

# Pollution of mycological surfaces in hospital emergency departments correlates positively with blood NKT CD3<sup>+</sup> 16<sup>+</sup> 56<sup>+</sup> and negatively with CD4<sup>+</sup> cell levels of their staff

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## Abstract

The aim of the present study was the assessment of the putative influence of yeast and filamentous fungi in healthcare and control (office) workplaces (10 of each kind) on immune system competence measured by NK (natural killer), CD4<sup>+</sup>, and NKT (natural killer T lymphocyte) cell levels in the blood of the personnel employed at these workplaces.

Imprints from floors and walls were collected in winter. The blood was taken in spring the following year, from 40 men, 26 to 53 years old, healthcare workers of hospital emergency departments (HED), who had been working for at least five years in their current positions, and from 36 corresponding controls, working in control offices. Evaluation of blood leukocyte subpopulations was done by flow cytometry. The qualitative analysis of the surface samples revealed a prevalence of strains belonging to *Aspergillus* spp. and *Penicillium* spp. genus. There was no statistically significant difference between the level of NKT; however, the percentage of NK cells was lower in the blood of HED workers than in the blood of offices personnel. Spearman analysis revealed the existence of positive correlation ( $r = 0.4677$ ,  $p = 0.002$ ) between the total CFU/25 cm<sup>2</sup> obtained by imprinting method from walls and floors of HED and the percentage of NKT (CD3<sup>+</sup>16<sup>+</sup>56<sup>+</sup>) lymphocytes collected from the blood of their personnel, and negative correlation ( $r = -0.3688$ ,  $p = 0.019$ ) between this parameter of fungal pollution and the percentage of CD4<sup>+</sup> lymphocytes in the blood of HED staff. No other correlations were found.

**Key words:** fungi, emergency departments, healthcare workers, offices staff, NKT blood lymphocytes, NK cells, CD4 lymphocytes.

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## Introduction

This is the second part of our studies on the immunological status of medical emergency workers exposed for many years to hazardous biological agents and on the immune system of their corresponding controls. Previously, we reported the existence of a negative correlation between the number of phagocytising monocytes in the blood of hospital emergency departments (HED) staff and the number of fungi (colony-forming units – CFU) collected by imprinting method from the surfaces. Surprisingly, the similar study performed in control offices and their staff

revealed positive correlation between above parameters [1]. It was difficult to explain this discrepancy. Persons from both examined groups had been working for at least five years at the present location and, at least at the moment of comparative sampling, mycological pollution of surfaces was similar and generally low. Moreover, the number of phagocytising monocytes in the blood of personnel did not differ between HED and office locations. However, the obtained results might suggest the existence of some intrinsic disturbances in innate defence cell subsets.

In this paper we present the results of the examination of natural killer (NK) cell CD4<sup>+</sup> and natural killer T lymphocyte

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(NKT) levels in the blood of these HED workers and their corresponding controls from control offices. NK and NKT lymphocytes belong to the group of cells participating in the mechanisms of innate cell-mediated immunity, together with granulocytes, monocytes, macrophages, and dendritic cells. Cells from this group recognise microbial patterns without prior sensitization and react rapidly to the pathogen. Natural killer cells are cytotoxic against neoplastic and pathogen-infected host cells [2]. In response to stimuli, they produce interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and also act directly against some extracellular pathogens.

Natural killer T cells are innate-like T lymphocytes restricted by the CD1d molecule that presents self and exogenous glycolipids. NKT after activation can immediately secrete a high amount of IFN- $\gamma$  and other cytokines and are able to activate NK cells, T cells, B cells, and dendritic cells (DCs). They are possibly responsible for induction or activation of innate as well as adaptive immunity and play an important role in protecting the host against infectious pathogens [2-4].

### Material and methods

The study of mycological contamination was conducted as previously described [1]. Briefly, the samples were collected from 10 Warsaw hospital emergency departments (HED) and in 10 control locations (office spaces), and included imprints of floors and walls. 90 samples were collected from 10 hospitals, and 90 samples from 10 control offices. In the imprinted method a special applicator with media (Count Tack CT SI – Biomerieux) was dedicated for fungi. These samples were taken from the flat surfaces of walls and floors. Next the biological material was put into a plastic container and transported to the microbiological laboratory.

