MAJOR ARTICLE

Immunopathogenesis and Diagnosis of Tuberculosis and Tuberculosis-Associated Immune Reconstitution Inflammatory Syndrome during Early Antiretroviral Therapy

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Background. In many settings, the benefits of antiretroviral therapy (ART) are reduced by the high early incidence of tuberculosis and tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS).

Methods. We used tuberculin skin testing and the QuantiFERON-TB Gold In-Tube assay to investigate cellular immune responses to purified protein derivative (PPD) and region of difference 1 (RD1) antigens during the first 24 weeks of ART.

Results. TB-IRIS and ART-associated tuberculosis occurred in 15 of 75 (20%) and 11 of 231 (4.8%) participants at risk, respectively. Greater increases in interferon γ (IFN-γ) and skin test responses to PPD were seen at week 24 and 12 in participants with TB-IRIS ($P \leqslant .04$), respectively. Raw IFN-γ responses to RD1 antigens and PPD corrected for pre-ART $CD4^+$ T cell counts were higher at all time points in individuals with ART-associated tuberculosis ($P < .001$) and were associated with areas under receiver operator characteristic curves of 0.90 for RD1 (95% confidence interval [CI], 0.78–1.00) and 0.92 for PPD (95% CI, 0.83–1.00) for the diagnosis of ART-associated tuberculosis. Pre-ART IFN-γ responses enabled stratification of participants into groups with risks of subsequent tuberculosis of 0.7%, 9.3%, and 30.0%.

Conclusions. Type 1 effector T cell responses are prominent in ART-associated tuberculosis, but additional immune defects may be more important in paradoxical TB-IRIS. IFN-γ release assays may contribute to the prediction and diagnosis of tuberculosis during early ART.

Human immunodeficiency virus (HIV) infection is associated with immune activation and depletion of $CD4^+$ T cells, which leads to defects in immune responses [1, 2]. The use of combination antiretroviral therapy (ART) is associated with repletion of circulating $CD4^+$ T cells and improvement in immune function, which results in a decrease in the incidence of opportunistic infections [3–5]. However, some individuals experience clinical disease associated with a prominent inflammatory response to an opportunistic pathogen after commencing ART, which is commonly referred to as immune reconstitution inflammatory syndrome (IRIS).

"Paradoxical" tuberculosis-associated IRIS (TB-IRIS) is characterized by initial diagnosis of and treatment for tuberculosis, followed by initiation of ART and subsequent clinical deterioration [6]. Previous studies have suggested that IRIS results from disregulated restoration of a pathogen-specific immune response [7–15]. However, no large prospective study of the immunopath-

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ogenesis of TB-IRIS has been reported to date. A significant proportion of people treated in settings with a high burden of tuberculosis develop active tuberculosis after initiating ART [16, 17], a condition termed "ART-associated tuberculosis" [6]. It is not known to what degree the clinical presentation of this condition is driven by microbial virulence in the setting of persisting immunodeficiency versus immunopathological immune responses to occult active *Mycobacterium tuberculosis* infection [18].

Interferon γ (IFN-γ) release assays (IGRAs) measure IFN-γ production by T cells in response to antigens encoded by genes from the region of difference 1 (RD1) domain of the *M. tuberculosis* genome. While a rapidly growing body of knowledge is informing the use of these assays in the diagnosis of latent tuberculosis [19], less is known regarding their performance in individuals with HIV infection [20–23] or their utility in the diagnosis of active tuberculosis [24–28]. No studies have been reported that address the performance of these assays or of the tuberculin skin test (TST) in the diagnosis of ART-associated tuberculosis or paradoxical TB-IRIS. We sought to investigate the immunopathogenesis and diagnosis of paradoxical TB-IRIS and ART-associated tuberculosis, using TSTs and an IGRA in a setting with limited resources and a high burden of tuberculosis.

METHODS

We performed a prospective cohort study at the National Center for HIV/AIDS, Dermatology and Sexually Transmitted Diseases Social Health Clinic, an ambulatory HIV clinic in Phnom Penh, Cambodia. Participants were HIV-1 antibody positive, ⩾18 years old, commencing ART, known to have no previous use of ART, and willing and able to provide written informed consent. Study participants were recruited from December 2005 through March 2007 and followed up for 24 weeks. The study was approved by the Cambodian National Ethics Committee and the University of New South Wales Human Research Ethics Committee.

