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RESPIRATORY INFECTIONS

Reduced interferon- γ release in patients recovered from Legionnaires' disease

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Background: *Legionella pneumophila*, a Gram negative intracellular pathogen, causes Legionnaires' disease (LD). Interferon (IFN)- γ is important for host defence against *L pneumophila* so reduced IFN- γ production capacity and/or responsiveness might render humans more susceptible to infection with *L pneumophila*.

Methods: Seventy seven patients who suffered from LD after a point source outbreak one year earlier participated in the study. Whole blood was incubated with non-specific stimuli (lipopolysaccharide (LPS) or interleukin (IL)-12) or specific stimuli (viable or heat killed *L pneumophila*) to evaluate IFN- γ production, and with IFN- γ to evaluate IFN- γ responsiveness. Expression of complement receptor 3 on monocytes was determined by flow cytometry. Thirty seven companions who were also exposed but had not developed LD served as controls.

Results: Patients released less IFN- γ than controls in response to stimulation with LPS (mean (SE) 393 (58) pg/ml v 914 (178) pg/ml; $p=0.001$) and IL-12 (96 (14) pg/ml v 177 (41) pg/ml; $p=0.058$). IFN- γ responsiveness, measured by release of IFN- γ inducible protein (IP)-10, tumour necrosis factor α , IL-12 production capacity, and monocyte expression of complement receptor 3, did not differ between patients and controls. IFN- γ release after stimulation with LPS and IP-10 release after stimulation with IFN- γ were weakly associated with severity of LD in the former patient group ($p=-0.3$, $p=0.011$ and $p=-0.3$, $p=0.037$, respectively).

Conclusion: These results suggest that impaired IFN- γ production may contribute to susceptibility to *L pneumophila* infection.

Legionella pneumophila, a Gram negative facultative intracellular pathogen, is the causative agent of Legionnaires' disease (LD). *Legionella* spp infect humans via inhalation of contaminated aerosols from waterborne environmental sources. Once *L pneumophila* enters the respiratory tract, bacteria invade mononuclear cells by binding to complement receptors (CR) 1 and 3, and they replicate predominantly in alveolar mononuclear phagocytic cells (MPC).^{1,2}

Resistance to *L pneumophila* lung infection is dependent on the induction of cellular immunity and is mediated by cytokines such as interferon (IFN)- γ ,^{3,4} tumour necrosis factor (TNF)- α ,⁵ and interleukin 12 (IL-12).^{6,7} The T helper 1 (Th1) cytokine IFN- γ activates MPC to inhibit *L pneumophila* replication^{3,4} and TNF- α acts synergistically with IFN- γ to promote cellular defence.⁸ IL-12 is a pivotal denominator of the balance between Th1 and Th2 lymphocyte subsets as it drives naive T cells into a Th1 direction.⁹ IL-12 is critical for resolution of replicative *L pneumophila* lung infection in mice.^{6,10}

In retrospective studies human factors that increased susceptibility to *L pneumophila* infection were smoking, presence of chronic obstructive lung disease, and the use of immunosuppressive drugs,^{11,12} and high fatality rates up to 50% have been reported in immunocompromised patients.¹¹ Impairments in cytokine mediated immune responses leading to increased susceptibility to *L pneumophila* infection have not yet been studied. We therefore measured in vitro cytokine production after stimulation of whole blood with *L pneumophila*, lipopolysaccharide (LPS), or IL-12 in patients who had experienced an episode of LD.

METHODS

Patients and study design

Seventy seven patients who suffered from LD during an outbreak with *L pneumophila* serogroup 1 at a flower show in

the Netherlands¹³ participated in the study which was performed 1 year after the outbreak. LD was diagnosed when a patient who visited the flower show had symptoms compatible with pneumonia, radiological signs of infiltration, and laboratory evidence of infection with *Legionella*. In 67 patients the laboratory evidence included: (1) isolation of *L pneumophila* from a respiratory sample, or (2) a fourfold rise in antibodies or seroconversion to positive IgM and/or IgG antibodies to *L pneumophila* in acute phase and convalescent phase serum samples, or (3) detectable *Legionella* antigens in a urine sample (Binax Now; Binax, Portland, Maine, USA). In 10 patients laboratory evidence included a positive polymerase chain reaction (PCR) test on sputum or a single high antibody titre, or no laboratory evidence provided that no other microorganism was identified.¹³ Thirty seven companions of the patients who also visited the flower show but had not developed LD served as controls. Blood was obtained from a patient and a control subject on the same occasion.

