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Persistent NK cell deficiency associated with pulmonary cryptococcosis

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Abstract
We describe pulmonary cryptococcosis in a 28-year-old previously healthy man. Exhaustive immunological investigations revealed a primary NK cell deficiency associated with a secondary impaired anti-*Cryptococcus* CD8 lymphocyte response and the expansion of a CD8Vβ14+T cell clone. This case illustrates the potential role of NK cells in immunity against *Cryptococcus*.

Keywords NK cells, NK cell deficiency, *Cryptococcus*, CD8 clonotype, Vβ14 repertoire

Introduction

Cryptococcus neoformans is an encapsulated fungus in the soil that is responsible for worldwide opportunistic infections that are uniformly fatal unless treated. Cryptococcosis causes more than 112,000 deaths annually, mainly in patients with acquired immunodeficiency syndrome (AIDS) [1]. Other causative immunodeficiencies include solid organ transplantation, hematological malignancies, CD4 lymphopenia, prolonged immunosuppressive therapy, and defined immunodeficiencies [2]. Immunocompetent patients may also be affected, though recent studies have begun to identify underlying immunodeficiencies, especially antibodies to GM-CSF or other defined inborn errors of immunity [2]. Natural killer (NK) cells are nonB- nonT-lymphocytes (Lc) known to participate in the body's defense against viruses and tumor cells, and human NK cell deficiency (NKD) is usually associated with viral infections, including *Herpesviridae* [3]. NK cells exist at the interface of innate and adaptive immunity and directly kill pathogens or tumoral cells, but are also essential to shaping adaptive immunity. While it is well known that NK cells can kill tumor or viral-infected cells, they have also been reported to play a role in the direct killing of fungi [4, 5], including *Cryptococcus sp* [6–8]. NK cells are restrained by Major Histocompatibility Complex (MHC) class I antigens (negative receptors) and triggered by many activation receptors. NK cells are responsible for the direct killing of *Cryptococcus*, and the receptors mediating this anti-cryptococcal activity are the activating receptor NKp30 and β1-integrin [9, 10]. More recently, it was shown that NKG2D, a central activating receptor, was involved in target recognition and killing of fungi, including *Cryptococcus* [11]. It is well established that mice with NK cell defects are susceptible to *Cryptococcus* [12, 13]. NK cells interact with the adaptive immune system, involving direct and indirect mechanisms [14, 15], including controlling T-cell clonal expansion after antigen stimulation. Human NK cell deficiency (NKD) is a very rare deficiency, which provides an important

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view to understanding the role of NK cells in humans. We describe, to our knowledge, the first case of pulmonary cryptococcosis in a 28-year-old male patient with a previously unknown NKD. The patient's innate NKD was associated with two other secondary immunological dysfunctions: a CD8 Vβ14T-cell expansion and an in vitro impaired anti-*Cryptococcus* CD8 response.

Material & methods

Lymphocyte immunophenotyping and NK cell enumeration

Lymphocyte phenotypes were performed on fresh whole-blood samples with the BD Multitest TBNK 6 C reagent. NK cells were defined as $CD3^{-}CD56^{+}$ cells.

NK cell perforin expression

Whole blood was incubated for 30 min with CD45, CD3, and CD56. Red blood cells were lysed, fixed, and permeabilized. Cells were incubated at + 4 °C for 30 min with PE-conjugated anti-perforin (IgG2b, clone DG9) or corresponding isotype control. After 2 washes, the cells were analyzed on a BD FACSCantoII cytometer.

NK cell degranulation and IFN-g production by NK and T cells

The study was performed using peripheral blood mononuclear cells (PBMCs). We monitored NK cell activation by assessing the ability of these cells to degranulate and produce IFN-g in response to several stimuli: major histocompatibility complex class I-negative human erythroleukemic K562 target cells (natural cytotoxicity) and mouse mastocytoma P815 cells coated with rabbit anti-mouse lymphocyte antibodies (Accurate Biochemicals, Westbury, NY; antibody-dependent cell cytotoxicity [ADCC]). Cell lines K562 and P815 were obtained from the American Type Culture Collection (Manassas, VA).

A total of 5×10^5 PBMCs per well were distributed in U-bottom 96-well plates and cultured in media alone or stimulated with target cells (2.5×10^5 per well) in the presence of Golgi Stop (1/1500; Becton Dickinson) and FITC-conjugated anti-CD107 monoclonal antibodies (anti-CD107a [IgG1, H4A3], anti-CD107b [IgG1, H4B4]; Becton Dickinson) for 4 h. Cells were then washed in phosphate-buffered saline supplemented with 2% fetal calf serum and 1 mM of EDTA and stained for 30 min at 4 °C with PerCP-Cy5.5–conjugated anti-CD3 antibody (BD), APC-conjugated anti-CD56 antibody (IgG1 N90; Beckman Coulter), and 2% normal mouse serum (Sigma Aldrich).

