MAJOR ARTICLE

# Mediators of Innate and Adaptive Immune Responses Differentially Affect Immune Restoration Disease Associated with *Mycobacterium tuberculosis* in HIV Patients Beginning Antiretroviral Therapy

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***Background.*** Initiation of antiretroviral therapy (ART) in human immunodeficiency virus patients with treated or unrecognized *Mycobacterium tuberculosis* infection may result in tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) or ART-associated tuberculosis (ART-TB), respectively. Both conditions appear to be immune restoration disease but their immunopathogenesis is not completely understood.

***Methods.*** Chemokines and cytokines produced by the innate immune system (CCL2, CXCL8, CXCL9, CXCL10, and interleukin 18 [IL-18]) were assayed in plasma from unstimulated whole blood cultures obtained from 15 TB-IRIS case patients, 11 ART-TB case patients, and matched control participants over 24 weeks of ART.

***Results.*** When compared with control participants, levels of IL-18 and CXCL10 were higher in TB-IRIS case patients ($P = .002$ and .006, respectively), whereas CCL2 was lower ($P = .006$). IL-18 level was higher in ART-TB case patients ($P = .002$), but CXCL10 was only marginally higher ($P = .06$). When TB-IRIS case patients were compared with ART-TB case patients, IL-18 was higher in ART-TB ($P = .03$), whereas CXCL10 was higher in TB-IRIS ($P = .001$). Using receiver operating characteristic curves, pre-ART levels of CCL2, CXCL10, and IL-18 were predictive of TB-IRIS and additive to IFN-$\gamma$ responses.

***Conclusions.*** Perturbations of the innate immune response to *M. tuberculosis* before and during ART may contribute to the immunopathology of TB-IRIS, whereas elevated IL-18 alone suggests adaptive immune responses predominate in ART-TB. These findings may have implications for therapy in TB-IRIS.

Tuberculosis is the most common opportunistic infection associated with human immunodeficiency virus (HIV) and the leading cause of morbidity and mortality in people living with HIV/AIDS in resource-limited settings [1]. Combination antiretroviral therapy (ART) reduces the incidence of tuberculosis in HIV-1–infected individuals [2–4]. However, initiation of ART may be associated with a paradoxical worsening of treated tuberculosis or presentation of new tuberculosis, conditions that have been referred to as tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) and ART-associated tuberculosis (ART-TB), respectively [5]. These presentations of *Mycobacterium tuberculosis* disease in HIV patients who have recently begun receiving ART have many characteristics of immune restoration disease (IRD), which we have argued results from the restoration of a pathogen-specific immune response that causes immunopathology [6–8].

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TB-IRIS is a significant obstacle to the introduction of ART in populations in which HIV and *M. tuberculosis* infections are common. The incidence varies from 7% to 43% of HIV patients with treated tuberculosis in different populations [9–17]. It has protean clinical manifestations including fever, weight loss, intrathoracic, intraabdominal, and cervical lymphadenopathy, worsening of pulmonary infiltrates, pleural effusions, peritonitis, meningitis, soft tissue and spleen abscesses, arthritis or osteomyelitis, and gastrointestinal disease [5]. Presentation is usually within the first month of ART [9, 12–19] but can be as late as 6 months after starting ART [11]; mean disease duration is 2.53 months [14, 20, 21]. Mortality rates are generally low but use of health resources in resource-poor countries is substantial [21, 22]. A greater understanding of the immunopathogenesis of this type of IRD may lead to improved treatments and risk reduction strategies.

Major risk factors for TB-IRIS have been identified as a low $CD4^+$ T cell count prior to initiation of ART [12, 14, 15], disseminated and extrapulmonary tuberculosis [11, 13, 14] and a short interval between starting antitubercular therapy and ART [10, 12, 17]. However, less is known about the underlying immunological mechanisms. Clinicopathological and immunological studies suggest that mycobacterial IRD is associated with the recovery of type 1 T helper cell (Th1) responses against mycobacterial antigens, which are characterized by increased delayed-type hypersensitivity skin test or T cell interferon-$\gamma$ (IFN-$\gamma$) responses to mycobacterial antigens [9, 15, 17, 23–25], and possibly $KIR^-V\delta2^+$ TCR $\gamma\delta^+$ T cell responses [24] but not with a deficiency of circulating regulatory T cells [23, 25, 26]. However, some studies have not demonstrated a relationship between TB-IRIS and IFN-$\gamma$ responses to *M. tuberculosis* antigens [27], and some could not demonstrate a temporal relationship with disease onset [17, 23]. One possible explanation for this is the involvement of innate immune responses.

We have previously reported on IFN-$\gamma$ responses to *M. tuberculosis* antigens in a cohort of Cambodian HIV patients with and without treated tuberculosis who began receiving ART [17]. Within the same cohort of patients, we examined levels of CCL2, CXCL10 (also known as IFN-$\gamma$-inducible protein 10 [IP-10]), CXCL9, CXCL8, and interleukin 18 (IL-18), which are critical for the initiation and maintenance of an effective immune response against *M. tuberculosis* infection [28]. We demonstrate that TB-IRIS in case patients is associated with differences in the production of IL-18, CXCL10, and CCL2 compared with control participants, suggesting that the innate immune response associated with *M. tuberculosis* is perturbed and contributes to the disease phenotype. In contrast, ART-TB was associated with increased production of IL-18, consistent with our previous findings that restoration of T cell IFN-$\gamma$ responses against *M. tuberculosis* antigens is prominent in this form of IRD [17].

