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BRIEF REPORT

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Associations of Plasma Cytokine and Microbial Translocation Biomarkers With Immune Reconstitution Inflammatory Syndrome

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A nested case-cohort study was performed in participants of a clinical trial of first-line human immunodeficiency virus treatments to investigate plasma biomarkers of inflammation and microbial translocation for their association with immune reconstitution inflammatory syndrome (IRIS). Fifty-one of 1452 participants with baseline CD4 count <350 cells/μL developed IRIS. Plasma from 51 IRIS cases, including 6 stratified by preenrollment CD4 count ≤200 cells/μL, were analyzed and compared to 94 non-IRIS controls. At baseline, CXCL10, lipopolysaccharide, soluble CD14, 16S ribosomal DNA, and interferon-α2 were associated with greater risk of IRIS. Systemic inflammation through persistent monocyte activation and microbial translocation appear to be important in IRIS pathogenesis.

Keywords. cytokines; microbial translocation; immune reconstitution inflammatory syndrome; IRIS.

Immune reconstitution inflammatory syndrome (IRIS) remains clinically important in human immunodeficiency virus (HIV)–infected individuals starting combination antiretroviral therapy (cART), particularly when cART is initiated in severely immunocompromised patients. There are varying reports on the frequency of IRIS in HIV-infected individuals starting cART, ranging from 11% to 25% [1].

The pathogenesis of IRIS is still unclear. A high pathogen load in severely lymphopenic patients may be important, but the events leading to the observed clinical manifestations are still being elucidated. An expansion of antigen-specific CD4 T cells has been observed in tuberculosis IRIS with secretion of proinflammatory cytokines such as interferon gamma (IFN-γ), interleukin 12 (IL-12), and tumor necrosis factor alpha (TNF-α) consistent with the type 1 T-helper (Th1) commitment [1]. In chronic HIV–infected individuals, damage to gut integrity resulting in microbial translocation into the circulation [2, 3] and increased expression of pathogen-specific Toll-like receptors [4, 5] have been implicated in the development of IRIS after ART initiation, but this is not well understood. Timely diagnosis and effective treatment strategies directed at controlling hyperactivation of the immune system in the management of IRIS could be facilitated by the identification of predictive markers. We conducted a nested case-cohort study among participants in a large randomized longitudinal clinical trial (AIDS Clinical Trials Group [ACTG] A5202) in a US-based cohort and investigated 18 potential plasma biomarkers of inflammation and microbial translocation for their association with IRIS.

METHODS

A5202 Study Design

ACTG A5202 (ClinicalTrials.gov NCT00118898) was a phase 3b, randomized equivalence study of ART-naive individuals that compared the efficacy and safety of efavirenz and atazanavir/ritonavir combined with tenofovir/emtricitabine or abacavir/lamivudine [6–8]. The human subjects committees of participating sites approved the study protocol, and written informed consent was obtained from study participants in adherence to the human experimentation guidelines of the US Department of Health and Human Services. IRIS events were identified using ACTG criteria [9] and included unmasking and paradoxical events. Events were identified prospectively by site investigators and retrospectively by the IRIS study team based on review of compatible presentations.

Stratified Case-Cohort Study Design

Participants for this nested case-cohort study were selected from A5202 participants who met the following inclusion criteria: (1) had a baseline CD4 count <350 cells/μL; (2) initiated study treatment; and (3) had available stored ethylenediaminetetraacetic acid plasma samples. We analyzed 100 randomly selected participants ("random subcohort"; 94 non-IRIS and 6 IRIS cases) and the remaining 45 IRIS cases. The random subcohort was stratified by preenrollment CD4 count ≤200 cells/μL, a potential confounder, to match the distribution in the IRIS cases.

