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First description of agonist and antagonist IP-10 in urine of patients with active TB

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ABSTRACT

Objectives: Biomarkers for tuberculosis (TB) diagnosis and clinical management are needed to defeat TB. In chronic hepatitis, patients not responding to interferon/ribavirin treatment had high levels of an antagonist form of IP-10. Recently, antagonist IP-10 has been shown to be involved also in TB pathogenesis. Here, we investigated IP-10 agonist/antagonist forms as potential inflammatory biomarkers to support TB diagnosis and monitoring.
Methods: Total IP-10 and its agonist/antagonist forms were measured by SIMOA digital ELISA in urine obtained from patients with active TB at baseline and after treatment. Healthy donors (HD) and patients with pneumonia were enrolled as controls.
Results: Patients with active TB had significantly higher levels of total and agonist IP-10 at baseline compared to HD; conversely, no differences were observed between IP-10 levels in active TB vs pneumonia. Moreover, in active TB a decline of total urine IP-10 was observed at therapy completion; agonist/antagonist forms reflected this decline although their differences were not statistically significant.
Conclusions: We showed for the first time that agonist/antagonist IP-10 forms are measurable in urine. IP-10 levels associate with TB and pneumonia disease, suggesting their association with acute inflammation. Further studies are needed to assess their role to monitor TB treatment efficacy.

Introduction

Tuberculosis (TB), with more than 10 million cases and 1.7 million deaths yearly (WHO, 2017), is still a global health priority. The gold standard for TB diagnosis is the isolation in culture of Mycobacterium tuberculosis in biological samples; however, culture is time consuming and does not accomplish the need of quickly and properly starting treatment, it is not always feasible in extra-pulmonary TB and it is difficult to carry out in resource-limited settings. Moreover, to predict the efficacy of anti-TB therapy, sputum conversion after 2 months of specific treatment is widely used in culture positive TB cases, though simpler and more rapid diagnostic assays would be useful (Petruccioli et al., 2016). Therefore, identification of new biomarkers for TB diagnosis and therapy monitoring will provide powerful tools to defeat TB.

In this context, Interferon Inducible Protein-10 (IP-10 or C-X-C motif chemokine 10, CXCL10) has been proposed as a biomarker for TB. IP-10 may be released by infected alveolar macrophages promoting the migration of Th1 cells to the site of infection (Kaufmann and Dorhoi, 2013; Lindestam Arlehamn et al., 2013; Moguche et al., 2017; O'Garra et al., 2013; Saha et al., 2013) through

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binding to CXCR3 expressed on T cells. Moreover, IP-10 is increased in plasma and urine of active TB patients (Goletti et al., 2010a; Goletti et al., 2010b; Vanini et al., 2012) and decreases after efficacious therapy (Azzurri et al., 2005; Chegou et al., 2013; Cannas et al., 2010), correlates with risk of progression to disease and TB disease severity (Azzurri et al., 2005; Petrone et al., 2018; Riou et al., 2012) and is easily detected as an inflammatory marker in both adults and children (Petrone et al., 2015; Petrone et al., 2016; Santos et al., 2018). Importantly, IP-10 is a key player in trained immunity, a protection phenomenon dependent on epigenetic modifications of monocytes (Joosten et al., 2018). Indeed, IP-10 production by nonclassical $CD14^{dim}$ monocytes strongly correlated with Bacillus Calmette–Guérin (BCG) growth reduction in the mycobacterial growth inhibition assay (MGIA) and pharmacological blockade of CXCR3 reverted mycobacterial growth control (Joosten et al., 2018). A prospective evaluation of IP-10 levels has been performed in Human Immunodeficiency Virus (HIV)-infected subjects with active TB where blood IP-10 levels have been demonstrated to be a useful early biomarker for treatment response, as it declines after 2 weeks of anti-TB specific treatment (Garcia-Basteiro et al., 2017).