Environmental samples culture and identification of fungi were performed as described previously [1].

Blood for immunological investigation was taken from 40 men, 26 to 53 years old, health care workers of HED departments, who had been working for at least five years in their current positions and from 36 corresponding controls, who worked in control offices, without a history of systemic, inflammatory, or immunological diseases. Evaluation of blood leukocyte subpopulations was done by haematological analyser and cytometry analysis.

Informed consent from study participants and permission from the Local Ethical Committee were obtained.

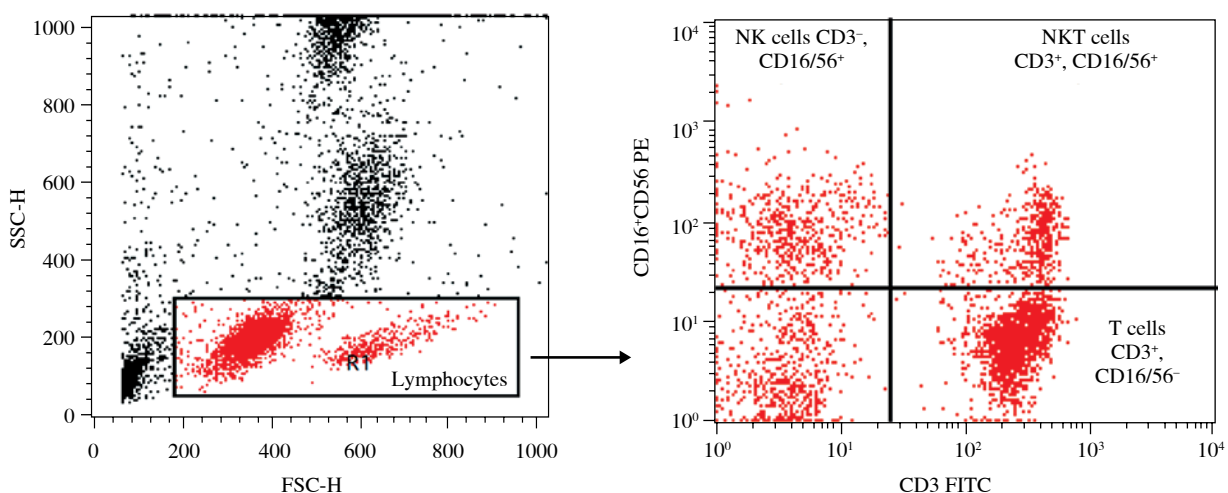
The total number of white blood cells was measured in EDTA blood samples using a haematological analyser in accordance with the manufacturer’s protocol (Exigo Vet, Boule Medical AB, Sweden). Subsequently, the cytometry assay was performed.

For examination of NK, NKT, and CD4 subsets, samples were labelled with CD3 FITC and CD16/56 PE or CD4 FITC antibodies (IMK Test, BD Biosciences) as previously described [5]. The samples were acquired by flow cytometry (FACS Calibur) and analysed in CellQuest Software (BD Biosciences). The NK, NKT, and CD4<sup>+</sup> results were presented as percentage of lymphocytes population (R1). Typical analysis of NK/NKT cell populations is shown in Fig. 1.

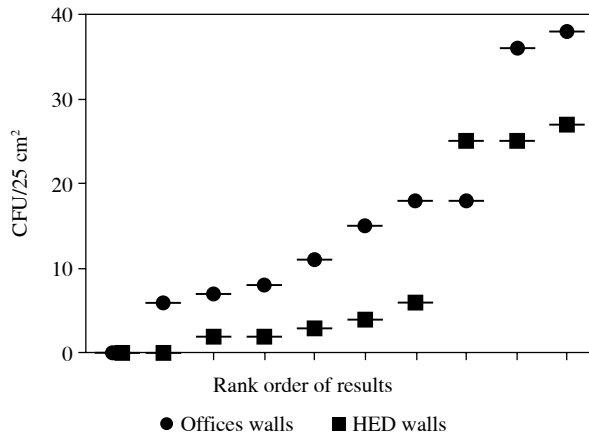
Statistical evaluation of the results was done by column statistics, Spearman correlation analysis, and Mann-Whitney test (GraphPadPrism).

