Flow Immunophenotyping of Benign Lymph Nodes Sampled by FNA: Representative With Diagnostic Pitfalls

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BACKGROUND: Fine-needle aspiration with flow cytometry (FNA-FC) is routinely used in the evaluation of lymph nodes suspicious for lymphoma, yet data comparing immunophenotype distributions and outliers in benign lymph nodes sampled by fine-needle aspiration (FNA) versus excision are lacking. **METHODS:** Flow cytometry data from 289 benign lymph node FNA cases were assessed for the overall antigen distribution, with a focus on outliers relevant to the diagnosis of lymphoma. Distributions and outlier proportions were compared with those of a separate cohort of 298 excisional biopsies. **RESULTS:** Compared with excisional biopsies, FNA specimens overrepresented CD3+ events (72% vs 63%), underrepresented CD19+ events (22% vs 29%), and had 25% fewer large cell–gated events. Normalized antigen distributions in FNA were equivalent to those in excisional biopsy. Twenty-three percent of FNA-FC cases exhibited an outlier, including a skewed kappa:lambda light-chain ratio, increased CD5+ or CD10+ B-cell events, a skewed CD4:CD8 ratio, and increased CD7 loss on T cells, with no significant differences in frequency or type in comparison with excisional specimens. Outliers for the light-chain ratio and T-cell antigens were enriched among older patients and included patients with a variety of autoimmune/rheumatologic conditions. **CONCLUSIONS:** Benign lymph node FNA yields flow immunophenotypes remarkably similar to those from excisional biopsies. Outlier flow immunophenotypes are identified in benign lymph nodes sampled by FNA at a frequency similar to that with excisional biopsies. Older patients, who have a higher baseline risk of lymphoma, are more likely to exhibit lymphoma-mimicking outliers such as a light-chain predominance on B cells and skewed CD4:CD8 ratios or increased CD7 loss on T cells, and they warrant additional diagnostic caution. ***Cancer Cytopathol* 2018;126:797-808.**

KEY WORDS: cytometry; flow; distribution; immunophenotype; informatics; lymph node; outlier

INTRODUCTION

Fine-needle aspiration with flow cytometry (FNA-FC) serves often as the initial diagnostic step and sometimes as the only diagnostic step in evaluating a clinically concerning lymph node. It is generally assumed that flow data from fine-needle aspiration (FNA) specimens are concordant with excisional biopsy data, but for most routine antigens, there are minimal published baseline flow data for FNA-FC[1–5] (the studies are summarized in Supporting Table 1) and no direct comparisons with excisional biopsy[6–18] (the studies are summarized in Supporting Table 2). The largest FNA-FC study to date reported quantitative data for only 4 antigens (CD7, CD4:CD8 ratios, and CD19), and another study that evaluated 10 clinically relevant antigens had just 7

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patients.[1,2] Pathologists lack data-driven guidance on how (or whether) to adjust their interpretation of FNA-FC data versus excisional biopsy and how to identify immunophenotypic outliers that could represent diagnostic pitfalls. This is especially pertinent because the interpretation of flow immunophenotyping without morphologic correlation has been shown to have a false-positive rate of 4.7%, mostly in the setting of reactive lymphoid hyperplasia.[19] FNA-FC has technical variabilities that differ from those encountered in excisional lymph node biopsies, such as transport media, trituration/disaggregation, and potential biases in patient selection and sampling sites.

This single-institution study represents a 15-year comparison of benign lymph node FNA-FC and benign lymph node excisional biopsy with flow immunophenotyping, with the majority of the cases having more than 2 years of clinical follow-up. Because of the long time period studied, many of the cases were analyzed with 4-color flow immunophenotyping; 8-color flow data were incorporated when available for documented outliers. The increasingly routine adoption of flow assays with 8, 10, or more colors alongside 4-color assays[20] means that experience and reporting are quite heterogeneous from laboratory to laboratory. Herein we provide data on the normal ranges that can be seen in routine clinical FNA-FC versus excisional biopsy.

MATERIALS AND METHODS

The study was approved by the Stanford University institutional review board. We included all lymph nodes with FNA-FC and a nonmalignant diagnosis between 2000 and 2015 and excluded cases deemed at the time to have insufficient cellularity or unacceptable contamination/necrosis. Two hundred eighty-nine benign lymph node FNA cases with concurrent flow cytometry immunophenotyping were identified and matched by sample site to 298 previously published benign lymph node excisional biopsies.[21] Over a mean follow-up time of 2.9 ± 2.4 years (with a minority of patients lacking follow-up), no patients developed a hematologic malignancy except for 1 patient who 4 years later was diagnosed with peripheral T-cell lymphoma and 1 patient who 1 year later was diagnosed with lambda light chain–restricted mantle cell lymphoma (discussed later). Flow cytometry studies were performed on 10-channel, 8-color FACSCanto flow cytometers (Becton Dickinson, San Jose, California) in 4- (n = 245) or 8-color cocktails (n = 44). To look comprehensively at 15 years of institutional data with extensive follow-up, we focused on the data available via 4-color flow cytometry in this study. Per institutional protocol, fresh tissues were placed into Roswell Park Memorial Institute medium. Specimens were processed routinely for flow cytometry with red cell lysis. Antibodies, all from BD Biosciences (San Jose, California), included clones HR1K (FMC7), 1-555-2 (lambda-monoclonal), TB28-2 (kappa-monoclonal), S5.2 (CD2), SK7 (CD3), SK3 (CD4), L17F12 (CD5), M-T701 (CD7), SK1 (CD8), HI1OA (CD10), NKP15 (CD16), SJ25C1 (CD19), L27 (CD20), S-HLC-1 (CD22), EBVCS-5 (CD23), HB7 (CD38), 2D1 (CD45), NCAM16.2 (CD56), and HNK-1 (CD57). Antibodies were combined in 4-color (fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein complex, and allophycocyanin) combinations (FMC7/CD23/CD45/CD22, lambda [monoclonal]/kappa [monoclonal]/CD45/CD19, CD8/CD3/CD45/CD19, CD7/CD3/CD45/CD2, CD57/CD8/CD45/CD3, CD16/CD3/CD45/CD56, CD20/CD10/CD45/CD19, and CD5/CD38/CD45/CD19) or 8-color (fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein complex, allophycocyanin, phycoerythrin–cyanine 7, allophycocyanin-H7, V500, and V450) combinations (lambda [monoclonal]/kappa [monoclonal]/CD5/CD38/CD10/CD20/CD45/CD19 and CD5/CD123 [or CD1a, CD30, CD10, or CD56]/CD4/CD2/CD8/CD3/CD45/CD7). Antigens were included if they were present in 40 or more cases in the cohort. Small cell (lymphocyte) and large cell populations were gated with CD45+ events with low forward and side scatter properties and with CD45+ events with higher forward scatter and low/moderate side scatter properties, respectively. Quadrant marker placement was performed with internal negative controls,[22] and data were analyzed with FCS Express (Becton Dickinson).

