Depressed CCL5 Expression in Human Pulmonary Tuberculosis

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CCL5/regulated on activation, normal T expressed and secreted production (RANTES) is a principal CC chemokine, and can activate macrophages and Th1 lymphocytes, however, little is known about the CCL5 profiles associated with active tuberculosis (TB). In this study, we investigated the production of CCL5 by the peripheral blood mononuclear cells (PBMCs) of patients with active pulmonary TB after stimulation with Triton X-100 soluble proteins (TSP) or the 30-kDa antigen. The profiles of cytokines/chemokines [CXCL8/interleukin (IL)-8, IL-12 p40, and interferon (IFN)-γ] were also examined by PBMCs from TB patients, and compared with those obtained from healthy tuberculin reactors (HTR). Concordant with earlier studies, IFN-γ production was significantly depressed in the PBMCs from TB patients compared with those from HTR. In addition, the CCL5, but not CXCL8, levels in the PBMCs from TB patients were significantly depressed after stimulation for 18 hr compared to those in the PBMCs from HTRs. The CCL5 release was not significantly correlated with the release of IFN-γ in the cells from TB patients and HTRs. Further, inhibitor studies show that the 30-kDa- or TSP-induced CCL5 mRNA expression is sensitive to inhibitors of mitogen-activated protein kinase kinase (MEK) 1/2 and Janus kinase (JAK) 2, but not p38, pathway activation, suggesting a MEK1/2- or JAK2-based mechanism is responsible for modulating of the CCL5 expression in human PBMCs. Collectively, these data suggest that TB patients show depressed production of CCL5 secretion, which can be modulated by MEK- and JAK2-based transcriptional regulatory mechanisms, in response to the mycobacterial antigens.

Key Words: Tuberculosis, CCL5, Interferon-γ, 30-kDa antigen, TSP antigen

INTRODUCTION

Tuberculosis (TB) is a chronic disease requiring the constant expression of cellular immunity to limit bacterial growth. A complex interaction between host and mycobacteria plays an important role for the regulation of the development of cellular immunity, which affects both protective and inflammatory roles (12). The immune cell interaction with Mycobacterium tuberculosis (Mt) includes binding of Mt to macrophage and dendritic cell receptors such as mannose receptor, toll-like receptors (TLRs), and DC-SIGN (29). Triggering of these receptors induces cell activation with the subsequent production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, which are to play multiple roles in immune and pathologic responses in TB (12). Various
Several cytokines and chemokines have been identified as critical factors in the protective immunity and pathophysiology of TB (18). Chemokines are critical in this response since they are both chemotactic and immuno-regulatory molecules (42). Chemokines are small-molecular-mass chemotactic cytokines (8–14 kDa) that mediate constitutive and inflammatory recruitment of leukocytes from the blood into tissues. Chemokines are grouped into four structurally related families (designated CC, CXC, C, or CX3C), determined by the numbers of amino acids that separate the two cysteine residues closest to the amino terminus (31). Among the many chemokines, CCL5/regulated-upon-activation, normal T-cell-expressed and -secreted chemokine (RANTES), a CC chemokine, is produced by a variety of cell types, including T cells and macrophages. CCL5 has a selective action as a monocyte/macrophage attractant in the airways and strongly induces migration of T cells with the memory phenotype (3). Expression of CCL5 has been detected predominantly in the macrophages and endothelial cells in the granulomatous lesions of both sarcoidosis and TB patients, suggesting that CCL5 production in delayed-type hypersensitivity granulomas may play a role in the selective accumulation of macrophages and memory T helper lymphocytes characterizing this type of cell-mediated immune reaction (9).

