Flow Cytometric Assessment of T-cell Chronic Lymphoproliferative Disorders
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As in the assessment of B-cell lymphoproliferative disorders, flow cytometry is often used in the evaluation of peripheral blood and bone marrow specimens for potential involvement by T-cell malignancies. Unlike immunoglobulin, however, with its dichotomously expressed kappa and lambda light chains, the T-cell antigen receptor (TCR) lacks an easily analyzed structural element that can provide a surrogate marker of clonality. As a result, abnormal T-cell populations are detected by flow cytometry through the identification of aberrant expression patterns of T-cell and natural killer cell (NK-cell)–associated antigens. These findings are then typically substantiated through the demonstration of T-cell clonality either through high complexity flow cytometric evaluation of the TCR or molecular genetic assessment of TCR gene rearrangements. One such example of the former is TCR-Vβ flow cytometry, in which fluorescently labeled antibody reagents to specific TCR β-chain variable region family members are used. In this assay, clonality can be determined when the T-cell population of interest expresses a predominance of a single TCR-Vβ, when compared with the reactive T-cells present.

The topics of aberrant immunophenotypes and TCR clonality raise two important caveats in diagnosing a T-cell lymphoproliferative disorder (TLPD). First, immunophenotypic “aberrancies” can be encountered in reactive T-cell populations. Second, reactive T-cell populations, particularly in the setting of chronic stimulation, may comprise a limited number of clonotypes (ie, are oligoclonal) and therefore are prone to producing false-positive clonal TCR rearrangement results. Given these confounding elements, it is imperative that one has a basic understanding of T-cell biology when
combining flow cytometric and molecular genetic findings to formulate a specific diagnosis.

T-cells are bone marrow–derived lymphocytes that share, as a unifying feature, the expression of a TCR heterodimer formed from the unique rearrangement of elements of the TCR genes [1]. This TCR heterodimer may comprise either α and β or γ and δ chains, and T-cells are thus broadly divided into αβ or γδ types (Fig. 1). These T-cell types are unique in their ontogeny and tissue distribution. αβ T-cells mature and differentiate in the thymus and constitute over 95% of all T-cells in adults. These αβ T-cells play a fundamental role in the generation and execution of antigen-specific immune responses and are found throughout the body being particularly prevalent in the circulation and lymph nodes. In contrast, γδ T-cells mature through a thymus-independent pathway and seem to have functions more closely linked to innate immunity (ie, immune responses not requiring antigen priming). The discrete niche γδ T-cells occupy in the immune system is belied by their somewhat unusual distribution because these cells primarily reside in mucosal sites and the spleen, areas where they can readily provide a “first line” of defense against environmental pathogens.

Although the TCR provides a single lineage-defining protein, there are several other proteins and protein complexes preferentially expressed by T-cells, which are of great use during flow cytometric evaluation. Perhaps foremost among these is CD3, a multimeric protein complex associated with the TCR heterodimer (see Fig. 1). TCR heterodimer expression is required for the assembly of the full CD3 complex, and conversely, assembly of the CD3 complex is required for expression of TCR heterodimer on the cell surface. Given the close relationship between these proteins, they are often considered as synonymous entities. NK-cells, by definition, lack the

![Antigens Expressed By T-cells](image)

Fig. 1. Schematic of T-cell antigen expression. All T-cells express a T-cell receptor heterodimer comprising either α and β or γ and δ chains, which is associated with the multiprotein CD3 complex (left). αβ T-cells also express either CD4 or CD8; the latter is a dimer comprised of either two α chains or an α and β chain (right). CD2, CD5, and CD7 are uniformly expressed by CD4 and CD8-positive T-cells.
TCR heterodimer and therefore the fully assembled CD3 complex; they do however express the CD3 ε and ζ chains. This is not of particular relevance for flow cytometric immunophenotyping because this method typically uses antibodies that recognize epitopes formed by fully assembled CD3. In contrast, the expression of these antigens by NK-cells is important when considering paraffin-embedded immunohistochemistry, because in this method, antibodies to CD3ε chain are typically used, and therefore NK-cells can be “CD3-positive” by this technique.

There are several other pan–T cell antigens apart from CD3 that are routinely analyzed in the clinical laboratory (see Fig. 1), including CD2, CD5, and CD7. Although these antigens are most frequently and strongly expressed by T-cells, they lack the complete T-cell lineage specificity of the CD3-TCR complex.

