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3 **Biomarkers of CD4+ T cell activation as risk factors for tuberculosis-associated**
4 **Immune Reconstitution Inflammatory Syndrome**

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ABSTRACT

Objective: Patients co-infected with HIV and *Mycobacterium tuberculosis* frequently experience a paradoxical worsening of tuberculosis (TB) symptoms early after the initiation of combination antiretroviral therapy (cART). This immune reconstitution inflammatory syndrome (TB-IRIS) can lead to significant morbidity and needs to be distinguished from TB recurrence due to ineffective treatment. We investigated whether plasma biomarkers could predict the occurrence of TB-IRIS.

Design: ANRS 129 BKVIR is a single-arm multi-center trial that enrolled 69 cART naive HIV-1 infected patients treated for TB. The patients received once daily tenofovir/emtricitabine/efavirenz first-line regimen. TB-IRIS cases (IRIS+) were validated by an Event Review Committee.

Methods: A panel of 26 plasma biomarkers was monitored longitudinally for 24 weeks from cART initiation onward, using multiplexed assays and high-sensitivity ELISA. Statistical analyses of biomarkers were adjusted for test multiplicity.

Results: A third of patients (n=23) experienced TB-IRIS. The inflammatory cytokines and chemokines IL-6, IL-8/CXCL8, IP-10/CXCL10, and TNF- α showed increased plasma levels at wk4 in IRIS+ patients (P<0.05 for each biomarker). The soluble IL-2 receptor sCD25, which is released upon CD4+ T cell activation, was significantly increased at wk0 in IRIS+ patients (P<0.05), and remained elevated throughout follow-up. IL-7, a key homeostatic cytokine for CD4+ T cells, showed a trend for higher values in the TB-IRIS group. Both sCD25 and IL-7 baseline levels were independently associated with a shorter time to TB-IRIS occurrence (P=0.005 and P=0.02, respectively).

Conclusions: These findings support a role for CD4+ T cell activation prior to massive inflammation in the development of TB-IRIS.

Keywords: Immune Reconstitution Inflammatory Syndrome; HIV; tuberculosis; biomarker; IL-7; soluble IL-2 receptor.

71 INTRODUCTION

72 Co-infection with *Mycobacterium tuberculosis* (*Mtb*) and HIV represents a major threat to
73 public health worldwide [1]. Tuberculosis (TB) is frequent and severe in HIV-infected
74 patients, who often present with atypical symptoms and extra-pulmonary *Mtb* dissemination
75 [2, 3]. TB treatment is more difficult to manage in HIV-infected patients due to
76 pharmacological interactions with antiretroviral drugs and to a higher frequency of multi-drug
77 resistant TB strains [4]. Moreover, the treatment of HIV and *Mtb* co-infected patients can be
78 complicated by the occurrence of an Immune Reconstitution Inflammatory Syndrome (IRIS),
79 which is manifested by a recurrence of TB symptoms after the initiation of combination
80 antiretroviral therapy (cART) [5, 6]. Two major forms of TB-IRIS can be distinguished,
81 including a "paradoxical" form occurring in patients who were previously treated for TB, and
82 an "unmasking" form occurring in patients not known to be infected with *Mtb* and in whom TB
83 symptoms are recognized only after cART initiation. TB-IRIS causes significant morbidity,
84 especially in resource-limited settings, and is life-threatening when associated with
85 meningeal or cerebral granulomatous reaction [7-9].

86
87 IRIS is thought to result from a rapid recovery of immune responses against opportunistic
88 pathogens, resulting in a massive inflammatory reaction directed against pathogen-loaded
89 tissues. The factors most commonly associated with IRIS occurrence are a high pathogen
90 burden and an advanced state of immunosuppression, as indicated by a very low CD4+ T
91 cell count at cART initiation [10, 11]. An immunological hallmark of IRIS is the rapid
92 expansion of CD4+ T cells specific for the causal opportunistic pathogen, while CD4+ T cells
93 specific for HIV remain stable [12, 13]. During TB-IRIS, CD4+ T cells specific for Purified
94 Protein Derivative (PPD) antigens show massive proliferation, activation, and release of Th1-
95 type cytokines [14, 15]. Macrophages also appear involved in IRIS pathogenesis, as
96 suggested by marked increases in inflammatory markers such as interleukin (IL)-6, tumor
97 necrosis factor (TNF)- α and C-reactive protein (CRP) [16-18] and by accumulation of CD68+
98 macrophages at IRIS lesion sites [19, 20]. The anti-inflammatory cytokine IL-10 is also
99 increased in the course of TB-IRIS, which may reflect the activation of immunoregulatory
100 mechanisms counteracting the deleterious effects of inflammation [21]. Finally, innate
101 immune cells other than macrophages may also play a role in IRIS pathogenesis, as
102 evidenced by an increase in KIR-negative $\gamma\delta$ T cells [14], a higher degranulation capacity of
103 natural killer cells [11], and a higher expression of TLR2 on myeloid dendritic cells [22] at
104 cART initiation in patients who subsequently develop TB-IRIS.

105
106 Though helpful IRIS clinical criteria have been validated by the International Network for the
107 Study of HIV-associated IRIS (INSHI) [7], the diagnosis of TB-IRIS is not straightforward [23,

108 24]. It is necessary to distinguish TB-IRIS from a recurrence of TB due to drug resistance or
109 non-observance of anti-mycobacterial treatment, and to rule out the onset of a new
110 opportunistic infection. Defining parameters predictive of IRIS occurrence represents an
111 important objective, as these may impact case management. Several plasma biomarkers
112 measured before or at the time of cART initiation have shown significant changes in patients
113 who subsequently developed TB-IRIS, though a consensus on the most informative markers
114 remains to be established. The combination of an increase in IL-18 and IP-10/CXCL10 with a
115 decrease of CCL2 was predictive of paradoxical TB-IRIS in a cohort of Cambodian patients
116 [25]. In two studies, markers of inflammation, such as CRP, D-dimer, or IL-6 were increased
117 at baseline in patients who developed IRIS [26, 27]. In a third study, increased levels of
118 plasma IFN- γ and CRP were predictive of unmasking TB-IRIS, but not of paradoxical TB-
119 IRIS [28]. Thus, while both Th1-derived cytokines and innate inflammatory markers appear
120 dysregulated in IRIS pathogenesis [16], there is a need to further define predictive markers of
121 IRIS and to characterize the chain of events leading to the cytokine storm characteristic of
122 TB-IRIS.

