Seminars in Diagnostic Pathology 37 (2020) 303–320

Contents lists available at ScienceDirect

Seminars in Diagnostic Pathology

journal homepage: www.elsevier.com/locate/semdp

Application of flow cytometry in the analysis of lymphoid disease in the lung and pleural space

May Fu, Malary Mani, Jaclyn Bradford, Weina Chen, Mingyi Chen, Franklin Fuda*

Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX

A B S T R A C T

Various types of lymphoid neoplasms can occur in the lung. Lung parenchyma, the pleura or the pleural cavity can be the primary site of a lymphoid neoplasm or can be involved secondarily as a result of systemic dissemination from a separate primary site. Recognition of pulmonary lymphoid neoplasms (PLN) has increased secondary to technological advances in the medical field. Multiparameter flow cytometry (FC) is a one of the diagnostic tools that serves an essential role in the detecting and categorizing PLNs. FC allows for rapid identification and immunophenotypic characterization of PLN. In this article, we discuss the role of FC in the diagnosis of the most commonly encountered PLNs as well as their basic clinicopathologic features. We briefly discuss the role of FC in identifying non-hematolymphoid neoplasms in lung specimens as well.

Introduction

Pulmonary lymphoid neoplasms (PLN) include lymphoma and plasma cell neoplasms. These neoplasms can arise primarily in the lung or secondarily as a result of systemic dissemination from a separate primary site. Primary pulmonary lymphoid neoplasms (pPLNs) are rare, accounting for only 0.3% of all primary lung neoplasms and less than 0.5% of all lymphoid neoplasms; however, the incidence is increasing, which is partly due to improved recognition through modern diagnostic advances.[1-4] Secondary pulmonary lymphoid neoplasms (sPLNs) are much more common with pulmonary involvement occurring in 24% of non-Hodgkin lymphoma and 38% of Hodgkin lymphoma from other sites of origin.[2] Depending on the subcategory of lymphoid neoplasm, secondary malignant pleural effusions are relatively common with up to 16% of patients developing pleural effusions during the course of the disease.[5-7] In some instances, the pleural cavity is the primary site of the lymphoid neoplasm. The most common pulmonary lymphoid neoplasm is extranodal marginal zone lymphoma (MALT lymphoma; accounting for 70% of pulmonary lymphoma) followed by diffuse large B-cell lymphoma (DLBCL; 12% of lymphoma).[1,8] Other lymphoid neoplasms involving the pulmonary system are relatively rare.

Multiparameter flow cytometry (FC) has become an essential diagnostic tool for rapid detection and categorization of pulmonary lymphoid neoplasms.[9] In this regard, the primary roles of flow cytometry are to assign lineage to cell populations, distinguish between reactive and neoplastic cell populations, and establish a differential diagnosis for neoplastic populations based on their immunophenotypic features. Lineage determination is aided through identification of lineage specific and/or associated markers, e.g., CD19 for B-lineage cells and CD3 for T-lineage cells. Distinguishing reactive from neoplastic populations is accomplished through recognizing aberrant patterns of marker expression, i.e. different-from-normal (DFN) patterns. DFN can involve: (i) aberrant over or under expression of normally expressed markers, (ii) asynchronous expression of normally expressed markers, (iii) expression of markers not normally expressed, and/or (iv) abnormal expansions of certain cell populations. Once an aberrant population is identified, the overall immunophenotype is then used to place a neoplastic population into a differential diagnosis. This differential diagnosis is used in conjunction with clinical, morphologic, and genetic features to establish the best final diagnosis.

In this review, we discuss general principles and methods of FC analysis along with common immunophenotypic features of various lymphoid neoplasms, and briefly non-hematolymphoid neoplasms that may involve the pulmonary system. These immunophenotypic features include markers that can be assessed by FC as well as some that can only be assessed through immunohistochemistry. The review contains information from the body of available literature as well as our single institutional experience. Case examples will be included to illustrate the use of FC in a wide range of PLNs and illustrate key immunophenotypic features of these malignancies.

Flow cytometry general principles

Multiparameter flow cytometry (FC) is a testing method that can rapidly measure physical and chemical properties of individual cells and/or particles. In clinical hematopathology, it is used to identify and

* Corresponding author.
E-mail address: franklin.fuda@utsouthwestern.edu (F. Fuda).

https://doi.org/10.1053/j.semdp.2020.06.006

Available online 30 June 2020

immunophenotypically characterize hematolymphoid neoplasms by determining the types and levels of molecules expressed on cell surfaces and intracellular compartments. FC can be performed on any tissue sample that can be processed into a single cell suspension.

Typical samples from the pulmonary system include bronchoalveolar lavage fluid (BAL), pleural fluid, and solid lung or pleural tissue. Malignant cells from these types of tissues degenerate relatively quickly. Therefore, acquisition and processing should be standardized within an institution to ensure time efficiency and accurate flow cytometry results.[9] These sample types should be quickly delivered to the laboratory. During transit, cellular degeneration can be reduced by keeping these samples near or at 2–8 °C (e.g., keeping the sample on ice).[9] Upon receipt, accessioning and processing of these sample types (similarly to cerebrospinal fluid, any body cavity fluid, and solid tissue from any body site) should be prioritized. In our laboratory, we perform at least 3 washes on tissue samples to ensure optimal clearance of cytophilic antibodies (see further discussion below). BAL and pleural fluid samples are first washed with a buffer solution. After the wash cycle is complete, the supernatant is decanted, and the cell button is re-suspended. For bloody samples, the RBCs are then lysed using ammonium chloride for 5 min, and the sample is rewashed two to three times.[10,11] The washed cells are then re-suspended in a 5% newborn calf serum RPMI media. The specimen is inspected for clumps of particles, and if visible the specimen is filtered through a mesh to remove them. Solid tissue biopsies require more vigorous processing, and special care is taken during the tissue digestion process to maximize cell recovery and minimize cell apoptosis. The tissue samples are removed from the liquid transport media and placed into a petri dish. The sample is then cut, and two touch imprint preparation slides are made. One of these is stained for cytologic evaluation, and the other is left unstained for possible reflex testing such as cytogenetic/molecular fluorescent in situ hybridization (FISH). The tissue sample is then gently broken up using a scalpel. The cells are re-suspended in the liquid media and transferred through a mesh filter into a clean conical to preserve cells of interest and surface markers. The sample is then washed and if necessary lysed in the same manner as the BAL or pleural fluid samples. After the RBC lysing (if necessary) and washing processes are complete for all sample types, a cell count is performed on an automated cell counter. If 300,000 cells or more are present, cellular viability using a 7-aminoactinomycin D (7-AAD) dye is determined. As part of the FC workup, two cytologic preparations, including the touch imprint preparation (when applicable) and a cytospin preparation, are made for each specimen using a Wright stain procedure. These cytologic preparations are used for triage and for a final collaborative interpretation.

Pulmonary tissue samples often have low cellularity. This is particularly true of BAL samples, pleural fluid samples and fine needle aspiration samples of solid tissue. Such low cellularity restricts the size of the assay to one or two FC tubes. Therefore, triage of these low cellular samples is an essential step to ensure an optimal diagnostic approach and is based on patient demographics, clinical history and disease suspicion along with a brief cytologic evaluation. When B-cell lymphoma is one of the major diagnostic considerations, surface immunoglobulin light chain (sIg LC) evaluation will be an indispensable part of the assay (Fig. 1). Abnormal sIg LC expression is a hallmark for identifying neoplastic B-cell populations by FC (Fig. 2). To ensure optimal assessment of sIg LC on low cellular samples, our laboratory performs a 20 min, 37 °C incubation/wash on any B-cell tubes containing antibodies for Ig LC assessment. The 37 °C incubation/wash aids in removal of cytophilic antibodies (i.e., antibodies bound nonspecifically via Fc receptors) that can create difficulty in sIg LC assessment (Fig. 3).[10] In our experience, cytophilic antibody interference may cause B-cell populations to appear double positive for sIg light chain kappa and lambda when viewing each sIg LC in a separate plot. When viewing sIg LC kappa and lambda in the same plot (i.e., kappa vs lambda; Fig. 1) the cytophilic antibodies may cause the B-cells to localize along the diagonal (Fig. 3), which is an area typically considered to be populated by B-cells that lack expression of sIg LC. It is important to note that certain antigens, such as CD23, may be lost through heated incubation.[10]

In addition to frequent low cellularity, pulmonary samples often contain a large number of mesothelial cells, histiocytes and/or degenerating cells. Mesothelial cells and histiocytes typically have high forward light scatter (FSC) and high orthogonal light scatter (SSC) correlating to their large cell size and cytoplasmic complexity, respectively. Degenerating cells show low to moderate FSC and low to high SSC. All three cell types typically show some level of nonspecific marker staining or autofluorescence that can potentially lead to erroneous labeling as neoplastic cells (Fig. 4a and b). Being mindful of such artifact and evaluating cytologic features on a concurrently prepared cytospin or touch preparation can be helpful in preventing misinterpretation. Alternatively, evaluation of neoplastic degenerating cells can sometimes be very helpful in cases where a viable neoplastic population represents only a small fraction of the total events collected or is completely absent (Fig. 5a and b). In this scenario, the degenerating cells may offer a clue to the presence of the neoplasm. Once again, cytologic evaluation is helpful in this situation.

For the examples in the following lymphoma discussions, FC data were acquired using CELLQuest software on 4-color BD FACSCalibur or 10-color BD FACSCanto™ flow cytometers (Becton Dickinson, San Jose, CA). BD antibodies (unless otherwise specified) conjugated to the appropriate fluorophores were used and included: CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD13, CD14, CD15, CD19, CD20, CD22, CD25, CD34, CD36, CD38, CD45, CD56, CD64, CD71, CD79a, CD138, surface polyclonal κ and λ light chains (DAKO/Agilent, Santa Clara, CA), HLA-DR, intracellular CD3, intracellular VS38 (DAKO/Agilent, Santa Clara, CA), intracellular myeloperoxidase (MPO) (Beckman Coulter, Brea CA), intracellular TdT, and intracellular κ and λ light chains. Cocktail tubes/panels with various combinations of antibodies were developed and employed in a disease and tissue dependent manner. A pathologist triage was performed to determine the specific panels used on individual cases. Data was analyzed using cluster analysis with Cytopaint Classic Software (Leukobyte, CA), as previously described.[12] A population was considered positive for a specific marker if at least 10% of the population exceeded an isotypic or autofluorescent control threshold, fluorescence minus one control threshold, or staining of a negative internal control population depending on the particular case. Positive staining patterns were further described according to intensity and heterogeneity of expression as partial positive, dim positive, variably positive, positive or bright positive.

Many different types of hematolymphoid neoplasms can involve the pulmonary system. While B-lineage neoplasms are the most common, a screening assay can be designed to allow for identification of any of the numerous possibilities. The screening assay for pulmonary tissue used at our institution includes 2 tubes (Table 1) with a total of 16 unique antibodies (i.e., CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD30, CD38, CD45, CD56, CD64, surface Ig lC kappa, and surface Ig LC), which allows for identification of essentially any hematolymphoid neoplasm. Additional reflex tubes are added as needed.

B-lineage neoplasms

B-lineage neoplasms include neoplasms of immature B-lineage cells (B-lymphoblastic leukemia/lymphoma), mature B-lineage cells (mature B-cell leukemia/lymphoma) and terminally differentiated B-lineage cells (plasma cell neoplasms). Assigning B-lineage to a population of hematolymphoid cells by multiparameter flow cytometry is aided through identification of one or more B-lineage specific or associated markers including CD19, CD20, CD22, CD79a, CD79b, and/or immunoglobulin light chain kappa and lambda. Assigning states of maturation and differentiation is accomplished by assessing markers of immaturity (i.e., CD34 and TdT) and assessing the expression patterns of markers such as CD10, CD20, CD38, VS38, CD45, CD138 and surface

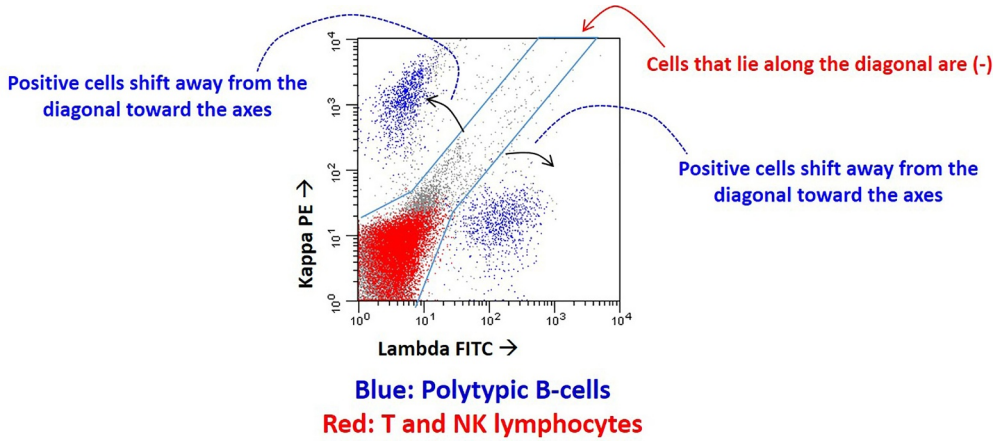

Fig. 1. Surface Ig Light Chain Plot. Evaluation of immunoglobulin light chain is very helpful in identifying B-cell neoplasms. When plotting kappa versus lambda for immunoglobulin light chain (either surface or cytoplasmic), traditional quadrant analysis may not be accurate. Rather, anything that lies along the diagonal is generally considered negative. Positive populations shift away from the diagonal toward the axes. Intensity of expression can be determined by the position along a particular axis with brighter populations lying further along the axis.

and cytoplasmic immunoglobulin light chain (Ig LC) and immunoglobulin heavy chain (Ig HC).

Familiarity with normal expression patterns for these and other markers enables identification of DFN in B-lineage neoplasms. Abnormality in Ig LC expression is a hallmark DFN for identifying abnormal B-lineage populations. Normal B-lineage cells express Ig LC as part of their B-cell receptor complex in a dynamic fashion (Fig. 6). Immature B-lineage cells known as hematogones undergo 3 stages of maturation with the earliest stages lacking sIg LC expression and later stages showing expression. Mature naive B-cells show moderate to bright levels of sIg LC expression; reactive germinal center B-cells show decreased to negative sIg LC expression; and memory B-cells presumably once again show moderate to bright levels. Plasma cells and plasmablasts have decreased to negative sIg LC expression and increased cytoplasmic Ig LC expression. In our experience CD30 (+) reactive B-cells/immunoblasts show moderate to decreased sIg LC and increased cytoplasmic Ig LC expression. Each B-lineage cell can only express one Ig LC, either kappa or lambda. Normal populations of B-lineage cells are made up of a mixture of kappa expressing cells and lambda expressing cells, i.e., they are polytypic with a normal ratio generally between 1 and 2 kappa to 1 lambda (i.e., kappa:-lambda = 1–2:1), although this ratio may vary among different laboratories. On the other hand, neoplastic B-lineage populations are clonal (i.e., generated from a single cell). Therefore, the cells of neoplastic B-lineage populations are typically monotypic, which means all cells in the population express the same immunoglobulin light chain. Alternatively, all the cells of a neoplastic B-lineage population may aberrantly lack expression of sIg LC.[13,14] Neoplastic B-cell populations that lack surface Ig LC expression may or may not exhibit restricted cytoplasmic Ig LC expression.[15] In some instances, surface Ig LC negative B-cell neoplasms may show abnormality in surface immunoglobulin heavy chain expression, which can be used as supportive evidence of aberrancy in these populations.[16]

Identifying a monotypic B-lineage population or an Ig LC negative B-cell population is a mainstay for identifying B-lineage neoplasms by FC. Monotypic B-lineage populations may be difficult to isolate when present in a background containing numerous polytypic, normal B-lineage cells. In such scenarios, an abnormal kappa:lambda ratio may be a clue for the presence of a B-lineage neoplasm. However, identifying an abnormal kappa:lambda ratio without identifying a distinct monotypic population should be considered nonspecific, since it can also be seen in reactive conditions. Noting the abnormal ratio as a nonspecific finding that can be seen in reactive conditions as well as B-lineage lymphoproliferative disorders in the interpretation will highlight these possibilities.

