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Flow-Cytometry Assessment of DNA Content Analysis and Immunophenotyping of Immune-Cells in Lymph-Node-Specimens as a Potential Diagnostic Signature of Aggressiveness in B-Non-Hodgkin Lymphomas

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Abstract: Introduction: Flow-cytometry (FC) is a powerful tool that can assist in lymphoma diagnosis in lymph node (LN) specimens. Although lymphoma diagnosis and classification are mainly based on tumor cell characteristics, surrounding cells are less employed in this process. Methods: We retrospectively investigated alterations in the ploidy status, proliferative cell fraction (PF) and the percentages of surrounding immune cells in 62 consecutive LN specimens with B-Cell Non-Hodgkin Lymphoma (B-NHL) and 26 gender and age mached reactive LN (RLN) specimens that were submitted for FC evaluation between 2019-2022. Results: B-NHL samples showed increased rate of DNA aneuploidy and PF, increased $CD45^+$ cells, lymphocytes and B-cells, and decreased T-cells compared to RLN. Compared with indolent B-NHLs, aggressive B-NHLs show increased DNA aneuploidy and PF, increased monocytes, immature-granulocytes, mature granulocytes, $CD8^+$ T-cells, Double-Negative-T-cells and Double-Positive-T-cells, and decreased $CD45^+$ cells, lymphocytes, $CD4^+$ T-cells and CD4/CD8 ratio. Receiver operating characteristic analysis determined integration of PF > 6.8%, immature-granulocytes > 0.9% and Double-Negative-T-cells >3.1% as optimal cutoffs with highest specificity and sensitivity in differentiating aggressive and indolent B-NHLs. Conclusions: These findings further strength the diagnostic value of DNA content analysis by FC and suggest the utilization of tumor surrounding immune cells in NHL diagnosis and classification.

Keywords: lymph-node (LN); aggressive-lymphoma; indolent-lymphoma; DNA-cell-cycle-indexing; immune-cells; tumor-microenvironment

1. Introduction

Flow cytometry (FC) is a diagnostic tool used for rapid multivariate analysis of liquid suspended cells that is frequently used in clinical laboratories around the world. FC allows rapid analysis of multiple characteristics in a large number of cells in a short amount of time (Brown and Wittwer, 2000). Cell analysis using FC is based on physical characters that include relative cell size and complexity, and on fluorescent-conjugated-antibody based detection of intracellular or cell surface

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proteins termed Cluster of Differentiation (CDs)(Martig and Fromm, 2022). In addition to CDs, the use of chemical markers for DNA such as propidium iodide (PI) or DRAQ5, could add important information on the cell cycle including the proliferation and apoptosis status of the cells (Brown and Wittwer, 2000; Martig and Fromm, 2022).

FC has a tremendous contribution to the diagnosis and classification of hematological malignancies such as leukemia and lymphoma (McKinnon, 2018). It has a central role not only in the identification and quantification of malignant cells in the specimens, but it also adds crucial information needed for the classification and differential diagnosis of leukemia and lymphomas subtypes (Gunduz *et al.*, 2013). Peripheral blood and bone marrow aspirates are naturally suitable specimens that are routinely screened in most clinical FC laboratories. In certain clinical FC laboratories including ours, cell suspension is also prepared from lymph-node (LN) biopsies or aspirates and routinely examined for lymphoma as part of the diagnostic process.

B-Cell-Non-Hodgkin's-Lymphomas (B-NHLs) comprise the largest group of lymphomas in the western world. Using FC for B-NHLs diagnosis in LN, the tumor B-cells are usually identified by their immunoglobulin (Ig) light-chain restriction and by their aberrant markers expression profile. These tumor B-cells are typically detected in the background of inflammatory lymphoid and myeloid derived immune cells that could be analyzed and characterized simultaneously by the FC screening tubes. Hence, in this clinical scenario, FC is an attractive tool as it can simultaneously detect and classify the Ig restricted lymphoma cells and cherecterise them in the mixed populations of remaining healthy cells (Rimsza and Jaramillo, 2014). This advantage becomes more evident in minimally invasive biopsies where there is little material, and the tissue architecture is not always preserved for performing an intact histological evaluation.

The biological features and clinical behavior of B-NHLs range from indolent (e.g., grade 1 Follicular lymphoma) to highly aggressive (e.g., Diffused Large B cell lymphoma or Burkitt cell lymphoma). The exact classification of a B-NHL subtype is very important for precision therapy. However, this process is not always clear. Problems become more evident when tissue biopsy is not available, inadequate, or not representative. In addition, in some B-NHLs with the same classification such as in mantle cell lymphoma (MCL) the disease behavior could range between indolent to aggressive (McKinnon, 2018), suggesting that clinical behavior is a spectrum rather than a fixed point. Furthermore, as we learn from the transformation of FL to Diffused-Large-B-cell-lymphoma (DLBCL), indolence seems to be a dynamic process that could change with the disease course and between different sites in the same patient (Gunduz *et al.*, 2013). Therefore, defining FC based measurable biomarkers that could help in fast and accurate monitoring of lymphoma subtype is essential.