Diagnostic tests. Before initiation of ART, after 4, 12, and 24 weeks of ART, and at the time of suspected paradoxical TB-IRIS or ART-associated tuberculosis, we performed QuantiFERON-TB Gold In-Tube (QFTGIT; Cellestis) assays in accordance with the manufacturer's instructions, using RD1 antigens (ESAT-6, CFP-10, and TB 7.7), positive mitogen (phytohemagglutinin), and negative controls. In a second nil tube, 3 drops of purified protein derivative (PPD; CSL) were added, in accordance with the instructions for the second-generation QuantiFERON-TB test [29]. Samples with an assay optical density >3.5 were diluted 1:4 or 1:8, and the assay was repeated. Laboratory scientists performing the assays were blinded to all clinical and skin test data.

Skin testing was performed using PPD (0.1 mL at a concentration of 10 U/mL) and *Mycobacterium avium* (0.1 mL at a concentration of 100 U/mL) antigens (CSL) at the same time points, with the exception of the test 4 weeks after initiating ART. Antigens were placed using the Mantoux method and read 48–72 h later by a trained study nurse blinded to QFTGIT results and study outcome status. A positive test result was defined as an area of induration ⩾5 mm in diameter.

Case definitions. The study's initial case definitions contributed toward and were replaced by the definitions proposed by the International Network for the Study of HIV-Associated IRIS (Figure 1) [6], with no modification required to case or control status of clinical events or participants. Participants who received treatment for tuberculosis at the time of ART initiation were defined as having "paradoxical TB-IRIS" or "no paradoxical TB-IRIS." Participants who did not receive treatment for tuberculosis at this time were defined as having "ART-associated tuberculosis" (any tuberculosis diagnosis after ART initiation) or "no tuberculosis." Suspected cases of paradoxical TB-IRIS or ART-associated tuberculosis were defined as events in which fever, lymphadenopathy, or abdominal pain were present and typical drug toxicity rash was absent. Events were assigned case or control status through independent review by 2 experienced HIV specialists.

Diagnosis of tuberculosis and initiation of tuberculosis treatment required assessment by both the senior HIV physician at the Social Health Clinic and a tuberculosis specialist from the National Center for Tuberculosis and Leprosy, using standard World Health Organization (WHO) case definitions [30]. Sputum samples were processed by the National Tuberculosis Laboratory. Liquid *M. tuberculosis* culture was under development at this laboratory but was not available by the end of the study. Chest radiographs were obtained routinely before ART and as clinically indicated, as were sputum smears for participants with cough. Episodes of abdominal pain were assessed using abdominal ultrasound.

Statistical methods. The incidence of paradoxical TB-IRIS and ART-associated tuberculosis was calculated and associated factors were identified using univariate and multivariate logistic regression with exact methods. Participants with paradoxical TB-IRIS were compared with those who also started ART during therapy for tuberculosis but did not develop paradoxical TB-IRIS. Similarly, participants with ART-associated tuberculosis were compared with those who also started ART without concomitant therapy for tuberculosis but did not develop ART-associated tuberculosis. We have previously reported elsewhere that IFN-γ responses to RD1 antigens were associated with $CD4^+$ T cell counts [31]. We also noted that background IFN-γ levels increased at the time of paradoxical TB-IRIS and ART-associated tuberculosis, presumably as a result of immune activation, and that the usual approach of analyzing the specific

Figure 1. Case definition of paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) [6].

I. Both of the following antecedent requirements must be met:
   A. The tuberculosis diagnosis was made before starting antiretroviral therapy (ART) and fulfilled World Health Organization criteria.
   B. Prior to ART initiation, the patient's condition stabilized or improved on appropriate treatment for tuberculosis (unless ART was started within 2 weeks of treatment for tuberculosis).

II. The following clinical criteria should be fulfilled:
   A. The onset of manifestations is within 3 months of ART initiation, reinitiation, or regimen change due to treatment failure.
   B. At least 1 of the following major or 2 of the following minor clinical criteria are required.
      1. Major criteria:
         a) New or enlarging lymph nodes, cold abscesses, or other focal tissue involvement
         b) New or worsening radiological features of tuberculosis
         c) New or worsening central nervous system tuberculosis
         d) New or worsening serositis
      2. Minor criteria:
         a) New or worsening constitutional symptoms
         b) New or worsening respiratory symptoms
         c) New or worsening abdominal pain accompanied by peritonitis, hepatomegaly, splenomegaly, or abdominal adenopathy

III. The following alternative explanations for clinical deterioration must be excluded:
   A. Failure of tuberculosis treatment due to tuberculosis drug resistance.
   B. Poor adherence to tuberculosis treatment.
   C. Another opportunistic infection or neoplasm.
   D. Drug toxicity or reaction.