While Ig LC abnormality is a hallmark of B-lineage neoplasms, it does not absolutely equate to a neoplastic and/or malignant process. Monotypic B-cell populations are not always monoclonal (e.g., the λ-light chain restricted plasmablasts of HHV-8 multicentric castleman's disease or λ-light chain restricted B-cells in atypical marginal zone

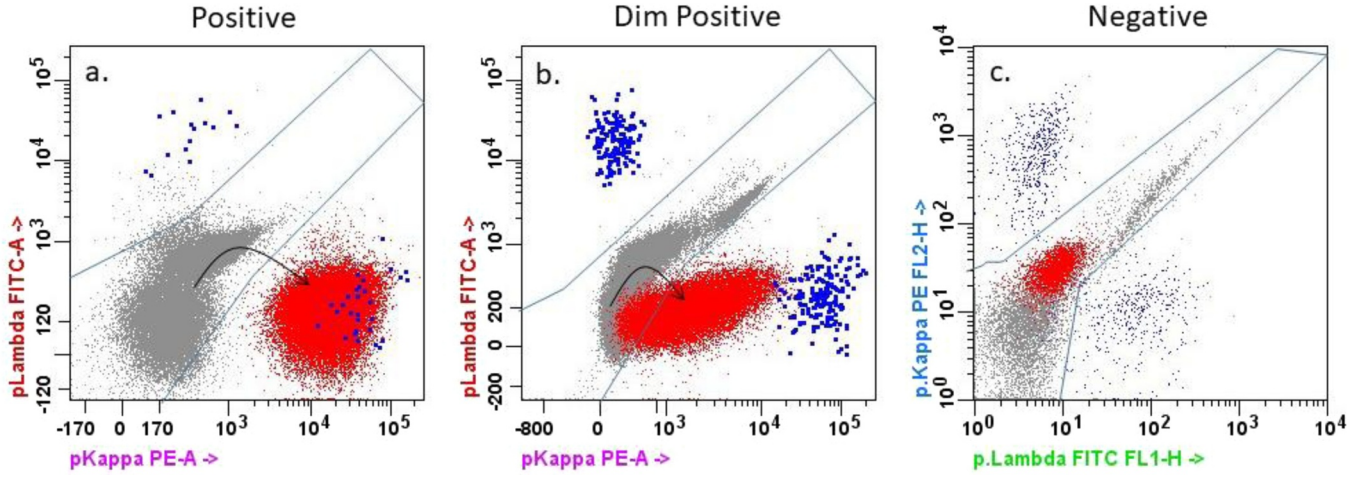

Fig. 2. Surface Ig Light Chain neoplastic expression patterns. The plots show three different types of abnormal expression patterns for surface Ig LC on populations of mature B-cell neoplasms (red). Normal polytypic B-cells are shown for reference in blue (a.) Mature B-cell lymphoma with kappa light chain restriction with normal staining intensity. (b.) Mature B-cell lymphoma with kappa light chain restriction with weak staining intensity. (c.) Mature B-cell lymphoma that lacks expression of surface Ig LC.

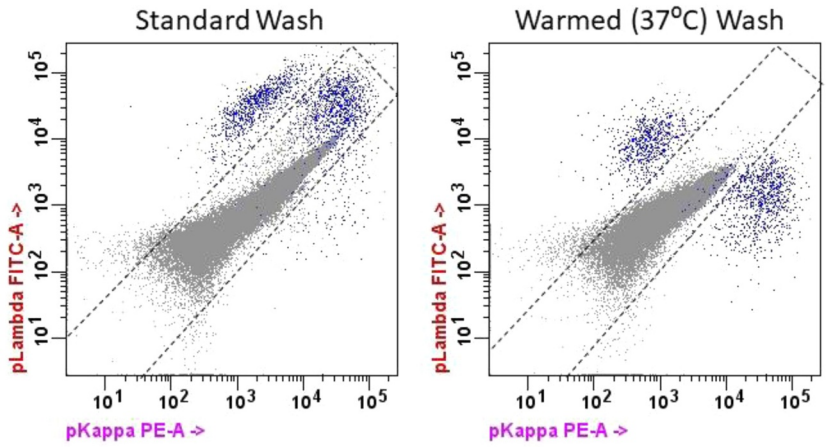

Fig. 3. Cytophilic antibody interference, surface Ig light chain staining: The plots show surface Ig light chain staining using polyclonal antibody reagent on a population of normal, polytypic, mature B-cells (blue). There appears to be cytophilic antibody staining on the B-cells in the left plot using a standard wash (see cells staining along the diagonal denoted by dashed box). This artifact resolves following a 37 ˚C incubation and wash in the plot on the right.

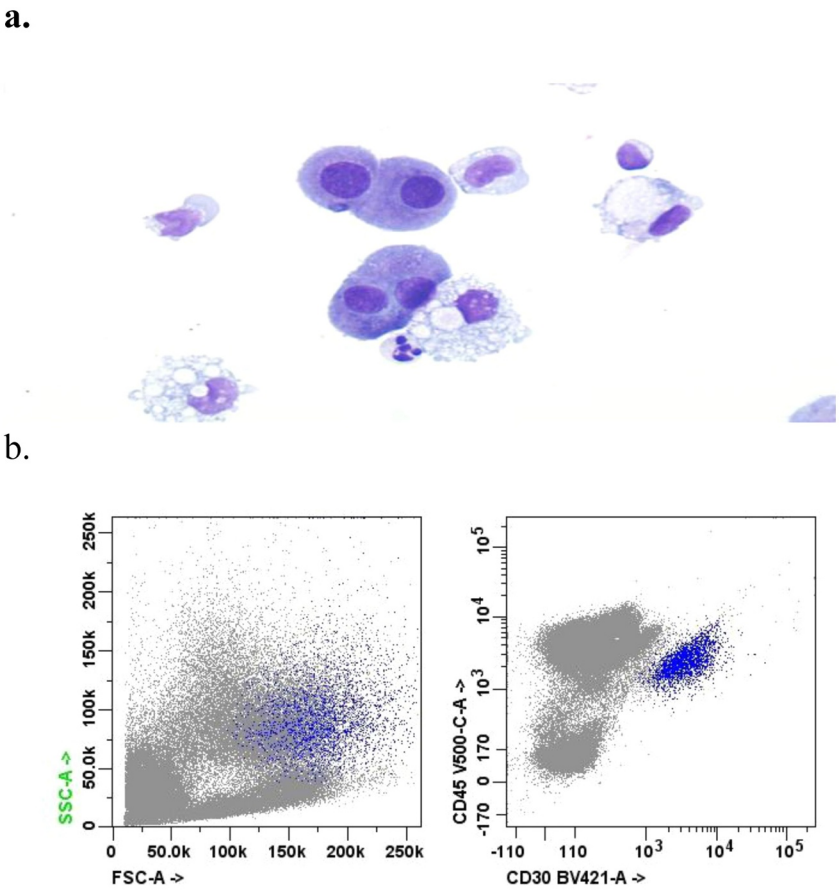

Fig. 4. Mesothelial cells, Pleural Fluid. (a.) Photomicrograph (50X objective) of cytospin preparation shows large mesothelial cells and histiocytes with small lymphocytes and neutrophils in the background. (b.) FC plots show mesothelial cells (blue) that appear to stain positive for CD45 and CD30. Both benign and malignant mesothelial cells are known to occasionally stain positive for CD30[95] but do not express CD45. These cells also show equivocal staining for additional markers such as CD4 (not shown). Such artifactual staining can be misinterpreted as a hematolymphoid neoplasm. Careful evaluation of the FC in association with the findings on the cytospin preparation, ensures that these cells are appropriately recognized as benign.

hyperplasia in children), and monoclonal B-cell proliferations are not necessarily malignant (e.g. monoclonal B-cell proliferations of undetermined significance that lack evidence of overt lymphoma and may never progress clinically).[96–100] Likewise, sIg LC negative B-cell populations are not always neoplastic and/or malignant. This is particularly true of B-cell populations in body fluids such as pleural fluids where populations of normal B-cells more commonly show decreased expression of sIg LC[17] and in the presence of follicular hyperplasia where germinal center B-cells show decreased expression of sIg LC. Apparent lack of surface Ig light chain staining can also be secondary to a technical artifact. If encountered and sample cellularity permits, performing a 37 ˚C wash to remove cytophilic antibodies and/or restaining with a different antibody preparation to account for epitope binding site alterations/polymorphisms (e.g., using a different clone or a polyclonal vs monoclonal reagent preparation) may resolve the artifact.[17] To summarize, abnormalities in Ig LC are very helpful in identifying neoplastic and malignant B-cell populations; however, these abnormalities are not definitively diagnostic on their own. It is imperative to interpret such findings in conjunction with other immunophenotypic features, particularly the lack or presence of other immunophenotypic aberrancy, as well as the overall clinical picture and morphologic/genetic studies when rendering a final diagnosis.

Similar to mature B-lineage non-Hodgkin lymphomas, plasma cell neoplasms also most commonly demonstrate Ig LC restriction. This is typically demonstrated by restricted cytoplasmic Ig LC, which may or may not be accompanied by surface Ig light chain expression. Rarely, an

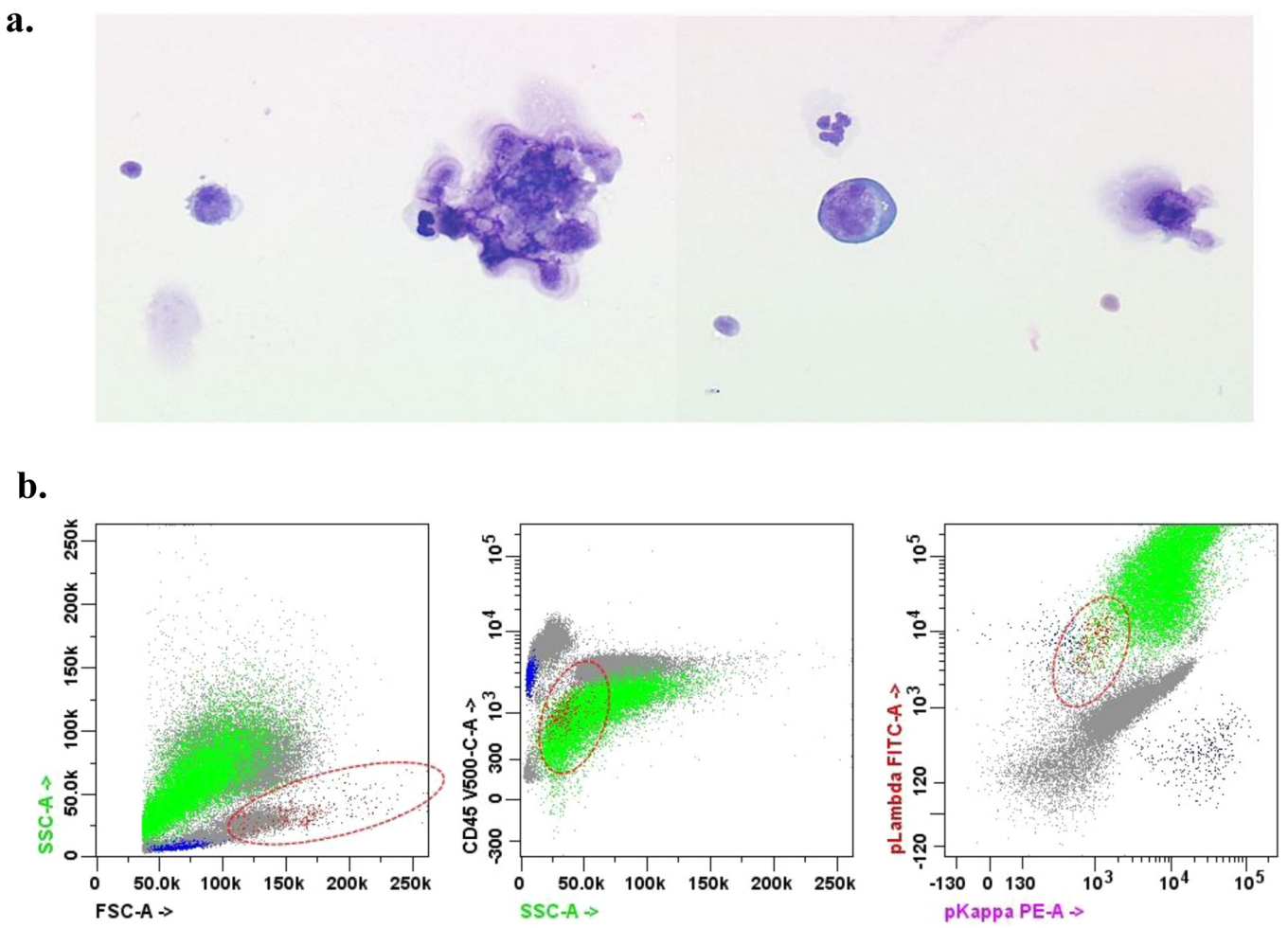

Fig. 5. Degenerating High Grade B-cell Lymphoma with *MYC* and *BCL-6* rearrangements, Lung Fine Needle Aspiration. (a) Photomicrograph (50X objective) of cytospin preparation showing predominantly degenerated cells and debris. There are rare atypical large cells with round nuclei, dispersed chromatin, multiple nucleoli, and a small amount of deeply basophilic cytoplasm with occasional vacuoles. (b.) FC shows a minute population (0.50% of total events collected) of cells (colored red and encircled) with high forward light scatter indicating large cell size and moderate orthogonal light scatter suggestive of some level of cytoplasmic complexity (i.e., vacuolation in this case). These cells could easily be missed given their very low number and their location outside of the typical lymphocyte gate on both FSC vs SSC and CD45 vs SSC plots. Additionally, these neoplastic cells show weak evidence of B-lineage differentiation (i.e., CD19 (predominantly -/few cells dim +) and CD20 (-); data not shown) making it very difficult to identify the cells. However, the cells clearly present themselves on the kappa versus lambda plot as lambda restricted large cells. Identification of this neoplasm is aided in this case by the large population of degenerated lymphoma cells (green), which show restricted lambda light chain staining. Viable normal, polytypic, small B-lymphocytes are colored blue as reference.

Ig LC nonproducing plasma cell neoplasm will be encountered that lacks staining for both surface and cytoplasmic Ig LC.

B-cell neoplasms are the most common type of hematolymphoid neoplasms involving the pulmonary system with extranodal marginal zone lymphoma of mucosa associated tissue being the most common subtype followed by diffuse large B-cell lymphoma and lymphomatoid granulomatosis. Many other B-cell neoplasms also involve the pulmonary system, albeit at very low rates. Subcategories of B-cell neoplasms show expected immunophenotypic features, of which many can be demonstrated through flow cytometry. Discussion of each of these is beyond the scope of this manuscript; however, typical immunophenotypes of the B-cell neoplasms most commonly encountered in pulmonary tissue specimens will be presented.

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)

MALT lymphoma is an indolent, extranodal B-cell lymphoma belonging to the larger subcategory of marginal zone lymphoma. MALT lymphoma generally develops in acquired mucosa associated lymphoid tissue in sites such as the stomach, salivary glands, thyroid and the lung.[8] Pulmonary MALT (pMALT) lymphoma arises in acquired bronchial mucosa associated lymphoid tissue and is sometimes referred to as bronchial mucosa or bronchus associated lymphoid tissue lymphoma (BALT lymphoma). While a rare disorder itself, it is the most common primary pulmonary lymphoma, accounting for over 70% of primary PLNs.[1-4,8,18] pMALT lymphoma almost exclusively affects adults with a

Table 1
Tissue Screening Panel (1) Screening B-cell tube; (2) Screening T-cell tube. *p* = polyclonal reagent.

