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Conflict of Interest Disclosure

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Abstract

In lymphomas arising from the germinal center, prognostic factors are linked to the myeloid compartment. In particular, high circulating monocyte or myeloid-derived suppressor cell counts are associated with poor prognosis for patients with high-grade B-cell lymphomas. Macrophages with an M2 phenotype are enriched within lymphoma tumors. However, the M1/M2 nomenclature is now deprecated and the clinical impact of this phenotype remains controversial. Across cancer types, myeloid cells are primarily thought to function as immune suppressors during tumor initiation and maintenance, but the biological mechanisms behind the myeloid signatures are still poorly understood in germinal center B-cell lymphomas. Herein, we describe the role and clinical relevance of myeloid cells in B-cell lymphoma and propose innovative approaches to decipher this complex cellular compartment. Indeed, characterization of this heterogeneous cell ecosystem has been largely accomplished with “low resolution” approaches like morphological evaluation and immunohistochemistry, where cells are characterized using a few proteins and qualitative metrics. High-resolution, quantitative approaches, such as mass cytometry, are valuable in order to better understand myeloid cell diversity, functions, and to identify potential targets for novel therapies.

Keywords

B-cell lymphoma; tumor microenvironment; Regulatory Myeloid Suppressor Cells; Myeloid-Derived Suppressor Cell; Tumor Associated Macrophage; Mass Cytometry

Précis

Mass cytometry deciphers the ecosystem of suppressive myeloid regulatory cells in

germinal center B-cell lymphomas

Abbreviations

CyTOF: cytometry by time-of-flight

DLBCL: diffuse large B cell lymphoma

FL: follicular lymphoma

HL: Hodgkin lymphoma

M-MDSC: monocytic myeloid derived suppressor cell

PMN-MDSC: polymorphonuclear myeloid derived suppressor cell

TAM: tumor associated macrophage

TME: tumor microenvironment

Treg: regulatory T cell

viSNE: visualization of t-distributed stochastic neighbor embedding

In B-cell lymphomas, the myeloid compartment contributes to prognosis

Lymphomas constitute a large group of cancer arising from lymphoid or extra-nodal tissues [1]. The nomenclature of these neoplasms regularly evolves, and currently comprises more than 30 distinct clinical, pathological, genetic, and molecular entities [2]. Altogether, around 10,000 new cases of lymphoma are diagnosed each year, worldwide, and 90% of these are B-cell lymphomas [3]. Diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and classical Hodgkin lymphoma (HL) represent 60% to 70% of B-cell lymphoma cases. Over the past decade of B-cell lymphoma research, the tumor microenvironment (TME) has emerged as a therapeutic target [3-8]. The composition of the TME is highly variable, and B-cell neoplasms can be categorized in terms of their dependence on cells in the TME [3]. For example, more than 90% of the cells in HL tumors are considered non-malignant TME and thought to support tumor cell growth [3]. Conversely, in Burkitt's lymphoma, tumor cells appear to be virtually independent from non-tumor cells signals [3]. The abundance of different TME cell subsets varies greatly between tumors and can include stromal cells, T cell subsets (including T helper cells [T_H1, T_H2], T follicular helper cells [T_{FH}], regulatory T cells [T_{reg}], and $CD8^{pos}$ T cells), B cells, and myeloid cells (including mast cells, macrophages, and myeloid-derived suppressor cells [MDSC]) [3, 9].

Despite the introduction of immunotherapy, treatment failure is still observed and emphasizes the need for prognostic biomarkers to better identify at-risk patients [10] and research into mechanisms of resistance. In B-cell lymphomas, some prognostic factors are linked to myeloid cell biology. In particular, gene expression profiling experiments in DLBCL, FL, and HL have revealed the presence of myeloid

cell-related prognostic signatures [4-6]. Currently, we still lack a full understanding of the biology that underlies these signatures. Prognostic factors that are evaluated at the time of diagnosis in peripheral blood or at the tumor site include: *i*) soluble factors such as soluble PD-L1 [11, 12], soluble CD163 [13], CCL17 [13], CXCL10 [14], and IL-10 [14], *ii*) number of myeloid cells or macrophages in the TME expressing $CD68^{pos}$ [6, 10, 15, 16] or $CD163^{pos}$ [16-18], and *iii*) number of circulating myeloid cells [17, 19-25]. In Rituximab era studies of DLBCL, FL, and HL, increases in circulating neutrophils [17, 19] and/or monocytes [17, 20-24] have been proposed as independent prognostic markers. In particular, we demonstrated that a regulatory subtype of monocytes, so-called M-MDSC (Monocytic-MDSC), is increased in the blood of DLBCL patients, as compared to healthy donors, and that this increase is correlated with poor prognosis [25].

