

# Skewing of the CD4<sup>+</sup> T-Cell Pool Toward Monofunctional Antigen-Specific Responses in Patients With Immune Reconstitution Inflammatory Syndrome in The Gambia

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**Background.** A common complication of starting antiretroviral therapy (ART) for human immunodeficiency virus (HIV) is the development of immune reconstitution inflammatory syndrome (IRIS) in approximately 25% of patients. Despite similarities with paradoxical reactions to tuberculosis and reversal reactions in leprosy, the exact mechanisms, and therefore potential determinants, of IRIS are still unknown.

**Methods.** In this longitudinal cohort study, we analyzed 20 patients who developed IRIS following initiation of ART and 16 patients who did not, matched for ART time point. Peripheral blood mononuclear cells were stimulated overnight with a positive control antigen and 2 tuberculosis-specific antigens (purified protein derivative [PPD] and ESAT-6/CFP10), followed by polychromatic flow cytometry for analysis of cytokine production from CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

**Results.** Responses to PPD were significantly higher in IRIS patients compared to controls during the IRIS time point, but CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses to the positive control stimulation were significantly lower in IRIS patients at all time points. Furthermore, whereas control patients had rejuvenated polyfunctional T-cell responses by 3 months after ART, IRIS patients were strikingly monofunctional (generally interferon  $\gamma$  alone), even up to 6 months of ART in response to all stimulations.

**Conclusions.** Our findings suggest that the peripheral T-cell responses to the underlying pathogen are exaggerated in IRIS patients but that the overall quality of the peripheral T-cell pool is significantly reduced compared to non-IRIS patients. Furthermore, these effects are apparent at least up to 3 months after cessation of IRIS.

**Keywords.** HIV; immune reconstitution inflammatory syndrome; tuberculosis; polyfunctional T cells; West Africa.

Current estimates suggest that in lower- and middle-income countries, there has been a significant improve-

ment with about half of all people living with human immunodeficiency virus (HIV) having access to antiretroviral therapy (ART) [1]. ART reduces the risk of tuberculosis in HIV type 1 (HIV-1)-infected patients by 70%–90% [2, 3] and was introduced in The Gambia in 2004. Currently the World Health Organization recommends starting an ART regimen when the CD4 count drops below 350 cells/ $\mu$ L [1], but implementation of these guidelines depends on diagnostic delays as well as the availability and access to treatment. In sub-Saharan Africa, CD4<sup>+</sup> T-cell counts <100 cells/ $\mu$ L are still commonly seen before ART is initiated, mainly due to late presentation at health facilities. It is anticipated that ART will become more accessible for a greater percentage

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of people living with HIV in the future; however, this will increase the incidence of ART-related diseases and complications. One such complication is immune reconstitution inflammatory syndrome (IRIS), in which an exacerbated immune response to an opportunistic infection (OI) occurs following the start of ART [4]. There are 2 broad categories of IRIS: paradoxical and unmasking. Paradoxical IRIS is characterized by clinical deterioration in symptoms from a previous OI that was or is being treated [5]. Unmasking IRIS occurs when an underlying OI newly presents and causes an exacerbated immune response following initiation of ART [5]. To date, 20 different infective agents have been shown to cause IRIS [6]; most are either (myco)bacterial (*Mycobacterium tuberculosis*, *Mycobacterium avium-intracellulare*, *Mycobacterium leprae*, and *Bartonella henselae*) or viral (hepatitis B and C, cytomegalovirus, and herpes simplex virus). Tuberculosis IRIS is immune reconstitution caused by *M. tuberculosis* and is the second most common form of IRIS (20%) worldwide but the number one cause in developing countries [7]. Unfortunately, diagnostic tests for the causative pathogens tend to have lower sensitivity in IRIS, as they are not necessarily replicating at the time they are recognized by the reconstituting immune system. This further complicates the distinction between IRIS and therapeutic failure of treatment for HIV or an opportunistic infection. Consequently, current diagnosis of IRIS requires extensive and complex evaluations to rule out other potential causes, which causes delay in instituting the appropriate patient management and treatment regimens. The way forward is to have clear parameters for predicting or diagnosing IRIS, but these are lacking today despite multiple studies performed in different countries.

Many studies have investigated cytokines and their role in the development of IRIS with hypercytokinemia as a proposed pathogenic mechanism [8]. The role of proinflammatory cytokines (ie, interleukin 2 [IL-2], tumor necrosis factor alpha [TNF- $\alpha$ ], interferon gamma [IFN- $\gamma$ ]) is central to this hypothesis as well as decreased levels of regulatory cytokines (interleukin 10, interleukin 4) [8]. A recent study in The Gambia showed an increase in effector CD4<sup>+</sup> T cells but no difference in regulatory T (Treg)-cell levels in IRIS compared to controls [9]. Treg cells are essential for maintaining the balance of the immune response with the outcome determined by the ratio of effector to Treg cells [10]; an elevated ratio results in increased disease-associated pathology. Despite the requirement for predictors of IRIS development, studies in Thailand, South Africa, and The Gambia looking at soluble mediators have seen no difference between IRIS and controls at the pre-ART time point [9, 11, 12]. Two recent studies have shown that presence of high IFN- $\gamma$  and TNF- $\alpha$  in cerebrospinal fluid [13] or plasma [14] could potentially predict development of IRIS, but these findings require validation. Few studies have analyzed cellular production of cytokines as a potential diagnostic indicator of IRIS.

