Selective Expansion of Polyfunctional Pathogen-Specific CD4+ T-cells in HIV-1-Infected Patients with Immune Reconstitution Inflammatory Syndrome

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This work was presented in part in an abstract format at the Conference on Retroviruses and Opportunistic Infections, February 2010, San Francisco CA.
Abstract

Since the introduction of highly active antiretroviral therapies (ART) the prognosis for HIV-1 patients has improved immensely. However, approximately 25% of patients can experience a variety of inflammatory symptoms that are collectively known as immune reconstitution inflammatory syndrome (IRIS). Studying the etiology and immunopathology of IRIS has been hampered by the fact that the symptoms and associated opportunistic infections (OI) are highly varied. We hypothesized that there is a common mechanism underlying IRIS pathogenesis and investigated a patient group with IRIS related to different pathogens. Functional and phenotypic characterization of PBMC samples was performed by polychromatic flow cytometry following in vitro stimulation with relevant antigenic preparations. In most patients, IRIS events were characterized by the robust increase of pre-existing polyfunctional, highly differentiated effector CD4⁺ T-cell responses that specifically targeted the antigens of the underlying co-infection. T-cell responses to HIV-1 or other underlying infections were not affected and did not differ between IRIS and non-IRIS patients. These data suggest that patients with IRIS do not have a generalized T-cell dysfunction; instead, IRIS represents a dysregulated CD4⁺ T-cell response against residual OI antigen. These studies were registered at www.clinicaltrials.gov as #NCT00557570 and #NCT00286767.

Keywords: Immune reconstitution inflammatory syndrome, ART, antigen-specific T-cells, mycobacteria, Th1 cytokines
Introduction

Immune reconstitution inflammatory syndrome (IRIS) is a severe clinical complication that manifests in approximately 25% of HIV-1 patients initiating highly active antiretroviral therapy (ART). Previous studies showed that the incidence of IRIS is higher in patients with advanced immunodeficiency who also have underlying opportunistic infections (OI). With ART becoming more widely available in populations with a high incidence of OIs, the occurrence of IRIS is likely to further increase in the near future. Thus, a better understanding of the immunopathology and identification of predictive biomarkers are of great clinical importance in order to develop targeted interventions.

Different types of IRIS have been identified, including “unmasking” IRIS, defined as a previously sub-clinical, undiagnosed OI occurring shortly after ART initiation, which cannot be explained by a *de novo* infection; “paradoxical” IRIS, defined as a worsening of a known condition for which the patient has been treated successfully (microbial cultures are often negative); and “autoimmune” IRIS, encompassing immune responses to self-antigens. This last category has received less attention in the literature, though there have been several reports of Graves’ disease associated with IRIS. In all three types, there is inflammation occurring during immune reconstitution that cannot be explained by drug toxicity or *de novo* infection. IRIS is largely a diagnosis of exclusion, but some criteria have been outlined by the AIDS Clinical Trials Group, as well as the International Network for the study of HIV-associated IRIS (INSHI).

The immunopathogenesis of IRIS remains unclear. IFN-γ-producing T-cells appear to be the main players driving the dysregulated inflammatory response but these findings are not consistent with those of other groups. In a study of *Cryptococcus*-associated IRIS, paucity of immune responses prior to ART appeared to predispose to
IRIS, possibly due to defective antigen (Ag) clearance and accumulation. Severely immunocompromised patients who are ART-naïve at OI diagnosis and those with low CD4 counts appear to be most susceptible to IRIS. Other risk factors include high HIV-1 RNA levels pre-ART, a more rapid initial HIV-1 RNA suppression, a high OI-derived antigen load at the time of ART initiation, a short time interval between diagnosis and/or treatment start of an OI and ART initiation, and a low body-mass-index. Nonetheless, reports are not consistent, and it has been suggested that risk factors might differ by mode of IRIS presentation and etiology.

Most studies to date trying to elucidate potentially diagnostic risk factors and/or biomarkers of IRIS have focused on patient groups with a particular opportunistic infection, such as Mycobacterium tuberculosis, Mycobacterium avium and intracellulare complex, Cryptococcus neoformans, or CMV. However, we hypothesized that there might be some common mechanisms underlying the development of IRIS in HIV-1 patients on ART, despite the highly variable clinical manifestations and variety of agents that can be associated with the syndrome.

