

A Case of *Pneumocystis jiroveci* Pneumonia in X-Linked Agammaglobulinaemia Treated with Immunosuppressive Therapy: A Lesson for Immunologists

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Key Words

Pneumocystis jiroveci pneumonia · X-linked agammaglobulinaemia · Naïve T cells · Lymphoproliferative response

Abstract

The case of a 20-year-old patient, affected by X-linked agammaglobulinaemia (XLA), who developed severe pneumonia from *Pneumocystis jiroveci* (formerly *Pneumocystis carinii*) (PCP), is reported. This infection usually affects patients with AIDS, children affected by severe combined immunodeficiency or hypogammaglobulinaemia with hyperimmunoglobulin M, or patients undergoing severe immunosuppression. The XLA patient developed PCP during therapy with steroids and cyclosporine A, carried out for several months, due to an extended skin vasculitis, accompanied by general symptoms. The pneumonia had a severe clinical course, requiring a long hospitalization. At the diagnosis of PCP, immunosuppressive therapy was suspended and the pa-

tient recovered after a long-term trimethoprim/sulfamethoxazole therapy. Immunological studies revealed an unexpected normal number of CD4+ and CD8+ T cells. The two subsets had an exclusive naïve phenotype (95% CD4+CD45RA+CD62L+ and 89% CD8+CD45RA+CD62L+ cells), with an absence of primed cells. Lymphoproliferative responses to *P. carinii* and recall antigens as well as to mitogens were extremely deficient. During the follow-up, memory cells appeared with recovery of the lymphoproliferative response to mitogens and maintained defective responses to antigens. This is one of the few reported XLA cases experiencing severe PCP. In this patient, the infection became clinically evident during immunosuppressive therapy. We believe that the absence of functional activities, despite a normal level of T lymphocyte counts, sustained this long-lasting infection. Thus, the CD4+ and CD8+ T cell count evaluation, without functional studies, may not be per se sufficient for predicting the risk of a severe clinical course of PCP in patients undergoing immunosuppression.

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Introduction

X-linked (Bruton's) agammaglobulinaemia (XLA) is a primary immunodeficiency, whose features include absence or small numbers of circulating mature B cells and severely reduced serum immunoglobulin (Ig) values of all classes. The mutated gene, localized on the long arm of the X chromosome, encodes for a cytoplasmic tyrosine kinase – Bruton's tyrosine kinase (Btk) [1, 2]. In general, affected subjects have normal T cells, both in number and function. The disease is characterized by recurrent bacterial infections, mostly due to capsulated pyogenes, while viral and fungal infections are less frequent [1]. Among primary immunodeficiencies, the hypogammaglobulinaemia with hyper-IgM (HIGM) is conversely frequently complicated by *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) [3] pneumonia (PCP), due to CD40 ligand (CD154) deficiency, which does not deliver a costimulus to activate T cells for antigen processing [4, 5]. PCP also occurs in patients affected by severe combined immunodeficiency [6]. Up to now, two patients with XLA were reported with PCP [7, 8]. Other clinical settings accounting for the emergence of PCP are immunodeficiency resulting from the human immunodeficiency virus (HIV) infection, when CD4 cells are below 200/ μ l [9], or resulting from immunosuppressive treatments in organ transplant recipients [10]. In both cases, a prophylaxis with trimethoprim/sulfamethoxazole (TMP/SMX) is recommended [10, 11].

The aim of the present study is to report a case of XLA, complicated by a severe and long-lasting PCP during immunosuppressive therapy, accompanied by a normal number of T lymphocytes but associated with the defect of functional activities.