CD2 is a T-cell–associated antigen that was first identified through its ability to cause sheep erythrocytes to adhere to these cells. It has since been recognized that CD2 is a cellular adhesion molecule, which binds CD58 and potentiates signal transduction through CD3ε [2,3]. This antigen is expressed by mature and thymic T-cells; other cells types that are CD2-positive include NK-cells and a subset of thymic B-cells [4,5]. CD2 is not typically expressed by other normal or abnormal hematopoetic cells and, as such, is generally a reliable indicator of T- or NK-cell lineage.

CD5 is a glycoprotein that, like CD2, was an early-characterized T-cell–associated antigen. This antigen is thought to participate in lymphocyte selection and activation and is uniformly expressed on mature and thymic αβ T-cells. In contrast, normal γδ T-cells may have diminished or absent CD5 expression (Roden and Morice, unpublished data, 2006). CD5 is also present on a subset of memory B-cells (B1a cells) and is aberrantly expressed in several B-cell chronic lymphoproliferative disorders, such as B-cell chronic lymphocytic leukemia [6]. This antigen is also found on select nonhematolymphoid cells including thymic epithelial cells.

CD7 is another antigen ubiquitously expressed by T-cells. This antigen is a member of the immunoglobulin gene superfamily that functions as a costimulatory molecule during T-cell activation [7]. CD7 is also found on several other cell types including B-cell and myeloid lineage precursors, NK-cells, and a subset of monocytes. Furthermore, although “aberrantly” diminished CD7 expression is a recognized feature of some TLPDs, particularly those arising in the skin, it can also be seen in reactive T-cell expansions secondary to viral infection, chronic inflammatory dermatoses, and drug-related cutaneous eruptions. These factors limit the specificity of “abnormal” patterns of CD7 expression as an isolated finding in TLPDs [8].

CD25, also referred to as the low-affinity receptor for interleukin-2 (IL-2), is actually the inducibly expressed α subunit of the complete IL-2 receptor complex, which also includes the constitutively expressed β and γ IL-2 receptor subunits (CD122 and CD123, respectively). High levels of CD25 on CD4-positive T-cells also seem to delineate a distinct regulatory subset
with the capacity to inhibit CD25 negative cells. CD25 is also expressed on a subset of circulating B-cells [9,10]. Assessment of CD25 expression has limited diagnostic utility, but its measurement has become more widespread because it has proved an attractive target for cell specific immunotherapy in TLPDs with the advent of new types of pharmacologic agents such as denileukin difitox (Ontak), an IL-2-diptheria toxin fusion protein [11,12].

Although T-cells can be broadly subcategorized by the TCR heterodimer expressed, there are several other phenotypic and/or functionally defined T-cell categories that are particularly relevant to the diagnostic flow cytometry laboratory. Chief among these is the distinction of the CD4-positive and CD8-positive αβ T-cell subsets. CD4 stabilizes TCR interactions with antigen presented by major histocompatibility complex (MHC) II, and therefore CD4-positive T-cells interact with MHC-II positive antigen presenting cells, such as B-cells and macrophages. These interactions usually cause the T-cells to elaborate cytokines requisite for normal immune responses, and therefore they are referred to as CD4-positive “helper” T-cells. In contrast, CD8 stabilizes TCR-MHC-I interactions and this interaction typically causes the T cell to destroy the antigen-positive cells. As such, CD8-positive cells are particularly important in killing cells infected by intracellular viral pathogens. Although CD8-positive T-cells produce several cytokines to orchestrate this type of immunity, they are generally referred to as “cytotoxic” or “killer” T-cells. This bimodal scheme is likely an oversimplification of a complex biological system, and certainly cells with overlap function between these groups can be identified. However, it does provide a useful and applicable context for considering mature TLPDs. γδ T-cells are typically either CD4 and CD8 double negative or CD8 positive; they will be considered with cytotoxic T-cells because they bear a close functional relationship to this T-cell type [1].

Lymphoproliferative disorders of CD4-positive T-cells

There are several CD4-positive TLPDs that preferentially involve the peripheral blood. In many of these disorders, there may be minimal lymph node or other tissue-based disease, and flow cytometric evaluation of a peripheral blood or bone marrow specimen is often the initial, and sometimes the only, diagnostic test performed. Although many of these disorders have prototypic immunophenotypic features, these features are rarely disease specific, and accurate diagnosis requires correlation with the clinical, morphologic, molecular genetic, and cytogenetic findings.