Tube	FITC	PE	PerCp 5.5	PE-Cy	APC	APC-R700	APC−H7	BV421	V500c	BV605
1.	CD2	CD3	CD5	CD56	CD4	CD64	CD8	CD30	CD45	CD7
2.	pLambda	pKappa	CD38	CD19	CD10	–	CD20	CD5	CD45	–

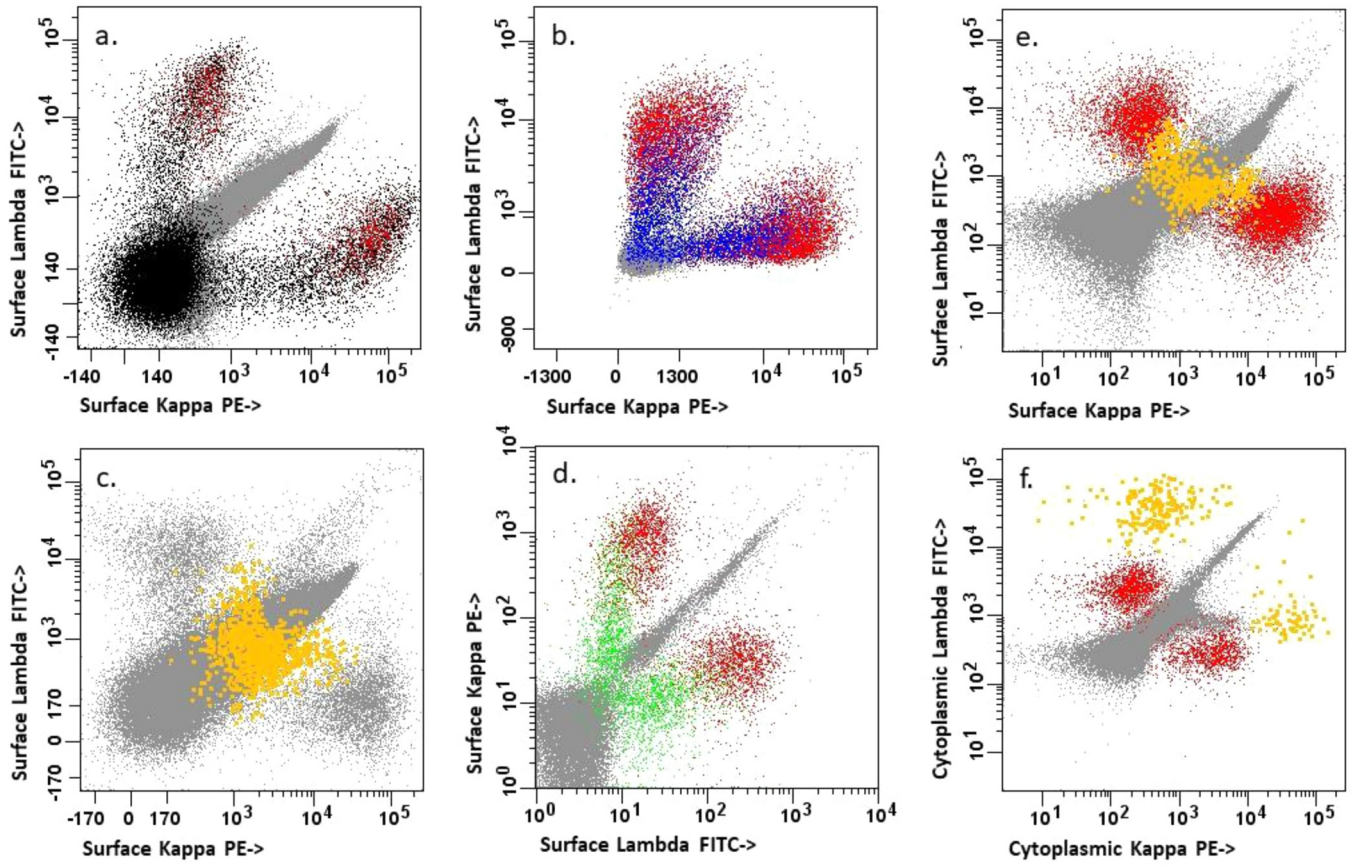

Fig. 6. Surface Ig Light Chain expression, various normal B-cells: (a.) Surface Ig LC expression goes from negative to bright positive as hematogones (black) transition from stage 1 to stage 3 of maturation; Mature naive and memory B-cells (red). (b.) Germinal center B-cells (blue) show decreased to negative sIg LC; Mature naive and memory B-cells (red). (c.) Plasma cells and plasmablasts (yellow) show low to negative sIg LC. (d.) CD30 (+) reactive B-cells/immunoblasts (green) show variable expression of sIg LC typically from moderate to dim; Mature naive and memory B-cells (red). (e. and f.) Mature B-lymphocytes (red) and Plasma cells (yellow) showing surface versus cytoplasmic Ig LC expression. Notably, positive staining of the B-lymphocytes in the cytoplasmic plot is in part due to surface staining rather than true cytoplasmic expression of Ig LC. The cells are permeabilized so that the reagent antibody can enter the cell, but the reagent antibody will also stain any surface Ig LC present. Note the shift in theintensityof staining (dim to bright) for the plasma cells from the surface to cytoplasmic plots indicative of true cytoplasmic expression. Alternatively, the B-lymphocytes exhibit decreased intensity of staining (bright to dim) indicative of low to absent cytoplasmic expression.

median patient age range between 60 and 70 years, although rare cases of patients under the age of 30 are reported.[1,3,19,20] It is notable for its indolent growth pattern and asymptomatic presentation. It is rarely associated with pleural effusions, which are seen in only about 10% of cases.[20,21] In up to 30% of patients, a history of an underlying chronic immunologic abnormality such as autoimmune disorders[3,4] or infections is noted.[4,22] While specific causative agents have been discovered in MALT lymphoma at other body sites, a specific causative agent associated with pMALT has not been identified; however, current studies to investigate possible candidates are underway.[8]

MALT lymphoma is characterized by small to medium-size B cells or monocytoid B cells. Plasma cell differentiation can be seen (Fig. 7).[23] Neoplastic cells infiltrating the bronchiolar or alveolar epithelial tissue form lymphoepithelial lesions, which morphologically can be an important diagnostic feature.[8,18] Immunophenotypically, pMALT lymphoma is similar to MALT lymphoma in other locations and other marginal zone lymphomas. pMALTs show pan B-cell antigen expression (CD19, CD20, CD22 and CD79a) with Ig LC abnormality (e.g., κ or λ light chain restriction) (Fig. 7) and are typically negative for CD5, CD10, CD23 and Cyclin D1.[8,18] Plasmacytic differentiation with monotypic plasma cells sharing similar light chain restriction is frequent (Fig. 7). While the neoplastic plasma cells of MALT lymphoma show some similarity to the neoplastic plasma cells of plasma cell neoplasms (e.g., $CD38^{Bright+}$, $CD138^{Bright+}$, $vs38^{Bright+}$, and a restricted pattern of cytoplasmic Ig $LC^{Bright+}$), they also typically exhibit a few distinct differences.[24] The plasma cells of MALT lymphomas are usually positive for CD45, CD19, and surface immunoglobulin light chain (in a restricted pattern) and negative for CD56, whereas the plasma cells of plasma cell neoplasms are more commonly negative for CD45, CD19, sIg LC and positive for CD56. This IP difference is useful in differentiating MALT lymphoma with pronounced plasma cell differentiation (i.e., composed entirely or almost entirely of neoplastic plasma cells without a clear neoplastic B-lymphocyte component) and plasma cell neoplasms such as plasmacytoma. However, it is important to be mindful that this immunophenotypic distinction is imperfect and final characterization of the neoplasm requires full clinicopathologic correlation. Notably, lack of plasmacytic differentiation and absence of autoimmune disease can be suggestive of a pulmonary MALT lymphoma harboring a t(11;18)(q21;q21) involving the *API2* and *MALT1*. The *API2/MALT1* fusion results in promotion of cell survival through prevention of apoptosis and is the most frequently occurring chromosomal translocation in pulmonary MALT lymphomas.[1] The main differential diagnosis for MALT lymphoma includes other low-grade B-cell lymphoma.

By flow cytometry, the aberrant B-lineage cells will reveal the above mentioned immunophenotypic features and will typically show low to moderate forward light scatter correlating with small to medium cell size. Higher forward side scatter is sometimes encountered in cases with the abundant cell cytoplasm, which can create the illusion of a large cell lymphoma. Correlation with morphology and genetics helps distinguish

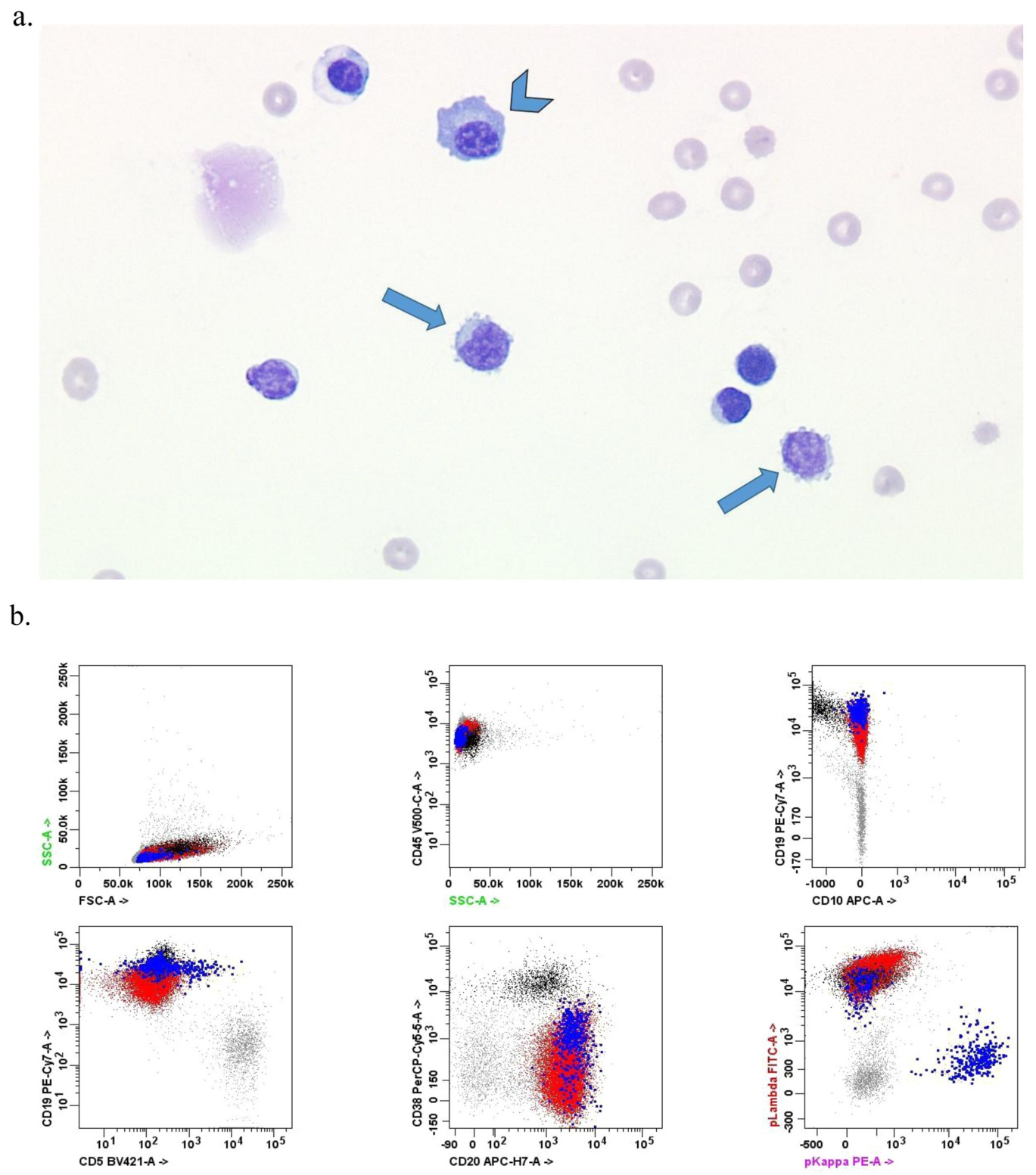

Fig. 7. Marginal Zone Lymphoma with plasmacytic differentiation, Lung Biopsy: (a) Photomicrograph (50X objective) of cytospin preparation shows medium-sized lymphoid cells with mature cytologic features (arrows) and plasma cells (arrow head). (b) FC shows a variably sized population of neoplastic B-cells (red events) by FSC and SSC which demonstrate co-expression of B-cell antigens CD19 and CD20 with surface lambda light chain restriction. These neoplastic cells are positive for CD45 and CD38 (partial and variable) and negative for CD5 and CD10. There is also a neoplastic population of plasma cells (black events) that are positive for CD19 and CD45 also with surface lambda light chain restriction. These plasma cells are negative for CD5, CD10, CD20 and CD56 (not shown). Residual normal B-cells (blue events) serve as a reference population.

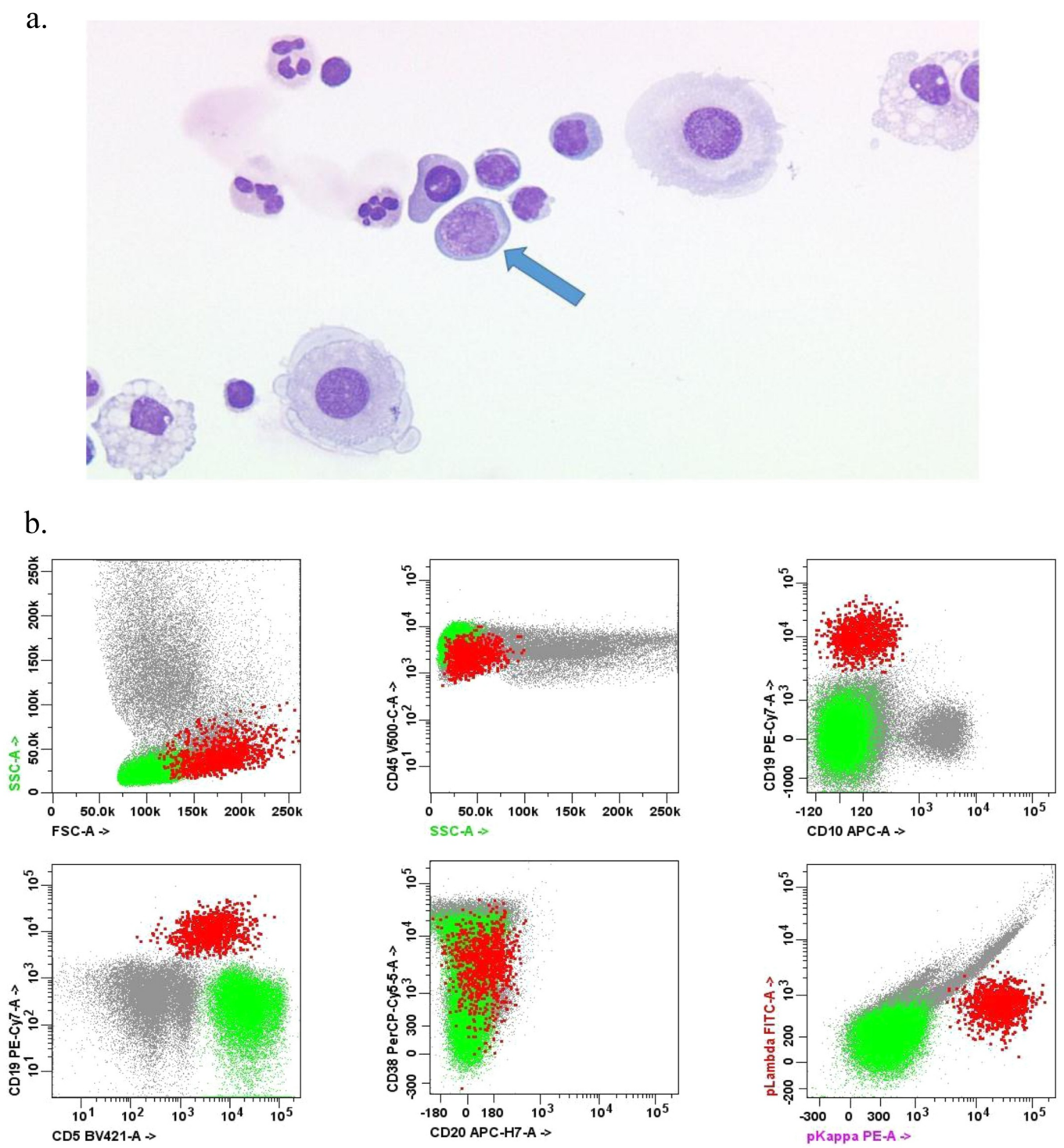

Fig. 8. Diffuse Large B-cell Lymphoma, Pleural Fluid: (a) Photomicrograph (50X objective) of cytospin preparation shows a large atypical lymphoid cell (arrow) with immunoblastic features including a round to oval nucleus, relatively open chromatin, a prominent nucleolus and moderate basophilic cytoplasm in a background of mesothelial cells, histiocytes, neutrophils and small lymphocytes. (b) FC shows large neoplastic cells (red events) in reference to normal T-lymphocytes (green events). The neoplastic cells are large in size with low to moderate cytoplasmic complexity by FSC and SCC parameters. IP analysis shows positivity for CD5, CD19, CD38, CD45, kappa light chain and negativity for CD10, CD20, and lambda light chain. The CD10 looks dim positive on the plot; however, this is artifactual secondary to autofluorescence of light from the large lymphoma cells into the FITC channel. Autofluorescent artifact is a common finding with large cells that needs to be recognized. If necessary, a control tube (e.g., a fluorescence minus one tube/FMO) can be performed to help determine true versus artifactual expression. This case represents secondary involvement of the pleural cavity by a primary breast DLBCL. The patient had been previously treated with anti-CD20 (rituximab) explaining the lack of CD20 staining.