In addition, CCL5 protein production was synergistically induced by both IFN-γ and TNF-α in human umbilical venous endothelial cells, whereas the Th2-type cytokines IL-4 and IL-13 significantly inhibit the CCL5 secretion (28). Further, IFN-γ deficient type 1 granulomas showed decreased levels of TNF-α and CCL5, suggesting that CCL5 production is associated with protective cytokines (6). Recent studies using various leukocyte populations (splenocytes, alveolar, and resident peritoneal macrophages) obtained from naïve and M. bovis bacillus Calmette-Guérin (BCG)-vaccinated animals demonstrated that cells from BCG-vaccinated guinea pigs and infected with Mtb produced elevated CCL5 mRNA and protein compared to infected cells from naïve animals (36). The same study also showed that both CCL5 mRNA and protein levels significantly increased after induction of tuberculous pleurisy in BCG-vaccinated guinea pigs (36). Furthermore, the interaction between CCL5 and CCR5 may be critical in regulating T cell functions, by mediating their recruitment and polarization, activation, and differentiation (42). Despite numerous studies indicate the role of CCL5 in vitro and in vivo, it remains elusive in the profiles of CCL5 production in peripheral mononuclear cells and its regulatory mechanism in human TB.

Mycobacterial infection leads to a signaling response by the host macrophage and subsequent production of pro-inflammatory mediators. In this response, macrophage signaling pathways are activated by infection with mycobacteria include the mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-regulated kinase (ERK) 1/2 and the calmodulin/calmodulin kinase pathway (19,32,33,43,44). Our recent study demonstrates that the 30-kDa Ag-induced CCL20 mRNA expression involves MAPKs- and NF-κB-dependent signaling (26). Following chemokine receptor aggregation on T cells, various tyrosine phosphorylation signaling cascades can be engaged, including the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, focal adhesion kinase (FAK) activation, MAPKs, and phosphatidylinositol 3-kinase activation (42).

In the present study, we analyzed the CCL5 secretion profiles of peripheral blood mononuclear cells (PBMCs) from active pulmonary TB patients after in vitro stimulation with two mycobacterial Ags, 30-kDa Ag and Triton X-100 soluble protein (TSP) from Mtb H37Rv. The CCL5 secretion
profiles were compared with those of healthy tuberculin reactors (HTR) as well as the other cytokine productions, such as IFN-γ, IL-12p40, and CXCL8. The results show that the expression of CCL5 is prominently down-regulated in PBMCs from TB patients compared to those from HTRs. Of importance, CCL5 release is not correlated with IFN-γ by PBMCs in HTR and TB patients. We also demonstrate that the 30-kDa- or TSP-induced CCL5 mRNA expression involves MEK1/2- and JAK2-dependent signaling.

**MATERIALS AND METHODS**

1. **Subjects**

Whole blood sample was obtained from a total of 20 TB patients and 20 HTRs. All subjects gave their informed consent before being enrolled in the study. The HTR subjects were recruited from medical college students at Konyang University Hospital, and they had no previous history of clinical TB. Skin reactions in healthy volunteers of more than 10 mm after an intradermal test with 2TU of PPD (RT23; Statens Serum Institut, Copenhagen, Denmark) were considered to be positive.

Active pulmonary TB patients participated in this study within 1 month of beginning first-line antituberculosis drug medication at the Konyang University Hospital (Daejeon, Korea). All of the patients in this study had parenchymal TB, and none had miliary or pleural TB. All patients had a positive sputum culture for Mtb. They had no previous history of diabetes mellitus or steroid therapy, and all were HIV-negative. Extensive clinical histories were obtained from the subjects and included data on age, sex, medical history, chest radiographic findings, sputum staining and culture results, drug sensitivities, treatment, and outcome. The study was reviewed and approved by the Institutional Research Board of Konyang University Hospital, and written informed consent was obtained from each participant.

2. **Purification of the 30-kDa Ag and isolation of the TSP Ag**

*Mtb* H37Rv strain (ATCC 27294) were grown in Sauton synthetic medium at 37°C for six weeks. Cells were harvested by centrifugation, washed twice with Tris buffered saline (TBS), used for TSP Ag isolation. The *Mtb* H37Rv culture supernatant was then filter-sterilized using 0.22 μm membrane filter, and used for 30-kDa Ag purification.