Event data were further analyzed with RStudio (RStudio, Inc [https://www.rstudio.com/]) and reported for the following antigen/antigen combinations: CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD22, CD23, FMC7, kappa, lambda, CD56, CD57, CD5+CD19+, and CD38+CD19+. T-cell antigen events normalized to CD3 levels were not reported for the large cell gate because T-cell antigen event counts were confounded by monocytes expressing dim CD4 and CD56 as well as aggregates of monocytes and T cells.[23] CD7 loss on T cells was estimated as the percentage of CD2+ events minus CD7+ events and included both T and natural killer (NK) cells, although T cells represented the majority of events. B-cell antigens and antigen combinations (CD10, CD20, CD22, FMC7, kappa, lambda, CD5+CD19+, and CD38+CD19+) were normalized to CD19, and T- and

NK-cell antigens (CD2, CD3, CD4, CD5, CD7, CD8, CD56, and CD57) were normalized to CD3.

Antigen event distributions are presented as group medians and first/third quartiles. Group differences between FNA-FC and excisional lymph node biopsy with flow cytometry were evaluated with the Mann-Whitney-Wilcoxon test corrected for multiple comparisons by control of the false-discovery rate.[24] Figures contain standard box and whisker plots, with upper and lower hinges indicating the 25th and 75th percentiles and upper and lower whiskers showing the highest and lowest values within 1.5 times the interquartile range of the hinge. In addition, notches (diagonal lines) show 1.58 times the interquartile range divided by the square root of the sample size. Outliers for kappa:lambda ratios were defined as greater than 3:1 or less than 1:1, and outliers for CD4:CD8 ratios were defined as greater than 10:1 or less than 1:1.[25] All other outliers for B- and T/NK-cell antigens were defined on the basis of excisional biopsy data as being greater than 1.5 times the interquartile range above the third quartile. With these definitions, outliers for B-cell antigens were identified as having 1 of the following: CD10+ events greater than 36.9% of CD19+ events in the lymphocyte gate, CD10+ events greater than 100% of CD19+ events in the large cell gate, CD5+CD19+ events greater than 28.5% of CD19+ events in the lymphocyte gate, and CD5+CD19+ events greater than 57.3% of CD19+ events in the large cell gate. T/NK-cell outliers were analyzed for the lymphocyte gate only and were defined as having 1 of the following: CD7 loss greater than 17.9% of CD2+ events, CD56+CD3+ events greater than 3.4% of CD3+ events, CD57+CD3+ events greater than 18.1% of CD3+ events, and CD56+CD3– events greater than 2% of CD45+ events. Cohort ages were compared with the Student *t* test, sex proportions were compared with a test of equal proportions, and location proportions were compared with the Pearson chi-square test.

RESULTS

FNA and excisional biopsy groups were matched by sample location (axillary, 20.7% vs 19.4% of the cohort; neck, 51.9% vs 51.0% of the cohort; inguinal, 8.9% vs 15.4% of the cohort; thoracic, 8.9% vs 7.0% of the cohort; and peritoneal/intra-abdominal, 9.3% vs 7.0% of the cohort; Table 1). The groups were similar in sex proportions, and the FNA group was older by a mean of 11 years (Table 1). The ratio of T cells to B cells sampled, measured as CD3:CD19, was significantly higher in FNA specimens than excisional biopsies (3.2:1 vs 2.1:1; $P < .01$; Fig. 1 and Supporting Table 3). FNA and excisional biopsy had similar median percentages of events in the lymphocyte gate (90% vs 91%; Table 1 and Supporting Table 3), and there was a significant 25% decrease in events in the yield of large cell–gated events (3% vs 4%; $P < .01$; Fig. 2 and Supporting Table 4).

TABLE 1. Study Cohorts: Clinical Characteristics and Sampling Site

	FNA (n = 289)	Excision (n = 298)	*P*
Age, mean ± SD, y	47 ± 18	36 ± 23	<.01
Male sex, % of total	38	51	.3
Sample locations, % of total			.2
Axillary	20.76	19.46	
Head/neck	51.90	51.00	
Inguinal	8.99	15.43	
Thoracic	8.99	7.04	
Peritoneal/ intra-abdominal	9.34	7.04	

Abbreviations: FNA, fine-needle aspiration; SD, standard deviation.

T- and NK-Cell Events

Compared with excisional biopsy–flow cytometry immunophenotyping, FNA-FC immunophenotyping showed a significantly increased median percentage of CD3+ T-cell events (72% vs 63%; $P < .01$) in the lymphocyte gate. T and natural killer T (NKT) antigen expression in the lymphocyte gate was essentially equivalent between the FNA and excisional biopsy groups when it was normalized to the percentage of CD3+ events (Supporting Table 3 and Fig. 1). T cell–related antigen events, normalized to CD3 levels in the large cell gate, were confounded by known dim CD4 and CD56 expression on monocytes and aggregation of monocytes and T cells and are thus not reported. NK-cell events were typically negligible (median, 1% CD56+CD3– events), and there was no difference between the FNA and excisional biopsy cohorts.