In germinal center B-cell lymphomas, myeloid regulatory cells are involved in the neoplastic process

The myeloid tumor microenvironment includes dendritic cells (DCs), MDSCs, and tumor-associated macrophages (TAMs). Few studies have evaluated associations between DCs and B-cell lymphoma prognosis. High numbers of plasmacytoid DCs in FL tumors were correlated with a good prognosis [26]. Consistent results were found in HL for $CD83^{pos}$ myeloid DCs [27] and in DLBCL for $CD1a^{pos}$ DCs [28]. Altogether, these results suggested DCs mediate anti-tumor immunity [26].

MDSCs are a heterogeneous population arising from polymorphonuclear cells (PMN-MDSC, $Lin^{neg}HLA\text{-}DR^{neg}CD33^{pos}CD11b^{pos}$) and from monocytes (M-MDSC, $CD14^{pos}HLA\text{-}DR^{low}$) [29]. MDSCs are primarily defined by immunosuppressive

functions [29, 30]. Additional markers, such as CD116, CD124, VEGF-R (vascular endothelial growth factor receptor), CD11c, CD11b, and PD-L1, are commonly expressed on MDSCs and play functional roles in MDSC-mediated immune regulation [30]. Interestingly, these markers are regulated by environmental signals, and MDSC phenotypes vary widely across tumors [31]. MDSC induction and expansion is mediated by soluble factors including VEGF (vascular endothelial growth factor), GM-CSF, M-CSF, S100A8/A9, IL-4, IL-6, and IL-10, which can be produced by tumor and/or surrounding cells, such as stromal cells, T cells, and macrophages [30]. Many of these environmental cues depend on STAT3, STAT6, and STAT1, and these transcription factors activate expression of genes involved in the blockade of the myeloid differentiation or in immune regulation. These multiple suppressive mechanisms converge to impair effector T cell and NK cell functions and also contribute to macrophage polarization towards what has classically been described as an M2 anti-inflammatory phenotype. In humans, myeloid regulatory mechanisms include: *i*) T_{reg} expansion, *ii*) depletion of amino acids essentials for T-cell metabolism by expression of arginase 1 or IDO, *iii*) production of reactive oxygen species (ROS) through expression of NADPH (Nicotinamide Adenine Dinucleotide Phosphatase) oxydase (*NOX2*), and *iv*) IL-10, TGFβ (transforming growth factor) release and PD-L1 expression [31, 32].

In most solid cancers studied (melanoma, renal, lung, liver, or prostate cancer), circulating MDSCs are increased and their abundance is correlated with tumor stage, tumor volume, and disease prognosis [33-35]. In hematological malignancies, the role of circulating MDSCs is less clear. An enrichment in circulating MDSCs has been described in myeloma [36] and T-cell lymphoma [37]. We recently showed, as others, that the number of circulating MDSCs constitutes a prognostic