IFN-γ response to RD1 antigens by subtracting the nonspecific background IFN-γ level partially cancelled out evidence of nonspecific and *M. tuberculosis*–specific immune responses during these events. We therefore performed post hoc analyses of the IFN-γ levels divided by the pre-ART $CD4^+$ T cell counts without subtraction of the background IFN-γ levels.

Continuous variables were grouped according to accepted cutoff points and variable distribution. Multivariate logistic regression models were constructed using the forward stepwise method. Results of the QFTGIT assays and TSTs were compared as binary outcomes by use of the χ^2 test and as continuous variables by use of the Wilcoxon rank sum test. Sensitivity, specificity, positive and negative predictive values, and likelihood ratios of QFTGIT and skin testing for the diagnosis of paradoxical TB-IRIS and ART-associated tuberculosis were calculated using receiver operating characteristic (ROC) curves and exact methods. All available data on the participants who consented and who received at least 1 dose of ART were included in analyses. Data were censored at the time of the last clinic visit for participants who died, were lost to follow-up, or transferred, and missing data were excluded from all analyses. Two-sided $P < .05$ was considered statistically significant. All analyses were performed using Stata software, version 10.0 (StataCorp), and were planned unless otherwise stated.

RESULTS

Cohort characteristics. Of 467 ART-naive adults invited to participate in the study, 130 (27.8%) declined and 31 (6.6%) did not receive $\geq$1 dose of ART during the recruitment period. The remaining 306 patients were included in these analyses and contributed a total of 1756 patient-months of follow-up. The median age of participants was 34 years, 138 participants (45.1%) were women, and 166 (54.3%) and 74 (24.2%) were in WHO Clinical Stages 3 and 4, respectively. The median pre-ART $CD4^+$ T cell count was 69 cells/μL, with 127 counts (41.5%) that were <50 cells/μL. Final participant disposition is shown in Figure 2.

Paradoxical TB-IRIS. ART was initiated during treatment for tuberculosis in 75 participants (24.5%) at a median of 49 d (interquartile range, 27–83 d) after start of treatment for tuberculosis. Of these 75 participants, 1 died (1.3%), 3 were lost to follow-up (4.0%), and 1 transferred (1.3%). Paradoxical TB-IRIS developed in 15 participants (20.0%) at a median of 10 d (range, 7–89 d) after ART initiation. A trend toward an association between TB-IRIS and initiation of ART <30 d after start of treatment for tuberculosis was seen (Table 1). No participant with paradoxical TB-IRIS died.

ART-associated tuberculosis. Of the 231 participants who

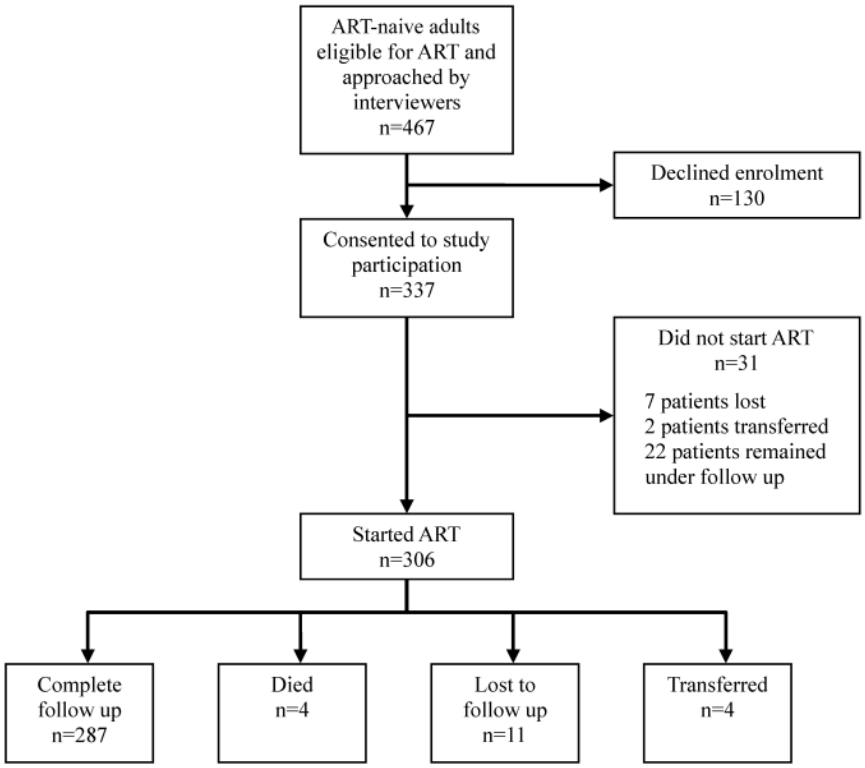

Figure 2. Disposition of study participants. ART, antiretroviral therapy.

