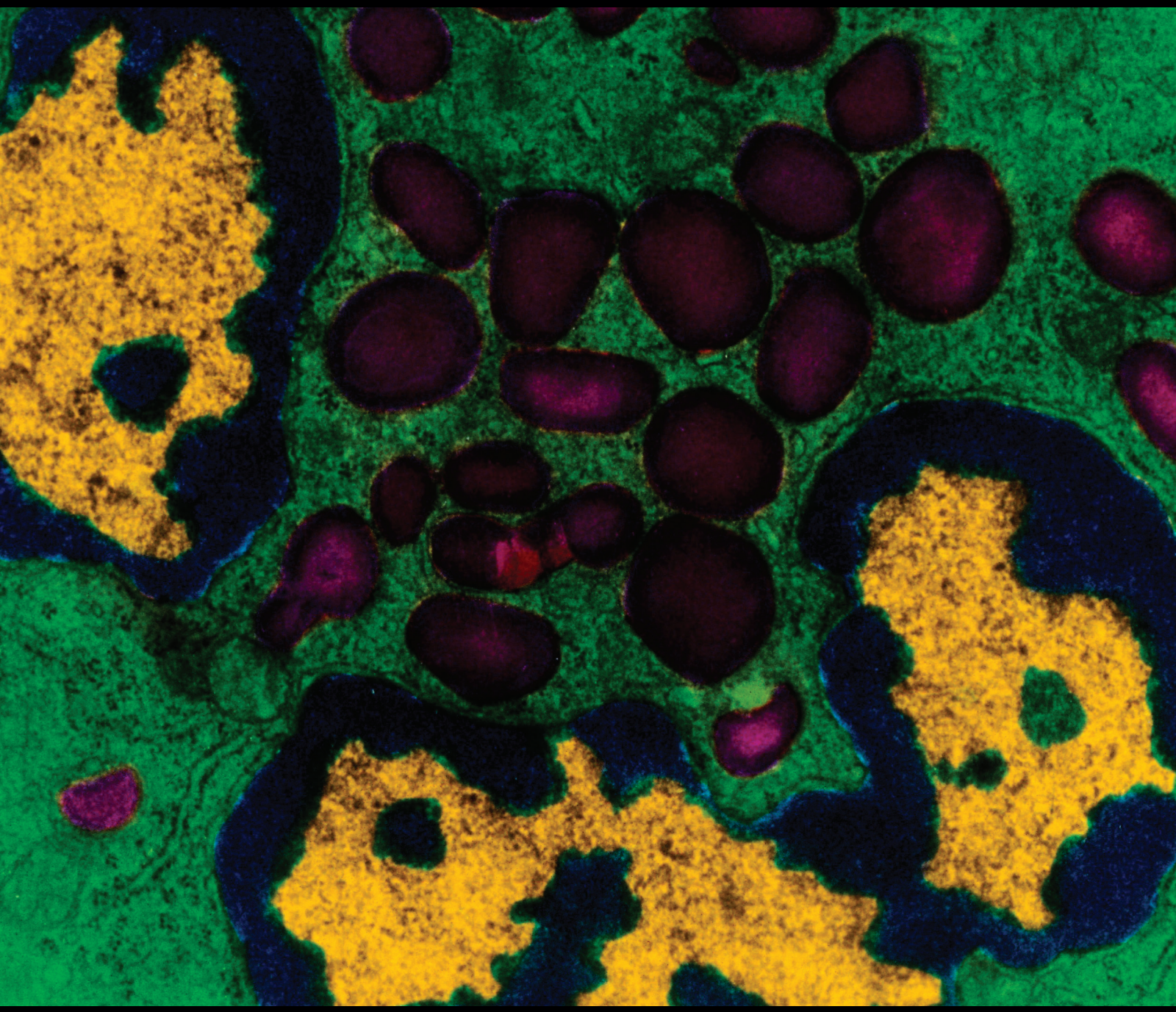
Mediators of Inflammation in Pulmonary Diseases

Guest Editors: Kostas Spiropoulos, Nikolaos Siafakas, Marc Miravitlles, Francesco Blasi, and Kiriakos Karkoulias



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Editorial Board

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Editorial

Mediators of Inflammation in Pulmonary Diseases

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Inflammation is supposed to play a great role in the pathogenesis of the most common diseases of the respiratory system. In Chronic Obstructive Pulmonary Disease (COPD), which is a major cause of morbidity and mortality all around the world, smoking mainly causes the initiation of the inflammatory process that leads to an impaired respiratory function. Moreover, in bronchial asthma there are numerous proinflammatory mediators that are responsible for the onset and the progression of the disease. Finally, pulmonary infections and especially tuberculosis result in the orchestration of an inflammatory process that targets the causative agent in order to protect the host.

L. M. O. Caram et al. have evaluated the levels of vitamin A in the serum and sputum and attempted to correlate it with known inflammatory markers, such as tumor necrosis factor alpha (TNF-α), interleukin- (IL-) 6, IL-8, and C-reactive protein (CRP) in 50 COPD patients and 50 individuals without COPD. The authors concluded that serum concentration of vitamin A is negatively associated with the presence of COPD and that it is positively associated with smoking status. Although COPD patients exhibited increased inflammation, these inflammatory markers were not associated with serum retinol concentrations.

The manuscript of W. Zhang et al. tested the effect of a novel γ-secretase inhibitor to promote Th17 cell differentiation in a mouse model of allergic asthma. Their findings suggest that the inhibitor directly regulates Th17 responses in the mouse model of allergic asthma that they used, making it potentially efficacious.

In their study, R. Rajajendram et al. tried a synthetic chalcone analogue in a murine model of asthma. Their results demonstrate a potential role of the substance in asthma, as the treatment with it inhibited eosinophilia, goblet cell hyperplasia, peripheral blood total IgE, and airway hyperresponsiveness in ovalbumin-sensitized and challenged mice. However, the tested nonsteroid potentially anti-inflammatory substance is far away from clinical use.

In the manuscript entitled "Binding of CXCL8/IL-8 to *Mycobacterium tuberculosis* Modulates the Innate Immune Response," A. Krupa et al. illustrate the role of IL-8 in the pathogenesis of tuberculosis (TB). The authors investigated the contribution of IL-8 in the inflammatory processes that are typically elicited in patients with TB. Their findings show that IL-8 seems to be the major chemokine responsible for recruiting T lymphocytes (CD3+, CD4+, and CD8+ T cells) and plays important role in the innate immunity against *Mycobacterium tuberculosis*.

Last but not least, the study of R. Baumann et al. investigated the IgA and IgG responses to mycobacterial protein antigens in subjects with latent tuberculosis, active tuberculosis, and healthy individuals. In their conclusions they present a new biomarker for the diagnosis of active pulmonary tuberculosis.

Acknowledgment

Special thanks are due to Dr. Dimosthenis Lykouras for his valuable help in this special issue.

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Research Article

A Subgroup of Latently *Mycobacterium tuberculosis* Infected Individuals Is Characterized by Consistently Elevated IgA Responses to Several Mycobacterial Antigens

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Elevated antibody responses to *Mycobacterium tuberculosis* antigens in individuals with latent infection (LTBI) have previously been linked to an increased risk for progression to active disease. Studies in the field focussed mainly on IgG antibodies. In the present study, IgA and/or IgG responses to the mycobacterial protein antigens AlaDH, NarL, 19 kDa, PstS3, and MPT83 were determined in a blinded fashion in sera from 53 LTBI controls, 14 healthy controls, and 42 active TB subjects. Among controls, we found that elevated IgA levels against all investigated antigens were not randomly distributed but concentrated on a subgroup of < 30%—with particular high levels in a small subgroup of ~ 5% comprising one progressor to active TB. Based on a specificity of 100%, anti-NarL IgA antibodies achieved with 78.6% sensitivity the highest accuracy for the detection of active TB compared to healthy controls. In conclusion, the consistently elevated IgA levels in a subgroup of controls suggest higher mycobacterial load, a risk factor for progression to active TB, and together with high IgG levels may have prognostic potential and should be investigated in future large scale studies. The novel antigen NarL may also be promising for the antibody-based diagnosis of active TB cases.

1. Introduction

Approximately one third of the world's population has latent infection with *Mycobacterium tuberculosis* [1]. Latent *M. tuberculosis* infection (LTBI) represents a considerable reservoir of future active disease and contagion. Risk factors include co-infection with human immunodeficiency virus (HIV), diabetes mellitus, low body weight, old age, or use of immunosuppressive medications. In immunocompetent individuals, the annual risk of progression is estimated to be greatest in the first 1 or 2 years after infection.

Preventing LTBI individuals from reactivation (before they become symptomatic and infectious) may constitute a major step towards the elimination of TB. Therefore, the revised global plan to stop TB (2011–15) [2] has set 2015 as the goal for point-of-care tests that can be used for the accurate detection of preclinical TB.

Bacterial load is associated with disease risk, and antibody levels against *M. tuberculosis* components may be biomarkers for load [3] as well as disease risk [4–6]. Stratification of TB suspects into groups of absent, low (smear-negative tuberculosis), and high (smear-positive tuberculosis) bacterial burden showed that antibody levels correlated with bacillary burden [4]. In the same study, data of the macaque model, which reproduces key features of latent TB in humans, showed that infection outcome was reflected by the antibody response in the latent infection group [4], thereby confirming previous animal studies [7–9]. Although studies were small,

increased specific antibody levels during the LTBI stage in humans also characterized progressors [5, 6]. Antibody-based tests for the diagnosis of active TB disease are often criticized for their lack of specificity in TB endemic regions [10], which is due to a high background prevalence of LTBI [11]. Clearly, further research is needed to elucidate whether *M. tuberculosis* specific antibody tests can determine active TB and cases at risk for progression and whether lack of specificity of antibody-based TB tests will turn out to be due to a high risk for an early stage of progression to active TB.

The low frequency of reactivation in immunocompetent LTBI individuals poses a challenge for the discovery of prognostic markers. Therefore, we chose to investigate the distribution of serologic responses, as a nonrandom distribution may point to a subgroup with higher bacterial load and an increased risk for future progression to disease. In a South African TB endemic population, we evaluated the serodiagnostic reactivity of L-alanine dehydrogenase (AlaDH) (Rv2780), nitrate/nitrite response transcriptional regulator NarL (Rv0844c), periplasmic phosphate-binding lipoprotein PstS3 (Rv0928), 19 kDa lipoprotein antigen precursor LpqH (Rv3763), and lipoprotein MPT83 (Rv2873). IgG responses to each of the two surface-exposed lipoproteins, 19 kDa and MPT83, are predominantly recognized in active TB sera and not in non-TB disease (NTBD) sera [4]. The 19 kDa antigen promotes binding to host cells and phagocytosis of mycobacteria [12], inhibits IFN-γ-induced killing of mycobacteria by macrophages [13], and induces macrophage apoptosis [14]. MPT83 elicits T cell proliferation of the majority of TB patients and is being considered as future subunit vaccine candidate [15]. The third lipoprotein, *M. tuberculosis* PstS3, which is involved in active transport of inorganic phosphate across the membrane (import), has not been investigated yet in subjects with LTBI for the serodiagnosis of *M. tuberculosis* [16]. However, PstS3 generates IFN-γ-producing cells in a more potent manner than the closely related 38 kDa (PstS1) [17], a major *M. tuberculosis* antigen [4, 18, 19]. The in TB serodiagnostics newly investigated protein antigen NarL is a putative nitrate response regulator involved in the regulation of anaerobic metabolism [20] and is part of the membrane fraction of *M. tuberculosis* [21]. IgG responses to the culture filtrate (and membrane) protein AlaDH are unable to distinguish untreated TB patients and controls in endemic settings [22]. AlaDH is present in *M. tuberculosis* but not in the vaccine strain *Mycobacterium bovis* BCG [21, 23]. It may play a role in cell wall synthesis as L-alanine is an important constituent of the peptidoglycan layer.

We focused on IgA antibodies because *M. tuberculosis*-specific IgA antibodies discriminated better than IgG antibodies between active TB and TB endemic controls in Africa [24] as well as between healthy close contacts of pulmonary TB patients and healthy individuals without such contact [25]. Moreover, IgA production was reported to be highly T-cell dependent [26], and a protective role for IgA was suggested in several murine models of mycobacterial infection, for example, [27].

2. Materials and Methods

2.1. Study Population. Sera utilized to probe antibody assays were from a retrospective serum bank collected from individuals in an epidemiological field site in metropolitan Cape Town in South Africa with a population of whom 99.7% are of mixed race. The incidence of new smear-positive TB in this community was 341/100 000 population in 2002 and the majority of people harbours latent infection [28]. In the study community, BCG vaccination (Danish strain, 1331, Statens Serum Institute, Copenhagen, Denmark) is routinely administered at birth since 1971. The study was approved by the Ethics Committee of the Faculty of Health Sciences at the Stellenbosch University and written informed consent was obtained from all participants or their legal guardians in the case of children.

Community Control Subjects. Inclusion criteria for all healthy control participants enrolled into the study were residence in the described community, absence of clinical signs of TB or other diseases, absence of prior TB, HIV negativity, and no pregnancy. Several parameters were employed to determine TB infection status: the Mantoux skin test, two different and independent commercial interferon-γ release assays (IGRAs) [the QuantiFERON TB Gold in Tube (QFT) (Qiagen, Hilden, Germany, Australia) and T-SPOT.*TB* (Oxford Immunotec, Abingdon, UK)], chest X-ray (CXR), and sputum AFB staining. We used >15 mm as cut off for Mantoux test positivity [29] and alternatively >5 mm [30] as described in more detail in the results section. Two IGRAs were used, as rates of positive results have been reported to differ between T-SPOT.TB and QuantiFERON-TB Gold (e.g. [31, 32]). Sixty-four consecutively recruited recent household contacts of active TB patients were part of a larger household contact study, and because the non-LTBI individuals constituted fewer than 20% of the contacts, three additional community controls with no known previous TB exposure were added and underwent the same investigations as the household contacts except the IGRA assays. The majority of LTBI subjects were followed up for the development of active TB disease within 2 years after their first recruitment, and one progressor (after 3 months) was identified according to hospital records.

TB Patients. Forty-two HIV-negative, Ziehl-Neelsen sputum smear-positive and BACTEC sputum culture-positive active pulmonary TB patients with no known multidrug resistance that were part of the same larger study as the controls, of which results have been published recently [33, 34], were included. Seven TB patients were excluded due to HIV-seropositivity ($n = 1$), NTM infection ($n = 2$), or concomitant illness, such as diabetes mellitus ($n = 4$). All TB patients were self-reporting, untreated cases, and all except 2 had a first episode of active TB.

2.2. Serum Preparation. Blood samples of control subjects and TB patients (at diagnosis prior to initiation of treatment) were taken. After transport of the blood samples to the laboratory (within 2 h of collection and at ambient conditions),

Table 1: Recombinant antigens of *M. tuberculosis* used in this study.

Protein name(s)	Rv number	Mol mass (kDa)	Expression vector	6-fold His-Tag	*E. coli* host strain
NarL	Rv0844c	23.9	pET22	N-terminal	BL21 (DE3)
AlaDH	Rv2780	38.7	pJLA604	—	CAG629
19 kDa glycolipoprotein, LpqH	Rv3763	16.0	pET26	C-terminal	BL21 (DE3)
PstS3	Rv0928	38.8	pET22	C-terminal	Rosetta (DE3)
MPT83, MPB83	Rv2873	24.9	pET21	C-terminal	BL21 (DE3) [pLysS]

Nitrate/nitrite response transcriptional regulatory protein NarL; secreted L-alanine dehydrogenase (AlaDH); 19 kDa lipoprotein antigen precursor LpqH; periplasmic phosphate-binding lipoprotein PstS3; cell surface lipoprotein MPT83.

serum was separated by centrifugation (1250 ×g for 7 min) and stored in aliquots at −80°C until use.

2.3. Antigen Cloning, Protein Expression, and Purification. The production of the functional *M. tuberculosis* L-alanine dehydrogenase (AlaDH) in the heat-induced strain *Escherichia (E.) coli* CAG629 (pMSK12) has been described previously [35]. For cloning, the genes of the remaining 4 protein antigens (Table 1) were amplified by PCR using primers with integrated restriction sites allowing the site-directed insertion of cleaved PCR-products into pET vectors (Novagen). All four genes were fused to sequence coding for 6-fold His-tag (Table 1). The genes were expressed in *E. coli* BL21(DE3) or in case of *pstS3* in *E. coli* Rosetta (DE3). The antigens were purified using standard chromatographic methods (affinity chromatography, ion exchange chromatography, size exclusion chromatography). Insoluble antigens were solubilized (refolded) from denaturating conditions (8 M urea) into buffers free of choatropic reagents. Further details regarding the protein purification are described in the supplements (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/364758).

2.4. Enzyme-Linked Immunosorbent Assay. Microtiter plates were coated with *M. tuberculosis* antigens and serologic antibody responses were determined using standard procedures as described in the supplements. Laboratory personnel performing the serodiagnosis assays were blinded to the clinical status of the patients or the controls. Subsequent record reviews were done by clinical staff to classify the individuals into clinical groups without knowledge of the serologic response phenotype.

2.5. Statistical Analysis. GraphPad Prism (Graph Pad, San Diego, CA, USA) was used to create graphs and statistical analyses were performed using Medcalc software (Kagi, Berkeley, CA). The t-test for independent samples or the Mann-Whitney test was used for the statistical comparison of 2 groups depending on the fact whether or not the data were normally distributed. Given the aim to develop a diagnostic test for TB with a specificity level above at least 90%, the experiments were analyzed by using specificity levels of ≥ 90%, as was done by other investigators [36, 37]. The Spearman rank test was used for correlation analyses. Generally, a two-tailed P value of $P \leq 0.05$ was considered significant.

3. Results

3.1. Classification of Study Participants according to Their Mycobacterium tuberculosis Infection Status. Sixty-four healthy household contacts of recently (within past 2 months) diagnosed active pulmonary TB patients underwent TST and 2 commercial IGRA tests, QFT, and T-SPOT.TB. Fifty-three household contacts were classified as LTBI due to positivity in TST (induration ≥ 15 mm) [29], QFT and/or T-SPOT.TB ($n = 53$; age range: 15–59 years; 58.2% females). The remaining 11 household contacts were classified as healthy controls (HC). As noninfected control individuals were rare in the described TB endemic settings [28], we added 3 healthy nonhousehold contacts (community controls with no known previous TB exposure) with TST-indurations of 0 mm, normal chest X-rays and AFB-negative-assisted sputum samples to the healthy control group in order to increase the statistical power ($n = 14$; age range: 10.7–55.2 years; 71.4% females). The Mantoux induration > 15 mm criterion was used for the definition of LTBI, as a large-scale study performed in a rural African population showed that the local environmental mycobacterial exposure is reflected in Mantoux indurations that cluster around 10 mm, whereas the *M. tuberculosis* exposures are reflected by indurations with a mode at 15–17 mm or larger [29]. Still, the exact definition of the LTBI status is hampered by the lack of a gold standard. According to Centers for Disease Control and Prevention (CDC) criteria Mantoux induration > 5 mm of recent TB household contacts can be considered as LTBI [30], and we alternatively considered this cut off for defining a positive tuberculin reaction: of the fourteen members of the original HC group, two subjects had Mantoux indurations between 5 mm and 15 mm, and both individuals had negative IGRA results. Therefore, in an alternative grouping of the controls, we transferred the 2 IGRA-negative individuals with Mantoux indurations between 5 and 15 mm from the HC group (HC*: $n = 12$) into the LTBI group (LTBI*: $n = 55$). The 42 smear-positive active pulmonary TB patients (age range: 18–55 years; 40.5% females) included in this study were recruited from the same community as the control participants.

3.2. Profile of Specific IgA and/or IgG Antibodies in an Endemic Setting. The 5 protein antigens NarL, AlaDH, 19 kDa, PstS3, and MPT83 (Table 1) were cloned and expressed in *E. coli* and purified using standard chromatographic methods. A

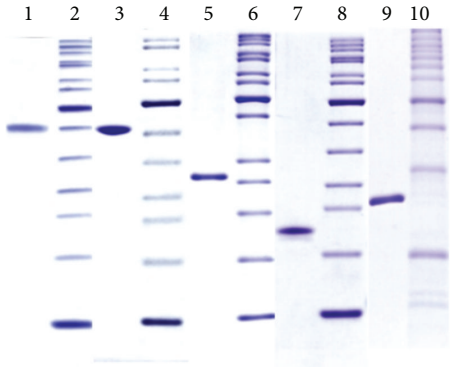

Figure 1: Quality control SDS-PAGE analysis (reduced) of the highly purified recombinant antigens (1 μg per lane). *Lane 1*: PstS3, *Lane 3*: AlaDH, *Lane 5*: MPT83, *Lane 7*: 19 kDa, *Lane 9*: NarL, *Lanes* 2, 4, 6, 8, and 10: molecular weight ladders (each corresponding to the antigen on the left side), *Lanes* 1–4: 15% Laemmli gels, and *Lanes* 5–10: 12% Laemmli gels. All stained with Coomassie brilliant blue R250.

Coomassie Brilliant Blue R-250 stained SDS-PAGE analysis of the 5 proteins is shown in Figure 1.

The 109 serum samples derived from 42 pulmonary TB patients, 53 LTBI controls, and 14 non-LTBI controls were tested in a blinded fashion for IgA responses specific to the 5 proteins as well as for IgG responses to AlaDH. Among controls, we found that elevated IgA levels against the investigated 5 antigens were not randomly distributed but concentrated on a subgroup of 28.4% ($n = 19$)—with particular high levels in a subgroup of 4.5% ($n = 3$), which comprised the progressor from latent infection to active TB. To graphically distinguish between those LTBI individuals with negative serum data (more than 70% of the controls) and those with moderately elevated and highly elevated serology, we used the mean ranks of IgA signals against the 5 antigens investigated to divide the LTBI subjects into the 3 subgroups LTBI (low IgA), LTBI (medium IgA) and LTBI (high IgA) (Figure 2). Furthermore, we statistically compared the non-LTBI controls with either the active TB patients or the LTBI subgroup with elevated IgA signals comprising the 2 subgroups LTBI (medium IgA) and LTBI (high IgA) (Table 2). Based on a cut-off referring to the 92.9-percentile of the healthy non-LTBI controls [92.9% (95% CI, 66.1–99.8%) specificity], the IgA response against the novel protein antigen NarL achieved with 81% (95% CI, 65.9–91.4%) the highest sensitivity for the detection of active TB patients, followed by anti-AlaDH IgA with 76.2% (95% CI, 60.5–87.9%) sensitivity and anti-19 kDa IgA with 64.3% (95% CI, 48.0–78.4%) sensitivity (Table 2). Moreover, based on a specificity of 100% (95% CI, 76.8–100%), anti-NarL IgA and anti-AlaDH IgA both detected 84.2% (95% CI, 60.4–96.6%) of the LTBI subgroup with elevated IgA signals, followed by anti-19 kDa IgA with 78.9% (95% CI, 54.4–93.9%) sensitivity (Table 2). Anti-NarL IgA, anti-19 kDa IgA, and anti-AlaDH IgA detected the LTBI (high IgA) subgroup, which comprised the progressor to active TB, with a distinct signal-to-noise ratio compared to the non-LTBI community controls (Figures 2(a), 2(b), and 2(f)). In contrast, the LTBI (high IgA) subgroup was indistinguishable from the non-LTBI community controls when using the IgG response to AlaDH (Figure 2(e)). Very similar results to those shown in Table 2 were obtained when using the alternative groups HC* and LTBI* (Table S1).

When testing for correlations between the antibody OD values in LTBI sera ($n = 53$) (Table 3 and Figure 3), we found strong correlations between the IgA responses and the 5 protein antigens [strongest correlation between anti-AlaDH IgA and anti-19 kDa IgA: Spearman's $r = 0.96$ (95% CI, 0.93–0.98); $P < 0.0001$ (Figure 3(a)); weakest correlation between anti-NarL IgA and anti-PstS3 IgA: $r = 0.82$ (95% CI, 0.71–0.89); $P < 0.0001$ (Figure 3(d))]. In contrast, we found no or negligible correlations of any of the IgA responses with the exemplarily tested IgG response to AlaDH [e.g., no correlation between anti-AlaDH IgA and anti-AlaDH IgG: $r = 0.21$ (95% CI, −0.06–0.46); $P = 0.128$ (Figure 3(c))] (Table 3). Very similar results to those shown in Table 3 were obtained when using the alternative group LTBI* (Table S2).

4. Discussion

In recent years it became increasingly clear that antibody-based diagnostics of active TB (often developed and tested in non-TB endemic countries) performed poorly in TB endemic settings [10] due to high background signals of antibodies in LTBI individuals [11]. In our study we also found that the presence of LTBI affected identification of active TB by serology. In particular, we found that among controls elevated IgA levels against the investigated 5 antigens were not randomly distributed but concentrated on a subgroup of <30%—with particular high levels in a small subgroup of ~5%. The consistently elevated IgA levels in a subgroup of controls suggest higher mycobacterial load, a risk factor for progression to active TB. Whether high IgA and/or IgG levels have prognostic potential should be investigated in future large scale studies.