T/NK-Cell Antigen Outliers

In the FNA group, multiple specimens had outlier values in T-cell subsets (CD4:CD8 ratios, CD7 loss, CD56+/CD3+ NKT cells, and CD57+/CD3+ T cells) as well as CD56+CD3– NK cells. Patients whose cases had 1 or more outliers in T/NKT cell–related antigens were significantly older than the remaining FNA cohort (mean, 58 vs 46 years; $P< .01$) but were not significantly different in terms of the CD45+ yield in the lymphocyte or large

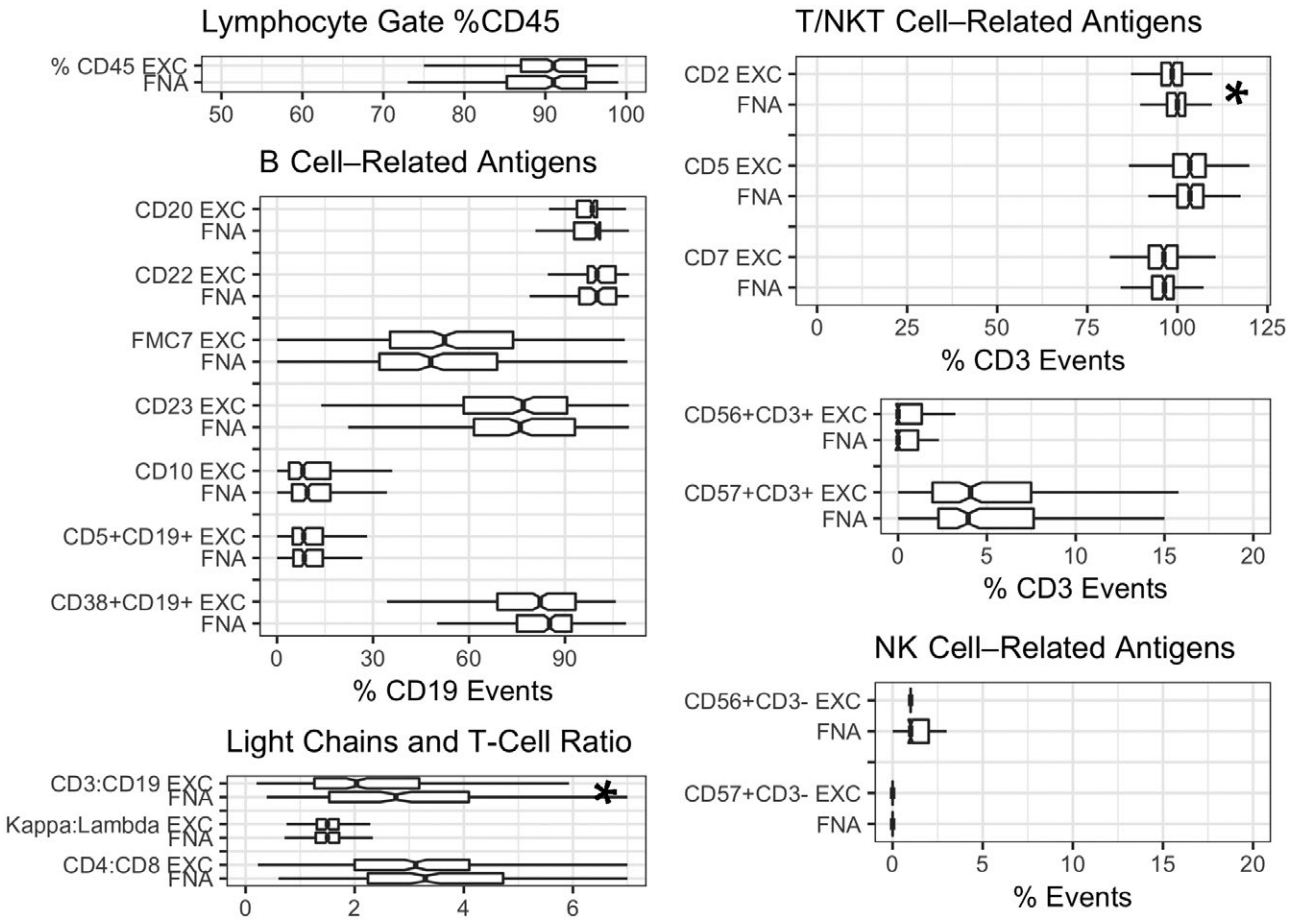

Figure 1. Lymphocyte gate: FNA versus EXC of benign lymph nodes. (*Left*) B cell–related antigens, normalized to the percentage of CD19+ B-cell events, and (*Right*) T/NKT cell–related antigens, normalized to the percentage of CD3+ T-cell events, are presented as notched boxplots, which show medians and quartiles. *$P < .05$ (FNA vs EXC). EXC indicates excisional biopsy; FNA, fine-needle aspiration; NK, natural killer; NKT, natural killer T.

cell gate. Moreover, 4.4% of the cases had CD4:CD8 ratios greater than 10:1; notably, these patients were also significantly older than the rest of the FNA group (mean, 65 vs 47 years; $P < .01$). Also, 1.2% of the FNA cases had a predominance of CD8+ T cells, with CD4:CD8 ratios less than 1:1. In a subanalysis, 3 of 4 FNA outliers and 6 of 11 excisional biopsy outliers had CD4:CD8 ratios below a stricter cutoff of 0.8:1. Among the FNA cases, 3.0% had a prominent loss of CD7 (median, 22% CD7– events); specimens in this group did not have concomitant outlier values of other T- or NK-cell antigens. The CD4:CD8 and CD7 loss outliers most divergent from the median had a variety of underlying clinical conditions, including human immunodeficiency virus lymphadenitis, tuberculosis, Sjogren's syndrome, inclusion body myositis, poorly differentiated papillary thyroid cancer, and amyloidosis (Table 2 and Supporting Table 5).

CD56+CD3+ NKT-cell populations were typically minute (median, 0.1% of CD3+ events), but 4.1% of cases had an outlier of increased CD56+/CD3+ NKT-cell populations (median, 7.6%). Increased CD57+/CD3+ NKT-cell populations were present in 4.7% of FNA specimens, with a median of 25% CD57+/CD3+ events in the outlier group. Unlike the overall FNA cohort, patients with increased CD56+/CD3+ events and/or increased CD57+/CD3+ events were predominantly male (5:3 and 8:1 male:female ratios). The NKT antigen outliers most divergent from the median had clinical conditions including an ongoing lupus-like autoimmune disorder with adenopathy, autoimmunity (primary biliary cirrhosis, systemic sclerosis, and diabetes) with adenopathy, pancreatic adenocarcinoma, and self-resolved submandibular lymphadenopathy with negative 10-year follow-up (Table 2 and Supporting Table 5).