### Results

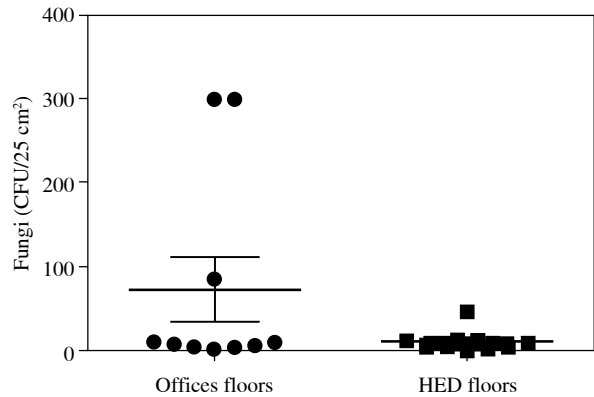
**Fungi examination.** The results of fungi quantitative analysis collected by imprint method from walls and floors are presented in Fig. 2 (walls) and Fig. 3 (floors). No statistically significant differences were observed between the values obtained for HED and office surfaces (Table 1 and 2).



**Fig. 1.** Typical dot plots of NK/NKT flow cytometry evaluation generated in CellQuest Software (BD Biosciences)



**Fig. 2.** Mycological pollution of walls in 10 control offices and in 10 hospital emergency departments



**Fig. 3.** Mycological pollution of floors of 10 control offices and 10 hospital emergency departments

**Table 1.** Statistical analysis of walls mycological pollution (CFU/25 cm<sup>2</sup>)

	Offices	HED
Number of values	10	10
Minimum	0.0	0.0
25% Percentile	6.750	1.500
Median	13.00	3.500
75% Percentile	22.50	25.00
Maximum	38.00	27.00
Mean	15.70	9.400
Std. Deviation	12.55	11.37
Std. Error	3.969	3.597
Lower 95% CI of mean	6.720	1.263
Upper 95% CI of mean	24.68	17.54
Sum	157.0	94.00
Mann-Whitney test		
<i>p</i> -value		0.1500
Exact or approximate <i>p</i> -value?	Gaussian approximation	
<i>p</i> -value summary		NS
Are medians significantly different? ( <i>p</i> < 0.05)		no
One- or two-tailed <i>P</i> value?		two-tailed
Sum of ranks in column A, B		124.5, 85.50
Mann-Whitney <i>U</i>		30.50

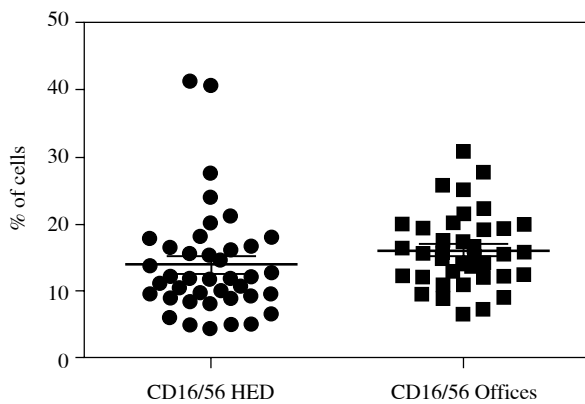
**Table 2.** Statistical analysis of floors mycological pollution (CFU/25 cm<sup>2</sup>)

	Offices	HED
Number of values	10	10
Minimum	2.000	0.0
25% Percentile	4.750	3.750
Median	8.500	6.500
75% Percentile	139.5	12.00
Maximum	300.0	45.00
Mean	73.20	10.60
Std. Deviation	122.1	12.77
Std. Error	38.61	4.039
Lower 95% CI of mean	-14.15	1.463
Upper 95% CI of mean	160.5	19.74
Sum	732.0	106.0
Mann-Whitney test		
<i>p</i> -value		0.4715
Exact or approximate <i>p</i> -value?	Gaussian approximation	
<i>p</i> -value summary		NS
Are medians significantly different? ( <i>p</i> < 0.05)		no
One- or two-tailed <i>p</i> -value?		two-tailed
Sum of ranks in column A, B		115.95
Mann-Whitney <i>U</i>		40.00

The qualitative analysis of the surfaces and air samples revealed a prevalence of genus *Aspergillus* spp. and *Penicillium* spp. The most common species were: *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium expansum*, and *Trichoderma koningii*. In two samples, one collected from a hospital emergency department and the other from a control location, *Aspergillus fumigatus* was found. One emergency department was a source of samples containing *Aspergillus flavus*. Furthermore, various species belonging to the *Candida* spp. were found in hospital emergency rooms as well as *Aspergillus niger*.