In several previous reports it has indeed been suggested, directly or indirectly, that progression to active TB may be predicted by increased specific antibodies levels. Certain TB-specific antibody responses decline significantly during successful therapy (and the time thereafter) when both bacterial load and risk of disease also decline [38, 39]. In inactive TB (defined by a positive response to the TST, negative sputum cultures, and abnormal but stable CXR findings), a special form of latent infection known to have increased risk of active TB, elevated specific antibody responses have been reported [40, 41]. Several independent studies showed that antibody responses to mycobacterial proteins were detectable months to years prior to the diagnosis of TB in persons infected with HIV, for example, [8], again suggesting that *in vivo M. tuberculosis* replication may begin long before progression to active TB becomes clinically detectable. Specific antibacterial antibodies have been shown to be present in sera obtained from *M. tuberculosis* H37Rv aerosol-infected rabbits, and guinea pigs both with preclinical TB [7, 8]. In mouse models of *M. avium* infection, susceptibility to infection correlated

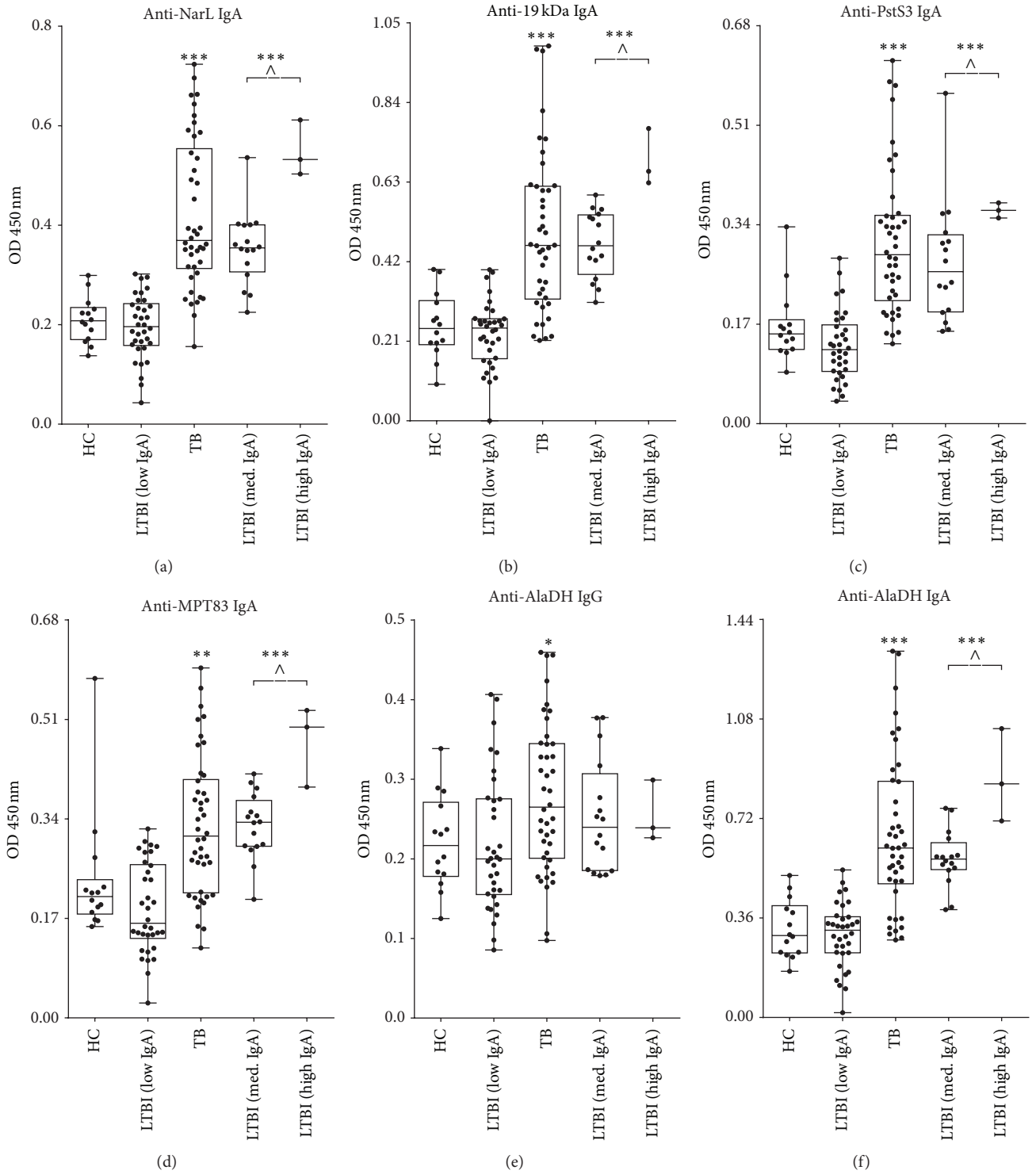

Figure 2: Box-and-whisker plots including individual points for the comparison of 109 TB or non-TB specimens showing the optical density (OD) values of the following selected antigens: anti-NarL IgA (a), anti-19 kDa IgA (b), anti-PstS3 IgA (c), anti-MPT83 IgA (d), anti-AlaDH IgG (e), and anti-AlaDH IgA (f). Values are shown for sera from 14 healthy controls (HC), 42 TB patients, and 3 different LTBI groups. The mean ranks of IgA signals against the 5 antigens investigated were used to group the LTBI group into the top 3 group [LTBI (high IgA); comprising the progressor to active TB disease], the following 16 mean ranks [LTBI (medium IgA)] and the remaining LTBI group [LTBI (low IgA)]. The symbol ∗ depicted above either the TB patients or the LTBI (high and medium IgA) group shows a significant difference of this group compared to healthy controls. A P value of ≤ 0.05 was judged significant and levels of significance were indicated as follows: $^{*}P = 0.01–0.05$, $^{**}P = 0.001–0.01$, $^{***}P < 0.001$.

Table 2: Specificities and sensitivities of single seroantigens to distinguish between healthy non-TB infected controls [HC ($n = 14$)] and either active TB patients ($n = 42$) or LTBI [with medium or high IgA levels ($n = 19$)].

Antigen	Ig class	TB versus HC		LTBI (elevated IgA) versus HC	
		Sens. (%) (95% CI) based on 92.9% (95% CI, 66.1–99.8%) spec.	Sens. (%) (95% CI) based on 100% (95% CI, 76.8–100%) spec.	Sens. (%) (95% CI) based on 92.9% (95% CI, 66.1–99.8%) spec.	Sens. (%) (95% CI) based on 100% (95% CI, 76.8–100%) spec.
NarL	A	81.0 (65.9–91.4)***	78.6 (63.2–89.7)***	84.2 (60.4–96.6)***	84.2 (60.4–96.6)***
MPT83	A	47.6 (32.0–63.6)$^{\#\#}$	2.4 (0.06–12.6)$^{\#\#}$	63.2 (38.4–83.7)$^{\#\#\#}$	0 (0.0–17.6)$^{\#\#\#}$
19 kDa	A	64.3 (48.0–78.4)***	64.3 (48.0–78.4)***	78.9 (54.4–93.9)***	78.9 (54.4–93.9)***
PstS3	A	61.9 (45.6–76.4)***	35.7 (21.6–52.0)***	57.9 (33.5–79.7)***	31.6 (12.6–56.6)***
AlaDH	G	42.9 (27.7–59.0)*	28.6 (15.7–44.6)*	26.3 (9.1–51.2) n. s.	15.8 (3.4–39.6) n. s.
AlaDH	A	76.2 (60.5–87.9)***	69.0 (52.9–82.4)***	89.5 (66.9–98.7)***	84.2 (60.4–96.6)***

Significance levels of P values: P values refer to the t-test (if appropriate on log-transformed data) or the Mann-Whitney test where indicated. A P value of ≤0.05 was judged significant and levels of significance were indicated as follows: * or $^{\#}P = 0.01$–0.05, ** or $^{\#\#}P = 0.001$–0.01, *** or $^{\#\#\#}P < 0.001$; asterisks refer to parametric tests, hashes to nonparametric tests.

vs. = versus; n. s. = not significant; sens. = sensitivity; spec. = specificity.

The mean ranks of the IgA signals against the 5 investigated antigens were used to group the LTBI group into the top 19 groups [LTBI with elevated mean IgA levels ($n = 19$)] and the remaining LTBI group [LTBI (low IgA)].

The data were analyzed by using specificity levels of ≥90%, as described by other investigators [36, 37].

Table 3: Spearman correlation between the antibody performances for 53 individuals with LTBI[a].

Anti-AlaDH IgG	Anti-AlaDH IgA	Anti-19 kDa IgA	Anti-PstS3 IgA	Anti-MPT83 IgA	
$r = 0.17$ (0.1–0.42) $P = 0.22$	$r = 0.93$ (0.87–0.96) $P < 0.0001$	$r = 0.91$ (0.85–0.95) $P < 0.0001$	$r = 0.82$ (0.71–0.89) $P < 0.0001$	$r = 0.89$ (0.81–0.93) $P < 0.0001$	anti-NarL IgA
—	$r = 0.21$ (−0.06–0.46) $P = 0.13$	$r = 0.26$ (−0.01–0.49) $P = 0.064$	$r = 0.31$ (0.04–0.53) $P = 0.025$	$r = 0.27$ (0.0–0.50) $P = 0.052$	anti-AlaDH IgG
—	—	$r = 0.96$ (0.93–0.98) $P < 0.0001$	$r = 0.86$ (0.77–0.92) $P < 0.0001$	$r = 0.83$ (0.72–0.90) $P < 0.0001$	anti-AlaDH IgA
—	—	—	$r = 0.88$ (0.80–0.93) $P < 0.0001$	$r = 0.83$ (0.73–0.90) $P < 0.0001$	anti-19 kDa IgA
—	—	—	—	$r = 0.88$ (0.80–0.93) $P < 0.0001$	anti-PstS3 IgA

[a]Spearman's coefficient r of rank correlation (95% CI for r).

with increased synthesis of specific anti-bacterial antibodies [9]. Integration of macaque and human proteome-scale antibody profiling data revealed dynamic characteristics of the antibody response in relation to bacillary burden and infection outcome [4]. Two individuals with elevated specific IgG responses originally categorized as LTBI were subsequently diagnosed to have culture-confirmed active TB within a few weeks of the serologic testing [6]. Moreover, among apparently healthy professional contacts of TB patients (or pathological specimens thereof), elevated specific IgG and/or IgM responses to mycobacterial antigens were determined in a subgroup of 9 individuals, of whom 4 (44.4%) developed active TB within one year after serological testing [5].

Clearly, future large scale, long term prospective studies in different TB endemic areas are needed to further evaluate and substantiate the validity of the hypothesis that certain specific anti-mycobacterial antibody responses are predictors of future active TB. If true, the reported low specificity of serodiagnostics for the detection of active TB would turn out to be due to a high risk for an early stage of progression to active disease and would then offer new opportunities to interrupt the cycle of transmission. If our findings are confirmed the clinical relevance would be as follows: a corresponding antibody-based test in endemic settings would then not differentiate between active TB cases on the one hand and latent or absent infection on the other hand. Instead, it would distinguish a high-risk group for preclinical or active TB from a group that is unlikely to progress to clinical disease. This would be of particular significance as such antibody tests could be developed into low-cost, point of care tests that may be used as screening tools, particularly in high TB burden low-resource settings. In such a clinical approach, the pre-screened high risk individuals could then be investigated using established assays such as X-ray, IGRA, sputum smear, sputum culture and GeneXpert MTB/RIF for final clinical assessment [3, 42]. Concerning the serodiagnostic results, those LTBI individuals showing the highest IgA or IgG antibody signals might have the highest risk to progress to active TB as their higher antibody levels may indicate higher bacterial loads. The detection of preclinical and early TB would have considerable clinical impact, as the identification, close monitoring, or early treatment of those LTBI with incipient disease would offer an opportunity to break the cycle of transmission and to prevent more serious

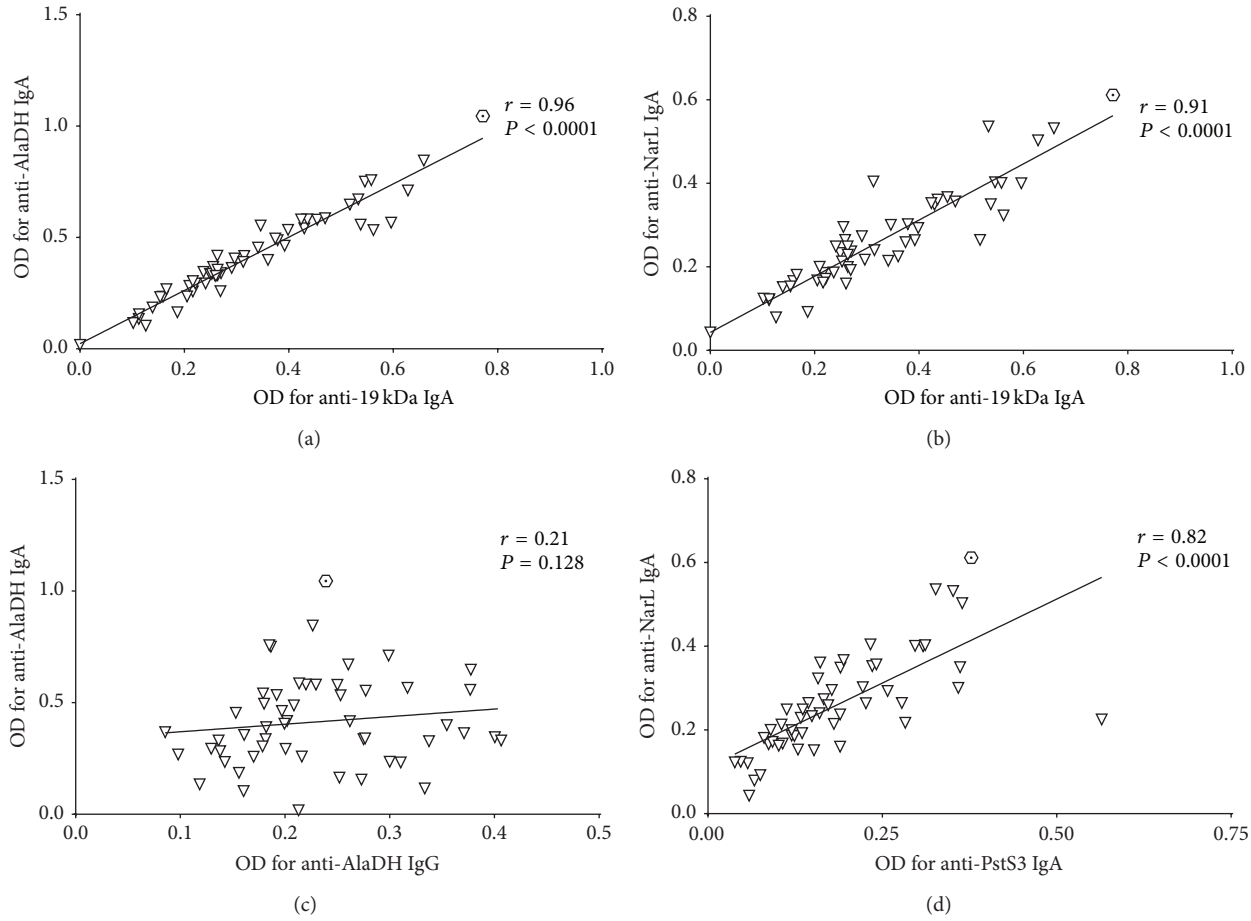

Figure 3: Correlations of IgA antibody levels in serum of latently *M. tuberculosis* infected individuals. Scatter graphs showing the relations between anti-AlaDH IgA and anti-19 kDa IgA (a), anti-NarL IgA and anti-19 kDa IgA (b) and anti-AlaDH IgA and anti-AlaDH IgG (c) as well as anti-NarL IgA and anti-PstS3 IgA (d) using the optical density (OD) values in diluted sera ($n = 53$). The correlation coefficient r and the P value were calculated using the Spearman rank test. The hexagon with a dot in its center characterizes the progressor to active TB, whereas all other latently *M. tuberculosis* infected individuals are characterized by the symbol ∇.

lung destruction. In addition, early treatment of LTBI may decrease the emergence of multidrug resistance- (MDR-) TB strains [43], as the low numbers and slow turnover of mycobacteria in LTBI presumably present less opportunity to develop resistance.

In the present study the LTBI subject with the highest IgA levels against NarL, AlaDH and 19 kDa developed active TB within 3 months after recruitment. Although this is in line with the working hypothesis, one has to be very careful with its interpretation as we are dealing only with a single case. Clearly, as stated above, further research is needed to investigate whether antibody responses to mycobacterial antigens hold any prognostic significance for subsequent development of active TB in individuals with LTBI as previously suggested [4–6, 8].

Though a recent metaanalysis showed that so far neither IGRAs nor the TST can discriminate the ~90% of persons with true LTBI from the ~10% who will develop active TB [44], promising tendencies are noteworthy also in this field [45, 46]. Subjects with high risk for preclinical TB might be monitored more closely in defined time intervals using the currently best available resources and should be treated where applicable.

Due to the low frequency of reactivation in immunocompetent LTBI individuals even in TB endemic settings, large-scale longitudinal human studies would have to be conducted to further investigate whether serologic responses to mycobacterial antigens hold any prognostic significance for subsequent development of active TB in individuals with LTBI (correlates of risk). The results of this study and previous human and animal studies [4, 5, 7–9, 38–41] suggest that this is a promising approach, particularly as the serological tests could be performed on existing stored sera from previous studies. Our results suggest that for these investigations besides IgG also IgA serology should be considered. Due to the important role of IgA antibodies in the lung, the additional consideration of sputum IgA levels might be meaningful. The development of point-of-care tests that can

be used as correlates of risk or as diagnostic for active TB would constitute a major advance.

5. Conclusions

In conclusion, as suggested in human studies and several animal models [4, 5, 7–9, 38–41], it remains a promising hypothesis that those latently infected individuals with antibody responses resembling those of active TB subjects are more prone to progression to active TB. Our finding of a nonrandom distribution of antibody responses among LTBI subjects suggests that the development of serodiagnostic kits for the determination of a high-risk group for preclinical or active TB in endemic settings may be possible. Furthermore, our results encourage the further investigation of IgA besides IgG responses in the field of serodiagnosis of active TB and possibly preclinical TB. Moreover, IgA antibodies against the novel antigen NarL were able to determine, besides the putative high-risk subgroup for preclinical TB (including highest levels for the actual progressor), the highest proportion of active TB patients.

Abbreviations

TB: Tuberculosis
LTBI: Latent *Mycobacterium tuberculosis* infection
HC: Healthy controls.

Conflict of Interests

The authors declare no conflicts of interests. LIONEX did not provide any financial support to the partners.

Authors' Contribution

Ralf Baumann and Susanne Kaempfer contributed equally to this work.

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Research Article

γ-Secretase Inhibitor Alleviates Acute Airway Inflammation of Allergic Asthma in Mice by Downregulating Th17 Cell Differentiation

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T helper 17 (Th17) cells play an important role in the pathogenesis of allergic asthma. Th17 cell differentiation requires Notch signaling. γ-Secretase inhibitor (GSI) blocks Notch signaling; thus, it may be considered as a potential treatment for allergic asthma. The aim of this study was to evaluate the effect of GSI on Th17 cell differentiation in a mouse model of allergic asthma. OVA was used to induce mouse asthma model in the presence and absence of GSI. GSI ameliorated the development of OVA-induced asthma, including suppressing airway inflammation responses and reducing the severity of clinical signs. GSI also significantly suppressed Th17-cell responses in spleen and reduced IL-17 levels in serum. These findings suggest that GSI directly regulates Th17 responses through a Notch signaling-dependent pathway in mouse model of allergic asthma, supporting the notion that GSI is a potential therapeutic agent for the treatment of allergic asthma.

1. Introduction

Asthma is an allergic disease characterized by airway inflammation, mucin hypersecretion, and airway hyperresponsiveness (AHR) [1]. Infiltration of $CD4^+$ T cells, eosinophils, mast cells, and B cells and their interaction with airway resident cells contribute to airway inflammation [2]. IL-17-producing (Th17) $CD4^+$ Th cells are important players in asthma pathogenesis [3, 4]. Numerous studies have shown an increase of Th17 cells in inflammatory airway [5, 6]. Therefore, suppressing Th17 response may be a novel therapeutic strategy for treating asthma. Notch signaling pathway is evolutionarily conserved. In mammalian, there are four Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five Notch ligands (Jagged1, Jagged2, Delta-like ligand (Dll)1, Dll3, and Dll4) [7]. Notch plays a crucial role in a broad spectrum of cellular activities such as proliferation, differentiation, and regulation of cell function [8]. Notch signaling is initiated when Notch receptors are engaged with a Notch ligand. A series of enzymatic reactions lead to the cleavage of the Notch receptor intracellular domain (NICD) which is translocated to the nucleus, where it binds with CSL/RBP-Jk to recruit Mastermind-like 1 protein. The newly formed complex then initiates the transcription of downstream genes.

Previously, we showed that Notch signal pathway regulates the proliferation and differentiation of $CD4^+$ T lymphocytes in a mouse model of asthma, indicating that Notch may be a potential target for treating asthma [9]. Others have reported that pharmacologic inhibitor of Notch signaling can reduce allergic pulmonary inflammation by modulating Th1 and Th2 responses [10, 11]. However, the exact protective mechanism of Notch signaling in asthma remains unknown. The present study aims to test whether GSI has therapeutic

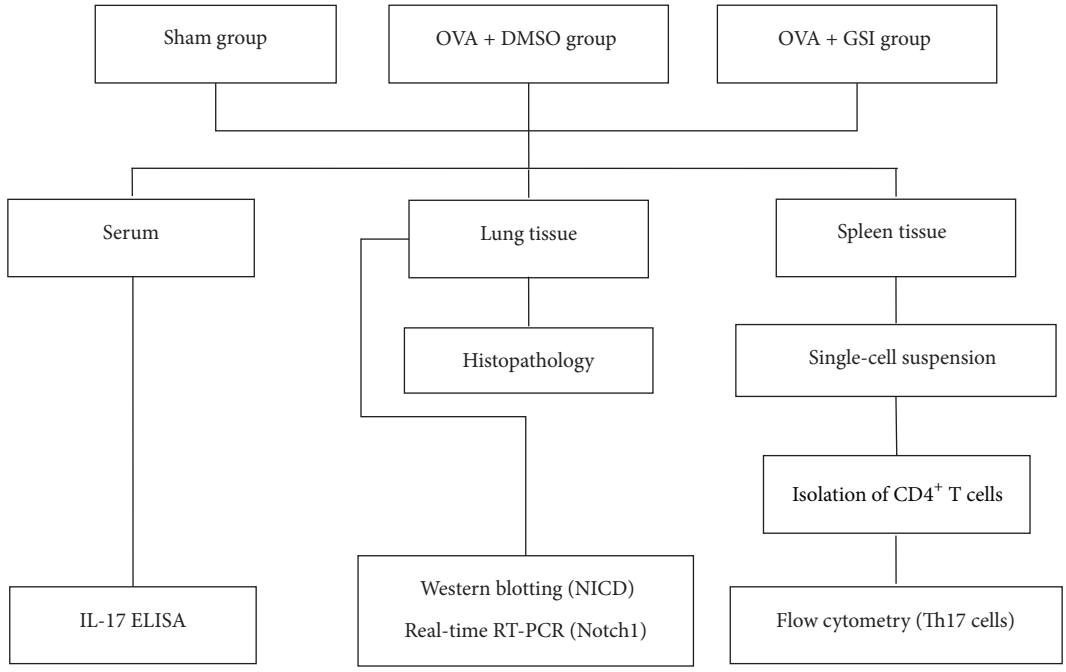

Figure 1: Flowchart of experimental design.

effects on the development of asthma through regulating Th17 mediated immune response.

2. Materials and Methods

Experimental design is outlined in Figure 1.

2.1. Animal Model of Asthma. Male BALB/C mice, 4 to 6 weeks old, weighing 20–22 g, were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and bred in pathogen-free environment in the animal center of Wenzhou Medical University. Animal experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC) of Wenzhou Medical University. OVA induced asthma was established as described previously [9]. Experimental animals were divided into three groups: sham group, OVA + DMSO (vehicle) group, and OVA + GSI (0.3 mg/kg) group. Mice were sensitized by i.p. injection of 10 μg ovalbumin (OVA) (Sigma, USA) emulsified in 20 mg Al $(OH)_3$ gel in 0.1 mL normal saline (NS) on days 1 and 13. They were then challenged with OVA (1 mg/mL) aerosol for 30 min daily for eight consecutive days from day 25 by Jet nebulizer (Pari IS-2 Jet nebulizer; PARI Respiratory Equipment). GSI L685,458 (Calbiochem, CA) was administered intranasally 30 minutes before each OVA challenge at 0.3 mg/kg as previously described [11]. The sham mice were sensitized and challenged with normal saline (NS) and treatment with dimethylsulfoxide (DMSO) as a control for GSI. Mice were sacrificed within 24 h after the last allergen challenge.

2.2. Histopathological Examination. At the time of sacrifice, the left lung tissue was first fixed with 4% paraformaldehyde for 4 h. It was then dehydrated in ethylic alcohol, embedded with paraffin, sectioned in 4 μm, and stained with haematoxylin and eosin (HE). Tissue slices were evaluated through light microscope (Nikon) by trained technician in a blind fashion.

The degree of allergic airway inflammation was scored according to the following histologic grading system (scored 0–4): absence of peribronchial inflammatory cells; a few scattered peribronchial inflammatory cells involving less than 25% of the circumference of the bronchus; focal peribronchial inflammatory cells infiltration not completely surrounding a bronchus (i.e., involving approximately 25%–75% of the circumference of the bronchus); one definite layer of peribronchial inflammatory cells completely surrounding a bronchus; 2 or more layers of peribronchial inflammatory cells completely surrounding a bronchus. In each lung section the mean peribronchial inflammatory score was determined by the sum of scores of all individual bronchioles in the section divided by the number of bronchioles [12].

2.3. Preparation of Splenic Single-Cell Suspension. The spleen tissue was fragmented into small pieces that were then pressed against nylon mesh with a plunger of a disposable syringe. Erythrocytes were lysed by red blood cell lysis buffer. Cells were washed in PBS.

2.4. Isolation of $CD4^+$ T Cells. $CD4^+$ T cells from splenic single-cell suspension were isolated by magnetic cell sorting by positive selection method using mouse $CD4^+$ T cell isolation kit (MACS, Miltenyi Biotec, Germany) according to the manufacturers' instruction. The purity of the cells was 92.04 ± 5.18%, as confirmed by flow cytometry analysis.

2.5. Flow Cytometry Analysis. FITC-labeled anti-mouse CD4 and PE-labeled anti-mouse IL-17A were used to detect Th17 cells. Matching IgG was used as isotype control. All antibodies were purchased from BD Bioscience, USA.

For Th17 cell analysis, $CD4^+$ T cells (1×10^6/mL) from the spleen tissue were stimulated for 4.5 hr with phorbol myristate acetate (PMA) at 100 ng/mL and Ionomycin at 1 μg/mL in the presence of 1.6 μg/mL Monensin (all from Beyotime, China). Cells were collected, washed, and surface-stained with FITC-labeled anti-CD4 antibody at 4°C for 20 min in the dark and resuspended in Fix/Perm solution according to the manufacturer's instruction (Invitrogen, USA). They were then stained intracellularly with PE-labeled anti-IL-17 antibody. After washing, cells were resuspended in fixation solution and subjected to FACScalibur flow cytometer (BD FACSCanto II, USA) analysis. Background fluorescence was assessed by the corresponding isotype control antibodies. Data were analyzed with WinMDI software.

2.6. Western Blotting. Lung tissue was fragmented and lysed in RIPA buffer with protease inhibitor mixture (Beyotime, China). A total protein of 20 μg was loaded into each well of a SDS-PAGE gel for separation by electrophoresis and then transferred onto nitrocellulose membrane. The resulting blots were blocked for 1 h with TBS Tween 20 containing 5% powder skim milk and then probed overnight at 4°C with anti-NICD (Abcam, UK). Blots were then washed three times and probed for 1 h with anti-rabbit HRP-conjugated antibody. β-actin mouse mAb (Beyotime, China) was used as the loading control. Immunostained proteins were detected by ECL.