For the isolation of TSP Ag, as previously described by Kim *et al.* (20), the cells were suspended in 100 ml TBS of 1% Triton X-100/10 mM phenylmethylsulphonyl fluoride (PMSF)/0.05% NaN3/1 mM EDTA and incubated with vigorously shaking for 18 hr at 37°C. The solubilized extracts by Triton X-100 were centrifuged at 10,000 × g for 1 hr at 4°C, and the supernatants were transferred in a clean bottle. The proteins in the supernatant were precipitated with ammonium sulfate (20 to 80% saturation), and pre-condensation with Triton X-114 was performed by repeated dilution and phase separation. The resultant aqueous phase was cleansed by the repeated addition of 11.4% Triton X-114 to a final concentration of 2%. After 3 repeats of 0 to 80 ammonium sulfate precipitation, the isolated Ag was finally applied to a column (Extracti-Gel D Detergent Removing Gel AffinityPak; Pierce, Rockford, IL, USA) to reduce the extraction of unwanted detergent from solutions containing proteins. The TSP Ag was stored in sterile aliquots at -20°C.

The 30-kDa Ag was purified from culture filtrate protein (CFP) of *Mtb* H37Rv (ATCC 27294), as previously described by Lee *et al.* (25). In brief, the native 30-kDa Ag was purified from the CFP by initial anion-exchange chromatography supported by Macro-Prep High Q (Bio-Rad, Hercules, CA, USA), ammonium sulfate precipitation, hydrophobic interaction chromatography (HIC), and second anion-exchange chromatography (UNO-Q6 column; Bio-Rad). The finally purified Ag was dialyzed against phosphate-buffered saline (PBS, pH 7.2) and applied to a column with immobilized polymyxin B (Detoxi-Gel Endotoxin Removing Gel; Pierce) to reduce the level of endotoxin, then filter sterilized, and frozen at -20°C. The endotoxin content in the each 30-kDa or TSP Ag preparation was < 0.02 ng/mg protein, determined by the limulus amebocyte lysate test (QCL1000; BioWhittaker, Walkersville, MD, USA). The protein concentrations were estimated using the bicinchoninic acid protein assay kit (Pierce), with bovine serum albumin
(BSA) as the standard.

3. Isolation of human PBMCs and Ag stimulation

PBMCs were isolated from whole blood by density sedimentation over Histopaque-1077 (Sigma, St. Louis, MO, USA). The PBMCs were suspended at a density of $1 \times 10^6$ viable cells/ml in complete medium [RPMI 1640 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco-BRL), sodium pyruvate, nonessential amino acids, 100 IU/ml penicillin G, and 100 μg/ml streptomycin]. PBMCs were then stimulated with 1.0 μg/ml 30-kDa or TSP Ag, and incubated at 37°C in a humidified, 5% CO₂ atmosphere until used for supernatant collection. LPS (1 μg/ml; Sigma) and phytohaemagglutinin (PHA) (1 μg/ml; Sigma) were used as positive controls for the Ag stimulation in this study.

4. Materials and reagents

The specific inhibitors of MEK, PD98059 and U0126, and p38 MAPK, SB203580 were purchased from Calbiochem (Darmstadt, Germany). The specific JAK2 kinase inhibitor AG490 was obtained from Mitsubishi Chemical Corp. (Tokyo, Japan). Dimethyl sulphoxide (DMSO; Sigma) was added to cultures at 0.1% (v/v) as a solvent control. All other reagents were purchased from Sigma, unless stated otherwise.

5. Enzyme-linked immunosorbent assay (ELISA) and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

A sandwich ELISA was used for detecting human IFN-γ, IL-12p40, and CXCL8 (BD PharMingen, San Diego, CA, USA), CCL5 (R&D System, Minneapolis, MN, USA) in culture supernatants. Assays were performed as recommended by the manufacturers. Cytokine concentrations in the samples were calculated using standard curves generated from recombinant cytokines, and the results were expressed in picograms or nanograms per milliliter. The difference between duplicate wells was consistently less than 10% of the mean.

For semi-quantitative RT-PCR analysis, total RNA was extracted from PBMCs using TRIzol (Invitrogen, Carlsbad, CA, USA), as previously described (44). Primer sequences were as follows: hCCL5 (forward: 5'-TTGCTCTTTGCTCTAGCTTGGGAG-3', reverse: 5’-GCAGCGCTTCAGAACGTCTTC-3’), β-actin (forward: 5’-ATCTGGACACCACACCTTCTACAATGAGCTGG-3’, reverse: 5’-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3’).

6. Statistical analysis

For statistical analysis, the data obtained from independent experiments were presented as the mean ± SD; they were analyzed using a Student t-test or simple linear regression analysis. P level < 0.05 was regarded as statistically significant.

