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Review Article

Tumor Microenvironment and Immune Effects of Antineoplastic Therapy in Lymphoproliferative Syndromes

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Lymphomas represent a wide group of heterogenic diseases with different biological and clinical behavior. The underlying microenvironment-specific composition seems to play an essential role in this scenario, harboring the ability to develop successful immune responses or, on the contrary, leading to immune evasion and even promotion of tumor growth. Depending on surrounding lymphoid infiltrates, lymphomas may have different prognosis. Moreover, recent evidences have emerged that confer a significant impact of main lymphoma's treatment over microenvironment, with clinical consequences. In this review, we summarize these concepts from a pathological and clinical perspective. Also, the state of the art of lymphoma's anti-idiotype vaccine development is revised, highlighting the situations where this strategy has proven to be successful and eventual clues to obtain better results in the future.

1. Introduction

Tumors are in permanent state of chronic inflammation, and lymphoproliferative syndromes are not only a collection of tumoral cells or a simple genetic disease. Tumoral cells may give and receive instructions from other structural components, the tumor microenvironment, which is composed by extracellular matrix, stromal cells, neoangiogenic vessels and overall, the cells and cytokines that constitute the tumoral immune response. These elements constitute a complex signalling network where a delicate balance exists between microenvironment and tumoral cells. The products of mutated or deregulated genes contribute to the growth and invasion of tumoral cells, as well as to the expression of proteins with the ability to stimulate the immune response. The immunogenic capacity of the tumor can be evaluated by means of the study of the reactive infiltration, which is mainly composed by innate immune cells, especially macrophages, also granulocytes, eosinophils and mast cells, and adaptive immune cells, especially cytotoxic T cells—CTLs-, the most important suppressor of tumoral growth and target for vaccine approaches.

The hypothesis of immunesurveillance postulates that one of the principal functions of the immune system would be recognizing neoplastic cells and eliminating them before they form tumors [1]. This affirmation implies that in the absence of an effective immune system there is a high risk of developing cancer. Truly, there is evidence involving the immune system in the protection from certain tumors, especially those associated with viral infections, tumors related with elderly, transplanted and immunosuppressed patients, and those lymphomas associated with Epstein-Barr virus (EBV), Kaposi's sarcoma, and human immunodeficiency virus (HIV).

Immune system is a nonlinear complex system, and its main function in cancer is acting as an effective suppressive

system of tumors. However, as it occurs in the immunology of infectious diseases, an adequate immune response with enough magnitude to eradicate the microorganism or harmful pathogen is necessary. Nevertheless, the system can behave in an ineffective manner, as the appearance of tumors in immunocompetent population shows. So, along with the concept of immunosurveillance as immune defensive process, the concept of immunostimulation also arises [2], meaning that the immune response might not only be ineffective but it might contribute actively to tumoral progression. Among different molecular distinctive alterations of the lymphoproliferative syndromes, the role of the microenvironment has been studied extensively, and lymphocytes (cytotoxic T cells—CTL- and native killer—NK-), macrophages, dendritic cells, and neutrophils constitute potential effectors of the antitumoral immunity. Nevertheless, in the last few years a huge amount of evidence has emerged suggesting that these cells can also promote the growth and the development of the neoplasia, and that the immune system not only can affect the tumor, but the tumor itself may also alter the host immune system.

The ability of the immune system to act as a double-edge weapon, protective or stimulating, indicates that tumoral clearance requires the effective coordination of the different elements of the immune system in an appropriate balance in quantity and quality. Therefore, current cancer research in lymphoproliferative syndromes and other tumors aims to develop methods to increase the effectiveness of host antitumor immune response. This inevitably leads to consider tumors as more than an accumulation of neoplastic cells; they might be more properly considered as a functional tissue immunologically mediated and formed by a complex tissue network in which neoangiogenesis, infiltrating immune competent cells, stromal cells, and a differentiated and specific extracellular matrix constitute the tumor microenvironment with the capacity of regulating cancer development [3]. The interplay between the host immune system, malignant cells, and all other components of tumoral stroma determine proliferation, invasion, angiogenesis, and remodelling of extracellular matrix and metastasis.

2. Cellular Microenvironment and Hematopoiesis

Lymphoid neoplasms are functionally connected tissues dependent on the microenvironment, determining morphology and tumor classification, clinical behaviour, prognosis, and immune response to the tumor [4]. Perhaps one of the greatest exponents of the maxim that tumors constitute caricatures of normal tissues from which they arise might be the lymphoproliferative syndromes. In physiological conditions, the production of cellular elements corresponding to the immune system is an elaborated process, into which a series of main cells evolve in a sequential way, in a process of differentiation of each of the hematopoietic series. Its shortcomings are implied in the pathogenesis of hematological malignancies [5].

The hematopoietic microenvironment is constituted by a three-dimensional complex and highly organized structure, which serves to regulate the location, proliferation, and function of the hematopoietic cells. This is established by stromal cells, extracellular matrix (ECM), cytokines, and chemokines. Among stromal cells, there are macrophages—derived from hematopoietic stem cells, fibroblasts, adipocytes, and osteoblasts. All of them are derived from mesenchymal stem cells in the bone marrow and from immature myeloid cells in the stromal tumor, along with the endothelia of the neoangiogenic tumor microvessels. The hematopoietic microenvironment not only has great importance in the physiology of hematopoiesis and the physiopathology of some leukaemia, but also in the formation of the intratumoral cell microenvironment, which structure corresponds to the ECM. ECM represents a biophysical filter that offers protection, nutrition, and cell innervation, giving way for immune response, angiogenesis, fibrosis, and tisular regeneration. Its disruption supposes a functional loss for nutrition, elimination, cell denervation, regenerative capacity, and wound healing. This also causes the loss of the immune response to pathogens, tumor cells, and toxins.

Stromal tumor cells derive from progenitors of the bone marrow, which are mobilized across the circulation until joining the tumor microenvironment [6], where they will develop across different cellular lines in endotelia, fibroblasts, histiocytes, and macrophages and finally constitute the tumoral stroma. Tumoral microenvironment is involved in the regulation of tumoral cell growth and the metastatic potential of the tumor, so it is determinant in the response to treatment [7]. The collaboration of one of these cells in particular, the macrophage, turns out to be essential in the process of migration, invasion, and tumor metastasis [8]. It seems that stem cells derived from the bone marrow represent the precursors of metastasis in distant sites, being those in charge to activate a suitable microenvironment, preparing an ideal niche to receive the tumoral cells [9]. Myeloid cells, with CTL suppressive activity, have a special importance because they are recruited by the soluble factors liberated by the proper tumor [10]. There, and in the shape of intratumoral inflammatory monocytes, $CD11b^+$ exert their powerful immunosuppressive action in a multistep process that could be interfered in each single step with the subsequent restoration of immune reactivity.

3. Antitumoral Immunity

Tumor cells can express strange molecules recognized by the immune system and the expression patterns of these antigens vary among different tumors. These tumor antigens can be tumor-specific antigens (TSA), which are exclusively expressed in tumor cells and easily evoke immune responses; characteristic tumor antigens are TSA expressed only in one or a few tumor clones, harbouring peculiar typical mutations of these tumors; tumor-associated antigens (TAA) expressed both in normal and tumoral cells are often unable to induce immune response owed to tolerance mechanisms. Finally viral antigens represent viral strange tumor proteins produced by oncogenic virus [11]. Tumor antigens recognized by T cells (generally $CD8^+$ lymphocytes) represent the

principal target of antitumoral immunity and are presented by MHC class I molecules; that is to say, that tumoral cells behave as antigen presenting cells (APC), presenting their own antigens to T cells. Naturally, professional APC can also present antigens to $CD4^+$ lymphocytes through MHC class II molecules. Tumors can be destroyed by means of specific CTL and an increase in tumoral immunogenicity is accompanied by the specific rejection of the tumor.

