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MAJOR ARTICLE

Emergence of Polyfunctional Cytotoxic CD4$^+$ T Cells in *Mycobacterium avium* Immune Reconstitution Inflammatory Syndrome in Human Immunodeficiency Virus-Infected Patients

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Background. Immune reconstitution inflammatory syndrome (IRIS) is an aberrant inflammatory response in individuals with advanced human immunodeficiency virus (HIV) infection, after antiretroviral therapy (ART) initiation. The pathogenesis of *Mycobacterium avium* complex (MAC)–associated IRIS has not been fully elucidated.

Methods. We investigated monocyte and CD4$^+$ T-cell responses in vitro, tumor necrosis factor (TNF) expression in tissues, and plasma cytokines and inflammatory markers, in 13 HIV-infected patients with MAC-IRIS and 14 HIV-uninfected patients with pulmonary MAC infection.

Results. Prior to ART, HIV-infected compared with HIV-uninfected patients, had reduced TNF$^+$ monocytes ($P = .013$), although similar cytokine (interferon gamma [IFN-γ], TNF, interleukin 2 [IL-2], and interleukin 17 [IL-17])–expressing CD4$^+$ T cells. During IRIS, monocyte cytokine production was restored. IFN-γ$^+$ ($P = .027$), TNF$^+$ ($P = .004$), and polyfunctional CD4$^+$ T cells ($P = 0.03$) also increased. These effectors were T-bet^{low}, and some expressed markers of degranulation and cytotoxic potential. Blockade of cytotoxic T-lymphocyte associated protein 4 and lymphocyte activation gene-3 further increased CD4$^+$ T-cell cytokine production. Tissue immunofluorescence showed higher proportions of CD4$^+$ and CD68$^+$ (monocyte/macrophage) cells expressed TNF during IRIS compared with HIV-uninfected patients. Plasma IFN-γ ($P = .048$), C-reactive protein ($P = .008$), and myeloperoxidase ($P < .001$) levels also increased, whereas interleukin 10 decreased ($P = .008$) during IRIS.

Conclusions. Advanced HIV infection was associated with impaired MAC responses. Restoration of monocyte responses and expansion of polyfunctional MAC-specific T-bet^{low} CD4$^+$ T cells with cytotoxic potential after ART initiation may overwhelm existing regulatory and inhibitory mechanisms, leading to MAC-IRIS.

Keywords. immune reconstitution inflammatory syndrome; IRIS; *Mycobacterium avium* complex; MAC; HIV.

Immune reconstitution inflammatory syndrome (IRIS) is an aberrant inflammatory immune response that can be observed after initiation of antiretroviral therapy (ART) in human immunodeficiency virus (HIV)–infected patients with advanced immunodeficiency [1, 2]. One of the pathogens that is associated with IRIS is *Mycobacterium avium* complex (MAC) [3–7]. Although the incidence of MAC infection has decreased dramatically in the era of combination ART [8], mortality from MAC infection remains high [9], and MAC-IRIS continues to complicate the management of HIV-infected patients upon initiation of ART [5, 9–11].

Appreciation of the pathogenesis of mycobacterial IRIS arises from data from patients with tuberculosis (TB) IRIS. The higher rates of IRIS in patients who initiated ART shortly after antimycobacterial treatment [12–14] and patients with positive sputum smears [15, 16], cerebrospinal fluid cultures [17], and higher levels of lipoarabinomannan in urine [18] suggest strong associations between antigen load and IRIS. Host immune responses, in particular, an exaggerated T-helper 1 (Th1) response, are thought to be critical in IRIS pathogenesis, given that patients with mycobacterial

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IRIS often have increased antigen-specific $CD4^+$ T-cell responses after in vitro stimulation [19, 20] and elevated plasma levels of inflammatory cytokines such as tumor necrosis factor (TNF) and interferon gamma (IFN-γ) [21, 22]. This is further supported by data from a murine model where MAC-infected T-cell–deficient mice can develop IRIS after transfer of $CD4^+$ T cells in a process that is dependent on the presence of IFN-γ–producing, antigen-specific $CD4^+$ T cells [23]. Monocytes also play a role in TB-IRIS pathogenesis. Transcriptional studies comparing gene expression in monocytes isolated from patients with and without TB-IRIS have found differences in expression in >100 genes [24]. Plasma biomarkers of monocyte activation including soluble CD163 and soluble tissue factor, as well as ex vivo monocyte cytokine production, were also elevated in patients with TB-IRIS at baseline, prior to ART initiation, and further increased during IRIS [16].

The pathogenesis of MAC-IRIS has not been clearly delineated. Defective regulatory T cells and higher frequency of MAC-specific $CD4^+$ T cells in comparison with healthy controls have been described previously [25]. The levels of MAC-specific $CD4^+$ T cells in comparison with patients with MAC infection, without HIV infection, are not known. It is thus unclear if responses of MAC-specific $CD4^+$ T cells and monocytes during MAC-IRIS represent a return to immune competency or aberrancy. In this study, we compared $CD4^+$ T-cell and monocyte responses in HIV-infected patients experiencing MAC-IRIS with HIV-uninfected patients with pulmonary MAC infection. Our data support an exuberant polyfunctional $CD4^+$ T-cell response by effectors with a distinct transcription factor profile (T-bet^{low}, Eomesodermin $[Eomes]^+$) and cytotoxic potential that is accompanied by myeloid cell activation and production of inflammatory cytokines in peripheral blood and tissues in MAC-IRIS.

MATERIALS AND METHODS

Study Design and Patient Cohort

ART-naive patients with a $CD4^+$ T-cell count <100 cells/μL enrolled in prospective studies at the National Institutes of Health (NIH) (NCT00286767, NCT02147405) were evaluated to identify those with MAC-IRIS. Similarly, HIV-uninfected patients with MAC infection enrolled in a natural history study of mycobacterial disease at the NIH (NCT00018044) were identified. All 3 studies were approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, and written informed consent was obtained from all participants prior to any study procedures.

Immunophenotyping and Stimulation Experiments

Cryopreserved peripheral blood mononuclear cells (PBMCs) were collected from a single time point in HIV-uninfected patients with MAC infection and at 3 time points in HIV-infected patients with MAC-IRIS; baseline (prior to ART initiation); during IRIS; and at week 48. Cells were then used for immunophenotyping and stimulation experiments, respectively (See Supplementary Methods).