123 124 125 **METHODS**

126 **Study population**

127 ANRS 129 BKVIR was a phase II single-arm multi-center trial that enrolled 69 antiretroviral
128 naive HIV-1 infected patients treated for TB in France [29]. Briefly, eligible patients were HIV-
129 1 infected individuals aged ≥ 18 years, with microbiologically or histologically confirmed TB,
130 treated with anti-tuberculosis drugs for less than 12 weeks, and naive of cART. The CD4+ T
131 cell counts were not a criterion for inclusion in the trial. Patients received a first-line cART
132 regimen consisting in a once-daily combination of tenofovir DF (300 mg/day)/emtricitabine
133 (200 mg/day) and efavirenz (800mg/day if treated by rifampin, otherwise 600 mg/day). TB-
134 IRIS cases (IRIS+) were classified according to the criteria defined by French et al. [30] and
135 by the INSHI [7]. All TB-IRIS cases were validated by an Event Review Committee, based on
136 a detailed review of medical charts. Written informed consent was obtained for all trial
137 participants. The ANRS 129 BKVIR trial and the BKVIR-CYTOK sub-study were approved by
138 the ethics committee "Comité de Protection des Personnes CPP Ile de France II".

139 140 **Biomarker measurements**

141 Biomarkers were monitored longitudinally at cART initiation, at week 0 (wk0), and at wk4,
142 wk12 and wk24 on plasma samples stored at -80°C . A panel of 26 biomarkers, including
143 homeostatic cytokines, inflammatory cytokines and chemokines, was tested by multiplex
144 assay (Milliplex, Millipore). The panel included Flt-3L; G-CSF; GM-CSF; IFN- $\alpha 2$; IFN- γ ; IL-1 α ;

145 IL-1 α ; IL-2; IL-4; IL-5; IL-6; IL-8/CXCL8; IL-9; IL-10; IL-12 (p40); IL-12 (p70); IL-15; IL-17; IP-
146 10/CXCL10; MCP-1/CCL2; MIP-1 β /CCL4; sCD40L; sIL-2R α ; TGF- α ; TNF- α ; VEGF. Plasma
147 samples collected on EDTA (100 μ l) were tested in duplicate for the 26 biomarkers
148 simultaneously, using a Luminex 100 reader (GMI, Inc.). Data was analyzed in the
149 Starstation 2.3 software (Applied Cytometry Systems). Concentrations were derived from
150 fluorescence intensities (FI) by 5-parameters logistic curves. The cytokine IL-7 was
151 measured separately on duplicate samples by a high-sensitivity ELISA assay (Quantikine HS
152 kit, R&D systems). Multiplex and ELISA assays were done in parallel on plasma samples
153 that were thawed just once to optimize the sensitivity of biomarker measurements.

154

155 **Statistical analysis**

156 Comparisons of clinical parameters between the IRIS+ and IRIS- groups were made by
157 Mann-Whitney tests for quantitative variables and χ^2 tests for qualitative variables.
158 Analyses of biomarkers were performed on fluorescence intensities (FI) and derived
159 concentrations. Descriptive analyses were done for all biomarkers, but statistical
160 comparisons were limited to 11 pre-defined biomarkers of interest (IFN- γ ; IL-1 α ; IL-1 α ; IL-12
161 (p40); IL-6; IL-7; IL-8; IP-10; sCD40L; sIL-2R α ; TNF- α). Levels of these biomarkers were
162 compared between the IRIS+ and IRIS- groups at four time points by Mann-Whitney tests,
163 with an adjustment of P-values for test multiplicity (adaptive false discovery rate, FDR) [31].
164 P-values based on FI were considered the primary analyses, given that statistical
165 comparisons of concentrations could be limited by missing or censored values [32]. Cox
166 proportional hazards models with a step-wise backward selection procedure were performed
167 to determine whether a panel of three biomarkers chosen on a pathophysiologic basis (IL-7;
168 IP-10; sIL-2R α) allowed to predict the time to IRIS occurrence. A logistic regression, that
169 modeled IRIS occurrence independently of time, was used as robustness analysis. The
170 kinetics of IP-10 decrease was evaluated with a linear mixed effect model. All statistical
171 analyses were performed with SAS software version 9.1.3 (SAS Institute Inc., Cary, NC,
172 USA).

173

174

175 **RESULTS**

176

177 ***Frequent and rapid occurrence of TB-IRIS***

178 The characteristics of the 69 patients co-infected with HIV-1 and *Mtb* are reported in Table 1.
179 Most patients were male and half of them (54%) were born in Sub-Saharan Africa. CD4+ T
180 cell counts were generally low (median: 74/mm³) and a majority of patients had disseminated
181 TB (71%), indicative of advanced immunodeficiency. A third of patients (33%, n=23)

182 developed TB IRIS after cART initiation. Five IRIS events were considered serious, including
183 two acute renal failures, two cerebral tuberculoma, including one with seizures and one with
184 intracranial hypertension, and one segmental bronchial compression. Baseline plasma HIV-1
185 RNA was slightly higher in patients with than without IRIS (5.5 versus 5.1 log₁₀ copies/mL,
186 p=0.020). The median period between start of anti-mycobacterial therapy and cART was
187 shorter in patients with than without IRIS (46 vs 61 days, p=0.026). Following cART initiation,
188 the median time to IRIS onset was 8 days (interquartile range (IQR): 5-14), and 87% of IRIS
189 cases occurred before wk4.