Most tumor antigens identified by specific CTL are products of aberrant gene expression and, surprisingly, are not mutated. Products of oncogenes and mutated tumor suppressor genes may also be presented in association with MHC class I and/or class II. Both $CD4^+$ and $CD8^+$ T cells can respond to the products of these genes such as mutated ras gene, p53 and bcr-abl; however, it appears that these responses are poorly protective. Other tumor antigens are encoded by both RNA and DNA viruses that are implicated in tumor development. Tumor cells synthesize and process viral peptide complexes that are presented bound to MHC class I, and therefore stimulate specific T cell responses. These antigens encoded by viruses are not tumor specific but are shared by all tumors induced by the same type of virus. In particular, the protective function of the immune system in controlling virus-induced tumor DNA is given by the high frequency of these tumors in immunosuppressed patients, such as EBV-associated lymphoma.

Effector systems of tumoral immunity are varied and exert different mechanisms of action. The main elements are T cells and especially $CD8^+$ cytotoxic T lymphocytes (CTLs) that destroy tumor cells via the triggering of apoptosis, and providing effective antitumor immunity *in vivo*. They are predominantly CD8 and carry out their function of surveillance by means of the recognition and destruction of potentially malignant cells, which express peptids derived from mutated cellular proteins or oncogenic viral proteins, presented in affiliation to MHC class I molecules. Tumor infiltrating lymphocytes (TIL) are mononuclear cells that infiltrate solid tumors. These include tumor-specific CTL. The $CD4^+$ helper T cells are traditionally considered as noncytotoxic, although new evidence is emerging against this concept [12]. Actually, at least four distinct CD4 T-cells subsets have been described: Th1, Th2, Th17, and Treg cells, each one with a unique cytokine secretion pattern and function [13]. Of course one of their primary roles is providing cytokines for the development of CTL, in addition to being able to secrete TNF and IFN-gamma, which can increase the expression of MHC class I by the tumor cell and therefore increase its sensitivity to CTL lysis. Immunoregulatory cytokines such as IL-10 and TGF-beta play an important role in immune tolerance, and it seems that suppressor effect of T $CD4^+CD25^+$ is independent of cytokines.

Among natural cytotoxic cells, natural killer cells (NK cells) can be activated directly by contact with the tumor or as a result of the stimulus provided by cytokines such as interferons, TNF, IL-2, and IL-12, released by tumor-specific T lymphocytes and macrophages; therefore, their activity is endowed with some degree of specificity. In addition, lymphokine-activated killer cells (LAK) are a group of NK cells derived from peripheral blood cells or TIL in patients with high concentrations of IL-2 and show a high capacity, nonspecific in this case, to lyse tumor cells.

Macrophages are cellular mediators capable of lysing tumor cells by releasing a large amount of lysosomal enzymes and reactive oxygen metabolites. Once activated they also produce cytokines such as tumor necrosis factor (TNF) that exerts its cytotoxic activity triggering apoptosis in a similar way to that mediated by Fas; it has indirect effects on tumor vasculature and vascular thrombosis and produces free radicals from which normal cells are protected by the secretion of superoxide dismutase, but not tumoral cells. Dendritic cells (DC) and other antigen presenting cells (APC) are dispersed between tissues as sentinels or alarm systems ready to detect the presence of foreign antigens. While in the tumor microenvironment IL-12 production tends to be suppressed, resulting in a decrease in Th1 activity, DCs represent probably the most important regulators of naïve T cells, with a great capacity to produce and release IL-12. In their process of polarization, DCs are under the influence of inflammatory mediators such as prostaglandins produced by macrophages, fibroblasts, and tumor cells. A new route of junction between innate and adaptive immunity through the interaction between DC and NK cells has been suggested [14]. Finally, it is believed that antibodies are less important than the T cells to mediate antitumor immune response. However, there are Ab responses specifically against viral Ag, as in patients with EBV-associated lymphomas.

The tumor microenvironment consists of a specific mixture of immune cells that express a distinctive profile for each tumor type [15], from which the efficacy of the immune response against the tumor is eventually derived. Especially polynuclear neutrophils, dendritic cells, macrophages, NK cells, and mast cells play an important functional role in preneoplastic and tumoral tissues. Differences in gene expression profiles of malignant cells in lymphoproliferative syndromes do not always determine the aggressiveness of the lymphoma, while recent contributions determine the increasing importance role of cellular microenvironment in prognosis and disease progression. Each phase of tumor development progresses according to specific signals. So, while the activation of the immune response in advanced stages may be beneficial to the host, its activation during early stages can stimulate tumor development. Although lymphoid cells infiltrating the tumors are often considered as cytotoxic to tumor cells, these cells often contribute to the oncogenic process, tumor growth, and dissemination. Specific cells are responsible for activating specific processes within the tumor tissue, as occurs with mast cells and tumor neovascularization. Dendritic cells and macrophages may provide growth factors to malignant cells, sometimes instigated by viral sequences from stromal cells and not tumor cells themselves. The same cell in different microenvironments can act differently, as befits its power of dialogue and dynamic response to stimuli from the stromal environment.

4. Immune Response in Lymphoproliferative Syndromes

Lymphoproliferative syndromes are mainly distinguished by specific clinical factors and characteristic molecular alterations of low- and high-growth fraction lymphomas. Lymphomas with smaller fraction of growth include follicular lymphoma (FL), marginal zone lymphoma (MZLs), mantle cell lymphoma (MCL), and chronic lymphocytic leukaemia B (B-CLL), which as a group share a paradoxical combination of advanced clinical stages associated with a low clinical aggressiveness. By contrast, lymphomas with high-growth fraction, including diffuse large B cell lymphoma (DLBCL) and Burkitt lymphoma (BL), are frequently associated with clinically localized stage but a high clinical aggressiveness. Each of these groups appears to accumulate specific molecular proliferative and apoptotic changes [16], but differences in gene expression profiles of malignant cells do not always determine the aggressiveness of the lymphoma.

A wide repertoire of specific cell subpopulations constitutes the tumor microenvironment of each lymphoproliferative syndrome [17], with important diagnostic, prognostic, and therapeutic implications [4] (Table 1). The nature, role, and specificity of effector cells that are capable of inhibiting the growth of T and B-cell lymphomas *in vitro* and *in vivo* in immunocompetent individuals have been studied extensively. The number and especially the activation status of infiltrating cells appear to be independent of the degree of malignancy in Hodgkin's lymphoma and various B and T cell non-Hodgkin's lymphomas [18]. The reactive microenvironment determines not only histological morphology and immune phenotype, but also the clinical outcome of lymphoproliferative syndromes. Some of them, such as HL, slow growing tumor as FL, fast growing tumor as DLBCL or T cell lymphomas, are going to be revised briefly in the next paragraphs (Table 1).

4.1. Hodgkin Lymphoma. As if tumoral entities were sculpted by the immune system, the presence of a characteristic inflammatory background not only distinguishes Hodgkin Lymphoma (HL) from other lymphomas, but even more, this is the main characteristic that makes HL a separate entity itself allowing its diagnosis. Tumor cells of HL-PLN and the TCRBCL are the same or very similar, nevertheless we classify these two diseases differently depending on the accompanying tumor microenvironment. Thus, HL and other germinal centre-derived lymphomas can be differentiated through their cellular microenvironment [19].