RESULTS

1. Purification of the 30-kDa Ag and isolation of the TSP Ag from Mtb H37Rv

The TSP protein, not a single protein, but a complex
protein is a cell wall-associated soluble protein from Mtb H37Rv, while 30-kDa Ag is a major secreted protein of Mtb H37Rv. The isolation of TSP protein from Mtb H37Rv was carried out by extraction with 1% triton X-100, 20 to 80% ammonium sulfate precipitation, 3 times of phase separation with triton X-114, followed by 3 times of 0 to 80% ammonium sulfate precipitation. The 30-kDa Ag was purified from 6-week-culture filtrates of Mtb H37Rv by initial anion-exchange chromatography, 0 to 40% ammonium sulfate precipitation, hydrophobic interaction chromatography (HIC), and second anion-exchange chromatography.

The SDS-PAGE analysis of purified 30-kDa Ag and isolated TSP Ag are shown in Fig. 1. As determined by the SDS-PAGE, purified 30-kDa Ag was at least 90% pure, while TSP Ag showed diverse molecular masses in the range from 10 to 100 kDa molecular weight (MW). From 460 g wet weight of bacilli (Mtb H37Rv), approximately 10 mg of TSP Ag was recovered. From 985 mg of the CFPs of Mtb H37Rv, approximately 40 mg of purified 30-kDa Ag was recovered.

2. IFN-γ and IL-12p40 production by PBMC stimulated with 30-kDa and TSP Ags in TB patients and HTRs

The induction of potent Th1 immune response is mainly involved in the generation of protective immunity against TB (12,29). We compared the Th1 cytokine, IFN-γ and Th1 driving cytokine, IL-12p40 induction to 30-kDa and TSP Ags in the PBMC from TB patients and HTRs. The

![Figure 2. IFN-γ (A) and IL-12p40 (B) production in PBMC from TB patients and HTRs in response to the 30-kDa and TSP Ags. The production of IFN-γ and IL-12p40 by PBMC from TB patients and HTRs was determined after in vitro 96 hr (IFN-γ) or 18 hr (IL-12p40) stimulation with the 30-kDa and TSP Ags (1 μg/ml). Data are plotted as individual points on the graph (TB patients, n=20; HTRs, n=20). The protein levels of IFN-γ and IL-12p40 in culture supernatants were measured by specific ELISA. *, p<0.05; **, p<0.01; ***, p<0.001. Un, unstimulated.](image)

![Figure 3. CCL5 (A) and CXCL8 (B) production in PBMC from TB patients and HTRs in response to the 30-kDa and TSP Ags. The production of CCL5 and CXCL8 by PBMC from TB patients and HTRs was determined after in vitro 18 hr stimulation with the 30-kDa and TSP Ags (1 μg/ml). Data are plotted as individual points on the graph (TB patient, n=20; HTR, n=20). The protein levels of CCL5 and CXCL8 in culture supernatants were measured by specific ELISA. *, p<0.05; **, p<0.01. Un, unstimulated.](image)
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kinetics of IFN-γ and IL-12p40 production in response to these Ags were monitored, so as to determine the time required to achieve maximal IFN-γ and IL-12p40 levels (data not shown). As shown in Fig. 2A, the mean IFN-γ production in PBMC from TB patients after in vitro stimulation with 30-kDa and TSP Ags was significantly depressed compared with the corresponding values of the HTRs. The levels of IFN-γ in a media control were also significantly depressed in TB patients as compared to those of HTR. However, the production levels of IL-12p40 following stimulation with 30-kDa and TSP Ags did not show significant differences between the TB patients and HTRs (Fig. 2B). Accordingly, these data clearly show that the induction of IFN-γ, but not of IL-12p40, by PBMCs from TB patients is down-regulated by mycobacterial 30-kDa or TSP Ag.

