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Original Article

Circulating cytokines, chemokines, and soluble CD molecules in *mycobacterium tuberculosis*-infected rhesus monkeys (*Macaca mulatta*), as determined by Luminex xMAP assays

Fangui Min[1], Jing Wang[1], Jinchun Pan[1], Shuwu Huang[1], Lan Zhang[2], Yu Zhang[1]

[1]*Guangdong Laboratory Animals Monitoring Institute, Guangdong Provincial Key Laboratory of Laboratory Animals, Guangzhou, P. R. China;* [2]*Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Department of Obstetrics and Gynecology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, P. R. China*

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Abstract: Previous studies have demonstrated that mycobacterium tuberculosis (MTB) infections might induce an imbalance of Th cells and cytokines. Serum concentrations of 43 cytokines or chemokines were measured by Luminex xMAP technologies in 30 healthy and 35 naturally MTB infected rhesus monkeys, evaluating their clinical relevance. Of the 11 proinflammatory cytokines, concentrations of 6 cytokines (GM-CSF, IFN-γ, IL15, IL17A, IL22, and sCD40) in MTB infections were significantly higher than those in controls ($P < 0.05$). However, concentrations of 92% (11/12) of anti-inflammatory cytokines showed no significant differences between controls and MTB infections ($P > 0.05$). Two pleiotropic cytokines (IL-6 and MIF) were substantially increased in MTB infections, compared to controls ($P < 0.05$). For the 14 chemokines, 4 chemokines (MCP-1, RANTES, CCL11, and IP-10) were significantly increased and 2 chemokines (CCL18 and CCL22) were significantly decreased in MTB infections, compared to controls ($P < 0.05$). Other cytokines or chemokines showed no significant differences between controls and MTB infections ($P > 0.05$). Th1/Th2 cytokine ratios revealed Th1/Th2 cytokines imbalances, characterized with a shift towards a predominance of Th1 cytokines in MTB infections. Significant positive correlation was observed between IFN-γ and IP-10 in either controls or MTB infections ($P < 0.05$). IFN-γ was correlated positively with IL-15 in MTB infections, but not with controls ($P < 0.05$). Similarly, RANTES was positively correlated with MCP-1 and MCP-4 in MTB infections, but not with controls ($P < 0.05$). The current study profiled multiple cytokine responses, indicating Th1/Th2 cytokine imbalances toward Th1 polarization in rhesus monkeys with natural MTB infections.

Keywords: Rhesus monkey, mycobacterium tuberculosis, cytokine, Th1/Th2 cytokine imbalance

Introduction

Mycobacterium tuberculosis (MTB) is one of the world's most ubiquitous pathogens. It is prevalent not only in humans [1], but also in non-human primates [2]. For non-human primates, old world monkeys are more often used in biomedical research than new world monkeys. Of the old world monkeys, rhesus monkeys (*Macaca mulatta*) are more susceptible to MTB than cynomolgus monkeys (*Macaca fascicularis*) [3-5].

In the course of MTB infections, immune cells, along with their cytokines and receptors, may affect immune activation or inflammation and immunity. Serial studies have suggested that Th1/Th2 imbalances are associated with development of tuberculosis [6-9]. Severity of infections is closely related to Th1 response. Lower Th1 responses indicate more severity of the disease. For human patients, about 10% of MTB infections develop active tuberculosis, with increased Th1 responses in resistance to MTB infections [7]. Most human patients with MTB have a lower Th1 response and/or a stronger Th2 response [8, 9]. Dysregulation of Th1 and Th2 cytokine responses has been regarded as a secondary effect of tuberculosis. Progression of the disease has been associated with