IP-10 is a chemokine with multiple properties and it is involved in many Th1-type inflammatory diseases such as neurological and autoimmune disorders (Liu et al., 2014; Antonelli et al., 2015), as well as Hepatitis C Virus (HCV) infection (Petrone et al., 2014), where it is an IL–28B independent negative predictive marker of efficacious therapy (Albert et al., 2011). In chronic hepatitis HCV, it has been demonstrated that patients not responding to conventional treatment with interferon/ribavirin, show high levels of an antagonist form of IP-10, which results from the enzymatic activity of dipeptidyl dipeptidase 4 (DPP4) (Casrouge et al., 2011). This antagonist form of IP-10 binds to CXCR3 but does not induce signalling. This is the first evidence for IP-10 antagonism in human disease and identifies a possible factor contributing to the inability of HCV clearance (Casrouge et al., 2011). Recently, it has been shown that IP-10 antagonism is also a feature of TB pathogenesis (Blauenfeldt et al., 2018). Indeed, it has been demonstrated in patients with active TB that IP-10 may be inactivated shortly after secretion at the site of TB disease by membrane bound DPP4, thus reducing its chemotactic activity on T cells.

The antagonist form of IP-10 is detectable in the plasma of TB patients. Urine is a biological sample easy to handle and with low risk to manage. Therefore, the aim of this study was to evaluate if IP-10 and its forms are detectable in urine, and if measurable, can they be exploited as useful biomarkers to evaluate the immune response in active TB at diagnosis and after therapy.

Materials and methods

Study participants

Patients with confirmed active TB were enrolled at INMI. Active TB was defined as “microbiological” based on i) a positive culture for Mtb from the sputum or broncholavage for pulmonary TB; ii) a positive Mtb −specific RNA amplification and/or NAT from biological specimens or presence of acid fast bacilli (AFB) in a tissue sample or by positive culture for Mtb in clinical samples for extra-pulmonary TB. Active TB was defined as “clinical” if the diagnosis was based on clinical and radiologic criteria including appropriate response to standard anti-TB therapy. For each patient with a diagnosis of active TB, the radiological disease severity was assessed by the radiologists evaluating chest X-ray at baseline. The following scale of severity was considered: 0: normal chest X-rays; 1: mild grade; 2: intermediate grade; 3: high grade (Petruccioli et al., 2017). Moreover, healthy donors (HD) and patients with pneumonia (either bacterial or viral) were enrolled as controls. Patients with pneumonia had a final diagnosis based on symptoms and signs of an acute lower respiratory tract infection, and could be confirmed by microbiological tests, clinical signs, a chest X-ray showing pulmonary lesions that is not due to any other cause, and successful treatment with therapy different from TB-specific drugs. Both TB and pneumonia patients were prospectively enrolled within 7 days (median 3 days) of starting the specific treatment and during treatment. In particular, TB patients were enrolled at baseline (T0), after two months of anti-TB treatment (T2) and at the end of therapy completion; in all of them anti-TB therapy was completed after 6 months (T6). Patients with pneumonia were treated for 14-21 days. We enrolled them at baseline and one month after the end of therapy completion (median 1 month, IQR: 1-3). A successful treatment completion with clinical-radiological and/or microbiological resolution of the disease defined a cured TB or pneumonia. Kidney diseases or urinary infections were excluded for all the subjects enrolled. The study was approved by the INMI Ethical Committee (pareri no 29/2014; 34/2010; 28/2014). All subjects provided written informed consent. We were inspired by the STARD criteria (Bossuyt et al., 2003) to test if our IP-10 species could be used as immune biomarkers for active TB diagnosis and monitoring.