Blocking Inhibitory Receptors Using Antibodies

PBMCs from HIV-infected patients with MAC-IRIS from baseline and IRIS time points were incubated with immunoglobulin G control or respective anti-inhibitory receptor antibodies, including anti–cytotoxic T-lymphocyte associated protein 4 (CTLA-4) antibody (Biolegend, clone L3D10, 5 μg/mL), anti–lymphocyte activation gene-3 (LAG-3) antibody (Abcam, clone 11E3, 20 μg/mL), anti–programmed cell death 1 (PD-1) antibody (Biolegend, clone EH12.2h7, 10 μg/mL), and anti–programmed cell death ligand-1 (PD-L1) antibody (Biolegend, clone 29E.2A3, 20 μg/mL), and stimulated with heat-inactivated, sonicated *M. avium* as described in the Supplementary Methods.

Confocal Microscopy of Lymph Node Aspirate and Bronchoalveolar Lavage

A subset of HIV-infected patients had lymph node fine-needle aspiration (n = 4) and bronchoalveolar lavage (BAL; n = 3) performed at the time of IRIS, and a subset of HIV-uninfected patients with MAC infection had BAL (n = 2) or lung fine-needle aspiration (n = 2) for diagnostic purposes. Tissue immunofluorescence was performed on these samples (Supplementary Methods).

Statistical Analysis

Comparisons between different time-points (baseline, IRIS, and week 48) in HIV-infected patients were analyzed using Wilcoxon signed-rank test. Comparisons between HIV-infected and HIV-uninfected patients were analyzed using Mann-Whitney test.

The inferential networks (host interactome) were generated from Spearman correlation matrices containing values of each biomarker measured in the plasma samples as well as frequencies of cytokine-producing monocytes or lymphocytes in PBMCs after in vitro MAC stimulation. In this analysis, each mediator is selected as a target, and the software program (JMP 13, SAS Institute) searches among the other mediators for those that are correlated, with the target calculating a correlation matrix using Spearman rank tests. Thus, the features related to the selected target are linked. The links shown in the networks represent highly statistically significant Spearman rank correlations ($P < .05$). We next employed bootstrapping (100×) of the correlation matrices. Only correlations remaining with $P < .05$ in at least 40 of 100 times were considered.

Statistical analyses were performed using R, JMP, and GraphPad Prism software.

RESULTS

Patient Characteristics

Thirteen HIV-infected patients with MAC-IRIS and 14 HIV-uninfected patients with pulmonary MAC infection with available cryopreserved PBMCs were identified from 3 observational cohorts. All HIV-infected patients were ART naive at baseline. Nine HIV-infected patients had unmasking MAC-IRIS and 4 had paradoxical MAC-IRIS. Patients with paradoxical MAC-IRIS had been treated for MAC infection for 3–7 days prior to the

initiation of ART. The median time between ART initiation and the onset of MAC-IRIS was 30 (interquartile range [IQR], 18–55) days. The majority of HIV-infected patients with MAC-IRIS were middle-aged black men, while the HIV-uninfected patients with MAC infections were mostly older white women (Table 1). In HIV-infected patients, median $CD4^+$ T-cell count was 8 (IQR, 5–38) cells/μL and plasma HIV-RNA was 5.4 (IQR, 4.7–5.7) $\log_{10}$ copies/mL at baseline. During IRIS, median $CD4^+$ T-cell count rose to 119 (IQR, 34–161) cells/μL and plasma HIV-RNA levels fell to 158 (IQR, 50–316) copies/mL (Supplementary Figure 1).

Monocyte Cytokine Responses to MAC Are Impaired in Advanced HIV Infection and Restored During IRIS

After in vitro stimulation with heat-inactivated MAC, PBMCs from HIV-infected patients at baseline had significantly lower percentage of TNF^+ ($P = .013$) and lower percentage of interleukin 1β $(IL\text{-}1\beta)^+$ ($P = .056$) monocytes compared with samples from HIV-uninfected patients (Figure 1A and 1B). During IRIS, there was a significant, though small, increase in the percentage of interleukin 6 $(IL\text{-}6)^+$ monocytes ($P = .039$, Figure 1C), and the percentage of TNF^+ and $IL\text{-}1\beta^+$ monocytes approached levels seen in HIV-uninfected patients (Figure 1A and 1B). After lipopolysaccharide stimulation, PBMCs from HIV-infected patients at baseline similarly showed a pattern of lower TNF^+ and $IL\text{-}1\beta^+$ monocytes; however, the percentage of $IL\text{-}6^+$ monocytes was significantly higher than in HIV-uninfected patients (Supplementary Figure 2*A*–*C*).

High Frequency of Polyfunctional, T-bet^{low}, and Cytotoxic MAC-Specific $CD4^+$ T Cells in HIV-Infected Patients During IRIS

At baseline, following in vitro stimulation with MAC, the percentages of cytokine-producing ($IFN\text{-}\gamma^+$, TNF^+, interleukin 2 $[IL\text{-}2]^+$, and interleukin 17 $[IL\text{-}17]^+$) $CD4^+$ T cells were low but were not significantly different between samples from HIV-infected and -uninfected patients (Figure 2A–E).

During IRIS, there was a significant increase in $IFN\text{-}\gamma^+$ ($P = .027$) and TNF^+ ($P = .004$) $CD4^+$ T cells (Figure 2B and 2C). Polyfunctional MAC-specific $CD4^+$ T cells expressing all 4 cytokines (IFN-γ, TNF, IL-2, IL-17; $P = .03$, Figure 2F) also increased. In contrast, there was no significant increase in cytomegalovirus-specific $CD4^+$ T-cell responses during the MAC-IRIS timepoint (Supplementary Figure 3).

To further characterize MAC-specific $CD4^+$ T cells during IRIS, the expression of T-bet (Figure 3A) and Eomes (Figure 3B) was measured. T-bet is a T-box transcription factor that regulates Th1 cell differentiation and IFN-γ production [26, 27]. Eomes is also a T-box transcription factor that is involved with immunologic memory and cytotoxicity [28]. In the setting of chronic viral infection and immune exhaustion, T-bet expression is reduced and Eomes expression is upregulated [29, 30]. As shown in Figure 3C, a large proportion of $IFN\text{-}\gamma^+$ $CD4^+$ T cells was $Eomes^+$ (median, 43.5% [IQR, 30.3%–52.4%]) and only a small proportion was $T\text{-}bet^+$ (median, 4.6% [IQR, 3.0%–13.0%]). A proportion of $IFN\text{-}\gamma^+$ $CD4^+$ T cells also expressed markers of degranulation [31] (CD107a and CD107b; median, 24.2% [IQR, 22.1%–40.8%]) and cytotoxic potential [32] (granzyme B; median, 6.6% [IQR, 3.0%–25.6%]). When perforin production was measured, 5%–9% of $IFN\text{-}\gamma^+$ $CD4^+$ T cells and 41%–72% of granzyme B^+ $IFN\text{-}\gamma^+$ $CD4^+$ T cells also expressed perforin (Figure 3D).