Informed consent was obtained from all participants in the study which was approved by the medical ethics committee of the Academic Medical Centre, Amsterdam, the Netherlands.

Clinical data collection and definitions

Data on the following variables were collected from the medical chart and by interview: age, sex, smoking (≥ 1 cigarette per day), use of systemic immunosuppressive medication (ongoing treatment with chemotherapy or steroids >10 mg/day), and the presence of underlying diseases such as chronic obstructive pulmonary disease (COPD), diabetes mellitus, and cardiovascular disease (which was considered present if cardiac medication was used).

For the assessment of severity of pneumonia we constructed a 5 point scale (0–4) using the minor criteria for severe community acquired pneumonia described by the American Thoracic Society.¹⁴ The following items each yielded

1 point: (1) respiratory rate >30/min, (2) chest radiograph showing bilateral involvement or involvement of multiple lobes, (3) shock (systolic blood pressure below 90 mm Hg or diastolic blood pressure below 60 mm Hg), (4) arterial oxygen tension (P_{aO_2}) <8 kPa or arterial oxygen saturation (S_{aO_2}) <92%. Severe LD was defined as a score of 2 or more.

Bacterial preparation

L. pneumophila serogroup 1 isolated during the outbreak¹³ was used for whole blood stimulation. Bacteria were grown on buffered charcoal yeast extract agar supplemented with α -ketoglutarate (BCYE- α , Difco, Detroit, MI, USA) for 72 hours from a passage 1 stock maintained at -80°C . The bacteria were suspended in pyrogen free RPMI 1640 (Bio Whittaker, Verviers, Belgium) and the suspension was adjusted spectrophotometrically to a concentration of 1×10^8 CFU/ml. Bacteria were killed by heating the suspension at 80°C for 30 minutes.

Whole blood stimulation

Whole blood stimulation was performed as described previously.¹⁵ Briefly, heparinised blood from patients and controls was collected aseptically. Whole blood diluted 1:1 in pyrogen-free RPMI 1640 was stimulated for 24 hours at 37°C with 5×10^7 CFU/ml live *L. pneumophila*, 5×10^7 CFU/ml heat killed *L. pneumophila*, LPS (from *E. coli* serogroup O111:B4; Sigma, St Louis, MO; final concentration 10 ng/ml), recombinant human IL-12 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands; final concentration 100 ng/ml), or recombinant human IFN- γ (CLB, final concentration 5 ng/ml). After incubation the supernatant was obtained by centrifugation and stored at -20°C until assays were performed.

Assays

IFN- γ and TNF- α (both CLB; detection limits 2.0 and 1.4 pg/ml, respectively) and IFN- γ inducible protein-10 (IP-10), IL-12p70, and IL-12p40 (R&D Systems, Abingdon, UK; detection limits 20 pg/ml, 1.6 pg/ml and 16 pg/ml) were measured by ELISA according to the instructions of the manufacturer. Leucocyte and differential cell counts were measured in EDTA anticoagulated blood by a STKR Coulter counter (Bedfordshire, UK).

