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Title: Cytokine/chemokine secretion for detecting tuberculosis in quantiferon supernatants from HIV^{+} and HIV^{-} children

Running title: Biomarkers of tuberculosis in HIV-infected children

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Sir,

The QuantiFERON-Gold In Tube assay (QFT) relies on the detection of IFN-gamma secreted by immune T-cells after whole blood stimulation with ESAT-6, CFP-10 and TB7-7 mycobacterium tuberculosis (M.tb) antigens (Ag). Although QFT has proved more specific than the Tuberculin-Skin test (TST) for detecting tuberculosis (TB) in countries where TB incidence is low [1], its use in pediatric tuberculosis (TB) is limited by the lower sensitivity of the test in immunocompetent children younger than 5 years old and in HIV-infected children [2].

In this journal, we recently reported the potential added value of combining IFN-gamma, IP-10 and IL-13 releases for diagnosing latent TB in 0-5 years old immunocompetent children [3]. Here, we further explored the potential added value of these biomarkers in HIV-infected children. The study was conducted in Ile-de-France (France), a low TB burden area where the sensitivity of QFT to diagnose active TB is impaired in HIV-infected children (Hormi et al. Ped Infect Dis J 2017 *in press*).

Among 63 consecutive HIV^+ children who were referred to our center (hospital Robert-Debré Paris-France) for QFT reactivity analysis between January 2011 and November 2015, residual plasma from QFT were available for 12 HIV^+/TB^+ co-infected children (stage III of HIV-infection, n=11/12). At QFT sampling, four HIV^+/TB^+ children had untreated active TB (culture-confirmed, n=3/4). Four had untreated latent TB. Four have had previous active TB. Two other groups of children not-infected with HIV (HIV^-) that were evaluated for QFT reactivity during the same period as HIV^+/TB^+ children were included as reference; i) Twelve HIV^-/TB^+ children were used for comparison with the HIV^+/TB^+ co-infected group. Inclusion of HIV^-/TB^+ children was based, as far as possible, on a HIV^+/TB^+, HIV^-/TB^+ case-control design. ii) Eleven age-matched children who were presumed not-infected (HIV^-/TB^-) based on one-year follow-up QFT and/or TST negativity and normal chest x-ray in the

absence of anti-TB treatment (11/11) following a recent contact with a not-infectious (not-bacillary) index case (10/11) were included to identify biomarkers that discriminate TB^+ from TB^- children. Informed consent was obtained from the parents and the study was approved by the Robert Debré hospital local ethic committee.

According to selection design, HIV^+/TB^+ and HIV^-/TB^+ clinical groups displayed similar demographic and clinical main characteristics (median age: 10y6m and 8y11m; M/F gender: 7/5 and 6/6; birth and/or a long stay in a country with a high TB burden 7/12 and 6/12; clear documentation of BCG-vaccination 10/10 and 9/12 in HIV^+/TB^+ and HIV^-/TB^+ children respectively). Also, TB status was similar in the two groups (ongoing active TB before anti-TB treatment, n=4/12 and 3/12, untreated latent TB or past active TB n=8/12 and 9/12 children in HIV^+/TB^+ and HIV^-/TB^- respectively).

Lower M.tb-specific QFT value was observed in HIV^+/TB^+ children (median: 0.63 IU/ml) than among HIV^-/TB^+ children, median: 9.42 IU/ml, p=0.007). Overall, the sensitivity of QFT was 91% among HIV^-/TB^+ children (one negative result: 0.03 IU/ml) but 58% among HIV^+/TB^+ children (five negative results ranging from 0.00 to 0.01 IU/ml).

The accuracy of 18 cytokines/chemokines for diagnosing TB were evaluated using the human 17-Plex and IP-10 kits (Bio-Rad, Laboratories Inc., Marnes-La-Coquette, France) as previously described [4]. Cytokine/chemokine concentrations were measured in residual plasmas (cryopreserved at -80°C until use) from QFT (QuantiFERON GIT, Cellestis). Plasmas from the 3 QFT tubes (not-stimulated, M.tb Ag-stimulated and PHA-stimulated) were analyzed for each child.

Five biomarkers showing either concentrations lying outside an interpretable range (IL-8, MCP-1, MIP-1beta) or similar levels in all culture conditions (IL-7, IL-12) were excluded from analysis.

As shown in table 1, stimulation with M.tb Ag was associated with increased expression levels of IL-2, IL-5, IL-13, IFN-gamma and IP-10 in plasmas from HIV^-/TB^+ but not in plasmas from HIV^-/TB^- children. ROC analysis of M.tb-specific cytokine/chemokine releases (value in the M.tb Ag-stimulated tube minus value in the corresponding not-stimulated tube) showed that IL-2, IL-5, IL-13 and IP-10, in addition to IFN-gamma could discriminate HIV^-/TB^+ from HIV^-/TB^- children (table 2).