One study has shown an increase in the proportion of activated tuberculin-specific IFN- $\gamma$ <sup>+</sup>TNF- $\alpha$ <sup>+</sup>CD4<sup>+</sup> T cells in IRIS patients compared to controls [15] at the IRIS time point. A more recent study showed a distinct increase in polyfunctional (IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>TNF- $\alpha$ <sup>+</sup>) CD4<sup>+</sup> (but not CD8<sup>+</sup>) T cells to the causative pathogen in IRIS patients at the time of IRIS, suggesting that the development of IRIS is solely driven by dominant responses to residual OI antigens [16].

In our previous work in non-IRIS patients [17] we saw a rapid increase in polyfunctional CD4<sup>+</sup> T-cell responses to tuberculin protein (purified protein derivative [PPD]) at 3 months post-ART, which was not seen for other antigens (ie, HIV<sub>gag</sub>). This suggests that, particularly in a tuberculosis-endemic setting where the majority of patients are latently infected with *M. tuberculosis*, rapid regeneration of the T-cell compartment will reveal dominant responses to *M. tuberculosis* antigens. To further investigate the mechanisms underlying development of IRIS, we compared CD4<sup>+</sup> and CD8<sup>+</sup> immune profiles in response to *M. tuberculosis* and general antigens before, during, and after development of IRIS.

## METHODS

### Patient Recruitment and Sample Collection

Patients presenting at the Genito-urinary clinic at the Medical Research Council Unit were clinically assessed by medical staff for IRIS using the criteria set out by Haddow et al [18]. Samples were available from 36 ART-naive patients who were recruited between 2005 and 2007, of whom 20 were from IRIS cases and the remaining 16 were used as controls. All patients had samples collected at baseline (prior to ART), during IRIS (2–3 months on ART for controls), and at 6 months of ART (approximately 3 months from IRIS cessation; labeled as post-IRIS on figures). Clinically, the patients were treated symptomatically for their IRIS diagnosis, with targeted antibiotic therapy in a minority of patients in whom a specific diagnosis (unmasking IRIS) was made. None of the patients received steroids. Ethical approval was obtained from The Gambia Government/Medical Research Council joint ethics committee, and all patients provided written informed consent.

### Antigenic Stimulation

Cryopreserved peripheral blood mononuclear cells were used in this study. After thawing, cells were stimulated overnight with PPD (10  $\mu$ g/mL; SSI, Denmark), ESAT-6/CFP-10 peptide pool (EC; 2.5  $\mu$ g/mL; Proimmune, UK), and anti-CD3/CD28 (positive control, 5  $\mu$ g/mL each; Becton Dickinson [BD]). Media alone was added to 1 tube as a negative (unstimulated) control. Each tube was also costimulated with CD28/CD49d (2  $\mu$ g/mL each; BD). Stimulation was performed for 2 hours at

37°C, with 5% CO<sub>2</sub> prior to addition of brefeldin A (10 µg/mL; Sigma) and incubated for a further 16 hours.

### Flow Cytometry Staining

Following antigenic stimulation, cells were stained with VIVID live-dead reactive dye (Invitrogen) incubated for 30 minutes and washed. Surface stain was then added (CD3 phycoerythrin [PE]–Cy7 [eBioscience, UK], CD4 allophycocyanin [APC]–Cy7 [eBioscience, UK], CD8 Pacific Blue [BD]) and incubated for 15 minutes at 4°C. Following washing, 150 µL of cytofix/cytoperm (eBioscience, UK) was added and incubated at 4°C for 15 minutes followed by a 20-minute incubation with Perm2 buffer (BD). Following washing, anticytokine antibodies were added to each tube (IFN-γ APC, TNF-α fluorescein isothiocyanate, and IL-2 PE; all from BD) and incubated at room temperature (in the dark) for 30 minutes. A final wash step was performed and cells were resuspended in 300 µL of wash buffer prior to acquisition.

### Flow Cytometry Acquisition and Analysis

Between 200 000 and 500 000 lymphocytes were acquired with a CyAn ADP (Beckman Coulter) flow cytometer following gating according to forward and side scatter plots. Flow cytometry data were analyzed using FlowJo software, version 9.2 (Treestar, Ashland, Oregon). After gating on singlets and live cells, CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells were gated. The percentage of different combinations of IL-2<sup>+</sup>, TNF-α<sup>+</sup>, and IFN-γ<sup>+</sup> cells following antigenic stimulation were calculated within the total population of CD4<sup>+</sup> or CD8<sup>+</sup> T cells and background subtracted. A threshold of 0.01% was used as described previously [19]; values below this were set to zero.

### Statistical Analysis

Group medians and distributions were analyzed using a Mann-Whitney *U* test or Kruskal-Wallis test followed by Dunn's post-test comparison. All analyses were performed with GraphPad Prism software, version 5 (Software MacKiev). Analysis of poly-functional T-cell responses and presentation of distributions was performed using SPICE version 5.2 (downloaded from <http://exon.niaid.nih.gov/spice>) [20]. Comparison of distributions was performed using the Wilcoxon rank-sum test and a partial permutation test as described [20].