190

191 ***Induction of inflammatory mediators during TB-IRIS***

192 Five biomarkers showed significant differences between the IRIS+ (n=23) and IRIS- (n=46)
193 groups of patients. Table 2 summarizes data for these markers at early time points (wk0 and
194 wk4). Four biomarkers characteristic of inflammation showed an increased plasma
195 concentration at wk4 in the IRIS+ group compared to the IRIS- group: IL-6, TNF- α , IL-
196 8/CXCL8, and IP-10/CXCL10 (Fig. 1). At cART initiation (wk0), the levels of these
197 inflammatory mediators did not differ between IRIS+ and IRIS- patients. IL-6, IL-8, and TNF-
198 α were rapidly induced in the IRIS+ group during the first weeks of cART (Fig. 1A).
199 Subsequently, IL-6 returned to baseline levels at wk12, while IL-8 and TNF- α showed a
200 trend for increased levels in the IRIS+ group throughout follow-up. Thus, IRIS was
201 characterized by a massive inflammation that persisted to some extent for up to 6 months.

202

203 The chemokine IP-10 did not follow the same kinetics as the other inflammatory mediators,
204 but rather decreased continuously from wk0 to wk12. In the IRIS- group, the IP-10 plasma
205 concentration decreased in parallel with HIV-1 RNA after cART initiation, while the decrease
206 appeared slower in the IRIS+ group (Fig.1B). Analysis by a linear mixed effect model showed
207 that, in the IRIS- group, the IP-10 concentration decreased significantly between wk0 and
208 wk4 (-384 pg/ml per week [95% confidence interval (CI): -612;-156], p=0.0013), as well as
209 between wk4 and wk12 (-158 pg/ml per week [95% CI: -222;-94], p<0.0001). The decrease
210 in IP-10 appeared less marked in the IRIS+ group (p=0.098) during the first 4 weeks, with a
211 mean loss of -52 pg/ml per week (95% CI: -374;+270). Between wk4 and wk12, changes in
212 IP-10 concentration were comparable between both groups (p=0.30), with a mean loss of -
213 216 pg/ml per week (95% CI: -305;-127) in the IRIS+ group. Thus, while baseline IP-10
214 levels did not differ between groups, the decrease after cART initiation tended to be slower in
215 the IRIS+ group. In contrast, the kinetics of HIV-1 RNA decrease was superimposable in
216 patients with and without IRIS (Fig. 1C), suggesting a comparable efficacy of cART regimen
217 in both groups.

218

219 ***Increased markers of CD4+ T cell activation in TB-IRIS patients***

220 The homeostatic cytokine IL-7 was studied because of its key role in CD4+ T cell survival
221 and proliferation [33]. As preliminary experiments showed that the sensitivity of IL-7 detection
222 was low in multiplexed assays, we measured IL-7 separately by a high sensitivity ELISA
223 assay. IL-7 showed a non-significant trend for persistently higher levels in the IRIS+ group at
224 wk0 and throughout follow-up (Fig. 2A). CD4+ T cell counts did not differ between the IRIS+
225 and IRIS- groups at wk0 as well as during the immune reconstitution phase (Fig. 2B). Thus,
226 the trend for higher plasma IL-7 levels in the IRIS+ group could not be explained by a smaller
227 CD4+ T cell pool that would have resulted in decreased IL-7 consumption.

228

229 The alpha chain of the IL-2 receptor, CD25/IL-2R α , is predominantly expressed by CD4+ T
230 cells and is released by proteolytic cleavage from the surface of activated T cells [34, 35].
231 The baseline plasma level of soluble CD25 (sCD25) was significantly higher in the IRIS+
232 group as compared to the IRIS- group, and showed further increase with a peak at wk4 and
233 a progressive decline thereafter (Fig. 4C). The concentrations of sCD25 remained
234 significantly higher in the IRIS+ group throughout follow-up, pointing to T cell activation as a
235 major contributor to IRIS pathogenesis.

236

237 ***IL-7 and sCD25 levels are independently associated with TB-IRIS occurrence***

238 We next studied whether the baseline concentration of the IL-7, sCD25, and IP-10 markers
239 were associated with the subsequent occurrence of TB-IRIS. Baseline values of two bio-
240 clinical parameters, hemoglobin and body mass index (BMI), were also included in the model
241 (supplementary Table 1), as these parameters had been found to be associated with TB-IRIS
242 occurrence in multivariable analyses of the BKVIR core trial [29]. In the final Cox proportional
243 hazards model (Table 3A), only IL-7 ($p=0.022$) and sCD25 ($p=0.005$) were independently
244 associated with TB-IRIS occurrence. Robustness analysis using a logistic regression model
245 yielded consistent results (Table 3A).

246

247 ***Baseline IL-7 determines inflammatory cytokine induction***

248 We then determined whether the baseline levels of IL-7 and sCD25, as well as hemoglobin
249 and BMI, were associated with the levels of the three inflammatory cytokines/chemokines
250 that were significantly increased at wk4 in the IRIS+ group (IL-6, IL-8, TNF- α). In the linear
251 regression models, IL-7 baseline values at wk0 were positively associated with IL-6, IL-8,
252 and TNF- α levels at wk4 (Table 3B). In contrast, no significant associations with sCD25
253 baseline levels were detected. Of the two clinical parameters, only baseline hemoglobin
254 showed an independent association with inflammatory cytokines levels at wk4. Taken

255 together, these findings suggested a role for IL-7 availability in the induction of inflammation
256 during TB-IRIS.