In the entire panel of cytokines/chemokines we examined, only IP-10 expression displayed a higher median level in the M.tb Ag-stimulated tube than in the not-stimulated tube among HIV^+/TB^+ children (table 1). ROC analysis confirmed IP-10 as a potential biomarker to discriminate HIV^+/TB^+ children from HIV^-/TB^- children (table 2). The IP-10 threshold-value that best discriminated TB^+ from TB^- children was however less elevated among HIV^+/TB^+ (912.9pg/ml) than among HIV^-/TB^+ children (3644pg/ml). Using the 912.9pg/ml value as the cut-off of positivity 3/5 HIV^+/TB^+ children with QFT negative results displayed a positive IP-10 release (active TB, n=1; past active TB, n=2) and the sensitivity of IP-10 (83%) was somewhat better than IFN-γ release in the multiplex assay (50%) or QFT (58%).

This study is the first that used excess QFT supernatants to compare M.tb-specific cytokine/chemokine releases in HIV^+/TB^+ and HIV^-/TB^- children. Our results showing defective IL-2, IL-5 and IL-13 responses to M.tb in co-infected children ($p \leq 0.002$) make a contribution to our knowledge in the immunopathology of pediatric HIV/TB co-infection. IP-10 response to M.tb appeared less affected by HIV and showed promise in improving QFT sensitivity. A recent meta-analysis concluded that the IP-10 test performs slightly better or comparable to QFT in HIV^+/TB^+ adults [5]. To our knowledge, only two previous pediatric studies regarding QFT and IP-10 performance included HIV^+ children. These two studies, both conducted in low/middle income countries and high TB burden area, concluded that both QFT and IP-10 release exhibited an equally poor performance in diagnosing TB [6, 7]. We

have no clear explanations for the discrepancies between these results and our findings. The impact of the TB burden in the different places where the studies were conducted should be investigated.

In conclusion, although combining IL-13 and IP-10 may improve QFT sensitivity for diagnosing TB in immunocompetent young children [3], only IP-10 showed promise for improving TB diagnosis in HIV^+ children. Larger pediatric cohorts are warranted to investigate whether measuring IP-10 in combination with IFN-γ may improve QFT sensitivity without compromising specificity in HIV^+/TB^+ children and invite to identify HIV^+ pediatric subgroups that could best benefit from IP-10 release implementation.

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Table 1

Table 1: Biomarker concentrations (pg/ml) in QFT plasmas from the not-stimulated and the M.tb-Ag stimulated tubes

	HIV $^{-}$ TB $^{-}$ children (n=11)			HIV $^{-}$ TB $^{+}$ children (n=12)			HIV $^{+}$ TB $^{+}$ children (n=12)		
Cytokine	**Not-stimulated**	**M.tb-Ag stimulated**	**P**	**Not-stimulated**	**M.tb-Ag stimulated**	**P**	**Not-stimulated**	**M.tb-Ag stimulated**	**P**
IL-1β	2603 (146 - 4297)	4434 (1196 - 6015)	0.2145	2532 (952 - 12172)	6301 (1453 - 13064)	0.0304	1908 (247 - 12583)	3469 (384 - 14078)	0.1572
IL-2	120 (42 - 306)	123 (53 - 566)	0.9320	104 (42 - 260)	2025 (110 - 9414)	**0.0001**	94 (39 - 233)	161 (65 - 7276)	0.0351
IL-4	123 (54 - 185)	115 (67 - 236)	0.6350	103 (56 - 258)	168 (72 - 263)	0.0531	109 (29 - 213)	130 (46 -222)	0.5067
IL-5	17 (10 - 178)	21 (1 - 334)	0.6860	13 (8 - 23)	70 (13 - 175)	**0.0002**	14 (12 - 15)	14 (11 - 115)	0.2024
IL-6	6328 (318 - 10641)	4920 (168 - 14831)	0.9770	5476 (441 -19763)	8063 (3513 - 23279)	0.2145	5181 (109 - 15827)	5782 (315 - 18514)	0.5834
IL-10	73 (17 - 179)	85 (28 - 195)	0.7950	55 (21 - 387)	75 (25 - 152)	0.8173	58 (46 - 222)	63 (49 - 160)	0.5440
IL-13	46 (23 - 188)	47 (23 - 408)	0.8173	26 (14 - 59)	524 (25 - 2005)	**0.0002**	26 (16 - 58)	41 (15 -1865)	0.1332
IL-17	433 (172 - 642)	348 (197 - 736)	0.5444	427 (139 - 767)	541 (211 - 959)	0.1939	506 (73 - 728)	524 (208 - 783)	0.5834
IFN-γ	86 (31 - 138)	80 (41 - 185)	0.7508	70 (29 - 213)	893 (205 - 4038)	**<0.0001**	66 (31 - 180)	99 (38 - 1159)	0.1410
TNF-α	465 (84 - 887)	552 (74 - 991)	0.6350	383 (160 - 1711)	1009 (208 - 17154)	0.0266	235 (29 - 1812)	240 (54 - 1763)	0.9770
IP-10	972 (174 - 13286)	1144 (277 - 13910)	0.8852	970 (348 - 1686)	18149 (1812 - 20299)	**<0.0001**	772 (198 - 3264)	3962 (654 - 24756)	**0.0036**
G-CSF	129 (40 - 298)	100 (39 - 155)	0.1939	106 (25 - 1046)	147 (27 -376)	0.4357	126 (25 - 785)	159 (29 - 710)	0.9370
GM-CSF	274 (178 - 679)	293 (138 - 394)	0.8173	203 (93 - 381)	324 (110 - 385)	0.0120	262 (87 - 339)	289(93 - 471)	0.7073