factor in DLBCLs [25, 38]. Poor prognostic significance of MDSCs has also been observed in indolent lymphoma [39], chronic lymphocytic leukemia [40, 41], and HL [38, 42]. While numerous suppressive mechanisms have been described in MDSC biology [31, 32], only few of these mechanisms have been explored in B-cell lymphomas (Figure 1). In DLBCLs, an increase of PMN-MDSC in peripheral blood has been reported [25, 38, 43]. We observed PMN-MDSC express arginase I, but we did not observe an association between MDSC abundance and clinical outcome, in contrast with a recent study [38]. This difference could be explained by subtle differences in expert gating strategies used for PMN-MDSC enumeration ($CD66b^{pos}CD33^{dim}$HLA-DR^{neg} [38] *vs.* $Lin^{neg}CD123^{low}$HLA-$DR^{neg}CD33^{pos}CD11b^{pos}$ [25]). Computational analysis of myeloid cells may provide an unbiased way to resolve this difference [44, 45]. Preparation of samples was also different in these studies. Our experiments were performed on whole peripheral blood and the other study was performed on the mononuclear cell fraction of peripheral blood [38]. The latter one is now recommended to better evaluate low density PMN-MDSCs [29]. M-MDSCs ($CD14^{pos}$HLA-DR^{low}) were detected in peripheral blood from DLBCL patients in 4 studies [25, 39, 46, 47]. Arginase I involvement in immune suppression was reported only in one study [39]. We demonstrated that the overall increase of monocytes in peripheral blood of DLBCL patients might be related to an increase of M-MDSCs. Indeed, gene expression profiling revealed a myeloid suppressive cell signature in peripheral blood characterized by expansion in circulating M-MDSC counts ($CD14^{pos}$HLA-DR^{low}). Interestingly, intact M-MDSCs function was demonstrated by suppression of T-cell response *in vitro*. M-MDSCs ($CD14^{pos}$HLA-DR^{low}) were compared to monocytes ($CD14^{pos}$HLA-DR^{high}) by transcriptomic analysis. In M-MDSCs, we found an overexpression of genes involved in MDSC biology such

as *IL4-R, IL6-R, RELB, STAT3, NFKB, CEBPβ, AIM2, TNFR2,* and *NOX2.* In DLBCL, the M-MDSC transcriptomic signature and the abundance of M-MDSC in peripheral blood were correlated with the international prognostic index and event-free survival. Finally, although arginase I and IDO activities were detected in plasma from DLBCLs, the suppressive activity of M-MDSC was not impaired *in vitro* in the presence of arginase- or IDO- inhibitors. Thus, we concluded that M-MDSC suppressive activity in DLBCL is independent of arginase I and IDO activity. This finding was supported by the observation that myeloid-dependent T-cell suppression could be ascribed to a release of IL-10 and S100A12 and an increase in PD-L1 expression [25] (Figure 1). MDSC counts were evaluated in two recent studies with a total of 53 indolent lymphomas (cases were primarily FL; also included were cases of mantle cell lymphoma, chronic lymphocytic leukemia, mucosa-associated lymphoid tissue lymphoma, and lymphoplasmacytoid lymphoma) [38, 39]. When compared to healthy donors, an increase in circulating PMN-MDSCs ($CD66b^{pos}CD33^{dim}HLA\text{-}DR^{neg}$) with arginase I activity was observed in a cohort of 31 indolent lymphomas [38]. M-MDSCs ($CD14^{pos}HLA\text{-}DR^{low}$) were detected in a cohort of 22 indolent lymphomas [39]. In HL, abundance of both PMN- and M-MDSCs was increased in peripheral blood [38, 42] and arginase I activity was increased.

It has been demonstrated in murine models of solid tumors that MDSCs also have the ability to differentiate into TAMs at the tumor site [48-50]. TAMs exhibit a so-called M2 phenotype, and are characterized by the expression of immunomodulatory molecules (such as PD-L1, B7-H4, and VISTA [V-domain Ig suppressor of T cell activation]), the production of immunosuppressive cytokines (like IL-10 and TGFβ), and the capacity to inhibit effector T cell functions via arginase 1 or IDO activities [50]. Within tissues, CD68 and CD163 are frequently proposed to define macrophage

subsets [50]. Macrophages, which are involved in tissue homeostasis and host defense, were historically split into classically activated or “M1” and alternatively activated or “M2”. M1 polarization occurs in response to IFN-γ or LPS stimulation and is associated with an increase of inflammatory cytokines and tumoricidal capabilities. In contrast, IL-4, IL-10, or IL-13 stimulation polarizes macrophages to an M2 phenotype associated with tissue repair, angiogenesis, and a lack of effective tumor immunity. In fact, these two types capture functions that are the extremes of a wide spectrum of overlapping polarization states that depend largely on programming from external stimuli [51-53].