started ART without concomitant therapy for tuberculosis, 3 died (1.3%), 8 were lost to follow-up (3.5%), and 3 transferred (1.3%). Eleven participants (4.8%) developed ART-associated tuberculosis during the first 6 months of ART (9.6 cases per 100 person-years). Four (36%) of these 11 participants had nonspecific symptoms at the time of ART initiation. Among those who were asymptomatic at the start of ART, the median time between initiation of ART and onset of symptoms was 10 d (range, 1–28 d). None of the variables described in Table 1 were significantly associated with ART-associated tuberculosis in univariate and multivariate analyses ($P \geqslant .11$). No participant with ART-associated tuberculosis died or went on to develop clinical evidence of a subsequent paradoxical reaction.

IFN-γ and skin test responses in participants with paradoxical TB-IRIS. Among participants who initiated ART during treatment for tuberculosis, the development of paradoxical TB-IRIS was not associated with statistically significant differences in IFN-γ responses to RD1 antigens ($P \geqslant .11$) (Figure 3*A*). In post hoc analyses of IFN-γ levels without subtraction of background IFN-γ levels and after correction for pre-ART $CD4^+$ T cell counts, participants who developed paradoxical TB-IRIS had statistically significantly greater responses to RD1 antigens before starting ART and at week 4 ($P = .03$ and .02, respectively) (Figure 3*B*).

Initiation of ART during treatment for tuberculosis resulted in an initial increase in IFN-γ responses to PPD, regardless of whether paradoxical TB-IRIS developed ($P \geqslant .11$ before and weeks 4 and 12 after ART initiation) (Figure 3*C* and 3*D*). These responses gradually increased in participants who developed paradoxical TB-IRIS compared to those who did not, reaching statistical significance 6 months after initiation of ART in both corrected and uncorrected analyses ($P = .02$ and .04, respectively).

Skin test responses to PPD followed a pattern similar to that of in vitro IFN-γ responses, with increased responses in both groups after initiation of ART but greater responses in the group who developed paradoxical TB-IRIS. Differences between the groups appeared earlier than was seen with IFN-γ responses ($P = .02$ and .01 for uncorrected and corrected week 12 comparisons, respectively) (Figure 3*E* and 3*F*). Skin test responses to the *M. avium* antigen were similar to but less marked than responses to PPD, and no significant differences were seen between participants who developed paradoxical TB-IRIS and those who did not (data not shown).

IFN-γ and skin test responses in participants with ART-associated tuberculosis. In participants not treated for tuberculosis at any time during the study period, IFN-γ responses to RD1 antigens and to PPD remained low over the first 6 months of ART, whereas in participants who received tuberculosis diagnoses during early ART, IFN-γ responses to RD1

Table 1. Factors Associated with Paradoxical Tuberculosis-Associated Immune Reconstitution Inflammatory Syndrome (TB-IRIS) in Patients Starting Antiretroviral Therapy (ART) during Therapy for Tuberculosis

Characteristic	No. (%) of patients with TB-IRIS	Univariate		Multivariate	
		OR	*P*	AOR (95% CI)	*P*
Total (*N* = 75)	15 (20.0)	…	…	…	…
Sex					
Male (*n* = 50)	11 (22.0)	1.00 (Ref)	…	…	…
Female (*n* = 25)	4 (16.0)	0.68	.78	0.67 (0.13–2.72)	.77
Age					
<35 years (*n* = 40)	8 (20.0)	1.00 (Ref)	…	…	…
⩾35 years (*n* = 35)	7 (20.0)	1.00	>.99	0.90 (0.23–3.38)	>.99
Site of tuberculosis					
Pulmonary (*n* = 28)	3 (10.7)	1.00 (Ref)	…	…	…
Extrapulmonary (*n* = 37)	11 (29.7)	3.46	.12	2.53 (0.52–16.56)	.33
Disseminated (*n* = 10)	1 (10.0)	0.93	>.99	0.72 (0.01–10.90)	>.99
Pre-ART $CD4^+$ count					
⩾50 cells/μL (*n* = 39)	7 (18.0)	1.00 (Ref)	…	…	…
<50 cells/μL (*n* = 36)	8 (22.2)	1.30	.86	0.98 (0.25–3.85)	>.99
Increase in $CD4^+$ count[a]					
<100 cells/μL (*n* = 38)	8 (21.1)	1.00 (Ref)	…	…	…
⩾100 cells/μL (*n* = 37)	7 (21.1)	0.88	>.99	1.03 (0.27–3.95)	>.99
Time to ART start					
⩾30 d (*n* = 48)	6 (12.5)	1.00 (Ref)	…	…	…
<30 d (*n* = 27)	9 (33.3)	3.44	.07	3.44 (0.93–13.65)	.07

NOTE. AOR, adjusted odds ratio; CI, confidence interval; OR, odds ratio; Ref, reference value.