257

258 **DISCUSSION**

259 In this study, we analyzed plasma biomarkers in naïve HIV-1 infected patients with confirmed
260 *Mtb* infection and who initiated cART. The high rate of TB-IRIS occurrence in this well-
261 characterized study population allowed the identification of biomarkers associated with IRIS.
262 The plasma levels of the cytokines and chemokines IL-6, IL-8, TNF- α , and IP-10 were
263 significantly increased during TB-IRIS, consistent with an underlying role of the inflammatory
264 response in the pathogenesis of IRIS. However, the biomarkers that predicted IRIS
265 occurrence at the time of cART initiation were those associated with CD4+ T cell activation
266 rather than the inflammatory mediators. Indeed, both IL-7 and sCD25 were independently
267 associated with TB-IRIS occurrence. IL-7 is a γc family cytokine that is considered as the
268 master regulator of CD4+ T cell homeostasis, by promoting T cell survival, activation, and
269 proliferation [36]. As such, IL-7 may contribute to the massive CD4+ T cell proliferation that
270 characterizes the acute phase of TB-IRIS [12, 13]. The soluble form of the IL-2 receptor,
271 sCD25, is primarily released by activated CD4+ T cells through proteolytic processing [34,
272 35], and is known to be increased in infectious, autoimmune, and malignant diseases
273 characterized by chronic T cell activation [34, 37, 38]. Thus, our findings support a key role
274 for CD4+ T cell activation in the pathogenesis of IRIS.

275

276 The sequential increase of sCD25 and IL-7 followed by inflammatory markers in patients with
277 TB-IRIS suggest a two-stage pathogenic process where CD4+ T cell activation precedes
278 inflammation. This notion is supported by the positive association between IL-7 baseline
279 values at wk0 and inflammatory cytokine values at wk4, a time point by which the majority of
280 IRIS-TB events had occurred. Advanced HIV infection is known to be associated with a
281 chronic activation of dysfunctional T cells that show signs of functional exhaustion and
282 increased apoptosis [39]. Abundant mycobacterial antigens present in TB-infected patients
283 may further drive the activation of CD4+ T cells that remain dysfunctional and fail to
284 proliferate during the phase of active HIV replication. Our findings suggest that patients with
285 higher levels of CD4+ T cell activation at cART initiation are more prone to develop TB-IRIS,
286 possibly because of a rapid functional recovery of this pre-activated CD4+ T cell population.
287 The notion of a prominent but abnormal CD4+ T cell activation prior to IRIS is supported by a
288 study showing increased levels of the inhibitory costimulatory molecules PD-1, LAG-3, and
289 CTLA-4 in CD4+ T cells of patients before IRIS occurrence [40].

290

291 Increased IL-7 levels may also prime CD4⁺ T cells for rapid proliferation once viral replication
292 is controlled. We and others have shown that IL-7 responses are impaired in CD4⁺ T cells of
293 patients with detectable HIV load, due to IL-7 receptor down-regulation [41] and perturbed IL-
294 7 downstream signaling [42, 43]. Importantly, functional IL-7 responses are recovered under
295 cART, allowing for the reconstitution of the CD4⁺ T cell pool. Higher IL-7 levels in patients
296 with IRIS likely contribute to the massive proliferation of *Mtb*-specific CD4⁺ T cells that
297 occurs once cART is initiated [14, 15]. Consistent with our results, two studies found
298 evidence for persistently elevated IL-7 concentrations in patients with IRIS [40, 44],
299 suggesting a prolonged effect of this cytokine. The determinants of increased IL-7 levels in
300 patients with IRIS remain to be elucidated. An inverse correlation between CD4⁺ T cell
301 counts and IL-7 levels has been documented in HIV-infected patients, suggesting that
302 increases in IL-7 result from a decreased consumption of this cytokine by CD4⁺ T cells [45].
303 Increased IL-7 levels would in turn promote CD4⁺ T cell survival and proliferation,
304 contributing to the reconstitution of CD4⁺ T cell population. While this homeostatic
305 mechanism is likely at work in IRIS patients, it is not sufficient to account for the higher IL-7
306 levels, given that CD4⁺ T cell counts were not significantly lower than in patients without
307 IRIS. IL-7 had long been thought to be constitutively produced by stromal cells within
308 lymphoid organs, but recent studies have revealed that hepatocytes are capable of inducible
309 IL-7 production when stimulated by type I interferons or inflammatory mediators [46]. In
310 addition, specialized lymph node and thymic stromal cells, as well as lymphatic endothelial
311 cells, can increase their IL-7 production in response to lymphopenia [47, 48]. Thus, the
312 possibility that patients with higher inducible IL-7 production may be more susceptible to TB-
313 IRIS warrants further investigation.

314

315 The nature of the cytokines and chemokines induced during TB-IRIS, including TNF- α , IL-6,
316 and IL-8, is indicative of inflammation mediated primarily by macrophages, though the
317 contribution of other innate cells and of stromal cells is also possible. These findings are in
318 agreement with studies showing increases in inflammatory mediators during IRIS induced by
319 *Mtb* [25, 28] and other opportunistic pathogens such as *Pneumocystis jiroveci* and
320 *Cryptococcus neoformans* [49, 50]. Considering that memory CD4⁺ T cells are potent
321 activators of macrophages [51], it is likely that the rapid recovery of CD4⁺ T cell helper
322 functions under cART contributes to the activation of macrophages loaded with *Mtb* and
323 underlies the massive inflammation characteristic of IRIS. This two-step scenario would
324 explain in particular why CD4⁺ T cell activation precedes that of macrophages in TB-IRIS.
325 This notion is also compatible with the model of IRIS pathogenesis recently proposed by
326 Barber *et al.*, based on experiments in T cell deficient mice infected with *M. avium* [17, 52]. In
327 this model, IRIS results from a dysregulated response of macrophages that were primed in

328 the absence of CD4+ T cell help, with the sudden transfer of CD4+ T cells leading to
329 macrophage hyper-responsiveness and tissue-destructive inflammation. While this model
330 strongly supports a role for macrophage dysregulation in IRIS pathogenesis, it does not
331 account for the increased activation of CD4+ T cells prior to IRIS. One possibility is that *Mtb*-
332 specific CD4+ T cells also undergo prolonged priming in suboptimal conditions during the
333 stage of active HIV replication, resulting in dysregulated Th1 responses after cART initiation.