Case Report

We report the case of a 20-year-old male patient, affected by XLA, whose diagnosis was established at 18 months of age, due to recurrent upper and lower respiratory tract infections and the absence of B lymphocytes and serum Ig in the peripheral blood. The diagnosis was later confirmed by genetic analysis (Btk mutation, G594E). The analysis was performed at the Central Italian Laboratory for the Study of Btk mutation, Department of Pediatrics, University of Rome Tor Vergata. A replacement therapy with intravenous Ig was started at the time of diagnosis and well tolerated. In June 2001 (at 16 years of age), painful nodular lesions on both legs were evident. The patient was referred to a hospital centre in his home town. A skin biopsy was performed, resulting in a diagnosis of nodular vasculitis. After 3 months, the patient developed asthenia, anorexia, continuous fever, with enlarged and confluent nodular lesions, which also extended to the

lateral abdominal area. The leg skin presented intense inflammatory symptoms, associated with leucocytosis, neutrophilia, increase in sedimentation rate, positive C-reactive protein and an increased level of fibrinogen. Low-dose steroids were started and a lymphocyte subset analysis, performed after a 1-month therapy, showed increased T helper cells (CD3+CD4+ 74%, 3,620 cells/ μ l). Ninety-nine percent of them had a naïve pattern (CD4+CD45RA+). At this time, no lymphocyte functional assays were performed. Subsequently, steroids were increased and cyclosporine A therapy was started (deflazacort 0.5 mg/kg/day, cyclosporine 4 mg/kg/day) and continued for 2 months. The steroid was then diminished to a dose of 0.2 mg/kg/day and the cyclosporine was maintained at the same dosage. After 4 months, a partial remission of the symptoms was observed. However, in December 2001, the patient manifested an episode of bronchopneumonia, which was treated with antibiotics (ceftriaxone i.v. 2 g/day for 10 days). After this episode, white blood cells (WBC), lymphocytes and CD4 levels were within the normal range. In February 2002, due to a new acute phase of severe dyspnoea, cough, high fever and a relapse of subcutaneous vasculitis, he was referred to our department and hospitalized. During hospitalization, leucocytosis (WBC 21,780 cells/ μ l, with 81% of neutrophils and 12.3% of lymphocytes) increased erythrocyte sedimentation rate (48 mm/h), and positive C-reactive protein values were found. Haemoglobin was 10.2 g/dl and blood iron 27 μ g/dl. Ferritin levels were 9 ng/dl. Routine liver and renal functional tests gave normal values. B and C hepatitis markers were negative. Complement fractions (C3 and C4) were in the normal range as well as the search for organ- and non-organ-specific autoantibodies. Total proteins were 5.5 g/dl, IgG 370 mg/dl, IgA <5.8 mg/dl, IgM <4.3 mg/dl and total IgE <17.8 mg/dl. Arterial blood gases gave results of PaO₂ 58 mm Hg and PaCO₂ 28.4 mm Hg. Chest X-ray and computed tomography scan showed pulmonary fibrosis and interstitial inflammation. The sputum as well as the bronchoalveolar lavage (BAL) fluid resulted positive for *P. carinii*, as assessed by indirect immunofluorescence, using a commercial kit (Monofluo™, Biorad, Hercules, Calif., USA). Immunosuppressive therapy was withdrawn and therapy with intravenous TMP/SMX (20 mg/kg/day and 100 mg/kg/day, respectively) was begun, with improvement in symptoms. After 2 weeks and ongoing clinical improvement, the therapy was switched to an oral regimen (TMP 160 mg and SMX 800 mg 4 times a day). After 1 week, BAL showed persistence of *Pneumocystis*. Thus, the intravenous treatment cycle was reintroduced, with clinical improvement. The patient was then discharged with prophylactic therapy (TMP/SMX 160/800 mg b.i.d. per os). The prophylaxis was continued for 1 year, despite that the BAL examination for *Pneumocystis* was three times negative, as well as the chest X-ray and computed tomography scan. The prophylaxis was continued because after steroid suspension, the patient developed a severe relapse of skin vasculitis associated with severe general symptoms. For this reason, in June 2002, the steroid (0.5 mg of prednisone/kg/day associated with 900 mg of salicylic acid, for 3 months) was reintroduced. The prednisone was then diminished to a dose of 0.25 mg/kg/day, and after 6 months, to a maintenance dose of 0.1 mg/kg/day for another year. During 2003, the skin vasculitis, according to clinical and ultrasonographic criteria, was completely cleared. The inflammatory parameters returned to normal values. The patient suspended all therapy for PCP and vasculitis, but continued the therapy with intravenous Ig at a dose

Table 1. WBC, lymphocyte number and lymphocyte subpopulations in the study patient, measured at different time points