2.7. Quantitative Real-Time RT-PCR Analysis. RNA was extracted from lung tissue using Trizol (Invitrogen, USA) according to the manufacturer's instruction. cDNA was synthesized by reverse transcription with oligo (dT) from total RNA. The quantitative real-time RT-PCR was performed using an ABI Step One Plus System (Applied Biosystems, USA) with QuantiFast SYBR Green PCR Kit (QIAGEN, Germany). GAPDH was used as internal control. Primers used were as follows: GAPDH, 5′-TGGCCTTCCGTGTTCCTAC-3′ (forward) and 5′-GAGTTGCTGTTGAAGTCGCA-3′ (reverse); Notch1, 5′-TGCCACAATGAGATCGGCTC-3′ (forward) and 5′-GAGTTGCTGTTGAAGTCGCA-3′ (reverse). Delta-Delta Ct method was used to express the fold induction of target mRNA after GAPDH normalization.

2.8. ELISA. The concentration of cytokines IL-17 in serum was assessed by standardized sandwich ELISA according to the manufacturer's protocol. The IL-17 kit was purchased from eBioscience, San Diego, USA.

2.9. Statistical Analysis. All data were expressed as Mean ± SEM. Differences between groups were analyzed for statistical significance by one-way analysis of variance using SPSS 13.0 software (SPSS Inc., Chicago, USA). Differences with a P value < 0.05 were considered statistically significant.

3. Results

3.1. GSI Ameliorates the Severity of Inflammation in OVA-Induced Asthma. To explore the effect of GSI on OVA-induced asthma, BABL/C mice were sensitized and challenged with OVA (or NS for sham mice). Those mice received GSI, a highly selective inhibitor of γ-secretase or vehicle (DMSO), during the challenge phase. Mice were sacrificed within 24 h after the last allergen challenge and lung tissue was fixed, embedded, and sectioned for HE staining. The degree of airway inflammation of HE-stained lung tissue was scored as described in Materials and Methods. As shown in Figure 2(a), OVA + DMSO group demonstrated significant infiltration of eosinophils and lymphocytes with marked thickening of airway wall and epithelial goblet cell metaplasia, as compared with the sham group. GSI treatment reduced such inflammation and airway wall thickening (Figure 2(b)). OVA-challenged mice showed an inflammation score of 3.25 ± 0.46 as compared to sham control (0.38 ± 0.52). GSI treated group showed a score of 1.88 ± 0.64 that is significantly lower than OVA-DMSO group (Figure 2(b), $P < 0.01$).

3.2. GSI Affects the Expression of Notch Signaling Component. To investigate the blockage effects of GSI on Notch signaling, mRNA expression of Notch1, a receptor of Notch signaling, was examined. As shown in Figure 3(a), OVA-challenged mice revealed enhanced Notch1 mRNA expression, as compared with the sham group (1.31 ± 0.13 versus 0.84 ± 0.13, $P < 0.01$). On the other hand, GSI treatment led to the reduction of Notch1 mRNA expression (0.92 ± 0.088 $P < 0.01$ comparing to OVA group). Consistent with this observation, OVA-challenged mice revealed increased NICD generation as compared to sham group (0.18 ± 0.02 versus 0.09 ± 0.01, $P < 0.01$). GSI treatment decreased NICD generation (0.06 ± 0.03) comparing to OVA group (Figure 3(b), $P < 0.01$). Results presented here suggest that GSI can effectively block Notch signaling.

3.3. GSI Decreases the Frequency of Th17 Cells in the Spleen of OVA-Induced Asthma Mice. To evaluate the effect of GSI treatment on Th17 cell expansion, splenic $CD4^+$ T cells were isolated by magnetic cell sorting. Th17 cells were identified by IL-17A staining. Sham group expressed a baseline Th17 cell frequency of 0.30 ± 0.16% of total splenic $CD4^+$ T cells. OVA-induced asthma mice revealed a significant increase of Th17 cells (2.43 ± 0.69%, $P < 0.01$, comparing to sham group). GSI treatment reduced Th17 cell frequency to 1.26 ± 0.85% which is statistically significant from OVA group ($P < 0.05$, Figure 4). This finding indicates that GSI reduces the development of Th17 cells.

3.4. GSI Treatment Reduces the Production of IL-17 of Asthma Mice. Th17 cells are the main source of IL-17. To further examine the function of such Th17 cells, serum levels of IL-17 were measured from OVA-induced asthma mice. As illustrated in Figure 5, sham group expressed a baseline level of IL-17 in serum at 48.07 ± 5.73 pg/mL. The IL-17 level was significantly elevated in OVA-induced asthma mice

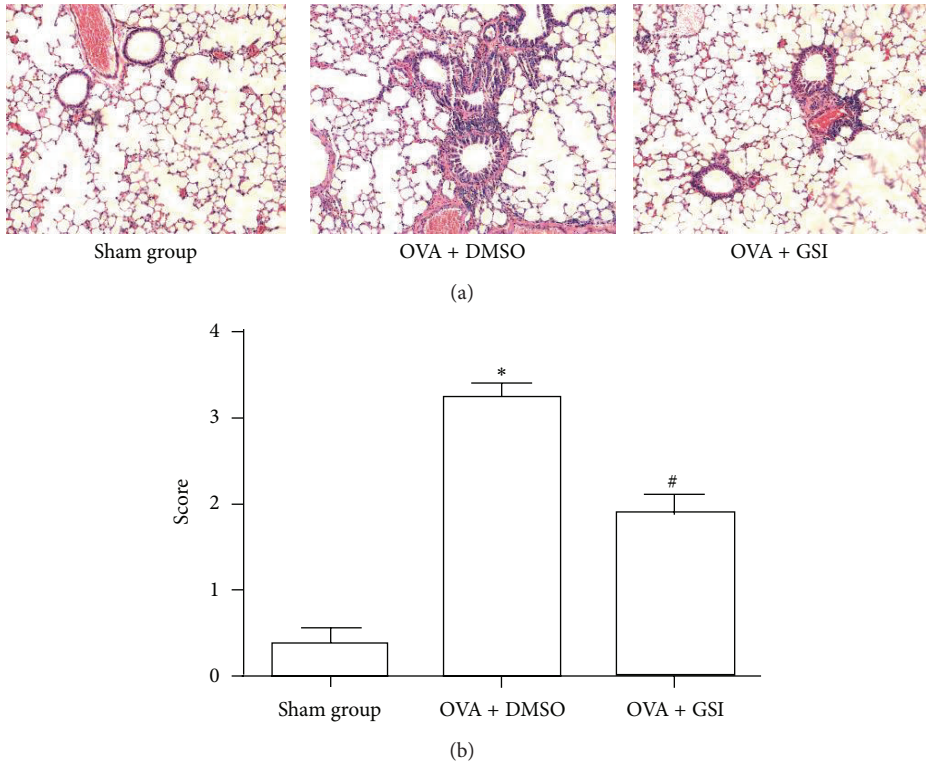

Figure 2: γ-Secretase inhibitor (GSI) reduced OVA-induced airway inflammation. (a) BALB/C mice were sensitized i.p. with OVA and challenged with OVA in the presence and absence of GSI. Mice were sacrificed within 24 hr after last challenge. Lung tissues were stained with haematoxylin and eosin and subjected to light microscope (×200) examination. (b) Semiquantitative pathology scores among sham, OVA, and OVA + GSI groups. Data expressed as Mean ± SEM. $N = 8$ mice per group. $^{*}P < 0.01$ compared with the sham group; $^{\#}P < 0.01$ compared with the vehicle group.

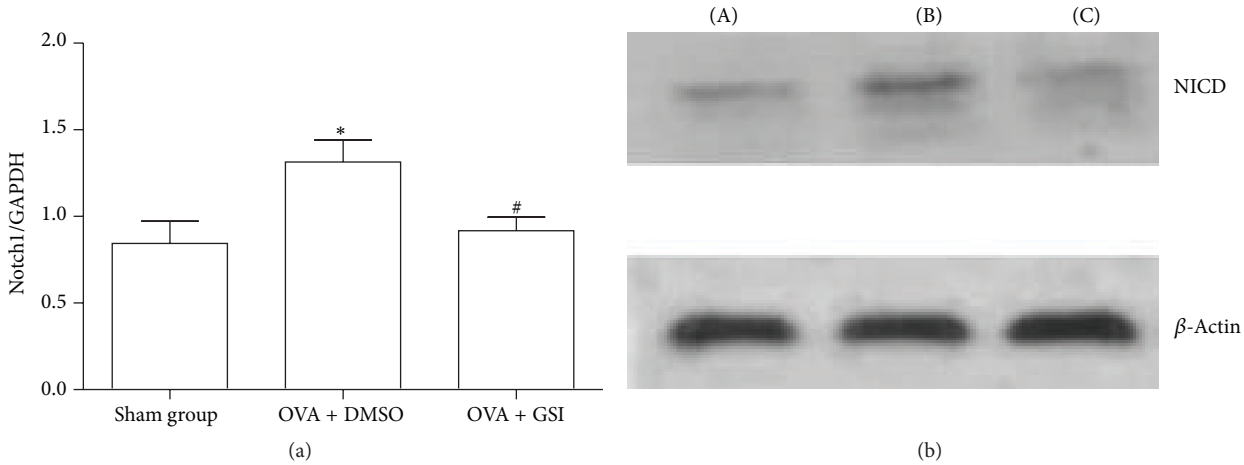

Figure 3: GSI decreased Notch1 and NICD. BALB/C mice were sensitized i.p. with OVA and challenged with OVA in the presence and absence of GSI. (a) The expression of Notch1 mRNA was evaluated by quantitative real-time RT-PCR. GAPDH was used as internal control. (b) Protein levels of NICD were examined by Western blotting. β-actin was used as a loading control. (A) Sham group; (B) OVA + DMSO; (C) OVA + GSI. Data expressed as Mean ± SEM. $N = 8$ mice per group. $^{*}P < 0.01$ compared with the sham group. $^{\#}P < 0.01$ compared with the vehicle group.

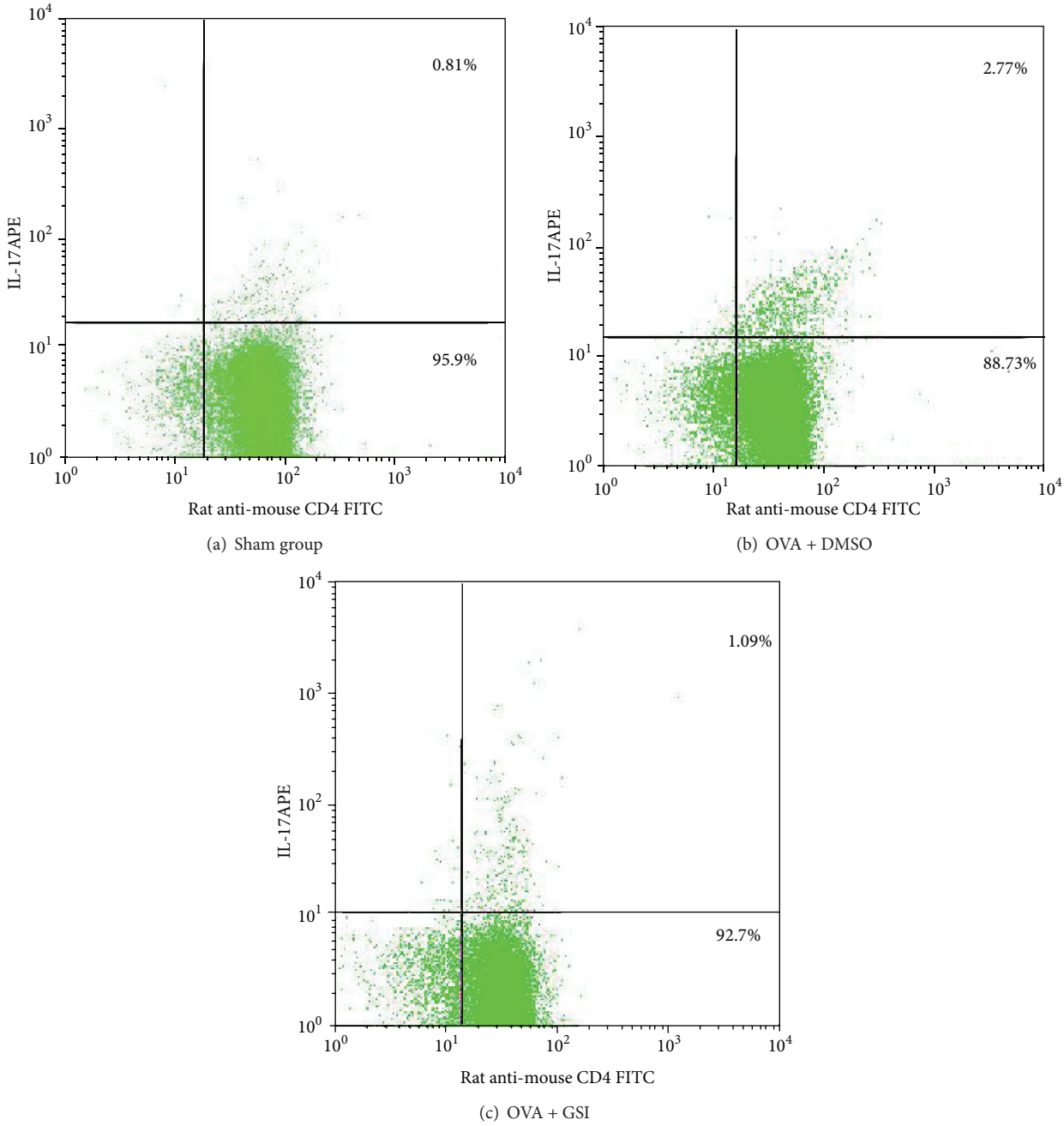

Figure 4: GSI administration resulted in reduced Th17 cell expansion. BALB/C mice were sensitized i.p. with OVA and challenged with OVA in the presence and absence of GSI. Splenic $CD4^+$ T cells were isolated by magnetic cell sorting. Th17 cells were examined by IL-17A staining and data were analyzed by flow cytometry. Dot plots show as percent of cells positive for CD4 and IL-17A staining. Graphs representative of one of eight experiments.

(120.09 ± 5.73 pg/mL, $P < 0.01$). GSI administration during challenge phase significantly reduced the IL-17 level to 81.82± 8.95 pg/mL, $P < 0.01$. These findings confirm the possibility that GSI downregulates IL-17 expression.

4. Discussion

The Notch signaling pathway is involved in many aspects of organ formation and cell function [7]. Dysregulation of Notch signaling may induce human disorders such as asthma. The effect of Notch signaling inhibition on the development of asthma has been addressed in several recent studies. Jin and colleagues reported that inhibition of Notch signal pathway by GSI alleviated the airway inflammation in OVA-induced asthma model and it was through the regulation of Th1 and Th2 responses [11]. Knockdown of the Notch l gene by small interfering RNA led to overproduction of IL-4 and IFN-γ, which played an important role in the pathogenesis of asthma

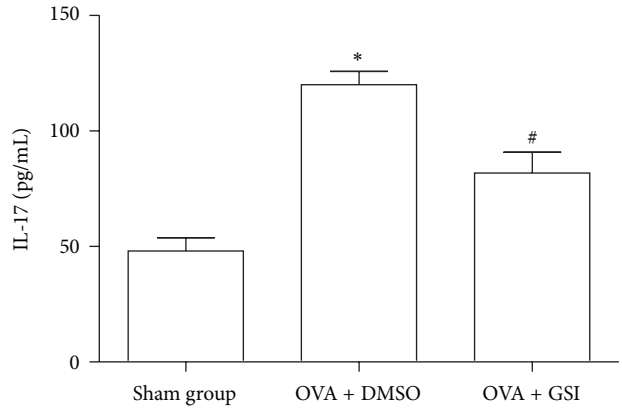

Figure 5: GSI administration reduced production of IL-17. Serum IL-17 levels were measured from sham, OVA, and OVA plus GSI groups using standardized sandwich ELISA. Data expressed here are Mean ± SEM. $N = 8$. $^{*}P < 0.01$ compared with the sham group. $^{\#}P < 0.01$ compared with the vehicle group.

[13]. However, the exact underlying mechanism is yet to be fully elucidated.

In the present study, we used GSI to block Notch signaling in a mouse model of asthma and demonstrated that in vivo administration of GSI effectively attenuated eosinophilic and lymphocyte infiltration in the airways and decreased goblet cell metaplasia. Furthermore, Notch inhibition by GSI reduced the frequency of Th17 cells in spleen and the serum levels of IL-17. Taken together, these findings demonstrate the effectiveness of GSI in animal models and strongly suggest that inhibition of Notch signaling could be an effective strategy for the treatment of asthma.

T helper 17 (Th17) cells play a critical role in adaptive immune responses through the production of cytokines, namely, IL-17A, IL-17F, and IL-22 [14]. Recent studies indicate that Th17 cells are active players in acute airway inflammation of allergic asthma. Marked elevation of IL-17A was detected in the sputum of severe asthma patients [15]. Excess IL-17-secreting cells were observed in the lung tissue of such patients as well [16]. Li et al. [17] suggested that the ratio of Th17 cells/$CD3^+$ T cells in peripheral blood was significantly increased in asthma patients compared with nonasthma individuals. Similar change was found in the present study; that is, the proportion of Th17 cells in isolated spleen $CD4^+$ T cells was significantly increased in OVA-induced asthma mice compared to the sham group. McKinley et al. reported that adaptive transferring of Th17 cells to an asthma mouse led to neutrophils infiltration and airway hyperresponsiveness, which is resistant to corticosteroid therapy [18]. IL-17 mediated airway inflammation may result in severe airway obstruction. The expression of IL-17A was also noticed in ozone exacerbated asthma [19]. Consistent with these findings, our investigation revealed that treatment with GSI markedly reduced serum IL-17 levels of asthma mice. Of note, Doe et al. [20] found that IL-17A was elevated in mild to moderate human asthma compared with healthy control, while it was not increased in severe asthma. Therefore, further research is needed to characterize the exact relationship between IL-17 and the severity of asthma.

Notch can directly regulate retinoic acid-related orphan receptor γt, an important transcription factor for Th17 differentiation [21]. The effect of GSI on autoimmune inflammatory disorders has been addressed in several studies. γ-Secretase inhibitor treatment can downregulate Th17 response and inhibit vascular inflammation [22]. Inhibition of Notch signaling by Notch3 antibody attenuates Th17-type responses, while treatment with Notch ligand Delta-like 1 promotes Th17 response [23]. In vitro knockdown of Notch and in vivo administration of GSIs result in reduced IL-17 production and substantially impede Th17-mediated disease progression in mouse model of multiple sclerosis [24]. GSIs have been actively tested in clinical trials for Alzheimer disease for their potential in blocking the generation of A peptide [25]. MRK003, a γ-secretase inhibitor, exhibits promising in vitro preclinical activity in multiple myeloma and non-Hodgkin's lymphoma [26]. After blocking Notch signaling in our mouse asthma model, we noticed decreased level of NICD, ameliorated airway inflammation, reduced serum IL-17 level, and improved clinical signs. Our data strongly suggest that inhibition of Notch signaling could be considered as an effective therapy for asthma. Of course, further preclinical and clinical research is needed to address such potential.

In conclusion, the current study proves that GSI administration inhibits Th17 differentiation, decreases IL-17 production, and alleviates airway inflammation in OVA-sensitized and OVA-challenged BALB/C mice. These results support the idea of considering GSI as a novel, effective antiasthma agent.

Abbreviations

Th:	T helper
GSI:	γ-Secretase inhibitor
AHR:	Airway hyperresponsiveness
Dll:	Delta-like ligand
NICD:	Notch receptor intracellular domain
IACUC:	Institutional Animal Care and Use Committee
NS:	Normal saline
DMSO:	Dimethylsulfoxide
HE:	Haematoxylin and eosin
PMA:	Phorbol myristate acetate
OVA:	Ovalbumin
Th17:	IL-17-producing $CD4^+$ Th cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Weixi Zhang and Xueya Zhang contributed equally to this study.

Acknowledgments

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Research Article

Serum Vitamin A and Inflammatory Markers in Individuals with and without Chronic Obstructive Pulmonary Disease

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Background. Vitamin A is essential for the preservation and integrity of the lung epithelium and exerts anti-inflammatory effects. *Objective.* Evaluating vitamin A in the serum and sputum and testing its correlation with inflammatory markers in individuals with or without COPD. *Methods.* We evaluated dietary intake, serum and sputum vitamin A, tumor necrosis factor alpha, interleukin- (IL-) 6, IL-8, and C-reactive protein in 50 COPD patients (age = 64.0 ± 8.8 y; FEV_1 (forced expiratory volume in the first second) (%) = 49.8 ± 16.8) and 50 controls (age = 48.5 ± 7.4 y; FEV_1 (%) = 110.0 ± 15.7). *Results.* COPD exhibited lower serum vitamin A (1.8 (1.2–2.1) versus 2.1 (1.8–2.4) μmol/L, $P < 0.001$) and lower vitamin A intake (636.9 (339.6–1349.6) versus 918.0 (592.1–1654.6) RAE, $P = 0.05$) when compared with controls. Sputum concentration of vitamin A was not different between groups. Sputum vitamin A and neutrophils were negatively correlated ($R^2 = -0.26$; $P = 0.03$). Smoking (0.197, $P = 0.042$) exhibited positive association with serum vitamin A. COPD was associated with lower serum concentrations of vitamin A without relationship with the systemic inflammation. *Conclusions.* Serum concentration of vitamin A is negatively associated with the presence of COPD and positively associated with smoking status. Sputum retinol is quantifiable and is negatively influenced by neutrophils. Although COPD patients exhibited increased inflammation it was not associated with serum retinol.

1. Introduction

Vitamin A is essential for the preservation of the integrity of the epithelium, and it exerts anti-inflammatory effects in the lungs. Vitamin A deficiency promotes and aggravates preexisting inflammation [1, 2]. Previous studies have demonstrated that the risk for chronic obstructive pulmonary disease (COPD) increased with decreasing levels of serum vitamin A [3, 4]. Furthermore, cross-sectional studies have reported that the intake and serum concentration of vitamin A are associated with the degree of airway obstruction in smokers and COPD patients, and the serum concentration of vitamin A is lower in patients with moderate or severe COPD than in nonsmoking controls [5, 6]. In addition, improvement in pulmonary function is achieved with vitamin A supplementation [5].

COPD is associated with chronic inflammation, and there is increasing evidence that systemic inflammatory mediators, such as C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin- (IL-) 8, and IL-6, are elevated in the peripheral blood of COPD patients [7, 8]. Some studies have demonstrated an association between systemic inflammation and a decrease in serum vitamin A concentrations [9–11]. Possible contributing mechanisms for the decrease in vitamin A during the course of inflammatory diseases include the decreased synthesis of retinol-binding protein by the liver and increased vascular permeability at sites of inflammation, thereby allowing leakage of retinol-binding protein to the extravascular space and the loss of the vitamin in the urine [12]. Another mechanism proposed to explain the decrease in the serum concentration of vitamin A is the increased demand to repair the damage caused by acute or chronic

inflammation [12]. We hypothesized that vitamin A concentration measured in the serum may be related to increased levels of inflammatory markers and may be associated with the presence of COPD. Thus, the aim of the present study was to evaluate vitamin A levels in the serum and sputum and to test whether the concentration of vitamin A is correlated with markers of inflammation in the peripheral blood of individuals with or without COPD. In addition, we measured, for the first time, vitamin A levels in induced sputum and tested whether the level of sputum vitamin A was associated with local markers of inflammation.

2. Material and Methods

We performed a cross-sectional study at the Botucatu Medical School, located in the city of Botucatu, Brazil, evaluating 50 clinically stable patients with COPD (GOLD II: 20 (40%), GOLD III: 12 (24%), and GOLD IV: 18 (36%)) and 50 controls (61% current smokers). COPD was diagnosed according to the criteria established by the Global Initiative for Chronic Obstructive Lung Disease [13]: a forced expiratory volume in 1 s/forced vital capacity (FEV_1/FVC) ratio <70% after the administration of a bronchodilator (400 μg of fenoterol) without significant reversibility (<11% predicted FEV_1 or 200 mL). All COPD patients were lifelong smokers (smoking history > 20 pack-years), and 39% were active smokers. The following exclusion criteria were applied: oral steroid use or COPD exacerbation in the last three months before enrollment in the study; diagnosis of another chronic or respiratory disease; and inability to understand the study protocol. All controls underwent routine clinical assessment, including spirometry and a chest X-ray. The Research Ethics Committee of the Botucatu Medical School approved the study design, and all participants provided written informed consent.

2.1. Pulmonary Function Tests and Oximetry. We determined the forced expiratory volume in the first second (FEV_1) and the forced vital capacity (FVC) based on the flow-volume curve obtained using a spirometer (Koko; Ferraris Respiratory, Louisville, CO) before and 20 min after inhalation of a beta 2-agonist (fenoterol, 400 μg). The highest value of at least three measurements, expressed as percentages of reference values, was selected [14]. Oxygen saturation (SpO_2%) was evaluated using a portable oximeter (Nonin Medical, Plymouth, MN).

2.2. Nutritional Assessment. The nutritional assessment included the measurement of height and weight, and body mass index (BMI) was calculated as (kg/m^2). The typical daily nutrient intake during the past six months was estimated using a food-frequency questionnaire [15]. The instrument listed 120 food and beverage items. Using the computer software NutWin, Nutrition Program (Information Health Science Center of São Paulo Federal University, São Paulo, Brazil, 2002), we converted dietary information into energy, protein, fat, and vitamin A intake values (retinol activity equivalent, RAE).

2.3. Blood Sampling and Sputum Induction. Fasting peripheral blood samples were collected in the early morning (between 8:00 and 10:00 a.m.), and the serum was stored at −80°C until analysis. We followed the European Respiratory Society recommendations for the induction and processing of sputum [16, 17] as previously described [18], and all procedures were conducted under red light.

2.4. Determination of Serum and Sputum Retinol Concentration. We used reverse-phase HPLC to measure retinol concentrations in the serum and induced sputum [19]. Aliquots of the serum and sputum supernatant were prepared for extraction [19] and assayed using a C18 column (Symmetry C18, 46 × 75 mm; 3.5 μm). The HPLC system consisted of a separation module (Waters Alliance 2695; Waters, Milford, MA) with a photodiode array detector (2996; Waters), which we set to 325 nm. The HPLC mobile phase was water-acetonitrile-tetrahydrofuran (30 : 50 : 20, by vol., with 1% ammonium acetate in water; solvent A) and water-acetonitrile-tetrahydrofuran (6 : 50 : 44, by vol., with 1% ammonium acetate in water; solvent B). The gradient procedure, at a 1 mL/min flow rate (16°C), was as follows: 2 min in 85% solvent A and 15% solvent B; 9–19 min in 17% solvent A and 83% solvent B; a 1min hold in 100% solvent B; and 21–30 min in 85% solvent A and 15% solvent B. We quantified the retinol concentration by determining peak areas in the HPLC chromatograms calibrated against known standard quantities. We corrected for extraction and handling losses by monitoring recovery of the internal standard.