### Immunological study

As was described previously [1], there were no statistical differences between emergency and office workers with respect to age, leukocytes number, and percentage of phagocytising granulocytes and monocytes. Presently, we found lower numbers of NK (CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup>) lymphocytes in the blood of HED workers (median value 11.87) than in the blood of control office staff (median value 15.49) (Fig. 4). No difference was observed between the percentage of NKT (CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>) cells in the blood of HED workers (median value 3.87%) and respective value of control office staff (4.26%).



Mann-Whitney test	
p-value	0.0209
Exact or approximate p-value?	Gaussian approximation
p-value summary	*
Are medians significant different? (p < 0.05)	yes
One- or two-tailed p-value?	two-tailed
Sum of ranks in column A, B	1318, 1609
Mann-Whitney U	497.5

**Fig. 4.** Lower number of NK cells in the blood of hospital emergency departments workers than in the blood of control offices personnel

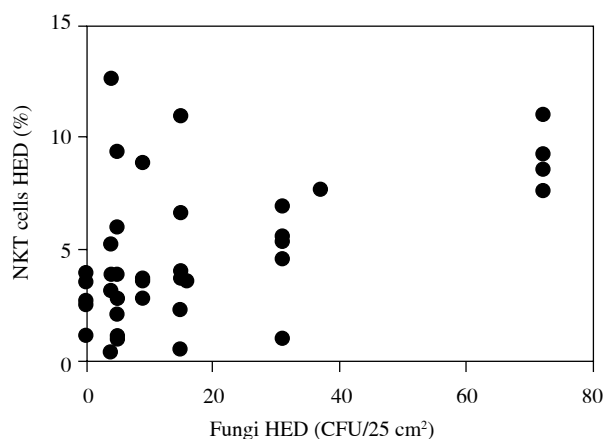
Spearman correlation analysis revealed significant positive correlation ( $r = 0.4677$ ;  $p = 0.002$ ) between the percentage of NKT cells in the blood of HED workers and the number of CFU/25cm<sup>2</sup> obtained from the walls and floors of hospital emergency departments (Fig. 5). No correlation was found in the case of control offices (Fig. 6) and their staff ( $r = -0.0251$ ;  $p = 0.8841$ ). Negative correlation was found between total number of fungi on surfaces in HED and % of CD4<sup>+</sup> lymphocytes in the blood of health care workers ( $r = -0.3688$ ;  $p = 0.019$ , Fig. 7) and no such correlation in office workers (Fig. 8).

### Discussion

Disruptions in immune system response may result in severe diseases, i.e. autoimmune diseases, hypersensitivity syndromes, or cancer. Such disruption may be a result of malnutrition [6], drug action [7], or chronic inflammatory conditions caused by microorganisms [8]. It is well known how the human immune system recognises and combats bacterial and fungal infection. However, there is still little information about the long-term effect of fungi exposure on the function and reactivity of the immune system. Pioneering experiments by Mierzwinska *et al.* revealed impairment of cell-mediated immune responses in patients with fungal prosthetic stomatopathies during long-term oral candidiasis. In these patients eradication of *Candida* infection resulted in normalisation of immunological cellular responses [9, 10].

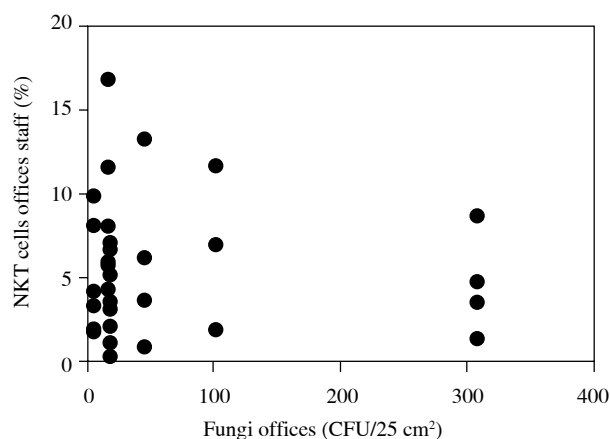
Fungi are a heterogeneous group of microorganisms. They are able to colonise both internal and external environments of the human body. The range of immune interactions with fungi is not fully recognised and is multidirectional. More than 400 mould species live in indoor environments [11]. Many of them are pathogenic in humans. *Penicillium*, *Aspergillus*, and *Candida* species secrete proteases disturbing anti-fungal immunity as well as immunotoxic and cancerogenic mycotoxins [12-17]. In mycoses nonspecific innate and acquired immunity play important roles in host defence. It involves neutrophils, monocytes, macrophages, dendritic and mast cells, as well as lymphocytes [18]. NK and NKT cells play a specific and exceptional role in this process.