3. CCL5 and CXCL8 production by PBMC stimulated with 30-kDa and TSP Ags in TB patients and HTRs

Chemokines including CCL2, CCL5 and CXCL8 are important molecules for the regulation of inflammatory cell influx to granuloma lesion of Mtb (5,7,34,45). Therefore, we next compared CCL5 and CXCL8 production by PBMC stimulated with either 30-kDa or TSP Ag in TB patients and HTRs. As shown in Fig. 3A, the mean CCL5 production by PBMC from TB patients was significantly decreased, compared with those from HTRs, at 18 hr-treatment with either 30-kDa or TSP Ag. However, the CXCL8 production induced by 30-kDa and TSP Ags in

![Figure 4](Figure 4. Correlation between IFN-γ and CCL5 or CXCL8 production in response to 30-kDa and TSP Ags in PBMC from TB patients and HTRs. The levels of each cytokine release after stimulation with the 30-kDa and TSP Ags were assayed by ELISA. Linear regression analyses were carried out to determine a possible correlation between CCL5 and IFN-γ production (for Panel A) and CXCL8 and IFN-γ production (for Panel B) in TB patients (n=12) and HTRs (n=18).)

PBMC from TB patients was significantly increased than corresponding values in HTRs at 18 h. Medium control level of CXCL8 was also significantly increased in PBMC from TB patients, when compared to those from HTR (Fig. 3B). Moreover, CXCL8 levels in monocyte, as a major source of the CXCL8 were also up-regulated in TB patients, when cultured with 30-kDa or TSP Ag (data not shown). There was no significant difference between 30-kDa and TSP Ags to CCL5 or CXCL8 production (p>0.1 for both Ags).

We also determined whether the individual CCL5 or CXCL8 production correlated with IFN-\(\gamma\) levels induced after 18-hr stimulation with the 30-kDa or TSP Ags. As shown in Fig. 4, there was no significant correlation between CCL5 and IFN-\(\gamma\) production by PBMCs from TB patients (r = -0.08, p<0.001, for 30-kDa Ag; r = 0.33, p<0.001, for TSP Ag) and from HTRs (r = 0.05 and p<0.001, for 30-kDa Ag; r = 0.17 and p<0.001, for TSP Ag), after in vitro stimulation with the 30-kDa or TSP Ags. In addition, CXCL8 production did not correlate with the production of IFN-\(\gamma\) by PBMC from TB patients (r = 0.03 and p = 0.59, for 30-kDa Ag; r = 0.14 and p = 0.11 for TSP Ag) and from HTRs (r = -0.10 and p<0.001, for 30-kDa Ag; r = -0.13 and p<0.001, for TSP Ag).

4. Either 30-kDa- or TSP-induced mRNA expression of CCL5 is dependent on MEK1/2- or JAK2 pathways in human PBMCs

Finally, we investigated the effects of the inhibition of MAPK activities on 30-kDa-or TSP-induced CCL5 mRNA expression in PBMCs. The 30-kDa-induced CCL5 mRNA expression was inhibited by JAK2 inhibitor AG490 in a dose-dependent manner when PBMCs were pretreated with AG490 for 1 hr prior to the 30-kDa stimulation. Similarly, AG490 pretreatment almost completely blocked CCL5 mRNA expression in response to the TSP Ag. Moreover, MEK1/2 inhibitor U0126 also decreased the 30-kDa- or TSP-induced CCL5 mRNA expression from PBMCs in a dose-dependent manner (n=4 experiments; Fig. 5). In contrast, SB203580, a selective inhibitor of p38 MAPK, had no significant effect on the CCL5 expression (Fig. 5).

**DISCUSSION**

Chemokines have been identified as key factors in orchestrating the development of protective TB granuloma (6,7). Previous studies showed that the elevated levels of CXCL8, CCL5, and CCL2 are observed in association with TB pleuritis, supporting the roles of chemokines that play a central role in a development of immunity against TB (7,11). In this study, we have focused on the chemokine profiles, specifically on the CCL5 and CXCL8 production, in human PBMCs from active pulmonary TB patients induced by 30-kDa and TSP Ags from Mtb H37Rv. To our knowledge, this is the first demonstration that the 30-kDa- or TSP-mediated CCL5 production is prominently down-regulated in PBMCs from TB patients, when compared with those from HTRs. In addition, individual secretion of CCL5 and CXCL8 was not correlated with that of IFN-\(\gamma\) in HTR or TB patients.

