Published in final edited form as:

Leuk Lymphoma. 2013 August ; 54(8): . doi:10.3109/10428194.2012.750723.

Impaired bactericidal but not fungicidal activity of polymorphonuclear neutrophils in patients with chronic lymphocytic leukemia

Dimitrios P. Kontoyiannis¹, Sarah P. Georgiadou¹, William G. Wierda², Susan Wright¹, Nathaniel D. Albert¹, Alessandra Ferrajoli², Michael Keating², and Russell E. Lewis¹

¹Department of Infectious Diseases, Infection Control and Employee Health, The University of Texas MD Anderson Cancer Center, Houston, TX

²Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX

Abstract

We examined the qualitative polymorphonuclear neutrophil (PMN)-associated immune impairment in patients with chronic lymphocytic leukemia (CLL) by characterizing phagocytic killing of key nonopsonized bacterial (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and fungal (*Candida albicans* and *Aspergillus fumigatus*) pathogens. Neutrophils were collected from 47 nonneutropenic CLL patients (PMN count > 1000/mm³), and age-matched and young healthy controls (five each). A subset of patients (13%) had prior or subsequent infections. We found that the CLL patients had diminished PMN microbicidal response against bacteria but not against fungi than did the controls. Compared to patients with effective PMN responses, we did not identify differences of basal PMN pathogen-associated molecular pattern receptor gene expression, soluble pathogen-associated molecular pattern gene expression, or inflammatory cytokine signatures in patients with impaired PMN responses when PMNs were analyzed in multiplex real-time polymerase chain reaction assays. However, differences in PMN microbicidal response against *A. fumigatus* in CLL patients were associated with the degree of hypogammaglobulinemia.

Keywords

CLL; neutrophil function; infection; innate immunity

Introduction

Bacterial and fungal infections are major causes of morbidity and mortality in patients with chronic lymphocytic leukemia (CLL). The pathogenesis of infections in these patients is multifactorial.¹⁻⁵ Hypogammaglobulinemia and chemotherapy-induced defects in adaptive cellular immunity have long been considered the signature immune deficits that predispose

Correspondence: Dimitrios P. Kontoyiannis, Department of Infectious Diseases, Infection Control and Employee Health, Unit 402, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030; telephone: (713) 792-6237; fax: (713) 745-6839; dkontoyi@mdanderson.org..

Authorship

Contribution: D.P.K. conceived the idea, performed data analysis, and wrote the manuscript. R.E.L. performed experiments and data analysis and wrote the manuscript. S.P.G. performed data analysis and wrote the manuscript. N.D.A. performed experiments. S.W. enrolled the patients. M.K., W.G.W., and A.F. critically reviewed the manuscript and contributed the patients.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Presented in part: Hematologic Malignancies Conference. 6th International Conference, Houston TX, October 2010.

patients to severe, life-threatening infections.¹⁻⁵ However, genetic or acquired deficiencies in innate cellular immune responses in heavily pretreated CLL patients may predispose them to severe, frequently fulminant infections.⁶⁻¹¹ Therefore, we sought to characterize the qualitative polymorphonuclear neutrophil (PMN)-associated immune impairment in CLL patients with a focus on differences in phagocytic killing by PMNs of key nonopsonized bacterial (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and fungal (*Candida albicans* and *Aspergillus fumigatus*) pathogens that frequently cause severe infections in CLL patients.

Study design

In this prospective, noninterventional single-institution study, blood samples were obtained from 47 outpatients with CLL at The University of Texas MD Anderson Cancer Center from August 1, 2006, to January 1, 2009. Patients with neutropenia (PMN count < 1000/mm³) or prior splenectomy were excluded. The patients' electronic medical records were reviewed for demographic characteristics, Rai stage, prior chemotherapy, and chemotherapy response. In addition, serum immunoglobulin (IgG, IgM, and IgA) levels and prior, current, and subsequent infections were recorded (minor mucosal infections such as herpes simplex virus reactivation were excluded). Five healthy age-matched and five healthy young volunteers were used as controls. The study protocol was approved by the MD Anderson Institutional Review Board.