## METHODS

***Case patients and control participants.*** Patients were recruited from the National Centre for HIV/AIDS, Dermatology, and Sexually Transmitted Diseases, Social Health Clinic, in Phnom Penh, Cambodia, and were a subgroup of those reported elsewhere [17]. Seventy-five patients were being treated for active tuberculosis upon starting ART and 15 (20%) developed TB-IRIS at a median time of 10 days (range, 7–89 days) after starting ART. Eleven (4.8%) of 231 patients with no prior history of tuberculosis developed ART-TB at a median time of 10 days (range, 1–28 days) after starting ART. We used case definitions of TB-IRIS and ART-TB proposed by the International Network for the Study of HIV-associated IRIS [5]. Each TB-IRIS and ART-TB case patient was matched with 2 control participants by sex, pre-ART $CD4^+$ T cell count, and tuberculosis history. The study was approved by the Cambodian National Ethics Committee and the human research ethics committees of the University of New South Wales and Royal Perth Hospital.

***Assay of chemokines and cytokines in plasma from whole blood cultures.*** Plasma from unstimulated tubes of Quantiferon-TB Gold in-tube (QFTGIT) assays (Cellestis) was collected pre-ART, after 4, 12, and 24 weeks of ART, and at the suspected time of TB-IRIS or ART-TB infection and cryopreserved at −80°C. Levels of CCL2, CXCL8, CXCL9, and CXCL10 were assayed in plasma using BD Cytometric Bead Array Flex Sets (BD Biosciences). In total, 300 events were collected per analyte using a BD FACSArray machine and BD FACSArray System Software version 1.0.3 (BD Immunocytometry Systems). Analysis was performed using FCAP Array Software version 1.0.1 (BD Biosciences). All samples were diluted 1:5, and the lowest limit of detection was 5 pg/mL. The coefficient of variance ranged from 5.1% to 10.1%, depending on the analyte measured.

IL-18 levels were measured in plasma at a 1:3 and 1:9 dilution by enzyme-linked immunosorbent assay (MBL) according to the manufacturer's instructions. Absorbance was read at 450 nm using an Asys HiTech Expert Plus version 1.2 spectrophotometer (Asys HiTech GmbH). Data were analyzed using Mikrowin 2000 software (Mikrotek Laborsysteme GmbH). The lowest limit of detection and coefficient of variance for the IL-18 assay was 15.6 pg/mL and 7.4%, respectively.

***Statistical analysis.*** Demographic characteristics of TB-IRIS and ART-TB case patients and control participants were assessed using the Mann-Whitney *U* test and Fisher exact test. Data were assessed for normality and lognormality using the Shapiro-Wilk *W* test and ln-transformed when necessary. Mean levels of CCL2, CXCL8, CXCL9, CXCL10, and IL-18 (all ln-transformed) within TB-IRIS and ART-TB case patients and their control participants were compared over 24 weeks of ART using longitudinal random effects regression models using max-

imum likelihood estimation. Levels of CCL2, CXCL8, CXCL9, CXCL10, and IL-18 were compared pre-ART and at the time of the TB-IRIS or ART-TB (or equivalent time-point post-ART for control participants) within each group using the Wilcoxon signed-rank test. Levels were compared between TB-IRIS and ART-TB case patients and control participants pre-ART and at the time of TB-IRIS or ART-TB infection using the Mann-Whitney *U* test. The predictive power of pre-ART levels of CCL2, CXCL10, and IL-18 was examined using receiver operating characteristic (ROC) curves. Correlations were estimated using Pearson *r* for ln-transformed variables. Analyses were performed using STATA software, version 11 (Stata) and Prism software, version 5.02 (GraphPad). Statistical significance was defined as $P < .05$.

## RESULTS

***Demographic characteristics of TB-IRIS and ART-TB case patients and control participants.*** The demographic characteristics of TB-IRIS and ART-TB case patients and control participants are described in Table 1. As can be seen, case patients and control participants were well matched for age, pre-ART $CD4^+$ T cell count, and change in $CD4^+$ T cell count during 24 weeks of ART.

***TB-IRIS and ART-TB were associated with increased levels of IL-18.*** Because TB-IRIS has been associated with increased numbers of circulating T cells that produce IFN-γ after stimulation with *M. tuberculosis* antigens [23], we examined levels of IL-18 because this is a potent macrophage-derived stimulator of IFN-γ production [29]. IL-18 levels in plasma from unstimulated QFTGIT assays were higher in TB-IRIS case patients ($n = 15$) over 24 weeks of ART than in control participants ($n = 30$) ($P = .002$; odds ratio [OR], 2.42; 95% confidence interval [CI], 1.37–4.26; longitudinal regression [LR]) (Figure 1*A*) and at the time of TB-IRIS ($P < .01$; Mann-Whitney) (Figure 2*A*). ART-TB case patients ($n = 11$) also had significantly higher levels of IL-18 compared with control participants ($n = 22$) during 24 weeks of ART ($P = .002$; OR, 2.42; 95% CI: 1.37–4.26; LR) (Figure 1*B*), but levels did not differ between ART-TB case patients and control participants at the time of ART-TB (Figure 2*A*). IL-18 levels were then compared in TB-IRIS and ART-TB case patients and found to be significantly lower in TB-IRIS case patients during 24 weeks of ART ($P = .03$; OR, 0.41; 95% CI: 0.19–0.90; LR) (Figure 3*A*). This did not reflect a difference in HIV disease severity because pre-ART $CD4^+$ T cell counts and $CD4^+$ T cell recovery were similar in TB-IRIS and ART-TB case patients ($P = .80$ and 0.39; Mann-Whitney test) (data not shown).