Quantitation of Inflammation Markers, Immune Activation, and Gut Microbial Translocation in Plasma

Samples collected at baseline (pre-ART) were tested. The cytokines tumor necrosis factor alpha (TNF-α), colony-stimulating

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factor 2 (CSF-2), interferon gamma (IFN-γ), interferon alpha 2 (IFN-α2), interleukin (IL) 1β, IL-2, IL-6, IL-10, IL-12, IL-17, chemokine ligand 2 (CCL2), and chemokine ligand 8 (CXCL8) were determined in undiluted plasma using Milliplex cytokine magnetic bead panel in the MAGPIX instrument (Luminex). Soluble TNF-α receptors (sTNF-RI and sTNF-RII) were determined using a bead panel in a laser-based instrument (Luminex). CXCL10 was determined using a Mesoscale Discovery instrument. Lipopolysaccharide (LPS) was measured using Limulus amebocyte lysate chromogenic endpoint assay (Lonza Group Ltd) in plasma diluted 1:5 in endotoxin-free water and heat-inactivated at 80°C for 10 minutes prior to the assay. LPS concentration in the samples was calculated in relation to an *Escherichia coli* endotoxin standard. Soluble CD14 was quantified in plasma (diluted 400-fold) by human sCD14 immunoassay (R&D Systems, Minneapolis, Minnesota). The 16S ribosomal DNA (16S rDNA) assay was performed as previously described [2, 3]. No-template controls were used each time to ensure there were no false-positive reactions. Circulating 16S rDNA copies/μL plasma was calculated using a standard curve generated from serial dilutions of plasmid DNA containing known copy numbers of template (courtesy of Dr Danny Douek, National Institutes of Health).

Statistical Methods

Each run in the laboratory had a lower detection limit. Biomarkers with >20% of values below or at the highest lower limit of all runs were dichotomized, and association measures compared above to below or at this higher limit. Biomarkers with ≤20% of values below or at this limit were categorized into tertiles, and measures of association compared the middle and upper tertiles to the lower tertile. If there was evidence for nonlinearity, the model using tertiles was considered primary. Otherwise, values below the detection limit of the run were imputed at the limit, and models with continuous linear terms were fitted. TNF-α, sTNF-RI, sTNF-RII, granulocyte colony-stimulating factor, CCL2, CXCL10, LPS, and sCD14 were $\log_{10}$ transformed; the estimates of association are presented per intertertile range. Univariable Cox models, stratified by preenrollment CD4 cell count and weighted for the case-cohort design [10], evaluated associations between baseline biomarkers, and time to first IRIS sign/symptom. Covariables, baseline HIV type 1 (HIV-1) RNA, CD8 percentage, history of AIDS, and randomized treatment arm, associated with IRIS in a previous analysis of the A5202 cohort [11], were adjusted for in multivariable models. Sensitivity analyses in the low stratum (preenrollment CD4 count ≤200 cells/μL) additionally adjusted for CD4 cell count as a continuous variable and accounted for a CD4 count <50 cells/μL and nucleoside reverse transcriptase inhibitor (NRTI) treatment interaction. *P* values <.05 were considered statistically significant. No adjustment for multiple testing was made. Statistical analyses were conducted in SAS version 9.2 software.

RESULTS

Overview of the Population

Among 1452 participants meeting the inclusion criteria for this nested case-cohort study, 51 developed IRIS within the first year of ART initiation (3.9 cases/100 person-years). A total of 55 IRIS-related events were reported on these 51 participants (Supplementary Table 1). Thirty-seven cases were identified prospectively and 14 through retrospective review. The first IRIS sign/symptom occurred a median (25th, 75th percentile) of 4.4 (2.0, 9.4) weeks after ART initiation, with 24 (47%) occurring within 4 weeks, 20 (39%) between 4 and 16 weeks, and 4 (14%) between 16 and 43 weeks. Baseline characteristics prior to ART initiation for the 145 participants selected for the nested case-cohort study are provided in Table 1.