334

335 Of note, a study of TB-IRIS found that a combination of increased IL-18 and IP-10 levels,
336 with decreased CCL2 levels, was predictive of TB-IRIS occurrence [25]. These findings
337 suggest that some but not all inflammatory mediators may be induced prior to IRIS,
338 consistent with macrophage dysregulation. We did not detect increased IP-10 levels at
339 baseline in the present study, possibly because patients in the BKVIR trial had slightly higher
340 CD4+ T cell counts, or because of differences in experimental setup, as we measured IP-10
341 directly in plasma rather than after overnight culture [25]. Interestingly, IP-10 showed a
342 different kinetics than other inflammatory mediators, with a high concentration at baseline
343 and a continuous decrease that paralleled that of the viral load. The induction of IP-10 in
344 situations of high viral load, which has also been reported during primary HIV infection [53],
345 may be explained by the facts that IP-10 is a chemokine primarily induced by type 1 and type
346 2 interferons [54], and that HIV is both a direct inductor of type 1 interferons through the
347 innate response and of type 2 interferon through the adaptive response. The observation that
348 IP-10 levels decreased more slowly in patients with IRIS suggests that the persistence of an
349 interferon response may contribute to the pathogenesis of IRIS. Persistently high levels of IP-
350 10 in patients with IRIS is a consistent finding, as it has been observed in TB-IRIS [16, 25]
351 but also in IRIS triggered by other pathogens. For instance, HIV/HBV coinfecting patients who
352 develop hepatic flares after cART initiation show a persisting increase of IP-10, while patients
353 without signs of hepatic inflammation maintain moderate levels of the chemokine [55]. As
354 HIV loads are rapidly decreasing during IRIS, the persistence of IP-10 may not be virally
355 driven, but may rather reflect a strong IFN- γ -dependent adaptive response. In the present
356 study, while we did not detect significant differences between the IRIS+ and IRIS- groups in
357 IFN- α 2 and IFN- γ levels, we noted a trend for higher IFN- γ values at wk4 in IRIS patients.
358 Approaches based on the analysis of IFN- γ induction after PPD antigenic stimulation in vitro
359 have documented a consistent increase in IFN- γ secretion during TB-IRIS [12, 13, 15, 56].
360 Thus, the persistence of high IP-10 levels may be a sensitive indicator of an ongoing Th1
361 response. Considering that IP-10 plays an important role in the differentiation of Th1 cells
362 within lymphoid tissues [57], and in the recruitment of effector T cells to inflamed tissues [54],
363 it is likely that this chemokine contributes to a positive feedback loop driving Th1 cell

364 activation during TB-IRIS. Our study has limitations due to the relatively limited size of the
365 cohort studied and the fact that patient peripheral blood cells were not available for analyses.
366 Therefore, it will be important in future studies to include an analysis of CD4+ T cell
367 phenotype and cytokine secretion capacity, to confirm a predominant role of activated Th1
368 cells in triggering and sustaining TB-IRIS.

369

370 Patients who developed TB-IRIS in the ANRS 129 BKVIR trial had slightly higher viral loads
371 than patients who did not show signs of TB-IRIS. One reason may be that HIV directly
372 contributes to the abnormal activation that primes immune cells for TB-IRIS. In addition, a
373 higher viral burden may be associated with a more advanced stage of TB, and hence with a
374 higher load of *Mtb* antigens available to prime a dysregulated immune response.
375 Mycobacteria can in turn promote HIV replication through the release of TNF- α [58], resulting
376 in synergistic interactions between the two pathogens. Patients in the IRIS+ group also had a
377 shorter delay between the initiation of anti-TB therapy and cART than patients in the IRIS-
378 group, a finding in agreement with published studies [23, 59-61], and which may reflect the
379 lower load of *Mtb* antigens in patients who have been treated for a longer time with
380 antituberculous drugs. Importantly, large clinical trials have demonstrated that even though
381 shortening the delay to cART to 2-4 weeks increases the risk of TB-IRIS in HIV/*Mtb*
382 coinfecting patients, this approach is beneficial in patients with very low CD4+ T cell counts
383 (<50 cells/mm³), as it limits AIDS-associated mortality [59-61]. Thus, as early cART emerges
384 as the strategy of choice in HIV/*Mtb* coinfecting patients with advanced immunosuppression,
385 it becomes all the more important to validate predictive markers that can help focus limited
386 medical resources on patients at high risk of TB-IRIS.

387

388 In conclusion, this study shows that patients at high-risk of TB-IRIS have signs of preexisting
389 CD4+ T cell activation, as indicated by increased circulating levels of sCD25 and increased
390 availability of IL-7. After cART initiation, TB-IRIS patients showed rapid increases of the
391 inflammatory mediators IL-6, IL-8, TNF- α , and IP-10 in plasma. These findings support a role
392 for CD4+ T cell activation prior to massive inflammation in the development of TB-IRIS.
393 Further validation of these results on an independent cohort of patients should help
394 determine the potential of sCD25 and IL-7 as predictive biomarkers of patients at high risk of
395 TB-IRIS.