Table 1. Information of selected cytokines and panels

Kits	Number	Cytokines
Panel 1	6	TNF RII, IL19, RANTES, MIF, MPO, Galectin-3
Panel 2	37	IFN-γ, TNF-α, TNF RI, GM-CSF, IL1β, IL1RA, IL1 RII, IL2, IL2 Rα, IL4, IL5, IL6, IL6 Rα, IL8, IL10, IL15, IL17A, IL22, MCP-1, MIP-1β, MCP-3, CCL11, MCP-4, CCL18, CCL22, CCL27, CXCL1, CXCL4, IP-10, CXCL12, CXCL13, sCD14, sCD27, sCD30, sCD40, sCD56, B7-H1

reduced Th1 and enhanced Th2 cytokine response. Chemokines, also called chemotactic cytokines, are critical regulators of leukocyte-mediated inflammation and immunity during MTB infections [10, 11].

Although non-human primates have been widely used for tuberculosis research, cytokine balances and profiles of chemokines have rarely been reported. Previous studies have reported a correlation between IFN-γ and IP-10 in non-human primates with MTB infections [12]. The current study expanded the circulating profiles of multiple cytokines and chemokines in MTB-infected rhesus monkeys.

Materials and methods

Negative controls

Thirty healthy rhesus monkeys from Guangdong Blooming-Spring Biological Technology Development Co. Ltd. [license number SCXK (Yue) 2014-0027] were tested free of tuberculosis, B virus, and simian retrovirus. Serum was collected and stored at -80°C.

MTB infections

In recent years, 35 rhesus monkeys were confirmed to possess natural MTB infections, according to repeated tuberculin skin testing (TSTs), necropsy, and bacterial culturing. Sera were collected from every infected monkey and stored at -80°C.

Multiplex cytokine analysis

Serum concentrations of multiple cytokines were measured using Luminex xMAP technologies. Multiplexed immunoassay panels, containing 43 cytokines (including chemokines), were equipped by R&D Systems. **Table 1** shows the information of selected cytokines and multiplexed immunoassay panels. Analytes with overlapping bead regions or other incompatibilities would be placed in their own assays. Thus, 43 cytokines were divided into 2 panels. Six cytokines were included in Panel 1 (Kit Lot Number: L122016) and 37 cytokines were involved in panel 2 (Kit Lot Number: L122018). Testing was conducted in accordance with manufacturer protocol.

Data analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). The normality of distribution for quantitative variables was checked with the Shapiro-Wilk tests. Non-normal data are expressed as medians (25%, 75% percentile). Comparisons between groups were conducted using non-parametric Mann-Whitney tests. Correlation between different cytokines was analyzed using Spearman's rank correlation analysis. *P*-values < 0.05 indicate statistical significance.

Results

Laboratory parameters

Basic concentrations of serum cytokines, chemokines, and soluble CD molecules in controls (n = 30) and MTB infections (n = 35) were measured and compared (**Table 2**). According to their roles in immune response, they were classified into subgroups, including proinflammatory cytokines, anti-inflammatory cytokines, pleiotropic cytokines, and chemokines.

For the 11 proinflammatory cytokines, 9 were increased in MTB infections. Six cytokines (GM-CSF, IFN-γ, IL15, IL17A, IL22, sCD40) in MTB infections were significantly higher than in controls ($P < 0.05$). IL-1β and CD14 showed a slight decrease in MTB infections, but differences were statistically not significant ($P > 0.05$). Of the 12 anti-inflammatory cytokines, significant differences between controls and MTB infections were observed in IL-1R II ($P < 0.05$). Two pleiotropic cytokines (IL-6 and MIF) were substantially increased in MTB infections,

Table 2. Basic concentrations (pg/mL) of cytokines, chemokines, and soluble CD molecules in healthy and MTB infections

