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Dejiang Zhou,* Yun Shen,* Laura Chalifoux,† David Lee-Parritz,† Meredith Simon,† Prabhat K. Sehgal,† Leiya Zheng,* Matilda Halloran,* and Zheng W. Chen2*

It has recently been proposed that Mycobacterium tuberculosis may enhance the pathogenicity of HIV infections and accelerate the course of HIV disease. This hypothesis has been tested in the present study using a simian immunodeficiency virus of macaques (SIVmac)/Mycobacterium bovis bacille Calmette-Guérin (BCG)-coinfected macaque model. Naive and chronically SIVmac-infected monkeys were evaluated. Following BCG inoculation, the SIVmac-infected monkeys exhibited the dominant responses of East Campus, Boston, MA 02215. E-mail address: zchen@bidmc.harvard.edu

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1 Address correspondence and reprint requests to Dr. Zheng W. Chen, Harvard Medical School, Beth Israel Deaconess Medical Center, 330 Brookline Avenue RE113, East Campus, Boston, MA 02215. E-mail address: zchen@bidmc.harvard.edu

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4 Abbreviations used in this paper: PPD, purified protein derivative; BCG, bacille Calmette-Guérin; CDR3, complementarity-determining region 3; SIVmac, simian immunodeficiency virus of macaques; PE, phycoerythrin; QC-PCR, quantitative competitive polymerase chain reaction; bDNA, branched DNA.

It is estimated that at least 6 million people worldwide are coinfected with Mycobacterium tuberculosis and HIV. These coinfections are associated with high rates of active tuberculosis, making tuberculosis one of the leading causes of death in HIV-infected individuals (1–3). Clinical studies have shown that HIV infection substantially increases the risk of developing both primary and reactivation tuberculosis (4–7). In addition, manifestations of tuberculosis are more severe and life threatening in HIV-infected persons (8). Furthermore, epidemiologic studies suggest that M. tuberculosis may have an impact on the course of HIV infections (9, 10).

It is hypothesized that tuberculosis may accelerate HIV disease through activation of the immune system, which may result in increased viral replication in HIV/M. tuberculosis-coinfected individuals. In vitro studies have shown that M. tuberculosis can up-regulate HIV-1 replication in chronically or acutely infected T or macrophage cell lines (11, 12). M. tuberculosis or purified protein derivative (PPD) can stimulate activation of the CD4+ lymphocytes from PPD-positive individuals, which results in increase in HIV-1 replication (13). Pleural fluids and lymphocytes from patients with tuberculous pleuritis can enhance HIV-1 replication in the in vitro system (14). Furthermore, a transient increase in plasma HIV RNA was noted in a small group of HIV-1-infected patients with active tuberculosis (13), although another study was unable to demonstrate such a change in virus loads in HIV-1-infected tuberculosis patients before and after antituberculosis treatment (15). However, it is not clear how a M. tuberculosis coinfection can compromise the immune system in HIV-infected individuals and whether M. tuberculosis-driven immune activation can accelerate HIV-1 disease. Additional in vivo studies are clearly needed to characterize the natural history of HIV-1/M. tuberculosis coinfection and the immunopathogenesis in HIV/M. tuberculosis-coinfected individuals.

SIV-infected nonhuman primates have proved to be powerful models for the study of AIDS (16–18). Using the SIV/macaque animal model, we have explored T cell responses to AIDS viruses and studied the AIDS immunopathogenesis (19–26). More recently, we reported that an SIVmac-infected monkey developed a...
tuberculosis-like disease and an accelerated SIVmac disease following *Mycobacterium bovis* BCG coinfection (25). This observation suggests that SIVmac/BCG-coinfected monkeys may be a valuable animal model for studying HIV-Mycobacterium interactions and the reciprocal impact on Mycobacterium- and HIV-induced diseases. In the present study, we utilized the SIVmac/BCG coinfection model to study the impact of mycobacterial coinfection on the AIDS virus-mediated disease. We found that BCG coinfection in SIVmac-infected monkeys resulted in a prolonged stimulation of T cell subpopulations, which correlated with marked changes in virus loads and accelerated SIVmac disease.

### Materials and Methods

**Animals and virus**

Rhesus monkeys (*Macaca mulatta*) were used in these studies. The monkeys used in the chronic SIVmac/BCG coinfection were 3 to 5 years old; those used in acute BCG infection were 1-year-old naïve animals. These animals were maintained in accordance with the guidelines of the Committee on Animals for Harvard Medical School and the Guide for the Care and Use of Laboratory Animals (46). All monkeys were inoculated i.v. with SIVmac 251 strain, as described previously (19).

**M. bovis BCG coinfection**

*M. bovis* BCG (Pasteur strain) was generously provided by Dr. Scott Koenig, MedImmune, Gaithersburg, MD. BCG was stored in liquid nitrogen and thawed immediately before inoculation. Four groups of monkeys were included for BCG infection studies: 1) 8 monkeys were infected with SIVmac for 3 to 18 mo and then coinoculated with BCG; 2) 2 naïve monkeys were inoculated simultaneously with SIVmac and BCG; 3) as controls, 4 normal monkeys not infected with SIVmac were infected with BCG alone; 4) as another controls, 10 monkeys were infected only with SIVmac in this study. Monkeys were inoculated i.v. with 10⁷ CFU of BCG. After BCG inoculation, the monkeys were followed for the signs of clinical illness. Monkeys that died from SIVmac/BCG coinfection were subjected to a necropsy study.