Date (year/month)	WBC 10 ³ /μl	Lymphocytes cells/μl	CD3+ cells/μl	CD3+ %	CD4+ cells/μl	CD4+ %	CD8+ cells/μl	CD8+ %	NK cells cells/μl	NK cells %	CD19+ %
2002/02	28,590	2,066	2,024	98	1,487	72	516	25	21	1	1
2002/03	8,850	1,381	1,298	94	925	67	290	21	n.d.	n.d.	0
2003/04	14,300	1,144	995	87	709	62	240	21	160	14	0
2005/02	8,350	1,430	1,187	83	581	49	332	28	157	11	0

Natural killer (NK) cells were defined as CD3–CD16+CD56+. n.d. = Not done.

of 400 mg/kg every 2 weeks, with IgG values consistently around 700 mg/dl, during the entire follow-up period, which has now lasted 32 months. No signs of PCP or vasculitis have been evident up to present (April 2005).

Methods

For the flow cytometric analysis of CD4+ and CD8+ T cell subsets, 500 μl of whole blood was lysed using 10 ml of Ortho lysing reagent (Ortho-Clinical Diagnostics, Raritan, N.J., USA), washed, labelled with a cocktail of four monoclonal antibodies for 30 min at 4°C, and fixed within 1 h from blood collection. The peripheral distribution of naïve and memory T cells was investigated by evaluating the differential expression of CD45RA and CD62L molecules on both CD4+ and CD8+ T cells, as previously reported [12]. Anti-CD4 allophycocyanin, anti-CD8 peridinin chlorophyll protein, anti-CD45RA fluorescein isothiocyanate (FITC), anti-CD62L phycoerythrin, and anti-HLA-DR and CD95 FITC were purchased from Becton Dickinson Immunocytometry Systems (San Jose, Calif., USA); FITC-labelled anti-Fas was obtained from Medical and Biologic Laboratories Co., Ltd., Nagoya, Japan. After staining, cells were washed once in phosphate-buffered saline containing 2% fetal bovine serum and analysed on a FACSCalibur cytofluorimeter (Becton Dickinson Immunocytometry Systems) using the Cell Quest software. To determine marker expression on CD4+ and CD8+ cells, total lymphocytes were first identified and gated by forward and side scatter. The cells were then additionally gated for CD4 or CD8 expression. For the lymphoproliferative (LP) assay, the peripheral blood was collected in EDTA vacutainer tubes. Peripheral blood mononuclear cell (PBMC) separation (Ficoll-Hypaque) was carried out within 4 h of withdrawal. For the LP assay, we used previously published protocols with rat-derived *P. carinii* antigens (PcAg) and human samples [13]. LP was carried out by measuring ³H-thymidine incorporation after 7 days of culture at 37°C in a 5% CO₂ incubator; 10⁵ PBMCs/well were incubated in medium alone (MED) or together with PcAg, influenza virus vaccine (FLU), or phytohaemagglutinin (PHA) as antigens in four replicates for each antigen. PcAg was added in a 10:1 ratio of trophozoite/PBMC. On day 6, the wells were pulsed with radioactive thymidine (Amersham) and harvested 18 h later, and the incorporated activity measured as counts per minute. For the PcAg preparation, trophozoites were obtained from *P. carinii*-free Sprague-Dawley rats infected with *P. carinii* by transtracheal in-

oculation and expanded onto human embryonic lung (HEL 299 – ATCC CCL137) cells sheeted on microcarrier beads in spinner flasks, as previously reported [13]. Pelletted microorganisms were washed twice in phosphate-buffered saline, counted on Giemsa-stained calibrated drops and used fresh or thawed after cryopreservation at a 10:1 ratio of trophozoites/PBMC. To monitor the purity of PcAg, aliquots were microscopically checked for morphology and cultured for bacteria and fungi; lipopolysaccharide contamination was excluded by Limulus assay (E-toxate; Sigma, Italy). European guidelines for animal experimentation were followed in the animal study. Other antigens included: MED = culture medium, no stimulator, negative control, background values; PHA = positive control for mitogenic activity (2.5 μg/ml; Murex, Rome, Italy); FLU = prepared with a mixture of A/Taiwan, A/Shanghai and B/Victoria (24 mg/ml); CD3 monoclonal antibodies OKT3 (25 ng/ml; Ortho Diagnostic Systems.); recall antigen = mannoprotein (Mp) of *Candida albicans* at a final concentration of 50 μg/ml. After 3 days (for PHA and CD3) and 6 days (for Mp) of cultures at 37°C in humidified 5% CO₂, 0.5 μCi of tritiated thymidine (³H-T, specific activity 25 Ci/mmol; Amersham, UK) was added to each well, to assess lymphoproliferation in response to PHA, anti-CD3 and Mp.

