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Natural Killer cell activation distinguishes *M. tuberculosis*-mediated Immune reconstitution syndrome (IRIS) from chronic HIV and HIV-MTB co-infection

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Abstract

Background—With increased access to antiretroviral treatment (ART), Immune Reconstitution Inflammatory Syndrome (IRIS) in Mycobacterium tuberculosis (MTB)-infected populations remains a clinical challenge. We studied a cross-sectional cohort of HIV-infected subjects in Johannesburg (South Africa) to help define the immune correlates that best distinguish IRIS from ongoing MTB cases.

Methods—We studied HIV+ subjects developing MTB-related unmasking IRIS (u-TB-IRIS) after ART initiation; control groups were HIV subjects and HIV-TB co-infected subjects with comparable ART treatment. Testing was conducted with whole blood-based 4-color flow cytometry and plasma-based Luminex cytokine assessment.

Results—NK cell activation, C-reactive protein and IL-8 serum concentration were significantly higher in u-TB-IRIS subjects as compared to both control groups. In addition, all MTB co-infected subjects, independent of clinical presentation, had higher neutrophils and T cell activation, together with lower lymphocytes, CD4⁺ T cell and myeloid DC counts. Using conditional inference tree analysis we show that elevated NK cell activation in combination with lymphocyte count characterizes the immunological profile of u-TB-IRIS.

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Conclusions—Our results support a role for innate immune effectors in the immunopathogenesis of unmasking MTB-related IRIS, and identify new immune parameters defining this pathology.

Keywords

Mycobacterium tuberculosis; IRIS; HIV; immune reconstitution; natural killer cells; CD69

Introduction

Approaches to expand treatment of HIV infection in sub-Saharan Africa have been complicated by the high prevalence of *Mycobacterium tuberculosis* (MTB) infection^{1, 2}. According to WHO, 1.37 million people worldwide have HIV/tuberculosis, accounting for 456,000 deaths every year, almost 25% of all HIV-related deaths. In addition to the challenge of combined drug toxicity, simultaneous treatment of HIV and MTB co-infection often results in immune reconstitution syndrome (IRIS), an unanticipated aggravation of tuberculosis symptoms in a previously controlled subject (paradoxical IRIS) or in unmasking of a previously latent infection (unmasking IRIS or ART-associated TB)^{3, 4}. Recent studies support initiating HIV treatment in TB-infected persons 2 weeks before initiation of anti-TB treatment (CAMELIA study⁵), and in November 2009, WHO recommended treating patients with antiretrovirals “as soon as possible” within the first 2 months following the start of tuberculosis treatment¹. As the risk of IRIS has been shown to be increased when ART is initiated together or shortly after anti-MTB treatment, these recommendations are likely to result in an even higher incidence of TB-IRIS in sub-Saharan Africa.

The immunopathogenesis of IRIS remains unclear; in addition to the wide variety of conditions associated with IRIS (reviewed in⁶), growing evidence indicates that even in *M. tuberculosis* infection the pathogenesis of paradoxical and unmasking IRIS may differ significantly: Elliot et al.⁷ showed that IFN- γ responses to RD1 antigen were greater in unmasking IRIS, whereas skin test response to PPD was greater in paradoxical IRIS. Haddow et al.⁸ recently reported that, while CRP serum levels are typically elevated in all forms of IRIS, levels of MCP-1 and IL-10 are reduced in paradoxical TB-IRIS, whereas cases of unmasking TB-IRIS have higher pre-ART IFN- γ , as well as higher TNF- α at the time of event. Finally, ex-vivo stimulation with mycobacterial antigens was shown to induce higher levels of IL-18 and CXCL10 and lower CCL2 in subjects with paradoxical TB-IRIS, whereas in unmasking TB-IRIS only IL-18 was markedly elevated⁹, suggesting a prevalence of adaptive responses in unmasking TB-IRIS. Subjects with active MTB infection have been shown to have increased T-cell activation (CD3⁺/HLA-DR⁺ cells), as well as decreased production of IL-2, IL-4, IL-5 and IL-10 IFN- γ and TNF- α ¹⁰ and higher serum IL-6 and IP-10¹¹. Importantly, serum TNF- α is increased in HIV/MTB co-infection¹², which also results in higher serum levels of IL-12 and neopterin as compared to MTB alone¹³. Based on this knowledge, a number of studies have focused on the detection of serum factors associates with IRIS presentation, demonstrating increased serum levels of IFN γ IL-6 and TNF α in association with IRIS^(14, 15, also reviewed in 16).