The contribution of TAM infiltration to B-lymphoma prognosis is hotly debated and TAM phenotypes are poorly characterized in human lymphoma [54]. Additionally, although TAMs have been associated with immunomodulation in other tumor types, their functional role has not yet been fully defined within the lymphoma microenvironment. In DLBCL, TAMs were defined in the TME as $CD68^{pos}$ or $CD68^{pos}CD163^{pos}$ and their prognostic impact remains controversial [15, 55, 56]. In particular, differing correlations with clinical outcome were observed and appeared to depend on the way TAMs were defined (*i.e.*, as $CD68^{pos}$, $CD163^{pos}$, or $CD68^{pos}CD163^{pos}$) and whether treatments included the anti-CD20 antibody Rituximab or not [57]. To date, these discrepancies have not been resolved in DLBCL and a definitive study employing standardized techniques is needed [57]. In FLs, TAMs were defined and enumerated as $CD68^{pos}$ or $CD163^{pos}$ cells and their correlation with clinical outcome has also been controversial. Indeed, a high TAM count correlated with poor prognosis in patients treated by chemotherapy [58], in agreement with their capacity to activate FL B cells through the release of IL-15 or the triggering of BCR signaling [59, 60]. In contrast, in a study of FL patients treated

with Rituximab [61-63], TAMs were associated with good prognosis. This apparent discrepancy might be explained by rituximab-dependent tumor cell phagocytosis mediated by macrophages that would otherwise be suppressive [64]. Interestingly, M2 macrophages, which bear many similarities to TAMs and may be an equivalent population, display a phagocytic capacity superior to classical pro-inflammatory M1 macrophages. This increased functional capacity is thought to be based in expression of receptors to the Fc portion of IgG isotype immunoglobulin (FcγRs), including high expression of CD32a/FcγRIIa [65]. Thus, TAMs exhibit treatment-specific roles in B-cell lymphomas, and the same might be expected to be observed for MDSCs. Detrimental immunosuppressive and tumor-promoting properties of MDSCs have been widely described in solid cancers during disease onset and, to a lesser extent, during chemotherapy treatment. It is currently unclear whether the efficacy of immunotherapeutic agents, such as cytotoxic antibodies, is modulated by MDSCs (and vice versa). As for newer approaches like immune checkpoint inhibitors, it is highly likely that TAMs play a role, since TAMs can express PD family ligands PD-L1 and PD-L2. However, the involvement of MDSCs is more speculative as their characterization is more recent and relies on deep phenotyping and functional assays. In HL, TAMs defined as $CD68^{pos}$ or $CD163^{pos}$ cells are associated with a shortened overall survival in the majority but not all of the studies [5, 66-68].

Mass cytometry clarifies the myeloid landscape

Although their phenotype is heterogeneous, myeloid regulatory cell characterization has classically relied on a small number of proteins, such as CD68 and/or CD163, to delineate TAM populations [69, 70]. MDSCs were originally defined by fluorescence cytometry using a small set of canonical markers that included

CD11b, CD15, CD33, HLA-DR, and CD14 [29]. High-resolution approaches such as mass cytometry can better characterize the diversity and function of MDSC and may help to reveal cell-specific features that may constitute potential targets for novel therapies [71-73]. Mass cytometry combined with high-dimensional analysis tools from machine learning, such as visualization of t-distributed stochastic neighbor embedding (viSNE) and spanning-tree progression analysis of density-normalized events (SPADE), is now considered a robust method to identify numerous and novel subsets within heterogeneous tissues, including blood and tumor [74-76]. Several studies using mass cytometry have explored immune compartments including B-, T-, NK-, or myeloid cells [74, 77-89]. In particular, Becher *et al.* developed a dedicated panel to characterize myeloid cells across eight mouse tissues, which revealed previously unidentified populations in mice using unsupervised analysis of mass cytometry [44, 79]. In humans, 2 different myeloid panels revealed underestimated TAM subtypes in renal cell carcinoma and lung adenocarcinoma [90, 91]. In particular, TAM subsets were identified expressing high level of HLA-DR, CD68, CD64, CD204, and CD38 or CD14, CD64, CD11c, and PPARγ (peroxisome proliferator-activated receptor gamma) in samples from renal or lung carcinoma, respectively.