Classification	Cytokines	Median (25%, 75% percentile) pg/ml		*P*-values*
		Controls (n = 30)	MTB infections (n = 35)	
Proinflammatory cytokines	TNF-α	8.61 (6.59, 9.55)	8.67 (7.33, 12.55)	*P* = 0.1148
	GM-CSF	4.03 (3.03, 5.21)	5.09 (4.11, 6.16)	*P* = 0.0353
	IFN-γ	62.98 (47.34, 108.2)	96.13 (79.55, 167.6)	*P* = 0.0002
	IL-1β	17.63 (14.60, 23.00)	15.32 (13.41, 21.89)	*P* = 0.1184
	IL-2	41.19 (29.02, 89.33)	58.06 (37.68, 154.1)	*P* = 0.1796
	IL-8	1052 (765, 1330)	1189 (927, 1337)	*P* = 0.4228
	IL-15	16.53 (12.60, 19.50)	19.65 (16.09, 45.82)	*P* = 0.0010
	IL-17A	29.00 (21.12, 36.03)	32.43 (28.13, 42.09)	*P* = 0.0226
	IL-22	7.42 (4.76, 10.10)	9.71 (7.42, 25.32)	*P* = 0.0031
	sCD14	52667 (52558, 52757)	52539 (50782, 52732)	*P* = 0.0539
	sCD40	31.97 (26.89, 47.11)	66.97 (39.16, 107.6)	*P* = 0.0002
Anti-inflammatory cytokines	IL-1RA	1345 (1041, 3090)	1332 (644, 3028)	*P* = 0.4986
	IL-4	121.48 (105.8, 136.0)	124.52 (109.2, 140.3)	*P* = 0.5492
	IL-5	20.88 (19.21, 22.42)	20.97 (20.06, 22.37)	*P* = 0.8676
	IL-10	8.42 (7.49, 10.18)	8.40 (7.38, 15.27)	*P* = 0.8728
	TNF RI	3694 (3269, 4522)	3641 (2947, 4746)	*P* = 0.6402
	TNF RII	1815 (1278, 2502)	1563 (1075, 2812)	*P* = 0.9089
	IL-1 RII	18475 (16318, 20916)	21123 (18594, 24063)	*P* = 0.0028
	IL-2 Rα	289.61 (240.2, 426.9)	263.08 (144.3, 548.7)	*P* = 0.4190
	IL-6 Rα	18629 (13305, 21715)	14898 (12507, 19004)	*P* = 0.1190
	IL-19	2503 (2109, 2839)	2318 (2082, 2423)	*P* = 0.1186
	sCD27	612 (456, 933)	633 (360, 1004)	*P* = 0.4190
	sCD30	100.42 (88.16, 130.9)	116.41 (94.72, 152.7)	*P* = 0.1109
Pleiotropic cytokine	IL-6	11.28 (9.27, 15.06)	22.25 (10.21, 53.18)	*P* = 0.0160
	MIF	6029 (4332, 9197)	17787 (10278, 25726)	*P* < 0.0001
Chemokines	MCP-1	98.14 (47.95, 156.8)	140.03 (104.3, 202.5)	*P* = 0.0109
	MIP-1β	268.56 (264.8, 287.7)	262.97 (249.9, 288.7)	*P* = 0.0902
	RANTES	1556 (841, 3599)	4501 (2758, 6630)	*P* < 0.0001
	MCP-3	1206 (986, 1513)	1101 (939, 1717)	*P* = 0.8831
	CCL11	198.79 (160, 360)	549.66 (224, 1151)	*P* = 0.0010
	MCP-4	59.91 (41.31, 90.16)	56.77 (28.73, 120.5)	*P* = 0.8014
	CCL18	1153 (881, 1455)	650 (394, 1120)	*P* = 0.0008
	CCL22	349.82 (212.1, 415.5)	96.09 (50.77, 122.7)	*P* < 0.0001
	CCL27	1279 (1009, 1495)	1152 (880, 1368)	*P* = 0.1171
	CXCL1	326.87 (269.9, 395.4)	335.19 (281.7, 436.9)	*P* = 0.5067
	CXCL4	3017 (2855, 3103)	2852 (2629, 3243)	*P* = 0.1440
	IP-10	536.77 (472.1, 617.4)	651.43 (530, 814)	*P* = 0.0134
	CXCL12	110.21 (109.83, 110.70)	110.04 (109.66, 112.00)	*P* = 0.2431
	CXCL13	85.86 (57.34, 101.1)	70.95 (57.80, 95.26)	*P* = 0.4265
Other cytokines	sCD56	167180 (141128, 183459)	162558 (133482, 174342)	*P* = 0.2096
	B7-H1	2199 (1908, 2717)	2500 (2197, 4434)	*P* = 0.0818
	MPO	7018 (6066, 8240)	7703 (5518, 10319)	*P* = 0.4578
	Galectin-3	224352 (176627, 248771)	191197 (148154, 235357)	*P* = 0.1426