Sample collection and IP-10 determination

Spot morning urine samples (10 mL) were collected in a vacutainer for urine (BD Bioscience, code 364915), then transferred to protease-inhibitor tubes p800 (BD Bioscences), aliquoted in 1 mL tubes and stored at −80 °C until use. Thawed urine samples were centrifuged at 10,000 g for 10 minutes at 4 °C, diluted 1:3 and tested for all forms of IP-10 (total IP-10), agonist IP-10 (long IP-10, 1-77 CXCL10) and antagonist IP-10 (short IP-10, 3-77 CXCL10) using in-house SIMOA ultrasensitive digital ELISA (Quanterix) as previously described (Meissner et al., 2015). Creatinine was measured to normalize IP-10 levels using a BioAssay Systems Quantichrom photometric assay and IP-10 levels were expressed as mg/g. Each sample was quantified in duplicate and a coefficient of variation (CV)% was calculated. The CVs median as a measure of test reproducibility were: 13%, 12%, 5.4% for long IP-10, short IP-10 and total IP-10 respectively; and 3.2% for creatinine. To characterize the dispersion of these CVs around related medians, a second CV% was calculated. The results were 68%, 95%, 96% and 245% respectively. SIMOA limit of detection (LOD) for each assay were as follows: long IP-10: 0.24 pg/mL; short IP-10: 0.31 pg/mL; total IP-10: 0.18 pg/mL. The SIMOA quantification of all IP-10 forms was performed blinded with respect to the diagnosis of the subjects enrolled. The results were analysed and interpreted by experienced researchers. No adverse events from performing sampling were registered.

Statistical analysis

Data were analysed with SPSS v.19 for Windows (SPSS Italia SRL, Bologna, Italy). The following statistics were calculated: medians and interquartile ranges (IQR) for continuous measures; Chi-square test for proportions; Kruskall-Wallis test for comparisons among several groups; Mann-Whitney U test for pairwise comparisons; Wilcoxon test for paired data; linear regression for slope analysis. A post-hoc correction was applied when relevant and p values <0.05 or <0.016 after the Bonferroni correction were considered significant. A receiver-operator characteristic (ROC) analysis was performed to describe the diagnostic ability and identify cut-off values of IP-10 forms. No other subgroups out of those declared in the above paragraph were analysed.

Results

Study population

Between November 2014 and December 2016, we enrolled 33 patients with active TB, 11 patients with pneumonia (either bacterial or viral) and 17 HD. Three subjects (one per group) were excluded as creatinuria was not available; therefore, the analysis was performed on 58 subjects: 32 patients with active TB, 10 patients with pneumonia and 16 HD (Table 1). Patients with active TB were part of a previous study (Blauenfeldt et al., 2018). As shown in Table 1, active TB patients mainly came from Eastern European countries whereas subjects with pneumonia and HD were mainly Italian. As a consequence, a significant difference was found regarding the BCG status of the subjects enrolled. Moreover, more than eighty percent of patients with active TB had a microbiological diagnosis and a high grade severity.

Patients with active TB have high urine levels of total IP-10 at baseline

Patients with active TB had significantly higher levels of all forms of IP-10 (total IP-10) at baseline compared to HD ($p < 0.0001$) but not compared to the levels detected in pneumonia patients (Figure 1). The SIMOA assay allows us to differentiate between long active IP-10 and the short antagonist form (2 amino acids cleaved at the N terminus). Therefore, utilizing SIMOA, we found that patients with active TB and with pneumonia had significantly higher levels of long IP-10 compared to HD ($p = 0.009$ and $p = 0.009$ respectively).

Based on these results we performed a ROC analysis for both long IP-10 and total IP-10 for the diagnosis of active TB. Significant area under the curve (AUC) results were obtained for both long and total IP-10 (AUC: 0.74, 95% confidence interval (CI): 0.59-0.88, $p = 0.008$ and AUC: 0.87, 95% CI: 0.76-0.98, $p < 0.0001$, respectively). The cut-off values of 20×10^{-5} mg/g for the long IP-10 and 15×10^{-5} mg/g for the total IP-10 identified active TB with 69% and 78% sensitivity and 81% and 94% specificity, respectively (Figure 2a, b). Moreover, as the long IP-10 form was significantly increased in patients with pneumonia compared to HD, we evaluated its accuracy for pneumonia diagnosis. The ROC analysis showed a significant AUC (AUC: 0.81, 95% CI: 0.63-1, $p = 0.008$) and a cut-off $\geq 19 \times 10^{-5}$ mg/g identified pneumonia with 80% sensitivity and 81% specificity (Figure 2c). No significant IP-10 differences were found between active TB and pneumonia patients, which was reflected in an AUC of 0.61 (95% CI: 0.43-0.79), 0.57(95% CI: 0.35-0.78), 0.70 (95% CI: 0.47-0.93), for antagonist, agonist and total IP-10, respectively (Figure 3a,b,c). Altogether these data indicate that IP-10 associates with an acute disease status independently of the type of disease evaluated.