Associations Between Biomarker Levels and IRIS

Higher baseline CXCL10 (hazard ratio [HR], 1.6 per tertile [95% confidence interval {CI}, 1.2–2.2], *P* = .005), LPS (HR, 1.7 per tertile [95% CI, 1.3–2.3], *P* < .001), sCD14 (HR, 2.1 per tertile [95% CI, 1.5–3.0], *P* < .001), 16S rDNA (HR, 1.4 per tertile [95% CI, 1.1–1.9], *P* = .017) and IFN-α2 (HR, 2.8 [95% CI, 1.4–5.6] for >10.9 vs ≤10.9 pg/mL, *P* = .003) were associated with a greater risk of IRIS (Supplementary Table 2). These results were supported by multivariable models adjusting for baseline HIV-1 RNA, CD8 percentage, history of AIDS, and randomized treatment arm. There was evidence that sTNF-RII (*P* = .012) and CCL2 (*P* = .005) had nonlinear relationships with risk of IRIS in univariable models, with the middle tertile having the lowest risk and the upper tertile the highest (sTNF-RII: HRs, 0.4 [95% CI, .2–1.0] for middle vs lower tertile and 1.6 [95% CI, .8–3.5] for upper vs lower tertile, overall *P* = .012; CCL2: HRs, 0.5 [95% CI, .2–1.3] for middle vs lower tertile and 2.1 [95% CI, 1.0–4.5] for upper vs lower tertile, overall *P* = .005). However, only the relationship for CCL2 remained significant in multivariable modeling. Figure 1A displays jitter plots of the baseline biomarker levels by IRIS status for those with statistically significant associations confirmed by multivariable modeling. All other biomarkers had *P* ≥ .15.

Sensitivity Analyses

In the low CD4 cell count stratum only (preenrollment CD4 count ≤200 cells/μL), models additionally adjusting for CD4 cell count as a continuous variable or a CD4 count <50 cells/μL and NRTI treatment interaction produced similar association estimates.

Discussion

In this nested case-cohort study, we evaluated plasma biomarker levels in HIV-infected participants who developed IRIS after ART initiation. The important findings were that at baseline, higher CXCL10, IFN-α2, microbial translocation markers (LPS and 16S rDNA), and markers of monocyte activation (sCD14) were associated with a greater risk of IRIS.

Table 1. Baseline Characteristics by Case-Cohort Group

	Random Subcohort (n = 100)		Remaining Cases	Total
Baseline Characteristics	No IRIS (n = 94)	IRIS (n = 6)	IRIS (n = 45)	(N = 145)
Male sex	74 (79)	5 (83)	36 (80)	115 (79)
Race/ethnicity				
White non-Hispanic	30 (32)	2 (33)	14 (31)	46 (32)
Black non-Hispanic	32 (34)	2 (33)	12 (27)	46 (32)
Hispanic	26 (28)	1 (17)	17 (38)	45 (31)
Other/unknown	6 (6)	1 (17)	2 (4)	9 (6)
Age, y, median (25th, 75th percentile)	39 (32, 48)	37 (27, 39)	41 (32, 46)	39 (32, 47)
HIV-1 RNA, log_{10} copies/mL, median (25th, 75th percentile)	4.7 (4.4, 5.3)	4.9 (4.8, 5.4)	4.9 (4.7, 5.5)	4.8 (4.6, 5.4)
CD4 count, cells/µL, median (25th, 75th percentile)	108 (35, 194)	28 (21, 37)	56 (26, 161)	84 (30, 184)
Preenrollment CD4 count ≤200 cells/µL	74 (79)	6 (100)	36 (80)	116 (80)
CD8 count, cells/µL, median (25th, 75th percentile)	645 (459, 861)	329 (296, 536)	577 (368, 998)	617 (446, 872)
CD8 percentage				
<50%	21 (22)	2 (33)	6 (13)	29 (20)
50%–59%	31 (33)	1 (17)	4 (9)	36 (25)
60%–69%	25 (27)	1 (17)	18 (40)	44 (30)
≥70%	17 (18)	2 (33)	17 (38)	36 (25)
History of an AIDS illness at study entry	24 (26)	4 (67)	22 (49)	50 (34)
Study treatment				
Efavirenz plus tenofovir/emtricitabine	26 (28)	0 (0)	9 (20)	35 (24)
Efavirenz plus abacavir/lamivudine	19 (20)	3 (50)	12 (27)	34 (23)
Atazanavir/ritonavir plus tenofovir/emtricitabine	23 (24)	0 (0)	12 (27)	35 (24)
Atazanavir/ritonavir plus abacavir/lamivudine	26 (28)	3 (50)	12 (27)	41 (28)

Data are presented as No. (%) unless otherwise indicated.

