the regression line and is used to describe the relationship between the dependent variable and predictor variables. The regression equation is of the form:

$$\hat{y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \ldots + \beta_k X_k$$

Where, dependent variable ($\hat{y}$) is the estimated value of the outcome. Constant ($\beta_0$) is the coefficient of the predictor variable when the independent variables are zero, $\beta_0$ is also called the intercept of the $\hat{y}$.

Independent variable (X) is the value of the predictor. Coefficients ($\beta_1, \beta_2, \beta_3 \ldots \beta_k$) represent the estimated change in mean response for each unit change in the independent value.

Multiple linear regressions test the linear relationships between one dependent variable and two or more predictors. Prior to fitting a linear regression model with all the predictors, forward selection approach was used which involved starting with no variables in the model, then adding the variables one by one and including them if they were statistically
significant (p value \leq 0.10). This was an automatic procedure, at each stage in the process, after a new variable was added; a test was made to check if some variables could be deleted without appreciably increasing the residual sum of squares. The procedure was ended when the measure was maximized or the available improvement falls below some critical value. The Least Squares were used in the regression model because it did not require a normal distribution for statistical analysis.

In the present study, the model selection was based on cell biologic function, the interaction of plasma level of FasL and Fas expression on memory B cell death, therefore the main effects included plasma level of FasL, Fas expression on memory B cells, and their interaction.

- Missing value data

Missing values in multivariate data is a common occurrence in statistical analysis. It could be an issue when the missing data is not at random. When the inputs are not random missing from data observations, it becomes very difficult to predict the desired value in prediction. Some of the widely used missing value techniques are Imputation, Full information maximum likelihood estimation, Indicator variable, and various ad hoc practices such as List wise deletion, Case wise depletion (Schafer 1998). In the present study, the missing data was assumed to be missing completely at random, because equipment malfunctioned or people got sick. Thus this assumed the probability that an observation was missing was unrelated to the value of any other values within the same variable or any other variables.
• Modeling building and diagnosis

Microbial LPS and HIV virus synergistically induce memory B cell death through Fas/Fas ligand cell death signaling pathway is the primary hypothesis in the present study. Due to the technical difficulties of measuring plasma level of bacterial LPS, defining plasma level of Fas ligand and the susceptibility (Fas) of memory B cells to Fas ligand were tested in vivo in the regression model.

Phase I. Plasma levels of Fas ligand and Fas expression on memory B cells were added as main predictors. The synergistic effects (interaction) of plasma levels of Fas ligand and Fas expression on memory B cells would expect to be significant (p < 0.05, two-sided) in this model.

Phase II. The primary analysis examined whether there was synergistic interaction for plasma levels of Fas ligand and Fas expression on memory B cells. That was, the percentage of memory B cell death expected to be higher than what predicted from just the additive individual effects of plasma levels of Fas ligand and Fas expression on memory B cells. In a more exploratory fashion, possible predictors (e.g. peripheral CD4 T cell counts) that may mediate how plasma levels of Fas ligand and Fas expression on memory B cells were tested their effects on the percentage of memory B cell death. Depends on the results in Aim1, If adding peripheral CD4 T cell counts to the model developed in Phase II reduced the association of plasma levels of Fas ligand and Fas expression on memory B cells with the percentage of memory B cell death, this result
would support further investigations into whether the percentage of memory B cell death was more directly related to plasma levels of Fas ligand and Fas expression on memory B cells.

The primary analysis used regression models to examine how plasma levels of Fas ligand and Fas expression on memory B cells were related to the percentage of memory B cell death in HIV+ treatment-naïve donors using HIV-negative donors as a control group. In addition, strong multiplicative interactions of plasma levels of FasL and Fas expression on memory B cells are expected to see. Other predictors—peripheral CD4+ T cell counts (provide help and potentially effect on B cell survival), plasma levels of HIV RNA and clinical characteristics [e.g. age (age will have an effect on B cell function (85)) and gender (different gender has different responses to TLR7 signals (86, 87))]—served as covariates to assess confounding effects. Data from all variables were graphed and the influential associations were ascertained in the regression modeling.

In the present study, SAS program “Proc Reg” provided least squares regression analysis to determine the relationship between the percentage of memory B cell death and independent variables. All calculations of Proc Reg used a forward method to systematically add independent variables and potential covariates with the most predictive power until remaining variables have a p value ≤ 0.10, no further variables can be entered with P < 0.05. Within the final analysis of the multiple linear regression, any association with a p value ≤ 0.05 was considered statistically significant. Importantly, all variables with biological functions were included in the model even if they were non-statistically significant.
The percentage of B cell death was not expected to correlate inversely with the numbers of B cells in circulation as in the model; these numbers were driven down by the influence of HIV RNA and bacterial products over time.

Experiments

• PBMC isolation
  1. Peripheral blood mononuclear cells (PBMC) were isolated from healthy and HIV-infected subjects by Ficoll-Hypaque.
  2. 10mL Ethylenediaminetetraacetic acid (EDTA) whole blood was spin down at 400x g for 10 minutes at room temperature. EDTA was used for blocking coagulation.
  3. Plasma was collected, aliquot, and stored at -80 degree for later analysis.
  4. A 50 mL Falcon tube was used to dilute remaining blood with RPMI1640 to 30mL, the diluted blood was mixed by pipetting.
  5. 10 ml Ficoll Hypaque solution was added and slowly overlay diluted blood on top of Ficoll Hypaque.
  6. The mixed Ficoll, blood and RPMI solution was spin at 400x g for 30 minutes at room temperature (no brake).
  7. Inter-phase PBMCs were harvested and transferred to a new 50mL tube, diluted with RPMI1640 to 50mL, and spin down at 400x g for 8 minutes at room temperature.

• In vitro assay
1. Supernatant from the above PBMC isolation was decanted and the pellet was re-suspended by completely media (10% Fetal bovine serum (FBS) + 1% L-glutamine + 1% antibiotics).
2. Cells were counted and added in a 96-well plate in 37°C incubator.
3. A concentration of $0.2 \times 10^6$/well isolated PBMC were cultured with medium, HIV virion, LPS or HIV plus LPS for 30 h in vitro.
4. Cells were harvested and examined for annexin V binding, Fas expression, CD27 and CD19 expression using flow cytometry.