Flow cytometry

Expression of CD11b and CD18 (CR3) on circulating monocytes was determined in heparinised blood. All procedures were performed at 4°C . Erythrocytes were lysed with isotonic NH_4Cl solution (155 mmol/l NH_4Cl , 10 mmol/l KHCO_3 , 0.1 mmol/l EDTA, pH 7.4) for 10 minutes. Incubations for fluorescence activated cell sorter (FACS) analysis were performed in 96-well V-shaped microplates (Greiner BV, Alphen a/d Rijn, the Netherlands). For staining, 3×10^5 cells/well were incubated with the following mouse anti-human monoclonal antibodies: FITC labelled anti-CD18 and phycoerythrin labelled anti-CD11b (both Pharmingen, San Diego, CA, USA). All FACS reagents were used in concentrations as recommended by the manufacturer, and all analyses were also conducted with the appropriate isotype controls (Pharmingen). Cells were incubated on ice for 30 minutes and washed twice with cold FACS buffer (PBS supplemented with 0.5% w/v bovine serum albumin (BSA), 0.3 mM EDTA, and 0.01% w/v sodium azide) and re-suspended in FACS buffer. Monocyte analysis was done by forward scatter and side scatter gating. The results are expressed as the mean fluorescence intensity of gated cells.

CD4+ and CD8 + lymphocytes were measured by FACS analysis using mouse anti-human CD3, CD4, and CD8 monoclonal antibodies (CLB).

Table 1 Positive results of diagnostic tests for Legionnaires' disease (LD) in study patients (n=77)

Positive diagnostic test for LD	No of patients
Sputum culture	9
Fourfold rise in titre/seroconversion	33
Urinary antigen test	50
Polymerase chain reaction	3
Single high titre $\geq 1:256$	6
None	7

Statistical analysis

Values are given as mean (SE) unless otherwise indicated. Differences between patients and controls were analysed by the χ^2 test, Student's *t* test, or the Mann-Whitney U test. Spearman's ρ was used to determine correlation coefficients. A *p* value of <0.05 was considered to be statistically significant.

RESULTS

Patient characteristics

A total of 77 subjects were studied 1 year after their episode of LD. The median age was 66 years (range 46–88) and 62% were men. The diagnosis of LD was based on epidemiological, clinical, and diagnostic features (table 1). Twenty patients (26%) suffered from severe LD (severity score ≥ 2), of whom eight had required mechanical ventilation. Underlying diseases such as COPD, cardiovascular disease, and diabetes mellitus were present in 10%, 30%, and 9%, respectively, of the patients. Two patients used immunosuppressive medication at the time of this study and 44% were active smokers. None of the patients had a history of recurrent infections. The median age of the control subjects was 64 years (range 47–78) and 32% were men. All control subjects denied underlying diseases except one who had diabetes mellitus.

Cytokine release

The number of leucocytes, lymphocytes, and CD4+ and CD8+ T lymphocytes did not differ between former patients and control subjects (*t* test, all $p > 0.05$, data not shown). Whole blood stimulation with LPS and IL-12 resulted in a lower IFN- γ release in patients than in controls (393 (58) pg/ml in patients v 914 (178) pg/ml in controls ($p = 0.001$) after stimulation with LPS and 96 (14) pg/ml v 177 (41) pg/ml ($p = 0.058$) after stimulation with IL-12, fig 1). In contrast, no difference in IFN- γ response was found between patients and controls after stimulation with live bacteria (204 (81) pg/ml and 107 (19) pg/ml, respectively, $p = 0.65$) or with heat killed bacteria (104 (21) pg/ml and 66 (8) pg/ml, respectively, $p = 0.73$).

Since former patients and control subjects were not completely comparable with regard to sex, smoking habit, and underlying diseases, we determined whether IFN- γ release was influenced by these factors. A comparison was made of IFN- γ release in patients below and above the median age and between patients with or without underlying conditions (Mann-Whitney U test; data not shown). None of the factors significantly affected IFN- γ release. The only exception was that active smokers had a higher IFN- γ release than non-smokers after stimulation with dead bacteria but not after stimulation with live bacteria, LPS, or IL-12. Differences in IFN- γ release between former LD patients and control subjects are therefore not likely to have resulted from differences between the groups with respect to sex, smoking habit, or underlying diseases.