Note: **p*-values were obtained by Mann-Whitney tests.

compared to controls ($P < 0.05$). Of the measured 14 chemokines, 5 (MCP-1, RANTES, CCL11, CXCL1, IP-10) were increased and 9 (MIP-1β, MCP-3, MCP-4, CCL18, CCL22, CCL27,

Table 3. Results of Th1/Th2 cytokine ratios

Th1/Th2 cytokine ratio	Median (25%, 75% percentile)		*P*-values*
	Controls (n = 30)	MTB infections (n = 35)	
TNF-α/IL4	0.068 (0.061, 0.077)	0.068 (0.058, 0.101)	*P* = 0.7857
IFN-γ/IL4	0.548 (0.3883, 0.9245)	0.88 (0.595, 1.311)	*P* = 0.0006
IL-1β/IL-4	0.154 (0.1213, 0.2038)	0.134 (0.113, 0.162)	*P* = 0.1492
IL-2/IL-4	0.3815 (0.2495, 0.6713)	0.445 (0.282, 1.133)	*P* = 0.2189
IL-15/IL-4	0.1398 (0.1064, 0.191)	0.2524 (0.1077, 0.393)	*P* = 0.0009
TNF-α/IL5	0.41 (0.34, 0.47)	0.415 (0.35, 0.553)	*P* = 0.2088
IFN-γ/IL5	3.204 (2.107, 5.24)	4.77 (3.965, 7.757)	*P* = 0.0001
IL-1β/IL-5	0.8755 (0.7105, 1.08)	0.723 (0.613, 1.086)	*P* = 0.1093
IL-2/IL-5	2.161 (1.549, 4.101)	2.801 (1.797, 6.611)	*P* = 0.1651
IL-15/IL-5	0.7824 (0.5856, 0.9444)	1.414 (0.8021, 2.195)	*P* = 0.0007
TNF-α/IL10	0.95 (0.81, 1.13)	0.975 (0.846, 1.321)	*P* = 0.4537
IFN-γ/IL10	7.348 (4.904, 12.69)	13.74 (7.842, 17.94)	*P* = 0.0018
IL-1β/IL-10	2.104 (1.656, 2.57)	1.807 (1.407, 2.824)	*P* = 0.1840
IL-2/IL-10	5.456 (3.158, 10.73)	6.904 (3.266, 12.07)	*P* = 0.3475
IL-15/IL-10	1.737 (1.406, 2.539)	2.826 (1.916, 5.426)	*P* = 0.0024

Note: **p*-values were obtained by Mann-Whitney tests.

CXCL4, CXCL12, CXCL13) were decreased in MTB infections. However, significant differences were only found in MCP-1, RANTES, CCL11, CCL18, CCL22, and IP-10 ($P < 0.05$). The remaining 4 cytokines (sCD56, B7-H1, MPO, Galectin-3) showed no significant differences between controls and MTB infections ($P > 0.05$).