2.5. Quantification of Inflammatory Mediators in the Serum and Supernatant of Induced Sputum. Were assessed tumor necrosis factor alpha (TNF-α), interleukin- (IL-) 6, and IL-8 levels in duplicate using high-sensitivity commercial enzyme-linked immunosorbent assay kits, according to the manufacturer's instructions (BioSource International Inc., Camarillo, CA). The lower detection limit was 0.09 pg/mL for TNF-α, 0.16 pg/mL for IL-6, and 0.39 pg/mL for IL-8. We assessed serum C-reactive protein (CRP) levels, also in duplicate, using a high-sensitivity particle-enhanced immunonephelometry (CardioPhase; Dade Behring Marburg GmbH, Marburg, Germany) with a lower detection limit of 0.007 mg/L.

2.6. Statistical Analyses. The mean ± SD or the median interquartile range (25–75%) was used to present the results according to the data distribution. The subjects were separated into two groups based on the diagnoses of COPD (absence or presence). When comparing the two study groups, an unpaired t-test was used for continuous variables, and the Mann-Whitney U-test was used for ordinal variables. The chi-square test or Fisher's exact test was used to evaluate the qualitative variables. For robust multiple linear regressions, clinically relevant variables were selected. The included categorical variables were sex (female = 0, male = 1), presence of COPD (absence = 0, presence = 1), interaction of IL-6 × CRP × TNF-α (was considered by multiplying the variables to construct one variable), and age, as a continuous variable.

All the data were analyzed using the software SigmaStat 3.2 (SPSS Inc., Chicago, IL, USA) and STATA. Statistical significance was defined as $P < 0.05$.

3. Results

In this study, 100 subjects were included in the analysis of the results. Sputum samples were obtained from COPD patients ($n = 50$) and smoker controls ($n = 19$). Table 1 presents the general characteristics of individuals grouped according to diagnoses (controls and COPD). Gender and active smoking status did not differ statistically between the groups. Patients with COPD exhibited lower values of spirometric variables, functional capacity, and fat-free mass (Table 1).

The analyses of inflammatory markers revealed higher serum concentrations of TNF-α ($P = 0.05$), IL-6 ($P < 0.001$), CRP ($P < 0.001$), neutrophils ($P < 0.001$), and leukocytes ($P < 0.001$) in COPD patients when compared with controls. IL-8 levels and lymphocytes did not exhibit a statistically significant difference between the groups (Table 2). In sputum, TNF-α (8.18 (1.53–33.98) versus 1.10 (0.38–6.86) pg/mL, $P = 0.01$) was higher in COPD patients than in smoker controls. Sputum IL-6, IL-8, neutrophils, and leukocytes did not differ between the groups. The airway and systemic inflammation (TNF-α, IL-6, IL-8, and CRP) in COPD patients according to GOLD stage were not different between groups (data not shown).

Table 3 presents the intake of protein, energy, and vitamin A and the concentration of vitamin A in the serum. COPD patients exhibited lower levels of protein ingestion in g/day ($P = 0.01$) and in g/Kg/day ($P = 0.01$) when compared with the controls. Furthermore, the vitamin A intake ($P = 0.05$) and the serum concentration of vitamin A ($P < 0.001$) were lower in the group with airway obstruction. The sputum concentration of vitamin A did not exhibit statistically significant differences (34.5 (8.1–57.6) versus 28.8 (18.0–66.6) μmol/L, $P = 0.38$) between groups. However, vitamin A and neutrophils were negatively correlated in the sputum ($R^2 = -0.26$; $P = 0.03$) (Figure 1).

Age, gender, smoking status, and the presence of COPD were included as independent variables in robust multiple linear regression to identify factors associated with vitamin A serum concentrations (Table 4). Smoking status (0.197, $P = 0.042$) exhibited a positive association with vitamin A concentrations, and the presence of COPD was associated with lower concentrations of vitamin A (−0.480, $P = 0.001$) (Table 4).

4. Discussion

The present study demonstrated that COPD patients exhibited lower intake and serum concentration of vitamin A when compared with controls. Furthermore, active smoking is positively associated with the serum concentration of vitamin A. Although our data confirm previous findings demonstrating that COPD patients exhibit higher concentrations of TNF-α, IL-6, CRP, neutrophils, and leukocytes when compared with controls, the serum concentration of vitamin A was not associated with inflammatory markers. The study also showed for the first time that vitamin A is quantifiable and is negatively influenced by the percentage of neutrophils in induced sputum.

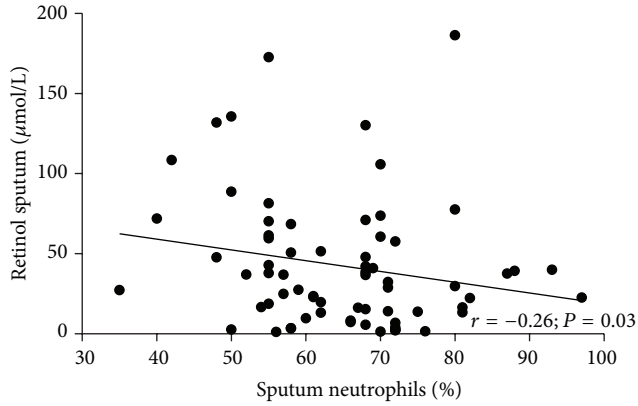

Figure 1: Correlation between retinol sputum and sputum neutrophil concentrations.

Table 1: General characteristics of individuals grouped according to diagnoses.

Variables	Controls ($n = 50$)	COPD ($n = 50$)	P value
Gender F/M (n)	21/29	20/30	0.80
Active smokers (%)	61	43	0.53
Pack/years	35.0 (26.2–41.5)	50.0 (30.0–60.0)	**0.01**
Age (years)	48.5 ± 7.4	64.0 ± 8.8	**<0.001**
FEV_1 (%)	110.0 ± 15.7	49.8 ± 16.8	**<0.001**
FEV_1/FVC (%)	81.0 (77.0–84.0)	48.0 (38.0–61.0)	**<0.001**
6 MWD (m)	562.5 (497.9–624.0)	411.0 (357.0–486.0)	**<0.001**
BMI (kg/m^2)	24.4 (22.5–26.5)	24.8 (22.3–27.6)	0.41
FFM (kg)	47.0 (39.4–53.4)	42.1 (37.3–45.6)	**0.01**
IFFM (kg/m^2)	17.4 (15.4–18.2)	16.3 (14.8–17.5)	0.06

Data are reported as the means ± SD or as the medians (interquartile range (25–75%)). F/M = female/male; FEV_1 = forced expiratory volume in the first second (% of predicted); FVC = forced vital capacity (% of predicted); 6 MWD: six-minute walk distance; FFM: fat-free mass; IFFM: index fat-free mass. The P values refer to COPD compared with the control (unpaired t-test or Mann-Whitney test and chi-square).

Lower serum concentrations of vitamin A among COPD patients when compared with controls and a significantly negative influence of COPD diagnoses on vitamin A concentrations have been previously described [3–5, 20]. Paiva et al. (1996) and Lin et al. (2010) demonstrated that the serum concentration of vitamin A was lower in patients with COPD than in controls [5, 21]. In addition, McKeever et al. (2008), using data from the Third National Health and Nutrition Examination Survey, demonstrated that higher serum levels of antioxidant vitamins (vitamin A) were independently associated with higher levels of FEV_1 [6].

The physiopathology for the lower serum concentrations of vitamin A in COPD remains unclear. Our results revealed

Table 2: Inflammatory markers in the serum of control subjects and COPD patients.

Variables	Control ($n = 50$)	COPD ($n = 50$)	P value
TNF-α (pg/mL)	3.9 (3.6–5.4)	4.5 (4.1–5.1)	**0.05**
IL-6 (pg/mL)	0.3 (0.2–0.6)	1.1 (0.8–1.9)	<**0.001**
IL-8 (pg/mL)	4.9 (3.4–7.1)	4.1 (3.3–7.2)	0.48
CRP (mg/L)	1.1 (0.6–2.2)	6.3 (2.7–9.3)	<**0.001**
Neutrophils (cell/mm^3)	3605 (2900–4342)	4712 (4037–5667)	<**0.001**
Lymphocytes (cell/mm^3)	1750 (1529–2090)	1720 (1470–2397)	0.94
Leukocytes (cell/mm^3)	6400 (5300–7300)	7600 (7000–9125)	<**0.001**

Data are reported as the median (interquartile range (25–75%)). TNF-α: tumor necrosis factor; IL-6: interleukin-6; IL-8: interleukin-8; CRP: C-reactive protein. The P values refer to COPD compared with the control (Mann-Whitney test and chi-square).

that vitamin A intake was lower in COPD patients when compared with controls, which could explain, at least in part, the lower serum concentration. Our data are consistent with the data of Lin et al. (2010), who reported that vitamin A intake (4053 ± 2447 versus 5988 ± 3451, $P = 0.009$ (calculated from diet/1000 kcal total energy)) was lower in COPD patients than in controls [21].

Some studies have demonstrated an association between systemic inflammation and a decrease in serum vitamin A concentrations [9–11]. Our results demonstrated that COPD patients exhibited higher concentrations of inflammatory markers (TNF-α, IL-6, CRP, neutrophils, and leukocytes) compared with controls, which are in agreement with previous findings [22–24]. No influence of airway obstruction severity on airway or systemic inflammation in COPD patients was observed in our study. Previous findings also showed no difference in inflammatory markers between COPD patients at different GOLD stages [25]. Analyzing data from the Third National Health and Nutrition Examination Survey, Stephensen and Gildengorin demonstrated that chronic pulmonary disease was associated with elevated serum CRP concentrations and that serum retinol was lower in subjects with elevated CRP concentrations [9]. In contrast, in our study, vitamin A concentrations were not associated with inflammatory markers. This may be because the inflammation in COPD patients seems to be of low grade and is not persistent in the majority of the patients [23, 26]. Godoy et al. (1996) followed up weight losers (WL) and weight stable (WS) COPD patients for 6 months. The authors reported that TNF-alpha concentrations were significantly higher in the WL COPD patients when compared with WS patients at baseline. However, this difference was not maintained after 6 months of follow-up [26]. Furthermore, a recent study evaluated the systemic inflammatory state (white blood cells (WBC) count and CRP, IL-6, IL-8, fibrinogen, and TNF-α) in 1,755 COPD patients, 297 smokers with normal spirometry and 202 nonsmoker controls, who were followed up for three years. According to Agustí et al. (2012), at baseline, 30% of COPD patients did not exhibit evidence of systemic inflammation, and only 16% exhibited persistent systemic inflammation [23].

We have showed a positive association between smoking status and serum vitamin A concentrations and that retinol in induced sputum is quantifiable and is negatively influenced by the percentage of neutrophils. Therefore, our data obtained in sputum confirm that inflammation may have a negative influence on the concentration of retinol in the airways [9, 10]. We also showed that retinol in induced sputum did not correlate with vitamin A intake and the concentration of retinol in the serum. In agreement with our findings Redlich et al. (1996) evaluated retinol in bronchoalveolar lavage (BAL) cells and the lung tissue of 21 patients with respiratory disease and demonstrated that retinol was detectable in the BAL cells but exhibited no relationship with tissue or dietary/serum concentrations. However, the retinol concentration in the BAL cells was the best predictor of vitamin A concentrations in the lung tissue [27]. The concentration of vitamin A on lung tissue was not performed in our study; however, the data obtained in the sputum may indicate a local relationship between inflammation and lower levels of vitamin A on lung tissue.

Higher concentrations of circulating vitamin A in smokers have not been reported in previous studies [28–30]. In fact, a study evaluating 12,741 volunteers (7,713 women, 35–60 years of age, and 5028 men, 50–60 years of age) showed that smoking had no effect on differences in serum retinol between nonsmokers, former smokers, and current smokers ($P = 0.987$) [29]. We evaluated only the serum concentrations and not the reserves of vitamin A and it is possible that the vitamin in the lung tissues has been liberated to the systemic circulation in smokers. Therefore, this is a limitation of our study and does not permit a conclusion about the influence of smoking on the vitamin A status. Higher levels of systemic inflammation (IL-6 and CRP) in COPD than in smokers found in our study and in the Eclipse study [23] and lower intake of vitamin A in COPD compared to smokers (636.9 (339.6–1349.6) versus 918.0 (592.1–1654.6), $P = 0.05$) can explain why COPD patients and not smokers present decrease in serum vitamin A. There were other possible limitations to this study. The analyses were performed using cross-sectional data and therefore valid inferences regarding causal pathways cannot be drawn. For future investigations, larger study populations are needed.

5. Conclusions

In conclusion, our results demonstrate that low intake and serum levels of vitamin A are associated with the presence of COPD. The serum concentration of vitamin A is positively associated with smoking status. Although COPD patients exhibited increased inflammation, these inflammatory markers were not associated with serum retinol concentrations. Furthermore, sputum retinol is quantifiable and is negatively influenced by the presence of neutrophils. A new contribution of our study was that sputum measurements may be a tool to evaluate the nutritional status of the retinol in the airways.

Table 3: Energy, protein, and vitamin A intake and serum vitamin A.

Variables	Control ($n = 50$)	COPD ($n = 50$)	P value
Energy (Kcal/day)	2545 (2034–2907)	2525 (1811–3092)	0.79
Energy (Kcal/kg/day)	39.4 (29.6–45.9)	36.7 (27.6–48.0)	0.73
Protein (g/day)	77.8 (64.7–102.1)	68.2 (43.5–89.4)	**0.01**
Protein (g/kg/day)	1.2 (0.9–1.5)	1.0 (0.7–1.4)	**0.01**
Vitamin A intake (RAE)	918.0 (592.1–1654.6)	636.9 (339.6–1349.6)	**0.05**
Vitamin A (serum) (μmol/L)	2.1 (1.8–2.4)	1.8 (1.2–2.1)	<**0.001**

RAE: retinol activity equivalent. Data are reported as the median (interquartile range (25–75%)). The P values refer to COPD compared with the control (Mann-Whitney test).

Table 4: Robust multiple linear regression to identify factors associated with vitamin A concentrations.

Vitamin A (serum)	Dependent variables	Standardized coefficients	P value
	Age (years)	0.068	0.620
	Gender (male)	−0.161	0.086
	Smoking status (yes)	0.197	**0.042**
	IL-6 × CRP × TNF-α (pg/mL)	0.112	0.247
	Presence of COPD (yes)	−0.480	**0.001**

$R^2 = 0.26$. For robust multiple linear regressions, clinically relevant variables were selected. The included categorical variables were gender (female = 0, male = 1), smoking status (absence = 0, presence = 1), presence of COPD (absence = 0, presence = 1), the interaction of IL-6 × CRP × TNF-α (was considered by multiplying the variables to construct one variable), and age as a continuous variable.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

L. M. O. Caram, R. A. F. Amaral, R. Ferrari, S. E. Tanni, C. R. Correa, S. A. R. Paiva, and I. Godoy conceptualized the study. L. M. O. Caram, R. Ferrari, and S. E. Tanni performed the statistical analysis. L. M. O. Caram, R. Ferrari, and I. Godoy analyzed the data and drafted the paper. R. A. F. Amaral and C. R. Correa contributed to the data collection. All authors contributed to the writing of this paper and read and approved the final draft of the paper.

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Research Article

Binding of CXCL8/IL-8 to *Mycobacterium tuberculosis* Modulates the Innate Immune Response

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Interleukin-8 (IL-8) has been implicated in the pathogenesis of several human respiratory diseases, including tuberculosis (TB). Importantly and in direct relevance to the objectives of this report quite a few findings suggest that the presence of IL-8 may be beneficial for the host. IL-8 may aid with mounting an adequate response during infection with *Mycobacterium tuberculosis (M. tb)*; however, the underlying mechanism remains largely unknown. The major goal of our study was to investigate the contribution of IL-8 to the inflammatory processes that are typically elicited in patients with TB. We have shown for the first time that IL-8 can directly bind to tubercle bacilli. We have also demonstrated that association of IL-8 with *M. tb* molecules leads to the augmentation of the ability of leukocytes (neutrophils and macrophages) to phagocyte and kill these bacilli. In addition, we have shown that significant amount of IL-8 present in the blood of TB patients associates with erythrocytes. Finally, we have noted that IL-8 is the major chemokine responsible for recruiting T lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$ T cells). In summary, our data suggest that the association of IL-8 with *M. tb* molecules may modify and possibly enhance the innate immune response in patients with TB.

1. Introduction

Mycobacterium tuberculosis (*M. tb*) is an infectious agent that claims about three million lives each year [1]. Pathological manifestations of tuberculosis (TB) result from disregulated inflammatory responses. In contrast to adaptive (acquired) immunity, developed after contact with an antigen and mediated by T lymphocytes, the innate immune system is not dependent on memory of a previous exposure and therefore has no specificity. Thus, neutrophils, monocytes, macrophages, and natural killer cells constitute cellular effectors of innate immunity. Impairment of the functional integrity of this pivotal arm of the immune system leads to an increase in host susceptibility to infection with *M. tb* [2].

Interleukin-8 (IL-8) displays two major biological activities: chemoattraction and activation of several types of white blood cells. These properties of IL-8 can have important clinical consequences by affecting the pathogenesis of severe infectious diseases, including mycobacterial infections such as TB. IL-8 plays a central role in normal immune response to *M. tb* and has been shown to be absolutely required for granuloma formation [3]. Monocytes and macrophages infected with *M. tb* may be primary producers of IL-8 during the course of TB [4–6]; however, neutrophils as well as

respiratory epithelial cells also have the ability to secrete this chemokine [3, 7, 8]. Moreover, IL-8 is most likely responsible for bringing neutrophils to sites of infection in patients with TB; for example, bronchoalveolar (BAL) fluids from these patients show a dramatic increase in neutrophil numbers which correlates with elevated concentrations of IL-8 [9, 10]. Similarly, the extent of expression of IL-8 mRNA in tuberculous lymph nodes is proportional to neutrophil infiltration [11]. The consequences of high levels of IL-8 secretion during pulmonary tuberculosis include the accumulation of neutrophils and the recruitment of T lymphocytes and monocytes [3, 12]. In addition, plasma IL-8 concentrations are higher in patients who died from TB than in survivors [9, 10]. On the other hand, IL-8 is required for effective host defense against *M. tb*. For instance, because of its angiogenic properties IL-8 could contribute to the development of new vessels that are found at the margins of tuberculous cavities during the healing process [13]. Moreover, Friedland et al. [14] have demonstrated that the inability to stimulate production of IL-8 *ex vivo* correlated with poor prognosis in patients with TB.

It is known that several cytokines and growth factors have the ability to directly bind to bacterial molecules [15–22]. Therefore, we hypothesized that IL-8 could interact with *M. tb* and modulate the proinflammatory properties of this pathogen, especially since high concentrations of IL-8 are typically detected in patients with active TB [9, 10].

2. Methods

2.1. Human Subjects. All studies involving human blood and bronchoalveolar lavage fluids were approved by Human Subjects Investigation Committees of University of Tyler Health Science Center and University of Cincinnati Medical Center. Informed consent was obtained from both healthy volunteers and patients. The diagnosis of TB was confirmed by positive culture or using nucleic acid amplification testing (PCR). The diagnosis of MAC lung disease was based on criteria published by the American Thoracic Society/Infectious Diseases Society of America which have symptomatic, radiographic, and microbiologic components, the latter being the most important [23].

2.2. Purification of Monocytes and T Lymphocytes. White blood cells containing monocytes and lymphocytes were prepared from blood of normal donors by centrifugation through Ficoll Paque Plus (Pharmacia, Piscataway, NJ). Monocytes were then separated from other cells, mainly lymphocytes, by adherence to plastic. The adherent cells (monocytes) were incubated overnight with heat-killed *M. tb* (ATCC, Rockville, MD) to induce cytokine production. Then, conditioned media were collected and stored for further analysis. T lymphocytes were obtained by incubation of nonadherent cells with magnetic beads conjugated to CD3 (Dynal, Lake Success, NY). A magnetic cell separator was used to positively select $CD3^+$ cells. $CD4^+$ and $CD8^+$ T lymphocytes were purified in a similar manner. The purity of the cells was tested by cytofluorometric analysis.

2.3. Chemotaxis. Chemotaxis of T lymphocytes was performed using Boyden chambers. Each chamber consisted of two compartments separated by a membrane. The lower compartment was filled with the stimulant, including conditioned media from *M. tb* stimulated monocytes and purified IL-8 alone (positive control). Then the membrane (a five-micron pore size polycarbonate filter, Nucleopore, Pleasanton, CA) was placed on the surface and the chamber was assembled. A 200 μL aliquot of the T cell preparation was added to the top of the filter (the top compartment), and the chambers were incubated at 37°C for 4 h. After the incubation, the chambers were centrifuged for 20 min at 1,200 rpm to achieve settling of T lymphocytes, which migrated through the filters, at the bottom of the lower chambers. Then, the filter was removed, and the cells were counted using a hemocytometer (according to the manufacturer's instructions). In some experiments conditioned media from stimulated monocytes were incubated overnight with an antibody against IL-8 (R&D Systems, Minneapolis, MN), or monocyte chemotactic protein-1 (MCP-1) (R&D Systems, Minneapolis, MN), or macrophage inflammatory protein-1α (MIP-1α) (Serotec Inc., Raleigh, NC), or control antibody (mouse IgG1; Sigma, Chemical Co., St. Louis, MO) prior to performing the chemotactic assay.

2.4. Neutrophil Infection and Viability Determination. Blood was drawn from healthy volunteers, and neutrophils were purified according to the protocol routinely used in our laboratory [24]. Neutrophils were maintained in RPMI-1640 media supplemented with 2 mM L-glutamic acid, 1 mM sodium pyruvate, and 10% fetal bovine serum (Sigma, St. Louis, MO) at 37°C and 5% CO_2. Cells were infected with *M. tbH37Rv* (ATCC, Rockwille, MD) at MOI of 1 : 10 for 30 min. Unbound bacteria were washed off and the cells incubated for an additional 3 h. After 3 h, infected neutrophils were lysed with 1 mL of 0.1% sodium dodecyl sulfate (SDS) in PBS. Appropriate dilutions of cell lysates were plated onto Middlebrook 7H10 agar supplemented with 10% Oleic Albumin Dextrose Catalase (OADC) enrichment. After 21 days of culture, the number of colony forming units (CFU) was counted. Killing index was calculated as percent of CFU at 30 min according to the following formula: ([CFU at 30 min − CFU at 3 hr] × 100/CFU at 30 min). In some experiments, *M. tbH37Rv* were incubated with IL-8 (final concentration 100 ng/mL per 5×10^7 molecules of *M. tb*) overnight at 4°C, washed with PBS, and then used for infection.

2.5. Macrophage Infection and Viability Determination. Human monocytic leukemia cell line THP-1 (ATCC, Manassas, VA) was maintained in RPMI-1640 media supplemented with 2 mM L-glutamic acid, 1 mM sodium pyruvate, and 10% fetal bovine serum (Sigma, St. Louis, MO) at 37°C and 5% CO_2. Prior to infection, THP-1 cells were exposed to 20 ng/mL phorbol-12-myristate-13-acetate (PMA) for 48 h to differentiate into macrophages (cells stop dividing and become adherent). THP-1 derived macrophages were infected with *M. tbH37Rv* (ATCC, Rockwille, MD) at MOI of 1 : 10 for 30 min. Unbound bacteria were washed off and the cells incubated for an additional 3 h. After 3 h, infected

macrophages were lysed with 1 mL of 0.1% sodium dodecyl sulfate (SDS) in PBS. Appropriate dilutions of cell lysates were plated onto Middlebrook 7H10 agar supplemented with 10% Oleic Albumin Dextrose Catalase (OADC) enrichment. After 21 days of culture, the number of colony forming units (CFU) was counted. Killing index was calculated as percent of CFU at 30 min according to the following formula: ([CFU at 30 min − CFU at 3 hr] × 100/CFU at 30 min). In some experiments, *M. tbH37Rv* were incubated with IL-8 (final concentration 100 ng/mL per 5×10^7 molecules of *M. tb*) overnight at 4°C, washed with PBS, and then used for infection.

2.6. Respiratory Burst (Fluorescence Assay). Neutrophils mounted on microscope slides and THP-1 derived macrophages growing on coverslips were incubated with anti-phospho-p40phox antibody (Sigma) followed by Alexa 568 conjugated secondary antibody. Stained cells were analyzed using a Nikon Eclipse TE2000-U inverted microscope with a UV filter set. Intensity scan was created using an Ultraview Program (Perkin Elmer, Waltham, MA).

2.7. Measurement of IL-8. IL-8 concentrations were measured in an ELISA assay using matched antibody pair (R&D Systems, Minneapolis, MN).

2.8. Western Blot. Western Blot was performed to detect binding of IL-8 to mycobacterial proteins. Bacterial extracts were loaded into a 4–15% gradient SDS-PAGE gel. After electrophoresis the gel was subjected to electrophoretic transfer to a nitrocellulose membrane. The membrane was then blocked and incubated with IL-8. Next, anti-IL-8 antibody was applied and followed by enhanced chemiluminescence (ECL) reagents (Biosource, Camarillo, CA). The membrane was then exposed to X-ray film (Fuji Super RX).

2.9. Interaction of IL-8 with M. tb Molecules. M. tb growing in Middlebrook 7H9 broth supplemented with OADC and 0.05% Tween 80 were incubated with IL-8 (100 ng/mL final concentration) overnight at 4°C. The presence of IL-8 attached to *M. tb* molecules was visualized using anti-IL-8 antibody followed by Alexa 568 conjugated secondary antibody. Heated killed *M. tb* molecules and bacteria not incubated with IL-8 served as controls. Fluorescence was analyzed using a Nikon Eclipse TE2000-U inverted microscope with a UV filter set.

2.10. Statistical Analysis. Differences between groups were analyzed by a simple one way analysis of variance (ANOVA), or if the data were not normally distributed by a Kruskal-Wallis ANOVA on ranks. The direct comparison between any two treatment groups was performed using Student's t-test, or the nonparametric Mann-Whitney test when the data sets were not normally distributed. A P value of 0.05 or less was considered significant. All statistics were performed using SIGMA STAT (SPSS Science Inc., Chicago, IL).

