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The Alveolar Microenvironment of Patients Infected with Human Immunodeficiency Virus Does Not Modify Alveolar Macrophage Interactions with *Streptococcus pneumoniae*

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We tested the hypothesis that HIV infection results in activation of alveolar macrophages and that this might be associated with impaired defense against pneumococcus. We compared alveolar macrophages and lymphocytes in 131 bronchoalveolar lavage samples from HIV-infected and healthy controls using inflammatory gene microarrays, flow cytometry, real-time PCR, and enzyme-linked immunosorbent assay (ELISA) to determine the pattern of macrophage activation associated with HIV infection and the effect of this activation on defense against pneumococcus. We used gamma interferon (IFN-γ) priming to mimic the cellular milieu in HIV-infected lungs. InnateDB and BioLayout 3D were used to analyze the interactions of the upregulated genes. Alveolar macrophages from HIV-infected adults showed increased gene expression and cytokine production in a classical pattern. Bronchoalveolar lavage from HIV-infected subjects showed excess $CD8^+$ lymphocytes with activated phenotype. Toll-like receptor 4 (TLR4) expression was increased in macrophages from HIV-infected subjects, but function was similar between the groups; lung lavage fluid did not inhibit TLR function in transfected HeLa cells. Alveolar macrophages from HIV-infected subjects showed normal binding and internalization of opsonized pneumococci, with or without IFN-γ priming. Alveolar macrophages from HIV-infected subjects showed classical activation compared to that of healthy controls, but this does not alter macrophage interactions with pneumococci.

Acute respiratory infections are a leading infectious cause of death in both children and adults worldwide. *Streptococcus pneumoniae* is the most common isolate in these infections and also has a unique association with HIV infection, resulting in a 20- to 100-fold increase in the incidence of pneumococcal infections in regions of high HIV seroprevalence (1). Increased pneumococcal carriage (2), decreased $CD4^+$ lymphocyte function in the respiratory mucosa (3), impaired immunoglobulin function (4), and altered immunoglobulin responses to pneumococcal antigens have all been shown to play a role in the increased susceptibility to pneumococcal infection among HIV-infected subjects, but the importance of alveolar macrophage dysfunction remains unclear. Phagocytosis of pneumococci by alveolar macrophages was unimpaired in HIV-infected subjects (5), but limited studies of macrophage cytokine responses showed abnormalities potentially significant in neutrophil recruitment (6).

Alveolar macrophages (AM) play an important role in respiratory tract homeostasis (7), including the early clearance of bacterial pathogens from the airspaces (8). Macrophage activation is normally reversible, plastic, and classified in 4 distinct patterns of activation (Fig. 1), termed innate, classical, alternative, and deactivated states (9). Innate and classical activation of macrophages are appropriate responses to pneumococcal infection (10). Innate activation of macrophages occurs following pathogen-associated molecular pattern interaction with Toll-like receptors (TLR) or stimulation from NK cells (11) and is characterized by an increase in reactive oxygen species. This has been demonstrated in pneumococcal responses using pneumolysin interactions with both TLR2 and TLR4 (12). Classical activation of macrophages occurs following activated cognate $CD4^+$ lymphocyte interactions with macrophages and is mediated by gamma interferon (IFN-γ) released by $CD4^+$ lymphocytes (13). Classical activation results in an augmented set of responses compared to innate activation, including increased expression of major histocompatibility complex class II (MHC-II), phagocytosis, antigen presentation, and microbicidal functions (9). Studies of human and murine $CD4^+$ lymphocytes provide indirect evidence that this mode of activation is important in pulmonary defense against pneumococci (14). Alternative activation or deactivation of alveolar macrophages could be expected to be detrimental to host defense against pneumococci (15). Alternative activation is mediated by interleukin 4 (IL-4) released by T-helper cells and is characterized by increased endocytosis and parasite killing (16). Macrophage activation states are also regulated by T-regulatory lymphocytes, with published data to support both deactivation (9) and alternative activation induced by T-regulatory cells (17).

Alveolar macrophages are vulnerable to HIV infection via CD4

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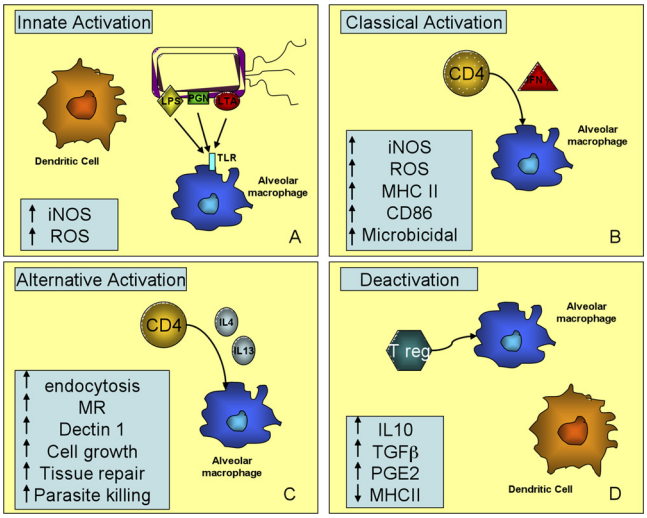

FIG 1 Macrophage activation states. Macrophages are activated in several patterns dependent on the antigenic stimulus, the receptor activated, coreceptor stimulation, and the cytokine milieu. (A) Innate activation results when pattern recognition, typically by Toll-like receptors (TLR), results in increased production of reactive nitrogen species by inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS). (B) Classical activation results when activated $CD4^+$ lymphocytes (T-helper 1 type) producing gamma interferon (IFN-γ) interact with macrophages. This interaction results in increased antigen presentation, increased coreceptor expression (including CD86), and increased microbicidal activity in addition to the features of innate activation. (C) Alternative activation results from T-helper 2-type activation of macrophages by CD4 lymphocytes. This alternative activation pattern results in parasite killing as well as increased endocytosis, mannose receptor (MR) expression, fibrosis, and tissue repair. Deactivation (D) results from the effect of regulatory T cells (Treg). Inhibitory factors, including interleukin 10 (IL-10) and transforming growth factor beta (TGF-β), result in downregulation of proinflammatory cytokines by the macrophage, prostaglandin E2 (PGE2) production, and decreased antigen expression, finally leading to apoptosis.

and chemokine receptors (18). HIV infection of alveolar macrophages is noncytopathic, but virus-derived gene products, such as Nef, Tat, and the gp120 envelope glycoprotein, alter gene transcription and translation, resulting in macrophage activation (19). In addition, bystander uninfected macrophages in HIV-infected subjects may be activated by either the action of cytokines released by virus-targeted $CD8^+$ cells (20) or by the effect of high levels of circulating bacterial products, including lipopolysaccharide (LPS), which result from a failure of mucosal defense in the gut (21) or by Treg depletion at certain stages of disease (22). The pattern of HIV-related alveolar macrophage activation and the effect of this activation on defense against pneumococcus have not been described.