Regardless of the classic clinical and pathological features, some studies have shown that the presence of activated CTLs (granzyme B^+) is associated with an unfavourable follow up of HL patients [20]. There is a predominance of activated $CD4^+$ T cells in the background of the tumor [21] and a high number of cytotoxic T lymphocytes [22] around the Hodgkin/Reed Sternberg cells (H/RS). $CD4^+$ T cells produce Th2 cytokines that could contribute to local suppression of the cellular immune response mediated by Th1. However, the categorization of $CD4^+$ T cells in Th1 and/or Th2 is an oversimplification [23] as regulatory T cells with $CD4^+CD25^+$ phenotype not only play a regulatory role of autoimmunity, but also have suppressive effects on the development of antigen-reactive lymphocytes associated with the tumor [24]. Functional and molecular characterization of these cells has been facilitated by the identification of markers such as FOXP3, which acts by converting naïve T cells $CD4^+CD25^-$ into the regulatory phenotype $CD4^+CD25^+$ [25]. These regulatory T cells can inhibit the production of IL-2 as well as upregulating the expression of IL-2Ra (CD25), delaying or blocking the activation of $CD8^+$ cells and NK cells against tumor antigens [26]. In HL, the immunosuppressive properties of regulatory T cells appear to be particularly important because of its large effect on cellular cytotoxicity represented by CTLs and NK cells. The presence of low numbers of $FOXP3^+$ cells and a consequent high rate of TIA-1^+ cells in the infiltrate represents an independent prognostic factor negatively affecting the survival of the disease. Furthermore, when the disease relapses and progresses, larger number of TIA-1^+ cells and lower proportion of $FOXP3^+$ on the reactive background of the tumor are also prone to be seen [27]. There is a significant loss of intratumoral $CD4^+$ T cells (an inversion of CD4/CD8 ratio) and a decrease of intratumoral activated CTLs in HIV-infected HL patients [28]. All these data are of interest due to the possibility to significantly expand tumor-induced $CD4^+$ Tregs by the application of therapeutic cancer vaccines [29].

A plausible explanation of the extensive inflammatory infiltrate present in HL could be the secretion of a variety of cytokines produced by both tumor cells and the surrounding stromal tissue. H/RS cells produce and secrete high amounts of chemokines, including TARC and MDC, which attract lymphocytes expressing the CCR4 receptor [30]. In addition, immune cells themselves can produce cytokines responsible for proliferation and tumor survival.

Within the complexity of the interactions between the inflammatory reaction and HL tumor cells, immune cells present in the infiltrate can modulate apoptosis and proliferation of tumor cells [31]. The antiapoptotic profile observed in H/RS cells is associated with a general increase in infiltrating $CD4^+$ T cells and a general decrease in infiltrating $CD8^+$ T lymphocytes, NK cells, and dendritic cells. The progression of G1/S tumoral phase and the high rate of proliferation are also strongly associated with higher infiltration of the overall immune response against the tumor [32]. These results point to the regulation of proteins involved in apoptosis and proliferation of tumor cells by direct interactions between these cells and the surrounding inflammatory microenvironment. This opens up new approaches for research and treatment of HL through the modulation of host immune response.

4.2. An Example of Low-Grade B-NHL, Follicular Center Cell Lymphoma (FL). FL is recognized as a disease of functional B cells, in which T-cell costimulation is essential in the maintenance and ongoing development of B-cell secondary follicles [33, 34].

Table 1: Type of immune infiltration in Lymphomas and prognosis.

Type of lymphoma	Microenvironment	Prognosis
HL	↑↑ activated CTLs (Granzyme B^+)	Unfavourable
	↑↑ TIA-1^+ cells	
	↓↓ $FOXP3^+$ cells	
FL	Type 1 immune response pattern	Longer survival
	↑↑ T lymphocytes and regulatory T cells ($FOXP3^+$)	Favourable outcome
	Type 2 immune response pattern	Shorter survival
	↑↑ tumor-associated-macrophages (TAM, $CD68^+$) and NK cells ($CD57^+$)	Poor prognosis
DLBCL	↑↑ activated $CD4^+$ T cells, dendritic cells and macrophages	Better prognosis
	Infiltrate greater than 20% of $CD4^+$ cells, including $CD45RO^+$	
	↑↑ $FOXP3^+$	
	Higher expression of Th1 than Th2	
	↓↓ IL-6 (Th2 response) during the first weeks after the therapy	Predict complete remission
	↓↓ TILs-$CD8^+$, ↑↑ activated CTLs	Poor prognosis
T and NK cell	↑↑ monocytes	Poor prognosis
	↓↓ $FOXP3^+$	Unfavorable
ALCL	↑↑ Granzyme B^+	Unfavorable
	↑↑ Granzyme B^+ and lack of expression of ALK	Poor prognosis

Since the FL represents the tumoral counterpart of germinal centre B cells and resembles its follicular architecture, the development of this lymphoma may be closely linked to interactions with cellular components of the microenvironment, including dendritic and T cells inside the follicle. It is said that the relationships between tumoral cells and microenvironment can follow three distinct patterns: a loss of interconnection with the immune response to the tumor, a dysfunctional environment, and a friendly, regulated coexistence of the malignant and immune cells [35]. FL seems a good example of the latter pattern, a disease usually indolent and with a long median survival in which at least 15% or greater may experience spontaneous remission, sometimes after acute viral illness and with rapid responses after vaccine therapy. In addition, tumor microenvironment but not tumor cells could be the fundamental key to choosing the most appropriate chemotherapeutic regimen for these patients [36].

At molecular level, survival of patients with FL appears to correlate with the characteristics of nonmalignant immune cells present in the tumor at diagnosis through two patterns of gene expression [15]. Type 1 immune response pattern is associated with longer survival and includes a complex mixture of T cells and other immune cells, while type 2 pattern is associated with shorter survival and includes genes that encode no markers of innate immune cells, primarily macrophages. The differences in the biology of the host immune response determine the clinical course and prognosis of patients with LF, and not the genetic alterations of the tumor cells themselves.

At cellular level, the relationship between cellular elements of specific and nonspecific cell-mediated immunity implies that FL is an immunologically functional disease in which an interaction between the tumor cells and the functional composition of the microenvironment determines their clinical behaviour [37]. The general mechanisms involved in FL tumor immunity have been principally attributed to $CD4^+$ T helper lymphocytes, $CD8^+$ cytotoxic T-cells (CTLs), NK cells, and macrophages. The presence of modulating $FOXP3^+$ T-cells has also proved to have an important role in the host immune response [38]. Taken as a whole, the results of these studies have highlighted the existence of two principal immune facts in which the presence of T lymphocytes and regulatory T cells is related to a favorable outcome, whereas the presence of tumor-associated macrophages (TAM) and NK cells is more usually associated with a poor prognosis. Dysfunctional immune profiles in the tumor microenvironment of FL seem to be attributed to the state of functionality of regulatory T cells, the presence of a particular subset of $CD57^+$ cells, and the reprogrammed immune cells such as TAMs [37, 38].

The favorable clinical impact of the high number of Tregs in FL may be due to a direct inhibitory effect on neoplastic B-cells [39] and the inhibition of tumor-infiltrating leukocytes that can facilitate tumor progression by secreting various growth factors and proteases. However, in epithelial carcinomas these cells correlated inversely with clinical outcome [40], representing the dominant immune escape mechanism early in the tumor progression but not in late phases [41]. These different behaviors seem to be secondary to different mechanisms of immune response regulation from Tregs in FL and in solid tumors. Tregs have numerous lymphoid targets, including $CD8^+$ T cells, B cells, NK cells, and dendritic cells. When the control of the immune response is misguided, Tregs cells can induce immunosuppressive mechanisms through the attenuation

of tumor-specific $CD8^+$ T-cell killing and restricted NKT cells.

In addition to $FOXP3^+$ Tregs cells, $CD57^+$ cells appear to represent another marker of general immune dysfunction in FL [42]. Unlike T cells and macrophages, a higher infiltration of $CD57^+$ cells appears to be related to unfavorable clinicobiological factors in FL patients [38]. CD57 is expressed on NK cells, one of the major effector cells in cellular cytotoxicity together with CTLs. The nonspecific inflammatory infiltrate ($CD57^+$ cells and $CD68^+$ macrophages) seems to be mainly involved in the control of growth and expansion of tumoral cells whereas the specific immune infiltrate ($CD4^+$ and $CD8^+$ T lymphocytes) seems to be mainly involved in the host immune response against the tumor and the main clinical features. Both systems seem to emerge directly associated with the capacity to disseminate tumoral cells, as shown by the greater infiltration of T lymphocytes observed in low-grade FL with spontaneous regression [43], the relatively low absolute number of T cells observed on transformation [44], and the role of naïve and memory T cells in downregulating tumor proliferation rate [45].