Pathogen-killing assays

PMNs from the patients' and controls' whole blood samples were purified using modified standardized methods¹² as described previously.^{13,14} PMN viability (>95%) was verified via trypan blue staining. PMNs were mixed with key nonopsonized bacterial (*S. aureus* [strain ATCC 29213] and *P. aeruginosa* [strain ATCC 27853]) and fungal (*C. albicans* [strain 600603] and *A. fumigatus* [strain Af293]) pathogens at a ratio of 10:1. For strain Af293, hyphae were generated by incubating 1 mL of 10⁵ conidia in RPMI plus 10% fetal calf serum (FCS) for 18-20 hours in a shaking incubator at 37°C, centrifuging vials (14000 rpm for 15 minutes), removing the supernatant and incubating the hyphae with 10⁶ neutrophils for 1 hour at 37°C in a shaking incubator (hyphae:PMN ratio, 1:10). Hyphae were incubated in RPMI plus 10% FCS media. Vials were then centrifuged (14000 rpm for 15 minutes) and aspirated to remove the supernatant, 1 mL of cold sterile water was added in each vial to induce hypotonic lysis of neutrophils, and the vials were vortexed again. Vials were then centrifuged and aspirated without disturbing the pellets before the addition of 0.5 mL of XTT reaction solution (1 mg/mL containing 125 microg menadione) and incubated at 37°C in a shaking incubator for 1 hour. After incubation, vials were centrifuged, and their supernatants were transferred in 100-μL aliquots to a 96-well microplate to determine their absorbance at 492 nm with a reference wave length of 690 nm (plate absorbance) using a microplate spectrophotometer.

For *C. albicans*, yeast cells were grown overnight to log phase and washed in phosphate-buffered saline, were separated into 1-mL aliquots containing 2×10^7 yeast cells, pelleted via centrifugation, and resuspended in RPMI plus 10% FCS medium containing 1×10^6 freshly isolated neutrophils (yeast:PMN ratio, 20:1).

For *S. aureus* and *P. aeruginosa*, bacterial cells were collected from 24-hour blood agar plates, washed in phosphate-buffered saline, and prepared as 200-μL aliquots containing 5×10^7 cells. To opsonize bacteria, 20 μL of heat-inactivated normal human serum (30 minutes in a 55°C water bath) was added to each aliquot containing bacterial cells, and the aliquots were incubated for 30 minutes at 37°C in a shaking incubator. Cells were then resuspended in 1 mL of RPMI plus 10% FCS containing 5×10^6 neutrophils (PMN:bacteria ratio, 1:10).

PMN-induced bacterial or hyphal damage was assessed using the following equation: percentage of hyphal damage = $[(1-X)/C] \times 100$, in which $\times$ is the optical density of the test wells and C is the optical density of the control wells. All pathogen killing data were performed in triplicate.

Gene expression assays of unstimulated PMNs

Total RNA was extracted from PMNs using a commercial kit (RNeasy; Qiagen). RNA concentrations and purity were determined using Agilent RNA 6000 Nano Kit chips analyzed with a 2100 Bioanalyzer (Agilent Technologies). cDNA from each sample was prepared from 25 ng of RNA using an RT² PreAMP cDNA Synthesis Kit (SA Biosciences) before gene expression analysis using an 84-gene real-time polymerase chain reaction array designed for analysis of human genes involved in host response to bacterial infections and sepsis (Human Innate and Adaptive Immune Responses Array; SA Biosciences). All expression data were normalized according to housekeeping genes and reaction controls in the plate and analyzed using a software program provided by SA Biosciences.

Statistical analysis

Pathogen-killing data for the patients and controls were compared using the Student *t*-test or Mann-Whitney test where appropriate. Expression data were compared with PMN killing indices for each pathogen using logistic regression analysis. *P* values less than 0.05 were considered indicative of statistical significance.

Results and discussion

The median age of the patients at diagnosis was 62 years (range, 38-87 years), and 27 (57%) of them were male (Table 1). The mean total follow-up duration ($\pm$ standard deviation [SD]) after blood collection was 21 ± 8 months. A small patient subset (6 [13%]) had a prior or subsequent history of documented infection.

We found that PMNs from the CLL patients were less effective than those from age-matched controls at killing *P. aeruginosa* and *S. aureus* ($P < .05$), but we observed no differences in *C. albicans* or *A. fumigatus* killing assays ($P > .05$) (Figure 1). We also observed no differences in PMN killing indices between young and old (age-matched) controls (data not shown). Furthermore, we grouped the patients with CLL according to PMN microbicidal response: no impaired response (PMN killing index > 0.5) and impaired response (PMN killing index < 0.5) compared to controls. Bivariate and logistic regression analysis failed to identify significant relationships among PMN pathogen-associated molecular pattern (PAMP) receptor gene expression, soluble PAMP gene expression, or inflammatory cytokine signatures associated with impaired versus no impaired PMN microbicidal response (supplemental Figures 1-3).

