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Cryptococcosis-IRIS is associated with lower *Cryptococcus*-specific IFN-gamma responses before antiretroviral therapy but not higher T-cell responses during therapy

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Abstract:

Background:

Cryptococcosis-associated immune reconstitution inflammatory syndrome (C-IRIS) may be driven by aberrant T-cell responses to cryptococcal antigens. We investigated this in HIV-infected patients with treated cryptococcal meningitis (CM) who commenced combination antiretroviral therapy (cART).

Methods:

Mitogen- and cryptococcal mannoprotein (CMP)-activated (CD25+CD134+) CD4+ T-cells and induced production of IFN-gamma, IL-10 and CXCL10 were assessed in whole blood cultures, in a prospective study of 106 HIV-CM co-infected patients.

Results:

Patients with paradoxical C-IRIS (n=27), compared to patients with no neurological deterioration (no-ND; n=63), had lower CMP-induced IFN-gamma production in 24-hour cultures of blood collected pre-cART and 4-weeks post-cART (p=0.0437 and 0.0257, respectively) and lower CMP-activated CD4+ T-cell counts pre-cART (p=0.0178). Patients who survived to 24-weeks had higher proportions of mitogen-activated CD4+ T-cells and higher CMP-induced CXCL10 and IL-10 production in 24-hour cultures pre-cART than patients who did not survive (p=0.0053, 0.0436 and 0.0319, respectively). C-IRIS was not associated with higher CMP-specific T-cell responses before or during cART.

Conclusion:

Greater preservation of T-cell function and higher CMP-induced IL-10 and CXCL10 production before cART are associated with improved survival on cART. Lower CMP-induced IFN-gamma production pre-cART but not higher CMP-specific T-cell responses after cART were risk factors for C-IRIS.

Introduction:

Cryptococcal meningitis (CM) is the leading cause of adult meningitis in southern and central Africa and particularly affects persons infected with HIV, accounting for 13-44% of HIV-related deaths [1]. Morbidity and mortality associated with this condition are affected by cryptococcosis-associated immune reconstitution inflammatory syndrome (C-IRIS), which has been reported in up to 50% of HIV-infected patients with treated CM who commence combination antiretroviral therapy (cART) [2]. Like TB-IRIS, the immunopathogenesis of C-IRIS is poorly understood; both are thought to be due in part to an aberrant interferon-gamma (IFN-gamma) driven Th1 response to disease-specific antigens [3]. Better understanding of C-IRIS pathogenesis is necessary to recognize high-risk patients and develop diagnostic and treatment strategies.

Currently, the diagnosis of C-IRIS remains a diagnosis of exclusion [4]. No definitive diagnostic marker or predictive signature has been adopted into clinical practice, outside of research settings [5]. Distinguishing C-IRIS from other causes of neurological deterioration (ND) after commencing cART is crucial as management and prognosis are vastly different. Analysis of T-cell responses to cryptococcal antigens may be informative.

Whole blood assays for detecting antigen-specific T-cells bear potential as a 'field-based assay' that would be particularly attractive in resource-limited settings. Readouts may include expression of activation markers on CD4+ T-cells and production of cytokines and chemokines by antigen-activated T-cells and other cells activated by these T-cells. Accordingly, co-expression of CD25 (interleukin-2 receptor-alpha chain, IL-2Rα) and CD134 (OX40) on CD4+ T-cells in whole blood incubated with antigens has been suggested as an alternative measure of activated and proliferating antigen-specific memory CD4+ T-cells [6,7].

Cryptococcal mannoproteins (CMP) are a group of heterogenous T-cell antigenic determinants isolated from *C. neoformans* [8]. CMPs stimulate lymphoproliferative responses and cytokine production in patients recovering from cryptococcosis [9,10], and have been shown experimentally

to induce the production of tumor necrosis factor-alpha (TNFα), IL-12 and IFN-gamma in human peripheral blood monouclear cells (PBMC) and murine macrophages co-cultured with T-cells [8,11-13].

We explored the use of a whole blood assay to detect CMP-reactive T-cells that expressed CD25 and CD134 or produced IFN-gamma as a potential 'field-based assay' for the prediction and diagnosis of paradoxical central nervous system (CNS) C-IRIS. CXCL10 (also known as IFN-gamma-inducible protein 10, IP-10) and IL-10 were also assayed in plasma from the whole blood cultures as the former is induced by IFN-gamma and the latter is an anti-inflammatory and regulatory cytokine.

Methods:

Patients

Patients with paradoxical CNS C-IRIS (n=27) and patients with no ND (n=63) were recruited in Durban, KwaZulu-Natal, South Africa from a prospective study of adult, HIV-infected, cART-naïve patients (n=106) experiencing their first episode of CM (positive cerebrospinal fluid (CSF) cryptococcal antigen (CrAg) or India ink test) who were treated with standard antifungal therapy. Patients demonstrated a good response to antifungal therapy before being commenced on cART and followed for 24-weeks, for episodes of neurological deterioration such as recurrence of headaches, new seizures or new neurological deficits [14]. Patients were excluded from enrollment if they were concurrently pregnant, had a severe coagulopathy (INR >2, platelets $<20x10^9$/L), had a previous history of CM or previous use of cART within the last 6 months (excluding cART taken as part of prevention for mother-to-child transmission) or were deemed likely to be unwilling to comply with study protocol. All patients provided written informed consent and the protocol was approved by ethics committees of the University of KwaZulu-Natal (BF053/09), Monash University (2009001224) and University of Western Australia (RA/4/1/2541).