Although in a non-restrictive cohort of FL patients was considered, the presence of $CD68^+$ TAM tended to be associated with an indolent clinical behavior and longer survival [46], $CD68^+$ TAM appeared to be associated with an unfavorable outcome for FL patients [47]. The specific subsets of activated macrophages evaluated by the expression of STAT1 may be considered as prototypic type 2 polarized macrophages reprogrammed to induce *in situ* immune suppression. TAMs seem to have a dual nature that appears to be specific to the tumor type. Depending on the microenvironment, they may either exhibit antitumor cytotoxic activity or facilitated tumor growth and progression while reinforcing the Th2-biased immune response [48]. Polarized M1 and M2 (or alternatively activated) macrophages differ in terms of receptor expression, effector function, and cytokine and chemokines production. Regardless of functional defects or absence of activation, in most of the cases TAM do not exhibit cytotoxic activity and facilitate tumor growth, angiogenesis, and metastasis, that is, Th1 immunosuppressive response. TAM products act in two manners to support tumor progression, on one hand, they support tumor growth angiogenicity and extracellular matrix degradation, and on the other hand, they suppress potential antitumor activities [49].

4.3. An Example of High-Grade B-NHL, Diffuse Large B Cell Lymphoma (DLBCL). Recent molecular studies show that survival of patients with DLBCL is influenced by immune cells, fibrosis, and angiogenesis of tumor microenvironment [50]. Stromal-1 signature genes encode components of the extracellular matrix and antiangiogenic factors, while stromal-2 signature genes encode markers of endothelial cells and key regulators of angiogenesis. This survival model reflects the character of nonmalignant cells in DLBCL, including TAMs and myeloid-derived suppressor cells.

At immunophenotypical level, the component of nonmalignant infiltrate can vary among the different subtypes of DLBCL, with the greatest exponent provided by the T cell/histiocyte rich B-cell lymphoma. The presence of an increased number of activated $CD4^+$ T cells [51] as well as dendritic cells and macrophages seems to predict a better prognosis of DLBCL. The DLBCL negative for gene expression of MHC II have few $CD8^+$ T cells infiltrating the tumor [52] and a high percentage of activated CTLs, both of them representing a powerful adverse prognostic factor [53]. A specific subgroup of patients with DLBCL defined in terms of host response has been identified [54]; in this type of response, an increased expression of NK/T cell, monocyte-macrophages, and dendritic cells (DCs) markers as well as inflammatory mediators can be observed. Moreover, those cases with an infiltrate greater than 20% of $CD4^+$ cells, including $CD45RO^+$, show a trend towards better survival. Although it is possible that the role of the microenvironment as a whole can be dual, depending on the tumor, the patient, and the functional status of the host immune system.

An effective cytotoxic response represented by a dense CTL infiltrate and numerous accompanying reactive cells including a high number of $FoxP3^+$ Treg cells seems to be accompanied by better prognosis in DLBCL [55]. The presence of interdigitating dendritic cells associated with infiltrating T cells is involved in coordinating the immune response. However, tumor cells also seem able to modulate the maturation of dendritic cells so they can remove the ability of these cells to process and present tumor antigens. A higher expression of Th1 than Th2 response has also been observed in patients who achieved complete remission [56], and a significant decrease in IL-6 (Th2 response) during the first weeks after therapy in patients with aggressive NHL seems to predict complete remission [57]. In these patients, a germinal center phenotype ($bcl\text{-}6^+/CD10^+$) is accompanied by a lower level of circulating IL-6 compared to activated phenotypes.

4.4. T and NK Cell Lymphomas. Frequently, in T-NHL, the microenvironment cellularity represents the bulk of the tumor, and the clinicobiological manifestations of disease reflect a deregulated immune response rather than the effect of tumoral cells. In PTCL as well as in AITL, a follicular helper T cell tumor, once again, there is not an association between gene clusters and their histological subtypes [58]. However, cells present in tumor microenvironment promote tumorigenesis and suppress host immunity. T cell lymphomas characteristically present a great number of monocytes that promote survival of malignant cells [59]. Another lymphoma characterized by the significant presence of reactive lymphocytes around tumor cells is anaplastic large cell lymphoma (ALCL), where infiltration of a high percentage of activated cytotoxic cells (Granzyme B^+) is an unfavorable prognostic marker [60], especially when combined with the lack of expression of ALK. The mechanisms by which tumor cells escape the CTL attack have been scarcely investigated, although among the postulated mechanisms, the downregulation of MHC I molecules, the expression of IL-10, the expression of FAS-L in tumor cells, overexpression of Bcl-2, and also the expression of PI9, an inhibitor of proteolytic activity of granzyme B, have been considered [61]. The $FoxP3^+$ Tregs cells

predicted improved clinical outcome in extranodal NK/T lymphomas, whereas a decreased number of these cells are more common in patients with poor performance status [62].

Therapeutic Applications of Immune Response. The immune system appears to be essential for therapeutic success if we consider that it can eliminate definitively residual cancer cells that remain after chemotherapy. In this sense, therapy applied during tumor escape phase can inhibit suppressive mechanisms of tumor-induced tolerance, boost T and/or B cells, or stress tumor cells in such a way that tumor cells become immunogenic and sensitive to lysis [63]. The simple reduction of the tumor mass by chemotherapy or surgical removal may also reduce its immunosuppressive properties, reversing tumor-induced immune tolerance and restoring the antibody- and cell-mediated immune responses [64].

On the relationship between the tumor and the immune system, the immunosuppressive side effects of massive chemotherapy should be reevaluated. A bidirectional interaction between tumor and inflammatory/immune cells is ultimately responsible for orchestrating the immunosuppressive network at the tumor site [65]. The manipulation of one of these partners may consistently influence the other. Looking for new modalities of cancer treatment, the induction of a potent and specific immune system has been described as a logical and reasonable strategy for controlling tumor evolution. The different strategies that have been used to improve immunity against tumors include vaccination to provide antigens to the patients' immune system, providing costimulatory signals on tumor cells, induction of cell death with cytotoxic drugs, sustaining immune effectors with NK, NKT, or DC adjuvant, and improving efficiency of cross-priming [66]. If anticancer immune responses dictate long-term therapeutic success, then local signs of antigen priming (DCs) or NK and T cell responses would correlate with favorable responses.

In conclusion, different entities of lymphoproliferative syndromes, independently whether they are HL or NHL, B or T cell lymphomas, and fast or slow growing tumours, have specific patterns of immune responses associated with their morphologic aspect, their immunophenotype, their clinicobiologic course, outcome and the probable response to the therapeutic drug used in their clinical management. Among the nonspecific immune response, a clear distinction can be made between activated and nonactivated CTL, with different significance in various lymphomas [4]. There is a specific machinery to control the tumor microenvironment, represented fundamentally by Treg cells, FoxP3$^+$, CD57$^+$ T cells, and TAMs. Chemotherapy can inhibit a tumor-promoting immune reaction but may in fact be an example of immunotherapy depressing mast cells and macrophages that secrete factors with the ability of promoting tumor growth. In the search of new modalities of lymphoma treatment, the induction of a specific immune response through effective immunotherapy appears to be a promising route of help for these patients and their clinicians.

5. Immune Effects of Antineoplastic Therapy in Lymphoproliferative Syndromes

We will discuss new evidence about immune microenvironment changes after antineoplastic treatment in lymphomas. Lymphomas represent a wide group of heterogenic diseases with different clinical behaviours, and nowadays, the three main options in lymphoma's armamentarium remain to be chemotherapy, radiotherapy, and passive immunotherapy (monoclonal antibodies). All these treatments have a relevant impact on the surrounding stroma and microenvironment.