Abbreviations: HIV-1, human immunodeficiency virus type 1; IRIS, immune reconstitution inflammatory syndrome.

This case-cohort study has notable strengths. First, the IRIS-associated pathogens included 29 viruses and 16 Mycobacteriaciae. Whereas outside the United States discussion of IRIS is dominated by *Mycobacterium tuberculosis*, this cohort allowed an examination of biomarkers associated with IRIS among a spectrum of agents. Second, prior T-cell activation along with a role for macrophages has been described in the setting of tuberculosis-associated IRIS [12]. In this case-cohort study, macrophage-associated biomarkers (CXCL10, CCL2) remained important for diverse pathogens, suggesting that the dominance of tuberculosis in IRIS-associated pathology is not just a matter of disease prevalence but rather points to a fundamental role for the macrophage in the pathogeneses of IRIS.

The study had some limitations. First, each assay had a different laboratory detection limit, and many cytokine levels were dichotomized to avoid imputation of >20% of values at the lower limit. This dichotomization reduced statistical power to observe an association between the cytokine levels and IRIS, and therefore a nonsignificant association for these cytokines could be explained by limited statistical power. Second, biomarkers analyzed may be related to one another and adjustment was not made due to complex relationships between the many biomarkers. Third, pre-ART CD4 cell count is an important risk factor for IRIS [1]. Our nested case-cohort study adjusted for CD4 count by stratifying on preenrollment CD4 count ≤200 vs >200 cells/µL as 80% of IRIS cases occurred in the lower CD4 count stratum. This design was chosen for efficiency but did not lend itself to further adjustment for baseline CD4 as a continuous variable in the complete nested case-cohort subset. Sensitivity analysis conducted in the low stratum only further accounting for CD4 produced similar results. Moreover, the fact that antiretrovirals could have played a role in inflammation cannot be overlooked. However, results were consistent after adjustment was made for the antiretrovirals used.

Although most patients who develop IRIS show reduced CD4 T-cell count, high levels of proinflammatory cytokines have been noted [13]. In our study, among all the cytokines and chemokines tested, only CXCL10 and IFN-α2 were associated with a greater risk of IRIS. The association of CXCL10 with IRIS was also observed in the early time-point (approximately 4 weeks post-ART) and remained higher in IRIS cases even at 48 weeks post–ART initiation (data not shown). CXCL10 is a potent proinflammatory chemokine released by a variety of cells and is thought to attract more IFN-γ–producing Th1 cells to the site of inflammation and thereby inhibit the anti-inflammatory Th2 cells [14]. IFN-α2, an inflammatory type I interferon, correlated with increased risk of IRIS at baseline in our study. It has been proposed that IFN-α2 induces CXCL10 production in monocyte-derived dendritic cells and enhances their capacity to attract effector T cells [15].