Reagents

R10 medium was made with 500mL RPMI 1640 without L-Glutamine (Lonza Walkersville Inc., Maryland) and supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria), 10mM L-glutamine, 50mM HEPES buffer and 50000unit/50000unit/125microgram Pen/Strep/Fungizone (all from Lonza Walkersville Inc.). Red cell lysis buffer was made by adding 7.7g ammonium chloride and 0.84g sodium bicarbonate to 1Liter of distilled water. Lyophilised CMP was purified from culture supernatants of *C. neoformans* acapsular strain Cap 67 as described [8] and was reconstituted with distilled water to a final concentration of 1mg/mL.

Whole blood assay of mitogen- and CMP-specific T-cell responses

Mitogen (Phytohemagglutinin, PHA) or CMP-antigen-reactive T-cells were detected in whole blood cultures. 250microliter of lithium heparin-anticoagulated freshly collected whole blood was mixed with 250microliter R10, in the presence of no additive (unstimulated control), polyclonal mitogen Gibco® PHA-M 1.5% (Life Technologies, Johannesburg, South Africa) or CMP 12.5microgram, and cultured for 24 hours at 37^0C in 5% CO_2. At 24 hours, 100microliter each of unstimulated, mitogen-stimulated and CMP-stimulated whole blood were lysed using a red cell lysis buffer, and stained with CD3 PE/Cy7, CD4 PerCP-Cy5.5, CD25 APC and CD134 PE (BD Biosciences, Franklin Lakes, New Jersey) based on the method of Zaunders et al [7]. Data acquisition was performed on a LSRII™ flow cytometer, using FACSDiva software (both, BD Biosciences, Franklin Lakes, New Jersey) and analyzed with FlowJo™ (TreeStar Inc., Ashland, Oregon). Corrected expression of CD25+CD134+ was calculated by subtracting the background expression on unstimulated cells from antigen-stimulated cells. To obtain an estimate of the CMP-specific CD4+ T-cell count, the proportion of CD4+ T-cells that were CD25+CD134+ in CMP-stimulated whole blood was multiplied by the absolute CD4+ T-cell count.

In addition to the analysis of CD4+ T-cell activation by flow cytometry, cultured plasma was collected from the set of 24-hour whole blood cultures, here termed “24h-Unstim”, “24h-mitogen” and “24h-CMP”. Also, 5-day cultures of 100microliter of whole blood mixed with 400microliter of R10, in the presence of no additive (unstimulated control), mitogen 1% or CMP 5microgram, here termed “5d-Unstim”, “5d-mitogen” and “5d-CMP” were also undertaken. Cultured plasma samples were cryopreserved at -80^{o}C.

Assay of cytokines and chemokines in plasma from whole blood cultures

Thawed plasma samples from cultured supernatants were analyzed for IFN-gamma, CXCL10 and IL-10 by customized multiplex Bio-Plex Pro™ Assays (Bio-Rad, Gladesville, Australia) using the Bio-Plex 200™ suspension array system and Bio-Plex Manager™ 5.0 software. The lower limits of detection for the cytokine assays were derived by extending the standard curve (after consultation with the manufacturer) and set at 7, 6 and 5 pg/mL for IL-10, IFN-gamma and CXCL10 respectively, ensuring these were above values for blank wells. Plasma from unstimulated, mitogen- and CMP-stimulated tubes was assayed on the same plate. Corrected cytokine or chemokine levels were calculated by subtracting the value for unstimulated blood from the value for mitogen- or CMP-stimulated blood. Negative corrected values were reported as zero, represented on log-axis graphs as 0.01.

Statistical analyses

Continuous variables were assessed for skew and summarized using mean and standard error or median and interquartile range (IQR) as appropriate and analyzed using either a t-test or a Wilcoxon rank-sum test. Predictors of time to C-IRIS were analyzed using univariable and multivariable Cox proportional-Hazards regression. Reported *p*-values are two-tailed and in these analyses $p<0.05$ was considered significant.

Overall differences for each variable were tested by Kruskal-Wallis. If a significant difference was seen, each pair of time points were tested separately using a Wilcoxon rank-sum test with Bonferroni adjustment of the p-level cut-off for multiple comparisons; where p-values below 0.0083 were considered significant. Curve comparison of survival analysis to 24-weeks was performed with log-rank (Mantel-Cox) test.

All analyses were performed using Stata v.12 (StataCorp, College Station, Texas) and GraphPad Prism® v5.

Results:

Summary of baseline patient demographics

Data were analyzed on the entire patient cohort (n=106), and subsets of patients experiencing C-IRIS (n=27) or no-ND (n=63), and survivors (n= 85) and non-survivors (n=21) at 24-weeks post-cART [15]. The C-IRIS group, when compared to the no-ND group, had lower CD4+ T-cell counts pre-cART (median 16 [IQR 6-53] vs.36 [16-83]cells/microliter,p=0.015), but there was no significant difference in age (median 34 vs. 33 years, p=0.704), HIV viral load (5.1 vs. 5.3$\log_{10}$copies/mL, p=0.322), CSF or serum cryptococcal antigen (p=0.947 and 0.756 respectively) [15].