T-cell prolymphocytic leukemia/chronic lymphocytic leukemia

T-cell prolymphocytic leukemia (T-PLL) is an uncommon, distinct lymphoproliferative disorder of postthymic mature CD4-positive T-cells [13]. T-PLL affects the middle aged to elderly and typically presents with
a striking lymphocytosis, anemia and thrombocytopenia. Splenomegaly is common and is less frequently accompanied by hepatomegaly, generalized adenopathy, and cutaneous eruptions. [14,15]. T-PLL is an aggressive lymphoproliferative disorder with a median survival of less than 1 year when treated with conventional chemotherapy. A subset of patients shows a more indolent course in which the disease may respond to anti-CD52 monoclonal antibodies [16,17].

The neoplastic T-cells of T-PLL show a wide morphologic spectrum ranging from cases with numerous prolymphocytes having large nuclei, reticular chromatin, and single prominent central nucleoli (most cases) to cases with increased numbers of small, cytologically bland lymphocytes (the minority of cases). Some cells may have irregular nuclei, and in rare cases, the degree of nuclear convolution may approximate that of Sezary cells. Some controversy persists regarding the nomenclature of this disorder as cases with small, bland nuclei have been termed the small cell variant of T-PLL by some authors and T-cell chronic lymphocytic leukemia (T-CLL) by others [18,19]. Although splenomegaly may be less prominent in cases with small lymphocyte cytology, the clinical behavior and prognosis are otherwise indistinguishable, and as a result, many use the combined moniker T-PLL/CLL to encompass all of the cytologic variants of this entity.

The neoplastic T-cells of T-PLL/CLL have an immunophenotype typical of mature postthymic T-cells with expression of CD2, CD3, CD5, and CD7. Aberrantly diminished or absent expression of these antigens in T-PLL/CLL is uncommon, although diminished staining intensity for CD3 may be seen. In addition, abnormally uniform or “tight-staining” for the T-cell–associated antigens is occasionally encountered in T-CLL/PLL (Fig. 2A). This staining pattern belies the clonal nature of the T-cell expansion and may be seen in other TLPDs as well. T-PLL/CLL lacks CD25 and, like other mature TLPDs, the thymocyte/blast-associated antigens CD1a, CD34, and TdT. Over 60% of cases are CD4+ and CD8-, the remaining are usually dual CD4+ and CD8+, although rare CD8+ and CD4- T-PLL/CLL have been described. CD8+ T-PLL/CLL lacks the properties of cytotoxic T-cells such as the presence of cytotoxic granules or the expression of NK-associated receptors.

T-cell clonality can be documented in virtually all T-PLL/CLL by either assessment of TCR-Vβ usage by flow cytometry (Fig. 2B) or analysis of TCR gene rearrangement by polymerase chain reaction or Southern blot. Directly documenting clonality in the cell population of interest along with a rapid turn-around time are two distinct advantages of TCR-Vβ flow cytometry [20]. In over 80% of T-PLL/CLL cases either an inv(14)(q11;q32) or a t(14;14)(q11;q32) can be identified. Both of these cytogenetic abnormalities lead to fusion of the TCL1 gene on chromosome 14q32.1 with the TCR α/δ gene(s) on chromosome 14q11, which causes overexpression of TCL-1 oncoprotein [21–23]. When a diagnosis of T-PLL/CLL is suspected, one should test for either TCL1/TCRα/δ fusion or

\[ \text{TCL1/TCRα/δ fusion or} \]
Fig. 2. Flow cytometry in a case of T-cell PLL/CLL. (A) Gating on the lymphocytes (upper, forward, and side light scatter) reveals increased numbers of CD4-positive T-cells (arrow, lower right). These cells do not show antigen loss; however, they have abnormally uniform staining intensity for T-associated antigens, particularly CD2 and CD5 (arrow, lower left). (B) Analysis of TCR-Vβ reveals the total CD3-positive T-cells (upper) and the CD3-positive, CD8-negative T-cells (lower) to have uniform, clonotypic expression of TCR-Vβ 13.2 (small arrows, right). The TCR-Vβ flow is a five-color assay that includes antibodies to CD3, CD8, and three TCR-Vβs labeled with FITC, PE, and dual fluoroisothiocyanate & phycoerythrin, respectively.
TCL-1 protein expression. In addition, an association of T-PLL/CLL with Ataxia Telangiectasia has been described, and in approximately 40% to 50% of cases, abnormalities of the Ataxia Telangiectasia Mutated gene on chromosome 11q23 can be detected by molecular genetic analyses [24].