3. Results

3.1. Human IL-8 Interacts with Tubercle Bacilli. It has been previously reported that the concentration of IL-8 increases in patients with active tuberculosis [9, 10, 25]. Kurashima et al. [9], for example, showed that the level of IL-8 in BAL fluid from TB patients was as high as 559.7 pg/mg albumin. Moreover, some bacterial pathogens can associate with proinflammatory factors [15–22]. Based on these facts we hypothesized that IL-8 could interact with tubercle bacilli. We first incubated IL-8 with the bacilli as described in Section 2 and used fluorescent microscopy to detect IL-8 bound to *M. tb* molecules. IL-8 associated with bacteria was visualized with a specific anti-IL-8 antibody followed by a secondary antibody conjugated with Alexa 568. *M. tb* cells cultured without IL-8 served as a negative control (Figure 1, top histogram). Tubercle bacilli coated with IL-8 and incubated only with the secondary antibody were used to demonstrate the specificity of anti-IL-8 antibody (Figure 1, bottom histogram). Microscope analysis revealed the presence of IL-8 attached to *M. tb* molecules (Figure 1, red histogram). We also used thermally killed tubercle bacilli in which the protein components were denatured to determine whether IL-8 binds to a surface protein of *M. tb*. We found that heat killed bacteria bound no IL-8 (Figure 1, second histogram from the bottom). This observation suggests that the mycobacterial "IL-8 receptor" may be a protein.

To further confirm the ability of IL-8 to be associated with *M. tb* molecules, mycobacterial cell fractions were separated on a SDS PAGE gel. We noted that IL-8 bound to the membrane and whole cell lysate fractions but not to the cytosolic fraction (Figure 2). Detection of free IL-8 (Figure 2, left panel) served as a control of specificity of the anti-IL-8 antibody.

3.2. The Direct Association of IL-8 with M. tb Cells Leads to the Enhancement of the Ability of Inflammatory Cells to Phagocytose and Kill M. tb. Neutrophils are among the first cells attracted to a site of inflammation and play a critical role in the restricting of bacterial spread and in controlling the initial replication of bacteria [26]. Further, neutrophils which accumulate in the area of infection possess antimycobacterial qualities. These include the ability to phagocytize bacteria and elicit the oxidative burst which results in the production of reactive oxygen intermediates and degranulation, leading to the release of potent antimicrobial enzymes [12, 27, 28].

We tested proinflammatory functions of *M. tb* molecules coated with IL-8 using purified neutrophils. Neutrophils purified from blood of healthy volunteers [24] were infected with tubercle bacilli coated with IL-8 (100 ng of IL-8 per 5×10^7 bacteria) or not coated. An index of phagocytosis was determined after 30 min of infection using fluorescent microscopy (*M. tb* conjugated with fluorescein—FITC). At least 100 neutrophils were evaluated. We also assessed phagocytosis by counting colony forming units (CFU). We found that phagocytic uptake of IL-8/*M. tb* complexes by neutrophils was substantially enhanced ($P < 0.01$). Accordingly, the phagocytic index (number of ingested bacteria per cell) was 5.0 for IL-8-coated bacteria (*M. tb*/IL-8) and 3.0 for

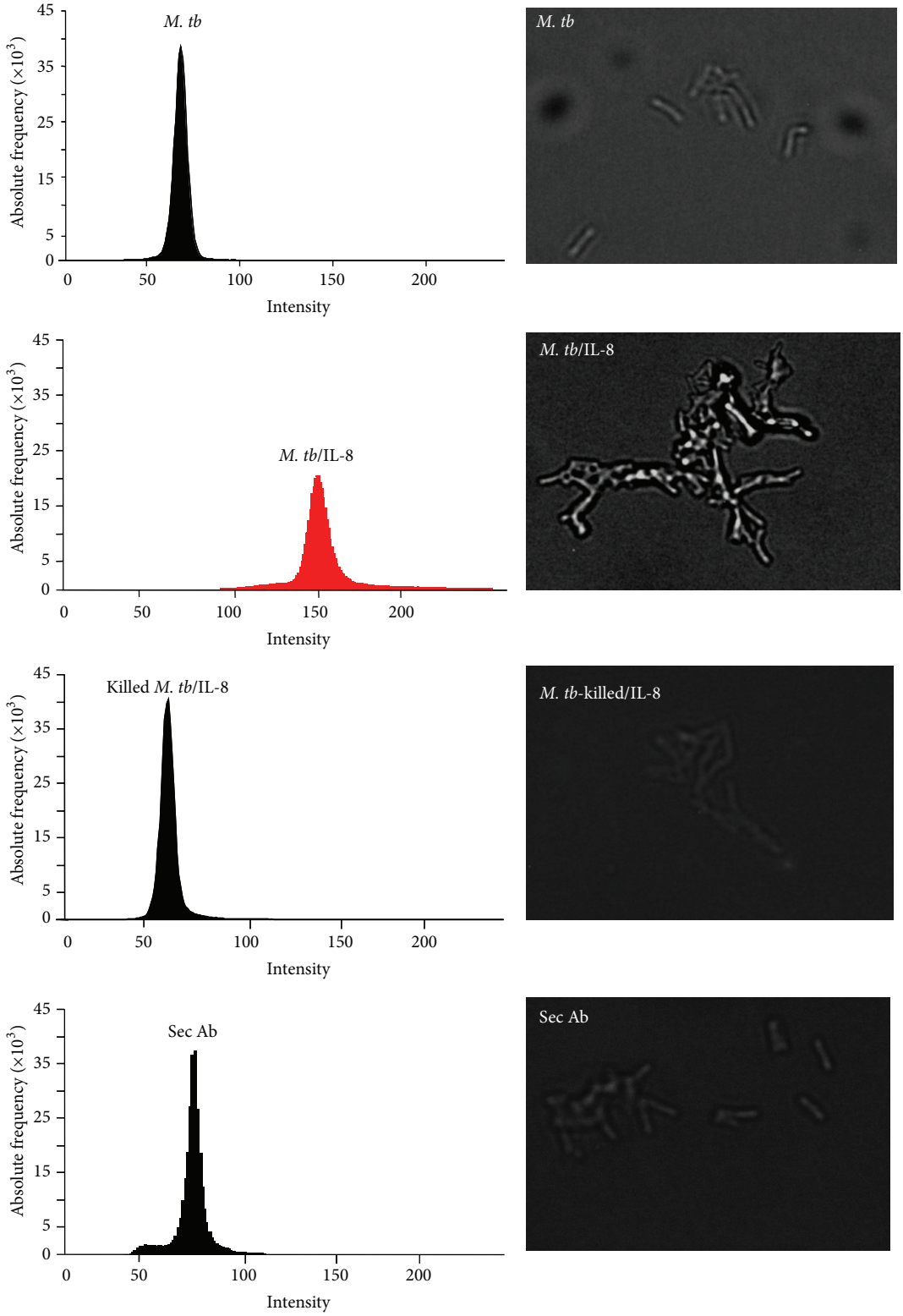

Figure 1: Binding of IL-8 to *M. tb*. Detection of IL-8 associated with *M. tb* using fluorescence microscopy.

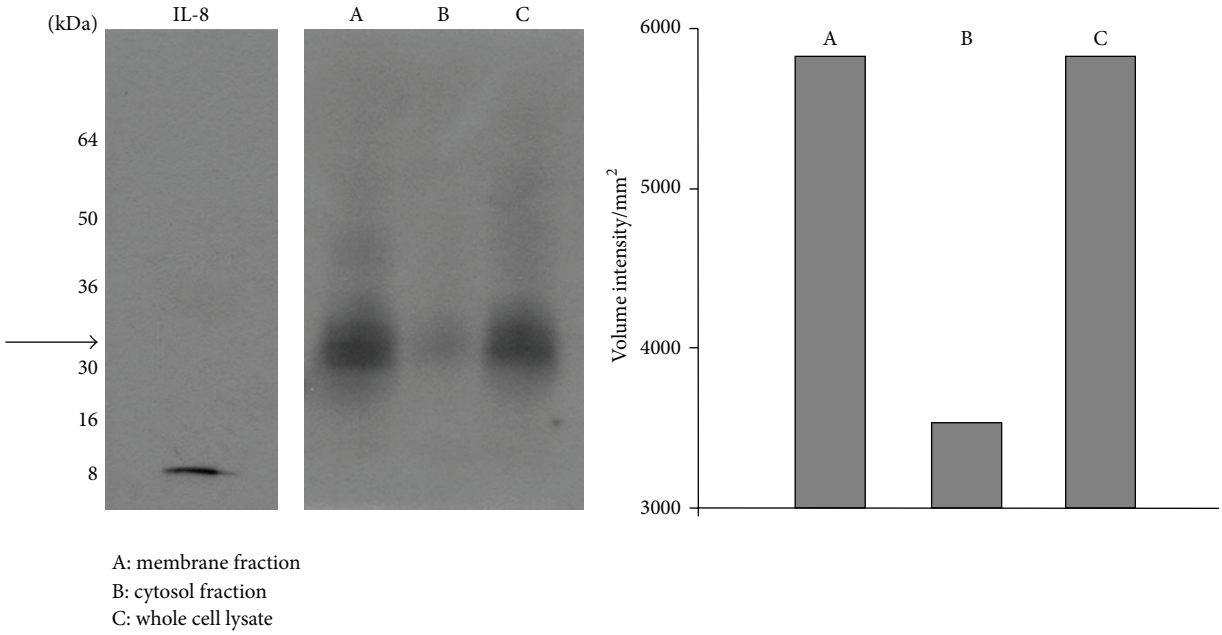

Figure 2: Binding of IL-8 to *M. tb*. Detection of IL-8 associated with cellular fractions of *M. tb* using Western Blot. The vertical bar chart depicts densitometric analysis of protein bands with Quantity One 1D Analysis Software (Bio-Rad).

control bacteria (*M. tb*). Therefore, the association of IL-8 with *M. tb* triggered a substantial increase (approximately 1.5 times) in the ability of neutrophils to phagocyte bacteria (Figure 3(a)). Furthermore, analysis of 3 h infection indicated that the presence of IL-8 downregulated the infectious potential of *M. tb*. As shown in Figure 3(b), neutrophils killed bacilli opsonized with IL-8 more effectively than IL-8-free bacteria. Analysis of the CFU showed that the total number of *M. tb* was lower at both time points (30 min and 3 h) for bacteria preincubated with IL-8 in comparison to the number of IL-8-free *M. tb* ($P < 0.05$ and < 0.01, resp.) This observation was further confirmed by calculating killing index as shown in Figure 3(c) ($P < 0.05$).

Macrophages are the second major group of inflammatory cells involved in the innate immunity response against microorganisms. They are implicated in phagocytosing and killing mycobacteria and are considered the primary host cells for mycobacteria [29]. THP-1 cells have become one of the most widely used cell lines to investigate biological functions of monocytes and macrophages as they relate to various diseases, including TB [30]. In our studies, THP-1 cells were differentiated into macrophages with PMA and then infected with *M. tb* coated with IL-8 (100 ng of IL-8 per 5×10^7 bacteria) or uncoated bacteria. Analysis of phagocytic index showed that the association of IL-8 with *M. tb* enhanced the ability of macrophages to ingest bacteria 1.22 times but this difference did not reach statistical significance ($P = 0.07$; Figure 4(a)). The analysis of the CFU at 3 h indicated that the total number of *M. tb*/IL-8 was substantially ($P < 0.001$) decreased in comparison to the number of IL-8-free *M. tb* bacilli (Figure 4(b)). However, the killing index did not differ for these 2 groups as shown in Figure 4(c) ($P = 0.054$).

The protein p40phox is a major constituent of NADPH-oxidase, which is a multicomponent enzyme system responsible for the oxidative burst [31, 32]. As a consequence of stimulation of inflammatory cells p40phox is phosphorylated and translocates from the cytosol to the cell membrane to form an enzymatic complex which produces oxygen radicals. Analysis of the level of phospho-p40phox in neutrophils and THP-1 derived macrophages infected with *M. tb* was performed using fluorescent microscopy. As shown in Figure 5(a), the presence of IL-8 associated with *M. tb* molecules caused the increase (approximately 1.38 times) in the level of pp40phox in neutrophils compared to chemokine-free bacteria but the difference was not statistically significant ($P = 0.28$). Similar observations were made using THP-1 derived macrophages stimulated with IL-8 coated or uncoated *M. tb* cells (Figure 5(b)). Analysis of fluorescence intensity showed that cells infected with *M. tb* opsonized with IL-8 appeared to be more potent (1.57 times) producers of phospho-p40phox ($P < 0.001$; Figure 5(b)).

3.3. IL-8 Concentration in Blood and Lung Fluids from TB Patients: Clinical Aspect of IL-8 in Tuberculosis. IL-8 concentrations have been measured by several investigators in both plasma and BAL fluids from TB patients. However, the results are quite variable [9, 10, 33–35]. Therefore we evaluated concentrations of this chemokine in blood and BAL fluids from patients with TB and for comparison in samples from patients with mycobacteriosis caused by *Mycobacterium avium-complex* (MAC). It has to be noted that IL-8 produced by white blood cells is present in blood in two forms: soluble (plasma IL-8) and cell-associated (IL-8 associated with red blood cells). It has been shown that a significant percentage of

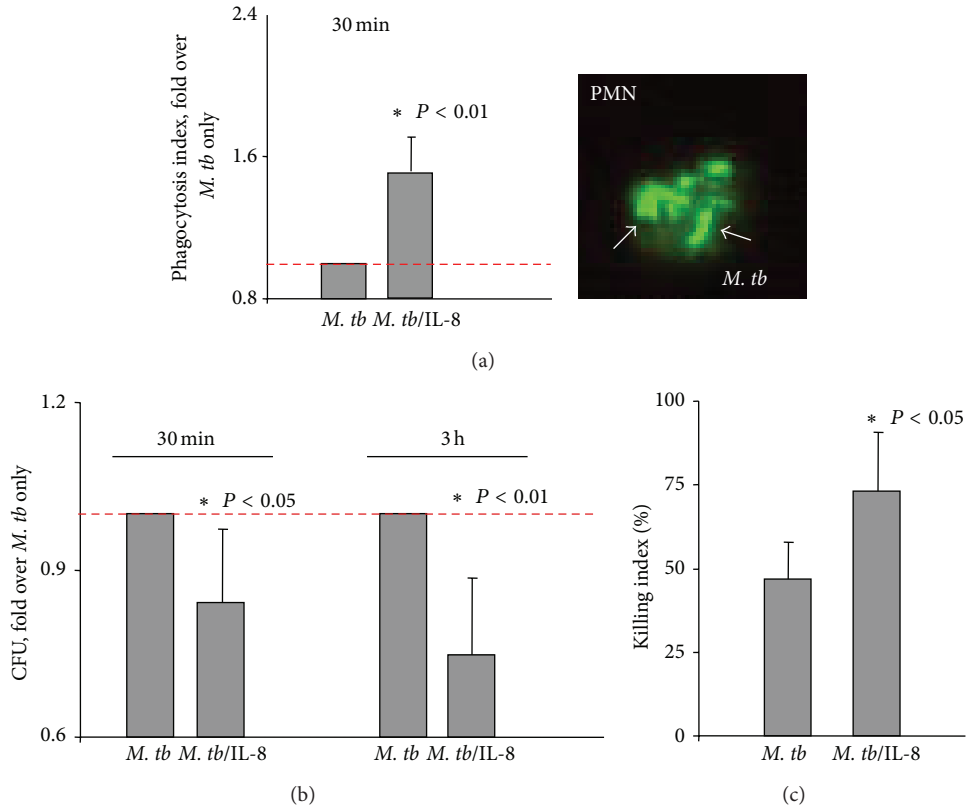

Figure 3: Effect of direct association of IL-8 with *M. tb* molecules on the ability of neutrophils to phagocytose and kill *M. tb*. (a) Phagocytosis of *M. tb* only (*M. tb*), or *M. tb* bound to IL-8 (*M. tb*/IL-8) presented as a phagocytosis index/increase fold over *M. tb* only. Image of neutrophil phagocyting *M. tb* conjugated with FITC photographed under fluorescent microscope. (b) Phagocytosis and killing of *M. tb* only (*M. tb*), or *M. tb* associated with IL-8 (*M. tb*/IL-8) calculated after 30 min and 3 h of infection using colony forming units (CFU) and presented as a CFU/fold over *M. tb* only. (c) Killing index (%) calculated for *M. tb* cytokine-free (*M. tb*) and *M. tb* associated with IL-8 (*M. tb*/IL-8) according to the formula ([CFU at 30 min − CFU at 3 h] × 100/CFU at 30 min).

Table 1: Concentration of IL-8 (pg/mL) associated with red blood cells in human blood from: normal subjects (7 subjects), patients infected with MAC (13 patients), and patients infected with TB (14 patients).

IL-8 associated with red blood cells (pg/mL)		
Normal	MAC	TB
53.8 ± 17.6	103.8 ± 82.5*	125.3 ± 91.3**

Values are means ± SD.
* $P < 0.05$ compared with normal.
** $P < 0.05$ compared with normal.

Table 2: Concentration of IL-8 (pg/mL) in human samples; **A**/IL-8 in human plasma from: normal subjects (7 subjects), patients infected with *Mycobacterium Avium Complex* (MAC, 13 patients), and patients infected with TB (14 patients).

IL-8 in plasma (pg/mL)		
Normal	MAC	TB
20.7 ± 26.2	64.0 ± 85.4*	64.1 ± 77.6**

Values are means ± SD.
* $P < 0.02$ compared with normal.
** $P < 0.02$ compared with normal.

IL-8 in blood is associated with red blood cells. Erythrocytes express a receptor that binds multiple chemokines, including IL-8 [36, 37]. We measured both types of IL-8 in blood from 7 healthy volunteers, 14 TB patients, and 13 patients infected with MAC. We showed for the first time that levels of IL-8 associated with red blood cells from TB patients, MAC patients, and healthy donors were significantly different ($P < 0.05$) (Table 1). In agreement with previous reports [24] we found that IL-8 concentrations were significantly higher in plasma from TB patients than in plasma from normal subjects ($P < 0.02$) (Table 2). Our data also demonstrated (Table 2) that amounts of IL-8 were substantially elevated in plasma from patients with MAC compared to the amounts of this chemokine in plasma from normal subjects. Moreover, we evaluated concentrations of IL-8 in BAL fluids from TB patients, patients with MAC, and healthy subjects (Table 3). The levels of IL-8 were significantly higher ($P < 0.001$) in the two patient groups. Previous reports also demonstrated that lung fluids from TB patients contained significant amounts of IL-8 [9, 10]. We also noted that concentrations of IL-8 were lower in plasma than in bronchoalveolar lung lavage fluids.

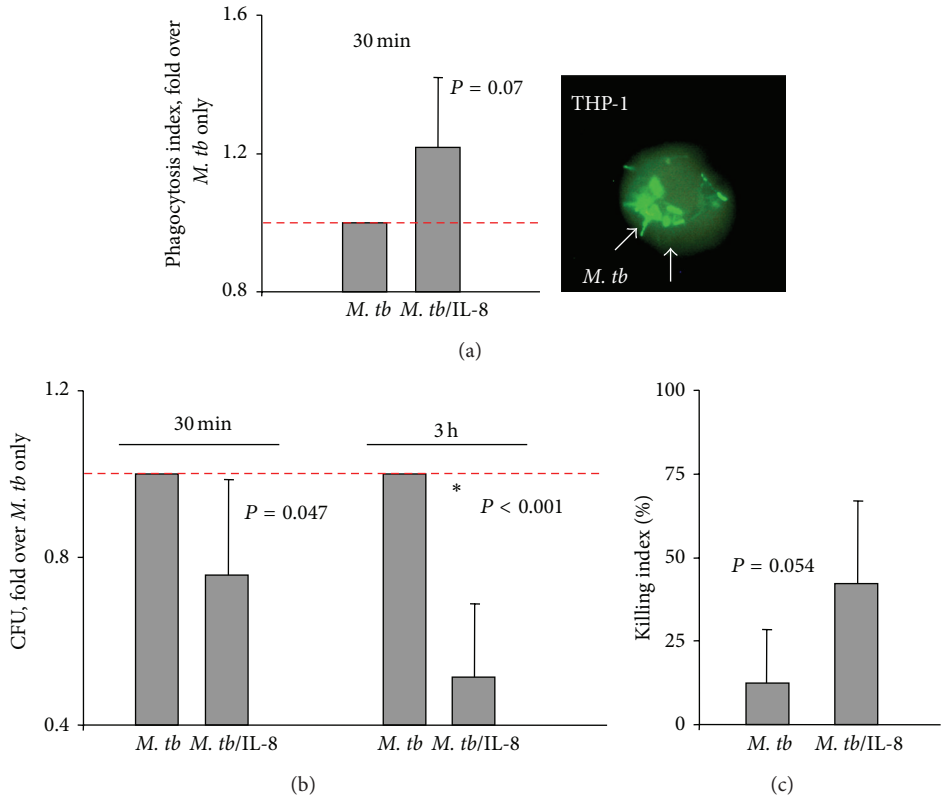

Figure 4: Effect of direct association of IL-8 with *M. tb* molecules on the ability of THP-1 cells to phagocytose and kill *M. tb*. (a) Phagocytosis of *M. tb* only (*M. tb*), or *M. tb* bound to IL-8 (*M. tb*/IL-8) presented as a phagocytosis index/increase fold over *M. tb* only. Image of THP-1 cell phagocyting *M. tb* conjugated with FITC photographed under fluorescent microscope. (b) Phagocytosis and killing of *M. tb* only (*M. tb*), or *M. tb* associated with IL-8 (*M. tb*/IL-8) calculated after 30 min and 3 h of infection using colony forming units (CFU) method and presented as a CFU/fold over *M. tb* only. (c) Killing index (%) calculated for *M. tb* cytokine-free (*M. tb*) and *M. tb* associated with IL-8 (*M. tb*/IL-8) according to the formula ([CFU at 30 min − CFU at 3 h] × 100/CFU at 30 min).

Table 3: Concentration of IL-8 (pg/mL) in lung fluids from: normal subjects (10 subjects), patients infected with MAC (10 patients), and patients infected with TB (15 patients).

IL-8 in lung fluids (pg/mL)		
Normal	MAC	TB
4.0 ± 4.4	141.3 ± 334.6*	365.6 ± 794.9**

Values are means ± SD.
*$P < 0.001$ compared with normal.
**$P < 0.001$ compared with normal.

3.4. IL-8 Triggers Chemotaxis of T Lymphocytes. Since the role of IL-8 as a chemoattractant for T lymphocytes remains controversial [38–41], we evaluated the ability of IL-8 to recruit these cells. We observed that IL-8 induced a significant chemotaxis of T lymphocytes (Figure 6). This response was highly reproducible and dose dependent (Figure 6). Moreover, both $CD4^+$ and $CD8^+$ T cell subsets play a central role in developing resistance to *M. tb* [42]; thus, $CD4^+$ and $CD8^+$ T lymphocytes were also tested in the chemotactic assay (Figure 6). These cells were isolated using magnetic beads conjugated to CD4 or CD8 (purity of >95%). We found that both $CD4^+$ and $CD3^+$ T cells reached maximum response at an IL-8 concentration of 10^{-10} M (Figure 6). The response of $CD8^+$ T lymphocytes was weaker than of other cell types and reached a maximum at an IL-8 concentration of 10^{-11} M (Figure 6).

TB is considered a granulomatous disease due to the accumulation of significant numbers of neutrophils, monocytes, and T lymphocytes in the infected area [2]. However, specific chemokines responsible for recruiting these cells have not yet been identified. We used conditioned media from human monocytes stimulated with heat-killed *M. tb* to evaluate the contribution of IL-8 to chemotactic responses of T lymphocytes. We noted that monocytes stimulated with *M. tb* produced approximately 100 ng/mL of IL-8. Conditioned media induced a significant chemotaxis of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Furthermore, we found that the chemotactic response of T lymphocytes was substantially inhibited by a monoclonal anti-IL-8 antibody ($P < 0.05$) and not by the control antibody. To examine the possibility that other chemokines, which are produced by the stimulated monocytes, contribute to the migratory activity of the conditioned media, we tested specific neutralizing antibodies against chemokines with known activity towards T cells. These include macrophage inflammatory protein-1α (MIP-1α) and macrophage chemotactic protein-1 (MCP-1). Our

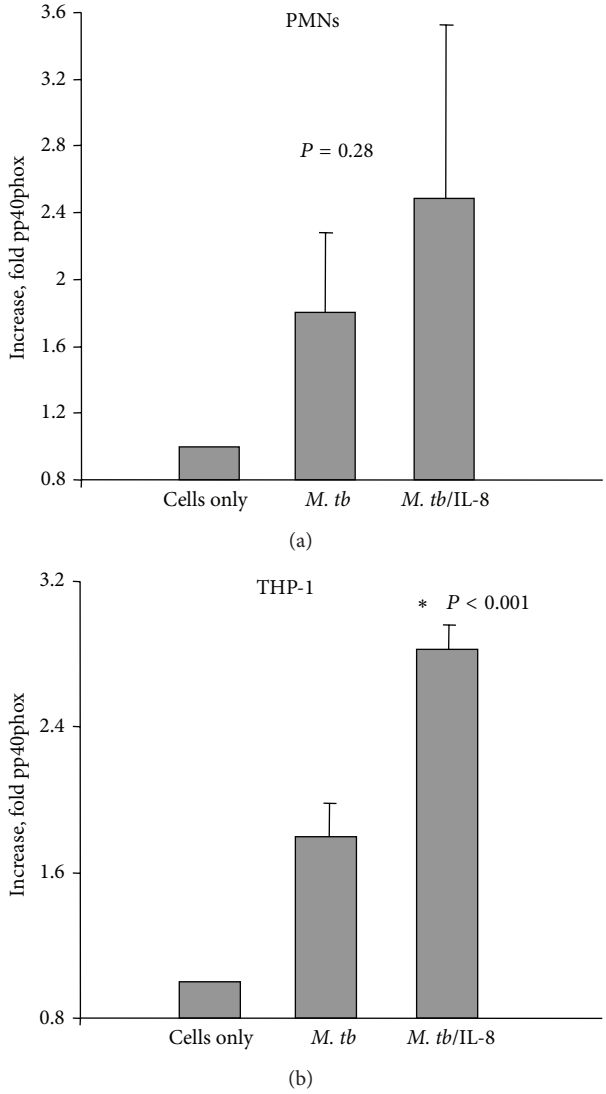

Figure 5: Respiratory burst presented as a level of pp40phox component detected using fluorescent microscopy. (a) Level of pp40phox molecule in neutrophils infected with *M. tb* cytokine-free (*M. tb*) or *M. tb* associated with IL-8 (*M. tb*/IL-8) presented as an intensity scan/increase fold over normal cells. (b) Level of pp40phox molecule in THP-1 cells infected with *M. tb* cytokine-free (*M. tb*) or *M. tb* associated with IL-8 (*M. tb*/IL-8) presented as an intensity scan/increase fold over normal cells.