5.1. Chemotherapy—The Anthracyclines Model. Chemotherapy remains the treatment modality of choice for most lymphomas, especially in advanced stages. Different cytotoxic drugs destroy tumor cells by apoptosis, a process mediated by the activation of caspases and exposure of phosphatidylserine residues in the outer leaflet of the cell [67]. Apoptosis destroys billions of cells in an adult lifetime as a consequence of physiologic tissue renewal and cell turnover without leading to any adverse inflammatory or autoimmune phenomena. Thus, programmed cell death has been traditionally considered as immunologically "bland" or nonimmunogenic. However, this theoretical assumption has not been confirmed in basic and translational research. Rather, it seems that apoptosis is a heterogeneous process, that under some circumstances may lead to immunogenic effects. Recent studies focus on apoptosis and tumor suppressor pathways in cancer and suggest that some chemotherapeutics may induce tumoral destruction which improves cancer cell recognition by the immune system [68, 69].

Anthracyclines remain one of the drugs of choice against lymphoproliferative diseases for Hodgkin's and Non-Hodgkin's Lymphomas and is included in most of the first line chemotherapy schedules. There is now clear evidence that anthracyclines may promote apoptosis in cancer cells with immunogenic effects through several mechanisms (Figure 1).

(1) Calreticulin. Anthracyclines facilitate the translocation of intracytoplasmic protein calreticulin (CRT) to the cell surface, inducing the apoptotic cell antigen presentation to Antigen Presenting Cells (APCs), in particular dendritic cells (DC), and stimulating specific antitumor T cell responses [70].

(2) High-Mobility Group Box 1 (HMGB1). Another immunogenic determinant of cell death is the pro-inflammatory factor HMGB1. HMGB1 is a nuclear protein that is released after necrotic cell death and, as recently reported, from dying cells during late stage apoptosis. After cell death induced by anthracyclines and alkylating agents, HMGB1 may be released in the stroma and act as a neo-antigen representing an immunogenic endogenous "danger signal", initiating an inflammatory response through binding Toll-Like Receptor

4 (TLR4) on DC (but not other DC receptors, such as RAGE or TLR2) [71]. MyD88 is one downstream effector of TLR4, and nowadays there is clear evidence which supports that immunogenicity triggered by anthracyclines is exclusively dependent on an intact TLR4-MyD88 signalling pathway [72].

(3) DNA Damaging Agents. Alkylating chemotherapeutic agents, such as cyclophosphamide, induce the expression of NKG2D ligands. NKG2D acts as an activating receptor on NK cells, $\gamma\delta$ T cells, NKT cells, and memory $CD8^+$ T cells, giving raise to the possibility that DNA damage response may induce immune system activation [68, 73].

(4) Secondary Necrosis. Although not specific for anthracyclines, when massive chemotherapy cell destruction occurs, the mechanisms of controlled apoptosis are overwhelmed and a secondary necrosis occurs triggering an inflammatory response mediated by the intracellular inflammation mediators release, as uric acid, heat shock proteins (HSP), and IL-12 [74].

(5) Cross-Presentation. Cross-presentation is a mechanism favoured by some antineoplastic drugs such as anthracyclines or gemcitabine. These drugs allow tumor antigens to be presented to MHC class I pathway through APCs, a pathway previously thought to be restricted to class II pathway. This mechanism allows tumor antigen presentation to both CD4 T and CD8 T cells which will subsequently identify and destroy the remaining tumour cells [75], but paradoxically apoptotic cells lead to secretion of VEGF that promote the proliferation of endothelial cells and other survival factors that stimulate extracellular matrix with many other implications.

(6) Eradication of Tumor Cells by Chemotherapy. Immune-inhibitory molecules released by tumor cells, such as interleukin-10 (IL-10) or tumor growth factor-β (TGF-β) which inhibit T cell activation, or IL-10, IL-6, and vascular endothelial growth factor (VEGF), involved in the maturation and differentiation of dendritic cells (DCs), are downregulated as a result of the use of chemotherapy after an effective cell destruction [76].

Therefore, emerging evidence led Lake and Robinson to announce a paradigm shift in the way of understanding the effects of chemotherapy on the surrounding stroma [76, 77]. CT can induce a highly potent immune response by increasing antigen (neoantigens) threshold and presentation (via APCs), with enhancement of T-cell response and generation of memory T cells. Other chemotherapeutics like cyclophosphamide, etoposide, and taxanes (docetaxel and paclitaxel) have also proved to have an immunogenic effect in preclinical models [78, 79]; however, evidence is scarce and further investigation is required. These new concepts may serve to consider chemotherapeutics like anthracyclines as less empirical and more specific drugs, and then customizing treatments taking into account its potential effects on microenvironment.

5.1.1. Dendritic Cells and GM-CSF. Among different molecules and cells activated as a result of immunogenic cancer cell death mediated by chemotherapy, antigen presenting cells (APCs), in particular dendritic cells (DC), seem to play an essential role. This is particularly true because tumor reactive T cells are often anergic because of inappropriate antigen exposure or owed to self-recognition. On the contrary, immunogenic tumor cell death mediated by some chemotherapies is characterized by a temporal sequence of events including early translocation of calreticulin to the cell surface, and thereafter interaction of CRT with multiple receptors on DC with apoptotic bodies phagocytosis, release and exposure of heat shock proteins, and late release of HMGB1 [68]. HMGB1 is able to bind to the TLR4 receptor on DC, which allows tumor derived antigens to be processed and presented along with MHC and costimulatory molecules on the surface of DC [68, 69]. These mechanisms altogether serve to trigger DC-mediated specific antitumor response, which may be enhanced by the use of costimulatory molecules. Costimulatory molecules provide additional or second signals for lymphocyte activation beyond those provided through the antigen receptor.

GM-CSF is one of the most important cytokines in cancer microenvironment [80]. GM-CSF has pleiotropic properties, including the mobilisation, differentiation, and function of dendritic cells, possibly by reversing the host's immune tolerance to its own tumor associated antigens, and by initiating (priming) immune responses for which immunologic memory has not been established, that is, activating the so-called naïve T cells. The proliferation of naïve lymphocytes during the first encounter with an antigen (primary immune response) generates both effector T and B cells and memory T and B cells. Memory cells enable a quantitatively and qualitatively superior secondary immune response to be mounted after a subsequent encounter with the same antigen [81].

GM-CSF has been studied in the clinical setting in relapsed follicular lymphoma, along with Rituximab, showing promising results. In a phase 2 trial, combination of rituximab with GM-CSF attained 70% overall response rate (ORR) and 39% complete response rate (CRR), which compares favourably with rituximab as single therapy against relapsed follicular lymphoma (CRR of 6%) [82]. This synergism can be explained, at least in part, by the antibody-dependent cellular cytotoxicity (ADCC) of Rituximab, which is enhanced by GM-CSF [83]. Interestingly, a molecular substudy was executed, demonstrating that this immunotherapy schedule increases the counts of numerous immune cells, particularly monocytes and granulocytes. However, there were no differences between CR and non-CR patients in the mean and ratio pre- or postimmunotherapy counts [82]. Some groups argue that maybe a better way of analyzing the impact of these therapeutic approaches would be determining the magnitude of accumulation of the effector cells at tumor sites, and not only blood levels, with semiquantitative measures through radiolabeling autologous granulocytes or mononuclear white blood cells with indium-111 labeled oxine [84]. The latter would be a highly interesting approach

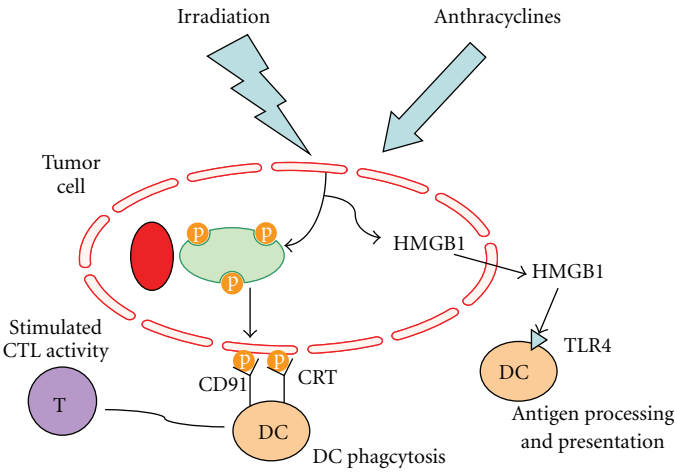

Figure 1: Immunogenic effects of anthracyclines and radiation. CRT—Calreticulin; DC—Dendritic cell; CTL—Cytotoxic T lymphocytes; TLR4—Toll-like receptor 4; HMGB1—High-mobility group box 1.

to monitor immune response *in vivo*, especially if it correlates with clinical efficacy.