• Ex vivo assay

1. Surface antibodies (CD20-PEcy5 and CD27-APC, and isotype controls) were added to 100ul EDTA coated blood.
2. Cultured in room temperature for 10 minutes
3. Then blood was lysed in lysis buffer and cultured in room temperature for 10 minutes
4. Added wash buffer and spin down
5. Decanted the supernatants
6. Fixed with 1% paraformaldehyde
7. Run in a FACScan flow cytometer (Becton-Dickinson)

• Cell apoptosis

1. Cells were wash by annexin staining buffer
2. Then stained with the annexin-V apoptosis reagents (BD Bioscience, CA) in room temperature for 15 minutes
3. Added annexin staining buffer 200ul
4. Directly analyzed by flow cytometry.

• Plasma level of Fas ligand
ELISA measured soluble Fas ligand in plasma.

1. Brought all reagents and samples to room temperature before use.
2. All samples, controls, and standards were assayed in duplicate.
3. Added 100 uL of Assay Diluent RD1S to each well.
4. Added 50 uL of Standard, control, or sample per well. Covered with the adhesive strip provided.
5. Incubated for 2 hours at room temperature.
6. Aspirated each well and washed, repeating the process three times for a total of four washes.
7. Washed by filling each well with Wash Buffer (400 uL) using a squirt bottle, manifold dispenser, or autowasher.
8. Completed removal of liquid at each step is essential to good performance.
9. After the last wash, removed any remaining Wash Buffer by aspirating or decanting.
10. Inverted the plate and blotted it against clean paper towels.
11. Added 200 uL of Fas Ligand Conjugate to each well. Covered with a new adhesive strip.
12. Incubated for 2 hours at room temperature.
13. Repeated the wash step. Added 200 uL of Substrate Solution to each well.
14. Incubated for 30 minutes at room temperature. Protected from light.
15. Added 50 uL of Stop Solution to each well. The color in the wells changed from blue to yellow.
16. Gently tap the plate to ensure thorough mixing.
17. Finally determined the optical density of each well, using a microplate reader set to 540 nm or 570 nm.

**Flow cytometry**

*Sample preparation*

1. For each 1 ml of blood, add 14 ml of room temperature FCM Lysing solution to lyse the red blood cells.

2. The cells did not lyse correctly if the solution was cold. Incubated for 5 minutes at room temperature on a rotator.

3. Centrifuged for 5 minutes at 1000 RPM for human blood. Carefully aspirated supernatant, then resuspended pellet in approximately 50 ml cold 1X PBS. Took a small sample to perform a cell count.

4. Centrifuged for 5 minutes at 1000 RPM for human blood.

5. Aspirated supernatant.

*Cell stimulation*

Stimulated cells as necessary.

*Stain preparation*

1. Once supernatant was aspirated from cell preparation, resuspended pellet in enough 1X PBS to have a final cell concentration of 10 million cells/ml.

2. Blocked by incubating the cell suspension with 1 mg of FcR blocking antibody per 1 ml of cell suspension for 10 minutes. Did not rinse. Proceeded with staining. Fixed and Permeabilized Cells for Intracellular Staining
3. Once supernatant was aspirated from cell preparation, resuspended pellet in enough 1X PBS to have a final cell concentration of 10 million cells/ml.

4. Block by incubating the cell suspension with 1 µg of FcR blocking antibody per 1 ml of cell suspension for 10 minutes.

5. Resuspended pellet in approximately 50 ml 1X PBS to wash away any excess blocking antibodies.

6. Centrifuged for 5 minutes at 1000 RPM.

7. Once supernatant was aspirated from cell preparation, resuspended pellet in FCM Fixation Buffer. Use 1mL per million cells.

8. Incubated for 30 minutes at room temperature on a rotator.

9. Centrifuged for 5 minutes at 1500-2000 RPM. Cells got more buoyant after fixation. If pellet was too small, spin again at a higher RPM, but did not exceed 3000 RPM.

10. Pour off supernatant. Cells may be lost if aspirating from this point on, so always decant. Used a quick motion and didn’t allow the supernatant to wash back and forth over the cells.

11. Resuspend pellet in approximately 50 ml 1X PBS to wash away any excess Fixation Buffer.

12. Centrifuged for 5 minutes at 1500-2000 RPM.

13. Decant supernatant. At this point, cells can be resuspend in a small amount of PBS and stored for up to 1 month at 4° C. To permeabilize at this time, proceeded to next step.
14. If cells had been stored in PBS, centrifuged for 5 minutes at 1500-2000 RPM and decant supernatant.

15. Broke up cell pellet and drop wise add the same amount of cold (stored at -20° C) FCM Permeabilization Buffer at 1 ml per 1 million cells. Vortexed while adding.

16. Incubated for 5 minutes only at RT on a rotator.

17. Immediately centrifuged for 5 minutes at 2000-2500 RPM.

   Cells were more buoyant after permeabilization and much care must be exercised to maintain volume of cells.

*Staining*

1. Labeled tubes.

2. Added 20 µl of fluorochrome-conjugated antibodies to tubes.

3. Added 100 µl of the prepared cell suspension (equal to 1 million cells) to each tube.

4. Vortexed and incubate for 15-30 minutes in a covered ice bucket.

5. To wash off excess antibody following staining, added 1.5-2 ml of 1X PBS to each tube.

6. Centrifuged in tabletop microfuge for 5 minutes at 2000 RPM.

   This speed should be increased to 3000 or 4000 RPM for intracellular staining.

7. Aspirated supernatant, being careful not to disturb pellet.

8. Resuspended pellets in 500 µl of 1% paraformaldehyde.

   Tubes can be stored in the dark for 24 hours (maximum for intracellular staining) to 1 week (maximum for surface staining).
The following monoclonal Abs were used for analysis in this study: anti-annexin V-FITC, anti-CD27-APC, anti-IgD-PE, Fas-PEcy5 and anti-CD20-PEcy5 and appropriate isotype-matched control monoclonal Abs.