Our first hypothesis was that the effect of HIV infection on the activation state of macrophages in the lung would fall into one of the typical patterns, both as a result of direct infection and the HIV-induced alterations in the alveolar milieu. In order to categorize the predominant activation state of alveolar macrophages in HIV-infected subjects as the innate, classical, or alternative activation pattern, we used flow cytometry, microarray, and real-time PCR (RT-PCR) to determine the phenotype of alveolar macrophages from HIV-infected subjects and healthy controls. Our second hypothesis was that the observed activation pattern in macrophages from HIV-infected subjects would be associated with an alteration in pulmonary defense against pneumococcus. We have previously observed normal opsonophagocytosis and killing of *S. pneumoniae* by alveolar macrophages obtained from patients with HIV infection (5). In this study, we extended that observation with new subjects and with the addition of a gamma interferon priming step. We then observed the association of HIV infection with cytokine responses to pneumococcal challenge *in vitro* and measured binding, internalization, and killing of opsonized pneumococci by human alveolar macrophages with and without gamma interferon priming. We also used InnateDB (23) in order to view the interactions that the upregulated genes have with one another. This gave us the ability to plot clear interaction pathways for any of the upregulated genes, which allowed us to gain a better understanding of how and why the regulation varied over time.

MATERIALS AND METHODS

Recruitment, consent, and bronchoalveolar lavage. Volunteers recruited by advertisement gave written, informed consent to an ongoing program of research on lung defense against infection. Consent to participation included chest X ray, HIV test, CD4 testing, and bronchoalveolar lavage. Inclusion criteria were asymptomatic adults (17 years or older, current nonsmokers) with a normal chest X ray. Bronchoscopy was carried out as previously described (24). Briefly, hand suction of 200 ml of warm saline lavage fluid resulted in a median return of 110 ml containing 1×10^7 cells from a subsegmental bronchus of the middle lobe.

Alveolar macrophage culture, microarrays, and microarray analysis. Alveolar macrophages were isolated (24) and cultured at a density of 1×10^6/well in 24-well plates for 48 h. Supernatant lavage fluid was stored at −80°C for later analysis. Macrophages were washed 3 times at the end of 48 h of culture to remove nonadherent cells (predominantly lymphocytes). Preparations of this type have been previously demonstrated to contain 98% viable alveolar macrophages (24). RNA extraction and sample preparation were performed according to the manufacturer's instructions (PowerScript reverse transcriptase; BD Biosciences), and the fluorescent cyanine dyes Cy3 and Cy5 (Amersham Biosciences) were incorporated into the modified aminoallyl UTP-cRNA.The hybridization mixture (50 μl) was pipetted onto the array slides (human inflammation arrays; MWG-Biotech AG, Ebersberg, Germany) and incubated for 18 h. Arrays were scanned on an Affymetrix 428 array scanner, and the image was analyzed using the ImaGene software.

The microarrays contained duplicate spots for 19 housekeeping genes and 136 inflammation-related genes. Using stringent criteria for image quality and hybridization success, mean log2 Cy3/Cy5 ratios were calculated for each gene. The signal from housekeeping genes was used for normalization. Three analyses were performed comparing samples from HIV-infected subjects and healthy controls: (i) time zero, to identify differences in basal gene expression levels; (ii) time 4 h after pneumococcal challenge, to compare gene expression after stimulation; (iii) using time zero and 4 h, the change in expression for each gene, within each individual, induced by pneumococcal challenge was calculated. Both a *t* test with an adjusted *P* value ($P < 1/n$, where n is the number of genes involved in the analysis) and a nonparametric method (25), with the false discovery rate set as less than one gene, were used. Only genes which were found to be significantly different using both methods were adjudged to be differentially expressed. Further analysis was performed on the microarray data using InnateDB, Cytoscape, and BioLayout 3D, which are all open-source software packages. InnateDB (23) was used to find the interactions, and Cytoscape and BioLayout 3D (26) were used to view these interactions first as a two-dimensional (2-D) model and then as a three-dimensional (3-D) web.

Bacterial challenge experiments. Type 1 *S. pneumoniae* cells, WHO reference laboratory strain SSISP 1/1 (Statens Seruminstitut, Copenhagen, Denmark) grown to mid-log phase in brain heart infusion (Oxoid, Unipath Ltd., Basingstoke, United Kingdom) with 10% fetal calf serum

(Bioclear Ltd.,Wiltshire, United Kingdom) and opsonized with pooled human serum as previously described (25), were added to alveolar macrophages at a multiplicity of infection of 10:1 for 15 min, after which nonadherent bacteria were washed off. Interaction of bound bacteria with the cells was then allowed to proceed for 24 h. RNA was extracted at 0 h (control well) and 4 h for real-time PCR and microarray analysis. Supernatants were collected at 0, 4, 8, and 24 h of stimulation for cytokine analysis and measurement of bacterial concentration.

RT-PCR, ELISA, and cytokine bead array assays. To validate gene expression, RNA was reverse transcribed (ImProm-II reverse transcription system; Promega) and used for quantitative real-time PCR (RT-PCR; Carbett Research Rotor-Gene 2000 real-time cycler) using the QuantiTect multiplex PCR mix (Qiagen) and QuantiTect gene-specific primer-probe combinations for human IL-8, TLR2, and NF-κB. Levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, and the chemokine IL-8 in culture supernatants were determined by cytometric bead array (BD Pharmingen, Oxford, United Kingdom) and confirmed by enzyme-linked immunosorbent assays (ELISA; R&D Systems, Abingdon, United Kingdom), in accordance with the manufacturer's instructions.

Flow cytometry for lymphocyte phenotype in lavage. Flow cytometric analysis of lavage cells was carried out in order to determine the HIV-associated changes in macrophage and T and B lymphocyte phenotypes. Alveolar macrophages were determined by forward and side scatter morphology and staining with CD206. Alveolar macrophages were costained with CD14, HLA-DR, CD69, CD80, CD86, CD40, CD49d, CD54, CD11b, and CD11c. T cell surface markers associated with activation (CD25, CD69) and adhesion (CD11b, CD62L, CD54, CD11a) were costained with CD3, CD4, and CD8. B cell surface markers associated with T cell interaction and immunoglobulin production (CD80, CD86, CD40) were costained with CD19 as a B cell marker.