We recently reported elevated frequencies of PD-1+ cells pre-ART, both within the CD4+ and CD8+ T-cell compartments, showing evidence of antigenic stimulation and a propensity for Th1 cytokine production. It was unclear whether the increased T-cell activation was due to HIV-1, the IRIS-associated pathogen or other co-pathogens. We addressed this question using a cohort of HIV-1-infected IRIS patients with variable IRIS-causing OIs, and HIV-1-infection contemporary controls. In order to exclude the baseline CD4+ T-cell count and persistent HIV-1 viremia as factors influencing outcome, only patients with <100 CD4+ cells/μl at baseline and <50 HIV-1 RNA copies/ml after one year of treatment were included in the present study. We found that most IRIS patients manifest an upsurge of pre-existing Ag-specific CD4+ T-cell responses that are
polyfunctional and are targeting exclusively the IRIS-associated pathogen and not HIV-1 itself or other co-infections.
Patients, Materials and Methods

Human Subjects and Sample Collection

67 HIV-1-infected subjects were enrolled and provided written informed consent at the Clinical Center of the National Institute of Allergy and Infectious Diseases, NIH, under an Institutional Review Board (IRB) approved protocol. Patient inclusion criteria have been described previously \(^{24}\). Briefly, all patients (1) were ART-naïve or had interrupted treatment for at least one year (n=4, plus n=2 who had previously received brief mono- or dual-therapy; one of these was an IRIS patient) with a viral rebound of >10,000 copies/ml, (2) had ≤100 CD4\(^+\) T-cells/\(μl\) at baseline, (3) suppressed their HIV-1 viral load to <500 copies/ml within one year of ART, and (4) had available PBMC samples taken pre-ART as well as after 1, 3, 6 and 12 months of ART. 19 patients developed IRIS episodes following commencement of ART, while 48 underwent uneventful immune reconstitution (Table 1). IRIS was defined according to the AIDS Clinical Trials Group criteria \(^8\). The median time to IRIS was 1.4 months (IQR 0.9-2.6).

Baseline characteristics, use of non-nucleoside reverse-transcriptase inhibitors (NNRTI) or protease inhibitors (PI), and ethnicity distribution was comparable between the IRIS and non-IRIS groups; the frequency of co-infections was more elevated in IRIS patients (Table 1). A subset of this cohort has been previously described in detail \(^{24}\).

Ethical Approval

All patients provided written informed consent prior to inclusion in accordance with the Declaration of Helsinki, and the study was performed according to an NIAID Institutional Review Board (IRB) approved protocol.
Determination of Plasma Viral Load, CD4+ and CD8+ T-cell Counts

Plasma HIV-1 viral loads (PVL), as well as CD4+ and CD8+ T-cell counts were determined in a Clinical Laboratory Improvement Amendment (CLIA) approved laboratory. The plasma viral load was measured using the ultrasensitive Quantiplex HIV-1 bDNA version 3.0 (Bayer).

CD4+ and CD8+ T-cell counts were determined by four-colour flow cytometry! The BD Multitest (BD Biosciences) that was used includes the following Abs: CD3FITC (clone SK7), CD4APC (clone SK3), CD8PE (clone SK1) and CD45PerCP (clone 2D1). Samples were acquired on a FACSCanto (BD Biosciences). CD4+ T-cell counts were calculated as % CD4+ CD3+ cells within CD45+ lymphocytes divided by 1% of the white blood cell count. The corresponding calculation was performed for CD8+ T-cell counts.

Sample Preparation and Ag-stimulation

Cryopreserved PBMC were thawed in pre-warmed RPMI 1640, 10% FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco; this medium will hereafter be referred to as complete RPMI), in the presence of 20 μg/ml benzonase nuclease (Novagen). Cells were rested in complete RPMI for 4-6 hours at 37°C, 5% CO2 and either left unstimulated (mock control) or stimulated overnight in 200 μl RPMI complete with different Ag-preparations (Table S1): 2 μg/ml CMV pp65 peptide pool, 2.5 μg/ml HBV surface Ag peptide pool, 0.8 μg/ml HHV-8 K12 and K15 peptide pool, 2.5 μg/ml HIV-1 Gag peptide pool (NIH AIDS Research and Reference Reagent Program, Germantown MD, USA), protein pool of HPV6 (1.6 μg/ml), HPV11 (3.2 μg/ml), HPV16 (3.2 μg/ml) and HPV18 (1.6 μg/ml) (Gardasil, Merck), 5.7 μg/ml JC Virus VP1 peptide pool, 2.5 μg/ml VZV IE62 peptide pool (New England Peptide), 5 μg/ml MAC bacterial lysate, 2.5 μg/ml TB PPD (Statens Serum Institute,
Denmark), 25 μg/ml *Cryptococcus neoformans* secreted mannoprotein \(^{29}\), 5 μg/ml *Histoplasma capsulatum* cell wall extract \(^{30}\), or 100 μg/ml *Strongyloides stercoralis* sodium deoxycholate (DOC)-soluble proteins from parasite lysate\(^{31,32}\). Stimulation cultures contained anti-CD49d and anti-CD28\(^{\text{PE-Cy5}}\) Ab (BD Biosciences). Monensin and Brefeldin A (BD Biosciences) were added after 2 hours of stimulation. Healthy donor PBMC were stimulated with SEB (Sigma) to serve as a positive control.