between these lymphomas. In pMALT with plasmacytic differentiation, a separate monotypic plasma cell population will be present also with the above mentioned immunophenotypic features and typically moderate to higher forward light scatter and moderate orthogonal light scatter.

Diffuse large B-Cell lymphoma (DLBCL)

Diffuse Large B-cell Lymphoma (DLBCL) represents the second most common type of pulmonary lymphoma, accounting for 12–20% of cases.[2-4] The demographic most commonly affected are older patients, 60 years of age or older. DLBCL is most commonly *de novo*, although cases also arise as transformation of marginal zone lymphoma,[25] chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and follicular lymphoma. Associated pleural effusion is a common finding.[3,5,6] Clinically, patients present with non-specific respiratory symptoms. Computed tomography (CT) of the chest can illustrate a diverse set of findings including single to multiple solid pulmonary nodules or masses, mediastinal enlargement, or cavitation.[26] Biopsy of tissue or pleural fluid sampling typically shows large atypical lymphocytes which exhibit either immunoblastic or centroblastic morphology[25] (Fig. 8). By FC, the tumor cells have moderate to high FSC indicative of larger cell size. Dependent on the cytoplasmic complexity (e.g., vacuolation) in the individual case, SSC can be low to high. Similar to DLBCL in other sites, pulmonary DLBCL typically shows positive B-cell antigen expression (CD19, CD20, CD79a) with monotypic light chain expression (Fig. 8), although roughly 25% of DLBCL's lack both kappa or lambda expression (i.e. surface Ig LC negative).[8] Bright to very bright CD38 expression can serve as a biomarker for detecting *MYC* abnormalities in DLBCL and high grade B-cell lymphoma with *MYC and BCL2* and/or *BCL6* rearrangements [previously known as double hit lymphomas (DHL) and triple hit lymphoma (THL)].[12] The expression of CD5 and/or CD10 varies for DLBCL. CD5 expression is relatively uncommon in DLBCL only occurring in approximately 5–10% of cases from all body sites. To our knowledge, the occurrence of CD5 positive primary pulmonary DLBCLs is exceedingly rare with only a few cass reported. The biologic characteristics of CD5 positive pulmonary DLBCL is not well characterized although in general these patients tend to exhibit a more aggressive clinical course with less favorable response to traditional therapeutic regimens in comparison to CD5 negative DLBCLs.[26] Despite combined chemotherapy regimens, the prognosis for pulmonary DLBCL remains poor with a median survival rate between 3 and 5 years.[25] FC can help identify immunophenotypic subsets of DLBCL, which is important for prognostic and therapeutic indications.

Lymphomatoid granulomatosis

Lymphomatoid granulomatosis (LYG) is an angiocentric and angiodestructive, EBV driven lymphoproliferative disorder primarily arising in the lungs. LYG has a male predominance and presents most commonly in immunocompromised patients or those on immunosuppressive agents such as methotrexate.[22] Imaging typically reveals multiple lung nodules which grossly display granulomatous lesions with central cavitation and necrosis. Microscopically, the nodules show a polymorphous infiltrate with variable numbers of large atypical cells. Angiocentric and angiodestructive features in the pulmonary blood vessels will be present. The atypical cells range from immunoblastic to plasmablastic morphology.[22] The atypical large cells show in situ hybridization for EBV-encoded small RNA (EBER) with pan B-cell antigen expression and variable CD30 positivity. Monoclonal cytoplasmic immunoglobulin expression may be seen in cases with extensive plasmacytoid differentiation. LYG has a tiered grading system from grade 1 to grade 3. Categorization is dependent on the proportion of EBV(+) large cells.

Very little is known regarding flow cytometry findings in LYG due its relative rarity. Similar to B-cell neoplasms with relatively few neoplastic B-cells (e.g., T-cell rich, histiocyte-rich DLBCL), the paucity of neoplastic cells in LYG could be challenging to identify by routine FC and would likely require a highly sensitive and directed assay.

Primary effusion lymphoma (PEL)

Primary Effusion Lymphoma (PEL) is an aggressive B-lineage non-Hodgkin lymphoma (medium survival of 4 months) that most commonly presents as a lymphomatous effusion involving pleural, pericardial or peritoneal cavities without a detectable solid mass.[5,8,22,27] Occasionally, PEL will present with both lymphomatous effusion and an extracavitary mass and rarely as an extracavitary/solid mass without malignant effusion (i.e. extracavitary PEL/PEL-EC).[27-29] The pleural cavity is the most common site of involvement with peritoneal and pericardial cavities making up the bulk of the remaining cases.[30] PEL can involve other “cavitary type tissue” as well, including cerebrospinal fluid and even intravascular space.[29,31-33] PEL has also been reported to be a possible complication of breast implants,[32,34] although reassignment of the rare reported case of breast implant associated PEL to breast implant associated anaplastic large cell lymphoma has been recently proposed.[35]

PEL is associated with human herpesvirus-8/Kaposi sarcoma associated virus (HHV-8/KSHV) infection and usually occurs in immunocompromised patients, often with very low absolute T cell counts (median T-cell count 139 cell/μL).[27] These patients are most commonly afflicted with HIV infection, but PEL also occurs in patients having other conditions associated with altered immunity (e.g., solid organ transplant recipients) and in the elderly, who can experience age related immunosenescence. PEL accounts for approximately 4% of HIV-associated NHL and less than 1% of non-HIV-related lymphomas.[36] Co-infection of HHV8 and Epstein Barr Virus (EBV) is common in PEL (80% of cases), and EBV likely plays a role in PEL pathogenesis; however, it is not required for diagnosis.[37-40] HHV-8 is the major etiologic agent for PEL and promotes lymphomagenesis by affecting multiple regulatory mechanisms including the viral interleukin-6 (IL-6) receptor signaling pathway.[22] Cytologically, the neoplastic cells can range from immunoblastic to plasmablastic to anaplastic. They often exhibit high grade features including large nuclei, prominent nucleoli, and abundant amphophilic or basophilic cytoplasm (Fig. 9). Occasional Reed-Sternberg-like cells can also be seen.[37] Clonal rearrangement of the immunoglobulin heavy chain (Ig HC) gene, consistent with B-lineage derivation, is typically identified by polymerase chain reaction (PCR), although B-cell clonality by PCR is not identified in all cases nor is it necessary for diagnosis. Notably, cases with both Ig HC gene rearrangements and T-cell Receptor (TCR) gene rearrangements have been reported as well as cases with TCR gene rearrangements alone.[22,32,40-42] While these rare latter cases could be considered T-cell PEL, the cell of origin in the vast majority of cases is believed to be a post germinal center, late-differentiated B-cell. This cellular origin is suggested by gene expression profiling showing transcriptomes closest to that of plasmablasts and plasma cell neoplasms and by the presence of somatic hypermutation of the Ig HC genes in most cases.[43-47]

Immunophenotypically, PEL usually expresses leukocyte common antigen (CD45) but typically lacks expression of lineage specific markers, i.e., PEL often shows a null immunophenotype. Therefore, PEL commonly lacks expression of B-lineage markers such as CD19, CD20, CD22, cCD79a, PAX-5, and surface and cytoplasmic immunoglobulin light chains[8,18] whereas markers that do not define lineage such as activation markers and markers associated with terminal B-cell/plasmacytic differentiation are often positive. Such markers include HLA-DR, CD30, CD38, CD71, CD138, VS38c, EMA, Mum-1 and Blimp-1[10,27,48,49] (Fig. 9). Germinal center markers CD10 and BCL-6 are typically negative. T-cell related markers are also typically negative; however, positive expression of T-lineage associated markers CD2, CD3, CD5, and CD7 can be seen, more commonly in PEL-EC, and may be diagnostically misleading. It is important to keep in mind that

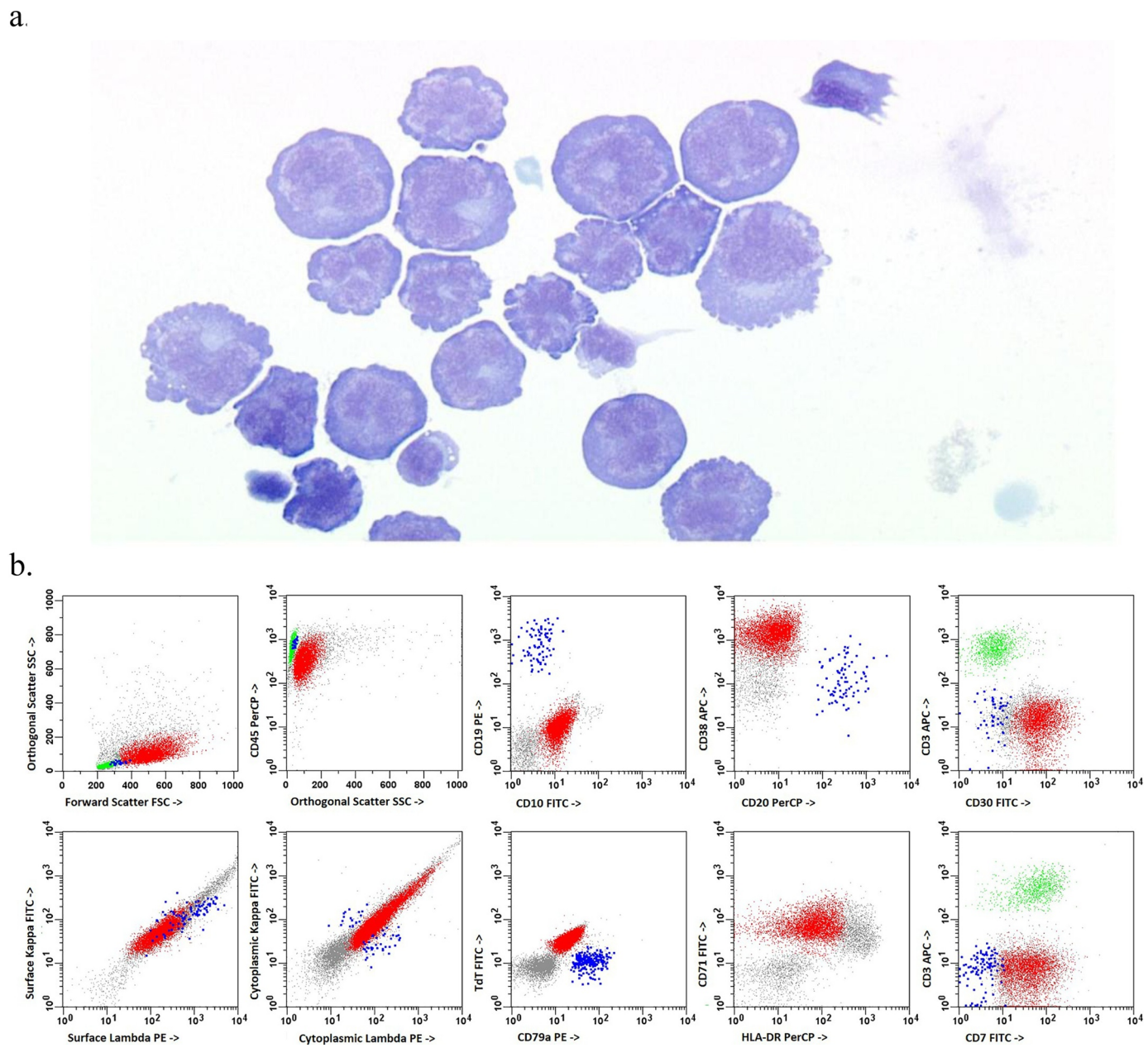

Fig. 9. Primary Effusion Lymphoma, Pleural Fluid: (a) Photomicrograph (50X objective) of cytospin preparation of a pleural fluid specimen shows large atypical lymphoid cells with round to slightly irregular nuclear contours, relatively open chromatin, prominent nucleoli and abundant basophilic cytoplasm. (b) FC shows large neoplastic cells (red), B-lymphocytes (blue), and T-lymphocytes (green). The neoplastic cells are CD45 (+) but show a null immunophenotype, i.e., negative for B-lineage markers CD19, CD20, CD22 (not shown), CD79a surface and cytoplasmic Ig LC and most T-lineage markers CD2 (not shown), CD3, CD4 (not shown), CD5, and CD8 (not shown). The cells also lack expression of CD10, CD34 (not shown) and TdT but are positive for nonspecific markers of activation, i.e., CD38, CD71 and HLA-DR. The neoplastic cells in this case showed positive expression of the T/NK lineage marker CD7. B-cell clonality was demonstrated by IGH PCR. The cells were HHV-8 positive via IHC on a cell block preparation. Notably, the B-lymphocytes (blue) lie along the diagonal in the surface Ig kappa vs lambda plot. This is likely an artifact due to the presence of cytophilic antibodies, as this resolved upon multiple washing cycles performed prior to cytoplasmic staining (see plot labelled cytoplasmic kappa vs lambda).

expression of T-lineage associated markers does not always indicate T-lineage derivation and that it can be seen aberrantly in PEL that is negative for T-cell clonality and positive for B-cell clonality by PCR.