A number of studies has assessed the contribution various cellular subsets to the pathogenesis of IRIS: increased antigen-specific responses in CD4⁺ Th1 cells, as well as KIR⁺ TCR $\gamma\delta$ ⁺ T cells¹⁷ and production of type 1 and pro-inflammatory cytokines¹⁸ have been reported in paradoxical TB-IRIS. Evidence for a pathogenetic role of regulatory T cells remains inconclusive^{19, 20}. In addition to triggering adaptive responses via TCR $\alpha\beta$ or $\gamma\delta$ ²¹, mycobacteria interact with cell surface receptors (e.g. in macrophages TLR2^{22, 23}, mannose receptor and complement receptor²⁴), increasing production of expression of nitric oxide²⁵

and TNF- α ²⁶. Markers associated with monocyte/macrophage function have been associated with IRIS presentation (e.g., IL-6 and related CRP production ²⁷) indicating that acute activation of innate effectors may be a distinct feature of IRIS ²⁸.

In this manuscript we present immune correlated of unmasking TB-IRIS, identifying innate immune parameters uniquely associated which may help diagnose and differentiate unmasking IRIS in HIV-TB co-infected control subjects.

Methods

Study subjects

Subjects were recruited at the Themba Lethu clinic, Johannesburg, ZA. All subjects received ART. Written informed consent was obtained for all participants; consent forms and procedures, as well as study protocol, were approved by the University of the Witwatersrand Ethics Committee and the Wistar Institute Institutional Review Board.

Study groups

107 subjects divided as follows

Group 1 - HIV control—58 HIV⁺ subjects; inclusion criteria: documented HIV infection, receiving first line ARV treatment for up to 3 months.

Group 2 - HIV/TB co-infection—31 HIV⁺ subjects with confirmed *M. tuberculosis* (MTB) infection and receiving first line ARV treatment for up to 3 months. These subjects were referred from surrounding primary TB clinics (site of first diagnosis) for initiation of ART and according to local guidelines after initiating anti-TB treatment.

Group 3 - uTB-IRIS—18 HIV⁺ subjects receiving first line ARV treatment for up to 3 months, and presenting with unmasking MTB-related IRIS at during scheduled or unscheduled follow-up visits, or referred from surrounding hospitals for treatment.

IRIS definition

Putative uTB-IRIS cases were classified based on a revision of the definition by Shelbourne et Al. ^{29, 30}. At protocol closure uTB-IRIS cases were reviewed according to the ACTG IRIS definition (2005 revision, summarized in supplemental table 1); all IRIS cases were observed in subjects with no prior known diagnosis of *M. tuberculosis* infection (“unmasking” IRIS). Subjects matching definitions 1–5 were classified as confirmed IRIS; subjects matching definitions 1–4 or 1, 2, 3 and 5 were classified as probable IRIS. Based on this assessment, uTB-IRIS diagnosis was classified as confirmed in 16 subjects, and probable in 2 subjects; 5 subjects were not confirmed and were not included in this analysis.

Variables assessed

A total of 96 variables was assessed:

Clinical variables—Treatment history, age, gender, ethnicity, weight were collected during the first visit. Full blood differential counts, CD4 and CD8 counts and blood chemistry were performed at the Department of Molecular Medicine and Hematology, University of the Witwatersrand, Johannesburg, ZA

Flow cytometry—Whole blood-based 4-color was performed at the same location using the following antibody panels: 1) T cell memory subsets: CD45RA, CD62L, CD3, CD4; 2)

T cell activation: CD38, CD28, CD3, CD4; 3) NK cells subsets: CD56, CD161, CD3, CD16; 4) NK cell activation: CD56, CD69, CD3, HLA-DR; 5) Plasmacytoid Dendritic cells (PDC): CD86, CD303/304, HLA-DR, CD197; 6) Myeloid Dendritic cells (MDC): CD1c, CD11c, CD19, CD197 *Soluble cytokines and acute phase reactants* were assessed in cryopreserved plasma using the following Invitrogen multiplex beads (Invitrogen, Carlsbad, CA) and analyzed on an Affimetrix Luminex platform (CFAR Immunology Core, University of PA). Multiplex 1: IL- β , IL-1R α , IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, TNF- α , IFN- α , IFN- γ , GM-CSF, MIP-1 α , MIP-1 β , IP-10, MIG, Eotaxin, RANTES, MCP-1, VEGF, G-CSF, EGF, FGF-basic, and HGF. Multiplex 2: human β 2 Microglobulin, Haptoglobin, CRP, and Gc Globulin.