**Flow Cytometry**

The reagent panel used in the present study was developed based on that described by Mahnke and Roederer \(^{33}\) and is given in Table S2. The following reagents were used: anti-CD3\(^{\text{APC-Cy7}}\) (clone SK7), anti-CD14\(^{\text{PacBlu}}\) (clone M5E2), anti-CD28\(^{\text{PE-Cy5}}\) (clone CD28.2), anti-IL-2\(^{\text{APC}}\) (clone MQ1-17H12) (BD Pharmingen), anti-IFN-γ\(^{\text{Ax488}}\) (clone B27; Reametrix), anti-CD31\(^{\text{PE-Cy7}}\) (clone WM59), anti-PD-1\(^{\text{biotin}}\) (clone EH12.2H7) (BioLegend), anti-CD127\(^{\text{PE-Cy5.5}}\) (clone R34.34; Immunotech Coulter), anti-TIM-3\(^{\text{PE}}\) (clone 344823; R&D Systems), and streptavidin\(^{\text{QD605}}\) (Invitrogen). Anti-CD4 (clone M-T477), anti-CD7 (clone M-T701), anti-CD8 (clone RPA-T8), anti-CD19 (clone HIB19), anti-CD27 (clone M-T271), anti-CD45RO (clone UCHL1), anti-CD57 (clone NK-1), anti-TNF (clone MAb11) (BD Pharmingen) and anti-CCR7 (clone 150503; R&D Systems) were conjugated in-house to QD800, QD705, QD585, pacific blue, QD655, QD545, QD565, Ax594, Ax680 (Invitrogen), respectively. Dead cells were detected with the live/dead fixable violet dead cell stain (Molecular Probes, Invitrogen). For intracellular staining, cells were treated with BD Cytofix/Cytoperm Permeabilization Solution (BD Biosciences). Data were acquired on an LSR II (BD Biosciences).

**Data Analysis**
Data were analyzed using FlowJo (Tree Star), Pestle (by M Roederer) and SPICE 5.1. The gating scheme is illustrated in Figure S3. All cytokine measurements were background subtracted, taking into account the frequency of cells producing cytokines in the absence of antigenic stimulation (mock control). For the phenotypic analysis of Ag-specific cells, only those samples with >10 cytokine-positive events and response magnitudes >3x that of the corresponding mock control were considered.

Statistical Analysis

A two-tailed t-test was used to compare the proportion of individuals using steroids between the two patient groups. Nonparametric tests were used for all other analyses (SAS version 9.2). Changes from baseline within each group (paired differences) were evaluated using the Sign test. Data comparisons of single measurements between groups (IRIS vs. non-IRIS vs. other IRIS) were performed using the Wilcoxon rank-sum test. Statistical comparisons of pie charts were performed in SPICE 5.1 software using 10,000 permutations. Given the exploratory nature of this study, there was no adjustment for multiple comparisons.
Results

Longitudinal Analysis of Total CD4+ and CD8+ T-cells During ART

Since IRIS episodes were experienced at different timepoints by individual patients, we defined time ranges for IRIS patients synchronized to the initiation of ART and their respective IRIS events (Table S3).

We first investigated the evolution of T-cell activation phenotypes and T-cell differentiation stages over the course of ART in IRIS and non-IRIS patients to determine whether these groups experienced differences in the overall reconstitution of T-cells during ART. Some of the phenotypes were previously investigated using different aliquots of a subset of these samples. Here we significantly extended those studies, synchronizing samples to the IRIS event (Table S3), and characterizing the pathogen-specific responses.

Even though statistically significant changes from baseline were observed over time in non-IRIS patients for many of the differentiation and activation markers investigated, comparable changes in IRIS patients were only rarely significant (Figure 1A-D). Nevertheless, no statistically significant differences were detected between the overall activation status or T-cell subset distribution of CD4+ T-cells between IRIS and non-IRIS patients over the course of the study, except for the previously described elevated frequencies of PD-1+ cells pre-ART in eventual IRIS patients (Figure 1A, C). PD-1+ cells were also elevated pre-ART in CD8+ T-cells, while the only statistically significant differences at the time of IRIS (tp3) were observed in the frequencies of CD28+ and CD127+ cells, both of which were decreased in IRIS patients (Figure 1B). Concordantly, a significantly more elevated proportion of highly differentiated (mainly TEM) CD8+ T-cells was found in IRIS patients during and early after (tp3-4) clinical IRIS
diagnosis (Figure 1D). Together, these data confirm our previous observations that IRIS events are associated with highly activated T-cells.