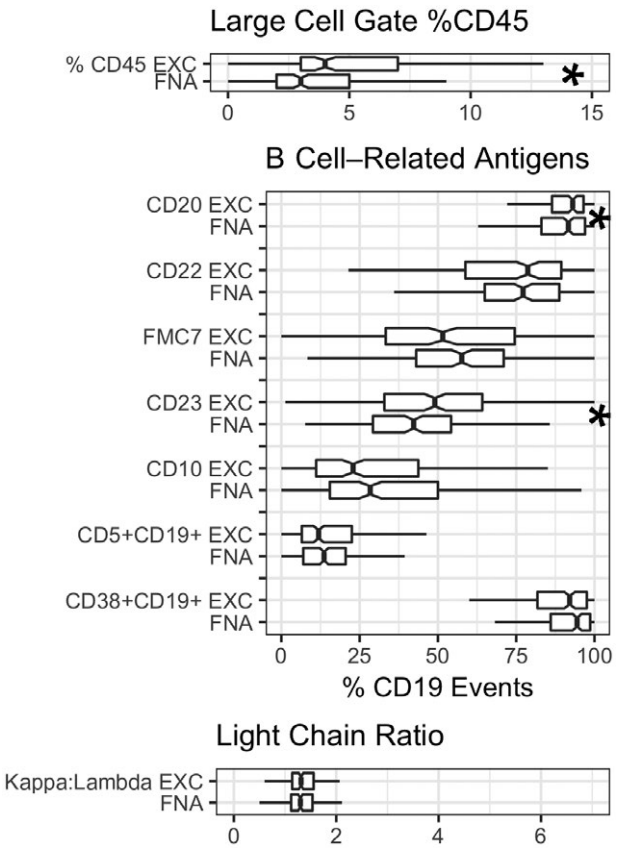

Figure 2. Large cell gate: FNA versus excision (EXC) of benign lymph nodes. Event percentages for B cell–related antigens are plotted as notched boxplots, which show medians and quartiles. *P < .05 (FNA vs EXC). EXC indicates excisional biopsy; FNA, fine-needle aspiration.

Most FNA cases had a low proportion of NK cells (median, 1% CD56+CD3– events), but 14.2% of FNA cases had an increased percentage of NK cells with a median of 4% CD56+CD3– events in the NK-cell outlier group. The NK antigen outliers also had significant increases in CD56+/CD3+ NKT-cell subsets (1.3% vs 0%; P < .05). The NK antigen outliers most divergent from the median had clinical conditions, including liver transplantation due to biliary atresia with new concern about a small bowel obstruction versus infection, severe perennial and seasonal allergies, and multilobular lung consolidation with mediastinal lymphadenopathy due to organizing pneumonia (Table 2 and Supporting Table 5).

B-Cell Antigens

FNA specimens slightly underrepresented CD19+ B-cell events in comparison with excisional biopsy in the lymphocyte gate (median, 22% vs 29%; P < .01), with no difference in the percentage of CD19+ events between the FNA and excisional biopsy groups in the large cell gate. B-cell antigen expression in the lymphocyte gate was not significantly different between the FNA and excisional biopsy groups when it was normalized to the percentage of CD19+ events (Supporting Table 3 and Fig. 1). In the large cell gate, FNA cases had slightly decreased CD20+ events (median, 95% vs 97%; P < .05) and CD23+ events (median, 42% vs 50%; P < .05) in comparison with excisional biopsy cases (Supporting Table 4 and Fig. 2).

B-Cell Antigen Outliers

At least 1 B-cell antigen outlier immunophenotype was present in 18.3% of the FNA specimens, including 6.8% of cases with kappa:lambda ratios greater than 3:1 or less than 1:1, 9.9% of cases with increased CD10 expression when it was normalized for CD19 expression, and 5.9% with prominent CD5+CD19+ B cells. Patients with any B-cell antigen outlier were not different from the remainder of the group in terms of age, but B-cell antigen outliers had significantly increased yields in the large cell gate in comparison with the rest of the FNA cases (median, 5% vs 3%; P < .05). Cases with kappa:lambda ratios greater than 3:1 or less than 1:1 in either the lymphocyte or large cell gate were from significantly older patients in comparison with the remaining FNA cases (mean, 55 vs 47 years; P < .05). Cases with increased CD5+CD19+ events in the lymphocyte gate (median, 38.8%) also had increased CD5+CD19+ events in the large cell gate in comparison with the rest of the FNA group (median, 35% vs 13%; P < .05).

Patients with CD10+ and CD5+CD19+ antigen outliers most divergent from the median ranged in age from 24 to 76 years and had various clinical courses, including common variable immunodeficiency (2 cases), lung cancer (2 cases), Castleman disease, invasive basal cell carcinoma, and resolving lymphadenopathy with negative follow-up (multiple cases). Five of the top 10 CD5/CD19 outliers had initial or add-on 8-color flow looking at light-chain restriction among CD5+ events. Of these, 4 were within the normal range, and 1 was lambda-predominant (0.5:1); this patient did not have a subsequent biopsy in our system and to our knowledge did not have a subsequent diagnosis of lymphoma. Of the top 10 CD10/CD19 outliers, 1 had initial or add-on 8-color flow looking at light-chain restriction among CD10+ events; this was within normal limits. Of the top 10 CD10/CD19 outliers, 3 had an excisional biopsy,