Mitogen-reactive CD4+ T-cells in whole blood increased over 24-weeks of cART but there was no change in CMP-reactive CD4+ T-cells

Blood collected prior to cART commencement demonstrated significantly higher proportions of activated (CD25+CD134+) T-cells after stimulation with mitogen (p<0.0001) and CMP (p<0.0001) compared to unstimulated tubes (Figure 1A). Over 24-weeks of cART, the proportion of activated CD4+ T-cells remained low in the unstimulated tubes while the proportion of mitogen-activated

CD4+ T-cells increased significantly (p<0.0001). In contrast, the proportion of CMP-activated CD4+ T-cells did not change over 24-weeks of cART (Figure 1B).

Mitogen- and CMP-induced cytokine and chemokine production before and after cART

In blood collected pre-cART, CMP-induced IFN-gamma was undetectable in 32.7% and 54.8% of the entire patient cohort after 24-hours and 5-days of stimulation, respectively. Levels of mitogen-induced IFN-gamma and IL-10 production in plasma after 24-hours and 5-days of stimulation increased on cART (all p=<0.0001) (Figure 2A; 2C) while levels of mitogen-induced CXCL10 increased following 5-days (p=0.0008) but not 24-hours of stimulation (Figure 2B). CMP-induced IFN-gamma production increased on cART following 5-days of stimulation (p=0.0001) (Figure 2D) and CMP-induced CXCL10 production decreased on cART following both 24-hours and 5- days of stimulation (p=0.0038 and 0.0023, respectively) (Figure 2E). There were no significant changes in CMP-induced IL-10 production over 24 weeks of cART (Figure 2F).

C-IRIS was associated with lower CMP-induced IFN-gamma production pre-cART but not with increased CMP-specific T-cell responses during cART

Having established the validity of the whole blood assay methods by examining the entire patient cohort, we compared T-cell responses in patients who experienced C-IRIS or no-ND. Proportions of mitogen-activated (CD25+CD134+) CD4+ T-cells did not differ between the groups before cART but were lower in the C-IRIS group after 24-weeks of cART (p=0.0077) (Figure 3A). Proportions of CMP-activated CD4+ T-cells did not differ between groups before or during cART (Figure 3B) though CMP-activated CD4+ T-cell counts were lower in the C-IRIS group pre-cART (p=0.0178) but not during cART (Figure 3C).

In whole blood cultures, the C-IRIS group when compared to the no-ND group had lower CMP-induced IFN-gamma production after stimulation for 24-hours pre-cART (p=0.0437; figure 4A) and after stimulation for 24-hours (p=0.0257) and 5-days, (p=0.0261) at 4 weeks after cART initiation

(Figure 4A and 4B). There were no differences in CMP-induced IL-10 or CXCL10 levels between these groups before and during cART.

Higher proportions of mitogen-activated CD4+ T-cells and greater production of CXCL10 and IL-10 in 24h-CMP cultures pre-cART were associated with improved 24-week overall survival

We next explored whether CMP- and mitogen-induced T-cell and cytokine and chemokine responses in whole blood cultures before cART were associated with patient survival during 24-weeks of cART. The proportion of mitogen-activated (CD25+CD134+) CD4+ T-cells in blood collected pre-cART was higher in survivors than non-survivors (median 22.5 [IQR 10.9-32.5] vs. 9.2 [4.2-22.4]%; p=0.0053; Figure 5A). In CMP-stimulated cultures, survivors had higher pre-cART CXCL10 production in 24-hour cultures (median 611.3 [143.4-1598.0] vs. 399.4 [0.01-987.8]pg/mL; p=0.0436) and higher IL-10 production in both 24-hour and 5-day cultures (median 588.2 [373.0-984.3] vs. 341.2 [91.9-675.7]pg/mL;p=0.0319; and 239.0 [109.1-427.7] vs.109.2 [57.9-267.4]pg/mL, p=0.0444, respectively; Figure 5B). There was no difference in CMP-induced IFN-gamma production between these groups.

Using survival analysis, patients in the lowest quartile for proportion of mitogen-activated (CD25+, CD134+) CD4+ T-cells pre-cART had a reduced 24-week survival compared with other patients (Hazard ratio HR 0.1386,95%CI 0.0454-0.4229;p=0.0005. Patients in the lowest quartile for 24h-CMP-induced CXCL10 and IL-10 production also had decreased survival (HR 0.2553,95%CI 0.0878-0.7426;p=0.0122 and HR 0.1837,95%CI 0.0422-0.7994;p=0.0239). When we analyzed these three parameters together, patients who had at least one of these parameters in the lowest quartile were less likely to survive to 24-weeks than those with all three parameters measured in the upper three quartiles (HR 0.2115,95%CI 0.0834-0.5367;p=0.0011) (Figure 6A). There were insufficient events to undertake a multivariable analysis of the relationship between pre-cART values for these parameters and survival.

