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REVIEW ARTICLE

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The utility of peripheral blood smear review for identifying specimens for flow cytometric immunophenotyping

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Abstract

Laboratory professionals are in an ideal situation to identify CBC and peripheral blood smear findings that raise the possibility of a hematolymphoid neoplasm, and based on this information make recommendations for additional studies, such as flow cytometric immunophenotyping. In some circumstances a definitive diagnosis can be established from the combined peripheral blood morphologic and immunophenotypic findings obviating the need for bone marrow evaluation, such as for chronic lymphocytic leukemia. Occasionally flow cytometric studies are superior to morphologic assessment, such as in screening for hairy cell leukemia or identifying lymphocytic-variant hypereosinophilia. However, there is increasing recognition of small immunophenotypically unusual or abnormal populations of peripheral blood cells, particularly in older patients, which are of uncertain clinical significance, such as monoclonal B lymphocytosis and T-cell clonopathy. Therefore, it is important to integrate peripheral blood smear review findings with the clinical and other information before recommending flow cytometry. In addition, it is important to recognize situations where the results of peripheral blood smear review and flow cytometric immunophenotyping do not explain the clinical findings.

KEYWORDS

Flow cytometry, Peripheral blood smear review

1 | INTRODUCTION

Advances in automated hematology analyzers have decreased the samples that require microscopic smear review or manual differential count. However, peripheral blood smear review remains an important diagnostic tool, and consensus guidelines have been developed for when such review should be triggered.[1,2] Peripheral blood smear review is useful for confirming instrument flags, such as for platelet clumps or blasts, occasionally establishing a definitive diagnosis, such as for malaria or acute leukemia, or more frequently suggesting a differential diagnosis and recommendations for next steps ie, a clinical consultation.

Flow cytometric immunophenotyping has a well-established role in the evaluation of peripheral blood, including monitoring CD4 positive T-cells in the setting of HIV infection, assessing red blood cells for hereditary spherocytosis, and evaluating white blood cells (WBC) as part of the multiparameter evaluation for hematolymphoid neoplasms. For the latter indication, WBC can be identified by their characteristic antigen expression and then assessed for phenotypic aberrancy and restriction.[3] The medical indications for peripheral blood flow cytometric immunophenotyping were summarized by a consensus panel in 2006, and include some abnormal CBC findings and clinical symptoms and signs that suggest the possibility of a hematolymphoid neoplasm.[4] Laboratory professionals are in an ideal situation to identify CBC and peripheral blood smear findings that raise the possibility of a hematolymphoid neoplasm, and based on this information make recommendations for additional studies, such as flow cytometric immunophenotyping. This article addresses the utility of peripheral blood smear review in identifying specimens for flow cytometric immunophenotyping.

2 | COMPLETE BLOOD CELL COUNT AND PERIPHERAL SMEAR REVIEW FINDINGS

2.1 | Overt lymphocytosis

Overt absolute lymphocytosis is most frequently composed of small mature lymphoid cells. In a child, the possibility of a mature lymphoid neoplasm is unlikely and therefore, it is important to consider reactive causes, such as pertussis. In addition, the possibility of acute leukemia should be considered, because lymphoblasts may mimic more mature cells. Often diligent review of the smear will reveal a few definitive blasts with characteristic finely distributed chromatin. However, if the smear review findings are not definitive, flow cytometric studies can be of great value in further investigating for phenotypic evidence of immaturity, such as expression of CD34 and TdT (Figure 1).

In an adult patient, with overt absolute lymphocytosis (perhaps >10×10^9/L) a lymphoid neoplasm is likely and flow cytometric characterization is of great value in reaching a definitive diagnosis and classification. For example, a diagnosis of chronic lymphocytic leukemia (CLL) can be established with a lymphocytosis composed of small, mature cells with dense block-type chromatin and a few prolymphocytes, along with demonstration of the following characteristic immunophenotype by flow cytometry: CD19+, CD20+ (dim), CD5+, CD10(−), CD23+, FMC-7(−), CD200+, sIg+ (dim).[5] However, if the appearance or immunophenotypic findings are atypical for CLL, other studies may be of interest, such as fluorescence *in situ* hybridization (FISH) studies for the IGH/CCND1 rearrangement characteristic of mantle cell lymphoma. For a specimen diagnostic of CLL, peripheral blood FISH studies can also be used to obtain prognostic information, such as the presence of 13q deletion, trisomy12, 11q deletion, and 17p/TP53 deletion, and molecular studies can be performed for immunoglobulin heavy chain variable region mutation. Therefore, an astute peripheral blood smear reviewer identifying findings suggestive of CLL could contact the ordering physician and make recommendations for additional testing, which might obviate the need for additional blood draws or bone marrow evaluation.