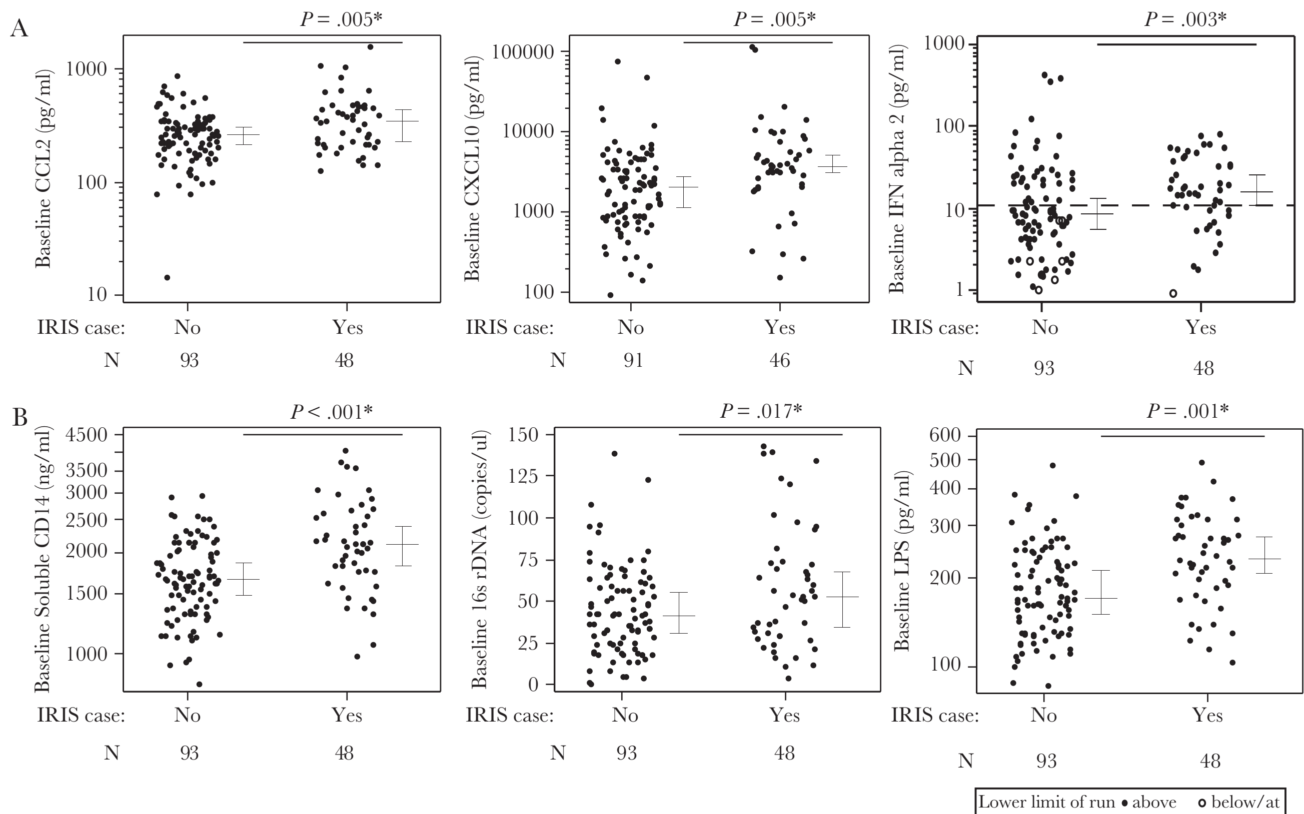

Figure 1. Baseline biomarkers by immune reconstitution inflammatory syndrome (IRIS) status. Circulating levels of chemokines (CCL2 and CXCL10) and cytokines (interferon [IFN] α2) (*A*), immune activation (soluble CD14), and markers of microbial translocation (16S ribosomal DNA [rDNA] and lipopolysaccharide [LPS]) (*B*) were quantitated in plasma from IRIS cases and controls. Biomarkers with significant ($P < .05$) associations confirmed by adjusted models are displayed. *P values from unadjusted models are given. Black lines display the median and intertertile range (33rd, 67th percentile). Dashed line displays detection limit, the highest lower limit of all runs.

Soluble CD14, a coreceptor for LPS and marker of monocyte activation, is a predictor of morbidity and mortality in HIV infection. We found an association between higher baseline levels of sCD14 and increased risk of IRIS. Gut microbial translocation is considered to be a major cause of persistent immune activation in HIV-infected people [2]. In this study, we found association between higher baseline levels of plasma LPS and 16S rDNA and increased risk of IRIS. These findings indicate that increased translocation of bacterial components into the systemic circulation in untreated patients could be contributing to the inflammatory response seen at the onset of IRIS events. LPS is a well-established marker of microbial translocation and 16S rDNA has emerged as a LPS core antigen marker [2, 3]. Higher baseline inflammation contributed by microbial translocation from the gut to the periphery leads to release in proinflammatory cytokines in HIV infection. ART initiation in the background of this inflammation can result in CD4 T-cell reconstitution leading to dysregulated responses to the inciting antigen/pathogen.

The results of this study suggest that persistent monocyte activation and bacterial translocation in immunosuppressed participants are important in the pathogenesis of IRIS and may be useful to predict risk for IRIS. Phenotypic analysis of T-cell subsets, function, and regulation in a nested matched case-control study by specific IRIS pathogen is ongoing for investigation of adaptive immune responses.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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