Moreover, the levels of short IP-10 and long IP-10 were moderately correlated in the active TB and pneumonia groups ($r_s = 0.5$ and 0.58, $p < 0.004$ and 0.08, respectively); the linear regression analysis showed no significant difference in the regression slopes of active TB patients compared to pneumonia patients (Supplementary Figure 1).

In active TB patients urine IP-10 is decreased at the end of specific therapy

We followed 11 patients with active TB over the course of anti-TB specific therapy and urine samples were collected 2 months after starting treatment (T2) and upon therapy completion (T6) (Figure 4, Table 2).

Quantitative analysis showed a significant decrease of total IP-10 levels at T6 compared to baseline ($p = 0.009$, Figure 4a) and to T2 ($p = 0.03$, Figure 4a); moreover, a decreased trend for both long and short IP-10 levels in patients with active TB during therapy was also observed, although not significant (Figure 4b-c). Urine IP-10 levels were also evaluated in 9 patients with pneumonia at baseline and at the end of specific chemotherapy (Figure 4, Table 3) but neither short/long IP-10, nor total IP-10 levels showed significant changes (Figure 4d-f).

Discussion

Biomarkers for active TB diagnosis and clinical management are needed to reduce TB incidence and recurrence. Moreover, it is

Table 1
Baseline information of study participants.

	Active TB	Pneumonia	HD	Total	p Value
N (%)	32 (55.2)	10 (17.2)	16 (27.6)	58 (100)	
Age Median (IQR)	39 (32-50)	52 (32-67)	40 (28-50)	40 (32-53)	0.20[a]
Sex N (%)					
Female	9 (28.1)	4 (40.0)	11 (68.8)	24 (41.4)	0.03[b]
Origin N (%)					**0.003**[b]
West Europe	9 (28.1)	8 (80.0)	15 (93.8)	32 (55.2)	
East Europe	16 (50.0)	2 (20.0)	0 (0)	18 (31.0)	
Asia	3 (9.3)	0 (0)	1 (6.2)	4 (7.0)	
Africa	2 (6.3)	0 (0)	0 (0)	2 (3.4)	
America	2 (6.3)	0 (0)	0(0)	2 (3.4)	
BCG N (%)					<**0.0001**[b]
Vaccinated	25 (78.1)	1 (10.0)	3 (18.8)	29 (50.0)	
QFT N (%)					<**0.0001**[b]
Positive	21 (65.7)	1 (10.0)	0 (0)	22 (37.9)	
Negative	5 (15.6)	5 (50.0)	13 (81.2)	23 (39.7)	
Indeterminate	1 (3.1)	0 (0)	0 (0)	1 (1.7)	
Not Done	5 (15.6)	4 (40.0)	3 (18.8)	12 (20.7)	
Microbiological Active TB	27 (84.4)	-	-	-	

N: number; TB: tuberculosis; IQR: Interquartile Range; BCG: Bacillus Calmette–Guérin; QFT: QuantiFERON Gold IN TUBE.
Values considered as statistically significant are indicated in bold.
[a] Kruskall-Wallis test.
[b] Chi-square test.