TLR expression in macrophages and effect of bronchoalveolar lavage on TLR function in transfected HeLa cells. TLR4 expression is a characteristic of both innate and classical macrophage activation. We designed three experiments to test the effect of HIV on TLR function in the HIV-infected alveolar milieu: (i) TLR4/MD2 expression was measured using real-time PCR on unstimulated alveolar macrophages from HIV-infected subjects and healthy controls using standard methods, (ii) alveolar macrophages from HIV-infected and healthy controls were stimulated with ultrapure LPS (Alexis), and (iii) TLR4- and MD2-transfected HeLa cells were challenged with ultrapure LPS (Alexis) as an agonist in the presence of BAL supernatant fluid from HIV-infected subjects and healthy controls. Reporter levels were measured using a dual-Luciferase system (Promega), and we compared the effect of replacing media with whole BAL fluid (from HIV and healthy controls) diluted 1:2 in medium for the agonist and control assays.

IFN-γ stimulation experiments. Alveolar macrophages were collected as described above. On day 1 and day 3, cells were primed for 72 h and 24 h, respectively, using antibiotic-free RPMI containing 100 ng/ml (1,000 IU/ml) recombinant human IFN-γ (Pharmingen, San Diego, CA). Control cells were mock primed with medium alone. Primed and mock-primed cells were challenged with opsonized type 1 *S. pneumoniae* on day 4. Immunofluorescence microscopy and killing assays were carried out as previously described (5).

Statistical analyses and ethical review. Categorical variables were compared using the χ^2 test. Gene expression data were considered statistically significant only when two statistical criteria were met: (i) the simple parametric (*t* test) test with adjusted *P* value and (ii) nonparametric sampling method (SAM) to compared difference in expression, with the false discovery rate set to zero. In laboratory experiments, parametric tests (*t* test) were used when data fulfilled criteria for a normal distribution (Shapiro-Wilks test), and nonparametric tests (rank sum) were used for non-normally distributed data. All statistical tests were carried out using Stata version 9 (Statacorp, Texas).

The studies described were reviewed by the South Sheffield Research Ethics Committee, the Liverpool School of Tropical Medicine Research Ethics Committee, and the College of Medicine Research Ethics Committee of the University of Malawi.

TABLE 1 Subject details

Characteristic	Value	
	HIV positive ($n = 37$ subjects)	HIV negative ($n = 41$ subjects)
Gender, male:female	24:13	30:11
Mean age in yrs (SD)	32.7 (7.73)	30.7 (10.5)
Median CD4 count in cells/μl (range)	276 (7–997)	715 (236–1240)
Median plasma viral load in copies/ml (range)	140,000 (0–850,000)	NA[a]
Median bronchoalveolar lavage fluid viral load in copies/ml (range)	130 (0–250,000)	NA
No. of current cigarette smokers	1	3
No. of former cigarette smokers	5	5

[a] NA, not applicable.

RESULTS

Recruitment of subjects, consent, and determination of HIV status. A cohort of 37 HIV-infected subjects but otherwise healthy adults and 41 healthy controls were recruited with written informed consent to participate in bronchoscopy-based studies of lung immunity. Subject details from this cohort are summarized in Table 1.

A total of 131 bronchoalveolar lavage procedures were carried out. There were no serious adverse events. Subjects consenting to more than one procedure had a minimum of 6 months between the procedures. The subsets of BAL fluid samples were used in each specific experiment.

Inflammatory gene expression in alveolar macrophages from HIV-1-infected subjects compared to healthy controls showed a classical pattern of activation. RNA extracted from alveolar macrophages 48 h after isolation from bronchoalveolar lavage fluid was analyzed using an inflammation array. There was robust data to compare the expression of 53 genes between pooled data from HIV-infected subjects and healthy controls. As shown in Table 2, 35 of the 53 genes were categorized *a priori* as being typical of classical activation. The remaining genes were categorized as being typical of alternative or deactivation pathways based on a review of the available literature for each gene. When the literature provided evidence that was contradictory, the genes were not categorized. When HIV-infected subjects and healthy controls were compared, there was found to be significant upregulation (minimum change of 1.88-fold) of 10 of the 35 classical activation signals in HIV-infected subjects compared to that of healthy controls (range, 1.88 to 4.93) but none of the 18 innate, alternative, or deactivation signals ($P < 0.0001$), as shown in Fig. 2A.

Using InnateDB, the 10 upregulated genes were further explored. These 10 genes upregulated prechallenge with pneumococcus linked to 242 other genes. In addition, a further 5 genes upregulated postchallenge with pneumococcus linked to 84 genes, creating a much smaller interaction web with more separation, as shown in Fig. 3. These interaction webs could then be used to follow the interaction pathways of specific genes, as demonstrated by Fig. 4.