## RESULTS

### Patient Characteristics

Demographic characteristics of the patients are summarized in Table 1. For the IRIS group, 75% were female with a median age of 38 years (interquartile range [IQR], 33–42 years; Table 1). Seventeen of the IRIS patients were infected with HIV-1, 2 were infected with HIV type 2 (HIV-2), and 1 patient

**Table 1. Patient Characteristics**

Group	IRIS	Non-IRIS
No.	20	16
Male to female ratio, No.	5:15	3:13
Age, y	38 (33–42)	38 (27–47)
HIV-1 only, No. (%)	17 (85%)	16 (100%)
HIV-2 only, No. (%)	2 (10%)	0
HIV-1 and HIV-2 dual infection, No. (%)	1 (5%)	0
CD4 pre-ART, cells/µL	65 (20–130)	80 (20–145)
CD4 at IRIS, cells/µL	180 (105–308)	170 (120–240)
CD4 increase at 6 mo from baseline, cells/µL	150 (120–330)*	100 (50–180)
Time to IRIS, d	21 (14–28)	NA
IRIS duration, d	49 (30–87)	NA
Viral load at baseline, log <sub>10</sub> copies/mL	5.9 (4.9–6.0)	5.5 (5.1–5.9)
Viral load at IRIS, log <sub>10</sub> copies/mL	2 (2–2.9)	2 (2–2.3)

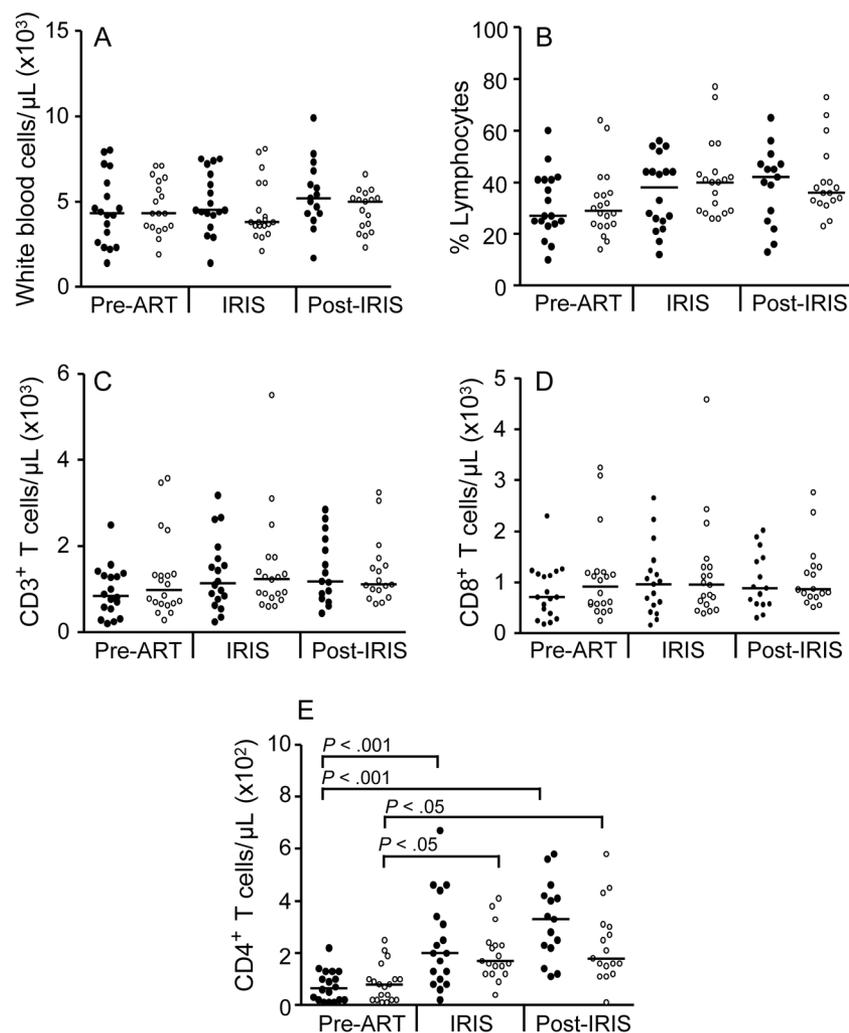
Data are expressed as median (interquartile range) unless otherwise specified. Abbreviations: ART, antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; IRIS, immune reconstitution inflammatory syndrome; NA, not applicable.

\**P* < .01.

was dually infected with HIV-1 and HIV-2 (Table 1). The median age of the control group was 38 years (IQR, 27–47 years), 81% were female, and all were infected with HIV-1 (Table 1). Time to diagnosis of IRIS was 21 days (IQR, 14–35 days) from initiation of ART with a median duration of 46 days (IQR, 31–85 days). Thus, the IRIS time point used throughout the results was compared with a 3-month ART time point for the controls. Viral load was significantly reduced in both groups to a median of <100 copies/mL by 3 months of ART (Table 1). The IRIS manifestations involved the lungs (45%), skin/mucosal surfaces (45%), or lymph nodes (10%), but pathogens were rarely identified. Eight IRIS patients had a prior history of tuberculosis, but no information on tuberculosis status was available for the controls. Supplementary Table 1 shows the clinical characteristics of the cohort (see also Table 2 [9]). Analysis of the 8 IRIS patients with prior tuberculosis history was compared to those without, and we saw no differences at any time point; thus, all results are presented as grouped analyses.

### Changes in Lymphocyte Subsets in IRIS and Control Patients Following Initiation of ART

No difference in total white blood cells, the percentage of lymphocytes, the percentage of absolute levels of CD3<sup>+</sup> T cells, or the percentage of absolute levels of CD8<sup>+</sup> T cells was seen in IRIS compared to controls at any time point (Figures 1A–D). No significant difference in CD4<sup>+</sup> T-cell counts was observed between IRIS and control groups at any time point; however,