**Isolation and fractionation of lymphocyte populations in blood and lymph nodes**

PBMC were isolated from EDTA blood of the monkeys using Ficoll/diatrizote gradient centrifugation. Peripheral lymph nodes were obtained by standard biopsy procedures before and after BCG inoculation, and were carefully teased to generate single-cell suspensions. CD4+ or CD8+ lymphocytes were purified using anti-CD4 or anti-CD8 Ab-conjugated Dynabeads (Dynal, Great Neck, NY), as described previously (19). PBMC or lymph node cells were incubated with these immunomagnetic beads for 30 min at room temperature and then selected in two cycles with a magnetic particle concentrator. Monocytes/macrophages in blood or tissues were purified by adherence to culture flasks through 1 h of incubation, as described previously (21). Monocytes/macrophages purified by this method contained less than 2% of CD4+ lymphocytes.

**mAbs and flow cytometric analysis**

The following anti-human CD mAbs that cross-reacted with corresponding macaque Ags were used: phycoerythrin (PE)-conjugated anti-monkey CD3 (FN18, Biosource, Camarillo, CA), PE-conjugated anti-human CD4 (Ortho Diagnostic Systems, Raritan, NJ), PE-conjugated anti-human CD8 (Dako, Carpinteria, CA). Whole blood staining was used following the instructions of the immunoysing kit, ImmunoPrep, from Coulter (Hialeah, FL). Single-cell suspensions from lymph nodes were stained using standard methods. Two-color flow cytometric analyses were performed on an XL flow cytometer (Coulter). Lymphocytes were gated by forward- and side-scatter characteristics, and up to 10,000 gated cells were analyzed.

**mRNA extraction and cDNA synthesis**

mRNA was extracted from unfractionated or fractionated lymphocytes using guanidinium thiocyanate and oligo(dT) spin columns (mRNA extraction kit, Pharmacia, Piscataway, NJ). The first-strand cDNA was synthesized in a 20-μl final volume at 42°C for 1 h using ~0.2–1 μg of mRNA, 1 μg of random hexanucleotides, and 5 U of reverse transcriptase (Promega, Madison, WI). The samples were heated for 5 min at 95°C to terminate the reaction.

**TCR-β CDR3 length analyses**

CDR3 length analyses were done by two methods: autoradiogram display after 3P labeling as described previously (22, 26); and spectratyping through fluorescence labeling using the Genescan analysis (28).

**Autoradiogram analysis of CDR3 lengths.** cDNAs were amplified by PCR for expression of 24 Vβ families using individual Vβ-specific primers and a Cβ-specific primer as described previously (22, 26). The second round of PCR was performed using nested Vβ primers and a Cβ primer, designed as described (26). The internal Cβ primer was labeled at its 5′ end with 3P. The TCR-β DNA from the first round PCR was amplified for 15 cycles under the following conditions: 95°C for 30 s; 55°C for 30 s; and 72°C for 30 s. The amplified TCR-β chains bearing various CDR3 lengths were visualized as a series of radialeabled bands, 3 bases apart, on a 6% polyacrylamide sequencing gel. The selected Vβ families that exhibited a change in CDR3 lengths were repeated at least once by the same method, followed by quantitation using the Genescan method.

**Spectratyping analysis of CDR3 lengths.** The PCR was done under the conditions as described above. The derived PCR products were then amplified in the second-round PCR using individual nested Vβ primers and the internal Cβ primer coupled with the Fam fluorophore (Applied Biosystems, Foster City, CA). The 15-cycle PCR was performed in a 15-μl volume with 0.2 μM concentrations of each primer under the same conditions as described above for autoradiogram analysis. One microliter of each reaction product was mixed with deionized formamide and a TMRA-500 size standard and then electrophoresed on a 5% acrylamide gel. Data were analyzed for size and fluorescence intensity using the Genescan software. Verifying experiments using the samples derived at three time points from four normal monkeys indicated that these CDR3 length analyses were highly reproducible. Further cloning and sequencing in conjunction with the CDR3 length display allowed for the prediction of CDR3 lengths, which were expressed as the predicted number of amino acids.

**Molecular cloning and sequencing of TCR-β CDR3 length-bearing cDNA**

This was done using PCR-based cloning technique (21, 23, 26). Briefly, selected Vβ-bearing cDNA exhibiting dominant CDR3 lengths was cut from the gel using the autoradiogram as a guidance. The cDNA in the gel piece was recovered by incubating the gel piece at 100°C for 5 min before the standard ethanol precipitation. The selected cDNA was then amplified by PCR for 30 cycles using nested Vβ-specific primers containing an EcoRI restriction site and the Cβ primer containing an XbaI restriction site. For a longitudinal clonotypic analysis, the same CDR3 length-bearing cDNAs from the CD4+ or CD8+ BCL PBL sampled at different time points from the monkeys were similarly isolated for cloning and sequencing. PCR was performed for 30 cycles as previously described (26). To minimize PCR-generated misincorporation, plaque-forming unit DNA polymerase was used in the PCR reactions. The PCR products were digested with EcoRI and XbaI and ligated into the plasmid pSP65 (Promega) for cloning and sequencing. At least 25 cDNAs were analyzed in each DNA sample. The frequency of the clonotypic sequences was determined based on the percentage of the CDR3-restricted clones in the total clones bearing the same CDR3 lengths.