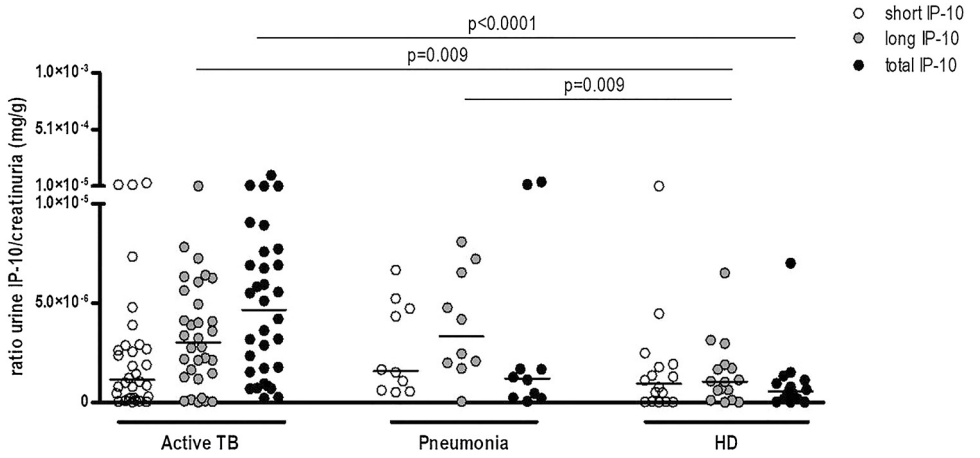

Figure 1. Increased levels of IP-10 and its agonist form in urine from patients with active TB and in subjects with pneumonia compared to HD. SIMOA digital ELISA was performed in urine; urine levels of IP-10 and its agonist/antagonist forms were normalized as ratio to creatinine and expressed as mg/g. The horizontal lines represent the median; statistical analysis was performed using the Mann-Whitney test with Bonferroni correction and p-value was considered significant if <0.016. Footnotes. IP-10: IFN-γ inducible protein 10; TB: tuberculosis; HD: healthy donors.

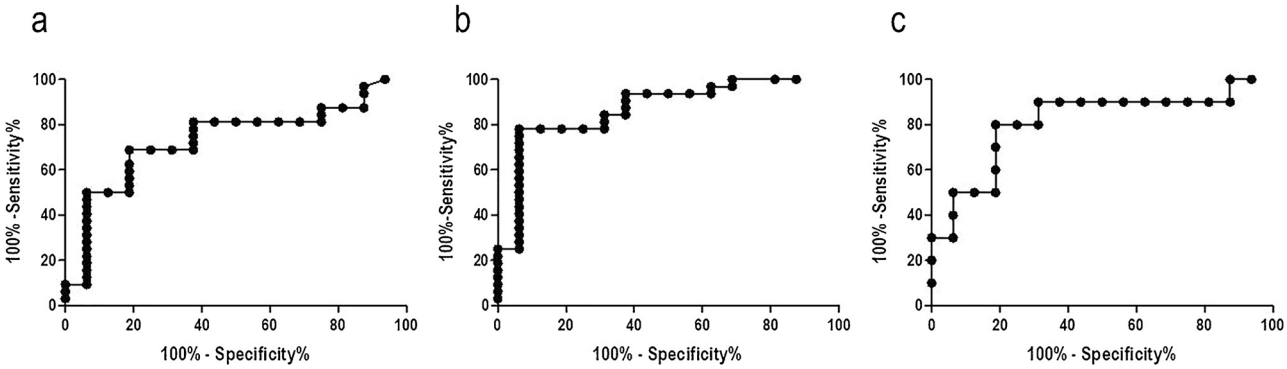

Figure 2. Receiver-operator characteristic (ROC) curves of the IP-10-based tests for discriminating active TB or pneumonia from healthy donors (HD). (a-b) ROC analysis between active TB and HD groups; significant area under curve (AUC) results were obtained for both long (a) and total (b) IP-10 (AUC: 0.74, 95% confidence interval (CI): 0.59-0.88, p = 0.008 and AUC: 0.87, 95% CI: 0.76-0.98, p < 0.0001, respectively). (c) ROC analysis between pneumonia and HD groups; significant AUC results (AUC: 0.83, 95% CI: 0.63-1, p = 0.008) were obtained for long IP-10. SIMOA was performed in urine; urine levels of IP-10 and its agonist/antagonist forms were normalized as a ratio to creatinine and expressed as mg/g.