Discussion:

We have undertaken the first large-scale analysis of cryptococcal-specific T-cell responses in HIV-infected patients with treated CM before and during the first 24-weeks of cART using assay methods with potential for translation into routine clinical settings. We established the validity of our assay methods by demonstrating a clear improvement in T-cell function in the entire cohort of patients over the first 6 months of cART as evidenced by a graduated increase in the proportion of mitogen-activated (CD25+CD134+) CD4+ T-cells and a rise in mitogen-induced IFN-gamma, CXCL10 and IL-10 production in whole blood cultures, consistent with previous studies demonstrating increased proliferative responses to mitogens and common antigens as a surrogate of immune recovery [16-18]. In addition, we demonstrated that a higher proportion of mitogen-activated CD4+ T-cells and higher CMP-induced production of CXCL10 and IL-10 in whole blood cultures pre-cART were associated with improved patient survival after 24 weeks of cART. While we did not detect higher CMP-activated CD4+ T-cells after cART in the C-IRIS group, these patients exhibited lower CMP-activated CD4+ T-cell count pre-cART, likely reflecting the lower total CD4+ T-cell count in this group. Patients with greater CD4+ T-cell depletion are known to have poorer antigen-specific proliferation [16].

The increase in mitogen-induced IFN-gamma and CXCL10 production on cART was associated with a decline in CMP-induced CXCL10 production, perhaps reflecting a declining effector memory T-cell response to a lower pathogen load. However, CMP-induced IFN-gamma responses in 5-day cultures increased during cART. The explanation for this discrepancy is unclear but it is possible that the relationship between CXCL10 and IFN-gamma is different in 5-day cultures.

More than 30% of patients in our study had undetectable CMP-induced IFN-gamma in whole blood cultures pre-cART. Notably, the C-IRIS group showed significantly lower CMP-induced production of IFN-gamma pre-cART and after 4 weeks of cART. It is well known that IFN-gamma plays a critical role in the control of cryptococcosis. In a murine model of pulmonary cryptococcosis, an increase in early IFN-gamma secretion from lung-associated lymph nodes was observed in cryptococcal-resistant

mice compared to cryptococcal-sensitive mice and both an anti-IFN-gamma and anti-IL-12 antibody resulted in reduced cryptococcal clearance [19,20]. Furthermore, mice lacking the IFN-gamma-receptor gene in a model of pulmonary cryptococcosis demonstrated uncontrolled fungal burden and disseminated disease despite enhanced pulmonary leucocytosis [21]. Higher CSF IFN-gamma levels in patients with CM were associated with improved rates of CSF cryptococcal clearance and survival [22]. The reduced CMP-induced IFN-gamma production in our patients may have contributed to the poorer cryptococcal clearance seen in the C-IRIS group [14,15], therefore predisposing them to C-IRIS. In support of this, a higher proportion of IFN-gamma+ CD4+ T-cells in HIV-infected patients with CM was associated with a lower fungal burden and a better survival rate at 2 weeks after cART [23]. In our study, CMP-induced IFN-gamma production in blood collected pre-cART was not associated with improved survival at 24 weeks, however, production of the IFN-gamma-inducible protein CXCL10 was. The discrepant association between IFN-gamma and survival may reflect the use of whole blood culture assays in our study compared to intracellular staining of T-cells in PBMCs in the study by Jarvis et al [23].

Two human studies have explored the promising role of adjunctive, short-course exogenous IFN-gamma therapy in HIV-infected patients with CM, with the more recent study showing significantly improved rates of CSF cryptococcal clearance [24,25] and a suggestion that the impact of exogenous IFN-gamma may have been greatest in patients with poor baseline cryptococcal-specific CD4+ T-cell memory responses [23]. Taken together, these findings suggest that patients with low IFN-gamma responses to CMP have a higher risk of developing C-IRIS and may be the group who would most benefit from adjunctive exogenous IFN-gamma therapy. Equally, those patients at risk of developing C-IRIS-related early mortality may potentially be detected by low or absent IFN-gamma or CXCL10 production using this simple assay of CMP-stimulated whole blood cultures.

In our study, the proportion of CMP-activated (CD25+CD134+) CD4+ T-cells did not distinguish C-IRIS patients from no-ND patients either before or after cART. This finding has several possible

explanations. First, T-cell responses to CMP may not be involved in the immunopathology of all cases of C-IRIS. In this study we only examined patients with paradoxical C-IRIS, whereas increased CMP-specific T-cell responses have mainly been reported in patients with 'unmasking' C-IRIS (21, 24). Second, C-IRIS events occurring in the CNS may have immune responses compartmentalized to the CNS and may not be detectable in blood. Third, whole blood assay methods may not be sensitive enough to detect CMP-reactive T-cells in patients with "paradoxical" C-IRIS, as appears to be the case in "paradoxical" TB-IRIS [26,27]. Importantly, Jarvis et al [23] also did not demonstrate an increase in CMP-reactive CD4+ T-cells after 4 weeks of cART using polychromatic flow cytometry. Finally, it is possible the CMP purified from the Cap67 acapsular *Cryptococcus spp.*mutant may not induce a robust T-cell response in patients infected with Cryptococcal strains found in South Africa. Different cryptococcal genotypes have been shown to induce different cytokine profiles [28].