***TB-IRIS was most clearly associated with increased levels of CXCL10.*** We next examined levels of chemokines generally induced by IFN-γ (CXCL10 and CXCL9) [30]. When compared with control participants, CXCL10 levels were higher in TB-IRIS case patients during 24 weeks of ART ($P = .006$; OR, 2.34; 95% CI: 1.27–4.32; LR) (Figure 1*C*) and at the time of TB-IRIS ($P < .05$; Mann-Whitney test) (Figure 2*B*). CXCL10 levels at the time of TB-IRIS were similar to pre-ART levels in case patients, whereas in control participants, levels measured at an equivalent time-point to TB-IRIS case patients were significantly lower than pre-ART levels ($P < .001$; Wilcoxon) (Figure 2*B*). In ART-TB, CXCL10 levels were higher in case patients than in control participants during 24 weeks of ART but the difference was not statistically significant ($P = .06$, OR, 1.59; 95% CI: 0.99–2.55; LR) (Figure 1*D*). No change in CXCL10

**Table 1. Demographic Characteristics of Case Patients and Control Participants with Tuberculosis-Associated Immune Reconstitution Inflammatory Syndrome (TB-IRIS) and ART-Associated Tuberculosis (ART-TB)**

| | TB-IRIS | | | ART-TB | | |
|---|---|---|---|---|---|---|
| Characteristic | Case patients | Control participants | *P* value | Case patients | Control participants | *P* value |
| Total | 15 | 30 | | 11 | 22 | |
| Sex | | | | | | |
| Male | 11 | 22 | | 8 | 16 | |
| Female | 4 | 8 | | 3 | 6 | |
| Age, years | 35 (26–54) | 36 (25–63) | .66 | 35 (28–45) | 38 (24–56) | .53 |
| Pre-ART $CD4^+$ cell count | 45 (1–133) | 44 (2–160) | .83 | 37 (1–227) | 38 (1–231) | .91 |
| Change in $CD4^+$ cell count[a], [b] | | | | | | |
| <100 cells/μL | 8 | 18 | >.99 | 4 | 10 | >.99 |
| ⩾100 cells/μL | 4 | 11 | >.99 | 6 | 11 | >.99 |

NOTE. ART, antiretroviral therapy.

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

[b] $CD4^+$ T cell count at week 24 was unavailable for 3 TB-IRIS case patients, 1 TB-IRIS control participant, 1 ART-TB case patient, and 1 ART-TB control participant. Fisher exact test was used for comparisons between change in $CD4^+$ cell count during 24 weeks of ART, and the Mann Whitney *U* test was used for all other comparisons.