Limitations and alternative approaches
The inter-assay variability of LPS Limulus amebocyte assay is about 25% (32), and there are technical difficulties to accurately conduct. Therefore, it was difficult to include in vivo LPS level as an independent variable in the regression model. Alternatively, the interplay between memory B cell apoptosis and defined cell death pathway involved in HIV/LPS-induced memory B cell death (Fas/FasL) were tested. The association between memory B cell death and Fas/Fas ligand may not exactly reflect the interaction between memory B cell death, HIV replication and plasma level of bacterial LPS, but may more accurately reflect biologic mechanisms of memory B cell death in HIV disease.
RESULTS

Data description

Table 1. Characteristics of HIV-negative control and HIV-infected subjects.

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 20)</th>
<th>Controls (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13 (65%)</td>
<td>7 (35%)</td>
</tr>
<tr>
<td>Female</td>
<td>7 (35%)</td>
<td>13 (65%)</td>
</tr>
<tr>
<td>Age no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30</td>
<td>5 (25%)</td>
<td>7 (35%)</td>
</tr>
<tr>
<td>30 - 39</td>
<td>6 (30%)</td>
<td>7 (35%)</td>
</tr>
<tr>
<td>40 - 49</td>
<td>7 (35%)</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>&gt; 50</td>
<td>3 (15%)</td>
<td>2 (10%)</td>
</tr>
</tbody>
</table>

Table 1 summarizes the distribution of the study subjects by age and gender. A total of 20 healthy controls and 20 viremic antiretroviral treatment-naïve HIV+ subjects were studied in the present project. Among controls, 13/20 were women, and among patients, 7/20 were women. The median age of cases was slightly higher, 39.5 (interquartile range (IQR): 28 – 42.75) than controls 32 (IQR: 29 – 44.5). This difference was not statistically significant (P > 0.05). The percentage of males in cases was higher than that in controls, this difference was marginally statistically significant (Chi-Square test, two-tailed test, P = 0.06). Although women were included more in control group than patient group, no clear evidence indicates that there is a gender difference in HIV disease progression.

Table 2. Dependent variable and independent variables.
### Controls (n = 20) vs Cases (n = 20) Median IQR (25-75) Median IQR (25-75) p value

<table>
<thead>
<tr>
<th>Metric</th>
<th>Median</th>
<th>IQR (25-75)</th>
<th>Median</th>
<th>IQR (25-75)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>%mBapop</td>
<td>20.70</td>
<td>15.83 - 27.93</td>
<td>43.00</td>
<td>24 - 48</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>FasGeomeanmB</td>
<td>29.45</td>
<td>24.88 - 37.48</td>
<td>152.00</td>
<td>57 - 239</td>
<td>0.02</td>
</tr>
<tr>
<td>%FasmB</td>
<td>72.50</td>
<td>60.25 - 79.75</td>
<td>83.50</td>
<td>66.5 - 93.88</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>PlasmaFasL</td>
<td>89.30</td>
<td>66.58 - 109.21</td>
<td>91.05</td>
<td>69.36 - 127.3</td>
<td>0.56</td>
</tr>
<tr>
<td>CD4 counts (cells/ul)</td>
<td>na</td>
<td>na</td>
<td>482.00</td>
<td>378.5 - 505</td>
<td></td>
</tr>
<tr>
<td>PlasmaViral Load (copies/ml)</td>
<td>na</td>
<td>na</td>
<td>12084.00</td>
<td>5190 - 33270</td>
<td></td>
</tr>
</tbody>
</table>

%A_Bapop: percentage of memory B cell apoptosis among total memory B cells  
FasGeomeanmB: Fas geometric mean of fluorescence intensity of memory B cells  
%FasmB: percentage of Fas-positive memory B cells among memory B cells  
PlasmaFasL: plasma level of Fas ligand (pg/ml)  
CD4 counts: peripheral CD4+ T cell absolute number (cells/ul)  
PlasmaViral load: plasma level of HIV RNA (copies/ml)  

A cross-sectional study was carried out to investigate the associations between risk factors and outcome of memory B cell death in HIV disease. The data included all subjects conducted for the outcome, memory B cell apoptosis, Fas expression on memory B cells and plasma level of Fas ligand. Viremic antiretroviral treatment-naïve HIV+ subjects were selected because they represent natural disease progression without drug interaction.

Overall, HIV-infected patients had enhanced memory B cell apoptosis (median 43.0% (IQR: 24.0 - 47.6) vs 20.7% (IQR: 15.8 - 27.9)), increased percentage of Fas-positive memory B cells (median 83.5% (IQR: 66.5 – 93.9) vs 72.5% (IQR: 60.3 - 79.8)) and Fas expression of geometric mean on memory B cells (median 152 (IQR: 57 - 239) vs 29.5 (IQR: 24.9 – 37.5)) compared to controls, however, plasma level of Fas ligand was at similar level compared to controls (median 91.05 pg/ml (IQR: 69.4 – 127.3) vs 89.3 pg/ml (IQR: 66.6 – 109.2)).
The synergistic effect of HIV and LPS on the induction of memory B cell death in PBMCs in vitro

To test cell apoptosis (Fig. 1), cells were tracked from annexin V and 7-Amino-actinomycin (7-AAD). Viable cells were annexin V negative and 7-AAD negative, early apoptotic cells (membrane integrity is present) were annexin V positive and 7-AAD negative, and end stage of apoptotic and death cells were annexin V and 7-AAD positive (Fig 1A). The movement of cells through these three stages suggested apoptosis (89). Since annexin V-binding represented apoptosis including early stage and late stage, for the next assay, only annexin V was used to define apoptotic cells.

The optimal concentration of HIV and LPS in vitro was titrated and was used in the later experiment. LPS was used at 20 ng/ml as optimal concentration and HIV was used at 150 ng/ml as optimal concentration. Importantly, concentrations of LPS (200 pg/ml) found to have a modest effect on memory B cell death in the presence of HIV were also observed in sera from HIV-1-infected viremic individuals (31), suggesting LPS could have a similar effect on memory B cell in vivo.