Our laboratory has extensive experience including DNA content analysis by PI, as part of the routine FC diagnostic workup. Our previous work demonstrated the applicability of S-phase and proliferating cell fraction (PF) determination by FC as a tool for differentiation between aggressive and indolent CD10 positive B-NHLs (Azoulay *et al.*, 2020). In the current study we aim to focus on LN biopsies and extend our previous observations to include specimens of CD10 positive and negative B-NHLs and reactive LN (RLN). Specifically, we would like to characterize differences in cell cycle parameters and relative incidences of surrounding immune cells between B-NHLs and RLN and to test their utility in discriminating between indolent and aggressive B-NHLs.

2. Results

2.1. Sample Characterization

Histopathological analysis determined B-Cell-Non-Hodgkin's-Lymphoma (B-NHL) in 62 specimens and reactive LN (RLN) in 26 specimens. We found no differences in the gender of the patients from which the specimens were isolated between B-NHL and RLN (F:M; 34:28 and 15:11 in B-NHL and RLN, Prob>chiSq 0.46). The age (years) of the patients from which the specimens were isolated showed similar median and range between B-NHL and RLN (65.65 and 27.4- 80 in BNHLs, and 59.5 and 44 - 89.8 in RLN, Prob>t 0.02). The distribution of the type of specimens (biopsy/fluid)

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showed no differences between B-NHL and RLN (biopsy: fluid, n and %; 41: 21 and 66: 34 in B-NHL, and 18:8 and 70:30 in RLN, Prob>chiSq 0.33).

2.2. Differences between Specimens with B-NHLs and RLN

The data of DNA content parameters and immune cell subpopulations between the specimens are summarized in Table 1. Differences between specimens of NHL and RLN were examined first. B-NHL samples demonstrated a high rate of DNA-aneuploidy as compared to specimens with RLN (% specimens with diploid/aneuploidy in B-NHL vs. RLN 70:30 vs. 100:0 respectively Prob>chiSq 0.001). All the aneuploid samples were hyper diploid. Specimens with B-NHL were found to have significantly higher proliferative cells fraction (PF) as compared with the RLN (8.95 ± 8.28 vs. 3.78 ± 2.09, *p value* = 0.002). Regarding immune cells, the percentages of $CD45^+$ cells of the total nucleated cells and the percentages of lymphocytes of the total $CD45^+$ cells were significantly higher in the B-NHLs specimens as compared to the RLN (91.3 ± 11.05 vs. 70.62 ± 29.03, *p value* < 0.0001 and 82.12 ± 20.07 vs. 68.64 ± 28.11, *p value* = 0.013 respectively). The percentages of B-cells of the total lymphocytes in specimens with B-NHLs were significantly higher compared to RLN (57.73 ± 22.75 vs. 39.16 ± 19.29, *p value* = 0.0004). Concurrently, the percentages of T-cells of the total lymphocytes were lower in specimens with B-NHLs compared to RLN (37.99 ± 19.42 vs. 55.81 ± 16.09, *p value* < 0.0001).

Table 1. Differences between specimens with RLN and B-NHL.

Parameter	All samples	RLN	B-NHL	P value BNHL vs. RLN
Ploidy % Diploid/Aneuploid	78:21	100:0	70:30	0.001
PF (% of total cells)	7.02 ± 7.11, 4.41 (0.85-43)	3.78 ± 2.09, 3.06 (1.31-9.98)	8.95 ± 8.28, 5.38 (1.12-43)	0.002
Total $CD45^+$ cells (% of nucleated cells)	83.98 ± 20.21, 93.14 (10-99.7)	70.62 ± 29.03, 81.05 (10-99.23)	91.3± 11.05, 95.16 (50-99.7)	<0.0001
Lymphocytes (% of total $CD45^+$ cells)	75.83 ± 24.6, 86.16 (4.4-98.53)	68.64 ± 28.11, 78.28 (4.4-96.42)	82.12 ± 20.07, 88.05 (8.33-98.53)	0.013
Monocytes (% of total $CD45^+$ cells)	3.31 ± 6.17 , 1.6 (0.1-60.3)	2.26 ± 2.45 , 1.25 (0.1-11)	2.7 ± 3.53 , 1.45 (0.1-16)	0.557
mGr (% of total $CD45^+$ cells)	7.51 ± 11.8, 3.8 (0-63)	9.47 ± 13.48, 4.35 (0.3-52.8)	6.26 ± 11.37, 2.5 (0.4-63)	0.255
iGr (% of total $CD45^+$ cells)	3.45 ± 6.31, 1.45 (0-60)	3.37 ± 3.56, 1.5 (0.1-12.2)	2.7 ± 3.43, 1.3 (0-16.2)	0.411