The differential diagnosis for PEL must include other hematolymphoid neoplasms that can present with effusion such as diffuse large B cell lymphoma (DLBCL), ALK(+) DLBCL, Burkitt lymphoma, HHV-8/KSHV-unrelated PEL-like or effusion based lymphoma, pyothorax-associated lymphoma, plasmablastic lymphoma, plasma cell neoplasms and the T-lineage anaplastic large cell lymphoma (ALCL).[49-51] Since PEL most commonly shows a null immunophenotype, its immunophenotypic features greatly aid in differentiating it from most of these neoplasms; however, ALK(+) DLBCL, plasmablastic lymphoma, plasma cell neoplasms, ALCL (which also often shows a null to near-null immunophenotype and expression of activation markers) or any lymphoma with a null immunophenotype can all resemble PEL clinically, cytologically and immunophenotypically. These types of lymphoma are often difficult to distinguish or even indistinguishable by flow cytometry . Differentiating PEL from these latter neoplasms is achieved by identifying HHV-8 viral infection in the nuclei of the malignant cells. HHV-8 is required for the diagnosis of PEL and is negative in all of these other hematolymphoid neoplasms. It is found in every PEL case and can be identified through immunohistochemical staining for HHV-8 LANA-

Table 2
Characteristic immunophenotypic features of B-lineage neoplasms with plasmacytoid features. While these features are the most characteristic for the neoplasms listed, individual neoplasms may show variation from these expression patterns. v, variable expression with some cases positive and some cases negative; -/+, variable expression more commonly negative than positive; +/-, variable expression more commonly positive than negative; †, typically negative although expression may occur more frequently in PEL-EC; ‡, typically lambda; ±, commonly positive in extraosseous infiltrates of multiple myeloma but more frequently negative in primary extraosseous plasmacytoma; EBER, EBV-encoded small RNA.

	CD45	B cell markers	Plasma cell markers	Cytoplasmic Ig LC	EBER	Other
PEL	+	CD19−, CD20−, CD79a−; †	CD138+, CD38+, MUM1++	–	++	CD30+, CD71+, HLA-DR+, ALK-1−, T-cell antigens† (CD2, CD3, CD5, CD7)−
HHV-8+ DLBCL	+/−	CD19v, CD20v, CD79a−	CD138−, CD38−/+, MUM1+/−	++	−	CD30−/+
Plasmablastic Lymphoma	−/+	CD19−, CD20−, CD79a−/+	CD138+, CD38+, MUM1+, VS38c+	+/−	++	CD30 +/−, CD56−/+
Plasma cell neoplasms	−/+	CD19−, CD20−, CD79a−	CD138+, CD38+, MUM1+, VS38c+	+	−	CD56 ±

1 expression or via DNA extraction and polymerase chain reaction amplification of HHV-8.[40,52,53]

PEL should likewise be differentiated from other HHV-8 positive hematolymphoid disorders including HHV-8-positive multicentric Castleman's disease (MCD), HHV-8-positive germinotropic lymphoproliferative disorder (GLPD), and HHV-8-positive diffuse large B-cell lymphoma, not otherwise specified (DLBCL, NOS) . Under influence of viral interleukin (IL)–6 and other factors, HHV-8 infected B-cells commonly transform into cells with plasmablastic features[29]; therefore, these HHV-8 infected neoplasms have many similarities including their immunophenotypic features. However, there are some immunophenotypic differences between these neoplasms that help distinguish them (Table 2 for PEL vs HHV-8 DLBCL). HHV-8(+) MCD plasmablasts, HHV-8(+) GLPD and HHV-8(+) DLBCL are characteristically CD138 negative and Ig LC positive (i.e., in a restricted fashion). HHV-8(+) MCD plasmablasts and HHV-8(+) DLBCL also frequently lack CD38 and CD30 expression but show variable expression of B-lineage markers such as CD20.[29]

It is also important to recognize that a single patient may be afflicted by more than one of these HHV-8(+) disorders sequentially or concurrently.[54] HHV8+ MCD often precedes HHV-8 DLBCL, PEL and PEL-EC. While HHV-8 DLBCL develops directly from HHV8+ MCD, there is currently no definite evidence that HHV8+ MCD evolves into PEL/PEL-EC.[29,55] Lastly, while FC is helpful in distinguishing between all of these neoplasms, it must be remembered that immunophenotypic features for all of these show variability. For instance, occasionally PEL may show expression of Ig LC or lack expression of CD138. There are even rare cases of PEL that show expression of B-lineage markers. Therefore, these types of neoplasms must be fully investigated using all available tools to render final and accurate diagnoses.

Post-transplant lymphoproliferative disorder (PTLD)

As the name suggests, PTLD arises as a consequence of immunosuppression after transplantation such as solid organ or stem cell allograft. Clinical presentation is highly variable and reflective of each institution's different patient population, allograft type and immunosuppressive regimens.[56] Shared similarities of PTLD include immunosuppression, EBV and cytomegalovirus infection.[3,4] The frequency of PTLD with lung transplant recipients is high risk (2.5%−8%)[22] which may be explained by the lung's increased immunosuppressive requirements as compared to other organs.[4] In solid organ transplants, PTLD can arise from the allograft, making for a difficult diagnostic picture since rejection and infection share similar clinical presentations. There are 4 main categorizations of PTLD: monomorphic PTLD (M-PTLD), polymorphic PTLD (P-PTLD), non-destructive PTLD, and Classic Hodgkin lymphoma PTLD. Appropriate categorization of PTLD is essential at time of diagnosis as it has therapeutic and prognostic implications. While tissue biopsy remains the gold standard for diagnosis, early detection and prompt intervention can be achieved with utilization of FC in correlation with clinical suspicion. Importantly, FC can help identify aberrant populations from reactive populations, making it extremely useful in cases of organ transplants.[56] The immunophenotype of the PTLD will mirror the type of non-Hodgkin lymphoma it represents.

Burkitt lymphoma (BL)

Burkitt Lymphoma is a mature B-cell lymphoma composed of medium-sized cells that is highly aggressive but often curable. According to the WHO classification,[22] no single morphologic, genetic or immunophenotypic parameters can be used as a gold standard for BL classification. Morphologically they are characterized by sheets of medium-sized lymphoid cells with high nuclear to cytoplasmic ratio, inconspicuous nucleoli, dispersed chromatin, and scant vacuolated basophilic cytoplasm. There are intervening scattered tingible-body

macrophages present giving the characteristic "starry sky" appearance.[25] Background necrosis, apoptotic bodies, and increased mitotic figures are typically be seen.

On FC analysis the neoplastic cells of BL will typically show intermediate forward light scatter (FSC) correlating with medium cell size but can show slightly higher FSC as well. Immunophenotypically, BL shows expression of B-cell antigens (e.g., CD19, CD20, CD22, CD79a, CD79b, and FMC-7), germinal center markers CD10 and BCL-6, and HLA-DR. BL most commonly shows restricted surface Ig LC expression, although a small proportion of BL cases completely lack surface Ig LC expression.[15] BL typically expresses MYC and CD43 but lacks MUM-1 expression by immunohistochemistry. BL also lacks expression of CD5, CD23, CD34, CD138, and TdT. BCL-2 is commonly negative or only weakly positive in a small proportion of cells, and CD200 is dim positive to negative.[57] BL often shows relatively high intensity of CD71 expression, a surrogate marker for proliferation index in BL. The proliferation index is very high in BL with positive Ki-67/Mib-1 staining in greater than 95% of neoplastic cells.[58] A very characteristic feature of BL is bright CD38 expression (i.e., level of germinal center B-cells or brighter), which is a feature often seen in B-cell lymphomas harboring a *MYC* translocation.[12,59,60]

Follicular lymphoma (FL)

Follicular Lymphoma is a germinal center derived B-cell neoplasm characterized by the t(14;18)(q32;q21) leading to BCL-2 gene overexpression. Histologically, this entity demonstrates a nodular growth pattern commonly involving lymph nodes but also occurs at extranodal sites including the spleen, bone marrow, peripheral blood, Waldeyer ring, or gastrointestinal tract. FL accounts for 20% of all lymphomas predominantly affecting adults, with a median age of 60.[16] Demonstration of a CD19(+), CD20(+) B-cell population with immunoglobulin light chain restriction and co-expression of CD10, a germinal center marker, and aberrant overexpression of BCL-2 are the diagnostic hallmarks of FL. This immunophenotypic profile by both FC and immunohistochemistry (IHC) has been established in nodal based FL,[61] however, slight differences can be seen in extranodal based FL. As follicular lymphoma leaves the microenvironment of the germinal center there can be diminished or loss of CD10 expression as often seen in bone marrow involvement.

Plasma cell neoplasms

Plasma cell neoplasms (PCNs) are neoplastic proliferations of plasma cells and are one of the most common forms of hematolymphoid neoplasms, representing 10% of all cases[22,62] PCNs are almost never found in children, and rarely in adults younger than 30 years old. About 90% of cases occur in patients over 50 years old, with a median age of 70 years old at the time of diagnosis.[22] The majority of cases originate and remain confined in the bone marrow (e.g., multiple myeloma); however, extramedullary involvement (e.g., extraosseous plasmacytoma) can occur which may signify advanced disease. Pulmonary involvement can be secondary or primary. Pleura invasion with pleural effusion can be seen in up to 6% of myeloma patients.[5,59,62] Morphologically, neoplastic plasma cells can exhibit mature features, such as abundant cytoplasm, eccentric nuclei and coarse chromatin (Fig. 10a) but can also display immature plasmablastic and pleomorphic appearance. General IP characteristics of PCNs include monotypic expression of cytoplasmic Ig LC while lacking expression of surface Ig. PCNs typically express bright CD38, bright VS38 and bright CD138 similar to normal plasma cell populations, but PCNs often aberrantly drop expression of CD45 and CD19.[22] Of the PCNs, multiple myelomas including extramedullary myeloma most commonly show aberrant expression of CD56 whereas primary extraosseous plasmacytomas often lack CD56 expression (Fig. 10b). Notably, plasma cells can be underrepresented in FC, especially in bone marrow specimens, and thus percentages of plasma cells used to establish a diagnosis should be generated from morphologic slides. Immunophenotypic differential diagnosis for plasma cell neoplasms include other lymphoid neoplasms with plasmacytic phenotypes and non-hematolymphoid neoplasms, as briefly discussed in other sections of this article (e.g., under the headings *Primary Effusion Lymphoma (PEL)* and *Non-hematolymphoid neoplasms and FC*). Plasma cell neoplasms involving the pulmonary system are also discussed in more detail in the article *Hematolymphoid Neoplasms with a Plasma Cell Phenotype* in the current journal.

T-lineage and NK-lineage neoplasms

T-lineage and NK-lineage neoplasms include neoplasms of immature cells (T-lymphoblastic leukemia/lymphoma and NK lymphoblastic leukemia/lymphoma) and mature cells (mature T-cell leukemia/lymphoma and mature NK cell neoplasms). Assigning T-cell lineage to a population of hematolymphoid cells by multiparameter flow cytometry is aided through identification of T-lineage specific or associated markers including CD2, surface CD3, cytoplasmic CD3, CD4, CD5, CD7, CD8, T-cell receptor (TCR) alpha/beta and TCR gamma/delta. NK cells share many of these markers but lack expression of surface CD3, CD4, and T-cell receptors. Additional routinely assessed markers seen on different subsets of normal T and NK cells include CD11b, CD16, CD56 and CD57. Assigning states of maturation and differentiation to T and NK cells is accomplished by assessing markers of immaturity [i.e., CD1a (in the context of T-lineage cells), CD34 and TdT] and assessing the expression patterns of T and NK lineage markers in association with markers such as CD10, CD38, CD45, CD45RA and CD45RO.[63-66] It is worth noting that the most commonly used antibody reagents for CD3 differ between flow cytometry (FC) and immunohistochemistry (IHC) in that the FC reagent is directed against the CD3 epsilon chain whereas the IHC reagent is directed against the CD3 zeta chain. Due to this difference, NK cells typically lack surface and cytoplasmic CD3 staining by flow cytometry but show positive cytoplasmic staining by IHC.

As with all hematolymphoid neoplasms identifying neoplastic populations of T and NK lymphocytes is accomplished by identification of DFN patterns of expression. This can be challenging with T and NK cells due to the potentially high number of normal/reactive subsets demonstrating immunophenotypic variation. Identifying neoplastic T and NK cell clones by FC can be aided through assessment of T-cell and NK cell receptors, in a fashion similarly to the role of Ig LC evaluation in B-cells. Traditionally, this has been accomplished through Vβ repertoire analysis for T-cells[67] and killer cell immunoglobulin-like receptor (KIR) analysis for NK cells.[68] However, these tests require a relatively high number of antibodies and are only occasionally performed due to the relatively low occurrence of T and NK cell neoplasms. Therefore, the number of clinical laboratories performing these tests is limited. More recently, a test for FC evaluation of T-cell clonality using T-cell receptor (TCR) β-chain constant regions has been developed that requires only one additional reagent antibody (TRBC1) making such testing feasible for many clinical FC laboratories.[69] Similar to B-cell expression patterns of sIg LC, normal TCRαβ T-cell populations are expected to exhibit comparable numbers of TRBC1-positive and TRBC2-positive subsets whereas TCRαβ T-cell neoplasms exhibit a restricted pattern of TRBC1 or TRBC2 expression. Incorporating the TCRBC1 reagent antibody in a T-cell tube facilitates identification of TCRαβ T-cell clones by FC.[69] While this approach cannot be used for TCRγδ T-cell population, the vast majority of T-cell malignancies derive from the TCRαβ T-cell subset making this approach applicable to most scenarios where a neoplastic T-cell population is suspected.[69]

Pulmonary involvement by T-cell and NK-cell neoplasms is very rare. The most common encountered at our institution is involvement of the pleural cavity by T-lymphoblastic leukemia/lymphoma, which is likely due to the proximity of the thymus to the lungs. Mature T-cell neoplasms are far less common.

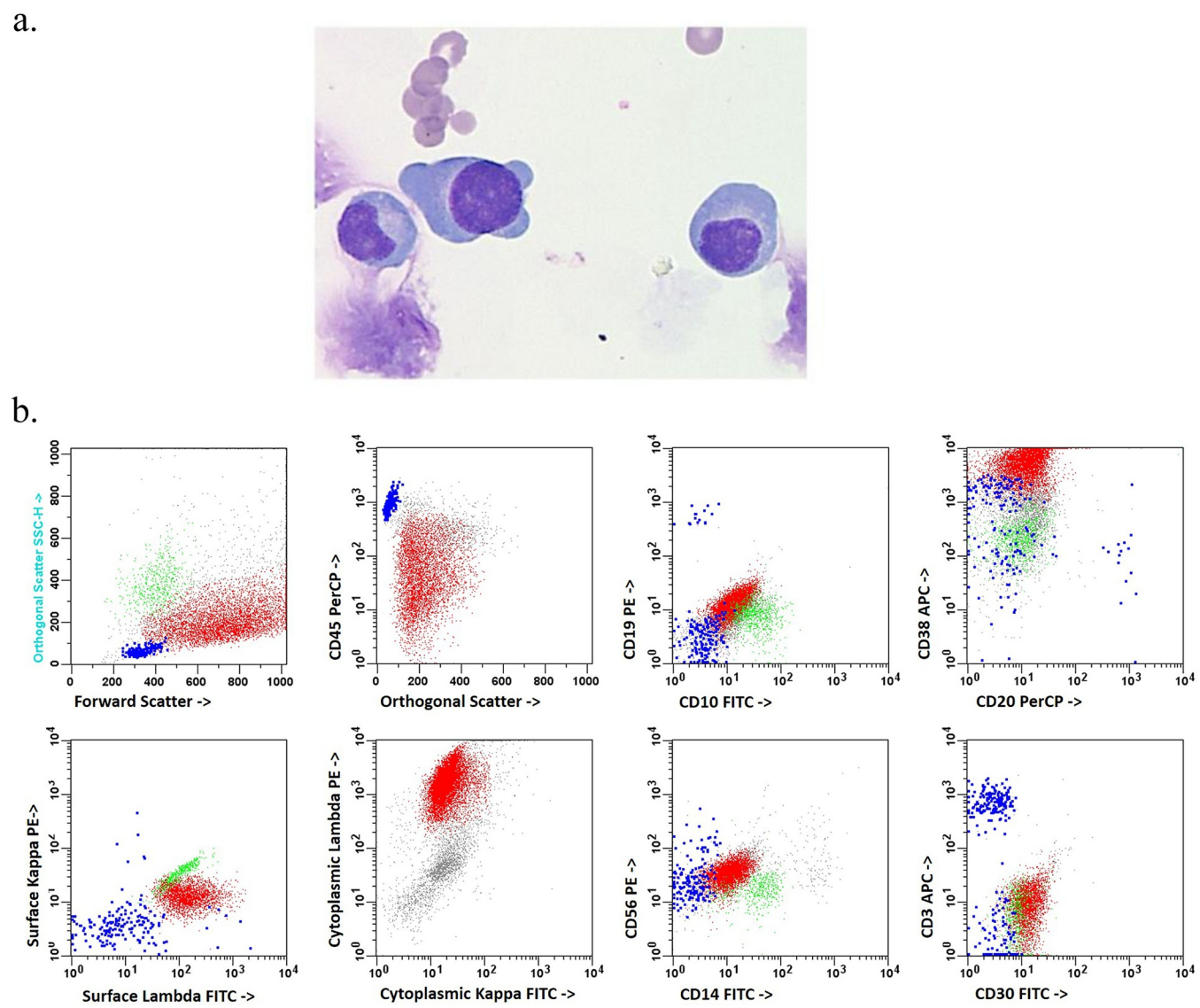

Fig. 10. Primary Extraosseous Plasmacytoma, Lung biopsy: (a.) Photomicrograph (50X objective) of a cytospin preparation of a pulmonary mass shows plasma cells with round, eccentrically placed nuclei, abnormally smooth chromatin, occasional nucleoli, abundant deep basophilic cytoplasm and perinuclear hofs. (b.) FC shows neoplastic plasma cells (red), T/B/NK lymphocytes (blue) and granulocytes (green). The plasma cells are negative for B-lineage markers CD19 and CD20 and show decreased to negative expression of CD45. The cells show features common for plasma cells such as bright CD38 and cytoplasmic Ig LC expression, which is restricted to lambda in this case. Note, that in contrast to the typical positive expression of CD56 by multiple myeloma in the bone marrow, this case is CD56 negative which is a feature often seen in primary plasmacytoma of the lung. In addition, this particular case shows positive dim expression of surface IG LC, which is a variation from the more commonly negative expression of sIG LC by plasma cell neoplasms. This latter finding highlights the fact that in actual practice neoplasms may show variation from characteristic immunophenotypes.