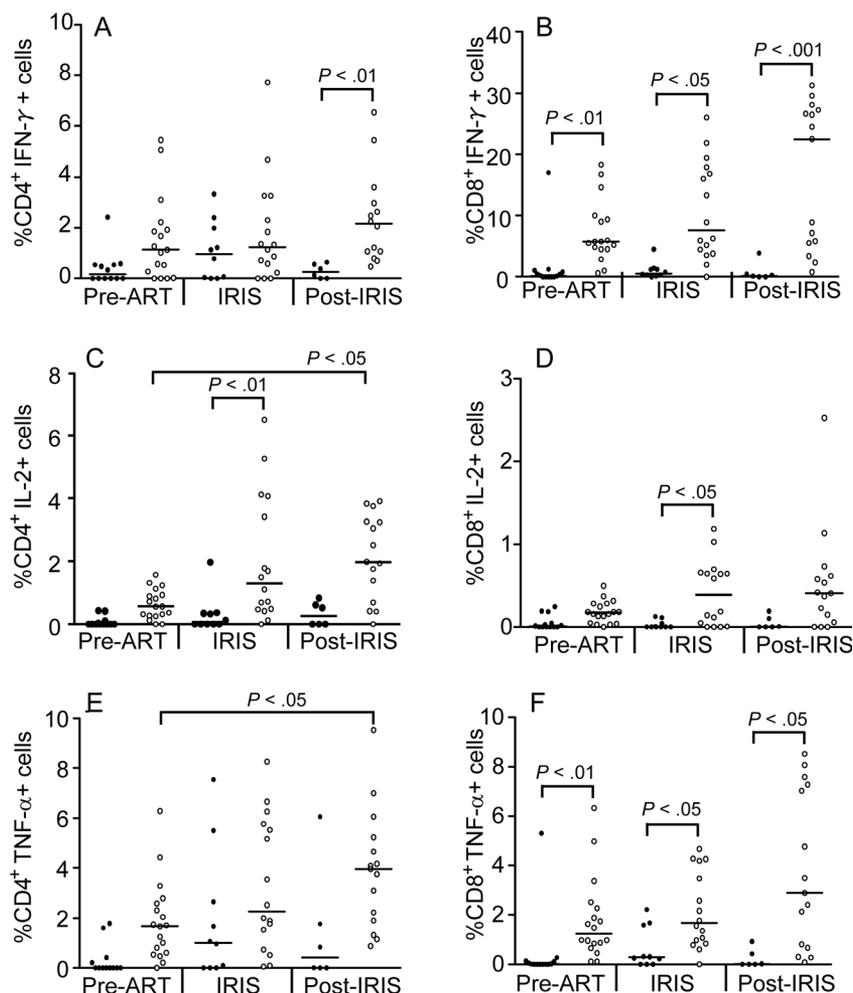
**Figure 1.** Analysis of total white blood cells, lymphocytes, and T-cell subset levels before and after antiretroviral therapy initiation in patients who developed immune reconstitution inflammatory syndrome (IRIS; closed circles) compared to non-IRIS controls (open circles). Data were analyzed using a Kruskal-Wallis test followed by Dunn's posttest comparison, and  $P$  values  $\leq .05$  are shown. Abbreviations: ART, antiretroviral therapy; IRIS, immune reconstitution inflammatory syndrome.

IRIS patients had a significantly higher rate of CD4<sup>+</sup> T-cell recovery post-ART compared to controls ( $P < .01$ ; Table 1). IRIS patients had a low CD4 nadir of 65 cells/μL (IQR, 20–130 cells/μL) prior to ART, and this rapidly increased to 200 cells/μL (IQR, 95–365 cells/μL) during IRIS ( $P < .001$ ; Figure 1E). For controls, pre-ART CD4 counts were comparable (80 cells/μL [IQR, 20–145 cells/μL]) and rose to 170 cells/μL (IQR, 120–240 cells/μL) at the same time point post-ART ( $P < .05$ ; Figure 1E).

#### Increased Specific but Not Generalized T-Cell Responses in IRIS Patients

Following stimulation with the positive control, there were no differences in the proportion of cytokine-positive CD4<sup>+</sup> T cells between IRIS patients and controls at the pre-ART time point

(Figure 2). However, the proportion of both CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and CD8<sup>+</sup>TNF- $\alpha$ <sup>+</sup> cells was significantly lower prior to ART in the patients who later developed IRIS ( $P < .01$  for both; Figure 2B and 2F). At the IRIS time point, patients with IRIS retained similar levels of CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and CD4<sup>+</sup>TNF- $\alpha$ <sup>+</sup> cells following the positive control stimulation compared to controls (Figure 2A and 2E). However, the proportion of CD4<sup>+</sup>IL-2<sup>+</sup> was significantly lower in patients with IRIS compared to controls ( $P < .01$ ; Figure 2C). Furthermore, IRIS subjects had very little response from CD8<sup>+</sup> T cells even at 6 months of ART (post-IRIS), whereas the controls had significantly higher proportions of CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup>, CD8<sup>+</sup>IL-2<sup>+</sup>, and CD8<sup>+</sup>TNF- $\alpha$ <sup>+</sup> cells at both 3 and 6 months of ART compared to the IRIS group (Figure 2B, 2D, and 2F). Interestingly, the IRIS group did not