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B-cells (% of total lymphocytes)	45.1 ± 24.11, 45 (0-94.9)	39.16 ± 19.29, 42.15 (0.1-76.6)	57.73 ± 22.75, 61.1 (0.41-94.9)	0.0004
NK-cells (% of total lymphocytes)	1.36 ± 2.62, 0.6 (0-20.5)	1.3 ± 1.42, 0.9 (0.1-7.5)	0.74 ± 1.4, 0.4 (0-10.6)	0.09
T-cells (% of total lymphocytes)	49.63 ± 21.44, 50.5 (4-90.3)	55.81 ± 16.09, 54.5 (16.4-80.4)	37.99 ± 19.42, 36.2 (4-81.3)	<0.0001
CD4 (% of T cells)	71.0 ± 15.26, 75.6 (10.1-96)	71.93 ± 16.16, 76.3(29-89.9)	70.62 ± 14.64, 72 (10.1-89.2)	0.711
CD8 (% of T cells)	27.83 ± 15.69, 22.5 (4.2-74)	26.21 ± 16.29, 21.2 (9.6-72)	28.58 ± 15.31, 24.6 (10.2-74)	0.518
CD4/CD8 ratio	3.85 ± 3.27, 3.35 (0.136-22.86)	3.98 ± 2.37, 3.59 (0.4-8.83)	3.44 ± 2.14, 2.95 (0.13-8.53)	0.305
DP T (% of T cells)	4.23 ± 4.06, 2.9 (0.24-21)	3.49 ± 2.73, 2.55 (0.4-11.6)	5.32 ± 4.88, 3.5 (0.3-21)	0.077
DN T (% of T cells)	4.18 ± 2.93, 3.4 (0.24-21)	3.92 ± 2.72, 3.1 (0.9-10.7)	4.65 ± 3.4, 3.7 (0.7-17.3)	0.332
NKT (% of T cells)	6.03 ± 8.53, 3 (0-50)	6.27 ± 6.68, 3.2 (0.1-22.5)	6.48 ± 10.42, 2.95 (0-50)	0.924

2.3. Differences between Specimens with Aggressive B-NHLs and Indolent B-NHLs

In this analysis we aimed to describe the differences between specimens with indolent B-NHLs and aggressive B-NHLs. The specimens with B-NHLs were divided into aggressive and indolent according to the accepted WHO criteria and their pathological record (the classification and prevalence of the B-NHLs in our study are summarized in supplementary Table 2). Regarding DNA content analysis parameters, specimens with aggressive B-NHLs demonstrated a high rate of DNA-aneuploidy compared to indolent B-NHLs (% specimens with diploid/aneuploid 51.5 : 48.5 vs. 89 : 11 in aggressive vs. indolent B-NHLs respectively Prob>chiSq 0.002). Specimens with aggressive B-NHLs were found to have significantly higher percentages of cells in PF compared to indolent B-NHLs (12.85 ± 9.35 vs. 4.08 ± 2.39, *p value* < 0.0001). Regarding to immune cells, the percentages of $CD45^+$ cells of the total nucleated cells and the percentages of lymphocytes of the $CD45^+$ cells were lower in the aggressive B-NHLs specimens compared to the indolent B-NHLs (88.13 ± 12.41 vs. 94.85 ± 8.32, *p value* = 0.019 and 74.35 ± 23.08 vs. 90.88 ± 11.22, *p value* = 0.001 respectively). The percentages of Monocytes, mature Granulocytes (mGr) and iGr of the $CD45^+$ cells were significantly higher in the aggressive B-NHLs specimens compared to the indolent B-NHLs (4.13 ± 4.32 vs. 1.03 ± 0.74, *p value* = 0.0005, 9.57 ± 14.69 vs. 2.58 ± 2.78, *p value* = 0.018 and 4.23 ± 4.07 vs. 0.93 ± 0.89, *p value* = 0.0001 respectively). The percentages of CD8 T-cells, DP T-cells and DN T-cells were significantly higher in aggressive B-NHLs specimens compared to the indolent B-NHLs (35.07 ± 16.00 vs. 20.75 ± 10.53, *p value* = 0.0002, 7.04 ± 5.93 vs. 3.21 ± 2.05, *p value* = 0.002, and 6.07 ± 3.91 vs. 3.01 ± 1.68, *p value* = 0.0004 respectively). The percentages of CD4 T-cells and the CD4/CD8 ratio were significantly lower in

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aggressive B-NHLs specimens compared to the indolent B-NHLs (64.32 ± 15.4 vs. 78.04 ± 9.97, *p value* = 0.002, and 2.61 ± 2.09 vs. 4.49 ± 1.79, *p value* = 0.0005 respectively).