**Adult T-cell leukemia/lymphoma**

Adult T-cell leukemia/lymphoma (ATLL) is a mature TLPD associated with the human T-cell lymphotropic virus-1 (HTLV-1) [25,26]. HTLV-1 is considered the cause of ATLL, although HTLV-1 infection alone is not a transforming event, and the time span between infection and progression to ATLL is often several decades. ATLL is most commonly encountered in geographic areas where HTLV-1 sero-positivity is endemic, such as southern Japan, the Caribbean, sub-Saharan Africa, and Central and South America [27–29].

The clinical features of ATLL vary. Typically, cases present as a systemic illness characterized by constitutional symptoms, prominent PB involvement, organomegaly, cutaneous eruption, adenopathy, and hypercalcemia [29,30]. This is often referred to as the “acute” form of ATLL and is associated with a particularly dismal prognosis. Infrequently, ATLL may have a less fulminate clinical presentation; included in this group are “chronic” and smoldering ATLL, which typically manifests with transient skin lesions and little peripheral blood involvement. The prognosis of these more indolent ATLL-variants is still poor with median 2-year survivals of 78% and 52% for the chronic and smoldering forms, respectively (compared with 20% for the acute ATLL) [29–32].

In the acute form of ATLL, peripheral blood examination often reveals increased numbers of lymphocytes with medium to large-sized polylobate nuclei (leading to the eponym “flower cells”) and deeply basophilic cytoplasm. Nucleoli may be prominent. The cytologic atypia is less pronounced in the chronic and smoldering variants.

The phenotype of ATLL is that of mature CD4-positive T-cells. Diminished expression of CD7 is a frequently described abnormality in ATLL; however this “aberrancy” has little disease specificity because it may be encountered in leukemic phase cutaneous T-cell lymphomas and in reactive T-cell populations [33]. The expression of the T-associated antigens CD2, CD3, and CD5 in ATLL usually differs little from normal cells. For these reasons, it may be difficult to identify the abnormal cells of ATLL by conventional flow cytometry alone, particularly in the more indolent disease forms with a low PB disease burden [29,34–36]. High levels of CD25 (IL-2 receptor α) expression may be a distinguishing feature of ATLL, and detection of this phenotype may not only aid in diagnosis, but may provide laboratory data to support the use of targeted biomodulator therapy. Other less commonly described immunophenotypic abnormalities include dual CD4 and CD8 expression and CD30 positivity in the large cells [34]. T-cell
clonality should be demonstrable in the acute and indolent disease forms. Given the nonspecific immunophenotype of ATLL, one must be vigilant to test for HTLV-1 when a CD4-positive TLPD is encountered and/or when the social or travel history indicates possible exposure [37].

**Sezary syndrome/cutaneous T-cell lymphoma**

The evaluation of peripheral blood specimens for potential involvement by cutaneous T-cell lymphoma (CTCL) is perhaps one of the most common indications for T-cell phenotyping in the clinical flow cytometry laboratory. This presents a diagnostic challenge, in part because of misunderstandings regarding the nomenclature for this group of TLPDs. Sezary syndrome (SS) refers to a distinct clinicopathologic entity in which there is erythrodermic CTCL associated with ectropion, plantar/palmar keratoderma, leukemic peripheral blood involvement, and lymphadenopathy. SS is aggressive and associated with a poor prognosis. In contrast, mycosis fungoides, which is more prevalent than SS, is typically indolent, localized, and shows limited peripheral blood involvement. Confusion is created, however, because there can be overlap between these disease entities with mycosis fungoides type CTCL progressing to a disease more akin to SS. In addition, there are reactive dermatoses that may clinically mimic erythrodermic CTCL and be associated with cytologically and immunophenotypically distinct circulating CD4 positive T-cells. In both the reactive states and peripheral blood involvement by CTCL, the elevation in the peripheral blood lymphocyte count is often modest [38]. These factors create difficulties in detecting and diagnosing peripheral blood involvement by CTCL. Although SS and mycosis fungoides represent distinct clinical entities, they are discussed together because the features of the tumor cells, when present, are similar in each.