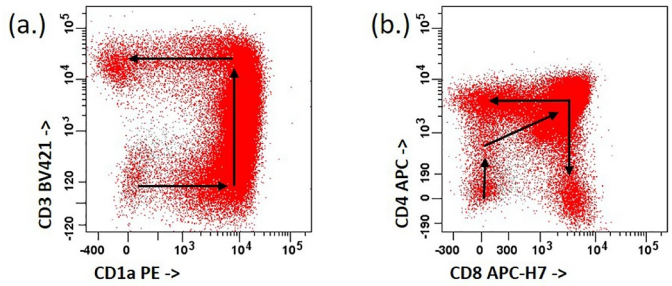

Fig. 11. Thymocytes, CD1a vs surface CD3 & CD4 vs CD8. Thymocytes go through the following patterns of marker expression from earliest to latest: (a.) CD1a (-)/CD3 (-) to CD1a (+)/CD3 (-) to CD1a (+)/CD3 (+) to CD1a (-)/CD3 (+) mature T cells and (b.) CD4(-)/CD8(-) to CD4(dim +)/CD8(-) to CD4(+)/CD8(+) to both CD4(+)/CD8(-) mature helper T-cells and CD4(-)/CD8(+) mature cytotoxic T-cells.

T-cell lymphoblastic lymphoma/leukemia (T-ALL)

T-ALL is a malignant neoplasm of immature T-cells (i.e., cells originating as thymocytes) that commonly presents with a rapidly expanding anterior mediastinal mass which can impinge upon respiratory function placing patients at risk for respiratory failure. Pleural and pericardial effusions are relatively common in T-ALL[5] and associated with an overall worse prognosis.[70] One of the main differential diagnoses of a patient presenting with a mediastinal mass includes lymphocyte-rich thymoma, which is an epithelial neoplasm containing non-neoplastic thymocytes. Ectopic and invasive thymoma can involve lung and pleural tissue.[71, 72, 73, 74, 75] Distinguishing between the benign thymocytes of thymoma and the malignant T-lymphoblasts of T-ALL can be especially challenging in specimens with limited material for morphologic evaluation (e.g., FNA or pleural fluid), given the similar cytologic features of thymocytes and lymphoblasts. In these scenarios,

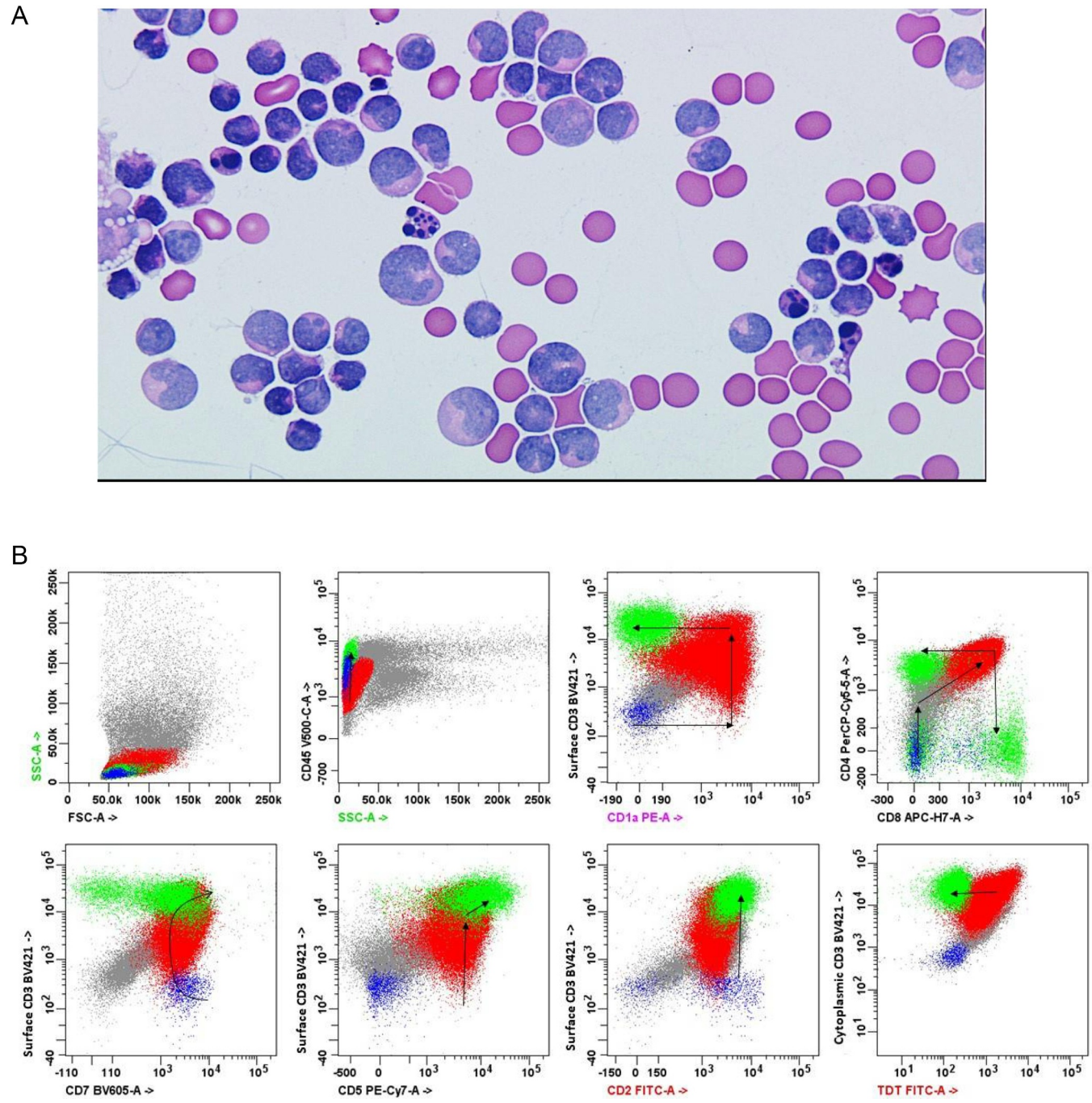

Fig. 12. T-lymphoblastic Leukemia/Lymphoma, Pleural Fluid. (a.) Photomicrograph (50X objective) of cytospin preparation shows variably sized lymphoblasts with round to irregular nuclei, dispersed chromatin, occasional nucleoli, and a small amount of cytoplasm with occasional vacuoles. (b.) FC shows T-lymphoblasts (red) with variable forward light scatter indicative of variable cell size and increased orthogonal light scatter secondary to cytoplasmic vacuolation. The lymphoblasts are CD1a (variably +), CD2 (dim +), surface and cytoplasmic CD3 (variably +), CD4 (+), CD5 (+), CD7 (+), CD8 (+), and TdT (+). These are all markers expressed by normal immature T-lineage cells (i.e., thymocytes). The normal pattern of expression for these markers thymocytes is illustrated by the arrows on the plots with the blunt end of the arrows representing the earliest thymocytes and the tips the most mature thymocytes. T-lymphoblasts (in red) show deviation from this expected expression pattern (i.e., DFN). In our experience, the majority of T-ALL can be identified by assessing CD1a vs surface CD3 and CD4 versus CD8 plots, which show DFN for these patterns in nearly every immature neoplastic T-cell population. Mature T-lymphocytes (green) and NK-lymphocytes (blue) are colored as reference populations.

flow cytometry is indispensable for differentiating thymocytes from T-lymphoblasts.[76,77] In their native location (i.e., the thymus), thymocytes follow a reproducible pattern of maturation for numerous markers.[63] This spectrum of maturation is very distinctive for CD1a versus surface CD3 and for CD4 versus CD8 with the earliest thymocytes being negative for all four markers. Thymocytes then progressively acquire each of these markers as they mature (Fig. 11). Deviation away from these patterns is very characteristic of T-ALL. Useful patterns of normal thymocyte expression for other markers are illustrated by the arrows in Fig. 12. Overall, the presence of a tight cluster of immature T-lineage cells, the lack of a spectrum of maturation, the expression of CD10 and/or CD34 with no surface CD3, CD2, or CD5 expression favors a diagnosis of T-LBL over thymoma.[65,78,79] There are a few reports that have assessed FC immunophenotypic features of ectopic thymocytes and

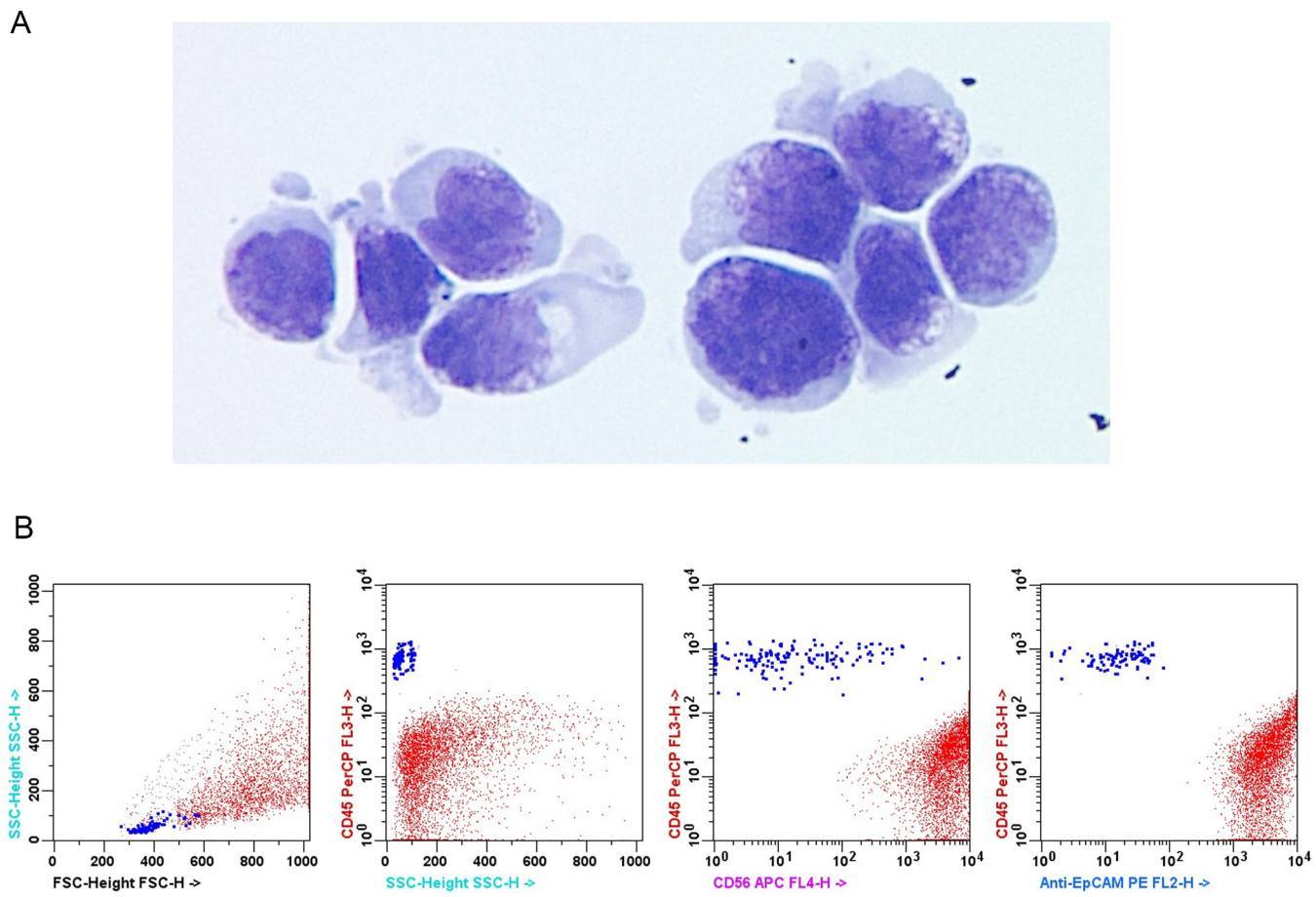

Fig. 13. Small Cell Carcinoma, Lung Biopsy: (a.) Photomicrograph (50X objective) of cytospin preparation of a pulmonary mass shows large cells with stippled to smooth chromatin, and moderate to abundant cytoplasm. These cells are present as single cells and in cohesive clusters with nuclear molding. (b.) FC from a pulmonary mass sample shows neoplastic cells (red) with predominantly intermediate to high forward light scatter indicative of larger cell size and low to high orthogonal light scatter. These cells lack expression of CD45 but show strong expression of CD56 and EpCAM (CD326). The cells lack all other hematolymphoid markers assessed (not shown in plots). Lymphocytes are shown in blue as a reference population.

have shown retention of the maturation spectrum for the markers tested[77,80]; however, when evaluating immature T-cell populations in sample sites from outside the thymus, it must be kept in mind that normal ectopic thymocytes have not been extensively evaluated. Therefore, DFN in pulmonary tissue must be carefully interpreted. In addition to abnormal patterns of normally expressed thymocyte markers (i.e., CD1a, CD2, CD3, CD4, CD8, CD10, CD34, CD45, TdT), T-ALL may aberrantly express markers typically associated with other cell lineages (e.g., CD79a, CD13, CD33, CD117, CD56). Once a population is determined to represent T-ALL, it should be labeled as either common T-ALL or early T-cell precursor acute lymphoblastic leukemia (ETP-ALL) due to potential differences in clinical management and prognosis. This is accomplished by evaluating the expression patterns of CD3, CD7, CD1a, CD8 and CD5 along with any stem cell or myeloid marker. As originally described, ETP-ALL must have unequivocal T-cell markers (i.e., it must express both CD7 and either surface or cytoplasmic CD3), CD1a and CD8 must be absent (i.e., expression must be in < 10% of the total T-lymphoblasts), CD5 must be dim compared to mature T-cells (i.e., MFI on T-lymphoblast population is at least 1 log lower or CD5 is expressed in less than 75% of the T-lymphoblasts), there must be 1 or more stem cell or myeloid associated marker (i.e., >10% of the T-lymphoblasts must express CD117, CD34, HLA-DR, CD13, CD33, CD11b, and/or CD65) and myeloperoxidase must be absent.[81,82]

Mature T-cell and NK-cell neoplasms

Mature T-cell hematolymphoid neoplasms may rarely infiltrate the lung and include neoplasms such as T-cell large granular lymphocytic leukemia, anaplastic large T-cell lymphoma, angioimmunoblastic T-cell lymphoma, and peripheral T-cell lymphoma, NOS. Extranodal NK/T-cell lymphoma, nasal type, very commonly involves the upper respiratory tract.[83] Due to various possible subsets of normal, reactive T and NK cells showing various immunophenotypic variations identifying neoplastic population of T-cells can be quite challenging and often requires a very in-depth analysis for deviations from normal T/NK-cell antigen patterns.[83,84] Historically, evaluation of T and NK-cell clonality by FC has been relatively complicated and not readily available in clinical FC laboratories. This has recently changed for identification of TCRγδ T-cell clones through inclusion of a single reagent antibody (TRBC1), as described in a previous section.