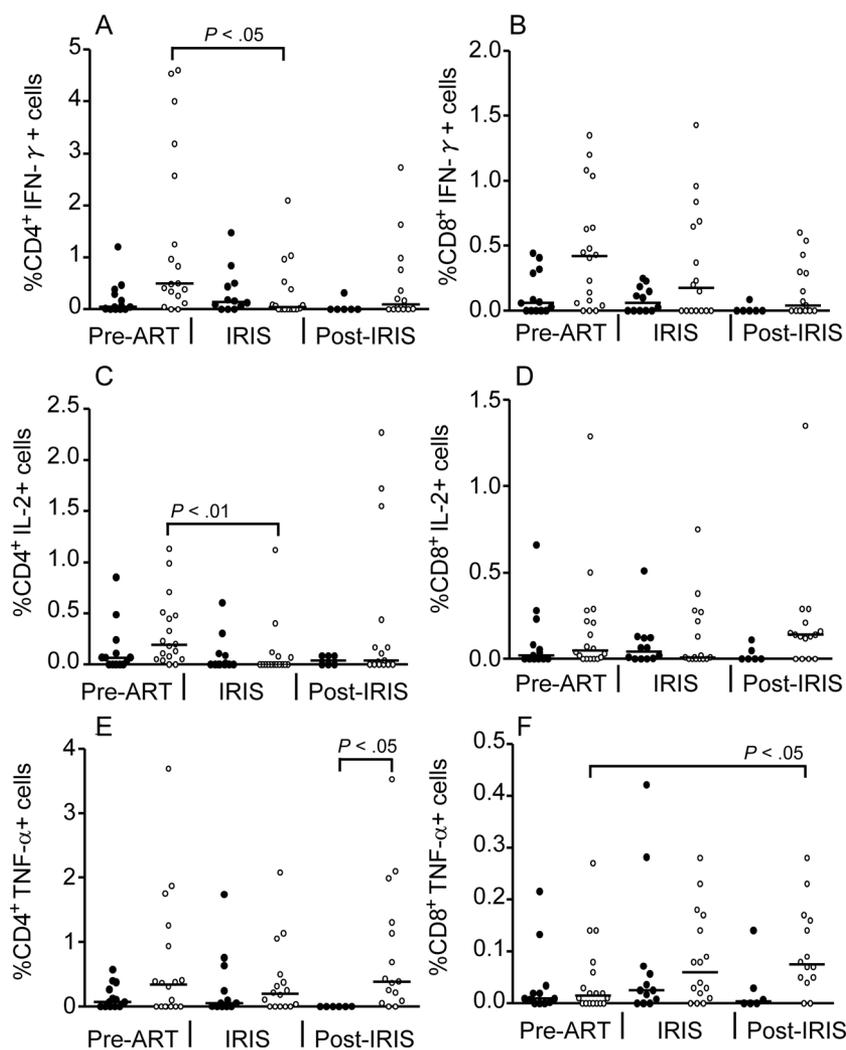
**Figure 2.** Immune reconstitution inflammatory syndrome (IRIS) patients show reduced CD4<sup>+</sup> and CD8<sup>+</sup> responses to general antigenic stimulation. Cryopreserved peripheral blood mononuclear cells were stimulated overnight using anti-CD3/CD28. Cells were analyzed by flow cytometry for interferon  $\gamma$ , tumor necrosis factor  $\alpha$ , and interleukin 2 expression within CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Bar indicates median of 20 IRIS (closed circles) and 16 non-IRIS (open circles) before, during, and after IRIS development. Data were analyzed using a Kruskal-Wallis test followed by Dunn's posttest comparison, and  $P$  values  $\leq .05$  are shown. Abbreviations: ART, antiretroviral therapy; IFN, interferon; IL-2, interleukin 2; IRIS, immune reconstitution inflammatory syndrome; TNF, tumor necrosis factor.

significantly increase overall cytokine production from CD4<sup>+</sup> or CD8<sup>+</sup> T cells up to 6 months of ART following stimulation with the positive control. Conversely, control patients had significantly higher levels of CD4<sup>+</sup>IL-2<sup>+</sup> and CD4<sup>+</sup>TNF- $\alpha$ <sup>+</sup> cells by 6 months post-ART compared to their pre-ART levels ( $P < .05$  for both; Figure 2C and 2E).

Analysis of responses to EC showed no differences between IRIS and control patients before ART and at the IRIS time point (both CD4<sup>+</sup> and CD8<sup>+</sup> T cells; all cytokines; Figure 3). However, there was a significantly higher proportion of CD4<sup>+</sup>TNF- $\alpha$ <sup>+</sup> cells at 6 months post-ART in control subjects compared to the IRIS group ( $P < .05$ ; Figure 3E). In patients who developed IRIS, there were few changes in CD4<sup>+</sup> or CD8<sup>+</sup> cytokine production following ART: the control patients

showed the majority of changes with a significant reduction in both CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and CD4<sup>+</sup>IL-2<sup>+</sup> cells at 3 months of ART compared to the pre-ART time point ( $P < .05$  and  $P < .01$ , respectively; Figure 3A and 3C). In addition, a significantly higher level of CD8<sup>+</sup>TNF- $\alpha$ <sup>+</sup> cells was seen following EC stimulation at 6 months post-ART compared to pre-ART for the control patients only ( $P < .05$ ; Figure 3F).

Despite the low level of overall cytokine production to the positive control stimulation (and significantly reduced response compared to controls), we saw a significantly higher proportion of PPD-specific CD4<sup>+</sup> cells producing IFN- $\gamma$  in patients with IRIS compared to controls following initiation of ART ( $P < .05$ ; Figure 4A). However, control patients had significantly higher levels of IFN- $\gamma$ <sup>+</sup> and TNF- $\alpha$ <sup>+</sup> CD8<sup>+</sup> cells at the same time point