TABLE 2 Microarray analysis

Name(s) of gene	Pattern	Fold increase in HIV
Intercellular adhesion molecule 1 precursor; *icam1*	Classical	
Interleukin 1, alpha proprotein; *il1a*	Classical	2.73
Interleukin 1, beta proprotein; *il1b*	Classical	2.16
Interleukin 8 precursor; *il8*	Classical	2.78
cd14 antigen precursor; *cd14*	Classical	
Interleukin 6 (interferon, beta 2); *il6*	Classical	2.27
Superoxide dismutase 2, mitochondrial; *sod2*	Classical	2.0
Vascular cell adhesion molecule 1 isoform A precursor; *vcam1*	Classical	
Tumor necrosis factor receptor superfamily, member 5 isoform 1 precursor; *tnfrsf5*	Classical	
Guanylate binding protein 1, interferon-inducible, 67 kd; *gbp1*	Classical	
Matrix metalloproteinase 12 preproprotein; *mmp12*	Classical	4.0
Myxovirus resistance protein 1; *mx1*	Classical	
Small inducible cytokine a2 precursor; *ccl2*	Classical	
Small inducible cytokine a3; *scya3*	Classical	
Chemokine (CC motif) ligand 4 precursor; *ccl4*	Classical	
Small inducible cytokine a5 precursor; *ccl5*	Classical	3.47
Toll-like receptor 2; *tlr2*	Classical	
Receptor-interacting serine-threonine kinase 2; *ripk2*	Classical	3.13
Nuclear factor of kappa light polypeptide gene enhancer in B cells 1 (p105); *nfkb1*	Classical	
bcl2-related protein a1; *bcl2a1*	Classical	
Guanylate binding protein 2, interferon-inducible; *gbp2*	Classical	
Small inducible cytokine a15 precursor; *ccl15*	Classical	1.8
Baculoviral *iap* repeat-containing 1; *birc1*	Classical	
Chemokine (CC motif) ligand 20; *ccl20*	Classical	
Triggering receptor expressed on myeloid cells 1; *trem1*	Classical	
Melanoma differentiation associated protein-5; *mda5*	Classical	
Molecule possessing ankyrin repeats induced by lipopolysaccharide; *mail*	Classical	
Leukotriene a4 hydrolase; *lta4h*	Classical	
Interferon gamma-induced precursor; *cxcl10*	Classical	
Complement factor b preproprotein; *bf*	Classical	
Serine (or cysteine) proteinase inhibitor, clade b (ovalbumin), member 2; *serpinb2*	Classical	
Prostaglandin-endoperoxide synthase 2 precursor; *ptgs2*	Classical	4.93
Interleukin 7 receptor precursor; *il7r*	Classical	
v-Jun avian sarcoma virus 17 oncogene homolog; *jun*	Classical	
Jun b proto-oncogene; *junb*	Classical	
Matrix metalloproteinase 9 preproprotein; *mmp9*	Alternative	
Ferritin, heavy polypeptide 1; *fth1*	Alternative	
Proteoglycan 1; *PRG1*	Alternative	
Interleukin 16 isoform 1 proprotein; *il16*	Alternative	
Homo sapiens immunoglobulin superfamily, member 6 *(igsf6)*; *mrna*	Alternative	
Heme oxygenase (decyclizing) 1; *hmox1*	Alternative	
Interleukin 11 precursor; *il11*	Deactivation	
Plasminogen activator, urokinase receptor; *plaur*	Deactivation	
Tumor necrosis factor (ligand) superfamily, member 13b; *tnfsf13b*	Deactivation	
Nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor alpha; *nfkbia*	Deactivation	
Integrin, beta 8; *itgb8*	Not categorized	
Interleukin 1 receptor antagonist isoform 1 precursor; *il1rn*	Not categorized	
Alpha 1 type vii collagen precursor; *col7a1*	Not categorized	
Chemokine (CXC motif) ligand 3; *cxcl3*	Not categorized	
Chemokine (CXC motif) ligand 5; *cxcl5*	Not categorized	
Traf-interacting protein tank isoform a; *tank*	Not categorized	
Homo sapiens growth arrest and DNA-damage-inducible, beta (gadd45b); *mrna*	Not categorized	
Tyrosine 3/tryptophan 5 monooxygenase activation protein, zeta polypeptide; *ywhaz*	Not categorized	

Inflammatory cytokine production is increased in HIV-infected compared to normal alveolar macrophages as determined by ELISA and real-time PCR. Cytokine levels were measured in supernatant culture fluid obtained from alveolar macrophages after 24 h in culture (Fig. 2B). Consistent with the microarray data, concentrations of IL-1 and IL-6 were raised in HIV-infected subjects compared to normal ($P < 0.01$ and $P < 0.05$, respectively), but concentrations of IL-8 were not significantly different. Real-time PCR showed that IL-1, IL-6, and IL-8 mRNA and Rip-k2 signal were significantly increased in HIV-infected subjects compared to in healthy controls (data not shown).

Higher levels of interleukin 1 and 6 were found in samples from HIV-infected subjects compared to healthy controls, as shown in

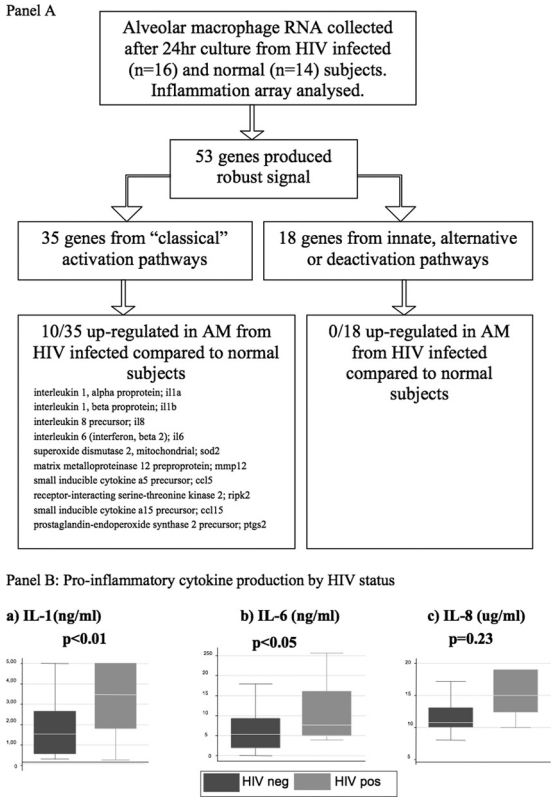

FIG 2 Alveolar macrophages from HIV-infected subjects show classical activation pattern gene expression and cytokine production. Alveolar macrophages freshly obtained from HIV-infected ($n = 16$) and normal ($n = 14$) subjects were cultured for 24 h. RNA was then extracted for proinflammatory array analysis, and cytokine concentration was measured in the culture supernatant. Panel A shows the analysis plan and results for the microarray data. A total of 53 genes from an inflammation array generated robust data after appropriate data normalization. A total of 35 of the 53 genes were defined *a priori* as being indicative of classical pattern activation or responses, and 18 of the 53 genes were indicative of either alternative, deactivation states, including tissue repair or nonspecific to any state. Ten of the 53 genes were differentially upregulated in HIV-infected subjects; all of these were in the classical pattern, and none were in a nonclassical pattern (χ^2 test; $P < 0.0001$). Real-time PCR showed that IL-1, IL-6, IL-8 precursor, and Rip-k2 signal were significantly increased in HIV-infected subjects compared to in normal subjects (data not shown). Panel B shows cytokine concentration in alveolar macrophage culture supernatant after 48 h in unstimulated culture. Concentrations of IL-1 and IL-6 were raised in HIV-infected subjects compared to normal subjects ($P < 0.01$ and $P < 0.05$, respectively), but concentrations of IL-8 were not significantly different, as discussed in the text.

Fig. 2B. Levels of IL-8 were also measured, and the increase seen in HIV-infected subjects was not found to be statistically significant.