IL-10 is a critical immunoregulatory molecule, and is able to down-regulate both innate and adaptive immunity, leading to increased susceptibility particularly, to intracellular pathogens, including *Cryptococcus spp*., and microbial persistence [29]. On the other hand, IL-10 may also protect hosts from exaggerated inflammatory and immune reactions and tissue injuries secondary to infections [29]. IL-10 is known to reduce IFN-gamma production in Th1 cells thereby amplifying deactivation of antigen-presenting cells [30], and is able to suppress all functions of monocytes/macrophages by interfering with antigen presentation, release of immune mediators and phagocytosis. The poorer survival rates seen in patients with lower CMP-induced IL-10 responses pre-cART, supports its role in containing the inflammation and tissue injury associated with CM.

Higher proportions of mitogen-activated CD4+T-cells in blood from patients pre-cART were also associated with improved survival at 24-weeks, suggesting that preservation of mitogenic response associated with CD4+ T-cell preservation pre-cART is important for survival in HIV-CM co-infected patients. Pre-cART measurement of these three parameters found to be associated with survival may identify those at risk for increased mortality and require enhanced clinical surveillance. Other

laboratory test predictors of C-IRIS and death previously reported have included perturbations of serum levels of several cytokines [31]. A direct comparison of all these biomarkers is warranted.

Our study has some limitations. Our C-IRIS and no-ND groups were not matched for CD4+ T-cell count pre-cART and this disparity may have contributed to the difference between our finding of a reduced IFN-gamma level in our C-IRIS patients compared to other studies. We chose to analyze our data collected strictly at scheduled timepoints prospectively, rather than reverse-matching at C-IRIS-event for an equivalent time-control. However, we believe our longitudinal follow-up of the cohort without selecting for equivalence in CD4+ T-cell count or reverse time-matching lends credence to our findings.

In conclusion, using whole blood assays of T-cell responses to CMP, we have shown that HIV-infected patients with treated CM who exhibit lower CMP-induced IFN-gamma production pre-cART are at increased risk of C-IRIS. The effect of adjunctive exogenous IFN-gamma should be trialed in patients who have low CMP-induced IFN-gamma responses as a means to enhance cryptococcal clearance and potentially to prevent C-IRIS. Further, preserved T-cell function, as demonstrated by mitogen-activated CD4+ T-cells, and increased IL-10 and CXCL10 responses in CMP-stimulated whole blood cultures pre-cART were associated with better survival at 24-weeks post cART. This assay might be incorporated into a risk-stratification algorithm in the management of HIV-CM co-infected patients.

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Authors' contribution:

MF, AL, CC and SRL designed the experiments; CC, TS, MF and SRL analyzed the data. CC, MF, SRL and SML wrote the manuscript. CC and SO performed the immunological assays. SML provided the cryptococcal mannoproteins. CC and BG conducted the clinical study. MF, SRL, CC, AL, TN, M-YSM, WC and JE were members of the protocol steering committee. All read and approved the final manuscript.

Conflict of interest:

All authors: none declared

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Figure legends

Figure 1: The percentage of CD4+ T-cells that express CD25+ and CD134+ in unstimulated cells or following stimulation with PHA/mitogen or CMP in whole blood collected from patients pre-cART (A) and over 24 weeks of cART (B). Data are shown as a box-and-whisker plot with the median, 25th and 75th percentiles shown as the middle, lower and upper lines of the box respectively. *p*-values are indicated as * p<0.0500, ** p<0.0100, *** p<0.0010 and **** p<0.0001.

Figure 2: Changes in mitogen- and CMP-induced cytokine and chemokine production before and after cART. Whole blood collected from patients prior to cART and over 24 weeks of cART was incubated with either mitogen (upper panel) or CMP (lower panel) for either 24-hours or 5-days. IFN-gamma, CXCL10 and IL-10 were assayed in plasma from the whole blood cultures. Corrected values (after subtraction of values for unstimulated cultures) are shown as a box-and-whisker plot with the median, 25th and 75th percentiles shown as the middle, lower and upper lines of the box respectively. *p*-values are indicated as * p<0.0500, ** p<0.0100, *** p<0.0010 and **** p<0.0001.

Figure 3: CMP- and mitogen-activated T-cells detected in whole blood from patients with C-IRIS or ND using flow cytometry. Percentage of CD25+CD134+CD4+ T-cells following stimulation with mitogen (A) or CMP (B) in whole blood from patients with C-IRIS (grey box) and no ND (open box) prior to and during 24 weeks of cART. Absolute numbers of CMP-activated CD25+CD134+CD4+ T-cells per microliter blood are also shown (C). The corrected data (after subtraction of values in unstimulated cultures) are shown as a box-and-whisker plot with the median, 25th and 75th percentiles shown as the middle, lower and upper lines of the box respectively. *p*-values are indicated as * p<0.0500, ** p<0.0100, *** p<0.0010 and **** p<0.0001.

Figure 4: CMP-induced IFN-gamma production in whole blood cultures from patients with C-IRIS or no-ND before and during cART. IFN-gamma levels in plasma after 24-hours (A) or 5-days (B) of culture are shown for patients with C-IRIS (grey box) or no ND (white box). The corrected data (after subtraction of values in unstimulated cultures) are shown as a box-and-whisker plot with the median, 25th and 75th percentiles shown as the middle, lower and upper lines of the box respectively. *p*-values are indicated as * p<0.0500, ** p<0.0100, *** p<0.0010 and **** p<0.0001.