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409

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413 L.R., and G.C. analyzed the data. L.C., C.B., and L.R. wrote the manuscript. All authors read
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606 **LEGEND TO THE FIGURES**

607 **Figure 1: Induction of inflammatory mediators during IRIS**

608 (A and B): The median and interquartile range (IQR) of biomarker concentrations are
609 reported in pg/ml for patients in the IRIS+ group (thick black line) and the IRIS- group (thin
610 grey line) at the following time points: wk0, wk4, wk12, and wk24. A significant P value
611 ($P < 0.05$) after adjustment for test multiplicity is indicated by an asterisk.

612 (C) The viral load in the IRIS+ and IRIS- groups, measured as the log₁₀ of the HIV-1 RNA
613 copies/ml plasma, was followed for 48 weeks after cART initiation at week 0. Median and
614 IQR are reported on the graph.

615

616 **Figure 2: Induction of CD4+ T cell activation markers before and during TB-IRIS**

617 (A and C): The median and interquartile range (IQR) of biomarker concentrations are
618 reported in pg/ml for patients in the IRIS+ group (thick black line) and the IRIS- group (thin
619 grey line) at the following time points: wk0, wk4, wk12, and wk24. A significant P value
620 ($P < 0.05$) after adjustment for test multiplicity is indicated by an asterisk. A P value < 0.01 after
621 adjustment for test multiplicity is indicated by a double asterisk.

622 (B) Changes in the number of CD4+ T cell lymphocytes per mm³ blood were followed for 48
623 weeks after cART initiation at week 0. Median and IQR are reported on the graph.

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Characteristics of patients			Total	IRIS+	IRIS-	P value*
			(N=69)	(N=23)	(N=46)	
Male	n	(%)	49 (71%)	16 (70%)	33 (72%)	0.85
Age (years)	median	(IQR)	43 (34-52)	48 (34-55)	39 (34-48)	0.47
Sub-Saharan African origin	n	(%)	37 (54%)	10 (43%)	27 (59%)	0.23
Body Mass Index (kg/m ²)	median	(IQR)	21 (19-24)	21 (17-24)	22 (19-24)	0.09
Disseminated tuberculosis	n	(%)	49 (71%)	19 (83%)	30 (65%)	0.13
CD4+ T cells at Wk0	median	(IQR)	74 (23-159)	69 (23-135)	85 (20-201)	0.52
HIV Viral load at Wk0 (log ₁₀ HIV-1 RNA copies/ml)	median	(IQR)	5.4 (4.9-5.9)	5.5 (5.2-6.1)	5.1 (4.6-5.7)	0.02
Delay between anti-TB therapy and ART (days)	median	(IQR)	56 (37-73)	46 (34-56)	61 (43-74)	0.03

Table 1: Clinical and virological characteristics of patients with and without TB-IRIS - ANRS 129 BKVIR-CYTOK study

Demographic, clinical, and virological characteristics are reported for all patients included in the study (Total), and separately for patients with and without TB-IRIS (IRIS+ and IRIS- groups, respectively).

*: Raw P values are reported. P values corresponding to significant differences (<0.05) between the last two groups are indicated in bold.

Time point	Biomarker	IRIS+		IRIS-		Raw P-value	Adaptive FDR P-value
		median (pg/ml)	IQR	Median (pg/ml)	IQR		
Wk 0	IL-6	4	[3-17]	3	[3-7]	0.22	0.23
Wk 0	IL-8	18	[3-31]	10	[3-21]	0.25	0.26
Wk 0	TNF- α	31	[22-50]	28	[20-41]	0.29	0.26
Wk 0	IP-10	3913	[2982-5748]	3830	[2411-5645]	0.43	0.32
Wk 0	IL-7	6	[5-11]	4	[2-7]	0.05	0.10
Wk 0	sCD25	127	[75-233]	69	[38-126]	0.02	0.047
Wk 0	IFN- γ	13	[8-35]	13	[8-22]	0.74	0.51
Wk 4	IL-6	10	[4-25]	4	[3-11]	<10⁻²	0.02
Wk 4	IL-8	28	[19-50]	13	[3-25]	<10⁻²	0.02
Wk 4	TNF- α	43	[27-75]	25	[15-41]	<10⁻²	0.02
Wk 4	IP-10	3226	[2078-6265]	1834	[1176-3567]	<10⁻²	0.03
Wk 4	IL-7	5	[3-10]	4	[2-7]	0.32	0.27
Wk 4	sCD25	253	[71-784]	53	[38-141]	<10⁻²	0.02
Wk 4	IFN- γ	27	[11-34]	12	[8-27]	0.08	0.11

Table 2: List of biomarkers that were increased in patients with TB-IRIS ANRS 129 BKVIR-CYTOK study

The median and interquartile range (IQR) of biomarker concentrations that were increased in TB-IRIS patients are reported for the wk0 (ART initiation) and wk4 time points. Concentrations are reported in pg/ml. P values obtained by comparing biomarker concentrations between the IRIS+ and IRIS- groups are reported before (raw P values) and after (adaptive FDR P values) adjustment for multiple comparisons. Significant P values (P<0.05) are reported in bold type.