Alveolar macrophages from HIV-infected subjects exist in an altered cellular milieu compared to healthy controls but have normal surface marker expression. Flow cytometry was used to determine the phenotype and relative numbers of $CD4^+$ and $CD8^+$ lymphocytes in BAL fluid as well as to categorize surface activation markers on alveolar macrophages. As shown in Fig. 5A, there were significantly fewer $CD4^+$ lymphocytes in BAL fluid obtained from HIV-infected subjects than from healthy controls. The $CD4^+$ lymphocytes present in each group showed identical activation marker and adhesion molecule expressions, however, as shown in Fig. 5B. BAL fluid samples from HIV-infected subjects had a significantly increased number of $CD8^+$ lymphocytes compared to that of samples from healthy controls, as shown in Fig. 5A. The $CD8^+$ lymphocytes from HIV-infected subjects showed a significant reduction in CD69 and CD25 expression, suggesting decreased activation, as well as a decrease in L-selectin (CD62L) expression, as shown in Fig. 5C. Alveolar macrophages obtained from HIV-infected subjects showed no difference in the percentages of cells expressing surface markers measured using flow cytometry, as shown in Fig. 5D. Mean fluorescent intensity was not recorded.

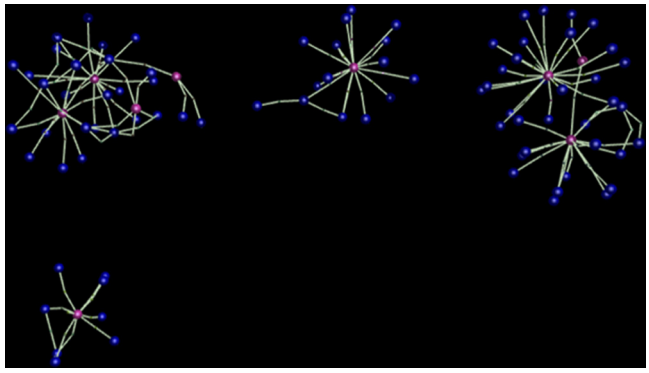

FIG 3 Gene interaction web after pneumococcal challenge. Shown are the interactions of the 5 upregulated genes after pneumococcal challenge. The pink nodes represent the 5 unregulated genes and their corresponding proteins, the blue nodes are other genes that the unregulated ones interact with, and the green lines represent an interaction of some kind; for example, MMP12 cleaves CXCL2.

Alveolar macrophages from HIV-infected subjects show increased TLR4 and MD2 expression but normal TNF production in response to LPS. TLR4 and MD2 gene expression was measured using real-time PCR (data not shown). Alveolar macrophages from HIV-infected subjects showed increased TLR4 and MD2 gene expression, consistent with an activated phenotype. Using ELISA and real-time PCR, the TNF signals produced in response to ultrapure LPS stimulation were similar for alveolar macrophages obtained from HIV-positive and HIV-negative donors, indicating that the increased TLR4 gene expression did not lead to increased cytokine production.

BAL supernatant fluid from HIV-infected subjects and healthy controls does not inhibit TLR4 function in TLR4/MD2-transfected HeLa cells. In order to determine if BAL fluid from HIV-infected subjects might inhibit the function of TLR4/MD2 on alveolar macrophages, we developed a model system using TLR4/MD2-transfected HeLa cells. Transfected cells stimulated with ultrapure *E. coli* LPS produced a luciferase signal (IL-8 promoter) in a reproducible and dose-dependent manner that was significantly different from cells stimulated with phosphate-buffered saline (PBS). Incubation with BAL fluid did not alter the signal in either the LPS- or PBS-stimulated limbs of these experiments (data not shown).

Alveolar macrophage cytokine mRNA response to *in vitro* challenge with opsonized *S. pneumoniae* is altered in HIV-infected subjects compared to that in normal controls, but there is no evidence of significant immunoparesis. Microarray analysis was performed on RNA obtained from alveolar macrophages that had been challenged *in vitro* with *S. pneumoniae* for 4 h. Using the same panel of inflammation-related genes as described for unstimulated macrophages described above, samples from HIV-in-

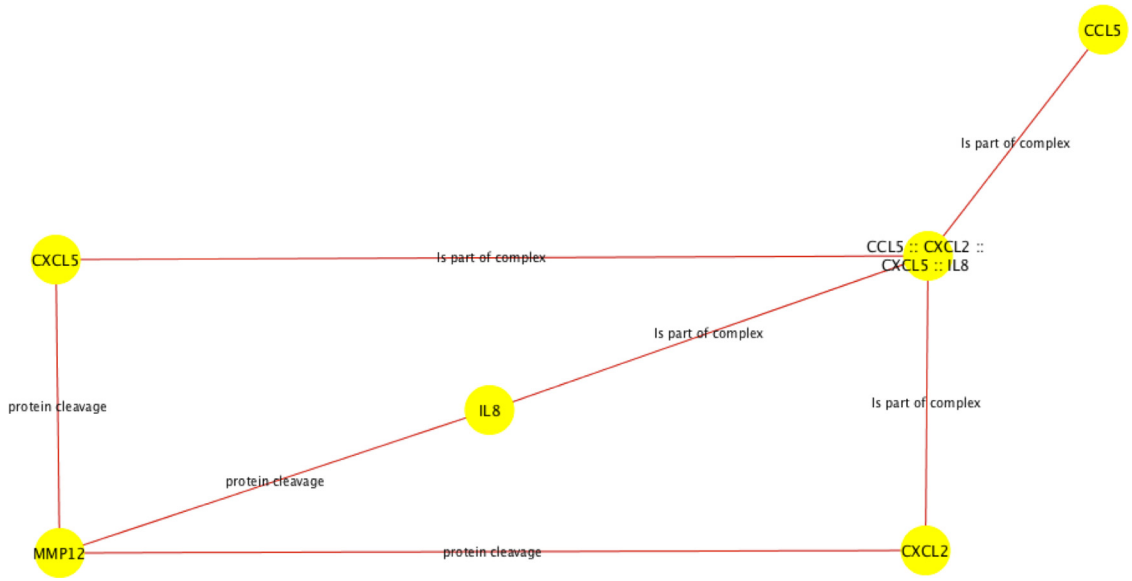

FIG 4 Nature of interaction pathway between MMP12 and CCL5. Shows the interaction pathway for MMP12 linking it to CCL5, and both were found to be upregulated in HIV-positive macrophages after pneumococcal challenge. The yellow nodes are the genes, and the red lines represent interactions and are labeled with the type of interaction occurring.

fected subjects and healthy controls were compared. Five genes showed differential upregulation between HIV-infected subjects and healthy controls, but 4 of these genes (shown in Table 3) were also differentially upregulated at baseline. Only chemokine ligand 3, which was not categorized in the *a priori* categorization, showed differential upregulation after pneumococcal challenge in HIV-infected subjects.