TABLE 2. Suspicious Antigen Outliers That Are Common in Benign Lymph Nodes

	Associated Conditions in This Study	Benign Lymph Nodes, (%)	Outlier Cutoff[1]
B-cell antigens			
Skewed kappa:lambda	Common variable immunodeficiency,	6.8	<1:1, >3:1
Increased CD10	lung cancer, basal cell carcinoma,	9.9	36.9 (% CD19)
Increased CD5+CD19+	Castleman disease, Sjogren's syndrome, lupus, amyloidosis, lymphadenopathy with no clear etiology	5.9	28.5 (% CD19)
T/NKT-cell antigens[2]			
Skewed CD4:CD8	HIV, tuberculosis, amyloidosis,	5.6	<1:1, >10:1
Increased CD7 loss	Sjogren's syndrome, papillary thyroid cancer	3.0	17.9 (% CD2)
Increased CD56+CD3+	Lupus, autoimmune disorders,	4.1	3.4 (% CD3)
Increased CD57+CD3+	adenocarcinoma	4.7	18.1 (% CD3)
NK-cell antigens			
Increased CD56+CD3−	Solid organ transplant, infection, allergy	14.2	2 (% gated)

Abbreviations: HIV, human immunodeficiency virus; NK, natural killer; NKT, natural killer T.

[1]Except for CD4:CD8 and kappa:lambda ratios, outliers are defined as >1.5 times the interquartile range above the third quartile. Outlier cutoffs exceed those previously published.

[2]T-cell antigens in large cell gates are not reported (confounded by monocyte binding and monocyte T-cell aggregation).

and all showed prominent CD10+ reactive germinal centers. One had 2 prior FNAs, which also showed an increased proportion of CD10/CD19 events. Of the top 10 CD5/CD19 outliers, none had an excisional biopsy, and none had prior FNAs at the same site. Two had subsequent FNAs at the same site. Six months later, 1 showed metastatic breast cancer but did not undergo flow immunophenotyping. A year later, 1 showed lambda light chain–restricted mantle cell lymphoma; the original case had a kappa:lambda ratio of 1.8:1 and only 2% CD5/19+ B cells, so even if the lymph node was involved, it would have likely been below the threshold of detection.

The patients in the light-chain outlier group most divergent from the median were adults (age range, 42-86 years) and had clinical conditions including Sjogren's syndrome, lupus, antiphospholipid syndrome, squamous cell carcinoma elsewhere, amyloidosis, and inclusion body myositis (Table 2 and Supporting Table 5). Of the top 10 kappa:lambda ratio outliers, 1 had prior FNA of the same site, which did not show the same light-chain abnormality; 1 had a subsequent excisional biopsy, which showed reactive germinal centers, and the kappa:lambda ratio was normal on the excisional biopsy.

Comparison of FNA and Excisional Biopsy Outliers

FNA cases had at least 1 outlier flow immunophenotype in 23% of cases and 2 or more outliers in 4.8% (Fig. 3). Among immunophenotypic outliers, B-cell abnormalities predominated and included increased CD19-normalized CD5+CD19+ or CD10+ events and skewed kappa:lambda light-chain ratios, but a significant proportion demonstrated T-cell abnormalities, including CD7 loss and skewed CD4:CD8 ratios. We compared the frequencies and types of outliers in the FNA group with those of the lymph node excision specimens; no significant differences in the overall outlier proportions (Fig. 3, top), the proportions of multiple-outlier cases (Fig. 3, middle), the proportions of individual outlier types (Fig. 3, bottom), the proportions of outliers in different anatomic locations (Supporting Fig. 1), or the outlier event distributions were found. There were no significant differences between the FNA and excisional biopsy cohorts in terms of the number of outliers, sex proportions, ages, or antigen event counts for all outlier types with the exception of older age in the CD5 outliers sampled by FNA versus excisional biopsy (Table 3). FNA cases with outlier immunophenotypes were reviewed to be assessed for cytomorphologic features that could explain the immunophenotypic aberrancies (ie, presumptive germinal center cells, collections of monotonous-appearing small lymphoid cells, and cells with minimal cytologic atypia such as mild nuclear membrane abnormalities; Fig. 4). B- and T-cell outliers were not predictable by cytomorphologic features.

TABLE 3. Antigen Outliers From FNA and Ex Bx: Lymphocyte-Gated Events

Kappa:Lambda Outliers		No.	Male:Female, No.	Age, mean (range), y	Kappa:Lambda
<1:1	FNA	4	1:3	55 (49-65)	0.7 (0.6-0.8)
	Ex Bx	3	1:2	54 (39-58)	0.8 (0.8-0.9)
>3:1	FNA	2	0:2	56 (48-64)	3.2 (3.2-3.3)
	Ex Bx	1	1:0	49 (49-49)	3.1
CD10 High Outliers		No.	Male:Female, No.	Age, y	CD10 (% CD19)
FNA		19	8:11	41 (33-65)	57 (47-69)
Ex Bx		27	12:15	44 (24-62)	44 (41-60)
CD5+CD19+ High Outliers		No.	Male:Female, No.	Age, y	CD5+CD19+ (% CD19)
FNA		12	5:7	41 (35-66)[1]	39 (35-64)
Ex Bx		24	17:7	5 (2-24)	35 (31-43)
CD7 Loss Outliers		No.	Male:Female, No.	Age, y	CD7 Loss (% CD2)
FNA		6	1:5	56 (52-61)	23 (19-36)
Ex Bx		10	5:5	41 (12-52)	30 (26-59)
CD4:CD8 Outliers		No.	Male:Female, No.	Age, y	CD4:CD8
<1:1	FNA	3	2:1	68 (61-77)	0.6 (0.6-0.7)
	Ex Bx	11	8:3	25 (5-36)	0.7 (0.7-0.8)
>10:1	FNA	11	5:6	66 (58-73)	11 (10-12)
	Ex Bx	6	3:3	65 (59-69)	12 (10-16)
CD56+CD3+ Outliers		No.	Male:Female, No.	Age, y	CD56+CD3+ (% CD3)
FNA		8	5:3	56 (51-59)	8 (4-10)
Ex Bx		14	7:7	51 (29-67)	6 (5-8)
CD57+CD3+ Outliers		No.	Male:Female, No.	Age, y	CD57+CD3+ (% CD3)
FNA		9	8:1	66 (44-70)	25 (23-28)
Ex Bx		16	12:4	49 (29-55)	22 (20-26)
CD56+CD3– Outliers		No.	Male:Female, No.	Age, y	CD56+CD3– (% CD45)
FNA		27	15:12	48 (36-63)	4 (3-6.5)
Ex Bx		32	18:14	39 (3-54)	4 (4-5)

Abbreviations: Ex Bx, excisional biopsy; FNA, fine-needle aspiration.