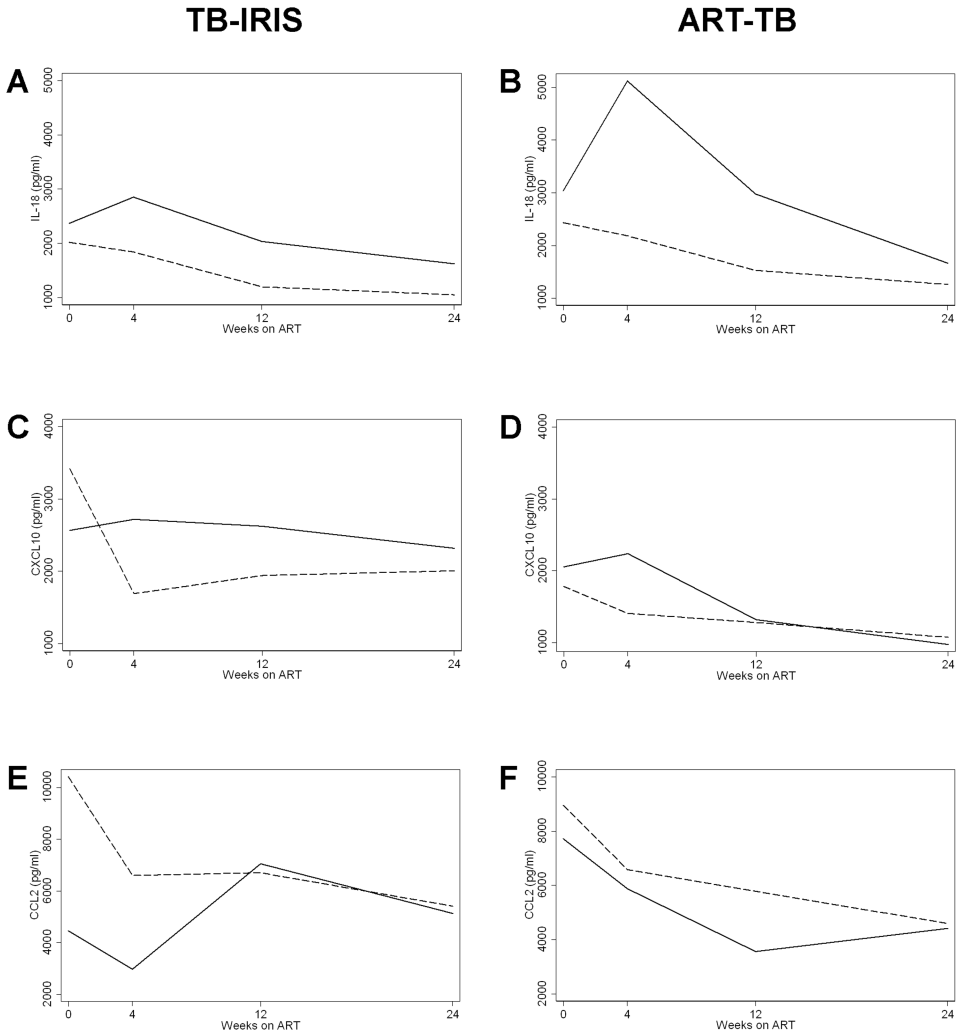


**Figure 1.** Longitudinal analyses of mean levels of interleukin 18 (IL-18), CXCL10, and CCL2 in tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) case patients and control participants (panels *A*, *C*, and *E*) and ART-associated tuberculosis (ART-TB) case patients and control participants (panels *B*, *D*, and *F*) over 24 weeks of antiretroviral therapy (ART). Case patients are denoted by a solid line, and control participants are denoted by a dashed line. Differences in mean levels of IL-18, CXCL10, and CCL2 between case patients and control participants were calculated using a longitudinal random effects regression model using maximum likelihood estimation.

levels between pre-ART and ART-TB timepoints was seen in ART-TB case patients or control participants (Figure 2*B*). Levels of CXCL10 were also significantly higher in TB-IRIS case patients than in ART-TB case patients ($P = .001$, OR, 7.37; 95% CI: 2.19–24.77; LR) (Figure 3*B*).

To determine whether the production of other IFN-γ-inducible chemokines was also increased in TB-IRIS, we assayed levels of CXCL9 and found that they did not differ between TB-IRIS case patients and control participants during 24 weeks of ART. There was no difference in levels of CXCL9 between ART-TB case patients and control participants or between TB-IRIS and ART-TB case patients during 24 weeks of ART, pre-ART or during the ART-TB episode. Levels of CXCL9 generally correlated with levels of CXCL10 within TB-IRIS and ART-TB case patients and control participants, although this was least notable for TB-IRIS case patients (data not shown).

***CCL2 levels were decreased in patients with TB-IRIS.*** The innate immune response to *M. tuberculosis* also includes production of chemokines that are chemotactic for monocytes [28, 31]. We therefore examined levels of CCL2 in plasma from the unstimulated whole blood cultures. CXCL8, which is chemotactic for neutrophils, was also examined for comparison. CCL2 levels were lower in TB-IRIS case patients than in control participants over 24 weeks of ART ($P = .006$, OR, 0.60; 95% CI: 0.41–0.87; LR) (Figure 1*E*), but levels did not differ significantly between ART-TB case patients and those of control participants ($P = .69$, OR, 1.07; 95% CI: 0.78–1.46; LR) (Figure 1*F*). Levels of CCL2 were significantly lower in TB-IRIS case patients pre-