5.2. Radiotherapy. Radiation therapy has been the backbone of lymphoma's treatment during decades. Though indications have diminished in the last years, radiotherapy still remains the treatment of choice for curative purposes in localized low-grade non-Hodgkin's lymphomas and Hodgkin's lymphoma, and even in high grade non-Hodgkin's lymphomas as a consolidation treatment after CT in sites of initial bulky disease.

Intrinsic radiosensitivity of malignant lymphocytes is extremely high; however, the underlying mechanisms which explain it are not fully elucidated. Recently, new evidence is emerging about some changes induced by radiation at a molecular level, which may provoke a type of cell death highly immunogenic [85]. Outside the field of treatment, radiation therapy can activate cells of the immune system to produce proinflammatory mediators of genomic instability [86]. Curiously, outcome of the inflammatory response triggered by radiation can be beneficial or detrimental depending upon the context, which is related with the type of macrophages activated (M1-proinflammatory or M2-woundhealing) [87]. Moreover, ionising radiation has different immune effects regarding the dose administered, so in the case of low doses, the final effect is mostly protumorigenic [88]. On the contrary, at higher doses with cytotoxic activity, cell death may induce tumoral neoantigens which can be embraced by dendritic cells, and thus activate an effective adaptive immune response [89]. As with anthracyclines, the two critical mediators of this process seem to be translocation of calreticulin to the cell surface and release of HMGB1 by the dying cells [85] (Figure 1). Both of them trigger danger signals which activate immune mechanisms. In addition, surviving cancer cells after radiation show increased expression of death receptors, adhesion molecules (ICAM-1), and major histocompatibility complex class I (MHC-I), which activate APCs [90, 91]. Once APCs are activated, essentially dendritic cells (DCs), they migrate to the tumor-draining lymph nodes, where naïve T cells can be activated through interaction with tumor-derived antigens presented by DCs. Preclinical studies have also revealed that irradiation of the tumour site may induce release of chemotactic cytokines, that regulate the transit of leukocytes, especially effector T cells, from blood into tumors [92].

Sequence of immune events generated by radiotherapy is critically important, since radiation of loco-regional lymph nodes, which is a common procedure in daily practice, may alter and disrupt the possibility of an effective immune response by depleting naïve T cells.

Immunogenicity of radiation therapy opens a new window of clinical research. Theoretically, molecules like anti-CTLA4 monoclonal antibodies, or costimulators such as GM-CSF, Interferons, or IL-2, may serve as boosters, amplifying immune effectors triggered by radiotherapy. So, if these new concepts are finally confirmed in the clinical setting, it will probably translate into a new way of administering radiotherapy in the coming future.

5.3. Passive Immunotherapy: The Rituximab Era. More than any other discovery, widespread use of monoclonal antibodies (mAbs) in daily practice has dramatically improved clinical results in terms of disease-free survival and overall survival in many types of lymphomas. This is especially true for rituximab, a chimeric monoclonal antibody targeting the CD20 antigen found on both normal B cells and on most low-grade and some high grade B-cell lymphomas [93]. It is effective as a single agent in induction and maintenance therapy, though it is primarily used in combination with standard chemotherapies in the treatment of patients with non-Hodgkin's B-cell lymphomas and chronic lymphocytic leukemia [93]. Although its mechanisms of action are not fully elucidated, rituximab can induce killing

of $CD20^+$ cells (95% of malignant B lymphocytes) via multiple mechanisms. Direct effects of rituximab encompass complement-dependent cytotoxicy (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), which are retained as the major mechanisms of action of rituximab on B-cell lymphomas. The indirect effects include structural changes, B-cell apoptosis, and sensitization of cancer cells to chemotherapy [83, 93].

The complement system can trigger three protease cascades known as the classical, mannose binding lectin (MBL) and alternative pathways. All three pathways converge at the C3 and C5 levels, leading to the formation of a membrane attack complex (MAC) that, if remains open, will directly induce targeted cell lysis by osmotic mechanisms [94]. Specifically, rituximab activates the classical complement cascade by interacting with C1q through its Fc region, exposed after binding with CD20 on the B-cell surface [95], forming MACs and subsequent cytolysis. Along with CDC, rituximab-mediated ADCC is important for the elimination of malignant B lymphocytes. ADCC triggers tumor cell killing through interaction between the Fc region of CD20 binding rituximab and FcγRs. The final effect is releasing of inflammatory and cytotoxic immune modulators, which lead to phagocytosis of targeted cancer cells by monocytes/macrophages and granulocytes/neutrophils, or lysis mediated by NK cells using the granzyme-perforin system [83, 96, 97]. Some cytokines may aid ADCC to enhance cytotoxicity and avoid antibody-targeted tumor resistance to innate immune cells. Again, GM-CSF has demonstrated *in vitro* enhancement of cytotoxicity upon lymphoma cells through upregulation of monocyte FcγRs [98]. Interleukin-2 (IL-2) activates selective immune effector cell responses associated with antitumor activity, since IL-2-activated NK cells strongly enhance activity of rituximab through ADCC in primary B-cell NHL [99]. Moreover, IL-2 acts as a chemokine, inducing activation and traffic of monocytes and NK cells to tumors. Other cytokines as IL-12 also synergize the rituximab effect by upregulating γ-interferon and other immune mediators, increasing NK cell lytic activity *in vitro* [100].

CDC-resistant cells may be sensitive to ADCC, and the same occurs with ADCC-resistant cells, that can be destroyed by CDC activation [83]. Nowadays, it is widely accepted that ADCC and CDC, the main mechanisms of action of rituximab against lymphoid cells, act synergistically by enhancing cytotoxicity in cancer cells through the ability of complement to promote inflammation and induce activation of innate immune effectors.

Besides the pure immunogenic effects of rituximab, other cytotoxic effects have been studied, in particular apoptosis induction and direct growth arrest. CD-20-rituximab crosstalk can redistribute lipid grafts of the cytoplasmic membrane and subsequently transactivate the Src family tyrosine kinase and the Fas-pathway, which results in initiation of downstream signaling pathways leading to a caspase-dependent apoptosis [18, 35, 101]. Moreover, rituximab downregulates the p38 mitogen-activated protein kinase (MAPK), nuclear factor (NF)-$\kappa\beta$, ERK-1/2, and Akt survival pathways, thus inhibiting the expression of antiapoptotic gene products (Bcl-2/Bcl-xL, and others) [102, 103]. Rituximab also induces caspase-independent apoptosis, through mechanisms still under investigation [104].