Statistical analysis

We assessed differences across groups for the 96 variables tested using the Kruskal-Wallis (KW) test; adjustment for multiple testing was performed using the Benjamini Yekutieli false discovery rate (FDR) method³¹ on the complete set of variables; variables with FDR adjusted p-values < 0.1 were considered significant; for these variables, differences between each group were assessed using an unadjusted Wilcoxon rank sum test. A conditional inference tree (CIT) framework³² (R “party” package, <http://cran.r-project.org/web/packages/party/index.html>, ctree function) was used to characterize combinations of variables associated with HIV infected, TB-HIV co-infected with no IRIS and HIV-TB co-infected with IRIS. Binary splits of all variables were considered and retained if the associated Bonferroni-adjusted p-value was less than 0.05. All statistical analysis was performed using R ver. 2.10.0³³.

Results

Patient characteristics

HIV control group (Group 1)—Median baseline (pre-ART) CD4 count was 118 (IQR = 49.5, 176). At the time of the study blood draw median CD4 count was 221 (IQR = 159, 339); 25 subjects had detectable VL (median = 1250, IQR = 602, 3170). ART was 3TC + D4T (58) and EFV (52) or NVP (2) or EFV + NVP (4); one subject also received LPV/r. Median time on ART was 44.5 days (IQR = 28, 57); one subject was a protocol deviation with treatment > 90 days. Median age was 34.5 (IQR = 31.25, 42), gender distribution was 76% female.

TB/HIV control group (Group 2)—ART was 3TC (31) + D4T (30) or AZT (1) and EFV (30) or EFV+NVP (1); median time on ART was 31 days (IQR = 27.5, 56.5). Median baseline (pre-ART) CD4 count was 60.5 (IQR = 11, 110). At the time of the study blood draw median CD4 count was 101 (IQR = 75, 220); 13 subjects had detectable VL (median = 4300, IQR = 1950, 20200). Median anti-TB treatment duration was 42.5 days (IQR = 27.75–64.25); all subjects were treated with a standard 4-drug regimen (Rifampin, Isoniazid, Pyrazinamide, and Ethambutol) for 8 weeks from diagnosis, followed by 2-drug (Rifampin, Isoniazid) maintenance for 16 weeks. Median age was 32 (IQR = 29.5, 37.5), gender distribution was 45% female.

Unmasking TB-IRIS group (uTB-IRIS, Group 3)—A detailed documentation of CD4 count, viral load, clinical presentation, diagnosis parameters and follow-up for this group is presented in Table 1; briefly, median baseline (pre-ART) CD4 count was 115 (IQR = 41, 145); as many baseline VL assessment could not be retrieved from the referring clinics, we interpreted all VL < 400 as fulfilling the requirement for virological response to ART. At the time of the study visit, the median CD4 count was 154 (IQR = 65.5, 231.5) and 6 subjects had detectable VL (median = 605, IQR = 278.5, 972.75). ART was 3TC (18) + D4T (17) or

AZT (1) and EFV (18). Additionally, subjects received TDF (4), FTC (3) or NVP (3). Median time on ART was 55 days (IQR = 31, 63.5); 2 subjects were protocol deviations with ARV treatment > 90 days. Median age was 38 (IQR = 30.5, 44), gender distribution was 70% female. 13 subjects had been hospitalized at the time of diagnosis; 11 subjects had pulmonary presentations, 2 of which had pleural involvement; the other 7 presentation were extra-pulmonary, 3 of which disseminated and 4 with lymphadenopathy (2 abdominal, 1 sub-mandibular and 1 cervical). 6-month clinical follow up was available for 14 subjects: 11 subjects improved on anti-MTB treatment, whereas 3 subjects died within 1 month of IRIS diagnosis (2 deaths attributed to MTB infection, 1 unclear). 4 subjects were lost to follow-up.