In addition, in 34 of the patients, for whom all clinical and microbiological data were available, we observed no significant differences in PMN microbicidal responses between those with and without prior or subsequent infections, although the former group was small (supplemental Table 1; supplemental Figure 4). However, we noted an inverse correlation ($P = .002$) between IgG serum concentration and PMN killing activity against *A. fumigatus* (supplemental Figure 5). In addition, we found a correlation between low serum IgG level and risk of infection (mean [$\pm$ SD] total IgG level: 480 ± 219 mg/dL in patients with infections and 986 ± 629 mg/dL in patients without infections; $P = .008$) (supplemental Figure 5).

Previous reports described defective neutrophil function and microbicidal mechanisms (e.g., myeloperoxidase deficiency, impaired chemotaxis) in CLL patients.⁶⁻¹¹ Our study demonstrated notable qualitative differences in phagocytosis against common bacterial but not fungal pathogens between the CLL patients and age-matched controls. Due to the small number of patients with infections, we were not able to identify a relationship between impaired PMN killing response and infection risk. Nevertheless, to our knowledge, this is the first study to show no differences in pattern recognition receptor expression and the proinflammatory response pathway between patients with impaired and no PMN microbicidal response.

In agreement with other investigators, we observed an association between hypogammaglobulinemia, the most commonly recognized inherent immune defect in CLL patients, and increased infection risk.¹⁶ Interestingly, in our study, the differences in PMN microbicidal response against *Aspergillus* species were linked with hypogammaglobulinemia than with altered pattern recognition receptor expression or cytokine response. Previously, investigators reported that phagocytosis of *Aspergillus* spp. is enhanced by opsonization and proinflammatory molecules, including immunoglobulins, complement, and mannose-binding lectins.¹⁷ Future studies comparing responses of PMNs to *Aspergillus* spp. at various IgG levels in patients with invasive aspergillosis would be interesting.

This preliminary study had several limitations; PMNs that were evaluated for gene expression assays were unstimulated, we used only one isolate of each bacterial or fungal pathogen and we had no epigenetic and proteomics data. In addition, the patient group with infections was small. Nevertheless, because currently available laboratory tests do not provide information on qualitative cellular immunity defects in CLL patients, a more pragmatic strategy for categorization of qualitative immunodeficiency may enhance infection risk stratification in CLL patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by a grant from the CLL Global Research Foundation to (D.P.K.). This research is supported in part by the National Institutes of Health through MD Anderson's Cancer Center Support Grant CA016672.

References

1. Dasanu CA. Intrinsic and treatment-related immune alterations in chronic lymphocytic leukaemia and their impact for clinical practice. *Expert Opin Pharmacother*. 2008; 9(9):1481–1494. [PubMed: 18518779]
2. Hamblin AD, Hamblin TJ. The immunodeficiency of chronic lymphocytic leukaemia. *Br Med Bull*. 2008; 87:49–62. [PubMed: 18755702]
3. Morrison VA. Infectious complications in patients with chronic lymphocytic leukemia: pathogenesis, spectrum of infection, and approaches to prophylaxis. *Clin Lymphoma Myeloma*. 2009; 9(5):365–370. [PubMed: 19858055]
4. Tsiodras S, Samonis G, Keating MJ, Kontoyiannis DP. Infection and immunity in chronic lymphocytic leukemia. *Mayo Clin Proc*. 2000; 75(10):1039–1054. [PubMed: 11040852]
5. Wadhwa PD, Morrison VA. Infectious complications of chronic lymphocytic leukemia. *Semin Oncol*. 2006; 33(2):240–249. [PubMed: 16616071]