**Quantitative competitive PCR (QC-PCR)**

A QC-PCR assay for measuring plasma HIV RNA was adapted to measure SIV RNA in plasma (29). In these experiments, a pSP72 plasmid containing the SIVmac gag fragment DNA (20) was used to create the mutant competitor by engineering a dominant CDR3 length. This plasmid was converted to linear DNA using an in vitro transcription system, as described previously (22). For viral RNA extraction, 200 μl of plasma from each time point were added to a tube containing 1800 μl of 20 mM Tris buffer and then ultra-centrifuged at 40,000 rpm for 70 min in a Sorval rotor. The pellets of viral particles were digested with protease K and DNase, and treated with phenol/chloroform for RNA extraction. The viral RNA was precipitated in ethanol and spun down for 15 min at 14,000 rpm. The extracted RNA was aliquoted into 6 different tubes, which contained individually 0, 1, 0.5, 10, 50, 100, 500, or 1000 copies of SIVmac gag competitor RNA. The RNA mixtures were reverse-transcribed to cDNA and competitively amplified by a 35-cycle PCR using a primer of SIVmac gag-specific primers. The sequences for these oligonucleotides were as follows: sense primer, 5′-TAA ATG CCT GGG TAA AAT-3′ (gag position 461–478 of SIVmac 251); antisense primer 5′-TGC TAT GGG GGT CTC TTG CTG G-3′ (gag position 752–774 of SIVmac 251). The amplified PCR products containing wild-type (314 bp) and competitor (239 bp) were separated on 2% agarose gels.
and measured for their densities in a GS 700 Imaging Densitometer (Bio-Rad, Richmond, CA). Quantitation was achieved by data analysis using Molecular Analyst system software (Bio-Rad). The coefficient variation of intra- and interassays using this protocol was <20%. The sensitivity of the QC-PCR was 1 × 10^3 RNA copies in 1 ml of plasma. As a complementary study, plasma SIV RNA was also quantitated by the branched DNA (bDNA) assay (Chiron, Emeryville, CA). This assay allows for detecting a minimum of 10^3 RNA copies/ml.

**PCR-based semi-quantitation for intracellular SIVmac RNA**

To analyze intracellular SIVmac RNA expression, purified CD4^+ lymphocytes and CD14^+ cells, as well as adherent monocytes, were subjected to mRNA extraction and cDNA synthesis. The 356-bp fragment was amplified from cellular cDNA by a 30-cycle PCR using a pair of SIVmac gag-specific primers as described above and previously (30). The PCR products were separated on a 2% agarose gel, transferred onto a nylon membrane, and then hybridized to a 32P-labeled internal oligonucleotide. The sequence of the hybridizing primer was 5′-GCC AGG ATT TCA GGC ACT GTC AGA AGG CTG-3′ (gap position 513–542 of SIVmac 251). The defined copies of SIV gag cDNA were always included as standards for the semi-quantitation. Radioactivity was quantitated using AMBIS 100 (22, 24, 26). To normalize SIV mac RNA expression levels, the housekeeping gene β-actin was similarly quantitated using the same amount of cellular cDNA. The sequences of actin oligonucleotides were as follows: sense primer, 5′-CCC CCA TGC CAT CCT GGC TCT G-3′; antisense primer, 5′-CAT GAT GGA GTT GAA GGT AGT TT-3′; hybridizing primer, 5′-GAC CTG ACT GAC TAC CTG ATG AAG ATC TCT AC-3′. The semi-quantitation was achieved by calculating the copy number of SIV gag RNA in 1.66 × 10^−16 M actin-containing cellular cDNA (∼10^6 cells).

**Quantitation of SIVmac Ags**

SIV virus loads in the acute infection was assessed by measuring SIV Gag p27 proteins in plasma using an Ag capture kit (Coulter).

**Statistical analysis**

Two methods of statistics were undertaken to determine whether BCG infection significantly enhanced a decline of CD4^+ PBL and accelerated the progression to clinical AIDS in SIVmac-infected monkeys. The paired Student t test, as described previously (19, 31), was used to examine the difference in decline rates of CD4^+ PBL before and after BCG coinfection in the monkeys chronically infected with SIVmac. The CD4^+ PBL decline rates in a SIVmac infection (before BCG coinfection) was calculated and expressed as the declining number of cells/μl/min using the data obtained from 2–6 mo of post-SIVmac infection through the time of pre-BCG inoculation. The decline rates in the SIVmac/BCG coinfection were derived from the CD4^+ PBL counts obtained from the different time points of entire course of BCG coinfection. In addition, the unpaired Student t test was utilized to analyze different decline rates of CD4^+ PBL between the SIVmac/BCG-coinfected monkeys and the monkeys infected with SIVmac alone. The CD4^+ PBL decline rates in the controls of SIVmac-infected monkeys were calculated using the CD4^+ PBL counts from 2–20 mo after SIVmac infection through the last time points of follow-up. Furthermore, the difference in the surviving time between the SIVmac/BCG-coinfected monkeys and the controls of the SIVmac-infected monkeys was also evaluated by the Student t test.