2.4. Integration of the Percentages of cells in PF, iGr and DN T-Cells Show Potential to Differentiate Specimens with Aggressive and Indolent B-NHL with High Specificity and Sensitivity

To check the potential of DNA content analysis parameters and immune cells subpopulations to differentiate between aggressive and indolent B-NHLs, we performed ROC analysis for all the parameters that show the most significant differences (i.e. *p value*<0.001) between the groups. We identified PF > 6.8% as an optimal cutoff value to discriminate between aggressive and indolent B-NHLs with the highest specificity (92.6%). We identified iGr > 0.9% and DN T-cells > 3.1% as optimal cutoff values to discriminate between aggressive and indolent B-NHLs with the highest sensitivity (88% and 82.3% respectively). (Optimal cutoffs for all the parameters analyzed by ROC are summarized in Table 3).

Table 2. Differences between specimens with aggressive and indolent B-NHLs.

Parameter	Aggressive	Indolent	P value Aggressive vs. Indolent
Ploidy % Diploid/Uneuploid	51.5 : 48.5	89:11	0.002
PF (% of total cells)	12.85 ± 9.35	4.08 ± 2.39	<0.0001
Total CD45⁺ cells (% of nucleated cells)	88.13 ± 12.41	94.85 ± 8.32	0.019
Lymphocytes (% of total CD45⁺ cells)	74.35 ± 23.08	90.88 ± 11.22	0.001
Monocytes (% of total CD45⁺ cells)	4.13 ± 4.32	1.03 ± 0.74	0.0005
mGr (% of total CD45⁺ cells)	9.57 ± 14.69	2.58 ± 2.78	0.0180
iGr (% of total CD45⁺ cells)	4.23 ± 4.07	0.93 ± 0.89	0.0001
B-cells (% of total lymphocytes)	54.28 ± 24.65	60.28 ± 18.38	0.307
NK-cells (% of total lymphocytes)	1.01 ± 1.87	0.46 ± 0.42	0.137
T-cells (% of total lymphocytes)	40.42 ± 21.77	36.43 ± 15.76	0.429
CD4 (% of T cells)	64.32 ± 15.40	78.04 ± 9.97	0.0002

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CD8 (% of T cells)	35.07 ± 16.00	20.75 ± 10.53	0.0002
CD4/CD8 ratio	2.61 ± 2.09	4.49 ± 1.79	0.0005
DP T (% of T cells)	7.04 ± 5.93	3.21 ± 2.05	0.002
DN T (% of T cells)	6.07 ± 3.91	3.01 ± 1.68	0.0004
NKT (% of T cells)	8.07 ± 13.25	4.99 ± 5.47	0.263

Table 3. Optimal cutoffs to differentiate between specimens with aggressive and indolent B-NHLs.

Parameter	Optimal Cutoff	AUC	% Sensitivity	% Specificity
PF	> 6.8%	0.827	67.6	92.6
CD8 T cells	> 30%	0.783	64.7	89
CD4 T cells	< 70%	0.799	73.5	86
CD4/CD8 ratio	< 2.92	0.796	76.4	82
DN T cells	> 3.1%	0.770	82.3	60
iGr	> 0.9%	0.882	88	37
Mono	> 1.5%	0.817	76.4	81.5

3. Discussion

In this study, we examined DNA content parameters and incidences of surrounding immune cells in specimens with RLN relative to specimens with B-NHLs, and in specimens with aggressive vs. indolent B-NHLs. We further analyzed optimal cutoffs with potential to differentiate them with high specificity and sensitivity. The comparison between RLN and B-NHLs showed DNA-hyperdiploidy in 30% of the B-NHLs and as expected, none of the RLN were detected with DNA aneuploidy. The high rate of DNA aneuploidy and the increased incidences of cells in PF in the specimens with B-NHLs are in line with our previous report that showed the tendency of aggressive B-NHLs to present with DNA aneuploidy and high proliferative activity (Azoulay *et al.*, 2020). The increased $CD45^+$ cells in B-NHLs may be explained by the relative reduced incidences of non-hematopoietic stromal cells and the increased proliferation and accumulation of the hematopoietic lymphoid tumor cells. Considering the type of lymphoid tumor cells in B-NHLs, the relative increase of B-cells and the corresponding decrease of T-cells that we observed compared to RLN are coherent.