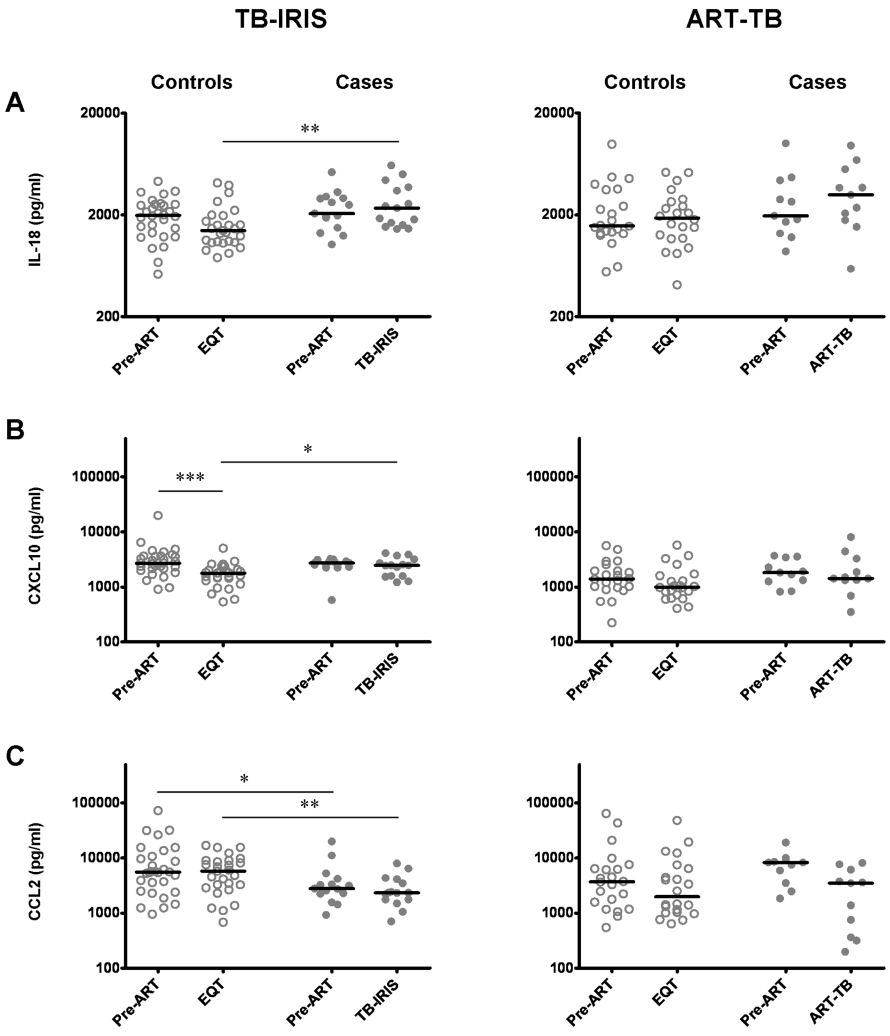


**Figure 2.** Comparison of levels of interleukin 18 (IL-18) (*A*), CXCL10 (*B*), and CCL2 (*C*) pre–antiretroviral therapy (ART) and at the time of immune restoration disease (IRD) in tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) and ART-associated tuberculosis (ART-TB) case patients (*filled circles*) and control participants (*open circles*). *$P < .05$; **$P < .01$; ***$P < .001$. Note that samples at the time of IRD were collected at a median time of 15 days after the IRD episode was diagnosed (range, 0–34 days). Samples in the control groups were selected at an equivalent time-point (EQT) post-ART for when TB-IRIS or ART-TB was diagnosed in matched case patients. Levels of IL-18, CXCL10, and CCL2 were compared within case patients or control participants using the Wilcoxon signed-rank test. Levels were compared between TB-IRIS and ART-TB case patients and control participants pre-ART and at the time of TB-IRIS or ART-TB using the Mann-Whitney *U* test.

ART and at the time of TB-IRIS than in control participants ($P < .05$ and $P < .01$; Mann-Whitney test) (Figure 2*C*). No differences were seen within ART-TB case patients and control participants (Figure 2*C*). In contrast, levels of CXCL8 did not differ within TB-IRIS and ART-TB case patients and their control participants during 24 weeks of ART, and levels of CCL2 and CXCL8 did not differ between TB-IRIS and ART-TB case patients during 24 weeks of ART (data not shown).

***Chemokine and cytokine levels pre-ART predicted TB-IRIS and ART-TB.*** Our finding that CCL2 production was lower in TB-IRIS patients pre-ART suggested that abnormalities of the innate immune system that are present prior to the commencement of ART may predispose an individual to the development of TB-IRIS. As levels of CCL2, CXCL10, and IL-18 were significantly different between TB-IRIS and ART-TB case patients and their control participants before and/or during ART, we investigated whether pre-ART levels of CCL2, CXCL10, and IL-18 alone or in combination were predictive of TB-IRIS or ART-TB after the commencement of ART using multivariate ROC curve analyses. We found that pre-ART levels of CCL2, CXCL10, and IL-18 alone showed moderate to good performance characteristics in the prediction of TB-IRIS (area under the curve [AUC], 0.79; 0.80; 0.72) (Figure 4*A*, 4*C*, and 4*E*). When CCL2, CXCL10, and IL-18 were combined, the ability to predict TB-IRIS was stronger (AUC, 0.86) (Figure 4*G*). Pre-ART levels of CCL2 alone and IL-18, CXCL10, and

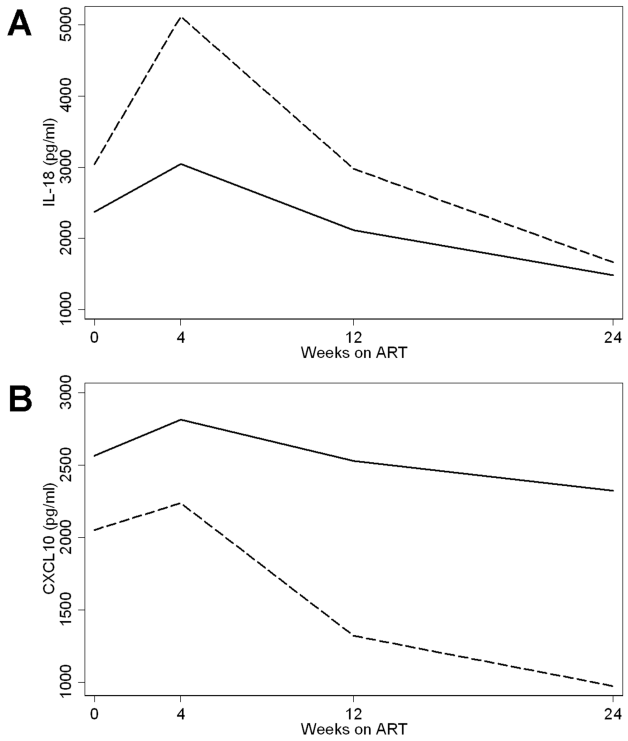


**Figure 3.** Longitudinal analyses of mean levels of interleukin 18 (IL-18) (*A*) and CXCL10 (*B*) in tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) (*solid line*) and ART-associated tuberculosis (ART-TB) case patients (*dashed line*) over 24 weeks of antiretroviral therapy (ART). Differences in mean levels of IL-18 and CXCL10 between case patients and control participants were calculated using a longitudinal random effects regression model using maximum likelihood estimation. It should be noted that due to differences in sample collection times between TB-IRIS and ART-TB case patients and the use of a longitudinal regression model, mean levels of IL-18 and CXCL10 differ slightly to the mean levels seen in case patients in Figure 1*A*–1*D*.