**Results**

**BCG infection resulted in a CDR3-dependent activation and expansion of selected Vβ-expressing cell subpopulations in normal monkeys not infected with SIVmac**

To optimally characterize BCG-driven T cell responses, normal monkeys not infected with SIVmac were inoculated with BCG and assessed for evidence of in vivo stimulation of selected Vβ-expressing CD4^+ and CD8^+ cell subpopulations. Purified CD4^+ and CD8^+ cells obtained from different time points after BCG inoculation were subjected to a molecular analysis of CDR3 lengths in each of 24 Vβ gene families. Interestingly, BCG infection resulted in a striking change in CDR3 lengths in selected Vβ-expressing CD4^+ and CD8^+ PBL subpopulations of the monkeys. CDR3 lengths used by selected Vβ family-expressing CD4^+ and CD8^+ PBL subpopulations underwent a change from gaussian distributions to one or two predominant lengths after the BCG inoculation (Fig. 1, A and B). This BCG-induced change in CDR3 lengths was most evident in PBL of the infected monkeys 1 to 3 wk after BCG inoculation.

To determine whether the change in CDR3 lengths represented clonal expansion of the selected Vβ-expressing cell subpopulations, we sought to examine the clonality of the CDR3 length-dominated CD4^+ and CD8^+ PBL subpopulations using the PCR-based cloning and sequencing strategy (21, 24, 25). As expected, Vβ9-bearing cDNA derived from CD4^+ and CD8^+ PBL obtained from monkey 269 before BCG inoculation revealed polyclonal sequences, indicating that the Vβ9-expressing PBL subpopulation was polyclonal before BCG coinfection. In contrast, oligoclonal and monoclonal sequences were dominant in Vβ9-bearing cDNA derived from CD4^+ and CD8^+ lymphocytes 2 wk after BCG infection. While the Vβ9^+ CD4^+ cell subpopulation used a Jβ1.5-bearing CDR3 length with 10 amino acids, the dominant Vβ9^+ CD8^+ cells employed the Jβ2.4-bearing CDR3, which was composed of 13 amino acids (Fig. 1C). These clonotypic sequences were undetectable in 30 cDNA clones derived from PBL of the monkey before and 2 mo after BCG inoculation. These results, therefore, provide molecular evidence suggesting that BCG Ags stimulate a dominant clonal expansion of CDR3-restricted Vβ-expressing CD4^+ and CD8^+ cell subpopulations during acute BCG infection of monkeys.

**BGC coinfection induced a prolonged clonal expansion of selected CDR3-bearing CD4^+ and CD8^+ T cell subpopulations in SIVmac-infected monkeys**

We then sought to determine whether BCG infection could result in similar activation and expansion of T cell subpopulations in SIVmac-infected monkeys. Eight monkeys previously infected with SIVmac for 3–18 mo were inoculated i.v. with BCG and examined for an alteration in CDR3 lengths. Like normal monkeys not infected with SIVmac, SIVmac-infected monkeys exhibited one or two dominant CDR3 lengths in selected Vβ-expressing CD4^+ and CD8^+ PBL subpopulations following BCG inoculation (Fig. 2, A and B). Furthermore, molecular analysis of the dominant CDR3 length-bearing β cDNA revealed that the clonal expansion, as seen in normal monkeys, was also identified in these selected Vβ-expressing cell subpopulations during the acute BCG coinfection of the SIVmac-infected animals (Fig. 2C). Surprisingly, while the clonal expansion was transiently identified in normal monkeys not infected with SIVmac, the clonotypic sequences in the selected CDR3 length-bearing Vβ cells were persistent during the chronic BCG infection and became dominant at the end stage of SIVmac/BCG coinfection (Fig. 2C). Nevertheless, these clonotypic sequences were not readily identified in the periods of pre-BCG inoculation (Fig. 2C), suggesting that the expansion of the CDR3-restricted cell subpopulations was driven by BCG Ags. These findings, therefore, suggested that BCG Ags stimulated a prolonged clonal expansion of the selected Vβ-expressing CD4^+ and CD8^+ PBL subpopulations in SIVmac-infected monkeys following the BCG coinfection.