Inhibition of antiapoptosis related pathways sensitizes B-cell NHL to undergo apoptosis and facilitates the proapoptotic effect induced by chemotherapy [105, 106]. The combination of rituximab and CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) is proving a highly effective combination in the treatment of NHL [93], with better clinical results of each treatment modality alone. It seems that synergism of this chemoimmunotherapy schedule relies, at least in part, in chemosensitization of drug-resistant NHL cells mediated by rituximab via selective downregulation of antiapoptotic factors through the type II mitochondrial apoptotic pathway [105, 106]. Moreover, new concepts about immunogenic apoptosis induced by chemotherapy may also contribute to explain the success of chemoimmunotherapy combinations.

6. Vaccines in Lymphoproliferative Diseases

Concept of vaccination is based on the fact that deliberate exposure to a harmless version of a pathogen generates memory cells, but not the pathologic sequelae of the harmful agent itself. In this way, the immune system is primed to mount a secondary immune response with strong and immediate protection against a new encounter with the pathogen in the future [107]. Active immunotherapy has been traditionally considered a promising approach in lymphoproliferative diseases, especially in low-grade lymphomas. In this sense, follicular lymphomas have demonstrated a high sensitivity to passive immunotherapy with Rituximab and Interferons, either alone or combined with chemotherapy [93]. Also, the indolent course of these diseases, with prolonged spontaneous remissions in up to 23% of patients, seems to be ascribed to immune regulation [108]. Finally, survival of patients with FL appears to correlate with gene expression signatures of tumor infiltrating lymphocytes (TILs) [109].

Vaccine strategies targeting LF have largely focused on using the tumor immunoglobulin molecule expressed on the surface of malignant B cells as an antigen. Antibodies can be produced as circulating or stationary molecules. The latter type has a hydrophobic transmembrane sequence that anchors the molecule in the B-cell membrane, where it functions as the B-cell receptor. Antibodies consist of two identical heavy chains and two identical light chains that are held together by disulfide bonds. The variable regions of the heavy and light chains of the tumor immunoglobulin contain unique determinants known as idiotype (Id) that are a collection of antigenic determinants selectively expressed in tumor cells and serve as tumor-specific antigens [110, 111]. Thus, idiotype vaccination can potentially induce effective polyclonal antibody and T-cell responses against malignant clonal B cells.

Induction of clinically relevant tumour-specific immunity was less frequent in animals with sizable tumour

burden [112]. Therefore, probably the best clinical setting for optimizing immunogenicity and achieving meaningful clinical results comes after a complete response after antineoplastic treatment. This is consistent with the way vaccines work in infectious diseases, without the harmful agent present at vaccination. Preclinical studies revealed that the tumour-specific Id is a weak self-antigen [110, 111]. To enhance immunogenicity, Id vaccine formulations require conjugation to a strongly immunogenic carrier protein, such as keyhole-limpet hemocyanin (KLH) [110]. In addition, using an immunological adjuvant as GM-CSF facilitates activation and recruitment of mature dendritic cells and induction of tumor-specific $CD8^+$ T cells [111]. Thus, most of the following clinical trials use KLH^+ GM-CSF to overcome immune tolerance.

6.1. Clinical Trials with Idiotype Vaccines. Kwak et al. conducted the first study of Id vaccination in humans [113]. It was a pilot study in which 41 FL patients, in complete response or minimal residual disease after chemotherapy, were immunized with subcutaneous injections of autologous purified tumor-derived immunoglobulin conjugated to KLH along with a standard emulsion adjuvant (Syntex adjuvant formulation 1). Results were successful in terms of biological efficacy, with a demonstrated 41% of specific anti-Id antibody, and clinical efficacy with 17% of cellular proliferative responses [113]. These promising data led the Biological Resources Branch, Development Therapeutics Program of the National Cancer Institute to initiate phase 2 trials to confirm safety, clinical efficacy, and good manufacturing practices (GMP) in order to start an eventual commercialization [114, 115]. Most phase 2 studies confirmed that vaccines were well tolerated and induced humoral and cellular immune responses with some clinical effects (clinical and molecular remissions). Results of phase 1 and 2 trials also suggested that biological and clinical efficacy may induce a clinical benefit, which is the capacity to influence on disease-free and overall survival. Inogés and coworkers showed that patients with FL in second CR after chemotherapy (not containing Rituximab) and being successfully vaccinated in biological terms had longer CR (more than 13 months) than the duration of the same patient's first CR obtained with standard chemotherapy with or without Rituximab [116]. These data, though achieved in a limited number of patients, are critically important because they suggest once again that the best clinical setting to employ vaccination is when there is minimal or no residual disease [116]. Hence, these encouraging results achieved with the Id-KLH^+ GM-CSF led to the initiation of three phase 3 trials to clarify its eventual clinical benefit in FL patients (Table 2).

6.2. Phase 3 Trials of Idiotype Vaccines. (i) First study sponsored by Genitope included FL patients treated with 8 cycles of first line CT with CVP schedule. Patients who achieved a complete or partial response were randomized in a 2 : 1 fashion to seven Id vaccine doses or a control arm with KLH and GM-CSF. Finally 192 patients were vaccinated and 95 received control treatment. Regarding the main endpoint, statistical significance was not found in terms of progression-free survival among both arms. However, it was observed that patients failing to mount an Id-specific humoral response had significantly worse results [117, 118].

(ii) Recently, Freedman et al. communicated the final results of another phase 3 trial sponsored by Favrille's in which patients treated with four doses of Rituximab who entered in CR, PR, or SD were randomized to an Id vaccine group ($n = 174$ patients) and a control group treated with GM-CSF ($n = 175$ patients). This trial not only failed in showing a better disease-free survival for vaccinated patients, but even demonstrated a statistically significant difference in favour of the control group treated with GM-CSF [119].

(iii) In the 2009 American Society of Clinical Oncology (ASCO) Annual Meeting, Schuster et al. presented phase 3 results on vaccine BiovaxID [120]. This trial, sponsored by Biovest, included patients in CR or CR unconfirmed after 6 cycles of CT with PACE or Rituximab plus CHOP. Again, randomization was done in two groups, Id vaccine (experimental) and KLH plus GM-CSF (control). Main objective was disease-free survival. Unfortunately, this study was halted in April 2008 with only 31,2% of patients included, owed to rituximab irruption and dominance in FL guidelines and clinical trials. Of 177 patients included in this trial, 60 relapsed while waiting for their vaccine, so conclusions were drawn from only 117 patients, 76 in the experimental and 41 in the control group. Median survival was statistically significant favouring treatment arm (44,2 versus 30,6 months; $P = .047$) and the main endpoint showed a 13,6-month increase in median disease-free survival for Id vaccine group [120].

6.3. Pitfalls and Clues in Vaccine Development. Though soluble protein idiotypic vaccination has provided proof of principle of biological and clinical efficacy, and even clinical benefit in some small clinical studies in FL, results of the three phase 3 trials mentioned above are disappointing and failed. However, there are many circumstances that may alter final results of these randomized trials.

(i) Two of the trials included patients irrespective of the quality of the response after CT. As suggested in preclinical models, disease's situation at vaccination seems to be crucial. When there is a sizable tumour burden, vaccines are less likely to be effective maybe because, among other mechanisms, remaining malignant cells still have the ability to secrete cytokines to evade immune recognition. Accordingly, it must be underscored that better clinical results have been obtained in clinical trials where a CR was previously achieved [114].

(ii) The Favrille's phase 3 trial employed four doses of Rituximab before vaccination [119]. Nowadays, in daily clinical practice, it is preferred using chemoimmunotherapy schedules at first line in fit patients. Hence, four doses of Rituximab may be considered as a suboptimal schedule with few complete responses. Besides this, Rituximab causes B cell depletion in normal and malignant cells, hence interfering in the initiation of humoral response. Final results of this

Table 2: Phase 3 trials of idiotype vaccines.