Median time on ART was not significantly different between groups [group 1 = 45 (IQR = 28;59), group 2 = 31 (IQR = 27;57) and group 3 = 52 (IQR = 27;64), $p = 0.3940$]; median age was also not significantly different between groups (not shown).

Immunologic characterization of unmasking TB-IRIS

To determine whether uTB-IRIS is characterized by a defined immunological and hematological profile, subjects classified as confirmed or probable uTB-IRIS (Group 3) were compared with both HIV-infected individuals (Group 1) and MTB-HIV co-infected individuals (Group 2) undergoing ART for comparable length of time. A summary of the primary variables evaluated is provided in Table 2 and those variables associated with uTB-IRIS are illustrated in Figure 1 (for complete list of primary and secondary variables assessed see Supplemental table 2). While NK cell numbers were similar across groups (not shown) uTB-IRIS subjects had a higher frequency of activated NK (CD69⁺ and CD69⁺/HLA-DR⁺) cells than either control group. In keeping with the clinical hyper-inflammatory state, uTB-IRIS subjects also had increased plasma levels of acute phase reactants (CRP and $\beta 2$ microglobulin), as well as a number of T cell, macrophage and mesenchymal derived pro-inflammatory cytokines (IL-6, IL-8, EGF and HGF).

The observed differences in NK cell activation (frequency of circulating CD69⁺ or CD69⁺/HLA-DR⁺ NK cells, Fig. 1A and B), CRP (fig 1C) and IL-8 (Fig. 1D) remained significant (adjusted $p < 0.1$) after adjustment for multiple testing.

Immune parameters associated with MTB infection

In addition to defining parameters specifically associated with uTB-IRIS, some variables were significantly different between Group 2 and 3 vs. Group 1, and were thus considered associated with MTB infection, independent of presentation (summarized in Fig. 2).

CD4 count was lower in MTB-infected subjects (Group 2 and 3) as compared to HIV-monoinfected subjects (Group 1, Fig 2D); importantly, in the same subjects lymphocyte count (Figure 2B) and % (Fig. 2A) were also lower than HIV monoinfecteds, suggesting that the CD4 depletion may be due to generalized lymphocytopenia. Neutrophil % (Group 1 = median 49.9, IQR 41–60.9; Group 2 = median 64.1, IQR 55.75–68; Group 3 = median 67, IQR 53.6–76.95. Fig. 2C), was higher in Group 2 and 3, supporting a skewing of the blood differential linked to lymphocytopenia.

MDC levels were observed to be significantly lower in MTB-infected (Group 2 and 3) than in HIV mono-infected subjects (Fig. 2H); conversely, IP10 plasma levels trended higher in the same groups (Table 2), in keeping with previous observations (^{11, 34}).

The frequency of HLA-DR-expressing T cells was significantly higher in Group 2 subjects as compared to both Group 1 and Group 2 controls, which had similar, lower levels of activated T cells (Fig. 2F). However, T cells co-expressing HLA-DR with the acute

activation marker CD69 had similar frequency in Group 2 and 3 (Fig 2E), which was higher than HIV-mono-infected subjects, suggesting a state of chronic activation. Plasma levels of the T-lymphocyte activation-associated marker soluble IL-2 receptor were also significantly higher in MTB-infected subjects as compared to mono-infected controls (Fig. 2G), further supporting the existence of an increased activation of the adaptive compartment in MTB-infected subjects, independent of clinical presentation.

Expression of HLA-DR on CD56⁺ NK-like T cells was also higher in Group 2 as compared to control Group 1, whereas the basophil count was lower in Group 2 than in Group 1 or 3; however, these differences were not significant after adjustment for multiple testing.

Use of Hematologic and Immunologic variables for the classification of unmasking MTB-related

The result of applying the conditional inference tree (CIT) framework³² to define combinations of variables characteristic of uTB-IRIS is given in Fig. 3: a frequency of CD69⁺ NK > 36.957 % was associated with an estimated probability of uTB-IRIS of 90% (node 5). In contrast, subjects with CD69⁺ NK frequency ≤ 36.957 % was associated with an estimated probability of uTB-IRIS of 90% (node 5). In contrast, subjects with CD69⁺ NK frequency ≤ 36.957% had a much lower risk of unmasking IRIS (12.5% and 6% for lymphocyte count ≤ 1.33 and > 1.33, respectively (node 4). Importantly, subjects in node 4 (CD69⁺ NK ≤ 36.957% and lymphocyte count > 1.33) had a cumulative estimated probability of TB infection of only 18%, suggesting that this combination of variables could also be potentially useful, in combination with other clinical tests, to assess the likelihood of MTB infection in HIV-infected individuals.