**BCG-induced activation of selected CDR3-bearing T cell subpopulations correlated with an increase in viral loads in the chronically SIVmac-infected monkeys**

BCG-induced T cell activation may increase the ability of AIDS virus to replicate in CD4^+ lymphocytes and macrophages. To address this issue, we examined whether CDR3-dependent activation of selected Vβ-expressing cell subpopulations resulted in a change in SIV loads in the SIVmac/BCG-coinfected animals. The change in intracellular and plasma SIV RNA was investigated using PCR-based quantitation and branch DNA assay, respectively (Fig. 3). As expected, the SIVmac-infected monkeys showed an apparent
increase in viral loads during the acute phase of BCG coinfection (Fig. 4). Increases of 3–100-fold in SIV cDNA in CD4<sup>+</sup> PBL or CD4<sup>+</sup> L.N. cells were detected in the SIVmac-infected monkeys 1–3 wk after BCG inoculations (Fig. 4A). Up to 6-fold increases in SIV RNA were detected in lymph node macrophages of the coinfected monkeys after BCG inoculation despite the lack of significant change in their blood monocytes (Fig. 4B). Consistent with the up-regulation of SIV RNA expression in CD4<sup>+</sup> T cells and macrophages was a marked increase in plasma SIV RNA in the SIVmac-infected monkeys (Fig. 4C). A maximum increase of 40- to 1000-fold in plasma RNA was identified by bDNA assay and QC-PCR in 6 of 8 SIVmac-infected monkeys following BCG inoculation (Fig. 4C). Furthermore, a sustained increase in virus loads in four of the infected monkeys coincided with the prolonged dominance of clonotypic sequences in CD4<sup>+</sup> lymphocytes, suggesting a correlation between the BCG-driven clonal activation and increased virus loads in the SIV/BCG-coinfected monkeys (Fig. 4D). Thus, these results suggest that BCG-induced T cell activation may be the driving force for the increased virus replication in the SIVmac-infected monkeys.

FIGURE 1. BCG infection resulted in dominant responses of CDR3-restricted CD4<sup>+</sup> and CD8<sup>+</sup> T cells in normal macaques not infected with SIVmac. A, Autoradiogram display of CDR3 lengths used by selected V<beta>-expressing PBL subpopulations of the monkey 269 before and after BCG inoculation. B, Histograms for spectrotyping analysis of CDR3 lengths used by selected V<beta>-expressing PBL subpopulations of monkey 148. Fragment lengths in nucleotides are displayed on the x-axis and fluorescence intensity on the y-axis. The numbers of nucleotides in different CDR3 lengths were determined in control experiments (see Materials and Methods) and were expressed as predicted number of amino acids. Only those altered V<beta> families from the entire repertoire (24 families) were shown for a change in CDR3 after BCG inoculation. C, Sequence and frequency analyses of the dominant CDR3 length-bearing cDNA as seen in A. The selected CDR3-bearing clones were generated from the dominant CDR3 bands and the corresponding bands revealed by the autoradiogram CDR3 display in cDNA from purified CD4<sup>+</sup> and CD8<sup>+</sup> PBL obtained from monkey 269 after BCG inoculation. The dominant nucleotide and amino acid sequences of the CDR3-restricted clones were shown on the top, with their frequencies (x-axis) on different days (y-axis) after BCG inoculation displayed in the graphics. All clones for a longitudinal analysis were derived from the individual V<beta> cDNA bearing the same CDR3 lengths. The frequency of the clonotypic sequences was expressed as percentage of the dominant clones in the total clones bearing the same CDR3 lengths.
FIGURE 2. See caption opposite page.
BCG coinfection enhanced a decline of CD4⁺ PBL counts in the SIVmac-infected monkeys and the naive animals simultaneously inoculated with SIVmac and BCG

Clinical studies have demonstrated that an enhanced decline of CD4⁺ PBL counts during the chronic HIV infection is indicative of the development of clinical AIDS (45). We therefore sought to determine whether BCG-driven T cell activation and associated increase in virus loads in SIVmac-infected monkeys resulted in an enhanced decline of CD4⁺ PBL and acceleration of SIVmac infection. Interestingly, the SIVmac/BCG-coinfected monkeys demonstrated an increased decline of CD4⁺ PBL counts (Fig. 5A), with the decline rates increasing from 200/μl/6 mo up to 1560/μl/6 mo a few months after BCG inoculation (Table I). The lymphocyte count of monkey 266, whose CD4⁺ in the blood was 980/mm³ before BCG inoculation, dropped to 600/mm³ 2 mo after BCG inoculation, and 1 mo later declined further to 200/mm³ (Fig. 5A).

The other coinfected monkeys also displayed such a rapid decline of CD4⁺ lymphocyte counts in the blood after the BCG inoculation (Fig. 5A, Table I). In contrast, the control SIVmac-infected monkeys had a slower decline of their CD4⁺ lymphocyte counts during the chronic SIVmac infection (Fig. 5B, Table I). The control monkeys infected with BCG alone did not show a decline of CD4⁺ PBL after BCG inoculation (Fig. 5C).