Peripheral blood mononuclear cells (PBMC) were cultured with HIV virion and bacterial LPS for 30 hours, memory B cells were gated on CD20+CD27+, apoptotic cells were tested by annexin V binding by flow cytometry. Enhanced Annexin V staining in memory B cells were detected by co-culturing with HIV and LPS, but not HIV or LPS alone (Fig. 1B). The median percentage (IQR: 25%-75%) of memory B cell apoptosis in PBMCs was 22 (17.1-30.8) vs 23.5 (19.3-29.8) vs
22.5 (16.5-28.8) vs 35.5 (26.8-44) for medium, in the presence of HIV, LPS or both respectively. Only the comparison between medium and both HIV and LPS was significant (P = 0.005). In contrast, the median percentage (IQR) of naive B cell apoptosis in PBMCs was 17.4 (12.1-26) vs 21 (13.3-30.8) vs 17.9 (13.5-27.4) vs 22 (15.2-27.5) for medium, in the presence of HIV, LPS or both respectively. None of the two comparisons was significant (P > 0.05). This experiment was done in 13 different healthy control donors. These results together suggested that HIV and LPS cooperated to induce memory B cell death in vitro.

Fig 1. Memory B cell apoptosis induced by HIV and LPS in PBMCs in vitro. PBMCs were cultured with HIV and/or LPS for 30 h. Naive B (A, nB, CD20+CD27-) and memory B (B, mB, CD20+CD27+) cells were evaluated for annexin V binding by flow cytometry. The percentages of annexin V binding were shown for nB and mB. Data shown were analyzed by a nonparametric Mann-Whitney test.
Monocyte-mediated memory B cell death

Human B cells however express low to undetectable levels of TLR4 and do not respond to its ligand LPS (90). Monocytes are the predominant cell population that express TLR4 in PBMCs (16, 91). Therefore memory B cells were tested their apoptosis by HIV and LPS in the mixed cell population with monocytes or in the purified population of B cells (Fig. 2). Monocytes and B cells were isolated from PBMCs using isolation kits from Miltenyi Biotec (Germany). The purity of monocytes was above 90% and the purity of B cells was above 99%. Memory B cell apoptosis was tested in the purified B cells or in the mixed cell population of B cells and monocytes in the presence of HIV and/or LPS for 30 hours. These results (Fig. 2) showed that monocytes were necessary to drive memory B cell death. The magnitude of apoptosis by HIV and LPS in B cells with monocytes (Fig. 2) is less than B cells in the PBMC (Fig. 1), suggesting that other cells besides monocytes also have effects on memory B cell apoptosis.
The induction of memory B cell apoptosis by HIV and LPS was mediated by Fas/FasL cell death pathway

Increasing evidence suggests that Fas/FasL cell death signal is involved in B cell death in HIV/SIV infection (41, 76, 92). Fas ligand is a cell killer that binds to its receptor Fas expressed on cell surface, and this Fas/FasL interaction can lead to cell apoptosis. To investigate the cell death signaling pathway, PBMCs were incubated with isotype antibody IgG1, blocking antibody against Fas, Fas ligand or both for four hours, then added medium, HIV, LPS or HIV+LPS and cultured another 30 hours. The induction of memory B cell death by HIV+LPS was reduced by blocking Fas/FasL signaling pathway (Fig. 3), suggesting that Fas/FasL pathway is mediated in memory B cell death induced by HIV/LPS in PBMCs.
HIV and LPS synergistically induce Fas expression on memory B cells, and Fas expression was related to the induction of memory B apoptosis in vitro.

Next, to investigate whether Fas expression is induced in memory B cells by HIV and LPS, and whether Fas surface expression on memory B cells is related to cell susceptibility to apoptotic induction, PBMCs were cultured in the presence of medium, LPS, HIV, HIV and LPS for 30 hours, Fas surface expression was tested on memory B cells, as well as memory B cell apoptosis was tested by annexin V binding by flow cytometry. These results indicate that Fas expression was increased in memory B cells in response to both HIV and LPS in PBMCs and its
induction was associated with the induction of memory B cell death through stimulation with HIV and/or LPS (Fig. 4, subtract medium values).

**Enhanced ex vivo Fas expression and apoptosis on memory B cells in viremic antiretroviral treatment-naïve HIV+ donors**

To investigate the interplay between memory B cell death and Fas/FasL cell death signal on memory B cells in vivo, the interplay between memory B cell death, circulating B cell counts (Fig. 5A), Fas expression on memory B cells (Fig. 5B and 5D), the percentage of memory B cell death (Fig. 5C) and plasma levels of FasL (Fig. 5E) were tested in vivo or ex vivo. These results indicate that memory B cells are activated, lose resistance to cell apoptosis and depleted in HAART-naïve HIV+ donors.

![Image](image_url)
Correlations between dependent variable and independent variables

Our data indicate that Fas/Fas ligand cell death pathway mediated memory B cell apoptosis by HIV and LPS in vitro, and in vivo or ex vivo, Fas expression was enhanced in HIV-infected subjects compared to controls, as well as memory B cells binding to annexin V was enhanced in HIV-infected subjects compared to controls, therefore the correlations between memory B cell death and these potential mediators, including Fas expression on memory B cells (the percentage of Fas-positive memory B cells or Fas geometric means), plasma levels of Fas ligand, CD4 T cell counts, gender and age were tested among all subjects (Table 3), HIV-negative subjects (Table 4) and HIV-infected subjects (Table 5).

In table 3 in all subjects, both Fas geomean expression ($r = 0.59$, $P < 0.001$) on memory B cells and plasma levels of Fas ligand ($r = 0.52$, $P < 0.001$) were significantly associated with memory B cell death, however, these correlations were not significant in separated groups (Table 4 and Table 5). Among HIV-infected patients, memory B cell death in each individuals were not significantly related to each covariance including CD4 T cell counts and plasma levels of HIV RNA (Table 5, or data not shown), suggesting that enhanced memory B cell death in HIV disease is related to Fas/Fas ligand cell death pathway compared to
controls, this effect may be not only mediated by Fas/Fas ligand cell death pathway, it may be more complex and including other factors.