Discussion

By comparing subjects with acute, unmasking TB-related IRIS to both HIV-infected and MTB/HIV-co-infected control subjects, we identified for the first time a number of innate response parameters that are uniquely associated with uTB-IRIS.

NK cell activation, as determined by surface expression of CD69 alone or with HLA-DR is significantly higher in uTB-IRIS. CD56⁺ NK cells have been reported to be involved in the host response to MTB, as indicated by the activation (HLA-DR and CD69 expression) and expression of chemokine receptors (including CXCR2, CCR1, 2 and 7 in CD56^{bright} and CCR5 on CD56^{dim}) on pleural NK cells from TB subjects, as well as their ability to migrate in response to IL-8, IP-10, MCP-1 and SLC³⁵. It is possible that the observed NK cell activation may result in part from their interaction with mycobacterial products (e.g. via TLR2,³⁶) as well as from enhanced chemokines production by infected/bystander macrophages at the sites of infection. Our observation that IL-8 is significantly increased in uTB-IRIS further supports this model, suggesting that IL-8 might be involved in the activation and chemotaxis of NK cells expressing CXCR1³⁷ or CXCR2³⁵.

Importantly, we identified a profile (CD69⁺ NK and lymphocyte count) that emphasizes the contribution of innate variables in classifying uTB-IRIS subjects in our cohort: further validation in an independent cohort, is needed to elucidate the generalizability of this finding.

We only observed a moderate raise in IL-6 in uTB-IRIS, which was significant only before adjustment for multiple testing. this is in contrast with prior studies in paradoxical TB-IRIS¹⁵ showing increased IL-6: this discrepancy may depend on the experimental design (cell culture supernatants of MTB-stimulated PBMC rather than serum/plasma) and IRIS presentation, as the pathogenesis of paradoxical TB IRIS is thought to be at least in part

separate from that of unmasking TB-IRIS^{3,7}. Further studies assessing the cellular subsets source of the cytokines associated with uTB-IRIS may help further define these differences.

While we observed an increased T-cell activation (HLA-DR/CD69 expression, plasma IL-2 receptor) in MTB co-infected subjects, uTB-IRIS subjects had levels of T-cell activation similar to HIV/MTB co-infected subjects, suggesting that T cell activation is driven by MTB infection, irrespective of clinical presentation.

In combination with the increased activation of NK cells in uTB-IRIS, these observations suggest that, in addition to the proposed role for adaptive immune responses⁷, which have also been shown to underlie paradoxical TB-IRIS^{17, 18}, innate immune effectors play a role in the pathogenesis of unmasking TB-related IRIS, by contributing to the Th1 response to M. Tuberculosis (NK cells) or producing pro-inflammatory mediators, as suggested by prior *in vitro* observations (reviewed in³⁸). Prior demonstration that NK cell rapidly achieve partial functional recovery upon ART initiation³⁹ further support their potential contribution to the pathogenesis of unmasking TB-IRIS.

While we could confirm the association of increased acute phase reactants (APRs) with uTB-IRIS in our cohort by assessing between-group differences, our results indicate that APRs are not the most useful variable to classify unmasking TB-IRIS, as they are outranked by NK cell activation and other hematological parameters (lymphocyte and neutrophil count) in our ctree analysis. It is possible that the chronic activation underlying both advanced HIV and MTB infection may result in some degree of elevation APRs, thus obfuscating their association with IRIS. Given the relatively small size of our cohort, larger studies will be required to confirm this result, particularly in reference to the cut-off values identified here, and to assess the usefulness of these markers in diagnosing other forms of IRIS, particularly in reference to different pathogens.

We have identified a number of immunological parameters associated with MTB infection, independent of IRIS presentation; among these are decreased lymphocyte and increased neutrophil counts. While prior studies in MTB-infected subjects in the same geographical area did not show alteration of these subsets⁴⁰, lymphopenia is a relatively common feature of MTB infection^{41, 42} it is likely that the advanced HIV co-infection, as well as ongoing anti-MTB treatment, may have contributed to the profile of our subjects. CD4⁺ T cell counts were also lower in MTB co-infected subjects; while MTB-related CD4⁺ T cell depletion has been reported^{41, 43, 44}.