If BCG coinfection increases the SIV pathogenicity, the enhanced SIVmac infection may be more apparent in the naive monkeys following an SIVmac/BCG coinfection. To test this possibility, two naive monkeys were inoculated simultaneously with SIVmac and BCG and then followed for signs of clinical illness, SIV p27 antigenemia and CD4⁺ PBL counts. As controls, two groups of normal monkeys were inoculated either with SIVmac or BCG alone. Surprisingly, the naive monkeys had their CD4⁺ PBL counts dropped from >1480/μl before infection to as low as 290/μl 2 wk after the simultaneous coinfection with SIVmac and BCG (Fig. 6A). The profound depletion of CD4⁺ PBL coincided with the persistent SIV p27 antigenemia in the SIVmac/BCG-coinfected but not the control SIVmac-infected monkeys (Fig. 6B). Moreover, the enhanced SIVmac infection appeared to be associated with BCG-induced immune activation. The monkey 347 developed T cell activation-related toxic shock syndrome characterized by severe dehydration...
and collapse in circulation on day 18 after the simultaneous SIVmac/BCG inoculation. The monkey had to be treated intensively with fluid infusion for a recovery from the shock syndrome. The other animal also developed a transient change in mental status. In contrast, the control SIVmac-infected or BCG-infected monkeys did not show any noticeable signs of clinical illness or such a marked depletion of CD4⁺ PBL during the acute infection (Fig. 6A). Thus, the results in the naive monkeys coinfected with SIVmac/BCG were consistent with those seen in the chronically coinfected monkeys, indicating that BCG coinfection induced the rapid decline of CD4⁺ PBL in SIVmac-infected monkeys.

Statistical studies (19, 31) were then performed to determine whether any significant enhancement of CD4⁺ lymphocyte decline and acceleration of disease progression could be discerned in the SIVmac/BCG-coinfected monkeys. When decline rates of CD4⁺ PBL before and after BCG coinfection were compared in the chronically coinfected animals, the paired Student t test indicated a significantly enhanced decline of CD4⁺ lymphocytes in the SIVmac/BCG-coinfected monkeys (p < 0.01). Similar results indicative of BCG-enhanced CD4⁺ PBL decline were also obtained when the decline rates of the coinfected animals were compared by the unpaired Student t test with those of the 10 control SIVmac-infected monkeys (p < 0.01). These results, therefore, provide in vivo evidence demonstrating that coinfection with M. bovis BCG can enhance the viral pathogenicity and accelerate SIVmac-induced disease in SIVmac-infected monkeys.

The enhanced decline of CD4⁺ PBL counts in SIVmac/BCG-coinfected monkeys resulted in the rapid progression to clinical AIDS

Since BCG coinfection accelerated SIVmac disease characterized by the enhanced decline of CD4⁺ PBL, we expected to see
FIGURE 4. BCG-induced T cell activation was correlated with a marked increase in SIV viral loads in the SIVmac-infected monkeys. A, SIV RNA expression in purified CD4⁺ PBL and lymph node cells obtained at the different time points before and after BCG inoculation. B, SIV RNA expression in monocytes/macrophages in PBL and lymph node cells obtained from different time points before and after BCG inoculation. C, Plasma SIV RNA measured by bDNA and QC-PCR before and after BCG inoculation. D, Correlation between the prolonged T cell activation and the increased SIV RNA in plasma of the SIVmac/BCG-coinfected monkeys.
that these coinfected monkeys died rapidly from clinical AIDS-like syndrome. The clinical course of AIDS is temporally compressed in the SIVmac-infected monkeys as compared with the HIV-1-infected humans, with animals usually dying 2–5 yr after infection. As expected, the enhanced decline of CD4\(^{+}\) PBL inevitably led to a rapid death in the SIVmac/BCG-coinfected monkeys. All SIVmac-infected monkeys died 2 to 7 mo after BCG inoculation (Table I). At the end stage of SIVmac/BCG coinfection, all these monkeys developed a disseminated BCG infection, as detected by acid-fast staining of tissue sections and PCR-based quantitation of BCG rRNA isolated from the tissues (25). Surprisingly, four coinfected monkeys developed disseminated tuberculosis-like disease. The BCG-induced tuberculosis was characterized clinically by diarrhea, colitis/peritonitis, and weight loss and pathologically by nonnecrotizing granulomas in multiple tissues, which has been described in some patients coinfected with HIV-1/M. tuberculosis (32). On the other hand, the SIVmac-induced disease was also apparent in the monkeys that died after SIVmac/BCG coinfection (Table I). Profound depletion of lymphocytes in lymphoid tissues, opportunistic infections, lymphoma, and the fatal thrombosis induced by SIVmac (16) were seen in these coinfected animals. In contrast, the control SIVmac-infected monkeys exhibited a natural course of SIVmac infection and survived for up to 5 years after the viral infection.

**FIGURE 5.** BCG coinfection resulted in an enhanced decline of CD4\(^{+}\) PBL counts in SIVmac-infected monkeys. A. Absolute numbers of peripheral blood CD4\(^{+}\) lymphocytes of the SIVmac-infected monkeys before and after BCG inoculation. Day 0 was the time for BCG inoculation whereas day minuses were those time points after SIVmac inoculation (before BCG inoculation) in the infected monkeys. In animals 320 and 315, CD4\(^{+}\) PBL counts before SIVmac infection were not available. B. Absolute numbers of peripheral blood CD4\(^{+}\) lymphocytes of the control SIVmac-infected monkeys before and after SIV inoculation. C. Absolute numbers of peripheral blood CD4\(^{+}\) lymphocytes of the control BCG-infected monkeys before and after BCG inoculation.
infection (Table I). The normal monkeys inoculated with BCG alone survived BCG infection without any noticeable signs of clinical illness (Table I). A statistical analysis of difference in surviving time between the coinfected monkeys and control SIVmac-infected animals indicated that BCG coinfection significantly shortened the course of SIV infection in the coinfected monkeys ($p < 0.001$).