Fine-needle aspiration–based flow cytometry of lymph nodes is comparable to excisional biopsy and exhibits similar outliers. The reference ranges provided in this study can aid pathologists and contribute in the long term to computational flow analysis and biomedical research.

[1] $P < .05$ (FNA vs Ex Bx).

DISCUSSION

We performed a large-scale evaluation of our 15-year institutional experience with FNA-FC in which we compared immunophenotypes obtained from benign lymph nodes by FNA and gold-standard excisional biopsy. After normalization for B- and T-cell contents, we found that FNA-FC very closely mirrored the distribution of immunophenotypes obtained from excisional specimens. Of crucial importance is the fact that not just the medians but also the distributions of immunophenotypes were very similar across a range of B, T, and NK antigens used in routine clinical practice; this means that FNA-FC of benign lymph nodes is no more prone to producing outlier immunophenotypic populations than flow cytometry performed on excised lymph nodes. These data emphasize the technical validity of FNA-based flow immunophenotyping and highlight potential pitfalls when one is evaluating a clinically suspicious lymph node.

The diagnosis of T- and NK-cell lymphomas is often difficult and is supported by aberrant antigen expression or loss. We found that 23% of benign lymph nodes that underwent FNA-FC immunophenotyping had at least 1 T/NK-cell antigen outlier,[26] including skewed CD4:CD8 ratios, CD7 loss, and expansion of NK(T) antigen–expressing subsets. In our study, 5.6% of benign FNA cases had CD4:CD8 ratios outside the expected range of 1:1 to 10:1; notably, though in general use, this is a wider range of normal values than reported in a prior study of T lymphoproliferative disorder immunophenotyping.[25] A prior study of FNA specimens of reactive lymph nodes showed a similar distribution of CD4:CD8 ratios with slightly increased CD4 and similar variability around the mean, but outlier cases were not discussed.[2] In addition, greater than 10% loss of CD7 expression was frequent (11% of cases). CD7 loss and skewed CD4:CD8 ratios may be caused by a variety of conditions, including smoking, immunodeficiency, and infection.[27,28] A prior study pooling benign, reactive, and B-cell lymphoma data found a similar background rate of T-cell antigen loss, including CD7 loss in greater than 20% of T cells.[26] CD10+ T cells are important mimics of follicular helper–type T-cell lymphoma,[5] and in our experience, small CD10+CD19– probable T-cell populations are routinely present in 4-color flow studies

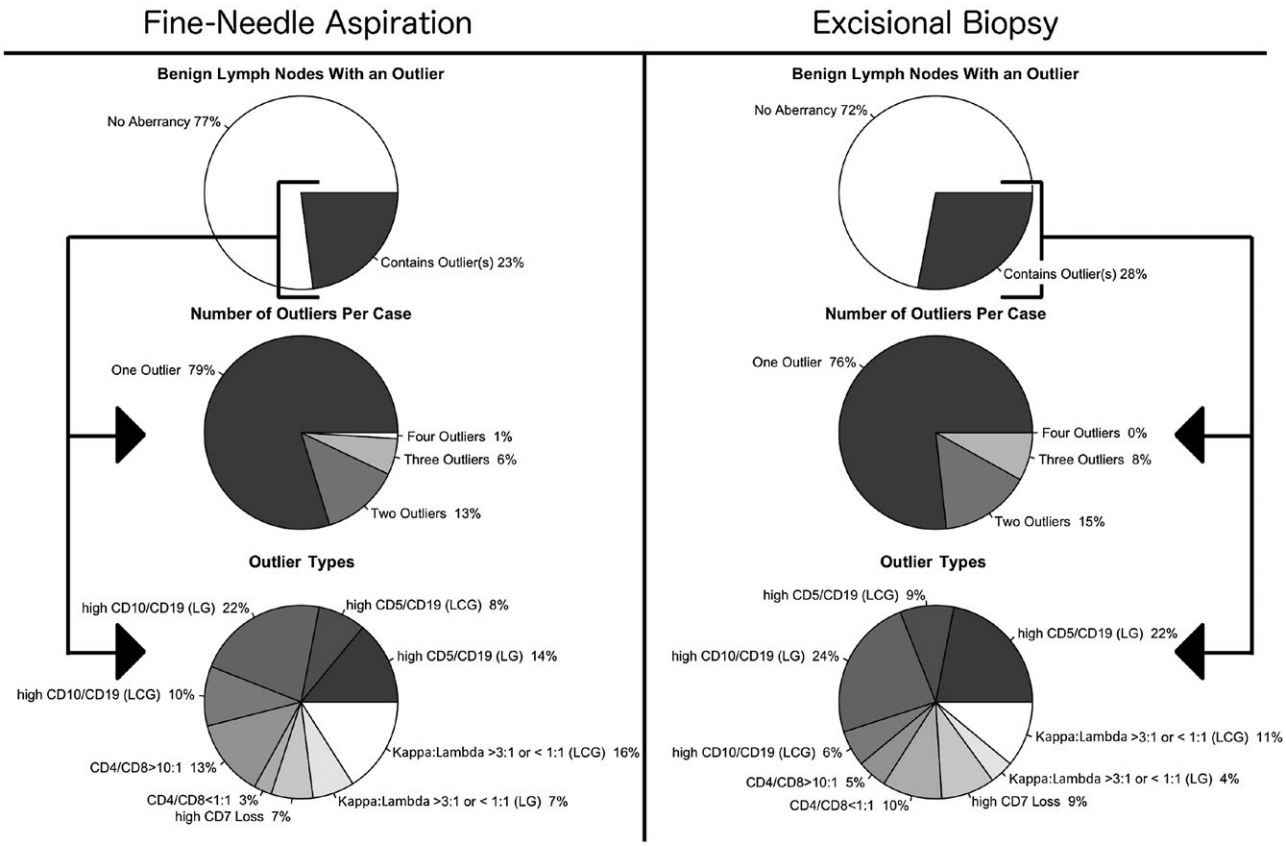

Figure 3. Distribution of immunophenotyping outliers for fine-needle aspiration with flow cytometry. LC indicates lymphocyte gate; LG, large cell gate.