Exuberant MAC-Specific $CD4^+$ T-Cell Responses During IRIS Were Not Attributed to an Abrupt Decrease in Inhibitory Signals or the Absence of Regulatory T Cells

Next, we sought to determine whether absence or reduction of inhibitory signals were associated with the massive increases

Table 1. Patient Characteristics

Characteristic	HIV Infected (n = 13)	HIV Uninfected (n = 14)	*P* Value
Median age, y (IQR)	37 (30–46)	59 (52–71)	<.001[a]
Female sex, No. (%)	3 (23)	13 (93)	<.001[b]
Race, No. (%)			
White	4 (31)	11 (79)	.02[b]
Black	9 (69)	1 (7)	
Asian	0	2 (14)	
Median baseline BMI, kg/m^2 (IQR)	21.4 (18.4–24)	22.2 (19–26.2)	.51[a]
MAC species identification			
Mycobacterium avium	9	3	
Mycobacterium intracellulare	1	0	
Both *M. avium* and *M. intracellulare*	0	10	
Unspeciated	2	1	
Negative culture	1	0	
Median baseline CD4 count, cells/μL (IQR)	8 (5–38)	NA	
Median baseline HIV RNA, $\log_{10}$ copies/mL (IQR)	5.4 (4.7–5.7)		

Abbreviations: BMI, body mass index; HIV, human immunodeficiency virus; IQR, interquartile range; MAC, *Mycobacterium avium* complex; NA, not available.

[a]Analysis was performed using Mann-Whitney test.

[b]Analysis was performed using Fisher exact test.

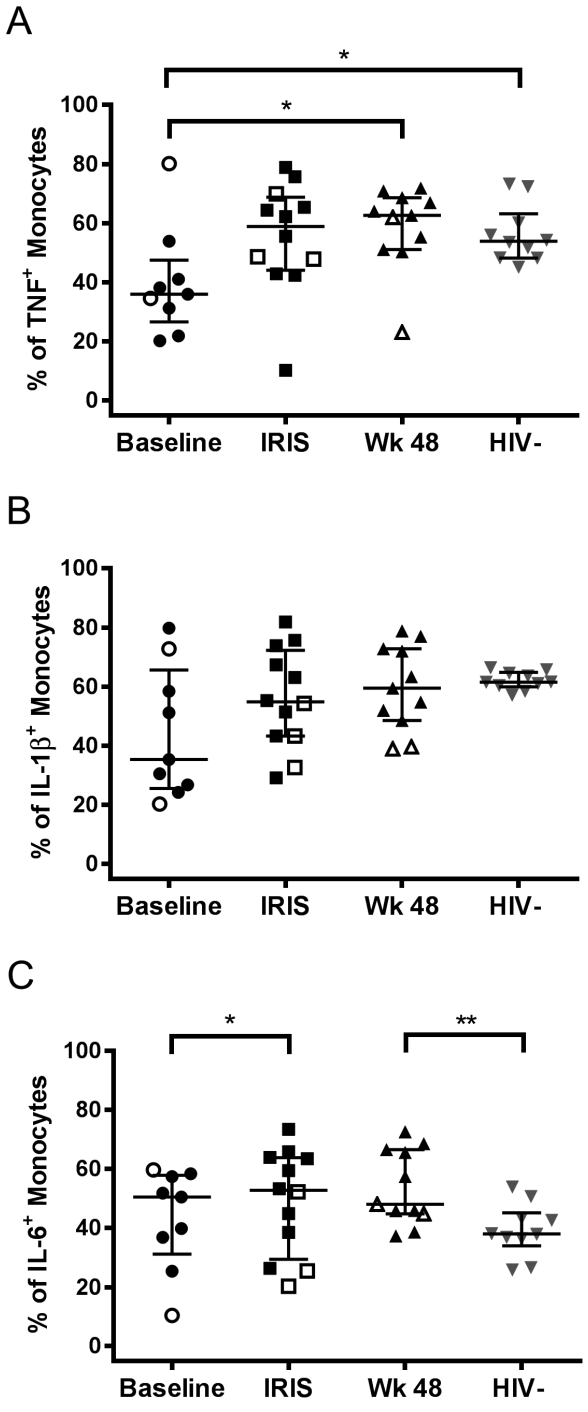

Figure 1. Production of inflammatory cytokines by peripheral blood monocytes in human immunodeficiency virus (HIV)–infected patients with *Mycobacterium avium* complex (MAC) immune reconstitution inflammatory syndrome (IRIS) and HIV-uninfected patients with MAC infection after stimulation with heat-inactivated MAC. Summary graphs of tumor necrosis factor (TNF)$^+$ (*A*), interleukin 1β (IL-1β)$^+$ (*B*), and interleukin 6 (IL-6)$^+$ (*C*) monocytes after 6 hours of stimulation with heat-inactivated MAC as measured by intracellular cytokine staining. The percentages of cytokine-producing monocytes between the 3 time points (baseline, IRIS, and week 48) within HIV-infected patients with MAC-IRIS were compared using Wilcoxon signed-rank test. Comparisons between HIV-infected patients and HIV-uninfected patients with MAC infection were made using Mann-Whitney test. Lines represent median and interquartile ranges, filled symbols denote patients with unmasking MAC-IRIS, and open symbols denote patients with paradoxical MAC-IRIS. **P* < .05, ***P* < .01.

in MAC-specific CD4$^+$ T-cell response. HIV-infected patients had a stable frequency of FOXP3$^+$ regulatory T cells (Tregs) over time (median of 8.3%, 8.5%, and 7.6% of CD4$^+$ T cells at baseline, IRIS, and week 48, respectively), that was not different from HIV-uninfected patients (median, 7.0% of CD4$^+$ T cells). The frequency of CD4$^+$ T cells expressing CTLA-4 (an inhibitory receptor that is induced following T-cell activation and suppresses T-cell responses [33]) was higher in HIV-infected patients at baseline (median, 9.3% of CD4$^+$ T cells [IQR, 4%–16%], P = .003) and during IRIS (median, 8.5% of CD4$^+$ T cells [IQR, 4.8%–21.9%], P = .002) when compared to HIV-uninfected patients (median, 3.1% of CD4$^+$ T cells [IQR, 2.5%–3.7%]). The levels of PD-1 (also an inhibitory receptor that is upregulated in exhausted cells [33]) expressing CD4$^+$ and CD8$^+$ T cells were also significantly higher in HIV-infected patients at baseline (median, 52% and 36% of CD4$^+$ and CD8$^+$ T cells, P < .001) and during IRIS (median, 45% and 35% of CD4$^+$ and CD8$^+$ T cells, P < .001) compared with HIV-uninfected patients (median, 8% and 11% of CD4$^+$ and CD8$^+$ T cells).