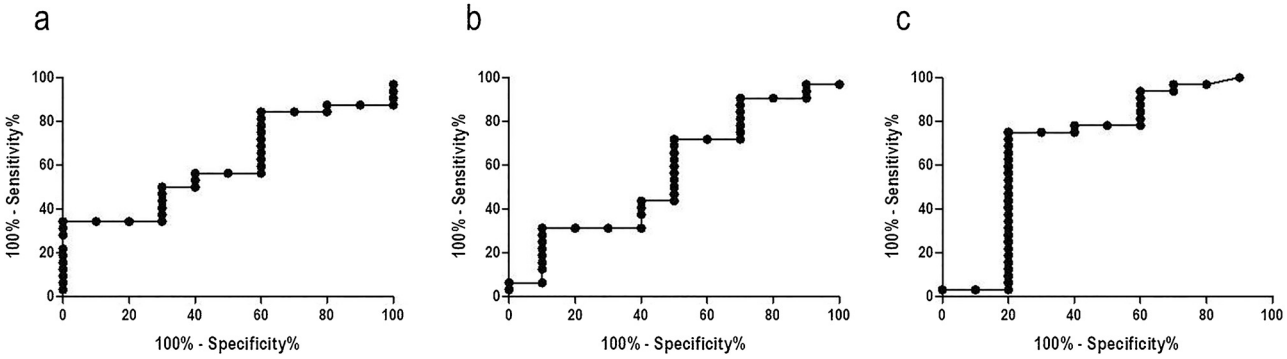

Figure 3. ROC analysis of the IP-10-based tests for discriminating active TB from pneumonia. (a) antagonist IP-10; (b) agonist IP-10; (c) total IP-10. AUC results of 0.61 (95% CI: 0.43-0.79), 0.57(95% CI: 0.35-0.78), 0.70 (95% CI: 0.47-0.93) were obtained. SIMOA was performed in urine; urine levels of IP-10 and its agonist/antagonist forms were normalized as a ratio to creatinine and expressed as mg/g.

important to design simple tools, useful for both adults and children and easily to be transferred to the patient bed as point-of-care tests. The IP-10 chemokine is well-suited to fulfil all these demands, and the recent characterization of post translationally modified IP-10 forms in TB (Blauenfeldt et al., 2018) have opened new opportunities to further evaluate its use as biomarker.

In this study we showed for the first time that IP-10 agonist and antagonist forms may be detected in urine. In line with our and other publications (Cannas et al., 2010; Petrone et al., 2015; Petrone et al., 2016), we confirmed that IP-10 is increased in the urine of patients with active TB but also in diseases different from TB such as pneumonia. Moreover, we demonstrated that levels of agonist