After 2% paraformaldehyde fixation and permeabilization, IFN-g production by activated NK cells was detected by incubation with PE-conjugated anti–IFN-g antibody (IgG1, 4 S-B3; BD) for 30 min at 4 °C. For CD107 and IFN-g, results were expressed as the percentage of the whole NK cell population displaying positive staining.

T cell proliferation

CFSE-stained PBMCs were cultured for either 4 days (mitogen activation) or 7 days (antigen activation). Proliferation was defined as the level of CFSE dilution within the T cell gate after stimulation. Mitogen concentration was 10 µg/ml PHA (Oxoid). Antigen concentrations were 10 µg/ml tetanus toxoid (Statens, Denmark) and 10 µg/ml cryptococcus antigen. CFSE dilution was quantified by using a BD FACSCanto II cytometer.

TCR vbeta repertoire analysis

The Vbeta repertoire was determined using **the** IOTest Beta Mark Kit by flow cytometry according to the manufacturer's instructions (Beckman Coulter).

Case report

A 28-year-old male patient was admitted in December 2008 for a nonproductive cough and non-calcified nodules found on chest X-ray and chest computed tomography (Fig. 1). The patient had no previous medical history of note. There was no peculiar exposure to pigeon droppings or soil contact. He presented without a fever, and the physical examination was normal. The standard biological results were within the normal ranges, notably a normal leukocyte count (including the monocyte count) and a C-reactive protein level of 2 mg/L. Bronchoscopy did not reveal a tumor, but the culture of bronchoalveolar lavage revealed a heavy growth of *C. neoformans.* The organisms were characterized by immunofluorescence *as C. neoformans var. grubii* (serotype A) at the National Reference Center Mycoses Invasives et Antifongiques, Institut Pasteur, Paris, France. Cryptococcal antigenemia, as well as fungal cultures, were negative in cerebrospinal fluid, blood, and urine; however, the clinical and radiological presentation and positive fungal cultures indicated a diagnosis of pulmonary cryptococcosis in an immunocompetent or mildly immunocompromised patient [16]. The patient was successfully treated with fluconazole 400 mg daily for 12 months, the clinical and radiographic abnormalities resolved (Fig. 1), and he remained clinically healthy for more than 10 years after the initial diagnosis without relapse or new infection.

Immune system evaluation

The patient was HIV-negative. Serology for toxoplasmosis, Epstein–Barr virus, *Herpesvirus* 6, and the *Varicella-Zoster* virus revealed past infections. Plasma protein electrophoresis immunoglobulin levels and complement assay results were normal. The patient had a normal response to the tetani vaccination [17]. The patient's

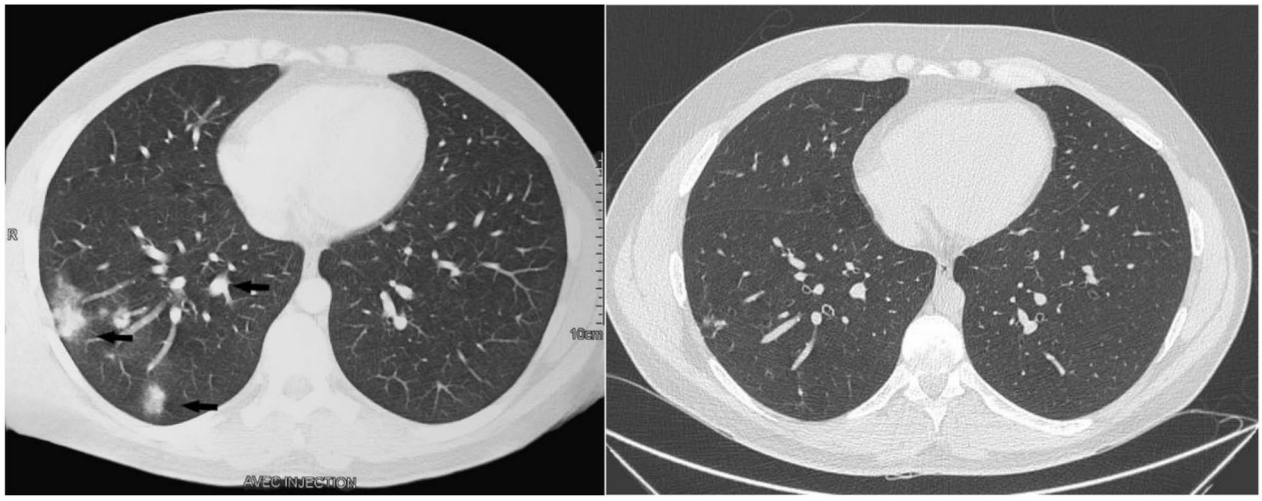

Fig. 1 Images of pulmonary nodules (arrows) related to *Cryptococcus* infection on chest computed tomography in 2009 (left). Regression of lesions 2 years later (right)