[a] Increase in $CD4^+$ T cell count from pre-ART to week 24.

antigens and to PPD increased dramatically ($P < .001$ and $P = .03$, respectively, at week 4) and then decreased. However, IFN-γ responses remained significantly higher in participants with ART-associated tuberculosis than in participants without tuberculosis ($P = .002$ and .01, respectively, at week 24) (Figure 3*A* and 3*C*). Correction of raw IFN-γ responses for baseline $CD4^+$ T cell counts strengthened the correlation with ART-associated tuberculosis ($P < .001$ at all time points) (Figure 3*B* and 3*D*).

Skin test responses to PPD were associated with ART-associated tuberculosis, with similar associations seen following the correction of skin test response levels for baseline $CD4^+$ T cell counts ($P = .02$ and .03 at week 12 and $P = .001$ and .004 at week 24 for uncorrected and corrected analyses, respectively) (Figure 3*E* and 3*F*). A slightly weaker association was seen between skin test responses to the *M. avium* antigen and ART-associated tuberculosis (data not shown).

Use of QFTGIT and TST as diagnostic tests for TB-IRIS and ART-associated tuberculosis. At the time that a suspected paradoxical TB-IRIS diagnosis was made neither a positive QFTGIT result nor a positive TST result (using the definitions of a positive result described above) was associated with events caused by paradoxical TB-IRIS ($P = .70$ for both). However, in ROC analysis, uncorrected TST results were associated with good performance characteristics for the diagnosis of paradoxical TB-IRIS (Table 2). Among patients with suspected ART-associated tuberculosis the association between a positive QFTGIT result and ART-associated tuberculosis was of borderline significance ($P = .046$). In ROC analysis, the IFN-γ responses to RD1 antigens and to PPD were associated with strong performance characteristics for the diagnosis of ART-associated tuberculosis (Table 2). Because fewer participants had TSTs performed at the time of the clinical event, the sample size was insufficient to investigate TST as a diagnostic test for suspected ART-associated tuberculosis.

The strong associations seen between corrected pre-ART IFN-γ responses and ART-associated tuberculosis led us to perform post hoc analyses of the ability of pre-ART tests to predict the subsequent development of this condition. Corrected IFN-γ responses to either RD1 antigens or PPD showed strong performance characteristics in the prediction of ART-associated tuberculosis (area under the curve, 0.81 [95% confidence interval {CI}, 0.75–0.86] and 0.81 [95% CI, 0.75–0.86], respectively), and threshold values identified to maximize discrimination (RD1 antigen level, 0.00625; PPD level, 0.0126) were able to stratify participants into a low-risk group with a risk