In Sezary’s [39] early descriptions of SS, peripheral blood involvement was detected through the identification of large, cytologically atypical lymphoid cells with markedly convoluted nuclei (so called “cellules monstrueuses”). The detection of lymphocytes with “cerebriform” nuclei has remained a fundamental element in evaluation for peripheral blood involvement by CTCL; however, as mentioned in the discussion of T-PLL/CLL, cells with these cytologic features may be seen in other TLPDs. Furthermore, circulating lymphocytes with small, cerebriform nuclei may be seen in reactive dermatoses and even PB specimens from normal adult controls [40]. For these reasons, morphologic correlation with flow cytometric T-cell phenotyping, T-cell clonality assessment, and the clinical features are all critical.

The CTCLs that commonly involve the peripheral blood are almost exclusively diseases of mature CD4-positive T-cells. Several immunophenotypic aberrancies have been described in circulating CTCL cells; most common among these is diminished expression of CD7, which can be seen in over 80% of cases [41–43]. In isolation, however, CD7 “aberrancy”
must be interpreted with caution because frequently only a subset of the neoplastic cells has decreased CD7 expression and a similar patterns of decreased or lost CD7 can be encountered in reactive T-cell populations. Other abnormalities that can be seen in CTCL include diminished expression of CD2 and/or CD3, which may be present in approximately one third of cases; loss of CD26; and abnormally diminished expression of CD4 [44,45]. Unusually bright expression of CD5 has also been described. These findings, though less common than decreased CD7 expression, have a much higher degree of disease specificity, and their presence should prompt vigilant assessment for CTCL or possibly another TLPD.

Establishing T-cell clonality is a requisite element in diagnosing peripheral blood involvement by CTCL given the clinical, cytologic, and immunophenotypic overlap with reactive dermatoses. T-cell clonality can be demonstrated either by molecular genetic assessment of TCR gene rearrangement or Vβ flow analysis. An advantage of the former is the results can be directly compared with results from a separately analyzed diagnostic skin biopsy to establish clonal identity between these disparate anatomic sites. These types of comparisons cannot be performed with Vβ flow, because this method is difficult to employ on tissue biopsies. A distinct advantage of Vβ flow, however, is that it can be used to directly demonstrate clonality in an immunophenotypically distinct T-cell population through combination with other antibody reagents. The detection of T-cell clonality, when associated with the presence of a CD4 positive T-cell population with a distinct immunophenotype (such as diminished CD3 or CD26), can allow a laboratorian to render a diagnosis of a TCLPD with relative confidence and raise the possibility of CTCL.

Angioimmunoblastic T-Cell lymphoma and other nodal based T-cell lymphomas

There are numerous manuscripts in the published literature espousing the value of flow cytometry in diagnosing lymph node or other tissue involvement by peripheral T-cell lymphomas. Although flow cytometry contributes to making these diagnoses, the reader is cautioned in several regards. Foremost is that the accurate interpretation of flow cytometry results requires correlation with the histopathologic findings in paraffin-embedded material. This is particularly crucial as T-cell immunophenotypes associated with TLPDs may also be seen in nonneoplastic T-cell populations. This concept is well-illustrated by a report from Cook and colleagues [46] in which T-cell coexpression of CD10, a feature associated with angioimmunoblastic T-cell lymphoma, was detected in the benign T-cells in reactive lymphoid hyperplasias and associated with B-cell malignancies. For this reason, T-cell phenotyping by flow cytometry should only be performed when there is ample tissue available, and the results must be interpreted in context of the concurrent histologic findings.
Among the lymph node–based mature TLPDs, flow cytometric findings are probably best described for angioimmunoblastic T-cell lymphoma (AITL). AITL typically affects adults and presents with a constellation of clinical features including generalized lymphadenopathy, organomegaly, anemia, polyclonal hypergammaglobulinemia, and cutaneous eruption [47–50]. Lymph node biopsy is required to establish a diagnosis of AITL, yet it is important to be cognizant of the immunophenotype of this disease because it often involves the peripheral blood and bone marrow, and the protean clinical manifestations may obfuscate the diagnosis.