CCL2 in combination showed moderate performance characteristics in the prediction of ART-TB (AUC, 0.69 for both) (Figure 4*B*–4*H*), whereas pre-ART levels of IL-18 and CXCL10 alone were weakly predictive of ART-TB (AUC, 0.60 and 0.58, respectively) (Figure 4*D*, 4*F*).

***Prediction of TB-IRIS and ART-TB increased when IFN-γ responses were modeled with CCL2, CXCL10, and IL-18.*** We have previously shown that IFN-γ responses to RD1 antigens and purified protein derivative (PPD) showed good performance characteristics in the diagnosis of ART-TB [17]. We therefore investigated whether pre-ART interferon γ (IFN-γ) responses to RD1 antigens or PPD alone or in combination with CCL2, CXCL10, and IL-18 improved the prediction of TB-IRIS or ART-TB. In this data set, pre-ART IFN-γ responses to RD1 antigens were moderately predictive of TB-IRIS (AUC, 0.76) (data not shown), whereas IFN-γ responses to PPD were weakly predictive (AUC, 0.60) (data not shown). Pre-ART IFN-γ responses to RD1 antigens and PPD alone were weakly predictive of ART-TB (AUC, 0.61 and 0.62) (data not shown). When pre-ART IFN-γ responses to RD1 antigens and PPD were combined with CCL2, CXCL10, and IL-18, the ability to predict TB-IRIS and ART-TB increased (AUC, 0.90 and 0.75, respectively) (Figure 5*A*, 5*B*).

## DISCUSSION

This substudy of a large prospective study of the immunopathogenesis of IRD associated with *M. tuberculosis* infection provides evidence that TB-IRIS is associated with perturbations of the innate immune response to *M. tuberculosis*. We have demonstrated that HIV patients who develop TB-IRIS have decreased levels of CCL2 before and after ART and increased levels of IL-18 and CXCL10 over 24 weeks of ART. These differences were also present at the time of TB-IRIS. Patients who develop ART-TB had higher levels of IL-18 and slightly increased CXCL10 levels. These data support our previous arguments that the immune response restored against *M. tuberculosis* antigens that causes the immunopathology of TB-IRIS partly results from factors that are additional to an increase in IFN-γ–producing T cells and that the immunopathogenesis of TB-IRIS and ART-TB may be different [17].

IL-18, in conjunction with IL-12, induces the production of IFN-γ, which activates macrophages to restrict the growth of *M. tuberculosis* [32]. Higher levels of IL-18 in TB-IRIS and ART-TB case patients than in control participants suggest that IL-18 may contribute to the sudden recovery of Th1 responses in these conditions. The effect of increased IL-18 levels in patients with TB-IRIS or ART-TB might be additionally increased by a deficiency of its natural antagonist IL-18–binding protein (IL-18BP) caused by HIV infection [33]. We examined this possibility by assaying pre-ART levels of IL-18BP and calculated free levels of IL-18 [34] in the TB-IRIS and ART-TB case patients and control participants reported here but did not find any differences (data not shown).

CXCL10 regulates trafficking of effector T cells and NK cells to sites of inflammation [35, 36] and has been implicated in the immunopathogenesis of TB, possibly by recruitment of effector T cells [28]. Our finding that CXCL10 levels are increased in TB-IRIS suggests that this condition is associated with increased recruitment of *M. tuberculosis*–specific effector T cells, a view that is strengthened by the finding that levels of CXCL8 (which is chemotactic for neutrophils) did not differ between TB-IRIS and ART-TB case patients and their control participants. It has been demonstrated that serum levels of CXCL10 are persistently increased in HIV patients with hepatitis B virus infection who develop a flare of hepatitis after starting ART (which was argued to be the result of IRD) [37]. We therefore suggest that increased CXCL10 production may be fundamental to the immunopathogenesis of some types of IRD and results in the recruitment of effector T cells, and possi-