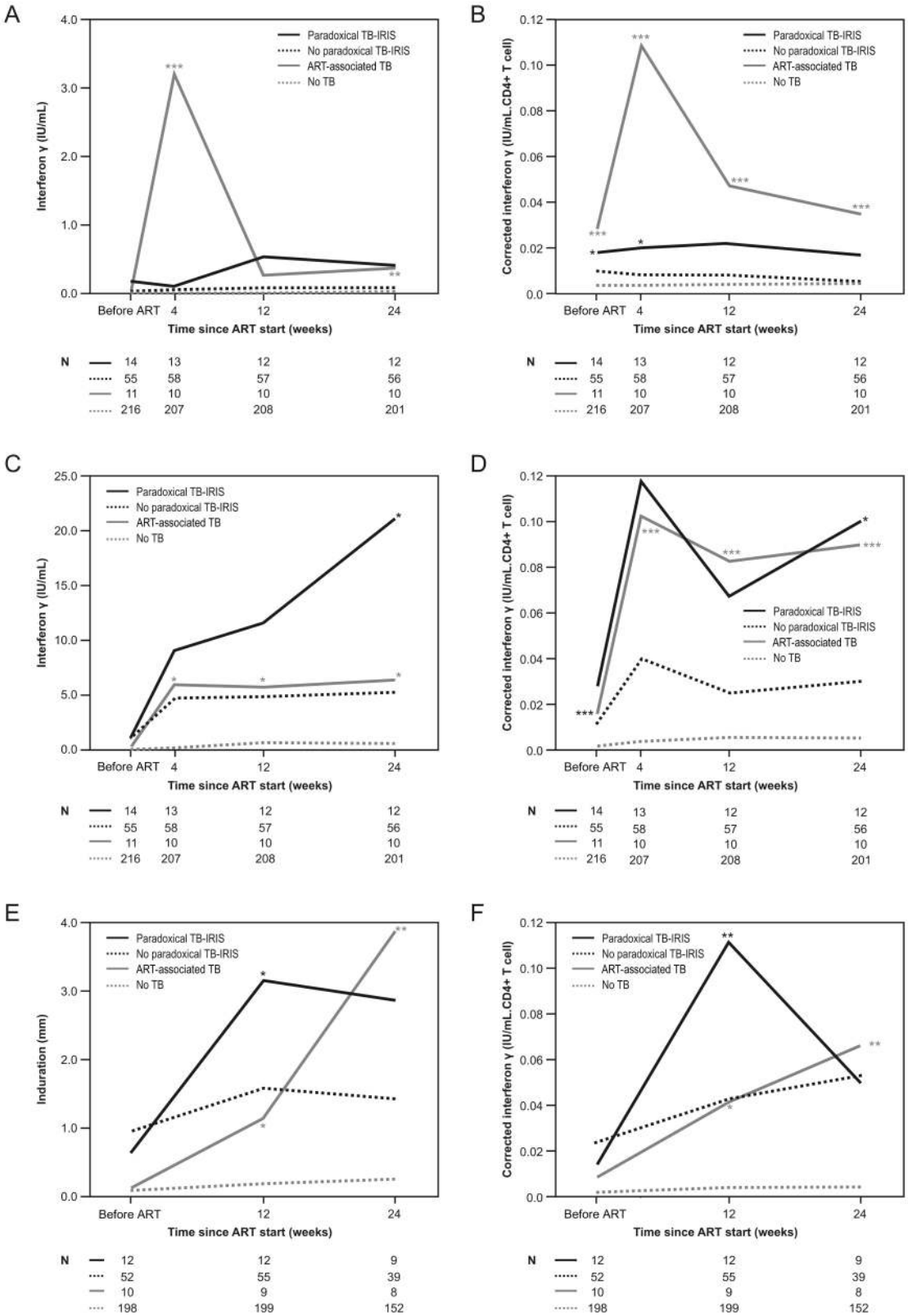

Figure 3. Mean interferon γ (IFN-γ) and skin test responses during 24 weeks of antiretroviral therapy (ART). *A*, IFN-γ responses to region of difference 1 (RD1) antigens. *B*, Raw IFN-γ responses to RD1 antigens corrected for pre-ART CD4⁺ T cell counts. *C*, IFN-γ responses to purified protein derivative (PPD). *D*, Raw IFN-γ responses to PPD corrected for pre-ART CD4⁺ T cell counts. *E*, Skin test responses to PPD. *F*, Skin test responses to PPD corrected for pre-ART CD4⁺ T cell counts. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$. TB, tuberculosis; TB-IRIS, tuberculosis-associated immune reconstitution inflammatory syndrome.

Table 2. Corrected Interferon γ (IFN-γ) Responses and Uncorrected Skin Test Responses at Time of Suspected Paradoxical Tuberculosis-Associated Immune Reconstitution Inflammatory Syndrome (TB-IRIS) or Antiretroviral Therapy (ART)–Associated Tuberculosis

Condition, antigen	No. of patients tested	No. of patients with true positive results	ROC AUC (95% CI)	Cutoff value, IU/L or mm	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Positive likelihood ratio	Negative likelihood ratio
Paradoxical TB-IRIS										
RD1	30	13	0.50 (0.31–0.69)	0.007	0.69	0.35	0.44	0.60	1.07	0.87
PPD	30	13	0.62 (0.41–0.77)	0.06	0.85	0.47	0.55	0.81	1.60	0.33
TST	15	7	0.82 (0.52–0.96)	6	0.71	0.88	0.84	0.77	5.71	0.33
ART-associated TB										
RD1	55	8	0.90 (0.80–0.97)	0.023	0.88	0.87	0.54	0.98	6.85	0.14
PPD	55	8	0.92 (0.80–0.97)	0.32	0.88	0.92	0.64	0.98	10.28	0.14

NOTE. For corrected IFN-γ responses, the raw IFN-γ level is divided by the pre-ART $CD4^+$ T cell count. Uncorrected skin test responses measure the skin induration. AUC, area under the curve; CI, confidence interval; PPD, purified protein derivative; RD1, antigens encoded by region of difference 1 in *Mycobacterium tuberculosis* genome; ROC, receiver operator characteristic; TB, tuberculosis; TST, tuberculin skin test.