Table 3. Correlation test in all subjects (Spearman correlation test)

<table>
<thead>
<tr>
<th></th>
<th>mBapop</th>
<th>FasL</th>
<th>PercentFas</th>
<th>GeomeanFas</th>
<th>age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
<td>n = 40</td>
</tr>
<tr>
<td>mBapop</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>r = 0.06</td>
<td>r = 0.05</td>
<td>r = 0.52</td>
<td>r = -0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.74</td>
<td>p = 0.73</td>
<td>p &lt; 0.001</td>
<td>p = 0.87</td>
<td></td>
</tr>
<tr>
<td>FasL</td>
<td>r = 0.59</td>
<td>r = 0.58</td>
<td>r = 0.03</td>
<td>r = 0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.84</td>
<td>p = 0.52</td>
<td></td>
</tr>
<tr>
<td>PercentFas</td>
<td>r = 0.05</td>
<td>r = 0.58</td>
<td>r = 0.58</td>
<td>r = 0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.73</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.10</td>
<td></td>
</tr>
<tr>
<td>GeomeanFas</td>
<td>r = 0.52</td>
<td>r = 0.03</td>
<td>r = 0.58</td>
<td>r = 0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.84</td>
<td>p &lt; 0.001</td>
<td>p = 0.47</td>
<td></td>
</tr>
<tr>
<td>age</td>
<td>r = -0.20</td>
<td>r = 0.10</td>
<td>r = 0.23</td>
<td>r = 0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.87</td>
<td>p = 0.52</td>
<td>p = 0.10</td>
<td>p = 0.47</td>
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</tr>
</tbody>
</table>

FasL: plasma level of Fas ligand (pg/ml)
PercentFas: percentage of Fas-positive memory B cells
GeomeanFas: Fas geo mean on memory B cells

Table 4. Correlation test in control subjects (Spearman correlation test)

<table>
<thead>
<tr>
<th></th>
<th>mBapop</th>
<th>FasL</th>
<th>PercentFas</th>
<th>GeomeanFas</th>
<th>age</th>
</tr>
</thead>
<tbody>
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<td>n = 20</td>
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<tr>
<td>mBapop</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>r = 0.25</td>
<td>r = -0.20</td>
<td>r = 0.06</td>
<td>r = 0.10</td>
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</tr>
<tr>
<td></td>
<td>p = 0.32</td>
<td>p = 0.40</td>
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<td>FasL</td>
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<td>r = -0.44</td>
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</tr>
<tr>
<td></td>
<td>p = 0.32</td>
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<td>p = 0.65</td>
<td>p = 0.05</td>
<td></td>
</tr>
<tr>
<td>PercentFas</td>
<td>r = -0.20</td>
<td>r = -0.21</td>
<td>r = 0.66</td>
<td>r = 0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p = 0.40</td>
<td>p = 0.38</td>
<td>p &lt; 0.001</td>
<td>p = 0.35</td>
<td></td>
</tr>
<tr>
<td>GeomeanFas</td>
<td>r = 0.06</td>
<td>r = -0.11</td>
<td>r = 0.66</td>
<td>r = 0.22</td>
<td></td>
</tr>
</tbody>
</table>
FasL: plasma level of Fas ligand (pg/ml)
PercentFas: percentage of Fas-positive memory B cells
GeomeanFas: Fas geometric mean on memory B cells

In figure 5, the percentage of Fas-positive memory B cells were not dispersed well in HIV-infected donors, in contrast, Fas geometric mean expression on memory B
cells were dispersed better in HIV-infected donors than controls. They are markers of Fas expression on memory B cells by different measurements. Therefore, Fas geometric mean expression on memory B cells was only selected in the final regression model as a marker for Fas expression.

A linear regression model indicated that Fas expression on memory B cells, plasma level of Fas ligand, and their interactions independently predict memory B cell apoptosis

In the present study, a linear regression model (forward regression) was analyzed for the predictors of memory B cell apoptosis.

Table 6. Independent variables include plasma level of FasL, Fas geometric mean expression on memory B cells, or HIV status among all subjects in a linear regression model by forward selection.

R-Square = 0.16, P = 0.008

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeomeanFas</td>
<td>0.07</td>
<td>0.03</td>
</tr>
</tbody>
</table>

GeomeanFas: geometric mean of Fas expression on memory B cells

R-Square = 0.007, P = 0.62

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td>0.05</td>
<td>0.1</td>
</tr>
</tbody>
</table>

FasL: Plasma level of Fas ligand (pg/ml)
R-Square = 0.35, P < 0.001

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIVstatus</td>
<td>20.9</td>
<td>4.4</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>

HIVstatus: HIV- or HIV+ subjects

R-Square = 0.17, P = 0.05

<table>
<thead>
<tr>
<th></th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td>0.06</td>
<td>0.1</td>
<td>0.52</td>
</tr>
<tr>
<td>GeomeanFas</td>
<td>0.07</td>
<td>0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>

FasL: Plasma level of Fas ligand (pg/ml)
GeomeanFas: geometric mean of Fas expression on memory B cells

Table 7. Independent variables include plasma level of FasL, Fas geometric mean expression on memory B cells and their interactions, and HIV status among all subjects in a linear regression model.

R-Square = 0.45, P = 0.001

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.001</td>
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<tr>
<td>HIVstatus</td>
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<td>6.3</td>
<td>0.008</td>
</tr>
<tr>
<td>GeomeanFas</td>
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<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>FasL</td>
<td>-0.25</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>FasL*GeomeanFas</td>
<td>0.003</td>
<td>0.001</td>
<td>0.01</td>
</tr>
</tbody>
</table>

FasL: Plasma level of Fas ligand (pg/ml)
GeomeanFas: geometric mean of Fas expression on memory B cells
HIVstatus: HIV- or HIV+ subjects

Table 8. Independent variables include plasma level of FasL, Fas geometric mean expression on memory B cells and their interactions among HIV-infected subjects alone in the linear regression model.
R-Square = 0.43, P = 0.057

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
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<tr>
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</tr>
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<td>GeomeanFas</td>
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<td>0.01</td>
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<td>FasL</td>
<td>-0.63</td>
<td>0.24</td>
<td>0.02</td>
</tr>
<tr>
<td>FasL*GeomeanFas</td>
<td>0.005</td>
<td>0.001</td>
<td>0.008</td>
</tr>
</tbody>
</table>

FasL: Plasma level of Fas ligand (pg/ml)
GeomeanFas: geometric mean of Fas expression on memory B cells

In table 7, adding HIV status made the model fit better (R-Square = 0.45, P = 0.001), as well as in table 8 among HIV+ subjects, the model also fits well (R-Square = 0.45, P = 0.057) without significantly effecting on the main predictors, suggesting that HIV status could play a role in the regression model. However, HIV status was significantly associated with the main effect, geometric mean expression of Fas (r = 0.75, P < 0.001, data not shown), and *in vitro* study, HIV and LPS enhanced Fas expression among memory B cells, therefore adding HIV status could over fit the model and HIV status was not included in the final model.