The production of IP-10 by various cell types is strongly dependent on IFN- γ and has been suggested to play a role in innate immunity. To examine IFN- γ responsiveness, IP-10 levels were measured after stimulation with IFN- γ . IP-10 release

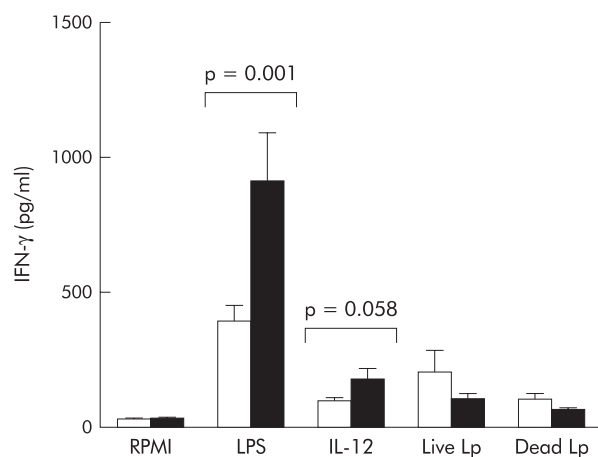


Figure 1 IFN- γ release after whole blood stimulation in former LD patients (open bars) and control subjects (solid bars). Whole blood was stimulated for 24 hours at 37°C with RPMI, *E coli* lipopolysaccharide (LPS, 10 ng/ml), IL-12 (100 ng/ml), and live and heat killed *L pneumophila* (5×10^7 CFU/ml). Data are mean (SE); p values by Mann-Whitney U test. Lp = *L pneumophila*.

was similar in patients (581 (83) pg/ml) and control subjects (541 (104) pg/ml, $p=0.41$), indicating that the response to IFN- γ was normal in both groups.

IFN- γ is predominantly produced by lymphocytes. To determine whether cytokine production by mononuclear cells is also decreased in former LD patients, TNF- α and IL-12 levels were measured. Stimulation with IFN- γ and IL-12 did not increase TNF- α release compared with incubation with RPMI alone (fig 2A). Stimulation with LPS and dead *L pneumophila* increased TNF- α levels in patients and control subjects to a similar extent (LPS: 1940 (156) pg/ml and 1434 (107) pg/ml, respectively ($p=0.23$); dead bacteria: 884 (95) pg/ml and 936 (96) pg/ml, respectively ($p=0.14$)). After stimulation with live *L pneumophila*, TNF- α production was significantly higher in patients than in controls (1463 (151) pg/ml and 962 (163) pg/ml, respectively, $p=0.028$).

IL-12p70, the active form of IL-12, is composed of a 40 kDa (p40) subunit and a 35 kDa (p35) subunit. IL-12p70 was undetectable after stimulation with all stimuli in both patients and controls (data not shown). The IL-12p40 subunit has no IL-12 activity but the dimer p40 has been shown to bind to the IL-12 receptor. After stimulation with LPS and viable or dead *L pneumophila*, IL-12p40 levels increased compared with RPMI incubation alone (fig 2B). However, the levels of IL-12p40 were similar in patients and control subjects (LPS: 615 (74) pg/ml and 595 (92) pg/ml, respectively ($p=0.87$); viable bacteria: 178 (27) pg/ml and 133 (35) pg/ml, respectively ($p=0.07$); dead bacteria: 192 (28) pg/ml and 273 (63) pg/ml, respectively ($p=0.33$)). IL-12p40 was undetectable after stimulation with IFN- γ .

IFN- γ release after stimulation with LPS and IP-10 release after stimulation with IFN- γ were weakly correlated with severity of pneumonia ($\rho=-0.3$, $p=0.011$ and $\rho=-0.3$, $p=0.037$, respectively). TNF- α release after stimulation with LPS was not significantly correlated with severity of pneumonia ($\rho=-0.17$, $p=0.14$).

Complement receptor 3 expression on monocytes

Thirteen representative patients and nine controls were randomly selected from the study group. Absolute number of monocytes were similar in patients and controls ($p=0.31$). No differences could be detected in CD18 and CD11b expression on monocytes between patients and controls (mean fluorescence CD18 538 (54) in patients ν 665 (106) in controls, $p=0.45$; mean fluorescence CD11b, 712 (81) ν 834 (117), $p=0.44$).