peripheral blood mononuclear cells (PBMCs) expressed normal pro-inflammatory cytokine synthesis (interleukin (IL)-1β, IL-6, or tumor necrosis factor α) in response to stimulation with lipopolysaccharide (LPS) or (IL-10 or IFN-γ) in response to phytohemagglutinin (PHA).

Lymphocyte immunophenotyping

The absolute lymphocyte count was 2,200 cells/mm^3, CD19$^+$ B cells were 176 cells/mm^3 (8%), CD3$^-$ CD56$^+$ NK cells were 7 cells/mm^3 (0.3%), and CD3$^+$ T cells were 1,943 cells/mm^3 (88%). Among the latter were 930 cells/mm^3 CD4$^+$ T cells and 971 cells/mm^3 CD8$^+$ T cells. No CD8 or CD4 T cell lymphopenia was detected. From 2009 to 2018, NK cells ranged between 7 and 33 cells/mm^3 (0.3–1.7%) (Supplementary Fig. 1A).

NK cell phenotype and functions

Two predominant NK cell subsets have been described according to the expression of CD56: a CD56^{bright} and a CD56^{dim}, respectively. The CD56^{dim} subset is the more mature subset that expresses high levels of CD16 and perforin. The patient's NK cell deficiency impacted the CD56^{dim} subset more than the CD56^{bright} subset (Supplementary Fig. 1 B 1 C). The patient's NK cells had normal activating receptor expression, including normal NKp30 expression (Supplementary Fig. 2). The perforin expression was normal, as were NK cell degranulation and interferon (IFN)-γ production in response to several stimuli [18] (Supplementary Fig. 3). These results suggest the existence of functional residual NK cells, albeit reduced in number.

In vitro lymphocyte proliferation assay using either PHA mitogen or antigens.

The patient's CD3 + T lymphocytes did not proliferate after incubation with 10 µg/mL of *Cryptococcus* antigen but did proliferate after PHA (Supplementary Figs. 4 and 5) or a tetanus protein challenge.

TCR Vβ repertoire analysis

Analysis of the TCR Vβ repertoire revealed a large subset of Vβ14 CD3$^+$CD8$^+$ lymphocytes, accounting initially for 52.4% of total circulating CD8 cells (Supplementary Fig. 6). This expanded CD8 clonotype was present during the follow-up and ranged from 52.4% in 2009 to 20% of total CD3$^+$CD8$^+$ lymphocytes in 2019 (Supplementary Fig. 7). In vitro stimulation of the VB14 CD8$^+$ clonotype for seven days with 10 µg/mL of *Cryptococcus* antigen did not induce lymphocyte proliferation (Supplementary Fig. 8).

Genetics

Whole-exome sequencing of the patient, his parents and his two siblings failed to identify pathogenic variants segregating with the disease.

Discussion

We describe a proven human case of cryptococcosis in a patient with an isolated NKD. The patient's clinical and radiological presentation was compatible with pulmonary cryptococcosis for an immunocompetent or mildly immunocompromised patient. Radiological manifestations of pulmonary cryptococcosis may include a lung mass (ranging from a simple nodule to a large cavitary mass) to multiple pulmonary nodules, as in our patient, to an interstitial process indicating widespread disseminated infection. The spectrum of clinical presentations is broad and depends on the host's immune status. Most frequent are cough, chest pain, sputum production and fever, although asymptomatic cases are described [2, 16].

We investigated the patient's anti-*Cryptococcus* defenses and demonstrated the persistence of a very low but functional NK compartment associated with a secondary impaired adaptive anticryptococcal CD8 + T cell immunity and the presence of a huge Vβ14 CD8 clonotype. Cryptococcosis has not been described among patients with isolated NKD. However, NKD and cryptococcosis have been described during more complex deficiencies such as AIDS [9], idiopathic CD4 + T-lymphocytopenia [19], and more recently in a young male with *C. neoformans* and Varicella Zoster meningitis and idiopathic hypereosinophilic syndrome, hypocomplementemia and low NK cells [20].