The comparison between aggressive and indolent B-NHLs showed significant increase in the rate of DNA aneuploidy and in the incidences of cells in PF in aggressive B-NHLs. These findings confirm, strengthen, and extend our previous observations in CD10 positive B-NHLs (Azoulay *et al.*, 2020), which further suggest the practical utility of DNA content analysis in B-NHLs classification. As was previously shown, the high rate of DNA aneuploidy in aggressive lymphomas may result

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from an unequal cell division (Tarte, 2017). Additionally, aggressive lymphoma cells are more likely to be found in a state of arrest in the different cell cycle stages of division without having completed the cell cycle properly (Wilkinson *et al.*, 2012). As was previously explained (Scott and Gascoyne, 2014), the high incidences of cells in PF in aggressive B-NHLs may be the consequence of accumulation of driver mutations that control cell division. In addition, increased proliferation could be promoted by signals and factors that are secreted from cells in the microenvironment of aggressive lymphomas (Yang *et al.*, 2006).

Analysis of the surrounding immune cells in our study demonstrated lower percentages of $CD45^+$ cells in aggressive B-NHLs compared to indolent B-NHLs. Stromal cell remodeling was shown to be the landscape of B-NHLs (Yang *et al.*, 2006). Interestingly, this single cell atlas of the human lymph node, demonstrated an increase of non-hematopoietic cells such as blood endothelial cells, follicular dendritic cells and marginal reticular cells and a decrease in lymphatic endothelial cells alongside the transformation of FL to DLBCL. As elucidated in this work, increased stromal cells in the LN of DLBCL reflects increased angiogenesis as an important mechanism that facilitates the lymphoma aggressiveness. Although it wasn't directly confirmed, our observation of lower percentages of $CD45^+$ cells in aggressive B-NHLs may be related to a relative increase percentages of non-hematopoietic stromal cells and thus supports this elucidation. In addition to reduced percentages of $CD45^+$ cells, we observed increased percentages of monocytes, mGr and iGr and relatively lower percentages of lymphocytes in aggressive B-NHLs compared to indolent B-NHLs. The high percentages of monocytes, mGr and iGr are in line with different reports showing the association of increased tumor infiltrating myeloid derived cells and myeloid suppressor cells with aggressive B-NHLs (Tzankov *et al.*, 2007; Menter and Tzankov, 2019). As was previously demonstrated, these cells can potentially contribute to lymphoma aggressiveness by the production of pro-angiogenic factors that facilitate stromal cell remodeling and angiogenesis (Kumar and Xu, 2018). Additionally, as group of tumor-suppressor cells (Kumar and Xu, 2018), monocytes, iGr and mGr can contribute to tumor aggressiveness by the secretion of inhibitory molecules that suppress the immune response and the activity of cytotoxic T-cells against the tumor cells (Tian *et al.*, 2019).

Corresponding with the increase of myeloid cells, our observations show significant alterations in the percentages of T-cells subsets, including reduced $CD4^+$ T cells, and relative increase of $CD8^+$ T cells, DP and DN T-cells in aggressive B-NHLs as compared to indolent B-NHLs. Of note, no significant difference in the percentage of CD4 or CD8 T-cells was demonstrated in RLN relative to B-NHL samples. In contrast, a significant difference was found when aggressive and indolent B-NHL samples were compared. Since RLN are very heterogeneous in its T-cell subpopulations, there may be no significance in the CD4 or CD8 populations in the raw assessment of B-NHL and RLN. The relative reduced percentages of CD4 T-cells and increased CD8 T-cells, corresponded with the low CD4/CD8 ratio in aggressive B-NHLs, are in line with previous report showing the increased infiltration of CD8 T-cells in DLBCL compared to FL (Augier *et al.*, 2010). However, as we never checked their functionality, we currently don't know if the infiltrated CD8 T-cells in our aggressive B-NHLs are immunologically exhausted or functional and could potentially act against the tumor cells when treated with immune-check point inhibitors (Wu *et al.*, 2022). In addition, as in other disease settings infiltrated CD8 T-cells were shown to promote pathological angiogenesis (Wu *et al.*, 2022), we could not exclude that these cells may also collaborate with non-hematopoietic stromal cells to increase angiogenesis in the microenvironment of aggressive B-NHLs. Parallel to CD8 T-cells, the increase percentages of DN and DP T cells which are known to have autoimmune activity (Yao *et al.*, 2019) may imply an increased autoimmune response against the tumor cells in aggressive B-NHLs. However, as these cells also have a regulatory role (Collins, Jacks and Pavletich, 1997) we could not exclude that these cells collaborate with myeloid cells to suppress the T-cell response against the tumor cells (A model summarizing our observations in LN of aggressive and indolent B-NHLs is presented in Figure 2) .