Mature T-cell neoplasms can be broadly separated into TCR alpha/beta and TCR gamma/delta neoplasms or CD4 (+) and CD8 (+) neoplasms with further refinement dependent on overall immunophenotypic features. Mature T-cell neoplasms with CD4/CD8 double negative and double positive immunophenotypes do occur but are even less common. FC findings of mature T and NK cell neoplasms is dependent on the particular neoplasm.

Non-hematolymphoid pulmonary neoplasms and FC

Non-hematolymphoid neoplasms are occasionally encountered in the flow cytometry lab when working up pulmonary samples. While clinical flow cytometry labs do not have the markers to fully characterize such neoplasms, routine flow cytometry can identify the presence of these neoplasms in many instances. Non-hematolymphoid neoplasms can often be identified on a forward light scatter (FSC) versus orthogonal light scatter (SSC) physical parameter plot and a CD45 versus SSC plot (Fig. 13). Non-hematolymphoid neoplasms consist of large cells that often show high forward light scatter and low to high orthogonal light scatter and lack nearly all markers routinely assessed by flow cytometry. In particular, non-hematolymphoid neoplasms lack leukocyte common antigen CD45, which is generally considered a hematolymphoid marker. Identification of a cell population with intermediate to high FSC that lacks CD45 should raise suspicion for a non-hematolymphoid neoplasm. It is important to remember that not all hematolymphoid neoplasms express CD45 (e.g., many plasma cell neoplasms and many B-lymphoblastic leukemia/lymphomas); however, most CD45 negative hematolymphoid neoplasms show expected expression patterns for other hematolymphoid and/or activation markers. Notably, non-hematolymphoid neoplasms may express some of the routinely assessed markers in FC such as CD10, CD30, CD138 and CD56 dependent on the cell of origin. Alternatively, non-hematolymphoid neoplasms may show artifactual positivity for various markers secondary to high autofluorescence. If warranted and enough sample is available, a fluorescence minus one (FMO) tube can be performed to determine staining thresholds for these typically large cells.

The absence of an abnormal CD45 (-) population with increased forward light scatter does not rule out the presence of a non-hematolymphoid neoplasm. This is quite evident when corresponding cytologic examination clearly shows the presence of non-hematolymphoid neoplastic cells. Such discrepancy may be due to the cohesive nature of certain non-hematolymphoid neoplasms (e.g., squamous cell carcinoma) or the fragility of the large tumor cells, presumably both resulting in very few neoplastic cells suspended singly in solution for flow cytometry analysis. However, sensitivity for non-hematolymphoid neoplasms of epithelial origin can be significantly increased using an epithelial marker in the analysis. This strategy can be employed in instances where clinical presentation or the triage on the imprint/cytospin preparation is suspicious for non-hematolymphoid neoplasm.

The most widely used epithelial marker in FC is EpCAM (CD326 or Ber-EP4), which is a cell membrane glycoprotein present on normal human epithelia and a wide range of epithelial neoplasms but not on normal or neoplastic mesothelial or hematopoietic cells.[85,86,87,88] Therefore, it is particularly useful in body fluid specimens such as pleural fluid, which normally contains mesothelial cells and hematolymphoid cells but not epithelial cells. It is also useful in other pulmonary tissue samples such as BAL and solid biopsies; however, these tissues will contain normal EpCAM positive epithelia that may be identified by FC.[89] EpCAM cannot distinguish between normal and malignant epithelial cells; therefore, cytologic preparations can be helpful in determining the nature of the epithelial cells. In cases where the epithelial cells are not seen on the cytologic preparations but are identified by FC, their presence can be noted but the significance must be described as undetermined. Notably, not all non-hematolymphoid neoplasms, including some carcinomas, express EpCAM.

In our laboratory, we use an antibody tube that contains CD45, CD56 and EpCAM for suspected non-hematolymphoid neoplasms (Fig. 13). The marker CD56 identifies neural cell adhesion molecule (NCAM), which is a member of the immunoglobulin super family expressed in highest concentrations on normal neurons, glia, skeletal muscle, NK lymphocytes and a subset of cytotoxic lymphocytes. Malignant neoplasms that often express CD56 include hematolymphoid neoplasms (e.g., myeloma, myeloid leukemia, blastic plasmacytoid dendritic cell neoplasms, NK and T cell neoplasms) as well as non-hematolymphoid neoplasms (e.g., neuroendocrine tumors, Wilms' tumor, neuroblastoma, pancreatic acinar cell carcinoma, pheochromocytoma, paraganglioma, small cell lung carcinoma, and the neuroectodermal/Ewing's sarcoma family of tumors).[90] A panel that includes a tube containing a CD45, CD56, and EpCAM combination can typically differentiate between a non-hematolymphoid and a hematolymphoid neoplasm. However, certain non-hematolymphoid neoplasms such as neuroendocrine tumors and hematolymphoid neoplasms such as plasma cell neoplasms or lymphomas with plasmacytic phenotypes may have the same CD45(-)/CD56(+)/EpCAM (-) expression profile but can typically be differentiated through cytologic features and/or assessment of other markers that may be included in the panel such as CD38, VS38, CD138 and Ig LC. Notably, expression of CD138 alone cannot distinguish between plasma cell neoplasms or lymphomas with plasmacytic phenotypes and various carcinomas such as prostate, colon, cervical, renal cell, and some forms of squamous cell carcinomas that also express CD138.[91] Not all of these carcinomas will show EpCAM positivity and not all plasma cell neoplasms will show CD56 positivity, which leads to a possible CD45(-)/CD56(-)/EpCAM(-) expression profile in both categories of neoplasia. The fact that pulmonary samples are often paucicellular further compounds the difficulty in differentiating such cases, as complete immunophenotypic work ups such as cytoplasmic evaluation of Ig LC may not be able to be performed. Such scenarios highlight the level of caution that must be taken when assigning significance to immunophenotypic features, especially in low cellular specimens. It also highlights the importance of a thorough investigation with correlation of immunophenotypic, clinical, morphologic and genetic features when establishing a final diagnosis.

Cases positive for carcinoma may lack EpCAM positive cells, which typically have high forward light scatter, but show EpCAM positive events with low FSC, which is an area that typically contains debris/mature red blood cells. These events may represent extracellular tumor microparticles that have broken off the neoplastic cells.[92] While they are unlikely to be whole tumor cells, these EpCAM positive “microparticles” can indicate the presence of non-hematolymphoid neoplasm. Inevitably, there will also be cases where there are CD45 (+) cells that stain EpCAM positive. This is also likely due to the presence of carcinoma microparticles adhered to hematolymphoid cells.[92] In summary, non-hematolymphoid neoplasms can be identified through FC, especially when combined with an imprint/cytospin triage/review of the sample. The intention of FC in the analysis of non-hematolymphoid neoplasms is to help identify the malignancy and to guide the pathologist the next steps of the work up.

Challenges/Pitfalls in the application of FC

Several commonly known PLNs such as classical Hodgkin lymphomas (CHLs) are not discussed extensively in this review because they are not typically identified by FC without special processing and specific reagents that are not performed or available in most clinical flow cytometry laboratories.[93] However, in the case of classical Hodgkin lymphoma, there are several nonspecific findings in the reactive background T-cells that are often seen in routine FC and that may raise suspicion for disease. These include an increased CD4 to CD8 ratio among T-lymphocytes and increased intensity of CD7 and CD45 staining on the CD4 (+) helper T-cells.[94] Hodgkin cells and Reed-Sternberg cells of CHL are also often identified cytologically on a cytospin preparation.

A few new challenges in FC analysis have arisen secondary to recently developed therapeutic approaches such as chimeric antigen receptor T-cell (CART) therapy and monoclonal antibody therapies. These types of therapies often interfere with traditional gating approaches for identifying neoplasms since they alter the expression patterns of the antigens they are directed against. Therefore, following such therapy new gating strategies using unconventional gating markers such as CD24 and CD22 for B-cell neoplasms or VS38 for plasma cell neoplasms may be required.