For adult patients with overt absolute lymphocytosis, several other mature lymphoid neoplasms should also be considered, and can often be distinguished by a combination of the morphologic appearance and immunophenotype. Follicular lymphoma and mantle cell lymphoma can be recognized by the presence of circulating abnormal small mature cells with irregular nuclear outlines, but are difficult to distinguish without additional information, such as the immunophenotype where: follicular is usually CD10+, CD5(−) and mantle cell lymphoma is often CD10(−), CD5+. A CD10(−), CD5(−) mature B-cell immunophenotype is less indicative of a specific subtype of lymphoid neoplasm, but if features of hairy cell leukemia (CD11c(−), CD25(−), CD103(−) are lacking and the cells display a villous appearance, the possibility of splenic marginal zone lymphoma can be raised, particularly if the patient has splenomegaly.[3,5]

Flow cytometric studies can also assist in the assessment for neoplasms of T- or NK-cells, but the morphologic appearance and immunophenotype are often less distinctive than for B-cell neoplasms. For example, the morphologic appearance and immunophenotype of T-cell prolymphocytic leukemia (PLL) are both quite variable, but this diagnosis should be considered in a patient with hepatosplenomegaly and rapidly increasing lymphocytosis composed of mature T-cells, particularly if there is a dual CD4+ and CD8+ phenotype, with no loss of pan-T-cell antigens.[3,5] In other circumstances where the phenotype is less distinctive, demonstrating the presence of the characteristic genotype of T-cell PLL, with TCL1 rearrangement, can assist in reaching a definitive diagnosis.

A diagnosis of large granular lymphocyte leukemia (LGLL) can be considered if there is overt lymphocytosis of large granular lymphocytes. In this setting flow cytometric studies can assist by distinguishing T-cell LGLL, usually CD2+, CD3+, CD5+(dim), CD7+(dim), CD8+, CD16+, CD57+, from a chronic lymphoproliferative disorder of NK-cell, usually CD2+, CD3(−), CD5(−), CD16+(dim/bright), CD56+/−,

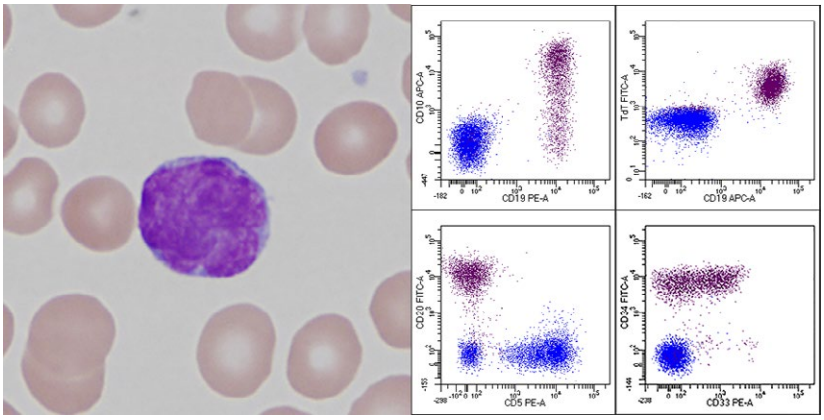

FIGURE 1 B lymphoblastic leukemia. A 5 year old boy with a persistent upper respiratory infection was found to be anemic and thrombocytopenic. Review of peripheral blood demonstrates few abnormal white blood cells, concerning for malignancy. Flow cytometric studies performed on the peripheral blood demonstrates B lymphoblasts (purple) identified as CD45+ (dim), low side light scatter events, with the following immunophenotype: CD19+, CD10+, TdT+, CD20+, CD5(−), CD34+, CD33+ (partial). Mature lymphoid cells identified as CD45+ (bright), low side light scatter events are shown for comparison (blue) [Colour figure can be viewed at wileyonlinelibrary.com]