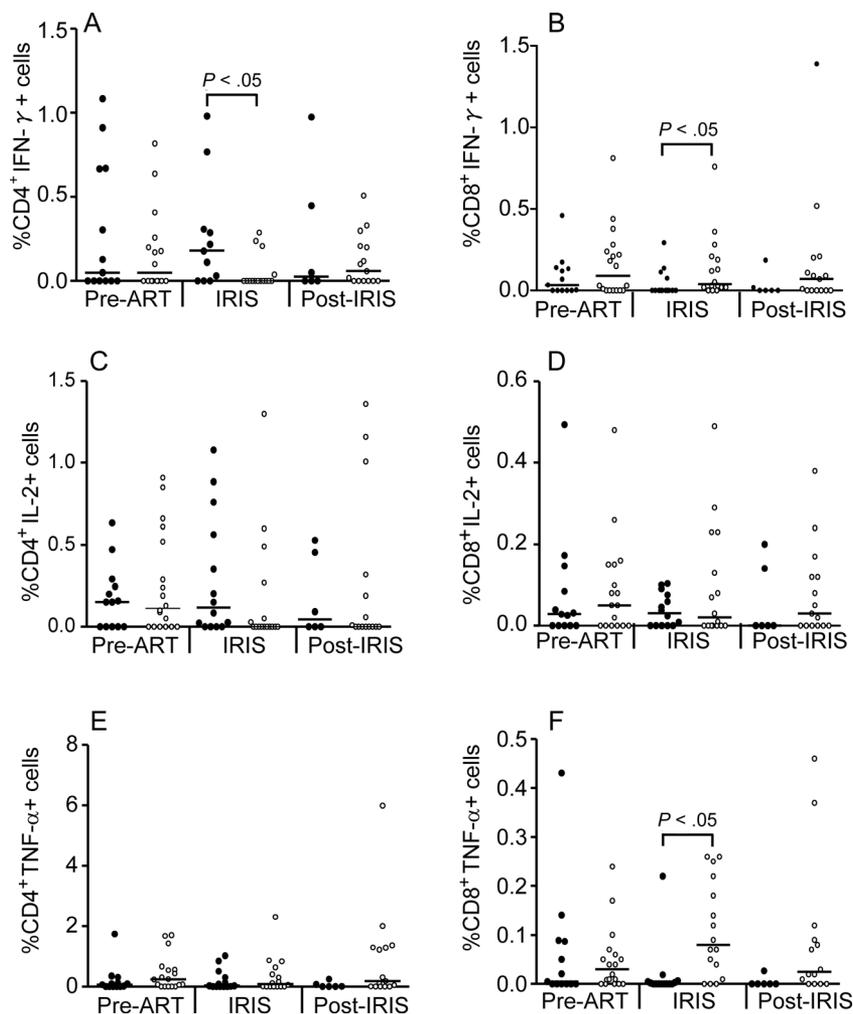
**Figure 3.** T-cell cytokine responses to ESAT-6/CFP-10 stimulation are similar in immune reconstitution inflammatory syndrome (IRIS) compared to non-IRIS subjects at all time points. Cryopreserved peripheral blood mononuclear cells were stimulated overnight using ESAT-6/CFP10 peptide pool. Cells were analyzed by flow cytometry for interferon  $\gamma$ , tumor necrosis factor  $\alpha$ , and interleukin 2 expression within CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Bar indicates median of 20 IRIS (closed circles) and 16 non-IRIS (open circles) before, during, and after IRIS development. Data were analyzed using a Kruskal-Wallis test followed by Dunn's posttest comparison, and  $P$  values  $\leq .05$  are shown. Abbreviations: ART, antiretroviral therapy; IFN, interferon; IL-2, interleukin 2; IRIS, immune reconstitution inflammatory syndrome; TNF, tumor necrosis factor.

( $P < .05$  for both; Figure 4B and 4F). No significant differences were seen at 6 months of ART between IRIS and control patients and no differences were observed within groups at any time point for any cytokine.

#### Reduction in CD4<sup>+</sup> T-Cell Polyfunctionality in IRIS Compared to Non-IRIS Patients

Analysis of the combinatorial profiles of cytokine-positive cells was performed using SPICE software (Figure 5). Results are based on only the cytokine-producing cells with analysis of any one cytokine, any 2 cytokines, and all 3 cytokines to determine differences in overall levels of polyfunctionality.

Prior to ART, control patients had significantly higher polyfunctional CD4<sup>+</sup> T-cell responses to the positive control stimulation compared to patients who developed IRIS ( $P = .037$ ; Figure 5A). This trend was also seen at 3 and 6 months post-ART ( $P = .021$  and  $P = .020$ , respectively; Figure 5A). Following PPD stimulation, control patients had significantly higher proportions of polyfunctional T cells at 3 and 6 months post-ART compared to IRIS patients, despite similar levels at baseline ( $P = .027$  and  $P < .001$ , respectively). Following EC stimulation, no differences were observed pre-ART or at 3 months of ART, but control patients had higher proportions of CD4<sup>+</sup> T cells producing any 2 cytokines at 6 months post-ART compared to IRIS patients ( $P = .001$ ; Figure 5A). Differences within CD8<sup>+</sup>