Discussion

Several studies in humans suggested that *M. tuberculosis* may increase the ability of HIV-1 to replicate in the CD4$^+$ T cells (11–14). More recently, Cheynier et al. (39) has reported that PPD-driven CD4$^+$ T cell activation can be associated with SIV quasispecies in SIV/BCG-coinfected monkeys. In the present study, we have found the following observations, which have not been documented in humans or animal models. 1) BCG induces a dominant expansion of CDR3-restricted CD4$^+$ and CD8$^+$ T cell subpopulations during the acute stage of BCG coinfection. 2) The prolonged T cell activation during the chronic BCG coinfection is correlated with the increase in virus loads as well as the enhanced decline of CD4$^+$ PBL counts in SIVmac-infected monkeys. 3) SIVmac/BCG coinfection in naive monkeys can result in T cell activation-related toxic shock syndrome and rapid death with the evidence of persistent SIV antigenemia and enhanced SIV disease. 4) BCG coinfection shortens the course of clinical AIDS and induces tuberculosis-like disease in SIVmac-infected monkeys.
results in the present study, therefore, provide in vivo evidence indicating that BCG can enhance the pathogenicity of the SIVmac infection and accelerate SIVmac-induced AIDS in macaques.

Interestingly, dominant responses of CDR3-restricted T cell subpopulations were transiently detected in the acute BCG infection of normal SIV-negative and SIVmac-infected monkeys. The transient expansion of CDR3-restricted T cell subpopulations preceded persistence of clonotypic sequences in the selected CDR3 length-bearing T cells. The dominant responses of CDR3-restricted T cell subpopulations may be driven by the high level of BCG Ags produced in the acute infection, since expansion of these cell subpopulations was associated with a resolution of BCG infection in normal monkeys not infected with SIVmac. In SIVmac-infected monkeys, however, the dominant responses of the CDR3-restricted T cell subpopulations preceded persistence of clonotypic sequences in the selected CDR3 length-bearing T cells. The transient expansion of CDR3-restricted T cell subpopulations was followed by an undetectability of clonotypic sequences after acute BCG infection in the normal monkeys not infected with SIVmac. In SIVmac-infected monkeys, however, the dominant responses of the CDR3-restricted T cell subpopulations preceded persistence of clonotypic sequences in the selected CDR3 length-bearing T cells. The decreased level of BCG loads during the chronic BCG coinfection may result in the reduced magnitude of clonal expansion in the coinfected monkeys. This notion is also supported by the finding that the clonal dominance in the selected CDR3 length-bearing T cells reoccurs at the time the animals die from BCG dissemination (Fig. 2C).

Our study demonstrated that BCG-driven activation and expansion of selected Vβ-expressing cell subpopulations were associated with the increase in intracellular SIV RNA expression as well as in plasma viremia in SIVmac-infected monkeys. The up-regulation of SIVmac replication in these coinfected animals was comparable with the transient increase in viremia observed in HIV-1-infected individuals following influenza vaccination or recall Ag immunization (35–38). Our results were also paralleled to the recent observation demonstrating that T cell activation can be a driving force for the selected variants of AIDS viruses (39, 40). The increase in virus loads in the SIV/BCG-coinfected monkeys may result from the BCG-driven activation and expansion of CD4+ T cells in that this increase was most evident at the time dominant expansion of CDR3-restricted T cell subpopulations was seen during the acute BCG infection. In addition, this increase in SIV loads was correlated with the prolonged clonal expansion driven by BCG in some of the coinfected monkeys. Furthermore, the increase in intracellular SIV RNA expression was more striking in CD4+ T cells than in macrophages during the acute BCG infection. Several possibilities may be considered as the explanation for the T cell activation-associated increase in the virus loads. The BCG Ags-activated CD4+ cells may become more infectable as a result of induction of an expression of chemokine receptors required for...
virus entry (41, 42). Moreover, activation signals in CD4+ T cells may trans-activate the latent virus and facilitate the virus replication (43). Finally, the proinflammatory cytokines produced by BCG Ags-activated CD4+ T cells may directly or indirectly up-regulate viral replication in SIVmac-infected CD4+ T cells (43). This cytokine-mediated up-regulation of SIV replication may also help to explain an increase in SIV RNA expression in tissue macrophages following BCG coinfection in the SIVmac-infected monkeys. BCG-infected macrophages may more readily be infected by SIVmac in the coinfected animals. In fact, increased viral production in macrophages has recently been reported in HIV-1-infected patients with Mycobacterium coinfection (44).