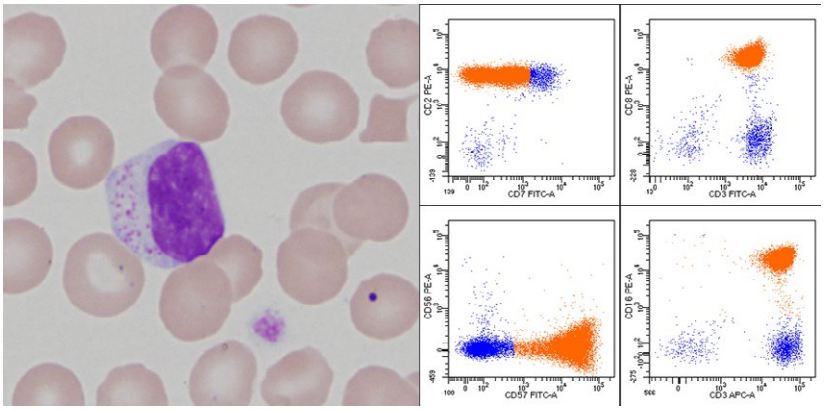

FIGURE 2 T-cell large granular lymphocyte leukemia (LGLL). An 82 year old man was found to have persistent lymphocytosis (absolute lymphocyte count 29.4×10^9/L). Peripheral blood smear demonstrates a uniform population of small mature lymphoid cells containing azurophilic granules. Flow cytometric studies performed on the peripheral blood with evaluation of lymphoid cells identified as CD45+ (bright), low side light scatter events, demonstrates a population of T-cells with an aberrant immunophenotype (orange): CD2+, CD7(−)/+(dim), CD3+, CD8+, CD56(−), CD57+, CD16+. These abnormal cells were also CD5(−) and CD4(−) (not shown). Other lymphoid cells are shown for comparison (blue) [Colour figure can be viewed at wileyonlinelibrary.com]

CD57(−).[6] In addition, flow cytometric studies can help to distinguish LGLL from more aggressive neoplasms, such as aggressive NK-cell leukemia[6-8] (Figure 2).

2.2 | Borderline lymphocytosis

The role of flow cytometry immunophenotyping is less well established in the setting of borderline lymphocytosis. For some disorders the peripheral blood appearance is distinctive, even when there are only a few circulating cells. For example, a diagnosis of Sezary syndrome can be reached with a combination of the large abnormal Sezary cells with characteristic nuclear folding, together with the following typical, but not unique T-cell phenotype: CD2+, CD3+, CD5+, CD7(−), CD4+, CD8(−).[3,5] For some other disorders with a low level of peripheral blood involvement, the immunophenotype is diagnostic, even without a morphologic correlate, such as for hairy cell leukemia: CD19+, CD20+ (bright), CD11c+ (bright), CD25+, CD103+, sIg+ (bright).[9] However, borderline lymphocytosis of small mature cells often demonstrates flow cytometric findings that are of uncertain clinical significance.

Monoclonal B-lymphocytosis (MBL) is the term coined for monotypic B-cells with an absolute count $<5.0\times10^9$/L, in the absence of clinical evidence of an overt lymphoid neoplasm. Although MBL may have a CD5(−), CD10(−) non-CLL-like and non-hairy cell leukemia-like immunophenotype, more information is known about MBL with a CD5+, CD10(−) CLL-like immunophenotype. CLL-like MBL is age related, with an incidence <2% below age 40 years, and approximately 7.5% above age 75 years, and is possibly due to immune senescence or persistent immune stimulation. Although CLL-like MBL was recognized because the findings were considered insufficient to initiate therapy for CLL, identification might be of clinical importance because patients are at increased risk of infection, and each year approximately 1% of patients with MBL will progress to overt CLL.[10]