We further investigated the role played by cellular inhibitory receptors in HIV-infected patients with MAC-IRIS by incubating PBMCs with antibodies against cell receptors that mediate inhibitory pathways of T-cell responses. Inhibition of CTLA-4, LAG-3, PD-1, and PD-L1 did not consistently increase baseline CD4$^+$ T-cell IFN-γ responses (Figure 4A–D). During IRIS, CTLA-4 (P = .012) and LAG-3 (P = .063) blockade were associated with increases in IFN-γ$^+$ CD4$^+$ T cells (Figure 4A and 4B). This suggests that the exuberant CD4$^+$ T-cell responses during IRIS are unlikely to be secondary to the absence of inhibitory receptor signaling. In fact, immune checkpoint inhibition is probably at work in IRIS, bridling CD4$^+$ T-cell responses to some degree.

CD4$^+$ T Cells and Monocyte/Macrophages Were Also Activated at Sites of IRIS Pathology

Many of the studies on mycobacterial IRIS have focused on peripheral blood but the inflammatory response is likely to be largely localized in tissues such as the lungs or the lymph nodes. Using confocal microscopy, we evaluated TNF expression in CD4$^+$ and CD68$^+$ cells in tissues at sites of IRIS pathology. We found that larger proportions of CD4$^+$ cells and CD68$^+$ monocytes/macrophages in tissue samples from HIV-infected patients with MAC-IRIS were expressing TNF when compared to HIV-uninfected patients with MAC infection (Figure 5). These data mirrored the TNF production seen in PBMCs during in vitro stimulation assays.

Systemic Inflammatory Response Was Evident During IRIS, With Highly Elevated Plasma IFN-γ and C-Reactive Protein Levels

Plasma levels of IFN-γ, TNF, interleukin 10 (IL-10), C-reactive protein (CRP), D-dimer, soluble CD14, and IL-2 were all higher in HIV-infected patients at baseline compared with HIV-uninfected patients (Figure 6A–E; Supplementary Figure 4*A* and