of benign lymph nodes; however, these subpopulations are not addressed in this study because of the lack of full immunophenotypic characterization available with 4-color flow. For example, follow-up 8-color studies for characterizing follicular helper T cells with markers for pan-T cells and CD279 (PD1) were not included, but they must be interpreted alongside clinical data to distinguish associated malignant conditions (eg, peripheral T-cell lymphomas) from reactive conditions (eg, progressive transformation of germinal centers, Epstein-Barr virus and human immunodeficiency virus lymphadenitis, and Rosai-Dorfman disease).[29,30]

NK- and NKT-cell malignancies can be associated with increases in the relevant populations on immunophenotyping.[31] In our study, 14.2% of benign lymph node FNA specimens had expanded CD56+CD3– NK populations, and 4.1% had expanded CD56+/CD3+ NKT-cell populations, which could potentially raise unnecessary concern about NK- or NKT-cell malignancies. Another 4.7% of cases had increased CD57+/CD3+ T-cell populations, and this potentially raises concerns about a cytotoxic T-cell or follicular helper T-cell lymphoma. We found that increases in CD57+/CD3+, CD56+/CD3+, and CD56+CD3– T- and NK-cell subsets are often seen together, and this provides a possible clue to the reactive nature of these expansions. Our finding of increased NK cells versus NKT cells is consistent with previous work showing increased NK cells in infection (accumulating in the paracortex around germinal centers and in the medulla)[32] but relatively infrequent lymph node homing of NKT cells in reactive conditions.[33]

The diagnosis of mature B-cell lymphoma depends heavily on detecting aberrant CD10 or CD5 co-expression and skewing of expected kappa:lambda light-chain ratios. We found these same aberrances in 18.3% of benign lymph nodes that underwent FNA-FC immunophenotyping. FNA specimens had kappa:lambda light-chain ratios skewing at a prevalence slightly higher than but not significantly different from the 1.3% prevalence

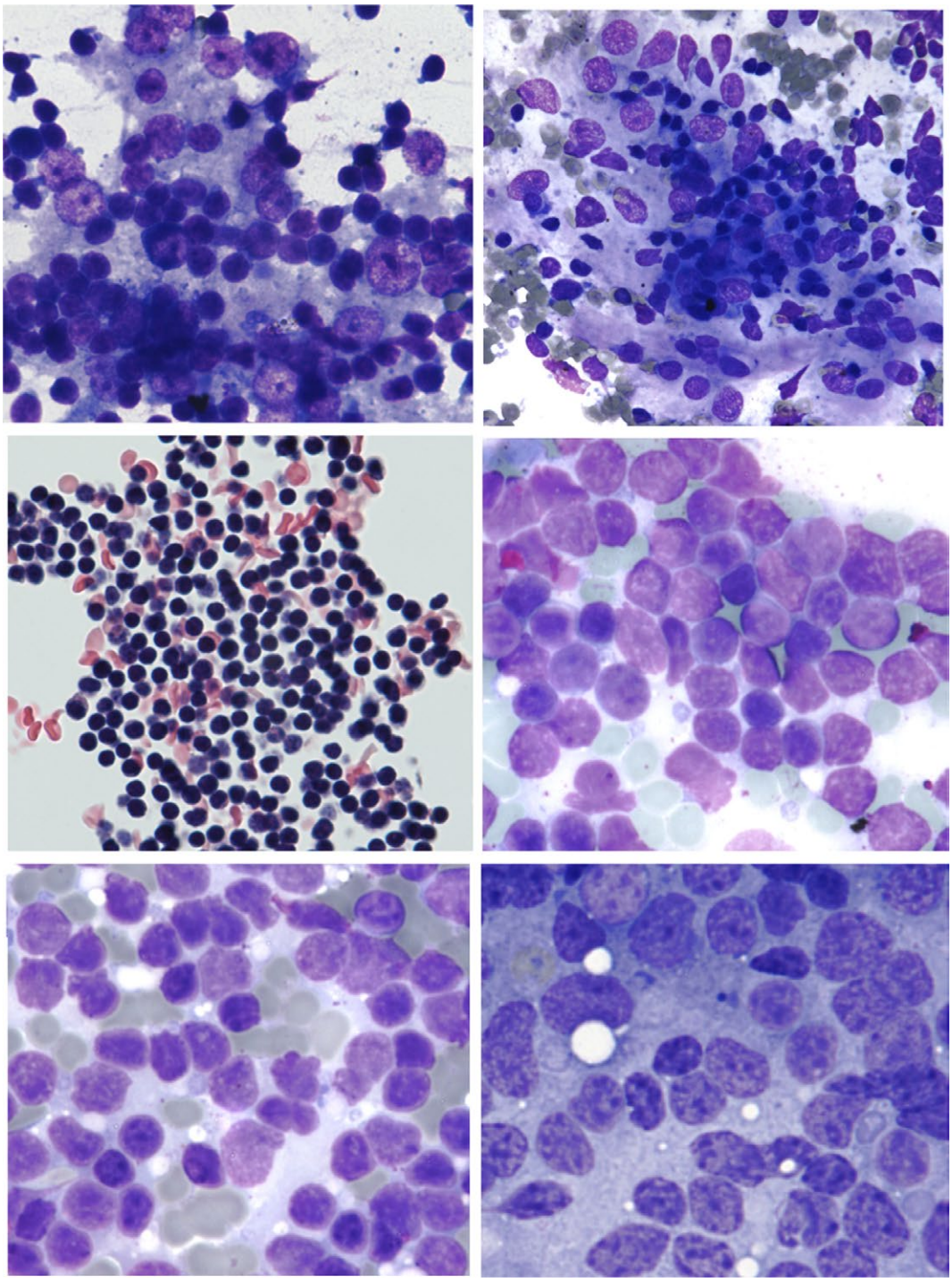

Figure 4. Benign lymph nodes sampled by fine-needle aspiration that had outlier flow immunophenotypes. Representative images show minimal atypia, including sampling of (*Top*) probable germinal centers with enlarged cells, (*Middle*) monotonous-appearing, expanded mature lymphocytes, and (*Bottom*) cells with mild nuclear contour irregularities and enlargement. B- and T-cell outliers were not predictable by cytomorphologic features.