In contrast to those reports studying a role of immune stimulation in AIDS virus pathogenesis (35–40), our studies in the SIV/BCG coinfection model provide important information concerning the clinical and pathological consequence of prolonged T cell activation in the coinfected individuals. The prolonged clonal expansion of T cells during the chronic BCG coinfection coincided with an enhanced decline of CD4+ PBL counts and accelerated progression to a clinical AIDS-like syndrome in SIVmac-infected monkeys. These findings suggest that BCG-driven T cell activation may be an underlying mechanism for the acceleration of SIVmac disease. BCG-induced T cell activation may increase the ability of SIV to deplete CD4+ T cells through up-regulating SIVmac replication. This correlation between the increased virus load and enhanced decline of CD4+ PBL counts was indeed observed in the naive monkeys coinfected with SIV/BCG (Fig. 6) as well as in some of the chronically coinfected animals (Figs. 4 and 5). On the other hand, the prolonged T cell activation in persistent BCG infection can certainly compromise and exhaust the immune system to a greater extent, which may inevitably result in an enhanced decline of CD4+ T cells and accelerated progression to a AIDS-like syndrome in the SIVmac/BCG-coinfected monkeys. This BCG-induced immune exhaustion may help to explain an accelerated decline of CD4+ PBL counts without sustained increases in viral loads in some coinfected monkeys.

The BCG-induced acceleration of the SIVmac disease was characterized by an enhanced decline of CD4+ PBL as well as rapid progression to a clinical AIDS-like syndrome in the coinfected monkeys. Like HIV-1-infected humans (45), chronically SIVmac-infected monkeys exhibited a slow decline of CD4+ PBL counts before BCG coinfection. Following BCG coinfection, the decline rates of CD4+ PBL can increase greatly to a level as high as 1560 cells/μl/6 mo. In fact, the accelerated decline of CD4+ PBL is observed in the HIV-1-infected patients at the time when they develop clinical AIDS (46). The fast decline of CD4+ PBL counts is also seen in the rapidly progressing persons infected with HIV-1 (46). However, Mycobacterium tuberculosis-induced acceleration of CD4+ PBL declines has not formally been demonstrated in HIV-1/M. tuberculosis-coinfected persons. The natural history of HIV/M. tuberculosis coinfection may not well be unveiled in the clinical setting, since anti-tuberculosis intervention must be initiated once tuberculosis is diagnosed in HIV-1-infected patients. Moreover, diagnosis of M. tuberculosis coinfection still remains a challenge in HIV-infected persons. These factors may underestimate the effect of tuberculosis on the immune system of the coinfected patients.

BCG-induced acceleration of SIV disease was more striking in the naive monkeys than in the chronically SIVmac-infected monkeys. A marked depletion of CD4+ PBL was noted in the naive monkeys 2 wk after the simultaneous SIV/BCG inoculation, whereas in the SIVmac-infected monkeys the increased decline of CD4+ PBL counts became apparent 2 to 4 mo after BCG coinfection. The difference in the pace of CD4+ PBL decline between these two groups of monkeys may be related to the status of SIVmac infection and the magnitude of BCG-driven T cell activation. In the naive monkeys, we have observed that SIVmac/BCG coinfection can lead to a toxic shock syndrome, a T cell activation-related disorder that we have seen in the monkeys challenged with a superantigen (26). Such a profound T cell activation can certainly render CD4+ T cells more infectable and destroyable during SIVmac/BCG coinfection. Moreover, the burst of viral replication may be more striking after the SIVmac/BCG coinfection in the monkeys naive for anti-SIVmac immune responses. This notion is indeed supported by the finding demonstrating the correlation between the CD4+ T cell depletion and prolonged SIV p27 antigenemia in the naive monkeys. In the chronically SIVmac-infected animals, however, the superimposed BCG infection may not be able to induce such a marked increase in virus burden in the presence of established immune responses to SIV. In addition, the magnitude of BCG-driven T cell activation may be lower in the SIVmac-infected monkeys, since the repertoire of T cells responding to BCG Ags is usually shrunk or depressed due to the global depletion of CD4+ T cells in the chronic infection.

Coincident with the accelerated decline of CD4+ PBL counts was a rapid progression to clinical AIDS-like syndrome in the SIVmac/BCG-coinfected monkeys. The SIVmac/BCG-coinfected monkeys died within 2 to 7 mo after BCG inoculation. The impact of mycobacterial coinfection is indeed twofold in these SIVmac/BCG-coinfected animals. The SIVmac-infected but not normal monkeys can develop disseminated BCG infection or tuberculosis-like disease following BCG coinfection. On the other hand, AIDS-like syndromes such as lymphocytic depletion in lymphoid tissues, opportunistic infections, lymphoma and SIVmac-induced arteriopathy, and thrombosis are observed in these monkeys that died from the SIVmac/BCG coinfection (16). Therefore, the coinfection can die from clinical AIDS that is linked to either Mycobacterium-related sequelae or SIVmac disease.

Thus, the results in SIVmac/BCG-coinfected monkeys indicate that BCG infection can accelerate a progression of SIVmac disease, strongly supporting the current hypothesis that mycobacterial coinfection can enhance viral pathogenicity as well as accelerate the progression to clinical AIDS in HIV-infected individuals. The findings in the present studies, therefore, suggest that the prolonged T cell activation driven by Mycobacterium may be a mechanism underlying the acceleration of AIDS virus-induced disease.

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References