Our study shows potential diagnostic utility for integration of PF, iGr and DN T-cells in differentiating specimens with aggressive and indolent B-NHLs by FC, with high specificity and sensitivity. These results support our proposal that surrounding immune cells could be employed in

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the process of lymphoma diagnosis and classification (Figure 1). From a clinical standpoint, there is a tendency towards a minimally invasive procedure for diagnosis of lymphoproliferative disorders. While an excisional biopsy may be a reasonable option for superficial lymphadenopathy, it is a more invasive procedure for deep-seated lesions, requiring inpatient surgery under full anesthesia, resulting in longer recovery time and a higher risk of complications than core-needle biopsy. Aggressive and indolent lymphomas differ in their clinical behavior and require different clinical follow-up and treatment strategies (Sansregret and Swanton, 2017). One of the advantages of FC is that it requires a minimal number of cells that may be achieved even through fine needle aspiration biopsy, which is even less invasive than core needle biopsy. Therefore, the added value of the combination of DNA content analysis and surrounding immune cell based biomarkers presented in the current study may enable the FC method to become a more informative and accurate diagnostic method for lymphoma diagnosis in minimally invasive specimens.

This study includes several limitations. First, the samples in this study include relatively small, non-homogenous representation of indolent lymphomas and lack of other aggressive lymphomas such as Burkitts lymphoma. There is also lack of comparison between MCLs with different clinical disease behaviour, different subtypes of FL and FL transformation to DLBCL. Second, we used manual and expert based gating strategy that is more flexible but exposed to self-biases and individual errors. Third, it was difficult to accurately gate and measure PF in DNA aneuploid samples, and differentiate and measure myeloid immune cells subsets by FC in some specimens, specially of aggressive BNHLs, due to non-specific staining and strong autoflorecence of the lymphoma cells. As regard to the lymphoid cells subset with the low incidences, there is an increase chance for mistakes in determination of their level specially in specimens with limited cell numbers. The lack of more detailed functional data regarding the immune cell subsets that were found are also limiting the study.

In conclusion, we have found significant alterations in the rate of DNA aneuploidy and proliferative fraction as well as in the percentages of myeloid and lymphoid immune cells subsets and emphasized their potential diagnostic utility. Further studies with prospective follow-up and correlations to prognostic clinical characteristics as well as the use of functional markers for angiogenesis and immune response will yield information that can be used to predict prognosis and accurate individual treatment for patients.

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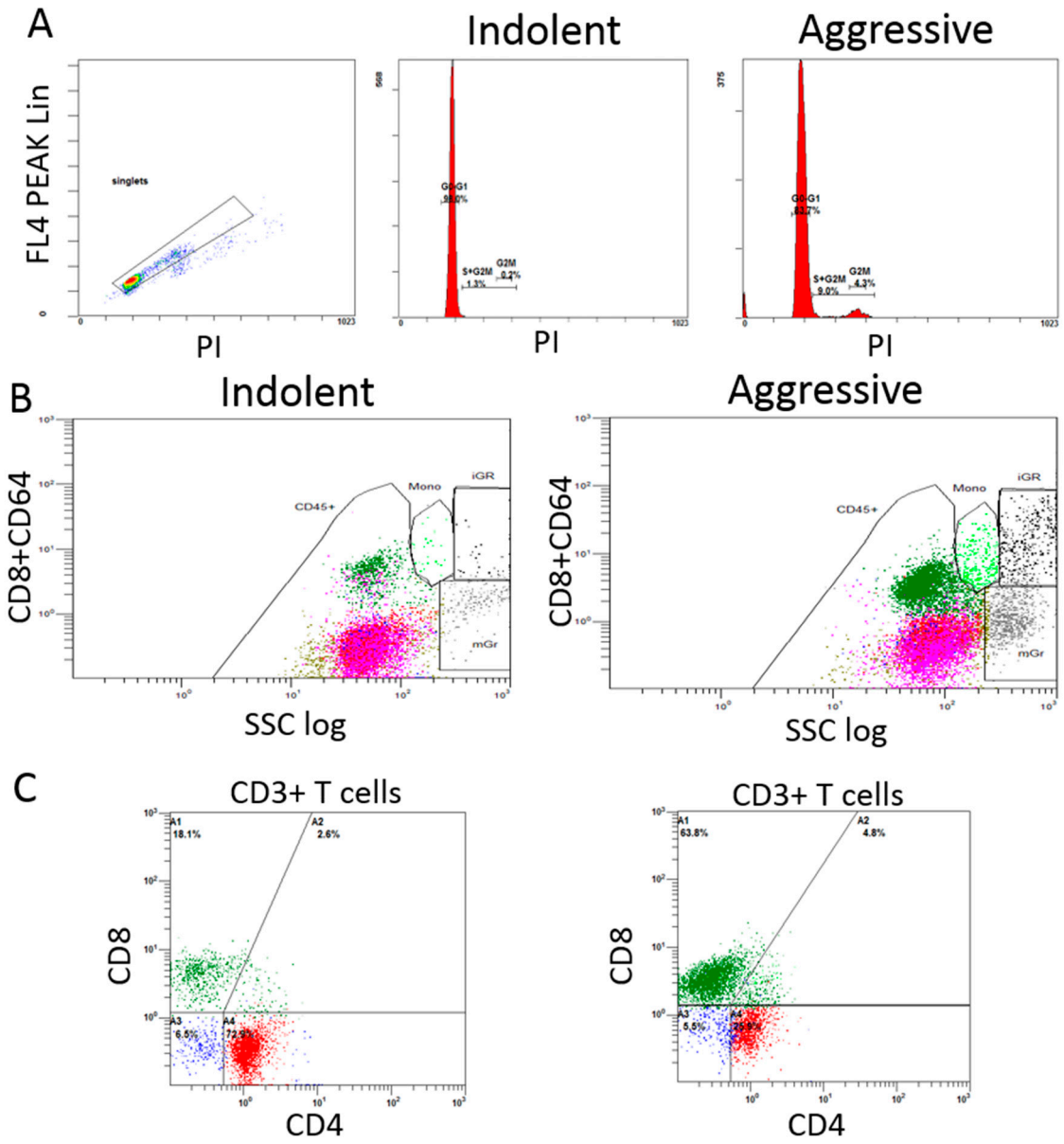