Figure 5: Pre-cART CMP-specific T -cell and cytokine/chemokine responses in patients who did (open box) or did not (grey box) survive the first 24-weeks of cART. (A) Percentage of CD4+ T-cells that expressed CD25+ and CD134+ when unstimulated or following stimulation with mitogen or CMP. (B). Levels of CMP-induced IFN-gamma, CXCL10 or IL-10 in plasma from 24-hour (left panel) or 5-day (right panel) whole blood cultures. The corrected data (after subtraction of values for unstimulated cultures) are shown as a box-and-whisker plot with the median, 25th and 75th percentiles shown as the middle, lower and upper lines of the box. *p*-values are indicated as * p<0.0500, ** p<0.0100, *** p<0.0010 and **** p<0.0001.

Figure 6: 24-week survival analysis for three immunological parameters measured in whole blood cultures prior to cART initiation. Survival was compared for patients in the upper three quartiles (solid line) and lowest quartile (broken line) for each of these parameters. Survival was analyzed for patients who demonstrated all 3 parameters in the upper three quartiles compared to patients with at least one parameter in the lower quartile (A).

Footnote Page

All authors have no commercial or other association that might pose a conflict of interest.

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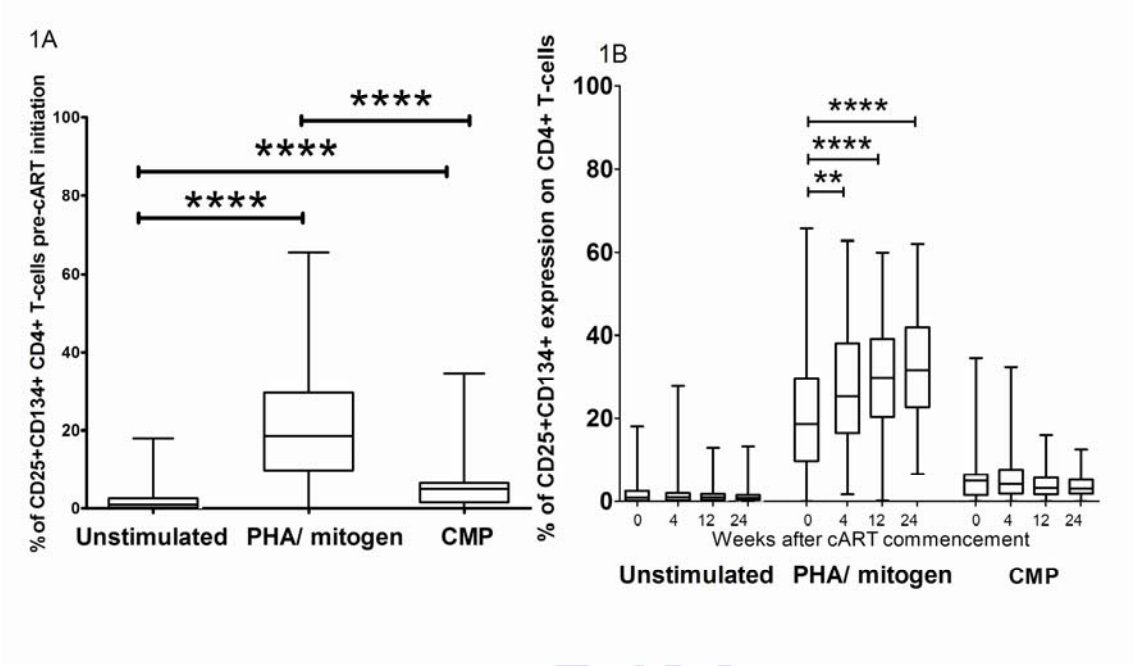
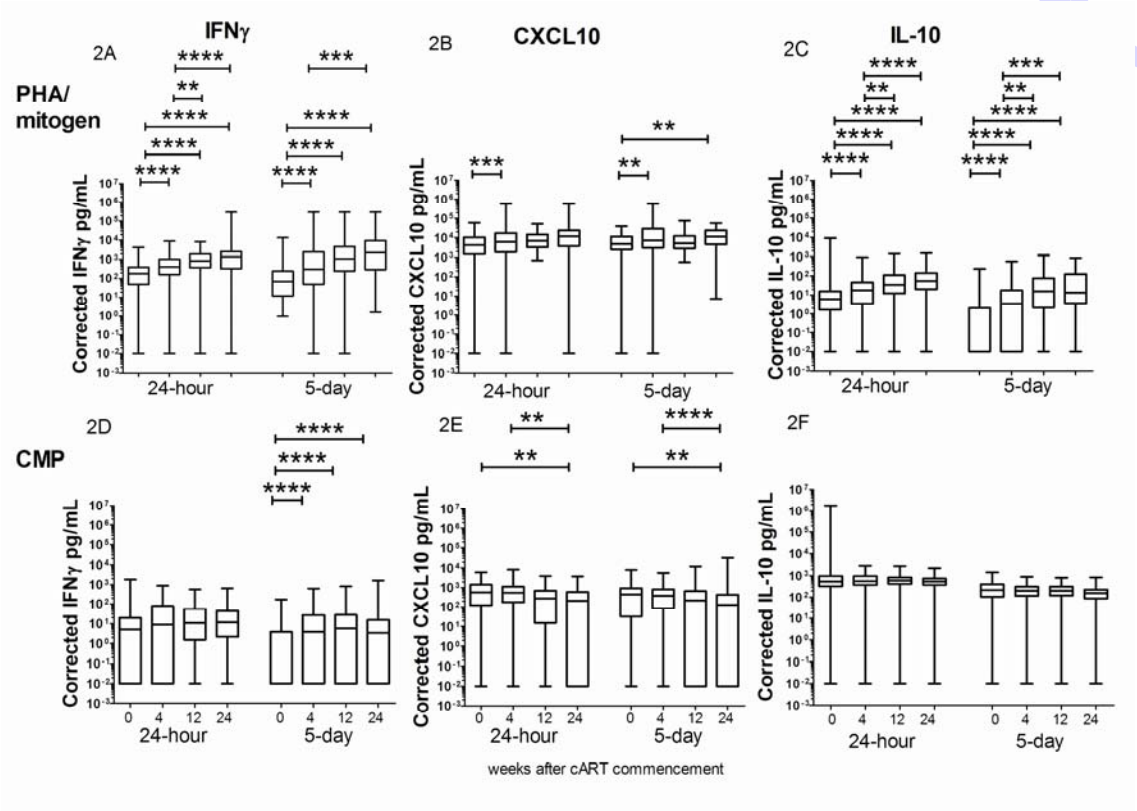
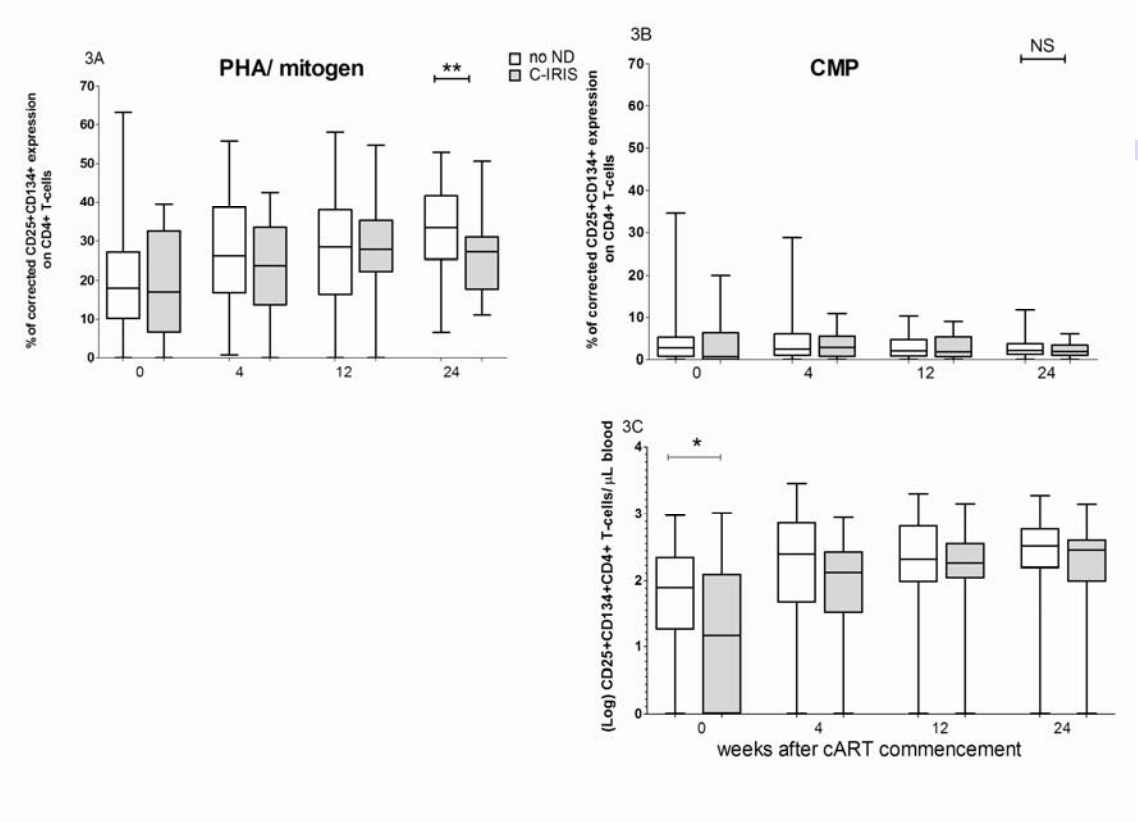
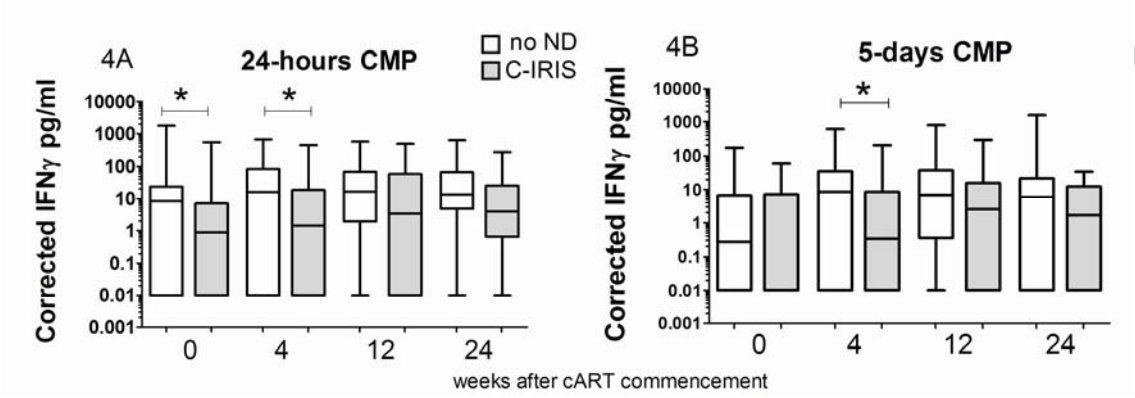
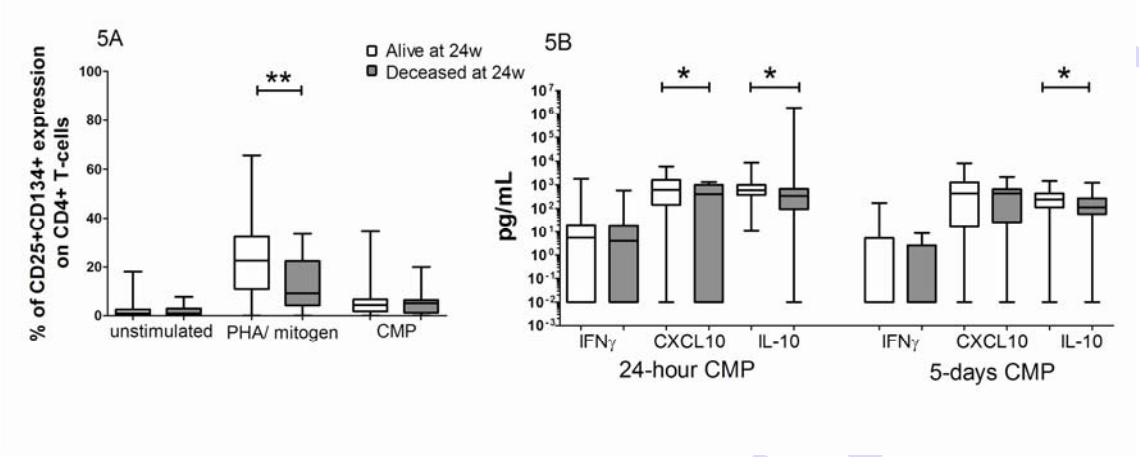
William Carr has moved to the Biology Department at Medgar Evers College, City University of New York since the completion of the study.