Th1/Th2 cytokine balances

Th1/Th2 cytokine ratios are a measure of Th1/Th2 cytokine balance, associated with infectious status. In the current study, Th1 (TNF-α, IFN-γ, IL-1β, IL-2, IL-15)/Th2 (IL-4, IL-5, IL-10) cytokine ratios were calculated and compared between controls and MTB infections (**Table 3**). Ratios between Th1 cytokines (IFN-γ, IL-15) and Th2 cytokines (IL-4, IL-5, IL-10) in MTB infections were significantly higher than those in controls ($P < 0.05$). No significant differences were observed in ratios of TNF-α, IL-1β, and IL-2, compared to Th2 cytokines (IL-4, IL-5, IL-10) between controls and MTB infections ($P > 0.05$).

Correlation between IFN-γ, IL-15, and IFN-γ-inducible chemokines

According to Th1/Th2 cytokine ratios, Th1/Th2 cytokine imbalances existed in MTB infections. These were characterized with a shift towards predominantly Th1 cytokines. This study analyzed correlation levels between Th1 cytokines (IFN-γ and IL-15) and IFN-γ-inducible chemokine (IP-10). Results are shown in **Figure 1**. Significant positive correlation was observed between IFN-γ and IP-10 in either controls or MTB infections ($P < 0.05$). IFN-γ was also positively correlated with IL-15 in MTB infections, but not with controls ($P < 0.05$). IL-15 was correlated positively with IP-10 in both controls and MTB infections, but differences were not significant ($P > 0.05$).

Correlation between RANTES and MCP-1, MCP-3, and MCP-4

Present results proved that, in MTB infections, cell-mediated immune response was strongly activated. This study further observed correlation between RANTES and MCP-1, MCP-3, and MCP-4 (**Figure 2**). Correlation between RANTES and MCP-1 and MCP-4 in controls was slightly negative ($P > 0.05$). However, RANTES was positively correlated with MCP-1 and MCP-4 in MTB infections ($P < 0.05$). No significant correlation between RANTES and MCP-3 was observed in either controls or MTB infections ($P > 0.05$).

Discussion

Cytokines are protein molecules produced by Th cells. They play a key role in regulating im-