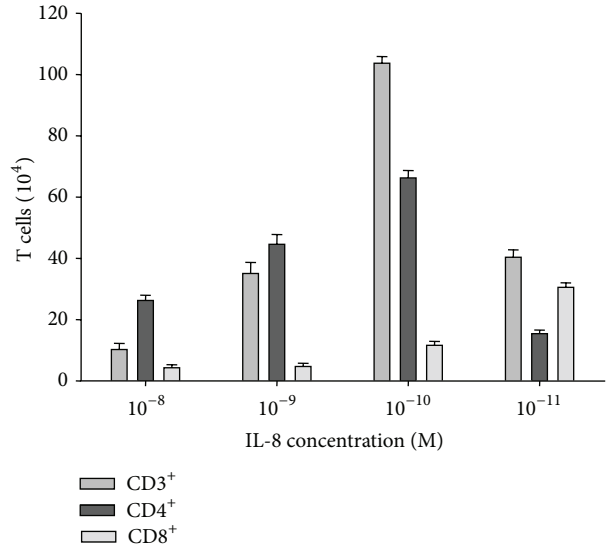

Figure 6: IL-8-induced chemotaxis of T lymphocytes.

results indicated that anti-MIP-1 α antibody or anti-MCP-1 antibody is less effective ($P < 0.05$) than anti-IL-8 antibody in suppressing the chemotactic response of $CD3^+$ (Figure 7(a)) as well as $CD4^+$ and $CD8^+$ T lymphocytes (Figures 7(b) and 7(c)).

4. Discussion

The function of IL-8 in the pathogenesis of TB is controversial and a major goal in the following studies was to investigate the involvement of IL-8 in modulating the immune and inflammatory responses during infection with *M. tb*. There are several studies showing that some growth factors, cytokines, or other mediators have the ability to interact with bacterial pathogens affecting the course of inflammation [15–22]. C3-binding molecules, for example, were detected on the surface of several intracellular pathogens, including *Legionella pneumophila*, *Chlamydia trachomatis*, *Leishmania*, and *Mycobacterium leprae* [18–20]. Bermudez et al. [21, 22] studied epidermal growth factor-binding protein in *Mycobacterium avium* and *M. tb*. In addition, Luo et al. [16] investigated TNF-α binding to *Shigella flexneri*. We analyzed the interaction between IL-8 and tubercule bacilli and showed for the first time that IL-8 could directly associate with *M. tb*. We also found that as a consequence of this interaction antimicrobial activities of neutrophils and macrophages were enhanced. Moreover, the amount of IL-8 that was used to coat the bacteria appears to be physiologically relevant (discussed in the next paragraph).

There is some information in the literature concerning the concentration of IL-8 in plasma and BAL fluids of patients with active TB [9, 10, 25, 33–35]. Sadek et al. [10] showed that levels of IL-8 were increased in BAL fluid by 8.9-fold, whereas we found that IL-8 concentrations were increased 91 times in BAL fluids of patient with TB in comparison to healthy subjects (Table 3). It is important to mention that the increase of IL-8 concentration would be even more significant considering the fact that the BAL method dilutes alveolar fluids by 50- to 100-fold [43].

In agreement with previous findings [44] we have shown that the concentration of IL-8 in plasma and BAL fluids from patients with MAC disease was significantly increased in comparison to normal subjects. It should be noted that we are the first group to show that high concentration of IL-8 associates with red blood cells in TB patients and subjects with MAC disease.

There are a limited number of studies reporting the role of IL-8 as a chemoattractant for T cells [38–41]. Xu et al. [38] showed that IL-8 was as potent as RANTES, MIP-1α,

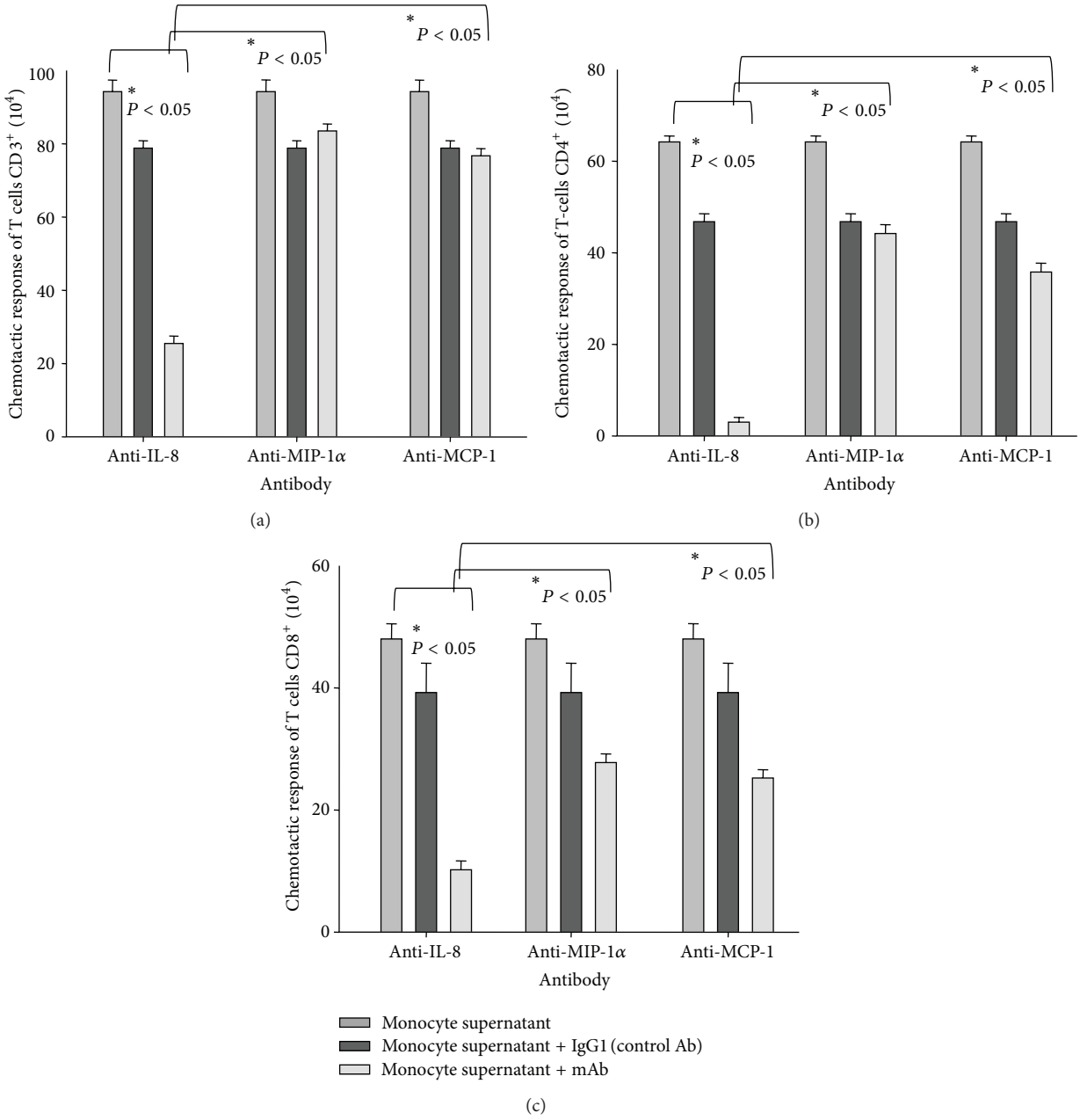

Figure 7: Chemotaxis of $CD3^+$ (a), $CD4^+$ (b), and $CD8^+$ (c) cells from a healthy subject triggered by conditioned media from *M. tb* stimulated monocytes. Effect of anti-IL-8, anti-MIP-1α, and anti-MCP-1 antibodies.

and MIP-1β in inducing chemotaxis of $CD3^+$ T lymphocytes. They tested several concentrations of IL-8 and found that the optimal concentration of IL-8 ranged from 10 to 50 ng/mL ($1–6 \times 10^{-9}$ M) [38]. We demonstrated that IL-8 present in culture media of monocytes stimulated with heat-killed *M. tb* was significantly more effective in inducing $CD3^+$ T cell chemotaxis than MIP-1α and MCP-1 (Figure 7). This is in contrast to Sadek et al. [10], who reported similar activity for IL-8, RANTES, MCP-1, and MIP-1α. Furthermore, we observed that the chemotactic migration of $CD3^+$ T cells was concentration dependent and reached a maximum response at an IL-8 concentration of 10^{-10} M (Figure 6).

Zachariae et al. [39] questioned the ability of IL-8 to attract $CD4^+$ and $CD8^+$ T lymphocytes equally well. In addition, the authors stated that IL-8 at concentration of 100 ng/mL (1.2×10^{-8} M) attracted $CD8^+$ T lymphocytes more efficiently. Taub et al. [40] demonstrated that IL-8 was a potent T lymphocytes migratory agent *in vivo* in SCID mice. However the effect which IL-8 had on T lymphocytes chemotaxis *in vitro* was indirect. According to the authors [40] IL-8 induced neutrophil stimulation and degranulation which further caused T cells migration. The neutralization analysis showed that the majority of neutrophil granule-induced T cell migration was not due to chemokines [40].

We found that IL-8 was a potent attractant for $CD4^+$ and $CD8^+$ T lymphocytes and that migration of these cells towards IL-8 reached a maximum at concentrations of 10^{-10} M and 10^{-11} M for $CD4^+$ and $CD8^+$ T cells, respectively (Figure 6).

In summary, chemokines, including IL-8, are produced in lungs in response to *M. tb* infection. They induce immune cell recruitment to the lungs and play an important role in granuloma formation. They also act as key regulators of host defense against *M. tb* infection. Our novel observations indicate that IL-8 has the ability to directly interact with *M. tb* and in this way enhance antimicrobial functions of proinflammatory cells, that is, macrophages and neutrophils.

Conflict of Interests

The authors declare no commercial or financial conflict of interests.

Authors' Contribution

Anna K. Kurdowska, Agnieszka Krupa, David Griffith, Robert P. Baughman, and Jaroslaw Dziadek were responsible for conception and design; Agnieszka Krupa, Marek Fol, Bozena R. Dziadek, Ewa Kepka, Dominika Wojciechowska, Anna Brzostek, and Agnieszka Torzewska were responsible for acquisition of data; Anna K. Kurdowska, Agnieszka Krupa, Marek Fol, and Bozena R. Dziadek were responsible for analysis and interpretation of data; David Griffith and Robert P. Baughman were responsible for providing patient samples; Anna K. Kurdowska and Agnieszka Krupa were responsible for the drafting of the paper; Anna K. Kurdowska, Agnieszka Krupa, and Jaroslaw Dziadek were responsible for the critical revision of the paper.

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Research Article

Inhibition of Epithelial CC-Family Chemokine Synthesis by the Synthetic Chalcone DMPF-1 via Disruption of NF-κB Nuclear Translocation and Suppression of Experimental Asthma in Mice

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Asthma is associated with increased pulmonary inflammation and airway hyperresponsiveness. The interaction between airway epithelium and inflammatory mediators plays a key role in the pathogenesis of asthma. *In vitro* studies evaluated the inhibitory effects of 3-(2,5-dimethoxyphenyl)-1-(5-methylfuran-2-yl)prop-2-en-1-one (DMPF-1), a synthetic chalcone analogue, upon inflammation in the A549 lung epithelial cell line. DMPF-1 selectively inhibited TNF-α-stimulated CC chemokine secretion (RANTES, eotaxin-1, and MCP-1) without any effect upon CXC chemokine (GRO-α and IL-8) secretion. Western blot analysis further demonstrated that the inhibitory activity resulted from disruption of p65NF-κB nuclear translocation without any effects on the mitogen-activated protein kinase (MAPK) pathway. Treatment of ovalbumin-sensitized and ovalbumin-challenged BALB/c mice with DMPF-1 (0.2–100 mg/kg) demonstrated significant reduction in the secretion and gene expression of CC chemokines (RANTES, eotaxin-1, and MCP-1) and Th2 cytokines (IL-4, IL-5, and IL-13). Furthermore, DMPF-1 treatment inhibited eosinophilia, goblet cell hyperplasia, peripheral blood total IgE, and airway hyperresponsiveness in ovalbumin-sensitized and ovalbumin-challenged mice. In conclusion, these findings demonstrate the potential of DMPF-1, a nonsteroidal compound, as an antiasthmatic agent for further pharmacological evaluation.

1. Introduction

Allergic asthma is a chronic inflammatory airway disorder characterized by bronchial hyperreactivity (BHR) to a wide variety of specific and nonspecific stimuli [1]. In most cases, the severity of BHR correlates with the level of airway inflammation, another hallmark of asthma that is associated with eosinophil infiltration, mucus hyperproduction, and increased production of T_H2 cytokines and allergen-specific IgE [2, 3]. The infiltration of eosinophils, mast cells, and T-lymphocytes into airway epithelia leads to airway inflammation, overproduction of mucus, and airway wall remodeling, which culminates in bronchial hyperreactivity and airway obstruction [4].

The airway epithelium acts as an essential controller of inflammatory, immune, and regenerative responses to allergens, viruses, and environmental pollutants that contribute to asthma pathogenesis [5]. The asthmatic airway epithelium produces chemokines that have prominent effects on leukocyte recruitment and activation. Increasing evidence suggests that the chemokine system coordinates leukocyte recruitment in the pathogenesis of many pulmonary diseases;

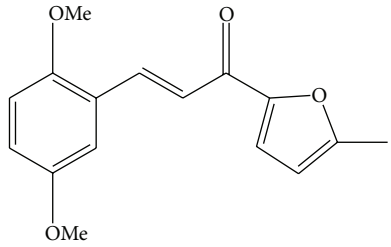

Figure 1: The chemical structure of DMPF-1.

hence, the accumulation and/or activation of leukocyte populations that drive the asthmatic response could be attenuated by blocking chemokine synthesis or chemokine receptors [6].

Chemokines are small cytokines (8 to 10 kDa) that are primarily involved in attracting and regulating leukocyte trafficking into the tissues, in a process called chemotaxis. To date, more than 40 chemokines have been classified into four subclasses according to their structure: CXC, CC, C, and CX3C. The two main groups are CXC (α-chemokines) and CC (β-chemokines). CXC chemokines such as IL-8 and growth-regulated oncogene-α (GRO-α) primarily target neutrophils and have mainly been related to acute inflammatory processes. On the other hand, CC chemokines such as eotaxin, RANTES, MCP-1 to MCP-4, MIP-1α, and MIP-1β primarily target monocytes, T cells, and eosinophils and are therefore of great relevance in asthma [7, 8].

Tumour necrosis factor-α (TNF-α) is a proinflammatory cytokine that has been implicated in many aspects of the airway pathology in asthma. TNF-α is produced predominantly by macrophages but also other immune cells such as T-lymphocytes, B-lymphocytes, dendritic cells, mast cells, neutrophils, and eosinophils as well as structural cells including fibroblasts, endothelial cells, epithelial cells, and smooth muscle cells. Once released in the airways, TNF-α activates the release of multiple inflammatory mediators, chemokines and cytokines, thus promoting ongoing inflammation [9]. Binding of TNF-α to its receptor initiates a variety of potential intracellular signaling cascades including activation of MAP kinase pathways (p38, MEK/ERK, and JNK) and proinflammatory transcription factors NF-κB and AP-1, depending on the exact tissue and the expression pattern of associated signaling machinery [10–12].

DMPF-1 (3-(2,5-dimethoxyphenyl)-1-(5-methylfuran-2-yl)prop-2-en-1-one; Figure 1) is a synthetic chalcone analogue. Chalcones, which belong to flavonoid family, are made up of two aromatic rings joined by a three-carbon α,β-unsaturated carbonyl system [13]. Studies have shown that synthetic and naturally occurring chalcones possess a diverse array of pharmacological activities including antimicrobial, antioxidant, anticancer, and anti-inflammatory properties [13, 14]. Furthermore, several chalcone derivatives have also been shown to inhibit airway inflammation and BHR in allergic asthma [15]. We have previously demonstrated that DMPF-1 inhibited nitric oxide production by LPS-stimulated RAW 264.7 murine macrophages [16]. However, the effect of DMPF-1 on airway inflammation has never been studied. Therefore, the objectives of this study were to determine the effect of DMPF-1 upon the synthesis of asthma-related proinflammatory chemokines in TNF-α-induced pulmonary epithelial cells and OVA-challenged BALB/c mice. We also attempted to determine the effect of DMPF-1 upon the proinflammatory NF-κB and MAPK signaling pathways in TNF-α-induced A549 cells. A final objective in the future involves assessment of the effect of orally administered DMPF-1 upon Th2 cytokine synthesis, eosinophilia, goblet cell hyperplasia, peripheral blood total IgE, and BHR in OVA-challenged BALB/c mice.

2. Materials and Methods

2.1. Synthesis of DMPF-1. The chalcone derivative DMPF-1 was chemically synthesized at the Institute of Bioscience, Universiti Putra Malaysia, by Claisen-Schmidt condensation reaction. NaOH (2.0 mmol, 40%) was added to a 250 mL single-necked round-bottom flask containing 2-acetyl-5-methylfuran (1.0 mmol) dissolved in methanol (20 mL) and stirred vigorously for 20 minutes in cold water. Then, 2,5-dimethoxybenzaldehyde (1.0 mmol) was added and the reaction mixture was stirred at room temperature for 24 hours. The completion of the reaction was assessed with thin layer chromatography (TLC). The mixture was acidified with concentrated HCl and the reaction mixture was transferred into a separating funnel containing 100 mL distilled water. The yellow layer was extracted with ethyl acetate and the solvent was concentrated in vacuum and dried over anhydrous sodium sulphate. The compound DMPF-1 (Figure 1) was purified by column chromatography using silica gel (100–200 mesh, Merck) and eluted with petroleum ether and ethyl acetate.

2.2. Cell Culture. Human A549 lung adenocarcinoma epithelial cells were obtained from the American Type Culture Collection (ATCC). These cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose, sodium pyruvate (1 mmol/L), L-glutamine (2 mmol/L), streptomycin (50 μg/mL), and penicillin (50 U/mL) at 37°C in a 5% CO_2 humidified incubator. The cells were generally maintained to a confluency of 80–90% and detached with trypsin-EDTA. Cell viability was always more than 90%, as determined by trypan blue dye exclusion. The concentration of cells was adjusted to 2×10^6 cells/mL prior to seeding. Cells were stimulated with 10 ng/mL of tumor necrosis factor-alpha (TNF-α) (PeproTech, USA) and cotreated with various concentrations of DMPF-1 in all experiments. DMPF-1 was dissolved in 100% dimethylsulfoxide (DMSO) and the final concentration of DMSO was always maintained at 0.1%.

2.3. Cell Viability Assay. Cytotoxicity of DMPF-1 was assessed by the MTT cytotoxicity assay. Following treatment with increasing concentrations of DMPF-1 (3.13–100 μM), the cells were incubated at 37°C in a 5% CO_2 humidified incubator for 24 hours. Then, the cell culture supernatant was removed and MTT solution (5 mg/mL) was added to each well and further incubated at 37°C, 5% CO_2 for 4 hours. Spent media containing the MTT solution was removed, and the formazan crystals were dissolved in 100 μL/well of DMSO.

The absorbance at 550 nm was measured with a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

2.4. Chemokine Immunoassay. Following 24-hour coincubation with 10 ng/mL TNF-α and increasing concentrations of DMPF-1, spent media was collected and stored at −80°C prior to chemokine immunoassay. The concentrations of MCP-1, IL-8, eotaxin-1 (BD Pharmingen, USA), RANTES, and GRO-α (RayBiotech Inc., GA) were quantified with commercially available sandwich ELISA kits. All assays were conducted according to the manufacturer's instructions.

2.5. Whole Cell, Nuclear, and Cytoplasmic Protein Extraction. Cells were grown until being confluent in 75 cm^2 tissue culture flasks. Culture media in the flask was discarded and cells were rinsed twice with ice-cold PBS (pH 7.4) and lysed with lysis buffer (125 mM, 4% SDS, 20% glycerol, 0.004% bromophenol blue, phosphatase inhibitor cocktail, and benzonase nuclease). After a 15 min incubation on ice, cells were scrapped out gently with a cell scrapper and boiled at 90–100°C for 5 min. Cell lysates were left to cool down before being centrifuged at 16000 g, 4°C, for 15 min. The supernatant was collected and stored at −80°C prior to analysis. Protein quantification was performed using the BCA assay kit (Pierce, USA).

Nuclear and cytoplasmic extractions were performed using the NucBuster Protein Extraction Kit (Novagen, CA) according to the manufacturer's instructions. Attached cells in the flasks were rinsed twice with ice-cold PBS (pH 7.4) and lysed with NucBuster Reagent 1. Cells were incubated on ice for 10 min and vortexed at high speed for 30 seconds. Cell lysates were centrifuged at 16,000 g, 4°C for 5 min, and the supernatant was collected as a cytosolic extract. The pellet was resuspended in NucBuster Reagent 2 containing protease inhibitor cocktail and DTT. The supernatant was collected as nuclear extract following centrifugation at 16,000 g, 4°C for 5 min. The concentration of protein in each sample was quantified with a BCA assay kit (Pierce, USA). Both cytosolic and nuclear extracts were stored at −80°C for further analysis.

2.6. Western Blot Analysis. Analysis of p38, p-p38, JNK, p-JNK, ERK, and p-ERK proteins was done using whole cell lysates while p65 NF-κB analysis was done on both cytosolic and nuclear extracts. Protein samples (20 μg) were loaded on 10% (for the analysis of p65 NF-κB) and 12% (for the analysis of p38, p-p38, JNK, p-JNK, ERK, and p-ERK) SDS-polyacrylamide gels. Gels were electrophoresed on a Mini-PROTEAN System (BioRad, CA) and blotted onto a PVDF membrane using a Trans-Blot Semi-Dry Transfer Cell (BioRad, CA). The membrane was blocked with 5% BSA for 1 hour prior to overnight incubation at 4°C with rabbit polyclonal antibody specific for p38 (1 : 500), p-p38 (1 : 500), JNK (1 : 500), p-JNK (1 : 500), ERK1 (1 : 1000), ERK2 (1 : 1000), p-ERK (1 : 500), and p65 NF-κB (1 : 1000). After washing three times with TBS-Tween, membranes were hybridized with HRP-conjugated donkey anti-rabbit secondary antibody (1 : 2500–5000) for 2 hours followed by three times washing with TBS-Tween. The same membrane was stripped and reprobed with HRP-conjugated mouse monoclonal antibody specific for β-actin (1 : 10,000) or rabbit anti-TFIIB polyclonal antibody (1 : 1000). Membranes were incubated with Super Signal West Femto Maximum Sensitivity Substrate Reagent (Pierce, Rockford, IL) for 5 minutes and bands were viewed under chemiluminescence on a Chemi-Smart gel documentation system (Vilber Lourmet, Marne-la-Vallee, France). Band intensities were quantified with Bio-Profil software (Celbio, Milan, Italy) and normalized by comparison to β-actin or TFIIB.

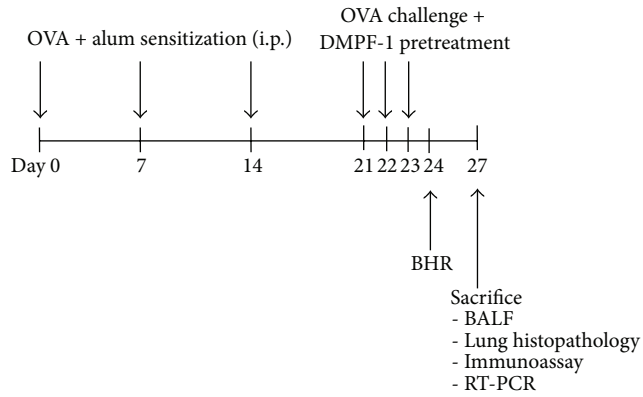

Figure 2: Schematic diagram of the *in vivo* experimental protocol. Mice were sensitized intraperitoneally with OVA (500 μg/mL) and 10% (w/v) alum on days 0, 7, and 14. On days 21, 22, and 23, sensitized mice were pretreated with DMPF-1 intraperitoneally one hour prior to challenge with aerosolized OVA (1%) (w/v). BHR was measured 24 hrs after the last OVA challenge (day 24). The mice were sacrificed on day 27 and BALF, serum, and lung tissues were collected for total and differential cell counts, histopathological evaluation, immunoassay, and RT-PCR.

2.7. Animals. Female BALB/c mice aged 8–10 weeks were used. The mice were housed at the Physiology Lab Animal Experimentation Room, which was maintained at 22–24°C with a 12-hour dark/light cycle, fed on a commercial lab animal pellet, and provided water *ad libitum*. All experiments were conducted according to protocols approved by the Animal Experimentation Ethics Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (Figure 2).

2.8. Ovalbumin-Induced Experimental Asthma. Mice were sensitized with 500 μg/mL ovalbumin (OVA) and 10% (w/v) aluminium potassium sulphate (alum) (Sigma-Aldrich, MO, USA) in 0.1 mL phosphate buffered saline (PBS). Sensitizations were administered intraperitoneally on days 0, 7, and 14 (weekly intervals). One week following the last sensitization, the mice were challenged with aerosolized OVA (1% (w/v) in PBS) for 30 minutes on three consecutive days (days 21–23). The aerosol exposure was performed in a chamber using an ultrasonic nebuliser (Omron, Japan). Naïve mice were sensitized with OVA and challenged with aerosols of PBS only.

2.9. Administration of DMPF-1. Four doses of DMPF-1 (0.2 mg/kg, 2 mg/kg, 20 mg/kg, and 100 mg/kg) were prepared in a vehicle consisting of 90% (v/v) distilled water, 5% (v/v) ethanol, and 5% (v/v) Tween-20. DMPF-1 was given to mice one hour prior to each OVA aerosolization intraperitoneally on days 21, 22, and 23. Mice administered with DMPF-1 appeared healthy, showed regular weight gain and activity levels similar to control mice, and had no ulceration at the injection sites. Dexamethasone (3 mg/kg) was used as a drug control, prepared in the same vehicle, and administrated through the same route. Preliminary studies showed that the vehicle used had no significant effect upon airway inflammation and mediator secretion.

2.10. Assessment of Bronchial Hyperresponsiveness (BHR). Bronchial hyperresponsiveness (BHR) was assessed by methacholine-induced airflow obstruction. BHR was measured at 24 hours (day 24) after the last OVA challenge. Conscious mice were placed unrestrained in a whole body plethysmograph system (Buxco Electronics, Inc., Troy, NY) and challenged with incremental doses of methacholine (6.25, 12.5, 25, and 50 mg/mL) (Sigma-Aldrich, MO, USA) in order to induce BHR. The mice were exposed to each dose of methacholine for 3 min and the readings were taken for 5 min after each nebulization. Bronchopulmonary resistance was expressed as enhanced pause (Penh).