With the aim to obtain a broad overview of the myeloid compartment in B-cell lymphoma, a panel dedicated to the myeloid compartment was defined and validated on *in-vitro* derived monocyte, MDSC, and macrophage subsets [45]. Sample preparation and analysis workflows were also optimized or developed [92-94]. Tumor tissues from DLBCL, FL, and reactive lymph nodes from healthy donors were analyzed (unpublished data). These studies revealed the diversity of the myeloid compartment in TME by enumerating MDSCs/TAMs and DCs and by characterizing

the specific pattern of myeloid cell enrichment in each disease (Figure 2). As with the studies of lung adenocarcinoma and renal cell carcinoma, mass cytometry revealed involvement of T cell subsets in B cell lymphoma [90, 91]. In particular, in DLBCL T_{regs} and CD8 effector memory were increased in the TME (unpublished data). Altogether, these studies demonstrated the feasibility of deep phenotyping human tissues and the value of mass cytometry in deciphering the myeloid compartment and relationships between immune cell subsets.

Challenges and future directions

In the near future, mass cytometry will be combined with state-of-the-art immunohistochemistry approaches [95, 96]. Such combinations allow detection of more than 30 parameters on histological samples and quantitative, high dimensional analysis that is coupled to knowledge of cell location and quantification of cell-to-cell positional relationships within tissue microenvironments (Table I). High-resolution imaging with 3D reconstruction after tissue clearing is a rapidly growing field [97]. These approaches are expected to be particularly valuable for studies of TAM biology in lymphomas. Notably, antibody based imaging and flow cytometry approaches allow the detection of transcription factors phosphorylation events (*e.g.*, phospho-STATs and cEBPβ) and other key molecules contributing to immune regulation (*e.g.*, Arginase I, NOX2, NOS2 [nitric oxide synthase], PD-L1, IL-10, TGF-β, and CD124) [29]. Although deep phenotyping does not replace functional assays (reviewed in [29]), the opportunity to simultaneously analyze numerous MDSC/TAM surface markers, transcription factors, signaling events, and immune regulation molecules will likely be a major step forward in clinical, personalized medicine studies of TAM/MDSC, where functional assays are impractical.

Controlling the expansion and accumulation of MDSCs and blocking MDSC suppressive functions, e.g. by targeting CSF1-R (colony stimulating factor 1 receptor) [98] or S100 family members [99], represent promising novel approaches in cancer therapy. As MDSC do not bear a single, cell-lineage specific, extracellular membrane marker, it is very difficult to deplete MDSC *in vivo* while sparing other myeloid cells. To date, the main target is CSF1-R, the receptor for M-CSF, as its expression is largely restricted to cells from the monocytic lineage. Numerous clinical trials investing the efficacy of blocking CSF-1R and its effector signaling cascade by means of kinase inhibitors or antagonistic monoclonal antibodies are ongoing [50, 100]. These studies should determine whether shutting down the CSF1/CSF1-R axis *in vivo* effectively removes MDSC, induces MDSC reprogramming, and/or skews MDSC differentiation. Some classical chemotherapeutic molecules, such as gemcitabine, 5-fluoro-uracile (5-FU) or lenalidomide, may also specifically trigger MDSC apoptosis *in vitro* and *in vivo* [101-103].

In B-cell lymphomas, there is a need to capture the biological features and immunological properties of regulatory myeloid cells and to understand the crosstalk between tumor cells, myeloid cells, and other immune cells. This knowledge will greatly accelerate the development of targeted treatments for patients with B-cell lymphoma. In the near future, the field is expected to benefit greatly from high-dimensional approaches, such as flow mass cytometry, imaging mass cytometry, and high-resolution microscopy with 3D reconstruction.

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Table I: Potential approaches for myeloid regulatory cells phenotype analysis

	Immunohisto -chemistry/-fluorescence	Flow cytometry	Mass cytometry
Main advantages	● Histology ● Direct cell-cell interactions ● Signaling (phosphoprotein, transcription factor)	● 10-18 parameters ● Single cell analysis ● Signaling (phosphoprotein, transcription factor) ● Dynamic (basal / after stimulation or co-culture) ● High dimensional analysis workflow ● Potentially combined with transcriptomic or functional analyses after sorting	● >40 parameters ● Single cell analysis ● Signalling (phosphoprotein, transcription factor) ● Dynamic (basal / after stimulation or co-culture) ● High number of parameters ● High dimensional analysis workflow
Limitations	● Expert interpretation needed ● Limited number of parameters ● No dynamic approach	● Dissociated cells	● Dissociated cells ● Expert analysis needed ● Number of cells required
Current developments	● High-resolution confocal microscopy and 3D reconstruction ● Tissue clearing	● Increase number of parameters, new fluorochromes	● Increase number of parameters, new metal tags ● New visualization tools ● Harmonization of analysis worflow ● Imaging mass cytometry