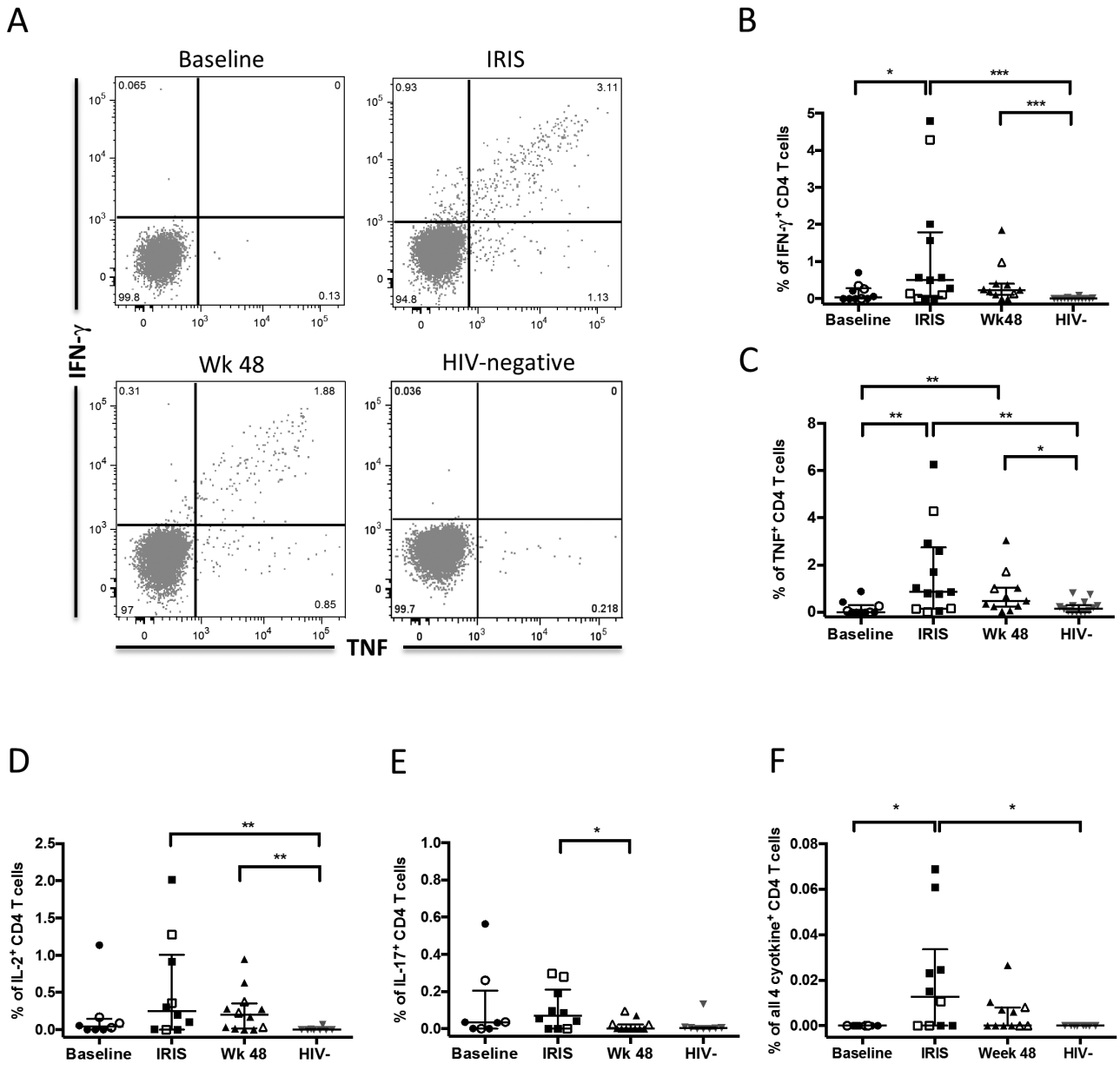

Figure 2. Production of cytokines by peripheral blood CD4$^+$ T cells in human immunodeficiency virus (HIV)–infected patients with *Mycobacterium avium* complex (MAC) immune reconstitution inflammatory syndrome (IRIS) and HIV-uninfected patients with MAC infection after stimulation with heat-inactivated MAC. Representative flow cytometry plots of intracellular cytokine staining of CD4$^+$ T-cell interferon gamma (IFN-γ) and tumor necrosis factor (TNF) production after stimulation with heat-inactivated MAC for 6 hours at baseline, IRIS, and week 48, and in an HIV-uninfected patient with MAC infection (*A*). Percentages of IFN-γ$^+$ (*B*), TNF$^+$ (*C*), interleukin 2 (IL-2)$^+$ (*D*), and interleukin 17 (IL-17)$^+$ (*E*) CD4$^+$ T cells, as well as polyfunctional CD4$^+$ T cells expressing all 4 cytokines (*F*) after stimulation with heat-inactivated MAC for 6 hours, were compared between the 3 time points (baseline, IRIS, and week 48) within HIV-infected patients with MAC-IRIS using Wilcoxon signed-rank test. Comparisons between HIV-infected and HIV-uninfected patients with MAC infection were done using Mann-Whitney test. Lines represent median and interquartile ranges, filled symbols are data from patients with unmasking MAC-IRIS, and open symbols are data from patients with paradoxical MAC-IRIS. *$P < .05$, **$P < .01$, ***$P < .001$.

4*B*). HIV-uninfected patients, in contrast, had higher plasma myeloperoxidase (MPO) levels (Supplementary Figure 4*C*). During IRIS, there was a significant increase in plasma IFN-γ ($P = .048$, Figure 6A), CRP ($P = .008$, Figure 6D), and MPO ($P < .001$, Supplementary Figure 4C). Increase in plasma IL-6 levels did not reach statistical significance ($P = .080$, Figure 6F), whereas IL-10 levels decreased ($P = .008$, Figure 6C) compared with baseline.

Interactions Between T-Cell, Monocyte, and Plasma Cytokine and Inflammatory Markers

Using inferential network analysis with bootstrapping, we examined the relationships, between cytokine-producing monocytes and CD4$^+$ T cells after in vitro MAC stimulation with plasma cytokine and inflammatory markers in HIV-infected patients with MAC-IRIS. At baseline (Supplementary Figure 4*D*), plasma IL-6 levels correlated with IFN-γ$^+$ CD4$^+$ T cells and plasma

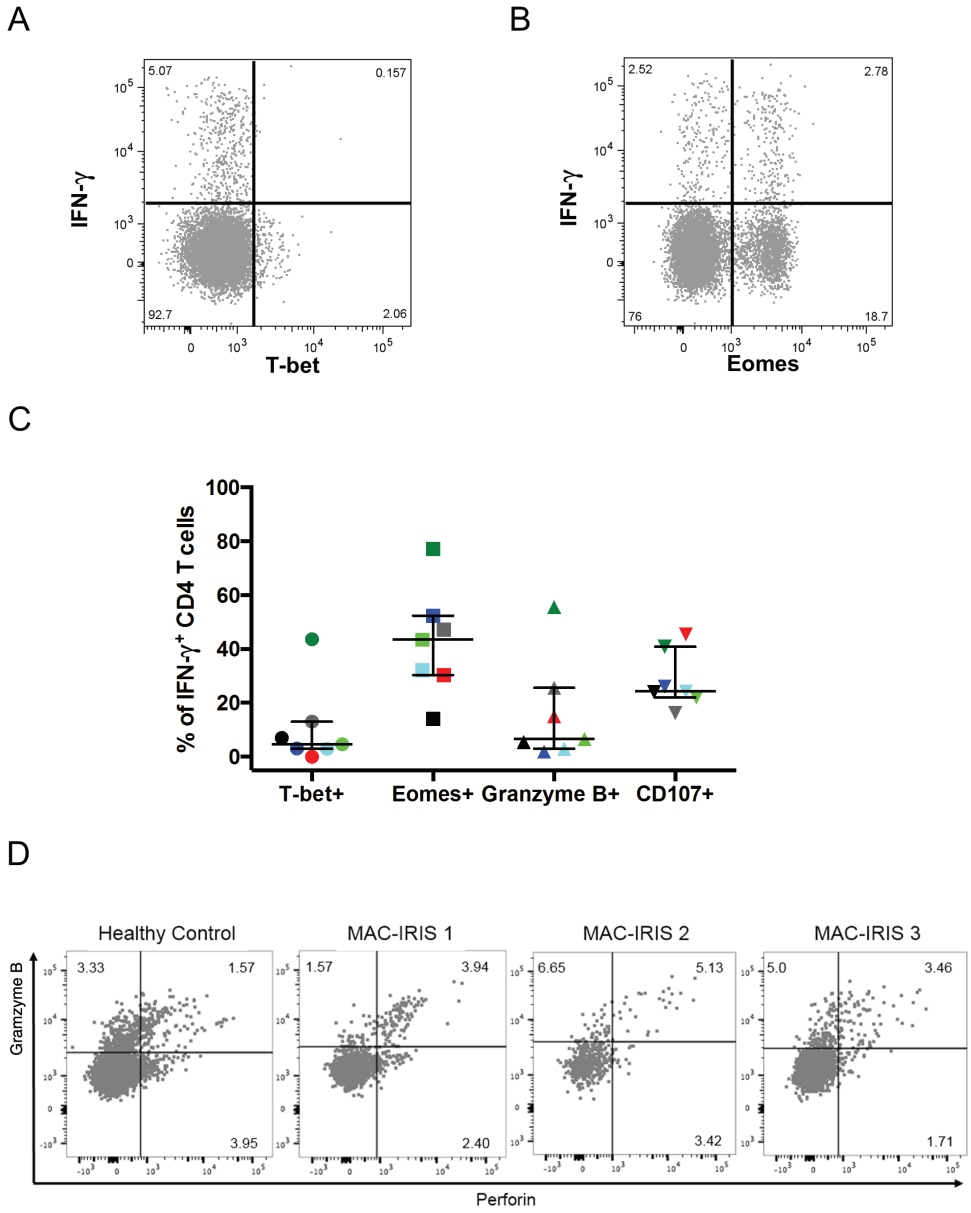

Figure 3. Expression of transcription factors and cytotoxicity markers in $CD4^+$ T cells of human immunodeficiency virus (HIV)–infected patients during *Mycobacterium avium* complex (MAC) immune reconstitution inflammatory syndrome (IRIS). Representative flow cytometry plots of T-bet (*A*) and Eomesodermin (Eomes; *B*) expression in interferon gamma (IFN-γ)$^+$ $CD4^+$ T cells after in vitro MAC stimulation in HIV-infected patients during MAC-IRIS are shown. IFN-γ^+ $CD4^+$ T cells in HIV-infected patients during MAC-IRIS were analyzed for T-bet, Eomes, granzyme B, and CD107 expression (*C*), with each color representing an individual patient (n = 7). Flow cytometry plots of granzyme B and perforin expression by IFN-γ^+ $CD4^+$ T cells after in vitro MAC stimulation in a healthy control and 3 HIV-infected patients during MAC-IRIS are also shown (*D*).