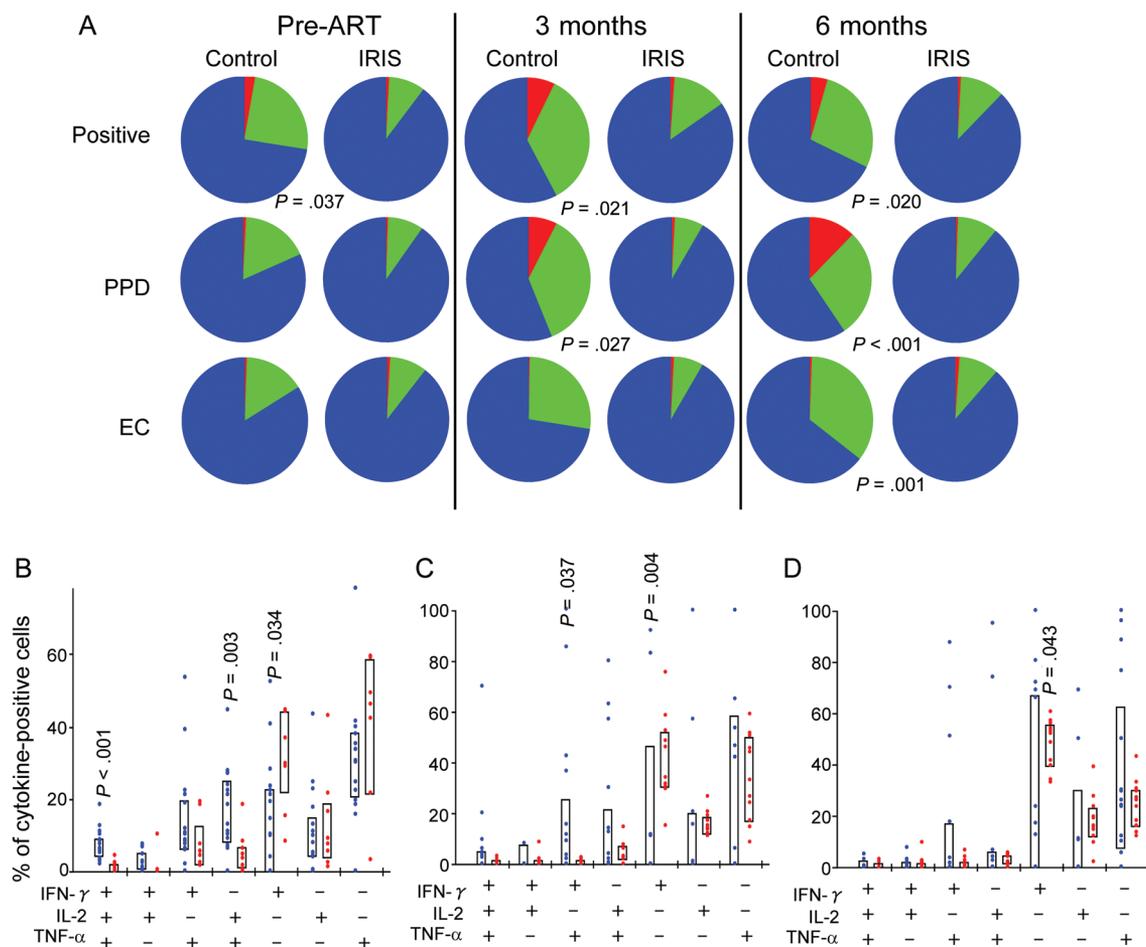


**Figure 4.** T-cell responses to purified protein derivative (PPD) are significantly increased in immune reconstitution inflammatory syndrome (IRIS) subjects at the IRIS time point. Cryopreserved peripheral blood mononuclear cells were stimulated overnight using PPD. Cells were analyzed by flow cytometry for interferon  $\gamma$ , tumor necrosis factor  $\alpha$ , and interleukin 2 expression within  $CD4^+$  and  $CD8^+$  T cells. Bar indicates median of 20 IRIS (closed circles) and 16 non-IRIS (open circles) before, during, and after IRIS development. Data were analyzed using a Kruskal-Wallis test followed by Dunn's posttest comparison, and  $P$  values  $\leq .05$  are shown. Abbreviations: ART, antiretroviral therapy; IFN, interferon; IL-2, interleukin 2; IRIS, immune reconstitution inflammatory syndrome; TNF, tumor necrosis factor.

T-cell cytokine-producing cells were seen only at the 6-month post-ART time point, with controls having significantly higher proportions of polyfunctional cells following all 3 stimulations compared to IRIS patients ( $P = .004$ ,  $P = .0001$ , and  $P < .001$ , respectively; data not shown).

Analysis of just the IRIS time point revealed further differences between IRIS and control groups (Figure 5B–D). Following the positive control stimulation, the control group had significantly higher levels of  $CD4^+$  cells producing all 3 cytokines ( $P < .001$ ) and  $CD4^+$  cells producing IL-2 and TNF- $\alpha$  together ( $P = .003$ ; Figure 5B). Whereas fewer polyfunctional cells were seen, IRIS patients had a significantly higher proportion

of  $CD4^+$  T cells producing only IFN- $\gamma$  following the positive control stimulation ( $P = .034$ ; Figure 5B). Following PPD stimulation, control patients had significantly higher proportions of IFN- $\gamma^+$ TNF- $\alpha^+$   $CD4^+$  cells compared to IRIS patients ( $P = .037$ ; Figure 5C) but similar to the positive control, IRIS patients had significantly higher levels of IFN- $\gamma^+$  $CD4^+$  (single-positive) cells ( $P = .004$ ; Figure 5C). The only difference following EC stimulation was, again, a significantly higher proportion of  $CD4^+$  cells producing only IFN- $\gamma$  in the IRIS group compared to controls ( $P = .043$ ; Figure 5D). The high proportion of IFN- $\gamma^+$  $CD4^+$  T cells was retained at 6 months post-ART in IRIS patients compared to controls for all 3 stimulations (data not shown).



**Figure 5.** Analysis of polyfunctional responses following  $\alpha$ CD3/CD28, purified protein derivative (PPD), or ESAT-6/CFP10 peptide pool (EC) stimulation. A, CD4<sup>+</sup> T cells were analyzed for different combinations of interferon (IFN)  $\gamma$ , tumor necrosis factor (TNF)  $\alpha$ , and interleukin 2 (IL-2). Blue indicates the sole production of any one cytokine, green indicates any 2 cytokines produced, and red indicates all 3 cytokines are being produced. Pies were analyzed using analysis of variance according to slice color, and  $P$  values  $\leq .05$  are shown. B–D, Analysis of the immune reconstitution inflammatory syndrome time point only following positive control (B), PPD (C), or EC (D) stimulation. Results are shown for all combinations of IFN- $\gamma$ , IL-2, and TNF- $\alpha$  in controls (blue) and immune reconstitution inflammatory syndrome (red) patients. Data were analyzed using Wilcoxon rank-sum test, and  $P$  values  $\leq .05$  are shown. Abbreviations: ART, antiretroviral therapy; EC, ESAT-6/CFP10 peptide pool; IFN, interferon; IL-2, interleukin 2; IRIS, immune reconstitution inflammatory syndrome; PPD, purified protein derivative; TNF, tumor necrosis factor.