**Longitudinal Analysis of HIV-1-specific T-cell Responses During ART**

We then set out to determine whether the HIV-1-specific T-cell response varied between the two patient groups. No significant differences were observed in terms of response magnitude, cytokine pattern or T-cell subset distribution within HIV-1 Gag-specific cells in either CD4+ (Figure 2A, 3A-B) or CD8+ T-cells (Figure 2B, 3C-D) at any of the timepoints analyzed. The same was true for the activation phenotype of HIV-1-reactive CD4+ or CD8+ T-cells in IRIS and non-IRIS patients (p>0.2 for CD28, CD31, CD57, CD127, PD-2 and TIM-3). Statistically significant decreases were observed over time in the magnitude of the HIV-1-specific CD8+ T-cell response after ART initiation in both patient groups (Figure 2B). Though the relative frequency of IFN-γ+ IL-2− TNF− cells decreased (Figure 3A) and that of T_{TM} cells among HIV-1-specific CD4+ T-cells increased (Figure 3B) increased over time in non-IRIS patients, the changes from baseline were not statistically different between patient groups.

**Elevated CD4+ T-cell Responses to Relevant Ags in Patients With Mycobacterial- or Fungal-associated IRIS Events**

In order to obtain sufficient patient numbers to perform statistical analyses, the results of stimulations with mycobacterial (MAC, TB) or fungal (Cryptococcus, Histoplasma) Ags were grouped together, as similar trends were observed when analyzing the Ags separately (Figure 4A-D). The magnitude of the CD4+ T-cell response was significantly elevated in IRIS patients experiencing episodes associated with mycobacterial or fungal infections compared to samples from non-IRIS patients, starting
at clinical IRIS diagnosis and lasting throughout follow-up (Figure 4E). Overall, the magnitude of these responses was significantly increased during IRIS (tp3) compared to pre-ART (tp1) in most IRIS patients. Patients with “other IRIS” events included patients with MAC-associated IRIS whose PBMC were also tested for reactivity to TB, which explains the slight increase in CD4+ T-cell responses at tp3 as PBMC from MAC+ individuals often show some measure of cross-reactivity to the TB Ag preparation (PPD) used. Nevertheless, CD4+ T-cell responses to the IRIS-associated Ag were significantly higher at later timepoints (tp4-5; Figure 4E) compared to non-associated mycobacterial or fungal Ags (other IRIS). The change from baseline (tp1) to tp3 was significantly different between patients with mycobacterial or fungal IRIS and non-IRIS patients (p=0.001), as well as other IRIS patients (p=0.012). Differences in changes from baseline to tp4 (p=0.002) and tp5 (p=0.009) were also found to be statistically significant between IRIS and non-IRIS patients.

Within patients with TB-associated IRIS, one patient exhibited elevated IRIS-Ag-specific CD4+ T-cell responses at the sampling timepoint closest after clinical IRIS diagnosis (tp3), while in the other one the response was similar compared to pre-ART, rising again at a later time point (Figure 4A). This could be due to the timing of PBMC sampling (23 versus 6 days after the onset of IRIS event), which could be indicative of variation of biologic phenomena or tissue redistribution of TB-specific CD4+ T-cells.

The patient with Histoplasma-associated IRIS experienced concomitantly a MAC-IRIS event (cervical lymphadenopathy) and we cannot exclude that the IRIS occurrence was mainly driven by MAC. However, since fungal elements were detected in the biopsy material, this IRIS event had been formally classified as being driven by both OIs. In this patient (Patient 56) the IRIS-associated Histoplasma-specific response
demonstrated but a 1.3-fold increase from pre-ART (tp1), whereas the MAC-associated response increased 14.8-fold in the same time span (Figure 4D, Fig.5A).

IRIS patients also demonstrated a significantly higher fraction of polyfunctional IFN-γ+ cells during the IRIS episode than non-IRIS patients at a comparable sampling timepoint (Figure 4F), as well as a larger proportion of T_EM cells (Figure 4G) among CD4+ T-cells reactive to the IRIS-associated Ag. However, no marked differences were observed in their activation phenotypes (Figure 4H).