2.11. Bronchoalveolar Lavage (BAL) Fluid Collection and Cell Counts. Mice were sacrificed on day 27 (three days after the final challenge dose of OVA). The trachea was cannulated with a 22 G feeding needle and tied with surgical thread. BAL fluid was obtained by flushing both lung lobes with 0.9 mL ice-cold PBS (pH 7.4). Flushing was done four times repeatedly in a slow manner. The bronchoalveolar fluid (BALF) was centrifuged (400 g, 10 min, 4°C) and the supernatant was stored at −80°C prior to quantification of cytokines and chemokines. The cell pellet was resuspended in PBS and cytosmears were prepared on a Hettich centrifuge (500 g, 5 min, 4°C) with cytospin adaptors. Smears were dried overnight and stained with Wright's Stain for differential cell counts. Total cell numbers were counted in a hemocytometer after 1 : 1 dilution of the cell suspension with trypan blue.

2.12. Lung Histopathology. Following BALF collection, the lungs and trachea of the mice were fixed with 0.9 mL of 10% formalin through the 22 G feeding needle that was cannulated into the lung. Lungs were then removed and kept in 10% formalin. Lung tissue was fixed in formalin for at least 72 hrs and cut into small pieces prior to tissue processing. The tissue was dehydrated with increasing percentages of ethanol and cleared with xylene on a Leica Automated Tissue Processor TP 1020 (Leica Instrument Gmb, Germany). Sections were embedded in paraffin wax for 24 hours. Embedded tissues were cut into 4 μm sections using a microtome (Leica, IL, USA). Tissue sections were deparaffinized prior to staining with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS). Slides were mounted with DPX mountant and covered with a glass coverslip. The entire slide was scanned initially under low magnification (100x) to count the total number of airways on each slide. All airways on the slide were counted except for those with internal perimeters of more than 500 μm. The range of airway internal perimeters was between 200 μm and 700 μm. Airways with a short/long diameter ratio less than 0.3 were considered as being tangentially cut and excluded in the study. H&E stain was used for the evaluation of cellular infiltration. The number of inflammatory cells in the peribronchial/perivascular regions (within 50 μm from the external perimeter of airways and blood vessels) of each section from the same animal was counted and divided by the number of airways/blood vessels found on each section. The same procedure was repeated on all experimental mice in the same group ($n = 10$) to get the mean number of infiltrated inflammatory cells in the peribronchial/peribronchial region. Three sections were counted for each animal. The numbers of total inflammatory cells per airway and blood vessel were obtained by adding the average number of cells from perivascular count/number of airways and peribronchial count/number of blood vessels. PAS stain was used for histopathological evaluation of goblet cell hyperplasia. The numbers of goblet cells in each airway were counted in a similar manner as mentioned above. To assess goblet cell hyperplasia, the sum of the number of goblet cells was divided by the total number of airways in each slide. The same procedure was repeated on all experimental mice in the same group ($n = 10$) to get the mean number of goblet cells of each group. Three sections were counted for each animal. All the counting in histological studies was carried out in a blinded fashion by two investigators in the laboratory.

2.13. Cytokine, Chemokine, and IgE Immunoassay. Concentrations of eotaxin, IL-4, IL-5 (BD Pharmingen, CA, USA), RANTES (RayBiotech Inc., GA), and IL-13 (R&D Systems, Minneapolis, MN) in BALF were quantified using sandwich EIA kits according to the manufacturer's instructions. Similarly, the serum level of total IgE was quantified with a commercially available EIA kit (BD Pharmingen, CA, USA).

2.14. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR). The total RNA of homogenized lung tissue was extracted using Qiagen RNeasy Plus Mini Extraction kit (Qiagen, USA) according to the manufacturer's instruction. RNA integrity was examined by formaldehyde agarose gel electrophoresis and concentrations were determined by UV spectrophotometry (DU 530 Life Science UV/Visible Spectrophotometer, Fullerton, CA). Master mix was prepared using Qiagen One-Step RT-PCR kit according to the manufacturer's instructions (Qiagen, USA). RNA (2 μg) was added as a template for reverse transcription at 50°C for 30 min, initial PCR activation at 95°C for 2 min, and final extension at 72°C for 10 min in an Eppendorf thermal cycler. PCR products were separated by electrophoresis through a 2% agarose gel, stained with ethidium bromide, and visualized with a gel imaging system under UV light (Vilber Lourmet, Marne-la-Vallée Cedex 1, France). Band intensities were quantified by Bio-Profil software (Celbio, Milan, Italy) and normalized by comparison to the RT-PCR products of glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

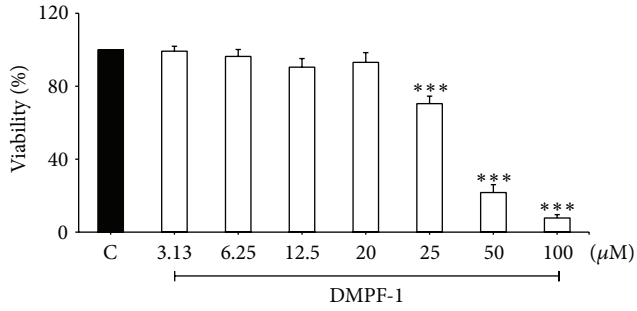

Figure 3: Cell viability of A549 cells following DMPF-1 treatment. Cells were treated with increasing concentrations of DMPF-1 for 24 hours. C stands for vehicle control. The values are expressed as mean ± SEM of three independent experiments performed in triplicate. *** $P < 0.005$, significantly different from the vehicle control.

mRNA. Oligonucleotide primers used for this experiment were published gene sequences.

2.15. Statistical Analysis. Statistical analyses were conducted using SPSS version 17.0. One-way ANOVA followed by Dunnett's post hoc test was used to determine statistical significance. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Cell Viability. An MTT cytotoxicity assay was performed to determine nontoxic concentrations of DMPF-1 to be used in subsequent *in vitro* experiments. Figure 3 shows that DMPF-1 significantly reduced the viability of A549 cells at 25 μM and above. Thus DMPF-1 was used at 20 μM and below for the subsequent assays.

3.2. Chemokine Secretion. Figure 4 shows significant inhibition of eotaxin-1, RANTES, and MCP-1 secretion by TNF-α-stimulated A549 cells following DMPF-1 treatment. However, no effect on the secretion of IL-8 and GRO-α was observed.

3.3. DMPF-1 Disrupts NF-κB but Not MAPK Signaling. When stimulated with TNF-α, p65NF-κB translocates from the cytoplasm into the nucleus (Figure 5). However, treatment with DMPF-1 significantly inhibited the translocation of p65 from cytoplasm into the nucleus. Figure 6 shows that DMPF-1 had no effect upon the phosphorylation of p38, ERK1/2, and JNK.

3.4. DMPF-1 Reduces Bronchial Hyperresponsiveness (BHR). BHR of mice was plotted as percentage increase of enhanced pause (Penh). Methacholine doses were increased from 6.25 mg/mL to 50 mg/mL while PBS was set as a baseline. Figure 7 shows that exposure to methacholine increased Penh in OVA-sensitized and OVA-challenged mice as opposed to naïve mice (OVA-sensitized and PBS-challenged). DMPF-1 treatment of OVA-challenged mice caused significant reduction of Penh at the highest methacholine dose (50 mg/mL). However, there was no clear dose-response effect among the treatment groups.

3.5. DMPF-1 Reduces Total and Differential Cell Counts in BALF. As shown in Figure 8, there was a significant increment of total cell counts in BALF of OVA-challenged mice compared to naïve mice due to a significant increase in the number of infiltrating eosinophils, neutrophils, and lymphocytes. The number of inflammatory cells recruited to the lung of OVA-challenged mice in response to DMPF-1 was markedly reduced compared to that of the OVA-challenged group. Differential cell counts indicated that the changes in total cell number resulted from an increase in the representation of eosinophils as a proportion of total white blood cells in OVA-challenged mice. Administration of DMPF-1 significantly reduced the influx of inflammatory cells of all three cell types especially eosinophils in BALF in comparison to the OVA-challenged group.

3.6. DMPF-1 Reduces Infiltration of Inflammatory Cells into Airways and Goblet Cell Hyperplasia. Inflammatory cell infiltration of the area surrounding the airways and blood vessels in the lung as well as goblet cell hyperplasia was evaluated histologically. Figures 9 and 10 show representative micrographs of both H&E- and PAS-stained sections. In comparison to naïve mice, OVA-sensitized and OVA-challenged mice showed robust pathological changes in allergic pulmonary inflammation which were characterized by extensive infiltration of eosinophils and mononuclear cells around airways and vessels with goblet cell hyperplasia. Mice treated with DMPF-1 demonstrated significant attenuation of pathological changes. To be more specific, H&E-stained sections in Figure 9 show that, in comparison to naïve mice (Figure 9(a)), there were a large number of inflammatory cells concentrated near the airways and in the perivascular and peribronchial areas of OVA-challenged mice (Figure 9(b)). However, the number was markedly decreased following treatment with increasing doses of DMPF-1 (Figures 9(d)–9(g)). Lung histology slides were also stained with PAS to show goblet cells. Figure 10 shows that OVA-challenged mice (Figure 10(b)) had most PAS-staining goblet cells compared to the naïve mice (Figure 10(a)). Similarly, mice treated with

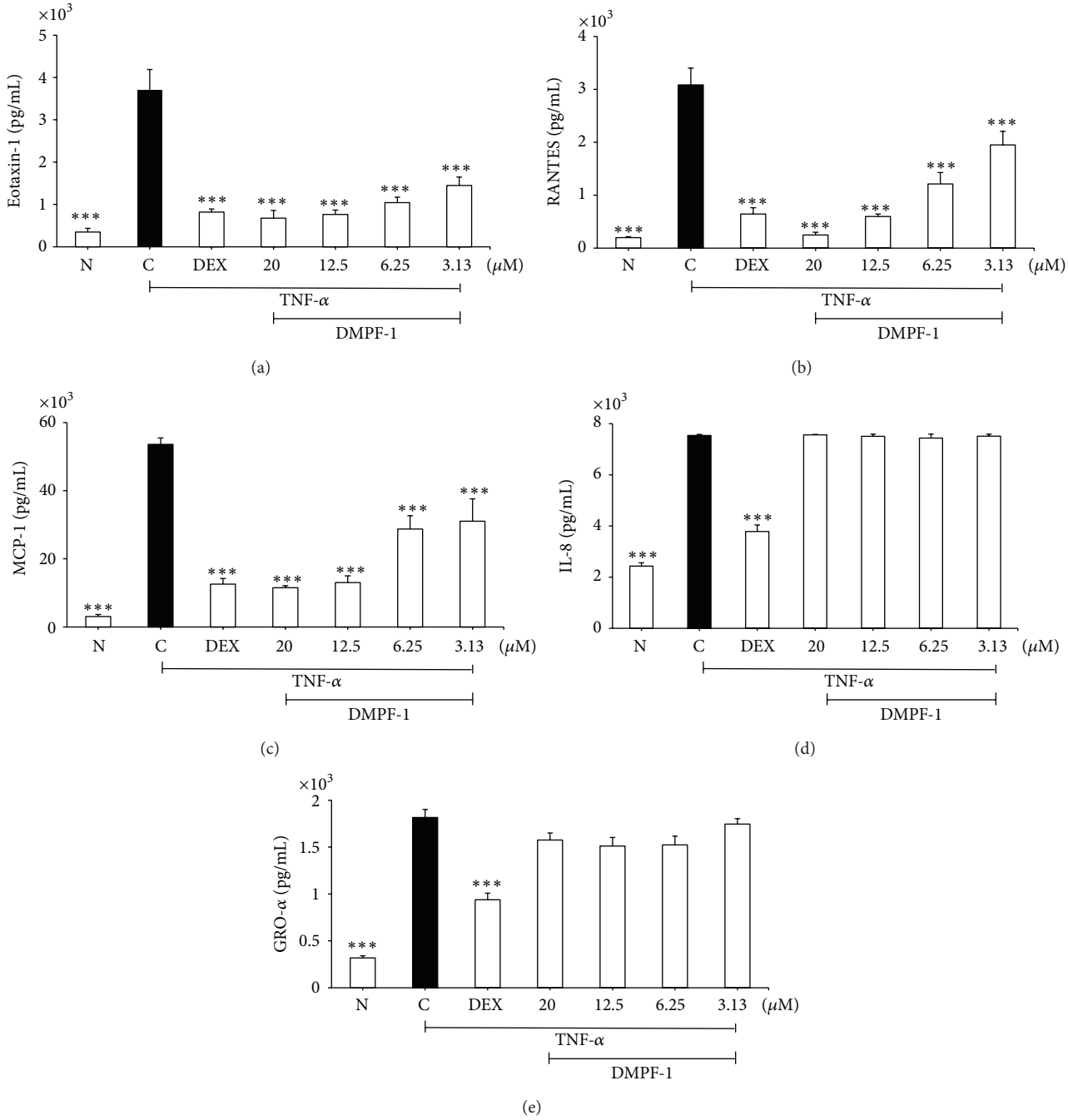

Figure 4: Effect of DMPF-1 on TNF-α-induced chemokine secretion by A549 cells. Cells were stimulated with 10 ng/mL of TNF-α and treated with increasing concentrations of DMPF-1 for 24 hours and the concentrations of (a) eotaxin-1, (b) RANTES, (c) MCP-1, (d) GRO-α, and (e) IL-8 were assayed by EIA. N stands for normal (without TNF-α-stimulation); C stands for vehicle control (TNF-α-stimulated). The values are expressed as mean ± SEM of three independent experiments performed in triplicate. $^{***}P < 0.005$, significantly different from the TNF-α-stimulated vehicle control.

increasing doses of DMPF-1 significantly reduced goblet cell hyperplasia (Figures 10(d)–10(g)).

3.7. DMPF-1 Attenuates Excessive Chemokine and Cytokine Synthesis in Lung Tissue and Reduces Total Serum IgE. In comparison to naïve mice, the levels of eotaxin, RANTES, IL-4, IL-5, and IL-13 significantly increased in BALF of OVA-challenged mice (Figures 11(a)–11(e)). However, following treatment with all doses of DMPF-1, the levels of eotaxin, RANTES, IL-4, IL-5, and IL-13 reduced markedly. Figure 11(f) shows that DMPF-1 had a similar inhibitory effect on IgE level found in the serum of OVA-challenged mice. To further determine whether DMPF-1 modulated eotaxin, RANTES, IL-4, IL-5, and IL-13 in OVA-challenged mice at the

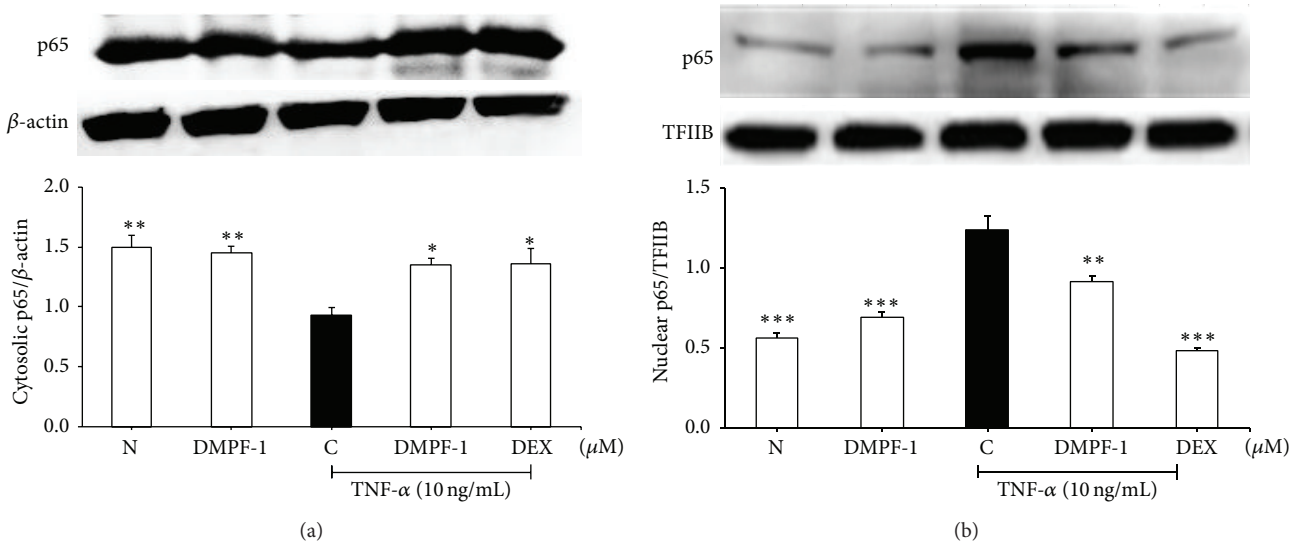

Figure 5: Effect of DMPF-1 on TNF-α-stimulated nuclear translocation of NF-κB p65 in A549 cells. Cells were treated with DMPF-1 (7.5 μM) or positive controls in the presence or absence of TNF-α for 1 hour. Cytosolic and nuclear fractions were subjected to Western blot analysis. (a) Cytosolic fraction in which protein levels of p65 were normalized to β-actin. (b) Nuclear fraction in which protein levels of p65 were normalized to TFIIB. The values are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{*}P < 0.01$, and $^{***}P < 0.005$, significantly different from the TNF-α-stimulated control group.

transcriptional level, their mRNA levels were determined by RT-PCR. As shown in Figure 12, OVA-challenged group had the highest mRNA expression in comparison to the naïve mice. However, these highly expressed mRNAs were significantly inhibited by all doses of DMPF-1.

4. Discussion

Airway inflammation is a dominant feature that leads to clinical symptoms of allergic asthma. The inflammatory response in the asthmatic airways involves a complex interplay of the respiratory epithelium, innate immune system, and adaptive immunity that initiates and drives a chronic inflammatory response. The respiratory epithelium is a major source of many mediators released during airway inflammation in allergic asthma, including Th2 cytokines and chemokines that activate many arms of the immune system [17]. Airway epithelium secretes many chemokines including RANTES, IL-8, eotaxin, and MIP-1α to recruit and activate leukocytes, as well as to induce the proliferation and survival of structural cells [18]. Due to the important role of chemokines in airway inflammation, we were interested in determining whether DMPF-1 may alter the synthesis of both CC and CXC chemokines.

Interestingly, DMPF-1 specifically inhibited CC chemokines including RANTES, eotaxin-1, and MCP-1 without inhibiting CXC chemokines such as IL-8 and GRO-α. This finding is interesting as CC chemokines have been demonstrated to target monocytes, T cells, and eosinophils that have been shown to have major relevance in the pathogenesis of asthma to CXC chemokines which are mainly related to acute inflammatory processes [19]. The results from Western blot analysis further proved that the specificity of DMPF-1 on CC chemokines may possibly be a result of the selective disruption on NF-κB pathway without affecting the MAPK pathway.

In comparison to the MAPK pathway, the NF-κB pathway indeed plays a more important role in the expression of CC chemokines (MCP-1, eotaxins, and RANTES). Previous studies suggested that the binding of p65 and c-Rel/p65 to the two NF-κB sites (A1 and A2 sites) of the human MCP-1 gene is important in elevating the transcription of this gene [20]. NF-κB-like regulatory sequence can also be found at the eotaxin promoter at position -68 relative to the transcription start site. The presence of NF-κB elements has been shown to be indispensable but not sufficient for TNF-α-stimulated chemokine gene expression. Maximal transcriptional activation requires binding sites for additional nuclear factors such as NF-IL-6 and/or SP-1 which are abundant within the eotaxin promoter region and are found to be proximal as well as distal to the eotaxin coding region while recognition sequences for the activator proteins AP-1 and AP-3 or for the phorbol-ester response element PEA3 are only found in more distal eotaxin promoter regions [21]. It was also reported that the RANTES promoter region contains four NF-κB binding sites at positions -30, -44, -213, and -579 relative to the transcription start site. Mutation on any of those NF-κB sites or coexpression of IκB alpha (cytoplasmic inhibitor of NF-κB) markedly reduced the promoter activity and expression of RANTES [22]. Site-directed mutagenesis also indicated that regulation of RANTES promoter activity requires intact NF-κB binding sites while the four putative activator protein-1 (AP-1) recognition sites were dispensable. This finding contradicts that for IL-8, which is a CXC chemokine that requires AP-1 and NF-κB recognition for its full induction

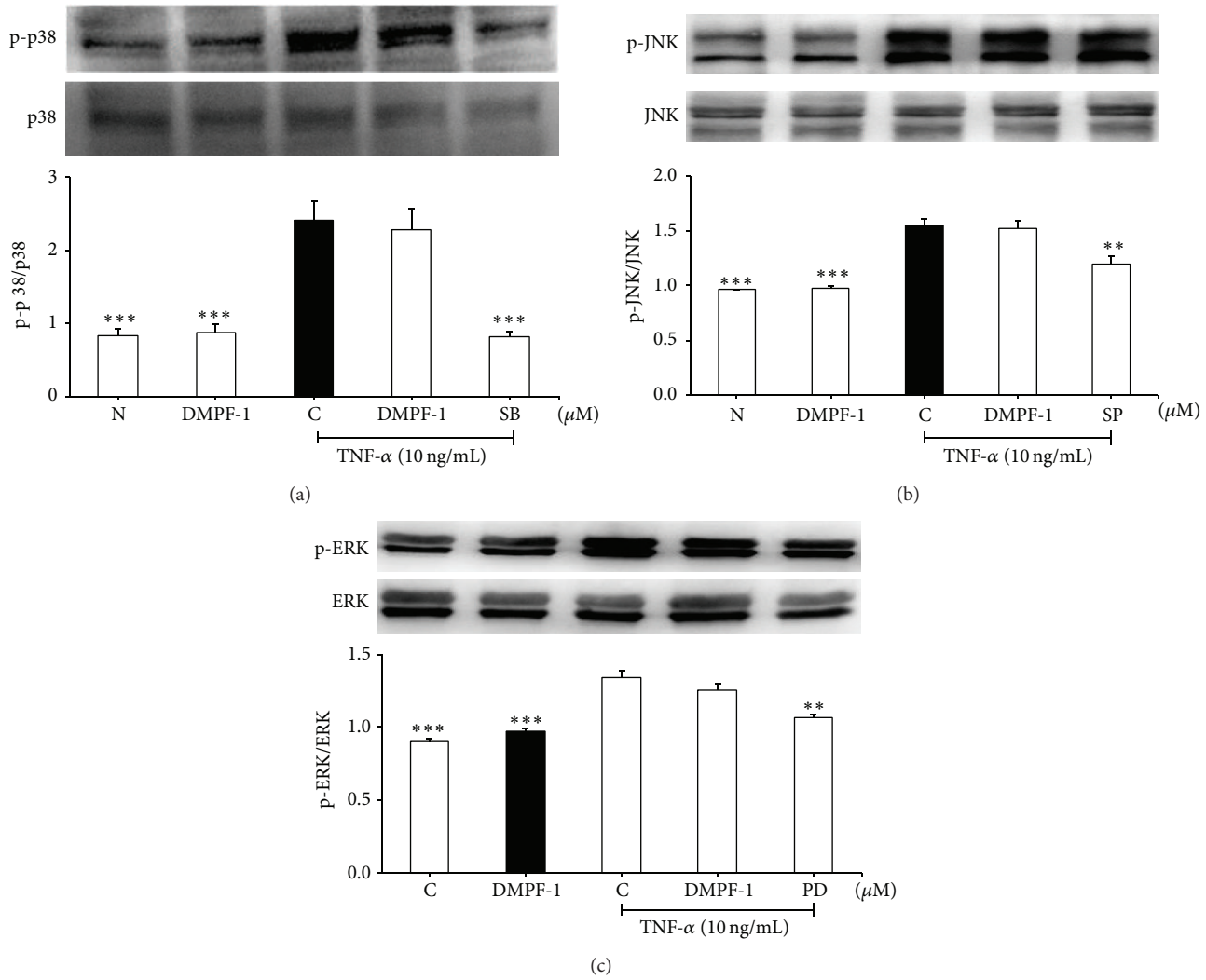

Figure 6: Effect of DMPF-1 on TNF-α-stimulated phosphorylation of MAP kinases in A549 cells. Cells were treated with DMPF-1 (7.5 μM) or positive controls in the presence or absence of TNF-α for 30 min. Whole cell protein extract was subjected to Western blot analysis. Expression levels of phosphorylated protein of (a) p38, (b) JNK, and (c) ERK were quantified and normalized to nonphosphorylated proteins. The values are expressed as mean ± SEM of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.005$, significantly different from the TNF-α-stimulated control group; SB: SB203580; SP: SP600125; PD: PD98059.

of promoter activity by TNF-alpha [23]. AP-1 is a sequence-specific transcription factor composed of members of the Jun and Fos families that mediate gene induction by the phorbol-ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA).

Three different types of MAPKs (ERK, JNK, and p38) contribute to induction of AP-1 activity through phosphorylation of a different substrate [24]. The less significant role of AP-1 in the expression of MCP-1, RANTES, and eotaxin-1 may explain the selective disruption of DMPF-1 on NF-κB pathway which eventually leads to specific inhibition of CC chemokines but not CXC chemokines.

These encouraging findings prompted us to further determine whether DMPF-1 could inhibit chemokine synthesis in an experimental animal model of allergic asthma. We also sought to determine the effect of DMPF-1 upon other prominent variables of asthma such as increased levels of Th2 cytokines, circulating IgE, airway goblet cell metaplasia, and bronchial hyperresponsiveness [25, 26]. The results from *in vivo* experiments showed that DMPF-1 significantly inhibited the synthesis of Th2 cytokines (IL-4, IL-5, and IL-13) and CC chemokines (eotaxin-1 and RANTES).

Eotaxin and RANTES are produced at high concentrations in asthmatic lungs and are the most important eosinophil chemoattractants in allergic inflammation when acting in synergy with IL-5. RANTES binds many CC chemokine receptors (CCRs), including CCR1, CCR3, and CCR5. On the other hand, eotaxin binds specifically to CCR3, which is highly expressed on eosinophils and has selective chemoattractant activity for eosinophils. In addition, eotaxin induces α4- and β1-integrin expression on eosinophils, allowing for firm adhesion of eosinophils to the endothelium and

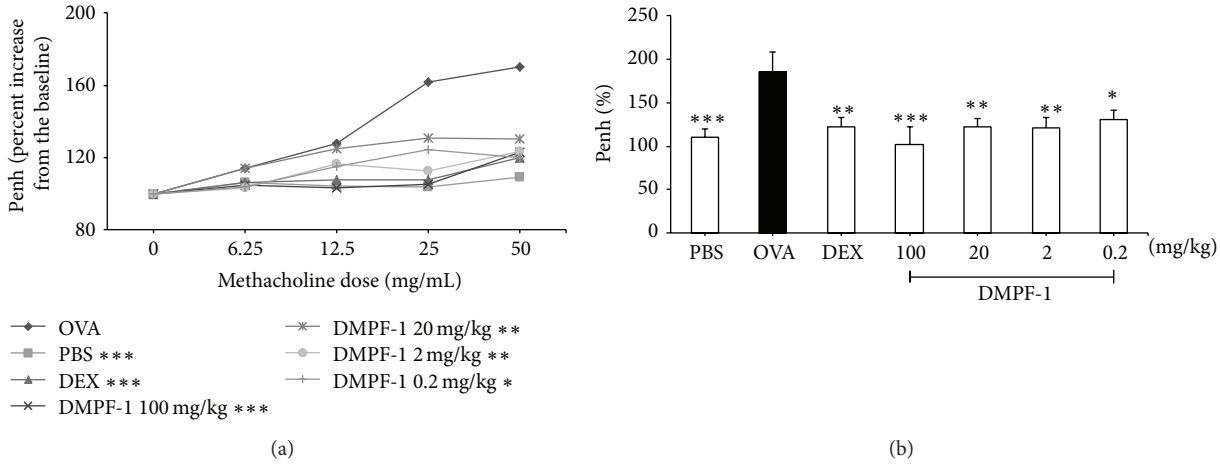

Figure 7: Effect of DMPF-1 on bronchial hyperresponsiveness to methacholine. Female BALB/c mice were sensitized and challenged with OVA 1 hour after treatment with various doses of DMPF-1. After 24 hours of the last OVA challenge, the mice were exposed to (a) increasing doses of methacholine (6.25, 12.5, 25, and 50 mg/mL) in an enclosed chamber for 3 min and the readings were taken for 5 min after each nebulization. (b) The Penh of all groups at the highest dose of methacholine. The values are expressed as mean ± SEM ($n = 10$). $^{***}P < 0.005$, significantly different from the OVA-challenged group.