In order to take the differential gene expression at baseline into consideration, a ratio was calculated to express the fold increase in gene expression between baseline ($t = 0$) and 4 h incubation ($t = 4$). Data from 42 genes were sufficiently complete to allow this ratio ($t = 4/t = 0$) to be compared between HIV-infected subjects and healthy controls. All of the fold changes in response to bacterial challenge were small, and only one gene was found to be significantly different by HIV status. HIV-infected subjects (fold change = 0.8) showed half of the fold change seen in expression of receptor-interacting serine-threonine kinase 2 (Ripk2) in samples from healthy controls (fold change = 1.6).

Alveolar macrophage cytokine response to *in vitro* challenge with opsonized *S. pneumoniae* is altered in HIV-infected subjects compared to that in healthy controls. Cytokine levels in alveolar macrophage supernatant were measured after 4, 14, and 24 h of challenge with opsonized *S. pneumoniae*. Levels of IL-1, IL-6, IL-8, and TNF-α in pg/ml are shown plotted as box plots for each time point by HIV status in Fig. 6. Using area under the curve as a summary statistic representing total cytokine production in each experiment, there was a greater production of IL-1 and TNF-α in HIV-infected subjects than in healthy controls. The total production of IL-6 and IL-8 was not significantly different using the area under the curve (statistics are in the figure legend), but levels of IL-6 were increased and IL-8 levels decreased in HIV-infected subjects compared to those in healthy controls at later time points.

Priming of alveolar macrophages with IFN-γ prior to *in vitro* challenge does not alter alveolar macrophage binding, internalization, or killing of *S. pneumoniae*. In view of the $CD8^+$ lymphocytosis seen in BAL fluid from HIV-infected subjects, we hypothesized that IFN-γ priming of alveolar macrophages *in vivo* by resident lung $CD8^+$ lymphocytes might be important in understanding the effect of HIV-associated macrophage activation on defense against pneumococcus. We therefore examined the effect of IFN-γ priming for 24 h before *in vitro* challenge with *S. pneumoniae*. As illustrated in Fig. 7, IFN-γ priming made no difference to the binding (upper panel), internalization (middle panel), or killing (lower panel) of opsonized pneumococci in either HIV-infected subjects' or healthy controls' alveolar macrophages.

DISCUSSION

Streptococcus pneumoniae is an important cause of recurrent pneumonia in HIV-1-infected individuals, with rates of pneumococcal lung disease correlating with increasing viral load and a decline in $CD4^+$ T cell counts in the periphery (28, 29. Alveolar macrophages are sentinel phagocytes in the lung that have defective function against some pathogens when infected by HIV (30–32).

We have shown here that HIV infection in untreated subjects (predominantly male) results in a classical pattern of activation in alveolar macrophages. Associated with this classical activation was increased TLR4/MD2 expression but no increase in TLR4-dependent TNF production. There was increased production of NF-κB-dependent cytokines in response to pneumococcus but no difference in the binding, internalization, or killing of opsonized bacteria. Unopsonized bacteria were not studied, as we have previously shown the critical importance of opsonization in the alveolar compartment (25), and most BAL fluid samples did not yield enough cells for experiments of this type to be carried out on both opsonized and unopsonized bacteria.

The first observation regarding classical activation is novel and consistent with the published literature on HIV infection in the lung (33) and in macrophages (34). In particular, classical activa-