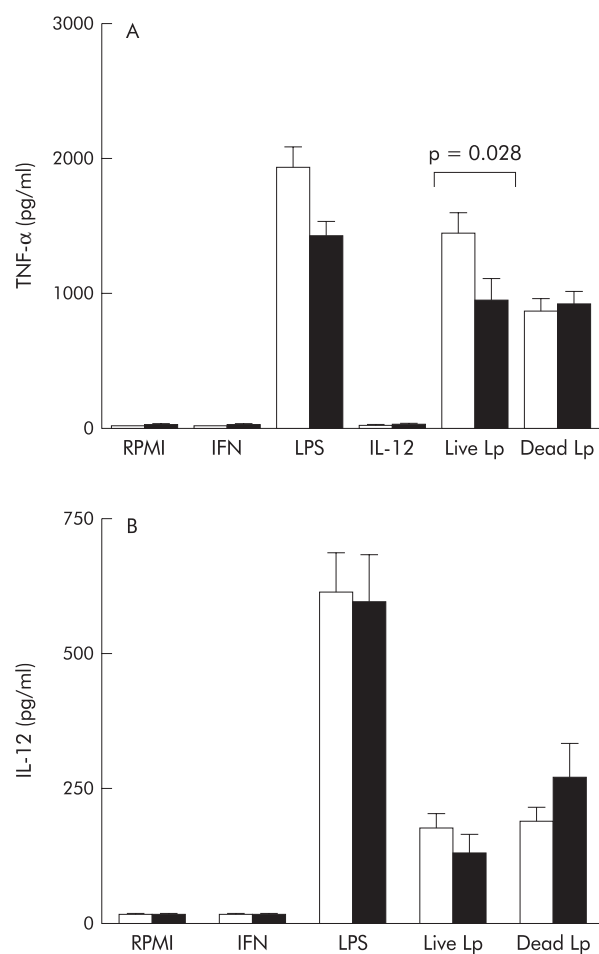


Figure 2 (A) TNF- α release and (B) IL-12p40 release after whole blood stimulation in former LD patients (open bars) and control subjects (solid bars). Whole blood was stimulated for 24 hours at 37°C with RPMI, IFN- γ (5 ng/ml), *E coli* lipopolysaccharide (LPS, 10 ng/ml), IL-12 (100 ng/ml), and live and heat killed *L pneumophila* (5×10^7 CFU/ml). Data are mean (SE); p values by Mann-Whitney U test. Lp = *L pneumophila*.

DISCUSSION

Cellular immunity is pivotal in host defence against *L pneumophila*. In vitro and in murine models a Th1 response, characterised by IFN- γ production, was found to be protective against replicative infection with *L pneumophila*.^{4 6 16 17} Although a relative predominance of Th1 type cytokines was seen in patients with active LD,¹⁸ interpretation of these data is hampered by the fact that, during acute infection, circulating levels of inflammatory mediators are usually high. To identify impairments in cytokine mediated immunity in patients with LD, we measured levels of IFN- γ , IP-10, and TNF- α after whole blood stimulation with LPS, IL-12, IFN- γ , and *L pneumophila* in patients who had LD a year earlier and in control subjects. IFN- γ levels were lower in former LD patients after incubation with LPS and IL-12. The response to IFN- γ , as measured by IP-10, as well as TNF- α production and IL-12p40 production was similar in patients and controls. Our data suggest that a decreased IFN- γ production capacity may be a risk factor for developing LD.

The control population consisted of partners or companions of the former LD patients who, together with the patients, had visited the flower show and thus were likely to have been exposed to *L pneumophila* to a similar extent. However, serological investigations within the control group were not performed. In a serological survey among more than 700 exhibitors who were present during every day of the flower

show, the prevalence of serologically positive subjects was only 12%.¹⁹ This suggests that a seronegative status does not exclude exposure.