AITL is a neoplasm of CD4-positive T-cells, which usually show unremarkable expression of CD2, CD5. These cells often have diminished or lost CD7 expression, although as in CTCL, this is usually only present in a subset of the neoplastic cells. Aberrantly diminished or lost expression of CD3 is also frequent in AITL [51]. This can create problems in gating on and identifying the abnormal cells, and for this reason Baseggio and colleagues [52] advocate an alternative gating strategy predicated on CD4+ T-cells when AITL is suspected. Aberrant coexpression of CD10 byAITCL cells can be identified by paraffin immunoperoxidase and flow cytometric techniques in up to 90% of cases, although it is usually expressed only by a subset of the neoplastic cells [51–54]. This immunophenotype seems to distinguish AITL from other mature TLPDs although, as previously mentioned, T-cell expression of CD10 can be detected in nonneoplastic T-cells. AITL is also positive for the cytokine CXCL13, although this nuclear antigen cannot currently be detected by flow cytometry. The expression of CD10 and CXCL13 lend credence to the evolving concept that AITL is a neoplasm of T-cells, which normally reside in and organize the germinal center [55,56].

Anaplastic large cell lymphoma, another nodal based T-cell lymphoma, has several noteworthy immunophenotypic features, including frequent aberrant expression of myeloid antigens and absent expression of T-cell–associated antigens. In a series of 19 anaplastic large cell lymphoma cases studied by flow cytometric immunophenotyping, Juco and colleagues [57] reported aberrant expression of at least one of the myeloid-associated antigens CD13, CD15, or CD33 in 60% of cases, with CD13 expression seen in 47% of the cases. In addition, CD3, CD5, and/or CD7 expression was present in only approximately 30% of cases, with CD2 being the most stably expressed T-cell–associated antigen, seen in 71% of cases [57]. Again, correlation of the flow cytometry with the tissue histopathology is critical in arriving at an accurate diagnosis because the limited expression of T-cell–associated antigens, the expression of myeloid lineage associated antigens, and the frequent extranodal presentation of anaplastic large cell lymphoma in pediatric patients may lead to confusion with extramedullary hematopoietic tumor. Myeloid antigen expression can be seen in other T-cell lineage neoplasms, most notably lymphoblastic tumors, which can exhibit similar immunophenotypic features including limited T-cell antigen expression and coexpression of CD13 and/or CD33.
Disorders of CD8-positive and other cytotoxic T-cells

In the past 10 to 15 years, several lymphoproliferative disorders of cytotoxic cells have been identified. These lymphomas have several characteristics in common including their rarity and their proclivity to arise in and involve extranodal sites. Given the predominant extranodal location, the biopsies obtained are typically small, and therefore flow cytometry is rarely performed. In contrast to these tissue-based diseases, T-cell large granular lymphocytic leukemia (T-LGL) is more prevalent and preferentially involves the peripheral blood and bone marrow. For these reasons, the evaluation of cytotoxic T-cells in the clinical flow cytometry laboratory most often centers on analysis for potential involvement by T-LGL.

T-large granular lymphocyte leukemia

The hallmark features of T-LGL are the presence of a distinct clonal population of cytotoxic T-cells in the peripheral blood and bone marrow that is accompanied by unexplained anemia and/or neutropenia. This disorder typically occurs in adults (median age 55 years), and an association with rheumatoid arthritis and other autoimmune phenomenon has long been recognized [58]. The lymphocytosis in T-LGL is usually modest (median lymphocyte count 7800 cells/μL), and the diagnosis is often first considered due to recurrent bacterial infections and/or fatigability, which are secondary to the disease-associated neutropenia and anemia, respectively [59].

The cytologic features of T-LGL are those of cytotoxic lymphocytes and are typified by the presence of cells with small bland nuclei and abundant cytoplasm containing variable numbers of azurophilic granules. Pronounced cytologic atypia in T-LGL is rare and when encountered should lead one to consider the possibility of a different, more aggressive lymphoproliferative disorder of cytotoxic lymphocytes. It is also noteworthy that the prominence of the cytoplasmic granularity varies between cases, and the cytoplasm of these cells is not always voluminous. For these reasons, performing absolute granular lymphocyte counts is problematic, and this is no longer emphasized as an important element of the diagnostic criteria.

Given the lack of distinguishing cytologic features in T-LGL, the identification of an immunophenotypically distinct cytotoxic T-cell population is a fundamental element in establishing the diagnosis. T-LGL is usually a disorder of CD8-positive αβ T-cells; although rare CD4-positive cases and γδ T-cell cases have been described [60]. By flow cytometry, T-LGL frequently shows abnormalities of T-cell antigen expression. Diminished or lost CD5 and CD7 expression is seen in approximately 90% and 80% of cases, respectively [61–63].