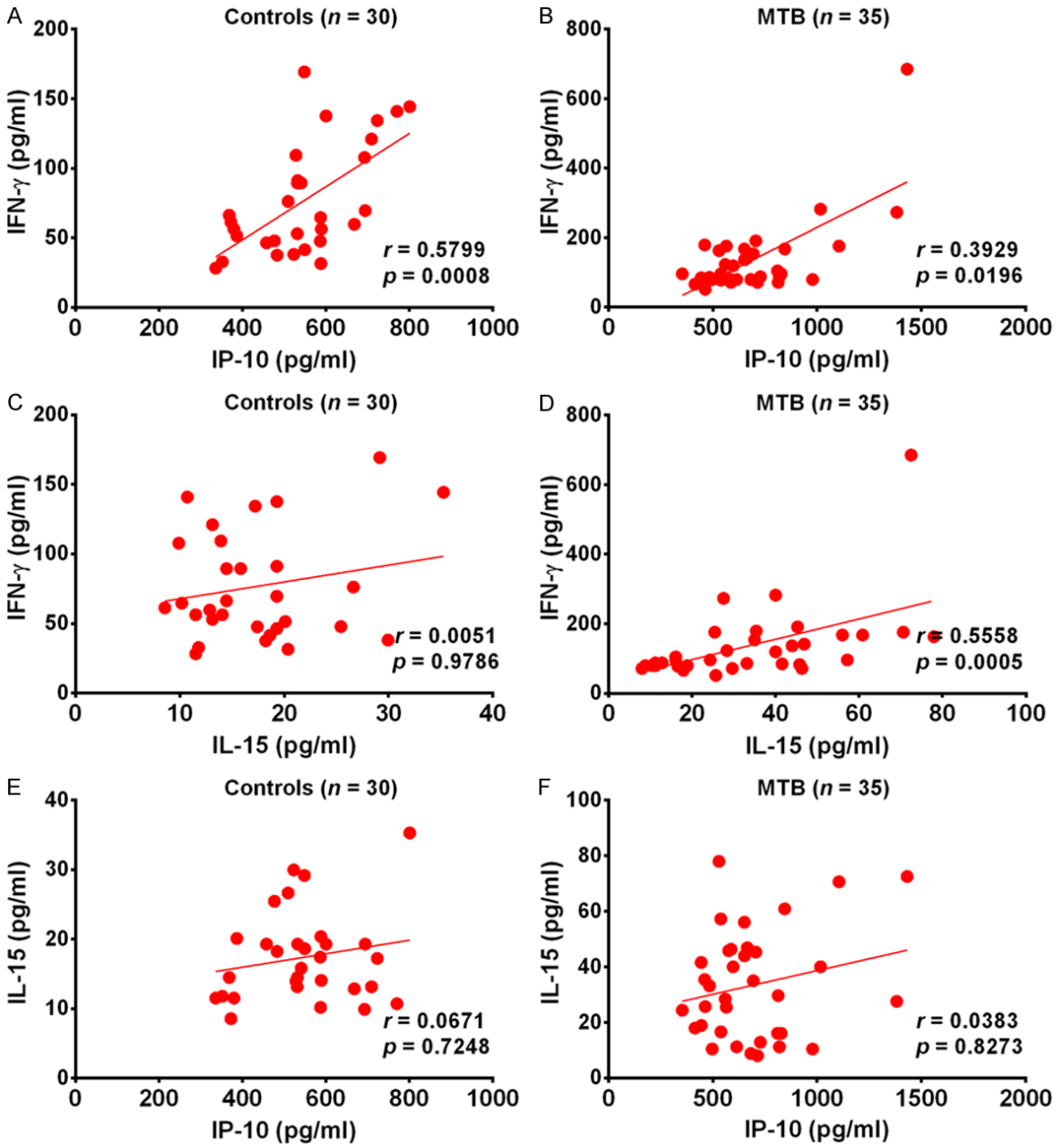

Figure 1. Correlation between IFN-γ and IL-15 and IP-10. Representing correlation of (A) IFN-γ and IP-10 in controls, (B) IFN-γ and IP-10 in MTB infections, (C) IFN-γ and IL-15 in controls, (D) IFN-γ and IL-15 in MTB infections, (E) IL-15 and IP-10 in controls, (F) IL-15 and IP-10 in MTB infections.

mune response and inflammation. Alteration in serum concentrations of various proinflammatory and anti-inflammatory cytokines has been reported to be associated with MTB infections in human patients [6-9]. In this study, most pro-inflammatory cytokines (6/11) were significantly evaluated in MTB infections, compared to controls. However, only 1 out of 12 anti-inflammatory cytokines was significantly higher in MTB infections, compared to controls. Two pleiotropic cytokines (IL-6 and MIF) were significantly increased in MTB infections, compared to controls. The other 4 cytokines exhibited no significant differences between MTB infections and controls. Chemokines are essential regulators of cell-mediated inflammation and immunity by transmigration of various immune cells to sites of infection [13]. As potent proinflammatory chemotactic cytokines, regulation of chemokines must, therefore, be tightly controlled.