Figures

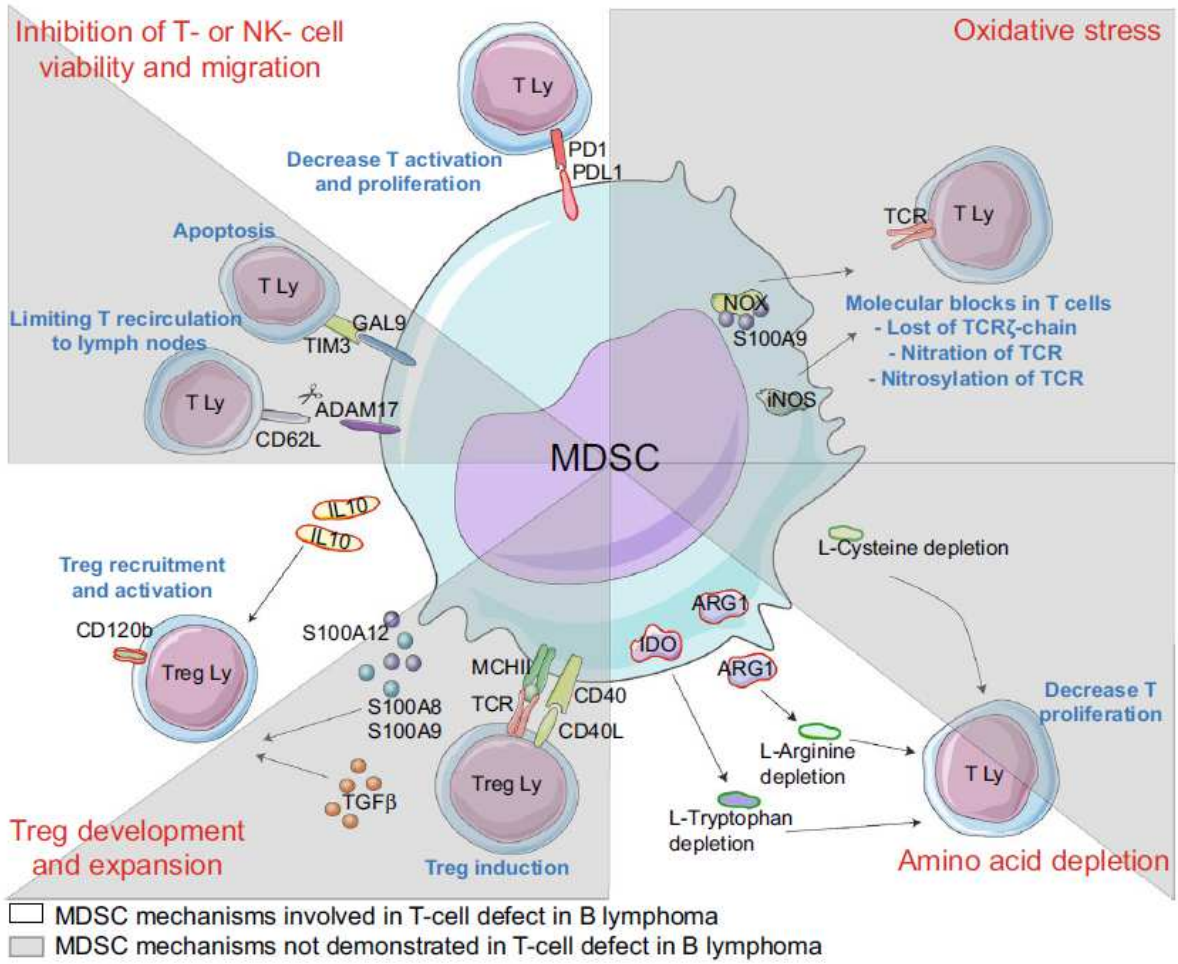

Figure 1: In B-cell lymphoma, myeloid regulatory cells engage various suppressive mechanisms