The study has a number of limitations, the first being the relatively small size of the cohort: while our confidence in the significance of the tests conducted is based on a conservative approach to multiple testing adjustment, it is possible that other parameters (e.g. IL-6 and IP-10 levels) would have been found to be significantly different using a larger cohort.

Secondly, our work focused exclusively on subjects with the unmasking form of TB-IRIS: further studies will be required to assess whether or not our findings apply also to other presentations (i.e. paradoxical IRIS) or pathogens.

It is also important to consider that, due to the fact that anti-TB treatment is initiated before ART in the vast majority of HIV-infected subjects, in order to match time on ART our MTB control group included only subjects undergoing anti-mycobacterial treatment, which limits the interpretation of the results due to the regressing MTB infection and the potential effects of anti-MTB drugs.

Another limitation is that our study assessed uTB-IRIS subjects at presentation, and lacked pre-treatment samples and extensive follow-up data, due to the fact that a) most uTB-IRIS

subject were referred as hospital inpatients, and b) most subjects in the MTB-HIV co-infection control group were referred from TB clinics in the geographical catchment of the Themba Lethu clinic. Partly due to acuteness of the clinical presentation, blood drawing was limited in 5 of uTB-IRIS group subjects, limiting the amount of flow cytometry data available for analysis.

Finally, the cross-sectional nature of the cohort did not address whether pre-treatment or early “on-treatment” elevation of cell activation may be predictive of IRIS onset; further prospective studies will be required to determine this point. A prospective study enrolling subject at the time of HIV diagnosis and with extended follow-up will therefore be required to confirm and extend these observations.

Conclusions

We sought to identify immune parameters that uniquely characterize MTB-related IRIS by studying a cohort of subjects with unmasking IRIS presentation. Our results indicate that elevated NK cell activation, together with increased plasma levels of acute phase reactants and IL-8, is uniquely associated with unmasking TB-related IRIS, supporting the hypothesis that innate immune effectors participate in the pathogenesis of IRIS. Based on our conditional inference tree classification we propose that NK activation and lymphocyte counts may be clinically useful to support a diagnosis of MTB-related IRIS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Variables associated with unmasking *M. tuberculosis* IRIS

Distribution of Flow cytometry (top panels) and multiplex array (middle and bottom panels)-derived variables in HIV-infected subjects (group 1), HIV/MTB-co-infected subjects (group 2) and uTB-IRIS subjects (group 3). For all indicated variables, boxes represent median, 25th and 75th percentiles and whiskers represent 5th and 95th percentiles. Brackets indicate significant differences between groups (unadjusted Wilcoxon test p < 0.05). Benjamini-Yekutieli FDR-adjusted Kruskal-Wallis test p < 0.1 for all variables.

Figure 2.

Variables associated with *M. tuberculosis* infection

Distribution of hematology, Flow cytometry and multiplex array-derived variables in study groups (see Fig. 1). For all indicated variables, boxes represent median, 25th and 75th percentiles and whiskers represent 5th and 95th percentiles. Full and dashed brackets indicate differences between groups with unadjusted Wilcoxon test $p < 0.05$ and $p < 0.07$, respectively. Benjamini-Yekutieli FDR-adjusted Kruskal-Wallis test $p < 0.1$ for all variables.

Figure 3.

Conditional partitioning inference tree (ctree) classification

Graphical representation of recursive conditional inference tree classification analysis: nodes 1, 2 and 4 represent significant (Bonferroni-adjusted $p < 0.05$) splits at the indicated thresholds (for CD69⁺ NK cells, values represent % of total NK cells). The relative frequency and numbers for each study group (see also fig. 1) are provided for the distal nodes (3, 5, 6 and 7) and illustrated by histograms; the percentages above the bars represent the relative frequency of subjects in group 1 (HIV control), group 2 (MTB-HIV co-infection) and 3 (uTB-IRIS) for each node.