Aberrant expression of NK-cell associated antigens is a pathognomonic feature of T-LGL that is best demonstrated by flow cytometry. Early
reports emphasized the importance of CD57 coexpression in the diagnosis of T-LGL. Although frequent in T-LGL, this finding lacks disease specificity as CD57 is also expressed by normal memory cytotoxic T-cells [61]. CD16, a low-affinity FcγRIII receptor normally found on NK-cells, is expressed in over 80% of T-LGL. Unlike CD57, CD16 is rarely expressed by normal T-cells, and the detection of a CD16-positive T-cell population comprising over 30% of the total T-cells present should lead one to consider T-LGL [64]. There are different CD16 isoforms with specific antibodies to each. In T & NK-cell flow cytometry, it is important to use antibodies to the integral membrane form of CD16 expressed by these cell types and not the glycoposphatidyl inositol membrane bound form found on granulocytes. CD56 is expressed in only a minority of T-LGL cases [61].

A group of receptors expressed by NK-cells and a subset of cytotoxic T-cells that recognize MHC I and related antigens on potential target cells have recently been described. These receptors, termed the NK-cell associated receptors or NK-associated receptors, are broadly divided into two types, the lectin-family receptors, which include the CD94/NKG2 heterodimeric complexes, and the killer cell immunoglobulin-like receptors (KIRs) [64]. Although a detailed discussion of the NK-associated receptor biology is beyond the scope of this manuscript, one should be familiar with these receptors because they provide valuable targets for analysis in the evaluation of T-LGL and other lymphoproliferative disorders of cytotoxic lymphocytes. Of particular interest in T-LGL are the KIR antigens. There are multiple different KIRs, each of which recognize specific MHC I ligands. Normal cytotoxic lymphocytes show a polymorphic pattern of KIR expression, whereas in T-LGL, restricted expression of a single KIR antigen is seen in approximately two thirds of cases (Fig. 3) [61,63,65]. This restricted pattern of KIR expression in T-LGL aids in characterization of the abnormal cell population and provides indirect evidence of clonality. CD94 expression is also seen in T-LGL, although the frequency of CD94 positivity varies between reports [61–63]. In comparison to the KIRs, the expression of CD94 is of somewhat limited utility in diagnosing T-LGL because this antigen is more commonly expressed by normal T-cells, and it does not provide a surrogate marker of clonality. Evaluation of CD94/NKG2 complexes may be particularly useful in evaluating NK-cell lineage lymphoproliferative disorders [66].

Of the TLPDs discussed in this manuscript, T-LGL may be the most problematic to diagnose because of the great degree of phenotypic overlap between T-LGL and reactive CD8-positive T-cells and the fact that many causes of reactive CD8-positive T-cell expansions, such as viral infections and myelodysplastic syndromes, can be associated with cytopenias. Furthermore, T-cell clonality may not serve as a “gold standard” for making this distinction, because these reactive processes may yield apparently clonal TCR gene rearrangement results due to the limited clonal diversity
in the reactive cell population. For these reasons, as with all of the TLPDs discussed, correlation of the flow cytometric and molecular genetic results with the other clinical and laboratory findings is critical. A diagnosis of T-LGL can be rendered with confidence when a T-cell population with the immunophenotypic and molecular genetic properties previously discussed is identified with associated cytopenias and in the absence of a potential inciting stimulus. In cases whereby there is concern that the T-cells are reactive and/or the immunophenotype or TCR gene rearrangement results are ambiguous, it is advisable to consider repeat evaluation after 6 months to 1 year. Persistence of the process can help establish a T-LGL diagnosis. Bone marrow evaluation can also aid in establishing a diagnosis of T-LGL and excluding other possible causes for the cytopenias and increase in CD8 positive T-cells [67]. If these types of clinicopathologic correlations are not assiduously performed, one is at risk of rendering an incorrect diagnosis.

**Hepatosplenic T-cell lymphoma**

When considering disorders of cytotoxic T-cells other than T-LGL, hepatosplenic T-cell lymphoma (HSTCL) is an entity, which, although rare, often enters into the differential diagnosis. Unlike T-LGL, HSTCL is an aggressive malignancy of cytotoxic T-cells that typically affects young men in the form of massive hepatosplenomegaly, anemia, thrombocytopenia, and B-symptoms. The prognosis in HSTCL is dismal and often fatal within a year of diagnosis [68,69]. Isochromosome 7q is a recurring cytogenetic abnormality seen in HSTCL [70].