A: Factors that predict IRIS occurrence**Cox proportional hazard model of time to IRIS occurrence**

Explanatory variables	Final multivariable model		
	HR	95% CI	P-value*
IL-7 level at wk0 (per additional log ₁₀ pg/ml)	5.03	(1.26 ; 20.00)	0.022
sCD25 level at wk0 (per additional log ₁₀ FI)	4.76	(1.59 ; 14.30)	0.005
IP-10 level at wk0 (per additional 1000 FI)	NA	NA	NA
Hemoglobin (per additional g/dL)	NA	NA	NA
BMI (per additional kg/m ²)	NA	NA	NA

Logistic regression model of IRIS occurrence

Explanatory variables	Final multivariable model		
	OR	95% CI	P-value*
IL-7 level at wk0 (per additional log ₁₀ pg/ml)	7.07	(1.12 ; 44.80)	0.038
sCD25 level at wk0 (per additional log ₁₀ FI)	6.12	(1.19 ; 31.40)	0.030
IP-10 level at wk0 (per additional 1000 FI)	NA	NA	NA
Hemoglobin (per additional g/dL)	NA	NA	NA
BMI (per additional kg/m ²)	NA	NA	NA

B: Factors that predict levels of inflammatory cytokines at wk4**Multiple linear regression model of IL-6 (log₁₀ FI) at wk4**

Explanatory variables	Beta	95% CI	P-value*
Intercept	1.98	(0.68 ; 3.28)	0.003
IL-7 level at wk0 (per additional log ₁₀ pg/ml)	0.26	(0.02 ; 0.50)	0.033
sCD25 level at wk0 (per additional log ₁₀ FI)	0.03	(-0.18 ; 0.25)	0.758
Hemoglobin at wk0 (per additional g/dL)	-0.07	(-0.13 ; -0.02)	0.015
BMI at wk0 (per additional kg/m ²)	-0.01	(-0.04 ; 0.01)	0.264

Multiple linear regression model of IL-8 (log₁₀ FI) at wk4

Explanatory variables	Beta	95% CI	P-value*
Intercept	2.54	(1.36 ; 3.73)	<0.001
IL-7 level at wk0 (per additional log ₁₀ pg/ml)	0.32	(0.10 ; 0.53)	0.005
sCD25 level at wk0 (per additional log ₁₀ FI)	-0.02	(-0.22 ; 0.18)	0.835
Hemoglobin at wk0 (per additional g/dL)	-0.06	(-0.11 ; 0.00)	0.036
BMI at wk0 (per additional kg/m ²)	-0.02	(-0.04 ; 0.00)	0.119

Multiple linear regression model of TNF- α (log₁₀ FI) at wk4

Explanatory variables	Beta	95% CI	P-value*
Intercept	2.31	(1.24 ; 3.38)	<0.001
IL-7 level at wk0 (per additional log ₁₀ pg/ml)	0.31	(0.12 ; 0.51)	0.002
sCD25 level at wk0 (per additional log ₁₀ FI)	0.07	(-0.11 ; 0.25)	0.444
Hemoglobin at wk0 (per additional g/dL)	-0.05	(-0.1 ; 0.00)	0.044
BMI at wk0 (per additional kg/m ²)	-0.01	(-0.03 ; 0.00)	0.129

Table 3: Baseline variables associated with IRIS occurrence (A) and with inflammatory cytokines at wk 4 (B)

ANRS 129 BKVIR-CYTOK study

(A): Logistic and Cox regression models were applied to determine whether a panel of 3 biomarkers chosen on a pathophysiologic basis (IL-7; IP-10; sCD25) at wk0 allowed to predict the occurrence of IRIS. Final multivariable models were obtained after step-wise backward selection procedure. Both models show that baseline sCD25 and IL-7 are independent predictors of IRIS occurrence.

The Cox proportional hazard model takes into account the time to IRIS occurrence, whereas the logistic model treats IRIS as a binary variable (occurrence of IRIS: yes or no). The hazard ratios (odds ratio in the logistic model) for the risk (odds in the logistic model) of developing IRIS per unit increase in the explanatory variables (measured at wk0) are reported.

(B) Multiple linear regression models were used to determine the variables predictive of the levels of three inflammatory biomarkers at wk4: IL-6, IL-8, and TNF- α . The full multivariable models are shown (no step-wise variable selection procedure). The parameter beta reflects the mean estimated change in the dependent variable (the given cytokine at wk4) per unit of the explanatory variable (measured at wk0).

*: Raw P values are reported. Significant P values are reported in bold. HR: hazard ratio. OR: odds ratio. CI: confidence interval. NA: not applicable. FI: fluorescence intensity. wk: week.

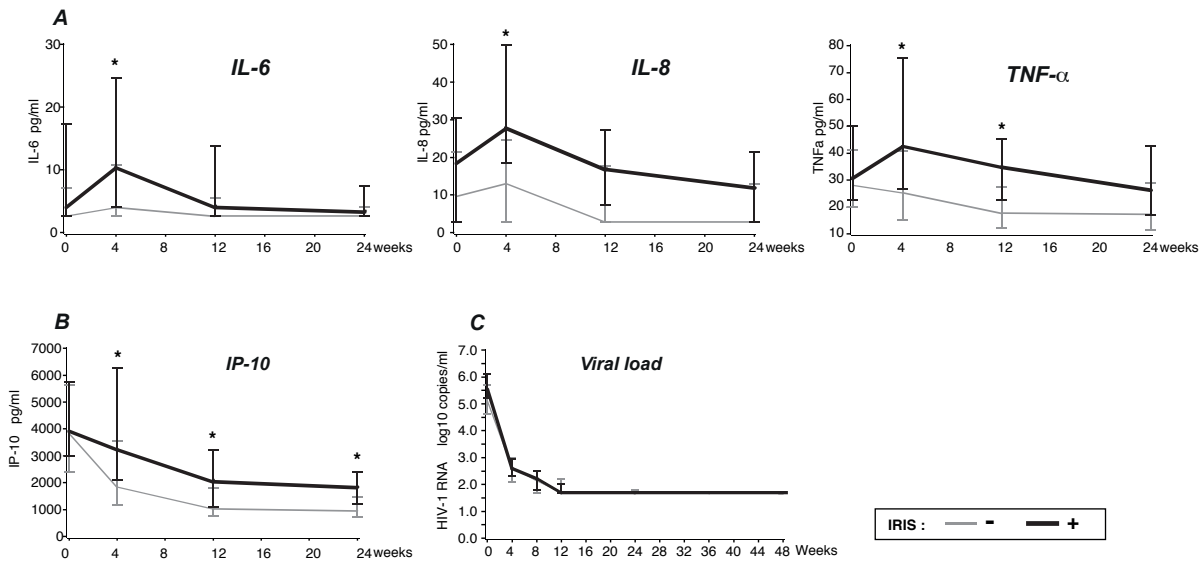


Figure 1: Induction of inflammatory mediators during IRIS ANRS 129 BKVIR-CYTOK study