CRP levels correlated with the percentages of IL-6^+ and IL-$1\beta^+$ monocytes. During IRIS (Supplementary Figure 4*D*), the percentage of TNF^+ $CD4^+$ T cells correlated with the percentage of TNF^+ monocytes, and both correlated with plasma TNF levels. Similarly, IL-6^+ monocytes and IFN-γ^+ $CD4^+$ T cells correlated with plasma IL-6 and IFN-γ levels, respectively.

DISCUSSION

In this study, we compared monocyte and $CD4^+$ T-cell immune responses after in vitro MAC stimulation between HIV-infected patients with MAC-IRIS and HIV-uninfected patients with MAC infection to determine whether the immune responses seen during IRIS represent a return to immune competency or aberrancy.

We observed that during IRIS there was restoration of TNF production in monocytes in response to MAC. Furthermore, MAC-specific $CD4^+$ T-cell responses increased substantially during IRIS, to levels much higher than HIV-uninfected patients with MAC infection. Similar increases in IFN-γ^+ and TNF^+ $CD4^+$ T cells, specific for the antigen responsible for the IRIS event during IRIS, have been well documented in patients with TB-IRIS [19, 20, 34]. During IRIS, MAC-specific $CD4^+$ T cells were

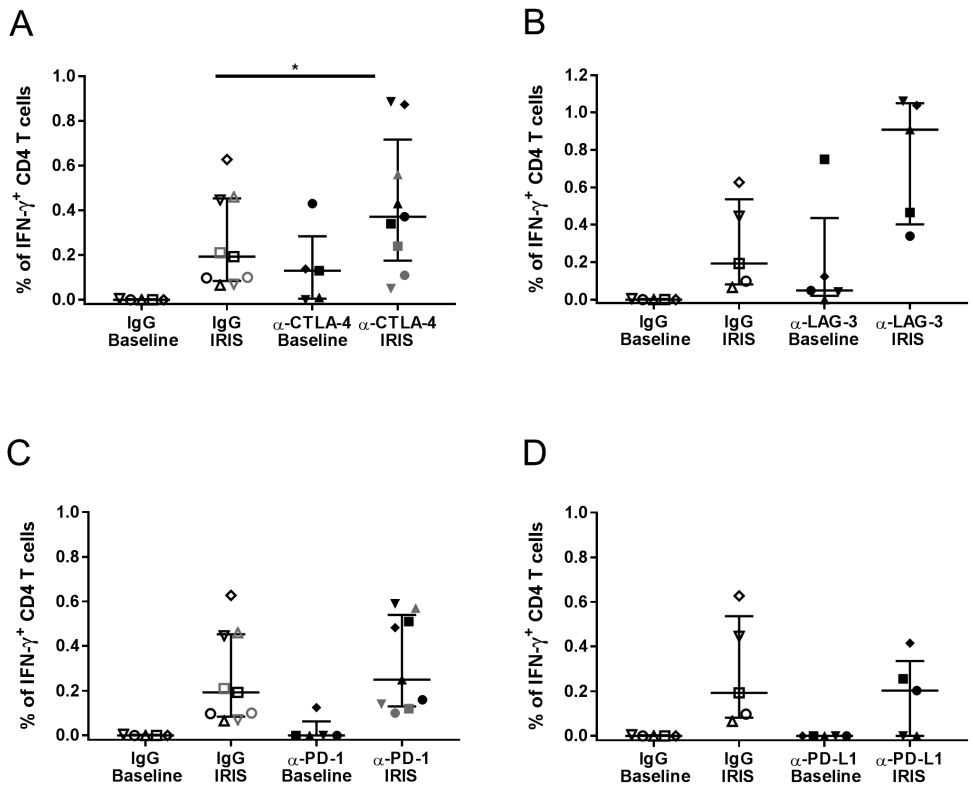

Figure 4. The effects of blocking inhibitory receptor signaling pathways on *Mycobacterium avium* complex (MAC)–specific CD4$^+$ T cells. The percentage of interferon gamma (IFN-γ)$^+$ CD4$^+$ T cells at baseline and immune reconstitution inflammatory syndrome (IRIS) following in vitro MAC stimulation in the presence of antibodies to inhibitory receptors (filled symbols), anti–cytotoxic T-lymphocyte associated protein 4 (CTLA-4) antibody (*A*), anti–lymphocyte activation gene-3 (LAG-3) antibody (*B*), anti–programmed cell death 1 (PD-1) antibody (*C*), and anti–programmed cell death ligand-1 (PD-L1) antibody (*D*), and control immunoglobulin G (IgG) (open symbols). Each symbol represents data from an individual patient. Patients with paired baseline and IRIS samples are denoted with black symbols. Patients with samples at IRIS timepoint only are denoted with gray symbols. *$P < .05$.

polyfunctional, expressing low levels of T-bet and high levels of Eomes. The T-bet^{low} Eomes^{hi} CD4$^+$ T-cell phenotype is associated with inhibitory receptor expression and immune exhaustion. This is likely secondary to underlying advanced HIV infection [29, 30]. A proportion of MAC-specific CD4$^+$ T cells also expressed markers of degranulation and showed cytotoxic potential.