NK cells participate in anti-mycotic response by direct or indirect mechanism. NK cells are able to induce apoptosis by Fas ligand or tumour necrosis factor ligands. Moreover, NK cells may release from granule content constitutively expressed proteins such as perforin, granzymes and granulysin. The released proteins can perforate the cell membrane (which leads to water influx and cell lysis) and trigger apoptosis [19]. NK cells may damage fungal cells also by a direct mechanism. They are able to produce several important cytokines, i.e. GM-CSF, RANTES, or IFN- $\gamma$ , which enhances migration, adherence phagocytosis of neutrophils and macrophages, maturation of dendritic cells, and proliferation of T-cell [20]. Addi-



Spearman $r$	0.4677
95% confidence interval	0.1778 to 0.6829
$p$ -value (two-tailed)	0.0020
$p$ -value summary	**
Exact or approximate $p$ -value?	Gaussian approximation
Is the correlation significant ( $\alpha = 0.05$ )?	yes

**Fig. 5.** Positive correlation between the number of fungi collected by imprinting method from walls and floors of hospital emergency departments and the level of NKT lymphocytes in the blood of HED workers



Spearman $r$	-0.02518
95% confidence interval	-0.3597 to 0.3151
$p$ -value (two-tailed)	0.8841
$p$ -value summary	NS
Exact or approximate $p$ -value?	Gaussian approximation
Is the correlation significant ( $\alpha = 0.05$ )?	no

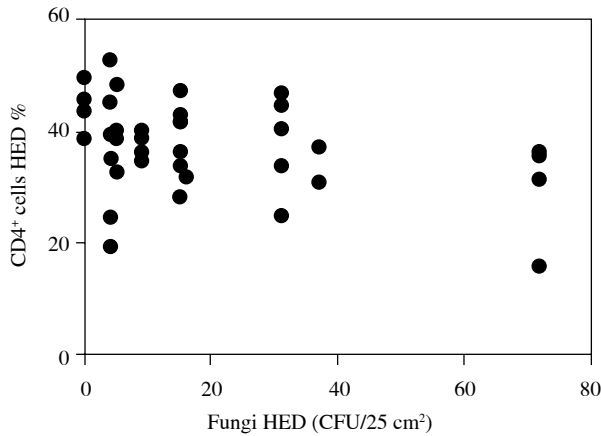
**Fig. 6.** No correlation between the number of fungi collected by imprinting method from walls and floors of offices spaces and NKT level in the blood of their workers

tionally, NK cells may induce CD4<sup>+</sup> T-cell response by MHC II class activation [21]. Fungi are able to disturb the response of immune system cells mainly by modulation of the regulatory network. This is connected with mycotoxin activity, which can inhibit phagocytosis and ROS produce in neutrophils, increase apoptosis in monocytes, and attenuate T-cell response [22]. It has been demonstrated that the level of NK cells plays an important role in fungal infection. Fungal mycotoxin may impair NK functionality. It has been presented that mycotic infection (i.e. *Aspergillus fumigatus* or *Candida albicans*) downregulates level of INF- $\gamma$ , GM-CSF, and TNF- $\alpha$  produced by NK cells [20]. In the present work we did not study the functionality of NK cells isolated from HED and office staff; however to fully understand the effect of long-time exposure on mycotic pollution this study should be made.