A borderline absolute lymphocytosis with clonal T-cells is less well recognized, but again appears to be age-related, most often occurring over 60 years of age. The term T-cell clonopathy of undetermined significance has been proposed, and hypothesized to be related to immune senescence, with a reduced repertoire of CD8+ T-cells and impaired efficacy of immunity to specific antigens, such as CMV.[11] On peripheral blood smear review there may be increased large granular lymphocytes (LGL) and/or small mature lymphocytes, and by flow cytometry there is often increased CD8+, or double CD4+ and CD8+ positive T-cells (Figure 3). Caution should be exercised in evaluating flow cytometric data for immunophenotypic aberrancy, since some non-neoplastic T-cell have an unusual phenotype, such as the normal subset of CD5(−), CD8+ T-cells.[12] It's worth emphasizing that borderline T-cell lymphocytosis is often clonal using flow cytometric and molecular diagnostic studies, but this finding does not equate to neoplasia and likely reflects limited heterogeneity of the immune response.[13,14] Following allogeneic stem cell, solid organ, and autologous transplant there is often a mild increase in LGL, with a cumulative incidence of 20% at 2 years following transplant.[15,16] It has been proposed that post-transplant T-cell lymphocytosis may be a result of constant antigenic stimulation and/or chronic infection, and demonstrates a strong association chronic GVHD and CMV status. Post-transplant lymphocytosis usually has a CD8+, CD57+ immunophenotype similar to LGLL and is mostly clonal, but unlike many cases of LGLL is not associated with splenomegaly or persistent neutropenia.

NK cell lymphocytosis of uncertain clinical significance has also been described, and may have restricted expression of KIR antigens, CD158a, CD158b, CD158e, and CD94/NKG2A, suggesting clonality.[6,17] Of interest, increased NK cells, cytotoxic T-cells and gamma-delta T-cells have been described at diagnosis in chronic myeloid leukemia (CML) and multiple myeloma, and are often monoclonal or oligoclonal. In addition, lymphocytosis has been described in patients with CML receiving treatment with the second generation tyrosine kinase inhibitor Dasatinib.[18]

Therefore, in the setting of borderline lymphocytosis without abnormal cells on review of peripheral blood smear, correlation with

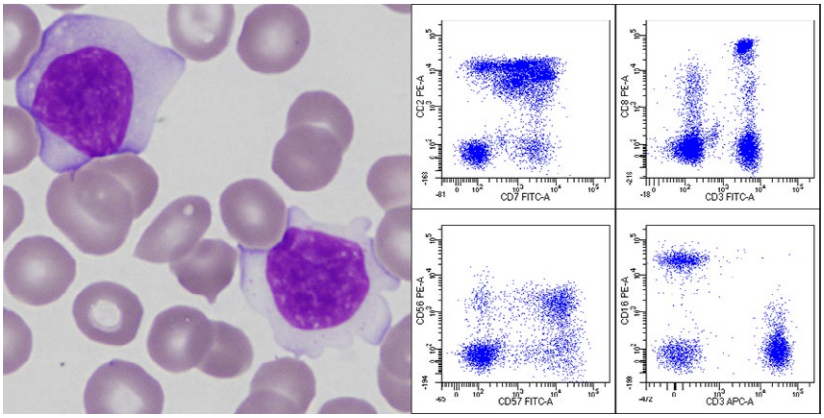

FIGURE 3 Persistent mild lymphocytosis of uncertain clinical significance. An 82 year old woman was found to have persistent mild lymphocytosis (most recent absolute lymphocyte count 5.6×10^9/L). Peripheral blood smear demonstrates increased mature lymphoid cells, including some large granular lymphocytes. Flow cytometric studies performed on the peripheral blood with evaluation of lymphoid cells identified as CD45+ (bright), low side light scatter events, demonstrates heterogeneous populations of cells, including CD16+, CD3(−) NK-cells and multiple subsets of CD3+ T-cells with variable expression of CD56 and CD57 and absence of CD16. Further analysis of NK-cells demonstrated the following phenotype CD56+, CD57+ (partial), CD94/NKG2a heterodimers+(partial), and polytypic for expression of the KIR antigens CD158a, CD158b and CD158e [Colour figure can be viewed at wileyonlinelibrary.com]

the clinical findings is essential in order to avoid unnecessary flow cytometry.