Despite being termed a lymphoma, HSTCL typically shows a “leukemic” pattern of organ infiltration with preferential involvement of the splenic red pulp and liver and bone marrow sinusoids. Circulating tumor cells are common, although the peripheral blood disease burden is usually low at presentation and increases with disease progression. Typically, the tumor cells are monomorphic, intermediate in size, have round to slightly irregular nuclei, inconspicuous nucleoli, and variably granulated cytoplasm [68]. The degree of cytologic atypia in HSTCL is usually greater than that seen in T-LGL.

Unlike many other TLPDs, HSTCL shows an unusual tendency express $\gamma\delta$ TCR heterodimer, although bona fide cases with $\alpha\beta$ TCR have been recognized (Fig. 4) [71]. Although the $\gamma\delta$ and $\alpha\beta$ TCR HSTCL subtypes may exhibit some clinical differences with the latter showing a tendency to occur in females in the fourth and fifth decades of life, they share common immunophenotypic attributes [72]. Like T-LGL, HSTCL often shows diminished expression of CD5 and coexpression of CD16 (see Fig. 4). In contrast to T-LGL, HSTCL is frequently CD56 positive and does not express CD57, although CD57 positivity may be encountered in HSTCL arising in the pediatric age group [71,72].
Fig. 3. Flow cytometry in a case of T-LGL. (A) Gating on the lymphocytes (upper) reveals increased numbers of CD8-positive T-cells (arrow, lower right) with aberrantly lost expression of CD5 (arrow, lower left) and CD7 (lower middle). (B) These CD8-positive T-cells were found to aberrantly express the NK-associated antigen CD16 (upper). This feature is used for selective gating on the abnormal cells in a three-color KIR assay, which demonstrates the abnormal cells to have restricted expression of the KIR antigen CD158b (lower middle, arrow). These cells do not express the KIR antigen CD158e (also referred to as KIR p70, lower left). Note that the staining pattern suggests that the cells also have dim coexpression of CD158a (lower left); expression of multiple KIR antigens can be seen in a small subset of T-LGL. Restricted KIR expression is strongly associated with clonality in T-LGL disorders, which in this case was documented by TCR-Vβ flow (C) with uniform TCR-Vβ1 positivity both the total CD3-positive T-cells (upper) and CD3-positive, CD8-positive (lower) T-cells (small arrows, right).
expression in HSTCL is frequent. KIR expression also seems to be a feature common to HSTCL cases; although in contrast to T-LGL and other described cytotoxic lymphocyte disorders, HSTCL shows an unusual tendency to simultaneously express multiple KIR isoforms (Fig. 4C). It has yet to be determined if this pattern of KIR expression is a distinguishing feature of HSTCL [72].

As with other TLPDs, the distinction of HSTCL from T-LGL and other T-cell neoplasms as well as reactive γδ T-cell expansions ultimately requires correlation of the flow cytometry results with the clinical, histologic, and cytogenetic features.

Summary

In writing a review manuscript of TLPD flow cytometry, it is straightforward to catalog the described immunophenotypes of the various entities. Conversely, it is much more difficult to convey when the detection of an immunophenotypically distinct T-cell population indicates a TLPD. In disorders like T-PLL/CLL, the striking lymphocytosis and immunophenotypic homogeneity provide incontrovertible evidence of malignancy. Unfortunately, in many other disorders such as CTCL and T-LGL, the observed clinical, immunophenotypic, and TCR gene rearrangement results may overlap considerably with reactive conditions. Thus, the diagnosis of a T-cell lymphoproliferative disorder requires synthesis of clinical,
Fig. 4. (A) Flow cytometry in a case of hepatosplenic T-cell lymphoma reveals the presence of abnormal \( \gamma \delta \) T-cells (arrow lower right) with loss of CD5 (arrow, lower left). These cells are preferentially found in the large lymphoid gate (forward and side light scatter, upper); (B) the small lymphoid gate (upper) predominantly contains immunophenotypically unremarkable \( z \beta \) T-cells with only a minor population of CD5-negative \( \gamma \delta \) T-cells (small arrows). (C) Three-color analysis of the CD5-negative hepatosplenic T-cell lymphoma cells in another case reveals the abnormal cells to uniformly express the KIR antigens CD158a, CD158b, and CD158c/p70.
histologic, immunophenotypic, and molecular information. No one methodology in isolation can address the complexities of this heterogeneous disease category.

References


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