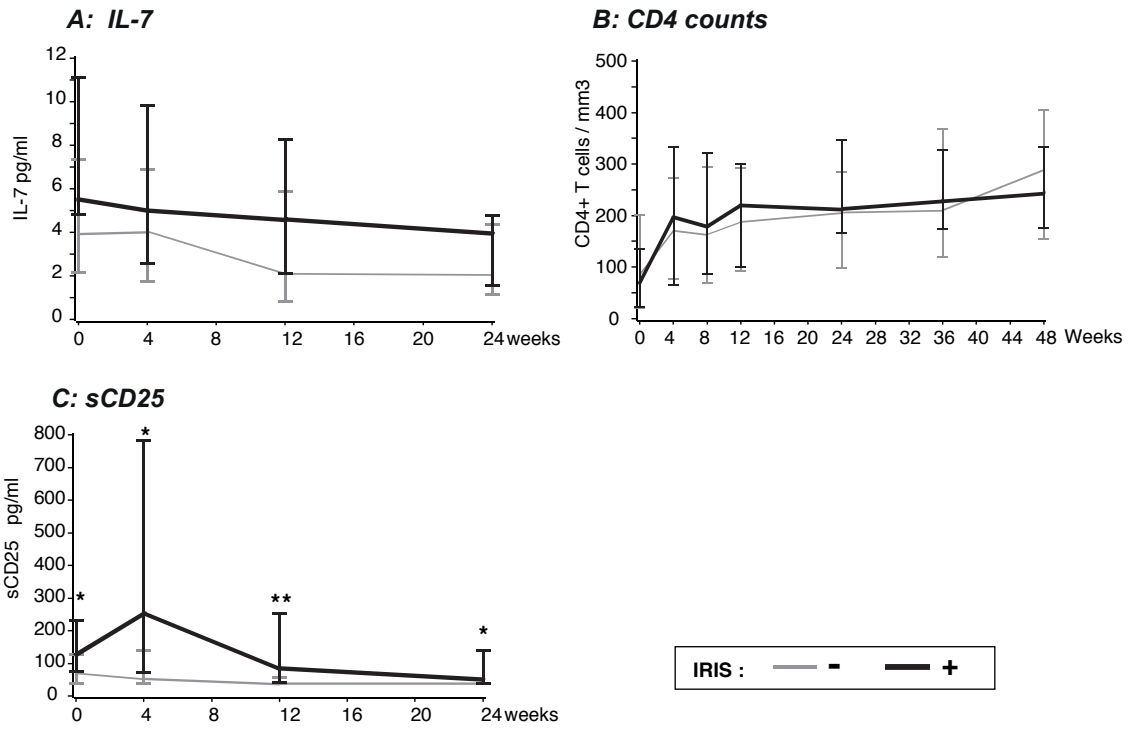


Figure 2: Induction of CD4+ T cell activation markers before and during TB-IRIS ANRS 129 BKVIR-CYTOK study

Cox proportional hazard models of time to IRIS occurrence

Explanatory variables	Univariable models		
	HR	95% CI	P-value*
IL-7 level at wk0 (per additional log ₁₀ pg/ml)	3.69	(0.97 ; 14.00)	0.055
sCD25 level at wk0 (per additional log ₁₀ FI)	3.40	(1.28 ; 9.05)	0.014
IP-10 level at wk0 (per additional 1000 FI)	1.06	(0.90 ; 1.23)	0.495
Hemoglobin (per additional g/dL)	0.78	(0.58 ; 1.04)	0.095
BMI (per additional kg/m ²)	0.89	(0.78 ; 1.02)	0.096

Logistic regression models of IRIS occurrence

Explanatory variables	Univariable models		
	OR	95% CI	P-value*
IL-7 level at wk0 (per additional log ₁₀ pg/ml)	5.57	(1.03 ; 30.10)	0.046
sCD25 level at wk0 (per additional log ₁₀ FI)	4.75	(1.13 ; 19.90)	0.033
IP-10 level at wk0 (per additional 1000 FI)	1.07	(0.87 ; 1.31)	0.517
Hemoglobin (per additional g/dL)	0.71	(0.50 ; 0.99)	0.047
BMI (per additional kg/m ²)	0.86	(0.74 ; 1.00)	0.056

Supplementary Table 1: Baseline variables associated with IRIS occurrence - Univariable models

ANRS 129 BKVIR-CYTOK study

(A): Logistic and Cox regression models were applied to determine whether a panel of 3 biomarkers chosen on a pathophysiologic basis (IL-7; IP-10; sCD25) at wk0 allowed to predict the occurrence of IRIS. The initial univariable models are reported in this table.

The Cox proportional hazard model takes into account the time to IRIS occurrence, whereas the logistic model treats IRIS as a binary variable (occurrence of IRIS: yes or no). The hazard ratios (odds ratio in the logistic model) for the risk (odds in the logistic model) of developing IRIS per unit increase in the explanatory variables (measured at wk0) are reported.

*: Raw P values are reported. Significant P values are reported in bold. HR: hazard ratio. OR: odds ratio. CI: confidence interval. NA: not applicable. FI: fluorescence intensity. wk: week.

Supplementary File 1:

ANRS 129 BKVIR TRIAL GROUP

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ANRS 129 BKVIR-CYTOK STUDY GROUP

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