The robust MAC-specific CD4$^+$ T-cell response during IRIS was also not secondary to a dramatic reduction in cell-associated inhibitory signals. The frequency of Tregs remained stable from baseline to IRIS and was comparable to patients without HIV infection. This is consistent with data from patients with TB-IRIS [35]. Weakly suppressive Tregs have previously been found in patients with MAC infection [25]. We were not able to test regulatory T-cell suppressive function. Other inhibitory receptors, such as PD-1 and CTLA-4, were present during IRIS, at levels higher than HIV-uninfected patients. Blocking of inhibitory receptors had minimal effects on the percentage of IFN-γ$^+$CD4$^+$ T cells at baseline. In contrast, CTLA-4 and LAG-3 blockade were associated with increases in the percentage of IFN-γ$^+$ CD4$^+$ T cells during IRIS, suggesting that some degree of immune checkpoint control was active during IRIS. Plasma IL-10 levels dropped during IRIS when compared to baseline, a finding also noted by other studies [36, 37]. IL-10 has immunoregulatory and anti-inflammatory effects and can directly suppress T-cell activation and cytokine production [38, 39]. This supported the hypothesis that regulatory and inhibitory signals, though present, might have been inadequate to balance the exaggerated inflammatory responses [40]. In addition to in vitro data, we also found higher percentage of TNF-expressing CD4$^+$ and CD68$^+$ myeloid cells at the sites of IRIS pathology (lymph nodes and BAL). Furthermore, increased levels of IFN-γ and CRP were found in plasma, consistent with published data on patients with TB-IRIS [21, 22, 36]. Inferential network analysis further supported the association between cytokine production by monocytes and CD4$^+$ T cells and plasma cytokine levels, suggesting that the exuberant CD4$^+$ T-cell responses seen after in vitro MAC stimulation may also be present in vivo and may potentially be the predominant driver of local and systemic immune activation and inflammation in MAC-IRIS.

It was surprising that the percentage of cytokine-producing CD4$^+$ T cells after in vitro MAC stimulation were low in HIV-uninfected patients with MAC infection. In HIV-uninfected patients, MAC infection was localized to the lungs; nonetheless, there was no evidence to suggest sequestration of TNF$^+$ CD4$^+$ T cells in the tissue samples collected. Furthermore, it is likely that the antigen load would have been quite different between HIV-uninfected patients with pulmonary MAC infection and

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Patient	Tissue	% of TNF+ CD4+ cells	% TNF+ CD68+ cells
HIV-1	LN	31	86
HIV-2	LN	8	77
HIV-3	LN	42	84
HIV-4	LN	35	36
HIV-4	BAL	50	90
HIV-5	BAL	0	48
HIV-6	BAL	33	100
non-HIV-1	BAL	0	41
non-HIV-2	BAL	0	11
non-HIV-3	Lung	11	43
non-HIV-4	Lung	9	3

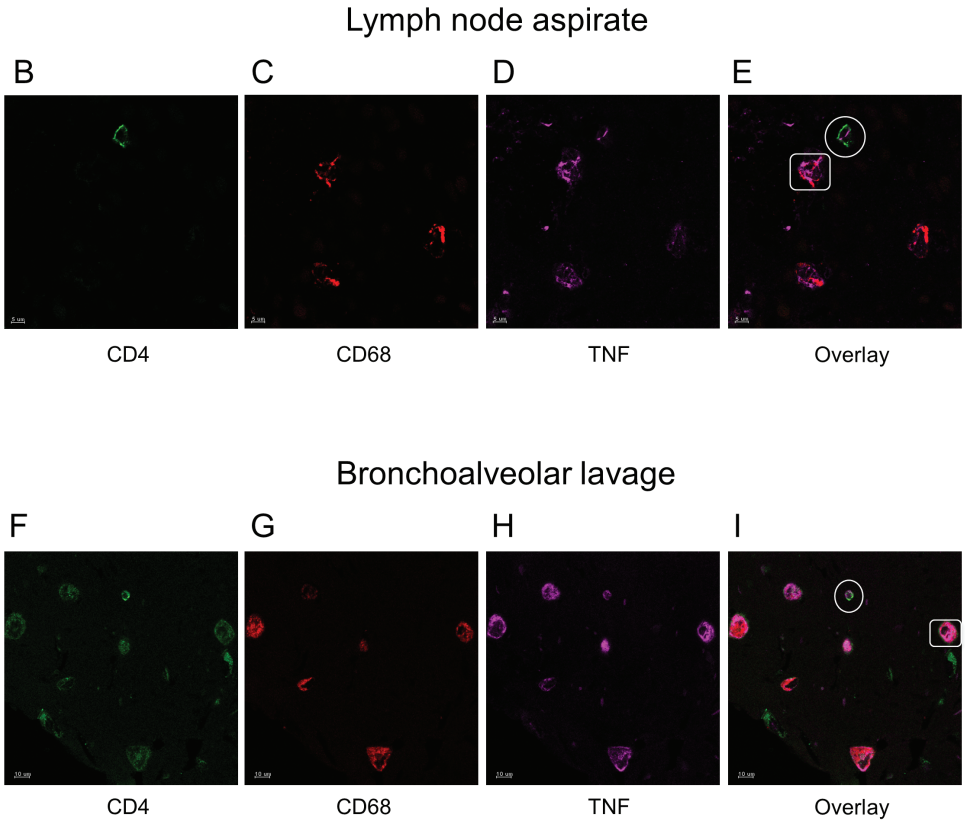

Figure 5. Evaluation of tumor necrosis factor (TNF) expression in tissue CD68$^+$ monocytes/macrophages and CD4$^+$ cells. Percentages of CD4$^+$ cells and CD68$^+$ monocytes/macrophages expressing TNF in lymph node (LN) aspirate, bronchoalveolar lavage (BAL), and lung fine-needle biopsy specimens from human immunodeficiency virus (HIV)–infected patients with *Mycobacterium avium* complex (MAC) immune reconstitution inflammatory syndrome and HIV-uninfected patients with MAC infection were evaluated by confocal microscopy (*A*). Representative images from LN aspirate (*B–E*) and BAL (*F–I*) showing CD4$^+$ (green) cells (*B* and *F*), CD68$^+$ (red) cells (*C* and *G*), TNF$^+$ (pink) cells (*D* and *H*), and overlay of the 3 colors (*E* and *I*). Examples of CD4$^+$ cells and macrophages expressing TNF were circled and boxed (*E* and *I*), respectively.

HIV-infected patients with disseminated MAC infection. On the other hand, reduced MAC-specific CD4$^+$ T cells might have contributed to the development of MAC infection in these patients given the critical role of IFN and TNF in the protection against MAC infection [41, 42]. These patients have been extensively investigated and no known immunodeficiencies were identified; the MAC infections did not disseminate and remained localized in the lung. It is also possible that myeloid responses (neutrophils, macrophages) may be playing a significant role as suggested by the high plasma MPO levels.