Table 9. Independent variables include plasma level of Fas ligand, Fas geometric mean expression on memory B cells and their interactions among all subjects in a linear regression model (final model)

R-Square = 0.29, P = 0.01

<table>
<thead>
<tr>
<th></th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FasL</td>
<td>-0.19</td>
<td>0.14</td>
<td>0.19</td>
</tr>
<tr>
<td>GeomeanFas</td>
<td>-0.18</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td>FasL*GeomeanFas</td>
<td>0.003</td>
<td>0.001</td>
<td>0.03</td>
</tr>
</tbody>
</table>

FasL: Plasma level of Fas ligand (pg/ml)
GeomeanFas: geometric mean of Fas
FasL*GeomeanFas: interaction between plasma Fas ligand and Fas Geomean expression on memory B cells
When plasma level of Fas ligand and Fas geometric mean expression on memory B cells were selected as independent variables in the model, only Fas geometric mean expression on memory B cells was significantly positively associated with the estimated percentage of memory B cell apoptosis (Table 8, $P = 0.02$), this was consistent with the association between the dependent variable and Fas geometric mean expression (Table 3, $r = 0.52$, $P < 0.001$), suggesting that the Fas death receptor expression is important for the susceptibility of cell death by FasL.

Moreover, when plasma level of Fas ligand, Fas geometric mean expression on memory B cells, and their interaction were selected as independent variables in the model, neither Fas expression by geometric mean on memory B cells (Table 9, $P = 0.12$) nor plasma level of Fas ligand (Table 9, $P = 0.19$) was significantly associated with the percentage of memory B cell death, but their interaction significantly related to the dependent variable (Table 9, $P = 0.03$), also the regression model fits well in the dataset (R-Square = 0.29, $P = 0.01$), suggesting that their interaction independently and positively predict memory B cell apoptosis. The regression equation is shown below:

$$m\text{Bapop} = 41 - 0.19\text{FasL} - 0.18\text{GeomeanFas} + 0.003\text{FasL} \cdot \text{GeomeanFas}$$

From this model, if plasma level of Fas ligand is 89.3 pg/ml, Fas geometric mean is 29.45 the same as shown the median values in controls (Table 2), then the estimated percentage of memory B cell apoptosis is 26.6%. If plasma level of Fas ligand is fixed as 89.3 pg/ml, every 10 units of increases in Fas geometric mean
will result in about 1 more percentage of apoptotic memory B cells. In table 2, plasma levels of Fas ligand were similar in controls and patients (89.3 pg/ml vs 91.05 pg/ml); Fas geometric mean expression was lower in controls than in patients (29.45 vs 152). From this model, Fas expression as geometric means on memory B cells in patients are about 120 higher than controls, thus patients should have 12% more apoptotic memory B cells than controls. In the observed values in table 2, controls have 20.7% apoptotic memory B cells versus patients have 43% apoptotic memory B cells ex vivo, suggesting that other mechanisms also contribute to memory B cell death in HIV disease. In conclusion, Fas geometric mean expression plays an important role for the susceptibility of memory B cells to die by Fas ligand (Table 8 and 9), and plasma level of Fas ligand also involves in memory B cell death because of its interaction with the receptor (Fas geometric mean expression, table 3 and 9). The predictive effect of FasL, Fas and their interaction on memory B cell death in the final regression model among all subjects (Table 9) was much stronger than those effect in the model including HIV status (Table 7 and table 8), suggesting that HIV status could be a partial confounder.

Table 10. Adding age in the regression model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>50.3</td>
<td>17.6</td>
<td>0.008</td>
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<tr>
<td>age</td>
<td>-0.22</td>
<td>0.27</td>
<td>0.41</td>
</tr>
<tr>
<td>GeomeanFas</td>
<td>-0.17</td>
<td>0.11</td>
<td>0.13</td>
</tr>
<tr>
<td>FasL</td>
<td>-0.2</td>
<td>0.14</td>
<td>0.17</td>
</tr>
<tr>
<td>FasL*GeomeanFas</td>
<td>0.0025</td>
<td>0.001</td>
<td>0.03</td>
</tr>
</tbody>
</table>

R-Square = 0.31, P = 0.02
Table 11. Adding peripheral CD4+ T cell counts in the regression model

R-Square = 0.43, P = 0.12

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>102.9</td>
<td>27.8</td>
<td>0.003</td>
</tr>
<tr>
<td>CD4ab</td>
<td>-0.01</td>
<td>0.03</td>
<td>0.75</td>
</tr>
<tr>
<td>GeomeanFas</td>
<td>-0.42</td>
<td>0.15</td>
<td>0.02</td>
</tr>
<tr>
<td>FasL</td>
<td>-0.62</td>
<td>0.25</td>
<td>0.03</td>
</tr>
<tr>
<td>FasL*GeomeanFas</td>
<td>0.004</td>
<td>0.002</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 12. Adding gender in the regression model

R-Square = 0.30, P = 0.03

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>44.4</td>
<td>15.2</td>
<td>0.007</td>
</tr>
<tr>
<td>Gender</td>
<td>-2.8</td>
<td>5.6</td>
<td>0.62</td>
</tr>
<tr>
<td>GeomeanFas</td>
<td>-0.19</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>FasL</td>
<td>-0.2</td>
<td>0.15</td>
<td>0.17</td>
</tr>
<tr>
<td>FasL*GeomeanFas</td>
<td>0.003</td>
<td>0.001</td>
<td>0.03</td>
</tr>
</tbody>
</table>

FasL: Plasma level of Fas ligand (pg/ml)
GeomeanFas: geometric mean of Fas
FasL*GeomeanFas: interaction between plasma Fas ligand and Fas Geomean expression on memory B cells
CD4ab: Peripheral CD4 T cell count

Other potential variables were tested in the regression model. Plasma level of HIV RNA was highly related to Fas geometric mean expression on memory B cells (Table 5, r = 0.57, P = 0.008), group (HIV status) was related to Fas geometric mean expression on memory B cells (r = 0.75, P < 0.001, data not shown), therefore they were not independent variables (confounders) and were not included in the final regression model. Moreover, adding age (Table 5, P = 0.41), CD4 T cell counts (Table 6, P = 0.75), or gender (Table 7, P = 0.62) as independent variables in the final regression model did not have a significant
association with the dependent variable. Thus CD4 T cell count, age and gender were not included in the final model.