Results

Table 1 summarizes the lymphocyte subpopulation distribution during the study periods. The WBC count was increased at the time of hospitalization at our department, with a reduction after anti-PCP therapy. The same was found for the absolute lymphocyte count and T cell subpopulations. The CD4+ T cell count was always >900/μl during hospitalization and decreased during the follow-up. The low initial levels of CD3–CD16+CD56+ natural killer cells were conversely increasing. The extremely low level of CD19+ cells, during the entire study period, was consistent with the diagnosis of XLA. To further characterize T lymphocyte subsets, due to the finding of a PCP in a patient who underwent immunosuppressive therapy but still showed a normal number of CD4+ T cells, the naïve or memory status of the cells

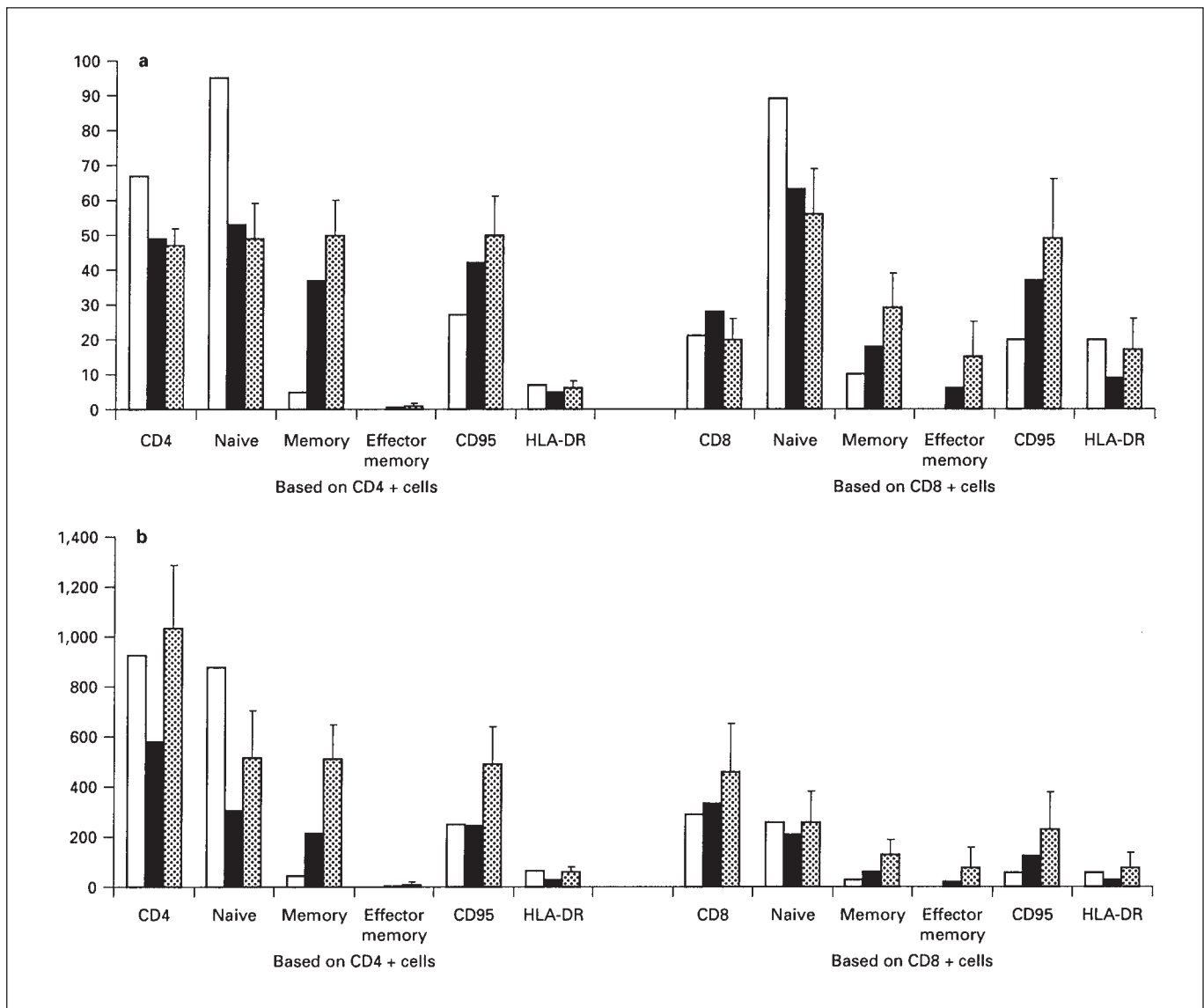


Fig. 1. Distribution of T lymphocytes and their naïve and memory subsets. **a** Data are reported in percentages for CD4+ and CD8+ cells, respectively. **b** Data are reported in absolute numbers (cells/ μ l) for CD4+ and CD8+ cells, respectively. $\square$ = Phenotype performed during immunosuppression; $\blacksquare$ = phenotype after immunosuppression; $\square$ = values in age-sex matched healthy controls with the standard deviation. CD45RA+CD62L+ were defined as naïve cells, CD45RA-CD62L- and CD45RA-CD62L+ as memory cells, and CD45RA+CD62L- as effector memory cells.