The use of HIV-uninfected patients with MAC infection as controls enabled the delineation of immune responses in IRIS from immune responses in active MAC infection without HIV infection. A limitation of this study was the lack of HIV/MAC coinfected patients who did not develop IRIS as controls as MAC infection in the absence of IRIS is uncommon in our cohort, though incidence in the literature has been variable [9, 43]. We also considered the use of HIV-infected patients without MAC infection as controls, but with only 1 in 6 individuals in the United States being sensitized [44], the absence

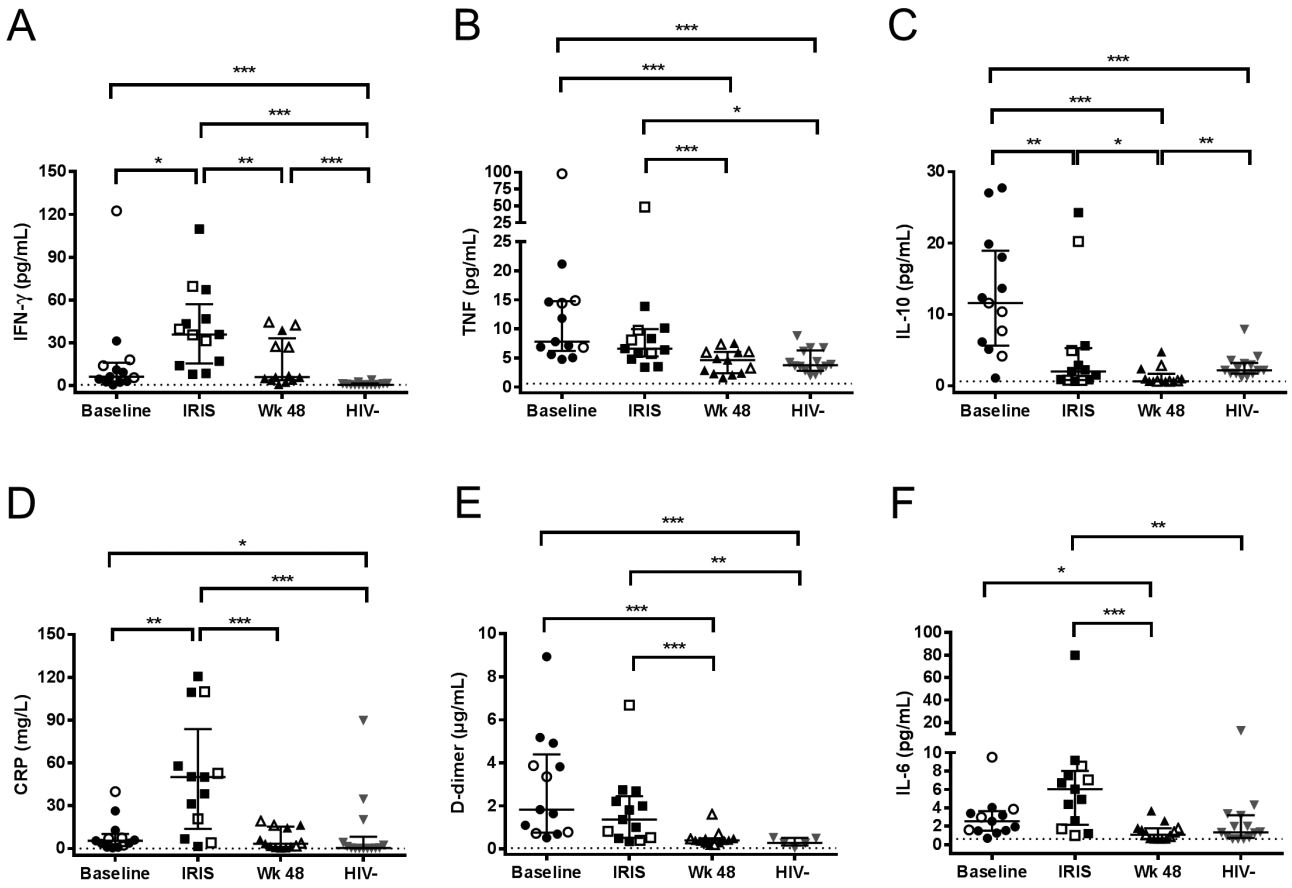

Figure 6. Plasma levels of cytokines and inflammatory markers in human immunodeficiency virus (HIV)–infected and HIV-uninfected patients with *Mycobacterium avium* complex (MAC) infection. Levels of plasma interferon gamma (IFN-γ; *A*), tumor necrosis factor (TNF; *B*), interleukin 10 (IL-10; *C*), C-reactive protein (CRP; *D*), D-dimer (*E*), and interleukin 6 (IL-6; *F*) were compared between the 3 time points (baseline, immune reconstitution inflammatory syndrome [IRIS], and week 48) among HIV-infected patients with MAC-IRIS using Wilcoxon signed-rank test. Comparisons between HIV-infected patients and HIV-uninfected patients with MAC infection were made using Mann-Whitney test. Lines represent median and interquartile ranges, filled symbols are data from patients with unmasking MAC-IRIS, open symbols are data from patients with paradoxical MAC-IRIS, and dotted lines represent lower limit of detection of the marker assayed. *$P < .05$, **$P < .01$, *** $P < .001$.

of MAC-specific $CD4^+$ T cells could be an effect of lack of exposure.

Although this study had a relatively small sample size, it represents the largest cohort of MAC-IRIS patients with prospective study design, long-term follow-up, and PBMC collection. We studied patients with unmasking and paradoxical MAC-IRIS, although patients with paradoxical MAC-IRIS only received MAC therapy for 3–7 days prior to ART initiation and thus the antigen load at ART initiation was probably not substantially different. Furthermore, our data revealed no obvious differences in results between unmasking and paradoxical IRIS.

In conclusion, MAC-IRIS is characterized by the restoration of TNF^+ monocytes and exuberant cytokine-producing $CD4^+$ T cells in vitro after stimulation with MAC, inflammatory cytokine production at the tissue sites of IRIS pathology, and systemic inflammation. We thus postulate that HIV infection with profound immunodeficiency leads to impairment in cytokine production by monocytes and macrophages, resulting in an inability to effectively control MAC infection, leading to accumulation of antigens [2]. Vigorous restoration of MAC-specific T-bet^{low} cytokine-producing $CD4^+$ T cells with cytotoxic potential in consort with monocyte/macrophage responses ensues after ART initiation, overwhelming available regulatory and inhibitory mechanisms and leading to IRIS.

Supplementary Data

Supplementary materials are available at *Clinical 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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