NK cells are non-B non-T-cell $CD3^{-}CD56^{+}$ lymphocytes and are the third largest contingent of blood lymphocytes (4–15%) [3]. Most circulating NK cells are cytotoxic and express a low level of CD56 as well as an immunoglobulin G Fc receptor, FcγRIIIA (CD16), and are termed as $CD56^{dim}$ NK cells in contrast with the more immature $CD56^{bright}$ NK cells [3]. $CD56^{dim}$ NK cells constitute the majority of peripheral blood NK cells [21, 22] NK cells are located at the interface of innate and adaptive immunity with three main functions: cytotoxicity, chemokine and cytokine production, and the regulation of immunity through contact-dependent costimulatory and regulatory mechanisms [3, 4, 15]. In infectious diseases, NK cells act against certain elusive T-cell pathogens—particularly *Herpesviridae*—that negatively regulate MHC class 1–infected cells [3]. NK cells are negatively regulated by MHC class I antigens, while they are triggered by many activation receptors. NK cells are implicated in eliminating pathogens such as viruses but are also involved in the immune response against *Cryptococcus spp* [7, 8]. NK cells directly kill *Cryptococcus* using the NKp30, β1-integrin and NKG2D activating receptors [9, 11]. In human pathology, NKD syndrome is divided into classical and functional deficiencies [3]. Our patient had a classic NKD with a stable defect over time and an opportunistic infection that was unlike any previously described NKD cases. NK cells account for nearly 10% of the lung lymphocyte population. NKD may, therefore, favor fungal lung infections such as infection with *Cryptococcus*, which penetrates the body through the respiratory tract.

The residual NK cells displayed normal NKp30 receptors, IFN-gamma production, degranulation, and perforin expression. However, severe NK cell lymphopenia could lead to a global loss of cytotoxicity, which could have affected the destruction of fungi dependent on perforin, as previously described [4]. NK cells do not only exhibit direct target destruction but also act as regulators of other immune agents via cytokine synthesis and are thus of utmost importance to mounting the robust Th1 response necessary to fight *Cryptococcus* infection [14]. Our patient had an impaired CD8 T cell response to *Cryptococcus*. The patient's T cells did not proliferate after in vitro stimulation by a cryptococcal antigen suspension. Cytotoxic CD8 cells play an important role in the host's immune response to *C. neoformans* [5, 7]. This defective CD8 response appeared related, i.e. secondary to the primary NKD, resulting in a weak IFN-γ production, leading to an impaired DC IL-12 production and an impaired TH1 response against *C. neoformans* [8, 14].

Due to an initial slight increase in the CD8 population, we analyzed TCRVβ, revealing a chronic expansion of a Vβ14 CD8 + clone. The clonotype persisted during the follow-up, though with a slight decrease (the patient stopped his follow-up in 2019). Such clonal CD8 expansion has already been described in healthy subjects and many nonmalignant disease states including immunodeficiencies [23, 24]. These clonotypes do not represent malignant proliferation. In the literature, it is hypothesized that these clonal expansions may reveal reactive CD8 lymphocytes with specificity for the antigens of common pathogens, particularly *Herpesviridae*—which are responsible for recurrent reactivation [24]. Immunologic memory in these CD8 + T cell clonotypes is necessary to maintain the host pathogenic equilibrium relationship. Still, it can also lead to a reduction in the T-cell repertoire by inhibiting other antigen-specific lymphocytes [24]. NK cells modulate T-cell memory differentiation and regulate and kill recently activated proliferative CD8 + T cells [14]. NKD could, therefore, have led to the persistence of the patient's CD8 clonotype due to a lack of killing of expanded clonotype cells. The possible negative effect of a preexisting clonotype reducing the NK cell compartment seems less plausible. This phenomenon has not previously been described and should have affected other lymphocyte compartments. Due to the diversity of peptides and HLAs, the antigen responsible for the patient's Vβ14 clonotype expansion is difficult to assess. We showed that this clonotype, though this measure was performed only once during the follow-up, did not proliferate with a challenge using *Cryptococcus* antigen.

In conclusion, this case report describes a new type of classical NKD phenotypically characterized by isolated lung cryptococcosis resulting from a complex deficiency affecting both innate (NKD) and adaptive immunity. This case confirms in human pathology the central role of NK cells in *Cryptococcus* clearance via innate and adaptive immunity.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

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Author contributions
MM wrote the manuscript. FV, SSL, CF, CD, CP, CM performed all the analyses and prepared the figures and tables. MM, FV and CF conceptualized and designed the study. All the authors reviewed and revised the manuscript. All the authors have read and approved the final version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Consent for publication
Written consent for publication was obtained from the patient.

Competing interests
The authors declare no competing interests.

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