of contracting tuberculosis during the first 6 months of ART of 1 in 146 (0.7%) and 1 in 140 (0.7%) for RD1 antigens and PPD, respectively, and a higher risk group with a risk of 10 in 85 (11.8%; odds ratio [OR], 19.33 [95% CI, 2.43–153.90]; $P = .005$) and 10 in 91 (11.0%; OR, 17.16 [95% CI, 2.16–136.51]; $P = .007$) for RD1 antigens and PPD, respectively. A second threshold value of adjusted IFN-γ responses to RD1 antigens (0.11) was able to further stratify the higher risk group into a medium-risk group with a risk of 7 in 75 (9.3%; OR, 14.92 [95% CI, 1.80–123.71]; $P = .01$) and a high-risk group with a risk of 3 in 10 (30.0%; OR, 62.14 [95% CI, 5.71–675.98]; $P = .001$).

DISCUSSION

In this study, we demonstrated the rapid development of disease associated with *M. tuberculosis* infection in many individuals who started ART in a setting with a high tuberculosis burden. The rapid increase in IFN-γ responses to RD1 antigens and to PPD suggests a significant contribution from ART-induced restoration of type 1 helper T cell responses in the pathogenesis of ART-associated tuberculosis and a possible role for IGRAs in the diagnosis and prediction of tuberculosis during early ART. In contrast, paradoxical TB-IRIS was associated with a relatively slow increase in IFN-γ responses, with no difference between case patients and control patients until 24 weeks, suggesting the importance of other aspects of the restoration of *M. tuberculosis*–specific immune responses in the pathogenesis of this syndrome.

Paradoxical TB-IRIS was common, occurring in one-fifth of participants who started ART during treatment for tuberculosis, and it was usually evident within 2 weeks of ART initiation. Earlier initiation of ART tended to increase the risk of paradoxical TB-IRIS, but this was not associated with any mortality in this study. Our findings therefore support arguments that early initiation of ART in patients with HIV infection who receive treatment for tuberculosis improves patient survival [32, 33]. Despite the common understanding that IRIS predominantly occurs in individuals with advanced immunodeficiency, we did not find any association between paradoxical TB-IRIS and pre-ART $CD4^+$ T cell count, consistent with most [34–38] but not all [39] previous studies. However, this may be due to low variability in pre-ART $CD4^+$ T cell counts in this study.

In this prospective study, we found that IFN-γ responses to PPD, as measured by a whole-blood IGRA, increased after initiation of ART in participants who received treatment for tuberculosis regardless of TB-IRIS status, which is consistent with findings from a South African study [40] and in contrast to findings from an earlier study conducted in France [10]. Our analysis extended through the first 6 months of ART and demonstrated a further increase in IFN-γ response only in the subgroup that developed paradoxical TB-IRIS. Taken together, these findings suggest that IFN-γ responses to PPD in individuals with paradoxical TB-IRIS gradually increase to levels above the response levels seen in other individuals who commence ART during treatment for tuberculosis. This contrasts strongly with the rapid onset of clinical disease in individuals who develop paradoxical TB-IRIS, suggesting that additional immunological defects (such as impaired regulatory cell function during ART-induced restoration of *M. tuberculosis*–specific immune responses) may be the primary pathogenic mechanism leading to paradoxical TB-IRIS [14, 15, 41]. The proportion of circulating $CD4^+$ T cells expressing the transcription factor FoxP3 (a marker of regulatory T cells) is not reduced in patients with TB-IRIS [40, 42]. However, impaired function of regulatory T cells [43] or depletion and/or dysfunction of cells of the innate immune system remain possibilities.

This study demonstrated an earlier and clearer differentiation of the group of participants with paradoxical TB-IRIS by means of skin test responses to PPD. Skin test responses are reflective of a delayed-type hypersensitivity reaction that includes both effector and regulatory immune responses, which supports the hypothesis that an overall imbalance in regulatory and effector

immune responses may be more central than increased IFN-γ responses alone to the etiology of paradoxical TB-IRIS. Furthermore, ROC analyses suggested that skin testing, but not QFTGIT, may play a role in the diagnosis of paradoxical TB-IRIS.

We also found that, both before ART initiation and after 4 weeks of ART, corrected IFN-γ responses to RD1 antigens were higher in individuals with paradoxical TB-IRIS than they were in individuals without paradoxical TB-IRIS. Because these individuals started ART earlier after the initiation of treatment for tuberculosis, these transiently higher responses may be attributable to a greater burden of viable *M. tuberculosis* organisms at these times, which supports the hypothesis that earlier initiation of ART results in a greater antigenic stimulus for the development of paradoxical TB-IRIS.