in excisional biopsies.[21] Light chain–skewed specimens may be enriched for germinal center B cells (Fig. 2) because 1) there was significant kappa:lambda skewing in cases with increased CD19-normalized CD10+ events in the current study (Fig. 1) and 2) previous research has shown that 1% of reactive lymph nodes contain light chain–restricted CD10+ germinal center B cells, which represent at least 20% of total B cells.[34] Notably, this

study also demonstrated that nonmalignant light-chain ratios are wider (0.13:1 to 12.5:1) when the analysis is restricted to follicular B cells.[34] Light-chain restriction is a diagnostic clue for B-cell lymphoma but is also reported in reactive follicular hyperplasia, Castleman disease, and normal childhood tonsils.[35] Studies of the autoimmune disease Hashimoto thyroiditis caution that light-chain imbalance can be a common but insensitive marker. These studies have demonstrated light-chain imbalances generally within 1:1 to 5:1, but with some cases positive for clonality only by polymerase chain reaction and not by flow cytometry.[36] The diagnosis of B-cell lymphoma also involves flow immunophenotyping assessment for co-expression of CD5 or CD10, and increased levels of CD10+ or CD5+ B cells were previously reported in roughly 5% of benign excised lymph nodes.[21] Similarly to excisional biopsies, 6.9% and 4.4% of FNA cases (lymphocyte gate) in the current study had outlier increased levels of CD10+ and CD5+ B-cell events. This is an important potential pitfall because CD10+ and CD5+ B cells are conceptualized as minority populations in benign lymph nodes.[35]

Overall flow immunophenotype distributions from FNA and excisional biopsy specimens are remarkably similar, with the exception of slightly lower percentages of events in the large cell gate and relative enrichment for T-cell events in FNA specimens. Normalized B and T antigen expression is nearly identical overall between the FNA-FC immunophenotyping and excisional biopsy groups in both the lymphocyte and large cell gates. We note that this excellent performance of FNA-FC immunophenotyping must be interpreted in the context of studies performed at a tertiary care center with onsite adequacy evaluations by cytopathologists, which have been shown to markedly reduce nondiagnostic rates from 20% to 1.35%.[37]

The relative enrichment for small lymphocyte-gate T-cell events in FNA-FC immunophenotyping is difficult to ascribe to 1 underlying cause, but we suspect that this involves relatively increased paracortical sampling rather than peripheral blood contamination. Although peripheral blood, in comparison with lymph nodes, contains more T cells and fewer B cells at a median ratio of 5.2:1,[38] which is consistent with the relative enrichment for T-cell events in FNA-FC immunophenotyping in comparison with excisional biopsy, this does not account for the unchanged CD4:CD8 ratio and unchanged percentage of NK-cell events in FNA-FC. Peripheral blood typically has a higher proportion of CD8+ T cells than lymph nodes, with a reported median CD4:CD8 ratio of approximately 1.3:1.[38] The paracortex, in contrast, is highly enriched for T cells, has a CD4:CD8 ratio of 3-4:1, and lacks significant numbers of NK cells.[32,39] If peripheral blood contamination were a major factor in the overrepresentation of T cells in lymph nodes, the CD4:CD8 ratio would be expected to decrease. In fact, no such decrease in the CD4:CD8 ratio was observed. In addition, cases were excluded from the study if they were deemed at the time of diagnosis to have unacceptable levels of blood contamination. This relative overrepresentation of the paracortex may be due to either technical differences or a selection bias for lymph nodes with a greater paracortical component in the FNA group. For example, the FNA group was skewed toward older patients and may also have been skewed toward more accessible or less clinically suspicious lymph nodes, which may have a low component of activated follicles and proportionally greater representation of paracortex. A third possible cause of decreased large-gate events is enhanced fragility or circuit sticking due to FNA specimen processing.

A limitation of the current study's use of 4-color flow cytometry is an inability to detect some subpopulations that may be informative in the identification of aberrant lymphocyte subpopulations and in future studies of reactive lymphadenopathies. For example, the expansion of CD5+ or CD10+ B cells would not increase suspicion for B-cell lymphoma if these same populations were shown to be polytypic on flow assays with 8 or more colors. By the same token, if they do prove to be outliers by the light-chain ratio, there may be increased temptation to diagnose lymphoma. Efforts such as those by the Children's Oncology Group and EuroFlow use 8-color flow cytometry and have successfully reduced the assay's technical variability (eg, tandem fluorophores and complex compensation) by standardizing antibody vendors, flow cytometers, and analysis software. These efforts have led to significant advances in areas such as the detection of minimal residual disease.[40] Standardization would also be of great utility in developing reference ranges for 8- and 10-color FNA-FC that could be used to guide pathologists. The current study, albeit mostly 4-color, is the largest study and the first direct comparison of data distribution and outliers

between FNA-FC and excisional biopsy that can be used to guide the interpretation of routine and outlier data.[41] We have demonstrated the representativeness of FNA-based flow cytometry data with respect to both distributions and outliers that may represent diagnostic pitfalls. With minor caveats, pathologists can interpret FNA-FC data similarly to flow cytometry data derived from excisional biopsies. Special caution should be taken not to overinterpret single- or even double-outlier populations in the absence of an excisional biopsy, especially in older patients and in patients with underlying autoimmune/rheumatologic disease.

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AUTHOR CONTRIBUTIONS

Gregory D. Scott: Conceptualization, data curation, formal analysis, investigation, methodology, software, visualization, writing–original draft, and writing–review and editing. **Hubert D. Lau:** Data curation, formal analysis, and writing–review and editing. **Jason H. Kurzer:** Writing–review and editing. **Christina S. Kong:** Writing–review and editing. **Dita A. Gratzinger:** Conceptualization, methodology, supervision, and writing–review and editing.

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