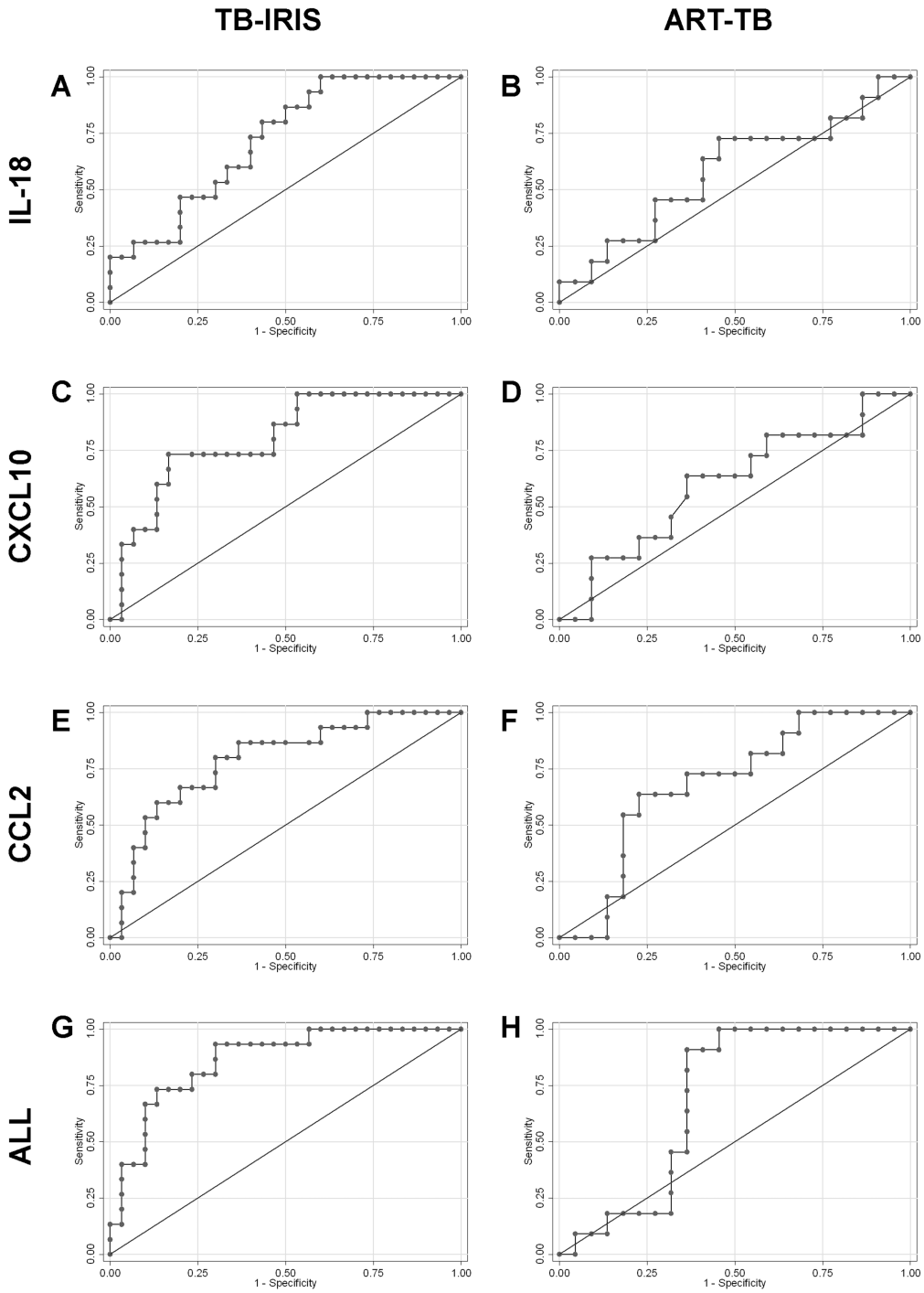


**Figure 4.** Receiver operating characteristic curves for the prediction of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) and ART-associated tuberculosis (ART-TB) using pre–antiretroviral therapy (ART) levels of CCL2 (*A*, area under the curve [AUC], 0.79; *B*, AUC, 0.69), CXCL10 (*C*, AUC, 0.80; *D*, AUC, 0.60), interleukin 18 (IL-18) (*E*, AUC, 0.72; *F*, AUC, 0.58) alone and CCL2, CXCL10, and IL-18 in combination (*G*, AUC, 0.86; *H*, AUC, 0.69).

bly natural killer (NK) cells, to tissues infected by the triggering pathogen.

Inhibition of CXCL10 can reduce T cell recruitment to affected tissues in various experimental models of inflammatory disease, including cerebral malaria [38], autoimmune encephalomyelitis [39], and colitis [40]. Although full details of the process are lacking, our hypothesis that CXCL10 may mediate effector T cell recruitment to tissues infected by mycobacteria

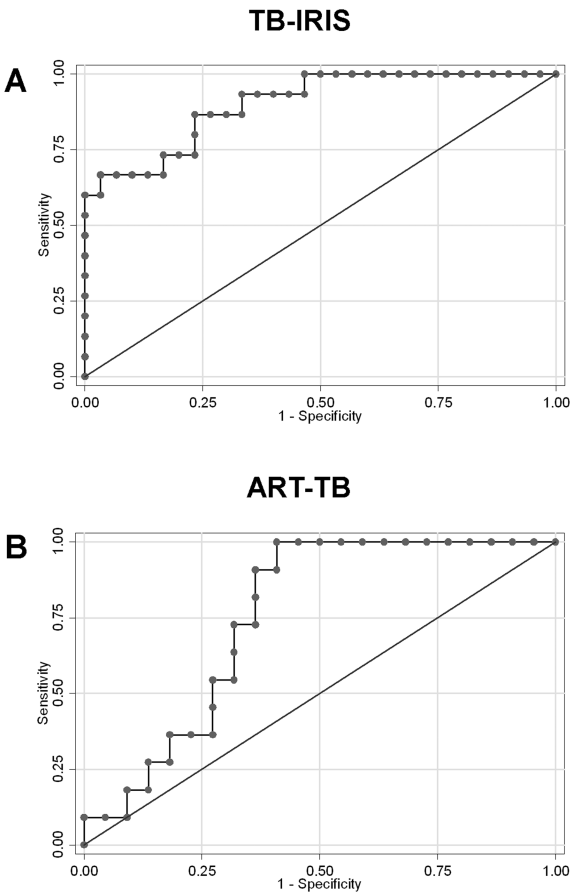


**Figure 5.** Receiver operating characteristic curves for the prediction of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) (*A*, area under the curve [AUC], 0.90) and ART-associated tuberculosis (ART-TB) (*B*, AUC, 0.75) when pre–antiretroviral therapy (ART) interferon $\gamma$ responses to RD1 antigens and purified protein derivative are modeled with pre-ART levels of CCL2, CXCL10, and interleukin 18.