It is likely that active tuberculosis during early ART is a heterogeneous entity with variable contributions from microbial virulence in the setting of immunodeficiency versus ART-induced immunopathology. In some participants with ART-associated tuberculosis, nonspecific symptoms were present at the time of ART initiation, which suggests a significant contribution from pre-ART immunodeficiency. In other participants with an episode of ART-associated tuberculosis in the first 6 months of ART, the median time from start of ART to onset of symptoms was 10 d, which is identical to the median time to onset of paradoxical TB-IRIS. This concentration of tuberculosis cases with onset in the initial days of ART suggests a significant contribution from the restoration of *M. tuberculosis*–specific immune responses in the pathogenesis of ART-associated tuberculosis.

Previous studies involving predominantly untreated HIV-seropositive individuals have demonstrated an association between active tuberculosis and IFN-γ responses to either RD1 antigens or PPD that were measured using enzyme-linked immunospot assays [24–28]. This study differed from previous studies, in that we followed individuals prospectively over time and specifically investigated IFN-γ responses in HIV-infected individuals during the rapid immunological changes that occur during early ART. Participants who developed tuberculosis during this time demonstrated strong and rapid increases in IFN-γ responses to RD1 antigens and PPD and in skin test responses to PPD, suggesting that a type 1 helper T cell immune response contributes to the clinical presentation of tuberculosis during early ART.

Correction of IFN-γ response levels for baseline $CD4^+$ T cell counts strengthened the association between ART and active tuberculosis in most of the studies conducted to date [24, 25, 27, 28]. We report the first data, to our knowledge, on this analytical approach with the use of a commercially available whole-blood IGRA or during early ART, and we found that the association with ART-associated tuberculosis was strengthened, which is consistent with an association between *M. tuberculosis*–specific effector memory T cell responses and absolute number of circulating $CD4^+$ T cells. The association between skin test responses and ART-associated tuberculosis was not altered, possibly because of the broader immunological responses contributing to delayed-type hypersensitivity responses.

At the time of onset of suspected ART-associated tuberculosis, analysis of IFN-γ responses measured by QFTGIT assays and corrected for baseline $CD4^+$ T cell counts demonstrated strong performance characteristics. If these performance characteristics are confirmed in subsequent studies, then these assays could contribute significantly to the diagnosis of ART-associated tuberculosis and the reduction of the high mortality seen during early ART in many settings with a high tuberculosis burden [34]. The longitudinal design of our study also enabled us to examine the ability of pre-ART IFN-γ responses to predict the development of ART-associated tuberculosis. Corrected pre-ART IFN-γ responses predicted ART-associated tuberculosis, and threshold values were chosen to maximize discrimination enabled tuberculosis risk stratification. These data constitute proof of concept that *M. tuberculosis*–specific immune responses in individuals who have not yet begun ART predict subsequent development of active tuberculosis during early ART. If these data are validated in an independent study population, then they may enable novel strategies for presumptive treatment for tuberculosis prior to initiation of ART.

This study had a number of limitations. The definition of paradoxical TB-IRIS remains difficult despite the publication of an international consensus definition [6], because no formal case validation study has been conducted. ART-associated tuberculosis may have been underdiagnosed because of a lack of *M. tuberculosis* cultures during the study period, and drug-resistant tuberculosis may have been the cause of some episodes of clinical deterioration during treatment for tuberculosis. However, the prevalence of multidrug-resistant tuberculosis is relatively low in Cambodia [39], and all episodes were self-limited with no evidence of ongoing progression of tuberculosis and no deaths. The generalizability of this study is high, given the characteristics of our study population, the widespread use of ART, and the availability of treatment regimens for tuberculosis. Nevertheless, this study was based at a single ambulatory clinic, and there may be important contextual factors to take into account, particularly the prevalence of latent *M. tuberculosis* infection in the population, when applying these results in other settings.

This prospective cohort study afforded an opportunity to observe the interplay between tuberculosis infection and disease and enhanced *M. tuberculosis*–specific cellular immune responses during early ART and enabled insights into the pathogenesis of paradoxical TB-IRIS and ART-associated tuberculosis. We conclude that both of these conditions are associated

with restoration of an immune response against *M. tuberculosis* antigens but that the immunopathogenic mechanisms appear to be different. Type 1 helper T cell responses are prominent in ART-associated tuberculosis, but additional immune defects may be important in paradoxical TB-IRIS. IGRAs may contribute to the diagnosis of ART-associated tuberculosis and enable strategies to prevent the considerable burden of TB-associated morbidity and mortality during early ART.

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