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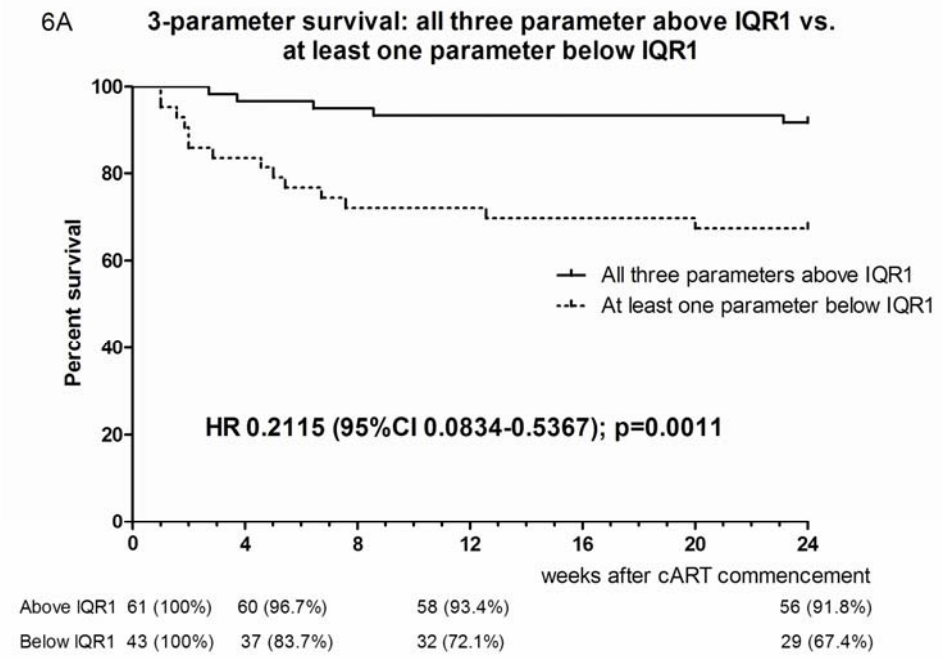

Above IQR1	61 (100%)	60 (96.7%)	58 (93.4%)	56 (91.8%)
Below IQR1	43 (100%)	37 (83.7%)	32 (72.1%)	29 (67.4%)