was assessed, as well as the functional responses to a PcAg, to recall antigens and mitogens. In 2002, 95% of CD4+ and 89% of CD8+ T lymphocytes showed a naïve pattern (fig. 1). The effector memory subtype was reduced, mainly in the CD8+ T cell fraction. During the follow-up, the naïve cells progressively decreased, both in percentage and absolute number, and the memory

cells increased. The HLA-DR activation marker and CD95, which detects the Fas expression, were represented in normal range, both in CD4+ and CD8+ T cell subsets, thus arguing against Fas-mediated apoptotic events. The results were also consistent during the follow-up. A relevant decrease in the LP responses to all antigens and mitogens was found during immunosuppression, where-

Table 2. Lymphocyte proliferation tests to different stimuli

Antigen	During immuno-suppressive therapy		After immuno-suppressive therapy		Normal value
	cpm	SI	cpm	SI	
MED	979		870		
PHA	7,247	7.40	34,500	39.65	>25
OKT3	4,098	4.18	31,760	36.50	>15
FLU	1,916	1.96	4,322	4.96	>4
Trophozoites from <i>P. carinii</i>	8,723	8.91	n.d.		>10
MED	3,899.3		2,278		
Mp	6,694.86	1.72	9,876	4.33	>5

Values for Mp antigens were obtained after 6 days of incubation. Thus, the corresponding MED values are given. cpm = Counts per minute; SI = stimulation index; n.d. = not done.

as an improvement in mitogens was found thereafter. Defective responses persisted to FLU and Mp antigens (table 2).

Discussion

PCP has previously been reported only in 2 XLA patients [7, 8]. Here, we describe an XLA patient who developed a severe and long-lasting PCP during immunosuppressive therapy, despite a normal CD4⁺ T cell count. Defective LP responses were found. This case raises several issues about diagnostic and therapeutic management.