The IFN- γ response to LPS is presumably indirect as a result of LPS induction of lymphocyte activating monokines such as IL-12 and TNF that cause lymphocytes and natural killer (NK) cells to release IFN- γ . The impaired IFN- γ response to exogenous IL-12 and the increase in TNF- α and IL-12p40 after stimulation with LPS argue against the possibility that impaired monokine release is the sole explanation for impaired IFN- γ release in former LD patients. The data are also consistent with a possible defect in lymphocyte IL-12 binding capacity (IL-12R). To confirm the finding that IFN- γ production in patients with LD is impaired in comparison with controls, it would be interesting to perform additional tests with different non-specific stimuli in a dose response experiment.

IP-10 is a CXC chemokine which binds to the CXCR3 receptor and specifically targets T lymphocytes and NK cells.²⁰ It is important in the innate immune response to bacterial infection, probably by attracting CXCR3 positive Th1 cells to the site of inflammation, and it is largely IFN- γ dependent.²¹ We found that IP-10 release after whole blood stimulation with IFN- γ was similar in patients and controls, suggesting that IFN- γ responsiveness is normal in former LD patients.

Phagocytosis of *L pneumophila* by mononuclear phagocytic cells is mediated by CR1 and CR3 which selectively bind to the major outer membrane of the *Legionella* bacterium.²² If bacteria are opsonised with complement C3 or antibodies, phagocytosis is enhanced but, in the absence of complement or antibodies, *L pneumophila* still binds to monocytes via CR1 and CR3.² Expression of CR3 on mononuclear cells from patients and controls was not different, suggesting that the extent of CR3 expression is not involved in the susceptibility to *L pneumophila*. However, other factors may be involved in phagocytosis of *L pneumophila*, since bacterial growth after complement-independent attachment has been observed in guinea pig alveolar macrophages and cell lines.²

Although former LD patients had less IFN- γ release after stimulation with LPS or IL-12 than controls, IFN- γ release was comparable after stimulation with live or heat killed *L pneumophila*. In addition, patients had a higher TNF- α response after stimulation with live *L pneumophila* than controls. This may be explained by a recall immune response. Mononuclear cells sensitised to *L pneumophila* have been shown to persist for up to 20 months after an episode of LD.²³ Supernatants from cultured monocytes of former patients stimulated with dead *L pneumophila* contained more monocyte activating cytokines and inhibited multiplication of *L pneumophila* significantly more than supernatants from monocytes from control subjects.²³ A memory response involving IFN- γ has also been described after mycobacterial infection or vaccination.²⁴ CD8+ lymphocytes from BCG vaccinated cattle produced more IFN- γ upon stimulation with BCG or PPD than lymphocytes from non-vaccinated cattle.²⁵ Thus, because of a recall response in former LD patients, higher levels of IFN- γ may be produced after stimulation with *L pneumophila* than after stimulation with non-specific stimuli such as LPS or IL-12 in comparison with controls. However, in this study we used whole blood for stimulation assays and we did not know about the presence of circulating antibodies to *L pneumophila* in patients and controls 1 year after exposure. This means that we cannot exclude the possibility that antibodies to *Legionella* antigens interfere with the outcome. It is not clear whether a memory response protects against recurrent disease with *L pneumophila* but, to our knowledge, no recurrence of LD has been described in immunocompetent individuals.

The inverse weak correlation between release of IFN- γ or IP-10 and the severity of LD is consistent with the idea that lower IFN- γ responses may be associated with worse LD. It has been shown that IFN- γ mediated inhibition of in vitro intracellular multiplication of *L pneumophila* is dose

dependent.³ Impaired IFN- γ production capacity after a non-specific stimulus such as LPS now and *L pneumophila* at the time of infection may therefore not only increase the susceptibility to development of LD, but may also influence the outcome.

IFN- γ plays a pivotal role in the host defence against LD. We have shown that former LD patients have a reduced capacity to produce IFN- γ on stimulation with LPS or IL-12 compared with control subjects who were also exposed to *L pneumophila* but did not develop the disease. These data suggest that an impaired capacity to produce IFN- γ may contribute to susceptibility to infection with *L pneumophila*.

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