**Data distribution**

- Residuals

  The difference between the observed value of the dependent variable \( y \) and the predicted value \( \hat{y} \) is called the residual \( e \). Each data point has one residual.

  \[
  e = y - \hat{y}
  \]

- Residual Plots

  The residual plot was shown in figure 6, which Y- axis represents the residuals and X- axis represents the dependent or independent variables. The residual plot shows randomly dispersed around the horizontal axis, suggesting that a linear regression model is appropriate for the data (Fig 6).

Fig. 6 Plots of the residuals
FasGeomean: Fas geometric mean expression on memory B cells

PlasmaFasL: Plasma level of FasL (pg/mL)

mBapop: Percentage of apoptotic cells in memory B cells

fasg_pla: The interaction between Fas geometric mean expression and plasma level of FasL
Table 13. Effect of outliers in the regression model among all subjects

A linear regression model including all data points

R-square = 0.29, p = 0.01

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>41.1</td>
<td>13.6</td>
<td>0.005</td>
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<tr>
<td>GeomeanFas</td>
<td>-0.18</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td>Fas ligand</td>
<td>-0.19</td>
<td>0.14</td>
<td>0.19</td>
</tr>
<tr>
<td>FasL*GeomeanFas</td>
<td>0.003</td>
<td>0.001</td>
<td>0.027</td>
</tr>
</tbody>
</table>

A linear regression model excluding two outliers

R-square = 0.32, p = 0.01

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>9.67</td>
<td>11.4</td>
<td>0.4</td>
</tr>
<tr>
<td>GeomeanFas</td>
<td>0.19</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>FasL</td>
<td>0.12</td>
<td>0.12</td>
<td>0.33</td>
</tr>
<tr>
<td>FasL*GeomeanFas</td>
<td>-0.001</td>
<td>0.001</td>
<td>0.35</td>
</tr>
</tbody>
</table>

FasL: Plasma level of Fas ligand (pg/ml)
GeomeanFas: geometric mean of Fas
FasL*GeomeanFas: interaction between plasma Fas ligand and Fas Geomean expression on memory B cells

First, as shown by quantile versus residual, the model fits pretty well. There are two outliers (HIV+ subjects) shown in figure 6 with high residuals dispersed around one side of the horizontal axis. In table 13, through R-square and P value are similar, excluding two outliers resulted in loss of significant association between FasL and Fas interaction and the outcome, suggesting that these two outliers significantly influence the model. This could be a potential problem. There
was no evidence these outliers should be excluded in the model based on their clinical characteristics. Therefore the final model indicates all data with the caveat that there is a small sample size with influential observations.

In summary, *in vitro* assays, neither bacterial LPS nor HIV virus, but synergistically induced memory B cells death, this effect may be mediated by monocytes; the percentage of memory B cell death was significantly associated with Fas expression on memory B cell surfaces. *In vivo* or *ex vivo*, memory B cells from HIV-infected donors had higher percentage of apoptosis, higher Fas surface expression, similar plasma levels of Fas ligand compared to controls. Fas geometric mean expression was highly correlated to the percentage of apoptotic memory B cells. By linear regression analysis, Fas geometric mean expression on memory B cells, plasma level of Fas ligand, and their interactions independently predict the susceptibilities of memory B cells to die *in vivo*. These results suggest that bacterial LPS and HIV virus *in vivo* may work together to induce memory B cell apoptosis and depletion through Fas/Fas ligand cell death pathway.
DISCUSSION

This cross-sectional study found that HIV replication and microbial LPS synergistically induce memory B cell apoptosis. This effect was through cell death signal pathway Fas/Fas ligand. From the above analyses, we identified plasma level of Fas ligand, Fas geometric mean expression on memory B cells, and their interactions as possible predictors in vivo for memory B cell death by a linear regression model.

The linear least squares method is the simplest and most commonly applied form of linear regression; it is a way to find the best fitting straight line through a set of points. The least squares method is a non-parametric method. Instead of solving the equations exactly, minimizing the sum of the squares of the residuals is used in the least square. The least squares have important statistical interpretations. The residuals are the differences between the observations and the model, and are used to find outliers. If appropriate probabilistic assumptions about underlying error distributions are made, least squares produce the maximum-likelihood estimate of the parameters.

In the present project, a cross-sectional study was conducted. The advantages of cross-sectional study are relatively inexpensive, less time to conduct, no loss to follow-up, and many outcomes and risk factors can be assessed. The disadvantages of cross-sectional study are difficult to make causal inference; one time point may generate bias.

Figure 7. A model for HIV-related humoral immunodeficiencies
In this model (Fig. 7, Solid lines represent proved directions; dashed lines represent predicted directions), HIV infection results in enhanced apoptosis of gut epithelial cells which allows microbial translocation from the damaged gut (31, 93-95). The hypothesis is that HIV infection and microbial translocation are the key causes of profound humoral immune dysregulations, which include memory B cell depletion, decreased antigen-specific antibody production and increased B cell polyclonal activation. While microbial TLR ligands (e.g. TLR9 ligation) can directly stimulate B cells and induce polyclonal activation as reflected in enhanced autoreactive antibodies in HIV infection (34, 96), memory B cell apoptosis is supposed to be induced by TLR ligands (e.g. LPS) in cooperation with HIV. These TLR ligands stimulate B cells and may drive activation-induced cell death (AICD). As a consequence of memory B cell death and lack of CD4 T cell help, B cell antibody production is impaired in response to recall antigens and vaccinations in chronic HIV infection. Reduced antigen-specific IgA levels in the mucosal sites might also contribute to the “leaky” gut thereby permitting greater translocation of microbial TLR ligands into the systemic circulation. Released bacterial products
lead to a vicious cycle of memory B cell death followed by reduced protection from microbial translocation, and as a consequence the progressive memory B cell losses seen in HIV disease. Therefore memory B cell depletion and dysfunction in HIV disease play an important role in immuno-deficiency, impaired vaccine responses and enhanced opportunistic infection.