Figure 1. Representative histograms of DNA content analysis and flow-cytometry dot plots of surounding immune cells in indolent and aggressive B-NHL. A. Representative histograms of DNA content analysis. Left plot show the gating strategy for excluding debris from the DNA content analysis as lymphoma samples frequently contain significant debris underlying all phases of the cell cycle. B. Total $CD45^+$ cell component within the total nucleated cells (total $CD45^+$) and the percentages of lymphocytes ($CD45^+/SSC^{low}$) within the total $CD45^+$ cell component were determined using CD45 vs. lin SSC gating strategy (not shown). Representative plots showing the gating strategy for determining the level of Monocytes ($CD45^+/CD64^{high}/SSC^{dim}$), Mature Granulocytes ($CD45^+/CD64^{low}/SSC^{high}$) and Immature Granulocytes ($CD45^+/CD64^{high}/SSC^{high}$) within the total $CD45^+$ cell component. C. The percentages of B-cells ($CD19^+$), T-cells ($CD3^+$) and NK cells ($CD3^-/CD56^+$) were determined within the total lymphocytes in the specimens (not shown). Representative plots showing the gating strategy for determining the percentages of $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+/CD8^+$ double positive T-cells (DPT) and $CD4^-/CD8^-$ double negative T-cells (DNT) within the total T-cells.

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4. Materials and Methods

4.1. Case Selection

The study included a retrospective analysis of FC data on consecutive LN specimens that were isolated from patients who were presented with lymphadenopathy and underwent a diagnostic biopsy at the Galilee Medical Center (Nahariya, IL) between the years 2019-2022. The FC analysis was carried out on cell suspension that was prepared from fresh open or needle biopsy LN specimens and/or fluid that was taken from the LN area. Only specimens with a definitive diagnosis of B-NHL or reactive lymph-node (RLN) according to pathological evaluation were included in the study. Specimens with T-cell lymphoma, Hodgkins-Disease and non-hematological malignancies were excluded from the current study. Specimens with inadequete cells for FC analysis (i.e. mistakenly placed in preservative) or specimens with incomplete FC data were excluded from the study.