The patient developed a vasculitis which was accompanied by constitutional symptoms and required immunosuppressive therapy, and this most likely induced the onset of clinically evident PCP. In this case, the cause of the vasculitis is unknown, and even its pathogenesis is not clear and may be multifactorial. Several immunological mechanisms underlie vasculitis [14], leading to the deposition of immune complexes in the affected organs. The immune mechanisms include activation of inflammatory responses, triggered by either infectious or chemical agents, including drugs [14]. Moreover, intravenous Ig therapy may represent an additional mechanism through the engagement of the Fc gamma receptor [14]. Vasculitis is very rare in patients with Btk deficiencies, whereas a corticosteroid-responsive lymphoid vasculitis has been reported in the X-linked lymphoproliferative syndrome [15]. In our patient, the Btk mutation has been unequivocally demonstrated, thus ruling out the possibil-

ity of another primary immunodeficiency. The vasculitis required immunosuppressive therapy, which raises the question if this therapy may account, per se only, for the reduction in memory T cells or if other mechanisms may operate. Our patient showed an increase in naïve T cells, thus ruling out the hypothesis of thymic insufficiency. The hypothesis of a telomerase shortening may be active, but we had no chance to test it. Naïve T cells were increased at the beginning of the immunosuppressive therapy, which was based on steroids only. In animal models, corticosteroids, used at pharmacologic doses, trigger thymocyte apoptosis, induced by T cell activation [16]. However, in our patient, no activated cells were found, since the HLA-DR expression on T cells was normal. Thus, the absence of memory T cells seems not fully dependent on the immunosuppressive therapy only. Another possibility may be the recruitment of memory cells at inflammation sites, including the vasculitic skin. Unfortunately, the T cell immunophenotype was not investigated in the patient's skin biopsy, since it has been performed in another clinical centre. The study on the distribution of naïve and memory T cells has not been extensively performed in primary immunodeficiencies. In particular, there are no findings in primary immunodeficiency patients with associated autoimmune diseases, which, in several cases, may be triggered by infectious agents. In particular, in these settings, studies on the possible role of regulatory cells were still not addressed. Several types of regulatory cells are triggered by infectious agents. As a result, a protective immunity or an autoimmune disease can develop [for a review, see ref. 17]. This may be mirrored in the peripheral blood or in the inflammation sites

by disturbances in lymphocyte subpopulation distribution and may contribute, on the one side, to the self-maintaining of the autoimmune diseases and, on the other, to the pathogenesis of the deficiency in some cell subsets, like activated T cells. Besides HIGM, severe combined immunodeficiency and XLA [3, 5, 7, 8], PCP has been reported in acquired immunosuppressed patients, where it usually emerges when the CD4 cell count is below or slightly above 200/ μ l [9, 10, 18]. In animal models, the immune system is involved in both the protection and physiopathology of the lung damage caused by *P. carinii*. In the human system, besides HIGM, no clear models are available. In HIV infection, the disease usually appears when the CD4 cell count is below 200/ μ l, even if some cases with higher CD4 levels have been reported [19]. Our patient, despite immunosuppressive therapy which included the use of steroids and subsequently cyclosporine A, presented a high and unexplained CD4 cell count. After several months of immunosuppressive therapy, he showed a PCP. His circulating CD4+ as well as CD8+ T cells had an exclusive naïve phenotype. Thus, the patient had no circulating cells primed to drive effective immune responses, including the lymphoproliferative ones. Based on clinical and immunological results from the follow-up, memory T cells may have been recruited on disease onset at the site of infection, being thus undetectable in the circulation. This is consistent with recent findings in children with a life-threatening varicella course [20]. Interestingly, the children who were not suffering from any known primary immunodeficiency had a reduced natural killer

cell number at the time of the study and an exclusive presence of circulating naïve T cells, as found in our patient. However, in the latter cases, a genetic background explaining the disease severity was not ruled out. Finally, in our case, an improved functional activity to mitogens was found, whereas a defective one was still present to recall antigens. This is reminiscent of previous findings in HIV-infected patients [21] and argues in favour of the need to explore functional activities, in addition to the lymphocyte enumeration, and to define the role of the immune system more carefully for disease management.

In conclusion, the CD4 lymphocyte count is usually sufficient to predict a subsequent development of PCP in categories at risk. However, in the presence of other factors, including immunosuppressive therapies, the number of CD4+ T cells >200/ μ l may not be sufficient to abort *Pneumocystis* infection. Thus, a more detailed analysis of the naïve and memory status of the T cells as well as of functional tests must be undertaken to introduce, where necessary, a prompt prophylactic regimen, especially in patients with primary or secondary immunodeficiencies, requiring long-lasting immunosuppressive therapy.

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