References

1. Chilosi M, Zinzani PL, Poletti V. Lymphoproliferative lung disorders. *Semin Respir Crit Care Med.* 2005;26(5):490–501.
2. Sohani A, Ferry J. Lymphomas and lymphoproliferative diseases of the lung. *Diagnostic Histopathology.* 2014;20.
3. Tang VK, Vijhani P, Cherian SV, Ambelil M, Estrada YMRM. Primary pulmonary lymphoproliferative neoplasms. *Lung India.* 2018;35(3):220–230.
4. William J, Variakojis D, Yeldandi A, Raparia K. Lymphoproliferative neoplasms of the lung: a review. *Arch Pathol Lab Med.* 2013;137(3):382–391.
5. Bode-Lesniewska B. Flow Cytometry and Effusions in Lymphoproliferative Processes and Other Hematologic Neoplasias. *Acta Cytol.* 2016;60(4):354–364.
6. Ahmed S, Shahid RK, Rimawi R, et al. Malignant pleural effusions in lymphoproliferative disorders. *Leuk Lymphoma.* 2005;46(7):1039–1044.
7. Vega F, Padula A, Valbuena JR, Stancu M, Jones D, Medeiros LJ. Lymphomas involving the pleura: a clinicopathologic study of 34 cases diagnosed by pleural biopsy. *Arch Pathol Lab Med.* 2006;130(10):1497–1502.
8. Borie R, Wislez M, Antoine M, Cadranel J. Lymphoproliferative Disorders of the Lung. *Respiration.* 2017;94(2):157–175.
9. Tighe RM, Redente EF, Yu YR, et al. Improving the Quality and Reproducibility of Flow Cytometry in the Lung. An Official American Thoracic Society Workshop Report. *Am J Respir Cell Mol Biol.* 2019;61(2):150–161.
10. Ortolani C. *Flow Cytometry of Hematological Malignancies.* Wiley; 2011.
11. Britt Z ODM, Mills D. Surface staining for kappa and lambda, how many washes are sufficient? You might be surprised. *International Clinical Cytometry Society.* 2015;VI (3) Published Sept 7 2015.
12. Alsuwaidan A, Pirruccello E, Jaso J, et al. Bright CD38 Expression by Flow Cytometric Analysis Is a Biomarker for Double/Triple Hit Lymphomas with a Moderate Sensitivity and High Specificity. *Cytometry B Clin Cytom.* 2019;96(5):368–374.
13. Li S, Eshleman JR, Borowitz MJ. Lack of surface immunoglobulin light chain expression by flow cytometric immunophenotyping can help diagnose peripheral B-cell lymphoma. *Am J Clin Pathol.* 2002;118(2):229–234.
14. Kaleem Z, Zehnbauer BA, White G, Zutter MM. Lack of expression of surface immunoglobulin light chains in B-cell non-Hodgkin lymphomas. *Am J Clin Pathol.* 2000;113(3):399–405.
15. Kelemen K, Braziel RM, Gatter K, Bakke TC, Olson S, Fan G. Immunophenotypic variations of Burkitt lymphoma. *Am J Clin Pathol.* 2010;134(1):127–138.
16. Grier DD, Al-Quran SZ, Cardona DM, Li Y, Braylan RC. Flow cytometric analysis of immunoglobulin heavy chain expression in B-cell lymphoma and reactive lymphoid hyperplasia. *Int J Clin Exp Pathol.* 2012;5(2):110–118.
17. Horna P, Olteanu H, Kroft SH, Harrington AM. Flow cytometric analysis of surface light chain expression patterns in B-cell lymphomas using monoclonal and polyclonal antibodies. *Am J Clin Pathol.* 2011;136(6):954–959.
18. Borie R, Wislez M, Antoine M, Copie-Bergman C, Thieblemont C, Cadranel J. Pulmonary mucosa-associated lymphoid tissue lymphoma revisited. *Eur Respir J.* 2016;47(4):1244–1260.
19. Bi L, Li J, Dan W, Lu Z. Pulmonary MALT lymphoma: a case report and review of the literature. *Exp Ther Med.* 2015;9(1):147–150.
20. Couto C, Martins V, Ribeiro V, et al. Primary Pulmonary MALT Lymphoma: a Case Report and Literature Review. *Biomed Hub.* 2019;4(3):1–5.
21. Bachuwa G, Naik P, Campe J, Lecea N, Congdon D. Ninety-one year old: oldest patient reported with pulmonary mucosa-associated lymphoid tissue lymphoma and rare association with pleural effusion. *Geriatr Gerontol Int.* 2012;12(1):149–151.
22. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood.* 2016;127(20):2375–2390.
23. Crapanzano JP, Lin O. Cytologic findings of marginal zone lymphoma. *Cancer.* 2003;99(5):301–309.
24. Seegmiller AC, Xu Y, McKenna RW, Karandikar NJ. Immunophenotypic differentiation between neoplastic plasma cells in mature B-cell lymphoma vs plasma cell myeloma. *Am J Clin Pathol.* 2007;127(2):176–181.
25. Pina-Oviedo S, Weissferdt A, Kalhor N, Moran CA. Primary Pulmonary Lymphomas. *Adv Anat Pathol.* 2015;22(6):355–375.
26. Wang T, Zhang M, Sun J, et al. A rare case of primary pulmonary diffuse large B cell lymphoma with CD5 positive expression. *Open Med (Wars).* 2016;11(1):49–51.
27. Foster WR, Bischin A, Dorer R, Aboulafia DM. Human Herpesvirus Type 8-associated Large B-cell Lymphoma: a Nonserous Extracavitary Variant of Primary Effusion Lymphoma in an HIV-infected Man: a Case Report and Review of the Literature. *Clin Lymphoma Myeloma Leuk.* 2016;16(6):311–321.
28. Guillet S, Gerard L, Meignin V, et al. Classic and extracavitary primary effusion lymphoma in 51 HIV-infected patients from a single institution. *Am J Hematol.* 2016;91(2):233–237.
29. Chadburn A, Said J, Gratzinger D, et al. HHV8/KSHV-Positive Lymphoproliferative Disorders and the Spectrum of Plasmablastic and Plasma Cell Neoplasms: 2015 SH/EAHP Workshop Report-Part 3. *Am J Clin Pathol.* 2017;147(2):171–187.
30. Castillo JJ, Shum H, Lahijani M, Winer ES, Butera JN. Prognosis in primary effusion lymphoma is associated with the number of body cavities involved. *Leuk Lymphoma.* 2012;53(12):2378–2382.
31. Chadburn A, Hyjek E, Mathew S, Cesarman E, Said J, Knowles DM. KSHV-positive solid lymphomas represent an extra-cavitary variant of primary effusion lymphoma. *Am J Surg Pathol.* 2004;28(11):1401–1416.
32. Said JW, Tasaka T, Takeuchi S, et al. Primary effusion lymphoma in women: report of two cases of Kaposi's sarcoma herpes virus-associated effusion-based lymphoma in human immunodeficiency virus-negative women. *Blood.* 1996;88(8):3124–3128.
33. Crane GM, Xian RR, Burns KH, Borowitz MJ, Duffield AS, Taube JM. Primary effusion lymphoma presenting as a cutaneous intravascular lymphoma. *J Cutan Pathol.* 2014;41(12):928–935.
34. Kim Y, Leventaki V, Bhaijee F, Jackson CC, Medeiros LJ, Vega F. Extracavitary/solid variant of primary effusion lymphoma. *Ann Diagn Pathol.* 2012;16(6):441–446.
35. Lyapichev KA, Medeiros LJ, Clemens MW, et al. Reconsideration of the first recognition of breast implant-associated anaplastic large cell lymphoma: a critical review of the literature. *Ann Diagn Pathol.* 2020;45:151474.
36. Kaplan LD. Human herpesvirus-8: kaposi sarcoma, multicentric Castleman disease, and primary effusion lymphoma. *Hematology Am Soc Hematol Educ Program.* 2013;2013:103–108.
37. Jaffe ES, Arber DA, Campo E, Harris NL, Quintanilla-Martinez L. *Hematopathology.* 2017.
38. Cesarman E. Gammaherpesviruses and lymphoproliferative disorders. *Annu Rev Pathol.* 2014;9:349–372.
39. Dunleavy K, Wilson WH. How I treat HIV-associated lymphoma. *Blood.* 2012;119(14):3245–3255.
40. Manzano M, Patil A, Waldrop A, Dave SS, Behdad A, Gottwein E. Gene essentiality landscape and druggable oncogenic dependencies in herpesviral primary effusion lymphoma. *Nat Commun.* 2018;9(1):3263.
41. Kalogeraki A, Haniotis V, Karvelas-Kalogerakis M, Karvela-Kalogeraki I, Psyllaki M, Tamiolakis D. Primary effusion lymphoma with aberrant T-cell phenotype in an iatrogenically immunosuppressed renal transplant male: cytologic diagnosis in peritoneal fluid. *Diagn Cytopathol.* 2015;43(2):144–148.
42. Coupland SE, Charlotte F, Mansour G, Maloum K, Hummel M, Stein H. HHV-8-associated T-cell lymphoma in a lymph node with concurrent peritoneal effusion in an HIV-positive man. *Am J Surg Pathol.* 2005;29(5):647–652.
43. Jenner RG, Maillard K, Cattini N, et al. Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphoma has a plasma cell gene expression profile. *Proc Natl Acad Sci U S A.* 2003;100(18):10399–10404.
44. Klein U, Gloghini A, Gaidano G, et al. Gene expression profile analysis of AIDS-related primary effusion lymphoma (PEL) suggests a plasmablastic derivation and identifies PEL-specific transcripts. *Blood.* 2003;101(10):4115–4121.
45. Fan W, Bubman D, Chadburn A, Harrington Jr WJ, Cesarman E, Knowles DM. Distinct subsets of primary effusion lymphoma can be identified based on their cellular gene expression profile and viral association. *J Virol.* 2005;79(2):1244–1251.
46. Hamoudi R, Diss TC, Oksenhendler E, et al. Distinct cellular origins of primary effusion lymphoma with and without EBV infection. *Leuk Res.* 2004;28(4):333–338.
47. Matolcsy A, Nador RG, Cesarman E, Knowles DM. Immunoglobulin VH gene mutational analysis suggests that primary effusion lymphomas derive from different stages of B cell maturation. *Am J Pathol.* 1998;153(5):1609–1614.
48. Fernandez-Trujillo L, Bolanos JE, Velasquez M, Garcia C, Sua LF. Primary effusion lymphoma in a human immunodeficiency virus-negative patient with unexpected unusual complications: a case report. *J Med Case Rep.* 2019;13(1):301.
49. Ogata M, Imamura T, Mizunoe S, Ohtsuka E, Kikuchi H, Nasu M. Natural killer cell-type body cavity lymphoma following chronic active Epstein-Barr virus infection. *Am J Hematol.* 2003;73(2):126–130.
50. Nakatsuka S, Yao M, Hoshida Y, Yamamoto S, Iuchi K, Aozasa K. Pyothorax-associated lymphoma: a review of 106 cases. *J Clin Oncol.* 2002;20(20):4255–4260.
51. Alexanian S, Said J, Lones M, Pullarkat ST. KSHV/HHV8-negative effusion-based lymphoma, a distinct entity associated with fluid overload states. *Am J Surg Pathol.* 2013;37(2):241–249.
52. Brimo F, Michel RP, Khetani K, Auger M. Primary effusion lymphoma: a series of 4 cases and review of the literature with emphasis on cytomorphologic and immunocytochemical differential diagnosis. *Cancer.* 2007;111(4):224–233.
53. Narkhede M, Arora S, Ujjani C. Primary effusion lymphoma: current perspectives. *Onco Targets Ther.* 2018;11:3747–3754.
54. Guerrero C, Jain T, Kelemen K. HHV-8-Associated Lymphoproliferative Disorders and Pathogenesis in an HIV-Positive Patient. *Case Rep Hematol.* 2019;2019:4536157.
55. Pan ZG, Zhang QY, Lu ZB, et al. Extracavitary KSHV-associated large B-Cell lymphoma: a distinct entity or a subtype of primary effusion lymphoma? Study of 9 cases and review of an additional 43 cases. *Am J Surg Pathol.* 2012;36(8):1129–1140.
56. Li X, Li N, Yang T, Chen Z, Hu J. Lymph Node Flow Cytometry as a Prompt Recognition of Ultra Early Onset PTLD: a Successful Case of Rituximab Treatment. *Case Rep Hematol.* 2015;2015:430623.
57. Challagundla P, Medeiros LJ, Kanagal-Shamanna R, Miranda RN, Jorgensen JL. Differential expression of CD200 in B-cell neoplasms by flow cytometry can assist in diagnosis, subclassification, and bone marrow staging. *Am J Clin Pathol.* 2014;142(6):837–844.
58. McGowan P, Nelles N, Wimmer J, et al. Differentiating between Burkitt lymphoma and CD10+ diffuse large B-cell lymphoma: the role of commonly used flow cytometry cell markers and the application of a multiparameter scoring system. *Am J Clin Pathol.* 2012;137(4):665–670.
59. Rodig SJ, Vergilio JA, Shahsafaei A, Dorfman DM. Characteristic expression patterns of TCL1, CD38, and CD44 identify aggressive lymphomas harboring a MYC translocation. *Am J Surg Pathol.* 2008;32(1):113–122.
60. Maleki A, Seegmiller AC, Uddin N, Karandikar NJ, Chen W. Bright CD38 expression is an indicator of MYC rearrangement. *Leuk Lymphoma.* 2009;50(6):1054–1057.
61. Sorigue M, Santos-Gomez M, Comes M, et al. Flow Cytometry in the Differential Diagnosis of CD10-Positive Nodal Lymphomas. *Lab Med.* 2019.
62. Kanagarajah P, Cao K, Chen L, Rumbak M. Malignant Multiple Myeloma of the Pleura. *J Bronchology Interv Pulmonol.* 2018;25(4):332–334.
63. Kroft SH. Role of flow cytometry in pediatric hematopathology. *Am J Clin Pathol.* 2004;122(Suppl:S19–32).
64. van Dongen JJ, Lhermitte L, Bottcher S, et al. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. *Leukemia.* 2012;26(9):1908–1975.

65. Li S, Juco J, Mann KP, Holden JT. Flow cytometry in the differential diagnosis of lymphocyte-rich thymoma from precursor T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma. *Am J Clin Pathol.* 2004;121(2):268–274.
66. Renoux VM, Zriwil A, Peitzsch C, et al. Identification of a Human Natural Killer Cell Lineage-Restricted Progenitor in Fetal and Adult Tissues. *Immunity.* 2015;43(2):394–407.
67. Tembhare P, Yuan CM, Morris JC, Janik JE, Filie AC, Stetler-Stevenson M. Flow cytometric immunophenotypic assessment of T-cell clonality by vbeta repertoire analysis in fine-needle aspirates and cerebrospinal fluid. *Am J Clin Pathol.* 2012;137(2):220–226.
68. Schonberg K, Hejazi M, Uhrberg M. Protocol for the clonal analysis of NK cell effector functions by multi-parameter flow cytometry. *Methods Mol Biol.* 2012;903:381–392.
69. Shi M, Jevremovic D, Otteson GE, Timm MM, Olteanu H, Horna P. Single Antibody Detection of T-Cell Receptor alphabeta Clonality by Flow Cytometry Rapidly Identifies Mature T-Cell Neoplasms and Monotypic Small CD8-Positive Subsets of Uncertain Significance. *Cytometry B Clin Cytom.* 2020;98(1):99–107.
70. Chang MH, Kim SJ, Kim K, et al. Clinical features and treatment outcomes of adult B- and T-lymphoblastic lymphoma: results of multicentre analysis in Korea. *Leuk Lymphoma.* 2009;50(7):1119–1125.
71. Detterbeck FC, Parsons AM. Thymic tumors. *Ann Thorac Surg.* 2004;77(5):1860–1869.
72. Dashottar S, Singh AK, Suhag V, Sunita BS. Ectopic thymoma presenting as a large intrathoracic mass. *J Cancer Res Ther.* 2015;11(4):1033.
73. Yan B, Lim D, Petersson F. Ectopic cervical thymoma: a report of two cases of a rare entity frequently misdiagnosed on fine needle aspiration cytology and frozen section. *Head Neck Pathol.* 2010;4(2):152–156.
74. Yamazaki K, Yoshino I, Oba T, et al. Ectopic pleural thymoma presenting as a giant mass in the thoracic cavity. *Ann Thorac Surg.* 2007;83(1):315–317.
75. Benveniste MF, Korst RJ, Rajan A, Detterbeck FC, Marom EM. International Thymic Malignancy Interest G. A practical guide from the International Thymic Malignancy Interest Group (ITMIG) regarding the radiographic assessment of treatment response of thymic epithelial tumors using modified RECIST criteria. *J Thorac Oncol.* 2014;9(9 Suppl 2):S119–S124.
76. Li W, Morgan R, Ali R, Nieder R, Wheeler L. Flow Cytometric Detection of Intrathyroidal Ectopic Thymic Tissue in Thyroid FNA Samples: report of Three Cases. *Am. J. Clin. Pathol.* 2016;146(suppl_1).
77. Escobar FA, Pantanowitz L, Picarsic JL, et al. Cytomorphology and sonographic features of ectopic thymic tissue diagnosed in paediatric FNA biopsies. *Cytopathology.* 2018;29(3):241–246.
78. Yu GH, Vergara N, Moore EM, King RL. Use of flow cytometry in the diagnosis of lymphoproliferative disorders in fluid specimens. *Diagn Cytopathol.* 2014;42(8):664–670.
79. You MJ, Medeiros LJ, Hsi ED. T-lymphoblastic leukemia/lymphoma. *Am J Clin Pathol.* 2015;144(3):411–422.
80. Aguayo-Figueroa L, Golightly MG, Hu Y, Cohen HL, Wilson TA. Cytology and flow cytometry to identify ectopic thymic tissue masquerading as a thyroid nodule in two children. *Thyroid.* 2009;19(4):403–406.
81. Coustan-Smith E, Mullighan CG, Onciu M, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. *Lancet Oncol.* 2009;10(2):147–156.
82. Campana D. Early T-cell Precursor Acute Lymphoblastic Leukemia (ETP-ALL): identification and Clinical Significance. https://www.cytometry.org/public/educational_presentations/Campana.pdf. Accessed.
83. Jevremovic D, Olteanu H. Flow Cytometry Applications in the Diagnosis of T/NK-Cell Lymphoproliferative Disorders. *Cytometry B Clin Cytom.* 2019;96(2):99–115.
84. Chen W, Kesler MV, Karandikar NJ, McKenna RW, Kroft SH. Flow cytometric features of angioimmunoblastic T-cell lymphoma. *Cytometry B Clin Cytom.* 2006;70(3):142–148.
85. Patriarca C, Macchi RM, Marschner AK, Mellstedt H. Epithelial cell adhesion molecule expression (CD326) in cancer: a short review. *Cancer Treat Rev.* 2012;38(1):68–75.
86. Went PT, Lugli A, Meier S, et al. Frequent EpCam protein expression in human carcinomas. *Hum Pathol.* 2004;35(1):122–128.
87. Pillai V, Cibas ES, Dorfman DM. A simplified flow cytometric immunophenotyping procedure for the diagnosis of effusions caused by epithelial malignancies. *Am J Clin Pathol.* 2013;139(5):672–681.
88. Dorwal P, Moore H, Stewart P, Harrison B, Monaghan J. CD326 (EpCAM) testing by flow cytometric BerEP4 antibody is a useful and rapid adjunct to histopathology. *Cytometry B Clin Cytom.* 2018;94(3):536–541.
89. Acosta M, Pereira J, Arroz M. Screening of carcinoma metastasis by flow cytometry: a study of 238 cases. *Cytometry B Clin Cytom.* 2016;90(3):289–294.
90. Cornfield D, Liu Z, Gorczyca W, Weisberger J. The potential role of flow cytometry in the diagnosis of small cell carcinoma. *Arch Pathol Lab Med.* 2003;127(4):461–464.
91. Ramalingam P, Adeagbo B, Bollag R, Lee J, Reid-Nicholson M. Metastatic hepatocellular carcinoma with CD138 positivity: an unusual mimic of multiple myeloma? *Diagn Cytopathol.* 2008;36(10):742–748.
92. Pillai V, Dorfman DM. Flow Cytometry of Nonhematopoietic Neoplasms. *Acta Cytol.* 2016;60(4):336–343.
93. Grewal RK, Chetty M, Abayomi EA, Tomuleasa C, Fromm JR. Use of flow cytometry in the phenotypic diagnosis of hodgkin's lymphoma. *Cytometry B Clin Cytom.* 2019;96(2):116–127.
94. Wu D, Thomas A, Fromm JR. Reactive T cells by flow cytometry distinguish Hodgkin lymphomas from T cell/histiocyte-rich large B cell lymphoma. *Cytometry B Clin Cytom.* 2016;90(5):424–432.
95. Dabir S, Kresak A, Yang M, Fu P, Wildey G, Dowlati A. CD30 is a potential therapeutic target in malignant mesothelioma. *Mol Cancer Ther.* 2015;14(3):740–746.
96. Du Ming-Qing, et al. Kaposi sarcoma–associated herpesvirus infects monotypic (IgM lambda) but polyclonal naive B cells in Castleman disease and associated lymphoproliferative disorders. *Blood.* 2001;97(7):2130–2136. https://doi.org/10.1182/blood.v97.7.2130 In press.
97. Vafaii P, DiGiuseppe JA. Detection of B-cell populations with monotypic light chain expression in cerebrospinal fluid specimens from patients with multiple sclerosis by polychromatic flow cytometry. *Cytometry B Clin Cytom.* 2014;86(2):106–110. https://doi.org/10.1002/cyto.b.21099 In press.
98. Attygalle Atyoma, et al. Atypical marginal zone hyperplasia of mucosa-associated lymphoid tissue: A reactive condition of childhood showing immunoglobulin lambda light chain restriction. *Blood.* 2004;104(10):3343–3348. https://doi.org/10.1182/blood-2004-01-0385.
99. Kroft Steven. Monoclones, monotypes, and neoplasia pitfalls in lymphoma diagnosis. *Am J Clin Pathol.* 2004;121(4):457–459. https://doi.org/10.1309/RR8A-XUWM-9FYX-178A.
100. Fend F, et al. Early lesions in lymphoid neoplasia: Conclusions based on the Workshop of the XV. Meeting of the European Association of Hematopathology and the Society of Hematopathology, in Uppsala, Sweden. *J Hematop.* 2012;5(3) https://doi.org/10.1007/s12308-012-0148-6.