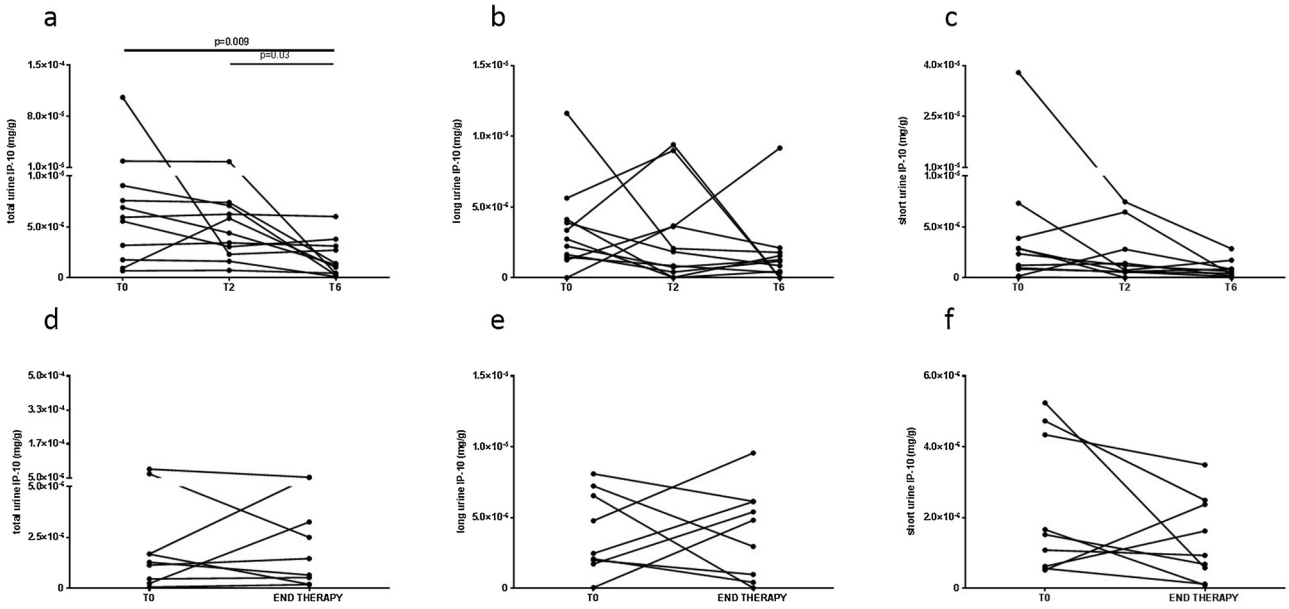

Figure 4. Decrease of urine IP-10 in patients with active TB during anti-TB specific therapy. (a) total IP-10; (b) agonist IP-10; (c) antagonist IP-10 levels in patients with active TB during anti-TB specific therapy; (d) total IP-10; (e) agonist IP-10; (f) antagonist IP-10 levels in patients with pneumonia during therapy. SIMOA was performed in urine; urine levels of IP-10 and its agonist/antagonist forms were normalized as a ratio to creatinine and expressed as mg/g. Statistical analysis was performed using the Wilcoxon test for paired data and p-value was considered significant if <0.05. Footnotes. IP-10: IFN-γ inducible protein 10; T0: baseline; T2: 2 months therapy; T6: end therapy.

Table 2
IP-10 levels in active TB patients during anti-TB specific treatment.

	Anti-TB therapy follow-up (months)		
	T0	T2	T6
Short IP–10 mg/g	24×10^{-5}	75×10^{-6}	47×10^{-6}
Median (IQR)	(87×10^{-6} -39×10^{-5})	(56×10^{-6}-28×10^{-6})	(14×10^{-6}-88×10^{-6})
Long IP–10 mg/g	28×10^{-5}	19×10^{-5}	12×10^{-5}
Median (IQR)	(15×10^{-5}-41×10^{-5})	(41×10^{-6}-37×10^{-5})	(37×10^{-6}-18×10^{-5})
Total IP–10 mg/g	59×10^{-5}	44×10^{-5}	13×10^{-5}
Median (IQR)	(18×10^{-5}-91×10^{-5})	(23×10^{-5}-71×10^{-5})	(42×10^{-6}-31×10^{-5})

TB: tuberculosis; T0: baseline; T2: 2 months therapy; T6: end-therapy; IP-10: Interferon Inducible Protein-10; IQR: Interquartile Range.

Table 3
IP-10 levels in patients with pneumonia during specific treatment.

	Therapy follow-up	
	T0	End therapy
Short IP-10 mg/g	15×10^{-5}	93×10^{-6}
Median (IQR)	(59×10^{-6}-45×10^{-5})	(35×10^{-6}-24×10^{-5})
Long IP-10 mg/g	25×10^{-5}	48×10^{-5}
Median (IQR)	(19×10^{-5}-69×10^{-5})	(70×10^{-6}-61×10^{-5})
Total IP-10 mg/g	13×10^{-5}	15×10^{-5}
Median (IQR)	(35×10^{-6}-13×10^{-5})	(36×10^{-6}-53×10^{-5})

T0: baseline; IP-10: Interferon Inducible Protein-10; IQR: Interquartile Range.