Unusually Vigorous CD4+ T-cell Responses to the IRIS-associated Ag During IRIS Episodes

When we examined the evolution of CD4+ T-cell responses to different Ags in individual IRIS patients, we found dramatic increases only to the IRIS-associated Ag (with the exception of TB in MAC-IRIS patients due to the poor specificity of PPD) (Figure 5A)\textsuperscript{35}. A comparable analysis of non-IRIS patients did not reveal any significant changes in CD4+ T-cell responses over time including pathogens the subjects were known to have been infected with (Figure 5B).

CD8+ T-cell responses to the relevant Ag of IRIS patients with mycobacterial- or fungal-associated IRIS episodes did not show corresponding dramatic increases (Figure S2A-D) as observed in the CD4+ subset (Figure S1A-D), but that could partially be due to the predominant use of proteins as stimulants. Interestingly though, even in a case where IRIS was linked to CMV, a virus inducing both robust CD4+ than CD8+ T-cell responses\textsuperscript{36}, CMV-specific CD4+ T-cells were substantially boosted in frequency (Figure S2A) while CMV-specific CD8+ T-cells showed only a moderate increase during the IRIS episode (Figure S2B), even though a peptide pool was used for stimulation. Notably, both CD4+\textsuperscript{37} and CD8+\textsuperscript{38} T-cell responses are thought to be pivotal in controlling JCV. The
patient with JCV-associated unmasking IRIS did not produce any detectable JCV-specific CD4+ T-cell responses before the IRIS episode; proximally to the IRIS event, over 2% of total CD4+ T-cells were JCV-specific (Figure S2C, open symbol at tp4: 2.3 months after clinical IRIS diagnosis). JCV-specific CD8+ T-cell responses demonstrated a vigorous increase directly after commencing ART that dampened early during the IRIS event and increased again thereafter (Figure S2D). This indicates an involvement of CD8+ T-cells in the pathogenesis of PML-IRIS although the increase in the CD8+ T-cell response during the IRIS event (tp3 to tp4) was lower in magnitude (2.1 fold) than the corresponding delayed CD4+ T-cell response (-0.3% to 2.1%). These data underscore a possible significant involvement of CD4+ T-cells in IRIS pathogenesis in some cases linked to viral pathogens.

Other IRIS-associated OI-specific T-cell responses were investigated (HBV, HHV-8, HPV, Strongyloides, VZV), but did not yield conclusive data.

Finally, proportionately more IRIS patients (26%) received glucocorticosteroid treatment directly pre-ART or within the first 6 months of ART compared to patients with uneventful immunoreconstitution (6.3%) (p=0.0223). Such treatment could reduce the overall magnitude of cytokine-responses measured. Notably, no significant changes were observed in HIV-1-specific CD8+ T-cell responses, while CD4+ T-cell responses were only moderately reduced in glucocorticosteroid-treated patients after 3 (p=0.013) and 12 months (p=0.035) of ART. This indicates that the differences observed between IRIS and non-IRIS patients were not the result of glucocorticosteroid treatment.
Discussion

In this report we show that, in the majority of cases, IRIS events are characterized by the expansion of highly differentiated, polyfunctional CD4⁺ T-cell responses directed against the underlying IRIS-associated infection. This exuberant CD4⁺ T-cell response is restricted to the OI-specific compartment, as T-cell responses directed against HIV-1 or other co-infections did not differ between IRIS and non-IRIS patients. Thus, IRIS does not appear to either be due to, or result in, a global T-cell defect.

Early reports focusing on viral infections correlated the development of IRIS to the frequency or cell count of CD8⁺ cells 39,40. However, in our study the CD4⁺ T-cell response to the IRIS-associated Ag was significantly boosted during the IRIS event, while CD8⁺ T-cell responses were only moderately affected, if at all, suggesting that IRIS occurs primarily due to a hyper-activation of CD4⁺ T-cells. This was true even for CMV, to which strong CD8⁺ T-cell responses are typically observed: during CMV–associated unmasking IRIS, a far more dramatic amplification occurred in the CD4⁺ T-cell compartment.