Author/Sponsor	Idiotype	Comparison	Pretreatment	Patient status prevaccination	End Point	Results
Levy et al. [118] Genitope	Recombinant	2/1 randomization in first line	8 cycles of CVP	First CR or PR	PFS	P = n.s
Freedman et al. [119] Favrille	Recombinant	2/1 randomization in first line	4 doses of Rituximab	First CR, PR or SD	TTP	P = n.s
Schuster et al. [120] Biovest	From hybridoma	2/1 randomization in first line	6 cycles of PACE or CHOP-R	Firsr CR or CRu	DFS	P = .045

phase III trial suggest poorer results for the experimental arm and probably this is the consequence of an early vaccination, before B-cell counts after rituximab treatment were recovered.

However, there is still scarce evidence in vaccination after rituximab-containing immunochemotherapy schedules. Neelapu et al. [121] communicated data of a pilot trial in 26 patients with mantle cell lymphoma treated with EPOCH-R, followed 12 weeks later with five monthly vaccinations of autologous tumor-derived Id-KLH$^+$GM-CSF. As expected, after chemoimmunotherapy, peripheral blood B cells were completely depleted in all patients. Recovery was detected at 6 months, returning to baseline levels at 12 months. CD4$^+$ T cell counts decreased only slightly after CT and recovered 3 months later, by the start of vaccination. CD8$^+$ T cell counts did not change substantially. Curiously, after rituximab administration, antibody responses against KLH and Id were detected in 74% (17 out of 23) and 30% (7 out 23) of patients, respectively. Humoral responses were delayed and correlated with the recovery of B cells following the administration of rituximab, especially after the fourth or fifth vaccination.

The results of this pilot study are extremely important, because it demonstrates that vaccination after rituximab treatment is feasible and can induce delayed humoral responses. Taking into account that Id vaccine production takes some months, it would be interesting to design clinical trials in which Id-vaccine was administered between 6 and 12 months after a chemoimmunotherapy schedule containing rituximab.

(iii) Control group in the three phase 3 trials used KLH$^+$GM-CSF or GM-CSF. It is uncertain whether these compounds may induce an immune response against lymphoid cells by themselves. In particular, GM-CSF is a cytokine with highly immunogenic properties that has even demonstrated clinical efficacy in the clinical setting in FL, in combination with Rituximab, and in other solid neoplasms [122]. So, it is arguable if KLH$^+$GM-CSF or GM-CSF alone represents an ideal control group with neutral immune effects.

(iv) Follicular lymphoma is such a heterogenic disease with a different and unpredictable evolution. Moreover, host's immune response to vaccines is also heterogeneous in every single patient. Therefore, there are many sources of uncontrollable variability that make idiotypic vaccination in FL such a difficult strategy to reach success in randomized clinical trials, where the methodology remains rigorously dictated by statistics and clinical benefit in the overall population.

(v) As previously mentioned, several mechanisms may explain the low clinical effectiveness reported. One of the main reasons lies in the inability of immune cells to infiltrate and become activated after an encounter with tumor antigen *in vivo*. Moreover, it seems that tumors do not express costimulatory molecules or produce the inflammatory microenvironment necessary to activate effector cells with the ability to eradicate tumors [123, 124]. Therefore, the development of methods to activate antitumor immune cells by stimulating APCs and generate long-term memory cells, probably with the aid of costimulators, is one of the future challenges for definitively integrating tumor vaccines into the antineoplastic arsenal. In this sense, GM-CSF has demonstrated clinical activity when used alone [125] (melanoma) and in combination with other agents (follicular lymphoma, colorectal and breast cancer [82, 126, 127]). Among these protocols, GM-CSF administration is prolonged, ranging from 5 to 14 days, yet in vaccine trials GM-CSF is commonly used in a short course of three or four doses. So, safety and efficacy data encourage the prolonged administration of maintenance boosters (GM-CSF, Interleukin-2, etc.), especially once there is biological evidence of an immune response successfully triggered.

(vi) Regardless of the method used, Id vaccine production is expensive and time consuming. In fact, the NCI/Biovest study loosed more than 30% of patients included because of a relapse while waiting for the vaccine production. This is especially worrying because it predicts serious difficulties in an eventual extensive clinical use. Other sources of vaccination are under development with membrane proteoliposomes, or tumor cell-based vaccines transduced with GM-CSF, CD40-activated or HSP-96 [111]. These new formulations are under clinical investigation, mostly in phase 1 trials and have the advantage of targeting multiple tumor antigens with a shorter production time.

(vii) Therapeutic armamentarium in LF is changing, and knowledge of the immune effects of the new therapies employed may be of critical importance for clinical trials with Id-vaccines. Recently, Yttrium-90 Tositumomab Tiuxetan (Zevalin^R) has been approved by the FDA in first line of LF, as consolidation after CT [128]. Zevalin^R is a CD-20-directed radiotherapeutic antibody with several mechanisms of action. In addition, in the 2009 annual meeting of

the American Society of Hematology (ASH), results of a phase III trial in low-grade lymphomas, comparing CHOP-R and Bendamustine-R, have been communicated, providing better progression-free survival (median PFS 54,9 versus 34,8 months; HR 0.57, $P = .00012$) and a better toxicity profile for the experimental arm [129]. These data could oust the standard CHOP-R regimen in brief, so anthracyclines could be out of first line treatment in the coming future.

Even though clinical results in many types of lymphomas, including LF, that have improved over the years owed to introduction of rituximab and chemoimmunotherapy schedules, there is still room for improvement; yet many patients relapse and finally die as a consequence of their disease. Thus, once confirmed proof of principle of biological and clinical efficacy of vaccine-therapy, these results might not be overlooked nor neglected by physicians, since it may translate into a prolonged disease-free survival, and eventually the recovery of some LF populations. Although history of vaccines in Oncology has been extremely disappointing, reminding the myth of Minotaurus, with every little step forward followed by a new frustration, new insights into this strategy may hopefully obtain better and surprising results, and so finding out the Ariadne's thread which eventually leads to see the end of this complex and challenging labyrinth.

7. Conclusions and Final Remarks

Tumorigenesis is a multistep process leading to the progressive transformation of normal human cells into highly malignant derivatives. Thus, mechanisms of oncological diseases are extremely complex, with several alterations at multiple sites. Hanahan and Weinberg, in an effort to synthesize the huge body of knowledge in cancer research, postulated six essential alterations in cell physiology [130]. Among them, the insensitivity to antigrowth signals, evasion of apoptosis, sustained angiogenesis, and tissue invasion and metastasis seem to be intrinsically related to microenvironment dysregulations. Lymphomas constitute an excellent model for microenvironment translational research. These tumors might be considered as a functional tissue immunologically mediated and formed by a complex tissue network in which the imbalance of homeostasis between the host immune system, malignant cells, and all other components of tumoral stroma determine proliferation, invasion, angiogenesis, and remodelling of extracellular matrix and metastasis. Moreover, the distinctive profile of immune cells in the surrounding stroma leads to a wide repertoire of specific cell subpopulations which constitute the specific tumor microenvironment of each lymphoproliferative syndrome. In the last few years, the critical importance of these findings and correlation with prognosis and clinical results has been recognized.

Recent evidence has emerged that confers new properties to antineoplastic treatments against lymphomas, in relation with microenvironment changes. This is the case of some chemotherapeutics like anthracyclines or radiotherapy that may induce tumoral destruction with the ability of improving cancer cell recognition by the immune system, and thus enhancing the possibility of a successful immune response. In addition, these "new discoveries" in the mechanisms of action of classic antineoplastic treatments might be the basis of the synergism of the new combined chemoimmunotherapy strategies in lymphomas that include passive immunotherapy with the monoclonal antibody Rituximab. Finally, active immunotherapy with anti-idiotype vaccines, though still far from daily practice integration, has demonstrated clinical efficacy in some subpopulations of patients. Fine tune approaches in vaccine development and a better design of vaccine clinical trials are needed to definitely elucidate the role of active immunotherapy in lymphoproliferative syndromes.

Conflict of Interests

Authors declare no conflict of interests.

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T. Álvaro and L. de la Cruz-Merino have contributed equally to this article.

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