CCL5 (\(\beta\)-chemokine) are not only chemoattractants of T cells and macrophages, activating and expanding T cell populations (2), but also coactivators of macrophages, thus induces a Th1 response (10). Previous studies reported that
CCL5 contributes to the inflammatory milieu through the secretion of additional cytokines and mediators by recruiting mononuclear cells and neutrophils into the airway (30). In human TB, the levels of CXCL8, CCL5, and CCL2 were increased in bronchoalveolar lavage fluids from TB patients during acute and convalescent stages (21). Indeed, human alveolar macrophages are likely to encounter Mtb firstly thus actively release CCL2, CCL3 (MIP-1α), CCL4, CCL5, IP-10, and CXCL8 (34,35). Moreover, in a guinea pig model, neutralizing CCL5 resulted in lower macrophage accumulation, reduced levels of IFN-γ, TNF-α and CCL5 mRNA in pleural effusion cells, and diminished lymphocyte proliferation (36). Together with our current data, altered regulation of CCL5 production can be considered to reflect ineffective immune responses during human TB.

We found that CXCL8 production was increased by PBMCs from TB patients, compared with those from HTR. These data partly confirmed our previous studies, in which newly diagnosed TB patients elicited up-regulated expression of CXCL8 by PBMCs in response to the 30-kDa Ag, than multidrug-resistant TB patients or healthy controls did (24). CXCL8 is produced by a variety of cell types, including monocytes, fibroblasts, lymphocytes, epithelial and endothelial cells, and is a potent chemoattractant and activator of neutrophils and T cells (22,45). Previous studies reported that CXCL8 is necessary for the early accumulation of leukocytes in the skin and the development of a delayed type hypersensitivity reaction in the rabbit model of TB (23). In addition, circulating serum levels of CXCL8 were significantly higher in patients who subsequently died of TB, along with proinflammatory cytokines IL-6 (13). Casola et al. have also reported that CXCL5 transcription level is activated only after stimulation with TNF-α, but not IFN-γ (4). Therefore, these results imply that CCL5 and IFN-γ secretion is differently regulated in human immune cells in response to mycobacterial Ags.

Agonist stimulation of TLRs leads to the activation of MAPK cascades, including ERK1/2 (17). These signaling pathways are implicated in activation of the inflammatory and antimicrobial innate immune response to mycobacteria (17). We previously showed that the 30-kDa Ag-induced CCL20 mRNA expression involves ERK1/2, p38, and NF-κB-dependent signaling (26). In addition, ERK1/2, but not that of p38, pathway is required for the secretion of CCL2 from human monocytes infected with Mtb H37Rv (38). The present study shows that the 30-kDa- or TSP-mediated CCL5 mRNA expression is dependent on ERK1/2, but not p38, signaling pathways. We also found that CCL5 mRNA expression was significantly modulated by specific inhibitors of JAK2. The activation of the non-receptor tyrosine kinases JAK1 and JAK2, and subsequent phosphorylation of the signal transducer and activator of transcription 1 (STAT1) are central components of the cell-
ular responses to IFN-γ. Phosphorylated STAT1 dimerizes, translocates to the nucleus, and induces the transcriptional activation of multiple target genes (8,15). Previous studies reported that the infection of J774 murine macrophage cells with *M. bovis* BCG inhibited IFN-γ-stimulated STAT1 activation and tyrosine phosphorylation of JAK1 and JAK2 through the induction of SOCS-1 and SOCS-3 (16). In addition, *M. avium* infection of RAW 264.7 macrophages inhibited the JAK/STAT pathway activation stimulated by IFN-γ (14). As our data demonstrate that JAK2 pathway is crucial for the induction of the 30-kDa- or TSP-mediated CCL5 mRNA expression and that the CCL5 expression is down-regulated by PBMCs from TB patients, the JAK/STAT pathways might play a role as a potential molecular mechanism for the poor IFN-γ and CCL5 responses during human TB. Further studies are needed to clarify the exact molecular mechanism underlying the depressed CCL5 secretion during TB infection.

Collectively, results from our study suggest that altered CCL5 production is observed in PBMCs from active pulmonary TB patients, which might play an important role in immunopathogenesis during TB. There was no direct link between IFN-γ and CCL5 or CXCL8 levels. In addition, MEK1/2 and JAK2, but not p38, pathways are crucially involved in the CCL5 mRNA expression.

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