Table 1

clinical characteristics of IRIS group subjects

ID	Pre-ART CD4	Event CD4	Pre-ART VL	Event VL	Time on ART	Localization	Presentation	Diagnostic method	Hospitalized	Outcome
1	na	181	na	<50 *	55	pulmonary	Weight loss, Productive cough, Night sweats	sputum	no	Improved with anti TB treatment
4	176	58	230000	78	47	pulmonary, pleural	Worsening crepitation, Dyspnea, Weakness	x-ray, pleural aspirate	yes	Death **
5	28	263	na	<50	87	pulmonary	N/A	sputum	no	Improved with anti TB treatment
6	145	162	200000	185	28	pulmonary	Night sweats, Weight loss, Loss of appetite	x-ray	yes	Improved with anti TB treatment
7	117	78	220000	651	23	pulmonary	Cough, Weakness	x-ray	yes	LTFU
8	33	73	1200000	<400	64	pulmonary	Weight loss, Night sweats, Cough	x-ray	no	Improved with anti TB treatment
9	101	125	na	<400	63	pulmonary	Fatigue, Fever, Shortness of breath	x-ray	yes	Death *
10	52	55	14000	1080	25	pulmonary	Cough, Night sweats, Weight loss	x-ray	yes	Improved with anti TB treatment
11	121	89	2500	<400	29	pulmonary, pleural	Cough, Fever, Dyspnea	x-ray, pleural aspirate	yes	Death *
14	115	327	na	<400	62	pulmonary	Fever, Weight loss, Chest pain	x-ray	yes	Improved with anti TB treatment
15	170	200	9300	559	27	abdominal	Abdominal pain	ultrasound	yes	Improved with anti TB treatment
16	64	436	50000	<50	111	disseminated	Shortness of breath, Fever, Rigors	x-ray	yes	Improved with anti TB treatment
17	na	21	na	<400	52	disseminated	Weight loss, abdominal pain, Fever, Cough, Generalized lymphadenopathy	ultrasound	yes	LTFU

ID	Pre-ART CD4	Event CD4	Pre-ART VL	Event VL	Time on ART	Localization	Presentation	Diagnostic method	Hospitalized	Outcome
18	22	29	na	<400	51	pulmonary	Cough, Night sweats, Headache	x-ray	yes	LTFU
21	146	305	na	1222	33	disseminated	Cough, Chest pain, Night sweats, Weight loss, Tender Hepatomegally	Bactec	yes	Improved with anti TB treatment
22	41	154	na	<400	57	submandibular	Swollen submandibular glands	aspirate	no	LTFU
25	9	44	na	<400	55	abdominal	Pancytopenia, grade 4 LFT, Abdominal lymphadenopathy, Splenic microabscesses	ultrasound	yes	Improved with anti TB treatment
26	124	321	119981	<400	140	cervical	Bilateral cervical lymphadenopathy	aspirate	no	Improved with anti TB treatment

* Undetectable VL at the time of IRIS presentation was considered as fulfilling IRIS diagnostic criteria, independent of prior measurements