raises the possibility that blocking the production or effects of CXCL10 might be a therapeutic approach for the prevention or treatment of TB-IRIS. Studies of inflammatory cell migration in human blood-brain barrier–derived endothelial cells have demonstrated that statin therapy reduces inflammatory cell recruitment, in part by decreasing CXCL10 production [41]. Statin therapy also suppresses CXCL10 production in Crohn's disease [42], and drugs that inhibit CXCR3, the main receptor for CXCL10, are under development [43]. Furthermore, effector T cells also express CCR5, and preliminary data suggest that the function and/or trafficking of T cells is affected by CCR5 inhibitor therapy for HIV infection [44, 45]. Suppression of CXCL10 production might also be an effect of corticosteroid therapy in TB-IRIS.

To explain the increased production of CXCL10 in TB-IRIS case patients compared with control participants, we investigated interleukin 10 (IL-10) production in unstimulated and PPD-stimulated whole blood cultures, because IL-10 can suppress STAT transcription factors for specific promoter motifs on IFN-$\alpha$ and IFN-$\gamma$ inducible genes [46]. However, we found no differences in IL-10 production between TB-IRIS and ART-TB case patients and control participants (data not shown). This may be because TB-IRIS and ART-TB do not reflect IL-10 deficiency, or our culture conditions may have been inadequate to detect PPD-induced IL-10 production. Additional investigation of this issue is needed.

Production of CCL2 is induced by *M. tuberculosis* antigens in the regional lymphoid tissue of patients with pulmonary tuberculosis [47], and CCL2 is critical for the recruitment of monocytes to the pleural space of people with tuberculosis [48, 49]. Data from a murine model of pulmonary cryptococcal infection also show that CCL2 affects trafficking of macrophages and T cells to the lung [50]. Our finding that CCL2 levels were lower in TB-IRIS case patients than in control participants before and after ART suggests that monocyte recruitment might be impaired and predispose individuals to TB-IRIS, possibly by increasing the burden of *M. tuberculosis*.

We have previously shown that pre-ART IFN-$\gamma$ responses to *M. tuberculosis* antigens may aid in the prediction and diagnosis of ART-TB [17]. Here, ROC curve analyses showed that the development of TB-IRIS and, to a lesser extent, ART-TB could be predicted by pre-ART levels of CCL2, CXCL10, and IL-18 and were additive to the predictive power of IFN-$\gamma$ responses to RD1 antigens and PPD. Therefore, it is likely that both adaptive and innate immune responses to *M. tuberculosis* are occurring before ART is commenced but are ineffective in controlling the infection, and that perturbations of the innate immune response contribute to the immunopathology of TB-IRIS during ART. Our findings also suggest that pre-ART levels of CCL2, CXCL10, and IL-18 may reflect different aspects of the immunopathogenesis of TB-IRIS because a combination of data on all 3 cytokines improved the prediction of TB-IRIS. Furthermore, the difference in the ability of CCL2, CXCL10, and IL-18 to predict TB-IRIS or ART-TB supports our hypothesis that the immune mechanisms involved in the development of TB-IRIS and ART-TB may be different [17].

The differences over 24 weeks between IL-18 and CXCL10 levels in HIV patients who developed TB-IRIS or ART-TB (Figure 3) also suggests that the immunopathogenesis of the 2 conditions differ. Increased levels of IL-18 in ART-TB case patients compared with TB-IRIS case patients might be explained by a predominant adaptive immune response, which includes the pronounced increase in IFN-$\gamma$ responses to RD1 antigens that we have previously described [17] and which unmasks *M. tuberculosis* infection following the commencement of ART. In contrast, higher levels of CXCL10 in TB-IRIS case patients than in ART-TB case patients may result in increased recruitment of effector T cells to sites of treated *M. tuberculosis* infection.

Our study has some limitations that need to be considered.

The number of TB-IRIS and ART-TB case patients was relatively small, and we did not distinguish patients with differing disease presentations. The definition of TB-IRIS and ART-TB is problematic because no formal case validation study has been performed. In this study, we used an international consensus definition as the basis for diagnosing TB-IRIS and ART-TB [5]. It is unclear whether the levels of immune mediators in plasma from blood cultured for 18 to 24 h reflect ex vivo production from cells in the cultured blood or in vivo production prior to collection of the blood, or both. This should be examined in future studies. Due to the difficulties in defining latent *M. tuberculosis* infection, we also do not know how many of the ART-TB control group were ever exposed to *M. tuberculosis*, how many had resolved previous infection, and how many harbored latent infection. Future studies should address this through careful analysis of clinical histories.

These data provide an important insight into the immunopathogenesis of TB-IRIS and ART-TB, contributing to improved diagnosis and management of this condition. New diagnostic and treatment strategies for TB-IRIS might be implemented on the basis of the results of this study, which could reduce the morbidity and mortality associated with this condition. This is particularly important because the incidence of TB-IRIS and ART-TB is expected to increase sharply as ART becomes more readily available in regions of the world that have a high incidence of HIV and *M. tuberculosis* infection.

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