## DISCUSSION

This is the first study in West Africa to delineate the cellular source of cytokines following antigenic stimulation in HIV-positive subjects who develop IRIS following ART initiation. The most striking finding from this study was the suppressed responses to positive control stimulation (mainly CD8<sup>+</sup> T cells) even at 6 months of ART in IRIS patients (3 months following IRIS resolution) compared to controls. Conversely, IRIS patients had a significantly higher proportion of IFN- $\gamma$ -producing CD4<sup>+</sup> T cells following PPD stimulation compared to controls during IRIS, which decreased following IRIS resolution. Together with a rapid increase in absolute levels of CD4<sup>+</sup> T cells, this suggests that development of IRIS in The Gambia (a tuberculosis-

endemic setting) is associated with specific pathogen-related T-cell responses from predominantly CD4<sup>+</sup> T cells, with a reduction in ability to respond to general stimulation.

In line with a previous study on the same cohort [9], we saw few differences between subjects prior to ART initiation. However, we did see significantly lower production of TNF- $\alpha$  and IFN- $\gamma$  from CD8<sup>+</sup> T cells following positive control stimulation in subjects who developed IRIS compared to those who did not prior to ART. Despite similar levels of absolute CD8<sup>+</sup> T-cell levels at all time points of analysis, a decreased responsiveness from CD8<sup>+</sup> T cells was seen up to 3 months after cessation of IRIS and indicates a potential role for CD8<sup>+</sup> T cells in development of IRIS.

Despite attempts to characterize IRIS, a definitive diagnosis remains intangible. As seen in previous studies, the rate of

increase in CD4<sup>+</sup> counts in the IRIS patients was significantly higher than in controls and increased even further at 6 months of ART (at least 3 months following cessation of IRIS). Associated with this increase in CD4<sup>+</sup> T cells, we saw a significant increase in CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T-cell levels following stimulation with PPD in IRIS compared to controls, despite a very low response to the positive control stimulation. Our finding is similar to a recent study in a non-tuberculosis endemic environment, which shows a bias in response to the IRIS-associated antigen at the IRIS time point [16]. However, in our study, the quality of the immune response to all antigens was restricted in IRIS patients: polyfunctional (IFN- $\gamma$ <sup>+</sup>IL-2<sup>+</sup>TNF- $\alpha$ <sup>+</sup>) T-cell responses (both CD4<sup>+</sup> and CD8<sup>+</sup>) were significantly lower in IRIS patients at least up to 6 months of ART, whereas IRIS patients had significantly higher levels of CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> single-positive cells following all stimulations at the IRIS time point compared to controls. These differences likely reflect differences in study setting (ie, tuberculosis-endemic vs nonendemic), study group (ie, defined IRIS vs all-cause IRIS), and analysis (grouped antigen responses vs tuberculosis only).

The present study had a number of limitations. The majority of patients had suffered from tuberculosis previously, but none were taking tuberculosis therapy at the time of ART initiation. More importantly, few patients who suffered IRIS received a definitive diagnosis of the pathogen; thus, the IRIS group was defined as all-cause IRIS [9] and will therefore dilute out responses seen for just tuberculosis IRIS subjects. Additionally, data on the tuberculosis status of the control group were not available, which raises the question whether differences were associated with differences in tuberculosis sensitization. However, there was no difference in response to ESAT-6/CFP-10 between IRIS and controls. Responses to EC are not influenced by BCG vaccination or environmental mycobacteria; therefore, a similar response to this antigen for both groups suggests a similar level of exposure. Another limitation was the small cohort, making it difficult to perform a separate analysis of paradoxical vs unmasking IRIS, which would be useful for future diagnostic purposes.

It is not clear from this study how CD4<sup>+</sup> monofunctional responses and reduction in CD8<sup>+</sup> responsiveness, particularly to our positive control stimulation, will affect the pathology at the IRIS time point, but dominance of an IFN- $\gamma$ -only response may contribute to the reduced level of T-cell responsiveness after IRIS. For instance, homeostatic mechanisms would indicate that increased IFN- $\gamma$ -only cells will reduce (proportionately) levels of IL-2<sup>+</sup> cells, which are essential for maintaining the T-cell pool and controlling viremia in HIV-1 infection [21]. The dominant response in the IRIS subjects was a highly significant rebound of CD4<sup>+</sup> T cells, most of which produced only IFN- $\gamma$  after stimulation. However, despite increases in this

subset at the IRIS time point, by the 6-month time point, IRIS subjects had significantly lower levels of cytokine-producing cells compared to controls, possibly due to immune exhaustion. Although there were no differences in total CD8<sup>+</sup> cells at any time point, we did see a significant reduction in the functional capacity of the CD8<sup>+</sup> T-cell pool, which will also contribute to the dysregulation of the CD4<sup>+</sup> response. However, larger cohorts of more clearly defined IRIS patients are needed to further understand the pathology of IRIS.

In summary, we have shown a slow recovery of general T-cell responses (both CD4<sup>+</sup> and CD8<sup>+</sup>) but specific increases in monofunctional CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> responses to tuberculosis antigens during IRIS. These findings provide insight into the underlying causes of IRIS and may be useful for identifying patients at risk of developing IRIS.

## Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (<http://cid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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