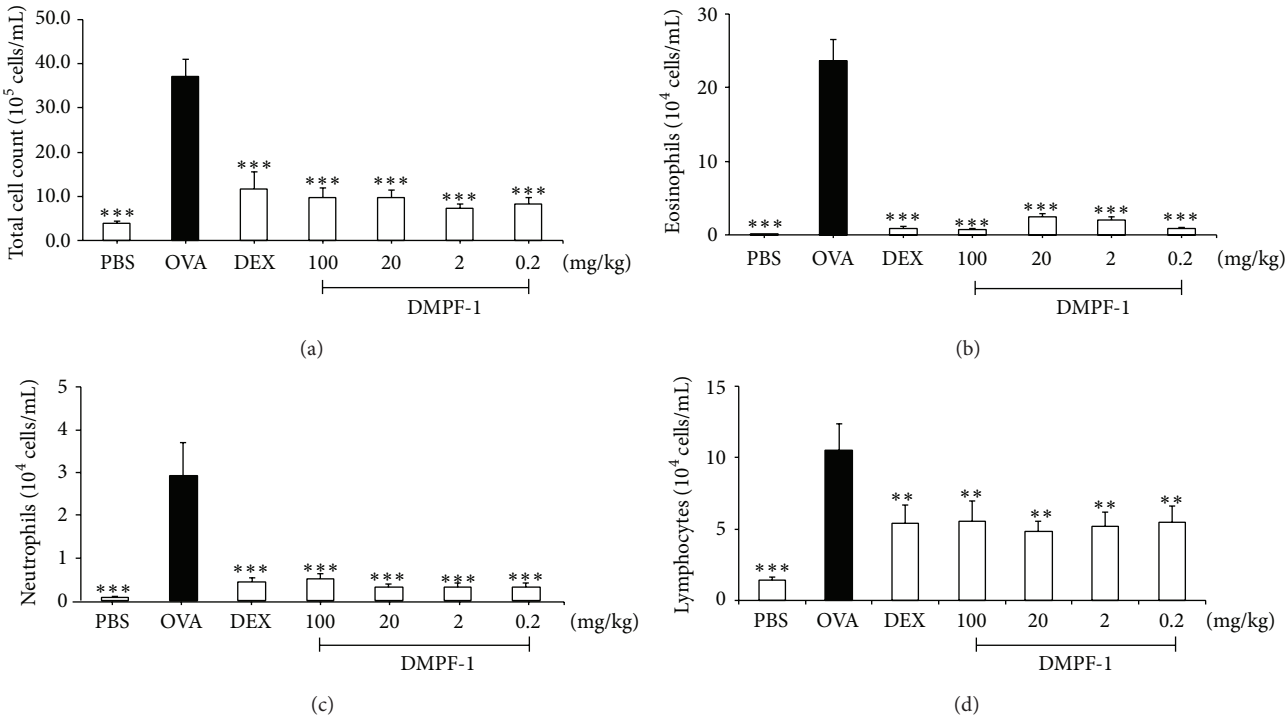

Figure 8: The effect of DMPF-1 on the total and differential cell count in BALF of OVA-sensitized mice. The numbers of (a) total leukocytes and (b) eosinophils, (c) neutrophils, and (d) lymphocytes. The values are expressed as mean ± SEM ($n = 10$). $^{***}P < 0.005$, significantly different from the OVA-challenged group.

transmigration into the site of inflammation [27]. Not only chemokines but also Th2 cytokines such as IL-4 are able to induce eotaxin and are crucial in upregulating the expression of endothelial vascular cell adhesion molecule-1 (VCAM-1) that interacts with very late antigen-4 (VLA-4) to promote the rolling and adhesion of circulating eosinophils to endothelial cells which can then be attracted into target tissues by IL-5 and chemokines [28]. IL-5 induces differentiation and proliferation of bone marrow eosinophils, promotes blood eosinophilia, activates eosinophils, and prolongs their survival [29, 30]. IL-13 is one of the most potent inducers of eotaxin and is another Th2 cytokine that induces VCAM-1

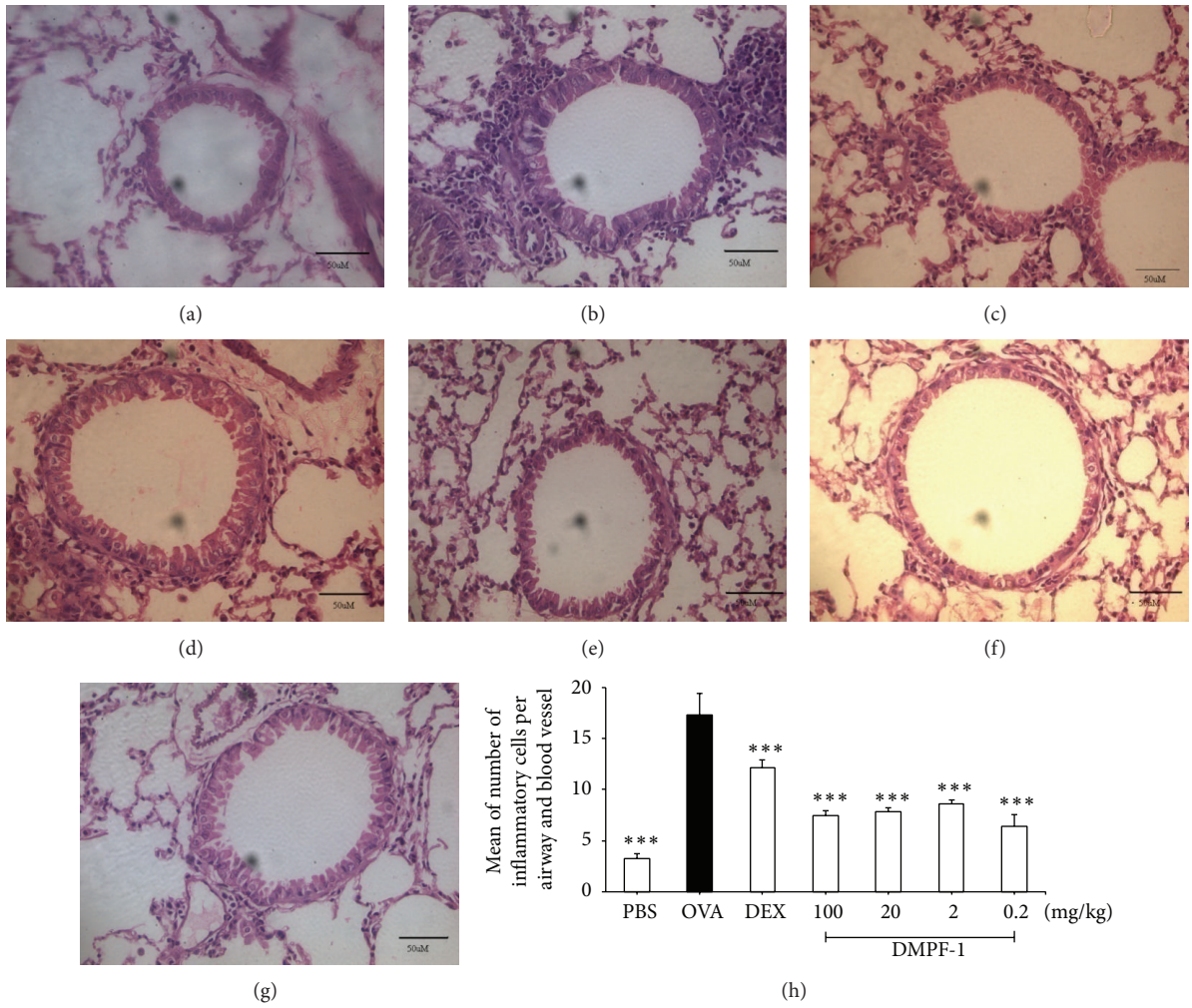

Figure 9: Representative hematoxylin and eosin- (H&E-) stained lung sections. Airways and blood vessels of (a) naïve mice, (b) OVA-challenged mice, (c) dexamethasone-treated mice, (d) 100 mg/kg DMPF-1-treated mice, (e) 20 mg/kg DMPF-1-treated mice, (f) 2 mg/kg DMPF-1-treated mice, and (g) 0.2 mg/kg DMPF-1-treated mice. These photos were taken under 40x objective (400x magnification) using light microscope (Bar = 50 μm). This experiment used 10 mice per group ($n = 10$). (h) For quantitative analysis of infiltration of inflammatory cells, the numbers of total inflammatory cells per airway and blood vessel were obtained by adding the average number of cells from perivascular count/number of airways and peribronchial count/number of blood vessels. The values are expressed as mean ± SEM ($n = 10$). $^{***}P < 0.005$, significantly different from the OVA-challenged group.

expression on vascular endothelium, activates eosinophils, and promotes their differentiation [31]. All these cytokines can be produced by Th2 cells and detected in BALF of asthmatic subjects and show synergistic effects on the induction of lung eosinophilia. DMPF-1 was demonstrated to significantly inhibit Th2 cytokine and eotaxin synthesis and it reduced eosinophilic infiltration.

The role of Th2 cells in allergic inflammation is not limited to their capacity to promote infiltration of eosinophils and other inflammatory cells to the target tissues. Th2 cells secrete IL-4, IL-5, IL-9, IL-10, and IL-13, which are involved in antibody synthesis [32]. Similarly, chemokines are important in allergy and asthma not only for their role in regulating leukocyte recruitment, but also for their role in regulation of IgE synthesis [33]. The inhibitory effect of DMPF-1 upon the synthesis of both Th2 cytokines and CC chemokines possibly explains the suppression of serum IgE in treated mice.

Bronchial hyperresponsiveness (BHR, defined by exaggerated airflow obstruction in response to bronchoconstrictors), goblet cell hyperplasia, and mucus overproduction are important hallmarks that contribute to airway obstruction in bronchial asthma. IL-13 is responsible for mucus hypersecretion by goblet cells and induces hyperplasia of goblet cells. The resulting hypersecretion of mucus associated with goblet cell hyperplasia causes airway narrowing and thus contributes to airflow obstruction. Together with IL-13, other Th2 cytokines such as IL-4, IL-5, and IL-9 induce mucus hypersecretion through upregulation of goblet cell hyperplasia and contribute to the increase of BHR [34, 35]. The decrease in bronchial hyperresponsiveness following

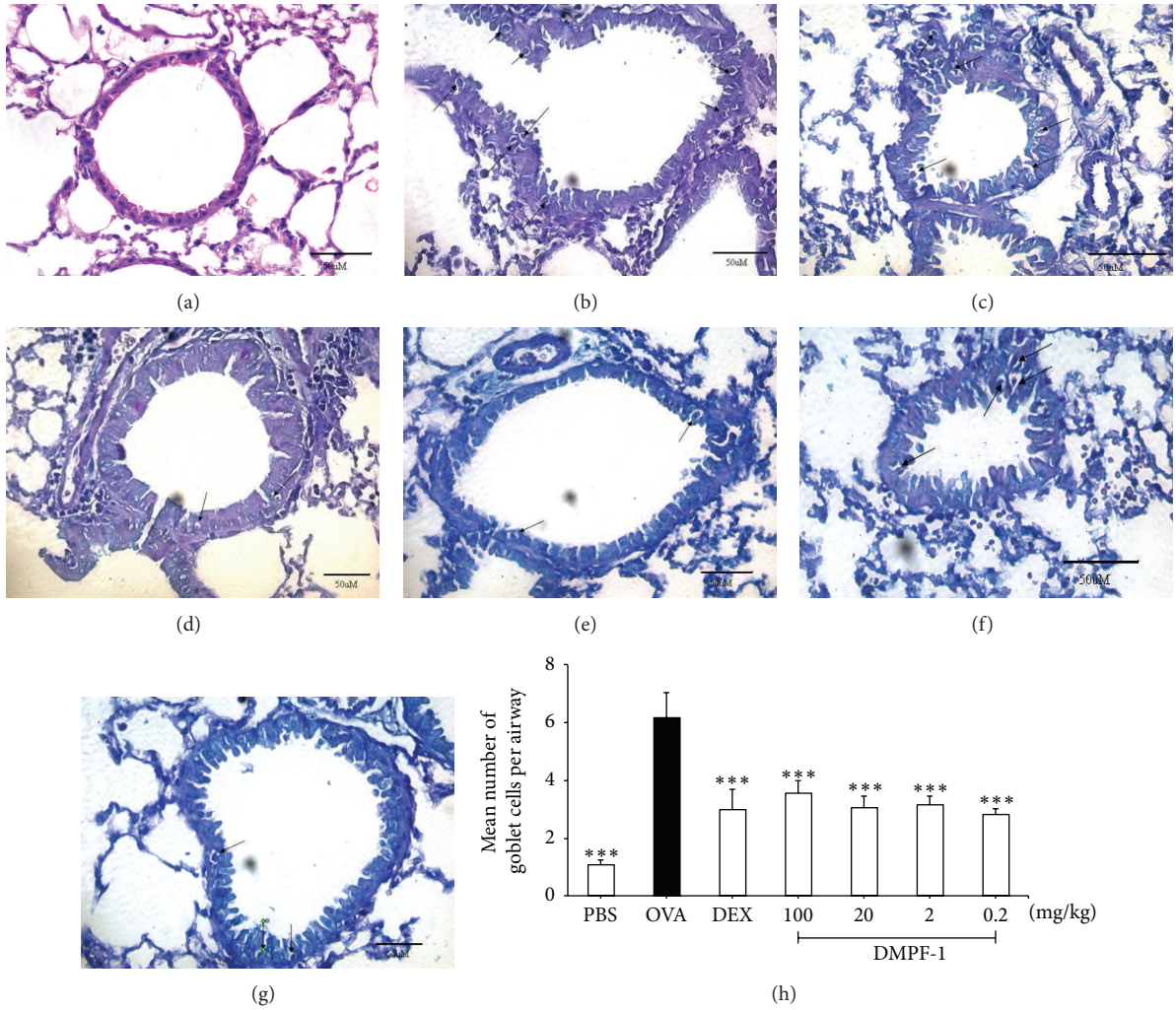

Figure 10: Periodic acid Schiff- (PAS-) stained lung tissue. Airways of mice taken from (a) naïve mice, (b) OVA-challenged mice, (c) dexamethasone-treated mice, (d) 100 mg/kg DMPF-1-treated mice, (e) 20 mg/kg DMPF-1-treated mice, (f) 2 mg/kg DMPF-1-treated mice, and (g) 0.2 mg/kg DMPF-1-treated mice. The goblet cells are indicated by black arrows. These photos were taken under 40x objective (400x magnification) using light microscope (Bar = 50 μm). This experiment used 10 mice per group ($n = 10$). (h) For quantitative analysis of goblet cell hyperplasia, the number of goblet cells in each airway was counted and the sum of the goblet cells was divided by the total number of airways in each slide. The values are expressed as mean ± SEM ($n = 10$). $^{***}P < 0.005$, significantly different from the OVA-challenged group.

DMPF-1 treatment may be related to inhibition of IL-13 synthesis which has an indirect effect upon mucus hypersecretion.

Although significant effects of DMPF-1 on BHR were noted, we do acknowledge the limitations of noninvasive whole body plethysmography in the assessment of BHR. The use of Penh, a unitless parameter derived mathematically from the respiratory waveform produced by whole-body plethysmography, allows noninvasive and repeated evaluation of airway hyperresponsiveness in unrestrained mice. However, using Penh to evaluate airway resistance is controversial as it may be modified by factors that are not directly related to bronchoconstriction, such as movement, humidity, and temperature as well as upper airway resistance [36–38]. Despite the fact that Penh can be influenced by breathing patterns, many investigators identified a correlative relationship between Penh and airway resistance and suggested that Penh may still be suitable as a preliminary technique [39–41]. Nevertheless there have also been arguments on the validity of invasive methods as this approach requires the need for surgical tracheostomy, thus precluding repeated measurements, the needs for anesthesia, mechanical ventilation, and expertise in handling which may alter baseline breathing patterns. The use of either invasive or noninvasive approaches as the sole indicator of BHR still remains under intense debate. However, in order to gain a more definitive insight of the effect of DMPF-1 on BHR, further investigations on the pharmacology of DMPF-1 will employ both noninvasive and invasive methods.

It is interesting to note that although DMPF-1 demonstrated a dose-dependent inhibitory effect on chemokine synthesis by TNF-α-stimulated A549 cells, this was not

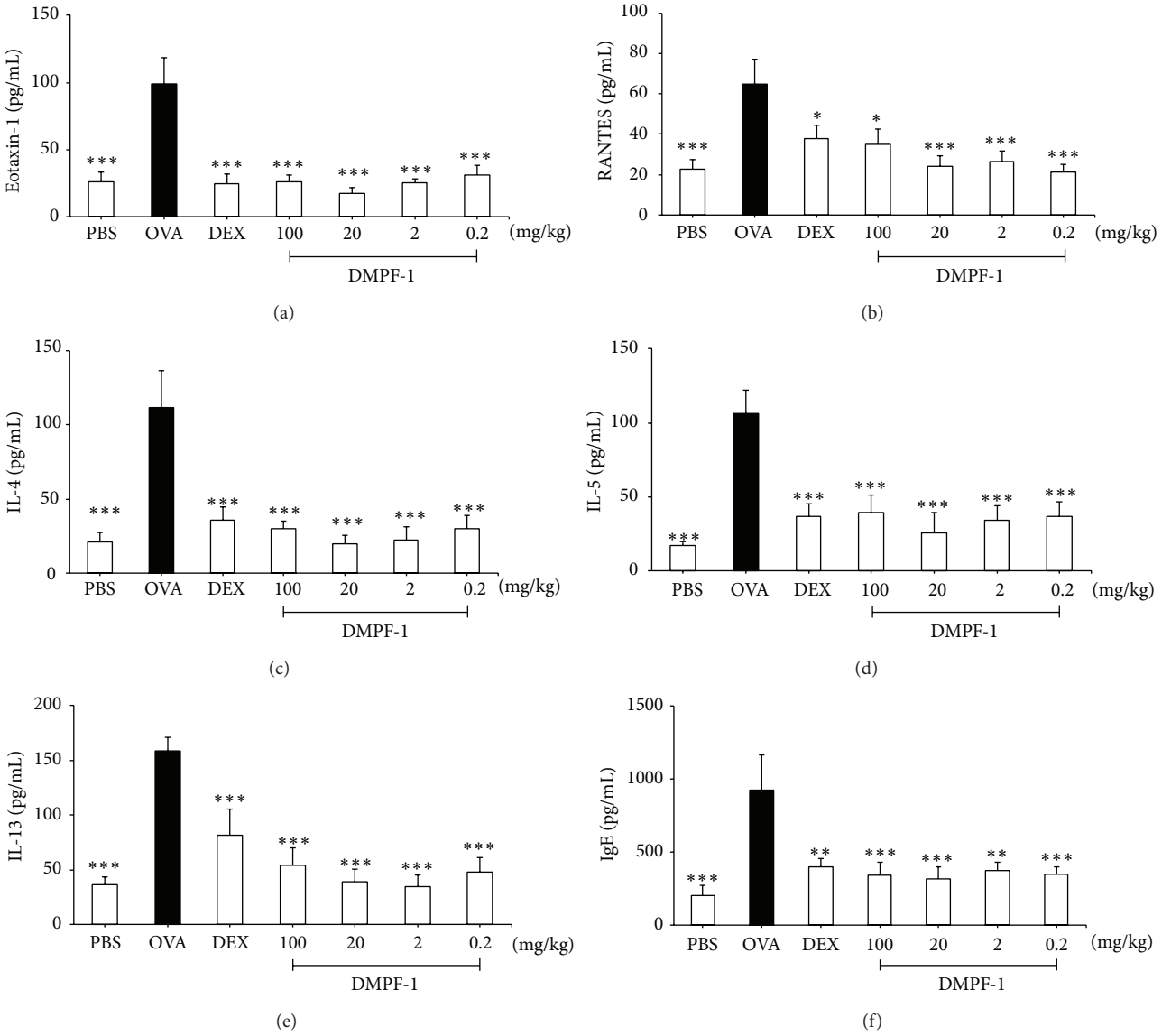

Figure 11: Effect of DMPF-1 on the level of chemokines and Th2 cytokines in the BALF and IgE in the serum of OVA-sensitized mice. After the mice were sacrificed, the BALF was collected and used to measure the level of (a) eotaxin-1, (b) RANTES, (c) IL-4, (d) IL-5, and (e) IL-13 whereas serum from the collected blood was used to measure the (f) IgE level by using ELISA kits according to manufacturer's protocol. The values are expressed as mean ± SEM ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$, significantly different from the OVA-challenged group.

observed amongst variables measured in our animal model. Obviously dosing regimens cannot be directly translated from cell to animal studies and we would be interested in broadening our dose spectrum in future studies. In conclusion, we have demonstrated that DMPF-1 inhibits the synthesis of CC chemokines *in vitro* and have further confirmed this effect in a standard model of murine allergic asthma. Doses of 0.2 mg/kg and above are effective in alleviating common variables of pulmonary dysfunction in the above model and it is possible that these effects are partly due to inhibition of NF-κB nuclear translocation. Our findings demonstrate that DMPF-1 has potential for further pharmacological evaluation as a new potential nonsteroidal drug lead for the control of asthma.

Conflict of Interests

The authors do not have any conflict of interests regarding the publication of this paper.

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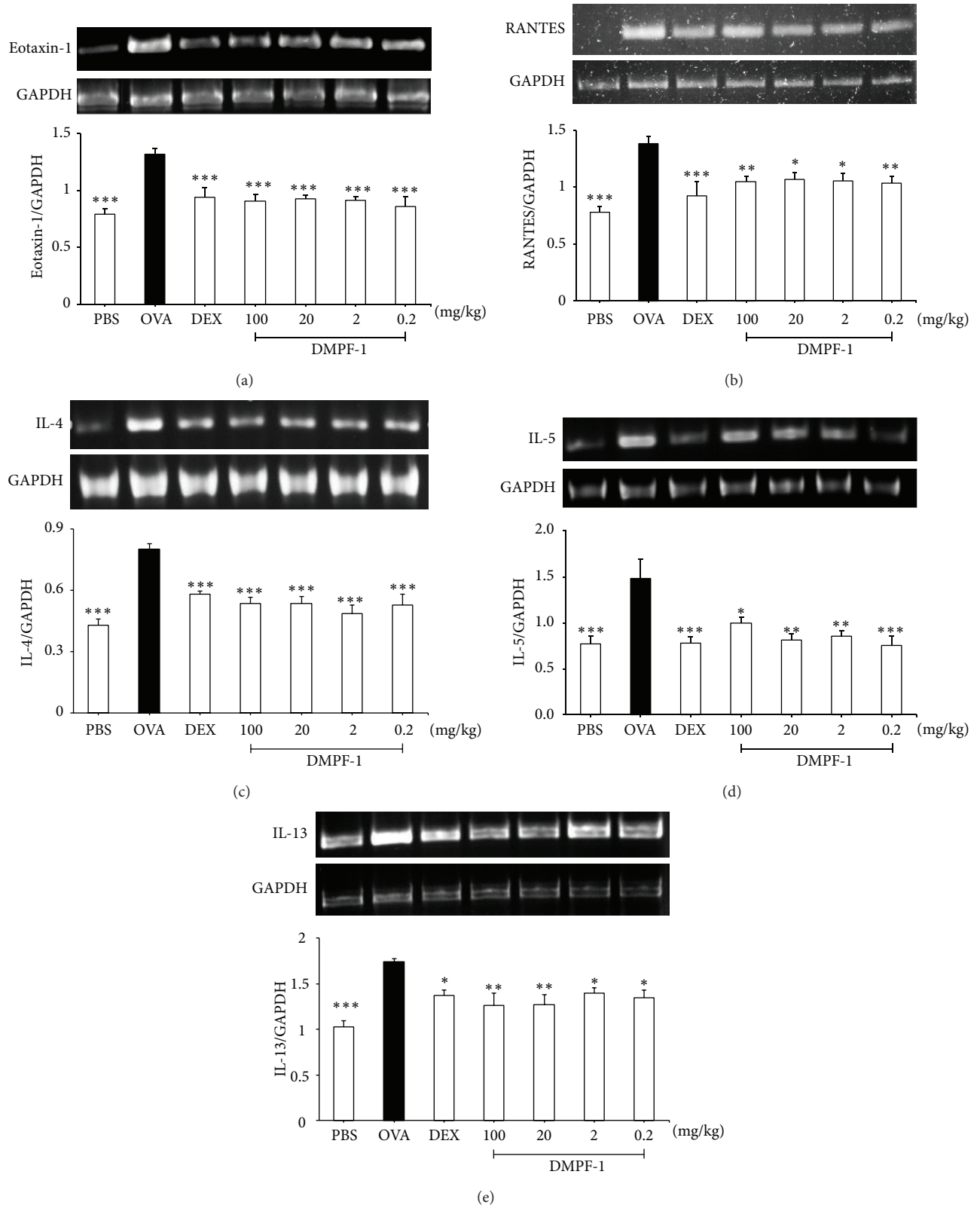

Figure 12: Effect of DMPF-1 on the mRNA level of chemokines and Th2 cytokines on OVA-sensitized mice. Expression levels of mRNA were determined by RT-PCR analysis. After the mice were sacrificed, the lung tissue was collected and the mRNA levels of (a) eotaxin-1, (b) RANTES, (c) IL-4, (d) IL-5, and (e) IL-13 were measured. The values are expressed as mean ± SEM ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$, significantly different from the OVA-challenged group.

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