In previous studies, IRIS was linked to increased IFN-γ⁺ 10,11,19,41, IL-2⁺ 11 and TNF⁺ 19 T-cell responses, especially in the case of TB-associated IRIS. Since some of these studies have been based on total PBMC ELISpot data, the association might have been underestimated, as CD4⁺ T-cells appear to be the main players in the immunopathology of IRIS. In agreement with previous studies 10,11,19, we observed an exuberant CD4⁺ T-cell response to the IRIS-associated Ag. In the case of mycobacterial and fungal Ags, the response was significantly more elevated than in non-IRIS patients or in individuals experiencing IRIS events related to other causative agents. The most important observation in terms of cytokine-producing CD4⁺ T-cells appears to be the change in IFN-γ⁺ cells from baseline to the IRIS timepoint: a significant increase was
detected in mycobacterial/fungal-IRIS patients, resulting in increased proportions of polyfunctional cells (IFN-γ+ IL-2+ TNF+ and IFN-γ+ IL-2− TNF+), while in non-IRIS patients the proportion of IFN-γ+ (mainly single positive) cells decreased during the same time frame. Furthermore, as previously reported for TB-IRIS patients 19, we found that the frequency of TEM cells among CD4+ T-cells reactive to the IRIS-associated OI was dramatically increased at the time of IRIS. Thus, the functional and phenotypic analyses support the conclusion that highly activated cells (polyfunctional, late differentiation stage) are enriched in IRIS patients’ CD4+ T-cells specific to the IRIS-OI-relevant Ag during clinical episodes. This was particularly evident in cases of mycobacterial and cryptococcal IRIS. However, no predictive alteration in Ag-specific T-cell responses was identified at baseline, and there was no apparent (antigen-specific or general) T-cell defect pre-IRIS.

The dysregulated inflammatory immune responses occurring during IRIS have been hypothesized to stem from a combination of poor clearance of OI-related Ags due to HIV-1 co-infection 14 and a reversal of the HIV-1-induced CD4+ T-cell suppression upon commencing ART 42. In this context, TB-specific CD4+ T-cell responses rapidly decrease upon HIV-1 infection 43, which could contribute to the lack of Ag-clearance. Furthermore, progressive multifocal encephalopathy (PML) was shown to occur in HIV-1− individuals after withdrawal of unrelated immunosuppressive treatment 44. This, as well as other examples of paradoxical inflammatory reactions 45, demonstrates that IRIS-like phenomena can occur independently of lymphopenia and may instead be solely driven by changes in the activation status of T-cells and their abrupt recovery from immunosuppression in the presence of accumulated residual Ag of a partially treated (paradoxical) or untreated (unmasking) infection.
We did not identify any phenotypic alterations at the time of IRIS in either total or Ag-specific (to HIV-1 or the IRIS-associated OI) CD4+ T-cells. In contrast, total CD8+ T-cells of IRIS patients demonstrated delayed recovery of CD28 and CD127 expression, which is likely a result, rather than a cause, of the inflammatory response occurring during IRIS. However, PD-1 expression was elevated pre-ART in IRIS patients both on CD4+ and CD8+ T-cells and many of those cells expressed the co-stimulatory ICOS and inhibitory CTLA-4 and LAG-3 molecules. Together, these data are consistent with a high level of activation and a potentially reduced functionality of CD4+ T-cells in vivo pre-ART in IRIS patients.

Though altered levels of regulatory T-cells (T_{REG}) have not been reported in IRIS patients, it has been proposed that the impairment of T_{REG} to suppress proliferation of responder T-cells reported during IRIS events could at least in part explain the exuberant inflammatory T-cell responses observed in IRIS patients. However, our finding that T-cell responses against other pathogens than the IRIS-associated one were not significantly altered is inconsistent with this hypothesis.

In summary, our data suggest a common mechanism for the development of many IRIS occurrences that is independent of the associated opportunistic infection, whereby rapidly expanded Ag-specific CD4+ T-cells mount a polyfunctional inflammatory response to residual Ag of previously existing and inadequately controlled opportunistic infections once the HIV-1-induced functional inhibition is reversed by ART.
Acknowledgments

We thank Joanne Yu for Ab conjugations and titrations, Jessica Hodge for assistance in panel development, Catherine Rehm and JoAnn Mican for help with PBMC samples, Brian O Porter for providing clinical information, and Drs. Joe Casazza, Guislaine Carcelain, Gene Major, Molly Perkins, Daniel Douek, Stuart Levitz, George Deepe and David Abraham for their generosity in sharing their reagents. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, NIH.

Authorship

Contribution: IS, AS, MR, YDM and LRVA developed the study concept and experimental design. IS and GR were responsible for clinical supervision of patients and PBMC sampling. Experiments and data analyses were performed by YDM and JHG, while statistical analyses were done by RDS and YDM. The manuscript was written by YDM and IS. All authors were involved in manuscript review and editing.

Conflict-of-interest disclosure: The authors have no financial or other conflicts of interest.