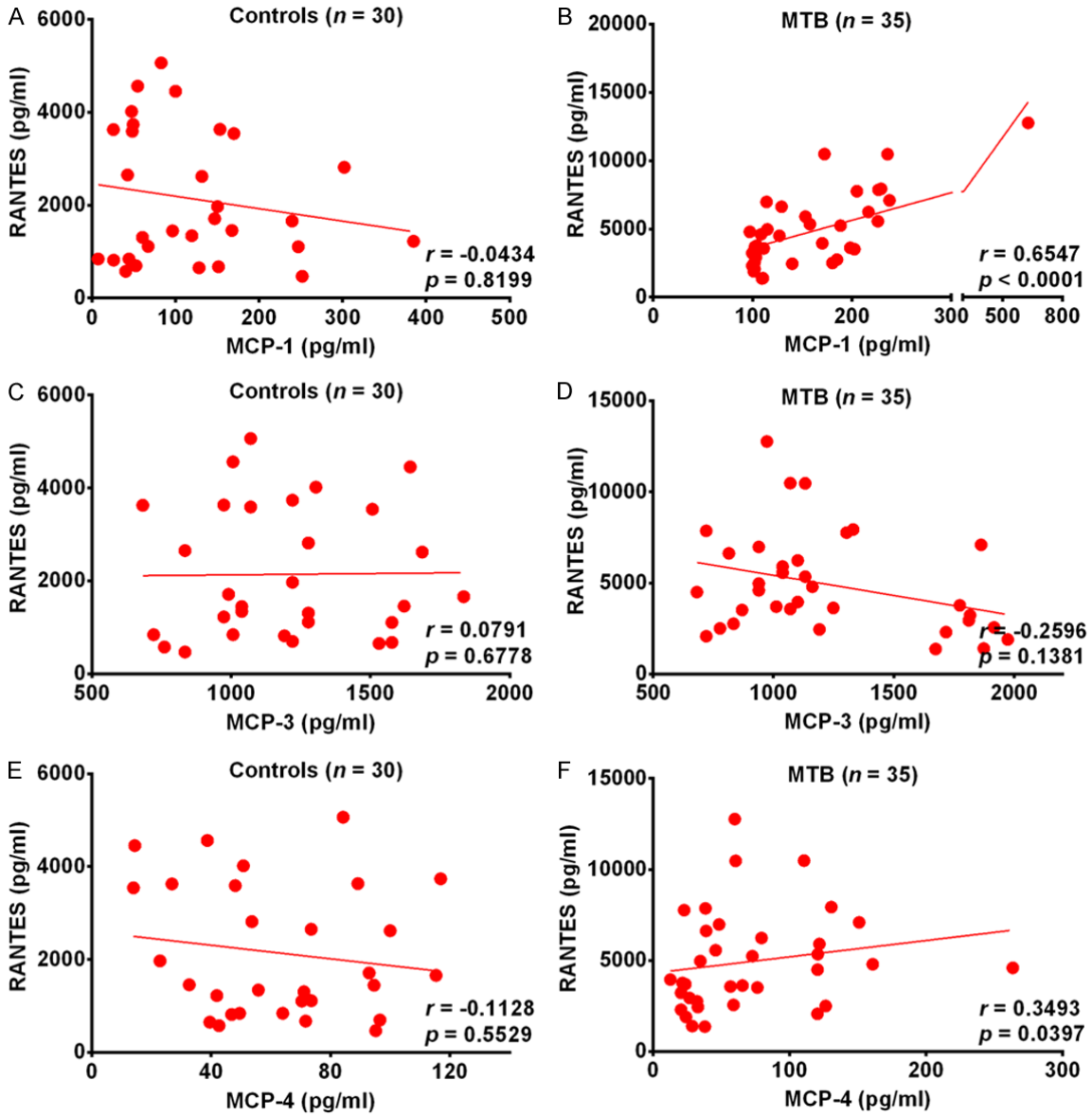

Figure 2. Correlation between RANTES and MCP-1, MCP-3, and MCP-4. Representing correlation of (A) RANTES and MCP-1 in controls, (B) RANTES and MCP-1 in MTB infections, (C) RANTES and MCP-3 in controls, (D) RANTES and MCP-3 in MTB infections, (E) RANTES and MCP-4 in controls, (F) RANTES and MCP-4 in MTB infections.

Of the 14 chemokines, 5 chemokines were markedly increased in MTB infections, compared to controls. Results suggest that those MTB-infected monkeys might be at a stage of resistance to MTB.