4.2. FC Analysis

FC was performed on fresh specimens that were collected in 0.9% NaCl solution (B. Braun Melsungen, Germany) without any preservative. Tissue biopsies were mechanically processed into a single cell suspension in phosphate buffer saline (PBS) or RPMI 1640 (Biological Industries, Beit-Haemek LTD, Israel) within 24h of isolation, and washed and suspended in PBS before staining. In case of aspirations from the LN area, the sample was centrifuged, and cell pellet was suspended in PBS before staining. For staining, samples of 50 µL cell suspension (containing approximately $1X10^4$ - $1X10^5$ cells) were placed in a separated polypropylene FACS tubes. For DNA cell cycle analysis, the cells immediately stained for DNA content, using a Coulter DNA prep REAGENT Kit [5] according to the manufacturer's instructions. Briefly, 50 µL of reagent containing detergent were added and vortexed for 15 sec. Then, reagent containing the dye and the red cell lysing solution was added and vortexed for 8 sec. Samples were read using a Beckman Coulter Navios flow cytometer instrument using the FL3 channel and selection of singlet events was done using FL4 peak against FL3 and FL4 time of flight (TOF) against FL3. Peripheral blood leukocytes of healthy donors were used as a calibration standard to determine the G_0/G_1 peak of DNA diploid with X-median of approximately 200 in a linear scale. DNA index (DI) as well as the estimation of cells in S and G_2M cell cycle compartments (Proliferative Fraction) were performed by an expert manual gating analysis. For immune cell population screening we added 7 µL of our lymphocyte screening antibody cocktail containing; CD7 Pacific-Blue, CD45 PE-Cy7, CD56 PE-Cy5, CD3 ECD, CD64+CD8 PE and CD19+CD4 FITC (All mAb from Beckman Coulter Inc. Brea CA) into a new sample of 50 µL cell suspension. After 10 min incubation at room temperature protected from light, the samples were washed and suspended in 500 µL PBS. The samples were acquired on the flow cytometer and at least 20,000 nucleated $CD45^+$ cells were recorded. Using CD45/SSC gating strategy, we determined and recorded the percentages of; Total $CD45^+$ cell component within the total nucleated cells, the percentages of lymphocytes ($CD45^+/SSC^{low}$), Monocytes ($CD45^+/CD64^{high}/SSC^{dim}$), Mature Granulocytes ($CD45^+/CD64^{low}/SSC^{high}$) and Immature Granulocytes ($CD45^+/CD64^{high}/SSC^{high}$) within the total $CD45^+$ cell component. We also determined and recorded the percentages of B-cells ($CD19^+$), T-cells ($CD3^+$) and NK cells ($CD3^-/CD56^+$) within the total lymphocytes and the percentages of $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+/CD8^+$ double positive T-cells (DPT), $CD4^-/CD8^-$ double negative T-cells (DNT) and $CD3^+/CD56^+$ NK-T cells within the total T-cells.

4.3. Statistical Analysis

We compared the percentages of cells in proliferative fraction (PF) and the percentages of all immune cell populations by using T-test for comparing 2 independent variables or ANOVA test for multivariable comparison. The Pearson chi-square analysis was used to compare non-parametric variables between groups. A Receiver Operating Characteristic (ROC) analysis was generated and the area under the curve was calculated to evaluate the optimal cutoffs of the variables between indolent and aggressive lymphomas, with the highest degree of sensitivity and specificity. All statistical analyses were performed using JMP (SAS Inc.) statistical software.

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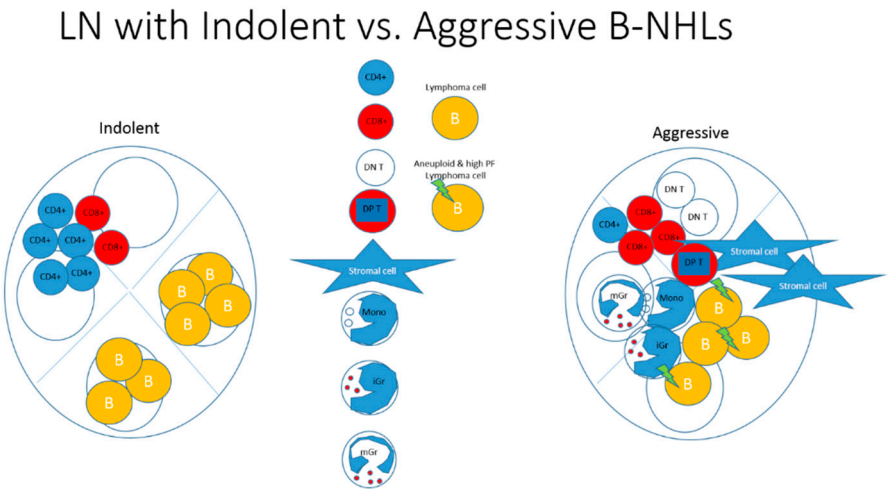

Figure 2. Compared with indolent B-NHLs, aggressive B-NHLs show increased DNA aneuploidy and PF, increased monocytes, immature-granulocytes, mature granulocytes, $CD8^+$ T-cells, Double-Negative-T-cells and Double-Positive-T-cells, and decreased lymphocytes and $CD4^+$ T-cells and decreased total CD45+ cells which may indicate increased $CD45^-$ cells such as stromal cells.

Conflicts of Interest: All the authors declare no conflict of interest.

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