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References


8. Network. ADoIRISIACTG.


Table 1. Patient Cohort Characteristics

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<th>IRIS</th>
<th>non-IRIS</th>
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<tr>
<td>N</td>
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<td>48</td>
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<tr>
<td>Age at ART initiation*</td>
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<td>CD8+ cells / μl*</td>
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<td>52.1</td>
</tr>
<tr>
<td>PI</td>
<td>31.6</td>
<td>47.9</td>
</tr>
<tr>
<td>Ethnicity, %</td>
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<tr>
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<td>47.9</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Caucasian</td>
<td>15.8</td>
<td>20.8</td>
</tr>
<tr>
<td>mixed</td>
<td>10.5</td>
<td>12.5</td>
</tr>
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</table>

* median (inter-quartile range); PVL – plasma viral load; § p – paradoxical IRIS, u – unmasking IRIS; ¶ only those OI associated to IRIS events in this study were considered; # p=0.0138 (none of the other parameters were statistically different between patient groups; individual infections were not tested for statistical significance); NNRTI – non-nucleoside reverse-transcriptase inhibitors; PI – protease inhibitors.
Figure Legends

Figure 1  Longitudinal analysis of total CD4+ and CD8+ T-cell phenotypes of IRIS and non-IRIS patients during ART. Characteristics of total CD4+ (A, C) and CD8+ T-cells (B, D) were analyzed in PBMC sampled from IRIS (red) and non-IRIS patients (blue) before as well as after 1, 3, 6 and 12 months of ART. (A, B) Activation phenotype and presence of recent thymic emigrants (RTE; in CD4+ T-cells only). (C, D) Representation of T-cell differentiation states. T-cell differentiation subsets were defined by expression of CD45RO (“RO”), CCR7 (“R7”) and CD27 (“27”). T_{NV} – naïve; T_{CM} – central memory; T_{TM} – transitional memory; T_{EM} – effector memory; T_{TE} – terminal effector. T_{CM*}, T_{TM*} and T_{TE*} are phenotypically-defined populations that are not described in the literature, but that arise by this gating scheme; their activation phenotype and cytokine potential most closely resembles that of T_{CM}, T_{TM} and T_{TE}, respectively, hence their nomenclature. Graphs show interquartile ranges, median bars, as well as individual data points. Grey boxes highlight the first timepoint within 3 months of clinical manifestation of IRIS, while dashed lines separate pre-ART from on-ART samples. All timepoints were compared between patient groups (results indicated in black above bars/pies), as well as to corresponding pre-ART measurements within each patient group (results are color-coded and indicated below bars/pies): * p<0.01, ** p<0.001, *** p<0.0001.

Figure 2  The Magnitude of HIV-1 Gag-specific T-cells Does Not Differ Significantly Between IRIS and Non-IRIS Patients. The total response magnitude, measured by production of IFN-γ and/or IL-2 and/or TNF, generated by HIV-1 Gag
reactive CD4+ (A) and CD8+ T-cells (B) of IRIS and non-IRIS patients were compared at the five analysis timepoints. Graphs show interquartile ranges, median bars, as well as individual data points. Dashed lines separate pre-ART from on-ART samples, while grey boxes highlight samples from IRIS patients within 3 months of clinical IRIS onset. All timepoints were compared between patient groups (no statistically significant differences found) and to corresponding pre-ART measurements within each patient group (red asterisks: IRIS; blue asterisks: non-IRIS; indicated below graphs): * p<0.01, ** p<0.001.

**Figure 3**  
Cytokine Pattern and Phenotype of HIV-1 Gag-specific T-cells Do Not Differ Significantly Between Patient Groups. HIV-1 Gag reactive CD4+ (A-B) and CD8+ T-cells (C-D) of IRIS and non-IRIS patients were compared at the five analysis timepoints. (A, C) Cytokine pattern. Relative proportion of total HIV-1 Gag-reactive cells producing each possible combination of the cytokines measured. (B, D) Differentiation state. Dashed lines separate pre-ART from on-ART samples, while grey boxes highlight samples from IRIS patients within 3 months of clinical IRIS onset. All timepoints were compared between patient groups and to corresponding pre-ART measurements within each patient group (no statistically significant differences found). Individual pie segments were also compared between timepoints within each patient group (indicated within relevant pie segments): * p<0.01, ** p<0.001. i.d. – insufficient data – not enough samples met inclusion criteria.