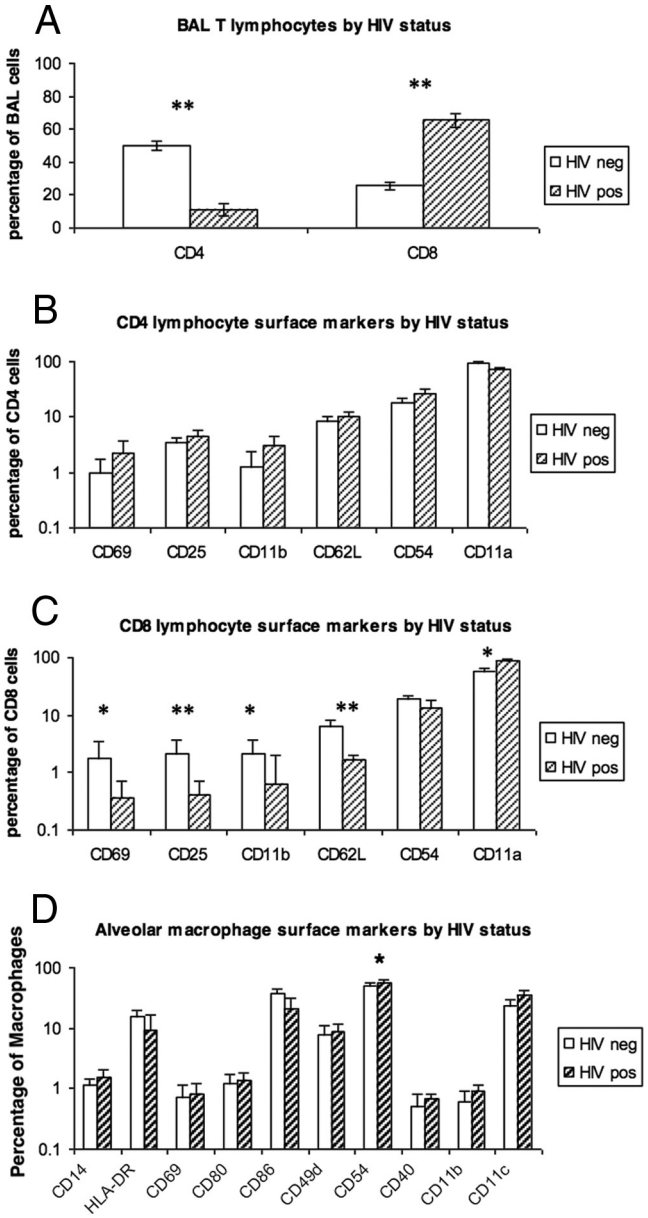

FIG 5 Alveolar macrophages from HIV-infected subjects are found in an altered cellular milieu and show increased TLR4 gene expression. Fresh bronchoalveolar lavage (BAL) samples obtained from HIV-infected ($n = 19$) and normal ($n = 23$) subjects were analyzed using a flow cytometric panel to describe cell type and surface marker phenotype. BAL fluid from HIV-infected subjects had a greater percentage of lymphocytes than normal subjects (20.4% versus 10.7%; $P = 0.009$; data not shown), and BAL fluid from HIV-infected subjects had a reduced percentage of CD4 lymphocytes (11.2% versus 50% of CD3 cells; $P < 10$), with an increased percentage of CD8 lymphocytes (65.3% versus 25.6% of CD3 cells; $P < 10$ compared to normal subjects [panel A]). The phenotype of CD8 cells showed reduced activation in HIV-infected subjects compared to that in normal subjects (C), but the phenotypes of CD4 lymphocytes (B) and alveolar macrophages (D) were the same in HIV-infected and normal subjects. Surface TLR expression could not be detected by flow cytometry, but using real-time PCR, there was increased TLR4 and MD2 gene expression in AM from HIV-infected ($n = 11$) compared to normal ($n = 12$) subjects (data not shown). Phenotype marker percentages on flow cytometry and RT-PCR-normalized copy number data were not normally distributed. Data are shown as geometric means with standard error and statistical significance tested using Wilcoxon rank sum tests (*, $P < 0.05$; **, $P < 0.01$).

TABLE 3 Genes differentially expressed in alveolar macrophages from HIV-positive subjects relative to controls 4 h after exposure to pneumococcal challenge

Mean fold change	Gene symbol	Gene name
2.6	*cxcl3*	Chemokine (CXC motif) ligand 3
5.5	*ccl5*	Small inducible cytokine a5 precursor
3.7	*il6*	Interleukin 6 (interferon, beta 2)
2.0	*il1b*	Interleukin 1, beta proprotein
4.5	*mmp12*	Matrix metalloproteinase 12 preproprotein

tion is consistent with published measurements of raised cytokine levels in bronchoalveolar lavage fluid (35) and also with upregulation of receptors critical in phagocytosis of *Mycobacterium tuberculosis* (36). Several cell functions characteristic of activation (reactive oxygen species, reactive nitrogen species) have not been measured, but the functions described are representative of important macrophage capacity in defense against pneumococcal infection. We have not shown a difference in macrophage surface marker expression in this study. This may have been because of methodological issues, particularly that (i) there were high levels of autofluorescence in these cells, making use of the fluorescein isothiocyanate (FITC) channel problematic; and (ii) we did not compare fluorescent intensity using a continuous variable.

Classical activation of macrophages is consistent with the upregulation of Toll-like receptors (TLR) that we have shown in this study, as this form of activation augments the innate response and there is cellular plasticity between the two states (13). The lack of TNF release in response to ultrapure LPS stimulation is also consistent with two published mechanisms. First, Tachado and others have shown that HIV inhibits TLR4 release of TNF by an extracellular signal-regulated kinase (ERK)-dependent mechanism (37). Second, TLR desensitization following viral infection has been shown to be an important mechanism linking viral infection and subsequent bacterial pneumonia (38). Macrophages have also been shown to be important in the regulation of endotoxin-related inflammation in a rat model, where macrophage depletion resulted in excess inflammation (39).

Does alveolar macrophage activation in HIV infection matter? Classical activation of alveolar macrophages does not result in protracted pneumonitis in HIV-infected patients; regulatory mechanisms, such as IKKβ, have been shown to be anti-inflammatory and to regulate macrophage responses in this context (40, 41). It is possible, however, that delayed apoptosis of alveolar macrophages may result in impaired defense against resolving pneumococcal infection (42). The raised BAL fluid cytokine levels observed by others (20) and the altered cytokine profile demonstrated here may result in altered neutrophil recruitment (6, 43) but are more likely to contribute to the altered lymphocyte profile seen in BAL fluid from HIV-infected subjects discussed below.

HIV infection of the lung results in alveolar lymphocytosis (20). In this study, we showed that the $CD8^+$ cells causing this alveolitis have an activated phenotype. This observation and the observations of others (34) led us to test the hypothesis that differential alveolar macrophage function against pneumococcus might be seen in the context of gamma interferon priming. We have confirmed our previous published observations without IFN-γ priming (5) and have now extended that observation to