Studies pertaining to immune response to fungi in residential houses have shown increases in lymphocyte B and T levels (including CD4) with associated decreases in NK levels in exposed residents [23, 24]. The role of NKT lymphocytes in anti-tumour immune defence is widely known. Except for secretion cytokines characteristic for Th2 in humoral defence or INF- $\gamma$  and TNF, they show direct cytolytic activity eliminating cancer target cells [25-27]. In

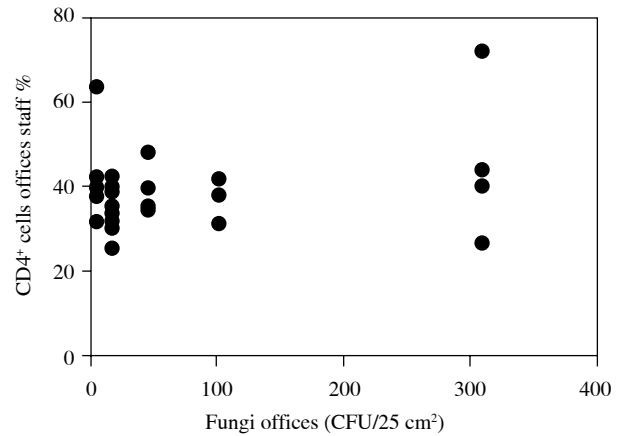
the present work we examined the effect of mycological pollution in work areas on the level of NK cells in the blood of their staff. The study was conducted in 10 Warsaw hospital emergency departments (potentially with increased levels of dangerous pathogens) and in 10 control offices. The analysis of general fungal pollution did not reveal significant differences between colony-forming unit numbers in HED and control offices. These fungi were able to produce mycotoxins floating in the air, which may affect immune response in exposed personnel. Interestingly, despite the similar fungal contamination in control (offices) and study (hospital) environments, we found a positive correlation between the fungal contamination in the study environment (hospital) and NKT levels in the study group (rescue personnel) (Fig. 5), while there was no such correlation in the control group (Fig. 6). It was also shown that levels of NK cells were lower in the study group than in the control group (Fig. 4). We noticed a negative correlation between CD4 lymphocyte levels in hospital personnel and fungal contamination levels but no such relation in the control group (Figs. 7 and 8).

CD4 lymphocytes play a key role in antifungal protection [28]. Differentiation of naive CD4 to Th1 and Th17 strengthen this response. CD4 lymphocytes take an active



Spearman <i>r</i>	-0.3688
95% confidence interval	-0.6162 to 0.05514
<i>p</i> -value (two-tailed)	0.0192
<i>p</i> -value summary	*
Exact or approximate <i>p</i> -value?	Gaussian approximation
Is the correlation significant ( $\alpha = 0.05$ )?	yes

**Fig. 7.** Negative correlation between the number of fungi collected by imprinting method from walls and floors of hospital emergency departments and the percentage of CD4<sup>+</sup> lymphocytes in the blood of HED workers



Spearman <i>r</i>	-0.08700
95% confidence interval	-0.2582 to 0.4125
<i>p</i> -value (two-tailed)	0.6139
<i>p</i> -value summary	NS
Exact or approximate <i>p</i> -value?	Gaussian approximation
Is the correlation significant ( $\alpha = 0.05$ )?	no

**Fig. 8.** Lack of correlation between the number of fungi collected by imprinting method from walls and floors of offices and the percentage of CD4<sup>+</sup> lymphocytes in the blood of offices staff

part in induction of allergic reaction by fungi Helper T lymphocytes are engaged in cell-type reaction to massive and long-term exposures to fungi. Many fungi are intracellular pathogens and trigger cell-type response (Th1-associated). On the other hand, immune response insufficiency with relevant decrease in CD4 count may cause susceptibility to fungal infection, e.g. in AIDS patients. Th1-associated response plays a pivotal role in fungal infection. Th1 lymphocytes secrete INF- $\gamma$  that activates phagocytes (neutrophils, macrophages) [29]. One could expect an increase in CD4<sup>+</sup> count after fungal exposure or at least its positive correlation with CD4<sup>+</sup>. A possible explanation of our findings is that exposure to pathogenic fungi together with other unidentified factors, prolonged for many years, may change this trend. Our observations may be partly dependent on other confounding variable modifying rescuers' immune response, altering the response to protracted fungal exposure. Taking into consideration the specificity of the life-saver's job, the authors suspect the influence of chronic stress and fatigue.

*The authors declare no conflict of interest.*

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