** Death occurred within 1 month of IRIS diagnosis

Table 2

Summary of variables assessed¹

Variable	Group 1			Group 2			Group 3			Kruskal-Wallis test p			Wilcoxon test		
	N	Median		N	Median		N	Median		Unadjusted	FDR-adjusted		G1 vs G2	G1 vs G3	G2 vs G3
Red blood cells ($\times 10^9/\text{ml}$)	57	3.96	31	3.67	18	3.22	0.00647	0.18815	0.147	0.00464	0.02327				
Hemoglobin	56	11.95	31	11.4	18	9.95	0.02768	0.52264	0.55248	0.03007	0.04446				
Lymphocyte Count ($\times 10^6/\text{ml}$) ²	58	1.56	31	1.14	18	0.85	0.00001	0.00642	0.00002	0.00126	0.64072				
Lymphocyte % ²	58	34.25	31	26	18	18.8	0.00006	0.01466	0.00016	0.00165	0.22499				
Neutrophil count ($\times 10^6/\text{ml}$)	58	2.34	31	2.74	18	3.03	0.03173	0.52264	0.18335	0.00731	0.05758				
Neutrophil % ²	58	49.9	31	64.1	18	67	0.00009	0.01466	0.00018	0.00235	0.19848				
Basophil count ($\times 10^6/\text{ml}$)	52	0.02	30	0.01	14	0.02	0.00919	0.24492	0.00282	0.54115	0.07461				
K ⁺ (mMol/l)	58	4.3	31	4	18	4.25	0.03160	0.52264	0.00875	0.46576	0.17216				
C-reactive protein (ng/ml) ²	29	5392	31	10600	16	17128.5	0.00040	0.03904	0.08622	0.00007	0.00507				
$\beta 2$ microglobulin (ng/ml)	29	114.5	31	137	16	192.5	0.04351	0.64963	0.67871	0.0123	0.0144				
GC Globulin (ng/ml)	29	16672	31	17539	16	16895	0.02202	0.47160	0.01142	0.649	0.05106				
IL-8 (pg/ml) ²	29	32.11	31	37.65	16	81.42	0.00129	0.06386	0.13701	0.00090	0.00467				
Soluble IL-2R (pg/ml) ²	29	208.68	31	503.12	16	385	0.00129	0.06386	0.00051	0.01319	0.93736				
IL-6 (pg/ml)	29	13.68	31	17.77	16	34.3	0.00309	0.10335	0.12755	0.00238	0.00254				
IP-10 (pg/ml)	29	242.55	31	438.4	16	500	0.00314	0.10335	0.00274	0.0029	0.66951				
HGF (pg/ml)	29	440.3	31	440.3	16	793.5	0.00942	0.24492	0.9646	0.00549	0.00159				
MIG (pg/ml)	29	734.1	31	1059.9	16	931.6	0.00608	0.18788	0.00133	0.03628	0.94843				
EGF (pg/ml)	29	120.2	31	120.9	16	89.8	0.03579	0.57040	0.9587	0.0108	0.00998				
IL-15 (pg/ml)	29	61.8	31	78.4	16	74.7	0.03117	0.52264	0.00845	0.09213	0.82091				
IL-13 (pg/ml)	29	62.6	31	85	16	36.5	0.02291	0.47160	0.01367	0.64908	0.06638				
IFN- γ (pg/ml)	29	38.2	31	58.6	16	19.4	0.02116	0.47160	0.0274	0.42523	0.04257				
pre-ART CD4 ⁺ T cell count (cells/ml)	55	118	26	60.5	17	108	0.02287	0.47160	0.0084	0.37787	0.13281				
CD4⁺ T cell count (cells/ml) ²	57	221	31	101	18	140	0.00063	0.05217	0.00038	0.01555	0.77153				
CD45RA ⁺ /CD62L ⁺ (% of CD4 ⁺ T cells)	47	19.29	26	31.92	13	19.49	0.02914	0.52264	0.01777	0.53588	0.03444				
CD8 ⁺ T cell count (cells/ml)	57	860	31	612	17	479	0.00254	0.09858	0.00294	0.01267	0.60842				

Variable	Group 1			Group 2			Group 3			Kruskal-Wallis test p			Wilcoxon test		
	N	Median		N	Median		N	Median		Unadjusted	FDR-adjusted		G1 vs G2	G1 vs G3	G2 vs G3
HLA-DR ⁺ T cells (% of T cells) ²	46	33.72		26	53.34		13	28.75		0.00082	0.05778		0.00015	0.93522	0.01642
CD69 ⁺ /HLA-DR ⁺ T cells (% of T cells) ²	46	2.96		26	5.06		13	4.37		0.00104	0.06386		0.00016	0.06291	0.98828
HLA-DR ⁺ NKT cells (% of CD56 ⁺ T cells)	46	23.26		26	36.48		13	30.11		0.01177	0.29085		0.00576	0.06607	0.89479
CD69 ⁺ /HLA-DR ⁺ (% of CD56 ⁺ T cells)	46	2.95		26	5.67		13	5.77		0.03831	0.59146		0.02258	0.0856	0.60664
CD69 ⁺ NK cells (% of NK) ²	47	13.48		26	15.37		12	44.11		0.00038	0.03904		0.23036	0.00008	0.00059
CD69 ⁺ /HLA-DR ⁺ NK cells (% of NK cells) ²	47	7.69		26	9.4		12	19.29		0.00173	0.07767		0.25889	0.00083	0.00367
Myeloid DC (cells/ml) ²	48	0.13		26	0.06		13	0.05		0.00259	0.09858		0.00330	0.01269	0.57130

¹ Only variables with unadjusted K.W. $p < 0.05$ are reported; please see supplemental table for a complete report of all variables

² FDR-adjusted Kruskal-Wallis ANOVA $p < 0.1$