**Figure 4**  
Elevated CD4+ T-cell Responses to Relevant Ags in Patients With Mycobacterial- or Fungal-associated IRIS Events. CD4+ T-cell responses to TB, MAC, *Cryptococcus neoformans* and *Histoplasma capsulatum* were analyzed. For this purpose, patients were divided into three groups: those with IRIS events to
mycobacterial- or fungal-associated OI and stimulated with the relevant Ag (mycobacterial/fungal associated IRIS; red), those with IRIS events to other Ags, which can include mycobacterial/fungal Ags, but stimulated here with IRIS-irrelevant Ags (other IRIS; pink), and non-IRIS patients (non-IRIS; blue). Each data point represents stimulation with one Ag only. Shown are the response magnitudes by Ag: TB (A), MAC (B), Cryptococcus neoformans (C) and Histoplasma capsulatum (D), as well as grouped for all four Ags (E). A-D: Longitudinal data points are only connected for IRIS patients with IRIS manifestations associated to the given Ag. Cytokine pattern (F), T-cell subset distribution (G) and activation phenotype (H; tp3 only) of CD4+ T-cells reactive to TB, MAC, Cryptococcus neoformans or Histoplasma capsulatum were also determined. Grey boxes highlight the first timepoint within 3 months of clinical manifestation of IRIS, while dashed lines separate pre-ART from on-ART samples. All groups were compared within timepoints to the mycobacterial/fungal-associated IRIS group (color-coded asterisks above bars or pies), as well as to corresponding pre-ART measurements within groups (red asterisks: IRIS; blue asterisks: non-IRIS; indicated below graphs or pies). In (C), the number of samples per pie are indicated (see Materials and Methods; these also apply to data in (D)). i.d. – insufficient data; * p<0.05, ** p<0.01, *** p<0.001.

**Figure 5  During ART, Only Frequencies of CD4+ Cytokine-producing T-cells Specific to IRIS-associated Ags Increase Dramatically.** PBMC samples from five IRIS patients (A) and five non-IRIS patients (B) were stimulated with MAC, Histoplasma capsulatum, Cryptococcus neoformans, TB, CMV, JCV or HIV-1. The IRIS-associated opportunistic infection and other known infecting pathogens tested for in the present assays are indicated for each patient. The number of stimulations performed with each PBMC sample was determined by the number of cells available, and priorities were given
to those Ags that a given patient was known to have been exposed to. Grey boxes highlight the first timepoint within 3 months of clinical manifestation of IRIS, while dashed lines separate pre-ART from on-ART samples. T-cell responses to IRIS-associated Ags are indicated by bold lines. IRIS patients were selected for illustration if stimulation data were available for at least two Ags and at least four time-points. If two Ags fulfilled these criteria, all other stimulations with at least two data points were shown for that patient. Non-IRIS patients were selected according to the above criteria, as well as having known to be exposed to at least one of the Ags being tested for. 1 – tp1; 2 – tp2; 3 – tp3; 4 – tp4; 5 – tp5.
Figure 1

A. CD7, CD28, CD31, CD57, CD127, PD-1, TIM-3, RTE

B. CD7, CD28, CD31, CD57, CD127, PD-1, TIM-3

C. IRIS, non-IRIS

D. IRIS, non-IRIS

Figure 2

A. % of total CD4+ T-cells

B. % of total CD8+ T-cells

IRIS, non-IRIS
Figure 3

A

IRIS

non-IRIS

B

IRIS

non-IRIS

C

IRIS

non-IRIS

D

IRIS

non-IRIS

Figure 4

A

% of total CD4+ T-cells

B

% of total CD4+ T-cells

C

% of total CD4+ T-cells

D

% of total CD4+ T-cells

E

% of total CD4+ T-cells

F

% of total CD4+ T-cells

G

% of total CD4+ T-cells

H

% of total CD4+ T-cells

IFN-γ

IL-2

TNF

differentiation

Mycobacterial / Fungal IRIS

other IRIS

non-IRIS

Mycobacterial / Fungal IRIS

other IRIS

non-IRIS

% of total CD4+ T-cells
Figure 5

A

Patient 1
IRIS: Cryptococcus

Patient 55
IRIS: MAC

Patient 56
IRIS: MAC, Histoplasma

Patient 61
IRIS: MAC, Other: CMV enterocolitis

Patient 69
IRIS: CMV

% of total CD4+ T-cells

B

Patient 59
Cryptococcal meningitis

Patient 60
Disseminated histoplasmosis

Patient 83
CMV retinitis

Patient 93
CMV oesophagitis

Patient 99
TB

% of total CD4+ T-cells

Legend:
- CMV
- Cryptococcus
- Histoplasma
- HIV
- JCV
- MAC
- TB

Timeline:
1 2 3 4 5
Selective expansion of polyfunctional pathogen-specific CD4+ T cells in HIV-1–infected patients with immune reconstitution inflammatory syndrome

Yolanda D. Mahnke, Jamieson H. Greenwald, Rebecca DerSimonian, Gregg Roby, Lis R.V. Antonelli, Alan Sher, Mario Roederer and Irini Sereti