The cause and the consequence of microbial TLR ligand translocation and B cell dysfunction in HIV infection are not defined. In addition to providing help for antigen-specific humoral responses (28), TLRs are also reportedly involved in autoimmune diseases which are present in HIV infection, as well as impairing antigen-specific antibody production (26, 34, 97). The mechanisms of hyperimmunoglobulinemia in HIV-1 infection are only partially known (34, 56). Antiretroviral therapy (e.g. protease inhibitor) may cause autoimmune diseases (98). On the other hand, TLR ligands, especially bacterial CpG DNA (TLR9 agonists, the main TLR on B cells) as a result of viral enteropathy have potent abilities to promote polyclonal activation of B cells independent of other cell help (19, 99). Thus long term exposure to microbial TLR ligands (probably also including HIV) may cause the hyperactivation seen in hyperimmunoglobulinemia or a reduced ability to produce antigen-specific antibodies in HIV disease. However, it is clear that patients with other diseases where microbial translocation across the gut occurs also have B cell dysfunction. For example, in inflammatory bowl diseases (IBD), both autoimmune diseases (B cell dysfunction) and enhanced microbial translocation are present (100). Here TLR ligands alone (not in cooperation with HIV) are proposed to activate the immune system and induce autoimmune diseases, but without B cell depletion. In HIV infection, HIV itself may play a role in B cell differentiation and development (57, 101), however, CD4 T
cells are suggested to be necessary to induce autoantibody (102). In the present study, the results indicate (Fig 1) that TLR ligands plus HIV (but not TLR ligands alone) induce memory B cell apoptosis giving rise to the mechanisms of memory B cell depletion and probably impaired function in HIV infection. Thus, the association of B cell dysfunction and microbial translocation may be different in HIV disease (B cell depletion with reduced function) as compared to other diseases (e.g. in IBD, B cell activation appears to result in autoimmune diseases and gut damage). As neither CD4 lymphopenia nor cell-mediated immune deficiency are recognized concomitants of untreated inflammatory bowel disease (IBD) (100), it would appear that the virus maintains a central role in cellular and humoral immunodeficiency.

The interplay between HIV and LPS may have important consequences for immune function. Microbial translocation is recognized as a driver for persistent immune activation. However, persistent immune activation doesn’t have to result in humoral immuno-deficiency as in inflammatory bowel diseases (IBD). Microbial TLR agonists are recognized important for maintaining normal immune function in human and mice. Sterile environment will cause immune failure in mice. However, too much microbial TLR ligands may be associated to autoimmune diseases. Chronic immune activation is shown as the best predictor for HIV disease progression, and microbial LPS play an important role in chronic immune activation and CD4 T cell gain after HAART in HIV disease. Our results of the interplay between HIV, LPS and memory B cell death suggest that blocking microbial translocation from the damage gut may be important to restore humoral immunity, enhance vaccine responses and prevent opportunistic infection.
Importance of this model:

1. It proposes a model for broad activation, depletion and dysfunction of memory B lymphocytes in HIV infection.
2. It recognizes a role for both HIV replication and microbial translocation.
3. It provides a pathway for humoral immune impairment that may be amenable to therapeutic intervention.

Limitations of this model:

1. It may be difficult to determine the causality between memory B cell death and predictive variables.
2. Sample size was small, this could effect on the power.
3. HIV status not included in the regression model could be a problem.
4. Two outliers had significant effects on the regression model.
5. The mechanisms of memory B cell depletion and dysfunction may be more complex. Impaired maturation and differentiation from naïve B cells to memory B cells due to insufficient CD4 T cell help may be also responsible for memory B cell decline in HIV infection. However, the intrinsic B cell dysfunction (e.g. enhanced Fas expression and frequency of apoptotic B cells ex vivo in periphery in HIV infection) suggests that B cells, especially memory B cells are stimulated, activated and driven to death in HIV infection.
6. LPS assay has higher internal and external variability, thus give us the limitation to address the association between risk factors and the outcome. Fas expression on memory B cells and plasma level of Fas ligand on B cell death were tested in a linear multiple regression instead of HIV and LPS.
7. Other cell killing pathways may also contribute to memory B cell death since the final regression model cannot fully explain the \textit{in vivo} observations.

Nevertheless, among most viremic HAART-naive patients, enhanced memory B death was coincident with heighten levels of Fas expression on memory B cells and levels of bacterial LPS. Importantly, HAART-treated patients have significantly reduced memory B cell death and Fas expression on memory B cells even with heightened HIV replication (data not shown), suggesting that HIV may require high levels of LPS to synergistically induce Fas expression on memory B cells \textit{in vivo}. However, the help of CD4 T cells for B cell survival may also play a role for memory B cell death.

The loss of memory B cells in HIV disease may have important clinical implications. For example, enhanced apoptosis and reduced number of memory B cells may result in reduced vaccine responsiveness and may contribute to impaired integrity of the gut mucosa. The latter might lead to a vicious cycle of memory B cell death followed by reduced protection from microbial translocation, and as a consequence the progressive loss of memory B cells seen in HIV disease. In the future, the goal of our research is to investigate the function of memory B cells and plasma cells in mucosal sites in SIV/HIV infection. Therapeutic methods will be investigated for prevention of B cell loss and dysfunction, such as suppression of microbial translocation by mucosal protector (e.g. sulfhydryl compounds (103, 104)) or probiotic microbes before SIV infection, and test the hypothesis that SIV/HIV itself rarely induces B cell death unless cells are also exposed to TLR ligands such
as LPS in animal models. If our model is an accurate reflection of pathogenesis, a therapeutic strategy (e.g. mucosal protector/microbes, inhibition of TLR signals or even blockage of Fas/FasL interactions) might help reverse B cell dysfunction and depletion, thereby helping to maintain normal immunity and control viral replication at peripheral and mucosal sites since antiviral Abs have been shown to be necessary to control SIV infection (43, 105, 106). In addition, improving humoral responses at mucosal sites might also help to prevent microbial translocation and attenuate chronic immune activation in HIV infection.
REFERENCES