6. Jayaswal U, Roper S, Roath S. Impairment of neutrophil chemotaxis by serum from patients with chronic lymphoproliferative disease. *J Clin Pathol.* 1983; 36(4):449–453. [PubMed: 6833512]
7. Kansu E, Akalin E, Civelek C, Tekesin O, Laleli Y, Firat D. Serum bactericidal and opsonic activities in chronic lymphocytic leukemia and multiple myeloma. *Am J Hematol.* 1986; 23(3):191–196. [PubMed: 3766522]
8. Siegbahn A, Simonsson B, Venge P. The chemokinetic inhibitory factor (CIF) in serum of CLL patients: correlation with infection propensity and disease activity. *Scand J Haematol.* 1985; 35(1): 80–87. [PubMed: 3901243]
9. Zeya HI, Keku E, Richards F 2nd, Spurr CL. Monocyte and granulocyte defect in chronic lymphocytic leukemia. *Am J Pathol.* 1979; 95(1):43–54. [PubMed: 219699]
10. Itala M, Vainio O, Remes K. Functional abnormalities in granulocytes predict susceptibility to bacterial infections in chronic lymphocytic leukaemia. *Eur J Haematol.* 1996; 57(1):46–53. [PubMed: 8698131]
11. Nagler A, Obedeau N, Tatarsky I, Hocherman I, Merzbach D, Carter A. Chemiluminescence response of polymorphonuclear leucocytes to serum-opsonized zymosan--a predictive parameter for infections and prognosis in multiple myeloma patients. *Clin Lab Haematol.* 1988; 10(4):365–375. [PubMed: 2855047]
12. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl.* 1968; 97:77–89. [PubMed: 4179068]
13. Lamaris GA, Lewis RE, Chamilos G, et al. Caspofungin-mediated beta-glucan unmasking and enhancement of human polymorphonuclear neutrophil activity against *Aspergillus* and non-*Aspergillus* hyphae. *J Infect Dis.* 2008; 198(2):186–192. [PubMed: 18500936]
14. Chamilos G, Lewis RE, Lamaris G, Walsh TJ, Kontoyiannis DP. Zygomycetes hyphae trigger an early, robust proinflammatory response in human polymorphonuclear neutrophils through toll-like receptor 2 induction but display relative resistance to oxidative damage. *Antimicrob Agents Chemother.* 2008; 52(2):722–724. [PubMed: 18025115]
15. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. *Blood.* 2003; 102(7):2660–2669. [PubMed: 12829592]
16. Itala M, Helenius H, Nikoskelainen J, Remes K. Infections and serum IgG levels in patients with chronic lymphocytic leukemia. *Eur J Haematol.* 1992; 48(5):266–270. [PubMed: 1644158]
17. Marr KA, Koudadoust M, Black M, Balajee SA. Early events in macrophage killing of *Aspergillus fumigatus* conidia: new flow cytometric viability assay. *Clin Diagn Lab Immunol.* 2001; 8(6): 1240–1247. [PubMed: 11687470]

Figure 1. Lower microbicidal response rates for bacteria but not fungi in CLL patients than in age-matched controls

The graphs show the mean response rates ($\pm$ SD) in controls versus CLL patients. The groups were compared using a two-sided, nonpaired Student *t*-test (* $P < .05$).

Table 1
Demographic, clinical, and laboratory characteristics of the 47 patients

Characteristic	n (%)
Mean age, years (range)	62 (38-87)
Sex	
Male	27 (57)
Mean follow-up duration, months (range)	64 (2-416)
Rai stage	
0	22 (47)
1	8 (17)
2	9 (19)
3	2 (4)
4	6 (13)
CLL status *	
Complete remission	13 (28)
Partial remission	9 (19)
No remission	10 (21)
Not available or newly diagnosed	15 (32)
Chemotherapy	
Previous	24 (51)
Current	10 (21)
History of prior or subsequent infections [†]	6 (13)
Mean neutrophil count, $\times 10^3/\text{mm}^3$ (range) *	3.3 (1.0-6.5)
Mean β_2 microglobulin level, mg/dL (range) *	3.2 (1.4-11.1)
Mean immunoglobulin level, mg/dL (range) *	
IgG	870 (176-3590)
IgA	89 (15-274)
IgM	40 (4-201)
Mean albumin level, g/dL (range) *	4.2 (3.1-5.2)

* At enrollment.

[†] Patient 1: *Escherichia coli* urinary tract infection, varicella-zoster viral infection, and bacterial sinusitis. Patient 2: bacterial sinusitis, upper respiratory infection, and skin and soft tissue infection. Patient 3: recurrent central venous catheter coagulase-negative *Staphylococcus* bacteremia, α -hemolytic *Streptococcus* bacteremia, *P. aeruginosa* pneumonia, and *Nocardia* pneumonia. Patient 4: invasive mold sinusitis, *P. aeruginosa* sinusitis, *Streptococcus pneumoniae* pneumonia, *Enterococcus* urinary tract infection, varicella-zoster viral infection, and *Cytomegalovirus* viremia. Patient 5: bacterial pneumonia and α -hemolytic *Streptococcus* bacteremia. Patient 6: bacterial pneumonia without pathogen isolation.