M.tb-Ag: *Mycobacterium-antigens* contained in QFT (ESAT-6, CFP-10 and TB7.7)
Results are given as median, (range) concentrations (pg/ml).
p-values below 0.0038 thresholds (Mann-Whitney test comparing not-stimulated with M.tb-Ag stimulated values after Bonferroni adjustment for 13 tests) are highlighted in bold.

Table 2

Table 2: Accuracies of IL-2, IL-5, IL-13, IFN-γ and IP-10 releases in the diagnosis of TB in HIV $^{-}$/TB^{+} (A) and HIV^{+}/TB^{+} (B) children

(A)

HIV $^{-}$ TB $^{+}$ children (n=12)								
Biomarker	**AUC**	**p**	**Cut-off value(pg/l)**	**Sensitivity (%)**	**95% CI**	**Specificity (%)**	**95%CI**	**Incidence Positivity n (%)**
IL-2	0.962	**0.0001**	175.7	91.67	61.52 to 99.79	90.91	71.51 to 100	11 (91.67)
IL-5	0.886	**0.0010**	9.6	91.67	61.52 to 99.79	90.91	58.72 to 99.77	11 (91.67)
IL-13	0.947	**0.0002**	60.8	91.67	61.52 to 99.79	90.91	58.72 to 99.77	11 (91.67)
IFN-γ	1.000	**<0.0001**	111.4	100	73.54 to 100	100	71.51 to 100	12 (100)
IP-10	0.977	**0.0001**	3644.0	91.67	61.52 to 99.79	90.91	58.72 to 99.77	11 (91.67)

(B)

HIV $^{+}$ TB $^{+}$ children (n=12)								
Biomarker	**AUC**	**p**	**Cut-off value (pg/l)**	**Sensitivity (%)**	**95% CI**	**Specificity (%)**	**95%CI**	**Incidence Positivity n (%)**
IL-2	0.651	0.218	115.5	41.67	15.17 to 72.33	90.91	58.72 to 99.77	5 (41.66)
IL-5	0.594	0.441	7.4	58.33	27.67 to 84.83	54.55	23.38 to 83.25	1 (8.33)
IL-13	0.678	0.148	32.5	33.33	9.25 to 65.11	81.82	48.22 to 97.72	4 (33.3)
IFN-γ	0.704	0.096	47.85	50	21.09 to 78.91	81.82	48.22 to 97.72	6 (50)
IP-10	**0.825**	**0.008**	912.9	83.33	51.59 to 97.91	81.82	48.22 to 97.72	**10 (83.33)**

AUC: Area under curve. 95% CI: 95% Confidence Interval.

Receiver operating characteristics (ROC) curves were generated by plotting biomarker release values observed in HIV-/TB- children (specificity) against the corresponding values in HIV^{-}/TB^{+} (A) or HIV^{+}/TB^{+} (B) children (sensitivity). Only those analytes with significant ROC curves for HIV^{-}/TB^{+} or HIV^{+}/TB^{+} group are shown.

Specific release (value in the *M.tb* Ag tube minus value in the corresponding not-stimulated tube) was used in ROC curve analyses in agreement with the current literature.

Cut-off values were determined on the basis of the highest likelihood index (Youden's Index).

Sensitivity and specificity are expressed as a percentage.

Groups with AUC ≥0.70 and p<0.05 after ROC analysis are highlighted in bold.