2.3 | Atypical/variant or abnormal lymphoid cells and plasma cells

Peripheral blood smear review may identify cells with an appearance that is so abnormal it indicates malignancy, such as a uniform population of large cells, or smaller cells with marked nuclear irregularities. In this situation, flow cytometry can assist with indicating a specific subtype of lymphoid neoplasm. As mentioned above, there are some lymphoid neoplasms with a relatively distinct morphologic appearance, such as in Sezary syndrome. Adult T-cell leukemia/lymphoma (ATLL) can also be recognized when there are peripheral blood lymphoid cells with a characteristic "flower cell" appearance due to marked nuclear indentations, particularly when associated with the following characteristic immunophenotype: CD2+, CD3+, CD5+, CD7(−), CD4+, CD25+ (bright).[3,5] However, the morphologic appearance of Sezary syndrome and ATLL may not be characteristic, and the immunophenotype of either neoplasm is not unique. Therefore, there are times that peripheral blood flow cytometry may be insufficient to establish a diagnosis, but can narrow down the diagnostic possibilities and suggest the need for additional specimens.

Peripheral blood smear review may also identify atypical lymphoid cells with a reactive appearance, such as associated with a viral infection. This finding does not require flow cytometric studies, and in the correct clinical context can be valuable for establishing a diagnosis of infectious mononucleosis.[19] Therefore, it is important to report these finding with clarity eg, "atypical, reactive-appearing lymphoid cells", rather than "abnormal lymphoid cells, concerning for a lymphoproliferative disorder".

Plasma cells may be present in the peripheral blood as part of a reactive condition, where they typically form part of a heterogeneous mixture of lymphoid cells. Circulating plasma cells may also be seen in plasma cell myeloma and lymphoma with plasmacytic differentiation. Recognition of plasma cell leukemia is important, as defined by circulating neoplastic plasma cells representing >20% of total WBC or with an absolute count $>2\times10^9$/L, and is associated with a poor prognosis. Circulating plasma cells have also been reported to indicate a poor prognosis for patients with smoldering myeloma.[20] Peripheral blood smear findings that raise the possibility of a neoplastic process with plasmacytic differentiation include lack of the heterogeneous mixture of atypical/reactive appearing lymphoid cells described above, rouleaux suggesting the presence of a serum paraprotein, or the presence of morphologically abnormal plasma cells. Abnormal plasma cells may have prominent nucleoli, less clumped chromatin than normal plasma cells, or intranuclear inclusions. Flow cytometric studies can assist in identifying plasma cells, through expression of bright CD138 and CD38, and assessing for an aberrant immunophenotype, such as CD56+, CD19(−) plasma cells.[21]

2.4 | Blasts and other immature cells

Peripheral blood blasts, promyelocytes and promonocytes can be seen in both reactive and neoplastic disorders. Morphologic features may assist in determining their etiology, with reactive conditions often displaying a complete spectrum of cells at different stages of maturation and associated neutrophil toxic changes, whereas a neoplasm might be recognized by Auer rods or morphologically abnormal cells. For many specimens the etiology of circulating blasts remains uncertain after smear review, and flow cytometric studies can assist by evaluating for an aberrant immunophenotype, such as myeloid blasts with abnormal expression of CD7 or TdT. However, the combined morphologic and immunophenotypic findings are usually insufficient to establish a diagnosis of a specific myeloid neoplasm. For example myeloid blasts with an aberrant immunophenotype might be seen in

acute myeloid leukemia (AML), mixed phenotype acute leukemia, a myelodysplastic syndrome or primary myelofibrosis, and therefore often requires bone marrow evaluation for diagnosis. The presence of immature B- or T-lymphoblasts in the peripheral blood is considered an abnormal finding, although there are rare reports of circulating normal B-cell progenitors (hematogones). However a circumstance that might require caution is the identification of very rare B-lymphoblasts in the setting of CML. Although the latter finding is very worrisome for blasts crisis, there have been reports of patients with CML with rare bone marrow B lymphoblasts identified by flow cytometry who do not develop progressive disease.[22] Therefore, bone marrow examination is probably warranted.