During the infection period, immune responses to MTB are involved not only in protection against infections but also in tissue damage [8, 14]. Th1 immune response plays a crucial role in the acquisition of resistance to MTB. Progressive diseases always fail to generate Th1 responses and/or induce stronger Th2 responses, causing Th1/Th2 imbalances to Th2. Th1 cytokines and Th2 cytokines are crucial for Th1 and Th2 cell-mediated immune response. The current study evaluated Th1/Th2 cytokines ratios in MTB infections and controls. Five Th1 cytokines (TNF-α, IFN-γ, IL-1β, IL-2, and IL-15) and 2 Th2 cytokines were (IL-4, IL-5, and IL-10) were included. Significant increases of Th1 (IFN-γ and IL-15)/Th2 (IL-4, IL-5, and IL-10) cytokine ratios were observed in MTB infections, compared to controls, indicating Th1/Th2 cyto-

kine imbalances toward Th1 polarization in MTB infections. Determined by cytokine response due to imbalances or deficiencies in the cytokine network, infectious statuses of MTB-infected rhesus monkeys were mainly at the stage of primary (initial) infection or active TB. This was always accompanied by augmented humoral responses resistant to MTB infections.

Mounting evidence has suggested identified that IFN-γ is crucial for anti-MTB immune response. IFN-γ-inducible chemokine (IP-10) is comparable to IFN-γ as a diagnostic marker of tuberculosis [15]. IL-15 has been identified as an IFN-γ inducing cytokine that plays an important role in Th1 response [16]. Thus, the current study analyzed potential association between IFN-γ and IL-15 and IP-10. In a previous report, IFN-γ and IP-10 were measured using ELISA kits. Results were lower than those measured by Luminex xMAP technologies in the current study [12]. However, significant positive correlation was observed between IFN-γ and IP-10 in either controls or MTB infections in this study, as previously reported [12]. IFN-γ was positively correlated with IL-15 in MTB infections, but not with controls ($P < 0.05$). Present findings also support IFN-γ and IP-10 as dominant and valuable proinflammatory cytokines.

Regarding migration and survival of monocyte subsets, CXC and CC types of chemokines and their receptors play a critical role. Previous evidence has demonstrated that RANTES, MCP-1, MCP-3, and MCP-4 are associated with tuberculosis [13, 17-20]. The current study analyzed correlation between RANTES and MCP-1, MCP-3, and MCP-4. Although no significant correlation between RANTES and MCP-1, MCP-3, and MCP-4 was found in controls, RANTES was positively correlated with MCP-1 and MCP-4 in MTB infections ($P < 0.05$). Enhanced monocyte migration might exist in MTB infections, acting against infections.

Current findings demonstrate that most MTB-infected monkeys were at the course of anti-infection immune response to MTB. This was distinct from human patients. Today, rigorous surveillance procedures of tuberculosis are utilized to locate infections, testing each captive monkey in breeding farms semi-annually. Most infected monkeys are diagnosed in no more than 6 months. Thus, most MTB-infected monkeys may be infectious during the stage of primary infection or active MTB infection.

Profiles of peripheral cytokines and chemokines in human tuberculosis patients have been demonstrated in numerous studies. However, few have been reported for MTB-infected monkeys. The current study profiled peripheral cytokines and chemokines in naturally MTB-infected rhesus monkeys, investigating Th1/Th2 cytokine ratios and performing correlation analysis between different cytokines. The current study provides evidence for the study of immunity and diagnosis of tuberculosis.

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Disclosure of conflict of interest

None.

Address correspondence to: Lan Zhang, Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Department of Obstetrics and Gynecology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, P. R. China. Tel: 86-20-84106820; E-mail: 51326169@qq.com; Yu Zhang, Guangdong Laboratory Animals Monitoring Institute, Guangdong Provincial Key Laboratory of Laboratory Animals, Guangzhou, P. R. China. Tel: 86-20-84106803; E-mail: zhangyugzh@hotmail.com

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