IP-10 in urine were increased in patients with active TB or pneumonia compared to healthy subjects, reflecting ongoing and active secretion. The ROC analysis showed that both the agonist IP-10 and the total IP-10 detected are associated with a status of acute disease, independently of TB or pneumonia status, as previously shown by us and others for the total IP-10, in blood and urine samples (Santos et al., 2018).

The assessment of treatment response in active TB patients is currently based on sputum reversion and improvement of clinical symptoms and often of the radiology lesions after 2 months of therapy. However, cultures seem inadequate as the gold standard, as several studies showed recurrent TB in treated patients despite sputum reversion at the end of the anti-TB therapy as well as active TB lesions post-treatment (Goletti et al., 2018; Malherbe et al., 2016). Several biomarkers for treatment response based on either pathogen or host features have been taken into consideration, however, none have been validated yet. Therefore, additional tests and new biomarkers for predicting treatment efficacy are needed. Blood IP-10 has been demonstrated as an early predictor of treatment response as its levels rapidly decrease in the first week of treatment, mainly in microbiologically confirmed active TB with or without HIV infection (Garcia-Basteiro et al., 2017; den Hertog et al., 2015; Chung et al., 2016). In this study we found declining levels of total and agonist/antagonist IP-10 forms in urine of patients with active TB during specific therapy but not in patients with pneumonia, suggesting that modifications of IP-10 forms during therapy may be associated to TB disease. However the decline of the agonist/antagonist IP-10 forms was not significant, likely due to the small number of subjects evaluated. This supports the hypothesis that IP-10 is expressed at the site of TB infection, diffuses to the blood and is excreted in the urine (Blauenfeldt et al., 2018), however the low levels found in urine compared to blood remains a challenge. In this study, the absolute levels of urine IP-10 were below the LOD in 14% and 8.8% of samples from active TB or pneumonia patients respectively. The values below the LOD were found mainly for agonist/antagonist forms underpinning the

difficulty determining them in this sample. Therefore, the use of a more concentrated sample, such as urine collected over 24-hours, may improve IP-10 detection and help in clarifying its kinetics during therapy.

Further studies should include the evaluation of agonist and antagonist IP-10 in both microbiological and clinical TB as well as shorter follow up times to fully understand their modulation and the potential accuracy as biomarkers to predict treatment response. Furthermore, the identification of new additional post-translational modifications of IP-10 and their role in TB pathogenesis may also be explored. Importantly, the "end therapy" time point differs between active TB and pneumonia patients (6 months vs 1 month), however, different drug regimens are expected for these diseases; shorter time courses during therapy, as previously described (Garcia-Basteiro et al., 2017), will better highlight the differences of IP-10 and its agonist/antagonist levels in urine.

In conclusion, we showed for the first time that agonist/antagonist IP-10 forms are measurable in urine. Increased IP-10 levels associate with both TB and pneumonia disease, suggesting that it is associated with acute inflammation. Further studies are needed to assess their role as tools to monitor TB treatment efficacy.

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Conflict of interest

DG has been a consultant and presented talks for Quidel and Qiagen in 2018. The other authors do not report any conflict of interest.

Ethical approval

The study was approved by the INMI Ethical Committee (pareri no 29/2014; 34/2010; 28/2014). All subjects provided written informed consent.

Author contributions

LP analysed and interpreted data, wrote the manuscript; VB developed and optimized SIMOA for IP-10 detection and analysed the patient samples; VV processed urine samples, FP, DG and GC enrolled patients with active TB and pneumonia and healthy donors; IP, GD, FC, GMF and TB participated in the interpretation of data; MR participated in data analysis and interpretation; DD developed SIMOA assays for specific detection of IP-10 agonist and antagonist forms, supervised assay implementation and participated to the interpretation of data; DG designed the study, coordinated and supervised the project, enrolled patients, coordinated sample handling, interpreted data, contributed to the writing of the manuscript. All authors discussed the results and approved the final version of the manuscript.

Declarations of interest

DG has been a consultant and presented talks for Quidel and Qiagen in 2018. The other authors do not report any conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2018.09.001.

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