2.5 | Monocytosis

Absolute monocytosis can be seen in reactive conditions and neoplastic disorders, such as chronic myelomonocytic leukemia (CMML) and AML with monocytic differentiation. A relative monocytosis that includes immature cells can also be seen with recovery from bone marrow suppression. Unfortunately, both peripheral blood smear review and flow cytometric evaluation are often of limited value in distinguishing reactive and neoplastic monocytes. Probably the most definitive peripheral blood smear finding is overt leukocytosis with a uniform population of promonocytes (considered blasts equivalents) indicative of AML with monocytic differentiation. However, the presence of mostly mature monocytes is less predictive, because AML with monocytic differentiation often has fewer immature cells in the peripheral blood than the bone marrow and therefore, may mimic CMML or a reactive monocytosis. Flow cytometry can assist in evaluating monocytes for an abnormal immunophenotype, such as expression of CD56, which has been reported in the majority of CMML.[23] However, these findings are not specific for CMML, being present in some non-neoplastic monocytes.[23] A recent study reported an increase in the classical CD14+, CD16(−) monocyte subset in CMML in comparison to controls and reactive monocytosis, but these findings need to be confirmed.[24]

2.6 | Eosinophilia

Absolute eosinophilia usually represents a reactive process, but can also be part of a myeloid or lymphoid neoplasm. Flow cytometric studies can assist in identifying a lymphoid neoplasm with associated reactive eosinophilia, particularly if abnormal lymphoid cells are identified on peripheral blood smear review. Immunophenotyping is also of value for the group of myeloid and lymphoid neoplasms with eosinophilia and rearrangements of PDGFRA, PDGFRB, or FGFR1 genes, particularly for lineage assignment.[5] In the setting of a hypereosinophilic syndrome (absolute eosinophil count >1.5×10^9/L for more than 6 months and evidence of associated tissue damage), flow cytometric studies might also be of interest to identify lymphocyte-variant hypereosinophilia. Lymphocyte-variant hypereosinophilia represents the combination of an abnormal T-cell population demonstrated by peripheral blood lymphocyte flow cytometric immunophenotyping or T cell receptor gene rearrangement studies, together with reactive eosinophilia, probably as a result of cytokine production. Immunophenotypic findings include abnormal T-cells lacking CD4 or CD8, or lacking CD3, sometimes together with lack of CD7 or increased expression of CD5, and expression of the markers of activation CD25 and HLA-DR. Patients with lymphocyte-variant hypereosinophilia usually present with skin manifestations, and often have systemic manifestations. Therefore, flow cytometry evaluation of peripheral blood might be of interest in a patient with eosinophilia, even if no morphologically abnormal cells are identified on smear review, but correlation with the clinical information is essential.[25]

2.7 | Neutrophilia and basophilia

Flow cytometric immunophenotyping is of limited value in the assessment of absolute basophilia and neutrophilia. Although immunophenotypic abnormalities have been described in CML, molecular studies for the characteristic BCR/ABL gene rearrangement are usually more important.[5]

2.8 | Pancytopenia, and other cytopenias

Peripheral blood flow cytometry may be of value in patients with pancytopenia, particularly if there is clinical suspicion for hairy cell leukemia, such as due to associated monocytopenia and splenomegaly. The immunophenotype of hairy cell leukemia is very distinctive and can be detected at very low levels in the peripheral blood by flow cytometry, even when not detected by smear review.[9] If circulating blasts are present, flow cytometry might be of interest to evaluate for phenotypic abnormalities that might provide evidence of an underlying hematolymphoid neoplasm, particular if there is hesitation to perform bone marrow evaluation. Flow cytometric studies also have a role in the assessment for a myelodysplastic syndrome, and may detect abnormalities in the peripheral blood, but these studies are usually reserved for bone marrow specimens.[26]

3 | CONCLUSION

In summary, the combination of manual peripheral blood smear review and flow cytometric studies provides very useful information for the evaluation for lymphoid neoplasms and acute leukemia. In some circumstances a definitive diagnosis can be established from the combined peripheral blood morphologic and immunophenotypic findings, obviating the need for bone marrow evaluation, such as for CLL. Occasionally flow cytometric studies are superior to morphologic assessment, such as in screening for hairy cell leukemia or identifying lymphocytic-variant hypereosinophilia. However, there is increasing recognition of small immunophenotypically unusual or abnormal populations of peripheral blood cells, particularly in older patients, which are of uncertain clinical significance, such as monoclonal B lymphocytosis and T-cell clonopathy. Therefore, it is important to integrate peripheral blood smear review findings with the clinical and other information before recommending flow cytometry. In addition, it is

important to recognize discordance between the clinical information and peripheral blood smear review and flow cytometric findings, such as patients with angioimmunoblastic T-cell lymphoma who may not have circulating lymphoma cells and present with peripheral blood polyclonal plasmacytosis and hypergammaglobulinemia.[27]

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