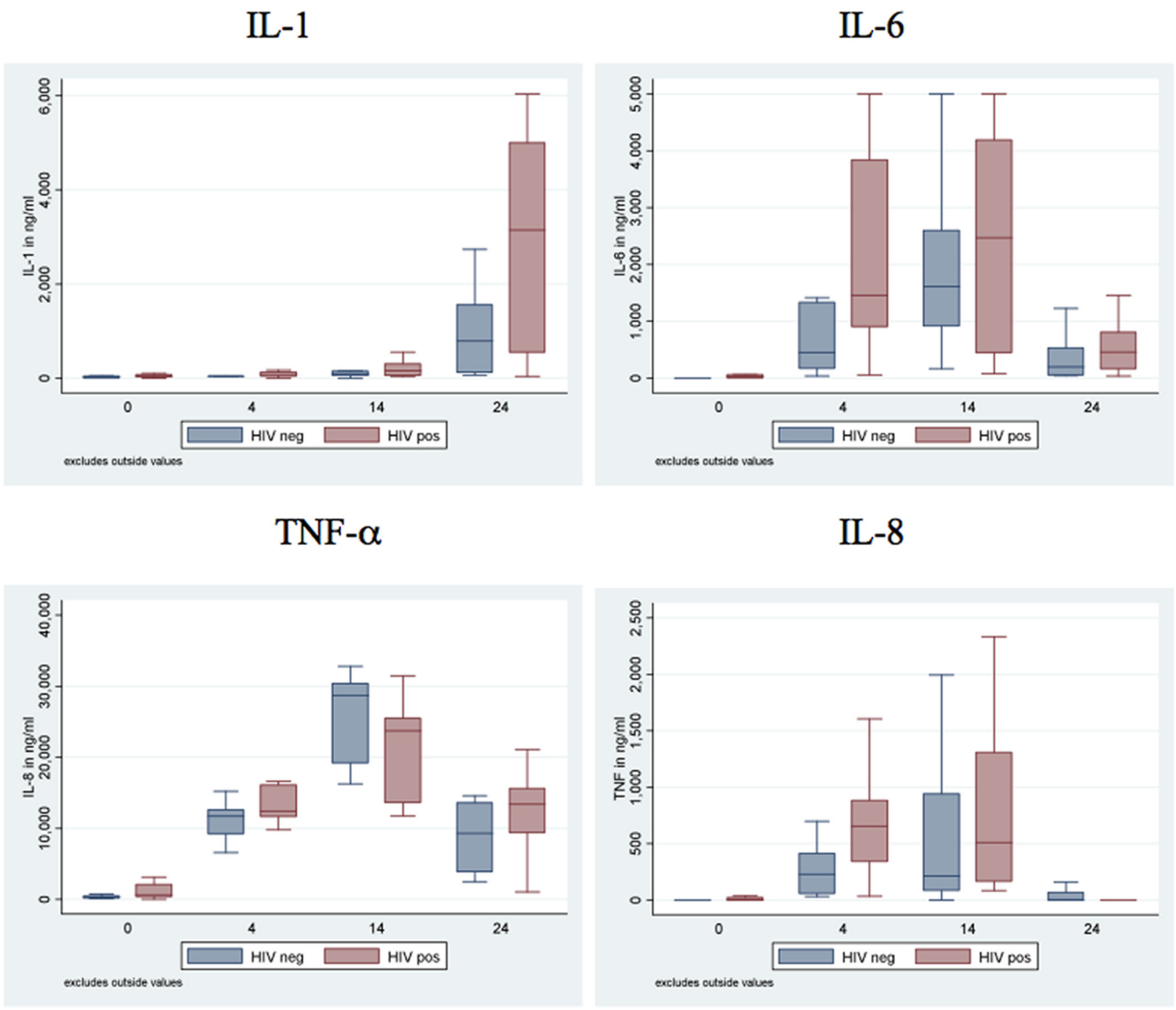

FIG 6 Alveolar macrophages from HIV-infected subjects show altered cytokine production in response to *S. pneumoniae* compared to that of normal subjects. Alveolar macrophages were challenged *in vitro* at a multiplicity of infection of 10 using opsonized type 1 *S. pneumoniae* and cytokine concentrations measured by ELISA in culture supernatants over a 24-h time course. The production of cytokine over the whole time course was estimated using the area under the concentration-time curve and shown to be greater in macrophage supernatants from HIV-infected subjects than from normal subjects for IL-1 ($P = 0.04$) and TNF-α ($P = 0.05$). Measured IL-6 concentrations were also higher at most time points in HIV-infected supernatants, but the areas under the curves were not significantly different ($P = 0.13$). The change in IL-8 concentration over time following bacterial challenge showed a blunted response in macrophages from HIV-infected subjects, but the area under the curve was not significantly different from that of normal subjects ($P = 0.98$).

show no difference in alveolar macrophage function against pneumococcus using IFN-γ-primed alveolar macrophages from either HIV-infected subjects or healthy controls. These data are consistent with observations using *Salmonella* spp. (44) and suggest that IFN-γ levels in the alveolar milieu are sufficient for optimal alveolar macrophage function. It is likely that the major immunodeficiency in the lung specific to pneumococcus following HIV infection is CD4 depletion and, in particular, a loss of Th17 cells (3). The cytokine production is altered in HIV, but the functional significance of this is unclear, as there is a surprising lack of correlation with mycobacterial load (45).

A major difficulty in interpreting the findings in this study is the fact that a minority of alveolar macrophages will be actively infected with HIV. We have previously shown that BAL fluid viral loads are very low, and others have published that the percentage of cells showing active infection is low (<20%) (20). The study design used here has obtained pooled data regarding macrophage gene expression, cytokine production, and bacterial opsonophagocytosis. Individual cell effects were not measured. The overall defense of the lung and the alveolar milieu in particular is a product of the combined effect of lymphocyte, macrophage, and epithelial influences on innate and acquired immune functions. This study suggests that the classical activation of macrophages seen in HIV-infected adults does not have important functional significance with regard to pneumococcal disease and supports the opinion that the focus of attention in HIV-related disease

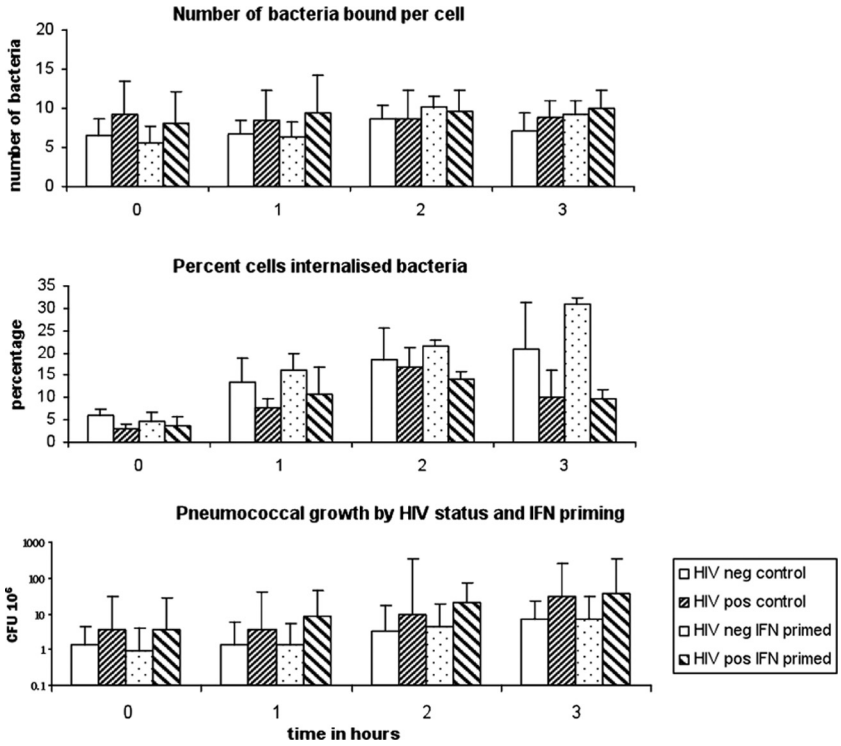

FIG 7 Alveolar macrophages from HIV-infected subjects show normal phagocytosis and killing of *Streptococcus pneumoniae* both before and after gamma interferon priming. Alveolar macrophages from HIV-infected subjects exist in an altered cellular milieu that may be deficient in T-helper support. Alveolar macrophages from HIV-infected and normal subjects were primed using gamma interferon-enriched medium for 24 h and during the *in vitro* challenge with *S. pneumoniae*. There was no difference between macrophages from HIV-infected compared to normal subjects in either interferon-primed or unprimed experiments in the number of bacteria bound per cell (top), the percentage of macrophages binding bacteria (data not shown), the percentage of cells internalizing bacteria (middle), or the concentration of pneumococci in the supernatant at each time point measured in CFU (bottom).

should be toward restoring competent mucosal immunity (46). It is encouraging that HAART does result in some mucosal immune reconstitution, but concern remains that once CD4 populations are severely depleted, full reconstitution does not occur (3).

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