This figure is adapted from Gabrilovich *et al.* [32] and depicts mechanisms involved in MDSC immunosuppression. Mechanisms already described in B-cell lymphoma are unshaded whereas mechanisms not explored are shaded in grey. MDSCs can inhibit T cell responses through various mechanisms, 1) interference with T/NK cell migration and viability, 2) generation of oxidative stress by ROS and NO production, 3) deprivation in essential amino acids for growth and differentiation, and 4) Treg development and expansion. Ly: lymphocyte; NOX: NADPH oxidase complex; iNOS: inductible nitric oxide synthase; ARG1: arginase 1. Figure prepared using tools from Servier Medical Art (http://www.servier.com/Powerpoint-image-bank).

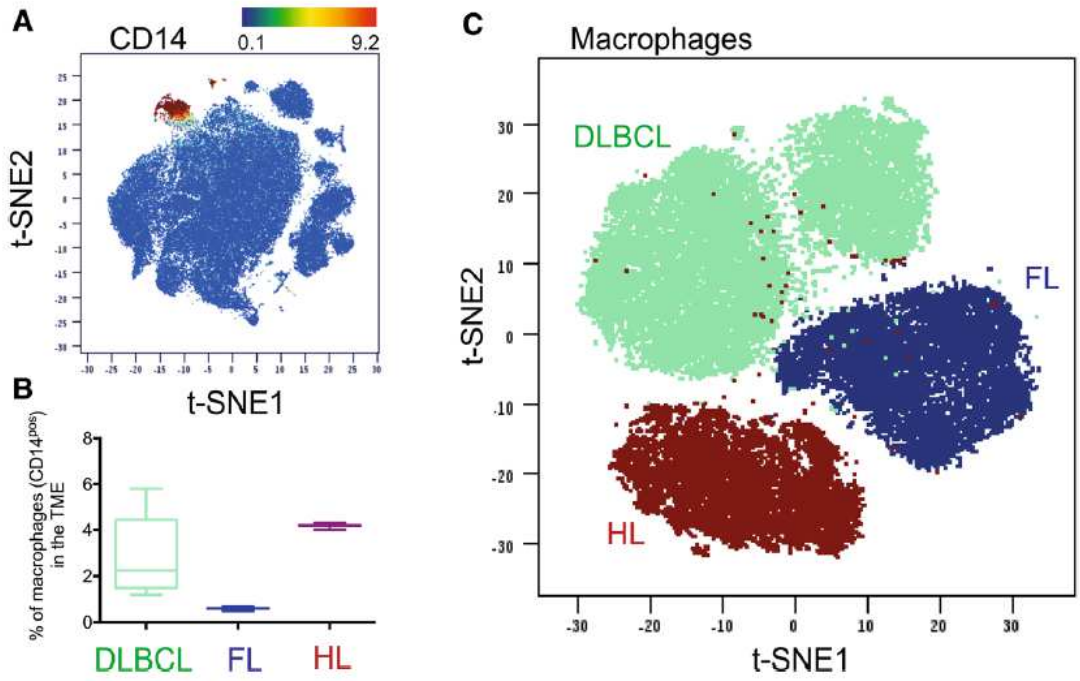

Figure 2: Mass cytometry reveals contrasting myeloid cell phenotypes between and within three lymphoma tumors

Cryopreserved cells from B-cell lymphomas (DLBCL, HL, and FL) were stained with a panel dedicated to the myeloid compartment [45]. A- After acquisition on CyTOF, cells were parsed by ViSNE to define B-, T-, NK-, and myeloid- cells following a workflow analysis already described [45, 94]. B- Due to the frequency of macrophages cells in TME from B-cell lymphoma, $5x10^5$ to $1x10^6$ viable cells were acquired on CyTOF. C- Macrophages from patients were then analyzed jointly by viSNE. A representative example of viSNE analysis is shown for myeloid cells from 3 patients: 1 DLBCL (green), 1 HL (blue), and 1 FL (brown) demonstrating differences in myeloid cell phenotype across these B-cell lymphomas.